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 characterized by vascular defects. 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 mainly restricted to the dorsal aorta. Knockdown of Sox7 or Sox18 alone 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 on double morphants revealed that Sox7 and Sox18 are dispensable for the initial specification and positioning of the major trunk vessels. However, morphants display ectopic expression of the venous Flt4 marker in the dorsal aorta and a concomitant reduction of the artery-specific markers EphrinB2a and Gridlock. The striking similarities between the phenotype of Sox7/Sox18 morphants and Gridlock mutants strongly suggest that Sox7 and Sox18 control arterial–venous identity by regulating Gridlock expression.

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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 signaling 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 from members of the Ets family (Patterson and Patient, 2006; Pham et al., 2007), very few transcription 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 \textit{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 \textit{ragged} (Ra) mice, which display defective cardiovascular and hair follicle formation, mutations in the transactivation domain of \textit{Sox18} lead the protein to act in a dominant-negative manner (Pennisi et al., 2000b). As mice null for \textit{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 \textit{Sox} proteins from the same subgroup, that can account for the mild phenotype observed in these mice. In pathological situations however, the \textit{Sox18} null mice present reduced tumor vascularization and subsequent growth, suggesting that interfering with \textit{Sox18} function impairs tumoral angiogenesis and growth (Young et al., 2006). The role of \textit{Sox18} in angiogenesis is further supported by the observation that \textit{Sox18} is detected in granulation tissue during wound healing in adult mice (Darby et al., 2001). During mouse embryogenesis, \textit{Sox18} expression is 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, \textit{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 \textit{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 \textit{Sox7} has been less documented. During mouse development, \textit{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 \textit{Sox7} and \textit{Sox18} function in the vasculature remains to be elucidated. In zebrafish, although blood circulation is established early, passive oxygen diffusion allows embryos showing important vasculogenetic 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 \textit{Sox7} and \textit{Sox18} in vascular development.

Results

Cloning of the zebrafish \textit{Sox7} and \textit{Sox18} genes

In the process of decrypting the role of \textit{Sox7} and \textit{Sox18} genes in zebrafish vasculogenesis, we searched the zebrafish orthologs by mining EST and genomic databases. This \textit{in silico} search allowed us to identify the \textit{Sox7} and \textit{Sox18} genes, both present as a single copy in the zebrafish genome. We amplified the \textit{Sox7} and \textit{Sox18} cDNAs by RT-PCR on mRNAs ranging from 2 hpf to 31 hpf. The predicted amino acid sequences indicate that zebrafish \textit{Sox18} (XP_694383) and \textit{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-\textit{Sox18} and Dr-\textit{Sox7} are the true orthologs of the human genes as the phylogenetic tree clearly shows that the zebrafish \textit{Sox18} and \textit{Sox7} proteins fit into the \textit{Sox18} and \textit{Sox7} clade, respectively (Fig. 1B). Moreover synteny occurs between the zebrafish and the human chromosomal regions harboring the \textit{Sox18} and \textit{Sox7} genes. Indeed, the zebrafish \textit{Sox18} gene, located on chromosome 23, is directly adjacent to the \textit{teca2} gene like its human counterpart which is located on chromosome 20. Similarly, Dr-\textit{Sox7} and Hs-\textit{Sox7} locus on chromosome 20 and 8, respectively share the same gene organization (\textit{Sox7}, \textit{PinX1}, \textit{Q96KT1} and \textit{MTMR9}) (data not shown).

\textit{Sox7} and \textit{Sox18} have overlapping and distinct expression patterns in the endothelial compartment

We next assessed \textit{Sox7} and \textit{Sox18} expression during zebrafish embryogenesis. Whole mount \textit{in situ} hybridizations performed on zebrafish embryos at different stages of development showed no expression of \textit{Sox7} and \textit{Sox18} before the end of gastrulation (data not shown). \textit{Sox7} and \textit{Sox18} transcripts are first detected at bud stage and are located in bilateral stripes in the posterior lateral plate mesoderm (Figs. 2A, 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 \textit{Sox7} and \textit{Sox18}-stained embryos (Figs. 2B, G). During these early stages, \textit{Sox18} transcripts are expressed at lower levels than \textit{Sox7}. At 18 somites stage, \textit{Sox7} and \textit{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. 2C, H). At 24 hpf, by the time circulation starts in zebrafish, \textit{Sox7} and \textit{Sox18} are expressed in the vasculature within the head, trunk and tail (Figs. 2D, I). Closer inspection of the staining reveals that \textit{Sox18} expression is detected in the dorsal aorta, the intersomitic vessels and in the axial vein (Fig. 2J). \textit{Sox7} expression is also observed in the dorsal aorta and the intersomitic vessels but its expression becomes strongly reduced in the vein at that stage (Fig. 2E). In addition, two discrete stripes are detected in the hindbrain with the \textit{Sox7} probe. 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 \textit{Sox7} and \textit{Sox18} are expressed. We then performed double fluorescent \textit{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 \textit{Sox7} perfectly colocalizes with Flk1$^+$ cells in two subpopulations of cells.
Fig. 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 (NP_035576), 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. The accession numbers for the sequences are as follow: Dr-Sox7 (AF168614), Dm-Sox7 (AJ250955), Dm-Sox18 (AJ251580). Dm: Drosophila melanogaster.
One domain of Flk+/Sox7+ expressing cells is located in the presumptive dorsal aorta (1 in Figs. 2K–P). 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. 2K–P) 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, at 18 S stage, Sox7 and Sox18, like Flk1, become restricted to the endothelial lineage.

**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 a morpholino antisense knockdown approach to uncover possible defects in the vasculature caused by Sox 7 and Sox 18 interference. We used morpholinos designed to target Sox7 or Sox18 ATG and inhibit translation (MO1 Sox7, MO1 Sox18). These were injected either alone or in combination in fertilized eggs and the phenotype analyzed at 28 hpf, after the onset of circulation (Fig. 3). No morphological nor circulation defects were observed in embryos injected with either Sox7 or Sox18 morpholino, despite the fact that these two morpholinos are very effective in blocking translation (see Supplementary Fig. 1A). In contrast, combined doses of MO1 Sox7 and MO1 Sox18 resulted in a consistent phenotype, in which the embryos morphology 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

Fig. 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 (4 ng of each) prevents trunk circulation in morphants. Sox7 or Sox18 knockdown alone shows no effect. (B) Left panel: Microangiography analyses to assess the functional integrity of the vasculature of 48 hpf control and Sox7/Sox18 morphants. The main axial vessels of double morphants embryos, injected with 4 ng of MO1 Sox18 and 4 ng of MO1 Sox7, show no uptake of the fluorescent dye whereas circulation in the head is not affected. Right panel: Normal Fli1 expression in Sox7/Sox18 knockdown embryos reveals no vascular apparent defect in the posterior vasculature at 28 hpf. (C) Lateral views of the trunk and tail, shown with anterior to the left, of embryos at 28 hpf. As evidenced by whole mount in situ with specific riboprobes, no significant difference in the vasculogenic expression of Fli1, Tie1, Tie2 is observed between control embryos and Sox7/Sox18 double morphants. There is a mild decrease in Fli1 expression at that particular stage but not at earlier time points (data not shown).
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. 3A). Control embryos, injected with 5-base mismatch Sox7 and Sox18 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

![Image](https://example.com/image1.png)

**Fig. 4.** Sox7/Sox18 morphants display abnormal arterial–venous specification and arteriovenous shunts. Except in panels I, J, all embryos shown resulted of injection of regular doses of control or Sox7/Sox18 morpholinos (4 ng of each) and are fixed at 28 hpf. Pictures and sections are made at the trunk level. (A–D) *In situ* staining with the venous-specific marker Flt4 shows an ectopic expression in the dorsal aorta while the domain corresponding to the artery-specific marker EphrinB2a (panels E–H) is reduced. Panels C, D, G, H are confocal images of transverse sections of fluorescent embryos labeled by *in situ*. (I, J) Transverse sections of embryos injected with high doses of Sox7/Sox18 morpholinos (6 ng of each). As revealed by Flt4 expression, the vein is widely expanded, at the expense of the artery. (K) Expression of the artery markers Notch3, hRT and Dll4 is not affected in double morphants. (L) There is an abolition of Gridlock expression in the double morphants within the aorta while its expression in the brain was not affected. (M) White arrows mark the proximal aorta at the level where the paired lateral dorsal aortae normally fused into a single tube. 28 hpf *Tg(fli1:EGFP)*^Y1^ control fishes show well differentiated proximal aorta whereas morphants display strong dysmorphogenesis of the proximal aorta. (N) Hematoxylin–eosin stained sections reveal arteriovenous shunts in the trunk region in Sox7/Sox18 morphants. NC = notochord; DA = dorsal aorta; AV = axial vein.
demonstrated by performing a microangiography analysis in which we injected a fluorescent dye into the sinus venous of Sox7/Sox18 morphants and, as control, of Sox7mut/Sox18mut morphants at 48 hpf (Fig. 3B, left panels).

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 (MO2Sox7) and one second translational Sox18 morpholin (MO2Sox18) whose effectiveness was confirmed (see Supplementary Fig. 1B). When both were simultaneously injected, they produced a similar absence of blood circulation phenotype (Fig. 3A).

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. 3B, right panels). 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 morphants (Fig. 3C). These included the general markers of endothelial cells Fli1, Flk1 and some endothelium-specific receptor tyrosine kinases markers such as Tie1 and Tie2 (Brown et al., 2000; Lyons et al., 1998). 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 arterial or venous endothelial cell identity, Sox7/Sox18 morphants were analyzed for the expression of the arterial marker EphrinB2a and the venous marker Flt4 (Lawson et al., 2001). 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 (Fig. 4, panels A–D). Furthermore, a slight, but reproducible, reduction of EphrinB2a signal was also observed in the dorsal aorta of morphants (Fig. 4, panels E–H). We next tested the expression of other artery markers such as Notch3, Dil4, hRT and Gridlock (Mailhos et al., 2001; Szeto et al., 2002; Zhong et al., 2001). Signals corresponding to Notch3, Dil4 and hRT appeared normal in Sox7/Sox18 morphants, suggesting that some arterial differentiation has occurred (Fig. 4K). In contrast, Gridlock expression within the aorta was totally abolished in the double morphants at 28 hpf while its expression in the brain was not affected (Fig. 4L). Taken together, these data indicate that Sox7 and Sox18 act upstream of Gridlock and specify arterial–venous identity. We could besides emphasize the transfillating phenotype by injecting higher doses of Sox7 and Sox18 morpholinos (6 ng of each). Figs. 4I, J shows that, using higher doses, the vein is drastically expanded at the expense of the artery.

In an 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 28 hpf Sox7/Sox18-injected embryos revealed strong dysmorphogenesis of the proximal aorta, at the level where the two paired lateral dorsal aortae fuse into a single tube (Fig. 4M) (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. 4N). In the control sections, the dorsal aorta and the axial vein are always clearly distinguishable. In contrast, in morphants, demarcations between the artery and the vein are poorly defined, resulting, at some levels of the trunk axis, in arteriovenous shunts. These morphogenetic defects are probably the cause of the lack of circulation in the trunk and tail. Taken together, these data indicate that Sox7 and Sox18 are required for proper arterial–venous identity and are necessary to allow primary vessels to display clear arteriovenous demarcations.

Hematopoietic 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 next verified whether the other lineages were disturbed in double morphants. To test whether the blood lineage was affected, 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) (Fig. 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/Sox18-28 hpf injected embryos (data not shown).

Sonic Hedgehog and VEGF signaling are required for Sox7 expression

Previous studies have shown that arterial endothelial differentiation is dependent on a regulatory cascade involving three signaling pathways, Sonic Hedgehog (Shh), VEGF and Notch signaling (Gering and Patient, 2005; Lawson et al., 2002). Shh signaling is first required for the migration of the dorsal aorta progenitors from the lateral plate to the midline. Afterwards, Sonic Hedgehog induces the expression of VEGF
Fig. 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.

Fig. 6. Influence of Shh, VEGF and Notch signaling on *Sox7* and *Sox18* expression. All embryos are shown at 24 hpf, and pictures were taken from lateral views at the trunk level. (A) *Sox7* expression is lost in *Smu* mutant embryos and in embryos chemically impaired with cycloamine whereas *Sox18* expression is maintained. (B) Embryos treated with a VEGF receptor tyrosine kinase inhibitor show no expression of *Sox7*. *Sox18* still appears expressed in these embryos. (C) *Sox7* and *Sox18* expression are maintained in *Mindbomb* mutants that are impaired in the Notch signalling.
in the somites which will signal presumptive arterial cells to upregulate Notch3 expression. This leads to the activation of EphrinB2α and to the downregulation of Flt4 expression in the dorsal aorta at 24 hpf.

To determine where Sox7/Sox18 are placed in this regulatory cascade, we tested their expression in embryos defective in these pathways (Fig. 6). We first analyzed the expression pattern of Sox7 and Sox18 in the Smu mutant embryos, in which the Shh co-receptor Smoothened is mutated. In Smu embryos, the angioblasts fail to migrate towards the midline to form the dorsal aorta under the notochord/hypochord and do not display any sign of arterial differentiation. Expression of Sox7 was not detected in the trunk of Smu embryos (Fig. 6A) while its expression was not disturbed in the head vessels as well as in the two stripes of the hindbrain (data not shown). On the other hand, Sox18 remains expressed in the angioblasts. Secondly, we tested Sox7 and Sox18 expression in embryos treated with cycloamine, a drug known to block all Shh signaling. That treatment does not interfere with angioblast migration but disturbs arterial differentiation as seen by the loss of the arterial markers Gridlock, Notch3, hrT and ephrinB2α (Gering and Patient, 2005). Duplicate experiments showed that treatment of embryos from 90% epiboly with 50 μM cycloamine led to a loss of Sox7 expression in the trunk at 24 hpf. Here again, Sox18 expression was retained in drug-treated embryos (Fig. 6A).

Thirdly, to examine the requirement of VEGF signaling for Sox7 and Sox18 induction, we treated 90% epiboly embryos with a VEGF receptor tyrosine kinase inhibitor (Hennequin et al., 1999). Embryos incubated with 5 μM of drug failed to show arterial expression of Sox7 at 24 hpf, whereas Sox18 remained expressed (Fig. 6B). As control to these experiments and, as previously published, we found no arterial expression of ephrinB2α in embryos that had undergone exposure to cycloamine or the VEGF receptor tyrosine kinase inhibitor.

Finally, we tested whether Sox7 and Sox18 lie downstream of the Notch pathway by analyzing their expression in the zebrafish Mindbomb mutants (Mib), that lack an E3 ubiquitin ligase crucial for Notch signal activation (Jiang et al., 1996). These mutant embryos show a loss of some arterial markers such as EphrinB2α and Notch3 while others are retained, like Gridlock and hrT. In situ experiments showed that wild-type siblings and embryos homozygous for mib+/- both exhibited similar expression of Sox7 and Sox18, whereas EphrinB2α expression was, as reported, abolished in the mib−/− mutants (Lawson et al., 2001). Taken together, these data indicate that Sox7 expression in the artery is dependent of Shh and VEGF but not of Notch signaling, while Sox18 does not seem dependent of any of these pathways.

**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. We show that the two genes are expressed very early during angioblast cell differentiation and that they act redundantly to control arterial–venous identity, by at least in part, activating Gridlock in the arterial vessels.

Functional redundancy between F-subgroup Sox factors has been previously suggested by the contrasting phenotypes of the Sox18 null mice compared to the ragged mutant mice that carry mutations in the transactivation domain of Sox18. Indeed, mice null for Sox18 have only a mild coat defect but no developmental vascular-associated phenotype (Pennisi et al., 2000a) while ragged mice display defective cardiovascular and hair follicle formation. This suggested that the ragged mutations lead to the synthesis of a modified Sox18 protein acting in a dominant-negative manner. The mild phenotype of Sox18 null mice could be explained by functional redundancy amongst some Sox proteins from the same subgroup such as Sox7 and Sox17 (Pennisi et al., 2000b). Redundancy between Sox17 and Sox18 has been recently demonstrated in mice as Sox17+/−/Sox18−/− mutant mice display reduced postnatal neo-vascularization and early cardiovascular defects (Matsui, 2006; Sakamoto et al., 2007). In developing mouse embryos, Sox17 is indeed transiently expressed in some endothelial cells in addition to its well-known expression in endodermal cells. In zebrafish, it seems unlikely that such redundancy occurs as Sox17 expression is clearly restricted to the endodermal cells and has not been detected in the vasculature (Alexander and Stainier, 1999). Sox7, on the other hand, presents an overlapping expression pattern with Sox18 in the zebrafish vasculature and acts redundantly with it. As Sox7 is also expressed in the vasculature in mice it would be interesting to test similar functional redundancy of Sox7 and Sox18 in that animal model (Sakamoto et al., 2007).

Combined knockdown of Sox7 and Sox18 in zebrafish embryos led to a lack of circulation specifically in the trunk and the tail while circulation in the head was not affected. Cardiac contractility and size appeared to be normal and no difference in the signal of a heart-specific Cmc12 riboprobe could be detected on 28 hpf double morphant embryos (data not shown) indicating that the lack of circulation cannot be linked to a heart defect. The trunk circulation arrest is rather due to strong dysmorphogenesis of the proximal aorta and to multiple arteriovenous shunts along the axial vessels. A 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 signaling (Brown et al., 2000; Lawson et al., 2002). For example, mutants of the VEGF pathway itself often display lack of circulation, poorly formed dorsal aorta, reduced EphrinB2α expression and A–V shunts (Covassin et al., 2006; Lawson et al., 2002; Nasevicius et al., 2000; Ober et al., 2004). In zebrafish, the first markers of angioblast differentiation appear right after gastrulation in the lateral plate mesoderm (Fouquet et al., 1997; Liang et al., 2001; Thiss and Zon, 2002). It is noteworthy that Sox7 and Sox18 are both detected during that early specification stage, suggesting that they play a key role in vasculogenesis. The angioblasts migrate toward the midline in two waves (Jin et al., 2005). The arterial endothelial precursors migrate first and form a separate chord of angioblasts under the notochord/hypochord. Then, a second migration wave occurs, that generates the venous
vessels located between the aorta and the endoderm. Based on their different migration waves and on their final location, arterial and venous endothelial cells receive different signals from the neighbouring tissues. Arterial identity is controlled by a signaling cascade where Sonic hedgehog secreted by the notochord/hypochord induces VEGF expression in the adjacent somites, which in turns induce Notch signaling in the dorsal aorta (Lawson et al., 2002). 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 the dorsal aorta, axial vein and intersomitic vessel 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, Flt1, Tie1 and Tie2. This implies that there is no significant defect in angioblasts differentiation, proliferation and migration, nor in the coalescence of these cells at the midline. Instead, the main axial vessels remain non-functional as a consequence of a lack of proper arterial–venous identity. They fail to fully express EphrinB2a but still exhibit normal expression of other arterial markers such as hRT, Notch3 and Dll4, indicating that some arterial differentiation can still occur in the double morphants. Importantly, they totally lack Gridlock expression in the artery. Gridlock has been reported to be an important determinant of the arterial–venous fate through repression of venous differentiation (Zhong et al., 2001). In Sox7/Sox18 morphants, two indications allow us to believe that both genes also act primarily to repress the venous fate. Firstly, we observe an ectopic expression of the venous marker Flt4 in the dorsal aorta of morphants at 24 hpf, at a time where its expression should normally be downregulated to allow the artery to fully acquire arterial characteristics. Secondly, Sox7 and Sox18 morpholinos led to an increase of venous Gridlock expression. In Sox7/Sox18 morphants, two indications allow us to believe that both genes also act primarily to repress the venous fate. Firstly, we observe an ectopic expression of the venous marker Flt4 in the dorsal aorta of morphants at 24 hpf, at a time where its expression should normally be downregulated to allow the artery to fully acquire arterial characteristics. Secondly, Sox7 and Sox18 morpholinos led to an increase of venous Gridlock expression. In Sox7/Sox18 morphants, two indications allow us to believe that both genes also act primarily to repress the venous fate. Firstly, we observe an ectopic expression of the venous marker Flt4 in the dorsal aorta of morphants at 24 hpf, at a time where its expression should normally be downregulated to allow the artery to fully acquire arterial characteristics. Secondly, Sox7 and Sox18 morpholinos led to an increase of venous Gridlock expression.

Materials and methods

Zebrafish maintenance

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

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 Sox7 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. Sequences of the primers used in this study are the following:

```plaintext
BP448: ATGAATATATCTCTGATCTAGTTGC
BP449: TTATCTCTGTAATGCAGCAGCTGTGA
BP479: CAGTTCTCAGCCAGCCAGGTGTGC
BP480: CCGGCTCCACTCGCTTCATCTTCT
BP458: CATGGCGGCTCTCGTCTGAAGTGCG
BP459: TGCGGTATTTGAGGATGCTGTTAGTG
BP481: GTGGAGGAGACCGGACGCTGTCGC
BP482: AAGCGGCGGTGTTACCTGAGTGTGC
OB65: CCCCAGATTCAAGTACAGTACAGACAGTGCTG
OB66: CCCGAAATTCCTGGCCTTTGAGCACAATCGACT
O76: TCGCCCTTTCCTCCACATATGG
OI32: GGGCCCATGGAGCAGATAGACATACACAGCAG
OI33: CCCGGATACCTGAGCTTCTTAACCCACCGC
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Sox7 and Sox18 5′ UTR fusion construct and mRNA synthesis

Polymerase chain reaction (PCR) using the primers OB65 and OB66 was performed to amplify 79 bp of the 5′ UTR and the first 38 nucleotides of the coding sequence of Sox18. Similarly, the primers O132 and O133 were used to amplify 161 bp of the 5′ UTR of Sox7 followed by the first 30 nucleotides of the coding sequence. The resulting BamHI/NcoI digested fragments were cloned in frame with the GFP coding sequence in the pCS2+ construct. The Sox7 and Sox18 5′ UTR–GFP fusion constructs were linearized with NotI. Sp6 RNA polymerase was used for in vitro synthesis of capped mRNA (Ambion, Austin, TX).
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 to or splice junctions. Their sequences are as follow:

- M01 Sox7: AATACGACTTATCAGAGGCGCCAT
- M02 Sox7: CAACGTAAAATCTTACCAAGACCC
- M03 Sox18: CAGATATTCTACCCAGGACGAC
- M04 Sox18: ACAGGATTTAAGGAACTGTTGCT

Five-base mismatch M01mut Sox7 (AATACGACATATGAGGCGCAT), M01mut Sox18 (CACATATTTGATTCACCAACACC) or standard control MO were used as controls.

They were dissolved in 1× Danieau buffer at 2 mM and microinjected at the 1–2 cell stage. Regular analyses were performed on double morphant embryos injected with 4 ng of each morpholino (M01 Sox7 and M01 Sox18), at a dose where the phenotype was very penetrant. However, in Figs. 4I, J, as stipulated, a higher dose of 6 ng of each morpholino was used to exacerbate the phenotype of artery–vein transfiguring (see text for details).

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 or DNP labeling mix (Roche) according manufacturer’s instructions. They were subsequently purified on NucAway spin columns (Ambion) and ethanol-precipitated. The Gridlock probe was made by amplifying a fragment corresponding to the 3′ UTR region of the cDNA with the following primers: Grtf: 5′-gttctctactatgcgaagct-3′, Grirle: 5′-gtgaaagtctgtgacttgaaaggt-3′. The PCR fragment was cloned in pCRII-Topo (Invitrogen, CA) and sequenced before transcription.

Microangiography analysis

For microangiography analysis, isocastinB4 (Molecular Probes) was dissolved at 5 μg/μl in 0.3× Danieau buffer 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 digoxigenin labeling mix (Roche) or DNP labeling mix (Roche) according manufacturer’s instructions. They were subsequently purified on NucAway spin columns (Ambion) and ethanol-precipitated. The Gridlock probe was made by amplifying a fragment corresponding to the 3′ UTR region of the cDNA with the following primers: Grtf: 5′-gttctctactatgcgaagct-3′, Grirle: 5′-gtgaaagtctgtgacttgaaaggt-3′. The PCR fragment was cloned in pCRII-Topo (Invitrogen, CA) and sequenced before transcription.

Drug treatments

Cyclosporine and VEGFR-2 tyrosine kinase inhibitor (number: 676475) were obtained from Calbiochem and were used at a concentration of 50 μM and 5 μM respectively. Zebrafish embryos were soaked in 24-well plates and were treated with the drugs from 90% epiboly, until they were processed for in situ experiments. As control for both experiments, embryos from the same batches were treated with DMSO or ethanol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.028.

References


