Small RNA Sequencing and Functional Characterization Reveals MicroRNA-143 Tumor Suppressor Activity in Liposarcoma

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Abstract

Liposarcoma remains the most common mesenchymal cancer, with a mortality rate of 60% among patients with this disease. To address the present lack of therapeutic options, we embarked upon a study of microRNA (miRNA) expression alterations associated with liposarcomagenesis with the goal of exploiting differentially expressed miRNAs and the gene products they regulate as potential therapeutic targets. MicroRNA expression was profiled in samples of normal adipose tissue, well-differentiated liposarcoma, and dedifferentiated liposarcoma by both deep sequencing of small RNA libraries and hybridization-based Agilent microarrays. The expression profiles discriminated liposarcoma from normal adipose tissue and well-differentiated disease from dedifferentiated disease. We defined over 40 miRNAs that were dysregulated in dedifferentiated liposarcomas in both the sequencing and the microarray analysis. The upregulated miRNAs included two cancer-associated species (miR-21 and miR-26a), and the downregulated miRNAs included two species that are highly abundant in adipose tissue (miR-143 and miR-145). Restoring miR-143 expression in dedifferentiated liposarcoma cells inhibited proliferation, induced apoptosis, and decreased expression of BCL2, topoisomerase 2A, protein regulator of cytokinesis 1 (PRC1), and polo-like kinase 1 (PLK1). The downregulation of PRC1 and its docking partner PLK1 suggests that miR-143 inhibits cytokinesis in these cells. In support of this idea, treatment with a PLK1 inhibitor potently induced G2/M growth arrest and apoptosis in liposarcoma cells. Taken together, our findings suggest that miR-143 re-expression vectors or selective agents directed at miR-143 or its targets may have therapeutic value in dedifferentiated liposarcoma. Cancer Res; 71(17); 5659–69. ©2011 AACR.

Introduction

Liposarcoma, the most common soft tissue sarcoma, accounts for 20% of adult sarcoma cases (1). It is classified into 5 subtypes constituting 3 biological groups, the most common of which consists of well-differentiated liposarcomas (WDLS) and dedifferentiated liposarcomas (DDLS). WDLS/DDLS is characterized by chromosome 12q amplification in approximately 90% of cases (2). DDLS are thought to arise from WDLS because the dedifferentiated component of these tumors always coexists with an adjacent region of WDLS and because WDLSs often progress and recur as DDLS. Surgical resection is the primary treatment for WDLS/DDLS, but local recurrence is common and more than 60% of patients eventually die from these tumors. For retroperitoneal disease, the most common anatomic location, DDLS has higher rates of local and distant recurrence and a 6-fold higher mortality than WDLS (3). DDLS/DDLS is largely resistant to conventional chemotherapy and radiotherapy, so there is a pressing need to develop new targeted therapies for patients with recurrent disease.

The search for oncogenes and tumor suppressor genes has recently expanded to include small RNAs, including miRNAs. These are RNAs of 20 to 24 nucleotides that bind to the 3’ untranslated region (UTR) of target mRNAs to inhibit translation and decrease mRNA stability (4–6). An individual miRNA may regulate hundreds of transcripts directly or indirectly (7), affording each miRNA extensive control over cellular functions. MicroRNA (miRNA) expression is dysregulated in...
multiple human cancers, including lung, breast, prostate, and gastrointestinal cancers (8, 9). Some of the dysregulated miRNAs seem to be tumor suppressors, and therefore miRNA re-expression vectors could be considered as a therapeutic for tumors that underexpress the miRNA. Other miRNAs seem to be oncogenes (10), so anti-miRNAs (11) could be considered as a therapeutic for tumors that overexpress the miRNA. Therefore, we sought to identify dysregulated miRNAs and their target genes in liposarcoma. MicroRNA expression in WDLS, DDLS, and normal adipose tissue was analyzed by small RNA sequencing and hybridization-based Agilent microarrays. We then characterized the role in liposarcoma for a strongly downregulated miRNA, miR-143, through phenotypic analysis of liposarcoma cells re-expressing miR-143, profiling of genes regulated by miR-143, and pharmacologic inhibition of one of the pathways implicated.

Materials and Methods

Sequence data analysis, quantitative real-time reverse transcription PCR, in situ hybridization, proliferation assays, fluorescence-activated cell-sorting (FACS) analysis of DNA content using 4’,6-diamidino-2-phenylindole, immunohistochemical staining, immunoblots, luciferase reporter assays, apoptosis analysis, and cell-cycle analysis with propidium iodide are described in Supplementary Data.

Patient sample acquisition

Informed consent was obtained and the project was approved by the MSKCC Institutional Review Board. Patient characteristics are shown in Supplementary Table S1. Tumor and normal adipose tissue samples obtained during surgical resection were snap-frozen in liquid nitrogen and embedded in cryomolds.

Cell culture

Liposarcoma cell lines were established from tissue samples obtained from consenting patients: LPS141 and DDLS8817 from DDLS samples and WD0082 from a WDLS sample. Comparative genomic hybridization array studies confirmed that these cell lines contain the 12q amplification. Primary human adipose tissue-derived stromal/stem cells (ASC) were isolated from subcutaneous fat tissue samples from consenting patients as described (12). The cell lines were maintained in Dulbecco’s modified Eagle’s medium HG:F12 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin and kept at 37°C in 5% carbon dioxide. Media promoting differentiation included the above plus 100 nmol/L insulin, 1 µmol/L dexamethasone, 250 µmol/L 3-isobutyl-1-methylxanthine, 33 µmol/L biotin, 77 µmol/L pantethonic acid, and 5 µmol/L rosiglitazone. All cultures were Mycoplasma free.

RNA isolation

Cryomolds (0.5 × 1 × 1 cm) were macrodissected under the supervision of a pathologist as described (13) to ensure subtype uniformity and to eliminate necrotic/normal tissue. Tumor samples were then lysed with QIAzol lysis reagent and homogenized using Mixer Mill MM 300 (Retsch). Cell line samples were trypsinized, washed once in phosphate-buffered saline (PBS), then lysed in QIAzol. For all samples, total RNA was purified using standard phenol-chloroform extraction and ethanol precipitation. Cell line miRNA used in quantitative PCR was purified with the miRNeasy Mini Kit (Qiagen).

Microarray analysis

The Agilent Human miRNA Microarray was used to compile gene expression profiles of 34 DDLS, 32 WDLS, and 17 normal fat samples. The miRNA array images were quantitated using Agilent’s Feature Extraction program, which provides integrated gene-level signals for each miRNA. The gene-level values were then transformed with the generalized log function (to handle negative values) and normalized using the VSN package from Bioconductor.

Illumina arrays were used to generate triplicate gene expression profiles in LPS141 and DDLS8817 cell lines at 2.5 and 3.5 days following infection with miR-143 or scramble lentiviruses, and in untreated controls. The expression profiles were processed using the LUMI package (Illumina microarray data analysis, Bioconductor) with the default options: background subtraction, normalization with the quantile method, and log_{2} transformation of expression values. Data were normalized using the variance-stabilization method (lumiExpresso).

For both Agilent and Illumina data, LIMMA (linear models for microarray data; Bioconductor) was used to compute differential expression, and the false discovery rate (FDR) method was used for multiple testing corrections.

Solexa sequencing

Small RNA cDNA libraries were prepared from 22 DDLS, 22 WDLS, and 11 normal fat tissue samples as described (14). In 20-µL reactions, 2 µg total RNA was ligated to 100 pmol adenylated 3’ adapter containing a unique pentamer barcode at the 5′ end (Supplementary Table S2) using 1 µg Rnl2(1-249) K227Q [purified from Escherichia coli containing pET16b-Rnl2 (1-249)K227Q (Addgene)] in 50 mmol/L Tris-HCl, pH 7.6; 10 mmol/L MgCl2; 10 mmol/L 2-mercaptoethanol; 0.1 mg/mL acetylated bovine serum albumin (Sigma-Aldrich); and 15% dimethyl sulfoxide for 16 hours on ice. After ligation, up to 20 samples bearing unique barcodes were pooled and purified on a 15% denaturing polyacrylamide gel. RNAs of 45 and 50 nucleotides were excised from the gel, eluted, and ligated to 100 pmol 5’ oligonucleotide adapter (gaucagacucagucucgacgac) as described above for the 3’ adapters, except that reactions contained 0.2 mmol/L ATP and RNL1 instead of RNL2(1-249)K227Q and were incubated for 1 hour at 37°C. Ligated small RNAs were purified on a 12% polyacrylamide gel, reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen), and amplified by PCR. The forward primer was AATGATACGGCACAGCAGACGTTCAGAGATCTTCACGGTCAAGACAGGCAA; reverse transcription and reverse primer was CACAGGCAAAGCCGACACGCTATGA. On average 200,000 (range 9,000–680,000) sequence reads of miRNAs were obtained per sample.
**In situ hybridization**

DDLS cells and ASCs were grown on chamber slides and treated as described in Supplementary Methods (15).

**miRNA re-expression**

Re-expression was based on the miRexpress Lentiviral microRNA system (Open Biosystems). The vectors consisted of pLKO.1 with inserting precursors for miR-143, miR-145, or Scr, a scramble control sequence not targeting any known human genes. These constructs were produced by transient cotransfection of 293T cells (American Type Culture Collection) with 10 μg of pLKO.1-derived plasmid, 9 μg of packaging plasmid pSPAX2, and 1 μg of envelope plasmid pMD2.G. Cells (in 10-cm² Petri dishes) were transfected using Lipofectamine 2000 (Invitrogen). Infectious viral supernatants were collected at 48, 72, and 96 hours after transfection, pooled, and concentrated by centrifugation using an Amicon Ultra-15 100K cutoff filter device (Millipore). LPS141 and DDLS8817 cells were then infected with lentiviruses containing miR-143, miR-145, or Scr sequence. Puromycin (Sigma) was used to select infected cells.

**Proliferation assays**

Proliferation of LPS141 and DDLS8817 cells was evaluated in triplicate samples by estimation of DNA content using the CyQuant Cell Proliferation Kit (Molecular Probes) as detailed in Supplementary Methods. The results per condition were normalized to the earliest time point (day 2 for lentiviral infection, day 0 for drug treatment).

**Analysis of gene expression changes after miR-143 re-expression**

To analyze correlations between motifs in mRNA 3’ UTRs and expression changes upon miR-143 re-expression, we subjected Illumina microarray results (for miR-143 vs. untreated and for miR-143 vs. Scr in DDLS8817 and LPS141 cells at 2.5 and 3.5 days postinfection) to 2 different methods, a linear-regression–based analysis, miRReduce (16), and a non-parametric motif correlation analysis (17). We identified genes consistently upregulated or downregulated at 2.5 days after miR-143 re-expression by requiring significant differential expression (P < 0.05, unadjusted LIMMA moderated t test; ref. 18) in at least 3 out of the 4 comparisons (miR-143 re-expression in LPS141 and DDLS8817 each compared with untreated and Scr controls). The combined set of dysregulated genes was analyzed for enrichment of known molecular interactions using NetBox, which uses known pathway and protein–protein interactions extracted from Pathway Commons (19) to identify connected genes in the input list (19). NetBox settings were shortest-path threshold = 2, p-value cutoff = 0.05. Statistical significance of the size of the resulting gene module and degree of connection within it was tested by comparison with 1,000 randomly selected sets of 268 genes.

**Measurement of DNA replication**

DNA replication was quantified using incorporation of bromodeoxyuridine (BrdU). Briefly, cultured cells were incubated with fresh medium containing 10 μmol/L BrdU (Sigma) for 1 hour. Cells were washed with PBS and fixed in 70% ethanol at −20 °C. Following fixation, cells were rinsed with PBS and DNA was denatured with hydrochloric acid and Triton X-100. Cells were then resuspended in 0.1 mol/L sodium borate and were stained with fluorescein isothiocyanate-conjugated mouse anti-BrdU antibody (BD Pharmingen) for 1 hour at room temperature. A 488-nm laser was used for excitation and fluorescence was measured (FACSCalibur, Becton Dickinson). Percentage of BrdU incorporation was measured using FLOWJO Flow Cytometry Analysis Software.

**Results**

**MicroRNA profiles discriminate adipose tissue, WDLS, and DDLS**

We profiled miRNA expression in 83 samples of WDLS, DDLS, and normal adipose tissue (hereafter normal fat) using Agilent microarrays. In 52 of these samples and in 1 additional WDLS sample and 2 additional normal fat samples, we also profiled miRNA expression using deep sequencing of small RNA libraries. To compare the 2 profiling platforms, we first found those miRNAs that were significantly differentially expressed in DDLS compared with normal fat in the sequencing analysis. Among those miRNAs, fold changes in sequence count were highly correlated with fold changes in microarray expression value (Supplementary Fig. S1), indicating that statistically significant ratios are consistent in the 2 technologies.

Unsupervised consensus clustering of the miRNA profiles from deep sequencing revealed 3 distinct clusters (Fig. 1A). These clusters corresponded exactly to DDLS, WDLS, and normal fat, except for one WDLS sample that clustered with the DDLS samples. This WDLS sample was isolated from a locally recurrent retroperitoneal tumor that, within a year of complete resection, subsequently recurred as a DDLS. These miRNA profiles therefore discriminate liposarcomas from normal fat tissues, and WDLS from DDLS.

The miRNAs that were strongly dysregulated in WDLS and DDLS are listed in Tables 1 and 2. A number of them had relatively high expression levels (in terms of the percentage of microRNA sequence reads) in either normal fat or in DDLS cells, suggestive of functional significance.

**miR-143 and miR-145 are underexpressed in WDLS and DDLS**

The miRNA most frequently sequenced in normal fat libraries was miR-143, with 7.75% of the total miRNA reads (Tables 1 and 2). This miRNA was strongly downregulated in liposarcomas compared with normal fat (3.3- and 7.9-fold change in WDLS and DDLS, respectively; Fig. 1B). miR-145, which is transcribed from the same bicistronic primary transcript (20), followed a similar pattern of downregulation: 2.8-fold in WDLS (though this was not statistically significant) and 6.6-fold in DDLS.

In addition to their dysregulation in human tumors, both miR-143 and miR-145 were downregulated in DDLS and WDLS cell lines relative to ASCs. Both quantitative PCR (Fig. 1C) and deep sequencing (Fig. 1D) showed downregulation in the two
DDLS cell lines (DDLS8817 and LPS141) and the WDLS line (WD0082). In most cases, expression of these miRNAs in the liposarcoma cell lines was less than 5% of the level in ASCs. Furthermore, in situ hybridization showed substantially weaker perinuclear miR-143 staining in DDLS cells than in ASCs (Fig. 1E).

Re-expression of miR-143 inhibits proliferation and induces apoptosis

We next re-expressed miR-143 and miR-145 in the 2 DDLS cell lines using a lentiviral expression system. Quantitative PCR showed increased expression in cells re-expressing each miRNA compared with cells expressing Scr control (all \( P < 0.001 \); Fig. 2A). By 2 days after lentivirus infection, levels of miR-143 and miR-145 were similar to or somewhat higher than the levels in ASCs. In an analysis of cell proliferation, re-expressing miR-143 was antiproliferative relative to control by day 6 in DDLS cells (\( P < 0.001 \)). Re-expressing miR-145, however, had no effect on proliferation (Fig. 2B). To assess apoptosis, we were unable to use propidium iodide staining because our lentivirus-infected cells express turbo red fluorescent protein. Instead, we used FACS to analyze DNA content, and found that re-expression of miR-143 induced a 2.9-fold increase in the frequency of cells with subG1 DNA content, whereas miR-145 had little effect (Supplementary Fig. S2). We also measured apoptosis using immunohistochemical staining of cleaved caspase-3 and immunoblot analysis of cleaved caspase-3 and cleaved PARP. By these assays as...
well, re-expression of miR-143, but not miR-145, induced apoptosis (Fig. 2C and D; Supplementary Fig. S3). Re-expression of miR-143 resulted in a 26-fold or more increase in the percentage of liposarcoma cells with caspase-3 cleavage \( (P < 0.001) \) and an accompanying increase in PARP cleavage.

Re-expression of miR-143 decreases entry into S phase and inhibits mitosis

To assess the effects of miR-143 re-expression on the cell cycle, we assessed BrdU incorporation and phosphorylated histone H3 immunohistochemistry. BrdU incorporation was significantly decreased with miR-143 re-expression relative to Scr and miR-145 re-expression, indicating decreased entry into S phase (Fig. 2E). Phosphorylated histone H3 was also significantly decreased with miR-143 re-expression relative to Scr and miR-145 (Fig. 2F, Supplementary Fig. S4), signifying decreased mitosis.

miR-143 downregulation has no clear role in blocking adipocyte differentiation

To determine the mechanism for the proliferative and apoptotic effects of miR-143, we first examined whether miR-143 downregulation is involved in the block of adipogenesis in DDLS cells. Recently, miR-143 levels were found to be increased in differentiating adipocytes, and antisense inhibition of miR-143 in preadipocytes blocked differentiation (21). Similarly, we found that miR-143 expression increased 2-fold during adipocyte differentiation (Fisher test, \( P < 1 \times 10^{-15} \); Fig. 1D). Nevertheless, miR-143 re-expression in DDLS cells grown in media promoting differentiation did not cause any increase in the expression of adipocyte differentiation markers (CEBPA, FABP4, and PPARG; Supplementary Fig. S5).

miR-143 regulates a gene module involved in apoptosis, DNA replication, and cytokinesis

We continued our investigation into how miR-143 affects proliferation and apoptosis with a systematic exploration of mRNA expression changes driven by miR-143 in both DDLS cell lines. Microarray analysis showed 437 genes significantly differentially expressed \( (P < 0.05, \text{Limma moderated } t\text{-test}) \) in the DDLS8817 cell line and 819 genes in the LPS141 cell line after miR-143 re-expression (measured relative to both Scr and untreated controls). Of these, 268 genes were significantly differentially expressed in both cell lines (125 downregulated and 143 upregulated genes).

In both DDLS cell lines, the expression changes after miR-143 expression correlated more strongly with the miR-143 seed sequence than with any other 3’UTR sequence motif (see Materials and Methods), validating miR-143’s effect in our experiments. Similarly, in DDLS8817 cells re-expressing miR-143 versus Scr, miRNAs with miR-143 target sites (predictions from TargetScan) in their 3’UTRs were significantly downregulated compared with miRNAs lacking seeds at both 2.5 days \( (P < 0.001, \text{Wilcoxon 1-sided test}) \) and 3.5 days.

<table>
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<tr>
<th>MicroRNA</th>
<th>Mean clone count</th>
<th>Frequency of cloning</th>
<th>Fold change (WDLS versus normal fat)</th>
<th>FDR</th>
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**Table 1. Differentially expressed microRNAs in WDLS compared with normal fat tissue samples (FDR < 0.05, fold change ≥3)**

\[^{a}\]\ miR-199a-3p and miR-199a-5p are both encoded by 2 genes, miR-199a-1 and miR-199a-2. miR-199a-2 is in a cistron with miR-214, which was not upregulated in WDLS (or in DDLS). Therefore, most of the upregulation likely originates from miR-199a-1, which is monocistronic.
In LPS141 cells, miR-143 mRNA targets showed a similar trend of downregulation (2.5 days: $P = 0.09$; 3.5 days: $P < 1.0$).

To explore functional programs that may be controlled by miR-143 in these cells, we evaluated the 268 differentially expressed genes for enrichment of molecular interactions using a computational network-based approach (NetBox) that takes into account known signaling pathways and protein–protein interactions. This analysis revealed a module of 24 connected genes (Fig. 3), 22 of which were among the differentially expressed genes. The number of affected genes and interactions was significantly higher than expected by chance ($P = 0.04$). This result suggests that the phenotypic changes of miR-143 loss may be driven through the pathway and the

### Table 2. Differentially expressed microRNAs in DDLS compared with normal fat tissue samples (FDR < 0.05, fold change ≥3)

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<td>0.01</td>
<td>-12.2</td>
<td>8.1E-07</td>
</tr>
<tr>
<td>miR-652</td>
<td>32</td>
<td>0.01</td>
<td>-9.9</td>
<td>1.0E-11</td>
</tr>
<tr>
<td>miR-365</td>
<td>62</td>
<td>0.02</td>
<td>-4.4</td>
<td>1.3E-08</td>
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<tr>
<td>miR-190</td>
<td>43</td>
<td>0.02</td>
<td>-5.9</td>
<td>6.3E-10</td>
</tr>
</tbody>
</table>

*miR-143 and miR-145 cocistronic.*

*miR-451 and miR-144 are cocistronic.*

($P < 10^{-8}$). In LPS141 cells, miR-143 mRNA targets showed a similar trend of downregulation (2.5 days: $P = 0.09$; 3.5 days: $P < 1.0 \times 10^{-5}$).

To explore functional programs that may be controlled by miR-143 in these cells, we evaluated the 268 differentially expressed genes for enrichment of molecular interactions using a computational network-based approach (NetBox) that takes into account known signaling pathways and protein–protein interactions. This analysis revealed a module of 24 connected genes (Fig. 3), 22 of which were among the differentially expressed genes. The number of affected genes and interactions was significantly higher than expected by chance ($P = 0.04$). This result suggests that the phenotypic changes of miR-143 loss may be driven through the pathway and the
protein–protein interactions implied by this gene set. This gene module contained 9 predicted miR-143 targets based on sequence complementarity: CENPM, PRC1, BCL2, PTPN2, HMGA2, TOP2A, CDC2, CDC25B, and TSC1 (Supplementary Table S3).

Importantly, the gene module includes genes involved in apoptosis (BCL2), DNA replication (TOP2A), and cytokinesis (PRC1, PLK1, CDC25B, ECT2, and CDC2; refs. 22–24). Of these BCL2, TOP2A, PRC1, CDC25B, and CDC2 are predicted direct targets of miR-143. We used PCR and Western blots to validate the downregulation of BCL2, TOP2A, PRC1, and PLK1 in DDLS cells re-expressing miR-143 (Fig. 4, Supplementary Fig. S6).

Compared with Scr, miR-143 expression reduced mRNA levels of all 4 genes in both LPS141 and DDLS8817 cell lines (P < 0.001 for all except BCL2 in DDLS8817, which was not significant). miR-143 re-expression reduced BCL2, topoisomerase 2A (TOP2A), polo-like kinase 1 (PLK1), and protein regulator of cytokinesis 1 (PRC1) protein levels in DDLS cells. In addition, luciferase reporter assays showed that miR-143 directly regulates the 3′UTRs of TOP2A and PRC1 (Supplementary Fig. S7).

**PLK1 inhibition in DDLS cells is antiproliferative and proapoptotic**

Because miR-143 regulates key members of the central spindle regulatory pathway, we tested whether selective inhibition of this pathway would have antiproliferative or proapoptotic effects in DDLS cells. PLK1, a regulator of this pathway, is abundant in DDLS cells relative to ASCs (Fig. 4C). We utilized a pharmacologic inhibitor of PLK1, BI 2536 (Selleck Chemicals; ref. 25), which is currently in phase II clinical trials and has shown limited success in the treatment of multiple solid tumors and chronic myeloid leukemia (26, 27). Treatment with BI 2536 at 20 nmol/L resulted in complete growth inhibition of LPS141 cells and an 83% reduction in cell number of DDLS8817 cells, without affecting proliferation of ASCs (Fig. 5A). A 2-day treatment induced a 3- to 4.5-fold increase in apoptosis in DDLS8817 and LPS141 cells, respectively, compared with untreated controls (P < 0.001), but induced no change in apoptosis in ASCs (Fig. 5B). Furthermore, the 2-day treatment induced G2–M cell-cycle arrest (Fig. 5C).
Discussion

We have shown that sequencing-based miRNA expression profiles discriminate normal fat, WDLS, and DDLS. MicroRNA-143, which is abundant in normal adipose tissue, is underexpressed in WDLS, and its expression decreases further as tumors progress to DDLS. These findings imply that miR-143 upregulation in an early event in liposarcomagenesis, but that further loss may play a role in dedifferentiation. Esau and colleagues previously showed that miR-143 levels increase in differentiating adipocytes and that miR-143 is required for differentiation (21). We found as well that miR-143 levels increase in differentiating adipocytes; however, re-expression of miR-143 is insufficient to restore differentiation in the cellular context of DDLS. Therefore, while miR-143 loss may produce effects through inhibition of differentiation, miR-143 restoration alone is unable to reverse this phenotype in DDLS cells.

Re-expression of miR-143 but not its co-cistronic partner miR-145 inhibits DDLS cell proliferation and induces apoptosis. miR-143 re-expression also decreases entry into S phase and inhibits mitosis. The effects of miR-143 on proliferation, apoptosis, and cell-cycle progression may be explained by a gene network targeted by miR-143, including direct miR-143 targets BCL2, TOP2A, and PRC1 and the indirect target PLK1. This network includes genes involved not only in cell proliferation and apoptosis, but also in cytokinesis. Pharmacologic inhibition of PLK1, a regulator of cytokinesis, induces apoptosis and cell-cycle arrest in DDLS cells.

Prior work has implicated miR-143 as a tumor suppressor in other cancers. In bladder cancer, miR-143 inhibits cancer growth through antagonizing the expression of RAS (28). Both miR-143 and miR-145 are downregulated in colon cancer and in B-cell malignancies, such as chronic lymphocytic leukemia and Burkitt’s lymphoma (29), miR-143 in particular seems to be a tumor suppressor in colorectal cancer, acting through downregulation of KRAS, a mediator of the mitogen-activated protein kinase cascade (30). In these other cancer types, the main effect of miR-143 re-expression is induction of proliferation without induction of apoptosis. In contrast, in liposarcoma cells we detect significant induction of apoptosis after only 48 hours of miR-143 re-expression.

We have shown that miR-143 downregulates BCL2, TOP2A, and PRC1 in DDLS cells. BCL2 encodes a mitochondrial membrane protein that blocks apoptosis and has been implicated in multiple human cancers. It was recently identified as a direct target of miR-143 in osteosarcoma (31) and bears at least 2 predicted miR-143 target sites (Supplementary Table S3). The repression of BCL2 may contribute to miR-143’s proapoptotic properties, but, given miR-143’s lack of significant effect on BCL2 in one of the two DDLS cell lines, this may not apply to all DDLS tumors.

TOP2A is an enzyme controlling the topologic state of DNA and involved in transcription and DNA replication. It is upregulated in multiple human cancers such as breast and prostate cancer, and its expression is associated with androgen resistance and decreased survival in prostate cancer. Our group has found that TOP2A expression is upregulated 43-fold in DDLS relative to normal fat (Supplementary Table S4). Furthermore, TOP2A upregulation in liposarcomas was associated with decreased distant recurrence-free survival. Short hairpin RNA knockdown of TOP2A causes induction of apoptosis and decreased invasive ability of DDLS cells (32), underscoring its importance in this disease.

To date, the regulation of TOP2A in DDLS and other tumors has been poorly defined. Our study provides the first evidence that miR-143 regulates TOP2A and that this regulation may partially explain miR-143’s antiproliferative and proapoptotic effects. In addition, miR-143’s regulation of TOP2A, a key enzyme of DNA replication, may explain the decreased progression into S phase after miR-143 restoration.

TOP2A is a target of anthracycline-based chemotherapeutic agents, such as doxorubicin, which alter TOP2A catalysis, generating high levels of DNA breaks that then trigger cell death pathways. These agents, however, are not completely TOP2A-specific, and their use is limited by high toxicity and poor efficacy in DDLS. The present results suggest that rational screening for more potent and specific TOP2A-targeted agents may lead to more effective therapy for patients with liposarcoma.
PRC1 and PLK1 are crucial regulators of cytokinesis. PRC1, a docking partner of PLK1, recruits PLK1 to the central spindle during anaphase (22). Suppression of PRC1 expression (33, 34) or chemical inhibition of PLK1 (35) results in mitotic failure, and both proteins are required for proper cytokinesis. Our group has previously found that PRC1 is upregulated 24-fold in DDLS relative to normal fat (13). We showed here that miR-143 negatively regulates PRC1 and PLK1 expression, that miR-143 restoration inhibits mitosis in DDLS cells, and that PLK1 inhibition via drug treatment leads to mitotic arrest and cell death. These observations, together with the microarray and network analysis implicating miR-143’s regulation of other genes involved in cytokinesis (ECT2, CDC2, and CDC25B), suggest that miR-143 plays an important role in regulating cytokinesis in liposarcoma cells. Furthermore, they imply that PLK1 inhibitors may have potential for the treatment of DDLS.

Key to our study has been elucidation of the complex regulatory network of miR-143. Consistent with the observation that microRNAs target a multitude of genes, each of which has a small but significant effect on the overall phenotype conferred by the microRNA, we identified a strongly connected network regulated by miR-143. Interestingly, expression levels of 10 of the 24 genes in the network are associated with decreased distant recurrence-free survival (ref. 32; Supplementary Table S4). In addition, 9 of these 10 genes are upregulated significantly in DDLS relative to normal fat (13). The 24-gene network controls several important cellular functions ultimately converging in cell growth and survival. Although agents targeting individual miR-143 targets may provide useful therapy for DDLS, our results suggest therapy targeting the entire dysregulated gene network via delivery of miR-143 to DDLS tumors may provide more effective therapy.

Figure 4. Downregulation of A, TOP2A, B, PRC1, and C, PLK1 after miR-143 re-expression. Levels of miRNAs (left) were measured by quantitative PCR 2.5 days after lentiviral infection and are shown relative to ASCs. Protein levels of TOP2A, PRC1, and PLK1 were assessed by Western blot analysis at day 2 after lentivirus infection in LPS141 and at day 4 in DDLS8817.
Disclosure of Potential Conflicts of Interest

T. Tuschl is a cofounder and scientific advisor to Alnylam Pharmaceuticals and an advisor to Regulus Therapeutics. The other authors disclosed no potential conflicts of interest.

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References


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