Differential Regulation of Vascular Endothelial Growth Factors by Promoter-targeted shRNAs

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Vascular endothelial growth factors (VEGFs) and their receptors (VEGF-R) are central regulators of vasculogenesis, angiogenesis, and lymphangiogenesis. They contribute to many vascular-related pathologies, and hence VEGF-targeted therapies have been widely sought after. In this study, the authors investigated the ability of promoter-targeted small hairpin RNAs (shRNAs) to regulate VEGF-A, VEGF-C and VEGF-R1 in different cell lines. The authors identified shRNAs that can upregulate hVEGF-C at both the mRNA and protein levels, and differentially regulate hVEGF-A depending on the cell type. Likewise, the authors identified shRNA that downregulated VEGF-R1 gene expression. Hence, promoter-targeted shRNAs can affect endogenous gene expression not only bimodally, but also differentially in a cell-type specific manner. Importantly, all three genes tested were regulated by at least one shRNA, supporting the idea that nuclear RNA interference is a widespread phenomenon. The level of regulation across the panel of shRNAs varied maximally from a 2.2-fold increase to a 4-fold decrease. This level of change should be useful in fine-tuning and modulating target gene expression, which for potent molecules, such as VEGF-A and VEGF-C, can be very beneficial. These promoter-targeted shRNAs may facilitate the design and development of targeted, context-dependent strategies for both pro- and antiangiogenic therapies for the treatment of vascular-related pathologies.

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Introduction

Small RNAs (sRNA) targeting promoters and subsequently regulating gene expression were observed in plants1 and subsequently in mammalian cells.2 Although originally, inhibition of gene expression by the promoter-targeted sRNA was observed, soon after gene activation by these sRNA was demonstrated.3 Importantly, these sRNA have since then been shown to be active not only in vitro3–11 but also in vivo in mouse models of hind-limb ischemia10 and myocardial infarction.11 In addition, a functional outcome of promoter-targeted regulation of tumor-related genes has been demonstrated in cancer models.9,13–15

The sequence requirements4,5,16 of activating RNAs have been characterized and their mechanisms of action have been determined to some extent,17 but these still need to be clearly defined. To date, downstream modifications of epigenetic marks have been observed, in particular histone modifications characteristic of the active marks.4,3,4,10,16 However, although DNA methylation of the targeted promoters was observed with silencing sRNA,7,19,30 changes in DNA methylation were not observed for activating sRNA.3 These sRNA have demonstrated some dependence on RNAi factors. In particular, they have been associated with a requirement for Argonaute (Ago), especially Ago2,3,5,6,10,21 and GW182.22 Furthermore, in some cases they have induced enrichment of RNA polymerase II at the activated promoters23,24 or have been associated with specific heterogenous nuclear ribonucleoproteins.25 In other instances binding to an antisense transcript overlapping the activated promoter has been observed6,23,24 and proposed as a putative regulatory mechanism.

Although the mechanisms of promoter-targeted sRNA still need to be elucidated, the potential of these as therapeutic agents is obvious. Of particular interest is the activating sRNA, since only a few systems exist that can upregulate endogenous gene expression, in contrast to gene silencing which can be efficiently achieved by traditional RNAi and antisense technologies. Importantly, promoter-targeted sRNA can simultaneously regulate the expression of multiple isoforms generated from the targeted gene12 and thereby can maintain a better physiological balance of the isoforms. One of the genetic targets to which this type of regulation would be very useful is the vascular endothelial growth factor (VEGF) family of proteins.

The VEGF family consists of proteins which are critical for vascular growth and homeostasis. In particular, VEGF-A mediates angiogenesis and vasculogenesis,26 whereas VEGF-C regulates lymphangiogenesis.27 The biological effects of these factors are mediated via their receptors VEGF-R1, 2, and 3.28 Because of their important roles in vasculature, VEGFs are putative therapeutic targets for many different pathologies, such as ischemic diseases and vulnerable atherosclerotic plaques29 as well as cancer.30 However, the therapeutic requirements are different depending on the targeted disease, i.e., there is a need for both pro- and antiangiogenic therapies.31 The dichotomy of required therapies subsequently makes promoter-targeted sRNA an appealing strategy. In this study the authors’ intention was to test the...
effectiveness of promoter-targeted short hairpin (sh)RNAs in the regulation of endogenous genes of the VEGF family, including VEGF-A, VEGF-C, and VEGF-R1. The authors’ second intention was to test these shRNAs in a variety of cell types to address potential issues of cell specificity.\textsuperscript{3,10}

Results

To study the regulation of promoter-targeted shRNAs on VEGF-A, VEGF-C or VEGF-R1 cells were transduced with viral-like particles encapsulating lentiviral vectors (LVs) encoding for specific shRNAs under the hU6 promoter and coexpressing enhanced green fluorescent protein (eGFP) under the human phosphoglycerate kinase-1 promoter. The transduction efficiency of the cells as indicated by eGFP expression was monitored by flow cytometry. The authors used MOI of 10–20 to achieve transduction efficiencies of ≥80%. There were no significant differences in the transduction efficiencies of different viral-like particles as compared with the control transductions in any of the cell types tested (data not shown).

Regulation of the VEGF-C gene

The regulation of the VEGF-C gene by promoter-targeted shRNAs was investigated and the authors identified one shRNA that upregulated VEGF-C (Figure 1). Specifically, shRNA516 upregulated hVEGF-C mRNA and protein levels in PC3 following 4 days of transduction and the secreted protein further accumulated up to day 7 (Figure 1a). Likewise, shRNA516, which has 100% homology with both the mouse and the human VEGF-C promoters, significantly increased VEGF-C mRNA expression in the human primary cells, human umbilical vein endothelial cells, and in the mouse endothelial cell line, C166 (Figure 1b). In addition, a mouse VEGF-C promoter sequence specific shRNA, shRNA348, significantly increased VEGF-C mRNA in C166 cells. Of note, expression of

![Figure 1](https://example.com/figure1.png)

Figure 1  Promoter-targeted shRNA regulation of vascular endothelial growth factor (VEGF)-C. (a) Human PC3 were transduced with LV coding for shRNA targeting the VEGF-C promoter (LV-sh516, LV-sh914), or scrambled shRNA (LV-shCTRL) or LV not expressing a shRNA (LV-GFP). Cells and media were harvested following 4 and 7 days of transduction. The VEGF-C mRNA expression was determined by RT-qPCR (dotted diagrams) and the secreted VEGF-C protein was quantified by ELISA (bar diagrams). (b) Human umbilical vein endothelial cells (HUVECs) and mouse C166 were transduced with LV-shCTRL, LV-sh348, LV-sh516, or LV-sh914, and VEGF-C mRNA expression was determined by RT-qPCR following 7 days of transduction. Data in bar diagrams are presented as the mean ± SEM (n = 5–7) and were analyzed by analysis of variance (ANOVA) and Tukey’s multiple comparison tests. Nonhomogenous data (mRNA) were analyzed by Kruskal–Wallis and Dunn’s multiple comparison tests. Statistical significance (P < 0.05) between groups is indicated by (*). LV, lentiviral vector; shRNA, small hairpin RNA; VEGF, vascular endothelial growth factor.
a scrambled shRNA (shCTRL) did not affect hVEGF-C mRNA and protein levels, as there were no differences between LV-GFP and LV-shCTRL (Figure 1a). In addition, hVEGF-A was measured in the 7-day media of PC3 samples, and no change in hVEGF-A was observed following hVEGF-C promoter-targeted shRNA treatment (data not shown), suggesting that the changes are specific for hVEGF-C.

Regulation of the VEGF-A gene
The regulation of VEGF-A gene by promoter-targeted shRNAs was investigated in both PC3 and EAhy.926 cells. Activity of shRNAs targeting the VEGF-A promoter was observed. In EA.hy926 cells, hVEGF-A mRNA was significantly increased by shRNA1212 (Figure 2). In addition, there was a corresponding increase in the frequency and median of detectable secreted hVEGF-A protein in EA.hy926-conditioned media with shRNA1212. In PC3 cells, shRNA674 resulted in significantly reduced expression of hVEGF-A mRNA and protein (Figure 2).

Regulation of the VEGF-R1 gene
The regulation of VEGF-R1 expression was investigated, and the authors observed that shRNA223 downregulated VEGF-R1 mRNA expression in C166 (Figure 3). The VEGF-R1 mRNA was reduced by shRNA223 as early as 4 days after transduction and was maintained for at least 7 days in C166. The reduced VEGF-R1 mRNA corresponded to significantly lower cellular VEGF-R1 protein expression (Figure 3). In addition, shRNA223 downregulated hVEGF-R1 mRNA expression (0.6785 ± 0.044-fold change, n = 5) in EAhy.926 cells.

Effect of combined treatment of activating and repressing shRNAs
To address the effects of coexpression of both activating and repressing shRNAs in the same cells, the authors tested previously published LV-shRNAs targeting mouse VEGF-A promoters, LV-451 and LV-856, which upregulate and downregulate, respectively, mouse VEGF-A mRNA in mouse C166 cells.10 C166 cells were cotransduced with both LV-451 and LV-856 (MOI 10), and mouse VEGF-A mRNA was measured after 4 days. Combined expression of both shRNA-451 and -856 resulted overall in an unchanged expression of mouse VEGF-A (fold change 1.22 ± 0.094, n = 3, P = 0.25) as compared to LV-shCTRL transduced cells. These data demonstrate that coexpression of activating and suppressing...
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Figure 3 Promoter-targeted shRNA regulation of VEGF-R1. Mouse C166 cells were transduced with LV coding for shRNA targeting the VEGF-R1 promoter (LV-sh223), or scrambled shRNA (LV-shCTRL). Cells and media were harvested following 4 and/or 7 days of transduction. The VEGF-R1 mRNA expression was determined by RT-qPCR (dotted diagrams) and the secreted or cellular VEGF-R1 protein (day 7) was quantified by ELISA (bar diagrams). Data in bar diagrams are presented as the mean ± SEM (n = 5–6) and were analyzed by analysis of variance (ANOVA) and Tukey’s multiple comparison tests. Nonhomogenous data (mRNA) were analyzed by Kruskal–Wallis and Dunn’s multiple comparison tests. Statistical significance (P < 0.05) between groups is indicated by (*).

shRNAs can balance each other’s effects on gene expression and suggest that shared cellular factors are not limiting, as both shRNAs were functional.

Effects of active shRNAs on histone methylation and acetylation of targeted promoters
Previously, promoter-targeted shRNA have been shown to affect histone modifications at the target site, and hence to delineate the mechanisms of regulation by promoter-targeted shRNA, the authors investigated the histone methylation and acetylation status of the genomic DNA at the targeted promoters. Figure 4 represents the chromatin immunoprecipitation (ChIP) analysis of these cells at the specific site of the targeting shRNA as well as an additional promoter site. Antibodies recognizing both active (H3K27ac, H4K5ac) and inactive (H3K27me3) histone markers were used in the ChIP analysis. The authors observed a consistent trend for a higher association of the activating markers in C166 and PC3 with activating shRNA at the shRNA target site but not at other promoter areas (Figure 4). In contrast, shRNA674 which suppressed hVEGF-A expression in PC3 cells (Figure 2) resulted in a reduced association of active histone mark H4K5ac at the targeted promoter site when compared with cells expressing control shRNA (Figure 4). In addition, shRNA674 resulted in an increased association of H3K27me3 suppressing mark both at the targeted site and at other sites within the hVEGF-A promoter (Figure 4).

Discussion
This study demonstrates differential regulation of multiple genes of the VEGF family by promoter-targeted shRNAs in various cell types. The authors demonstrated novel upregulation of VEGF-C and downregulation of VEGF-R1, in addition to dual regulation of hVEGF-A. Importantly, all three genes tested were regulated by at least one shRNA that had been designed to target their promoters. This supports the idea that nuclear RNA interference, as defined by promoter targeting, is a more general phenomenon associated with both upregulation and downregulation of the target genes and may be a more widespread concept than classical RNAi in the cytoplasm. Evidence is accumulating that factors involved in RNAi can be found in the nucleus, and hence nuclear generated shRNAs may be processed in the nucleus.

Importantly, in this study the authors demonstrated upregulation of gene expression from their endogenous promoters by the shRNAs. These promoter-targeted shRNAs may become useful tools for cell-type specific gene regulation in multicellular organisms. Although the mechanisms underlying the cell specificity of the shRNA activity remain to be elucidated, these may be multifactorial and may include variation in promoter methylation status, differential expression of noncoding RNAs or promoter miRNAs, or modulation of transcriptional regulatory complexes. In particular, DNA methylation has been observed to play a role in the activity of promoter-targeted sRNA. However, this effect was limited to shRNA inducing transcriptional gene silencing and not activation. In addition, differential expression of an antisense RNA or promoter RNA of the gene of interest may affect the shRNAs activity. Earlier, promoter-targeted sRNA modulation of Progesterone receptor, low-density lipoprotein, and cyclooxygenase-2 were linked to promoter-targeted sRNA binding of an antisense transcript or long noncoding RNA overlapping the promoters of these genes. These interactions were proposed as a putative mechanism of transcriptional regulation either by degradation of the promoter RNA or by interference with a putative scaffolding function. Alternatively, differential expression of promoter-targeting miRNAs may affect promoter-targeted shRNA activity. Recently, Ago-bound sRNA from proximal promoters (named TSS-miRNA) were found globally. Thus, promoter-targeted shRNAs may interfere with these promoter-specific miRNA targets to induce regulation. Furthermore, it was also shown that sRNA targeting cyclooxygenase-2 promoter could upregulate not only cyclooxygenase-2, but also phospholipase A2 potentially because of interrupted looping of chromosomal DNA by which these two genes are coregulated. This raises a
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possibility whether shRNA can in a similar fashion interfere with or augment enhancer activity by affecting its looping to the gene promoter(s).

Overall, our study demonstrates regulation of members of the VEGF family by promoter-targeted shRNAs. The level of regulation was often moderate which in the case of hVEGF-A can be beneficial, because earlier hVEGF-A therapies tested in the clinics have been associated with unwanted edema because of the strong potency of this molecule. Hence, these promoter-targeted shRNAs can be used for fine-tuning or modulating target gene expression. Careful assessment of any particular promoter-targeted shRNA has to take place to define their cell specificity. However, they have a clear potential for cell-type specific, context-dependent therapeutic applications.

Materials and methods

Vectors and lentiviral production. The promoter targeted shRNA sequences were designed using the Dharmacon siDESIGN Center algorithm (http://www.dharmacon.gelifesciences.com/design-center). Specifically, of the algorithm-derived siRNA sequences, the authors chose those ones that did not overlap GC rich areas or known

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Table 1 List of promoter-targeted small hairpin RNA (shRNAs) specified with their gene promoters, distance from TSS and sequence

| Name   | Targeted gene promoter | Distance from TSS (human) | Distance from TSS (mouse) | Targeted sequence 5′- | Targeted sequence 3′- |
|--------|------------------------|---------------------------|---------------------------|-----------------------|-----------------------|
| sh674  | hVEGF-A                | -328                      |                            |                       |                       |
| sh1212 | hVEGF-A                | -321                      |                            |                       |                       |
| sh1657 | hVEGF-A                | -299                      |                            |                       |                       |
| sh348  | mVEGF-C                | -287                      |                            |                       |                       |
| sh516  | mVEGF-C                | -287                      |                            |                       |                       |
| sh914  | mVEGF-C                | -287                      |                            |                       |                       |
| sh223  | mVEGF-R1               | -287                      |                            |                       |                       |
| shCTRL | —                      | -287                      |                            |                       |                       |

TSS, transcriptional start site; VEGF, vascular endothelial growth factor.
transcription factor binding sites in the promoter. The selected shRNA sequences with reference to the genes TSS and a mismatched control sequence are listed in Table 1. The shRNAs were cloned under the hU6 promoter in the context of the third generation HIV-1 derived LVs coexpressing eGFP under the human phosphoglycerate kinase-1 promoter [LV-hU6shRNAAXXX-hPGK-GFP]. The viral-like particles encapsulating the LV-hU6shRNAAXXX-hPGK-GFP vectors were prepared using standard calcium phosphate transfection in 293T-cells as described earlier.

Cell culture and transductions. The following cell lines were used in this study: C166 (ATCC: CRL-2583), EA.hy926,39 and PC3. All cell lines were cultured in their specified media (RPMI for PC3 and DMEM for remaining cell lines; Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100µg/ml streptomycin. Primary human umbilical vein endothelial cells were either isolated from umbilical cords obtained from the maternity ward of Kuopio University Hospital, Kuopio, Finland, with the approval of local Ethics Committee, or purchased from Gibco (C-003-5C; Grand Island, NY).

Protein analysis. Fresh cells or frozen cell pellets were lysed as described earlier in the presence of 1x complete protease inhibitor (Roche Diagnostics Oy, Espoo, Finland). The total protein content of the precleared cell lysates were quantified using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. The VEGF-A, VEGF-C, or VEGF-R1 content in cell lysates or conditioned media was quantified using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions. Specifically, human VEGF-A and VEGF-C were assayed using relevant Quantikine ELISA kits (R&D Systems), the human and mouse VEGF-R1 were measured using DuoSet ELISA development kits (R&D Systems), and Mouse VEGF-C using mVEGF-C ELISA kit (MyBioSource, San Diego, CA).

ChIP assay. ChIP assays were performed as described earlier. The chromatin was sheared by sonication in the presence of 30 second on/30 second off cycle for 15 minutes with Bioruptor resulting in 500–1000 bp chromatin fragments. Antibodies against trimethylated histone H3K27 (H3K27me3, 07-449), acetylated histone H3K27 (H3K27ac, 07-360) and acetylated histone H4K5 (H4K5ac, 07-327) as well as rabbit IgG serum

### Table 2: List of Taqman assays for gene expression and chromatin immunoprecipitation analysis

| Gene assay name | Assay code | Forward primer 5′- | Reverse primer 5′- | FAM probe 5′-FAM 3′-NFQ |
|-----------------|------------|---------------------|---------------------|------------------------|
| hVEGF-A         | Hs00900555_m1 | —                   | —                   | —                      |
| mVEGF-A         | Mm00437304_m1 | —                   | —                   | —                      |
| hVEGF-C         | Hs01099203_m1 | —                   | —                   | —                      |
| mVEGF-C         | Mm00437310_m1 | —                   | —                   | —                      |
| hVEGF-R1        | Hs01052961_m1 | —                   | —                   | —                      |
| mVEGF-R1        | Mm00438890_m1 | —                   | —                   | —                      |
| 674probe        | HVEGFAP674   | GGGCCATTAGACGACATACC | CTCATCTGAGCCCCATCTTCT | CCAACAGCCAGAACATG     |
| 983probe        | HVEGFAP983   | ATGGTGAGAGGTTGAGAAAGGG | TCAGGATCCCTGGACTTCT | CAGGCTACAGGCTCT     |
| 1212probe       | HVEGFAP1212  | GGGCAGCTGGGCTCTACCA | GCCCTGGTATGAACTACCTA | CAGCATTAAAGGACGTC     |
| 516probe        | HVEGFCP516   | GAGGAGGGCTGAGACGAGA | GCCGTTTCTCTACTACAGA | CCGGCGCTTTCTCTACAGA |
| 914probe        | HVEGFCP914   | GCGGCGCTCAGACGAGA | AGGTAAGAGGCTCAGAAAACCCA | ACATGCGGAGCTCCC |
| 348probe        | MVEGFCPROM348 | TCCCTCTCCGACACCCCT | CCGGCTTTCTCTCTACAGA | CCGGCTTTCTCTCTACAGA |
| TSSprobe        | MVEGFCTSS    | GCCCAAGCCAGAGAGCGC | GAGGACAGAGCCGCCAGAGTG | CCGCTTCTCTTCTACAGA |

FAM, 6-carboxyfluorescein; NFQ, non fluorescent quencher; VEGF, vascular endothelial growth factor.
(12–370) and Magna ChiP Protein A Magnetic beads were from Merck Millipore.

ChiP DNA samples were assayed by qPCR as described earlier. Results were normalized with respect to inputs as follows: derived ΔCt, which is a subtraction of the threshold cycles input from the IP samples [Ct(immunoprecipitated DNA) – Ct(input)] and then calculated fold change using the formula [2^−ΔΔCt] and subsequently % input.

Statistics. Statistical analysis of the data was performed using GraphPad Prism (5.03; GraphPad Software, San Diego, CA). The data are presented as mean ± SEM unless otherwise stated. When the data conformed to homogeneity of variance, then either one-way analysis of variance (ANOVA) or two-tailed Student’s t-test was performed; otherwise, non-parametric tests were used, including Kruskal–Wallis with Dunn’s post hoc test or Wilcoxon matched-pair signed rank test. The specific tests used for each data set are indicated in the figure legends. Statistical significance was ascribed at P < 0.05 and is indicated by (*).

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