SOX-18 controls endothelial-specific claudin-5 gene expression and barrier function

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In multicellular organisms, epithelial and endothelial cell layers exert specific and selective control over the passage of water and solutes, thus allowing formation and maintenance of compartments that differ in fluid and solute composition. The paracellular aspect of this control is mainly attributed to tight junction strands, which require members of the claudin family of four-pass transmembrane proteins for their formation (9). Both in vivo and in vitro studies have provided evidence that claudins are major determinants in the selectivity of epithelial and endothelial tight junctions (11, 38, 41, 42). Several claudins were found to exhibit specific expression patterns in vivo (14, 30, 31, 38), whereas expression of other claudins is more ubiquitous (Ref. 30, review in Ref. 39). In addition, junction strands generally contain multiple claudin species that may interact in homo- and heterophilic ways, both within and between junction strands (10). Thus the view emerges that claudin expression patterns could account for the diverse functional characteristics of the resulting barriers. This paradigm implies an important role for mechanisms that establish and maintain correct spatial distribution and local concentration of claudins. Indeed, several reports provide evidence that functional mutations in claudin-encoding genes or knockout of individual claudin genes result in specific pathologies as a consequence of localized barrier dysfunction (12, 14, 34, 38). The contribution of transcriptional control to the regulation of claudin gene expression under normal and pathological conditions has been the subject of a, as yet, limited number of reports (17, 26, 29, 33, 36).

Claudin-5 was reported to be an endothelial-specific member of the claudin family (30, 31). Expression levels were found to vary among specific parts of the vasculature, being particularly high in lung and brain. Accordingly, the relative contribution of claudin-5 to the overall barrier of endothelium seems to vary (7, 34). Our laboratory reported that, in cultured endothelial cells, expression of claudin-5 occurs only after cells have reached confluence, which seemed relevant in relation to control of junction formation (7).

Here, we present a comparative analysis of transcriptomes from sparse and postconfluent endothelial cell cultures that was performed to search for genes relevant in endothelial cell barrier formation. Sex-determining region Y-box (SOX)-18, an endothelial-specific transcription factor, shows strong induction in postconfluent cells. In silico analysis of the claudin-5 (CLDN5) promoter reveals an evolutionary conserved SOX consensus binding site. Overexpression of SOX-18 and a dominant-negative mutant thereof, as well as SOX-18 silencing, affects levels of claudin-5. Using a newly developed lentiviral promoter-reporter system, we dissect the CLDN5 promoter and show the relevance of SOX-18 expression and a SOX consensus binding site in endothelial-specific regulation and timing of expression of the CLDN5 gene. Impedance measurements show that the barrier function of SOX-18-silenced endothelial cells is impaired. The data presented here point at a nonredundant function of SOX-18 in controlling endothelial barrier formation.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described (15, 20). The culture medium was composed of medium 199 (Invitrogen, Paisley, UK), supplemented with 20% (vol/vol) fetal bovine serum, 50 μg/ml heparin (Sigma, St. Louis, MO), 12.5 μg/ml endothelial cell growth supplement (Sigma), and 100 U/ml penicillin/streptomycin (Invitrogen). All culture surfaces were fibronectin coated.

Microarray probe synthesis and hybridization procedures. Total RNA was isolated using the TRizol reagent (Invitrogen) and enriched

for poly(A)^+ RNA using the Oligotex mRNA Minikit (Qiagen, Hilden, Germany). Aminooilyl-labeled first-strand cDNA probes were synthesized from 0.5-μg poly(A)^+ RNA using SuperScript II (Invitrogen), with a molecular ratio of aminooilyl-UTP (Sigma) to UTP of 4:1. Labeled cDNA was purified using the QIAquick PCR purification kit (Qiagen), and Cy3 or Cy5 monoreactive dyes (Amersham Biosciences, Piscataway, NJ) were coupled according to the manufacturer’s instructions. Purified Cy3- and Cy5-labeled cDNAs were hybridized to the microarrays for 20 h at 40°C in microarray hybridization solution (Amersham) and 35% (vol/vol) formamide (Sigma). Microarrays were glass based containing 60-mer oligonucleotide sequences (Sigma/Compugen Library), which represents 18,650 human genes (Micro Array Department, Swammerdam Institute of Life Sciences, Amsterdam, the Netherlands). Microarray probe synthesis and hybridization procedures were performed in triplicate, using a dye-swap procedure. Images were acquired using the Agilent-II scanner (Agilent Technologies, Palo Alto, CA) and processed by ArrayVision 8.0 software (Imaging Research, St. Catharines, ON, Canada). Background-subtracted intensities were Loess Normalized (Limma Package, Bioconductor Software, http://www.bioconductor.org) and imported into the Rosetta Resolver database and analysis software (Rosetta Biosoftware, Seattle, WA). Data are available at http://www.ncbi.nlm.nih.gov/projects/geo accession number GSE9334.

Semiquantitative real-time RT-PCR. Total RNA was isolated using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Total RNA (0.5 μg) was used for reverse transcription on (dT)12-18 primer using SuperScript II (Invitrogen), according to the manufacturer’s instructions. PCR reactions were performed in 15-μl reactions on an iCycler system (Biorad Laboratories, Veenendaal, The Netherlands). β-Actin levels were used to normalize levels of the assayed mRNAs. Primer sequences are as follows: CLDN5, (forward) 5′-GCC AGC TTT TAG TCC TCT CAC CCC-3′, (reverse) 5′-GAG CCG CCG CCG CAG GAA G-3; zonula occludens 1 (ZO-1), (forward) 5′-GCC CCC GAA GAA GAA-3′; α-actin (forward) GGA AAA TCG TGT GTG ACA TTA AG, (reverse) TGT GTG GTC GTA AG GTC TTT G; ELK-3, (forward) 5′-GAC GAG CCT TAG TCT AGT GTC GGA-3′, (reverse) 5′-TGA GAG TTA GAA GAA AGG ATC G-3; and SOX-18, (forward) 5′-GAC ATG GAA CCA AAC ATA CAC G-3′, (reverse) 5′-GAC AGT GAA CCA AAC ATA CAC G-3′.  

Lentiviral SOX-18 overexpression. The entire human SOX-18 open reading frame (ORF) cDNA was obtained by RT-PCR from full-length human SOX-18 open reading frame clone 5589289 (Geneservice, Cambridge, UK) using primers (forward) 5′-ACCTGGCAAGGCTGGAATGCAAGGATC-3′ and (reverse) 5′-CAATTCTCA GCCGAGTGCAGCGG-3′, incorporating Psrl and EcoRI restriction sites (underlined). The resulting amplicon was cloned in pGEM-T Easy vector (Promega, Madison, WI) and then transferred, using Psrl-EcoRI sites, to the pRL-cPPT-CMV-X2-PRE-SIN vector (kindly provided by Dr. J. Seppen, Department of Experimental Hepatology, Academic Medical Center, Amsterdam, The Netherlands) and verified by sequencing. Similarly, a COOH-terminal truncated variant, similar to the ragged opossum (Ra^-) mutant of the mouse ortholog, was constructed by introducing an LEU266TER mutation, using 5′-GCAATTCTCATGGGACGTCAGCGG-3′ and 5′-GCAATCTCA CCGGAGTGCAGCGG-3′ as reverse primer in PCR. Lentivirus were generated in human embryonic kidney (HEK)-293 T cells, as described (32, 43), and virus-containing supernatants were titrated on HUVEC to determine the titers needed to transduce >90% of the cells. The otherwise identical vector, but without SOX-18 cDNA, was used to generate mock viruses for control transductions. Freshly isolated or first-passage HUVEC cultures were transduced in normal growth medium at ~80% confluency during 24 h after which they were used for further experimentation.

Immunochemistry. Primary endothelial cells were grown on fibronectin-coated coverslips. Cells were washed with serum-free medium at 37°C and fixed for 15 min with methanol at room temperature. Phosphate-buffered saline was used as incubation and wash buffer and was supplemented with 1% (wt/vol) bovine serum albumin during antibody incubations. Antibodies were diluted according to the manufacturer’s instructions (anti-VE-cadherin: BD Biosciences, Erembodegem, Belgium; anti-ZO-1 and anti-claudin-5: Invitrogen). Signals were detected using anti-rabbit Cy3 (Jackson Immuno Research, West Grove, PA). Coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA), and images were recorded using a Zeiss axioplan2 microscope and Image-Pro software (CyberMedia, Silver Spring, MD).

Electrophoresis and immunoblotting. Cells were harvested by scraping in a buffer containing 50 mM Tris·Cl, pH 7.6, 1% (vol/vol) Triton X-100, 60 mM octyl β-D-glucopyranoside (Sigma), 150 mM NaCl, and supplemented with mammalian protease inhibitor cocktail (Sigma). Samples were denatured by boiling in sample buffer in the presence of 1% (wt/vol) SDS. Equal amounts of protein were used for SDS-PAGE followed by transfer to nitrocellulose for immunoblot analysis. Incubation and wash buffer contained 10 mM Tris·Cl, pH 7.4, 150 mM NaCl, 0.05% (vol/vol) Tween 20, and was supplemented with 5% (wt/vol) nonfat dry milk during blocking of the filter and incubation with antibodies. Primary antibodies were diluted according to the manufacturer’s instructions. The following antibodies were used: anti-claudin-5 polyclonal antibody (Invitrogen) and anti-SOX-18 polyclonal antibody (Sigma). Signals were visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), in combination with a chemiluminescence substrate (enhanced chemiluminescence, General Electric). Quantification of signals was performed using ImageQuant software (Amersham Biosciences, UK).

RNA silencing. Silencing of the SOX-18 mRNA was achieved by delivery of annealed silencer RNA (siRNA) 5′-GGGUUCAUUUUUUU- GAAGG-3′ (Ambion, Silencer Pre-designed siRNA, no. 109098), using oligofectamine (Invitrogen), according to the manufacturer’s instructions. 5′-CAGUCGUGUAGCCUGGGUG-3′ was used as nonspecific control.

Promoter-reporter constructs. CLDN5 promoter fragments were amplified from HUVEC chromosomal DNA using standard PCR procedures. Sequences of primers used for amplification, their position relative to the CLDN5 transcription start site, and their chromosomal localization can be found in Supplementary Table S2. (The online version of this article contains supplemental data.) The resulting amplicons were cloned in pGL3-basic (Promega) using KpnI-Xhol restriction sites and sequence verified.

The lentiviral vector pRRL-cPPT-CMV-X2-PRE-SIN (kindly provided by Dr. J. Seppen, Department of Experimental Hepatology, Academic Medical Center, Amsterdam, The Netherlands) was modified for use as carrier of promoter-reporter cassettes as follows: a unique NotI restriction site was eliminated by cleaving with NotI, filling of the overhangs, and religation. Next, a Nhel-CloI fragment containing the cytomegalovirus (CMV) promoter and the multiple cloning site was replaced by a new, synthetic multiple cloning site. The resulting lentiviral vector was then used to accommodate promoter-reporter cassettes, excised from existing plasmids.

First, a cassette containing Renilla luciferase under control of the constitutive herpes simplex virus thymidine kinase promoter was cloned. Second, CLDN5 promoter-reporter cassettes were transferred from pGL3-basic (Promega) as NotI-Sall fragments containing successively a synthetic pA/transcriptional pause site, a CLDN5 promoter fragment, the firefly luciferase reporter gene, and the simian virus 40 (late) polyadenylation signal. The resulting lentiviral CLDN5 promoter-firefly luciferase reporter vector was then packaged, in the presence of a small amount (5% wt/wt) of the herpes simplex virus thymidine kinase Renilla luciferase vector, using HEK-293 T cells as described (32, 43). Virus-containing supernatants were used for transductions, and luciferase activities were measured after at least 3 days,
using the dual luciferase system (Promega). Renilla luciferase activities were used to normalize firefly luciferase activity.

Electric cell impedance sensing. Confluent HUVEC layers were monitored by electrical cell impedance sensing (ECIS) (13). HUVEC were cultured on gold electrodes (type 8W10E+, Applied Biophysics, Troy, NY) coated with fibronectin. One day after seeding, when the layers were near confluence, siRNA transfections were performed, and impedance measurements were initiated at 4 kHz.

Statistical analysis. Data are reported as means ± SD. Differences in mean values were analyzed using Student’s t-test. Differences were considered significant at the P < 0.05 level.

RESULTS

Genes induced in postconfluent cultures of HUVECs. In an attempt to select genes that, analogous to CLDN5, are specifically expressed in postconfluent endothelial monolayers and encode gene products that, based on further in silico analysis may be involved in junction formation, we compared microarray mRNA expression profiles of sparse- and postconfluent monolayers of primary human endothelial cells. Previously, we have shown that, under the applied culture conditions, claudin-5 was not detectable in sparse cells and showed induction only after cells had reached confluence. VE-cadherin, a transmembrane component of endothelial adherens junctions (22), and ZO-1, a cytoplasmic protein connecting multiple transmembrane junction proteins to the actin cytoskeleton (8, 19), both showed basal expression under sparse conditions and a moderate increase, compared with claudin-5, when cells became confluent (7). Microarray data from three different isolates of HUVEC were analyzed by unpaired Cyber-T statistical analysis and corrected for false discovery rate by the Benjamini-Hochberg method (5). Comparison between sparse cultures and postconfluent cultures revealed a large set (N = 511) of reproducibly differential genes at statistical significance P < 0.05. In addition to established endothelial cell-specific markers as endoglin, endothelin 1, von Willebrand factor, and plasminogen inactivator 1 (SERPIN1), this set contains several genes encoding proteins involved in junction formation, platelet endothelial cell adhesion molecule, tight junction protein 1/ZO-1, and VE-cadherin (CDH5), thus showing consistency with our laboratory’s earlier findings (7). The corresponding list of genes is given in Supplemental Table S1. We validated the analysis by performing a semiquantitative RT-PCR assay in which PCR reaction products were gel analyzed at intervals of five amplification cycles. In addition to the three well-described genes ZO-1, CDH5, and CLDN5, we tested 10 additional genes, with microarray P values ranging from 1.14E-2 to 1.19E-3. From these genes, 9 out of 10 showed specific cell density-dependent induction, thus independently confirming the result of our microarray analysis (Supplemental Fig. S1).

Interestingly, in our set of differentially expressed genes, signals of SOX-18 and ELK-3 show the highest confluent-to-sparse signal ratios. PCR analysis suggests an on/off type of switch, rather than gradual differences in expression levels, as observed for all other genes tested. This was further confirmed by semiquantitative real-time RT-PCR (Fig. 1). SOX-18 is a member of the SOX gene family that shows endothelial expression in nascent blood vessels and hair follicles. Mutations in SOX-18 are associated with ragged phenotypes in mice and hypotrichosis-lymphedema-telangiectasia (HLTS) in humans. In both cases, defects in vascular (lymphatic) development are accompanied by diffuse (lymph) edema (18, 35). ELK-3 is an ETS ternary complex transcription factor that, like many other members of the ETS family, is expressed in the endothelium of the developing vasculature (3, 4). An interspecies comparative analysis of the CLDN5 promoter revealed the presence of a single evolutionary conserved SOX consensus transcription factor binding site between position −183 and −167 relative to the CLDN5 transcription initiation site. Like many other endothelial-specific genes, the CLDN5 promoter contains multiple conserved ETS consensus sites, i.e., four sites between position −165 and −74 relative to the CLDN5 transcription initiation site (Supplemental Table S2). The importance of ETS transcription factors in endothelial biology is well established and comprises junction formation via regulation of CDH5 (24). Here, we tested the hypothesis that SOX-18 is involved in timing and endothelial specificity of the CLDN5 promoter.

Endothelial specificity of the CLDN5 promoter and SOX-18 expression. Endothelial-specific expression of claudin-5 has been described in tissue of mouse- and human origin (25, 30, 31, 34). We examined whether transcriptional regulation is involved in endothelial specificity and whether this regulation can be studied under in vitro conditions. We developed a lentivirus-based system that allowed highly efficient transduction of CLDN5-promoter-reporter constructs in virtually any cell type, thus making long-term stable expression from a chromosomal environment in both homologous and heterologous cell types possible. Based on in silico analysis, an evolutionary conserved hypothetical core promoter comprising 300 bp upstream of the CLDN5 transcription start site and including the conserved SOX binding site was defined (Supplemental Table S2). This fragment was cloned upstream of the firefly luciferase gene in a viral vector, and virus particles were packaged for transduction of several types of primary cells and cell lines, as described in MATERIALS AND METHODS. In HUVEC, the hypothetical CLDN5 core promoter increased luciferase activity 42-fold, relative to a promoter-less luciferase control virus. As shown in Fig. 2A, the CLDN5 core promoter induced
transcriptional activity at a considerably higher rate in HUVEC compared with primary arterial smooth muscle cells, as well as CaCo, HeLa, and HEK-293 cell lines.

Next, we examined cell specificity of SOX-18 gene expression by measuring its mRNA levels in different cell types, using real-time semiquantitative RT-PCR, and compared its actin-normalized relative mRNA expression to that of CDH5 and CLDN5, two established endothelial-specific junction components, ELK-3, of unknown cellular specificity, and ZO-1, a ubiquitously expressed component of the junctional plaque. As shown in Fig. 2B, SOX-18 is exclusively expressed in endothelial cells. ELK-3 expression is preferentially in endothelial cells, and ZO-1 shows preferred expression in endothelial/epithelial cell types. Together, these data point at cell-specific transcriptional control of CLDN5 expression under culture conditions and may suggest a role for SOX-18 in this control.

SOX-18 specifically increases claudin-5 expression at the mRNA and protein level. We examined possible effects of SOX-18 on claudin-5 expression levels by lentiviral transduction of human SOX-18 encoding cDNA into a native, human vascular cell type (7). In addition, we tested a COOH-terminally truncated mutant of SOX-18 (ΔCT), which was analogous to the dominant-negative Raop mutant of the mouse ortholog (21, 35). SOX-18 ΔCT was constructed by introducing a LEU266TER mutation, resulting in a truncated SOX-18 protein. In Raop, a frame shift at this position leads to missense coding and premature truncation. SOX-18 and SOX-18 ΔCT cDNA was cloned into a lentiviral vector under control of the CMV promoter, and infectious virus particles were produced and titrated, as described in MATERIALS AND METHODS. At the RNA level, SOX-18 expression was typically between 15- and 25-fold increased (data not shown). Immunostaining on Western blot detected SOX-18 and SOX-18 ΔCT at 46 kDa and between 34 and 40 kDa, respectively (Fig. 3A). Comparison to predicted sizes of 41 kDa (Swiss prot. no. p35713) and 30 kDa suggests posttranslational modification of, as yet, unknown nature. Using immunofluorescence microscopy, we examined expression and localization of wild-type SOX-18 and SOX-18 ΔCT, as well as their possible effects on claudin-5, VE-cadherin, and ZO-1 expression in HUVEC. Figure 3B shows that both overexpressed wild type and COOH-terminally truncated SOX-18 localize to the nucleus, as does endogenous SOX-18. SOX-18 ΔCT signals are slightly higher and show less variation in signal intensity compared with overexpressed wild-type SOX-18. In both cases, signals are considerably higher than that observed for endogenous SOX-18, and nuclear localization seems more pronounced. Relative to vector and SOX-18 ΔCT transduced cells, signals of claudin-5 are increased in SOX-18 transduced cells. As expected, claudin-5 fluorescence concentrates at the areas of cell-cell contact. Neither VE-cadherin nor ZO-1 expression seems affected by SOX-18 or SOX-18 ΔCT. Next, we examined effects of SOX-18 overexpression on mRNA levels of CLDN5, ZO-1, and CDH5 by real-time semiquantitative RT-PCR in both postconfluent cells and sparse cells, i.e., in the absence and presence of endogenous SOX-18, respectively. As shown in Fig. 3C, in postconfluent cells, CLDN5 mRNA expression is significantly increased compared with mock-transduced cells, whereas ZO-1 and CDH5 mRNA levels remain unaltered, thus confirming, at the RNA level, the observations made by immunofluorescence microscopy. Under sparse conditions, SOX-18 overexpression does not have an effect on any of the mRNA levels measured. The effects of SOX-18 ΔCT on CLDN5 mRNA expression were limited; a small decrease (n = 6, mean value relative to control 0.75, P value 0.03) was observed, likely due to a dominant-negative effect on endogenous transcriptional activity from SOX F family members. Finally, we quantified claudin-5 protein levels of SOX-18, SOX-18 ΔCT, and mock transduced cells on Western blot (Fig. 4). We could detect significantly increased claudin-5 levels in SOX-18 overexpressing cells, whereas claudin-5 levels were significantly decreased in SOX-18 ΔCT cells, thus substantiating our results obtained by immunofluorescence and semiquantitative PCR. Together, the finding that SOX-18 overexpres-
SOX-18 regulates expression of the claudin-5 gene

Fig. 3. Lentiviral overexpression of SOX-18 and SOX-18 ΔCt in HUVEC. A: SOX-18 overexpression detected by Western blotting. Four days after lentiviral transduction of HUVEC, lysates were analyzed by immunoblotting with SOX-18 antibodies. Mock, transfected with virus containing vector without SOX-18 insert; SOX-18, transduction with virus containing the SOX-18 open reading frame (ORF) under control of the cytomegalovirus (CMV) promoter; SOX-18 ΔCt, transduction with virus encoding a (Leu266Ter) SOX-18 mutant under control of the CMV promoter. Equal loading was confirmed with anti-α tubulin antibodies. B: effect of SOX-18 and SOX-18 ΔCt overexpression on fluorescence patterns of SOX-18, claudin-5, VE-cadherin, and ZO-1. After transduction with either mock, SOX-18 ΔCt, or SOX-18 virus (rows), cells were seeded on fibronectin-coated glass coverslips and grown for 4 days. Cells were fixed and stained with antibodies against SOX-18, claudin-5, VE-cadherin, and ZO-1 (columns) and a Cy-3-conjugated secondary antibody. Recordings were made using a Zeiss axioplan2 microscope, with settings optimized for SOX-18 transduced cells. Subsequently, mock and SOX-18 ΔCt transduced cells were recorded with identical settings. SOX-18 transduced cells show increased fluorescence of claudin-5 compared with mock and SOX-18 ΔCt-transduced cells. VE-cadherin and ZO-1 fluorescence are not affected by SOX-18. Bar: 60 μm. C: effect of overexpression of SOX-18 on mRNA levels of ZO-1 (TJP1), CDH5, and CLDN5 under sparse and confluent conditions. Cells were transfected with virus containing the SOX-18 ORF under control of the CMV promoter. As control, transductions with virus containing vector without insert were performed (mock). Three days after transduction, either growth was continued (confluent), or cells were reseeded at a fivefold lower density (sparse). Cells were then grown another 24 h before harvest. mRNA levels were determined using real-time semiquantitative RT-PCR. Values were normalized for β-actin and plotted as value relative to mock. SOX-18 transduced cells show significantly increased levels of CLDN5 mRNA under confluent conditions but not under sparse conditions. Means ± SD from 3 (ZO-1 and CDH5) or 6 (CLDN5) HUVEC isolates are shown. *Significant differences with mock transductions are indicated, $P < 0.05$.

A SOX consensus binding site in the CLDN5 promoter is required for SOX-18 enhanced transcriptional activity. We tested whether the SOX-18-induced increase in claudin-5 expression was due to increased transcriptional activity from the CLDN5 promoter. To this purpose, the 300-bp hypothetical core promoter that was used to establish endothelial specificity of CLDN5 promoter activity (Fig. 2) was now tested in either the presence or absence of SOX-18 or SOX18 ΔCt overexpression. In addition, a larger fragment comprising 1,000 bp upstream of the CLDN5 transcription start was tested. These fragments were cloned in a lentiviral reporter vector and transduced into HUVEC, as described in MATERIALS AND METHODS. Twenty-four hours after this transduction, cells were transduced with SOX-18, SOX-18 ΔCt, or mock virus. Three days after the second transduction, lysates were prepared for measurement of reporter activity. As shown in Fig. 5, the effects of SOX-18 and SOX-18 ΔCt overexpression on reporter activity are essentially the same as observed earlier for claudin-5 mRNA and protein, indicating that SOX-18 enhances claudin-5 expression via transcriptional activation of the CLDN5 promoter. In addition, the viral promoter-reporter system reliably reflects the behavior of the endogenous CLDN5 promoter in response to SOX-18 or SOX-18 ΔCt.

We then further fragmented the CLDN5 promoter along lines of evolutionary conservation (Fig. 6A and Supplemental Table S2). Immediately upstream of the transcription start site, the well-conserved 300-bp hypothetical core promoter was identified, whereas overall sequence conservation shows a sharp decline further upstream, as well as downstream of the transcription start. Within the conserved 300-bp core promoter fragment (designated −300), we identified three clusters of conserved transcription factor binding sites (Fig. 6B and Supplemental Table S2). A promoter-deletion analysis was performed in which these clusters were covered by fragments −300, −230, −148, and control (empty reporter vector). Two additional short sequences that each contain two overlapping transcription factor binding sites are located in the −490 and −362 fragments. Reporter constructs were transduced into HUVEC, which was followed after 24 h by transduction with either SOX-18 or mock virus. Reporter activity was measured after 3 days. As can be inferred from Fig. 6B, both basal (endogenous) and SOX-18-enhanced transcriptional activity...
In particular, members of the SOX-F subfamily (SOX-18, -17, -16, and -7) that are thought to display functional redundancy in endothelial cells (27, 37) are candidates. Inspection of our microarray data revealed that, in addition to the SOX-18 gene, SOX-17, -2, -3, and -6 genes are expressed. In contrast to SOX-18, expression of none of the other SOX genes mentioned showed significant dependence on cell density.

We specifically targeted SOX-18 expression by transfecting double-stranded SOX-18-siRNAs into HUVEC and monitored their effect by measuring mRNA levels for SOX-18 and its potential target, CLDN5, by real-time semiquantitative RT-PCR. As a negative control, we examined expression of ZO-1, which we had found not to be affected by overexpression of SOX-18. Compared with control siRNA, we observed that SOX-18-siRNA induced efficient silencing of SOX-18, accompanied by a profound decrease of CLDN5 mRNA levels, whereas ZO-1 mRNA levels remained unchanged (Fig. 7A). These data support the view that SOX-18 is the specific member of the SOX family involved in transcriptional control of CLDN5. Western blotting of cell lysates from non-sense and SOX-18 siRNA-treated cells (Fig. 7B) further substantiated these results. Strikingly, the decrease of CLDN5 at the protein level is more pronounced than at the mRNA level. Our CLDN5 real-time RT-PCR has been proven to accurately measure CLDN5 mRNA expression in a wide dynamic range (Fig. 1 and Ref. 7). We speculate that stability of claudin-5 protein is decreased upon SOX-18 silencing. A decrease of claudin-5 concentration in the cell membrane may result in a lower degree of claudin strand formation and, hence, in lower stability. Thus specific silencing of SOX-18 expression reveals a nonredundant and obligatory role for SOX-18 in the control of CLDN5 expression.

are maintained in the consecutive promoter fragments −490, −362, −300, and −230. However, a significant decrease in both basal and SOX-18-enhanced reporter activity is observed upon deletion of the −230 to −149 fragment, which contains, among others, the conserved SOX consensus binding site. The importance of this site was confirmed by testing the effect of local point mutations. In the 300-bp reporter construct, we converted the SOX core sequence CAAT to either TGAT or TGTG and measured the effect on reporter activity after transduction of either SOX-18 or mock virus. Figure 6C shows that, both under conditions of SOX-18 overexpression and under control (mock) conditions, point mutations in the core SOX binding site cause a significant decrease in reporter activity. These findings imply that the SOX site is involved not only in the enhanced transcriptional activity from the CLDN5 promoter upon overexpression of SOX-18, but also in basal, endogenously directed transcription of the CLDN5 gene.

RNA silencing of SOX-18 expression specifically reduces basal, endogenous transcription from the CLDN5 promoter. Our findings from the previous section are consistent with the hypothesized role of SOX-18 in regulation of the CLDN5 promoter. However, given the fact that members of the SOX family tend to bind to a common consensus site (23, 28), the question as to which particular SOX protein is involved in basal CLDN5 expression still has to be answered conclusively. In particular, members of the SOX-F subfamily (SOX-18, -17, -16, and -7) are thought to display functional redundancy in endothelial cells (27, 37) are candidates. Inspection of our microarray data revealed that, in addition to the SOX-18 gene, SOX-17, -2, -3, and -6 genes are expressed. In contrast to SOX-18, expression of none of the other SOX genes mentioned showed significant dependence on cell density.

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Fig. 4. Effect of lentiviral overexpression of SOX-18 and SOX-18 dCt on claudin-5 expression in HUVEC. A: claudin-5 protein detected by Western blotting. Four days after lentiviral transduction of HUVEC, lysates were analyzed by immunoblotting with claudin-5 antibodies. SOX-18, transduction with virus containing the SOX-18 ORF under control of the CMV promoter; SOX-18 dCt, transduction with virus encoding a (Leu266Ter) SOX-18 mutant under control of the CMV promoter; mock, transduced with virus containing vector without insert. Equal loading was confirmed with anti-α-tubulin antibodies. B: quantitative representation of the experiment shown under A. SOX-18 values are normalized on α-tubulin. SOX-18 induces an increase in claudin-5 protein levels, whereas SOX-18 dCt has the opposite effect. Means ± SD from 6 HUVEC isolates are shown. Significant differences with mock transductions are indicated: *P < 0.05, **P < 0.01.

Fig. 5. Overexpression of SOX-18 enhances transcription directed by the CLDN5 promoter. 1,000-bp and 300-bp fragments upstream of the CLDN5 transcription start site were cloned upstream of a firefly luciferase reporter gene on a lentiviral vector, which was then packaged into lentiviral particles. During packaging, a small amount of a similar vector carrying the constitutive HSV-TK promoter in front of the Renilla luciferase reporter gene was spiked for normalization. Resulting virus was used to transduce HUVEC. In a subsequent second round of transduction, SOX-18 or SOX-18 dCt under control of the CMV promoter were introduced. As a control, virus containing the viral expression vector without insert was transduced (mock). After 3 days, cells were lysed, and luciferase activities were measured. Normalized promoter activity was plotted relative to mock transduced cells. SOX-18 enhances transcriptional activity of the CLDN5 promoter. Means ± SD from 4 HUVEC isolates are shown. *Significant differences with mock transductions are indicated: *P < 0.05.
RNA silencing of SOX-18 expression results in impairment of endothelial barrier function as measured by ECIS. To investigate whether SOX-18 regulates integrity of HUVEC barriers, transendothelial electrical resistance of confluent endothelial monolayers was measured in time using ECIS (13). Two days after transfection with SOX-18 siRNA, cell shape became elongated, but monolayers remained intact, as observed by phase-contrast microscopy (Fig. 7C). Sixty hours after transfection, resistance was significantly reduced in SOX-18 siRNA cells compared with control-treated cells and had dropped to levels observed in nonmatured monolayers (Fig. 7D). The complete ECIS time course graph is available as Supplemental Fig. S2.

**DISCUSSION**

Previously, our laboratory has reported the transcriptional upregulation of several genes involved in endothelial barrier...
endothelial barriers was suggested by reports on SOX-18 mutations in vascular anomalies in ragged (Ra) mice (21, 35) and in the human HLTS syndrome (18). The associated pathologies are characterized by coat defects, chylous ascites, edema, and cardiovascular dysfunction. All four described mouse mutations, and one out of three mutations described for HLTS have in common that they encode truncated versions of the SOX-18 protein with impaired trans-activation activity. These truncated SOX-18 proteins are thought to interfere, in a dominant-negative fashion, with other members of the SOX F subfamily (21, 27, 35, 37).

Here, we observed that, of several cell types tested, only HUVEC expressed SOX-18. Overexpression of SOX-18 and a dominant-negative derivative thereof, SOX-18 ΔC7, induce an increase and decrease of claudin-5 expression, respectively. We observed that the human CLDN5 promoter displayed cell-specific activity and identified a potential SOX binding site. Controlled deletion analysis of the CLDN5 promoter, as well as mutational analysis of the potential SOX binding site, showed that the latter is required for both basal and SOX-18 overexpression driven CLDN5 expression. Finally, silencing of SOX-18 showed that endogenous SOX-18 is essential for basal CLDN5 transcription. These findings identify the CLDN5 gene as an important SOX-18 target.

SOX-18 is a member of the SOX gene family that shares a conserved high-mobility group domain. Individual members are responsible for regulation of cell fate and differentiation processes (review in Ref. 23) and show characteristic spatiotemporal expression patterns during development and adulthood. SOX-18 was found in endothelial cells of pre- and postnatal developing vasculature in mice (16, 27, 35). These observations are consistent with our view that SOX-18 regulation of the CLDN5 promoter contributes to endothelial cell specificity of CLDN5 expression.

Here, one may speculate that CLDN5, being a target of SOX-18, may be involved in pathologies associated with HLTS in humans and ragged mutations in mice, as these result from dominant-negative SOX-18 mutants. The phenotype of CLDN5−/− mice, however, shows no overlap with the ragged phenotype: CLDN5−/− mice have a normally developed vasculature, do not show edema or vascular leakage, but only display partial failure of the blood-brain barrier (34). Indeed, the importance of claudin-5 in non-blood-brain barrier endothelium seems limited (7, 34). Thus, despite the fact that we identify CLDN5 as SOX-18 target gene, it seems unlikely that CLDN5 is a major effector gene in pathologies associated with HLTS or ragged phenotypes. However, ECIS measurements applied to SOX-18-silenced HUVEC monolayers show impaired barrier function, which is in marked contrast to the lack of a barrier-related phenotype in SOX-18−/− mice (35). Apparently, masking of effects resulting from the loss of SOX-18 function by redundant activity of other members of the SOX F subfamily, as observed in mouse knockout models (27, 35, 37), does not occur under our culture conditions. Thus we show that loss of SOX-18 function results in a severely compromised endothelial barrier function. A future inventory of SOX-18-downstream genes might identify potential effector genes.

At the subcellular level, Claudins are functionally associated with tight junction complexes via interaction with ZO proteins (19). In epithelial cells, this interaction plays a role in spatiotemporal control of tight junction formation (40). Initially,
ZO-1 and ZO-2 accumulate on primordial adherence junctions between adjacent cells. In a later stage, claudins are recruited to these structures where their polymerization into functional strands is facilitated (1, 2, 40). Therefore, it may be speculated that claudin genes are subject to regulatory mechanisms that exert temporal control during junction formation. Consistent with earlier findings (7), our data reveal tight control of SOX-18 over CLDN5 transcription, which is dependent on cell density and thus coincides with junction formation. Under sparse conditions, when endogenously expressed SOX-18 is absent, we observed no significant effect of SOX-18 overexpression on CLDN5 mRNA levels, suggesting that additional, obligatory transcription factors are lacking. Alternatively, the CLDN5 promoter may be actively repressed under sparse conditions. Under confluent conditions, the response of the CLDN5 gene to SOX-18 overexpression is limited, likely due to the background of endogenous SOX-18. Silencing of endogenous SOX-18 in postconfluent cells revealed an essential and nonredundant role for SOX-18 in CLDN5 gene expression. This observation, together with the observed induction of SOX-18 in postconfluent cells, reveals a potent mechanism for temporal control of CLDN5 during junction formation. These findings are consistent with the paradigm that SOX family members are key regulators in spatiotemporal control of gene expression (23).

Recent studies have shown that the regulation of claudin gene expression seems to have diverged greatly, in marked contrast to the highly conserved sequence and structural homology of the claudin proteins, thus allowing a specific regulation of claudin genes. In the present study, we now identify SOX-18 as an essential regulator of endothelial claudin-5 expression, as well as barrier function, supporting the view that individual claudin genes are controlled by unique transcriptional regulatory mechanisms to achieve correct tissue-specific and temporal expression.

GRANTS

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