Effect of Disrupted SOX18 Transcription Factor Function on Tumor Growth, Vascularization, and Endothelial Development

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Background: The growth of solid tumors depends on establishing blood supply; thus, inhibiting tumor angiogenesis has been a long-term goal in cancer therapy. The SOX18 transcription factor is a key regulator of murine and human blood vessel formation. Methods: We established allograft melanoma tumors in wild-type mice, Sox18-null mice, and mice expressing a dominant-negative form of Sox18 (Sox18RaOp) (n = 4 per group) and measured tumor growth and microvessel density by immunohistochemical analysis with antibodies to the endothelial marker CD31 and the pericyte marker NG2. We also assessed the effects of disrupted SOX18 function on MCF-7 human breast cancer and umbilical vein endothelial cell (HUVEC) proliferation by measuring BrdU incorporation and by MTS assay, cell migration using Boyden chamber assay, and capillary tube formation in vitro. All statistical tests were two-sided. Results: Allograft tumors in Sox18-null and Sox18RaOp mice grew more slowly than those in wild-type mice (tumor volume at day 14, Sox18 null, mean = 486 mm³, 95% confidence interval [CI] = 345 mm³ to 627 mm³, P = .004; Sox18RaOp, mean = 233 mm³, 95% CI = 73 mm³ to 119 mm³, P < .001; versus wild-type, mean = 817 mm³, 95% CI = 643 mm³ to 1001 mm³) and had fewer CD31- and NG2-expressing vessels. Expression of dominant-negative Sox18 reduced the proliferation of MCF-7 cells (BrdU incorporation: MCF-7Ra = 20%, 95% CI = 15% to 25% versus MCF-7 = 41%, 95% CI = 35% to 45%; P < .013 and HUVECs (optical density at 490 nm, empty vector, mean = 0.46 versus Sox18 mean = 0.29; difference = 0.17, 95% CI = 0.14 to 0.19; P < .001) compared with control subjects. Overexpression of wild-type SOX18 promoted capillary tube formation of HUVECs in vitro, whereas expression of dominant-negative Sox18 impaired tube formation of HUVECs and the migration of MCF-7 cells via the disruption of the actin cytoskeleton. Conclusions: SOX18 is a potential target for antiangiogenic therapy of human cancers. [J Natl Cancer Inst 2006;98:1060–7]

The concept of antiangiogenic cancer therapy was first proposed more than 30 years ago (1), but success in this field has been limited. Recent phase 3 trials with bevacizumab (Avastin; Genentech, San Francisco, CA), the humanized monoclonal antibody to vascular endothelial growth factor (VEGF), have validated this concept where time of progression to disease in patients with metastatic renal cell carcinoma and colorectal cancer has been extended substantially (2,3). Further development of this approach, e.g., through the use of specific gene-based strategies to combat a range of cancers, hinges on a growing understanding of the molecular regulation of tumor angiogenesis.

Evidence abounds that the molecular mechanism used by tumor cells to generate a vascular network is a recapitulation of the developmental angiogenic process (4). The signaling pathways that mediate vascular development have been well characterized; endothelial cell tropic factors, such as VEGF, platelet-derived growth factor, fibroblast growth factors, epidermal growth factor, and their receptors, have been identified as key angiogenic regulators (5). However, comparatively little is known about the transcriptional regulation of endothelial cell development and function during angiogenesis.

SOX18, a member of the Sry-related HMG box–containing (SOX) family of transcription factors (6), is expressed transiently in endothelial cells during the development of all blood vessels in the mouse embryo (7,8). We have previously shown that SOX18 participates in the VEGF–Flk1 pathway of endothelial cell activation, interacts with the muscle and endothelial transcription factor MEF2C, and activates expression of VCAM1, which is required for endothelial cell function (9,10). SOX18 binds to the common SOX target sequence (A/T)ACAA(A/T)G and contains a discrete transcriptional transactivation domain (11).

The importance of Sox18 for vascular development is revealed by the vascular defects caused by SOX18 mutations in humans and mice. In humans, SOX18 mutations result in hypotrichosis–lymphedema–telangiectasia syndrome (12), characterized by localized vascular leakage and edema. Also, vascular and coat anomalies in ragged (Ra) mutant mice result from mutations in Sox18 (13–15). Among the four allelic forms of Ra, the most severe phenotype is conferred by ragged-opossum (RaOp). Mice heterozygous for this allele show severe vascular and hair follicle anomalies that mimic hypotrichosis–lymphedema–telangiectasia syndrome, and homozygotes die in utero, possibly from gross vascular dysgenesis (16). RaOp mice express a dominant-negative mutant form of Sox18 (Sox18RaOp) that does not interact effectively with the endothelial partner protein MEF2C; this failure to interact blocks angiogenic target gene activation and compromises vascular development (9). In contrast, loss of function of Sox18 by gene targeting in mice does not result in a vascular phenotype (17), possibly reflecting genetic redundancy due to the coexpression of the highly related Group F Sox genes Sox7 and Sox17 in developing vasculature (18–20). These findings therefore strongly suggest that the Sox18RaOp mutant protein exerts its dominant-negative effect on SOX7 and SOX17 in addition to SOX18.
Recent studies have shown that all three members of the Group F Sox genes are misexpressed in a variety of human tumor cell lines (21–23). In this study we investigated SOX18 expression in the vasculature of both human and murine tumors, and we tested whether insufficiency and/or disruption of Sox18 would restrict neoangiogenesis in and inhibit growth of induced allograft tumors. We also examined the role of SOX18 in endothelial cell differentiation, migration, and proliferation to illuminate the mechanism by which SOX18 might mediate physiologic and pathologic angiogenesis.

**Materials and Methods**

**Melanoma Allograft Assay**

Six- to 10-week-old wild-type mice on a C57BL/6J background; Sox18 mutant mice from the RaOp:C57BL/6J strain, obtained from the Jackson Laboratory (Bar Harbor, ME), and mice null for Sox18, on a 129/Sv-CD1 mixed background previously generated in this laboratory (17) were maintained in-house (University of Queensland). Procedures involving mice and their care conformed to institutional and federal guidelines (University of Queensland Animal Ethics Committee). Mouse B16 melanoma cells (American Type Culture Collection [ATCC], Manassas, VA) (106) were suspended in phosphate-buffered saline (PBS; 0.2 M sodium phosphate and 0.8% saline, pH 7.2) and were injected bilaterally into the flanks of mice using a 30-gauge needle (n = 4 mice per strain). Tumor growth was monitored every 48 hours, and tumor volumes were calculated by measuring length and width of each tumor with calipers, according to the Anticancer Drug Development Guide (24). Tumor volume data were calculated and graphed as mean tumor volumes and 95% confidence intervals (CIs). Mice were killed by cervical dislocation at 14 days postgraft or when tumor volumes approached 1000 mm3, in accordance with ethical requirements. The experiment was conducted three times. One mouse, heterozygous for the dominant-negative Sox18 mutation RaOp, from each experiment was maintained for 40 days to ascertain any delayed tumor growth.

**In Situ Hybridization Analysis of Sox Gene Expression in Allografts, Human Colorectal Carcinomas, and Mouse Embryos**

Human colorectal tumors (n = 4) were supplied from the Royal Brisbane and Women’s Hospital in collaboration with Queensland Institute of Medical Research (QIMR). Tumors were collected in accordance with the regulations of University of Queensland Ethics Committee. Mouse allografts (two mice from each strain) were assayed.

Mouse embryos generated from timed 129/Sv-CD1 matings were staged according to the number of tail somites present. To examine Sox expression in endothelial cells lining the posterior dorsal aorta and in the anterior neural medial axial artery, we used stage 9dpc and stage 11.5dpc embryos.

For in situ hybridization, we used standard protocols on multiple samples; in brief, samples were fixed overnight in 4% paraformaldehyde, dehydrated in methanol, and embedded in wax. Sections (8 mm thick) were mounted serially on Superfrost Plus slides (Lomb Scientific, Taren Point, Australia). Slides were dewaxed and hybridized overnight with digoxigenin (DIG)-labeled riboprobes for Sox18, -17, and -7 that were synthesized with the DIG Labeling kit (Roche, Castle Hill, Australia) according to the manufacturer’s instructions. Probe primer sequences used were as previously described (8,20,22). Slides were then incubated with antidigoxigenin antibody coupled to alkaline phosphatase (1:2500; Roche) until color reaction was seen.

**Immunohistologic Analysis of Sox18 Expression**

Excised allograft tumors (n = 2 per mouse) were fixed for 1 hour in 4% paraformaldehyde in PBS, washed overnight in 30% sucrose–PBS, and frozen in compound embedding medium (OCT; Sakura, Tokyo, Japan). Sections (10 μm thick) were mounted on Superfrost Plus slides. Slides were blocked with 10% heat-inactivated sheep serum (Trace Scientific, Clayton, Australia) and 2% bovine serum albumin in PBS and then incubated with mouse monoclonal anti-CD31 (1:300; BD-Pharmin, Palo Alto, CA), mouse monoclonal anti–NG2-Chondroitin (1:100; Chemicon, Boronia, Australia), or mouse monoclonal anti-SOX18 (1:100; Chemicon) overnight at 4 °C. Slides then incubated for 1 hour with either Oregon Green-conjugated anti-mouse immunoglobulin G (IgG) or streptavidin–horseradish peroxidase–conjugated polyclonal anti-mouse IgG (1:100, Zymed, San Francisco, CA) and examined by fluorescence microscopy or light microscopy (to visualize the development of dianminobenzidine tetrahydrochloride staining), respectively. Tissues were washed three times with PBS between each step.

**Generation of an Adenoviral Vector Overexpressing Sox18**

The Sox18 cDNA was excised from pCMV-SPORT6-SOX18 (NIH Mammalian Gene Collection, Bethesda, MD) with MluI and subcloned into the EcoRV site of the shuttle vector pAdTrack-CMV (Qbiogene, Carlsbad, CA). Recombinant adenovirus expressing Sox18 was constructed using the pAdEasy system according to the manufacturer’s protocol (Qbiogene). Viral titers are reported as log10 dose giving 50% tissue culture infection per milliliter of culture, and viral particle number was measured at an optical density at 600 nm. Human umbilical vein endothelial cells (HUVECs; ATCC) were isolated and cultured in gelatin-coated flasks in M199 medium with Earle’s balanced salts and 0.68 mM glutamine, 20 mM HEPES, 20% fetal calf serum, 15 μg/mL endothelial cell growth supplement (BD Biosciences, Bedford, MA), 50 U/mL penicillin, 50 μg/mL streptomycin, and 15 μg/mL heparin (Sigma, St. Louis, MO). For capillary tube formation experiments, HUVECs were grown to 80% confluence and infected with an amount of pAdEasy empty vector or pAdEasy-Sox18 virus particles to achieve 90%–100% infection efficiency and that yielded a similar level of green fluorescence protein expression when measured by fluorescence-activated cell sorting analysis.

**Transient Transfection of HUVECs with Sox18RaOp and Proliferation Assay**

HUVECs were grown in human endothelial serum-free medium (Invitrogen, Mt. Waverly, Australia), supplemented with bFGF (20 ng/mL), epidermal growth factor (10 ng/mL), and human plasma fibronectin (10 μg/mL). HUVECs were transfected with Sox18, Sox18RaOp-expressing pCDNA3.1 (Invitrogen), or empty vector using Lipofectamine 2000 (Invitrogen) or TransPassD2 reagent (Biolabs), according to the manufacturers’
instructions. HUVECs were grown in complete growth medium to 80% confluence before assays were performed.

For proliferation assays, transfected HUVECs were suspended in complete medium and plated at 4 × 10^5 cells/well in quadruplicate in each of two 96-well plates (one each for day 0 and day 3) and incubated at 37 °C. Soluble tetrazolium salt (MTS) cell viability assays were performed according to the manufacturer’s protocol (Promega, Madison, WI) to measure the number of viable cells. In brief, 30 μL of MTS reagent was added to each well (150 μL of medium) and incubated at 37 °C for 2 hours. The absorbance at 490 nm was determined on days 0 and 3 using an enzyme-linked immunosorbent assay plate reader.

Matrigel Capillary Tube Formation Assay and Virtual Northern Blot Analysis

Wells of a 96-well flat-bottom plate were each coated with 100 μL of Matrigel (Becton Dickinson, Franklin Lakes, NJ), which was allowed to polymerize at 37 °C for 1 hour. HUVECs (4 × 10^4) that were infected with pAdEasy empty vector or pAdEasy-Sox18 virus were resuspended in complete medium containing phorbol myristate acetate (20 ng/mL) and anti–α2β1 integrin (30 μg/mL) and plated on the Matrigel-coated wells. Photographs were taken at regular intervals over 24 hours. Experiments were performed in triplicate.

Virtual northern blot analysis was used to measure Sox18 and cyclophilin (CycA) mRNA expression during capillary tube formation. In brief, total RNA was isolated using TRIzol (Life Technologies, Melbourne, Australia) and reverse transcribed using Omniscript reverse transcriptase (QIAGEN, Valencia, CA). cDNA was amplified quantitatively using the SMART polymerase chain reaction cDNA synthesis procedure using oligo(dT) primers (Clontech, Palo Alto, CA). cDNA was subjected to 0.9% agarose gel electrophoresis and Southern transfer to Hybond-N nylon membranes (Amersham Bioscience, Little Chalfont, St. Giles, UK). We have previously demonstrated the linearity of virtual northern blots for measuring magnitudes of differential gene expression (25). Cloned cDNA fragments for Sox18 and CycA (prepared as above) were polymerase chain reaction amplified using T7 and T3 primers and the products labeled with [α-32P]dATP using a MegaPrime Kit (Amersham Biosciences). Probes were incubated with membranes at 65 °C for 2 hours in ExpressHyb Hybridization Solution (Clontech) and hybridized DNA was visualized and signals quantified within a linear range using a Typhoon 9410 PhosphorImager and ImageQuant 3.3 software (Molecular Dynamics, Amersham Biosciences).

MCF-7 Proliferation and Migration Assays

MCF-7 cells (ATCC) were maintained at 60%–80% confluence in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1 mM sodium pyruvate and 10% fetal calf serum at 37 °C and 5% CO2. To measure proliferation, 10^4 MCF-7 cells were plated in triplicate in DMEM and cultured until they reached approximately 60% confluence. Cells were treated with or without 25 ng/mL of VEGF (Sigma, Perth, Australia) and cultured for 16 hours. Cells were then assayed for proliferation using the BrdU labeling and Detection Kit I (Roche). The percentage of positive cells was counted in a blind assay in 10 fields per group. Each experiment was performed three times.

For migration assays, MCF-7 cells were transfected with Sox18RaOp-expressing pcDNA3.1 and stable transfectants selected with G148 gentamicin (GIBCO-BRL, Melbourne, Australia) at 800 μg/mL. Single colonies were expanded and tested for Sox18RaOp expression by immunohistochemistry. Stably expressing Sox18RaOp (MCF-7Ra) and parental cells (10^5) were added to the upper well of a Boyden chamber containing an 8.0-μm-pore-size polycarbonate membrane separating two 6.5-mm Transwell chambers (Costar). The upper wells were placed into the lower chambers, which contained 0.75 mL of DMEM. VEGF (50 ng/mL) was added to the upper or lower well to act as a chemoattractant. After 4 hours at 37 °C in 5% CO2, nonmigratory cells on the membrane surface in the upper chamber were removed with a cotton swab, and the cells that had traversed the membrane onto the lower surface were fixed and stained with the fluorescent nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) at 10 μg/mL for 15 minutes. Membranes were mounted on glass slides, and cells in three random fields per slide were counted in a double-blind assay. Three independent experiments were performed.

Immunoblot Analysis of Signaling Proteins

Cells were lysed directly into Laemmli buffer (containing 1% sodium dodecyl sulfate and 5% β-mercaptoethanol), and protein concentrations were determined by comparison to known standards by gel electrophoresis. Equal amounts of protein (10 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% acrylamide gels and electrophoretically transferred to nylon membranes (Hybond N+; Amersham Biosciences). Standard immunoblot analysis was performed using rabbit polyclonal anti–α-tubulin (1:2000; Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti–phospho- histone H1 (1:1000; Upstate Biotechnology, Charlottesville, VA), rabbit polyclonal anti-SAPK/JNK (1:1000), and rabbit polyclonal anti-phospho SAPK/JNK (1:1000; Cell Signaling Technology). Bound antibody was detected using polyclonal goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Laboratories), and antigen–antibody complexes were visualized using the ECL chemiluminescence reagent (Pierce, Rockford, IL).

Labeling of Actin Cytoskeleton

To visualize filamentous actin, MCF7 and MCF7Ra cells were stained with Texas Red X-Phalloidin (1:2000; Molecular Probes, Invitrogen) according to the manufacturer’s protocol. Nuclei were counterstained with DAPI (10 μg/mL).

Statistical Analysis

Statistical significance of differences between paired groups was determined using the Student’s t test. Variance between multiple groups was calculated using analysis of variance. All statistical tests were two-sided, and P<.05 was considered statistically significant.

RESULTS

SOX18 Expression During Developmental and Oncogenic Vascularization

We first investigated SOX18 expression during tumor angiogenesis. SOX18 protein was detected in neovascular endothelial cells in human colorectal tumors (n = 4) and in mouse melanoma.
lated proteins and that the Ra O p mutant form of Sox18 acts in a dominant-negative manner to interfere with the function of all members of Sox subgroup F with similar sequence and structural features (6), were coexpressed in vascular endothelial cells during developmental angiogenesis in wild-type mice (Fig. 1, B). This finding supports the hypothesis, based on molecular studies (16,19), that the lack of overt vascular phenotype observed in Sox18−/− mice is due to functional redundancy between these related proteins and that the RaOp mutant form of Sox18 acts in a dominant-negative manner to interfere with the function of all three transcription factors during angiogenesis.

**Allograft Tumor Growth in Mice Heterozygous for RaOp or Null for Sox18**

We next investigated whether impaired SOX18 function would inhibit the vascularization and subsequent growth of tumors. Cells from an aggressive mouse melanoma cell line (B16) were injected subcutaneously, at two posterior dorsal sites, into mice heterozygous for RaOp (RaOp+/-), mice null for Sox18 (Sox18−/−), and wild-type mice, siblings of the RaOp/+ cohort (n = 4 per group). Ten days after injection, tumors from the three strains of mice were noticeably different in size (Fig. 2, A). After 14 days, tumors in wild-type mice were large (mean volume = 817 mm3, 95% CI = 643 mm3 to 1001 mm3; Fig. 1), and the experiment was terminated for ethical reasons. At this time, tumors in Sox18−/− mice were smaller (mean volume = 486 mm3, 95% CI = 345 mm3 to 627 mm3;  P < .001; Fig. 1) than those in wild-type mice. Tumor growth in RaOp+/- mice was dramatically slower than that of wild-type mice (mean volume = 233 mm3, 95% CI = 73 mm3 to 119 mm3;  P < .001; Fig. 2). Tumors from RaOp+/- mice were histologically dense, whereas those from Sox18−/− and wild-type mice were markedly less so (data not shown). Some RaOp+/- mutant mice bearing allograft tumors were maintained to the end of the 40-day observation period, with minimal tumor growth and no sign of morbidity.

**Microvessel Density in Allograft Tumors**

To establish whether impaired tumor growth was associated with a paucity of neovascularature, histologic sections of the tumors were stained using monoclonal CD31 and NG2 chondroitin antibodies to visualize endothelial cells and vascular pericytes, respectively (26,27). NG2-stained pericytes are associated only with mature endothelial vasculature and as previously reported; proportionally less of this mature vasculature was observed in the tumor microenvironment (28). However, CD31 is expressed in both tumorigenic lymph and angiogenic endothelial cells of both early and mature vasculature. Tumors from wild-type mice had abundant vasculature, whereas those from Sox18−/− and RaOp+/- mice had relatively smaller and sparser vessels (Fig. 3, A). Counts of CD31- and NG2-positive vessels with clear lumens in the plane of the section in three separate tumors from each of the RaOp+/-, Sox18−/−, and wild-type mice confirmed a dramatic reduction in microvessel numbers in RaOp+/- and, to a lesser extent,
cells. *, **

intervals of vessel numbers in 30

Stained blood vessels were counted in each

tubation, and lowest at 6 hours, when cell migra-

( Fig. 4, A ), an interval that corresponds to active migration of the

pression of Sox18 mRNA was highest between 0.5 and 1 hour

24 hours

expression antibody to invade the substrate and form tubular networks over

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nant-negative form of SOX18 produced in the Ra O p/+  mutant

coinciding with capillary tube maturation ( Fig. 4, A and B ).

Expression levels increased again between 6 and 24 hours,

ing angiogenesis, we used an in vitro tube formation assay, in

The means and 95% confidence intervals of vessel numbers in 30 fields are shown for CD31- and NG2-positive cells. *, P = .001, as determined using Student’s t test and analysis of variance.

in Sox18−/− mice (Fig. 3, B ). These data, together with the known role of SOX18 in vascular development, strongly suggest that the observed tumor growth inhibition was a direct consequence of impaired microvessel development leading to dramatically reduced vasculature in the tumors.

Effect of SOX18 and Sox18RaOp on Tube Formation and Cell Migration in Human Endothelial Cells

To investigate the role of SOX18 in endothelial function during angiogenesis, we used an in vitro tube formation assay, in which HUVECs grown on a collagen matrix stimulated by the addition of phorbol myristate acetate and anti-α2β1-integrin antibody to invade the substrate and form tubular networks over 24 hours (29). We first established, using virtual northern blotting, that human Sox18 is expressed during this process and that expression of Sox18 mRNA was highest between 0.5 and 1 hour (Fig. 4, A), an interval that corresponds to active migration of the HUVECs into the matrix, and lowest at 6 hours, when cell migration rates slow and endothelial tubes start to form (30,31). Expression levels increased again between 6 and 24 hours, coinciding with capillary tube maturation (Fig. 4, A and B).

Next, we transfected HUVECs with Sox18RaOp, the dominant-negative form of SOX18 produced in the RaOp/− mutant mouse. Such HUVECs showed a dramatic reduction in tube formation compared with that in cells transfected with empty vector (Fig 4, C). Large continuous capillary tubes formed in the cells that were transfected with the empty vector, whereas the tubes formed in the cells transfected with Sox18RaOp were short and often failed to join up into a network.

We also assessed capillary tube formation in HUVECs in which overexpression of wild-type SOX18 was achieved by adenoviral infection. Infection efficiencies in excess of 90% were achieved using the pAdEasy system, quantified by fluorescence-activated cell sorting analysis. Tubular networks formed and progressed more quickly in cells that overexpressed wild-type Sox18 than in vector controls, being noticeable by 3 hours in Sox18 cells (Fig. 4, C) compared with 4–6 hours (data not shown) in control cells. The results of this gain-of-function assay complement both the Sox18RaOp in vitro overexpression data and the results of the in vivo loss-of-function analyses described above, and they suggest a role for SOX18 in facilitating tube formation, which in turn may be due to effects of SOX18 on the rate of endothelial cell migration, proliferation, and/or assembly into vessels.

Effect of Sox18RaOp Expression on Migration and Proliferation In Vitro

To investigate the effect of SOX18 dysfunction on cell proliferation and migration, we established from one colony of transfected MCF-7 mammary adenocarcinoma cells a line stably expressing Sox18RaOp (MCF-7Ra). All cells of this clonal line expressed RaOp at similar levels (data not shown). Previous studies have established that proliferation and migration in both endothelial and MCF-7 cells occur via similar mechanisms and that MCF-7 cells express elevated levels of Sox18 (21,32,33).

MCF-7Ra and parental MCF-7 cells were grown in the presence or absence of VEGF for 24 hours, and the rate of proliferation

Fig. 3. Vasculature within melanoma allograft tumors grown in Sox18−/−, RaO p/+ , and wild-type age-matched male mice. B16 mouse melanoma cells were injected subcutaneously into mice, and tumor vasculature was examined by using immunohistochemical staining with anti-CD31 and anti-NG2 chondroitin antibodies. A) Frozen sections (10 μm) of tumors were stained with mouse monoclonal anti-CD31 antibody for total vessel count and mouse monoclonal anti-NG2 chondroitin antibody to label pericytes. Both antibodies show quantitative and qualitative differences in the vasculature of the tumors in the three genotypes (arrowheads indicate stained cells). Scale bar = 100 μm. B) Stained blood vessels were counted in each field. The means and 95% confidence intervals of vessel numbers in 30 fields are shown for CD31- and NG2-positive cells. *, P = .001, as determined using Student’s t test and analysis of variance.

Fig. 4. Sox18 and Sox18RaOp expression and in vitro capillary tube formation in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were infected with adenovirus empty vector (EV) or that expressing wild-type Sox18. A) Sox18 mRNA levels during capillary tube formation in vitro. Total RNA from adenovirus-infected HUVECs was isolated at the times (hours) shown and analyzed for Sox18 and cyclophilin A (CYCA) expression using virtual northern blot analysis. Duplicate virtual northern blots (Expt A and B) were performed. B) RNA levels in A were quantified using a phosphorimager; Sox18 mRNA levels were normalized to that of CYCA, and the fold induction was plotted over time. C) HUVECs expressing wild-type Sox18 or empty vector (left) and HUVECs transiently transfected with empty vector or with a dominant-negative form of Sox18 expressing construct (RaOp) (right) that were induced to undergo capillary tube formation in vitro were photographed every 30 minutes. Data presented at 1.5, 3, 4, and 21 hours (arrows, tube formation). Scale bar = 200 μm.
was then analyzed. MCF-7Ra cells proliferated at a slower rate than parental MCF-7 cells in the presence of VEGF (BrdU incorporation, mean = 20%, 95% CI = 15% to 25% versus mean = 41%, 95% CI = 35% to 45%; \( P = .013 \); Fig. 5, A).

We also carried out MTS proliferation assays using monolayer cultures of HUVECs that were transiently transfected with the Sox18RaOp expression construct. Again, we observed a substantial decrease (approximately 33%) in cell viability of HUVECs transfected with Sox18RaOp-expressing construct compared with cells transfected with empty vector (optical density at 490 nm, mean = 0.27 versus mean = 0.46; difference = 0.19, 95% CI = 0.145 to 0.235; \( P = .001 \); Fig. 5, B).

We next examined the effect of SOX18 on several biochemical pathways associated with cell migration and/or proliferation. Immunoblot analysis of protein extracts revealed that MCF-7Ra and HUVECs transiently transfected with murine Sox18RaOp had lower levels of H1-histone phosphorylation than parental MCF-7 cells and HUVECs transfected with empty vector, characteristic of cells accumulating in G1 phase of the cell cycle (Fig. 5, C) (34, 35). Because JNK activity has previously been implicated in proliferative and migratory control pathways in these and other cell types (33, 36), we next examined levels of JNK phosphorylation. Although we observed no difference in JNK phosphorylation between MCF-7Ra and parental cells, JNK phosphorylation was substantially reduced in HUVECs transiently transfected with Sox18RaOp compared with empty vector control subjects. These data suggest that the dominant-negative (i.e., Sox18RaOp) form of SOX18 exerts a negative effect on cell proliferation and migration. However, HUVECs may be more sensitive to the mechanism by which this interference occurs, as demonstrated by more severe disruption of the mitogen-activated protein kinase signaling pathway in HUVECs compared with that in MCF-7 cells. Although these experiments do not fully exclude the possibility that the altered behavior of the MCF-7Ra cells was due in part to the process of selecting the clones, the similarities with the observed effects in HUVECs suggest that selection is not the case.

To further investigate the functional link between Sox18 expression and cell migration, we next examined the effect of Sox18 and Sox18RaOp in migrating cells. In Boyden chamber migration assays, we observed a decrease in MCF-7Ra cell migration of 60% compared with parental cells in the presence of VEGF (Fig. 6, A). Similar results were...
observed in three independent experiments (MCF-7Ra, mean = 99 cells migrated through membrane at 4 hours versus MCF-7, mean = 204 cells, difference = 105 cells, 95% Cl = 72 cells to 139 cells; \( P = .001 \)).

To investigate the possible involvement of disruption to the actin cytoskeleton in the observed changes in cell migration, we compared actin assembly in MCF-7Ra and MCF-7 cells (33,36). In wild-type cells, polymerized actin was observed in a rigid cytoskeleton at the cell periphery and in parallel transcellular stress fibers (Fig. 6, B). In MCF-7Ra cells, lower levels of actin were observed at the cell periphery and fewer actin stress fibers formed (Fig. 6, B). These differences in actin organization suggest that SOX18 regulates cell migration by influencing actin cytoskeleton organization. This regulation may not involve a direct effect on the actin cytoskeleton; rather, it may be indicative of a role for SOX18 in regulating the expression of genes involved in cell motility and migration.

**DISCUSSION**

Angiogenesis is essential for cancer progression and is one of the characteristics of metastases and poor patient prognosis. Direct angiogenesis inhibitors such as angiotatin and bevacizumab specifically target vascular endothelial cells, preventing them from proliferating or migrating or from avoiding cell death in response to proangiogenic signals (37–39). Here we have identified Sox18 as a gene expressed in endothelial cells during the initial steps of tumor vascularization and demonstrate that interfering with its function inhibits blood vessel formation and subsequent tumor growth.

In this study, mice heterozygous for the dominant-negative Sox18 mutation RaO\( \text{p} \) or null for Sox18 showed reduced tumor growth when injected with cells cultured from a mouse B16 melanoma line. The reduction in tumor size corresponded to a reduction in microvessel density. Microvessel density is strongly associated with metastatic risk and poor prognosis in most, although not all, tumor types (40), and although microvessel density is not necessarily a good indicator of therapeutic efficacy, a reduction in microvessel density after treatment generally leads to an improved prognosis (40,41). Our observations, therefore, suggest that the ability of the host endothelial cells to respond to proangiogenic signals is perturbed in RaO\( \text{p} \) and Sox18-null mice, resulting in fewer microvessels in the tumor and subsequent tumor growth inhibition.

We found that SOX18 also plays a role in regulating cell migration and subsequent vascular tube formation. When Sox18RaO\( \text{p} \) was overexpressed in HUVECs, tube formation was disrupted, and when SOX18 was overexpressed, tube formation was augmented, suggesting that SOX18 promotes vascularization, possibly by increasing endothelial cell migration rates. Previous studies have suggested that endothelial cells migrate into a soft matrix by using a rigid actin cytoskeletal structure, which then undergoes reversible remodeling before tubelike structures can form (42). Extra SOX18 may promote this activity. Conversely, the forced overexpression of the dominant-negative form of Sox18 disrupts the migration of MCF-7 cells by causing a breakdown of the rigid cytoskeletal assembly, characterized by less actin at the cell periphery and fewer transcellular stress fibers. Although this disruption may not impede cell spreading, adhesion, or survival on a rigid two-dimensional surface, it may contribute to the observed compromised ability of MCF-7Ra cells to migrate in response to a chemoattractant such as VEGF.

Also, the reduced rate of proliferation observed in both HUVECs and MCF-7 cells expressing Sox18RaO\( \text{p} \) may also contribute to the decrease in tumor microvessel density. Levels of phosphorylated histone H1 normally increase and peak as cells enter M phase, with levels of phosphorylation being lowest in G\( \text{1} \) phase (34). The reduced rate of proliferation of MCF-7Ra cells (seen via BrdU incorporation) corresponded to a decrease in the amount of phosphorylated histone H1 in these cells, suggesting that they are accumulating in G\( \text{1} \) phase and not dividing. Similarly, in HUVECs transiently transfected with a construct to overexpress the murine Sox18RaO\( \text{p} \) form of Sox18, a decrease in the levels of phosphorylated histone H1 was observed. Our observations imply that inhibiting SOX18 function may also restrict the number of endothelial cells present in tumor neovascularization by reducing their rate of proliferation. In accordance with this assertion, data recently reported indicate that the overexpression of SOX18 in endothelial cells may contribute to vascular cell growth in advanced human coronary atherosclerotic lesions, which can be reduced by blocking Sox18 expression in these cells (43).

Interestingly, phosphorylation of JNK was decreased in HUVECs expressing the Sox18RaO\( \text{p} \) dominant-negative form of Sox18, but not in MCF-7Ra cells. The inhibition of JNK phosphorylation contributes to impaired cell migration and proliferation in endothelial cells (36,44). The apparent difference in the response of the two cell lines suggests that HUVECs are more sensitive to SOX18, and therefore also to the Sox18RaO\( \text{p} \) form of Sox18, signifying that HUVECs may have more angiogenic-related cofactors within their nuclei than do MCF-7 cells.

Although this work highlights Sox18 as a potential target for an antiangiogenic agent, several obstacles remain. The design of specific transcription factor inhibitors and their delivery to the correct cells are real challenges. Recent advances in the use of peptidomimetics, antisense oligonucleotides, and small-molecule inhibitors may provide an avenue for mimicking the ability of Sox18RaO\( \text{p} \) to interfere with Sox18, \(-17\) and \(-7\) activity simultaneously during tumor angiogenesis (45). Such specific drug design in combination with targeted delivery systems offers real potential for exploiting Sox18 as an antiangiogenic target (46).

Regardless of these issues, it is clear that targeting the genes involved in endothelial cell function is likely to provide important adjunct approaches to current available anticancer therapies. In particular, genes involved in the early development, differentiation, and assembly of endothelial cells into a neovasculature are likely to be of value in this context, because interfering with their function is less likely to compromise the function of existing mature vasculature. Studies in mice have shown that Sox18 is active in developing embryonic vasculature but not in mature blood vessels (17). Here we have established that interference of SOX18 function by using a dominant-negative allele of Sox18 (RaO\( \text{p} \)) severely restricted tumor growth in vivo and that this inhibition coincided with decreased tumor microvessel density and reduced cell migration and proliferation in vitro. The degree of tumor inhibition is all the more remarkable given that Sox18 function is only partially compromised in heterozygous RaO\( \text{p} \) mutant mice (15). The inhibition of tumor growth in a mouse model, together with recent advances in targeted, systemic gene delivery to tumor neovasculature (46), indicates that SOX18 may be a useful target for human cancer therapy.

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NOTES

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