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Snail modulates cell metabolism in MDCK cells

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ABSTRACT

Snail, a repressor of E-cadherin gene transcription, induces epithelial-to-mesenchymal transition and is involved in tumor progression. Snail also mediates resistance to cell death induced by serum depletion. By contrast, we observed that snail-expressing MDCK (MDCK/snail) cells undergo cell death at a higher rate than control (MDCK/neo) cells in low-glucose medium. Therefore, we investigated whether snail expression influences cell metabolism in MDCK cells. Although gylcolysis was not affected in MDCK/snail cells, they did exhibit reduced pyruvate dehydrogenase (PDH) activity, which controls pyruvate entry into the tricarboxylic acid (TCA) cycle. Indeed, the activity of multiple enzymes involved in the TCA cycle was decreased in MDCK/snail cells, including that of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDH2), succinate dehydrogenase (SDH), and electron transport Complex II and Complex IV. Consequently, lower ATP content, lower oxygen consumption and increased survival under hypoxic conditions was also observed in MDCK/snail cells compared to MDCK/neo cells. In addition, the expression and promoter activity of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inhibits the activity of PDH, was increased in MDCK/snail cells, while expression levels of glutaminase 2 (GLS2) and ATP-citrate lyase (ACLY), which are involved in glutaminolysis and fatty acid synthesis, were decreased in MDCK/snail cells. These results suggest that snail modulates cell metabolism by altering the expression and activity of key enzymes. This results in enhanced glucose dependency and leads to cell death under low-glucose conditions. On the other hand, the reduced requirements for oxygen and nutrients from the surrounding environment, might confer the resistance to cell death induced by hypoxia and malnutrition.

1. Introduction

Snail is a zinc-finger transcription factor known to induce epithelial-to-mesenchymal transition (EMT), in which epithelial cells acquire the characteristics of mesenchymal cells with migratory properties. EMT contributes to the formation of many tissues during embryonic development and to the acquisition of invasive properties in epithelial tumors [1]. Snail expression has been detected in a large number of human carcinomas [2]. In human cancer, aberrant expression of snail is correlated with invasive growth potential [3]. Snail also has other functions in addition to the induction of EMT. Snail arrests the cell cycle by increasing the expression of p21 and confers resistance to cell death induced by serum depletion [2,3]. Resistance to cell death conferred by snail provides a selective advantage to migratory cells during tumor dissemination [2]. Recently, snail has been reported to implicate in metabolic stress-induced necrosis. Knockdown of snail significantly inhibited necrosis induced by glucose depletion, and snail is presumed to facilitate necrosis by promoting mitochondrial ROS production [4]. We observed a higher rate of cell death in MDCK cells expressing snail (MDCK/snail) than in control MDCK/neo cells in low-glucose medium and hypothesized that snail might also play a role in cellular metabolism. Because snail expression has been detected in a large number of human carcinomas, we investigated whether aberrant snail expression alters cellular metabolism, a commonly observed characteristic of cancer cells.

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High glycolytic rates and reduced mitochondrial oxidation are commonly observed in cancer cells [5]. Increased *de novo* lipid synthesis [6] and increased glutaminolysis [7] are also key features of the metabolic profile of cancer cells. In proliferating cells, citrate, a TCA cycle metabolite, is continually exported to the cytoplasm and used for the generation of acetyl-CoA, which serves as a precursor of lipid synthesis. Therefore, replenishment of TCA intermediates is necessary, and glutamines serve as both a replenishment substrate and a carbon source for energy production [5].

We found that although MDCK/snail cells exhibited decreased amounts of oxidative phosphorylation, they did not exhibit increased glycolysis. Furthermore, compared to MDCK/neo cells, MDCK/snail cells expressed lower levels of ATP-citrate lyase (ACLY) and glutaminase 2 (GLS2), which are involved in fatty acid synthesis [6] and glutaminolysis [7]. These results suggest that snail might downregulate these metabolic pathways and confer resistance to cell death induced by hypoxia and malnutrition. They also raise the possibility of a new role for snail in tumor progression.

2. Materials and methods

2.1. Cell culture and reagents

MDCK cells were transfected with either a hemagglutinin (HA)-tagged human snail expression vector (snail) or a control vector (neo) using the calcium phosphate method and designated MDCK/snail 1, MDCK/neo 1, MDCK/snail 2, or MDCK/neo 2. MDCK cells and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) containing 5.5 mM glucose and 10% fetal calf serum (FCS). MitoTracker was purchased from Molecular Probes, Inc. (Eugene, OR, USA).

2.2. Cell survival

MDCK cells (2×10^4) were seeded in 96-well plates in DMEM containing various concentrations of glucose and 10% dialyzed FCS. After 24–96 h incubation, the medium was removed, and the remaining adherent cells were stained with 0.1% crystal violet and dissolved with 0.5% Triton X-100 in PBS. The optical density was measured at 595 nm. Cell number was calculated from a standard curve (cell number versus absorbance).

2.3. Measurement of glucose and lactate in the medium

MDCK cells (2×10^4) were seeded in 96-well plates in DMEM containing 5.5 mM glucose and 10% FCS. Aliquots were removed at various time points and assayed for glucose content with a D-glucose measurement kit (GAHK20; Sigma–Aldrich) in accordance with the manufacturer's protocol. Lactate levels were measured as described previously [8].

2.4. Measurement of intracellular ATP

MDCK cells (2×10^4) were seeded in 96-well plates. The next day, the medium was changed to DMEM containing 10% dialyzed FCS with or without glucose. Intracellular ATP levels were measured at various time points using a cellular ATP measuring reagent (Toyo Ink; Tokyo, Japan). Luminescence was measured using a luminometer (TD-20/20; Turner Design, Sunnyvale, CA, USA).

2.5. Measurement of mitochondrial membrane potential by flow cytometry

MDCK cells (5×10^5) were seeded in 60 mm dishes. After 24 h, the cells were incubated for 30 min in serum-free DMEM containing MitoTracker. The cells were washed with PBS and then analyzed using a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA). Fluorescence was detected in the FL3 channel, as described previously [9].

2.6. Measurement of pO_2

MDCK cells (5×10^6) were harvested and suspended in Hanks buffer containing 5.5 mM glucose. The pO_2 level of the medium was measured with an MTS 5000S test system (M T Giken, Tokyo, Japan), as reported previously [10].

2.7. Mitochondrial fractionation for TCA cycle enzyme assay

MDCK cells (4×10^6) were seeded in a 100 mm dish. After 48 h incubation, the intact mitochondrial fraction was prepared as described previously [11]. Mitochondria were sonicated, either in a sonicating water bath for 30 s or by an ultrasonic disruptor (TOMY UD-200).

2.8. Measurement of TCA enzyme activities

The activities of hexokinase (HK) [12], phosphofructokinase (PFK) [13], pyruvate kinase (PK) [14], lactate dehydrogenase (LDH) [15] and glucose 6-phosphate dehydrogenase (G6PDH) [16] were determined as described previously. The activities of NAD^+-specific isocitrate dehydrogenase (IDH3), keto-glutarate dehydrogenase (KGDH), SDH, malate dehydrogenase (MDH), citrate synthase (CS), and Complex I were measured as described previously [17]. The activities of IDH2 [18], aconitase (ACO2) and

Table 1
Primers sequences of RT-PCR.

Gene name	Oligonucleotides
Human gene	
Snail	5′-TTCCAGCAGCCCTACGACCAGA-3′
	5′-GCCTTTCCCACTGTCCTCATC-3′
Dog gene	
ACC	5′-ATGAATGGTTCATGCGTAGA-3′
	5′-CTCATCAGTGAGAGTAGGGT-3′
ACLY	5′-ATCCCTGCAAGGAAAGAGTG-3′
	5′-CAGGGATGCCTTCAGCTATG-3
ACO2	5′-TCCGTGTTCCCCTACAACCA-3′
	5′-TTAGGACCTGTGCGTAGCCA-3
ALT1	5′-CGCATCTTGCAGGCATGTGG-3′
	5′-CAGCTTCAGCACCGTCACGA-3′
Beta actin	5′-TAAGGACCTGTATGCCAACAC-3′
	5′-CGTACTCCTGCTTGCTGATCC-3′
GAPDH	5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′
	5′-CATGTAGGCCATGAGGTCCACCAC-3′
GDH	5′-GGTGTTAAGATCAATCCCAA-3′
	5′-AACGTTTTATCTCCAAACCC-3′
GLS2	5′-CCCTATCCACAAGTTCACCA-3′
	5′-TGGATCGCATCTTTGAGGAT-3
IDH2	5′-GACCAGGTCACCATAGACTC-3′
	5′-AAGTCTGTGGCCTTGTACTG-3′
PDH	5′-ATTTCAAGCTTAAGCTTACAACATGG-3′
	5′-TCCAGATCTACAGTAGGCGG-3′
PDK1	5′-TGAACGCTTGTGAAAAGACC-3′
	5′-ACCAATTGAACTGATGGAGT-3′
SDHA	5′-GGTGGCACTTCTACGACACC-3′
	5′-GGTGGCACTTCTACGACACC-3′
SDHB	5′-GATTAAGAATGAAATTGACTC-3′
	5′-TACTGAGCATAGAAGTTGCTC-3′
SDHC	5′-CTCTTCCCATGGCAATGTCC-3′
	5′-GGGAATCTTCAGGCCTTTCC-3′
SDHD	5′-TAGAACCCAGCACATTCACC-3′
	5′-TTGACAGCTTTCTGCAAACC-3′
SREBP	5′-CCTGTTCGACCCACCGTATG-3′
	5′-ATGGGCAGCTTCTCAGTGTC-3′

fumarase [19], Complex II [20], and Complex V [21] were measured as described previously.

2.9. Measurement of Complex IV (COX) activity

Intact isolated mitochondria were used to determine cytochrome c oxidase activity using a cytochrome c oxidase assay kit (CYTOCOX-1; Sigma–Aldrich).

2.10. Measurement of pyruvate dehydrogenase (PDH) activity

MDCK cells (4×10^5) were seeded in a 60 mm dish. After 48 h, cell extracts (50–100 μg of protein) were prepared and PDH activity was measured using a PDH enzyme activity dipstick assay kit (Abcam Inc.).

2.11. RT-PCR analysis and real-time PCR analysis

RNA was extracted with a commercial kit (ISOGEN; Wako, Osaka, Japan) and reverse-transcribed using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Beta-actin or GAPDH was used as an internal control. The primers are listed in Table 1. cDNA was subjected to real-time PCR with Syber premix Ex Taq (Takara, Ohtsu, Japan). Data were analyzed according to the relative standard curve and normalized relative to beta-actin mRNA.

2.12. Measurement of promoter activity

MDCK and HepG2 cells were seeded (1×10^5 cells/well) into 24-well plates and transfected with the human PDK1 reporter vector [22] and the pRL-CMV vector. HepG2 cells were co-transfected with pCAGGS-snail vectors (snail) or an empty pCAGGS vector (neo). After 24 h incubation, both firefly and Renilla luciferase activities were measured using a dual luciferase reporter assay kit (Promega, Madison, WL, USA), as described previously [23].

2.13. Statistical analyses

Statistical analysis was performed by Student's *t*-test for independent samples. Comparisons between groups with respect to survival under hypoxia and enzyme activities were performed by one-way analysis of variance (ANOVA) and Scheffe's *F* test. Data are expressed as the means ± s.e.m. Statistical significance is indicated by asterisks ($^*P < 0.05$ and $^{**}P < 0.01$).

3. Results

3.1. Survival of MDCK/snail cells is decreased in low-glucose medium

Although several reports have demonstrated that snail inhibits cell death [2,3], we observed massive cell death in MDCK/snail cells cultured in DMEM with 5.5 mM glucose (Fig. 1A). However, enhanced cell death was not observed when MDCK/snail cells were cultured with 16 mM glucose (Fig. 1A). 2-Deoxyglucose (2DG) is a glucose analogue and an inhibitor of glycolysis [24]. MDCK/snail 1 cells were more sensitive to 2DG than MDCK/neo 1 cells when cultured in glucose-free DMEM (Fig. 1B). Thus, MDCK/snail 1 cells appeared to have an increased glucose dependency. Therefore, we examined whether snail expression enhances glycolysis in MDCK cells. To exclude the possibility that the phenotype (altered cellular metabolism) was due to specific clonal variation of MDCK/snail cells, we created a second MDCK/snail clone (MDCK/snail 2). Neither MDCK/snail 1 nor MDCK/snail 2 cells exhibited increased glucose consumption or lactate accumulation compared to their respective controls (MDCK/neo 1 and MDCK/neo 2; Fig. 1C and D). Consistent with this result, the activities of enzymes involved in glycolysis, such as HK, PK and LDH, were not enhanced by snail expression in

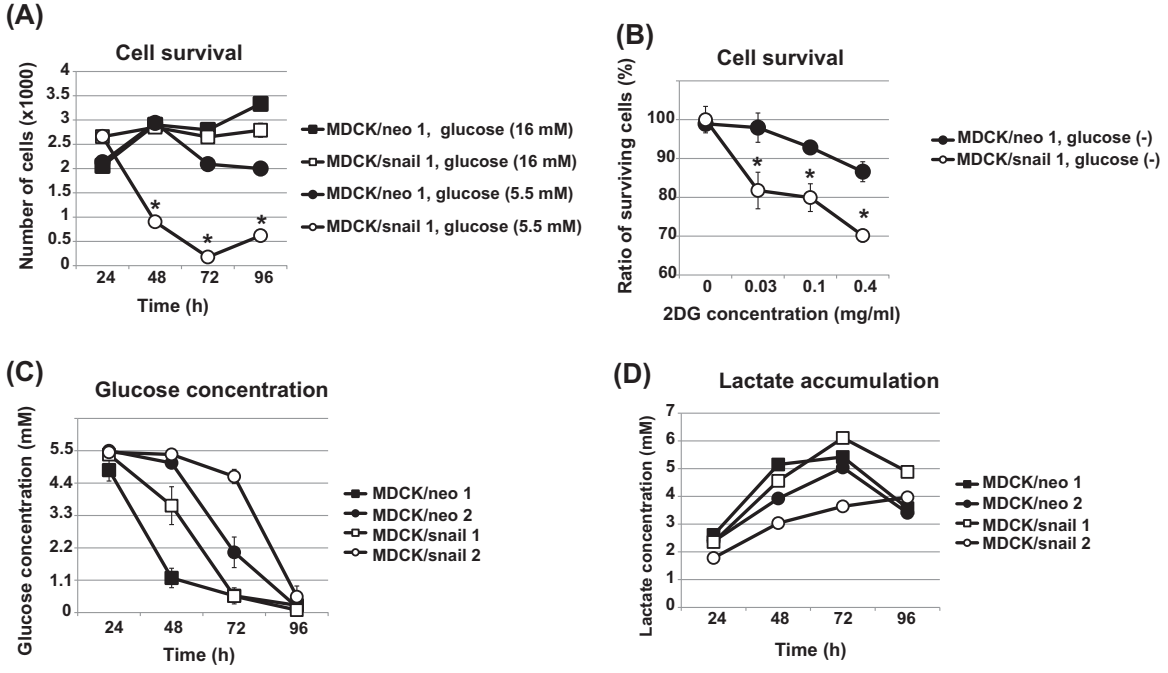

Fig. 1. MDCK/snail cells exhibit increased cell death under low-glucose conditions, but not enhanced glycolysis. (A) Survival of MDCK cell lines. MDCK cells (2×10^4) were cultured in DMEM containing 5.5 mM glucose or 16 mM glucose. (B) Effect of 2DG on the survival of MDCK cells cultured in glucose-free DMEM supplemented with dialyzed FCS. Values are the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from the corresponding MDCK/neo cells (Student's *t*-test). (C) Glucose concentration and (D) lactate concentration in culture medium at the indicated times.

Table 2
Activities of enzymes involved in glycolysis, TCA cycle, and oxidative phosphorylation.

Enzyme	Activity	
	MDCK/neo 1	MDCK/snail 1
HK	35.8 ± 1.8	35.6 ± 2.3
PFK	132.2 ± 39.2	312.8 ± 62.0
PK	341.0 ± 16.7	351.9 ± 28.6
LDH	32090 ± 596	32980 ± 732
G6PDH	242.5 ± 18.8	248.5 ± 25.3
CS	548.0 ± 22.2	750.7 ± 33.7
NAD^+-IDH	7.2 ± 0.1	7.2 ± 0.3
KGDH	1.2 ± 1.8	0.7 ± 0.1
MDH	36.6 ± 7.6	42.6 ± 0.9
Fumarase	68.9 ± 4.6	65.5 ± 11.5
Complex I	41.9 ± 13.8	29.6 ± 8.6
Complex V (ATPase)	1474.2 ± 277.6	1335.6 ± 158.7

Values are expressed as nmol min^{-1} (mg protein)$^{-1}$ and represent the means ± s.e.m. Each experiment was performed 3–7 times.

MDCK cells (Table 2). In addition, the activity of G6PDH, which catalyzes the rate-limiting step in the pentose phosphate pathway [25], was comparable between MDCK/neo and MDCK/snail cells (Table 2). These results show that MDCK/snail cells exhibit increased glucose dependency but not enhanced glycolysis.

3.2. MDCK/snail cells exhibit a lower mitochondrial membrane potential, decreased oxygen consumption, and lower intracellular ATP content

Because MDCK/snail cells demonstrate increased glucose dependency, we investigated whether MDCK/snail cells also exhibit decreased levels of oxidative phosphorylation. First, we measured the ATP content in MDCK cells. When cultured in glucose-free DMEM, the level of intracellular ATP in MDCK/neo cells decreased by 50% over 48 h, but then stabilized for up to 96 h. However, under the same conditions, almost no intracellular ATP could be detected in MDCK/snail cells after a 24 h incubation (Fig. 2A). Next, we measured the mitochondrial membrane potential by staining MDCK cells with MitoTracker. The proportion of cells with a low mitochondrial potential (M1 fraction) was increased in MDCK/snail cells compared to control cells (Fig. 2B–D). We then determined oxygen consumption by measuring pO_2 levels in the cell medium. As expected, the oxygen consumption rate was lower in MDCK/snail 1 cells than in MDCK/neo 1 cells when they were supplemented with glucose (Fig. 2E–G). Consequently, the survival of MDCK/snail cells under hypoxic conditions (0.8% O_2 for a 96 h incubation) in the presence of 10 mM glucose was greater than that of MDCK/neo cells under the same conditions (Fig. 2H). These results demonstrate that MDCK/snail cells

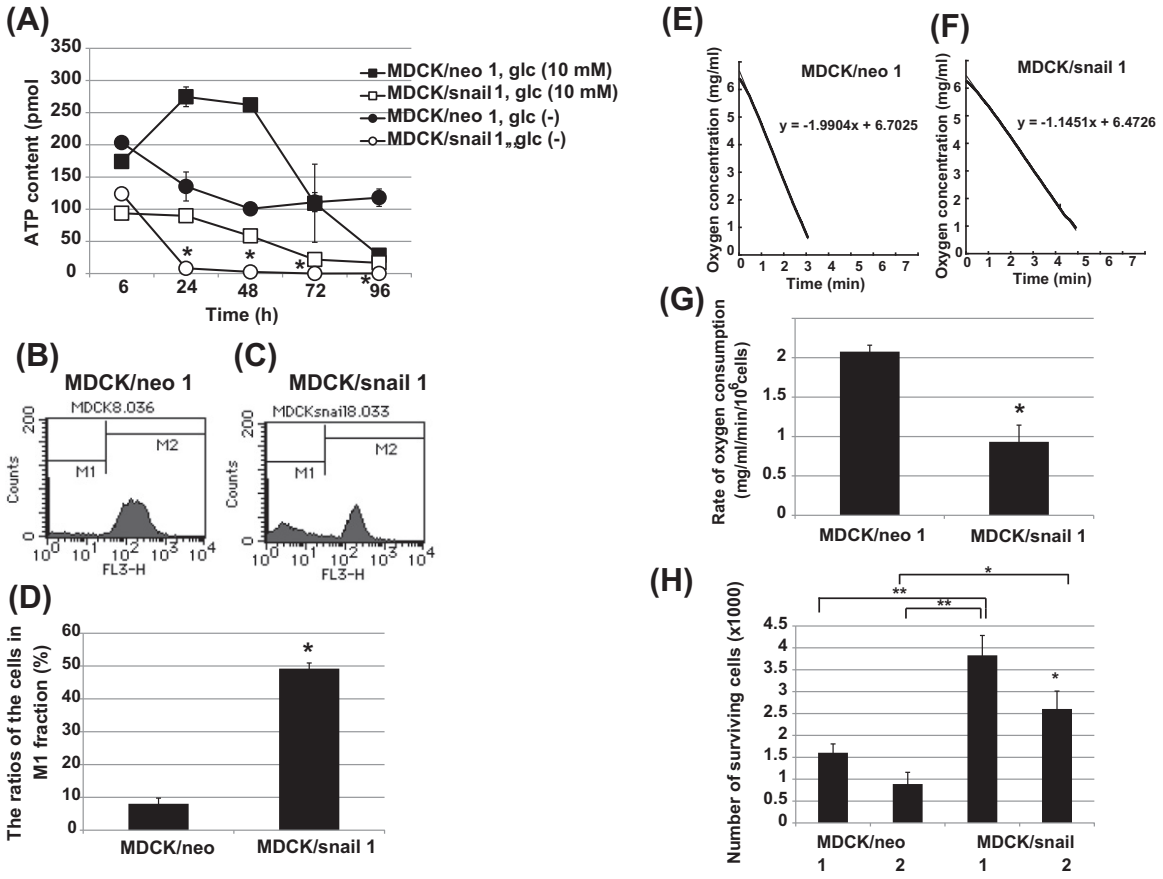

Fig. 2. MDCK/snail cells exhibit reduced mitochondrial membrane potential, oxygen consumption and ATP production. (A) Intracellular ATP contents in MDCK cells cultured in DMEM without or with 10 mM glucose (glc). Values are the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from the corresponding control cells (Student's *t*-test). (B and C) Flow cytometry analysis of mitochondrial membrane potential with MitoTracker Red staining. (D) The percentage of cells with lower mitochondrial membrane potential in the M1 fraction. Data are expressed as the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from MDCK/neo cells (Student's *t*-test). (E and F) Respiratory traces of O_2 consumption in the medium of MDCK/neo and MDCK/snail cells. (G) Rate of oxygen consumption in MDCK/neo and MDCK/snail cells. Data are expressed as the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from MDCK/neo cells (Student's *t*-test). (H) Cell survival following exposure to 0.8% O_2 for 96 h in DMEM containing 10 mM glucose. Comparison of cell survival rates between groups in hypoxia was performed by one-way analysis of variance (ANOVA) and Scheffe's *F* test. Data are expressed as the mean ± s.e.m. Statistical significance is indicated by an asterisk ($^*P < 0.05$ and $^{**}P < 0.01$).

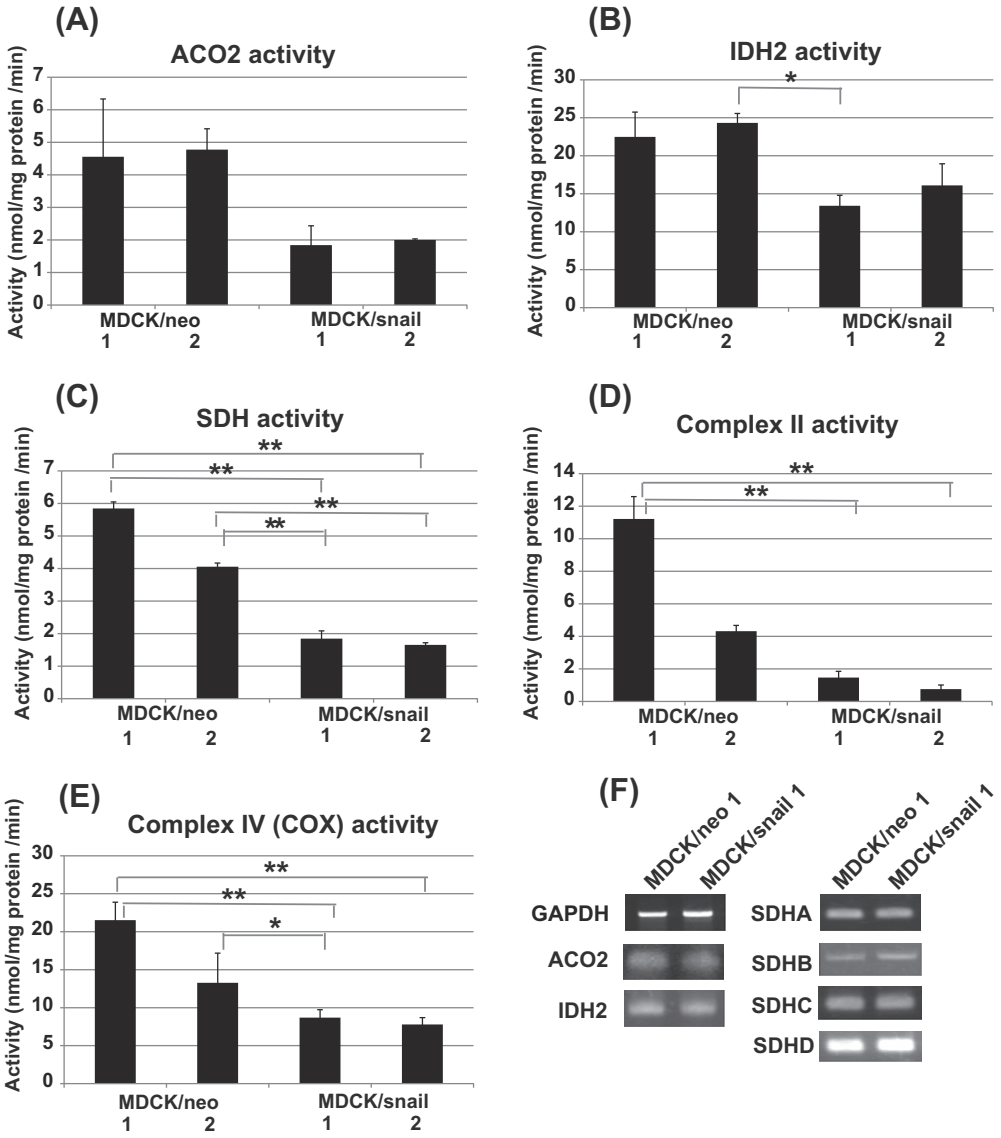

Fig. 3. TCA cycle enzymes and electron transport complexes in MDCK/snail cells exhibit reduced activities. (A–E) Enzyme activities in isolated mitochondrial fractions of MDCK cells. Values are expressed as the mean ± s.e.m. in nmol (mg mitochondrial protein)$^{-1}$ min^{-1}. Comparison between groups was performed by one-way analysis of variance (ANOVA) and Scheffe's *F* test. Statistical significance is indicated by an asterisk ($^{*}P < 0.05$ and $^{**}P < 0.01$). (F) Enzyme expression as determined by RT-PCR. Representative bands of RT-PCR analyses of indicated genes. Each experiment was performed at least four times.

exhibit decreased levels of oxidative phosphorylation and are resistant to hypoxia-induced cell death.

3.3. The activities of TCA cycle enzymes and electron transport complexes are decreased in MDCK/snail cells

To elucidate the source of decreased oxygen consumption and ATP generation in MDCK/snail cells, we measured the activities of TCA cycle enzymes and electron transport complexes. Indeed, the activities of ACO2, IDH2, SDH, Complex II, and Complex IV (COX) were decreased in MDCK/snail cells (Fig. 3A–E). IDH2 catalyzes the conversion of isocitrate to α-ketoglutarate, a rate-limiting step of the TCA cycle [18]. SDH is a part of both the TCA cycle and an electron complex known as Complex II, which oxidizes succinate to fumarate [26]. Because snail is a transcription factor, we investigated whether snail attenuated the expression of these enzymes. However, the expression levels of ACO2, IDH2 and SDH were comparable between MDCK/snail and MDCK/neo cells (Fig. 3F).

3.4. PDH activity is decreased in MDCK/snail cells

Because the expression levels of TCA cycle enzymes were unchanged, we hypothesized that the reduced availability of substrate, such as acetyl-coA, might decrease the activities of these enzymes. To explore this possibility, we determined PDH activity. PDH converts pyruvate to acetyl-CoA and controls pyruvate entry into the TCA cycle, and thus sits at the interface between glycolysis and glucose oxidation [5]. PDH activity was decreased (Fig. 4A). However, the expression of PDH was not decreased in MDCK/snail cells compared to control cells (Fig. 4B).

3.5. Expression of PDK1 is increased in MDCK/snail cells

PDH activity is tightly regulated by PDK1 via inhibitory phosphorylation of PDH alpha subunit [5]. We found that the expression of PDK1 mRNA in MDCK/snail cells was higher than in MDCK/neo cells (Fig. 4B and D).

3.6. Promoter activity of PDK1 is enhanced by expression of snail

Because the expression of PDK1 correlated with the expression of snail (Fig. 4C and D), we next asked whether snail expression affects the promoter activity of PDK1. As shown in Fig. 4E, PDK1 promoter activity in MDCK/snail 1 cells was 23 times higher than in MDCK/neo 1 cells. Furthermore, PDK1 promoter activity in snail-transfected HepG2 (HepG2 + snail) cells was 7.6 times higher than that of mock-transfected HepG2 (HepG2 + neo) cells (Fig. 4F). These results suggest that snail modulates PDH activity by enhancing PDK1 expression.

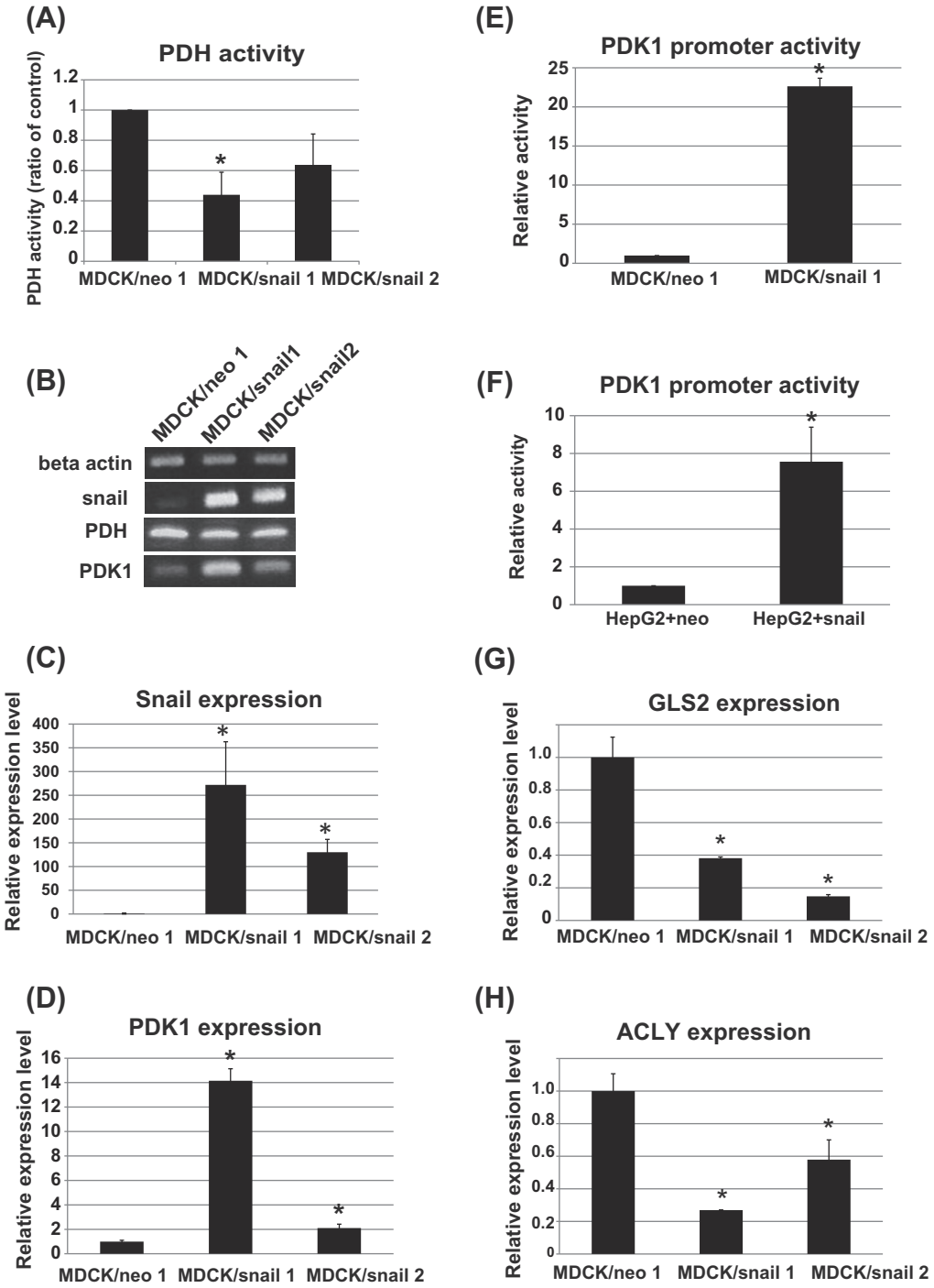

Fig. 4. MDCK/snail cells exhibit enhanced PDK1 promoter activity and expression and decreased PDH activity. (A) PDH activity in MDCK cells. PDH activity was measured using a PDH enzyme activity dipstick assay kit. (B) Gene expression of *Snail*, *PDH* and *PDK1* in MDCK cells. Representative bands of RT-PCR analyses of the indicated genes. (C and D) Quantitative RT-PCR analyses of *Snail* (C) and *PDK1* (D) expression in the indicated cells. Values are given as the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from MDCK/neo cells (Student's *t*-test). (E and F) PDK1 promoter activity in MDCK (E) and HepG2 (F) cells. MDCK/neo and MDCK/snail cells were transfected with 1 μg of a human PDK1 reporter vector and 20 ng of a pRL-CMV vector. HepG2 cells were co-transfected with pCAGGS-Snail vectors (HepG2 + Snail) or an empty pCAGGS vector (HepG2 + neo). Data are expressed as the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from the corresponding control cells. (G and H) MDCK/snail cells exhibit decreased expression of GLS2 and ACLY. Quantitative RT-PCR analyses of GLS2 (G) and ACLY (H) expression the in respective cells. Values are presented as the mean ± s.e.m. Asterisks indicate a statistical difference ($P < 0.05$) from MDCK/neo cells (Student's *t*-test).

3.7. Snail affects the expression of enzymes involved in glutaminolysis and fatty acid synthesis

Glutamines serve as a replenishment substrate for the TCA cycle and as a carbon source for energy production. Glutamine is converted to glutamate by GLS, and glutamate is then converted into alpha-ketoglutarate by either aminotransferase (ALT) or glutamate dehydrogenase (GDH), which can then enter the TCA cycle [27]. We determined the expression of these enzymes and found that the expression of GLS2 was decreased in MDCK/snail cells (Fig. 4G).

In fatty acid synthesis, citrate is converted into acetyl-coA by ACLY, and acetyl-coA is then carboxylated to malonyl CoA by acetyl-CoA carboxylase (ACC). Most of the enzymes involved in fatty acid biosynthesis are regulated by sterol regulatory element-binding proteins (SREBPs) [28]. We determined the expression of ACLY, ACC and SREBPs and found that ACLY expression was reduced in MDCK/snail cells (Fig. 4H). These results suggest that MDCK/snail cells exhibit reduced amounts of glutaminolysis and fatty acid synthesis.

4. Discussion

Aberrant snail expression has been detected in a large number of human cancers [2]. Cancer cell metabolism is associated with increased glycolysis and reduced mitochondrial oxidation, a phenomenon known as the Warburg effect [24]. Recently, it was reported that inhibition of PDH activity contributes to the Warburg effect [5]. PDH inhibition occurs via enhanced expression of PDK1, which results in inhibitory phosphorylation of PDH. Consequently, knockdown of PDK-1 restores PDH activity and decreases hypoxic cell survival [5]. In this study, we demonstrated that snail increases PDK1 promoter activity and expression in MDCK/snail cells, and confirmed that PDH activity in MDCK/snail cells is lower than in MDCK/neo cells. Under hypoxic conditions with sufficient glucose, MDCK/snail cells exhibited enhanced cell survival compared with MDCK/neo cells. Because snail expression has been detected in a large number of human carcinomas, snail activity might contribute to the Warburg effect.

In addition to reduced mitochondrial oxidation and high glycolytic rates, cancer cells exhibit increased glutaminolysis [27] and high rates of *de novo* lipid synthesis [28]. Compared with MDCK/neo cells, MDCK/snail cells did not exhibit increased levels of glycolysis. Furthermore, MDCK/snail cells showed reduced expression of GLS2 and ACLY, which might lead to reduced glutaminolysis and fatty acid synthesis. We can assume that downregulation of these metabolic pathways would decrease the requirement for nutrients and O_2 from the environment and would therefore confer resistance to cell death under conditions of malnutrition or hypoxia. Indeed, snail has been reported to decrease cell death induced by serum depletion in MDCK cells [2]. Therefore, snail might provide a selective advantage to migratory cells during tumor dissemination. On the other hand, reduced oxidative phosphorylation and glutaminolysis would increase the glucose dependency of MDCK/snail cells, which would then lead to massive cell death under low-glucose conditions.

Our findings raise the possibility of a new function for snail in tumor progression, which may have clinical relevance: because increased snail expression enhances cellular dependency on glycolysis, treatment of snail-expressing tumors with glycolytic inhibitors may represent a novel treatment option.

IDH2 catalyzes the conversion of isocitrate to alpha-ketoglutarate [18], which is required for prolyl-4-hydroxylase (PHD) activity. In turn, PHD promotes HIF-1alpha degradation [29]. Therefore, reduced IDH2 activity might contribute to PHD inactivation and HIF-1α stabilization. Because PDK1 is an HIF-1alpha regulated gene [30], snail might enhance PDK1 expression not only via a direct effect on its promoter, but also by stabilizing HIF-1alpha.

Although MDCK cells are a non-transformed cell line, these cells were selected to study the role of snail in cell metabolism because typical cancer cells have already had alterations in metabolism, the effect of snail on changes in metabolism would not be clear in these cell types. Using a non-transformed cell type allowed us to look at the effect of snail without confounding effects from other cancer-related cellular alterations. Still, there is a possibility that the role of snail might differ in different cellular contexts.

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References

[1] A. Barrallo-Gimeno, M.A. Nieto, The Snail genes as inducers of cell movement and survival: implications in development and cancer, Development 132 (2010) 3151–3161.
[2] S. Vega, A.V. Morales, O.H. Ocaña, et al., Snail blocks the cell cycle and confers resistance to cell death, Genes Dev. 18 (2004) 1131–1143.
[3] M. Kajita, K.N. McClinic, P.A. Wade, Aberrant expression of the transcription factors Snail and slug alters the response to genotoxic stress, Mol. Cell. Biol. 24 (2004) 7559–7566.
[4] C.H. Kim, H.M. Jeon, S.Y. Lee, et al., Implication of Snail in metabolic stress-induced necrosis, PLoS One 6 (2011) e18000.
[5] T. McFate, A. Mohyeldin, H. Lu, et al., Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells, J. Biol. Chem. 283 (2008) 22700–22708.
[6] N. Zaidi, J.V. Swinnen, K. Smans, ATP-citrate lyase: a key player in cancer metabolism, Cancer Res. 72 (2012) 3709–3714.
[7] D. Daye, K.E. Wellen, Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis, Semin. Cell Dev. Biol. 24 (2012) 362–369.
[8] E.P. Marbach, M.H. Weil, Rapid enzymatic measurement of blood lactate and pyruvate. Use and significance of metaphosphoric acid as a common precipitant, Clin. Chem. 15 (1967) 314–325.
[9] M. Kalbácová, M. Vrbacký, Z. Drahota, et al., Comparison of the effect of mitochondrial inhibitors on mitochondrial membrane potential in two different cell lines using flow cytometry and spectrofluorometry, Cytometry A 52 (2003) 110–116.
[10] F. Hirai, S. Motoori, S. Kakinuma, et al., Mitochondrial signal lacking manganese superoxide dismutase failed to prevent cell death by reoxygenation following hypoxia in a human pancreatic cancer cell line, KP4, Antioxid. Redox Signal. 6 (2004) 523–535.
[11] R. Ikeda, X.F. Che, M. Uchiyama, et al., 2-Deoxy-d-ribose inhibits hypoxia-induced apoptosis by suppressing the phosphorylation of p38 MAPK, Biochem. Biophys. Res. Commun. 342 (2006) 280–285.
[12] N. Taneja, P.E. Coy, I. Lee, et al., Proinflammatory interleukin-1 cytokines increase mesangial cell hexokinase activity and hexokinase II isoform abundance, Am. J. Physiol. Cell Physiol. 287 (2004) C548–C557.
[13] Y.Q. Liu, K. Tornheim, J.L. Leahy, Shared biochemical properties of glucotoxicity and lipotoxicity in islets decrease citrate synthase activity and increase phosphofructokinase activity, Diabetes 47 (1998) 1889–1893.
[14] E. Iori, R. Millioni, L. Puricelli, et al., Glycolytic enzyme expression and pyruvate kinase activity in cultured fibroblasts from type 1 diabetic patients with and without nephropathy, Biochim. Biophys. Acta 1782 (2008) 627–633.
[15] M. Haraguchi, S. Torii, S. Matsuzawa, et al., Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2, J. Exp. Med. 191 (2000) 1709–1720.
[16] N.C. Yang, K.C. Jeng, W.M. Ho, et al., DHEA inhibits cell growth and induces apoptosis in BV-2 cells and the effects are inversely associated with glucose concentration in the medium, J. Steroid Biochem. Mol. Biol. 75 (2000) 159–166.
[17] A.C. Nulton-Persson, L.I. Szweda, Modulation of mitochondrial function by hydrogen peroxide, J. Biol. Chem. 276 (2001) 23357–23361.
[18] D.T. Hartong, M. Dange, T.L. McGee, et al., Insights from retinitis pigmentosa into the roles of isocitrate dehydrogenases in the Krebs cycle, Nat. Genet. 40 (2008) 1230–1234.
[19] S. Melov, P. Coskun, M. Patel, et al., Mitochondrial disease in superoxide dismutase 2 mutant mice, Proc. Natl. Acad. Sci. USA 96 (1999) 846–851.

[20] V. Rhein, G. Baysang, S. Rao, et al., Amyloid-beta leads to impaired cellular respiration, energy production and mitochondrial electron chain complex activities in human neuroblastoma cells, Cell. Mol. Neurobiol. 29 (2009) 1063–1071.
[21] H. Schägger, K. Pfeiffer, The ratio of oxidative phosphorylation complexes I–V in bovine heart mitochondria and the composition of respiratory chain supercomplexes, J. Biol. Chem. 276 (2001) 37861–37867.
[22] Z. Wang, Y. Iwasaki, L.F. Zhao, et al., Hormonal regulation of glycolytic enzyme gene and pyruvate dehydrogenase kinase/phosphatase gene transcription, Endocr. J. 56 (2009) 1019–1030.
[23] M. Haraguchi, T. Okubo, Y. Miyashita, et al., Snail regulates cell-matrix adhesion by regulation of the expression of integrins and basement membrane proteins, J. Biol. Chem. 283 (2008) 23514–23523.
[24] R.H. Xu, H. Pelicano, Y. Zhou, et al., Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia, Cancer Res. 65 (2005) 613–621.
[25] C.J. Ramnanan, K.B. Storey, Glucose-6-phosphate dehydrogenase regulation during hypometabolism, Biochem. Biophys. Res. Commun. 339 (2006) 7–16.
[26] J.J. Brière, J. Favier, V. El Ghouzzi, et al., Succinate dehydrogenase deficiency in human, Cell. Mol. Life Sci. 62 (2005) 2317–2324.
[27] F. Weinberg, R. Hamanaka, W.W. Wheaton, et al., Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity, Proc. Natl. Acad. Sci. USA 107 (2010) 8788–8793.
[28] C.R. Santos, A. Schulze, Lipid metabolism in cancer, ATP-Citrate Lyase: a key player in cancer metabolism, FEBS J. 279 (2012) 2610–2623.
[29] C. Hartmann, J. Meyer, J. Balss, et al., Type and frequency of *IDH1* and *IDH2* mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1010 diffuse gliomas, Acta Neuropathol. 118 (2009) 469–474.
[30] C. Muñoz-Pinedo, N. El Mjiyad, J.E. Ricci, Cancer metabolism: current perspectives and future directions, Cell Death Dis. 3 (2012) e248, http://dx.doi.org/10.1038/cddis.2011.123.