Essential Role of Apelin Signaling During Lymphatic Development in Zebrafish

Jun-Dae Kim, Yujung Kang, Jongmin Kim, Irina Papangeli, Hyeeseon Kang, Jingxia Wu, Hyekyung Park, Emily Nadelmann, Stanley G. Rockson, Hyung J. Chun, Suk-Won Jin

Objective—Apelin and its cognate receptor Aplnr/Apj are essential for diverse biological processes. However, the function of Apelin signaling in lymphatic development remains to be identified, despite the preferential expression of Apelin and Aplnr within developing blood and lymphatic endothelial cells in vertebrates. In this report, we aim to delineate the functions of Apelin signaling during lymphatic development.

Approach and Results—We investigated the functions of Apelin signaling during lymphatic development using zebrafish embryos and found that attenuation of Apelin signaling substantially decreased the formation of the parachordal vessel and the number of lymphatic endothelial cells within the developing thoracic duct, indicating an essential role of Apelin signaling during the early phase of lymphatic development. Mechanistically, we found that abrogation of Apelin signaling selectively attenuates lymphatic endothelial serine–threonine kinase Akt 1/2 phosphorylation without affecting the phosphorylation status of extracellular signal–regulated kinase 1/2. Moreover, lymphatic abnormalities caused by the reduction of Apelin signaling were significantly exacerbated by the concomitant partial inhibition of serine–threonine kinase Akt/protein kinase B signaling. Apelin and vascular endothelial growth factor-C (VEGF-C) signaling provide a nonredundant activation of serine–threonine kinase Akt/protein kinase B during lymphatic development because overexpression of VEGF-C or apelin was unable to rescue the lymphatic defects caused by the lack of Apelin or VEGF-C, respectively.

Conclusions—Taken together, our data present compelling evidence suggesting that Apelin signaling regulates lymphatic development by promoting serine–threonine kinase Akt/protein kinase B activity in a VEGF-C/VEGF receptor 3–independent manner during zebrafish embryogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: apelin protein, zebrafish ■ zebrafish

Lympathic vessels have essential roles in maintaining the homeostasis of interstitial body fluid and facilitating immune responses in vertebrates. In addition, lymphatic vessels have been associated with progression of diverse diseases in humans, including tumor metastasis and obesity. Malformation or obstruction of lymphatic vessels cause an accumulation of interstitial fluid resulting in the swelling of extremities, pathological conditions collectively categorized as lymphedema, which affect >140 million people worldwide. During development, lymphatic endothelial cells (LEC), the main component of lymphatic vessels, first appear around E10 in mice, embryonic week 6 to 7 in humans, and 3 days postfertilization (dpf) in zebrafish. Lineage tracing and in vivo time lapse analyses in mouse and zebrafish support the model proposed by Florence Sabin that the LECs may originate from ECs in the cardinal vein.

Apelin and its receptor Aplnr/Apj regulate a wide range of developmental and physiological processes. Although Apelin is widely expressed during development, the expression of Aplnr/Apj is more restricted. In mouse, Apj is strongly expressed within the cardiovascular system as early as E8.0. Similarly, aplnra and aplnrb are specifically expressed in developing ECs and cardiomyocytes in zebrafish. Consistent with the expression pattern, Apj knockout mice display various cardiovascular defects and are embryonic lethal in certain genetic background. Zebras have 2 Apelin receptors, aplnra and aplnrb. Although aplnra and aplnrb are broadly expressed in areas including heart primordial cells and lateral plate mesoderm during early development, their expression gradually become restricted to blood vessel in late development. After 24 hours postfertilization, only venous ECs in the trunk region express detectable levels of aplnra, suggesting that Apelin signaling may be essential for blood ECs (BECs) and LECs. Mutations in aplnrb cause similar developmental defects in zebrafish. Despite its proposed role in cardiovascular development, current analyses on Apelin signaling predominantly aim to address its role in vascular physiology, and consequently, the function...
of Apelin signaling in the development of lymphatic vessels remains largely unknown.

In this report, we examined the function of Apelin signaling in developing LECs and found that Apelin signaling provides a nonredundant function to induce serine–threonine kinase Akt/protein kinase B (AKT) activation, therefore serving as an essential signaling input to promote lymphatic development. Abrogation of Apelin signaling caused severe defects of the lymphatic structure in zebrafish embryos by substantially decreasing the phosphorylation of AKT1/2, independent of vascular endothelial growth factor-C (VEGF-C) signaling. Our data presented here support the idea that Apelin signaling is essential for proper lymphatic development.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Apelin Signaling Modulates the Proper Lymphatic Vessel Formation During Zebrafish Development

To delineate the roles of Apelin signaling in vascular development of zebrafish, we first attenuated the level of Aplnra by morpholino-mediated knockdown of aplnra (Figure IA–ID in the online-only Data Supplement). Because proper circulation is essential for lymphatic development, a suboptimal dose of aplnra morpholino was injected (1.8 ng/embryo) to avoid the early cardiac abnormalities that have been previously reported17,19 and assess the role of Apelin signaling specifically in lymphatic development (Figure ID in the online-only Data Supplement). The general morphology and the rate of heartbeat at 4 dpf in embryos injected with 1.8 ng of aplnra morpholino were comparable with those of control morpholino–injected embryos (Figure IA and Figure IB and IC in the online-only Data Supplement). In addition, patterning of intersegmental vessels (ISVs), the dorsal aorta, and the cardinal vein, was clearly visible in 4 dpf control morpholino–injected embryos, similar structures were absent in aplnra morpholino–injected embryos (Figure 1B). In addition, the number of nEGFP+/mCherry+ LECs within the thoracic duct in aplnra morpholino–injected embryos was greatly decreased (Figure 1C), suggesting that LECs are more sensitive to attenuated level of Apelin/Aplnr signaling activity. Similarly, the number of VEGFR3+ LECs in the diaphragm of Apf+/− mice was significantly reduced (Figure IE and IF in the online-only Data Supplement), suggesting that the role of Apelin/Aplnr signaling on lymphatic development may be conserved within vertebrate species.

We next determined whether the attenuation of the cognate ligand of Aplnr, Apelin, would cause comparable defects in lymphatic development. To ensure that the lymphatic defects found in apln morpholino–injected embryos are not secondary to the previously reported cardiac defects,17,19 the amount of morpholino was titrated. Injection of 2.7 or 5.4 ng per embryo effectively blocked the normal splicing of apln transcript (Figure IIA in the online-only Data Supplement) without causing obvious morphological defects in axis formation, rate of heartbeat, or blood vessel formation (Figure IIB and IIC in the online-only Data Supplement). In contrast, the formation of parachordal vessels (arrows), which give rise to the presumptive LECs, was severely affected by partial knockdown of apln (Figure 2A and 2B). At 3 dpf, embryos injected with 2.7 ng of apln morpholino completely lacked parachordal vessel. Consistent with the defects in

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Figure 1. Lack of aplnra activity causes lymphatic vessel defects. A, Gross morphology (top) and vascular structures (bottom) in 4 days postfertilization (dpf) aplnra morpholino (MO)–injected embryos. B, The loss of lymphatic endothelial cells (LEC) in thoracic duct (white arrows) in 4 dpf aplnra MO–injected embryos. C, Quantification of the number of LECs in control and aplnra MO–injected embryos. LECs within the thoracic duct between 8th and 15th somites were counted. All embryos shown have Tg(fli1a:nEGFP);Tg(kdrl:mCherry) double transgenic background to visualize blood ECs (shown as yellow) and LECs (shown as green). CV indicates cardinal vein; DA, dorsal aorta; and ISV, intersegmental vessel. Scale bars, 400 μm (A) and 50 μm (B).
parachordal vessels, we found that the number of LECs in 4 dpf apln morpholino–injected embryos was decreased in a dose-dependent manner (Figure 2C and 2D). Consistent with these findings, microlymphangiography demonstrated that embryos with a reduced level of Apelin signaling do not develop proper lymphatic vessels (Figure III in the online-only Data Supplement).

Because LECs emerge from venous ISVs, 12 it is possible that a decrease in Apelin signaling activity may limit the number of forming venous ISVs and indirectly influence the number of LECs. To evaluate this possibility, we examined the effects of Apelin signaling on the formation of venous ISVs by counting the number of ISVs that were directly connected to the cardinal vein (venous ISV connections) at 4 dpf. Although the number of LECs was substantially decreased by injecting a low concentration of apln morpholino (0.9 and 1.8 ng/embryo), the number of venous ISV connections was largely unaffected (Figure 2E and 2F), suggesting that Apelin signaling is likely to directly regulate lymphatic development in zebrafish embryos.

To further confirm that the lymphatic defects in apln morpholino–injected embryos are directly caused by the attenuation of Apelin signaling, we attempted a phenotypic rescue of apln morpholino–injected embryos by injecting synthetic apln mRNA. Because apln mRNA injection can cause cardiac defects, 17 the amount of mRNA was titrated. Although a high dose of apln mRNA caused severe cardiac defects, injection of 8 pg apln mRNA per embryo did not cause any phenotypic defects (Figure IV A and IVB in the online-only Data Supplement). When coinjected, 8 pg apln mRNA successfully restored the number of LECs in apln morpholino–injected embryos (Figure 2G and 2H), suggesting that the reduction in the number of LECs in apln morpholino–injected embryos was not a secondary effect of general developmental delays caused by morpholino injection. Taken together, our data suggest that proper activity of Apelin/Aplnra signaling is essential for lymphatic development in zebrafish embryos.

Apelin Signaling Modulates AKT Activity in Human and Zebrafish LECs

It has been reported that Apelin promotes the migration of BECs and LECs in cell culture. 23 Consistent with the previous report, knockdown of APLNR in human LECs (hLECs) adversely affected the migration of hLECs. In a scratch wound...
assay. APLNR small interfering RNA (siRNA)–treated hLECs displayed a significantly attenuated migratory behavior compared with control siRNA–treated hLECs (Figure 3A and 3B). The migration defect of APLNR siRNA–treated hLECs was likely caused by a reduced level of Apelin signaling because addition of exogenous Apelin 13 was not able to alleviate the migration defects in APLNR siRNA–treated hLECs (Figure 3A and 3B).

To identify the downstream effectors of Apelin signaling in LECs, we examined the level of phospho-AKT1/2 and phospho–extracellular signal–regulated kinase 1/2 (ERK1/2) in APLNR siRNA–treated hLECs (Figure 3C). Because it has been reported that ERK1/2 and AKT, which function as downstream effectors for several G protein–coupled receptors including APLNR,16,27,28 are essential for modulating the development of lymphatic vessels,1,3 it is tempting to speculate that ERK1/2 and AKT may be involved in downstream of Apelin signaling during lymphatic development. Although the level of phospho-ERK1/2 did not change in APLNR siRNA–treated hLECs compared with control siRNA–treated cells, the level of phospho-AKT1/2 was significantly decreased on APLNR deprivation (Figure 3C–3E). Moreover, stimulation with Apelin ligand in hLECs led to an increased level of phospho-AKT1/2 in a dose-dependent manner (Figure V in the online-only Data Supplement). Similarly, attenuation of Apelin signaling by morpholino injection drastically reduced the level of phospho-AKT1/2 in developing zebrafish. Although phospho-AKT1/2 is strongly detected within ECs in the dorsal aorta and cardinal vein of 48-hour postfertilization control morpholino–injected embryos, it is largely absent in apln morpholino–injected embryos (Figure 3F). Therefore, it seems that Apelin signaling may function as a major stimulus for AKT1/2 phosphorylation in both cell culture and in vivo. Previously, Aplnr have been reported to function independent of Apelin ligand.19 Therefore, we also examined the level of phospho-AKT1/2 in apln morpholino–injected embryos and found that phospho-AKT1/2 was substantially reduced in these embryos, suggesting that phosphorylation of AKT1/2 is dependent on Apelin/Aplnr signaling (Figure 3F).

Figure 3. Apelin signaling is essential for human lymphatic endothelial cell (hLEC) migration and maintenance of phospho–serine–threonine kinase Akt 1/2 (p-AKT1/2). A, Representative images of scratch wound healing assay using hLEC. Black lines demarcate the boundaries of scratch wounds. The recovery of the wound was substantially attenuated in APLNR small interfering RNA (siRNA)–treated hLECs compared with control siRNA–treated hLECs. B, Quantification of the migration of hLECs. C, Lack of Apelin signaling selectively attenuated the level of p-AKT1/2 in hLECs grown in complete growth medium 3 days after siRNA treatment. Experiments were performed as triplicates. D, Quantification of the relative levels of p-AKT1/2 and total AKT (tAKT) in control or APLNR siRNA–treated hLECs. E, Quantification of the levels of phospho–extracellular signal–regulated kinase 1/2 (p-ERK1/2) and total ERK1/2 (t-ERK1/2) in control or APLNR siRNA–treated hLECs. F, Confocal image showing a transverse section of 48 hours postfertilization control morpholino (MO; top), apln MO (middle), or aplnra MO (bottom) injected Tg(fli1a:nGFP) embryos. Developing ECs within dorsal aorta (DA) and cardinal vein (CV) contain a high level of p-AKT1/2 (arrowheads). Lack of Apelin signaling selectively abrogated the presence of p-AKT1/2 in ECs without affecting the p-AKT1/2 in nearby blood cells (arrowhead). Scale bar, 10 μm. N.S. indicates not significant.
Apelin and AKT Activity Functionally Cooperate for Zebrafish Lymphatic Vessel Formation

To further substantiate the link between AKT activity and Apelin signaling in LECs, we manipulated the level of AKT activity in zebrafish embryos and examined the effects on developing LECs. Because manipulation of AKT activity at earlier developmental stages may compromise the specification of arterial and venous ECs, we used previously reported chemical antagonists of AKT to induce temporal AKT inhibition. Treatment with either 10 μmol/L LY294002 (phosphoinositide 3-kinase inhibitor) or 2 μmol/L Torin1 (mammalian target of rapamycin complex 1 and 2 inhibitor), both of which inhibit AKT phosphorylation, drastically reduced the number of LECs in 4 dpf zebrafish embryos (Figure 4A–4D). Moreover, the lymphatic defects caused by a partial reduction of Apelin signaling activity were significantly exacerbated by administering a suboptimal dose of the aforementioned chemical antagonists of AKT (Figure 4E–4G). Although embryos injected with 0.9 ng of apln morpholino or treated with 5 μmol/L of LY294002 contained an average of 5.6±0.65 and 6.2±0.79 LECs, respectively, embryos that were injected with 0.9 ng of apln morpholino and treated with 5 μmol/L of LY294002 had significantly fewer LECs (3.8±1.11), representing a further ≈40% reduction the in number of LECs compared with single manipulations (Figure 4E and 4F). Similar effects were also observed in embryos that were injected with 0.9 ng of apln morpholino and treated with 1 μmol/L of Torin1 (≈40% reduction; Figure 4E and 4G).

Apelin and VEGF-C Signal Independently in Zebrafish Lymphatic Development

Previously, it has been reported that AKT activity within ECs can be induced by VEGF-C signaling, which is the key stimulus for lymphatic development. We next tested whether Apelin and VEGF-C signaling converge at the level of AKT or function redundantly to promote differentiation and maintenance of LECs. Attenuation of Apelin signaling did not influence the expression of VEGF-C signaling components, including expression of vegfc or its receptor flt4 (Figure VIA in the online-only Data Supplement). Similarly, inhibition of VEGF-C signaling activity did not affect the expression of apln, aplnra, or aplnrb (Figure VIB in the online-only Data Supplement). However, coinjection of apln and vegfc morpholino with suboptimal doses exacerbated the lymphatic phenotypes (Figure 5A and 5B), indicating that Apelin and VEGF-C signaling may synergistically promote lymphatic development. We next examined whether Apelin signaling is sufficient to compensate for the loss of VEGF-C signaling. Ectopic activation of Apelin signaling by synthetic mRNA...
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injection was unable to alleviate the lymphatic defects in vegfc morpholino–injected embryos (Figure 5C and 5D). In a similar manner, ectopic expression of Vegfc by synthetic mRNA injection was unable to restore the lymphatic abnormalities caused by reduced Apelin signaling (Figure 5E and 5F). The inability of apln mRNA to alleviate the defects of vegfc morpholino–injected embryos and vegfc mRNA to apln morpholino–injected embryos collectively suggest that Apelin and Vegfc signaling may function in a nonredundant manner in lymphatic development.

Considering that both Apelin and Vegfc signaling can activate AKT to induce lymphatic development, but seem to have nonredundant functions, it is possible that Vegfc and Apelin signaling may have distinct functions; although Vegfc may potentiate presumptive LECs within venous vascular beds, Apelin signaling may successively promote differentiation of LECs in later developmental stages. Alternatively, it is possible that Apelin and Vegfc signaling may induce AKT activity in a temporally distinct manner. To examine this possibility, we analyzed the expression of apln, aphnra, vegfc, and vegfr3 within the first 5 days of development in zebrafish embryos (Figure 6A). Although the expression level of vegfc gradually increased and then stabilized, the expression level of vegfr3 precipitously dropped by 2 dpf, indicating that the activity of Vegfc-Vegfr3 signaling may reduce to the basal level when LECs emerge from venous ISVs (Figure 6A). In contrast, the expression of apln and aphnra continuously increased during the first 5 days of development (Figure 6A and Figure VIC in the online-only Data Supplement), suggesting functions of Apelin signaling may be required later in development than Vegfc signaling.

**Discussion**

Considering the expression pattern of Apelin signaling components during development, and their role during lymphatic regeneration, we concluded that Apelin signaling provides key regulation during lymphatic development. In this report, we have demonstrated that Apelin signaling–induced
AKT activity is essential for differentiation of LECs during development. AKT activity has been implicated in both developmental and pathological lymphangiogenesis.\textsuperscript{34,37,38} For instance, targeted deletion of Akt1, Akt2, or Akt3 in mouse caused a significant reduction in the number of developing LECs and led to defects in lymphatic valve formation.\textsuperscript{37,39} In addition, Kaposi’s sarcoma associated herpesvirus activates AKT to induce ectopic lymphatic structures.\textsuperscript{40,41} Although AKT can be activated by diverse signaling inputs,\textsuperscript{42,43} attenuating Apelin signaling substantially decreased the phosphorylation of AKT in LECs, both in cell culture and developing zebrafish embryos. Therefore, it seems that Apelin signaling may function as a main inducer of AKT activity within LECs. Moreover, our data suggest that Apelin signaling may coordinate with VEGF-C signaling, an essential prolymphangiogenic signaling pathway,\textsuperscript{34,36,39} to maintain the proper level of AKT activity in LECs. During development, components of Apelin and VEGF signaling seem to be expressed at distinct stages, suggesting that these 2 prolymphangiogenic signaling pathways may activate the same downstream effectors, but their activity may be temporally separated during development (Figure 6B).

Although Apelin signaling is required for AKT activation in LECs and the disruption of the Apelin-AKT signaling cascade drastically reduced the number of LECs in zebrafish, ectopic AKT activation by chemical agonists (data not shown) and ectopic expression on Vegfc (Figure 5E) were not sufficient to restore the number of LECs in zebrafish embryos with compromised Apelin signaling. Therefore, it is likely that the prolymphangiogenic role of Apelin signaling may be transduced by additional effectors. ERK1/2, which are known to be activated by VEGF signaling in LECs,\textsuperscript{35,36} do not seem to mediate Apelin signaling in LECs because lack of Apelin signaling did not affect the phosphorylation status of ERK1/2 in hLECs (Figure 3C). Ongoing work in the laboratory is examining the role of other serine–threonine kinases, including ERK5, Raf, and PKA, as potential downstream mediators of Apelin signaling in LECs. Convergence of Apelin and VEGF-C at AKT, as well as divergent signaling pathways activated by these distinct signaling cascades, likely provide the necessary cues that ensure proper lymphatic development.

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Disclosures

None.

References


factor-C signaling to promote lymphatic fate. Despite importance of Apelin/APJ signaling in diverse biological processes, its role during lymphatic development is relatively unknown. We


vessel integrity. Apelin inhibits diet-induced obesity by enhancing lymphatic and blood


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Alternatively, Apelin/APJ signaling may synergize with vascular endothelial growth factor-C signaling to promote lymphatic fate.
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Supplementary Methods

MATERIALS AND METHODS

Husbandry, morpholino (MO) injection, and chemical treatment of zebrafish
Zebrafish (Danio rerio) were maintained as previously described1. The follow strains were used in this research in addition to wild-type; Tg(fli1a:nEGFP)y7, Tg(kdrl:mCherry)s843, and TgBAC(prox1a: KalT4-UAS:uncTagRFP)nim5. Control, apelin2, aplnra, and vegfc3 MOs were used (Genetools). MOs were injected with phenol-red (0.1%) in zebrafish embryos at the 1~2 cell stage. The sequences of MO used were apln MO; 5′-AACAGCCGTCAGCTCCCGACTTAC-3′, aplnra MO; 5′-CGCGTTGGCTTTGAGTCCTTCTTGA-3′, and vegfc MO; 5′-GAAAATCCAAATAAGTGCATTTTAG-3′. For chemical inhibition of AKT activity, LY294002 (S1105; Selleckchem), a PI3K inhibitor4 and Torin1 (S2827; Selleckchem), an ATK and mTORC1 inhibitor5, were used. Embryos were treated with different amounts of LY294002 and Torin1 dissolved in DMSO from 48hpf to 4dpf, and imaged with confocal microscopy to evaluate the effects of treatments on developing lymphatic vessels.

Rescue experiment by the injection of synthetic apln mRNA
To achieve the in vitro transcription of apln mRNA, apln full length DNA was amplified from 2 dpf zebrafish cDNA and cloned into the pCS2P+ vector (Addgene; Plasmid 17095). pCS2P+ plasmid containing apln full length DNA was cut by NotI, transcribed by SP6 RNA polymerase and purified by SP6/T7 transcription kit according to the manufacturer’s protocol (Roche). Synthetic apln mRNA was diluted in RNase free water to make the working solution at a concentration of 16ng/ml, and injected at the concentration of 8~16pg/embryos into control or apln MO-injected embryos at the 1~2 cell stage. The PCR primer sequences for apln full length DNA are as follows: Forward primer, 5′-ATGAATGTGAAGATCTTGACG-3′; Reverse primer, 5′-TGCCTTGCTGTAGAATGGCA-3′.

Semi-quantitative conventional and quantitative real-time PCR
The total RNA from wild-type, control and apln MO-injected embryos was extracted at several developmental time points using an RNeasy mini kit (Qiagen) and reverse transcribed by High Capacity cDNA Kit according to the manufacturer’s protocol (Life Technologies). Semi-quantitative conventional and quantitative real-time PCR were conducted according to the manufacturer’s protocol. The sequences of PCR primers used in this study are; apln forward, 5′-ATGTGAAATCTCTGACGGTC-3′; apln reverse, 5′-TGCCCTTCGCTCAAGATGGCA-3′; aplnra forward, 5′-GGCTTCCTCTACAAATCATC-3′; aplnra reverse, 5′-AGCCCTTCTTCCAGATCC-3′; aplnrb forward, 5′-TGCTGACTTCTGACAGTA-3′; aplnrb reverse, 5′-CATCATACTCCTGGTG-3′; vegfc forward, 5′-CATGGCCAAAGGAATCGAG-3′; vegfc reverse, 5′-GGTGTTGCATCAACAGC-3′; vegfr3/flt4 forward, 5′-GCCGCTGGTATATTCAAGAA-3′; vegfr3/flt4 reverse, 5′-GCAGTGTGGTACCAAGGAA-3′; actb1 forward, 5′-ATGGAGATGTCTGGCGATCTC-3′; actb1 reverse, 5′-ACCCAGACTCTTACACATC-3′.

Whole mount in situ hybridization and immunohistochemistry
Whole mount in situ hybridization was performed as previously reported6. The RNA probe for aplnra was labeled with dig using a DIG-labeling kit (Roche) by in vitro transcription method1. For immunohistochemistry, phospho-AKT1/2 antibody (cell Signaling;#4060) was used in cryo-sectioned embryos (10mm width per slice).

Human lymphatic endothelial cell (hLEC) culture and scratch migration assay
Human microvascular EC-dLyAd-adult human dermal lymphatic microvascular ECs (HMVEC-dLyAd-HDLECs; CC-2812), (hLECs), were obtained from Lonza. Cells were cultured at 36.5 °C in a 5% CO₂ incubator in the growth medium EGM-2 (Lonza) containing 2% fetal bovine serum (FBS) (Lonza). For experimental treatments, hLECs (passages 3–5) were grown to 80–90% confluence. For gene silencing, siRNAs (Stealth siRNA, Life Technologies) were transfected using RNAiMAX (Life Technologies) using manufacturer’s protocols.

For scratch migration assay, hLECs were incubated on 6-well plates in EGM-2 medium overnight. hLECs were transfected with control siRNA (Life Technologies, Stealth RNAi) or APJ siRNA (Life Technologies, HSS100324) for 48 hours and were then starved for 6 hours with EBM-2 with 0.5% FBS medium. Monolayers of hLECs were scratched with a universal blue pipette tip and the widths of the scratches in four fields per well were captured using a TS100 Nikon microscope. Cells were incubated for 14 hours in starvation medium containing PBS (Lonza) or 1µM Apelin-13 (Sigma). The same fields were captured again after migration. Differences in the widths of scratches before and after migration were calculated. The width of one field is an average of the widths at three different places in the same field. The means and SEM of triplicate wells were calculated. All experiments were replicated at least three times, with similar results.

**Western blot assay**

Proteins (20µg) in soluble lysate were resolved by TGX precast gel (Cat# 456-9036) from Bio-Rad then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in 3% BSA in PBS containing 0.1% Tween-20 (PBST) overnight at 4 °C and probed with antibodies specific to phospho-Akt(Ser473) (Cat# 4060), Akt (pan) (Cat# 4691), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cat# 4377), p44/42 MAPK (Erk1/2) (Cat# 9102), or GAPDH (Cat# 2118) from Cell Signaling Technology. Subsequently, the membrane was thoroughly washed in PBST, and incubated with HRP-conjugated secondary antibody (Cat# 7074) from Cell Signaling Technology for 1 hour at room temperature. Finally, the membrane was developed by enhanced chemiluminescence detection method (Thermo Scientific).

**Confocal Image Acquisition, Processing and Quantification**

Zebrafish embryos were anesthetized, plated and oriented laterally on a glass bottom dish. Image acquisition from zebrafish was performed using a Nikon confocal microscope and merged Z-stack images by MBF ImageJ. Three dimensional reconstructions of confocal images were performed by Velocity 3D Image Analysis Software (PerkinElmer). The number of LECs in developing TDs of zebrafish embryos was individually counted from the trunk region that presented by 7 somites, between from somite boundary 8 or 9 to 15, on Z-stacked confocal images. For proper quantification, experiments were repeated at least three times. Quantification graphs were generated by PRISM or Microsoft Excel programs. Results were evaluated by two-tailed and unpaired Student’s t test and each error bar represents the standard error of the mean (SEM). p values are as follows: *p<0.05, **p<0.01, ***p<0.001.

**REFERENCES**


Supplementary Figures

**Figure I.** Attenuation of Aplnra signaling selectively affects lymphatic development in zebrafish and mouse embryos. (A) Validation of the MO efficacy. Since *aplnra* is a single exon gene, we generated a construct, pEGFP-N1-5’UTR-*aplnra* plasmid (50pg/embryo), which encodes GFP containing 5’UTR from *aplnra* locus. Co-injection of this construct with control MO did not attenuate expression of GFP, while co-injection with *aplnra* MO (2.7ng/embryo) abolished the GFP expression. Embryos were imaged at shield stage. (B) Quantification on the percentage of embryos expressing GFP. (C) The partial knock-down of *aplnra* did not affect the heart rate in zebrafish embryos. Embryos were injected the *aplnra* MO (1.8ng/embryo) at 1-2 cell stage and heart beating rate was measured by 4dpf. (D) The late LEC development (6.5 dpf) was perturbed in *aplnra* KD zebrafish embryos. (E) The number of VEGFR3+ LECs and lymphatic vessel formation were substantially reduced in the diaphragm of P5 *Apj*−/− mouse embryos. (F) Quantification on the number of lymphatic vessel branches in P5 wild-type and *Apj*−/− mice. Images were taken from 3 individual wild-type and *Apj*−/− embryos. ISV, inter-segmental vessel; DA, dorsal aorta; CV, cardinal vein. White arrows point individual LEC in TD. Scale bars are 600 µm in (A) and 50 µm in (D).
Figure II. The validation of *apln* anti-sense morpholino (MO) and phenotype of the knock-down (KD) embryos of Apelin. (A) *apln* MO efficiency was evaluated by RT-PCR. The splicing aberrant forms of *apln* transcript (*apln_aberrant*) were increased by high dose *apln* MO injection. Total RNA from control and *apln* MO injected embryos was extracted at 2 dpf. (B) The partial KD embryos of Apln by MO injection had the normal body morphology and vascular lecture at 4 dpf. (C) Heart beating rate of control and *apln* MO injected embryos were counted at 4 dpf. Experiments were triplicated. ISV, inter-segmental vessel; DA, dorsal aorta; CV, cardinal vein. Scale bar is 400 µm.
Figure III. Lymphatic function is compromised in zebrafish embryos with reduced Apelin/Aplnr signaling

Micro-lymphangiography was performed to determine function of lymphatic vessels in apln or aplnra MO-injected embryos. Briefly, FITC-dextran (wt 2,000,000) was injected to the subcutaneous space into control (right), apln (middle) or aplnra (right) MO-injected embryos. MOs were injected at 1-2 cell stage and FITC-dextran was injected at 4.5dpf. Embryos were imaged after one hour of FITC-dextran injection. While control MO-injected embryos readily absorb FITC-dextran, apln or aplnra MO-injected embryos failed to do so, indicating lack of lymphatic function in these embryos. ISV, inter-segmental vessel; DA, dorsal aorta; CV, cardinal vein; TD, thoracic duct. Scale bar is 50 µm.
Figure IV. The phenotypes of Apln over-expression. (A) Representative images for the phenotype of the Apln overexpression by synthetic *apln* mRNA injection. Embryos were injected synthetic *apln* mRNA at 1-2 cell stage and pictured at 48 hpf. Arrows point the cardiac edema. (B) Percentage of embryos having the cardiac edema after the injection of *apln* mRNA was counted at 48hpf. Total experimented embryos were gathered from 3 individual experiment set.
Figure V. Dose and time dependent activation of AKT1/2 by Apelin stimulation in human lymphatic endothelial cells (hLECs) (A) Dose dependent induction of phospho-AKT1/2 by Apelin ligand. hLECs were treated with incremental dose of Apelin after 1 day of starvation, and phospho-AKT1/2, total AKT1/2, and GAPDH were detected after 1 hour of Apelin treatments. (B) Time course of Apelin mediated activation of phospho-AKT1/2. hLECs were treated with 1µM Apelin after 1 day starvation, and phospho-AKT1/2, total AKT1/2, and GAPDH were detected at 15, 30, 60, 180, and 360 minutes after Apelin stimulation. (C) Validating the effects of APLNR siRNA in hLECs.
Figure VI. Expressions of Apelin and Vegfc signaling components. (A) The vegfc and vegfr3/flt4 expressions in Apln KD embryos were evaluated by quantitative real-time PCR at 48hpf. The vegfc and vegfr3/flt4 were not changed by Apln KD in zebra fish embryos. (B) The apln, aplnra and aplnrb expressions in Vegfc KD embryos were evaluated by quantitative real-time PCR at 48hpf. These genes were not changed by Vegfc KD in zebrafish embryos. (C) Expression of aplnra and apelin at late developmental stages detected whole mount in situ hybridization. At 48hpf, aplnra is expressed in cardinal vein (CV), while aplin is strongly expressed within somites (sm). After 72hpf, expression of both genes are difficult to detect, possibly due to the technical limitations.