Differentially Regulated Micro-RNAs and Actively Translated Messenger RNA Transcripts by Tumor Suppressor p53 in Colon Cancer

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Abstract

Purpose: The aim of this study was to investigate the role of p53 in regulating micro-RNA (miRNA) expression due to its function as a transcription factor. In addition, p53 may also affect other cellular mRNA gene expression at the translational level either via its mediated miRNAs or due to its RNA-binding function.

Experimental Design: The possible interaction between p53 and miRNAs in regulating gene expression was investigated using human colon cancer HCT-116 (wt-p53) and HCT-116 (null-p53) cell lines. The effect of p53 on the expression of miRNAs was investigated using miRNA expression array and real-time quantitative reverse transcription-PCR analysis.

Results: Our investigation indicated that the expression levels of a number of miRNAs were affected by wt-p53. Down-regulation of wt-p53 via small interfering RNA abolished the effect of wt-p53 in regulating miRNAs in HCT-116 (wt-p53) cells. Global sequence analysis revealed that over 46% of the 326 miRNA putative promoters contain potential p53-binding sites, suggesting that some of these miRNAs were potentially regulated directly by wt-p53. In addition, the expression levels of steady-state total mRNAs and actively translated mRNA transcripts were quantified by high-density microarray gene expression analysis. The results indicated that nearly 200 cellular mRNA transcripts were regulated at the posttranscriptional level, and sequence analysis revealed that some of these mRNAs may be potential targets of miRNAs, including translation initiation factor eIF-5A, eIF-4A, and protein phosphatase 1.

Conclusion: To the best of our knowledge, this is the first report demonstrating that wt-p53 and miRNAs interact in influencing gene expression and providing insights of how p53 regulates genes at multiple levels via unique mechanisms.

The tumor suppressor gene *p53* is one of the key regulators of cell cycle control and apoptosis and has been named the guardian of the genome (1). In addition to its function as a transcription factor, p53 also acts as an RNA-binding protein capable of regulating its own mRNA translation (2). As an RNA-binding protein, p53 regulates the expression of other cellular mRNA transcripts at the posttranscriptional level (3). p53 also influences apoptosis by accumulating to mitochondria (4, 5).

With the recent discovery of noncoding RNAs [micro-RNAs (miRNA) and small interfering RNAs (siRNA)] and their function as translational regulators, it is clear that miRNAs play important roles in regulating gene expression. The notion that miRNAs regulate gene expression at the translational level is based on the study of the first two miRNAs, *lin*-4 and *let*-7, in *Caenorhabditis elegans*. *Lin*-4 attenuates the translation, but not the mRNA level, of two target genes, *lin*-14 and *lin*-28, by imperfect base pairing to complementary sequences in the 3′ untranslated region of the target mRNAs (6, 7). Translational regulation has been extensively studied in plant biology (8). In plants, translational regulation provides acute responses due to sudden environmental changes and this process is highly reversible and energy efficient. Translational control also provides the same advantage for mammalian systems, in particular during genotoxic stress (9).

The central concept of translational regulation is that gene expression may be controlled by the efficiency of translation of a given mRNA in the absence of a corresponding change in the steady-state level of that mRNA. Translational regulation provides the cell with a more precise, immediate, and energy-efficient way of controlling expression of proteins, and can induce rapid changes in protein synthesis without the need for transcriptional activation and subsequent mRNA processing steps. In addition, translational control also has the advantage of being readily reversible, providing the cell with great flexibility in responding to various cytotoxic stresses.

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Little is known, however, how miRNAs are regulated at the transcriptional level. After transcription, pre-miRNAs are processed by Dicer complex to their corresponding mature miRNAs. We hypothesize that p53 may also mediate certain miRNAs expression due to its function as a transcription factor. In addition, p53 may also affect other cellular mRNA gene expression at the translational level either via its mediated miRNAs or due to its own RNA-binding function. This hypothesis is partially supported by a recent report from O'Donnell et al. (10) showing that c-Myc regulated a number of miRNAs, and two of the miRNAs regulated E2F expression. c-Myc is a helix-loop-helix leucine zipper transcription factor that regulates an estimated 10% to 15% of genes in the human genome.

Translational control has been shown to play a key role in oncogenesis (9). One of the examples is thymidylate synthase, one of the important targets for fluoropyrimidine-based anticancer therapy (11). Another example is vascular endothelial growth factor, which was shown to be regulated, at least in part, at the translational level (12). More importantly, *p53*, the critical tumor suppressor gene, was also regulated at the translational level (2). However, the RNA-binding function of p53 and its potential for regulating other downstream genes has not been fully elucidated.

The main function of miRNAs is to regulate gene expression at the translational level. Although the exact function of most of the newly discovered miRNAs and siRNAs are just emerging, their ability to regulate cell proliferation and cell death has been recently shown (13). Recent reports have shown that expression of miRNAs can be altered in cancer (14). With the recent discovery of the function of miRNA as translational attenuators, we have reasoned that there might be a potential interaction between miRNAs and p53 because of the dual function of p53 as a transcription factor and RNA-binding protein, and the roles of both in the translational regulation process.

Therefore, we chose to explore the potential relationship between the transcription factor function of p53 and miRNA expression in a colon cancer–related context, as *p53* is one of the most frequently altered tumor suppressor genes in colon cancer due to mutations and deletions. The human HCT-116 (wt-p53) and HCT-116 (null-p53) colon cancer cell lines were chosen as model systems to investigate the role of p53 on the expression of miRNAs. HCT-116 (null-p53) cell line was developed via targeted deletion using homologous recombination using HCT-116 (wt-p53) cells (15). This model has been used extensively for the investigation of p53 functions in cell cycle control and apoptosis (15–18). We expect that the functional miRNAs are localized in the actively translated polyribosome complexes (19). Hence, we have investigated the effect of wt-p53 on miRNAs and their translationally regulated mRNA targets by isolating both actively translated mRNA transcripts and miRNAs from polyribosome complexes from these two colon cell lines. The effect of p53 on miRNA expression and on the expression levels of both steady-state and actively translated mRNA transcripts were analyzed. Our study indicated that the expression levels of a number of miRNAs were affected by wt-p53. Down-regulation of wt-p53 via siRNA abolished the effect of wt-p53 in regulating miRNAs in HCT-116 (wt-p53) cells. Global sequence analysis revealed that >46% of the 326 miRNA putative promoters contain potential p53-binding sites, suggesting that some of these miRNAs were potentially regulated directly by wt-p53. Nearly 200 cellular mRNA transcripts were regulated at the posttranscriptional level, and sequence analysis revealed that some of these mRNAs may be potential targets of miRNAs.

Materials and Methods

Cell lines and reagents. The HCT-116 (wt-p53) and HCT-116 (null-p53) cell lines were a gift from Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD) and were described in detail previously (15, 16). Both cell lines were maintained in McCoy's medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mmol/L L-Glutamine, and antibiotics. All cell lines were grown at 37°C in a humidified incubator with 5% CO_2. 5-Fluorouracil (5-FU) was purchased from Sigma (St. Louis, MO).

Isolation of steady-state total mRNA and actively translated mRNA transcripts. The procedures for isolating steady-state total mRNA and actively translated mRNA transcripts were described in detail previously via sucrose gradient ultracentrifugation (20). The activated translated mRNA transcripts were isolated from pooled polysome fractions (fractions 7-13) using Trizol-LS Reagent (Invitrogen, Carlsbad, CA).

mRNA transcript expression analysis using microarray. CodeLink UniSet Human 20 K Bioarray (GE Healthcare/Amersham Biosciences, Piscataway, NJ), containing ~20,289 gene probes, was used to generate gene expression profiles of both steady-state total mRNAs and actively translated mRNAs isolated from HCT-116 (wt-p53) and HCT116 (null-p53). All reagents and protocols were provided by GE Healthcare/Amersham Biosciences. Double-stranded cDNAs were generated using 2 μg RNA from each sample. After purification, the double-stranded cDNAs were used as templates to generate cRNA via an *in vitro* transcription reaction using T7 RNA polymerase and biotin-11-UTP (Perkin-Elmer, Boston, MA). Biotin-labeled cRNA (10 μg) was fragmented and hybridized to a UniSet Human 20 K Bioarray. The arrays were washed and stained with Cy5-streptavadin. After washing, the dried slides were scanned by Axon GenePix Professional 4200A microarray scanner using Genepix Pro 5.1 software. The images were grided by Codelink 4.1 software (GE-Healthcare/Amersham Biosciences). Contaminated and irregularly shaped spots were removed before the data files were analyzed. GeneSpring Software 7.2 (Agilent, Palo Alto, CA) was used for the final gene expression analysis. Under Cross-Gene Error Model, normalization step was done in two steps: (*a*) "per chip normalization," in which each measurement was divided by the 50th percentile of all measurements in its array, and (*b*) "per gene normalization," in which all the samples were normalized against the specific samples (controls). The results were filtered by flags and 4-fold cutoff. The expression profiles were compared using one-way ANOVA analysis with $P < 0.05$.

Mature miRNA expression analysis using miRNA array. The cDNA synthesis procedures for miRNA quantitation using total RNAs was based on method described by Elbashir et al. (21). Actively translated RNAs from HCT-116 (wt-p53) and HCT-116 (null-p53) cells was size-fractionated using an YM-100 column (Millipore, Billerica, MA) and 0.5 μg size-fractionated RNA were used for the ligation of adaptor sequences. The sequences of the adaptors are as follows: 5′-AAAGGAGGAGCTCTAGaua-3′ and 5′-(P)ugGCCTATAGTGAGTCGTATTATTT-3′. Uppercase letters denote deoxyribonucleotides and lower case letters denote ribonucleotides. The adaptors were ligated to the size-fractionated RNA with subsequent gel fractionation steps. Following ligation, the samples were converted to cDNA using a primer complementary to the 3′-adaptor (5′-TAATACGACTCACTATAGGCCA-3′). The cDNA was amplified by PCR using the above-mentioned oligonucleotide as a reverse primer and a forward primer matching the adaptor (5′-AAAGGAGGAGCTCTAGATA-3′). The cDNA was amplified

by PCR and digested with *Xba*I to remove the majority of the 5′ adaptor sequence. The miRNA expression analysis was conducted based on the protocol of Rossetta Genomics (Rehovot, Israel) and Icoria (Research Triangle Park, NC; ref. 22). The array was constructed based on the Sanger Database, containing a total of 247 known miRNAs. cDNA labeled with either Cy3-CTP or Cy5-CTP was generated from HCT-116 (wt-p53) and HCT-116 (null-p53) using the low-input linear amplification kit (Agilent) according to the protocol of the manufacturer. Hybridized microarrays were scanned using the Agilent LP2 DNA Microarray Scanner at 10 μm resolution. Microarray images were visually inspected for defects. The expression of miRNAs was analyzed using Feature Extraction Software (Agilent). The signal of each probe was set as its median intensity. The threshold for reliable probe signals was set at 1,500. Clustering analysis was done using CLUSTER 3.0/TreeView software (23).

Real-time quantitative reverse transcription-PCR analysis for mRNA expression. Real-time quantitative reverse transcription-PCR (qRT-PCR) analysis was done using total RNAs isolated from HCT-116 (wt-p53) and HCT-116 (null-p53) cells and RNAs isolated from both cell lines treated with 10 μmol/L 5-FU for 24 hours. Real-time qRT-PCR primers and probes for *p53* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were purchased from Applied Biosystems, Inc. (Foster City, CA). qRT-PCR was done on an ABI 7500HT instrument under the following conditions: 25°C, 10 minutes; 37°C, 2 hours for reverse transcription; and 95°C, 10 minutes; 95°C, 15 seconds; 60°C, 1 minute for PCR. The reaction was done up to 40 cycles ($n = 3$). The gene expression ΔC_T value of p53 from each sample was calculated by normalizing with internal house keeping gene *GAPDH* and relative quantitation values were plotted.

Real-time qRT-PCR analysis for miRNA expression. Real-time qRT-PCR analysis was done using total RNAs isolated from HCT-116 (wt-p53) and HCT-116 (null-p53) cells and RNAs isolated from both cell lines treated with 10 μmol/L 5-FU for 24 hours. The miRNA sequence-specific RT-PCR primers for hsa-miR-30a-5p, hsa-miR-181b, hsa-let-7g, hsa-miR-26a, hsa-let-7b, has-miR-15b, has-miR-27a, has-miR-200c, has-miR-191, has-miR-30c, and endogenous control 5S rRNA were purchased from Ambion (Austin, TX). Real-time qRT-PCR analysis was done on an ABI 7500HT instrument using *mir*Vana qRT-PCR miRNA Detection kit (Ambion) under the following conditions: 37°C, 30 minutes; 95°C, 10 minutes of reverse transcription; 95°C, 3 minutes; 95°C, 15 seconds; 60°C, 35 seconds. The reaction was done up to 40 cycles ($n = 3$). The gene expression ΔC_T values of miRNAs from each sample were calculated by normalizing with internal control 5S rRNA and relative quantitation values were plotted.

Decreasing p53 expression via siRNA knockdown. siRNA molecules were purchased from Dharmacon Research (Lafayette, CO), including p53, positive control (Lamin A/C), and mismatch control. Oligofect-AMINE-mediated transfection of siRNA was carried out in six-well tissue culture plate according to instructions of the manufacturer (Invitrogen). Transfection mixtures containing either 100 or 400 nmol/L siRNA and 8 μL OligofectAMINE in 200 μL Opti-MEM (Invitrogen) were added directly to preincubated cells in 800 μL Opti-MEM. Cells were then incubated for 4 hours and cultured further in McCoy's medium supplemented with 10% fetal bovine serum. Cells were harvested after 48 hours of transfection and total cellular proteins were isolated for Western immunoblot analysis.

Western immunoblot analysis. Western immunoblot analysis was used to characterize the expression of p53 protein after gene knockdown by siRNA and 5-FU treatment in HCT-116 (wt-p53) cells and HCT-116 (null-p53) cells. Equal amounts (15 μg) of protein extracts from each sample were resolved by SDS-PAGE on 12.5% gels by the method of Laemmli (24). Proteins were probed with mouse anti-p53 monoclonal antibody (1:1,000 dilution), α-tubulin (1:3,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a horseradish peroxidase–conjugated secondary antibody (1:1,000 dilution, Bio-Rad, Hercules, CA). Proteins were visualized with a chemiluminescence detection system using the Super Signal substrate (Pierce, Rockford, IL).

Identification of putative p53-binding site(s) at the miRNA promoters. To identify potential p53-binding sites related to human miRNAs, a set of putative miRNA promoters were extracted by defining 5 kb upstream region of each miRNA precursor. The miRNA genomic coordinates of 326 annotated miRNAs were identified from the miRBase (25). In contrast to protein coding gene, where 1 to 2 kb immediately upstream of the transcription start site are usually used as promoters, instead we chose a 5 kb region upstream of each miRNAs because it is well known that the nuclear transcripts of miRNAs are longer than the known pre-miRNA hairpin precursor that is documented in the databases, and therefore transcription start sites still remain undefined.

Results and Discussion

In this study, a comprehensive analysis was provided for gene expression regulated by wt-p53 at multilevels using human colon cancer cell lines HCT-116 (wt-p53) and HCT-116 (null-p53). This includes steady-state total mRNAs, actively translated mRNAs, and small noncoding miRNAs. The global regulatory network regulated by wt-p53 was revealed, which included transcription, posttranscription, and translation. HCT-116 (wt-p53) and HCT-116 (null-p53) cell lines provide a well-controlled

Table 1. Expression analysis of miRNAs using MIRCHIP2 array in HCT-116 (wt-p53) and HCT-116 (null-p53) cells

Up-regulated miRNA	Fold-change	Down-regulated miRNA	Fold change
Hsa-miR-30a-5p	+32.56	Hsa-miR-15b	−126.34
Hsa-miR-181b	+11.87	Hsa-miR-27a	−60.76
Hsa-miR-372	+6.57	Hsa-miR-200c	−58.30
Hsa-let-7g	+4.93	Hsa-miR-191	−56.39
Hsa-miR-26a	+2.27	Hsa-miR-30c	−50.12
Hsa-let-7b	+2.26	Hsa-miR-25	−49.24
Hsa-miR-296	+2.26	Hsa-miR-107	−48.55
Hsa-miR-30a-3p	+2.00	Hsa-miR-339	−46.52
Hsa-miR-21	+1.89	Hsa-miR-125a	−39.97
Hsa-miR-132	+1.58	Hsa-miR-27b	−32.84
Hsa-miR-181a	+1.34	Hsa-miR-23a	−28.88
Hsa-miR-320	+1.05	Hsa-miR-10a	−3.51

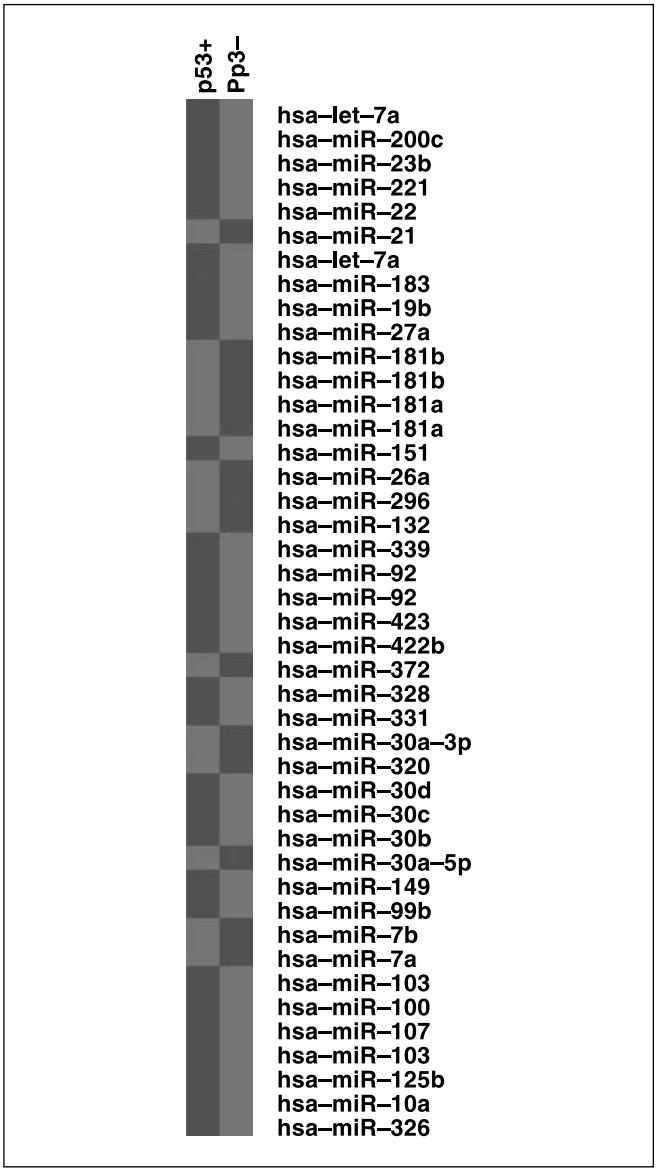

Fig. 1. Hierarchical clustering analysis of miRNA expression (*light gray,* overexpressed genes; *dark gray* underexpressed genes). miRNAs were isolated from actively translated RNA population in HCT-116 (wt-p53) and HCT-116 (null-p53) cells using gel fractionation. The level of miRNAs were quantified with miRNA array analysis. The expression of miRNAs were analyzed using Feature Extraction Software. The signal of each probe was set as its median intensity. The threshold for reliable probe signals was set at 1,500. Clustering analysis was done using CLUSTER 3.0/TreeView software (23).

and ideal *in vitro* model. The *wt-p53* gene in HCT-116 (wt-p53) cells was completely inactivated by targeted deletion using homologous recombination to create HCT-116 (null-p53) cells (15). Although the rate of cell proliferation seemed to be similar in HCT-116 (p53-null) cells compared with HCT-116 (wt-p53) cells in culture, knocking out wt-p53 has already provided HCT-116 (null-p53) cells with certain potential growth advantages under stress conditions. In this report, we discovered that the expression of a number of miRNAs and mRNAs have been affected by the deletion of wt-p53 in HCT-116 (null-p53) cells. We believe some of these altered miRNA and mRNA expression will provide surviving advantage to the HCT-116 (null-p53) cells after genotoxic stress.

Due to the function of miRNAs as translational regulators, we have reasoned that the active population of miRNAs must be localized in the polysomes. The miRNAs were isolated from actively translated RNA population using gel fractionation and the level of miRNA expression was quantitated with miRNA array analysis. We found that 11 miRNAs were up-regulated by wt-p53 and nearly 43 miRNAs were down-regulated by wt-p53 (Table 1). Hierarchical clustering analysis of miRNA expression is shown in Fig. 1. The large number of down-regulated miRNAs is intriguing because it has been predicted that some miRNAs might function as oncogenes due to their suppressive activity (26). We speculate that, as an RNA-binding protein, p53 might affect the recruitment of certain miRNA molecules to the actively translated mRNAs complex. RNA-binding protein tends to interact with a conserved stem-loop secondary structure rather than conserved sequence (27). This is consistent with the fact that most of the miRNAs contain conserved stem-loop structure. On the other hand, p53 acts as a transcription factor to up-regulate certain miRNAs and many downstream cellular mRNAs, including cyclin-dependent kinase inhibitor *p21* gene expression during genotoxic stress. Based on various miRNA target prediction algorithms, it is predicted that roughly 30% of all genes are regulated by miRNAs (28). The prediction points out the potential functional significance of wt-p53-mediated noncoding miRNAs. Several up-regulated miRNAs, such as hsa-miR-181b and hsa-miR-132, have been shown to alter the process of cell proliferation (29). Hsa-miR-21 was shown in a recent report to play a role in regulating apoptosis in human glioblastoma cells (13). The down-regulated miRNAs by antisense against hsa-miR-191 caused increased cell proliferation in HeLa cells, which contain a p53 deletion. In contrast, down-regulating hsa-miR-191 in A549 human lung cancer cells decreased cell proliferation (29). We analyzed the status of p53 in A549 cell lines and the results indicated that A549 cells contain *wt-p53* gene. It seems likely, therefore, that there might be a

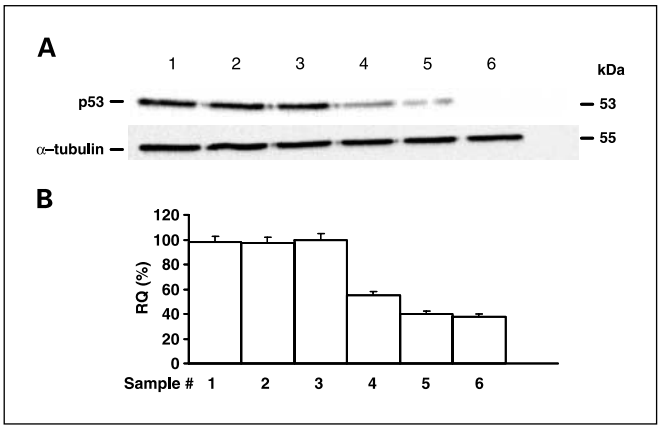

Fig. 2. *A,* effect of p53 siRNA knockdown on the expression of wt-p53 via Western immunoblot analysis. HCT-116 (wt-p53) cells were transfected with siRNA mismatch control and wt-p53-specific siRNA and the expression levels of wt-p53 were quantified via Western immunoblot analysis [*lane 1,* control; *lane 2,* mismatch control; *lane 3,* Lamin A/C positive control; *lane 4,* 100 nmol/L siRNA; *lane 5,* 400 nmol/L siRNA; *lane 6,* HCT-116 (null-p53)]. *B,* real-time qRT-PCR analysis of has-miR-26a expression [*n* = 3; *lane 1,* control; *lane 2,* mismatch control; *lane 3,* Lamin A/C positive control; *lane 4,* 100 nmol/L siRNA; *lane 5,* 400 nmol/L siRNA; *lane 6,* HCT-116 (null-p53)]. 5S rRNA was used as internal standard for expression normalization.

Table 2. Prediction of putative p53-binding sites of miRNA promoters

miRNA	Site position (upstream miRNA)	Gap	Sequence	Site score
Shorter gap sites				
Hsa-let-7b	828	0	AGCCATGTCT...CTTCTTGTCT	87.56
Hsa-mir-26a-1	3,108	2	CAGCAAGACT...GGGCAAGAGC	86.96
Hsa-mir-26a-2	3,024	0	GCCCTTGCCC...CTGCTTGTCT	86.30
Hsa-mir-372	3,661	3	CGCCATGTTG...AGGCTAGTCT	84.81
Hsa-let-7b	3,628	1	TCGCATGCCT...TGTCTTGCTG	83.73
Hsa-mir-181b-1	1,420	0	AGCCAAGCTT...TGGCATGACT	82.44
Hsa-mir-200c	2,183	2	AGACAAGGAG...GAGCAAGGGT	81.59
Hsa-mir-26a-2	174	3	CAGCATGTTG...AGTCAAGTTC	80.06
Longer gap sites				
Hsa-mir-200c	3,807	11	ATACAAGCCG...AGGCAAGTCC	89.80
Hsa-mir-181b-1	1,470	7	AAACATGTCC...CAACTTGCCT	89.00
Hsa-mir-181b-1	4,594	6	GAACTAGCCC...GGCCATGTTT	88.70
Hsa-mir-26a-2	4,840	13	AAGCAAGCAC...GAGCAAGACT	87.77
Hsa-let-7g	1,620	11	AGGCTTGCCT...CAGCAAGCGC	86.69
Hsa-mir-26a-2	4,066	12	CAGCTTGCTT...TGCCATGCCC	85.20
Hsa-mir-26a-2	4,070	8	TTGCTTGCCC...TGCCATGCCC	84.06
Hsa-mir-26a-1	4,496	6	ACGCAAGTCC...TCCCATGTCC	83.60
Hsa-mir-26a-1	4,496	12	ACGCAAGTCC...GTCCTTGCTT	83.29
Hsa-mir-26a-2	4,846	7	GCACAAGATC...GAGCAAGACT	83.12
Hsa-mir-27a	2,060	11	CCTCATGCCT...GAGCTTGGTT	81.88
Hsa-let-7b	2,624	10	GGGCATGGGG...TAGCATGCCG	80.81
Hsa-mir-26a-2	4,863	12	GAGCAAGACT...TGTCTAGTCT	80.66
Hsa-mir-26a-1	1,437	9	TGCCTTGTTC...GGGCATGCAG	80.52
Hsa-mir-26a-1	4,512	4	TCCCATGTCC...TTTCTTGCTT	80.33
Hsa-mir-181b-2	1,330	6	AAACATGAAT...TGACATGCTG	80.28

connection between the function of hsa-miR-191 and the status of p53. The results may help us to further explain the complex biology and function of miRNAs. It shows that in this case, at least the status of tumor suppressor gene function has to be taken into consideration, not just the expression levels of miRNAs.

To confirm that the expression changes of miRNAs were specific due to the status of the p53, the expression of wt-p53 was reduced in HCT-116 (wt-p53) cells by treatment with p53-specific siRNA. The level of the wt-p53 protein was decreased by 80% after treatment with both 100 and 400 nmol/L siRNA in HCT-116 (wt-p53) cells using Western immunoblot analysis (Fig. 2A). The decrease in p53 expression is sequence specific compared with the siRNA controls (*lanes 1* to *3*, and p53-specific siRNA treatment in *lanes 4* and *5* in Fig. 2A). The expression of selected miRNAs were compared in control HCT-116 (wt-p53) cells and cells treated with p53 siRNA. As an example, decreasing p53 expression via siRNA treatment significantly decreased hsa-miR-26a expression quantified via miRNA-specific qRT-PCR analysis (Fig. 2B). This result confirmed the miRNA array results that difference in hsa-miR-26a expression was directly related to the status of wt-p53 expression.

To investigate the potential function of p53 as a transcription factor of some of these miRNAs, the potential p53-binding sites of miRNA promoters were analyzed using bioinformatics approach. The p53-binding site is a dimer, comprising of two monomers, each is 10 nucleotides long, with a variable spacer that can range between 0 to 13 nucleotides (30). The consensus sequence of the monomer is RRRCWWGYYY (R = G or A, W = T or A, Y = C or T; ref. 31), but there are well-documented sites that deviate from this consensus, such as the p53-binding site in the MDM2 promoter (32). Our program uses the following scheme to scan a given sequence for p53-binding sites: the first step uses TFBS (33) to scan the given sequence for a match to the monomer Position-Specific Scoring Matrix, taken from TRANSFAC (Position-Specific Scoring Matrix accession no. M00761; ref. 34), above a given score S. Then, our program searches for dimer sites (i.e., for pairs of monomer sites with a gap of maximum length G). In addition, the program may screen out dimer sites that deviate from the core consensus of the p53 site. It can disqualify sites on any deviation from the consensus, or allow a single deviation, according to the user's choice.

First, we searched for p53 sites related to the 10 candidate miRNAs: hsa-miR-30a, hsa-miR-181b, hsa-let-7g, hsa-let-7b, hsa-miR26a, hsa-miR-15b, hsa-miR-27a, hsa-miR-200c, hsa-miR-25, and hsa-miR-372 (Table 1). In fact, two of the candidates, hsa-miR-30a and hsa-miR-181b, are each transcribed from two distinct genomic loci, and thus our list of candidate contained 12 promoters. We first used a cutoff score $S = 80$, allowed only short gaps of less than four nucleotides, and demanded that both monomers in each dimer will perfectly match the core consensus. Using these variables, we identified p53-binding sites in 6 of 12 promoters (hsa-miR-181b-1, hsa-let-7b, hsa-miR26a-1, hsa-miR26a-2, hsa-miR-200c, and hsa-miR-372), which correspond to 5 of 10

candidate miRNAs. We relaxed the initial variables and checked for sites in more candidate promoters. Hsa-miR-25 had a perfect consensus site in a score of 78, and another site that deviates from the consensus with a score of 84. In addition, hsa-miR-30a also has a consensus site with a deviation. When we relaxed the gap variable and searched for sites with gap up to 13 nucleotides, two more candidates, hsa-let-7g and hsa-miR-27a, were revealed to contain a perfect consensus site. The results are summarized in Table 2. Overall, we found putative p53 sites for 10 of the 12 candidate promoters, which correspond to 9 of 10 candidate miRNAs that we have checked.

The entire set of 326 miRNA putative promoters were screened for p53 potential binding sites using the first variable configuration (i.e., $S = 80$, $G = 3$) and perfect core-consensus match. The search resulted in 187 sites in the promoters of 130 unique miRNAs. To assess the significance of this result, we repeated the same search on 1,000 sets of 326 reshuffled miRNA promoters. Out of 1,000 reshuffled sets, a mean of 47 and maximum of 69 unique promoters contain at least one p53 site, as opposed to 130 in real promoters. This clearly indicates that relative to randomized versions of the miRNA promoters, the real promoters contain a very high number of p53-binding sites ($P < 0.001$). In summary, the search for p53 sites in the putative promoters of the set of candidate miRNAs resulted in 9 of 10 candidates (or 10 of 12 distinct promoters) containing at least one potential p53-binding site. When looking at the entire set of

Table 3. Expression analysis of steady-state total mRNA transcripts in HCT-116 (wt-p53) and HCT-116 (null-p53) cells

Genbank accession no.	Gene ID	Fold change	Biological function
Increased genes			
NM_000474	*TWIST*	+34.30	Cell differentiation; chromosome organization and biogenesis
NM_001225	*CASP4*	+20.22	Apoptosis; proteolysis and peptidolysis
NM_012427	*KLK5*	+11.13	Epidermis development; proteolysis and peptidolysis
NM_004172	*SLC1A3*	+11.07	L-Glutamate transport; dicarboxylic acid transport
NM_005930	*MGEA6*	+10.92	RNA processing
D16350	*SAH*	+10.07	Metabolism; regulation of blood pressure
NM_004864	*PLAB*	+9.75	Cell-cell signaling; signal transduction
NM_025048	*FLJ22684*	+9.46	Neuropeptide signaling pathway
AB029015	*PLCL2*	+9.11	Intracellular signaling cascade; lipid metabolism
NM_016135	*TEL2*	+9.00	Organogenesis; regulation of transcription
NM_006017	*PROML1*	+8.88	Visual perception
NM_002923	*RGS2*	+8.54	Cell cycle; G-protein signaling pathway; signal transduction
M23419	*EIF5A;TNNI3*	+7.09	Protein biosynthesis; translational initiation
NM_003633	*ENC1*	+6.99	Development; neurogenesis
AK023349	*Nup43*	+4.97	Intracellular protein transport
NM_005596	*NFIB*	+4.89	DNA replication; regulation of transcription, DNA dependent
NM_012198	*GCA*	+3.96	Membrane fusion
NM_001654	*ARAF1*	+3.92	Cell growth and/or maintenance; intracellular signaling cascade
NM_001387	*DPYSL3*	+3.88	Neurogenesis; signal transduction
NM_000043	*TNFRSF6*	+3.80	Apoptosis; immune response; protein assembly; signal transduction
NM_000389	*CDKN1A*	+3.55	Cell cycle; apoptosis; cell proliferation; regulation of CDK activity
BC007613	*CRMP1*	+3.14	Neurogenesis; nucleobase, nucleoside, nucleotide and nucleic acid metabolism
AK057343	*ZNF131*	+3.11	Regulation of transcription, DNA-dependent
NM_052966	*C1orf24*	+3.08	Protein folding
Decreased genes			
NM_014178	*HSPC156*	−21.83	Vesicle-mediated transport
NM_000582	*SPP1*	−19.53	T-helper 1 type immune response; antiapoptosis; cell-cell signaling
NM_001147	*ANGPT2*	−13.04	Angiogenesis; cell growth and/or maintenance; signal transduction
NM_003121	*SPIB*	−11.76	Regulation of transcription from Pol II promoter
BC010398	*PMPCB*	−7.41	Proteolysis and peptidolysis
NM_000310	*PPT1*	−5.26	Neurogenesis; protein modification; visual perception
NM_000465	*BARD1*	−4.59	Protein ubiquitination
NM_016611	*KCNK4*	−4.29	Ion transport; potassium ion transport
NM_001901	*CTGF*	−3.50	DNA metabolism; cell adhesion; cell growth; cell motility
NM_003925	*MBD4*	−3.34	Base-excision repair
NM_005627	*SGK*	−3.09	Apoptosis; phosphorylation; response to stress; sodium ion transport
NM_005834	*TIMM17B*	−3.06	Protein-mitochondrial targeting
NM_013961	*NRG1*	−3.06	Cell differentiation; embryonic development; neurogenesis

miRNAs in the database, we get clear indications that these results are not random and contain a high rate of true positives (estimates at ~64%, when looking at a 1,000 reshuffled sets of promoters as an indication to the rate of false positives). It is, therefore, possible that additional miRNAs may be regulated by p53.

The gene expression of steady-state total mRNA transcripts from both HCT-116 (wt-p53) and HCT-116 (null-p53) cells was

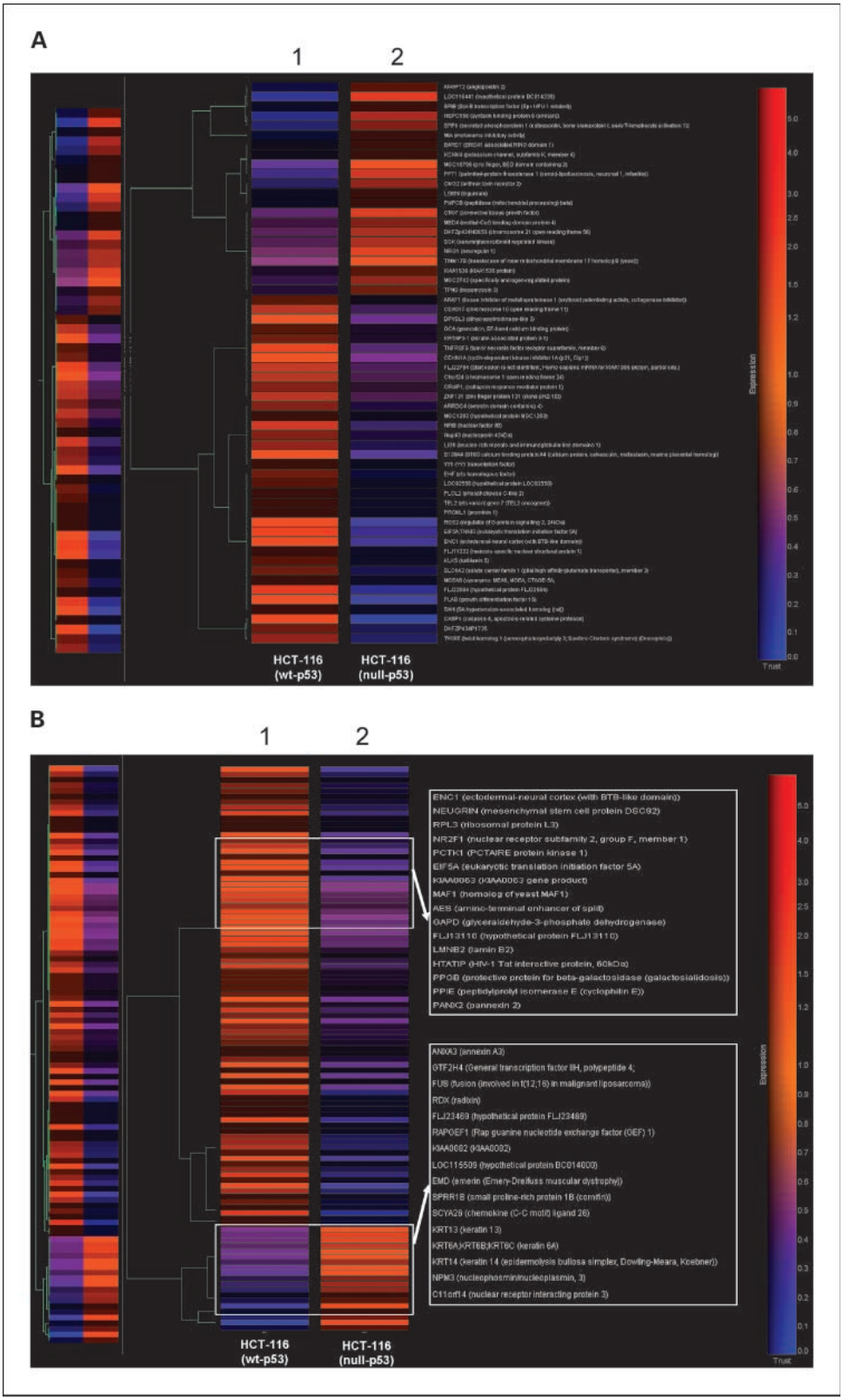

Fig. 3. *A,* hierarchical clustering analysis of steady-state total mRNAs expression profile between HCT-116 (wt-p53; *lane 1*) and HCT-116 (null-p53; *lane 2*) cells via microarray expression analysis (*red,* overexpressed genes; *blue,* underexpressed genes). *B,* hierarchical clustering analysis of actively translated mRNAs expression profile between HCT-116 (wt-p53; *lane 1*) and HCT-116 (null-p53; *lane 2*) cells (*red,* overexpressed genes; *blue,* underexpressed genes).

Table 4. Expression analysis of actively translated mRNA transcripts in HCT-116 (wt-p53) and HCT-116 (null-p53) cells

Genbank accession no.	Gene ID	Fold change	Biological function
Increased genes			
NM_016292	*TRAP1*	+19.01	Protein folding
NM_014474	*ASML3B*	+13.51	Carbohydrate metabolism
NM_001654	*ARAF1*	+12.79	Cell growth and/or maintenance; intracellular signaling cascade
NM_000178	*GSS*	+11.20	Amino acid metabolism; glutathione biosynthesis; neurogenesis
NM_001225	*CASP4*	+8.83	Apoptosis; proteolysis and peptidolysis
NM_001402	*EEF1A1*	+8.44	Protein biosynthesis; regulation of translation
NM_000918	*P4HB*	+7.86	Electron transport
NM_004335	*BST2*	+7.28	Cell proliferation; cell-cell signaling; development; immune response
NM_006743	*RBM3*	+6.85	RNA processing
NM_004046	*ATP5A1*	+6.83	ATP synthesis – coupled proton transport
NM_000546	*TP53*	+6.58	Apoptosis; DNA recombination; DNA repair; cell cycle; transcription
NM_004317	*ASNA1*	+6.54	Anion transport; response to arsenate
NM_004127	*GPS1*	+6.19	c-Jun-NH_2-kinase cascade; cell cycle; inactivation of mitogen-activated protein kinase
NM_000291	*PGK1*	+6.13	Glycolysis
NM_003752	*EIF3S8*	+6.12	Protein biosynthesis; regulation of translational initiation
NM_005500	*SAE1*	+5.85	Protein ubiquitination
NM_000107	*DDB2*	+5.70	Nucleotide-excision repair
AK024835	*CNN2*	+5.68	Cytoskeleton organization; smooth muscle contraction
NM_003915	*CPNE1*	+5.62	Lipid metabolism; vesicle-mediated transport
NM_006374	*STK25*	+5.53	Phosphorylation; response to oxidative stress; signal transduction
NM_017916	*FLJ20643*	+5.44	Metabolism
NM_006400	*DCTN2*	+5.35	Cell proliferation; microtubule-based process; mitosis
NM_000474	*TWIST*	+5.19	Cell differentiation; chromosome organization and biogenesis
NM_005030	*PLK*	+5.09	Mitosis; protein amino acid phosphorylation; regulation of cell cycle
BC033103	*INPP5E*	+5.04	Proteolysis and peptidolysis
NM_032272	*MAF1*	+4.91	Regulation of transcription, DNA-dependent
NM_001970	*EIF5A*	+4.85	Protein biosynthesis; translational initiation
NM_006201	*PCTK1*	+4.73	Protein amino acid phosphorylation; regulation of cell cycle
NM_005654	*NR2F1*	+4.66	Regulation of transcription, DNA-dependent; signal transduction
NM_003633	*ENC1*	+4.62	Development; neurogenesis
NM_016016	*CGI-69*	+4.60	Transport
NM_000967	*RPL3*	+4.58	Protein biosynthesis
NM_016645	*NEUGRIN*	+4.56	Neuron differentiation
NM_018658	*KCNJ16*	+4.38	Ion transport; potassium ion transport
NM_004864	*PLAB*	+4.33	Cell-cell signaling; signal transduction
NM_003624	*RANBP3*	+4.29	Small GTPase-mediated signal transduction
NM_004559	*NSEP1*	+4.28	Regulation of transcription; response to pest/pathogen/parasite

analyzed and genes with known functions are listed in Table 3 and hierarchical clustering analysis is shown in Fig. 3A. The list contains many genes involved in cell cycle control (TWIST, CASP4, and CDKN1A) and altogether 63 genes were affected by the deletion of wt-p53. It is interesting to note that the expression of SPIB, a regulator of transcription from Pol II promoter, is decreased by 11-fold in HCT-116 (wt-p53) cells. It has been reported that transcription of miRNAs are mediated by RNA polymerase II (35), which could help to explain another potential regulatory mechanism of miRNAs with decreased expressions listed in Table 1.

As an RNA-binding protein, p53 regulates gene expression at the posttranscriptional level. miRNAs also regulate gene expression at posttranscriptional level. The changes in the rate of mRNA translation will not be captured by just quantifying steady-state total mRNA levels. Therefore, it is critical to analyze gene expression using actively translated mRNA transcripts. The gene expression profiles of actively translated mRNA transcripts from both HCT-116 (wt-p53) and HCT-116 (null-p53) cells were analyzed, and genes with known functions are listed in Table 4. Hierarchical clustering analysis is shown in Fig. 3B. The results indicated that 107 genes were affected at the level of posttranscriptional control, many of which are related to RNA processing (RBM3) and protein synthesis (EEF1A1, EIF3S8, EIF5A, and EIF4A; Table 4). These changes may be mediated by p53 at the posttranscriptional level via direct or indirect manner and some of the genes that were indirectly regulated posttranscriptionally

Table 4. Expression analysis of actively translated mRNA transcripts in HCT-116 (wt-p53) and HCT-116 (null-p53) cells (Cont'd)

Genbank accession no.	Gene ID	Fold change	Biological function
NM_012427	*KLK5*	+4.14	Epidermis development; proteolysis and peptidolysis
NM_001085	*SERPINA3*	+4.12	Inflammatory response; regulation of lipid metabolism
NM_021734	*SLC25A19*	+4.12	Deoxynucleotide transport
NM_000175	*GPI*	+4.10	Carbohydrate metabolism; gluconeogenesis; glycolysis; hemostasis
NM_001908	*CTSB*	+4.10	Proteolysis and peptidolysis
NM_002923	*RGS2*	+4.01	Cell cycle; G-protein signaling pathway; signal transduction
NM_000687	*AHCY*	+3.95	One-carbon compound metabolism
NM_000190	*HMBS*	+3.94	Heme biosynthesis
NM_013388	*PREB*	+3.93	Regulation of transcription, DNA dependent
NM_005410	*SEPP1*	+3.92	Response to oxidative stress
NM_015679	*CLONE24922*	+3.87	RNA processing
NM_021008	*DEAF1*	+3.82	Regulation of transcription, DNA dependent
AK000822	*DKFZP564M182*	+3.75	Protein biosynthesis
NM_000182	*HADHA*	+3.74	Fatty acid metabolism
NM_002611	*PDK2*	+3.70	Glucose metabolism; signal transduction
NM_005802	*TP53BPL*	+3.68	Protein ubiquitination
AF016266	*TNFRSF10B*	+3.59	Caspase activation; electron transport; induction of apoptosis
NM_001536	*HRMT1L2*	+3.58	Signal transduction; defense response; methylation
NM_133455	*LOC129080*	+3.52	Phosphate transport
NM_001640	*APEH*	+3.51	Proteolysis and peptidolysis
NM_001416	*EIF4A1*	+3.50	Protein biosynthesis
NM_006388	*HTATIP*	+3.33	Regulation of transcription, DNA-dependent; transcription
NM_006112	*PPIE*	+3.29	Protein folding
NM_000308	*PPGB*	+3.28	Intracellular protein transport; proteolysis and peptidolysis
NM_002046	*GAPD*	+3.24	Glucose metabolism; glycolysis
NM_001130	*AES*	+3.24	Wnt receptor signaling pathway; development; transcription
Decreased genes			
NM_014178	*HSPC156*	−27.25	Vesicle-mediated transport
NM_006533	*MIA*	−15.34	Cell proliferation
NM_006993	*NPM3*	−7.58	Protein folding
NM_005554	*KRT6A*	−6.62	Ectoderm development
NM_002274	*KRT13*	−5.75	Epidermis development
NM_003125	*SPRR1B*	−4.81	Epidermis development
NM_000117	*EMD*	−4.76	Muscle contraction; muscle development
NM_006072	*SCYA26*	−4.69	Cell-cell signaling; immune response; signal transduction
BC014000	*LOC115509*	−4.18	Regulation of transcription, DNA dependent
NM_024710	*FLJ23469*	−3.88	Metabolism
NM_002906	*RDX*	−3.47	Cytoskeletal anchoring

may be mediated via miRNAs. We attempted to match the potential mRNA targets with several miRNAs using predictive software (miRNada; refs. 28, 36), and the results are shown in Fig. 4. These results were based on the improved prediction rules by reducing the number of G:U wobbles and increase the high match scale factor from 2 to 4 at position 2-8 from the 5′ end of miRNA (rather than position 1-11). Interestingly, the expression levels of translation initiation factor 4A and 5A were altered by the expression of p53. This change may be mediated by miRNAs such as hsa-miR-15b and hsa-miR-125a, respectively, based on target prediction analysis. It is possible that miRNAs are joining force with wt-p53 to help regulating gene expression at multiple levels.

To further confirm the functional significance of the p53 in regulating translation, both HCT-116 (wt-p53) and HCT-116 (null-p53) cells were treated with 10 μmol/L 5-FU for 24 hours. 5-FU is one of the main anticancer compounds used in treating colorectal cancer. The regulation of p53 was known to be controlled at the posttranscriptional level (37). To validate our approach, the expression level of p53 was analyzed by Western immunoblot and real-time qRT-PCR analysis. The wt-p53 protein level was induced after 5-FU treatment in HCT-116 (wt-p53) cells (Fig. 5A). However, the level of wt-p53 mRNA was not changed by 5-FU treatment (Fig. 5B). These results, taken together, suggest that the up-regulation of wt-p53 after 5-FU exposure is indeed due to posttranscriptional regulation. These data clearly point out the importance of

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3'_ACAUUUGGUACUA-C-ACGACGAU_5':hsa-miR-15b
___||.|.:|.:||.|.|.||:||||:___
5'_TGGATGCAGTGCTCGCTGTTGCTG_3': eIF-4A

3'_gugUCCAAUUUCCCAGAGUCCcu_5':hsa-miR-125a
___...|.||.|.||.|.|||||||..___
5'_gcgATGTCATAGCGGCTCAGGat_3': Heat Shock Protein 5A

3'_cUUUCUCU-GGCCAAGUGACACu_5':hsa-miR-128b
___.:||||||.||.|.||||.|||.___
5'_aGAAGAGATCCTGATCACGGTGc_3': eIF-5A

3'_gUGUUUAAGCC---UAGAUGUCCCAu_5':hsa-miR-10a
___.:|.|:.|.||...:|.||:|||||.___
5'_aGCCAGGTGGGCCTGTATATAGGGTc_3': Protein phosphatase 1,
                                  subunit 12C
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Fig. 4. miRNA target prediction for hsa-miR-15b, hsa-miR-125a, hsa-miR-128b, and hsa-miR-10a. Potential mRNA targets with several miRNAs using predictive software (miRNada; refs. 28, 36).

analyzing gene expression using actively translated mRNAs in addition to the steady-state total mRNAs. Several miRNAs was also up-regulated in response to wt-p53 induction after 5-FU treatment based on real-time qRT-PCR analysis. The expression analysis of hsa-miR-26a in response to 5-FU treatment is shown in Fig. 5C. The expression level of hsa-miR-26a was increased by nearly 2-fold in HCT-116 (wt-p53) cells in response to the increasing expression of wt-p53 after 5-FU exposure. In contrast, there was only a slight increase in HCT-116 (null-p53) cells after 5-FU treatment. These results further support the functional significance of wt-p53 on miRNA expression.

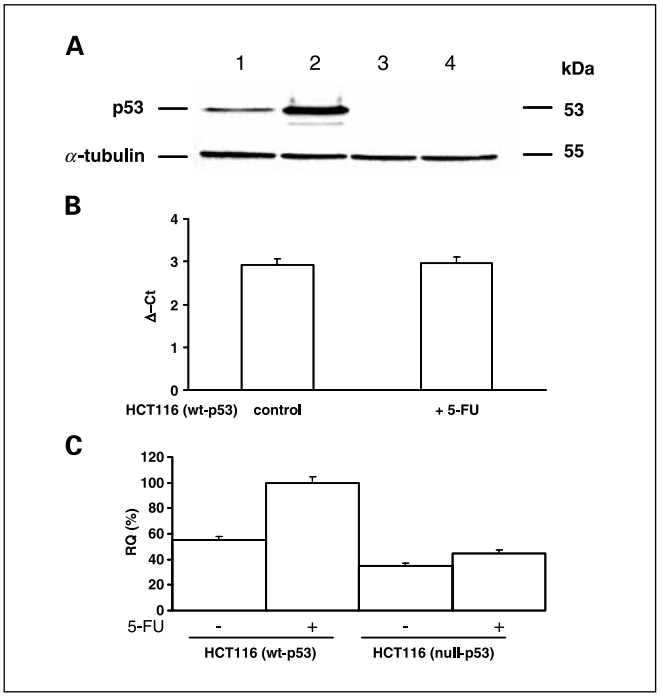

Fig. 5. *A,* effect of 5-FU treatment on the expression of p53 via Western immunoblot analysis [*lane 1,* control HCT-116 (wt-p53); *lane 2,* HCT-116 (wt-p53) cells treated with 10 μmol/L 5-FU for 24 hours; *lane 3,* control HCT-116 (null-p53); *lane 4,* HCT-116 (null-p53) cells treated with 10 μmol/L 5-FU for 24 hours]. *B,* expression of wt-p53 mRNA from HCT-116 (wt-p53) control cells and cells treated with 10 μmol/L 5-FU for 24 hours analyzed by real-time qRT-PCR analysis ($n = 3$). The expression of housekeeping gene *GAPDH* was used as internal control. *C,* effect of 5-FU treatment on hsa-miR-26a expression analyzed by real-time miRNA qRT-PCR ($n = 3$) and 5S rRNA was used as internal standard. The ratio of target miRNA and 5S rRNA was used to calculate the relative expression.

Based on our results, we provide a flow diagram to illustrate the new aspects of the regulatory function of wt-p53 (Fig. 6) to better understand the complexity of the regulatory network mediated by wt-p53. Wt-p53 not only regulates posttranscriptional and translational events via its RNA-binding function, but also acts as a typical transcription factor to regulate a number of cellular mRNAs at the transcriptional level. The new aspect of this regulatory network is that wt-p53 also regulates a number of noncoding miRNAs at the transcriptional level. Therefore, it is very likely that wt-p53 also regulates certain cellular mRNA translation through its mediated miRNAs. Wt-p53 also enhances apoptosis via directly accumulating to mitochondria (4, 5).

In conclusion, we describe here a comprehensive gene expression analysis to provide evidence that wt-p53 regulates gene expression at multiple levels due to its diverse functions. Wt-p53 not only regulates gene expression as a transcription factor to induce mRNA expression, but also influences miRNA expression by direct or indirect manner in this colon cancer cell line model. Wt-p53 also affects gene expression at posttranscriptional levels either through miRNAs or its RNA-binding capability. Some of the miRNAs have been shown to play roles in cell proliferation and apoptosis (29). We have also identified a number of wt-p53-regulated mRNAs at both transcriptional and posttranscriptional levels and some of these genes are candidate targets for miRNAs. The understanding of the complicated molecular networks regulated by wt-p53 is crucial in further elucidation of gene regulation.

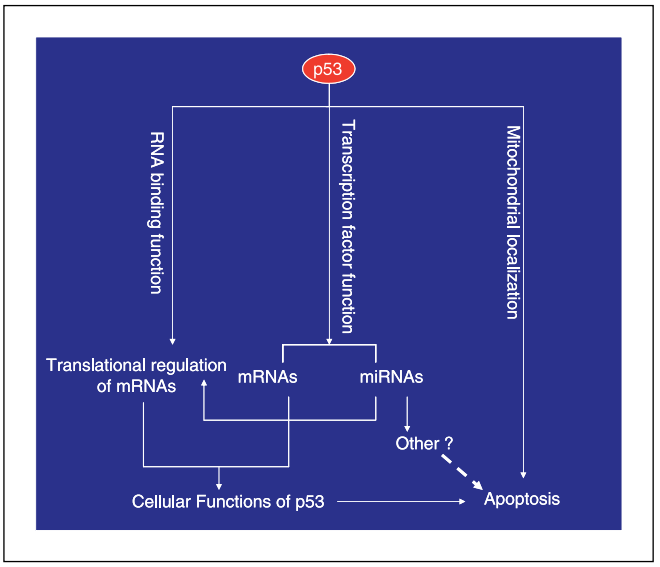

Fig. 6. Schematic illustration of p53 regulatory pathways. Wt-p53 not only acts as a typical transcription factor to regulate a number of cellular mRNAs at the transcriptional level, but also regulates gene expression at posttranscriptional and translational events via its RNA-binding function. In addition, wt-p53 regulates a number of noncoding miRNAs at the transcriptional level thereby influences certain cellular mRNAs translation through its mediated miRNAs. Wt-p53 also influences apoptosis pathway via accumulation to the mitochondria.

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