

Functional linkage between mitochondrial electron transport, glycolysis, and AMP-activated protein kinase signaling underlying cancer cell survival

Momoko Uchida

Showa Medical University Graduate School of Pharmacy

Masato Higugrashi

Showa Medical University Graduate School of Pharmacy

Hidetsugu Nakagawa

Showa Medical University Graduate School of Pharmacy

Fumihiro Ishikawa

Showa Medical University

Kazunori Mori

Showa Medical University Graduate School of Pharmacy

Motoko Shibamura

`smotoko@pharm.showa-u.ac.jp`

Showa Medical University Graduate School of Pharmacy

Research Article

Keywords: respiratory chain complex, mitochondrial electron transport, glycolysis, AMP-activated protein kinase

Posted Date: January 12th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8003013/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Molecular and Cellular Biochemistry on October 15th, 2026. See the published version at <https://doi.org/10.1007/s11010-026-05770-0>.

Abstract

Mitochondrial metabolism has emerged as a potential target for cancer therapy because of its essential role in cell proliferation and survival beyond ATP production. However, translation into clinical practice is challenging owing to several limitations, including the difficulty in distinguishing cancerous mitochondria from noncancerous mitochondria. The heterogeneity of cancerous mitochondria further complicates this effort. Herein, we focused on cancerous mitochondria exhibiting low DNA/RNA levels and respiratory function compared with those exhibited by normal cells and determined the effect of low mitochondrial respiratory activity on cancer cells. Interestingly, mitochondria low-type (mt-Low) cancer cells derived from hepatocellular carcinoma were selectively and highly sensitive to electron transport chain (ETC) inhibition. Specifically, cells died under the treatment with the ETC inhibitors, rotenone and antimycin A, at lower doses compared with cells exhibiting normal respiratory activity (mt-Normal), although ATP levels were sustained in both types of cells under these conditions. In mt-Normal cells, glycolysis increased and AMP-activated protein kinase was activated upon ETC inhibition, which critically contributed toward cell survival. However, mt-Low cells could not induce these responses, which resulted in cell death. Based on these results, therapeutics targeting respiratory function have emerged as promising precision medicines for mt-Low cancers. Similar to conventional ETC inhibitors, small interfering RNAs targeting core subunits of respiratory complex I or III were effective in inhibiting cell proliferation (complex I and III) and survival (complex I) of mt-Low cancer cells, encouraging pioneering anticancer approaches using the next-generation modality.

Introduction

Studies on cancer metabolism, which have been inspired by Otto Warburg's discovery of aerobic glycolysis, have substantially progressed over the several decades to further elucidate the metabolic phenotype of cancer cells [1, 2]. The molecular mechanisms explaining how cancer cells reprogram metabolism to cope with the increased anabolic demands for incessant and durable cell proliferation are more understandable [1–5]. Accordingly, our view on mitochondrial metabolism in cancer cells has also evolved from the original concept that misinterpreted aerobic glycolysis as a consequence of irreversible mitochondrial respiratory defects in cancer cells. Mitochondria in most tumors are intact and actively performing oxidative phosphorylation (OXPHOS) [1, 6, 7]. Furthermore, mitochondrial respiration is indispensable for cell proliferation. Thus, cell proliferation is impeded by pharmacological or genetic inhibition of electron transport chain (ETC), specifically, respiratory complex I and III [8–12].

From a conventional perspectives, ETC activity likely affects cell proliferation through ATP generation and/or reactive oxygen species (ROS) release; however, because of the diverse roles of ETC beyond ATP generation, including metabolite production through TCA cycle flux and pyrimidine synthesis pathway for macromolecule biosynthesis, alternatives are also possible. Recent studies have highlighted the importance of ETC in the production of electron acceptors, which fulfill a specific metabolic requirement to support cell proliferation. For example, de novo synthesis of aspartate, a key biosynthetic precursor of proteins, and purine and pyrimidine nucleotides, is critically dependent on NAD⁺ regeneration by ETC

complex I [10, 13]. Recently, we found that NAD⁺ regenerated by complex I regulates p21^{Cip1} expression and cancer cell proliferation through SIRT activity [14]. Ubiquinol oxidation to ubiquinone by ETC complex III is also required for tumor growth [9].

Evidence supporting the role for ETC in cell proliferation has led to attempts that target ETC for cancer therapy; however, the translation of various ETC inhibitors into clinical practice has been challenging. Thus far, clinical trials have failed because of setbacks, such as dose-limiting toxicity, lack of potency and specificity, even when preclinical results were promising [15–19]. These failures have emphasized the importance of discriminating between noncancerous and cancerous mitochondria, or identifying and focusing on specific phenotypes of cancerous mitochondria and targeting their distinct vulnerabilities for the development of safe and effective mitochondrial-based therapies.

According to a recent mitochondrial phenotyping study, the expression/function of the OXPHOS complex is consistently lower in cancer tissues compared with matched normal tissues across several cancer types [20]. Genome-wide analyses of mitochondrial DNA (mtDNA) and mitochondrial RNA (mtRNA) showed a significant decrease in mtDNA copy number on average in 7 of 15 cancer types and a concomitant decrease in mtRNA levels [21]. These results suggest that the sizeable proportion of cancerous mitochondria exhibit a functionally lower mtDNA/RNA/OXPHOS phenotype compared with their normal counterparts in numerous types of cancers. This difference can be potentially exploited to develop mitochondrial medicines that can differentiate between cancerous and normal mitochondria as well as specifically target cancer cells with lower mitochondrial activity.

Our recent study of hepatocellular carcinoma (HCC) cell lines revealed that over half (4 of 7) of them exhibited a mt-Low phenotype with lower mtDNA/RNA and membrane potential compared with non-malignant hepatocytes [22], which agreed with the previous studies. Similarly, 61% (17 of 28) of in vivo HCC tissues were mt-Low cases [22]. In this study, we characterized the metabolic traits of these HCC cell lines, especially focusing on mt-Low cells. Interestingly, mt-Low HCC cells are highly and selectively sensitive to ETC inhibition. Mechanistically, the sensitivity of mt-Low cancer cells to ETC inhibition results from the loss of a sufficient functional linkage between ETC, glycolysis, and AMP-activated protein kinase (AMPK) signaling.

Materials and methods

Materials

Antimycin A (AMA) and rotenone (Rot) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and Sigma-Aldrich, Inc. (St Louis, MO, USA), respectively. Staurosporine and 2-Deoxy-D-glucose were purchased from Wako Pure Chemical Corporation (Osaka, Japan). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and oligomycin A (OligoA) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Compound C and metformin were purchased from Merck KGaA

(Darmstadt, Germany) and Enzo Life Sciences, Inc. (Farmingdale, NY, USA), respectively. AICAR and A-769622 (A-7) were purchased from FUJIFILM and Adooq Bioscience LLC (Irvine, CA, USA), respectively.

Cell culture

The cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) or the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and maintained in their respective media for subculture. For the experiments, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Shimadzu Diagnostics Corp., Tokyo, Japan) supplemented with 10% fetal bovine serum to standardize the nutrient supply.

Cell proliferation and viability assay

The cells were seeded at a density of 5×10^4 or 1×10^5 cells in 12-well plate. Total and viable cell numbers were counted with a Countess II Automated Cell Counter (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cell viability was assessed by performing trypan blue staining.

Measurements of lactate and ATP levels

Cells (1.5×10^5 or 2×10^5) were seeded into a single well of a 12-well plate. Lactate released into the culture medium was measured using the Lactate Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The culture supernatant was collected and diluted 10-fold with water and analyzed. The working solution was incubated with the supernatant for 30 min at 37°C, and the absorbance was measured at 450 nm using a Varioskan Lux (Thermo Fisher Scientific Inc.) microplate reader. The remaining cells were lysed, and intracellular ATP levels were measured using an ATP determination kit (Thermo Fisher Scientific, Inc.) as described previously [23].

Measurement of glucose consumption

Cells (0.5×10^5 or 1×10^5) were plated in a single well of a 12-well plate. The following day, the cells were treated with or without reagents after aliquots of the supernatant were collected (sup 0 h). After 12 h, the supernatants (i.e., medium; sup 12 h) were collected from each well, and the cells were counted. Glucose concentrations in the sup (0 h) and (12 h) were measured using the Glucose Assay Kit-WST (Dojindo Laboratories) based on the manufacturer's instructions, and glucose consumption ($\mu\text{mol/h}$) was calculated from the difference in glucose levels between sup (0 h) and sup (12 h), normalized with 10^6 cells.

LDH release assay

Lactate dehydrogenase (LDH) activity retained in cells and released into the culture media was determined using the LDH-Cytotoxic Test wako (Wako Pure Chemical Industries, Ltd.) according to manufacturer's instructions. Briefly, 0.5×10^5 or 1×10^5 cells were seeded into a single well of a 12-well plate and treated with the reagent the following day. After 24–72 h, the medium was centrifuged, and the resulting supernatant and pellets were collected. The remaining cells in each well were solubilized with phosphate buffer solution (PBS) containing 0.2% Tween-20 together with the cells pelleted from the medium. Equivalent aliquots of the supernatant and cell lysate were incubated with the coloring solution after clearing by centrifugation, and the absorbance at 570 nm was measured using a Varioskan Lux (Thermo Fisher Scientific, Inc.). Fresh medium and PBS/0.2% Tween were used as a control for the supernatants and cell lysates, respectively. LDH release was calculated as the ratio of LDH activity in the medium to the total LDH activity.

Western blotting

Western blotting was performed as previously described [22]. The primary antibodies used are listed in Table S1. The specific bands were quantified using ImageJ software (version 1.53 k, National Institutes of Health, Bethesda, MD, USA) to compare densities.

RNA interference

Small interfering RNA (siRNA) (FlexiTube siRNA) was purchased from Qiagen (Venlo, the Netherlands). Transfection of the siRNA was achieved using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Inc.) as described previously [14].

Statistical analysis

All results are reported as the mean $\pm$ standard deviation with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Comparisons between two groups were analyzed using a two-tailed Student's *t*-test. For multiple comparisons, the data were analyzed using one-way ANOVA with Bonferroni's post hoc correction to identify datasets that differed from the control data. A *p*-value of < 0.05 was considered significant.

Results

Selective and high sensitivity of mt-Low HCC cells to ETC inhibition

We previously categorized seven HCC cell lines into an mt-Normal or mt-Low phenotype based on normal or low mtDNA/RNA content and membrane potential compared with primary hepatocytes [22]. Oxygen consumption rates confirmed that the respiratory activities were higher and lower in mt-Normal cells and mt-Low cells, respectively (Fig. S1). We further characterized the metabolic properties of the

two groups (mt-Normal cells; HepG2, JHH-1, and JHH-6, mt-Low cells; HLF, HuH-7, JHH-2, and JHH-4). Specifically, we disrupted respiratory activity using the established ETC inhibitors, AMA and Rot, which inhibit complexes III and I, respectively, and observed the response of the mt-Normal and mt-Low cells. In the experiments, all cell lines were cultured in DMEM throughout the study to standardize the nutrient supply.

The mt-Normal cells were largely tolerable to inhibitor treatment within the concentration range used in the experiments (IC₅₀ of AMA and Rot are 16 nM and 56 nM, respectively [24]). However, the inhibitors, especially AMA, were remarkably cytotoxic to the mt-Low cells, except JHH-2, at lower doses, where the majority of the mt-Normal cells were alive (Fig. 1A). Therefore, the sensitivity to the ETC inhibition was markedly different between the two groups. However, the sensitivities to FCCP and OligA, which interfere with ATP synthesis at the final step of OXPHOS, were indistinguishable between the two groups and independent of respiratory levels of the cells (Fig. 1B). Moreover, they were comparably tolerable to FCCP and OligA treatment over the concentration range used in this study (IC₅₀ of FCCP and OligA; 4.8–8.5 μ M and 0.21–3.8 μ M, respectively [25, 26]). Sensitivity to staurosporine (IC₅₀; 0.3 μ M [27]), a representative toxic substance, was also independent of the groups (Fig. 1B). Collectively, the results indicated the selective and high sensitivity of mt-Low HCC cells to ETC inhibition.

The sensitivity to ETC inhibition is dependent upon a variety of cellular processes that sustain energy homeostasis. Because a major role of ETC is to support ATP synthesis through OXPHOS, the ATP synthetic potential of cells is the most likely determinant of sensitivity to ETC inhibition. However, this was unlikely because ATP levels were sustained for several hours following AMA treatment in mt-Normal and mt-Low cells (Fig. S2A), possibly because of metabolic plasticity as described below. Moreover, in contrast to the intolerance toward ETC inhibition, the mt-Low and mt-Normal cells were tolerant to direct ATP synthesis inhibition by FCCP and OligA (Fig. 1B), suggesting that they are equally adaptable for the inhibition of OXPHOS-driven ATP synthesis.

Cellular antioxidant capacity is likely an alternative determinant of sensitivity to ETC inhibition. The inhibition of ETC by Rot and AMA accompanies ROS production [28, 29]. Accordingly, cells require a sufficient antioxidant defense during ETC inhibition. Therefore, we quantified total glutathione (GSH) levels as a major antioxidant with the ratio of GSH/glutathione disulfide. Both parameters varied among the HCC cell lines at steady-state levels and during AMA treatment, but appeared unrelated to the sensitivity to ETC inhibition or mt-Normal/Low status (Fig. S2B-C).

Among cellular signaling, PI3K/AKT pathway plays an important role in cell survival and is frequently implicated in resistance to toxic substances [30, 31]. This may be relevant to atypical resistant phenotypes of JHH-2 to these inhibitors (Fig. 1). As shown in Fig. S2D, AKT was distinctly activated in JHH-2. In conclusion, the sensitivity of the mt-Low HCC cells to ETC inhibition was independent of ATP synthetic potential, antioxidant defense capacity, and PI3K/AKT survival signaling, except JHH-2.

Differences in glycolysis availability between mt-Normal and mt-Low HCC cells

To gain insight into determinants of sensitivity to ETC inhibition in the mt-Low cells, we compared fundamental metabolic characteristics, glycolytic/mitochondrial ATP synthetic capacity and glycolysis availability, between mt-Normal and mt-Low HCC cells. We first evaluated glycolytic/mitochondrial ATP synthetic capacity by disrupting the two synthetic pathways using 2-deoxy-D-glucose (2-DG) or OligA, which inhibit OXPHOS and glycolytic ATP synthesis, respectively. Consistent with the above observation of sustained ATP levels under ETC inhibition (Fig. S2A), the results indicated that the cancer cells in both groups are highly metabolically plastic and flexibly generate ATP through glycolysis or OXPHOS in response to the availability of these two pathways (Fig. 2A). Alternatively, a surplus quantity of ATP may be normally reserved in cells [32].

Conversely, glycolysis availabilities, which are evaluated by measuring lactate release from cells in the presence of OligA, were different between the two groups. During OligA treatment, in which OXPHOS is inhibited, cells generally enhance glycolysis by mobilizing their reserve capacity for glycolysis to compensate for OXPHOS, releasing more lactate into the medium. Accordingly, the amount of released lactate in the presence of OligA reflects a maximum level of glycolysis availability of cells, and the difference from the control conditions without OligA treatment corresponds to the reserve capacity for glycolysis. In this study, lactate release was significantly increased during OligA treatment in mt-Normal HepG2 and JHH-1 cells (Fig. 2B); however, the release was only marginally increased in mt-Low cells, such as HLF and JHH-4, under these conditions, suggesting that mt-Low cancer cells lack a sufficient reserve capacity for glycolysis or are mechanistically defective in upregulating glycolysis. In summary, the mt-Normal and mt-Low cells were similarly able to synthesize ATP by glycolytic and mitochondrial pathways, but differed in the upregulation of glycolysis during ETC inhibition.

Upregulation of glycolysis is important for cell survival during limited ETC activity

Based on the above results, the difference between mt-Normal and mt-Low cells with regard to sensitivity for ETC inhibition may be attributed to their difference in glycolysis availability. Consistent with this idea, the mt-Normal cells, which are resistant to ETC inhibition, exhibited enhanced glycolysis during ETC inhibition with AMA (Fig. 3A), concomitant with increased glucose consumption (Fig. 3B). However, glycolysis and glucose consumption were only slightly enhanced in mt-Low cells under these conditions (Fig. 3A, B).

These results suggested that if glycolysis were sufficiently upregulated, mt-Low cells could resist ETC inhibition, similar to mt-Normal cells. In fact, the mt-Low cells became tolerant of AMA treatment (Fig. 3C; viability) when glycolysis was enhanced following glucose supplementation into the medium (Fig. 3C; lactate assay). Conversely, glycolysis inhibition with 2-DG markedly decreased cell survival (Fig. 3D). These results highlight the importance of upregulating glycolysis for cell survival during limited ETC activity.

In addition to glucose, supplementation with other biosynthetic precursors or intermediates was also effective at mitigating cell death induced by ETC inhibition. Pyruvate and uridine significantly decreased

LDH release and cell death at supra-physiological concentrations during ETC inhibition by Rot treatment (Fig. 3E). When ETC was inhibited by AMA, cell death was partially relieved by pyruvate supplementation (Fig. 3F). These results suggest that metabolic pathways driven by glucose and other metabolites substitute for ETC function, which is important for cell survival.

Activation of AMP-activated protein kinase underlies cell survival during limited ETC activity

AMPK is an energy-sensing kinase, which is activated by various metabolic stresses that lower cellular energy levels, and has various roles in cellular energy homeostasis [33]. In the HCC cell lines, an active form of AMPK phosphorylated at threonine 172 was observed at various levels during ETC inhibition (Fig. 4A-B). Interestingly, the activation levels of AMPK correlated with the resistance of the HCC cell lines to the inhibitors. For example, the levels were higher in JHH-1, HepG2, and JHH-4 compared with that of HLF during Rot treatment (Fig. 4A), which mirrored their viability under these conditions (Fig. 1A). During AMA treatment, JHH-1 and JHH-2, both of which were remarkably resistant to the treatment, exhibited higher activation levels compared with the others (Fig. 4B). In JHH-1, robust and sustained AMPK activation was consistently observed in response to AMA, whereas AMPK activation was moderate in HuH-7 and JHH-4 cells, even when AMA was treated with higher doses for longer periods (Fig. 4C-D). These correlations between the activation of AMPK and cell viability suggest that AMPK underlies cell survival during ETC inhibition.

To substantiate the role of AMPK in cell survival directly, we inhibited AMPK by an inhibitor and siRNA or activated AMPK with activators, and observed their effects on cell fates. The treatment with compound C, a well-known AMPK inhibitor, reversed the resistance phenotype of the mt-Normal JHH-1 and HepG2 cells during AMA treatment (Fig. 5A). Similarly, cell survival was reduced by knockdown of AMPK with siRNA (Fig. 5B). Conversely, the AMPK activator A-7, which potently activated AMPK among all the activators tested in the study, significantly promoted the resistance phenotype of the cells (Fig. 5C). These results highlight the important role of AMPK activation in cell survival during ETC inhibition. Collectively, it was most likely that glycolysis upregulation and AMPK activation were the determinants of sensitivity to ETC inhibition.

Effects of siRNA targeting the subunits of respiratory complex I and III on cell survival and proliferation

Clinically, the selective and high sensitivity or vulnerability of mt-Low cells to ETC inhibition may represent a therapeutic target for mt-Low cancers. As substitutes for small molecular inhibitors, including Rot, AMA, and others, for which clinical trials were discontinued, we evaluated the efficacy of siRNAs targeting core subunits of respiratory complex I and III to inhibit cell survival and/or the proliferation of mt-Low HCC cells. We knocked down *NDUFV1* (NADH: ubiquinone oxidoreductase core subunit V1) and *NDUFS1* (NADH: ubiquinone oxidoreductase core subunit S1) of complex I, and *URCRFS1* (ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1) of complex III. *NDUFV1* and *NDUFS1* constitute the electron input module, which accepts electrons from NADH through flavin mononucleotide and sequentially transfers them along a chain of iron-sulfur (FeS) clusters in complex I.

UQCRCF1 is a subunit that receives an electron from ubiquinol and transfers it to cytochrome c_1 , thereby playing an essential enzymatic role in complex III.

All three siRNAs downregulated the expression of the target gene (Fig. S3) and showed cytostatic effects on mt-Low JHH-4 and HLF cells as evidenced by decreased cell numbers (Fig. 6A). Moreover, siRNAs for *NDUFV1* and *NDUFS1* exhibited cytotoxic effects and decreased cell viability. These results suggest that siRNAs targeting the core subunits of complex I and III, in particular those of complex I, are promising therapeutics for the mt-Low-type cancers.

Discussion

For the successful development of mitochondrial-targeted therapeutics, it is necessary to characterize cancerous mitochondria in detail, identify distinctive and druggable features or vulnerabilities, and target them specifically. Based on previous studies, cancerous mitochondria exhibit heterogeneous mtDNA/RNA content and OXPHOS function [20, 21, 34, 35]. Toward the goal of developing mitochondrial precision medicine, we focused on the mt-Low-type of cancer, which occurs in approximately half of all cancer types [21, 22]. We found that the mt-Low-type of cancer cells are distinctively vulnerable to the ETC inhibition. Previously, the preferential effect of OXPHOS inhibition on mitochondria was a concern with respect to normal OXPHOS activity in healthy organs, particularly those with a high reliance on OXPHOS, rather than tumor mitochondrial flux [20]. Our results indicate that it is possible to preferentially compromise tumor mitochondria flux while sparing healthy cells by targeting ETC functions in the case of mt-Low-type cancers.

Mechanistically, mt-Low cancer cells are unable to upregulate glycolysis efficiently during ETC inhibition, resulting in cell death. The mt-Low cells may be forced to leverage glycolytic flux at nearly maximum levels because of their intrinsically low respiratory activity; therefore, it is likely that they cannot afford to upregulate glycolysis in response to ETC inhibition. By supplementing the culture medium with glucose, glycolysis was upregulated in mt-Low cells, and cell death was mitigated (Fig. 3D). This highlights the importance of glycolysis upregulation for cell survival under ETC-limited conditions.

Supplementation with pyruvate also ameliorated cell death induced by the ETC inhibition. Uridine was also effective in rescuing Rot-treated cells (Fig. 3E). In AMA-treated cells, only pyruvate was effective (Fig. 3F). The difference in effectiveness of uridine in relieving cell death induced by Rot or AMA may be attributed to the difference in TCA metabolite abundance, which was affected by Rot and AMA treatment as previously reported [10]. According to a recent study [36], OXPHOS dysfunction resulted in higher lactate/lower pyruvate abundance, lower levels of TCA intermediates and aspartic acid, and increased glycerol-3-phosphate levels. Interestingly, these metabolite imbalances were normalized by combined supplementation of pyruvate and uridine. Taken together, these beneficial effects of nutrient supplementation on cell viability during ETC inhibition suggest the compensatory network between ETC functions, glycolysis, and pyruvate/uridine metabolism. Therefore, a functional linkage has emerged

between glycolysis, pyruvate/uridine metabolic pathways, and respiratory capacities, which is important as a safeguard for cell survival during ETC dysfunction.

AMPK coordinates metabolic pathways and maintains energy homeostasis through the phosphorylation of various substrates [33]. It also promotes mitochondrial biogenesis and dynamics [37]. By performing these roles, AMPK contributes to cell survival under low-energy conditions and mitochondrial dysfunction. In this study, the mt-Normal cells activated the kinase in response to ETC inhibition as previously reported (Fig. 4A, B) [33, 37–39]. Conversely, the mt-Low HCC cells were unable to activate AMPK. A series of experiments using the inhibitor, the activators, and siRNA support the role for AMPK in cell survival during ETC inhibition.

Although AMP is the most common activator of AMPK, a non-canonical mechanism independent of AMP [33] is suggested to be predominantly involved in activating AMPK in the ETC-inhibited HCC cells, because the ATP levels were unchanged (Fig. S2A) and among the activators tested, A-7 was the most prominent activator in the HCC cells. Unlike AICAR and metformin, A-7 activates AMPK by a different mechanism than AMP [40, 41]. Recently, glycolysis was found to be a key regulator of AMPK activation [42]. In this study, a correlation was observed between the ability of cells to activate AMPK and upregulate glycolysis, suggesting that the glycolytic pathway regulates its activation. However, in another report, AMPK activation was suggested to upregulate glycolysis [43, 44]. Thus, the relationship between glycolysis and AMPK activation is context-dependent and requires further study.

Oligonucleotide therapeutics represent a next-generation modality in medicine. In this study, we evaluated potential and efficacy of siRNAs targeting respiratory complex I and III for anticancer therapeutics. Complex I was targeted by siRNAs against the core subunits, *NDUFV1* and *NDUFS1*. Treatment with these siRNAs inhibits NADH oxidation and electron transfer along a chain of FeS clusters, which is the initial step in electron transfer for complex I [45]. Rot inhibits ubiquinone reduction following electron transfer [46]. The two siRNAs decreased cell viability, similar to Rot (Fig. 6A). siRNA targeting *UQCRCF1*, the Rieske FeS protein in complex III, was also effective in inhibiting cell proliferation; however, it could not induce cell death. AMA has both cytostatic and cytotoxic effects. Complex III (*bc₁* complex; ubiquinol cytochrome c oxidoreductase) contains three redox subunits along with the Rieske FeS protein. It catalyzes electron transfer from ubiquinol to cytochrome c. AMA inhibits electron transfer between the redox subunits, cytochrome *b* and *c* [47], whereas *UQCRCF1* knockdown inhibits electron transfer between ubiquinol and cytochrome *c₁* [48]. These results suggest that electron transfer between cytochrome *b* and *c* is critical for cell survival rather than that between ubiquinol and cytochrome *c₁*.

In conclusion, this study encourages continuous efforts in pursuing an ETC inhibition approach as an effective anticancer strategy. It is worth re-attempting the clinical trials with the previous inhibitors by restricting the study to a patient cohort/subset with the mt-Low-type cancer. Along with small molecule inhibitors, siRNAs targeting complex I and III exerted cytostatic and/or cytotoxic effects on HCC cells, particularly on mt-Low cells. After massive but unfruitful efforts at developing ETC-targeted medicine, pioneering approaches from a different perspective are expected in this field.

Abbreviations

A-7, A769622; AMA, antimycin A; AMPK, AMP-activated protein kinase; C.C, compound C; ETC, electron transport chain; FeS, iron-sulfur; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; GSH, glutathione; GSSG, glutathione disulfide; HCC, hepatocellular carcinoma; OligA, oligomycin A; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; Rot, rotenone; siRNA, small interfering RNA; TCA, tricarboxylic acid cycle; 2-DG, 2-deoxy-D-glucose.

Declarations

Acknowledgements: We thank Yui Shikama, Yuki Tsuchiya, Kohei Naka, Saki Fujiwara, Ken Hirabayashi, Yudai Suto, Misato Narisawa, Maho Betsuyaku, Eiji Funayama, Yoshihiro Watanabe, Takane Watari, Saki Kikuchi, and Ayaka Saito for their contribution to this work as part of their bachelor's degrees.

We would also like to thank Enago (www.enago.jp) for the English language review.

Authorship contributions: Uchida M: Investigation, Data curation, Writing—original draft. Higugrashi M: Investigation, Data curation, Writing—original draft. Nakagawa H: Writing—review and editing. Ishikawa F: Writing—review and editing. Mori K: Supervision, Writing—review and editing. Shibamura M: Conceptualization, Project administration, Funding acquisition, Supervision.

Funding: JSPS KAKENHI Grant Number 21K06809.

Data availability: No datasets were generated or analyzed during this study.

Conflict of interest The authors declare no conflict of interest.

References

1. Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* 11(5):325–337
2. Vaupel P, Multhoff G (2021) Revisiting the Warburg effect: historical dogma versus current understanding. *J Physiol* 599(6):1745–1757
3. DeBerardinis RJ, Chandel NS (2016) Fundamentals of cancer metabolism. *Sci Adv* 2(5):e1600200
4. Finley LWS (2023) What is cancer metabolism? *Cell* 186(8):1670–1688
5. Pavlova NN, Zhu J, Thompson CB (2022) The hallmarks of cancer metabolism: Still emerging. *Cell Metab* 34(3):355–377
6. Weinhouse S (1956) On respiratory impairment in cancer cells. *Science* 124(3215):267–269
7. Zong WX, Rabinowitz JD, White E (2016) Mitochondria and Cancer. *Mol Cell* 61(5):667–676
8. Han YH, Kim SH, Kim SZ, Park WH (2008) Antimycin A as a mitochondrial electron transport inhibitor prevents the growth of human lung cancer A549 cells. *Oncol Rep* 20(3):689–693

9. Martinez-Reyes I, Cardona LR, Kong H, Vasan K, McElroy GS, Werner M et al (2020) Mitochondrial ubiquinol oxidation is necessary for tumour growth. *Nature* 585(7824):288–292
10. Sullivan LB, Gui DY, Hosios AM, Bush LN, Freinkman E, Vander Heiden MG (2015) Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. *Cell* 162(3):552–563
11. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E et al (2014) Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife* 3:e02242
12. Zhang X, Fryknas M, Hernlund E, Fayad W, De Mito A, Olofsson MH et al (2014) Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. *Nat Commun* 5:3295
13. Birsoy K, Wang T, Chen WW, Freinkman E, Abu-Remaileh M, Sabatini DM (2015) An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis. *Cell* 162(3):540–551
14. Higurashi M, Mori K, Nakagawa H, Uchida M, Ishikawa F, Shibamura M (2025) Respiratory complex I-mediated NAD(+) regeneration regulates cancer cell proliferation through the transcriptional and translational control of p21(Cip1) expression by SIRT3 and SIRT7. *Mol Oncol* 19(6):1775–1796
15. Sanchez M, Gastaldi L, Remedi M, Caceres A, Landa C (2008) Rotenone-induced toxicity is mediated by Rho-GTPases in hippocampal neurons. *Toxicol Sci* 104(2):352–361
16. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 3(12):1301–1306
17. Trotta AP, Gelles JD, Serasinghe MN, Loi P, Arbiser JL, Chipuk JE (2017) Disruption of mitochondrial electron transport chain function potentiates the pro-apoptotic effects of MAPK inhibition. *J Biol Chem* 292(28):11727–11739
18. Janku F, LoRusso P, Mansfield AS, Nanda R, Spira A, Wang T et al (2021) First-in-human evaluation of the novel mitochondrial complex I inhibitor ASP4132 for treatment of cancer. *Invest New Drugs* 39(5):1348–1356
19. Yap TA, Daver N, Mahendra M, Zhang J, Kamiya-Matsuoka C, Meric-Bernstam F et al (2023) Complex I inhibitor of oxidative phosphorylation in advanced solid tumors and acute myeloid leukemia: phase I trials. *Nat Med* 29(1):115–126
20. Boykov IN, Montgomery MM, Hagen JT, Aruleba RT, McLaughlin KL, Coalson HS et al (2023) Pan-tissue mitochondrial phenotyping reveals lower OXPHOS expression and function across cancer types. *Sci Rep* 13(1):16742
21. Reznik E, Miller ML, Senbabaoglu Y, Riaz N, Sarungbam J, Tickoo SK et al (2016) Mitochondrial DNA copy number variation across human cancers. *Elife*. ;5
22. Maruyama T, Saito K, Higurashi M, Ishikawa F, Kohno Y, Mori K et al (2023) HMGA2 drives the IGFBP1/AKT pathway to counteract the increase in P27KIP1 protein levels in mtDNA/RNA-less cancer cells. *Cancer Sci* 114(1):152–163

23. Mori K, Uchida T, Fukumura M, Tamiya S, Higurashi M, Sakai H et al (2016) Linkage of E2F1 transcriptional network and cell proliferation with respiratory chain activity in breast cancer cells. *Cancer Sci* 107(7):963–971
24. Zuberek M, Paciorek P, Rakowski M, Grzelak A (2022) How to Use Respiratory Chain Inhibitors in Toxicology Studies-Whole-Cell Measurements. *Int J Mol Sci.* ;23(16)
25. Han YH, Moon HJ, You BR, Kim SZ, Kim SH, Park WH (2009) Effects of carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone on the growth inhibition in human pulmonary adenocarcinoma Calu-6 cells. *Toxicology* 265(3):101–107
26. Lysenkova LN, Saveljev OY, Grammatikova NE, Tsvetkov VB, Bekker OB, Danilenko VN et al (2017) Verification of oligomycin A structure: synthesis and biological evaluation of 33-dehydrooligomycin A. *J Antibiot (Tokyo)* 70(8):871–877
27. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F (1986) Staurosporine, a potent inhibitor of phospholipid/Ca⁺⁺ dependent protein kinase. *Biochem Biophys Res Commun* 135(2):397–402
28. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA et al (2003) Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* 278(10):8516–8525
29. Panduri V, Weitzman SA, Chandel NS, Kamp DW (2004) Mitochondrial-derived free radicals mediate asbestos-induced alveolar epithelial cell apoptosis. *Am J Physiol Lung Cell Mol Physiol* 286(6):L1220–L1227
30. Glaviano A, Foo ASC, Lam HY, Yap KCH, Jacot W, Jones RH et al (2023) PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Mol Cancer* 22(1):138
31. Locasale JW, Cantley LC (2011) Metabolic flux and the regulation of mammalian cell growth. *Cell Metab* 14(4):443–451
32. Kilburn DG, Lilly MD, Webb FC (1969) The energetics of mammalian cell growth. *J Cell Sci* 4(3):645–654
33. Steinberg GR, Hardie DG (2023) New insights into activation and function of the AMPK. *Nat Rev Mol Cell Biol* 24(4):255–272
34. Yuan Y, Ju YS, Kim Y, Li J, Wang Y, Yoon CJ et al (2020) Comprehensive molecular characterization of mitochondrial genomes in human cancers. *Nat Genet* 52(3):342–352
35. Kopinski PK, Singh LN, Zhang S, Lott MT, Wallace DC (2021) Mitochondrial DNA variation and cancer. *Nat Rev Cancer* 21(7):431–445
36. Adant I, Bird M, Decru B, Windmolders P, Wallays M, de Witte P et al (2022) Pyruvate and uridine rescue the metabolic profile of OXPHOS dysfunction. *Mol Metab* 63:101537
37. Herzig S, Shaw RJ (2018) AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol* 19(2):121–135

38. Ma X, Li Z, Ma H, Jiang K, Chen B, Wang W et al (2025) Rotenone inhibited osteosarcoma metastasis by modulating ZO-2 expression and location via the ROS/Ca(2+)/AMPK pathway. *Redox Rep* 30(1):2493556
39. Han SY, Jeong YJ, Choi Y, Hwang SK, Bae YS, Chang YC (2018) Mitochondrial dysfunction induces the invasive phenotype, and cell migration and invasion, through the induction of AKT and AMPK pathways in lung cancer cells. *Int J Mol Med* 42(3):1644–1652
40. Sanders MJ, Ali ZS, Hegarty BD, Heath R, Snowden MA, Carling D (2007) Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. *J Biol Chem* 282(45):32539–32548
41. Kim J, Yang G, Kim Y, Kim J, Ha J (2016) AMPK activators: mechanisms of action and physiological activities. *Exp Mol Med* 48(4):e224
42. Guo S, Zhang C, Zeng H, Xia Y, Weng C, Deng Y et al (2023) Glycolysis maintains AMPK activation in sorafenib-induced Warburg effect. *Mol Metab* 77:101796
43. Wu SB, Wei YH (2012) AMPK-mediated increase of glycolysis as an adaptive response to oxidative stress in human cells: implication of the cell survival in mitochondrial diseases. *Biochim Biophys Acta* 1822(2):233–247
44. Domenech E, Maestre C, Esteban-Martinez L, Partida D, Pascual R, Fernandez-Miranda G et al (2015) AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest. *Nat Cell Biol* 17(10):1304–1316
45. Pagniez-Mammeri H, Loublier S, Legrand A, Benit P, Rustin P, Slama A (2012) Mitochondrial complex I deficiency of nuclear origin I. Structural genes. *Mol Genet Metab* 105(2):163–172
46. Pereira CS, Teixeira MH, Russell DA, Hirst J, Arantes GM (2023) Mechanism of rotenone binding to respiratory complex I depends on ligand flexibility. *Sci Rep* 13(1):6738
47. Li H, Zhu XL, Yang WC, Yang GF (2014) Comparative kinetics of Qi site inhibitors of cytochrome bc1 complex: picomolar antimycin and micromolar cyazofamid. *Chem Biol Drug Des* 83(1):71–80
48. Fernandez-Vizarra E, Zeviani M (2018) Mitochondrial complex III Rieske Fe-S protein processing and assembly. *Cell Cycle* 17(6):681–687

Figures

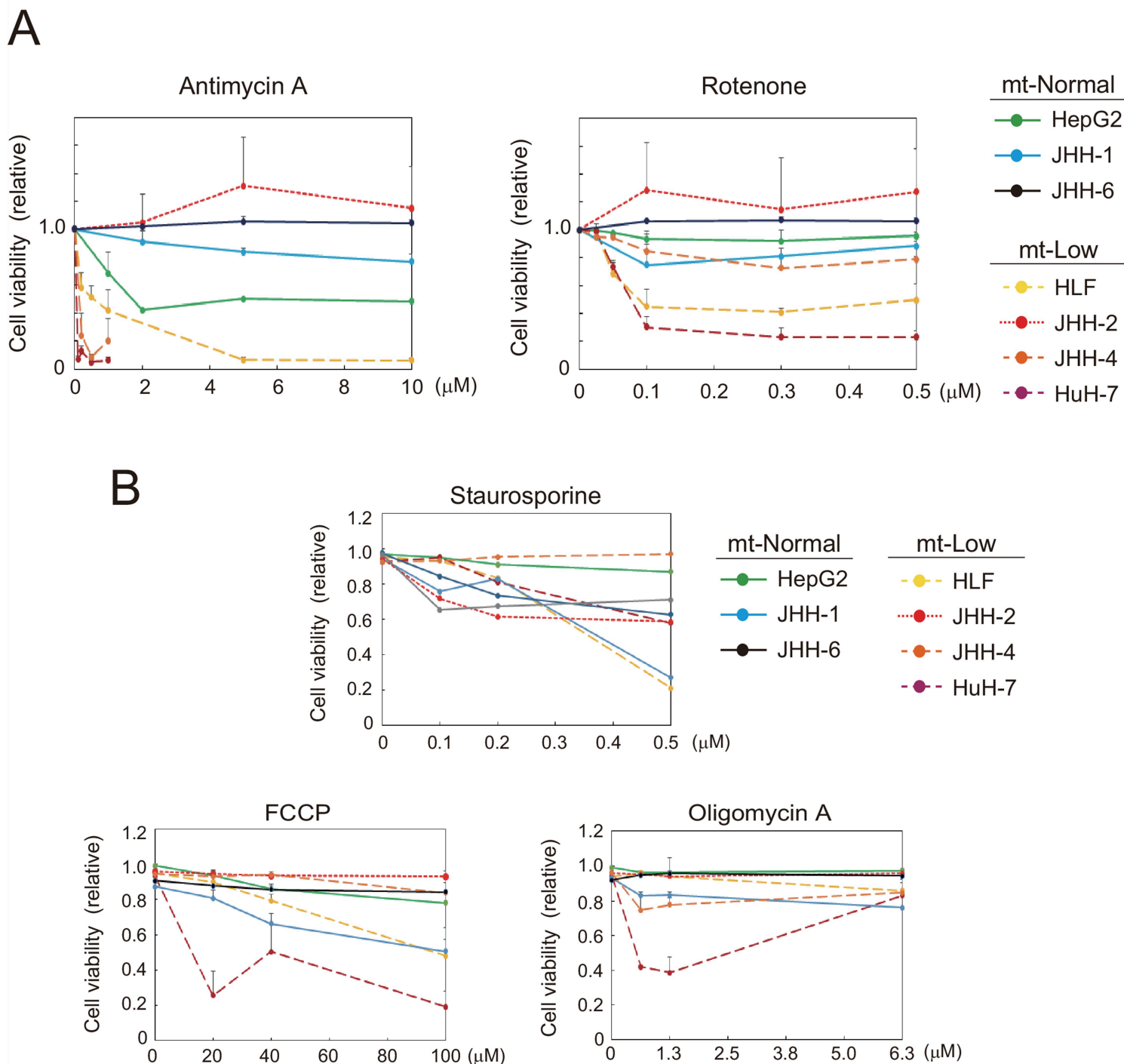
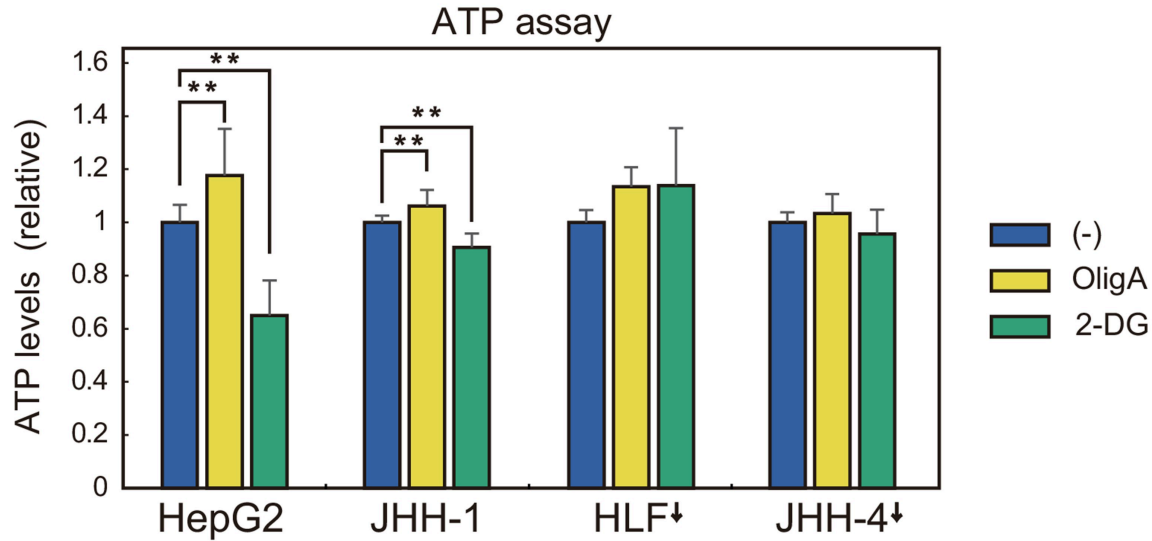
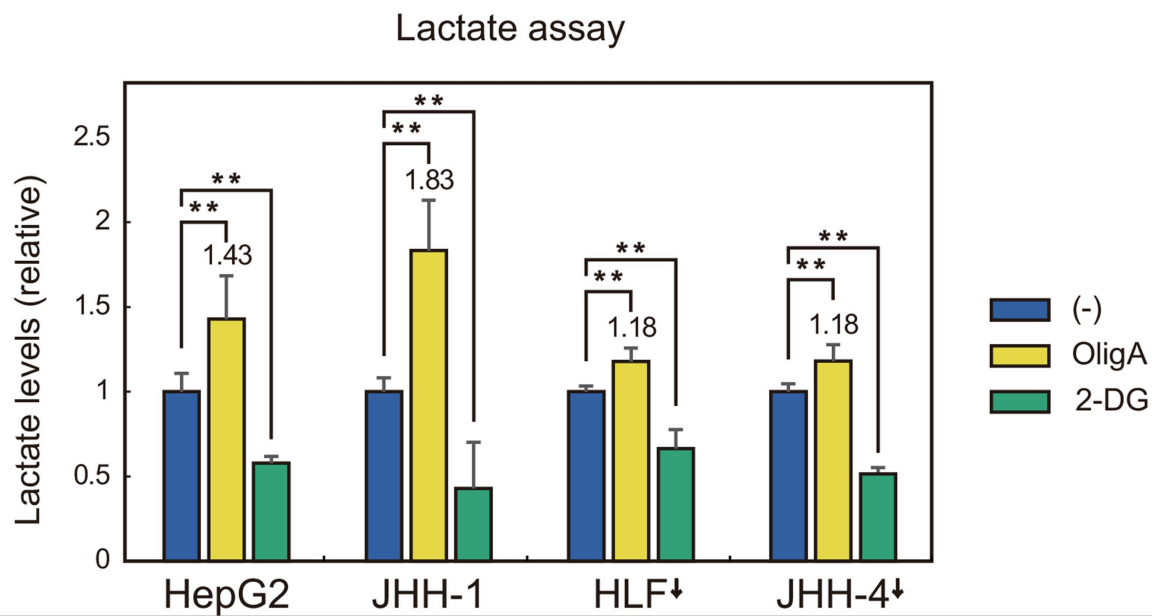


Figure 1

Sensitivities to the inhibition of electron transport chain complexes and oxidative phosphorylation in mt-Normal and mt-Low HCC cells. **A, B** Measurement of cell viability. In **A**, cells were treated with antimycin A and rotenone, and in **B**, with staurosporine, FCCP, and oligomycin A at the indicated concentrations for 24 h. Straight lines; mt-Normal cells, dotted lines; mt-Low cells. Plots represent the means $\pm$ standard deviation from two independent experiments with triplicate measurements for each experiment.

A**B****Figure 2**

ATP synthetic capability and glycolysis availability of mt-Normal and mt-Low cells. **A, B** Cells were treated with 1.25 mM oligomycin A (OligA) or 22.5 mM 2-deoxy-D-glucose (2-DG) for 5 h. The cell lysates and culture supernatants were used for measuring ATP (**A**) and lactate (**B**) levels. The levels are shown as ratios to the vehicle-treated control (-). Cells marked with a downward arrow represent mt-Low cells. Bars represent the means $\pm$ standard deviation from three independent experiments with triplicate measurements for each experiment. ** $P < 0.01$ vs. (-).

Fig. 3

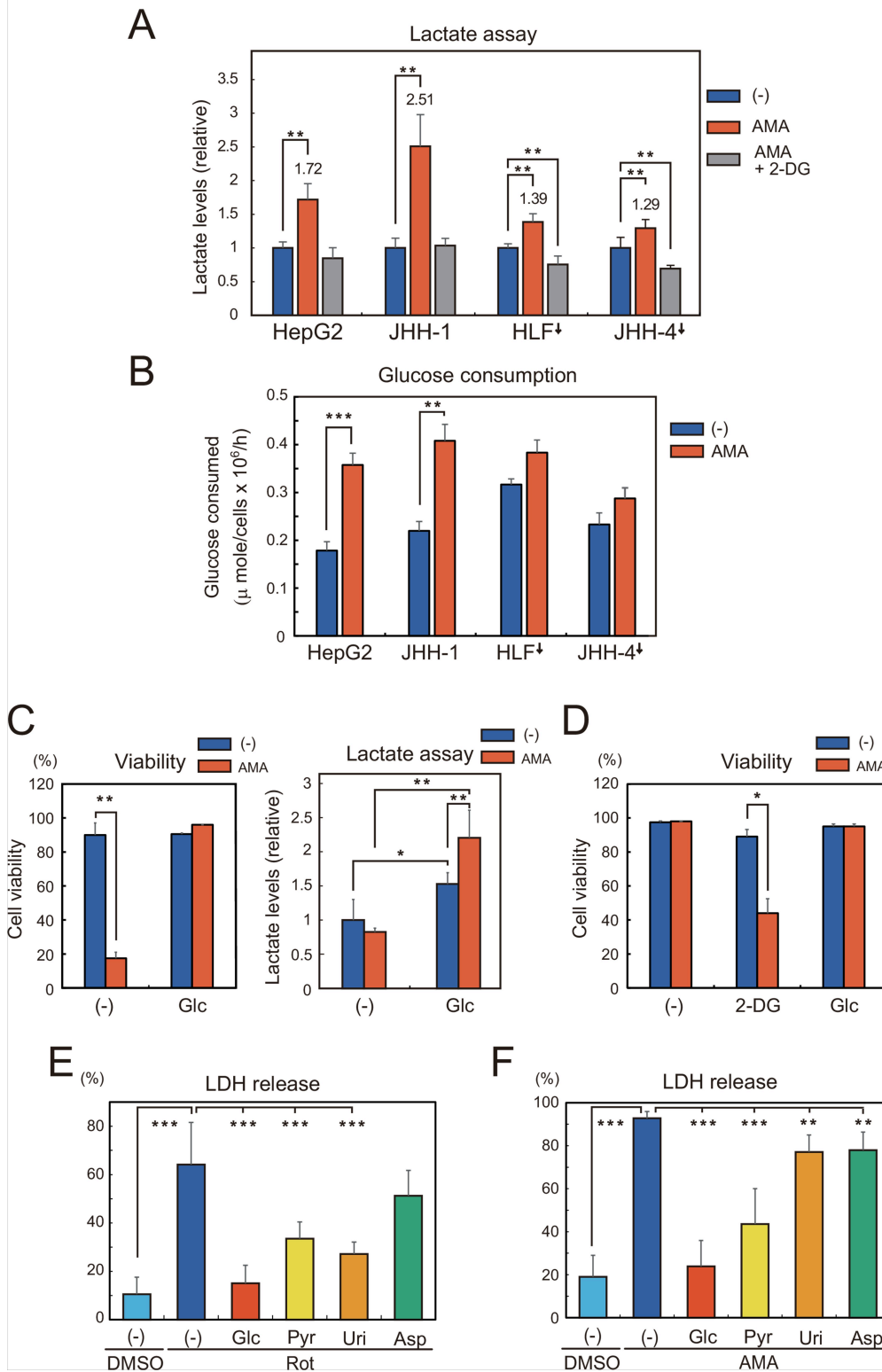


Figure 3

Effects of glycolysis upregulation and nutrient supplementation on cell survival during ETC inhibition. **A** Lactate levels in the culture supernatants were measured. Cells were treated with 0.2 mM antimycin A (AMA) with or without 22.5 mM 2-deoxy-D-glucose (2-DG) for 4 h. The values are expressed relative to the vehicle-treated control (-). Bars represent the means $\pm$ standard deviation from at least three independent experiments with triplicate measurements for each experiment. **B** Glucose consumption

was measured in cells treated with 0.2 mM AMA for 12 h. Cells marked with a downward arrow represent mt-Low cells. Bars represent the means $\pm$ standard deviation from two or three independent experiments with triplicate measurements for each experiment. **C** In lactate assay, JHH-4 cells were treated with or without 10 mM D-glucose (Glc) for 24 h, followed by treatment with 0.2 mM AMA for 9 h. Lactate release into the culture medium was measured as described above. Values are expressed relative to the vehicle-treated control (-). **C, D** Cell viability (%) was determined in JHH-4 cells treated with 0.2 mM AMA for 48 h in culture medium supplemented with 10 mM Glc (**C**) and in HLF cells treated with 0.2 mM AMA for 17 h in culture medium supplemented with 10 mM Glc or 2-DG (**D**). Bars represent means $\pm$ standard deviation from three independent experiments with triplicate measurements for each experiment. **E, F** HLF (**E**) and JHH-4 (**F**) cells were treated with 0.2 mM Rot (**E**) and 0.2 mM AMA (**F**) or vehicle (dimethyl sulfoxide: DMSO) for 48 h in medium supplemented with 10 mM Glc, 5 mM pyruvate (Pyr), 0.2 mM uridine (Uri), and 20 mM aspartate (Asp). LDH release assay was conducted to measure the LDH levels in the culture medium and cell lysate. The ratios (%) of LDH released into the medium to the total amount are shown. Bars represent the means $\pm$ standard deviation from at least three independent experiments with triplicate measurements for each experiment. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ vs (-).

Fig. 4

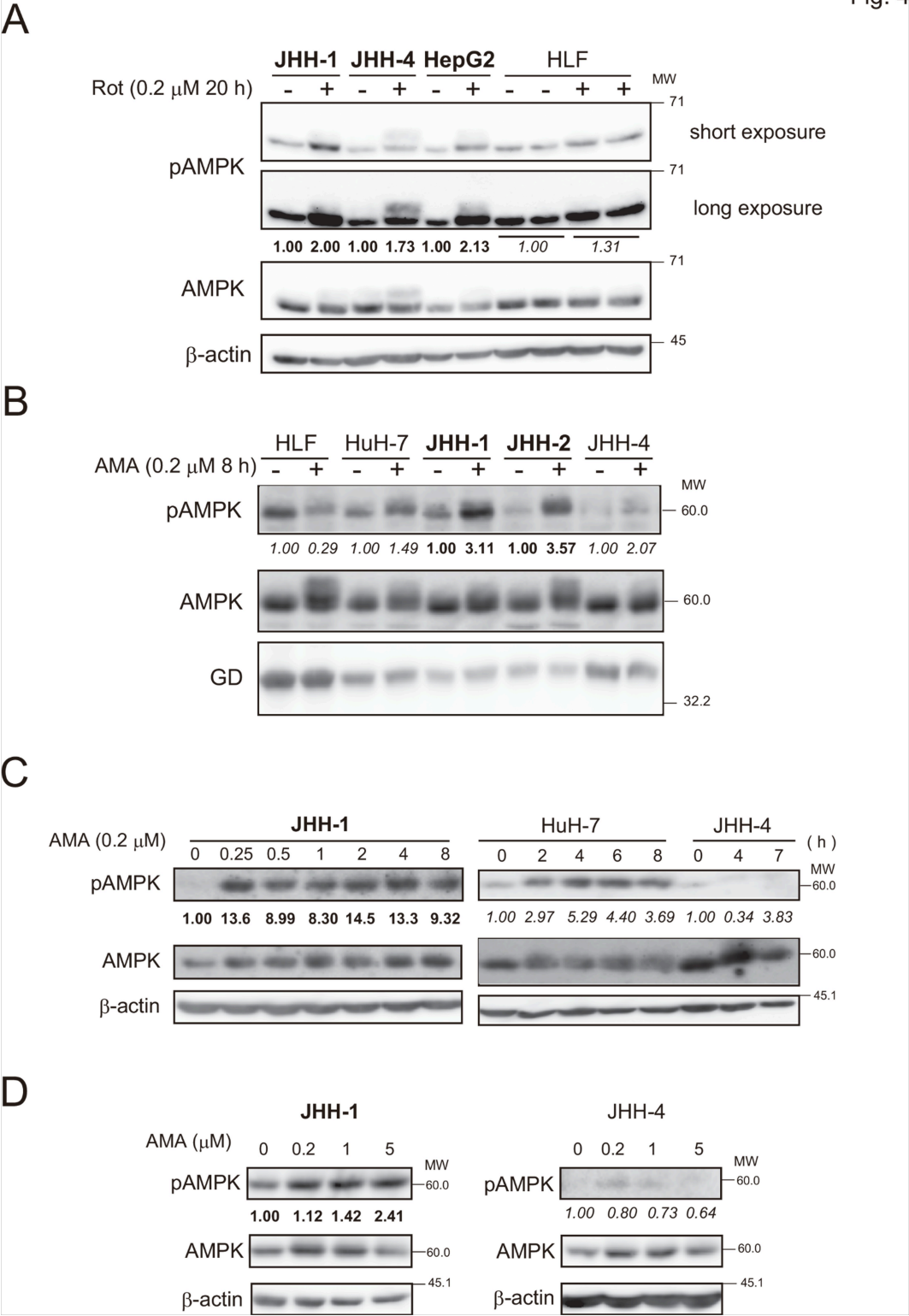


Figure 4

AMPK activation in response to ETC inhibition. **A, B** Cells were treated with 0.2 mM rotenone (Rot) for 20 h (**A**) and 0.2 mM antimycin A (AMA) (**B**) for 8 h and analyzed by western blotting using the indicated antibodies. **C, D** Cells were treated with 0.2 mM AMA for the indicated periods (**C**) and with AMA at the indicated concentrations for 8 h (**D**). Band intensities for AMPK phosphorylated at threonine 172 (pAMPK) measured using ImageJ software are shown relative to the control [(-) or 0 h] after

normalization with those for total AMPK. b-actin and glyceraldehyde-3-phosphate dehydrogenase (GD) were used as the loading controls. Cells marked with bold and plain letters indicate the resistant and sensitive phenotypes, respectively, to cell death following treatment with Rot or AMA (Fig. 1A). Representative results from three independent experiments are shown.

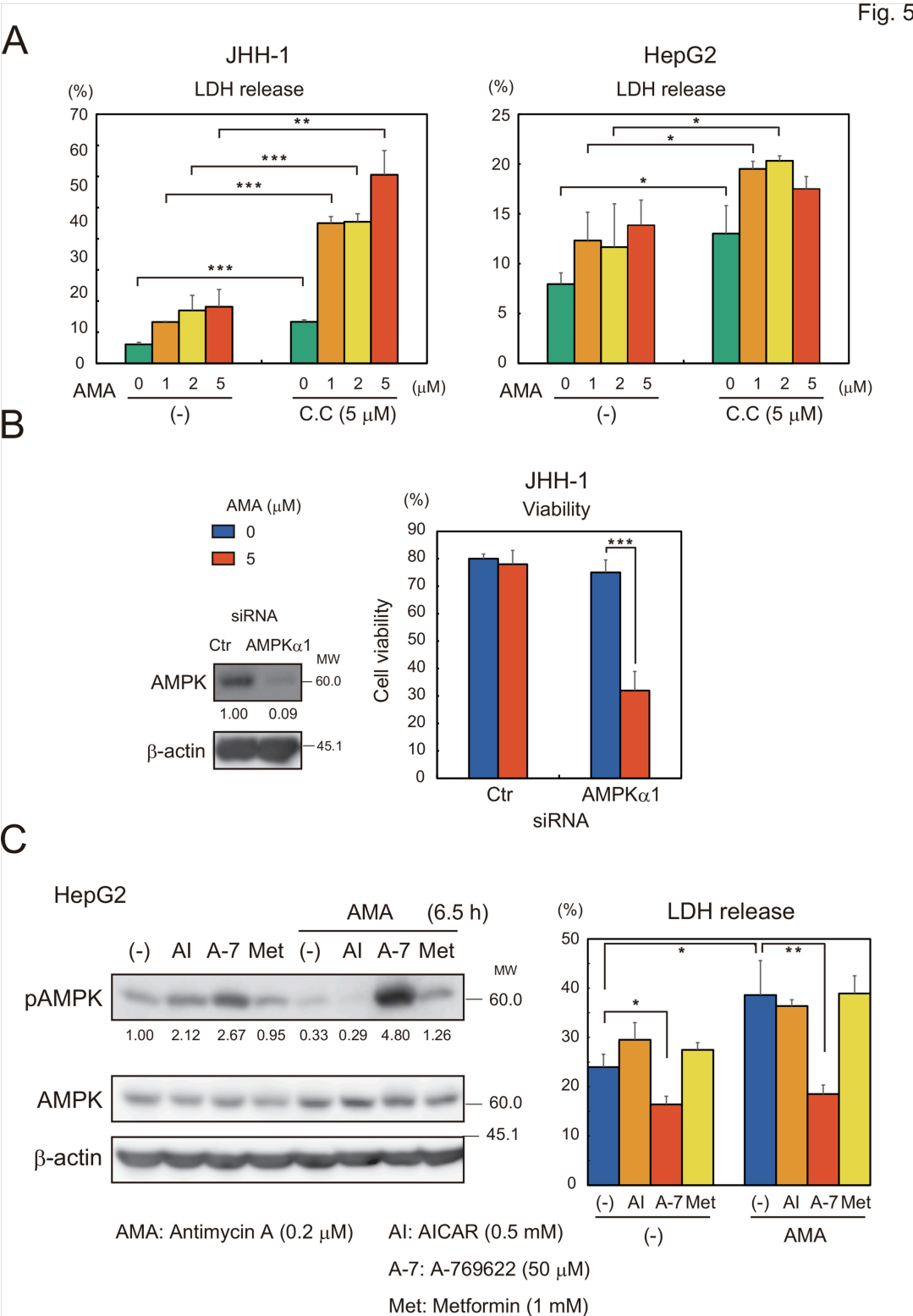


Figure 5

Effects of activation and inhibition of AMPK on cell survival under ETC inhibition. **A** JHH-1 (left) and HepG2 (right) cells were pretreated with 5 mM compound C for 1 h, followed by treatment with antimycin A (AMA) at the indicated concentrations for 24 h. LDH release assay was performed, and the ratios (%) of LDH released into the medium to the total amount are shown. **B** JHH-1 cells were incubated with 50 nM siRNA for AMPK α 1 for 3 days, followed by treatment with 5 mM AMA for 20 h, and cell viability (%) was measured. **C** HepG2 cells were pretreated with 0.5 mM AICAR, 50 mM A-7, and 1 mM metformin for 1 h, followed by treatment with 0.2 mM AMA. After 6.5 h (left), western blot analysis was performed using the indicated antibodies. After 40 h (right), LDH release assay was performed. b-actin was used as the loading control. Representative blots from two independent experiments are shown. Bars represent the means $\pm$ standard deviation from two or three independent experiments with triplicate measurements for each experiment. *** P < 0.001, ** P < 0.01, and * P < 0.05 vs (-).

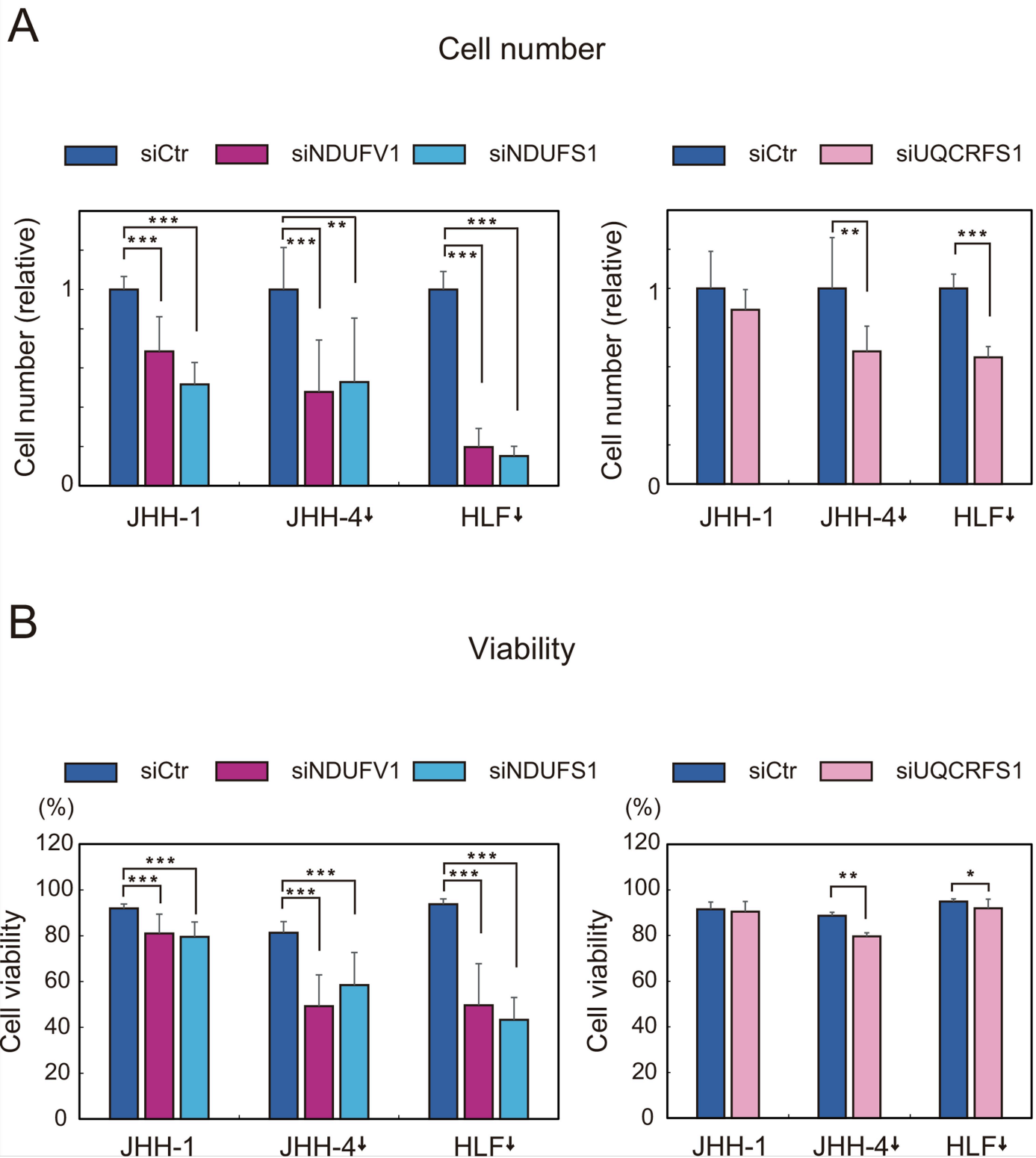


Figure 6

Effects of siRNA for the core subunits of complex I and III on cell proliferation and survival. **A, B** Cells marked with a downward arrow represent mt-Low cells. The cells were incubated with 50 nM siRNAs for the core subunits of respiratory complex I (*NDUFV1* and *NDUFS1*) and III (*UQCRFS1*) or negative control siRNA (Ctr) for 3 days, and cell numbers (**A**) and cell viability (%) (**B**) were determined. Bars represent the

means $\pm$ standard deviation from three independent experiments with triplicate measurements for each experiment. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs siCtr.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [graphicalabstract.eps](#)
- [Supplementarylegends.docx](#)
- [FigS1.eps](#)
- [FigS2.eps](#)
- [FigS3.eps](#)
- [TableS1Antibody.eps](#)
- [TableS2Primer.eps](#)