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# Repression of Vascular Endothelial Growth Factor A in Glioblastoma Cells Using Engineered Zinc Finger Transcription Factors

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## ABSTRACT

**Angiogenic factors are necessary for tumor proliferation and thus are attractive therapeutic targets. In this study, we have used engineered zinc finger protein (ZFP) transcription factors (TFs) to repress expression of vascular endothelial growth factor (VEGF)-A in human cancer cell lines. We create potent transcriptional repressors by fusing a designed ZFP targeted to the VEGF-A promoter with either the ligand-binding domain of thyroid hormone receptor $\alpha$ or its viral relative, vErbA. Moreover, this ZFP-vErbA repressor binds its intended target site *in vivo* and mediates the specific deacetylation of histones H3 and H4 at the targeted promoter, a result that emulates the natural repression mechanism of these domains. The potential therapeutic relevance of ZFP-mediated VEGF-A repression was addressed using the highly tumorigenic glioblastoma cell line U87MG. Despite the aberrant overexpression of VEGF-A in this cell line, engineered ZFP TFs were able to repress the expression of VEGF-A by >20-fold. The VEGF-A levels observed after ZFP TF-mediated repression were comparable to those of a nonangiogenic cancer line (U251MG), suggesting that the degree of repression obtained with the ZFP TF would be sufficient to suppress tumor angiogenesis. Thus, engineered ZFP TFs are shown to be potent regulators of gene expression with therapeutic promise in the treatment of disease.**

## INTRODUCTION

Angiogenesis, the process by which new capillary blood vessels grow, is of critical importance to tumor progression and therefore is an attractive target in the treatment of cancer (1). Solid tumors, for example, require neovascularization for growth beyond a diameter of ~3 mm (2), and this angiogenic response is correlated with increased tumorigenicity (3). Vascular endothelial growth factor (VEGF)-A, which is a potent effector of angiogenesis, is overexpressed in a variety of human cancers, including highly invasive brain tumors (4). Expression of VEGF-A is usually tightly regulated in response to physiological conditions. Up-regulation (which occurs in response to hypoxia) can induce the proliferation of capillary endothelial cells, leading to remodeling of pre-existing capillaries or the growth of additional capillary networks (5). Constitutive high-level VEGF-A expression, as seen in many human cancers, provokes aberrant remodeling and capillary growth and thus facilitates tumor progression. Additionally, VEGF-mediated capillary and endothelial remodeling appear to be integral steps in facilitating tumor metastasis via the host blood supply (6, 7). Conversely, the inhibition of VEGF-induced angiogenesis has been shown to suppress tumor growth *in vivo* (8). Repression or inhibition of VEGF-A function thus presents a potential avenue for anticancer therapy.

Misregulation of gene expression leading to constitutively high VEGF-A levels, for example, is a hallmark of cancer (1). Tumor-specific transcriptional activity has been linked to the aberrant action of chromatin-based modulators of gene regulation typified by histone deacetylation activities in leukemia (9) and histone methyltransferase enzymes in prostate cancer (10). Indeed, a cancer-specific transcriptional circuit is evidenced in the up-regulation of VEGF-A in glioblastoma (11). Treatments that correct such defects at a transcriptional level are limited. Engineered zinc finger protein (ZFP) transcription factors (TFs) represent a novel class of potential therapeutic agents that invoke the natural mechanisms of gene control and offer the ability to regulate a specific gene at its endogenous location in the genome (12). In this regard, we have previously used engineered ZFP TFs to specifically up-regulate the expression of the endogenous VEGF-A gene both in human cell lines (13) and in whole animal models of angiogenesis (14). It remained unclear, however, whether a designed ZFP TF repressor would be sufficient to reduce the high levels of VEGF expression associated with vascularizing tumors to a therapeutically relevant degree. In the present study, we have now used these DNA-binding proteins to inhibit VEGF-A expression in a variety of human cancer cell lines. We show that ZFP TFs can repress VEGF-A expression at the protein and mRNA levels in HEK293 cells. To address the therapeutic potential of ZFP TF-mediated VEGF-A repression, we investigated the potency of this reagent in the highly tumorigenic human glioblastoma line U87MG. Using a stable U87MG cell line engineered to exhibit inducible expression of the ZFPA-vErbA protein, we demonstrate that expression of VEGF-A can be repressed to a level that parallels that of a nonangiogenic, low-tumorigenic glioblastoma line (U251MG). These data establish that specific engineered ZFP TFs can potently inhibit the expression of VEGF-A in a therapeutically relevant model system.

## MATERIALS AND METHODS

**Cell Culture and Transient Transfections.** HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum in a 5% $CO_2$ incubator at 37°C. Transfection experiments were carried out as described previously (15). Charcoal-filtered fetal bovine serum (HyClone Laboratories) was used as indicated. HEK293 cells were adapted to growth in charcoal-filtered serum for 1 week before use. Cell proliferation and viability were determined by the WST-1 assay (Roche), according to the manufacturer's recommendations.

**Secreted VEGF-A Protein ELISA.** Secreted VEGF-A in the tissue culture media by transfected HEK293 cells was assayed after 48 h using a human VEGF-A ELISA kit (R&D Systems) in duplicate according to the manufacturer's recommendations.

**Assembly of Expression Constructs Carrying ZFP-TR$\alpha$ and ZFP-v-ErbA Chimeras.** The ligand-binding domain of chicken (*Gallus gallus*) thyroid hormone receptor $\alpha$1 (TR$\alpha$1; NR1A1) was amplified from a clone of full-length TR$\alpha$1 in pT7TS (16) using the following primers: forward primer, 5′-AAG-GAT-CCA-TCT-CCG-TGG-GCA-TGG-CCA-TGG-3′; and reverse primer, 5′-AAA-AGC-TTA-CAC-CTC-CTG-GTC-CTC-GAA-GACC-3′. These primers isolate amino acids 114–408 of the receptor (GenBank accession number M24748), introduce an in-frame *Bam*HI site at the 5′ end, and

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**Notes:** This paper is dedicated to the memory of our colleague and friend, Dr. Alan P. Wolffe. The present address for C. O. Pabo is 257 Thockmorton Avenue, Mill Valley, CA 94941.

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replace the amber stop codon in native TR$\alpha$ with an ochre stop codon and a *Hin*dIII site. The cognate segment of v-ErbA (17, 18) was amplified from a clone of full-length v-ErbA in pT7TS (16) with the same forward primer used for TR$\alpha$ and the following reverse primer: 5′-AAA-AGC-TTA-CAC-CTC-CTG-GGG-GGA-GAG-CTC-3′. The PCR products were gel purified, digested with *Bam*HI and *Hin*dIII, and cloned into pcDNA3 expression vectors (Invitrogen) carrying designed ZFPs that regulate the VEGF gene (13). By analogy with domain architecture in native nuclear hormone receptors, this cloning scheme introduces the nuclear hormone receptor ligand-binding domain (LBD) and the cognate segment of v-ErbA at the COOH terminus of the ZFP moiety. These constructs were named pCMV-ZFPA-vErbA and pCMV-ZFPA-TR$\alpha$, respectively. All constructs were confirmed by sequencing.

**Immunoblot Detection of ZFP Fusion Proteins.** For immunoblot analysis of ZFP protein expression, cells were lysed 72 h after transfection in RIPA (Santa Cruz Biotechnology) according to the manufacturer's recommendations. Briefly, samples were boiled in 2× Laemmli sample buffer and resolved by SDS-PAGE, followed by Western blotting using an anti-TR$\alpha$ antibody (Santa Cruz Biotechnology). Immunoblots were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) as described previously (19).

**Quantitative Real Time-PCR Analysis of VEGF-A mRNA Expression (TaqMan).** HEK293 cells were lysed, and total RNA was prepared using the High Pure RNA isolation kit (Roche) according to the manufacturer's recommendations. RNA (25 ng) was used in real-time quantitative reverse transcription-PCR analysis using TaqMan chemistry in a 96-well format on an ABI 7700 Sequence Detection System machine (Perkin-Elmer Life Sciences) as described previously (20). Primers and probes used were as described previously (13). The results were analyzed using Sequence Detection System Version 1.6.3 software (Perkin-Elmer Life Sciences).

**Chromatin Immunoprecipitation (ChIP).** ChIP was performed using the ChIP assay kit according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY), as described previously (15). The abundance of specific DNA fragments was quantified using real-time PCR (TaqMan) as described above, omitting the reverse transcription reaction step. The relative abundances of the various VEGF-A genomic fragments were calculated relative to an internal glyceraldehyde-3-phosphate dehydrogenase genomic probe set. All primers sets used have been described previously (15).

**Generation of Stable Cell Lines.** The parental T-REx-U2OS cell line (Invitrogen) was cultured in DMEM supplemented with 10% FCS in a 5% $CO_2$ incubator at 37°C. To generate stable Tet-inducible cell lines expressing the ZFPA-vErbA transcription factor, the coding region from the pCMV-ZFPA-vErbA vector was subcloned into pcDNA4/TO (Invitrogen) through the *Afl*II and *Xho*I restriction sites. The resulting construct pTO-ZFPA-vErbA was transfected into the TREx-U2OS cell line using LipofectAMINE 2000 (Invitrogen). After 2 weeks of selection in culture medium containing 400 $\mu$g/ml Zeocin (Invitrogen), single cell-derived stable lines were obtained, and individual lines were tested for ZFPA-vErbA induction and repression of VEGF-A by the addition of 1 ng/ml doxycycline (DOX) to the culture medium. The identical procedure was performed for the U87MG T-REx line, except that the parental line itself was first created by transfection of U87MG cells with the TetR expression construct pcDNA6/TR (Invitrogen).

***In Vitro* DNase I Footprinting of ZFP-A Binding Site.** The fragment used for DNase I footprinting studies comprised bases +334 to +566 relative to the start site of transcription of the human VEGF-A gene. For DNA fragment isolation and labeling for footprinting studies, the plasmid pGLPVFH (13) was digested with *Eag*I followed by dephosphorylation with shrimp alkaline phosphatase (Roche). This DNA was then digested with *Sac*I, and the resultant 232-bp fragment was gel purified, and 0.4 pmol of the fragment was labeled with $^{32}$P at the (dephosphorylated) *Eag*I end via incubation with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (New England Biolabs). The ZFP-maltose-binding protein fusion used for DNase I footprinting studies was prepared as described previously (13). The DNase I footprinting experiment was performed as follows: (*a*) binding reactions were assembled containing 2.5 fmol of labeled template DNA and varying amounts of ZFP (30, 60, 120, 240, or 480 fmol) in 12 $\mu$l of binding buffer [100 mM KCl, 10 mM Tris (pH 8), 10 mM DTT, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 10% (w/v) glycerol, 0.02% (w/v) IGEPAL detergent (Sigma), and 0.02% (w/v) BSA]; (*b*) reactions were incubated for 1 h at room temperature; (*c*) 6 $\mu$l of a DNase I solution containing 7 $\mu$g/ml DNase I (Worthington product DPRF) in binding buffer was added; (*d*) samples were incubated at room temperature for 90 s; and (*e*) after addition of 18 $\mu$l of a formamide gel loading buffer, samples were heated at 80°C for 10 min and then placed on ice for 3 min before electrophoresis on a denaturing 6% acrylamide gel. Bands were visualized via exposure to a Molecular Dynamics PhosphorImager screen. The location of the footprint was confirmed via co-running of a dideoxy sequencing ladder (data not shown).

## RESULTS

**ZFP-Mediated Repression of the Endogenous VEGF-A Gene.** To achieve the transcriptional repression of the VEGF-A gene, we used previously designed zinc finger DNA-binding domains that have been shown to activate the transcription of the VEGF-A promoter when fused to either the herpes simplex virus transactivator VP16 or RelA/p65 nuclear factor $\kappa$B transactivation domains (13). To create transcriptional repressors, these zinc finger DNA-binding domains were combined with either the LBD of TR$\alpha$ or vErbA. In the absence of ligand, TR$\alpha$ functions as a transcriptional repressor via the interaction of its LBD with the histone deacetylase complex (HDAC)-associated corepressor complexes, N-CoR and SMRT (21–23). Addition of the cognate ligand converts this natural transcriptional regulator into a potent transcriptional activator by displacing the co-repressor complexes and effecting the recruitment of the p160, p300/CBP, and mediator classes of coactivator (reviewed in Ref. 24). In contrast, v-ErbA, although able to efficiently recruit the N-CoR/HDAC corepressor complex, carries mutations within the LBD that abolish hormone binding (25–27). Thus v-ErbA, in contrast to TR$\alpha$, is a constitutive repressor, unable to activate transcription in the presence of ligand.

The region encompassing the LBD of TR$\alpha$, or the cognate portion of v-ErbA, was cloned COOH-terminal to the engineered ZFP DNA-binding domain, thus conserving the general architecture of the nuclear hormone receptors (Fig. 1*A*). For regulation of the endogenous *VEGF-A* gene, these two effector domains were fused to the DNA-binding domains from three of the previously described VEGF-A ZFP activators [VZ +434, VZ +42/+530, and VZ-573 (13), which are referred to here as ZFPA, ZFPB, and ZFPC, respectively]. We first determined whether these ZFP TFs carrying the TR$\alpha$ LBD domain or its viral relative, v-ErbA, could invoke the transcriptional repression of VEGF-A. Because standard serum used in cell culture growth media contains trace quantities of thyroids and retinoids that could cause TR$\alpha$ to act as a transcriptional activator, we first adapted HEK293 cells to growth in media containing charcoal-filtered serum in which all traces of such hormones are removed. Plasmid DNA encoding the ZFPA-TR$\alpha$ LBD fusion was transiently transfected into HEK293 cells maintained in hormone-depleted media, and the VEGF-A mRNA expression levels were compared with the results of a transfection in which the cells were maintained in standard media. Additionally, the ZFPA-vErbA chimera was tested in parallel because this domain is a constitutive repressor unable to bind hormone and thus should be unaffected by trace levels of ligand. As shown in Fig. 1*B*, ZFPA-TR$\alpha$ represses VEGF-A mRNA levels by ~50% despite background VEGF-A gene expression from nontransfected cells. This repression was observed only when the HEK293 cells were maintained in hormone-free media (compare charcoal-treated *versus* standard). As expected, the ZFPA-vErbA fusion proved to be an equally potent repressor of VEGF-A mRNA levels even in the presence of trace hormone (Fig. 1*B*).

The potent yet hormone-independent characteristics of the vErbA domain make it a potentially more useful *in vivo* effector than its native TR$\alpha$ relative. We thus tested constructs with the vErbA domain fused to the ZFPB and ZFPC zinc finger DNA-binding domains. Fig. 1*C* demonstrates that all three ZFP-vErbA fusions were capable of

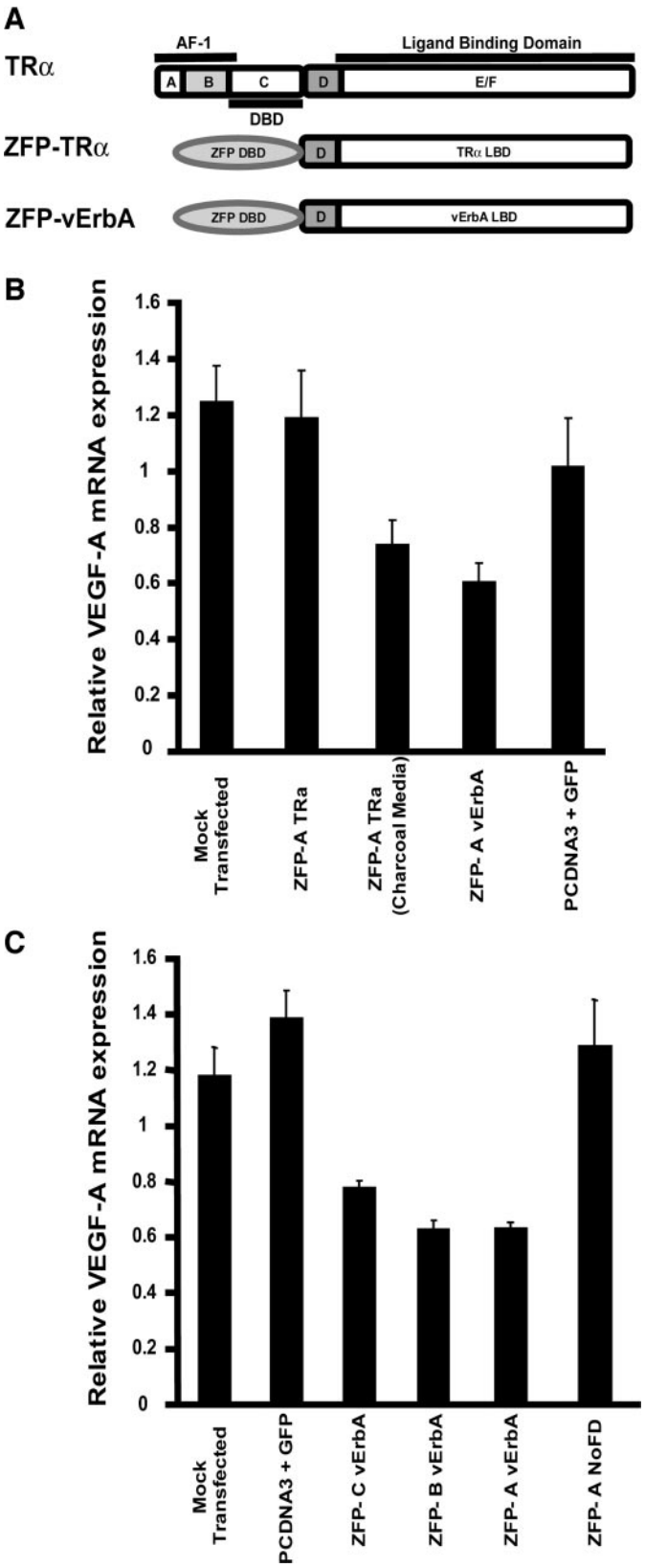



Fig. 1. Zinc finger protein (ZFP)-thyroid hormone receptor $\alpha$ (TR$\alpha$) and ZFP-vErbA fusions repress endogenous vascular endothelial growth factor (VEGF)-A gene expression in a ligand-dependent manner. *A*, a schematic representation of the domain structure of the class II nuclear hormone receptors and of the ZFP-fusions that were designed. Domains labeled *A* and *B* are the AF-1 activation domain, *C* is the DNA-binding domain (DBD), *D* is a flexible linker region, *E/F* represents the ligand-binding domain (LBD) [which in the case of TR$\alpha$ but not vErbA also contains the AF-2 activation domain]. The *bottom drawings* indicate the structure of the engineered ZFP DNA-binding domain fusions indicating the regions of the vErbA or TR$\alpha$ LBD used in relation to the natural receptor (*top*). *B*, ZFP-TR$\alpha$ and ZFP-vErbA fusions down-regulate *VEGF-A* mRNA expression. HEK293 cells were transiently transfected with the plasmids indicated. A green fluorescent protein (GFP) expression plasmid (pcDNA3+GFP) and mock (no DNA) transfection serve as controls. ZFP-TR$\alpha$ was transfected into cells grown in either standard media or ligand-depleted (charcoal-filtered) media. Total RNA was isolated, and *VEGF-A* mRNA levels were determined by real-time PCR (TaqMan) 72 h after transfection. *VEGF-A* mRNA levels were normalized relative to an internal control of *GAPDH* mRNA and are expressed as this ratio. Results are representative of at least three independent experiments, with the means and SDs shown. *C*, ZFP-vErbA fusions constitutively repress *VEGF-A* mRNA expression. HEK293 cells were transiently transfected with the indicated plasmid DNA and controls, as described in *B*, together with a ZFPA construct lacking any functional domain (*ZFPA No FD*). Relative *VEGF-A* mRNA expression levels were determined 72 h after transfection by real-time PCR (TaqMan), as described in *B*.

driving a ~50% repression in the expression of VEGF-A mRNA in transient transfection experiments. No VEGF-A repression was observed by transfection with control plasmids expressing green fluorescent protein alone or with a plasmid encoding the ZFP-A DNA-binding domain alone (Fig. 1*C*). We conclude that ZFP-vErbA fusions that bind the VEGF-A promoter can effectively repress gene expression in transient transfection experiments.

**Potent Dose-Dependent ZFP-Mediated Regulation of VEGF-A Gene Expression in Stable Inducible Cell Lines.** To eliminate the contribution of untransfected cells in the transient transfection assays of repression, we constructed stable cell lines in which the T-REx system (Invitrogen) provided inducible expression of the ZFP TF. We first created vectors that placed ZFPA-vErbA expression under the control of a TetO-regulated cytomegalovirus promoter and then introduced them into the U2OS T-REx cells by transfection and antibiotic selection. After the isolation of single cell-derived clones, we tested for DOX-dependent repression of the *VEGF-A* gene. Multiple clones capable of DOX-inducible VEGF-A repression were obtained, and the results from one such ZFPA-vErbA U2OS T-REx clone are shown in Fig. 2*A*. Increasing DOX concentrations led to a dose-dependent repression of VEGF-A expression as measured at both the mRNA and protein levels. This decrease was paralleled by a DOX-dependent increase in ZFPA-vErbA expression as demonstrated by immunoblotting (Fig. 2*B*). Indeed, a striking correlation was observed between the level of the ZFPA-vErbA expressed and the degree of VEGF-A protein or mRNA down-regulation (compare Fig. 2, *A* and *B*). Importantly the magnitude of target gene repression increased significantly in the stable inducible cell line system. For example, at the 1 ng/ml DOX concentration, an ~81% reduction in VEGF-A protein was observed (Fig. 2*A*), greatly exceeding the ~50% repression observed in the transient assays (Fig. 1, *B* and *C*). ZFPA-vErbA fusions are thus shown to be potent, dose-dependent regulators of VEGF-A expression.

**ZFPA-vErbA Binds to Its Intended Targeted Site *in Vivo*.** To confirm that ZFPA acts through its intended binding site, we conducted *in vitro* DNAse I footprinting experiments using recombinant, bacterially expressed ZFPA. As probe, a $^{32}$P-labeled VEGF-A promoter fragment encompassing the intended binding site (+434) and comprising the sequence from +334 to +566 relative to the start site of transcription of the human VEGF-A gene was used (Fig. 3*A*). As shown in Fig. 3*B*, increasing quantities of recombinant ZFPA protein resulted in both a region of protection from DNAse I cleavage (marked by a *vertical bar*) and a new enhanced cleavage site (marked by an *arrow*) directly at the intended binding site. Thus, ZFPA binds to the +434 site *in vitro*.

To provide *in vivo* validation of ZFPA binding, we performed ChIP assays on the ZFPA-vErbA U2OS T-REx cell line using antibodies specific for the FLAG epitope carried by the ZFP transcription factor. Precipitated DNA was then analyzed with three sets of VEGF-A-specific primer pairs: two regions (+305 to 355 and −4 to +69) both

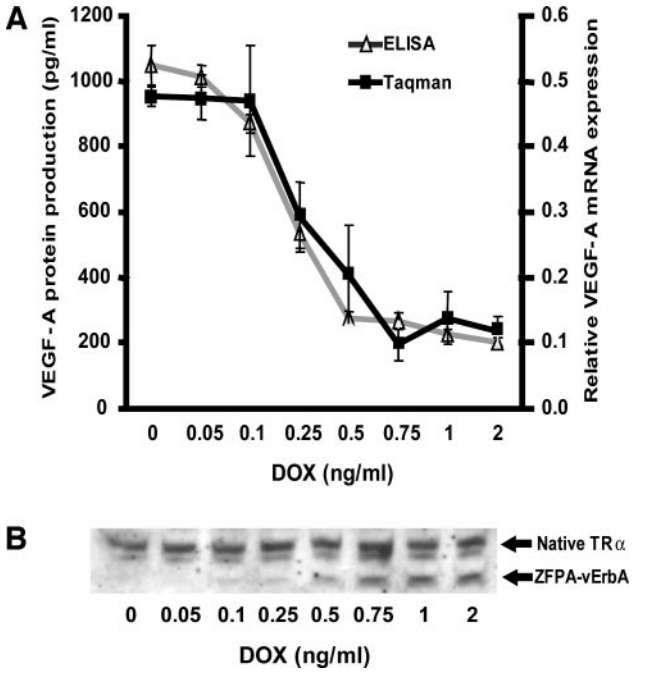



Fig. 2. A stable inducible zinc finger protein (ZFP) A-vErbA cell line exhibits potent ZFP dose-dependent repression of vascular endothelial growth factor (VEGF)-A expression. *A*, a stable inducible U2OS T-REx ZFPA-vErbA cell line demonstrates ZFP dose-dependent repression of VEGF-A expression. Equivalent numbers of U20S T-REx ZFPA-vErbA cells were grown in standard growth media supplemented with the indicated concentration of the doxycycline (DOX) for 72 h before harvesting. The relative levels of secreted VEGF-A protein (*right axis*) were determined using an ELISA assay kit (R&D Systems), and the corresponding VEGF-A mRNA levels (*left axis*) were determined via quantitative real time-PCR (TaqMan). Results are representative of at least three independent experiments. *B*, ZFPA-vErbA expression from the U2OS T-REx ZFP-vErbA line is DOX dependent. Immunoblot of U2OS T-REx ZFP-vErbA cells treated with the indicated concentrations of DOX inducer for 72 h. Expression of the ZFPA-vErbA chimera was detected via immunoblot using an anti-thyroid hormone receptor $\alpha$ (TR$\alpha$) antibody. Endogenous TR$\alpha$, which also reacts with this antibody, was used as an internal loading control (*top band*).

in close proximity to the ZFP binding site (+434); and a third primer set located upstream of the transcription initiation site (–563 to –494). The experiment was internally controlled using primers for exon 7 of the glyceraldehyde-3-phosphate dehydrogenase locus. Sequence composition directly across the +434 site prevents the use of a primer pair that spans this site precisely. The results of this analysis are shown in Fig. 3*C*. At a DOX concentration of 10 ng/ml [which is in excess of that necessary to detect the expression of ZFPA-vErbA protein and repression of the VEGF-A gene (Fig. 2, *A* and B)], a clear enrichment of signal (~40-fold) was observed with the primer sets directly adjacent to the intended binding site. A greatly reduced but nonetheless robust enrichment of ~12-fold was observed for the (−4 to +69) probe set, consistent with the proximity of these primers relative to the +434 site, and the observed average fragment length of ~500 bp for the genomic DNA after sonication. In contrast, no enrichment was detected for a third VEGF-A-specific primer set (–563 to –494) located ~1 kb upstream of the intended site (Fig. 3*B*). Significantly, no enrichment was detected across all primer sets at a DOX concentration of 0.1 ng/ml, at which neither detectable expression of the ZFPA-vErbA fusion nor VEGF-A repression was observed (Fig. 2, *C* and *D*). No signal was obtained from control samples prepared in the absence of the antibody (data not shown). Whereas accurate resolution of the location of ZFP binding is not technically possible by ChIP experiments, these data, taken together with *in vitro* DNAse I footprint (Fig. 3*B*), are consistent with ZFPA binding to its intended site within the VEGF-A promoter.

**ZFPA-vErbA Targeted Repression Is HDAC Dependent and Results in Promoter-Specific H3 and H4 Deacetylation.** TR$\alpha$ and vErbA repress transcription via their interaction with the HDAC-associated N-CoR/SMRT corepressor complex (16, 28). We therefore wished to determine whether the ZFPA-vErbA-mediated repression of VEGF-A required the same HDAC-dependent mechanism used by this domain at its natural target genes. A number of different HDACs have been shown to functionally interact with the N-CoR complex; in

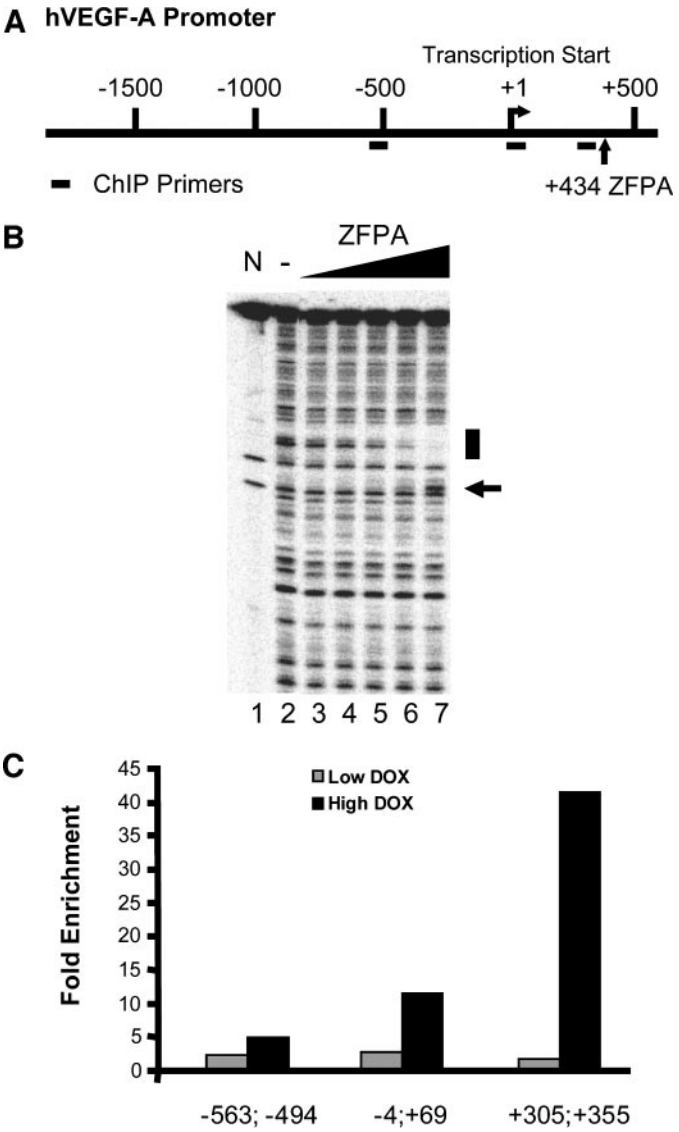
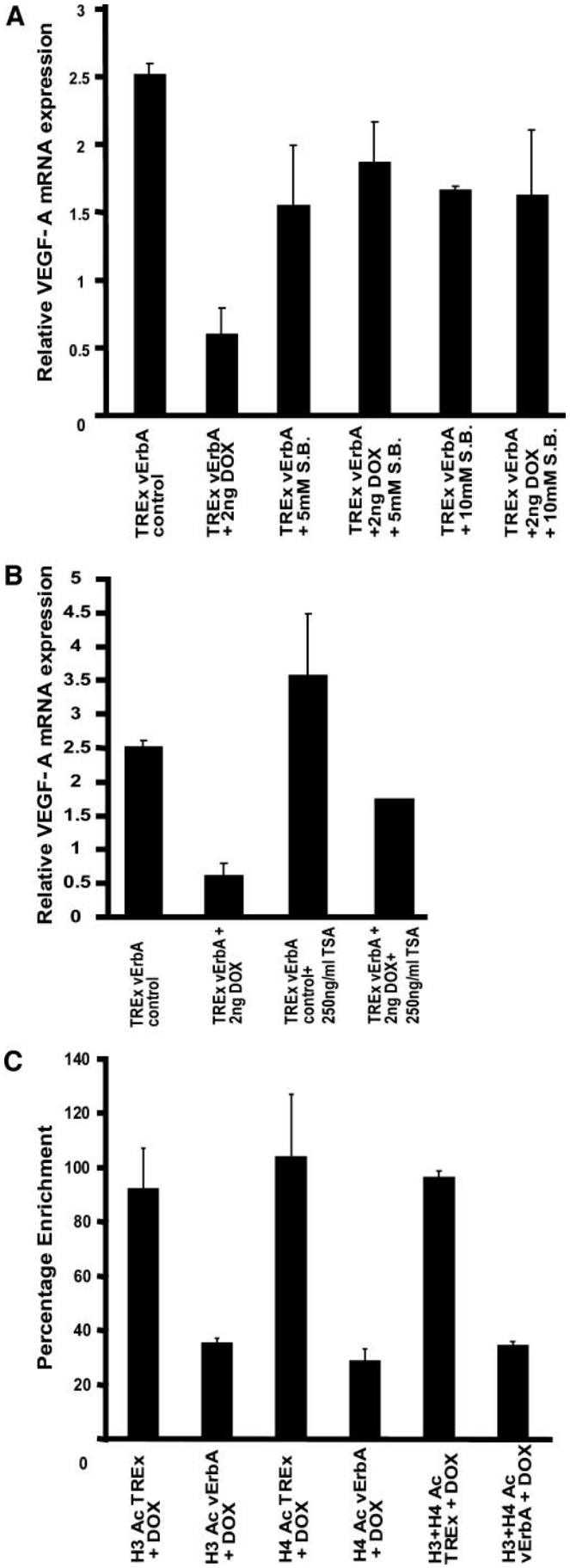



Fig. 3. Zinc finger protein (ZFP) A binds specifically to its intended target site *in vitro* and *in vivo*. *A*, schematic of the vascular endothelial growth factor (VEGF)-A promoter region, indicating the designed ZFPA binding site (+434), the transcription start site (+1), and the relative positions of the DNA primer sets used for chromatin immunoprecipitation (ChIP). *B*, *in vitro* DNase I footprinting of the VEGF-A promoter region containing the ZFPA binding site. DNase I digestion of a $^{32}$P-labeled VEGF-A promoter DNA fragment in the presence of increasing amounts of ZFPA shows a clear region of protection (*solid block*) and an area of enhanced cleavage (*arrow*) specifically at the intended binding site for ZFP-A. *Lane 1*, no DNAse I; *Lane 2*, DNAse I no protein; *Lanes 3–7*, as *Lane 2* but contain 30, 60, 120, 240, or 480 fmol of ZFPA, respectively. *C*, ZFPA-vErbA binds the VEGF-A promoter *in vivo*. U2OS T-REx ZFPA-vErbA cells induced with low (0.1 ng/ml) or high levels (10 ng/ml) of doxycycline (DOX) for 48 h were assayed for ZFPA-vErbA binding by ChIP with an antibody against the FLAG-epitope tag carried by the ZFP TF and primers specific for different regions of the VEGF-A promoter as indicated. Enrichment was quantified by PCR/Taqman analysis, and the results are expressed as the fold increase of enrichment relative to a control primer pair within the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Results are representative of two independent experiments with SDs of <20%. No enrichment was observed with preimmune serum (data not shown).




particular HDAC3 resides in the N-CoR complex targeted by TR$\alpha$/v-ErbA *in vivo* (16, 23, 29–32). To address the question of whether HDAC activity was required for the repression function of ZFPA-vErbA, we used the broad-spectrum HDAC inhibitors trichostatin A and sodium butyrate. The ZFPA-vErbA U2OS T-REx cell line was cultured in the presence (2 ng/ml) or absence of DOX in media that contained the indicated concentrations of each inhibitor, and the effect on VEGF-A transcriptional repression was determined at the mRNA level. Fig. 4*A* shows the effect of sodium butyrate addition. This HDAC inhibitor almost completely abolishes the repression of VEGF-A mediated by the ZFPA-vErbA fusion. Analysis of the effects of trichostatin A treatment was confounded by a slight elevation in the basal levels of VEGF-A caused by the addition of this inhibitor. Nevertheless, the repression of VEGF caused by ZFPA-vErbA was significantly reduced in the presence of trichostatin A also (Fig. 4*B*). These data suggest that at least part of the repression function of the v-ErbA domain used in these assays is HDAC dependent.

To confirm that the ZFPA-vErbA-driven repression resulted from histone deacetylation *in vivo*, we performed ChIP assays on the ZFPA-vErbA U2OS T-REx cell line with antibodies specific for acetylated histone H3 and/or acetylated H4. Precipitated DNA was then analyzed with primers specific for the +350 region of the VEGF-A gene adjacent to the ZFPA-binding site and internally controlled using primers for exon 7 of the glyceraldehyde-3-phosphate dehydrogenase locus. As demonstrated in Fig. 4*C*, expression of ZFPA-vErbA results in a significant reduction in the levels of acetylated-H3, acetylated-H4, and the combination acetylated-H3 and acetylated-H4 at the VEGF-A promoter relative to controls (non-DOX treated). No change in the level of histone acetylation was detected at the nontargeted p16 gene (data not shown). Similar results were obtained using primers specific for the +1 site, indicating that the deacetylated region encompassed the start site of VEGF-A transcription (data not shown). Taken together, the HDAC inhibitor and ChIP experiments demonstrate that ZFPA-vErbA repression utilizes histone deacetylase activity, presumably recruited to the promoter through the known interaction of vErbA with the N-CoR complex. Thus, ZFPA-vErbA is shown to repress the expression of a targeted gene via a mechanism indistinguishable from that used by endogenous class II nuclear hormone receptors.

**ZFPA-vErbA Reduces VEGF-A Expression in the Glioblastoma Cell Line U87MG to Nonangiogenic Levels.** VEGF-A is dramatically overexpressed in highly vascularizing tumorigenic lines, such as the glioblastoma U87MG (33). Notably, the expression of VEGF-A protein is some ~20-fold higher in U87MG cells compared with HEK293 cells, a result paralleled at the mRNA level, suggesting

Fig. 4. Zinc finger protein (ZFP) A vErbA represses via a histone deacetylase-dependent mechanism. *A*, the histone deacetylase inhibitor sodium butyrate inhibits ZFPA-vErbA-mediated repression of vascular endothelial growth factor (VEGF)-A. U2OS T-Rex ZFPA-vErbA stable inducible cells were grown in the absence or presence of 2 ng/ml doxycycline (DOX) in the presence of the indicated concentrations of sodium butyrate (*S.B.*) for 72 h and assayed for *VEGF-A* mRNA expression via quantitative real time-PCR/TaqMan as described in Fig. 1*B*. *B*, trichostatin A partially inhibits ZFPA-vErbA-mediated repression of vascular endothelial growth factor (VEGF)-A expression. U2OS T-Rex ZFPA-vErbA stable inducible cells were were grown in the absence or presence of 2 ng/ml DOX and 250 ng/ml trichostatin A (*TSA*) as indicated for 72 h and assayed for *VEGF-A* mRNA expression via quantitative real time-PCR/TaqMan as described in Fig. 1*B*. *C*, ZFPA-vErbA induces nucleosome deacetylation at the VEGF-A promoter. U2OS T-REx and U2OS T-REx ZFPA-vErbA cells were grown in standard media or induced with 5 ng/ml DOX for 72 h before being assayed for histone H3, histone H4, and H3/H4 acetylation levels by ChIP with primers specific for the +350 VEGF-A promoter region. Enrichment was quantified by PCR using TaqMan chemistry and determined relative to a control primer pair within the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as described in Fig. 3*C*. Values shown are the percentage acetylation relative to noninduced controls, which were arbitrarily set to 100%. Results are representative of at least two independent experiments, with means and SDs shown.

transcriptional misregulation as the cause (data not shown). We therefore wished to determine whether the ZFP-based approach to gene regulation could be used to repress VEGF-A expression in this therapeutically relevant model. In particular, it remained unclear as to whether a heterologous repression domain would be sufficient to overcome the cancer-specific transcriptional circuitry that drives aberrant VEGF overexpression in this line (11).

To address this question, U87MG cells were transiently transfected with plasmid DNA encoding vErbA fusions to each of the three VEGF-A-specific ZFP DNA-binding domains (ZFPA, ZFPB, and ZFPC). As shown in Fig. 5*A*, all three ZFP-vErbA fusion constructs significantly reduced the expression level of VEGF-A protein, from ~7000 to ~3500 pg/ml. This striking reduction was observed despite a transfection efficiency of only ~50% as determined by cotransfection with a plasmid expressing green fluorescent protein. To eliminate the contribution to VEGF-A levels from these untransfected cells, a U87MG T-REx cell line was constructed and shown to provide tightly regulated DOX-inducible regulation of control vectors without effect on growth rate or VEGF-A expression levels (see below). The vectors used earlier (Fig. 2) that place ZFPA-vErbA expression under the

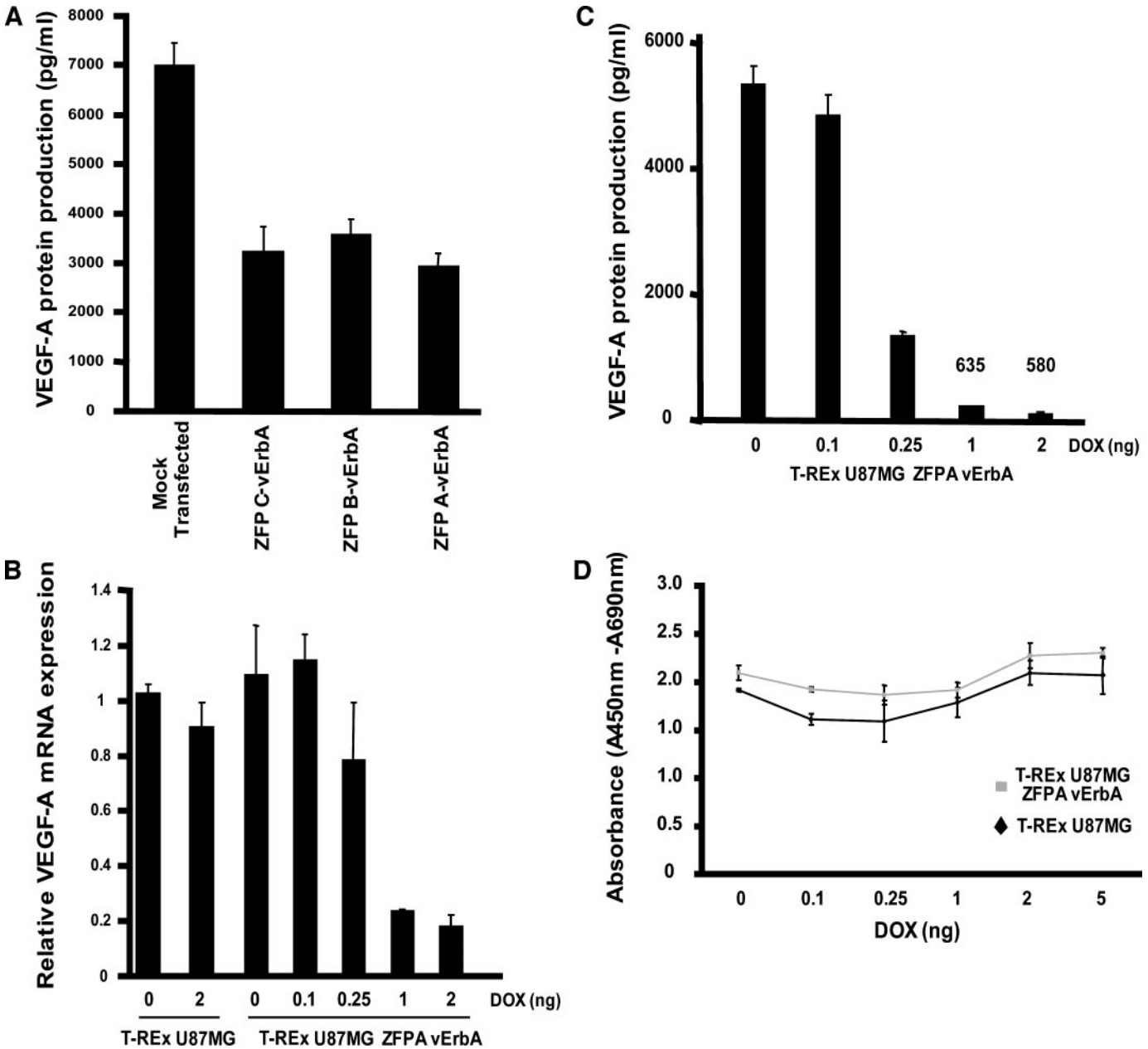



Fig. 5. Zinc finger protein (ZFP) A-vErbA functions as a potent repressor in the vascular endothelial growth factor (VEGF)-A-overexpressing U87MG glioblastoma cell line. *A*, ZFP-vErbA fusions can repress VEGF-A overexpression in U87MG glioblastoma cells. U87MG cells were transiently transfected with the indicated plasmids and assayed for VEGF-A protein secretion using a human VEGF-A ELISA assay kit. As a control, a transfection with no DNA (*Mock*) was done in parallel. Transfection efficiency was assessed in each independent experiment via the use of a green fluorescent protein (GFP) expression plasmid. For U87MG cells, ~50% GFP-positive cells were observed. *B*, stable inducible U87MG T-REx ZFPA-vErbA cells demonstrate a potent and ZFP-dependent repression of *VEGF-A* mRNA. U87MG T-REx ZFPA-vErbA cells or the parental U87MG T-REx line were induced with the indicated amount of doxycycline (DOX), and relative *VEGF-A* mRNA expression levels were determined 72 h after induction by TaqMan as described in Fig. 1*B*. *C*, stable inducible U87MG T-REx ZFPA-vErbA cell lines demonstrate a potent and ZFP-dependent repression of VEGF-A protein. U87MG T-REx ZFPA-vErbA cells were induced with the indicated amount of DOX, and the relative VEGF-A proteins levels were determined 72 h after induction by ELISA (R&D Systems). *D*, ZFP TF expression does not affect cell proliferation. A WST-1 cell proliferation assay was performed on the U87MG T-REx parental and U87MG T-REx ZFP-A vErbA cell lines after treatment with the indicated concentrations of DOX 72 h after DOX induction. Conversion of the WST-1 substrate is shown as the $A_{450\ nm}$-$A_{690\ nm}$ reading, a reduction in the levels of WST-1 conversion would indicate reduced proliferation. *Light gray squares* and *lines* plot the T-REx U87MG ZFPA-vErbA line, whereas *dark diamonds* and *lines* plot the parental line, T-REx U87MG.

control of a TetO-regulated cytomegalovirus promoter were then used to transfect this U87MG T-REx cell line. Single cell-derived cell lines were isolated and tested for DOX-dependent repression of the VEGF-A gene. Fig. 5*B* demonstrates that induction of ZFPA-vErbA expression by treatment with increasing DOX concentrations resulted in a dramatic and dose-dependent repression of VEGF-A mRNA expression, whereas the identical DOX treatment of the parental U87MG T-REx cell line had no effect on VEGF-A expression. Concordantly, VEGF-A protein levels dropped from ~5500 to ~300 pg/ml in the U87MG T-REx ZFPA-vErbA cell line (Fig. 5*C*). To rule out a potential reduction in VEGF-A expression as a result of nonspecific cell growth effects caused by the DOX induction process, the cell proliferation and viability of the U87MG T-REx cell lines were measured. At all DOX concentrations used, for both the ZFP-expressing and parental T-REx U87MG lines, no change was observed in viability or proliferation rate (Fig. 5*D*). This indicated that neither the presence of DOX nor the expression of the ZFP was affecting cell viability or the rate of proliferation, confirming that any effect on VEGF-A resulted from a direct consequence of promoter-specific transcriptional repression. Thus ZFPA-vErbA is shown to be a potent inhibitor of VEGF-A expression in the glioblastoma line U87MG.

Finally, to provide a measure of the potential therapeutic success of this degree of repression, we sought to compare the repressed levels of VEGF-A expression in U87MG cells with the levels observed in cell lines not capable of the aggressive vascularization. In this regard, the cancer cell line U251MG has been shown to be of low tumorigenic potential but, importantly, can be converted into a line of demonstrably high tumorigenicity by transfection with expression vectors encoding the $VEGF_{121}$ and/or $VEGF_{164}$ cDNAs (34). In this particular case, it appears that the VEGF-A levels are a key determinant of tumorigenicity and, importantly, that the basal levels of VEGF-A expressed by this line are insufficient for this process (34). We therefore directly compared the levels of VEGF-A in the ZFPA-vErbA U87MG T-REx cell line after DOX induction with those of the nontumorigenic U251MG cell line. Additionally, we compared these levels with a second nontumorigenic human glioblastoma line, T98G (35), which also expresses low levels of VEGF-A (11). Fig. 6 demonstrates that the repressed levels of VEGF-A in U87MG cells mediated by the ZFPA-vErbA fusion are ~20-fold reduced compared with non-DOX-treated controls. Moreover, this ZFP TF repressed level of VEGF expression, measured at ~300 pg/ml, is comparable to the ~350 pg/ml observed in both the U251MG and T98G cell lines. Thus the degree of ZFP-mediated VEGF-A repression observed in the therapeutically relevant glioblastoma cell line U87MG would be predicted to reduce or even ablate the tumorigenic potential of this cancer cell line *in vivo*.

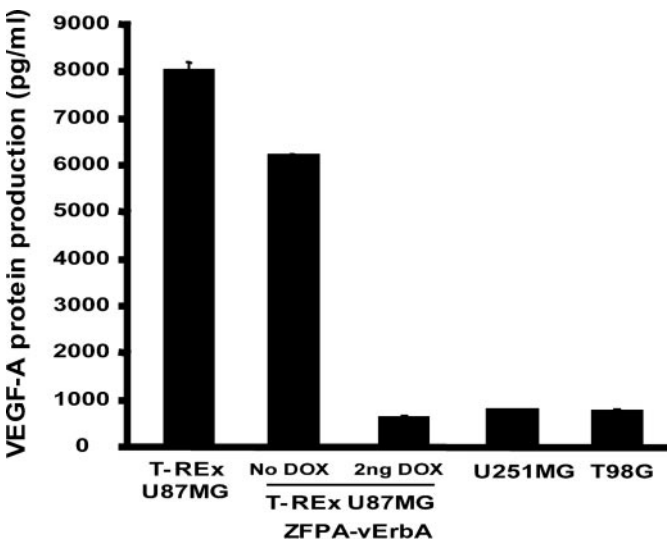



Fig. 6. A therapeutic level of vascular endothelial growth factor (VEGF)-A repression is driven by zinc finger protein (ZFP) A-vErbA in the glioblastoma U87MG cell line. Glioblastoma cell lines U251MG, T98G, U87MG T-Rex, and U87MG T-REx ZFPA-vErbA were seeded at equal density and grown in the presence or absence of 2 ng/ml doxycycline (*DOX*), as indicated. Secreted VEGF-A protein levels were assayed by ELISA (R&D Systems) 72 h after induction, and the resulting expression levels were corrected for differences in cell number between the different cell lines.

## DISCUSSION

ZFP TFs are shown here to be potent transcriptional repressor proteins able to achieve a dramatic knockdown in the mRNA and protein levels of a target gene critical for cancer growth. In this study, we have used both the LBD of TR$\alpha$ as well its constitutive non-ligand-binding viral relative v-ErbA to specify repression function. As expected, ZFP targeted repression driven by the ZFPA-vErbA protein was both HDAC dependent and resulted in the deacetylation of histones at the targeted promoter. This mechanism of repression is analogous to that driven by native TR$\alpha$ or vErbA, suggesting that the endogenous corepressor complexes N-CoR/SMRT are efficiently recruited to the gene of interest in a manner determined by the DNA binding specificity of the ZFP. This result is not limited to the nuclear hormone receptor family because we have previously demonstrated that a ZFP histone methyltransferase catalytic domain fusion is capable of directing the transcriptional repression of the target gene in a H3K9 methylation-dependent manner (15). These results highlight the modularity of ZFP-effector fusion proteins and demonstrate the utility of these chimeras in the dissection of the regulatory mechanisms used by the domain itself or in the pathways controlled by the targeted gene.

The repression of VEGF-A in the glioblastoma cell line U87MG is of particular note. The ability to drive such a dramatic down-regulation of VEGF-A expression in this cell line demonstrates the potency of this natural mechanism of transcriptional regulation. Indeed, whereas antisense (33, 36, 37) or RNA-interference-based mechanisms (38) of mRNA degradation must bind the many copies of the mRNA transcript, the ZFP approach targets the promoter itself. Indeed, ZFPA is shown here to bind to its intended site at the promoter (Fig. 3). Importantly, irrespective of the VEGF-A mRNA expression levels, just two such target sites (one in each allele of the VEGF locus) exist per cell. The concentration of ZFP TF necessary to maintain this down-regulation is therefore likely to be independent of the rate of target gene expression. Moreover, the ZFP TF approach can be used to drive either the up-regulation (13) or down-regulation (15) of the VEGF-A target gene. This may be of considerable therapeutic value because the proangiogenic properties of VEGF-A, for example, are of potential benefit in the treatment of numerous cardiovascular conditions, and ZFP TFs able to up-regulate this gene have already shown promise in animal models of angiogenesis (14).

From the antiangiogenic perspective, U87MG cells represent a well-documented highly vascularizing glioblastoma-derived cell line of demonstrated tumorigenicity (33). U251MG cells, by contrast, are of low angiogenicity and tumorigenicity but, importantly, can be switched into a highly aggressive angiogenic and tumorigenic mode if transfected with a cDNA expressing VEGF-A (34). Thus, for U251MG cells, the level of VEGF-A itself appears to be the limiting factor with regard to malignancy and therefore provides a benchmark with which to score the success of our gene repression. As described above, the ~20-fold reduction in VEGF-A expression in U87MG cells obtained after ZFP TF induction resulted in U251MG-like amounts of residual VEGF-A production. These data suggest that the

degree of repression driven by the ZFP TF would be sufficient to dramatically reduce the tumorigenic potential of U87MG cells *in vivo*. Indeed, earlier work using adenoviral delivery of an antisense reagent against $VEGF_{164}$ demonstrated that a ~50% reduction in secreted VEGF-A from U87MG cells was sufficient to cause the suppression of tumor growth *in vivo* but not tumor regression (37). The drug-inducible ZFPA-vErbA U87MG T-Rex cell line developed in this work provides a unique opportunity to dissect the *in vivo* role of VEGF-A in malignancy. The ability to determine the timing, duration, and extent of VEGF-A regulation *in vivo* by the addition or removal of the appropriate concentration of DOX will doubtless expand our understanding of the pivotal role played by VEGF-A in cancer angiogenesis.

ZFP TFs, which target VEGF-A transcription rather than the extracellular VEGF-A protein or its receptors, represent a novel therapeutic approach to antiangiogenic treatment. The ultimate success of such an approach is limited by the available modes of delivery and the assumed necessity to hit the majority (if not all) of the malignant cells. However, the development of viral systems capable of tumor cell-specific replication (39, 40) demonstrates the progress being made in this regard. Such vector systems present antiangiogenic ZFP TF reagents with an attractive targeting capability. Indeed, given the positive roles played by the proangiogenic factors in important physiological processes such as wound healing (41), a local correction in VEGF-A levels may be superior to the use of a systemically delivered small molecule drug.

Designed zinc finger transcription factors may be applied to the study of any gene of interest (12). Indeed, ZFP TFs have been documented to control the expression of a wide range of genes implicated in cancer such as VEGF-A (13–15), ErB-2 and ErB-3 (42), oncogenic chromosomal rearrangements (43), and specific genes involved in the replication of both the HIV-1 (44) and herpes simplex virus-1 (45) viral genomes. ZFP TFs thus provide powerful tools with direct therapeutic potential for the study and treatment of disease.

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# Repression of Vascular Endothelial Growth Factor A in Glioblastoma Cells Using Engineered Zinc Finger Transcription Factors

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