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p53 regulates glucose metabolism by miR-34a

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ABSTRACT

Cancer cells rely mainly on glycolysis rather than mitochondrial respiration for energy production, which is called the Warburg effect. p53 mutations are observed in about half of cancer cases, and p53 controls the cell cycle and cell death in response to cellular stressors. p53 has been emphasized as a metabolic regulator involved in glucose, glutamine, and purine metabolism. Here, 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.

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 [1–3]. 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 [4–6].

p53, a well-known tumor suppressive transcription factor, performs diverse roles regulating the mammalian cell stress response [7–9]. Indeed, inactivation of p53 is the most frequent alteration in many types of human malignancies and has been reported in about half of human cancers [10]. p53 has a well-established role in regulating the cell cycle, apoptosis, and senescence, but recent studies have revealed a role for p53 in metabolism [11,12]. 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) [13–17].

Furthermore, p53 transactivates several microRNAs, particularly the miR-34 family targets broad spectrum genes involved in the cell cycle, apoptosis, and DNA damage [18–22]. MicroRNAs (miRNAs) are a family of noncoding RNAs (18–22 nucleotides) that play important roles in physiological processes [23,24]. Mature miRNAs regulate target gene expression by degrading mRNA or disturbing protein translation [25]. A specific miRNA has multiple targets, and one gene can be regulated by multiple miRNAs [26]. miRNAs modulate cell proliferation, cell death, differentiation, apoptosis, and cell signaling pathways [27,28]. In addition, several studies have shown that miRNAs have functions involved in energy metabolism including glucose and lipid metabolism and amino acid biogenesis [29,30]. For example, miR-122 affects hepatic cholesterol and fatty acid metabolism by regulating genes in the cholesterol biosynthesis pathway [31], and miR-143 controls glycolysis by targeting hexokinase 2 [32,33].

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 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.

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In this study, we showed that p53-inducible microRNA, miR-34a, directly targets glycolytic enzymes and pyruvate dehydrogenase kinase 1 (PDK1), a mediator between glycolysis and mitochondrial oxidative phosphorylation, ultimately leading to the regulation of glucose metabolism.

2. Materials and methods

2.1. 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 previously [17,34]. 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.

2.2. 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) [35], 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).

2.3. 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 Supplementary Table 1. To measure endogenous miR-34a, the Taqman microRNA assay kit was purchased from Applied Bioscience.

2.4. 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 KH_2PO_4, 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 $MgCl_2$, 2.27 mM glucose, 0.4 mM NADP, 8.93 mM ATP, 1.33 mM $NaPO_4$, 60 mM KCl, 0.5 mM EDTA, 1 U/ml G6PDH), and then the OD at λ = 340 nm was measured for 15 min. 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 $MgCl_2$, 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.

2.5. 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).

2.6. 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 h.

2.7. Lactate production measurement

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

2.8. 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 min. After centrifugation at 1000*g* for 5 min, 25 μL of the supernatant was analyzed using the ATP Bioluminescence Assay Kit CLS II (Roche) and luminometer (TECAN).

3. Results and discussion

3.1. 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. 1A and Fig. S1A). In addition, mRNA levels of *phosphoglucomutase* (*PGM*), *phosphoglycerate dehydrogenase* (*PHGDH*), and *pyruvate dehydrogenase 1* (*PDK1*) decreased by p53. PGM is an enzyme necessary for

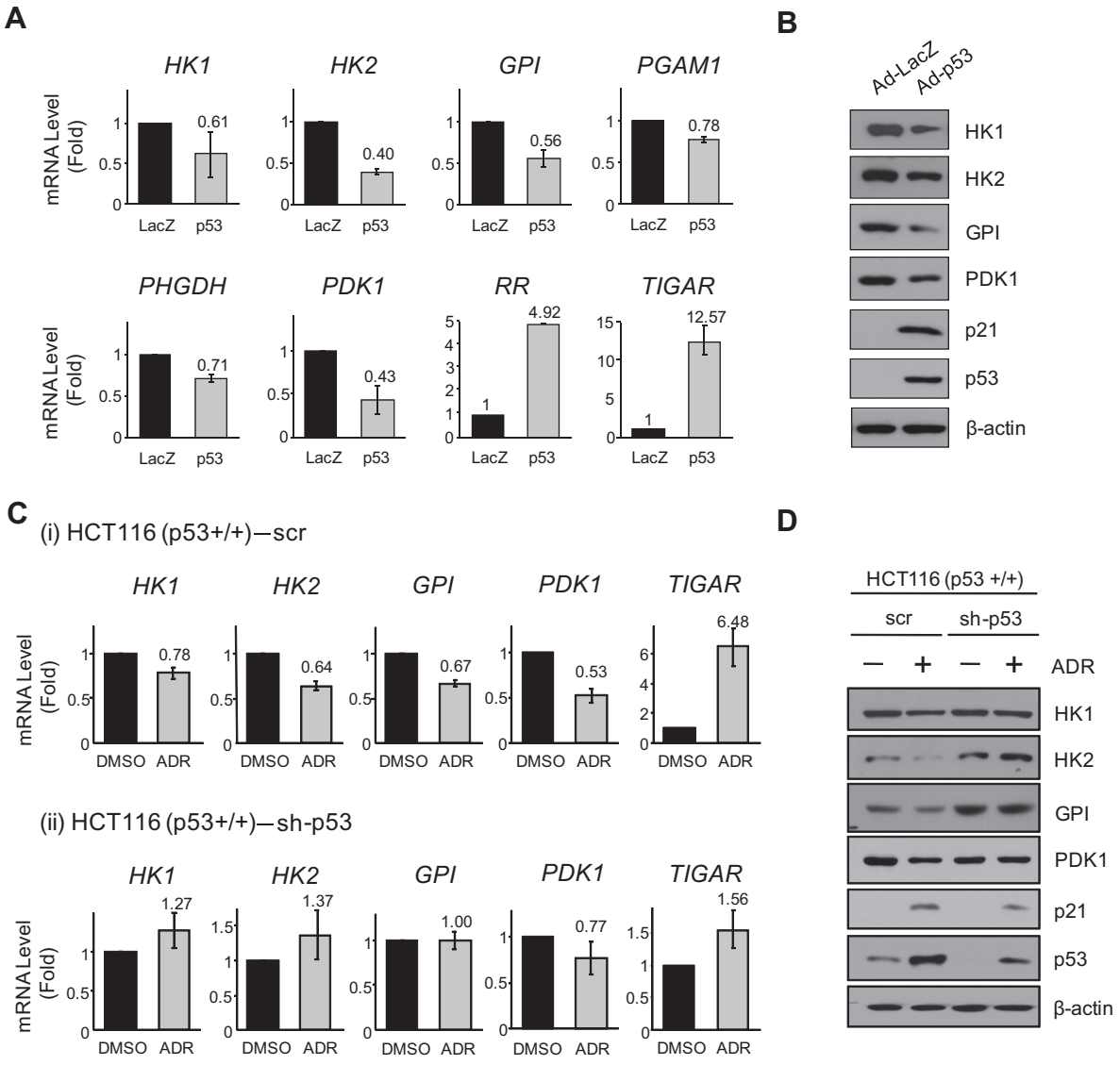

Fig. 1. p53 downregulates the expression of glucose metabolic enzymes. (A) *p53* null H1299 cells were infected with adenovirally expressed LacZ (control) or p53 for 24 h and mRNA level of indicated genes was quantified by qRT-PCR. (B) H1299 cells were infected as in (A), and protein levels were analyzed by immunoblotting using the indicated antibodies. (C) 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, Sigma) for 24 h. Indicated mRNA expression was quantified by qRT-PCR as in (A). (D) HCT116 cells were infected and treated as in (C), and total protein extracts were analyzed by immunoblotting using the indicated antibodies.

glycogen biosynthesis [36], and PHGDH is the first enzyme in the serine biosynthesis pathway branching from glycolysis [37]. PDK is a negative regulator of pyruvate dehydrogenase and decreases oxidation of pyruvate in mitochondria [38]. Consistent with this result, HK1, HK2, GPI, and PDK1 protein levels decreased with p53 expression (Fig. 1B). 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 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. 1C and D). 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. 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.

3.2. 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 [18,39]. 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. 1). HK1, HK2, GPI, and PDK1 transcription was repressed when H1299 cells were infected with the adenovirally expressed form of miR-34a (Fig. 2A), which led to a decrease in protein levels (Fig. 2B). However, the reduced expression of PGAM1 by p53 activation in Fig. 1A 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. 2C).

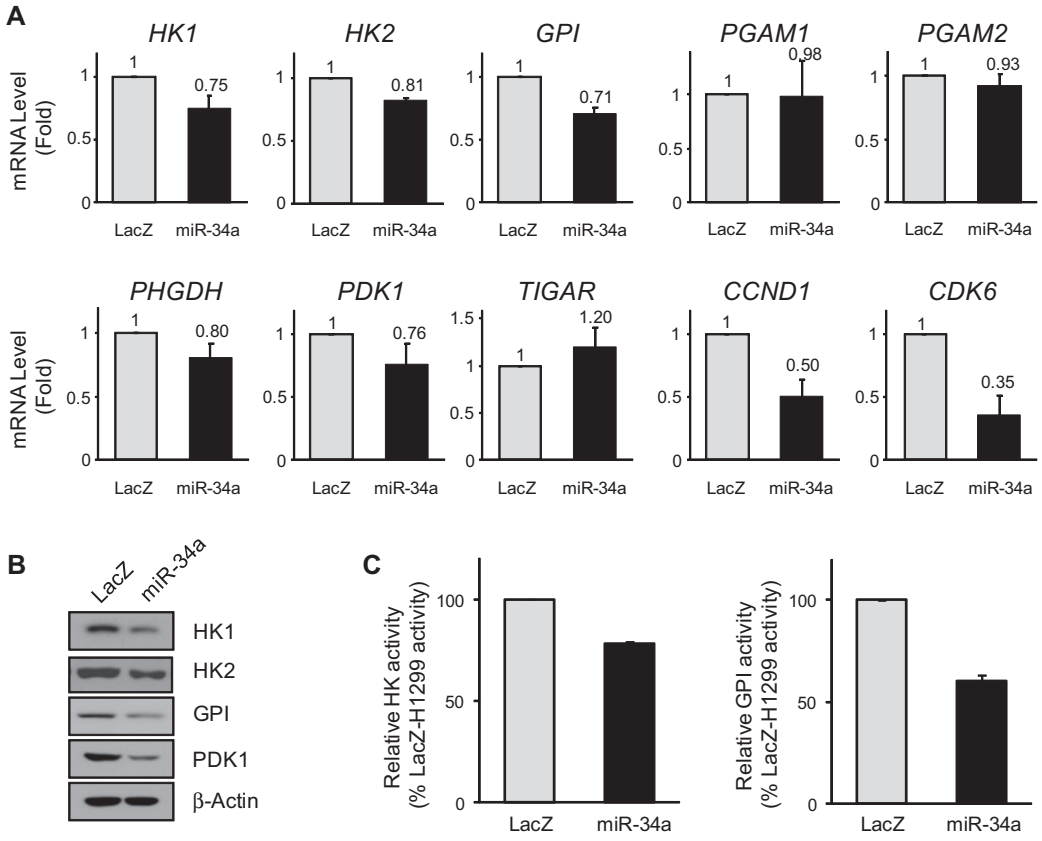

Fig. 2. miR-34a downregulates the expression and activity of glucose metabolic enzymes. (A) H1299 cells were infected with adenovirus expressing miR-34a or LacZ for 72 h, and mRNA levels of indicated genes were quantified by qRT-PCR. (B) Cell lysates were immunoblotted with the indicated antibodies under the same conditions. (C) H1299 cells were infected as in (A), and HK and GPI enzymatic activity was measured using glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay.

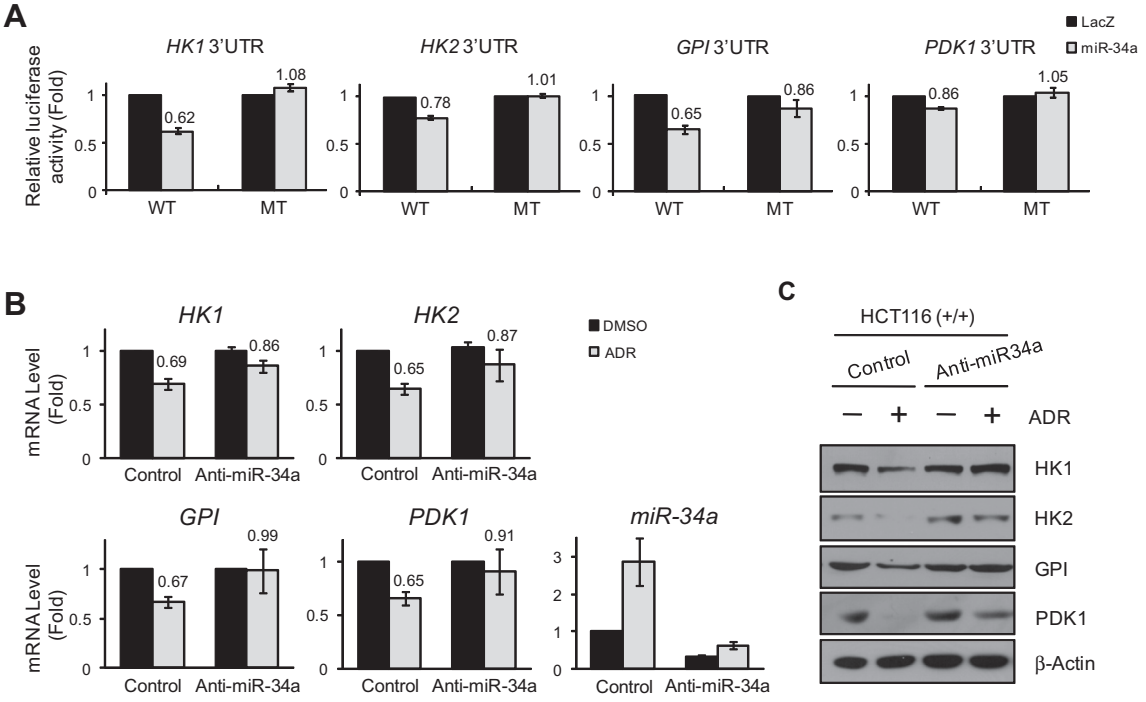

Fig. 3. miR-34a directly targets glucose metabolic enzymes. (A) H1299 cells were transfected with pGL3UC luciferase vector including either the wild-type (WT) or mutant of the miR-34a response element (MT) within the 3′ UTR of the indicated genes). Alignment of the hsa-miR-34a sequence and the 3′ UTR of indicated mRNAs is described in Supplementary Fig. 2. 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 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. miR-34a level was measured using the Taqman microRNA assay. Total protein extracts were analyzed by immunoblotting using the indicated antibodies under the same conditions as (B).

3.3. 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. 3A and Fig. S2).

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. 3B), and protein levels of these enzymes were consistent with those of mRNA (Fig. 3C), indicating that miR-34a directly regulates HK1, HK2, GPI, and PDK1.

3.4. 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

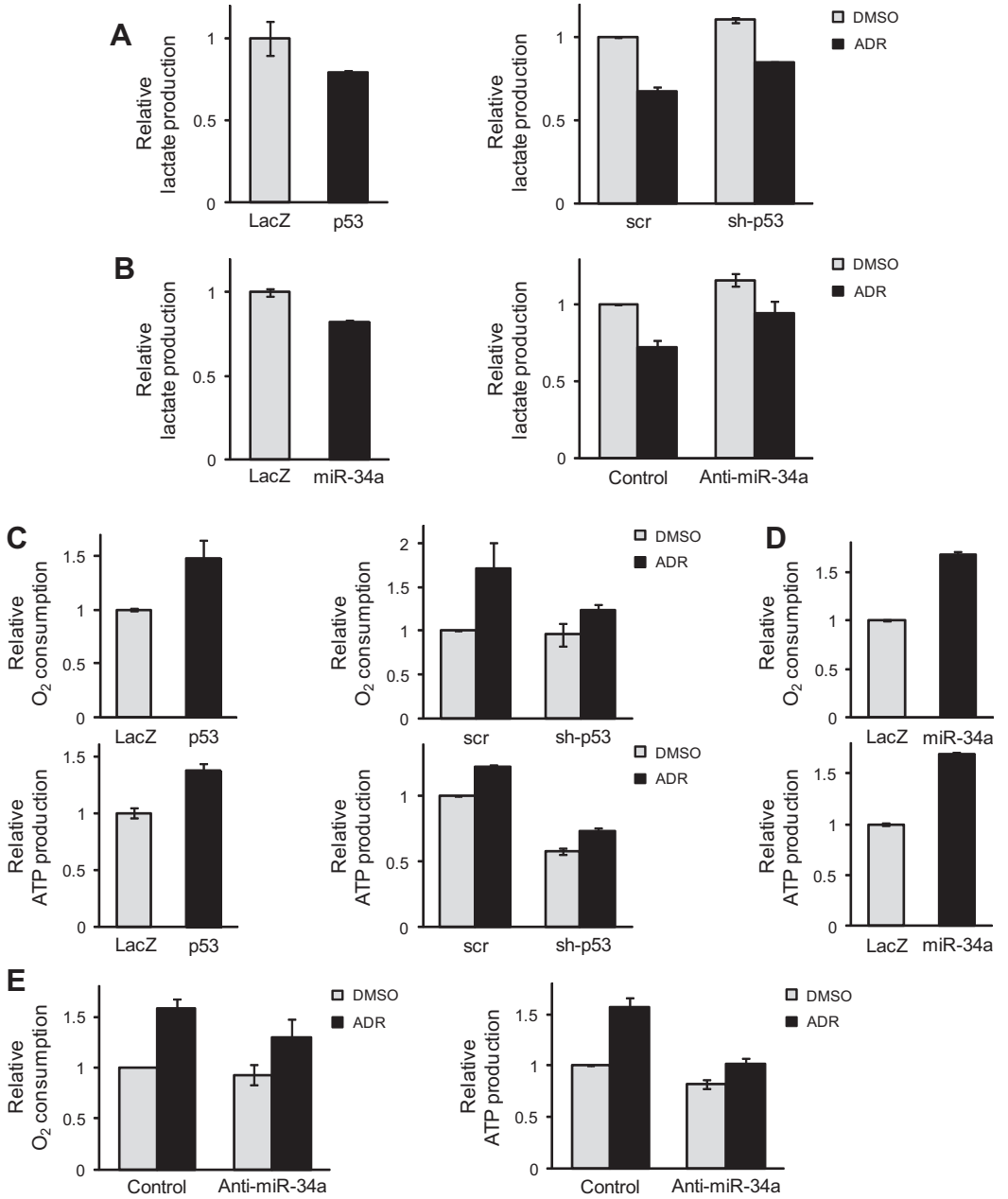

Fig. 4. p53 regulates glucose metabolism by miR-34a. (A) H1299 cells were infected with the adenovirus expressing LacZ or p53 for 24 h, and secreted lactate was measured in media, and secreted lactate was measured under the same conditions as Fig. 1C. (B) H1299 cells were infected with adenovirus expressing LacZ or miR-34a for 72 h, and secreted lactate was measured in media. 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. (C) Under the same conditions as in (A), the oxygen consumption rate of the cells was monitored continuously for 15 min, and cellular ATP production was measured using the ATP Bioluminescence Assay Kit CLS II. (D) 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 (C). (E) 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 (C).

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. Lactate production was decreased by p53 under both conditions, whereas p53 knockdown moderated a reduction in lactate production under DNA damaging conditions (Fig. 4A). As was the case with p53 activation, miR-34a overexpression or induction of endogenous miR-34a following adriamycin treatment decreased lactate production (Fig. 4B). 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 O_2 consumption and ATP production under the same conditions as in Fig. 4A. In contrast to the decrease in lactate production, O_2 consumption and ATP production increased in p53-infected H1299 cells (Fig. 4C). The results of mitochondrial oxygen consumption and ATP production in adriamycin-treated HCT116 cells was the same as p53 overexpression. Mitochondrial 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. 4D). The increase in mitochondrial respiration by miR-34a was confirmed using a miR-34a specific inhibitor (Fig. 4E). Taken together, these data suggest that p53 regulates the expression of glucose metabolic enzymes and substantive glucose metabolism through p53-inducible microRNA, miR-34a.

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 [40,41], and GPI is secreted as an autocrine tumor motility factor [42,43]. Moreover PDK1 activity increases in hypoxic cancer cells, and PDK1 inactivates pyruvate dehydrogenase by phosphorylation [44]. PDK1 is important to connect cytosolic glycolysis and mitochondrial oxidative phosphorylation [6].

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 [32,45] and p53 regulating glucose metabolism by TIGAR, and SCO2 [13,15], 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.043.

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