Repression of Vascular Endothelial Growth Factor Expression by the Zinc Finger Transcription Factor ZNF24

Jay Harper,¹,² Li Yan,³ Robyn M. Loureiro,³ Jinmin Wu,¹,² Jianmin Fang,³ Patricia A. D’Amore,¹ and Marsha A. Moses¹,²,³

¹Vascular Biology Program, Children’s Hospital Boston and ²Department of Surgery and ³Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts; ⁴Oncology Research, Centocor R&D, Inc., Malvern, Pennsylvania; and ⁵Department of Preclinical Oncology and Immunology, Cell Genesys, Inc., San Francisco, California

Abstract
Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis. Although many positive regulators of VEGF have been identified, relatively little is known regarding the negative regulation of VEGF expression. We identified a zinc finger transcription factor, ZNF24, that may repress VEGF transcription. An inverse correlation between expression of VEGF and ZNF24 was observed in a series of independent studies. ZNF24 was up-regulated in angiogenic tumor nodules where VEGF expression is significantly decreased compared with preangiogenic nodules. In human breast carcinoma cells cultured under normoxic conditions, ZNF24 levels were significantly up-regulated whereas VEGF levels were low. In contrast, VEGF was significantly increased in hypoxic cells whereas ZNF24 was down-regulated. The same inverse correlation between ZNF24 and VEGF was also observed in 70% of matched cDNA pairs of normal and malignant tissues from human colon and breast biopsies. Over-expression of ZNF24 resulted in a significant down-regulation of VEGF, whereas silencing of ZNF24 with small interfering RNA led to increased VEGF expression. Cotransfection of ZNF24 and a VEGF promoter luciferase reporter construct in cancer cells resulted in a significant decrease in VEGF promoter activity. Taken together, these data suggest that ZNF24 is involved in negative regulation of VEGF and may represent a novel repressor of VEGF transcription. [Cancer Res 2007;67(18):8736–41]

Introduction
Vascular endothelial growth factor (VEGF) is required for physiologic angiogenesis during development as shown by the homozygous or heterozygous ablation of the VEGF gene and the results from the use of VEGF antagonists in developmental studies (1, 2). Increased VEGF expression is associated with many angiogenesis-dependent pathologies, such as cancer (reviewed in ref. 3). Increased expression was investigated via ZNF24 overexpression, silencing, and a zinc finger transcription factor belonging to the family of SCAN box domain–containing Krüppel-like Cys2-His2 zinc finger transcription factors (14, 16). It contains four zinc finger motifs that encode putative DNA binding domains. The ZNF24 gene is localized to chromosome 18q12.1 (14–16), a region frequently deleted in colorectal carcinomas, suggesting a possible role in the negative regulation of tumor growth (17, 18). ZNF24 was shown to possess transrepression activity of the GAL4 promoter in Chinese hamster ovary and NIH-3T3 cells (14); however, little else is known regarding its role in gene expression.

In the present study, ZNF24 was identified, via microarray analyses, as a gene that was differentially expressed in two independent systems: an in vivo tumor model that reliably recapitulates the transition to the angiogenic phenotype (19, 20) and an in vitro system analyzing hypoxia-inducible gene expression. In both studies, an inverse correlation between ZNF24 and VEGF was observed. ZNF24 mRNA expression was also inversely correlated with VEGF expression in matched pairs of human colon and breast tumors compared with adjacent normal tissue. The functional relationship between ZNF24 and VEGF expression was investigated via ZNF24 overexpression, silencing, and VEGF promoter analyses. Our results suggest that ZNF24 may be involved in the negative regulation of VEGF expression and that it may function as a novel transcriptional repressor of the VEGF gene.

Materials and Methods
Tumor model and cell culture. Preangiogenic and angiogenic tumors were established and harvested as previously described (19, 20). Human MDA-MB-231 and MCF-7 breast carcinoma cells and U87 MG glioblastoma cells (American Type Culture Collection) were cultured in DMEM (Invitrogen/Life Technologies) supplemented with 10% bovine calf serum (HyClone) and 1% glutamine/penicillin/streptomycin (Invitrogen/Life Technologies) at 37°C, 5% CO₂. For hypoxia experiments, MDA-MB-231 cells were cultured in a hypoxia chamber (Model MIC-101, Billups-Rothenberg, Inc.) in 1% O₂, 5% CO₂ and 94% N₂ for 24 h. For conditioned media experiments, cells were washed with PBS, and the media changed to serum-free DMEM, 1% glutamine/penicillin/streptomycin, 24 h before collection. Conditioned media and/or RNA was harvested 24 h after culture under either normoxic or hypoxic conditions.
Microarray analyses. RNA was purified from tumor nodules and cells using the RNeasy kit (Qiagen) according to manufacturer's protocol. cDNA synthesis, cDNA synthesis and labeling, and hybridization to microarrays were done by the Gene Array Technology Center at Brigham and Women's Hospital (Boston, MA). The human U95A GeneChip array (Affymetrix) and the MIROMAX human cDNA array (New England Nuclear) were used in these experiments. Analysis of hybridization data was done with GeneChip 3.1 expression analysis software (Affymetrix).

Semiquantitative reverse transcription–PCR. Reverse transcription was done using SuperScriptII RNase H− (Invitrogen) according to manufacturer’s protocols. Serial dilutions of cDNA were prepared, normalized against β-actin, then analyzed via PCR to validate microarray results. The following primers were used: ZNF24 primers (F 5′-GGTTTGAAGAAA-GAGAAATCC-3′, R 5′-AGGTTTTCCTGATTGTC-3′; 374 bp product). The inverse correlation between ZNF24 and VEGF was also confirmed using real-time qPCR (Fig. 1C).

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Results

Inverse correlation between ZNF24 and VEGF. In the first study, we used a tumor model that reliably recapitulates the transition to the angiogenic phenotype in vivo (19). Transcriptional profiling of preangiogenic and angiogenic tumor nodules was done to identify genes that were differentially expressed during the transition to the angiogenic phenotype. Analysis of preangiogenic and angiogenic tumor gene expression identified ZNF24 as a gene that was up-regulated in angiogenic nodules. This result was confirmed via several rounds of qRT-PCR done on normalized serial dilutions of cDNA synthesized from each set of nodules (Fig. 1A). Conversely, VEGF expression was significantly up-regulated in avascular nodules compared with vascular nodules (Fig. 1A), an observation that confirms our previously reported results (20). The inverse correlation between expression of ZNF24 and VEGF was also confirmed using real-time qPCR (Fig. 1C).

Interestingly, the same inverse correlation between ZNF24 and VEGF was observed in transcriptional profiling experiments aimed at identifying hypoxia-inducible changes in gene expression of MDA-MB-231 human breast carcinoma cells. ZNF24 was down-regulated in cells cultured under hypoxia, where VEGF is up-regulated, and up-regulated in cells cultured under normoxic conditions, where VEGF is down-regulated (Fig. 1B). The inverse correlation between ZNF24 and VEGF, observed in these two separate and independent systems, suggested that this transcription factor may be involved in negative regulation of VEGF expression.

Expression of ZNF24 and VEGF in matched tumor/normal cDNA pairs. ZNF24 is localized to chromosome 18q12.1, a region that is mutated in a variety of human cancers, including colorectal carcinomas and invasive breast cancer suggesting a potential tumor suppressor role for this transcription factor (17, 18, 21). Based on this finding, matched cDNA pairs of normal and malignant colon and breast tissues were purchased from Clonetech and normalized to ribosome binding protein-9. Semiquantitative reverse transcription–PCR (qRT-PCR) was done on these samples with VEGF and ZNF24 primers described above using the reaction hotstart at 94° C 5 min, then 94° C 1 min, 60° C 30 s, and 72° C 30 s for 20 to 30 cycles, followed by extension at 72° C 10 min. PCR products were analyzed via gel electrophoresis.

Real-time quantitative PCR. Real-time quantitative PCR (qPCR) was done using the DNA Engine Opticon II real-time PCR detection system (Bio-Rad Laboratories/MJ Research). cDNA synthesis from preangiogenic and angiogenic tumor nodule RNA was done as described above. qPCR glyceraldehyde-3-phosphate dehydrogenase primers are R 5′-ACTGACGAGGAGGGCATATG-3′, R 5′-GCCACAGGATTGGCTT-GAAGA-3′. PCR reaction with Platinum PCRSuperMix (Invitrogen) were tested at 94° C 30 s, 54° C 30 s, 72° C 30 s for 20 to 30 cycles, followed by extension at 72° C 10 min. PCR products were analyzed via gel electrophoresis.

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Establishment of MDA-MB-231 clones that overexpress ZNF24. Full-length human ZNF24 was cloned from MDA-MB-231 cells into the expression vector pcDNA3.1/V5-His-TOPO using the Topo-TA cloning kit (Invitrogen) according to manufacturer's protocols and verified by sequencing. Expression of ZNF24 is the control for cytomegalovirus (CMV) promoter. The primers used to generate the full-length ZNF24 cDNA: F 5′-CACATGTCGACAGCTGAGTA-3′, R 5′-AACCTCCAAACATTCTGAA-3′. The expression vector was transfected into MDA-MB-231 cells with Effectene (Qiagen) according to manufacturer's protocols. Clones overexpressing ZNF24 (MDA-MB-231-ZNF24) were selected with G418 (500 μg/mL; Invitrogen) and overexpression of ZNF24 verified by qRT-PCR.

Squamous cell carcinoma. MCF7 human breast carcinoma cells were transiently transfected with 100 nmol/L of ZNF24 small interfering RNAs or control small interfering RNAs (Dharmacon, Inc.) with DharmaFECT transfection reagent (Dharmacon, Inc.). RNA was purified from transfected cells after 24 to 48 h in culture, and ZNF24 and VEGF expression was analyzed by qRT-PCR as described above.

Determination of VEGF synthesis. MDA-MB-231 and MDA-MB-231-ZNF24 cells were cultured under either normoxic or hypoxic conditions, as described above, in the presence of 0.1% bovine serum albumin (Intergen). After 24 h, conditioned media samples were normalized against protein concentration (Micro BCA protein assay, Pierce) and VEGF concentrations determined by Quantikine human VEGF immunoassay (R&D Systems).

VEGF promoter reporter assays. MDA-MB-231 cells were plated in antibiotic-free media, cultured for 24 h, then transiently cotransfected with equimolar ratios of either a ZNF24 or LacZ expression vector and with either VEGF promoter luciferase constructs or a control vectors using FuGENE 6 (Roche Diagnostics) according to manufacturer's protocols. After 48 h, with a medium change at 24 h, the cells were lysed with Passive lysis buffer (Promega) according to manufacturer's protocols. Luciferase activity in lysates was assayed with the Dual luciferase reporter assay system (Promega) according to manufacturer's protocols, and results were normalized to transfection efficiency of a third cotransfected thymidine kinase–driven renilla luciferase vector.

Data from the matched cDNA pair analyses are consistent with the results from the microarray analyses. The strong inverse correlation between ZNF24 and VEGF expression observed in vitro and in vivo in these multiple systems and the fact that the chromosomal locus of the ZNF24 gene is mutated in several types of cancer suggest that ZNF24 may represent a potential tumor suppressor that may function to negatively regulate the expression of VEGF.

Down-regulation of VEGF in response to overexpression of ZNF24. The correlative data on inverse expression patterns of ZNF24 and VEGF from multiple systems suggested that this transcription factor may be involved in the negative regulation of VEGF. To better determine the functional relationship between ZNF24 and VEGF expression, MDA-MB-231 cells that stably overexpress ZNF24 were established. These cells and parental MDA-MB-231 cells were cultured under either normoxic or hypoxic conditions for 24 h. To ascertain the effect of overexpression of ZNF24 on VEGF secretion, the levels of secreted VEGF in conditioned media from these cell lines were assayed using human VEGF ELISA kits. VEGF protein levels were significantly reduced in multiple MDA-MB-231 clones compared with parental cells (Fig. 3A) or transfection control cells (data not shown). Comparable inhibition of VEGF secretion was observed whether the cells were cultured under normoxic or hypoxic conditions.

Silencing of ZNF24 leads to up-regulation of VEGF. To further verify the inverse correlation observed between expression of ZNF24 and VEGF, ZNF24 expression was knocked down with small interfering RNAs. The correlative data on inverse expression patterns of ZNF24 and VEGF from multiple systems suggested that this transcription factor may be involved in the negative regulation of VEGF. To better determine the functional relationship between ZNF24 and VEGF expression, MDA-MB-231 cells that stably overexpress ZNF24 were established. These cells and parental MDA-MB-231 cells were cultured under either normoxic or hypoxic conditions for 24 h. To ascertain the effect of overexpression of ZNF24 on VEGF secretion, the levels of secreted VEGF in conditioned media from these cell lines were assayed using human VEGF ELISA kits. VEGF protein levels were significantly reduced in multiple MDA-MB-231 clones compared with parental cells (Fig. 3A) or transfection control cells (data not shown). Comparable inhibition of VEGF secretion was observed whether the cells were cultured under normoxic or hypoxic conditions.

Decreased VEGF promoter activity in response to overexpression of ZNF24. ZNF24 is a Cys2-His2 transcription factor with four Krüppel-like zinc finger motifs that encode putative DNA binding domains. Therefore, the ability of this transcription factor to repress transcription of VEGF was investigated. VEGF promoter luciferase reporter constructs (Fig. 3C) were constructed and used to analyze the effect of overexpression of ZNF24 on VEGF promoter activity. MDA-MB-231 cells were cotransfected with the VEGF promoter construct or control constructs and either ZNF24 or control LacZ expression plasmids. Cotransfection with VEGF promoter constructs and ZNF24 resulted in a significant decrease in VEGF promoter activity compared with cells that were transfected with the LacZ vector (Fig. 3D). These data indicate that overexpression of ZNF24 results in down-regulation of VEGF promoter activity and suggests that ZNF24 may negatively regulate expression of VEGF via transcriptional repression of the VEGF gene.

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Taken together, these data suggest that ZNF24 may be involved in transcriptional repression of VEGF.

Repression of genes, including VEGF, can occur via a number of mechanisms, including, but not limited to, repression of transcription, a decrease in mRNA stability, or premature degradation of the synthesized protein (22). We hypothesized that ZNF24 was functioning as a transcriptional repressor for a number of reasons. First, the C-terminal domain of ZNF24 contains four Krüppel-like Cys2-His2 zinc finger motifs that encode putative DNA-binding domains (14). Second, ZNF24 was previously reported to possess transcription repression activity against a GAL4 reporter plasmid in mammalian Chinese hamster ovary and NIH3T3 cells (14). Finally, another member of the SCAN domain–containing Cys2-His2 transcription factor family, ZNF174, has been characterized as a novel transcriptional repressor of both the platelet-derived growth factor-B and transforming growth factor-β promoters (23). Therefore, based on the secondary structure of ZNF24, the reported transcriptional repression by ZNF24 and the fact that ZNF24 and ZNF174 belong to the same transcription factor family, we designed our studies to determine whether ZNF24 is involved in repression of VEGF transcription. The effect of ZNF24 on VEGF transcription was investigated by analyzing the effect of overexpression of ZNF24 on the transcriptional activity of the VEGF promoter using VEGF promoter luciferase constructs. Overexpression of ZNF24 resulted in a significant decrease in luciferase activity, which corresponds to decreased VEGF promoter activity, supporting our hypothesis that ZNF24 may negatively regulate VEGF expression through transcriptional repression.

Negative regulation of VEGF transcription by ZNF24 could occur via a number of mechanisms. For example, ZNF24 may bind the VEGF promoter directly either by itself, as part of a transcriptional repression complex, or indirectly as part of a complex that binds the promoter via one of the other molecules comprising that complex. The N-terminal region of ZNF24 contains a SCAN box domain, a leucine-rich domain that has been implicated as an oligomerization domain mediating protein-protein interactions to form homoligomers or heteroligomers with other proteins containing SCAN domains (24–26). Therefore, it is possible that ZNF24 comprises part of a transcriptional repression complex that binds the VEGF promoter via the DNA-binding domains of ZNF24 and/or other potential DNA-binding molecules within the complex. Alternatively, ZNF24, either alone or as part of a complex, may bind the VEGF promoter at a site that hinders binding of transcriptional activators of VEGF, such as HIF-1 and Sp1. It is also possible that ZNF24 indirectly inhibits VEGF promoter activity by sequestering a transcriptional activator in a fashion similar to the inhibition of VEGF expression by the von Hippel Lindau gene product that sequesters Sp1 and thus prevents it from activating VEGF transcription (9). Finally, ZNF24 may act as a transcriptional activator, turning on the expression of a separate transcription factor, for example, which in turn is responsible for repression of VEGF. These potential mechanisms are currently being investigated in our laboratory.

The data presented here show that VEGF expression is negatively regulated by ZNF24 or a ZNF24-mediated mechanism, suggesting a tumor suppressor role for ZNF24. ZNF24 was down-regulated in 60% of the colon carcinomas and in 100% of the breast carcinomas that we analyzed compared with respective adjacent normal tissues. Down-regulation of ZNF24 expression in tumors is consistent with our hypothesis that ZNF24 may be involved in negative regulation of tumor growth by repressing expression of
VEGF. ZNF24 may be down-regulated to promote VEGF expression during tumor progression and other angiogenesis-dependent diseases characterized by aberrant VEGF expression. Furthermore, ZNF24 is localized to a chromosomal locus (18q12) that is mutated in a number of human cancers, such as colorectal carcinomas (17, 18), head and neck cancer (27), invasive breast cancer (21), gastric cardia adenocarcinomas (28), testicular germ cell tumors (29), and a number of hematopoietic malignancies (30). The high incidence of mutations within this chromosomal locus strongly suggests the presence of potential tumor suppressor genes within this region. To date, no mutations in ZNF24 have been associated with malignancies; however, given its negative effect on VEGF expression, it would be informative to knockout ZNF24 expression or construct dominant negative ZNF24 mutations to determine the result on repression of VEGF and on subsequent angiogenesis and/or tumor progression.

Whereas it seems that ZNF24 may be involved in the negative regulation of VEGF expression, it is unclear how expression of ZNF24 is regulated. The data presented here indicate that ZNF24 is down-regulated during conditions in which HIF-1 is activated, for example in preangiogenic tumor nodules and in cells cultured under hypoxia, suggesting that expression of ZNF24 might be regulated by hypoxia. Hypoxia induces increased VEGF expression through activation or up-regulation of positive regulators of VEGF expression, such as HIF-1 (8) and Ets-1 (31). However, it is also possible that negative regulators of VEGF expression, such as ZNF24, could be down-regulated, augmenting the increase in VEGF production. Elucidating the hypoxia-mediated and nonhypoxia-mediated mechanisms for regulating expression of ZNF24 would be informative, both in terms of understanding the regulation of this transcription factor and in characterizing potentially novel mechanisms involved in the negative regulation of VEGF. It is important to note that although expression of ZNF24 may be regulated by hypoxia, the way in which ZNF24 represses expression of VEGF seems to be hypoxia-independent. A comparable decrease in VEGF expression was observed whether MDA-MB-231-ZNF24 cells were cultured under normoxic or hypoxic conditions. Although VEGF expression was decreased regardless of oxygen conditions, the overall VEGF expression was still higher in cells cultured under hypoxia compared with normoxic conditions, suggesting that hypoxia-inducible expression of VEGF can still occur in MDA-MB-231-ZNF24 cells, albeit, at a significantly decreased rate.

Overexpression of ZNF24 results in decreased expression of VEGF with an associated decrease in VEGF promoter activity. These data suggest that ZNF24 may represent a novel transcriptional repressor of VEGF and, as such, may be an addition to the relatively small number of negative regulators of VEGF expression that have been identified. Elucidating the mechanism and consequence of VEGF repression by ZNF24 may be useful in the development of therapeutic approaches to treating angiogenesis-dependent pathologies by turning VEGF on or off, using ZNF24 as the switch. Recently, it has been shown that gene therapy experiments using artificially designed zinc finger transcription factors can activate expression of VEGF in vivo, leading to stimulation of angiogenesis (31, 32). The potential exists for the development of similar technologies based on the inactivation of VEGF expression via ZNF24.

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References


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