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PINK1-dependent and Parkin-independent mitophagy is involved in reprogramming of glycometabolism in pancreatic cancer cells

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ABSTRACT

Cancer cells rely on glycolysis to generate ATP for survival. However, inhibiting glycolysis is insufficient for the eradication of cancer cells because glycolysis-suppressed cells undergo metabolic reprogramming toward mitochondrial oxidative phosphorylation. We previously described that upon glycolytic suppression in pancreatic cancer cells, intracellular glycometabolism is shifted toward mitochondrial oxidative phosphorylation in an autophagy-dependent manner for cellular survival. Here, we hypothesized that mitophagy, which selectively degrades mitochondria via autophagy, is involved in mitochondrial activation under metabolic reprogramming. We revealed that glycolytic suppression notably increased mitochondrial membrane potential and mitophagy in a pancreatic cancer cell model (PANC-1). PTEN-induced kinase 1 (PINK1), a ubiquitin kinase that regulates mitophagy in healthy cells, regulated mitochondrial activation through mitophagy by glycolytic suppression. However, Parkin, a ubiquitin ligase regulated by PINK1 in healthy cells to induce mitophagy, was not involved in the PINK1-dependent mitophagy of the cancer glycometabolism. These results imply that cancer cells and healthy cells have different regulatory pieces of machinery for mitophagy, and inhibition of cancer-specific mechanisms may be a potential strategy for cancer therapy targeting metabolic reprogramming.

1. Introduction

Healthy mammalian cells produce ATP as an energy source for survival from both glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). In contrast, cancer cells mainly obtain ATP from glycolysis even when oxygen is available [1]. Glycolysis is less efficient but faster at generating ATP than OXPHOS. Therefore, increased glycolysis is effective for the rapid growth and proliferation of cancer cells. However, it has been suggested that mitochondrial function is not impaired in cancer cells [2], indicating that it should be investigated further.

Cancer therapies that target transporters and enzymes in the glycolytic pathway have been developed. For instance, the hexokinase inhibitor 2-deoxyglucose (2-DG) suppresses the proliferation of numerous cancer cells in vitro [3]. However, monotherapy with a hexokinase inhibitor has limited efficacy in vivo, whereas 2-DG increases the anti-tumor effect of adriamycin and paclitaxel, which decreases the volume of human tumors in vivo [4]. Considering this, we speculate that inhibiting glycolysis suppresses the growth of cancer cells but is insufficient for their eradication, while combination therapy using a glycolytic inhibitor with other anticancer drugs is more effective in suppressing tumors. The metabolic pathways upregulated in glycolysis-suppressed cancer cells have been extensively studied to understand why inhibiting glycolysis is insufficient in suppressing tumors. For instance, glycolytic suppression in human colon adenocarcinoma and mouse melanoma reprogrammed the cells to rely on OXPHOS and mitochondrial ATP production to maintain their viability [5]. However, the mechanisms that regulate the metabolic reprogramming of cancer cells and maintain their survival remain unclear.

We previously described that upon glycolytic suppression in multiple types of cancer cells, intracellular glycometabolism is reprogrammed toward mitochondrial OXPHOS in an autophagy-

Abbreviations: 2-DG, 2-deoxyglucose; ARIH1, Ariadne RBR E3 ubiquitin protein ligase 1; CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazone; BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3; CQ, chloroquine; OXPHOS, oxidative phosphorylation; PINK1, PTEN-induced kinase 1; ROS, reactive oxygen species.

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dependent manner to ensure their survival [6,7]. Notably, in pancreatic cancer cells, glycolytic suppression-dependent mitochondrial activation and ATP production have been observed [7]. Autophagy is a nonselective degradation process, which provides amino acids during nutrient starvation. Autophagy can also selectively degrade specific proteins and organelles, such as mitophagy, which is the selective degradation of mitochondria [8]. In particular, PTEN-induced kinase 1 (PINK1)-Parkin-dependent mitophagy has been well characterized [9]. PINK1 and Parkin are important drivers of mitophagy in neurons, and mutations in the genes that encode these proteins are one of the causes of Parkinson's disease [10]. PINK1, a serine/threonine kinase, is stabilized on depolarized mitochondria, and recruits Parkin, an E3 ubiquitin ligase, from the cytoplasm to the mitochondrial outer membrane [11]. These mitochondria are subsequently degraded to amino acids through various processes. The mechanism of PINK1-Parkin-dependent mitophagy is well characterized in healthy cells, and recent reports have shown that Parkin expression is downregulated in various types of cancer cells [12], implying that cancer cells have a different mechanism of mitophagy than healthy cells.

Mitophagy regulates the mitochondrial quantity and quality to meet metabolic demand [13]. When OXPHOS is activated, the small GTPase Rheb is recruited to the mitochondrial outer membrane, promoting mitophagy to maintain ATP production from mitochondria [14]. Furthermore, activation of AMP-activated protein kinase through energy stress activates mitophagy via phosphorylation of unc-51 like autophagy activating kinase 1/2, a mammalian protein kinase required for the induction of autophagy, thus controlling mitochondrial homeostasis and cell survival [15].

Taken together, we hypothesize that mitophagy regulates metabolic reprogramming and cell survival through the maintenance of mitochondrial quality and ATP production in cancer cells under glycolysis-suppressed conditions. In this study, to clarify the underlying mechanisms of mitophagy and those that regulate metabolic reprogramming, we investigated the involvement of mitophagy in metabolic reprogramming in OXPHOS by suppressing the glycolytic pathway in pancreatic cancer cells.

2. Materials and methods

2.1. Materials

Carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP; Sigma-Aldrich, St. Louis, MO, USA), and chloroquine (CQ; Sigma-Aldrich) were dissolved in ultrapure water or dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan).

2.2. Cell culture

PANC-1 cells (RIKEN Cell Bank, Tsukuba, Japan) and 293A cells (Thermo Fisher Scientific, Hanover Park, IL, USA) were maintained in RPMI-1640 medium (Nacalai Tesque) and high glucose Dulbecco's modified eagle's medium (Nacalai Tesque), respectively, supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and antibiotics (Nacalai Tesque). Cryopreserved primary human hepatocytes were purchased from Oriental Yeast (Tokyo, Japan). In each experiment, PANC-1 cells were cultured in normal (2 g/L) or low-glucose (0.2 g/L) RPMI-1640.

2.3. Evaluation of mitophagy

Mitophagy was evaluated using mtKeima-Red as previously described [7]. A schematic diagram explaining the process involving mtKeima-Red is provided in Fig. 1A. We calculated the ratio of excitation intensity at 555 nm (red) and 488 nm (green). To produce the adenovirus, nucleotide sequence encoding mtKeima-Red was subcloned into pAd/CMV/V5-DEST Gateway Vector (Thermo Fisher Scientific), and then the linearized vector was transfected into 293A cells according to the manufacturer's instructions. The virus was purified using Vivapure AdenoPACK 20 (Sartorius, Goettingen, Germany). Titers were confirmed using the Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountain View, CA, USA).

2.4. RNA isolation and quantitative real-time PCR

Total RNA was isolated using Sepasol-RNA I reagent (Nacalai Tesque) and reverse-transcribed using Rever Tra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The resulting cDNA was mixed with THUNDERBIRD SYBR qPCR Mix (Toyobo) and subjected to quantitative real-time PCR using a LightCycler Nano System (Roche, Mannheim, Germany) using the following primers: peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α forward, 5'-agcgaagatgaaagtgataaa-3' and reverse, 5'-aaagaagaacaagaaggagaca-3'; β-actin forward, 5'-TTCAACACCCCAGCCATGTACG-3' and reverse, 5'-GTGGTGGTGAAGCTGTAGCC-3'.

2.5. Citrate synthase assay

The enzymatic activity of citrate synthase was measured using a spectrophotometric method and normalized to each protein concentration as described previously [16].

2.6. MitoTracker staining

In total, 1 μM Hoechst 33342 (Thermo Fisher Scientific) and 100 nM MitoTracker Green FM (Thermo Fisher Scientific) were added to PANC-1 cells for the final 30 min of incubation. Cells were washed once and imaged immediately in serum/phenol red-free RPMI-1640 medium in a 37 °C and 5% CO_2 incubator using a Zeiss Axio Observer Z1 (Carl Zeiss, Oberkochen, Germany) inverted epifluorescence microscope at 63 × magnification.

2.7. Construction of PINK1-FLAG and the dominant-negative plasmid

For the preparation of the PINK1-FLAG expression vector, we used cDNA reverse transcribed from the total mRNA of PANC-1 cells, amplified the coding region of PINK1 with a carboxy-terminal 3 × FLAG tag, and inserted it into pcDNA3.1. PINK1 dominant-negative expression vector (PINK1 mutant)-FLAG was generated via site-directed mutagenesis of two bases of PINK1-FLAG (682 T→G, 1204 A→G).

2.8. Western blotting

Western blotting analysis was performed as previously described [7]. The membranes were blocked and probed with primary antibodies specific for PINK1, Parkin, LC3B, and GAPDH (Cell Signaling Technology Beverly, MA, USA).

2.9. Immunostaining

Cells were fixed in methanol, and immunostained with Monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich) and TOM20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as well as with a secondary antibody conjugated to an Alexa Fluor dye (Life Technologies). Nuclei were stained with TO-PRO-3 iodide (Life Technologies). Fluorescence was detected using a Carl Zeiss LSM700 laser scanning confocal microscope.

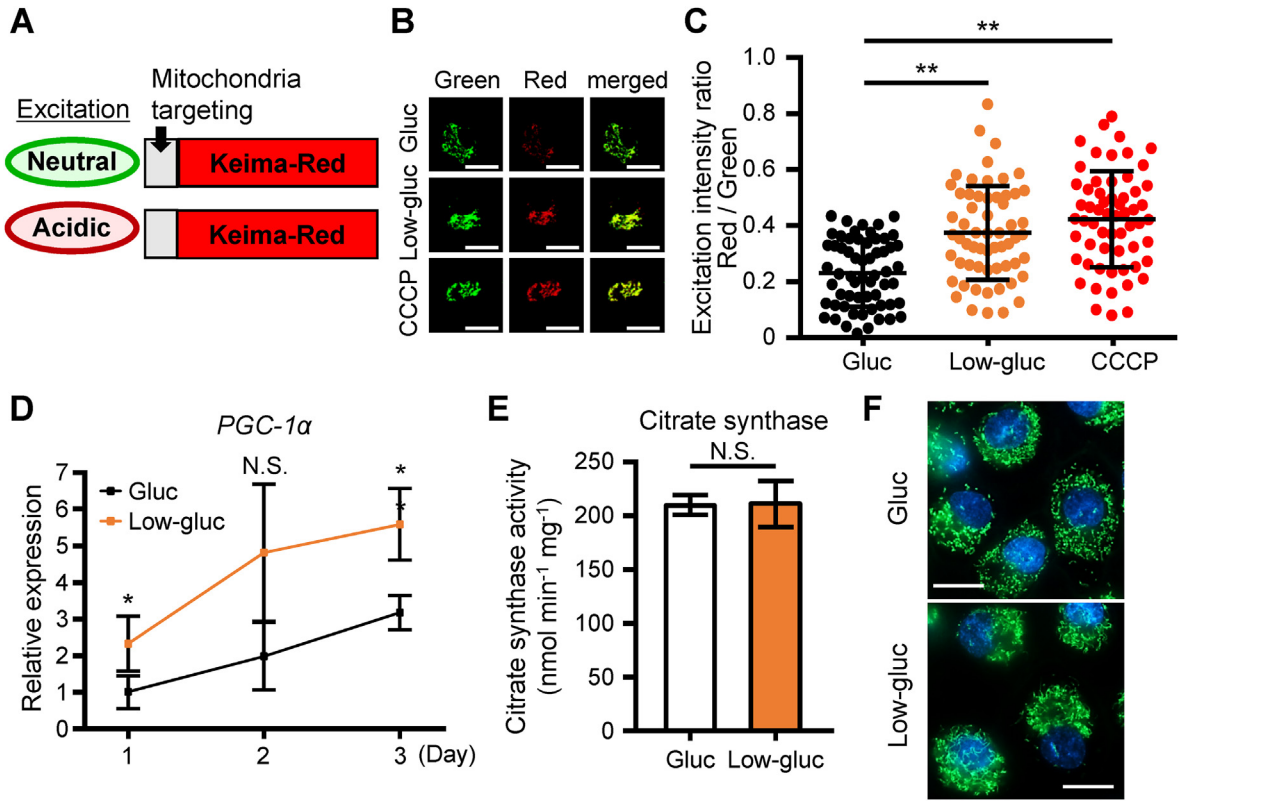

Fig. 1. Glycolytic suppression upregulates mitophagy. (A) Diagram showing excitation of mtKeima-Red at different pH values. (B, C) PANC-1 cells cultured in glucose (Gluc) or low-glucose (Low-gluc) medium for 72 h were transfected with mtKeima-Red plasmid, and then cultured for an additional 48 h. Positive control cells were treated with CCCP for the last 24 h. Cells were then observed using confocal microscopy. Scale bars, 20 μm **P < 0.01. (D) After PANC-1 cells were cultured in Gluc or Low-gluc medium for the indicated day(s), the mRNA expression of PGC-1α was quantified and normalized to that of β-actin. *P < 0.05. N.S., not significant. (E) PANC-1 cells were cultured in Gluc or Low-gluc medium for 72 h and lysed with hypotonic buffer. 40 μg of lysate was used for citrate synthase activity measurements. The absorbance at 412 nm was monitored for 3 min. N.S., not significant. (F) PANC-1 cells cultured in Gluc or Low-gluc medium for 72 h were stained with MitoTracker Green (green) and Hoechst 33342 (blue). Scale bars, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.10. RNA interference targeting PINK1 and Parkin

PANC-1 cells were transiently transfected with PINK1- and Parkin-targeting siRNAs (Japan Bio Services, Saitama, Japan) (siPINK1 and siParkin, respectively) using Lipofectamine 2000. The sequences of the two oligonucleotide strands of the siRNA duplex were as follows: siPINK1 (knockdown efficacy >95%), 5'-GCUG-GAGGAGUAUCUGAUATT-3'; siParkin (knockdown efficacy >88%), 5'-UGUAAAGAAGCGUACCAUTT-3'. MISSION® siRNA Universal Control #1 (Sigma-Aldrich) was used as the control siRNA (siControl).

2.11. Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated as previously described [7]. Mitochondrial membrane potential was measured by calculating the fluorescence signal intensity ratio of the polymer (red) to monomer (green) in each cell.

2.12. Statistical analyses

All data are expressed as the mean ± standard deviation of at least three independent experiments unless otherwise indicated. Statistical analyses were performed using the Student's *t*-test or analysis of variance followed by the Bonferroni test, where applicable.

3. Results

3.1. Glycolytic suppression upregulates mitophagy

As per our previous study [7], PANC-1 cells were used as a pancreatic cancer model in this study. Based on our previous findings [7], we used a low-glucose culture medium to suppress glycolysis. To visualize mitophagy, we constructed a mtKeima-Red vector encoding mitochondrial-targeted Keima-Red. Keima-Red has an excitation spectrum, which varies according to pH (Fig. 1A). When mitochondria are exposed to acidic conditions, similar to the condition in lysosomes, the spectrum of the excitation wavelength of the mitochondria-targeted fluorescent probe shifts from green to red. Here, a mitochondrial uncoupler CCCP, was used as a positive inducer of mitophagy. We observed that glycolytic suppression significantly increased red fluorescence signal intensity, which indicated the increased number of mitochondria in acidic conditions, suggesting that mitophagy was induced under the metabolic reprogramming to OXPHOS (Fig. 1B and C).

Next, to evaluate mitochondrial biogenesis, we quantified the mRNA level of PGC-1α, which is a transcriptional coactivator that induces mitochondrial biogenesis [17]. The expression of PGC-1α was higher in cells in a low-glucose medium than in those in a normal glucose medium (Fig. 1D). However, mitochondrial content, assessed by citrate synthase activity, was unchanged (Fig. 1E). Morphologically, microscopic analysis revealed that low-glucose cultured PANC-1 cells had more elongated (tubular) and less fragmented mitochondria than normal glucose cultured ones (Fig. 1F),

implying highly functional and active mitochondria. These results indicated that glycolytic suppression induced mitophagy and mitochondrial biogenesis to maintain the high function in PANC-1 cells.

3.2. PINK1 localizes on mitochondria due to glycolytic suppression

In mammalian cells such as neurons or hepatic cells, PINK1-dependent mitophagy is involved in mitochondrial quality control [10,18]. PINK1-dependent mitophagy is induced in a mitochondrial membrane potential-dependent manner and contributes to mitochondrial homeostasis. Therefore, first, we investigated the subcellular localization of PINK1. In healthy mitochondria, PINK1 is transported to the mitochondrial inner membrane, where it is cleaved by Presenilin-associated rhomboid-like protein and rapidly degraded in a proteasome-dependent pathway. However, in damaged mitochondria, the transport of PINK1 to the mitochondrial inner membrane and subsequent cleavage are suppressed; thus, PINK1 is stabilized on the mitochondrial outer membrane [11]. In the current study, we observed that PINK1 was co-localized on TOM20, a mitochondrial outer membrane marker, following glycolytic suppression (Fig. 2A). This result indicated that a part of PINK1 was stabilized on the damaged mitochondria, making it digestible by mitophagy.

Next, we evaluated the expression level of PINK1 following glycolytic suppression. Based on our previous results that mitochondrial membrane potential is increased following glycolytic suppression [7], we hypothesized that the majority of PINK1 would be cleaved and degraded after glycolytic suppression. The results showed that PINK1 expression was reduced following glycolytic suppression, and CCCP treatment increased PINK1 expression (Fig. 2B). In addition, to evaluate the correlation between mitochondrial quality and PINK1 expression, the cells were exposed to an autophagy inhibitor CQ, which also inhibits mitophagy. The decreased expression of PINK1 following glycolytic suppression was partially inhibited upon CQ treatment (Fig. 2B). These results indicated that PINK1 was recruited to the mitochondria of the glycolysis-suppressed PANC-1 cells and its expression might be regulated in a mitochondrial membrane potential-dependent manner.

3.3. Mitophagy and mitochondrial activation following glycolytic suppression are dependent on PINK1

To confirm whether mitophagy and mitochondrial activation following glycolytic suppression are dependent on PINK1, we knocked down endogenous PINK1 using siRNA. To evaluate mitophagy, mtKeima-Red adenovirus was used to infect PANC-1 cells. The results showed that mitophagy following glycolytic suppression was significantly inhibited when PINK1 expression was suppressed (Fig. 3A and B), suggesting that PINK1 was involved in the mitophagy of glycolysis-suppressed PANC-1 cells. Next, to investigate mitochondrial function, we evaluated mitochondrial membrane potential using JC-1 staining. The accumulation of the polymeric form of JC-1 indicated a high uptake of the stain into the mitochondria, corresponding to a high mitochondrial membrane potential [19]. As expected, following control siRNA transfection, glycolytic suppression led to an increase in the ratio of the polymeric (red) to monomeric (green) form of the JC-1 stain, whereas the ratio decreased when PINK1 was knocked down (Fig. 3C and D). These findings implied that PINK1 positively regulated mitophagy induced by glycolytic suppression, and PINK1-dependent mitophagy was essential for maintaining mitochondrial membrane potential.

3.4. Parkin is not involved in the activation of mitophagy and mitochondrial activity under glycolytic suppression

It has been reported that autophosphorylation of PINK1 at Ser228 and Ser402 is required for Parkin recruitment on the mitochondrial outer membrane [20]. Thus, we transfected a dominant-negative construct of wildtype PINK1, in which the autophosphorylation sites of the two serine residues were replaced with alanine (PINK1 mutant). Remarkable mutant PINK1 expression was observed using the PINK1 antibody (data not shown), whereas induced mitophagy was not suppressed following the introduction of the PINK1 mutant (Fig. 4A). This result indicated that PINK1 autophosphorylation, which was necessary for the activation of Parkin, was not involved in mitophagy induced by glycolytic suppression. In addition, Parkin expression in PANC-1 cells was rarely observed compared to that in healthy cells (human hepatocytes [18]) (Fig. 4B).

Furthermore, knocking down Parkin using siRNA confirmed that Parkin was not essential for mitophagy in the glycometabolism of pancreatic cancer cells. Parkin-knockdown cells were infected with mtKeima-Red adenovirus and their mitophagy was evaluated. As opposed to the results obtained after knocking down PINK1, mitophagy was still induced when Parkin was knocked down in glycolysis-suppressed PANC-1 cells (Fig. 4C). Similarly, mitochondrial membrane potential was not affected by Parkin knockdown (Fig. 4D). These findings suggest that Parkin is not involved in mitophagy induced by glycolytic suppression in PANC-1 cells.

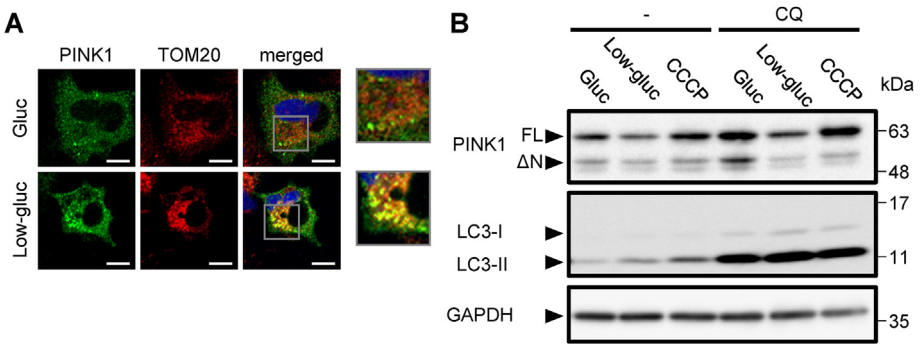

Fig. 2. PINK1 is localized on mitochondria following glycolytic suppression. (A) PANC-1 cells cultured in glucose (Gluc) or low-glucose (Low-gluc) medium for 72 h were transfected with PINK1-FLAG and cultured for another 72 h. Scale bars, 10 μm. (B) PANC-1 cells cultured in Gluc or Low-gluc medium for 72 h were transfected with PINK1-FLAG and cultured for another 72 h. Cells were treated with or without CQ for the last 48 h. FL represents the full-length PINK1; ΔN represents its cleaved form.

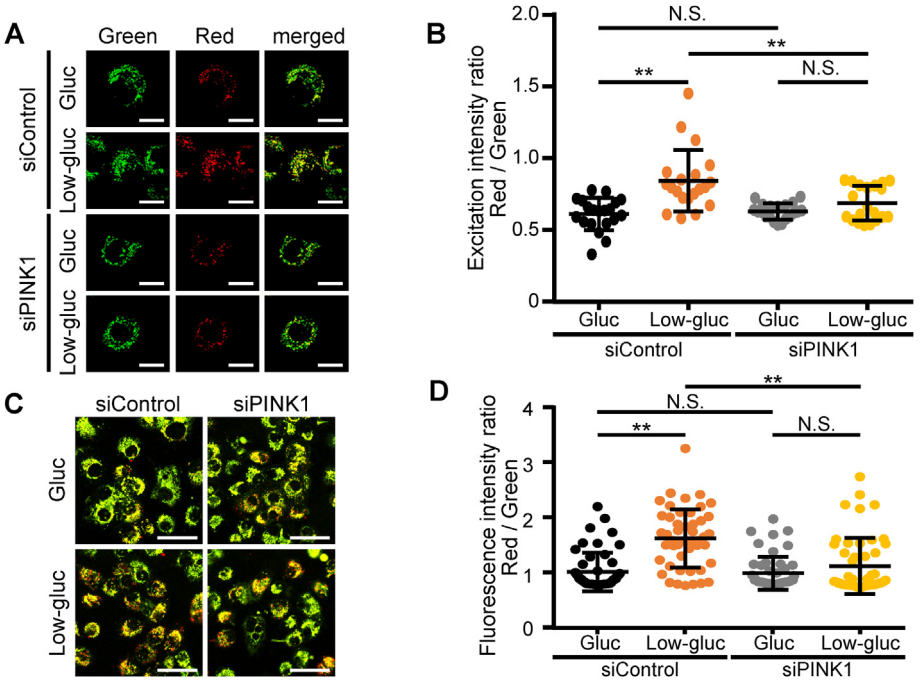

Fig. 3. Mitophagy and mitochondrial activation following glycolytic suppression are dependent on PINK1. (A, B) PANC-1 cells transfected with siControl or siPINK1 were cultured in glucose (Gluc) or low-glucose (Low-gluc) medium for 48 h, then infected with mtKeima-Red adenovirus for another 48 h and observed using confocal microscopy. Scale bars, 20 μm **P < 0.01. N.S., not significant. (C, D) PANC-1 cells transfected with siControl or siPINK1 were cultured in Gluc or Low-gluc medium for 96 h, then stained with JC-1 and observed using confocal microscopy. Representative images show merged images of polymeric (red) and monomeric (green) JC-1. Scale bars, 50 μm **P < 0.01. N.S., not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

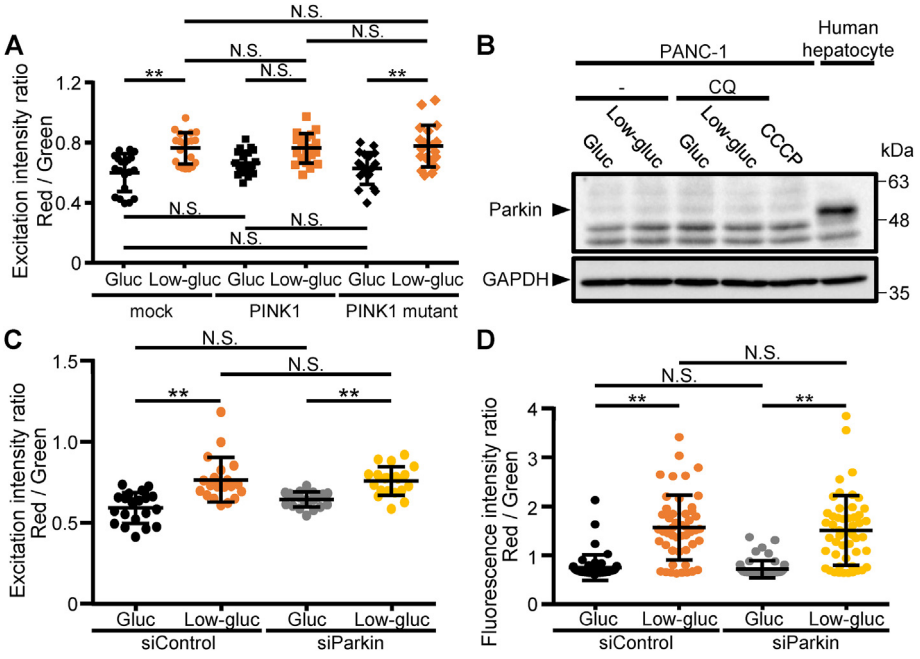

Fig. 4. Parkin is not involved in the activation of mitophagy and mitochondrial function under glycolytic suppression. (A) PANC-1 cells cultured in glucose (Gluc) or low-glucose (Low-gluc) medium for 72 h were transfected with LacZ (mock), PINK1-FLAG (PINK1), or dominant-negative mutant (PINK1 mutant), and cultured for another 24 h, then infected with mtKeima-Red adenovirus for another 48 h and observed using confocal microscopy. Scale bars, 20 μm **P < 0.01. N.S., not significant. (B) PANC-1 cells cultured in Gluc or Low-gluc medium for 72 h. (C) PANC-1 cells transfected with siControl or siParkin were cultured in Gluc or Low-gluc medium for 48 h, then treated with mtKeima-Red adenovirus for another 48 h and observed using confocal microscopy. Scale bars, 20 μm **P < 0.01. N.S., not significant. (D) PANC-1 cells transfected with siControl or siParkin were cultured in Gluc or Low-gluc medium for 96 h, then stained with JC-1 and observed using confocal microscopy. Scale bars, 50 μm **P < 0.01. N.S., not significant.

4. Discussion

We revealed that glycolytic suppression induces mitophagy in PANC-1 pancreatic cancer cells. Here, when PINK1 was knocked down, mitophagy was inhibited and mitochondrial membrane potential was reduced, indicating that PINK1 plays an essential role

in mitophagy in the glycolysis-suppressed PANC-1 cells. On the contrary, when the Parkin was knocked down, which is a key regulator of PINK1-dependent mitophagy in healthy cells, mitophagy was not affected. These results suggest that different regulatory types of machinery control mitophagy in pancreatic cancer and healthy cells.

Parkin expression is downregulated in many cancer cells [12], and Parkin is unlikely to be involved in mitophagy in cancer cells. A recent report has shown that Ariadne RBR E3 ubiquitin-protein ligase 1 (ARIH1), an E3 ligase in the RBR family along with Parkin, controls the mitophagy of damaged mitochondria in a PINK1-dependent manner and promotes therapeutic resistance [21]. This study showed that PINK1 autophosphorylation is not necessary for mitophagy in PANC-1 cells, while ARIH1 is phosphorylated at Ser/Thr residue through the induction of CCCP-mediated mitophagy [21]. Another study showed that PINK1 and BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), a mitophagy receptor, interact with each other [22]. BNIP3, which is localized on the mitochondrial outer membrane, promotes the recruitment of PINK1 to the mitochondrial outer membrane, leading to PINK1-Parkin-dependent mitophagy [22]. BNIP3 induces mitophagy when it is recognized by LC3. Thus, we can speculate that mitophagy is induced by the accumulation of BNIP3 on the mitochondrial outer membrane through direct interaction with PINK1, without PINK1 autophosphorylation, and subsequent promotion of polyubiquitination. Further studies are required to completely understand mitophagy induced by glycolytic suppression in cancer cells, as well as the roles of mitophagy receptors and the involvement of polyubiquitination. Inhibiting specific factors in cancer cells should improve the efficacy and safety of anticancer drug treatments.

A recent study has shown that mitophagy is involved in resistance to anticancer drugs. For instance, doxorubicin produces mitochondrial reactive oxygen species (ROS) via its firm bond with mitochondrial OXPHOS complex-I and inhibits topoisomerase [23]. Furthermore, to prevent mitochondrial damage, cancer stem cells can induce BNIP3-like (BNIP3L)-dependent mitophagy and remove the damaged mitochondria to reduce mitochondrial ROS levels, leading to therapeutic resistance [24]. Another study has shown that depletion of the FUN14 domain-containing 1 protein inhibits the proliferation of cancer cells, induces apoptosis, and enhances sensitivity to cisplatin and ionizing radiation in cervical cancer cells [25]. Both reports concluded that the cancer cells survive, as they overcome the damage due to therapy, by removing damaged mitochondria via mitophagy. We suggest that the combination of anticancer drug therapy and suppression of mitophagy may improve the efficacy of cancer treatment.

In contrast, as PINK1 and Parkin are important for mitophagy in neurons [10,26], broad suppression of mitophagy may lead to severe side effects in healthy tissues. Therefore, effective treatments should aim to target cancer cell-specific mechanisms of mitophagy to reduce the side effects in healthy tissues or organs. This study shows that PINK1-dependent mitophagy is involved in the reprogramming of glycometabolism via the activation of mitochondria in pancreatic cancer cells. Elucidation of cancer-specific mechanisms of mitophagy and inhibition will provide a new strategy of cancer therapy targeting metabolic reprogramming.

Declaration of competing interest

The authors have no competing interests to declare.

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