MicroRNA-21 Targets the Tumor Suppressor Gene Tropomyosin 1 (TPM1)*

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MicroRNAs are small noncoding RNA molecules that control expression of target genes. Our previous studies show that mir-21 is overexpressed in tumor tissues compared with the matched normal tissues. Moreover, suppression of mir-21 by antisense oligonucleotides inhibits tumor cell growth both in vitro and in vivo. However, it remains largely unclear as to how mir-21 affects tumor growth, because our understanding of mir-21 targets is limited. In this study, we performed two-dimensional differentiation in-gel electrophoresis of tumors treated with anti-mir-21 and identified the tumor suppressor tropomyosin 1 (TPM1) as a potential mir-21 target. In agreement with this, there is a putative mir-21 binding site at the 3′-untranslated region (3′-UTR) of TPM1 variants V1 and V5. Thus, we cloned the 3′-UTR of TPM1 into a luciferase reporter and found that although mir-21 down-regulated the luciferase activity, anti-mir-21 up-regulated it. Moreover, deletion of the mir-21 binding site abolished the effect of mir-21 on the luciferase activity, suggesting that this mir-21 binding site is critical. Western blot with the cloned TPM1-V1 plus the 3′-UTR indicated that TPM1 protein level was also regulated by mir-21, whereas real-time quantitative reverse transcription-PCR revealed no difference at the mRNA level, suggesting translational regulation. Finally, overexpression of TPM1 in breast cancer MCF-7 cells suppressed anchorage-independent growth. Thus, down-regulation of TPM1 by mir-21 may explain, at least in part, why suppression of mir-21 can inhibit tumor growth, further supporting the notion that mir-21 functions as an oncogene.

MicroRNAs (miRNAs)2 are a class of naturally occurring small noncoding RNAs that regulate gene expression by targeting mRNAs for translational repression or cleavage (1, 2). Like protein-coding mRNAs, miRNAs are transcribed as long primary transcripts in the nucleus. However, unlike protein-coding mRNAs, miRNAs are subsequently cleaved to produce stem-loop-structured precursor molecules of ~70 nucleotides in length (pre-miRNAs) by the nuclear RNase III enzyme Drosha (3). The pre-miRNAs are then exported to the cytoplasm, where the RNase III enzyme Dicer further processes them into mature miRNAs (~22 nucleotides). Thus, miRNAs are related to, but distinct from, short interfering RNAs (siRNAs) (4, 5). A key difference between siRNAs and miRNAs is that siRNAs require almost identical sequences to targets to exert their silencing function, whereas miRNAs bind through partial sequence homology to the 3′-untranslated region (3′-UTR) of target genes. Because of this unique feature, a single miRNA has multiple targets. Thus, miRNAs could regulate a large fraction of protein-coding genes, and as high as 30% of all genes could be miRNA targets (6).

As a new layer of gene regulation mechanism, miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation, and apoptosis (7, 8). Hence, deregulation of miRNA expression may lead to a variety of disorders. Aberrant expression of miRNAs in cancer has been well documented (7). Apparently, miRNAs may function as tumor suppressors or oncogenes by targeting oncogenes or tumor suppressor genes (9). In this regard, tumor-suppressive miRNAs are usually underexpressed in tumors. For instance, let-7 is down-regulated in lung cancer (10, 11). Furthermore, more than 60% of investigated patients suffering from B-cell chronic lymphocytic leukemia (B-CLL) have been reported to show a deletion at chromosome 13q14 where the mir-15 and mir-16 genes are located; these genes are under-represented in many B-CLL patients (12). Deregulation of miRNAs has also been reported in many other types of cancers. However, although miRNAs have been the subject of extensive research in recent years, the molecular basis of miRNA-mediated gene regulation and the effect of these genes on tumor growth remain largely unknown because of our limited understanding of miRNA target genes.

Identification of miRNA target genes has been a great challenge. Computational algorithms have been the major driving force in predicting miRNA targets (13–15). The approaches are mainly based on base pairing of miRNA and target gene 3′-UTR, emphasizing the location of miRNA complementary elements in 3′-UTR of target mRNAs, the concentration in the seed (6–8 bp) of continuous Watson-Crick base pairing in the 5′ proximal half of the miRNA, and the phylogenetic conservation of the complementary sequences in 3′-UTRs of ortholo-
gous genes. However, evidence suggests that perfect seed pairing may not necessarily be a reliable predictor for miRNA-target interactions (16), which may explain why many predicted target sites are nonfunctional. A recent study also suggests that there may be at least three types of miRNA-mRNA interactions in mammals (17). Hence, with few exceptions, large portion of the physiologic targets for miRNAs remain to be identified or verified experimentally.

In this study, we analyzed tumors derived from breast cancer MCF-7 cells treated with antisense mir-21 oligonucleotide (anti-mir-21) or the negative control by two-dimensional differentiation in-gel (2-DIGE) and identified the tumor suppressor tropomyosin 1 (TPM1) as a putative mir-21 target. Subsequent experiments confirmed that mir-21 down-regulated expression of TPM1, whereas anti-mir-21 up-regulated its expression through the mir-21 binding site at the 3′-UTR region. Furthermore, ectopic expression of TPM1 suppressed anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 cells (obtained from American Type Cell Collection, Manassas, VA) were grown in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM glutamine, 100 units of penicillin/ml, and 100 µg of streptomycin/ml (Cambrex). MCF10A cells (ATCC) were grown in serum-free mammary epithelial growth medium (from Cambrex) supplemented with 100 ng/ml cholera toxin (EMD Biosciences, San Diego, CA). 293T cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (Cambrex) supplemented with 10% fetal bovine serum. All cells were incubated at 37 °C in a humidified chamber supplemented with 5% CO₂.

Reagents—Anti-mir-21 (AM17000, ID No. AM10206) and the negative control (AM17010) were purchased from Ambion (Austin, TX). Anti-TPM1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Transfection—Transfection of MCF-7 cells was performed with OptiFect reagent (Invitrogen) following the manufacturer’s protocol. Briefly, the cells were seeded in 6-well plates at 30% confluence on the day before transfection. Three µg of TPM1-expressing plasmid or control vector was used for each transfection in antibiotic free Opti-MEM medium (Invitrogen). Transfection of 293T cells (ATCC) were performed using the calcium phosphate method as described previously (18). The negative control oligonucleotide or anti-mir-21 oligonucleotide (both from Ambion) at 50 nM or 3 µg of appropriate plasmid (otherwise indicated) was used for each transfection. Transfection efficiency was monitored by spiking GFP-expressing vector or β-galactosidase-expressing vector when necessary.

Detection of Mature mir-21 by TaqMan Real-time PCR—TaqMan miRNA assays (ABI, Forest City, CA) used the stem-loop method (19, 20) to detect the expression level of mature mir-21. For RT reactions, 10 ng total RNA was used in each reaction (15 µl) and mixed with the RT primer (3 µl). The RT reaction was carried out under the following conditions: 16 °C for 30 min; 42 °C for 30 min; 85 °C for 5 min; and then held on 4 °C. After the RT reaction, the cDNA products were diluted at 150X; and 1.33 µl of the diluted cDNA was used for PCR reac-

FIGURE 1. Identification of differentially expressed proteins from tumors treated with anti-mir-21 or negative control by 2-DIGE. Tumors were harvested, frozen and processed as described under “Experimental Procedures.” Protein was labeled with Cy3 (green) for negative control and Cy5 (red) for anti-mir-21, respectively. Isoelectric focusing was carried out at pH 3–10, and the two-dimensional separation was carried out in an 8–14% gradient SDS-PAGE. A, protein profiles of tumor samples treated with negative control or anti-mir-21. B, gel image revealing differential expression of proteins in the control and treated samples after merging. Protein spots shown in red are presumably due to up-regulation by anti-mir-21, and 10 such spots are circled. The molecular weight and pH markers are also indicated. T, TPM1; S, SELENBP1; I, ITGB4BP. C and D, up-regulation of the endogenous TPM1 in tumor samples by anti-mir-21 as detected by Western blot. Values in D are means of three separate experiments ± S.E. **, p < 0.01; N, negative control; A, anti-mir-21.
**TPM1 Is Targeted by mir-21**

**A**

| 192 | TPM1 Coding region | 1046 |
|-----|---------------------|------|
| 756 | 828 | 1246 |
| 1042 | 1090 |

**Variant 1**

**Variant 5**

**B**

**Putative mir-21 binding site**

AGUUGUAGUCAGAC-UAUUCGA-5′  
5′-TCAATATACACACTGTGTTAGCTTACAGTGTTT-3′ (1239)  
TPM1

**C**

**Relative Luciferase Activity**

| pGL3-control | Luc-TPM1-V1-UTR |
|--------------|-----------------|
| 1.0          | T**+**          |
| 0.8          |                |
| 0.6          |                |
| 0.4          |                |
| 0.2          |                |
| 0.0          |                |

**FIGURE 2. Down-regulation of luciferase activity of Luc-TPM1-V1-UTR.** A, schematic description of TPM1 variants 1 (GenBank™ accession number NM_000366.5) and 5 (GenBank™ accession number NM_00366.5). The coding region is shown in the open box from nucleotides 192–1406. B, alignment of the TPM1 UTR mir-21 binding site from variants 1 and 5 with mir-21, displayed in 3′ to 5′. C, luciferase activity of Luc-TPM1-V1-UTR compared with that of the pGL3 control vector, which was carried out in 293T cells. ***, p < 0.01.

Detection of TPM1 mRNA—To detect relative levels of TPM1 transcription, qRT-PCR was performed using the Cyber Green method under the following conditions: 94 °C for 3 min followed by 30 cycles of 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 0.5 min. PCR primers were TPM1-5.1, sense, 5′-CTCTCAACGATATGACTATCCCCATTTCC-3′, and TPM1-3.1, antisense, 5′-TTTTTTTAGCTTACACAGTGTTT-3′. Both were purchased from Sigma-Genosys (Woodland, TX).

Constructs—To construct a plasmid expressing mir-21, we first modified pCMV-Myc (Clontech, Mountain View, CA) by deleting the Myc tag by PCR. We then amplified a 500-bp DNA fragment carrying pre-mir-21 from MCF10A genomic DNA using PCR primers mir-21-5.1, 5′-GAATTCCTGATTGAACT-TGTTCAATTT-3′ where the EcoRI site is underlined, and mir-21-3.1, 5′-GTTACCAATTAGACTATCCCCATTCTCACA-3′, where the KpnI site is underlined. The amplified fragment was first cloned into pCR8 (Invitrogen) and was subsequently cloned into this modified pCMV vector at the EcoRI and KpnI sites.

Full-length TPM1 plus 3′-UTR was amplified from MCF-7 cells using primers TPM1-EcoRI-5.1, 5′-GAATTCCTGAGCCATCAAGAAGAAGA-3′, and TPM1-UTR-NotI-3.1, 5′-GCGGCCCCTTACAATGTGCATTTTATATTCC-3′, then cloned into pCR8, and finally subcloned into the original pCMV-Myc. The 250-bp 3′-UTR region of TPM1 was also amplified from MCF-7 cells using primers TPM1-UTR-XbaI-5.1, 5′-TCTAGACTCTCAACGATATGGACTTCA-3′, and TPM1-UTR-XbaI-3.1, 5′-TCTAGATTTTTTCTGCTTACACAGTGTT-3′, using the same approach described above, and was finally cloned into pGL3 control vector (Promega, Madison, WI) at the XbaI site.

To construct a plasmid expressing the GFP-TPM1 fusion protein (pEFGP-TPM1 + UTR), we also used primers TPM1-R1-5.1 and TPM1-UTR-NotI-3.1 as indicated above. This fragment was finally cloned into pEFGP-C3 (Clontech) at the EcoRI and NotI sites in-frame with the GFP coding region.

To clone the 3′-UTR of TPM1 into a GFP reporter, which was different from the GFP fusion construct, we first modified the pEFGP-C3 by introducing a stop codon in the front of the multiple cloning sites by PCR and then cloning the TPM1-UTR fragment into EcoRI site of this modified vector. All PCR products were verified by DNA sequencing before cloning into the final destination vectors.

**Luciferase Assay**—293T cells were seeded in 6-well plates and transfected with luciferase reporters using the calcium phosphate method as described above. After transfection, the cells were split into 12-well plates (in duplicates) and harvested for luciferase assays 24 h later using a luciferase assay kit (Promega) according to the manufacturer’s protocol. β-Galactosidase was used for normalization.

**Cell Growth Assay**—After transfection with vector control or TPM1-expressing vector, the cells were seeded into 96-well plates at 2500 cell/well. The MTT assay was used to determine relative cell growth as described previously (21).

**Anchorage-independent Assay**—To determine anchorage-independent growth of transfected cells, the cells were grown in soft agar according to a published method (22). Briefly, 1 day after transfection with TPM1, cells were harvested and mixed with tissue culture medium containing 0.7% agar to result in a final agar concentration of 0.35%. Then, 1-ml samples of this cell suspension were immediately plated in 12-well plates covered with 0.6% agar in tissue culture medium and cultured at 37 °C with 5% CO₂. To assess cell viability before plating in soft agar, cell number was determined by trypan blue staining in Vi-Cell XR (Beckman Coulter, Fullerton, CA).

**Western Blot**—Total protein was isolated from tumor samples or 293T cells transfected with an appropriate plasmid in cell lysis buffer (20 mM Tris, p 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Protein concentration was measured using the Bio-Rad protein assay kit. The membrane was first probed with antibodies against Myc (Applied Biomaterials) or GFP
(Clontech), and then with anti-β-actin antibody (Sigma-Aldrich). Secondary antibodies were labeled with either Alexa Fluor 680 (Invitrogen) or IRDye800 (Rockland Immunochemicals, Gilbertsville, PA). Signals were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

**Animal Work**—Female nude (nu/nu) mice (4–5 weeks old) were purchased from Harlan (Indianapolis, IN) and were maintained in the Southern Illinois University School of Medicine’s accredited animal facility. All animal studies were conducted in accordance with National Institutes of Health animal use guidelines and a protocol approved by the Southern Illinois University Animal Care Committee. Exponentially growing MCF-7 cells were harvested, mixed with 50% Matrigel (BD Biosciences) at 15 million cells/ml, and injected (1.5 million cells/spot) into mammary pads of female nude mice. To facilitate tumor growth, a 0.72-mg 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) was implanted beneath the back skin. Tumors usually appeared 1 week after inoculation when anti-mir-21 or negative control oligonucleotide was delivered to tumor sites by injecting 50 nl (50 nM) of the oligonucleotide carrying 12 nl of Optifect. One week later another injection of the same amount was performed. Tumor size was monitored every other day; 4 weeks after inoculation of MCF-7 cells, tumors were harvested, weighed, and frozen immediately in a −80 °C freezer.

**Proteomic Analysis of Tumor Samples**—Tumor samples that were harvested and stored at −80 °C were sent directly for 2-DIGE and mass spectrometry analysis, as a service provided by Applied Biomics (Hayward, CA). Total protein was extracted and labeled with either Cy3 or Cy5. Isoelectric focusing in the first dimension was carried out at pH 3–10, and in the second dimension was carried out in 8–14% gradient SDS-PAGE. Differentially expressed proteins were cut out and subjected to trypsin digestion before mass spectrometry analysis.

**Statistical Analysis**—Data are expressed as means ± S.E., and p < 0.01 is considered as statistically significant by Student’s t test.

**RESULTS**

**Suppression of Tumor Growth by Anti-mir-21**—We have previously shown that transient transfection of MCF-7 cells with anti-mir-21 causes tumor growth inhibition in a xenograft carcinoma mouse model (21). Thus, we asked here whether intratumoral delivery of anti-mir-21 has the same effect on tumor growth. Tumors treated with anti-mir-21 grew substantially smaller in size than those treated with the negative control; tumors treated with anti-mir-21 revealed a lower level of Ki-67 staining compared with the vector control (not shown). This is...
consistent with the previous finding (21), suggesting that suppression of tumor growth likely occurs because of reduced cell proliferation, increased apoptosis, or both as suggested previously (21, 23). Therefore, these results not only support the notion that mir-21 is an oncogenic miRNA but also imply that anti-mir-21 has a therapeutic potential.

TPM1 Is Targeted by mir-21

TPM1 is overexpressed in many types of tumors, suggesting its role in cancer development, the underlying mechanism of mir-21-mediated tumorigenesis is still unclear largely because of limited knowledge about mir-21 targets. Although various computer-aided algorithms have predicted many putative mir-21 targets, these targets have not been validated experimentally. Because miRNAs are believed to regulate gene expression mainly through translational repression in mammalian cells, we thought to determine the differential expression of proteins from the tumor samples after treatment with anti-mir-21. Protein was extracted from tumors derived from MCF-7 cells treated with either the negative control (labeled with Cy3) or anti-mir-21 (labeled with Cy5). Unlike conventional two-dimensional gels in which two samples are run in separate gels, this method separates two samples labeled with different fluorescent dyes in a single gel, thus eliminating gel-to-gel variation and allowing for easy comparison of relative expression levels. After separating the proteins by isoelectric focusing and SDS-PAGE, we found that several proteins were either up-regulated or down-regulated as shown by either red or green color, respectively (Fig. 1). This result, in fact, is in agreement with the finding that mir-122 also causes up-regulation or down-regulation of many proteins (24), because some of these differentially expressed proteins may be due to the secondary effect of miRNA regulation. Analysis of another pair of tumor samples harvested from different mice revealed an almost identical pattern to that of Fig. 1, suggesting the reproducibility of this method. We are particularly interested in those proteins up-regulated by anti-mir-21 because they are potential direct targets for mir-21. We picked 10 protein spots that were up-regulated more than 2-fold in the tumor samples treated with anti-mir-21 compared with the negative control; these are circled in Fig. 1B. Mass spectrometry analysis identified seven of them with a good score. Among them, three proteins have been implicated in tumorigenesis: TPM1 (25), integrin-β4-binding protein (ITGβ4BP), (26) and selenium-binding protein-1 (SELENBP1) (27). Therefore, we tested these three genes by cloning their UTRs into a luciferase reporter. V, vector (pCMV); 21, pCMV-mir-21; N, negative control oligonucleotide; A, anti-mir-21. **, p < 0.01.

TPM1 Is Up-regulated in Anti-mir-21-treated Tumor Samples as Detected by 2-DIGE Analysis—Although mir-21 is overexpressed in many types of tumors, suggesting its role in cancer development, the underlying mechanism of mir-21-mediated tumorigenesis is still unclear largely because of limited knowledge about mir-21 targets. Although various computer-aided algorithms have predicted many putative mir-21 targets, these targets have not been validated experimentally. Because miRNAs are believed to regulate gene

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A) GFP

GFP + Hoechst

GFP-TPM1

GFP-TPM1 + Hoechst

B) GFP-TPM1 + Vector

GFP-TPM1 + mir-21

C) 

GFP-TPM1

β-actin

FIGURE 5. Regulation of expression of the GFP-TPM1 fusion protein by mir-21. A, expression of GFP or GFP-TPM1 in 293T cells. The plasmids pEGFP-C3 and pEGFP-TPM1+UTR were first introduced into 293T cells. One day later, cells were seeded on coverslips and grown for 16 h. Cells were stained with Hoechst dye and examined under a fluorescence microscope. Note that GFP-TPM1 fusion protein is present exclusively in the cytoplasm as compared with GFP, which is present throughout the cell. B and C, effect of mir-21 on expression of the GFP-TPM1 fusion protein as determined by fluorescence microscopy (B) or Western blot (C). Co-transfection with β-galactosidase-expressing vector indicated a comparable transfection efficiency between GFP-TPM1+UTR with vector control and GFP-TPM1+UTR with mir-21.

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and -4, encoded by different genes (28). TPM1 has seven variants through alternative splicing. Coincidently, TPM1 variants 1 and 5 carry a putative mir-21 binding site, as predicted by the Sanger miRNA data base target search program (Fig. 2A). Variant 1 differs from variant 5 in a sequence coding for 24 amino acids and also by lacking an additional 48 nucleotides upstream of the 3'-UTR (Fig. 2A). The potential base pairing between mir-21 and TPM1 3'-UTR is shown in Fig. 2B. Thus, we tried to amplify this UTR region of both variants from MCF-7 cells, which, however, appeared to express only TPM1 variant 1. Hence, we cloned this variant 1 3'-UTR into pGL3 control vector. As shown in Fig. 2C, the luciferase activity in 293T cells for Luc-TPM1-V1-UTR was about 20% less than that of pGL3 control vector, suggesting that TPM1 3'-UTR carries a regulatory element(s).

To confirm that this regulatory region is mir-21 specific, we transfected 293T cells with the same Luc-TPM1-UTR plasmid along with either the pCMV vector or the mir-21-expressing plasmid. The ectopic expression of mir-21 was confirmed by TaqMan real-time PCR, which revealed about a 4-fold higher mir-21 expression in the mir-21-transfected cells than in vector control (Fig. 3A). In contrast, anti-mir-21 reduced mir-21 by almost 50%, as determined by the same method (Fig. 3B). We then transfected the 293T cells with various amounts of mir-21-expressing vector. As shown in Fig. 3C, reduction of luciferase activity by mir-21 was dose-dependent, suggesting that this regulation is specifically responsive to mir-21. In contrast, mir-21 had no effect on Luc-TPM1-V4-UTR, which is derived from variant 4 and lacks the mir-21 binding site (Fig. 3D). In addition, we tested the effect of anti-mir-21 on the luciferase activity of Luc-TPM1-V1-UTR. As expected, mir-21 suppressed the luciferase activity, whereas anti-mir-21 increased the luciferase activity (Fig. 4E), further suggesting that expression of TPM1 is specifically regulated by mir-21. To determine the role of the mir-21 binding site in regulating its expression, we deleted the mir-21 binding site in variant 1 (Luc-TPM1-V1-UTR-d). As shown in Fig. 3F, neither mir-21 nor anti-mir-21 had any effect on the luciferase activity, highlighting the importance of this mir-21 binding site.

To ensure that down-regulation of luciferase activity by mir-21 was not due to the reporter we used, we made similar reporter constructs in EGFP vector. In this case, we cloned TPM1-V1-UTR into the EcoRI site of the modified pEGFP-C3 (see “Experimental Procedures”). Consistent with the luciferase data, the level of EGFP-TPM1-V1-UTR was reduced by mir-21 but was increased by anti-mir-21, as measured either by Western blot or fluorescence microscopy (not shown).

mir-21 Regulates TPM1 at the Translational Level—Translational repression is a major mechanism of miRNAs to regulate gene expression (29). To determine whether mir-21 also suppresses TPM1 through translational repression, we cloned the full-length TPM1 plus the 3'-UTR into pCMV-Myc. Expression of Myc-tagged TPM1 was confirmed by anti-Myc antibody (Fig. 4A). Importantly, although ectopic expression of mir-21 significantly reduced TPM1 protein, anti-mir-21 enhanced TPM1 protein (Fig. 4, B and D). To further determine the importance of the mir-21 binding site, we did similar experiments with pCMV-Myc-TPM1+UTR-d in which the mir-21-binding site was deleted. Deletion of this site abolished the effect of mir-21 or anti-mir-21 on TPM1 expression at the protein level (Fig. 4, C and E). However, despite the effect of mir-21 or anti-mir-21 on TPM1 at the protein level, no effect on the TPM1 mRNA level was detected by real-time qRT-PCR for pCMV-Myc-TPM1+UTR (Fig. 4F). Therefore, these results suggest that the mir-21 binding site present in the TPM1-UTR region is critical for mir-21-mediated regulation at the translational level.

In addition, we made a GFP fusion construct with the full-length TPM1 plus the 3'-UTR (GFP-TPM1+UTR). We first confirmed its expression of its fusion protein by Western blot (not shown). Fluorescence microscopy clearly showed a distinguished subcellular localization of GFP-TPM1. Although we detected the green protein all over the cell for GFP alone, GFP-TPM1 fusion protein was localized exclusively to the cytoplasm (Fig. 6A). Moreover, mir-21 also reduced the GFP fusion protein expression, as detected by fluorescence microscopy (Fig.1).
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5B), which was confirmed by Western blot (Fig. 5C). In this case, we co-transfected the cells with GFP-TPM1 and β-galactosidase plasmid, confirming a comparable transfection efficiency between GFP-TPM1+UTR with vector and GFP-TPM1+UTR with mir-21. Thus, even though the mir-21 binding site is away from the GFP (separated by TPM1 coding region), it is still functional. This result further indicates that TPM1 is a mir-21 target.

Overexpression of TPM1 Suppresses Cell Growth in Vitro and Anchorage-dependent Growth—Because previous studies have indicated that suppression of TPM1 is a prominent feature of many transformed cells, and TPM1 functions as a tumor suppressor (30), we first tested whether overexpression of TPM1-V1 affects cell growth. Thus, pCMV-Myc-TPM1-V1 was transiently transfected to MCF-7 cells and their growth determined by MTT assays. We found that overexpression of TPM1-V1 suppressed cell growth in a time-dependent manner (Fig. 6A). For instance, although there was no difference between vector control and TPM1-V1 during the first 2 days after transfection, by days 3 and 4 after transfection we detected that cells transfected with TPM1-V1 grew more slowly than the vector control, with about 20% inhibition. To determine whether TPM1-V1 affects anchorage-independent growth, we grew MCF-7 cells transfected with either vector control or pCMV-Myc-TPM1-V1 in the soft agar medium. As shown in Fig. 6B, the number of colonies from MCF-7 cells transfected with pCMV-Myc-TPM1-V1 was significantly lower than that of vector control. Of interest, although in vitro cell growth inhibition was about 20%, a greater effect was seen on inhibition of colony formation (almost 50%; Fig. 6B). Furthermore, the size of the colonies from the cells transfected with pCMV-Myc-TPM1-V1 was much smaller than those of vector control (Fig. 6, C and D). These results are consistent with the finding that expression of TPM1 induces anoikis (30), thus providing further evidence that TPM1 is a tumor suppressor. Accordingly, identification of TPM1 as a mir-21 target gene may explain at least in part why suppression of mir-21 can inhibit tumor growth, as we have demonstrated previously (21).

DISCUSSION

It is now well known that miRNAs regulate a variety of cellular pathways through regulation of expression of multiple target genes (4). In this regard, mir-21 has been suggested to function as an oncogene because it is overexpressed in many types of tumors compared with the normal tissues (21, 23, 31, 32). Furthermore, suppression of mir-21 inhibits cell growth, possibly through activation of apoptosis pathways (21, 23). However, it largely remains to be determined as to how a specific miRNA affects these pathways, in particular, regarding miRNA-associated oncogenesis, because little is known about the physiologic targets of mir-21. Our study indicates that TPM1 is one such target. As a tumor suppressor, TPM1 has been shown to play a role in suppression of the malignant phenotype (25, 33, 34). Thus, identification of TPM1 as a mir-21 target gene provides a possible explanation of why suppression of mir-21 can inhibit tumor growth (21).

In animals, miRNAs are believed to bind through partial homologous sequence to a target gene at 3’-UTR, causing translational repression. This notion is supported by two well characterized miRNA target genes that play a critical role in cancer, ras and bcl-2. In the former case, let-7 binds to the 3’-UTR of ras and causes its translational repression by 8 bases of homology (11). Similarly, mir-16 directly targets bcl-2 at the 3’-UTR by a same mechanism (35). Apparently, both are tumor-suppressive miRNAs. With regard to oncogenic miRNAs, a relatively limited number of target genes
has been characterized experimentally, although there is overwhelming information on putative targets predicted by different algorithm programs. For instance, the Sanger miRNA data base target search reveals >900 targets for mir-21, which is not consistent with the prediction of about 100 target genes per single miRNA (36). Furthermore, we previously tested several of the putative mir-21 targets such as FasL and CDC25A by Western blot, but none of them seem to be regulated by mir-21 (21). Therefore, it is very likely that only a small fraction of predicted targets may be true targets, and thus it would be a daunting task to validate them. Accordingly, we took an alternative approach, i.e. proteomics, because a major action of miRNAs is thought to be at the translation (29).

Several lines of evidence indicate that TPM1 is a mir-21 target. First, TPM1 expression is increased in tumors treated with anti-mir-21. Second, the ability of mir-21 to regulate TPM1 protein expression is likely direct, as it binds to the 3′-UTR region of TPM1 mRNA with complementarity to the mir-21 seed region (Fig. 2B). Third, Luc-TPM1-V1-UTR is specifically responsive to mir-21 overexpression or anti-mir-21. Finally, deletion of the mir-21 site abolishes its mir-21 regulation. Although miRNAs may regulate protein expression by accelerating messenger RNA degradation and/or inhibiting transcription from existing messenger RNA (37), our results suggest that mir-21 inhibits TPM1 protein translation, as steady state TPM1 mRNA levels are not affected by mir-21 or anti-mir-21.

Tropomyosins are widely distributed in all cell types associated with actin such that they serve as actin-binding proteins and stabilize microfilaments (38). In animals, four known tropomyosin genes code for diverse isoforms that are expressed in a tissue-specific manner and regulated by an alternative splicing mechanism (28). Suppression of TPM1 and TPM2 has been reported in malignant cells, suggesting a role for these proteins in neoplastic transformation (25, 39). In addition, transfection of tropomyosins into viral oncogene-transformed rodent cells suppresses tumorigenic phenotypes (40, 41). Moreover, TPMs regulate both microfilament organization and anchorage-independent growth, highlighting the importance of TPMs in cell transformation (33). TPMs belong to the class II tumor suppressor genes (42), because expression of TPMs is apparently subject to epigenetic regulation (43, 44); these genes are structurally intact in their sequences but are underexpressed or unexpressed due to down-regulation or silencing in transcription or translation.

Epigenetic modification of TPM expression seems to involve several cellular factors. One such factor is methylation. For instance, treatment of cancer cells with demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) increases mRNA levels of TPM1 (43). Importantly, such treatment can restore transforming growth factor-β induction of TPM1 and formation of stress fibers, thus altering the transforming growth factor-β tumor suppressor function (43). Interestingly, MCF-7 cells express little TPM1 (34), and inhibition of DNA methyl transferase with 5-aza-dC alone does not induce TPM1 expression (44). However, combined treatment of the histone deactylase inhibitor trichostatin A and 5-aza-dC results in readily detectable expression of TPM1 (44), suggesting that acetylation may also be involved in regulating TPM1 expression. Thus, this study provides another potential mechanism of posttranscriptional regulation of TPM1 expression, ultimately modulating cell transformation and tumor cell growth.

Interestingly, the 3′-UTR region of TPMs alone may also play a role in tumor suppression. For instance, constitutive expression of RNA from the 3′-UTR suppresses anchorage-independent growth and tumor formation in a nondifferentiating mutant myogenic cell line (45), although the 3′-UTR of TPMs alone may not be sufficient to cause tumor suppression or may not be required for tumor suppression in other types of cells (46, 47). Nevertheless, it would be of interest to determine whether oncogenic miRNAs such as mir-21 interact with this region. If this interaction exists, we would expect that overexpression of this 3′-UTR region might deplete a pool of such miRNAs in the cell, leading to tumor suppression.

In summary, TPM1 expression can be regulated by mir-21. This study extends our knowledge about the regulation of TPM1, a tumor suppressor protein. Thus, in addition to epigenetic regulation, as mentioned above, TPM1 is also regulated at the translational level by miRNAs. Given that a single miRNA has multiple targets, we believe that mir-21 also has many targets. It is our expectation that more mir-21 targets will be identified in the near future with the same proteomic approach such that we will be better able to understand the molecular basis of mir-21-mediated tumorigenesis.

REFERENCES

1. Pillai, R. S. (2005) RNA 11, 1753–1761
2. Zamore, P. D., and Haley, B. (2005) Science 309, 1519–1524
3. Kim, Y. N. (2005) Nat. Rev. Mol. Cell. Biol. 6, 376–385
4. Bartel, D. P. (2004) Cell 116, 281–297
5. Fitzgerald, K. (2005) Curr. Opin. Drug Discovery Dev. 8, 557–566
6. Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005) Cell 120, 15–20
7. Croce, C. M., and Calin, G. A. (2005) Cell 122, 6–7
8. Chen, C. Z., Li, L., Lodish, H. F., and Bartel, D. P. (2004) Science 303, 83–86
9. Chen, C. Z. (2005) N. Engl. J. Med. 353, 1768–1771
10. Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y., Mitsudomi, T., and Takahashi, T. (2004) Cancer Res. 64, 3753–3756
11. Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K. L., Brown, D., and Slack, F. J. (2005) Cell 120, 635–647
12. Calin, G. A., Dumitruc, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., and Croce, C. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15524–15529
13. Stark, A., Brennecke, J., Russell, R. B., and Cohen, S. M. (2003) PLoS Biol. 1, e60
14. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003) Cell 115, 787–798
15. Kiriakidou, M., Nelson, P. T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., and Hatzigeorgiou, A. (2004) Genes Dev. 18, 1165–1178
16. Didiano, D., and Hobert, O. (2006) Nat. Struct. Mol. Biol. 13, 849–851
17. Smalheiser, N. R., and Torvik, V. I. (2006) Methods Mol. Biol. 342, 115–127
18. Mo, Y. Y., and Beck, W. T. (1999) Exp. Cell Res. 252, 50–62
19. Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., Lao, K. Q., Livak, K. J., and Guegler, K. J. (2005) Nucleic Acids Res. 33, e179
20. Lao, K., Xu, N. L., Yeung, V., Chen, C., Livak, K. J., and Straus, N. A. (2006) Biochem. Biophys. Res. Commun. 343, 85–89
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21. Si, M. L., Zhu, S., Wu, H., Lu, Z., Wu, F., and Mo, Y. Y. (2006) *Oncogene*, in press
22. Finlay, T. H., Tamir, S., Kadner, S. S., Cruz, M. R., Yavelow, J., and Levitz, M. (1993) *Endocrinology* **133**, 996–1002
23. Chan, J. A., Krichevsky, A. M., and Kosik, K. S. (2005) *Cancer Res.* **65**, 6029–6033
24. Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005) *Nature* **438**, 685–689
25. Cooper, H. L., Feuerstein, N., Noda, M., and Bassin, R. H. (1985) *Mol. Cell. Biol.* **5**, 972–983
26. Sanvito, F., Vivoli, F., Gambini, S., Santambrogio, G., Catena, M., Viale, E., Veglia, F., Donadini, A., Biffo, S., and Marchisio, P. C. (2000) *Cancer Res.* **60**, 510–516
27. Chen, G., Wang, H., Miller, C. T., Thomas, D. G., Gharib, T. G., Misek, D. E., Giordano, T. J., Orringer, M. B., Hanash, S. M., and Beer, D. G. (2004) *J. Pathol.* **202**, 321–329
28. Lees-Miller, J. P., and Helfman, D. M. (1991) *BioEssays* **13**, 429–437
29. Engels, B. M., and Hutvagner, G. (2006) *Oncogene* **25**, 6163–6169
30. Raval, G. N., Bharadwaj, S., Levine, E. A., Willingham, M. C., Gheary, R. L., Kute, T., and Prasad, G. L. (2003) *Oncogene* **22**, 6194–6203
31. Iorio, M. V., Ferracin, M., Liu, C. G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriani, M., Fabbrì, M., Campiglio, M., Menard, S., Palazzo, J. P., Rosenberg, A., Musiani, P., Volinia, S., Nenci, I., Calif, G. A., Querzoli, P., Negrini, M., and Croce, C. M. (2005) *Cancer Res.* **65**, 7065–7070
32. Rololo, C., Missiaglia, E., Hagan, J. P., Falconi, M., Capelli, P., Bersani, S., Calif, G. A., Volinia, S., Liu, C. G., Scarpa, A., and Croce, C. M. (2006) *J. Clin. Oncol.* **24**, 4677–4684
33. Boyd, J., Risinger, J. I., Wiseman, R. W., Merrick, B. A., Selkirk, J. K., and Barrett, J. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11534–11538
34. Mahadev, K., Raval, G., Bharadwaj, S., Willingham, M. C., Lange, E. M., Vonderhaar, B., Salomon, D., and Prasad, G. L. (2002) *Exp. Cell Res.* **279**, 40–51
35. Cimmino, A., Calif, G. A., Fabbri, M., Iorio, M. V., Ferracin, M., Shimizu, M., Wojcik, S. E., Aqelain, R. I., Zufo, S., Dono, M., Rassenti, L., Alder, H., Volinia, S., Liu, C. G., Kipps, T. J., Negrini, M., and Croce, C. M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 13944–13949
36. Brennecke, J., Stark, A., Russell, R. B., and Cohen, S. M. (2005) *PLoS Biol.* **3**, e85
37. Valencia-Sanchez, M. A., Liu, J., Hannon, G. J., and Parker, R. (2006) *Genes Dev.* **20**, 515–524
38. Perry, S. V. (2001) *J. Muscle Res. Cell Motil.* **22**, 5–49
39. Bhattacharya, B., Prasad, G. L., Valverius, E. M., Salomon, D. S., and Cooper, H. L. (1990) *Cancer Res.* **50**, 2105–2112
40. Takenaga, K., and Masuda, A. (1994) *Cancer Lett.* **87**, 47–53
41. Prasad, G. L., Fuldner, R. A., and Cooper, H. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 7039–7043
42. Jones, P. A., and Laird, P. W. (1999) *Nat. Genet.* **21**, 163–167
43. Varga, A. E., Stourman, N. V., Zheng, Q., Safina, A. F., Quan, L., Li, X., Sossey-Alaoui, K., and Bakin, A. V. (2005) *Oncogene* **24**, 5043–5052
44. Bharadwaj, S., and Prasad, G. L. (2002) *Cancer Lett.* **183**, 205–213
45. Rastinejad, F., Conboy, M. J., Rando, T. A., and Blau, H. M. (1993) *Cell* **75**, 1107–1117
46. Janssen, R. A., and Mier, J. W. (1997) *Mol. Biol. Cell* **8**, 897–908
47. Braverman, R. H., Cooper, H. L., Lee, H. S., and Prasad, G. L. (1996) *Oncogene* **13**, 537–545