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RESEARCH

High-dose vitamin C promotes mitochondrial biogenesis in HCT116 colorectal cancer cells by regulating the AMPK/PGC-1α signaling pathway

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Abstract

Background Mitochondrial dysfunction is closely associated with cancer development. Colorectal cancer (CRC) cells often exhibit altered energy metabolism, characterized by increased glycolysis and reduced oxidative phosphorylation. Enhancing mitochondrial biogenesis and function may represent a promising therapeutic approach. High-dose vitamin C has demonstrated anti-tumor properties and the ability to reverse the Warburg effect, but its role in regulating mitochondrial biogenesis and function remains unclear.

Methods We evaluated the altered mitochondrial functional status of HCT116 colorectal cancer cells compared to FHC colorectal epithelial cells, assessed the effects of high-dose vitamin C on mitochondrial biogenesis and function in HCT116 cells, and explored the underlying regulatory mechanisms.

Results HCT116 cells exhibited mitochondrial dysfunction compared to FHC cells, including decreased expression of electron transport chain complexes III and IV, reduced TFAM levels, and lower mtDNA content. Vitamin C treatment significantly enhanced mitochondrial biogenesis and function, as reflected by increased AMPK phosphorylation, upregulation of PGC-1α, SOD2, NRF2, TFAM, MT-CYB, and MTCO1, elevated mtDNA content, restored membrane potential, enhanced oxidative phosphorylation, and reduced glycolytic activity. Furthermore, vitamin C markedly suppressed HCT116 cell viability and clonogenic capacity, while these effects were substantially diminished by cotreatment with Compound C.

Conclusion This study demonstrates that high-dose vitamin C ameliorates mitochondrial dysfunction and promotes mitochondrial biogenesis and function in colorectal cancer cells through activation of the AMPK–PGC-1α signaling pathway, thereby suppressing tumor cell proliferation. These findings suggest that vitamin C may serve as a promising therapeutic agent for targeting mitochondrial metabolism in colorectal cancer.

Keywords Vitamin C · Colorectal cancer · Mitochondrial biogenesis · AMPK · PGC-1α

Introduction

Mitochondria are central organelles responsible for cellular energy production, metabolic regulation, and signal transduction. In addition to their well-known roles in ATP synthesis and lipid and nucleic acid metabolism, mitochondria also contribute to tumor development and metastasis (Chen et al. 2024; Zhou et al. 2024; Wang et al. 2024a). One of the hallmarks of cancer is metabolic reprogramming, whereby most tumor cells rely on aerobic glycolysis (the Warburg effect) rather than oxidative phosphorylation for rapid ATP production (Warburg 1956). This metabolic shift provides a proliferative advantage by supplying both energy and biosynthetic intermediates. However, whether this glycolysis-dominated phenotype is accompanied by significant

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changes in mitochondrial function, and how these changes influence the tumor metabolic network, remain incompletely understood.

Mitochondria are highly dynamic organelles that constantly undergo biogenesis and mitophagy, processes collectively known as mitochondrial quality control (MQC) (Song et al. 2021). MQC maintains mitochondrial homeostasis by coordinating the generation of new mitochondria and the selective clearance of damaged ones, thus sustaining energy supply and preventing mitochondrial dysfunction (Pickles et al. 2018). Dysregulated MQC—particularly impaired mitophagy mediated by the PINK1/Parkin pathway—leads to the accumulation of dysfunctional mitochondria, oxidative stress, and genomic instability, all of which contribute to tumorigenesis (Narendra and Youle 2024). Conversely, enhancing mitochondrial biogenesis through the PGC-1α–NRF2–TFAM axis can restore oxidative phosphorylation and inhibit tumor progression (Lebleu et al. 2014; Gureev et al. 2019). Therefore, MQC plays a pivotal role in shaping tumor metabolism and response to therapy.

Among the key processes governing MQC, mitochondrial biogenesis is a highly intricate mechanism that requires the coordinated expression of genes from both nuclear and mitochondrial genomes (Jornayvaz and Shulman 2010; Scarpulla 2008). A central regulator of this process is peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), whose reduced expression has been closely linked to metabolic dysfunction and unfavorable tumor cell outcomes (Puigserver and Spiegelman 2003; C L et al. 2016; Wang et al. 2024b). PGC-1α expression is influenced by numerous signaling pathways, among which adenosine monophosphate-activated protein kinase (AMPK) serves as a critical upstream modulator (Trefts and Shaw 2021). AMPK, known for its role in maintaining cellular energy homeostasis and metabolic function, has attracted attention as a promising therapeutic target for inhibiting tumor progression and limiting cancer cell proliferation (Jiang et al. 2019; AMPK can suppress tumorigenesis and the Warburg effect[J]. 2013).

Colorectal cancer (CRC) remains one of the deadliest cancers worldwide (Dekker et al. 2019). As the main digestive and absorptive organ of the body, the colorectum is essential for maintaining nutrient metabolic processes. The metabolic profile of colorectal cancer cells exhibits a Warburg effect that is highly dependent on glycolysis (Yu et al. 2023), but whether their mitochondria are altered in this process is not fully understood. Moreover, clinical research has shown a reduction in mitochondrial DNA (mtDNA) copy number in patients with CRC, indicating that impaired mitochondrial function could be a key contributor to CRC progression (Haupts et al. 2021).Consequently, enhancing mitochondrial biogenesis is emerging as a potential therapeutic approach for addressing various conditions, such as cancer, cardiovascular disorders, and insulin resistance.

Ascorbic acid (VC), a naturally occurring nutrient abundant in fresh fruits and vegetables, plays an essential role in human health (Levine et al. 1996). Studies indicate that individuals diagnosed with cancer often face a heightened risk of significantly decreased or even deficient plasma VC concentrations, but VC supplements can be used as biological response modifiers or adjunctive medications to improve their health (White et al. 2020). Interestingly, high levels of VC (mM concentration) were selectively cytotoxic to cancer cells but not to normal cells. (Yu et al. 2023; Chen et al. 2005).

Existing studies on VC's anti-cancer mechanisms have focused on its roles in oxidative stress, immune regulation, and epigenomic reprogramming (Chen et al. 2005). Interestingly, additional research has demonstrated that VC can influence glucose metabolism in HCT116 cells and suppress their proliferation by mitigating the Warburg effect (Yu et al. 2023). These findings collectively highlight the potential of VC as an effective adjunct therapy for managing cancer and its related complications.

Despite its known biological functions, the direct relationship between VC and mitochondrial activity remains poorly understood, and whether VC directly targets mitochondria in cancer cells has yet to be clarified. Therefore, this study was conducted to examine the functional changes in mitochondria within HCT116 cells, assess the impact of VC on the mitochondrial function in HCT116 cells, and explore the underlying regulatory mechanisms using this cell model.

Our study suggests that although mitochondrial function is reduced in HCT116 colorectal cancer cells compared to FHC cells, VC, as a metabolic modulator, may partially restore these functions by promoting mitochondrial biogenesis. Notably, VC treatment improved mitochondrial function in HCT116 cells, but total energy production in cancer cells decreased rather than increased.

Materials and methods

Cell culture and reagents

HCT116 human colorectal cancer cells and FHC normal colon epithelial cells were purchased from Procell Biotechnology. HCT116 cells were cultured in McCoy's 5 A medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humidified

incubator with 5% CO_2. FHC cells were maintained under the same conditions but cultured in DMEM supplemented with 10% FBS. To ensure consistency, cells derived from similar tissues were used for comparative analyses.

Drug treatment

Sodium ascorbic (VC) was obtained from Sigma (A4034). To evaluate the effects of VC on mitochondrial biogenesis, HCT116 cells were treated with VC for 1 h at indicated concentrations. Additionally, to explore the involvement of AMPK in this mechanism, some cells were co-treated with the AMPK-specific inhibitor Dorsomorphin (Compound C, HY-13418–40, MedChemExpress) at a final concentration of 5 μM.

Electron microscopy observation

To visualize mitochondrial morphology, cells were harvested and fixed promptly in a solution containing 2.5% glutaraldehyde at 4 °C for 2 h. After fixation, the cells were embedded in 1% agarose and then subjected to post-fixation with 1% osmium tetroxide at 25 °C for 2 h. The specimens were dehydrated using a graded ethanol series and subsequently infiltrated with EMBed 812 resins for 5–8 h. The embedded samples were then placed in an embedding mold and cured overnight at 37 °C.Ultra-thin sections with a thickness of 60–80 nm was prepared using an ultra-microtome. Sections were stained for 8 min in the dark with a 2% uranium acetate alcohol solution, followed by staining with 2.6% lead citrate under CO2-free conditions for an additional 8 min. After staining, the copper grids were placed on grid holders and allowed to air-dry overnight at room temperature. Imaging was conducted using a transmission electron microscope (Hitachi HT7800, Tokyo, Japan) at magnifications of 4000 ×and 15,000 ×. The number of mitochondria was manually counted, while mitochondrial length was measured using Fuji software. All electron micrographs were evaluated by blinded observers to ensure unbiased analysis of the experimental conditions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from both types of cells utilizing TRIzol reagent (GK20008, GlpBio). Complementary DNA (cDNA) was synthesized via reverse transcription with RT MasterMix for qPCR II (HY-K0511 A, MedChemExpress). The synthesized cDNA was further analyzed via qRT-PCR on a CFX-96™ real-time PCR system (Bio-Rad, USA), employing the SYBR Green qPCR Master Mix (Universal) (HY-K0501 A, MedChemExpress). The primer sequences used for amplification were custom-designed and synthesized by Sangon Biotech, with detailed information provided in Table 1.

Western blot analysis

Protein extracted from HCT116 and FHC cells after treatment using RIPA lysis buffer containing protease inhibitors. The lysates were clarified by centrifuging at 12,000 ×g for 5 min at a low temperature of 4 °C.Protein concentrations in the supernatants were determined using a BCA assay. Equivalent amounts of protein were denatured, resolved by SDS-PAGE, and transferred onto PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% skim milk in TBST and then incubated with primary antibodies overnight at 4 °C. Primary antibodies used in this study included: PGC-1α (Proteintech Group, #66369–1-Ig), SOD2 (Abcam, #ab68155), NRF2 (Abmart, #T55136 F), AMPK (Abcam, #ab32047), p-AMPK (Abcam, #ab23875), MT-CYB (Abmart, #TD2372S), MTCO1 (Abcam, #ab14705), mtTFA (Abcam, #ab307302), β-Actin (Affinity, #AF7018), and GAPDH (Abmart, #M20006 F). Antibodies were diluted according to the manufacturers' protocols. After washing, the membranes were incubated with HRP-conjugated

Table 1 Oligonucleotide primers used in this study

1	PGC-1α primers (forward: 5'-TCGTCATCCACCTCCTCGTTCTC-3'; reverse: 5'-TGCTGCTGCTGCTGCTGTTG-3');
2	NRF2 primers (forward: 5'-TCCAAGTCCAGAAGCCAAACTGAC-3'; reverse: 5'-GGAGAGGATGCTGCTGAAGGAATC-3');
3	TFAM primers (forward: 5'-GTGCTGAGTGCCCTGGGAAG-3'; reverse: 5'-GCCAAGACAGATGAAAACCACCTC-3');
4	SOD2 primers (forward: 5'-CCCGACCTGCCCTACGACTAC-3'; reverse: 5'-AACGCCTCCTGGTACTTCTCCTC-3');
5	18S rRNA primers (forward: 5'-CTTTGGTTCCTCAAGCACCTCCTG-3'; reverse: 5'-GAGGGATGCCATCGCTAATACCAC-3');
6	mtND1 primers (forward: 5'- TCCACACTAGCCGAAACAAACCG −3'; reverse: 5'- GCGAATGGTCCTCCTGCGTATTC −3');

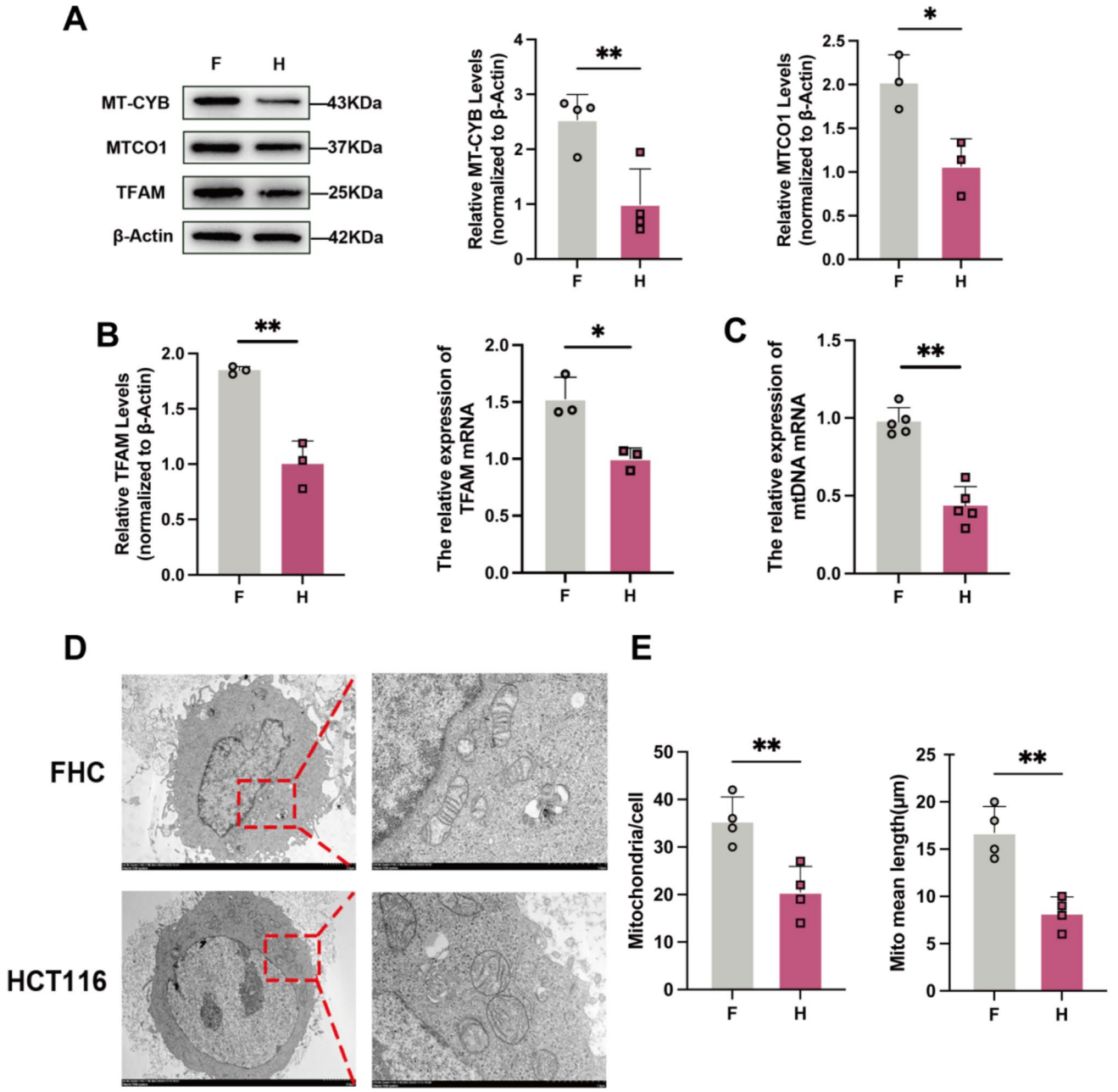


Fig. 1 HCT116 cells have reduced mitochondrial function and biogenesis and increased mitochondrial damage compared to FHC cells. **A** wb bands of MT-CYB, MTCO1 and TFAM in FHC and HCT116, quantitative analysis of protein levels normalized to β-Actin (mean ± SD).; **B** Protein levels and mRNA levels of TFAM; **C** Mitochondrial-to-nuclear DNA (mtDNA/nDNA) ratio; **D** Representative transmission electron microscopy images of mitochondria in FHC and HCT116 with Scale bars of 2 μm, 1 μm (magnification, ×4,000, ×15,000); **E** Quantification of mitochondrial mean length, number, presented as mean ± SEM based on observations from three cells

secondary antibodies, including Anti-mouse IgG (Affinity, #S0002) and Anti-rabbit IgG (Affinity, #S0001). Protein bands were visualized, and their intensities were quantified using ImageJ software, with β-Actin or GAPDH as internal controls for normalization. Semi-quantitative analysis was performed by calculating the grayscale intensity ratio of each target protein band to β-Actin or GAPDH.

Mitochondrial DNA (mtDNA) content analysis

mtDNA content was determined using qRT-PCR with primers targeting mitochondrial NADH dehydrogenase 1 rRNA and nuclear 18S rRNA. The ratio of mtDNA to nuclear DNA (nDNA) was calculated to reflect mitochondrial content. Total DNA was isolated according to standard protocols provided by the manufacturer.

Oxygen consumption rate (OCR)

Mitochondrial respiration was assessed using the Extracellular Oxygen Consumption Assay Kit (ab197243, Abcam) in accordance with the provided instructions. Fluorescence signals were detected using a preheated ThermoFisher Multifunctional Microplate Reader (3020–252) maintained at 37 °C. Data were collected at 1.5-min intervals over a total period of 120 min, with excitation and emission wavelengths set at 380 nm and 650 nm, respectively.

Extracellular acidification rate (ECAR)

The glycolytic activity of treated cells was assessed using the Extracellular Acidification Assay Kit (ab197244, Abcam). ECAR measurements were conducted under the same conditions as OCR analysis, with fluorescence recorded at wavelengths of 380 nm/615 nm over 120 min.

ATP content

Cells were seeded into 6-well plates at a density of 5×10^5 cells per well and allowed to adhere. Subsequently, they were treated with the appropriate drugs as outlined in the experimental design and incubated in the specified medium for predetermined durations. At designated time points, both the supernatant and cell samples were collected. The ATP content was then quantified using the ATP assay kit (Beyotime Technology, S0027) according to the manufacturer's protocol.

Mitochondrial membrane potential assay (JC-1)

Mitochondrial membrane potential ($\Delta\Psi m$) is the marker of mitochondrial health, which we assessed in HCT116 cells using the JC-1 probe (Beyotime, C2003S). JC-1 aggregates in mitochondria, forming red fluorescent aggregates in polarized membranes, but as green fluorescent monomers when the membrane potential is depolarized. Cells were stained with JC-1 dye at 37 °C for 20 min in a dark environment, after that, wash with JC-1 buffer, and then observed under the laser confocal microscope (Andor2000). The fluorescence intensities of red and green channels were measured, and the ratio of red to green fluorescence was calculated to evaluate mitochondrial membrane potential.

Mitochondrial permeability transition pore (mPTP) opening

mPTP opening was evaluated using the calcein-AM/$CoCl_2$ assay (Beyotime, C2009S) following the manufacturer's protocol. After treat with 1 μM calcein-AM, HCT116 cells were incubated at 37 °C for 35 min in the dark. followed by washing and treatment with $CoCl_2$ to quench cytosolic fluorescence. The remaining fluorescence, indicating mitochondrial calcein-AM retention, was detected using a confocal microscope (Andor2000). Reduced fluorescence intensity represented increased mPTP opening.

MTS assay

Cell viability was determined by the MTS assay (Promega, #G1112). Cells (4,000/well) were seeded in 96-well plates and treated with experimental conditions. After 24 h post-treatment, absorbance at 490 nm was measured to assess viability.

Cell colony formation

HCT116 cells were seeded in 6-well plates, allowed to adhere, and then treated with experimental drugs. After 14 days of culture, colonies were fixed with 4% paraformaldehyde, stained with crystal violet, and imaged.

Statistical analysis

All experiments were performed at least triplicate to ensure reliability, and data are presented as mean ± standard deviation (SD). GraphPad Prism 10 software was used for statistical analysis. Student's t-test was used for two-group comparisons; one-way ANOVA followed by Tukey's post-hoc test was used for multiple groups. Repeated-measures two-way ANOVA was used for time-dependent data. $P < 0.05$ was considered statistically significant.

Results

Compared to FHC, HCT116 has lower levels of key oxidative phosphorylation proteins and reduced mitochondrial quality

Mitochondrial dysfunction and reduced biogenesis are commonly observed in various cancer cells. Our study demonstrated that, compared to normal colonic epithelial cells (FHC), HCT116 tumor cells displayed reduced protein expression of mitochondrial oxidative phosphorylation complex III (MT-CYB) and complex IV (MTCO1) (Fig. 1A). TFAM, a critical regulator of mitochondrial biogenesis, stabilizes mitochondrial DNA (mtDNA) and promotes its replication and transcription in a dose-dependent manner, thereby increasing mtDNA copy number. In HCT116 cells, both mRNA and protein levels of TFAM were markedly lower than those in FHC cells (Fig. 1B),

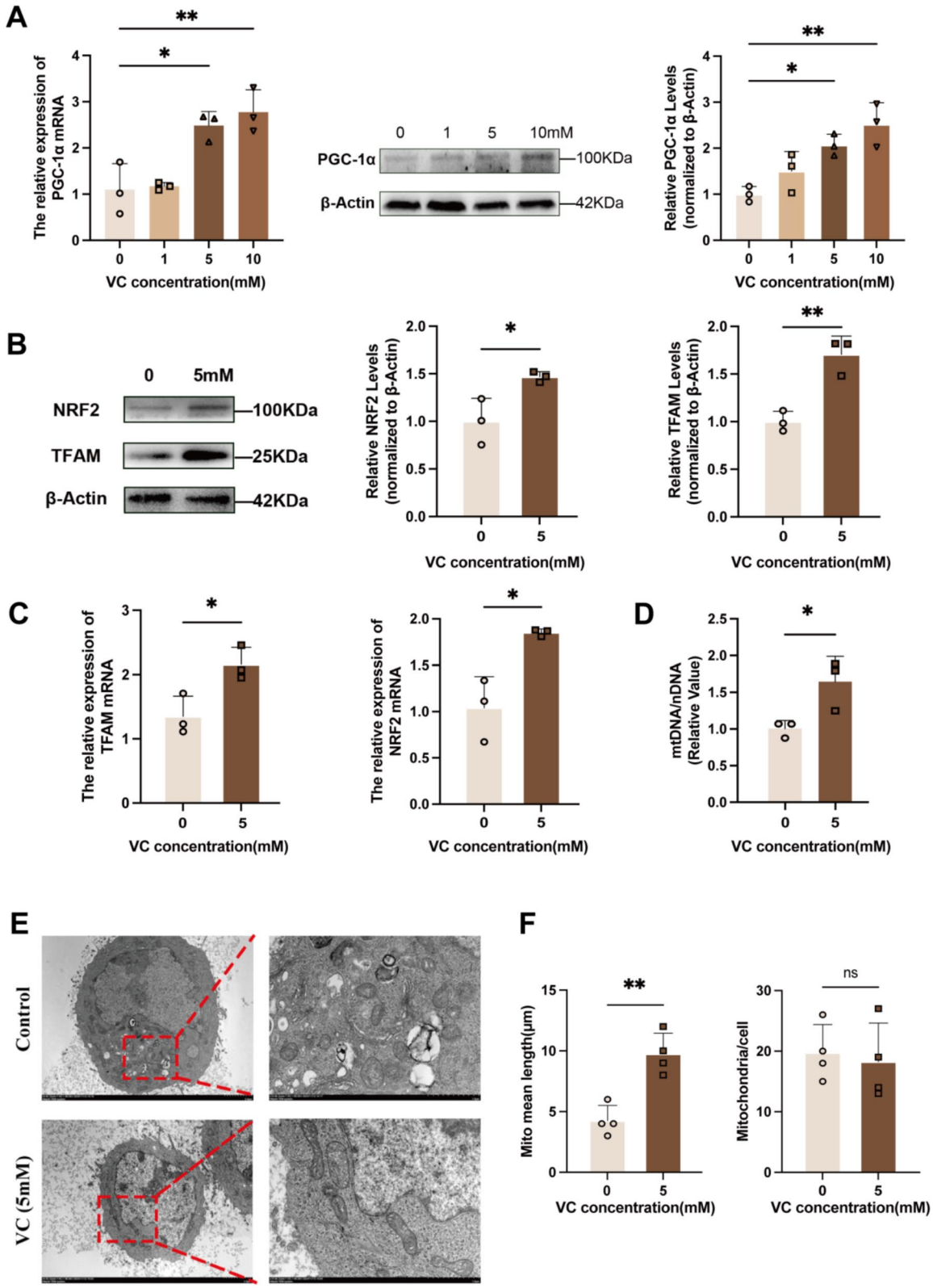


Fig. 2 VC up-regulated mitochondrial biogenesis related genes (**A**) mRNA levels and WB bands of PGC-1α with increasing concentrations of VC; **B** WB bands of NRF2 and TFAM; **C** mRNA levels of NRF2 and TFAM; **D** mtDNA/nDNA ratio; **E** Representative transmission electron microscopy images of mitochondria from HCT116 treated with or without VC. Scale bars 2 μm, 1 μm (magnification, ×4,000, ×15,000); **F** Quantitative analysis of mitochondrial parameters, including mean length, count, proportion of damaged mitochondria, and mitochondria-containing autophagosomes, presented as mean ± SEM from three individual cells

accompanied by a corresponding decrease in mtDNA content (Fig. 1C). Transmission electron microscopy further revealed that, in HCT116 cells, mitochondria exhibited smaller size, reduced numbers, increased damage, and elevated autophagy compared to FHC cells (Fig. 1 D, E).

High-dose vitamin C up-regulates the mitochondrial biogenesis pathways

PGC-1α functions as a co-transcriptional regulator that drives mitochondrial biogenesis. It also plays a central role in cellular responses to shifts in energy metabolism, as well as in regulating mitochondrial quantity and function. In our study, treatment of HCT116 cells with increasing concentrations of VC (0, 1, 5, and 10 mM) for 1 h, resulting in a dose-dependent increase in both mRNA and protein levels of PGC-1α (Fig. 2A), suggesting that high-dose VC stimulates both the transcriptional activity and translation of PGC-1α in these cells. Although 10 mM VC further increased PGC-1α expression, 5 mM was sufficient to elicit a significant effect without inducing potential toxicity or altering normal cell metabolism.

The PGC-1α-NRF2-TFAM signaling pathway is well-established as a critical axis for mitochondrial biogenesis. Given this, we further explored the effect of VC on the expression of two pivotal downstream targets, NRF2 and TFAM. Treatment with 5 mM VC significantly increased protein levels of NRF2 and TFAM (Fig. 2B), as well as the mRNA levels (Fig. 2C) in HCT116 cells. This indicates that high-dose VC activates key transcriptional programs involved in mitochondrial biogenesis.

To assess whether these transcriptional changes translate to enhanced mitochondrial biogenesis, we measured mtDNA content. D-loops, critical regions for mtDNA heavy and light strand transcription initiation, are known to be regulated by TFAM, which facilitates promoter activation and transcription initiation. Consistent with the observed upregulation of TFAM expression, we found that treatment with 5 mM vitamin C significantly increased the mitochondrial D-loop/18S rRNA ratio (Fig. 2D), indicating an elevation in mtDNA copy number. This suggests that high-dose vitamin C promotes mitochondrial biogenesis by upregulating regulatory factor expression and promoting mtDNA replication.

High-dose vitamin C promotes AMPK-mediated mitochondrial biogenesis

AMPK plays a pivotal role in regulating mitochondrial biogenesis by activating PGC-1α. To investigate the underlying mechanisms by which vitamin C promotes mitochondrial biogenesis, we assessed AMPK phosphorylation and the subsequent expression of key regulators, including PGC-1α, NRF2, and TFAM in HCT116 cells under different treatment conditions.

Vitamin C treatment markedly increased AMPK phosphorylation, as well as the protein expression levels of PGC-1α, NRF2, and TFAM (Fig. 3A, D). These changes were accompanied by upregulation of their corresponding mRNA levels, as revealed by qPCR analysis (Fig. 3B), suggesting enhanced transcriptional activity of these mitochondrial biogenesis regulators.

Importantly, these effects were abolished by Compound C, a selective AMPK inhibitor. Treatment with Compound C significantly suppressed both the protein (Fig. 3A, D) and mRNA expression (Fig. 3B) of PGC-1α, NRF2, and TFAM. In line with these findings, the vitamin C-induced increase in mtDNA content (Fig. 3C) was also negated by Compound C, further confirming the AMPK-dependency of this process.

Moreover, vitamin C treatment elevated the protein levels of mitochondrial electron transport chain complexes III and IV (Fig. 3D), indicating enhanced oxidative phosphorylation capacity. These effects were similarly reversed by Compound C, supporting the notion that vitamin C-mediated improvements in mitochondrial function rely on AMPK signaling.

Notably, high-dose vitamin C also significantly increased the expression of SOD2 at both the mRNA and protein levels (Fig. 3A, B). As a PGC-1α-regulated antioxidant enzyme, SOD2 plays a crucial role in maintaining mitochondrial redox homeostasis. The upregulation of SOD2 may represent a compensatory response to increased ROS levels induced by vitamin C, linking mitochondrial biogenesis with oxidative stress adaptation.

Collectively, these findings demonstrate that the AMPK–PGC1α–NRF2–TFAM signaling axis is essential for vitamin C-induced mitochondrial biogenesis and functional enhancement in HCT116 cells.

High-dose VC treatment improves mitochondrial function in HCT116

To evaluate the effects of high-dose VC on mitochondrial function and cellular metabolism in HCT116 cells, we examined several critical markers of mitochondrial activity. The parameters assessed included mitochondrial respiration (OCR), glycolytic activity (ECAR), intracellular ATP levels, membrane potential ($\Delta\Psi m$), and mPTP opening.

As shown in Fig. 4A, treatment with 5 mM VC significantly increased OCR, indicating enhanced mitochondrial oxidative phosphorylation (OXPHOS) capacity. However, while vitamin C treatment improved mitochondrial OXPHOS, it concurrently inhibited ECAR (Fig. 4B), a measure of glycolytic activity, resulting in an overall reduction in ATP production (Fig. 4C). This suggests that vitamin C shifts cellular energy metabolism from glycolysis to

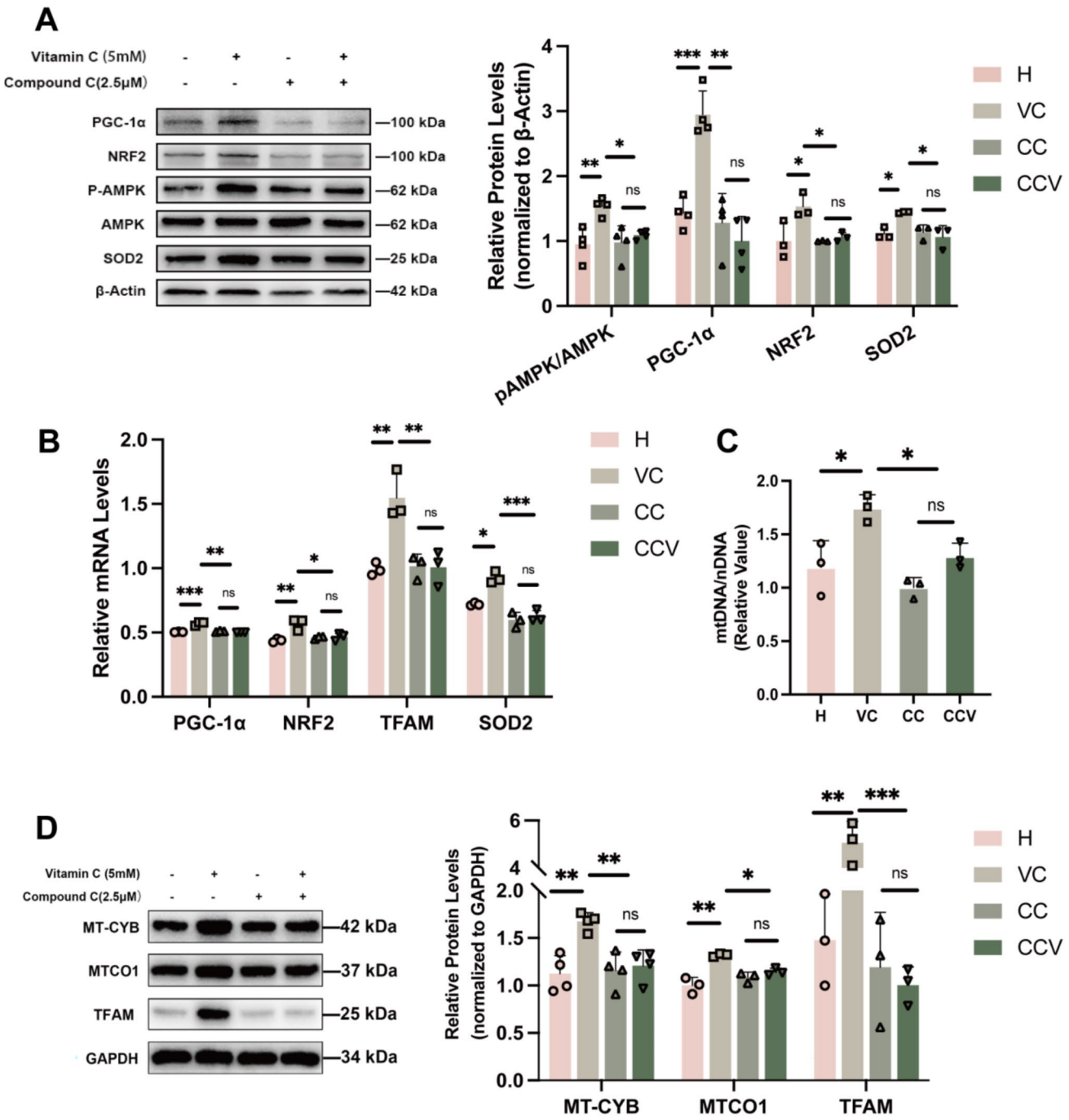


Fig. 3 Vitamin C Activates AMPK Signaling to Enhance Mitochondrial Biogenesis. H = Blank Control; VC = 5 mM Vitamin C; CC = 5 μM Compound C; CCV = 5 mM Vitamin C + 5 μM Compound C. **A** WB bands of Phosphorylated AMPK, AMPK, PGC-1α, NRF2 and SOD2. The p-AMPK/t-AMPK ratios were quantified, and normalized protein levels relative to β-Actin are presented as mean ± SD; **B** mRNA levels of PGC-1α, NRF2 and TFAM and SOD2; **C** Relative Value of mtDNA/nDNA; **D** Mitochondrial oxidative phosphorylation related protein expression, semi-quantified and normalized to GAPDH, are shown as mean ± S

mitochondrial respiration but does not fully compensate for the loss of ATP generated through glycolysis.

Furthermore, JC-1 staining revealed that vitamin C improved mitochondrial membrane potential (Fig. 4D), indicating stabilized mitochondrial function. However, mPTP opening assays showed no significant differences across groups (Fig. 4E), suggesting that vitamin C treatment does not adversely affect mitochondrial integrity under the tested conditions.

Treatment with Compound C (5 μM for 24 h) abolished the effects of vitamin C on OCR, ECAR, ATP content, and mitochondrial membrane potential, highlighting the critical role of AMPK activation in mediating the mitochondrial functional improvements induced by vitamin C.

Consistently, functional assays demonstrated that vitamin C suppressed cell viability and colony-forming ability in an AMPK-dependent manner (Fig. 4F, G)), indicating that its regulatory effect on mitochondrial metabolism is coupled with tumor growth inhibition.

Discussion

Cancer remains a major public health challenge and mitochondrial dysfunction is recognized as one of its key hallmarks (Lu et al. 2015). Tumor cells are reprogrammed metabolically to adapt to their increased energy and biosynthetic demands, and a notable feature of this reprogramming is the shift toward increased glycolytic activity, even with sufficient oxygen, which is accompanied by a suppression of oxidative phosphorylation—a phenomenon commonly known as the Warburg effect (Gogvadze et al. 2008). In addition to these metabolic changes, mitochondrial dysfunction promotes immune evasion and metastasis by impairing p53-mediated anticancer mechanisms, reducing ROS-induced cytotoxicity, and altering the tumor microenvironment and immune checkpoints (Kh and Km 2009; Trachootham et al. 2009; Hanahan and Weinberg 2011). Targeting mitochondrial dysfunction offers a promising therapeutic strategy to address metabolic and non-metabolic vulnerabilities in cancer.

In this study, we observed that HCT116 colorectal cancer cells exhibit significant mitochondrial dysfunction compared with normal FHC cells, characterized by reduced expression of ETC complexes III and IV, decreased TFAM levels, and lower mtDNA copy number. These findings are consistent with prior reports linking impaired mitochondrial biogenesis to colorectal cancer progression (Haupts et al. 2021). Since mitochondrial quality control (MQC) is maintained by a balance between biogenesis and mitophagy, disruption of this equilibrium may contribute to altered energy metabolism and tumorigenesis (Lu et al. 2009).

Given that tumor cells exist in nutrient-rich microenvironments and exhibit enhanced uptake of glucose, amino acids, and lipids, they are highly dependent on metabolic homeostasis (Martinez-Outschoorn et al. 2017). This metabolic plasticity, while advantageous for tumor survival, may paradoxically create therapeutic vulnerabilities—a concept supported by our findings. Our results suggest that this dependence may also represent a vulnerability: restoring mitochondrial biogenesis and function could disrupt this balance and inhibit tumor growth.

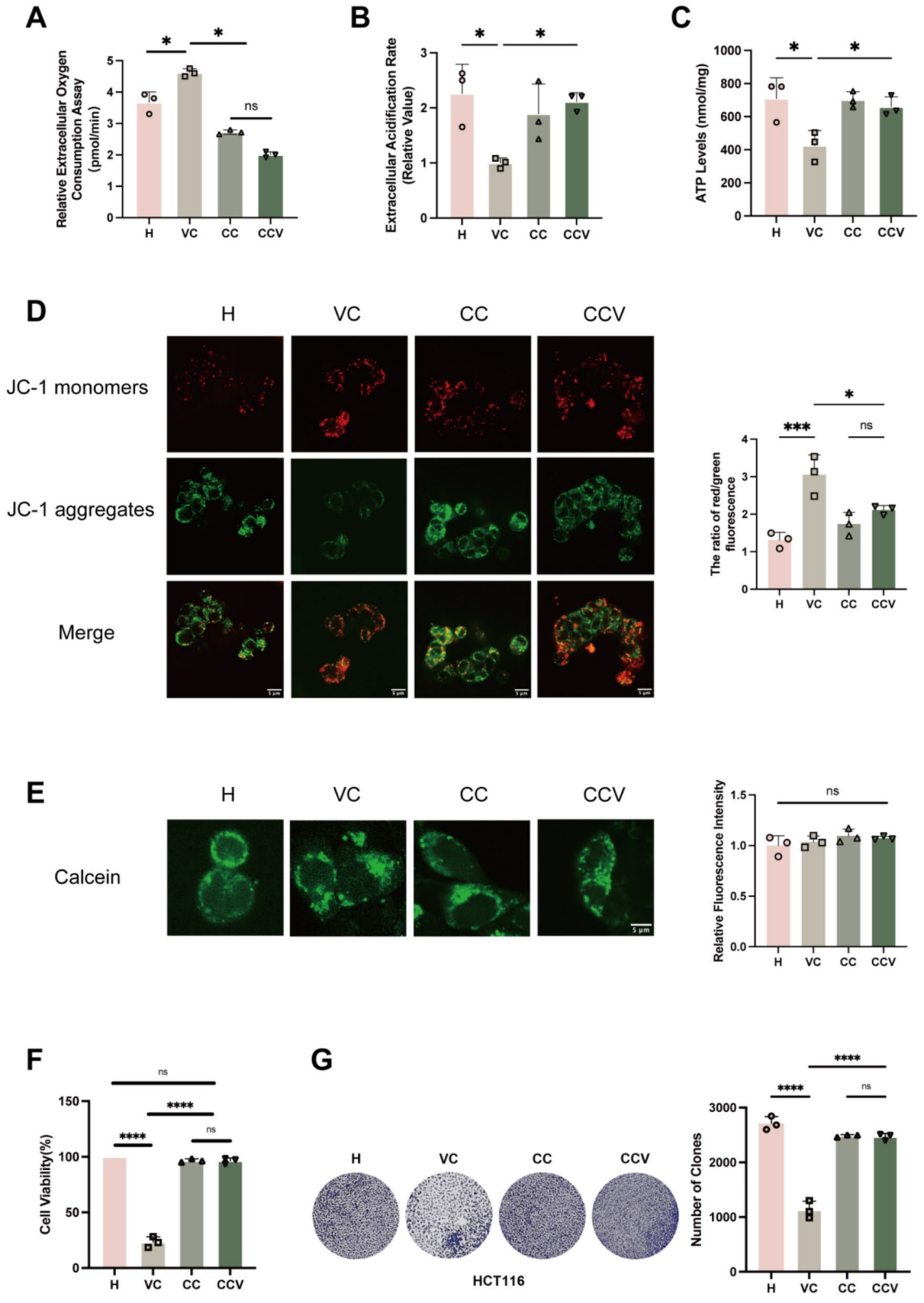
We further examined whether vitamin C (VC) affects mitochondrial biogenesis. VC treatment upregulated PGC-1α expression in a dose-dependent manner (0, 1, 5, 10 mM), and 5 mM VC also increased NRF2 and TFAM levels. PGC-1α, as a master regulator of mitochondrial biogenesis, activates NRF2, which in turn promotes TFAM transcription (Jornayvaz and Shulman 2010). TFAM then translocates into mitochondria to regulate mtDNA replication and transcription. Thus, the PGC-1α–NRF2–TFAM axis is essential for orchestrating mitochondrial biogenesis.

In addition, VC treatment increased expression of SOD2, a mitochondria-specific antioxidant enzyme regulated by PGC-1α (St-Pierre et al. 2006). SOD2 catalyzes the dismutation of superoxide anions into hydrogen peroxide and oxygen, thereby maintaining mitochondrial redox homeostasis. This suggests that VC not only promotes mitochondrial biogenesis but may also initiate adaptive antioxidant responses—possibly in response to elevated ROS levels induced by high-dose VC.

While this study focused on mitochondrial biogenesis, the potential impact of VC on mitophagy remains unclear. It has been reported that excessive mitochondrial biogenesis without efficient mitophagy may lead to mitochondrial dysfunction and metabolic stress. Therefore, future studies should examine whether VC modulates mitophagy pathways such as PINK1/Parkin signaling to fully clarify its role in MQC.

VC treatment also resulted in enhanced mitochondrial DNA (mtDNA) content and membrane potential. These effects were found to be dependent on the AMPK-PGC-1α signaling pathway, as the inhibition of AMPK with Compound C markedly reduced the impact of VC. Furthermore, although VC increased mitochondrial membrane potential, no significant changes in the mitochondrial permeability transition pore (mPTP), such as alterations in apoptotic regulation or calcium homeostasis, were observed under different VC treatment conditions. These findings suggest that VC treatment restored mitochondrial biosynthesis and respiratory function, an effect that was largely independent of mPTP kinetics, highlighting its potential for targeting mitochondrial dysfunction in cancer therapy (Mnatsakanyan et al. 2017).

Over the last two decades, extensive research has shown that high concentrations of VC exert significant anti-tumor effects while sparing normal cells (González-Montero et al. 2022), and clinical trials have shown that VC can enhance the efficacy of traditional therapies (Yang et al. 2017; Liu et al. 2023).Moreover, many cancer patients have reduced or even deficient plasma VC (Wang et al. 2022). Unlike its antioxidant effects under physiological conditions, high-dose VC exhibits pro-oxidant activity in tumor cells (Yun et al. 2015). This pro-oxidant effect may potentiate cellular oxidative stress by altering the ATP/AMP ratio or increasing intracellular reactive oxygen species (ROS) levels, which may


A
Relative Extracellular Oxygen Consumption Assay (pmol/min)
H
VC
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B
Extracellular Acidification Rate (Relative Value)
C
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JC-1 monomers
JC-1 aggregates
Merge
The ratio of red/green fluorescence
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E
Calcein
Relative Fluorescence Intensity
F
Cell Viability(%)
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G
HCT116
Number of Clones

◂**Fig. 4** AMPK-mediated effects of VC on mitochondrial energy metabolism and function in HCT116. H = Blank Control; VC = 5 mM VC; CC = 5 μM Compound C; CCV = 5 mM VC + 5 μM Compound C. **A** Oxygen consumption rate (OCR) displayed as mean ± SEM from three independent measurements. **B** Extracellular acidification rate (ECAR). **C** Cellular ATP content. **D** JC-1 staining (40 ×) to evaluate mitochondrial membrane potential, with the red/green fluorescence ratio representing ΔΨm. Results are shown as mean ± SEM from three independent experiments. **E** mPTP opening assay (40 ×), with green fluorescence intensity indicating mPTP activation. **F** Cell viability was measured by MTS assay. **G** Colony formation ability was assessed by clonogenic assay. Data are presented as mean ± SD from at least three independent experiments

directly or indirectly activate AMPK (Trachootham et al. 2009; Hardie et al. 2012). In addition, moderate VC-induced ROS production may directly activate PGC-1α, further promoting mitochondrial biosynthesis (Biogenesis et al. xxxx). Although we did not measure mitochondrial ROS directly, the observed increase in AMPK phosphorylation supports the possibility that VC activates mitochondrial biogenesis via oxidative stress signaling.

In addition to its effects on mitochondrial biosynthesis, VC treatment significantly increased mitochondrial oxygen consumption and improved respiration. However, ATP assays revealed a paradoxical decrease in cellular ATP levels after VC treatment. This result is consistent with ECAR findings indicating that glycolysis is diminished in HCT116 cells, resulting a metabolic shift, favoring oxidative phosphorylation over glycolysis (Yun et al. 2015). Although mitochondrial respiration was restored, it failed to fully compensate for the diminished glycolytic flux, leading to an overall reduction in cellular ATP levels and energy output. This metabolic shift likely induces energetic stress, thereby sensitizing cancer cells to growth suppression. This energy imbalance may contribute to the tumor inhibition, illustrating the multifaceted effects of VC on cancer cell metabolism.

Indeed, functional assays (MTS and colony formation) confirmed that VC significantly inhibits HCT116 cell growth. Importantly, these effects were reversed by AMPK inhibition, reinforcing the critical role of AMPK in regulating both mitochondrial metabolism and tumor suppression.

In conclusion, high-dose VC enhances mitochondrial biogenesis, regulates redox balance, and reprograms energy metabolism in colorectal cancer cells through the AMPK/PGC-1α signaling axis. By restoring mitochondrial function while suppressing glycolysis, VC disrupts tumor metabolic homeostasis and inhibits proliferation. These findings

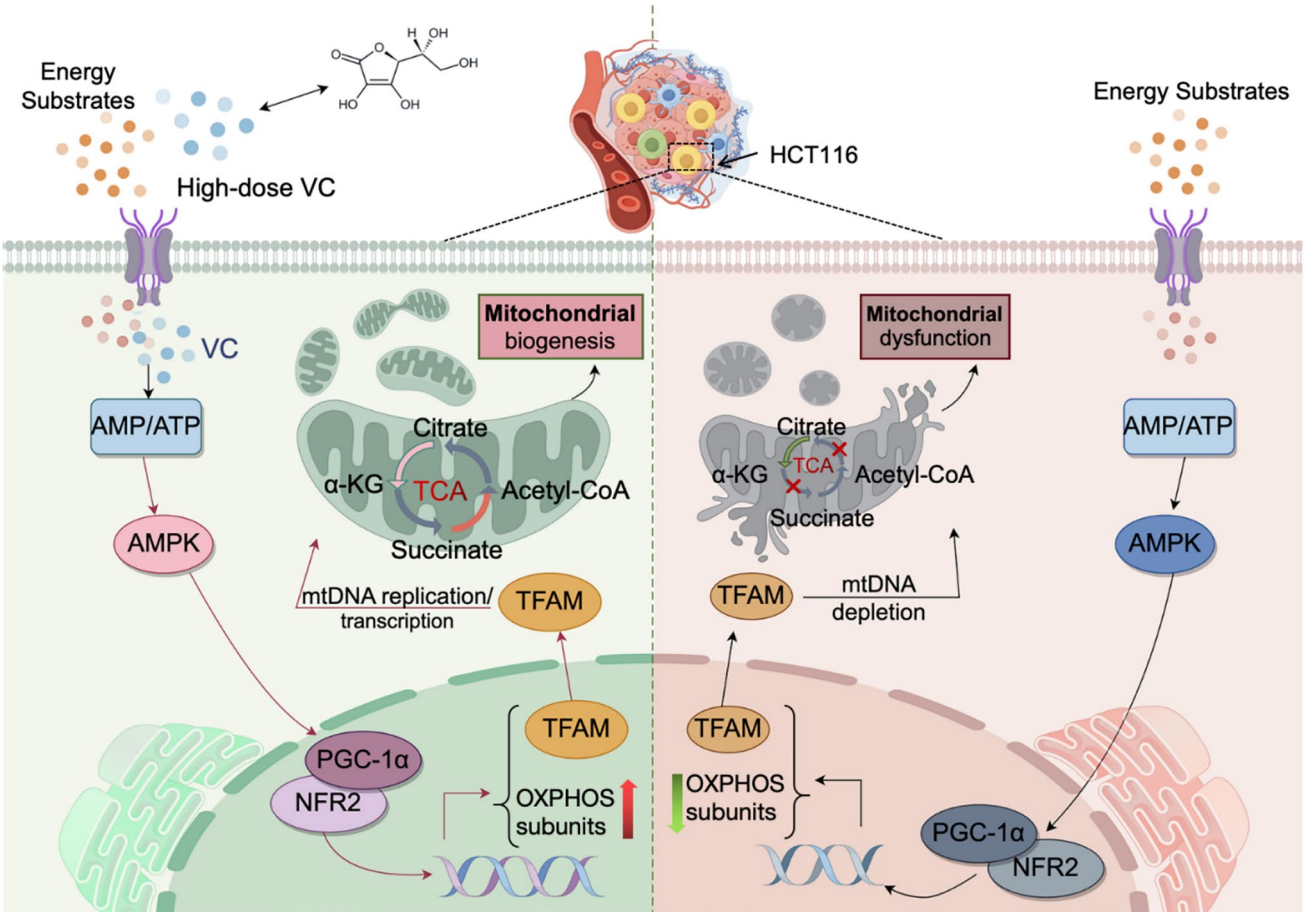

Fig. 5 The schematic representation of the AMPK-PGC-1α-NRF2-TFAM pathway activation by high-dose vitamin C

highlight VC's potential as a metabolic modulator in anti-tumor therapy.

This work has limitations. Our results were obtained from a single cell line (HCT116), and whether they apply to other tumor types remains to be determined. Additionally, while we identified the AMPK/PGC-1α pathway as a key mechanism, upstream regulatory signals, including mitochondrial ROS, need further investigation. While this study focused on mitochondrial metabolism, we acknowledge that evaluating VC's impact on migration would further strengthen its therapeutic relevance. This represents an important direction for future work. However, we acknowledge the importance of evaluating VC's impact on tumor invasiveness and will consider this in future studies. The long-term effects and clinical relevance of high-dose VC also require validation. Using an integrated approach combining mitochondrial function assays and pathway-specific inhibition provided robust mechanistic insights. Despite these limitations, our findings offer novel insights into the metabolic reprogramming effects of VC and support its potential as an adjunctive strategy in cancer treatment (Stine et al. 2022).

Conclusions

This study provides compelling evidence that high-dose vitamin C promotes mitochondrial biogenesis and enhances mitochondrial function in HCT116 colorectal cancer cells through activation of the AMPK-PGC1α–NRF2–TFAM signaling axis. Our findings highlight VC's dual capacity to reverse Warburg metabolism and restore mitochondrial function, positioning it as a promising metabolic modulator for adjuvant cancer therapy. Further investigation into its clinical application as an adjuvant treatment is warranted. (The proposed mechanism is summarized in Fig. 5).

Author contributions Ruiyang Hong contributed to the conception and design of the study, conducted the experiments, analyzed the data, and wrote the manuscript. Minsu supervised the research, provided critical intellectual input, and revised the manuscript. Mou Zou , Jia Huang contributed to data analysis and interpretation, as well as drafting sections of the manuscript. Dongyu Zhou assisted with experimental procedures and helped with the data analysis. Yun Liang contributed to manuscript revisions and interpretation of the results. All authors reviewed and approved the final manuscript for publication.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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