miR-192 Regulates Dihydrofolate Reductase and Cellular Proliferation Through the p53-miRNA Circuit

Bo Song[#,1], **Yuan Wang**[†,#,1], **Kenji Kudo**[*], **Elaine J. Gavin**[*], **Yaguang Xi**[*], and **Jingfang Ju**[#,†,‡]

[#]*Translational Research Laboratory, Department of Pathology, Stony Brook University Medical Center, Stony Brook, 11794*

[†]*School of Medicine, Wuhan University, P. R. China*

[*]*Cancer Genomics Laboratory, Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36688*

Abstract

Purpose—The purpose of this study is to investigate the molecular mechanism of *miR-192* in colon cancer.

Experimental Design—Human colon cancer cell lines with different p53 status were used as our model system to study the impact of *miR-192* on cell proliferation, cell cycle control and mechanism of regulation.

Results—Our results show that one of the key *miR-192* target genes is dihydrofolate reductase (DHFR). *miR-192* impacts cellular proliferation through the p53-miRNA circuit. Western immunoblot analyses indicated that the expression of DHFR was significantly decreased by *miR-192*. Further investigation revealed that such suppression was due to translational arrest rather than mRNA degradation. More profound inhibition of cellular proliferation was observed by ectopic expression of *miR-192* in colon cancer cell lines containing wild type p53 than cells containing mutant p53. Thus, the effect of *miR-192* on cellular proliferation is mainly p53 dependent. Over-expression of *miR-192* triggered both G1 and G2 arrest in HCT-116 (wt-p53) cells but not in HCT-116 (null-p53) cells. The cell cycle check point control genes p53 and p21 were highly over-expressed in cells that over-expressed *miR-192*. Endogenous *miR-192* expression was increased in HCT-116 (wt-p53) and RKO (wt-p53) cells treated with methotrexate, which caused an induction of p53 expression. Chromatin immunoprecipitation (ChIP)-qPCR analysis revealed that the p53 protein interacted with the *miR-192* promoter sequence.

Conclusion—These results indicate that *miR-192* may be another miRNA candidate that is involved in the p53 tumor suppressor network with significant impact on cell cycle control and cell proliferation.

[‡]Corresponding Author: Jingfang Ju, Ph.D., Associate Professor, Co-Director of Translational Research Laboratory, BST L9, Room 185, Department of Pathology, Stony Brook University Medical Center, Stony Brook, NY 11794-8691, Office phone: (631)-444-3598, Office fax: (631)-444-3424, E-mail: jiju@notes.cc.sunysb.edu
[1](Bo Song and Yuan Wang contribute equally to this work)

Statement of Translational Relevance.

We provide experimental evidence in this study that *miR-192* is another candidate microRNA that is directly involved in the regulation of a key anticancer target Dihydrofolate Reductase (DHFR). Over expression of *miR-192* suppresses the cellular proliferation and restored cell cycle control. The expression and function of the *miR-192* is largely dependent on the presence of functional wild type p53. This raises the potential of using *miR-192* as a novel therapeutic option for treating cancer via an effective delivery system either alone or in combination with anti-folate compounds. *miR-192* may also serve as a potential biomarker for clinical prognosis.

Keywords

miR-192; dihydrofolate reductase; p53; cell proliferation

INTRODUCTION

miRNAs are non-coding, single-stranded RNAs of ~22 nucleotides, processed from larger pre-miRNAs by the RNase III enzyme, Dicer, into miRNA duplex complexes (1). One strand of this duplex can associate with the RNA-induced silencing complex (RISC), with the other strand generally degraded by cellular nucleases (1). The miRNA-RISC complex has been shown to bind to specific mRNA targets resulting in translational repression or cleavage of these mRNA sequences. miRNAs modulate protein expression by promoting RNA degradation, inhibiting mRNA translation, and also affecting transcription. Currently, there are over 450 human miRNAs that have been identified and the final number will likely be much higher (2). Although miRNA-mediated mRNA degradation occurs in mammals, in most cases, the impact of miRNA on its targets are thought to use a secondary mechanism of gene regulation via imperfect base-pairing to the 3′-untranslated regions (*3′-UTRs*) of the mRNA targets. This results in the repression of target gene expression post-transcriptionally, most likely at the translational level of gene expression (3-5). Such translational regulation provides the cell with a more precise, immediate and energy-efficient way of controlling expression of a given protein (6). Such regulation can induce rapid changes in protein synthesis without the need for transcriptional activation and subsequent steps in mRNA processing. Additionally, the translational control of gene expression has the advantage of being readily reversible, providing the cell with great flexibility in responding to various cytotoxic stresses (7). Clearly, it is essential to know not only the expression levels of individual mRNAs, but also to what extent mRNAs are being translated into their corresponding proteins.

Post-transcriptional control mediated by miRNAs has become an area of intense research over the last few years. The notion of miRNAs mediating gene expression at the translational level is based on the study of the first two identified miRNAs, *lin-4* and *let-7*, in Caenorhabditis elegans (8). The *lin-4* miRNA 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′-UTR* of the target mRNAs (3,8). Although the exact function of many newly discovered miRNAs are just emerging, their ability to regulate cell proliferation and cell death has been recently demonstrated (9). Recent reports have shown that the expression of miRNAs is altered in cancer cells, and that some miRNAs can function as translational attenuators (5).

The first indication that miRNAs may function as tumor suppressors was derived from a study by Calin *et al.*, who found that *miR-15a* and *miR-16-1* were commonly deleted in >65% of patients with B-cell chronic lymphocytic leukemia (CLL) (10). Further, Cimmino *et al.* showed that *miR-15a* and *miR-16-1* negatively regulated *Bcl2*, an anti-apoptotic protein that is often over-expressed in many tumor types (11). Several expression profiling studies have also reported de-regulated miRNAs in colon cancer, breast cancer and other types of solid tumors (2,12-14).

miRNAs are shown to be critical in oncogenesis, and alterations in miRNA expression are found to be associated with several human cancers (9,10,14-16). miRNAs can act as either oncogenes or tumor suppressor genes based on their mRNA targets (14). We have previously identified that the expression of a number of miRNAs was altered due to the loss of p53 tumor suppressor gene (12). Subsequently, a number of groups have reported that *miR-34* was directly involved in the p53 tumor suppressor network and regulated directly by p53 (17-19). Based on these results, we reasoned that there should be additional miRNAs that are involved in the

p53 tumor suppressor network. By performing an *in silico* analysis coupled with experimental validations to search for these potential miRNAs, we report here the discovery that the *miR-192* promoter contains a conserved p53 binding site and that one of the key *miR-192* target genes is dihydrofolate reductase (DHFR), an important target for antifolate based anti-cancer chemotherapy such as methotrexate (MTX) (20). We also experimentally confirmed that the downstream pathway of *miR-192* in cell cycle control is mediated through increased p53 expression by the induction of p21. Many studies have indicated that expression of DHFR is regulated at least in part at the translational level (21-26). Recently, Mishra et al. reported that a *miR-24* binding-site polymorphism in DHFR gene led to MTX resistance (27). Translational control provides cells with acute response to growth condition changes and it is readily reversible (28). We provide evidence that *miR-192* is another player in the p53-miRNA circuit and contributes to the regulation of cell cycle control and cellular proliferation.

Materials and Methods

Cell Culture and Reagents

The human colon cancer cell lines HCT-116 (wt-p53) and HCT-116 (null-p53) were gifts from Professor Bert Vogelstein (The Johns Hopkins University), and were maintained in McCoy's 5A medium (Gibco Laboratories). The other two human colon cancer cell lines, RKO (wt-p53) and HT-29 (mut-p53) were obtained from the American Type Culture Collection. The HT-29 (mut-p53) cell line has a missense mutation in codon 273 of p53 resulting in an Arg to His substitution. RKO (wt-p53) and HT-29 (mut-p53) cells were maintained in Eagle's Minimum Essential Medium and Iscove's Modified Dulbecco's Medium at ATCC respectively. All the media were supplemented with 10% dialyzed fetal bovine serum (HyClone Laboratories, Inc.). All cell lines were grown at 37°C in a humidified incubator with 5% CO_2. MTX was purchased from Sigma-Aldrich.

Transfections of miRNA and siRNA Specific to Dihydrofolate Reductase

HCT-116 (wt-p53), HCT-116 (null-p53), RKO (wt-p53), and HT-29 cells (2×10^5) were plated in six-well plates, and transfected with 100 nM of either *miR-192, miR-24* precursors or non-specific control *miR* (Ambion) after 24 h with Oligofectamine (Invitrogen) according to the manufacturer's instructions. *siRNA* specific to DHFR (ON-TARGET plus SMARTpool L-008799-00-0010, human DHFR, NM_000791) was purchased from Dharmacon and transfected with Oligofectamine (Invitrogen) according to the manufacturer's protocols at a final concentration of 100 nM. *siRNA* specific to DHFR was used as the positive control. *miR-24*, a recently reported miRNA that also targets DHFR (27), was also used as a positive control.

RNA Isolation

Total RNA, including miRNA, was isolated from cell lines by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions at 24 h after transfection.

Real Time qRT-PCR Analysis of miRNA

cDNA synthesis was carried out with the High Capacity cDNA synthesis kit (Applied Biosystems) using 10 ng of total RNA as template. The miRNA sequence-specific RT-PCR primers for *miR-192, miR-24* and endogenous control RNU6B were purchased from Ambion. Real-time quantitative reverse transcription-PCR (qRT-PCR) analysis was carried out using Applied Biosystems 7500 Real-Time PCR System. The PCR master mix containing TaqMan 2× Universal PCR Master Mix (No Amperase UNG), 10× TaqMan assay and RT products in 20 μl volume were processed as follows: 95°C for 10 min, and then 95°C for 15 sec, 60°C for 60 sec for up to 40 cycles (n=3). Signal was collected at the endpoint of every cycle. The gene

expression ΔC_T values of miRNAs from each sample were calculated by normalizing with internal control RNU6B and relative quantitation values were plotted.

Real Time qRT-PCR Analysis of mRNA Expression

cDNA was synthesized with the High Capacity cDNA synthesis kit (Applied Biosystems) using 2μg of total RNA as the template and random primers. Real-time qRT-PCR analysis was done on the experimental mRNAs. The PCR primers and probes for DHFR, and the internal control gene GAPDH were purchased from Applied Biosystems. qRT-PCR was performed on an ABI 7500HT instrument under the following conditions: 50°C, 2 min of reverse transcription; 95°C, 10 min; 95°C, 15 s; 60°C, 1 min for up to 40 cycles (n =3).

Western Immunoblot Analysis

At 48 h after transfection with *miR-192, miR-24* precursors or non-specific control miRNA, the cells were scraped and lysed in RIPA buffer (Sigma). Equal amount of proteins were resolved by SDS-PAGE on 12% gels by the method of Laemmli (29), and transferred to polyvinylidene fluoride membranes (BIO-RAD Laboratories). The membranes were then blocked by 5% nonfat milk in TBS-T (Tris-buffered saline and 0.5% Tween-20) at room temperature for 1 h. The primary antibodies used for the analysis included mouse anti-DHFR mAb (1:250, BD Bioscience), mouse anti-p53 mAb (1:1000, DO-1), mouse anti-p21mAb (1:1000, F-5), and mouse anti-α-tubulin mAb (1:1000, TU-02) purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated antibodies against mouse or rabbit (1:1000, Santa Cruz Biotechnology) were used as the secondary antibodies. Protein bands were visualized with a chemiluminescence detection system using the Super Signal substrate.

MTX Cytotoxicity

HCT-116 (wt-p53) cells were plated in 96-well plates at 1×10^3 cells/well in triplicate. They were transfected with *miR-192* precursor, non-specific control miRNA, or *siRNA* against DHFR in 100 μl of medium. Twenty-four hours later, MTX in 100 μl medium (final concentration 25 nM) was added, and incubated for 72 h. 10 μl of WST-1 (Roche Applied Science) was added to each well. After 2 h incubation, absorbance was measured at 450 and 630 nm respectively (n=3). Non-specific control miRNA alone was used as a negative control, and *siRNA* incubation with MTX was used as a positive control.

Cell Proliferation Analysis

HCT-116 (wt-p53), HCT-116 (null-p53), RKO (wt-p53), and HT-29 cells were plated in 96-well plates in triplicate at 1×10^3 cells/well after transfection with *miR-192* precursor or non-specific control miRNA. Cells were cultured for 24, 48, 72, 96 h. The absorbance at 450 and 630 nm was measured after incubation with 10 μl of WST-1 for 2 h.

Cell Cycle Analysis

HCT-116 (wt-p53) and HCT-116 (null-p53) cells were transfected with *miR-192* precursor and the non-specific control miRNA described as above. At 36 h after transfection, cells were harvested and resuspended at $0.5\text{-}1\times10^5$ cells/ml in modified Krishan buffer (17,18) containing 0.1% sodium citrate and 0.3% NP-40 and kept at 4°C. Before being analyzed by flow cytometry, cells were treated with 0.02mg/ml RNase H and stained with 0.05mg/ml propidium iodide (Sigma).

MTX Treatment

HCT-116 (wt-p53), HCT-116 (null-p53), RKO (wt-p53), and HT-29 cells were seeded in six-well plates at a density of 2×10^5 cells per well and then incubated with or without MTX (25

nM). The cells were collected at 24 h after incubation, and total RNA and protein were extracted respectively. The subsequent real-time qRT-PCR of miRNA and western immunoblot analysis for p53 and α-tubulin were performed as described above.

Chromatin Immunoprecipitation (ChIP) and qPCR Analysis

To show that p53 protein directly interacts with the *miR-192* promoter region, we performed ChIP-qPCR analysis using p21, a known cell cycle regulator transcriptionally regulated by p53, as a positive control. Mouse monoclonal antibody (DO-1) against p53 (Santa Cruz Biotechnology) was used for immunoprecipitation of the p53 binding complex. Non-related antibody α-tubulin (TU-02, Santa Cruz Biotechnology) was used as a negative control. Immunoprecipitation was performed based on the manufacturer's protocols of Active Motif. The primer sequences for the *miR-192* promoter and the p21 promoter are listed as follows:

miR-192 promoter (forward primer): 5′-AGCACCTCCCATGTCACC-3′

miR-192 promoter (reverse primer): 5′-CAAGGCAGAGCCAGAGC-3′

p21 promoter (forward primer): 5′-GCTGGTGGCTATTTTGTCCTTGGGC-3′

p21 promoter (reverse primer): 5′-CAGAATCTGACTCCCAGCACACACTC-3′

Conserved P53 Binding Mir-192 Promoter Reporter Activity Assay

Luciferase reporter assay was used to determine the transcriptional activation of conserved p53 binding promoter of miR-192. pGL3-Basic promoterless luciferase reporter plasmid (Promega) was used in this study. Double stranded DNA oligonucleotides of conserved p53 binding sequence of miR-192 was synthesized and annealed and cloned upstream of firefly luciferase in the pGL3-Basic plasmid (miR-192-pGL3). The p53 binding site oligonucleotide (underline) contains *MluI* at 5′-end and *BglII* sequence (*italic*) at the 3′-end (5′-A*CGCGT*CCATGTCACCACCAGGGGTCGCCATGCCTCCTGGCCTTGCCCAGC*AGATCT*-3′). Control vector and miR-192-pGL3 vector were transfected into both HCT-116 (wt-p53) cells and HCT116 (null-p53) cells. To further induce p53 expression, tranfected HCT-116 (wt-p53) cells and HCT116 (null-p53) cells were also treated with 5 μM 5-FU for 24h. The promoter activity of each construct was quantified by dual luciferase assay (Promega) 24 h after transfection. Firefly luciferase activity was normalized with *Renilla* luciferase internal control under each condition.

Statistical analysis

All experiments were repeated at least twice. Statistical significance was evaluated by Student's t test (two tailed) comparison between two groups of data. Asterisks indicate significant differences of experimental groups compared with the corresponding control condition. Statistical analysis was done using GraphPad Prism software (GraphPad, Inc.). Differences were considered statistically significant at *P < 0.05*.

Results and Discussion

miRNA regulates the mRNA translation rate by perfect or imperfect base pairing with the 3′-*UTR* regions of their targets (1). It has been predicted that one miRNA can potentially regulate translation of up to hundreds of mRNAs (1). This has created a challenge for experimentally validating miRNA specific targets. Previous studies from our laboratory have discovered that nearly half of the miRNAs promoter regions contain putative p53 binding site(s) (12). To further validate the significance of some of these candidate miRNAs, we took a systematic approach by first analyzing which miRNA may target critical anticancer target genes. We also select the miRNA candidates with high predicted ranking scores of p53 binding promoter. This allows us to focus on *miR-192* with DHFR as one of its predictive target, an important

anticancer target. DHFR is the key enzyme responsible for intracellular folate metabolism and a target of MTX (30). The regulation of DHFR is complex and includes post-transcriptional control by an auto feedback mechanism (31).

Translational regulation of DHFR expression by *miR-192*

We investigated the roles of miRNAs in regulating the expression of DHFR. Based on the structural analysis of *3'-UTR* of the DHFR gene and miRNA target analysis (TargetScan, PicTar, miRnaDa), we identified several miRNAs which potentially interact with the *3'-UTR* region of DHFR mRNA. Bioinformatic analysis of the secondary structure of the *3'-UTR* of the DHFR mRNA and miRNA binding sites led us to reduce the candidate miRNAs to a small number. This allows us to efficiently identify miRNAs that involved in regulating key targets like DHFR.

Fig. 1A shows the target sequence of *3'-UTR* of the DHFR mRNA that interacts with *miR-192*. To experimentally confirm that the expression of DHFR was regulated by *miR-192*, a *miR-192* precursor was transfected into HCT-116 (wt-p53) cells. A non-specific *miR* was used as a negative control. It has been reported that the expression of DHFR is regulated by *miR-24* (27). We thus used both a DHFR *siRNA* and *miR-24* as positive controls. Over-expression of *miR-192* and *miR-24* was confirmed by real time qRT-PCR analysis using U6 RNA to normalize the expression (supplemental data file 1). The expression of DHFR protein was analyzed using Western immunoblot analysis and the results are shown in Fig. 1B. Over-expression of *miR-192* clearly decreased the expression of DHFR protein (Fig. 1B, lane 4). The potency of *miR-192* for decreasing DHFR expression was comparable to *miR-24* (Fig. 1B, lane 5). We also analyzed the expression level of DHFR mRNA using real time qRT-PCR analysis and the results (Fig. 1C) indicated that there was no reduction in DHFR mRNA expression by *miR-192* (lane 4) and *miR-24* (lane 5). Thus, the suppression of DHFR expression was regulated at the translational level without the degradation of DHFR mRNA. By contrast, the decreased expression of DHFR by *siRNA* was clearly caused by mRNA degradation (lane 3, Fig. 1C).

miR-192 sensitizes HCT-116 (wt-p53) cells to MTX

Because DHFR is a target for MTX, the increased expression of *miR-192* may also contribute to the sensitivity to MTX treatment. With equal molar concentration of MTX at 25 nM (IC-10), cell proliferation was reduced by 10% in non specific control *miR* treated cells (Fig. 2, lane 2). However, cell proliferation was reduced by nearly 70% in cells transfected with *miR-192*, demonstrating a synergistic effect in combination with MTX (Fig. 2, lane 4). By contrast, cells treated with siRNA against DHFR were inhibited by 55% (Fig. 2, lane 3). The more potent effect of *miR-192* plus MTX compared to siRNA targeting specifically to DHFR suggests that *miR-192* may also target additional mRNA targets through imperfect base pairing. Future studies are clearly needed to systematically identify additional *miR-192* regulated mRNA transcripts.

Effect of overexpression of *miR-192* on cellular proliferation

To assess the functional significance of *miR-192*, we evaluated the impact of *miR-192* on cellular proliferation using HCT-116 (wt-p53), HCT-116 (null-p53), RKO (wt-p53) and HT-29 (mut-p53) colon cancer cell lines. A non-specific *miR* was used as a negative control. Our results show that the overexpression of *miR-192* can suppress cellular proliferation in HCT-116 (wt-p53) cells by over 55% (n=3) (Fig. 3A) and RKO (wt-p53) cells by 48% (n=3) (Fig. 3B), with less impact on HCT-116 (null-p53) (15%, n=3) (Fig. 3C) and HT-29 cell lines (24%, n=3) (Fig. 3D). By contrast, the non-specific control *miR* has no effect on cellular proliferation, indicating that this effect caused by *miR-192* is highly specific. These results clearly show that the effect of *miR-192* on the inhibition of cellular proliferation is more potent in colon cancer

cell lines containing wild type p53 than in the p53-null or mutant p53 cell lines, further indicating that *miR-192*'s function depends on the status of p53.

Impact of cell cycle control by *miR-192*

To determine whether the *miR-192's* impact on cellular proliferation was related to cell cycle control, we analyzed the effect of *miR-192* on cell cycle control by flow cytometry using HCT-116 (wt-p53) and HCT-116 (null-p53) cells transfected with non-specific control *miR* or *miR-192*. Our results show that *miR-192* increases G1/S ratio (>2-fold) and G2/S ratio (>3-fold) in HCT-116 (wt-p53) cells (Fig. 4A). By contrast, this effect has not been observed in HCT-116 (null-p53) cells (Fig. 4B). Thus, the cell cycle analysis data is highly consistent with the cell proliferation results that the function of *miR-192* is dependent on the presence of wild type p53 for cell cycle control.

Effect of *miR-192* on cell cycle control genes

To further analyze the cell cycle control genes involved in *miR-192* overexpression, we analyzed a number of cell cycle control genes (p53, p21, Bax). Fig. 5 shows the results of Western immunoblot analysis in HCT-116 (wt-p53) cells (Fig. 5A) and RKO (wt-p53) cells (Fig. 5B). Ectopic expression of *miR-192* increased the expression of the p53 protein (Fig. 5A, lane 4) over 10-fold and p21 10-fold. By contrast, *siRNA* against DHFR (Fig. 5A, lane 3) did not cause an increase in expression of p53 and p21. The expression of Bax was not altered by *miR-192,* indicating that *miR-192* may not trigger apoptosis. Similar results were obtained for RKO (wt-p53) cells (Fig. 5B, lane 3, *miR-192,* lane 1, non-specific *miR*; lane 2, *siRNA* of DHFR). It has been well characterized that the induction of the p53 dependent cell cycle check point control gene p21 is the key to trigger cell cycle arrest at both the G1 and G2 phase (32, 33). Thus, our results further confirm the notion that the function of *miR-192* is clearly dependent on the status of wild type p53.

miR-192 expression was dependent on p53

With ectopic over-expression of *miR-192* by transfection, both HCT-116 (wt-p53) and RKO (wt-p53) cells undergo cell cycle arrest at the G1 and G2 phase leading to decreased cellular proliferation. Bioinformatic analysis also reveals that there is a putative p53 binding site in the *miR-192* promoter region. To confirm the direct regulatory relationship with p53, we performed the following experiments. First, the expression of the p53 protein was induced by treatment with MTX. The induction of p53 protein expression by MTX treatment in HCT-116 (wt-p53) and RKO (wt-p53) cells (Fig 6A) caused a significant increase of *miR-192* expression (Fig 6B). By contrast, MTX treatment in HCT-116 (null-p53) and HT-29 (mut-p53) cells did not cause any change in the expression of *miR-192* (Fig 6B). Furthermore, the expression level of miR-192 in HCT116 (wt-p53) cells was nearly 4-fold higher than HCT-116 (null-p53) cells (supplemental data file 4). These results suggest that the endogenous expression of *miR-192* depends on the wild type p53 after genotoxic stress by MTX treatment. These findings combined with the data in Fig. 5, which shows that p53 is induced by ectopic expression of *miR-192,* indicate that there might be a positive feedback loop between p53 and *miR-192* to ensure proper cell cycle control. miR-192 may down regulate some key cell cycle related genes to trigger p53 induction. Further studies are currently underway to define the detailed mechanism of this positive feedback loop between miR-192 and p53.

We have predicted in our previous study that the promoter site of *miR-192* contains a well conserved p53 binding sequence (12). The binding sequence is 5′-CGCCATGCCT...GGCCTTGCCC-3′ with a 3-base gap with a ranking site score of 90 based on TFBS (34). Our results are subsequently confirmed by a recent report using a similar type of analysis which shows that the promoter of *miR-192* contains a p53 binding site (35). To experimentally confirm a direct interaction between the p53 protein and the *miR-192* promoter,

we utilized ChIP-qPCR analysis to isolate p53 bound chromosome DNA. The isolated p53 specific binding DNA was PCR amplified using primers which span the predicted p53 binding sites of the *miR-192* promoter or the positive control p21 promoter transcriptionally regulated by p53 protein. We show that the p53 protein directly interacts with the *miR-192* promoter based on ChIP-qPCR analysis with a 4-fold enriched signal with p53 specific monoclonal antibody compared to the non-specific antibody control DNA (supplemental data file 2A-B). These results validate our bioinformatic prediction of the existence of a conserved p53 binding site at the promoter region of *miR-192* (12). We further demonstrated directly that the conserved p53 binding site at the promoter region of miR-192 can activate luciferase expression only in HCT116 (wt-p53) cells. The activation was further enhanced by induced p53 expression in HCT (wt-p53) cells treated with 5-FU. By contrast, the induction of luciferase activity was completely absent from the HCT116 (null-p53) cells (supplemental data file 3). This suggests that *miR-192*, like *miR-34*, is another miRNA that is involved in the p53 tumor suppressor network. It is well established that p53 is one of the most frequent altered tumor suppressor gene in colorectal cancer (36). We hypothesize that the potential function of multiple miRNAs involved in p53 tumor suppressor network is to provide the p53 with greater flexibility in rapidly responding to different growth condition changes, perhaps by having unique miRNAs (e.g. *miR-34, miR-192*) mediate the regulation of the key mRNA targets. We also discovered recently that the expression of *miR-192* was decreased in a panel of colorectal tumor specimens compared to the adjacent normal tissues (data not show). This is consistent with a recent report by Schetter *et al.* showed that *miR-192* was one of the miRNAs with reduced expression in a large cohort of colon cancer patient samples, further supporting the potential impact and clinical relevance of *miR-192* in colon cancer (37). We speculate that the decrease or loss of the suppressive function of *miR-192* in colon cancer may be an important factor related to cell cycle control and chemosensitivity to anti-folate based therapy.

In conclusion, our study provides evidence that *miR-192* is another candidate microRNA that is directly involved in the regulation of a key anticancer target DHFR. The expression and function of the *miR-192* is largely dependent on the presence of functional wild type p53. This raises the potential of using *miR-192* as a novel therapeutic option for treating cancer via an effective delivery system either alone or in combination with anti-folate compounds. Further studies are needed to identify additional *miR-192* mediated targets and its relationship with other miRNAs involved in the same pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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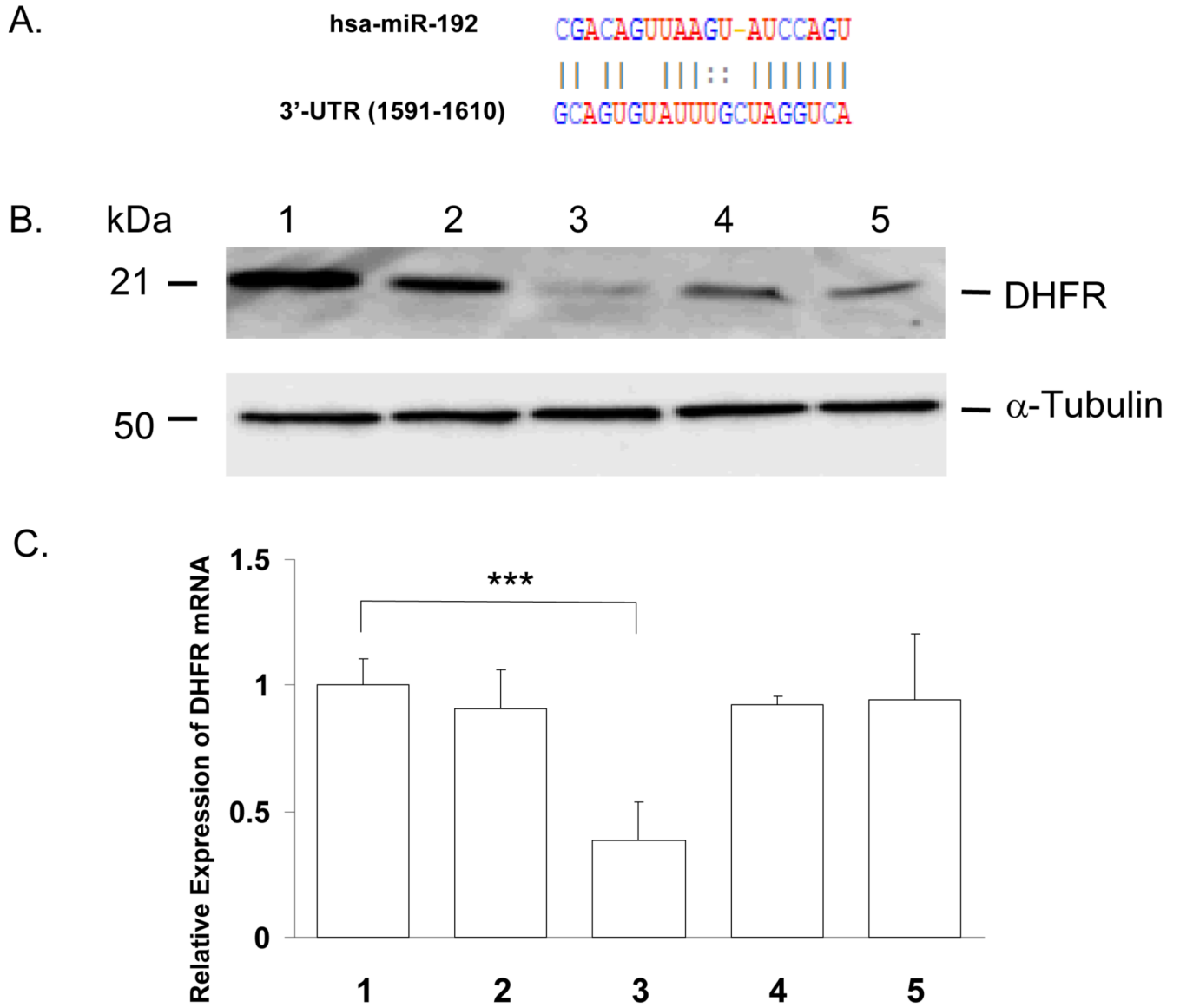
A.

hsa-miR-192

CGACAGUUAAGU-AUCCAGU

|| || |||:: ||||||||

3'-UTR (1591-1610)   GCAGUGUAUUUGCUAGGUCA

B.

C.

Figure 1.
The *miR-192* binding site at 3′-UTR of DHFR mRNA (A). Western immunoblot analysis of DHFR protein expression levels in HCT-116 (wt-p53) cells transfected with *miR-192* (lane 1, vehicle control; lane 2, non specific-miR control; lane 3, DHFR *siRNA* positive control; lane 4, *miR-192*; lane 5, positive control miR-24), α-tubulin was used as protein loading control (B). Expression analysis of DHFR mRNA in HCT-116 (wt-p53) cells by real time qRT-PCR analysis (lane 1, vehicle control; lane 2, non-specific *miR* control; lane 3, DHFR *siRNA* positive control; lane 4, *miR-192*; lane 5, positive control miR-24). GAPDH was used as internal standard for normalization (C) (*** *P < 0.0001*).

1. Non specific control miR
2. Non specific control miR+MTX
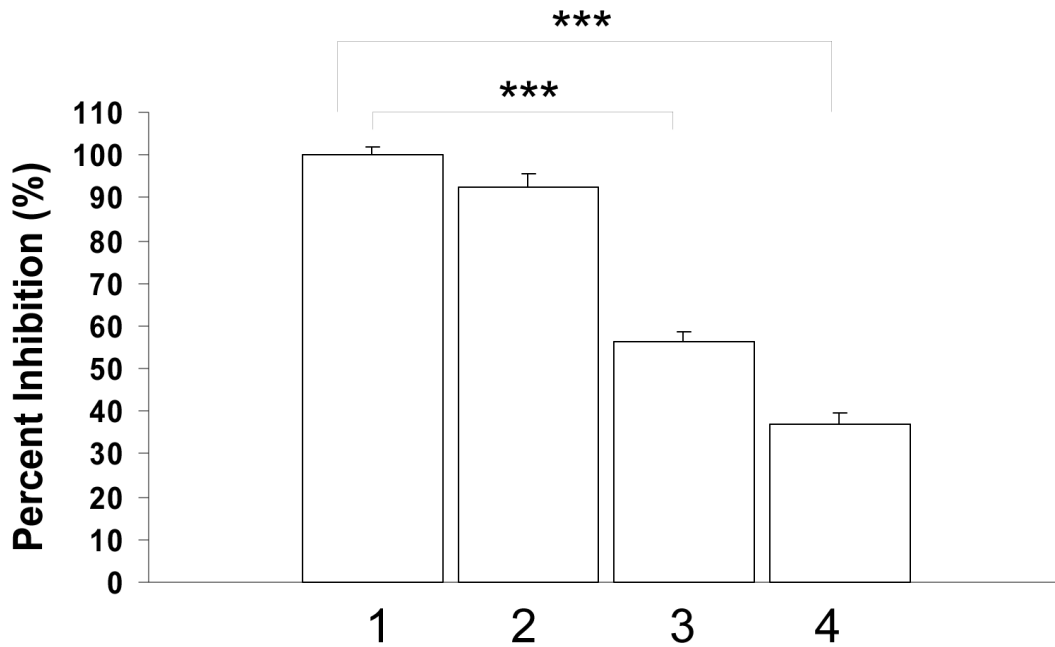**3. siRNA of DHFR+MTX**
4. miR-192+MTX

Figure 2.
Impact of *miR-192* on cell proliferation with MTX treatment in HCT-116 (wt-p53) cells transfected with DHFR specific *siRNA* or *miR-192* (lane 1, non-specific control *miR*; lane 2, 100 nM non-specific control *miR* + 25 nM MTX; lane 3, 100 nM DHFR *siRNA* + 25 nM MTX; lane 4, 100 nM *miR-192* + 25 nM MTX) (* *P < 0.006*).
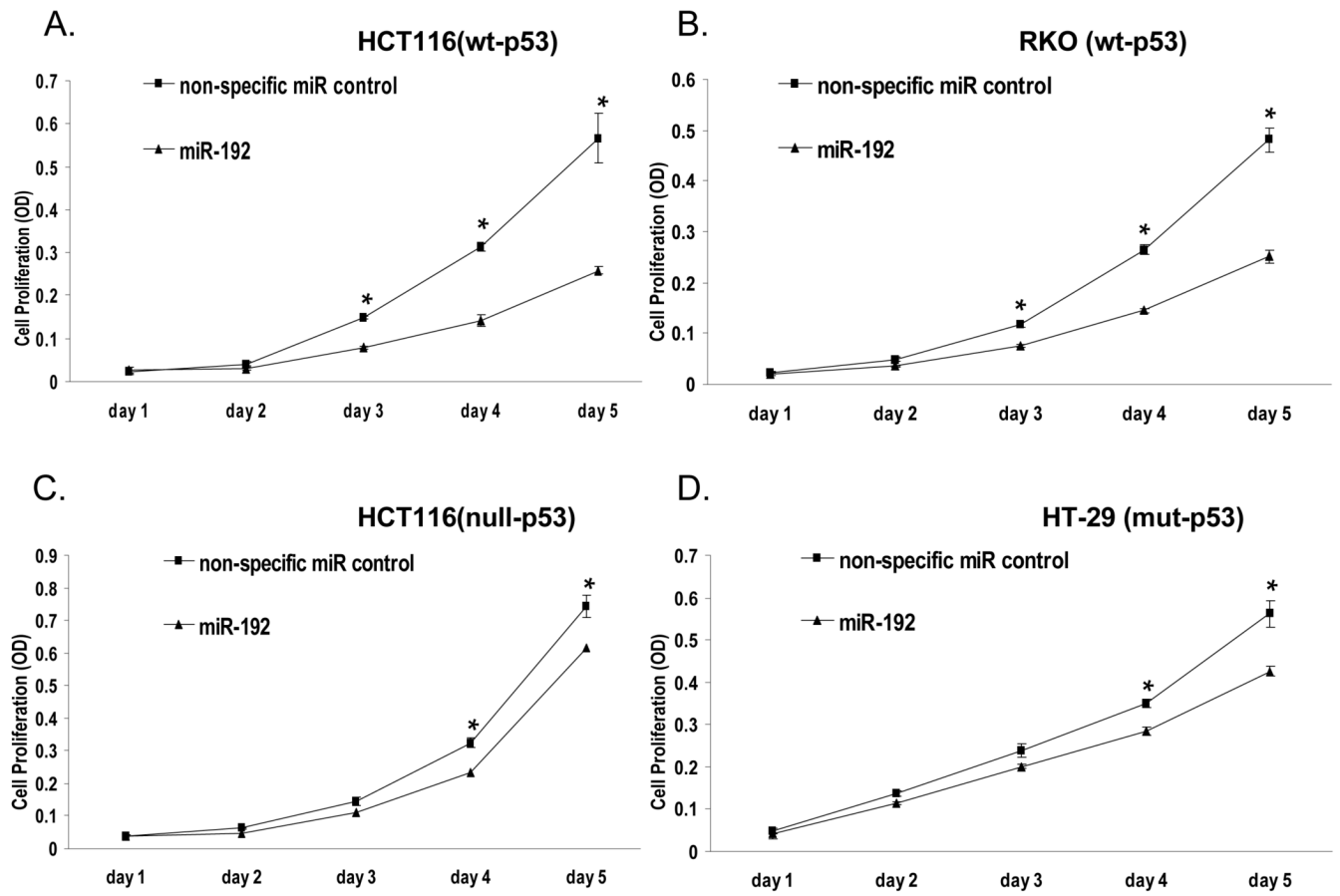
Figure 3.
Impact of *miR-192* on cell proliferation using WST1 assay in HCT-116 (wt-p53) cells (A) (*
P < 0.001), RKO (wt-p53) cells (B) (* *P < 0.001*), HCT-116 (null-p53) cells (* *P < 0.02*) (C),
HT-29 (mut-p53) cells (D) (* *P < 0.029*). Each cell type was transfected with 100 nM non-
specific control *miR* or *miR-192*; cell numbers were determined by the WST-1 assay.
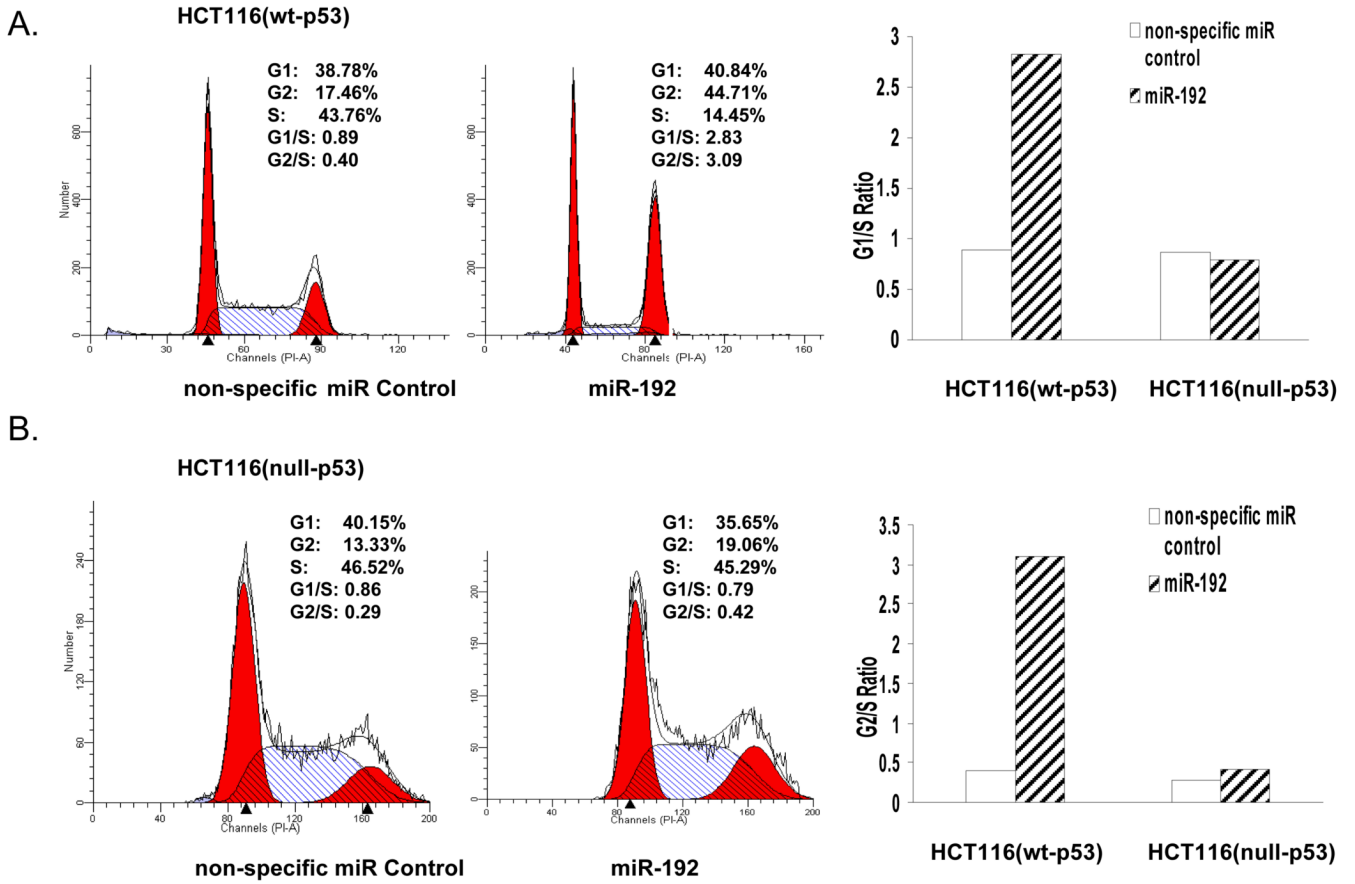
Figure 4.
Cell cycle analysis by flow cytometry in HCT-116 (wt-p53) (A) or HCT-116 (null-p53) (B) transfected with 100 nM non-specific *miR* or *miR-192*. The bar graphs show the fold increase of G1/S and G2/S ratio in both HCT-116 (wt-p53) and HCT (null-p53) cells transfected with *miR-192*.
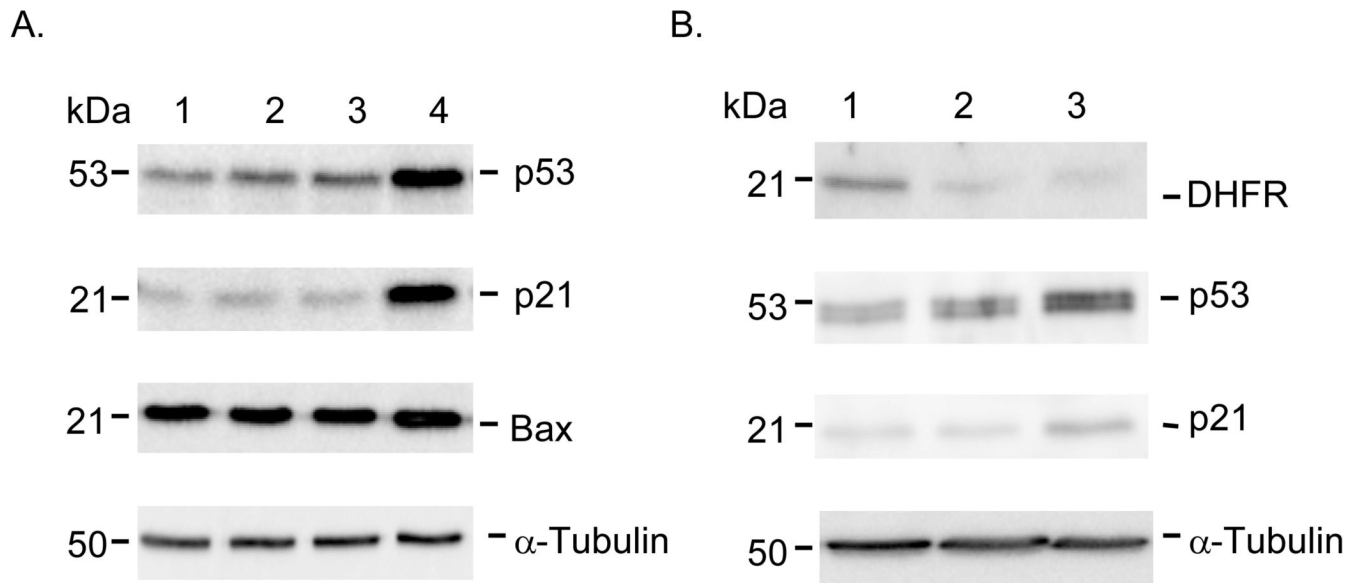
A.

B.

Figure 5.
Western immunoblot analysis of p53, p21 and Bax expression in HCT-116 (wt-p53) cells (lane 1, vehicle control; lane 2, non specific control; lane 3, DHFR siRNA positive control; lane 4, 100 nM *miR-192*). α-tubulin was used as a protein loading control. B. Western immunoblot analysis of p53 and p21 expression in RKO (wt-p53) cells (lane 1, non-specific *miR* control; lane 2, DHFR *siRNA* positive control; lane 3, 100 nM *miR-192*). α-tubulin was used as a protein loading control.

A.

| | HCT116 (wt-p53) | RKO (wt-p53) | HCT116 (null-p53) | HT-29 (mut-p53) |
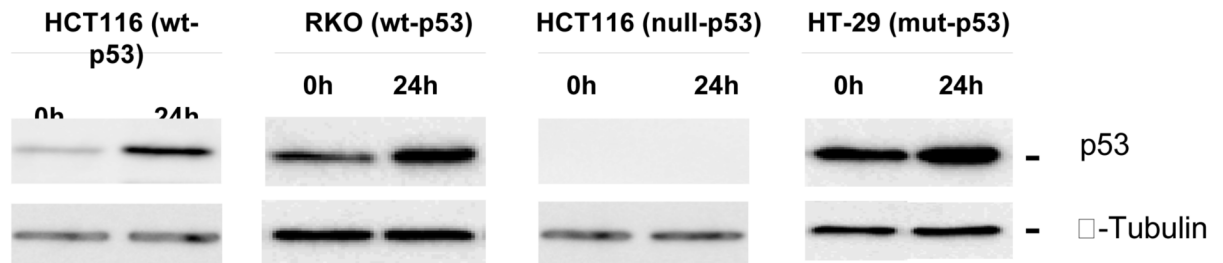
B.
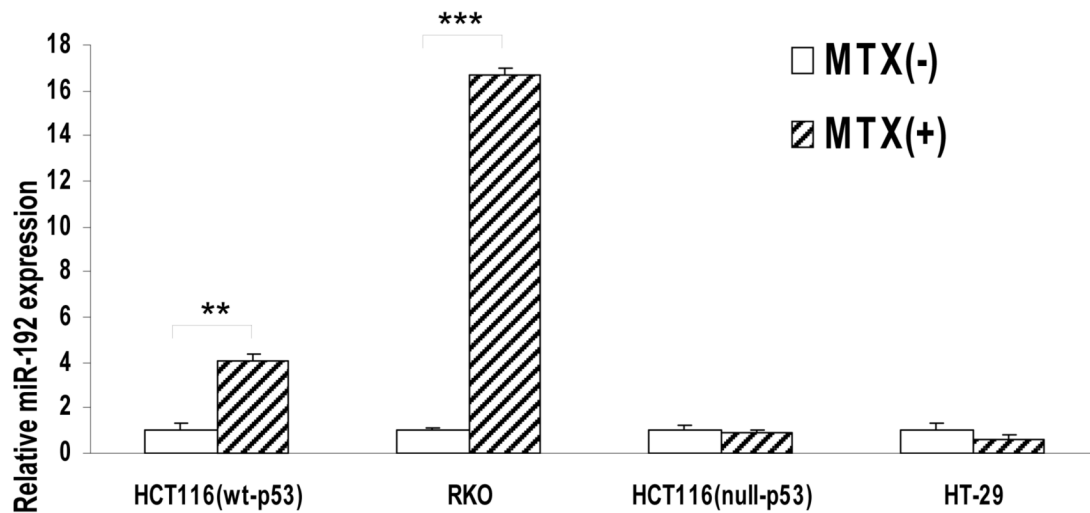
Figure 6.
(A) Effect of p53 induction by MTX treatment on the expression of endogenous *miR-192* expression in HCT-116 (wt-p53) cells (** *P < 0.001*), RKO (wt-p53) cells (*** *P < 0.0001*), HCT-116 (null-p53) cells and HT-29 (mut-p53) cells. The p53 protein expression levels were analyzed by Western immunoblot analysis and α-tubulin was used as a protein loading control. (B) The expression of endogenous mature *miR-192* was analyzed by real time qRT-PCR analysis using RNU6B as an internal standard for normalization (open bar, control; dashed bar, plus MTX treatment).