Efficient Regulation of VEGF Expression by Promoter-Targeted Lentiviral shRNAs Based on Epigenetic Mechanism
A Novel Example of Epigenetherapy

Mikko P. Turunen, Tiia Lehtola, Suvi E. Heinonen, Genet S. Assefa, Petra Korpisalo, Roseanne Girnary, Christopher K. Glass, Sami Väisänen, Seppo Ylä-Herttuala

Rationale: We studied a possibility that shRNAs can lead to transcriptional gene activation at the promoter level via epigenetic mechanism.

Objective: The purpose of this study was to test the effects on vascular endothelial growth factor (VEGF-A) expression by promoter targeted small hairpin RNAs (shRNAs) in vitro and in experimental animals in vivo using stable local lentiviral gene transfer.

Methods and Results: One shRNA was identified which strongly increased VEGF-A expression in C166 endothelial cells at mRNA and protein level whereas another shRNA decreased VEGF-A expression. Quantitative chromatin immunoprecipitation analysis revealed that the repressing shRNA caused epigenetic changes, which increased nucleosome density within the promoter and transcription start site and led to repression of VEGF-A expression. Epigenetic changes caused by the activating shRNA were opposite to those caused by the repressing shRNA. These results were confirmed in vivo in an ischemic mouse hindlimb model after local gene transfer where VEGF-A upregulation achieved by promoter-targeted shRNA increased vascularity and blood flow.

Conclusions: We show that lentivirus-mediated delivery of shRNA molecules targeted to specific regions in the mVEGF-A promoter either induce or repress VEGF-A expression via epigenetic modulation. Thus, we describe a new approach of gene therapy, epigenetherapy, based on an epigenetic mechanism at the promoter level. Controlling transcription through manipulation of specific epigenetic marks provides a novel approach for the treatment of several diseases. (Circ Res. 2009;105:604-609.)

Key Words: endothelial cells | gene regulation | gene therapy | transcription factors | transcriptional regulation | vascular endothelial growth factor

Small RNAs have been studied in lower organisms for a long period of time, but only recently their importance in mammals has been recognized. In human cells, RNA-directed transcriptional gene silencing (TGS) can be initiated through small interfering RNAa (siRNAs) and requires Argonautes (Ago) 1 and 2.1 However, it is not known whether these mechanisms are also functional in normal and pathological tissues in vivo and what mechanisms are involved in siRNA-mediated TGS. It has been proposed that antisense strands of siRNAs have a role in writing the histone code and regulation of gene expression by interaction with promoter-associated RNA variants that are present at the promoter sites.2 An alternative mechanism has recently been described in which noncoding promoter-specific transcripts function to recruit an RNA binding protein, which in turn inhibits CREB-binding protein (CBP)/p300 histone acetyltransferase activity.3 Interestingly, activation of gene transcription by small RNAs in human cells has also been reported,4,5 and RNA-directed DNA methylation seems to require dsRNAs that are cleaved into small RNAs similar to those that guide mRNA degradation in RNA interference (RNAi).6

We wanted to evaluate the possibility to modulate vascular endothelial growth factor (VEGF-A) expression by designing small hairpin RNAs (shRNAs) that are complementary to different regions at VEGF-A promoter and test their functionality in vitro and in experimental animals in vivo by stable local lentiviral gene transfer. VEGF-A was selected as the target gene because it is an important factor in many
To analyze whether alterations in the VEGF-A production correlated with mRNA levels, we analyzed C166 cells by TaqMan RT-PCR, which showed a similar profile of VEGF-A mRNA expression (Figure 1c). Morphological changes were also observed. C166 cells gradually lost adherence and ability to grow when transduced with LV-856 (data not shown). Because autocrine VEGF-A signaling is required for endothelial cell survival, the observed strong growth arrest and morphological changes caused by inhibition of VEGF-A production is not surprising.

shRNA Targeting Alters Chromatin Activity Both at the mVEGF Promoter and Coding Region

To determine whether the shRNA targeting resulted in epigenetic changes within the VEGF-A promoter in C166 cells, we performed chromatin immunoprecipitation (ChIP) analysis using antibodies against H3K4me2, H3K9me2, and H3K9ac (Online Figure Ia through Ic). The targeted regions as well as the TSS of the VEGF-A gene were studied with respect to changes in histone modifications. The promoter and coding region of the VEGF-A gene in untreated C166 cells were highly methylated at H3K4 and acetylated at H3K9 (Online Figure Ib). On the other hand, H3K9 methylation levels at both promoter and coding region were nearly undetectable. These data suggest that in untreated C166 cells, chromatin within the VEGF-A promoter is readily accessible to transcription factors, which regulate VEGF-A transcription. LV-856 caused demethylation of H3K4me2 and deacetylation of H3K9ac within the targeted promoter region and TSS, but had no detectable effects on H3K9me2 level within the studied regions. Interestingly, LV-451 did not affect the level of H3K4me2 within the targeted promoter region, but increased the level of H3K4me2 at TSS to some extent. No other effects on the histone modifications could be observed attributable to LV-451. LV-599 had no effects on the chromatin activity either at the promoter or at the coding region.

In MS1 cells, ChIP analysis revealed that histone acetylation and methylation levels within the VEGF-A promoter and coding region were very similar to those of C166 cells. However, LV-856 and LV-451 did not result in any detectable changes in chromatin activity (Online Figure Ic). Thus, LV-856

Methods

An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Vector Constructs

The shRNA sequences were designed by using Dharmagon siDESIGN Center algorithm (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx). The chosen target sequences were: CGT-TCTAGTGGCACAATA (LV-451), CTGCCGACACTCAAGAATCATCA (LV-599), GACGCCGTGGTCTCCATGTGA (LV-856), and CAAAGAGAGAGAGAGAGAGAGAGAGAGA (LV-353). The number in parentheses refers to the first nucleotide in the sequence of the shRNA relative to the transcription start site (TSS) of mouse VEGF-A promoter (U41383) (Figure 1a). As controls we used a mis-matched sequence that lacks any similarity to the murine sequence and a lentivirus (LV) encoding only green fluorescent protein (GFP) without an shRNA cassette. The third generation human immunodeficiency virus 1 (HIV-1)-based LV-PGK-GFP-U6shRNA vectors were prepared by standard calcium phosphate transfection method in 293T cells as described.

Results

shRNAs Targeting Distal and Proximal mVEGF-A Promoter Affect Gene Expression

C166 cells (yolk sac derived mouse endothelial cells) were transduced with LVs encoding shRNAs targeting different areas of the mouse VEGF-A promoter. As monitored by ELISA assay (Figure 1b), LV-856 caused a major decrease in VEGF-A production. On the other hand, LV-451 caused a substantial increase in VEGF-A expression. Other constructs did not show any major effects on the VEGF-A production. To analyze whether alterations in the VEGF-A production correlated with mRNA levels, we analyzed C166 cells by TaqMan RT-PCR, which showed a similar profile of VEGF-A mRNA expression (Figure 1c).
and LV-451 resulted in epigenetic changes within the VEGF-A promoter and TSS in C166 cells but not in MS1 cells.

These findings directed us to study more precisely the distal and proximal promoter and TSS of the VEGF-A gene. For this purpose, a set of new PCR primers and hydrolysis probes were designed which covered the locations −856, −451, and TSS (Online Table II), and quantitative ChIP (qChIP) assays were performed for C166 cells. In addition to the above covalent histone modifications, H3K4me3 and H3K27me3 were studied. Moreover, nucleosome positioning was studied by using an antibody targeted against the C terminus of histone H3 (H3CT). According to our results, LV-856 resulted in an enrichment of nucleosomes within the distal and proximal promoter as well as TSS. LV-451 had no effects on the nucleosome enrichment (Online Figure II).

To find out how fast the epigenetic changes took place within VEGF-A gene after transductions, we studied the H3K4me2 levels after multiple treatment times (Online Figure III). According to our data, the LV-856 decreased the H3K4me2 level already in 14 hours after transductions both at the distal promoter and TSS and the repressive effect remained visible also in 24 hours and 7 days after transductions. The LV-451 caused increased H3K4me2 level in 14 hours after transductions at TSS, but not at the proximal promoter. In 24 hours after transductions, the activating effect was significant both at the promoter and TSS and in 7 days the H3K4me2 level was further increased at TSS but decreased at the proximal promoter.

Taken together, these data suggest that LV-856 represses VEGF-A transcription by decreasing H3K4me2 and H3K9ac levels and increasing H3K9me2 and H3K27me3 levels, which results in a higher nucleosome density. Instead, LV-451 results in an increased H3K4me2 and H3K4me3 levels and a more accessible chromatin, which facilitates increased transcription.

**shRNA Targeting Attracts Transcription Factors to the mVEGF-A Promoter**

Our ChIP results suggested that LV-856 and LV-451 recruited histone modifying proteins to the promoter. To verify this, we examined the recruitment of Ago2, histone demethylase lysine-specific demethylase 1 (LSD1), steroid receptor coactivator 1 (SRC-1), and histone acetyltransferase CBP to the proximal and distal promoter as well as to TSS by using qChIP assays. Our results suggested that LV-856 recruited Ago2 to the targeted promoter region and both LV-856 and LV-451 resulted in the recruitment of Ago2 to TSS (Online Figure IV). LV-856 clearly decreased the recruitment of both SRC-1 and CBP to the distal promoter and TSS. LV-451 increased the recruitment of both SRC-1 and CBP at proximal promoter and TSS. LV-856 caused a significant increase in LSD1 levels both at the targeted promoter region and TSS. Instead, LV-451 had no effect on the LSD1 recruitment.

These data suggested that Ago2 is important for both shRNA-mediated upregulation and downregulation of VEGF-A in C166 cells. LV-451 seems to promote recruitment of activating transcription factors to the promoter and TSS, which results in increased VEGF-A expression. The repressive effects of LV-856 are achieved via increased recruitment of LSD1 and decreased recruitment of activating transcription factors to the promoter and TSS of VEGF-A.

**shRNA Targeting Results in Epigenetic Changes In Vivo**

Although the results obtained in cultured cells were very promising, a question still remained regarding whether shRNA targeting could have any effects on mVEGF-A transcription in vivo. To answer this question, we injected LV-856, LV-451, and LV-GFP control vector to mouse hindlimbs which were made ischemic at the time of the injection.9 ELISA analysis from the skeletal muscles showed the same effect as was seen in C166 cells (Figure 2a). Also, TaqMan RT-PCR showed a similar profile of VEGF-A mRNA expression from skeletal muscles as was seen in C166 cells (Figure 2b).

To analyze whether shRNA-mediated epigenetic modulation of the VEGF-A promoter was present also in vivo, we performed qChIP analysis using skeletal muscle tissues from transduced mice with antibodies against H3K4me2, H3K4me3, H3K9me2, H3K27me3, H3K9ac, and H3CT. According to our results, LV-856 and LV-451 showed similar epigenetic profiles in vivo as was observed in C166 cells. LV-856 caused enrichment of H3K9me2, H3K27me3, and nucleosome density and simultaneously decreased H3K4me2 and H3K9ac both at the targeted promoter region and TSS of VEGF-A (Figure 3). The effects of LV-451 were opposite to those of LV-856, as it enriched H3K4me2 and H3K4me3, and decreased H3K9me2 both at the targeted promoter region and TSS. However, no clear effects on H3K9ac, H3K27me2, or nucleosome density were observed. Thus, our in vivo qChIP results are in a good agreement with those obtained in C166 cells. The ChIP analysis with chromatin extracted from healthy untreated muscle tissues did not reveal any epigenetic changes within VEGF-A gene, suggesting that the shRNA-dependent epigenetic changes were limited to the treated muscle (data not shown).

To evaluate therapeutic potential of lentiviral transfer of promoter targeted shRNAs, we repeated in vivo experiments with VeVo ultrasound analysis of blood flow in ischemic...
hindlimbs 7 days after operation and transduction (Figure 4a-d). LV-856 did not have any effect as compared to control muscles but LV-451 caused a significant increase in vascularity and improved blood flow. This demonstrates that the manipulation of epigenetic state of tissue by promoter-targeted shRNAs can have therapeutic effects in vivo.

Discussion
Small RNAs have become valuable tools in molecular biology. They also show significant promise as therapeutic agents. Small RNAs have been used to knock down target genes by RNA-induced silencing complex (RISC)-mediated mRNA cleavage, but their effects on chromatin have remained unclear. Certain covalent modifications of aminoacids at the N-terminal histone tails have been linked to gene activation or repression. Together these covalent modifications form a histone code. According to the histone code, important indicators for gene activation are methylation of histone H3K4 and acetylation of histone H3K9. On the other hand, methylation of H3K9 and H3K27me3 are involved in gene repression. Methylation of H3K4 has been found as a preferred site in transcriptionally active macronuclei of *Tetrahymena*, which also correlates with histone acetylation.13 It is presumed that reversal of the H3K4 methylation has an important biological impact on normal development and human diseases.14 In previous reports, dsRNA targeting has been linked to histone modifications or DNA methylation,5,15–19 although histone modifications sometimes appear to be dispensible for dsRNA-mediated modulation of transcription.1 In this study we show that shRNA-mediated modulation of mVEGF-A transcription also involves histone modifications both in cultured cells and in vivo. According to our data, shRNA LV-856 can mediate the removal of both H3K4 methylation and H3K9 acetylation at specific regions of mVEGF-A promoter resulting in transcriptional repression of the gene. On the other hand, shRNA LV-451 increases H3K4 methylation at the proximity of TSS, which increases transcriptional activity. Thus, we show an important link between shRNA-mediated epigenetic changes of mVEGF-A promoter and transcriptional repression/activation.

The mechanism whereby dsRNA targeting of a given gene promoter affects transcriptional activity is still largely unknown. It has been reported that targeting of E-cadherin promoter can result in either increased or repressed transcription.5,17 This is in agreement with our observations that mVEGF expression can be either induced or repressed by shRNAs targeting different promoter locations.

Our ChIP scanning results suggested that shRNA targeting resulted in epigenetic changes within VEGF-A promoter and TSS in vivo. For quantitative ChIP analysis, chromatin was extracted from quadriceps muscles of transduced mice and immunoprecipitated with antibodies against H3K4me2, H3K4me3, H3K9ac, H3K9me2, H3K27me3, or H3CT. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and nonspecific IgG results by using formula \[2^{-\Delta\Delta C_P}\text{specific antibody}/2^{-\Delta\Delta C_P}\text{nonspecific IgG}\], where \(\Delta C_P\) is the \(C_P(\text{immunoprecipitated DNA})-C_P(\text{input})\) and \(C_P\) is the cycle where the threshold is crossed. Two-tailed paired Student t test was performed using Prism4.0c software, and probability values of the fold enrichments were calculated in reference to GFP (*\(P<0.01\) to 0.05, **\(P<0.001\) to 0.01, ***\(P<0.001\)). In each panel, \(n\) is at least 6. Error bars indicate SD.

Figure 3. shRNA targeting results in epigenetic changes within VEGF-A promoter and TSS in vivo. For quantitative ChIP analysis, chromatin was extracted from quadriceps muscles of transduced mice and immunoprecipitated with antibodies against H3K4me2, H3K4me3, H3K9ac, H3K9me2, H3K27me3, or H3CT. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and nonspecific IgG results by using formula \[2^{-\Delta\Delta C_P}\text{specific antibody}/2^{-\Delta\Delta C_P}\text{nonspecific IgG}\], where \(\Delta C_P\) is the \(C_P(\text{immunoprecipitated DNA})-C_P(\text{input})\) and \(C_P\) is the cycle where the threshold is crossed. Two-tailed paired Student t test was performed using Prism4.0c software, and probability values of the fold enrichments were calculated in reference to GFP (*\(P<0.01\) to 0.05, **\(P<0.001\) to 0.01, ***\(P<0.001\)). In each panel, \(n\) is at least 6. Error bars indicate SD.
RNAi is thought to locate in the cytoplasm, Ago2 has been shown to be present at promoters during TGS and it seems to be critical for both dsRNA-mediated activation and repression of transcription in cultured cells.1,5,21 According to our qChIP results, Ago2 was recruited to targeted promoter as well as to TSS of mVEGF-A both in the case of shRNA-mediated transcriptional activation and repression in cultured cells.

Recently described histone demethylase, LSD1, can alternatively demethylate H3K4me2 or H3K9me2, contributing to transcriptional repression or activation, respectively.22,23 Although the protein associations that direct LSD1-dependent demethylation of H3K9 are not yet defined, the repression functions of LSD1 are related to its association to multi protein complexes, which contain histone deacetylase activity.24,25 We showed by qChIP assays that LV-856 caused enrichment of LSD1 at the targeted promoter region and TSS in cultured cells. These results are in a good agreement with the observation that LV-856 resulted in demethylation of H3K4 and deacetylation of H3K9 at the targeted promoter region and TSS. In the light of results presented here, we propose a model that may explain how shRNA targeting influences mVEGF-A transcription (Figure 4e). We suggest that after RISC has bound to shRNA in the cytoplasm, the complex goes through processing, in which histone modifying transcription factors are attached. After that the complex is transported back into the nucleus. The complex recognizes the target sequence in the genome and modulates gene activity by regulating transcription via histone acetylation and methylation.
tors to the VEGF-A promoter and TSS. Although shRNAs were designed so that they were not directly targeted to any known or predicted transcription factor binding sites, a few sites, including AP-1 and SP-1, are close to shRNA target sequences (Online Figure V). If the complex formed by shRNA and transcription factors is recruited to the promoter in a very close proximity to an important binding site, it may prevent normal regulation of transcription. Thus, we cannot fully exclude the possibility that shRNA targeting influences mVEGF-A transcription via interfering other transcription factors at the mVEGF-A promoter. Importantly, effects mediated by shRNAs are limited to the target tissue because in nontreated muscle of LV-856—and LV-451–treated mice shRNA targeting did not cause epigenetic changes within VEGF-A promoter and TSS in vivo (Online Figure VI). Furthermore, LV-599 and LV335, which did not cause effects on mVEGF expression (Figure 1b and 1c), also had no effects on key proteins at mVEGF TSS or promoter area (Online Figure VII) nor changed H3K4me2 enrichment at mVEGF TSS (Online Figure VIII).

Taken together, we show that lentivirus-mediated delivery of shRNA molecules targeted to specific regions in the mVEGF-A promoter either repress or induce VEGF-A expression via epigenetic modulation. This significantly expands the therapeutic use of small RNAs and suggests new possibilities for the regulation of gene expression in vitro and in vivo. Controlling transcription through manipulation of specific epigenetic marks would provide a novel tool for molecular medicine. Downregulation of VEGF-A expression is favorable in the treatment of many cancers, whereas VEGF-A upregulation has been used for the treatment of muscle ischemia and cardiovascular diseases. Here we show that vascularity and blood flow in ischemic hindlimbs can be increased using promoter targeted shRNAs. Thus, epigenetic control of gene expression via delivery of promoter-targeted shRNAs could become an important new therapeutic approach for the treatment of several diseases.

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Disclosures
M.P.T., G.S.A., and R.G. are employed by Ark Therapeutics Oy.

References
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Supplemental Material

Extended Materials and Methods

Cell culture

The MS1 and C166 (ATCC: CRL-2583) cells were cultured in DMEM containing fetal bovine serum (FBS, 10% for C166 and 5% for MS1), 100 units/ml penicillin and 100 ug/ml streptomycin.

mRNA and protein expression assays

GFP fluorescence was measured by flow cytometry using CANTO II (Becton Dickinson) and by fluorescence microscopy. Quantitative determination of VEGF in medium was determined by a specific murine VEGF-A enzyme-linked immunosorbent assay (Quantikine VEGF-ELISA kit; R&D Systems). For realtime TaqMan RT-PCR total RNA of C166-GFP cells was extracted by Trizol Reagent (Gibco BRL) and treated with DNase I (Promega). RNA was reverse transcribed to cDNA by M-MuLV reverse transcriptase (MBI Fermentas). mVEGF-A mRNA levels were measured by real time PCR (ABI PRISM 7700 detection system, Applied Biosystems) using Taqman® Gene expression assay (assay ID: Mm00437304_m1, Applied Biosystems). β-actin mRNA levels were (Mouse ACTB Endogenous Control (VIC®/MGB Probe, Primer Limited, part number: 4352341E, Applied Biosystems) used as an endogenous amplification control for normalization.

In vivo treatments

Mice were fed ad libitum with a normal chow diet (R36, Lactamin, Sweden). At the age of 8 weeks the mice were anesthetized subcutaneously using xylazine (10 mg/kg) and ketamine (80 mg/kg) and unilateral hindlimb ischemia was induced by ligating the left femoral artery proximal to the bifurcation of the proximal caudal femoral vein. Immediately after operation gene transfers were performed by injecting 30 µl of lentivirus (titer 2-4*10^9 TU/ml) encoding shRNAs (-856 or -451) and green fluorescent protein (GFP) or GFP only. Animals were sacrificed 5 or 10 days after the operation using carbon dioxide. Muscle samples were divided in three parts: samples for histology were immersionfixed in 4% paraformaldehyde for 4 h and embedded in paraffin. Samples for ELISA were snap frozen in liquid nitrogen. Samples for ChIP were collected as described below. All experiments were approved by the Experimental Animal Committee of the University of Kuopio.

Ultrasound imaging of muscle vasculature

Vasculature in the transduced and contralateral intact calf muscles was measured with VeVo 770 and 704 transducer (VisualSonics) using the Power Doppler mode (power 100%, RF-cycle 5, gain 25, velocity medium, wall filter 15, scan speed 15, priority 100, intensity range maximum 53 and minimum 19). Video clips containing approximately 50 frames were captured and the vascularity index (normalized to the area of the muscle) in three evenly separated frames was quantified with VeVo 770 measurement software (VisualSonics). The results are represented as group means of ratios to intact values to reduce measurement dependent variation.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described. Antibodies against dimethylated histone H3K4 (H3K4me2, 07-030), trimethylated histone H3K4 (H3K4me3, dimethylated histone H3K9 (H3K9me2, 07-523), trimethylated histone H3K27 (H3K27me3, 07-449), acetylated histone H3K9 (H3K9ac, 07-352) and histone H3 C-terminus (H3CT, 07-690) were from Upstate Biotechnology (Lake Placid, NY, USA). Nonspecific IgG (sc-2027) and antibodies against SRC-
1 (sc-7216) and CBP (sc-369) were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). Antibodies against Ago2 (ab32381) and LSD1 (ab17721) were from Abcam (Cambridge, UK).

In ChIP scanning analysis, the chromatin templates were analyzed by semi-quantitative real-time PCR. For each of the 8 regions within the mVEGF-A promoter or coding region, specific primer pairs were designed (Supplementary Table 1), optimized and controlled by running PCR with 25 ng genomic DNA (input) as a template. When running immuno-precipitated DNA (output) as a template, the following PCR profile was used: 10 min at 95 °C, 34 cycles of 30 s at 95 °C, 20 s at 56°C and 20 s at 72 °C, and the final extension for 10 min at 72 °C. The PCR products were separated by electrophoresis in 2 % agarose gels. Gel images were scanned in a FLA-3000 reader (Fuji, Tokyo, Japan) and analyzed using Image Gauge software (Fuji, Tokyo, Japan).

In in vivo ChIP, the mice were sacrificed (see above), their quadriceps excised and washed once with ice cold PBS and cross-linked with formaldehyde (final concentration 1%) for 15 min at RT. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M and incubation for 5 min. The tissues were washed twice with ice cold PBS and homogenized with Potter-Elvehjem homogenizer on ice. The cells were collected by centrifugation and the pellet was dissolved in 1 ml of ChIP lysis buffer (1 % SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1). The chromatin was shared by sonication for 15 min with Bioruptor resulting in 400-800 bp chromatin fragments and immunoprecipitated as described2.

Quantitative PCR analysis of immunoprecipitated DNA
Quantitative PCR analysis was performed by using BHQ1-FAM hydrolysis probes provided by Eurogentec (Seraing, Belgium). The sequences of primers and hydrolysis probes are listed in Supplementary Table 2. The qPCR reaction was performed with LightCycler480 apparatus (Roche Diagnostics, Mannheim, Germany) using following PCR profile: 10 min at 95 °C, 50 cycles of 20 s at 95 °C, 1 min at 60°C. Results were normalized with respect to inputs and fold enrichments relative to non-specific IgG results were calculated using the formula \(2^{-\Delta C_{p}}\)specific antibody / \(2^{-\Delta C_{p}}\)non-specific IgG, where \(\Delta C_{p}\) is the \(C_{p}(\text{immunoprecipitated DNA}) - C_{p}(\text{input})\) and Cp is the cycle where the threshold is crossed. Two-tailed, paired Student's t-test was performed using Prism4.0c software and P-values of the fold enrichments were calculated in reference to GFP (* P=0.01 to 0.05, ** P= 0.001 to 0.01, *** P< 0.001).

Supplementary references


**Supplementary table 1.** PCR primers used in ChIP scanning analysis. Sequences and location relative to the TSS (+1) are shown.

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<th>Location</th>
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**Supplementary table 2.** PCR primers and hydrolysis probes used in quantitative ChIP analysis. Sequences and location relative to the TSS (+1) are shown.

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Online Figure Legends

Supplementary Figure 1 shRNA targeting results in epigenetic changes within VEGF-A promoter and TSS in C166, but not in MS1 cells. ChIP analysis was done using antibodies against H3K9me2, H3K9ac or H3K4me2. Chromatin was extracted from C166 or MS1 cells, which were transduced with different vectors using MOI 10, 7 days time points. (a) Location of PCR products and different shRNAs within the mVEGF promoter. Red boxes indicate positions of LV-856, LV-599, and LV-451. (b) ChIP analysis of C166 cells. (c) ChIP analysis of MS1 cells.

Supplementary Figure 2 Epigenetic changes within VEGF-A promoter and TSS in C166 cells. Chromatin was extracted from C166 cells, which were transduced with different vectors using MOI 10, 7 days time point. ChIP assays were performed using antibodies against H3K4me2, H3K4me3, H3K9ac, H3K9me2, H3K27me3 or H3CT. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and non-specific IgG results by using formula \[ \frac{2^{(\Delta\text{Cp})\text{specific antibody}}}{2^{(\Delta\text{Cp})\text{non-specific IgG}}} \], where \( \Delta\text{Cp} \) is the \( \text{Cp(immunoprecipitated DNA)} - \text{Cp(input)} \) and Cp is the cycle where the threshold is crossed. Two-tailed, paired Student's t-test was performed using Prism4.0c software and P-values of the fold enrichments were calculated in reference to GFP (* P=0.01 to 0.05, ** P= 0.001 to 0.01, *** P< 0.001). In each panel, n is at least 6. Error bars indicate S.D.

Supplementary Figure 3 Epigenetic changes within VEGF-A at TSS (a), distal promoter (b) and proximal promoter (c) in C166 cells after short treatment times. Chromatin was extracted from C166 cells, which were transduced with different vectors using MOI 10 for 14 h. Timepoints for sample collections were 14 h, 24 h and 7 days. ChIP assays were performed using antibody against H3K4me2. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and non-specific IgG results by using formula \[ \frac{2^{(\Delta\text{Cp})\text{specific antibody}}}{2^{(\Delta\text{Cp})\text{non-specific IgG}}} \], where \( \Delta\text{Cp} \) is the \( \text{Cp(immunoprecipitated DNA)} - \text{Cp(input)} \) and Cp is the cycle where the threshold is crossed. Two-tailed, paired Student's t-test was performed using Prism4.0c software and P-values of the fold enrichments were calculated in reference to GFP (* P=0.01 to 0.05, ** P= 0.001 to 0.01, *** P< 0.001). In each panel, n is at least 4. Error bars indicate S.D.

Supplementary Figure 4 Recruitment of transcription factors to VEGF-A promoter and TSS in C166 cells. Chromatin was extracted from C166 cells, which were transduced with different vectors using MOI 10, 7 days time point. ChIP assays were performed using antibodies against Ago2, SRC-1, CBP or LSD1. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and non-specific IgG results by using formula \[ \frac{2^{(\Delta\text{Cp})\text{specific antibody}}}{2^{(\Delta\text{Cp})\text{non-specific IgG}}} \], where \( \Delta\text{Cp} \) is the \( \text{Cp(immunoprecipitated DNA)} - \text{Cp(input)} \) and Cp is the cycle where the threshold is crossed. Two-tailed, paired Student's t-test was performed using Prism4.0c software and P-values of the fold enrichments were calculated in reference to GFP (* P=0.01 to 0.05, ** P= 0.001 to 0.01, *** P< 0.001). In each panel, n is at least 6. Error bars indicate S.D.

Supplementary figure 5 Known and predicted binding sites of transcription factors around LV-856 and LV-451. The binding sites were determined using net-based program AliBaba2.1 by Niels Grabe. Location and sequences of LV-856 and LV-451 within VEGF-A promoter are shown in red color.
Supplementary Figure 6 shRNA targeting does not cause epigenetic changes within VEGF-A promoter and TSS in vivo in non-treated muscle of LV-856 and LV-451 treated mice. The chromatin was extracted from quadriceps muscles of transduced mice and immunoprecipitated with antibodies against H3K4me2 and H3K9me2. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and non-specific IgG results by using formula $2^{-\Delta\text{Cp}}$ specific antibody / $2^{-\Delta\text{Cp}}$ non-specific IgG, where $\Delta\text{Cp}$ is the $\text{Cp}_{\text{immunoprecipitated DNA}} - \text{Cp}_{\text{input}}$ and Cp is the cycle where the threshold is crossed. In each panel, n is at least 3. Error bars indicate S.D.

Supplementary Figure 7 qChIP analysis of the effects of LV-599 and LV355 targeting at mVEGF promoter and TSS in C166 cells. Chromatin was extracted from C166 cells, which were transduced with different vectors using MOI 10, 7 days time point. ChIP assays were performed using antibodies against Ago2, LSD-1, H3K9ac, H3K27me3, or H3CT. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and non-specific IgG results by using formula $2^{-\Delta\text{Cp}}$ specific antibody / $2^{-\Delta\text{Cp}}$ non-specific IgG, where $\Delta\text{Cp}$ is the $\text{Cp}_{\text{immunoprecipitated DNA}} - \text{Cp}_{\text{input}}$ and Cp is the cycle where the threshold is crossed. In each panel, n is at least 3. Error bars indicate S.D.

Supplementary Figure 8 qChIP analysis of the effects of shRNA targeting to the H3K4me2 enrichment at mVEGF promoter, TSS and coding region in C166 cells. Chromatin was extracted from C166 cells, which were transduced with different vectors using MOI 10, 7 days time point. ChIP assays were performed using antibody against H3K4me2. Quantitative PCR was performed with LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Results were normalized with respect to input and non-specific IgG results by using formula $2^{-\Delta\text{Cp}}$ specific antibody / $2^{-\Delta\text{Cp}}$ non-specific IgG, where $\Delta\text{Cp}$ is the $\text{Cp}_{\text{immunoprecipitated DNA}} - \text{Cp}_{\text{input}}$ and Cp is the cycle where the threshold is crossed. In each panel, n is at least 3. Error bars indicate S.D.
Supplementary Fig. 1

(a)  

(b)  

(c)
Supplementary Fig. 2
Supplementary Fig. 3

**a**

- 14 hours
- 24 hours
- 7 days

**b**

- 14 hours
- 24 hours
- 7 days

**c**

- 14 hours
- 24 hours
- 7 days

GFP -856 to -451

H3K4me2 enrichment

250 bp

mVEGF-A

TSS

-856

-451

TSS

-856

-451

TSS

-451

GFP -856 to -451

H3K4me2 enrichment

Supplementary Fig. 3
Supplementary Fig. 4

250 bp

-856  -451  TSS

Ago-2 enrichment

SRC-1 enrichment

CBP enrichment

LSD-1 enrichment

* p < 0.05
** p < 0.01
Supplementary Fig. 6

![Diagram showing enrichment levels of H3K4me2 and H3K9me2 at different sites relative to TSS.](image-url)
Supplementary Fig. 8