Regulation of VEGF-A Expression in Endothelial Cells by Transcriptional Gene Activation or Transcriptional Gene Silencing: Analysis of Genome Wide Transcriptional Response

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Abstract

Vascular endothelial growth factor (VEGF-A) is an important gene in many diseases, such as cancer and cardiovascular diseases. We investigated changes in genome-wide gene expression patterns in murine endothelial cells when the expression of VEGF-A was epigenetically modulated using promoter targeted small hairpin RNAs (shRNAs). Murine endothelial cells were transduced with lentiviral vectors expressing shRNAs that are complementary to the gene promoter. Gene expression array experiments were conducted to investigate genome-wide mRNA expression changes caused by up- and downregulating VEGF-A. There were several hundreds of differentially expressed genes according to the applied statistical criteria. We noticed that the effects of downregulating VEGF-A were much more wide-spread than the effects of VEGF-A upregulation. Our *in silico* analysis revealed that a number of different biological processes are altered due to epigenetic effects on VEGF-A expression. One of the main regulators of VEGF-A mediated transcriptional response was found to be transcription factor ATF-4. This is the first study showing the transcriptional response to epigenetic modification of VEGF-A expression in endothelial cells. Epigenetic gene regulation represents a natural gene regulatory mechanism and these results reveal previously unknown implications of VEGF-A regulation in endothelial cells.

Keywords: Transcriptional gene silencing; Transcriptional gene activation; Endothelial cell; Microarray

Introduction

Vascular endothelial growth factor (VEGF-A) is a key regulator of angiogenesis in many diseases, such as cancer and cardiovascular diseases [1]. For that reason it is not surprising that its effects on endothelial cells have been studied by several approaches, such as microarray analysis [2] and next generation sequencing [3]. For example, Shin et al. used microarray analysis to study transcriptional response to human dermal lymphatic endothelial cells treated with recombinant human VEGF-A165 [4]. For the inhibition of VEGF/VEGFR signaling, Bevacizumab has been used in a mouse xenograft model of endometrial cancer [5]. However, manipulation of VEGF-A expression, either upregulating the expression by delivering VEGF-A expression cassettes and recombinant proteins or inhibiting it by using RNAi or antibodies, is somewhat artificial in terms of endothelial biology. Recently it has become evident that small RNAs, such as miRNA, siRNA or shRNA, can regulate target gene expression via promoter targeted epigenetic modifications [6-8]. These processes have been studied in plants for a long time, but when Morris et al. [6] found that small RNAs directed to the gene promoters induce transcriptional gene silencing (TGS) and a little later Li et al. [7] showed that they can also induce transcriptional gene activation (TGA), their potential as therapeutics was quickly realized. However, the specificity and safety of these promoter targeted small RNAs must be evaluated before clinical trials.

Modulating the expression levels of mouse VEGF-A was achieved by transducing mouse endothelial cells with lentiviral vectors that express different shRNAs. These molecules are complementary to the promoter of mouse VEGF-A gene, and they are known to either increase or decrease VEGF-A expression by causing epigenetic changes in the promoter region as described previously [8,9]. Epigenetic gene regulation is a natural mechanism for altering gene expression during

development and adaptation to new situations. In this study, we analyzed gene expression responses in endothelial cells after epigenetic manipulation of VEGF-A and bioinformatically predicted possible off-target effects of these shRNAs. This data is important for the future development of this technique for clinical gene therapy.

Methods

An expanded Materials and Methods section is available in the Data Supplement.

Vector constructs

The construction of lentiviral (LV) shRNA expression vectors has been described [8]. The used target sequences were: CGTTCT-CAGTGCCACAAAT (-856) and GACGCGTGTTCATGTGA (-451). The number in parenthesis refers to the first nucleotide in the sequence of the shRNA relative to the TSS of mouse VEGF-A promoter (U41383). As a control we used LV encoding only green fluorescent protein (GFP) without a shRNA cassette. The third generation HIV-1 based LV-PGK-GFP-U6shRNA vectors were prepared by standard calcium phosphate transfection method in 293T cells as described [10].

Transductions, sample preparation and Affymetrix analysis

C166 cells were transduced with lentiviral vector expressing either

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upregulating shRNA, downregulating shRNA, or LV control (referred in this report as “VEGF-A UP”, “VEGF-A DOWN” and control, respectively). Cells were transduced at 20% confluency using MOI 10 and samples were collected 7 days after the transduction. Sample preparation, hybridization and scanning were all done using standard Affymetrix protocols. The gene expression patterns of each group of cells were measured with 4 expression arrays (Affymetrix GeneChip Mouse Genome 430 2.0 Array), totaling 12 arrays.

Statistical Analysis

After preprocessing there were expression data for 17370 genes. From this set, we identified and filtered out those expression profiles where all absolute expression levels were in the lowest 10 percent of the data set. Statistical scores for these genes would be too inaccurate. 1518 genes were excluded based on this criterion. The data were then divided into three sets of four arrays each: downregulation, upregulation and control experiments. For each gene, we computed two fold changes and two differential expression p-values corresponding to “VEGF-A DOWN” experiment vs. control and “VEGF-A UP” experiment vs. control. P-values were computed with two-tailed t-test with permutation testing (10,000 permutations). To correct for multiple hypothesis testing related errors, we computed false discovery rates (FDR) using the Storey-Tibshirani procedure [11]. False discovery rate is defined as the expected ratio of the number of false positives to the total number of positive calls in a differential expression analysis between two groups of samples. The q-values, which measure the minimum FDR that occurs when calling the test significant, were also computed.

A gene is here considered to be differentially expressed if it shows both statistical and biological significance. Using p-values as a measure of statistical significance ($p < 0.01$) and fold change (FC) ($\log_2(\text{FC}) > 0.5$ for the “VEGF-A DOWN” experiment, and $\log_2(\text{FC}) > 0.25$ for the “VEGF-A UP” experiment) as a measure of biological significance, the differentially expressed genes were computed for both experiments. A more stringent fold change criterion was applied in the “VEGF-A DOWN” experiment because these expression profiles deviated considerably more from the control profiles.

Gene ontology and pathway analysis

Gene ontologies, pathways affected and regulatory transcription factors were analyzed using Ingenuity Pathway Analysis tool, (IPA, www.ingenuity.com) by analyzing only the datasets of genes that we found significantly regulated by our own analysis.

qRT-PCR

cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Real-time quantitative PCR was performed with a LightCycler 480 apparatus (Roche) using TaqMan Gene Expression Assays (Applied Biosystems) for ACTB (4352663), Tnnt2 (Mm01290256_m1), Kdr (Mm004401111_m1), Vegfa (Mm00437306_m1), Ccl2 (Mm00441242_m1), Selp (Mm00441295_m1), Mmp13 (Mm00439491_m1), Angptl4 (Mm00480431_m1) and LightCycler 480 Probes Master (Roche). PCR cycling conditions were: 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. Fold changes were calculated using the formula $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{shRNA}) - \Delta\text{Ct}(\text{GFP})$, and $\Delta\text{Ct} = \text{Ct}(\text{Target gene}) - \text{Ct}(\text{ACTB})$. Ct is the cycle at which the threshold line is crossed.

Results

Based on the selected criteria, there were 326 overexpressed genes and 469 downregulated genes in the “VEGF-A UP” experiment; and

Figure 1: Volcano plots. Upper panel shows a volcano plot for the downregulation experiment and lower panel for the upregulation experiment. The log-ratio of mean gene expression is plotted on the x-axis and negative logarithm of p-value is plotted on the y-axis. Orange dotted lines correspond to the p value threshold ($p < 0.01$) and fold change threshold (0.5 for downregulation experiment and 0.25 for upregulation experiment). Darker blue dots, which exceed both thresholds, are significantly differentially expressed genes, and light blue dots that fail to exceed either threshold are not differentially expressed.

Figure 2: Boxplot of VEGF-A log-intensities. VEGF-A expression is clearly elevated in the upregulation experiment (VEGF-up) compared to the control group (Control). Similarly, the expression of VEGF-A is significantly repressed in the downregulation experiment (VEGF-down). Red lines mark median values, edges of the boxes are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

618 overexpressed genes and 587 downregulated genes in the “VEGF-A DOWN” experiment. In Figure 1, volcano plots for both experiments are shown. The spread of data points in the volcano plot is notably wider in the “VEGF-A DOWN” experiment, indicating a more widespread

effect of VEGF-A modulation.

In Figure 2, VEGF-A intensities are plotted for each of the three experiments. There is a marked downregulation of VEGF-A in the downregulation experiment and a more modest upregulation of VEGF-A in the upregulation experiment. In Figure 3, we performed hierarchical clustering (with Euclidean distance and Ward's linkage) to show that the arrays cluster correctly together according to the experiments.

It can be noted that arrays from the upregulation experiment cluster more closely with the control experiments, which means that the genome-wide effects of the downregulating shRNA are stronger. The relative expression levels of the differentially expressed genes were hierarchically clustered to investigate the proportions of up- and downregulated genes in the two experiments (Figure 4).

Figure 3: Dendrogram plot of hierarchical cluster tree of arrays. Arrays were hierarchically clustered (with Euclidean distance and Ward's linkage) to examine the similarities between samples. All arrays cluster tightly according to the experiment. Noteworthy, however, is that arrays from the upregulation experiment (blue cluster) seem to be considerably more similar to the controls (red) than arrays from the downregulation experiment (green). This indicates that the effects of VEGF-A downregulation are more widespread than the effects of upregulation.

Figure 4: Clustered expression ratios of differentially expressed genes. Expression of each gene was normalized against the mean of control samples and then hierarchically clustered (with Euclidean distance and Ward's linkage) to reveal the patterns of differential expression. There are significantly more over- (red) and underexpressed (green) genes in the downregulation experiment (VEGF-down) compared to the samples from the upregulation experiment (VEGF-up). Interestingly, there are also a few genes that are either repressed or elevated in both of the experiments.

Entrez ID	Gene	Fold change	T-statistic	P-value	FDR	Q-value
14955	H19	-25,50	10,5	0,0017	0,0040	0,0040
12353	Car6	-18,86	33,8	0,0001	0,0039	0,0038
21956	Tnnt2	-18,58	27,6	0,0002	0,0040	0,0039
21952	Tnni1	-10,71	11,1	0,0015	0,0040	0,0040
216188	Aldh1l2	-10,05	93,6	0,0000	0,0020	0,0020
50706	Postn	-8,83	20,8	0,0003	0,0040	0,0039
107869	Cth	-8,31	27,0	0,0002	0,0040	0,0039
17022	Lum	-8,13	14,6	0,0008	0,0040	0,0040
20210	Saa3	-7,18	18,4	0,0005	0,0040	0,0040
108151	Sema3d	-7,10	29,2	0,0001	0,0039	0,0039
11607	Agtr1a	-6,77	7,4	0,0029	0,0042	0,0042
70726	Angptl6	-6,66	21,3	0,0003	0,0040	0,0039
66695	Aspn	-6,55	15,8	0,0007	0,0040	0,0040
20319	Sfrp2	-6,40	13,3	0,0010	0,0040	0,0040
14734	Gpc3	-6,20	13,8	0,0009	0,0040	0,0040
18787	Serpine1	-6,01	17,2	0,0006	0,0040	0,0040
66246	Osgep	-4,83	13,1	0,0010	0,0040	0,0040
654812	Angptl7	-4,74	15,7	0,0007	0,0040	0,0040
70544	5730437N04Rik	-4,74	46,0	0,0000	0,0037	0,0037
17844	Mup5	4,99	-10,7	0,0016	0,0040	0,0040

64929	Scel	5,04	-36,8	0,0001	0,0039	0,0038
22403	Wisp2	5,44	-55,7	0,0000	0,0036	0,0036
27226	Pla2g7	5,62	-17,3	0,0005	0,0040	0,0040
234564	Ces1f	5,72	-20,0	0,0004	0,0040	0,0040
17178	Fxyd3	6,10	-21,8	0,0003	0,0040	0,0039
56047	Msln	6,16	-51,0	0,0000	0,0037	0,0037
116872	Serpib7	6,56	-56,7	0,0000	0,0036	0,0036
235345	4833427G06Rik	6,65	-21,6	0,0003	0,0040	0,0039
15245	Hhip	6,96	-33,2	0,0001	0,0039	0,0039
108096	Slco1a5	7,35	-4,9	0,0056	0,0048	0,0048
20527	Slc2a3	7,38	-107,1	0,0000	0,0000	0,0000
13107	Cyp2f2	7,69	-20,2	0,0004	0,0040	0,0039
108078	Olr1	8,37	-24,5	0,0002	0,0040	0,0039
77055	Krt76	9,13	-21,9	0,0003	0,0040	0,0039
16691	Krt8	10,20	-4,9	0,0056	0,0048	0,0048
16542	Kdr	10,52	-43,5	0,0000	0,0038	0,0038
56811	Dkk2	12,95	-17,5	0,0005	0,0040	0,0040
21950	Tnfsf9	19,76	-28,3	0,0001	0,0040	0,0039
270097	Vat1l	34,74	-26,3	0,0002	0,0040	0,0039

Table 1: Twenty most downregulated and twenty most upregulated genes in response to downregulation of VEGF-A by TGS.

Entrez ID	Gene	Fold change	T-statistic	P-value	FDR	Q-value
386463	Cdsn	3,26	-13,2	0,0003	0,0103	0,0103
12353	Car6	2,62	-20,4	0,0001	0,0100	0,0100
66222	Serpib1a	2,57	-12,5	0,0003	0,0105	0,0104
110454	Ly6a	2,29	-13,6	0,0002	0,0103	0,0103
17772	Mtm1	2,22	-9,2	0,0007	0,0109	0,0109
235345	4833427G06Rik	2,13	-7,0	0,0013	0,0116	0,0115
59083	Fetub	2,12	-11,1	0,0004	0,0106	0,0106
11468	Actg2	2,05	-6,7	0,0014	0,0117	0,0116
20706	Serpib9b	2,05	-53,3	0,0000	0,0051	0,0051
13380	Dkk1	2,00	-9,3	0,0006	0,0109	0,0108
107869	Cth	1,92	-9,3	0,0006	0,0108	0,0107
57875	Angptl4	1,90	-11,3	0,0004	0,0107	0,0105
103172	Chchd10	1,88	-7,7	0,0010	0,0112	0,0112
16669	Krt19	1,82	-4,9	0,0030	0,0141	0,0141
17386	Mmp13	1,75	-5,7	0,0021	0,0126	0,0126
228139	P2rx3	1,75	-7,4	0,0011	0,0113	0,0113
56772	Mlt11	1,74	-8,8	0,0007	0,0109	0,0109
69219	Ddah1	1,74	-14,7	0,0002	0,0102	0,0101
16149	Cd74	1,74	-3,8	0,0068	0,0207	0,0207
22041	Trf	-2,28	16,6	0,0001	0,0102	0,0100
15109	Hal	-2,28	10,2	0,0005	0,0107	0,0107
66695	Aspn	-2,29	10,2	0,0005	0,0107	0,0107
20344	Selp	-2,32	23,4	0,0000	0,0093	0,0089
17392	Mmp3	-2,40	7,7	0,0010	0,0112	0,0112
11676	Aldoc	-2,41	16,4	0,0001	0,0103	0,0100
20319	Sfrp2	-2,41	9,0	0,0007	0,0110	0,0109
20309	Cxcl15	-2,41	22,1	0,0000	0,0098	0,0097
19215	Ptgds	-2,50	8,2	0,0009	0,0111	0,0111
17879	Myh1	-2,55	6,5	0,0015	0,0117	0,0117
11816	Apoe	-2,58	10,3	0,0005	0,0108	0,0107
11606	Agt	-2,59	11,3	0,0004	0,0106	0,0105
12623	Ces1g	-2,60	12,0	0,0003	0,0106	0,0105
16000	Igf1	-2,71	8,5	0,0008	0,0110	0,0110
20296	Ccl2	-2,89	22,0	0,0000	0,0099	0,0097
96875	Prg4	-2,90	13,9	0,0002	0,0103	0,0103
20306	Ccl7	-2,95	18,4	0,0001	0,0101	0,0100
14955	H19	-2,99	7,1	0,0012	0,0115	0,0115
78896	1500015O10Rik	-3,20	4,3	0,0045	0,0164	0,0164
104158	Ces1d	-5,37	20,0	0,0001	0,0100	0,0100

Table 2: Twenty most upregulated and twenty most downregulated genes in response to upregulation of VEGF-A by TGA.

Clearly, the downregulation experiment yielded in much stronger expression patterns than the overexpression experiment. There are only a few genes that are differentially expressed to the same direction in both experiments. In Tables 1 and 2, the most differentially expressed genes are listed with appropriate statistical scores. Full lists of differentially expressed genes can be found in the supplementary Table 1 and 2.

Down regulation of VEGF-A

Genes that are linked to cancer (154 genes), cardiovascular disease (83 genes), developmental disorders (45 genes), inflammatory response (121 genes) and genetic disorders (67 genes) are significantly regulated in response to the downregulation of VEGF-A in endothelial cells. Top 5 molecular and cellular functions associated are: cell growth and

STAT1 and C/EBP transcription factors are activated in response to VEGF-A downregulation and SRF, SMAD3, HIF1A, ATF4 and TRIM24 transcription factors are inhibited by VEGF-A downregulation (Figure 5).

Genes that are linked to cancer (120 genes), cardiovascular disease

Transcription factors ATF4, TP73, NOTCH1 and TRIM24 are activated upon the epigenetic upregulation of VEGF-A and the expression of HNF1A and TOB1 is inhibited. As in the case of

downregulation of VEGF-A, these transcription factors regulate a number of genes that are further regulating other genes and signaling pathways (Figure 6).

qRT-PCR validation of differentially expressed key genes

We validated the expression of top upregulated and downregulated genes (Tnnt2, Kdr, Ccl2, Selp, Mmp13 and Angptl4) from both groups by using qRT-PCR analysis. The expression changes were very similar for all genes as in microarray analysis. Also the differential expression of VEGF-A was confirmed using qRT-PCR analysis (Figure 7).

Possible off-target effects of shRNAs

Non-specific binding of shRNA sequences to mouse gene promoters was evaluated genome-wide by aligning the shRNA binding sequences to the mouse genome (NCBI37/mm9). We then compared the gene expression levels of the genes closest to these putative binding sites to find out if they might be regulated by the shRNAs. Importantly, the predicted off-target genes showed a very little change in their expression (Table 3).

Therefore, the genes regulated in this study are likely affected by either up- or downregulation of VEGF-A and not by off-targets of shRNAs.

Discussion

Vascular endothelial growth factor A (VEGF-A) is a mitogen that acts on endothelial cells and has various effects, including mediation of angiogenesis and vasculogenesis. VEGF-A is also known to have effects on vascular permeability, endothelial cell proliferation, migration, and inhibition of apoptosis [12–14]. Hypoxia has been shown to induce VEGF-A expression [15]. VEGF-A has two receptors, VEGFR-1 (FLT-1) and VEGFR-2 (FLK-1/KDR) that mediate its effects. VEGFR-2 seems to be the major mediator of angiogenesis and vascularization [16], whereas VEGFR-1 has been suggested to negatively regulate angiogenesis during embryogenesis, and positively in adulthood [17]. VEGF-A induces autophosphorylation of VEGFR-2, after which PLC γ binds to VEGFR-2, activating the MAPK/ERK cascade and

Gene	Distanceto TSS (bp)	Mismatches	shRNA	FC up	FC down
Prkcz	21	5:A>C,12:G>T,13:C>T	down	NaN	NaN
Vegfa	437	9:C>A	down	1,280103	-1,537277
Mir3110	458	2:G>C,4:G>C,17:A>G	down	NaN	NaN
3632454L22Rik	528	0:T>G,3:G>A,10:T>C	up	1,000995	-1,009152
Vegfa	843	None	up	1,280103	-1,537277
Ces3a	975	1:G>C,12:T>G,14:C>G	up	NaN	NaN
Rap2c	1059	5:C>T,10:C>G,16:T>A	up	1,171336	1,098138
Cldn24	1696	2:A>C,4:A>C,5:C>G	down	NaN	NaN
Sart3	2160	0:A>C,9:C>T,10:A>G	up	-1,067785	-1,088687
Kank4	2165	0:G>C,16:G>A	up	-1,44748	-1,171926
Utrn	2588	11:A>G,18:A>T	down	1,089009	1,082528
Defb2	13474	0:A>C,10:C>G	up	-1,003149	-1,005893
Neat1	17106	0:T>G,18:T>A	up	-1,045212	1,462386
F730016J06Rik	19668	1:A>C,17:A>T	up	NaN	NaN
Dppa4	20860	0:T>C,12:T>C	up	1,048477	1,11234
Nat15	23343	7:G>T,8:G>C	up	1,026753	1,256342
Ogfod1	25050	1:T>C,18:T>A	up	1,021149	1,066103
Dpcd	37770	2:C>G,9:T>A	down	-1,042219	1,276962

Table 3: Analysis of putative shRNA binding sites by promoter scanning. Possible off-target genes show little change in gene expression.

proliferation of endothelial cells [18]. VEGF-A also activates other signaling pathways, such as Akt/PI3K [18]. VEGF-A has been associated with various diseases including cancer, cardiovascular diseases, and retinopathy [19–21]. It has been suggested as a therapeutic agent in the treatment of myocardial ischemia by overexpressing it in the heart via viral vectors [1]. Also, VEGF-A inhibition has been studied as a potential target in cancer [22].

In this study, it was found that both the activation and inhibition of VEGF-A in endothelial cells resulted in distinctive transcriptional profiles and widespread effect in the gene expression patterns of hundreds of genes. Many of the regulated genes are in turn regulated by transcription factors. One of these transcription factors, Activating transcription factor 4 (ATF4, tax-responsive enhancer element B67, CREB2) was found to be involved in the transcriptional response of both epigenetic activation and inhibition of VEGF-A. The role of CREB2 in the regulation of VEGF-A is very interesting and warrants further studies. We have previously found H3K27 to be the most important histone residue for the epigenetic regulation of mouse VEGF-A expression [8,9]. Also, we have shown that CBP/CREB interaction inhibitor abolishes the shRNA-mediated upregulation of VEGF-A. The targeting strand of this shRNA contains one mismatch (G to T) in the middle part of the sequence and this area in the murine VEGF-A promoter contains a conserved cAMP response element (CRE). CREB, which binds to CRE [23], regulates the H3K27 acetylation by binding CREB binding protein (CBP)/p300 and CREB Regulated Transcription Coactivator (CRTC) [24]. When cells were treated with CBP-CREB interaction inhibitor, the shRNA-mediated VEGF-A upregulation was reduced to the level of shRNA control [9].

It has become obvious that in addition to targeting mRNAs in the cytoplasm as in the canonical RNAi, small non-coding RNAs can target also the genome in the nucleus, and therefore all promoter areas should be checked for possible off-target effects. In this study we observed only minor gene expression changes in genes that were close to possible off-target sites in genome. Therefore, the gene expression changes observed with both TGS and TGA were most likely due to the regulation of VEGF-A and the action of transcription factors that are regulated. Taken together, this study analyzed the transcriptional profile of mouse endothelial cells in response to epigenetic up- or downregulation of VEGF-A and provides new leads for further analysis of VEGF-A signaling and its biological actions.

Data Accessibility

GEO files for review can be found at: <http://www.ncbi.nlm.nih.gov/geo/query/GSE47444>

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