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Original Article

Repressing malic enzyme 1 redirects glucose metabolism, unbalances the redox state, and attenuates migratory and invasive abilities in nasopharyngeal carcinoma cell lines

Fang-Jing Zheng[1,2], Hao-Bin Ye[1,2,4], Man-Si Wu[1,2], Yi-Fan Lian[1,2], Chao-Nan Qian[1,2,3] and Yi-Xin Zeng[1,2]

Abstract

A large amount of nicotinamide adenine dinucleotide phosphate (NADPH) is required for fatty acid synthesis and maintenance of the redox state in cancer cells. Malic enzyme 1 (ME1)-dependent NADPH production is one of the three pathways that contribute to the formation of the cytosolic NADPH pool. ME1 is generally considered to be overexpressed in cancer cells to meet the high demand for increased *de novo* fatty acid synthesis. In the present study, we found that glucose induced higher ME1 activity and that repressing ME1 had a profound impact on glucose metabolism of nasopharyngeal carcinoma (NPC) cells. High incorporation of glucose and an enhancement of the pentose phosphate pathway were observed in ME1-repressed cells. However, there were no obvious changes in the other two pathways for glucose metabolism: glycolysis and oxidative phosphorylation. Interestingly, NADPH was decreased under low-glucose condition in ME1-repressed cells relative to wild-type cells, whereas no significant difference was observed under high-glucose condition. ME1-repressed cells had significantly decreased tolerance to low-glucose condition. Moreover, NADPH produced by ME1 was not only important for fatty acid synthesis but also essential for maintenance of the intracellular redox state and the protection of cells from oxidative stress. Furthermore, diminished migration and invasion were observed in ME1-repressed cells due to a reduced level of Snail protein. Collectively, these results suggest an essential role for ME1 in the production of cytosolic NADPH and maintenance of migratory and invasive abilities of NPC cells.

Key words Nasopharyngeal carcinoma, malic enzyme 1, low glucose, NADPH

Nasopharyngeal carcinoma (NPC) is a human malignancy derived from epithelial cells. NPC is prevalent in South China, Southeast Asia, and several countries in North Africa, with an annual incidence 25 times higher than that in most other areas of the world[1]. Diet [2], Epstein-Barr virus (EBV) infection[3], and genetic factors[4-6] have been implicated in the etiology of NPC.

Cancer cells primarily generate energy by undergoing a high rate of glycolysis followed by lactic acid fermentation in the cytosol (the Warburg effect), in contrast to normal cells that have a comparatively low rate of glycolysis followed by the oxidation of pyruvate in the mitochondria[7]. Cancer cells also possess a high level of *de novo* fatty acid synthesis even when there are sufficient amounts of fatty acid in the surrounding microenvironment[8]. This synthesis requires a large amount of nicotinamide adenine dinucleotide phosphate (NADPH) and acetyl-coenzyme A, which can be provided through glucose metabolism[9]. Therefore, a large amount of glucose is required for the maintenance and proliferation of cancer cells. However, when cancer

Authors' Affiliations: [1]State Key Laboratory of Oncology in South China, Guangzhou, Guangdong 510060, P. R. China; [2]Department of Experimental Research, [3]Department of Nasopharyngeal Carcinoma, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong 510060, P. R. China; [4]Department of Pathology, University of Rochester Medical Center, Rochester, NY, USA.

Corresponding Authors:
Yi-Xin Zeng, Chao-Nan Qian, State Key Laboratory of Oncology in South China, 651 Dongfeng Road East, Guangzhou, Guangdong 510060, P. R. China;
Email: zengyx@sysucc.org.cn, qianchn@sysucc.org.cn.

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cells form solid tumors, the tumor center is distant from the surrounding blood vessels and thus is often deprived of oxygen and glucose[10]. How cancer cells manage to survive under these adverse conditions is not fully understood.

Malic enzyme 1 (ME1) is a cytosolic protein. Using $NADP^+$, ME1 converts malate to pyruvate and produces NADPH, which is essential for fatty acid synthesis. ME1-dependent NADPH production is one of the three pathways that contribute to the cytosolic NADPH pool. Although it is generally considered to be overexpressed in cancer cells[11], there are only a few references regarding the role of ME1 in cancer cells. These studies include findings that show significantly high ME1 activity in breast cancer cell lines[12,13] and the flux of ME1-produced NADPH as high as the flux of glucose-6-phosphate dehydrogenase (G6PD) in glioblastoma cells[14].

Reactive oxygen species (ROS) are chemically reactive molecules, such as NO, H_2O_2 and O_2^-, which contain oxygen and are natural byproducts of the normal metabolism of oxygen. ROS have been reported as both a stimulant for cell proliferation and an inducer of cell death[15-18]. There are several systems responsible for maintenance of the intracellular redox state, including the reduced glutathione (GSH)-oxidized glutathione (GSSG) system, the thioredoxin system, and the NADPH oxidase (NOX) system. These systems protect cells from outside oxidative stress induced by molecules such as peroxidase, superoxide, and oxygen-free radicals.

In this study, we show that ME1 activity was high in NPC cell lines and that repressing ME1 had profound effects on cellular glucose metabolism, the response to oxidative stress, and migration and invasion.

Materials and Methods

Cell culture

The SV40T-immortalized nasopharyngeal epithelial cell line NP-69 was cultured in Defined Keratinocyte-SFM supplemented with Defined Keratinocyte-SFM Growth Supplement (Invitrogen, Grand Island, NY, USA). NPC cell lines CNE-2, CNE-1, SUNE-1, and HONE-1 were kept in the Department of Experimental Research at the Sun Yat-sen University Cancer Center. The NPC cell lines were maintained in DMEM medium (1000 mg/L D-glucose) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin at 37℃ with 5% CO_2.

shRNA plasmid transfection

Short hairpin RNA (shRNA) plasmids targeting ME1 were constructed using a pSUPER retro puro vector (Oligoengine, Seattle, WA, USA): 5'-GGGCATATTGCT-TCAGTTCTTCAAGAGAGAACTGAAGCAATATGCCC-3' for sh#1 and 5'-GAGAGACAGCAATTGAACATTCAAG-AGATGTTCAATTGCTGTCTCTC-3' for sh#5. The sequence of scrambled shRNA was 5'-GCGCGCTTTG-TAGGATTCGTTCAAGAGACGAATCCTACAAAGCGCG-C-3'. Lipofectamine 2000 (Invitrogen) was used for shRNA vector transfection. Cells transfected with scrambled shRNA were used as control cells.

Malic enzyme 1 activity assay

ME1 activity was assayed using fresh cell extracts. Briefly, at 72 h after transfection with a scrambled shRNA plasmid or with shRNA plasmids targeting ME1, CNE-2 cells were collected and resuspended in 20 mmol/L Tris-HCl lysis buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% (*v*/*v*) Triton X-100, and 0.1% SDS, and supplemented with protease inhibitor mix. The suspension was incubated on ice for 30 min and then centrifuged at 12000 × g for 10 min at 4℃. The supernatant containing the total cell extract was collected, and the protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The extracts were added to a reaction system containing 70 mmol/L Tris-HCl buffer, pH 7.4, 5 mmol/L $MnCl_2$, 0.3 mmol/L $NADP^+$, and 3 mmol/L L-malate (Megazyme International Ireland, Wicklow, Ireland). The absorbance at 340 nm (A_{340}) was read every 1 min for up to 15 min using SpectraMax M5 Multi-Mode Microplate Readers (Molecular Devices, Sunnyvale, CA, USA). The resulting slope of A_{340} versus time was normalized to the protein content of the total lysate.

Immunoblot analysis

Aliquots of 20 μg of total cell extract were resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were incubated with one of the following primary antibodies: ME1 (Sigma, St. Louis, MO, USA), Snail (Cell Signaling Technology, Boston, MA, USA), E-cadherin (Cell Signaling), FASN (Sigma), p-Akt (Ser473) (Cell Signaling), Akt (Cell Signaling), p-GSK-3β (Cell Signaling), GSK-3β (Cell Signaling), Tubulin (Sigma), or Actin (Sigma). The membranes were incubated with primary antibody overnight at 4℃, followed by HRP-conjugated secondary antibody treatment at room temperature (Promega, Madison, WI, USA). The protein-antibody complexes were visualized using an enhanced Phototope TM-HRP Detection Kit (Cell Signaling) and exposed to a Kodak medical X-ray processor (Kodak, Rochester, NY, USA).

Cell growth assay

At 12 h after shRNA transfection, CNE-2 cells were trypsinized, plated into 6-well plates (5×10^4 cells/well), and cultured in different concentrations of glucose. The medium was changed every 2 days. Four days later, the cells on the plates were rinsed with phosphate buffer solution (PBS), fixed with methanol for 10 min, stained with 1% crystal violet for 20 min, and dried at room temperature. Each well was washed with 1 mL of 33% acetic acid. The absorbance of the wash liquid was then measured at 570 nm (A_{570}).

Lactate production

The CNE-2 cell media were collected for lactate concentration analysis using an L-lactic acid assay kit (Megazyme International Ireland, Wicklow, Ireland) according to the manufacturer's protocol.

Isocitrate dehydrogenase activity assay

An isocitrate dehydrogenase (IDH) activity assay was performed as previously described[19]. Briefly, protein extracts from CNE-2 cells were added to an enzyme reaction system containing 100 mmol/L Tris-HCl buffer (pH 7.5), 1.3 mmol/L $MnCl_2$, 0.33 mmol/L EDTA, 0.1 mmol/L β-$NADP^+$, and 0.1 mmol/L D-(+)-threo-isocitrate. A_{340} was read every 1 min for up to 15 min. The resulting slope of A_{340} versus time was normalized to the protein content of the total cell extract.

G6PD and PGD activity assay

G6PD and phosphogluconate dehydrogenase (PGD) activities were determined as previously described [20]. Briefly, protein extracts from CNE-2 cells were added to a reaction system containing 55 mmol/L Tris-HCl buffer (pH 7.5), 18.5 mmol/L $MgCl_2$, 0.18 mmol/L $NADP^+$, and 1.8 mmol/L glucose-6-phosphate for G6PD activity, or containing 55 mmol/L Tris-HCl buffer (pH 7.5), 1.68 mmol/L $MgCl_2$, 0.15 mmol/L $NADP^+$, and 0.34 mmol/L 6-phosphogluconate for PGD activity. A_{340} was read every 1 min for up to 15 min. The resulting slope of A_{340} versus time was normalized to the protein content of the total cell extract.

NADPH quantification

NADPH concentration in CNE-2 cells was measured using an $NADP^+$/NADPH quantification kit (Biovision, Milpitas, CA, USA) according to the manufacturer's protocol.

Caspase-3 activity assay

Caspase-3 activity in CNE-2 cells was determined using a caspase-3 activity kit (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's protocol, which quantitates the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp-p-nitroanilide into the yellow formazan product p-nitroaniline.

Glucose uptake detection

Glucose uptake was measured using the fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG). Briefly, CNE-2 cells were incubated in medium with a final concentration of 10 μmol/L 2-NBDG for 1 h at 37℃ with 5% CO_2. The cells were collected, washed with PBS twice, and resuspended in PBS for flow cytometric measurement using a Cytomics FC 500 (Beckman, Indianapolis, IN, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Mitochondrial membrane potential detection

Mitochondrial membrane potential was assessed by the retention of rhodamine 123 (Rh123). CNE-2 cells were incubated in medium containing 1 μmol/L Rh123 at 37℃ with 5% CO_2 for 30 min. The fluorescence intensity was determined at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Cytomics FC 500.

Cellular ATP measurement

Cellular ATP was measured using a firefly luciferase-based ATP assay kit (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. Briefly, CNE-2 cells were schizolysised and centrifuged at 12 000 × g for 5 min. In 96-well plates, 10 μL of each supernatant was mixed with 100 μL of ATP detection solution at a working dilution. Luminance (RLU) was measured using the Spectra Max M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Total ATP levels were expressed as nmol/mg of protein according to the manufacturer's instructions.

Determination of ROS generation

ROS levels in CNE-2 cells were detected by measuring the oxidative conversion of cell permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) to fluorescent dichlorofluorescein (DCF) using a Cytomics FC 500 (Beckman) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

GSH and GSSG measurement

The content of GSH and GSSG in CNE-2 cells was measured using a glutathione assay kit (Biovision, Milpitas, CA, USA) according to the manufacturer's protocol.

Cell migration and invasion

Cell migration was measured using 6.5 mm Transwell chambers (8 μm pore size; Costar, Corning Incorporated Life Sciences, Lowell, MA, USA). CNE-2 cells were suspended in DMEM, and 3×10^4 cells were added to the upper chamber. For analysis of cell invasion, the upper Transwell chamber was coated with 5 mg of Matrigel (Sigma) diluted in cold water and allowed to air dry. After 24 h of incubation, nonmigrating or noninvading cells were removed mechanically from the upper chamber using a cotton swab. Cells that migrated or invaded into the lower surface of the Transwell membrane were fixed in methanol for 10 min, stained with DAPI (Sigma), and quantified by fluorescence microscopy.

Statistical analysis

Statistical analysis was performed using an unpaired Student's *t* test. A *P* value of < 0.05 was considered significant.

Results

NPC cell lines exhibited high ME1 activity

We chose four NPC cell lines (CNE-2, CNE-1, SUNE-1, and HONE-1) and one nasopharyngeal epithelial cell line (NP-69) to compare the activity of ME1. Equal amounts of protein extracted from these cell lines were subjected to an ME1 activity assay. All NPC cell lines had higher ME1 activity than NP-69 (Figure 1A). The protein level of ME1 was positively correlated with its activity (Figure 1B). Of the four NPC cell lines, CNE-2 had the highest ME1 activity and thus was selected for investigating the role of ME1 in NPC.

Glucose induced higher ME1 activity

ME1 was reported to be up-regulated in mice consuming a high-carbohydrate diet [21]. We wondered whether ME1 would be influenced by carbohydrate in CNE-2 cells. Glucose (5, 30, and 50 mmol/L) was used as an inducing carbohydrate source. Glucose dose-dependent induction of ME1 activity was observed in CNE-2 cells (Figure 1C). The increase in ME1 activity was consistent with an increase in ME1 protein (Figure 1D). We also found that pyruvic acid, the glycolysis product of glucose, induced ME1 activity and increased ME1 protein level (Figures 1E and 1F). These data indicate that glucose induces ME1 activity and expression through glycolysis.

High incorporation of glucose was observed in ME1-repressed cells, and the excessive glucose was not for energy production

Because ME1 could be induced by glucose, we asked whether glucose metabolism would be affected by ME1. We constructed several shRNA plasmids targeting ME1, transfected them into CNE-2 cells, and selected the two with the highest knock down efficiency. The loss of enzymatic activity and decreased protein levels demonstrated the high efficiency of the two shRNA plasmids (Figures 2A and 2B). A fluorescent glucose analog, 2-NBDG, was used to detect the amount of glucose uptake. To our surprise, approximately 1.6 times more glucose was incorporated into ME1-repressed cells than into control cells (Figure 2C).

Next, we tried to determine the fate of the excessive glucose influx. First, we detected a slight increase of mitochondrial membrane potential in ME1-repressed cells (Figure 2D). However, in view of the amount of glucose uptake, this slight increase did not justify the major result. Thus, the excessive glucose was largely not participating in oxidative phosphorylation.

Second, cell lactate production was analyzed to determine whether glucose was subjected to glycolysis. We demonstrated slightly reduced lactate production in ME1-repressed cells (Figure 2E), which suggested that this pathway also could not account for our observations.

Finally, the ATP levels were measured to see whether glucose was used for energy production. However, no significant difference in ATP concentration was found between ME1-repressed and control cells (Figure 2F). Thus, neither of these two major pathways for glucose consumption could account for the excess glucose uptake. Furthermore, from the results of the ATP concentration analysis, we concluded that glucose was not used for energy production.

Enhancement of the pentose phosphate pathway in ME1-repressed cells

ME1 has been reported to be essential for cytosolic NADPH production[14]. When this function is blocked, the concentration of cytosolic NADPH decreases. Thus, we postulated that the excessive glucose was used to compensate for NADPH loss through other pathways. To

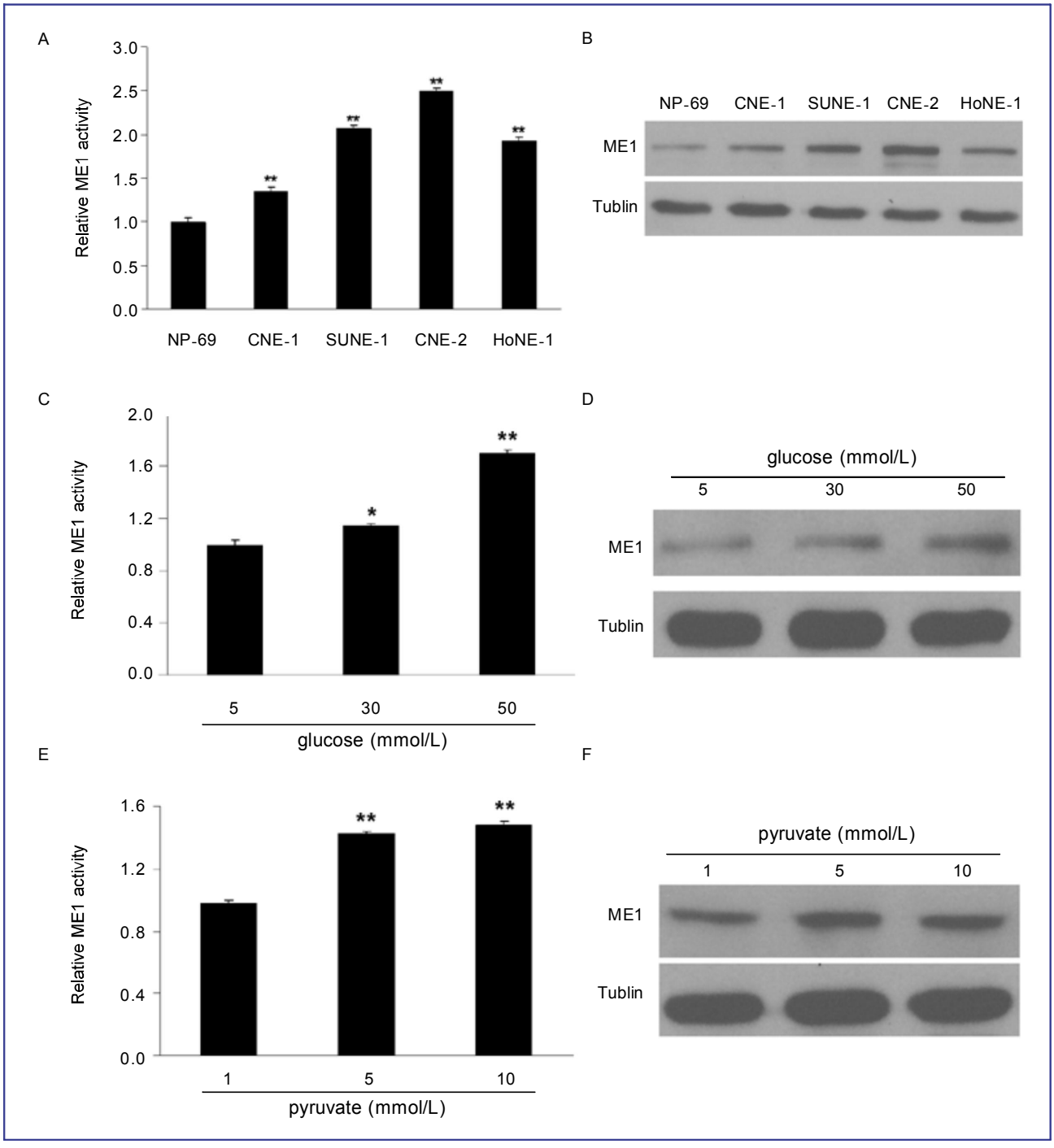

Figure 1. **Nasopharyngeal carcinoma (NPC) cell lines exhibit high malic enzyme 1 (ME1) activity, and ME1 activity can be induced by carbohydrates in CNE-2 cells.** A, ME1 activity is higher in NPC cell lines than in NP-69 cells. Protein extracts from each cell line were added to the enzymatic reaction buffer. The absorbance at 340 nm (A_{340}) was recorded per minute. Enzyme activity was determined by the slope of A_{340} versus time normalized to the protein content of the total cell extract. *, $P < 0.05$; **, $P < 0.005$. B, equal amounts (20 μg) of protein extract from each cell line were subjected to western blot analysis. Tubulin was used as a loading control. C and D, cells were cultured in medium containing 5, 30, or 50 mmol/L glucose for 24 h. They were then collected, and protein was extracted for an enzyme activity assay and western blot analysis. E and F, cells were cultured in glucose-free medium containing 1, 5, or 10 mmol/L pyruvate for 24 h. They were then collected, and protein was extracted for an enzyme activity assay and western blot analysis.

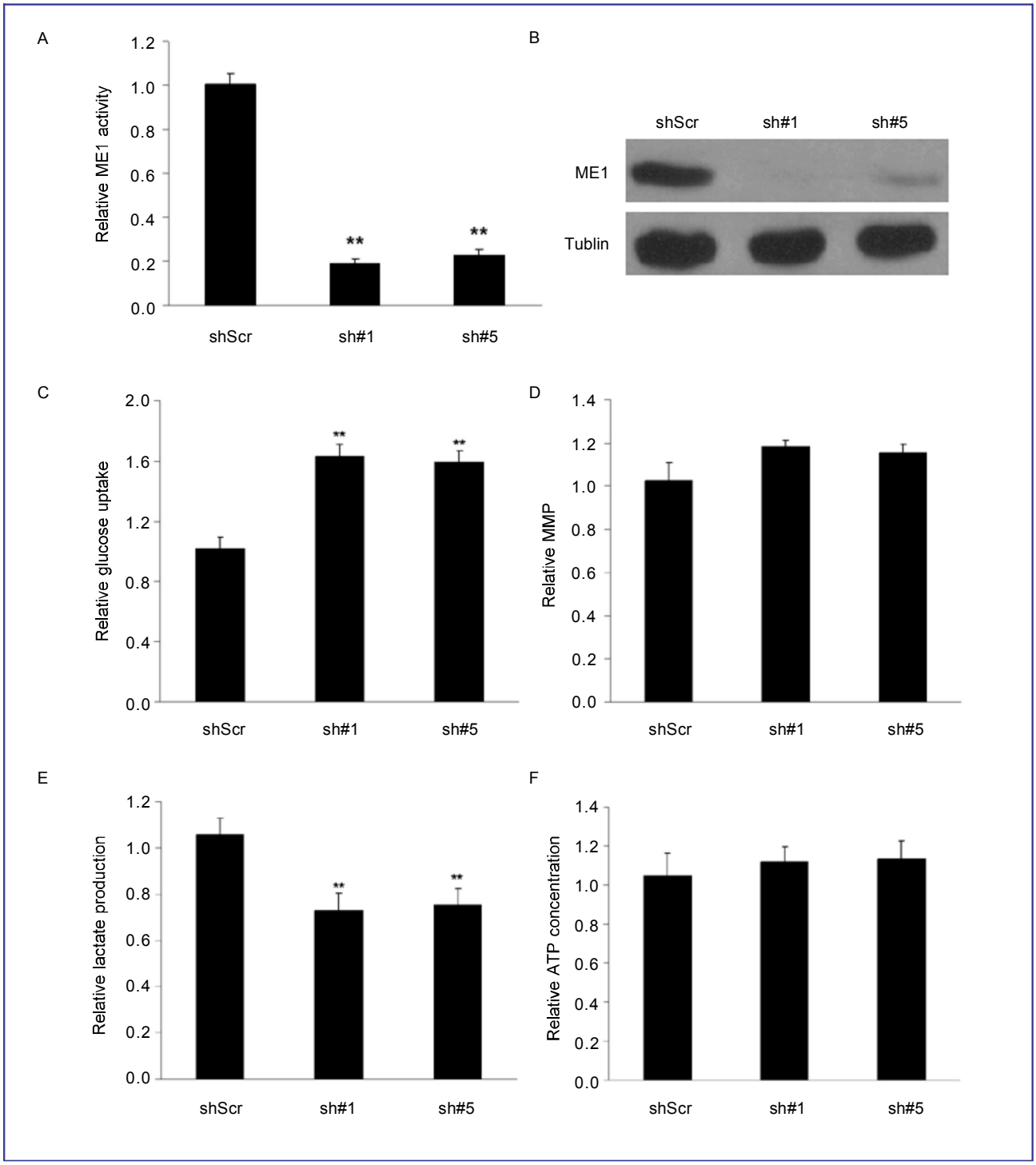

Figure 2. **Higher incorporation of glucose in ME1-repressed cells.** CNE-2 cells were transfected with a scrambled short hairpin RNA (shRNA) plasmid or with shRNA plasmids targeting ME1, and were subjected to the below experiments at 72 h after transfection. A and B, cells were collected, and protein was extracted for enzyme activity assay and western blot analysis. shScr, scrambled shRNA. C, cells were incubated in media with 10 μmol/L 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) for 1 h and then were collected for flow cytometric measurement. D, cells were incubated in medium containing 1 μmol/L rhodamine 123 (Rh123) for 30 min and then were collected for flow cytometric measurement of mitochondrial membrane potential (MMP). E, fresh medium was added, and was collected 12 h later for a lactate production assay. F, cells were washed with cold phosphate buffer solution and schizolysed for ATP detection. **, $P< 0.005$.

test this assumption, the activities of three enzymes that are vital for NADPH production were measured. We found that IDH1 activity was approximately 25% lower in ME1-repressed cells than in control cells, whereas G6PD activity was approximately 40% higher and PGD activity was approximately 35% higher in ME1-repressed cells (Figures 3A-C). To demonstrate the importance of ME1 in NADPH production within NPC cells, CNE-2 cells were cultured in glucose-free medium supplied with pyruvate, which can be used as carbohydrate source when glucose is absent [22]. Under this condition, there was no substrate for the pentose phosphate pathway to produce NADPH, but substrates for two other pathways that produce NADPH would not be affected. We observed that control cells survived under this condition, but ME1-repressed cells died (Figure 3D).

ME1 is essential for cell survival under lowglucose conditions

Next, we examined the NADPH concentration in ME1-repressed and control cells cultured in medium containing different glucose concentrations. With sufficient glucose (30 or 50 mmol/L), there were no significant differences in NADPH concentration between ME1-repressed and control cells. However, when the glucose concentration was lower (2 or 5 mmol/L), obvious differences were observed (Figure 4A). These results indicated that under low-glucose condition, there was not enough substrate for NADPH production.

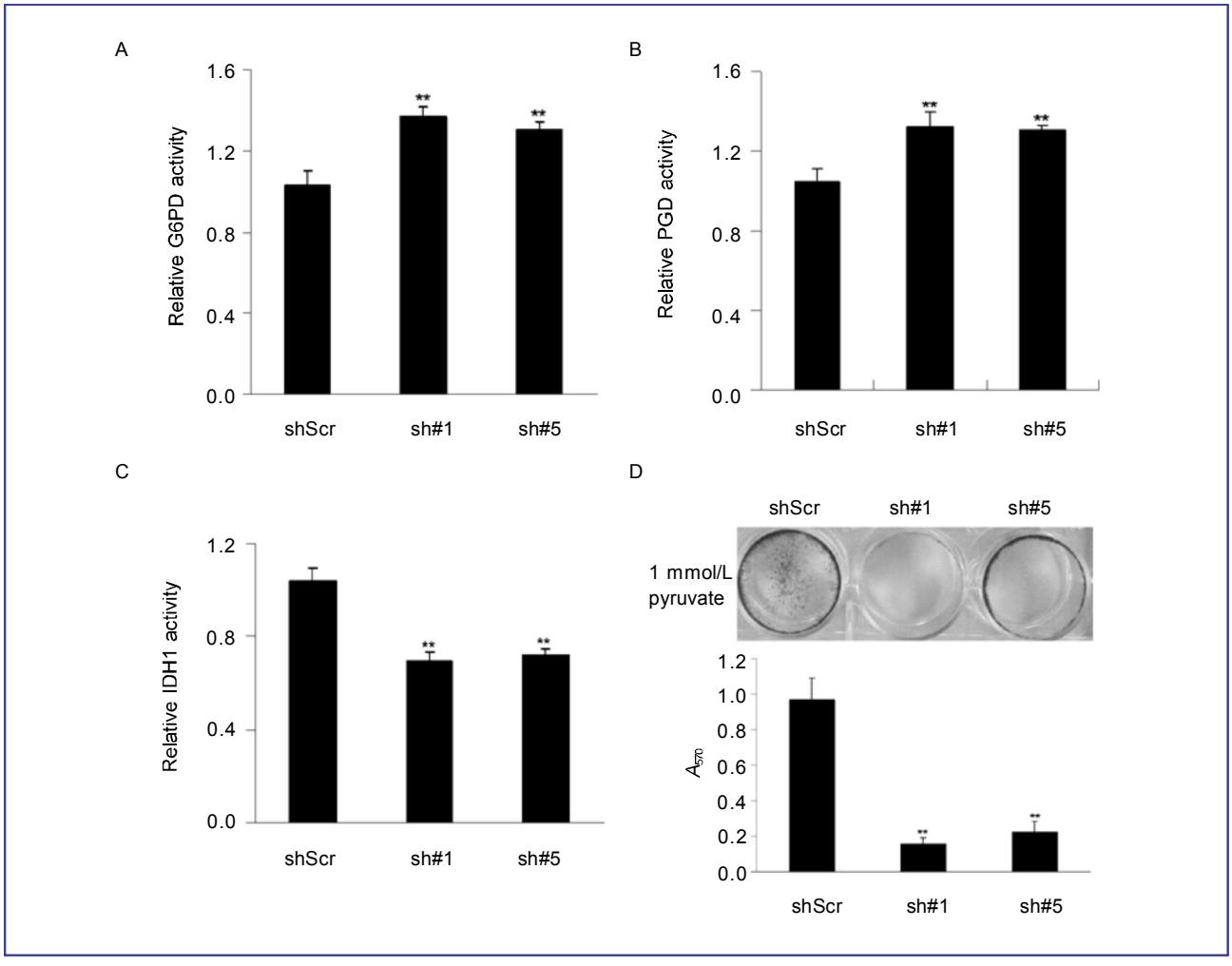

Figure 3. **Enhancement of the pentose phosphate pathway in ME1-repressed cells.** At 72 h after shRNA transfection, cells were collected for protein extraction. The protein extracts were assayed for glucose-6-phosphate dehydrogenase (G6PD) (A), phosphogluconate dehydrogenase (PGD) (B), and isocitrate dehydrogenase 1 (IDH1) activity (C). D, at 12 h after transfection, cells were plated in 12-well plates (5×10^4 cells/well) and cultured in glucose-free medium supplied with 1 mmol/L pyruvate. The medium was changed every 2 days. Four days later, the cells were fixed and stained with crystal violet, and the wash liquid was measured for absorbance at 570 nm (A_{570}).

Tumors are usually glucose-deprived[10], and higher demands for glucose would adversely affect cancer cells under this condition. As expected, we found that ME1-repressed cells did not survive as well as control cells did under low-glucose condition (Figure 4B). Higher caspase-3 activity was also observed in ME1-repressed cells under this condition (Figure 4C). Interestingly, compared with 5 mmol/L glucose, 2 mmol/L glucose did not lead to lower ME1 activity (Figure 4D) or lower ME1 protein level (Figure 4E).

ME1-dependent NADPH production was essential for maintenance of the intracellular redox state and protected cells from oxidative stress

Cancer cells encounter several stresses, including oxidative stress. The GSH-GSSG system is a cellular system that protect cells from oxidative stress and keep the intracellular balance of the redox state[23]. NADPH is a reactant that participates in the conversion of GSSG to GSH. Because ME1 could regulate the NADPH level, we wondered whether the GSH-GSSG system was affected by repression of ME1. The results showed that the GSH content and GSH/GSSG ratio were significantly reduced in ME1-repressed cells (Figures 5A and 5B). The GSH-GSSG system protects cancer cells from outside oxidative stress. To test the role of ME1 in the response of CNE-2 cells to oxidative stress, ME1-repressed cells were treated with 1.5 mmol/L H_2O_2 for indicated time, and then intracellular ROS level was measured using the fluorescent probe DCFH-DA. In the first hour, ROS level in ME1-repressed cells was dramatically increased compared to that in control cells (Figure 5C). Although it dropped thereafter, ROS level was still much higher in ME1-repressed cells than in control cells (Figure 5C). Additionally, we found a large number of dead cells in the ME1-repressed group when the duration of H_2O_2 treatment was prolonged to 24 h. Correspondingly, the enzyme activity assay demonstrated much higher caspase-3 activity in ME1-repressed cells (Figure 5D).

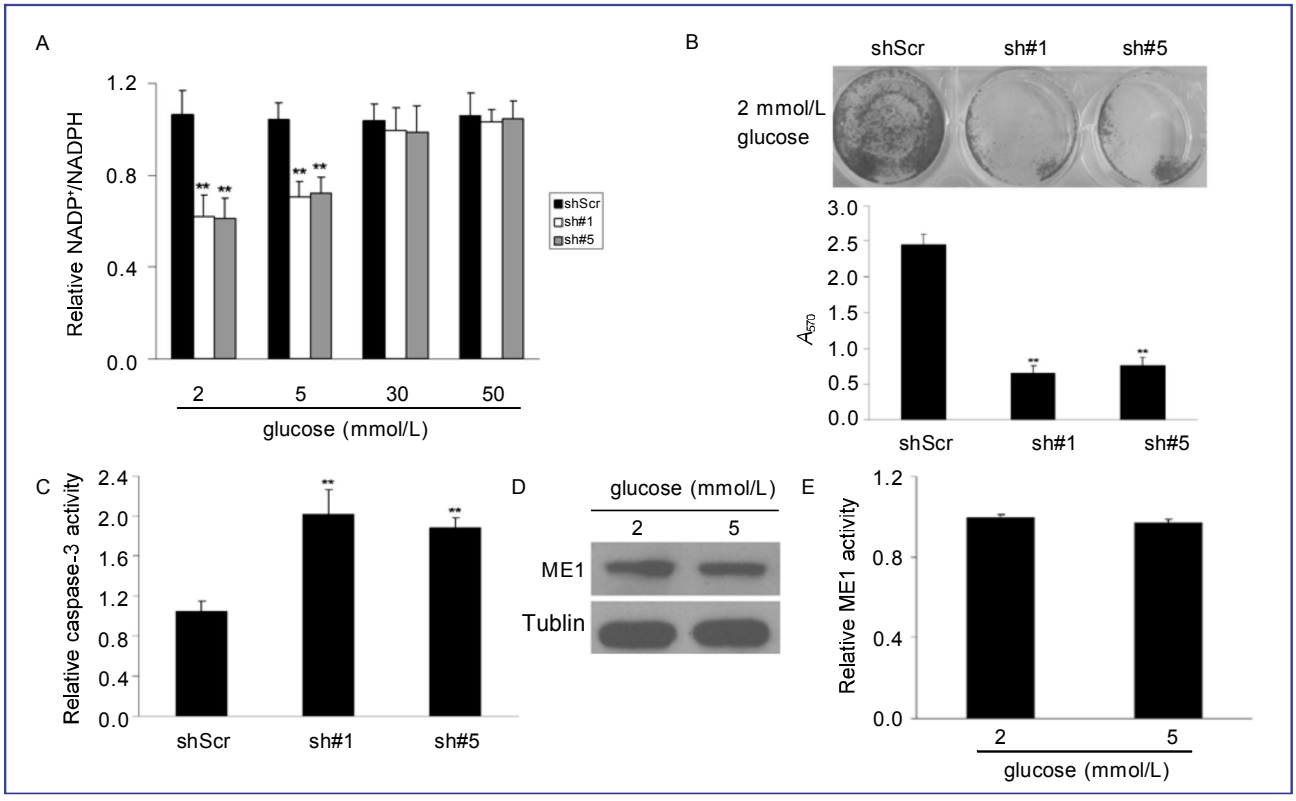

Figure 4. **ME1 enabled cell survival under low-glucose condition.** A, at 72 h after shRNA transfection, ME1-repressed and control cells were cultured in medium containing glucose at indicated concentrations for 24 h and then were collected to measure nicotinamide adenine dinucleotide phosphate (NADPH) and $NADP^+$ concentration. B, at 12 h after shRNA transfection, ME1-repressed and control cells were plated in 12-well plates (5×10^4 cells/well) and cultured in medium with 2 mmol/L glucose. The medium was changed every 24 h. Four days later, the cells were fixed and stained with crystal violet, and the wash liquid was measured for A_{570}. C, at 72 h after shRNA transfection, ME1-repressed and control cells were cultured in medium containing 2 mmol/L glucose for 24 h and then were collected for caspase-3 activity assay. D and E, cells were cultured in medium containing 2 or 5 mmol/L glucose for 24 h and then were collected for an enzyme activity assay and western blot analysis.

Repressing ME1 led to lower Akt activity and reduced cell migration and invasion

Akt is involved in many metabolic events [24,25] and promotes cell survival [26]. Fatty acid synthesis has been reported to have a profound effect on the regulation of Akt [27]. Because ME1 is essential for fatty acid synthesis, we tested whether the Akt pathway was affected in ME1-repressed cells, and found a lower level of p-Akt in ME1-repressed cells (Figure 6A). Two substrates of p-Akt, GSK-3β and fatty acid synthase (FASN) [28], were also examined. As expected, the protein levels of p-GSK-3β and FASN were down-regulated in ME1-repressed cells (Figure 6A). Snail, a transcription factor that regulates adhesive protein expression including E-cadherin [29], is regulated by GSK-3β [30]. We detected a lower level of Snail in ME1-repressed cells (Figure 6B), which indicated that ME1 might influence cell migration and invasion. Using a transwell system, we functionally demonstrated that fewer cells in the ME1-repressed group migrated or invaded into the lower surface of the transwell membrane (Figures 6C and 6D).

Discussion

In this study, we show an important role of ME1 in cytosolic NADPH production within NPC CNE-2 cells. We compared ME1 activity in CNE-2 cells with that in other cancer cell lines, including MCF-7, HepG2, HeLa, Raji, and U2OS cells, and found that ME1 activity was much higher in CNE-2 cells (data not shown). This result indicated the significant status of ME1 in CNE-2 cells.

Our study showed that glucose induced ME1 in a dose-dependent manner in CNE-2 cells, suggesting that carbohydrates can induce ME1 activity *in vitro*. We used another type of carbohydrate, pyruvate, to see whether ME1 activity would still be induced. To our surprise, pyruvate could also induce ME1 activity in a dose-dependent manner in CNE-2 cells. High carbohydrate

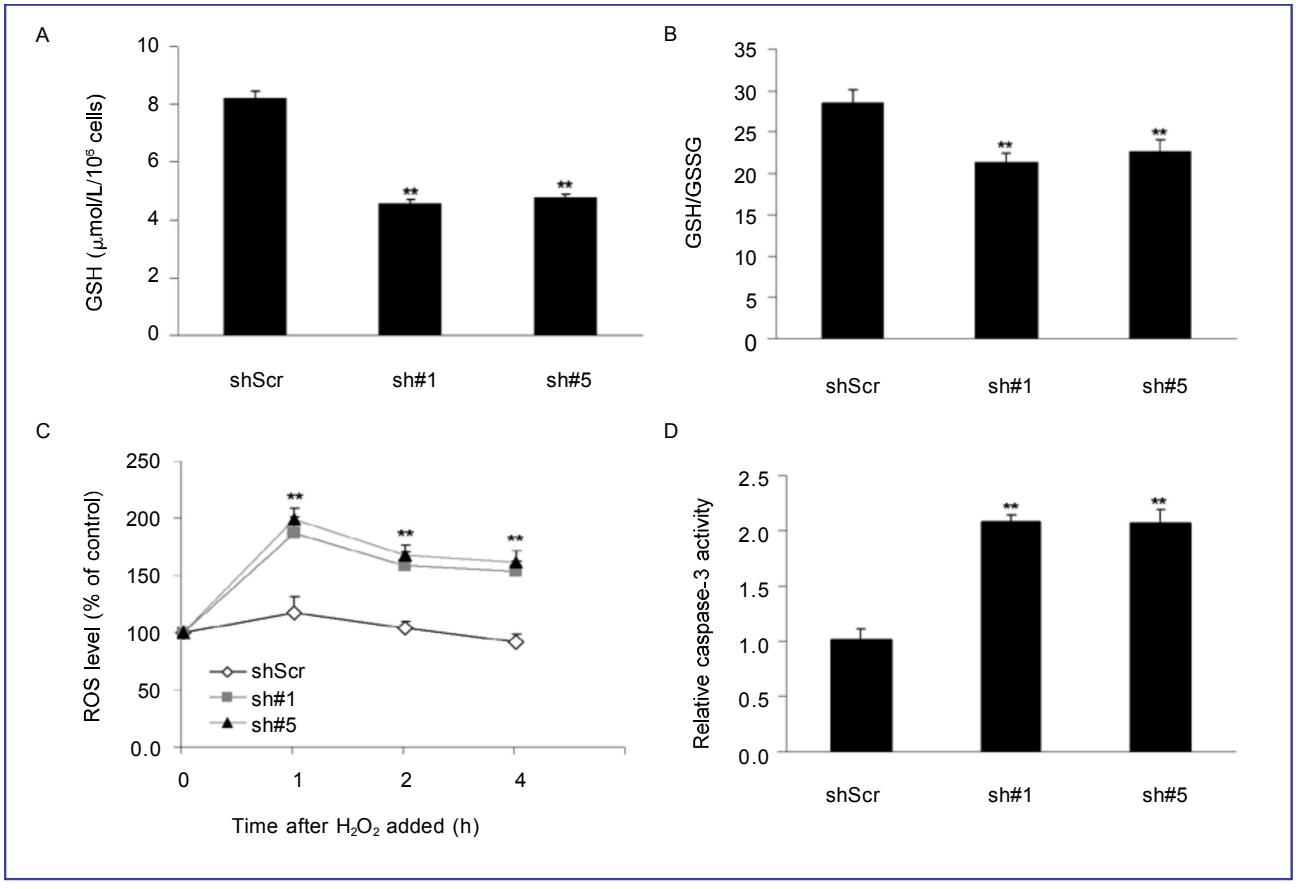

Figure 5. **ME1 functions as a keeper of the intracellular redox state.** At 72 h after shRNA transfection, cells were subjected to the below experiments. A and B, glutathione (GSH) content and the GSH/glutathione-oxidized glutathione (GSSG) ratio were measured in ME1-repressed and control cells. C, intracellular reactive oxygen species (ROS) level was measured in ME1-repressed and control cells after treatment of 1.5 mmol/L H_2O_2 at the indicated time points using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). D, caspase-3 activity was measured in ME1-repressed and control cells after treatment of 1.5 mmol/L H_2O_2 for 24 h.

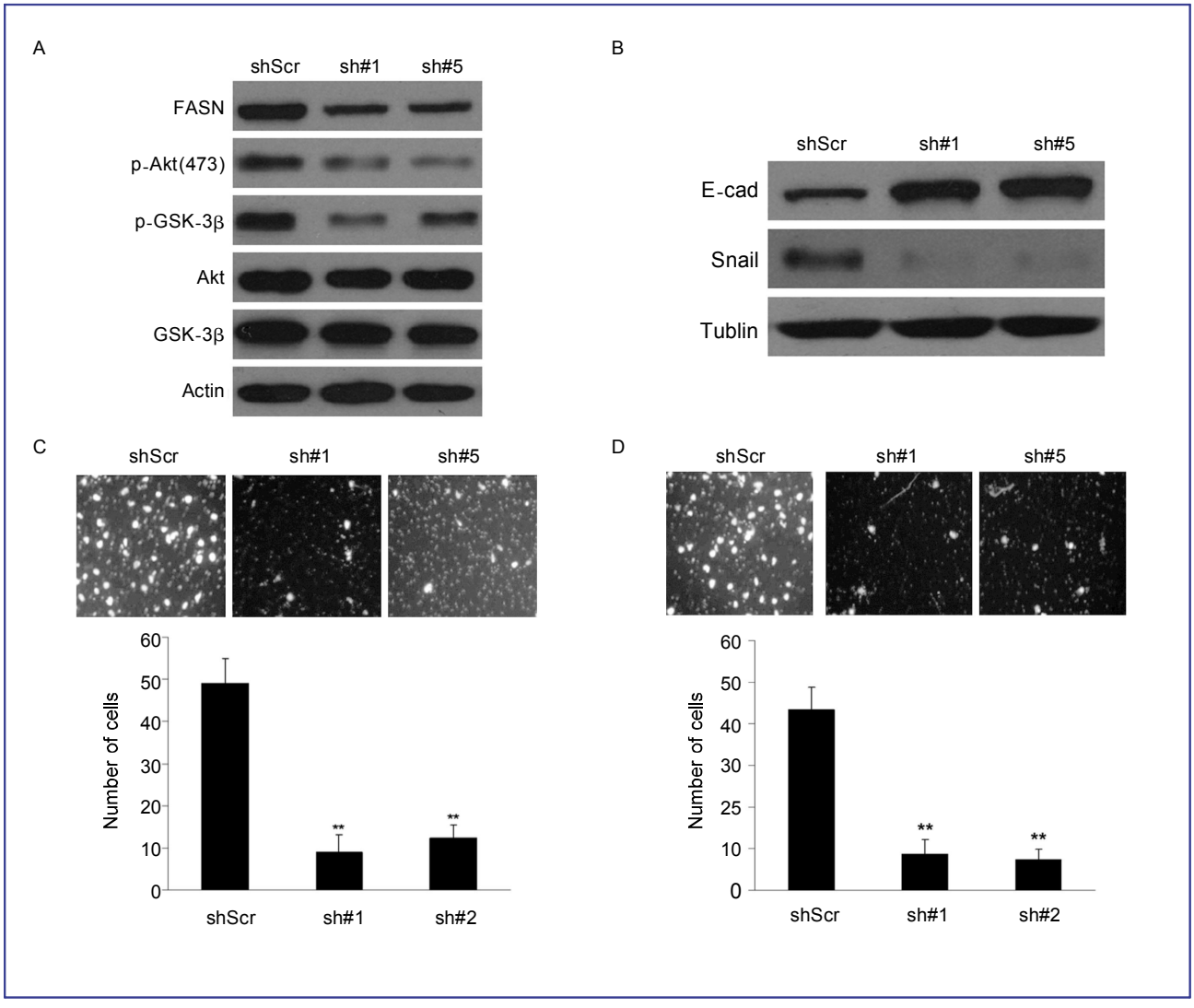

Figure 6. **Repressing ME1 reduces cell migration and invasion.** A and B, at 72 h after shRNA transfection, cells were collected for western blot analysis, and proteins were detected with the indicated antibodies. C and D, at 48 h after shRNA transfection, 3×10^4 cells were added to the upper chambers without (C) or with (D) Matrigel. After 24 h of incubation, cells that migrated to the lower surface of the Transwell membrane were fixed, stained with DAPI, and quantified by fluorescence microscopy.

diet-induced ME1 activity was thought to be a response to insulin change *in vivo*. Exactly how carbohydrates induce ME1 activity *in vitro* remains to be further studied.

A significantly higher incorporation of glucose was observed in ME1-repressed cells. We studied two major pathways for glucose consumption in cancer cells: oxidative phosphorylation and glycolysis. To our surprise, we found a slight increase in mitochondrial membrane potential and reduced levels of lactate production. The increase in mitochondrial membrane potential could be explained by a higher flux of malate into the mitochondria because the concentration of malate outside the mitochondria was much higher due to the repression of ME1. Malate would participate in the tricarboxylic acid cycle and oxidative phosphorylation and thus lead to increased mitochondrial membrane potential. A similar result has been observed when ATP citrate lyase (ACL) is repressed[31]. Because of a higher flux of citrate toward the mitochondria, ACL-repressed cells exhibited higher mitochondrial membrane potential. Because the Akt pathway is reported to be involved in regulation of glycolysis, the decreased lactate production could be attributed to a lower level of p-Akt in ME1-repressed cells[32].

The two major pathways for glucose consumption were shown not to use the excess glucose, thus

requiring another more reliable explanation. We detected higher activity of the enzymes that are responsible for NADPH production in the pentose phosphate pathway in ME1-repressed cells. The need to compensate for the loss of ME1-produced NADPH provides a rational explanation for the high incorporation of glucose. We are unable to explain the lower activity of IDH1. The fact that ME1-repressed cells could not survive in a glucose-free medium supplied with pyruvate revealed the vital role of ME1 in NADPH production in CNE-2 cells. A study conducted in rat insulinoma cells has shown that there were no differences in the activity of G6PD and IDH1 between ME1-repressed and control cells [33]. However, this result could be due to differences between species or various tissues. The regulation mechanism of these enzymes in ME1-repressed cells remains to be further studied.

ME1 is regarded as an important enzyme for NADPH production in the cytosol [14]. However, only under low-glucose condition did we detect a decrease in NADPH. Under high-glucose condition, there was enough glucose both for NADPH production through the pentose phosphate pathway and for other biological events.

A high demand for glucose to compensate for NADPH loss in ME1-repressed cells would be adverse for cancer cells cultured under low-glucose condition. We observed a lower tolerance to low glucose in ME1-repressed cells. Contrary to the glucose dose-dependent induction of ME1, the constant ME1 activity under low-glucose condition indicated that ME1 was vital in this situation.

Many types of antitumor drugs have the potential to increase intracellular ROS level and thus cause damages to the membrane system, protein, or DNA, leading to the eventual death of cancer cells [34-37]. Thus, systems that are responsible for scavenging ROS are vital for the survival of cancer cells treated with different types of antitumor drugs. We found a significant decrease of GSH content in ME1-repressed cells, which suggested that ME1 may act as an important enzyme for drug-resistant cancer cells. H_2O_2 treatment showed extraordinary impairment in ME1-repressed cells, demonstrating the importance of ME1 as a keeper of the intracellular redox state. The role of ME1 in drug resistance in cancer cells is worthy of further study.

Akt activity has been reported to be regulated by cellular fatty acid synthesis [27,38-40]. Reduced NADPH level would lead to down-regulation of fatty acid synthesis and thus would affect Akt activity. Conversely, cellular fatty acid synthesis is regulated by Akt [28]. Consistent with this result, decreased level of FASN was detected in ME1-repressed cells.

We attributed the down-regulated migration and invasion of ME1-repressed cells to a decreased level of the protein Snail, which is regulated by GSK-3β. However, there may be other factors that contribute to this phenomenon. A large amount of glucose was subjected to the pentose phosphate pathway, while glycolysis was decreased in ME1-repressed cells. Lactate produced from glycolysis is secreted from cells and creates a microenvironment of acidity that is important for migration and invasion [41-44]. Thus, it is possible that decreased glycolysis caused by a readjustment of glucose flux is partially responsible for the reduced migration and invasion. In addition, differences of expression level and activity of ME1 among different NPC cell lines showed a pattern: well differentiated cell lines, such as CNE-1 and HONE-1, demonstrated lower ME1 activity and expression level when compared with poorly differentiated cell lines, such as CNE-2 and SUNE-1.

In conclusion, our results suggest that NADPH production in CNE-2 cells largely depends on ME1. Consequently, repressing ME1 would have a profound impact on glucose metabolism, the cell redox state, and cell migration and invasion.

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