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A novel transcriptional complex on the VE-cadherin promoter regulated the downregulation of VE-cadherin in the Down Syndrome Candidate Region 1 Isoform 1L-mediated angiogenesis

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Abstract

Angiogenesis is critical for many diseases. Previously, we reported that Down Syndrome Candidate Region 1 isoform 1L (DSCR1–1L) was one of the most up-regulated genes in endothelial cells induced by VEGF and histamine, and regulated endothelial cell proliferation and Matrigel angiogenesis in mice. However, it was not known whether DSCR1–1L regulated angiogenesis *in vivo* and what was the molecular mechanism underlying it. In this study, gene knockdown and overexpression models were established to study the role of DSCR1–1L in angiogenesis in vivo. Further, the downstream regulatory target of DSCR1–1L was explored with molecular biological methods in vascular endothelial cells. We found that DSCR1–1L shRNAs significantly inhibited angiogenesis induced by VEGF in mice ($p < 0.0001$). In the

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gain-of-function assay, overexpression of DSCR1–1L cDNA in mouse endothelium of EC-FH-DSCR1–1L transgenic mice was sufficient to induce angiogenesis significantly ($p < 0.01$). DSCR1–1L regulated angiogenesis in the early stage by down-regulation of the VE-cadherin expression through targeting its transcription, but not mRNA stability. Three DSCR1–1L-targeted DNA elements in the VE-cadherin promoter were identified by promoter reporter assays, among which, a novel specific transcriptional complex was found. The DNA sequence (CTTCTG) in the VE-cadherin promoter was identified to directly interact with proteins by Electrophoresis Mobility Shift Assays and DNase I footprint assay. Hence, DSCR1–1L is an excellent therapeutic target for angiogenic diseases through down-regulating the formation of a novel transcriptional complex on the VE-cadherin promoter. DSCR1–1L shRNAs and cDNA have the potential to be developed for clinical application. Our results also contribute significantly to the field of mechanistic studies.

Keywords

Down Syndrome Candidate Region 1 isoform 1L; angiogenesis; promoter; VE-cadherin

Introduction

Angiogenesis is a sophisticated multi-step process of new blood vessels formation with the coordinated participation of different cells and factors (1). It is a hallmark of many diseases, including cancer, wound healing, inflammation, and ischemic heart disease. Among many angiogenic factors, vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis and associated microvascular permeability to plasma proteins (2–6). Anti-VEGF neutralizing antibodies and VEGFR kinase/multiple kinase inhibitors have been successfully developed and widely used in the clinic (review in (7)). However, anti-angiogenic therapy faces the problems of insufficient efficacy (8–17), resistance and intrinsic refractoriness (15,18,19), in addition to their toxic side effects (20). Therefore, it is desirable to identify other targets of angiogenesis.

In our gene profiling study, we identified that Down Syndrome Candidate Region 1 isoform 1L (DSCR1–1L) was one of the most up-regulated genes in human umbilical vein endothelial cells (HUVECs) induced by Vascular endothelial growth factor (VEGF) for one hour (21). The DSCR1 gene (aliases: DSCR1, ADAPT78, CSP1, DSC1, MCIP1, RCN1) encodes four different mRNA transcripts / isoforms that have different N-terminuses encoded by each of the first four exons, and a common C-terminus encoded by exons 5–7 (22,23). The transcription of Down Syndrome Candidate Region 1 isoform 4 (DSCR1–4) is controlled by a promoter located between exon 3 and exon 4, which is different from the promoter that regulates the transcriptions of isoforms 1, 2 and 3 (22–25). The N-terminuses of DSCR1 isoform 1 (DSCR1–1), isoform 3 (DSCR1–3) and isoform 4 (DSCR1–4) proteins contain 84, 3, and 29 amino acid residues, respectively, while the mRNA transcript of isoform 2 is most likely not translated into protein because it lacks a methionine starting site (22,23).

The DSCR1 isoforms have different expression patterns, functions and regulatory mechanisms (22,23). DSCR1–1L was found to play a protective role against cell stress

(26–28). DSCR1–4 played an inhibitory role in cardiac and skeletal muscle hypertrophy and angiogenesis (21,29–35). As far as we know, prior to our report published in 2006 (21), nothing was known about the role of DSCR1–1L in angiogenesis. We systematically analyzed the function of DSCR1 isoforms 1L, 3 and 4 in angiogenesis by overexpressing their respective proteins and their specific siRNAs (21). We found that DSCR1–1L and DSCR1–4 had antithetical effects on the angiogenic responses (21). The overexpression of DSCR1–1L protein significantly increased the HUVEC proliferation and Matrigel angiogenesis in mice in the presence and absence of VEGF, whereas the HUVEC proliferation and Matrigel angiogenesis in mice induced by VEGF were strikingly inhibited with a DSCR1–1L-specific siRNA (21). In contrast, the overexpression of DSCR1–4 protein strikingly inhibited HUVEC proliferation and Matrigel angiogenesis in mice induced by VEGF, whereas DSCR1–4-specific siRNA (D4Si) stimulated such responses in the presence and absence of added VEGF (21). The overexpression of DSCR1–3 protein had no effect on the HUVEC proliferation and Matrigel angiogenesis in mice in either the presence or absence of VEGF (21). DSCR1–1L was highly expressed in human tumor vasculature, but not detected in tumor cells or in normal tissues (ovaria and kidney) (21,24). However, it was not known whether DSCR1–1L regulated angiogenesis *in vivo* and the molecular mechanism underlying it.

In this study, we found that knocking down DSCR1–1L inhibited, but overexpresion of DSCR1–1L cDNA in mouse endothelium is sufficient to induce angiogenesis. DSCR1–1L is a cytosolic protein and controls angiogenesis by down-regulation of VE-cadherin expression via decreasing the VE-cadherin promoter activity, but not 3'untralation region (3'UTR) activity. DSCR1–1L inhibited the formation of a transciprtion complex that contained a novel oligonucleotide element in the VE-cadherin promoter. Therefore, DSCR1–1L is an excellent therapeutic target for angiogenesis.

Materials and Methods

Materials

VEGF (Catalog # DY-493) was purchased from R&D Systems (Minneapolis, MN, USA). Trypsin/EDTA (Catalog #: CC-5012) and Trypsin Neutralization Solution (Catalog # T1426) were purchased from Lonza (Walkersville, MD, USA). Actinomycin D (Catalog # A-1410) and FITC conjugated lectin (Catalog # 712–095-153) were the products of Sigma (Sigma-Aldrich, Inc. Burlington, MA). The antibodies against VE-cadherin (Catalog # sc-6458), CD31 (Catalog # sc-1505), c-Jun (Catalog # sc-7345), HA (Catalog # sc-7392), and β-actin (Catalog # sc-8432) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The α-tubulin antibody (Catalog # a01410) was the product of Genscript (Piscataway, NJ, USA). A customized DSCR1–1L antibody was produced by NeoBioLab (Woburn, MA) and validated as described in the Supplemental Data because the DSCR1–1L antibody used in our previous studies (21) was discontinued.

Cell culture

Primary HUVECs (Catalog #: CC-2517) purchased from Lonza (Walkersville, MD) were cultured on plates coated with 30 μg/ml vitrogen (Collagen Biomaterials, Palo Alto, CA)

in endothelial basic medium (EBM, Catalog #: CC-3121) with the EGM-SingleQuots Kit (Catalog #: CC-4143) (Lonza, Walkersville, MD). The HUVECs at passages 5 were used for all experiments. At 80% confluence, HUVEC was incubated in EBM containing 0.1% fetal bovine serum (FBS) for 24 hours and then used for subsequent experiments. B16F1 cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal bovine serum. At 80% confluence, B16F1 cells were used for subsequent experiments.

The construction of DSCR1–1L shRNAs

This study utilized three DSCR1–1L shRNAs, hu-shD1L, mu-shD1L and hu/mu-shD1L, that specifically knocked down DSCR1–1L in the species of human, mouse, both of human and mouse, respectively. The shRNA oligonucleotides were cloned to the lentiviral vector pLKO.1 to produce lentiviruses following the instructions provided by Addgene (Cambridge, MA). HUVECs and mouse melanoma B16F1 cells were transduced with the lentiviruses expressing shGFP as a control, hu-shD1L, mu-shD1L, and hu/mu-shD1L, respectively. Sixty hours later, the RNAs were isolated and subjected to quantitative real-time reverse transcription-PCR (RT-PCR). After confirmation of the specificity, each DSCR1–1L shRNA was cloned to the pENTR1A-stuffer vector and transferred to the adenovirus pAd/PL-DEST vector to prepare adenoviruses following the instructions provided by Invitrogen (Carlsbad, CA). The adenoviruses expressing DSCR1–1L shRNAs were used to infect human and mouse cells, respectively.

Proliferation assay

As described previously (36), HUVECs were seeded in 96-well plates. Twenty-four hours later, the cells were transduced with the viruses as indicated. Forty-eight hours later, the cells were serum-starved with EBM containing 0.1% FBS for 48 hours, and treated with and without VEGF (10 ng / ml) for 24 hours. Cell Counting Kit-8 reagents (Dojindo Molecular Technologies, Inc. Washington. D.C) were added to each well. The plates were incubated for 3 hours before the absorbance at 450 nm was measured using a microplate reader.

Monolayer migration assay.

Monolayer migration assay was carried out as described previously (36). Briefly, HUVECs (6×10^4 cells/well) were seeded in 6-well plates. Twenty-four hours later, the cells were transduced with the viruses as indicated. Forty-eight hours later, the cells were serum-starved with EBM containing 1% FBS for 24 h, respectively. Scratch wounds were generated with a 200 μl pipette tip and photographed immediately at 0 h. The cells were stimulated either with or without VEGF (10 ng/ml) for 16 h and photographed. The cells that migrated to the wound area were counted. The results are expressed as mean ± SD from 6 views.

In vivo VEGF-induced angiogenesis models

The assay was carried out according to the consensus guidelines (37,38). Both male and female Nu/Nu mice (4- to 5-week-old) purchased from the National Institutes of Health (NIH) were injected subcutaneously (s.c.) in each ear with the adenovirus mixtures

containing 10^8 PFU of the non-replicating adenoviral vectors expressing mouse VEGF (Ad-VEGF) and either the shGFP as a control, or one of the indicated shDSCR1–1Ls (9.5×10^9 OFU). The injections with the viruses without ad-VEGF were repeated every three days. The ears were photographed daily using a Wild M400 Macroscope with Digital Camera Capability, Lightglass Optics (Cedar Crest, NM) from the injection day (Day 0) to Day 9. The ear tissues were collected for the quantitative analysis of vessels at day 9. The experiments were repeated in triplicates.

The generation of the transgenic mice, in which Flag-HA fused DSCR1–1L cDNA was inducibly expressed in the mouse endothelial cells

The Flag-HA fused DSCR1–1L cDNA was sub-cloned into the pUHO-10.3 expression vector (kindly provided by Dr. Hermann Bujard, Zentrum Für Molekulare Biologie, der Universität Heidelberg) under the control of a tetracycline-regulated promoter (Tet-O or tet responsive element TRE) that could be activated by the tet off-transactivator tTA in a tet-bound form. The TRE-FH-D1L mice in the Fvb genic background were generated with the linearized DNA fragment containing the promoter-targeted gene SV40poly A by the Transgenic Core at BIDMC, and crossed with the transgenic VE-cad-tTA mice that specifically expressed the transactivator tTA in mouse endothelium obtained from Dr. Laura Benjamin (39) to produce the EC-FH-DSCR1–1L double transgenic mice. Tetracycline was provided in the drinking water (1.5 g tetracycline, 50 g sucrose per liter tap water) to prevent the expression of the trans-gene. The six-week-old EC-FH-DSCR1–1L mice and their control wild type littermates (males and females) were provided with the tetracycline-free water as indicated to induce the expression of the transgene.

Miles microvessel permeability assay.

Six days after tetracycline was removed from the mouse drinking water, mouse flank skin hair was clipped and depilated one day before the permeability experiments. The mice were anesthetized with avertin (tribromoethanol, 200 mg/kg) and injected intravenous (iv). via the tail vein with 0.2 ml of 0.5 % Evans blue dye in saline. VEGF was injected intradermally (id). into the flank skin. The tissues were harvested and photographed after 30 min.

Histology, immunohistochemistry and immunofluorescence staining.

The fresh frozen tissues were cut into 5 μm thick sections, fixed with cold acetone for 10 min, and then air-dried. The sections were blocked in the TBS with 1% BSA at room temperature for 2 hours, incubated with the antibodies in TBS with 1% BSA at 4 overnight, rinsed with the TBS 3 times, and then treated with 0.3% H_2O_2 for 15 minutes. Next, the slides were incubated with IgG-HRP (1:500 dilution; Catalog # SC-2004; Santa Cruz, CA) in TBS-1%BSA at room temperature for 1 hour, rinsed with TBS 3 times, and stained with contents from a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). The pictures (RGB) were taken with an OLYMPUS BX41 Microscope, OLYMPUS DP72 camera system, and CellSens Standard 1.8.1 software (OLYMPUS). The vessel density and the ear thickness were measured with an Image J software.

The HUVECs with and without VEGF (10 ng/ml) stimulation were fixed with 4% paraformaldehyde and quenched with PBS-0.1 M glycine. Following the permeabilization

with 0.1% (v/v) Triton X-100 in PBS and the blocking with 5% donkey serum, the samples were incubated with the specific primary antibodies (1:100 dilution) at 4°C overnight. After the incubation with fluorescence conjugated second antibodies (1:200) at RT for 30min, each sample was added with one drop of Vectorshield with DAPI. All of the fluorescence images were captured by the Zeiss AXIOCAM MRC system and analyzed with the ImageJ software 1.44p (National Institutes of Health, USA).

The effect of actinomycin-D on transcription

Twenty-four hours after seeded on plates, the HUVECs (3×10^5 cells/plate) were infected with the adenoviruses expressing DSCR1–1L, or Lac Z as a control. Sixteen hours later, the cells were treated with actinomycin-D (10 μg/ml). The RNA was collected at 8 hours.

The construction of the VE-cadherin reporter plasmid and its mutants

The 1,124bp VE-cadherin promoter, containing the nucleotides between −1,031bp and +93bp relating to the first base pair of the reported VE-cadherin cDNA was obtained through PCR from human genomic DNA with the forward primer vep-1031F and the reverse primer vep+93R. The PCR product was digested with the restriction enzymes XhoI and HindIII and cloned to the luciferase pGL3-basic vector (Promega Corporation, Madison, WI) to generate the plasmid VEp-1031. All of the deleted mutants were created similarly with the VEp-1031 as the template. For the triplicated mutants, the triplicated oligonucleotides were sub-cloned upstream of the VEp341, with the MluI and XhoI restriction enzymes in the 5’- and 3’-end, respectively. For the VE-cadherin 3’ untranslated region (VE-3’UTR) reporter plasmid, the 1,769bp PCR product was cloned from the human cDNA with the forward primer hVE-cad-3UTR-F1 and the reverse primer hVE-cad3UTR-R1769, which was then digested with the restriction enzymes XbaI and BamHI and cloned into the 3’UTR region of the VEp-341. All of the plasmids were confirmed with DNA sequencing by GENEWIZ. All of the primer sequences were listed in the primer section.

Luciferase assay

Twenty-four hours after being seeded in a 24-well plate, the HUVECs (3×10^4 cells / well) were washed with Minimum Essential Media (MEM) (Life Technologies, Grand Island, NY) three times, transfected with promoter luciferase constructs (4 wells per group), and then infected with the adenoviruses as indicated. For the transfection, the VE-cadherin promoter construct (495 ng / well) was added into 25 μl of MEM. The pRT-SV40 luciferase vector, serving as the internal control (5 ng / well), was added into 25 μl of MEM, and then added into the mixture of the promoter constructs. TransIT2020 (0.5 μl; Mirus Bio LLC, Madison, WI) was then added to the DNA mixture. The transfection mixture was incubated at room temperature for 20 minutes and added to the cells. Four hours later, the cells were infected with the adenoviruses overexpressing DSCR1–1L, or Lac Z as a control, in the EBM containing 0.1% FBS. After being incubated for 20 hours, the cells were lysed and subjected to luciferase analysis with a Dual-Luciferase Reporter Assay System (Catalog # E1960; Promega Corporation, Madison, WI), following the manufacturer’s instructions. The luciferase activity in each well was normalized to the internal luciferase activity. The data were expressed as the ratio of luciferase activity in DSCR1–1L-expressing cells to that in Lac Z-expressing cells in terms of average fold changes.

The preparation of nuclear and cytoplasmic extracts

The nuclear and cytoplasmic extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Catalog # 78835; Thermo Fisher Scientific, Rockford, IL). The cell membranes were lysed in 200 μl of the ice-cold Cytoplasmic Extraction Reagent I (CER I) for 10 minutes and placed on ice. Then, the ice-cold Cytoplasmic Extraction Reagent II (CERII) (11 μl) was added. The cells were incubated for 1 min. and centrifuged at 4 °C for 10 min. The supernatants were collected as cytoplasmic fractions. The insoluble pellets that contained the nuclei were washed two times with PBS to remove the remaining cytoplasmic proteins. The washed pellets were resuspended in 100 μl of ice-cold Nuclear Extraction Reagent (NER), incubated on ice for 40 min., and centrifuged (16000 × g) at 4 °C for 10 min. The supernatant was used as the nuclear extract for EMSA. For the footprint assay, the volume of NER was decreased to 40 μl to obtain more concentrated nuclear extracts. The protein concentration was determined using DC™ Protein Assay Kit II (Cat. No. 5000112, Bio-Rad, Hercules, CA).

Electrophoresis Mobility Shift Assays (EMSA)

The 40 bp long DNA oligonucleotides of D1LTR, SREBP, and MyoG in the VE-cadherin promoters were synthesized, labeled with biotin as probes with a Biotin 3’ end DNA labeling kit (Catalog # 89818; Thermo Fisher Scientific) following the manufacturer’s instruction, annealed, and incubated with the nuclear extracts isolated from HUVECs. The probe sequences were listed in the Primer Section. EMSA was performed using a LightShift Chemiluminescent EMSA kit (Catalog # 20148; Thermo Fisher Scientific) as described previously (24). Briefly, 2 μl of nuclear extracts and 20 fmol of biotin end-labeled probes were incubated in a 20 μl reaction mixture (10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 0.05% NP-40). Either poly dI·dC (50 ng / μl) or Sheared Salmon Sperm DNA (ssDNA, 200 μg / mL) was included in the nuclear protein-DNA reaction mixtures as an unspecific blocking agent. The DNA-protein complexes were resolved by electrophoresis on a 6% poly-acrylamide gel in 0.5×TBE at 100 V and 4°C for 1–1.5 h, and subsequently transferred to the positively charged nylon membranes in the 0.5×TBE at 380 mA and 4 for 1 h. The membranes were exposed to UV light radiation (120 $mJ*cm^{-2}$, 1 min.) to cross-link the DNA to the membrane. The detection of the biotin-labeled DNA probe was performed using a Chemiluminescent Nucleic Acid Detection Module (Catalog # 89880; Thermo Fisher Scientific, Waltham, MA), in accordance with the manufacturer’s Instruction. The unlabeled wild type probes and the scrambled mutant probes were used to validate the binding specificity.

DNase I footprint assay

DNase I footprint assays were performed similarly to the method described by Zianni et al (40). Briefly, the fluorescent 6-carboxy-fluorescein (FAM) labeled probes that encompassed the bases between −701 and −392 upstream of the human VE-cadherin cDNA, including the target site, were amplified using the primers FP-5’FAM-vep-701F (5’-ACATCAGAAGCAGAGCTGG-3’) and FP-vep-392R (5’-GTTTAAGGTGC TTGTCCACC-3’). After agarose gel electrophoresis, the FAM-labeled probes were purified with a QIAquick gel extraction kit (Qiagen) and quantified with a NanoDrop 2000

(Thermo). The amount of 100 fmol (20 ng) fluorescently labeled probes was incubated with 15 μl of nuclear proteins in a total volume of 95 μl in the same buffer as that for EMSA. Bovine serum albumin (BSA) was used for the control experiment. After incubating for 20 min at 25°C, an amount of 5 μl solution containing DNase I (Catalog # M6101; Thermo Fisher Scientific, Waltham, MA) and freshly prepared $CaCl_2$ was added to the reaction mixture to a final volume of 100 μl (1.15 U DNase I, 2 mM $CaCl_2$), followed by further incubation for 5 min. at 25°C, based on the results obtained from the optimization experiments using various concentrations. The mixture was heat-inactivated at 75 for 10 min. The DNA fragments were purified with the QIAquick PCR Purification kit (Catalog # 28104; Qiagen, Hilden, Germany) and eluted with 30 μl of ddH_2O immediately. The digested DNA and sequencing reaction products were analyzed with an ABI 3730×l DNA analyzer (Applied Biosystems, Foster City, CA). The sequences were then analyzed with a Peak Scanner software (Applied Biosystems, Foster City, CA) to determine the protected sequences in the DNase I digestion map.

Animal welfare

All of the animal experiments were performed in compliance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Statistical analysis

Results were presented as mean ± SD. ANOVA and student's t-test were employed to determine the statistical significance. The p values less than 0.05 were considered as statistically significant.

Results

Down Syndrome Candidate Region 1 Isoform 1L shRNAs inhibited angiogenesis in vivo.

In order to facilitate the studies in the human cells and the mouse angiogenesis model *in vivo*, we designed three shRNAs, hu-shD1L, mu-shD1L and hu/mu-shD1L, that specifically knocked down the DSCR1–1L expression in the species of human, mouse, and both of human and mouse, respectively. RNA was isolated from 1) HUVECs that were transduced with shGFP as a control, hu-shD1L and hu/mu-shD1L, and 2) mouse melanoma B16F1 cells used to test the specificity of siRNA targeting the mouse species were transduced with shGFP as a control, hu/mu-shD1L and mu-shD1L. The Realtime PCR data showed that the DSCR1–1L mRNA was greatly knocked down in HUVECs by hu-shD1L and hu/mu-shD1L, and in B16F1 cells by hu/mu-shD1L and mu-shD1L (Fig. 1A). We further tested the specificity of the DSCR1–1L shRNAs. Immunoblotting with the DSCR1–1L antibody was carried out on cellular extracts that were isolated from human melanoma A375 and mouse melanoma B16F1 cells transduced with shGFP as a control, hu-shD1L, mu-shD1L and hu/mu-shD1L. As shown in Figure 1B, hu-shD1L greatly inhibited the DSCR1–1L expression in human A375sm cells, but not in mouse B16F1 cells; mu-shD1L inhibited the DSCR1–1L expression in B16F1 cells, but not in A375sm cells; hu/mu-shD1L knocked down the DSCR1–1L expression in both A375sm and B16F1 cells (Fig. 1B).

In the Ad-VEGF-induced angiogenesis in mouse ears or other normal tissues, the different subtypes of abnormal blood vessels identified in human tumors were reproduced (38). The blood vessels induced by Ad-VEGF are more amenable to analyze and visible than that in a tumor system. Mouse ears were injected i.d. with Ad-VEGF plus adenoviruses expressing shGFP as a control, mu-shD1L or hu/mu-shD1L as indicated. Angiogenesis was readily detected in the mouse ears injected with VEGF + shGFP, but was greatly inhibited in the presence of mu-shD1L or hu/mu-shD1L (Fig.1C). The RNA was isolated and then subjected to RT-PCR with the specific DSCR1–1L primers. The DSCR1–1L mRNA was highly induced by VEGF, and the induction was greatly inhibited in the presence of mu-shD1L and hu/mu-shD1L (Fig.1D). The ear tissues were collected, fixed and immuno-stained with an antibody against CD31. The vessel numbers were counted. The vessel density was greatly reduced in the ears treated with mu-shD1L and hu/mu-shD1L (Fig.1E, top panels of panel I, and panel II). Further, the ear thickness (arrows on the bottom panels of panel I) that correlated to edema was measured and was found significantly reduced in ears treated with mu-shD1L or hu/mu-shD1L (Fig.1E bottom panels of panel I, and panel III). These data clearly indicated that DSCR1–1L shRNAs inhibited angiogenesis and its associated edema *in vivo*.

Overexpression of DSCR1–1L in mouse endothelium induced angiogenesis and its associated microvessel permeability.

The transgenic TRE-FH-DSCR1–1L mice were generated as described in the section of Material and method and outlined in Figure 2 (Fig.2A). Six out of 70 screened mice were identified to contain the transgene, among which, mouse clones 11, 12 and 15 were used to generate EC-FH-DSCR1–1L double transgenic mice (Fig.2B). The RNA was isolated from the hearts of EC-FH-DSCR1–1L mice and their control littermates 6 days after tetracycline was withdrawn from drinking water, and was subjected to RT-PCR. The expression of DSCR1–1L mRNA was increased about 2.5 × fold in the EC-FH-DSCR1–1L mice as compared to that of their control littermates (Fig.2C, left panel). The increase of DSCR1–1L in the transgenic mice was comparable to the endogenous DSCR1–1L levels in HUVECs induced by VEGF (Fig.2C, right panel). The heart tissues were immuno-stained with the antibodies against Flag and the vessel markers, CD31 and lectin. The expression of Flag-fused protein was readily detected in the vasculature of the heart tissues in the EC-FH-DSCR1–1L mice, but not in the control mice (Fig.2D, b vs. a). The vessel density and the average vessel area by analysis of the lectin staining were increased in the hearts of the EC-FH-DSCR1–1L mice as compared to that of the control wild type mice (Fig.2D, d vs. c, f vs. e; and panels g and h). Most recently, we reported that DSCR1–1L was induced by angiogenic factors with microvessel permeability, including VEGF-A^{165}, VEGF-A^{121} and histamine, but not those without microvessel permeability, including bFGF, PlGF and PDGF (25). Further, the results from previous section Figure 1E suggested that shDSCR1–1Ls inhibited edema. Therefore, we examined the microvessel permeability in EC-FH-DSCR1–1L mice. Evan's blue was injected i.v. via the tail vein to the EC-FH-DSCR1–1L mice and their wildtype control littermates 6 days after tetracycline was withdrawn. HBSS and VEGF were injected i.d. in the mouse flank skin. Half an hour after the Evan's blue injection, the tissues were dissected and photographed. The data showed that the basal level of tracer accumulation in the lungs (a and b), mesenteries (c and d), and flank skin (e and f), and the

acute level of tracer accumulation induced by VEGF (g and h) were increased in the EC-FH-DSCR1–1L mice (Fig.2E). Therefore, overexpression of DSCR1–1L in mouse endothelium was sufficient to induce angiogenesis and its associated microvessel permeability.

DSCR1–1L regulated angiogenesis via down-regulation of the VE-cadherin expression.

The above studies clearly indicated that DSCR1–1L regulated angiogenesis in the early stage by modification of proliferation and migration. In adult vessels, vascular integrity is maintained by endothelial cell-endothelial cell (EC-EC) junctions. EC junction proteins had also been implicated in altering vascular permeability and cell migration (41–43). Cellular proteins were isolated from HUVECs that were transduced as indicated. The immunoblot analysis indicated that the expression of DSCR1–1L cDNA and shRNAs greatly down-regulated and up-regulated the protein level of VE-cadherin, respectively (Fig.3A). In the RNA isolated from the HUVECs that were transduced with DSCR1–1L cDNA and shRNA, the VE-cadherin mRNA was also down-regulated and up-regulated by DSCR1–1L cDNA and shRNA, respectively (Fig.3B). Then, we studied whether DSCR1–1L regulated HUVECs proliferation and migration through the down-regulation of VE-cadherin. We used the loss-of-function assay by preventing the down-regulation of VE-cadherin with the overexpression of its cDNA (44). The HUVECs expressed DSCR1–1L were transfected with the retroviruses expressing the VE-cadherin cDNA and Lac Z as control, and subjected to proliferation and migration assays. The overexpression of VE-cadherin cDNA significantly inhibited the proliferation and migration in HUVECs induced by DSCR1–1L (Fig.3C). Our data clearly indicated that DSCR1–1L down-regulated the expression of VE-cadherin, and that VE-cadherin played important roles in the DSCR1–1L-induced angiogenesis.

DSCR1–1L did not regulate the mRNA stability and the 3’UTR activity of VE-cadherin, although it localized in cytosol.

So far, nothing was known about the molecular feature of DSCR1–1L, although we found that DSCR1–1L increased NFAT transcriptional activity (21). Therefore, we examined the cellular localization of DSCR1–1L. The serum-starved HUVECs were stimulated with or without VEGF, fixed and immunostained with the DSCR1–1L antibody. DSCR1–1L was detected in the cytosol, but not in the nuclei (Fig.4A). To further confirm the cytosolic localization of DSCR1–1L, we isolated the cytosolic and nuclear fractions from HUVECs. The immunoblot analysis with a DSCR1–1L antibody showed that DSCR1–1L localized in the cytosolic, but not in the nuclear, fraction (Fig.4B, top panel). The cytosolic and nuclear fractions were confirmed by immunoblotting with the antibodies against α-tubulin and c-Jun that were markers for cytosolic and nuclear fractions, respectively (Fig.4B, middle and bottom panels). Our data indicated that DSCR1–1L localized in the cytosol.

RNA was isolated from HUVECs that were transduced with Lac Z as control or DSCR1–1L, and then treated with or without actinomycin-D, a transcription inhibitor. With RT-PCR analysis, DSCR1–1L down-regulated VE-cadherin RNA in the absence of actinomycin-D (Fig.4C, 1 vs. 2). However, in the presence of actinomycin-D, the VE-cadherin mRNA in control Lac Z expressing cells was decreased to the level similar to that in the HUVECs expressing DSCR1–1L without the actinomycin-D treatment (Fig.4C, 3 vs. 2). There was no difference in the VE-cadherin expression in the cells expressing Lac Z and

DSCR1–1L in the presence of actinomycin-D (Fig.4C, 3 vs. 4). These data suggested that DSCR1–1L regulated transcription but not mRNA degradation of VE-cadherin. To further confirm that DSCR1–1L did not regulate VE-cadherin mRNA stability, we studied whether DSCR1–1L regulated the activity of mRNA 3'untranslated region (3'UTR) that controlled mRNA stability. HUVECs were transfected with a SV40 promoter luciferase reporter (pGL3-reporter) plus an internal reporter control plasmid for equal transfection, then infected with the adenoviruses expressing Lac Z as control and DSCR1–1L cDNA. The cells were lysed and subjected to luciferase analysis with a Dual-Luciferase® Reporter Assay System, following instructions provided by the company. The promoters' luciferase activities in each sample were normalized to the internal luciferase activity. The data were expressed as the relationship of luciferase activity in DSCR1–1L-expressing cells to that in Lac Z-expressing cells in terms of average fold changes. DSCR1–1L decreased the luciferase activity regulated by the SV40 promoter (Fig.4D, lane 2 vs. 1), indicating that the SV40 promoter could not be used to study the 3'UTR activity regulated by DSCR1–1L. We constructed the luciferase reporter containing VE-cadherin basic promoter (VEp-341). After being transfected to the HUVECs expressing DSCR1–1L or Lac Z as control, VEp-341 was not regulated by DSCR1–1L (Fig.4D, lane 3 vs. lane 4). Then, the 3'UTR of VE-cadherin was cloned to the VEp-341 luciferase reporter to obtain a VEp-341+3'UTR reporter. The HUVECs that were transduced with DSCR1–1L or Lac Z as control were transfected with the VEp-341+3'UTR reporter together with an internal reporter control plasmid. There was no significant difference in the luciferase activity of VEp-341+3'UTR in the HUVECs transduced with DSCR1–1L or Lac Z as control (Fig.4D, lane 5 vs. 6). The data indicated that DSCR1–1L did not regulate the mRNA stability and the 3'UTR activity of VE-cadherin.

DSCR1–1L downregulated VE-cadherin expression by targeting transcription via 3 transcriptional cis DNA elements in VE-cadherin promoter.

A VE-cadherin promoter luciferase reporter, pGL3-VEp-1031/93, was cloned, which contained the DNA sequences between −1031 and +93 related to the transcription starting site of VE-cadherin. The pGL3-VEp-1031/93 and an internal luciferase control were transfected to HUVECs expressing Lac Z as a control and DSCR1–1L. DSCR1–1L significantly down-regulated the pGL3-VEp-1031/93 activities about 50% (Fig.5A, bars 1). Then, a serial of deletion mutants, pGL3-VEp-823/93, pGL3-VEp-581/93, pGL3-VEp-511/93, pGL3-VEp-456/93, and pGL3-VEp-391/93, were cloned, which contained the promoter regions of −823, −581, −511, −456, and −391, respectively (Fig.5A). The down-regulation of VE-cadherin promoter activity by DSCR1–1L was decreased from 50% in pGL3-VEp-1031/93 to 10% in pGL3-VEp-823/93 and pGL3-VEp-581/93 (Fig.5A, bars 1 vs. 2 and 3). Hence, there was a DSCR1–1L targeting site within the region between −1031 and −823. The pGL3-VEp-511/93 did not respond to DSCR1–1L regulation, indicating that another DSCR1–1L targeting site located in the region between −581 and −511 (Fig.5A, bars 4). Furthermore, the pGL3-VEp-456/93 maintained about a 20% down-regulating response to DSCR1–1L, which was nullified through more deletions as in the pGL3-VEp-391/93, suggesting that there were negative and positive regulation sites in the regions of −551/−456 and - 456/−391, respectively (Fig.5A, bars 5, 6 and 7). These data showed that there were three different DSCR1–1L-targeted segments, −1031/−823, −581/−511 and −456/−391, in VE-cadherin promoter.

Because there was a significant reduction in response to DSCR1–1L regulation between pGL3-VEp-1031/93 and pGL3-VEp-823/93, two mutants, pGL3-VEp-958/93 and pGL3-VEp-888/93, were cloned. While the pGL3-VEp-958/93 mutant contained about 50% down-regulation activity in responding to DSCR1–1L, the pGL3-VEp-888/93 mutant lost the response to DSCR1–1L (Fig.5B, top panel). Then, mutants of pGL3-VEp-938/93 and pGL3-VEp-908/93 were cloned. Both of the pGL3-VEp-938/93 and pGL3-VEp-908/93 mutants had no response to DSCR1–1L, indicating that the DSCR1–1L targeting site might be located within the DNA region between −958 and −938 (Fig.5B, middle panel). Analysis of potential transcription factor binding sites suggested that this DNA region between −958 and −938 of the VE-cadherin promoter contained a putative binding site for the transcription factor, sterol regulatory element-binding protein (SREBP). To further confirm that the putative SREBP site between −958 and-938 in VE-cadherin promoter contained the DSCR1–1L responding activity, a mutant pGL3-VEp-341+3×SREBP was cloned, which contained three repeats of the −958 and-938 sequence upstream of the VEp-341. DSCR1–1L was unable to down-regulate the luciferase activity of Vep-341, but decreased the luciferase activity of VEp341+3×SREBP (Fig.5B, bottom panel). The data indicated that SREBP was required and sufficient for the response of VE-cadherin promoter to DSCR1–1L.

To study the DNA fragments between −581 to −511 of VE-cadherin promoter, pGL3-VEp-541/93 was cloned. DSCR1–1L was unable to down-regulate pGL3-VEp-541/93 luciferase activity (Fig. 5C, top panel). The data suggested that the DSCR1–1L targeting element localized between −581 to −541, within which, pGL3-VEp-561/93 was cloned. DSCR1–1L downregulated the pGL3VEp-561/93 luciferase activity (Fig.5C, middle panel). The data indicated that the 21 oligonucleotides of −561 and −541 in the VE-cadherin promoter was the DSCR1–1L targeting site. But none similar DNA sequence was found in the transcriptional data bases. Therefore, we named this DNA fragment as DSCR1–1L targeting region (D1LTR). To test whether D1LTR was sufficient to regulate DSCR1–1L activity, we cloned pGL3-VEp-341+3×D1LTR that contained three repeats of D1LTR sequence upstream of the VEp-341. DSCR1–1L decreased the luciferase activity of VEp341+3×D1LTR, but not Vep-341 (Fig.5C, bottom panel). Therefore, a novel transcriptional cis element, D1LTR, was discovered, which was required and sufficient for the response of VE-cadherin promoter to DSCR1–1L.

For the DNA region between −456 and −391 of the VE-cadherin promoter, pGL3-VEp-421/93 was cloned. DSCR1–1L significantly decreased its luciferase activity, suggesting that the DSCR1–1L-targeting element localized between −421 and −391 (Fig.5D, top panel). Then pGL3-VEp-411/93 and pGL3-VEp-401/93 were cloned. pGL3-VEp-411/93 did not response to DSCR1–1L, while pGL3-VEp-401/93 did (Fig.5D, middle panel). Hence, the DSCR1–1L targeting site located between −421 and −411 of VE-cadherin promoter. Data base analysis showed that the DNA element between −421 and −411 of VE-cadherin promoter contained a MyoG binding site. Then the three repeats of MyoG were cloned upstream of Vep-341 to obtain VEp341+3×MyoG. DSCR1–1L significantly decreased the luciferase activity of VEp341+3×MyoG (Fig.5D, bottom panel). Therefore, MyoG was another DNA element in VE-Cadherin promoter regulated by DSCR1–1L.

Identification of a specific transcriptional complex down-regulated by DSCR1–1L

We carried out an electricity mobility shift assay (EMSA). The D1LTR, SREBP, and MyoG oligonucleotides of the VE-cadherin promoters were synthesized, labeled with biotin, annealed, and incubated with the nuclear extracts isolated from HUVECs. A shift band was detected in the presence of the nuclear proteins for each oligo (Fig.6A, lane 1 vs. lanes 2, 9 and 13). The signals of the protein/D1LTR complex was gradually decreased by the increase of the unlabeled wild type oligonucleotides in the protein/DNA mixtures, but not affected by the mutant oligonucleotides (Fig.6A, lanes 2–8). Thus, D1LTR was specifically interacted with protein(s). However, for the MyoG/protein complexes, both the wild type and mutant unlabeled probes were able to decrease the intensity of the shifted band, indicating that this MyoG/protein complex was not specific (Fig.6A, lanes 9–11). This non-specific MyoG/protein complex was further confirmed by the inclusion of ssDNA (Fig.6A, lane 12). Similarly, the SREBP/protein complex was found non-specific with ssDNA (Fig.6A, lane 14). Then, we studied whether DSCR1–1L regulated the formation of the D1LTR/protein complex. The nuclear extracts isolated from the HUVECs that were transduced with or without Lac Z as a control and DSCR1–1L were used for the EMSA with the D1LTR oligonucleotides. The data showed that the DSCR1–1L expression reduced the formation of the D1LTR/protein complex (Fig.6A, lanes 16 and 17). Therefore, DSCR1–1L decreased the formation of a specific D1LTR/protein complex, but the specific MyoG/protein and SREBP/protein complexes were not found by this method.

The fluorescent 6-carboxy-fluorescein (FAM) labeled probes were incubated with and without nuclear proteins, and then partially digested with DNase. The purified oligo mixtures were sequenced. The signals corresponding to the DNA sequence between −556 and −551 (CTTCTG) of the VE-cadherin promoter were greatly reduced, indicating that the oligonucleotides, CTTCTG, were protected from DNase digestion by nuclear proteins (Fig.6B). Therefore, oligonucleotide sequence of CTTCTG directly interacted with protein.

Discussion

So far, most studies about DSCR1 gene have been focused on DSCR1–4. Previously, we reported that DSCR1–1L was upregulated by angiogenic factors, including VEGF and histamine, and was expressed in the vasculature of human cancer tissues, but was not detectable in the normal vessels (21). Further, knocking down DSCR1–1L inhibited HUVEC proliferation and migration induced by VEGF and histamine (21,25). These findings suggested that DSCR1–1L played a role in angiogenesis. In this study, we found that knocking down the expression of DSCR1–1L with novel DSCR1–1L shRNAs inhibited the angiogenesis induced by VEGF in mice. Further, the expression of DSCR1–1L in mouse endothelium was sufficient to induce angiogenesis and its associated microvessel permeability. With this novel *in vivo* model, our data strongly confirmed our previous findings that DSCR1–1L was a pro-angiogenic factor (21), and suggested that DSCR1–1L was a therapeutic target for diseases, in which pro-angiogenesis was a promoting factor, including ischemic diseases.

So far, the functions of DSCR1–1L have been overlooked, not even to mention the molecular mechanism underlying it. Previously, we reported that DSCR1–1L regulated

angiogenesis by activating the calcineurin-NFAT axis, unlike DSCR1–4 that inhibited this pathway (21,29–35). Here, we found that DSCR1–1L down-regulated the expression of VE-cadherin mRNA and protein in HUVECs. In the loss-of-function assay, the VE-cadherin overexpression prevented the HUVEC proliferation and migration induced by the expression of DSCR1–1L. Our data indicated that VE-cadherin was another down-stream target of DSCR1–1L.

VE-cadherin, which was involved in various pathological processes, could suppress proliferation, cell motility and the germination of new blood vessels (45). Our results were consistent with previous studies. DSCR1–1L shRNAs up-regulated VE-cadherin to prevent angiogenesis. In quiescent endothelial cells, VE-cadherin bound to VEGFR2 to keep VEGFR2 dephosphorylated, thereby maintaining the quiescent state of cell proliferation (46). In the absence of VE-cadherin, there were persistent pseudopodia in endothelial cells to maintain the movement and migration (47). In addition, VE-cadherin was also involved in transcriptional signaling. It had been found that many VE-cadherin partners (β-catenin, p120-catenin, and plaque globin) could be transported to the nucleus to regulate gene transcription (48). The biological functions of DSCR1–1L by regulating VE-cadherin may contain these downstream mechanisms, which should be verified by further research.

We further found that although DSCR1–1L was a cytosolic protein, it did not regulate the 3’UTR activity of VE-cadherin. These data suggested that DSCR1–1L did not regulate mRNA stability. Instead, we found that the transcription inhibitor actinomycin-D blocked the down-regulation of VE-cadherin induced by DSCR1–1L. Moreover, DSCR1–1L down-regulated the VE-cadherin promoter activity, presumably via three different DNA regions, among which, a specific transcriptional complex containing a novel DNA fragment, D1LTR, was identified. DSCR1–1L inhibited the formation of the D1LTR transcription complex. Since the D1LTR sequence does not have a NFAT binding site, it rules out the possible involvement of NFAT in DSCR1–1L-induced VE-cadherin downregulation. The exact transcription factor(s) that binds to the novel cis-element remains to be identified. The transcriptional complexes containing SREBP and MyoG elements will be further explored in the future.

In summary, DSCR1–1L regulated angiogenesis *in vitro* and *in vivo*, suggesting it may represent a novel therapeutic target for angiogenesis. DSCR1–1L shRNAs will be developed into therapeutics. The advantage of targeting DSCR1–1L as compared to other tools, such as VE-cadherin, is that DSCR1–1L also controls other pathways, such as NFAT (49). The findings that DSCR1–1L down-regulated VE-cadherin by targeting a novel transcriptional complex contribute significantly to the field of mechanism studies. Therefore, these studies have significant impacts on basic research and have translation potentials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3'UTR	3' untranslated region
D1LTR	DSCR1–1L targeting region
DSCR1–1L	Down Syndrome Candidate Region 1 isoform 1L
DSCR1–3	Down Syndrome Candidate Region 1 isoform 3
DSCR1–4	Down Syndrome Candidate Region 1 isoform 4
EMSA	electricity mobility shift assay
FAM	fluorescent 6-carboxy-fluorescein
HUVECs	human umbilical vein endothelial cells
SREBP	sterol regulatory element-binding protein
VEGF	vascular endothelial growth factor
RT-PCR	quantitative real-time reverse transcription-PCR
DMEM	Dulbecco's Modified Eagle Medium
iv	intravenous
id	Intradermal
VE-3'UTR	VE-cadherin 3' untranslated region
MEM	Minimum Essential Media

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Highlights

- Down Syndrome Candidate Region 1 Isoform 1L shRNAs inhibited angiogenesis in vivo.
- overexpression of DSCR1–1L cDNA in mouse endothelium was induce angiogenesis.
- DSCR1–1L down-regulated the VE-cadherin transcription, but not mRNA stability.
- A novel specific transcriptional complex regulated by DSCR1–1L was found.
- The DNA sequence was identified to directly interact with proteins.

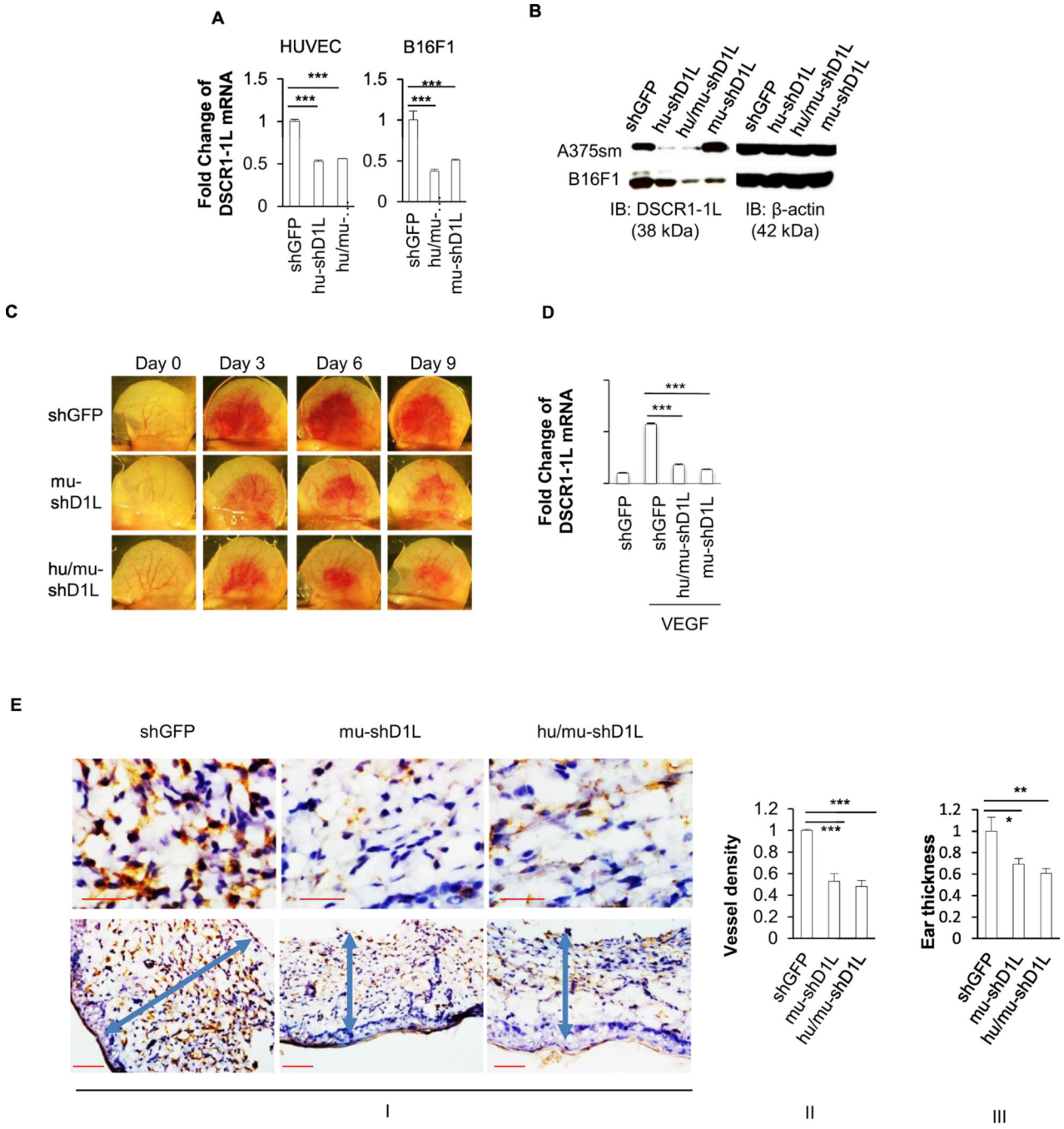

Figure 1. The angiogenesis in mice were inhibited by DSCR1–1L shRNAs.

A) The RNA isolated from the HUVECs (left panel) and the B16F1 cells (right panel) that were transduced as indicated were subjected to RT-PCR with the specific human (left panel) and mouse (right panel) DSCR1–1L specific primers (n=4); **B)** The cellular extracts isolated from the human A375sm cells (top panels) and the mouse B16F1 cells (bottom panels) that were transduced with the viruses expressing shGFP as a control, hu-shD1L, hu/mu-shD1L, and mu-shD1L were subjected to immunoblotting with the antibodies against DSCR1–1L (left panel), and β-actin as the protein equal loading control (right panel); **C)**

Nu/Nu mouse ears were injected s.c. with the adenovirus mixture containing VEGF+shGFP (top panels), VEGF+mu-shD1L (middle panels) and VEGF+hu/mu-shD1L (bottom panels). The ears were photographed on days 0, 3, 6 and 9. Data represent one of 4 mouse ears in each group, all of which exhibited similar pattern; **D)** RNA were isolated from the ears and subjected to RT-PCR with the DSCR1–1L specific primers (right panel, n=4); **E)** The ear tissues were collected and fixed for immunohistochemically staining with CD31 antibody. Magnification bars are 50 μm and 200 for the top and bottom panels, respectively (panel I). (Data represent one of 20 views per group). The vessel density (panel II) and the thickness of ears (panel III) were measured and plotted (n=20 views from 4 mice in each group); *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. All experiments were repeated three times.

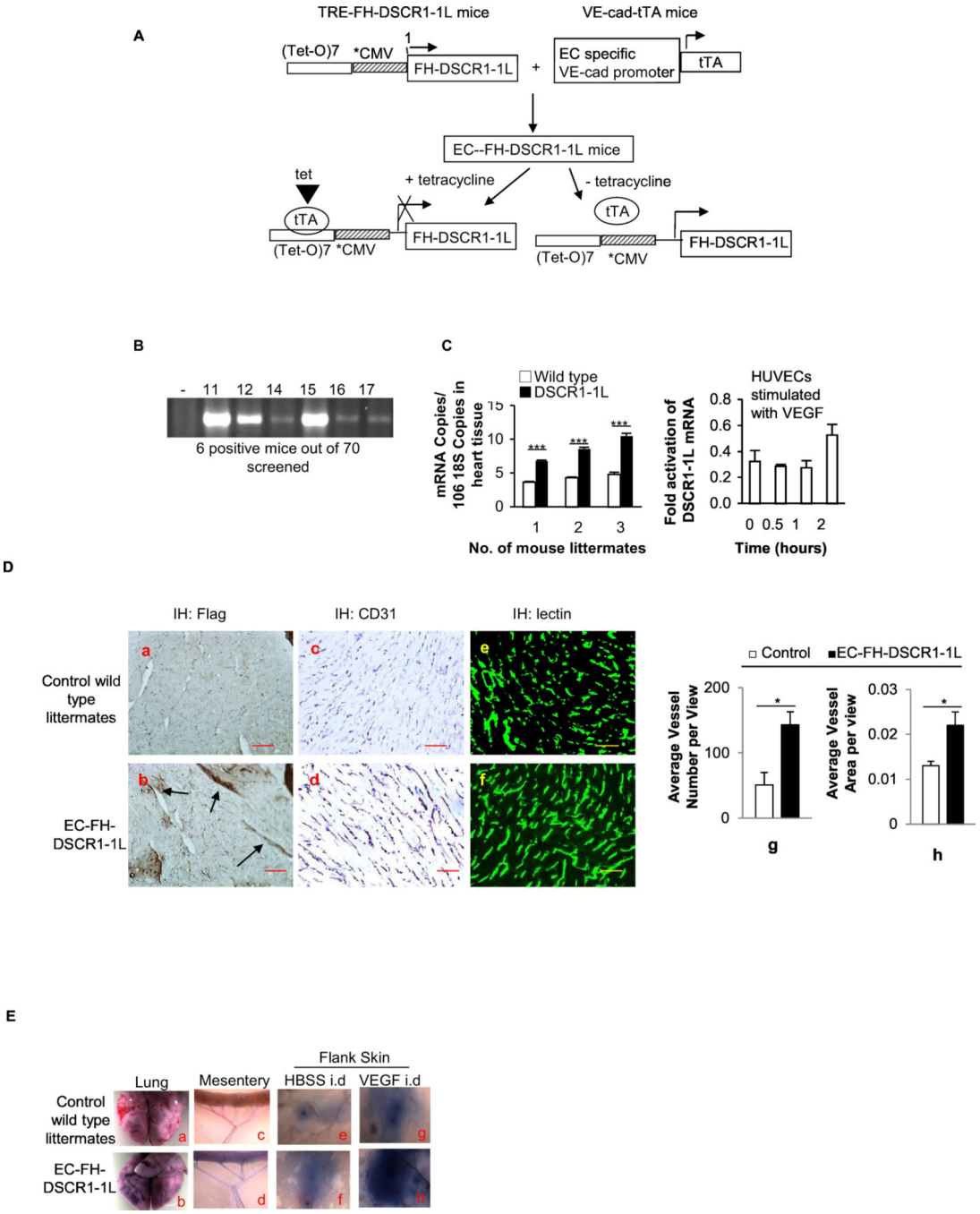

Figure 2. The expression of DSCR1–1L in mouse endothelium induced angiogenesis and microvessel permeability.

A) Diagram to illustrate the strategy for the generation of inducible transgenic mice; **B)** The gel electrophoresis of PCR products with the genomic DNA as the template and the primers that amply the transgene; **C)** The RT-PCR with the RNA isolated from the transgenic mice and the wild type control mice (left panel) and the HUVECs stimulated with VEGF (right panel). n=3 for Realtime PCR. **D)** Immunohistochemically staining of FH-DSCR1–1L protein (a and b), CD31 (c and d), and lectin (e and f) in the heart tissues of the wild type

mice (top panels) and the EC-FH-DSCR1–1L mice (bottom panels). Magnification bars are 50 μm. The vessel density (panel g) and the vessel area (panel h) were measured and plotted using the data of lectin staining (n=40 from 4 mice / group); **E)** The macroscopic images of lungs (a, b), mesentery (c, d), and flank skin without (e, f) and with VEGF treatment (g, h). *$p < 0.05$, ***$p < 0.001$.

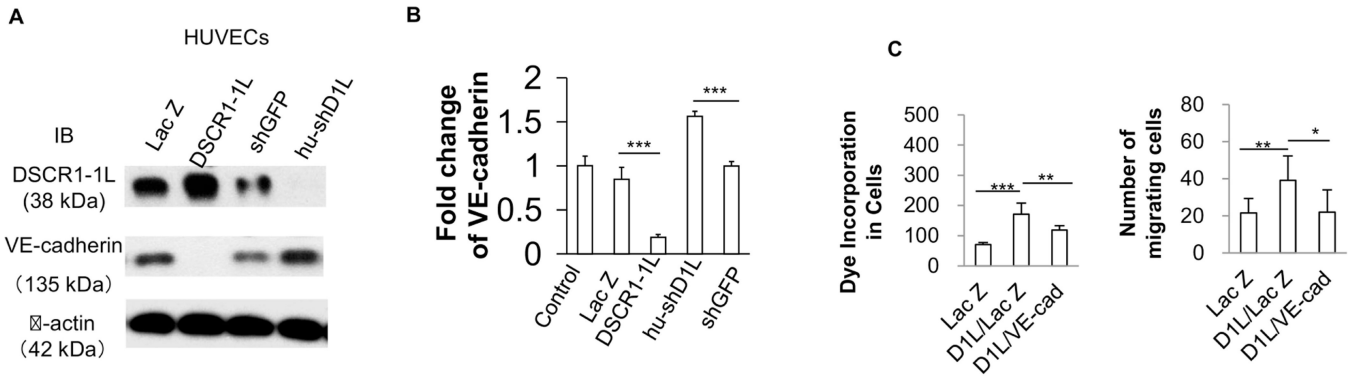

Figure 3. VE-cadherin was a downstream target of DSCR1–1L.

A) The cellular extracts isolated from the HUVECs that were transduced with Lac Z as a control, FH-DSCR1–1L, shGFP as a control for shRNAs, and hu-shD1L were subjected to Immunoblotting with the antibodies against DSCR1–1L (top panel), VE-cadherin (middle panel) and β-actin as the equal protein loading control (bottom panel); **B)** The RNA isolated from the HUVECs that were transduced with Lac Z as a control, FH-DSCR1–1L, hu-shD1L and shGFP as a control were subjected to RT-PCR with the VE-cadherin specific primers (n=4); **C)** HUVECs were transduced as indicated and subjected to proliferation assay (left panel, n=6) and monolayer migration assay (right panel, n=8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The experiments were repeated three times.

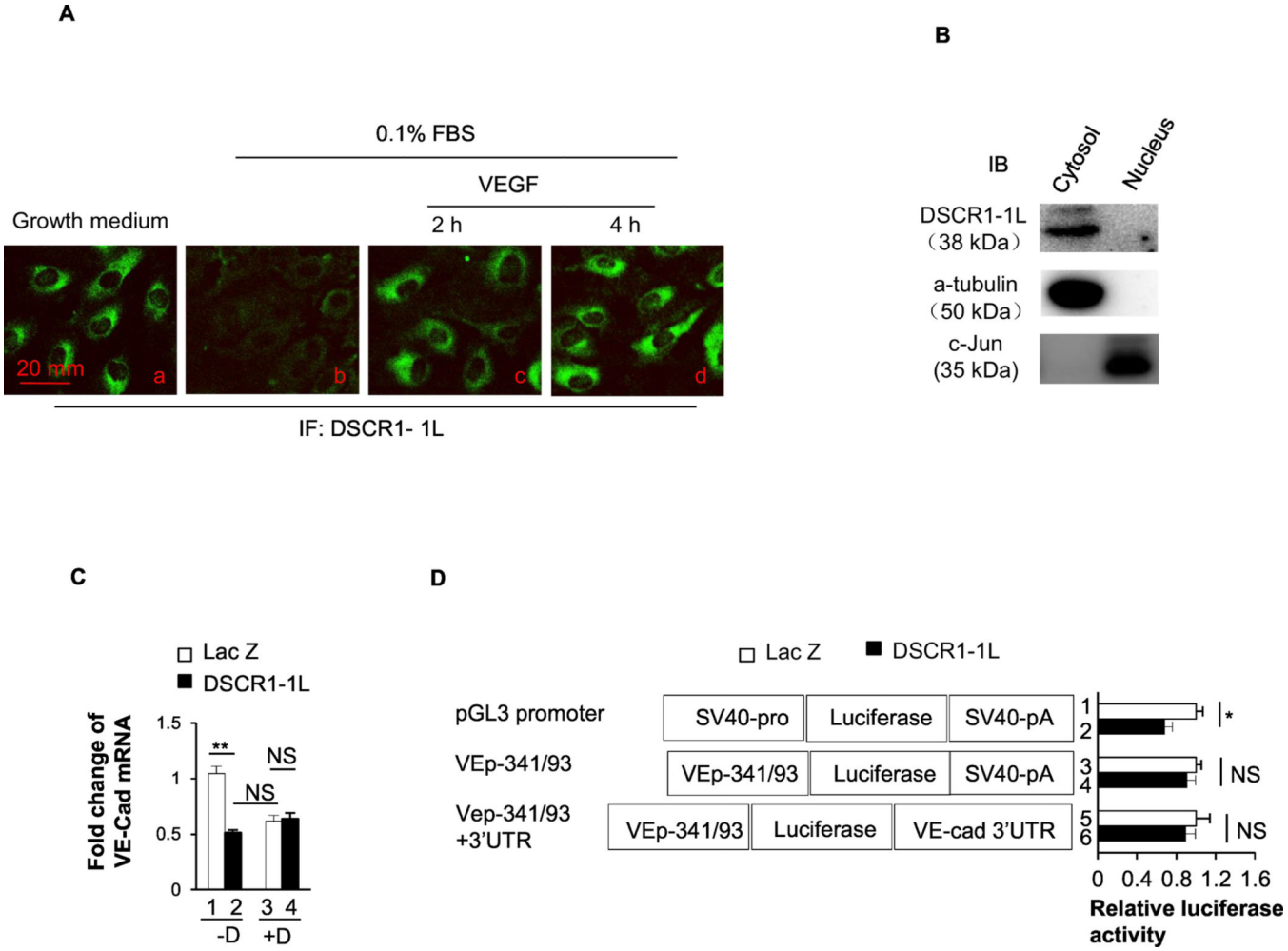

Figure 4. DSCR1–1L was a cytosolic protein, but did not regulate the mRNA stability and the 3'UTR activity of VE-cadherin.

A) HUVECs were grown in growth medium (a). Quiescent HUVECs were treated with vehicle (b) or VEGF for 2 hours (c) or 4 hours (d). The cells were then immunostained with a DSCR1–1L antibody; **B)** Cytosolic and nuclear fractions were isolated from HUVECs and subjected to immunoblot with the antibodies against DSCR1–1L (top panel), cytosolic marker tubulin (middle panel) and nuclear marker c-Jun (bottom panel); **C)** The RT-PCR with VE-cadherin specific primers and the RNA isolated from the HUVECs expressing Lac Z as a control (unfilled bars) or DSCR1–1L (filled bars), and treated with (+D) or without (-D) actinomycin-D (n=3 for Realtime PCR); **D)** The luciferase assays from the HUVECs that were transduced with Lac Z (unfilled bars) or DSCR1–1L (filled bars), and then transfected as indicated (n=4). *p<0.05, **p<0.01, NS, no significant difference. The experiments were repeated three times.

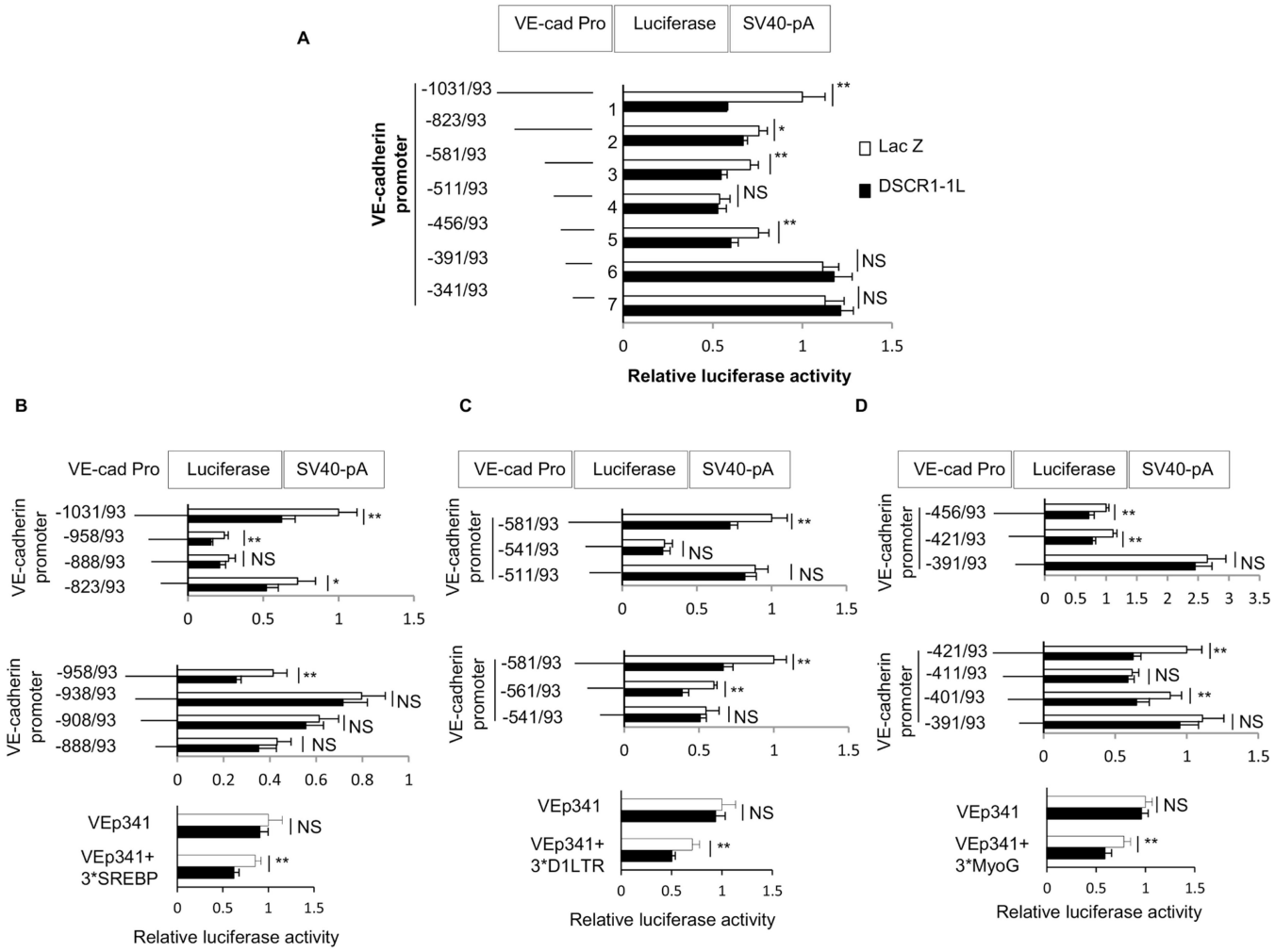

Figure 5. DSCR1–1L inhibited VE-cadherin transcription.

A) - D) The luciferase assays from the HUVECs that were transfected as indicated, and then transduced with Lac Z as a control (unfilled bars) or DSCR1–1L (filled bars) (n=4). *p<0.05, **p<0.01, NS, no significant difference. The experiments were repeated three times.

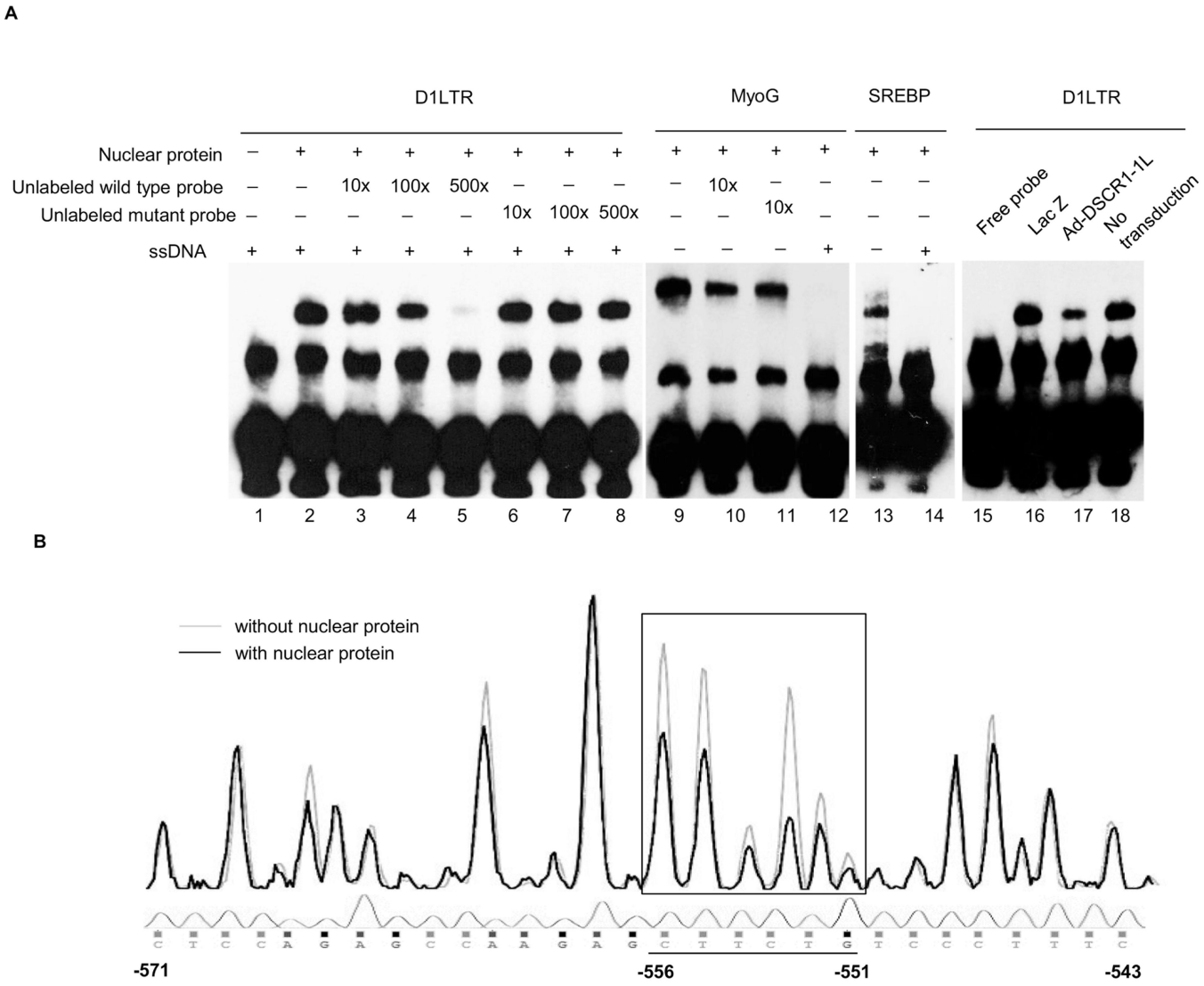

Figure 6. Identification of a specific transcriptional complex down-regulated by DSCR11L.

A) Electrophoresis mobility shift assay (EMSA); **B)** DNase footprint assay. Data represent 3 independent experiments.