MicroRNA-21 Targets a Network of Key Tumor-Suppressive Pathways in Glioblastoma Cells

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Abstract

MicroRNA dysregulation is observed in different types of cancer. MiR-21 up-regulation has been reported for the majority of cancers profiled to date; however, knowledge is limited on the mechanism of action of miR-21, including identification of functionally important targets that contribute to its proproliferative and antiapoptotic actions. In this study, we show for the first time that miR-21 targets multiple important components of the p53, transforming growth factor-β (TGF-β), and mitochondrial apoptosis tumor-suppressive pathways. Down-regulation of miR-21 in glioblastoma cells leads to derepression of these pathways, causing repression of growth, increased apoptosis, and cell cycle arrest. These phenotypes are dependent on two of the miR-21 targets validated in this study, HNRPK and TAp63. These findings establish miR-21 as an important oncogene that targets a network of p53, TGF-β, and mitochondrial apoptosis tumor suppressor genes in glioblastoma cells. [Cancer Res 2008;68(19):8164–72]

Introduction

With over 500 microRNA (miRNA) genes identified experimentally in the human genome and a plethora of computationally predicted mRNA targets, it is believed that these small RNAs have a central role in diverse cellular and developmental processes. Therefore, aberrant expression of miRNA genes could lead to human disease, including cancer (1–3). Several studies have confirmed that miRNAs regulate cell proliferation and apoptosis (2, 4, 5).

In recent years, several profiling studies have reported dysregulation of miRNAs in cancer (6–8). The majority of these profiling studies have reported increased levels of miR-21 in most human cancers (6, 8–10). High levels of miR-21 were first reported in glioblastoma tumors and cell lines (6). In the same study, inhibition of miR-21 by LNA or 2′O-Me-miR-21 led to increased caspase-dependent apoptosis, suggesting that miR-21 could be acting as an oncogene to inhibit apoptosis in glioblastoma cells (6). Further studies showed that miR-21 had the same antiapoptotic role in other cancer types, such as cholangiocarcinomas and breast cancer cells (10, 11). Recently, a study determined that miR-21 knockdown in glioblastoma cells significantly repressed tumor formation *in vivo* (12).

Gliomas are the most common primary tumors in the brain and are divided into four clinical grades on the basis of their histology and prognosis. Grade IV glioblastoma is the most malignant of all brain tumors and is almost always fatal. Glioblastoma either arise *de novo* or progress from lower grade to higher grade over time. The treatment strategies for this disease have not changed appreciably for many years, and most are based on a limited understanding of the biology of the disease (13).

Although miR-21 is emerging as a novel oncogene, its mechanism of action is largely unknown. Three miR-21 targets have, thus far, been described: phosphatase and tensin homologue (PTEN), programmed cell death 4 (PDCD4) and Tropomyosin 1 (TPM1; refs. 10, 14–16). However, these targets do not fully elucidate the antiapoptotic function of miR-21 in glioblastomas and other types of cancer. *PTEN*, a well-studied tumor suppressor gene is inactive in a large number of cancers where miR-21 is up-regulated, including glioblastomas (17, 18). On the other hand, knowledge is limited on the contribution of PDCD4 and TPM1 to the malignant phenotype of glioblastomas.

The p53 tumor suppressor is at the center of many cellular pathways that respond to DNA damage, improper mitogenic stimulation, and cellular stress. p53 is activated by such signals and can facilitate growth arrest, promote apoptosis, or mediate DNA repair in a context-dependent manner (19). The importance of p53 in preventing tumor formation is indicated by the presence of mutations in the p53 pathway in nearly all cancers (20). Additionally, there are two homologues of p53, tumor protein p63 (TP73L) and tumor protein p73 (TP73), which also perform many of the p53 functions (19). Studies have shown that p53 activation is mediated by several different factors that respond to oncogenic stimuli and assist p53 by either stabilizing it or acting as cofactors in transcriptional activation or repression of certain genes to promote cell cycle arrest and apoptosis (21). These factors include tumor protein p53 binding protein 2 (TP53BP2), topoisomerase I binding (TOPORS), junction-mediating and regulatory protein (JMY), heterogeneous nuclear ribonucleoprotein K (HNRPK), and death-associated protein 6 (DAXX; refs. 21–26). A recent publication revealed a correlation between miR-21 and some transcripts that are up-regulated in response to p53 activation; however, they failed to determine the mechanism of interaction between miR-21 and p53 (15).

Transforming growth factor-β (TGF-β) is the quintessential growth inhibitory cytokine that can also induce apoptosis. As a mediator of growth arrest, it can activate the cyclin-dependent kinase (CDK) inhibitor *CDKN1A* (p21) and repress the growth-promoting transcription factor *c-MYC*. In addition, DAXX has been implicated as a mediator of TGF-β apoptotic signals by virtue of its physical association with TGF-β receptor II/III (TGFBR2/3) and its requirement for TGF-β–induced apoptosis (27). These various components of the TGF-β apoptotic program ultimately couple the TGF-β signal to the core components of the cell death machinery.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Recently, a computational study showed a correlation between miR-21 and certain components of the TGF-β pathway in a model of epithelial cell plasticity underlying epidermal injury and skin carcinogenesis (28).

Cellular apoptosis to a large extent is mediated by a pathway of mitochondrial outer membrane permeabilization, which involves several proteins including voltage-dependent anion channel 1 (VDAC1) and peptidylprolyl isomerase F (PPIF), resulting in release of cytochrome *c*. Cytochrome *c* interacts with cytosolic apoptotic peptidase activating factor 1 (APAF1), which in turn activates caspase-9 and subsequently caspase-3/7. This intrinsic mechanism of apoptotic activation is disrupted in many cancers, which evade apoptosis (29).

The goal of our study is to decipher the mechanism of action of miR-21 in glioblastomas by identifying some of the most essential tumor-suppressive targets of miR-21 in addition to observing downstream effects with important functional implications. This report is the first to show that miR-21 is in the nexus of direct and indirect negative regulation of a plethora of genes involved in p53, TGF-β, and apoptotic signaling, which establishes its function as an oncogene.

Materials and Methods

Antibodies, reagents, and plasmids. The following antibodies were used for immunoblotting: rabbit DAXX (Santa Cruz), β-actin (Sigma), mouse APAF1 monoclonal (R&D Systems), and mouse p53 monoclonal (BD Biosciences), rabbit polyclonal TAp63 (Biolegend), rabbit polyclonal p21 (Santa Cruz), goat anti-TGFBR2 (Abcam). Secondary antibodies used include goat anti-mouse secondary Alexa Fluor 680 (Molecular Probes), goat anti-rabbit IRDye 800CW, and donkey anti-goat IRDye 800CW (LI-COR).

Plasmids for lentiviral preparation, pLVTHM, psPAX2, and pMD2.G, were obtained from addgene (Trono Lab). Lentiviral production and titration were performed, as previously published (30).

Doxorubicin (Calbiochem) was used at 1, 12.5, 25, and 50 μmol/L. Nocodazole (Calbiochem) was used at 250 ng/mL.

Cell culture and Western blots. Human U251, U87, and HeLa cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomicyn (Invitrogen) in a 5% CO_2 humidified incubator at 37°C. Cells were lysed using RIPA buffer supplemented with protease inhibitors. Protein concentration was estimated by the bicinchoninic acid protein assay kit (Pierce). Protein was then separated on a 4% to 20% Novex tris-glycine gels (Invitrogen), transferred to a nitrocellulose membrane, incubated with the relevant antibodies, and detected with fluorescence-conjugated antibodies. Bands were visualized using Li-Cor Odyssey IR imager (LICOR).

Transfections. To down-regulate miR-21 in U251 and U87 cells, we used 30 pmol LNA-miR-21 (locked bases underlined) TCAACATCAGTCTGATAAGCTA (Sigma Proligo), 2′-O-me-miR-21 (Anti-miR-21; Ambion), or a respective nonsilencing scrambled sequence CATTAATGTCGGACAACTCAAT. Transfections were performed using Lullaby small interfering RNA (siRNA) transfection reagent (OZ Biosciences) according to the manufacturer's protocol. At 24 h later, cells were lysed for immunoblotting and/or RNA.

For siRNA/LNA dual transfections, we also used 30 pmol of siRNA and LNA, respectively. siRNA target sequences HNRPK AAUAUUAAGGCUCUCCGUACA, TAp63 GAUGGUGCGACAAACAAGA, and Scramble UGCGGAUUCUAUCUGUGAU.

RNA isolation and real-time PCR analysis. RNA was extracted by using the miRVana isolation kit (Ambion) followed by Turbo DNase treatment (DNA-free, Ambion). For mRNA profiling, reverse transcription (RT) was performed by using Superscript II first strand synthesis kit (Invitrogen). Real-time PCR to assay mRNA levels was performed in an Applied Biosystems PRISM 7900HT Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems). To check miRNA levels, RT was performed using MMLV reverse transcriptase and the RT primers for miR-21 and control RNU6B (Applied Biosystems). Taqman real-time PCR reaction was performed using Fast TaqMan Universal Master Mix and the TaqMan probe and forward primer for miR-21 or RNU6B. All primers were provided by Applied Biosystems. All reactions were performed according to manufacturer's protocols. Normalizations for mRNA and miRNA real-time PCR were performed using the Ct of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RNU6B, respectively.

Dual luciferase assay. Firefly luciferase reporter vector pMIR-Report (Ambion) was used for these experiments. Downstream of the luciferase gene, we inserted a 60-bp sequence of the 3′ untranslated region (UTR) of the gene of interest containing the miR-21 predicted target site. Assay was performed as previously published (31).

Cell growth assays. U251 cells were seeded in 96-well plates at 8,000 per well and transfected with 50 nmol/L of LNA-miR-21 (Sigma Genosys), 50 nmol/L of negative control LNA-scramble (Sigma Genosys), or transfection reagent alone. Cell Titer Glo (Promega) reagent was used to determine growth at days 0, 1, 2, 3, and 4. Results represent the means of three separate experiments.

Apoptosis assays. For caspase-3/caspase-7 assays, U251 and U87 cells were seeded in 96-well plates 72 h before assay and transduced using virus-expressing miR-21 or negative control scrambled. At 24 h later, three different concentrations 0/12.5/25/50 μmol/L of doxorubicin were added to the cells. At 24 h later, caspase-3/caspase-7 Glo (Promega) reagent was used to determine relative apoptosis. Results represent the means of three separate experiments.

For Annexin V assays, we harvested U251 and U87 cells 24 and 48 h posttransfection and used the Annexin V-PE Apoptosis Detection kit (Biovision) to detect Annexin V. Results were obtained using Guava EasyCyte Flow Cytometer (Guava Technologies). The amount of Annexin V is given as a fold change of the LNA-mir-21 treated relative to the LNA-scr cells.

Cell cycle analysis. Cells were transfected with 50 nmol/L LNA-scram or LNA-miR-21. U251 and U87 cells were synchronized with nocodazole (250 ng/mL) and/or doxorubicin (1 μmol/L) 24 and 48 h posttransfection, respectively. Cells were released 16 to 20 h later, and cell cycle distributions were assayed by ethanol fixation overnight and propidium iodide (PI) staining followed by fluorescence-activated cell sorting using Guava EasyCyte Flow Cytometer (Guava Technologies).

Target prediction and network analysis. Conserved and nonconserved targets of miR-21 were identified using TargetScan 3.1. List of predicted targets was obtained from TargetScan data download.[3] Total number of human miR-21 targets from TargetScan 3.1 is 1,398, including duplicates.

The Ingenuity Pathway Analysis 3.0 (Ingenuity Systems[4]) was used to analyze the list of predicted miR-21 targets. Filtering was performed to remove duplicates and genes with no annotation in ingenuity pathway analysis (IPA), resulting in a list of 765 network eligible genes. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of genes were then algorithmically generated. GO analysis was also performed using IPA.

Statistical analysis. Statistical analysis was either determined by ANOVA or Student's *t* test. Statistical significance is displayed as $P < 0.05$ (*) or $P < 0.01$ (**).

Results

MiR-21 predicted targets form network of interactions. Given the potential of miRNAs to regulate a large number of cellular transcripts, we decided to take a broad approach to identify targets and determine the mechanism by which miR-21 acts as an oncogene in cancer cells. We first chose to assemble a

[3] http://jura.wi.mit.edu/targetscan/mamm_31/mamm_31_data_download/Conserved_Family_Info.txt.zip

[4] http://www.ingenuity.com

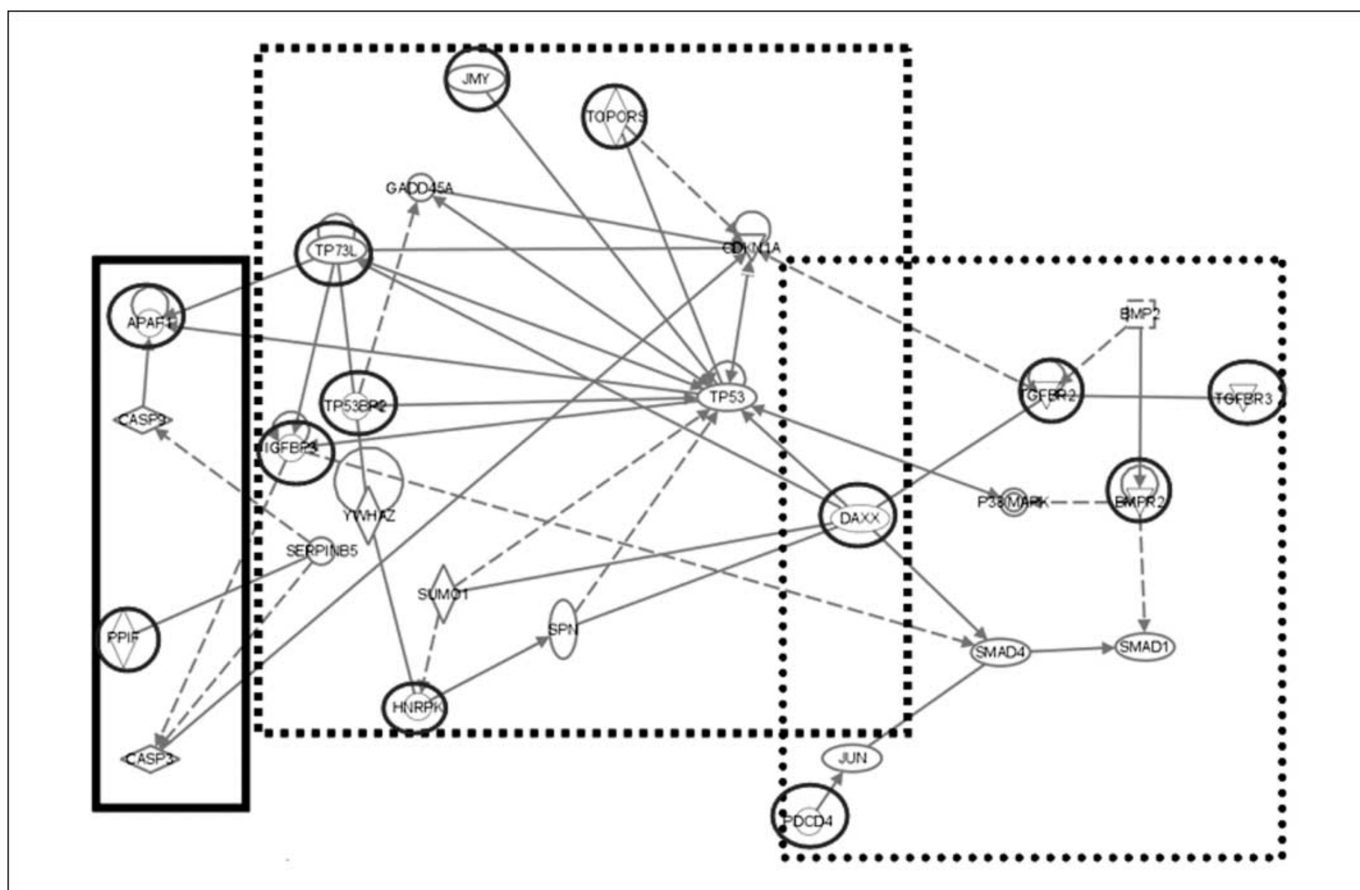

Figure 1. MiR-21 predicted targets form a network of interactions. Network of interactions was constructed using IPA software. The input was all the TargetScan predicted miR-21 targets (conserved and nonconserved) that had cell growth, proliferation, and apoptosis annotations. The genes in bold circles are the predicted targets. Dashed lines annotate unknown transitions in the interactions. Round, square, and solid triangles indicate TGF-β, p53, and mitochondrial apoptosis genes, respectively. IPA provided network score, 34.

list of miR-21 predicted targets using TargetScan 3.1, which included both conserved and nonconserved targets in humans. Using IPA, we performed GO analysis to identify the subset of predicted targets (Supplementary Table S1) that are involved in regulating growth or apoptosis. Interestingly, among the statistically significant annotations, we found headings for cell growth and proliferation, cell death, cancer, and cell cycle (Supplementary Table S1). We then chose the genes from the above annotations and used IPA to determine whether these predicted targets formed a network of interactions. To construct the network, we included additional nodes which were not predicted as targets. Interestingly, we observed that there were three distinct networks in the interactome, which included genes from the TGF-β, p53, and mitochondrial apoptotic pathway (Fig. 1).

Several genes within this TGF-β pathway were predicted targets of miR-21, including TGFBR2, TGFBR3, and DAXX. The TGF-β pathway is known to induce apoptosis in cancer cells and also during development in response to TGF-β ligand binding to its receptors, TGFBR2 and TGFBR3, which in turn can inhibit growth and activate apoptosis either through activation of SMAD transcription or DAXX, respectively (27).

p53 pathway predicted targets included the tumor-suppressive homologue of p53, TP73L (TAp63), and activating cofactors of p53, such as JMY, TOPORS, HNRPK, and TP53BP2 (19, 21–26). All the above genes are known to associate with p53 and assist in the transcriptional activation of antiproliferative and proapoptotic genes in response to DNA damage. Other p53-related targets include genes transactivated by p53 in response to DNA damage.

The mitochondrial-apoptotic pathway genes included important apoptotic inducers, such as APAF1, caspase-8, and proteins that control mitochondria permeability and cytochrome *c* release, such as VDAC1 and PPIF (29).

MiR-21 suppresses major components of key tumor-suppressive pathways. Often, miRNAs can reduce the level of target transcripts (32, 33). Therefore, we assayed the mRNA levels of miR-21 predicted targets and other genes in the network by real-time PCR in response to miR-21 down-regulation (Supplementary Table S2). We chose to perform these experiments in U251, a glioblastoma multiforme cell line that contains the highest levels of miR-21 compared with all cancer cell lines that we assayed by single-plex real-time PCR analysis (data not shown).

To observe changes in the transcripts of genes that are repressed by miR-21 in U251 cells, we had to achieve a significant down-regulation of miR-21 levels. To determine the best method to down-regulate miR-21, we tested both 2′-O-me-miR-21 and LNA-miR-21 (Supplementary Fig. S1). We observed a much greater knockdown with the use of LNA-miR-21 and decided to use it for our further studies.

Upon down-regulation of miR-21 with LNA, we observed significant increases ranging from 2-fold to 16-fold in the levels of major components of the p53/TGF-β/apoptotic pathways, including many of the predicted targets (Fig. 2). The p53 pathway was derepressed to a great extent in response to miR-21 knockdown. Several p53 cofactors and activators, JMY, TP53BP2, HNRPK, and TOPORS, which are predicted targets of miR-21, increased significantly in addition to up-regulation of p53 and its other two homologues, TP73L (TAp63) and TP73. Additionally, we observed increases in several transactivation targets of p53, such as IGFBP3, TNSFRSF10B, NOXA, p21 (CDKN1A), and GADD45A, which are expected due to the increase of p53 and its homologues (Fig. 2*A*; refs. 19, 34). However, some of this deregulation may occur through direct targeting by miR-21, for example, IGFBP3 contains a predicted miR-21 target site; therefore up-regulation could occur because IGFBP3 is a direct target of miR-21.

We observed significant changes in the levels of many TGF-β pathway genes, including receptors TGFBR2/TGFBR3, their ligands TGFB1/TGFB2, DAXX, and the transcription factor SMAD3, which is activated in response to TGFB1/2 binding to the receptors (Fig. 2*B*). In addition, PDCD4, which is involved in mediating TGF-β–induced apoptosis, increased ∼8-fold (35). From these transcripts, only the two receptors and DAXX are predicted targets of miR-21. The up-regulation of all these TGF-β pathway genes could also explain the increase in p21, which is also transactivated by SMAD2/3/4 (27).

Several of the genes involved in mitochondrial apoptosis were also up-regulated in response to miR-21 down-regulation, including APAF1, PPIF, and caspase-3 (CASP3). APAF1 and PPIF are both predicted targets of miR-21 (Fig. 2*C*). Up-regulation of these transcripts is significant because of their importance in mediating mitochondrial apoptosis by cytochrome *c* release in response to a large number of upstream apoptotic stimuli (29).

A subset of the derepressed transcripts, APAF1, BMPR2, PDCD4, and p21 were reported to be up-regulated in MCF-7 breast cancer cells with LNA-miR-21 treatment (15). This confirms that many of the transcripts we identified may also be regulated by miR-21 in other types of cancer.

MiR-21 target validation. A selection of candidate targets whose mRNA levels were deregulated in response to miR-21 down-regulation was validated by luciferase and Western blotting. To test

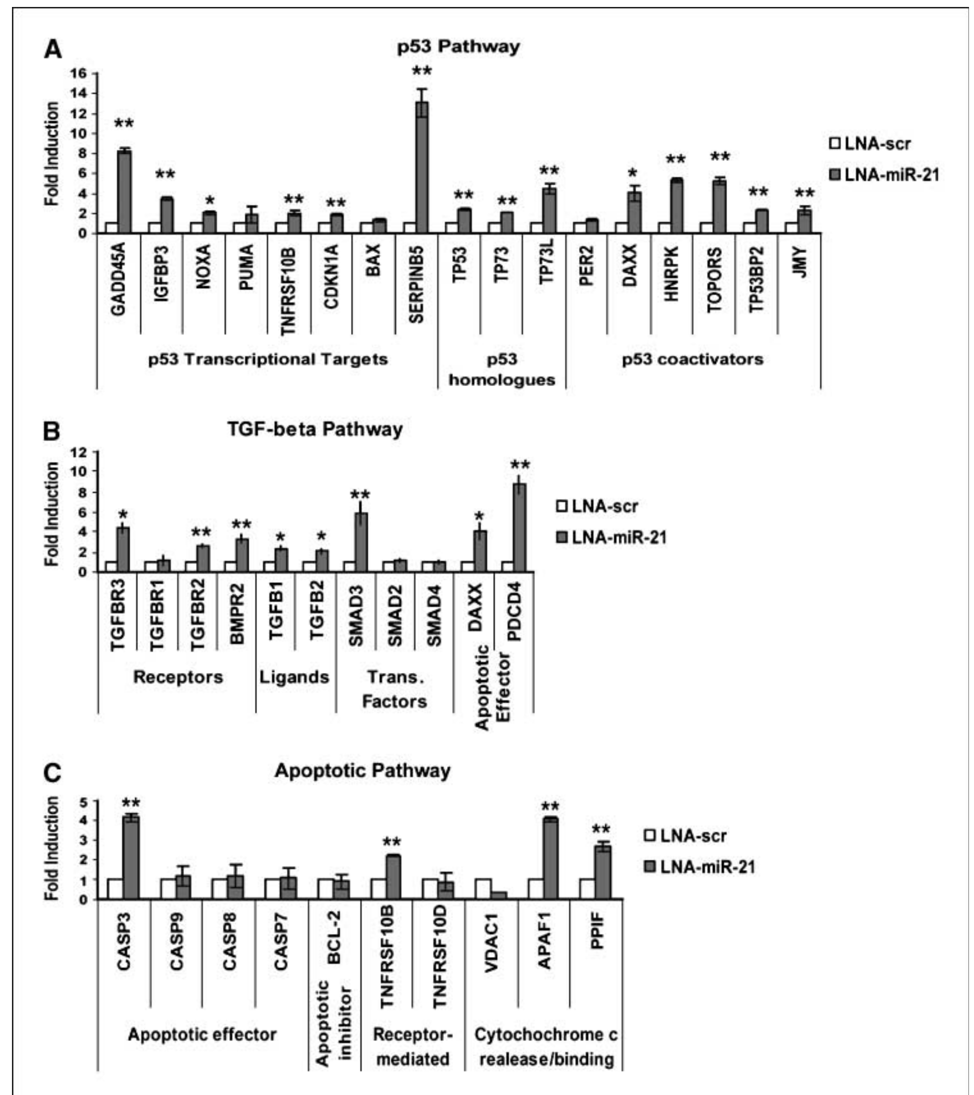

Figure 2. mRNA levels of various p53, TGF-β, and mitochondrial apoptosis pathway genes, including several predicted targets. By real-time PCR analysis, we determined the levels of genes upon transfection of negative control LNA-scr (50 nmol/L; *white column*) or LNA-miR-21 (50 nmol/L; *gray column*) in U251 cells. MiR-21 knockdown derepresses genes in the following: *A,* p53 pathway, JMY, TOPORS, IGFBP3, HNRPK, DAXX, SERPINB5, TP53BP2, and TP73L; *B,* TGF-β pathway, TGFBR2, TGFBR3, PDCD4, and DAXX upon transfection of negative control LNA-scr (50 nmol/L; *white column*) or LNA-miR-21 (50 nmol/L; *gray column*) in U251 cells (expression of each gene was normalized to that of GAPDH); *C,* mitochondrial apoptosis pathway, caspase-3/7/8/9, BCL-2, TNFRSF10B/D, VDAC1, APAF1, and PPIF. Expression of each gene was normalized to that of GAPDH. Student's *t* test was performed. *, $P < 0.05$; **, $P < 0.01$.

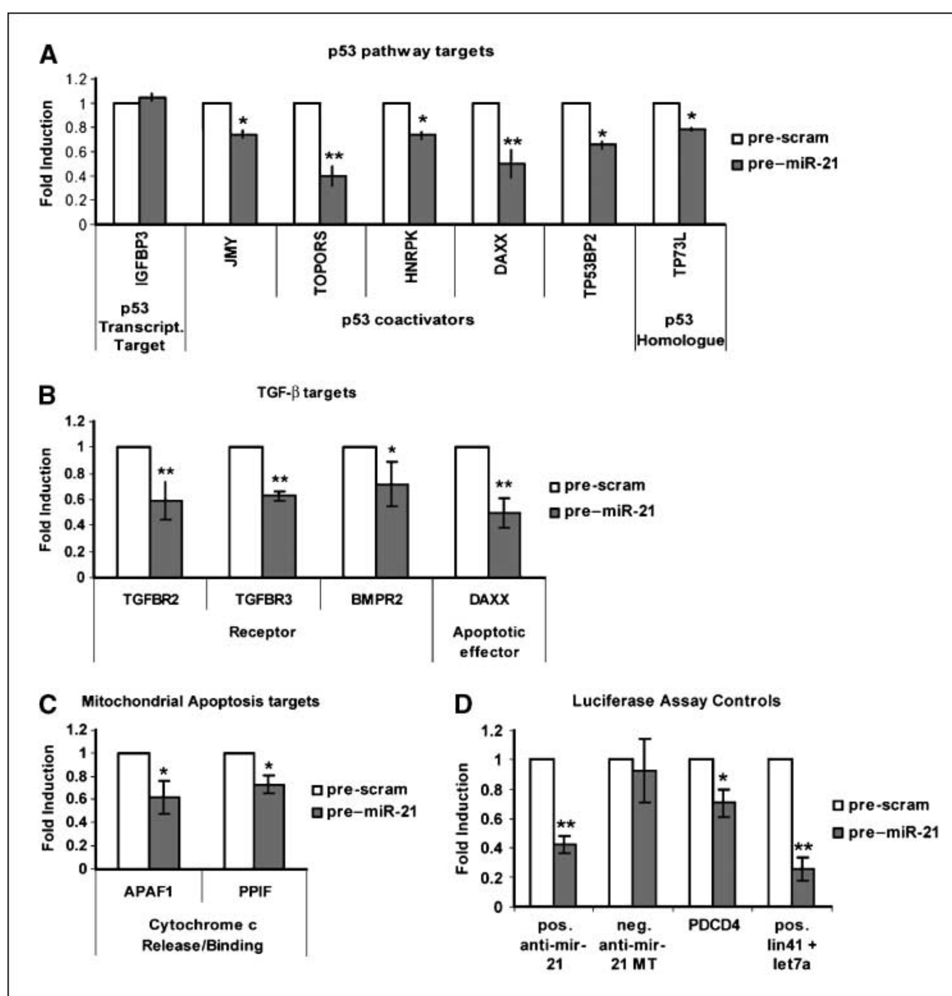

Figure 3. MiR-21 targets genes in the p53, TGF-β, and mitochondrial apoptosis pathway. Prescram (50 nmol/L; *white column*) or pre–miR-21 (50 nmol/L; *gray column*). The targets that were tested are as follows: *A,* p53 pathway genes IGFBP3, JMY, TOPORS, HNRPK, DAXX, TP53BP2, and TP73L; *B,* TGF-β components, TGFFBR2/3, BMPR2, and DAXX; *C,* mitochondrial apoptosis genes APAF1 and PPIF. *D,* assay controls include a 3′ UTR completely complementary to mature miR-21, PDCD4, a prevalidated target, a negative control with three mutations in the seed region, and lin-41, a known target of let-7a. Student's *t* test was performed. *, $P < 0.05$; **, $P < 0.01$.

whether the regulation of the predicted target transcripts, whose levels changed, was direct, we fused 60-bp sequences of the 3′ UTR from each candidate containing the predicted miR-21 target site to a firefly luciferase gene. Cotransfection with pre-miR-21, but not prescrambled sequence, specifically decreased luciferase levels of the reporter of JMY, TGFBR2, TGFBR3, HNRPK, TP73L (TAp63), APAF1, BMPR2, TOPORS, DAXX, TP53BP2, and PPIF (Fig. 3*A–C*). On the other hand, we did not observe a change in luciferase levels for IGFBP3. A positive control reporter bearing a sequence exactly complementary to miR-21 exhibited a 70% decrease in luciferase activity (positive anti–miR-21). Three base mutations within the miR-21 binding site in the positive control reporter completely abolished the effect of the miRNA (negative anti–miR-21). A known target of miR-21, PDCD4, showed a ∼30% decrease in luciferase activity (14, 15). An additional positive control required cotransfection of let-7a miRNA and its target, a lin-41 whole 3′ UTR construct, which induced a 70% decrease in luciferase (36).

On transfection of LNA-miR-21 into U251 cells, we observed the expected 2-fold to 3-fold increase in protein level of each target examined (Fig. 4*A*). Therefore, the target validation and the changes in transcript levels of the targets translate to changes in protein levels. We also observed increases in proteins, such as p53, DNA damage-activated p53 (Ser^{15}), and p21, which are not targets but are indirectly negatively regulated by miR-21 (Fig. 2).

To verify that the changes we observed in the targets were not cell line or cancer type–specific, we overexpressed miR-21 in HeLa cells where the levels of miR-21 are moderate compared with U251 (data not shown). Upon ectopic overexpression of miR-21 by a lentiviral system, we observed significant decreases in the levels of APAF1, DAXX, TAp63 (TP73L), and TGFBR2 compared with the empty vector negative control. However, we did not detect any changes in p53 or its downstream target p21, which could potentially be explained due to the effect of HPV-E6, a viral antigen that inhibits p53 dissociation from MDM2 (37). Taken together, miR-21 dysregulation significantly changes the protein levels of its targets in both glioblastoma and cervical cancer cells.

MiR-21 is necessary for suppression of apoptosis in glioblastoma cells. The antiapoptotic role of miR-21 has been reported in several different studies. Our real-time PCR and protein data suggest that, upon down-regulation of miR-21 in U251 cells, the levels of targets in the TGF-β, p53, and apoptotic pathway, which have known proapoptotic effects, increase significantly (19, 27, 29). Therefore, we tested the effect of miR-21 down-regulation on the apoptosis of U251 and the additional glioblastoma cell line U87. On transfection of LNA-miR-21 in U251 and U87 cells, we observed 1.5-fold to 2-fold increase in the levels of an early marker of apoptosis, Annexin V, after 24 and 48 hours, respectively, compared with the negative control LNA-scrambled (Fig. 5*A*). Additionally, we

looked at the effect of 2′-O-Me-mir-21 treatments on the levels of Annexin V; however, the increase of apoptotic cells in this case was significantly less when compared with the effect of LNA-miR-21 (Supplementary Fig. S2*A*). The increased apoptosis confirmed that miR-21 is acting as a suppressor of cell death in glioblastoma cells. Furthermore, the differential effect of 2′O-Me-miR-21 and LNA-miR-21 indicated the necessity for a sufficient magnitude of decrease in miR-21 levels to cause significant phenotypic effects.

We then tested whether some of the validated mir-21 targets were required for the apoptotic phenotype, which we observe when mir-21 is down-regulated by addition of LNA-mir-21 to U251 cells. To test this, we performed siRNA against validated miR-21 targets TAp63 and HNRPK, which were chosen based on their key position in the network (Fig. 1). We used siRNA sequences that were previously tested and published along with a scramble negative control siRNA (22, 38). Once we confirmed that the siRNA sequences were effective at down-regulating the mRNA of the target genes (data not shown), we introduced the siRNA sequences with LNA-mir-21 or LNA-scr in U251 cells and assayed the levels of Annexin V 24 hours posttransfection. There was ~25% decrease in Annexin V induction in siRNA-TAp63 and siRNA-HNRPK–treated cells (Fig. 5*B*). These results indicate that HNRPK and TAp63 are essential for the antiapoptotic function of miR-21.

MiR-21 suppresses p53-mediated apoptosis in U251 cells. The targeting of important p53 cofactors by miR-21 and the up-regulation of essential mediators of p53 tumor suppression in response to miR-21 knockdown led us to the hypothesis that miR-21 may be inhibiting p53-mediated apoptosis. To test this hypothesis, we compared apoptosis induction by addition of different doses of doxorubicin (adriamycin), a chemotherapeutic drug widely used to activate p53-mediated apoptosis by DNA damage in the presence or absence of miR-21 overexpression (39). For both U251 and U87 cell lines, cells expressing the scrambled control showed a predicted dose-dependent increase in caspase-3/caspase-7 activity, whereas the cells expressing miR-21 did not respond to the drug treatment even at higher doses (Fig. 5*C*). This result indicates that miR-21 up-regulation in cancer cells is sufficient to inhibit p53-mediated apoptosis, thus protecting the cells from p53-mediated apoptosis in response to DNA damage induced by a chemotherapeutic agent.

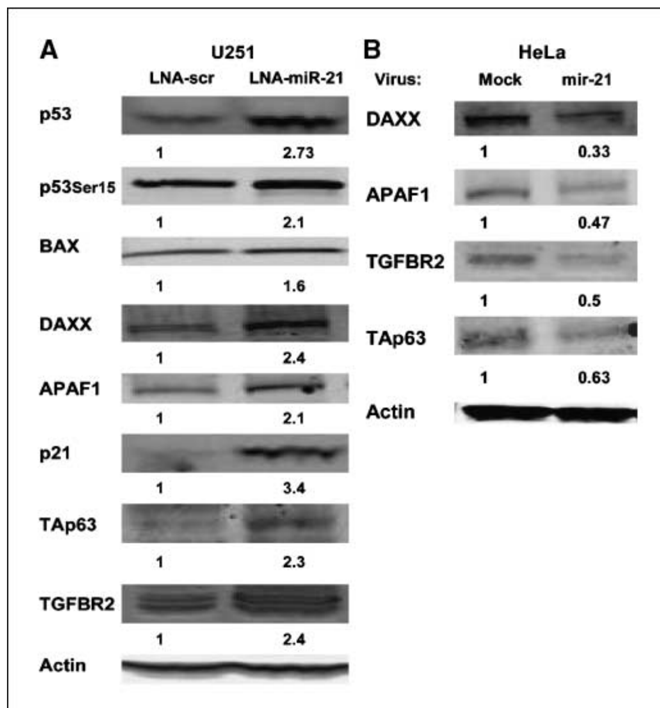

Figure 4. MiR-21 knockdown increases protein levels of key tumor suppressor genes. *A,* immunoblotting of U251 cell lysates upon treatment with LNA-miR-21 (50 nmol/L) and a negative control LNA-scr (50 nmol/L). We checked levels of miR-21 targets DAXX, APAF1, TAp63 (TP73L), TGFBR2, and nondirect targets p53 and p21. *B,* changes in the protein levels of the indicated miR-21 targets were confirmed in HeLa cell lysates in response to miR-21 up-regulation using a lentiviral expression vector of miR-21 and an empty negative control vector. Fold change was determined after normalization to actin and is indicated below the blots.

MiR-21 knockdown suppresses growth of glioblastoma cells. Our mRNA, protein, and luciferase data indicate a broad dysregulation of many important tumor-suppressive genes with known effects on cellular growth. The observed increase in TGF-β pathway components in addition to p53, p21, and GADD45A suggests a potential modulation of cell growth in addition to apoptosis (Figs. 2 and 4*A*). To test the effect of miR-21 on the growth of U251 and U87 cells, we knocked down miR-21 using LNA-miR-21 in both U251 and U87 cells and assayed cell growth over 4 days. Interestingly, we observed a significant suppression in growth of cells in which miR-21 was knocked down compared with negative controls, LNA-scrambled, and the mock (Fig. 6*A*). This retarded growth continued throughout the 4 days with the most significant difference observed on day 3. The negative control LNA-scrambled and mock did not show any significant growth difference, suggesting that the LNA treatment itself does not affect growth. These results suggest that miR-21 plays an essential role in the proliferative capacity of glioblastoma cells.

MiR-21 knockdown led to cell cycle arrest. To test whether the effect on cell growth is occurring due to cell cycle arrest, another hallmark of p53 function, we decided to see the effect that miR-21 knockdown has on the cell cycle of U251 and U87 cells. We transfected both cell lines with LNA-miR-21 or LNA-scr; 24 or 48 h later, cells were synchronized using doxorubicin (G_2-M phase) and nocodazole (M phase); and 20 to 24 h later, we performed cell cycle analysis by PI staining. Addition of LNA-miR-21 (50 nmol/L) caused a significant increase in the population of cells in G_0-G_1 phase compared with LNA-scr treatments in both U251 and U87 cells (Fig. 6*B*). For U251 cells, we observed a 21% and 25% increase in cells arrested at G_0-G_1 with nocodazole and doxorubicin synchronization, respectively, in the cells where miR-21 was knocked down (Fig. 6*B* and Supplementary Fig. S2*B*). These experiments show that miR-21 is required for progression through the G_0-G_1 phase of the cell cycle in glioblastomas cells.

To determine whether HNRPK and TAp63 contribute to the cell cycle arrest observed in response to mir-21 down-regulation, we used the same siRNAs previously tested in the Annexin V assays. At 24 h posttransfection of each siRNA with LNA-scr or LNA-miR-21, we added nocodazole to synchronize cells. At 16 to 20 h later, we harvested the cells and performed PI staining for cell cycle analysis. In the presence of siRNA-HNRPK and siRNA-TAp63, we observed a 2-fold decrease in the proportion of cells arrested in G_0-G_1 in response to miR-21 down-regulation. Therefore, HNRPK and TAp63 are important targets for miR-21–mediated cell cycle progression.

Discussion

The emerging significance of miRNAs in cancer has spiked major interest in recent years resulting in numerous cancer profiling studies. In this study, we have addressed the mechanisms by which

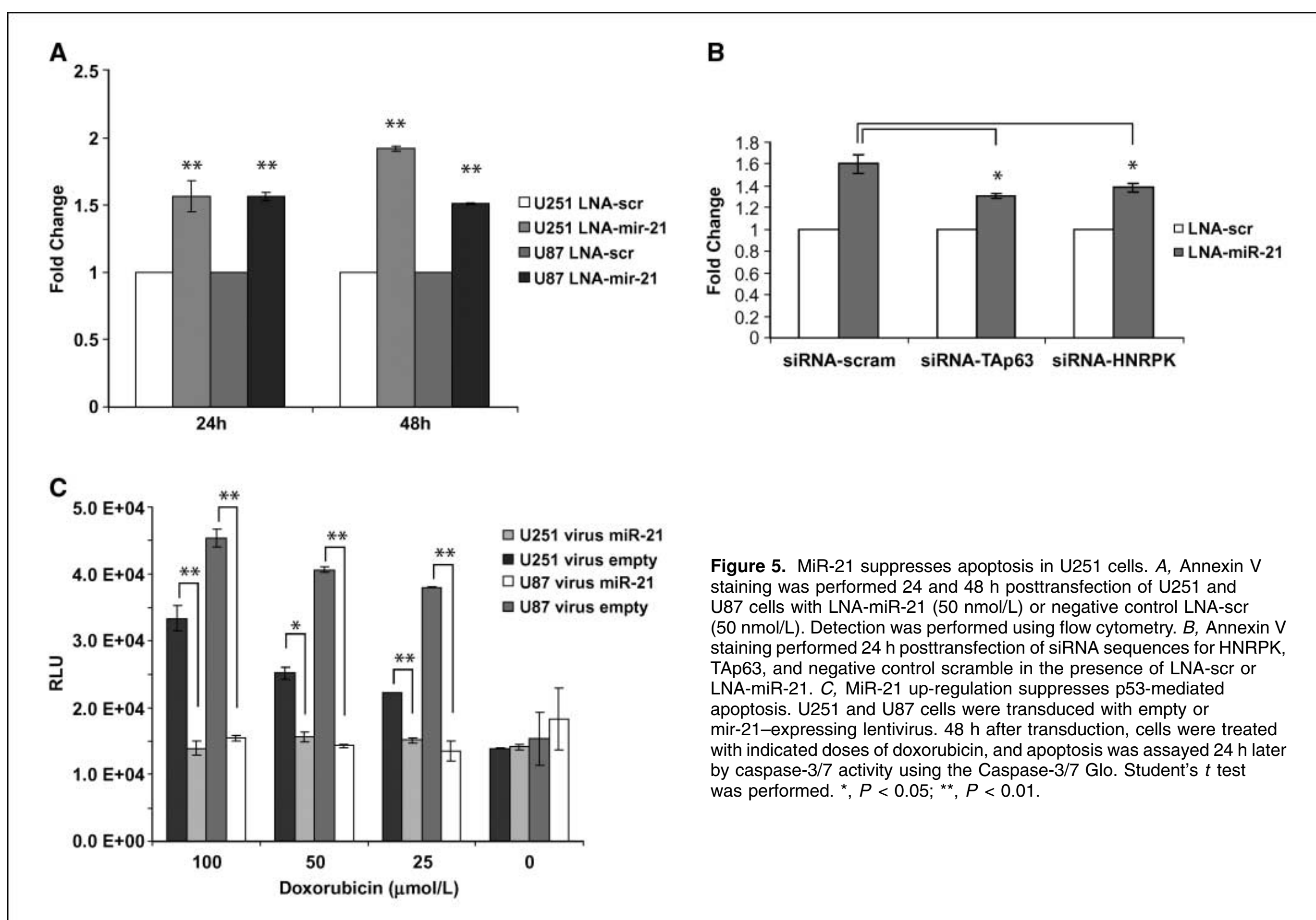

Figure 5. MiR-21 suppresses apoptosis in U251 cells. *A,* Annexin V staining was performed 24 and 48 h posttransfection of U251 and U87 cells with LNA-miR-21 (50 nmol/L) or negative control LNA-scr (50 nmol/L). Detection was performed using flow cytometry. *B,* Annexin V staining performed 24 h posttransfection of siRNA sequences for HNRPK, TAp63, and negative control scramble in the presence of LNA-scr or LNA-miR-21. *C,* MiR-21 up-regulation suppresses p53-mediated apoptosis. U251 and U87 cells were transduced with empty or mir-21–expressing lentivirus. 48 h after transduction, cells were treated with indicated doses of doxorubicin, and apoptosis was assayed 24 h later by caspase-3/7 activity using the Caspase-3/7 Glo. Student's *t* test was performed. *, $P < 0.05$; **, $P < 0.01$.

miR-21 contributes to the transformed state of glioblastoma cells by identifying targets in important tumor-suppressive pathways, such as p53, TGF-β, and mitochondrial apoptosis. These data were first reported in our poster at the miRNAs in Cancer Keystone meeting (Papagiannakopoulos et al., 2007).

Our approach links putative targets around a function and succeeds in identifying not only multiple targets but uncover an entire network under miRNA control. One gets no sense of the thematic relationship among targets from currently popular *in silico* search algorithms for miRNA targets. Current methods tend to identify targets one at a time, which leads to highly skewed misunderstandings of miRNA function and is a hopelessly exhaustive task. We know that miRNAs have hundreds of targets; however, this fact must be understood in the context of the somewhat dichotomous observation that an miRNA sometimes seems to have its predominant phenotypic effect on a single mRNA. First, by getting a foothold on a functional network of genes, one can systematically analyze each component of the network for direct and indirect effects of the miRNA, which in our case was miR-21.

The miR-21 targets we identified included p53 homologue, p63, p53 activators JMY, TOPORS, TP53BP2, DAXX, and HNRPK, which can stabilize p53 protein levels by interfering with MDM2 and/or act as p53 transcriptional cofactors, assisting p53 in transactivating genes that can induce apoptosis and growth arrest (21–26). These targets are required for p53 tumor-suppressive activity; therefore, by targeting these genes, miR-21 can impair p53 response to stimuli, such as DNA damage. MiR-21 repressed p53-mediated apoptosis in response to chemotherapeutic (doxorubicin)–induced DNA damage, therefore contributing to drug resistance in glioblastoma cells. MiR-21 suppression of the p53 response may take place in most cancers where miR-21 is up-regulated, and this oncogenic mechanism may be even more significant contributor to malignancy in tumors with wild-type p53. Regulation of these p53-related targets by miR-21 may potentially explain previous observations in breast cancer cells, where p53-transactivated genes were up-regulated in response to miR-21 down-regulation (15).

The regulation of the TGF-β pathway is also critical for the transformed state of glioblastomas, particularly due to the fact that gliomas become resistant to TGF-β. This resistance may be partly due to the negative regulation of multiple components of the pathway of miR-21, including the indirect regulation of TGFB1/2 and the direct targeting of the receptors TGFBR2/3 and the apoptotic mediator DAXX. DAXX, which can stabilize p53 in addition to mediating TGF-β apoptosis, plays a role in both pathways and, therefore, may be a key target in disrupting cross-talk between the two pathways (Supplementary Fig. S3). The functional convergence of p53 and TGF-β pathways has been previously reported by several studies, which reported assistance and necessity of p53/p63/p73 for TGF-β–mediated transactivation of certain antiproliferative and proapoptotic factors, such as p21

(40, 41). Interestingly, a transcriptional target of the functional convergence of these pathways is SERPINB5, the transcript most highly up-regulated in response to miR-21 knockdown (Fig. 2*A*; ref. 42). Derepression of the p53 pathway in response to miR-21 down-regulation may assist in the derepression of the cytostatic response of TGF-β signaling, leading to cell cycle arrest and apoptosis.

We have shown that miR-21 regulates sets of genes, and the regulation is direct through targeting of certain transcripts, and in other cases, the effects are indirect. This finding is in agreement with limited data from other laboratories that suggests that many miRNAs have evolved to regulate multiple components of certain pathways, thus mediating a more effective negative regulation on those pathways (33). These data are also consistent with findings in glioblastomas that significant effects of miRNAs occur indirectly rather than by direct targeting (24, 43).

The phenotypic effects observed upon down-regulation of miR-21 in glioblastoma cells reflect the significant repression of multiple components of the p53, TGF-β, and apoptotic pathways by miR-21. We were able to show these effects in two established glioblastoma cells lines, U251 and U87. When miR-21 levels were decreased, we observed a significant increase in apoptotic cells, as has been previously reported (6). In addition, miR-21 down-regulation significantly represses growth and leads to cell cycle arrest at G_0-G_1, which can be explained by the up-regulation of p21 (Figs. 2*A* and 4*A*), a protein that is known to regulate cell cycle checkpoints in response to its transactivation by p53. In accordance, we observe a significant increase in the protein levels of p53 (Ser^{15}; Fig. 4*A*), which occurs in response to DNA damage and can mediate cell cycle arrest at G_0-G_1 by transactivation of p21 (19). Both the apoptotic and cell cycle phenotypes, which we observed in the absence of miR-21, are partly dependent on two miR-21 targets, HNRPK and TAp63. Both of these targets play an essential role in the oncogenic function of miR-21. HNRPK is a known coactivator of p53 in response to DNA damage, assisting in the transactivation of p21 and GADD45A (22). TAp63 is a homologue of p53 and is known to mediate both cell cycle arrest and apoptosis (19, 38). However, the inability of their knockdown to completely rescue the proapoptotic and cell cycle arrest phenotypes, which are observed in the absence of miR-21, suggests that there are other critical miR-21 targets involved in mediating miR-21 oncogenic phenotypes.

The effects we describe when we knockdown miR-21 suggest that miR-21 lies embedded in a nexus of tumor suppression genes and has multiple specific targets in the network that lead to the observed phenotypes. These broad effects enable miR-21 to act as a key oncogene modulating cell growth, desensitizing cells to apoptosis and cell cycle arrest (Supplementary Fig. S3). Cellular mechanisms that control apoptosis, cell growth, and cell cycle function as efficient fail-safe mechanisms to prevent cancer development, despite the trillions of somatic cell divisions that occur during a human lifetime. Cancer cells therefore only survive

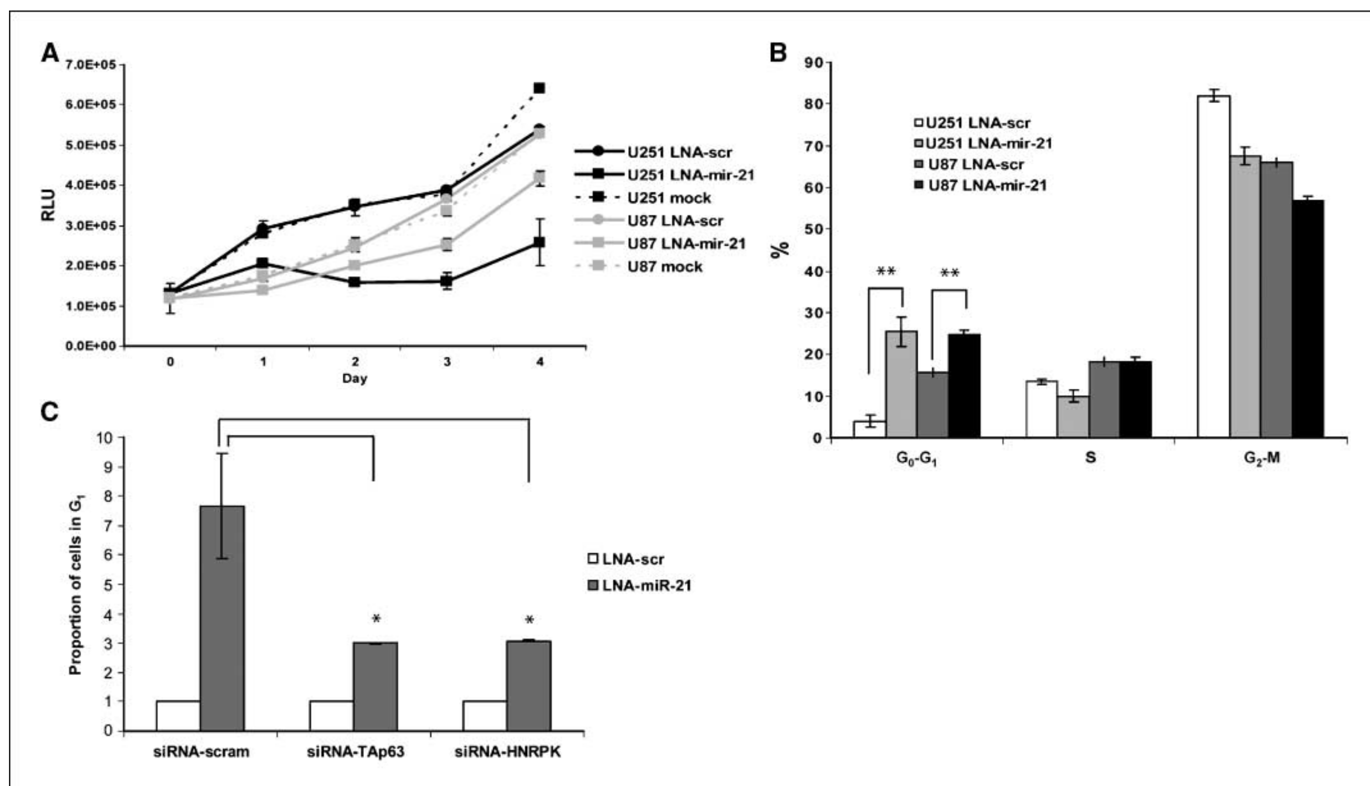

Figure 6. MiR-21 knockdown suppresses U251 cell growth and leads to cell cycle arrest. *A,* U251 (*black*) and U87 (*gray*) cells were transfected with LNA-miR-21 (50 nmol/L) or LNA-scr (50 nmol/L) and mock with just transfection reagent. Cell growth was determined using Cell Titer Glo assay performed at day 0, 1, 2, 3, and 4. *B,* U251 cells were transfected with LNA-miR-21 (50 nmol/L) or LNA-scram (50 nmol/L), and cells were synchronized using nocodazole (250 ng/mL). U251 (24 h) and U87 (48 h) cells were harvested for cell cycle analysis using PI staining 20 and 24 h, respectively, after addition of nocodazole. *C,* PI staining performed 24 h posttransfection of siRNA sequences for HNRPK, TAp63, and negative control scramble in the presence of LNA-scr or LNA-miR-21. Data represent proportion of U251 cells in G_0-G_1. The amount of cells accumulating in G_1 is given as a fold change of the LNA-mir-21 treated relative to the LNA-scr cells. Student's *t* test was performed. *, $P < 0.05$; **, $P < 0.01$.

if the apoptotic response is dampened or disrupted, cell cycle checkpoints are evaded, and unrestricted cell proliferation occurs (44). Due to the functional relationship among miR-21 targets, changes in the level of miR-21 is likely to dampen the robustness of a highly interconnected tumor-suppressive network and result in global regulation or dysregulation of the network functions. Our data suggest that miR-21 up-regulation may be a key step leading to oncogenesis in glioblastoma and other types of cancer. Furthermore, targeted down-regulation of miR-21 in human tumors and particularly glioblastoma, which are fatal, could prove of high therapeutic value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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