Sox18 Genetically Interacts With VegfC to Regulate Lymphangiogenesis in Zebrafish

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Objective—Lymphangiogenesis is regulated by transcription factors and by growth factor pathways, but their interplay has not been extensively studied so far. We addressed this issue in zebrafish.

Approach and Results—Mutations in the transcription factor–coding gene SOX18 and in VEGFR3 cause lymphedema, and the VEGFR3/Fit4 ligand VEGFC plays an evolutionarily conserved role in lymphangiogenesis. Here, we report a strong genetic interaction between Sox18 and Vegfc in the early phases of lymphatic development in zebrafish. Knockdown of sox18 selectively impaired lymphatic sprouting from the cardinal vein and resulted in defective lymphatic thoracic duct formation. Sox18 and the related protein Sox7 play redundant roles in arteriovenous differentiation. We used a novel transgenic line that enables inducible expression of a dominant-negative mutant form of mouse Sox18 protein. Our data led us to conclude that Sox18 is crucially involved in lymphangiogenesis after arteriovenous differentiation. Combined partial knockdown of sox18 and vegfc, using subcritical doses of specific morpholinos, revealed a synergistic interaction in both venous and lymphatic sprouting from the cardinal vein and greatly impaired thoracic duct formation.

Conclusions—This interaction suggests a previously unappreciated crosstalk between the growth factor and transcription factor pathways that regulate lymphangiogenesis in development and disease. (Arterioscler Thromb Vasc Biol. 2013;33:1238-1247.)

Key Words: lymphangiogenesis ■ lymphedema ■ Sox transcription factors ■ vascular development ■ zebrafish

The lymphatic system is a major component of the vertebrate vasculature and plays key roles in tissue fluid homeostasis, fat absorption, and the immune response. Lymphatic vessel function and dysfunction contribute to the progression of several pathological conditions, such as tumor metastasis, lymphedema, obesity, and inflammation. Despite its relevance for human health and disease, lymphangiogenesis has been far less studied than blood vessel angiogenesis. Hereditary (primary) lymphedema is a rare genetic disorder commonly caused by inherited mutations in genes that regulate crucial pathways during the development of the lymphovascular system. Several genes have been identified in lymphedema, including VEGFR3 (Milroy disease), FOXC2 (lymphedema distichiasis syndrome), HGF/MET, GJC2, PTPN14, GATA2, and KIF11.

Hypertrichosis—lymphedema—telangiectasia is a human syndrome displaying mutations in the transcription factor SOX18 and is characterized by the association of heritable alopecia, lymphedema, and vascular malformations. The spontaneous ragged (Ra) mutant mice represent the murine counterpart of the hypertrichosis—lymphedema—telangiectasia syndrome. The most severe Ra mutation, ragged-opossum (RaOp), and the Sox18 null mice, in a pure C57BL/6 (B6) background, showed gross subcutaneous edema at 13.5 days after coitum and died after 14.5 days after coitum, precluding study of lymphatic physiology beyond that stage. Recent findings in mice demonstrated that Sox18 directly activates the transcription of Prox1, a master regulator of lymphatic endothelial cells (LEC), specification, by binding to its proximal promoter.

In mammals, according to the currently widely accepted model, lymphatic vessels arise by direct sprouting of precursors from the cardinal veins to give rise to the early lymph sacs, which remodel and sprout to give rise to the entire lymphatic vasculature. Very recently, it has been shown that a combination of cellular processes that involve ballooning from the cardinal vein and migration as single cells are necessary to establish the lymphatic vascular plexus. A venous origin has been shown also for the recently discovered zebrafish lymphatic system that shares key molecular regulators (including the vascular endothelial growth factor VEGFC and its receptor VEGFR3/Fit4) with the mammalian system. Recently Cebe1 (Collagen and calcium-binding EGF domains-1), identified in zebrafish as an important regulator of lymphangioblast budding, has been found to be mutated.
in human patients affected by Hennekam Lymphangiectasia Lymphedema syndrome,\(^2^2,2^3\) clearly demonstrating that the zebrafish model system could directly aid in defining the molecular basis of human lymphangiopathies.

In zebrafish, developmental angiogenesis occurs in 2 different waves: during the first wave, primary sprouts from the dorsal aorta (DA) give rise to intersomitic vessels (ISVs) from \(\approx 22\) hours postfertilization (hpf); during the second wave, half of the sprouts from the vein will convert arterial ISVs into venous ISVs (vISVs), whereas the other half gives rise to a pool of lymphatic precursors at the horizontal myoseptum (HMS),\(^1^9,2^0\) the parachordal lymphangioblasts (PLs), from \(\approx 32\) hpf.\(^2^1,2^5\) The lymphatic thoracic duct (TD), the main lymphatic vessel described in zebrafish,\(^1^9,2^0\) is then generated by the venous endocardial cell differentiation is more impaired than arterial differentiation in sox18/sox7 double morphants.\(^2^6\)

We found that both sox18 and sox7 are expressed in the developing axial and ISVs, and in the developing head vasculature at 29 hpf (Figure 1Aa, 1Aa’, 1Ab, and 1Ab’). However, at later stages of development, the 2 genes are differentially expressed in the PCV: at 36 hpf, only sox18 expression is clearly detectable in the PCV by in situ hybridization (ISH), whereas both sox18 and sox7 are expressed in the DA (Figure 1Ac, 1Ac’, 1Ad, and 1Ad’). Given the venous origin of LECs, we decided to evaluate the role played by Sox18 and Sox7 in the early phases of lymphatic development.

Knockdown of sox18 Affects TD Formation

The analysis of TD formation is commonly used to study lymphatic development in zebrafish (Materials and Methods in the online-only Data Supplement). To knockdown sox18 or sox7, we injected splice-blocking morpholinos (sox18-MO2 and sox7-MO2) and translation-blocking morpholinos (sox18-MO1 and sox7-MO1)\(^2^6\) into tg(fli1a:EGFP)\(^1\) embryos, where both blood and lymphatic vessels are labeled.\(^1^9\) Larvae were subdivided into phenotypic classes of increasing severity, ranging from fully formed to completely absent TD, to account for the variability of the lymphatic defects (Materials and Methods in the online-only Data Supplement).

We optimized the dose of sox18-MOs to produce relevant defects in TD formation, while minimizing morphological or circulatory defects that would interfere with TD analysis (data not shown). The injection of sox18-MO2 at 1 pmol/embryo specifically impairs TD formation at 5 dpf (Figure 1B and 1C; Table I in the online-only Data Supplement). In contrast, most control larvae showed fully formed TD (Figure 1C; Table I and Figure I in the online-only Data Supplement). Similar defects in TD formation resulted from the injection of an independent morpholino targeting sox18 (sox18-MO1) although with lower penetrance (Figure IIA and IIB in the online-only Data Supplement). Moreover, sox18 RNA rescues the lymphatic phenotype of sox18 morphants in a dose-dependent manner (Figure 1B and 1C; Table I in the online-only Data Supplement), supporting the specificity of these phenotypes (Figure 1C; Table I in the online-only Data Supplement).

We next analyzed the effects of sox7 knockdown on lymphatic development. Using 2 different morpholinos at the maximal doses allowing robust blood circulation, we found that the knockdown of sox7 caused only minor defects in TD formation (Figure IIC and IID in the online-only Data Supplement and data not shown). The coinjection of even low doses of sox18- and sox7-MOs blocks blood circulation in the trunk, due to impaired arteriovenous differentiation and arteriovenous shunt formation\(^2^9\); therefore, we could not fully investigate whether knocking down sox7 exacerbates the lymphatic phenotype of sox18 morphants.

When dealing with a subfamily of SOX genes, it is important to check for background-dependent effects, like strain-specific compensatory upregulation of other Sox family members when one is knocked down or out.\(^1^4,2^9\) We analyzed the expression

Materials and Methods

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

Results

sox18, but not sox7, Is Expressed in the Cardinal Vein During Lymphatic Precursor Sprouting

Sox18 and Sox7 play redundant roles in arteriovenous differentiation of endothelial cells in zebrafish,\(^2^6,2^8\) Simultaneous partial knockdown of both genes, but not single partial knockdowns, causes multiple fusions between the major axial vessels (the DA and the PCV) because of an incomplete acquisition of arteriovenous identity by endothelial cells. In particular,

Vein During Lymphatic Precursor Sprouting

sox18 and vegfc in Early Lymphatic Development

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levels of sox7 in sox18 morphants at various developmental stages (18–20 somites, 24 hpf and 36 hpf), before or around early lymphatic development, and we did not observe any significant changes (Figure IIIa–IIIi in the online-only Data Supplement).

Our data thus point to an important role of Sox18 in lymphatic development, whereas Sox7 does not seem to play such a prominent role in this process and has not been analyzed any further in this study.

**Knockdown of sox18 Impairs the Sprouting of Lymphatic Precursors From the Vein, but Does Not Alter vISV Angiogenesis**

We next sought to better characterize early steps of lymphatic development in sox18 morphants. In the secondary wave of zebrafish angiogenesis, half of the sprouts from the vein anastomose with and convert arterial derived ISVs into vISVs, whereas the other half gives rise to a pool of lymphatic precursors at the myoseptum, the PLs (also called lymphangioblasts or parachordal chain cells).\(^{19,21,24,25,30}\) This process involves several molecular players and is known to be controlled by VEGF-C/VEGFR3 signaling.\(^{24,25,31}\)

Knockdown of sox18 caused a reduction of \(\approx40\%\) in the total number of sprouts from the vein scored at 1.5 dpf, which is compatible with an effect limited to lymphangiogenic sprouting (Figure 2A). Knockdown of vegfc caused, instead, a much more drastic reduction (Figure 2A), pointing to an overall impairment of secondary sprouting, as already reported in literature.\(^{11}\) These data indicate considerably more specificity to the sox18 morphants phenotype we observe here.

Lymphatic precursors, originating from the PCV, are transiently residing at the HMS before migrating ventrally or dorsally to give rise to TD and other trunk lymphatic vessels.\(^{19,21}\) We directly scored PLs at the HMS in circulating sox18 morphants and found a significant decrease at 56 hpf (5.1\(\pm\)0.5 versus 8.3\(\pm\)0.2 in controls, Figure 2B).

We next scored vISVs in circulating control embryos and sox18 morphants at 2.5 dpf. In 3 independent experiments, we found that sox18 knockdown did not significantly alter vISV numbers (Figure 2C). Additionally, in separate experiments, we scored for a/v ISVs at 2.5 dpf and kept morphants for further TD scoring at 5 dpf. This enabled us to calculate,
a posteriori, the number of a/v ISVs at 2.5 dpf in sox18 morphants showing different degrees of lymphatic defects at 5 dpf. We found that sox18 morphants with more severe TD defects and those either unaffected or with minor TD defects showed comparable vISV numbers (Figure IVA and IVB in the online-only Data Supplement).

These analyses confirm that sox18 knockdown, at the morpholino dose we chose to avoid circulatory defects, impairs lymphatic development, without significantly altering the venous component of the secondary angiogenic wave.

Heat-Shock Inducible Overexpression of Mouse Sox18 Ragged Opossum Inhibits Zebrafish PL Development Postarteriovenous Segregation

Sox18 plays a role in early arteriovenous differentiation and, theoretically, a ubiquitous knockdown across all developmental stages (morpholino approach) could induce PL and TD defects secondarily to a mild arteriovenous defect that we might not be able to score at a basic morphological level.

To inhibit Sox18 activity at stages subsequent to arteriovenous differentiation, we generated a transgenic line for the temporally inducible inhibition of its transcriptional activity. RaOp is the strongest of the 4 known ragged mutant alleles, all coding for Sox18 mutant proteins with an intact DNA-binding domain but compromised transactivation ability.32–34 Ragged mutant proteins act in a dominant-negative fashion, preventing the binding of redundant SoxF factors (ie, Sox7 and Sox17) to Sox18 target genes.

Sox18RaOp dominantly interferes with Sox18–, 7–, and 17–regulated transcription in mouse embryonic lymphangiogenesis.14,29 We took advantage of this mutant allele and cloned the mouse Sox18RaOp cDNA, fused in frame with the mCherry coding sequence, under the control of the hsp70l promoter (Figure 3A). We used Tol2-mediated transgenesis35 to generate a stable zebrafish tg(hsp70l:Sox18RaOp mCherry) line.

We performed staged heat shocks and observed the nuclear accumulation of mCherry protein by 3 to 4 hours (but not 2 hours) after heat-shock treatment (data not shown) using confocal microscopy. The tg(hsp70l:Sox18RaOp mCherry) line was crossed to tg(fli1a:EGFP)y1 or tg(fli1a:EGFP)y1;tg(fli1enh,RFP). Therefore, half of the GFP+ progeny carries the inducible transgene, and the other half of the GFP+ progeny serves as nontransgenic controls, alongside non-heat-shocked transgenic controls. Heat shock was performed at 24, 29, 36, 48, and 72 hpf, and embryos were separated based on mCherry expression at 3 to 4 hours after heat shock.

Heat-shock induction of Sox18RaOp at 24 hpf led to significant cardiovascular defects and a general (not lymph-) edema phenotype by 5 dpf (Figure VA in the online-only Data Supplement), attributable to circulatory defects, thus further
Heat shock at 29 hpf also led to a highly significant reduction in the number of vISVs with respect to control embryos (Figure 3E, 3F, 3H, 3I, and 3K). Such a reduction was not detectable in sox18 morphants (Figure 2C, and Figure IVA and IVB in the online-only Data Supplement). This prompted us to analyze the expression level of vegfc in the Sox18RaOp induced transgenic embryos, because Vegfc/Vegfr3 signaling is crucial for venous and lymphatic sprouting.21,31 Heat shock at 29 hpf resulted in a reduction of vegfc expression in these embryos (Figure VC in the online-only Data Supplement). This reduction may contribute to the severe phenotypes observed on overexpression of the dominant-negative Sox18 mutant protein, but these may also be due to a more generally impaired input of SoxF proteins.

These experiments show that dramatic PL defects were observed when Sox18RaOpmCherry overexpression was induced at 29 hpf, which leads to the expression of nuclear mCherry protein by 32 to 33 hpf. The milder phenotype with 36 hpf heat shocks suggests a critical period for SoxF activity in lymphatic development between 32 and 40 hpf, during the period of PL sprouting from the cardinal vein.

**sox18 and vegfc Genetically Interact in Zebrafish TD Formation**

To gain insight into the molecular events governing the early phases of lymphatic development, we decided to analyze the interplay between sox18 and vegfc, a growth factor crucial for this process.19,20,31

We reproduced the lymphatic phenotype associated with knockdown of vegfc and then constructed a dose–response curve by injecting several doses of vegfc-MO to identify a critical range (Figure VI in the online-only Data Supplement). This led us to define a subcritical dose of vegfc-MO (0.06 pmole/embryo) to be used in coinjection experiments along with a subcritical dose of sox18-MO2 (0.5 pmole/embryo).

The single injection of these low doses of sox18 and vegfc morpholinos caused no gross morphological or lymphatic abnormalities (Figure 4; Figure VII in the online-only Data Supplement). On the contrary, coinjection of subcritical doses led to severe defects in TD development (Figure 4): almost 70% of coinjected larvae showed a total absence of TD or the presence of only 10% to 30% TD+ segments (Figure 4B; Figure VII in the online-only Data Supplement). The synergistic effect of the coinjection can also be obtained even when cutting by half the subcritical doses of sox18-MO2 and vegfc-MO (0.25 and 0.03 pmole/embryo, respectively; Figure VIII A and VIIIIC in the online-only Data Supplement), and TD formation is drastically affected also by coinjecting through the DA and PCV, however the circulation through ISVs was abnormal at 2.5 dpf. Embryos heat shocked at later time points showed normal circulation.

Heat shock at 29 hpf led to a near complete loss of PLs at the HMS at 56 hpf, whereas 36 and 48 hpf heat shocks led to milder reductions in PL numbers (Figure 3G and 3J; Table IV in the online-only Data Supplement). Later heat shock at 72 hpf had no effect on later TD formation (data not shown). Non-heat-shocked transgenic animals and heat-shocked non-transgenic controls showed no phenotype (Figure 3B, 3D–3F, and data not shown).

Heat shock at 29 hpf also inhibits parachordal lymphangioblast (PL) development and a/vISV scoring in a double transgenic tg(fl/flaRFP) background (Figure VII in the online-only Data Supplement). The synexpression is shown in Figure VI B (online-only Data Supplement), D–I, PL development and a/vISV scoring in a double transgenic tg(fl/flaRFP) background at 56 hpf. D and G, Representative images of PL development visualized by tg(fl/flaRFP) expression in tghsp70::Sox18RaOp mCherry transgenic embryos without heat shock (D) and after 29 hpf heat shock (G). Arrows indicate the presence of PLs, and asterisk indicates the absence of PLs at the horizontal myoseptum. E and H, arterial ISVs (aISVs; white arrowheads) analyzed in tghsp70::Sox18RaOp mCherry transgenic embryos without heat shock (E) and after 29 hpf heat shock (H). F and I, merge of images shown in D, E and H, respectively, vISVs are only visible without heat-shock. J and K, Quantification of PL development and vISVs after heat shock at 29, 36, and 48 hpf. Cherry positive embryos are transgenic induced animals, sorted based on visual scoring 3 to 4 hours after heat shock. Cherry negative embryos, that is, either non-heat-shocked transgenic or heat-shocked nontransgenic embryos, serve as controls. Analyses after 29 hpf heat shock were performed in a double transgenic tgfli1a:EGFP::tg(flt1::mRFP) background, after 36 and 48 hpf heat shocks in a tgfli1a:EGFP:: background. ***P<0.001 vs control embryos; *P<0.05 vs control embryos. Raw data are reported in Table IV (online-only Data Supplement).
a low dose of vegfc-MO (0.06 pmoles/embryo, Figure 4) with a subcritical dose of an independent sox18 morpholino (sox18-MO1, 0.5 pmoles/embryo), that does not largely affect TD formation when injected on its own (Figure VIIIB in the online-only Data Supplement).

Moreover, synergistic defects in TD formation were also obtained by simultaneous partial knockdown of sox18 and of the VegfC receptor gene flt4 (Figure IX in the online-only Data Supplement), coinjecting low doses of sox18-MO2 (0.5 pmoles/embryo) and flt4-MO (0.06 pmoles/embryo).

To determine whether Sox18 and VegfC cross-regulate at the mRNA level, we analyzed by ISH whether the mRNA levels of sox18 or vegfc are perturbed by the knockdown of vegfc or sox18, respectively. sox18 transcripts did not show any significant reduction in vegfc morphants nor did vegfc expression show changes in sox18 morphants (Figure X in the online-only Data Supplement). In addition, we found that the simultaneous partial knockdown of sox18 and vegfc does not alter sox7 expression (Figure III in the online-only Data Supplement). These data suggest that the interactions observed here are not occurring at the level of embryonic transcription of these genes.

The overexpression of a dominant-negative Sox18 mutant protein resulted in a reduction of vegfc expression, whereas the knockdown of sox18 did not produce detectable changes in the vegfc ISH signal. These data might imply other SoxF proteins in the regulation of vegfc. Alternatively, they might imply that a more pronounced reduction in Sox18 than the one caused by knockdown is needed to produce an alteration in vegfc levels.

To further address the molecular basis of the Sox18/VegfC interplay, we coinjected vegfc RNA while knocking down sox18 by morpholino injection: overexpression of vegfc partially rescued TD formation defects in sox18 morphants (Figure XIA in the online-only Data Supplement). The reverse experiment, namely sox18 RNA injection in vegfc morphants, did not cause any amelioration of the TD phenotype (Figure XIB in the online-only Data Supplement).

Taken together, our data for the first time suggest a relationship between the growth factor pathways that specifically regulate lymphangiogenesis (VegfC/Negr3 signaling) and the transcriptional pathways that modulate lymphangiogenesis.

**Figure 4.** The simultaneous partial knockdown of sox18 and vegfc severely affects thoracic duct (TD) formation. A, Confocal analysis of circulating larvae at 5 dpf revealed that partial knockdown of sox18 or vegfc with subcritical doses of either sox18- or vegfc-MOs (0.5 pmol and 0.06 pmol, respectively) does not affect TD formation, as observed in tg(fli1a:EGRFP)Y1 control larvae injected with std-MO (white arrowheads). On the contrary, TD formation is largely impaired by the coinjection of the same subcritical doses of both MOs (sox18+vegfc double partial morphants; white asterisks). B, The bar chart shows the percentage of larvae grouped in phenotypic classes with increasing TD defects. TD formation defects observed in sox18+vegfc double partial morphants are greater than the mere sum of the defects observed in single partial morphants, indicating a synergistic effect of MOs on coinjection. The number and percentage of embryos belonging to each class are reported in Table V (online-only Data Supplement); statistical analysis is shown in Figure VII (online-only Data Supplement). DA indicates dorsal aorta; PCV, posterior cardinal vein; and TD, thoracic duct.

To test how robust these observations are, we decided to examine the interaction with independent molecular markers of the vasculature. We performed ISHs with the pan-endothelial marker cdh5 and some venous specific markers, such as dab2, ephB4, and flt4 around 29 hpf. Hybridization signals for these molecular probes were comparable in sox18 morphants and in combined partial sox18 and vegfc morphants with respect to controls, suggesting that blood endothelial cells were largely unaffected (Figure 6A; Figure XIIA in the online-only Data Supplement).

Next, we examined venous and lymphatic precursor sprouting using the lyve1 marker\(^{21, 17}\) in ISHs at 2 dpf. lyve1+ sprouts from the PCV were clearly visible in controls (Figure 6Bb, white arrows) but severely reduced or absent in sox18 morphants and in combined partial sox18 and vegfc morphants (Figure 6Bd, 6Be, and 6Bg). We subdivided morphants to better describe their phenotypes in terms of presence/absence and length of lyve1+ sprouts (Figure 6B). Normal lyve1+ sprouts were detectable in most control embryos, but in only \(\approx 10\%\) of sox18 morphants and 5% of combined partial sox18-vegfc morphants (Figure 6Bh). Interestingly, among sox18 morphants, the prevalent phenotype was that of embryos with
The simultaneous partial knockdown of sox18 and vegfc impairs sprouts from the vein and reduces parachordal lymphangioblast (PL) and venous intersomitic vessel (vISV) numbers. 

A. We scored the number of sprouts from the vein on one side of sox18-vegfc double partial morphants (injected with 0.5 pmol sox18-MO2 + 0.06 pmol vegfc-MO) and plotted here mean values considering 10 segments/embryo of circulating tg(fli1a:EGFP)\(^3\) embryos at 1.5 dpf. When subcritical doses of sox18 and vegfc-MOs were coinjected, we scored slightly less than half of the total number of venous-derived sprouts present in controls (**P<0.001 vs std-MO; see Table IIA in the online-only Data Supplement). B. We next scored the presence/absence of PLs at the horizontal myoseptum: we analyzed 10 consecutive trunk segments of circulating tg(fli1a:EGFP)\(^3\) embryos at 2.5 dpf, only on one side. PL+ segments are marked with an arrowhead, segments devoid of PLs with an asterisk. Confocal analysis shows that the number of PL+ segments at the horizontal myoseptum is reduced in sox18-vegfc double partial morphants (b) if compared with control embryos (a). C. Mean values are plotted in the bar chart (see Table IIB in the online-only Data Supplement). We show here the same control embryos data present in Figure 2, because we performed a unique set of 3 experiments to analyze single sox18 morphants and double sox18-vegfc morphants.

Discussion

In the past few years, zebrafish has emerged as a very potent system to study lymphangiogenesis.\(^{18,19}\) Overall, the zebrafish lymphatic system shares several morphological, functional, and molecular characteristics with mammals. Since the initial description of the zebrafish lymphatic system in 2006,\(^{19,20}\) a handful of molecular players have been shown to be evolutionarily conserved, but much remains to be elucidated and we are far from a complete picture of the degree of conservation of molecular pathways from zebrafish to human lymphangiogenesis. This prompted us to study the role of sox18 in zebrafish lymphatic development, because SOX18 mutations are associated with lymphedema in patients affected by the hypotrichosis–lymphedema–telangiectasia syndrome, and studies in mouse placed Sox18 very high in the hierarchy of transcription factors governing LEC differentiation.\(^{40}\)

Sox18 belongs to the Sox F group of Sry-related high mobility group box transcription factors, also comprising the closely related Sox7 and Sox17 proteins. Sox proteins of the same subfamily tend to be biochemically interchangeable in vitro, and the relevance of individual Sox genes for a specific process is often linked to their differential expression in vivo.\(^{14,29}\)

Ours and other groups have reported that sox7 and sox18 are coexpressed in angioblasts and endothelial cells of the forming vasculature, and that they play redundant roles in arteriovenous differentiation.\(^{26–28}\) We show here that sox7 stops being expressed earlier than sox18 in the axial vein, whereas both genes are still expressed in the DA, and that Sox18 specifically regulates lymphatic development.

In mice, Sox18 acts in concert with CoupTFII to drive the transcription of Prox1.\(^{14,44}\) The polarized expression of sox18 precedes that of Prox1 in a subset of cells along the dorsolateral aspect of the cardinal vein at 9 days after coitum,\(^{13}\) whereas no polarized expression of CoupTFII has been reported so far. Remarkably, we have no evidence of a polarized expression of sox18 within the PCV, when secondary sprouts are arising from the dorsal aspect of the axial vein in zebrafish.

Although quite prominent and statistically highly significant, the degree of impairment in TD formation we observe in sox18 morphants versus control embryos is less striking than that observable when vegfc is fully knocked down. Our analysis is limited to circulating morphants without gross morphological abnormalities, and this sets an upper limit to the dose of sox18 morpholino we use. Hence, the strong but not full impairment in TD formation in sox18 morphants could be the result of a submaximal dose of MO in these experiments.
Lymphatic precursors sprout from the vein at $\approx 1.5$ dpf and account for approximately half of the total sprouts, the other half consisting of venous sprouts that will connect to arterial ISVs and convert them into venous ISVs. Our results point to a specific impairment of lymphangiogenic sprouting from the cardinal vein in $sox18$ morphants, whereas secondary venous angiogenic sprouting is not substantially perturbed. Importantly, several pan-endothelial ($cdh5$) or venous markers ($dab2$, $ephB4$, $flt4/vegfr3$) are unaffected in $sox18$ morphants, whereas the venous/lymphatic endothelial marker $lyve1$ is altered: $lyve1^+$ sprouts are either shorter or reduced in number, up to totally absent in the most severely affected $sox18$ morphants, providing an independent molecular assay for our morphological observations. Notably, PL defects in $sox18$ morphants do not seem to be secondary to overt venous differentiation problems. These data together imply a specific role of Sox18 in the early phases of lymphatic differentiation and sprouting in zebrafish.

Injection of an uncaged morpholino at very early stages of embryo development leads to a constitutive knockdown of gene function. We used a complementary approach, based on an inducible overexpression of a dominant-negative mutant form of mouse Sox18 in a newly developed stable transgenic line, to interfere with zebrafish SoxF proteins function in a temporally regulated way. A series of heat shocks enabled us to conclude that Sox18 and, possibly, other SoxF proteins function at the time of lymphatic precursor emergence from the PCV and regulate PL sprouting post-arteriovenous differentiation.

Although many signaling pathways have been implicated in lymphangiogenesis, it has been pointed out that most of...
their effects may be secondary to the induction of VEGF-C/D in a variety of cell types.\(^1\) VEGF-C/VEGFR3 signaling has an established and evolutionarily conserved role in lymphatic development.\(^{1,10,20,31,43}\) The current literature holds that the specification of LEC fate and the sprouting of LECs from the PCV are regulated independently.\(^{1,44,45}\) Our findings that Sox18 and Vegfc show strong genetic interaction in zebrafish lymphatic development challenge this model and mandate a careful mechanistic analysis of this interaction in vertebrate model systems. Specifically, we show that embryos co-injected with subcritical doses of morpholinos against sox18 and vegfc, which produce little or no effect when injected separately, display severe PL and TD defects. This observation seems to be highly specific because the trunk vascular tree does not show abnormalities at the morphological or molecular marker levels. Interestingly, whereas the single knockdown of sox18 does not perturb venous sprouts but only PLs, double partial knockdown of sox18 and vegfc impacts more generally on all secondary angiogenesis from the vein, thus possibly revealing a combined role of both vegfc and sox18 and does not perturb venous whereas the single knockdown of sox18 at the morphological or molecular marker levels. Interestingly, the transcriptional activity of the residual SOX18 protein. These observations may point to a post-translational modification mechanism, which remains to be studied. Interestingly, vegfc overexpression ameliorates TD formation in embryos where sox18 is knocked down, possibly suggesting that the transcriptional activity of the residual Sox18 protein is positively modulated by enhanced VegfC/Vegfr3 signaling. Whatever the mechanism may be, it is clear from the data presented here that the strong Sox18–VEGFC interplay in lymphangiogenesis is evolutionarily conserved and points to a novel molecular mechanism in lymphangiogenesis that remains to be further investigated.

Sox18 expression is not required for the maintenance of the lymphatic identity in mammals, whereas under pathological conditions, such as tumor growth, Sox18 is critical for tumor-induced angiogenesis and lymphangiogenesis.\(^{46,47}\) Both Sox18 and VEGF-C/VEGFR3 are promising targets for inhibition of tumor lymphangiogenesis.\(^{4,54}\) Our findings uncover a novel interplay between a key transcription factor and one of the most potent lymphangiogenic growth factors, hence opening new potential therapeutic avenues.

**Note added in proof:** A mutation in VEGFC has just been reported in a patient affected by Milroy-like primary lymphedema.\(^{49}\)

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**Disclosures**

None.

**References**


**Significance**

This work reveals for the first time a conserved role for the transcription factor Sox18 in lymphatic development in a non-mammalian organism, thus strengthening the use of zebrafish as a potent system to study the molecular network at the basis of lymphangiogenesis. Our data reinforce the notion that Sox18 controls the early phases of lymphangiogenesis. Transcription factors and growth factor pathways have been implicated in lymphangiogenesis, but their interplay has not yet been extensively studied. The genetic interaction we observe points to a so far poorly characterized role of the VEGFC growth factor pathway in the modulation of the activity of a key transcription factor that regulates lymphangiogenesis.
Sox18 Genetically Interacts With VegfC to Regulate Lymphangiogenesis in Zebrafish

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“Sox18 genetically interacts with VegfC to regulate lymphangiogenesis in zebrafish”

Cermenati et al.

Materials and Methods

Zebrafish lines and maintenance
Zebrafish were raised and maintained according to established techniques \(^1\). The following strains were used: AB (from the Wilson lab, UCL, London, UK), tg(\textit{fli1a}:EGFP)\(^{y1}\) (from the Lawson lab, University of Massachusetts Medical School, MA), tg(\textit{flt1}\(^{enh}\):RFP) line \(^3\).

MO/RNA microinjections
Antisense morpholinos (MOs; Gene Tools, Philomath, OR) used in this study were already described: \textit{sox18}-MO1, \textit{sox18}-MO2, \textit{sox7}-MO1, \textit{sox7}-MO2, \(^4\); \textit{vegfc}-MO \(^5\); \textit{flt4}-MO \(^6\). MOs, diluted in Danieau buffer \(^7\), were injected at 1- to 2-cell stage. Escalating doses of each MO were tested for phenotypic effects; as control for unspecific effects, each experiment was performed in parallel with a std-MO (standard control oligo) with no target in zebrafish embryos. We usually injected 1 pmol/embryo of \textit{sox18}-MO2, 0.5 pmol/embryo of \textit{vegfc}-MO, and 0.8 pmol/embryo of \textit{sox18}-MO1, \textit{sox7}-MO1 and \textit{sox7}-MO2. For combined knockdown experiments, we injected \textit{sox18}-MO2 and \textit{vegfc}-MO at 0.5 and 0.06 pmol/embryo or 0.25 and 0.03 pmol/embryo, respectively; \textit{sox18}-MO2 and \textit{flt4}-MO at 0.5 and 0.06 pmol/embryo, respectively.

For \textit{sox18} RNA, a \textit{SmaI}-XbaI fragment from pBSKS\(^+\)\textit{sox18} \(^4\), containing zebrafish \textit{sox18} cDNA, was subcloned into pCS2\(^+\); the resulting plasmid was digested with \textit{NotI} and transcribed with Sp6 RNA Polymerase (Roche). Rescue experiments were performed with the coinjection, into 1-cell stage embryos, of 1 pmol/embryo \textit{sox18}-MO2 and 25 or 50 pg/embryo \textit{sox18} RNA diluted in Danieau buffer.

For \textit{vegfc} RNA injections, plasmid pCS2+\textit{vegfc} \(^8\) was digested with \textit{NotI} and transcribed with Sp6 RNA polymerase. As an internal control, we wanted to check if \textit{vegfc} RNA could rescue the severe lymphatic phenotype in \textit{vegfc} morphants. Given the full complementarity of \textit{vegfc}-MO to \textit{vegfc} RNA, all injections with \textit{vegfc} RNA were carried out using two independent needles: the same 1-cell stage embryos were injected first with 1 nl of \textit{vegfc} RNA (50 pg) and then with 1 nl of \textit{vegfc}-MO or \textit{sox18}-MO2 (0.5 or 1 pmol, respectively).
**Specificity of sox18/sox7-MOs**

All sox18/sox7-MOs used in this study had been previously described. Specificity of the splicing morpholinos (sox18-MO2, sox7-MO2) had been addressed by checking through RT-PCR that each splicing morpholino was altering processing of its target pre-mRNA, without affecting the splicing of the other sox transcripts (as shown in Figure S4 of the above mentioned paper). The efficacy of the translation blocking MOs (targeting the AUG region or the 5’UTR) could not be directly tested due to lack of specific antibodies. However, we could show that three independent sets of MOs (sox18-MO1+sox7-MO1, sox18-MO2+sox7-MO2, sox18-MO4+sox7-MO4) when coinjected at low doses, but not when injected separately, were producing the same circulatory phenotype in the trunk region. Taken together, these observations strongly support the notion that all sox18/sox7-MOs presented in Cermenati et al., 2008 are specific for their targets.

The sequences of the sox18/sox7-MOs used in this study are reported below. For splicing MOs, sequences complementary to the intron sequence of the pre-mRNA target are shown in lowercase letters:

- sox18-MO1 5’-TATTCATTCCAGCAAGACCAACG-3’
- sox18-MO2 5’-gtgagtgctttacCCAGCATTTTAC-3’
- sox7-MO1 5’-ACGCACTTATCAAGGCGCCATGTG-3’
- sox7-MO2 5’-gttataatctttacCAAGCATCTTGC-3’

**Phenotypic analysis**

The analysis of TD formation is commonly used to study lymphatic development in zebrafish. We analyzed TD formation by scoring its length along 10 consecutive trunk segments, up to the anus, in tg(fli1a:EGFP) larvae at 5 dpf, as previously described. Due to the variability of the lymphatic phenotype, the analyzed larvae were distributed into five phenotypic classes of increasing severity: fully formed TD (normal TD), TD present in 7-9 segments (70-90% TD), 4-6 segments (40-60% TD), 1-3 segments (10-30% TD) and absent TD. At 1.5 to 2.5 dpf, we scored 10 segments in the same region also to analyze the number of sprouts from the vein, PLs and a/v ISVs. All larvae analyzed in this study were circulating to avoid secondary defects that would interfere with TD phenotypes.
Production of \(\text{tg}(hsp70l:Sox18^{RaOp} \text{ mCherry})\) transgenic line

The inducible construct consisting of an in frame fusion of the \(Sox18^{RaOp}\) cDNA with mCherry, under the control of the \(hsp70l\) promoter, was generated with Gateway system (see below for detailed description). The construct is sketched in Figure 3A. The pDestTol2CG-Ragged plasmid was injected with Tol2 transposase RNA (25ng/µl) to generate \(\text{tg}(hsp70l:Sox18^{RaOp} \text{ mCherry})\) lines. Founders, crossed with \(\text{tg}(fli1a:EGFP)^{y1}\) or \(\text{tg}(fli1a:EGFP)^{y1};\text{tg}(flt1^{enh}:RFP)\), were heat shocked at 37-38°C for 1 hour and embryos sorted for mCherry expression 3-4 h post-heat shock to confirm induction of construct expression. PLs and a/vISVs were counted at 54-56 hpf and the presence of TD was scored at 5 dpf.

Construct for \(\text{tg}(hsp70l:Sox18^{RaOp} \text{ mCherry})\) transgenic line

The \(RaOp\) mutation was introduced into a mouse \(Sox18\) cDNA using site-directed mutagenesis (Quickchange Lightning Site-Directed Mutagenesis Kit, Stratagene) with the primers Sox18ragmut f (5'-GAGCCTGGCGAGGCTCCTTCTTCCCA-3') and Sox18ragmut r (5'-TGGAAGAAGGAGCCTCGCCAGGCTC-3'). This cDNA sequence was amplified using the primers attB1kozacsox18F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGACCATGCAGAGATCGCCGCCCGGC-3') and sox18ragged-nostop-attB2R (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGTTTGGCCAGTGCCACGTGGT-3') to remove the stop codon and clone into a Gateway entry vector. The amplified insert was cloned into DONR211 donor using Gateway technology to generate a pME-\(Sox18^{RaOp}\) middle entry vector.10, 11 p5E-\(hsp70l\) 5'entry clone, p3E mCherry-pA 3' entry clone, pME-\(\text{ragged}\) middle entry vector and pDestTol2CG were combined to generate pDestTol2CG-\(\text{ragged}\) where \(Sox18^{RaOp}\) is fused with mCherry and under the control of the \(hsp70l\) promoter.

In situ hybridization and imaging

Whole-mount in situ hybridizations (ISHs) were carried out essentially as described.4 For ISHs on AB embryos, we synthesized probes as described in the following papers: \(sox18\) and \(sox7^4\), \(cdh5^{12}\) and \(vegfc^8\). \(ephB4\) was kindly provided by R. Patient.
For ISHs on tg(fli1a:EGFP) embryos, to avoid background problems as reported on the dedicated web page at zfin.org and recently published, we generated probes using the following primers and templates:

<table>
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<th>probe</th>
<th>primer sequence</th>
<th>template</th>
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<tr>
<td>sox18</td>
<td>5’-GGAGCCAGGAGTTACAAAACAC-3’&lt;br&gt;5’-CTAATACGACTCATATAGGGCTCCATATGTGCACCAGACTTC-3’</td>
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<td>4</td>
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<tr>
<td>sox7</td>
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<td>Image clone 7045912</td>
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<tr>
<td>flt4</td>
<td>5’-CAAGTCGACGGATGATAGA-3’&lt;br&gt;5’-CTAATACGACTCATAAGGGGAATGTGGGCCACCCAGTG-3’</td>
<td>pBSflt4</td>
<td>14</td>
</tr>
<tr>
<td>dab2</td>
<td>5’-GCTCTTGCTGCTCGTCTCC-3’&lt;br&gt;5’-CTAATACGACTCACAATAGGGGATGCAGCAAGCGACATTC-3’</td>
<td>pBK-CMVdab2</td>
<td>15</td>
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<tr>
<td>lyve1</td>
<td>5’-AAGGGTTTTGGTGCCATGTC-3’&lt;br&gt;5’-CTAATACGACTCATAAGGGGATGATGTGCATGCTGCATTC-3’</td>
<td>Image clone 679881</td>
<td>16</td>
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</table>

Images were taken with a Leica MZFLIII epifluorescence stereomicroscope equipped with a DFC 480-R2 digital camera and the LAS imaging software (Leica, Wetzlar, Germany). Confocal microscopy was performed on a Leica TCS SP2 AOBS microscope, equipped with an argon laser, or a Zeiss 510 microscope. Images were processed using the Adobe Photoshop software (Adobe, San Jose, CA) or Imaris software packages.

**Histological sections**

For histological analysis after ISH, embryos were re-fixed in 4% PFA, dehydrated, wax embedded, sectioned (8 µm) with a microtome (Leitz 1516) and stained with eosin. Images were taken with a Leica microscope equipped with a Leica 480 digital camera and the LAS software (Leica, Germany).

**Statistical analysis**

Statistical analyses were performed with Student’s t-test or one-way ANOVA followed by Dunnett’s Multiple Comparison post-test, when needed, using GraphPad PRISM version 5.0 (GraphPad, San Diego, CA). In the graphs, * and ** mark statistically significant data with a p value <0.05 and <0.01, respectively. Statistically highly significant data, with a p value <0.001, are marked by ***.
Quantitative RT-PCR analysis

Quantitative Real Time reverse-transcriptase (qRT) polymerase chain reaction (PCR) analysis was performed on RNA extracted at 32.5 hpf from pools of around 30 Cherry+ embryos of the tg(hsp70l:Sox18RaOp mCherry) line heat-shocked at 29 hpf or the same number of Cherry- non heat-shocked controls. Total RNA was isolated with the RNAeasy Minikit (Qiagen) and reverse-transcribed with the Superscript III kit (Invitrogen). Quantitative PCR was performed in technical triplicates using SYBR Green PCR mastemix (Applied Biosystem) according to manufacturer’s instructions. Gene expression was normalized to hprt1; relative fold-changes were calculated by the comparative Ct or ΔΔCt method, where Ct stands for threshold cycle.

Primers were as follows: vegfc-qF 5’-ACCCTACCTACCGGATCATG-3’, vegfc-qR 5’-TCAAAACAACGTCTTGCTGATG-3’; cdh5-qF 5’-AAGCCCAATGTTGACCTAAT-3’, cdh5-qR 5’-ATGGTAACACGCTAGTGCC-3’; hprt1-qF1 5’-ATCATGGACCGAACTGAACG-3’, hprt1-qR1 5’-AGCGATCACTGTTGCGATTA-3’.
sox18 and vegfc in early lymphatic development

References for Materials and Methods

Figure I. Knockdown of *sox18* leads to a statistically highly significant increase in TD defects. 

(A) In the bar chart, we present cumulative data from all the different experiments we performed analyzing TD formation at 5 dpf in control larvae (std-MO) and in larvae injected with 1pmol of *sox18*-MO2. All analyzed tg(*flila*:EGFP)y1 larvae (276 controls and 211 *sox18* morphants) were circulating. 

(B-F) For all phenotypic classes, based on the analysis of 10 consecutive trunk segments per larva, there is a statistically highly significant difference between *sox18* morphants and control larvae. In particular, we observed a decrease in the percentage of *sox18* morphants with fully formed TD (B) and an increase in the percentages of *sox18* morphants with TD present in 7-9 segments (C), 4-6 segments (D), 1-3 segments (E), or with no TD (F).

*** = p<0.001 vs std-MO, ** = p<0.01 vs std-MO.
Figure II

Figure II. Knockdown of **sox18** with an independent MO produces also a lymphatic phenotype, while knockdown of **sox7** causes only very minor lymphatic defects. (A,B) We analyzed TD formation in control larvae (std-MO), and in larvae injected with an independent morpholino against **sox18** (**sox18-MO1**), at 5 dpf. Circulating **tg(fli1a:EGFP)** larvae were analyzed by scoring the presence/absence of TD within 10 consecutive intersomitic segments (bar chart B). Also in **sox18-MO1** morphants TD development is defective (white asterisks in A) if compared with control embryos (white arrowheads in A), although the defects observed are less pronounced than those obtained with **sox18-MO2**. (C,D) We analyzed TD formation at 5 dpf in control larvae (std-MO), and in larvae injected with **sox7-MO2**. Circulating larvae were analyzed by scoring the presence/absence of TD within 10 intersomitic segments (bar chart D). In **sox7-MO2** morphants TD development is similar to std control morphants (white arrowheads in C).
**Figure III**

Figure III. The expression of sox7 is largely unaffected in sox18 morphants. The expression of sox7 was analyzed by ISH at 18-20 somites (a,d,g), 24 hpf (b,e,h) and 1.5 dpf (c,f,i) and we found no gross differences between control embryos (a,b,c), sox18 single morphants (d,e,f) and sox18-vegfc double partial morphants (g,h,i). All embryos were of the tg(fli1a:EGFP)y1 line. Images were taken at 40X (a,b,d,e,g,h) or 63X magnification (c,f,i), lateral views anterior to the left. White arrowhead: DA, black arrowhead: PCV.
Figure IV. a/v ISV numbers are not significantly impaired even in the most severely affected sox18 morphants and in sox18 and vegfc single partial morphants. (A,B) We performed a set of three experiments counting the number of arterial and venous intersomitic vessels in circulating sox18 morphants and controls at 2.5 dpf. The tg(fli1a:EGFP)y1 embryos were kept separate to analyze their individual TD phenotype at 5 dpf and subdivided into phenotypic classes. (A) t-test analysis revealed no significant variations between aggregated sox18 morphants and controls. (B) The comparison of a/v ISV numbers between sox18 morphants with mild and severe TD formation defects (TD present in 7-10 and 0-6 segments, respectively) did not highlight statistically significant differences. (C) We analyzed circulating sox18 and vegfc single partial morphants at 2.5 dpf scoring the number of a/v ISVs in the trunk region. In sox18-MO2 injected embryos (0.5 pmol), no gross differences were observed if compared to controls. vegfc single partial morphants display a slight decrease in the number of vISVs (and consequently a small increase in the number of aISVs), but these variations are not statistically significant.

Raw data are presented in Table III.
Figure V

A

<table>
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<tr>
<th>non-transgenic</th>
<th>tg(hsp70::Sox18RaOp mCherry) NO HS</th>
<th>tg(hsp70::Sox18RaOp mCherry) HS 24 hpf</th>
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<td>HS 24 hpf</td>
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48hpf

B

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<tr>
<th>tg(hsp70::Sox18RaOp mCherry) NO HS</th>
<th>tg(hsp70::Sox18RaOp mCherry) HS 29 hpf</th>
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</table>

5dpf

C

<table>
<thead>
<tr>
<th>cdh5</th>
<th>vegfc</th>
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<tbody>
<tr>
<td>![Graph](cdh5 график)</td>
<td>![Graph](vegfc график)</td>
</tr>
</tbody>
</table>

sox18 and vegfc in early lymphatic development
Figure V.

(A) \(tg(hsp70l:Sox18^{RaOp} \text{mCherry})\) embryos following heat shock at 24 hpf display cardiovascular defects and generalized oedema. (A, upper panels) Gross morphology of embryos at 48 hpf following 24 hpf heat shock. Genotype and treatment indicated above in Supplementary Materials and Methods and in the main text (see Materials and Methods). Arrow indicates cardiac oedema in \(tg(hsp70l:Sox18^{RaOp} \text{mCherry})\) embryos post heat shock. (A, lower panels) Gross morphology of \(tg(hsp70l:Sox18^{RaOp} \text{mCherry})\) larvae (as indicated on the left) compared with non-transgenic larvae (right) at 5 dpf, post heat shock at 24 hpf.

(B) \(tg(hsp70l:Sox18^{RaOp} \text{mCherry})\) embryos following heat shock at 29 hpf display PL defects. General trunk views of non heat-shocked controls and heat-shocked embryos at 56 hpf visualized by \(tg(fli1a:EGFP)^{y1}\) expression. Red arrows point to PL+ segments, asterisks mark absence of PLs.

(C) Heat-shock induced overexpression of Sox18\(^{RaOp}\) results in reduced \(vegfc\) expression. A representative qRT-PCR experiment is shown here (experimental details are described above in Supplementary Materials and Methods). The pan-endothelial \(cdh5\) gene shows no gross difference between non heat-shocked and heat-shocked embryos, while \(vegfc\) expression is reduced post heat shock.
sox18 and vegfc in early lymphatic development

Figure VI

Figure VI. Injection of vegfc morpholino dose-dependently affects TD formation. The sub-critical dose of vegfc-MO that causes only mild defects in TD formation (0.06 pmol) was identified through analysis of a dose-response curve. Circulating tg(flila:EGFP)\textsuperscript{y1} larvae were analyzed by scoring the presence/absence of TD within 10 consecutive intersomitic segments at 5 dpf, and subdivided into phenotypic classes. The bar chart in the figure shows that raising the morpholino dose of 50% to 100% (0.09 and 0.13 pmol) the percentage of embryos with most severe TD defects is at least 10-fold greater than what observed with the sub-critical vegfc-MO dose (0.06 pmol).
Figure VII

**Figure VII.** Combined partial knockdown of *sox18* and *vegfc* leads to a statistically significant increase of severely affected larvae. We performed statistical analysis on TD formation data of control larvae, *sox18* and *vegfc* single partial morphants and *sox18*-*vegfc* double partial morphants (see Figure 4). We observed a statistically significant decrease in the percentage of *sox18*-*vegfc* MOs double injected larvae that have a normal TD with respect to both control larvae and single partial morphants (A). This is linked to the statistically significant increase in the percentage of larvae belonging to the most affected classes, *i.e.* with 1-3 TD segments (D) or absent TD (E). No significant variations were found among controls and double/single partial morphants in the classes with mild TD defects, *i.e.* TD present in 7-9 (B) or in 4-6 segments (C). *** = p<0.001 vs std-MO; ** = p<0.01 vs std-MO; * = p<0.05 vs std-MO.
sox18 and vegfc in early lymphatic development

Figure VIII

A

B

C

n=48  n=62  n=54  n=43

n=35  n=20  n=36

sox18-MO  0.5pmol  0.5pmol  0.06pmol

vegfc-MO  0.03pmol  0.03pmol

std-MO

1pmol

1pmol

10-30% TD

40-60% TD

70-90% TD

normal TD
Figure VIII. TD formation is synergistically affected even when halving the subcritical doses of sox18-MO2 and vegfc-MO or coinjecting suboptimal doses of sox18-MO1 and vegfc-MO.

(A) The subcritical doses of sox18 and vegfc MOs showed in Figure 4 were reduced by 2-fold to analyze TD formation in sox18 and vegfc single or double morphants at 5 dpf. Only the coinjection but not the single injection of sox18- and vegfc-MOs (0.25pmol and 0.03pmol respectively) causes noticeable TD formation defects. Statistical analysis is shown below (C).

(B) We also analyzed TD formation in control larvae (std-MO), and in larvae injected with a subcritical dose of sox18-MO1 (0.5 pmol) or coinjected with subcritical doses of sox18-MO1+vegfc-MO (0.5 + 0.06 pmol). The bar chart shows that TD formation is severely impaired only in double partial morphants.

(C) Statistical analysis of the data in bar chart A. We observed a statistically significant decrease in the percentage of sox18-vegfc double partial morphants that have a normal TD with respect to both control larvae and single partial morphants. This is linked to the statistically significant increase in the percentage of larvae belonging to the most affected classes, i.e. with 1-3 TD+ segments or absent TD. No significant variations were found among controls and double/single partial morphants in the classes with mild TD defects. *** = p<0.001, ** = p<0.01, * = p< 0.05 vs std-MO.
Figure IX. TD formation is synergistically affected by the coinjection of suboptimal doses of *sox18-MO2* and *flt4-MO*. When injected separately, the subcritical dose of *sox18-MO2* (0.5pmol) does not lead to major TD formation defects, as observed in controls, while *flt4-MO* affects TD formation in a sizeable manner even at the suboptimal dose (0.06pmol). Nevertheless, the defects observed upon coinjection of the two MOs are much more severe than the mere sum of the defects observed when they are injected separately. Circulating *tg(*fli1a*:EGFP)* larvae were analyzed at 5 dpf by scoring the presence/absence of TD within 10 consecutive intersomitic segments.
Figure X. The expression of *sox18* is not altered in *vegfc* morphants and the expression of *vegfc* appears unaffected in *sox18* morphants. Hybridizations with *sox18* probe were performed on std-MO (a,b,e) and *vegfc*-MO injected embryos (c,d,f). We also performed *vegfc* ISHs on control embryos and *sox18* morphants (g,h). No gross differences were found at the indicated developmental stages. Images were taken at 40X magnification, lateral views anterior to the left.
Figure XI

The injection of *vegfc* RNA partially rescues the lymphatic phenotype of *sox18* morphants. We analyzed TD formation in 5 dpf larvae of the *tg(fli1a:EGFP)* line. Circulating larvae were analyzed by scoring the presence/absence of TD within 10 consecutive intersomitic segments along the trunk and subdivided into phenotypic classes.

(A) The injection of 50 pg of *vegfc* RNA drastically reduces the severe TD formation defects of *vegfc* morphants (internal control, left bar chart) and it partially rescues TD formation defects of *sox18* morphants (right bar chart), although not as efficiently as *sox18* RNA (see Figure 1).

(B) The injection of 50 pg of *sox18* RNA rescues TD formation defects of *sox18* morphants (internal control), but it does not reduce the severe TD formation defects of *vegfc* morphants.

The number and percentages of larvae belonging to each phenotypic class are reported in Table VI.
sox18 and vegfc in early lymphatic development

Figure XII

Figure XII. Analysis of sox18 morphants and sox18-vegfc double partial morphants by ISHs. 
(A) ISHs were carried out with the pan-endothelial marker cdh5 (a-c) and the venous markers dab2 (d-f), ephb4 (g-i) and flt4 (j-l) in sox18 single morphants and in sox18+vegfc double partial morphants of the tg(fli1a:EGFP)y1 line. Around 29 hpf, their expression is largely unaffected. Images a-c, d-f, j-l are lower magnifications of embryos showed in Figure 6A (ephb4 ISHs are only shown here). The numbers of embryos with the pictured ISH signal among those analyzed in a typical experiment are shown in the lower left corner of each panel. Red arrows: DA; blue arrows:
PCV; black arrowheads: ISVs. (B) ISHs were carried out on 2 dpf tg(fli1a:EGFP)^y1 morphants with the pan-endothelial marker cdh5 (a,c,f) and the zebrafish ortholog of the mammalian lymphatic marker LYVE1 (b,d,e,g). lyve1+ sprouts are reduced in single sox18 morphants and almost absent in sox18-vegfc double partial morphants. Images are lower magnification of embryos showed in Figure 6B. Black arrows: ISVs, white arrows: lyve1+ sprouts, white arrowhead: lyve1+ sprouts reduced in length, asterisk: absence of lyve1+ sprouts.
Table I

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<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>sox18-MO2 1pmol + sox18 RNA25 pg</th>
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<tr>
<td>100% TD</td>
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<td>70-90% TD</td>
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<tr>
<td>absent TD</td>
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<td>10/56 (18%)</td>
<td>5/41 (12%)</td>
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TD analysis upon injection of sox18-MO2 and sox18-MO2 + sox18 RNA. The number (and percentage) of larvae belonging to each phenotypic class (shown in the bar chart in Figure 1) is reported; data were gathered in three independent experiments. The number of larvae with almost completely formed TD (normal TD and 70-90% TD) is nearly doubled in sox18-MO2+sox18-RNA (50pg) coinjected embryos compared with sox18 morphants. We also report a very strong reduction of the more affected classes (absent to 30% TD).

Data gathered in additional independent rescue experiments of sox18 morphants with sox18 RNA are shown in Table VI B.
sox18 and vegfc in early lymphatic development

Table II

<table>
<thead>
<tr>
<th>A</th>
<th>sprouts from the vein</th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>vegfc-MO 0.5pmol</th>
<th>sox18-MO2 0.5pmol + vegfc-MO 0.06pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>8.1000</td>
<td>5.0000</td>
<td>2.0630</td>
<td>3.7810</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.2054</td>
<td>0.4082</td>
<td>0.5735</td>
<td>0.4746</td>
</tr>
<tr>
<td></td>
<td>number</td>
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<td>n=33</td>
<td>n=16</td>
<td>n=32</td>
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<table>
<thead>
<tr>
<th>B</th>
<th>PL+ segments</th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>vegfc-MO 0.5pmol</th>
<th>sox18-MO2 0.5pmol + vegfc-MO 0.06pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>8.2670</td>
<td>5.1380</td>
<td>1.8670</td>
<td>4.4670</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.2346</td>
<td>0.5246</td>
<td>0.4145</td>
<td>0.5419</td>
</tr>
<tr>
<td></td>
<td>number</td>
<td>n=30</td>
<td>n=29</td>
<td>n=30</td>
<td>n=30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>vISV</th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>sox18-MO2 0.5pmol + vegfc-MO 0.06pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>5.1500</td>
<td>4.7963</td>
<td>3.2424</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.1423</td>
<td>0.1895</td>
<td>0.2275</td>
</tr>
<tr>
<td></td>
<td>number</td>
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<td>n=66</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>vISV</th>
<th>std-MO 1pmol</th>
<th>vegfc-MO 0.5pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>4.9500</td>
<td>1.1500</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.2348</td>
<td>0.3500</td>
</tr>
<tr>
<td></td>
<td>number</td>
<td>n=20</td>
<td>n=20</td>
</tr>
</tbody>
</table>

Sprouts from the vein, PL+ segments and a/v ISVs scores of sox18 and vegfc single morphants. Raw data used for the bar charts shown in Figure 2A and 5A (A), Figure 2B and 5B (B), and Figure 2C and 5C (C) are reported here. Only circulating embryos are indicated. All data presented here were obtained in three independent experiments, except for vISV scores in vegfc morphants and control embryos. SEM: standard error of the mean. Statistical analysis was performed on the complete data sets.
### Table III

#### A

<table>
<thead>
<tr>
<th></th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>vISV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>4.8550</td>
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<td>n=52</td>
</tr>
<tr>
<td>aISV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>5.0910</td>
<td>5.3080</td>
</tr>
<tr>
<td>SEM</td>
<td>0.1428</td>
<td>0.1933</td>
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</table>

#### B

<table>
<thead>
<tr>
<th></th>
<th>sox18-MO2 1pmol unaffected/mild</th>
<th>sox18-MO2 1pmol severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>vISV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>4.7330</td>
<td>4.3640</td>
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<tr>
<td>SEM</td>
<td>0.2296</td>
<td>0.3193</td>
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<tr>
<td>number</td>
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<td>n=22</td>
</tr>
<tr>
<td>aISV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>5.1000</td>
<td>5.5910</td>
</tr>
<tr>
<td>SEM</td>
<td>0.2317</td>
<td>0.3267</td>
</tr>
<tr>
<td>number</td>
<td>n=30</td>
<td>n=22</td>
</tr>
</tbody>
</table>

#### C

<table>
<thead>
<tr>
<th></th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 0.5pmol</th>
<th>vegfc-MO 0.06pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>vISV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>4.7000</td>
<td>5.1360</td>
<td>4.2920</td>
</tr>
<tr>
<td>SEM</td>
<td>0.1933</td>
<td>0.2307</td>
<td>0.2210</td>
</tr>
<tr>
<td>number</td>
<td>n=20</td>
<td>n=22</td>
<td>n=24</td>
</tr>
<tr>
<td>aISV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>5.2500</td>
<td>4.8640</td>
<td>5.6670</td>
</tr>
<tr>
<td>SEM</td>
<td>0.1902</td>
<td>0.2307</td>
<td>0.2225</td>
</tr>
<tr>
<td>number</td>
<td>n=20</td>
<td>n=22</td>
<td>n=24</td>
</tr>
</tbody>
</table>

**a/v ISVs counts.** Raw data used for the bar chart shown in Figure IV are reported here. The data were collected in three independent experiments. SEM: standard error of the mean.
**Table IV**

<table>
<thead>
<tr>
<th>PL+ segments</th>
<th>29hpf HS Cherry+</th>
<th>29hpf HS Cherry-</th>
<th>36hpf HS Cherry+</th>
<th>36hpf HS Cherry-</th>
<th>48hpf HS Cherry+</th>
<th>48hpf HS Cherry-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>5.1860</td>
<td>0.5278</td>
<td>4.3100</td>
<td>2.6490</td>
<td>5.2900</td>
<td>3.5710</td>
</tr>
<tr>
<td>SEM</td>
<td>0.3478</td>
<td>0.2373</td>
<td>0.4801</td>
<td>0.4123</td>
<td>0.4179</td>
<td>0.6346</td>
</tr>
<tr>
<td>number</td>
<td>n=43</td>
<td>n=36</td>
<td>n=42</td>
<td>n=37</td>
<td>n=31</td>
<td>n=21</td>
</tr>
</tbody>
</table>

**vISV**

<table>
<thead>
<tr>
<th></th>
<th>29hpf HS Cherry+</th>
<th>29hpf HS Cherry-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>4.9150</td>
<td>2.6040</td>
</tr>
<tr>
<td>SEM</td>
<td>0.1395</td>
<td>0.3060</td>
</tr>
<tr>
<td>number</td>
<td>n=59</td>
<td>n=77</td>
</tr>
</tbody>
</table>

**PL+ segments and vISVs counts after heat-shock induced expression of murine Sox18<sup>RaOp</sup>.**

Raw data used for the bar chart shown in Figure 3 are indicated here.

**Table V**

<table>
<thead>
<tr>
<th>std-MO 1pmol</th>
<th>sox18-MO2 0.5pmol</th>
<th>vegfc-MO 0.06pmol</th>
<th>sox18-MO2 0.5pmol + vegfc-MO 0.06pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% TD</td>
<td>48/52 (92%)</td>
<td>34/45 (76%)</td>
<td>30/37 (81%)</td>
</tr>
<tr>
<td>70-90% TD</td>
<td>4/52 (8%)</td>
<td>8/45 (18%)</td>
<td>5/37 (14%)</td>
</tr>
<tr>
<td>40-60% TD</td>
<td>0/52 (0%)</td>
<td>0/45 (0%)</td>
<td>2/37 (5%)</td>
</tr>
<tr>
<td>10-30% TD</td>
<td>0/52 (0%)</td>
<td>0/45 (0%)</td>
<td>0/37 (0%)</td>
</tr>
<tr>
<td>absent TD</td>
<td>0/52 (0%)</td>
<td>3/45 (6%)</td>
<td>0/37 (0%)</td>
</tr>
</tbody>
</table>

**TD formation analysis of sox18 and vegfc single/double partial morphants.** The number (and percentage) of larvae belonging to each phenotypic class shown in the bar chart of Figure 4 is reported here.
Table VI

**A, left bar chart**

<table>
<thead>
<tr>
<th>Phenotypic Class</th>
<th>std-MO 1pmol</th>
<th>vegfc-MO 0.5pmol</th>
<th>vegfc-MO 0.5pmol+ vegfc RNA50 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% TD</td>
<td>15/17 (88%)</td>
<td>0/13 (0%)</td>
<td>6/29 (21%)</td>
</tr>
<tr>
<td>70-90% TD</td>
<td>2/17 (12%)</td>
<td>0/13 (0%)</td>
<td>4/29 (14%)</td>
</tr>
<tr>
<td>40-60% TD</td>
<td>0/17 (0%)</td>
<td>1/13 (8%)</td>
<td>6/29 (21%)</td>
</tr>
<tr>
<td>10-30% TD</td>
<td>0/17 (0%)</td>
<td>0/13 (0%)</td>
<td>2/29 (6%)</td>
</tr>
<tr>
<td>absent TD</td>
<td>0/17 (0%)</td>
<td>12/13 (92%)</td>
<td>11/29 (38%)</td>
</tr>
</tbody>
</table>

**A, right bar chart**

<table>
<thead>
<tr>
<th>Phenotypic Class</th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>sox18-MO2 1pmol+ vegfc RNA50 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% TD</td>
<td>47/53 (89%)</td>
<td>11/60 (18%)</td>
<td>27/68 (40%)</td>
</tr>
<tr>
<td>70-90% TD</td>
<td>6/53 (11%)</td>
<td>18/60 (30%)</td>
<td>19/68 (28%)</td>
</tr>
<tr>
<td>40-60% TD</td>
<td>0/53 (0%)</td>
<td>17/60 (28%)</td>
<td>11/68 (16%)</td>
</tr>
<tr>
<td>10-30% TD</td>
<td>0/53 (0%)</td>
<td>7/60 (12%)</td>
<td>7/68 (10%)</td>
</tr>
<tr>
<td>absent TD</td>
<td>0/53 (0%)</td>
<td>7/60 (12%)</td>
<td>4/68 (6%)</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Phenotypic Class</th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>sox18-MO2 1pmol+ sox18 RNA50 pg</th>
<th>vegfc-MO 0.5pmol</th>
<th>vegfc-MO 0.5pmol+ sox18 RNA50 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% TD</td>
<td>34/47 (78%)</td>
<td>5/34 (15%)</td>
<td>16/32 (50%)</td>
<td>0/38 (0%)</td>
<td>1/56 (2%)</td>
</tr>
<tr>
<td>70-90% TD</td>
<td>13/47 (28%)</td>
<td>8/34 (23%)</td>
<td>10/32 (32%)</td>
<td>2/38 (5%)</td>
<td>2/56 (3.5%)</td>
</tr>
<tr>
<td>40-60% TD</td>
<td>0/47 (0%)</td>
<td>14/34 (41%)</td>
<td>3/32 (9%)</td>
<td>3/38 (8%)</td>
<td>6/56 (11%)</td>
</tr>
<tr>
<td>10-30% TD</td>
<td>0/47 (0%)</td>
<td>1/34 (3%)</td>
<td>0/32 (0%)</td>
<td>7/38 (19%)</td>
<td>2/56 (3.5%)</td>
</tr>
<tr>
<td>absent TD</td>
<td>0/47 (0%)</td>
<td>6/34 (18%)</td>
<td>3/32 (9%)</td>
<td>26/38 (68%)</td>
<td>45/56 (80%)</td>
</tr>
</tbody>
</table>

TD analysis upon injection of vegfc RNA in vegfc and sox18 morphants (A) and of sox18 RNA in sox18 and vegfc morphants (B). The number (and percentage) of larvae belonging to each phenotypic class (shown in the bar charts in Figure XI) is reported here.
Table VII

<table>
<thead>
<tr>
<th>lyve1+ sprouts</th>
<th>std-MO 1pmol</th>
<th>sox18-MO2 1pmol</th>
<th>sox18-MO2 0.5pmol + vegfc-MO 0.06pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal sprouts</td>
<td>33/50 (66%)</td>
<td>6/54 (11%)</td>
<td>3/60 (5%)</td>
</tr>
<tr>
<td>reduced sprouts number</td>
<td>11/50 (22%)</td>
<td>21/54 (39%)</td>
<td>14/60 (23%)</td>
</tr>
<tr>
<td>shorter sprouts</td>
<td>6/50 (12%)</td>
<td>13/54 (24%)</td>
<td>20/60 (33%)</td>
</tr>
<tr>
<td>absent sprouts</td>
<td>0</td>
<td>14/54 (26%)</td>
<td>23/60 (39%)</td>
</tr>
</tbody>
</table>

Analysis of lyve1+ sprouts in sox18 morphants and sox18-vegfc double partial morphants.

Numbers (and percentages) of embryos shown in Figure 6B are reported here. These are cumulative data gathered in three independent experiments.

Among controls, the vast majority of embryos showed normal lyve1+ sprouts (33/50 std-MO injected embryos). On the contrary, normal lyve1+ sprouts were detectable in only about 10% of sox18 morphants and 5% of combined partial sox18-vegfc morphants (6/54 and 3/60, respectively), while the remaining morphants were variably affected. Among sox18 morphants, the prevalent phenotype we observed is the reduction of lyve1+ sprout number, accounting for almost 40% (21/54 vs 11/50 in controls), followed by embryos characterized by lyve1+ sprouts of reduced length (13/54 vs 6/50 in controls) and embryos devoid of lyve1+ sprouts (14/54; this class is not represented in controls). Among combined partial sox18-vegfc morphants, the absence of lyve1+ sprouts in the trunk characterized about 40% of the embryos (23/60), about 30% of them showed lyve1+ sprouts of reduced length (20/60 vs 6/50 in controls), while the percentage of embryos with a reduced number of lyve1+ sprouts was comparable to controls (14/60 vs 11/50)