Mitochondrial Sirt3 supports cell proliferation by regulating glutamine-dependent oxidation in renal cell carcinoma

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Mitochondrial Sirt3 supports cell proliferation by regulating glutamine-dependent oxidation in renal cell carcinoma

Directed by Professor Kyung-Sup Kim

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ABSTRACT

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Clear cell renal carcinoma (RCC) is the most common malignancy arising in the adult kidney. It exhibits increased aerobic glycolysis and low mitochondrial respiration due to *von Hippel-Lindau* gene defects and constitutive hypoxia-inducible factor-α expression. Although the "Warburg effect" is considered to be the metabolic hallmark of tumor metabolism, recent studies have elucidated the importance of mitochondrial functions even in cells with minimal mitochondrial biogenesis, such as RCC cells. Sirt3 is a major mitochondrial deacetylase that is involved in the regulation of various types of energy metabolism. The role of Sirt3 as a tumor

suppressor or oncogene in human cancer depends on the cell type. Here, we demonstrate that Sirt3 expression was increased in the mitochondrial fraction of human RCC tissues. Sirt3 depletion by lentiviral short-hairpin RNA, as well as the stable expression of the inactive mutant of Sirt3, inhibited cell proliferation and tumor growth in xenograft nude mice, respectively. Furthermore, mitochondrial pyruvate, which was used for oxidation in RCC, might be derived from glutamine, but not from glucose and cytosolic pyruvate, due to depletion of mitochondrial pyruvate carrier and the relatively high expression of malic enzyme 2. Depletion of Sirt3 suppressed glutamate dehydrogenase activity, leading to impaired mitochondrial oxygen consumption. Our findings suggest that Sirt3 plays a tumor-progressive role in human RCC by regulating glutamine-derived mitochondrial respiration, particularly in cells where mitochondrial pyruvate usage is severely compromised.

Key words : renal cell carcinoma, Sirt3, mitochondrial oxidation, glutamate dehydrogenase

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Ⅰ. INTRODUCTION

Clear cell renal carcinoma (RCC) is the most common malignancy arising in the adult kidney. It is characterized by a defect in the tumor suppressor gene, *von Hippel-Lindau*, and the stable expression of hypoxia-inducible factor-α (HIFα), independent of oxygen availability [1]. The constitutive stabilization of HIF-α is known to play a central role in renal carcinogenesis by stimulating glycolytic gene expression, which facilitates the glycolytic flux; this is known as the "Warburg effect" [2]. In addition, RCC is metabolically reprogrammed toward low mitochondrial oxidative phosphorylation, which results from decreases in mitochondrial DNA, respiratory

chain activities, and oxidative phosphorylation-related proteins [3-5].

Although increased aerobic glycolysis is a characteristic of tumor cells, many studies have specified the importance of mitochondrial functions, including the tricarboxylic acid (TCA) cycle, which replenishes biosynthetic intermediates for lipid and amino acid synthesis, as well as oxidative phosphorylation, in proliferating tumor cells through glutaminolysis [6]. Therefore, understanding how mitochondrial metabolic pathways are regulated in tumor cells with defective mitochondria is of great interest. Focus has also been placed on how cancer cells can balance both the glycolytic pathway and mitochondrial functions and adapt to different sources of metabolic stress, such as energy and oxygen depletion, to sustain cell growth.

Protein acetylation is an important type of posttranslational modification that regulates mitochondrial function and various types of cellular metabolism [7,8]. Sirt3 is the primary mitochondrial acetyl-lysine deacetylase and is closely associated with metabolism in many cellular functions and human pathologies, including metabolic syndromes and cancer [9,10]. It deacetylates many mitochondrial proteins, such as acetyl-CoA synthetase 2, long-chain acyl-CoA dehydrogenase, isocitrate dehydrogenase 2, glutamate dehydrogenase, mitochondrial pyruvate carrier 1, and pyruvate dehydrogenase, to augment their activity for metabolic homeostasis [11-13]. In addition, Sirt3 mediates redox status by activating detoxification, thus protecting mitochondria against oxidative damage [14,15].

Recently, the role of Sirt3 in carcinogenesis has been studied, although its role as a tumor suppressor or oncogene remains controversial. In human

hepatocellular carcinoma (HCC), lung adenocarcinoma, breast cancer, and gastric cancer, Sirt3 expression is downregulated when compared with normal tissues, and the overexpression of Sirt3 inhibits cell growth and induces cell death[16-20]. Contrary to these observations, Sirt3 protein is highly expressed in oral squamous cell carcinoma (OSCC), and knockdown of Sirt3 decreases cell survival and proliferation in esophageal cancer and non-small cell lung carcinoma [21-24]. However, the role of Sirt3 in RCC has not been described previously.

Present study provides evidence supporting the tumor-progressive role of Sirt3 in RCC. Inactivation of Sirt3 diminished tumor cell growth and impaired the glutamine-derived oxygen consumption of mitochondria. Furthermore, the suppressed expression of mitochondrial pyruvate carrier (MPC) in human RCC led to a severe defect in glucose and pyruvate oxidation. These findings suggest that Sirt3 plays a critical role in RCC by activating glutamate dehydrogenase (GDH) for the efficient utilization of glutamine by mitochondria.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

Human RCC cell lines (A498, Caki1, Caki2, and 786-0) were obtained from A.T.C.C. (Manassas, VA, U.S.A.). All reagents related to animal cell culture were purchased from Life Technologies (Big Cabin, OK, USA). Caki1 and Caki2 cells were cultured in modified McCoy's 5A medium, A498 cells were cultured in Eagle's Minimum Essential medium, and 786-0 cells were maintained in Dulbecco's modified Eagle's medium. All media contained 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured at 37°C in a 5%-CO_2 humidified atmosphere.

2. Biopsies

All human kidney biopsies that were stored in liquid nitrogen and held by the Department of Urology (College of Medicine, Yonsei University) were used for this study. All studies were undertaken with approval from the Institutional Review Board at Yonsei University.

3. Cell counting

Cells (1×10^4 cells/well) were plated on 12-well plates and counted every 24 hr for 5 days using ADAM-MC (NANOENTEK, South Korea).

4. Quantitative real-time PCR

Total RNA was isolated from human tissues or cultured cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Human tissues were homogenized by TissueLyser II (Qiagen, Hilden, Germany). For quantitative real-time PCR, cDNAs were synthesized from 4 μg of total RNA using random hexamer primers and SuperScript reverse transcriptase II (Invitrogen) following the manufacturer's instructions. Diluted cDNAs were analyzed for qPCR using the SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers, and this was then subjected to RT-PCR quantification using the ABI PRISM 7300 RT-PCR System (Applied Biosystems).

Table 1. Primers used for real-time PCR

Gene symbol	Genbank Accession No.	Sequence
Sirt3	NM_012239	FW: AGCTCATGGAACCTTTGCCT RV: CCAAAGAACACAATGTCGGG
Sirt4	NM_012240	FW: GGCGAGAAACTTCGTAGGCT RV: CAAAGCATCCACATTTTGGG
Sirt5	NM_012241	FW: ACTGTGATTTATGTCTAGTGGTGGG RV: AAATGAAACCTGAATCTGTTCGTAG
COX Ⅳ	NM_001861	FW: ACCGCGCTCGTTATCATGTG RV: CTTGGAGGCTAAGCCCTGGA

NDUFA4	NM_002489	FW: TGCGTCTGGCATTGTTCAA RV: TCCAGGGCTCTGGGTTATTTC
PGC-1α	NM_013261	FW: GGACAGAACTGAGGGACCGT RV: GCAGCAAAAGCATCACAGGT
MPC1	NM_016098	FW: GTGCGGAAAGCGGCGGACTA RV: GGCAGCAATGGGAAGACCCCA
MPC2	NM_001143674	FW: TACCACCGGCTCCTCGATAAA RV: TATCAGCCAATCCAGCACACA
TBP	NM_003194	FW: TGTTTCTTGGCGTGTGAAGATAACC RV: AGAAACCCTTGCGCTGGAACTCGTC

5. Overexpression or knockdown assay

For the stable overexpression of *hSirt3*, the fragment encoding the full-length cDNA of Sirt3 was cloned into the pLL-CMV-puro lentiviral vector. The short-hairpin RNA (shRNA) technology platform (Sigma-Aldrich MISSION® shRNA Bacterial Glycerol stock) was used to stably knock down gene expression. For the knockdown of *hSirt3*, MISSION® shRNA lentiviral plasmids contained a U6 promoter transcribing non-target shRNA and hSirt3 shRNAs (TRCN000038892; Sigma-Aldrich, St.Louis, MO, USA). Plasmid DNAs were transfected into human embryonic kidney (HEK293T) cells, along with a lentiviral packaging mix consisting of an envelope and packaging vector to produce lentiviruses packed with hSirt3 cDNA or hSirt3 shRNA cassettes, according to the manufacturer's instructions.

Positive cells harboring hSirt3 cDNA or the hSirt3 shRNA cassette were selected by 0.2 μg/ml puromycin (Sigma-Aldrich) selection after infection.

6. Isolation of mitochondria

Cells were prepared and harvested with STE buffer (0.25 M sucrose, 1 mM EGTA, 50 mM Tris-HCl [pH 8.0], protease inhibitor, 0.02% digitonin), after which they were homogenized several times by insulin syringes and centrifuged at 1,000×g for 5 min at 4°C to separate the nuclei and unbroken cells. Human tissues were also homogenized by TissueLyser II with STE buffer. Then, the supernatant was collected in a new tube and centrifuged at 10,000×g for 20 min at 4°C to isolate the pellet containing mitochondria. The pellet was resuspended in 1% NP40 buffer containing protease inhibitors.

7. Enzymatic assay

Mitochondrial samples were subjected to a coupled enzymatic assay. The GDH activity reaction mixture contained 100 mM Tris-HCl, 5 mM L-glutamic acid, 2 mM *nicotinamide adenine dinucleotide* (NAD^+), 0.25 mM adenosine diphosphate (ADP), and 150 mM hydrazine. Absorbance was measured at 340 nm on a spectrometer (Infinite F200 pro, Tecan).

8. Western blot analysis

Cultured cells were washed twice with ice-cold phosphate-buffered saline and

harvested in whole-cell lysis buffer (1% sodium dodecyl sulfate, 60 mM Tris-HCl, pH 6.8). Protein concentrations were measured by the bicinchoninic acid assay. Equal amounts of protein extracts were subjected to SDS/PAGE and transferred onto nitrocellulose transfer membranes (Whatman). The membranes were blocked in 5% (w/v) non-fat Difco™ skimmed milk (BD Biosciences), followed by incubation with the primary antibodies in 1% bovine serum albumin. The following antibodies were used: anti-Sirt3, cytochrome c oxidase subunit (COX) IV, Mitochondrial pyruvate carrier (MPC) 1, MPC2 (Cell Signaling), NDUFA4, Glutamate dehydrogenase (GDH) (Abcam), adenosine triphosphate (ATP) synthase (Novex), HIF-2α (Novus), Heat shock protein (HSP) 60, Malic enzyme (ME) 2 (Santa Cruz Biotechnology), and α-tubulin (Merck Millipore).

9. Xenografts

Cells were implanted into BALB/c nude mice (Orient Bio, South Korea) at 1×10^6 cells/mouse in 50% Matrigel (BD Biosciences, San Jose, CA, USA). Control A498 and Sirt3-overexpressed A498 cells were injected into each flank of each mouse. Tumors were measured weekly by caliper for 5 weeks.

10. Oxygen consumption measurements

Metabolic activity was performed in standard, 24-well Seahorse microplates using the XF 24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA). Cells were plated onto a 24-well Seahorse assay plate, grown overnight.

One hour prior to the assay, cells were incubated in assay medium (D5030, Sigma, with 10% FBS) containing 10 mM D(+)-glucose (Sigma-Aldrich) and 10 mM sodium pyruvate (Gibco) or 2 mM L-glutamine (Gibco) or dimethyl-2-oxoglutarate (Sigma-Aldrich), with pH 7.4.

11. Statistical analysis

All results are expressed as the mean ± standard deviation of at least three independent experiments.

Ⅲ. RESULTS

1. Expression of Sirt3 in human RCC tissues

Although the role of Sirt3 has been studied in various human malignancies [16,18-22,25-29], its precise role in RCC has not been investigated. To determine the expression pattern of Sirt3 in matched human normal kidney and advanced RCC tissues, Sirt3 protein expression was examined by western blotting (Fig. 1A). Due to the stabilization of hypoxia-inducible factor (HIF) by VHL defects and low mitochondrial biogenesis, RCC is known to be glycolytic [3,4]. Therefore, we assessed levels of HIF2α, along with several mitochondrial proteins. As shown in Fig. 1A, mitochondrial proteins, such as Sirt3, ATP synthase, COX IV, and NDUFA4, are reduced in RCC tissues compared with normal kidney tissues, whereas the level of α-tubulin, a cytosolic protein, was unchanged. To evaluate the difference of these gene transcriptions, we performed real-time polymerase chain reaction (PCR) analysis (Fig. 1B). Notably, RCC tissues showed greater-than-twofold decreases in the mRNA levels of COX IV, peroxisome proliferator-activated receptor gamma co-activator 1α□and NDUFA4, whereas Sirt3 mRNA expression decreased to a lesser extent. Sirt3 mRNA levels were much less decreased than mRNA level of other mitochondrial Sirtuin family, Sirt4 and Sirt5, in cancer tissues compared with those in matched normal kidney tissues.

To check the level of Sirt3 in mitochondria, we utilized fractionated mitochondria from tissues to compare Sirt3 protein levels after normalization to COX IV. Interestingly, Sirt3 expression in RCC was increased compared with that in paired

normal kidney tissues (Fig. 1C). Conversely, levels of NDUFA4, which is a component of complex IV of the electron transport chain (ETC), were markedly decreased in mitochondrial fractions of RCC. Similar levels of heat shock protein (Hsp) 60 were also observed between normal and cancer tissues after normalization to COX IV. These data indicate that protein levels of Sirt3 in the mitochondria are increased in the RCC.

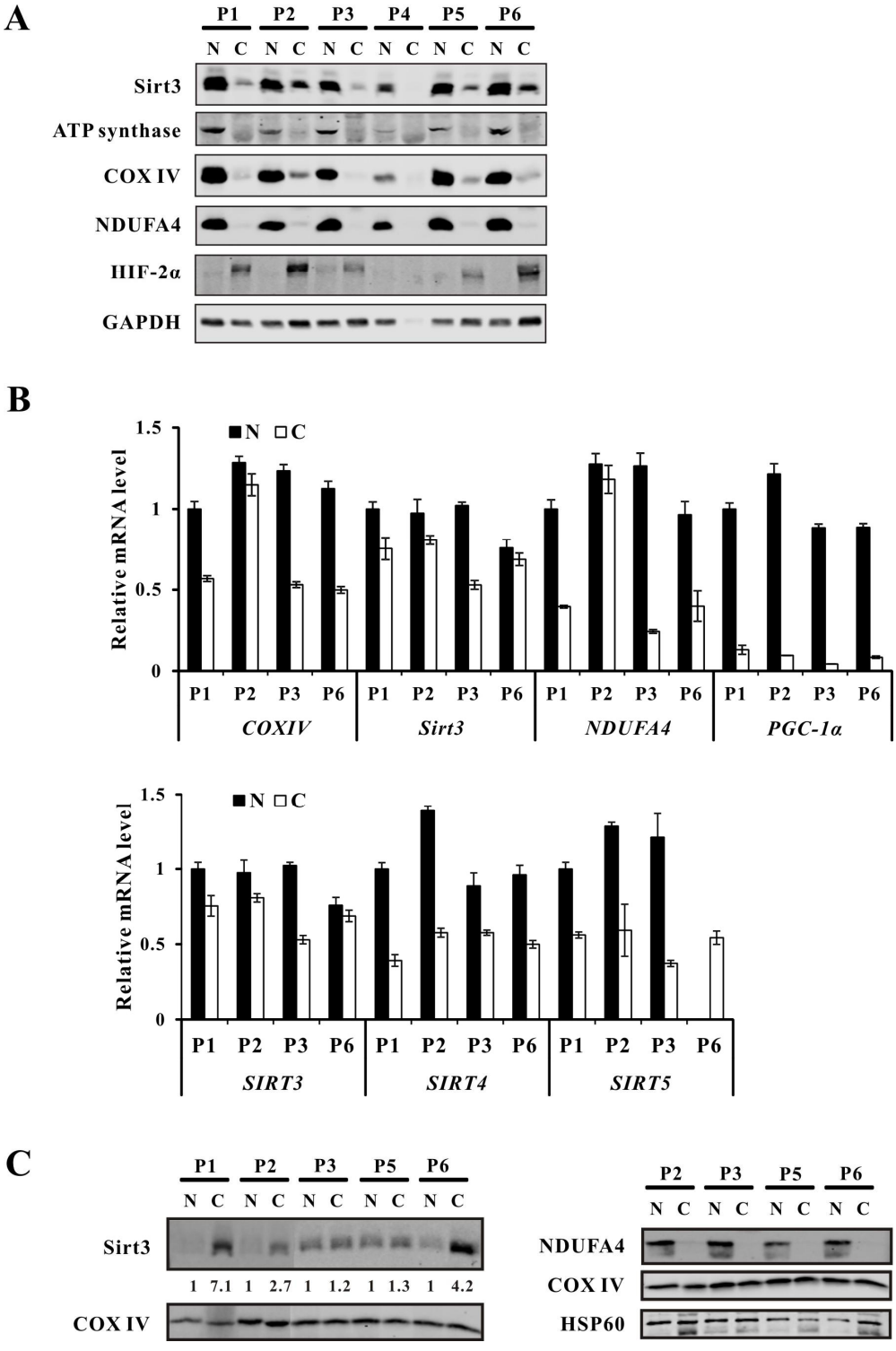


Figure 1. Expression of sirtuin 3 (Sirt3) in human renal cell carcinoma (RCC) tissues.

(A) Western blots of Sirt3 expression in paired tissues (N=normal; C=cancer; P=patient) from RCC patients (P=6). Adenosine triphosphate (ATP) synthase, cytochrome c oxidase subunit IV (COX IV), and NDUFA4 are mitochondrial marker proteins. GAPDH was used as the loading control. (B) Quantitative polymerase chain reaction (qPCR) analysis of the expression of mitochondrial genes in normal and cancer tissues. PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1α. Gene expression values were normalized to TBP and are presented as the mean ± S.D. (C) Western blots of mitochondrial extracts from normal and cancer tissues. COX IV was used as the loading control. Hsp60, heat shock protein 60.

2. Sirt3 is required for the maintenance of cell proliferation

Recent studies have focused on the importance of mitochondrial functions, including the ETC in tumor growth, and several studies have shown that glutamine-derived carbons are necessary for rapidly growing cells[30]. Therefore, we hypothesize that the increased expression of Sirt3 in mitochondria is essential for maintaining RCC tumor growth. To delineate the role of Sirt3 in cell proliferation, we used the shRNA lentivirus to knock down Sirt3. As shown in Fig. 2, Caki2 cells with stably depleted Sirt3 have reduced cell proliferation compared with negative control lentiviral shRNA (shNC)-infected cells.

A recent report documented that increased reactive oxygen species in Sirt3 null mouse embryonic fibroblasts (MEFs) stabilizes HIF1α, resulting in the activation of HIF target genes and cell proliferation [28]. Thus, we tested whether the suppressed cell growth in Sirt3-reduced cells might due to changes in the levels of HIF1α and HIF2α, which are important transcription factors in RCC [31]. There were no noticeable changes in HIF1α and HIF2α protein levels. This was possibly due to the fact that Caki2 cells have mutated VHL, which results in the constitutive activation of both HIF proteins (Fig. 2A). These data indicate that Sirt3 supports Caki2 RCC cell proliferation in a HIF1α-independent manner.

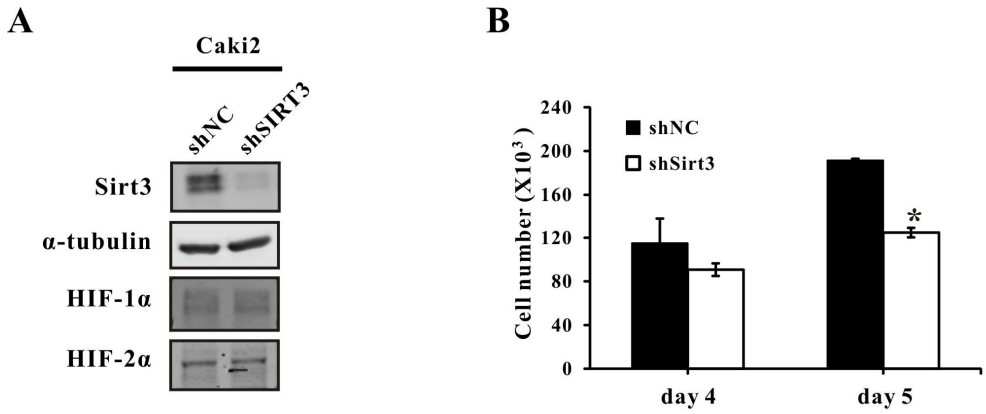


Figure 2. Sirt3 knockdown reduces cell growth.

(A) Western blots of Caki2 cells that were stably transduced with control short-hairpin RNA (shRNA) (shNC) or Sirt3 shRNA (shSirt3). The protein levels of Sirt3, hypoxia-inducible factor (HIF)-1α, and HIF-2α were assessed. α-tubulin was used as the internal control. (B) Caki2 stable cells were cultured in McCoy medium under normoxic conditions. Cell numbers were counted at 4 and 5 days using an Adam Cell Counter. $^{*}p < 0.05$ by the Student's two-tailed t-test. All bar graphs are plotted as mean ± standard deviation (SD).

3. RCC cells expressing deacetylase-inactive Sirt3 show less tumorigenesis *in vivo*

To further investigate the effects of Sirt3 on the growth of RCC cells *in vivo*, we performed xenograft assays in nude mice. We generated A498 cell lines that were stably transduced with lentiviruses harboring empty vector (Mock), wild-type Sirt3 (WT), or deacetylase-inactive Sirt3 (H248Y) which exhibits a dominant-negative effect on cellular acetylated status and its functional consequences [13,32] (Fig. 3A). Transduced cells were then subcutaneously implanted into nude mice. Three stable cell lines developed tumors, and the size of the tumors was measured weekly. Interestingly, overexpression of deacetylase-inactive Sirt3 exhibited significantly reduced tumor growth compared with tumors that developed from cells expressing Mock or WT (Fig. 3B). In addition, there was no noticeable difference in tumor growth rate and size between Mock- and WT-expressing cell-derived tumors (Fig. 3B-D). These results suggest that the deacetylase activity of Sirt3 promotes RCC tumor growth.

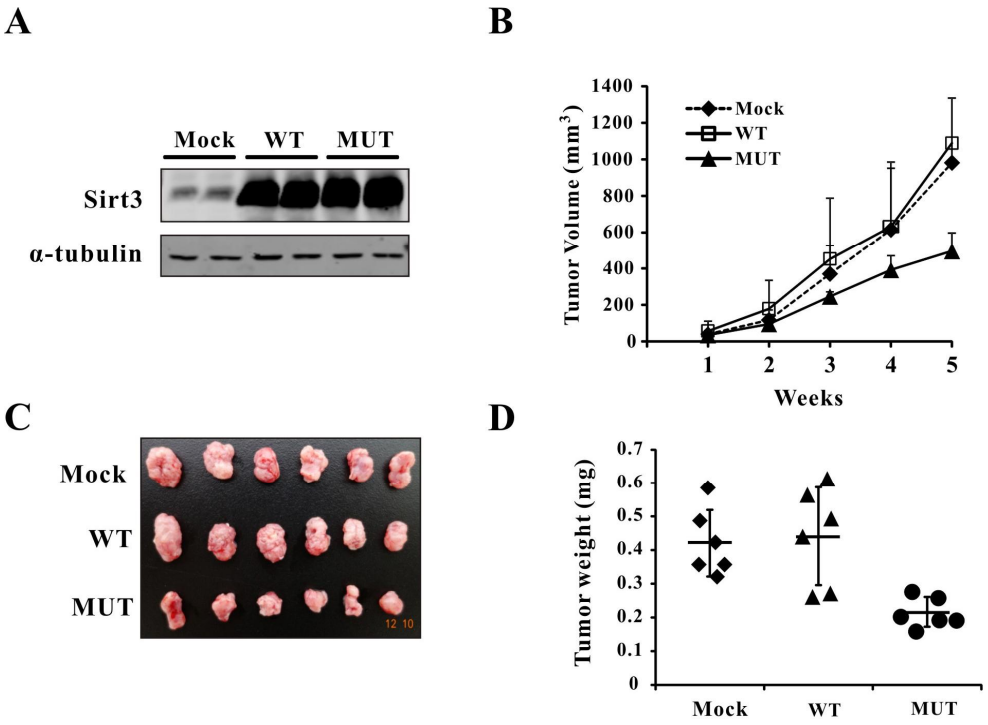


Figure 3. A498 cells expressing deacetylase-inactive Sirt3 inhibit tumorigenesis *in vivo*.

(A) Western blots of Sirt3 expression in A498 cells that were transduced with a control lentivirus vector (Mock), wild-type Sirt3 (WT), or a deacetylase-inactive form of Sirt3 (MUT), in which a 248-amino acid residue was changed from a histidine to tyrosine. (B and C) Tumor growth of A498 (n=6) xenografts, as determined by caliper measurement (mean ± standard error of the mean), and representative A498 xenograft tumor images. Tumor size was monitored every 7 days for 5 weeks. (D) Tumor weights were examined at the experimental endpoint. All bar graphs are plotted as mean ± SD.

4. Sirt3 regulates glutamine-dependent oxidation in RCC

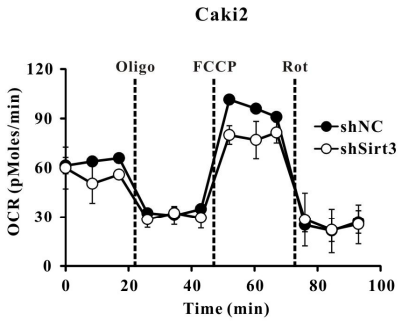
Sirt3 has been known to play a role in regulating the rate of mitochondrial ATP synthesis by controlling the activities of metabolic enzymes that contribute to various cellular functions [9,10]. Because Sirt3 suppression impaired cell proliferation and the tumorigenesis of RCC cells (Fig. 2 and Fig. 3), we assumed that Sirt3 is required for meeting cellular demands, such as ATP and macromolecules, for rapid cell growth. Therefore, we assessed the effects of Sirt3 on mitochondrial respiration by measuring the oxygen consumption rate (OCR). Notably, Sirt3 knockdown in Caki2 cells suppressed the OCR in complete medium containing 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine (Fig. 4A). In the glutamine-deficient medium, the OCR of shNC and shSirt3 cells was severely impaired (Fig. 4B). These results imply that Caki2 cells may have lost the ability to use glucose and pyruvate as a carbon source for mitochondrial ATP synthesis. Furthermore, Sirt3 appeared to affect the glutamine-derived OCR of mitochondria (Fig. 4A).

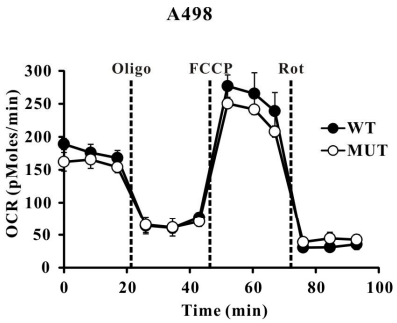
To investigate Sirt3-dependent substrate utilization for mitochondrial respiration, we checked the OCR that was derived from glutamine, which is the most abundant amino acid in blood and also an essential carbon source for anabolic processes. Importantly, the maximal OCR in 2 mM glutamine-containing media (without glucose and pyruvate) was significantly suppressed by either the knockdown of Sirt3 or the expression of the deacetylase-inactive mutant (Fig. 4C-D).

Collectively, these results suggest that glutamine is a predominantly utilized carbon source for mitochondrial oxygen consumption in both Caki2 and A498 cells.

Furthermore, Sirt3 was a critical regulator of glutamine usage for mitochondrial respiration.

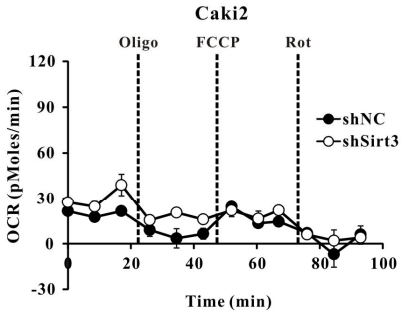
A

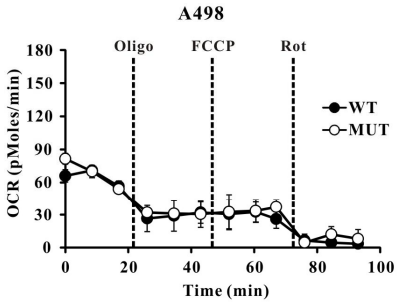




Glucose	Pyruvate	Glutamine
10 mM	1 mM	2 mM

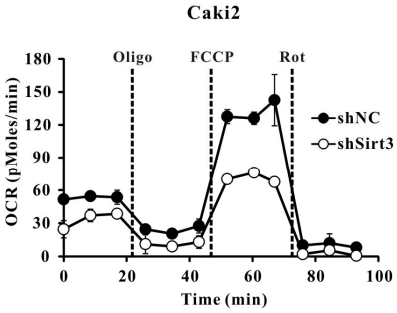
B

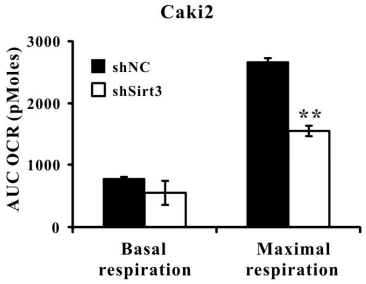




Glucose	Pyruvate	Glutamine
10 mM	1 mM	0 mM

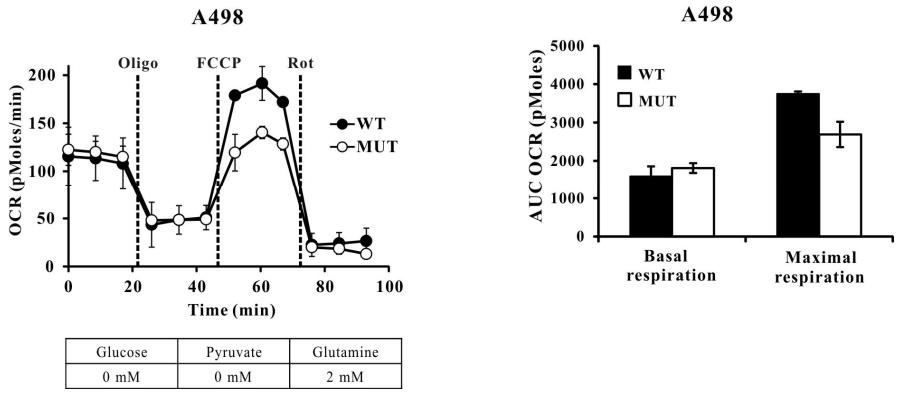
C





Glucose	Pyruvate	Glutamine
0 mM	0 mM	2 mM

D



Glucose	Pyruvate	Glutamine
0 mM	0 mM	2 mM

Figure 4. Sirt3 regulates glutamine-dependent oxidation in RCC.

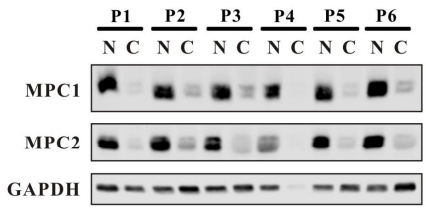
The oxygen consumption rate (OCR) was measured using the Seahorse XF analyzer in assay medium, which was supplemented with individual carbon sources, for 1 hour. The assay medium contained the substrate-free base medium, along with 10 mM D-glucose, 1 mM sodium pyruvate, and/or 2 mM L-glutamine. (A) The OCR of shNC-, shSirt3-transduced Caki2 cells and WT-, and MUT-transduced A498 cells in medium containing glucose, sodium pyruvate, and glutamine. (B) The OCR of shNC-, shSirt3-transduced Caki2 cells and WT-, and MUT-transduced A498 cells in glutamine-free medium. (C and D) The OCR of shNC-, shSirt3-transduced Caki2 cells and WT-, and MUT-transduced A498 cells in L-glutamine-supplemented medium. Basal respiration and maximal respiration were calculated. FCCP, Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone; Rot, rotenone.

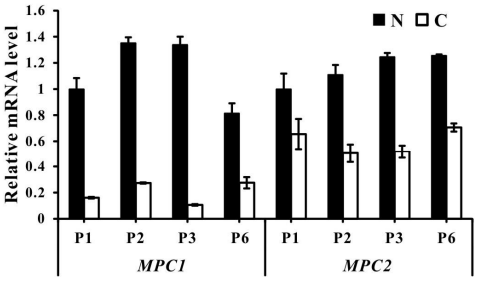
5. Expression of MPC1/2 in RCC

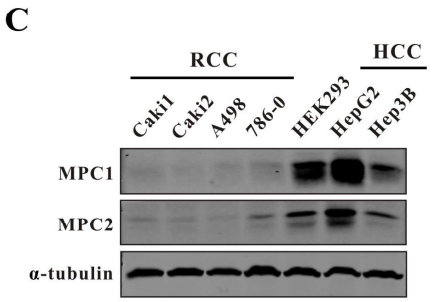
Pyruvate is a critical metabolite that plays a role in various metabolic pathways [33]. As shown in Fig. 4B, RCC cell lines are unable to use glucose and pyruvate as electron donors for oxidative phosphorylation, probably because HIF induces the expressions of lactate dehydrogenase A (LDHA), which favorably converts pyruvate to lactate, and pyruvate dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH) activity, thus resulting in the suppression of mitochondrial pyruvate utilization.

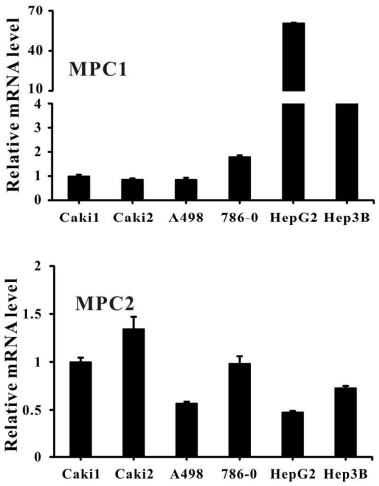
However, recent reports demonstrated that MPC, which is composed of MPC1 and MPC2, is required to import pyruvate into mitochondria. This process could be essential for mitochondrial pyruvate usage [34]. Therefore, we asked whether MPC expression is absent in RCC, because glucose- and pyruvate-derived OCRs were severely compromised in RCC cell lines.

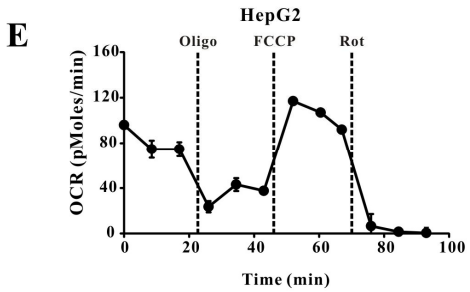
The expression levels of two MPC genes were markedly reduced in human RCC tissues (Fig. 5A and 5B). Moreover, protein levels of MPC1 and MPC2 were severely decreased in RCC cell lines when compared with HEK293 cells and HCC cell lines (Fig. 5C). The mRNA level of MPC1 was also markedly reduced in RCC cell lines without comparable changes in MPC2 mRNA levels (Fig. 5D). Furthermore, as shown Fig. 5E, we observed glucose- and pyruvate-dependent OCR of HepG2 cells compared with Caki2 cells.











Glucose	Pyruvate	Glutamine
10 mM	1 mM	0 mM

Figure 5. Expression of MPC1 and MPC2 in human RCC tissues and cell lines.

(A) Western blots of MPC1 and MPC2 expressions in paired tissues (N=normal; C=cancer; P=patient) from RCC patients. GAPDH was used as the loading control. (B) Quantitative PCR analysis of MPC1 and MPC2 expression in normal and cancer tissues. Gene expression values were normalized to TBP and are presented as the mean ± S.D. (C) Total protein extracts were prepared from RCC and hepatocellular carcinoma (HCC) cell lines and analyzed by immunoblotting. (D) MPC1 and MPC2 mRNA levels in indicated RCC and HCC cell lines. (E) The OCR of HepG2 cells in glutamine-free medium.

6. Glucose-dependent oxidation is disturbed in RCC through the downregulation of MPC

Next, we investigated whether the reduced expression of MPC contributes to impaired pyruvate oxidation in Caki2 cells. Both MPC genes were re-introduced into Caki2 cells (Fig. 6A), and pyruvate-dependent oxidation was assessed. As shown in Fig. 6B, upon the expression of both MPC genes, Caki2 cells show enhanced pyruvate oxidation in both basal and maximal respiration. To confirm that the elevated OCR was specifically mediated through MPC expression, we treated cells with an MPC inhibitor, UK5099. UK5099 treatment completely suppressed the elevating effects of MPC on the OCR (Fig. 6C). These results suggest that a defect in MPC may block mitochondrial pyruvate utilization in RCC.

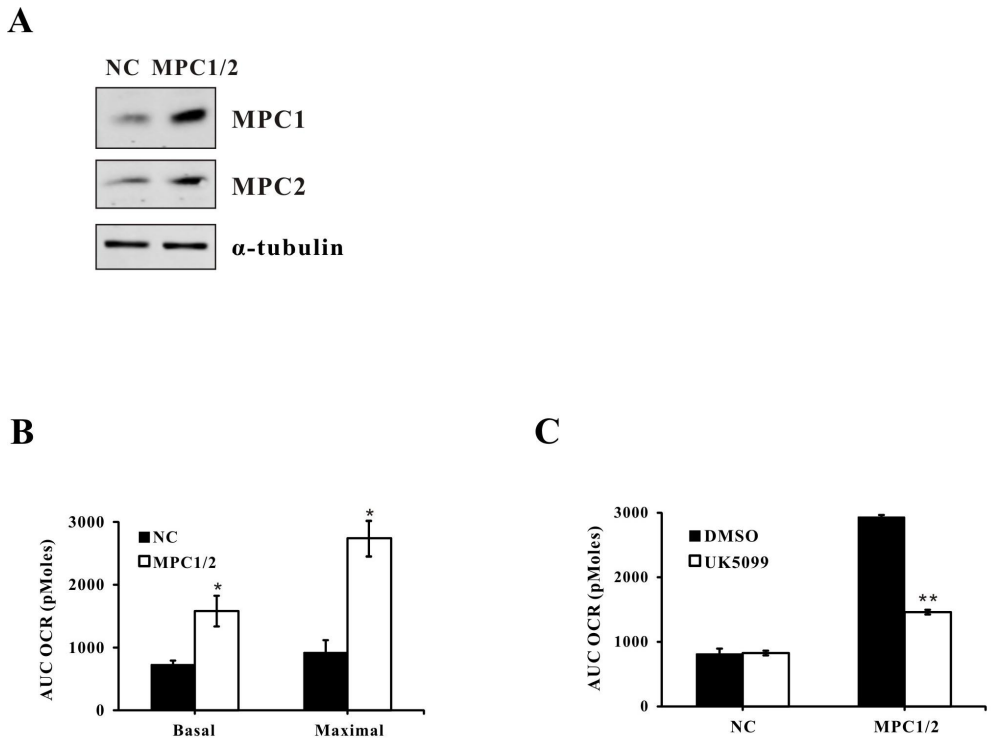


Figure 6. Glucose-dependent oxidation is disturbed in RCC through downregulation of mitochondrial pyruvate carrier (MPC).

(A) Western blots of Caki2 cells that were stably transduced with control lenti virus vector or MPC1/2. The protein levels of MPC1, and MPC2 were assessed. α-tubulin was used as the internal control. (B) Basal and maximal OCR of NC- and MPC1/2-transduced Caki2 cells in substrate-free base medium, which was supplemented 10 mM sodium pyruvate. (C) Maximal OCR of NC- and MPC1/2-transduced Caki2 cells in the presence and absence of the MPC inhibitor, UK5099. All bar graphs are plotted as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$ by Student's two-tailed t-test.

7. Sirt3 regulates GDH activity to mediate glutamine-derived mitochondrial oxidation in cells with defective mitochondria

Glutamine is the most abundant amino acid and meets nitrogen demand, which is imposed by nucleotide synthesis or the maintenance of nonessential amino acid pools. Many recent studies have highlighted glutamine as an essential carbon source for fueling mitochondrial metabolism in rapidly dividing cancer cells via the oxidative or reductive pathway, and many cancer cells exhibit high glutamine uptake [30]. Importantly, it has been proposed that glutamine-derived carbons and electrons from TCA cycles are required for cell growth and proliferation in the absence or presence of glucose, although both hypoxic cells and VHL-deficient cells exhibit a preference for glycolytic metabolism to generate energy [35].

Based on our observation that Sirt3 knockdown severely inhibited glutamine oxidation (Fig. 4), we hypothesize that Sirt3 is critical for driving glutaminolysis to mediate mitochondrial oxygen consumption in cells where mitochondrial pyruvate utilization is compromised.

GDH, which produces α-ketoglutarate (αKG) from glutamate, has been shown to be deacetylated and activated by Sirt3 [12,36]. To see if this occurs in RCC, we measured the activity of GDH in mitochondrial fractions. In Caki2 cells, depletion of Sirt3 significantly suppressed GDH activity without affecting GDH protein expression (Fig. 7A). In A498 cells, stable expression of the catalytically inactive Sirt3 mutant (MUT) significantly inhibited the activity of the enzyme compared with Mock and WT (Fig. 7B).

To test the possibility that decreased glutamine-derived OCR in Sirt3-depleting cells occurs due to reduced GDH activity, we measured the OCR in the presence of membrane-permeable dimethyl-αKG (DMKG). As shown in Fig. 7C, treatment with DMKG completely restores the reduction in mitochondrial OCR, thus implying that the inhibition of glutamine-derived OCR by Sirt3 inhibition is mostly due to the impaired GDH-catalyzed conversion of glutamine to αKG. Collectively, these results suggest that Sirt3 plays an important role in supplying glutamine as a carbon source in the mitochondrial oxidative pathway.

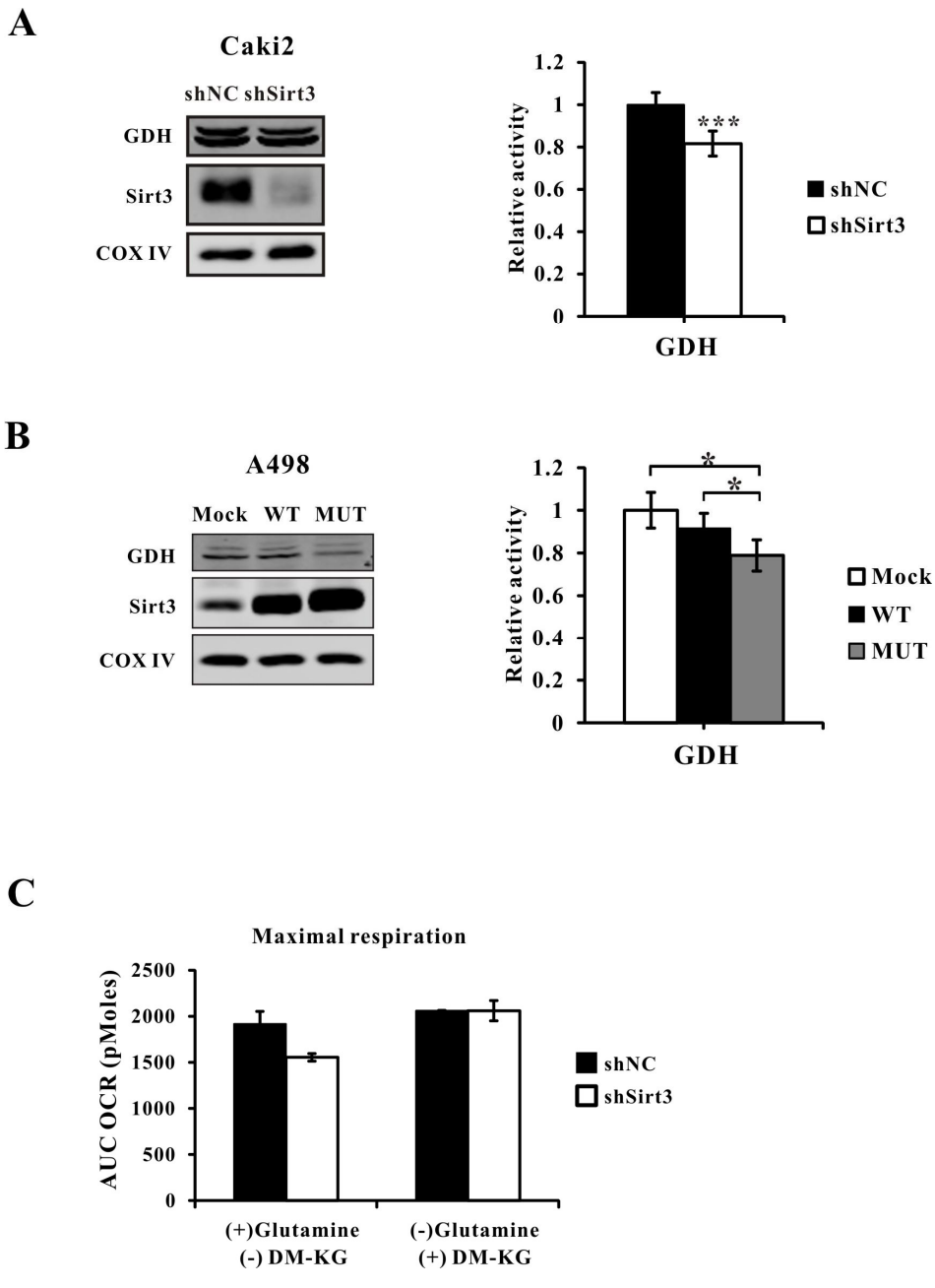


Figure 7. Sirt3 regulates glutamate dehydrogenase (GDH) activity to mediate glutamine-derived mitochondrial oxidation in cells with defective mitochondria.

(A and B) Western blots of mitochondrial extracts from Caki2 and A498 cells. COX IV was used as the loading control. Effects of GDH activity in mitochondria that were extracted from shNC-transduced Caki2 cells, shSirt3-transduced Caki2 cells, Mock-transduced A498 cells, WT-transduced A498 cells, and MUT-transduced A498 cells (C) The OCR was measured in assay medium supplemented with 2 mM dimethyl-2-oxoglutarate (DM-KG) or 2 mM L-glutamine. All bar graphs are plotted as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p<0.001$ by Student's two-tailed t-test.

8. Mitochondrial pyruvate can be supplied by ME2 in glutaminolysis in RCC cell lines

In addition to GDH, malic enzyme 2 (ME2) catalyzes the oxidative decarboxylation of malate to yield pyruvate. This process might be important when cytosolic pyruvate is not transported into mitochondria and can also contribute to mitochondrial glutamine oxidation. Interestingly, mitochondrial fractions of human RCC tissues exhibited significantly increased levels of ME2 protein (Fig. 7D). Furthermore, ME2 knockdown with siRNA transfection in Caki2 cells significantly inhibited cell growth (Fig. 7E). These data suggest that ME2 may play a critical role in supplying pyruvate to use in glutaminolysis in RCC.

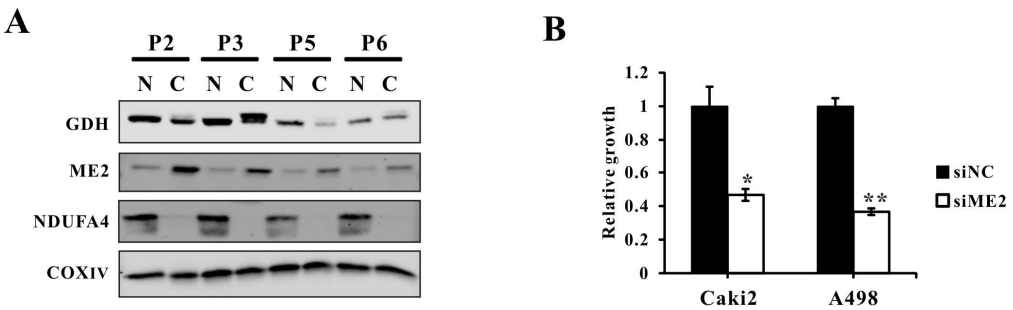


Figure 8. ME2 depletion inhibits cell proliferation.

(A) Western blots assessing mitochondrial enzymes in mitochondrial extracts from normal and cancer tissues. COX IV was used as the loading control. (B) Cell numbers of Caki2 cells that were transfected with control siRNA (siNC) or malic enzyme 2 (ME2) small-interfering RNA (siME2) for 72 hours. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p<0.001$ by Student's two-tailed t-test.

Ⅳ. DISCUSSION

Sirt3, a mitochondrial acetyl-lysine deacetylase, is a critical factor for maintaining mitochondrial integrity and function. Although the role of Sirt3 in carcinogenesis as a tumor suppressor or an oncogene has been investigated in various human cancers, its function in RCC is largely unknown. In this study, we demonstrate that Sirt3 acted as a positive regulator of human RCC by regulating glutamine-dependent mitochondrial respiration.

We show that the mitochondrial fractions of human advanced RCC tissues exhibited increased Sirt3 expression compared to mitochondrial fractions of normal kidney tissues. The discrepancy in Sirt3 mRNA and protein expression in whole-cell lysates might be due to low mitochondrial contents in human RCC, as demonstrated in several reports [3-5,37]. Our results also reveal that there was no consistent expression pattern of mitochondrial proteins. After normalization to COX IV, NDUFA4 remained downregulated, whereas Sirt3 was upregulated. Therefore, not all mitochondrial proteins were reduced in cells where mitochondrial biogenesis is minimized. Similarly, other studies have shown that re-introducing wild-type VHL to VHL-mutated RCC cells did not rescue all mitochondrial proteins [4,5].

Our findings suggest that Sirt3 is required for RCC cell growth. Knockdown of Sirt3 inhibited cell growth in a HIF-independent manner. In addition, overexpression of dominant-negative mutant Sirt3 (H248Y) attenuated xenograft tumor growth *in vivo*. Several mechanisms by which Sirt3 supports tumor growth have been proposed. The Sirt3-mediated interaction between Ku70 and bcl-2-like

protein 4 (Bax) protects cardiomyocytes from stress-induced apoptotic cell death [38]. Furthermore, the interaction between Sirt3 and nicotinamide mononucleotide adenylyl transferase 2 promotes mitochondrial function and cell proliferation [23]. In addition, Sirt3 abrogates growth arrest and p53 senescence activity in bladder carcinoma cells [39] and inhibits apoptosis in OSCC cells [21]. However, we did not observe apoptotic effects in Sirt3-inactivated cells.

Importantly, we found that the loss of Sirt3 was associated with a severe reduction in glutamine-dependent mitochondrial respiration. It should also be noted that Sirt3 inhibition did not completely block glutamine-derived OCR. This may have been due to either the incomplete depletion of Sirt3 in cells or the partial involvement of Sirt3 in mitochondrial glutamine utilization. Next, we propose that Sirt3 stimulates glutamine-dependent mitochondrial respiration by promoting the activity of GDH. Sirt3 depletion suppressed GDH activity, and supplementation with αKG completely rescued glutamine-derived OCR. However, we cannot exclude the possibility that other sirtuins may also affect glutamine-dependent mitochondrial oxidation. In particular, Sirt4 is known to inactivate GDH through ADP ribosylation [40].

The importance of glutaminolysis in RCC cells was signified by severely compromised glucose- and pyruvate-derived mitochondrial respiration. We propose that the low expression of MPC in RCC contributes to the blockade of mitochondrial pyruvate utilization. In our study, re-introduction of MPC enhanced the pyruvate-dependent OCR. These effects were blunted by an inhibitor of MPC, although the involvement of LDHA or pyruvate dehydrogenase kinase (PDK) on mitochondrial

pyruvate usage in RCC needs to be elucidated.

To maintain mitochondrial respiration under the condition of minimized cytosolic pyruvate, mitochondrial pyruvate must be supplied through glutaminolysis. Notably, RCC tissues exhibited elevated expression of ME2, which produces pyruvate from malate in the mitochondria. ME2 knockdown severely impaired cell proliferation. A previous study showed that accumulated malate increases reactive oxygen species and the $NADP^+$/NADPH ratio in A549 cells. Furthermore, a decrease in ATP inhibits cell proliferation and induces cell death [41]. In our study, both Caki2 and A498 cells exhibited severe blockade of pyruvate-derived OCR. However, further studies examining the effects of exogenous pyruvate supplementation on ME2 loss-inhibited cell proliferation are warranted.

Collectively, these data suggest that Sirt3 plays a tumor-progressive role in human RCC by regulating glutamine-derived mitochondrial respiration, particularly in cells where mitochondrial pyruvate usage is severely compromised. Because human RCC shows highly glycolytic and reduced mitochondrial functions, targeting the glycolytic pathway could be a promising treatment for RCC. However, a deeper understanding of the mechanisms driving altered metabolic pathways is essential for future therapeutic interventions.

Ⅴ. CONCLUSION

Mitochondrial fractions of human advanced RCC tissues exhibited increased Sirt3 expression compared to those of normal kidney tissues. Knockdown of Sirt3 inhibited RCC cell proliferation in a HIF-independent manner and overexpression of dominant-negative mutant Sirt3 attenuated tumorigenesis *in vivo*. In Caki2 cells, deletion of Sirt3 suppressed GDH activity without affecting GDH protein expression and consequently reduced glutamine-dependent oxidation. Glucose-dependent oxidation is disturbed in RCC because of downregulation of MPC expression. Low expression of MPC in RCC contributes to the blockade of mitochondrial pyruvate utilization. These suggest that Sirt3 plays a critical role in RCC by activating GDH for the efficient utilization of glutamine by mitochondria.

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ABSTRACT (IN KOREAN)

Sirt3가 콩팥세포암의 생존과 세포증식에 미치는 영향

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최 지 은

대부분의 콩팥세포암 (clear cell renal carcinoma)은 성인의 신장에서 나타난다. 콩팥세포암은 특이적으로 von Hippel-Lindau (VHL) 유전자의 결함으로 인해 hypoxia-inducible factor-a (HIF-a)의 발현이 높아지며, 이로 인해 미토콘드리아 호흡은 억제되고 호기성 해당작용을 통한 에너지 합성이 증가한다. 비록 'Warburg effect'가 종양의 대사에서 가장 대표적인 특징으로 알려져 있지만, 콩팥세포 암종과 같이 미토콘드리아 생물발생이 최소화된 종양에서도 미토콘드리아의 기능의 중요성이 밝혀지고 있는 추세이다. Sirt3는 미토콘드리아 유전자들의 탈아세틸화에 중요하게 작용하며, 세포의 에너지 대사를 조절한다고 알려져 있다. 하지만, Sirt3가 암 성장을 억제시키거나 촉진시키는지는 암세포 종에 따라 다르며 아직 콩팥

세포암에서의 역할은 보고되지 않았다. 본 연구에서는 사람의 콩팥세포암 조직에서 미토콘드리아 내의 Sirt3의 발현이 정상 콩팥 조직보다 증가되어 있는 것을 확인하였다. 콩팥세포암에서 Sirt3 발현의 역할을 밝히고자 Short-hairpin RNA를 통해 Sirt3의 발현을 억제하거나 또는 유전자 변이를 통해 기능을 약화시켰다. 그 결과 세포의 성장이 억제되고, Xenografts를 통한 생체 내 (*in vivo*) 실험 결과 누드마우스에서 종양 형성을 억제시켰다. 또한 콩팥세포암에서 mitochondrial pyruvate carrier (MPC) 유전자의 발현이 낮으며 malic enzyme (ME) 2의 발현은 높게 관측되는데, 미토콘드리아 산소 소모량 측정과 ME2의 small interfering RNA (siRNA)를 적용한 세포 성장 측정을 통해 미토콘드리아 산화과정에 사용되는 미토콘드리아의 피루베이트는 글루코스가 아닌 글루타민으로부터 ME2를 거쳐 공급된다는 것을 알 수 있었다. 따라서 Sirt3의 발현이 억제되면 glutamate dehydrogenase (GDH)의 활성을 억제하여 미토콘드리아의 기능을 손상시킨다고 추정할 수 있다. 요약하자면 본 논문에서 Sirt3는 콩팥세포암의 종양 형성에 필요하며, 이는 글루타민에 의한 미토콘드리아 호흡을 조절함으로써 이루어진다는 것을 밝혔다.

핵심되는 말: 콩팥세포암, Sirt3, 미토콘드리아 산화, glutamate dehydrogenase