The Oncogenic microRNA-27a Targets Genes That Regulate Specificity Protein Transcription Factors and the G_2-M Checkpoint in MDA-MB-231 Breast Cancer Cells

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Abstract

There is evidence that specificity proteins (Sp), such as Sp1, Sp3, and Sp4, are overexpressed in tumors and contribute to the proliferative and angiogenic phenotype associated with cancer cells. Sp1, Sp3, and Sp4 are expressed in a panel of estrogen receptor (ER)–positive and ER-negative breast cancer cell lines, and we hypothesized that regulation of their expression may be due to microRNA-27a (miR-27a), which is also expressed in these cell lines and has been reported to regulate the zinc finger *ZBTB10* gene, a putative Sp repressor. Transfection of ER-negative MDA-MB-231 breast cancer cells with antisense miR-27a (as-miR-27a) resulted in increased expression of *ZBTB10* mRNA and decreased expression of Sp1, Sp3, and Sp4 at the mRNA and protein levels and also decreased activity in cells transfected with constructs containing Sp1 and Sp3 promoter inserts. In addition, these responses were accompanied by decreased expression of Sp-dependent survival and angiogenic genes, including *survivin, vascular endothelial growth factor* (*VEGF*), and *VEGF receptor 1* (*VEGFR1*). Moreover, similar results were observed in MDA-MB-231 cells transfected with *ZBTB10* expression plasmid. Both as-miR-27a and *ZBTB10* overexpression decreased the percentage of MDA-MB-231 cells in S phase of the cell cycle; however, *ZBTB10* increased the percentage of cells in G_0-G_1, whereas as-miR-27a increased the percentage in G_2-M. This latter response was associated with induction of *Myt-1* (another miR-27a target gene), which inhibits G_2-M through enhanced phosphorylation and inactivation of cdc2. Thus, the oncogenic activity of miR-27a in MDA-MB-231 cells is due, in part, to suppression of *ZBTB10* and *Myt-1*. [Cancer Res 2007;67(22):11001–11]

Introduction

Specificity protein 1 (Sp1) was the first transcription factor identified (1, 2) and is a member of the Sp/Krüppel-like factor (KLF) family of transcription factors (3–6). These proteins are characterized by their COOH-terminal domains, which contain three C_2H_2-type zinc fingers that recognize GC/GT boxes in promoters of target genes. The NH_2-terminal domains of Sp/KLF proteins are highly variable in both structure and function, and many KLF proteins are truncated in this region. Some Sp/KLF members are critical for embryonic development, and knockout of *Sp1, Sp3*, and *Sp4* genes in mice results in embryolethality or multiple developmental deficits (7–10). Sp and KLF proteins cooperatively interact with one another and other transcription factors on GC-rich promoters to activate or inhibit diverse classes of mammalian and viral genes that play a critical role in regulating cellular homeostasis (11).

The tissue- and age-dependent expression of Sp proteins in humans and laboratory animal models has not been extensively investigated; however, several studies report that Sp1 protein is overexpressed in tumor versus nontumor tissues (12–19). For example, in gastric cancer, Sp1 expression was observed in tumor cells, whereas in stromal and normal glandular cells Sp1 expression was either weak or nondetectable (12). Moreover, survival of gastric cancer patients increased with decreasing Sp1 protein expression. Malignant transformation of human fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression and the transformed cells formed tumors in athymic nude mouse xenografts, whereas Sp1 knockdown gave cells that were nontumorigenic in the same mouse xenograft model (19).

Although Sp1 is widely expressed in tumors, there is increasing evidence that Sp3 and Sp4 are also expressed in cancer cells and contribute to Sp-dependent procarcinogenic responses (20–25). Using RNA interference, it was shown that Sp1 knockdown using a small inhibitory RNA for Sp1 (iSp1) inhibited G_0-G_1 to S phase progression in MCF-7 breast cancer cells (25). Small inhibitory RNAs for Sp3 (iSp3) and Sp4 (iSp4) were used along with iSp1 to show that in pancreatic cancer cells, Sp1, Sp3, and Sp4 proteins regulated expression of vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGFR1 or Flt), and VEGFR2 (KDR; refs. 20–22). Moreover, Sp3 acted as a repressor of p27 in pancreatic cancer cells (21), indicating that overexpression of Sp proteins in cancers contribute to their proliferative and angiogenic phenotype. The underlying factors associated with high expression of Sp proteins such as Sp1, Sp3, and Sp4 in tumors are not well understood, and we hypothesized that microRNAs (miRNA) may play a role in mediating overexpression of these transcription factors in tumors and cancer cells. MiRNAs are small noncoding RNAs that regulate expression of genes by specifically interacting with 3′ untranslated regions (UTR) of target gene mRNAs to repress translation or enhance mRNA cleavage (26, 27). Scott and coworkers (28) observed that miRNA-27a (miR-27a) suppressed *ZBTB10*/RINZF expression, and this novel zinc finger protein inhibits Sp1-dependent activation of the gastrin gene promoter (29). This suggests that miR-27a suppression of *ZBTB10*, a Sp repressor, may contribute to the overexpression of Sp proteins in tumors/cancer cells. MiR-27a is widely expressed in cancer cells and, in this study, miR-27a and Sp1, Sp3, and Sp4 mRNAs were detected in six estrogen receptor (ER)–positive and ER-negative breast cancer cell lines, and levels of

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ZBTB10 mRNA were lower than Sp mRNAs. Using the highly aggressive and metastatic ER-negative MDA-MB-231 breast cancer cell line as a model, we showed that transfection of antisense miR-27a (as-miR-27a) or overexpression of *ZBTB10* resulted in a dramatic decrease in Sp mRNA and protein expression, and this was accompanied by decreased levels of Sp-dependent antiapoptotic and angiogenic genes and induction of apoptosis. These results show a role for miR-27a in mediating overexpression of Sp proteins in breast cancer cells through regulation of the Sp repressor *ZBTB10*. In addition, miR-27a also facilitates cancer cell proliferation through suppression of *Myt-1*, which blocks cell cycle progression at G_2-M. These results suggest that miR-27a is oncogenic in these cell lines.

Materials and Methods

Chemicals, antibodies, plasmids, and reagents. Antibodies against Sp1, Sp4, Sp3, VEGF, VEGFR1, survivin, cdc2, and phosphorylated cdc2 were obtained from Santa Cruz Biotechnology. The polyclonal antibody for an ELISA assay for ZBTB10 was obtained from Imgenex Corp. The antibody for and poly(ADP-ribose) polymerase (PARP) was purchased from Cell Signaling Technology. Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega. LipofectAMINE 2000 reagent was supplied by Invitrogen. Western lightning chemiluminescence reagent was obtained from Perkin-Elmer Life Sciences. miRNA mirvaRNA extraction kits and the reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosciences. Primers for Sp1, VEGF, VEGFR1, survivin, ZBTB10, MYT1, and Wee1 were purchased from Integrated DNA Technologies Technologies. Primers for Sp3 and Sp4 were obtained from Qiagen; as-miRNA-27a, miR-27a mimic, and scrambled miRNA were from Dharmacon; and the ZBTB10 expression vector and empty vector (pCMV6-XL4) were from Origene. Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Cuidad and Veronique Noe (University of Barcelona, Barcelona, Spain). RNase, propidium iodide, sodium citrate, and Triton X-100 were obtained from Sigma-Aldrich.

Cell lines. Human mammary carcinoma cell lines MDA-MB-231, MDA-MB-435, BT474, SKBR3, MCF-7, and ZR-75 were obtained from the American Type Culture Collection. Cell lines were maintained in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in the presence of 5% CO_2.

Western blot analysis. MDA-MB-231 cells (4×10^5) were seeded in six-well plates in DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS for 16 h and then transfected with pCMV6-XL4-ZBTB10 expression plasmid or as-miRNa-27a using LipofectAMINE 2000 (Invitrogen) according to manufacturer's protocol. After 24 h, whole-cell lysates were obtained using high-salt buffer [50 mmol/L HEPES, 500 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton X-100 (pH 7.5)], and 5 μL/mL Protease Inhibitor Cocktail (Sigma-Aldrich). Protein samples were incubated at 100°C for 2 min, separated on 10% SDS-PAGE at 120 V for 3 to 4 h in 1× running buffer [25 mmol/L Tris base, 192 mmol/L glycine, and 0.1% SDS (pH 8.3)], and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 0.9 V for 1.5 h at 4°C in 1× transfer buffer (48 mmol/L Tris-HCl, 39 mmol/L glycine, and 0.025% SDS). The PVDF membrane was blocked in 5% TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] with gentle shaking for 30 min and was incubated in fresh 5% TBST-Blotto with primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody in 5% TBST-Blotto for 2 to 3 h. The membrane was washed with TBST for 10 min, incubated with chemiluminescence substrate (Perkin-Elmer), and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak).

ELISA for ZBTB10 protein. Cell lysates were diluted 1:10 with carbonate buffer (pH 9.6) in 96-well plates and incubated for 12 h at 4°C. Plates were then washed with PBS (pH 7.2) containing Tween 20% (0.05%) and rinsed with water. Plates were blocked with 3% nonfat milk powder in PBS (pH 9.6) followed by incubation with the primary ZBTB10 antibody (Imgenex) for 1 h. Plates were then washed and incubated with a secondary horseradish peroxidase–linked goat–anti-rabbit antibody (Santa Cruz Biotechnology). After washing, the antibody binding was visualized with OPD substrate (Sigma-Aldrich) in citrate buffer (pH 5.0) for 20 min. The reaction was stopped with 2.5 mol/L sulfuric acid, and absorbance was determined at 490 nm using a Fluostar Optima (BMG).

Semiquantitative RT-PCR. MDA-MB-231 cells were transfected with either as-miRNA-27a or with pCMV6-XL4-ZBTB10 expression plasmid using LipofectAMINE 2000 following the manufacturer's protocol. Total RNA was extracted using RNeasy Mini kit (Qiagen), and 2 μg of RNA were used to synthesize cDNA using Reverse Transcription System (Promega). Primers were obtained from Integrated DNA Technologies and used for amplification were as follows: Sp1 (sense 5′-ATGGGGGCAATGGTAATGGTGG-3′; antisense 5′-TCAGAACTTGCTGGTTCTGTAAG-3′), Sp3 (sense 5′-ATGACTGCAGGCATTAATGCCG-3′; antisense 5′-TGTCTCTTCAGAAACAGGCGAC-3′), Sp4 (sense 5′-ATGGCTACAGAAGGAGGGAAAAC-3′; antisense 5′-TTGACCAGGGGTGGAAGAATTAC-3′), ZBTB10 (sense 5′-GCTGGATAGTAGTTATGTTGC-3′; antisense 5′-CTGAGTGGTTTGATGGACAGAG-3′), VEGF (sense 5′-CCATGAACTTTCTGCTGTCTT-3′; antisense 5′-ATCGCATCAGGGGCACACAG-3′), VEGFR1 (sense 5′-ATGGAGCGTAAGAAAGAAAAAATG-3′; antisense 5′-TCAAGTACCTCCTTTTCTCACAT-3′), survivin (sense 5′-ATGGCCGAGGCTGGCTTCATC-3′; antisense 5′-ACGGCGCACTTTCTTCGCAGTT-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense 5′-ACGGATTTGGTCGTATTGGGCG-3′; antisense 5′-CTCCTGGAAGATGGTGATGG-3′). PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transillumination.

Transfection with as-miRNA-27-a or ZBTB10 expression vector. Transfection with 0 to 250 nmol/mL as-miRNA-27a (Dharmacon) and 0 to 4 μg/mL ZBTB10 expression plasmid pCMV6-XL4 vector (Origene) was done using LipofectAMINE 2000 according the manufacturer's protocol when cells were 50% to 60% confluent. The controls for the as-miR-27a experiment used an equal amount of a nonspecific oligonucleotide and, in the ZBTB10 overexpression experiment, we used the empty vector as a control. After transfection for 5 h, the transfection mix was replaced with complete medium and incubated for different times as indicated.

Promoter transfection and luciferase assays. Cells were transfected with constructs essentially as previously described (22). In brief, cells were plated in 12-well plates at 1×10^5 per well in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After growth for 16 to 20 h, 0.4 μg of reporter gene constructs was transfected using LipofectAMINE (Invitrogen) reagent according to the manufacturer's protocol. This transfection was done concurrently with the transfection of as-miR-27a, miR-27a mimic, or ZBTB10 expression vector as described above. For transfection experiments using as-miR-27a or miR-27a mimic, the control cells (0) were transfected with a scrambled oligonucleotide (100 or 250 nmol/mL), which matched the highest concentration of the as-miR-27a or miR-27a mimic used in the experiment. After transfection for 5 h, the transfection mix was replaced with complete medium and incubated for 19 h. Cells were then lysed with 100 μL of 1× reporter lysis buffer, and 30 μL of cell extract were used for luciferase assays. Lumicount was used to quantitate luciferase activity and the luciferase activities were normalized to protein concentration. β-Galactosidase could not be used to normalize for transfection efficiency because as-miR-27a and ZBTB10 directly affected β-galactosidase activity in MDA-MB-231 cells.

Real-time PCR analysis of mRNAs and miRNAs. miRNA was extracted using the mirvaRNA extraction kit (Applied Biosystems). Quantification of miRNA (RNU49, RNU6B, miRNA-27a, miRNA let7a, miRNA 17-5p) was done using the Taqman miRNA kit (Applied Biosystems) according to the manufacturer's protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression. For mRNA analysis, cells were transfected as described previously. Total RNA was isolated using the RNeasy Protect Mini kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was carried out with the SYBR Green PCR Master Mix from PE Applied Biosystems on an ABI Prism 7700 Sequence Detection System (PE Applied

Biosystems) using 0.5 μmol/L of each primer and 2 μL cDNA template in each 25 μL reaction. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: one cycle of 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative C_T method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies (20). The following primers were used:

MYT1 (F): 5′-CCTTCCAAGAGTAGCTCCAATTC-3′.
MYT1 (R): 5′-GCCGGTAGCTCCCATATGG-3′.
Sp1 (F): 5′-TCACCTGCGGGCACACTT-3′.
Sp1 (R): 5′-CCGAACGTGTGAAGCGTT-3′.
TBP (F): 5′-TGCACAGGAGCCAAGAGTGAA-3′.
TBP (R): 5′-CACATCACAGCTCCCCACCA-3′.
WEE1 (F): 5′-TTGCGCCTTGCCCTCACA-3′.
WEE1 (R): 5′-TTGATCTCCATTTCTCGGAAGAG-3′.
ZBTB10 (F): 5′-GCTGGATAGTAGTTATGTTGC-3′.
ZBTB10 (R): 5′-CTGAGTGGTTTGATGGACAGA-3′.

Primers for Sp3 and Sp4 were purchased from Qiagen.

Cell proliferation and fluorescence-activated cell sorting analysis. Cells were transfected with as-miRNA-27a or the ZBTB10 expression vector. Twenty-four hours after the transfection, cells were trypsinized, syringed, and collected by centrifugation. Cells were resuspended in staining solution [50 μg/mL propidium iodide, 30 units/mL RNase, 4 mmol/L sodium citrate, and Triton X-100 (pH 7.8)] and incubated at 37°C for 10 min. Sodium chloride solution was added to a final concentration of 0.15 mol/L. Stained cells were analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems) using Cell Quest (Becton Dickinson Immunocytometry Systems) acquisition software as previously described (30). For cell proliferation studies, cells were transfected with as-miR-27a or scrambled oligonucleotide using LipofectAMINE 2000 (Invitrogen); medium was change after 5 h, and 72 h later cells were counted using a Coulter Z1 cell counter (Beckman Coulter).

Results

In preliminary studies, we identified miR-27a expression in multiple cancer cell lines by Northern blot analysis (data not shown), and Fig. 1*A* summarizes expression of miR-27a relative to miR-49 in ER-positive ZR-75, BT474, and MCF-7 and ER-negative MDA-MB-435, MDA-MB-231, and SKBR3 breast cancer cell lines. U6 small nuclear RNA, which was expressed in all cell lines, was used to normalize relative expression of miR-27a. Similar results were obtained using miR-49 as a normalizing RNA. The miR let-7 family are cancer-associated tumor suppressors (31–35), and Fig. 1*A* also compares miR-27a and let-7a expression in breast cancer cells. Levels of the former miR tended to be higher in all six breast cancer lines. Using a similar approach, we also determined expression of Sp1, Sp3, Sp4, and *ZBTB10* mRNA (relative to TBP mRNA), and the Sp mRNAs were more highly expressed than *ZBTB10* mRNA in the six cell lines (Fig. 1*B*). Previous studies show that Sp1, Sp3, and Sp4 proteins are expressed in breast cancer cells, and results in Fig. 1*C* show expression of these three proteins in all six ER-positive and ER-negative breast cancer cell lines. Relative levels of Sp protein expression varied between cell lines, and this was particularly evident for Sp4, which was lowest in MCF-7 and MDA-MB-231 cells among the ER-positive and ER-negative breast cancer cell lines, respectively. In addition, we compare expression of Sp proteins in T47D cancer cells and normal MCF10A breast cancer cells and it was evident that Sp1, Sp3, and Sp4 are more highly expressed in cancer versus noncancer cell lines.

The role of miR-27a in mediating expression of *ZBTB10* and the downstream effects of this Sp repressor were investigated using a miR-27a antisense construct in MDA-MB-231 cells. Results in Fig. 2*A* show that as-miR-27a decreased miR-27a expression after treatment for 24 h and similar results were observed after 12 h. It is also possible that decreased miR-27a in cells transfected with as-miR-27a may be due to interference with PCR amplification or sequestration of miR-27a by the antisense oligonucleotide, which decreases PCR amplification due to inaccessibility of miR-27a (36). In contrast, expression of miR-7a and miR-17-5p were unchanged, demonstrating the specificity of the antisense construct. Transfection with as-miR-27a in MDA-MB-231 cells also increased *ZBTB10* mRNA levels, and this was accompanied by decreased expression of Sp1, Sp3, and Sp4 mRNA levels (Fig. 2*B*). Figure 2*C* summarizes the effects of as-miR-27a on transactivation in MDA-MB-231 cells transfected with pSp1For4, which contains the −751 to −20 region of the Sp1 gene promoter (37). The results show the dose-dependent decrease in luciferase activity in MDA-MB-231 cells transfected with pSp1For4 and 12.5 to 100 nmol/mL as-miR-27A, and we observed that as-miR-27a decreased luciferase activity in MDA-MB-231 cells transfected with Sp1For4 and the Sp1For2 and Sp1For1 deletion constructs. Transfection of different amounts of as-miR-27a also decreased luciferase activity in MDA-MB-231 cells transfected with pSp3For5, which contains the −417 to −38 region of the Sp3 gene promoter (38), and as-miR-27a also inhibited transactivation in cells transfected with pSp3For5 and the deletion construct pSp3For2 (Fig. 2*D*).

We also investigated the effects of miR-27a on expression of Sp proteins and Sp-regulated proteins. Results in Fig. 3*A* show that in MDA-MB-231 cells transfected with the as-miR-27a (100 nmol/mL), there was decreased expression of Sp1, Sp3, and Sp4 proteins (Fig. 3*A*). In contrast, transfection with miR-27a mimic enhanced expression of Sp1, Sp3, and Sp4 proteins (Fig. 3*B*) and showed a direct correlation between miR-27a and Sp protein expression. Previous studies have shown that Sp proteins regulate expression of survival and angiogenic genes (20–25, 34). Results in Fig. 3*C* show that after transfection of cells with as-miR-27a (100 nmol/mL), there was an increase in PARP cleavage and a decrease in expression of the antiapoptotic protein survivin and the antiangiogenic VEGF and VEGFR1 proteins and a parallel decrease in survivin, VEGF, and VEGFR1 mRNA (Fig. 3*D*). These results are consistent with decreased expression of Sp proteins (Fig. 3*A*) because *VEGF*, *VEGFR1*, and *survivin* are Sp-dependent genes (20–25, 39).

Because miR-27a regulates *ZBTB10*, which suppresses Sp-dependent gastrin promoter activation (29), we investigated the direct effects of *ZBTB10* by overexpressing this gene in MDA-MB-231 cells. Results in Fig. 4*A* show that in MDA-MB-231 cells transfected with a *ZBTB10* expression plasmid, there was increased *ZBTB10* mRNA levels, and this was accompanied by decreased Sp1, Sp3, and Sp4 mRNA levels. Using an ELISA assay, we also showed that ZBTB10 protein was also increased. The effects of *ZBTB10* on Sp1 and Sp3 promoter constructs are shown in Fig. 4*B* and *C*. The results show that overexpression of *ZBTB10* decreases transactivation in MDA-MB-231 cells transfected with pSp1For4, and similar decreases were observed after transfection with the deletion constructs pSp1For4, pSp1For2, and pSp1For1. *ZBTB10* also decreased transactivation in MDA-MB-231 cells transfected pSp3For5 (Fig. 4*C*), and these results were comparable with the effects of as-miR-27a using these same constructs (Fig. 2*C* and *D*). However, *ZBTB10* did not decrease luciferase activity in cells transfected with pSp3For2 and this differs from the effects of as-miR-27a on this same construct, suggesting that as-miR-27a may repress Sp3 expression by activation of ZBTB10 and possibly other

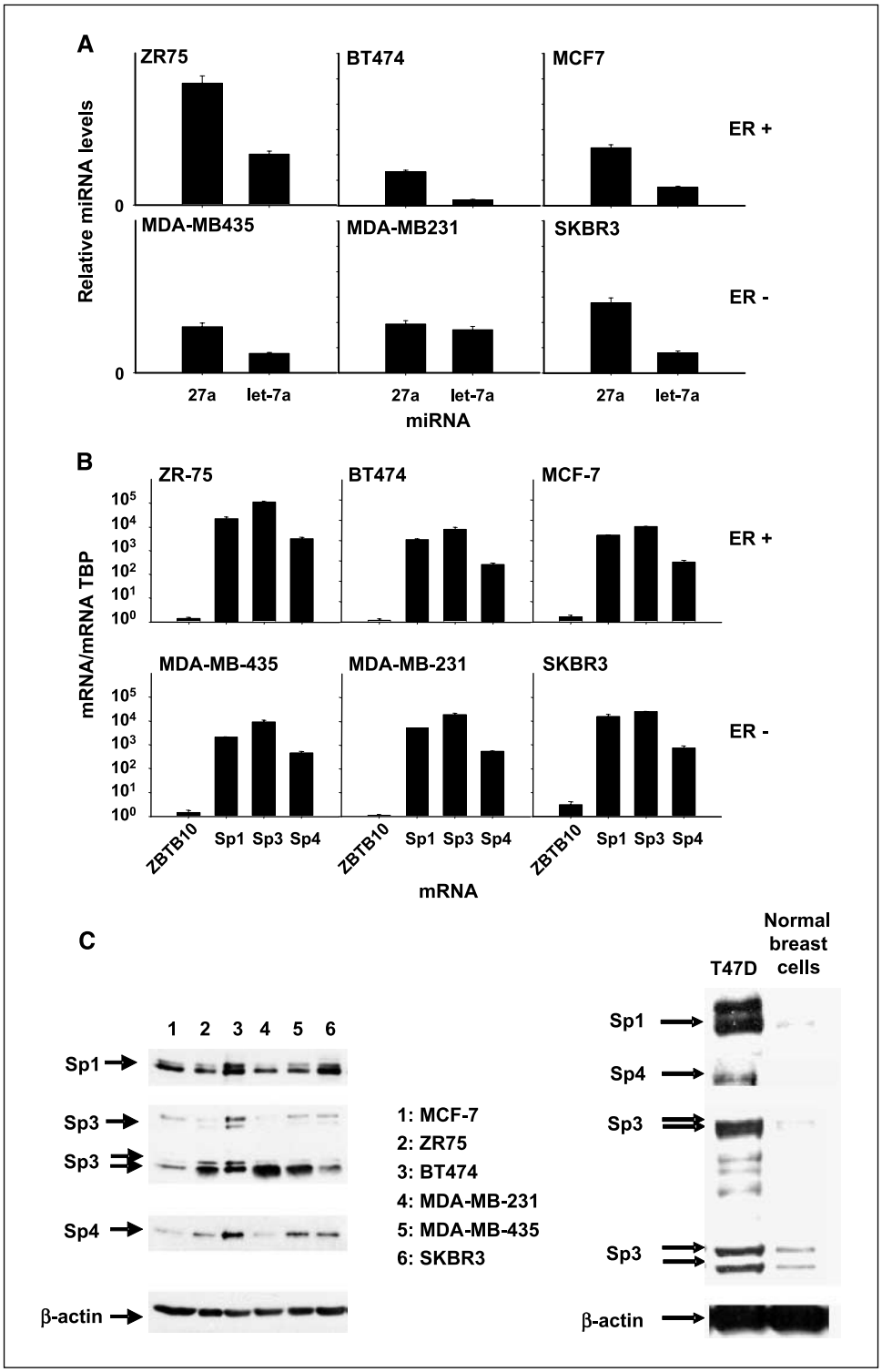

Figure 1. Expression of miR-27a, ZBTB10, and Sp mRNAs and Sp proteins in breast cancer cell lines. *A,* relative expression of miR-27a and let-7a. Expression of miR-27a and let-7a miRNAs in ZR-75, MCF-7 BT474, MDA-MB-435, MDA-MB-231, and SKBR3 breast cancer cell lines was determined by real-time PCR as described in Materials and Methods. *Columns,* means for three replicate determinations for each cell line; *bars,* SE. miR-27a and let-7a levels were normalized to U6 snRNA. *B,* expression of ZBTB10 and Sp mRNA levels in breast cancer cells. Expression of ZBTB10, Sp1, Sp3, and Sp4 mRNA levels in the panel of six breast cancer cell lines was determined by real-time PCR as described in Materials and Methods. *Columns,* means for three replicate determinations for each cell line; *bars,* SE. *C,* Sp protein expression. Whole-cell lysates were obtained from the panel of breast cancer cell lines. Sp1, Sp3, and Sp4 protein expression was determined by Western blot analysis as described in Materials and Methods. Relative expression of proteins Sp1, Sp3, and Sp4 was also determined in normal MCF10A cells and compared with T47D cells.

proteins that do not act through GC-rich motifs. In parallel studies, we also investigated the effects of *ZBTB10* expression on Sp protein and on Sp-dependent angiogenic and survival genes/proteins. Figure 5*A* shows that *ZBTB10* expression in MDA-MB-231 cells significantly decreased expression of Sp1, Sp3, and Sp4 proteins. Moreover, this was accompanied by decreased expression of VEGF, VEGFR1, and survivin mRNA levels (Fig. 5*B*) and their corresponding proteins (Fig. 5*C*). Moreover, decreased expression of these proteins was accompanied by activation of apoptosis as evidenced by caspase-dependent PARP cleavage. Thus, as-miR-27a and overexpression of *ZBTB10* gave complementary results in MDA-MB-231 cells and decreased expression of Sp proteins (and promoters) and Sp-dependent genes.

We also compared the effects of transfected as-miR-27a and *ZBTB10* expression plasmid on MDA-MB-231 cell cycle progression as determined by fluorescence-activated cell sorting (FACS)

analysis. Results in Fig. 6*A* show that the percentage of MDA-MB-231 cells in G_0-G_1, S, and G_2-M phases of the cell cycle were 55%, 29%, and 16%, respectively. Transfection with 25, 100, or 250 nmol/mL as-miR-27a significantly decreased the percentage of cells in S phase and increased the percentage in G_2-M phase with minimal effects on the percentage of cells in G_0-G_1. Moreover, in a parallel experiment, as-miR-27a also decreased cell proliferation. In contrast, *ZBTB10* overexpression (1.0–4.0 μg/mL) resulted in an increase of cells in G_0-G_1 and a decrease of cells in S phase (Fig. 6*B*), and these data are comparable with results of RNA interference with Sp1 knockdown in MCF-7 cells, which also resulted in inhibition of G_0-G_1 to S phase progression (25). The differences between as-miR-27a and *ZBTB10* overexpression on percentage distribution of MDA-MB-231 cells in different phases of

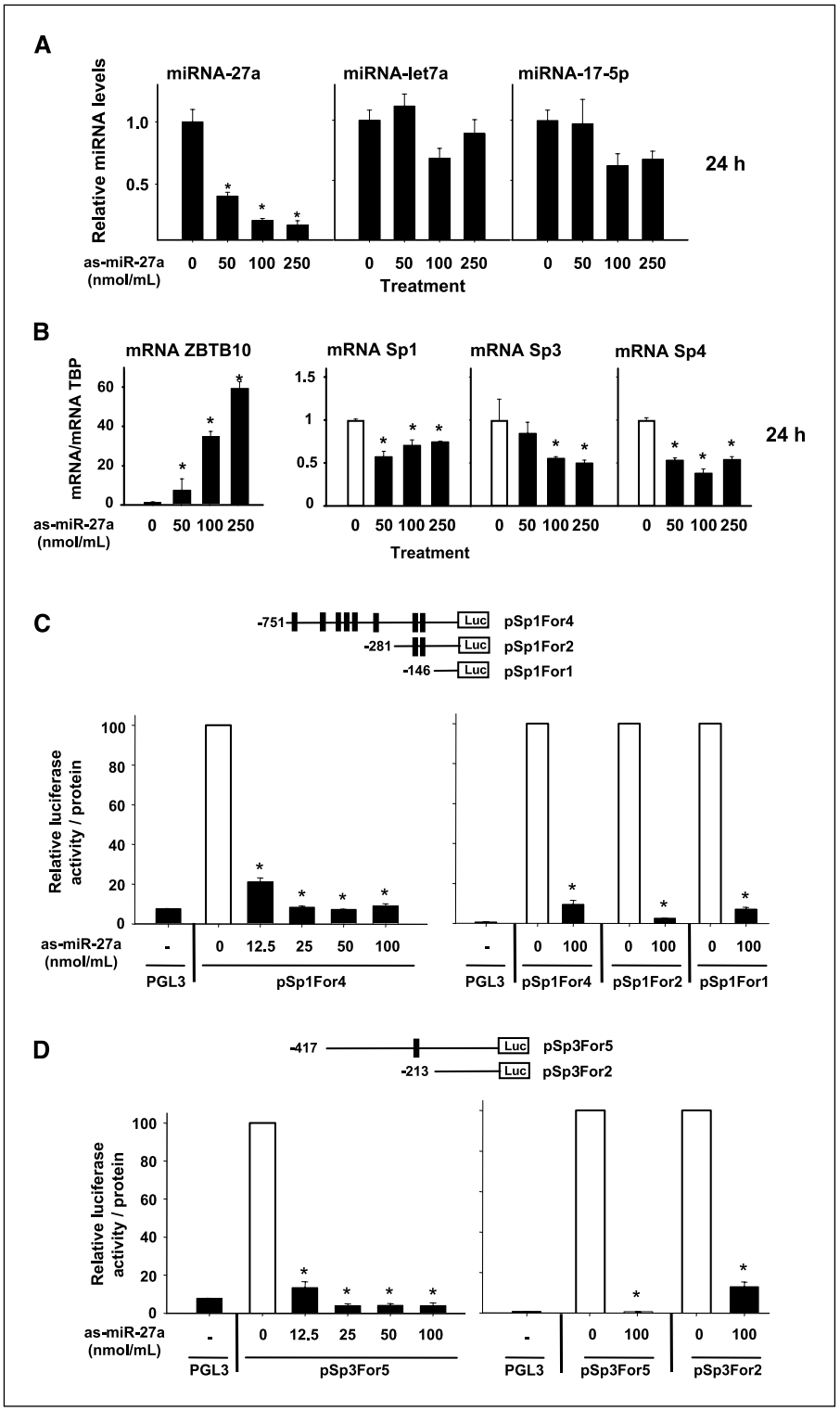

Figure 2. Effects of as-miR-27a on ZBTB10, Sp1, Sp3, and Sp4 expression in MDA-MB-231 cells. *A,* specificity of as-miR-27a. MDA-MB-231 cells were transfected with 0 to 250 nmol/mL as-miR-27a and analyzed for miR-27a, let-7a, and miR-17-5p expression by real-time PCR as described in Materials and Methods. *Columns,* means for three replicate determinations for each miRNA; *bars,* SE. *, $P < 0.05$, significantly decreased activity. *B,* as-miR-27a decreases ZBTB10 and Sp expression. MDA-MB-231 cells were transfected with 0 to 250 nmol/mL as-miR-27a and after 24 h, ZBTB10, Sp1, Sp3, and Sp4 mRNA levels were determined by real-time PCR as described in Materials and Methods. *Columns,* means; *bars,* SE. *, $P < 0.05$, significantly decreased expression. As-miR-27a decreases Sp1 (*C*) and Sp3 (*D*) promoter activity. MDA-MB-231 cells were transfected with as-miR-27a and Sp promoter constructs, and luciferase activity was determined as described in Materials and Methods. *Columns,* mean for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased activity. A scrambled oligonucleotide was transfected in the 0 treatment group in *A* and *B* (250 nmol/mL) and *C* and *D* (100 nmol/mL).

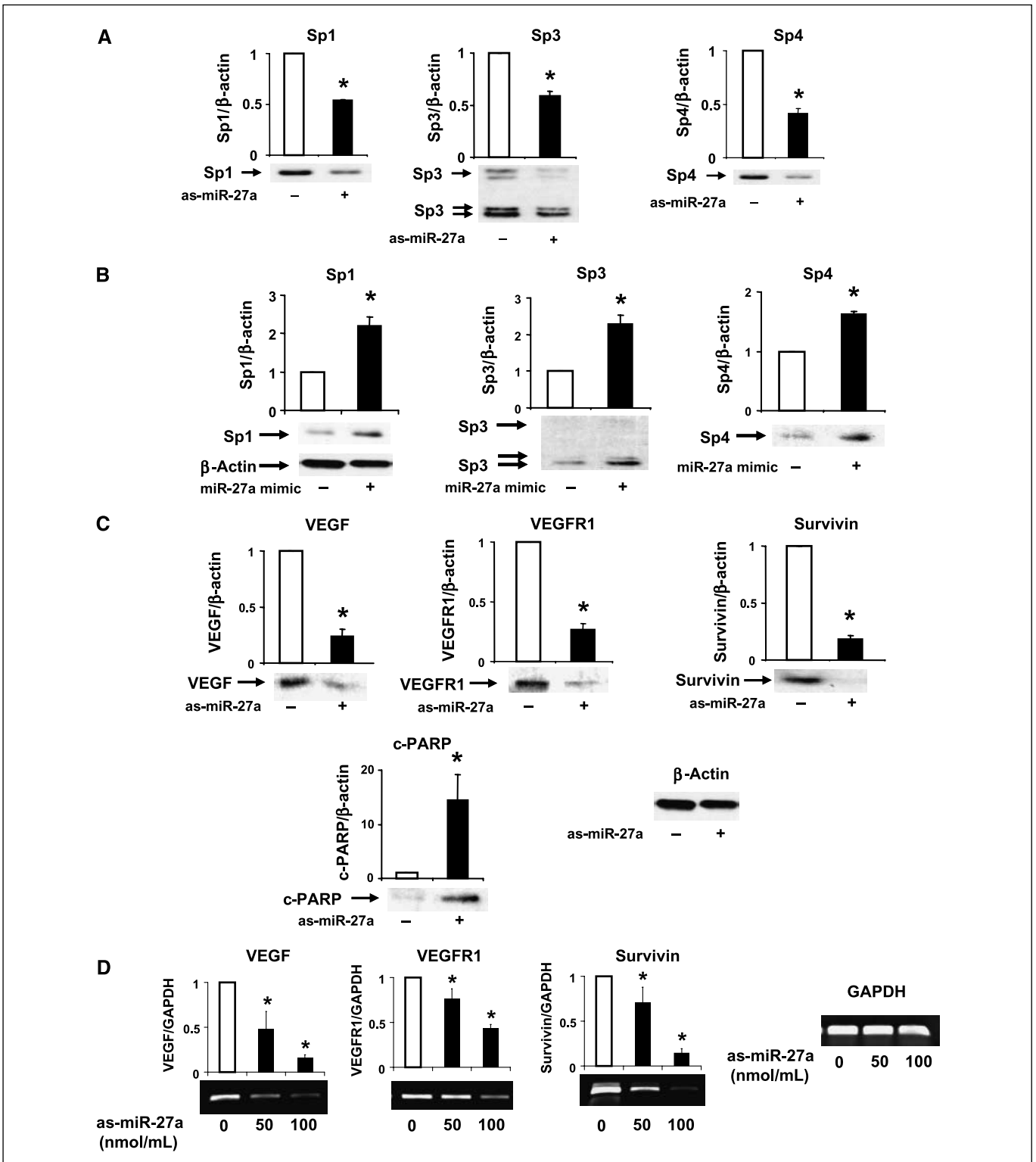

Figure 3. Role of miR-27a in expression of Sp and Sp-dependent genes/proteins. As-miR-27a decreases (*A*) and miR-27a mimic (*B*) increases Sp1, Sp3, and Sp4 proteins. MDA-MB-231 cells were transfected with 100 nmol/mL as-miR-27a or the miR-27a mimic (100 nmol/mL) and after 24 h, whole-cell lysates were analyzed by Western blot analysis for Sp1, Sp3, and Sp4 proteins as described in Materials and Methods. Relative protein expression was normalized to β-actin and levels in control cells were set at 1.0. *Columns,* means for at least three replicate determinations for each treatment group; *bars,* SE. *, significantly decreased (*A*) or increased (*B*) protein expression. *C,* expression of Sp-dependent proteins/responses. MDA-MB-231 cells were transfected with 100 nmol/mL as-miR-27a and protein expression was determined as described in *A. Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased expression. *D,* effects of as-miR-27a on survivin, VEGF, and VEGFR1 mRNA expression. MDA-MB-231 cells were transfected with as-miR-27a and mRNA levels were determined by real-time PCR as described in Materials and Methods. *Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased activity. The 0 treatment or untreated groups in *A* to *D* were transfected with 100 nmol/mL of scrambled oligonucleotide and served as controls for the experiments.

the cell cycle suggest that miR-27a may also modulate expression of genes/proteins that inhibit cells from progressing past G_2-M phase. The list of possible targets for miR-27a (40–43) includes two genes, *Wee-1* and *Myt-1*, that inhibit cdc2 and the cdc2/cyclin B–mediated G_2-M phase progression. Results summarized in Fig. 6*C* show that as-miR-27a also increases *Myt-1* (but not Wee-1) mRNA levels. Moreover, as-miR-27a also enhanced phosphorylation of cdc2 as determined using an antibody against phosphotyrosine-15 of cdc2, which is the inactive phosphorylated cdc2 (Fig. 6*D*). These results account for the effects of as-miR-27a on blocking cells at G_2-M in the cell cycle and show that in addition to *ZBTB10, Myt-1* is also regulated by miR-27a in MDA-MB-231 cells, and suppression of this G_2-M checkpoint gene by miR-27a contributes to the proliferative phenotype of these ER-negative breast cancer cells. These results show that miR-27a is an oncogenic miRNA through suppression of *ZBTB10* and *Myt-1*, and transfection of as-miR-27a induces multiple growth inhibitory, apoptotic, and antiangiogenic genes and pathways in MDA-MB-231 cells.

Discussion

MiRNAs are a class of small RNAs that play an important role in regulating expression of genes in various living systems. MiRNAs are found in plants and animals and are derived from longer than 70- to 100-base hairpin nucleotide precursors by cytoplasmic RNA III dicer-dependent cleavage to give ~22 nucleotide duplexes (26, 27, 44–47). One strand of the RNA duplex is degraded and the remaining strand serves as a functional miRNA that binds to 3′-UTR of target mRNAs to inhibit translation or induce mRNA degradation. The extent of miRNA regulation of human genes is unclear; however, computational studies that match miRNA sequences that overlap with 6 to 8 bp

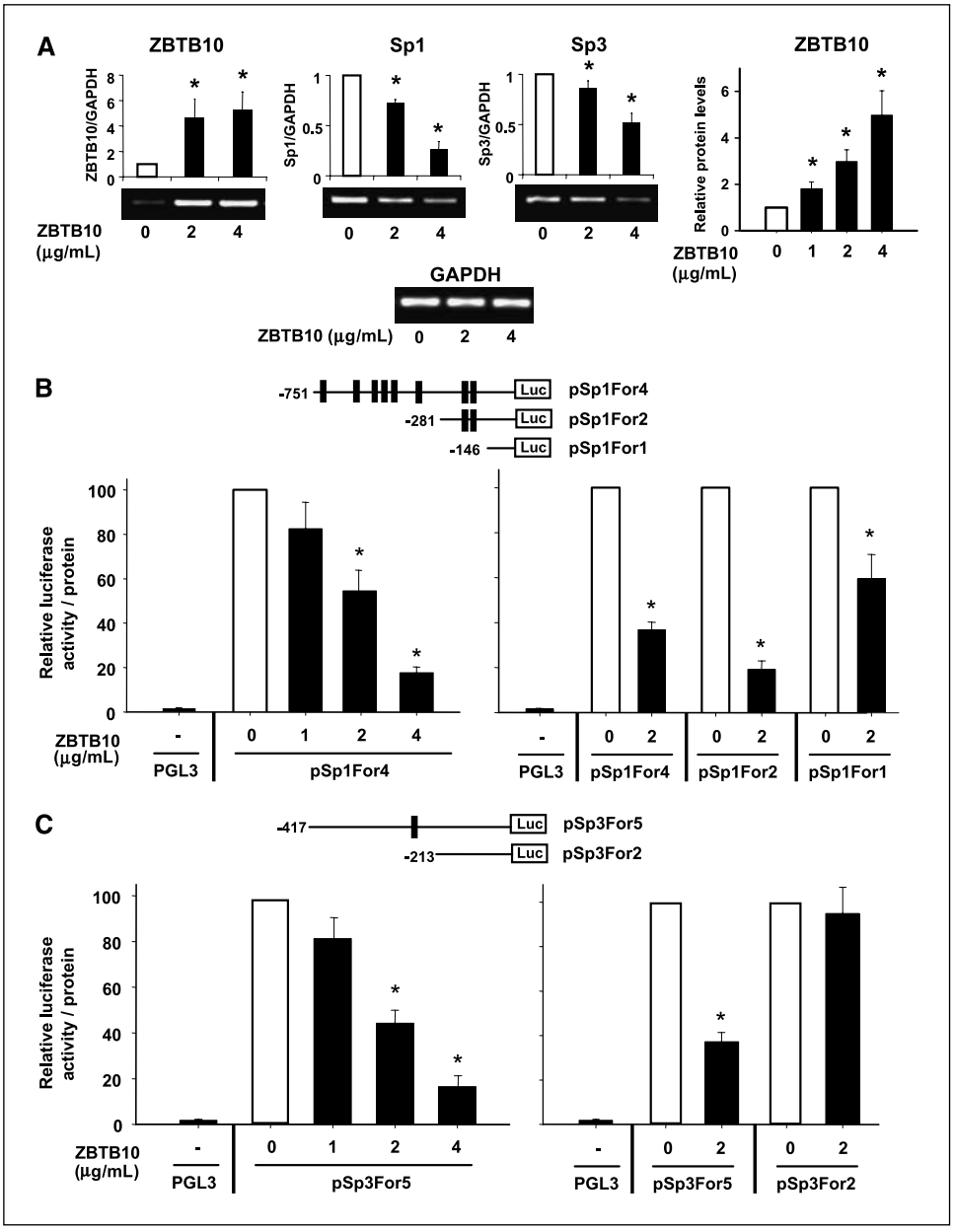

Figure 4. Effects of ZBTB10 on Sp and Sp-dependent gene expression. *A,* ZBTB10 expression decreases Sp mRNA levels and increases ZBTB10 protein. MDA-MB-231 cells were transfected with different amounts of ZBTB10 expression plasmid and after 24 h, mRNA or protein was extracted and levels of expression were determined by semiquantitative RT-PCR or ELISA as described in Materials and Methods. *Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased/increased mRNA levels (normalized to GAPDH mRNA) or ZBTB10 protein compared with cells not transfected with ZBTB10. ZBTB10 expression decreases Sp1 (*B*) and Sp3 (*C*) promoter activity. MDA-MB-231 cells were transfected with various constructs, and ZBTB10 expression plasmid and luciferase activities were determined as described in Materials and Methods. The empty vector (pCMV6-XL4; 4 μg/mL) was used in cells transfected with pSp1For4 and pSp3For5. *Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased activity.

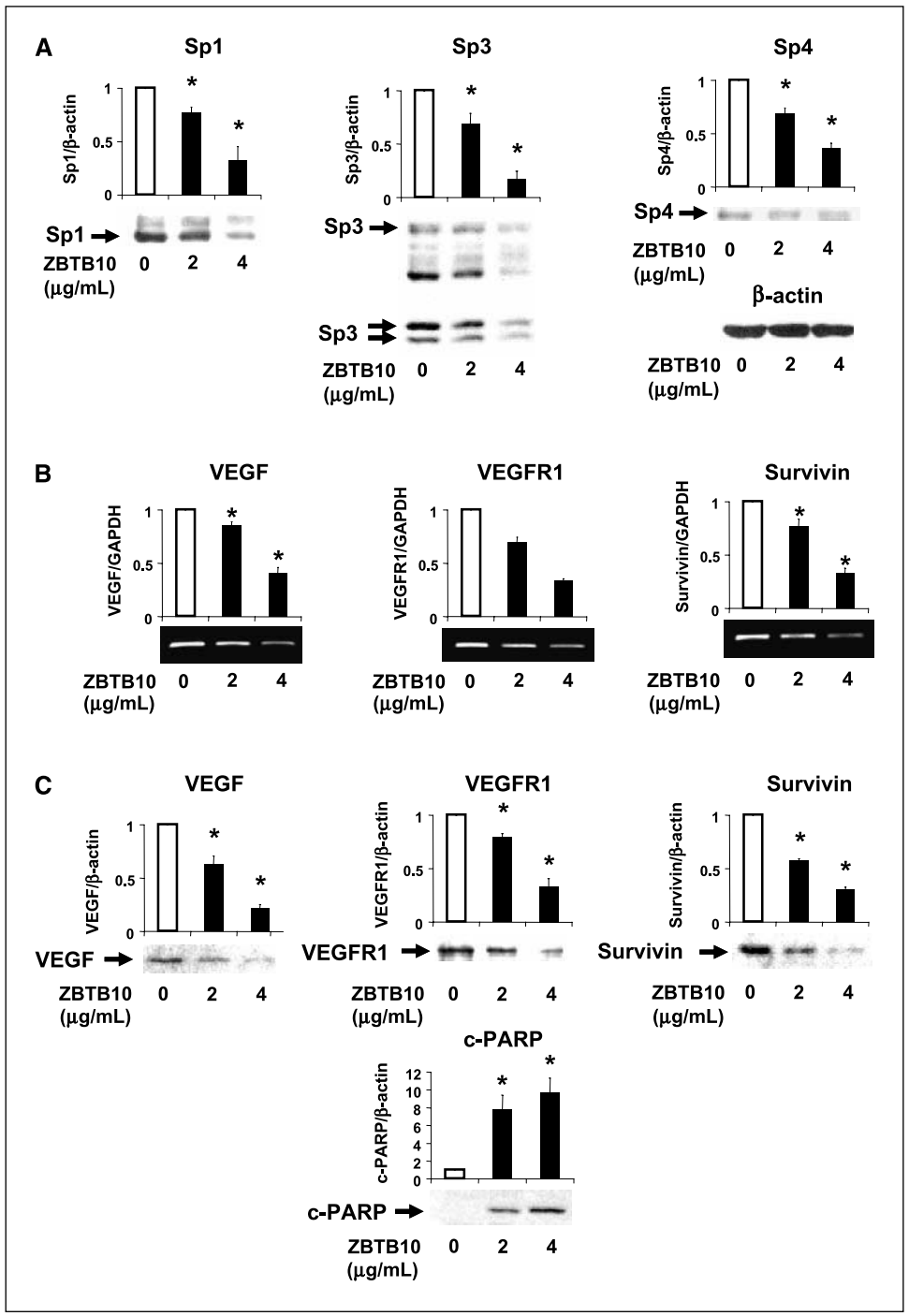

Figure 5. ZBTB10 decreases expression of Sp proteins and Sp-dependent angiogenic and survival genes. *A,* ZBTB10 expression decreases expression of Sp1, Sp3, and Sp4 proteins. MDA-MB-231 cells were transfected with ZBTB10 expression plasmid and after 24 h, whole-cell lysates were analyzed by Western blots as described in Materials and Methods. *Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased protein expression. ZBTB10 decreases expression of angiogenic/survival genes (*B*) and proteins (*C*). MDA-MB-231 cells were transfected with ZBTB10 expression plasmid and after 24 h, mRNA and protein were extracted and analyzed by semiquantitative RT-PCR and Western blots, respectively, as described in Materials and Methods. *Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significantly decreased activity.

in the 3′ UTR of human mRNAs suggest that each miRNA can potentially interact with an average of 200 target mRNAs (41, 42). The precise functions of individual miRNAs and their mRNA partners in normal and diseased tissue are being extensively investigated. Recent reports on transgenic animals deficient in specific miRNA expression have shown the critical physiologic importance of these regulatory RNA molecules (48–51). For example, miR-208 is a cardiac-specific miR and knockdown of miR-208 in mice significantly modulates expression of both α- and β-myosin heavy chain expression and cardiac response to both stress and hypothyroidism (48).

MiRs have been extensively investigated in tumors and cancer cells, and tumor-specific miR profiles have been identified for applications in diagnosis and prognosis of cancers (52–55). It is also clear that miRs exhibit both tumor-suppressor and oncogenic activities and have been directly linked to regulation of genes involved in these pathways. For example, miR-21 interacts with the 3′ UTR of the tumor-suppressor tropomyosin 1 (TPM1) in breast cancer cells, and miR-21–dependent down-regulation of TPM1 expression by miR-21 plays a role in the oncogenic activity of this miR (56).

Overexpression of Sp proteins in tumors and cancer cell lines has been linked to their regulation of Sp-dependent growth-promoting,

survival, and angiogenic genes (20–25), and we hypothesized that an oncogenic miR may contribute to Sp protein overexpression. Moreover, a recent report showing that miR-27a suppressed the "putative" Sp repressor *ZBTB10* (28) suggested that miR-27a may play a role in overexpression of Sp proteins. Tillotson (29) previously reported that like Sp1, *ZBTB10* interacts with GC-rich Sp binding sites; however, in Schneider SL2 cells, *ZBTB10* does not directly activate a GC-rich promoter construct but significantly decreases Sp1-dependent activation of this construct. Our studies in a panel of breast cancer cell lines has identified miR-27a expression in these cells along with relatively high levels of Sp1, Sp3, and Sp4 mRNA compared with lower levels of *ZBTB10* mRNA expression (Fig. 1). Sp1, Sp3, and Sp4 proteins are also expressed in breast cancer cells as previously reported (57), and levels of Sp4 were highly variable among this panel of breast cancer cells (Fig. 1*C*).

The potential role of miR-27a in regulating expression of Sp1, Sp3, and Sp4 and Sp-dependent genes/proteins was determined in MDA-MB-231 cells transfected with as-miR-27a (Figs. 2 and 3) or *ZBTB10* expression plasmid (Figs. 4 and 5). The results from both studies were complementary; as-miR-27a and *ZBTB10* overexpression decreased Sp1, Sp3, and Sp4 mRNA and protein levels and as-miR-27a also increased *ZBTB10* mRNA. In addition, we also showed that in MDA-MB-231 cells transfected with GC-rich Sp1 and Sp3 promoter constructs, decreased activity was observed after cotransfection with as-miR-27a or *ZBTB10* overexpression. The only exception to the parallel effects of *ZBTB10* and as-miR-27a was the failure of *ZBTB10* to decrease expression of luciferase activity in cells transfected with a construct (pSp_3For2; Fig. 4*C*) that did not contain GC-rich sites. This suggests that another protein/gene suppressed by miR-27a may be involved and this is currently being investigated. In parallel studies, we observed that decreased expression of Sp1, Sp3, and Sp4 was also accompanied by decreased expression of angiogenic genes (*VEGF* and *VEGFR1*) and survivin, and this was accompanied by increased caspase-dependent PARP cleavage (apoptosis). Previous studies have shown that Sp proteins regulate expression of *VEGF, VEGFR1*, and survivin and induce apoptosis (20–25, 39). However, in this study, which involves *ZBTB10*-dependent repressive effects at GC-rich gene

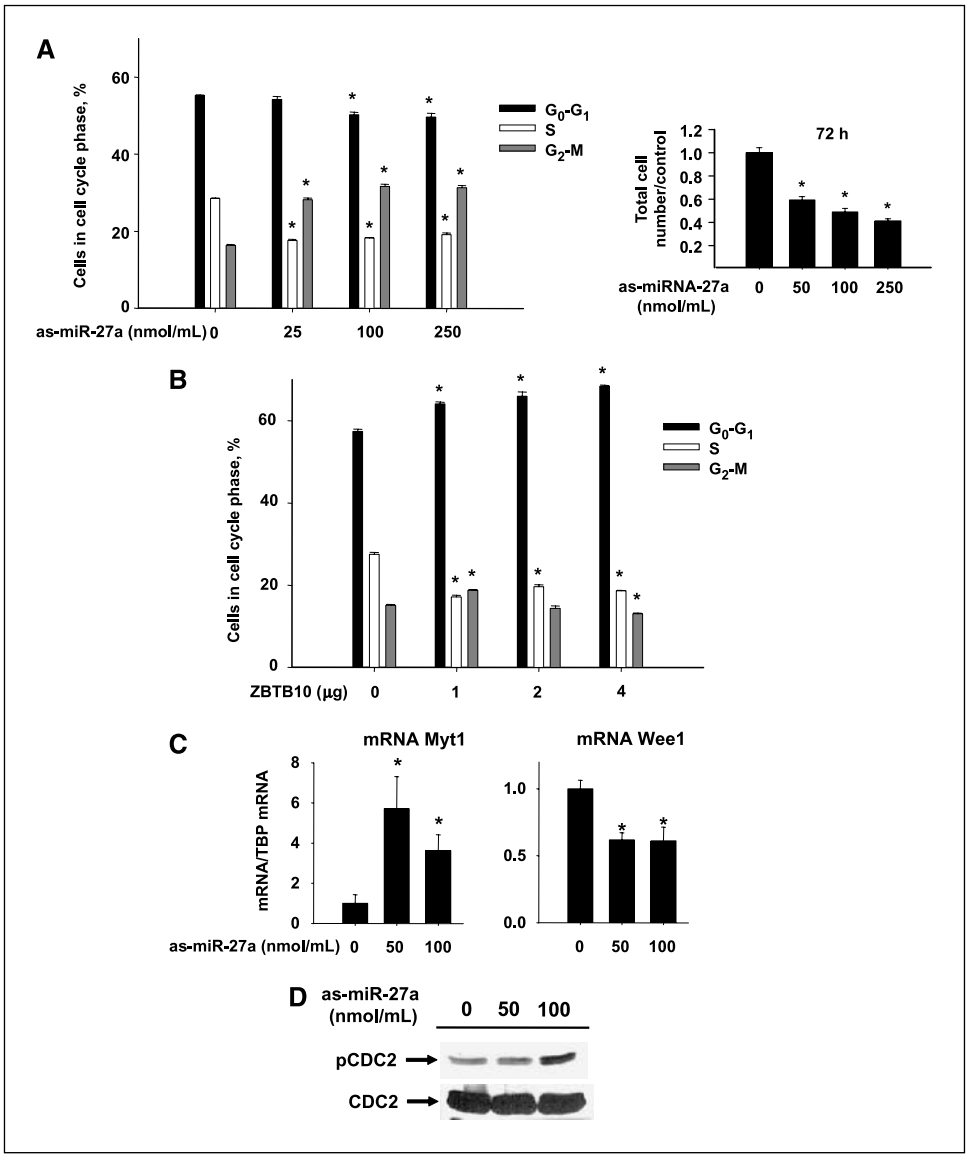

Figure 6. Modulation of cell proliferation and the cell cycle by as-miR-27a and ZBTB10. MDA-MB-231 cells were transfected with different concentrations of as-miR-27a (*A*) or ZBTB10 expression plasmid (*B*). The effects of as-miR-27a on cell proliferation were determined after 72 h. In addition, after 24 h, cells were examined by FACS analysis and the percentage of cells in G_0-G_1, S, and G_2-M phases of the cell cycle were determined as described in Materials and Methods. *Columns,* means for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significant changes induced by as-miR-27a or ZBTB10 compared with control cells. Effects of as-miR-27a on cdc2 inhibitors Myt-1 and Wee-1 mRNA (*C*) and cdc2 protein phosphorylation (*D*). MDA-MB-231 cells were treated for 24 h with as-miR-27a (50 or 100 nmol/mL), and mRNA levels (real-time PCR) and cdc2/phospho-cdc2 protein (Western blot) were determined as described in Materials and Methods. *Columns,* mean mRNA levels for three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significant induction. Phosphorylated cdc2 (*D*) was increased by ~2-fold in replicate experiments. The 0 treatment groups in *A, C*, and *D* were transfected with 100 nmol/mL of scrambled oligonucleotide.

promoters, decreased *VEGF, VEGFR1*, and *survivin* expression are undoubtedly due to both decreased Sp proteins and the direct effects of *ZBTB10* on these genes/gene promoters as previously described for the gastrin promoter (29).

The effects of as-miR-27a and *ZBTB10* overexpression on distribution of MDA-MB-231 cells in G_0-G_1, S, and G_2-M phases of the cell cycle (Fig. 6) were different. *ZBTB10* overexpression blocked G_0-G_1 to S phase progression (Fig. 6*B*) and these results were consistent with previous studies in MCF-7 cells using RNA interference (small inhibitory RNA for Sp1), which also inhibited the same step in the cell cycle (25). Although transfection of as-miR-27a in MDA-MB-231 cells also decreased cell proliferation and the percentage of cells in S phase, the major effect was the accumulation of cells in G_2-M. The failure of cells to undergo mitosis is associated with a G_2-M block and decreased activity of cdc2/cyclin B, which is required for this phase of the cell cycle. Interestingly, two genes, *Wee-1* and *Myt-1*, that are important regulators of cdc2/cyclin B activity are also potential targets of miR-27a because their 3′ UTRs contain potential miR-27 docking sites. Both *Wee-1* and *Myt-1* are kinases that phosphorylate residues on cdc2, resulting in the inhibition of cdc2/cyclin B–dependent initiation of mitosis (58–60), and results in Fig. 6*C* show that as-miR-27a induces *Myt-1* but not *Wee-1* gene expression. This response is accompanied by an increase in *Myt-1* protein and increased phosphorylation of cdc2 (Fig. 6*D*), which is consistent with the as-miR-27a–induced accumulation of MDA-MB-231 cells in G_2-M.

Our results show that miR-27a exhibits oncogenic activity through regulating *ZBTB10*, which, in turn, results in overexpression of Sp proteins and Sp-dependent genes that are important for cell survival and angiogenesis. However, we also observed that miR-27a suppresses the cdc2/cyclin B inhibitor *Myt-1* in MDA-MB-231 cells and thereby facilitates breast cancer cell proliferation by repressing a gene that blocks cancer cell division by arresting cells at G_2-M. Thus, the oncogenic activity of miR-27a in MDA-MB-231 cells is due, in part, to suppression of *ZBTB10* and *Myt-1*. Current studies are investigating other targets for miR-27a and development of novel approaches for modulating cellular expression of miR-27a in cancer cells and tumors and thereby inhibiting cancer growth through blocking the activity of this oncogenic miRNA.

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The Oncogenic microRNA-27a Targets Genes That Regulate Specificity Protein Transcription Factors and the G $_2$-M Checkpoint in MDA-MB-231 Breast Cancer Cells

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