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

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ABSTRACT

Members of the claudin family constitute tight junction strands and are major determinants in specificity and selectivity of paracellular barriers. Transcriptional control of claudin gene expression is essential to establish individual claudin expression patterns and barrier properties. Using full genome expression profiling we now identify SRY (sex determining region Y)-box 18 (SOX18), a member of the SOX family of high mobility group (HMG) box transcription factors, as one of the most differentially induced genes during establishment of the endothelial barrier. We show that overexpression of SOX-18 and a dominant negative mutant thereof, as well as SOX18 silencing, greatly affects levels of claudin-5 (CLDN5). The relevance of an evolutionary conserved SOX-binding site in the CLDN5 promoter is shown using sequential promoter deletions as well as point mutations. Furthermore, SOX18 silencing abrogates endothelial barrier function as measured by Electric Cell-Substrate Impedance Sensing (ECIS). Thus, an obligatory role for SOX-18 in the regulation of CLDN5 gene expression in an endothelial-specific and cell-density dependent manner is established, as well as a crucial, non-redundant role for specifically SOX-18 in formation of the endothelial barrier.

INTRODUCTION

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 4-pass transmembrane proteins for their formation (Furuse et al., 1998). Both *in vivo* and *in vitro* studies have provided evidence that claudins are major determinants in selectivity of epithelial and endothelial tight junctions (Furuse et al., 2001; Simon et al., 1999; van Itallie et al., 2001; Yu et al., 2003). Several claudins were found to exhibit specific expression patterns *in vivo* (Gow et al., 1999; Morita et al., 1999a; Morita et al., 1999b; Simon et al., 1999) whereas expression of other claudins is more ubiquitous (Morita et al., 1999; review in Turksen and Troy, 2000). In addition, junction strands generally contain multiple claudin species that may interact in homo- and heterophilic ways both within and between junction strands (Furuse et al., 1999). 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 knock-out of individual claudin genes result in specific pathologies as a consequence of localized barrier dysfunction (Furuse et al., 2002; Gow et al., 1999; Nitta et al., 2003; Simon et al., 1999). The contribution of transcriptional control to regulation of claudin gene expression under normal and pathological conditions has been subject of an, as yet, limited number of reports (Ikenouchi et al., 2003; Lui et al., 2007; Miwa et al., 2000; Niimi et al., 2001; Sakaguchi et al., 2002).

Claudin-5 was reported to be an endothelial-specific member of the claudin-family (Morita et al., 1999a; Morita et al., 1999b). 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 (Fontijn et al., 2006; Nitta et al., 2003). We reported that in cultured endothelial cells, expression of claudin-5 occurs only after cells have reached confluency, which seemed relevant in relation to control of junction formation (Fontijn et al., 2006).

Here, we present a comparative analysis of transcriptomes from sparse and post-confluent endothelial cell cultures that was performed to search for genes relevant in endothelial cell barrier formation. SOX18, an endothelial-specific transcription factor shows strong induction in post-confluent cells. *In silico* analysis of the CLDN5 promoter reveals an evolutionary

conserved SOX consensus binding site. Overexpression of SOX-18 and a dominant negative mutant thereof as well as SOX18 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 an SOX consensus binding site in endothelial-specific regulation and timing of expression of the CLDN5 gene. Impedance measurements show that the barrier function of SOX18-silenced endothelial cells is impaired. The data presented here, point at a non-redundant 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 (Horrevoets et al., 1999; Jaffe et al., 1973). The culture medium was composed of medium 199 (Invitrogen, Paisley, Scotland), supplemented with 20% (v/v) 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, Paisley, Scotland) and enriched for polyA$^+$ RNA using the Oligotex mRNA Minikit (Qiagen, Hilden, Germany). Aminoallyl (AA)-labeled first strand cDNA probes were synthesized from 0.5 µg polyA$^+$ RNA using SuperSript II (Invitrogen) wih a molecular ratio of aminoallyl-dUTP (Sigma,St Louis, MO) to dUTp of 4:1. Labeled cDNA was purified using the QIAquick PCR purification kit (Qiagen), and Cy3 or Cy5 mono-reactive 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 hours at 40ºC in Microarray Hybridization Solution (Amersham) and 35% (v/v) 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-substracted 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 GSE 9334.

Semi-quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was isolated using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. 0.5 µg of total RNA was used for reversed transcription on (dT)12-18 primer using Superscript II (Invitrogen) according to the maufacturer's instructions. PCR reactions were performed in 15µl reactions on an iCycler system (Biorad Laboratories, Veenendaal, The Netherlands). β-actin levels were used to normalize levels of the assayed mRNAs. Primer sequences: CLDN5: (forward) 5'-CCC AGA GGC TCC TGC TGA C-3', (reverse) 5'-GCA GAT TCT TAG CCT TCC CAC TCC-3'. VE-cadherin (CDH5): (forward) 5'-CGT GAG CAT CCA GGC AGT GGT AGC-3', (reverse) 5'-GAG CCG CCG CCG CAG GAA G-3'. Zonula ocludens 1 (ZO-1): (forward) 5'-CCC GAA GGA GTT GAG CAG GAA ATC-3', (reverse) 5'-CCA CAG GCT TCA GGA ACT TGA GG-3'. β-actin (forward) GGG AAA TCG TGC GTG ACA TTA AG, (reverse) TGT GTT GGC GTA AG GTC TTT G. ELK3: (forward) 5'-GAG CAG CCT TAG TCC AGT TG-3', (reverse) 5'-TGA GAG TTT GAA GAA AGC AGT

AC-3'. SOX18: (forward) 5'-TTC GAC CAG TAC CTC AAC TGC-3', (reverse) 5'-GAC ATG GAA CCA AAC ATA CAC G-3'.

Lentiviral SOX18 overexpression. The entire human SOX18 open reading frame (ORF) cDNA was obtained by RT-PCR from full-length image clone 5589289 (Geneservice Ltd., Cambridge, UK) using primers (forward) 5'-AACTGCAGCCCAGCTGGAATGCAGAGATC-3' and (reverse) 5'-CGAATTCCTAGCCGGAGATGCACGCGC-3', incorporating PstI and EcoRI restriction sites (underlined). The resulting amplicon was subcloned in pGEM-T Easy vector (Promega, Madison, WI), and then transferred, using PstI- EcoRI sites, to the pRRL-cPPT-CMV-X2-PRE-SIN-IRES-eGFP vector (kindly provided by Dr. J. Seppen, department of Experimental Hepatology, Academic Medical Center, Amsterdam, The Netherlands) and verified by sequencing. Likewise, an C-terminal truncated variant, similar to the *ragged opossum* (Ra^{op}) mutant of the mouse orthologue was constructed by introducing an LEU266TER mutation, using 5'-CGAATTCCTAGGGAGCCCCGTAGCAACCGCC-3' as reverse primer in PCR. Lentiviruses were generated in HEK293T cells as described (Naldini et al., 1996; Zufferey et al., 1998) 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 approximately 80% confluency during 24 h after which they were used for further experimentation.

Immunofluorescence. 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 (PBS) was used as incubation- and wash buffer and was supplemented with 1% (w/v) bovine serum albumin (BSA) 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: Invirogen, Paisley, Scotland. 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% (v/v) 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% (w/v) SDS. Equal amounts of protein were used for SDS-PAGE followed by transfer to nitrocellulose for immuno-blot analysis. Incubation- and wash buffer contained 10 mM Tris-Cl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-20 (TBS-T) and was supplemented with 5% (w/v) non-fat 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-Sox18 polyclonal

antibody (Sigma). Signals were visualized using horseradish peroxidase -conjugated secondary antibodies (Bio-Rad, Hercules, CA) in combination with a chemiluminiscent substrate (ECL, General Electric, U.K). Quantification of signals was performed using ImagQuant software (Amersham Biosciences, U.K.).

RNA silencing. Silencing of the SOX18 mRNA was achieved by delivery of annealed silencer RNA (siRNA) 5'-GGGUUACAUUUUUGAAGCA-3' (Ambion, Silencer Pre-designed siRNA # 109098), using oligofectamine (Invitrogen) according to the manufacturer's instructions. 5'-CAGUCGCGUUUGCGACUGG-3' was used as non-specific 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 2. The resulting amplicons were cloned in pGL3-basic (Promega) using KpnI-XhoI 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 re-ligation. Next, a NheI-ClaI fragment containing the cytomegalovirus (CMV) promoter and the multiple cloning site were 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 (HSV-TK) promoter was cloned. Second, CLDN5 promoter-reporter cassettes were transferred from pGL3-basic (Promega) as NotI-Sal fragments containing succesively a synthetic pA/transcriptional pause site, a CLDN5 promoter fragment, the Firefly luciferase reporter gene and the simian virus 40 (SV40) (late) pA signal. The resulting lentiviral CLDN5 promoter-Firefly luciferase reporter vector was then packaged, in the presence of a small amount (5% w/w) of the HSV-TK Renilla luciferase vector, using HEK293T cells as described (Naldini et al., 1996; Zufferey et al., 1998). Virus-containing supernatants were used for transductions and luciferase activities were measured after at least three 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) (Giaever and Keese, 1991). HUVEC were cultured on gold electrodes (type 8W10E+, Applied Biophysics, Troy, NY, USA) coated with fibronectin. One day after seeding, when the layers were near confluence, siRNA transfections were performed and impedence measurements were initiated at 4 kHz.

Statistical analysis. Data are reported as mean ± standard deviation (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 post-confluent cultures of human umbilical vein endothelial cells (HUVEC). In an attempt to select genes that, analogous to CLDN5, are specifically expressed in post-confluent 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 post-confluent 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 confluency. VE-cadherin, a transmembrane component of endothelial adherens junctions (Lampugnani et al., 1992) and ZO-1, a cytoplasmic protein connecting multiple transmembrane junction proteins to the actin cytoskeleton (Furuse et al., 1994; Itoh et al., 1999) both showed basal expression under sparse conditions and a moderate increase, as compared to claudin-5, when cells became confluent (Fontijn et al., 2006). 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 (Baldi and Long, 2001). Comparison between sparse cultures and post-confluent 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 (ENG), endothelin 1 (EDN1), von Willebrand factor (VWF) and plasminogen inactivator 1 (SERPIN1), this set contains several genes encoding proteins involved in junction formation; platelet/endothelial cell adhesion molecule (PECAM1), tight junction protein 1/ zona occludens 1 (TJP1/ ZO-1) and VE-cadherin (CDH5), thus showing consistency with our earlier findings (Fontijn et al., 2006). The corresponding list of genes is given in Supplementary Table 1. We validated the analysis by performing a semi-quantitative 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 (Supplementary Figure1).

Interestingly, in our set of differentially expressed genes, signals of SOX18 and ELK3 show the highest confluent/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 semi-quantitative real-time RT-PCR (Figure 1). SOX18 is a member of the SOX gene family that shows endothelial expression in nascent blood vessels and hair follicles. Mutations in SOX18 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 (Irrthum et al., 2003; Pennisi et al., 2000). ELK3 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 (Ayadi et al., 2001a; Ayadi et al., 2001b). An inter-species 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. 4 sites between position -165 and -74 relative to the CLDN5 transcription initiation site (Supplementary Table 2). The importance of ETS transcription factors in endothelial biology is well-established and comprises junction formation via regulation of CDH5 (Lelièvre et al., 2000). Here, we tested the hypothesis that SOX-18 is involved in timing and endothelial specificity of the CLDN5 promoter.

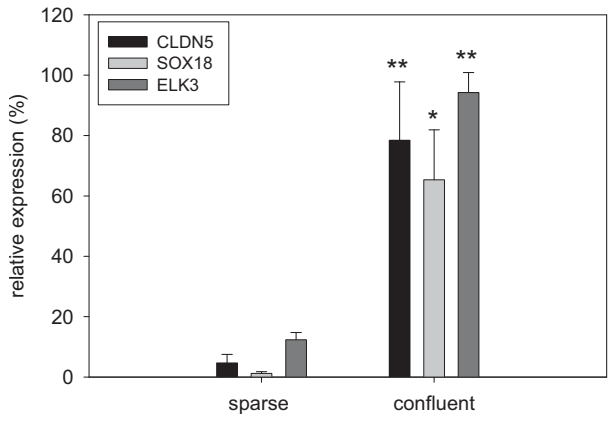


Figure 1. Expression of CLDN5, SOX18 and ELK3 as function of HUVEC density.. Sparse: HUVEC were seeded at $1x10^4$ per cm^2 and grown for 24 h. Confluent: cells were seeded at $3x10^4$ per cm^2 and grown to confluency after which culture was continued for 48 hours before RNA was harvested. Specific mRNAs were measured using semi-quantitative RT-PCR. Results were normalized for ß-actin and expressed as percentage of the maximum value measured for the specific mRNA. CLDN5, SOX18 and ELK3 were significantly upregulated in confluent cells as compared to sparse cells. Means ± SD from four HUVEC isolates are shown

Endothelial specificity of the CLDN5 promoter and SOX18 expression. Endothelial-specific expression of claudin-5 has been described in tissue of mouse- and human origin (Liebner et al., 2000; Morita et al., 2000a; Morita et al., 2000b; Nitta et al., 2003). 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 celltype, 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 (Supplementary Table 2). 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 promoterless luciferase control virus. As shown in Figure 2a, the CLDN5 core promoter induced transcriptional activity at a considerably higher rate in HUVEC as compared to primary arterial smooth muscle cells as well as CaCo-, HeLa- and HEK293 cell lines.

Next, we examined cell-specificity of SOX18 gene expression by measuring its mRNA levels in different cell types, using real time semi-quantitative RT-PCR, and compared its actin-normalized relative mRNA expression to that of CDH5 and CLDN5, two established endothelial-specific junction components, ELK3, of unknown cellular specificity, and ZO-1, an ubiquitously expressed component of the junctional plaque. As shown in Figure 2b, SOX18 is exclusively expressed in endothelial cells. ELK3 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 SOX-18ΔCt which was analogous to the dominant-negative *ragged opossum* (Ra^{op}) mutant of the mouse orthologue (James et al., 2003; Pennisi et al., 2000). SOX-18 ΔCt was constructed by introducing a LEU266TER mutation, resulting in a truncated SOX-18 protein. In Ra^{op} a frameshift at this position leads to missense coding and premature truncation. SOX18- and SOX18ΔCt cDNA was cloned into a lentiviral vector under control of the CMV promoter and infectious virus particles were produced and titrated as described

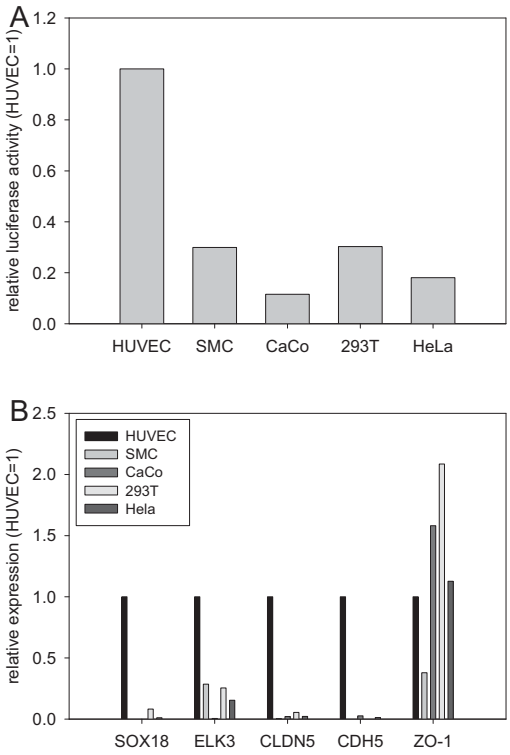
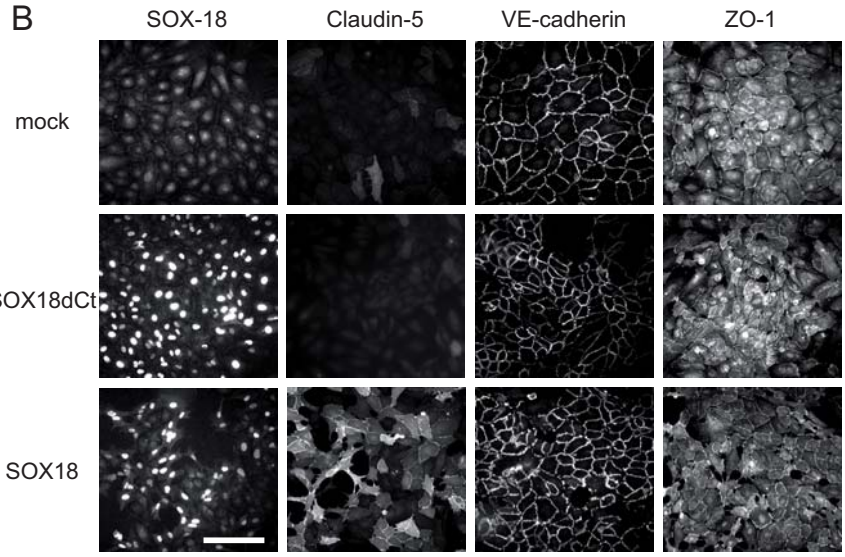


Figure 2. Endothelial specificity of claudin-5 promoter activity and SOX18 expression. (A) An evolutionary conserved fragment of 300 bp upstream of the CLDN5 transcription start site was cloned upstream of the Firefly luciferase reporter gene in an lentiviral vector which was then packaged into lentiviral 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. Both types of luciferase were measured after at least 48 hours. Ratios of Firefly luciferase over Renilla luciferase are plotted relative to ratios in HUVEC. (B) Approximately 48 hours after the cells had reached confluency, RNA was harvested and SOX18, ELK3, CLDN5, CDH5 and ZO-1 mRNA was measured by semi-quantitative RT- PCR. Values were normalized for β-actin mRNA and are expressed relative to HUVEC.

in materials and methods. At the RNA level, SOX18 expression was typically between 15 and 25-fold increased (data not shown). Immunostaining on Western blot detected SOX-18 and SOX-18ΔCt at 46 kDa and between 34- and 40 kDa, respectively (Figure 3a). Comparison to predicted sizes of 41 kDa (Swiss Prot. No. p35713) and 30 kDa, suggests post-translational modification of, as yet, unknown nature. Using immunofluorescence microscopy, we examined expression and localization of wild-type SOX-18 and SOX-18ΔCt, 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 C-terminally truncated SOX-18 localize to the nucleus, as does endogenous SOX-18. SOX-18ΔCt signals are slightly higher and show less variation in signal intensity compared to 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-18ΔCt 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-18ΔCt. Next, we examined effects of SOX-18 overexpression on mRNA levels of CLDN5, ZO-1 and CDH5 by real-time semi-quantitative

Figure 3. Lentiviral overexpression of SOX-18 and SOX-18dCt 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: transduced with virus containing vector without SOX18 insert. SOX18: transduction with virus containing the SOX18 ORF under control of the CMV promoter. SOX18dCt: transduction with virus encoding an (Leu266Ter) SOX-18 mutant under control of the CMV promoter. Equal loading was confirmed with anti-α tubulin antibodies.



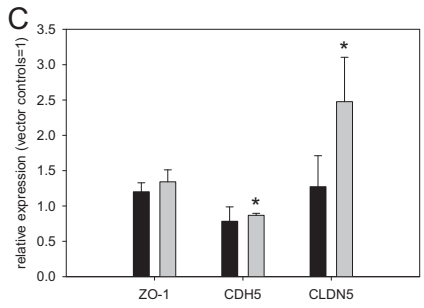


Figure 3 (continued). Lentiviral overexpression of SOX-18 and SOX-18dCt in HUVEC. (B) Effect of SOX18 and SOX18dCt overexpression on fluorescence patterns of SOX-18, claudin-5, VE-cadherin and ZO-1. After transduction with either mock-, SOX18-dCt or SOX18 virus (rows), cells were seeded on fibronectin-coated 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 an Cy-3-conjugated secondary antibody. Recordings were made using an Zeiss axioplan2 microscope with settings optimized for SOX18 transduced cells. Subsequently, mock- and SOX18dCt transduced cells were recorded with identical settings. SOX18 transduced cells show increased fluorescence of claudin-5 as compared to mock- and SOX18dCt-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, CDH5 (VE-cadherin) and CLDN5 (claudin-5) under sparse (black bars) and confluent (grey bars) conditions. Cells were transduced with virus containing the SOX18 ORF under control of the CMV promoter. As control, transductions with virus containing vector without insert were performed (mock). Three days after transduction, growth was either continued (confluent) or cells were re-seeded at a fivefold lower density (sparse). Cells were then grown another twenty-four hours before harvest. mRNA levels were determined using real-time semi-quantitative RT-PCR. Values were normalized for β-actin and plotted as value relative to mock. SOX18 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. * $P<0.05$.

RT-PCR in both post-confluent cells and sparse cells, i.e., in the absence and presence of endogenous SOX-18, respectively. As shown in Figure 3c, in post-confluent cells, CLDN5 mRNA expression is significantly increased as compared to 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 sox18ΔCt 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-18ΔCt- and mock- transduced cells on Western blot (Figure 4). We could detect significantly increased claudin-5 levels in SOX-18 overexpressing cells whereas claudin-5 levels were significantly decreased in SOX-18ΔCt cells, thus substantiating our results obtained by immunofluorescence and semi-quantitative PCR. Together, the finding that SOX-18 overexpression enhances CLDN5 gene expression, and the observed endothelial specificity of SOX18 expression support the view that SOX-18 is an important determinant in endothelial-specific expression of CLDN5.

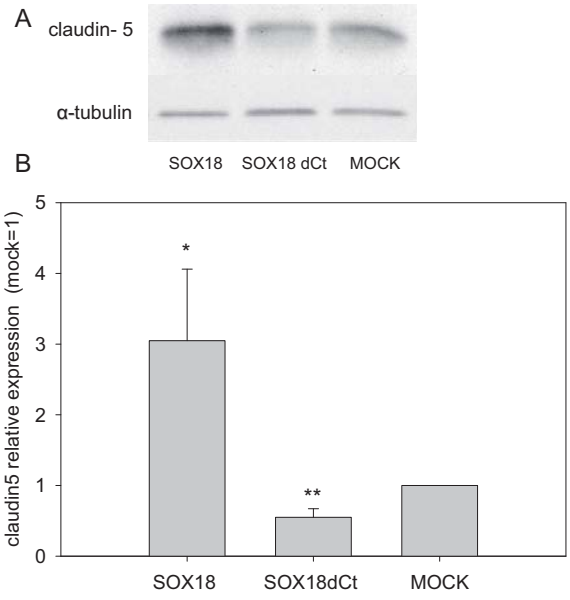


Figure 4. Effect of lentiviral overexpression of SOX-18 and SOX-18dCt on claudin-5 expression in HUVEC. (A) Claudin-5 protein detected by Western blotting. Four days after lentiviral transduction of HUVEC, lysates were analyzed by immunoblotting with claudin-5 antibodies. SOX18: transduction with virus containing the SOX18 ORF under control of the CMV promoter. SOX18dCt: transduction with virus encoding an (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-18dCt 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.

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 (figure 2) was now tested in either the presence or absence of SOX18 or SOX18ΔCt overexpression. In addition, a larger fragment comprising 1000 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 SOX18-, SOX18ΔCt- or mock virus. Three days after the second transduction, lysates were prepared for measurement of reporter activity. As shown in Figure 5, the effects of SOX-18 and SOX-18ΔCt 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-18ΔCt.

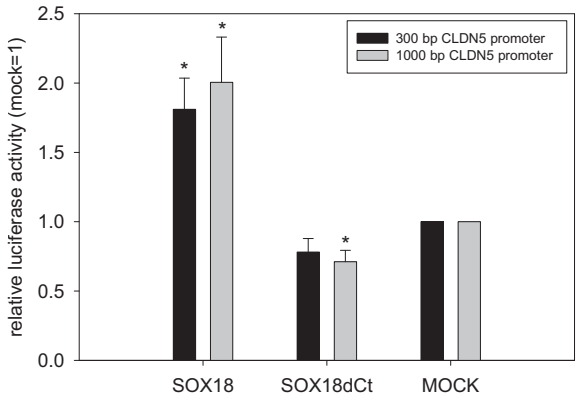


Figure 5. Overexpression of SOX-18 enhances transcription directed by the CLDN5 promoter. (A) 1000 bp and 300 bp fragments upstream of the CLDN5 transcription start site were cloned upstream of a Firefly luciferase reporter gene on a lentiviral vector which was then packaged into lentiviral particles. 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, SOX18 or SOX18dCt under control of the CMV promoter were introduced. As a control, virus containing the viral expression vector without insert was transduced (mock). After three 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 and ± SD from 4 HUVEC isolates are shown. Significant differences with mock transductions are indicated. *P<0.05.

We then further fragmented the CLDN5 promoter along lines of evolutionary conservation (Figure 6a and Supplementary Table 2). 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 (Figure 6b and Supplementary Table 2). 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 hours by transduction with either SOX18- or mock virus. Reporter activity was measured after three days. As can be inferred from Figure 6b, both basal (endogenous) and SOX-18-enhanced transcriptional activity 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, amongst 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 not only involved

Figure 6 (next page). Stimulation of the CLDN5 promoter by SOX-18 is critically dependent on an evolutionary conserved SOX-binding site. (A) Evolutionary conservation of the CLDN5 locus. Pre-aligned genome sequences for CLDN5 from human, mouse, rat and dog were analyzed for evolutionary conservation of regulatory sequences using the VISTA browser (http://genome.llbl.gov/vista) The boxed plot represents organization and evolutionary conservation (human versus mouse) of the CLDN5 locus using an 50 bp sliding window. Next, the Genomatix suite (http://www.genomatix.com) was used to identify conserved potential transcription factor binding sites within the conserved regions. Potential SOX, ETS and LEF binding sites are indicated, for a complete overview of conserved transcription factor binding sites, see Supplementary Table 2. (B) Progressive 5'deletion of the CLDN5 promoter. Deletion fragments of the CLDN5 promoter were generated by PCR (Supplementary Table 2). The resulting fragments were cloned upstream of the 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 second round of transduction, SOX18 under control of the CMV promoter was introduced. As a control, virus containing the viral expression vector without insert was transduced (mock). After three days, cells were lysed and luciferase activities were measured. Normalized values of Firefly luciferase activity (Ffluc) over Renilla luciferase activity (Rluc) are plotted. The region -230 to – 147 is critical in both basal and SOX-18-induced CLDN5 transcription. Means ± SD from 3 HUVEC isolates are shown. Significant differences between SOX18- and mock transductions are indicated. * $P<0.05$, ** $P<0.01$.

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.

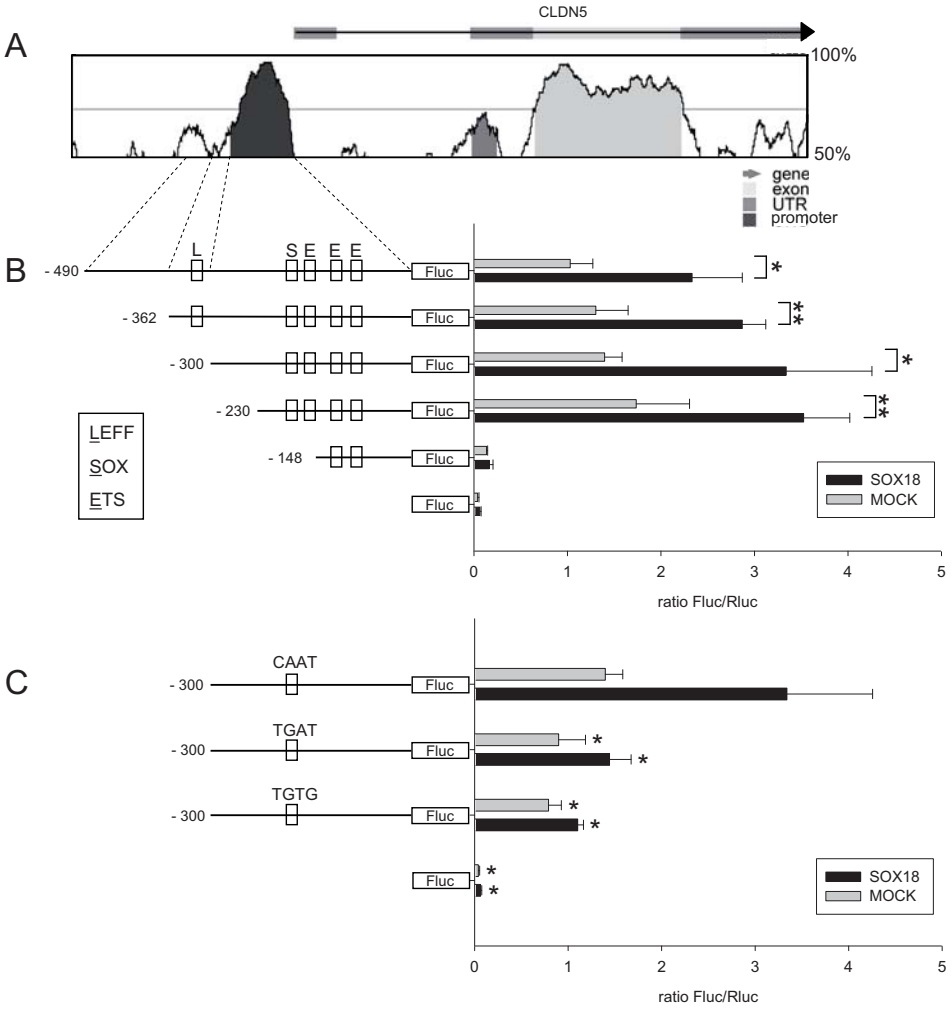


Figure 6 (continued). Stimulation of the CLDN5 promoter by SOX-18 is critically dependent on an evolutionary conserved SOX-binding site. (C) In the –300 CLDN5 upstream fragment, mutations in the core CAAT sequence of an evolutionary conserved SOX-binding site were introduced using PCR. The resulting 300 bp TGAT and 300 bpTGTG fragments were cloned and tested for their promoter activity in presence and absence of overexpressed SOX-18, described under (B). Normalized values of Firefly luciferase activity (Ffluc) over Renilla luciferase activity (Rluc) are plotted. Means ± SD from 3 HUVEC isolates are shown. Significant differences with the 300 bp CAAT fragment are indicated. * P<0.05

RNA-silencing of SOX18 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 (Lefebvre et al., 2007; Mertin et al., 1990), 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 and -7) that are thought to display functional redundancy in endothelial cells (Matsui et al., 2006; Sakamoto et al., 2007) are candidates. Inspection of our microarray data revealed that in addition to the SOX18 gene, SOX17, -2, -3 and -6 genes are expressed. In contrast to SOX18, expression of none of the other SOX-genes mentioned showed significant dependence on cell density.

We specifically targeted SOX18 expression by transfecting double stranded SOX18-siRNAs into HUVEC and monitored their effect by measuring mRNA levels for SOX18 and its potential target, CLDN5, by real time semi-quantitative 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 to control siRNA, we observed that SOX18-siRNA induced efficient silencing of SOX18, accompanied by a profound decrease of CLDN5 mRNA levels, whereas ZO-1 mRNA levels remained unchanged (Figure 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 SOX18 siRNA treated cells (Figure 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 proven to accurately measure CLDN5 mRNA expression in a wide dynamic range, (Figure 1 and Fontijn et al., 2006). We speculate that stability of claudin-5 protein is decreased upon SOX18 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 SOX18 expression reveals a non-redundant and obligatory role for SOX-18 in the control of CLDN5 expression.

RNA-silencing of SOX18 expression results in impairment of endothelial barrier function as measured by ECIS. To investigate whether SOX18 regulates integrity of HUVEC barriers, transendothelial electrical resistance (TEER) of confluent endothelial monolayers was measured in time using ECIS (Giaever and Keese, 1991). Two days after transfection with SOX18 siRNA, cell shape became elongated but monolayers remained intact as observed by phase-contrast microscopy (Figure 7c). Sixty hours after transfection, resistance was significantly reduced in SOX18 siRNA cells as compared to control treated

cells and had dropped to levels observed in non-matured monolayers (Figure 7d). The complete ECIS timecourse graph is available as Supplementary Figure 2.

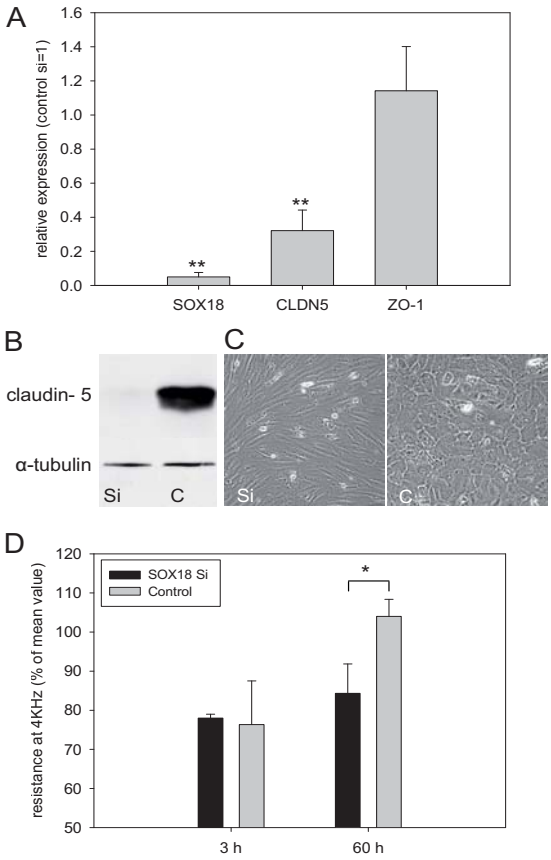


Figure 7. Effects of SOX18 silencing in HUVEC. (A) Effects of SOX18 silencing on SOX18, CLDN5 and ZO-1 mRNA. HUVEC cultures were treated with complexes of oligofectamine and either SOX-18 Si- or control double stranded RNA oligonucleotides. Two days after transfection, RNA was isolated and SOX18, CLDN5 and ZO-1 mRNA levels were determined using real-time semi-quantitative RT-PCR. Values were normalized for β-actin and are plotted relative to control double-stranded RNA. SOX18 and CLDN5 mRNAs are significantly reduced wheras ZO-1 mRNA is not affected. Means ± SD from 3 HUVEC isolates are shown. Significant differences with control double-stranded RNA are indicated. ** P<0.01. (B) Claudin-5 protein detected by Western blotting. HUVEC cultures were treated with complexes of oligofectamine and either SOX18 Si- or control double stranded RNA oligonucleotides, indicated as Si and C, respectively. Two days after transfection, lysates were analyzed by immunoblotting with claudin-5 antibodies. Equal loading was confirmed with anti-α tubulin antibodies. SOX18 Si transfected cells show decreased levels of claudin-5 protein. (C) Phase contrast microscopy of HUVEC 48h after SOX18- Si RNA (Si) or control (C) transfections. (D) ECIS measurement of SOX18-silenced HUVEC. Cultures were treated as under (B) and development of barrier function was monitored using ECIS. Resistance measured at 4KHz at the times indicated is plotted as percentage of the mean resistance, 100% corresponds to 1196 Ohm. Significant differences with control double stranded RNA are indicated. * P<0.05.

DISCUSSION

Previously, we have reported the transcriptional upregulation of several genes involved in endothelial barrier formation during maturation of endothelial monolayers (Fontijn et al., 2006). In the present study, SOX18 emerged as one of the most strongly induced genes in an analysis of transcriptomes from sparse versus post-confluent endothelial cells. A role for SOX18 in control of endothelial barriers was suggested by reports on SOX18 mutations in vascular anomalies in *ragged* (*Ra*) mice (James et al., 2003; Pennisi et al., 2000) and in the human hypotrichosis-lymphedema-telangiectasia (HLTS) syndrome (Irrthum et al., 2003). 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 (James et al., 2003; Matsui et al., 2006; Pennisi et al., 2000; Sakamoto et al., 2007).

Here, we observed that of several cell types tested, only HUVEC expressed SOX18. Overexpression of SOX-18 and a dominant negative derivative thereof, SOX-18ΔCt, 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 SOX18 showed that endogenous SOX-18 is essential for basal CLDN5 transcription. These findings identify the CLDN5 gene as an important SOX-18 target.

SOX18 is a member of the SOX gene family that share a conserved high-mobility-group domain. Individual members are responsible for regulation of cell fate and differentiation processes (review in Lefebvre et al., 2007) and show characteristic spatiotemporal expression patterns during development and adulthood. SOX-18 was found in endothelial cells of pre- and post natal developing vasculature in mice (Hosking et al., 2001; Matsui et al., 2006; Pennisi et al., 2000). 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 BBB (blood-brain barrier) (Nitta et al., 2003). Indeed, the importance of claudin-5 in non-BBB endothelium seems limited (Fontijn et al., 2006; Nitta et al., 2003). 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 SOX18-silenced HUVEC monolayers show impaired barrier function, which is in marked contrast to the lack of a barrier-related phenotype in $SOX18^{-/-}$ mice (Pennisi et al., 2000). Apparently, masking of effects resulting from the loss of SOX18 function by redundant activity of other members of the Sox F subfamily, as observed in mouse knock-out models (Matsui et al., 2006; Pennisi et al., 2000; Sakamoto et al., 2007), does not occur under our culture conditions. Thus, we show that loss of SOX18 function results in a severly compromised endothelial barrier function. A future inventory of SOX18-downstream genes might identify potential effector genes.

At the subcellular level, claudins are functionally associated with tight junction complexes via interaction with ZO-proteins (Itoh et al., 1999). In epithelial cells, this interaction plays a role in spatiotemporal control of tight junction formation (Umeda et al., 2006). 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 (Adams et al., 1996; Ando-Akatsuka et al., 1999; Umeda et al., 2006). Therefore, it may be speculated that claudin genes are subject to regulatory mechanisms that exert temporal control during junction formation. Consistent with earlier findings (Fontijn et al., 2006), 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 SOX18 in post-confluent cells revealed an essential and non-redundant role for SOX-18 in CLDN5 gene expression. This observation, together with the observed induction of SOX18 in post-confluent 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 (Lefebvre te al., 2007).

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 local barrier functions. Promoters of mouse claudin-3, -4 and -7 genes were repressed by Snail during epithelial-to-mesenchyme transition (Ikenouchi et al., 2003). Tcf4 binding elements in the 5' flanking region of the claudin-1 gene were found to be responsible for regulation by the β-catenin/Tcf-complex in colorectal cancers (Miwa et al., 2000). Overlapping GATA/NF-Y binding sites were found to be responsible for dual transcriptional control of CLDN11 in the blood-testis barrier (Lui et al., 2007). Tissue-specific regulation was described for CLDN18 in lung by T/EBP/NKX2.1 homeodomain transcription factor (Niimi et al., 2001). Sakaguchi et al reported on the role of two intestine-specific Caudal-type homeobox proteins, CDX1 and CDX2, and a member of the POU homeodomain family, TCF1 (HNF-1α), in regulation of the CLDN2 gene in intestine and liver (Sakaguchi et al., 2002). 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 mechanims to achieve correct tissue-specific and temporal expression.

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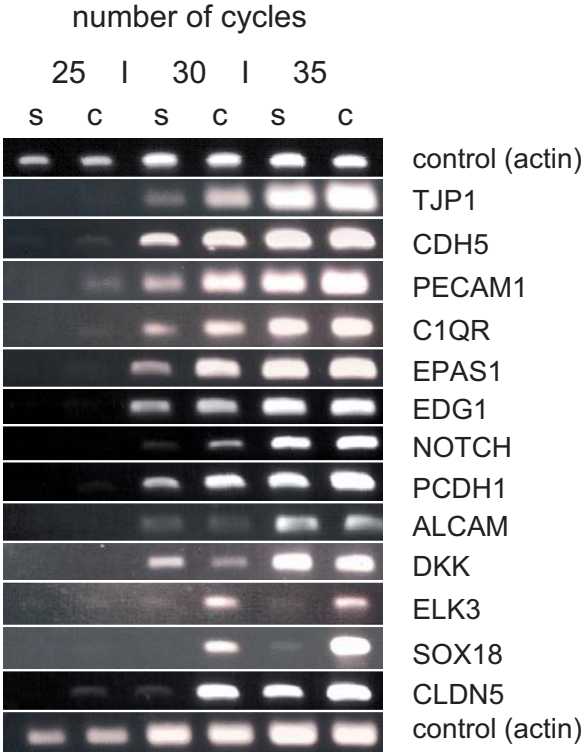
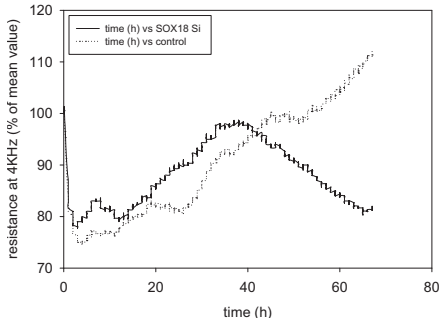
SUPPLEMENTARY MATERIAL

Supplementary table 1: the reader is referred to http://ajpheart.physiology.org/cgi/content/full/01248.2007/DC1



Supplementary figure 1. Validation of a microarray comparison of transcriptomes from sparse- versus confluent HUVEC using semi-quantitative RT-PCR. Reverse-transcribed RNA from sparse HUVEC (S) and confluent HUVEC (C) was amplified using primersets specific for the genes indicated. PCR reaction products were gel-analyzed at intervals of five amplification cycles. In addition to the three well-described genes TJP-1, CDH5 and CLDN5, specific cell density-dependent induction is shown for C1QR, EPAS, EDG1, NOTCH, PCDH1, ELK3, and SOX18. DKK is repressed, whereas ALCAM does not show regulation



Supplementary figure 2. ECIS measurement of SOX18-silenced HUVEC. HUVEC cultures were treated with complexes of oligofectamine and either SOX18 Si- or control double stranded RNA oligonucleotides and development of barrier function was monitored using ECIS. Resistance measured at 4KHz is plotted as percentage of the mean resistance, 100% corresponds to 1196 Ohm.

PCR primer TFB	position 5' of transcription start (bp.)	strand	sequence (core in capitals)	position chr.22
490 fwd.	**490-471**	**m**	**cga ggt acc cca gga ggg aga aag tca tc**	**17893331 17893350**
AP4R	466-450	p	ggtcaCAGAtgtgggag	
RP58	464-452	m	cccaCATCtgtga	
362 fwd.	**362-342**	**m**	**cgg ggt acc tca gtc cag ggc ctg gag ttc**	**17893202 17893222**
FKHD	336-320	m	ttactggaAACAaaggg	
NFAT	334-316	m	actGGAAacaaaggggccg	
LEFF	333-317	m	ctggaaaCAAAggggcc	
300 fwd.	**300-279**	**m**	**cga ggt acc aga act act aga aag ggg ctg g**	**17893139 17893160**
HESF	254-240	p	ggggCACGcggctcc	
HESF	253-239	m	gagccgcGTGCcccc	
EGRF	249-233	p	tcaggTGGGggcacgcg	
PLAG	248-228	p	GAGGctcaggtgggggcacgc	
HEN1	247-227	p	tgaggctCAGGtgggggcacg	
ZBPF	247-225	m	cgtgcccCCCAcctgagcctcagg	
ZFHX	243-231	m	cccccACCTgagc	
HESF	243-229	m	cccccaCCTGagcct	
AP2F	235-221	m	tgaGCCTcaggggggc	
230 fwd.	**230-210**	**m**	**cga ggt acc ctc agg ggg ccc gga gtg tcc**	**17893070 17893090**
MOKF	211-191	m	ccacaccagtggaCCTTtcga	
RORA	208-190	p	ctcgaAAGGtccactggtg	
HEAT	204-180	p	ccagccatttctCGAAaggtccact	
HEAT	203-179	m	gtggacctttCGAGaaatggctggg	
BCL6	200-184	p	ccaTTTCtcgaaaggtc	
YY1F	197-179	p	cccagCCATttctcgaaag	
SORY	183-167	p	tctgcaCAATggcccag	
TEAF	170-158	p	gggCATTcttctg	
STAT	168-150	p	ggatTTCCgggcattcttc	
P53F	167-147	m	aagAATGcccggaaatcccgc	
HICF	167-155	m	aagaaTGCCcgga	
STAT	166-148	m	agaaTGCCcggaaatcccg	
ETSF	165-145	m	gaatgcccGGAAatcccgcgc	
NFKB	158-146	p	cgcgggatTTCCg	
148 fwd.	**148-128**	**m**	**cga ggt acc gcg cct ccc tcc tcc agc aag**	**17892988 17893008**
GCMF	128-118	p	agCCCCcatcc	
ETSF	125-105	p	ggccaggaGGAAgagcccca	
NFKB	124-112	m	gggggctcTTCCt	
ZF35	121-109	p	aggaggAAGAgcc	
XBBF	112-94	m	tcctggccagGAAActcca	
ETSF	111-91	m	cctggccAGGAaactccaagt	
NFKB	104-92	p	cttggagtTTCCt	
MYT1	97-85	m	tccAAGTtggctt	
ETSF	95-74	p	caccctccGGAAgccaacttg	
EKLF	86-70	m	ttccggaGGGTggcctg	
PLAG	81-61	m	GAGGgtggcctgggggctggg	
RREB	81-67	p	cCCCAggccaccctc	
AP2F	77-63	m	gtgGCCTgggggctg	
SP1F	72-58	m	ctggGGGCtgggtg	
rev.	**22-1**	**p**	**a gtc ctc gag aaa ggg aca cgg agg ggc tgt g**	**17892861 17892882**

Supplementary table 2. The CLDN5 promoter. Evolutionary conserved transcription factor binding sites (TFBs) with their position relative to the transcription start site, polarity and sequence. Bold: primers used for generation fragements for promoter-reporter assays, their position relative to the transcription start site, polarity, sequence and position on chromosome 22. All forward (fwd.) primers were used in combination with a single reverse (rev.) primer. Underlined in forward primers: additional sequences, introducing an KpnI site for cloning in pGL3-basic, underlined in reverse primers: additional sequences, introducing an XhoI site for cloning in pGL3-basic.