Redundant Roles for Sox7 and Sox18 in Arteriovenous Specification in Zebrafish

Robert Herpers, Esther van de Kamp, Henricus J. Duckers,* Stefan Schulte-Merker*

The specification of arteries and veins is an essential process in establishing and maintaining a functional blood vessel system. Incorrect arteriovenous specification disrupts embryonic development but has also been diagnosed in human syndromes such as hypotrichosis–lymphedema–telangiectasia, characterized by defects in blood and lymphatic vessels and associated with mutations in SOX18. Here we characterize the role of sox7 and sox18 during zebrafish vasculogenesis. Sox7 and sox18 are specifically expressed in the developing vasculature, and simultaneous loss of their function results in a severe loss of the arterial identity of the presumptive aorta which instead expresses venous markers, followed by dramatic arteriovenous shunt formations. Our study identifies members of the Sox family as key factors in specifying arteriovenous identity and will help to better understand Hypotrichosis–lymphedema–telangiectasia and other diseases.

Arteriovenous (AV) specification and differentiation are two critical events required for the progression of vascular development and function, as evidenced by diseases such as hypotrichosis–lymphedema–telangiectasia and hereditary hemorrhagic telangiectasia, which have been associated with mutations in SOX18 and endoglin/activin-like receptor kinase-1, respectively.1–3 To study the process of AV specification, zebrafish embryos have proven particularly useful. Following the specification of arterial and venous cell types,4 endothelial cells coalesce into cord-like midline structures and subsequently reshape into tubes.5 However, although several signaling molecules and transcription factors6–8 have been implicated in these processes, we are still only beginning to understand their regulation. In an attempt to identify new factors involved in the regulation of AV specification and vasculogenesis, we analyzed the function of sox7 and sox18, which, together with the endodermally expressed sox17, form the Sox-F (Sry-related HMG box) family of DNA-binding proteins.9 Members of the Sox-F family play crucial roles during the formation of definitive endoderm,10 hematopoietic stem cell regulation,11 and cardiovascular development.12 Here we show temporal and spatial overlap of sox7 and sox18 expression and identify functionally redundant roles for these genes during vascular development in zebrafish embryos. Our results demonstrate a novel role for sox7 and sox18 in specifying the molecular identity of endothelial cells in their commitment to arterial tissues during blood/vessel formation.

Materials and Methods

Zebrafish Husbandry
Zebrafish (Danio rerio) were raised as described.13 Transgenic lines used were Tg(vegfr4: gfp)14 and Tg(fli1a:gfp)15 and Tg(fli1a:gfp)16–14

Histological Procedures
In situ hybridization and immuno-histochemistry were performed as described.5,13 The riboprobes used are specified in the online data supplement, available at http://circres.ahajournals.org.

Morpholino Injections and Microangiographs
Procedures are specified in the online data supplement.

Results and Discussion

Expression Analysis for sox7 and sox18
We first examined embryonic expression of sox7 and sox18. RT-PCR expression analysis (Figure I in the online data supplement) revealed that sox7 and sox18 are provided maternally. In situ hybridization showed that sox7 and sox18 transcripts localized to the lateral mesoderm at 12 hours postfertilization (supplemental Figure I). Reminiscent of migrating angioblasts, these presumptive precursor cells localized to the midline during somitogenesis (supplemental Figure I) and finally homed to the endothelium of the axial, head, and intersegmental vessels at 26 hpf (Figure 1A and 1B). Cells expressing sox7 and sox18 are likely endothelial based on the expression pattern of these genes at later stages and the absence of mesodermal sox7 and sox18 expression in cloche mutants and etsrp morphants,8 both of which lack the endothelial lineage (supplemental Figure II). In addition, sox7-expressing cells were found in rhombomeres at 26 hpf (Figure 1A, arrowhead), whereas sox18 expression was observed in the eye and retina (Figure 1B, arrowhead). The expression patterns of zebrafish sox7 and sox18 closely resemble the expression pattern of Sox18 in mice.12

Morpholino Knockdown Analysis of sox7 and sox18
Embryos injected with morpholinos (MOs) targeting sox7 or sox18 individually (two independent MOs for each gene; see supplemental Figure III for specificity tests) did not show any apparent morphological defects, or loss of endothelial cells (supplemental Figure I), or loss of circulation (supplementary Movies 2 to 3). Strikingly, on
Simultaneous injection of low amounts of both sox7- and sox18-MO, virtually all double knockdowns (dKDs) exhibited a loss of circulation in the posterior part of the embryo, whereas cardiac contractile function was normal (Figure 1E; supplementary Movie 1). Later, blood accumulated in a short circulatory loop near the heart leading to pericardial edema (>2 days postfertilization; not shown). Endothelial cells were specified in sox7/sox18-dKDs as demonstrated by fli1a:gfp expression, but we noticed poor segregation of artery and vein (compare supplemental Figure Ij and Im, insets). In addition, in microangiographs, the major axial vessels in the posterior part of sox7/sox18-dKDs were not filled with dye at 2 days postfertilization (Figure 1C and 1D). Dye injected into the sinus venosus drained from the heart into the posterior cardinal vein (PCV) rather than the dorsal aorta (DA) (Figure 1D, arrow). We conclude that combined loss of sox7 and sox18 function results in a severe disturbance of circulation.

**Artery/Vein Specification and Vascular Tube Formation in sox7/sox18-dKDs**

To further investigate this phenotype, we analyzed the expression of several molecular markers in sox7/sox18-dKDs compared with uninjected control embryos or silent heart morphants. No alteration was detectable in the primitive erythroid lineage marker gata1 (supplemental Figure IV), vegf receptors 2 and 4, or pan-endothelial markers like tie2, cdh5, and fli1a (not shown). However, we observed a dramatic decrease in the expression of arterial markers notch3, ephrinB2a (Figure 2A through 2D), and dll4 (supplemental Figure IV) and a concurrent increase in the expression of venous markers dab2 and flt4 in arterial tissues, such as the DA and intersegmental vessel (supplemental Figure IV and Figure 2C and 2F, respectively). These results suggest a key role for sox7 and sox18 in specifying the arterial fate of endothelial cells. A possible shift in AV identity attributable to the lack of circulation was excluded by analyzing silent heart morphants, which showed no alteration in
marker gene expression (Figure 2F and supplemental Figure VI). This demonstrates that *sox7* and *sox18* are essential regulators of AV identity.

To better understand the lack of lumen formation observed in *sox7/sox18*-dKD embryos, we next examined transverse sections of *sox7/sox18*-dKD embryos, uninjected control embryos, and silent heart morphants. The nonvascular morphology in *sox7/sox18*-dKDs (Figure 3C) was completely normal, suggesting a vessel-specific phenotype. Uninjected controls and silent heart morphants exhibited normal segregation and lumenization of axial vessels (Figure 3A, 3B, 3D, and 3E). In all *sox7/sox18*-dKDs (n = 16), we observed stretches of normal and physically separated axial vessels, alternating with regions where only a single PCV was present. At particular locations in *sox7/sox18*-dKDs, the DA apparently fused with the PCV (Figure 3C and 3F; see also supplemental Figure V). We conclude that the combined loss of Sox7 and Sox18 function disrupts AV specification and leads to severe shunt formation.

Our study provides novel insights into the molecular roles of *sox7* and *sox18*, which are essential to the specification of the molecular identity of the dorsal aorta during embryogenesis and possibly during later stages of life. These findings, for the first time, offer direct insights into the molecular consequences of Sox function in endothelial cells at the in vivo level. Understanding the requirement for Sox7 and Sox18 in the process of arteriovenous specification might help to better understand syndromes such as hypotrichosis–lymphedema–telangiectasia and hereditary hemorrhagic telangiectasia.

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References


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Materials and Methods

Zebralish husbandry

Zebralish (Danio rerio) embryos were obtained from wild-type strains and raised at 28°C as previously described [1]. The transgenic line Tg(vegfr4: gfp)$^{s843}$ [2], originally referred to as Tg(flk1:EGFP)$^{s843}$ [3] was obtained from Didier Stainier (San Francisco, CA, USA). The transgenic line Tg(fli1a:gfp)$^{y1}$ [4] was obtained from Brant Weinstein (Bethesda, ML, USA). Homozygous mutant cloche [5] or scl (tal1$^{t21384}$) [2] embryos were obtained by incrossing these fish.

RT-PCR analysis

cDNA was synthesized from RNA isolated from 0, 1, 2.5, 6, 8, 10 and 24 hours post fertilization old embryos using the SuperScript II RT Kit (Invitrogen). Primers used for reverse transcriptase PCR were:

sox7-Fwd: 5’-ACCAGCTGCTCACTCAAAC-3’
sox7-Rev: 5’-GATCTCTGAAGACCCTGACG-3’
sox18-Fwd: 5’-ACACTTCCGAGACCTCCAC-3’
sox18-Rev: 5’-GGTCAAACTCAATCCTGTCC-3’

Whole-mount in situ hybridization and immuno-histochemistry

Whole-mount in situ hybridization was performed as previously described [2]. Previously described riboprobes used in this manuscript were: notch3, ephrinB2a, dll4, dab, flt4, vegf-receptors 2 and 4, gata1, tie2, cdh5, fli1a [2, 6-11]. Bacterial clones for sox7 and
sox18 were obtained from Open Biosystems (http://www.openbiosystems.com). Embryos were mounted in glycerol and documented with a Zeiss axioplan mounted with a Leica DFC 480 camera.

Immuno-histochemistry was performed as previously described [12]. Briefly, embryos were fixed overnight with 2% paraformaldehyde and embedded in 4% low melting point agarose (Invitrogen). Embedded embryos were cut using a HM650V vibratome (Microm) into 250µm sections. Filamentous actin was visualized with rhodamine phalloidin (Fluka). Nuclei were visualized with 4,6-diamino-2-phenylindole (DAPI) (Sigma). Processed samples were mounted in Aquamount improved (BDH laboratory supplies) and imaged using a Leica TCS SPE confocal microscope.

Morpholino injections and microangiograms

Morpholinos (MOs) were obtained from Gene Tools (http://www.gene-tools.com) and diluted in water containing 0.2% phenol red. One cell stage embryos were injected (maximum volume of 2nl) as described [13]. Live embryos were anaesthetized using MS222 [1] and mounted in a glass-bottom dish containing 0.4% agarose. Images were taken with a LEICA CLSM SP2 AOBS confocal microscope. Confocal microangiography was performed as described [14]. Embryos were injected with silent heart [15], etsrp [16], or sox7 and sox18 specific morpholinos. Morholino (MO) sequences were:

MOsox7-ATG: 5’-CGCACCTTATCAGAGCCGCCATGTGC-3’
MOsox18-ATG: 5’-ATATTCATTCCAGCAAGACCAACAC-3’
MOsox7-UTR: 5’-CTGTCAAAACTTAGGCTTCCTTTTG-3’
MO_{sox18-UTR}: 5'- AGCAAGCTGTTGCTTTGAGTAAAG-3’

MO_{silent heart}: 5’-CATGTTTGCTCTGATCTGACACGCA-3’

MO_{etsrp}: 5’-CACTGAGTCTTATTTACATATC-3’

References


Movie Legends

**Supplementary movie 1.**
Phase-contrast movie showing circulation in trunk and tail of an uninjected control embryo at 2.5dpf (top, dorsal is up) and loss of circulation in trunk and tail of a sox7/sox18-dKD embryo at 3dpf (bottom, ventral is up). Anterior is to the right.

**Supplementary movie 2.**
Phase-contrast movie showing circulation in trunk and tail of an uninjected control embryo at 2.5dpf (top, dorsal is up) and a sox7-morphant embryo at 2.5dpf (bottom, dorsal is up). Anterior is to the left.

**Supplementary movie 3.**
Phase-contrast movie showing circulation in trunk and tail of an uninjected control embryo at 2.5dpf (top, dorsal is up) and a sox18-morphant embryo at 2.5dpf (bottom, dorsal is up). Anterior is to the left.
Figure Legends

Supplementary figure 1: Sox7 and sox18 expression and lack of trunk circulation in sox7/sox18-dKDs

a-c. Expression profile of sox7 (a) At 12hpf, sox7 is expressed in the lateral mesoderm of the embryo. (b) At 18hpf, sox7 expression is found in a cord-like structure (future DA and PCV) and in presumptive migrating angioblasts (arrowheads). (c) At 26 hpf (lateral view), sox7 expression is restricted to the endothelial cells of the main axial (white arrow), head (black arrow) and intersegmental (white arrowhead) vessels, as well as to two rhombomeres (black arrowhead).

d-f. Expression profile of sox18 (d) At 12hpf, sox18 is expressed within the lateral mesoderm of the embryo. (e) At 18hpf, sox18 expression is found in a cord-like structure and in presumptive migrating angioblasts (arrowheads). (f) At 26 hpf (lateral view), sox18 expression is restricted to endothelial cells of the main axial (white arrow), head (black arrow), and intersegmental (white arrowhead) vessels, as well as to the eye (black arrowhead).

(g) RT-PCR expression analysis showed maternal expression of both sox7 and sox18, expression levels for sox18 however are lower at 0, 1 and 2.5 hours post fertilization compared to expression levels for sox7. Sox7 and sox18 mRNA expression shown for 0, 1, 2.5, 6, 8, 10 and 24 hours post fertilization old embryos.

h-i. Microangiographies at 2.5 dpf. Major axial vessels in the posterior part of sox7/sox18-dKDs were not filled with dye.

j-m. Confocal images (30hpf) of (j) an uninjected fli1a:gfp embryo, (k) a vegfr4:gfp embryo injected with 10ng of sox7-MO, (l) a vegfr4:gfp embryo injected with 10ng of sox18-MO and (m) a fli1a:gfp embryo injected with 5ng of both sox7-MO and sox18-MO. Insets show
magnifications at the posterior level of the trunk. (m, inset) Note the partial fusion of the DA and PCV in sox7/sox18-dKDs. DA, dorsal aorta; dKD, double knock-down; MO, morpholino; PCV, posterior cardinal vein.

**Supplementary figure 2: Sox7 and sox18 expression in cloche mutants, scl mutants and etsrp morphants**

Whole mount in situ hybridization expression analysis for sox7 and sox18 in embryos defective in the blood lineage, the endothelial lineage or both. Mesodermal sox7 and sox18 expression was lost in cloche mutants, which lack nearly all blood and endothelial cells. Early sox7 and sox18 expression in scl mutants, defective in dorsal aorta as well as blood formation, appeared normal. Mesodermal sox7 and sox18 expression was vastly reduced in etsrp morphants, which lack the endothelial lineage only. Based on these results and the expression pattern of these genes at later stages we conclude that sox7 and sox18 expression is restricted to the endothelial lineage and does not contribute to other cell lineages derived from the mesoderm.

**Supplementary figure 3: Morpholino specificity controls**

MOsox7-ATG-, MOsox18-ATG-, MOsox7-UTR- and MOsox18-UTR-binding sites were cloned into pCS2+, which contained a transcript encoding for GFP, upstream of the translation initiation site. Translation of capped mRNA was efficiently blocked by the respective MOs, demonstrating efficacy and specificity (sox7-MO does not block sox18 translation) of the reagents used. Results shown for MOsox7-UTR- and MOsox18-UTR; similar results were
obtained with MO$_{sox7-ATG}$-, MO$_{sox18-ATG}$. Given the efficacy and specificity of the MOs used, we did not perform rescue experiments by co-injection of $sox7$- or $sox18$-mRNA.

**Supplementary figure 4: Arterial specification is perturbed in $sox7$/$sox18$-dKDs**

(a-d). Uninjected control embryos; (e-h. $sox7$/$sox18$-dKDs. All embryos at 26hpf. (a,e) $gata1$, (b,f) $dll4$, (c,g) $dab2$, (d,h) $flt4$. (a,e) Levels of $gata1$ appear normal in $sox7$/$sox18$-dKDs. (b,f) Note the dramatic reduction in expression levels of the arterial marker $dll4$ in the dorsal aorta (brackets) in $sox7$/$sox18$-dKDs. (c,d; g,h) Ectopic expression of the venous markers $dab2$ and $flt4$ in the dorsal aorta (brackets) and intersegmental vessels (white arrow) in $sox7$/$sox18$-dKDs. dKD, double knock-down.

**Supplementary figure 5: Vascular tube formation is perturbed in $sox7$/$sox18$-dKDs**

The lack of lumen formation observed in $sox7$/$sox18$-double knock downs (dKDs) was examined in both sagital and transverse 7µm thick sections of $sox7$/$sox18$-dKDs and uninjected control (UIC) embryos. a. Sagital section of the tail section of a 30hpf $sox7$/$sox18$-dKD (n=10) showing shunt formation between DA and PCV, indicated by the arrow. No shunts were observed in a comparable number of UIC embryos (data not shown). b-f. Consecutive transverse sections of the midtrunk region of a 30hpf $sox7$/$sox18$-dKD, showing the loss and reappearance of the physical boundary between the DA and PCV, indicated by the arrows. This is a representative case from 10 examined $sox7$/$sox18$-dKDs. No shunts were observed in UIC embryos (n=10) (data not shown). Sections were stained with hematoxylin and eosin. Scale bar in b-f 25 µm.
Supplementary figure 6: Arteriovenous specification is unaltered in sox7-, sox18- and silent heart morphants

a,b. Uninjected control (UIC) embryos; c-d. sox7-morphants; e-f. sox18-morphants; g-h. silent heart-morphants. Expression analysis of 26 hpf old embryos using the markers (a,c,e,g) ephrinB2a or (b,d,f,h) flt4 as a probe. Sox7- and sox18-morphants show normal levels of expression for ephrinB2a and flt4 compared to sox7/sox18-double knock downs (Figure 2 and Supplementary Figure 4), confirming the observed redundancy amongst sox7 and sox18. Expression levels of the markers tested appeared normal in silent heart-morphants, excluding the possibility of a shift in arteriovenous identity caused by a lack of circulation.
**Supplementary figure 1:** *Sox7* and *sox18* expression and lack of trunk circulation in *sox7/sox18-dKDs*
Supplementary figure 2: Sox7 and sox18 expression in cloche mutants, scl mutants and etsrp morphants
Supplementary figure 3: Morpholino specificity controls
Supplementary figure 4: Arterial specification is perturbed in sox7/sox18-dKDs
Supplementary figure 5: Vascular tube formation is perturbed in sox7/sox18-dKD s
Supplementary figure 6: Arteriovenous specification is unaltered in *sox7*-, *sox18*-
and silent heart morphants