Sox7 and *Sox17* are strain-specific modifiers of the lymphangiogenic defects caused by *Sox18* dysfunction in mice

Brett Hosking[1,*], Mathias François[1,*], Dagmar Wilhelm[1], Fabrizio Orsenigo[2,3], Andrea Caprini[2,3], Terje Svingen[1], Desmond Tutt[1], Tara Davidson[1], Catherine Browne[1], Elisabetta Dejana[2,3] and Peter Koopman[1,†]

Developmental defects caused by targeted gene inactivation in mice are commonly subject to strain-specific modifiers that modulate the severity of the phenotype. Although several genetic modifier loci have been mapped in mice, the gene(s) residing at these loci are mostly unidentified, and the molecular mechanisms of modifier action remain poorly understood. Mutations in *Sox18* cause a variable phenotype in the human congenital syndrome hypotrichosis-lymphedema-telangiectasia, and the phenotype of *Sox18*-null mice varies from essentially normal to completely devoid of lymphatic vasculature and lethal, depending on the strain of the mice, suggesting a crucial role for strain-specific modifiers in this system. Here we show that two closely related Group F Sox factors, SOX7 and SOX17, are able to functionally substitute for SOX18 in vitro and in vivo. SOX7 and SOX17 are not normally expressed during lymphatic development, excluding a conventional redundancy mechanism. Instead, these genes are activated specifically in the absence of SOX18 function, and only in certain strains. Our studies identify *Sox7* and *Sox17* as modifiers of the *Sox18* mutant phenotype, and reveal their mechanism of action as a novel mode of strain-specific compensatory upregulation.

KEY WORDS: Genetic modifier, Sox genes, *Prox1*, Lymphangiogenesis, HLT syndrome, Mouse

INTRODUCTION

The expression of different phenotypes caused by the same mutation is commonly attributed to genetic modifiers (Nadeau, 2001). Modifier genes represent an important but poorly understood component of genetic regulatory circuitry, and attempts to identify and study genetic modifiers have become a priority in functional genomics. A number of modifier loci have been identified in humans and mice by polymorphism mapping, but in only a very few cases have the gene(s) responsible been pinpointed and their molecular functions characterized (for a review, see Rivera and Tessarollo, 2008).

In mice, genetic background is well known to influence the phenotype resulting from gene inactivation by homologous recombination (gene knockout). Many examples exist in which a knockout phenotype may be mild or imperceptible on one genetic strain, but may reveal itself after the same mutation is bred onto a different background, typically C57BL/6 (Barthold, 2004). This phenomenon is usually attributed to strain-specific modifiers, but typically, the genes responsible remain unidentified, and hence little is known about how modifiers are involved at the mechanistic level in modulating the expressivity of the mutant phenotype.

Sox18 encodes a transcription factor implicated in the development of hair follicles and blood and lymphatic vasculature. Like other members of the Sox family, SOX18 selectively binds the heptameric consensus DNA sequence, $5'-^A/_T^A/_TCAA^A/_TG-3'$, by virtue of a 79 amino acid high-mobility group (HMG) domain (Hosking et al., 1995; Hosking et al., 2001b). SOX18 also is able to activate transcription via a trans-activation domain C-terminal adjacent to the HMG domain (Hosking et al., 1995). Dominant and recessive mutations of *SOX18* have been found to underlie the human hereditary syndrome hypotrichosis-lymphedema-telangiectasia (HLT) (Irrthum et al., 2003). Physical features of this complex disorder are sparse hair, absence of eyebrows, telangiectasia in various sites and lymphedema, particularly manifest in the limbs. In the case of dominant HLT, nonsense mutations cause premature truncation of the SOX18 trans-activation domain. Recessive mutations cause amino acid substitutions that affect the DNA-binding HMG domain, and are therefore most likely loss-of-function mutations.

We recently showed using cellular and in vivo genetic assays in mice that SOX18 is absolutely required for activating lymphatic *Prox1* transcription and initiating lymphatic vascular development from blood endothelial cell precursors (Francois et al., 2008). *Sox18* is a member of the Group F Sox subfamily, a group of three genes (*Sox7*, *Sox17* and *Sox18*) encoding proteins with remarkably similar primary structure (Bowles et al., 2000). As is often the case with different members of the same Sox subfamily (Wegner, 1999), *Sox7*, *Sox17* and *Sox18* share some domains of expression that suggest functional overlap: for example, all three genes are known to be expressed in endothelial cells during vascular development (Young et al., 2006). These observations raise the possibility that SOX7 and/or SOX17 might interact with the SOX18 pathway in some developmental and disease contexts.

In the present study, we sought to explore the role(s) that *Sox7* and/or *Sox17* might play in lymphatic vascular development. We found that although SOX7 and SOX17 are both able to activate the *Prox1* promoter in vitro and in vivo, neither is normally active during lymphatic development, indicating that their relationship with *Sox18* in this system is not one of simple redundancy. However, these genes were upregulated in the absence of *Sox18* function, and specifically on a mixed background but not on C57BL/6, indicating that they act as strain-specific modifiers of the *Sox18* lymphatic phenotype.

[1]Institute for Molecular Bioscience, The University of Queensland, Brisbane, Qld 4072, Australia. [2]IFOM, FIRC Institute of Molecular Oncology, Via Adamello 16, 20139 Milan, Italy. [3]Department of Biomolecular Sciences and Biotechnologies, School of Sciences, University of Milan, 20129 Milan, Italy.

*These authors contributed equally to this work
†Author for correspondence (e-mail: p.koopman@imb.uq.edu.au)

MATERIALS AND METHODS

Mouse strains

Sox18-null embryos were genotyped as previously described (Pennisi et al., 2000a). Backcrossing of *Sox18* null mice from the original mixed background (129/CD1) was accomplished by 11 generations of inbreeding onto a pure B6 background. Single (*Prox1*-GFP) and double (*Prox1*-GFP/*HoxB2 SoxF*) transgenic mice were produced by pronuclear injection of constructs into CBA/B6 F1 oocytes. Transgenic embryos were screened by PCR. Procedures involving animals conformed to institutional guidelines (University of Queensland Animal Ethics Committee).

Immunofluorescence

Immunofluorescence was performed as previously described (Wilhelm et al., 2005). Adult ear samples and embryos were dissected and fixed overnight in 4% PFA. Primary antibodies in blocking solution were added and incubated for 24 hours at 4°C. Samples were washed for 6 hours in washing solution (1% BSA, 1% DMSO in PBSTx) and incubated for 16 hours with secondary antibodies in blocking solution. Confluent H5V cells were fixed in 4% PFA for 15 minutes at room temperature. Cells were permeabilized with PBSTx (0.3%) for 5 minutes at 4°C and then blocked in PBS/5% BSA for 30 minutes at room temperature.

Fluorescent section in situ hybridization/immunofluorescence

Probes for *Sox7, Sox17* and *Sox18* were constructed and fluorescent section in situ hybridization was performed as described previously (Kanai-Azuma et al., 2002; Katoh, 2002; Pennisi et al., 2000b). Slides were boiled for 5 minutes in antigen unmasking solution (Vector), blocked in 10% heat-inactivated sheep serum in PBTx and hybridized with anti-PROX1 antibody overnight at 4°C. After washing, slides were incubated with secondary antibody diluted in blocking solution, washed, incubated with DAPI (1:2000 in PBTx) for 5 minutes, washed in PBTx, and mounted in 80% glycerol in PBS. Sections were examined by confocal microscopy using a Zeiss LSM 510 META confocal microscope.

Antibodies

Antibodies were used in the following dilutions: rabbit polyclonal anti-mouse PROX1 (Covance), 1:2500; mouse monoclonal anti-mouse α-tubulin (Sigma), rabbit polyclonal anti-LBX2 (Chemicon), 1:500; rabbit polyclonal anti-mouse SOXF (Kindly provided by Dr Y. Kanai, Tokyo University), 1:1000; rabbit polyclonal anti-mouse LYVE-1 (Fitzgerald Industries, Concord. MA), 1:1000; rat anti-mouse CD31 (BD Pharmingen) 1:1000; mouse monoclonal anti-MYC Tag antibody (9B11, Cell signaling), 1:2000; rabbit polyclonal anti-GFP (Molecular Probes), 1:1000. Secondary antibodies anti-rat IgG Alexa Fluor 594, anti-rabbit IgG Alexa Fluor 488, anti-hamster IgG Alexa Fluor 488, anti-biotin IgG Alexa Fluor 594, and anti-biotin IgG Alexa Fluor 488 (Molecular Probes) used at 1:200, and anti-rabbit IgG HRP conjugated (Zymed Laboratories) 1:2000.

Embryonic stem cell culture

Embryonic stem (ES) cells were differentiated to endothelial cells as previously described (Balconi et al., 2000). Undifferentiated stocks were mildly trypsinized and suspended in Iscove's modified Dulbecco medium with 15% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 450 μM monothioglycerol, 10 μg/ml insulin, 50 ng/ml human recombinant VEGF-A_{165} (Peprotech), 2 U/ml human recombinant erythropoietin (Cilag AG), 100 ng/ml human bFGF (Genzyme). Cells were seeded in bacteriological Petri dishes and cultured for 11 days at 37°C with 5% CO_2 and 95% relative humidity.

Quantitative PCR analysis

Wild-type and $Sox18^{-/-}$ embryos on B6 and mixed background strains were collected and the trunks dissected at 11.5 days post-coitum (dpc). Total RNA was isolated and 1 μg reverse transcribed with random hexamers (High-Capacity cDNA Archive Kit; Applied Biosystems) according to the manufacturer's instructions. cDNA (5 ng) was amplified in triplicate in a reaction volume of 15 μl using TaqMan Gene Expression Assay (Applied Biosystems) and an ABI/Prism 7900 HT thermocycler, using a pre-PCR step of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 s at 60°C. Preparations of RNA template without reverse transcriptase were used as negative controls. C_t values were normalized to *Gapdh* or *18S* (Spagnuolo et al., 2004).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out as previously described (Hosking et al., 2004). Two double-stranded oligonucleotides were constructed, containing either of the SOX-binding sites (underlined) and surrounding sequences from the *Prox1* promoter at –1135 bp (SoxA) 5′-GGTTCCCCCGCCCCCAGACAATGCTAGTTTGCATACAAAG-3′ and at –813 bp (SoxB) 5′-ACAGTCCATCTCCCTTTCTAACAATTGAAG-TCACCAAATG-3′.

Plasmids

The mouse *Prox1* promoter fragment encompassing nucleotides –3952 to –1 driving GFP expression, along with SoxA and SoxB mutants have been previously described (Francois et al., 2008). *Prox1* promoter deletion clones were generated by PCR amplification using the –1 primer (5′-CCCTCGAGGTGCCATCTTCAAAAGCTCGTCA-3′) and a primer at position –822 (5′-GGGGTACCTCCCTTTCTAACAATTGAAGTCA-CCAAAT-3′). *Myc-Sox* and *Myc-Hoxc10* constructs were generated using the plasmid pcDNA3 glo-myc (kindly provided by Dr P. Berta). *Myc-Sox* was subcloned into pDB22.0 (Sham et al., 1993) containing the 2Kb *HoxB2* enhancer element. *Sox* cDNAs were subcloned into pGEX-1N as previously described (Hosking et al., 1995).

Cell culture and transfection experiments

Mouse mesenteric lymph node endothelial cells (mlEnd) were created as previously described (Sorokin et al., 1994) and obtained from Dr Jennifer Gamble. Cells were transfected using a combination of Lipofectamine 2000 (Invitrogen) and CombiMag (Chemicell) for 20 minutes (according to the manufacturers' instructions) on a magnetic plate at room temperature. Cells were harvested 24 hours after transfection, and luciferase assays were carried out as previously described (Hosking et al., 2001a). H5V murine endothelial cells were cultures as previously described (Garlanda et al., 1994).

Lentiviral vector preparation

To generate HIV3-PGK vector, the *Pgk* promoter from pRetro/SUPER (Brummelkamp et al., 2002) was ligated into the lentiviral plasmid pRRLsin.PPTs.hCMV.GFPwpre (Follenzi et al., 2000) in place of the CMV promoter. Both *Sox7* and *Sox17* cDNAs were subcloned in HIV3-PGK and lentiviral vectors produced as described (Dull et al., 1998). Lentiviral and packaging plasmids were kindly donated by L. Naldini, HSR-TIGET, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy.

Chromatin immunoprecipitation

A total of 1×10^7 mlEnd cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Chromatin immunoprecipitation (ChIP) was performed as previously described (Wilhelm et al., 2005). Primers 5′-GGCAAGGGTGTTTCCTTTTT-3′ and 5′-GGCACAGACATGCACT-GTCC-3′ were used in a 22-cycle PCR reaction (annealing temperature 50°C).

Western blotting

TM3 cells were transfected with *Sox7*, *Sox9*, *Sox17* or *Sox18* expression constructs, and proteins were extracted in 150 μl of 2× sample buffer (125 mM Tris, pH 6.8; 4% SDS; 20% glycerol and 5% β-mercaptoethanol). Samples were then heated at 100°C for 5 minutes to denature proteins before 1D SDS-PAGE and immunoblotting using primary antibody overnight at 4°C. HRP-conjugated secondary antibody followed by chemiluminescence detection with Super Signal West Pico reagent (Pierce, Rockford, IL, USA) were used to reveal protein expression.

RESULTS

Strain dependence of the *Sox18*-knockout phenotype

We have shown previously that *Sox18* plays a crucial role in activating the *Prox1* gene and inducing differentiation of the lymphatic vasculature from blood endothelial precursors during

mouse development (Francois et al., 2008). Curiously, homozygous inactivation of *Sox18* (*Sox18*^{−/−}) causes only a mild coat phenotype on a mixed 129/CD1 genetic background (Pennisi et al., 2000a). However, when we bred this same mutation onto a pure C57BL/6 (B6) background by 11 generations of backcrossing, extensive subcutaneous edema developed in homozygotes (Fig. 1A), and embryos died at 14.5 dpc (Fig. 1B). Mild edema was observed only rarely in homozygous knockout embryos on the mixed background, and these mice survived to adulthood (Fig. 1). Heterozygous mutation (*Sox18*^{+/−}) caused gross mispatterning of adult ear lymphatic vasculature on a B6 background (Fig. 1C), but not on a mixed background (data not shown), whereas even homozygous mutation caused only an intermediate level of ear lymphatic mispatterning on a mixed background (Fig. 1C). The dramatic difference in *Sox18*-mutant phenotypes depending on background strain suggested the existence of one or more strain-specific modifier genes operating in this system.

SOX7 and SOX17 can activate SOX18 targets
We reasoned that the Group F Sox factors SOX7 and/or SOX17 might act interchangeably with SOX18 during the establishment of the lymphatic vasculature on a mixed background. This hypothesis is based on published findings of redundant roles of *Sox17* and *Sox18* in early cardiovascular development (Sakamoto et al., 2007) and postnatal angiogenesis in mice (Matsui et al., 2006), and of

Fig. 1. Severity of the *Sox18*-mutant phenotype depends on the genetic background. (**A**) Breeding of the *Sox18*^{−/−} mutation from its original mixed (129/CD1) background onto a pure B6 background revealed an edematous phenotype in mouse embryos (right panel, thick arrow) rarely observed on a mixed background (middle panel, thin arrow). (**B**) *Sox18*^{−/−} and *Sox18*^{+/−} mixed background mice all survived to adulthood (*n*=6), whereas *Sox18*^{−/−} B6 mice all died in utero around 14.5 dpc, and only 32% of *Sox18*^{+/−} B6 mice (*n*=19) survived. (**C**) Strain-dependent lymphatic mispatterning in adult *Sox18*-mutant mice. Immunofluorescence imaging of wild-type (mixed background), homozygous *Sox18*^{−/−} (mixed background) and heterozygous *Sox18*^{+/−} (B6 background) adult ear tissues using antibodies to the lymphatic marker LYVE-1. Large-diameter, blunt-ended lymphatic vessels were visible in wild type (left panel), whereas heterozygous *Sox18*^{+/−} (B6 background) mice showed small, highly branched lymphatic vessels (right panel). Homozygous *Sox18*^{−/−} (mixed background) mice displayed an intermediate phenotype (middle panel). Scale bars: A, 2 mm; C, 100 μm.

Sox7 and *Sox18* in arterio-venous specification in zebrafish development (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). We therefore undertook a series of experiments to test whether SOX7 and/or SOX17 might also activate *Prox1* expression during lymphangiogenesis.

First, we tested the effects of lentiviral-mediated overexpression of *SoxF* genes in differentiating mouse ES cells, which can differentiate into endothelial cells in vitro (Balconi et al., 2000; Liersch et al., 2006). As a control, ES cells were infected with lentivirus encoding the inert marker green fluorescent protein (GFP). Differentiation of GFP-infected ES cells for 11 days in culture resulted in an induction of the lymphatic marker *Prox1*, expression of which was significantly increased by overexpression of SOX7 or SOX17 (Fig. 2A). These results show that SOX7 and SOX17 stimulate lymphatic marker expression during the differentiation of ES cells along an endothelial-specific pathway, in a similar fashion to SOX18 (Francois et al., 2008).

We tested whether this effect was due to direct regulation of *Prox1* expression. Using EMSA, we tested the binding of SOX7 and SOX17 to the *Prox1* promoter elements SoxA (−1135 to −1130 bp relative to the translation start site) and SoxB (−813 to −808 bp). These elements are known to be important for SOX18 regulation of *Prox1* expression (Francois et al., 2008). Bacterially expressed GST-SOX7 and SOX17 fusion proteins bound strongly to both sites (Fig. 2B). Specificity of binding was confirmed by competing the interaction with increasing amounts of unlabeled oligonucleotide (Fig. 2B). Further, ChIP experiments indicated that SOX7 and SOX17 bind specifically to the native *Prox1* promoter in the mouse mesenteric lymph node endothelial cell line mlEnd (Sorokin et al., 1994). Anti-MYC antibody was able to precipitate *Prox1* promoter fragments from mlEnd cells transfected with Myc-SoxF expression constructs but not those transfected with Myc-Hoxc10 constructs as a negative control (Fig. 2C).

We also determined whether SOX7 or SOX17 could trans-activate the *Prox1* promoter driving a luciferase reporter gene in mlEnd cells (Francois et al., 2008). Overexpression of SOX7 and SOX17 led to a significant increase in luciferase activity (Fig. 2D) compared with controls. Deleting the SoxA-binding site (Fig. 2D), or disruption of either SOX-binding site by directed mutagenesis (Fig. 2E), abolished *Prox1* transcription in this assay. The finding that SOX7 and SOX17, like SOX18, can bind to and activate transcription from the *Prox1* promoter further supports the hypothesis that SOXF factors are capable of acting interchangeably during lymphatic endothelial development.

To demonstrate that SOX7 and SOX17 are able to induce *Prox1* expression in vivo, we made double-transgenic mice harboring both a *Prox1*-GFP reporter construct (Francois et al., 2008), and a second construct capable of driving either SOX7 or SOX17 expression ectopically under the control of a modified *HoxB2* enhancer (Davenne et al., 1999; Sham et al., 1993) (Fig. 3). The modified *Hoxb2* enhancer has been used in previous studies to test the molecular consequences of ectopic gene expression in transgenic mice; it is expressed in the second and third branchial arches, lateral plate mesoderm, otic vesicle and hindbrain rhombomeres 3, 4 and 5 during mouse development (Bell et al., 1997). Misexpression of *Sox9* using this enhancer results in upregulation of the chondrocyte marker *Col2a1* (Bell et al., 1997), and misexpression of *Sox18* using the same element stimulates expression of a number of lymphatic endothelial markers (Francois et al., 2008) (M.F., B.H. and P.K., unpublished), indicating that cells in at least some sites of *Hoxb2* enhancer expression are developmentally naïve and capable of differentiating into different cell types.
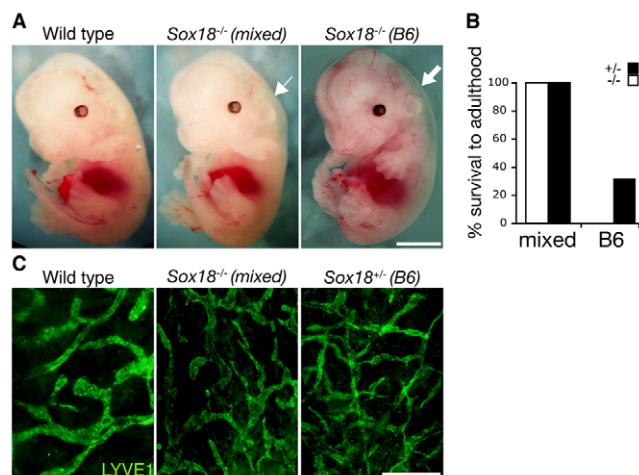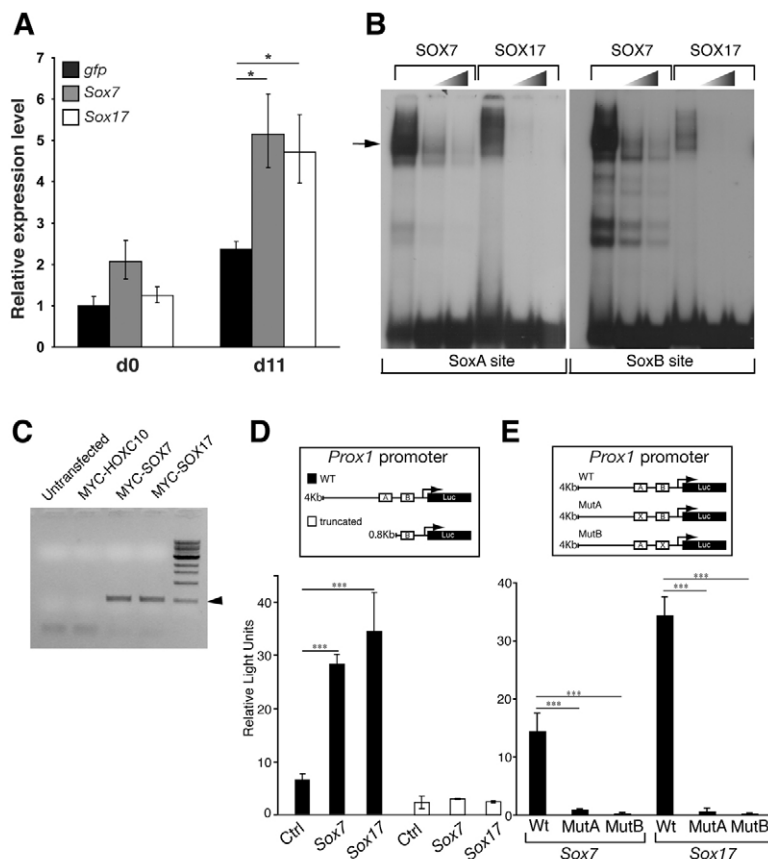
Fig. 2. SOX7 and SOX17 can regulate the lymphatic endothelial gene *Prox1*. (**A**) Quantitative RT-PCR analysis of *Prox1* expression levels in ES cells infected with lentiviral vectors encoding GFP (negative control; black bars), SOX7 (gray bars) or SOX17 (white bars) and induced to differentiate for 11 days. The figure represents a typical experiment of at least three performed. Data are means±s.e.m. of six replicates, and are shown relative to GFP. *$P<0.01$ (Student's *t*-test). (**B**) EMSA using purified GST-SOX7 or GST-SOX17 and radiolabeled oligonucleotides containing either the SoxA-binding site at –1135 bp (left panel) or the SoxB-binding site at –813 bp (right panel) in the *Prox1* promoter. SOX7 and SOX17 bound to both oligonucleotides (arrow). Specificity of binding was demonstrated by using a 25- or 50-fold molar excess of nonradioactive competitor oligonucleotide (graduated triangles). (**C**) ChIP in mlEnd cells using anti-MYC antibodies to precipitate chromatin fragments from untransfected cells or cells transfected with Myc-SoxF expression constructs, or an unrelated negative control Myc-Hoxc10 expression construct. Amplification of the region containing SoxA and SoxB sites from the *Prox1* promoter was seen only in cells transfected with Myc-SoxF expression vectors (arrowhead). (**D**) Reporter gene assays in which mlEnd cells were transfected with luciferase constructs containing a full-length (black bars) or truncated (white bars) *Prox1* promoter without (Ctrl) or with a *Sox7* or *Sox17* expression vector. Transcription was enhanced in cells overexpressing *Sox7* or *Sox17*, and only in promoter constructs containing the SoxA-binding site at –1135 bp. (**E**) Reporter gene assays in which mlEnd were transfected with luciferase constructs containing mutated SoxA or SoxB responsive elements in the *Prox1* promoter (MutA and MutB, respectively). Transcription was abolished when either site was mutated. Figures represent a typical experiment of three performed. Data are means±s.d. of three replicates. ***$P<0.01$.

Ectopic expression of *Sox7* or *Sox17* driven by the *Hoxb2* enhancer caused a corresponding ectopic upregulation of the *Prox1*-GFP reporter construct in linear groups of cells at the level of the hindbrain adjacent to the facio-acoustic ganglia in double-transgenic embryos (Fig. 3, arrows). These embryos did not show upregulation of SOX18 in these sites, indicating that reporter expression is stimulated directly by SOX7 and SOX17 and not indirectly via effects on SOX18 (see Fig. S1 in the supplementary material). Control transgenic mice carrying *Prox1*-GFP reporter alone did not express the reporter in these sites (see Fig. S2 in the supplementary material). These findings indicate that both SOX7 and SOX17 proteins can induce *Prox1* expression in vivo, and that this effect is mediated directly on the proximal 4 kb *Prox1* promoter fragment used in this study.

Sox7 and *Sox17* are not expressed during normal lymphangiogenesis

The hypothesis that *Sox7*, *Sox17* and *Sox18* might act redundantly during lymphangiogenesis further requires prima faciae that the three genes are co-expressed in lymphatic precursors. At 10.5 dpc, SOX18 and PROX1 are co-expressed in a dorsolateral subset of cells in the cardinal vein and in a population of cells migrating from this area (Francois et al., 2008). We used in situ hybridization for *Sox7* and *Sox17* mRNAs combined with immunofluorescence to detect PROX1 protein during lymphatic vascular sprouting (Fig. 4). In wild-type embryos of any genetic background, *Sox18* was clearly co-expressed with PROX1 in lymphatic endothelial precursors (Fig. 4A,B) but, surprisingly, *Sox7* and *Sox17* expression was undetectable (Fig. 4D,E,G,H). However, blood vascular endothelial

cells of the dorsal aorta robustly expressed all three genes (Fig. 4C,F,I). The lack of appreciable *Sox7* and *Sox17* expression in PROX1-positive lymphatic progenitor cells indicates that these genes are not normally involved in early establishment of the lymphatic vasculature.

Strain-specific activation of *Sox7* and *Sox17* in *Sox18*-mutant mice

We also tested the possibility that one or both genes might be upregulated specifically in the absence of SOX18 function, using an antibody that detects all three SoxF but not other Sox proteins (see Fig. S3 in the supplementary material). This antibody allowed us to study the expression of SOX7 and/or SOX17 in *Sox18*^{−/−} embryos on mixed and pure B6 backgrounds, using PROX1 expression during the migration of lymphatic endothelial precursor cells from the cardinal vein at 10.5 dpc as a point of reference (Fig. 5). In *Sox18*^{−/−} B6 embryos, which exhibit the severe lymphatic phenotype, expression of SOX7 and SOX17 was not detected in cells in the dorsal aspect of the cardinal vein, where lymphatic endothelial precursor cells are normally found (Fig. 5A). Consequently, PROX1 was not expressed in these cells, indicating a complete loss of PROX1-positive lymphatic progenitor cells on the B6 genetic background (Fig. 5B). PECAM-1-positive blood vascular endothelial cells of the dorsal aorta were clearly positive for SOX7 and/or SOX17 in these embryos (Fig. 5A).

By contrast, in *Sox18*^{−/−} embryos on a mixed background, SOX7 and SOX17 were clearly upregulated in cells migrating from the cardinal vein (Fig. 5D), allowing these cells to also express PROX1 and marking them as lymphatic endothelial progenitors (Fig. 5E).
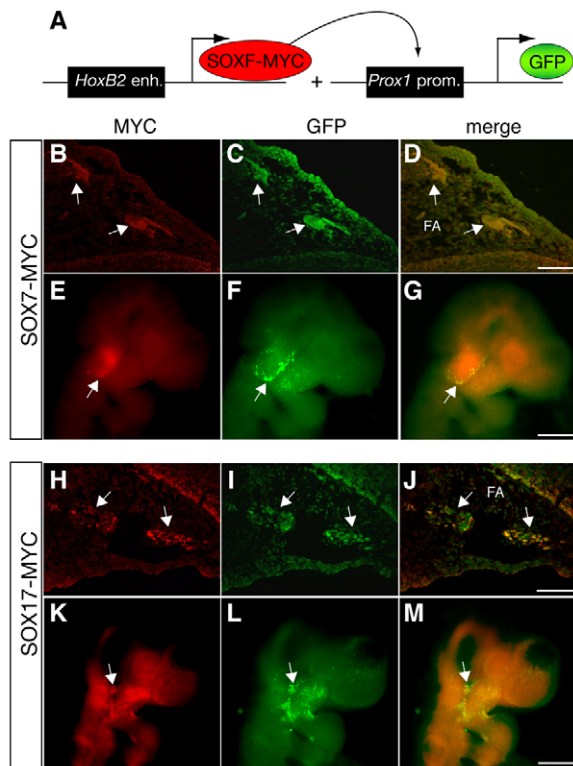
Fig. 3. Misexpression of SOX7 and SOX17 triggers ectopic expression of a *Prox1* reporter in vivo. (**A**) Constructs used in transient transgenic experiments. (**B-M**) Ectopic expression of MYC-SOX7 (B-G), or MYC-SOX17 (H-M), in the facio-acoustic neural ganglion complex (arrows) resulted in activation of the *Prox1* promoter driving GFP expression in the same subpopulation of cells (arrows). (B,E,H,K) Myc antibody, (C,F,I,L) GFP antibody, (D,G,J,M) merged images. Scale bar, 100 µm in E-G, K-M; 20 µm in B-D, H-J. Enh, enhancer; FA, facio-acoustic neural ganglion complex; prom, promoter.

Fig. 4. *Sox18*, but not *Sox7* or *Sox17*, is co-expressed with PROX1 during early lymphangiogenesis. Fluorescent in situ hybridization for individual Group F *Sox* mRNAs (red) combined with immunodetection of PROX1 expression (green) during the migration of lymphatic endothelial precursor cells from the cardinal vein at 10.5 dpc. (**A**,**B**) *Sox18* expression was detected in PROX1-positive cells lining and sprouting from the cardinal vein during early lymphatic development (arrows). (**D**,**E**,**G**,**H**) Expression of *Sox17* (D,E) and *Sox7* (G,H) was completely absent from PROX1-positive cells in and around the cardinal vein. (**C**,**F**,**I**) Blood vascular endothelial cells from the dorsal aorta expressed all F-group Sox genes. Similar results were obtained on all genetic backgrounds tested. B and C are magnified images of boxes indicated in A, E and F are magnified images of boxes indicated in D, and H and I are magnified images of boxes indicated in G. Scale bars: 100 µm in A,D,G; 20 µm in B,C,E,F,H,I. CV, cardinal vein; D, dorsal; DA, dorsal aorta; M, medial.

Quantitative real-time RT-PCR analysis confirmed an overall increase in *Sox7* and *Sox17* mRNA levels in trunk regions of *Sox18*-knockout embryos on a mixed but not a B6 background (Fig. 5C,F). These findings, together with our experiments that indicate a shared ability of SOXF factors to activate *Prox1* gene expression, indicate that SOX7 and SOX17 are able to act as modifiers of the *Sox18*⁻/⁻ phenotype on a mixed genetic background.

DISCUSSION

Attempts to map, identify and study modifier genes have become an important goal of mammalian functional genomics, due to the confounding effects of modifiers on genotype-phenotype relationships, the important role modifiers play in developmental and physiological regulatory circuitry, and their bearing on the penetrance and expressivity of human disease. In this study, we identify *Sox7* and *Sox17* as genetic modifier genes that profoundly affect the severity of the lymphangiogenic phenotype caused by *Sox18* mutation in different strains of mice. Although these genes have the ability to act redundantly with *Sox18* at the biochemical level, their mode of action is not one of simple redundancy, because these genes are not normally expressed together during lymphangiogenesis. Instead, they act by a strain-specific compensatory upregulation mechanism.
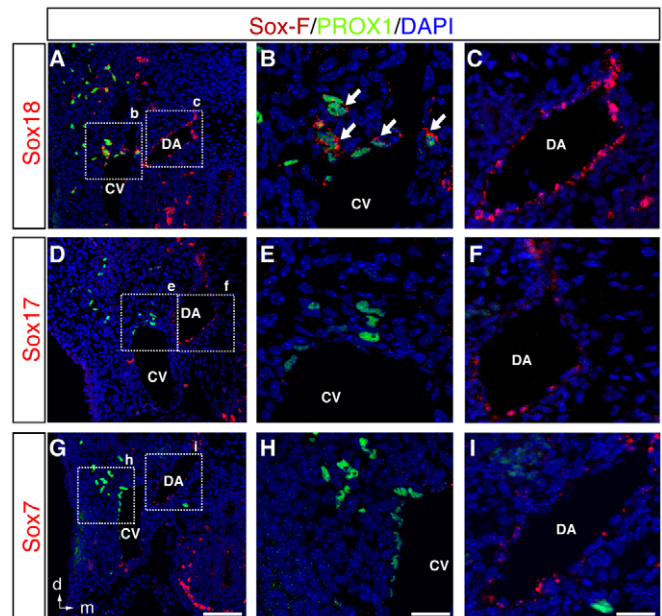
Although the mode of action of modifier genes has remained unclear, they can be envisaged to operate in a number of possible ways. For example, they may act downstream of the mutant gene (a phenomenon known as epistasis), or upstream (hypostasis); the modifier may encode a partner required for activity of the gene product, or encode a factor functionally interchangeable with the gene product. A recent study of modifiers of MeCP2 function in *Drosophila* identified several modifier genes that act in parallel pathways, and are therefore able to compensate for abnormal phenotypes caused by aberrant MeCP2 activity (Cukier et al., 2008). In another study, genetic modifiers affecting the Huntington's disease phenotype were identified as interacting partners for the huntingtin protein (Kaltenbach et al., 2007). Our present results indicate that SOX7 and SOX17 proteins are able to act interchangeably with SOX18 under certain conditions to ameliorate the lymphangiogenic phenotype in *Sox18* mutant mice, adding to the known mechanisms for modifier gene action.

Strain-specificity of modifiers, a frequent phenomenon in mouse studies, is commonly thought to be determined by differences in protein expression levels or activity between two or more strains. However, concrete examples of these phenomena have remained elusive. Our present data reveal strain-specific differences in upregulation of *Sox7* and *Sox17* transcription in response to *Sox18*
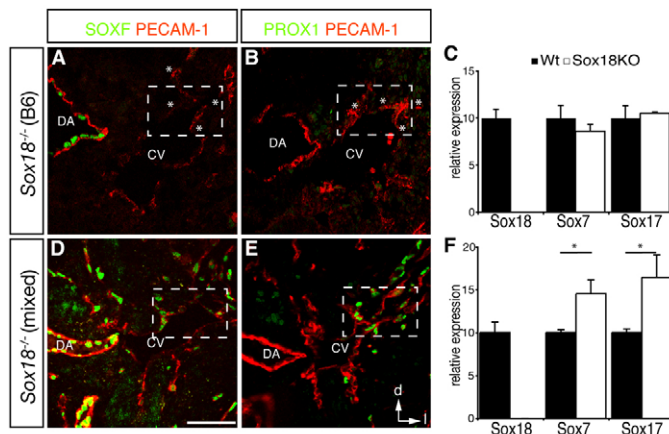
Fig. 5. SOX7 and SOX17 are upregulated during lymphangiogenesis in the absence of *Sox18* on a mixed background. (**A**,**D**) Immunofluorescence on 10.5 dpc *Sox18*-null embryos either on a B6 (A) or a mixed (D) background using the endothelial marker PECAM-1 (red) and the SOX-F antibody (green). On the mixed background, SOX7 and SOX17 were observed in the dorsal part of the cardinal vein (D, rectangle) whereas these transcription factors were absent on the B6 background (A, rectangle). SOX7 and/or SOX17 expression was detected in blood endothelial cells of the dorsal aorta. (**B**,**E**) Immunofluorescence on sections using PECAM-1 (red) and the lymphatic marker PROX1 (green) revealed expression of PROX1 in *Sox18*−/− mixed embryos (E, rectangle) and a complete loss of PROX1 expression in *Sox18*−/− B6 embryos (B, rectangle). (**C**,**F**) Quantitative RT-PCR analysis of *Sox7* and *Sox17* mRNA expression levels relative to *18S* from the trunks of *Sox18*−/− B6 (C; white bars, *n*=6) and *Sox18*−/− mixed background embryos (F, white bars, *n*=3), compared with wild-type embryo trunks (Wt, black bars). *$P<0.05$ (Student's *t*-test). Scale bar: 20 μm. CV, cardinal vein; D, dorsal; DA, dorsal aorta; L, lateral.

loss of function. An important challenge for the future will be to identify regulatory mechanisms for these genes and how these mechanisms differ between strains.

The ability of *Sox7* and *Sox17* to act as strain-specific modifiers in the context of lymphangiogenesis contrasts with their roles in the development of blood vessels and hair follicles. Neither organ system appears to be seriously compromised in *Sox18*-null mice on a mixed background (Pennisi et al., 2000a), although both are clearly compromised in HLT patients (Irrthum et al., 2003). Preliminary analysis of embryos carrying the *Sox18*-null mutation on a pure B6 background has not revealed any gross hemorrhage and hemangioma, evidently because all three *SoxF* genes are constitutively expressed during blood vessel development in mice and act redundantly in this process (Young et al., 2006). In recent studies, redundant roles for SoxF genes have been established in arterio-venous specification (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008) and fetal and postnatal angiogenesis (Matsui et al., 2006; Sakamoto et al., 2007).

Less is known regarding the expression of *Sox7* and *Sox17* during hair follicle development. *Sox18* is expressed in the mesenchyme underlying the hair follicle placode, and continues to be expressed during the invagination of the hair follicle (Pennisi et al., 2000b). Moreover, *ragged-opossum* homozygous mutant embryos lack any vibrissae or pelage hair follicles (Pennisi et al., 2000b). *Sox18*-null mice on a mixed background displayed only a mild hair follicle phenotype (Pennisi et al., 2000a), and *Sox18*−/− B6 embryos show no obvious defect in hair follicle development (M. Downes, M.F. and

P.K., unpublished data). These observations suggest that *Sox7* and/or *Sox17* encode constitutively expressed factors that act redundantly with SOX18 rather than acting as strain-specific genetic modifiers of a *Sox18*-induced hair follicle phenotype.

Our data provide a partial explanation for the differences between *Sox18* mutant phenotypes in humans and mice. In HLT patients, SOX18 mutations can be either recessive missense changes that debilitate the HMG DNA-binding domain, or dominant frameshift mutations that allow DNA binding but preclude transcriptional trans-activation (Irrthum et al., 2003). Clearly the phenotype caused by the human recessive mutations in HLT (sparse hair, lymphedema and microvascular hemorrhage) (Irrthum et al., 2003) is more severe than that of the *Sox18* knockout on a mixed background (normal with altered ratio of hair follicle types) (Pennisi et al., 2000a), but less severe than the *Sox18* knockout on a B6 background [lymphedema and fetal lethality (Francois et al., 2008) (and present study)]. Further, the variability displayed in all three components of HLT has been ascribed to modifier genes (Irrthum et al., 2003). Our present findings suggest that SOX7 and/or SOX17 are likely to modulate the severity of at least the lymphangiogenic aspects of the HLT phenotype. Similar mechanisms may operate in a broader range of human diseases, and so our findings have implications for the emerging concept of therapeutic modulation of genetic modifier activity in disease prevention or treatment (Nadeau, 2001).

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2385/DC1

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