

MicroRNA miR-183 Functions as an Oncogene by Targeting the Transcription Factor *EGR1* and Promoting Tumor Cell Migration

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Abstract

The transcription factor *EGR1* is a tumor suppressor gene that is downregulated in many cancer types. Clinically, loss of *EGR1* translates to increased tumor transformation and subsequent patient morbidity and mortality. In synovial sarcoma, the SS18-SSX fusion protein represses *EGR1* expression through a direct association with the *EGR1* promoter. However, the mechanism through which *EGR1* becomes downregulated in other tumor types is unclear. Here, we report that *EGR1* is regulated by microRNA (miR)-183 in multiple tumor types including synovial sarcoma, rhabdomyosarcoma (RMS), and colon cancer. Using an integrative network analysis, we identified that miR-183 is significantly overexpressed in these tumor types as well as in corresponding tumor cell lines. Bioinformatic analyses suggested that miR-183 could target *EGR1* mRNA and this specific interaction was validated *in vitro*. miR-183 knockdown in synovial sarcoma, RMS, and colon cancer cell lines revealed deregulation of a miRNA network composed of *miR-183-EGR1-PTEN* in these tumors. Integrated miRNA- and mRNA-based genomic analyses indicated that miR-183 is an important contributor to cell migration in these tumor types and this result was functionally validated to be occurring via an *EGR1*-based mechanism. In conclusion, our findings have significant implications in the mechanisms underlying *EGR1* regulation in cancers. miR-183 has a potential oncogenic role through the regulation of 2 tumor suppressor genes, *EGR1* and *PTEN*, and the deregulation of this fundamental miRNA regulatory network may be central to many tumor types. *Cancer Res*; 70(23); 9570–80. ©2010 AACR.

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that posttranscriptionally regulate gene expression (1) and play significant roles in maintaining normal cellular functions (2). Deregulation of miRNA expression leads to diverse disease types, including cancers (3) as exemplified by their differential expression in carcinomas (4), sarcomas (5, 6), and hematologic tumors (7). They have been demonstrated to regulate the expression levels of major cancer-related genes and hence may be useful in the treatment of cancer (8, 9). Furthermore, miRNAs are also stably present in serum; as a consequence, they are being investigated as potential cancer-specific biomarkers (10). It has been proposed that depending on the role

of the mRNA targets, miRNAs can function either as tumor suppressors or as oncogenes (11).

Losses of common tumor suppressor genes are implicated in a wide range of cancers. For example, decreased levels of tumor suppressor genes, such as *EGR1* and *PTEN*, have been associated with tumors such as sarcomas, colon tumors, and brain tumors (12–15). On the basis of this general concept, we hypothesized that transcriptional perturbations in specific miRNA(s) observed across multiple tumor types regulate the levels of important tumor suppressors in these tumor types. Recently, we developed a comprehensive database to catalog the miRNA expression profiles in various sarcoma types (www.oncomir.umn.edu; ref. 6). This provided a platform to compare the expression profiles across different tumors (synovial sarcoma, rhabdomyosarcoma, and colon cancer) and identified miR-183 as a potential oncogene in these tumor types.

As a member of an evolutionarily conserved miRNA cluster (miR-183, miR-96, and miR-182), miR-183 is located on human chromosome 7 and has been implicated in key cellular functions such as neurosensory development (16). In general, miRNAs pair with mRNA targets within the 3'UTR (untranslated region; ref. 17). A recent study, however, has shown that miR-183 can bind to the coding region of betaTrCP1 to regulate its expression (18). Previously, we and other groups have shown that miR-183 is significantly upregulated in colon cancer (19, 20). In the current study,

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>). A.L. Sarver and L. Li contributed equally to this work.

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using a network analysis, we identified similar overexpression patterns of miR-183 in sarcomas such as synovial sarcoma and rhabdomyosarcoma (RMS). To gain better insight into the convergent expression of miR-183, we performed *in vitro* target and functional characterization, as well as integrative genomic analyses. This revealed that miR-183 functions as a potential oncogene in specific sarcoma types and in colon cancer by directly or indirectly regulating *EGRI* and *PTEN* expression levels, respectively. In addition, we show that knockdown of miR-183 in the corresponding tumor cell lines affect cellular migration.

Materials and Methods

Details of constructs and primers are given as Supplementary Information.

Network analysis

We have previously generated miRNA expression profiles for over 300 sarcomas representing 22 sarcoma types (6) and for 80 colon tumors compared to 28 normal colon tissues (19). Using both these comprehensive data sets, we obtained miRNAs that showed a greater than 2-fold increase in colon tumors in comparison with normal colon tissues and also selected miRNAs that showed a greater than 2-fold increase in RMS relative to normal skeletal muscle. Furthermore, we also obtained miRNAs that showed a greater than 4-fold increases in synovial sarcoma. In all the cases, differentially expressed miRNA were required to be statistically significant following Bonferroni correction to the multiple testing problem. Cytoscape (21) was used to visualize the network generated by using all the selected miRNAs and tumors as nodes. The network is presented to highlight the miRNAs that are common between all the selected tumor nodes.

Cell lines and culture conditions

Synovial sarcoma cell lines SYO-1 and FUJI; colon cancer cell lines HCT116 and DLD1; Alveolar RMS (ARMS) Rh30, and embryonal RMS (ERMS) JR1 cells were used in this study. Synovial sarcoma cell lines FUJI and SYO-1 that were authenticated by the presence of SSX-SYT fusion transcript were obtained from Dr. Torsten Nielsen (University of British Columbia, originally established in Dr. Kazuo Nagashima, Hokkaido University School of Medicine, and Dr. Akira Kawai, National Cancer Centre Hospital, respectively). ARMS cell line Rh30 was obtained from ATCC (CRL-2061), and ERMS cell line JR1 was provided by Dr. Peter Houghton (St. Jude's Children's Research Hospital). Colon cancer cells HCT116 and DLD1 were kindly provided by Drs. Clifford Steer and David Largaespada (University of Minnesota), respectively. RMS and colon cancer cell lines were not authenticated in our laboratory.

Reporter constructs

Two sets of luciferase reporter systems (*psiCHECKTM2*-based vectors and *EGRI* 3'UTR reporter) were used to validate miRNA and mRNA pairing and targeting.

Cell transfection and dual-luciferase reporter assay

Three modes of transfection (Lipofectamine 2000, HiPerFect, and Attractene transfection reagents) were used in this study. The Dual-Luciferase Reporter Assay System (Promega) was used to measure luciferase activity of cells transfected with either *psiCHECK2* or *EGRI*-3'UTR-sGG and pGL4.73 control vector using a Synergy2 luminometer (BioTek).

Quantitative real-time PCR

Real-time PCR was performed on LightCycler 480 (Roche) using miScript real time PCR kit (Qiagen). Total RNA was extracted from tumor samples or cultured cells using a mirVana total RNA isolation kit (Ambion, Applied Biosystems; ref. 5).

Total protein extraction and Western blotting

Standard protein extraction and Western blotting techniques were used for the analysis.

Tumor tissue samples and RNA isolation

Frozen sarcoma tissue samples were obtained through the tissue procurement facility of the University of Minnesota. Total RNA was isolated from 75 to 100 mg of frozen tissue using the miRvana total RNA isolation kit.

Genome-wide mRNA expression profiling

mRNA expression profiles were studied using the human HT-12 Beadchip (Illumina Inc.; ref. 22). Heat maps were generated from the CDT files generated by Cluster3.0 using Java Treeview (23).

Wound healing/scratch test

Wound healing experiments were conducted on the basis of the protocol of Wang et al. (24), with minor modifications.

Cell proliferation assay

CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used according to manufacturer's instruction with minor modification.

Cell migration and invasion assay

Cell migration assay (Cultrex, Trevigen) and CultureCoat 24-well BME-coated cell invasion assay (Cultrex, Trevigen) were used to investigate cell migration as well as invasion following the manufacturer's instructions with minor modifications.

Cell-cycle assay

After anti-miR-183 treatment, cells were harvested by trypsin and washed with PBS. Seventy percent of precold (-20°C) ethanol in PBS was used to resuspend the cells and cells were stored at -20°C at least 30 minutes. Cells were centrifuged to remove ethanol, washed with PBS, and then resuspended with 2 $\mu\text{g}/\text{mL}$ of propidium iodide (MP Biomedicals) and 200 $\mu\text{g}/\text{mL}$ of RNase A (Qiagen) in PBS. Cell-cycle assay was conducted on BD FACSCalibur (BD Biosciences) and analyzed with Flowjo 7.5.5.

Results

miR-183 is deregulated in a wide range of tumor types and in the corresponding tumor cell lines

Following a comprehensive analysis of data from a large scale miRNA expression profiling experiment (6), we noticed that diverse types of malignant tumors, such as synovial sarcoma, RMS (ARMS and ERMS), and colon cancer, share a common set of perturbations in miRNA transcript levels relative to normal tissues and other sarcoma types. To further investigate this initial observation and systematically define the most central miRNAs relevant to tumorigenesis, we generated a network using tumors and their corresponding miRNAs as nodes connected by edges representing statistically significant expression fold increases in the miRNAs observed in these tumors. Analyses of the network topology revealed that miR-183 was upregulated in all 4 tumors, suggesting that miR-183 plays a central role in the tumorigenesis process (Fig. 1A). miR-183 was the only miRNA that occupied a central position in the network of all 4 tumor types. Another candidate, miR-135b, was induced in 3 of the tumor types analyzed (excluding ERMS). In addition, miR-183 cluster members miR-96 and miR-182 were also found at increased levels in both synovial sarcoma and colon cancer (Fig. 1A). Relative to corresponding normal/control tissues, miR-183 levels were increased in colon cancer (2.6-fold), ERMS (4-fold), ARMS (5-fold), and synovial sarcoma (10-fold). These increases were all highly statistically significant even after Bonferroni correction to the multiple testing problem ($P < 0.00004$; Fig. 1B).

Next, we proceeded to determine whether the cell lines (SYO-1, FUJI, RH30, JR1, DLD1, and HCT116) derived from synovial sarcoma, RMS, and colon cancer shared the high levels of miR-183 expression and thus could be useful for further *in vitro* functional analysis. miR-183 was 4- to 20-fold higher in the cell lines relative to normal skeletal muscle as measured by qRT-PCR, recapitulating the tumor tissue miR-183 upregulation (Fig. 1C). miR-182 was also shown to have increased levels in these cell lines.

To further examine whether the overexpressed miR-183 and its cluster member miR-182 are functionally active in the tumor cell lines, we generated luciferase reporter constructs containing reverse complementary sequences to miR-183 and miR-182, as well as their corresponding scrambled sequences, fused to the 3'UTR of luciferase. Luciferase levels decreased ~3-fold in FUJI (synovial sarcoma) cells following transfection of reporter constructs with sequences complementary to either miR-183 or miR-182, whereas the scrambled constructs showed luciferase levels similar to the empty vector (Fig. 2A). These data indicate that miR-183 and miR-182 are functionally relevant and are actively involved in sequence-specific target regulation in these cells.

miR-183 regulates *EGRI* levels through binding its 3'UTR

We then hypothesized that the significant increases in miR-183 transcript levels act mechanistically via attenuation

of the transcript level/translational status of a tumor suppressor. Examination of the miRGen miRNA target database V3.0 (25) revealed that 1,207 genes have the potential to interact with miR-183, as predicted by at least 1 of the target prediction algorithms. To further narrow the miR-183 target gene list, we analyzed the consensus target gene list predicted by multiple target prediction methods. Forty-four mRNAs were predicted to interact with miR-183 by at least 4 different target prediction algorithms (Supplementary Information 1).

EGRI, a tumor suppressor gene, was among the top ranking target genes for miR-183. We have previously reported decreases in *EGRI* levels in synovial sarcoma (15) and others have implicated decrease in *EGRI* in a wide range of tumors (26–29). To verify whether *EGRI* is a bona fide target of miR-183, we transfected a reporter containing the 3'UTR of *EGRI* in a variety of tumor cell lines, such as FUJI and SYO-1 (synovial sarcoma) and RH30 and JR1 (RMS), all of which expressed high levels of miR-183. The principle behind this experiment is that if *EGRI* is a genuine target of miR-183, transfection of a reporter construct containing the *EGRI*-3'UTR should show relative decreases in the luciferase activity compared to the empty control vector. As expected, all 4 tumor cell lines tested with the *EGRI*-3'UTR reporter showed 2- to 10-fold decrease in luciferase activity in comparison to the control cells (Fig. 2B), supporting our hypothesis that miR-183 can potentially regulate *EGRI*.

Further to determine whether the 3'UTR of *EGRI* directly interacts with miR-183, we transfected surrogate HEK293 cells with the *EGRI*-3'UTR reporter construct alone and compared these cells to HEK293 cells cotransfected with reporter and miR-183 mimics. We observed a significant decrease in luciferase levels following cotransfection, suggesting a direct interaction of miR-183 with the 3'UTR of *EGRI* (Fig. 2C). On the contrary, cotransfection with miR-183 mimics harboring 2 point mutations served as negative controls and showed minimal decrease in reporter activity. To provide further evidence that miR-183/*EGRI* 3'UTR interaction is direct, we introduced synonymous mutations in both the 3'UTR sequences in the reporter construct as well as the miR-183 mimics and performed similar cotransfection experiments. The modified reporter/mimic combination with perfect base pairing led to significant decreases in reporter activity, confirming the existence of a direct interaction between miR-183 and the 3'UTR of *EGRI* (Fig. 2C).

Knockdown of miR-183 leads to increases in *EGRI* and *PTEN* mRNA levels

Next, we determined that whether a decrease in miR-183 expression would modulate *EGRI* at the transcriptional level. For this, we knocked down miR-183 levels using anti-miR-183 in a series of tumor cell lines (SYO-1, FUJI, RH30, JR1, DLD1, and HCT116) that we previously showed had elevated levels of miR-183. We achieved up to 80% knockdown of miR-183 in the anti-miRs-treated cells (Supplementary Information 2). Treatment with anti-miR-183 resulted in a greater than 2-fold increases in *EGRI* transcript levels in SYO-1 and HCT116 cells, showed no significant changes in *EGRI* mRNA levels in FUJI, JR1, or RH30

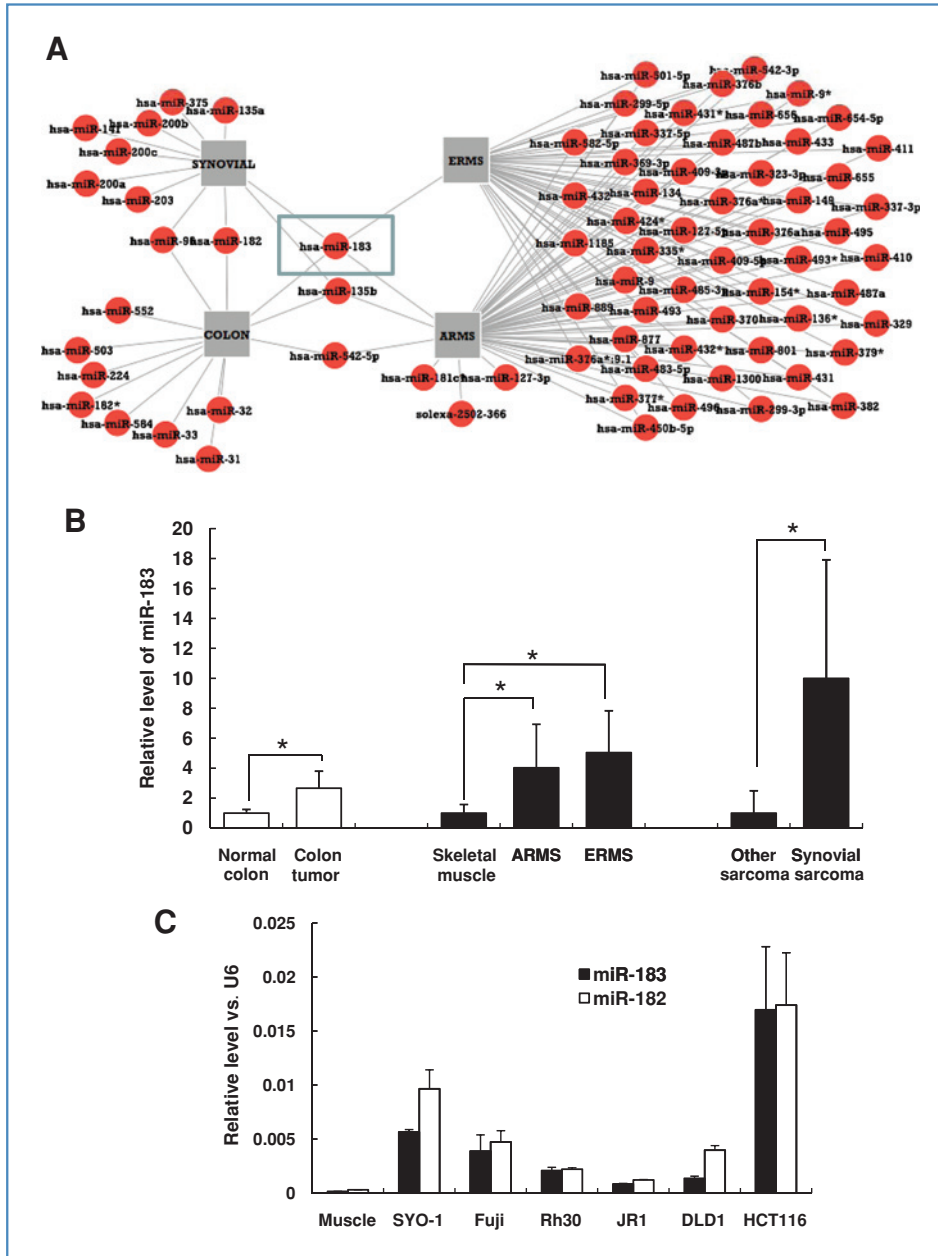


Figure 1. miR-183 is significantly upregulated in multiple tumor types. A, network analysis of miRNAs significantly increased in synovial sarcoma, RMS (ARMS and ERMS), and colon cancer. Significantly upregulated miRNAs from colon cancer are shown relative to normal colon (fold changes > 2); ARMS and ERMS are shown relative to skeletal muscle (fold changes > 4); and synovial sarcoma is shown relative to other sarcoma types (fold changes > 4). All miRNAs were highly significant as determined using a Bonferroni correction for multiple comparisons. miR-183 was the only miRNA induced in all 4 tumor types included in this analysis. B, relative fold change of miR-183 derived from miRNA profiling data. Open columns, average fold change in colon cancer cells ($n = 80$) relative to normal colon tissue ($n = 28$; P value = $1.0e-15$); gray columns, ARMS ($n = 19$; p -value = $2.28e-5$) and ERMS ($n = 23$; p -value = $1.46e-6$) relative to normal skeletal muscle ($n = 7$); and black columns, synovial sarcoma ($n = 16$; P value < $1.0e-15$) relative to other sarcomas ($n = 73$). C, quantitative real-time PCR analysis of miR-183 in synovial sarcoma, RMS, and colon cancer cell lines. miR-183 levels were normalized to those of normal skeletal muscle. Note the 4- to 8-fold elevated expression of miR-183 in all the tumor cells, consistent with the observation from tumor tissues.

cells, and showed a decrease in *EGR1* mRNA levels in DLD1 cells as measured by qRT-PCR (Fig. 3A). *EGR1* is a transcriptional activator of *P TEN* (30). Hence, we analyzed whether knockdown of miR-183 can increase *P TEN* mRNA, in these cell lines. Treatment with anti-miR-183 resulted in increases in

P TEN levels of a greater than 2-fold in SYO-1, FUJI, RH30, DLD1 and HCT116 cells but did not affect the transcript levels in JR1 cells (Fig 3A). These results suggest that miR-183 affects both *EGR1* mRNA transcript levels as well as *EGR1* protein levels via translational regulation.

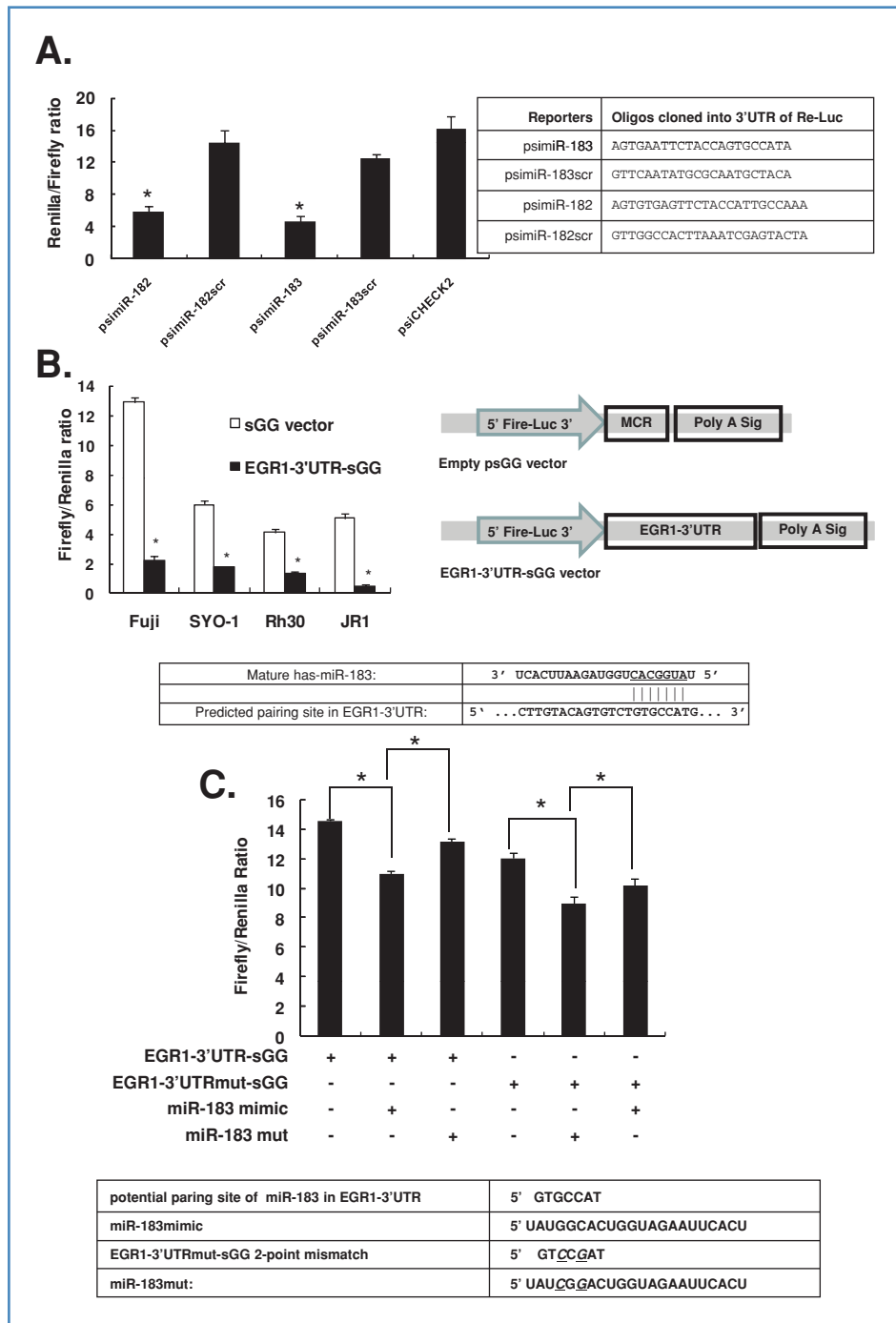


Figure 2. Luciferase reporter assays validating the interaction between miR-183 and *EGR1*. A, the synovial sarcoma cell line FUJI was transfected with psiCHECK2-based luciferase reporters. In psimiR-182 and -183, oligonucleotides complementary to mature miR-182 and miR-183 respectively were cloned into the reporters to fuse an artificial target for the miRNAs to the 3'UTR of the *Renilla* luciferase gene. In psimiR-182scr and -183scr, corresponding scrambled oligos were used as control reporters. *, $P < 0.05$, compared with corresponding scrambled controls. B, the 3'UTR of *EGR1* was added onto the 3' end of the *Firefly* luciferase gene in the vector to form a fused 3'UTR, which was used as a sensor for miRNAs that targeted the *EGR1* 3'UTR. Empty sGG 3'UTR vector was used as control. pGL4.73, with the *Renilla* luciferase gene, was cotransfected as an internal normalization control. *, $P < 0.05$, compared with empty sGG vector. Schematic representation of empty sGG vector and *EGR1*-3'UTR-sGG vector constructs. C, luciferase reporter assay using HEK293 cells to show the direct interaction of miR-183 with the 3'UTR of *EGR1*. Similar to B, the 3'UTR (or its mutant with 2 point mutations in the potential miR-183 binding site) of *EGR1* were cloned into the sGG vector at the 3' end of the *Firefly* luciferase gene, which permitted expression of a *Firefly* luciferase transcript with the *EGR1* 3'UTR (or its mutant). *EGR1*-3'UTR-sGG (or *EGR1*-3'UTR mut-sGG) was cotransfected with miR-183mimic or miR-183mut (2 point mutations in the seed region, perfectly matching the potential pairing site of *EGR1*-3'UTR mut-sGG) into HEK293T cells. pGL4.73 with the *Renilla* luciferase gene was cotransfected as an internal control. *, $P < 0.05$.

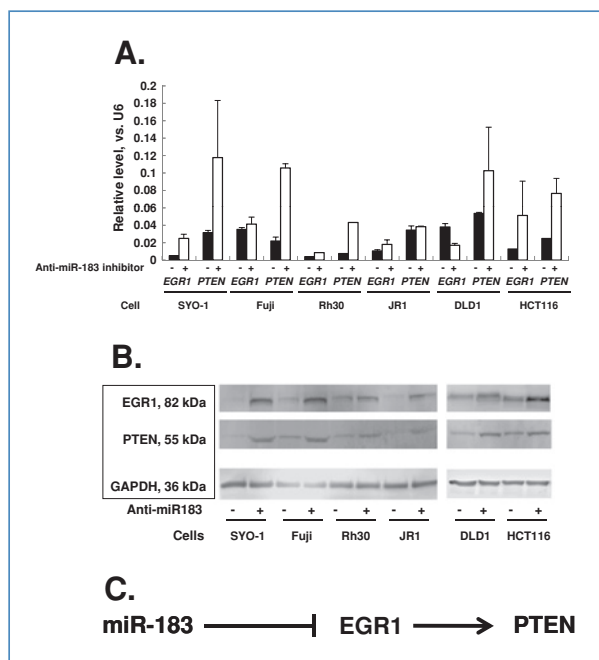


Figure 3. Anti-miR-183 treatment induces *EGR1* and *PTEN* in tumor cells. A, quantitative real-time PCR of *EGR1* and *PTEN* in synovial sarcoma, RMS, and colon cancer cell lines before and 48 hours after transfection with anti-miR-183. mRNA levels were normalized to GAPDH. B, Western blotting of *EGR1* and *PTEN* in synovial sarcoma, RMS, and colon cancer cell lines before and 48 hours after transfection with anti-miR-183. C, schematic representation of the *miR-183*–*EGR1*–*PTEN* network in tumorigenesis.

Treatment with anti-miR-183 leads to increase in *EGR1* protein levels

Because we observed increased *PTEN* mRNA levels in cell lines that did not show *EGR1* mRNA increases, we hypothesized that the regulation of *EGR1* by miR-183 occurs more generally via a translational block. To confirm this, we determined the protein levels of *EGR1* and its transcriptional target *PTEN* following anti-miR-183 treatment in FUJI and SYO-1 (synovial sarcoma), Rh30, and JR1 (RMS), and DLD1 and HCT116 (colon cancer) tumor cell lines. Immunoblotting for both *EGR1* and *PTEN* showed 2- to 5-fold increases in these protein levels following anti-miR-183 treatment, indicating that miR-183 acts generally through attenuation of the translation of *EGR1* (Fig. 3B). Therefore, removal of the *EGR1* translational block by decreasing miR-183 in the tumor cells leads to direct increases in *EGR1* protein as well as its transcriptional target *PTEN*, both of which are tumor suppressors. These results indicate that miR-183 regulates a gene network involving *EGR1* and *PTEN* in these tumors (Fig. 3C).

Genome-wide transcriptional response in tumor cell lines following miR-183 knockdown

miR-183 is predicted to have hundreds of mRNA targets, and its regulation may vary depending on the target availability in a specific cell type. To take a broader look at the role of miR-183 in tumorigenesis, we examined the genome-wide

transcriptional profiles of 3 different tumor cell lines, SYO-1 (synovial sarcoma), HCT116 (colon cancer), and JR1 (RMS), following exposure to anti-miR-183 at concentrations that resulted in increase in *EGR1* protein levels. This resulted in a gene expression signature involving 114 genes that was observed in all 3 cell lines ($P < 0.05$ and fold change > 1.5 ; Fig. 4A). Analyses of the biological functions associated with these 114 genes using Ingenuity Pathways Analyses (<http://www.ingenuity.com>) showed that differentially expressed genes were enriched in functions documented to be involved in neoplasia and tumorigenesis. Notably, these differentially expressed genes that were common in all 3 tumor cell lines were also enriched in functions related to cell migration (Table 1 and Supplementary Information 3).

mRNAs and miRNAs that exhibit positive and negative correlation with miR-183 *in vitro* are conserved in primary tumors

Global transcriptome analysis revealed that knockdown of miR-183 is capable of modulating the transcriptional profiles of tumor cell lines grown *in vitro*. Hence, we hypothesized that similar transcriptional modification may occur *in vivo* modulated by the elevated miR-183 levels. To assess this possibility, we profiled the mRNA expression of 12 synovial sarcomas and tumor samples from 16 RMS patients, in which we also determined the miR-183 levels. First, we identified the miRNAs that showed expression correlations with miR-183 levels. In both tumor types, miR-96 and miR-182, which are in a cluster with miR-183 on chromosome 7, were positively correlated with miR-183 expression levels (Fig. 4B and D). In addition, we expected that the sets of mRNAs that correlated positively and negatively with miR-183 expression levels could further define the functional significance of miR-183 in tumorigenesis. Overall, 219 and 347 genes were positively correlated with miR-183 and 120 and 606 genes were negatively correlated with miR-183 in synovial sarcoma and RMS tumors, respectively (Fig. 4C and E). Analyses of the overlapping genes between these 2 data sets showed that 34 genes were highly correlated with miR-183 in both these tumor types. For the majority of these genes (70%), the correlations were in the same direction, a result not likely to have occurred if this overlap was occurring randomly. Ingenuity pathway analysis of mRNAs that showed high correlations with miR-183 were highly enriched in genes previously studied in relation to colon cancer. Intriguingly, these analyses also showed enrichment in genes involved in cell migration for both synovial sarcoma and RMS data sets independently (Table 1 and Supplementary Information 3).

Anti-miR-183 treatment leads to decreases in tumor cell migration

On the basis of our observations that 1) miR-183 knockdown in tumor cell lines led to changes in the transcript levels of genes involved in migration and 2) transcript lists that showed high correlation with miR-183 levels in tumor tissues were also enriched in genes associated with cell migration, we examined the capability of the tumor cell lines to migrate following anti-miR-183 treatment using a scratch/wound healing assay. The migratory potential of

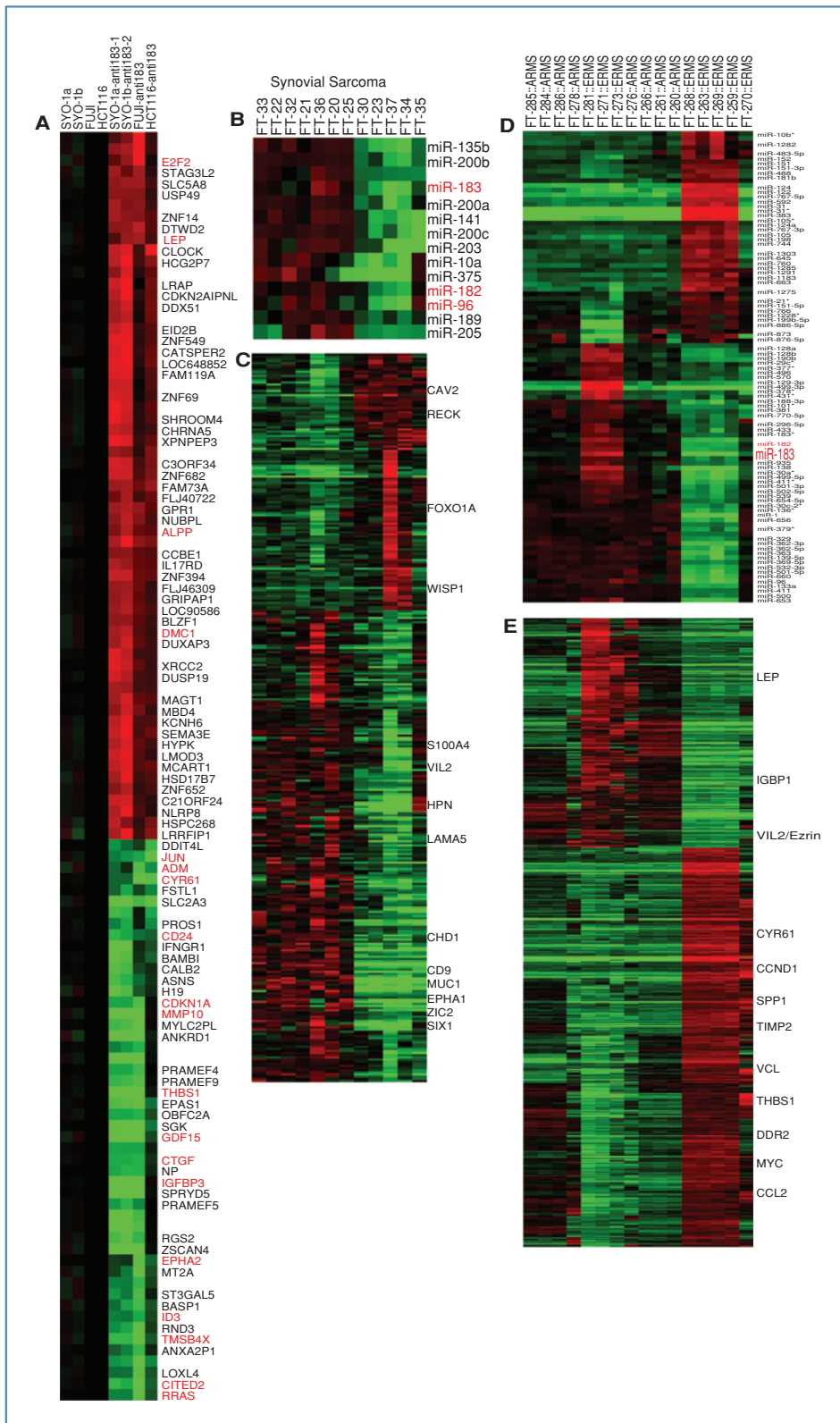


Figure 4. Genome-wide transcriptome profiling analyses implicate miR-183 in cellular migration. A, mRNAs whose transcript levels changed following treatment with anti-miR-183. In each of the 3 tumor cell lines used, the response to anti-miR-183 is shown relative to the basal state of the cell line so that the common effect of miR-183 can be visualized across the experiment. The heat map contains transcripts that had a *P* value < 0.05 and a fold change > 1.5 across all experiments carried out in cell lines as described within the figure. Genes implicated in cell proliferation/migration are shown in red. B, miRNA and C, mRNA that correlated both positively and negatively with miR-183 expression levels, with correlations > 0.7 and < 0.7, in different synovial sarcoma tumors. Expression levels were calculated from miRNA data as described in the S-MED (www.oncomiR.umn.edu), and mRNA transcript levels were obtained from the same set of tumors. Correlation coefficients were calculated using transcript data, which showed variance greater than 0.1 within the synovial sarcoma dataset, to ensure that correlations were calculated in "measurable" data. D, miRNAs and E, mRNAs that correlated both positively and negatively with miR-183 expression levels in different ERMS and ARMS tumors.

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Table 1. Biological functions significantly overrepresented in the gene sets

Pathway	Benjamini–Hochberg P-value
A. Functions enriched in genes significant to miR-183 knockdown	
Movement of cells	3.93E-04
Invasion of cell lines	2.35E-03
B. Functions enriched in genes that correlate to miR-183 levels in synovial sarcoma	
Rhabdomyosarcoma	1.94E-03
Invasion of tumor cell lines	7.15E-03
Migration of muscle precursor cells	1.83E-02
Colorectal cancer	1.83E-02
Migration of cells	2.18E-02
C. Functions enriched in genes that correlate to miR-183 levels in RMS	
Colorectal cancer	2.69E-04
Migration of brain cancer cell lines	2.39E-03
Migration of connective tissue cells	4.86E-03
Invasion of eukaryotic cells	1.21E-02
Migration of cell lines	1.34E-02

anti-miR-183-treated cells was determined by measuring the distance between the 2 scratched edges and by the presence of migrated cells in the scratched area. In synovial sarcoma cells (SYO-1), anti-miR-183 treatment led to decreases in cell migration, 24 hours following initiation of the scratch (Fig. 5A). In addition, we also tested colon cancer cells DLD1, and noticed similar effects after anti-miR-183 treatment (Fig. 5A). In both the anti-miR-183-treated cell lines, the initial gap between the scratched edges was comparable to untreated controls.

To quantify the effect observed in the scratch/wound healing assay, we measured cell migration as well as invasion using transwell assays in DLD1 and SYO-1 cells treated with anti-miR-183. Statistically significant decreases were observed in the DLD1 cell line following anti-miR-183 treatment in both the migration and invasion assays (Fig. 5B). A decrease was also observed in the SYO-1 cells although the SYO-1 cells showed higher migratory potential prior to anti-miRs-183 treatment (Fig. 5C).

***EGR1* knockdown functionally rescues the anti-miR-183 migration phenotype**

To determine whether *EGR1* was involved in anti-miR-183-mediated migration, we cotransfected DLD1 and SYO-1 cells with both anti-miR-183 and *EGR1* siRNA (Fig. 5B and C). Cotransfection experiments rescued the phenotype observed following anti-miR-183 treatment alone consistent with miR-183 controlling migration through control of *EGR1* levels. We also tested whether *EGR1* transcriptional target *PTEN* could rescue the effects of anti-miR-183 treatment. Cotransfection of both anti-miR-183 and *PTEN* siRNA showed attenuation in DLD1 cells although this result was not as robust in the SYO-1 cells (Fig. 5B). These results are consistent with a network whereby increased miR-183 in tumors leads to decreases in key tumor suppressors *EGR1* and *PTEN* which is functionally relevant to cell migration/invasion.

Finally, to determine whether increase in miR-183 is involved in additional functions we carried out proliferation, cell-cycle assays, and apoptosis after treating with anti-miR-183 in DLD1 and/or SYO-1. Significant changes in functional properties were not observed in these treated cells (Supplementary Information 4).

Discussion

Elevated levels of miR-183 are observed in a wide range of tumors, such as colon cancer, synovial sarcoma and RMS (ARMS and ERMS), and this deregulation is also maintained in cell lines derived from corresponding tumors. Thus, overexpression of miR-183 may represent a central and fundamental event in the tumorigenesis/transformation process. By a 2-pronged approach (tumor cell lines and HEK293 cells), we determined that *EGR1* is a functional target for miR-183. Further, knockdown of miR-183 led to significant increases in *EGR1* and *PTEN* protein levels in all the tumor cell lines tested. This indicates that miR-183 regulates *EGR1* primarily through translational repression. Because *EGR1* can function as a tumor suppressor (31, 32) and decreased *EGR1* levels have been correlated strongly with tumor formation and are involved in the transformation process (28, 33), our data provide a mechanistic explanation for the increased expression of miR-183 observed across several tumor types. Hence, it is tempting to conclude that miR-183 acts as an oncogene via the attenuation of the translation of *EGR1*.

EGR1 expression is positively correlated with the levels of *PTEN* (30, 34). As *PTEN* is also predicted to interact with miR-183 (miRGen 3.0), regulation of *PTEN* expression may occur either directly through the interaction with miR-183 or indirectly via the transcriptional activation by *EGR1* or by a combination of these 2 mechanisms. Collectively, it is possible that miR-183 can regulate *PTEN* expression. Alternate mechanisms may also account for the loss of *EGR1* expression

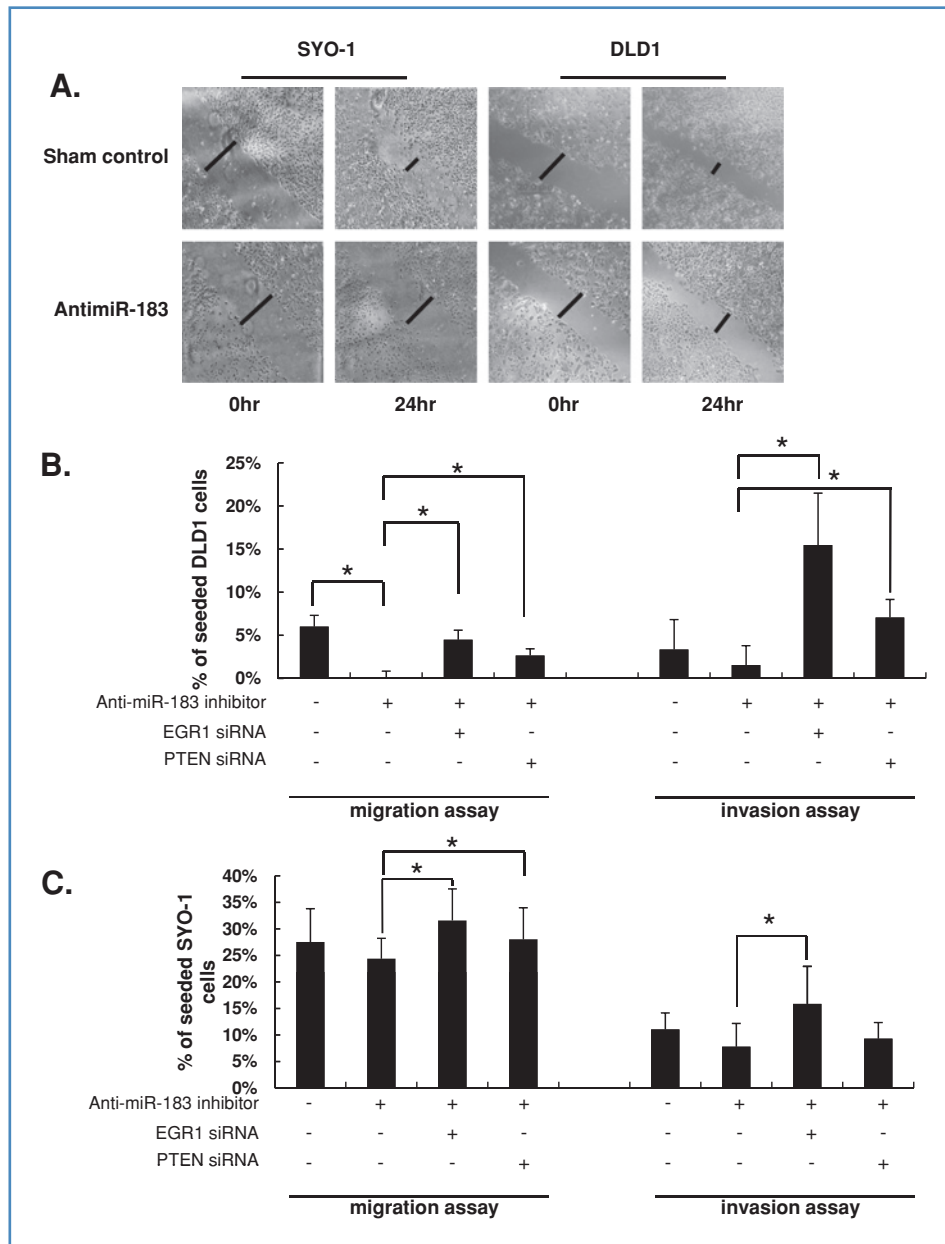


Figure 5. Migration and invasion assays using SYO-1 and DLD1 cells. A, wound healing experiment using SYO-1 and DLD1 tumor cells transfected with anti-miR-183. Wound healing was observed and photographed at 50 \times magnification 24 hours after scratching. Note the loss of migration potential in tumor cells treated with anti-miR-183 in both sarcoma (SYO-1) and colon cancer (DLD1) cells. Migration and invasion assays in DLD1 (B) and SYO-1 (C) cells. Both *EGR1* siRNA and *PTEN* siRNA significantly rescued loss of migration/invasion following anti-miR-183 treatment in DLD1 cells. Similar effects were observed in SYO-1 cells. *, $P < 0.05$.

found in various tumor tissues and tumor cell lines. For example, SS18-SSX1/2 fusion in synovial sarcoma regulates *EGR1* expression by recruiting polycomb genes to the *EGR1* promoter (15). Hence, increase in miR-183 observed in synovial sarcoma may work in combination with other mechanisms to improve the effectiveness of *EGR1* modulation.

EGR1 has been reported to function as an oncogene in prostate cancer cells (35) and decreases in *EGR1* level are implicated in synovial sarcoma (15). This dual role of *EGR1* as

either an oncogene or a tumor suppressor points to the need to better classify the similarities and differences that exist between tumor types. In this study, we show that significant number of miRNAs differentially expressed in synovial sarcoma are also differentially expressed in colon cancer and RMS. Notably, mRNAs that correlated with miR-183 expression in synovial sarcoma are also significantly enriched in genes that have been previously studied in colon cancer (19) and RMS (6). Specifically, the *miR-183-EGR1-PTEN* network

that we characterized in this study appears to be similarly deregulated in the above 3 tumors. It has been shown that synovial sarcoma has a myogenic origin (36). The similarities that we observed among synovial sarcoma and both types of RMS tumors further support the earlier observation (33). Our data also suggest that a common tumorigenic mechanism involving upregulation of miR-183 is at play in each of these tumor types.

The tumor microenvironment is highly complex. The lack of complete overlap between the genes that correlated with miR-183 levels in tumor samples of patients and the genes that responded transcriptionally following anti-miR-183 *in vitro* treatment underscores this complexity. The environmental cues and migration signals experienced by cell lines *in vitro* and tumor microenvironments *in vivo* are very different, so it is not surprising that responses to a central oncogenic signal are different in these different environments. In addition, miR-183 was more abundant in the majority of synovial sarcomas than in other tumors studied, and this variation in levels may have been affected by additional endogenous complexities not present in a knock-down experiment, so it is probably naive to expect more complete overlap between these gene lists. However, commonalities were seen between these experiments, and the gene/transcript lists in all 3 expression experiments were enriched in cell migration-based functions. For example, *VIL2/Ezrin* levels were positively correlated with miR-183 transcript levels in both synovial sarcoma (0.77) and RMS tissues (0.82). Ezrin has been implicated in the migration of RMS (37) and osteosarcoma (38). The homeobox gene *SIX1* was among the genes most highly correlated with miR-183 (0.927) in synovial sarcoma, and overexpression of *SIX1* has been demonstrated to promote breast cancer metastasis (39). Furthermore, *CYR61* was correlated with miR-183 levels in RMS and was reduced after anti-miR-183 treatment. *CYR61* protein increases the migration of cultured endothelial cells (40). In addition to a wide range of tumor tissues, increased levels of miR-183 have been reported in a number of stem cell lines (41). miR-183 levels decrease following stem cell differentiation, an effect that is consistent with the

concept that tumors utilize developmental states to escape cellular control.

The transcriptional responses following anti-miR-183 treatment as well as the mRNAs that correlate with miR-183 implicates miR-183 in cell migration. This observation is further confirmed by our *in vitro* functional analysis, which showed that reduction in miR-183 leads to a decrease in migration in colon cancer and synovial sarcoma cell lines. In addition, our results show that *EGR1* knockdown rescues the loss of cellular migration observed following anti-miR-183 treatment, consistent with our *miR-183-EGR1-PTEN* model that the migration effect of anti-miR-183 functions predominantly through this network. This suggests that pharmaceutical intervention leading to decreases in oncogenic miR-183 levels, either by direct anti-miR treatment or by indirect mechanisms producing transcript decreases, may be useful in treating tumors with upregulated miR-183. miR-183-targeted treatments, should they become a reality, may hold the promise of decreased side effects currently associated with conventional chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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