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의학박사 학위논문

microRNA-34a를 통한 전사인자 p53의 퓨린과 당 대사 조절

p53 regulates purine and glucose metabolism by microRNA-34a

2014년 02월

서울대학교 대학원
의과학과 의과학전공
김 화 연

A thesis of the Degree of Doctor of Philosophy

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February 2014

The Department of Biomedical Sciences

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College of Medicine

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지도 교수 윤 홍 덕

이 논문을 의학박사 학위논문으로 제출함

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p53 regulates purine and glucose metabolism by microRNA-34a

by

Hwa Yeon Kim

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science
at Seoul National University College of Medicine

December 2013

Approved by Thesis Committee:

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ABSTRACT

p53 is a well-known transcription factor that controls cell cycle arrest and cell death in response to a wide range of stresses. Moreover, p53 has been emphasized as a metabolic regulator involved in glucose, glutamine and fatty acid. p53 mutations are observed in about half of cancer cases, and metabolic abnormalities are a distinct feature in tumor cells. Cancer cells rely mainly on glycolysis rather than mitochondrial respiration for energy production, which is called the Warburg effect.

First, we demonstrated that p53-inducible microRNA-34a (miR-34a) repressed inosine 5'-monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme of de novo GTP biosynthesis. Nucleotide biosynthesis is also critical for cell proliferation and the cell division cycle. Nonetheless, little is known about whether p53 regulates nucleotide biosynthesis. Treatment with anti-miR-34a inhibitor relieved the expression of IMPDH upon DNA damage. Ultimately, miR-34a-mediated inhibition of IMPDH resulted in repressed activation of the GTP-dependent Ras signaling pathway. In summary, we suggest that p53 has a novel function in regulating purine biosynthesis, aided by miR-34a-dependent IMPDH repression.

Second, we demonstrated metabolic changes in cancer that occurred through p53. We found that p53-inducible microRNA-34a (miR-34a) repressed glycolytic enzymes (hexokinase 1, hexokinase 2, glucose-6-phosphate isomerase), and pyruvate dehydrogenase kinase 1. Treatment with an anti-miR-34a inhibitor relieved the decreased expression in these enzymes

following DNA damage. miR-34a-mediated inhibition of these enzymes resulted in repressed glycolysis and enhanced mitochondrial respiration. The results suggest that p53 has a miR-34a-dependent integrated mechanism to regulate glucose metabolism.

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Keywords: p53, miR-34a, IMPDH, Hexokinase, Glucose-6-phosphate isomerase, Pyruvate dehydrogenase kinase 1

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LIST OF ABBREVIATIONS

miR-34a	**MicroRNA-34a**
IMPDH	**Inosine 5’-monophosphate dehydrogenase**
ADR	**Adriamycin**
HK	**Hexokinase**
GPI	**Glucose-6-phosphate isomerase**
PDK1	**Pyruvate dehydrogenase kinase 1**
TIGAR	**TP53-induced glycolysis and apoptosis regulator**
CCND1	**Cyclin D 1**
CDK6	**Cyclin-dependent kinase 6**

CHAPTER 1

General introduction

The tumor suppressor p53 plays diverse roles in the response to cellular stress (Fig. 1-1) (1, 2). Besides the well-known functions of p53 in the cell cycle, apoptosis and aging, recently described functions of p53 as metabolic regulator suggest roles in normal cellular homeostasis as well as in cancer cell homeostasis (3). For example, p53 can modulate glycolysis, mitochondrial respiration, glutaminolysis and fatty acid oxidation (Fig. 1-2) (4-7).

Furthermore, p53 transactivates several microRNAs, particularly the miR-34 family targets broad spectrum genes involved in the cell cycle, apoptosis and DNA damage (Fig. 1-3) (8-11). MicroRNAs (miRNAs) are a family of noncoding RNAs (18–22 nucleotides) that play important roles in physiological processes (12, 13). Mature miRNAs regulate target gene expression by degrading mRNA or disturbing protein translation (14). A specific miRNA has multiple targets, and one gene can be regulated by multiple miRNAs. miRNAs modulate cell proliferation, cell death, differentiation, apoptosis and cell signaling pathways (15, 16). In addition, several studies have shown that miRNAs have functions involved in energy metabolism including glucose and lipid metabolism and amino acid biogenesis (17, 18). For example, miR-122 affects hepatic cholesterol and fatty acid metabolism by regulating genes in the cholesterol biosynthesis pathway (19), and miR-143 controls glycolysis by targeting hexokinase 2 (20, 21).

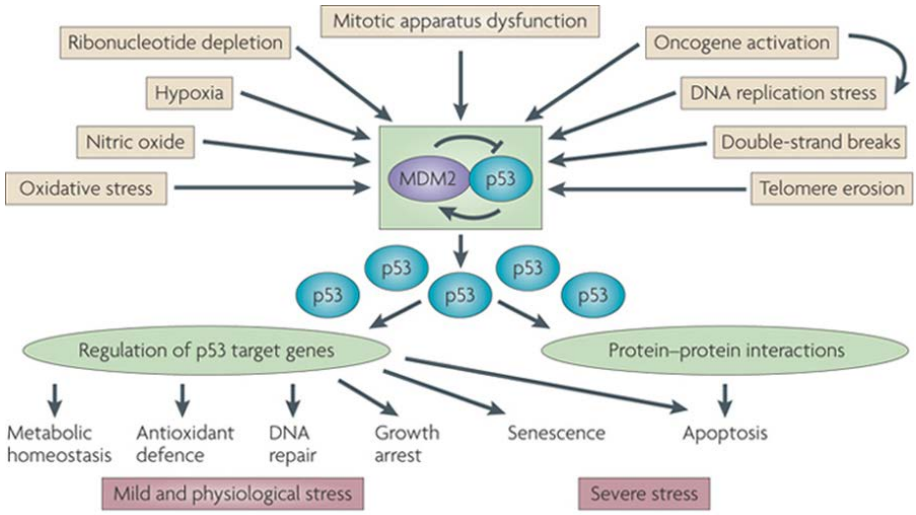

Figure 1-1. Simplified scheme of the p53 pathway.

Under normal conditions, it maintains constantly low steady-state p53 levels and activity by MDM2. Various stress signals impinge on this central loop to release p53 from MDM2-mediated inhibition. This increases p53 protein levels and activity, inducing various subsets of target genes. Severe stress induces apoptosis and senescence, whereas milder stress leads to a transient growth arrest coupled with an attempt to deal with the cause of stress and repair the damage caused by it. Recent evidence indicates that p53 also has an important role in enabling the cell to adjust its metabolism in response to mild normal physiological fluctuations (2).

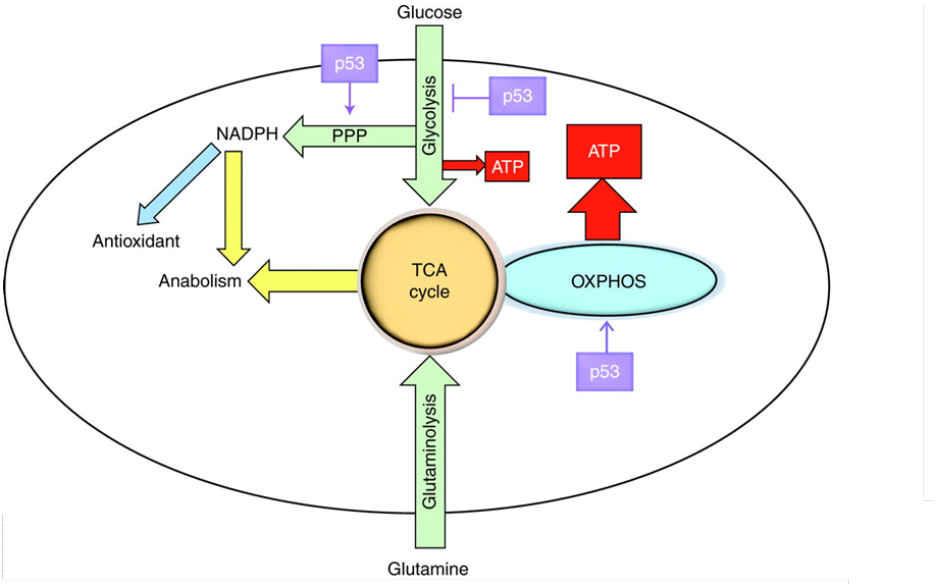

Figure 1-2. The main energy-generating metabolic pathways, and their regulation by p53.

By promoting oxidative phosphorylation and repressing glycolysis, p53 might oppose the Warburg effect that is seen in many cancers. Induction of the pentose phosphate pathway would also provide survival functions and may contribute to anabolic pathways necessary for damage repair (7).

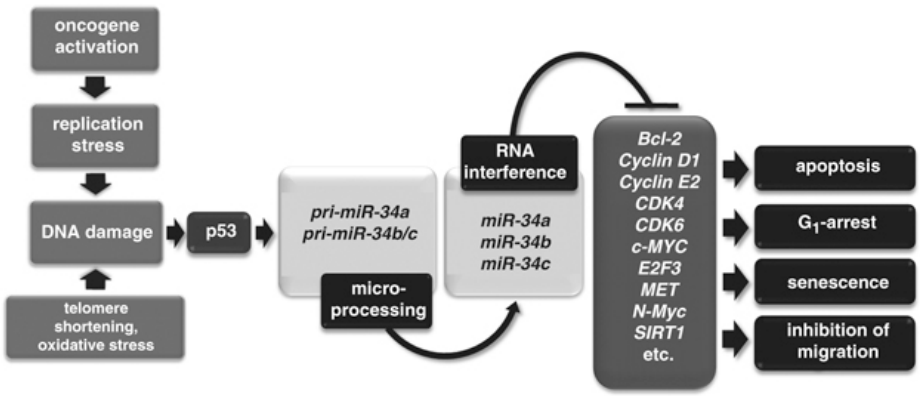

Figure 1-3. The miR-34 family as mediator of tumor suppression by p53.

After cellular stresses p53 is activated through ATM-kinases and transactivates target genes. The primary transcripts of the induced miR-34 genes are processed by DROSHA and DICER complexes. The mature miRNA mediates inhibition of translation or RNA degradation of the various targets (11).

In these studies, we focused on the roles of p53-miR-34a pathway concerning several cellular metabolic processes.

CHAPTER 2

A p53-inducible microRNA-34a downregulates Ras signaling by targeting IMPDH

1. INTRODUCTION

IMPDH is a rate-limiting enzyme in the de novo synthesis of purine nucleotides (Fig. 2-1) (22). Due to its role in GTP biosynthesis, IMPDH has been implicated in the oncogenic functions of various cancers. Numerous studies have demonstrated a role for IMPDH in cancer, with strong correlation between IMPDH activity and proliferating properties (23).

Until now, two isoforms of IMPDH (IMPDH1 and IMPDH2) have been identified, each consisting of 514 amino acids with 84% sequence identity (24). Whereas IMPDH1 is constitutively expressed in normal cells, IMPDH2 is selectively up-regulated in proliferating cells, especially in leukocytes and tumor cells (25, 26). Moreover, accumulation of IMPDH2 is reported in human ovarian cancer, leukemic cells from patients with chronic granulocytic/ lymphocytic and acute myeloid leukemias (27, 28). According to these clinical results, imbalance of purine metabolism occurs in cancer cells. For instance, up-regulation of purine metabolic enzymes like IMPDH, GMP synthase (GMPS), adenylosuccinate synthetase (ADSS), adenylosuccinase (ADSL), AMP deaminase (AMPD) and amidophosphoribosyltransferase (ATase) was identified in transplantable hepatomas in rat (29). Interestingly, overall reduction of guanine nucleotide pools by IMPDH inhibition influences the interruption of DNA and RNA synthesis, cell cycle arrest, differentiation and cell death (30). Therefore IMPDH is an attractive target for anticancer drugs, antiviral drugs and immunosuppressive chemotherapy (31, 32).

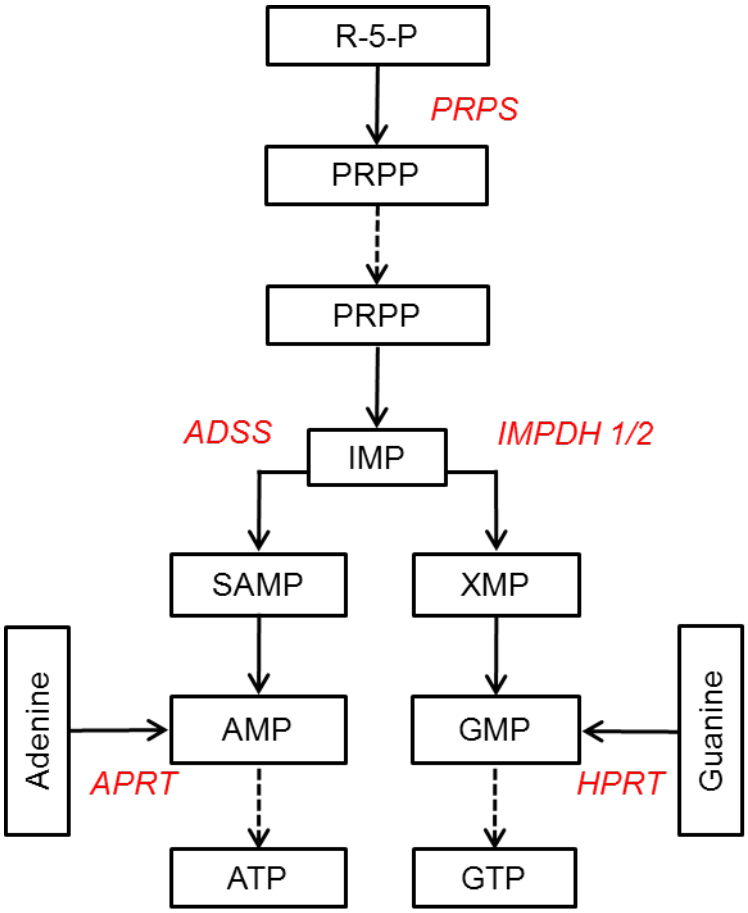

Figure 2-1. Schematic representation of purine metabolism.

Considering that p53 is associated with various metabolic pathways and abnormal metabolism is a distinct feature in tumor cells, we speculate that p53 has a regulatory function in the purine metabolic pathway. Although IMPDH has been suggested as a rate-determining factor for p53-dependent growth regulation and for p53-dependent asymmetric self-renewal (33, 34), the precise molecular mechanism for direct regulation of the purine metabolic pathway by p53 has not been fully elucidated yet. Thus, we investigated the mechanism for p53-dependent purine metabolism regulation.

2. MATERIALS AND METHODS

1. Cells

HEK293, H1299 and HCT116 cells were obtained from ATCC. H1299/RasG12V stable cells were selected using G418 (1 mg/ml) in H1299 cells transiently transfected with pcDNA3-RasG12V plasmid. Cells were cultured in DMEM containing 10% (v/v) fetal bovine serum and 50 U/ml of streptomycin and penicillin.

2. Plasmids and antibodies

The expression vector for full-length IMPDH2 was generated by inserting PCR fragments into pCMV-Tag2B-Flag. The antibodies used in this study were as follows: anti-Flag (M2) and anti-β-actin monoclonal antibodies were purchased from Sigma; anti-p53 (DO-1), anti-p21 (sc-6246), anti-c-jun (sc-1694) from Santa Cruz Biotechnology; anti-IMPDH1 (ab33039) from Abcam; anti-Ras (610001) from BD Transduction Laboratories™; anti-His-Tag (#2366), anti-JNK (#9258), anti-phospho-JNK (#9251), anti-phospho-c-jun (#9164) from Cell Signaling.

3. Quantitative real-time PCR (q-PCR)

Total RNA was isolated using QIAzol lysis reagent (Qiagen) and was reverse-transcribed with reverse transcriptase (AMV-XL reverse transcriptase,

Takara) using random hexamers (Takara). mRNA levels were quantified by qRT-PCR using DyNAmo HS qPCR master mix (Thermo Scientific) and monitored with the iQ5 Real-Time PCR Detection System (Bio-Rad). Gene expression was defined by the threshold cycle (Ct), and relative expression levels were calculated using the 2-Ct method. The primers for real-time PCR were described in Table 2-1. To measure endogenous miR-34a, the Taqman microRNA assay kit was purchased from Applied Bioscience.

4. Luciferase assay

500 ng of pGL3UC luciferase vector including either 3' UTR of IMPDH1 (with the WT or mutant miR-34a response element) or IMPDH2 CDS region (425-814 nucleotides) were transfected by Lipofectamine (Invitrogen). At 4-hr post-transfection, cells were infected with adenovirus expressing miR-34a or LacZ (control). Luciferase assays were performed with the dual luciferase reporter assay system (Promega) 72 hr after adenovirus infection and were normalized to renilla luciferase.

5. Northern blot

Northern blot was performed as described previously (35). The sequence of the probe for miR-34a was 5'- acaaccagctaagacactgcca-3'.

gene	sequence
Human IMPDH1	forward: 5'-TTGCTGAGAAGGACCACAC-3'
	reverse: 5'-TCATTGACGATAGGCAGCTTC-3'
Human IMPDH2	forward: 5'-GGGCATCATCTCCTCCAGGG-3'
	reverse: 5'-TGCTGCGCTGCAGAATTTCA-3'
Human PRPS	forward: 5'-TGCAGAGCCGGCTGTCCTAA-3'
	reverse: 5'-ACAAGCACCATGCGGTCCAC-3'
Human ADSS	forward: 5'-ATGGCGTTCGCCGAGACCTA-3'
	reverse: 5'-CCACCTTCCCTTTGCCTTCG-3'
Human APRT	forward: 5'-CACCCCAGGCGTGGTATTCA-3'
	reverse: 5'-TCCAGGGAATAGGAGGCCCA-3'
Human HPRT	forward: 5'-GACCAGTCAACAGGGGACAT-3'
	reverse: 5'-AACACTTCGTGGGGTCCTTTTC-3'
Human p21	forward: 5'-GCAGACCAGCATGACAGATTT-3'
	reverse: 5'-GGATTAGGGCTTCCTCTTGGA-3'
Human TIGAR	forward: 5'-TCGGGAAAGGAAATACGGGG-3'
	reverse: 5'-TCCACGCATTTTCACCTGGTC-3'
Human β-actin	forward: 5'-ACGTTGCTATCCAGGCTGTGCTAT-3'
	reverse: 5'-TTAATGTCACGCACGATTTCCCGC-3'

Table 2-1. Primers used in Chapter 2.

6. IMPDH activity assay

Cell lysates were added to 150 μL assay buffer (100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 150 mM KCl and 1 mM EDTA). The reaction was initiated by the addition of substrates (1 mM IMP and 2.5 mM NAD^+), and then IMPDH activity was measured by monitoring the production of NADH at 340nm using a spectrophotometer (36).

7. Treatment of miR-34a inhibitor

Anti-miR-34a inhibitor (anti-miR-34a) and control inhibitor were purchased from PANAGENE INC. Prior to adriamycin (0.4 μg/ml) treatment, HCT116 cells were treated with 0.5 μM anti-miR-34a for 24 hr.

8. Purification of bacterial proteins

cDNA of IMPDH2 was inserted into pRSET-A, and c-Raf (RBD) was subcloned into pGEX-5X-1. Purification of recombinant GST-fused c-Raf (RBD) and His-IMPDH2 was performed as described previously (37).

9. Virus production

Ad-LacZ and Ad-p53 amplification and treatment were performed as described previously (38). Ad-miR-34a was subcloned into pAd/CMV/V5-DEST (Invitrogen) using the gateway system. For lentiviral-mediated RNA interference, pLKO-IMPDH1, IMPDH2 and p53 were purchased from Open Biosystems.

3. RESULTS

1. p53 downregulates IMPDH

To examine the role of p53 in purine metabolism, we first measured the mRNA level of genes involved in the purine metabolic pathway (Fig. 2-1). With adenoviral expression of p53 in p53-null H1299 cells, successful p53-mediated transactivation was confirmed by increasing amounts of mRNA of well-known p53 target genes (p21 and TIGAR) (Fig.2-2A) (39, 40). Interestingly, mRNA levels of IMPDH1 and IMPDH2 specifically decreased with p53 overexpression, compared to other metabolic enzymes in the purine biosynthetic pathway (Fig. 2-2A and Fig. 2-3). Consistent with this result, the protein level of IMPDH was also down-regulated by p53 expression (Fig. 2-2B). Since IMPDH1 and IMPDH2 share 84% amino acid homology, it is difficult to discriminate between endogenous IMPDH1 and IMPDH2 by immunoblot with commercially-available antibody. In addition, both recombinant IMPDH2 purified from bacteria and overexpressed Flag-IMPDH2 extracted from cell lysates were detectable with anti-IMPDH1 antibody (Fig. 2-4). Next, to confirm whether endogenous p53 can regulate the stability of IMPDH1 and IMPDH2, the protein level of IMPDH was investigated in wild-type p53-harboring HCT116 cell lines, with or without adriamycin treatment. As adriamycin treatment induced p53 stabilization and p21 expression, it reduced the protein level of IMPDH (Fig. 2-5A). However when HCT116 cells were infected with lentivirus encoding shRNA specific for p53 (shp53), there was no change in IMPDH protein level upon adriamycin treatment. The change in mRNA level of IMPDH1 and IMPDH2,

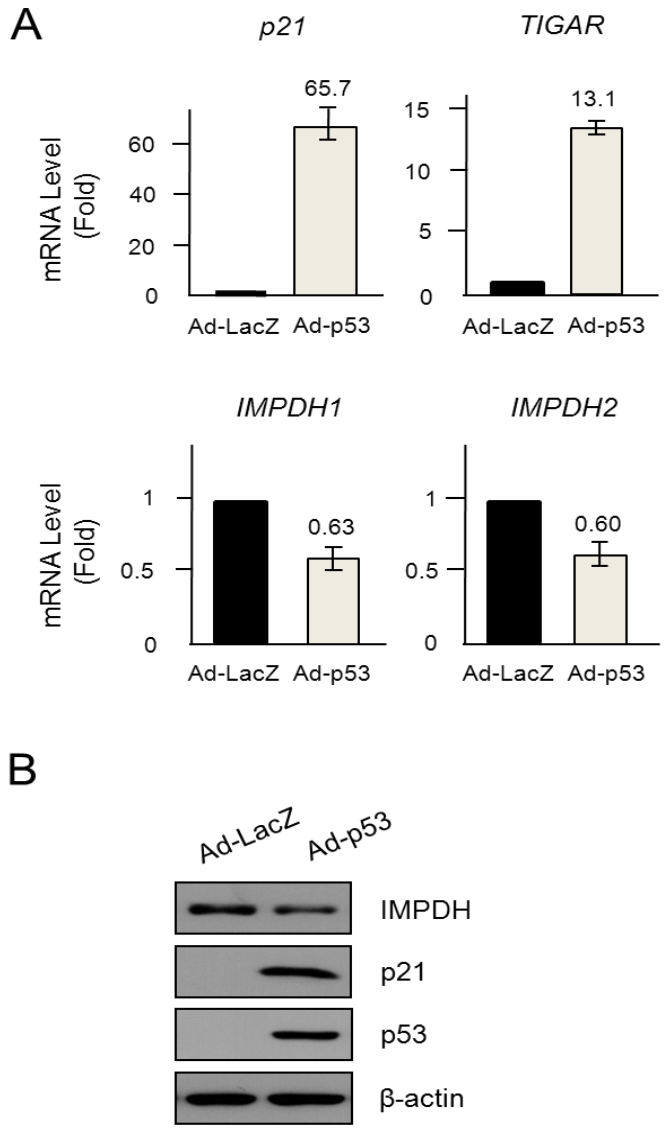

Figure 2-2. Over-expression of p53 reduces IMPDH expression.

(A) p53 decreases mRNA levels of *IMPDH1* and *IMPDH2*. After adenoviral expression of p53 in *p53* null H1299 cells for 24 hr, mRNA levels of indicated genes were measured by qRT-PCR. (B) Confirmation of protein levels of IMPDH, p21 and p53 under the same conditions as (A).

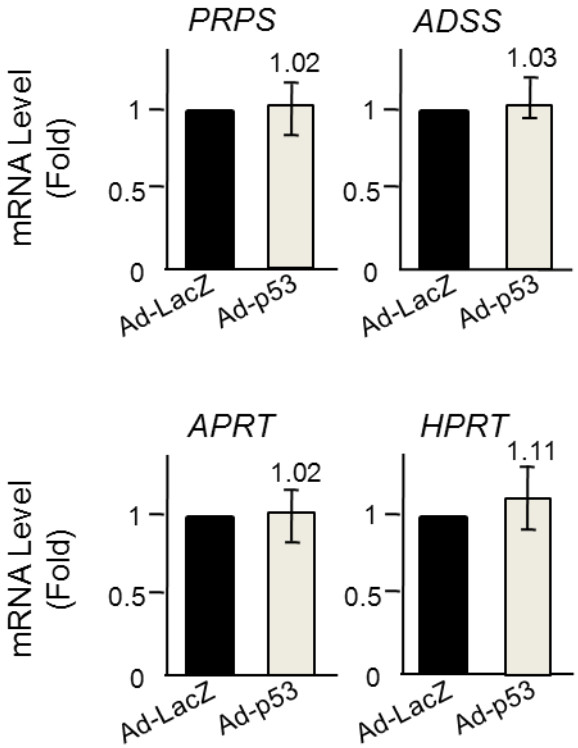

Figure 2-3. Other purine metabolic enzymes are not affected by p53 over-expression.

In same conditions with Fig. 2-2, mRNA levels of *PRPS* (phosphoribosyl pyrophosphate synthetase), *ADSS* (adenylosuccinate synthetase), *APRT* (adenine phosphoribosyltransferase) and *HPRT* (hypoxanthine phosphoribosyl- transferase) were measured by qRT-PCR.

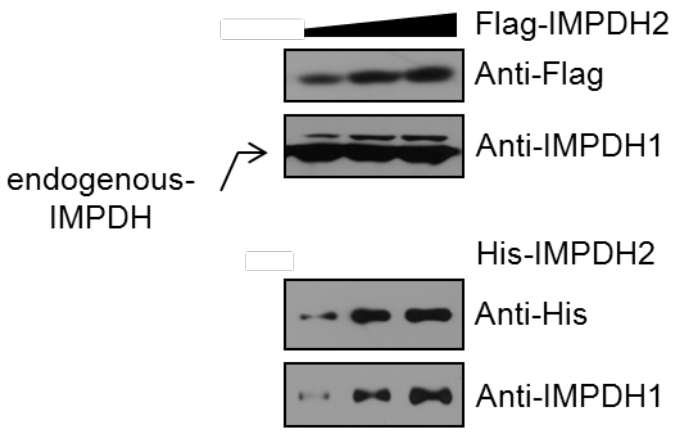

Figure 2-4. anti-IMPDH1 antibody recognizes IMPDH2 protein.

Both mammalian expressed Flag-IMPDH2 and bacterial expressed $(His)_6$-IMPDH2 were detectable with anti-IMPDH1 antibody.

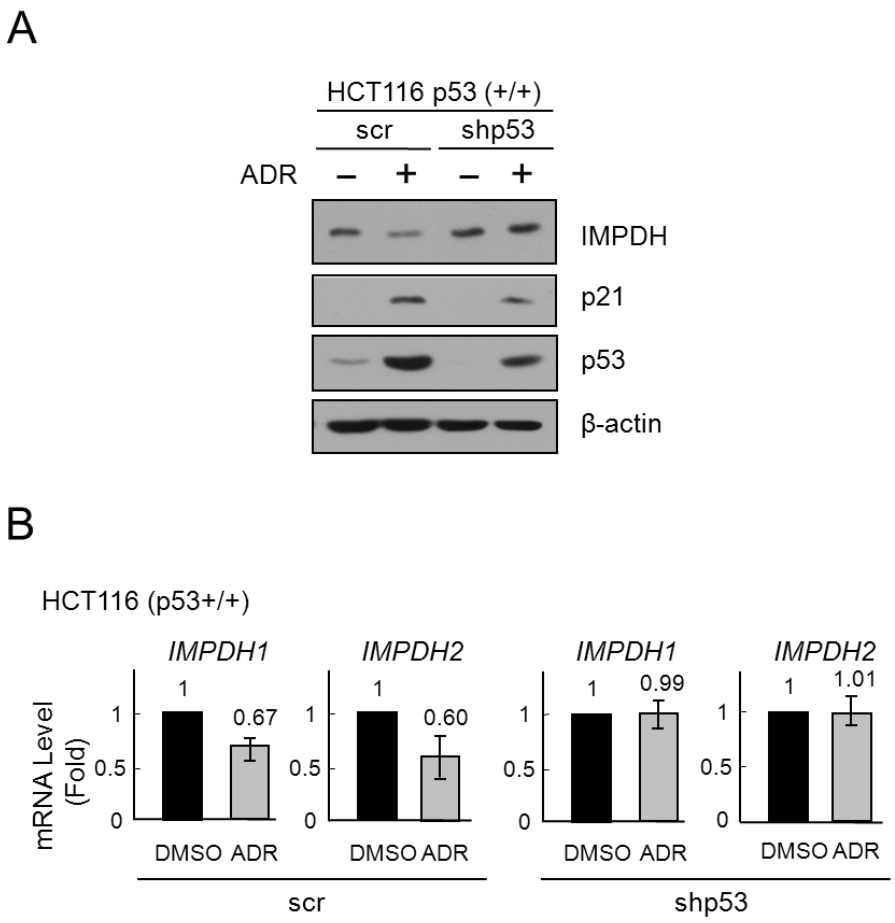

Figure 2-5. Transactivation of endogenous p53 reduces expression of IMPDH.

Pre-infected HCT116 (wild-type p53) cells with control (scr; scrambled) or shp53 (p53-knockdown) were treated with DMSO (control) or adriamycin (ADR, 0.4μg/ml) for 24 hr. (A) Cell lysates were then immunoblotted with anti-IMPDH, anti-p21 and anti-p53 antibodies. (B) The mRNA levels of *IMPDH1*, *IMPDH2* measured by qRT-PCR.

when HCT116 cells were infected with lentivirus encoding shRNA specific for p53 (shp53), there was no change in IMPDH protein level upon adriamycin treatment. The change in mRNA level of IMPDH1 and IMPDH2 was also consistent with the change in protein level of IMPDH (Fig. 2-5B). However, the mRNA levels of other purine metabolic enzymes were not changed upon p53 transactivation (Fig. 2-6).

2. miR-34a inhibits IMPDH function

The major consequence of p53 activation upon DNA damage is the induction of a variety of target genes to cope with genotoxic stress (41). Moreover, p53 can regulate non-coding RNAs (ncRNAs) like microRNAs (miRNAs) or long intergenic non-coding RNAs (lincRNAs) (42, 43). Importantly, transcriptional repression of specific genes by p53 is regulated by p53-targeted miRNAs, like miR-34a (8). Thus we speculated whether p53-inducble miRNA may regulate transcriptional repression of IMPDH. Intriguingly, we found a putative miR-34a targeting sequence in the 3' UTR of IMPDH1 mRNA (Fig. 2-7). To verify that miR-34a can directly interact with mRNA of IMPDH1, an adenovirally expressed form of miR-34a was first generated and validated with northern blot (Fig. 2-8). Then the IMPDH1 3' UTR region containing the putative miR-34a target sequence was inserted into a mammalian luciferase reporter vector. Through point-mutation of the miR-34a targeting region within IMPDH1 3'UTR, we concluded that miR-34a directly targets the putative site within the 3'UTR of IMPDH1 (Fig. 2-9).

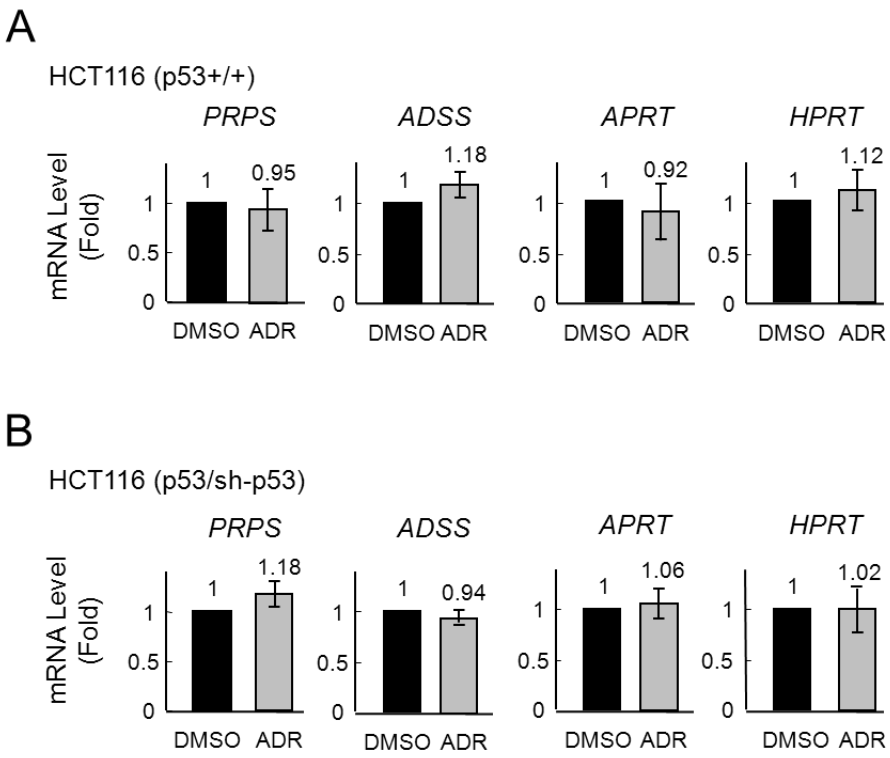

Figure 2-6. Other purine metabolic enzymes are not affected by p53 transactivation.

mRNA levels of four enzymes above was not changed under the same conditions as Fig. 2-5.

Figure 2-7. miR-34a target site within 3'UTR of *IMPDH1*.

The underlined sequence on *IMPDH1* 3'UTR was mutated by site-directed mutagenesis (*IMPDH1-MUT*).

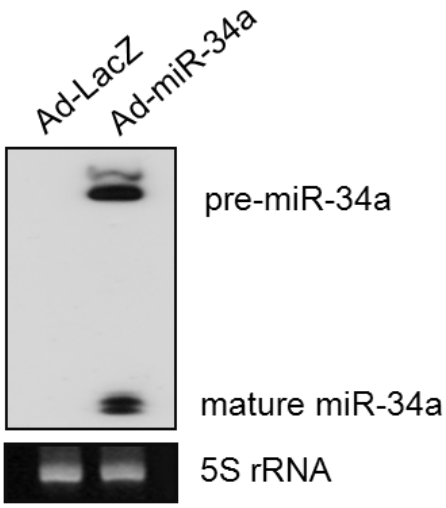

Figure 2-8. Generation of adenovirally expressed miR-34a.

The expression of miR-34a was confirmed by northern blot analysis. 5s rRNA was shown as a loading control using RT-PCR.

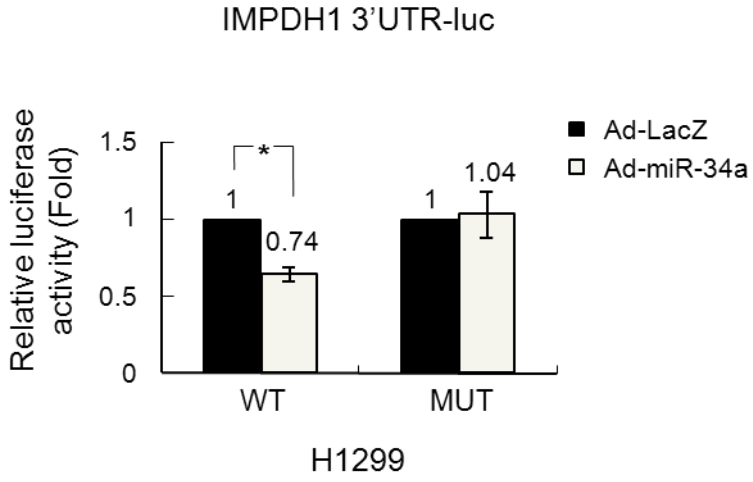

Figure 2-9. 3'UTR of *IMPDH1* is a direct target of miR-34a.

H1299 cells were transfected with a reporter vector containing the partial 3'UTR of *IMPDH1* wild type (*IMPDH1-WT*) or mutant (*IMPDH1-MUT*). Luciferase activities were normalized to renilla luciferase activities. *P < 0.05.

Unlike in IMPDH1, we failed to find a miR-34a target sequence in the 3'UTR of IMPDH2. However, target sequences of microRNAs occasionally exist within the protein coding region (44, 45), and we identified the putative miR-34a target sequence in exon 7 of IMPDH2 (Fig. 2-10A). Therefore, we tested whether miR-34a targets the IMPDH2 exon 7 fragment harboring the miR-34a target sequence using luciferase reporter gene. As a result, miR-34a directly targets IMPDH2 through a CDS-located miR-34a-binding site (Fig. 2-10B). We next investigated whether the binding of miR-34a to IMPDH mRNA effectively represses transcription. When the adenovirally expressed form of miR-34a was infected into H1299 cells, transcriptional repression of both IMPDH1 and IMPDH2 was observed, especially in proportion to the adenoviral infection time (Fig. 2-11). Moreover, the transcriptional repression of IMPDH1 and IMPDH2 was directly linked to translational inefficiency (Fig. 2-12). If IMPDH expression was transcriptionally inhibited by miR-34a-mediated repression, the enzymatic activity of IMPDH should be decreased upon miR-34a activation. As expected, a decrease in cellular IMPDH activity was observed by adenoviral infection of miR-34a (Fig. 2-13).

3. miR-34a downregulates IMPDH upon DNA damage

To show the direct effect of miR-34a on IMPDH inactivation, specific anti-miR-34a inhibitor (anti-miR-34a) was used for antisense inhibition of miR-34a. We first confirmed that anti-miR-34a can specifically inhibit endogenous

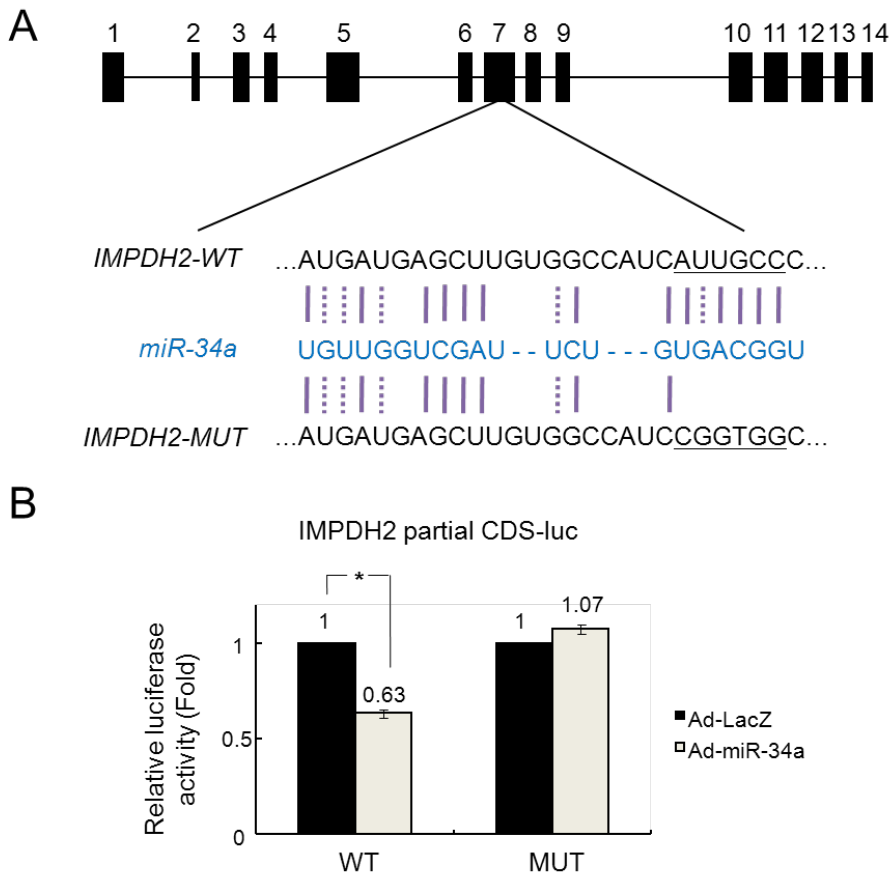

Figure 2-10. miR-34a directly targets IMPDH2 through a CDS-located miR-34a-binding site.

(A) Targeting site of miR-34a located in coding region (exon 7) of *IMPDH2-WT*. The underlined sequence on IMPDH2 was mutated by site-directed mutagenesis (*IMPDH2-MUT*). (B) miR-34a directly targets IMPDH2 through coding region. H1299 cells were transfected with reporter vector containing wild type (*IMPDH2-WT*) or mutant (*IMPDH2-MUT*) of IMPDH2 exon 7. Luciferase activities were normalized by renilla luciferase activities. *P < 0.05.

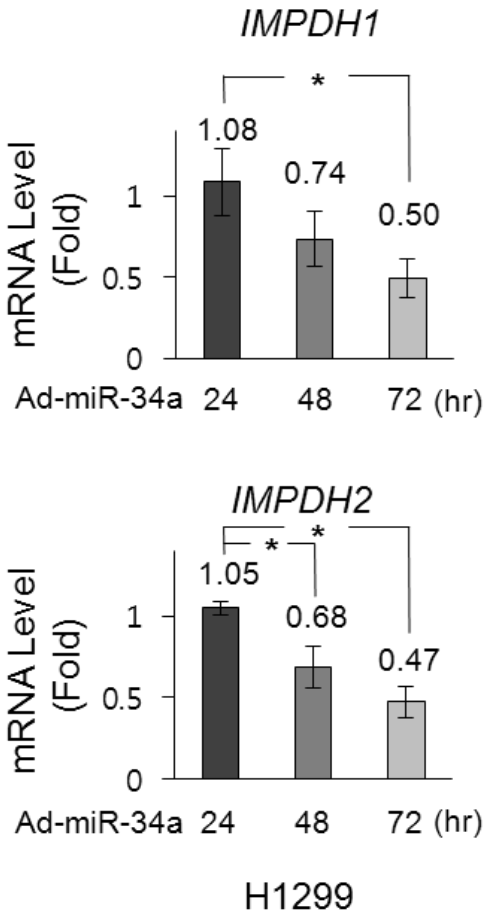

Figure 2-11. miR-34a decreases mRNA levels of IMPDH1 and IMPDH2.

In proportion to time of infection for Ad-miR-34a, IMPDH mRNA levels were reduced compared to Ad-LacZ infection control. *P < 0.05.

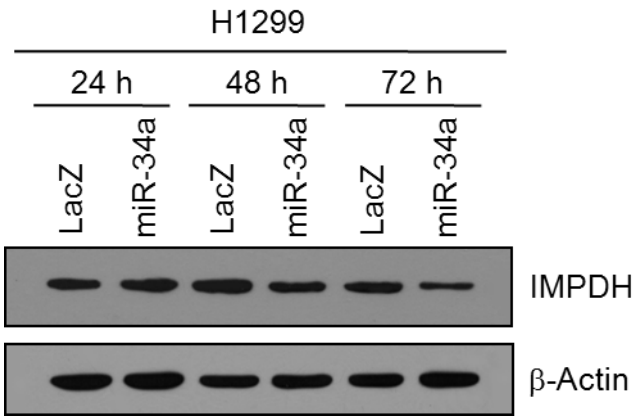

Figure 2-12. miR-34a represses expression of IMPDH in protein level.

In proportion to time of infection for Ad-miR-34a, IMPDH protein levels were reduced compared to Ad-LacZ infection control.

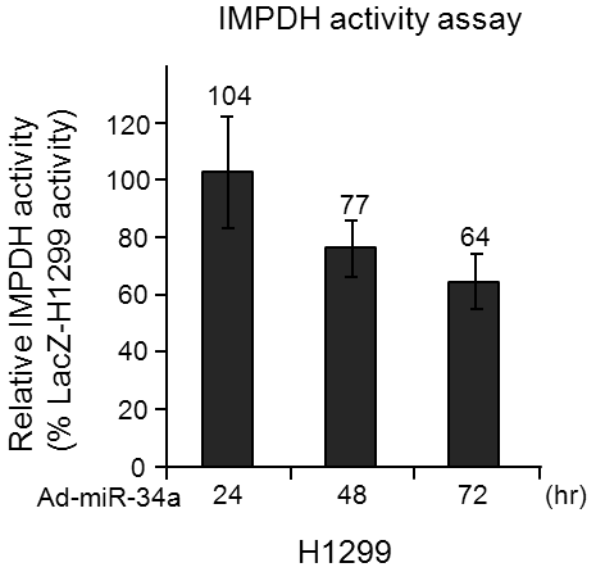

Figure 2-13. Enzymatic activity of IMPDH decreases upon miR-34a activation.

In proportion to time of infection for Ad-miR-34a, IMPDH activity was measured by monitoring the production of NADH at 340nm using a spectrophotometer.

miR-34a expression under conditions of DNA damage (Fig. 2-14). Notably, treatment of anti-miR-34a into HCT116 cells increased the expression of IMPDH1 and IMPDH2 mRNA under quiescent and DNA-damaging conditions (Fig. 2-15). The expression pattern of IMPDH was consistent with that of its mRNA (Fig. 2-16), indicating that miR-34a directly regulates IMPDH transcription.

4. miR-34a-mediated IMPDH repression affects GTP-dependent Ras signaling pathway

To understand the physiological context of miR-34a-dependent inactivation of IMPDH, we wanted to observe the effect of miR-34a on GTP-dependent Ras signaling pathway. We first investigated whether miR-34a interrupts the production of cellular GTP, the final product of the IMPDH-mediated purine metabolic pathway, by observing the interaction between Ras and Raf. A GST-fusion protein of Raf-RBD (Ras-binding domain, 1-149 amino acids) has long been used as an affinity precipitation assay to quantitate the level of activated Ras bound to GTP (37). As expected, the GST-Raf-RBD-Ras complex formation was significantly reduced in HCT116 cells upon DNA damage (Fig. 2-17, left). However, treatment with guanosine, which would replenish GTP pools via the salvage pathway, restored GST-Raf-RBD-Ras complex formation, even under DNA damaging conditions. Similarly, overexpression of miR-34a reduced the formation of GST-Raf-RBD-Ras

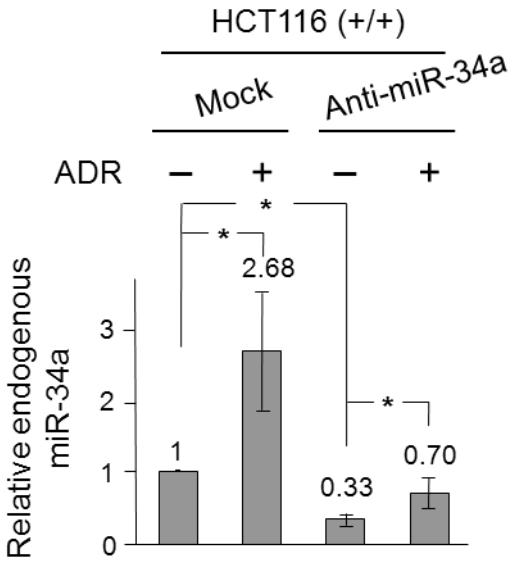

Figure 2-14. miR-34a transactivation upon DNA damage is impaired by anti-miR-34a inhibitor (anti-miR-34a).

Prior to adriamycin treatment, HCT116 cells were treated with anti-miR-34a (0.5 μM) or control inhibitor for 24 hr. Endogenous level of miR-34a was quantified by TaqMan MicroRNA Assay. *P < 0.05.

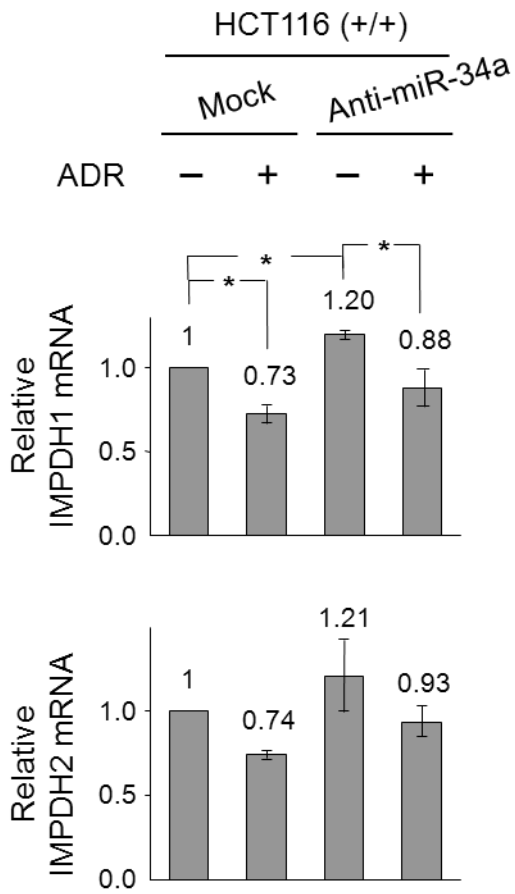

Figure 2-15. DNA damage-dependent repression of IMPDH transcription is relieved by miR-34a inhibition.

In the same conditions as Fig. 2-14, mRNA levels of *IMPDH1* and *IMPDH2* were measured by qRT-PCR. *P < 0.05.

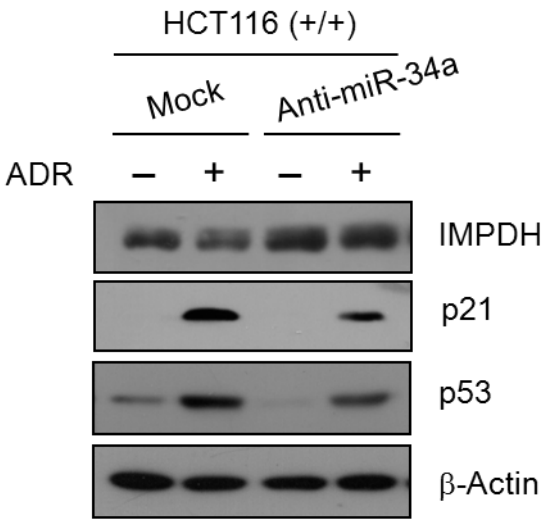

Figure 2-16. DNA-damage dependent repression of IMPDH in protein levels is moderated by miR-34a inhibition.

Prior to adriamycin treatment, HCT116 cells were treated with anti-miR-34a (0.5 μM) or control inhibitor for 24 hr. IMPDH protein level was confirmed by immunoblotting.

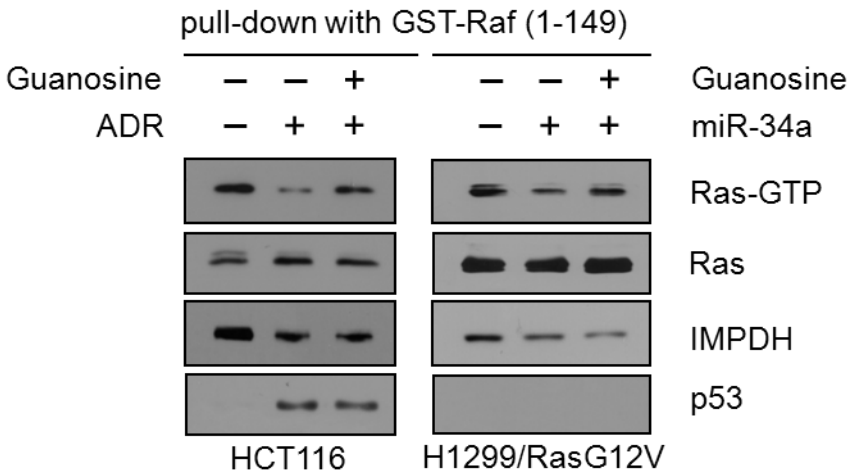

Figure 2-17. GTP-bound forms of Ras decrease upon DNA damage or miR-34a transactivation and can be partially restored by guanosine treatment. HCT116 cells were treated with adriamycin and with or without guanosine (25 μM) for 24 hr. H1299/RasG12V stable cells were infected with Ad-miR-34a for 48 hr and then treated with guanosine (25 μM) for 24 hr. These cell lysates were pulled-down with GST-Raf RBD, and then immunoblotted with anti-Ras antibody.

complex; however, guanosine-treatment prevented miR-34a-mediated deformation between Ras and Raf (Fig. 2-17, right).

Activation of Ras triggers a signaling cascade that leads to activation JNK by MKK4-dependent phosphorylation on Thr 183/185, and consequent phosphorylation of c-Jun at Ser 63/73 (46, 47). To elucidate whether inactivation of IMPDH can affect Ras-dependent signaling cascade, we investigated the phosphorylation levels of JNK and c-Jun in H1299 stable cells expressing the active form of Ras mutant, Ras G12V, after infection of either adenovirally-expressed miR-34a or lentivirally-expressed shIMPDH. Consistent with previous results, either miR-34a expression or knockdown of IMPDH inhibited Ras-dependent phosphorylation of JNK and c-Jun, which implies that inactivation of IMPDH depleted the cellular GTP required for Ras signaling activation (Fig. 2-18). Consequently, Ras-dependent AP-1 transactivation was significantly decreased by overexpression of miR-34a and knockdown of IMPDH in the AP-1 luciferase reporter gene assay (Fig. 2-19). We next determined the expression level of downstream target genes of activated c-Jun, CCNA2 and CDK4 mRNA using quantitative RT-PCR. As expected, miR-34a significantly reduced the mRNA levels of these genes under quiescent and Ras-activated conditions (Fig. 2-20). We concluded that the p53-inducible microRNA, miR-34a, can regulate the GTP-dependent Ras signaling pathway by targeting the purine metabolic enzyme, IMPDH.

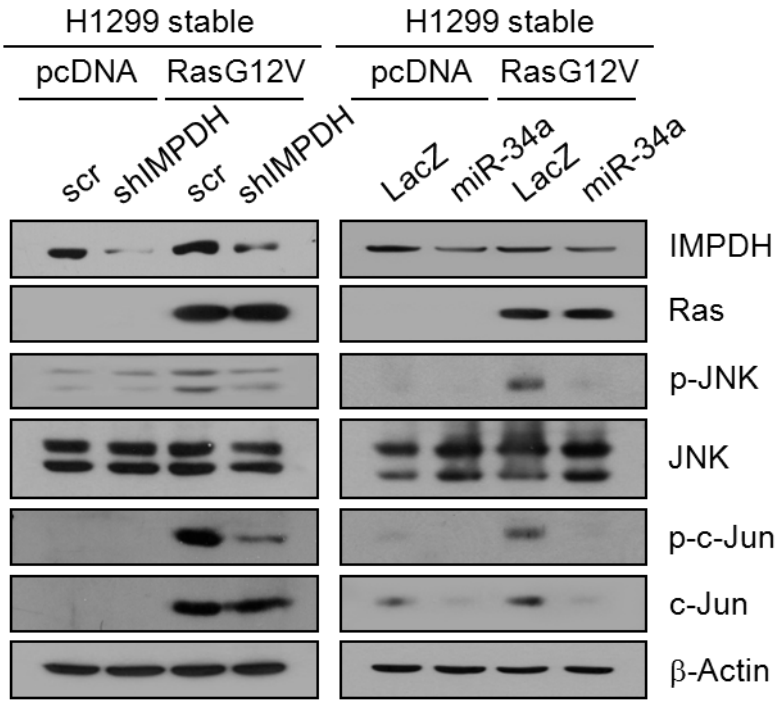

Figure 2-18. miR-34a-mediated IMPDH repression affects GTP-dependent Ras signaling pathway.

H1299/pcDNA or H1299/RasG12V stable cells were infected with lentiviral shIMPDH (*left*) or adenoviral miR-34a (*right*). These cell lysates were immunoblotted with antibodies of Ras-downstream factors; anti-JNK, anti-phospho-JNK (Thr183/Thr185), anti-c-Jun and anti-phospho-c-Jun (Ser63/Ser73).

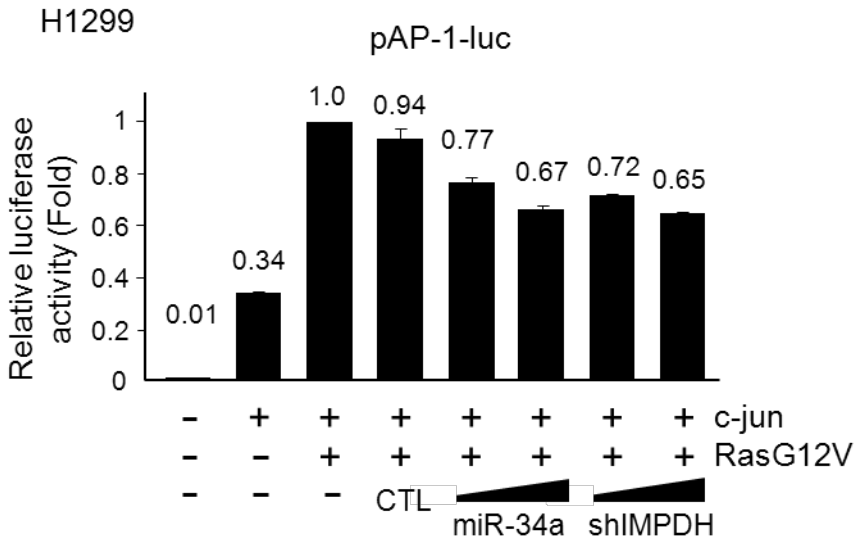

Figure 2-19. pAP-1 transactivation is repressed by miR-34a transactivation or direct IMPDH inhibition.

H1299 cells were transfected with pAP-1-luciferase vector along with c-Jun and RasG12V expression plasmid. At 4-hr post-transfection, cells were infected with either miR-34a-expressing adenovirus or shIMPDH-expressing lentivirus for 72 hr. Relative luciferase activity was measured using dual luciferase reporter assay.

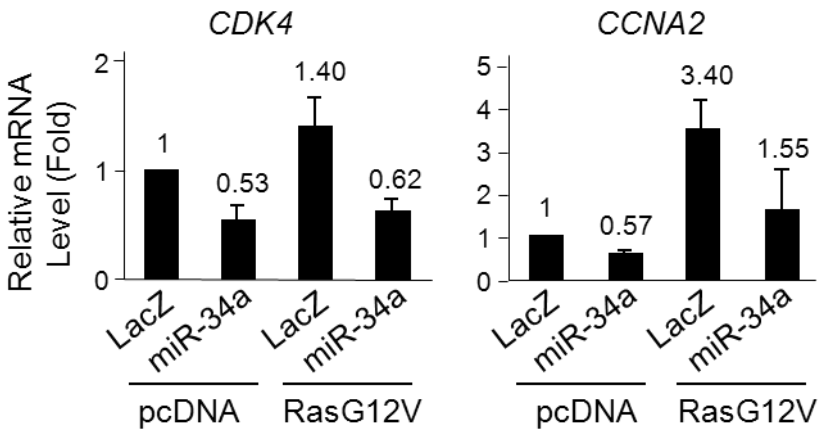

Figure 2-20. c-Jun target genes, *CCNA2* and *CDK4*, are repressed by miR-34a expression.

H1299/pcDNA or H1299/RasG12V stable cells were infected with Ad-LacZ or Ad-miR-34a for 72 hr. mRNA levels of each gene were quantified by qRT-PCR.

4. DISCUSSION

The altered metabolism in cancer provides clues as to how a normal cell can acquire pathogenesis and progress to cancer cells. For example, altered glucose metabolism in cancer cells contributes to tumorigenesis by supporting rapid proliferation (48). Interestingly, the latest cancer research has focused on unveiling novel metabolic functions of classical tumor suppressors such as p53 (3). From this point of view, metabolic change can occur in a cancer cell by uncontrolled p53 regulation. When considered together with the function of p53 in controlling cell cycle progression and apoptosis, this idea can offer a promising new strategy for cancer treatment. For example, TIGAR, a p53-inducible regulator of glycolysis and apoptosis, suggested a new ability of p53 to modulate glycolysis (39). The 6-phosphofructo-1-kinase, a key enzyme in the glycolytic pathway, is known to activated by fructose 2, 6-biophosphate (Fru-2,6-P_2) (49). As enhanced TIGAR expression upon p53 activation lowers the intracellular levels of Fru-2,6-P_2, slower glycolysis directs glucose to an alternative pathway like the pentose phosphate pathway (PPP). In addition, p53 has been found to regulate aerobic respiration via the novel transcription target SCO2, synthesis of cytochrome oxidase 2 (50). The SCO2 gene is a key regulator of the cytochrome C oxidase complex, which is essential for mitochondrial respiration. Because p53 induces TIGAR gene transcription to slow glycolysis and SCO2 gene transcription to enhance mitochondrial respiration, loss of p53 activity might result in the Warburg effect: extensive anaerobic glycolysis and impaired oxygen utilization for ATP generation in mitochondria (48). Moreover, glutaminase 2 (GLS2) was characterized as a p53 target gene that increases the production of glutamate and α-

ketoglutarate, which can enhance mitochondrial respiration and ATP generation, and guanidinoacetate methyltransferase (GAMT) was identified as a new p53 target gene that connects p53 to creatine metabolism (5, 6).

p53 regulates the members of the miR-34 family, which target genes involved in the cell cycle, apoptosis, DNA damage and senescence (8-10, 42). However, little is known about whether the p53-inducible miR-34 family directly regulates cellular metabolism. p53 governs a variety of cellular events such as apoptosis, cell cycle and DNA repair, which are directly connected with nucleotide biosynthetic metabolism. Nonetheless, there is no direct evidence about whether p53 controls the nucleotide biosynthetic pathway. Previously, it was reported that p53 negatively regulates the expression of IMPDH, but it is still unclear whether p53 directly regulates IMPDH through its binding to the promoter of the IMPDH gene (33). Thus, we investigated whether p53 may regulate purine nucleotide metabolism by targeting IMPDH.

IMPDH catalyzes a critical step in the de novo biosynthesis of guanine nucleotides and converts inosine 5'-monophospate (IMP) to xanthine 5'-monophospate (XMP) in a nicotinamide dinucleotide (NAD)-dependent manner. GTP, the final product of IMPDH-mediated purine metabolic pathway, modulates biochemical reactions associated with numerous cellular functions (51). Notably, GTP participates in DNA and RNA synthesis and hence cell proliferation, and is therefore essential for cellular signal transduction, energy transfer and microtubule dynamic instability (52-54). Judging from the importance of GTP in diverse processes, the regulation of GTP metabolism is an important factor in cellular homeostasis.

Until now, several studies have found higher IMPDH activity in human tumors or transformed cells (26). Moreover, the linear relationship between IMPDH activity and growth rates in diverse tumors supports the result of increased IMPDH expression in the malignant phenotype.

In addition, several groups have reported the non-enzymatic function of IMPDH; IMPDH binds both RNA and DNA in vivo, in a manner independent of catalytic activity (55). Indeed, IMPDH is found in the nuclei of human cells, and a few experiments suggest that IMPDH has a role in replication, transcription and translation. For example, IMPDH interacts with polyribosomes through a subdomain and is associated with translating rhodopsin mRNA (56). Moreover, the evidence that recruitment of IMPDH to actively transcribed genes mediates CTD phosphorylation of RNA polymerase II in yeast supports a new cellular function of IMPDH (57).

In this study, we demonstrate a novel mechanism whereby p53 regulates purine metabolism through a p53-miR-34a-IMPDH pathway. The significance of this finding is attributed to the identification of IMPDH as a miR-34a target and the novel function of p53 as purine metabolic regulator. Consequently, miR-34a-mediated inhibition of IMPDH perturbs the GTP-dependent Ras signaling pathway. Finally, monitoring the relationship of p53 with purine metabolism in human cancers may suggest a new therapeutic strategy.

CHAPTER 3

p53 regulates glucose metabolism by miR-34a

1. INTRODUCTION

One of the major distinguishing features between normal and cancer cells is altered glucose metabolism. Most cancer cells depend on aerobic glycolysis for energy production rather than pyruvate oxidation, even if oxygen is plentiful (58-60). Although cytosolic glycolysis produces a lower ATP yield than mitochondrial respiration, tumorigenic cells show an accelerated rate of glycolysis and increased lactate production compared to those of normal cells, this phenomenon is called Warburg effect (48, 61, 62). The mutations in oncogenes and tumor suppressor genes are known to be responsible for malignant transformation, and the Warburg effect is considered to be a result of these mutations.

p53, a well-known tumor suppressive transcription factor, has a well-established role in regulating the cell cycle, apoptosis and senescence, but recent studies have revealed a role for p53 in metabolism (3, 4). p53 has effects on glycolysis (via TP53-induced glycolysis and apoptosis regulator; TIGAR, phosphoglycerate mutase; PGAM), mitochondrial respiration (synthesis of cytochrome c oxidase 2; SCO2), glutamine metabolism (glutaminase 2) and purine metabolism (inosine 5′-monophosphate dehydrogenase) (5, 39, 50, 63, 64). Considering that a metabolic change, called the Warburg effect, is key event to understand tumor progression and that p53 is involved in glucose metabolic processes and malignancy, we hypothesized that p53 has a novel regulatory mechanism for the Warburg effect. Although p53 has been suggested as a glucose regulating metabolic

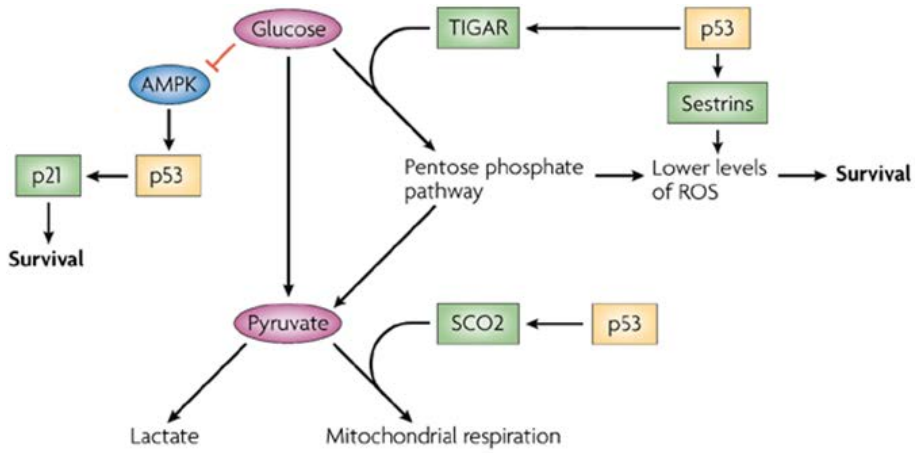

Figure 3-1. p53 and metabolism.

Several functions of p53 reduce the flux through the glycolytic pathway and increase oxidative phosphorylation p53 has been shown to induce the expression of the copper transporter SCO2, which is required for the assembly of cytochrome c oxidase. This allows p53 to enhance oxidative phosphorylation. And p53-inducible TIGAR (Tp53-inducible glycolysis and apoptosis regulator) decreases the activity of 6-phospho-1-kinase, a key glycolytic enzyme, thereby diverting the major glycolytic pathway into the pentose phosphate pathway (65).

factor, the precise mechanism for direct regulation of glycolysis and mitochondrial respiration by p53 has not been fully elucidated. Thus, we investigated the mechanism for the p53-dependent metabolic switch in cancer cells.

2. MATERIALS AND METHODS

1. Cells and virus infections

H1299 (p53 null) and HCT116 (wild-type p53) cells were obtained from ATCC. Cells were cultured in DMEM (Welgene) containing 10% (v/v) fetal bovine serum and 50 U/ml of streptomycin and penicillin (Gibco). Ad-LacZ, Ad-p53 and Ad-miR-34a amplification and treatment were performed as described in Chapter 1. For lentiviral-mediated RNA interference, pLKO-p53 was purchased from Open Biosystems. HCT116 cells were infected with lentivirally expressed control (scr; scrambled) or sh-p53 (p53-knockdown) using Polybrene (H9268; Sigma) for 48 h.

2. Western blot and antibodies

Whole cell lysates were obtained with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% (v/v) NP-40), and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Atto), and probed with specific antibodies. The antibodies used in this study were as follows: mouse monoclonal anti-HK1 (ab55144; Abcam), rabbit monoclonal anti-HK2 (#2867; Cell Signaling Technology), rabbit polyclonal anti-GPI (ab68643; Abcam), rabbit polyclonal anti-PDK1 (KAP-PK112; Stressgen Bioreagents), mouse monoclonal anti-p21 (sc-6246; Santa Cruz Biotechnology), mouse monoclonal anti-p53 (sc-126; Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (A5441; Sigma).

3. Quantitative real-time PCR (q-PCR)

Total RNA purification, cDNA synthesis, and qRT-PCR were performed as described in Chapter 2. The primers for real-time PCR were described in Table 3-1. To measure endogenous miR-34a, the Taqman microRNA assay kit was purchased from Applied Bioscience.

4. Hexokinase and glucose-6-phosphate isomerase activity measurement

HK and GPI enzymatic activity was measured using glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay. For the HK activity assay, cells were lysed with lysis buffer (45 mM Tris-Cl pH 8.2, 50 mM KH2PO4, 10 mM glucose, 0.5 mM EDTA, 0.2% Triton X-100), and 50 μL of the lysates were added to 150 μL assay buffer (40.6 mM Tris-Cl pH 8.5, 10.27 mM MgCl2, 2.27 mM glucose, 0.4 mM NADP, 8.93 mM ATP, 1.33 mM NaPO4, 60 mM KCl, 0.5 mM EDTA, 1 U/ml G6PDH), and then the OD at λ = 340 nm was measured for 15 minutes. For GPI activity assay, cells were lysed with lysis buffer (50 mM sodium phosphate pH 7.0, 0.1% (v/v) Triton X-100) and 10 μL of the lysates were added to 90 μL assay buffer (100 mM Tris-Cl pH 8.0, 10 mM MgCl2, 1 mM fructose-6-phosphate, 0.3 mM NADP, 1 U/ml G6PDH), and then the OD at λ = 340 nm was measured as in HK activity assay.

human HK1	forward	5′-GAGGAACCAATTTCCGTGTGCTGCT-3′
	reverse	5′-CCTTTGATCCCCATGTAGTCCAAGA-3′
human HK2	forward	5′-ATCCCTGAGGACATCATGCGA-3′
	reverse	5′-CTTATCCATGAAGTTAGCCAGGCA-3′
human GPI	forward	5′-AGGCTGCTGCCACATAAGGT-3′
	reverse	5′-CCAAGGCTCCAAGCATGAAT-3′
human PGAM1	forward	5′-TCAAGGAGGGGAAACGTGTA-3′
	reverse	5′-GGTTCAGCTCCATGATAGCC-3′
human PGAM2	forward	5′-ACGGGGAGGAGCAGGTGAAG-3′
	reverse	5′-GCTCTCGCAGGTGGGGAGTT-3′
human PFKM	forward	5′-GAAGCCACCTGGGAGAGCGT-3′
	reverse	5′-GAACGGAAGGTGTCAGCCCC-3′
human ALDA	forward	5′-CGGGAAGAAGGAGAACCTG-3′
	reverse	5′-GACCGCTCGGAGTGTACTTT-3′
human ALDC	forward	5′-GATTGTAGCCCCGGGCAAAG-3′
	reverse	5′-GCACTGAACAGGACCTGGCG-3′
human GAPDH	forward	5′-CCGTCTAGAAAAACCTGCC-3′
	reverse	5′-GCCAAATTCGTTGTCATACC-3′
human PGK	forward	5′-AAGTGAAGCTCGGAAAGCTTCTAT-3′
	reverse	5′-AGGGAAAAGATGCTTCTGGG-3′
human ENO1	forward	5′-GCCTCCTGCTCAAAGTCAAC-3′
	reverse	5′-AACGATGAGACACCATGACG-3′
human PKM2	forward	5′-TCATGAGGGGGTTCGGAGGT-3′
	reverse	5′-CGGTTGCACCGTCCAATCAT-3′
human LDHA	forward	5′-ACCCAGTTTCCACCATGATT-3′
	reverse	5′-CCCAAAATGCAAGGAACACT-3′
human PGM1	forward	5′-TAAGGAGGAGGGCCAAACGC-3′
	reverse	5′-ACCATGGTGGCGACTTGCTG-3′
human PHGDH	forward	5′-GGCTCAATGGAGCTGTCTTC-3′
	reverse	5′-TTCAGTCACATGCTGCTTCC-3′
human PDK1	forward	5′-ATTCAAGTTCATGTCACGCTGG-3′
	reverse	5′-TTTCCTCAAAGGAACGCCAC-3′
human RR	forward	5′-GAGCGCTTTAGTCAGGAGGT-3′
	reverse	5′-CGATGAGAATTTGAAAGCCA-3′
human TIGAR	forward	5′-TCGGGAAAGGAAATACGGGG-3′
	reverse	5′-TCCACGCATTTTCACCTGGTC-3′
human CCND1	forward	5′-CGTGGCCTCTAAGATGAAGG-3′
	reverse	5′-CTGGCATTTTGGAGAGGAAG-3′
human CDK6	forward	5′-TGCACAGTGTCACGAACAGA-3′
	reverse	5′-ACCTCGGAGAAGCTGAAACA-3′

Table 3-1. Primers used in Chapter 3.

5. Luciferase assay

The 3′ UTR fragment of the metabolic genes was amplified by PCR from human cDNA (positions 217–558 of HK1 3′ UTR, positions 715–1102 of the HK2 3′ UTR, positions 12–264 of GPI 3′ UTR, positions 146–540 of PDK1 3′ UTR) and was inserted into pGL3UC luciferase reporter vector. The mutant of the miR-34a response element within the 3′ UTR of the indicated genes was generated by site-directed mutagenesis. H1299 cells were transfected with 5 ng of renilla luciferase vector and 500 ng of pGL3UC luciferase vector including either the wild-type (WT) or mutant of the miR-34a response element (MT) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. At 4 h post-transfection, cells were infected with adenovirally expressed miR-34a or LacZ. Luciferase activity was measured with the dual luciferase reporter assay system (Promega) 72 h after adenovirus infection and was normalized to renilla luciferase activity using TECAN Infinite M200 luminometer (Tecan).

6. Treatment of miR-34a inhibitor

Anti-miR-34a inhibitor (anti-miR-34a) and control inhibitor were treated as described in Chapter 1. Prior to adriamycin (0.4 μg/ml) treatment, HCT116 cells were treated with 0.5 μM anti-miR-34a for 24 h.

7. Lactate production measurement

Secreted lactate was measured in media using a Lactate assay kit (BioVision) according to the manufacturer's instructions**.**

8. Oxygen consumption rate and ATP production measurement

1×10^6 cells were harvested by trypsinization and then resuspended in 200 μL of culture medium. The oxygen consumption rate of the cells was monitored continuously for 15 min using a Clark-type oxygen electrode (Instech Laboratories). To measure cellular ATP production, 3×10^5 cells were collected and resuspended in 500 μL of ATP assay buffer (100 mM Tris-Cl pH 7.75, 4 mM EDTA) and boiled for 2 minutes. After centrifugation at 1000 × g for 5 minutes, 25 μL of the supernatant was analyzed using the ATP Bioluminescence Assay Kit CLS II (Roche) and luminometer (TECAN).

3. RESULTS

1. p53 downregulates glucose metabolic enzymes

To examine the role of p53 associated with the Warburg effect, we first measured the mRNA level of genes involved in the glycolytic and biosynthetic pathways branching off of glycolysis. Successful p53-mediated transactivation was confirmed by increased mRNA of well-known p53 target genes related to metabolism (ribonucleotide reductase; *RR* and *TIGAR*) following adenoviral expression of p53 in *p53*-null H1299 cells. Among the glycolytic enzymes, the mRNAs of *hexokinase 1* (*HK1*), *hexokinase 2* (*HK2*), *glucose-6-phosphate isomerase* (*GPI*), *aldolase C* (*ALDC*), *phosphoglycerate mutase 1* (*PGAM1*) and *lactate dehydrogenase A (LDHA)* specifically decreased in association with p53 overexpression compared to that of other glucose metabolic enzymes (Fig. 3-2 and Fig. 3-3). In addition, mRNA levels of *phosphoglucomutase* (*PGM*), *phosphoglycerate dehydrogenase* (*PHGDH*) and *pyruvate dehydrogenase 1* (*PDK1*) decreased by p53. PGM is an enzyme necessary for glycogen biosynthesis (66), and PHGDH is the first enzyme in the serine biosynthesis pathway branching from glycolysis (67). PDK is a negative regulator of pyruvate dehydrogenase and decreases oxidation of pyruvate in mitochondria (68). Consistent with this result, HK1, HK2, GPI and PDK1 protein levels decreased with p53 expression (Fig. 3-4). Next, to investigate whether endogenous p53 can regulate the expression of these enzymes, we confirmed the mRNA levels of *HK1*, *HK2*, *GPI* and *PDK1* in a

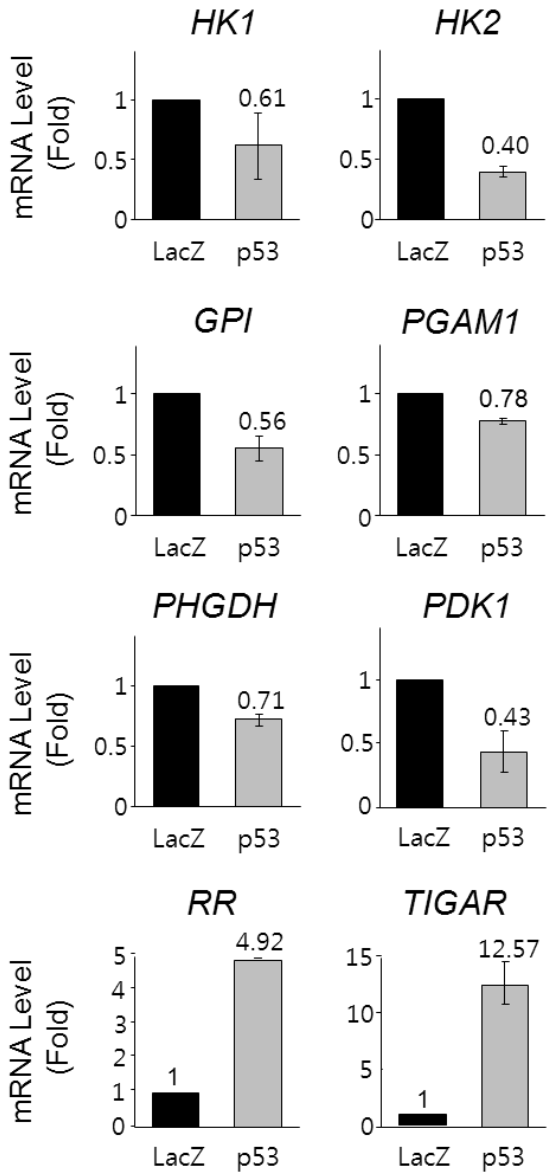

Figure 3-2. p53 downregulates the expression of several glucose metabolic enzymes.

p53 null H1299 cells were infected with adenovirally expressed LacZ (control) or p53 for 24 hr and mRNA level of indicated genes was quantified by qRT-PCR.

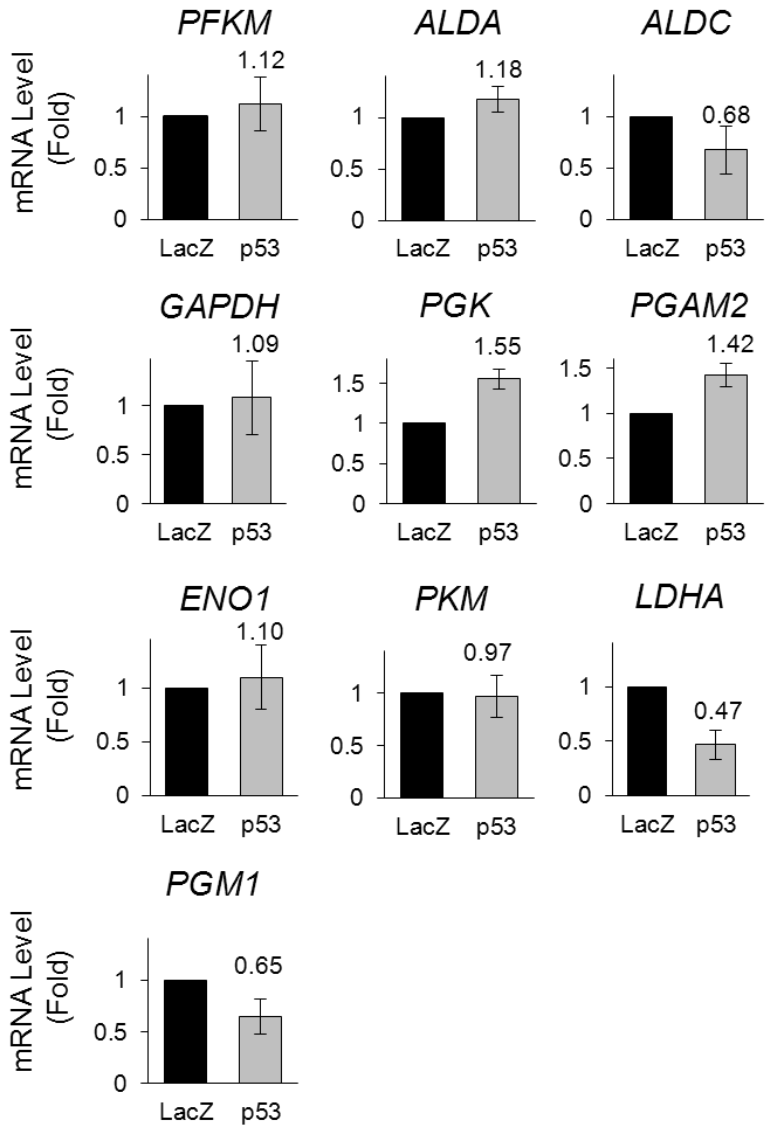

Figure 3-3. Expression changes of other glucose metabolic enzymes by p53.

H1299 cells were infected with the adenovirus expressing LacZ or p53 for 24 h, mRNA expression of indicated genes was quantified as in Fig. 3-2.

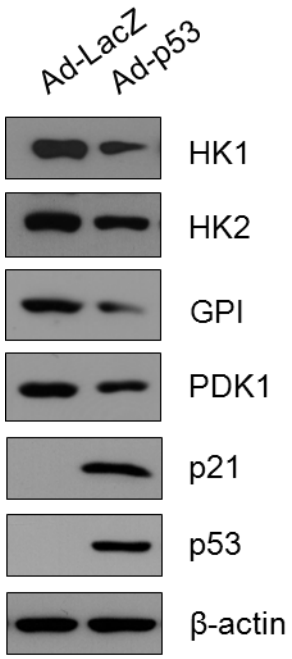

Figure 3-4. HK1, HK2, GPI and PDK1 protein levels decrease with p53 expression.

H1299 cells were infected with Ad-LacZ or Ad-p53 for 24 hr and protein levels were analyzed by immunoblotting using the indicated antibodies.

wild-type p53-harboring HCT116 cell line, with or without adriamycin treatment. As adriamycin treatment transactivated p53, it reduced both mRNA and protein levels of HK1, HK2, GPI and PDK1 (Fig. 3-5 and Fig. 3-6). However, when HCT116 cells were infected with lentivirus encoding shRNA specific for p53 (sh-p53), no decrease in expression levels of these enzymes was observed following adriamycin treatment. Additionally, we confirmed repression of PGAM1, PHGDH and PGM1 upon DNA damage in mRNA level (Fig. 3-7). These data demonstrate that transactivated p53 regulates HK1, HK2 and GPI expression, which are the first and second steps in the glycolytic process, and that PDK1 is an important enzyme connecting glycolysis with mitochondrial oxidative phosphorylation.

2. miR-34a inhibits glucose metabolic enzymes

The consequence of activating p53 following DNA damage is the induction of various target genes and non-coding RNAs such as miRNAs or long intergenic non-coding RNAs (43, 69). In particular, transcriptional repression of specific genes by p53 can be elucidated by p53-target miRNAs, such as miR-34a. Thus, we speculated that p53-inducble miR-34a may regulate transcriptional repression of metabolic enzymes reduced by p53 (Fig. 3-2). HK1, HK2, GPI and PDK1 transcription was repressed when H1299 cells were infected with the adenovirally expressed form of miR-34a (Fig. 3-8), which led to a decrease in protein levels (Fig. 3-9). However, the reduced

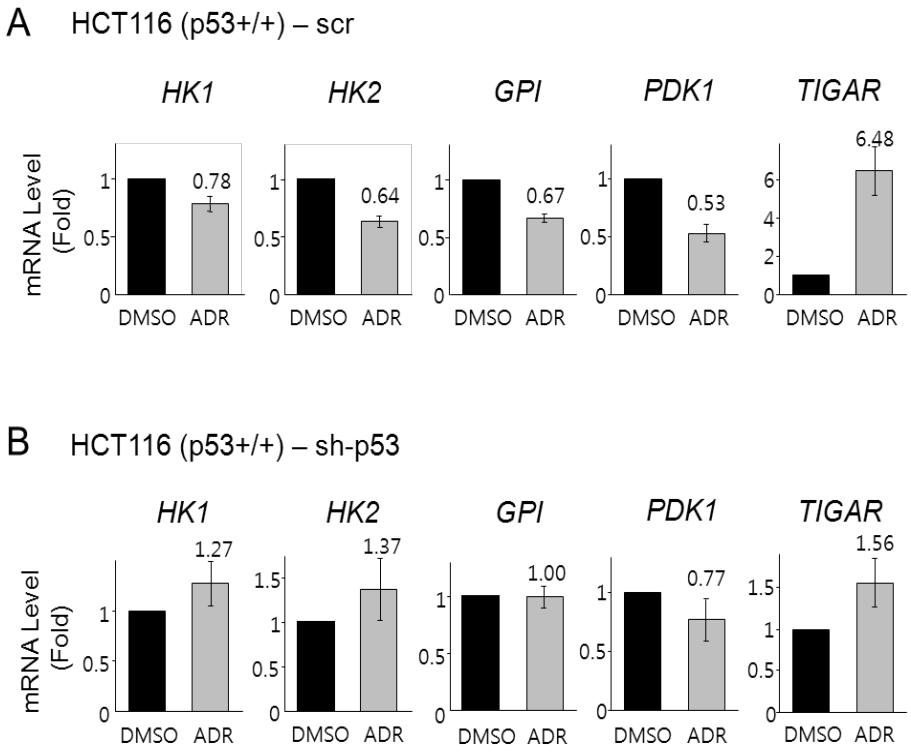

Figure 3-5. Transactivated p53 regulates mRNA levels of HK1, HK2, GPI and PDK1.

HCT116 (wild-type p53) cells were infected with lentivirally expressed control (scr; scrambled) or sh-p53 (p53-knockdown) for 24 h, and then treated with DMSO (control) or adriamycin (ADR, 0.4 μg/ml) for 24 h. Indicated mRNA expression was quantified by qRT-PCR.

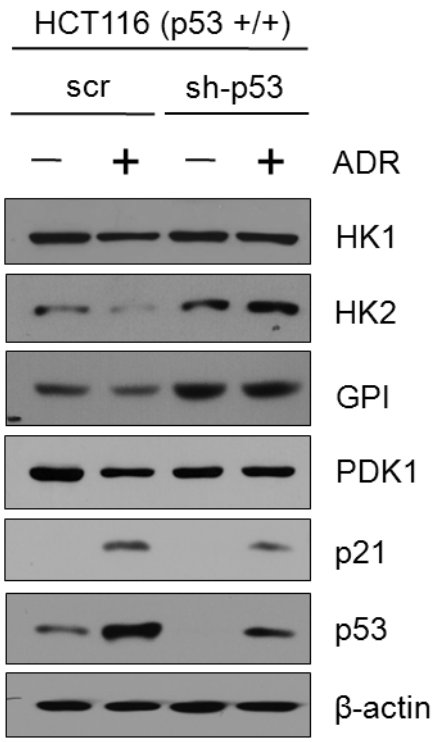

Figure 3-6. Transactivated p53 regulates expression of HK1, HK2, GPI and PDK1 in protein levels.

HCT116 cells were infected and treated as in Fig. 3-5, and total protein extracts were analyzed by immunoblotting using the indicated antibodies.

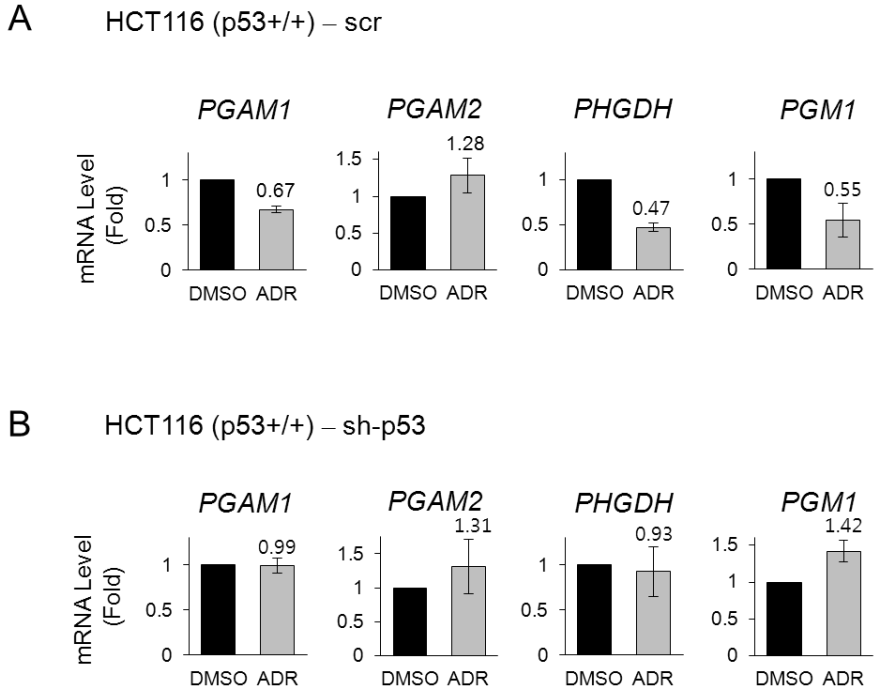

Figure 3-7. Expression changes of PGAM1, PGAM2, PHGDH and PGM1 by endogenous p53 transactivation.

HCT116 cells were infected with lentivirally expressed scr or sh-p53, after 24 h media were changed with DMSO or ADR for 24 h. The mRNA levels of indicated genes were quantified by qRT-PCR.

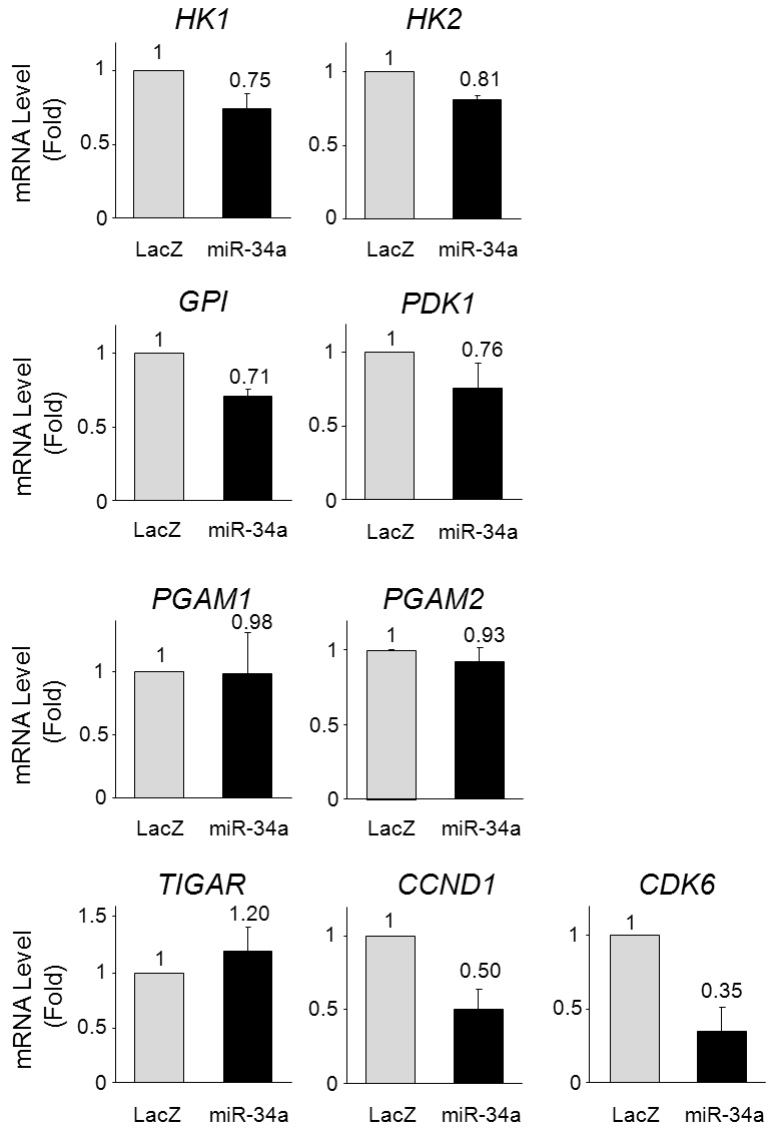

Figure 3-8. miR-34a downregulates the expression of HK1, HK2, GPI and PDK1.

H1299 cells were infected with adenovirus expressing miR-34a or LacZ for 72 h, and mRNA levels of indicated genes were quantified by qRT-PCR.

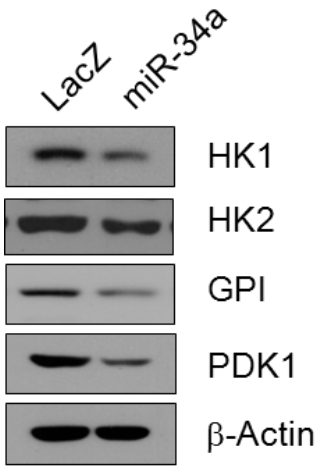

Figure 3-9. miR-34a represses the protein levels of HK1, HK2, GPI and PDK1.

Cell lysates were immunoblotted with the indicated antibodies under the same conditions as in Fig. 3-8.

expression of PGAM1 by p53 activation in Fig. 3-2 could not be confirmed by adenoviral infection of miR-34a. Moreover, a decrease in hexokinase and GPI cellular activity was observed in miR-34a infected cells compared to that in control cells (Fig. 3-10).

3. miR-34a directly regulates HK1, HK2, GPI and PDK1 expression

We searched for a miR-34a target sequence within the 3′ untranslated region (UTR) of these enzymes using computer analysis to verify that miR-34a can directly target HK1, HK2, GPI and PDK1 mRNAs. Intriguingly, we found a putative miR-34a target site in the 3′UTRs of the HK1, HK2, GPI and PDK1 mRNAs. We constructed a luciferase reporter vector containing the putative miR-34a binding site in the 3′UTRs to test whether miR-34a repressed these enzymes through the targeting sequence. We concluded that miR-34a directly targets the 3′UTR of HK1, HK2, GPI and PDK1 through a point-mutation in the miR-34a target site within the 3′UTR region of these enzymes (Fig. 3-11 and Fig. 3-12).

A specific miR-34a inhibitor (anti-miR-34a) was used for antisense inhibition of miR-34a to investigate whether decreased HK1, HK2, GPI and PDK1 expression is a direct effect of miR-34a. Decreased HK1, HK2, GPI and PDK1 mRNA following DNA damage was not observed in anti-miR-34a treated cells (Fig. 3-13), and protein levels of these enzymes were consistent

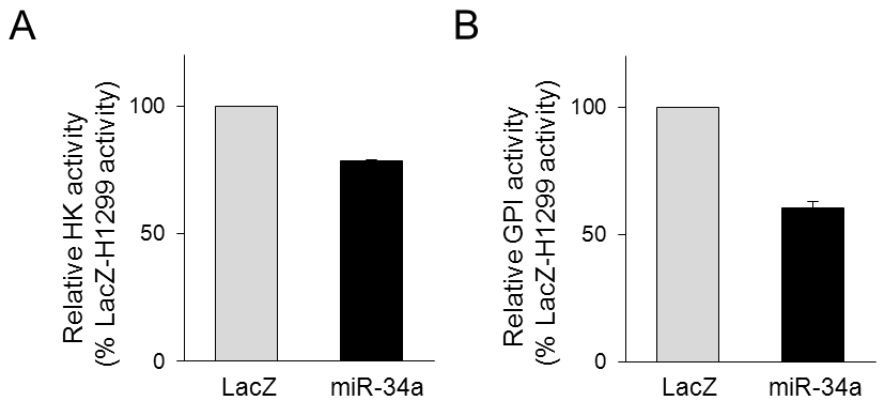

Figure 3-10. miR-34a decreases enzymatic activities of HK and GPI.

H1299 cells were infected as in Fig. 3-8, and HK and GPI enzymatic activity was measured using glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay.

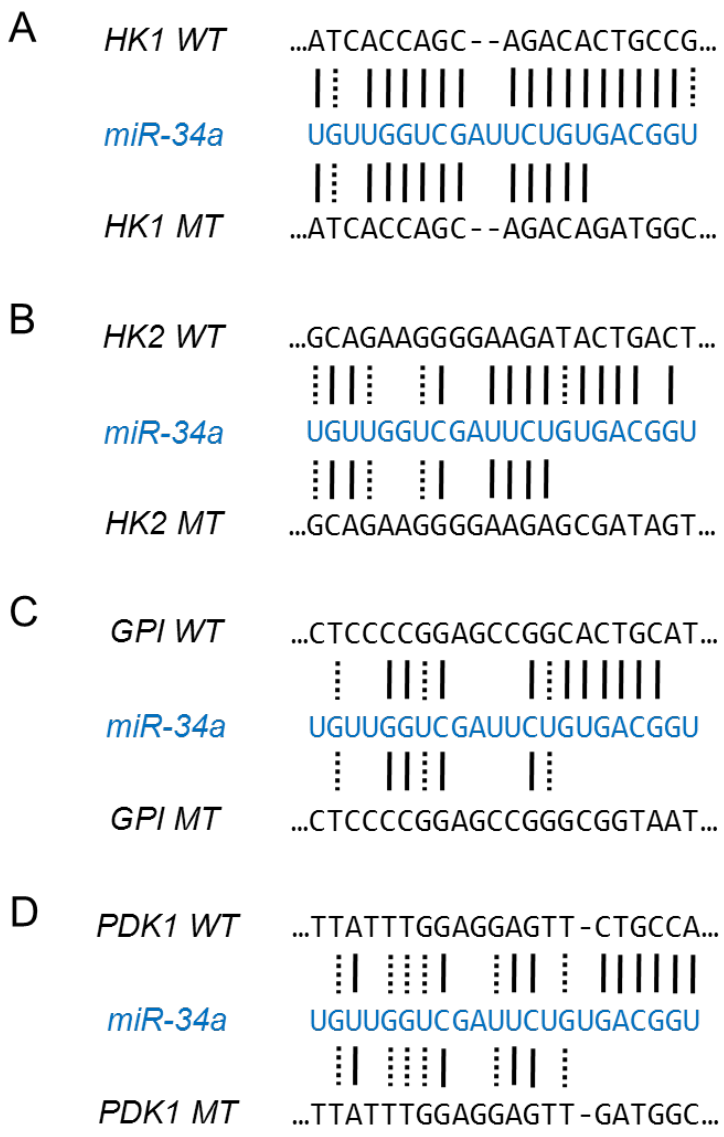

Figure 3-11. Alignment of the hsa-miR-34a sequence and 3' UTR of indicated mRNAs.

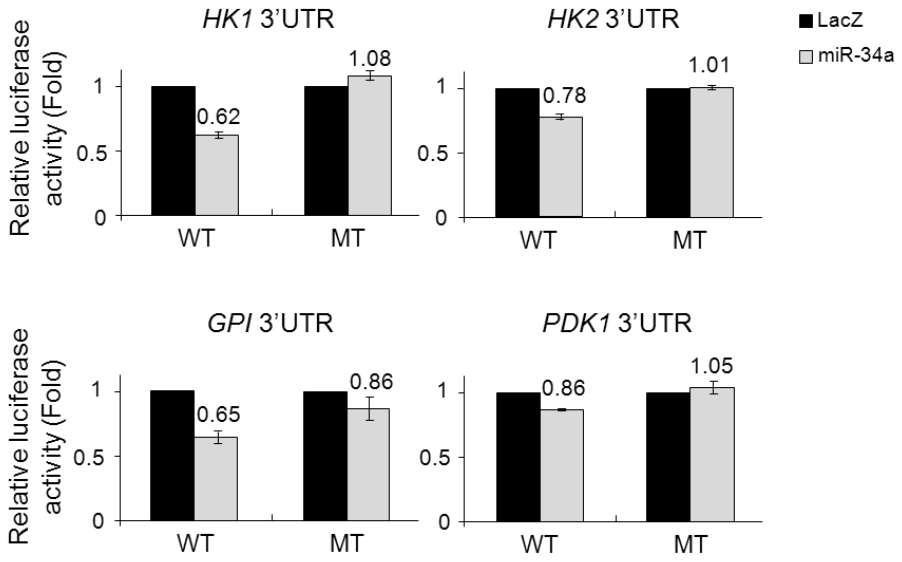

Figure 3-12. miR-34a directly targets 3'UTR of HK1, HK2, GPI and PDK1.

H1299 cells were transfected with pGL3UC luciferase vector including either the wild-type (WT) or mutant of the miR-34a response element (MT) within the 3′ UTR of the indicated genes. At 4 h post-transfection, cells were infected with adenovirally expressed miR-34a or LacZ. Luciferase activity was measured with the dual luciferase reporter assay system 72 h after adenovirus infection and was normalized to renilla luciferase activity.

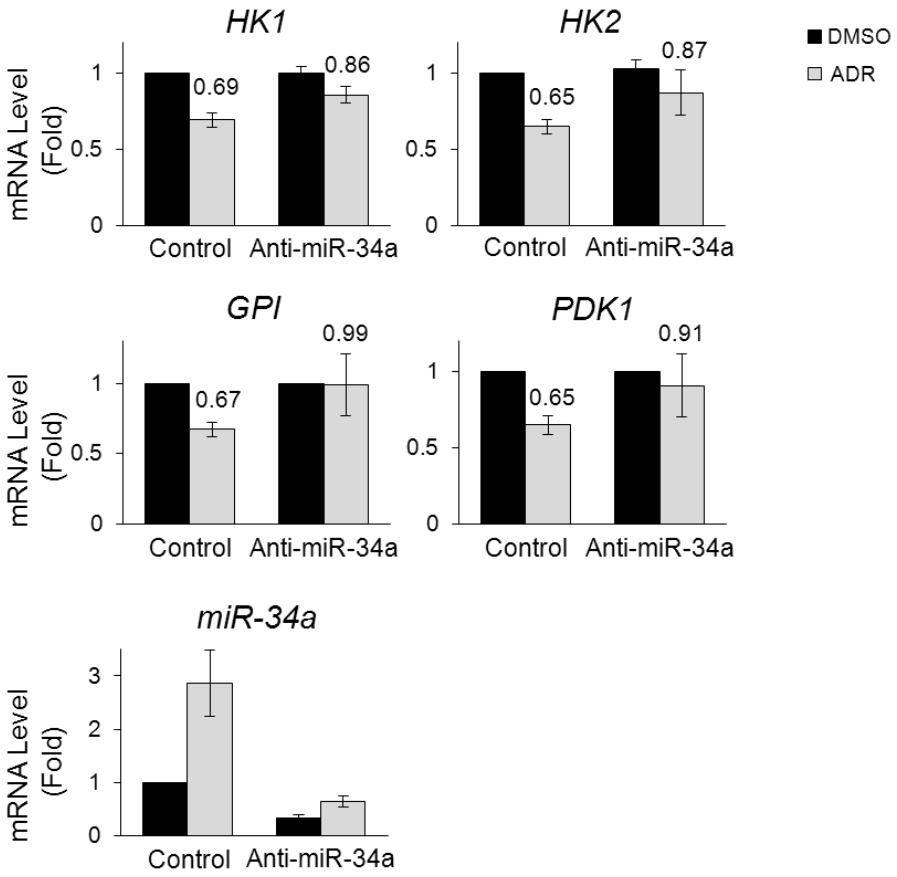

Figure 3-13. p53 transactivation-dependent repression of HK1, HK2, GPI and PDK1 transcription is impaired by miR-34a inhibition.

Prior to adriamycin (0.4 μg/ml) treatment, HCT116 cells were treated with 0.5 μM miR-34a inhibitor (anti-miR-34a) or a negative control inhibitor (Control) for 24 h. mRNA expression of the indicated genes was quantified by qRT-PCR.

with those of mRNA (Fig. 3-14), indicating that miR-34a directly regulates HK1, HK2, GPI and PDK1.

4. The p53-miR-34a pathway controls glucose metabolism

We wanted to observe whether the glycolytic enzyme and PDK1 expression changes lead to a substantive change in glucose metabolism by activating p53 or miR-34a. We first measured lactate production, an end-product of glycolysis, by overexpressing p53 or transactivating endogenous p53 following DNA damage (Fig. 3-15). Lactate production was decreased by p53 under both conditions, whereas p53 knockdown moderated a reduction in lactate production under DNA damaging conditions (Fig. 3-15B). As was the case with p53 activation, miR-34a overexpression or induction of endogenous miR-34a following adriamycin treatment decreased lactate production (Fig. 3-16). Treatment with the miR-34a specific inhibitor attenuated the reduced lactate production by the DNA damaging agent. These data suggest that that decreased expression of HK1, HK2 and GPI by p53-inducible miR-34a led to a decreased glycolysis rate.

Next, we examined mitochondrial respiration by measuring O2 consumption and ATP production under the same conditions as in Fig. 3-15. In contrast to the decrease in lactate production, O2 consumption and ATP production increased in p53-infected H1299 cells (Fig. 3-17). The results of mitochondrial oxygen consumption and ATP production in adriamycin-treated HCT116 cells was the same as p53 overexpression. Mitochondrial

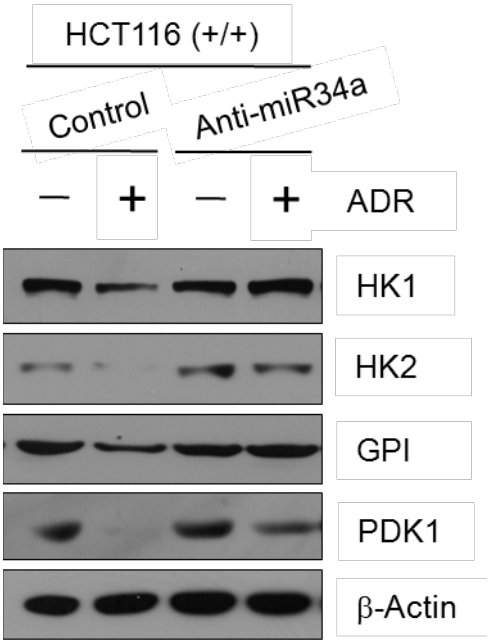

Figure 3-14. DNA damage-dependent repression of HK1, HK2, GPI and PDK1 is moderated by miR-34a inhibition.

Total protein extracts were analyzed by immunoblotting using the indicated antibodies under the same conditions as in Fig. 3-13.

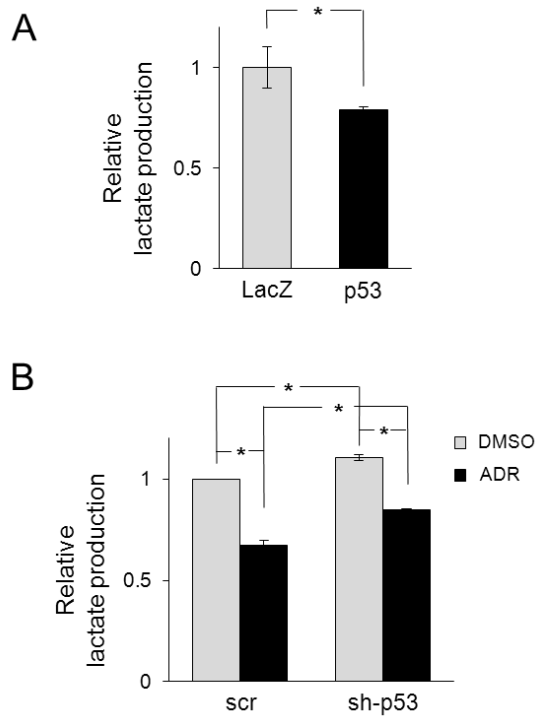

Figure 3-15. Lactate production is decreased by overexpressing p53 or transactivating endogenous p53 following DNA damage.

(A) H1299 cells were infected with the adenovirus expressing LacZ or p53 for 24 h, and secreted lactate was measured in media using a Lactate assay kit according to the manufacturer's instructions. (B) HCT116 cells were infected with lentivirally expressed control (scr; scrambled) or sh-p53 (p53-knockdown) for 24 h, and then treated with DMSO (control) or adriamycin (ADR, 0.4 μg/ml) for 24 h. As in (A), secreted lactate was measured in media. *$P < 0.05$.

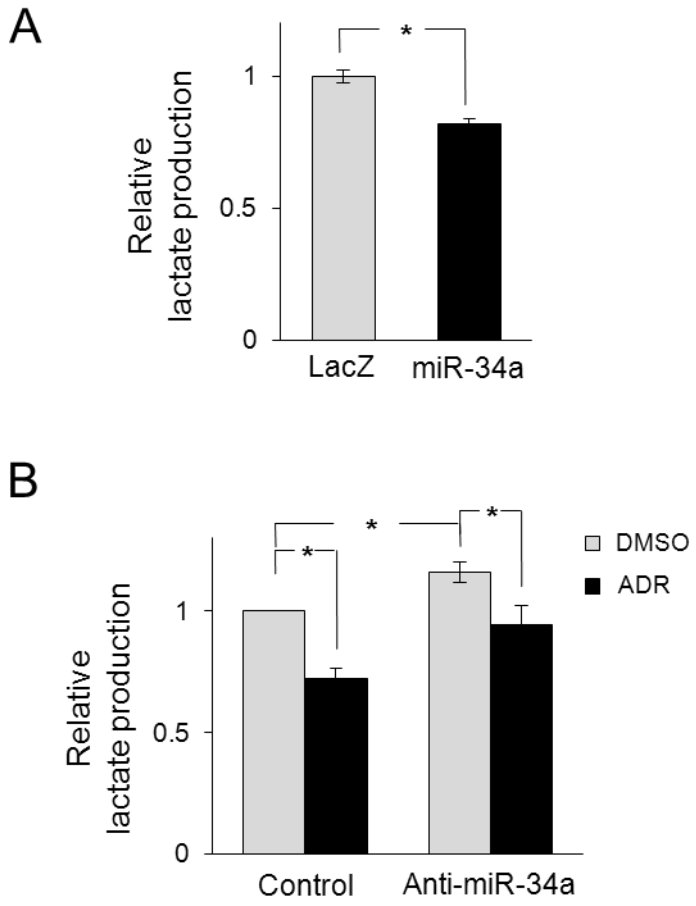

Figure 3-16. Lactate production is decreased by miR-34a, and inhibition of miR-34a alleviates reduction of lactate production.

(A) H1299 cells were infected with adenovirus expressing LacZ or miR-34a for 72 h, and secreted lactate was measured in media. (B) HCT116 cells were treated with 0.5 μM of miR-34a inhibitor or a negative control inhibitor for 24 h, then DMSO or ADR was added for 24 h, and secreted lactate was measured in media. *$P < 0.05$.

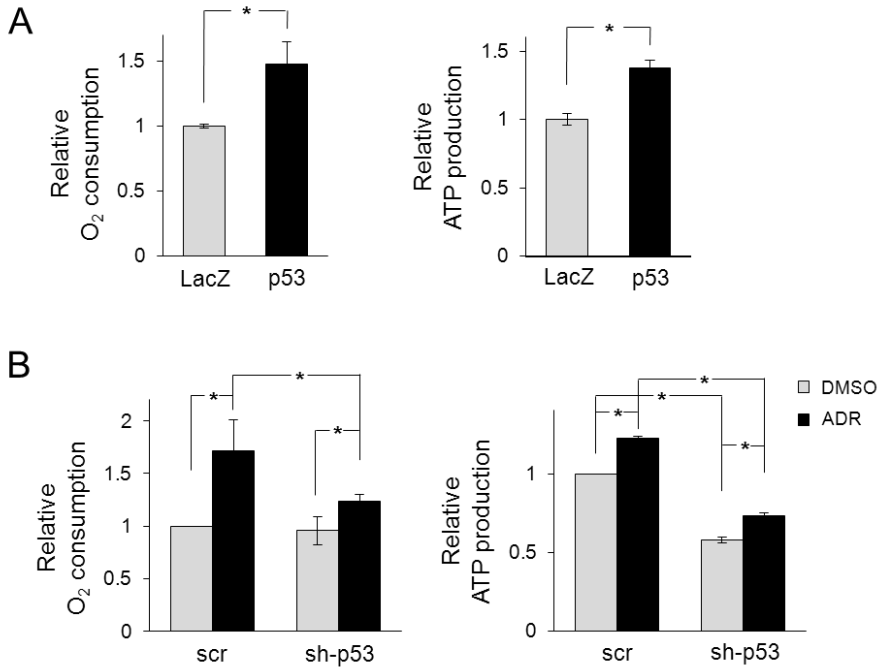

Figure 3-17. O_2 consumption and ATP production are decreased by overexpressing p53 or transactivating endogenous p53 following DNA damage.

Under the same conditions as in Fig. 3-15, cells were resuspended in 200 μL of fresh culture medium. The oxygen consumption rate of the cells was monitored continuously for 15 min using a Clark-type oxygen electrode. To measure cellular ATP production, cells were resuspended in ATP assay buffer and analyzed using the ATP Bioluminescence Assay Kit CLS II. *$P < 0.05$.

respiration in sh-p53-infected HCT116 cells increased less than that in control cells (scrambled) following DNA damage. The effect of miR-34a on mitochondrial oxygen consumption and ATP production was consistent with that of p53 (Fig. 3-18). The increase in mitochondrial respiration by miR-34a was confirmed using a miR-34a specific inhibitor (Fig. 3-19). Taken together, these data suggest that p53 regulates the expression of glucose metabolic enzymes and substantive glucose metabolism through p53-inducible microRNA, miR-34a.

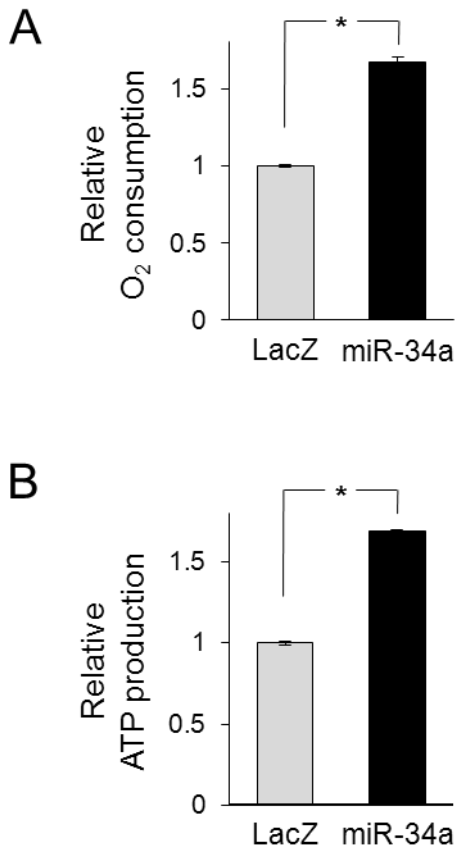

Figure 3-18. miR-34a increases O_2 consumption and ATP production.

H1299 cells were infected with the adenovirally expressed LacZ or miR-34a of 72 h, and oxygen consumption and ATP production were measured as in Fig. 3-17. *P < 0.05.

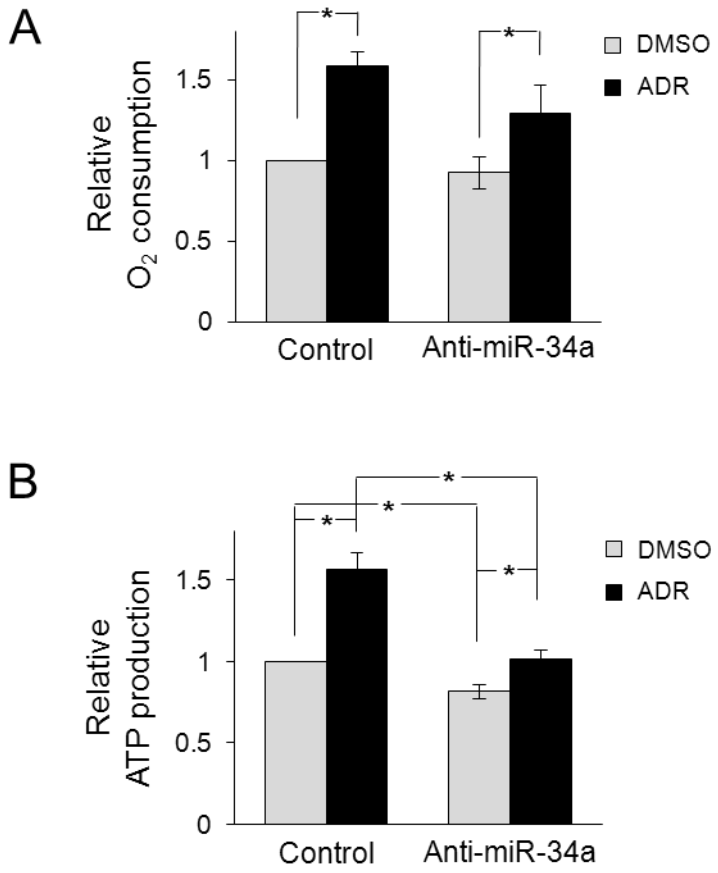

Figure 3-19. Increase of O_2 consumption and ATP production upon DNA damage is moderated by treatment of miR-34a inhibitor.

HCT116 cells were treated with the control inhibitor or miR-34a inhibitor. After 24 h, cells were treated with DMSO or ADR for 24 h, and oxygen consumption and ATP production were measured as in Fig. 3-17. *P < 0.05.

4. DISCUSSION

We found that p53 regulated several glycolytic enzymes (HK1, HK2, GPI, ALDC and PGAM1), and enzymes in the biosynthetic pathway branching from glycolysis (PGM1 and PHGDH). Of these, we focused on the regulation of HK1, HK2 and GPI because the mitochondrial hexokinases, HK1 and HK2, are highly elevated in rapidly-growing malignant cells compared to that in normal cells (70, 71), and GPI is secreted as an autocrine tumor motility factor (72, 73). Moreover PDK1 activity increases in hypoxic cancer cells, and PDK1 inactivates pyruvate dehydrogenase by phosphorylation (74). PDK1 is important to connect cytosolic glycolysis and mitochondrial oxidative phosphorylation (62). And the expression of PGAM1 was downregulated by p53 activation, but not by miR-34a. It was presumed to be regulated through another mechanism

We found that all of these enzymes were regulated by p53 and miR-34a. A single microRNA, miR-34a, regulated glycolysis and mitochondrial respiration by targeting multiple genes, such as HK1, HK2, GPI and PDK1. Furthermore, physiological changes following miR-34a transduction were confirmed by measuring lactate production, oxygen consumption and ATP production. Altered metabolism in cancer might be interpreted through the action of p53-miR-34a. Although several studies have investigated miRNAs targeting glycolytic enzymes (20, 75) and p53 regulating glucose metabolism by TIGAR and SCO2 (39, 50), the molecular mechanism to understand the Warburg effect by p53 has not been fully elucidated. We propose a novel

mechanism whereby p53 regulates glucose metabolism via miR-34a. This integrated miR-34a mechanism offers a promising new approach and strategy for cancer therapy.

CHAPTER 4

Conclusion

In these studies, I suggested a new mechanism that p53 regulates cellular metabolic processes. I showed that p53-inducible microRNA, miR-34a, directly targets both IMPDH isoforms, and treatment of anti-miR-34a inhibitor relieved the repression of IMPDH upon DNA damage. Ultimately, miR-34a-mediated inhibition of IMPDH resulted in repressed activation of GTP-dependent Ras signaling pathway.

And miR-34a directly targets glycolytic enzymes (HK1, HK2 and GPI) and pyruvate dehydrogenase kinase 1 (PDK1), a mediator between glycolysis and mitochondrial oxidative phosphorylation. The repression of these enzymes ultimately led to the regulation of glucose metabolism. In other words, p53 regulates the expression of glucose metabolic enzymes and substantive glucose metabolism by miR-34a. p53-miR-34a transactivation lowers glycolytic rate and enhances mitochondrial respiration.

In summary, p53 has a novel function in regulating purine biosynthesis and glucose metabolism, aided by miR-34a-dependent gene repression. p53 plays broad roles as a metabolic regulator by adjusting expression of diverse target genes through miR-34a.

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ABSTRACT IN KOREAN

국문 초록

p53 은 잘 알려진 전사조절인자로서, 광범위한 스트레스 상황에 반응하여 세포 주기 지연과 세포사멸을 조절한다. 게다가 최근에는 p53 의 당, 글루타민, 지방산 대사에 관여하는 대사 조절인자로서의 기능이 주목받고 있다. 실제로 p53 유전자의 돌연변이는 절반에 가까운 종양에서 발견되고 있고, 종양세포의 두드러진 특징 중에 하나가 대사조절이 비정상적으로 나타난다는 점이다. 특히 종양세포는 에너지를 생산함에 있어서 미토콘드리아 호흡보다는 해당작용에 의존하는 Warburg 효과가 나타난다.

그리고 뉴클레오티드 생합성은 세포 증식과 세포분열 주기에 있어서 필수적임에도 불구하고 p53 이 뉴클레오티드 생합성을 조절하는 지에 대한 보고는 거의 없었다.

첫번째로 우리는 p53 에 의해 유도되는 microRNA-34a (miR-34a)가 de novo GTP 생합성과정에서 중요한 속도 조절 효소인 inosine 5'-monophosphate dehydrogenase (IMPDH)를 억제함을 밝혔다. miR-34a 억제제를 처리하자 DNA 손상시 나타나던 IMPDH 발현 저하가 경감되었다. 궁극적으로, miR-34a 를 매개하는 IMPDH 의 억제가 GTP-의존적인 Ras 신호 경로의 활성을 억제한다. 요약하자면 우리는 p53 이 miR-34a 를

통한 IMPDH 의 억제를 통해 퓨린 생합성을 조절하는 새로운 기능을 제시하였다.

그리고 두번째로는 종양에서 나타나는 비이상적 대사 변화를 p53 을 통해 설명하고자 하였다. 우리는 miR-34a 가 몇몇의 해당작용 효소들 (hexokinase 1, hexokinase 2, glucoase-6-phosphate isomerase)과 pyruvate dehydrogenase kinase 1 을 억제함을 발견했다. 마찬가지로 miR-34a 억제제를 처리하자 DNA 손상시 나타나던 이 효소들의 발현 저하가 경감되었다. 이러한 miR-34a 를 매개하는 효소들의 억제가 해당작용을 저해하고 미토콘드리아 호흡을 증가시킴을 알 수 있었다. 이 결과들은 p53 이 miR-34a 를 통해 통합적인 기전으로 당 대사를 조절함을 제시한다.

주요어 : p53, miR-34a, IMPDH, Hexokinase, Glucose-6-phosphate isomerase , Pyruvate dehydrogenase kinase 1

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