Genomic Landscapes of Noncoding RNAs Regulating VEGFA and VEGFC Expression in Endothelial Cells

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ABSTRACT Vascular endothelial growth factors (VEGFs) are best known as key regulators of angiogenesis and lymphangiogenesis. Although VEGFs have been promising therapeutic targets for various cardiovascular diseases, their regulatory landscape in endothelial cells remains elusive. Several studies have highlighted the involvement of noncoding RNAs (ncRNAs) in the modulation of VEGF expression. In this study, we investigated the role of two classes of ncRNAs, long ncRNAs (lncRNAs) and enhancer RNAs (eRNAs), in the transcriptional regulation of VEGFA and VEGFC. By integrating genome-wide global run-on sequencing (GRO-Seq) and chromosome conformation capture (Hi-C) data, we identified putative lncRNAs and eRNAs associated with VEGFA and VEGFC genes in endothelial cells. A subset of the identified putative enhancers demonstrated regulatory activity in a reporter assay. Importantly, we demonstrate that deletion of enhancers and lncRNAs by CRISPR/Cas9 promoted significant changes in VEGFA and VEGFC expression. Transcriptome sequencing (RNA-Seq) data from lncRNA deletions showed downstream factors implicated in VEGFA- and VEGFC-linked pathways, such as angiogenesis and lymphangiogenesis, suggesting functional roles for these lncRNAs. Our study uncovers novel lncRNAs and eRNAs regulating VEGFA and VEGFC that can be targeted to modulate the expression of these important molecules in endothelial cells.

KEYWORDS VEGF, VEGFC, atherosclerosis, eRNA, IncRNA
yet been successful (15). Hence, new therapeutic avenues for angiogenesis are required. Moreover, little is known about the regulatory landscape of endothelial cells in the vasculature. Thus, a better understanding of the angiogenic regulatory network in endothelial cell function is needed for therapeutic advances. The angiogenic regulatory network can encompass many layers of regulation, and one of these is at the transcriptional level. In recent years, we have come to appreciate the complexity of transcriptional regulation and the role of noncoding RNAs (ncRNAs) in this process (17).

Protein-coding genes comprise less than 3% of the human genome, while the remaining 97% was once thought to be transcriptionally inactive. However, advances in sequencing techniques have revealed that the majority of the human genome is transcribed as noncoding RNA, and importantly, these regions also harbor the majority of disease-linked variants (18). The biggest class of ncRNA is comprised of long ncRNAs (lncRNAs), which are commonly defined as noncoding transcripts longer than 200 nucleotides (nt) (19). lncRNAs are a diverse class of transcripts that may overlap genes and be intergenic (lincRNAs) (20) and that can also originate from enhancer regions (eRNAs) (21). Although the estimated number of lncRNAs in humans is about 100,000, the functions of the vast majority remain uncharacterized (22). However, the use of next-generation sequencing (NGS) techniques allows identification of novel ncRNAs.

Recent studies have suggested that lncRNAs act to regulate genes in cis and in trans in a transcriptional or posttranscriptional manner during development, differentiation, and human diseases (17, 23). lncRNAs can regulate transcriptional activity in cis by recruiting chromatin-regulating complexes and thereby regulating the chromatin state (24). Furthermore, the cis-regulatory effect can be due to the transcription event itself or enhancer-like activities of the DNA sequence (25). trans-acting lncRNAs can recruit chromatin modifiers affecting the expression of multiple genes (26) and regulate transcription factor binding and RNA polymerase activity (27). Moreover, trans-acting lncRNAs can also act at a posttranscriptional level, regulating alternative splicing (28), mRNA decay (29), and regulation of translation (30). Additionally, studies have shown that lncRNAs participate in genomic organization (31).

One class of lncRNAs that are of particular interest is the enhancer RNAs. These RNAs arise from enhancers, which are defined as DNA sequences that increase the expression of protein-coding genes and can function in a cell type-specific manner (32), independently of their orientation and position to the target gene (33). Enhancer RNAs are usually less than 2 kb in length, are bidirectionally transcribed, and lack either splicing or polyadenylation (34). Some studies have shown that eRNAs regulate gene expression (35), promote enhancer-promoter looping (36), and bind to chromatin modifiers (37), hence remodeling chromatin conformation. Furthermore, eRNAs can recruit transcription factors (38) and regulate transcriptional machinery (39). Genetic variance in enhancers can thus modify the binding of transcription factors, leading to improper gene expression and susceptibility to disease (40).

The aim of this study was to decipher novel noncoding RNAs participating in the regulation of VEGFs in endothelial cells in order to provide a better understanding of this complex angiogenic regulatory network. Regulating VEGFs would be important to reverse neovascularization of atherosclerotic plaques, while revascularization of ischemic tissues would represent an important therapeutic strategy for treating atherosclerotic complications. Therefore, novel insights into the transcriptional landscape of VEGF genes may prove valuable for future therapeutic applications. By using next-generation sequencing data from endothelial cells, we identified novel eRNAs and IncRNAs that interact with VEGFA and VEGFC promoters. We discovered six and three putative enhancers for VEGFA and VEGFC, respectively, and identified active enhancers using reporter assays. We also demonstrated that deletion of VEGFA enhancer 5 (VEGFA-E5) and VEGFC enhancer 3 (VEGFC-E3) decrease VEGFA and VEGFC gene expression, respectively. Furthermore, global run-on sequencing (GRO-Seq) data allowed us to identify one lncRNA transcribed antisense to the VEGFA gene (VEGFA-LNC) and one lncRNA located 120 kb upstream of VEGFC (VEGFC-LNC) in human umbilical vein endothelial
cells (HUVECs). Deletion of VEGFA-LNC increased VEGFA expression, while deletion of VEGFC-LNC decreased VEGFC expression. Finally, we studied the genome-wide effects of these deletions using transcriptome sequencing (RNA-Seq) and found that they affected gene ontology (GO) pathways implicated in endothelial cell biology. Thus, our results uncover novel noncoding RNAs participating in the regulation of VEGF gene expression.

RESULTS

Identification and characterization of VEGFA enhancers. To identify putative regulatory elements of the VEGFA gene in endothelial cells, we started by identifying genomic loci that displayed enhancer features (defined by H3K27ac and H3K4me1), eRNA expression as determined by GRO-Seq, and chromatin interactions with the VEGFA promoter as determined by chromosome conformation capture (Hi-C) data in HUVECs. We identified six putative VEGFA enhancers, which were named VEGFA-E1 to VEGFA-E6 (Fig. 1A). The closest enhancer to the VEGFA transcription start site (TSS) was VEGFA-E1 (kb −13). The other five putative enhancers, VEGFA-E2, VEGFA-E3, VEGFA-E4, VEGFA-E5, and VEGFA-E6, were located +250 kb, +270 kb, +290 kb, +305 kb, and +334 kb downstream of the TSS, respectively. To determine the regulatory activities of the putative enhancers, we assessed their ability to induce reporter gene expression either in the context of the endogenous VEGFA promoter or a thymidine kinase (TK) minimal promoter. Enhancer activity was evaluated in primary human endothelial cells, HUVECs, and two additional endothelial cell lines, EA.hy926 and immortalized human aortic endothelial cells (TelOHAECs). Of the six characterized enhancers, only VEGFA-E1 was able to induce significant luciferase expression in all three endothelial cell lines (Fig. 1B). Importantly, VEGFA-E1 significantly activated the VEGFA promoter, as demonstrated by increased luciferase expression, varying between a 2- and a 5.8-fold increase in the different endothelial cells. The remaining putative enhancers (VEGFA-E2 to VEGFA-E6) were unable to induce any notable activation of luciferase expression in endothelial cells.

Assessment of hypoxia-inducible factor 1α (HIF-1α) chromatin immunoprecipitation sequencing (ChIP-Seq) data (41) indicated that VEGFA-E5 overlaps a HIF-1α binding site, and it may therefore be activated by hypoxia. Cells transfected with either the VEGFA-E1 or VEGFA-E5 vector were incubated under hypoxia or normoxia, and luciferase activity was assayed after 24 h. In line with our predictions, VEGFA-E5 was able to induce significant expression of luciferase following hypoxia, whereas VEGFA-E1 was not (Fig. 1C). Collectively, two enhancers (VEGFA-E1 and VEGFA-E5) out of the six predicted by a combination of Hi-C and GRO-Seq data were able to induce reporter gene expression, VEGFA-E1 constitutively and VEGFA-E5 in response to a hypoxia stimulus. Based on these results, we selected these two enhancers for further analysis.

Identification and characterization of VEGFC enhancers. Next, we applied the same approach detailed above to identify VEGFC enhancers. We identified three candidate enhancers, termed VEGFC-E1, VEGFC-E2, and VEGFC-E3, located 46 kb, 70 kb, and 110 kb upstream of the VEGFC TSS, respectively (Fig. 2A). The regulatory activities of these enhancers were also tested in the context of luciferase reporter gene expression in the three different endothelial cell types (data not shown). Results showed that all three enhancers were able to induce luciferase expression in the context of a minimal promoter in HUVECs (Fig. 2B). Among these, VEGFC-E3 had the highest activation, exhibiting a 6- to a 15-fold increase in reporter gene expression, followed by VEGFC-E1 (3.7- to 6-fold) and VEGFC-E2 (1.5- to 2-fold). In addition, both VEGFC-E3 and VEGFC-E2 demonstrated further activation in the context of the endogenous VEGFC promoter. Previous studies reported histone marks in enhancers to be highly correlated with cell type-specific gene expression (42), and a combination of multiple histone marks allows the characterization of chromatin states (43). Thus, we next compared the chromatin states of the identified VEGFC enhancers in different cell types using Encode data. Interestingly, the activities of these enhancers were not limited to endothelial cells but were also present in muscle cells and fibroblasts, among others. However, these
FIG 1 VEGFA enhancer identification and reporter assay. (A) UCSC browser image depicting the VEGFA locus and six putative enhancers (VEGFA-E1 to VEGFA-E6) in HUVECs. Enhancer regions are highlighted in blue, and arcs below the tracks depict significant interactions from Hi-C data. Coordinates of cloned enhancers are as follows: VEGFA-E1 (chr6: 43720993 to 43722024), VEGFA-E2 (chr6: 43940393 to 43941036), VEGFA-E3 (chr6: 44008921 to 44010132), VEGFA-E4 (chr6: 44026392 to 44027694), VEGFA-E5 (chr6: 44040639 to 44041514), and VEGFA-E6 (chr6: 44072050 to 44073251). (B) Luciferase reporter assay of the activities of the six putative enhancer regions either in the context of the TK minimal promoter or the VEGFA promoter. Each reporter construct was transfected into HUVECs, EA.hy926 cells, or TeloHAECs and assayed after 48 h. (C) Activities of VEGFA enhancers 1 and 5 (VEGFA-E1 and VEGFA-E5) under hypoxia stimulus. Cells transfected with the VEGFA-E1 and VEGFA-E5 vectors and control vectors were incubated under either hypoxia or normoxia, and luciferase activity was assessed after 24 h. All luciferase data are represented as fold changes from firefly luciferase activity (normalized to Renilla luciferase activity) induced by enhancer vectors over the firefly signal obtained with their respective control vectors. Error bars represent means ± standard errors of the means (SEM) (n = 3). *, P value < 0.05; **, P value < 0.001; ***, P value < 0.0001 (by one-way ANOVA and Dunnett’s multiple-comparison test).
enhancers were inactive in other cell types, including monocytes and lymphoblastoid and liver cells (Fig. 2C).

Validation of VEGFA and VEGFC enhancer activity at their endogenous loci. To experimentally validate the functional role of the VEGFA (VEGFA-E1 and VEGFA-E5) and VEGFC (VEGFC-E1, VEGFC-E2, and VEGFC-E3) enhancers in driving coding gene expression, we used CRISPR/Cas9 to delete the enhancer regions in EA.hy926. By PCR screening and sequencing, we confirmed independent cellular clones carrying homozygous deletions of the target enhancers (data not shown) and characterized their effects on VEGFA and VEGFC gene expression by quantitative reverse transcription-PCR (RT-qPCR) in different clones. From the results, we did not observe any effect of VEGFA-E1 deletion on VEGFA expression (Fig. 3A), in contrast to the observed VEGFA-E1 activity in the reporter assays (Fig. 1B). On the other hand, deletion of VEGFA-E5 resulted in a notable decrease (2.3-fold decrease) in VEGFA expression (Fig. 3A). Likewise, VEGFC-E3 deletion induced a significant decrease in VEGFC expression (2.5-fold) (Fig. 3B), in accordance with the reporter assay results (Fig. 2B). Deletion of VEGFC-E1 and VEGFC-E2, on the other hand, did not induce any notable effect on VEGFC expression (Fig. 3B). Additional validations using antisense oligonucleotides targeting the VEGFC eRNA transcribed from VEGFC-E3 showed that knockdown of the eRNA did not seem to have an effect on VEGFC expression (Fig. 3C), suggesting that the RNA transcript might not be needed
FIG 3 VEGFA and VEGFC expression upon enhancer deletions. (A) RT-qPCR analysis of VEGFA expression in EA.hy926 clones with deletions of VEGFA-E1 (ΔVEGFA-E1) or VEGFA-E5 (ΔVEGFA-E5) and control (CTL) cells that were transfected with only gRNAs. The bar graphs represent the averages of data from 4 and 6 deletion clones of VEGFA-E1 and VEGFA-E5, respectively. The data are represented as means ± standard deviations (SD) (n = 3). *** P value < 0.0001 (Student’s unpaired two-tailed t test). (B) RT-qPCR analysis of VEGFC expression in EA.hy926 clones with deletion of VEGFC-E1 (ΔVEGFC-E1), VEGFC-E2 (ΔVEGFC-E2), and VEGFC-E3 (ΔVEGFC-E3). The bar graphs represent the averages of data from 3 and 6 deletion clones of VEGFC-E1 and VEGFC-E2, respectively. For ΔVEGFC-E3, no clonal selection was done, as the deletion efficacy was 100%; thus, a pool of transfected cells was considered for expression analysis. To replicate this experiment, two different pairs of gRNAs, gRNA_P1 and gRNA_P2, for VEGFC-E3 deletion were used. The data are means ± SD (n = 3). *** P value < 0.001 (Student’s unpaired two-tailed t test). (C) RT-qPCR analysis of VEGFC expression where eRNA was knocked down using ASOs. ASO C1 and 2 are the different antisense oligonucleotides targeting the VEGFC-E3 eRNA. The data are means ± SD (n = 3). * P value < 0.05; *** P value < 0.0001 (one-way ANOVA and Dunnett’s multiple-comparison test). (D) Correlation plot displaying the log number of reads per kilobase of transcript per million mapped reads (RPKM) of the enhancer VEGFA-E5 versus that of VEGFA and of the enhancer VEGFC-E3 versus that of VEGFC from GRO-Seq data. EC, endothelial cells.
for the enhancer effect. Taking these observations together, we conclude that VEGFA-E5 and VEGFC-E3 contribute to the endogenous transcriptional expression of VEGFA and VEGFC, respectively.

To further investigate the role of the VEGFA-E5 and VEGFC-E3 enhancers in the regulation of VEGFA and VEGFC expression, respectively, we correlated the transcriptional activities of the enhancers with their putative target gene across a diverse set of human cells. Previous studies have shown that eRNA expression is highly linked to open chromatin state (indicated by DNase-hypersensitive sites) and regulatory activity (42), allowing evaluation of the chromatin state of the enhancers in cell types that express various levels of VEGFs (such as cardiomyocytes, smooth muscle cells, fibroblasts, and epithelial cells). For this, we collected numerous public (44–73) GRO-Seq data sets encompassing 44 cell types across 384 samples and studied the expression of eRNAs from the identified enhancer regions. The results (Fig. 3D) suggest that eRNA expression correlates well with VEGF expression throughout cell types and in response to hypoxic stimulation. The results corroborate the functional relevance of these enhancers for VEGF expression.

**Nearby IncRNAs play a role in the regulation of VEGF gene expression.** Further assessment of HUVEC GRO-Seq data allowed us to identify other transcripts around the VEGFA and VEGFC loci. As with the vast majority of mammalian gene TSSs (74), the VEGFA and VEGFC promoters were characterized by promoter-associated divergent transcription (Fig. 4). In addition, we detected two IncRNAs located nearby the VEGFA and VEGFC genomic loci, which we named VEGFA-LNC and VEGFC-LNC, respectively. Similarly to the coding gene TSSs, the IncRNA TSSs were marked by H3K4me3, a histone modification commonly associated with promoter elements. VEGFA-LNC is transcribed in the antisense orientation with respect to the VEGFA gene, with its TSS located in an intron ~4 kb downstream of the VEGFA TSS (Fig. 4A). Interestingly, VEGFA-LNC and its TSS are conserved in many other cell types, such as THP1 monocytes and MCF-7 cancer cells (Fig. 4B). Furthermore, VEGFA-LNC was also shown to be expressed in mouse thioglycolate-elicited macrophages (TGM) and C166 mouse endothelial cells, also supporting species conservation (data not shown). We also identified a transcript downstream of VEGFC-E3 with strong H3K27ac, H3K4me3, and GRO-Seq signals, which we named VEGFC-LNC. VEGFC-LNC is located ~120 kb upstream of the VEGF TSS, and it is transcribed in an opposing direction relative to that of the VEGFC transcript (Fig. 4C). However, VEGFC-LNC was specific to endothelial cells and not conserved (data not shown). Previous studies have shown that IncRNAs can hold coding potential and exert their biological function through small peptides (75). Thus, we next evaluated the coding potential of VEGFA-LNC and VEGFC-LNC using the CPPred prediction tool (76). CPPred integrates several features, such as open reading frame (ORF) length and coverage and Fickett score, among others, to predict the coding potential. From the results obtained, VEGFA-LNC and VEGFC-LNC were not predicted to have coding potential (data not shown).

To test the regulatory activities of these IncRNAs, we carried out CRISPR/Cas9-mediated deletion of the IncRNA promoters in the EA.hy926 cell line. VEGFA-LNC and VEGFC-LNC deletions were confirmed by genotyping and RT-qPCR of VEGFC-LNC expression levels (data not shown), and confirmed homozygous clones for each IncRNA deletion were chosen for further assessment of the effects on VEGFA and VEGFC gene expression. RT-qPCR expression analysis in different clones demonstrated that deletion of VEGFA-LNC led to a 1.8-fold increase in VEGFA mRNA expression (Fig. 5A), whereas a 1.6-fold decrease in the expression of VEGFC was observed upon deletion of VEGFC-LNC (Fig. 5B). The effects on VEGF gene expression were consistent between the clones (data not shown), suggesting that there is minimal clonal variation. Furthermore, degradation of VEGFA-LNC with two different antisense oligonucleotides promoted upregulation of VEGFA’s expression by 1.7- to 2-fold in EA.hy926 cells and by 1.2- to 1.6-fold in TeloHAECs, supporting the results obtained from VEGFA-LNC’s deletion (Fig. 5C), whereas targeting VEGFC-LNC with an antisense oligonucleotide did not alter VEGFC expression (data not shown).
not affect VEGFC expression (Fig. 5D), suggesting a transcript-independent effect. Collectively, these data suggest possible roles of VEGFA-LNC and VEGFC-LNC in transcriptional regulation, participating in the downregulation of VEGFA and upregulation of VEGFC, respectively.

To determine if the action is predicted to be nuclear and/or cytoplasmic, we next quantified the amount of VEGFA-LNC and VEGFC-LNC in the nuclear and cytoplasmic...
FIG 5 Regulatory function of VEGFA-LNC and VEGFC-LNC. (A and B) RT-qPCR analysis of VEGFA and VEGFC expression in control cells or in cell clones where VEGFA-LNC (ΔVEGFA-LNC) and VEGFC-LNC (ΔVEGFC-LNC) were deleted. The bar graphs represent the averages of data from 5 deletion clones of VEGFA-LNCs or VEGFC-LNC. (C) Expression analysis of VEGFA in EA.hy926 cells and TeloHAECs where VEGFA-LNC was knocked down with an ASO. ASO_A1 and ASO_A2 represent two different ASOs targeting different sites of the VEGFA-LNC. Data are represented as means ± SD (n = 3). *, P value < 0.05; ***, P value < 0.0001 (one-way ANOVA and Dunnett's multiple-comparison test). (D) Significance was evaluated with Student's unpaired, two-tailed t test (n = 3). (E) Bar plot showing the log, fold change of nuclear versus cytoplasmic fractions of VEGFC-LNC, along with other well-known nuclear and cytoplasmic transcripts. Statistical significance was evaluated with Student's unpaired two-tailed t test (n = 3). *** P value < 0.001. (F) Correlation plot displaying the log, RPKM of VEGFA-LNC versus VEGFA or VEGFC-LNC versus VEGFC from public HUVEC and HAEC GRO-Seq data. ND*, not detected.
fractions in HUVECs using RNA-Seq. The results (Fig. 5E) demonstrated that VEGFC-LNC was expressed mainly in the nucleus (average cpm, 8) with other known nuclear lncRNAs (XIST, MALAT1, NEAT1, and H3F3A), whereas VEGFA-LNC expression was too low to be evaluated (average cpm in the cytoplasm, 0.02). This strongly suggests that VEGFC-LNC acts in the nucleus and that VEGFA-LNC may play either a role in the cytoplasm or a cotranscriptional role in the nucleus. If the lncRNA regulates transcription in the nucleus, we expect the lncRNA expression in GRO-Seq (which detects nascent RNAs that can also exhibit low stability) to be inversely correlated with VEGF expression. To test this, we used all public GRO-Seq data from stimulated HUVECs and HAECs to measure the correlation between the coding gene and lncRNA expression (Fig. 5F). Our results demonstrated a significant positive correlation between VEGFC-LNC and VEGFC expression, supporting shared mechanisms of transcriptional regulation. On the other hand, a trend of anticorrelation was detected for VEGFA-LNC and VEGFA, although this was not significant. All together, our results suggest that the activating effect of VEGFC-LNC and repressing effect of VEGFA-LNC may be due to cotranscriptional cis mechanisms.

Functional evaluation of VEGFA-LNC and VEGFC-LNC by RNA-Seq. To further explore whether the VEGFA-LNC and VEGFC-LNC deletions had other genome-wide cis or trans effects, we carried out RNA-Seq on three knockout clonal lines for each region. Deletion of VEGFA-LNC resulted in 708 differentially expressed genes (DEGs), of which 484 (68%) were downregulated and 224 (32%) were upregulated (Fig. 6A; see Table S1 in the supplemental material). By the selected thresholds (a fold change [FC] of at least 1.5 but not more than 2, and a false-discovery rate [FDR] of less than 0.05), however, VEGFA itself was not among the DEGs. We further identified potential cis targets among these, such as NFkB1, KLHDC3, and SUPT3H, located within 1 Mb of VEGFA-LNC, suggesting that the majority of the downstream effects of VEGFA-LNC are likely mediated by trans mechanisms.

To test if VEGFA-LNC could regulate the expression of these genes (Table S1) by directly interacting within their promoter, we used Triplex Domain Finder software (77) to find regions of DNA binding domains (DBDs) within VEGFA-LNC that are able to bind to target promoters via triple-helix formation. One region of VEGFA-LNC, spanning bp 2412 to 2464, was predicted to significantly bind within the triplex target DNA site (TTS) of 31 genes via triplelexes (MDGA2, SIGLEC15, DCLK1, CCL26, RGS17, ADAMTS9, DHRS13, KCNQ3, CACNA2D1, ACANG6, STOX2, CCR4, CD300C, OSCAR, VASN, TRIM29, FAM133A, KLHDC8B, EFCAB6, PRR9, LRG1, EFR3B, KDF1, CAPN8, GCSAML, KR2, IL36A, NEDD9, HDAC9, and GAL3ST4 [data not shown]). Of those genes, 11 were upregulated and 20 downregulated upon VEGFA-LNC deletion (Table S1). Interestingly, some of these genes, such as OSCAR, TRIM29, RGS17, VASN, LRG1, NEDD9, IL36a, ADAMTS9, and HDAC9, were previously reported to be involved in regulating proliferation in different cell lines and endothelial cells (78–86).

In order to determine the relevant biological functions affected by the VEGFA-LNC deletion, gene ontology (GO) analysis was performed separately to select for upregulated and downregulated genes. In this analysis, redundant GO terms were ignored, and the top-most-informative GO terms were selected based on P values. The top five enriched biological terms associated with DEGs upon VEGFA-LNC deletion are shown in Fig. 6B. The upregulated genes were involved in functional categories related to the type I interferon (IFN) signaling pathway, virus response, ATP response, purine nucleoside metabolism, and negative regulation of cell proliferation. Among these, we identified CEBP2, MX01, CLM1, IFIT3, IFITM1, PTEN, PTK2B, SSTR5, SPRY1, and the known factors implicated in VEGF pathways, such as LEPR (87), HIP3A (GO identifier, 0001944), PTEN (88), LRG1 (82), MDK (89) (GO identifier, 0001944), and HOXA1 (90). On the other hand, downregulated genes were centered on categories related to cell adhesion, circadian rhythm, extracellular matrix (ECM) organization, negative regulation of DNA binding transcription factor activity, negative regulation of blood coagulation, and response to cyclic AMP (cAMP). We were also interested in the identification of potential upstream regulators that may explain the observed differential expression. To do so, we used IPA (Ingenuity Pathway Analysis; Qiagen) to identify both activated and inhibited upstream regulators. The list of top upstream regulators associated with DEGs following VEGFA-LNC deletion that we extracted is shown in Table 1.
The top 5 upstream regulators that appeared on this list include three cytokines (IFN-γ, IFN-α, and prolactine [PRL]), one transcription factor (IRF7), and one growth factor (transforming growth factor β1 [TGFB1]). All together, our results suggest that some of the downstream effects of VEGFA-LNC deletion may be mediated by VEGFA’s altered expression, but the majority may act through other pathways, such as interferon signaling.

The same criteria described above were applied to study the DEGs upon VEGFC-LNC deletion (Fig. 6A; Table S1). Deletion of VEGFC-LNC resulted in a total of 520 DEGs, of which 298 (57%) and 222 (43%) were upregulated and downregulated, respectively.
We also used Triplex Domain Finder software to find potential DNA binding domains within VEGFC-LNC that could bind to the promoters of the DEGs (Table S1). Using this approach, we identified 37 DBD regions; however, none of these were statistically significant.

We further performed GO analysis to identify biological processes associated with DEGs upon VEGFC-LNC deletion. The top biological terms identified with upregulated genes were associated mainly with extracellular matrix (ECM) organization, NF-κB signaling, apoptotic signaling, ubiquitin-dependent catabolism, and cell adhesion, while downregulated genes were related to nucleosome assembly, immune response, protein complex assembly, cell division, and keratinization (Fig. 6B). Here again, several of these pathway-related genes have been linked to VEGFC-mediated effects, including CCBE1 (91), ITGA4 (92), NOTCH4 (93), ANGPTL4 (94), and HMOX1 (95). We could not predict any potential VEGFC-LNC downstream cis target, as the closest gene among the DEGs was ~5 Mb distant from the VEGFC-LNC TSS. Furthermore, we identified several upstream regulators that could explain the observed DEGs upon VEGFC-LNC deletion, shown in Table 2. Among these, the TP53 transcription factor was the most significant upstream regulator, and it was predicted to be activated. Similarly, several growth factors, including TGFB2, TGFB1, VEGF, and EGF, were predicted to be activated. This suggests that VEGFC-LNC may mediate substantial effects through VEGF signaling, although alternative mechanisms may also exist.

Effect of lncRNAs and eRNA deletion on cell proliferation and migration. In order to investigate whether the identified lncRNAs and eRNA have an effect on cell proliferation and migration and to confirm the RNA-Seq data of the lncRNA-deleted clones, we performed cell proliferation and migration assays. Proliferation assay results demonstrated that deletion of VEGFA-LNC significantly reduced cell proliferation (Fig. 6C), while VEGFC-LNC and enhancer deletions did not show any notable effect on cell proliferation (Fig. 6C). These results support our findings from the RNA-Seq analysis of VEGFA-LNC deletion, where a negative effect on cell proliferation was observed among biological processes associated with upregulated genes upon deletion. On the other hand, scratch wound assays did not show any effect on the cell migration of the clones carrying VEGFA-LNC and VEGFC-LNC deletions (data not shown).

**DISCUSSION**

In our study, we performed in-depth characterization of the genomic loci around VEGFA and VEGFC genes and identified novel enhancers and lncRNAs that play a role in their expression in endothelial cells. While the enhancers clearly upregulated gene

| Upstream regulator | Molecule type | Activation state | Z-score  | P value |
|--------------------|---------------|-----------------|----------|---------|
| IFN-γ              | Cytokine      | Activated       | 2.2      | 2.41E-19|
| IRF7               | Transcription regulator | Activated | 4.1      | 1.84E-14|
| PRL                | Cytokine      | Activated       | 3.8      | 8.00E-14|
| IFN-α (group)      | Cytokine      | Activated       | 3.1      | 2.69E-13|
| TGFβ1              | Growth factor | Inhibited       | −4.6     | 1.50E-12|
| STAT2              | Transcription regulator | Activated | 2.1      | 2.64E-12|
| STAT1              | Transcription regulator | Activated | 2.7      | 5.75E-12|
| IL1RN              | Cytokine      | Inhibited       | −2.9     | 8.92E-12|
| IFNA2              | Cytokine      | Activated       | 3.5      | 3.26E-10|
| PML                | Transcription regulator | Activated | 3.9      | 2.14E-09|
| TREM1              | Transmembrane receptor | Inhibited | −4.1     | 3.90E-09|
| BTK                | Kinase         | Inhibited       | −3.2     | 5.54E-09|
| SMAD4              | Transcription regulator | Inhibited | −3.1     | 1.21E-08|
| IFNA1/IFNA13       | Cytokine      | Activated       | 2.7      | 1.67E-08|
| IRF1               | Transcription regulator | Activated | 2.7      | 1.23E-07|
| ATXN3              | Peptidase      | Activated       | 2.0      | 5.19E-07|
| PD98059            | Kinase inhibitor | Activated | 2.0      | 5.40E-07|
| IRF8               | Transcription regulator | Inhibited | −2.8     | 7.17E-07|
| F2                 | Peptidase      | Inhibited       | −2.8     | 1.19E-06|
expression, IncRNAs demonstrated various functions. CRISPR/Cas9 deletion and genome-wide RNA-Seq demonstrated several downstream targets of IncRNAs, including factors related to endothelial functions, such as angiogenesis and cell proliferation.

In this study, more enhancers were predicted from Hi-C and GRO-Seq data than were found to have activity in the reporter assay. A similar discrepancy has previously been reported (96), and enhancer regions with no activity in reporter assays may still have a biological role in gene regulation, acting as seed regions in order to bring important factors in three dimensions (3D) to activate others (97). Furthermore, the identified enhancers induced varied expression of the reporter genes among different cell lines, which can be explained by the different availabilities of transcription factors between cell types (32). Interestingly, Hi-C data also showed interactions among the enhancers, in accordance with previous studies where enhancer interactions were detected within the range of 1 kb to 10 Mb (98). Enhancer interactions have been reported to be cooperative and exhibit eRNA expression correlation as well as target gene correlation with the number of enhancers. Therefore, it was interesting to note that not all enhancers that were functionally active in the context of the reporter assay displayed endogenous activity on their target genes. To this end, only deletion of VEGFA-associated enhancer (VEGFA-E5) and VEGFC-associated enhancer (VEGFC-E3) resulted in the downregulation of their target genes. This may be due to the episomal context of the reporter plasmids, which does not recapitulate the long-range regulatory interactions of the endogenous chromatin (99). Supporting this, VEGFA-E5’s endogenous effect on VEGFA did not require hypoxia, contrasting with the reporter assay results. In addition, the resolution of 10 kb of the Hi-C data constitutes a potential limitation of our study, making some interactions, such as those between the enhancer VEGFC-E1 and the VEGFC promoter, not evident. Thus, future studies are needed to better understand the cooperativity and hierarchy of enhancers activating VEGF expression.

Interestingly, enhancers VEGFA-E5 and VEGFC-E3 were not the closest ones to their respective gene promoters, in accordance with the enhancer hallmark that they can act independently of their distance from and orientation to the target genes and exert their function at large distances by looping mechanisms (100). Additionally, knockdown of eRNA transcribed from VEGFC-E3 with antisense oligonucleotides (ASOs) did not result in any notable effects in VEGFC expression, suggesting that the transcript itself is not required for transcriptional regulation. This is in line with previous findings.

### Table 2: Top 20 upstream regulators upon deletion of VEGFC-LNC

| Upstream regulator | Molecule type      | Activation | Z-score | P value       |
|--------------------|--------------------|------------|---------|---------------|
| TP53               | Transcription regulator | Activated  | 3.61    | 4.25E–09      |
| TGFB2              | Growth factor       | Activated  | 2.90    | 4.14E–08      |
| TGFB1              | Growth factor       | Activated  | 2.59    | 6.22E–07      |
| LY394002           | Kinase inhibitor    | Inhibited  | −2.53   | 1.04E–06      |
| TNF                | Cytokine            | Activated  | 3.21    | 2.18E–06      |
| F2                 | Peptidase           | Activated  | 3.37    | 2.88E–06      |
| VEGF               | Growth factor       | Activated  | 2.17    | 4.86E–06      |
| EDN1               | Cytokine            | Activated  | 2.54    | 4.93E–06      |
| TP63               | Transcription regulator | Activated  | 2.23    | 5.92E–06      |
| U0126              | Kinase inhibitor    | Inhibited  | −2.51   | 9.20E–06      |
| ERK                | Kinase              | Activated  | 2.35    | 2.21E–05      |
| EGF                | Growth factor       | Activated  | 2.11    | 5.93E–05      |
| IFN-β              | Cytokine            | Activated  | 2.17    | 6.72E–05      |
| P38 MAPK           | Kinase              | Activated  | 3.09    | 1.17E–04      |
| JNK                | Transcriptional regulator | Activated  | 2.40    | 1.43E–04      |
| HIF1A              | Transcription regulator | Activated  | 2.93    | 1.77E–04      |
| NFAT               | Transcription regulator | Activated  | 2.41    | 3.31E–04      |
| ATF4               | Transcription regulator | Activated  | 2.15    | 8.19E–04      |
| EGFR               | Kinase              | Activated  | 2.07    | 1.21E–03      |
| AP-1               | Transcription regulator | Activated  | 2.18    | 1.24E–03      |
where instead of the RNA transcript itself, the DNA regulatory elements were found to be more important for function (96, 101).

Our study has also identified two IncRNAs, VEGFA-LNC and VEGFC-LNC, that may provide additional mechanisms regulating VEGFA and VEGFC expression, respectively, in endothelial cells. We showed that deletion of VEGFA-LNC resulted in a transcriptional increase in VEGFA, suggesting that VEGFA-LNC may participate in the transcriptional interference of the VEGFA locus. In line with previously reported data, IncRNAs can induce transcription interference by displacement of transcription factors near the promoter, nucleosome reposition over the promoter, and obstruction of RNA polymerase II (Pol II) (17, 23, 102). Interestingly, VEGFA-LNC was also conserved in mouse cells, suggesting its potential evolutionary importance in VEGFA regulation. Upregulation of VEGFA expression would be beneficial for the treatment of diseases that would benefit from angiogenesis, and this could be achieved by targeting VEGFA-LNC. In vivo studies using ASOs directed to IncRNAs have already achieved successful results in reducing multiple myeloma (103); thus, ASO targeting of VEGFA-LNC opens up new therapeutic possibilities.

A recent study also identified two short antisense VEGFA IncRNAs that regulate VEGFA expression in hypoxia, which were shown to be upregulated along with VEGFA and localized to its promoter and upstream elements (104). Currently, it is unclear whether these IncRNAs are spliced from the longer VEGFA-LNC described here, which initiates transcription from the 1st intron of the VEGFA gene, or whether these IncRNAs represent different transcripts. However, supporting the latter option, knockdown of these VEGFA IncRNAs with ASOs downregulated VEGFA expression, suggesting a mechanism of action that is opposite from that of VEGFA-LNC (104).

Analysis of RNA-Seq data from VEGFA-LNC or VEGFC-LNC deletions identified differentially expressed genes on the same and different chromosomes, indicating large genome-wide effects. Importantly, none of the observed differentially expressed genes coincided with the off-target genes predicted for the guide RNAs (gRNAs) used. The genome-wide effects induced by IncRNAs previously reported include modulation of target gene expression in close proximity to cis-acting regulatory mechanisms or elsewhere in the genome via trans-acting mechanisms (23, 25). In order to differentiate among these options, we used ASOs to perform a knockdown of VEGFA-LNC and found that VEGFA was upregulated almost at the same activation level as achieved in the VEGFA-LNC deletion clonal cell lines. Indeed, this finding suggests that the regulatory activity of VEGFA-LNC on VEGFA may be mediated by a cis mechanism that is dependent on the RNA transcript itself. However, a recent study reported that ASOs targeting the proximal region of a transcript can also trigger premature termination (105), thus providing an alternative explanation for the results. In support of the cotranscriptional role of VEGFA-LNC, we found VEGFA-LNC transcript levels to be nearly undetectable in regular RNA-Seq analyses, which, unlike with GRO-Seq, reflects the pool of stable RNAs in a cell. This suggests that the large trans effects evidenced by the majority of the downstream target genes being located on different chromosomes may be driven by the cis effects on VEGFA, NFKBIE, KLHDC3, and SUPT3H. For example, NFKBIE and VEGFA have been previously shown to be associated with the type I IFN signaling pathway (106, 107), which was the top gene ontology category induced by VEGFA-LNC promoter deletion. Moreover, type I IFNs have been shown to inhibit endothelial cell proliferation, another of the top-regulated categories after VEGFA-LNC deletion (108). Still, we cannot rule out the possibility that for the 5% of the genes regulated by VEGFA-LNC, the effect is mediated by the transcript itself. In fact, IncRNAs can exert their function by regulating chromatin topology, by acting as a scaffold, or by acting as decoys of proteins, such as chromatin-remodeling complexes or transcription factors and other RNAs, such as microRNAs (miRNAs) (109). Interestingly, among the differentially expressed genes predicted to directly interact with VEGFA-LNC, we found genes known to participate in cell proliferation and endothelial function. Among these, ADAMTS9 has been shown to act as an endogenous angiogenesis inhibitor (110, 111). In addition,
the role of HDAC9 has been studied in HUVECs, with which overexpression and knockdown studies reduced and increased the tube formation and sprouting capacity of the cells, respectively (86, 112). Moreover, a positive-feedback loop regulation among the HDAC9 and VEGFA genes has been proposed in previous studies, and in vivo animal models confirmed the role of HDAC9 in vessel formation (86). Regulation of ADAMTS9 and HDAC9 expression may thus represent mechanisms by which VEGFA-LNC affects cellular proliferation, which warrants future studies.

Unlike with VEGFA-LNC, deletion of VEGFC-LNC decreased the transcription of VEGFC expression, suggesting a transcriptional activation of VEGFC by VEGFC-LNC. This may be due to VEGFC-LNC interacting with chromatin remodeling factors and transcription factors, thereby inducing expression of nearby or distant genes (23, 113). In line with this, our RNA-Seq data showed that VEGFC-LNC is predominantly expressed in the nucleus, supporting the hypothesis that it may directly act on the VEGFC locus. Alternatively, the DNA regulatory elements may be important for VEGFC expression (96). Indeed, VEGFC-LNC knockdown did not affect VEGFC expression, suggesting that the transcript itself is not required to mediate the effect. Certainly, the VEGFC gene, enhancers, and the lncRNA are all located within the same regulatory space based on Hi-C data, and the removal of any of the components of the regulatory hub can lead to a decrease in transcriptional output (114). Inhibition of VEGFC expression may be beneficial for the treatment of diseases with excess vascularization, such as cancer. Furthermore, we have also noticed the presence of an lncRNA next to the VEGFC enhancer 1 and 5 (115); they regulate cell cycle control, induction of apoptosis, chromatin remodeling, global run-on sequencing (GRO-Seq) data from HUVECs (44) were used to locate genomic regions that interact with the VEGFA-LNC and VEGFC-LNC downstream target genes, and apoptosis signaling linked factors were distinctively associated with VEGFC-LNC downstream targets. In addition, among both VEGFA-LNC and VEGFC-LNC downstream pathway genes, some were linked to VEGFA and VEGFC pathways, such as those involved in angiogenesis and lymphangiogenesis. On the other hand, negative regulation of cell proliferation was found to be affected by a subset of genes upregulated upon VEGFA-LNC deletion. Together, these data suggest a possible implication of VEGFA-LNC and VEGFC-LNC in endothelial biology.

In conclusion, this study uncovers novel insights into the regulatory landscape of lncRNAs of VEGFA and VEGFC in endothelial cells. In this study, by integrating Hi-C and GRO-Seq data as well as reporter assays and genomic deletions, we identified novel as well as functional enhancers and lncRNAs that regulate VEGFA and VEGFC. These previously unknown VEGFA and VEGFC regulatory elements might be targeted to modulate VEGFA and VEGFC expression in endothelial cells. We provide a better understanding of the complex interplay of ncRNAs with gene regulation that might open up novel therapeutic approaches in the future.

**MATERIALS AND METHODS**

Enhancer identification and vector construction. Chromosome conformation capture (Hi-C) and global run-on sequencing (GRO-Seq) data from HUVECs (44) were used to locate genomic regions that interact with the VEGFA and/or VEGFC promoters and to demonstrate eRNA transcription, respectively. Putative candidate enhancers, together with VEGFA and VEGFC promoters, were amplified from HUVEC genomic DNA by PCR with Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, Waltham, MA) and specific primers. VEGFA enhancers 1 and 5 (VEGFA-E1 and VEGFA-E5) were amplified using the following forward (FW) and reverse (RV) primer pairs (sequences are 5’ to 3’): VEGFA-E1-FW, GGGTGGGCTAGTTAAGCT; VEGFA-E1-RV, CCTGTCATTGGGTAGAAAT; VEGFA-E5-FW, CAGAGGGTCAACAAAACTG; and VEGFA-E5-RV, GCAGGGACGCTGTGGTGT. VEGFC enhancers 1 (VEGFC-E1), 2 (VEGFC-E2), and 3 (VEGFC-E3) were amplified using the primer pairs VEGFC-E1-FW, ACCGCCAATTGTGATGGTG; VEGFC-E1-RV, AACCCTCTGAACCTCACGT; VEGFC-E2-FW, CAGAGGGTCAACAAAACTG; VEGFC-E2-RV, CAGAGGGTCAACAAAACTG; VEGFC-E3-FW, CAGAGGGTCAACAAAACTG; and VEGFC-E3-RV, CAGAGGGTCAACAAAACTG. VEGFA and VEGFC promoters (chromosome 6 [chr6] 43735395 to 43738046 and chr4 177713777 to 177717239, respectively) were cloned into the Kpn/ EcoRV sites of the pGL4.10 (Promega, Madison, WI) firefly luciferase vector to produce pGL4.10-VAp and pGL4.10-Vcp, respectively. The candidate enhancers were subsequently cloned into the Sall site of
pGL4.10-Vap or the pGL4.10-Cvp vector and into pGL4.10-TK, which contained the thymidine kinase (TK) minimum promoter that drives the expression of the firefly luciferase reporter gene.

**Cell lines and maintenance.** The human endothelial cell lines EA.hy926 (CRL-2922; ATCC, Manassas, VA) and TeloHAEC (CRL-4052; ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM)–high glucose (D5671; Sigma, Saint Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and in vascular cell basal medium (PCS-100-030; ATCC) supplemented with a vascular endothelial cell growth kit (PCS-100-041; ATCC), respectively. HUVECs were isolated by collagenase digestion (116) from umbilical cords obtained from the maternity ward of the Kuopio University Hospital, with approval from the Research Ethics Committee of the Northern Savo Hospital District. Prior written consent was obtained from the participants, and the experiments were performed according to the relevant regulations and the Declaration of Helsinki (117). HUVECs were cultured on 0.05% (Sigma)-coated culture plates and maintained in endothelial cell growth medium (EGM) supplemented with EGM SingleQuots (CC-3124; Lonza, Basel, Switzerland). Hypoxia was induced by incubating the cells in a Ruskinn InVivo2 400 hypoxia incubator in the presence of 1% O2 and 5% CO2.

**Transfection and reporter assay.** To perform transfection, cells were seeded into 96-well cell culture plates (PerkinElmer, Turku, Finland) at a density of 1.2 × 10^4 cells per well and transfected 1 day later with Lipofectamine 3000 (Thermo Fisher Scientific) in antibiotic-free medium. Individual wells were cotransfected with 90 ng of an individual enhancer vector construct and 10 ng of a Renilla luciferase expression vector (pGL4.75 [hrLuc/CMV]; Promega), which was used for normalization. Transfected cells were maintained in their specific growth media, and a luciferase reporter assay was performed 48 h after transfection using a dual-luciferase reporter assay system (E1910; Promega). The luciferase activity induced by a candidate enhancer was measured using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany). The luciferase signal was first normalized to the Renilla luciferase signal and subsequently to the signal obtained from the control plasmid (plasmid without the enhancer).

**CRISPR/Cas9-mediated enhancers and IncRNA deletion.** CRISPR/Cas9-mediated deletion of target regions was performed using the Alt-R CRISPR-Cas9 system (Integrated DNA Technology [IDT], Coralville, IA). Briefly, CRISPR/Cas9 single gRNAs (Table 3) flanking the target enhancers and IncRNAs were designed using an online CRISPR design tool (http://chopchop.cbu.ubc.ca/) and ordered from IDT as CRISPR RNAs (crRNAs). These crRNAs were annealed to a fluorescently labeled trans-activating CRISPR RNA (tracrRNA; ATTO 550 no. 1075927; IDT) and complexed with Cas9 endonucleases (HiFi Cas9 nuclease V3, no. A31186; Thermo Fisher Scientific) to form the ribonucleoprotein complex (RNP). The RNP complexes were then delivered into EA.hy926 cells by reverse transfection using Lipofectamine CRISPRMAX reagent (MAX00003; Thermo Fisher Scientific) by following the IDT genome-editing protocol. Two days later, transfected cells were harvested and analyzed for expression with CytoFLEX to determine the transfection efficiency and thereafter diluted for clone selection. Cell medium was refreshed every 4 days until single discrete colonies were formed, picked, and expanded for genotyping and expression analysis. As the negative control, the cells were transfected with gRNAs without Cas9 proteins, and they went through the same clonal selections as actual deletions for 2 weeks. In order to analyze enhancers and IncRNA deletions of EA.hy926 clones, genomic DNA was extracted using a Nucleospin tissue kit (Macherey-Nagel, Düren, Germany) and amplified by PCR using specific primers (Table 3) flanking the deletion sites and PCR master mix (K0172, 2×; Thermo Fisher Scientific). PCR products were then analyzed by electrophoresis in 1% agarose gel. The deletion was further confirmed by Sanger sequencing of the PCR product.

**ASOs.** Antisense oligonucleotides (ASOs) are short single-stranded DNA oligonucleotides that target RNA by complementarity, leading to RNAse H-mediated degradation of the target RNA. ASOs were designed to target 2 different regions of the VEGFA IncRNA (ASO A1 sequence [5′→3′, TCTGTCGCTTAGTGG] and ASO A2 sequence [5′→3′, GAGAAGTGGACAGTGG]), belonging to intrinsic regions of the VEGA; two regions of the VEGF enhancer 3 eRNA, one for the forward strand (ASO VEGFC-E3-1 sequence [5′→3′, AACTTGAAGACAAATAAA]) and one for the reverse strand of the eRNA (ASOs VEGFC-E3-2 sequence [5′→3′, GATGTTAACAAGAAG]), and one region of the VEGFC IncRNA (ASO C1 sequence [5′→3′, GACGACCGAGGAAGC]). ASO control B (LG000000002-FDA; Qiagen, Hilden, Germany) was used as a negative control. EA.hy926 cells and TeloHAECs were seeded into 12-well plates and transfected at 70% confluence with GapmeR LNA ASOs (Qiagen). ASOs were diluted in serum-free medium and transfected into EA.hy926 cells and TeloHAECs at a final concentration of 100 nM, using 3 μl of Lipofectamine 3000 (Thermo Fisher). Cells were washed, and 400 μl of serum-free medium was added with 100 μl of the transfection complex. The medium was changed after 24 h, and cell lysis was performed 48 h after the transfections. RNA and reverse transcription (RT) followed by quantitative real-time PCR (qPCR) were performed as described in the "RNA isolation and expression analysis" section below, but DNase treatment was done with DNase I (Thermo Scientific) instead.

**TDF analysis.** The triplex-forming potential between the IncRNAs and the top 80 differentially expressed genes upon CRISPR deletions was evaluated using Triplex Domain Finder (TDF) (77). A promoter test was used to identify DNA-binding domains (DBDs) within the IncRNAs with the potential to form triple helices in the promoter regions of candidate target genes (referred to as TTS, a triplex target DNA site). The promoter test compares the binding events of the DBD within the promoters of candidate genes (target promoters) with the binding events in the remaining promoters of the genome (nontarget promoters). Default settings, with the hg19 genome and triple helix binding size (∼) set to 20, were used.

**RNA isolation and expression analysis.** Total RNA was isolated from the cells using an RNAeasy minikit (74104; Qiagen) and DNase treated with the RNase-free DNase set (79254; Qiagen). RNA was reverse transcribed with random primers using a RevertAid first-strand cDNA synthesis kit (K1621; Thermo Fisher Scientific). Expression analysis was achieved by (RT-qPCR) using a human VEGFA (Hs00900055_m1; Thermo Fisher Scientific) and human VEGFC (Hs01099203_m1; Thermo Fisher Scientific) TaqMan assay or specific primers for VEGFC-LNC (5′→3′, FW, CCAGAGCCTCAACCTTGATCT; and Rev, TGGTCATCCTGTGTCCCTAAA) with the FastStart Universal SYBR green master (RoX) assay
TABLE 3 List of gRNA sequences and genotyping primers used in CRISPR deletions

| Primer name | Sequence (5'→3') |
|-------------|------------------|
| VEGF-A1_left (gRNA) | GACATGATCAACCTATAGA |
| VEGF-A1_right (gRNA) | GGTGAGCGGCGCTATACAT |
| VEGF-A5_left (gRNA) | GAGAGCCAGGTCAAGCTAGT |
| VEGF-A5_right (gRNA) | GTGAGGTGGCGGAGGGGAGC |
| VEGF-C1_left (gRNA) | GTATTGTGACTTGCACAGAC |
| VEGF-C1_right (gRNA) | GCTGTGCTAAGGTAATTCAT |
| VEGF-C1_left (gRNA) | GATGTATCGTAATGCTAAGC |
| VEGF-C1_right_p2 (gRNA) | TGCATCAAGGCTGGATAAAT |
| VEGF-C1_left_p2 (gRNA) | GAACTACCCAGCATGCCAC |
| VEGF-C1_right_p1 (gRNA) | TGATGCACTTAATCTCTC |
| VEGF-C1_left_p1 (gRNA) | TCTCTACATATTTAGACC |
| VEGF-C2_right (gRNA) | TGACTACGAAATGGGAATTGGA |
| VEGF-C2_left (gRNA) | AAGGCTGGGCAGATTCTACA |
| VEGF-C2_left (gRNA) | CGGACACCCAGCTTGCCTA |
| VEGF-E3_del_RV (genotyping) | CAGACAGGTCCTTGCTGTATATTT |
| VEGF-E3_del_FW (genotyping) | AAGGAACAGTTTACATAGGTCACG |
| VEGF-E2_del_RV (genotyping) | TGACTACGAAATGGGAATTGGA |
| VEGF-E2_del_FW (genotyping) | AAGGCTGGGCAGATTCTACA |
| VEGF-E1_del_RV (genotyping) | AACCCTTCTGAACCTCACGT |
| VEGF-E1_del_FW (genotyping) | ACCGCAATTGTGATGTTTGGA |
| VEGF-E5_del_RV (genotyping) | GCAGGGAGCTGGTCTGTTT |
| VEGF-E5_del_FW (genotyping) | CAGAGGGGTCAAACAACTGG |
| VEGF-LNC_right (gRNA) | GCTGTGCTAAGGTAATTCAT |
| VEGF-LNC_left (gRNA) | GATGTATCGTAATGCTAAGC |
| VEGF-LNC_right (gRNA) | GCTGTGCTAAGGTAATTCAT |
| VEGF-LNC_left (gRNA) | GATGTATCGTAATGCTAAGC |
| VEGF-LNC_right (gRNA) | GCTGTGCTAAGGTAATTCAT |
| VEGF-LNC_left (gRNA) | GATGTATCGTAATGCTAAGC |
| VEGF-LNC_right (gRNA) | GCTGTGCTAAGGTAATTCAT |
| VEGF-LNC_left (gRNA) | GATGTATCGTAATGCTAAGC |
| VEGF-LNC_right (gRNA) | GCTGTGCTAAGGTAATTCAT |
| VEGF-LNC_left (gRNA) | GATGTATCGTAATGCTAAGC |
transcripts in nuclear and cytoplasmic fractions, XIST, MALAT1, NEAT1, VEGFC-LNC, and VEGFA-LNC) or “count exons” (to quantify coding transcripts in nuclear and cytoplasmic fractions, H3F3A, APOE, TUBB, ATP5F1, and GLUL), using a custom annotation file containing the coordinates of our lncRNAs of interest.

For GRO-Seq analysis, public data sets (shown in Table 4) were quantified with HOMER v4.10 (122) using analyzeRepeats.pl with the `-strand 1`, `-rpkm`, and `-count genes` options and a custom annotation file containing the coordinates of our enhancers (VEGFA-E5 and VEGFC-E3) and lncRNAs (VEGFA-LNC and VEGFC-LNC) of interest. For eRNAs, both strands were quantified, and the sum of the counts was used for further analysis. Samples where transcripts were expressed (above 0.2 RPKM in both the eRNA/lncRNA and VEGF transcripts) were reported.

**Proliferation assay.** Cell proliferation was assessed with the real-time cell analyzer multiplate (RTCA MP) instrument (xCELLigence; ACEA Biosciences, Inc.). Briefly, three clones of EA.hy926 ΔVEGFA-LNC, ΔVEGF-LNC, ΔVEGFA-E5, and ΔVEGFC-E3 were seeded in E-plate 16 plates (ACEA Biosciences, Inc.) in duplicate wells at a density of 5 × 10^4 cells/well in a total volume of 200 μl of culture medium. The plate was subsequently placed in an RTCA MP located in the cell culture incubator, and the change in cell proliferation was recorded every 2 h for a period of 52 h.

**Migration assay.** Several clones of EA.hy926 ΔVEGFA-LNC and ΔVEGF-LNC knockouts and control cells were seeded in duplicates at a density of 4 × 10^4 cells/well in IncuCyte Imagelock 96-well plates (Essen BioScience) 24 h prior to the assay. Wounds were effected with the WoundMaker (Essen BioScience) according to the manufacturer’s instructions. Images of the wounds were acquired every 2 h by Incucyte (Essen BioScience) and analyzed with the IncuCyte ZOOM 96-well scratch wound cell migration software. Relative wound density was automatically calculated by measuring the cell density of the wound region relative to the density outside the wound area.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism v 5.03. For each experiment three biological replicates were used, and the difference in means was analyzed with one-way analysis of variance (ANOVA) and Dunnett’s multiple-comparison test or Student’s unpaired two-tailed t test. The statistical significance was defined as a P of <0.05 (indicated with an asterisk).

### TABLE 4 Public data sets used in this study

| Accession no. | Data set | Original publication |
|---------------|----------|----------------------|
| GSE39089      | ChIP-Seq (HIF1a) | Mimura et al. (41) |
| GSE29611      | ChIP-Seq (H3K27ac and H3K4me1) | Dunham et al. (18) |
| GSE52642      | Hi-C and GRO-Seq | Kaikkonen et al. (45) |
| GSE92375      | GRO-Seq | Bouvy-Liivrand et al. (46) |
| GSE48759      | GRO-Seq | Kaikkonen et al. (123) |
| GSE136813     | GRO-Seq | Linna-Kuosmanen et al. (47) |
| GSE118530     | GRO-Seq | Moreau et al. (48) |
| GSE94872      | GRO-Seq | Niskanen et al. (44) |
| GSE103530     | GRO-Seq | Kuosmanen et al. (124) |
| GSE13518      | GRO-Seq | Core et al. (49) |
| GSE27823      | GRO-Seq | Wang et al. (50) |
| GSE38140      | GRO-Seq | Galbraith et al. (51) |
| GSE39878      | GRO-Seq | Wang et al. (52) |
| GSE41009      | GRO-Seq | Sigova et al. (53) |
| GSE41323      | GRO-Seq | Danko et al. (54) |
| GSE41324      | GRO-Seq | Danko et al. (54) |
| GSE43070      | GRO-Seq | Jin et al. (55) |
| GSE51225      | GRO-Seq | Luo et al. (56) |
| GSE51633      | GRO-Seq | Liu et al. (57) |
| GSE53964      | GRO-Seq | Allen et al. (58) |
| GSE60454      | GRO-Seq | Core et al. (59) |
| GSE62046      | GRO-Seq | Andersson et al. (60) |
| GSE62296      | GRO-Seq | Meng et al. (61) |
| GSE66448      | GRO-Seq | Niskanen et al. (62) |
| GSE83860      | GRO-Seq | Malinen et al. (63) |
| GSE84432      | GRO-Seq | Toropainen et al. (64) |
| GSE67519      | GRO-Seq | Teppo et al. (65) |
| GSE67540      | GRO-Seq | Heinäniemi et al. (66) |
| GSE101803     | GRO-Seq | Kourtis et al. (67) |
| GSE96859      | GRO-Seq | Franco et al. (68) |
| GSE102819     | GRO-Seq | Gao et al. (69) |
| GSE86165      | GRO-Seq | Andrysik et al. (70) |
| GSE91011      | GRO-Seq | Williamson et al. (71) |
| GSE67295      | GRO-Seq | Stender et al. (72) |
| GSE117086     | GRO-Seq | Viiri et al. (73) |
Data availability. The RNA-Seq data generated in this study have been submitted to the NCBI Gene Expression Omnibus under accession number GSE141669. The public data sets used in this study are shown in Table 4.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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I.M., V.T.B., M.U.K., N.L.-K., H.N., and S.Y.-H. designed the research. I.M., V.T.B., H.N., and K.H. performed the experiments and analyzed the data. N.L.D. and P.R.M. analyzed RNA-Seq data. I.M., V.T.B., M.U.K., and N.L.-K. wrote the manuscript in consultation with the other authors. All authors revised, read, and approved the final manuscript.

We declare that there is no conflict of interest.

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