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

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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'-AGGARTCAAGG-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.
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 4 hours in washing solution (1% BSA, 1% DMSO in PBSTx) and incubated for 16 hours with secondary antibodies in blocking solution. Confluent HSF 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/imunofluorescence
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 10 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 overnight at 4°C) and then blocked in PBS/5% BSA for 30 minutes at room temperature. After washing, slides were incubated for 16 hours with secondary antibodies in blocking solution. Samples were washed for 6 hours in washing solution (1% BSA, 1% DMSO in PBSTx) and added and incubated for 24 hours at 4°C. HRP-conjugated secondary antibody followed by chemiluminescence detection with Super Signal West Pico reagent (Pierce, Rockford, IL, USA) at 60°C. Preparations of RNA template without reverse transcriptase were used as negative controls. Ct 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′-GGTCCCCGCCCCCAGACATCTGGTTCGATAAAG-3′ and at −813 bp (SoxB) 5′-ACAGTCCTCTCCTCTTACTAACATTTGAG-TCAACCAAATG-3′.

Plasms
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′-CCCTCGAGGTGTCATCCTTCAAAGCTGTC-3′) and a primer at position −822 (5′-GGGGATCCCTCTTCTTCAAATTTGAAGTC-3′). Myc-Sox and Myc-Hox10 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 (Chemimmobil) 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). HSF murine endothelial cells were cultured as previously described (Garlanda et al., 1994).

Lentiviral vector preparation
To generate HIV3-PGK vector, the Pgy 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 1×10³ 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′-GCAAGGGTTTCTCTTCTTTT-3′ and 5′-GGCACAGACATGCACTGTCC-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 (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 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 Sox7 (−1135 to −1130 bp relative to the translation start site) and Sox8 (−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.
Ectopic expression of Sox7 or Sox17 driven by the Hoxh2 enhancer caused a corresponding ectopic upregulation of the Prox1-GFP reporter construct in lineages 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 facie 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).
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
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).

Acknowledgements
We thank P. Berta, Y. Kanai, J. Gamble and L. Naldini for gifts of reagents. This work was supported by the National Health and Medical Research Council of Australia, the Queensland Cancer Fund, the Associazione Italiana per la Ricerca sul Cancro (Italy), the European Community, and the Australian Research Council. Confocal microscopy was performed at the Australian Cancer Research Foundation Dynamic Imaging Centre for Cancer Biology. P.K. is a Federation Fellow of the Australian Research Council.

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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