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Essential Role of Apelin Signaling during Lymphatic Development in Zebrafish

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Abstract

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 (BECs) and lymphatic endothelial cells (LECs) in vertebrates. In this report, we aim to delineate the functions of Apelin signaling during lymphatic development.

Approaches 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 (PaCV) and the number of LECs 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 AKT1/2 phosphorylation without affecting the phosphorylation status of ERK1/2. Moreover, lymphatic abnormalities caused by the reduction of Apelin signaling were significantly exacerbated by the concomitant partial inhibition of AKT signaling. Apelin and Vascular Endothelial Growth Factor-C (VEGF-C) signaling provide a non-redundant activation of AKT during lymphatic development, as over-expression 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 AKT activity in a VEGF-C/VEGFR3 independent manner during zebrafish embryogenesis.

Keywords

Apelin signaling; Lymphatic development; AKT; Zebrafish

INTRODUCTION

Lymphatic vessels have essential roles in maintaining the homeostasis of interstitial body fluid and facilitating immune responses in vertebrates [1–3]. In addition, lymphatic vessels

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DISCLOSURES
None

have been associated with progression of diverse diseases in humans, including tumor metastasis and obesity [4, 5]. 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 more than 140 million people worldwide [6–9]. During development, lymphatic endothelial cells (LECs), the main component of lymphatic vessels, first appear around E10 in mice, embryonic week 6 to 7 in humans, and 3 days post-fertilization (dpf) in zebrafish [10–13]. 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 endothelial cells (ECs) in the cardinal vein [12, 14, 15].

Apelin and its receptor Aplnr/Apj regulate a wide range of developmental and physiological processes [16–19]. While Apelin is widely expressed during development, the expression of Aplnr/Apj is more restricted [16]. In mouse, *Apj* is strongly expressed within the cardiovascular system as early as E8.0 [20]. Similarly, *aplnra* and *b* are specifically expressed in developing ECs and cardiomyocytes in zebrafish [17, 19]. Consistent with the expression pattern, *Apj* knockout mice display various cardiovascular defects and are embryonic lethal in certain genetic background [20–22]. Zebrafish have two Apelin receptors, *aplnra* and *aplnrb* [17, 19]. While *aplnra* and *b* 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 [19, 23]. After 24 hours post-fertilization (hpf), only venous endothelial cells in the trunk region express detectable levels of *aplnra* [23], suggesting that Apelin signaling may be essential for blood (BECs) and lymphatic endothelial cells (LECs). Mutations in *aplnrb* cause similar developmental defects in zebrafish [17, 19]. Despite its proposed role in cardiovascular development, current analyses on Apelin signaling predominantly aim to address its role in vascular physiology [16, 24–26], 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 non-redundant function to induce 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 VEGF-C signaling. Our data presented here support the idea that Apelin signaling is essential for proper lymphatic development.

Materials and Methods

Please see supplemental file.

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 (MO) mediated knock-down of *aplnra* (Figure IA-D in the online-only Data Supplement). Since proper circulation is essential for lymphatic development, a suboptimal dose of *aplnra* MO was injected (1.8ng/embryos) to avoid the early cardiac abnormalities that have been previously reported [17, 19] and assess the role of Apelin signaling specifically in lymphatic development (Fig. S1D). The general morphology and the rate of heartbeat at 4dpf in embryos injected with 1.8ng of *aplnra* MO were comparable to those of control MO-injected embryos (Fig. 1A, Figure IB and IC in the online-only Data Supplement). In addition, patterning of inter-segmental vessels (ISVs), the

dorsal aorta, and the cardinal vein were not affected in 4dpf embryos injected with 1.8ng of *aplnra* MO (Fig. 1A).

Although the vasculature was unaffected in *aplnra* MO-injected embryos, these embryos did contain profound defects in lymphatic vessels (Fig. 1B and 1C). To visualize developing LECs, we utilized *Tg(fli1a:nEGFP);Tg(kdrl:mCherry)* double transgenic lines, which contain nEGFP^{+}/mCherry^{+} BECs (appearing as yellow in the merged figures) and nEGFP^{+}/mCherry^{-} LECs. While the developing thoracic duct, located between the dorsal aorta and the cardinal vein, are clearly visible in 4dpf control MO-injected embryos, similar structures were absent in *aplnra* MO-injected embryos (Fig. 1B). In addition, the number of nEGFP^{+}/mCherry^{-} LECs within the thoracic duct in *aplnra* MO-injected embryos was greatly decreased (Fig. 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 *Apj*$^{-/-}$ 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* MO-injected embryos are not secondary to the previously reported cardiac defects [17, 19], the amount of MO was titrated. Injection of 2.7 or 5.4ng 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; PaCV) which give rise to the presumptive LECs, were severely affected by partial knock-down of *apln* (Fig. 2A and 2B). At 3dpf, embryos injected with 2.7ng of *apln* MO completely lacked PaCV. Consistent with the defects in PaCVs, we found that the number of LECs in 4dpf *apln* MO-injected embryos was decreased in a dose-dependent manner (Fig. 2C and 2D). Consistent with these findings, micro-lymphangiography demonstrated that embryos with a reduced level of Apelin signaling do not develop proper lymphatic vessels (Figure III in the online-only Data Supplement).

Since 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 4dpf. While the number of LECs was substantially decreased by injecting a low concentration of *apln* MO (0.9 and 1.8ng/embryo), the number of venous ISV connections was largely unaffected (Fig. 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* MO injected embryos is directly caused by the attenuation of Apelin signaling, we attempted a phenotypic rescue of *apln* MO-injected embryos by injecting synthetic *apln* mRNA. Since *apln* mRNA injection can cause cardiac defects [17], the amount of mRNA was titrated. While a high dose of *apln* mRNA caused severe cardiac defects, injection of 8pg *apln* mRNA per embryo did not cause any phenotypic defects (Figure IVA and IVB in the online-only Data Supplement). When co-injected, 8pg *apln* mRNA successfully restored the number of LECs in *apln* MO injected embryos (Fig. 2G and 2H), suggesting that the reduction in the number of LECs in *apln* MO injected embryos was not a secondary effect of general developmental delays

caused by MO 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 [25]. Consistent with the previous report, knockdown of *APLNR* in human LECs (hLECs) adversely affected the migration of human LECs (hLECs). In a scratch wound assay, *APLNR* siRNA-treated hLECs displayed a significantly attenuated migratory behavior compared to control siRNA-treated hLECs (Fig. 3A and B). The migration defect of *APLNR* siRNA-treated hLECs was likely caused by a reduced level of Apelin signaling since addition of exogenous Apelin 13 was not able to alleviate the migration defects in *APLNR* siRNA-treated hLECs (Fig. 3A and B).

To identify the downstream effectors of Apelin signaling in LECs, we examined the level of phospho-AKT1/2 and phospho-ERK1/2 in *APLNR* siRNA-treated hLECs (Fig. 3C). Since 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 downstream of Apelin signaling during lymphatic development. While the level of phospho-ERK1/2 did not change in *APLNR* siRNA-treated hLECs compared to control siRNA-treated cells, the level of phospho-AKT1/2 was significantly decreased upon APLNR depravation (Fig. 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 MO injection drastically reduced the level of phospho-AKT1/2 in developing zebrafish. While phospho-AKT1/2 is strongly detected within ECs in the dorsal aorta and cardinal vein of 48hpf control MO-injected embryos, it is largely absent in *apln* MO-injected embryos (Fig. 3F). Therefore, it appears that Apelin signaling may function as a major stimulus for AKT1/2 phosphorylation in both cell culture and *in vivo*. Previously, Aplnrs have been reported to function independent of Apelin ligand [18]. Therefore, we also examined the level of phospho-AKT1/2 in *apln* MO-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 (Fig. 3F).

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. Since manipulation of AKT activity at earlier developmental stages may compromise the specification of arterial and venous ECs [29–31], we utilized previously reported chemical antagonists of AKT to induce temporal AKT inhibition. Treatment with either 10μM LY294002 (Phosphoinositide 3-Kinase (PI3K) inhibitor) [32] or 2μM Torin1 (mTORC1/2 inhibitor) [33], both of which inhibit AKT phosphorylation, drastically reduced the number of LECs in 4dpf zebrafish embryos (Fig. 4A–4D). Moreover, the lymphatic defects caused by a partial reduction of Apelin signaling activity was significantly exacerbated by administering a suboptimal dose of the aforementioned chemical antagonists of AKT (Fig. 4E–4G). While embryos injected with 0.9ng of *apln* MO or treated with 5μM of LY294002 contained an average of 5.6±0.65 and 6.2±0.79 LECs respectively, embryos that were injected with 0.9ng of *apln* MO and treated with 5μM of LY294002 had significantly fewer LECs (3.8±1.11), representing a further ~40% reduction the in number of LECs compared to single manipulations (Fig. 4E and 4F). Similar effects were also observed in embryos that were injected with 0.9ng of *apln* MO and treated with 1μM of Torin1 (~40% reduction; Fig. 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 [34–36], which is the key stimulus for lymphatic development [37, 38]. We next tested whether Apelin and VEGF-C signaling converge at the level of AKT or function redundantly to promote differentiation and/or 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, co-injection of *apln* and *vegfc* MO with suboptimal doses exacerbated the lymphatic phenotypes (Fig. 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 injection was unable to alleviate the lymphatic defects in *vegfc* MO injected embryos (Fig. 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 (Fig. 5E and 5F). The inability of *apln* mRNA to alleviate the defects of *vegfc* MO-injected embryos and *vegfc* mRNA to *apln* MO-injected embryos collectively suggest that Apelin and Vegfc signaling may function in a non-redundant manner in lymphatic development.

Considering that both Apelin and Vegfc signaling can activate AKT to induce lymphatic development, but appear to have non-redundant functions, it is possible that Vegfc and Apelin signaling may have distinct functions; while 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*, *aplnra*, *vegfc*, and *vegfr3* within the first five days of development in zebrafish embryos (Fig. 6A). While the expression level of *vegfc* gradually increased and then stabilized, the expression level of *vegfr3* precipitously dropped by 2dpf, indicating that the activity of Vegfc-Vegfr3 signaling may reduce to the basal level when LECs emerge from venous ISVs (Fig. 6A). In contrast, the expression of *apln* and *aplnra* continuously increased during the first five days of development (Fig. 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 [16, 25, 26], it is likely 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 [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 [37, 39]. In addition, Kaposi's sarcoma associated herpesvirus (KSHV) activates AKT to induce ectopic lymphatic structures [40, 41]. Although AKT can be activated by diverse signaling inputs [42, 43], attenuating Apelin signaling substantially decreased the phosphorylation of AKT in LECs, both in cell culture and developing zebrafish embryos. Therefore, it appears 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 pro-lymphangiogenic signaling

pathway [34, 36, 39], to maintain the proper level of AKT activity in LECs. During development, components of Apelin and Vegfc signaling appear to be expressed at distinct stages, suggesting that these two pro-lymphangiogenic signaling pathways may activate the same downstream effectors, but their activity may be temporally separated during development (Fig. 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 (Fig. 5E) were not sufficient to restore the number of LECs in zebrafish embryos with compromised Apelin signaling. Therefore, it is likely that the pro-lymphangiogenic role of Apelin signaling may be transduced by additional effectors. ERK1/2, which are known to be activated by Vegfc signaling in LECs [35, 36], do not seem to mediate Apelin signaling in LECs, since a lack of Apelin signaling did not affect the phosphorylation status of ERK1/2 in hLECs (Fig. 3C). Ongoing work in the lab 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations

AKT	Serine–threonine kinase Akt/Protein kinase B
BEC	Blood vessel endothelial cell
dpf	days post-fertilization
ERK	Extracellular signal regulated kinase
HM	Horizontal myoseptum
hLEC	Human lymphatic endothelial cell
LEC	Lymphatic endothelial cell
MO	Morpholino
mTORC1/2	mammalian target of rapamycin complex 1 and 2
PaCV	Parachordal vessel
PI3K	Phosphoinositide 3-kinase

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SIGNIFICANCE

Despite importance of Apelin/APJ signaling in diverse biological processes, its role during lymphatic development is relatively unknown. We find that Apelin/APJ signaling is essential to induce and maintain phospho-AKT1/2 level in lymphatic endothelial cells therefore, positively regulates lymphatic development. Moreover, Apelin/APJ signaling may synergize with VEGF-C signaling to promote lymphatic fate.

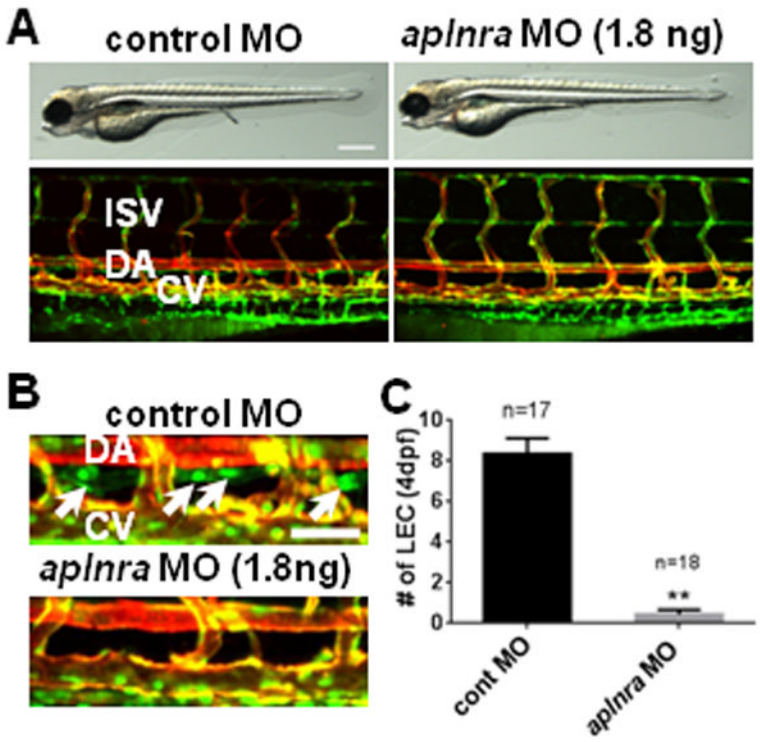

Fig. 1. Lack of *aplnra* activity causes lymphatic vessel defects

(**A**) Gross morphology (top panels) and vascular structures (bottom panels) in 4dpf *aplnra* MO-injected embryos. (**B**) The loss of LECs in thoracic duct (white arrows) in 4dpf *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 BECs (shown as yellow) and LECs (shown as green). DA, dorsal aorta; CV, cardinal vein; ISV, inter-segmental vessel. Scale bars are 400μm (A) and 50μm (B).

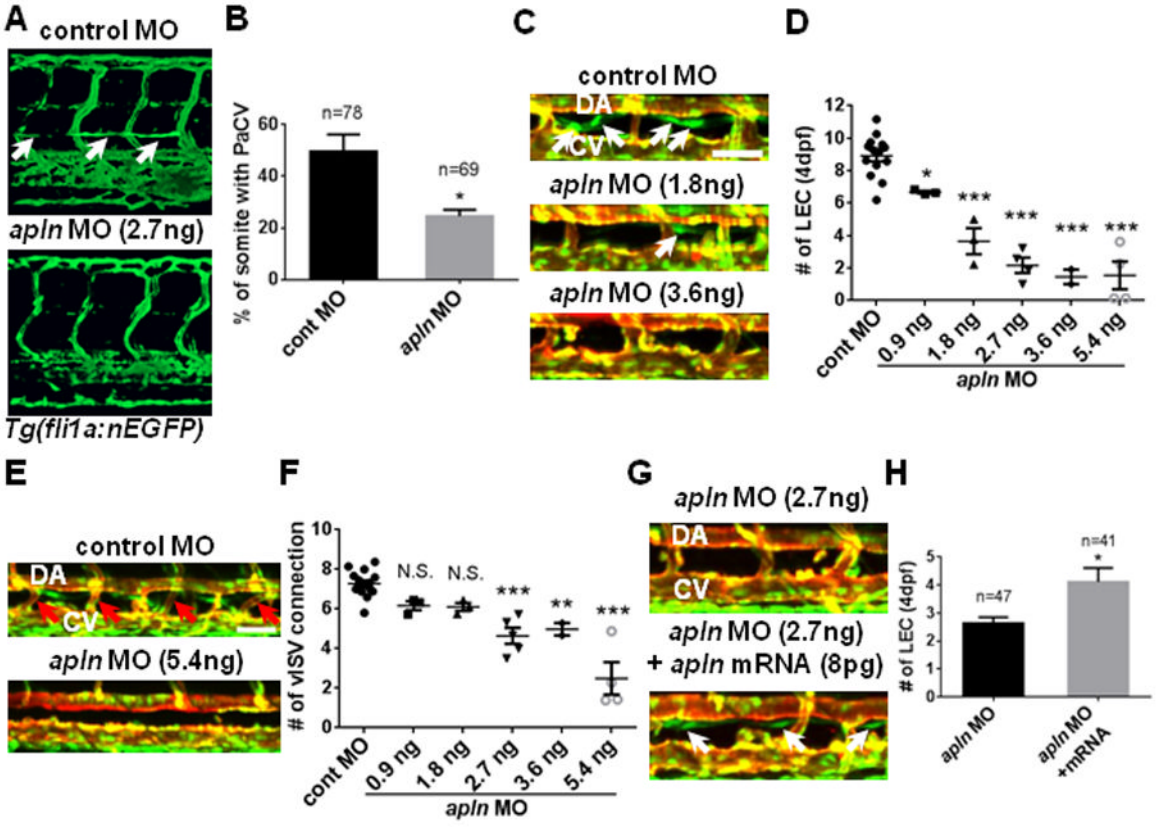

Fig. 2. Apelin is required for zebrafish lymphatic vessel formation

(**A**) The loss of parachordal vessel (white arrows) in 3dpf *apln* MO-injected embryos in *Tg(fli1a:nEGFP)* transgenic background. (**B**) Quantification of the formation of parachordal vessel (PaCV) at 3dpf. (**C**) Representative images of 4dpf *apln* MO-injected embryos in the trunk region. White arrows point to LECs within the thoracic duct. The deficit in the number of LECs progressively worsened as the dose of *apln* MO increases. (**D**) Quantification of the number of LECs in control and *apln* MO-injected embryos at 4dpf. LECs located between 8th and 15th somites were counted. Each dot represents an individual set of experiments. Total number of embryos are; control MO=96, *apln* MO 0.9ng=26, 1.8ng=30, 2.7ng=37, 3.6ng=19, and 5.4ng=29. (**E**) The loss of venous ISVs (red arrows; vISV) in 4dpf embryos injected with a high dose of *apln* MO. (**F**) Quantification of the number of vISVs in control and *apln* MO-injected embryos. Each dot represents an individual set of experiments. Total number of embryos are; cont MO=103, *apln* MO 0.9ng=26, 1.8ng=30, 2.7ng=48, 3.6ng=19, and 5.4ng=27. (**G**) Over-expression of synthetic *apln* mRNA can rescue lymphatic defects in *apln* MO-injected embryos. White arrows point to LECs in embryos co-injected with synthetic *apln* mRNA and *apln* MO. (**H**) Quantification of the number of LECs at 4dpf in embryos injected with *apln* MO alone and in embryos co-injected with *apln* MO and synthetic *apln* mRNA. DA, dorsal aorta; CV, cardinal vein. Scale bars are 50μm.

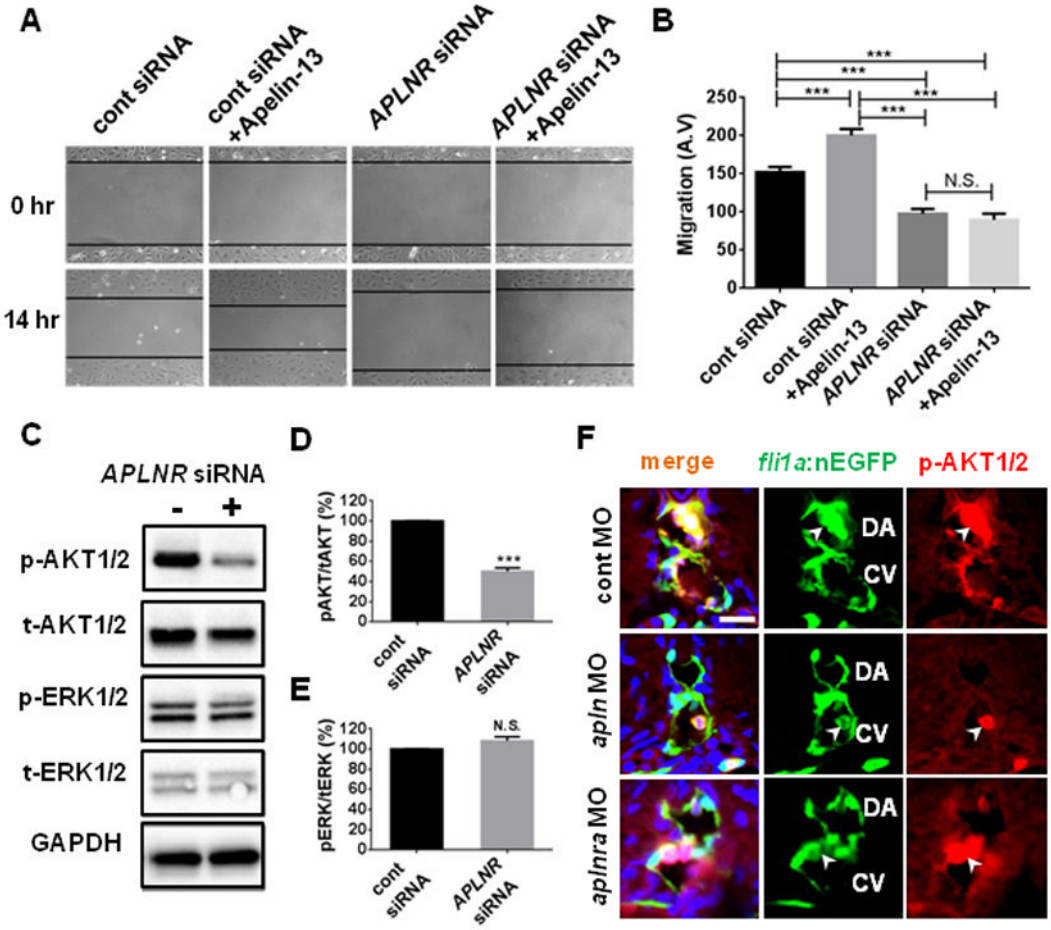

Fig. 3. Apelin signaling is essential for hLEC migration and maintenance of phospho-AKT1/2

(**A**) Representative images of scratch wound healing assay using human LEC (hLEC). Black lines demarcate the boundaries of scratch wounds. The recovery of the wound was substantially attenuated in *APLNR* siRNA-treated hLECs compared to control siRNA-treated hLECs. (**B**) Quantification of the migration of hLECs. (**C**) Lack of Apelin signaling selectively attenuated the level of phospho-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 phospho-AKT1/2 and total AKT in control or *APLNR* siRNA-treated hLECs. (**E**) Quantification of the levels of phospho-ERK1/2 and total ERK1/2 in control or *APLNR* siRNA-treated hLECs. (**F**) Confocal image showing a transverse section of 48hpf control MO (top), *apln* MO (middle), or *aplnra* MO (bottom) injected *Tg(fli1a:nGFP)* embryos. Developing endothelial cells (EC) within DA and CV contain a high level of phospho-AKT1/2 (arrowheads). Lack of Apelin signaling selectively abrogated the presence of phospho-AKT1/2 in ECs without affecting the phospho-AKT1/2 in nearby blood cells (arrowhead). Scale bar is 10μm.

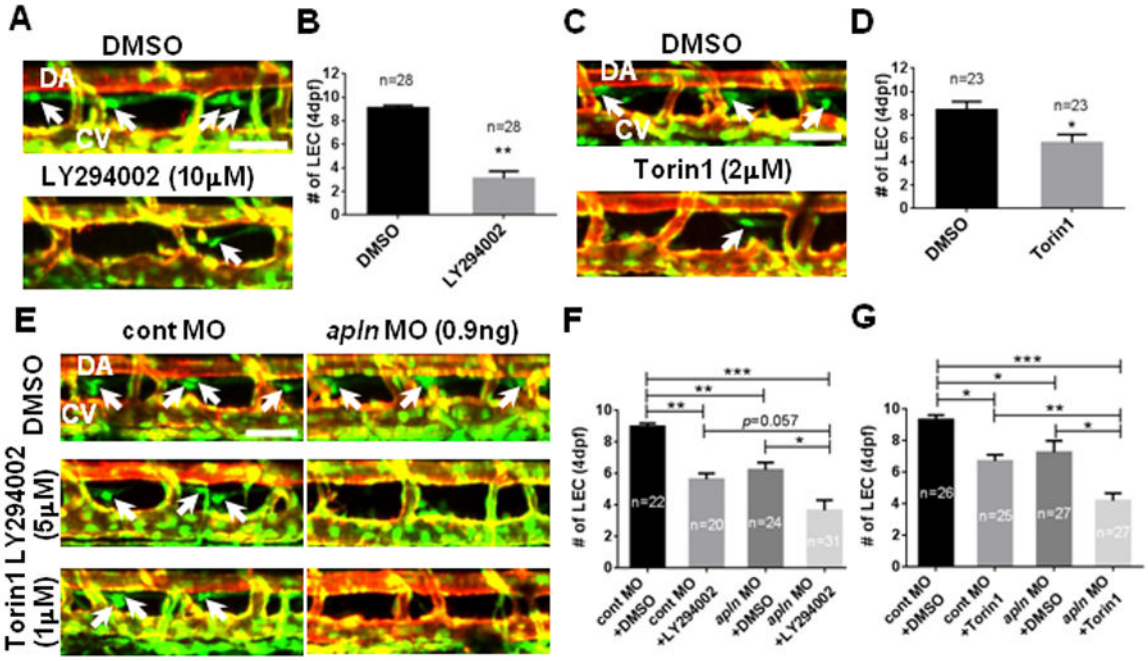

Fig. 4. Apelin and AKT cooperate to promote lymphatic development in zebrafish

(**A**) The number of LECs (white arrows) was substantially decreased in embryos treated with a chemical inhibitor of PI3K (LY294002) between 48hpf and 4dpf, therefore, with an attenuated level of AKT activity. (**B**) Quantification of the number of LECs in 4dpf DMSO or 10μM LY294002 treated embryos. **(C)** The number of LECs (white arrows) in embryos treated with Torin1 between 48hpf and 4dpf, a chemical antagonist against another upstream regulator of AKT, mTORC1/2, was significantly decreased. (**D)** Quantification of the number of LECs in 4dpf DMSO or 2μM Torin1 treated embryos. (**E**) Apelin and AKT may function within the same pathway. Treating embryos injected with a low dose of *apln* MO with a suboptimal dose of LY294002 (5μM) or Torin1 (1μM) exacerbated the effects of *apln* MO on the number of LECs (arrows). (**F**) Quantification of the number of LECs in control or *apln* MO injected embryos treated with either DMSO or LY294002. (**G**) Quantification of the number of LECs in control or *apln* MO injected embryos treated with either DMSO or Torin1. DA, dorsal aorta; CV, cardinal vein. Scale bars are 50μm.

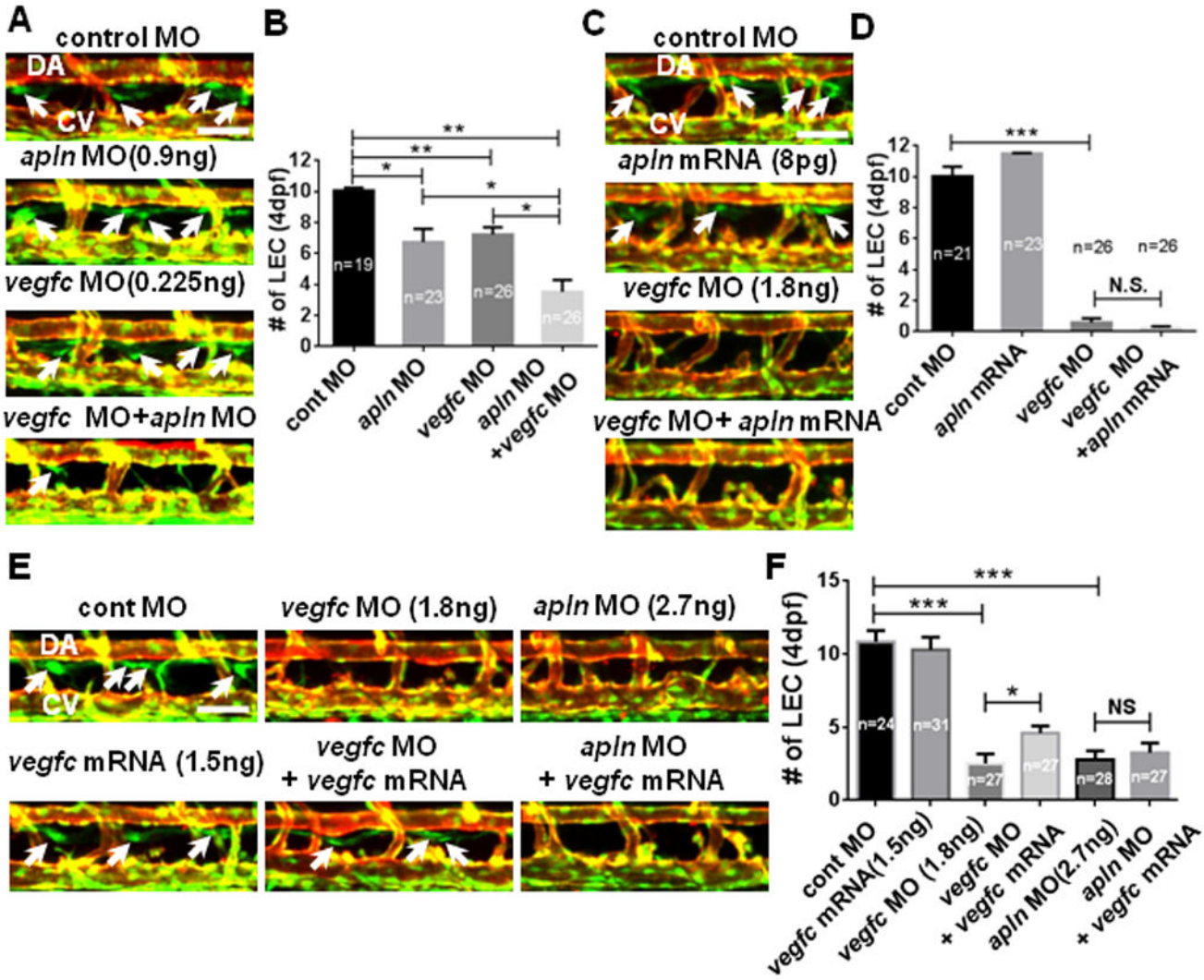

Fig. 5. Apelin and Vegfc signaling are not redundant but functionally synergize to facilitate lymphatic development in zebrafish

(**A**) Apelin and Vegfc signaling may synergistically promote lymphatic development. 4dpf embryos co-injected with low doses of *apln* and *vegfc* MOs contained a substantially decreased number of LECs (arrows) compared to control or single MO injected embryos. (**B**) Quantification of the number of LECs. (**C**) Apelin signaling may have a non-redundant role with Vegfc signaling during LEC development. Over-expression of Apelin by mRNA injection did not alleviate lymphatic defects in 4dpf *vegfc* MO-injected embryos. (**D**) Quantification of the number of LECs. (**E**) Representative images for the lymphatic vessel phenotype in 4dpf *Tg(fli1a:nEGFP);Tg(kdrl:mCherry)* embryos injected with *apln* and *vegfc* MOs and *vegfc* mRNA. *vegfc* mRNA injection at 1–2 cell stage only rescues the defects caused by Vegfc knockdown, but not Apln knock-down. (**F**) Quantification of LECs. DA, dorsal aorta; CV, cardinal vein. Scale bars are 50μm.

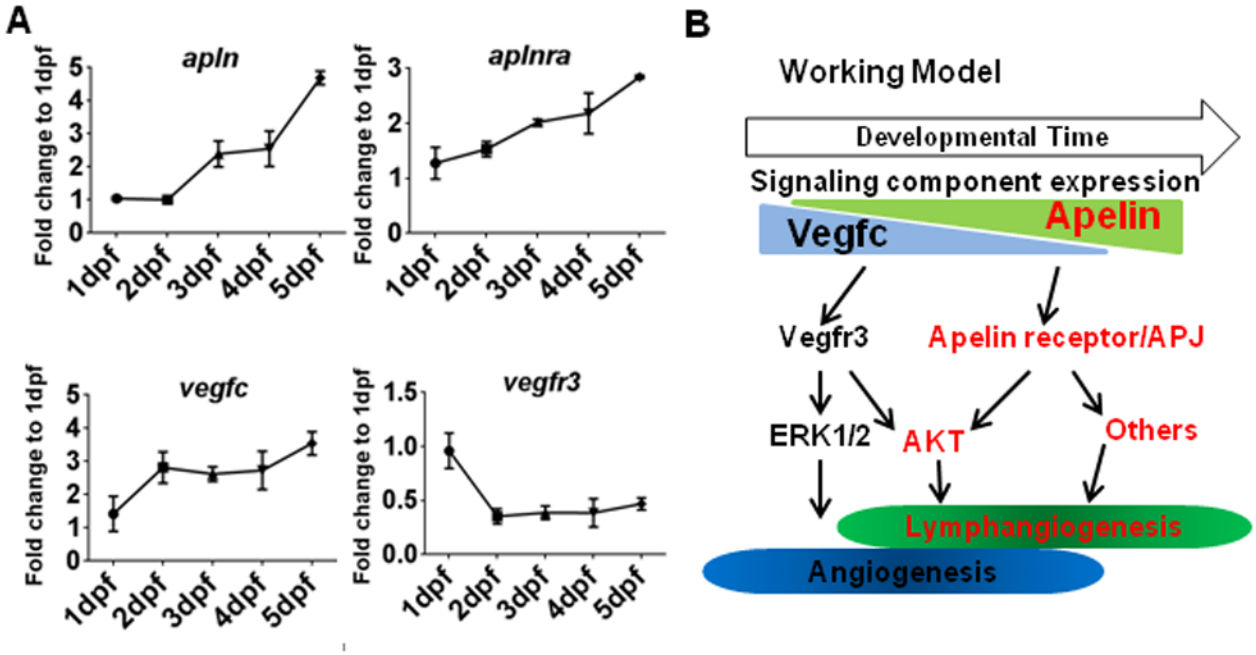

Fig. 6. Apelin and Vegfc signaling is temporally separated for lymphatic vessel formation in zebrafish

(**A**) Expression profiles of *apln*, *aplnra*, *vegfc*, and *vegfr3* during the first five days of zebrafish development, normalized to *actb1*. The expression of *apln* and *aplnra* gradually increased over time, while the expression of *vegfc* and *vegfr3* is either constant or attenuated. (**B**) Schematic working model. Apelin and Vegfc signaling appear to have non-overlapping functions during lymphatic development. While functionally redundant in activating AKT, Apelin and Vegfc signaling may be temporally separated and seem to activate specific sets of downstream effectors.