

Mir-509-5p joins the Mdm2/p53 feedback loop and regulates cancer cell growth

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Although the Mdm2/p53 interaction has been well documented, it is not clear whether there are new microRNAs participating in this regulatory network. Here, we provide evidence that miR-509-5p, which is downregulated in a subset of newly diagnosed cervical cancer and hepatocellular carcinoma tissues compared with the adjacent nontumor tissue, can be activated by p53 through binding the promoter of miR-509-5p and it suppresses the growth and invasion/migration of cervical cancer and hepatoma cells by regulating apoptosis and the G1/S-phase transition of cell cycle. Furthermore, Mdm2 was identified to be a target of miR-509-5p by targeting its 3'-UTR. Restoration of Mdm2 abrogated the cell phenotypes induced by miR-509-5p. Moreover, ectopic expression of miR-509-5p in HeLa and QGY-7703 cells repressed the expression of Mdm2, subsequently enhancing its p53-activating effects. These results suggest that miR-509-5p is a new regulator of Mdm2/p53 pathway and may play a key role in cancer development.

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MiRNAs are small, endogenous, noncoding RNAs that have been shown to be crucial post-transcriptional regulators of gene expression. MiRNAs typically silence multiple genes instead of a single gene, as miRNAs target the 3'-untranslated regions (3'-UTR) of mRNAs by partial sequence homology, leading to mRNA degradation or translation repression.¹ It is predicted that an average miRNA can target hundreds of mRNAs. Evidence suggests that miRNAs are often deregulated in human malignancies and can function as either oncogenes or tumor suppressors in a subset of cancers,² such as B-cell chronic lymphocytic leukemia,³ breast cancer,⁴ lung cancer,⁵ hepatocellular carcinoma⁶ and gastric adenocarcinoma.⁷ Despite the overwhelming evidences suggesting that miRNAs could play a causal role in human malignancies, the mechanisms underlying the deregulation of miRNAs and miRNA-mediated gene silencing that leads to cancer development are still poorly understood. It has been found that miRNA expression is regulated by many factors, such as MyoD, Mef2,⁸ TGF- β ⁹ and p53. It has also been shown that the miR-34 family is induced by the tumor suppressor p53 and elicits p53-like responses, such as senescence, cell cycle arrest and apoptosis, depending on the cell type.¹⁰

The tumor suppressor p53 is a master regulator controlling diverse cellular pathways by either activating or repressing downstream genes, such as S100A2 and TP53AP1.¹¹ Studies identified that p53 can be regulated by other proteins, such as HIPK2,¹² HDAC,¹³ p14ARF, Mdm2, Cop-1 and Pirh-2.¹⁴ Among them, murine double minute 2 (Mdm2), an E3 ubiquitin ligase, has been well documented. Mdm2, which is overexpressed in many human tumors, can mediate p53 ubiquitination for proteasomal degradation, resulting in a

decreased level of p53 in the cells.^{15–17} Thus, p53 can be antagonized by Mdm2 that offers a therapeutic strategy.¹⁸

Recent studies found that miRNAs are involved in the Mdm2/p53 pathway;^{19,20} for example, miR-605 can impair the Mdm2/p53 feedback loop.²¹ The goal of this study was to determine whether there are new miRNAs that participate in the Mdm2/p53 pathway. Using microarray analysis, we identified that miR-509-5p was significantly upregulated by p53. We further showed p53 induced the expression of miR-509-5p by interacting with miR-509-5p promoter. Ectopic expression of miR-509-5p inhibited cervical cancer and hepatocellular carcinoma cell proliferation and invasion. Furthermore, Mdm2 can be directly targeted and downregulated by miR-509-5p, and this resulted in the increase of the p53 protein level that subsequently repressed the cell cycle and induced apoptosis. These results suggest that, as a new member of the p53 regulatory network, miR-509-5p provides a direct link between Mdm2 and p53 in the regulatory network.

Results

Regulation of miR-509-5p after induction of p53. To determine the miRNAs that are regulated by p53, miRNA microarray analysis was used to examine the alterations in miRNA expression after overexpression of p53 in HeLa cells. The results showed that there were 19 differently expressed miRNAs, including 10 upregulated or 9 downregulated miRNAs (Figure 1a). We further confirmed the expression of these miRNAs by quantitative reverse transcription-PCR (qRT-PCR; Supplementary Figure S1). The results indicated that these miRNAs may be involved in cancer cell

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Abbreviations: ASO, antisense methoxy-modified nucleic acid oligo; ChIP, chromatin immunoprecipitation; CLL, chronic lymphocytic leukemia; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; Mdm2, murine double minute 2; PI, propidium iodide; P/S, penicillin/streptomycin; p53REs, p53 response elements; 3'-UTR, 3'-untranslated region

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development. Because p53 primarily acts to activate the transcription of tumor suppressive genes, we analyzed the miRNAs that were upregulated following p53 overexpression. Among these differential miRNAs, miR-509-5p was the most prominently upregulated.

Inspection of the putative miR-509-5p promoter using MatInspector module of the Genomatix software suite (Bayerstr, Germany) further suggested that p53 is a potential miR-509-5p regulator, because we identified two candidate p53-binding sites within a 800-bp DNA fragment upstream to the pre-miR-509-5p. Therefore, we examined whether endogenous p53 promotes the expression of miR-509-5p in cervical cancer (HeLa and Caski) and hepatocellular

carcinoma (HCC) cell lines (QGY-7703 and HepG2). The tumor suppressor p53 can be activated by genotoxic stress, such as doxorubicin (doxo) or serum starvation. QRT-PCR and western blot analysis showed that p53 expression was significantly upregulated in cells treated with doxo or serum starvation (Figures 1b and c, left panel; Figure 1e and f, top panel). We further confirmed the qRT-PCR result of p53 expression in four cell lines treated with 0.5 or 1.0 $\mu\text{g/ml}$ doxo by another two primers (Supplementary Figure S2). Given that p21 is the downstream target of p53,²² we also examined p21 protein level and found the expression of p21 was increased when p53 was induced by doxo or serum starvation (Figure 1e and f, bottom panel). Accordingly, we observed that

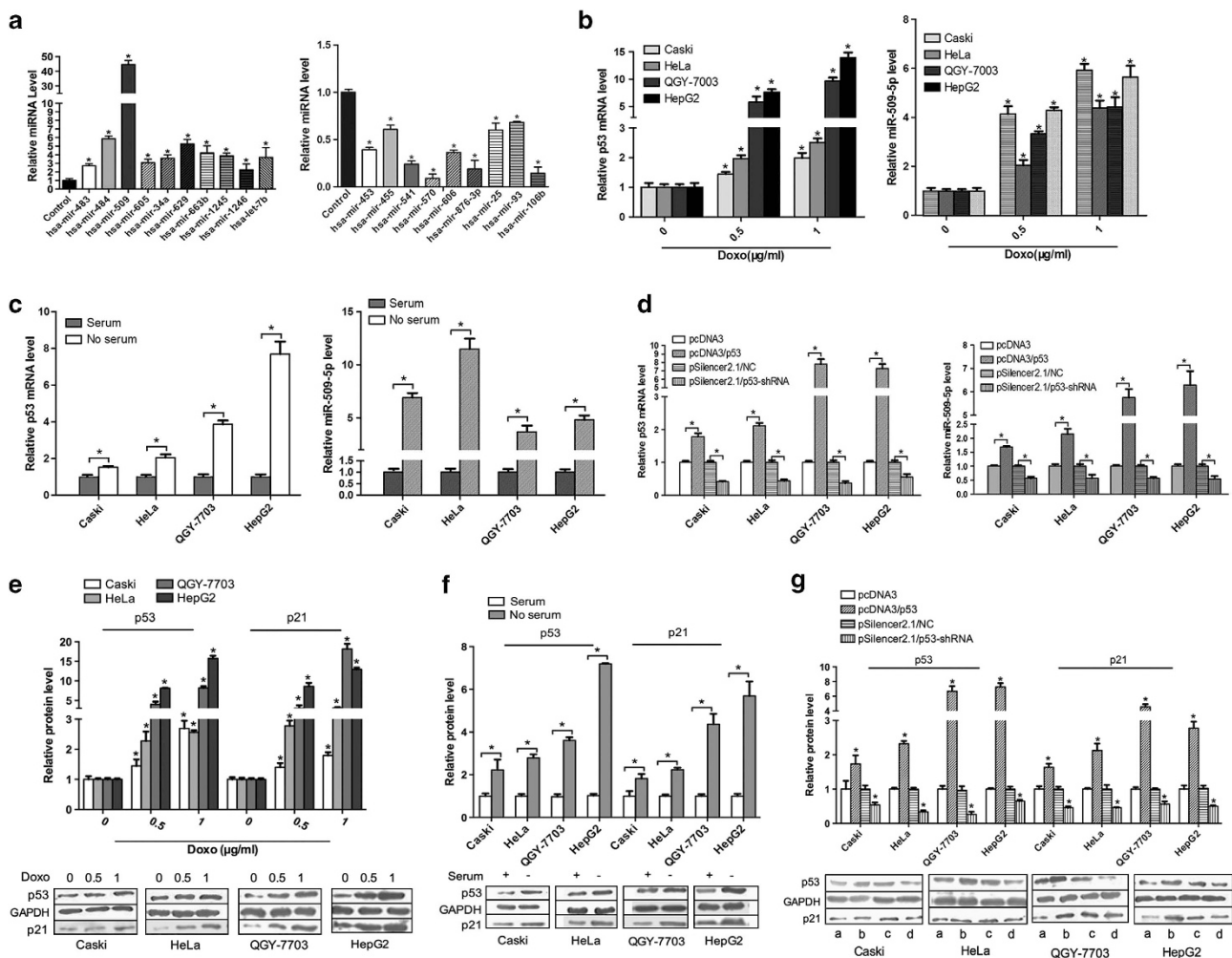


Figure 1 The miR-509-5p expression is modulated through the p53 pathway. (a) HeLa cells were transfected with pcDNA3/p53, and microarray was used to detect the differentially expressed miRNAs. The upregulated and downregulated miRNAs are shown. (b) p53 was induced in HeLa, Caski, QGY-7703 and HepG2 cells treated with 0.5 or 1.0 $\mu\text{g/ml}$ doxo for 16 h (left panel). Under this condition, miR-509-5p was induced in the four cell lines (right panel). (c) Cells were grown in medium containing 10% or no FBS for 24 h. p53 was induced in the four cells grown under serum starvation conditions (left panel), and miR-509-5p was induced in the four cell lines (right panel). (d) The qRT-PCR analysis showed that the level of p53 mRNA increased or decreased (left panel) following transfection with pcDNA3/p53 or pSilencer2.1/p53-shRNA, respectively, and miR-509-5p expression was also subsequently induced or reduced in these four cell lines (right panel). (e) Western blot analysis showed that p53 was induced in HeLa, Caski, QGY-7703 and HepG2 cells treated with 0.5 or 1.0 $\mu\text{g/ml}$ doxo for 16 h. Accordingly, increased p21 protein expression level was also detected. GAPDH was used as an internal control. (f) Western blot analysis was performed to examine p53 and p21 protein expression level in the four cell lines (HeLa, Caski, QGY-7703 and HepG2 cells) grown in medium containing 10% or no FBS for 24 h. GAPDH was used as an internal control. (g) The gain or loss of p53 protein expression level was measured in cells (HeLa, Caski, QGY-7703 and HepG2 cells) transfected with pcDNA3/p53 or pSilencer2.1/p53-shRNA as well as control plasmid by western blot analysis. The protein expression of p21 was also detected. GAPDH was used as an internal control. a, b, c and d represent the cells transfected with pcDNA3, pcDNA3/p53, pSilencer2.1 and pSilencer2.1/p53-shRNA, respectively (* $P < 0.05$)

doxo or serum starvation treatment led to the upregulation of miR-509-5p expression (Figures 1b and c, right panel). Furthermore, the gain or loss of p53 expression was used to alter the p53 expression in both mRNA and protein levels (Figure 1d, left panel; Figure 1g, top panel) and subsequently resulted in p21 protein-level alterations (Figure 1g, bottom panel). Knockdown of p53 resulted in the significant repression of miR-509-5p in the cells (Figure 1d, right panel), whereas overexpression of p53 resulted in a substantial induction of miR-509-5p expression in all cell lines (Figure 1d, right panel). These results demonstrate an important role of p53 in the induction of miR-509-5p in human cervical cancer and HCC cells.

p53 directly binds the promoter of miR-509-5p at the p53 response element. To determine whether p53 activates the miR-509-5p promoter, we cloned a 800-bp putative miR-509-5p promoter carrying two p53 response elements (p53REs) into a pGL3 basic vector to generate pMir-509p-Luc-1

(Figure 2a). We tested pMir-509p-Luc-1 in HeLa cells. As shown in Figure 2b, overexpression of p53 increased miR-509-5p promoter activity by 2.8-fold, whereas knock-down of p53 decreased the activity by 0.4-fold. We also identified that doxo can induce the increase of the luciferase intensity of the miR-509-5p promoter (Figure 2c). Furthermore, a series of deletion constructs were generated to determine whether the two elements directly interact with p53 to mediate the induction of the miR-509-5p promoter activity by p53. We showed that the deletion of p53RE2 abolished the induction activity, whereas the deletion of p53RE1 had no effect on p53-mediated induction of the miR-509-5p promoter activity, suggesting that only p53RE2 was responsible for p53-mediated miR-509-5p induction (Figure 2d). To further confirm the role of p53RE2 in miR-509-5p induction, chromatin immunoprecipitation (ChIP) assay was performed. Consistent with the luciferase results, we detected specific p53RE2 PCR product (Figure 2e) but not from p53RE1 after p53 overexpression. Quantification by qRT-PCR at the

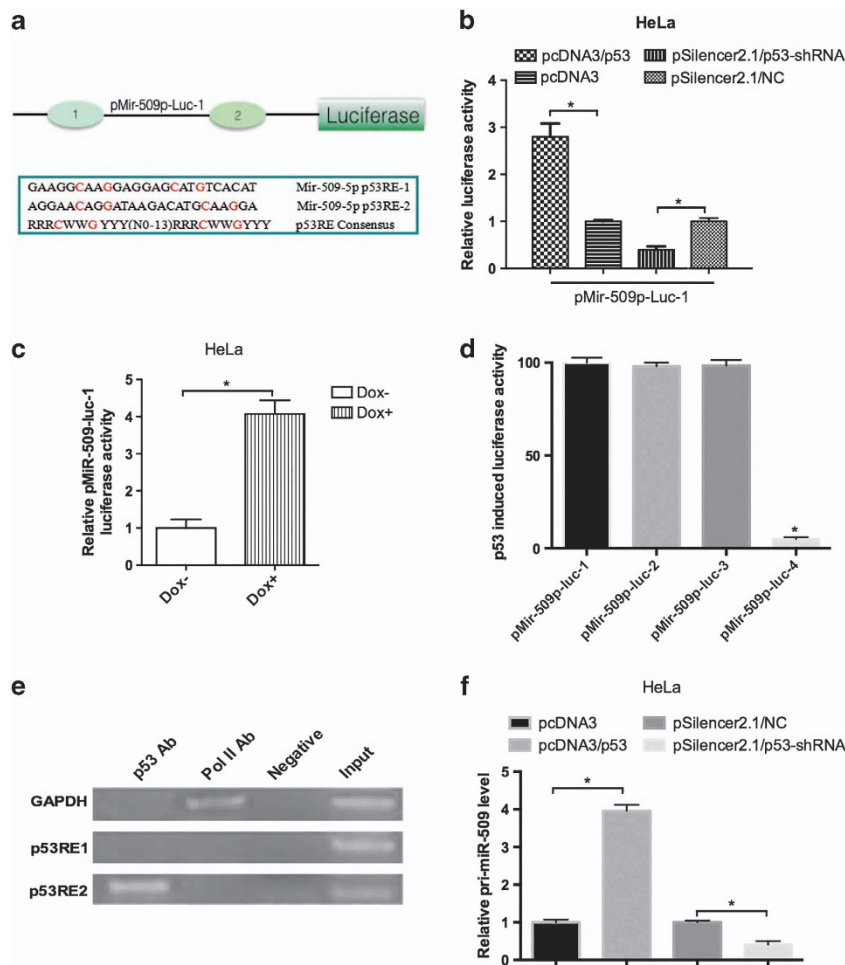


Figure 2 p53 induces the miR-509-5p promoter activity. (a) A schematic description of the putative miR-509-5p promoter with two potential p53 response elements, p53RE1 and p53RE2, is shown compared with the p53RE consensus, where R = A or G; W = A or T and Y = C or T. C and G in red are the conserved nucleotides. (b) pMir-509p-Luc-1 luciferase assays in HeLa cells with overexpression or knockdown of p53. (c) pMir-509p-Luc-1 luciferase assays in HeLa cells treated with 1 μ g/ml doxo. (d) Deletion analysis identifies p53RE2-mediated p53-induced miR-509-5p promoter luciferase activity. pMir-509p-Luc-2: p53RE1 was deleted; pMir-509p-Luc-3: the upstream sequence of pRE2 was deleted; pMir-509p-Luc-4: pRE2 sequence was deleted. (e) ChIP assay shows that p53 directly interacts with p53RE2. GAPDH sequence that can bind to Pol II antibody serves as a positive control. Normal mouse IgG was used as the negative control. (f) miR-509-5p primary transcript was detected by qRT-PCR (* P < 0.05)

miR-509-5p primary transcript level was further analyzed. Increased accumulation of miR-509-5p primary transcript was detected in cells overexpressing p53, and knockdown of p53 significantly repressed the expression of the miR-509-5p primary transcript (Figure 2f). Therefore, the results suggest that p53 induces miR-509-5p expression by directly interacting with its promoter.

MiR-509-5p suppresses cancer cell growth, migration and invasion *in vitro*. Given the ability of p53 to induce miR-509-5p, miR-509-5p is expected to have an impact on the malignant behaviors of cancer cells. First, we constructed a plasmid to overexpress miR-509-5p and synthesized an ASO (antisense methoxy-modified nucleic acid oligo) of miR-509-5p (ASO-miR-509-5p). QRT-PCR analysis revealed approximately threefold higher expression of miR-509-5p in the miR-509-5p-transfected cells and a 50% reduction in the ASO-treated cells compared with the control cells (Figure 3a). MTT and colony formation assays were performed to analyze the cell viability and growth capacity. The results indicated that miR-509-5p suppressed HeLa, QGY-7703 and HepG2 cell viability and their long-term proliferative capacity (Figures 3b and c), whereas ASO-miR-509-5p exhibited the opposite effects (Figures 3b and c). To determine whether miR-509-5p can affect the migration and invasion capacities of the cells, we detected changes in cell motility in the pri-miR-509-transfected cells using Transwell assays. The upregulation of miR-509-5p significantly suppressed cell migration of the HeLa and QGY-7703 cells, as measured by the Transwell assay in the absence of Matrigel. However, cell migration was significantly promoted when the cells were transfected with ASO-miR-509-5p compared with the ASO-NC-transfected cells (Figures 3d and e). Furthermore, Transwell assays performed in the presence of 2 mg/ml Matrigel showed that overexpression of miR-509-5p decreased cell invasion compared with the control cells. In contrast, an increase in the invasion ability of the ASO-miR-509-5p-transfected cells was observed compared with the ASO-NC-transfected cells (Figures 3f and g). Therefore, miR-509-5p exerts suppressive effects on cell growth, invasion and migration in human cervical cancer and HCC cell lines.

Mdm2 is a target of miR-509-5p-mediated post-transcriptional repression. Bioinformatics analyses predicted that Mdm2 might be a putative target gene of miR-509-5p (Figure 4a). First, we examined the expression levels of miR-509-5p, Mdm2 and p53 to determine their reciprocal correlation. As expected, miR-509-5p and p53 were generally expressed at low levels, whereas Mdm2 was generally upregulated in the cervical cancer and HCC tissues compared with their adjacent nontumor tissues (Figure 4b). QRT-PCR (Figure 4c) and western blot (Figure 4d) analyses confirmed that the ectopic expression of miR-509-5p substantially suppressed Mdm2 expression, and the results were also confirmed by another two primers (Supplementary Figure S3). To further determine the specificity of the miR-509-5p/Mdm2 interaction, we used ASO-miR-509-5p to knock down its level. As shown in Figures 4c and d, ASO-miR-509-5p was able to enhance Mdm2 expression.

Furthermore, to determine whether miR-509-5p directly targets Mdm2, we constructed an EGFP reporter carrying the Mdm2 3'-UTR with a putative miR-509-5p-binding site. EGFP expression was reduced by ~40% in the miR-509-5p-transfected cells compared with the vector control group (Figure 4e). This suppression of EGFP expression was abolished in the cells co-transfected with the Mdm2 3'-UTR-mut (Figure 4e), in which the seed sequence was mutated (Figure 4a). However, EGFP activity was increased in the cells co-transfected with ASO-miR-509-5p and the EGFP reporter carrying Mdm2 3'-UTR, whereas ASO-miR-509-5p had no effect on the activity of the Mdm2 3'-UTR-mut construct. We confirmed Mdm2 mediated the miR-509-5p-induced cell phenotype alterations, because ectopic expression of Mdm2 counteracted the suppressive effects of miR-509-5p on cell viability and proliferation in HeLa and QGY-7703 cells (Figures 4f and g).

To provide a more definitive function of Mdm2 in miR-509-5p-mediated proliferation, we used Mdm2 siRNA to transfect HeLa cells. Consistent with the role of miR-509-5p, knockdown of Mdm2 resulted in a suppressed proliferation of HeLa cells (Supplementary Figure S4). Furthermore, miR-509-5p did not have further growth inhibitory effects in Mdm2 siRNA-transfected cells (Figures 4h and i). Therefore, miR-509-5p acts as a tumor suppressor in cervical cancer and HCC cells by directly targeting Mdm2, and this specific silencing of Mdm2 by miR-509-5p accounts, at least in part, for the miR-509-5p-mediated inhibition of cervical cancer and HCC cell growth.

Implication of miR-509-5p participation in the Mdm2/p53 signaling pathway. Mdm2 is a well-known oncogene that plays an important role in the ubiquitination and subsequent proteasomal degradation of the p53 protein, and Mdm2 may repress the cancer cell cycle and promote apoptosis.²³ However, the underlying mechanism of p53 regulation by miR-509-5p is not understood. To determine whether miR-509-5p mediated the alteration of p53 expression, we ectopically expressed miR-509-5p in HeLa cells. The results revealed that miR-509-5p induced the accumulation of p53, and antagonizing miR-509-5p repressed p53 expression (Figure 5a, bottom panel). Furthermore, we confirmed that the ubiquitinated p53 was reduced when miR-509-5p was overexpressed, whereas cells transfected with ASO-miR-509-5p had increased level of ubiquitinated p53 (Figure 5a, top panel). As HeLa cells express HPV E6 that mediates p53 polyubiquitination and degradation, we examined whether miR-509-5p affected the expression of E6. The result showed that E6 expression was not affected by miR-509-5p (Supplementary Figure S5). Moreover, we selected an HPV negative cell line C33A and confirmed miR-509-5p can also inhibit Mdm2 and activate p53 expression significantly (Supplementary Figure S6). We further defined whether miR-509-5p regulated p53 mRNA and protein levels by downregulating Mdm2 expression during doxo treatment. ASO-miR-509-5p induced Mdm2 expression and caused reduction of the p53 level by 60%. However, when the ASO-miR-509-5p-transfected cells were treated with doxo, the Mdm2 expression was reversed by 30% (compared with ASO-miR-509-5p group with no doxo induction), and the p53 level was reversed by 40% (Figure 5b).

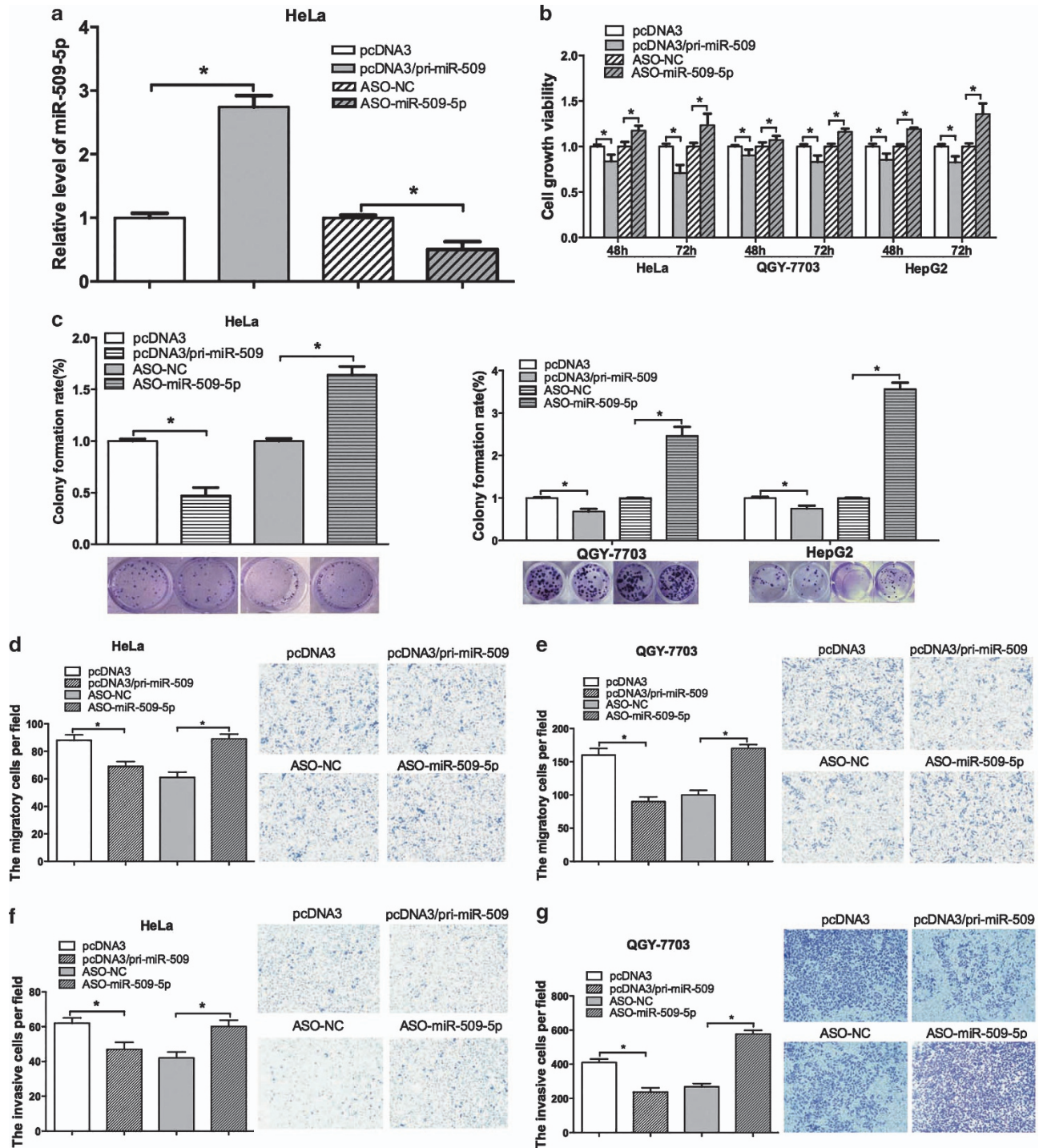


Figure 3 miR-509-5p serves as a tumor suppressor. (a) qRT-PCR was performed to examine the alterations of miR-509-5p expression after transfection with pri-miR-509 and ASO-miR-509-5p in HeLa cells. U6 snRNA was used as an internal control. (b and c) The effects of overexpression or knockdown of miR-509-5p on cell viability were detected using an MTT assay, and the long-term effects on proliferative capacity were examined after transfection using a colony formation assay in HeLa, QGY-7703 and HepG2 cells. (d and e) Transwell migration assays were performed with HeLa and QGY-7703 cells transfected with pri-miR-509, ASO-miR-509-5p and the corresponding control vectors. Transwell assays without Matrigel demonstrated that miR-509 significantly decreased the migration of HeLa and QGY-7703 cells when compared with the control vector groups. The results were consistent when miR-509-5p was depleted. (f and g) Transwell assays with Matrigel demonstrated that miR-509 overexpression significantly promoted the invasion of HeLa and QGY-7703 cells when compared with the control vector groups. The results were consistent when miR-509-5p was depleted (* $P < 0.05$)

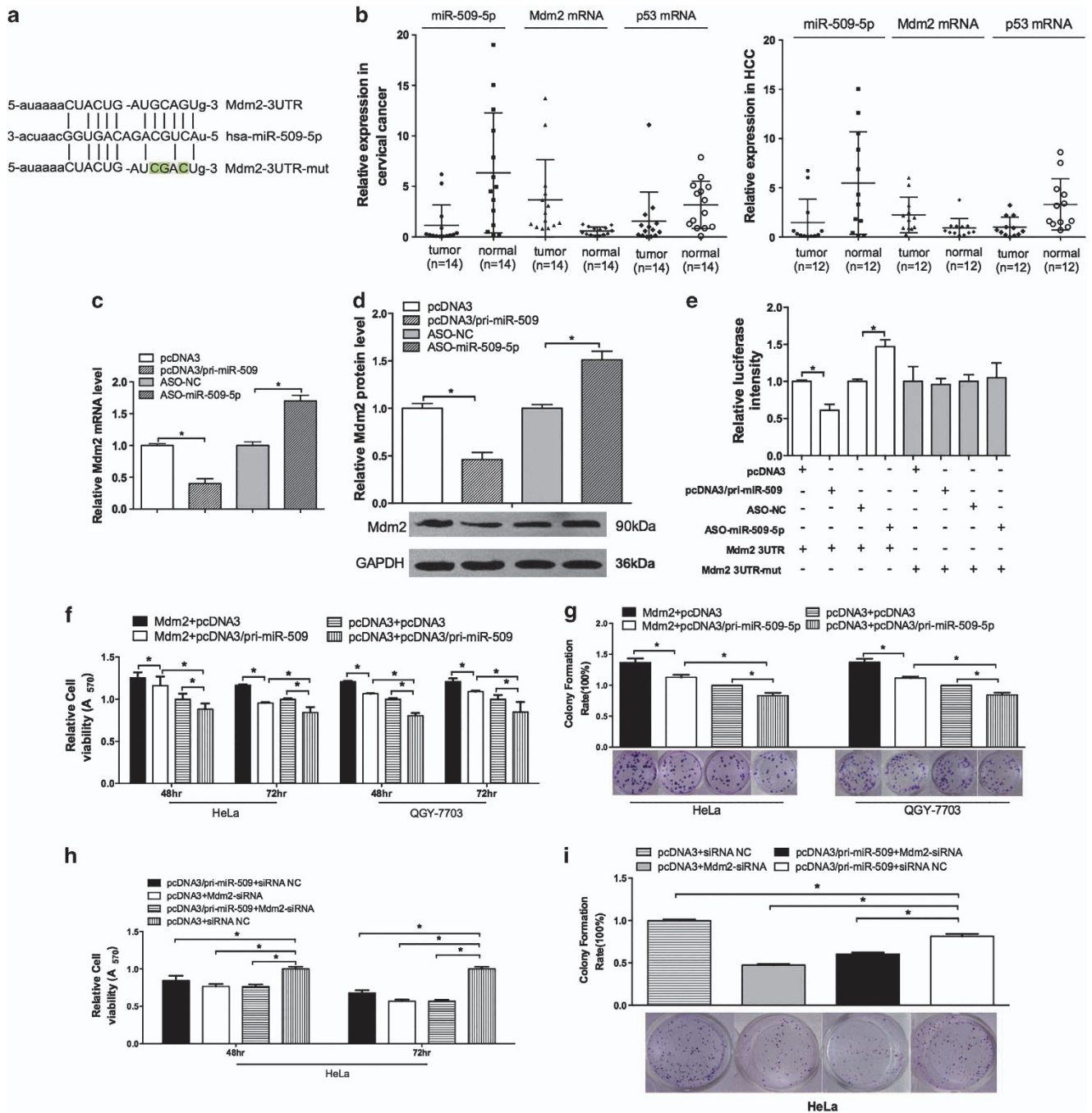


Figure 4 miR-509-5p directly targets Mdm2 and inhibits its expression. (a) A schematic of the bioinformatics predicted seed region in the 3'-UTR of Mdm2 is shown above; the mutated 3'-UTR used in this study is also shown. (b) Expression of miR-509-5p, Mdm2 and p53 in the 14 pairs of matched cervical tumor specimens and in the 12 pairs of matched HCC tissues. (c) qRT-PCR showed the suppression of Mdm2 expression by miR-509-5p. The HeLa cells were transfected with the empty vector or miR-509-5p for 24 h before harvesting. (d) Western blot analysis was used to detect Mdm2 protein expression when HeLa cells were transfected with pcDNA3/pri-miR-509 or ASO-miR-509-5p. (e) The effect of miR-509-5p or ASO-miR-509-5p on the EGFP activity of EGFP-Mdm2-3'-UTR and EGFP-Mdm2-3'-UTR-mut, in which the putative miR-509-5p-binding site was mutated. For the EGFP reporter assays, HeLa cells were transfected with a miR-509-5p expression vector or ASO-miR-509-5p oligo and then harvested for lysis 48 h after transfection. The MTT assay (f) and colony formation assay (g) were performed to assess the HeLa and QGY-7703 cell growth alterations in the rescue experiment to further confirm that miR-509-5p acts as a tumor suppressor. (h and i) MTT and colony formation assays were performed to detect the effect of miR-509-5p in Mdm2-siRNA-transfected cells (* $P < 0.05$).

We then examined the effect of miR-509-5p-mediated p53 promotion on the levels of p21 and bak, both of which are direct targets of p53.²² As shown in Figures 5c and d, miR-509-5p overexpression led to increased mRNA and protein

levels of p21 and bak protein, whereas ASO-miR-509-5p reduced the p21 and bak expression levels. Given that p53 plays a critical role in the regulation of p21 and bak, we performed cell cycle analysis in the miR-509-5p-transfected

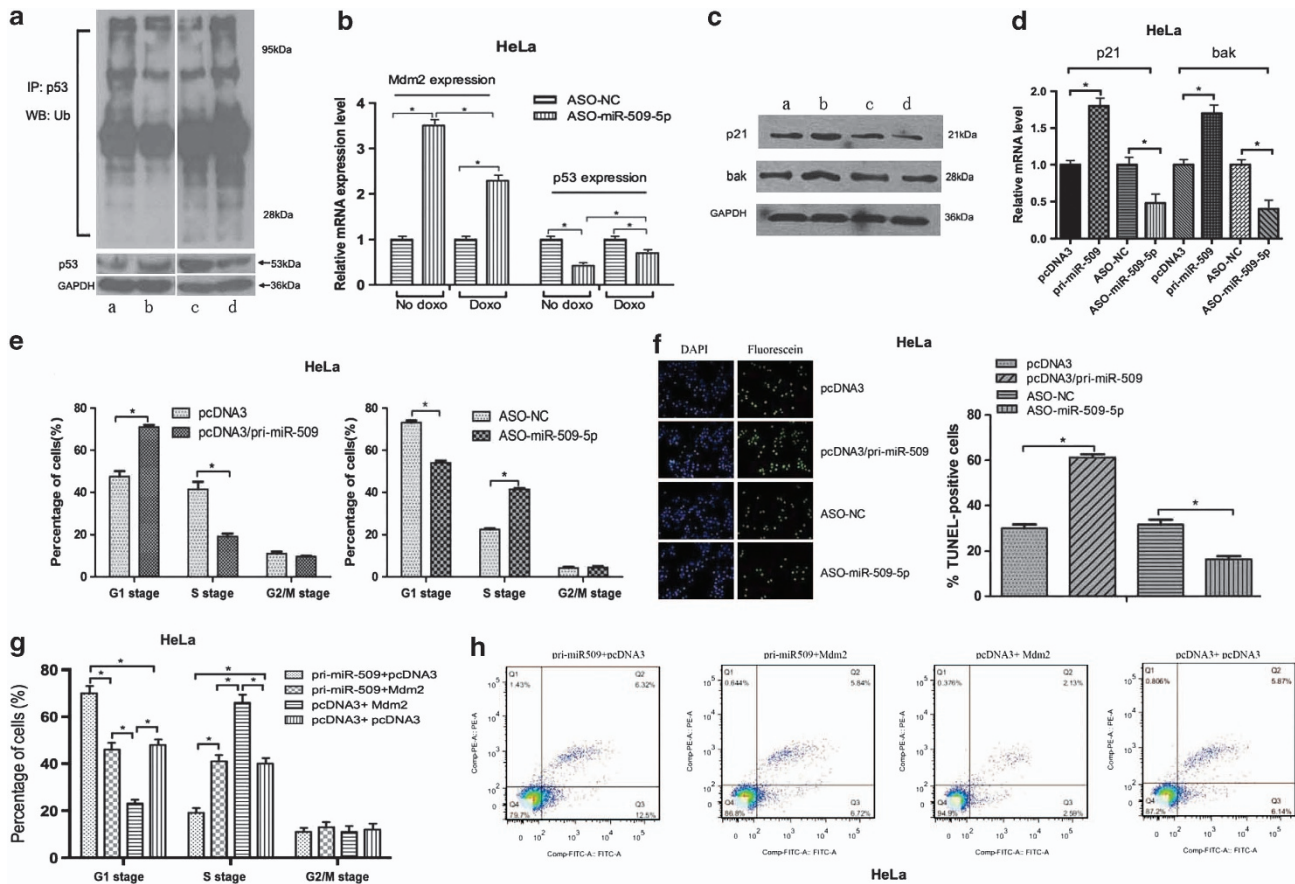


Figure 5 Role of miR-509-5p in the regulation of cell cycle and apoptosis. (a) Overexpression of miR-509-5p enhanced p53 ubiquitination, and inhibition of miR-509-5p by ASO-miR-509-5p attenuated endogenous p53 ubiquitination in HeLa cells (top panel). The bottom panel shows the effect of miR-509-5p or ASO-miR-509-5p on p53 expression. a, b, c and d represent the cells transfected with pcDNA3, pcDNA3/pri-miR-509, ASO-NC and ASO-miR-509, respectively. (b) The mRNA level of Mdm2 and p53 in the presence of ASO-miR-509-5p with or without treatment of doxo. (c) The HeLa cells were treated with miR-509-5p or ASO-miR-509-5p for 48 h and were then lysed. Western blot analysis was used to detect p21 and bak expression. a, b, c and d represent the cells transfected with pcDNA3, pcDNA3/pri-miR-509, ASO-NC and ASO-miR-509, respectively. (d) qRT-PCR was used to detect the mRNA levels of p21 and bak in HeLa cells. (e and f) Cell cycle progression and apoptosis were analyzed using FACS and TUNEL assay in HeLa cells. (g and h) Rescue experiment to detect miR-509-5p effect in Mdm2-transfected HeLa cells (**P*<0.05)

cells. We found that miR-509-5p increased the percentage of cells in G1 phase by ~35% and reduced the S-phase cell population by ~53% (Figure 5e, left panel); however, ASO-miR-509-5p reduced the G1-phase cell population by ~24% and increased S-phase cell population by ~51% (Figure 5e, right panel). Furthermore, miR-509-5p enhanced HeLa cell apoptosis, whereas ASO-miR-509-5p reduced HeLa cell apoptosis (Figure 5f).

To further investigate whether miR-509-5p affected cell cycle and apoptosis was mediated by Mdm2, Mdm2 was transfected into miR-509-5p-transfected HeLa cells. We observed that miR-509-5p inhibited cell cycle and induced apoptosis was rescued in the presence of Mdm2 (Figures 5g and h). Therefore, these results collectively indicate that miR-509-5p suppresses the Mdm2-mediated ubiquitination and degradation of p53, and the accumulated p53 induces cell cycle arrest and promotes apoptosis.

Discussion

MiRNAs have been implicated in many critical biological processes. The aberrant expression of miRNAs observed in

various human cancers suggests that miRNAs may play important roles in tumorigenesis.^{24,25} For example, it has been reported that miR-15a and miR-16-1 are downregulated in B-cell chronic lymphocytic leukemia (CLL),²⁶ and miR-143 and miR-145 are downregulated in colorectal cancer.²⁷ Amplification or overexpression of the miR-17-92 cluster and miR-155 was reported in lymphomas.²⁸ In addition, >50% of human miRNA genes are located in cancer-associated genomic regions or at fragile sites.^{29,30} In this study, we found that miR-509-5p can suppress the cell growth capacity of human cervical cancer and HCC cell lines, as well as delay the G1/S-phase transition of the cell cycle and facilitate apoptosis. miR-509-5p is generally downregulated in human cervical cancer and HCC tissues compared with the adjacent nontumor tissues, suggesting that miR-509-5p functions as tumor suppressor and plays an important role in tumorigenesis.

Because of the low expression and cell growth suppression of miR-509-5p in cervical cancer and HCC cells, we wondered whether it was under the control of the tumor suppressor p53 that acts as a transcriptional regulator of numerous genes involved in cell cycle arrest, apoptosis and senescence.

The p53 target genes can be classified as those that primarily induce cell cycle arrest (e.g., the cyclin-dependent kinase inhibitor CDKN1A/p21) and those that mediate apoptosis (e.g., bak);³¹ thus, we speculated that p53 may induce miR-509-5p expression. It has been shown that p53 regulates the expression of a set of specific miRNAs. For example, miR-34a has been shown to be a direct transcriptional target of p53, and this miRNA has been shown to regulate cellular apoptosis and senescence.³² The p53 protein binds to the promoter of miR-34a and increases the expression of miR-34a in response to stress. Inactivation of endogenous miR-34a strongly inhibits p53-dependent apoptosis. To determine whether p53 regulates the expression of more miRNAs, we used a miRNA array to screen for the differential expression of miRNAs when p53 was overexpressed in HeLa cells. Among the 19 differentially expressed miRNAs, the upregulation of miR-509-5p was the most significant change. Furthermore, p53 mediated upregulation of miR-509-5p in cervical cancer and HCC cell lines under stress conditions, such as doxo or serum starvation, as well as following overexpression or depletion of p53. We also confirmed that p53 can directly bind and activate the promoter of miR-509-5p using luciferase reporter and chip assays.

Generally, miRNAs exert functions by binding to the 3'-UTR of the target gene to repress its expression.¹ To identify the target genes of miR-509-5p, we first used bioinformatic analysis to predict a 100 candidate target genes of miR-509-5p. Combining the prediction results and the function of miR-509-5p described above, Mdm2 was chosen as a target gene for further study. Using an EGFP reporter assay, we confirmed the direct binding of miR-509-5p to the Mdm2 3'-UTR; qRT-PCR and western blot analyses showed that miR-509-5p downregulates Mdm2 expression in cervical cancer and HCC cell lines. Furthermore, qRT-PCR showed the inverse correlation between miR-509-5p and Mdm2 expression in the cancer and adjacent nontumor tissues in clinical specimens of cervical cancer and HCC. Moreover, ectopic expression of Mdm2 counteracted the phenotypes induced by miR-509-5p in the cancer cell lines and knockdown of Mdm2 had similar effects compared with overexpression of miR-509-5p. These results suggest that Mdm2 is a direct and functional target gene of miR-509-5p.

As an E3 ubiquitin ligase, Mdm2 binds to p53 to mediate its degradation; in addition, Mdm2 is overexpressed in many human tumors to antagonize p53 function that plays an important role in cancer initiation and development.^{15,16,33} Although some proteins have been found to regulate Mdm2 expression in cancer cells, such as PTEN,³⁴ IGF-1³⁵ and p53, miRNAs such as miR-605 can also target Mdm2 to repress its expression,³⁶ resulting in the formation of a regulatory network. Here, we identified a new regulator of the Mdm2/p53 regulatory loop. Our results indicate that depletion of miR-509-5p increases the level of Mdm2 protein and p53 ubiquitination, whereas overexpression of miR-509-5p decreases p53 ubiquitination that in turn influences the expression of the p53 target genes, p21 and bak. Altogether, p53 can directly bind to its consensus element (p53RE2) in miR-509-5p promoter that results in the induction of miR-509-5p and in turn represses Mdm2 activity to increase the p53 level. Thus, miR-509-5p, Mdm2 and p53 form a positive

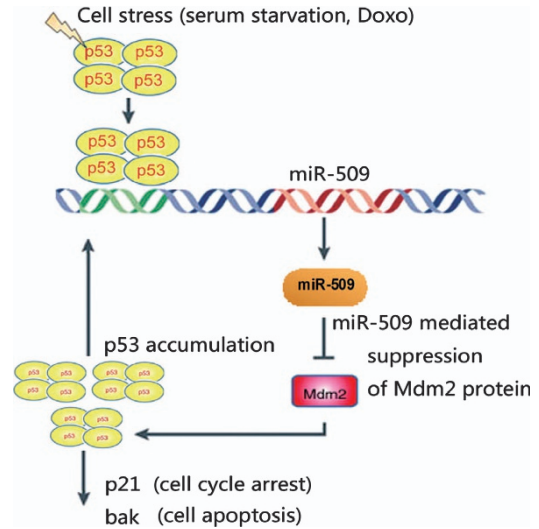


Figure 6 A proposed model describing the participation of miR-509-5p to form a positive feedback loop. In response to cellular stress, miR-509-5p expression is enhanced because of p53 activation, and this miR-509-5p upregulation then directly targets and represses Mdm2 expression and increases the level of p53 protein, leading to the activation of p21 and bak, therefore inducing cell cycle arrest and apoptosis

feedback loop (Figure 6) that provides new insights into the p53-mediated regulation of oncogenesis.

In summary, we have demonstrated here that (1) p53 directly binds to the promoter of miR-509-5p and activates its expression; (2) miR-509-5p suppresses HeLa, QGY-7703 and HepG2 cell growth; (3) miR-509-5p post-transcriptionally represses Mdm2 expression; (4) overexpression of miR-509-5p increases the transactivation activity of p53, resulting in the upregulation of p21 and bak; and (5) miR-509-5p induces cell cycle repression and apoptosis through Mdm2/p53 pathway. Together, our results have defined a mechanism by which miR-509-5p participates in the Mdm2/p53 regulatory pathway and have provided new insights into cervical cancer and HCC tumorigenesis, thus providing the basis for the development of miRNA-targeted therapies for cervical cancer and HCC.

Materials and Methods

Human tissue specimens and cell lines. Cervical cancer tissue specimens from 14 cervical cancer patients were obtained from the Tumor Bank Facility of Tianjin Medical University Cancer Institute and Hospital and National Foundation of Cancer Research (TBF of TMUCIH and NFCR). Fourteen pairs of fresh specimens were collected at the Cancer Center of Sun Yat-sen University of Medical Science, including human liver tumor and adjacent nontumor tissue from patients undergoing hepatectomy for HCC, in accordance with the ethical standards of the institutional committee. These tissue were stored frozen at -80°C according to a standard procedure. The human cervical cancer cell lines (HeLa and Caski) and HCC cell line (QGY-7703) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml penicillin/streptomycin (P/S); HepG2 cells were grown in MEM- α supplemented with 20% FBS and 1000 U/ml P/S. These human cancer cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2 .

Cell transfection. The cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection efficiency was monitored by fluorescence microscopy 48 h after transfection with pcDNA3/EGFP.

RNA preparation and qRT-PCR. RNA extraction of the cells or tissue samples was performed using the mirVana miRNA Isolation Kit (Ambion, Austin,

TX, USA) according to the manufacturer's instructions. Large RNAs (>200 nt) and small RNAs (<200 nt) were separated and purified in this procedure. For miRNA detection, 2 µg of small RNA was reverse transcribed to cDNA using M-MLV (Promega, Madison, WI, USA). QRT-PCR analysis for miR-509-5p was performed in triplicate with the SYBR Premix Ex Taq TM kit (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's instructions. The expression of miR-509-5p was normalized to the levels of U6 RNA. To detect other genes, 5 µg of large RNA was reverse transcribed to cDNA using oligo(dT) primers and M-MLV. QRT-PCR was used to determine the expression levels of Mdm2, p53, E6, p21 and bak using the primers described in Table 1. The levels of these transcripts were normalized to the levels of β-actin.

Plasmid construction. To construct a plasmid expressing miR-509-5p, a 400-bp DNA fragment carrying pri-miR-509-5p was inserted between the *Bam*HI and *Hind*III sites of pcDNA3. The EGFP reporter vector pcDNA3/EGFP was constructed as previously described. The 3'-UTR, including the target sites, of Mdm2 was amplified by qRT-PCR and cloned downstream of EGFP between the *Bam*HI and *Eco*RI sites. Similarly, the 3'-UTR mutant, which contained mutated miR-509-5p-binding sites, was cloned into pcDNA3/EGFP between the same sites. The primer sequences used for qRT-PCR amplification are shown in Table 1. Sequences that were inserted into the plasmid were verified by DNA sequencing.

To clone the putative miR-509-5p promoter, we amplified a DNA fragment (800 bp) from the genomic DNA of HeLa cells. PCR primers were p509-NheI-S and p509-XhoI-AS (Table 1). The PCR product was then subcloned into pGL3 basic (Clontech, Mountain View, CA, USA). The similar strategy was used to make deletion constructs. PCR primer sequences are in Table 1. All PCR products were verified by DNA sequencing.

Cell biology assays. For the MTT assay, HeLa, QGY-7703 and HepG2 cells were seeded in 96-well plates at a density of 4000 cells/well. After 48 h of transient transfection, the cells were incubated with 15 µl of MTT at a final concentration of 0.5 mg/ml at 37°C for another 4 h. The medium was then removed, and the precipitated formazan was dissolved in 100 µl of dimethyl sulfoxide (DMSO). After shaking for 15 min, the absorbance at 570 nm (A 570) was detected using a Quant universal microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

For the colony formation assay, after transfection, the cells were counted and seeded in 12-well plates (in triplicate) at a density of 100 cells/well. The culture medium was replaced every 3 days. Colonies were counted only if they contained more than 50 cells, and the number of colonies was counted from day 6 of seeding after the cells were stained with crystal violet. The rate of colony formation was calculated using the following equation: colony formation rate = (number of colonies/number of seeded cells) × 100%.

For the *in vitro* cell invasion and migration assays, the assays were performed using Transwell chambers (pore size of 8 µm; Costar, Corning, NY, USA) with or without 2 mg/ml Matrigel (Clontech). The transfected cells were resuspended in serum-free medium, and 200 µl of the cell suspension (4 × 10⁴ cells) was added to the upper chamber. Complete medium was added to the bottom wells of the chambers. For the screen, the cells that had not migrated after 20 h were removed, and the cells that adhered to the lower membrane of the inserts were counted, as previously described. Images of three different fields were taken. The mean of triplicate assays for each experimental condition was used. For the invasion assays, the cells that had traversed to the lower membrane after 24 h were counted.

MiRNA microarray. Cloning and sequence amplification of the microRNAs was performed using a microRNA labeling technique. Briefly, 30 µg of purified

Table 1 Oligonucleotides used in this study

Name	Sequence (5'-3')
miR-509-5p RT	GTCGTATCCAGTGCAGGGTCCGAGGTGACTGGATACGACTGATTGC
U6 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACAAAATATGGAAC
miR-509-5p Fwd	TGCGGTACTGCAGACAGTGGCAA
U6-Fwd	TGCGGGTGTCTCGCTTCGGCAGC
Reverse	CCAGTGCAGGGTCCGAGGT
pri-miR-509-S	CGAGGATCCAATGGCCACCCACAATTG
pri-miR-509-AS	CGGAATTCTGAGGTAGTCCAGCATGGAAG
P509-NheI-S	GCCTAGCTAGCGTGTTCATCCCGTGCCTGTTACC
P509-XhoI-AS	ACGAACCTCGAGCTGCAGAATCCAATCCAC
pMir-509p-Luc-2- S	CAGTGGTACCCTTACATGGATGGCGACAGGCA
pMir-509p-Luc-2- AS	ACGAACCTCGAGCTGCAGAATCCAATCCAC
pMir-509p-Luc-3- S	CCGGAATTCGTTAATGCTTTGCAAGTAGCAATGTGA
pMir-509p-Luc-3- AS	ACGAACCTCGAGCTGCAGAATCCAATCCAC
pMir-509p-Luc-4- S	GCCTAGCTAGCGTGTTCATCCCGTGCCTGTTACC
pMir-509p-Luc-4- AS	CCGGAATTCCTCACATTGCTACTTGCAAAGCATTAAC
Mdm2-509-3'-UTR-S	CGCGGATCCTCTCAAAGGTTAGGTGGAC
Mdm2-509-3'-UTR-AS	CGGAATCCCTGAACCTTCCGTAGTCCTT
Mdm2-509-3'-UTR-MS	TAAACTACTGATCGACAGAAGACAGTTGAA
Mdm2-509-3'-UTR-MA	TTCAACTGTCTTCTGTCGATCAGTAGTTTTA
Mdm2- primer1-S	GCGGAAGACAGTGGTGAAC
Mdm2- primer1-AS	AGCTGGAGTAGTCGCTCTGC
Mdm2- primer2-S	CAGGAATCATCGGACTCAGG
Mdm2- primer2-AS	GTTCACTTACACCAGCATCA
Mdm2- primer2-S	AGGGAAGAAACCCAAGAC
Mdm2- primer2-AS	CATACTGGGCAGGGCTTA
p53-primer1-S	CCCAAGCAATGGATGATTTGA
p53-primer1-AS	GGCATTCTGGGAGCTTCATCT
p53-primer2-S	CACTGCCCAACAACACCAGCTCCT
p53-primer2-AS	GTCTGAGTCAGGCCCTTCTGTCTTG
p53-primer3-S	TAACAGTTCCTGCATGGGCGGC
p53-primer3-AS	AGGACAGGCACAACACGCACC
HpV E6-S	ACACGGCGACCCTACAAG
HpV E6-AS	TTTTCTGCTGGATTCAACG
bak sense	CTCAGTTCTCTCCCTTCC
bak antisense	CTCCCTACTCCTTTTCCC
p21 sense	TTTGATTAGCAGCGGAACAAGGAGT
p21 antisense	TGGAGAAACGGGAACCAGGACAC
β-Actin-S	CGTGACATTAAGGAGAAGCTG
β-Actin-AS	CTAGAAGCATTTGCGGTGGAC

small RNA was fractionated using an 8 M urea denaturing 15% polyacrylamide gel, and the RNAs 18–26 nt in length were retrieved and purified. Two oligonucleotide adaptors were then ligated to the 5' and 3' ends of the retrieved RNA using T4 RNA ligase (Fermentas, Waltham, MA, USA). The following adaptor sequences were used: 5' adaptor 5'-CTGTAGGCACCATCAAx-3' (x:DMT-O- C3-CPG) and 3' adaptor 5'-ACTCGAGAAUCCGAAA-3'. A cDNA library was generated by reverse transcription using M-MLV (Promega) with the primer 5'-TTGATGGTGCC TACAG-3' that was reversed complementary to the 5' adaptor. The products were amplified using asymmetric PCR. After the lower-content primers were exhausted, the higher-content primers, which were labeled with Cy3 (for the control group) or Cy5 (for the experimental group), produced single-stranded DNA (ssDNA) samples. The following PCR primers were used: sense 5'-ACTCGAGAATTCG-GAAA-3' and antisense 5'-Cy3/ Cy5-CACCTTGATGGTGCTACAG-3'. A 3 × miRNA Hybridization Buffer (Ambion) was added to the PCR products. Following incubation at 95°C for 3 min, the solution was added on the slide that contained 640 human mature miRNA-associated probes with each probe present in triplicate. Hybridization was carried out for 18–24 h at 42°C, and the slide was washed and then scanned using the ScanArray Express microarray acquisition system of Packard Biochip Technologies (Waltham, MA, USA). All of the oligonucleotide sequences were purchased from Integrated DNA Technology (Coralville, IA, USA).

Cell cycle and apoptosis assays. Transfected cancer cells were seeded into six-well plates and incubated for 24 h in complete medium. Next, the cells were deprived of serum for 48 h and then returned to complete medium for an additional 24 h. All cells were collected by centrifugation, fixed in 95% ethanol, incubated at –20°C overnight and washed with phosphate-buffered saline (PBS). Then, the cells were resuspended in 1 ml of FACS solution (PBS, 0.1% Triton X-100, 60 µg/ml propidium iodide (PI), 0.1 mg/ml DNase free RNase and 0.1% trisodium citrate). After a final incubation on ice for 30 min, the cells were analyzed using a FACS Calibur flow cytometer (Beckman Coulter, Brea, CA, USA). A total of 10 000 events were counted for each sample.

For the TUNNEL assay, the cells were transfected with pcDNA3/pri-miR-509-5p or aso-miR-509-5p and were seeded into triplicate wells on a 14-well slide (Cell-Line/Erie Scientific Co., Portsmouth, NH, USA) at 48 h post transfection. Next, cell apoptosis was detected using the *In situ* Cell Death Detection Kit and Fluorescein (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. DAPI staining was used to determine the number of nuclei and to assess the gross cellular morphology. For Annexin V assay, miR-509-5p or Mdm2 expression plasmid was transfected into HeLa cells. After 48 h, DNA content was determined by PI staining as described by Hwang *et al.*,³⁷ and Annexin V staining was performed with the Vybrant Apoptosis Assay Kit (Invitrogen).

Detection of p53 ubiquitination. Ubiquitination activity was determined using an *in vitro* ubiquitination assay. Cell extracts prepared from the transfected cells were immunoprecipitated with anti-p53 antibody. The presence of ubiquitin-conjugated p53 proteins in the immunoprecipitates was detected by immunoblotting with an anti-ubiquitin antibody.

Western blot analysis. Cells were lysed in RIPA buffer, and the lysates were analyzed using a standard western blot procedure. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous loading control. The following primary antibodies were used: polyclonal rabbit anti-human Mdm2, p53, p21 and bak (Saierbio, Tianjin, China).

ChIP assay. The ChIP assay was performed using EpiQuik™ Chromatin Immunoprecipitation Kit from Epigentek Group Inc. (Brooklyn, NY, USA). Protein-DNA complexes were immunoprecipitated with p53 antibody, a positive control antibody (RNA polymerase II), a negative control normal mouse IgG. GAPDH primers was used as a positive DNA sequence to demonstrate the efficacy of the kit reagents and protocol. RNA polymerase II is considered to be enriched in the GAPDH gene promoter that is expected to be undergoing transcription in most growing mammalian cells, and can be immunoprecipitated by RNA polymerase II, but not by normal mouse IgG. DNA from these samples was then subjected to PCR analysis. Primer sets for p53RE1 were miR509-P53site1-S: 5'-CTGTATTAG CCCATTTTC-3'; miR509-P53site1-AS: 5'-TTTGCCCTGTCGCATCC-3'; primer sets for p53RE2 were miR509-P53site2-S: 5'-CAAGTAGCAATGTGAAAAGG-3'; miR509-P53site2-AS: 5'-CTGCAGAATCCAATCCAC-3'.

Statistical analysis. Student's *t*-test was used to analyze the significance of the differences between sample means obtained from three independent

experiments. The differences were considered statistically significant when **P* < 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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