MiR-155 Is a Liposarcoma Oncogene That Targets Casein Kinase-1α and Enhances β-Catenin Signaling

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Introduction

The adipogenic origin liposarcomas constitute the most common soft tissue sarcoma histologies, comprising approximately 15% to 20% of this relatively uncommon malignancy cohort (1). This group is composed of 3 categories as per the 2002 WHO guidelines (ref. 2: i) well-differentiated and dedifferentiated liposarcoma (WDLPS/DDLPS; ii) myxoid and round-cell liposarcoma; and (iii) pleomorphic liposarcoma. The focus of this study is WDLPS/DDLPS, the most common liposarcoma histologic subtype. The molecular hallmark of WD/DDLPS is the presence of one or more supernumerary circular ("ring") and/or giant rod chromosomes containing highly amplified (more than 10-fold in most cases) DNA sequences localized to the 12q13-q21 region (3). WDLPS arise in the deep soft tissues and lack a described precursor lesion. Although WDLPS developing in the periphery (e.g., extremities) commonly exhibit a favorable outcome and rarely dedifferentiate, cases arising in the retroperitoneum or deep soft tissues of the pelvis and abdomen exhibit a more dismal natural history. Retroperitoneal/abdominal WDLPS are usually large upon discovery and frequently cannot be excised with microscopically negative surgical margins (4). Although lacking metastatic capacity, the avid propensity of retroperitoneal/abdominal WDLPS for local recurrence is associated with considerable morbidity and mortality; adjuvant and neoadjuvant nonsurgical approaches are generally not effective (5). Approximately 25% to 40% of retroperitoneal/abdominal WDLPS will ultimately manifest a dedifferentiated pattern (DDLPS; 6). Dedifferentiation, as originally described by Evans in 1979 (7), is defined as an area within a well differentiated lesion lacking characteristic lipomatous features with the appearance of an intermediate- to high-grade sarcoma. About 90% of DDLPS are diagnosed de novo, as a component of a primary presenting lesion, whereas approximately 10% are identified in the context of a recurrent WDLPS (6). DDLPS are significantly more aggressive than pure WDLPS, exhibiting a local recurrence rate of more than 80%, a distant metastasis rate of up to 20%, and a 5-year disease-specific survival rate of 40% to 60% despite an aggressive surgical approach combined with systemic chemotherapy (8). There is a pressing need for improved DDLPS management strategies; consequently, enhanced knowledge of molecular deregulations underlying DDLPS biology is critical.

In the past decade, much attention has focused on the tumorigenesis and cancer progression impact of miRNAs. miRNAs are a class of evolutionally conserved noncoding small RNAs of 18- to 24-nucleotides in length; they participate in posttranscriptional gene expression regulation through mRNA

Abstract

Liposarcoma can be an aggressive, debilitating, and fatal malignancy. In this study, we identified miRNAs associated with the differentiation status of liposarcoma to gain insight into the basis for its progression. miRNA expression profiles determined in human tumors and normal fat specimens identified a dedifferentiated tumor expression signature consisting of 35 miRNAs. Deregulated miRNA expression was confirmed in a second independent sample cohort. The miR-155 was the most overexpressed miRNA and functional investigations assigned an important role in the growth of dedifferentiated liposarcoma cell lines. Transient or stable knockdown of miR-155 retarded tumor cell growth, decreased colony formation, and induced G1-S cell-cycle arrest in vitro and blocked tumor growth in murine xenografts in vivo. We identified casein kinase 1α (CK1α) as a direct target of miR-155 control which enhanced β-catenin signaling and cyclin D1 expression, promoting tumor cell growth. In summary, our results point to important functions for miR-155 and β-catenin signaling in progression of liposarcoma, revealing mechanistic vulnerabilities that might be exploited for both prognostic and therapeutic purposes. Cancer Res; 72(7); 1751–62. ©2012 AACR.

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degradation, translational inhibition, or chromatin-based silencing mechanisms (9). miRNAs exert multiple biologic functions, including development, differentiation, apoptosis, and cell proliferation (9). miRNA expression is commonly altered in cancer, contributing to tumor initiation, progression, and metastasis (10); miRNAs can function either as tumor suppressors or oncogenes (oncomiRs; ref. 10). Accordingly, there is great interest in developing therapeutic strategies to target tumor–driving miRNA deregulations. Insight into the role of altered miRNA expression in WD/DDLPS is currently limited; to date, apparently only 2 studies have investigated specific WD/DDLPS-associated miRNA altered expression and/or function (11, 12). The goal of this study was to further enhance our current knowledge about the role of miRNAs in WD/DDLPS. Using a miRNA array platform an expression signature consisting of 4 overexpressed and 31 downregulated miRNAs was found to differentiate WD/DDLPS from normal fat. One of the overexpressed miRNAs we identified (miR-155) was further evaluated for its role in promoting DDLPS growth in vitro and in vivo and its molecular mechanisms of function were elucidated.

Materials and Methods

Cell lines and reagents

The human DDLPS cell lines: lipo224, lipo224B, lipo246, lipo573B, and lipo863B (all originating from surgically resected human retroperitoneal DDLPS) were established in our laboratory as recently reported (13). The previously described human DDLPS cell line LPS141 was kindly provided by Dr. Jonathan Fletcher (Brigham and Women’s Hospital, Boston, MA; ref. 14). Cells were cultured and passaged in Dulbecco’s modified Eagle medium (DMEM) supplemented with glucose and 10% FBS. Authentication of DDLPS cell lines was conducted using short-tandem repeat DNA fingerprinting (Supplementary Table S1) as previously described (13). Human white adipocytes (PA) primary cultures were purchased from PromoCell. PA were differentiated into adipocytes per manufacturer’s instructions using a commercial adipocyte differentiation media [(serum-free media containing insulin, dexamethasone, IBMX, t-thyroxine, ciglitazone, and heparin and adipocyte nutrition media (3% fetal calf serum) supplemented media containing: insulin, dexamethasone, and 3-isobutyl-1-methylxanthine)]. Adipogenic differentiation was confirmed via Oil red O staining as previously described (13).

Commercially available antibodies were used for Western blot analyses or immunohistochemical detection of CK10 (Novus Biologicals), β-catenin (BD Transduction Laboratories and BD Biosciences), phospho beta-catenin (Ser 45) and Foxo3a (Cell Signaling), cyclin D1 (Santa Cruz Biotechnology) Ki67 (Dako); Cleaved caspase 3 (BioCare medical) and β-actin (Santa Cruz Biotechnology)

miRNA expression profiling and miR-155 in situ hybridization

For miRNA expression profiling, frozen tissues acquired under an Institution Research Board approved protocol derived from 17 surgically resected WD/DDLPS samples, 5 lipoma samples, and 8 frozen normal fat tissues were used. In all cases WD/DDLPS histology, as initially clinically diagnosed, was confirmed by a soft tissue and bone pathologist (AJL). Furthermore, in 11 of the cases 12q15/MDM2 FISH was done as previously described (13) and showed 12q15/MDM2 amplification as would be expected (Supplementary Fig. S1); in the remaining 6 cases hybridization was not successful. Tissue was homogenized in TRIzol reagent (Invitrogen) and miRNA fraction was purified from total RNA using RT2 qPCR-Grade miRNA isolation kit (SABiosciences) per manufacturer’s protocol. Deidentified samples were submitted to Exiqon ( Vedbaek) for quality control and high-throughput miRNA profiling; all experiments were conducted as per facility protocols. In brief, RNA quality was assessed by an Agilent 2100 Bioanalyzer profile; only samples with RNA Integrity Numbers (RIN) values more than 7 were included in the final analysis. One microgram total RNA from sample and reference RNA (a mixture of RNA from all samples included) were labeled with Hy3 and Hy5 fluorescent label, respectively, using the miRCURY LNA Array Power Labeling Kit. Next, Hy3-labeled samples and a Hy5-labeled reference RNA samples were mixed pair-wise and hybridized to the miRCURY LNA array version 11.0 containing capture probes targeting all miRBASE version 13 (Sanger Institute, Hinxton, Cambridge, UK) registered human, mouse, and rat miRNAs. Hybridization was carried out according to the miRCURY LNA array manual using a Tecan HS4800 hybridization station (Tecan). Post hybridization microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) to prevent potential bleaching of the fluorescent dyes. Next, slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and image analyses were carried out using the ImageGene 8.0 software (BioDiscovery). Quantified signals were normalized using the global Lowess (Locally Weighted Scatterplot Smoothing) regression algorithm. Raw and normalized miRNA array data were uploaded to GEO (GSE31045). A tissue microarray (TMA) consisting of cores derived from WD/DDLPS FFPE specimens (representing 59 different patients) and 8 normal fat control samples was constructed as previously described (15). In situ hybridization was conducted using double DIG–labeled mercury LNA miRNA probe (Exiqon) and a U6 control probe. Tissue slides were hybridized for 2 hours at 55°C on Discovery Ultra (Ventana Medical Systems). The digoxigenins were then detected with a polyclonal anti-DIG antibody and alkaline phosphatase–conjugated secondary antibody (Ventana) using NBT-BCIP (Ventana) as the substrate.

Quantitative real-time PCR, Western blot analyses, immunohistochemistry, and cellular growth–related assays

These assays were all conducted as previously described (13, 16). More detailed information is provided in Supplementary Data.

Transfection/transduction procedures

Transient miR-155 knockdown was conducted using peptide nucleotide acid (PNA)-based oligomers specifically
targeting has-miR-155 (Panagene): a nontargeting scrambled construct was used as control. Anti-miR-155 PNA sequence is RBBQBKBKR-OO-CTATGCAGATTAGCATA, and scramble control sequence is RBBQBKBKR-OO-ATATTGTCGGACAA. RBBQBKBKR is the cell penetrating peptide. Transfection procedure followed manufacturers’ instructions; Lipofectamine 2000 was used as transfection reagent (Invitrogen). A miRNA sponge method was used to stably knockdown miR-155 as previously described (17). In Brief, long DNA oligos containing 7 repeats of the antisense miR-155 sequence were synthesized and ligated into retroviral pSuperior.retro.neo-GFP vector with HindIII and BglII (New England Biolabs) to create expression plasmids. MiR-155 prime sequences are Forward (BglII): 5′-agggagcatctcatatatgaaccttttaataattagataggggttaatgctaattagataggggt-3′; Reverse (HindIII): 5′-gataaagtttaataattagatagttggtttatgttgatagtatggtgttgaatt-3′. Retroviruses were generated by cotransfection of the packing plasmids pCGP and pVSV-G (gifted by Dr. Zweidler-McKay, Dept. of Pediatrics, MD Anderson Cancer Center, Houston, TX) and the above expression plasmids into 293T cells. DDLPS cell lines were cultured in 6-well plates to which 8 μg/mL polybrene and virally infected supernatant was added for 4 hours and after 48 hours selected by fluorescence-activated cell sorter (FACS) for GFP expression. siRNAs (20 nmol/L) pools targeting β-catenin L-003482-00-0005 and control nontargeting constructs D-001810-01-05; Thermo Scientific) were introduced into cells using Lipofectamine 2000 (Invitrogen) per manufacturer’s instructions. Briefly, 2 × 10^5 cells were plated in each well of a 6-well plate and incubated overnight. A mixture of siRNA (20 nmol/L) and Lipofectamine 2000 (10 μL) diluted in DMEM was added for 24 hours, followed by incubation in regular medium. Knockdown was confirmed by Western blot analysis. Forced cyclin D1 expression was conducted using a pCMV–cycin D1 plasmid (Addgene): empty pCMV plasmid was used as control. Of note, DDLPS cell lines used for transfection/transduction all exhibited relatively high miR-155 expression and were selected on the basis of ease of transfection/transduction and high transfection/transduction efficiency. Lipo246 was specifically selected for stable knockdown as this cell lines exhibits 100% tumor take when injected to immunocompromised mice.

Luciferase reporter assays

pMIR-REPORT plasmids containing miR-155 target sequence in the Foxo3a 3′-untranslated region (3′-UTR) region and mutant miR-155 target sequence were kindly provided by Dr. Cheng (18). pRL plasmid was used as internal control. To clone the 3′-UTR region of CK1α cDNA, the following primers were synthesized: CK1α forward: 5′-atcctctgagATCTCAGT-CAACCTGCGAA-3′ and CK1α reverse: 5′-gccaggggctc-CAAGGTTGAAGGCACTGTGTC-3′. PCR was used to amplify the target 3′-UTR sequence containing the potential binding site of miR-155. The PCR product was inserted into a psiCHECK-2 vector (Promega) using the XhoI and NotI sites. Luciferase reporter assays were conducted as previously described (19). In Brief, DDLPS cells were transfected with the relevant constructs and internal controls using Lipofectamine 2000 (Invitrogen). After 48 hours, cells were lysed using passive lysis buffer; firefly and Renilla luciferase activities in cell lysates were determined using a Dual-luciferase assay kit (Promega) on DTX800 Multimode Detector (Beckman Coulter). Reporter assay results are depicted as relative luciferase activities; that is, average ratios of Firefly luciferase to Renilla ± SD for pMIR-REPORT Foxo3a constructs and average ratios of Renilla luciferase to Firefly ± SD for pCHECK-2 CK1α construct.

In vivo DDLPS xenograft experiments

All animal procedures/care was approved by UTMDDACC Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH “Guide for the Care and Use of Laboratory Animals.” Further information is provided in Supplementary Data.

Statistical analysis

The goal of the miRNA gene expression array statistical analysis was to identify the most significantly differentially expressed miRNAs comparing human WD/DDLPs and normal fat specimens. For each miRNA array probe, 2-tailed t test between human WD/DDLPs and normal fat specimens was calculated (using log-transformed data). The difference in Log Median Ratios (ΔLMR) between sample groups was calculated. Java TreeView represented the results as a heatmap diagram.

Cell culture–based assays and quantitative real-time PCR (qRT-PCR) analyses were repeated at least twice and mean ± SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, 2-sample t tests were used to assess the differences. Differences in xenograft size and weight between xenograft groups at study termination were assessed using a 2-tailed Student’s t test. Significance was set at P ≤ 0.05.

Results

High-throughput miRNA expression profiling reveals potential WD/DDLPs-associated deregulated miRNAs

The small RNA fraction isolated from 8 frozen human normal fat (NF) samples and 17 WD/DDLPs samples was characterized using miRNA expression arrays. Statistical analysis identified 4 miRNAs to be significantly (P < 0.0001, fold change >1.5) overexpressed in WD/DDLPs as compared with NF. Thirty-one miRNAs were downregulated in WD/DDLPs compared with normal tissues. A heatmap based on a 2-way unsupervised hierarchical clustering of tissue samples and miRNAs is depicted in Fig. 1A. A complete list of individual deregulated miRNAs is provided in Supplementary Table S2. Two of the 4 upregulated miRNAs have previously reported to have potential cancer promoting functions (i.e., oncomiRs), whereas 11 of the downregulated miRNAs (39%) have been shown to harbor tumor suppressor functions (Supplementary Table S2). In addition, 4 (11%) of the miRNAs identified in our study were recently found to be potentially deregulated in WD/DDLPs (based on massively parallel sequencing analysis), including...
miR-143, the focus of the previously published study (Supplementary Table S2; 11).

Next, expression levels of 3 of the deregulated miRNAs identified (miR-155, miR-10b, and miR-126) were assessed via qRT-PCR in an independent set of frozen human samples (NF ¼ 10, WD/DDLPS ¼ 20). As depicted in Fig. 1B, miR-155 was found to be, on average, significantly (P < 0.05) overexpressed in WD/DDLPS samples as compared with NF (and lipomas; Supplementary Fig. S2), whereas miR10b and miR126 were both downregulated in tumor tissues, recapitulating the expression pattern noted in high-throughput profiling experiments. Together, these data validated miRNA expression array analyses and justified further investigation of individual identified miRNAs for their role in DDLPS biology.

**MiR-155 contributes to enhanced DDLPS cell growth in vitro and in vivo**

Of the several WD/DDLPS-associated deregulated miRNAs discovered above, miR-155 was chosen for further comprehensive investigation. This selection aligned with our ultimate goal of identifying DDLPS-associated molecular deregulations that can be used for therapeutic targeting consonant with recent development of compounds targeting oncomiRs as anticancer strategies (20). On the basis of expression profiling results, miR-155 was the most statistically significantly overexpressed miRNA identified in DDLPS compared with NF as was also confirmed, as depicted above, in an independent set of frozen human samples. Furthermore, we have conducted miR-155 in situ hybridization on a liposarcoma focused TMA (Fig. 1C).
Results showed that miR-155 expression is markedly enhanced in WD/DDLPS samples (50/59 (85%) of samples exhibited high miR-155 expression) as compared with NF samples (high miR155 expression was noted in only one sample (12.5%)). MiR-155 was previously found to act as an oncogene in several hematologic and epithelial origin cancers (21–23); however, its contribution to DDLPS biology remains to be elucidated. First, we evaluated the expression of miR-155 in a panel of DDLPS cell lines and normal human adipocyte cell cultures. In concordance with the results obtained from the evaluation of human samples, high levels of miR-155 were noted in DDLPS cell lines as compared with adipocytes (Fig. 2A). Next, DDLPS cell miR-155 expression was knocked down using an antisense construct to determine the contribution of this miRNA to cell growth; qRT-PCR analyses confirmed a significant decrease in miR-155 expression after knockdown when compared with cultures transfected with a nontargeting scrambled construct used as control (Fig. 2B). Decrease in miR-155 function was validated using a previously described miR-155 responsive luciferase reporter system consisting of FOXO3a 3′-UTR constructs (18); increase in reporter-driven luciferase expression was noted in miR-155 knocked down cells compared with control (Fig. 2B). Most importantly, this functional knockdown abrogated DDLPS cell growth and decreased clonogenic capacity (Fig. 2C), suggesting that miR-155 contributes to DDLPS cell growth.

To further determine the potential impact of miR-155 on DDLPS tumorigenesis in vivo, this miRNA was stably knocked down in DDLPS cells using a retroviral pSuperior.retro.neo/GFP miR-155 sponge construct with empty vector as control. Stably transduced cells were isolated using GFP sorting. qRT-PCR and reporter assays were used to confirm functional knockdown (Fig. 3A). Similar to the findings above, stable miR-155 knockdown resulted in decreased DDLPS cell growth and colony formation (Fig. 3B). Furthermore, miR-155 knockdown resulted in marked G1 cell-cycle arrest in stably transduced DDLPS cells (Fig. 3B). However, no evidence of miR-155 knockdown-induced apoptosis was noted in Annexin V/PI FACS analyses (data not shown). Next, the growth of anti-miR-155–transduced Lipo246 cells was evaluated in vivo. As depicted in Fig. 3C, miR-155 knockdown xenografts showed slower growth and a significantly decreased volume and tumor weight at study termination compared with tumors originating from Lipo246 cells stably transduced with control vector [1,031 mm3 ± 608 vs. 185 mm3 ± 40 (P < 0.05) and 0.5 g ± 0.2 vs. 0.08 g ± 0.01 (P < 0.05)]. qRT-PCR confirmed decreased miR-155 expression in anti-miR-155–transduced DDLPS xenografts (Fig. 3D); Ki67 immunohistochemical analysis showed a marked decrease in DDLPS cell proliferation in these tumors compared with controls (Fig. 3D). No significant increase in cleaved caspase-3 expression was found in miR-155 knockdown xenografts (data not shown). In sum, these loss-of-function experiments supported an oncogenic role for miR-155 in DDLPS contributing to proliferation and cell-cycle progression in vitro and in vivo.

Casein kinase 1α (CK1α) is a novel miR-155 target; a potential molecular mechanism driving Wnt/β-catenin pathway deregulation in WD/DDLPS

Next, we wanted to identify the molecular mechanisms underlying miR-155 oncogenic function in DDLPS. Previous studies have reported Foxo3α and SCOS1 as miR-155 targets in
epithelial origin cancer (18, 24). Therefore, we evaluated the impact of miR-155 knockdown on the expression of these targets in DDLPS. Western blot analyses failed to show increased Foxo3a expression in response to miR-155 knockdown (Supplementary Fig. S3). Similarly, no increase, and even a decrease in SOCS1 expression was observed (qRT-PCR; Supplementary Fig. S3). Realizing that other novel miR-155 targets might be operative in DDLPS, we conducted a search of the miRNA Target Prediction database miRGen (25). Among multiple potential targets, this *in silico* search identified CK1α, a key regulator of the Wnt/β-catenin pathway, to be a predicted miR-155 target, containing a highly conserved motif (833-847 bp, NM_001892.4) matching with the miR-155 "seed" sequence (7 nucleotide: gcauuaa; Fig. 4A). Taking into account the well-

Figure 3. MiR155 knockdown inhibits DDLPS xenograft growth in vivo. A, stable miR-155 in DDLPS cells using a retroviral sponge construct was confirmed using qRT-PCR (top) showing marked decrease in miR-155 expression. A miR-155 reporter activity assay (WT and mutated Foxo3a 3’-UTR luciferase construct; bottom) showed increased firefly luciferase expression (normalized to Renilla luciferase) in response to miR155 inhibition after the transfection of the WT construct, but no difference when a mutated reporter construct was used (results in lipo246 cells are shown). These data further validated knockdown efficacy; B, similar to the effects of transient miR-155 knockdown, stable blockade was found to decrease the growth and colony forming capacity of DDLPS cells. Moreover, miR-155 knockdown inhibits DDLPS G1 cell-cycle progression (PI staining/FACS analysis; bottom); C, anti-miR-155-transduced lipo246 cells were evaluated for local growth in vivo. miR-155 knocked down xenografts exhibited slower growth, decreased size at study termination [left graph; *P < 0.05], and a significantly decreased weight [right graph; *P < 0.05] at study termination as compared with control empty vector–transduced xenografts; D, qRT-PCR confirmed decreased miR-155 expression in xenografts derived from anti-miR-155-transduced Lipo246 cells (top) and ki67 IHC analysis showed decreased tumor cell proliferation in these xenografts (images were captured at ×200 magnification). Graphs represent the average of at least 3 repeated experiments ± SD; * denotes statistically significant effects (*P < 0.05).
established importance of the CK1α/β-catenin axis in tumorigenesis and tumor progression, we selected to focus on this potential downstream target. Western blot analysis showed increased CK1α protein expression in both lipo246 and lipo224B cells stably transduced with anti–miR-155 (Fig. 4A). To confirm an interaction between miR-155 and the 3′-UTR of the CK1α mRNA, a luciferase reporter system consisting of a wild-type CK1α 3′-UTR miR-155 binding region containing construct and a mutated construct were developed (sequences are depicted in Fig. 4A). These were used to determine whether miR-155 directly impacts CK1α expression. DDLPS cells stably transduced with anti–miR-155 exhibited increased WT reporter activity as compared with control vector–transduced cells, although no significant difference in luciferase readout was found when the mutated construct was evaluated (Fig. 4A). These results suggested that CK1α is a direct miR-155 target. To further confirm that miR-155-induced modulation of CK1α is of clinical relevance, we assessed the protein expression levels of this target in a cohort of snap-frozen human DDLPS (i.e., miR-155 overexpressing; n = 8) and normal fat (n = 8) samples via Western blot analyses. As depicted in Fig. 4B DDLPS exhibit markedly lower CK1α expression.

In the “off” (inactive) state of Wnt/β-catenin pathway signaling, CK1α binds and phosphorylates β-catenin at serine 45, consequentially leading to the ubiquitination and degradation of this protein (26). To determine whether the above noted impact of miR-155 on CK1α expression functionally impact β-catenin signaling, the expression of phosphorylated β-catenin (S45), β-catenin, and cyclin D1 (a β-catenin downstream target) protein levels in miR-155 knocked down DDLPS cell lines were examined. As depicted in Fig. 4C, an increase in phosphorylated β-catenin expression and a marked decrease in β-catenin and cyclin D1 were found. These cell culture-
based observations were further confirmed to occur in vivo: immunohistochemical analysis showed decreased β-catenin and cyclin D1 expression in xenografts derived from miR-155 knocked down DDLPS cells (Fig. 4C).

To determine whether CK1α expression correlates with the expression of β-catenin and cyclin D1 in human samples, we used the Western blot panel depicted in Fig. 4B and evaluated the expression of these potential downstream targets. As shown in Fig. 5A increased β-catenin/cyclin D1 expression can be identified in DDLPS as compared with NF, especially in tumor samples expressing lower CK1α. Although β-catenin/cyclin D1 overexpression and enhanced signaling has been identified as a common molecular event in several malignancies (27), little is known about the status of these proteins in WD/DDLPS. To that end, we have assessed the expression of these proteins in our TMA. β-Catenin expression was identified in 48 WD/DDLPS samples (81%) to varying levels: low expression was noted in 52% of positively staining tumors and moderate to high expression in 48%. In contrast only one NF samples expressed β-catenin to a low level. Similarly, 95% (n = 56) of WD/DDLPS samples exhibited cyclin D1 expression: low levels were identified in 29% of positively staining tumors and 91% expressed moderate to high levels. None of the NF samples expressed cyclin D1 as per our scoring schema (Fig. 5B). Taken together, our data identified CK1α as a novel miR-155 target and suggested that miR-155 overexpression enhances the activation of β-catenin in these malignancies.

**MiR-155 pro-WD/DDLPS effects are driven by cyclin D1**

The above findings led to the hypothesis that miR-155 DDLPS-associated oncogenic functions are mediated via enhanced β-catenin signaling. To further investigate this possibility, the impact of β-catenin overexpression on DDLPS cell growth was evaluated in loss-of-function experiments. Knockdown was achieved using a pool of siRNA constructs specifically targeting β-catenin; nontargeting constructs were used as controls (Fig. 6A). A marked decrease in cyclin D1 was noted after β-catenin knockdown, confirming the effects of this genetic manipulation on β-catenin signaling (Fig. 6A). Most importantly, β-catenin knockdown resulted in a significant (P < 0.05) decrease in DDLPS cell growth (Fig. 6B) and in pronounced G1 cell-cycle arrest (Fig. 6C). These functional effects align with those identified as a consequence of miR-155 knockdown.

To determine whether miR-155 regulates DDLPS cell growth, at least in part, via β-catenin signaling (i.e., through cyclin D1) rescue experiments were conducted. Cyclin D1 was forcefully expressed into anti-miR-155–transduced lipo246 cell and empty vector–transduced cells (Fig. 6D); the effect on cell growth and cell-cycle progression was determined. As depicted in Fig. 6D, increased cyclin D1 expression was able to overcome the growth and cell-cycle progression blockade imposed by
miR-155 knockdown. Together, these data supported a role for β-catenin signaling/cyclin D1 in the DDLPS protumorigenic, proproliferative effects mediated via miR-155 overexpression.

Discussion
This study highlights several interconnected DDLPS-associated molecular deregulations of potential major translational and clinical relevance. Specifically, we found that miR-155 is highly expressed in human WD/DDLPS and contributing to the tumorigenic phenotype of these unfavorable malignancies via a previously not described regulation of CK1α which results in activation of the β-catenin pathway. Multiple lines of evidence strongly support the significant role that miR-155 plays in tumorigenesis and progression, acting predominantly as an oncogenic miRNA (oncomiR; 18, 21–24, 28). MiR-155 overexpression has been previously identified in several hematologic (e.g., B-cell lymphomas, acute myelocytic leukemia; refs. 22, 29) and epithelial origin carcinomas (e.g., colon, papillary thyroid, pancreatic ductal adenocarcinoma, breast, and lung [21, 30–36]). Moreover, miR155 as a molecular prognosticator has been identified in pancreatic carcinoma and...
lung cancer, in which high expression levels are associated with poor patient outcome (36, 37). Supporting role of miR-155 as an oncomiR is the finding that mice genetically engineered to express miR-155 demonstrated a role for miR-155 in inducing B-cell–specific promoter activity (38). Similarly, transduction of miR-155 in hematopoietic stem cells was shown to result in the development of a myeloproliferative disorder (39). Loss-of-function experiments conducted in several cancer models determined a role for miR-155 in tumor cell proliferation, migration, and invasion (18, 24, 28). To the best of our knowledge, our study is the first to report a role for miR-155 in mesenchymal origin solid malignancy.

miRNAs are robust regulators of gene expression functioning by directly inducing mRNA degradation or either repressing the translation targeted mRNAs (9). Translational inhibition may occur via mechanisms such as mRNA uncapping that lead to increased mRNA turnover and decreased target gene expression. OncomiRs such as miR-155 directly target and repress the expression of tumor suppressor or tumor suppressor–like genes (23). To date, several miRNAs coding for transcriptional regulatory proteins, receptors, kinases, and nuclear and DNA binding proteins have been identified as miR-155 direct targets in the cancer context. For example, miR-155 was shown to induce B-cell malignancies by targeting SHP and C/EBPβ (40); miR-155 regulation of TP53INP1 was found to be a major mediator of pancreatic cancer development (41). Additional cancer–associated targets include FOXO3, RHOA, MSH2, MSH6, MLH1, SOCS1, and others (18, 24, 42, 43). Interestingly, several targets including Meis1, c-MAF, AID, interleukin-1, IKKe, and Ets-1 have been identified to be regulated by miR-155 in the context of the immune and hematopoietic systems but have not been shown to have a role in miR-155–mediated oncogenesis (23). Together, these insights possibly suggest that miR-155 only negatively regulates a small number of dominant mRNA targets in a particular disease context and in a cancer cell type–specific manner. Identifying the dominant pathways/targets regulated by an miRNA in a given malignancy may shed light on molecular deregulations driving tumor biology. In this study, we identified CK1α as a novel miR-155 direct target, possibly implicating β-catenin signaling as a DDLPS molecular driver.

Human β-catenin, a homolog of the Drosophila Armadillo protein, is a multifunctional protein that plays essential roles in development and tissue maintenance (27). β-Catenin signaling is tightly regulated in normal cells (44). Generally, cytoplasmic β-catenin levels are kept low via continuous ubiquitin–proteasome–mediated degradation of this protein, a process which is regulated by a multiprotein complex containing CK1α, axin, APC, and GSK-3β (27). CK1α and GSK-3β mediate the degradation of β-catenin molecules by phosphorylating specific amino N-terminal residues (Ser45 by CK1α, and Thr41, Ser 37, and Ser33 by GSK-3β in this sequential order), thereby marking the protein and rendering it as recognizable by β-transducin repeat–containing protein (β-TrCP), a component of the E3 ubiquitin ligase, leading to its degradation by the 26S proteasome complex (27). Physiologic Wnt–pathway activation as well as pathologic aberrant, signaling (e.g., secondary to CK1α downregulation as shown in this study) results in β-catenin stabilization, increased expression, and enhanced activity leading to the overexpression of downstream effectors such as cyclin D1 (27). β-Catenin pathway deregulation has been frequently noted in multiple cancer types, in which aberrant signaling is subverted to provide advantages in growth and survival (44). However, the role of β-catenin in liposarcomagenesis and progression has not been widely studied. A recent report identified upregulated canonical WNT signaling in a large cohort of human sarcomas including liposarcoma (45). Sakamoto and colleagues evaluated a small human DDLPS sample cohort (n = 12 human specimens) and reported β-catenin protein overexpression in 42% of cases; 17% of samples harbored CTNNB1 (the gene encoding for β-catenin) mutations (46). Our studies further support a role for this pathway in DDLPS: β-catenin and its downstream effector cyclin D1 were found to be overexpressed in all human DDLPS cell lines as compared with preadipocytes and adipocytes and were shown to induce DDLPS cell proliferation and cell-cycle progression, thereby supporting further investigation of this pathway in DDLPS. Although we have focused our study on the impact of decreased CK1α expression on β-catenin signaling, CK1α has also been shown to regulate other cancer contributory targets including MDM2, p53, and E2F1 proteins (47, 48). MDM2 amplification is a hallmark of WD/DDLPS contributing to inception of these malignancies (3). Consequently, miR155: CK1α contribution to MDM2 activity may warrant further investigation.

The possibility that miRNA deregulations can be exploited as nodes of tumor vulnerability, that is, can form the basis for novel anticancer therapies, is an exciting potential strategy to modulate gene expression programs in an immediate and reversible manner (20). Several types of antisense-based miRNA inhibitors have been developed to target oncomiRs, seeking to turn off their expression; for example, “antagomirs,” locked nucleic acid oligonucleotides, and various types of 2′-O-modified oligonucleotides (20). Such approaches have shown initial promise in various preclinical cancer models. Our study shows a potential role for miR-155 in DDLPS biology and supports further development and testing of anti–miR-155 therapeutic strategies for this malignancy. However, miR-155 plays important roles in physiologic immune surveillance modulating TLR- or IFNβ-mediated innate immune responses and affecting T-, B-, and dendritic cell functions (23). Thus, systemic miR-155 inhibition could negatively affect immune proficiency. To overcome such concerns, DDLPS-specific delivery agents; for example, viral vectors or tumor-targeted nanoparticles, may be applicable. Alternatively, strategies targeting miR-155–induced downstream effects in DDLPS, that is, β-catenin signaling, might abrogate possible deleterious side effects of miR-155 inhibition. On the basis of data presented in our study, switching off β-catenin signaling upon which DDLPS cells seem to depend could potentially elicit significant antitumor effects. However, agents directly downregulating β-catenin are not yet available, and such interventions, if developed, may not be clinically useful if they compromise normal β-catenin physiologic functioning. For these reasons, approaches that target specific β-catenin protumorigenic downstream effects rather than directly downregulate β-catenin per se may
be more applicable. This study highlights a potential role for the β-catenin downstream effector cyclin D1 in DDLPS. Cyclin D1 inhibitors are currently under development and at least one agent (ON 013105) has recently reached phase I clinical testing (49) after showing preclinical efficacy in several cancer models in vitro and in vivo (50). Our study supports evaluation of such compounds in DDLPS experimental models.

In conclusion, we found that miR-155 functions as an oncomiR in DDLPS by targeting CK1α, consequently resulting in enhanced β-catenin/cyclin D1 expression and driving DDLPS cell proliferation and cell-cycle progression. Together, these data suggest novel DDLPS-associated molecular deregulations that could be exploited for development of more effective therapies on behalf of patients afflicted by these devastating malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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