The VCAM-1 Gene That Encodes the Vascular Cell Adhesion Molecule Is a Target of the Sry-related High Mobility Group Box Gene, Sox18*

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VCAM-1 (vascular cell adhesion molecule-1) and Sox18 are involved in vascular development. VCAM-1 is an important adhesion molecule that is expressed on endothelial cells and has a critical role in endothelial activation, inflammation, lymphatic pathophysiology, and atherogenesis. The Sry-related high mobility group box factor Sox18 has previously been implicated in endothelial pathologies. Mutations in human and mouse Sox18 lead to hypotrichosis and lymphedema. Furthermore, both Sox18 and VCAM-1 have very similar spatio-temporal patterns of expression, which is suggestive of cross-talk. We use biochemical techniques, cell culture systems, and the ragged opossum (RaOP) mouse model with a naturally occurring mutation in Sox18 to demonstrate that VCAM-1 is an important target of Sox18. Transfection, site-specific mutagenesis, and gel shift analyses demonstrated that Sox18 directly targeted and trans-activated VCAM-1 expression. Importantly, the naturally occurring Sox18 mutant attenuates the expression and activation of VCAM-1 in vitro. Furthermore, in vivo quantitation of VCAM-1 mRNA levels in wild type and RaOP mice demonstrates that RaOP animals show a dramatic and significant reduction in VCAM-1 mRNA expression in lung, skin, and skeletal muscle. Our observation that the VCAM-1 gene is an important target of SOX18 provides the first molecular insights into the vascular abnormalities in the mouse mutant ragged and the human hypotrichosis-lymphedema-telangiectasia disorder.

The formation of blood vessels occurs through two distinct mechanisms, vasculogenesis and angiogenesis (1). Vasculogenesis leads to the vascularization of the endodermally derived organs such as lung, intestine, spleen, liver, and stomach, whereas angiogenesis involves the vascularization of the organs of mesodermal and ectodermal origin such as the limbs, kidney, and the brain. It also gives rise to the intersomatic and vertebral arteries (2).

We have recently implicated Sox18 in vascular development (10). Sox18 is a member of the Sry-related HMG box-containing (Sox) family of transcription factors. SOX proteins bind to DNA in a sequence-specific manner via the HMG domain, with all the proteins characterized to date binding to the heptameric motif (A/T)CAAA(A/T)G (11). We have shown previously that Sox18 binds to the consensus sequence AACAAAG and trans-activates a heterologous promoter containing this element (12).

The Sox family displays both overlapping and distinct spatio-temporal expression patterns during embryogenesis and development. Aberrant Sox expression, mutation, or disruption leads to a number of diseases; for example, Sry and Sox9 are involved in sex reversal (13, 14).

The situation is similar for Sox18. In situ analysis of Sox18 has demonstrated expression in the mesenchyme underlying the developing hair follicle, in the presumptive heart, and in the developing vasculature (10). The naturally occurring mouse mutant ragged (Ra) of which there are four allelic variants, Ra, ragged Jackson (Radj), ragged-like (Ragl) and RaOP, all contain mutations in Sox18 (15). All these mutants display defects in hair and skin development. However, most life threatening is the generalized edema suffered by these animals (16), which is probably due to lymphatic aberrations (17–19).

Recently, a report has been published describing the investigation of several mutations in SOX18 and the hypotrichosis-lymphedema-telangiectasia (HLT) disorder in humans (20). Patients present with early onset alopecia of the scalp and lymphedema. The most severe lymphatic abnormality presented was non-immune hydrops fetalis (of unknown etiology). Telangiectasia was present in only some of the patients studied, as were other anomalies such as thinness and transparency of the skin, hydrocele, and cutaneous papular vascular lesions.

One of the major functions for the blood and lymphatic vascular system is to provide efficient access for leukocytes and other immune system molecules to all tissues of larger animals. The immune system responds to damage or illness via the accumulation of leukocytes, leading to a localized inflammation in the diseased area. This inflammatory response is necessarily tightly coordinated, as the lack of control can itself lead to various diseases, for example arthritis, psoriasis, multiple sclerosis, asthma, atherosclerosis, and allergy (21–24). Of the multitude of proteins involved in the immune system response, the cell adhesion molecules play a major role in mediating immune function and inflammation (25).

Vascular cell adhesion molecule-1 (VCAM-1), a member of VCAM-1; IRF-2, interferon regulatory factor-2; Ra, ragged; RadJ, ragged Jackson; Ragl, ragged-like; RaOP, ragged opossum; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; aa, amino acids.
the immunoglobulin gene superfamily, is expressed on the cell surface of activated endothelia, in the skin (26, 27), and in developing muscle and the lung (28–30). VCAM-1 recognizes the integrin receptors αvβ3 (31) and αvβ2 (32, 33) present on monocytes, eosinophils, and lymphocytes, whereas VCAM-1-deficient embryos die in utero due to abnormalities in chorio-allantoic fusion (34). In vivo animal studies demonstrated that attenuated VCAM-1 function in mice provided protection against atherosclerosis. For example, mice with reduced (not ablated) VCAM-1 expression and function crossed with low density lipoprotein receptor LDLR−/− mice prone to atherosclerosis produce animals resistant to atherogenesis (35).

Much of the previous research has focused on the control of VCAM-1 expression via extracellular signals. For example, lipopolysaccharide and cytokines such as interleukin-4 (IL-4), tumor necrosis factor-α (TNFα) (36, 37), interferon-γ (IFNγ), transforming growth factor-β1 (TGFβ1) (38), granulocyte-macrophage colony-stimulating factor (GM-CSF) (39), and vascular endothelial growth factor (VEGF) (40) cause an increase in the level of expression of VCAM-1 in endothelia as well as in other cell types, whereas angiopoietin 1 (ang1) can repress the activation of VCAM-1 by vascular endothelial growth factor (41).

In contrast, transcriptional regulation of VCAM-1 is less well understood. Most notable is the role of interferon regulatory factor-2 (IRF-2) in the control of VCAM-1 expression in muscle (42).

Sox factors act as critical regulators of organ ontogeny via the modulation of expression of particular target genes. Surprisingly, relatively few target genes for this family of transcription factors have been described in the literature (11). Therefore, to elucidate the function of Sox18 in the blood and lymphatic vascular system, the major site of expression and of abnormality in the ragged animal, it is important to find target genes for this factor in this organ.

Our study demonstrates that native Sox18 (and not the mutant ragged form) is able to induce the activity of the VCAM-1 promoter. In biochemical assays we identified and characterized three Sox18 binding sites. However, we demonstrated that only the SoxB site at −715 is necessary for VCAM-1 transcription. In vivo validation of this data was obtained from the RoOP animals that have very significantly reduced levels (8–40-fold) of VCAM-1 expression (and not other adhesion molecules, including ICAM-1 and ICAM-2, JCAM, NCAM-1, and PECAM-1).

**Materials and Methods**

**RNA and cDNA Preparation**—RNA for realtime PCR was isolated from organs of RoOP and wild-type adult male siblings as described previously (45), with the exception that the lyophilized RNA was purified by processing ~100 μg through a Qiagen RNeasy mini column. During this purification process the RNA was DNase-treated. Quantitation of the purified RNA was carried out as described previously (45). First strand cDNA synthesis was carried out using 5 μg of purified RNA and primed with oligo(dT)m, using the Superscript III enzyme and the supplied manufacturer’s protocol.

**Real Time PCR**—Target cDNA levels were quantitated by real time RT-PCR using an ABI Prism™ 7700 Sequence Detector system utilizing SYBR Green I (Molecular Probes). 2 μl of each of 20 μM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl2, 200 μM each of dGTP, dATP, and dCTP, 400 μM dUTP, 5 units of uracil-N-glycosylase, 500 nM ROX reference dye (Invitrogen), and 200 nM each forward and reverse primers. For mus musculus primer sequences for VCAM-1 are as follows: forward, 5′-TGACAGTCCCATGCTTGTA-3′; reverse, 5′-ACCTGCGACGGCATATT-3′.

**Promoter Construction Generation**—The sequence of the murine VCAM-1 promoter has been published elsewhere (46) and is available on NCBI (accession number U42327). Mouse genomic DNA (C57BL/6) was a gift from J. Rowland, and 10 ng was used in the outer PCR amplification to generate the VCAM-1 promoter. Nested PCR was necessary for amplification of the 1895-bp promoter that has been published previously. Outer primers were constructed at the published upstream limit of murine VCAM-1 promoter at −1895 bp and within the first exon at 19 bp. The outer PCR was performed with 100 ng of each primer (~1895, 5′-GGCGGTACCGATCTCATAGACCGGAG-3′; and 19, 5′-CGGATTCTTTGACCACTATGTTTTT-3′), 1.25 units of Phusion, and 0.2 mM dNTPs in a final volume of 50 μl containing the supplied buffer (Promega). Hot start PCR was performed at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The inner primers were constructed such that the 5′-primer at ~1889 contained the restriction enzyme site KpnI (~1887, 5′-GGCGGTACCATGACCGGAGTCTTT-3′), whereas, the 3′-primer had the restriction enzyme site XhoI (~1, 5′-GCTTTGCGTATCTATTGACCGAGAAG-3′). 1 μl of the outer PCR was combined with buffer B and 0.25 units of polymerase mix from the Fail-Safe PCR system (Epicenter) and 100 ng of each inner primer in a final volume of 10 μl. The VCAM-1 inner PCR profile was the same as that used for the outer PCR.

The product was digested with KpnI and XhoI, inserted into pGL2- Basic (pGL2B-Promega), and sequenced completely via automated sequencing using the ABI system and reagents. This construct was used as the parental plasmid to generate all other sub-clones. Deletion clones were generated either by restriction digests or PCR amplification. VC1219 was generated by restriction digest with PstI and KpnI, and the vector plus the remaining insert were then blunt-ended with Klenow (New England Biolabs) and religated. Both VC754 and 504 were generated via PCR amplification with the primers 5′-GACGTTCGTT- CATCAGGACATTGGTACAAA-3′ and 5′-GGCGGTACCTTTTGTTGAAAAG-3′, respectively. The 3′-primer was the inner primer −1 from the initial nested PCR. The PCR profile was essentially the same as that done for the nested PCR, with the exception that the annealing temperature was 55°C. Site-directed mutagenesis of the VCAM-1 promoter was carried out in vivo via the Quiq-Change® II kit following the manufacturer’s protocol. The primers used for the −1569 mutation are 5′-ATGACATGACATGACGTTGC- TCTTAC-3′ and 5′-CTAGGACACCTAATGATGTCATGTCAT-3′. The primers used for the −715 mutation are 5′-GCTTTGGGATCATCAACAAACAAA-3′ and 5′-TTTTGTTTTTATGATGTCATGTCGCC-3′.

**Cell Culture and Transient Transfections**—COS-1 (simian fibroblast) and SVEC4-10 (mouse high venule endothelial) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in 6% CO2 (mouse skeletal muscle) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum in 6% CO2. Cells for transfection were grown in 24-well dishes to 50% confluence before being transiently transfected. The reporter plasmid and 1 μg of the expression plasmids in 0.5 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum by a liposome-mediated procedure. Briefly, SVEC4-10 cells were transfected using N-[1-(2,3-dioloxyleoyl)]-N,N,N-trimethylethammonium propanemethylsulfate (DOTAP) (Roche Molecular Biochemicals) in quantities 8-fold (v/w) the total amount of DNA, whereas COS-1 and C3A cells were transfected using 15 μl of DOTAP and 10 μl of 1,3-di-oloxyleoyl-2-(6-carboxypermyl)-propylamidid (DOSPER) (Roche Molecular Biochemicals). 24 h post-transfection the medium was replaced, and the cells were grown for a further 24–48 h. Each experiment represented at least two sets of independent quadruplicates to overcome the variability inherent in transfections. Cells were harvested and assayed for luciferase activity as described previously (47).

**Electrophoretic Mobility Shift Assay (EMSA)**—We have described previously the cloning and protein expression of a murine Sox 18 fusion construct with glutathione-S-transferase (GST) using the pGEX-1 bacterial expression vector (12). Briefly, Escherichia coli DH5α cells containing this vector were induced for 1–2 h with 0.5 mM isopropyl thiogalactoside after the cells had grown to an A600 of 0.6. The pelleted cells were sonicated, and the cleared lysate containing the fusion protein was loaded onto glutathione-agarose columns in Dignam buffer C (containing protease inhibitors) (47). After extensive column washing, the fusion protein was eluted with Dignam buffer C, 50 mM reduced glutathione.

Probes used in all EMSAs were annealed and then radiolabeled using γ-32P and T4 polynucleotide kinase (PNK). The oligonucleotide sequences used as probes are as follows: −1569, 5′-TTTTGACATGACGTTGCAGGACTTCTC-3′, 5′-GAGGACACTGCAATCTGTTTTT-3′. The probe sequence was added to the 5′ end of the PCR product as a BglII linker.

**Electrophoresis**—The proteins were resolved on 15% SDS-polyacrylamide gels and transferred to nitrocellulose (Bio-Rad). The membranes were blocked in 5% nonfat dry milk-TBST and immunoblotted overnight with primary antibodies. The antibodies used were anti-glutathione-S-transferase monoclonal (Santa Cruz) and anti-Sox18, which is a kind gift from J. Rowland.
Fig. 1. Schematic representation of the murine VCAM-1 promoter and the positions of elements important for this study. A schematic diagram of the full-length VCAM-1 promoter construct cloned and used in this investigation is shown. Of particular note are the three putative Sox binding sites displayed as bold boxes and denoted as SoxA, SoxB, and SoxC. The core heptameric Sox sequence is displayed below each box with an arrow to indicate the direction of each binding site as compared with the direction of transcription. All numbers designate important elements or clones referred to in the text. Luc refers to the luciferase reporter gene, F1 ori denotes the origin of replication of filamentous phage for generation of single-stranded DNA, and Amp represents the ampicillin-resistance gene.

ATGTCATAAAA-3' (bottom strand); −715, 5'-GGCTGGGGGCattgtcAA-ACAAAAAG-3' (top strand) and 5'-CTTTTGTTTGacatGCCCCAGCC-3' (bottom strand); −491, 5'-GAAAGAAGacacetTTTTATTTTTTAAATTG-CAAAATGCAATTTCTT-3' (top strand) and 5'-AAGAAATGCAATTGCAA-TTAAAAAATAAAtgattctCTCCTTC-3' (bottom strand).

The bases in lowercase letters represent the putative Sox binding sites. All EMSA experiments were carried out in a total of 20 μl in Dignam Buffer C containing 1–2 ng of T4 polynucleotide kinase-labeled probe and 2 μg of the purified, bacterially expressed GST-Sox 18. The assays were incubated at room temperature for 20 min and electrophoresed through a 6% (polyacrylamide/bisacrylamide; 20:1) gel in 80 mM Tris borate and 2 mM EDTA. Gels were briefly soaked in 10% acetic acid, dried, and autoradiographed.

Competition EMSAs were carried out basically as above with the exception that unlabelled probe was added to the reaction in 20–80-fold molar excess as compared with the labeled probe. Both the probe and an unlabeled competitor were added to the reaction at the same time and then incubated and electrophoresed as before. For these experiments, the unlabeled competitor in each reaction was the same double-stranded oligonucleotide as the probe used in that reaction.

RESULTS

Cell-specific Transcription of the Mouse VCAM-1 Is Regulated by the 5'-Upstream Flanking Sequences—The naturally occurring Sox18 mutations in mice and humans (15, 20) display defects in hair and skin development. However, the most life threatening is the generalized edema caused by lysosomal vascular dysfunction (17–19). Hence, we were particularly interested in the identification of Sox18 target genes that play a role in lymphatic function and thus may allow us to better understand the molecular basis of the ragged phenotype.

We hypothesized that VCAM-1 was regulated by SOX18. Therefore, to test this supposition we examined the ability of VCAM-1 promoter sequences to direct the expression of the Luc gene in muscle, endothelial, and fibroblast cells. For this purpose, we designed primers to amplify the complete published murine VCAM-1 sequence (46) and cloned the sequenced amplified product into the promoterless pGL2-Basic luciferase reporter vector. This plasmid was denoted as PGL2-VC1889 and encompasses 1889 bp immediately upstream of the murine VCAM-1 translation start codon (Fig. 1). To test if Sox18 could trans-activate the VCAM-1 promoter, we transfected it into fibroblasts and endothelial and skeletal muscle cell lines (Fig. 2). Transfection of pGL2-VC1889 into COS-1 fibroblasts and SVEC4-10 high venule endothelial cell lines demonstrated that the upstream promoter sequences of the VCAM-1 gene confer high level expression in a cell-specific manner. For example, pGL2-VC1889 activity in COS-1 fibroblasts is ~5-fold greater than that of pGL2-Basic and 100-fold less than that of the constitutively active SV40 promoter. In contrast, in endothelial SVEC cells pGL2-VC1889 activity is ~50-fold greater than that of pGL2-Basic and is similar in activity to the very efficient SV40 promoter. This is consistent with our previous study that reported a high level of Sox18 expression in the nuclei of SVEC4-10 (48). Similarly, when the VCAM promoter was transfected into proliferating C2C12 myoblasts, the VCAM-1 promoter activity was ~50-fold greater than that of pGL2-Basic (data not shown), as has been reported previously (44). In conclusion, the efficient cell specific expression of VCAM-1 is consistent with the in vivo expression profile of VCAM-1.
The VCAM-1 Gene Is a Target of the Sry-related HMG Box Gene Sox18 as Shown by the Identification of Three SOX18 Binding Sites—VCAM-1 expression can be stimulated via cytokines (e.g. tumor necrosis factor-α) (43), mitogens (e.g. vascular endothelial growth factor) (49), and transcription factors (e.g. IRF-2 and Oct-1) (42, 43). We investigated whether SOX18 could trans-activate the mouse VCAM-1 promoter in COS-1 fibroblasts. Co-transfection of a Sox18 expression construct with the pGL2-VC1889 promoter resulted in a moderate 5-fold activation (Fig. 3A). This activation is dose-dependent, as increasing amounts of Sox18 augmented the trans-activity of the VCAM-1 promoter (Fig. 3B). Thus, we demonstrate here for the first time that Sox18 trans-activates the promoter of VCAM-1.

SOX and SRY proteins contain HMG domains that bind DNA in a sequence-specific manner. These proteins have been reported to bind in vitro and in vivo to sites with a core motif resembling (A/T)(A/T)CAA(A/T)G (for review, see Ref. 11). We have reported previously that SOX18 can bind and trans-activate a core consensus motif of AACAAAG (12). As an initial step in the investigation to examine whether SOX18 was able to bind the murine VCAM-1 promoter, we searched the sequence of the promoter using MatInspector (50) and found three potential Sox binding sites (Fig. 1). Of these, the two at −491 (SoxC) and −1569 (SoxA) contain a perfect core consensus site (AACAAT) for the binding of Sox/Sry transcription factors. The core sequence of the site at −715 (SoxB) is slightly different from the consensus, with a Ga t position 1 (GACAAT). Interestingly, it has been reported that SOX17 can bind this sequence from a pool of otherwise random oligonucleotides (51).

To investigate whether GST-SOX18 can interact with the putative SoxA, SoxB, and SoxC binding sites, we performed an EMSA using 32P-labeled oligos that consist of the Sox binding site in addition to some surrounding sequence (see “Materials and Methods”). As seen in Fig. 4A, the SOX18 protein bound strongly to all three putative Sox sites in the VCAM-1 promoter (arrow). Lanes 1–3 (counting from the left) delineate that the GST protein alone can not contribute to the binding of these oligonucleotides. All oligos were 32P-radiolabeled, combined with GST-Sox18, and then electrophoresed on 6% polyacrylamide gels. B, EMSA competition assays with up to 50-fold molar excess of “cold” unlabeled oligos were used delineate the specificity of SOX18 binding to the Sox motifs.

FIG. 3. Sox18 activates the VCAM-1 promoter. A, co-transfection of COS-1 cells with control vectors (pSG5, an expression vector and pGL2B, a luciferase reporter vector) and with pSG5 containing Sox18 (Sox18) and pGL2B containing 1889 bp of the proximal mVCAM-1 promoter (VC1889). B, co-transfection of increasing amounts of Sox18 leads to the dose-dependent expression of the mVCAM-1 reporter (VC1889). 1, 2, and 3 μg of Sox18 expression vector were transfected in lanes 2–4, 6 and 7, and 8, respectively (counting from the left). The results are expressed as the mean ± S.D. of at least two independent quadruplicates.

FIG. 4. Sox18 binds to multiple sites in the VCAM-1 promoter. A, a bacterially produced GST-Sox18 fusion protein binds strongly as a single band to all putative Sox sites in the VCAM-1 promoter (arrow). Lanes 1–3 (counting from the left) delineate that the GST protein alone can not contribute to the binding of these oligonucleotides. All oligos were 32P-radiolabeled, combined with GST-Sox18, and then electrophoresed on 6% polyacrylamide gels. B, EMSA competition assays with up to 50-fold molar excess of “cold” unlabeled oligos were used delineate the specificity of SOX18 binding to the Sox motifs.
VCAM-1 Is an Important Target of Sox18

The ragged Alleles of SOX18 Cannot trans-Activate VCAM-1 Expression—We have reported previously that Sox18 is mutated in all four alleles of ragged (Ra, Rap, Ragl, and RaOP). ragged is a mouse mutant that displays hair follicle, vascular, and lymphatic abnormalities (10, 15). All four mutants consist of a missense mutation that occurs in the activation domain (aa 252–345) for Ra (aa 313), Rap (aa 312), and Ragl (aa 323), whereas the RaOP (aa 349) mutation is 4 bp C-terminal of this.

The functional analysis correlated with the in vitro binding data. For example, we examined the ability of the mutant mSoxA and mSoxB motifs (Fig. 6D) to compete for SOX binding relative to the wild-type motifs in EMSA competition assays.

Fig. 6E demonstrates that the ability of the mutated Sox motifs to compete for SOX binding is certainly compromised relative to the native motifs. Fig. 6E demonstrates that even at 80-fold molar excess the mutated sequences very inefficiently compete for SOX protein binding. These experiments demonstrate that SoxB is necessary for trans-activation by Sox18 and that significant trans-activation of the VCAM-1 promoter by Sox18 is associated with efficient binding to the Sox motif.

trans-activation values and S.D. values were derived from 3 independent quadruplicates.

Fig. 5. Deletion analysis defines the cis-acting region between −754 and −504 as important in Sox18-mediated trans-activation in all cell lines tested. A, schematic representation of the deletion constructs. B, pGL2-Basic and the unidirectional deletion constructs of the VCAM-1 promoter cloned into pGL2-Basic were co-transfected into COS-1 fibroblasts with either pSG5 (empty vector) or pSG5-Sox18. Relative luciferase activity is compared with the luciferase activity obtained after co-transfection of pGL2-Basic with pSG5 (empty vector alone) and arbitrarily set to 1. C, the data from panel B is re-presented as the fold activation of the VCAM-1 promoter constructs by Sox18. Fold activation is expressed relative to the activation of the empty luciferase vector, pGL2-Basic, and co-transfected with the expression vector, pSG5-Sox18, which was arbitrarily set to 1. The mean luciferase fold activation values and S.D. values were derived from 2–3 independent quadruplicates.

The Sox B site at −715 bp Is Essential for trans-Activation by Sox18—In an attempt to further elucidate which sites were necessary for the in vitro trans-activation of VCAM-1 in fibroblast cells, we produced a series of 5’ unidirectional deletions in the mVCAM-1 promoter. Three deletion constructs were produced, namely pGL2-VC1215, pGL2-VC754, and pGL2-VC504, which contained 1215, 754, and 504 nucleotides, respectively, of 5’ flanking sequences cloned upstream of the luciferase reporter in pGL2-Basic.

By transfecting the full-length and deleted promoters in the absence/presence of the Sox18 expression vector in COS-1 cells, we could demonstrate that VC1889, VC1215, and VC754 were trans-activated by Sox18 (Fig. 5A). In contrast, deletion of the sequences from −715 to −504 completely ablated SOX18-mediated trans-activation. This suggested that the cognate Sox18 binding site at −715 was necessary for trans-activation. Moreover, the most proximal Sox binding site (−504 bp) could not independently support or mediate trans-activation. Interestingly, the sequences between −189 and −715 seem to contain elements that confer repression of the activity of the VCAM-1 promoter. This repressive activity may be due to the putative octamer sites present in this region.

To directly identify which of the two potential Sox sites in the VCAM-1 promoter at −1569 and −715 bp are necessary for Sox18 mediated trans-activation, we used site-directed mutagenesis (Fig. 6A). Two bases in the middle of each of the heptameric Sox sites were mutated (Fig. 6D). Transfection of the clone VC1889-mSoxA (Fig. 6, B, and C) demonstrated that the mutation of the distal SoxA binding site at −1569 did not compromise Sox18-mediated trans-activation of the VCAM-1 promoter. In dramatic contrast, mutation of Sox site B at −715 bp in the construct VC1889-mSoxB, completely abrogated the basal activity of the promoter and also ablated the Sox18-mediated trans-activation of the murine VCAM-1 promoter. These results, in addition to those from the deletion experiments, show that the Sox site at −715 bp in the murine VCAM-1 promoter is necessary for trans-activation by Sox18.

We have reported previously that Sox18 is mutated in all four alleles of ragged (Ra, Rap, Ragl, and RaOP). ragged is a mouse mutant that displays hair follicle, vascular, and lymphatic abnormalities (10, 15). All four mutants consist of a missense mutation that occurs in the activation domain (aa 252–345) for Ra (aa 313), Rap (aa 312), and Ragl (aa 323), whereas the RaOP (aa 349) mutation is 4 bp C-terminal of this.

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2 B. M. Hosking, personal observation.
domain. After the frameshift mutation, all alleles encode nonsense protein until it truncates at aa 435 (15). In an effort to gain a better understanding of the molecular events occurring at the VCAM-1 promoter, we investigated the effect of the ragged constructs in co-transfection with the VCAM-1 promoter.

**Fig. 6.** Site-directed mutation analysis identifies the Sox binding site B (-715 bp) as important in Sox18-mediated trans-activation of the VCAM-1 promoter in all cell lines tested. A, schematic representation of the mutated constructs. The bold boxes with crosses represent the mutated (m) putative Sox binding sites. B, pGL2Basic and the site-directed mutations (m) of the VCAM-1 promoter cloned into pGL2Basic were co-transfected into COS-1 fibroblasts with pSG5 (empty vector) or pSG5-Sox18 (Sox18). Relative luciferase (Luc.) activity is compared with the luciferase activity obtained after transfection of pGL2Basic with the empty vector pSG5 and arbitrarily set to 1. C, the data from B is re-presented as the fold activation of the VCAM-1 promoter constructs by Sox18. Fold activation is expressed relative to the activation of the empty luciferase vector pGL2Basic co-transfected with the expression vector pSG5-Sox18, which was arbitrarily set to 1. The mean luciferase fold activation values and S.D. values were derived from 2–3 independent quadruplicates. D, schematic describing the native (wt) and mutated (m) SoxA and B motifs. E and F, EMSA competition assays with 20–80-fold molar excess, demonstrating that the mutated oligos are unable to efficiently compete for SOX18 binding. wt, wild type.
Transfection analysis in fibroblasts using the full-length VCAM-1 promoter demonstrate the inability of the four alleles of *ragged*, *Ra*, *RaJ*, *Ragl*, and *RaOP*, to trans-activate the VCAM-1 promoter as compared with wild type Sox18 (Fig. 7A, lanes 12–16). Furthermore, co-transfection of the VCAM reporter construct with equal amounts of wild type Sox18 and any of the four *ragged* mutants leads to complete repression of VCAM-1 trans-activation, suggesting that these mutants act in a dominant-negative fashion. This result is also observed in endothelial and skeletal muscle cells (data not shown).

**VCAM-1 Expression in Lung, Skeletal Muscle, and Skin Is Significantly Reduced in the Ragged Mouse**—The phenotype of *ragged* mice, abnormal lymphatic system and hair follicles, is similar to that of humans with the disorder referred to as hypotrichosis-lymphedema-telangiectasia (20). Our *in vitro* and transfection analysis established Sox18 as crucial for the trans-activation of VCAM-1 expression in cells and show that *ragged* proteins target and prevent VCAM-1 activation by Sox18.

Of the four alleles of *ragged*, *RaOP* is the most deleterious to the survival of the offspring. The *RaOP* heterozygotes resemble the homozygotes of the other three allelic forms and generally display a hairless and edematous phenotype (52, 53). Therefore, we utilized the *RaOP* mouse model to investigate the *in vivo* effect of this mutant form of Sox18 on the expression levels of VCAM-1. By using real time RT-PCR with RNA from lung, skin, and skeletal muscle, organs which exhibit high expression of Sox18 and VCAM-1 (10, 26–30, 48), we demonstrated aberrant VCAM-1 expression in this mutant. Fig. 8 vividly demonstrates the effect that the *RaOP* mutation exerts on the expression of VCAM-1 in these tissues. In the wild type lung, VCAM-1 is abundantly expressed, in contrast to the mutant animal, which expresses 5-fold less. Moreover, the *RaOP* mutation extensively abrogates expression of VCAM-1 in skeletal muscle and skin, with levels of repression up to almost 40-fold (the data and normalization are described in Table I). Moreover, the specificity of aberrant VCAM-1 mRNA expression in the *ragged* mouse is highlighted by our observation that ICAM-1 and −2, JCAM, NCAM-1, and PECAM-1 mRNA expressions are unaffected in *Ra* animals (data not shown). Thus, the *in vivo* evidence is in strong concordance with the *in vitro* and cell culture analyses. In conclusion, we provide strong evidence that the VCAM-1 gene is a primary target of SOX18 and that its expression is significantly compromised by all *ragged* Sox18 mutant proteins.

**Discussion**

We present evidence that Sox18 regulates the expression of the important VCAM-1 that parallels the pattern of expression of Sox18. VCAM-1 is involved in the inflammatory response, and its aberrant regulation and/ or expression has been implicated in edema and atherogenesis.

This validated our Sox18 target gene rationale based on overlapping spatio-temporal expression profiles. For example, the Sox18 gene is expressed in the vasculature, hair follicle, allantois, and the yolk-sac blood islands during embryogenesis (10) with wide ranging expression in the adult, notably in the lung and hind limb skeletal muscle (45). This rationale was further developed by an examination of the *ragged* mouse abnormalities. The four allelic variants of the *Ra* phenotype are characterized by hypotrichosis (54), chylous ascites, and edema from mesenteric lymphatic dysfunction (18, 19), consistent with aberrant VCAM-1 expression. Moreover, VCAM-1 is an important counter receptor or ligand for α4 integrins (31) and is expressed in the developing allantois, the mesoderm of the yolk sac, and the hematopoiesis sites in the yolk sac (32, 55), similar to Sox18. Furthermore, VCAM-1 is expressed in multiple organs (56) and has an important role in skeletal muscle (28, 29) and blood vascular and lymphatic endothelia (46, 57–59).

We observed that the murine VCAM-1 promoter demonstrated cell-specific transcription in a manner similar to that reported previously (43, 44). We identified three potential Sox binding sites in the VCAM-1 promoter and utilized EMSA assays to show that Sox18 bound efficiently and in a sequence-specific manner to all three potential Sox binding sites. However, a series of 5′ unidirectional deletions and mutagenesis demonstrated that the SoxB motif is necessary and critical for SOX18-dependent trans-activation and basal expression of the VCAM-1 promoter.

The effect of mutation at the SoxB site at nucleotide position −715 bp on basal activity in the cell types tested was an unexpected aspect of this investigation, as it has been previously reported that expression of the human VCAM-1 promoter is dependent on the IRF-2 element in muscle (44). This element is positioned between the TATA box at −32 bp and the translation start site in the human promoter (42). Furthermore, the IRF-2 site is conserved in the murine promoter in the same region. Our study suggests that, in the context of the first 1889 bp of murine VCAM-1 promoter, the SoxB binding site at −715 bp is necessary and sufficient for the basal and Sox18-activated expression of VCAM-1 in fibroblasts, endothelial, and skeletal muscle cells.

In the context of understanding the *Ra* phenotype, we observed all *ragged* allelic forms of mutant SOX18 (*Ra*, *RaJ*, *Ragl*, and *RaOP*) failed to activate the VCAM-1 promoter. Furthermore, the *ragged* forms of SOX18 have the ability to ablate the *trans-activation* of VCAM-1 expression in the presence of native SOX18. These results suggest that *ragged* SOX18 proteins act in a dominant negative fashion, i.e. they are able to interfere with the role of the wild type protein. A similar situation has been reported for the Sox10 mutation in the human disease called the Waardenburg syndrome (60, 61), which results in the expression of a protein that is prematurely truncated shortly after the HMG box. This mutant has been shown to act in a dominant negative fashion by virtue of its
ability of other SOX proteins to modulate the activity of VCAM-1, and this hypothesis is currently under investigation.

In choosing to study VCAM-1, we were interested in elucidating aspects of the molecular mechanisms mediating edema in ragged mice and in humans with HLT syndrome. It has been reported that an overexpression of VCAM-1 leads to inflammation and an edema that is characterized by an immune infiltrate (63–65). The ragged animal has been characterized with an edematous phenotype, and, yet, we have demonstrated here a marked reduction in the level of VCAM-1 expression. This suggests that the edema present in the ragged animal may not be linked to the expression of VCAM-1. If this were indeed the case, then it could be expected that the fluid retained in the interstitial spaces of the ragged animal would not consist of immune infiltrate. In agreement with this hypothesis, Wallace (19) has reported that the composition of the chyle present in some of the ragged homozygous neonates contained very few monocytes and macrophages. This conclusion is also supported by the fact that no edema was reported in mice targeted for VCAM-1 (35). These results suggest that misregulation of VCAM-1 is not the source of the edema and that, potentially, the lack of immune cells in the chyle of the ragged animals may be due to the repression of VCAM-1 on the endothelia of the lymphatics. However, we cannot rule out that edema is the result of aberrant VCAM-1 expression, including overexpression or repression/ablation.

In conclusion, we show that SOX18 is essential for the expression of the cell adhesion molecule VCAM-1 (and not other adhesion molecules) in cell culture and animal models. The uncontrolled overexpression of VCAM-1 is associated with many chronic immune diseases such as atherosclerosis, arthritis, and asthma (66–68). In fact, much literature now reports the use of drugs that modulate VCAM-1 expression to successfully reduce immune infiltrate and the consequent edema in many diseases (69–71). We have shown that the −715 bp SoxB binding site regulates the VCAM-1 promoter activity and suggest that this site and Sox18 are therapeutic targets for the modulation of VCAM-1 expression and the control of inflammation.

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