Redundant roles of *Sox17* and *Sox18* in postnatal angiogenesis in mice

Toshiyasu Matsui1,*, Masami Kanai-Azuma2,*, Kenshiro Hara1, Shogo Matoba1, Ryuji Hiramatsu1, Hayato Kawakami2, Masamichi Kurohmaru1, Peter Koopman3 and Yoshiakira Kanai1,‡

1Department of Veterinary Anatomy, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
2Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan
3Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia

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Summary

*Sox7, Sox17* and *Sox18* constitute group F of the *Sox* family of HMG box transcription factor genes. Dominant-negative mutations in *Sox18* underlie the cardiovascular defects observed in *ragged* mutant mice. By contrast, *Sox18*+/– mice are viable and fertile, and display no appreciable anomaly in their vasculature, suggesting functional compensation by the two other *SoxF* genes. Here, we provide direct evidence for redundant function of *Sox17* and *Sox18* in postnatal neovascularization by generating *Sox17*+/–-*Sox18*+/– double mutant mice. Whereas *Sox18*+/– and *Sox17*+/–-*Sox18*+/– mice showed no vascular defects, approximately half of the *Sox17*+/–-*Sox18*+/– pups died before postnatal day 21 (P21). They showed reduced neovascularization in the liver sinusoids and kidney outer medulla vasa recta at P7, which most likely caused the ischemic necrosis observed by P14 in hepatocytes and renal tubular epithelia. Those that survived to adulthood showed similar, but milder, vascular anomalies in both liver and kidney, and females were infertile with varying degrees of vascular abnormalities in the reproductive organs. These anomalies corresponded with sites of expression of *Sox7* and *Sox17* in the developing postnatal vasculature. In vitro angiogenesis assays, using primary endothelial cells isolated from the P7 livers, showed that the *Sox17*+/–-*Sox18*+/– endothelial cells were defective in endothelial sprouting and remodeling of the vasculature in a phenotype-dependent manner. Therefore, our findings indicate that *Sox17* and *Sox18*, and possibly all three *SoxF* genes, are cooperatively involved in mammalian vascular development.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/119/17/3513/DC1

Key words: *Sox17, Sox18*, Liver, Kidney, Angiogenesis, Mouse

Introduction

The *Sry*-related HMG box (*Sox*) gene family was first identified through its homology to the high-mobility group (HMG) box of the sex-determining factor gene *SRY* (Gubbay et al., 1990). Members of this gene family encode transcription factors that regulate the specification of cell types and tissue differentiation in various developmental processes (Pevny and Lovell-Badge, 1997; Wegner, 1999; Bowles et al., 2000). Phylogenetic analyses have revealed that this family can be subdivided into ten groups, A to I, based on homologies both within and outside the HMG box domain (Soulier et al., 1999; Bowles et al., 2000).

*Sox17* belongs to *Sox* group F, together with *Sox7* and *Sox18*. Analyses of *Sox17* expression have revealed activity in the visceral and definitive endoderm of post-implantation mouse embryos (Kanai-Azuma et al., 2002), and in various cell types including vascular endothelial cells (this study) and spermatogenic cells at postnatal and adult stages (Kanai et al., 1996). *Sox17* heterozygous knockout mice are viable and fertile, but *Sox17*–null embryos show dysmophogenesis of the definitive endoderm, which results in embryonic lethality before embryonic day 10.5 (E10.5) (Kanai-Azuma et al., 2002). Because *Sox17* orthologues are also important for endoderm formation in frog (Hudson et al., 1997; Clements and Woodland, 2000; Clements et al., 2003; Sinner et al., 2004) and fish (Alexander and Stainier, 1999; Stainier, 2002), these data strongly indicate that *Sox17* has a conserved, crucial role in endoderm formation in vertebrates (Tam et al., 2003). However, the role of *Sox17* in vascular endothelial cells and spermatogenic cells, where *Sox17* is activated at the late embryonic, postnatal and adult stages, remains unclear.

Another member of *Sox* group F, *Sox18*, is implicated in the regulation of blood vessel development. *Sox18* mutations in mice cause cardiovascular and hair follicle defects in the *ragged* (*Ra*) series of spontaneous mutants, which includes the four allelic variants *ragged* (*Ra*), *ragged-Jackson* (*RaJ*), *ragged-like* (*Ragl*) and *ragged-opossum* (*RaOp*) (Pennisi et al., 2000a; James et al., 2003). *Ra, RaJ* and *Ragl* homozygotes exhibit edema, chylous ascites and cyanosis at birth and, in addition to hair and skin defects, commonly die before weaning (Carter and Phillips, 1954). Homozygous embryos of *RaOp*, the most severely affected variant (Green and Mann, 1961), display superficial haematoma and dilation, distention or rupture of peripheral embryonic blood vessels, which results in their lethality before E11.0 (Green and Mann, 1961; Pennisi et al., 2000a). Similar point mutations in human *SOX18*...
underlie recessive and dominant hypothyroidism-lymphedema-telangietasia (HLT), a hair, vascular and lymphatic disorder, confirming the role of Sox18 in these processes (Irrthum et al., 2003).

Surprisingly, Sox18-null mice are viable and fertile, and show no obvious cardiovascular abnormalities, although they display a mild coat defect (Pennisi et al., 2000b). It has been suggested that the point mutations found in ragged mice and in dominant forms of human HLT generate a SOX18 protein that acts in a dominant-negative fashion to interfere with the functions of wild-type SOX18 and of related proteins that play a role in vascular, hair follicle or lymphatic development or function, whereas defects in Sox18-null mice are masked by the compensatory action of related SOX factors (James et al., 2005; Boon et al., 2005).

In this study, to elucidate the possible redundant roles of Sox17 and Sox18 in vascular endothelial cells, we generated Sox17+/–-Sox18–/– double mutant mice, and compared their phenotypes with those of the Sox17–/– mice. The double heterozygous (Sox17+/–-Sox18+/-) mutants were viable and fertile in both sexes, and displayed no appreciable histopathological and physiological defects in the mixed genetic background (25% C57BL/6, 25% 129sv and 50% CD1 strains; data not shown). Some of the Sox17+/–-Sox18+/– mutant pups died before puberty; males of those that survived to the adult stage were fertile, but females were infertile and displayed a drastic decrease in sexual cycling and a prolonged anestrus by vaginal smear test (data not shown). In the intercross breeding between Sox17+/–-Sox18+/– males and Sox18+/– females, the Sox17+/-Sox18–/– mice were viable at P7 in the expected Mendelian distribution, but approximately 55% of the Sox17+/-Sox18–/– mutants died postnatally before P21 (Table 1).

Postnatal lethality and female infertility of Sox17+/-Sox18–/– double mutants
To investigate the possible functional redundancy of Sox17 and Sox18 in vascular development, we generated variations of Sox17+/-Sox18–/– double mutants, and compared their phenotype with that of Sox18–/– single mutants. The double heterozygous (Sox17+/-Sox18+/-) mutants were viable and fertile in both sexes, and displayed no appreciable histopathological and physiological defects in the mixed genetic background (25% C57BL/6, 25% 129sv and 50% CD1 strains; data not shown). Some of the Sox17+/-Sox18+/– mutant pups died before puberty; males of those that survived to the adult stage were fertile, but females were infertile and displayed a drastic decrease in sexual cycling and a prolonged anestrus by vaginal smear test (data not shown). In the intercross breeding between Sox17+/-Sox18+/– males and Sox18+/– females, the Sox17+/-Sox18–/– mice were viable at P7 in the expected Mendelian distribution, but approximately 55% of the Sox17+/-Sox18–/– mutants died postnatally before P21 (Table 1).

Abnormal vasculature in the Sox17+/-Sox18–/– double mutant mice that survived to adulthood
Gross anatomical and histopathological examination of adult Sox17+/-Sox18–/– double mutants revealed vascular anomalies in the liver, kidney and reproductive organs as compared with their Sox17+/-Sox18+/- single mutant littermates (Fig. 2, supplementary material Fig. S2).

The Sox17+/-Sox18–/– livers were small and jaundiced (Fig. 2A). Blood vessels were visible at the edge of the liver lobules in the Sox18+/- livers under a dissecting microscope, but not in the Sox17+/-Sox18–/– livers (Fig. 2B). Dilation of some surface hepatic veins was also apparent. Histopathological and corrosion-cast SEM analyses revealed atrophy of the hepatocytes and poor development of the sinusoidal microvasculature (i.e. a sparse capillary net with narrow lumen) in the Sox17+/-Sox18–/–, livers, compared with that of the Sox18+/- livers (Fig. 2C-E).

The Sox17+/-Sox18–/– kidneys displayed atrophy in the outer medulla but no appreciable defect in the cortex and inner medulla (Fig. 2F-H). In the outer medulla, vascular bundles of the vasa recta, which normally run radially from the corticomedullary junction towards the inner medulla, were missing (Fig. 2G-I). Such dysmorphogenesis of the outer medulla probably caused the hydronephrosis seen in variable
Fig. 1. Whole-mount in situ hybridization analysis showing Sox17 (left panels) and Sox18 (right panels) expression in developing blood vessels of mouse organogenic embryos. (A,B) Embryos at early somite stage [embryonic day 8.5 (E8.5)]; whole embryos (A) and histological sections (B). Arrowheads indicate small blood vessels around hindgut. (C-H) Embryos at early organogenic stage (E9.5); whole embryo (C,D) and histological sections (E-H). (I-K) Embryos at E11.5; whole embryo (I) and histological sections (J,K). Sox18-positive endothelial cells appear to be more widely distributed in the smaller microvasculatures (arrowheads in D,E,K). The red broken arrows (A,C,I) and boxed area (C,E,G,J) indicate the planes of sections and the magnified area in the designated panel, respectively. da, dorsal aorta; fg, foregut; gb, presumptive gallbladder; hg, hindgut; isv, intersomitic vessels; nt, neural tube. Bars, 50 μm.

Table 1. Postnatal lethality of Sox17+/–-Sox18+/– double mutant mice*

<table>
<thead>
<tr>
<th>Postnatal day</th>
<th>Mating type (female × male)</th>
<th>Sox17+/– × Sox18+/–</th>
<th>Sox18+/– × Sox17+/–-Sox18+/–</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>Sox17+/–-Sox18+/–</td>
<td>Sox18+/–</td>
</tr>
<tr>
<td>P0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>93 (47.0%)</td>
</tr>
<tr>
<td>P7</td>
<td>n.d.</td>
<td>n.d.</td>
<td>82 (48.2%)</td>
</tr>
<tr>
<td>P21</td>
<td>49 (45.8%)</td>
<td>58 (54.2%)</td>
<td>126 (30.0%)</td>
</tr>
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</table>

*Number of the live neonates of each genotype in the breedings between Sox18+/– males and Sox17+/– females (left) and between Sox17+/–-Sox18+/– males and Sox18+/– females (right). Numbers in parentheses represent the survival rate of each genotype per total offspring; n.d., not determined.
degrees in the Sox17+/-Sox18+/- kidneys (asterisks in Fig. 2F). Vascular-cast SEM analysis revealed that, in the Sox17+/-Sox18+/- kidneys, the smaller capillaries of presumptive interbundle plexus mainly occupy the affected outer medulla, and they also directly branch into the inner medulla vasa recta (right in Fig. 2I,J).

In the reproductive organs, scrotum became protruded and enlarged after puberty in all Sox17+/-Sox18+/- males that...
survived to adulthood (Fig. 2K). This was mainly caused by the abnormal lipid deposition around the spermatic cords, where the pampiniform plexus was less convoluted and enlarged relative to single-mutant Sox18+/– males (Fig. 2L, supplementary material Fig. S2A). Ovaries in Sox17+/–-Sox18+/– females were markedly smaller than Sox18+/– ovaries (Fig. 2M,N). In these affected ovaries, reduced numbers of growing follicles and corpora lutea were also evident. A drastic reduction of small blood vessels located near the developing follicles was confirmed by the histopathological analysis (supplementary material Fig. S2B-D).

By contrast, the Sox17+/–-Sox18–/– mutants showed no appreciable defects in their skin, hair and hair follicles, which had previously been reported to be severely affected in Sox18 ragged mutants (Pennisi et al., 2000a). Moreover, we could not detect any edema, which is common to all four ragged variants. No differences in other organs were found between the Sox17+/–-Sox18+/– and Sox18–/– mice, including lung, heart, intestine, stomach, pancreas, spleen, skeletal muscle, eye, cerebellum and cerebrum.

Liver atrophy and kidney dysmorphogenesis are likely to cause the postnatal lethality in approximately half of the Sox17+/–-Sox18–/– pups

To investigate the possible cause of the postnatal lethality in approximately half of the Sox17+/–-Sox18–/– pups (Table 1), we histopathologically examined various organs of the Sox17+/–-Sox18–/– mice from postnatal day 0 (P0) to P14. Sox17+/–-Sox18–/– neonates from P0 to P3 (n=26) exhibited no histological defect in any organs we examined. However, in the Sox17+/–-Sox18–/– mice sampled at P7-P14, approximately 55% of the pups (34 of 61) showed severe liver atrophy and variable degrees of outer medullar hypoplasia in the kidneys (Fig. 3). At P7-P14, the affected livers exhibited atrophy of the hepatocytes with vacuole degeneration (Fig. 3A-D). The affected Sox17+/–-Sox18–/– kidneys at P7-P14 also displayed hypoplasia of the outer medulla, which may result in varying degrees of hydronephrosis (Fig. 3E,G). Their outer medulla showed dysmorphogenesis, in which the running patterns of both renal tubules and vasa recta appeared to be irregular (Fig. 3F,H).

The defects in the P7-P14 livers and kidneys were similar to, but more severe than those of the Sox17+/–-Sox18–/– mice that survived to adulthood (Fig. 2A-J). Moreover, the frequency of these defects at P7-P14 (approximately 55%) was similar to that of the postnatal lethal phenotype in the Sox17+/–-Sox18–/– mice before P21 (survival rate at P21 was 42.7%, 126 of 295 mice; Table 1). In the Sox17+/–-Sox18–/– genotype, all of the P14 mutant mice with such severe defects of liver and kidney were slender and weak when compared with the Sox17+/–-Sox18–/– littermates without a phenotype (data not shown), suggesting imminent demise. In fact, these defects of liver and kidney were recognizable by autopsy of the P7-P21 Sox17+/–-Sox18–/– cadavers shortly after death (n=7). It is, therefore, reasonable to conclude that the described disorders in the livers and/or kidneys are probably the cause of postnatal lethality in approximately half of the Sox17+/–-Sox18–/– mice.

In the following experiments, in order to separately investigate the phenotypes of the presumptive postnatal lethal group from those of the presumptive survivors, the Sox17+/–-Sox18–/– pups sampled at P7-P14 were classified into two groups: (1) Severe-phenotype group of presumptive postnatal lethal phenotype, displaying atrophy in the liver and kidney and, (2) mild-phenotype group of presumptive survivors without any appreciable defects in these organs before P14.

Ischemic necrosis of parenchymal cells in livers and kidneys of the Sox17+/–-Sox18–/– mice at P7

To assess the hypoxic response of the parenchyma, we carried out anti-HIF (hypoxia-inducible factor)-1α staining in the affected livers and kidneys of the Sox17+/–-Sox18–/– mutant mice at P7. Positive signals were observed heterogenously in the nucleus of hepatocytes in the Sox18–/– livers at P7 (left in Fig. 4A,B). In affected livers of Sox17+/–-Sox18–/– mice, the HIF1α-positive hepatocytes appeared to have moderately

Fig. 3. Liver atrophy and kidney outer medulla hypoplasia in the Sox17+/–-Sox18–/– neonates at P7 and P14. (A-D) HE-staining of the affected Sox17+/–-Sox18–/– livers at P7 (A,B) and P14 (C,D) showing atrophy of the hepatocytes (asterisks in A,B and whole area in C,D). cv, central vein; hc, hematopoietic cells. (E-H) HE-staining of the affected Sox17+/–-Sox18–/– kidneys at P7 (E,F) and P14 (G,H), showing the hypoplasia of the outer medulla that might result in variable degrees of hydronephrosis. In the affected Sox17+/–-Sox18–/– kidneys, their outer medulla showed dysmorphogenesis (E,G), in which the running patterns of vasa recta appeared to be irregular (arrowheads and arrows in F,H). ct, cortex; im, inner medulla; om, outer medulla; rp, renal papilla. The boxed area encompasses the area magnified in the designated panel. Bars, 50 μm (for A-D,F,H) and 100 μm (for E,G).
increased in number (the ratio of HIF1α-positive hepatocytes versus total hepatocytes is 60.0±2.8% in Sox17+/−,Sox18+/− mice versus 45.9±5.6% in Sox18+/− mice; Fig. 4A,B). Moreover, immunoblot analysis confirmed increased expression of HIF1α protein in the liver of Sox17+/−,Sox18+/− mice in a phenotype-dependent manner (Fig. 4C). By contrast, no positive signals for anti-HIF1α staining were detectable even in the region of the atrophic outer medulla of the affected kidneys of Sox17+/−,Sox18+/− mice (supplementary material Fig. S3). Although immunoreactive deposits were observed in the nucleus of some renal tubular epithelia and glomeruli located in the kidney cortex, no appreciable expression was detected in Sox18+/− and affected Sox17+/−,Sox18+/− kidneys.

These findings suggest a severe hypoxic-ischemic state (or, alternatively, higher sensitivities to hypoxia) in the liver compared with the outer medulla region of the kidney in the affected Sox17+/−,Sox18+/− pups.

Ultrastructural analysis confirmed ischemic necrosis of parenchyma in affected Sox17+/−,Sox18+/− livers and kidneys. In the affected Sox17+/−,Sox18+/− livers at P7, both sinusoidal lumina and Disse’s space were found to be enlarged (right in Fig. 5A-C). Their surrounding hepatocytes were smaller, and some of them displayed necrosis and vacuolation with swollen mitochondria and dilated endoplasmic reticula in the Sox17+/−,Sox18+/− livers (asterisks in Fig. 5A,B). Affected kidneys at P7 showed no significant morphological defects (Fig. 5D,E). However, some of the renal tubular epithelia displayed necrotic morphology in the outer medulla of the affected Sox17+/−,Sox18+/− kidneys (asterisks in Fig. 5F).

Expression profiles of Sox7, Sox17, and Sox18 in vascular endothelial cells of neonatal livers and kidneys

Histopathological analyses of the Sox17+/−,Sox18+/− mutants indicated redundant functions of Sox17 and Sox18 in the postnatal angiogenesis of liver sinusoids and kidney outer medulla. To investigate their expression patterns in developing blood vessels of these organs, we carried out section in situ hybridization using neonatal wildtype mice (Fig. 6A,B). In situ hybridization analysis using livers at P3 to P7 revealed that Sox17 and Sox18 were expressed in the endothelial cells of central veins and their closely associated sinusoidal vessels, in addition to high levels of expression in the hematopoietic cells (Fig. 6A). However, no appreciable signals for Sox7 were detected in the sinusoidal vasculature at these stages.

In kidneys at P3-P7, Sox17 transcripts were found in the small and intermediate blood vessels in the cortex and the vasa recta in outer and inner medulla, but not in the microvasculature inside the glomeruli (Fig. 6B). Sox18 transcripts were more widely distributed in the developing vasculature than those of Sox17 in the kidneys at the same stage. Sox18 expression was found not only in the small vasculature of the cortex and the vasa recta of outer and inner medulla, but also in the glomeruli (Fig. 6B). In contrast to presumptive coexpression of Sox17 and Sox18 in the outer medulla vasa recta, no Sox7 expression was detected in the outer medulla region of the kidney at the same stage, although a weak signal was found in the cortex including the glomeruli (right in Fig. 6B).

To ascertain the tissue-specific expression profiles of each Sox group F gene, we further examined the relative expression levels in various tissues of wild-type mice at P7 and adult stages by semi-quantitative reverse transcriptase (RT)-PCR. All three SoxF genes were expressed highly in lungs, and moderately in hearts at both P7 and adult stages (Fig. 6C and data not shown). Moderate Sox18 expression was observed in all tissues examined, including kidney and liver. Interestingly, in the P7 liver and kidneys and the adult ovaries (i.e. the affected organs of the Sox17+/−,Sox18+/− mutants), the expression levels of both Sox17 and Sox7 were found to be relatively lower than that of Sox18. The P7 liver (i.e. the most severely affected organ of Sox17+/−,Sox18+/− mutants) showed the lowest level of Sox7 expression of the various tissues.
which was consistent with the present section in situ hybridization data showing only weak signals detectable in hematopoietic cells (right in Fig. 6A).

Reduced Vcam1 expression in vascular endothelial cells of hepatic sinusoids and kidney vasa recta of the Sox17+/–-Sox18–/– neonates

To address the influence on gene expression by the loss of one Sox17 allele in the Sox18-null background, we quantitatively examined the expression levels of several endothelial cell markers (Vcam1, Tiel1 and Tiel2) and angiogenic factors (Ang1 and Ang2) in the livers and kidneys of the Sox17+/–-Sox18+/– and Sox18–/– mice at P7 using quantitative real-time RT-PCR (Fig. 7). Vcam1 expression levels were significantly reduced in both livers and kidneys of the Sox17+/–-Sox18+/– severe-phenotype group when compared with the Sox18–/– group. By contrast, there was no significant difference in expression levels of Tiel1 and Tiel2 in livers or kidneys among these three groups. In the affected Sox17+/–-Sox18+/– livers, Ang1 expression levels were significantly reduced, whereas Ang2 expression showed a much higher level compared with that of the Sox18–/– group. This indicates an imbalance of Ang1 and Ang2 expression, but an unchanged expression of their receptors in the Sox17+/–/Sox18+/– livers.

Immunohistochemical analyses also confirmed reduced expression of VCAM1, but not TIE2, in both liver and kidney of the severe Sox17+/–-Sox18+/– group at P7. Anti-PECAM1 staining revealed sparse and dilated liver sinusoids (Fig. 8A), and irregular vasa recta of the kidney outer medulla (Fig. 8E,F) in the severe Sox17+/–-Sox18+/– group at P7. However, anti-PECAM1 immunoreactivity showed no difference in the microvasculatures between the severe Sox17+/–-Sox18+/– and Sox18–/– groups. By contrast, anti-VCAM1 staining revealed drastically reduced signal intensity in the sinusoids and central veins of the affected Sox17+/–-Sox18+/– livers compared with Sox18–/– livers (Fig. 8B). In the Sox18–/– kidneys, strong VCAM1 staining was found in the glomerular afferent and efferent arterioles of the cortex (Fig. 8G) and in the vasa recta of the outer medulla (Fig. 8G,H). In the affected Sox17+/–-Sox18+/– kidneys, normal VCAM1-positive staining was observed in the glomerular arterioles of the cortex, but a drastic reduction in intensity was displayed in the vasa recta of the outer medulla (broken arrows in Fig. 8H). Moreover, we could not find any changes in VCAM1 protein expression either in the unaffected organs (e.g. heart, lung and spleen) of the severe Sox17+/–-Sox18+/– group, or in the liver and kidney of the mild phenotype group (data not shown). In contrast to the reduced expression of VCAM1 in the affected microvasculature, anti-TIE2 immunostaining showed no difference in immunoreactivity of the liver sinusoidal vasculature between the severe Sox17+/–-Sox18+/– and Sox18–/– groups (Fig. 8C,D). TIE2 expression was observed predominantly in the vasa recta of the outer medulla in the Sox18–/– kidney. In the Sox17+/–-Sox18+/– kidney at P7, TIE2 expression was found as it is in wild type, in the vasa recta of the affected outer medulla (arrows in Fig. 8I,J).
Fig. 6. Section in situ hybridization (A,B) and semi-quantitative RT-PCR (C) analyses showing the expression profiles of Sox17, Sox18 and Sox7 in the livers, kidneys and other organs of the wildtype mice at P7 and adult stages. (A) The sections of the wildtype livers at P7, showing that Sox17 and Sox18, but not Sox7, are expressed in the endothelial cells of central veins (cv) and their closely associated sinusoidal vessels (arrowheads). Lower panels show the magnified view of central veins. cv, central veins; hc, hematopoietic cell. (B) The sagittal sections of the wildtype kidneys at P7, showing the expressions of Sox17 and Sox18, but not Sox7, in some populations of the outer medulla vasa recta (arrowheads and inset in bottom panels). Bottom panels show the magnified view of the outer medulla region and the outer medulla vasa recta (inset). ct, cortex; gl, glomerulus; im, inner medulla; om, outer medulla. (C) Semi-quantitative RT-PCR analyses showing relative expression levels of Sox17, Sox18 and Sox7 in various tissues of the wild-type mice at P7 and adult stages. Arrows indicate position of Sox products. Bars indicate position of G3pdh products as an internal control. Bars, 50 μm.

Fig. 7. (A-E) RT-PCR analysis showing the differences of Vcam1 (A), Tie1 (B), Tie2 (C), Ang1 (D) and Ang2 (E) transcript levels in the livers (left) and kidneys (right) of severe Sox17<sup>+</sup>-Sox18<sup>+</sup> (▲), mild Sox17<sup>+</sup>-Sox18<sup>+</sup> (△) and Sox18<sup>-/-</sup> (○) groups at P7. Vertical axis represents relative expression levels of each vascular endothelial cell marker gene per G3pdh amplicon ratio; horizontal axis represents the severe or mild Sox17<sup>+</sup>-Sox18<sup>+</sup> group or the Sox18<sup>-/-</sup> group. Each value indicates the expression level in each sample (one neonate); average of the values indicate the bar. Each asterisk on the average bar indicates a significant difference (*P<0.05; **P<0.01) compared with the data of the Sox18<sup>-/-</sup> group.
In addition, histopathological analyses using anti-α-SMA (a marker for vascular smooth muscle cells) and anti-LYVE-1 (a marker for lymphatic endothelial cells) antibodies revealed no appreciable defects in developing arteries and lymphatic vessels of both livers and kidneys in the severe Sox17+/− Sox18−/− groups at P7 (supplementary material Fig. S4).

Aberrant angiogenesis in livers and kidneys of the Sox17+/− Sox18−/− mice at P7
To further investigate the vascular defects in affected livers and kidneys of the severe-phenotype group, we carried out quantitative analyses of capillary density (PECAM1-positive area over total parenchymal area; Fig. 9C) and capillary number (PECAM1-positive cell number (nucleus number) over total parenchymal area; Fig. 9D) using sections immunostained with anti-PECAM-1 antibody (Fig. 9A,B). Both capillary density and capillary number of vascular endothelial cells in the affected Sox17+/− Sox18−/− livers showed a significant reduction when compared with the Sox18−/− livers at P7 (left in Fig. 9C,D). However, in the Sox17+/− Sox18−/− livers, capillary density was more severely affected than capillary number (approximately 55% and 75% reduction in capillary density and capillary number, respectively, of the levels of Sox18−/− livers). In kidneys of the severe Sox17+/− Sox18−/− group, the density of vascular endothelial cells in the outer medulla region was significantly lower than that in the Sox18−/− kidney. However, we could not detect a significant difference (P=0.196) in PECAM-1-positive cell number in the outer
medulla region between the Sox17+/−Sox18−/− and Sox18−/− kidneys. There were no differences in density or cell number in the cortex and inner medulla regions of the Sox17+/−Sox18−/− and Sox18−/− kidneys (Fig. 9C,D).

Since a significant reduction in PECAM-1-positive cell number was detectable in the severely affected Sox17+/−Sox18−/− livers (Fig. 9D), we further examined the proliferative index of liver sinusoidal endothelial cells by BrdU labeling. No defects were observed in their mitotic activity, and the proliferative index of sinusoidal endothelial cells was slightly higher in the affected Sox17+/−Sox18−/− livers than in Sox18−/− livers (supplementary material Fig. S5A,B). TUNEL analysis also revealed no significant changes of apoptotic cells in the sinusoids of the severely affected Sox17+/−Sox18−/− livers (supplementary material Fig. S5C,D). Such a severe reduction of capillary density (rather than of vascular cell number) suggests that the Sox17+/−Sox18−/− vasculatures have some defects in endothelial cell migration and/or sprouting rather than proliferation and maintenance.

Aberrant in vitro angiogenesis of the primary vascular endothelial cells isolated from the affected Sox17+/−Sox18−/− livers

To measure angiogenic activity of the Sox17+/−Sox18−/− endothelial cells, we first carried out in vivo invasion assays assessing the ability of microvasculatures to invade a synthetic extracellular matrix, Matrigel, implanted under the dorsal skin of the adult mice. However, we could not find any significant changes in the number and running patterns of the neovasculatures within the Matrigel in Sox17+/−Sox18−/− double mutants compared with the Sox18−/− single mutant (supplementary material Fig. S6A,B and data not shown). This is probably due to the less-affected endothelial cells located in the dermis of the Sox17+/−Sox18−/− mutants, because no defects were found in the skins, dermis and their vasculatures in neonatal and adult Sox17+/−Sox18−/− mutants (data not shown).

Finally, to directly examine the activity of the affected Sox17+/−Sox18−/− endothelial cells, we isolated vascular endothelial cells from Sox18−/− and Sox17+/−Sox18−/− livers at P7, and carried out in vitro angiogenesis assays by using Matrigel-coated 96-well plates (Fig. 10A,B). The reconstitution activities of the endothelial cells into vascular-like structures were morphometrically quantified as number of aggregated nodes per area, branch number per node, and vessel length. The vascular endothelial cells of the Sox18−/− livers were observed to migrate and aggregate together into small scattered nodes 1.5-2 hours after plating onto the Matrigel. The nodes were also found to connect to each other through endothelial cell processes, forming vascular network-like structures 3-4 hours after plating. The Sox17+/−Sox18−/−
endothelial cells of both severe and mild phenotype groups showed proper migration and aggregation into small nodes within 2 hours of plating. However, they displayed poor reconstitution of vascular-like structures even at 12 hours, especially in the severe phenotype group. Quantitative analyses in 6-hour cultures of liver vascular endothelial cells revealed no significant change in the node number per area among three groups (left in Fig. 10C). However, the Sox17+/–-Sox18–/– endothelial cells of the severe phenotype group displayed a marked reduction in both branch number and vessel length compared with those of the Sox18–/– endothelial cells (middle and right in Fig. 10C). The Sox17+/–-Sox18–/– cells of the mild phenotype group also displayed a moderate decrease in both branch number and vessel length. Therefore, it is likely that the affected Sox17+/–-Sox18–/– endothelial cells appear normal in the first migration and aggregation steps but are defective in sprouting and remodeling of the aggregated endothelial cells into vessel structures during in vitro angiogenesis.

Discussion

Sox18 and Sox7 were previously shown to be expressed in vascular endothelial cells of developing blood vessels in several vertebrate species including mice (Pennisi et al., 2000a; Olsson et al., 2001; Takash et al., 2001; Fawcett and Klymkowsky, 2004). Here, we have demonstrated that Sox17 is also activated in vascular endothelial cells of mouse late-organogenesis-stage embryos, suggesting that all three members of Sox group F are coexpressed in some populations of developing blood vessels in mice. Moreover, our genetic analyses showed that the loss of one Sox17 allele in a Sox18-null background causes defective neovascularization in livers and kidneys postnatally. Moreover, in vitro angiogenesis assays demonstrated defective sprouting and remodeling of primary endothelial cells isolated from affected Sox17+/–-Sox18–/– livers. Since Sox17 and Sox18 are coexpressed in the affected vasculature of both liver sinusoid and kidney outer medulla, these findings provide evidence that Sox17 and Sox18 are jointly required in vascular endothelial cells for correct postnatal angiogenesis in mice. This, in turn, indicates that Sox17 can compensate for loss of Sox18 function in developing vascular endothelial cells in mice.

Interestingly, our study demonstrates that the Sox17+/–-Sox18–/– mutants display vascular abnormalities in an organ/site-specific manner. In postnatal angiogenesis-deficient mice, vascular insufficiency and its associated defective organ growth were found in various organs, including the unaffected organs and sites in the Sox17+/–-Sox18–/– mice (Gerber et al., 1999; Mattot et al., 2002; Thurston et al., 2005; Malik et al., 2006). Therefore, the restricted pattern of vascular defects in the Sox17+/–-Sox18–/– mutants is unlikely to reflect the organ/site-dependent heterogeneous angiogenic activities during postnatal development. Interestingly, the present in situ hybridization analysis showed similar but distinct vascular expression patterns of Sox group F genes during embryogenesis, with Sox17 showing delayed onset of activation and lower levels of expression in the presumptive immature microvasculature compared with Sox18 and Sox7. Semi-quantitative RT-PCR analyses also displayed tissue-dependent expression level of each Sox subgroup F member: both Sox17 and Sox7 were expressed at relative low levels in the affected organs (i.e. kidneys and livers) compared with those in the unaffected ones (i.e. lung and heart). Moreover,
the section in situ hybridization analysis showed no Sox7 signals but considerable Sox17 expression in the liver sinusoids and outer medulla vasa recta of the wild-type neonates. Therefore, it appears that the affected blood vessels mainly express Sox17 but not Sox7 in the neonates, in addition to sufficient but relative lower level of Sox17 transcripts in these vessels. Such heterogeneity of Sox17 and Sox7 expressions in the microvasculature probably explains the organ/site-specific vascular defects in Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutant mice.

**Ragged** mutants, including the four allelic variants Ra, Ra(J), Ral and RaOp, arose spontaneously by mutations in the Sox18 gene in crossbred strains of mice. In contrast to the lack in vascular abnormality in Sox18<sup>−/−</sup> mice, the ragged mutant mice show cardiovascular and hair follicle defects due to frameshift-inducing point mutations in Sox18 (Penissi et al., 2000a; Penissi et al., 2000b). Since the ragged-mutant SOX18 protein is capable of acting in a dominant-negative fashion (Hosking et al., 2001), repression of Sox17 function by the Sox18 ragged mutations might partially cause the ragged phenotypes, which are similar to those of Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mice. The RaOp allele, the most severely affected variant (Green and Mann, 1961), displays superficial hematomata and dilation, distention or rupture of peripheral blood vessels at embryonic stage (Green and Mann, 1961; Penissi et al., 2000b). By contrast, the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutants did not show any defects at embryonic stages. However, they displayed vascular defects in postnatal microvasculatures, including the vasodilation of neonatal liver sinusoids and adult pampiniform venous plexus. This suggests that defective formation of new blood vessels and vasodilation are common phenotypes in the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> and RaOp mutants.

We found that expression levels of VCAM1 are significantly reduced in the affected vasculatures of the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutants compared with those in the Sox18<sup>−/−</sup> single mutants. A similarly reduced VCAM1 expression has previously been shown in the RaOp mutants (Hosking et al., 2004). Interestingly, SOX18 was also shown to directly target and trans-activate Vcam1 expression through its promoter sequences. Since the SOX18 protein of the ragged mutant attenuated the expression and activation of Vcam1 in vitro (Hosking et al., 2004), it is likely that Vcam1 expression in RaOp mutants is partially due to the dominant-negative repression of Sox17 function by the mutant SOX18 protein in ragged. Moreover, this study demonstrated that the defective VCAM1 expression is restricted to the affected microvasculatures of the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutants, further suggesting a correlation between the organ/site-dependent vascular defects and the defective Vcam1 expression in the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutants. However, the reduced expression of Vcam1 is unlikely to be the sole cause of defective angiogenesis in the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutants, because the Vcam1-null mutants were previously shown to display neither edema nor vascular defects at postnatal and adult stages (Gurtner et al., 1995).

Sprouting angiogenesis is generally thought to involve activation of specific signal transduction pathways including the VEGF-FLK1 and ANG-TIE2 systems in vascular endothelial cells (Yancopoulos et al., 2000; Ferrara, 2003). Both pathways have been shown to play crucial roles in postnatal angiogenesis in various organs (Suri et al., 1998; Gerber et al., 1999; Gale et al., 2002; Mattot et al., 2002; Pitera et al., 2004; Ward et al., 2004; Malik et al., 2006; Thurston et al., 2005). VEGF signaling is required for the proliferation and maintenance of newly formed microvasculature, especially in the hepatic sinusoidal vessels during postnatal development and liver regeneration (Gerber et al., 1999; Kraizier et al., 2001; Ross et al., 2001). Our BrdU-incorporation and TUNEL-labeling analyses revealed no influence on the proliferation and maintenance of sinusoidal endothelial cells, but a drastic reduction of sinusoidal vessels in the severely affected Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> livers (Fig. 9; supplementary material Fig. S5). Moreover, because no defects were detectable in FLK1 and VEGF expressions in the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> livers (supplementary material Fig. S7), these findings suggest that Sox group F genes act independently, or downstream, of the VEGF-dependent proliferation process during the postnatal neovascularization. This is consistent with previous data, suggesting that SOX18 acts downstream of the VEGF-FLK1 system (Penissi et al., 2000a; Downes and Koopman, 2001).

ANG-TIE2 signaling is known to be involved in the endothelial cell migration and sprouting in collaboration with VEGF signaling (Hanahan, 1997; Holash et al., 1999; Yancopoulos et al., 2000; Jain, 2003). ANG2, a major ligand of TIE2, has been shown to be dispensable for embryonic angiogenesis but specifically required for normal postnatal vascular remodeling and lymphatic patterning (Gale et al., 2002). Overexpression of ANG1, another major ligand of TIE2, experimentally induced blood vessel enlargement without endothelial cell sprouting in neonatal mice (Suri et al., 1998; Thurston et al., 1999; Ward et al., 2004; Thurston et al., 2005). In the quantitative RT-PCR analysis described here, Ang1 was significantly downregulated, while Ang2 was upregulated in the affected Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> livers. Such unbalanced expression of Ang1 and Ang2 should favor vascular destabilization and endothelial sprouting and remodeling. Since the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> vascular endothelial cells were defective in endothelial sprouting and remodelling, as shown by in vitro angiogenesis assay, aberrant expression of Ang1 and Ang2 might be partially due to the positive response to compensate the defective endothelial remodeling in the affected Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> livers. The ANG-TIE2 system might also be involved in the formation of vascular bundles during postnatal kidney development. This is because TIE2 and ANG2 are highly and specifically expressed in the vascular bundles and their associated stromal region of neonatal kidneys, respectively (Yuan et al., 1999; Yuan et al., 2000; Pitera et al., 2004). Although our RNA and immunohistochemical analyses did not show defects in the expression of Tie2 itself in the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutants, the identification and characterization of target genes will be required to resolve a potential linkage between SOX group F factors and the ANG-TIE2 system in postnatal angiogenesis.

### Materials and Methods

**Animals and genotyping**

Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mutant animals were generated by interbreeding Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mice that were obtained by crossing Sox17<sup>−/−</sup> (Kanai-Azuma et al., 2002) and Sox18<sup>−/−</sup> (Penissi et al., 2000b) mice. Since approximately half of the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup> mice died before postnatal day 21 (P21; Table 1), the Sox17<sup>−/−</sup>-Sox18<sup>−/−</sup>-mutant neonates at P7-P14 were classified into severe (presumptive postnatal lethal) and mild (presumptive survivors to adulthood) phenotypic groups by anatomical or histological examination of their liver and kidneys, as described in Results.
Histology and transmission electron microscopy
Various tissues of the mutant mice were fixed in Bouin’s solution for 4 hours and embedded in paraffin. Sections were cut 5 μm thick, and stained with hematoxylin and eosin for routine histological examination. For transmission electron microscopy, tissues were fixed in Karnovsky’s solution, post-fixed in 1% OsO4 and then embedded in EPON 812. Ultrathin sections were analyzed using an electron transmission microscope (JEOL 1010, Tokyo, Japan).

Vascular casting and scanning electron microscopy
Corrosion vascular casting was performed as described by Kondo et al. (Kondo et al., 1993). In brief, mice were perfused and fixed with 2.5% gluteraldehyde-PBS. Their vascular system was injected through the thoracic aorta with MEROX CL resin (Dainippon Ink, Japan) and left at room temperature for 30 minutes to allow polymerization and tempering of the resin. The livers and kidneys were immersed in 20% KOH, washed in water and air-dried. The freeze-dried casts were examined under a S4000 scanning electron microscope (Hitachi, Tokyo, Japan).

In situ hybridization
Whole-mount and section in situ hybridization were performed as described (Kanai et al., 1996; Kanai-Azuma et al., 1999), using published RNA probes for Sox7 (Taniguchi et al., 1999), Sox17 (Kanai-Azuma et al., 2002) and Sox18 (Pensini et al., 2000a).

Immunohistochemistry and immunoblotting
Rat monoclonal anti-PECAM1 (1:200 dilution; clone MEC13.3, Pharmingen, CA), rabbit anti-VCAM1 (1:50 dilution; Santa Cruz; CA), rabbit anti-TIE2 (1:50 dilution, Santa Cruz) and rabbit anti-HIF1α (1:50 dilution; Santa Cruz) antibodies were used in this study. The specificity of anti-PECAM1 and anti-TIE2 antibodies has been shown previously (Weerth et al., 2003; Cho et al., 2004, respectively). Acetonefixed cryosections were used for anti-PECAM1 and anti-TIE2 staining, whereas PFA-fixed, deparaffinized sections were used for anti-VCAM1- and anti-HIF1α staining. The reactions were visualized with an ABC kit (Vector Laboratories, CA) or Tyramide Signal Amplification kit (NEN Life Science Products, MA) as previously described (Satoh et al., 2004).

For in vitro quantification of angiogenesis, sections immunostained with anti-PECAM1 antibody were counterstained with hematoxylin. The number of microvessels was calculated and compared in the Sox17+/−, Sox18+/− and Sox17+/Sox18−/− mutants.

Semi-quantitative and quantitative RT-PCR analyses
RNA was isolated from mouse tissues using an Isogen RNA isolation kit (Nippon Gene, Japan). After treatment with DNase I, RNA was reverse-transcribed using an oligo(guanylic acid) (dT) primer with a Superscript II cDNA synthesis kit (Invitrogen, CA). For semi-quantitative RT-PCR for Sox7, Sox17 and Sox18 expression, Sox and Gapdh (internal control) were amplified in the same tube using the following primer sets: Sox7 (forward: 5’-CAAGTCCAGCAGAACGCTTTTCAATCA-3’; reverse: 5’-ACGTCGAGGCATTCGACGAGGTTG-3’), Sox17 (forward: 5’-CACTGTTCTCCACGAGCAGGAGCTTAC-3’, reverse: 5’-AGGCGGAGGTCAAAGGTGCTTACGAC-3’), Sox18 (forward: 5’-CACTGTTCCACGAGCAGGAGCTTAC-3’, reverse: 5’-AGGCGGAGGTCAAAGGTGCTTACGAC-3’); Gapdh (forward: 5’-TGAAACTGCTGTAAGGAGTATGGGC-3’, reverse: 5’-CTGATGTCGATGGTTCCAC-3’). PCR was performed with 30-32-cycle amplifications at 95°C for 30 seconds, 83°C for 30 seconds, and 72°C for 30 seconds.

For quantitative real-time RT-PCR of Vcam1, Tie1, Tie2, Ang1 and Ang2, all specific primers and probe sequences were purchased from Applied Biosystems (Foster City, CA). Amplification of the Gapdh gene was used to standardize the amount of RNA in each reaction mixture (Taqman control reagents, Applied Biosystems). PCR was performed using an ABI Prism 7900HT sequence detector with 40-cycle amplifications at 95°C for 15 seconds, 60°C for 1 minute followed by enzyme activation at 92°C for 2 minutes.

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