MOLECULAR AND CELLULAR BIOLOGY, Aug. 2010, p. 3902–3913 Vol. 30, No. 15
0270-7306/10/$12.00 doi:10.1128/MCB.01237-09

Regulation of the Expression and Activity of the Antiangiogenic Homeobox Gene *GAX/MEOX2* by ZEB2 and MicroRNA-221▽

Yun Chen,[1]† Malathi Banda,[3] Cecilia L. Speyer,[3] Jennifer S. Smith,[3] Arnold B. Rabson,[2] and David H. Gorski[3]*

Division of Surgical Oncology, UMDNJ-Robert Wood Johnson Medical School, Cancer Institute of New Jersey, 195 Little Albany St., New Brunswick, New Jersey 08901[1]; Departments of Molecular Genetics, Microbiology, and Immunology and Pathology and Laboratory Medicine, UMDNJ-Robert Wood Johnson Medical School, Cancer Institute of New Jersey, 195 Little Albany St., New Brunswick, New Jersey 08901[2]; and Breast Cancer Biology Program, Barbara Ann Karmanos Cancer Institute, and Department of Surgery, Wayne State University School of Medicine, 4100 John R St., Detroit, Michigan 48201[3]

Received 14 September 2009/Returned for modification 21 October 2009/Accepted 17 May 2010

Tumors secrete proangiogenic factors to induce the ingrowth of blood vessels from the stroma. These peptides bind to cell surface receptors on vascular endothelial cells (ECs), triggering signaling cascades that activate and repress batteries of downstream genes responsible for the angiogenic phenotype. To determine if microRNAs (miRNAs) affect regulation of the EC phenotype by *GAX*, a homeobox gene and negative transcriptional regulator of the angiogenic phenotype, we tested the effect of miR-221 on *GAX* expression. miR-221 strongly upregulated GAX, suggesting that miR-221 downregulates a repressor of GAX. We next expressed miR-221 in ECs and identified ZEB2, a modulator of the epithelial-mesenchymal transition, as being strongly downregulated by miR-221. Using miR-221 expression constructs and an inhibitor, we determined that ZEB2 is upregulated by serum and downregulates GAX, while the expression of miR-221 upregulates GAX and downregulates ZEB2. A mutant miR-221 fails to downregulate ZEB2 or upregulate *GAX*. Finally, using chromatin immunoprecipitation, we identified two ZEB2 binding sites that modulate the ability of ZEB2 to downregulate *GAX* promoter activity. We conclude that miR-221 upregulates *GAX* primarily through its ability to downregulate the expression of ZEB2. These observations suggest a strategy for inhibiting angiogenesis by either recapitulating miR-221 expression or inhibiting ZEB2 activation.

Interactions between a tumor and its surrounding stroma are critical to cancer progression and metastasis (8). In order to grow beyond a diameter of 1 mm, tumors must be able to induce their hosts to provide them with a blood supply. This process, tumor-induced angiogenesis, is critical to the growth and metastasis of malignant tumors, and the acquisition of the ability to induce angiogenesis, known as the "angiogenic switch," is a critical step in malignant transformation (16, 17). Multiple oncogenes and signaling pathways have been implicated in the acquisition of the angiogenic phenotype (1, 3–5, 12), but most have one feature in common, namely, that they result in tumor cells secreting proangiogenic factors. At the vascular endothelial cell (EC) level, the process of angiogenesis involves complex temporally coordinated changes in global gene expression in response to alterations in the balance between pro- and antiangiogenic factors (2, 32). Proangiogenic factors such as vascular endothelial growth factor (VEGF) bind to cell surface receptors and activate signaling pathways, the end result of which is the binding of transcription factors to promoters of genes whose modulation is necessary for the angiogenic phenotype.

One potential regulator of the angiogenic phenotype is the class of RNA molecules known as microRNAs (miRNAs). These short (~22-nucleotide) noncoding RNAs function in general as inhibitors of protein expression and have been implicated in many human diseases, including cancer, heart disease, psoriasis, and neurodegenerative disorders (20, 25, 30, 41, 42). Recently, they have also been implicated in the regulation of angiogenesis. For example, knocking out Dicer, the rate-limiting enzyme controlling the maturation of miRNAs, results in embryonic lethality, likely due to impaired embryonic angiogenesis (22, 43). Consistent with this result, depleting Dicer in human umbilical vein endothelial cells (HUVECs) and microvascular endothelial cells results in impaired migration and capillary formation on the reconstituted basement membrane (33, 34). In addition, specific miRNAs have been implicated in regulating angiogenesis. For example, miR-221 inhibits EC proliferation and migration (35) while miR-130a promotes the angiogenic phenotype in ECs by downregulating expression of the homeobox proteins HOXA5 and GAX, both of which inhibit EC activation (6, 28).

Of the two corresponding homeobox genes, *GAX* (also known as *MEOX2*) (10, 11), possesses characteristics suggesting that it is a possible master regulatory gene controlling the angiogenic phenotype in ECs. The *GAX* gene encodes a homeodomain-containing transcription factor which we originally isolated from a rat aorta cDNA library (10) but later

* Corresponding author. Mailing address: Breast Cancer Biology Program, Barbara Ann Karmanos Cancer Institute, and Department of Surgery, Wayne State University School of Medicine, 4100 John R St., Detroit, MI 48201. Phone: (313) 576-8298. Fax: (313) 576-9322. E-mail: gorskid@karmanos.org.

† Present address: Center for Pharmacogenomics, Washington University in St. Louis, 660 S. Euclid, Campus Box 8220, St. Louis, MO 63110.

▽ Published ahead of print on 1 June 2010.

found to be expressed in vascular ECs as well (9). *GAX* expression, maximal in quiescent vascular cells but rapidly downregulated in response to serum (9, 26) or proangiogenic and proinflammatory factors (26), inhibits EC proliferation (9, 26) and angiogenesis in both *in vitro* and *in vivo* models (26). In fibroblasts, its expression induces INK4a-dependent senescence (19). Two mechanisms through which GAX exercises its activities have been implicated in vascular ECs. First, GAX upregulates $p21^{WAF1/CIP1}$ expression by binding directly to its promoter (7, 31) and to an enhancer located 15 kb upstream from the $p21^{WAF1/CIP1}$ gene transcriptional start site (7), inhibiting EC proliferation and maintaining the cell in G_0/G_1. The second mechanism is that GAX expression downregulates NF-κB activity (26). Given these activities, we initially predicted that, if *GAX* were indeed a master regulatory gene, its levels should be increased by miR-221 relative to those induced by miR-130a, the latter of which downregulates *GAX* (6). We further hypothesized that, because miR-221 expression would be more likely to result in the upregulation rather than the downregulation of GAX, it would have to accomplish this either through a mechanism similar to that of miR369-3, which, depending on cell cycle, can either repress or activate transcription (40), or through the downregulation of a negative regulator of *GAX*.

Here, we present evidence supporting the hypothesis that miR-221 expression does indeed upregulate GAX expression and that it does so by downregulating an inhibitor of GAX expression. In addition, we provide evidence suggesting that the intermediary gene responsible is *SIP1*/*ZEB2*, which encodes a member of the δEF-1, or ZEB, family of two-handed zinc finger nuclear factors, whose members are characterized by a homeodomain flanked by two separate, highly conserved zinc finger clusters (29, 39). Knowing that ZEB2 acts primarily as a transcriptional repressor (38), we identified and confirmed multiple ZEB2 binding sites that mediate the downregulation of GAX. Although ZEB2 has been implicated in regulating the epithelial-to-mesenchymal transition (EMT) in other cell types including various epithelial malignancies (37), it has not, to our knowledge, been implicated in regulating the EC phenotype during angiogenesis. Our observations suggest not only that is ZEB2 a direct target of miR-221 but also that its downregulation by miR-221 leads to the upregulation of GAX expression, resulting in the inhibition of EC proliferation, migration, and angiogenesis. These observations further suggest that ZEB2 might serve as a molecular target for the antiangiogenic therapy of cancer and other diseases or disorders whose pathophysiology is driven by excessive angiogenesis.

MATERIALS AND METHODS

Cells and cell culture. Human umbilical vein endothelial cells (HUVECs) and EGM-2 medium were obtained from BioWhittaker (Walkersville, MD), and cells were cultured according to the manufacturer's instructions (6, 7, 9, 26).

Plasmid, adenoviral, and retroviral constructs. (i) *GAX*. The construction of the Flag-tagged GAX expression vector (pcDNA3.1-*GAX*) has been described before (7). In addition, serial fragments upstream of the *GAX* promoter were isolated from HUVECs by PCR and cloned upstream of luciferase in the pGL3 vector to produce pGAX-Luciferase: pGL3-*GAX1* (bp −8472 to +13), pGL3-*GAX2* (bp −8472 to +13, bp −7137 to −3293 deleted), pGL3-*GAX3* (bp −2206 to +13), pGL3-*GAX4* (bp −2206 to +13, bp −1902 to −1269 deleted), and pGL3-*GAX5* (bp −882 to +13). pGL3-*GAX3* contains three SIP1/ZEB2 binding consensus sequences and was further mutated by mutagenesis PCR. All plasmid inserts were sequenced completely, and protein expressions were verified by Western blotting.

(ii) *ZEB2*. The ZEB2 cDNA containing the 3′ untranslated region (UTR) and the miR-221 binding site contained within the 3′ UTR was amplified by PCR (sense, 5′-AAG AAT TCG ATG AAG CAG CCG AT-3′; antisense, 5′-TTC GAG CAT GGT CAT TTT CA-3′) and inserted into the pcDNA3.1 expression vector with a Flag tag at the 5′ end to add a Flag tag at the N-terminal end of the expressed protein. This expression vector was designated pcDNA3.1-*ZEB2*. Additionally, the ZEB2 coding region containing the 3′ UTR without a miR-221 binding site was amplified by PCR (sense, 5′-AAG AAT TCG ATG AAG CAG CCG AT-3′; antisense, 5′-AGT TTG GCT ACA TTT TTA TTC GAG C-3′) and inserted into the pcDNA3.1 expression vector with a sequence encoding an N-terminal Flag tag (pcDNA3.1-*ZEB2*mt). The full-length *ZEB2* 3′ UTR containing the miR-221 target sequence was cloned by PCR and inserted into the psiCHECK2 dual luciferase reporter plasmid (Promega, Madison, WI) fused in frame to the 3′ end of the cDNA encoding *Renilla reniformis* luciferase to produce psiCHECK2-*ZEB2*-3′-UTR (sense PCR primer, 5′-TGC TTG ATG GAG CAC AAG AC-3′; antisense, 5′-TTC GAG CAT GGT CAT TTT CA-3′). The control vector psiCHECK2-ZEB2-3′-UTRmt was constructed similarly using the same *ZEB2* 3′ UTR sequence, but with the miR-221 target sequence excised (sense, 5′-TGC TTG ATG GAG CAC AAG AC-3′; antisense, 5′-AGT TTG GCT ACA TTT TTA TTC GAG C-3′).

(iii) miR-221. A fragment containing miR-221 was cloned by PCR (sense, 5′-ACT TGC AAG CTG AAC ATC CA-3′; antisense, 5′-CGG TCC TTT CTC TGC ACT CT-3′) and inserted into pcDNA3.1 to produce pcDNA3.1-miR-221. To make pcDNA3.1-miR-221mt, we mutated the miR-221 core sequence in pcDNA3.1-miR-221 by using mutagenesis PCR (sense, 5′-GAT TTC TGT GTT CGT TAG GCA ACA GGC TAC CTG GAA ACA TGT TCT CC-3′; antisense, 5′-GGA GAA CAT GTT TCC AGG TAG CCT GTT GCC TAA CGA ACA CAG AAA TC-3′). In addition, we used a 2′-*O*-methyl-modified oligo-RNA antisense oligonucleotide (5′-UCU GAA AAG AGC UGA AAC CCA GCA GAC AAU GUA GCU UCG AGA UUC GUC U–3′) to inhibit miR-221 expression. For a negative control scrambled RNA oligonucleotide, we used the anti-miR negative control (Applied Biosystems, Foster City, CA).

Gene expression and inhibition assays. All transfections of HUVECs except those with the miR-221 inhibitor were carried out using Trans-IT Jurkat transfection reagent (Mirus Bio Corporation, Madison, WI) according to a modification of the manufacturer's instructions described in our previous work (6, 7). The total mass of DNA used for transfections was kept constant. To accomplish this, for transfections containing different doses of plasmid, the appropriate amount of empty vector was added to make up the difference. Additionally, for promoter assays, the dual luciferase assay (Promega, Madison, WI) with *Renilla* luciferase, under the control of the simian virus 40 (SV40) promoter (pRL-SV), was used as a control for transfection efficiency. For the miR-221 inhibitor, the 2′-*O*-methyl-modified oligo-RNA antisense oligonucleotide or a scrambled control was transfected into HUVECs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to our previously published methods for inhibiting a different microRNA (6).

Protein detection. Cell extracts from treated and control ECs were subjected to electrophoresis through 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes as previously described (6, 7, 26). GAX and ZEB2 protein levels were detected using either rabbit polyclonal anti-GAX or rabbit polyclonal anti-ZEB2 (LifeSpan Biosciences, Inc., Seattle, WA) antibodies, and levels were normalized to tubulin using monoclonal anti-α-tubulin (LifeSpan Biosciences). Bands were visualized by chemiluminescence using the ECL-Plus reagent (Amersham, Piscataway, NJ).

Real-time qRT-PCR. RNA was isolated as described previously (6, 7, 26) and then used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA was then subjected to real-time quantitative reverse transcriptase PCR (qRT-PCR) utilizing TaqMan probes to determine GAX and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels as previously described (6, 7, 26). Either a Cepheid SmartCycler thermocycler with associated SmartCycler version 2.0 software or a Bio-Rad iCycler iQTM was used, depending on the specific experiment, to analyze the data and determine the threshold count (C_T). PCR cycles started with an initial 1.5-min denaturation period at 95°C, followed by 30 to 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 20 s, and extension at 72°C for 30 s. Each sample was run in triplicate, and C_T was determined for the target gene. GAX levels were normalized to GAPDH using the $\Delta\Delta C_T$ method, as we have done previously (6, 7, 26). The Qiagen QuantiTect SYBR green PCR kit (Stratagene, CA) was used to perform the real-time PCR to detect miR-221 potential targets *HOXB5*, *HOXC10*, *ZEB2*, *HIPK1*, and *DHAC4* using *GAPDH* as the housekeeping gene control. The PCR program was 45 cycles of denaturation at 95°C for 30 s,

TABLE 1. Primer sequences for quantitative real-time PCR of putative miR-221 targets

Sequence name	Primer	
	Orientation	Sequence (5′–3′)
HoxB5	Forward	GCA GAC TCC GCA AAT ATT CC
	Reverse	TGT CCT TCT TCC ACT TCA TGC
HoxC10	Forward	AAC ATC TGG AAT CGC CTC AG
	Reverse	GTC AGC CAA TTT CCT GTG GT
ZEB2	Forward	AAT AAG GGA GGG TGG AGT GG
	Reverse	ATG CTG ACT GCA TGA CCA TC
POU3F2	Forward	ATG TGC AAG CTG AAG CCT TT
	Reverse	CTC ACC ACC TCC TTC TCC AG
HIPK1	Forward	TGT CCA AGA TCA GCA TCA GC
	Reverse	AGT GGA ATA AGG GCT GCT CA
DHAC4	Forward	AGA AGC AGC AGA TCC AGA GG
	Reverse	TGC CCT TCT CCT TGT TCT TG

TABLE 2. Primer sequences for chromatin immunoprecipitation

Sequence name	Primer	
	Orientation	Sequence (5′–3′)
ChIP primer1	Forward	CTG AGC AGC TGG TGA ACA AA
	Reverse	CAT CCG TGT GAA AGA TCG AA
ChIP primer2	Forward	TGC GAA GCC ACTA TTC TGC
	Reverse	GGG TAC AGC AAA CTG GGC TA
ChIP primer3	Forward	TAG CCC AGT TTG CTG TAC CC
	Reverse	ACC TGT CGC ATC AGG AGA AT
ChIP primer4	Forward	GGA CAC AAT CGC TTG GAA AT
	Reverse	GAA GGC GTG AGT CTG AAA GG
ChIP primer5	Forward	GGA CAC AAT CGC TTG GAA AT
	Reverse	GAA GGC GTG AGT CTG AAA GG
ChIP primer6	Forward	CCC CTG AAA GCA GTT CTC TG
	Reverse	CCA CCA CCC TCT GTC ACT TT

annealing at 60°C for 30 s, and extending at 72°C for 45 s. The primer sets used are listed in Table 1.

Migration and tube formation assays. Migration and tube formation assays were carried out as previously described (6, 7, 14). For migration assays, HUVECs were cotransfected with pcDNA3.1 control empty vector, pcDNA3.1-*GAX*, pcDNA3.1-miR-221, or pcDNA3.1-*GAX* plus pcDNA3.1-miR-221 and plated on 8.0-μm-pore-size gelatin-coated, polycarbonate membranes in 24-well plates (5×10^4/well) and migration through the membranes toward 10% fetal bovine serum (FBS) was measured after 4 h. Cells were then fixed with Diff-Quik stain (Dade Behring, Deerfield, IL) and photographed for counting five high-power fields (hpf) per well. For tube formation assays, HUVECs were cotransfected as above. After 18 h, 2×10^5 cells were plated in six-well plates coated with low growth factor Matrigel (BD Biosciences, San Jose, CA). They were then incubated overnight in the presence of serum and 10 ng/ml VEGF165 (R&D Systems, Minneapolis, MN). Under a low-power field (×50), the number of tubes, which are defined as projections that connect two cell bodies, was determined for each well for at least five fields by an observer blind to the experimental groups according to previously described protocols (6, 7, 26).

Dual luciferase reporter assays. Luciferase reporter assays were performed using the vector psiCHECK2-*ZEB2*-3′-UTR or psiCHECK2-*ZEB2*-3′-UTRmt. Cells were grown to approximately 80% confluence in 6-well plates and cotransfected with psiCHECK2-*ZEB2*-3′-UTR, psiCHECK2-*ZEB2*-3′-UTRmt, or psiCHECK2 empty vector plus 0.5 μg pcDNA3.1-miR-221 as described previously (6). Cells were incubated with the transfection reagent-DNA complex for 3 h, and then the medium was replaced with fresh EBM and supplements (Cambrex Corporation, East Rutherford, NJ) containing 0.1% FBS or 2.0% FBS overnight. Firefly and *Renilla* luciferase activities were evaluated using the dual luciferase reporter assay system (Promega, Madison, WI), with *Renilla* luciferase activity normalized to firefly luciferase activity. In the case of luciferase assays using pGAX luciferases, luciferase activity was normalized to *Renilla* luciferase activity as previously reported (6, 7).

Northern blotting. Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) and separated using 8 M urea–15% denatured polyacrylamide gel electrophoresis (PAGE), transferred to nylon membranes (Ambion, Foster City, CA), cross-linked with UV light, and then baked in a vacuum at 80°C for 1 h. Probes (miR-221, 5′-GAA ACC CAG CAG ACA ATG TAG CT-3′; U6, 5′-GCA GGG GCC ATG CTA ATC TTC TCT GTA-3′) were labeled with [γ-^{32}P]ATP, and filters were hybridized with the probe according to previously published methods (10, 31) except that UltraHyb Oligo (Ambion, Austin, TX) was used as the hybridization solution. Membranes were exposed at −80°C to Kodak BioMax MR film using an intensifying screen.

ChIP assay. Chromatin immunoprecipitation (ChIP) experiments utilizing HUVECs expressing ZEB2 and mutant ZEB2 (ZEB2mt) were carried out based on previously published methodology for GAX (7). Briefly, HUVECs in 100-mm dishes were transfected with either pcDNA3.1-ZEB2 or pcDNA3.1-ZEB2mt for 2 days and then incubated in fresh EGM-2 media (Cambrex, MD) with 0.1% FBS or 2.0% FBS overnight. After overnight incubation, the HUVECs were cross-linked with formaldehyde and the cell lysates were harvested. Protein-DNA complexes were then isolated by immunoprecipitation (IP), and the nuclei was lysed to release the chromatin. Chromatin samples were sonicated on ice to an average length of 600 bp and then pelleted by centrifugation. The supernatant was precleared with blocked protein A beads (Sigma, St. Louis, MO), and target protein-DNA complexes were immunoprecipitated by adding blocked anti-Flag antibody beads (Sigma, St. Louis, MO) to each sample. To the IP input control, blocked protein G beads were added. The antibody-protein-DNA complexes were collected by centrifugation, washed, and eluted using elution buffer (Sigma). Cross-linking was reversed with 5 M NaCl, added to a final concentration of 0.3 M, followed by heating at 65°C for 5 h. Finally, DNA was purified using a DNA gel extraction purification kit (Qiagen, Valencia, CA).

To detect the ZEB2 enrichment on chromatin upstream of *GAX*, ChIP primers (Table 2) were designed based on the SIP1/ZEB2 binding consensus sequence CACCT upstream of *GAX*. CHIP primer 2 and primer 9 were designed as negative controls to amplify fragments that did not contain the consensus sequence CACCT. Each primer set was designed to amplify approximately 200-bp fragments. Purified DNA for each ChIP assay was subjected to PCR under the following conditions: initial denaturation at 94°C for 2 min, then 35 cycles at 94°C for 30 s (denaturation), 60°C for 30 s (annealing), and 72°C for 45 s (extension). PCR products were subjected to gel electrophoresis through 2% agarose gels, and bands were visualized using ethidium bromide.

Statistics and data analysis. For all studies except the miR-221 inhibitor assays, experiments were repeated at least three times and statistical analysis was determined either by one-way analysis of variance (ANOVA) or unpaired Student's *t* test, as appropriate, depending on the number of experimental groups. For the miR-221 assays, experiments were repeated twice and statistical analysis was also performed using a one-way ANOVA.

RESULTS

miR-221 inhibits HUVEC migration and tube formation in an additive fashion with GAX. We have previously reported that GAX expression inhibits EC activation, proliferation, migration, and tube formation through its ability to induce $p21^{WAF1/CIP1}$ expression and downregulate NF-κB (7, 9, 26). Consequently, we wished to test the ability of miR-221 to inhibit migration and tube formation in HUVECs and to assess whether there was detectable synergy with GAX. To this end, HUVECs were transfected with either pcDNA3.1-*GAX*, empty vector, or an expression vector driving miR-221 expression (pcDNA3.1-miR-221), after which we measured migration through polycarbonate membranes and tube formation on reconstituted basement membrane (Fig. 1). miR-221 strongly

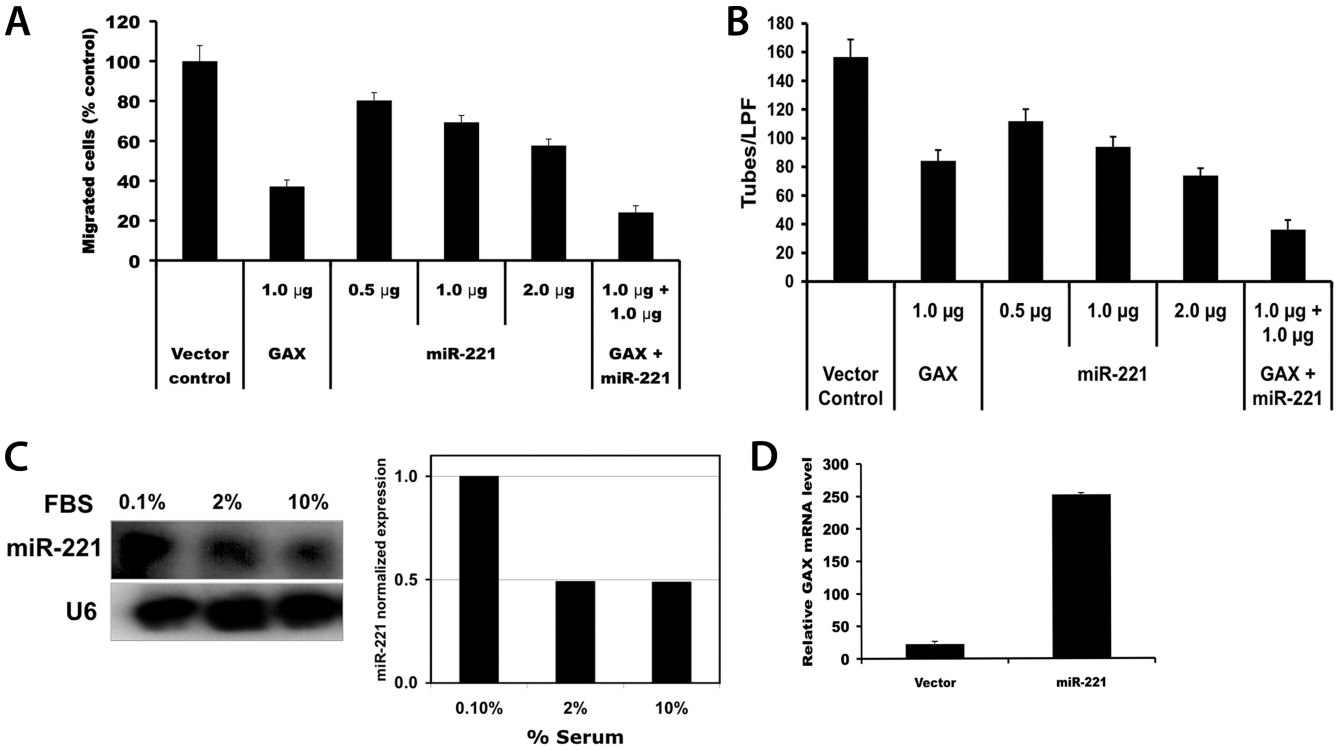

FIG. 1. miR-221 inhibits migration and tube formation in ECs and upregulates *GAX* expression. (A) GAX and miR-221 inhibit EC migration. The effects of GAX and miR-221 expression on *in vitro* measures of angiogenesis were measured. First HUVECs were transfected with either GAX, miR-221, or a combination of the two and allowed to incubate overnight, after which they were plated on 8-μm polycarbonate membranes coated with gelatin, and migration toward serum was measured as described in Materials and Methods. The percentages of cells that migrated through the polycarbonate membrane were measured as described previously (6, 7, 14). GAX, miR-221, and miR-221 plus GAX all resulted in significant decreases in migration ($P < 0.01$). (B) GAX and miR-221 inhibit EC tube formation on a reconstituted basement membrane. Similarly, the effect of expressing either GAX, miR-221, or a combination of the two was determined. HUVECs were transfected similarly with either GAX, miR-221, or a combination of the two, and tube formation on a reconstituted basement membrane was measured. GAX and miR-221 inhibited both migration and tube formation in an additive fashion. GAX, miR-221, and miR-221 plus GAX all produced significant decreases in tube formation ($P < 0.01$). (C) miR-221 expression is downregulated by serum. HUVECs were plated in various concentrations of serum and allowed to incubate overnight as described in Materials and Methods, after which they were harvested for RNA isolation. Consistent with a role in inhibiting a factor that represses GAX expression in high serum, miR-221 expression decreased markedly with increasing serum concentration. A representative experiment is shown. (D) miR-221 upregulates GAX expression. Randomly cycling HUVECs were transfected with pcDNA3.1-miR-221 or control empty vector at various doses, and RNA was harvested to measure GAX expression. miR-221 markedly upregulated GAX expression ($P < 0.001$).

inhibited not only EC migration (Fig. 1A), an effect that appeared to be additive with that of GAX, but also tube formation in a similar fashion (Fig. 1B). We conclude from this result that miR-221 appears to play an inhibitory role in EC activation during angiogenesis whose magnitude is comparable to that of the role of *GAX* and therefore wished to determine whether its expression altered *GAX* expression.

miR-221 upregulates *GAX* expression. Based on previous reports that miR-221 expression impairs postnatal angiogenesis (24, 27, 36), we were interested in determining the effect of expressing miR-221 on *GAX* regulation. Consequently, we first examined whether miR-221 expression could be downregulated by serum in HUVECs. Consistent with the ability of serum to downregulate *GAX* expression, miR-221 expression was also downregulated, by at least 2-fold, in response to increasing the serum concentrations from 0.1% to 10% (Fig. 1C). This suggests a role for miR-221 in inhibiting a putative factor that mediates the downregulation of *GAX* expression by serum stimulation. To confirm this, we transfected randomly cycling HUVECs in growth medium containing serum with pcDNA3.1-miR-221 or the control empty vector and examined *GAX* mRNA levels. Twenty-four hours after transfection, *GAX* mRNA levels were strongly upregulated (Fig. 1D), suggesting that miR-221 acts to inhibit EC migration and angiogenesis, at least in part through upregulating *GAX* expression. Based on these observations, we next hypothesized that the mechanism by which miR-221 upregulates *GAX* expression likely involves the downregulation of an intermediary protein that normally represses *GAX* expression when cells are proliferating.

miR-221 downregulates *ZEB2* expression, and ZEB2 in turn downregulates GAX expression. In an attempt to identify candidate proteins that might mediate the observed upregulation of GAX through the expression of miR-221, we next performed an *in silico* search for putative direct targets of miR-221 using the PicTar algorithm (http://pictar.mdc-berlin.de) (15, 21) and identified multiple potential targets. We decided to concentrate first on transcription factors identified, including those encoded by *HOXB5*, *HOXC10*, *ZEB2*, *DHAC4*, *POU3F2*, *HIPK1*, and *DHAC4*, postulating that any potential inhibitor of *GAX* expression downregulated by miR-221 would likely dem-

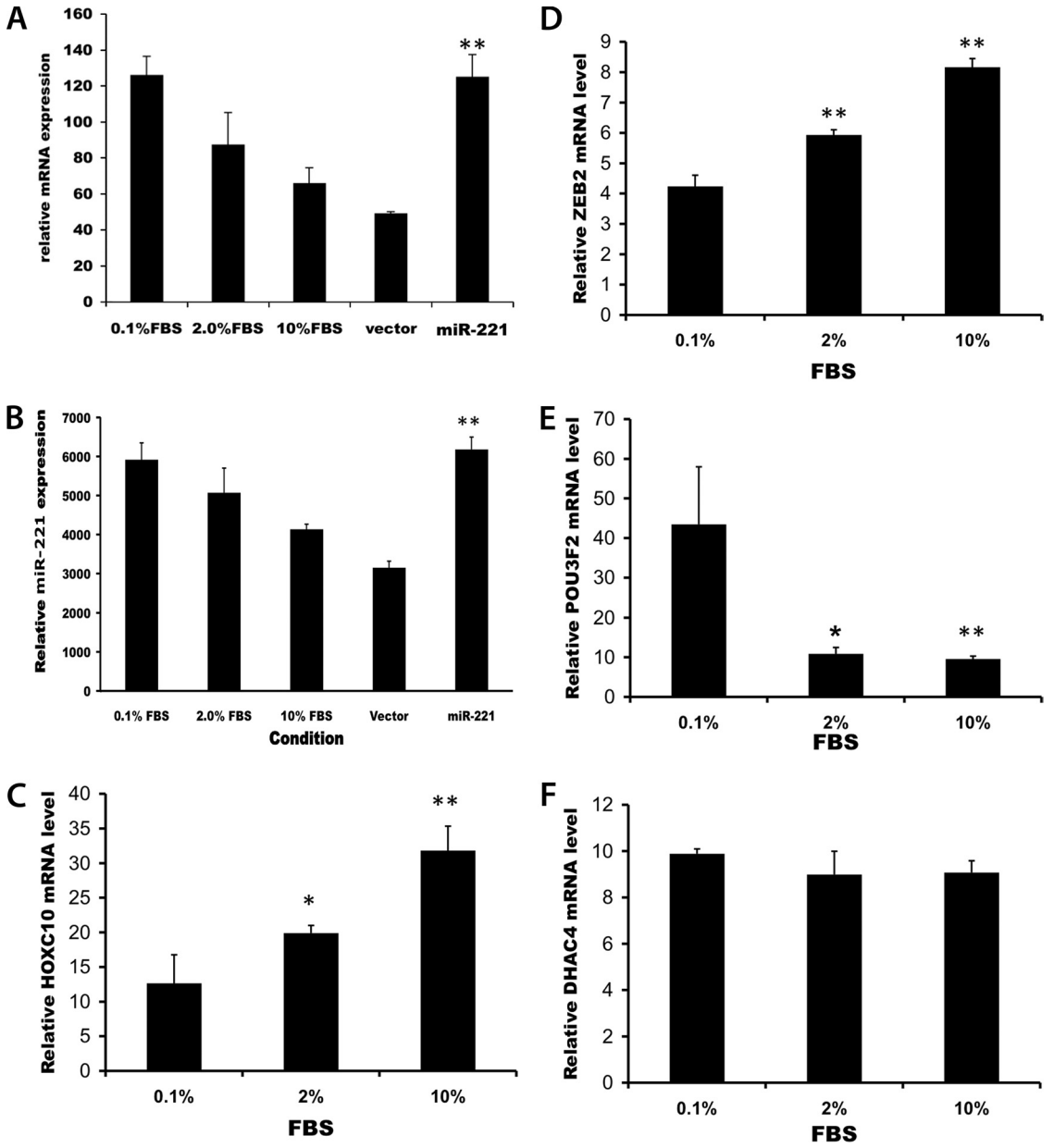

FIG. 2. Effect of serum stimulation on candidate genes that might mediate the effect of miR-221 on *GAX* expression. After performing an *in silico* search for putative direct targets of miR-221 using the PicTar algorithm as described in Materials and Methods, we identified multiple potential miR-221 targets that might mediate its ability to upregulate GAX. Randomly cycling HUVECs were exposed to various concentrations of serum overnight and harvested for RNA isolation, after which levels of various transcripts were measured by qRT-PCR. (A) GAX; (B) miR-221; (C) HOXC10; (D) ZEB2; (E) POU3F; (F) DHAC4. The experiment was repeated a total of three times. *, $P < 0.05$; **, $P < 0.01$ (all graphs). Note that, for panels A and B, levels of GAX and miR-221 from additional plates of cells treated at the same time and under the same conditions as the others have been included on the graph in order to show that the upregulation of miR-221 and GAX due to vector-driven miR-221 expression is roughly comparable to what is observed due to changes in serum concentration.

onstrate the opposite regulation in serum compared with *GAX*, i.e., would be upregulated by serum stimulation (26, 31). Consequently, these putative miR-221 targets were screened for regulation by serum (Fig. 2) and any regulation observed was compared to how serum regulates *GAX* (Fig. 2A) and miR-221 (Fig. 2B). Of the putative targets of miR-221 identified, only *HOXC10* and *ZEB2* were upregulated by serum (Fig. 2C and D, respectively) while POU3F2 was downregulated by serum (Fig. 2E). In contrast, neither *DHAC4* (Fig. 2F) nor any of the other putative targets (not shown) was detectably regulated by serum.

In order to test which of the serum-downregulated transcripts might be miR-221 targets, we cotransfected HUVECs with pcDNA3.1-miR-221 and then isolated the cells for real-time qRT-PCR for the appropriate transcript. Expanding on earlier observations, we noted that *GAX* expression was upregulated in a plasmid dose-dependent fashion (Fig. 3A), although to a somewhat lesser degree than previously observed

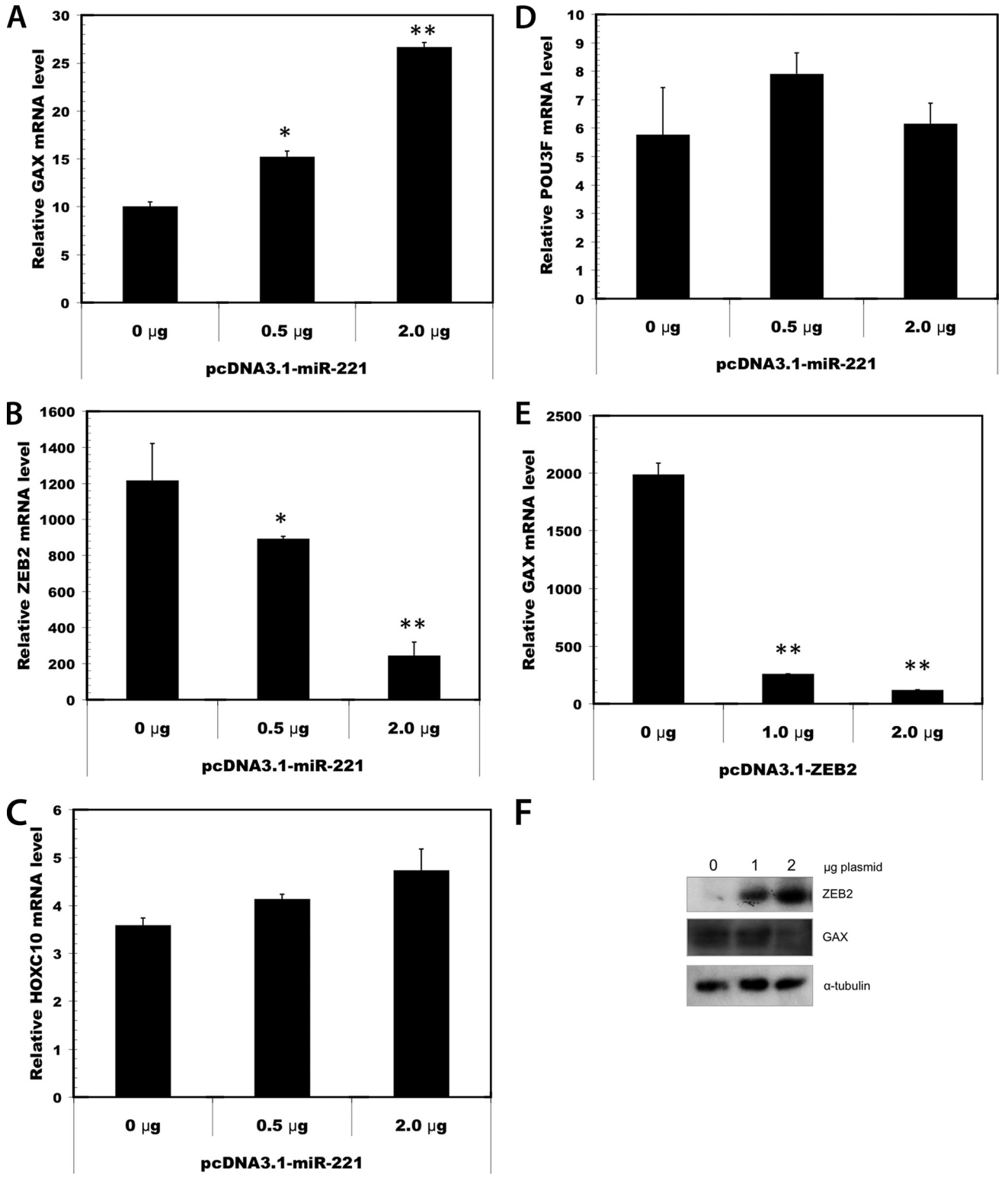

FIG. 3. miR-221 downregulates ZEB2, and in turn ZEB2 downregulates GAX expression. In order to determine whether any of the transcripts identified as having miR-221 consensus binding sites were in fact targets of miR-221, we transfected HUVECs with various amounts of pcDNA3.1-miR-221, with pcDNA3.1 empty vector control added in order to keep the plasmid mass constant in all groups. After 24 h, cells were harvested for total RNA, which was subjected to qRT-PCR. (A) miR-221 upregulates GAX expression. *, $P < 0.01$; **, $P < 0.002$ (also for panel B). (B) ZEB2 is strongly downregulated by miR-221. (C) HOXC10. (D) POU3F. Neither HOXC10 nor POU3F was regulated by miR-221. All experiments were repeated at least three times. (E) Real-time qRT-PCR for GAX mRNA expression. **, $P < 0.001$. HUVECs were transfected with a ZEB2 expression vector as described in Materials and Methods and incubated overnight, after which they were harvested for RNA and protein. (F) Western blot of protein from the same cells using the indicated antibodies.

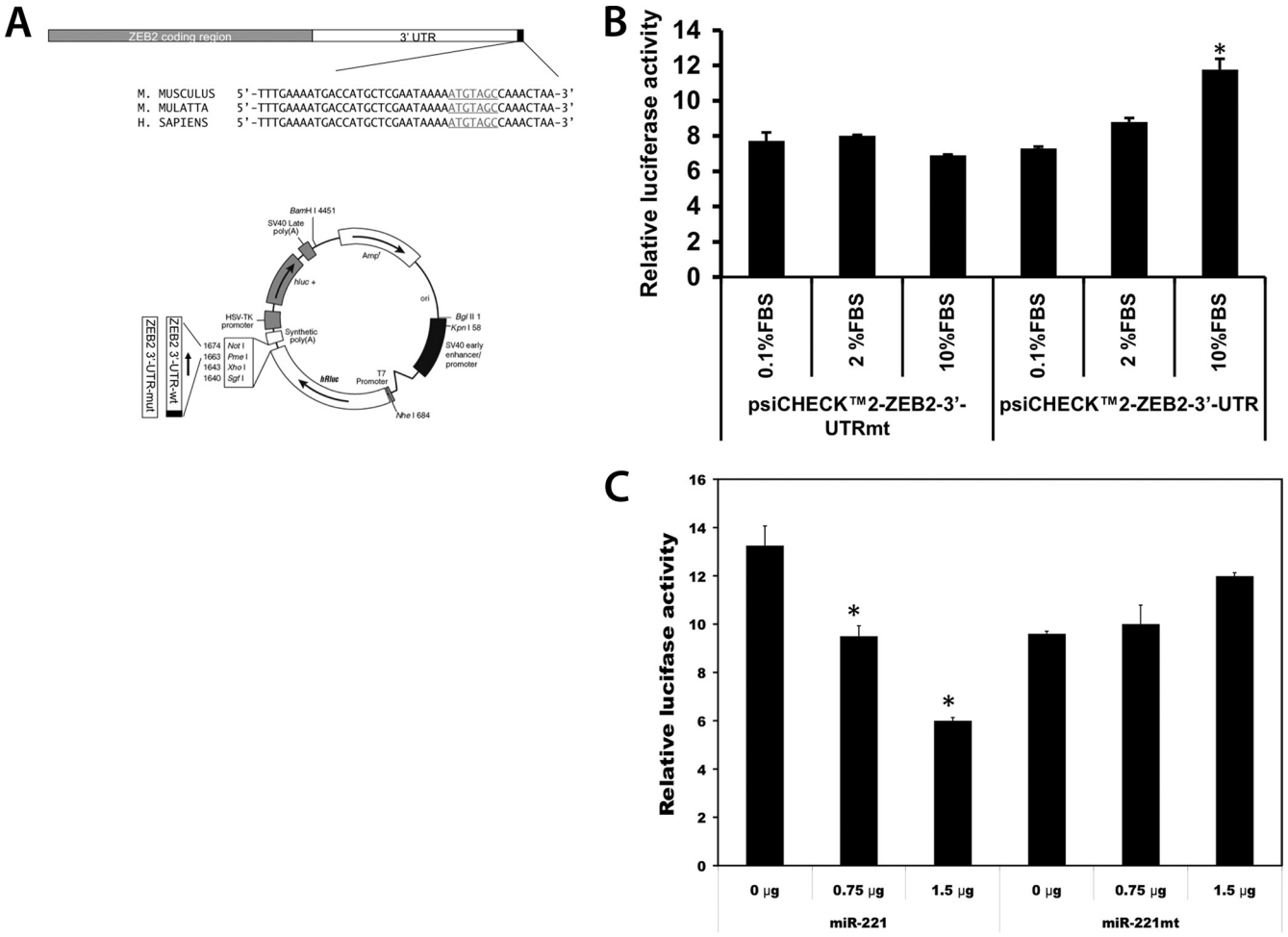

FIG. 4. The miR-221 binding site from the 3′ UTR of *ZEB2* confers serum responsiveness in HUVECs. (A) Construction of the psiCHECK2-ZEB2-3′-UTR reporter plasmid. The miR-221 consensus binding site is underlined. (B) Serum downregulates expression of the psiCHECK2-ZEB2-3′-UTR reporter. HUVECs were transfected with either psiCHECK2-*ZEB2*-3′-UTR or psiCHECK2-*ZEB2*-3′-UTRmt and then incubated for 24 h in media containing various concentrations of FBS, after which cells were harvested for the dual luciferase assay. (C) miR-221 downregulates the activity of psiCHECK2-*ZEB2*-3′-UTR. HUVECs were cotransfected with pcDNA3.1-miR-221 or pcDNA3.1-miR-221mt and psiCHECK2-*ZEB2*-3′-UTR, incubated 24 h in standard media, and then harvested for dual luciferase activity. miR-221 inhibited the activity of psiCHECK2-*ZEB2*-3′-UTR in a plasmid dose-dependent fashion, while miR-221mt did not. ∗ (B and C), $P < 0.01$.

(Fig. 1D), while *ZEB2* was strongly downregulated with increasing levels of transfected miR-221 plasmid (Fig. 3B). In contrast, neither *HOXC10* nor *POU3F2* demonstrated miR-221-dependent regulation (Fig. 3C and D). From these results, we concluded that, of these potential molecular targets of miR-221, ZEB2 was the most promising potential target of miR-221.

We next decided to test directly whether ZEB2 regulates GAX expression in ECs. HUVECs were transfected with pcDNA3.1-*ZEB2*, incubated overnight, and then harvested for isolation of RNA, which was subjected to real-time qRT-PCR (Fig. 3E), and protein, which was subjected to Western blotting (Fig. 3F). ZEB2 expression markedly downregulated *GAX* expression in a dose-dependent manner. From this we concluded that ZEB2 is likely to be an intermediary through which miR-221 exerts its effect on *GAX* expression.

miR-221 expression confers serum responsiveness to a reporter gene. Next, to determine if the putative miR-221 binding site in the 3′ UTR of the *ZEB2* cDNA might contribute to the downregulation of *ZEB2* expression, the sequence containing the miR-221 binding site was cloned into the psiCHECK2 vector at the 3′ end of the *Renilla* luciferase gene to produce psiCHECK2-*ZEB2*-3′-UTR (Fig. 4A). Similarly, a mutant *ZEB2* 3′ UTR, in which the core consensus binding sequence was deleted, was used to produce psiCHECK2-*ZEB2*-3′-UTRmt. HUVECs were transfected with either psiCHECK2 empty vector, psiCHECK2-*ZEB2*-3′-UTR, or psiCHECK2-*ZEB2*-3′-UTRmt, exposed to media containing different concentrations of serum, and incubated overnight. Luciferase activities were measured in cells transduced with psiCHECK2-*ZEB2*-3′-UTR or psiCHECK2-*ZEB2*-3′-UTRmt and normalized to those of corresponding HUVECs transduced with the empty vector to correct for transfection-related nonspecific changes in luciferase activity. Exposure to FBS resulted in a significant increase in luciferase activity in HUVECs transfected with psiCHECK2-*ZEB2*-3′-UTR but not with psiCHECK2-*ZEB2*-

3′-UTRmt (Fig. 4B), consistent with the downregulation of miR-221 expression we had observed previously (Fig. 2B). Next, we constructed pcDNA3.1-miR-221mt by mutating the core sequence as described in Materials and Methods. We then cotransfected HUVECs with psiCHECK2-ZEB2-3′-UTR and either pcDNA3.1-miR-221 or pcDNA3.1-miR-221mt. Consistent with our previous observations, psiCHECK2-*ZEB2*-3′-UTR exhibited a significant dose-dependent decrease in luciferase activity in response to miR-221 but was completely unresponsive to miR-221mt (Fig. 4C). From these results we conclude that miR-221 binds to the consensus sequence found in the 3′ UTR of *ZEB2* and can thus downregulate *ZEB2* expression in conditions of low serum.

Loss of *ZEB2* responsiveness to miR-221 attenuates the upregulation of *GAX*. In order to verify whether it is indeed *ZEB2* that mediates the ability of miR-221 to upregulate *GAX*, two mutants were tested. First, we constructed a *ZEB2* mutant lacking the miR-221 binding sequence in its 3′ UTR and placed it in pcDNA3.1 (pcDNA-3.1-ZEB2mt). HUVECs were then cotransfected with either miR-221 or miR-221mt and either *ZEB2* or *ZEB2*mt, and ZEB2 and *GAX* message levels were determined. It was observed that miR-221 downregulates *ZEB2* expression (Fig. 5A) and increases *GAX* expression (Fig. 5B), consistent with previous results. However, miR-221mt fails both to downregulate *ZEB2* (Fig. 5A) and upregulate *GAX* (Fig. 5B), strongly suggesting that it is the ability of miR-221 to downregulate ZEB2 that is responsible for the upregulation of GAX in serum. To test whether this is the case without using exogenously expressed ZEB2 or miR-221, we next incubated subconfluent HUVECs in 0.1% FBS overnight in order to maximize GAX expression and then treated them for 24 h with either a 2′-*O*-methyl-modified oligo-RNA antisense inhibitor of miR-221 or a scrambled control, both at 100 nM, after which we harvested total RNA. Inhibition of miR-221 expression was verified by Northern blotting (not shown). Consistent with our previous results, we observed that blocking miR-221 activity resulted in a significant downregulation of GAX expression comparable to that observed with 10% FBS (Fig. 5C).

ZEB2 represses *GAX* transcription through multiple upstream consensus binding sites. To understand how ZEB2 inhibits GAX expression, we employed PCR to clone 8 kb of promoter sequence upstream from the transcriptional start site of the GAX gene 5′ to the luciferase reporter gene in the pGL3 vector to produce pGL3-*GAX1*-Luc. Four additional serial deletions of the *GAX* promoter sequence based on the location of seven ZEB2/SIP1 consensus binding sites in pGL3-*GAX1*-Luc were also cloned into pGL3 (Fig. 6A). We tested which of these consensus binding sites conferred serum responsiveness to the luciferase reporter by transfecting HUVECs with this series of deletions in either 0.1% FBS, 10% FBS, or 10 ng/ml VEGF. Only transfection with pGL3-*GAX3*-Luc resulted in decreased luciferase activity in the presence of serum (Fig. 6B and C) or VEGF (Fig. 6B), suggesting that one or more of the six ZEB2/SIP1 binding sites in the GAX3 fragment (Fig. 6A) are mediators of *GAX* downregulation by ZEB2. To confirm that *ZEB2* can repress *GAX* transcription through one or more of the *ZEB2*/*SIP1* binding sites in the *GAX3* fragment, HUVECs were cotransfected with pGL3-*GAX3*-Luc and various amounts of pcDNA3.1-*ZEB2*, after

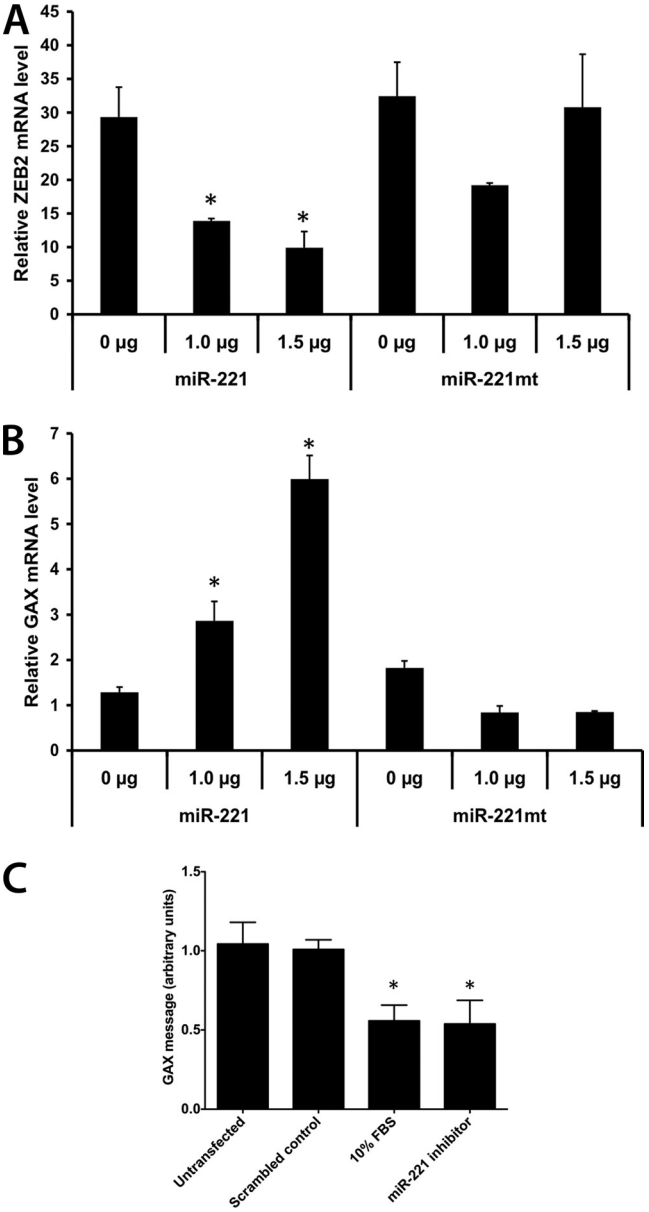

FIG. 5. Loss of ZEB2 responsiveness to miR-221 attenuates the induction of *GAX*. In order to determine whether ZEB2 can mediate the ability of miR-221 to upregulate GAX, we constructed a *ZEB2* mutant lacking the miR-221 binding sequence in its 3′ UTR and placed it in pcDNA3.1 (pcDNA-3.1-ZEB2mt). We then cotransfected HUVECs with either miR-221 or miR-221mt and either *ZEB2* or *ZEB2*mt, after which we measured both ZEB2 and GAX RNA expression. For cotransfections with *ZEB2* or *ZEB2*mt and increasing miR-221 or miR-221mt, pcDNA3.1-ZEB2 or pcDNA3.1-*ZEB2*mt plasmid mass was kept constant at 0.5 μg in order to standardize *ZEB2* expression as much as possible. (A) Effect of miR-221 and miR-221mt on ZEB2 expression. pcDNA3.1-ZEB2 (0.5 μg) was cotransfected with increasing miR-221 or miR-221mt. * (A and B), $P < 0.01$. (B) *GAX* expression using same total RNA isolates as for panel A. (C) Effect of inhibition of miR-221 on *GAX* expression. HUVECs incubated in low-serum media to maximize *GAX* expression were treated with 2′-*O*-methyl-modified oligo-RNA antisense oligonucleotides. *GAX* expression was downregulated approximately 2-fold compared to that of the scrambled oligonucleotide control. *, $P < 0.015$.

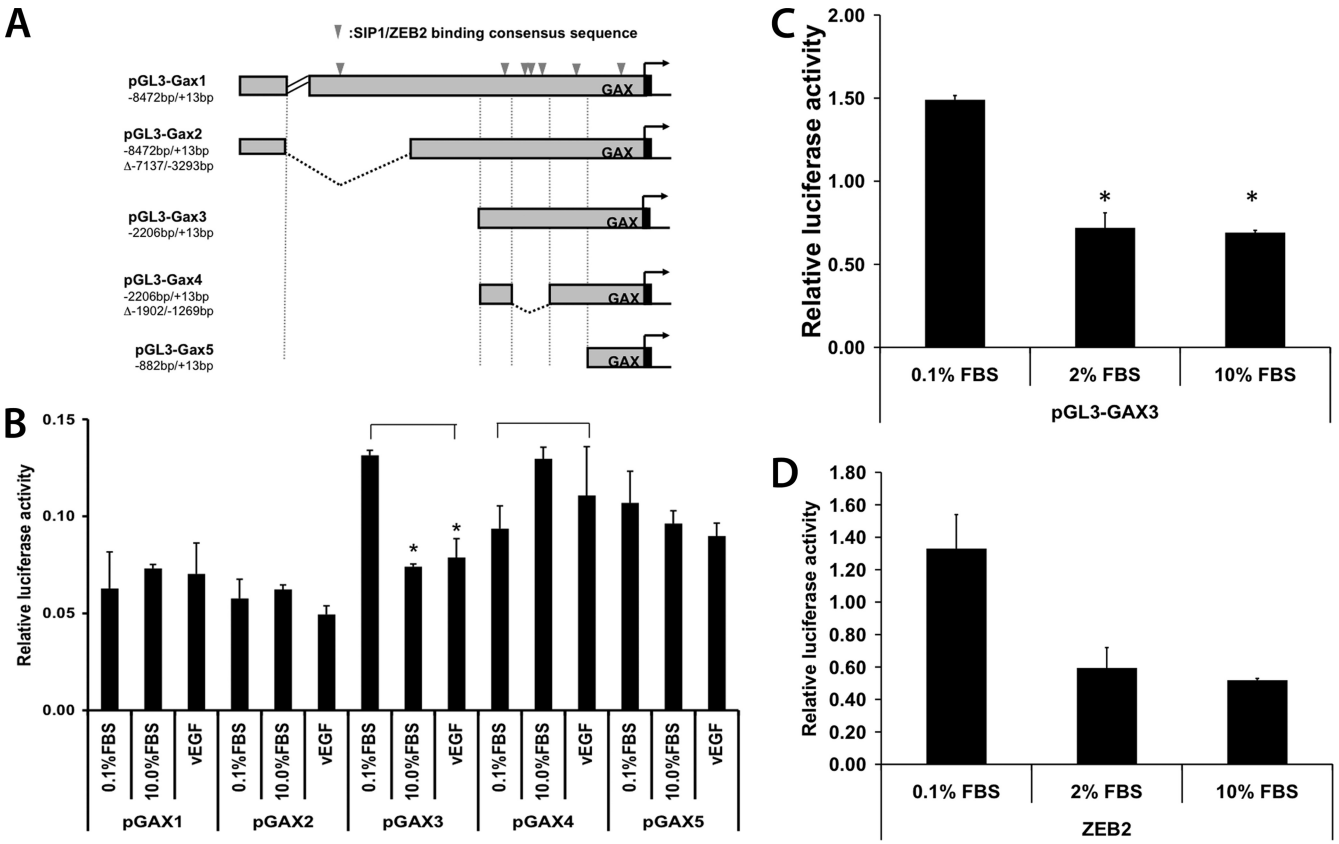

FIG. 6. ZEB2 represses *GAX* promoter activity through multiple upstream consensus binding sites. (A) *GAX* promoter constructs. To understand how ZEB2 inhibits GAX expression, we isolated serial promoter DNA fragments containing ZEB2 consensus binding sites located upstream of the *GAX* transcriptional initiation site. (B) Luciferase activity of GAX deletion mutants. Only *GAX3* demonstrated significant downregulation by serum and VEGF. * (B and C), $P < 0.01$. (C) GAX3 activity is downregulated by serum in a dose-dependent manner. Increasing serum concentrations resulted in decreasing GAX3 activity. (D) ZEB2 inhibits GAX3 activity. Cotransfection of HUVECs with pcDNA3.1-*ZEB2* and pGL3-*GAX3* resulted in a significant plasmid dose-dependent downregulation of luciferase activity.

which the cells were allowed to incubate overnight and the cell lysates were isolated for a dual luciferase assay. Consistent with the hypothesis that the binding of ZEB2 to one or more of the ZEB2/SIP1 binding sites in the GAX3 segment represses transcription, increasing *ZEB2* when cotransfected with pGL3-GAX3-Luc resulted in decreasing luciferase activity (Fig. 6D). The luciferase activity of pGL3 reporter plasmids in which GAX1, GAX2, GAX4, and GAX5 were placed upstream from luciferase was not affected by cotransfection with pcDNA3.1-*ZEB2* (not shown).

ZEB2 downregulates GAX by binding to two ZEB2/SIP1 consensus binding sites in *GAX3*. To identify which of the six candidate ZEB2 binding sites in the *GAX3* fragment ZEB2 binds in order to inhibit *GAX* promoter activity, we performed chromatin immunoprecipitation (ChIP) assays using primers sets 1 to 6 designed to span all six binding sites (Fig. 7A). Primer sets 1, 2, 5, and 6 (Table 1) demonstrated no binding of ZEB2 to the amplified segment containing the ZEB2 consensus binding sequence, while primer sets 3 and 4 bound ZEB2 by ChIP (Fig. 7B). Expressing *ZEB2* resulted in enrichment of the PCR product for primer 4 and the appearance of a new band for primer 3, suggesting that endogenous ZEB2 was binding to ZEB2 site C and possibly site B as well. To determine the functional significance of the three closely spaced ZEB2 binding sites (designated A, B, and C) located on fragment *GAX3*, we next used site-directed mutagenesis to mutate each of these three binding sites (Fig. 7C) to produce pGL3-*GAX3*mut1 through pGL3-*GAX3*mut3 reporter plasmids. Site A served as a negative control, given that there was no ChIP binding with primer set 2. HUVECs were transfected with *ZEB2* and pGL3-GAX3mut1, pGL3-*GAX3*mut2, or pGL3-*GAX3*mut3 in order to determine whether deleting any of the three ZEB2 binding sites contained therein would abrogate the downregulation of *GAX* promoter activity by ZEB2. In this experiment, pGL3-*GAX4* was used as a negative control because it contains no *ZEB2* binding sites. *ZEB2* binding site A had no effect on this downregulation, whereas deleting either site B or C resulted in the attenuation or abrogation of the downregulation of *GAX3*-dependent luciferase activity by *ZEB2* (Fig. 7D). Indeed, the deletion of site C produced a pattern of luciferase activity with increasing *ZEB2* that was virtually indistinguishable from that observed with pGL3-*GAX4*, which has no *ZEB2* binding sites.

DISCUSSION

The process of angiogenesis is critical to tumor growth beyond the limits imposed by the diffusion of nutrients and oxy-

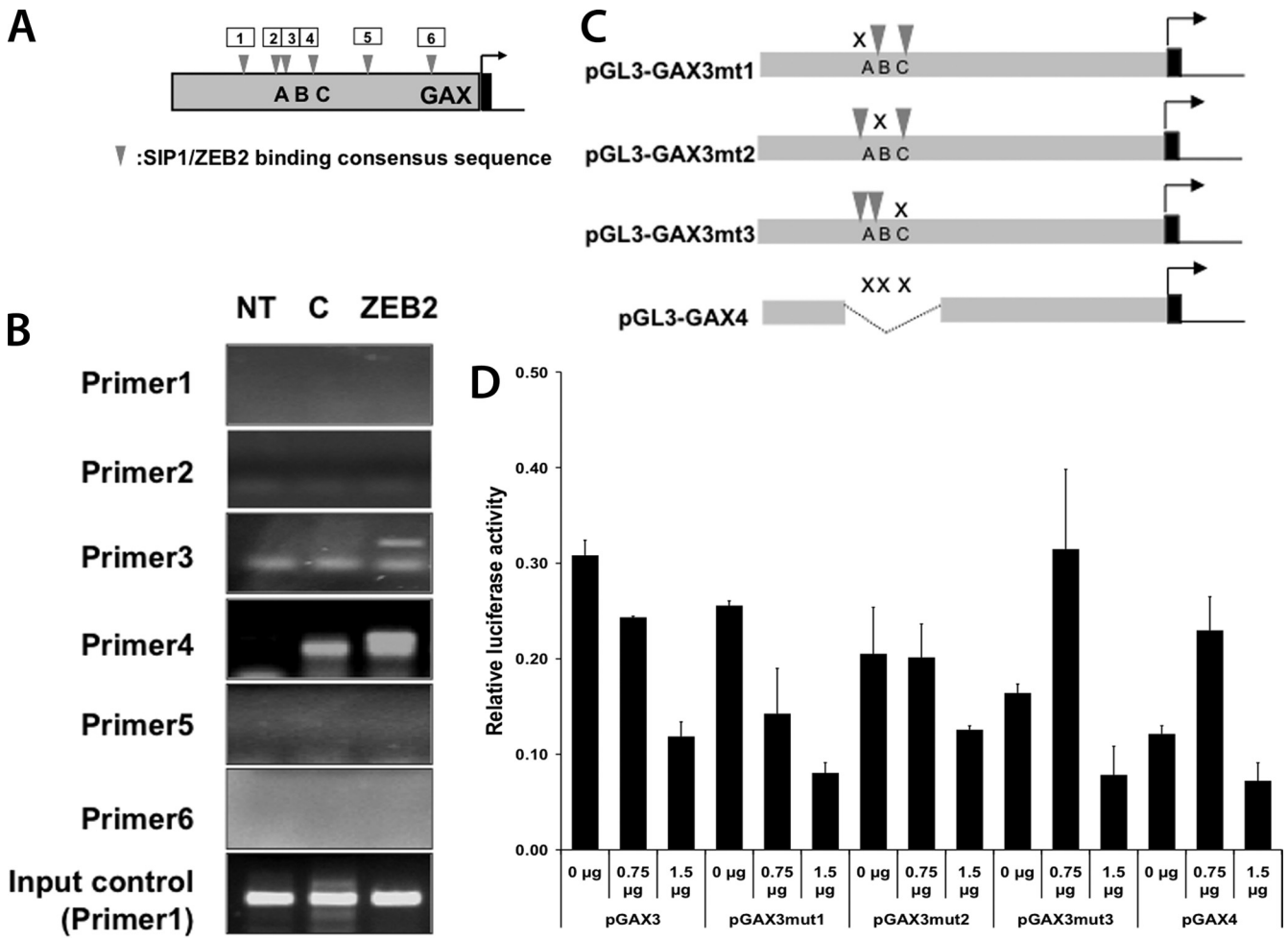

FIG. 7. ZEB2 binds to two ZEB2/SIP1 consensus binding sites in *GAX3*. ChIP experiments were performed in order to identify candidate ZEB2/SIP1 sites to which ZEB2 binds in the GAX upstream chromatin. (A) Primer set design for ChIP. Primers were designed for six ZEB2 binding sites. (B) Primer sets 3 and 4 demonstrate ZEB2 binding in ChIP. These results suggest that ZEB2 sites B and C are the sites to which ZEB2 binds (C, control, empty vector; NT, no treatment/transfection; *ZEB2*, *ZEB2* transfection.). (C) Construction of *GAX3* mutants. ZEB2 sites A, B, and C were serially deleted by site-directed mutagenesis. (D) Effect of mutating site A, B, or C on GAX3 activity. HUVECs were cotransfected with ZEB2 (0.5 μg) plus increasing amounts of the indicated pGAX3-containing mutants or pGAX4 as a negative control. Deleting site B or C on GAX3 abolished the ability of ZEB2 to downregulate GAX3 activity in cotransfection experiments.

gen in an aqueous medium (1, 3–5, 12, 16, 17). To overcome these limits, tumors acquire the ability to secrete peptides that stimulate the ingrowth of new blood vessels in order to supply themselves with oxygen and nutrients necessary for proliferation. The end target of these peptide growth factors is the vascular endothelial cell (EC), which expresses cell surface receptors that bind these peptides and thereby activate intracellular signaling cascades. In turn, these signaling cascades modulate the function of transcriptional regulators that activate and inhibit the downstream batteries of genes necessary to induce the angiogenic phenotype in ECs. The homeodomain-containing protein GAX represents one such protein that, we and others have suggested, plays a major role in regulating this transition (6, 7, 9, 10, 26). This is based on evidence from studies of ECs implicating it in inhibiting angiogenesis, NF-κB signaling (26), G_0/G_1 cell cycle arrest (7, 10, 31), transcriptional activation of INK4a (19), and upregulation of $p21^{WAF1/CIP1}$ expression through direct binding to an upstream enhancer (7).

Recently, we became interested in how GAX might be regulated by microRNAs. Specifically, we noted that there were two binding sites for miR-130a in the 3′ UTR of *GAX*, which led us to test whether miR-130a regulated *GAX* and thereby influenced the angiogenic phenotype of ECs (6). We found that miR-130a did indeed downregulate *GAX* and that miR-130a's induction by serum and proangiogenic factors implicated it as a candidate to mediate the rapid downregulation of *GAX* expression that occurs after ECs are stimulated with serum or VEGF. Furthermore, inhibiting miR-130a blocks this downregulation of *GAX*. Because miR-221 was implicated as a microRNA that antagonizes the angiogenic phenotype in ECs (35, 36), we decided to determine whether miR-221 affects *GAX* expression. Consistent with the previously reported antiangiogenic activity of miR-221/222 (35, 36) and the known antiangiogenic activity of *GAX* (6, 7, 9, 26), we observed that miR-221 was downregulated by serum (Fig. 2B). As would be predicted if miR-221 has a significant role in inhibiting angio-

genesis, miR-221 expression also strongly upregulated *GAX* expression in vascular ECs (Fig. 3A). These results are also in accord with the proposed role of miR-221 in inhibiting EC migration in response to hyperglycemia (24) and in inhibiting EC migration and proliferation (27, 33).

Because microRNAs most commonly function by binding to an mRNA and either inducing its degradation or inhibiting its translation (18), we postulated that miR-221 likely targets an intermediary protein that represses *GAX* expression. An *in silico* search returned multiple candidates, but only two were regulated by serum in an opposite fashion compared to *GAX*, and only one of them (ZEB2) downregulated *GAX* expression in cotransfection experiments (Fig. 3E and F). Further investigation revealed that miR-221 downregulates *ZEB2* through an miR-221 consensus binding site in the 3′ UTR of *ZEB2* (Fig. 4 and 5) and that ZEB2 inhibits *GAX* expression by binding to two ZEB2/SIP1 consensus binding sites in the upstream chromatin from *GAX* (Fig. 6 and 7). We therefore conclude from our observations that miR-221 upregulates expression of the antiangiogenic homeobox gene *GAX*, largely through its ability to downregulate *ZEB2*.

Because ZEB2 acts primarily as a transcriptional repressor (38), our results are consistent with its function as thus far understood, but they are intriguing because they are, to the best of our knowledge, the first to implicate ZEB2 as a potential regulator of the process of angiogenesis. Although ZEB2 has been implicated in regulating the EMT in other cell types, including a number of epithelial malignancies (37), ZEB2 has not been previously suggested as a regulator of the EC phenotype during angiogenesis. Consequently, very little is known about its role in ECs. However, because ZEB2 is also known as SMAD1-interacting protein 1 (SIP1), it is tempting to speculate that its regulation of GAX may also implicate GAX in modulating transforming growth factor β (TGF-β) activity in ECs, given that different TGF-β receptors modulate pro- and antiangiogenic activities, with ALK1 stimulating EC proliferation and migration through Smad1/5 gene transcription, an activity consistent with the downregulation of GAX by *ZEB2* (13, 23).

Given the role of ZEB2 in the EMT and dedifferentiation of epithelial cell types, with their conversion to a more mesenchymal state, in retrospect it is probably not surprising that GAX would be a target of ZEB2, given that GAX behaves in a manner consistent with a potential master regulatory protein controlling the angiogenic phenotype of vascular ECs. Moreover, the ability of ZEB2 to downregulate *GAX* suggests a potential reason why the upregulation of *GAX* after serum withdrawal is slower than *GAX* downregulation in the presence of serum. In the former case, our results suggest that miR-221 downregulates ZEB2, thus relieving its transcriptional repression of *GAX* and allowing GAX levels to accumulate again over several hours, whereas in the latter case miR-130a rapidly downregulates *GAX* mRNA, which has a short half-life (6, 10).

Finally, our observations suggest that ZEB2 might serve as a molecular target for the antiangiogenic therapy of cancer and other angiogenesis-driven diseases; indeed, given the role of ZEB2 in EMT, it is possible to envision that targeting this gene might treat both the cancer, by inhibiting EMT, and the angiogenesis provoked by the cancer by preventing the downregulation of *GAX* expression.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute (1 R01 CA111344) and the Department of Defense (DAMD17-03-1-0292).

REFERENCES

1. **Arbiser, J. L., M. A. Moses, C. A. Fernandez, N. Ghiso, Y. Cao, N. Klauber, D. Frank, M. Brownlee, E. Flynn, S. Parangi, H. R. Byers, and J. Folkman.** 1997. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc. Natl. Acad. Sci. U. S. A. **94:**861–866.
2. **Bell, S. E., A. Mavila, R. Salazar, K. J. Bayless, S. Kanagala, S. A. Maxwell, and G. E. Davis.** 2001. Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. J. Cell Sci. **114:**2755–2773.
3. **Bergers, G., and L. E. Benjamin.** 2003. Tumorigenesis and the angiogenic switch. Nat. Rev. Cancer **3:**401–410.
4. **Bergers, G., R. Brekken, G. McMahon, T. H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, and D. Hanahan.** 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat. Cell Biol. **2:**737–744.
5. **Care, A., F. Felicetti, E. Meccia, L. Bottero, M. Parenza, A. Stoppacciaro, C. Peschle, and M. P. Colombo.** 2001. HOXB7: a key factor for tumor-associated angiogenic switch. Cancer Res. **61:**6532–6539.
6. **Chen, Y., and D. H. Gorski.** 2008. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. Blood **111:**1217–1226.
7. **Chen, Y., A. D. Leal, S. Patel, and D. H. Gorski.** 2007. The homeobox gene GAX activates p21WAF1/CIP1 expression in vascular endothelial cells through direct interaction with upstream AT-rich sequences. J. Biol. Chem. **282:**507–517.
8. **Folkman, J.** 2002. Role of angiogenesis in tumor growth and metastasis. Semin. Oncol. **29:**15–18.
9. **Gorski, D. H., and A. J. Leal.** 2003. Inhibition of endothelial cell activation by the homeobox gene Gax. J. Surg. Res. **111:**91–99.
10. **Gorski, D. H., D. F. LePage, C. V. Patel, N. G. Copeland, N. A. Jenkins, and K. Walsh.** 1993. Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G_0/G_1 transition in vascular smooth muscle cells. Mol. Cell. Biol. **13:**3722–3733.
11. **Gorski, D. H., and K. Walsh.** January 1999. Growth arrest homeobox gene. U.S. patent 5,856,121.
12. **Goumans, M. J., F. Lebrin, and G. Valdimarsdottir.** 2003. Controlling the angiogenic switch: a balance between two distinct TGF-β receptor signaling pathways. Trends Cardiovasc. Med. **13:**301–307.
13. **Goumans, M. J., G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras, and P. ten Dijke.** 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J. **21:**1743–1753.
14. **Goydos, J. S., B. Mann, H. J. Kim, E. M. Gabriel, J. Alsina, F. J. Germino, W. Shih, and D. H. Gorski.** 2005. Detection of B-RAF and N-RAS mutations in human melanoma. J. Am. Coll. Surg. **200:**362–370.
15. **Grun, D., Y. L. Wang, D. Langenberger, K. C. Gunsalus, and N. Rajewsky.** 2005. microRNA target predictions across seven Drosophila species and comparison to mammalian targets. PLoS Comput. Biol. **1:**e13.
16. **Hanahan, D.** 1997. Signaling vascular morphogenesis and maintenance. Science **277:**48–50.
17. **Hanahan, D., and J. Folkman.** 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell **86:**353–364.
18. **He, L., and G. J. Hannon.** 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. **5:**522–531.
19. **Irelan, J. T., A. Gutierrez Del Arroyo, A. Gutierrez, G. Peters, K. C. Quon, L. Miraglia, and S. K. Chanda.** 2009. A functional screen for regulators of CKDN2A reveals MEOX2 as a transcriptional activator of INK4a. PLoS One **4:**e5067.
20. **Kocerha, J., S. Kauppinen, and C. Wahlestedt.** 18 June 2009. MicroRNAs in CNS disorders. Neuromol. Med. [E-pub ahead of print.] doi:10.1007/s12017-009-8066-1.
21. **Krek, A., D. Grun, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel, and N. Rajewsky.** 2005. Combinatorial microRNA target predictions. Nat. Genet. **37:**495–500.
22. **Kuehbacher, A., C. Urbich, A. M. Zeiher, and S. Dimmeler.** 2007. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. Circ. Res. **101:**59–68.
23. **Lebrin, F., M. Deckers, P. Bertolino, and P. Ten Dijke.** 2005. TGF-beta receptor function in the endothelium. Cardiovasc. Res. **65:**599–608.
24. **Li, Y., Y. H. Song, F. Li, T. Yang, Y. W. Lu, and Y. J. Geng.** 2009. MicroRNA-221 regulates high glucose-induced endothelial dysfunction. Biochem. Biophys. Res. Commun. **381:**81–83.
25. **Lu, M., Q. Zhang, M. Deng, J. Miao, Y. Guo, W. Gao, and Q. Cui.** 2008. An analysis of human microRNA and disease associations. PLoS One **3:**e3420.
26. **Patel, S., A. D. Leal, and D. H. Gorski.** 2005. The homeobox gene Gax

inhibits angiogenesis through inhibition of nuclear factor-κB-dependent endothelial cell gene expression. Cancer Res. **65:**1414–1424.
27. **Poliseno, L., A. Tuccoli, L. Mariani, M. Evangelista, L. Citti, K. Woods, A. Mercatanti, S. Hammond, and G. Rainaldi.** 2006. MicroRNAs modulate the angiogenic properties of HUVECs. Blood **108:**3068–3071.
28. **Reijntjes, S., S. Stricker, and B. S. Mankoo.** 2007. A comparative analysis of Meox1 and Meox2 in the developing somites and limbs of the chick embryo. Int. J. Dev. Biol. **51:**753–759.
29. **Remacle, J. E., H. Kraft, W. Lerchner, G. Wuytens, C. Collart, K. Verschueren, J. C. Smith, and D. Huylebroeck.** 1999. New mode of DNA binding of multi-zinc finger transcription factors: deltaEF1 family members bind with two hands to two target sites. EMBO J. **18:**5073–5084.
30. **Roldo, C., E. Missiaglia, J. P. Hagan, M. Falconi, P. Capelli, S. Bersani, G. A. Calin, S. Volinia, C. G. Liu, A. Scarpa, and C. M. Croce.** 2006. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J. Clin. Oncol. **24:**4677–4684.
31. **Smith, R. C., D. Branellec, D. H. Gorski, K. Guo, H. Perlman, J. F. Dedieu, C. Pastore, A. Mahfoudi, P. Denefle, J. M. Isner, and K. Walsh.** 1997. p21CIP1-mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene. Genes Dev. **11:**1674–1689.
32. **St Croix, B., C. Rago, V. Velculescu, G. Traverso, K. E. Romans, E. Montgomery, A. Lal, G. J. Riggins, C. Lengauer, B. Vogelstein, and K. W. Kinzler.** 2000. Genes expressed in human tumor endothelium. Science **289:**1197–1202.
33. **Suarez, Y., C. Fernandez-Hernando, J. S. Pober, and W. C. Sessa.** 2007. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ. Res. **100:**1164–1173.
34. **Suarez, Y., C. Fernandez-Hernando, J. Yu, S. A. Gerber, K. D. Harrison, J. S. Pober, M. L. Iruela-Arispe, M. Merkenschlager, and W. C. Sessa.** 2008. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proc. Natl. Acad. Sci. U. S. A. **105:**14082–14087.
35. **Suarez, Y., and W. C. Sessa.** 2009. MicroRNAs as novel regulators of angiogenesis. Circ. Res. **104:**442–454.
36. **Urbich, C., A. Kuehbacher, and S. Dimmeler.** 2008. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. Cardiovasc. Res. **79:**581–588.
37. **Vandewalle, C., J. Comijn, B. De Craene, P. Vermassen, E. Bruyneel, H. Andersen, E. Tulchinsky, F. Van Roy, and G. Berx.** 2005. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. Nucleic Acids Res. **33:**6566–6578.
38. **van Grunsven, L. A., C. Michiels, T. Van de Putte, L. Nelles, G. Wuytens, K. Verschueren, and D. Huylebroeck.** 2003. Interaction between Smad-interacting protein-1 and the corepressor C-terminal binding protein is dispensable for transcriptional repression of E-cadherin. J. Biol. Chem. **278:**26135–26145.
39. **van Grunsven, L. A., A. Schellens, D. Huylebroeck, and K. Verschueren.** 2001. SIP1 (Smad interacting protein 1) and deltaEF1 (delta-crystallin enhancer binding factor) are structurally similar transcriptional repressors. J. Bone Joint Surg. Am. *83-A*(Suppl. 1)**:**S40–S47.
40. **Vasudevan, S., Y. Tong, and J. A. Steitz.** 2007. Switching from repression to activation: microRNAs can up-regulate translation. Science **318:**1931–1934.
41. **Volinia, S., G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris, and C. M. Croce.** 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc. Natl. Acad. Sci. U. S. A. **103:**2257–2261.
42. **Yang, B., H. Lin, J. Xiao, Y. Lu, X. Luo, B. Li, Y. Zhang, C. Xu, Y. Bai, H. Wang, G. Chen, and Z. Wang.** 2007. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat. Med. **13:**486–491.
43. **Yang, W. J., D. D. Yang, S. Na, G. E. Sandusky, Q. Zhang, and G. Zhao.** 2005. Dicer is required for embryonic angiogenesis during mouse development. J. Biol. Chem. **280:**9330–9335.