國立臺灣大學生命科學院生命科學系

碩士論文

Department of Life Science

College of Life Science

National Taiwan University

Master Thesis

微核醣核酸(*miR-19c*)在斑馬魚血管發育扮演角色之探討

The role of *miR-19c* in zebrafish vessel development

廖穎嫻

Ying-Hsien Liao

指導教授：李士傑 博士

Advisor: Shyh-Jye Lee, Ph.D.

中華民國 104 年 6 月

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口試委員會審定書

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本論文係廖穎嫻君（學號 R02B21005）在國立臺灣大學生命科學系、所完成之碩士學位論文，於民國 104 年 6 月 10 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

碩士班生活隨著論文的完成也即將告一段落，就像是學習長泳的一段過程。起初從斷斷續續的五百公尺就氣喘吁吁急著上岸，到不中斷也不太累持續往上加的一公里、兩公里，也並非一下子節節高升這麼容易，有的只是累績的一些自己的東西罷了。過程中，可以學習很多，而成長幅度更是超過自己的想像。當中或許會有令人喘不過去的呼吸聲、不預期喝了可怕的泳池水、莫名被重踢的無影腳，但這些其實都不需在意。另外也發現到了，游泳的速度、快慢無從與任何人比較，必須有著你自己的步調繼續哪怕只是一百公尺的進步，最後發現累積的這些小小公里數會送給你許多可愛的果實。

兩年來，謝謝我最親愛的爸爸、媽媽和姐姐與其他家人們，毫不保留的給了我好多愛、支持與鼓勵，看到你們高興得說著與我為榮是我持續努力下去的動力。另外也非常感謝在實驗室中教導與幫助我的老師、學長姐、同學及學弟妹，一起討論各種學術問題都是很開心、很充實的時光。另外還有許多站在岸上真心為我加油、喝采，珍貴珍藏的朋友們，謝謝你們。

最後，我想最寶貴的，是莫過於自己的一點成長及進步，或許還不夠好不夠多，但我會帶著這些豐富的果實繼續在未來努力，保持我的速度游下去， 然後迎向下一次的上岸。

中文摘要

血管及淋巴管是循環系統的基本構造，但其在脊椎動物之研究常因皮膚及其他阻隔而增加其困難度。斑馬魚由於其胚胎透明、並有幾種血管螢光魚品系易於研究，近年來已成為血管新生(angiogenesis) 及淋巴管新生(lymphangiogenesis)的主流模式動物之一。微核醣核酸(miRNAs)在血管新生及淋巴管新生中扮演重要角色，前人研究發現幾種 miRNAs 在斑馬魚血管內皮細胞高量表現，並發現其中之一 *miR-221* 在血管新生成有其功能，但其他 miRNAs 所扮演角色則仍未知。在本論文中，我重複前人試驗發現在利用流式細胞儀分選出的內皮細胞中，一些 miRNAs 的表現量於產卵後 22-48 小時有顯著的變化。其中 *miR-19c* 被發現在 22-48 小時有顯著的上升情形，由已知的研究中知道這個時間點與血管和淋巴管發育皆有所相關，所以我大膽假設 *miR-19c* 可能參與血管發育或淋巴管發育。為了釐清這個問題，我以血管螢光轉基因斑馬魚為材料，利用 morpholino/miRNA 抑制劑與 miRNA 模擬物(mimic)分別抑制或增加 *miR-19c* 的表現來探討 *miR-19c* 在斑馬魚發育過程中所扮演的角色。我發現增加 *miR-19c* 的表現會造成淋巴血管系統缺陷，但不管是抑制或增加 *miR-19c* 的表現皆不明顯影響血管系統。利用微核醣核酸目標預測資料庫(Target Scan)與定量即時聚合酶鏈鎖反應(real-time PCR)，我發現 transforming growth factor beta 1a (*tgfβ1a*)為 *miR-19c* 的目標基因之一，且增加 *miR-19c* 表現可降低 *tgfβ1a* 表現量，但 *tgfβ1a* 在淋巴管新生成之功能則尚待釐清。總體而言，本研究結果顯示，*miR-19c* 可能經由 *tgfβ1a* 來影響斑馬魚淋巴管的發育。

關鍵字：斑馬魚、血管新生發育、淋巴管新生發育、微核醣核酸、*miR-19c*

Abstract

Vessel formation, including angiogenesis and lymphangiogenesis, paves the structural foundation of circulation. The study of vessel formation in vertebrates often hinders by the invisibility, but the availability of transparent zebrafish embryos greatly accelerates our understanding in the field. With the addition of several vessel reporter lines, zebrafish has become one of the popular models to explore the regulatory mechanisms of angiogenesis and lymphangiogenesis. microRNAs (miRNAs) have been reported to be involved in regulating angiogenesis and lymphangiogenesis. In particularly, several miRNAs have been shown to be enriched in blood endothelial cells. However, except *miR-221*, the functional roles of other miRNAs remain unknown. Here, I first validated the enrichment of those miRNAs in endothelial cells from embryos at 22-48 h post fertilization. Interestingly, I found one of miRNAs, *miR-19c*, increases notably during 22-48 hpf. This time is associated with blood vessel and lymphatic vessel formation, I hypothesized that *miR-19c* may regulate blood or lymphatic vessel formation.

I performed loss and gain of function analyses by applying antisense morpholino/miRNA inhibitor and miRNA mimic against *miR-19c* to different transgenic zebrafish lines expressing GFP. I observed that overexpression of *miR-19c*

impaired lymphatic development, but no significant change in blood vascular system was observed in embryos. Transforming growth factor beta1a (*tgfβ1a*) is a potential target of *miR-19c* and known lymphangiogenesis regulator. I found significant reduction of the expression of *tgfβ1a* in embryos injected with *miR-19c* mimic. The functional link between *miR-19c* and *tgfβ1a* still needs to be investigated. Collectively, I conclude that *miR-19c* is indispensable to lymphatic development in zebrafish possibly via *tgfβ1a*.

Keywords: Angiogenesis, Lymphangiogenesis, Zebrafish, miRNAs, *miR-19c*

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Introduction

microRNA (miRNA) is a small non-coding RNA with 21-24 nucleotides in plants, animals and some viruses. They play critical roles in post-transcriptional modification to regulate RNA silencing and gene expression (Ambros, 2004; Wienholds et al., 2005). Mature miRNAs bind the 3' untranslated region (UTR) of target mRNAs, resulting in mRNA decay and/or translational blockage (Ambros, 2004; Kim et al., 2009). More and more studies indicate that miRNAs are potent regulators of genome. They have known functions in embryonic development, cell proliferation, cell differentiation, apoptosis, stress response, metabolism, several diseases and organogenesis, including cardiovascular development (Filipowicz et al., 2008; Fish et al., 2008; He et al., 2014; Jones-Rhoades and Bartel, 2004; Zhao and Srivastava, 2007).

miRNAs in vascular development

By deep sequencing, several miRNAs have been found to be enriched in blood endothelial cells (BECs) in zebrafish. Functional assay further revealed that knockdown of *miR-221* exhibits similar defects like loss of the tip cell-expressed gene *Flt4*. It suggests *miR-221* is essential for angiogenesis (Nicoli et al., 2012). The roles

of other BECs-enriched miRNAs, including *miR-19c*, *miR-181c* and *miR-20b* remain unclear. miRNAs can facilitate integration of a physiological stimulus with growth factor signaling in BECs to guide angiogenesis (Wang et al., 2008). Thus, it is worthwhile to study the functional roles of those miRNAs in vascular development.

In addition to their regulations on angiogenesis, miRNAs have also been reported to function in lymphatic vessel development. In *Xenopus* and zebrafish, the increase in *miR-31* causes defects in venous sprouting and lymphatic vascular development, thus indicating a pivotal role of *miR-31* as a negative regulator of lymphangiogenesis (Pedrioli et al., 2010). *miR-34a*, an inducer of apoptosis and cell cycle arrest, inhibits lymphatic metastasis potential of mouse hepatoma cells (Guo et al., 2011). However, more evidences are required to solidify the role of microRNAs in lymphatic vascular biology.

Vascular formation in zebrafish

The vasculature is a pivotal system in vertebrates. The blood vascular system transports and exchanges life essential gas, nutrients, metabolic residues and other materials to different tissues. Lymphatic system develops in a parallel manner with blood vessels to regulate mainly immune functions by draining lymph containing immune cells from tissues, and to absorb lipids from the gut and body fluids. Both

blood and lymphatic vessels are mainly composed of endothelial cells. In detail, formation of the vascular system in the zebrafish embryos contains two waves of angiogenic sprouting (Isogai et al., 2003). BECs of the primary wave sprout from the dorsal aorta (DA) at 22 hours post fertilization (hpf) to form the segmental arteries (SA). These BECs grow dorsally, reach the dorsal neural tube and connect with their neighbors from anterior and posterior segments to form the dorsal longitudinal anastomotic vessel (DLAV). The second wave is the development of lymphatic endothelial cells (LECs), which come from the posterior cardinal vein (PCV) to form lymphatic vessel (Yaniv et al., 2006).

Lymphangiogenesis is a process of the formation of new lymphatic vessels from pre-existing lymphatics (Jeltsch et al., 2003). In zebrafish, lymphatic vessel development starts at 32 hpf (Yaniv et al., 2006). The lymphatic vessels can be divided into four parts: facial lymphatic network, intestinal lymphatic network, lateral lymphatic network and trunk lymphatic network (Okuda et al., 2012). Until now, the trunk lymphatic network was most widely studied, which consists of the thoracic duct, intersomitic lymphatic vessels (ISLVs) and dorsal longitudinal lymphatic vessels (DLLVs) (Kuchler et al., 2006). Due to its big size and better visibility, the thoracic duct has often been used as a model to study lymphangiogenesis (Hogan et al., 2009; Kuchler et al., 2006). The thoracic duct is connected to an existing SA and is

transformed into segmental veins (SV). They can also reach the horizontal myoseptum (HM) to form parachordal lymphangioblasts (PLs). Between 60 and 84 hpf, PLs migrate ventrally to give rise to the ventral part of the ISLVs or dorsally to form the DLLVs (Hogan et al., 2009; Yaniv et al., 2006). The majority of PLs eventually migrates away from the horizontal myoseptum and contributes to the lymphatic vasculature (Hogan et al., 2009).

Molecular mechanisms of lymphangiogenesis

Lymphangiogenesis is balanced between pro- and anti-lymphangiogenic factors to maintain its homeostasis. VEGF, HGF and FGF2 signaling are well known pro-lymphangiogenic factors (Shin and Lee, 2014). They affect collagen and calcium binding EGF domains 1 (*CCBE 1*), vascular endothelial growth factor C (*VEGFC*), prospero-related homeobox gene 1 (*PROX1*), lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*) to regulate lymphangiogenesis (Mulligan and Weinstein, 2014). VEGFC and it receptor VEGFR3 play a key role during multiple developmental stages of lymphatic vessel. The sprouting of LECs from veins is VEGFC-dependent. VEGFR3 is expressed by all endothelial cells, including in PROX1-positive lymphatic precursors and blood vessel precursors (Tammela et al., 2008). The VEGFR3 co-receptor neuropilin 2 (NRP2) modulates the signaling pathways that are

activated in response to VEGFC and VEGFD (Stacker et al., 2014). PROX1 is the major regulator of LECs fate and the most reliable marker of LEC identity (Wigle and Oliver, 1999). Its activity is crucial for the appearance of the LEC progenitors to leave the CV during lymphatic development (Hagerling et al., 2013; Yang et al., 2012). PROX1 and its regulators COUP-TFII and SOX18 drive LECs specification in mice. The cooperative control of early LEC fate induction is intriguing during this process. (Srinivasan and Oliver, 2011). *LYVE1* is one of the most specific and commonly used mammalian lymphatic endothelial markers. *LYVE1* orthologue has also been found in zebrafish (Flores et al., 2010; Oliver, 2004). In additional to those well-known pro-lymphatic genes, we have previously identified a novel player, lysophosphatidic acid receptor 1 (Lpar1), which is essential for lymphangiogenesis (Lee et al., 2008).

Interferon-γ (IFN-γ)and transforming growth factor β (TGF-β) are anti-lymphangiogenic factors (Shin and Lee, 2014). The TGF-β signaling pathway is existed in a variety of cell types to regulate growth, differentiation, migration, proliferation, adhesion and apoptosis. It is also involved in angiogenesis during development and maintenance of vascular integrity in adults (von Bubnoff and Cho, 2001). In a lymphedema model, suppression of *TGF-β* increases lymphatic vessel formation (Avraham et al., 2010; Clavin et al., 2008). Expression of LEC markers, including *LYVE-1* and *Prox1*, is inhibited by *TGF-β*, but is enhanced by a *TGF-β* type

I receptor inhibitor (Oka et al., 2008). The balance of pro-lymphangiogenic and anti-lymphangiogenic factors could thus make up the nature of lymphatic vessels in various development stages.

In this thesis, using quantitative polymerase chain reaction (qPCR) analysis I observed significant increase in the expression of several previous known BECs-enriched miRNAs in FACS-sorted endothelial cells from zebrafish embryos at 22-48 hpf. In particular, I found *miR-19c* was increased notably at 48 hpf. This time is associated with blood vessel and lymphatic vessel formation. Therefore, I focused on characterizing the roles of *miR-19c* in blood or lymphatic vessel formation. Injection of *miR-19c* mimic, which elevated *miR-19c* activity, caused over proliferation in lymphatic vessels in zebrafish embryos. In addition, the expression of a potential target gene, *tgfβ1a* was notably down-regulated in *miR-19c* mimic-injected embryos. It implies that *miR-19c* may inhibit *tgfβ1a* expression to allow lymphangiogenesis during zebrafish development.

Materials and Methods

Zebrafish maintenance and embryos collection

Zebrafish (*Danio rerio*), were maintained in 10 L tanks with 14 h/10 h day/night cycles at 28.5 ℃. Embryos were collected and washed after natural spawning, and then were cultured in 0.3x Danieau's buffer (1x Danieau's buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.6 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 5 mM HEPES, in double-distilled H_2O, pH 7.6) at 28.5 ℃ for all experiments.

Transgenic zebrafish lines

I used two transgenic lines, including *Tg(kdrl:EGFP)* and *Tg(kdrl:mCherry)* cross *Tg(SAGFF27C;UAS:GFP)* to observe blood and lymphatic vessel development, respectively.

Microinjection

I used the Nanoliter jnjector (World Precision Instrument Inc., Sarasota, Florida) to microinject different reagents at 2.3 nL into 1-cell stage embryos. The injected embryos were cultured at 28.5 °C in 0.3x Danieau's buffer until examination.

Fluorescence-activated cell sorting (FACS)

Embryos were dechorionated with 25 mg/ml pronase in 0.3X Danieau's buffer and rinsed in calcium free ringer's buffer (116 mM NaCl, 2.9 mM KCl, 5 mM HEPES, pH 7.2) for 15 min to remove yolk. Embryos were then transferred to pre-warmed protease solution with 200 mg/ml collagenase P (Roche, Basel, Switzerland), incubated at 28.5 ℃ for 15 min to dissociate cells. Upon cell dissociation, the reaction was terminated by adding stop solution (6X solution containing 30% fetal calf serum (FCS) and 6 mM $CaCl_2$ in phosphate-buffered saline (PBS)). Cells were centrifuged to discard supernatant, rinsed and adjusted cell density to 10×10^6 cells/ml in suspension medium (Leibovitz medium L-15, contains 0.3 mM glutamine, 0.8 mM $CaCl_2$, penicillin 50 U/mL / streptomycin 0.05mg/mL, 5 % FCS). Cells suspension was subjected to a flow cytometer (BD Biosciences, Franklin Lakes, NJ) to sort GFP positive and GFP negative cells, transferred separately to 250 L RNAzol RT (Molecular Research Center Inc. , Cincinnati, OH) and stored at – 80 ℃.

RNA isolation, cDNA preparation, RT-PCR and qPCR for miRNA analyses

Total RNAs were isolated from embryos by using RNAzol RT and reversely transcribed to cDNA by a miScript ll RT Kit (QIAGEN, Venlo, Netherlands). qPCR was operated using the miScript SYBR Green PCR Kit (QIAGEN, Venlo, Netherlands)

in a CFX96 Q PCR machine (Bio-Rad, Hercules, California). *β-actin2* was used as an internal control for normalization.

miR-19c manipulation

miR-19c morpholino oligonucleotides (MOs) were custom-made by Gene Tools (Philomath, OR) according to following sequences:

miR-19c MO: 5'-CGAGTTTTGCATGGATTTGCACAGC-3'

Ctrl MO : 5'-CCTCTTACCTCAGTTACAATTTATA-3'

miR-19c mimic were custom-made by QIAGEN (Venlo, Netherlands) according to following sequences:

miR-19c mimic: 5'-UGUGCAAAUCCAUGCAAAACUCG -3'

Allstar negative control siRNA, cat: 1027281 (QIAGEN ,Venlo, Netherlands) was used as a control dsRNA.

miR-19c hairpin inhibitor and its mismatched controlled were custom-made by Dharmacon (Lafayette, CO) according to following sequences of mature *miR-19c* :

5'-UGUGCAAAUCCAUGCAAAACUGA-3'

cel-miR-67 confirmed to have minimal sequence identity with miRNAs in human, mouse, and zebrafish was ordered as a negative control.

cel-miR-67, mature sequence:

5’-UCACAACCUCCUAGAAAGAGUAGA -3’.

All the above were dissolved in Milli-Q water to their respective stock concentrations and the stock solutions were diluted in 1x Danieau’s buffer containing 0.5% phenol red prior to use and kept at 4 ℃.

Niflumic acid (NA, Selleckchem, Houston, TX) was dissolved in DMSO to their respective stock concentrations and diluted to their desired working concentration before use.

RNA isolation, cDNA preparation and qPCR for gene expression analyses

Total RNAs were isolated as described previously. Genomic DNAs were removed from the isolated RNAs using DNA-Free kit (Life technologies, Waltham, MA). To synthesize single-strand cDNAs, 2 μg of total RNAs, oligo dT primers, dNTPs, RNase inhibitor, 5x M-MLV reaction buffer and M-MLV reverse transcriptase (Promega, Madison, WI) were applied to RT-PCR. qPCR primer were custom made by Genomics (Taipei, Taiwan) according to following sequences:

efnb2a-F	GCG ACT CTT TGT GGA GAT ATT AC
efnb2a-R	GTT CGT GGT GTT CCA GTA TAT G
hey2-F	TCC ACA TCC ACC ACA TCC
hey2-R	AAA CGC TCC CAC TTC AGT
vegfc-F	CAC CCT GCG TGT CTG TCT AC
vegfc-R	CCT TGC TTG ACT GGA ACT GTG AT
prox1b-F	AGA TGT GGA AGA AGT GAA GG
prox1b-R	CTA ATT GAA GTG CTT ATT GTT GTG

lyve1b-F	CAC TTC TCC TGT CTC CAC ATC
lyve1b-R	ATG GAG GGT TTA TTG TTG TTT GG
lpar1-F	GCCAGATCGACTCACTATACG
lpar1-R	GCGAATCAAACTTCATAACCACAA
tgfβ1a -F	AAT TGC GTC TTC GGA TCA AG
tgfβ1a -R	ACA AAG CGA GTT CCC AGA TA
cox2a-F	TTG TGA TCG ATG TGC AAC TTT GT
cox2a-R	GCA ACA GTT GAT TGG CAT TTG AAA

* F and R refer to forward and reverse primer, respectively.

qPCR was operated by using SYBR Green Supermix (Bio-Rad) and CFX96 (Bio-Rad). qPCR data were normalized to a housekeeping gene, *β-actin2*.

Whole-mount *in situ* hybridization

Desired stages of embryos were fixed in 4% paraformaldehyde (PFA) in PBS overnight and then transferred into 100% methanol to store at -20 ℃. Antisense digoxigenin (DIG) *miR-19c* locked-nucleic acid (LNA) modified DNA oligonucleotide probes were custom-made by Exiqon (Franklin Lakes, NJ). Expression signals were performed by hybridization of alkaline phosphatase-coupled anti-DIG antibody (Roche Applied Science) on to DIG group of specific probe. The whole-mount in situ hybridization procedures were described by Thisse et al. (1993). For cryosections, whole-mount in situ stained embryos were treated in PBS containing 30% sucrose overnight, and fixed in Optimal Cutting Temperature compound (OCT) embedding medium (Sakura, Tokyo) at -20 ˚C. Next, the samples

were cut into 10-m frozen sections by a CM 1900 rapid sectioning cryostat (Leica, Heidelberg, Germany) and attached to poly-L-lysine-coated slides (Electron Microscopy Science, Ft. Washington, PA). All photographs were taken by a Canon EOS7D (Canon, Tokyo, Japan).

Microscopic imaging

Images were record by an epi-fluorescence microscope (Leica DM 5000 B) or a confocal microscope imaging system (Zeiss LSM780) (Leica, Heidelberg, Germany).

Statistical analysis

All data values were presented as mean value ± standard deviation and analyzed by paired-sample student t-test in Microsoft Excel.

Results

Expression analysis of blood endothelial cell-enriched microRNAs

Nicoli et al. (Nicoli et al., 2012) showed that several miRNAs, including *miR-150, miR-221, miR-181c, miR-20b, miR-107* and *miR-19c*, are enriched in BECs of 24-hpf zebrafish embryos. Only one of those miRNAs, *miR-221*, was reported to be essential in angiogenesis. To further study the roles of those miRNAs in zebrafish vessel development, I used FACS to sort out GFP+ and GFP- cells and extended the qPCR expression analysis of GEP+ cells to 22-48-hpf embryos to repeat their experiments (Fig. 1A). As shown in Fig.1B-G, significant changes in expression of all miRNAs examined were observed as compared to their expression at 22 hpf. The rises in expression peaked at 24 or 48 hpf with a 3 to 100 folds increases. *miR-19c* increased notably during 22-48 hpf, a period which is critical for the construction of blood vessel and lymphatic vessel, so I hypothesized that *miR-19c* may regulate blood and/or lymphatic vessel formation. To test this hypothesis, I set out to examine the roles of *miR 19-c* in vessel formation.

Expression patterns of zebrafish *miR-19c*

To examine the spatial and temporal expression patterns of *miR-19c* during

development, I used a locked-nucleic acid (LNA)-modified DNA oligonucleotide probe of *miR-19c* to perform whole-mount *in situ* hybridization (WISH) in zebrafish embryos from 24 hpf to 5 days post fertilization (dpf). At 24 hpf, *miR-19c* was ubiquitously expressed in the entire body except yolk and its extension and strongly expressed in anterior mainly head region (Fig. 2A)**.** At 48 hpf, the anterior expression of *miR-19c* was less but more restricted to eyes and notochord (Fig. 2B). At 3 dpf, the anterior expression of *miR-19c* was recovered and distinct expression retained in notochord (Fig. 2C). At 5 dpf, the anterior and notochord expressions of *miR-19c* were still preserved. In addition, *miR-19c* transcripts were notably found in notochord, swim bladder and thoracic duct (Fig. 2D, E). Furthermore, I made frozen transverse sections of the 5-dpf zebrafish at the trunk region. It showed that *miR-19c* was expressed in notochord. *miR-19c* also heavily expressed in the peripheral of notochord, which could be parachordal lymphatic vessel (PLV) (Fig. 2F). These results suggest that *miR-19c* may play a role during lymphangiogenesis.

Overexpressing *miR-19c* affects lymphatic but not blood vessel development

To explore the role of *miR-19c* in vasculature development, I performed gain or loss of function analyses by applying miRNA mimic or antisense morpholino (MO)/ miRNA inhibitor against *miR-19c*, respectively. Overexpression of *miR-19c* by

injecting *miR-19c* mimic notably caused edema in 3-dpf to 5-hpf larva. Edema appeared in pericardial sac, abdominal cavity and dermis in a portion of *miR-19c* mimic -injected larva. The severity of edema was classified into slight, mild and severe categories compared to normal-looking embryos (Fig. 3A). The total percentage of embryos with edema was 15.4%, 22.2% and 52.8% in untreated, control mimic and *miR-19c* mimic-injected embryos, respectively. The percentages of embryos in each category are shown in Fig. 3B. To examine the effects of different treatments on the expression of lymphatic marker genes, I seperated treated embryos into two groups, severe/mild v.s. slight/normal and found no difference in expression of lymphatic marker genes. In addition, I also detected a nearly 3-fold increase of *cox2a* expression in *miR-19c*-injected embryos (Fig. 3C). The *cox2a* is a master inflammatory gene. I suspected that *miR-19c* mimic-induced defects might be due to inflammation. Therefore, I incubated embryos-treated without or with control or *miR-19c* mimic with 20 and 50 nM niflumic acid (NA) (Houston, TX), which is a *cox2a* inhibitor (Knauf and Mann, 1984), but found that edema could not be alleviated by blocking *cox2a* activity (Fig. 3D).

Edema can be due to blood vascular leakage or dysfunction of lymphatic vessels. To examine the blood vessel integrity, I injected *miR-19c* mimic to embryos of *Tg(kdrl:egfp)* transgenic zebrafish with EGFP labeled blood endothelial cells that

clearly marked all blood vessels (Alex et al., 2010). At 24 hpf, the untreated control embryos showed that blood endothelial cells sprout massively from the dorsal aorta (DA) to form segmental arteries(SA) and dorsal longitudinal anastomotic vessel (DLAV) (Fig. 4A) as previously reported (Isogai et al., 2003). I found no significant change in blood vascular patterning in embryos-injected with control or *miR-19c* mimic (Fig. 4B,C). Inhibiting *miR-19c* with antisense morpholino (MO) or miRNA inhibitor also failed to affect blood vasculature development except a slight delay in the formation of DLAV (Fig. 4D-G).

To examine whether *miR-19c* mimic-induced edema was due to its effect on lymphangiogenesis, I used embryos from the cross of two transgenic lines *Tg(kdrl:mCherry)* and *Tg(SAGFF27C;UAS:GFP)* (Bussmann et al., 2010) to examine the development of both blood (mCherry signal from *Tg(kdrl:mCherry)*) and lymphatic (EGFP signal from *Tg(SAGFF27C;UAS:GFP)*) vessels. No notable change in blood vasculature was observed in 5-dpf *miR-19c* mimic-injected embryos (Fig. 5A, mCherry). In contrast, thoracic duct and intestinal lymphatic network of *miR-19c* mimic-injected embryos were notably larger with higher fluorescent intensity compared to those of untreated and control mimic-injected embryos (Fig. 5A, EGFP and Merged). The enhancement of thoracic duct was more evident at a higher magnification (compare those representative 400X images in Fig. 5A). To further

examine the changes in vasculature, I applied 3D rendering function of the Imaris software (Oxford Instruments, Abingdon, UK) to analyze the assembled confocal images. The merged reconstructed 3D images clearly showed the expansion of lymphatic but not blood vasculature (Fig. 5B). To have better views in all angles, rotational movies were made (see supplementary Movie 1 and 2 for the control mimic and *miR-19c* mimic-injected larva, respectively). To further quantify the changes, I plotted the changes in area along trunk and the profiles are presented in Fig. 5B (middle: blood vessel profile; right: lymphatic vessel profile). The relative volumetric changes in both blood and lymphatic vasculature are presented in Fig. 5C. In addition, *miR-19c* inhibitor-injected embryos had no significant difference (Fig. 6). It appeared that *miR-19c* mimic-injected larva have significant higher volume of lymphatic but not blood vasculature. Collectively, these results suggest that *miR-19c* may mediate the formation of lymphatic network.

Overexpression of *miR-19c* enhances expression of genes associated with lymphatic vessel development

To examine the regulation of lymphangiogenesis by *miR-19c*, I performed qPCR gene expression analysis against different lymphatic marker genes. I found elevated expression of well-known lymphatic marker genes, *prox1b*, *vegfC* and *lyve1b* in

embryos injected with *miR-19c* mimic compared to those injected with control mimic at 5 dpf. The expression of lysophosphatidic acid receptor 1 (*lpar1*), a novel lymphangiogenesis regulator identified by our group (Lee et al., 2008), was similarly elevated. In contrast, *efnb2a* and *hey2*, which are specifically expressed in blood vessels, showed no significant changes (Fig. 7). It suggests that *miR-19c* may affect lymphatic vessel gene expression presumably via suppressing anti-lymphangiogenesis factors.

miR-19c reduces _tgfβ1a_ but not _lpar1_ expression

To search for other possible targets of *miR-19c*, which may be associated to lymphangiogenesis, I have retrieved a list of potential targets of *miR-19c* using the TargetScan analysis (http://www.targetscan.org/fish_62/). A partial list containing genes related to vascular biology is shown in (Table 1). *Lpar1*, transforming growth factor, *tgf-β*, and others are on the list. In particular, the TGF-β is of interest because the TGF-β signaling pathway is involved in developmental angiogenesis (von Bubnoff and Cho, 2001) and is a anit-lymphangiogenic factor (Shin and Lee, 2014). The TargetScan analysis reveals three target sites in the 3'UTR of *tgfβ1a*, the sequence of three target sites on *miR-19c* are shown with indication of different complementary sites (Fig. 8A). qPCR analysis showed the reduction of *tgfβ1a*

expression in *miR-19c* mimic-injected embryos (Fig. 8B). It indicated that *tgfβ1a* may be the lymphatic associated target gene of *miR-19c*.

Discussion

MicroRNAs have emergent roles in regulating cardiovascular development (Nicoli et al., 2012). In this thesis, I clearly show that *miR-19c* specifically affect lymphatic vessels but not angiogenesis. Mechanistically, I provide further evidence to demonstrate that the regulation of lymphangiogenesis by *miR-19c* may be through repression of *tgfβ1*.

The importance of miRNAs in vessel development has been clearly demonstrated during the critical period between 22-48 hpf. In addition to previous study that only provided a single time point (24 hpf) expression data, my results showed the dynamic changes in expression of BEC-enriched miRNAs to further strengthens the roles of those miRNAs in vascular development (Nicoli et al., 2012). More importantly, I found t *miR-19c* does not influence blood vessel development. Instead, *miR-19c* mainly regulates lymphatic vessel. BECs and LECs share common molecular and functional traits that have made it difficult to distinguish between blood vessels and lymphatics, especially in the remodeling of vascular networks (Hogan et al., 2009). However, LECs differ from BECs in their molecular characteristics and physiological behaviors. This is exemplified by their distinct gene expression profiles and functional characteristics, as well as their independent specification and

development (Stacker et al., 2014). LECs are originated from the posterior cardinal vein (PCV) to initiate lymphatic vessel formation at 32 hpf (Yaniv et al., 2006). Although BECs and LECs share the same origin the onset of LEC differentiation clearly drive them to a different rout by distinct regulations. The specific effects of *miR-19c* mimic on lymphatic but not blood vessel further demonstrated the distinct regulation between two vessel systems. This distinct regulation gained further proof by my analyses showing that the expressions of lymphatic but not blood marker genes are affected by *miR-19c* mimic. However, the regulatory mechanism on lymphangiogenesis by *miR-19c* is still unclear.

Due to the limitation of stock concentration, the highest amount of *miR-19c* mimic I could inject into embryos was 288 pg. Although the penetrance of edema at this dosage was moderate (~ 55%), but it was nonetheless dosage-dependent that lower edema rate and severity were observed at lower dosages (data not shown). In addition, the formation of lymphatic vessels occurs both in normal and pathological conditions like inflammation, lymphedema and tumor metastasis (Schneider, 2006). The induction of *cox2a,* a master inflammatory gene (Seibert, 1994), expression by *miR-19c* mimic further imposes the possibility that edema might be due to inflammatory responses. To clarify this issue, I used a *cox2a* inhibitor, niflumic acid, to see if it can alleviate *miR-19c* mimic-induced edema. In fact, niflumic acid at the

dosages tested had no effect on the *miR-19c* mimic-induced phenotypes. It suggests that the *miR-19c* effect is irrelevant to inflammation. Furthermore, the functionality of thoracic duct can be tested using an intramuscular injection of rhodamine dextran to examine whether *miR-19c* mimic may damage the extracellular fluid uptake by thoracic duct.

We have previously identified *lpar1* as a novel regulator of lymphangiogenesis (Lee et al., 2008). Interestingly, *lpar1* is among the potential target genes of *miR-19c* (Table 1). However, the expression of *lpar1*in *miR-19*c-overexpressed fish was increased instead of decreasing. It suggests that *lpar1* may not a direct target of *miR-19c*. The elevation of *lpar1* expression is intriguing, since we know that *Lpar1* may be a pro-lymphangiogenesis factor that *miR-19c* may release the suppression of *lpar1* expression and then cause unregulated expansion of lymphatic network observed in this study. The observation that *miR-19c* could down-regulate the expression of its potential target *tgfβ1a* imposes a possibility that *tgfβ1a* may be the suppression that can regulate *lpar1* expression and thus lymphangiogenesis. It can be used in particular pathway rescue to know whether those downstream genes are involved in lymphangiogenesis via *miR-19c*.

Vascular development is an integral component of the organ development,

serving important roles in nutrient delivery, fluid homeostasis and immune cell trafficking (Berggreen and Wiig, 2013). It is very important to understand possible regulation in angiogenesis and lymphangiogenesis. From my results and previous studies, lymphatic vessel development may be regulated by *miR-19c* but not in blood vessel development, and may act through *tgfβ1a* to regulate lymphangiogenesis. Because the role of *tgfβ1a* in lymphangiogenesis in zebrafish is not very clear. To further answer this question, further studies of how *tgfβ1a* affect zebrafish lymphangiogenesis should be done using target protector morpholino (Staton and Giraldez, 2011) to validate the miRNA target. In addition, how *miR-19c* blocks TGF signaling to regulate downstream genes remains to be determined. To our knowledge, this is a novel function of *miR-19c* discovered and the first study implicating association between *miR-19c* and lymphangiogenesis.

References

Ambros, V. (2004). "The functions of animal microRNAs." Nature **431**(7006): 350-355.

Avraham, T., S. Daluvoy, J. Zampell, A. Yan, Y. S. Haviv, S. G. Rockson and B. J. Mehrara (2010). "Blockade of transforming growth factor-beta1 accelerates lymphatic regeneration during wound repair." Am J Pathol **177**(6): 3202-3214.

Bos, F. L., M. Caunt, J. Peterson-Maduro, L. Planas-Paz, J. Kowalski, T. Karpanen, A. van Impel, R. Tong, J. A. Ernst, J. Korving, J. H. van Es, E. Lammert, H. J. Duckers and S. Schulte-Merker (2011). "CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C in vivo." Circ Res **109**(5): 486-491.

Clavin, N. W., T. Avraham, J. Fernandez, S. V. Daluvoy, M. A. Soares, A. Chaudhry and B. J. Mehrara (2008). "TGF-beta1 is a negative regulator of lymphatic regeneration during wound repair." Am J Physiol Heart Circ Physiol **295**(5): H2113-2127.

Filipowicz, W., S. N. Bhattacharyya and N. Sonenberg (2008). "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" Nat Rev Genet **9**(2): 102-114.

Flores, M. V., C. J. Hall, K. E. Crosier and P. S. Crosier (2010). "Visualization of

embryonic lymphangiogenesis advances the use of the zebrafish model for research in cancer and lymphatic pathologies." Dev Dyn **239**(7): 2128-2135.

Franzosa, J. A., S. M. Bugel, T. L. Tal, J. K. La Du, S. C. Tilton, K. M. Waters and R. L. Tanguay (2013). "Retinoic acid-dependent regulation of miR-19 expression elicits vertebrate axis defects." FASEB J **27**(12): 4866-4876.

Gore, A. V., K. Monzo, Y. R. Cha, W. Pan and B. M. Weinstein (2012). "Vascular development in the zebrafish." Cold Spring Harb Perspect Med **2**(5): a006684.

Guo, Y., S. Li, J. Qu, S. Wang, Y. Dang, J. Fan, S. Yu and J. Zhang (2011). "MiR-34a inhibits lymphatic metastasis potential of mouse hepatoma cells." Mol Cell Biochem **354**(1-2): 275-282.

Hagerling, R., C. Pollmann, M. Andreas, C. Schmidt, H. Nurmi, R. H. Adams, K. Alitalo, V. Andresen, S. Schulte-Merker and F. Kiefer (2013). "A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy." EMBO J **32**(5): 629-644.

Hogan, B. M., F. L. Bos, J. Bussmann, M. Witte, N. C. Chi, H. J. Duckers and S. Schulte-Merker (2009). "Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting." Nat Genet **41**(4): 396-398.

Isogai, S., N. D. Lawson, S. Torrealday, M. Horiguchi and B. M. Weinstein (2003). "Angiogenic network formation in the developing vertebrate trunk."

Development **130**(21): 5281-5290.

Jeltsch, M., T. Tammela, K. Alitalo and J. Wilting (2003). "Genesis and pathogenesis of lymphatic vessels." Cell Tissue Res **314**(1): 69-84.

Jones-Rhoades, M. W. and D. P. Bartel (2004). "Computational identification of plant microRNAs and their targets, including a stress-induced miRNA." Mol Cell **14**(6): 787-799.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat Rev Mol Cell Biol **10**(2): 126-139.

Lee, S. J., T. H. Chan, T. C. Chen, B. K. Liao, P. P. Hwang and H. Lee (2008). "LPA1 is essential for lymphatic vessel development in zebrafish." FASEB J **22**(10): 3706-3715.

Kuchler, A. M., E. Gjini, J. Peterson-Maduro, B. Cancilla, H. Wolburg and S. Schulte-Merker (2006). "Development of the zebrafish lymphatic system requires VEGFC signaling." Curr Biol **16**(12): 1244-1248.

Mulligan, T. S. and B. M. Weinstein (2014). "Emerging from the PAC: studying zebrafish lymphatic development." Microvasc Res **96**: 23-30.

Nicoli, S., C. P. Knyphausen, L. J. Zhu, A. Lakshmanan and N. D. Lawson (2012). "miR-221 is required for endothelial tip cell behaviors during vascular development." Dev Cell **22**(2): 418-429.

Nicoli, S., C. Standley, P. Walker, A. Hurlstone, K. E. Fogarty and N. D. Lawson (2010). "MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis." Nature **464**(7292): 1196-1200.

Oka, M., C. Iwata, H. I. Suzuki, K. Kiyono, Y. Morishita, T. Watabe, A. Komuro, M. R. Kano and K. Miyazono (2008). "Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis." Blood **111**(9): 4571-4579.

Okuda, K. S., J. W. Astin, J. P. Misa, M. V. Flores, K. E. Crosier and P. S. Crosier (2012). "lyve1 expression reveals novel lymphatic vessels and new mechanisms for lymphatic vessel development in zebrafish." Development **139**(13): 2381-2391.

Oliver, G. (2004). "Lymphatic vasculature development." Nat Rev Immunol **4**(1): 35-45.

Pedrioli, D. M., T. Karpanen, V. Dabouras, G. Jurisic, G. van de Hoek, J. W. Shin, D. Marino, R. E. Kalin, S. Leidel, P. Cinelli, S. Schulte-Merker, A. W. Brandli and M. Detmar (2010). "miR-31 functions as a negative regulator of lymphatic vascular lineage-specific differentiation in vitro and vascular development in vivo." Mol Cell Biol **30**(14): 3620-3634.

Shin, K. and S. H. Lee (2014). "Interplay between Inflammatory Responses and Lymphatic Vessels." Immune Netw **14**(4): 182-186.

Srinivasan, R. S. and G. Oliver (2011). "Prox1 dosage controls the number of lymphatic endothelial cell progenitors and the formation of the lymphovenous valves." Genes Dev **25**(20): 2187-2197.

Stacker, S. A., S. P. Williams, T. Karnezis, R. Shayan, S. B. Fox and M. G. Achen (2014). "Lymphangiogenesis and lymphatic vessel remodelling in cancer." Nat Rev Cancer **14**(3): 159-172.

Tammela, T., G. Zarkada, E. Wallgard, A. Murtomaki, S. Suchting, M. Wirzenius, M. Waltari, M. Hellstrom, T. Schomber, R. Peltonen, C. Freitas, A. Duarte, H. Isoniemi, P. Laakkonen, G. Christofori, S. Yla-Herttuala, M. Shibuya, B. Pytowski, A. Eichmann, C. Betsholtz and K. Alitalo (2008). "Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation." Nature **454**(7204): 656-660.

Thisse, C., Thisse, B., Schilling, T.F., Postlethwait, J.H., 1993. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119, 1203-1215.

von Bubnoff, A. and K. W. Cho (2001). "Intracellular BMP signaling regulation in vertebrates: pathway or network?" Dev Biol **239**(1): 1-14.

Wienholds, E., W. P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H. R. Horvitz, S. Kauppinen and R. H. Plasterk (2005). "MicroRNA

expression in zebrafish embryonic development." Science **309**(5732): 310-311.

Wigle, J. T. and G. Oliver (1999). "Prox1 function is required for the development of the murine lymphatic system." Cell **98**(6): 769-778.

Yang, Y., J. M. Garcia-Verdugo, M. Soriano-Navarro, R. S. Srinivasan, J. P. Scallan, M. K. Singh, J. A. Epstein and G. Oliver (2012). "Lymphatic endothelial progenitors bud from the cardinal vein and intersomitic vessels in mammalian embryos." Blood **120**(11): 2340-2348.

Yaniv, K., S. Isogai, D. Castranova, L. Dye, J. Hitomi and B. M. Weinstein (2006). "Live imaging of lymphatic development in the zebrafish." Nat Med **12**(6): 711-716.

Ye, H., X. Liu, M. Lv, Y. Wu, S. Kuang, J. Gong, P. Yuan, Z. Zhong, Q. Li, H. Jia, J. Sun, Z. Chen and A. Y. Guo (2012). "MicroRNA and transcription factor co-regulatory network analysis reveals miR-19 inhibits CYLD in T-cell acute lymphoblastic leukemia." Nucleic Acids Res **40**(12): 5201-5214.

Zhao, Y. and D. Srivastava (2007). "A developmental view of microRNA function." Trends Biochem Sci **32**(4): 189-197.

Table

Table1. A list of potential targets of *miR-19c*-related to vascular biology.

Target gene	All target sites	8mer	7mer~	Total context+ score
mn	4	1	3	-0.81
rag1	3	2	1	-0.6
sgk1	2	2	0	-0.53
lamp2	4	1	3	-0.41
trpc6	1	1	0	-0.4
tgfβ1a	3	1	2	-0.34
lpar1	3	0	3	-0.29
sox18	1	0	1	-0.15
vegfb	1	0	1	-0.12
flt3	1	1	0	-0.11
kdr	1	0	1	-0.1
tbx20	2	2	0	-0.06

Figures

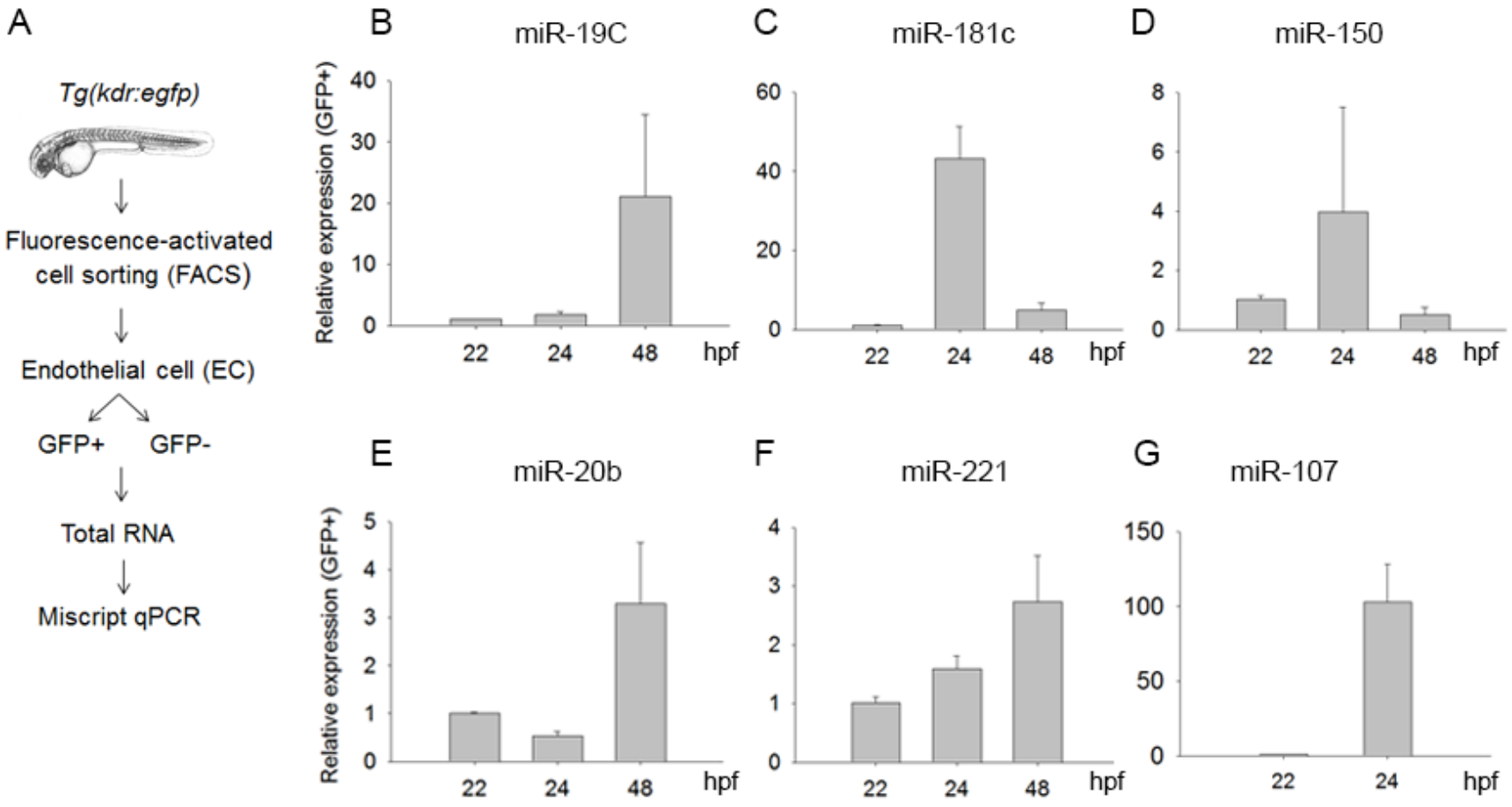

A
Tg(kdr:egfp)
Fluorescence-activated cell sorting (FACS)
Endothelial cell (EC)
GFP+
GFP-
Total RNA
Miscript qPCR
B
miR-19C
C
miR-181c
D
miR-150
E
miR-20b
F
miR-221
G
miR-107
Relative expression (GFP+)
22
24
48
hpf

Figure 1. *Expression analysis of blood endothelial cell-enriched microRNAs.*

(*A*) Blood endothelial cells (BECs, GFP+) and non-BECs (GFP-) were collected from *Tg(kdr:egfp)* embryos at 22-48 hours post fertilization (hpf) by fluorescence-activated cell sorting. Total RNAs were extracted from GFP+ or GFP- cells and subjected to Miscript Q-PCR for the expression analysis of designated BECs-enriched miRNAs. (*B-G*) The ratios of expression level of GFP+ cells were calculated and normalized to their respective ratio at 22-hpf. All treatments were significant from each other ($P < 0.001$).

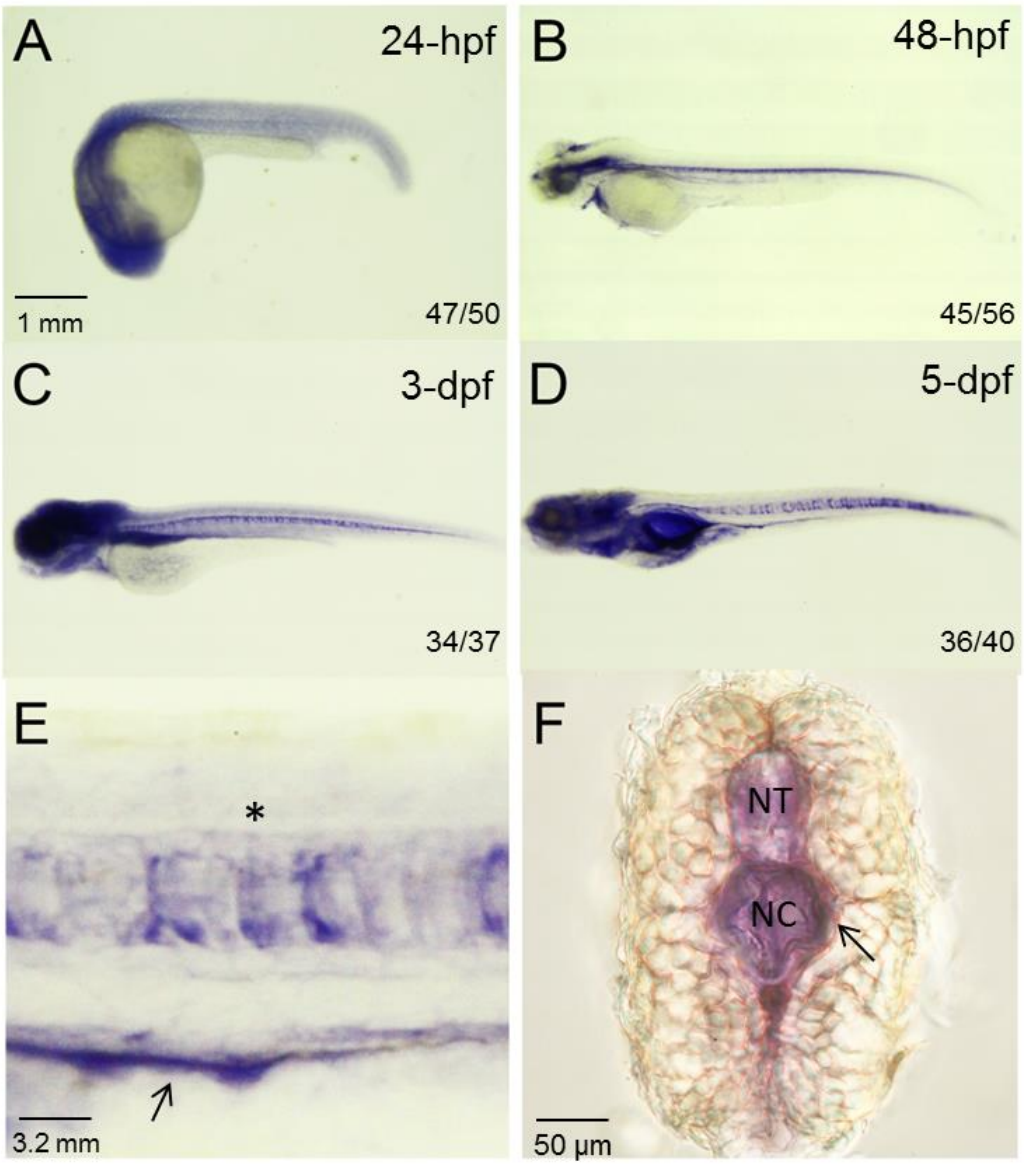


Figure 2. *miR-19c expresses in notochord and thoracic duct.*

(A-D) Whole-mount *in situ* hybridization against *miR-19c* was performed in embryos at designated stages by using a locked-nucleic acid–modified DNA oligonucleotide probes. Lateral views are shown. (*E*) Magnifications of the 5-dpf larval trunk region clearly show *miR-19c* signals in notochord (aster) and thoracic duct (arrow). (*F*)

Transverse section of 5 dpf fish at the mid-trunk reveal the abundance of *miR-19c* transcripts at the peripherals of neural tube and notochord. The numbers of embryos examined are presented at the lower right corner. Anterior to the left and dorsal to the top are in all graphs except in (*F*), which is anterior to the front. NT : neural tube ; NC : notochord.

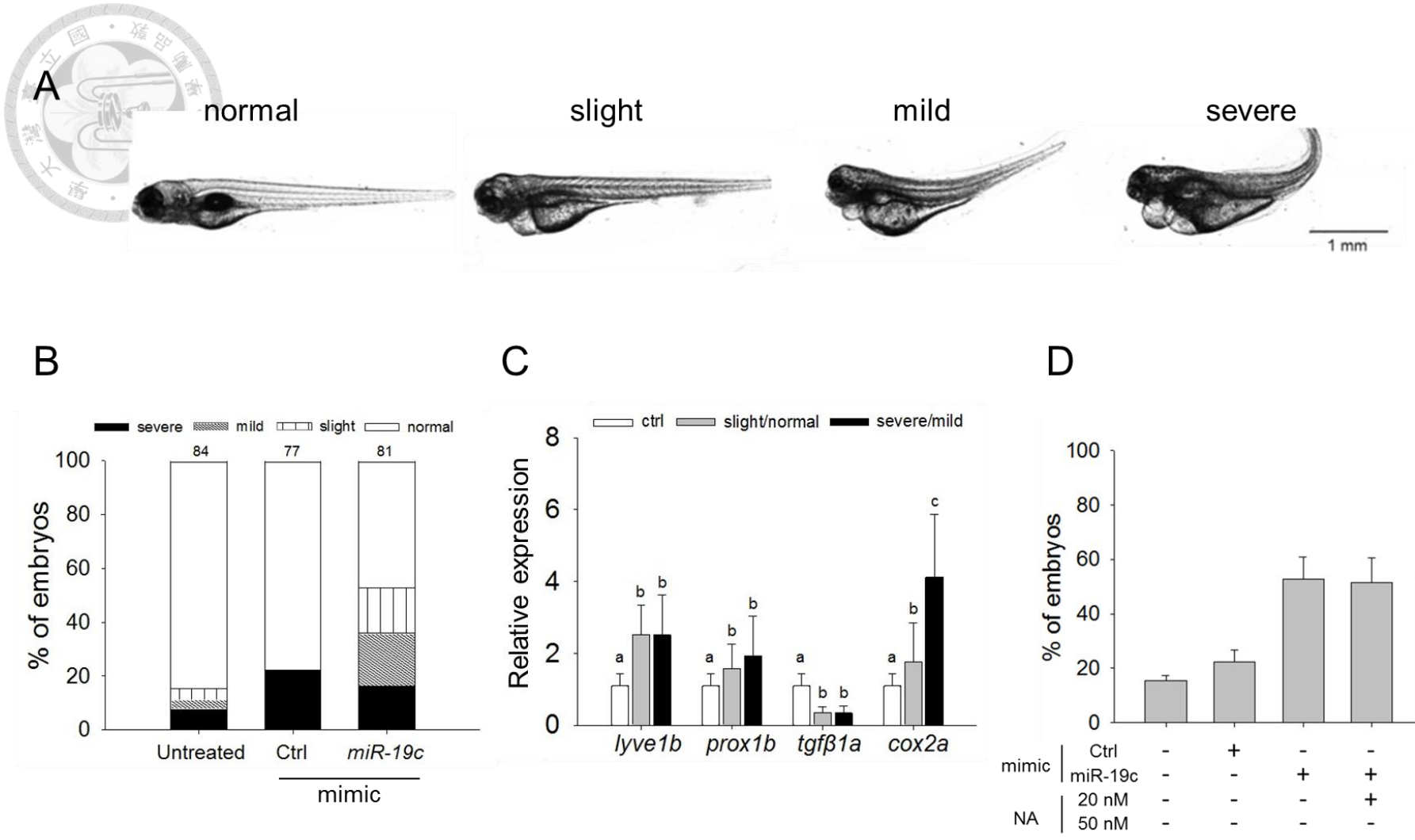
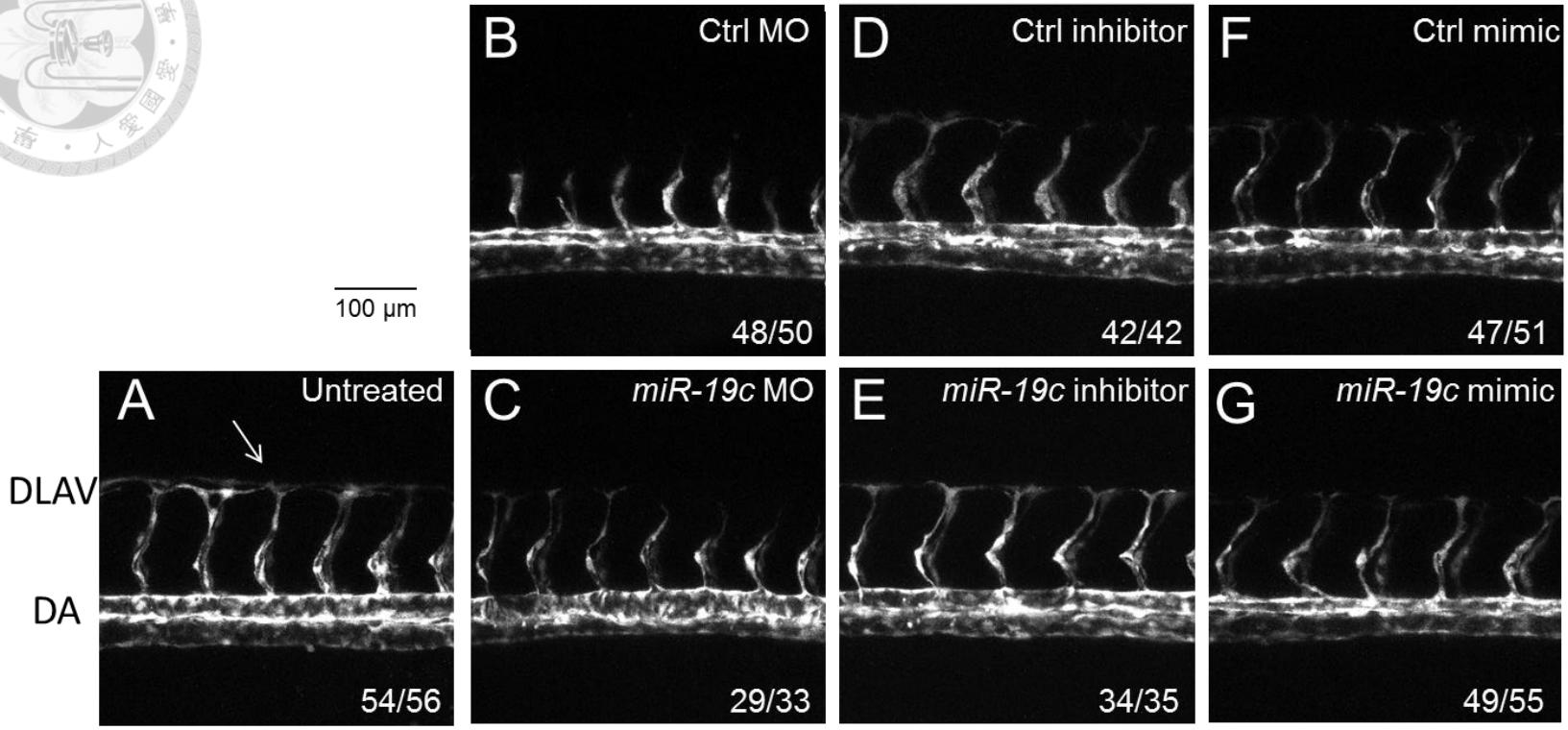

A
normal
slight
mild
severe
1 mm
B
severe
mild
slight
normal
84
77
81
100
80
60
40
20
0
% of embryos
Untreated
Ctrl
miR-19c
mimic
C
ctrl
slight/normal
severe/mild
Relative expression
8
6
4
2
0
a
b
b
a
b
b
a
b
b
a
b
c
lyve1b
prox1b
tgfβ1a
cox2a
D
100
80
60
40
20
0
% of embryos
mimic
Ctrl - + - - -
miR-19c - - + + +
NA
20 nM - - - + -
50 nM - - - - +

Figure 3. *Overexpression of miR-19c induces edema.*

Zebrafish embryos were injected without (untreated) or with 288 pg control mimic (Ctrl) or *miR-19c* mimic (*miR-19c*). Edema appeared in pericardial sacs of a portion of 5-dpf larva. The normal larva is shown in (A) and lava with different severity of edema are classified into slight, mild and severe as indicated. The percentages of embryo with edema with different severity are shown in (*B*). These experiments were repeated three times. The numbers of embryos analyzed are shown on the top of each bar. (*C*) Larva with severe/mild edema were pooled and those with slight edema were mixed with normal-looking larva. The expressions of indicated lymphatic marker genes and inflammatory marker gene in each group were measured by Q-PCR as described (Materials and Methods). The relative change of three groups (ctrl v.s. severe/mild v.s. slight/normal) are presented. *prox1b*, *vegfc*, *tgf*β1*a* are lymphatic marker genes; *cox2* is a inflammatory gene. a is significant from b and c.(*D*) Embryos were injected without or with 288 pg control mimic (Ctrl) or *miR-19c* mimic (*miR-19c*) in the absence or presence of 20 or 50 nM niflumid acid (NA). The percentages of embryos with edema are shown.


B
Ctrl MO
48/50
D
Ctrl inhibitor
42/42
F
Ctrl mimic
47/51
100 μm
A
Untreated
54/56
C
miR-19c MO
29/33
E
miR-19c inhibitor
34/35
G
miR-19c mimic
49/55
DLAV
DA

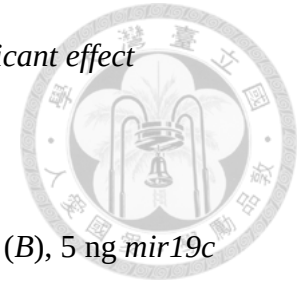


Figure 4. *Overexpression or inhibition of miR-19c shows no significant effect on blood vessel development.*

Tg(kdrl:egfp) embryos were treated without (*A*), with 5 ng ctrl MO (*B*), 5 ng *mir19c* MO (*C*), 1 fmol miR control inhibitor (*D*), 1 fmol *miR-19c* inhibitor (*E*), 288 pg control mimic (*F*) or 288 pg *miR-19c* mimic (*G*), cultured and imaged at 24 hpf under confocal microscopy. Representative photographs at lateral view are shown. Dorsal is up and anterior to the left. An arrow denotes the dorsal longitudinal anastomotic vessel (DLAV) in panel A.

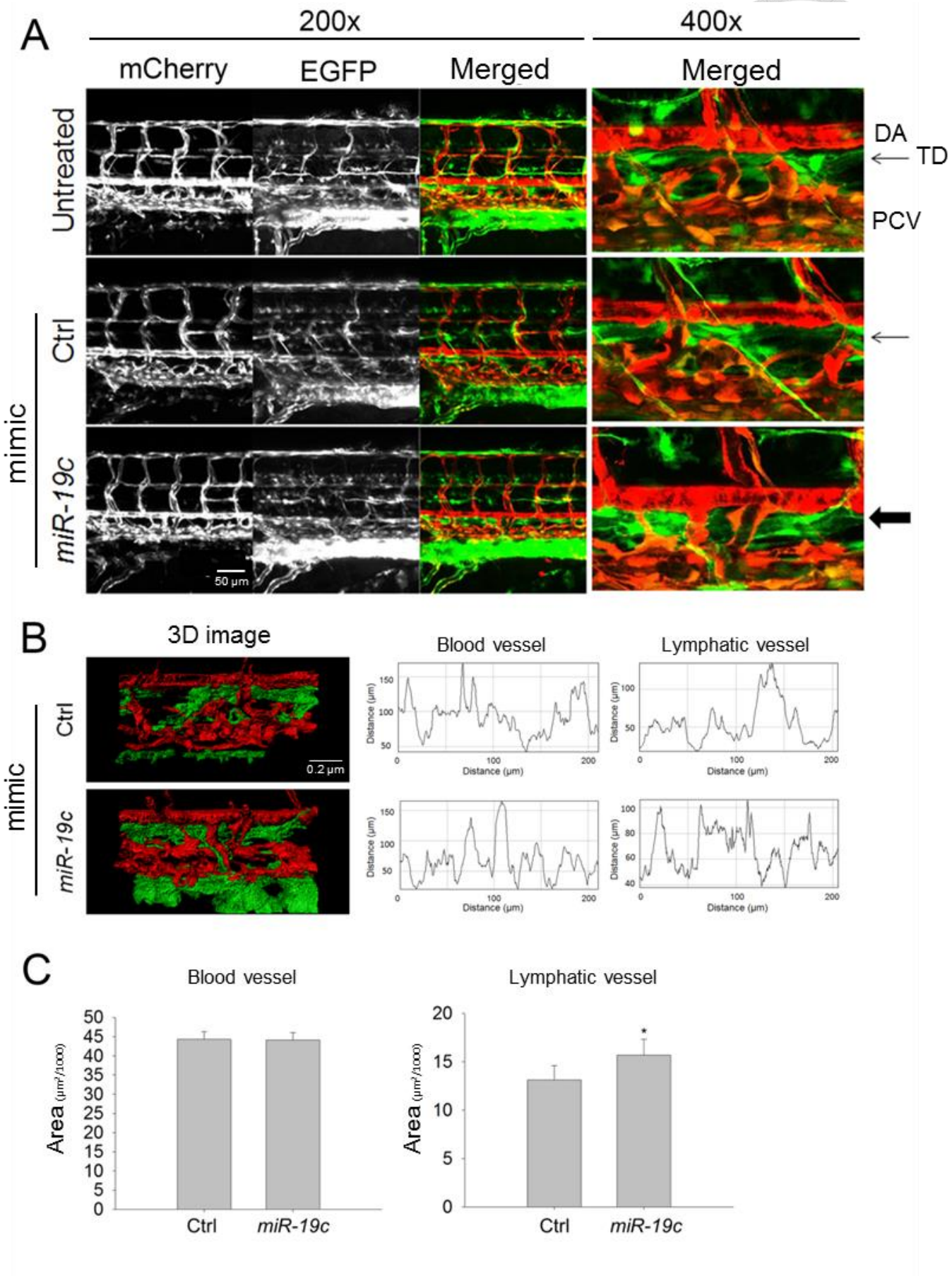

A
200x
400x
mCherry
EGFP
Merged
Merged
Untreated
mimic
Ctrl
miR-19c
DA
TD
PCV
50 μm
B
3D image
Blood vessel
Lymphatic vessel
Ctrl
miR-19c
0.2 μm
Distance (μm)
C
Blood vessel
Lymphatic vessel
Area (μm²/1000)
Ctrl
miR-19c
*

Figure 5. *Overexpressing miR-19c affects lymphatic but not blood vessel development.*

(*A*) *Tg(kdrl:mCherry* x *SAGFF27C;UAS;EGFP)* embryos were injected without (untreated) or with 288 pg control (Crtl) or *miR-19c* mimic (*miR-19c*), incubated for 5 days, imaged at mCherry (blood vessels) or EGFP (lymphatic vessels) channel and photographed under confocal microscopy. Superimposed images with pseudocolors (red: mCherry; green:EGFP) and different magnifications are shown on the right. Embryos are shown in lateral views, dorsal up and anterior to the right. Arrows point to thoracic ducts. The percentage of embryos examined are presented at untreated (23/24), ctrl (23/26) and *miR-19c* (18/31) respectively. (*B*) Using the Imaris software, the 3D rendering of two fluorescent signals were constructed. Representative 3D images for the control and *miR-19c* mimic treated larva are shown. The fluorescent intensity profile for blood and lymphatic vessels along the distance of trunk were plotted as shown (*C*) The area of each intensity profiles were measured and analyzed as shown (n = 28).

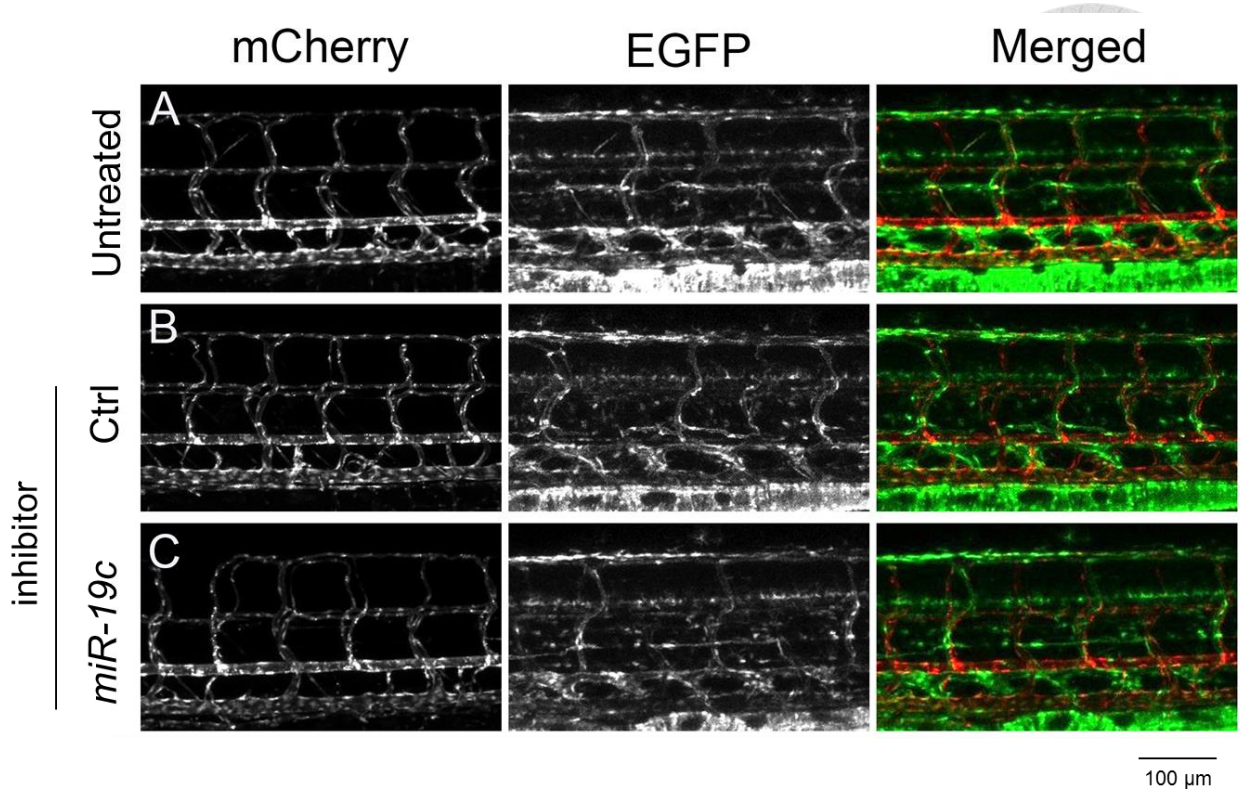


Figure 6. *Inhibiting miR-19c does not affect lymphatic vessel development.* *Tg(kdrl:mCherry x SAGFF27C;UAS;EGFP)* embryos were injected without (untreated) or with 288 pg control (Crtl) or *miR-19c* inhibitor (*miR-19c*), incubated, imaged, photographed and shown as described in Fig. 5. The percentage of embryos examined are presented at untreated (34/35), ctrl (28/30) and *miR-19c* (30/31) respectively.

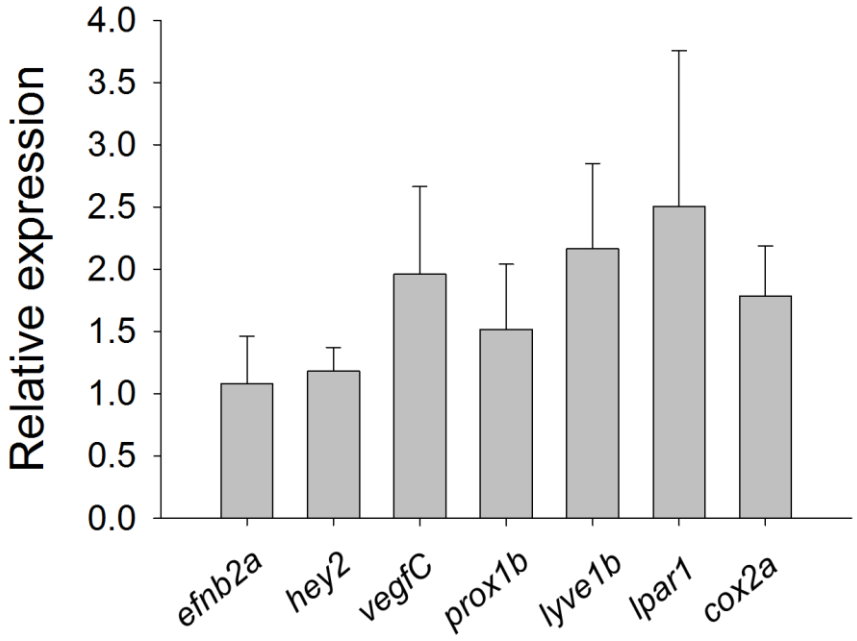


Figure 7. *Overexpression of miR-19c enhances expression of genes associated with lymphatic vessel development.*

Embryos were injected with control or *mir-19c* mimics as described previously and cultured until 5 dpf. Total RNAs were extracted from each sample, transcribed to cDNAs and then subjected to Q-PCR analysis against indicated genes. Data were normalized to an internal control *β-actin2*. The fold changes in expression of *miR-19c* v.s. control mimic were calculated and presented as mean ± standard deviation. *efnb2a and hey2* are blood vessel genes; *prox1b*, *vegfc*, *lyve1b* and *lpar1* are lymphatic marker genes; *cox2a* is a inflammatory gene.

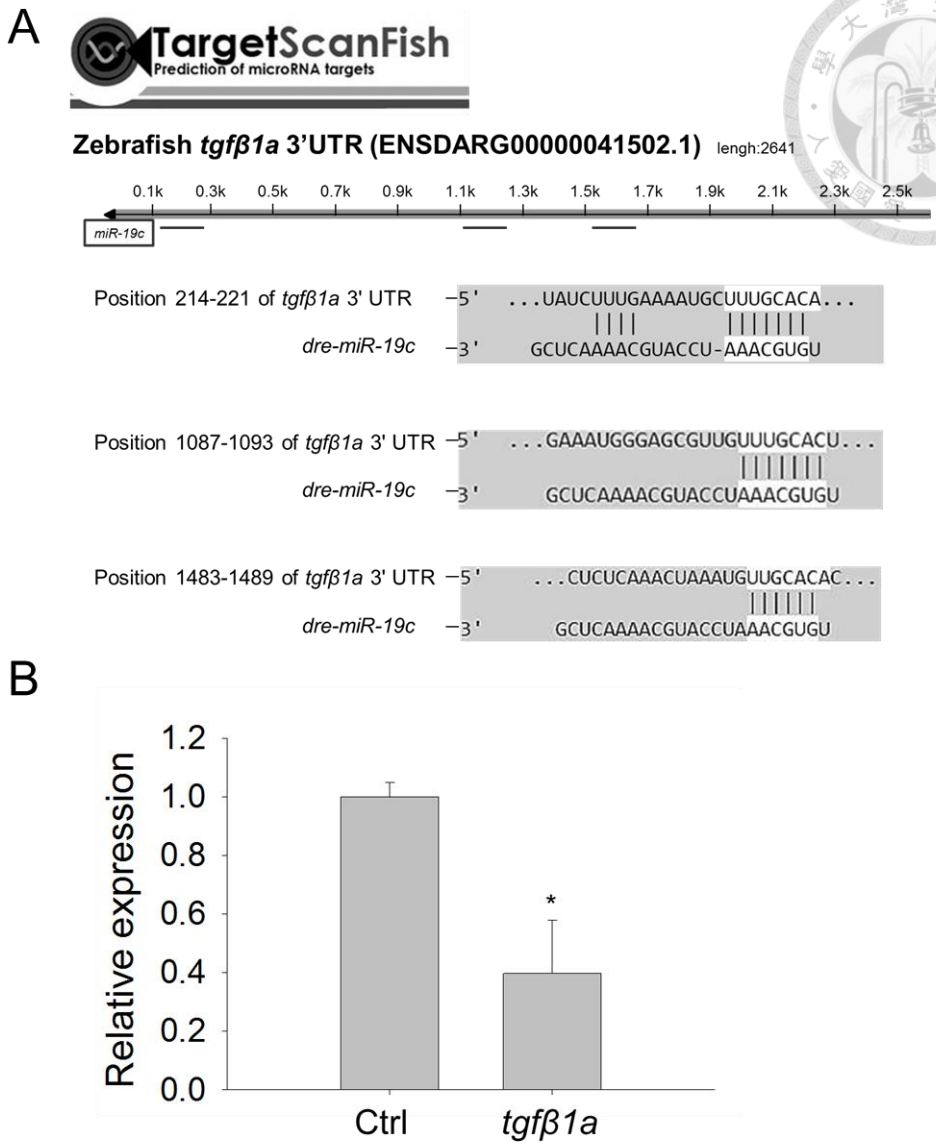


Figure 8. *The expression of tgfβ1a is reduced in embryos overexpressing miR-19c.*

(A) Using the TargetScanFish software, three *miR-19c* target sites (underlines) were found on the 3'UTR of *tgfb1a* gene. The sequence of three target sites on *miR-19c* are shown with indication of different complementary sites as shown. (*B*) Embryos were injected with control or *miR-19c* mimics, *tgfβ1a* expression level was analyzed by qPCR and presented as described in Figure. 7.