SOX FACTORS TRANSCRIPTIONALLY REGULATE ROBO4 EXPRESSION IN DEVELOPING VASCULATURE IN ZEBRAFISH

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Despite their importance as members of the Roundabout (Robo) family in the control of axonal and vascular patterning, the transcriptional regulation of these genes is poorly understood. In this study, we show that members of the Sry-related high mobility box (Sox) transcription factor family act as transcriptional regulators of roundabout4 (robo4), a Robo gene family member that participates in sprouting angiogenesis in vivo, in zebrafish. Double whole mount in situ hybridization analysis in zebrafish embryos revealed co-localization of the vascular relevant Sox factors sox7 or sox18 mRNA with robo4 transcripts in developing intersomitic vessels (ISVs). A 3 kb human ROBO4 promoter element was able to drive reporter expression in zebrafish to recapitulate the endogenous temporal ISV expression pattern of robo4. EMSA analysis confirmed binding of Sox18 to a canonical Sox binding site (from -1170 bp to -1176 bp) in the Robo4 promoter (3 Kb) and mutation analysis indicate that this site was partially responsible for Robo4 promoter activity in ECs. A combination of gain- and loss-of-function analysis identified Sox7 and Sox18 co-regulation of robo4 but not fli1a transcripts in zebrafish. Finally, Sox-mediated robo4 transcriptional regulation is conserved in the intersomitic vessels (ISVs), and is strikingly regulated with peak expression passing in a "wave" along the trunk axis from 19-29 somites (3), suggesting a high degree of transcriptional control of this gene product. Recently, a three kilobase (kb) human Robo4 promoter sequence has been identified that directs endothelial cell specific expression pattern in vivo and in vitro (5). In addition, a GA-binding protein [GABP]-binding element in the Robo4 promoter is necessary for evolution. These studies imply Sox-mediated transcriptional regulation of Robo4 in the developing embryonic vasculature.

INTRODUCTION

In developing vertebrates, neural and vascular patterning generate intricate branching networks that share several similar features (1). However, this connectivity is governed by a limited toolkit of signaling receptor systems. These systems must therefore be subject to exquisite control to achieve proper patterning and avoid miscues. Recently, members of the axon guidance family have shown both expression and functionality in the developing vasculature. Of the four distinct families of axon guidance signaling partners namely Slit-Robo, Ephrin-Eph, Netrin-Unc and Semaphorin-Plexin, our laboratory has focused on the Slit-Robo family members and their role in the vasculature.

Roundabouts (Robos), a class of cell surface receptors that were originally identified to function in axon guidance (2) have recently been implicated in providing critical directional information for migration of endothelial cells (ECs) (3,4). Four mammalian Robos are known of which the fourth member robo4 is expressed...
endothelial expression in vivo (6). However, to date little is known regarding transcription factors that are involved in regulating robo4 expression during embryonic vascular development. In this study, we provide evidence for Sox7 and Sox18 transcription factors as regulators of robo4 vascular expression during embryonic development in zebrafish. SoxF genes namely Sox7, Sox17 and Sox18 play pivotal roles in cardiovascular development including the orchestration of endothelial cell fate and cell differentiation (7). During embryonic mouse development, Sox7, Sox17 and Sox18 expression is evident in smaller branching vessels and ISVs (8-10) and zebrafish sox7 and sox18 are expressed in early angioblasts at lateral plate mesoderm and ISVs (11-13). To date, little is known in regards to transcriptional target for Sox F’s in the developing angiogenesis in vivo and this study provides evidence that suggests Robo4 may indeed serve as one candidate.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Zebrafish Stocks**

HUVECs were purchased from Lonza and maintained in Endothelial Cell Basal Medium-2 (EBM-2, Lonza) supplemented with Fetal Bovine Serum (FBS) (2%) and 2 U/mL Gentamycin. All HUVEC experiments were performed with cells in passage number 3 through 6. Mouse Robo4 cDNA (probe generation) and human Robo4 promoter-luciferase construct were kindly provided by Drs. Dean Y Li (University of Utah, USA) and William Aird (BIDMC, Harvard, USA). Dr. Monica Beltrame (Università degli Studi di Milano, Italy) provided the zebrafish Sox7 and Sox18 cDNAs. Zebrafish were grown and maintained at 28.5 °C in a 14 h day and 10 h night cycle. Mating was routinely carried out at 28.5°C and all embryos were staged according to established protocols. All zebrafish studies were performed under the MCW institutional guidelines (Animal Protocol Number 312-06-2).

**RNA/MO microinjections and in situ hybridization (ISH)**

Zebrafish sox7 and sox18 RNA were transcribed by T7 polymerase from linearized vectors containing the respective inserts in pcDNA3.1. For GOF experiments, 50-75 pg of capped RNAs were injected into the embryonic cell (1-cell stage). Digoxigenin (DIG)-labeled antisense RNA probes for fli, and robo4 were generated using a DIG RNA labeling kit (Roche). The MOs used for Sox7 and Sox18 were from previous publication (11), and were injected at a dose of 0.25pmol per embryo. The specificity and efficacy of the MOs used in this study has been reported previously (11).

For sox7 and sox18, chromogenic detection of single transcripts was carried out as described (14). For fluorogenic detection of two transcripts in co-expression studies, a DNP labeled robo4 probe was used together with DIG labeled sox7 and sox18 probes, respectively. Hybridized probes were visualized using peroxidase conjugated anti-DIG/DNP antibodies (1:1000, Roche), FITC- and Cy3 tyramides (1:100) from the TSA system (Perkin-Elmer) according to manufacturer’s instructions. Pictures were acquired using a Zeiss Observer Z1 inverted microscope (single staining) or a Zeiss LSM 510 confocal microscope. Confocal data were processed with AxioVision 6.8, and linear level adjustment was carried out with Adobe Photoshop CS. Details on double stainings are available upon request. The mouse probe for Robo4 was made as described previously (15). Mouse section in situ hybridization was performed on 7 µm sections of PFA-fixed, paraffin-embedded embryos. Sections were de-waxed, rehydrated and incubated in 5 mg/mL proteinase K for 20 min at room temperature. After washing in PBS, sections were re-fixed with 4% PFA for 10 min at room temperature, acetylated and pre-hybridized with hybridization solution (50% formamide, 5X SSC, 5X Denhardts, 250 mg/ml yeast RNA, 500 mg/ml herring sperm DNA) for 2 h at room temperature. Hybridization (hybridization solution + 0.5 µg/ml probe) was performed overnight at 60°C. Slides were washed in 5X SSC for 5 min, 0.2X SSC for 1 h at 60°C, 0.2X SSC for 5 min at room temperature and NT buffer (150 mM NaCl, 50 mM Tris-HCl pH7.5) for 5 min at room temperature, before incubating for 2 h with blocking solution (0.5% blocking powder (Roche) in NT buffer) in a humidified chamber. Anti-DIG antibody (Roche) at a 1:500 dilution in blocking solution was added to the slides and incubated overnight at 4°C. Unbound antibodies were removed by washing three times in NT buffer supplemented with 0.05% Tween 40.
Sections were equilibrated in detection buffer (0.1 M Tris pH8.0, 0.1 M NaCl, 10 mM MgCl₂) for 15 min at room temperature and incubated with color solution (BM purple, Roche) according to the manufacturer’s recommendations. Finally, sections were washed 2x 5 minutes in PBTx, and mounted in 80% glycerol in PBS. Stained sections were examined with an Olympus BX-51 microscope (DP-70 12Mp color camera).

**Electrophoretic mobility shift assay (EMSA)**

Oligonucleotides (oligos) synthesized by Integrated DNA Technologies (Coralville, IA) were used for DNA binding assays. Sequence information is provided in supporting information table. Double stranded probes were generated by heating equal molar amounts of each of the 5’→3’ oligo with its respective complementary oligo at 95°C for 10 min followed by cooling to RT for 1 h. Next, double-stranded oligos were labeled with DIG-11-ddUTP using recombinant terminal transferase (20 U/ml) in a final volume of 25 µl according to the DIG Gel Shift Kit, 2nd Generation instructions (Roche; Indianapolis, IN). EMSA was performed as we have previously described in detail (16). Briefly, DNA binding reactions were set up with 1 µg of nuclear or cytoplasmic proteins (HUVEC passage 3) and 0.08 pmol of the DIG-labeled wild-type or mutant Sox18 probe in our modified DNA binding buffer [20 mM Hepes (pH 7.6), 10 mM (NH₄)₂SO₄, 0.2% Tween-20, 30 mM KC1] (17) containing 1 µg [polyd(I-C)] and 0.1 µg poly L-lysine in a final reaction volume of 20 µl. For supershift assays, 2-3 µl SOX18 antibody (4 µg, EMSA certified, Santacruz) was added to the nuclear or cytoplasmic proteins prior to addition of the probe. For competition experiments, unlabeled double-stranded Sox18 WT oligo at final concentrations of 0.08, 0.8, 4.0, 8.0, 16.0 and 32.0 pmol were added simultaneously with 0.08 pmol DIG-labeled Sox18 WT probe to the binding reaction with 1 µg of nuclear protein. Control reactions were performed with the probe alone. Reactions were incubated at room temperature for 15 min after which samples were subjected to electrophoresis on a 6% DNA Retardation Gel (Invitrogen). Blotting on to a positively charged nylon membrane was followed by UV cross-linking. Blots were then incubated with AP-conjugated to anti-DIG antibody followed by addition of substrate CSPD. Fluor Chem HD from Alpha Innotech was used for chemiluminescence detection and quantification of bands was done using Image J software (http://rsb.info.nih.gov/ij/).

**Statistical Analysis**

Statistical analysis was performed using the Student’s t-test with Graph Pad Prism (GraphPad Software, La Jolla, CA) and Microsoft Office Excel 2010 software package. All data are presented as mean ± SEM (n and p-value are provided in each figure or legend).

**RESULTS**

In a microarray transcriptional profile study comparing lymphatic ECs isolated from ragged-opossum (RaOp⁻) mutant mice (RaOp⁻/⁻ mice) (18,19), which carries a mutation in Sox18 to lymphatic ECs obtained from WT mice, we observed an 8.5 fold decrease in Robo4 transcript levels with no detectable change in Robo1 and Robo2 transcript levels were detectable. To validate the microarray result, we performed robo4 ISH on E10.5 dpc sections of RaOp⁻/⁻ mice and WT mice. RaOp⁻/⁻ mice showed diminished robo4 expression in caudal vein ECs (Fig. 1B, white arrowhead) compared to WT mice (Fig. 1A). No change in robo4 expression was observed in dorsal aorta (DA) EC (Fig. 1A, black arrowhead) suggesting preferential venous loss of Robo4 expression in Sox18 KO mice. These results lead us to hypothesize that Sox18 transcription factor affects transcription of Robo4 gene during embryonic vascular development in vivo. To test this hypothesis, we investigated the Sox-mediated transcriptional regulation of robo4 gene in embryonic zebrafish development.

**Sox 7 and sox 18 transcripts are expressed prior to robo4 transcript expression and co-localize during embryonic zebrafish ISV development**

In zebrafish, two Sox factors namely Sox7 and Sox18 show redundant function during embryonic vascular development (11-13). We next performed whole mount ISH for sox7 (Fig. 2A-F) and sox18 (Fig. 2G-L) in zebrafish embryos ranging from 18-27 som in comparison to robo4 between 19-24 som (Fig. S1A-C). At 18-19 som, both sox7 (Fig. 2A) and sox18 (Fig. 2G, asterisk)
expression was observed in rostral ISV sprouts as they began to emerge and in axial vessel, DA. The robo4 expression at this time point was strong in notochord and was observed in angioblasts (Fig. S1A). As ISV development progresses, the expression of sox7 (Figs. 2D-F) and sox18 (Figs. 2I-L) followed a rostral-caudal temporal pattern resembling the pattern observed previously for robo4 (3) although the sox’s expression appears a somite or two early than robo4. We next investigated whether robo4, sox7 and sox18 transcripts were co-expressed in the vasculature during embryonic zebrafish development stages (19-24 som) where robo4 is expressed in ISVs (3). Confocal analysis of two-color fluorescence in situ hybridization of the zebrafish trunk regions shows that both sox7 (Fig. 2P) and sox18 (Fig. 2Q) transcripts were co-localized with robo4 transcript in the developing zebrafish vasculature. At this time point (24 hpf), sox7 expression was noticed predominantly in the leading ISV cell (Fig. 2P’), while sox18 expression was predominant in the axial vessels and the cell immediately ventral to the ISV leading cell (Fig. 2Q’). Our double ISH analysis clearly shows that both robo4 and individual sox (sox7/sox18) transcripts are co-localized on ISVs. Additional inverted microscope images are provided in Fig. S2A-F along with 3D surface rendering of the confocal picture that capture the regions of co-localization (Fig. S2G). Taking the ISV expression data together, it suggests that sox7 and sox18 are expressed in the rostral ISV sprouts prior to robo4 and could potentially influence the highly dynamic robo4 ISV expression observed in these stages.

**Sox18 induces Robo4 promoter activity via specific Sox binding site in vitro in ECs**

Because sox7 and sox18 were expressed prior to robo4 during ISV development, we investigated whether Sox TFs regulated robo4 ISVs expression level in zebrafish. To investigate this question, we utilized the 5’ flanking sequence element of the human Robo4 promoter, which was already shown to direct ECs specific expression in vivo (5,6). Further, bioinformatic analysis of this promoter indicated the presence of putative Sox18 TF binding consensus sequences (A/TA/TCAAA/TG) between -1170 to -1176 bp, which is conserved in mouse Robo4 promoter between -1339 to -1405 bp as well (Fig. S1E). We injected the 3 kb human Robo4 promoter-luciferase construct alone (50 pg) or in combination with sox18 mRNA (50 pg) at 1-cell stage into the embryonic cell and collected embryos at 18-21 som, 21-26 som, 26 som and 24-prim-6 stages for luciferase assays (Fig. 3A); developmental stages in which endogenous robo4 expression is highly dynamic. Embryos from each stage were lysed and assayed for luciferase activity. Remarkably, the exogenous human Robo4 promoter activity drives luciferase in a pattern reminiscent of endogenous robo4 expression in ISVs. We observed a bell shaped curve over the time course with maximal reporter activity at around 24-26 somite stage and returning to base line at 25 hpf-prim6 stage (Fig. 3A, dotted line). Interestingly, in sox18 mRNA co-injected embryos (Fig. 3A, black line), we found that the Robo4 promoter activity was increased at all time intervals when compared with basal levels (Fig. 3A, diamond bars), in essence replicating the bell shaped curve of promoter alone injected embryos but with higher luciferase values. Next, we generated four point mutants (SoxPM1, SoxPM2, SoxPM3, SoxPM4) (Fig. S1E), that had single nucleotide (SoxPM1) or multiple nucleotide (SoxPM4) substitutions at the putative Sox18 binding site in the Robo4 promoter. These constructs were individually transfected in HUVECs (Fig. 3B), and their activity compared to WT human Robo4 promoter (Fig. S1D). All constructs were transfected via electroporation or lipofectamine 2000 into HUVEC cells, and luciferase assays were performed from HUVEC lysates. Interestingly, two (SoxPM1 & SoxPM3) of the three point mutants in addition to SoxPM4 showed reduction in luciferase output (Fig. 3B) indicating that the putative Sox binding site on –1169 bp site is responsible in part for Robo4 promoter activity in ECs. All groups except SoxPM2 were statistically significant (p<0.05) when compared to WT human Robo4 promoter group.

To investigate if the endogenous sox7 or Sox18 proteins are responsible for the Robo4 promoter activity observed in human ECs, we utilized sox7 or sox18 gene specific efficacy-confirmed siRNAs (Santacruz and Fig. S2I) to knockdown endogenous Sox proteins and measured luciferase activity output of the hRobo4 promoter. The luciferase activity is greatly reduced from the Robo4 promoter co-transfected...
with Sox7/18 double siRNA sample when compared to Robo4 promoter-luciferase construct alone (Fig. 3C, P<0.05). To conclusively determine whether Sox7/18 proteins bind to putative Sox binding site in Robo4 promoter, we performed electrophoretic mobility shift assay (EMSA) (Fig. 3D) with nuclear extracts from ECs using WT Robo4 promoter oligo probe and Sox18 Mutant Robo4 promoter oligo probes (M1 & M2). As observed in EMSA blot, the nuclear proteins from HUVECs in WT probe lane clearly formed two complexes: complex 1 (slower migrating), and complex 2 (faster migrating) band (Fig. 3D). Interestingly, M1 mutant Robo4 promoter oligo probe also showed the presence of the two complexes albeit of lower intensity (Fig. 3D, M1 lane). Because the M1 mutant probe has additional Sox18 binding sites, which likely serves as alternate site for interaction, we generated a second mutant probe M2, where we mutated this site (Refer suppl. information for mutant probe sequences). Clearly, the M2 probe shows lower intensities in complex 2 (Fig. 3D, M2 lane). When the complex intensities in M1 and M2 probe lanes for nuclear protein extracts (N) were compared across three independent experiments to WT probe bands, we noticed a 70% and 90% decrease in complex 1 (M1: *P<0.001; M2: **P<0.0005) while a 50% and 70% decrease in complex 2 (M1: #P<0.001, M2: ##P<0.005) respectively for M1 and M2 probes (Fig. 3E). The M2 probe showed lower intensities for both complexes when compared with M1 probe. Both complexes also showed a dose-dependent reduction in intensity when unlabeled competitor probes was included in the EMSA reaction (Fig. S3B).

To determine whether Sox18 protein was present in the shifted complexes, we performed supershift assays with Sox18 antibody (Fig. 3D, Ab lane). Supershift results indicated the presence of Sox18 in both complex 1 (black arrow), and 2 (grey arrow) (Fig. 3D, lanes N, Ab). In addition, when binding reactions with the mutant (M1, data not shown) and WT probes (Fig. 3D, lane + Sox18 Ab) were performed in the presence of Sox18 antibody, the complex 1 band completely disappeared in WT probe lane (data not shown for M1), and the intensity of the complex 2 band decreased by 49% and 65% in reactions with M1 and WT probes, respectively. Collectively, these data suggest that Sox18 is part of the complex that binds to Robo4 promoter sequences and, in addition other co-factors may also be responsible in part for Robo4 promoter activity in ECs.

Taking the in vivo human Robo4 promoter activity across ISV development, ability of Sox18 to induce the Robo4 promoter robustly in vivo, point mutation analysis and Sox siRNA experiments in vitro, and the EMSA analysis data together we conclude that Sox7/18 are responsible in part for inducing Robo4 promoter activity in ECs in vivo and in vitro.

Sox GOF or LOF embryos shows complementary gain or loss of robo4 expression

To investigate if endogenous robo4 expression is modulated by Sox transcription factors, we performed gain (GOF) and loss-of-function (LOF) for sox7 and sox18 in zebrafish embryos. For GOF experiments, we injected sox7 or sox18 mRNA alone or in combination into 1-cell embryo and checked robo4 expression by ISH at 26~28 hpf. The robo4 ISV expression is enhanced in sox7+sox18 mRNA (Fig. 4E, white asterisk) injected embryos when compared to sox7 (Fig. 4B) or sox18 (Fig. 4C) or control mRNA (Fig. 4D) alone injected embryos at 24 hpf, which suggests co-operative interaction between the two factors in inducing robo4 ISV expression. Conversely, in LOF experiments, double knockdown of sox18/sox7 using MOs resulted in a diminished robo4 expression at 24 hpf (Fig. 4G) and 26 hpf (Fig. 4I) compared to control MO (Fig. 4F, 4H) injected embryos. At 24 hpf, 40 out of 42 (95%) sox18/sox7 double knockdown embryos show the phenotype depicted in Fig. 4G. At 26 hpf, 26 out of 27 double morphants showed diminished robo4 expression (Fig. 4I). Interestingly, the diminished expression was selectively observed in the ISVs (Fig. 4G, black arrowhead) with little to no qualitative change detected in the neural tube (Fig. 4G & 4F, NT, red arrowhead). We also checked fli1a expression in control MO (Fig. 4J & 4L) and sox7/18 double MO injected (Fig. 4K & 4M) 24 or 26 hpf embryos, and observed no change (Figs. 4J & K, black asterisk) in control (21 embryos) and double morphants (32 embryos) suggesting specificity of Sox regulation of robo4 ISV expression. Further, QPCR for robo4 and robo1 transcripts in Sox knockdown embryos shows selective down regulation of robo4 vs. robo1 transcripts (Fig.
S3C). This also suggests some level of Robo-specificity for Sox-mediated transcriptional regulation during development. Because robo4 expression is also observed prior to 24 hpf in non-vascular tissues such as neural tube (NT) and notochord (NO), we investigated the effect of sox7 or sox18 or double (sox7+sox18) mRNA injected embryos for robo4 transcript expression at 18 hpf (Fig. S4). At 18 hpf, little to no change was observed in robo4 expression in notochord. However, in neural tube (NT) (Figs. S4B-D, black asterisk) and midbrain hindbrain boundary (MHB) (Figs. S4B-D, red arrow), we observed an expansion in the robo4 expression domain in sox7 (Fig. S4B) or sox18 (Fig. S4C) or sox7+sox18 (Fig. S4D) mRNA injected embryos. Quantitation shows greater than 60% of injected embryos show strong overall robo4 induction qualitatively in MHB and NT (Fig. S4E) at 18 hpf. This result is not totally unexpected since Sox proteins are well known to function in neural development (20,21). At 24 hpf, quantitation was performed for robo4 induction in ISVs (Fig. S4F). The Sox GOF and LOF results when taken together show complementary changes in robo4 ISV expression in vivo, which is in agreement with the redundant function of Sox7 and Sox18 function in zebrafish vascular development (11). Taking the mouse data together from Fig. 1, this data also suggests that Sox-mediated transcriptional regulation of Robo4 is conserved across evolution.

**DISCUSSION**

This study identifies members of the Sox protein transcription factor family as putative regulators of Robo4 gene expression in vivo. The primary findings of this study include:

(a) Early temporal expression of sox7 and sox18 transcripts in DA prior to robo4 expression.
(b) Human Robo4 promoter activity is remarkably conserved in its behavior in zebrafish and correlates well with vascular robo4 expression pattern in vivo.
(c) A polar regulation of robo4 expression via Sox in zebrafish and mice.
(d) Sox7 and Sox18 along with temporal and spatial specific co-factors regulate robo4 transcript expression in the vasculature.

Whole mount ISH for sox7 and sox18 during zebrafish ISV development shows a preponderance of sox7 transcript in the tip of the leading sprout and sox18 transcript in the base of the sprout with prominent expression in axial vessels. Further, both sox7 and sox18 transcripts appear in the rostral sprouts earlier than robo4 expression suggesting regulatory mechanisms of Sox and Robo in ISV sprouting process. In terms of robo4, ISV expression is tightly controlled across a short temporal window and the expression is observed along the length of the entire sprout as well as the DA. This data argues that different Robo-Sox combinations are involved in ISV cell development in vivo. Perhaps, Robo4 and Sox7 in the tip while Robo4 and Sox18 in the base of the sprout function together to direct and maintain ISV sprout formation. Whether the expression levels of sox transcripts correlate with protein expression and function is not known. Interestingly, the polar distribution of sox transcripts in zebrafish ISVs suggests polar regulation of robo4 transcript, which is curiously observed in mice. The robo4 expression in Ra15 mutant mice is selectively down regulated in vein but not in artery suggesting selective regulatory function of Sox-mediated Robo4 induction in vein. It is worthwhile to note that the site of robo4 expression in vein is the area where emergence of Sox18-mediated Prox-1(E cells is noted (22), postulating a role of Robo4 in lymphatic ECs cell directional sprouting. Since lymphatic ECs derive from venous ECs (23) it is tempting to speculate that the selective regulation of Sox-mediated Robo4 induction in venous ECs is potentially the event that triggers the directional migration of Prox-1 LECs from vein. In terms of evolution, both mouse and human Robo4 promoter share a Sox18 binding site proximal to the start site (-1170 in human and -1339 in mouse). Because robo4 ISV expression is dynamically controlled across a short temporal window (18-29 hpf), this would suggest tight control of Robo4 promoter activity during this time frame. In fact, a previously published 3kb human Robo4 promoter (5,6) that shows endothelial-restricted expression in mice behaves similarly in zebrafish across the short temporal window of robo4 ISV expression. This remarkable correlative behavior is enhanced when exogenous sox18 mRNA is provided and is diminished when
the Sox18 binding site is mutated. Although the Robo4 promoter activity is not lost completely in the absence of Sox18 binding site, which is expected because other sites on the promoter such as GABP binding element has also been shown previously to be necessary for endothelial expression in vivo (6). In fact, at least three distinct experimental evidence points to the role of co-factors that participate with Sox in regulating robo4 ISV expression. (a) Mutation of Sox binding site on Robo4 promoter (Fig. 3B) does not result in complete loss of Robo4 promoter activity in ECs, (b) The EMSA study shows that Sox18 mutant probe (M1) clearly binds to non-Sox nuclear proteins, which is not blocked by Sox18 antibody (data not shown) and, (c) Sox GOF single mRNA and double mRNA injected embryos (Fig. 4A-D) show differential robo4 transcript regulation at 18 hpf (individual Sox’s and double Sox’s) (Fig. S4) and 24 hpf (only double Sox’s) (Fig. 4) presumably mediated by different sets of co-factors expressed at these time points during embryonic development. The 24 hpf double mRNA data is in agreement with redundant function of Sox’s in vascular development in zebrafish (11).

In this study, we provide convincing data in three species, each of which independently provides evidence that Sox transcription factors putatively regulate robo4 gene expression. In each of these systems there is ample published evidence of conservation of the molecular mechanisms of vascular development for Sox and Robo’s (3,7,9,11,13,15,18,24). In addition, the genes investigated in our study are well conserved at the sequence level between the species. In sum, the presented zebrafish, mouse, and human endothelial cell data make a substantive body of evidence for conservation of this observation in development. This study for the first time provides a molecular link between Sox and Robo family in vascular development, a paradigm recently shared by another axon guidance gene of the ephrin-Eph family (25).

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FOOTNOTES

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Abbreviations: bp: basepair, CV: Caudal Vein, DA: Dorsal aorta, EC: endothelial cell, flil: friend leukemia integration, hpf: hours post fertilization, HUVEC: Human Umbilical cord Vein Endothelial Cells ISVs: intersomitic vessels, kb: kilobases, MO: morpholino, Op: Opossum, Robo: human roundabout, robo4: zebrafish roundabout4, som: somite, WT: wild type, vessel

FIGURE LEGENDS

Fig. 1. Sox18 knockout mice show polar loss of Robo4 expression in caudal vein.
A and B are transverse section of robo4 in situ 10.5dpc embryos (WT and Ra{{OP^-}}). The expression of robo4 is detected in the endothelium of both DA and CV in dorsolateral polarized fashion. The black arrowhead shows robo4 expression in the dorsal aorta (DA), and white arrowhead shows the expression in the caudal vein (CV). A’ and B’ are respective high power images of the panels in A and B respectively with the dotted line outlining the DA and CV. ISH was performed on 3 WT and 3 Ra{{OP^-}} homozygous embryos.

Fig. 2. Montage of Robo4, Sox7 and Sox18 endogenous expression across embryonic zebrafish development.
Whole mount for sox7 (A-F), and sox18 (G-L) ISH embryos were performed as indicated in the materials and method section. Embryos were positioned with anterior (A) to the left, posterior (P)
to the right and dorsal (D) to the top and ventral (V) to the bottom as indicated by the orientation bars. Embryos were staged according to the somite numbers as indicated in the respective panel. Asterisks (black and white) indicate ISVs in the zebrafish trunk region. da: dorsal aorta, y: yolk, ye: yolk extension. P and Q are whole mount two-color confocal fluorescent sox7 or sox18 (red) with robo4 (green) ISH images of 24 hpf zebrafish trunk. White arrows indicate ISVs co-localized for robo4 and sox7/18 transcript. P’ and Q’ are higher magnification of regions highlighted by white brackets in P and Q panels respectively.

**Fig. 3.** Robo4:Sox7/18 transcriptional regulation. A is graphical representation of Sox18-induced Robo4 promoter activity in vivo. Details of the experimental design are provided in the materials and method section. Dotted line denotes the baseline exogenous Robo4 promoter activity. Black line denotes exogenous Robo4 promoter activity in sox18 over-expression (sox18 mRNA) embryos. Line along the x-axis indicates empty control pGL3 vector injected embryos. The graph is a representative experimental data set and each time interval contained 20-25 embryos. This experiment was performed twice with identical trends and error bars have not been provided due to the high variation in the luciferase values from one experiment to the next. B shows in vitro luciferase assays in HUVECs for Robo4 promoter point mutants (PM 1-4) in comparison to hRobo4WT promoter. Error bars represent SEM from three independent experiments and all luciferase values are shown as fold comparison to hRobo4 WT promoter sample. All sample groups were compared to hRobo4 WT promoter groups and all samples except hRobo4PM2 were statistically significant at *p<0.05. C shows Robo4 promoter activity in control (lacZ siRNA) and sox7 and sox18 knockdown (sox7 plus sox18 siRNA) ECs. For panels B and C, HUVECs were transiently transfected with WT-Robo4 and Robo-4 PM1-4 (4 mutants) constructs or WT hRobo4 promoter (1.5 μg) and lacZ and sox7 plus sox18 siRNA (240 nM each) for 36 h and the promoter activity was determined as a function of luciferase activity. Luciferase (firefly) readings were normalized to the control and data from 3 independent experiments is compiled together. **p<0.05 was determined by two-tailed statistical analysis. D shows EMSA of DNA binding reactions with Sox18 mutant (M1 & M2) and WT probes incubated with nuclear (N) or cytoplasmic (C) proteins from ECs, in the presence or absence of Sox18 antibody (Ab). The black and grey arrows indicate the shifted complex 1 and 2, respectively. The complex 1 - top band (black arrow) is diminished in intensity in mutant M1 probe N lanes, and is absent in mutant M2 probe and wild type probe plus Ab lanes. E shows the chemiluminescence values of the complex 1 and 2 bands (arbitrary unit) measured by Fluor Chem HD, and band intensities of the two complexes in M1 and M2 probe EMSA relative to WT probe EMSA are shown. Data is from 3 independent EMSA reactions, and error bars represent SEM (Complex 1-M1: *p=0.0009, M2: **p=0.0001; Complex 2-M1: #p=0.0076, M2: ##p<0.0018).

**Fig. 4.** Sox GOF and LOF show reciprocal change in robo4 transcript expression but no changes in fli expression. A-E shows robo4 ISH trunk expression between 26-28 hpf zebrafish embryo microinjected with control (A & D), sox7 (B), sox18 (C), and sox7+sox18 mRNA (E) (50 pg each). A-E shows strong robo4 expression in the neural tube. However, sox7+sox18 double mRNA injected embryos show strong robo4 expression in the ISV (asterisk). Experiments were repeated 3 independent times and pictures are representative of 15 embryos per injection group. Additional quantification is provided in Fig S2F. F-M show robo4 ISH trunk expression in 24 hpf (F, G) and 26 hpf (H, I) and fli at 24 hpf (J, K) and 26 hpf (L, M) zebrafish embryos injected with control MO (F, H, J, L) and sox7 + sox18 double MO (G, I, K, M). The inset numbers in panels F, G, H, I, J, K, L and M indicate number of embryos that show similar phenotype as in the image. Asterisk in J and K and black arrowhead in G indicate ISV expression. Red arrowhead in F and G indicate neural tube (NT) robo4 expression.
Figure 1
Figure 2
Figure 3

A

Luciferase Units

- Control pGL3 Vector
- hRobo4 promoter 50 pg
- hRobo4 promoter 50 pg + Sox18 mRNA 50 pg

B

Luciferase Units

Mouse hR1 hR2 hR3 hR4

C

Luciferase Units

pGL3 Vector hRobo4 promoter hRobo4 WT promoter + Sox17 + Sox18 siRNA (240 nM each)

D

Probe Sox18 Ab + + + + + + + + + +

E

Relative Densitometry Units

Complex 1

- WT
- M1
- M2

Complex 2

- WT
- M1
- M2
Figure 4

Control MO  sox7/18 double MO

F  G  24 hpf  14/15  14/14  26 hpf
H  I  26 hpf  11/11  12/13  12/12  16/16  26 hpf
J  K  26 hpf  12/12  16/16  12/12  20/20  16/16  26 hpf
Sox factors transcriptionally regulate ROBO4 expression in developing vasculature in Zebrafish

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