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Zebrafish Sox7 and Sox18 function together to control arterial-venous identity

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Abstract

Sox7 and Sox18 are members of the F-subgroup of Sox transcription factors family and are mostly expressed in endothelial compartments. In humans, dominant mutations in Sox18 are the underlying cause of the severe hypotrichosis-lymphedema-telangiectasia disorder. However little is known about which vasculogenic processes Sox7 and Sox18 regulate *in vivo*. We cloned the orthologs of *Sox7* and *Sox18* in zebrafish, analysed their expression pattern and performed functional analyses. Both genes are expressed in the lateral plate mesoderm during somitogenesis. At later stages, *Sox18* is expressed in all axial vessels whereas *Sox7* expression is preferentially restricted to the dorsal aorta. Knockdown of *Sox7* or *Sox18* alone by a morpholino strategy failed to reveal any phenotype. In contrast, blocking the two genes simultaneously led to embryos displaying dysmorphogenesis of the proximal aorta and arteriovenous shunts, all of which can account for the lack of circulation observed in the trunk and tail. Gene expression analyses performed with general endothelial markers in double morphants revealed that *Sox7* and *Sox18* are dispensable for the initial positioning of the major trunk vessels. In contrast, expression of the artery-specific *EphB2A* marker and of the venous marker *Flt4* was substantially altered, indicating that Sox7 and Sox18 are specifically required together for arterial-venous differentiation. Parler de SHH, VEGF

Keywords: zebrafish; *Sox7*; *Sox18* ; artery-vein

Introduction

Vasculogenesis is the process of *de novo* blood vessel formation that involves migration and differentiation of precursors of endothelial cells, in response to local cues (such as growth factors and extracellular matrix) (Risau, 1997). In angiogenesis, new blood vessels sprout from pre-existing ones and further remodelled to form mature blood vessels (Flamme et al., 1997). In zebrafish, as in other species, blood vessel development is an exquisite process that requires genetic interactions between several signalling molecules, ligands, tyrosine kinase-type receptors and their downstream effectors (Weinstein, 2002a). A wealth of information concerning key players of the VEGF pathway or molecules involved in vessel guidance is now available (Bielenberg and Klagsbrun, 2007; Goishi and Klagsbrun, 2004; Torres-Vazquez et al., 2004). However, aside members of the Ets family (Patterson and Patient, 2006; Pham et al., 2007), very few trancription factors have been shown to participate in blood vessel development.

Members of the SRY-related high mobility group box (Sox) superfamily encode transcription factors involved in developmental processes. Sox proteins bind the heptameric consensus sequence 5'-(A/T)(A/T)CCA(A/T)G-3'via their HMG domain. To date, the vertebrate *Sox* family comprises about 26 genes that can be classified into 7 subgroups (A-G) based on sequence similarity (Bowles et al., 2000). *Sox7*, *Sox18* and *Sox 17* belong to the F-subgroup and have been implicated in endothelial pathologies (Downes and Koopman, 2001; Matsui et al., 2006; Sakamoto et al., 2007).

Several recessive and dominant mutations in the *Sox18* gene are the underlying cause of the severe human hypotrichosis-lymphedema-telangiectasia disorder which is characterized by chronic edema at the extremities and abnormal dilation of capillary and arterioles (Irrthum et al., 2003). In the mutant *ragged* (Ra) mice, which display defective cardiovascular and hair follicle formation, mutations in the transactivation domain of Sox18 lead the protein to act in a dominant-negative manner (Pennisi et al., 2000b). As mice null for Sox18 have only a mild coat defect but no developmental vascular-associated phenotype (Pennisi et al., 2000a), it was suggested that there is a functional redundancy amongst some Sox proteins from the same subgroup, that can account for the mild phenotype observed in these mice. In pathological situation however, the *Sox18* null mice present reduced tumor vascularization and subsequent growth, suggesting that interfering with Sox18 function impairs tumoral

angiogenesis and growth (Young et al., 2006). The role of Sox18 in angiogenesis is further supported by the observation that Sox18 is detected in granulation tissue during wound healing in adult mice (Darby et al., 2001). During mouse embryogenesis, *Sox18* expression can be observed in endothelial cells of blood vessels, in the heart and in the mesenchyme underlying the vibrissae and pelage hair follicle (Pennisi et al., 2000b). In human, as in mouse, *Sox18* expression is not restricted to embryonic development and can be detected in coronary atherosclerotic lesions (Garcia-Ramirez et al., 2005). Studies have also shown that Sox18 is essential for the expression of VCAM-1 (vascular cell adhesion molecule-1) and interacts with the muscle and endothelial transcription factor MEF2C (Hosking et al., 2001; Hosking et al., 2004).
The physiological function of Sox7 has been less documented. During mouse development, *Sox7* expression is mainly detected within cells of the developing vasculature such as the small branching vessels and intersomitic vessels, but also in the heart, lung and gut (Takash et al., 2001).
The mechanism underlying Sox7 and Sox18 function in the vasculature remains to be elucidated. In zebrafish, although blood circulation is established early, passive oxygen diffusion allows embryos showing important vasculogenic defects to survive for a long period of time (up to 4 days post-fertilization), thereby facilitating the characterization of the phenotype (Weinstein, 2002b). In this report, we therefore used the zebrafish as a valuable model for analysing the role of Sox7 and Sox18 in vascular development.

Results

Cloning of the zebrafish *Sox7* and *Sox18* genes

In the process of decrypting the role of *Sox7* and S*ox18* genes in zebrafish vasculogenesis, we searched the zebrafish orthologs by mining EST and genomic databases. We amplified the *Sox7* and *Sox18* cDNAS by RT-PCR on mRNAs ranging from 2 hpf to 31 hpf. The predicted amino acid sequences indicate that zebrafish Sox18 (XP_694383) and Sox7 (AY423014) present respectively 61 and 73 % identity over their entire sequence with their human orthologs with a nearly perfect conservation in the HMG box (Fig. 1A). Dr-*Sox18* and Dr-*Sox7* are the true orthologs of the human genes as the phylogenetic tree clearly shows that the zebrafish Sox18 and Sox7 proteins fit into the Sox18 and Sox7 clade, respectively (Fig. 1B). Moreover synteny occurs between the zebrafish and the human chromosomal regions harboring the *Sox18* and *Sox7* genes. Indeed, the zebrafish *Sox18* gene, located on chromosome 23, is directly adjacent to the *tcea2* gene like its human counterpart which is located on chromosome 20. Similarly, Dr-*Sox7* and Hs-*Sox7* locus on chromosome 20 and 8, respectively share the same gene organisation (*Sox7*, *PinX1*, *Q96KT1* and *MTMR9*) (data not shown).

Sox7 and *Sox18* have overlapping and distinct expression patterns in the endothelial compartment

We next assessed *Sox7* and *Sox18* expression during zebrafish embryogenesis. Whole mount *in situ* hybridizations performed on zebrafish embryos at different stages of development showed no expression of *Sox7* and *Sox18* before the end of gastrulation (data not shown). *Sox7* and *Sox18* transcripts are first detected at bud stage and are located in bilateral stripes in the posterior lateral plate mesoderm (Figs. 2A, A,F). During somitogenesis, these expression domains extend along the posterior part of the lateral mesoderm whereas a signal corresponding to the anterior lateral plate mesoderm also starts to appear in *Sox7* and *Sox18*-stained embryos (Figs. 2A, B,G). During these early stages, *Sox18* transcripts are expressed at lower levels than *Sox7*. At 18 hpf (hours post-fertilization), *Sox7* and *Sox18* transcripts are found in the head and in the presumptive axial vessels that start to coalesce and differentiate at the midline to generate the dorsal aorta and the axial vein (Figs. 2A, C,H). At 24 hpf, by the time circulation starts in zebrafish, *Sox7* and *Sox18* are expressed in the head vessels (Figs. 2A, D,I). In addition, two discrete stripes corresponding to the *Sox7* riboprobe are detected in the hindbrain. A signal corresponding to both genes is also seen more posteriorly, in the vasculature of the trunk and tail: in the axial vessels, in the forming intersomitic vessels and in the intermediate cell mass (ICM). Closer inspection of the staining reveals that *Sox18* expression can be detected in the dorsal aorta and in the axial vein (Fig. 2A, J). In contrast, *Sox7* expression is rather restricted to the dorsal aorta at that stage (Fig. 2A, E). As, in zebrafish, the posterior lateral plate mesoderm contributes notably to the formation of the vascular and the blood lineages, we next wanted to define more precisely in which compartment *Sox7* and *Sox18* are expressed. We then performed double fluorescent *in situ* hybridizations with *Flk1* and *Scl* riboprobes, that respectively mark endothelial cells and hemangioblasts, on wild-type embryos at 18 somite stage, at a time point where populations of cells expressing markers for different lineages have already spatially migrated and are clearly distinguishable (Brown et al., 2000; Gering et al., 1998). Transverse sections from double-stained embryos showed that *Sox7* perfectly colocalizes with $Flk1^+$ cells in two subpopulations of cells. One domain of $Flk^+/Sox7^+$ expressing cells is located in the presumptive dorsal aorta (1 in Fig. 2B) These cells no longer express *Scl* (compare sections in L

and O) and are totally committed to an endothelial fate. The other domain consists in two bilateral patches (2 in Fig. 2B) that still express *Scl* and that are located above the endoderm. These patches of cells most likely correspond to precursors of endothelial cells that will later contribute to the formation of the axial vein. Below the dorsal aorta, in a region corresponding to the ICM (arrows in O,P) there is a population of *Scl*-expressing cells that do not express *Sox7*. These cells will be committed to the blood lineage. Double staining with a *Sox18* specific riboprobe showed similar results (data not shown). From these experiments, we can therefore conclude that *Sox7* and *Sox18* are solely expressed in the endothelial compartment at that stage.

Inhibition of *Sox7* and *Sox18* leads to a lack of circulation in the trunk and tail

Because *Sox7* and *Sox18* are expressed in the lateral plate mesoderm and then in the axial vessels, we wondered whether they might have a role in vasculogenesis or angiogenesis. We therefore used the MO antisense knockdown approach to uncover possible defects in the vasculature caused by Sox 7 and Sox 18 interference. We decided to use morpholinos designed to target *Sox7* or *Sox18* ATG and inhibit translation (*MO1Sox7, MO1Sox18*). Efficacy of these MO was confirmed . These were injected either alone or in combination in fertilized eggs and the phenotype analyzed at 28 hpf, after the onset of circulation (Figure 3). No change between control and injected embryos was observed with morpholino knockdown of either *Sox7* or *Sox18*. In contrast, combined doses of *MO1Sox7* and *MO1Sox18* at 4ng each resulted in a consistent phenotype, in which the embryos looked overtly normal but in which circulation in the trunk and tail was never established. Circulation within the head vessels was not affected and the heart appeared to beat normally. The circulation defect eventually resulted in pericardial oedema at 3-4 dpf and these embryos died shortly thereafter. The phenotype is very penetrant as approximately 95% of the double morphants suffer from a lack of circulation (Fig. 3, A). Control embryos, injected with 5-base mismatch *Sox7* and *Sox1*8 morpholinos had normal blood cell circulation within the axial vessels, intersomitic vessels and the dorsal anastamosing longitudinal vessels. The lack of circulation in the trunk and tail of Sox7/Sox18 knockdown embryos was clearly demonstrated by performing a microangiography analysis in which we injected a fluorescent dye into the sinus venosus of Sox7/Sox18 morphants and, as control, of *Sox7mut*/*Sox18mut* morphants at 48 hpf (Fig. 3, B).
The specificity of the phenotype was further confirmed by the injection of another set of morpholinos: one that targets the second exon-intron junction of Sox7 (*MosplSox7*) and whose effectiveness was confirmed by RT-PCR (data not shown) and one translational Sox18 morpholino (*MO2Sox18*). When both were simultaneously injected, they produced a similar absence of blood circulation phenotype (Fig.3, A).

Inhibition of *Sox7* and *Sox18* affects arterial-venous identity

As a first step toward understanding the phenotype observed following inhibition of *Sox7* and *Sox18*, we performed microinjections into the *Tg*(*fli1:EGFP*)^{y1} transgenic line that drives *eGFP* expression into endothelial cells and their angioblasts precursors (Lawson and Weinstein, 2002). Carrying out knockdown experiments in the *Tg*(*fli1:EGFP*)^{y1} line shows that the *de novo* formation of the main head and axial vessels, and of the segmental arteries was largely unaffected in the double morphants in comparison with that in control embryos at 28 hpf (Fig. 3, C). In order to elucidate the cellular basis of the vascular defect, we next analyzed the expression of several known markers of endothelial differentiation by whole mount *in situ* on 28 hpf control and double *Sox7*/*Sox18* MO-injected embryos at a regular dose (4ng) (Fig. 4, A). These included the general markers of endothelial cells *Fli1*, *Flk1*, some endothelium-specific receptor tyrosine kinases markers such as *Tie1* and *Tie2*, and *hRT* and *VEGF* that are specifically expressed in the dorsal wall of the aorta and in the somites, respectively (Brown et al., 2000; Liang et al., 2001; Liao et al., 1997; Lyons et al., 1998; Szeto et al., 2002). The *in situ* experiments revealed a slight diminution of *Flk1* expression at 28 hpf in the double morphants while expression of the other genes was not affected. These data suggest that initial migration of angioblasts precursors correctly occurred and that the trunk vessels assemble normally. To test whether the phenotypic effect could reflect a lack of proper specification of the endothelial cells we then stained *Sox7*/*Sox18* MO-injected embryos with riboprobes corresponding to the arterial marker *EphB2a* and to the venous marker *Flt4* (Lawson et al., 2001). (Fig. 3,B). These experiments showed a clear ectopic expression of *Flt4* in the dorsal aorta, in addition to its normal restricted expression in the axial vein at that stage. Furthermore, a slight, but reproducible, reduction of *EphB2a* signal was also observed in the dorsal aorta of morphant embryos. As the venous state is the "default" state during artery-venous specification, arterial expression of *Flt4* is normally downregulated at 24 hpf to allow the dorsal aorta to fully acquire arterial characteristics (Lawson et al., 2001). This is not the case in *Sox7*/*Sox18* morphant embryos. These data strongly suggest that ablation of *Sox7* and *Sox18* prevents primary vessels to fully acquire arterial identity. In a attempt to explain why the blood does not circulate into the caudal part of the embryos, we then carefully checked vessels formation of *Sox7*/*Sox18* morphants into a *Tg*(*fli1:EGFP*) background. Closer inspection of 48hpf *Sox7*/*Sox18*-injected embryos revealed dysmorphogenesis of the proximal aorta, at the level where the two paired lateral dorsal aortae fuse into a single tube (Fig. 4C, I,K) (Isogai

et al., 2001). Furthermore, histological sections performed through the trunk of *Sox7/Sox18* morphants at 48 hpf also revealed some abnormalities in the morphology of the main axial vessels (Fig. 4C, J,L). In the control sections, the dorsal aorta and the axial vein are always clearly distinguishable. In contrast, in morphant embryos, demarcations between the artery and the vein are poorly defined, resulting, at some levels of the trunk axis, in arteriovenous shunts (as shown in panel L). These morphogenetic defects explain the lack of circulation in the trunk and tail. Taken together, these data strongly suggest that Sox7 and Sox18 are required for proper arterial identity and seem necessary to allow primary vessels to display clear arteriovenous demarcations.

Hematopoeitic precursors are not affected in *Sox7/Sox18* morphants

Since *Sox7* and *Sox18* are expressed in the lateral plate mesoderm at early somitogenesis stages, in the region where vessels, primitive hematopoietic stem cells, and pronephric development occurs, we wanted to verify whether there was no contribution of an other lineage to the phenotype observed in double morphant embryos.
To test whether the blood lineage was affected in the morphants, we examined the expression of two blood markers: *Scl/Tal1*, a marker of hemangioblast and *Gata1*, which is a typical marker for erythroid differentiation, at 9 and 18 somite stage (Detrich et al., 1995; Gering et al., 1998) (Figure 5). At 9 somite stage, we found that the pattern of *Scl* expression in the anterior and posterior lateral plate mesoderm was normal in morphants. Similarly, hybridization to probes for *Scl* and *Gata1* at 18 somite stage indicated that abolition of *Sox7*/*Sox18* has no effect on the expression of *Scl* and *Gata1*, which extends along the entire axis in the posterior region. These data suggested that formation of the hematopoietic precursors is unaffected. Finally, *in situ* hybridization results with the pronephric duct marker *Pax2.1* (Majumdar et al., 2000) showed no abnormal change in *Sox7/Sox18*-28hpf injected embryos (data not shown).

Discussion

To gain insights into the function of *Sox7* and *Sox18* in blood vessel development, we have examined the expression and the function of *Sox7* and *Sox18* in zebrafish embryogenesis. Both of these genes were cloned and functionally blocked by a morpholino-based knockdown strategy. We showed that *Sox7* and *Sox18* transcripts are detected as bilateral mesodermal stripes during somitogenesis. At later stages, *Sox18* is expressed in all axial vessels whereas *Sox7* is mostly located in the dorsal aorta and in the ICM. That restricted expression pattern in the vascular compartment is somewhat similar to the expression of *Sox7* and *Sox18* in other species, pointed by expression experiments performed in mice, human, and Xenopus (Fawcett and Klymkowsky, 2004; Pennisi et al., 2000b). However, *Sox7* and *Sox18* transcripts are also found in the heart of mice and Xenopus but were not revealed by our work in zebrafish embryos. That discrepancy is further intriguing as a recent report has described a partial inhibition of cardiogenesis in Xenopus embryos co-injected with *Sox7* and *Sox18* morpholinos, supporting the idea that the heart expression is functionally relevant in other species (Zhang et al., 2005). Furthermore, abolition of *Sox7* and *Sox18* in zebrafish did not reveal any heart-specific phenotype but led instead to an interesting vascular-associated phenotype as double morphant embryos lack circulation in the trunk and tail. Cardiac contractility and size appeared to be normal and no difference in the signal of a heart-specific Cmcl2 riboprobe could be detected on 28 hpf double morphant embryos (data not shown). Taken together and consistent with the expression pattern of Sox7 and *Sox18*, these data suggest that the lack of circulation cannot be linked to a heart defect.
Similarly to the mild phenotype observed in *Sox7* morphants in Xenopus embryos or in *Sox18* null mice, injection of a Sox7 or a Sox18 morpholino separately led to no apparent defect on embryogenesis, suggesting functional compensation by closely related factors. Our data, showing clear overlap in the expression of both *Sox7* and *Sox18* in the lateral mesoderm and then in the dorsal aorta was a first indication that they may act in a redundant fashion. We confirmed functional redundancy by showing that knockdown of both *Sox7* and *Sox18* affects artery-vein identity and blocks blood circulation in the trunk. Recently, murine Sox18 has been shown to act redundantly with Sox17, another factor of the F-subgroup of Sox proteins. In addition to its well-known expression in endodermal cells, *Sox17* is transiently expressed in some endothelial cells in the developing mouse embryos, and $Sox17^{+/-}$-$Sox18^{-/-}$ mutant mice display reduced postnatal neovascularization and early cardiovascular defects (Matsui et al., 2006; Sakamoto et al., 2007). In zebrafish, it seems unlikely that such redundancy occurs as *Sox17* expression is restricted to endodermal cells and has never been reported in the vasculature (Alexander and Stainier, 1999). Moreover, although previous studies performed in Xenopus had initially showed that surgical removal of the endoderm affects the formation of functional vascular vessels (Vokes and Krieg, 2002), experiments recently made in the zebrafish *Casanova* endodermless embryos (Kikuchi et al., 2001) that lack gut tube and do not express any markers of endoderm differentiation, clearly showed that the endoderm is dispensable for vascular differentiation (Jin et al., 2005).

Similar lack of circulation phenotype is found in many other mutant or morphant embryos described so far, many of which have impaired Shh, VEGF or Notch signalling (Brown et al., 2000; Lawson et al., 2002). Reasons for a failure to establish blood circulation can be multiple and can either result from abnormal early endothelial development or from improper formation or differentiation of immature vascular tubes. In zebrafish, vessel development commences with the apparition of common precursors of angioblasts and hematopoietic stem cells during gastrulation (Vogeli et al., 2006). During somitogenesis, these cells are located in the lateral plate mesoderm and migrate toward the midline while adopting either a vascular or a hematopoietic fate (Davidson and Zon, 2004; Thisse and Zon, 2002). Different waves of migration are involved in these dynamic differentiation processes (Jin et al., 2005). Interestingly, some lineage-tracing studies also suggest that the angioblasts are already specified before the 8 somite stage to adopt either an arterial or venous identity (Childs et al., 2002; Zhong et al., 2001). From the 18 somite stage, the arterial and venous angioblasts start to coalesce at the midline and undergo subsequent remodelling and differentiation to form distinct tubes at 26 somite stage. Shortly thereafter (around ~24 hpf), mature vascular tubes lumenize allowing the establishment of the circulation. Intersomitic vessels will eventually develop by angiogenic sprouting from the main axial vessels. Whole mount *in situ* experiments performed with general endothelial markers on 28 hpf *Sox7*/*Sox18* double morphant embryos have indicated that dorsal aorta, axial vein and intersomitic vessels development is correctly initiated. Primary vascular tubes are present at their proper location, and express several markers known to denote blood vessels morphogenesis, such as *Flk1*, *Fli1*, *VEGF*, *Tie1* and *Tie2*. This implies that there is no significant defect in early angioblasts differentiation, proliferation and migration, nor in the coalescence of these cells at the midline.

Instead, the main axial vessels probably remain non functional as a consequence of a lack of proper arterial-venous identity, as revealed by the decrease in the expression of the arterial marker *EphB2a* and the ectopic expression of the venous marker *Flt4* in the aorta. These data suggest that Sox7 and Sox18 function together to induce terminal differentiation of arterial progenitors and to repress the venous fate, by downregulating *Flt4* expression in the DA at 24hpf. Why *Sox18*, which is also expressed in the vein at all stages tested, does not downregulate *Flt4* in that compartment is an intriguing question. A plausible explanation is _ that arterial endothelial cells express additional signals necessary for Sox18 to downregulate *Flt4* expression. Interestingly, although *Sox7*/*Sox18* morphants fail to fully express *EphB2A*, they still exhibit expression of other arterial markers such as *hRT* and *Notch5* (unpublished observation). This indicates that, although both genes are required for a specific step during arterial differentiation, some arteriovenous differentiation can still occur in the double morphants.

Sox7/*Sox18* morphant embryos exhibit strong dysmorphogenesis of the proximal aorta and have poorly defined arterial-venous demarcations in a more distal region. There are strong similarities between the *Sox7*/*Sox18* morphants and the mutant *Gridlock*, in which trunk but not head circulation is affected (Zhong et al., 2000). Screening of the *Gridlock* mutants by chemical compounds now show that overexpression of VEGF is sufficient to suppress the *Gridlock* phenotype (Hong et al., 2006; Peterson et al., 2004). Mutants of the VEGF pathway itself often display lack of circulation, poorly formed dorsal aorta, reduced *EphB2a* expression and A-V shunts (Covassin et al., 2006; Lawson et al., 2002; Nasevicius et al., 2000; Ober et al., 2004). Whether *Gridlock* expression is downstream or independent of Notch signalling is still under debate in the literature; however it is now clear that Notch signalling is also a prime player in arterial development (Lamont and Childs, 2006; Torres-Vazquez et al., 2003). Similarly, Shh is proven to be the master regulator at the top of the cascade that leads to correct arterial-venous differentiation (Gering and Patient, 2005; Lawson et al., 2002). In light of our results, we can therefore hypothesize that *Sox7* and/or *Sox18* may have an effect on arterial differentiation by acting within the Shh-VEGF-Notch regulatory cascade. It would be of great interest, in future studies, to explore the genetic interactions between *Sox7* and *Sox18* and these signalling pathways.

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Material and Methods

Cloning of Sox7 and Sox18 orthologues from zebrafish

Sox7 and Sox18 partial cDNAs were cloned by two rounds of PCR performed on a pool of cDNAs ranging from 2 hpf to 31 hpf. The primers used for Sox18 amplification are BP448 and BP449 for the first PCR followed by a nested PCR with BP479 and BP480, giving rise to a fragment of 502 pb in the coding region. The primers used for Sox7 amplification are BP458 and BP459 for the first PCR followed by a nested PCR with BP481 and BP482, giving rise to a fragment of 805 pb in the coding part of the gene. These two fragments were cloned into a pGEMT-easy vector (Invitrogen) and used as template for preparing labelled antisense RNA probes. Sequence of the primers used in this study are the following:
BP448: ATGAATATATCTGAGTCTAGTTGC
BP449: TTATCCTGTAATGCAGGCGCTGTA
BP479: CAGTTCTCAGCCCAGCCAGGTTGC
BP480: CCGGCTCCACTCGCTTCATCTTCT
BP458: CATGGCGGCTCTGATAAGTGCG
BP459: TGGCGTTTATGAAATGCTGTAGTTG
BP481: GTGGAGGAAGCCGAGCGTCTGC
BP482: AAGCCGGCGTTACCTGGATGTGTC

Zebrafish maintenance

Embryos were collected, raised at 28°C under standard laboratory conditions, and staged as described (Kimmel et al., 1995).

Morpholino sequences and injections

The Sox7 and Sox18 morpholinos were designed by Gene Tools and are complementary to the 5'sequence near the translation start or to splice junctions. Their sequences are as follow:
MO1 Sox7: AATACGCACTTATCAGAGCCGCCAT
MOspl Sox7: CAACGTTAAAATCTTACCAAGCATC
MO1 Sox18: CAGATATATTCATTCCAGCAAGACC
MO2 Sox18: ACACGATTAAAGCAAGCTGTTGTCT
Five-base mismatch *MO1mut Sox7* (AATAGGCAGTTATGAGAGGCGCGAT), *MO1mut Sox18* (CACATATATTGATTGCACCAACACC) or standard control MO were used as controls.
They were dissolved in 1x Danieau buffer at 2mM and microinjected at the 1-2 cell stage at concentrations ranging from 1ng to 4ng per egg, either alone or combined. Analyses were done on embryos injected with 2ng of each morpholino.
Injected embryos were then grown in the presence of 0.003% 1-phenyl-2-thiourea until the desired stage, fixed overnight in 4% PFA and stored in 100% methanol until use.

Plasmids and probes

Antisense riboprobes were made by transcribing linearized cDNA clones with SP6, T7, or T3 polymerase using digoxigenin labeling mix (Roche) according manufacturer's instructions.
They were subsequently purified on NucAway spin columns (Ambion) and ethanol-precipitated.

Microangiography analysis

For microangiography analysis, isolectinB4 (Molecular Probes) was dissolved at 5µg/µl in 0.3x Danieau and microinjected into the sinus venosus as previously described (Isogai et al., 2001).

Wholemount *in situ* hybridizations, sectioning and imaging

Single wholemount *in situ* hybridizations were carried out as described with minor modifications (Thisse et al., 1993). For fluorescent *in situ* hybridization, the different antisense riboprobes were labelled with digoxygenin labeling mix (Roche) or DNP-11-UTP ribonucleotides (Roche). Hybridizations were subsequently performed using the TSAi Plus system kit from Perkin Elmer. Briefly, after permeabilisation, hybridization and washes, 28 hpf embryos were blocked in TNT buffer with 0.5% Blocking Reagent and then incubated with a peroxidase-conjugated anti-FITC preabsorbed antibody (1:200) for simple flurorescent *in situ*. The green color was developed with tyramide-FITC. For double *in situ* hybridizations, revelation was sequential: first, the green color was developed with tyramide-FITC. Then, after inactivation of the first antibody with H_2O_2, the embryos were incubated with peroxidase-conjugated anti-DIG preabsorbed antibody (1:200) and the red color was developed with tyramide-Cy3. Embryos were then extensively washed in TNT buffer.

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Following fluorescent wholemount *in situ* hybridization analyses, embryos were refixed in 1% paraformaldehyde, embedded in low-melting agarose and cut at 150 μm on a Campden Instruments vibratome. Transverse sections at the level of the trunk were incubated in TO-PRO-3 iodide solution and pictures were taken by using a Leica TCS SP2 inverted confocal laser microscope (Leica Microsystems, Germany). For multicolor imaging, FITC, Cy3, and TO-PRO-3® iodide were visualized by using an excitation wavelength of 488 nm, 543 nm and 633 nm respectively. The acquisition was set up to avoid any cross-talk of the three fluorescence emissions. Captured images were exported as TIFF format files and further processed using Adobe Photoshop and Illustrator CS2 for figure mounting.
To perform histological sections, MO-injected embryos were dehydrated, embedded into JB-4 plastic resin (Polysciences, Inc.), and sectioned at 10 μm on a Leica microtome. Sections were counterstained with hematoxylin-eosin following standard procedures

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Figure legends

Figure 1: **Alignment of vertebrate Sox7 and Sox18 peptidic sequences and phylogenetic tree.**
A. Residues identical in all proteins are shaded in yellow and those conserved in just some of them are shaded in blue. Similar aa are shaded in green. The HMG box is indicated by a line. The accession numbers for the sequences are as follow Dr-Sox7 (AY423014) Hs-Sox7 (NP_113627), Mm- Sox7 (NP_035576), Xl- Sox7 (D83649), Dr-Sox18 (XP_694383 with one modification at position 193 where D is replaced by A), Gg-Sox18 (NP_989640), Hs-Sox18 (NP_060889), Mm- Sox18 (AAB18615) Rn-Sox18 (NP_001019952). Dr: *Danio Rerio*, Hs : *Homo sapiens*. Mm : *Mus Musculus*, Xl : *Xenopus laevis*, Rn : *Rattus Norvegicus*, Gg : *Gallus gallus.*
B. Phylogenetic tree of the vertebrate Sox7 and Sox18 proteins.

Figure 2: Spatio-temporal distribution of the zebrafish *Sox7* and *Sox18* transcripts from bud stage to 28hpf.
A. A,F: Posterior views of bud stage embryos indicating that *Sox7* and *Sox18* are expressed in stripes corresponding to the LPM. **B,G:** Expression of *Sox7* and *Sox18* in the LPM persists throughout somitogenesis. Staining occurs at the anterior and the posterior parts of 5 somite embryos. **C,H:** At 18 somite stage, a signal corresponding to *Sox7* and *Sox18* is seen in the presumptive axial vessels. **D:** At 24 hpf, *Sox7* is mostly expressed in the DA, the head vessels, the ICM and in two stripes located in the hindbrain. Preferential expression of *Sox7* in the dorsal aorta is confirmed in the close-up view of the midtrunk vasculature shown in **E**.
I: *Sox18* transcripts can be detected in the head vasculature, the ICM, the dorsal aorta and the axial vein. **J**: Close-up view of the trunk showing *Sox18* expression in both axial vessels at 24 hpf.
Lateral (C,D,E,H,I,J) or dorsal views (B,G). Embryos are shown with anterior to the left. The developmental stage is indicated in each panel. DA: dorsal aorta; AV: axial vein; NC: notochord ; LPM : lateral plate mesoderm; ICM: intermediate cell mass
B. Double fluorescence for the vascular marker *Flk1* and *Sox7* on wild-type embryos at 18 somite stage. Confocal views of transverse vibratome sections show that $Sox7^+$ positive cells totally overlap with $Flk1^+$ cells. Double staining of *Scl* and *Sox7* show a population of Scl^+-$Sox7^-$ cells located in the presumptive ICM (arrow in P). **M** and **P** are merged views counterstained with Topro3 (blue).

Figure 3: Knockdown of *Sox7* together with *Sox18* leads to a loss of circulation in the trunk and tail.
A. Targeting both *Sox7* and *Sox18* using different sets of morpholinos prevents trunk circulation in morphants. *Sox7* or *Sox18* knockdown alone shows no effect.
B. Microangiography analyses to assess the functional integrity of the vasculature of 48 hpf control (a) and *Sox7*/S*ox18* morphants (b). The main axial vessels of double morphant embryos show no uptake of the fluorescent dye whereas circulation in the head is not affected.
C. Normal *Fli1* expression in *Sox7*/*Sox18* knockdown embryos reveals no vascular apparent defect in the posterior vasculature (compare a and b, and c and d) at 28 hpf.

Figure 4: *Sox7*/*Sox18* morphants display abnormal arterial-venous specification and arteriovenous shunts.
All embryos are at 28hpf, except as noted. **A**. Lateral views of the trunk and tail are shown with anterior to the left. As evidenced by whole mount *in situ* with specific riboprobes, no significant difference in the vasculogenic expression of *Fli1*, *Tie1*, *Tie2*, *hRT* and in the somitic expression of *VEGF* is observed between control embryos and *Sox7*/*Sox18* double morphants. There is a mild decrease in *Flk1* expression at that particular stage but not at earlier time points (data not shown) **B**. *In situ* staining with the venous-specific marker *Flt4* shows an expansion of the labeling (A-D) while the domain corresponding to the artery-specific marker *EphrinB2a* is reduced (E-H). C,D,G,H are confocal images of transverse sections of fluorescent embryos labelled by double *in situ*.
C. 48 hpf *Tg*(*fli1:EGFP*)^{y1} control fishes show well differentiated proximal aorta (arrow in **I**) whereas morphants display dysmorphogenesis at the level where the paired lateral dorsal aortae normally fused into a single tube (arrowheads in **K**). (**L**) Hematoxylin-eosin stained sections reveal arteriovenous shunts in the trunk region in Sox7/Sox18 morphants. NC= notochord; DA= dorsal aorta; AV=axial vein; OV=otic vesicles.

Figure 5: *Sox7* and *Sox18* are not involved in molecular pathways leading to the commitment of mesoderm to a blood fate
At early stages, formation of hematopoietic precursors appeared unaffected, as evident from normal *Scl* expression in the posterior LPM at 9 somite stage and from normal *Gata1* and *Scl* expression at 18 somite stage in *Sox7*/*Sox18* morphants.

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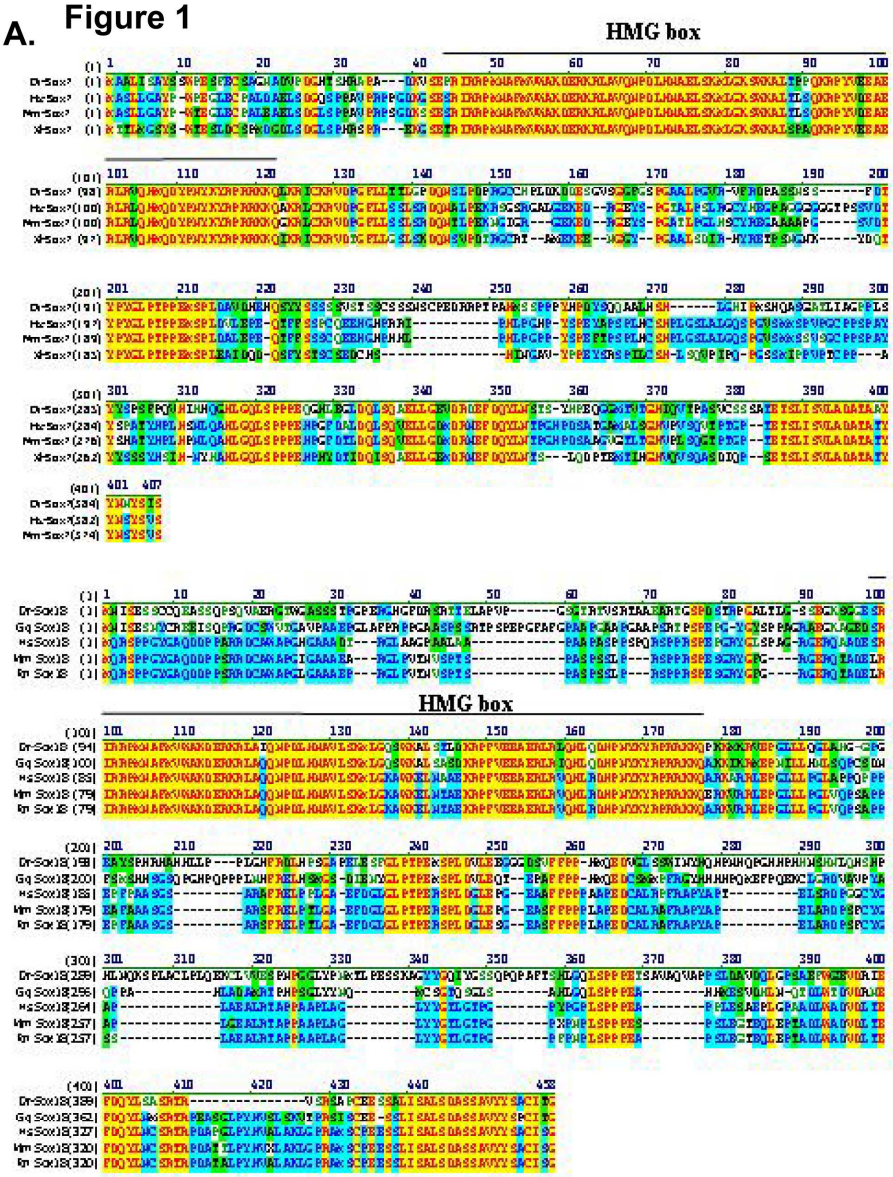
Figure 1

A.



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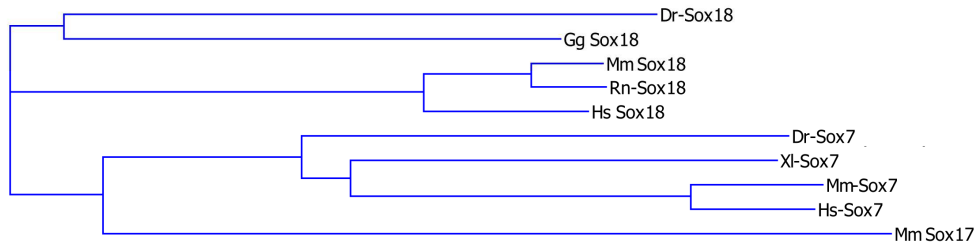

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Figure 2

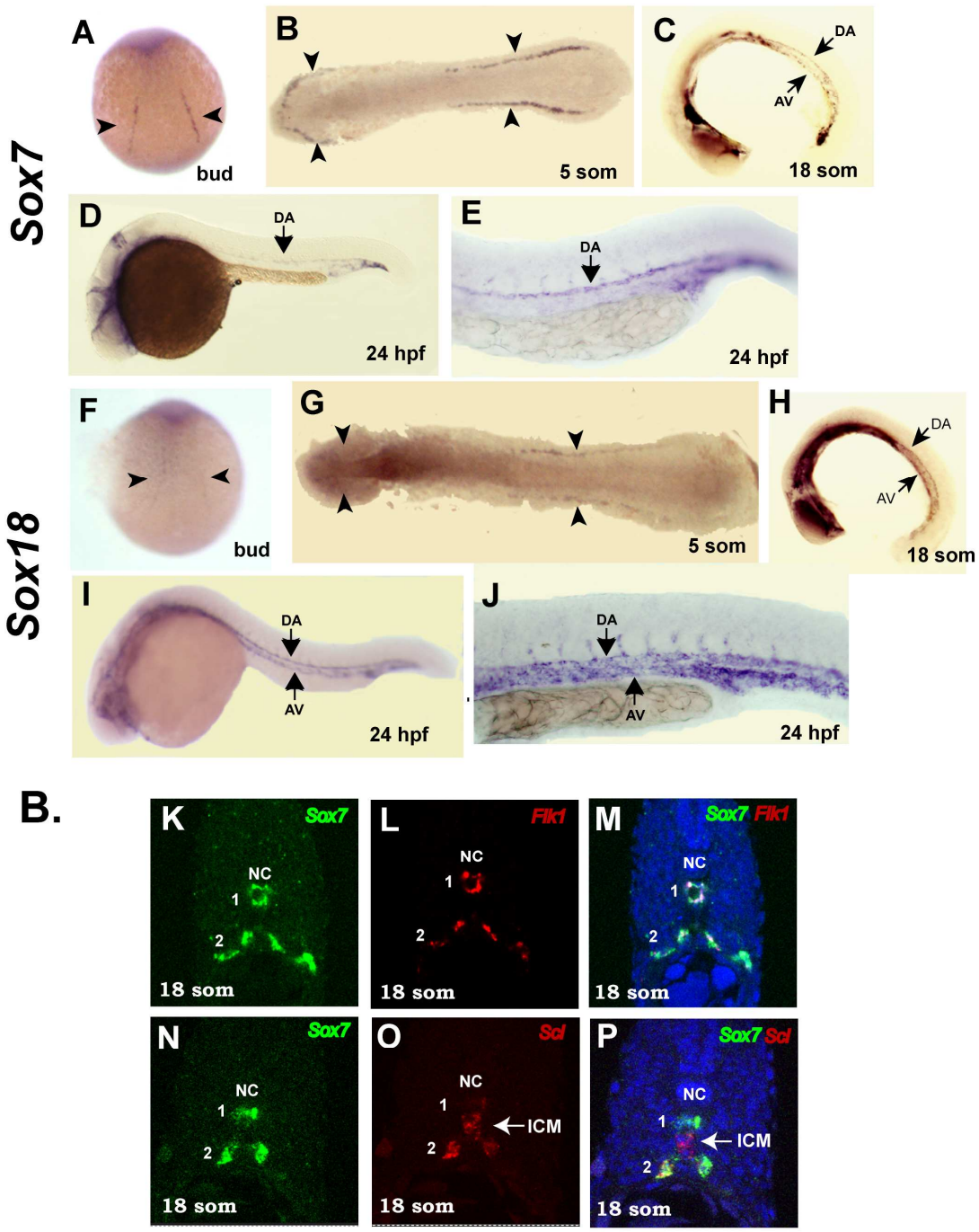

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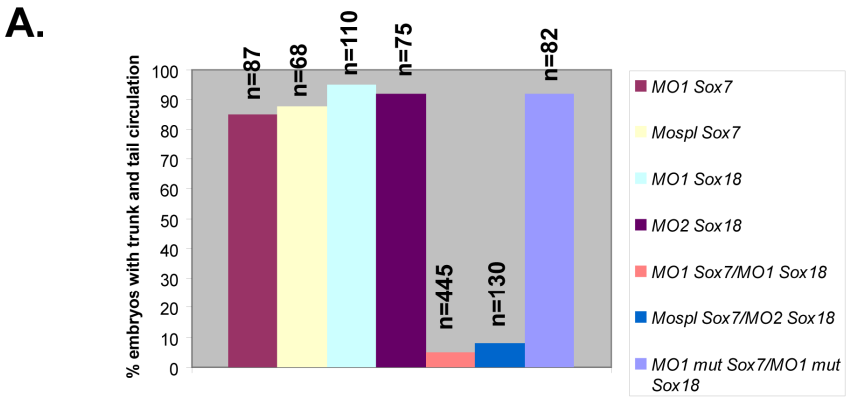
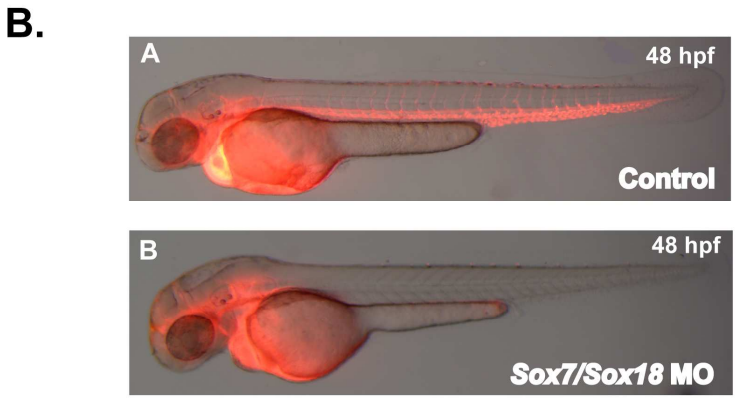
Figure 3





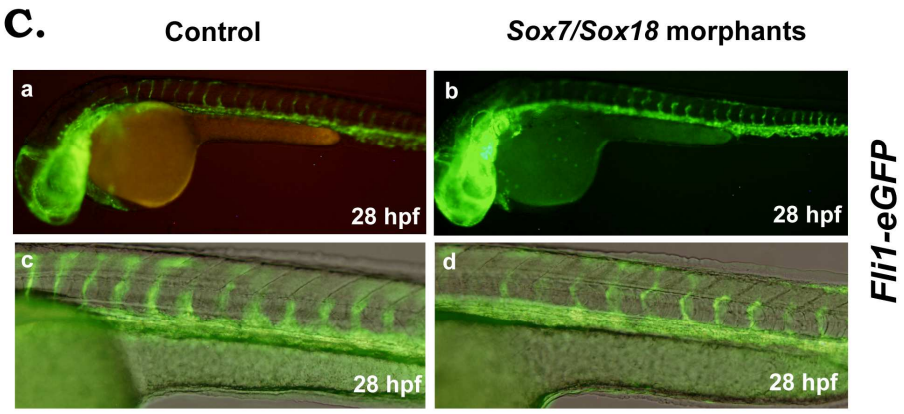


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Figure 4

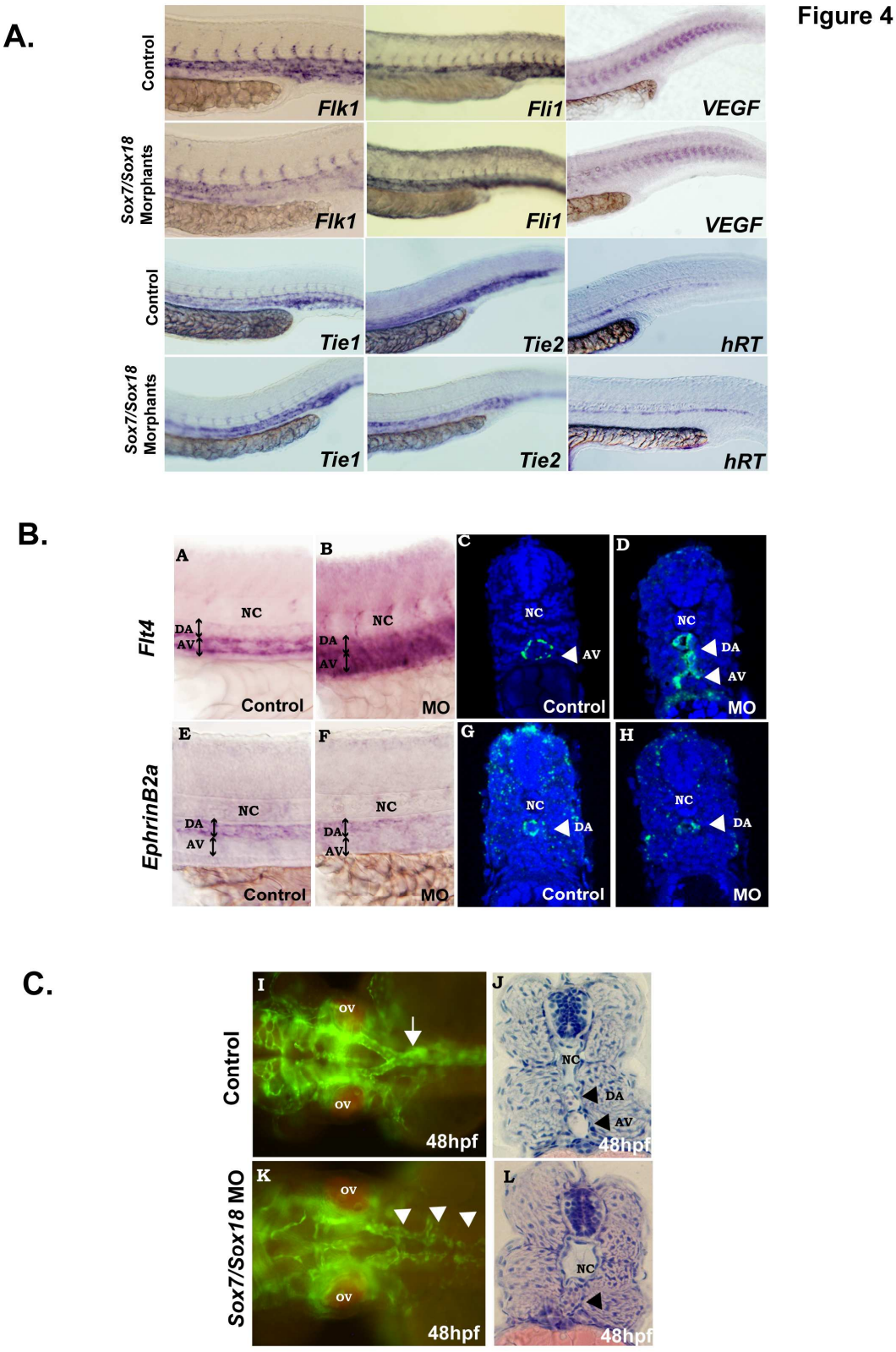

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Figure 5