Reprogramming of the MicroRNA Transcriptome Mediates Resistance to Rapamycin*

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The mammalian target of rapamycin (mTOR) is a central regulator of cell proliferation that is often deregulated in cancer. Inhibitors of mTOR, including rapamycin and its analogues, are being evaluated as antitumor agents. For their promise to be fulfilled, it is of paramount importance to identify the mechanisms of resistance and develop novel therapies to overcome it. Given the emerging role of microRNAs (miRNAs) in tumorigenesis, we hypothesized that miRNAs could play important roles in the cellular response to mTOR inhibitors. Long-term rapamycin treatment showed extensive reprogramming of miRNA expression, characterized by up-regulation of miR-17–92 and related clusters and down-regulation of tumor suppressor miRNAs. Inhibition of members of the miR-17–92 clusters or delivery of tumor suppressor miRNAs restored sensitivity to rapamycin. This study identifies miRNAs as new downstream effectors of miR-17-92 and related clusters and downstream-modulators of tumor suppressor miRNAs. Inhibition of members of the miR-17–92 clusters or delivery of tumor suppressor miRNAs restored sensitivity to rapamycin. This study identifies miRNAs as new downstream effectors of miR-17-92 and related clusters and downstream-modulators of tumor suppressor miRNAs. Inhibition of members of the miR-17–92 clusters or delivery of tumor suppressor miRNAs restored sensitivity to rapamycin. This study identifies miRNAs as new downstream effectors of miR-17-92 and related clusters and downstream-modulators of tumor suppressor miRNAs.

 clave:

Background: The role of miRNAs in the cellular response to mTOR inhibitors has never been tested.

Results: Rapamycin resistance is associated with up-regulation of oncogenic miRNAs and down-regulation of tumor suppressor miRNAs.

Conclusion: miRNAs influence the cellular response to mTOR inhibitors.

Significance: miRNAs are potential markers and novel targets for cancer therapy involving mTOR inhibitors.

The mammalian target of rapamycin (mTOR) is a master regulator of cell growth and division that is responsive to numerous signaling pathways including growth factors, nutrients, and energy supply. The PI3K/Akt/mTOR axis is a desirable target for the treatment of cancer because it integrates multiple pathways critical for cell growth. mTOR assembles into two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each of which has unique substrates (1–3). mTORC1 is composed of mTOR, Raptor, PRAS40, and mLAT8/GβL and regulates cell growth through downstream effectors such as the 40S ribosomal protein S6 kinase 1 (S6K1), and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (4). mTORC2 also contains mTOR, mLAT8/GβL, and the unique regulatory proteins Rictor, mSin-1, and Protor. mTORC2 regulates Akt phosphorylation on Ser-473 (5).

Rapamycin is a macrolide fungicide isolated from the bacteria Streptomyces hygroscopicus. When bound to the FK506-binding protein (FKBP12) it inhibits the serine/threonine kinase activity of mTORC1 as well as the translation of specific cell cycle miRNAs (required for G1 to S phase transition), ultimately leading to growth arrest (6). As opposed to mTORC1, mTORC2 is disrupted only by prolonged rapamycin treatment (>24 h) resulting in inhibition of Akt signaling. Rapamycin inhibits mTORC2 in only ~20% of the cancer cell lines studied (7). The mechanisms underlying mTORC2 inhibition in some tumors but not in others are not well understood.

Hyperactivation of the mTOR pathway due to PI3K or PTEN mutations, amplification of Akt, deregulation of tuberous sclerosis complex, or overexpression of eIF4E or S6K1 has been observed in some cancers (8–11). Hence, specific tumors may be particularly susceptible to mTOR inhibitors (12–14). Rapamycin and rapamycin analogues are being evaluated in clinical trials for the treatment of sarcomas, non-small cell lung cancer, neurofibromas, pancreatic cancer, hepatocellular carcinoma, and breast cancer (15–22). Importantly, the Food and Drug Administration has recently approved the use of a rapamycin analogue (everolimus) for the treatment of advanced renal cell carcinoma (11, 23).
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Given their potential in clinical oncology, there is considerable interest in the identification of tumor-related molecular markers that predict the efficacy of mTOR inhibitors (7, 11, 14, 24, 25). Predicting the efficacy of mTOR inhibitors in cancer therapy requires further elucidation of the signaling pathways that are inhibited by rapamycin and its analogues and the determination of the mechanisms underlying resistance to mTOR-targeted therapy.

miRNAs are small, non-coding functional RNAs, 22 nucleotides in length, that are transcribed by RNA polymerase II and occasionally by RNA polymerase III (26, 27). They act as gene suppressors by binding to the 3’-untranslated region (UTR) of target mRNAs to trigger either translation repression or mRNA degradation (28). miRNAs play an important role in tumorigenesis, and they have recently emerged as diagnostic and prognostic markers for successful therapeutic responses (29, 30).

Inhibition of mTOR affects gene expression by inhibiting transcription (31); however, the role of miRNA in the cellular response to mTOR inhibitor has not been tested. In the present study, we show that oncogenic miRNAs are significantly up-regulated and tumor suppressor miRNAs are down-regulated in rapamycin-resistant cells. Inhibitors of these oncogenic miRNAs (miR-17 and miR-19) or mimics of the tumor suppressor miRNAs (let-7) restored rapamycin sensitivity in resistant tumor cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—BC3H1 cells, a mouse brain tumor cell line with myogenic properties (ATCC), were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 20% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 mg/ml). Rapamycin-resistant cells (RR1 or RR3) were developed by culturing BC3H1 cells in the presence of 1 or 0.1 μM rapamycin, respectively, for 6 months as described previously (32). Primary vascular smooth muscle cells (VSMCs) were isolated from C56BL mice as described (33). The C2C12 mouse myogenic cell line (ATCC) and VSMC were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml).

**miRNA Array Analysis**—Total RNA was extracted from BC3H1 cells cultured in the presence of 100 nM rapamycin (BC3H1+R cells) or DMSO (BC3H1) for 24 h, and RR1 cells were cultured continuously in the presence of 1 μM rapamycin (RR1+R) or with DMSO for one week (RR1). All experiments were performed in quadruplicate. For each experiment, 1 μg of total RNA from each sample was labeled with Hy3™ dye and a reference RNA pool (consisting of a mixture of equal amounts of total RNA from BC3H1, BC3H1 + R, RR1 and RR1 + R cells) was labeled with Hy5™ dye using the miCURY™ labeling kit. The samples were hybridized to Exiqon miRCURY™ LNA arrays (version 10.0), which contained 417 mouse-specific capture probes (representing 417 miRNAs). Data analysis was performed within the R/Bioconductor statistical framework (34). We used the limma and Agi4 × 44PreProcess packages to preprocess the raw data and perform the quality controls and plots. Expression intensities were background-corrected using a convolution of normal and exponential distributions (’normexp’ method) (35, 36) with an offset of 50 and then normalized within each slide using loess normalization and between arrays using the QuanTiler method (37). One sample of the BC3H1 group did not pass the quality control tests and was therefore excluded.

We identified the differentially expressed miRNAs with the limma package. Following hypothesis testing, we corrected for multiple tests controlling for the false discovery rate using the Benjamini-Hochberg correction and an accepted α ≤ 0.05 (38).

**Gene Expression Data Analysis**—Total RNA was extracted from quadruplicates of BC3H1, BC3H1 + R, RR1 and RR1 + R cell cultures treated as described above. All RNA samples had a 28S/18S rRNA ratio of ~2 on ethidium bromide-stained 1.5% agarose gels and 260 nm/280 nm absorbance ratios between 1.9 and 2.1. Ten μg of each RNA sample was used for target preparation, using a one-cycle cDNA synthesis kit, and hybridized to GeneChip® Whole Mouse Genome 430 2.0 arrays (Affymetrix), which contain 45,000 probe sets representative of over 39,000 transcripts and variants from over 34,000 well-characterized murine genes. The arrays were washed and stained using the Affymetrix 450 Fluidics station and scanned using the GeneChip Scanner 3000, enabled for high resolution scanning. GeneChip® operating software v1.4 (Affymetrix) was used to determine the expression level and “present,” “absent,” or “marginal” calls for each probe set. The mRNA expression data were normalized with GC-RMA (39). To evaluate global changes in gene expression relative to miRNA dysregulation we plotted the empirical cumulative distribution function of changes in mRNA expression by subsets of transcripts. Transcript subsets were determined according to TargetScan predictions for the respective miRNAs (TargetScanMouse, release 6.0) (40). Differential mRNA expression was derived from Affymetrix array hybridization signals. Of 45,501 probe sets on the Affymetrix arrays, 45,037 had corresponding transcripts in the TargetScan database; the change in expression of genes in this list served as the reference distribution against which miRNA target subsets were evaluated. Cumulative distribution function plots and respective Wilcoxon rank sum tests were performed within the R framework.

**Quantitative Real-time PCR**—TaqMan microRNA assays (Applied Biosystems) were used to quantify mature miRNAs. cDNA was synthesized by priming with miRNA-specific looped primers or with snoRNA55 as an endogenous control. Total RNA (100 ng) extracted with the miRNeasy mini kit (Qiagen) was used for each reverse transcription reaction according to the manufacturer’s specifications, incubated for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C, and stored at 4 °C. A master mix of cDNA, TaqMan® Universal PCR Master Mix (P/N 4324018, Applied Biosystems), and TaqMan® MicroRNA assay. All reactions, excluding nontemplate controls and non-reverse transcribed controls, were run in triplicate and incubated in 96-well plates at 95 °C for 20 seconds followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds using the ABI 7500 Fast Real Time PCR detection system. Real-time PCR data were analyzed using the comparative CT method, with normalization against the expression of snoRNA55.

For gene expression analyses, 0.5 μg of total RNA was used to synthesize cDNAs using M-MuLV reverse transcriptase (New
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England Biolabs) according to the manufacturer’s specifications. Real-time PCR was carried out using a LightCycler 1.5 (Roche Applied Science) with the LightCycler TaqMan master mix. For Myc, we used universal probe 77 with 5'-CCTAGTGCTGATGAGAGAGACGACGACGGTGA-3' forward primer and 5'-TCTCTCCTCTCCTCTCTCTCTCTCTCTCTCT-3' reverse primer; for Tgif2 we used universal probe 106 with 5'-CCAGGTGTTTTGGGAAACCGGGTTGAA-3' forward primer and 5'-AGCCCTGCTGGTCTCCTAC-3' reverse primer; and for Thbs1 we used universal probe 9 with 5'-CAGGCCAAAGACCGGTTA-3' forward primer and 5'-CTGCATGAGGAGA-3' reverse primer. The amplification efficiencies were 1.909 for Myc, 1.993 for Tgif2, and 1.875 for Thbs1.

Cell Lysis and Immunoblotting Assays—Cells growing in 10-cm diameter dishes were rinsed once with cold PBS and lysed for 20 min in ice-cold buffer A (40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors (Roche Applied Science)) containing 0.3% CHAPS. After clearing of the lysates by centrifugation at 13,000 × g for 10 min, samples containing 50 μg of protein were resolved by SDS-PAGE, and proteins were transferred to PVDF and visualized by immunoblotting with antibodies against Akt, phospho-Ser-473 Akt, S6K1, phospho-Thr-389 S6K1, GSK, p-GSKβ, Myc, and GAPDH (Cell Signaling Technologies) using infrared-labeled secondary antibodies (1:10,000, LI-COR Biosystems).

Cell Transfections—BC3H1 or RR1 cells (5 × 10^4 cells/well in 24-well plates) were transfected in triplicate wells with Myc-specific siRNA or control siRNA (Origene Technologies, Inc.) at a final concentration of 10 nM or with miRIDIAN microRNA mimics or miRNA hairpin inhibitors (antagomirs, Dharmacon, Thermo Scientific) at a final concentration of 100 nM or as indicated using the DharmaFECT transfection reagent. 16 h later, cells were treated with or without 100 nM rapamycin and counted after 3 days of incubation. Myc cDNA (accession number NM_010849) was amplified using forward primer 5’-AAA-GAGCTCCTCGAGCTTTGGA-3’ and reverse primer 5’-TACCTCTACACAGGATCCAGCTCTCC-3’ inserted into pcDNA3.1 + plasmid (Invitrogen) using BamHI and EcoRI unique restriction sites. BC3H1 (5 × 10^4 cells/well in 24-well plates) were transfected with Myc-expressing plasmid or mock using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s specifications; total RNA was extracted after a 48-h incubation.

**RESULTS AND DISCUSSION**

Characterization of Rapamycin-resistant Cells—The potent anti-proliferative effect of mTOR inhibitors makes them attractive for cancer therapy, with numerous ongoing clinical trials evaluating their potential for treating malignancies (11). However, rapamycin and its analogues (temsirolimus and everolimus) have shown modest success in clinical trials, reflecting an incomplete understanding of mTOR functions. To investigate the role of miRNAs in the cellular response to mTOR inhibition, we studied the molecular changes associated with the development of rapamycin resistance in a murine myogenic cell line, BC3H1 (derived from a mouse brain tumor), and in rapamycin-resistant (RR1) cells developed in our laboratory by prolonged culturing of BC3H1 cells in the presence of 1 μM rapamycin without mutagenesis (32). As shown previously, rapamycin inhibited cell growth of the parental BC3H1 cells but not of the RR1 cells (Fig. 1A).

It has been proposed that the ability of rapamycin to inhibit both mTORC1 and mTORC2 accounts for its potent anti-proliferative effect (7, 41–43). To test whether the resistance to rapamycin was due to a lack of inhibition of mTORC1 or mTORC2, we compared the effect of 1- and 24-h treatment with 100 nM rapamycin on the phosphorylation of Thr-389-S6K1, a target of mTORC1 in exponentially growing BC3H1 or RR1 cells. Furthermore, we compared the phosphorylation of Ser-473-Akt and GSK targets of mTORC2 and Akt, respectively. One hour of rapamycin treatment inhibited mTORC1-mediated phosphorylation of S6K1 in both BC3H1 and RR1 cells. Because S6K1 normally suppresses the PI3K/Akt pathway (44, 45), inhibition of S6K1 by rapamycin resulted in increased phosphorylation of Akt and consequently GSKβ (Fig. 1B). Furthermore, 24 h of rapamycin treatment inhibited both mTORC1 and mTORC2 pathways in BC3H1 and RR1 cells, as shown by the dephosphorylation of Thr-389-S6K1, Ser-473-
Akt, and p-GSKβ. These results indicate that the mTORC1 and mTORC2 pathways are inhibited by rapamycin in the same fashion in both BC3H1 + R and RR1 + R cells. Therefore the development of rapamycin resistance observed in RR1 cells cannot be attributed to a lack of inhibition of mTORC1 or mTORC2.

Differential miRNA Expression in Rapamycin-sensitive and Rapamycin-resistant Cells—Global miRNA expression profiling was performed in BC3H1 cells treated with DMSO or 100 nm rapamycin for 24 h (BC3H1 and BC3H1 + R, respectively) or in RR1 cells cultured with DMSO for 1 week (RR1) or constantly treated with 1 μM rapamycin (RR1 + R). Unsupervised hierarchical clustering of normalized hybridization signals identified four distinct groups corresponding to the experimental conditions (Fig. 2A). One sample from the BC3H1 untreated group (BC3H1–3) was excluded for further analysis because it did not pass quality controls (Fig. 2A). Principle component analysis showed sample separation between rapamycin-sensitive cells and rapamycin-resistant cells on component 1 (PC1), whereas on component 2 (PC2) the sample separation was based on rapamycin treatment (Fig. 2B). The main contributors to the sample separation of the PC1 were 23 miRNAs, whereas 28 miRNAs contributed to PC2 (Fig. 2, C and D, and Table 1).

miRNAs Associated with Rapamycin Resistance—To identify miRNAs associated with rapamycin resistance, we evaluated the miRNA expression profiles of rapamycin-resistant and -sensitive cells by three pairwise comparisons: RR versus BC3H1, RR1 + R versus BC3H1 + R, and RR + R versus BC3H1. Twenty miRNAs were significantly changed (>2-fold, p < 0.001) in RR1 cells compared with BC3H1 cells (Table 2, left columns). RR1 + R cells showed significant changes (>2-fold, p < 0.001) in 18 miRNAs compared with BC3H1 + R and in 16 miRNAs when compared with BC3H1 (Table 2, middle and right columns). Sixteen miRNAs were similarly changed in all three comparisons (Table 2, shown in bold). The 10 up-regu-
lated miRNAs in resistant cells were miR-370 and members of the miR-17–92 and its related miR-106a–92 and miR-106b–25 clusters (miR-25 was increased 1.3-fold; data not shown), which have oncogenic properties (46–48). Among the 10 down-regulated miRNAs, some have been reported to have tumor suppressor properties (e.g., miR-143, miR-22, and miR-29a) (49–51). The expression levels of miR-17, miR-19b, miR-21, miR-22, miR-29a, and miR-143 in RR1 + R cells and BC3H1 were validated using quantitative RT-PCR and exhibited strong correlation with the microarray data (r = 0.78, CI = 0.069 to 0.976, p value 0.032) (Fig. 3A). To confirm the relationship between the differentially expressed miRNA and the rapamycin-resistant phenotype, we tested the expression of these miRNAs in another rapamycin-resistant cell colony (RR3, developed by culturing BC3H1 cells in the presence of 100 mM rapamycin for more than 6 months (32)) by qRT-PCR. The expression levels of all six miRNAs were similar to those found in RR1 cells (Fig. 3A), suggesting that the regulation of miRNAs is consistent across different rapamycin-resistant cells. These groups of oncogenic and tumor suppressor miRNAs may be involved in the switch from the rapamycin-sensitive to rapamycin-resistant phenotype. These data reveal for the first time that long-term rapamycin treatment is associated with extensive reprogramming of miRNAs expression, characterized by up-regulation of the miR-17–92 and related clusters and down-regulation of tumor suppressor miRNAs.

To evaluate whether the dysregulated miRNAs found in RR1 cells affect global gene expression we examined differential mRNA abundance across subsets of transcripts predicted to be targets of dysregulated miRNAs. Differential expression was assessed by comparing normalized Affymetrix array hybridization signals between RR1 and BC3H1 cells. miRNA target pre-
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FIGURE 3. Increased Myc and miR-17–92 cluster mediate the resistance to rapamycin. A, total RNA was extracted from BC3H1 cells treated for 24 h with DMSO or from RR1 or RR3 cells grown continuously in the presence of 1 μM or 100 nm rapamycin, respectively. Total RNA was extracted, and qRT-PCR was performed for the indicated miRNAs. Data shown are mean ± S.D., significantly different from DMSO-treated BC3H1 cells, * p < 0.01 by t test. B, cumulative distribution plots of fold changes in mRNA expression in RR1 versus BC3H1 cells. Separate curves are displayed for all miRNAs (black curve) or subsets of miRNAs predicted to be targeted by selected dysregulated miRNA (colored curves). The median change in expression for each miRNA subset is depicted by a respective dot on the x axis. C, total RNA was extracted from BC3H1 and RR1 cells treated as described in A, and qRT-PCR was performed for Myc, Tgf2, and Thbs1. Data shown are mean ± S.D., n = 3, significantly different from DMSO-treated BC3H1 cells; *, p < 0.01, by t test. D, representative immunoblots for the indicated proteins from whole cell extract of BC3H1 or RR1 cells treated with DMSO or with rapamycin, as indicated, for 24 h. E, GSEA plots of TGFβ positive response gene sets, which were repressed in RR1 + R cells: panel 1, PLASARI_TGFB1_TARGETS_10HR_UP, genes up-regulated in MEF cells upon stimulation with TGFB1 for 10 h; panel 2, LABBE_TGFβ1_TARGETS_UP, up-regulated genes in NMuMG cells (mammary epithelium) after stimulation with TGFB1; panel 3, PLASARI_TGFβ1_SIGNALING_VIA_NFIC_10HR_UP, genes up-regulated after 10 h of TGFB1 stimulation in MEF cells with NFIC knock-out versus wild type MEFs; panel 4, MCBRYAN_PUBERTAL_TGFβ1_TARGETS_UP, pubertal genes up-regulated by TGFB1; panel 5, TGFβ_UP.V1_UP, genes up-regulated in a panel of epithelial cell lines by TGFB1. F and G, RR1 cells were transfected with 10 nm Myc-specific siRNA, the indicated miRNA inhibitors (100 nm), or control and treated for 3 days with 100 nm rapamycin followed by cell count. Data shown are mean ± S.D., * significantly different compared with untreated cells (p < 0.001 by t test).

dictions were downloaded from TargetScan (mouse release 6.0). Fig. 3B shows that whereas the median log-fold change in gene expression in RR1 cells versus BC3H1 cells among all genes present on the arrays and listed in TargetScan was close to 0, the corresponding curves for miR-17 and miR-19 predicted target genes showed a statistically significant shift to the left (down-regulation). This shift implies a dominant down-regulation of miR-17 and miR-19 target genes in RR1 cells, in line with increased levels of these miRNAs. Conversely, the expression curves of miR-22 and miR-143 predicted targets showed a statistically significant shift to the right (up-regulation), in line with the decreased abundance of these miRNAs in RR1 cells (Fig. 3B). Although the median changes in expression portrayed in the above analyses are small, they are significant, in agreement with similar transcriptome-wide evaluations (52), and are likely affected by the relatively low specificity of miRNA target prediction as well as the secondary (indirect) effects of miRNA on target gene expression.

It has been shown that Myc represses the expression of numerous tumor suppressor miRNAs by direct binding to conserved promoter regions (53, 54) and also up-regulates the oncogenic miR-17–92 cluster, which in turn down-regulates anti-angiogenic proteins such as thrombospondin-1 (Thbs1) and connective tissue growth factor (CTGF) (53, 55). In addition, members of the miR-17–92 cluster have been shown to be potent inhibitors of TGFβ signaling by targeting multiple key effectors along the TGFβ signaling cascade as well as directly inhibiting TGFβ-responsive genes (56). To test whether the up-regulation of miR-17–92 cluster members is tied to the up-regulation of Myc, RT-PCR and immunoblotting were performed for Myc. Indeed, the expression of Myc was increased >15-fold at the mRNA level and >5-fold at the protein level (Fig. 3, C and D). Moreover, Thbs1 mRNA levels showed a decrease of >3-fold (Fig. 3C).

Interestingly, gene expression profiles of RR1 + R cells showed that the TGFβ pathway was greatly affected, according to Ingenuity Pathways Analysis (Ingenuity® Systems, p < 0.00022). Furthermore, of the 13 repressed TGFβ-responsive genes that harbor the miR-17–92 potential binding sites reported by Mestdagh et al. (56), we found 10 of 11 to be down-regulated in RR1 + R cells compared with BC3H1 cells (two genes were not represented on the mRNA chip). Similarly, curated gene set and oncogenic signature enrichment analysis of the RR1 + R versus BC3H1 expression arrays using GSEA (C2 and C6 gene sets) disclosed enrichment of several TGFβ target gene lists among mRNAs down-regulated in RR1 + R compared with BC3H1 cells, confirming suppression of TGFβ signaling in the former cell type (Fig. 3E). We next examined
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potential miR-17–92 binding sites upon 3′-UTR in members of the various TGFβ response gene sets listed above. To do so, we downloaded EIMMo mouse miRNA target predictions (January 2011 release) (58, 59) and cross-referenced with the genes in the pathway lists. Within the full set of genes upon the Affymetrix mouse 430A2 chip, the prevalence of the miR-17/20a, miR-18a, miR-19a/b, and miR-92a predicted sites was 10.4, 3.9, 6.8, and 5.2%, respectively, whereas 19.9% of the genes were predicted targets of at least one member of the miR-17–92 cluster. Table 3 lists the prevalence of miR-17–92 predicted targets within the enriched TGFβ response gene sets reported above and shows significant over-representation of miR-17–92 binding sites. Thus in RR1 + R transcription profiles we found evidence for the down-regulation of gene sets that have been shown by others to respond positively to TGFβ stimulation; within these gene sets, we report the enrichment of miR-17–92 binding sites and suggest that in addition to the down-regulation of TGFβ itself, targeting by miR-17–92 members may contribute to the repression of TGFβ-responsive genes as well. Interestingly, a significant increase in the mRNA levels of Tgf2 (TGFβ-induced factor homeobox 2, which represses the transcription of TGFβ-responsive genes) was found in both RR1 and RR3 + R cells (Fig. 3C). Tgf2 contains 3′-UTR target sites for miR-29 and miR-22 (TargetScan, release 6.2), which have been shown to be repressed by Myc (54). Thus our data are in agreement with the previous reports and indicate that the increase in the oncogenic miRNAs and decrease in tumor suppressor miRNA found in rapamycin-resistant cells may be attributed to the increase in Myc expression.

Because overexpression of Myc causes rapamycin resistance in prostate cancer cell lines (60), we wanted to determine whether down-regulation of Myc affects the response of RR1 cells to rapamycin and whether the miR-17–92 cluster that is downstream of Myc mediates the resistance to rapamycin in RR1 cells. To test this, we transfected RR1 cells with Myc-specific siRNA or with specific hairpin oligonucleotides to inhibit the endogenous miR-17 and other members of the cluster that share seed sequences such as miR-20a, miR-20b, miR-106a, miR-106b, and miR-93. In addition we used specific hairpin oligonucleotides to inhibit miR-19a and miR-19b. Notably, inhibition of Myc, miR-17, or miR-19 induced rapamycin sensitivity in RR1 cells (Fig. 3, F and G). Moreover, when both miR-17 and miR-19 were inhibited, sensitivity of RR1 cells to rapamycin was increased further (Fig. 3G). These findings extend previous observations of Myc-induced rapamycin resistance, suggesting that miR-17–92 cluster members may mediate Myc-induced resistance to rapamycin. Importantly, we found that inhibition of members of this miRNA cluster may restore sensitivity to rapamycin, an observation with significant implications for antitumor therapies.

### Table 4

miRNAs associated with rapamycin treatment

| Gene Name | P Value | Fold Change |
|-----------|---------|-------------|
| mmu-miR-224-2-5p | 3.5E-02 | 1.27 |
| mmu-miR-18a-3p | 3.1E-03 | 1.33 |
| mmu-miR-19a/b | 2.9E-03 | 1.64 |
| mmu-miR-19b | 2.2E-03 | 1.64 |
| mmu-miR-321-3p | 1.1E-03 | 0.77 |
| mmu-miR-321-5p | 1.1E-03 | 0.77 |
| mmu-miR-706-3p | 6.6E-10 | 0.40 |

* Sets 1–5, see respective sets in Fig. 3E; Set 6, TGFβ according to Ingenuity Pathways Analysis (Ingenuity® Systems); Set 7, KEGG TGFβ-signaling pathway.

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**TABLE 3**

Prevalence of predicted miR-17–92 cluster member binding sites upon TGFβ pathway and TGFβ-positive response gene sets

| Gene set | No. of genes in data set | miR-17/20a | miR-18a | miR-19a/b | miR-92a | miR-17–92 |
|----------|--------------------------|------------|---------|----------|---------|-----------|
| Affymetrix 430A2 | 13,790 | 10.4 | 3.9 | 6.8 | 5.2 | 19.9 |
| Set 1 | 161 | 21.1** | 11.8** | 11.81 | 7.5 | 38.5 |
| Set 2 | 87 | 12.6 (NS) | 5.7 (NS) | 13.81 | 6.9 (NS) | 26.4 (NS) |
| Set 3 | 40 | 37.5** | 2.5 (NS) | 12.5 (NS) | 10.0 (NS) | 45.0** |
| Set 4 | 156 | 23.7** | 5.1 (NS) | 17.9** | 9.0† | 34.6** |
| Set 5 | 128 | 18.8§ | 7.8§ | 13.3§ | 11.7§ | 38.3** |
| Set 6 | 25 | 24.0† | 12.0† | 24.0§ | 20.0§ | 48.0§ |
| Set 7 | 83 | 21.7 | 6.0 (NS) | 16.9 | 9.6† | 37.3** |

* Sets 1–5, see respective sets in Fig. 3E; Set 6, TGFβ according to Ingenuity Pathways Analysis (Ingenuity® Systems); Set 7, KEGG TGFβ-signaling pathway.

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**TABLE 4**

miRNAs associated with rapamycin treatment

| Gene Name | P Value | Fold Change |
|-----------|---------|-------------|
| mmu-miR-29b | 3.1E-02 | 1.23 |
| mmu-miR-7c | 1.2E-02 | 1.23 |
| mmu-miR-21a | 3.7E-03 | 1.34 |
| mmu-miR-125a-5p | 1.1E-02 | 1.24 |
| mmu-miR-127-3p | 7.5E-03 | 1.25 |
| mmu-miR-127-5p | 2.3E-04 | 1.25 |
| mmu-miR-125b-3p | 6.0E-04 | 1.27 |
| mmu-miR-140-3p | 1.2E-02 | 1.27 |
| mmu-miR-125b-5p | 2.6E-04 | 1.29 |
| mmu-miR-143a | 4.7E-04 | 1.29 |
| mmu-miR-700 | 4.4E-03 | 1.30 |
| mmu-miR-8-1 | 4.3E-01 | 1.30 |
| mmu-miR-24-2-3p | 3.1E-03 | 1.31 |
| mmu-miR-24-2-5p | 3.1E-03 | 1.31 |
| mmu-miR-125a-3p | 1.2E-02 | 1.33 |
| mmu-miR-122 | 1.8E-02 | 1.34 |
| mmu-miR-129b-3p | 5.0E-05 | 1.36 |
| mmu-miR-271 | 6.4E-05 | 1.36 |
| mmu-miR-26a | 2.2E-04 | 1.40 |
| mmu-miR-27a | 8.6E-06 | 1.43 |
| mmu-miR-26b | 1.3E-04 | 1.44 |
| mmu-miR-21 | 1.9E-04 | 1.47 |
| mmu-miR-28a | 2.1E-05 | 1.51 |
| mmu-miR-23a | 6.4E-05 | 1.56 |
| mmu-miR-762 | 2.3E-04 | 1.56 |
| mmu-miR-22 | 3.4E-04 | 1.68 |
| mmu-miR-151 | 2.4E-06 | 1.75 |
| mmu-miR-143 | 9.2E-04 | 1.79 |
| mmu-miR-20a | 4.4E-02 | 0.82 |
| mmu-miR-7a | 3.6E-04 | 0.79 |
| mmu-miR-603-5p | 3.7E-02 | 0.75 |
| mmu-miR-974 | 5.0E-05 | 0.74 |
| mmu-miR-320 | 2.2E-03 | 0.69 |
rapamycin sensitivity (Table 4, shown in bold). Notably, 33 of the 38 (~80%) significantly changed miRNAs in RR1 cells were up-regulated upon rapamycin treatment, and six of these belonged to the let-7 family of miRNAs. MiR-143, miR-22, miR-21, and miR-222 were also among the up-regulated miRNAs in response to short-term rapamycin treatment in RR1 cells.

Although not revealed by the miRNA arrays, qRT-PCR performed on RNA from BC3H1 and B3CH1/R cells showed increased levels (~1.5-fold) of let-7c, miR-21, miR-22, miR-23a, and miR-143 upon rapamycin treatment (Fig. 4A). To determine whether the changes in these miRNAs were cell line-specific, we tested the effect of 24-h rapamycin treatment in other myogenic cells such as primary murine vascular smooth muscle cells (VSMC, passage 5) and the C2C12 cell line. Indeed, all five miRNAs were up-regulated >1.5-fold by rapamycin treatment (Fig. 4A). These data imply that rapamycin treatment may affect miRNA expression in a similar manner across myogenic cell lines. The increase in miRNA expression upon short-term rapamycin treatment is likely the result of widespread changes in transcription and may be in part due to modifications (dephosphorylation) of RNA-binding proteins such as KSRP. In fact, it has been shown that KSRP, which is inactivated by PI3K/Akt signaling, interacts with Drosha and Dicer complexes to promote the biogenesis of a subset of miRNAs (61–63). Indeed, our short-term rapamycin treatment showed increases in the miRNAs that were reported to be affected by KSRP including let-7, miR-125a, miR-125b, miR-21, and miR-26a (61).

Numerous studies have shown that the let-7 family of miRNAs down-regulates oncogenes such as Myc, Ras, and HmgA2 and inhibits cell proliferation and tumorigenesis when overexpressed (64–69). To test whether the increase in the let-7 family of miRNAs antagonizes the expression of Myc, we used let-7c mimics or let-7c-specific inhibitors, and Myc levels were determined by RT-PCR. BC3H1 or RR1 cells transfected with let-7c mimic showed a ~40% decrease in Myc expression levels, whereas let-7c inhibitor showed a >1.3-fold increase in Myc expression (Fig. 4B). Moreover, overexpression of Myc in BC3H1 cells decreased the expression of let-7c, whereas inhibition of Myc expression in RR1 cells showed a 2-fold increase in let-7c expression (Fig. 4C). Because Myc was shown to induce the miR-17–92 cluster, BC3H1 cells overexpressing Myc induced the expression of miR-19 and miR-17, whereas inhibition of Myc in RR1 cells decreased the expression of miR-19 and miR-17 (Fig. 4, D and E). These data are in agreement with the previous reports and indicate that Myc and let-7 antagonize the expression of each other (54, 64). To test whether the inhibitory effect of rapamycin is mediated by increased expression of let-7 members, we used let-7a and let-7c mimics or specific inhibitors. BC3H1 cells transfected with let-7c or let-7a mimics showed an ~50% inhibition of pro-
MicroRNAs Mediate Resistance to Rapamycin

Lymphoblastic leukemia cell line BC3H1, which is sensitive to rapamycin, and its rapamycin-resistant variant R1 (RR1) were examined for the expression of tumor suppressor miRNAs mimics, or inhibition of onco-

target of miR-17–92 clusters and inhibits tumor suppressor miRNAs.

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