MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins

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Using highly sensitive microarray-based procedures, we identified eight microRNAs (miRNAs) showing robust differential expression between 31 laser-capture-microdissected nasopharyngeal carcinomas (NPCs) and 10 normal healthy nasopharyngeal epithelial samples. In particular, miRNA *mir-29c* was expressed at one-fifth the levels in tumors as in normal epithelium. In NPC tumors, the lower *mir-29c* levels correlated with higher levels of multiple mRNAs whose 3′ UTRs can bind *mir-29c* at target sequences conserved across many vertebrates. In cultured cells, introduction of *mir-29c* down-regulated these genes at the level of mRNA and inhibited expression of luciferase encoded by vectors having the 3′ UTRs of these genes. Moreover, for each of several genes tested, mutating the *mir-29c* target sites in the 3′ UTR abrogated *mir-29c*-induced inhibition of luciferase expression. Most of the *mir-29c*-targeted genes identified encode extracellular matrix proteins, including multiple collagens and laminin γ1, that are associated with tumor cell invasiveness and metastatic potential, prominent characteristics of NPC. Thus, we identify eight miRNAs differentially expressed in NPC and demonstrate the involvement of one in regulating genes involved in metastasis.

microarray | collagen | metastasis | miRNA

MicroRNAs (miRNAs) are short (≈22 nucleotides) noncoding RNAs involved in posttranscriptional silencing of target genes. In animals, miRNAs control expression of target genes by inhibiting translation, by degrading target mRNAs, or both, through binding to their 3′ UTRs with varying degrees of sequence complementarity (1). miRNAs have been found to regulate genes involved in diverse biological functions, including development, differentiation, proliferation, and stress response (2). Recently, a growing number of miRNAs have been implicated in cancers, including *mir-15* and *mir-16* in B cell chronic lymphocytic leukemias (3, 4); *mir-143* and *mir-145* in colorectal cancer (5); *mir-17-5p*, *mir-21*, *mir-125b*, *mir-145*, and *mir-155* in breast cancer (6, 7); *mir-19*, *mir-146*, *mir-181b*, *mir-221*, *mir-222*, and *mir-346* in thyroid cancer (8–10); and *mir-21* in glioblastoma (11). A significant number of miRNAs also have been mapped to cancer-associated genomic regions (12). Expression of miRNA *let-7* has been correlated with prognosis in lung cancer (13) and found to regulate Ras in the same tumor (14). Very recently, *mir-10b* has been shown to contribute to metastasis in breast cancer (15). Although many miRNAs have been implicated in regulating cancers, very few of their target genes, and hence their downstream mode of action, have been identified.

We developed a sensitive microarray-based assay to profile miRNA expression and used it to analyze human miRNAs in laser-microdissected tumor and normal cells from biopsies of a highly invasive cancer, nasopharyngeal carcinoma (NPC), and site-matched normal tissues. Eight miRNAs were differentially expressed. One of them, *mir-29c*, down-regulated in NPC, was shown to target multiple mRNAs encoding extracellular matrix proteins associated with cell migration and metastasis. Thus, reduced *mir-29c* in NPC tumors leads to increased accumulation of the mRNAs encoding these proteins, likely contributing to the invasive characteristic of this cancer.

Results

NPC is associated with EBV, is found prominently in people in South East Asia, and is highly invasive (16). We previously analyzed differential gene expression in NPC relative to normal nasopharyngeal epithelium that could underlie the properties of this tumor, which elucidated the contribution of EBV genes toward immune evasion of tumor cells in this cancer and further implicated DNA repair and nitrosamine metabolism mechanisms in NPC pathogenesis (17, 18). For the current study, we developed a sensitive method to measure miRNAs to determine whether they, too, could contribute to the properties of NPC. This method uses a microarray-based assay to profile miRNA expression from small samples of cells that can be isolated in pure form with laser-capture microdissection (LCM). Based on the unique 5′ ends of miRNAs, adaptors were ligated preferentially to miRNAs in a pool of total RNA, thus avoiding losses associated with size selection [supporting information (SI) Fig. S1]. The ligated miRNAs were copied into double-stranded cDNA and subsequently transcribed with T7 RNA polymerase. The amplified miRNA samples were probed with miRNA microarrays and detected by using signal enhancing fluorophores (Fig. S1; *Materials and Methods*). We analyzed the expression of 207 miRNAs (Table S1) from 31 laser-capture-microdissected NPC and 10 site-matched normal epithelial tissues.

miRNA expression values were normalized across samples and multiple statistical tests (Wilcoxon rank sum, *t* test on raw and log scale, each at 5% false discovery rate) were applied to establish their consistent differential expression between the 31 tumor and 10 normal samples (Table S2). To focus only on miRNAs showing robust differential expression, we excluded from analysis miRNAs expressed at low levels (quantile normalized expression value <800, Table S2) in both tumor and normal samples.

Eight cellular miRNAs had ≥5-fold differential expression between tumor and normal tissues. Six miRNAs (*mir-29c*, *mir-34b/c*, *mir-212*, *mir-216*, and *mir-217*) showed lower expression in

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Table 1. miRNAs differentially expressed between normal and NPC tumor tissues

miRNA	Normal* (n = 10)	Tumor* (n = 31)	Fold difference (tumor/normal)	Wilcoxon P value**
miR-29c	32,320	6,567	0.20	0.002
miR-34b	28,879	3,252	0.11	0.000
miR-34c	25,243	1,461	0.06	0.001
miR-212	4,363	885	0.20	0.000
miR-216	6,843	940	0.14	0.002
miR-217	4,212	351	0.08	0.000
miR-151	60	3,598	60.25	0.001
miR-192	71	1,573	22.02	0.000

*Each miRNA level is reported as the median of miRNA expression levels (microarray-normalized probe fluorescence) for all 10 normal or 31 tumor samples, respectively.

**Probability that a particular miRNA is not differentially expressed, based on Wilcoxon rank-sum comparison of all 310 possible tumor normal pairs.

Table 2. Fold changes in miRNA targeted mRNAs

miRNA	Target mRNA	Fold change (tumor/normal)
miR-29c	FLJ12505	6.34
miR-29c	COL4A1	5.24
miR-29c	COL4A2	4.58
miR-29c	COL3A1	4.14
miR-29c	COL1A2	4.10
miR-29c	COL5A2	4.05
miR-29c	FBN1	2.98
miR-29c	SPARC	2.93
miR-29c	COL15A1	2.92
miR-29c	FUSIP1	2.59
miR-29c	COL1A1	2.31
miR-29c	TFEC	2.27
miR-29c	IFNG	2.24
miR-29c	LAMC1	2.06
miR-29c	TDG	1.80
miR-34b/c	CCNE2	4.52
miR-34b/c	ATP11C	3.55
miR-34b/c	IQGAP3	3.14
miR-34b/c	SOX4	2.77
miR-34b/c	ARNT2	2.27
miR-34b/c	VEZATIN	2.07
miR-34b/c	E2F3	2.05
miR-212	SOX4	2.77
miR-212	EIF2C2	1.64
miR-216	LAMC1	2.06
miR-216	NFYB	1.85
miR-217	FN1	7.39
miR-217	ANLN	3.70
miR-217	EZH2	2.74
miR-217	FUSIP1	2.59
miR-217	POLG	2.57
miR-217	DOCK4	2.48
miR-217	HNRPA2B1	1.63

Fold change (tumors/normals) was averaged for mRNAs detected by multiple probe sets.

tumor cells, and two (*mir-151* and *mir-192*) had higher expression in tumors (Table 1). To determine how these eight differentially expressed miRNAs might contribute to tumor phenotypes, we searched for their potential regulatory targets using algorithms based on miRNA-mRNA complementarity and its evolutionary conservation [TargetScan (19) and PicTar (20)]. The target sites of miRNAs in mRNAs often are evolutionarily conserved, and considering such conservation increases the reliability of identifying targets (19). Because these target sites are identified by a minimum perfect complementarity of only 7–8 nt at the 5′ end of the miRNAs (the "seed" sequence), these algorithms produce many false-positive targets. In addition to regulating gene expression by inhibiting translation, which is thought to be the more common action of miRNAs, miRNAs can also regulate expression of a subset of their targets by decreasing mRNA stability (21–23). Such miRNA function should be evident in gene expression profiling data. Therefore, using our prior mRNA profiling (17), we sought bona fide targets among the large number of predicted target mRNAs of the eight highly differentially expressed miRNAs, by identifying those targets that accumulate differentially between tumor and normal samples. None of the predicted target mRNAs for *mir-151* and *mir-192* showed differential mRNA accumulation. However, we identified statistically significant differentially accumulating, candidate target mRNAs for the six miRNAs whose levels decreased in NPC (Table 2). The largest set of differentially expressed predicted targets was associated with *mir-29c*. *mir-29c* levels averaged one-fifth the level in NPC tumors as in normal nasopharyngeal epithelium (Table 1) and, correspondingly, the 15 differentially accumulating predicted *mir-29c* target mRNAs accumulated to 2- to 6-fold higher levels in NPC tumors (Table 2). Strikingly, 10 of these 15 differentially accumulating candidate target mRNAs of *mir-29c* were involved in extracellular matrix synthesis or its functions, including seven collagens, laminin γ1, fibrillin, and secreted protein, acidic, cysteine-rich (SPARC). Interestingly, two differentially expressed *mir-29c* targets, laminin γ1 and FUS-interacting protein (FUSIP1) mRNAs, also were predicted targets of *mir-216* and *mir-217*, respectively, which, like *mir-29*c, were down-regulated miRNAs in NPC tumors (Tables 1 and 2).

The seed sequence of *mir-29c* is identical to that of its two family members *mir-29a* and *mir-29b*. These three *mir-29* species vary in their last few 3′ end nucleotides. In addition, in close proximity to its seed sequence, *mir-29a* has a single-nucleotide difference from *mir-29b*/*c*, giving mir-29c an overlapping but distinct list of predicted target mRNAs. *mir-29a* is expressed at slightly higher levels than *mir-29c* in normal tissue, and its levels are moderately decreased in tumors. *mir-29b*, predominantly targeted to the nucleus (24), is expressed at one-fourth the level of *mir-29c* in normal nasopharyngeal epithelium. In NPC tumors, *mir-29b* and *mir-29c* have similar 4- to 5-fold decreased levels (Table S2). Thus, the levels of all three *mir-29* family members are decreased in tumors, implying parallel effects on their shared targets.

To test whether *mir-29c* indeed regulates the levels of the candidate target mRNAs, we transfected *mir-29c*'s precursor RNA into the epithelial cell lines HeLa and HepG2. The resulting changes in levels of the mature miRNA and its target mRNAs relative to their levels in untransfected cells were measured with real-time PCR (Table S3). The same cells were transfected in parallel with a control precursor miRNA, which when processed is unrelated to known miRNAs. In HeLa cells, eight potential *mir-29c* target mRNAs were detected at higher than background levels, and five of these were reduced significantly by *mir-29c* transfection: collagen 3A1, 4A1, 15A1, laminin γ1, and thymine-DNA glycosylase (TDG) (Fig. 1). In HepG2 cells, reductions were seen for four of these five mRNAs (Fig. 1), whereas the fifth, collagen 3A1 mRNA, was not detectable above background levels. Most of the reductions in mRNA levels were more pronounced in HepG2 than in HeLa cells, likely because of the lower basal level of these mRNAs in HepG2 (Table S4), the consequent higher miRNA:mRNA ratio posttransfection, and the attendant increased efficiency of mRNA down-regulation (25). In addition, HepG2 cells showed *mir-29c*-

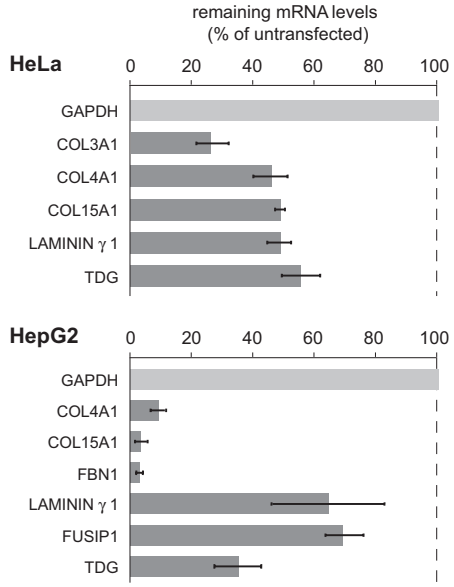

Fig. 1. *mir-29c* down-regulates accumulation of its target mRNAs. HeLa and HepG2 cells transfected with *mir-29c* precursor show lower levels of target mRNAs than untransfected cells as measured by quantitative real-time PCR normalized to GAPDH (Table S4). This down-regulation is more pronounced in HepG2 cells, possibly because of the lower basal level of these mRNAs in these cells, resulting in higher a miRNA:mRNA ratio posttransfection and attendant increased efficiency of mRNA down-regulation.

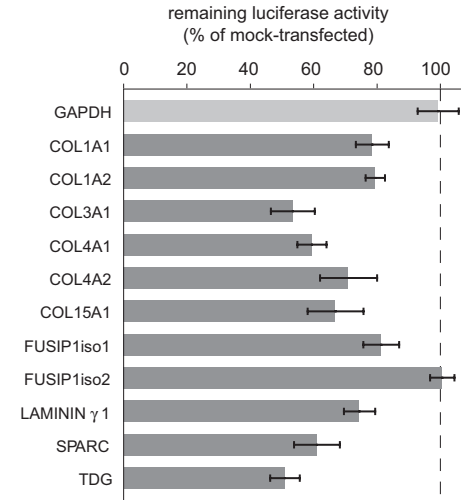

Fig. 2. *mir-29c* inhibits expression of luciferase with 3′ UTRs derived from *mir-29c*'s target genes. 3′ UTRs of target genes containing *mir-29c*-binding sites were cloned into vectors containing firefly luciferase that were transfected into HeLa cells. These cells were subsequently transfected with *mir-29c* precursor RNAs or mock-transfected. Compared with cells that were mock-transfected (set to 100%), *mir-29c* precursor-transfected cells show down-regulation in luciferase activity.

mediated reductions for two additional candidate, target genes, fibrillin 1, and FUSIP1 (Fig. 1). In all cases, these *mir-29c*-induced reductions significantly exceeded any changes resulting from parallel transfections of the randomized negative control precursor miRNA (Table S4), indicating the down-regulation was both specific to the sequence of the miRNA and unrelated to possible artifacts of transfection. In particular, introducing the miRNAs into HeLa or HepG2 cells did not elicit an IFN response, as evidenced by no significant changes in expression of mRNAs for IFN-activated gene OAS1 (Table S4). In addition, all control or *mir-29c*-transfected cultures had similar levels of GAPDH mRNA, an mRNA lacking target homology to *mir-29c*.

Next, we cloned the 3′ UTRs containing the *mir-29c* binding sites for 10 of its candidate target genes and their isoforms into a vector downstream of a firefly luciferase gene. In parallel, we cloned the GAPDH 3′ UTR, which is not a *mir-29c* target, downstream of luciferase as a control. HeLa cells were transfected with these constructs with or without subsequent *mir-29c* precursor RNA transfection. The 3′ UTRs of all of these 10 candidate target genes (Collagen 1A1, 1A2, 3A1, 4A1, 4A2, 15A1, FUSIP1iso1, laminin γ1, SPARC, and TDG) elicited significantly decreased luciferase activities (P values from 3×10^{-3} to 1.2×10^{-7}) in *mir-29c* transfected cells (Fig. 2). These inhibitions, ranging from ≈20% to 50%, are similar in magnitude to equivalent experiments involving transfection of miRNA precursors (26–28). In general, for each 3′ UTR, *mir-29c*-induced reductions in luciferase activity (Fig. 2) correlated well with the magnitude of the *mir-29c*-induced reduction in the level of the corresponding full mRNA (Fig. 1). Our findings with FUSIP1 provide additional support for the specificity of *mir-29c*. FUSIP1 has two isoforms, and only one of them (isoform1) is a potential target for *mir-29c*. The 3′ UTR of isoform2 did not support detectable inhibition of luciferase activity by *mir-29c*, whereas that of isoform1 led to statistically significant inhibition (P value $= 3 \times 10^{-3}$) (Fig. 2). Nucleotide substitutions disrupting the *mir-29c*-binding site(s) were introduced in the 3′ UTRs of collagen 1A1, 3A1, and 4A2 cloned downstream of the firefly luciferase gene (Fig. 3*A*). In every case, this disruption of the target binding sites for *mir-29c* abrogated the inhibition of luciferase activity by *mir-29c* (Fig. 3*B*). Thus, the predicted target sequences were responsible for the *mir-29c* sensitivity of these 3′ UTRs.

Discussion

We profiled miRNA expression in laser-microdissected NPC and normal surrounding epithelial cells using a sensitive assay able to detect miRNA expression from such small samples. Eight of 207 assayed miRNAs displayed >5-fold differential expression levels in NPC cells compared with surrounding normal epithelium (Table 1). Using bioinformatic approaches, we identified candidate target genes of these eight miRNAs. Next, we analyzed our previous mRNA expression profiling data of these same specimens (17) to identify candidate target genes that were differentially expressed, possibly because of action of these miRNAs. Among the differentially expressed candidate target genes of the eight miRNAs, those of *mir-29c* showed a group of 15 genes, 10 of which were extracellular matrix components involved in cell migration and metastasis (Table 2). In tumor cells, *mir-29c* levels were decreased >5-fold, whereas these mRNAs were up-regulated 2- to 6-fold.

Using multiple tissue culture-based assays (Figs. 1–3), we tested the regulation of these candidate target genes by *mir-29c*. Transfection and reporter assays confirmed regulation of 11 target genes by *mir-29c*. The results imply that the reduced levels of *mir-29c* in NPC tumors allowed the observed increase in mRNA levels of multiple extracellular matrix components, which as noted before would facilitate rapid matrix generation and renewal during tumor growth and the acquisition of tumor motility.

For many tumor cells, increased extracellular levels of collagens and/or laminins have been shown to induce increased invasiveness in culture and increased metastasis in animal models (29–36). Similarly, increased levels of collagens and laminins have been associated with an increased likelihood of clinical metastasis of multiple human solid tumors (37). This last study has elicited some controversy because of the possibility that the increased levels of mRNAs encoding extracellular matrix pro-

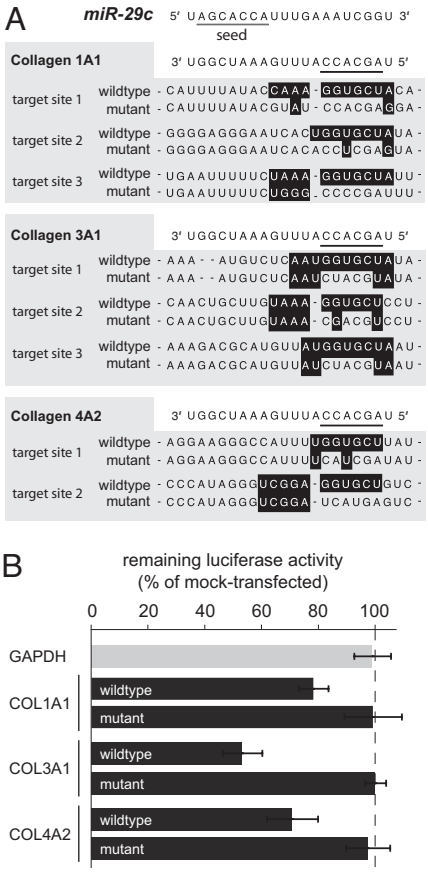

Fig. 3. Mutations disrupting the binding of *mir-29c* to the 3′ UTRs of three target genes block *mir-29c* mediated inhibition of the expression of these genes. (*A*) Black-boxed nucleotides in the mRNA sequence indicate the extent of basepairing with *mir-29c*, and in particular how the mutations disrupt basepairing with the *mir-29c* seed sequence. (*B*) The wild-type or mutated 3′ UTRs of target mRNAs were cloned into vectors containing firefly luciferase for expression in HeLa cells, which were subsequently transfected with precursor *mir-29c* RNA or mock-transfected. Luciferase activity was no longer affected by *mir-29c* in cells transfected with constructs containing the mutated target sequence.

teins in the studied tumor samples might have come from the stromal cells supporting those tumors (38). Our use of laser capture to isolate tumor cells essentially free of stromal contaminants (17) indicates that NPC tumor cells themselves upregulate mRNAs encoding collagens and laminins.

mir-29c is a member of the *mir-29* family that also includes the closely related *mir-29a* and *mir-29b*. In a recent report on cholangiocytes/cholangiocarcinoma, *mir-29b* was shown to regulate expression of the antiapoptotic protein Mcl-1 by inhibiting its translation without affecting Mcl-1 mRNA levels (26). Consistent with these observations, despite a 4-fold decrease in *mir-29b* levels in NPC tumor cells (Table S2), Mcl-1 mRNA levels also remained unchanged in NPC (17). It is feasible that the level of Mcl-1 protein is likewise increased in NPC tumor cells because of the reduced action of *mir-29b*, contributing to suppression of apoptosis. Increased aggressiveness in B cell chronic leukemia correlates with concerted reduction of the levels of *mir-29* and *mir-181* and increased expression of their common target, the B and T cell malignancy-specific oncogene TCL1 (39, 40). In NPC, TCL1 mRNA levels did not show a significant inverse correlation with *mir-29c* levels, possibly because *mir-29c* reduction was in part compensated in NPC by an increase in *mir-181* levels (Table S2).

The magnitude of the *mir-29c* effects reported here for target mRNAs (Fig. 2), ranging from ≈20% to 50% inhibition, is consistent with the effects of transfecting other single miRNAs (26–28). Frequently, multiple miRNAs target a single mRNA, thus increasing their effectiveness (27). For example, in neuroblastoma cells, three different miRNAs regulate the levels of a single protein (41). Similarly, two differentially expressed *mir-29c* targets, laminin γ1 and FUSIP1 mRNAs, are also predicted targets of *mir-216* and *mir-217*, respectively, which like *mir-29c* were down-regulated in NPC tumors. Moreover, in addition to down-regulating mRNA accumulation, the same miRNA(s) may inhibit translation of their target RNAs.

FUSIP1 is one of the identified *mir-29c* targets that does not encode an extracellular matrix component. FUSIP1 interacts with the oncoprotein FUS/TLS and has a role in general repression of RNA splicing (42), providing an additional mechanism for *mir-29c* to regulate gene expression. Another important gene regulated by *mir-29c* is thymine-DNA glycosylase (TDG), involved in DNA repair, a process frequently dysregulated in NPC and other cancers (18). In lung cancer, *mir-29* has a known epigenetic role in targeting expression of DNA methyltransferases (DNMT3A and -3B), and transfection of lung cancer cell lines with *mir-29* greatly reduces their potential to form tumors when engrafted into nude mice (28). *mir-29a* had the biggest effect on DNMT3A and -3B, because the largest rescue of these genes was brought about by silencing of *mir-29a* compared with the other members of the *mir-29* species. Consistent with the lack of differential expression of *mir-29a* in our NPC tumors (Table S2), we also did not observe changes in DNMT3A and -3B mRNA levels in NPC (17).

In conclusion, we provide mechanistic insights into miRNA functions in NPC, a tumor associated with high invasiveness. We have identified miRNAs that are aberrantly expressed in tumor cells compared with normal nasopharyngeal epithelium. Using several computational and tissue culture-based assays, we identified multiple genes whose expression is inhibited by one such aberrantly expressed miRNA, *mir-29c*. *mir-29c* is reduced in its levels in tumors leading to a concomitant increase in its target genes, which encode extracellular proteins and whose increased expression has been associated with increased invasiveness and metastasis of tumors. This decrease in the expression of *mir-29c* in NPC cells thus likely contributes to the invasive characteristic of these tumors.

Materials and Methods

miRNA Isolation and Amplification. NPC tissue sample collection and processing, including histopathology, laser-capture microdissection, and RNA extraction, have been described in detail (17). Institutional Human Subject Committee Review Boards of National Taiwan University, the University of Wisconsin-Madison, and the National Cancer Institute approved this study. Thirty-one NPC samples and 10 normal nasopharyngeal epithelium samples, including six normal tissues from NPC cases and four from biopsy-negative cases, were used for the present analysis of expression of miRNAs. miRNA was amplified from total RNA isolated from laser-microdissected/whole-tissue sections following Lau *et al.* (43). Briefly, 3′ linker (5′ AppCTG TAG GCA CCA TCA ATddC 3′, Integrated DNA Technologies) and 5′ linker (5′ ATC GTa ggc acc uga amino acid 3′ uppercase DNA; lowercase RNA, Dharmacon RNA Technologies) oligonucleotides were ligated and reverse-transcribed using SuperScript II (Invitrogen) and primer 5′ ATT GAT GGT GCC TAC 3′. The cDNA was amplified by PCR by using forward primer 5′ GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG TTC TCG TGT TCC GTT TGT ACT CTA AGG TGG AAT CGT AGG CAC CTG AAA 3′ and reverse primer 5′ ATT GAT GGT GCC TAC AG 3′ for 20 cycles. The forward PCR primer contained a 3′ region complementary to the 3′ end of the cDNA, an adjacent ''capture sequence'' (TTC TCG TGT TCC GTT TGT ACT CTA AGG TGG A) and a 5′ terminal T7 promoter. The PCR product was *in vitro*-transcribed by using T7 RNA polymerase producing a sense target for hybridization containing the complement of the capture sequence.

Microarray Construction. Microarray probes were antisense dimers of mature miRNA sequences taken from miRBase (http://microrna.sanger.ac.uk), previ-

ously the microRNA registry (44). Two hundred and seven probes for human miRNAs, as present in the miRBase of April 2005, and seven probes from *Drosophila melanogaster* miRNAs as controls (Table S1), all modified with a 5′ C6 amino linker to attach to aldehyde-coated slides (ArrayIt SuperAldehyde Substrates, Telechem International) were printed in quadruplicate with a BioRobotics MicroGrid II microarrayer (Genomic Solutions). Microarrays were printed by using 40-μM probe solutions in 2.4 $\times$ SSC and preprocessed according to the slide manufacturer's instructions.

Hybridization and Detection. The *in vitro*-transcribed targets were hybridized to the microarrays overnight at 55°C. Microarrays were washed and spin-dried, and a secondary hybridization for detection was carried out with Cy3 3DNA molecules with the capture sequence bound to the fluorophore (3DNA Array 900 Microarray detection kit, Genisphere). The 3DNA molecules each contain $\approx$900 Cy3 fluorophores and make possible the detection of hybridized short targets not detectable by conventional target labeling, because short targets like miRNAs incorporate very few fluorophores to allow detection. After the second hybridization for 4 h at 42°C, the arrays were again washed, dried, and scanned. Data were acquired with GenePix Pro 5.0 (Molecular Devices). All hybridization buffers, wash conditions, etc., were as provided by Genisphere.

Identification of Differentially Expressed miRNAs. Background-corrected raw-scale expression intensity values were obtained via GenePix Pro 5.0 (Molecular Devices) after minor manual adjustment to align and identify spots. Data from multiple microarrays were normalized by using a version of quantile normalization (45) in which the expression value at the pth quantile on the ith microarray was replaced by the median of pth quantiles across the set of all 41 microarrays. Gene-specific hypothesis tests were applied to the quantile-normalized data to assess differential expression between tumor and normal miRNA profiles. To minimize false-positive calls and retain robustness, we applied a Wilcoxon rank sum test, raw scale *t* test, and log scale *t* test and called a miRNA differentially expressed if it was significant by all three tests at the 5% false discovery rate. Gene-specific *P* values were converted to q-values (46); the list containing genes with q-value $\leq$5% is expected to have no $>$5% false positives. The above statistics for the miRNAs and their median expression values for the tumor and normal tissues are given in Table S2.

Target Predictions. Target mRNAs of the miRNAs were predicted by using PicTar (20) based on conservation in mammals (human, chimp, mouse, rat, and dog) and TargetScan (19). Targets predicted by both algorithms were considered in further analysis.

miRNA Transfections and Target Validations. miRNA precursor transfections and luciferase assays for validation of candidate target genes are given in *SI Text*.

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