Loss of the respiratory enzyme citrate synthase directly links the Warburg effect to tumor malignancy

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To investigate whether altered energy metabolism induces the Warburg effect and results in tumor malignancy, the respiratory enzyme citrate synthase (CS) was examined, silenced, and the effects analyzed. In human cervical carcinoma cells, RNAi-mediated CS knockdown induced morphological changes characteristic of the epithelial-mesenchymal transition (EMT). This switch accelerated cancer cell metastasis and proliferation in in vitro assays and in vivo tumor xenograft models. Notably, CS knockdown cells exhibited severe defects in respiratory activity and marked decreases in ATP production, but great increases in glycolytic metabolism. This malignant progression was due to activation of EMT-related regulators; altered energy metabolism resulted from deregulation of the p53/TIGAR and SCO2 pathways. This phenotypic change was completely reversed by p53 reactivation via treatment with proteasome inhibitor MG132 or co-knockdown of E3 ligase HDM2 and partially suppressed by ATP treatment. This study directly links the Warburg effect to tumor malignancy via induction of the EMT phenotype.

Most cancer deaths are caused by tumor metastases that are often resistant to conventional cancer therapies such as chemicals and irradiation. In a wide variety of carcinoma tumor cells, the phenotypic change from epithelial into mesenchymal cells (the so-called epithelial-mesenchymal transition, EMT) plays a decisive role during metastatic progression. In human cervical carcinoma cells, RNAi-mediated CS knockdown induced morphological changes characteristic of the epithelial-mesenchymal transition (EMT). This switch accelerated cancer cell metastasis and proliferation in in vitro assays and in vivo tumor xenograft models. Notably, CS knockdown cells exhibited severe defects in respiratory activity and marked decreases in ATP production, but great increases in glycolytic metabolism. This malignant progression was due to activation of EMT-related regulators; altered energy metabolism resulted from deregulation of the p53/TIGAR and SCO2 pathways. This phenotypic change was completely reversed by p53 reactivation via treatment with proteasome inhibitor MG132 or co-knockdown of E3 ligase HDM2 and partially suppressed by ATP treatment. This study directly links the Warburg effect to tumor malignancy via induction of the EMT phenotype.
was knocked down using RNAi-mediated gene silencing in HeLa and SiHa cells, two human cervical carcinoma cell lines that predominantly use aerobic respiration for ATP formation and display relatively low metastatic capability. The stable loss-of-function phenotype was then analyzed in detail, and these cell lines were used to further investigate the molecular mechanism underlying this phenomenon.

Results

Reduction of CS expression correlates with alterations in cellular bioenergetics. To establish the relationship between CS expression and tumorigenesis, we examined its expression level in a number of human cancer cell lines. Human cancer cell lines expressed varying levels of CS protein, ranging from clearly detectable to almost undetectable (Figure 1A). To further establish the connection between CS expression and tumor malignancy, we analyzed its expression level in normal and cancerous human cervical cell lines with different degrees of malignancy. A reduction in CS expression compared to a normal cervical cell line was observed in cervical cancer cell lines; the extent to which CS expression was reduced was directly proportional to the degree of malignancy of the cells (Figure 1B). To elucidate the possible role of CS expression in metabolic alterations, we examined the expression of genes involved in bioenergetic metabolism in the TCA cycle, glycolysis, and bioenergetic homeostasis in four selected cell lines (HeLa, SiHa, MCF7, and PC-3). Very low or nearly undetectable CS expression in MCF7 cells coincided with increased glycolytic enzyme expression and AMP-activated protein kinase (AMPK) activity (Figure 1C). Together, these results reveal that human cancer cell lines exhibit a wide spectrum of CS expression levels; reduced CS expression correlates with the switch of cellular bioenergetics from aerobic respiration to glycolysis, suggesting that there is a metabolic shift in energy production during tumorigenesis.

Knockdown of CS expression induces morphological change to the EMT phenotype. To directly investigate the involvement of CS in tumor malignancy, we generated a number of CS knockdown clones using the RNAi-mediated gene silencing technique in HeLa and SiHa cells, which predominantly use mitochondrial respiration for ATP production. Two different types of colonies were observed during the selection of CS-silenced clones in HeLa and SiHa cells. One colony type displayed the original epithelial morphology, while the other exhibited a fibroblast-like phenotype (Figure 2A). To examine CS expression in these two distinct colony types, immunofluorescence staining and Western blot analyses with an antibody specific for the CS protein were performed. The fibroblast-like cells exhibited an almost complete loss of CS expression, while the epithelial cells displayed normal CS expression levels (Figure 2B and C). CS activity in these two colonies was also assessed; a large decrease in CS activity was detected in the fibroblast-like cells compared to that of the epithelial cells (Figure 2D). These results show that inhibition of CS expression induces a fibroblast-like phenotype in both HeLa and SiHa cells. To characterize the behaviors and features of CS...
knockdown cells, we specifically selected four CS knockdown HeLa cell clones for further analyses (Figure 2E).

To analyze in detail the fibroblast-like phenotype induced by CS silencing, the formation of stress fibers by F-actin reorganization was examined using Alexa Fluor 488-conjugated phalloidin staining. Assembly and redistribution of F-actin stress fibers was observed in the CS knockdown cells as compared to mock- and vector-transfected cells (Figure 2F). Since this morphological change is similar to the EMT phenotype, we further examined the expression of EMT-related proteins, including E-cadherin, vimentin, α-smooth muscle actin (α-SMA), Snail, and Twist, using Western blot analysis. Expression of the epithelium-specific E-cadherin was strongly downregulated, whereas the mesenchyme-specific markers (vimentin and α-SMA) and transcription factors (Snail and Twist) were greatly upregulated after CS knockdown as compared to the mock- and vector-transfected cells (Figure 2G). In addition, the E-cadherin and vimentin expression patterns were confirmed by immunofluorescence staining (Figure 2H and I). These results indicate that cells with inhibited CS expression exhibit a typical EMT phenotype.

Silencing of CS expression greatly increases cell migration and proliferation in vitro. It has been well illustrated that cancer cells undergo EMT change, thus acquiring the ability to migrate and metastasize. Because the EMT phenotype induced by CS knockdown might promote cancer cell migration and invasion, these properties were examined. Greater migration was observed in CS knockdown cells as compared to mock- and vector-transfected cells in wound healing migration assays (Figure 3A). Using the Boyden chamber migration assay, the number of migrated CS-silenced cells was approximately five times higher than that of...
mock- and vector-transfected cells (Figure 3B). Furthermore, approximately ten times more CS-inhibited cells were invasive in the Matrigel invasion assay than mock- and vector-transfected cells (Figure 3C). These results reveal that the EMT phenotype induced by CS knockdown greatly accelerates cancer cell migration and invasion

In the wound healing migration assay, the incubation time required for cell confluence was much shorter in the CS-silenced cells than in the mock- and vector-transfected cells. To examine the short-term effects of CS knockdown on tumor cell growth, methyl thiazolyl tetrazolium (MTT) cell growth and bromodeoxyuridine (BrdU) incorporation assays were performed. Faster cell growth was detected in the CS knockdown cells as compared to the mock- and vector-transfected cells (Figure 3D and E). To evaluate the long-term effects of CS knockdown on tumor cell proliferation, colony formation and soft agar clonogenic assays were carried out. Anchorage-dependent and -independent clonogenic growth was greatly increased, resulting in increased colony size and number in the CS knockdown cells (Figure 3F and G). These results show that CS knockdown greatly increases tumor cell growth

Inhibition of CS expression markedly accelerates tumor malignancy

To directly evaluate the effect of CS silencing on tumor malignancy, a tumor xenograft model. Greater in vivo tumor formation and volume were observed in the CS-inhibited cells as compared to the mock- and vector-transfected cells (Figure 4A and Table 1). Rapid growth of the CS knockdown cells was detected with grafting of only 1 × 10⁵ cells per mouse after 8–14 days. Tail vein injection of cells (to determine their in vivo tumor metastatic potential) revealed higher and wider metastases of the CS-silenced cells as compared to the mock- and vector-transfected cells (Figure 4B and Table 2). Metastasis of the CS-inhibited cells was observed not only to lung but also to heart, kidney, liver, lymph node, mesentery, and subcutaneous tissues. In
Loss of CS expression causes severe defects in aerobic respiration but strongly upregulates glycolysis. Mitochondrial membrane potential (ΔΨm) is established by complexes I, III, and IV of the electron transport chain in aerobic respiration[24,25]. Thus, the level of ΔΨm corresponds to the level of mitochondrial respiration[26]. To evaluate the effect of CS knockdown on aerobic respiration, we measured ΔΨm in CS-silenced cells using Tetramethylrhodamine methyl ester (TMRM) staining assays. Strong staining was seen in the mock- and vector-transfected cells; however, little or no staining was detected in the CS-inhibited cells using this fluorescent probe, suggesting that CS knockdown impairs TCA cycle progression and, in turn, reduces mitochondrial respiration (Figure 5A). Because reactive oxygen species (ROS) are generated during aerobic respiration, decreased respiratory activity would reduce ROS production[27,28]. To further estimate the effect of CS silencing on mitochondrial respiration, ROS levels were measured using 5-(and-6)-chloromethyl-2',7'-dichlorodihydro fluorescein diacetate, acetyl ester (CM-H2DCFDA). Very low or no CM-H2DCFDA staining was detected in the CS-silenced cells compared to that of mock- and vector-transfected cells (Figure 5B). Together, these results clearly indicate that loss of the CS expression impairs mitochondrial function, which directly influences respiratory activity.

Because CS knockdown impaired TCA cycle progression and therefore aerobic respiration, the level of ATP production was assessed using the firefly luciferase ATP assay. Greatly reduced ATP levels were observed in the CS knockdown cells as compared to mock- and vector-transfected cells (Figure 5C). Because AMPK acts as an energy sensor, modulating metabolic stresses such as hypoxia and respiratory impairment[29,30], any stimuli that increase AMP or decrease ATP activate AMPK and subsequently increase glucose uptake[31]. To further examine the effect of decreased ATP generation, the level of phosphorylated AMPK (p-AMPK) in CS-inhibited cells was also analyzed. The level of p-AMPK was greatly increased in the CS-silenced cells compared to mock- and vector-transfected cells (Figure 5D). Together, these results clearly indicate that loss of CS expression induces bioenergetic alteration and enhances glucose uptake.

The extracellular signal-regulated kinase (ERK) pathway is a key component in the control of cell growth, survival, and motility[32]. This signaling is often upregulated in a diverse range of human cancers[33]. To examine whether CS silencing also activates the ERK signaling pathway, the level of phosphorylated ERK1 (p-ERK1) in CS-silenced cells was analyzed. The level of p-ERK1 was largely increased in the CS knockdown cells as compared to the mock- and vector-transfected cells (Figure 5D). These results reveal that inhibition of CS expression results in deregulation of cell growth signaling.

Altered metabolic enzyme expression may impair mitochondrial respiration and, in turn, activate or upregulate cytosolic glycolysis for cellular ATP generation[34]. To examine the effect of CS knockdown on bioenergetic metabolism, the expression of key enzymes involved in the TCA cycle, electron transport chain, and glycolysis was analyzed in the CS knockdown cells. Levels of the NAD+-specific isocitrate dehydrogenase α subunit (IDH3A), oxoglutarate dehydrogenase

addition, the metastasized tumor cells strongly expressed vimentin, while normal tissues, including lung and heart, displayed no vimentin expression (Figure 4C). Together, these results directly demonstrate that CS silencing greatly accelerates tumor malignancy, including increased tumor cell metastasis and proliferation.
of HIF prolyl hydroxylase (HPH)\textsuperscript{40–44}. In addition, this HIF-1 activation can directly trigger glycolytic enzyme expression, resulting in upregulated glycolysis.

The EMT switch induced by CS knockdown is reversed by p53 reactivation. Defects in the TCA cycle enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been shown to cause succinate or fumarate accumulation, resulting in the electron transport chain from complex I to V displayed no specific changes between CS-silenced, mock-transfected, and vector-transfected cells (Figure 5F). To further confirm the effect of LDH5 upregulation, LDH activity was measured in the CS knockdown cells. LDH activity greatly increased in the CS knockdown cells as compared to the mock- and vector-transfected cells (Figure 5G). To directly confirm the effect of increased LDH activity, the acidification of conditioned media from CS knockdown cells was examined for changes in color and pH. Compared to the mock- and vector-transfected cells, the color change and pH decrease of the conditioned media were greatly accelerated in the CS-silenced cells (Figure 5H).

Taken together, these results indicate that the inhibition of CS expression decreases ATP production and, in turn, activates AMPK/p38 MAPK signaling, resulting in increased glucose uptake and upregulated glycolysis.

EMT phenotype induced by CS knockdown is suppressed by ATP treatment. Inhibition of CS expression decreased ATP production and, in turn, activated AMPK/p38, MAPK, and ERK1 signaling, increased glycolytic activity, and downregulated p53/TIGAR and SCO2 pathways. In addition, the reactivation of p53 by either treatment with MG132 or co-knockdown of HDM2 completely reversed the EMT switch of CS-silenced cells (Figure 6C and E). In addition, the expression of p53, TIGAR, and SCO2 was partially restored after MG132 treatment and even slightly increased after HDM2 co-knockdown in CS-silenced cells as compared to mock- and vector-transfected cells (Figure 6D and F). These results clearly indicate that deregulation of the p53/TIGAR and SCO2 pathways is involved in the induction of the EMT phenotype by CS knockdown.

### Table 1: Stable CS knockdown accelerates tumor cell growth in vivo

| Cell line          | Number of mice | Day for first measuring tumor size | Tumor size at 20 days (mm\textsuperscript{3}) |
|--------------------|----------------|-----------------------------------|-------------------------------------------|
| Mock               | 6              | ND                                | 0                                         |
| HeLa-vector-1      | 6              | ND                                | 0                                         |
| HeLa-vector-2      | 6              | ND                                | 0                                         |
| HeLa-shCS1-1       | 12             | 8 [4/12], 11 [7/8], 14 [1/1]       | 2340.6 ± 752.1                            |
| HeLa-shCS1-2       | 12             | 8 [1/12], 11 [7/11], 14 [4/4]      | 2114.7 ± 806.9                            |
| HeLa-shCS1-3       | 12             | 8 [8/12], 11 [2/4], 14 [2/2]       | 1781.4 ± 457.6                            |
| HeLa-shCS1-4       | 12             | 8 [7/12], 11 [4/3], 14 [1/1]       | 1584.2 ± 511.2                            |

ND: no detectable tumor growth within 8 weeks after inoculation.

OGDH), hexokinase 1 and 2 (HK1 and HK2, respectively), and lactate dehydrogenase 5 (LDH5) were greatly increased in the CS knockdown cells as compared to the mock- and vector-transfected cells (Figure 5F). However, the expression levels of proteins involved in the electron transport chain from complex I to V displayed no specific changes between CS-silenced, mock-transfected, and vector-transfected cells (Figure 5F). To further confirm the effect of LDH5 upregulation, LDH activity was measured in the CS knockdown cells. LDH activity greatly increased in the CS knockdown cells as compared to the mock- and vector-transfected cells (Figure 5G). To directly confirm the effect of increased LDH activity, the acidification of conditioned media from CS knockdown cells was examined for changes in color and pH. Compared to the mock- and vector-transfected cells, the color change and pH decrease of the conditioned media were greatly accelerated in the CS-silenced cells (Figure 5H).

Taken together, these results indicate that the inhibition of CS expression decreases ATP production and, in turn, activates AMPK/p38 MAPK signaling, resulting in increased glucose uptake and upregulated glycolysis.

### Table 2: Stable CS knockdown increases tumor cell metastasis in vivo

| Organ          | HeLa-vector | HeLa-shCS1 |
|----------------|-------------|------------|
|                | Mock 1 2 3 4 6 |           |
| Lung           | 0/6 0/6 0/6 | 16/18 12/18 13/18 16/18 |
| Heart          | 0/6 0/6 0/6 | 6/18 6/18 8/18 6/18 |
| Kidney         | 0/6 0/6 0/6 | 0/18 2/18 3/18 3/18 |
| Liver          | 0/6 0/6 0/6 | 0/18 1/18 1/18 1/18 |
| Lymph node     | 0/6 0/6 0/6 | 0/18 0/18 2/18 3/18 |
| Mesentery      | 0/6 0/6 0/6 | 2/18 4/18 5/18 3/18 |
| Subcutaneous   | 0/6 0/6 0/6 | 3/18 3/18 2/18 2/18 |

EMT phenotype induced by CS knockdown is reversed by p53 reactivation. Defects in the TCA cycle enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been shown to cause succinate or fumarate accumulation, resulting in activation of hypoxia inducible factor 1 (HIF-1) due to inhibition of HIF prolyl hydroxylase (HPH).\textsuperscript{40–44} In addition, this HIF-1 activation can directly trigger glycolytic enzyme expression, resulting in upregulation of glycolysis.\textsuperscript{45} To determine whether the increased glycolytic activity observed in CS knockdown cells resulted from HIF-1 upregulation, the level of HIF-1α expression was analyzed in CS knockdown cells. In contrast to the positive control 1% O\textsubscript{2} treatment, expression of HIF-1α was not detected in the mock-, vector-transfected, or CS knockdown cells (Figure 6A). These results clearly indicate that the inhibition of CS expression does not induce a pseudo-hypoxic response, which activates HIF-1α expression.

Previous studies have demonstrated that the tumor suppressor p53 inhibits cytosolic glycolysis through induction of TP53-induced glycolysis and apoptosis regulator (TIGAR) and promotes mitochondrial respiration via activation of synthesis of cytochrome c oxidase 2 (SCO2).\textsuperscript{46–48} Therefore, deregulation or dysfunction of p53 is involved in the metabolic alteration of malignant tumors and, in particular, metastatic tumors. To examine the effect of CS knockdown on the p53/TIGAR and SCO2 pathways, we analyzed the expression of p53, TIGAR, SCO2, and E3 ligase HDM2 proteins in CS-silenced cells. The expression of all four proteins was decreased to very low levels in the CS knockdown cells as compared to the mock- and vector-transfected cells (Figure 6B). To investigate whether downregulation of the p53/TIGAR and SCO2 pathways is involved in the EMT change induced by CS silencing, p53 expression was reactivated by treatment with the proteosome inhibitor MG132 or co-knockdown of HDM2 in the CS-silenced cells. Both the MG132 treatment and HDM2 co-knockdown completely reversed the EMT switch of CS-silenced cells (Figure 6C and E). In addition, the expression of p53, TIGAR, and SCO2 was partially restored after MG132 treatment and even slightly increased after HDM2 co-knockdown in CS-silenced cells as compared to mock- and vector-transfected cells (Figure 6D and F). These results clearly indicate that deregulation of the p53/TIGAR and SCO2 pathways is involved in the induction of the EMT phenotype by CS knockdown.

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Figure 5 | CS knockdown causes severe defects in mitochondrial respiration, but increases glucose uptake and upregulates glycolytic metabolism. (A) ΔΨm assay of CS knockdown cells. Indicated cells were stained with TMRM and then analyzed using a flow cytometer. (B) ROS assay of CS knockdown cells. Cells were treated with CM-H2DCFDA and then analyzed using a flow cytometer. (C) ATP assay of CS knockdown cells. Total cell extracts prepared from indicated cells were subjected to ATP assay using ATP Bioluminescence Assay Kit CLSII. (D) Western blotting of Glut-1, Glut-3, p-AMPK, p-p38 MAPK, and p-ERK1 protein in CS knockdown cells. Total proteins isolated from indicated cells were blotted with antibodies as labeled. (E) Glucose uptake assay of CS knockdown cells. Cells were loaded with 2-NDBG and then analyzed using a flow cytometer. (F) Western blotting of proteins involved in the TCA cycle, electron transport chain from complex I to V, and glycolysis in CS knockdown cells. Total proteins isolated from indicated cells were blotted with antibodies as labeled. (G) Lactate dehydrogenase assay of CS knockdown cells. Cells were assayed for lactate dehydrogenase activity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay. (H) pH of conditioned media cultured with CS knockdown cells. Cells were cultured until confluent and then incubated in fresh media. The color and pH of the conditioned media were examined. The plotted data were averaged from three independent experiments, and the bars represent mean ± SD. The quantity of β-actin serves as a loading control.
Figure 6 | CS knockdown induced EMT switch is reverted by p53 reactivation. (A) Western blotting of HIF-1α expression in CS knockdown cells. Total proteins isolated from cells as indicated were blotted with antibodies for HIF-1α and β-actin. Proteins prepared from cells cultured in 1% O₂ for 24 h serves as positive control for hypoxic condition. (B) Western blotting of HDM2, p53, TIGAR, and SCO2 expression in CS knockdown cells. Total proteins isolated from indicated cells were blotted with antibodies as labeled. (C) Fluorescence imaging of stress fibers of CS knockdown cells treated with MG132. Cells were treated with 10 mM MG132 for 12 h and then stained with Alexa Fluor 488-conjugated phalloidin and DAPI. (D) Western blotting of HDM2, p53, TIGAR, and SCO2 proteins in CS knockdown cells treated with MG132. Cells as indicated were treated with 10 mM MG132 for 0, 6, and 12 h. Total protein extracts isolated from these cells were blotted with antibodies as indicated. (E) Morphological imaging of CS, HDM2, and CS/HDM2 knockdown cells. Cells with single CS, HDM2, or double CS/HDM2 knockdown as indicated were selected and imaged. (F) Western blotting of proteins involved in bioenergetic metabolism in single CS, HDM2, and double CS/HDM2 knockdown cells. Total protein extracts isolated from the indicated cells were blotted with antibodies as labeled. The level of β-actin serves as a loading control.
level in CS knockdown cells directly induces the EMT phenotype, resulting in increased tumor cell migration and proliferation.

Discussion

Defects in mitochondrial bioenergetics may promote tumor malignancy, forcing the cell to revert to the more primitive route of energy production through cytosolic glycolysis\(^7\)\(^-\)\(^9\). In this study, we demonstrate that inhibited CS expression in human cervical carcinoma cells is associated with a shift in cellular bioenergetics from aerobic respiration to glycolytic metabolism and simultaneously induces the EMT switch, resulting in accelerated tumor malignancy. Our results reveal that cells with the CS knockdown exhibit not only an almost complete loss of \(\Delta\psi_m\) but also very low levels of ROS and \(\text{H}_2\text{O}_2\), indicating that mitochondrial respiration is severely disrupted. In turn, cytosolic glycolysis is greatly accelerated, resulting in reduced ATP production. The decreased ATP levels directly activate AMPK/p38 MAPK signaling and upregulate Glut-1 and Glut-3 expression, which enhances glucose uptake and promotes glycolytic enzyme expression. Lactate dehydrogenase activity is consequently increased, leading to increased lactic acid production and accelerated acidification of the medium.

Notably, CS inhibition causes marked downregulation of p53, TIGAR, SCO2, and HDM2 expression, suggesting that the p53-mediated coordination of bioenergetic metabolism is deregulated in the CS knockdown cells. We also showed that CS knockdown directly induces the EMT phenotype through activation of Snail and Twist expression in human cervical cancer HeLa cells, resulting in accelerated tumor malignancy, including increased tumor cell proliferation and metastasis. However, this phenotypic switch can be completely reversed by treatment with MG132 or co-knockdown of HDM2 that reactivates the p53/TIGAR and SCO2 functions or temporarily suppressed by administration of ATP that downregulates the AMPK/p38 MAPK signaling, decreases glycolytic enzyme expression, and partially reactivates p53, TIGAR, and SCO2 expression. Taken together, these data indicate that the CS knockdown not only impaired the TCA cycle but also induced an EMT switch in human cervical carcinoma cell lines, resulting in markedly upregulated cytosolic glycolysis and accelerated tumor malignancy. This study provides the first evidence that the Warburg effect, induced by the alteration of bioenergetic enzymes, is directly linked to tumor malignancy via induction of the EMT phenotype.

A wide variety of carcinoma cells with the EMT switch have been shown to have cancer stem cell characteristics, resulting in acceler-
ated tumor malignancy and increased resistance to anticancer drugs. Loss of CS expression induced the EMT phenotype and resulted in malignant transformation, suggesting that the CS knockdown cells possess cancer stem cell properties. Therefore, to fully elucidate the potential mechanism of malignant transformation induced by knockdown of CS expression, it is essential to characterize the cancer stem cell behaviors in CS knockdown cells. In addition, it is important to test whether EMT change induced by loss of CS expression drives increased resistance to anticancer drugs.

Loss of the respiratory enzyme citrate synthase, which catalyzes the first reaction of the TCA cycle in respiratory metabolism, causes the bioenergetic switch from mitochondrial respiration to cytosolic glycolysis and accelerates tumor malignancy by inducing a change to the EMT phenotype. These metabolic alterations and malignant transformation result from deregulation of p53 functions and aberrant cell growth signaling. To our knowledge, the evidence provided by this study is the first to directly link the Warburg effect to tumor malignancy through induction of the EMT phenotype. In addition, the malignantly transformed human cervical cancer cells established in this study can serve as a tool for screening anticancer drugs that specifically target the Warburg effect.

**Methods**

**Cell culture.** Human breast cancer (MCF7), cervical cancer (HeLa and SiHa), colon cancer (COLO 205 and SW620), liver cancer (Hep G2), lung cancer (A549 and H23), brain cancer (U251 and N22), gastric cancer (MKN45), and B-cell cancer (BJAB) cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Human liver cancer (OC2) cells were originally obtained from Dr. R. C. Chang (Veterans General Hospital, Taipei, Taiwan). Human B-cell cancer (BJAB) cells were obtained from Dr. Y. S. Chang (Chang-Gung University, Taoyuan, Taiwan). Human normal primary cervical epithelial (normal), precancer (Z172 and Z183A), and cancer (Cx) cells were obtained from the National Cheng Kung University, Taiwan, Taiwan. All cells were cultured and maintained at 37 °C in a humidified incubator with 5% CO2. Under hypoxic conditions, cells were cultured in a modular incubator chamber (Billups-Rothenberg, del Mar, CA, USA) with 1% O2 and 5% CO2.

** Colony formation assay.** Cells were plated into 6-well plates at a density of 1 × 10³ cells per well. At 6 h before each incubation time point except on day zero (Day 0), the cells were treated with 10 µL of MITT solution (5 mg/mL) for 4 h, disrupted in 200 µL of dimethyl sulfoxide (DMSO), and measured at 590 nm using an ELISA reader (VERSAmax tunable microplate reader, Molecular Dynamics, Sunnyvale, CA, USA).

**BrdU incorporation assay.** Cells were seeded on 96-well plates at a density of 1 × 10³ cells per well in 200 µL of growth medium. At 6 h before each incubation time point except on day zero (Day 0), the cells were treated with 10 µL of MITT solution (5 mg/mL) for 4 h, disrupted in 200 µL of dimethyl sulfoxide (DMSO), and measured at 590 nm using an ELISA reader (VERSAmax tunable microplate reader, Molecular Dynamics, Sunnyvale, CA, USA).

**Western blot analysis.** Cells were harvested by scraping at 4 °C and lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) containing protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). Total protein extracts were separated by 12% SDS-PAGE and then electroblotted onto Immobilon-P membrane (Millipore, Billerica, MA, USA) using a semidry blotting system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blotted membranes were probed with mouse monoclonal antibodies specific for CS (DG34, Chemicon International, Temecula, CA, USA), HDM2 (human MDM2, SMP14; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (DO-1, Santa Cruz Biotechnology), HK1 (G-1, Santa Cruz Biotechnology), β-actin (Sigma-Aldrich Chemical, Saint Louis, MO, USA), E-cadherin (BD Biosciences, San Jose, CA, USA), Vimentin (V9, Sigma-Aldrich (G-1); Invitrogen), Glut-3 (G, Santa Cruz Biotechnology), and MitoProfile® Total OPTXPHOS Human WB Antibody Cocktail (ab10411, Abcam Inc, Cambridge, MA, USA); rabbit monoclonal antibody specific for AMPKα1 (Y365, Abcam), ERK1 (Y17, Epitomics, Burlingame, CA, USA) and p-ERK1 (Y187) (EP197T, Epitomics); rabbit polyclonal antibodies specific for IDH3A (Aviva Systems Biology, San Diego, CA, USA), p-AMPKα1 (threonine 172, Abcam), Snail (H-130, Santa Cruz Biotechnology), Twist (H-81, Santa Cruz Biotechnology), and Glut-1 (H-43; Santa Cruz Biotechnology); goat polyclonal antibodies specific for OGDH (a; KGDL; C-20; Santa Cruz Biotechnology), HIF2α (C-14, Santa Cruz Biotechnology), TIGAR (Y-20, Santa Cruz Biotechnology) and SCO2 (G-14, Santa Cruz Biotechnology); and sheep polyclonal antibody specific for LDH3 (LDH V, Abcam). Subsequently, the membranes were incubated with horseradish-peroxidase (HRP)–conjugated goat anti-mouse IgG (H + L) (Pierce Biotechnology, Rockford, IL, USA), goat anti-rabbit IgG–HRP (Santa Cruz Biotechnology), or rabbit anti-sheep-HRP. The bands recognized by specific antibodies were detected using the enhanced chemiluminescence system (GE Healthcare) according to the manufacturer’s instructions.
Boyd chamber migration assay. Cells were grown in 10 cm Petri dishes to 70–80% confluence and harvested with 0.1% trypsin (Bisohittaker Acambrex). The cells were seeded onto 8 μm pore size polycarbonate filters (Nuclepore Corp., Pleasanton, CA, USA) in a 48-well Boyd chamber (Neuro Probe Inc., Gaithersburg, MD, USA) at a density of 2.5 x 10⁴ cells per well and incubated for 6 h. The chemotactic migration of cells was induced by 10% FCS in the lower chamber. The migrated cells were fixed with 100% ethanol and stained with Livi’s staining solution. The stained cells were photographed and analyzed using an inverted phase-contrast microscope.

Matrigel invasion assay. Cells were cultured in 10 cm Petri dishes to 70–80% confluence and harvested with 0.1% trypsin (Bisohittaker Acambrex). The cells were plated onto an 8 μm invasion chamber at a density of 1 x 10⁴ cells per well and incubated for 8 h. The chemotactic invasion of cells was induced by 10% FCS placed in the lower chamber. The invasive cells were fixed with 100% ethanol and then stained with GIEMSA staining solution. The stained cells were photographed and analyzed by inverted phase-contrast microscopy.

In vivo tumor growth analysis. Cells (1 x 10⁴) were subcutaneously inoculated into the backs of NOD/SCID mice. Tumors were measured externally every 3 to 5 days using vernier calipers. The tumor volume was calculated as length x width x 0.52. After 20 or 60 days, mice were anesthetized with pentobarbital and photographed, after which they were sacrificed and the tumors removed and photographed. Animal experiments were approved and monitored by the Institutional Care and Use Committee of the National Cheng Kung University, Tainan, Taiwan. The IACUC approval number is 98092. Eight to 12-week-old female mice were used in all animal experiments.

In vivo tumor metastasis analysis. Cells (1 x 10⁴) were intravasally injected into the tail veins of NOD/SCID mice. After 20 days, the mice injected with stable CS knockdown cells appeared to be sick, so all mice were sacrificed and examined for tumor metastases. Their organs, particularly lungs and hearts, were removed, fixed in formalin, and processed for histological and immunohistochemical analyses.

Histological and immunohistochemical analyses. Mouse organs, particularly lungs and hearts, were embedded in paraffin blocks and processed into sections 4 μm thick. The organ sections were further processed with hematoxylin and eosin (H&E) or immunohistochemically stained using an anti-Vimentin-specific antibody (V9; DAKO, Glostrup, Denmark) and counterstained with hematoxylin. The antigen was visualized as a brown precipitate.

Aβ1-42 assay. Cells were seeded in 6-well plates at a density of 1 x 10⁴ cells per well. After 48 h, the cells were treated with 150 nM Tetramethylrhodamine methyl ester (TMRM) for 30 min in culture medium. The stained cells were quantitatively analyzed by flow cytometry using a FACSCalibur™ flow cytometer (Beckton Dickinson, San Jose, CA, USA).

ROS assay. Cells were inoculated onto 6-well plates at a density of 1 x 10⁴ cells per well. After 48 h, the cells were loaded with 10 μM M 2-[N-(7-chloro-2-naphthoyl)]-5-6 chloromethyl-2-

dichlorodihydro-fluorescein diacetate acetyl ester (CM-H2DCFDA; Molecular Probes Inc.), a D-glucose fluorescent analogue, and 3.3 mM glucose for 10 and 15 min and then incubated in fresh KRB buffer supplemented with 600 μM M 5-6 chloromethyl-2-

bicarbonate (KRB) buffer (129 mM NaCl, 5.0 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.0 mM CaCl₂, 1.2 mM MgSO₄, 10 mM HEPES, and 0.1% BSA; pH 7.4) for 15 min and then incubated in fresh KRB buffer supplemented with 600 μM M 2-[N-(7-

nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NDG; Molecular Probes Inc.), a D-glucose fluorescent analogue, and 3.3 mM glucose for 10 and 30 min. The stained cells were quantitatively analyzed by flow cytometry using a FACSCalibur™ flow cytometer (Beckton Dickinson).

Lactate dehydrogenase activity assay. Cells were inoculated onto 96-well plates at a density of 1 x 10⁴ cells per well in 200 μL culture medium for 2 h. The cells were directly lysed, and lactate dehydrogenase activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer’s instructions.

pH measurement in conditioned medium. Cells were seeded in 6-well plates at a density of 1 x 10⁴ cells per well. The cells were cultured until confluent and then incubated in fresh culture medium for 12 h. The colors and pH values of conditioned media were photographed using a Nikon D80 digital camera (Nikon Corp.) and measured using a FE20-FiveEasy™ pH meter (Metler Toledo, Zürchenbach, Switzerland).

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**Additional information**

**Competing financial interests:** The authors declare no competing financial interests.

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