

p53 Is Positively Regulated by miR-542-3p

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Abstract

The tumor suppressor p53 and miRNAs are linked through a complex network. Several miRNAs modulate p53 expression, while p53 regulates the transcription and/or biogenesis of several other miRNAs. Here, we report the development of a cell-based assay used with a library of human miRNA mimics in a high-throughput screen for miRNAs that modulate p53 expression. Overexpression of miRNA (miR)-542-3p in cancer cells elevated p53 expression, stimulated the expression of p53 targets, and inhibited cell proliferation. Mechanistically, miR-542-3p increased p53 protein stability by weakening interactions between p53 and its negative regulator MDM2. Furthermore, miR-542-3p suppressed ribosome biogenesis by downregulating a subset of ribosomal proteins such as RPS23, leading to upregulation of RPL11 and stabilization of p53. The 3' untranslated region in the RPS23 transcript contained a miR-542-3p-binding site, suggesting that RPS23 is a direct target of miR-542-3p. Our results define miR-542-3p as an important new positive regulator of p53 with potential applications in cancer treatment. *Cancer Res*; 74(12); 3218–27. ©2014 AACR.

Introduction

The p53 tumor suppressor, encoded by the *TP53* gene, functions mainly by transcriptionally regulating genes that govern many cellular events such as cell cycle, apoptosis, autophagy, senescence, metabolism, and angiogenesis. Genetic deletion or germline mutation of *TP53* leads to high incidence and early onset of cancer in both mice and human (1, 2). The *TP53* gene is mutated in about half of all human tumors, while tumors retaining wild-type *TP53* often have abnormal p53 function as a result of alterations occurring in regulators of p53 (reviewed in refs. 3, 4). Restoration of p53 function is thereby an attractive strategy for tumor management, and fully understanding the regulation of p53 is of particular interest in the field of cancer research (5).

The p53 protein level remains very low in unstressed cells, but is rapidly induced upon exposure to stimuli, such as DNA damage, hypoxia, nutrient deprivation, or oncogenic activation (6). Ribosomal stress, which can be induced by serum deprivation, growth contact inhibition, or actinomycin D treatment, also triggers the activation of p53. Upregulation of p53 protein in

response to ribosomal stress is largely due to the disruption of interaction between p53 and MDM2, an oncogenic E3 ligase that not only targets p53 for proteasome-mediated degradation, but also inhibits the transactivation activity of p53 (7, 8). Mechanistically, ribosomal stress reduces the expression of PICT1, which leads to the release of the ribosomal subunit RPL11 from its anchored sites in the nucleolus (9). The liberated RPL11 then interacts and sequesters MDM2 in the nucleus, thereby stabilizing and activating p53 (10). Furthermore, depletion of various ribosome subunit proteins by siRNAs can disrupt ribosome maturation and activate p53 due to sequestration of MDM2 by certain ribosome subunit proteins including RPL11, whose translation is increased upon depletion of RPS6 or RPS23 (11, 12). Thus, targeting the integrity of ribosome biogenesis may be a practical way to activate p53 for cancer treatment for *TP53* wild-type cancers (13, 14).

miRNAs are small noncoding RNAs that act as regulators of gene expression. Aberrant expression of miRNAs is often seen in cancer. miRNAs can function as tumor suppressors or oncogenes and modulate many aspects of carcinogenesis, such as cell proliferation, cell-cycle control, DNA repair, apoptosis, metastasis, and angiogenesis (15, 16). p53 modulates the expression of miRNAs by either activating the transcription of some miRNA-coding genes or modulating the biogenesis of a subset of miRNAs (17, 18). The expression of p53 is also under the control of several miRNAs. For example, miRNA (miR)-125b directly targets the 3' untranslated region (UTR) of p53 (19). Using a luciferase reporter driven by p53-binding motif, Park and colleagues screened a number of cancer-related miRNAs and identified miR-29 miRNAs as positive albeit indirect regulators of p53 expression through targeting p85- α and CDC42 (20). However, comprehensive analysis of miRNAs that regulate p53 expression has not been reported.

In the present study, we identify several miRNAs that regulate p53 expression. Among them, we describe miR-542-

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3p as a novel inducer of ribosomal stress and a potent positive regulator of p53 tumor suppressor.

Materials and Methods

Cell lines

U2OS, T98G, U118, LN229 (American Type Culture Collections), HCT116, HCT116 p53^{-/-} (Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD), and normal human foreskin fibroblasts (Dr. Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) were all grown in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 2 mmol/L L-glutamine. JHOC7 and OVISe cells (Dr. Hiroaki Itamochi, Tottori University, Japan) were cultured in RPMI medium with 10% FBS and 2 mmol/L L-glutamine. Cells were authenticated by short tandem repeat DNA profiling at Bio-Synthesis for U2OS, HCT116, T98G, U118, and LN229 and at CTAG for JHOC7 and OVISe. All cells were used for this study within 6 months of resuscitation.

Plasmids, siRNAs, miRNA mimics, and transfection

3'UTR of human RPS23 was amplified by PCR and cloned into pGL3-control (Promega) to obtain pGL3-RPS23 3'UTR plasmid. Putative binding site of miR-542-3p in RPS23 3'UTR was mutated using the QuikChange Site-Direct Mutagenesis Kit (Stratagene). RPS23 coding region was amplified by PCR and cloned into pLenti4-V5 vector (Invitrogen). miR-542-3p and the spanning sequences (150 bp on each end) were amplified and inserted into pSM30-GFP vector (a gift of Dr. Guangwei Du, University of Texas Health Science Center at Houston, Houston, TX) to generate miR-542-3p precursor. Specific siRNAs included siRPL11 (SMARTpool siGenome, Dharmacon), siRPS23 (5'-GCCATTAGGAAGTGTGTAA-3'), siRPS28 (5'-GTAAGTGA-GATGCTCCTTT-3'), siRPL22 (5'-CAAAGAGAGTTACGAATTA-3'; Sigma) and luciferase (siLuc, 5'-AACGTACGCGGAA-TACTTCGA-3', Qiagen). Delivery of plasmids, siRNAs, and miRNA mimics (Dharmacon) was done as described (21).

miRNA mimic library screening

Human miRIDIAN miRNA mimic library (v10.1, Dharmacon) was reversely transfected into U2OS cells followed by immunostaining with anti-p53 (DO-1, sc-126, Santa Cruz Biotechnology; 1:500) and Alexa594-conjugated secondary antibody (Invitrogen). Images were captured and processed as described (22, 23). Average intensity of p53 in nucleus was determined for calculation of Z-score values with the formula $Z = (X - \mu_{nc}) / \sigma$, where X was the score of individual sample, μ_{nc} was the mean of negative controls, and σ was the SD of the whole population. Average Z-scores from three independent screens were calculated.

Western blot analysis

Whole-cell extracts were obtained for SDS-PAGE as described (22). Primary antibodies included mouse anti-p53 (sc-126, Santa Cruz Biotechnology), RPS23 (sc-100837, Santa Cruz Biotechnology), RPL22 (sc-373993, Santa Cruz Biotechnology), RPS28 (14796-1-AP, Proteintech Group), MDM2 (04-1530, Millipore), MDM4 (sc-74468, Santa Cruz Biotechnology),

PIRH2 (sc-166293, Santa Cruz Biotechnology), p21 (554228, BD Pharmingen), Bax (H00000581-M01, Abnova), RPL11 (37-3000, Invitrogen), ARF (#2407, Cell Signaling Technology), and rabbit anti-PA28 γ (38-3800, Zymed), cleaved caspase-3 (#9661, Cell Signaling Technology), and actin (sc-1616-R, Santa Cruz Technology). Ponceau staining or actin was used as loading controls.

Immunoprecipitation

Cells were lysed in NP-40 lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP-40]. Precleared lysates (~300 μ g of protein) were incubated for 2 hours at 4°C with 2 μ g antibody of interest and then precipitated with 40 μ L of fresh Protein-A/G plus agarose beads (Santa Cruz Biotechnology) at 50% slurry overnight at 4°C. The beads were then pelleted, washed, and boiled in 2 \times Tris-glycine SDS sample buffer for Western blotting.

Real-time PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen) and reverse transcribed using the TaqMan microRNA or cDNA Reverse Transcription Kit (Applied Biosystems). The TaqMan MiRNA Assay or Gene Expression Kit was used for quantitative PCR. C_t values were used for quantification of transcripts. miRNA or p53 expression was normalized to the values of RNU24 or GAPDH.

RNA quality control and Illumina gene expression analysis

Total RNA was extracted using RNeasy Kit (Qiagen). The quality of RNA was analyzed using a bioanalyzer (Agilent) with the RNA Nano Kit. RNA was labeled and hybridized to the HT-12 v4 expression beadchip (Illumina), followed by analysis performed with annotations found in the lumiHumanAll.db package. Data from three independent transfections are available in the NCBI Gene Expression Omnibus database, Accession No. GSE47363.

Luciferase assay

Cells were cotransfected with miRNA mimics and pGL3-control firefly luciferase vectors containing empty or RPS23 3'UTR. pRL-TK *Renilla* plasmid was cotransfected as an internal control. Luciferase assays were performed 2 days post-transfection (21). Relative luciferase activity was calculated by normalizing the ratio of *Firefly/Renilla* luciferase to that of negative control-transfected cells.

Cell-cycle analysis

Cells were fixed with 70% ice-cold ethanol overnight, followed by staining for DNA content with 10 μ g/mL propidium iodide in PBS containing 10 μ g/mL RNase A. Flow-cytometric analysis was performed to determine the distribution of cell cycle on a FACSCanto system (BD Biosciences).

Statistical analysis

All the statistical analyses were performed with Student *t* test (paired two tailed). All results were expressed as mean \pm SD. *P* < 0.05 was considered significant.

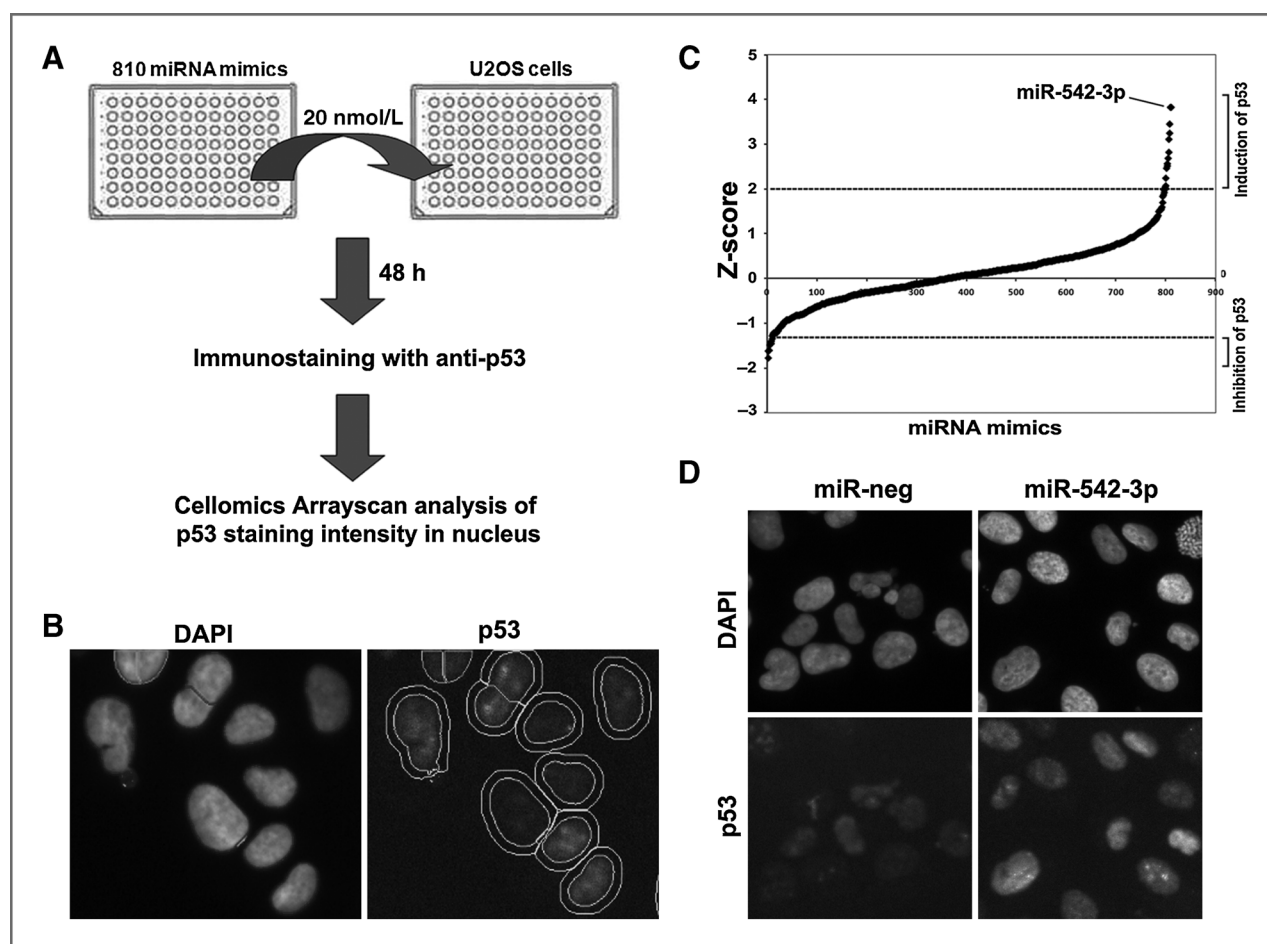


Figure 1. Identification of miRNAs that regulate p53 expression. A, scheme of miRNA gain-of-function screening for p53 immunofluorescence staining intensity in U2OS cells. B, representative images of p53 immunofluorescence staining. Nuclear regions were marked with circles (inner circle) based on 4', 6-diamidino-2-phenylindole staining. The outer circles were used to determine the intensity of p53 staining in cytoplasm (signal in ring region). C, Z-score plot of miRNA mimics in the regulation of nuclear p53 immunofluorescent intensity. Several miRNA mimics were identified to positively or negatively affect p53-staining intensity (Z -score >2 or <-1.2 ; Supplementary Table S1). miR-542-3p is the top hit that modulated p53-staining intensity in U2OS. D, representative images of p53 immunofluorescence staining in miR-neg or miR-542-3p-transfected cells.

Results

Identification of miR-542-3p as a positive regulator of p53 expression

To search for miRNAs that directly or indirectly modulate the expression of p53, we developed a high-throughput fluorescence microscopy cell-based assay. The average immunofluorescent staining intensity of p53 in the nucleus after transfection of miRNA mimics was used as a readout. The screening was performed using U2OS cells, an osteosarcoma cell line with wild-type *TP53*. We screened a human miRNA mimics library containing 810 miRNAs (Fig. 1A and B). As the basal level of p53 is low in U2OS cells, p53 siRNA caused a moderate reduction of nuclear p53 fluorescence intensity ($z = -1.94$). We set a threshold of $z < -1.2$ for miRNAs that significantly reduced the expression of p53 (Fig. 1C and Supplementary Table S1), which included fifteen potential novel negative regulators of p53 and two known negative regulators of p53, miR-125b ($z = -1.22$) and miR-504 ($z = -1.27$; refs. 19, 24). In contrast, our immunofluorescence intensity-based screen

more sensitively identified potential positive regulators of p53. Our positive control, the topoisomerase II inhibitor etoposide, strongly induced p53 fluorescent intensity ($z > 5$). We set a cutoff of $z > 2$ for miRNAs that significantly enhanced p53 expression, which included 13 potential novel positive regulators of p53 (Fig. 1C and Supplementary Table S1). As reactivation of p53 is therapeutically important for the treatment of cancers, we focused on miR-542-3p, which was the strongest p53 upregulating miRNA identified in the screen (Fig. 1C and D) and is downregulated in many cancer types such as colon, prostate, and lung cancers (25).

miR-542-3p disrupts p53-MDM2 interaction and increases the stability of p53 protein

In agreement with the screening result, overexpression of miR-542-3p by synthetic mimics markedly increased expression of p53 protein as detected by Western blotting in U2OS cells (Fig. 2A). In addition, transfection of a plasmid containing miR-542-3p primary sequence (pri-miR-542)

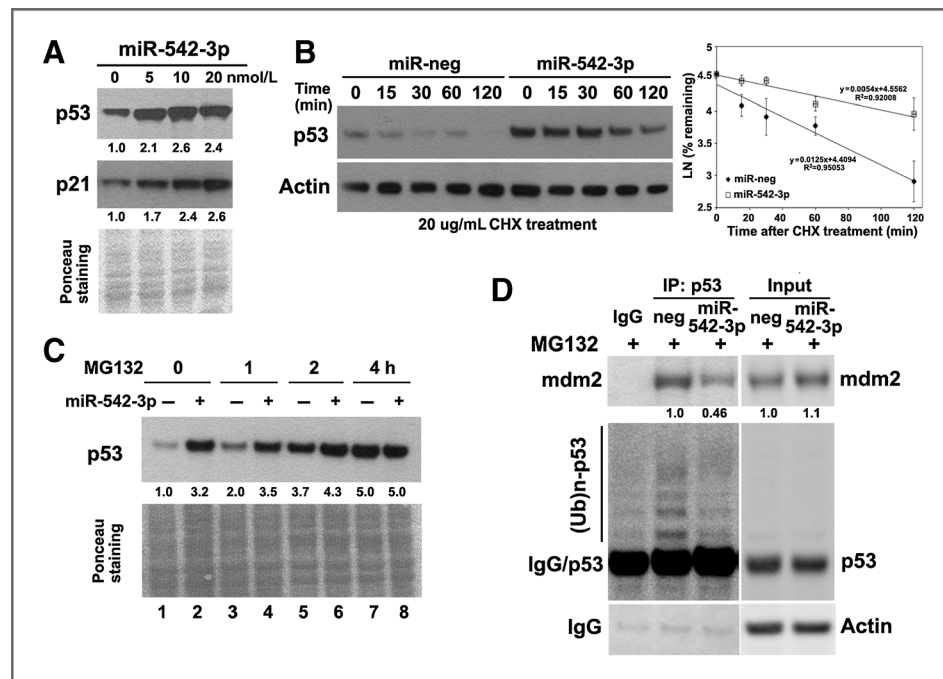


Figure 2. miR-542-3p increases the stability of p53 protein. **A**, miR-542-3p induces p53 at protein level. U2OS cells were transfected with negative or miR-542-3p mimics at indicated concentration. Cells were pelleted for Western blotting of p53 and p21. **B**, miR-542-3p increases the stability of p53 protein. U2OS cells transfected with either negative or miR-542-3p mimics at 10 nmol/L were reseeded and treated with protein synthesis inhibitor cycloheximide (CHX, 20 μ g/mL) to block *de novo* protein synthesis. Cells were harvested at indicated time for Western blot analysis. Half-life of p53 was determined by densitometric analysis of p53 bands from three independent experiments using the formula $t_{1/2} = -\ln 2/S$, where S represents the slope from each linear regression. **C**, miR-542-3p failed to further increase p53 level in the presence of MG132. U2OS cells transfected with either negative or miR-542-3p mimics at 10 nmol/L were reseeded and treated with MG132 (2 μ mol/L) before Western blot analysis of p53. **D**, miR-542-3p reduced MDM2–p53 interaction and p53 polyubiquitination. HCT116 cells transfected with either negative or miR-542-3p mimics were treated with MG132 (2 μ mol/L) for 4 hours. Cells were then lysed for immunoprecipitation with anti-p53. Immunoprecipitated proteins were subjected to Western blot analysis of MDM2 and polyubiquitinated p53 (Ub)n-p53. Quantitation of protein levels was labeled below the corresponding blots (same for Figs. 3–5).

efficiently produced miR-542-3p in U2OS cells and increased p53 expression (Supplementary Fig. S1). However, miR-542-3p did not increase p53 expression at the transcript level (Supplementary Fig. S2). To test whether miR-542-3p leads to stabilization of wild-type p53 protein, we treated U2OS cells with the protein synthesis inhibitor cycloheximide and evaluated the half-life of p53 protein. Overexpression of miR-542-3p increased the half-life of p53 from approximately 50 to 130 minutes in U2OS cells (Fig. 2B). Overexpression of miR-542-3p failed to further increase p53 level in U2OS cells treated with a proteasome inhibitor, MG132 (Fig. 2C, lanes 7 and 8). Furthermore, miR-542-3p induces p53 expression in both normal human fibroblast and HCT116 (wild-type p53, colon cancer), but not in MDA-MB-231 (breast cancer), T98G (glioma), and U118 (glioma) cells that harbor mutant p53 (26) with stabilized p53 expression (Supplementary Fig. S3). These data suggest that miR-542-3p leads to inhibition of proteasome-mediated degradation of wild-type p53 protein.

Because miR-542-3p increases the stability of p53, next we analyzed the integrity of p53 degradation pathway. Overexpression of miR-542-3p had no significant effect on the expression levels of either MDM2, the major E3 ligase targeting p53 for ubiquitin-proteasome-mediated degradation (7), or PIRH2, another E3 ligase that can target p53 for degradation (Supplementary Fig. S4; ref. 27). Moreover, miR-542-3p had no effect

on the expression of MDM4 and PA28 γ (Supplementary Fig. S4), two known positive regulators of p53–MDM2 interaction (4, 28). However, the interaction between p53 and MDM2 as well as the polyubiquitination of p53 were attenuated after overexpressing miR-542-3p in HCT116 cells (Fig. 2D), suggesting that miR-542-3p may stabilize p53 by affecting other regulators of p53–MDM2 interaction.

miR-542-3p induces ribosomal p53 response

To identify targets of miR-542-3p that lead to the disruption of p53–MDM2 interaction, we performed microarray-based expression profiling in control or miR-542-3p mimic-transfected U2OS cells using Illumina whole-genome gene expression platform. This analysis identified p53 signaling as the most altered pathway in miR-542-3p-overexpressing cells (Supplementary Tables S2 and S3). Noticeably, miR-542-3p overexpression reduced the level of 18S rRNA, the core of ribosome 40S rRNA subunit, by two-fold without affecting the level of 28S rRNA, the core of ribosome 60S rRNA (Fig. 3A). Treatment with low dose of actinomycin D, known to induce ribosome stress (29), strongly upregulated p53 level in U2OS cells (Supplementary Fig. S5). Therefore, miR-542-3p may suppress the maturation of 18S rRNA to induce the ribosomal p53 response.

Because ribosome subunits, especially the small ribosome subunit proteins (RPS), play important roles in 18S rRNA

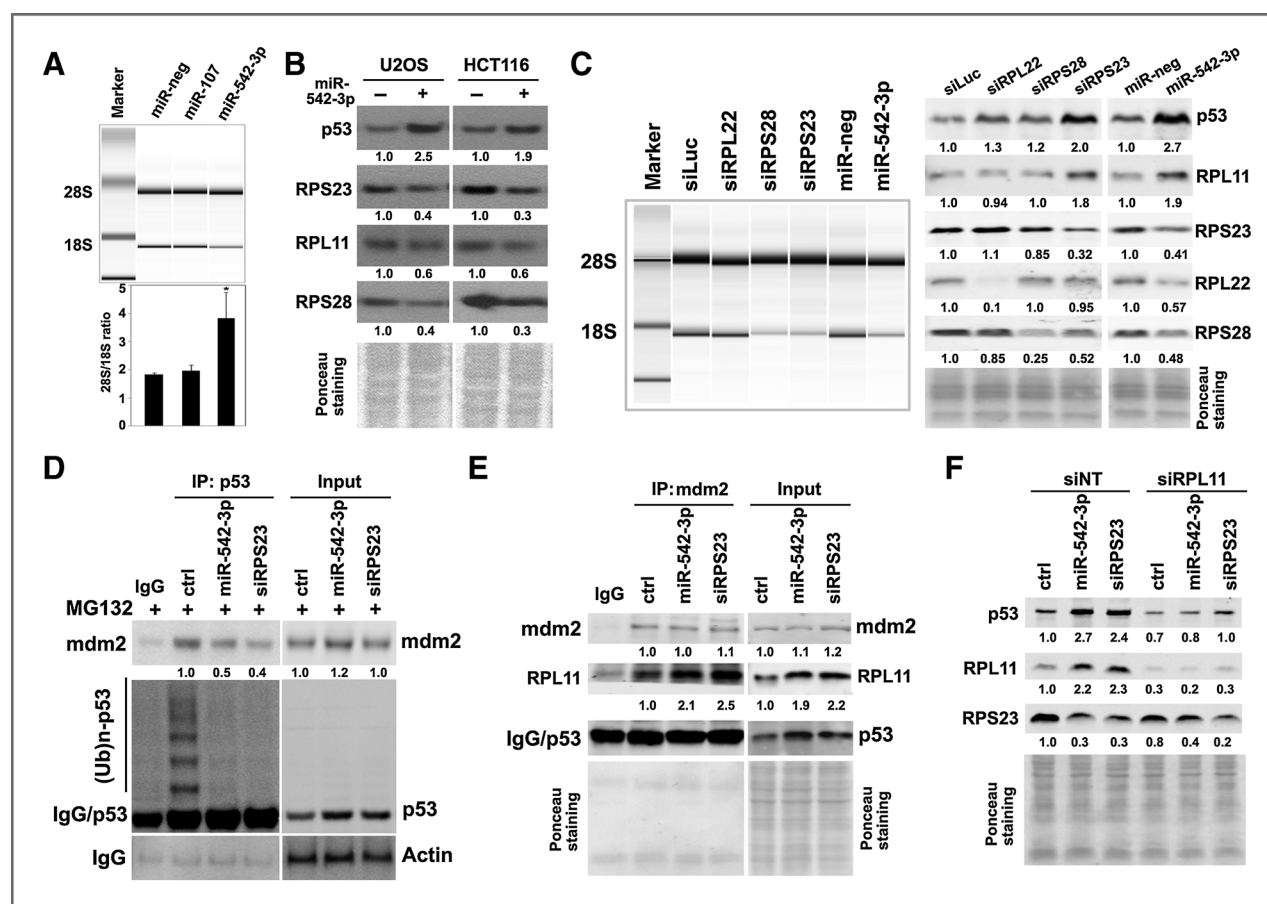


Figure 3. miR-542-3p induces ribosomal p53 response. **A**, miR-542-3p induces ribosome abnormality. U2OS cells were transfected as indicated. Forty-eight hours later, RNA was extracted and run on Agilent Bioanalyzer. The 28S/18S ratio was calculated from electropherogram data. **B**, miR-542-3p suppresses the expression of RPS23, RPS28, and RPL22. U2OS and HCT116 cells transfected with negative or miR-542-3p mimics were analyzed for the expression of ribosome subunits by Western blotting. **C**, effect of RPL22, RPS23, and RPS28 depletion by siRNA on 18S rRNA, RPL11, and p53 levels. U2OS cells transfected as indicated were collected for RNA quality analysis by Agilent Bioanalyzer (left) or Western blot analysis (right). **D**, depletion of RPS23 decreases MDM2–p53 interaction and p53 polyubiquitination. U2OS cells transfected, as indicated, were treated with MG132 (2 μ M/L) for 4 hours and then lysed for immunoprecipitation with anti-p53 followed by Western blot analysis. **E**, miR-542-3p and siRPS23 increase RPL11 and its association with MDM2. Cells transfected, as indicated, were lysed for immunoprecipitation with anti-MDM2 followed by Western blot analysis. **F**, depletion of siRPL11 reverses miR-542-3p and siRPS23-mediated p53 induction. U2OS cells were cotransfected as indicated were lysed for Western blot analysis. *, $P < 0.01$.

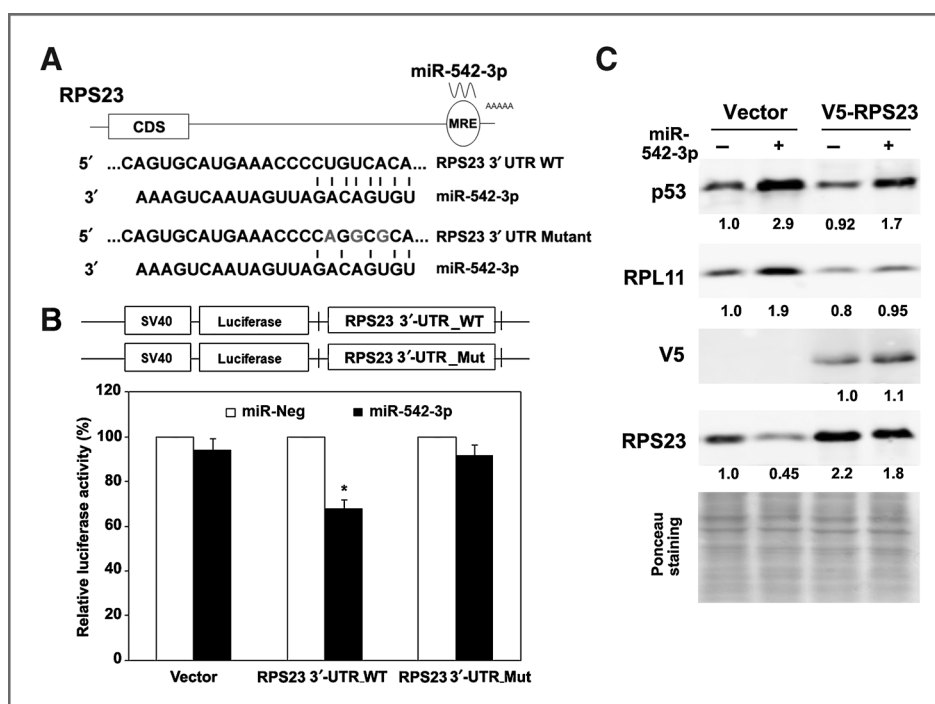
maturation (30), we first determined the effect of miR-542-3p overexpression on protein levels of ribosomal subunits RPS23, RPS28, and RPL22, whose transcript levels were robustly downregulated in miR-542-3p-overexpressing cells (Supplementary Table S4). The protein levels of RPS23, RPS28, and RPL22 were reduced in miR-542-3p-overexpressing U2OS and HCT116 cells (Fig. 3B). Next, we examined whether downregulation of these ribosome subunits can cause ribosome biogenesis defects in U2OS cells. In agreement with the essential roles of RPS23 and RPS28 in small ribosome subunit maturation, depletion of RPS23 and RPS28 by siRNAs robustly reduced 18S rRNA level without affecting 28S rRNA, whereas knockdown of RPL22 has no effect on production of both 28S and 18S rRNA (Fig. 3C, left). Importantly, RPS23 depletion induced p53 by two-fold, whereas depletion of RPS28 or RPL22 had very mild effect on p53 level (Fig. 3C, right). Similar to miR-542-3p overexpression, depletion of RPS23 weakened p53–MDM2 interaction and reduced p53 polyubiquitination (Fig. 3D). In

addition, miR-542-3p overexpression or RPS23 depletion induced RPL11 (Fig. 3C and Fig. 3E), a ribosomal protein known to sequester MDM2 and mediate p53 induction following RPS23 depletion (11), and the association between MDM2 and RPL11 (Fig. 3E). Depletion of RPL11 by pooled siRNAs almost completely blocked p53 induction upon miR-542-3p overexpression or RPS23 knockdown (Fig. 3F). Thus, our data suggest that miR-542-3p induces p53 mainly through suppression of RPS23, which leads to upregulation of RPL11 and sequestration of MDM2 by RPL11.

RPS23 is a direct target of miR-542-3p

The 3'UTR of the RPS23 transcript contains a predicted binding site of miR-542-3p by prediction algorithms MiRanda and TargetScan. To demonstrate whether RPS23 mRNA is a direct target of miR-542-3p, we first amplified fragments of RPS23 cDNA with PCR using multiple primer pairs (Supplementary Fig. S6A). As shown in Supplementary Fig. S6B, U2OS

Figure 4. RPS23 is a direct target of miR-542-3p. **A**, the putative miR-542-3p-binding site (wild-type or mutant) in the transcript of RPS23. **B**, miR-542-3p targets 3'UTR of RPS23. Wild-type or mutant RPS23 3'UTR were cloned into the pGL3 vector, as 3' fusions to the luciferase gene. U2OS were cotransfected with the indicated miRNA mimics and luciferase vectors. Luciferase activity was assayed 48 hours later and normalized to that of negative control-transfected cells from three independent experiments. **C**, ectopic RPS23 partially suppresses p53 induction by miR-542-3p. U2OS cells expressing vector or V5-tagged RPS23 coding region were transfected with negative or miR-542-3p mimics followed by Western blot analysis. *, $P < 0.01$.



cells do express the 3.3-kb long mRNA of RPS23 with a predicted miR-542-3p-binding site at C-terminus of 3'UTR that was confirmed by Sanger sequencing (Supplementary Fig. S6C). We then cloned 3'UTR of RPS23 mRNA downstream of the open-reading frame of the luciferase gene of pGL3 vector (RPS23 3'UTR; Fig. 4A) and transfected the construct together with miR-542-3p or negative control mimics into U2OS cells. miR-542-3p significantly downregulated luciferase activity of the construct fused with RPS23 3'UTR, while it did not affect that of the empty vector control (Fig. 4B). Mutation of the potential miR-542-3p-binding site in RPS23 3'UTR (RPS23 3'UTR mutant; Fig. 4A) completely abolished the inhibitory effect of miR-542-3p on luciferase activity (Fig. 4B), implying that RPS23 mRNA is a direct target of miR-542-3p.

Next, we expressed the V5-tagged RPS23 transcript lacking its 3'UTR in U2OS cells. The ectopic V5-tagged RPS23 was not affected by miR-542-3p expression as expected (Fig. 4C). miR-542-3p-mediated induction of RPL11 was largely diminished, and p53 induction by miR-542-3p was partially reverted in cells expressing V5-RPS23 (Fig. 4C), suggesting that direct targeting of the 3'UTR of RPS23 by miR-542-3p is at least partially responsible for miR-542-3p-mediated RPL11 and p53 induction.

miR-542-3p activates p53 pathway in MDM2-overexpressing or ARF-deficient cells

MDM2 amplification is an important mechanism, leading to suppression of p53 function in tumors with wild-type *TP53*, such as melanoma and ovarian clear cell carcinoma (31, 32). Stabilization of p53 by disrupting the MDM2-p53 interaction is an important therapeutic approach to target tumor cells with wild-type *TP53* and *MDM2* amplification (5). In addition, ARF deficiency is responsible for MDM2-mediated proteasomal degradation of p53 in a subset of cancer cells with wild-type

TP53 (33). To determine whether miR-542-3p can rescue p53 protein expression in MDM2-overexpressing or ARF-deficient cells, we tested two ovarian clear cell carcinoma lines, JHOC7 and OVI5E, and one glioblastoma cell line, LN229. JHOC7 and OVI5E expressed high levels of MDM2 and low amounts of wild-type p53 (unpublished data), whereas LN229 cells are ARF deficient and express wild-type p53 (34). Overexpression of miR-542-3p in JHOC7, OVI5E, and LN229 cells significantly increased p53 expression and reduced RPS23 levels (Fig. 5A). Furthermore, both HCT116 and U2OS cells are indeed ARF-silenced cell lines (35, 36). These data indicate that miR-542-3p may be useful in restoring the function of p53 in MDM2-overexpressing or ARF-deficient tumors.

Because p53 plays important roles in cell growth arrest and depletion of RPS23 also robustly inhibited cell growth of U2OS cells (Supplementary Fig. S7), we then determined whether miR-542-3p affected cell growth. Consistent with the activation of the p53 pathway (Supplementary Table S3), overexpression of miR-542-3p suppressed cell growth of U2OS, LN229, OVI5E, and JHOC7 cells significantly (Fig. 5B), which may be due to cell-cycle arrest at G₁ phase (Fig. 5C) mediated by p21 induction (Fig. 2A and Fig. 5A) and a mild induction of cell death (accumulation of cells at sub-G₁ phase; Fig. 5C). However, miR-542-3p still robustly inhibited growth of U2OS cells after reexpression of RPS23 (Supplementary Fig. S8) or p53 depletion by short hairpin RNA (Supplementary Fig. S9A), suggesting that the inhibition of growth by miR-542-3p can be mediated through multiple pathways. Interestingly, in HCT116 cells, overexpression of miR-542-3p caused a dramatic increase of cell death (sub-G₁, 26.4% vs. 8.7%; Fig. 5E and F) and an induction of Bax and cleaved caspase-3 levels (Fig. 5D). Although overexpression of miR-542-3p still inhibited the growth of p53^{-/-} HCT116 cells (Supplementary Fig. S9B), they

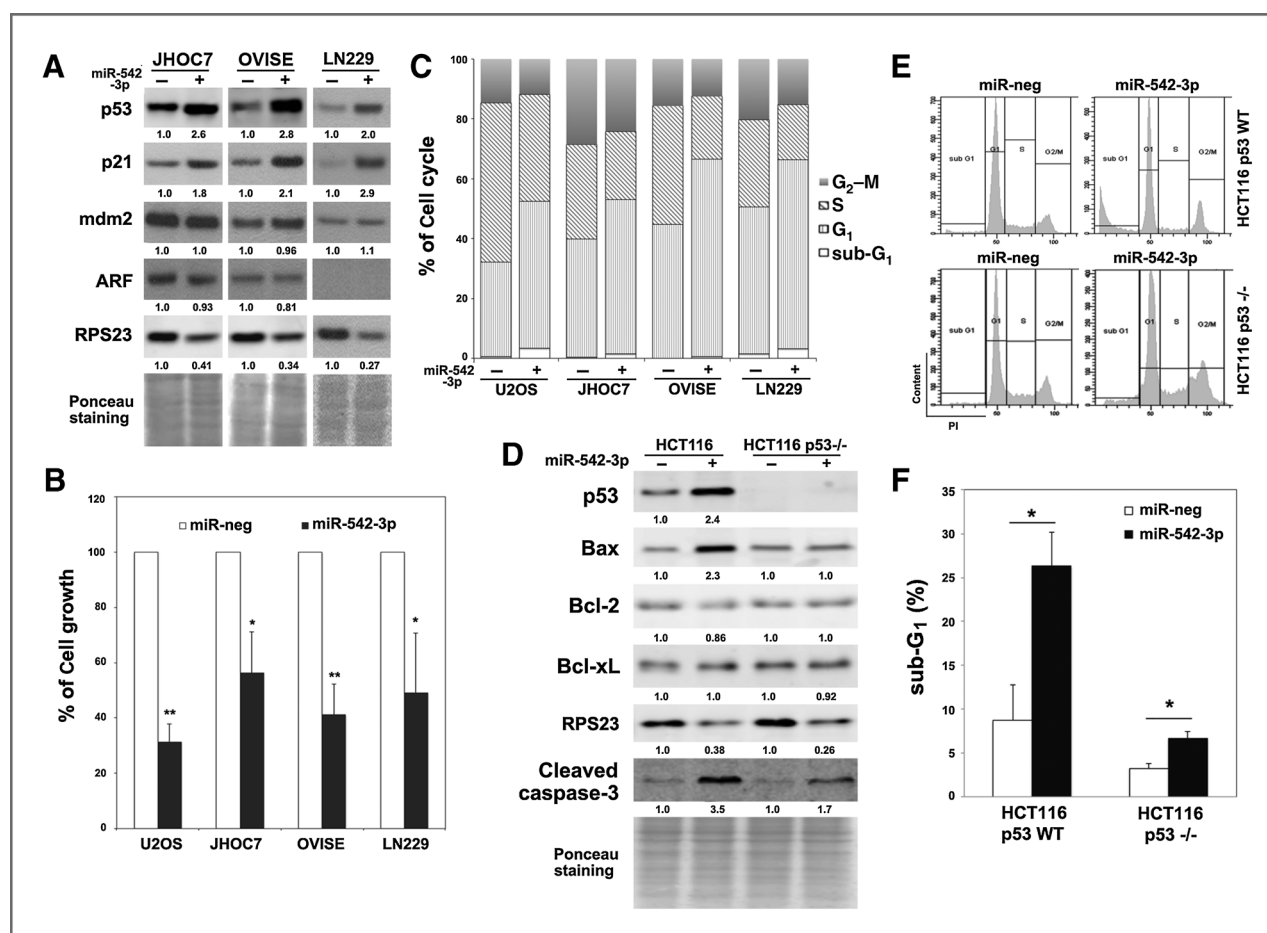


Figure 5. miR-542-3p activates p53 pathway in MDM2-overexpressing or ARF-deficient cells. **A**, miR-542-3p increases p53 in two ovarian clear cell carcinoma lines (JHOC7 and OVISE) and one glioma cell line (LN229). Cells were transfected with negative or miR-542-3p mimics followed by Western blot analysis. **B**, miR-542-3p inhibits cell growth. Cells were reseeded at 1×10^4 cells/well in 12-well plates, cultured for 5 days, and then fixed for crystal violet staining. Relative cell growth was calculated after resubstituting the plates. **C**, miR-542-3p induces G₁ cell-cycle arrest. Control or miR-542-3p-overexpressing cells were fixed and stained for cell-cycle profiling by FACS. **D–F**, p53 is crucial for miR-542-3p-induced cell death. **D** and **E**, HCT116 p53 WT and p53^{-/-} cells were transfected as indicated followed by Western blot (**D**) or cell-cycle analysis (**E**). **F**, percentage of cells in sub-G₁ was quantitated. *, $P < 0.05$; **, $P < 0.01$.

only mildly induced cell death in p53^{-/-} HCT116 cells (sub-G₁, 3.2% vs. 6.7%; Fig. 5E and F), implying that miR-542-3p-induced cell death operates largely through the p53 signaling pathway.

Discussion

The tumor suppressor p53 is an important therapeutic target as it is mutated or functionally inactivated in most human tumors. Multiple strategies have been developed to reactivate p53. For example, small molecules have been identified or designed to correct the folding of mutant p53, thus activating the function of p53, or to stabilize the wild-type p53 by protecting p53 from MDM2-mediated degradation (reviewed in ref. 37). Our current study demonstrates that miRNAs may represent another type of therapeutic agent that can stimulate p53 function. In particular, miR-542-3p stabilized wild-type p53 through disrupting MDM2-mediated degradation and thus may be useful in the treatment of tumors

harboring wild-type p53, in particular, those with either MDM2 amplification or ARF deficiency.

Accumulated evidence has linked miRNAs to the p53 pathway (reviewed in ref. 38). In particular, several studies identified a few miRNAs that serve as either negative regulators (miR-125b and miR-504) or positive regulators (miR-29, miR-34a, miR-122, miR-335, and miR-192/194/215) of p53 expression (19, 20, 24, 39–42). Using a miRNA library-screening platform, we have identified several miRNAs that can regulate the expression of p53. Although two known negative regulators of p53 (miR-125b and miR-504) showed a mild inhibitory role on p53 expression ($z < -1.2$) due to limited sensitivity of picking up negative regulators of p53 by immunofluorescence, it is surprising that our screening did not identify any miRNAs that were previously reported to induce p53 expression and activation. This discrepancy may be due to relatively low sensitivity of our screen. However, most of those miRNAs are not identified even when we lowered the cutoff to $z > 1$, suggesting that these miRNAs may not regulate p53 expression in U2OS cells.

In contrast, miR-542-3p induced p53 expression in a wide range of p53 wild-type cell lines.

The ribosome is a ribonucleoprotein, which is made of a complex of RNAs and proteins and serves as the site of protein synthesis. Accelerated ribosome biogenesis, reflected by enlarged nucleoli, is an important marker of aggressive tumor cells (43, 44). Tumor cells may be more dependent on their ability to produce ribosomes and are therefore potentially more vulnerable to compromised ribosomal function (14). Concordantly, targeting ribosome integrity by a specific RNA pol I inhibitor may have selective tumor-killing effect through induction of p53 (13, 45). Our study suggests that targeting ribosome integrity by a miRNA may be an alternatively attractive way to enhance p53 function. Consistent with a previous report that depletion of the ribosome subunit RPS23 by a siRNA activates p53 in an RPL11-dependent manner in A549 cells (11), we demonstrate that depletion of RPS23 by either siRNA or miR-542-3p strongly stimulated the function of p53 by increasing RPL11 level and enhancing RPL11–MDM2 association to disrupt MDM2-mediated p53 ubiquitination and degradation in U2OS cells. Interestingly, although RPS28 is essential for 18S rRNA biogenesis (Fig. 3C), depletion of RPS28 had no significant effect on the levels of p53 and RPL11 (Fig. 3C). This finding may reflect the differential roles of RPSs in controlling the biogenesis of small ribosome subunit (30), and raise the possibility that p53 may be triggered only if certain steps of 18S rRNA maturation is interrupted during small ribosome subunit biogenesis, which needs future investigation. It is also noteworthy that although miR-542-3p and RPS23 siRNA showed similar effects on RPS23 and 18S rRNA suppression, miR-542-3p had a stronger effect on p53 induction in U2OS cells (2.7 fold vs. 2 fold; Fig. 3C), and ectopic expression of RPS23 transcript lacking 3' UTR did not completely block miR-542-3p-mediated p53 induction, indicating that additional mechanism(s) will exist to activate p53 in miR-542-3p-overexpressing cells. Furthermore, miR-542-3p may have a selective effect on tumor cells as ectopic expression of miR-542-3p produced a much weaker effect on p53 expression in normal human foreskin fibroblasts (Supplementary Fig. S3). Future studies will be necessary to test this therapeutic selectivity.

The precursor of miR-542-3p, pre-miR-542, is cotranscribed with pre-miR-424 and pre-miR-503 as one transcript, which produces mature miRNAs, miR-542-5p, miR-542-3p, miR-424, and miR-503. It is predicted that the expression of this miRNA cluster can be controlled by transcriptional factors such as MAPK(46). Accordingly, miR-542-3p is repressed by c-Src-related signaling molecules, such as EGF receptor, Ras, and MAPKs, in HCT116 cells (25). It is thus not surprising that miR-542-3p is generally underexpressed in cancers such as colon, prostate, and lung cancers (25). Furthermore, silencing of this cluster can be mediated by promoter methylation, such as in prostate cancer (47).

Overexpression of miR-542-3p can inhibit cell growth and prevent tumor formation *in vivo* (25), which is consistent with our observation that the p53 pathway is robustly induced by overexpression of miR-542-3p. However, although miR-542-3p-mediated cell death is largely dependent on p53 (Fig. 5E and F), miR-542-3p can also suppress the cell growth of p53-

deficient tumor cells, such as p53-depleted U2OS cells, p53^{-/-} HCT116 cells (Supplementary Fig. S9), and SW480 cells (25), possibly because (i) ribosomal disruption can suppress cell growth in a p53-independent pathway (48) and (ii) miR-542-3p also inhibits survivin (BIRC5; ref. 49), ILK (25), and many components of the ERK/MAPKs pathway revealed by our gene expression analysis (Supplementary Table S2). Thus, it will be interesting to test whether delivery of miR-542-3p through nanoparticles or other technology is therapeutically applicable for treating tumors in a broad genetic background, regardless of p53 mutational status.

Interestingly, miR-542-5p, which shares the same precursor with miR-542-3p, also has tumor-suppressive functions in neuroblastoma (50). Lower expression of miR-542-3p is correlated with poor survival in patients with neuroblastoma (50). Ectopic expression of synthetic miR-542-5p decreases invasion of neuroblastoma cells *in vitro* and suppresses tumor growth and metastases in an orthotopic mouse xenograft model (46). Therefore, simultaneously rescuing the expression of miR-542-5p and miR-542-3p by lentiviral-based delivery of pre-miR-542 may have better tumor-suppressive effect in cancer treatment than expressing either miRNA alone.

Taken together, our studies identified miR-542-3p as a novel regulator of the p53 tumor suppressor via disruption of ribosome biogenesis. Our studies combined with other studies, provide support for an exploration of ectopic expression of miR-542-3p as a treatment strategy for cancers with wild-type TP53.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Wang, J.-W. Huang, D.G. Huntsman, T. Taniguchi
Development of methodology: Y. Wang, J.-W. Huang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Wang, J.-W. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, J.-W. Huang, M. Castella, D.G. Huntsman, T. Taniguchi
Writing, review, and/or revision of the manuscript: Y. Wang, M. Castella, D.G. Huntsman, T. Taniguchi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.G. Huntsman, T. Taniguchi
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