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Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth

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Current understanding of key transcription factors regulating angiogenesis is limited. Here we show that RNA-cleaving phosphodiester-linked DNA-based enzymes (DNAzymes), targeting a specific motif in the 5′ untranslated region of early growth response (Egr-1) mRNA, inhibit Egr-1 protein expression, microvascular endothelial cell replication and migration, and microtubule network formation on basement membrane matrices. Egr-1 DNAzymes blocked angiogenesis in subcutaneous Matrigel plugs in mice, an observation that was independently confirmed by plug analysis in Egr-1-deficient animals, and inhibited MCF-7 human breast carcinoma growth in nude mice. Egr-1 DNAzymes suppressed tumor growth without influencing body weight, wound healing, blood coagulation or other hematological parameters. These agents inhibited endothelial expression of fibroblast growth factor (FGF)-2, a proangiogenic factor downstream of Egr-1, but not that of vascular endothelial growth factor (VEGF). Egr-1 DNAzymes also repressed neovascularization of rat cornea. Thus, microvascular endothelial cell growth, neovascularization, tumor angiogenesis and tumor growth are processes that are critically dependent on Egr-1.

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a complex, multistep process involving endothelial basement membrane degradation, cell migration, proliferation, canalization, branching and maturation of neovessels. Angiogenesis is mandatory for tumor progression because it supplies oxygen and nutrients to the growing tumor, a concept first proposed 30 years ago[1]. It follows, then, that agents that interfere with blood vessel formation could be used to block tumor progression[2]. Examples of these include antagonists of angiogenic growth factors, receptors, integrins and proteolytic enzymes; some of these are currently under evaluation in various clinical trials[3].

Egr-1, first discovered as an immediate-early gene inducibly expressed in growth-quiescent fibroblasts exposed to serum[4], is a broadly expressed prototypical member of the Cys_2-His_2 zinc finger family of transcription factors[5]. Egr-1 is rapidly activated by multiple extracellular agonists (such as growth factors and cytokines) and environmental stresses (such as hypoxia, fluid shear stresses and vascular injury)[6]. Once activated, Egr-1 controls the expression of a diverse array of proangiogenic genes (encoding growth factors, cytokines, receptors, adhesion molecules and proteases)[5,7] through GC-rich, *cis*-acting elements in the promoter regions of these genes. Egr-1 is strongly coexpressed with proliferating cell nuclear antigen in von Willebrand factor–positive blood vessels in the chorioallantoic membrane assay (data not shown). Therefore, Egr-1 seemed *prima facie* to be an appealing therapeutic target given its capacity to integrate extracellular stimuli with changes in gene expression.

RESULTS

DNAzyme inhibits Egr-1 and endothelial growth

To begin to dissect the role of Egr-1 in angiogenesis, we evaluated the effect of two Egr-1 DNAzymes, DzF and DzA, on human microvascular endothelial cell proliferation. DNAzymes are cation-dependent enzymatic molecules composed entirely of DNA that can be engineered to cleave target mRNA in a gene-specific and catalytically efficient manner[8]. DzF and DzA each bear a 15-nucleotide catalytic domain of the 10-23 subtype[8], flanked by two 9+9 nucleotide arms that target the $A^{301}U$ and $G^{198}U$ sites in human Egr-1 mRNA, respectively. DzF and DzA each inhibited serum-inducible endothelial cell proliferation at 0.1 μM, with more potent inhibition by DzF (**Fig. 1a**). DzF inhibition was dose dependent and detectable at concentrations as low as 40 nM (data not shown). In contrast, DzFSCR, in which the order of nucleotides in the hybridizing arms of DzF was scrambled (SCR) without altering the catalytic domain, had no effect (**Fig. 1a**). DzF also inhibited endothelial cell migration in an *in vitro* scraping assay. Regrowth from the wound edge into the denuded zone 2 d after injury was inhibited 65% by DzF (0.1 μM), whereas DzFSCR had no effect (**Fig. 1b,c**).

We next evaluated the capacity of Egr-1 DNAzymes to inhibit capillary tubule formation by endothelial cells cultured on basement membrane matrices (Matrigel), wherein the cells undergo morphologic differentiation into an extensive network of capillary-like structures composed of highly organized three-dimensional cords[9]. Tubule formation in this model proceeds rapidly after plating, with maximal network formation after 8 h and gradual dissociation by 24 h (**Fig. 1d**). DzF caused profound sequence-specific inhibition of tubule formation

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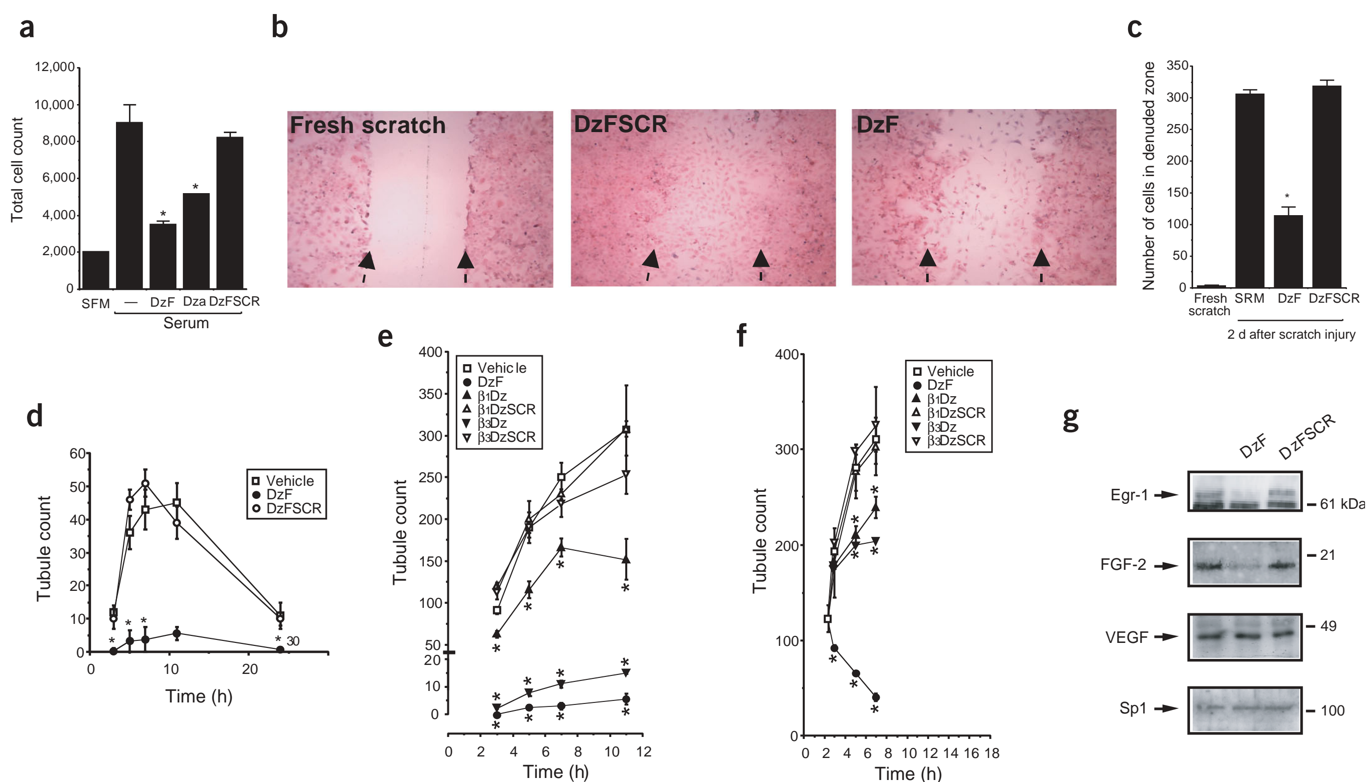

Figure 1 DNAzyme DzF inhibits human microvascular endothelial cell proliferation, migration, microtubule formation and Egr-1 expression. (**a**,**b**) Effect of DNAzymes on HMEC-1 proliferation after 3 d (**a**) and migration into denuded zone 2 d after scraping (**b**). Arrows indicate the wound edge. (**c**) Assessment of population of cells in denuded zone. (**d**–**f**) Microtubule formation on Matrigel after prior HMEC-1 transfection with DzF or DzFSCR and subsequent plating (**d**), or DzF, β_1Dz, β_1DzSCR, β_3Dz or β_3DzSCR and subsequent plating (**e**), or administration of DzF, β_1Dz, β_1DzSCR, β_3Dz or β_3DzSCR to pre-existing tubules (**f**). Tubules per field were counted under phase-contrast microscopy. (**g**) Western immunoblot analysis with indicated antibodies using extracts of human microvascular endothelial cells transfected with 0.1 μM DNAzyme and stimulated 1 h with serum. *, $P < 0.05$ relative to DzFSCR or vehicle group (Student's *t*-test). SFM, serum free medium; Srm, serum.

(**Fig. 1d**,**e**). It also induced time-dependent regression of pre-existing tubules (**Fig. 1f**). Comparison of DzF with DNAzymes targeting integrins β_1 or β_3 (ref. 10) revealed superior inhibition by the former (**Fig. 1e**,**f**). DzF, but not DzFSCR, virtually abrogated endothelial Egr-1 expression, whereas levels of transcription factor Sp1 did not change (**Fig. 1g**). Collectively, these findings indicate sequence-specific inhibition of microvascular endothelial cell proliferation, migration, three-dimensional capillary tube formation and Egr-1 protein expression.

DNAzymes block matrix neovascularization in mice

We next investigated whether Egr-1 is required for new blood vessel formation by injecting Matrigel as subcutaneous plugs into C57BL/6 mice, together with Egr-1 DNAzyme ED5, which cleaves mouse Egr-1 RNA in a time- and sequence-specific manner (**Fig. 2a**) and inhibits mouse microvascular endothelial cell proliferation and microtubule formation in a dose-dependent manner (data not shown). Significant blood vessel formation and inflammatory infiltrate was apparent in cross-sections of vehicle and ED5SCR groups 14 d after implantation, indicating the existence of a functional vasculature within these plugs. ED5 reduced vessel density and hemoglobin content in the plugs by over 50% (**Fig. 2b**).

We next conducted Matrigel plug analysis in a DNAzyme-free system in C57BL/6 mice deficient in Egr-1[11]. Plug vascularity 14 d after implantation was markedly impaired in these mice compared with their wild-type counterparts, with occasional inflammatory cells and fibroblasts apparent in a largely acellular matrix in the Egr-1-deficient cohort (**Fig. 2c**). We also evaluated the effect of the Egr-1 DNAzyme in the rat corneal model of neovascularization[12,13]. Implantation of recombinant VEGF-soaked disks into the normally avascular cornea generates a robust neovascularization response from the limbus toward the implant within 5 d (refs. 12,13). Conjunctival administration of ED5 after disk implantation strongly inhibited this angiogenic response (**Fig. 2d**,**e**). In contrast, neovascular density in the scrambled cohort was similar to that in the vehicle group (**Fig. 2e**). Thus, using complementary approaches, these data show that Egr-1 is required for angiogenesis.

DNAzyme inhibition of solid MCF-7 tumor growth in mice

We subcutaneously injected logarithmically growing MCF-7 breast carcinoma cells, with or without Egr-1 DNAzyme, into athymic Balb/c nude mice, and detected solid palpable tumors within 2–3 weeks. Tumor volumes in the ED5 group 60 d after implantation were fivefold smaller than those in the scrambled DNAzyme or vehicle groups. There was no difference in body weight between the groups (**Fig. 3a**,**b**). Immunohistochemical examination of cross-sections of resected tumors did not show TUNEL-positive apoptosis or necrosis but did indicate more erythrocyte-filled CD31$^+$ blood vessels in the ED5SCR and vehicle groups; these findings were confirmed by quantitative assessment of vascular densities (**Fig. 3c**,**d**). The lack of α-smooth muscle actin immunoreactivity in any group suggests that endothelial stabilization by way of α-smooth muscle actin–positive pericytes was not a feature in these capillaries. Local intratumoral delivery of ED5 twice a week also blocked solid MCF-7 tumor growth without adversely influencing body weight (**Fig. 3e**). Unlike DzF, which targets human Egr-1

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(ref. 14), ED5 inhibits MCF-7 tumor growth not by direct inhibition of MCF-7 proliferation but by blocking host angiogenesis (**Fig. 3f**).

We next examined systemic effects of twice-a-week intratumoral DNAzyme administration at the level of wound healing and hemostasis. No significant difference in the rate of wound closure after acute injury was detected between any groups (**Fig. 4a**). There was also no difference in mean clotting time of blood from these mice (**Fig. 4b**), nor was there any change in tissue factor concentration (**Fig. 4c**). Moreover, there was no change in circulating erythrocytes, hemoglobin, hematocrit, platelets, mean platelet volume or mean cell volume (**Fig. 4d**). MCF-7 tumor-bearing female mice from each group retained the capacity to reproduce after mating with male mice (data not shown). Virtually identical results were obtained with nude mice bearing solid A549 human lung carcinoma xenografts. ED5, but not its scrambled counterpart, blocked A549 growth without influencing wound healing or other hematologic parameters (data not shown). There was no overt evidence of skin erythema, lethargy or soft feces. Thus, it seems that Egr-1 DNAzymes inhibit solid tumor growth without compromising wound healing, hemostasis or reproduction.

DNAzyme inhibition of angiogenesis is mediated via FGF-2

FGF-2 has long been recognized as a potent inducer of angiogenesis[15]. FGF-2 stimulates Egr-1 expression in endothelial cells[16], and Egr-1 can in turn activate FGF-2 transcription[17,18]. We therefore hypothesized that Egr-1 DNAzyme inhibition is mediated, at least in part, by its blockade of endogenous FGF-2 expression.

Numerous studies have determined that MCF-7 tumor growth in mice is both FGF- and VEGF-dependent. For example, transfection of MCF-7 cells with FGF produced highly vascularized, estrogen-independent solid tumors when grown in nude mice as xenografts[19–21], which are markedly inhibited by the anti-angiogenic drug AGM-1470 (ref. 22). Moreover, solid MCF-7 tumor growth is strongly inhibited by overexpression of truncated dominant-negative FGF receptor[23]. Similarly, overexpression of VEGF in MCF-7 cells increased solid tumor growth and tumor angiogenesis, an effect neutralized by soluble VEGF receptor[24].

Egr-1 and FGF-2 were weakly, if at all, expressed in the invading endothelium of MCF-7 tumors treated with ED5 (**Fig. 5a**). In contrast, VEGF levels were not influenced by the Egr-1 DNAzyme (**Fig. 5a**), consistent with the inability of Egr-1 to regulate VEGF transcription[25,26]. Moreover, FGF-2 was poorly expressed in plug endothelium from Egr-1-deficient mice (**Fig. 5b**), whereas VEGF was expressed in plugs derived from both wild-type and Egr-1-deficient mice (**Fig. 5b**), despite differences in the size and number of blood vessels between these groups. In microvascular endothelial cells, western blot analysis revealed Egr-1 DNAzyme inhibition of FGF-2 but not VEGF (**Fig. 1g**), and comparative microarray analysis showed DNAzyme inhibition of FGF-2 but not VEGF mRNA (**Fig. 5c**). Our earlier observation that recombinant VEGF-inducible corneal angiogenesis is blocked by the Egr-1 DNAzyme (**Fig. 2d,e**) is supported by our demonstration that the DNAzyme inhibits VEGF-inducible endothelial Egr-1 and FGF-2 expression (**Fig. 5d**). Although Egr-1 is activated by exogenous VEGF, it has no influence on the *de novo* production of endogenous VEGF. This is the first demonstration of 'downstream inhibition' using a DNAzyme, whereby the expression of a given gene (such as FGF-2) is inhibited not because the DNAzyme recognizes a specific motif in the mRNA of that gene, but because expression of that gene is dependent on the DNAzyme target.

Based on these findings, we further hypothesized that tumor growth in the Egr-1 DNAzyme group could be rescued by exogenous FGF-2, as FGF-2 can activate Egr-1 (ref. 16). Bolus intratumoral administration of

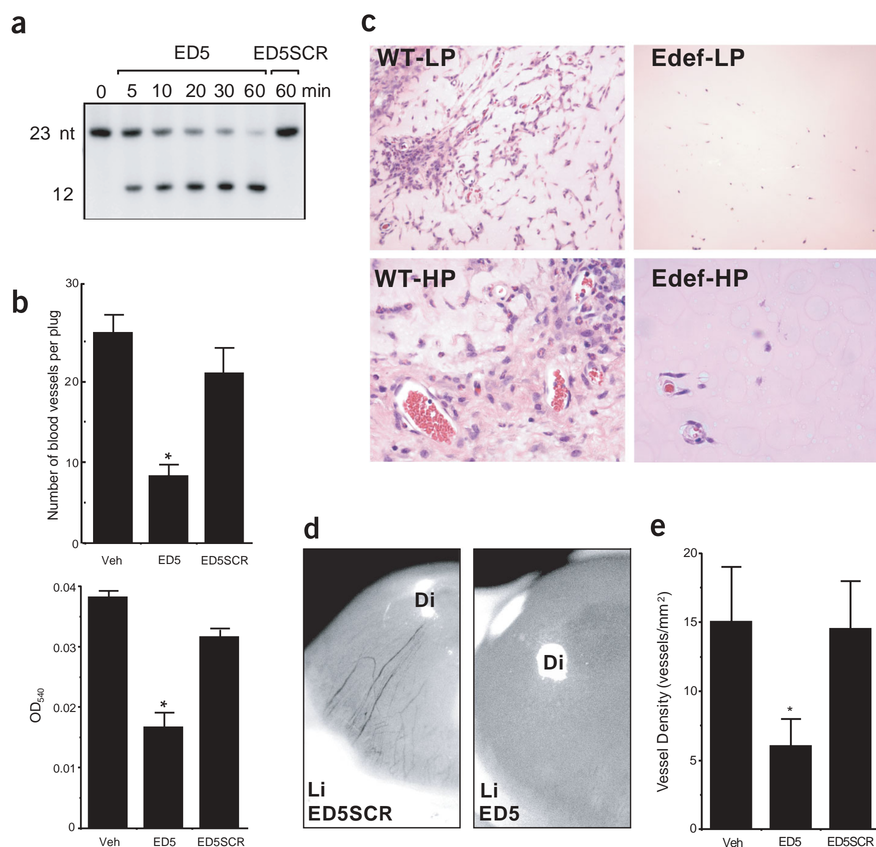

Figure 2 Egr-1 DNAzymes inhibit angiogenesis. (**a**) Cleavage of ^{32}P-labeled mouse Egr-1 RNA by ED5 but not ED5SCR. (**b**) Top, vascular density in H&E-stained cross-sections of Matrigel plugs 14 d after implantation. Bottom, assessment of hemoglobin content in plugs using Drabkin reagent. *, $P < 0.05$ relative to vehicle (Veh) by Student's *t*-test. (**c**) Representative cross-sections of H&E-stained plugs resected from wild-type (WT) and Egr-1-deficient (Edef) mice 14 d after implantation. LP, low power (×20); HP, high power (×60). Photomicrographs are representative of plug center. Erythrocyte-filled blood vessels in sections are indicated by bright red staining. (**d**) ED5 blocks neovascularization in rat corneas implanted with $VEGF_{165}$-containing filter discs, which would normally produce robust neovascular growth from the limbus toward the disc. Figure shows representative corneas 5 d after treatment with ED5 or ED5SCR. Di, disk; Li, limbus. Photographs were taken with high-resolution black-and-white film. (**e**) Number per unit and area (mm^2) occupied by new blood vessels in corneas 5 d after disk implantation and conjunctival delivery of DNAzyme or vehicle. *, $P < 0.05$ relative to DzFSCR or vehicle group (Student's *t*-test).

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Figure 3 Egr-1 DNAzyme inhibits human MCF-7 breast carcinoma growth as xenografts in nude mice through suppression of angiogenesis. (**a**) ED5 blocks solid MCF-7 tumor growth in athymic nude Balb/c mice 60 d after coadministration of 2×10^6 MCF-7 cells and DNAzyme or vehicle. Shown are gross tumors representative of ED5, ED5SCR and vehicle (Veh) groups immediately after resection. (**b**) Tumor volume (top) and body weight (bottom) after 60 d. (**c**) Cross-sectioned tumors stained with H&E. LP, low power (×20); HP, high power (×60). Representative blood vessels filled with $CD31^+$ erythrocytes are shown in bottom two panels. Arrows indicate blood vessels. (**d**) Vascular density in tumors treated with ED5, ED5SCR or vehicle. Density was calculated by quantitating blood vessels in three separate tumor cross-sections per group. (**e**) DNAzyme was administered intratumorally twice a week immediately after assessment of tumor volume and body weight. *X* axis denotes time since implantation of tumor suspension. Arrow indicates start of DNAzyme therapy. (**f**) DzF, but not ED5, inhibits MCF-7 cell proliferation. DNAzymes were used at 0.4 µM. *, $P < 0.05$ relative to control by Student's *t*-test.

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FGF-2 produced rapid solid tumor regrowth in the Egr-1 DNAzyme group but only modest growth in the scrambled DNAzyme group (**Fig. 5e**). FGF-2 also reversed DNAzyme inhibition of microvascular endothelial cell growth *in vitro* (**Fig. 5e**). Thus, Egr-1 DNAzyme inhibition of endothelial growth and tumor angiogenesis is reversible and mediated by FGF-2. That angiogenesis within FGF-transfected solid MCF-7 tumors is not associated with any increase in VEGF expression[21] supports these data.

To complement these findings, we evaluated the effect of ED5 on solid B16 tumor (malignant melanoma) growth. B16 growth and metastasis in mice seems to be critically dependent on the VEGF/VEGF receptor[27–29] but not the FGF-2/FGF receptor system[30]. We therefore hypothesized that ED5 would have no effect on B16 growth. Indeed, we found that B16 solid tumor growth was not influenced by ED5 or ED5SCR (**Fig. 6a**). In addition, the growth rate of B16 tumors was identical between wild-type and Egr-1-deficient mice (**Fig. 6b**). These findings further support the involvement of FGF-2, but not VEGF, in Egr-1 DNAzyme inhibition of tumor growth. A549 xenograft growth, like MCF-7 growth, was inhibited by the Egr-1 DNAzyme (data not shown) and is dependent on both FGF-2 (ref. 31) and VEGF (ref. 32).

DISCUSSION

This study provides the first evidence of the crucial role of Egr-1 in microvascular endothelial cell growth, neovascularization, tumor angiogenesis and tumor growth, with complementary analyses from our use of Egr-1 DNAzymes *in vitro* and *in vivo*, and Egr-1-deficient mice. DNAzymes targeting a given transcription factor may be useful in the identification of dependent genes, as the expression of FGF-2, an Egr-1-dependent gene whose mRNA was not the intended target, was strongly inhibited by the DNAzyme. Because Egr-1 controls the expression of other regulatory genes[33,34] strongly implicated in angiogenesis, such as transforming growth factors, platelet-derived growth factors, matrix metalloproteinases and even Flt-1 (VEGFR-1), strategies specifically targeting this master regulator may potentially be useful as clinical inhibitors of tumor growth. The possibility of unexpected toxicity could be minimized by local administration. The intratumoral route has received renewed attention in high-mortality cancers amenable to local delivery, such as breast, colorectal and lung cancers. There have been recent major advances in delivery modalities, including controlled release, preoperative chemotherapy and new drugs, thereby complementing the obvious advantages (higher local drug concentrations, reduced toxicity) of regional as opposed to systemic therapy[35].

In the clinical setting, an Egr-1 DNAzyme may inhibit both endothelial and tumor growth if Egr-1 is expressed in that tumor, just as Flt-1, the target of ANGIOZYME[13,36,37] (a 35-nucleotide modified ribozyme

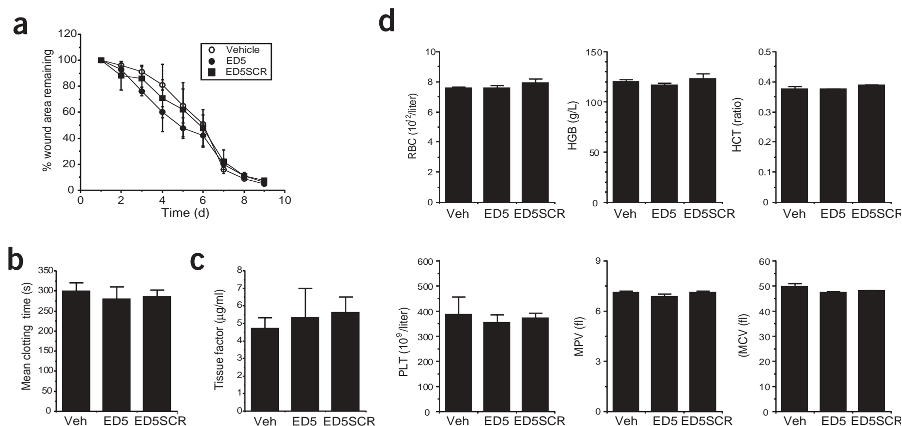

Figure 4 Intratumoral administration of Egr-1 DNAzyme has no adverse effect on wound healing or hemostasis. (**a**) Tumor-bearing athymic Balb/c nude mice receiving DNAzyme or vehicle twice a week were wounded by skin incision at day 22. *Y* axis indicates percent wound area (by digital caliper assessment) per mouse remaining as a function of time (*x* axis). (**b**) Mean clotting times of whole blood collected from DNAzyme-treated tumor-bearing mice as determined by coagulometry. (**c**) Concentration of tissue factor in plasma of tumor-bearing mice treated with DNAzyme or vehicle. (**d**) Hematological parameters in whole blood from tumor-bearing mice treated with DNAzyme or vehicle. RBC, erythrocytes; HBG, hemoglobin; HCT, hematocrit; PLT, platelets; MPV, mean platelet volume; MCV, mean cell volume; Veh, vehicle.

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currently in phase 2 clinical trials for several tumor types) is expressed not only in endothelial cells, but also in a wide variety of tumors[38,39]. This may provide a therapeutic advantage for gene-specific inhibition of Egr-1-dependent growth. Our studies, nonetheless, indicate that DNAzymes targeting human Egr-1 are at least fourfold more potent inhibitors of microvascular endothelial cell proliferation than of breast carcinoma cell proliferation. DNAzymes also closely parallel antiangiogenic synthetic oligodeoxynucleotides such as Macugen (EYE001), a 28-nucleotide aptamer[40] currently in phase 2 and 3 trials for age-related macular degeneration and phase 2 trials for diabetic retinopathy. DNAzymes have inherent physicochemical advantages over these nucleic acid–based agents, such as higher target site flexibility, minimal need for modification (phosphodiester), lower fragility and low cost of synthesis[41], suggesting that DNAzymes may well be a clinically appealing new class of therapeutic drug.

METHODS

Cell culture. Human microvascular endothelial cells-1 (HMEC-1) were cultured in MCDB131 with 10% FBS, L-glutamine (2 mM), hydrocortisone (1 µg/ml) and epidermal growth factor (10 ng/ml). MCF-7 breast carcinoma cells were cultured in RPMI with 10% FBS. Mouse brain microvascular endothelial cells (bEND-3) were cultured in DMEM with 10% FBS. FuGENE6 (Roche) was used for transfection; we consistently achieved >55% efficiency.

DNAzymes. DNAzymes were synthesized with a 3′-linked inverted T at the 3′ termini (Tri Link), conferring greater stability against nucleolytic degradation[42]. Reactions were performed in a 14-µl volume containing 10 mM $MgCl_2$,

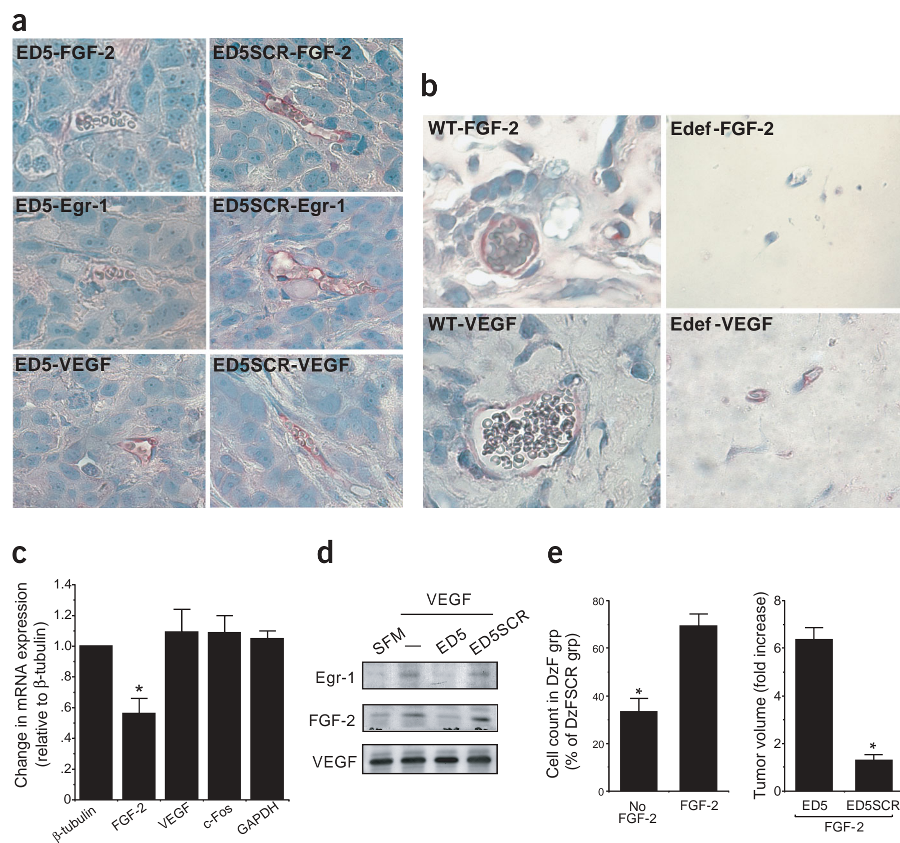

Figure 5 Egr-1 DNAzyme inhibition is mediated by Egr-1-dependent FGF-2 expression. (**a**) Immunohistochemical detection of FGF-2 and VEGF in cross-sections of MCF-7 tumors treated with ED5 or EDSCR. (**b**) Immunohistochemical detection of FGF-2 and VEGF in cross-sections of plugs resected from wild-type (WT) and Egr-1-deficient (Edef) mice 14 d after implantation. (**c**) Comparative microarray analysis using reverse-transcribed cDNA from human microvascular endothelial cells treated with DNAzyme and serum. *Y* axis shows DzF/DzFSCR ratio after normalization of DzF and DzFSCR values for each gene to β-tubulin. (**d**) VEGF-inducible Egr-1 and FGF-2 expression in mouse microvascular endothelial cells is blocked by ED5 but not ED5SCR. (**e**) Exogenous FGF-2 rescues microvascular endothelial cell growth and solid tumor growth from DNAzyme inhibition. Left, FGF-2 was added to cells just before a second DzF or DzFSCR transfection, producing a twofold increase in cell number in the DzF group (grp) after 48 h. *Y* axis represents population of cells in DzF group as a proportion of DzFSCR group. Right, FGF-2 was administered intratumorally to MCF-7 tumors treated with ED5, ED5SCR 60 d after tumor injection. *Y* axis represents fold induction in tumor volume after 7 d. *, $P < 0.05$ relative to DzFSCR (Student's *t*-test). SFM, serum-free medium.

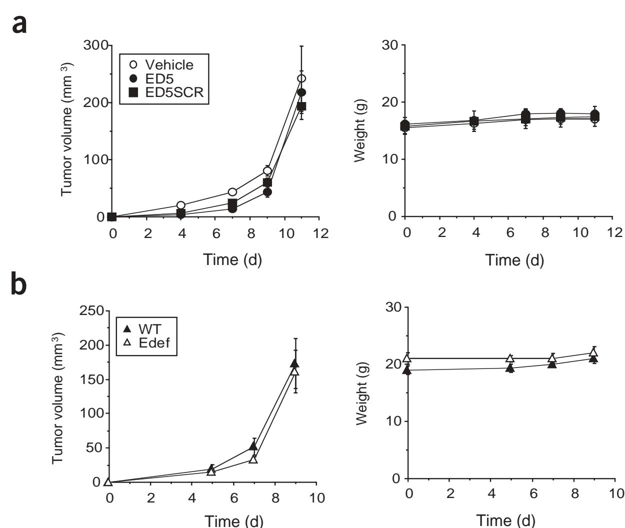

Figure 6 Solid B16 tumor growth in mice is Egr-1-independent. (**a**) Effects of ED5, ED5SCR or vehicle on subcutaneous B16 tumor growth. (**b**) Subcutaneous B16 tumor growth in wild-type and Egr-1-deficient mice. Tumor volume was assessed from caliper measurements.

5 mM Tris (pH 7.5), 150 mM NaCl, 1 μM [^{32}P]RNA substrate and 20 μM DNAzyme (1:20 substrate/DNAzyme ratio). Sequences were as follows: DzF, 5′-GCGGGGACAGGCTAGCTACAACGACAGCTGCATTi-3′; DzFSCR, 5′-GGA GCTGACGGCTAGCTACAACGAGATCGACGCTi-3′; DzA, 5′-CAGGGGA CAGGCTAGCTACAACGACGTTGCGGGTi-3′; $β_1$Dz ($β_1$ integrin DNAzyme), 5′-CAAGGTGAGGGCTAGCTACAACGAAATAGAAGTi-3′; $β_3$Dz ($β_3$ integrin DNAzyme), 5′-GAGTCCCATAGGCTAGCTACAACGAAAGACTTGAGTi-3′; $β_1$DzSCR (scrambled $β_1$ integrin DNAzyme), 5′-GCGAAGTGAGGCTAGCTA CAACGAGTAAAGTATi-3′; $β_3$DzSCR (scrambled $β_3$ integrin DNAzyme), 5′-CTAAGATCGCGGCTAGCTACAACGATGATGAGACATi-3′; ED5, 5′-CCGCT GCCAGGCTAGCTACAACGACCCGGACGTTi-3′; ED5SCR, 5′-GCCAGCCG CGGCTAGCTACAACGATGGCTCCACTi-3′; where Ti is a 3′ 3′-linked inverted T.

***In vitro* scraping.** Cells in eight-well chamber slides (Nunc) were transfected with 0.1 μM DNAzyme. After a second transfection, cells were injured by a deliberate scratch. Forty-eight hours after injury, cells were washed in PBS, fixed in 4% paraformaldehyde (vol/vol) and stained with H&E before photomicroscopy. Cell numbers in the denuded zone of each group were determined in triplicate in a blinded manner.

Western blotting. Cells in 100-mm plates were transfected with 0.1 μM DNAzyme. One hour after the second transfection in 5% FBS, 5 μg total lysate was immunoblotted with rabbit polyclonal antibody to Egr-1, rabbit polyclonal antibody to FGF-2, rabbit polyclonal antibody to Sp1 and rabbit polyclonal antibody to VEGF, with appropriate secondary antibodies and chemiluminescence (NEN).

Microarray analysis. We isolated mRNA from subconfluent HMEC-1 cells previously transfected with DNAzyme using poly(A)Quik mRNA isolation columns (Stratagene). We synthesized and hybridized cDNA probes to Atlas Human Cancer cDNA Array membranes before washing, phosphoroscreen exposure and analysis using AtlasImage (Clontech).

Cell proliferation. Cells were seeded into 96-well plates (TPP; 5,000 cells/well for HMEC-1, 5,000 cells/well for MCF-7 and 6,000 cells/well for bEND-3, in 10% FBS). After two DNAzyme transfections, cells were trypsinized and resuspended in 10 ml of Isoton II. Cells were counted using a Coulter counter.

Microtubule formation. HMEC-1 or bEND-3 cells in 100-mm plates were arrested in serum-free medium for 6 h before being transfected with DNAzyme. This pretransfection strategy was used for evaluation of Egr-1 and β-integrin DNAzymes as inhibitors of tubule formation[10]. Cells were trypsinized 18 h after transfection and 2×10^5 HMEC-1 or 2×10^5 bEND-3 cells were seeded into 96-well plates coated with 100 μl Matrigel (BD Biosciences). Alternatively, where indicated, DNAzyme was administered to pre-existing tubules (1 h after HMEC-1 cells were plated) with FuGENE6. Microtubules were quantitated by microscopy in a blinded manner.

Immunohistochemistry. Immunostaining was done using antibodies indicated as described[43].

Plug assay. All animal experimentation was approved by the University of New South Wales Animal Ethics Committee. C57BL/6 mice (6–8 weeks old) were injected subcutaneously (right flanks) with 515 μl of a solution containing Matrigel, FuGENE6 (2.5 μl), FGF-2 (0.5 μg total) and DNAzyme (750 μg). After 14 d, plugs were resected and fixed in paraformaldehyde, and 5-μm sections were stained with H&E. Alternatively, plugs were shaken overnight in water before 1 h of incubation with an equal volume of Drabkin reagent (Sigma) and colorimetric assessment at A540. In Egr-1-deficient mice or age-matched C57BL/6 controls, mice were injected with 500 μl of a solution containing Matrigel and FGF-2 (0.5 μg) and plugs were resected after 14 d.

Corneal neovascularization. Corneal surgery, intraconjunctival administration of DNAzyme and angiogenesis quantitation were done essentially as described[13]. Corneas of 7-week-old Sprague-Dawley rats were implanted with nitrocellulose disks (0.57 mm) soaked previously for 30 min with 1 μl of 30 μM $VEGF_{165}$ in 82 mM Tris-HCl (pH 6.9). DNAzyme (100 μg) or vehicle was administered into the conjunctiva adjacent to the disk after implantation. Five days after implantation, corneas were removed and photographed, and vascular densities were determined under microscopy.

Solid tumor models. Athymic Balb/c nude mice (6–8 weeks old) were injected subcutaneously with 2×10^6 logarithmically growing MCF-7 cells in 250 μl containing FGF-2 (0.5 μg), $MgCl_2$ (1 mM) and DNAzyme (750 μg). After 60 d, paraformaldehyde-fixed sections were stained with H&E or antibody to CD31. In the B16F10 xenograft studies, 5×10^5 B16 cells were injected subcutaneously, along with 750 μg DNAzyme and 200 μl Matrigel, into the dorsal midback region of 5-week-old C57BL/6 mice. Tumor volumes (mm^3) were determined using length × width × height × 0.52 after caliper measurement. Where indicated, we administered DNAzyme intratumorally. A 200-μl volume of MCF-7 human breast carcinoma cells (5×10^6) in Matrigel (50% vol/vol) were subcutaneously injected into the right dorsal region of Balb/c nude mice. A 17-β-estradiol pellet (0.72 mg/pellet; Innovative Research) was inserted subcutaneously into the left dorsal region, using a ten-gauge bevel, 2 cm from the site of injection. Fifteen days later, tumor-bearing mice were assigned randomly into three groups and injected intratumorally twice weekly with 20 μl of DNAzyme (20 μg), $MgCl_2$ (1 mM), FuGENE6 (1 μl) and DMEM (17.2 μl), or with vehicle alone.

Wound healing. The dorsal regions of anesthetized, tumor-bearing mice were wounded using a ten-gauge bevel. Intratumoral injections were continued as wound healing was monitored as a function of time. Wound areas (width × length) were measured by calipers and expressed as a percentage of initial wound area[44].

Blood coagulation and counts. Whole blood was collected by cardiac puncture into tubes containing 3.8% sodium citrate at a ratio of five parts blood to one part sodium citrate. Hematologic measurements were done by automated Sysmex (XE2100). Clotting times were determined by automated coagulometer (Diagnostica Stago) standardized for tissue factor procoagulant activity[45].

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COMPETING INTERESTS STATEMENT

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