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p21$^{WAF1/CIP1}$ deficiency induces mitochondrial dysfunction in HCT116 colon cancer cells

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ABSTRACT

p21$^{WAF1/CIP1}$ is a critical regulator of cell cycle progression. However, the role of p21 in mitochondrial function remains poorly understood. In this study, we examined the effect of p21 deficiency on mitochondrial function in HCT116 human colon cancer cells. We found that there was a significant increase in the mitochondrial mass of p21$^{-/-}$ HCT116 cells, as measured by 10-*N*-nonyl-acridine orange staining, as well as an increase in the mitochondrial DNA content. In contrast, p53$^{-/-}$ cells had a mitochondrial mass comparable to that of wild-type HCT116 cells. In addition, the expression levels of the mitochondrial biogenesis regulators PGC-1α and TFAM and AMPK activity were also elevated in p21$^{-/-}$ cells, indicating that p21 deficiency induces the rate of mitochondrial biogenesis through the AMPK-PGC-1α axis. However, the increase in mitochondrial biogenesis in p21$^{-/-}$ cells did not accompany an increase in the cellular steady-state level of ATP. Furthermore, p21$^{-/-}$ cells exhibited significant proliferation impairment in galactose medium, suggesting that p21 deficiency induces a defect in the mitochondrial respiratory chain in HCT116 cells. Taken together, our results suggest that the loss of p21 results in an aberrant increase in the mitochondrial mass and in mitochondrial dysfunction in HCT116 cells, indicating that p21 is required to maintain proper mitochondrial mass and respiratory function.

1. Introduction

p21$^{WAF1/CIP1}$ is a potent cyclin-dependent protein kinase inhibitor (CKI) and is the principal mediator of cell cycle arrest in response to DNA damage [1,2]. When DNA is damaged, p21 expression is upregulated by p53, and p21 then induces cell cycle arrest at the G1/S transition by inhibiting the activity of the cyclin E/CDK2 and cyclin D/CDK4/6 complexes [1,2]. p21 also inhibits cell cycle progression through a direct interaction with PCNA and indirect interactions with other cell cycle regulators [3]. In addition to its role in cell cycle arrest, p21 is also involved in many important cellular processes, including cell cycle progression, apoptosis, and transcriptional regulation through both p53-dependent and p53-independent pathways [4]. Previous studies have reported that p21 can protect cells against apoptosis in response to apoptotic stimuli [5,6]. Paradoxically, p21 promotes apoptosis in the context of certain cellular stresses [7]. It has been proposed that p21 inhibit DNA repair processes through a direct interaction with PCNA [8]. Although the critical role of p21 in cell cycle arrest and its anti-proliferative activities indicate that p21 acts as a tumor suppressor, recent studies suggest that p21 is able to promote cell proliferation and tumorigenesis under certain conditions [9–11]. These studies thus suggest that p21 has wide variety cellular and organismal functions that depend on the cellular context and circumstances. However, the role of p21 in mitochondrial function has not been investigated.

Mitochondria are multifunctional organelles whose activities are important in cellular proliferation and physiology. For example, mitochondria play essential roles in cellular energy (ATP) production via oxidative phosphorylation and in apoptosis via *cytochrome c* release [12,13]. Mitochondrial dysfunction is linked to several human diseases, including premature aging, diabetes mellitus and a variety of syndromes involving the muscles [12–14]. Cancer is also associated with mitochondrial dysfunction [14]. Various tumor cells actually exhibit differences in the number, morphology and function of mitochondria relative to normal cells [14]. These past studies indicate that mitochondrial dysfunction contributes to the development and progression of various human diseases, including cancer. Therefore, the identification of the proteins that regulate mitochondrial function is critical to understand the mechanisms underlying mitochondria-related diseases.

Numerous previous studies have demonstrated that p21 plays a critical role in tumor suppression [4]. Recently, it has been shown that mitochondrial function is regulated by tumor suppressors

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such as ATM and p53 (18–20). However, the role of p21 in mitochondrial function is largely unknown. In this study, we examined the role of p21 in mitochondrial function using wild-type, $p53^{-/-}$, and $p21^{-/-}$ HCT116 human colon cancer cells. We found that p21 deficiency resulted in functional defects in mitochondria and that the mitochondrial mass was significantly increased in $p21^{-/-}$ HCT116 cells.

2. Materials and methods

2.1. Cell culture and reagents

Wild-type, $p53^{-/-}$ and $p21^{-/-}$ HCT116 cells (kind gifts from Dr. Bert Vogelstein) were maintained in DMEM containing 10% FBS (Sigma, St. Louis). 10-*N*-nonyl-acridine orange (NAO) was purchased from Sigma–Aldrich. MitoTracker Red® CMXRos was obtained from Molecular Probes (Invitrogen, Carlsbad, CA).

2.2. MitoTracker staining

Cells were cultured on glass coverslips and stained with 100 nM MitoTracker Red® CMXRos. Cells were subsequently fixed in media containing 3.7% formaldehyde, and images of the stained cells were captured using an LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.3. Measurement of mitochondrial mass

To determine the mitochondrial mass, 1×10^6 cells were trypsinized and fixed with cold 70% ethanol. Subsequently, the cells were stained with 0.5 μM NAO for 15 min and then analyzed by flow cytometry using an FC500 cytometer and CXP software (Beckman Coulter, Inc., Brea, CA). The experiment was repeated at least three times, and the results are presented as the mean values with standard deviations.

2.4. Measurement of mitochondrial DNA content

To determine the mitochondrial DNA content, total genomic DNA including mitochondrial DNA was isolated as described previously [15]. The mitochondrial DNA content was quantified using quantitative real-time PCR with a probe that specifically recognizes the mitochondrial D-loop. The amount of 18S rDNA, used to quantify nuclear DNA, was simultaneously determined in the same reaction and was used as the internal control. Quantitative real-time PCR was performed as described previously [15]. The sequences of the primers used in this study were as follows: mitochondrial DNA (D-loop), 5′-GATTTGGGTACCACCCAAGTATTG-3′ and 5′-GTACAATATTCATGGTGGCTGGCA-3′; and nuclear DNA (18S rDNA), 5′-TAGAGGGACAAGTGGCGTTC-3′ and 5′-CGCTGAGCCAGT CAGTGT-3′.

2.5. Quantitative RT-PCR

To assess mRNA levels, total RNA isolation and real-time PCR were performed as described previously [15]. The amplification of β-actin was performed in the same reactions for all samples as an internal control, and the mRNA levels of other genes were normalized to the level of β-actin mRNA. The expression level of each mRNA was determined using the $2^{-\Delta CT}$-threshold cycle method. The primers used were as follows: TFAM, 5′-CGTTGGAGGGAA CTTCCTGAT-3′ and 5′-CCTGCCACTCCGCCCTATA-3′; hAMPKα1, 5′-TCGACAGAAGATTCGGAGCCTT-3′ and 5′-TTCATCCAGCCTTCCATT CTTACAGAT-3′; and hAMPKα2, 5′-CTGCCACAGACACCACTTTGTC-3′ and 5′-CAGGGTGACACAGGAGAACCAAG-3′.

2.6. Western blot analysis and antibodies

Cells were harvested and lysed in RIPA buffer and then subjected to Western blot analysis, as described previously [16]. Antibodies for PGC-1α (3G6), AMPK (23A3) and phospho-AMPK (T172) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The levels of actin protein were monitored as an internal loading control using anti-actin antibodies (Sigma). The intensities of the bands were quantified by densitometry using AlphaEaseFC 4.0 software (Alpha Innotech). The relative band intensities were calculated relative to the band intensity of HCT116 wild-type cells after normalization to the actin signal. The experiment was repeated at least three times, and the results are presented as the mean values with standard deviations.

2.7. Measurement of the cellular ATP level

Cellular ATP levels were measured using the ENLITEN ATP assay system kit (Promega Mannheim, Germany) according to the manufacturer's instructions using a LuBi microplate luminometer (Micro Digital Ltd., Seoul, South Korea). The absolute ATP levels were normalized to the protein concentration determined by the Bradford assay. The experiment was repeated at least three times, and the results are presented as the mean values with standard deviations.

2.8. Cell proliferation in galactose medium

To measure cell proliferation in galactose medium, 1×10^4 cells were seeded into 6-well plates and cultured in DMEM-galactose medium (DMEM glucose-free medium supplemented with 1 g/l galactose, 1 mM sodium pyruvate, 10% fetal bovine serum and 1% penicillin/streptomycin). The number of cells was counted every two days by trypan blue exclusion using a hematocytometer. To measure the colony-forming ability in galactose medium, 500 cells were seeded in 60 mm dishes and incubated in DMEM-galactose medium. The medium was changed every 3 days. After 2 weeks, the resulting colonies were fixed, stained with 2% methylene blue and counted. The experiments were repeated at least twice independently, and the results are presented as the mean values with standard deviations.

3. Results

3.1. Increased mitochondrial mass in p21-deficient HCT116 cells

To examine the role of p21 in mitochondria, we first examined the mitochondrial morphology of wild-type, $p53^{-/-}$, and $p21^{-/-}$ HCT116 cells after staining with MitoTracker Red dye. As shown in Fig. 1A, there were no significant differences in mitochondrial morphology among the $p53^{-/-}$, $p21^{-/-}$, and wild-type HCT116 cells. Interestingly, we found that $p21^{-/-}$ HCT116 cells had more mitochondria than the $p53^{-/-}$ and wild-type HCT116 cells (Fig. 1A). To determine the mitochondrial mass more precisely, HCT116 cells were stained with 10-*N*-nonyl-acridine orange (NAO), which can be used to determine the mitochondrial mass quantitatively because this compound binds to cardiolipin in the inner mitochondrial membrane [17]. The quantitative analysis of the NAO signal revealed that the mitochondrial mass of $p21^{-/-}$ HCT116 cells was approximately twofold higher than that of wild-type HCT116 cells, whereas $p53^{-/-}$ cells had a mitochondrial mass similar to that of wild-type HCT116 cells (Fig. 1B). Moreover, the mitochondrial DNA (mtDNA) content was also significantly increased in $p21^{-/-}$ HCT116 cells (1.8-fold) relative to $p53^{-/-}$ and wild-type HCT116 cells (Fig. 1C). These results suggest that both

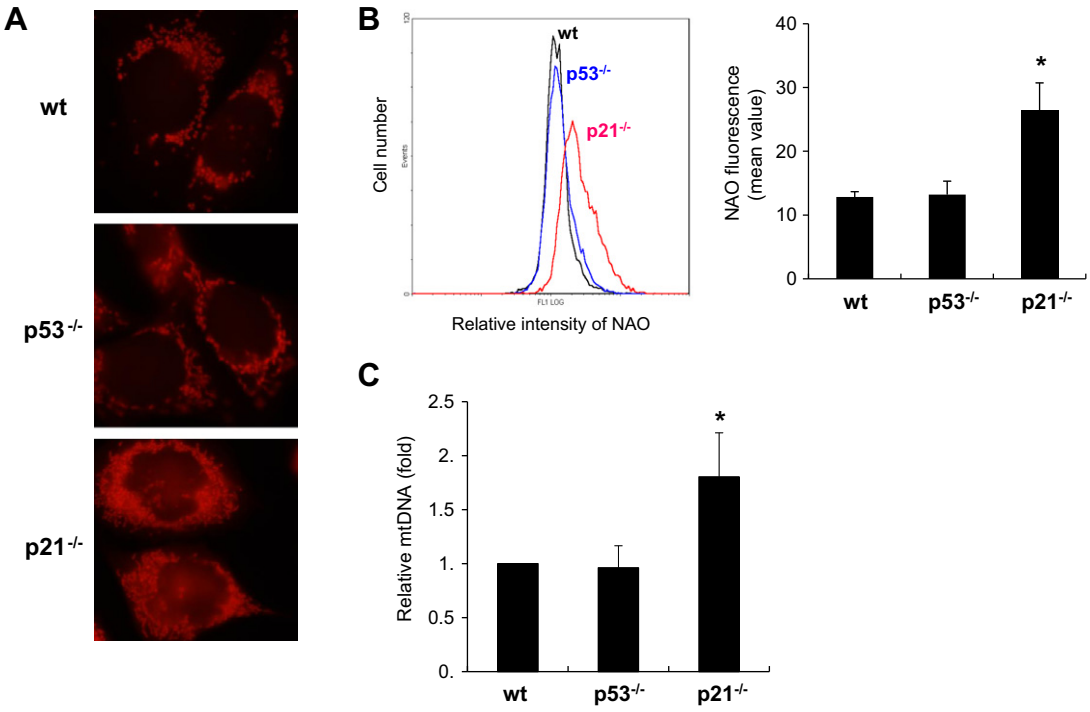

Fig. 1. Increase in mitochondrial mass in p21-deficient HCT116 cells. (A) To visualize mitochondria, wild-type (wt), p53$^{-/-}$, and p21$^{-/-}$ HCT116 cells were stained with MitoTracker Red (100 nM). Images were captured with a confocal microscope. (B) Mitochondrial mass was determined with NAO staining as described in Section 2. The data shown are representative of three independent experiments. The relative NAO fluorescence was measured in three independent experiments and is presented as the mean value with the standard deviation. (C) The intracellular mitochondrial DNA (mtDNA) content was analyzed by real-time PCR with a primer set targeting the mitochondrial DNA D-loop and a primer set targeting 18S rRNA as control for nuclear DNA. The mtDNA content of wild-type cells was defined as 1, and the relative mtDNA contents of p53$^{-/-}$ and p21$^{-/-}$ HCT116 cells were calculated. The relative mtDNA contents were measured in three independent experiments and are presented as the mean values with standard deviations. $^{*}p < 0.05$ compared with wild-type cells using Student's *t*-test.

the mitochondrial mass and the mitochondrial DNA content are increased in p21$^{-/-}$ HCT116 cells.

3.2. p21. deficiency induces the expression of mitochondrial biogenesis regulators in HCT116 cells

Mitochondrial mass and number are tightly regulated in the cell by several master regulators, including PGC-1α and TFAM [18]. The increased mitochondrial DNA content and mitochondrial mass in p21$^{-/-}$ cells suggested that p21 deficiency may induce mitochondrial biogenesis in HCT116 cells. To test whether the elevated mitochondrial mass in p21$^{-/-}$ HCT116 cells is associated with an increase in mitochondrial biogenesis, we next examined the expression level of PGC-1α, a master regulator of mitochondrial biogenesis. Western blot analysis using anti-PGC-1α antibodies revealed that the expression level of PGC-1α was significantly increased in p21$^{-/-}$ cells relative to the expression level in wild-type and p53$^{-/-}$ cells (Fig. 2A upper panel). The quantitative analysis of repeated experiments indicated that the PGC-1α levels were increased by approximately threefold in p21$^{-/-}$ cells (Fig. 2A lower panel).

Mitochondrial transcription factor A (TFAM) is an important regulator of mitochondrial DNA replication and transcription and has been shown to be controlled by PGC-1α [19]. We found that the mRNA level of TFAM was also increased, by approximately 1.6-fold, in p21$^{-/-}$ cells, whereas the TFAM mRNA level was decreased in p53$^{-/-}$ cells (Fig. 2B). These results suggest that p21 deficiency induces mitochondrial biogenesis through the upregulation of key mitochondrial biogenesis regulators, including PGC-1α and TFAM, in HCT116 cells.

3.3. Increase in AMPK activity in p21-deficient HCT116 cells

AMP-activated protein kinase (AMPK) has been shown to regulate mitochondrial biogenesis through PGC-1α [20]. To determine whether AMPK is involved in the elevation of mitochondrial mass in p21$^{-/-}$ cells, we first examined AMPK activation using an anti-phospho-Thr172-specific antibody for AMPK in wild-type, p53$^{-/-}$, and p21$^{-/-}$ HCT116 cells. Strikingly, we observed much stronger AMPK activation in p21$^{-/-}$ cells than in wild-type and p53$^{-/-}$ cells (Fig. 3A). In addition, the protein level of AMPK was also higher in p21$^{-/-}$ cells than in wild-type and p53$^{-/-}$ cells.

Because p21$^{-/-}$ cells exhibited an increase in the AMPK protein level, we next examined mRNA levels of AMPKα1 and AMPKα2 using quantitative real-time PCR. As shown in Fig. 3B, both the AMPKα1 and AMPKα2 mRNA levels were increased by approximately 1.5-fold in p21$^{-/-}$ cells relative to the levels in wild-type HCT116 cells, whereas the mRNA levels of AMPKα1 and AMPKα2 were decreased in p53$^{-/-}$ cells (Fig. 3B). These results suggest that the gene expression of AMPK is elevated in p21$^{-/-}$ cells and that the increase in AMPK activity may be responsible for the upregulation of PGC-1α and the increase in mitochondrial biogenesis in p21$^{-/-}$ HCT116 cells.

3.4. p21 deficiency induces defects in mitochondrial function in HCT116 cells

Mitochondria play important roles in cellular metabolism and energy production [12,13]. Previous studies have demonstrated that the induction of mitochondrial biogenesis leads to increases in the steady-state ATP level [18,21,22]. To determine whether the increase in mitochondrial biogenesis in p21$^{-/-}$ HCT116 cells

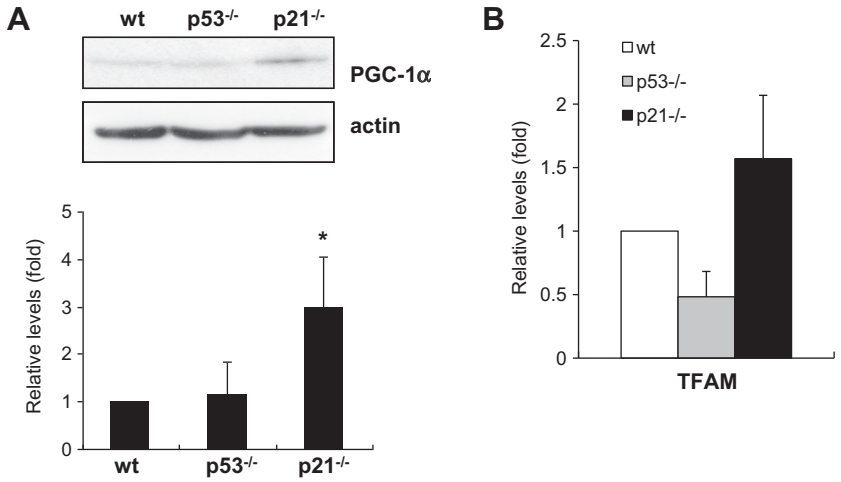

Fig. 2. Increase in the levels of mitochondrial biogenesis regulators in p21-deficient HCT116 cells. (A) Wild-type (wt), p53$^{-/-}$, and p21$^{-/-}$ HCT116 cells were lysed and subjected to western blotting using anti-PGC-1α and anti-actin antibodies. The data shown are representative of three independent experiments (upper panel). The band intensities were quantified by densitometry as described in Section 2. The relative band intensity values obtained in three independent experiments are presented as the mean values with standard deviations (lower panel). *$p < 0.05$ compared with wild-type cells using Student's *t*-test. (B) The mRNA levels of TFAM were measured by quantitative real-time PCR as described in Section 2. The relative TFAM mRNA levels obtained in three independent experiments are presented as the mean values with standard deviations.

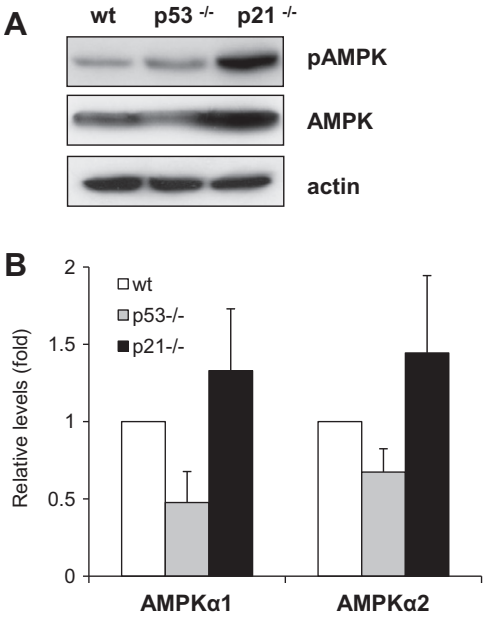

Fig. 3. Increase in the level of AMPK in p21-deficient HCT116 cells. (A) Wild-type (wt), p53$^{-/-}$, and p21$^{-/-}$ HCT116 cells were lysed and subjected to western blotting using anti-phospho-AMPK (Thr172), anti-AMPK, and anti-actin antibodies. The data shown are representative of three independent experiments. (B) The mRNA levels of AMPKα1 and AMPKα2 were measured by quantitative real-time PCR as described in Section 2. The relative TFAM mRNA levels obtained in three independent experiments are presented as the mean values with standard deviations.

is associated with an increase in ATP synthesis, we measured the cellular ATP level using an ATP bioluminescence assay. Interestingly, there were no significant differences in the ATP level among the wild-type, p53$^{-/-}$, and p21$^{-/-}$ HCT116 cells (Fig. 4A). Quantitative analysis of the results of three replicate experiments even indicated that the ATP level in p21$^{-/-}$ cells is slightly lower than that in wild-type HCT116 cells, although this difference was not statistically significant ($p > 0.05$).

The reduced ATP level in p21$^{-/-}$ cells suggested that the mitochondria in p21$^{-/-}$ HCT116 cells may have a functional defect. Previous studies have demonstrated that cells with defects in the mitochondrial respiratory chain exhibit growth impairments in galactose medium because cells grown in galactose medium rely primarily on oxidative phosphorylation to produce ATP [23,24]. To identify defects in mitochondrial function in p21-deficient cells, the proliferation levels of wild-type and p21$^{-/-}$ cells were measured in DMEM glucose-free medium supplemented with galactose. As shown in Fig. 4A, p21$^{-/-}$ cells showed a clear growth impairment relative to wild-type cells in galactose medium (Fig. 4B). In addition, a colony formation assay showed that p21$^{-/-}$ cells exhibited significantly reduced colony-forming ability relative to wild-type cells in the galactose medium (Fig. 4C). These results indicate that p21 deficiency is associated with defects in the mitochondrial respiratory chain in HCT116 cells.

4. Discussion

In this report, we describe our finding that p21 deficiency induces alterations in the number and function of mitochondria in HCT116 human colon cancer cells. First, p21$^{-/-}$ HCT116 cells exhibited an approximately twofold increase in the mitochondrial mass relative to wild-type cells, as measured by 10-*N*-nonyl-acridine orange (NAO) staining (Fig. 1B). The increase in the mitochondrial DNA (mtDNA) content (Fig. 1C) and in the levels of PGC-1α and TFAM, the master regulators of mitochondrial biogenesis, in p21$^{-/-}$ HCT116 cells (Fig. 2) confirmed that mitochondrial biogenesis is increased in p21$^{-/-}$ HCT116 cells. We also found that the expression of AMPK, an important upstream regulator of PGC-1α, was upregulated in p21$^{-/-}$ cells (Fig. 3), suggesting that the loss of p21 increases the level of mitochondrial biogenesis through the AMPK-PGC-1α pathway.

The increases in mitochondrial mass and mitochondrial DNA in p21$^{-/-}$ HCT116 cells suggest that p21 suppresses mitochondrial biogenesis. In addition, the data from our present study suggest that p21 may suppress mitochondrial biogenesis by inhibiting the gene expression of AMPKα1 and AMPKα2. It has been reported that p21 regulates other the expression of other genes by functioning as a transcription factor through direct protein–protein interactions. p21 associates directly with E2F1 and suppresses its transcriptional activity [25]. p21 also binds to the transcription factor STAT3 and

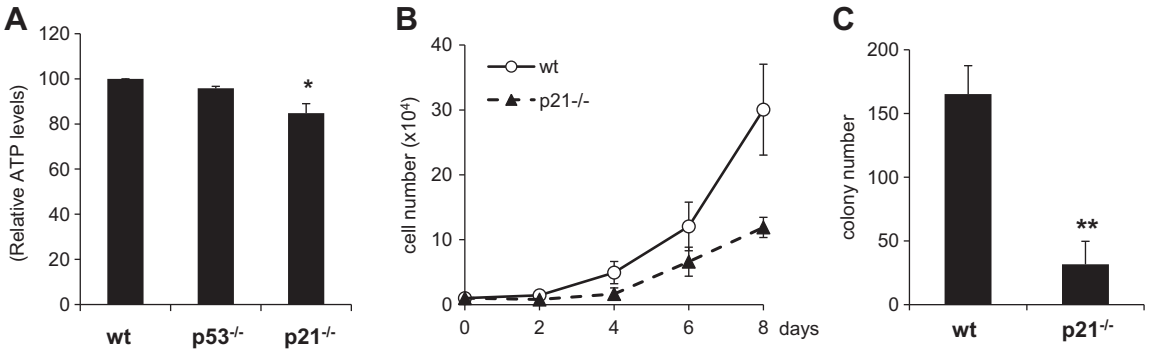

Fig. 4. Mitochondrial dysfunction in p21-deficient HCT116 cells. (A) Cells were homogenized, and the levels of intracellular ATP levels were determined by an ATP bioluminescence assay as described in Section 2. The relative cellular ATP levels obtained in three independent experiments are presented as the mean values with standard deviations. $^{*}p > 0.05$ compared with wild-type cells using Student's *t*-test. (B) A 1×10^4 wild-type (wt) or $p21^{-/-}$ HCT116 cells were seeded in 6-well culture plates and cultured in DMEM-galactose medium. The number of cells was counted every 2 days by trypan blue exclusion using a hematocytometer. (C) Approximately 500 wild-type (wt) or $p21^{-/-}$ HCT116 cells were seeded in 6-well culture plates and cultured in DMEM-galactose medium. After 2 weeks, the resulting colonies were fixed, stained with methylene blue and counted. The experiments were repeated at least twice independently, and the results are presented as the mean values with standard deviations. $^{**}p < 0.05$ compared with wild-type cells using Student's *t*-test.

represses its transcriptional activity, leading to the inhibition of STAT3-dependent gene expression [26]. In addition to directly binding to transcription factors, p21 also regulates transcriptional processes through interactions with transcription coactivators, such as p300 or CBP, which are essential cofactors for transcriptional activation. p21 positively regulates ERα-dependent transcription via an interaction with CBP [27,28]. Devgan et al. showed that p21 negatively regulates Wnt4 gene transcription by inhibiting the recruitment of p300 to the promoter [29]. Thus, p21 could inhibit AMPKα1 and AMPKα2 expression through its transcriptional regulatory function. Alternatively, p21 could inhibit AMPK gene expression indirectly by regulating an upstream pathway. Whether p21 suppresses AMPK transcription through direct binding or indirect action has yet to be determined. Interestingly, Valenti-Vega et al. recently showed that the loss of ATM results in an increase in the number of mitochondria due to defects in mitophagy [30]. Thus, although we showed in this study that the loss of p21 increased the expression levels of AMPKα1 and AMPKα2, it is still possible that p21 modulates mitochondrial mass through another mechanism. Future studies on this topic will provide a better understanding of how p21 regulates mitochondrial biogenesis.

Importantly, in this study, we found that p21 deficiency induces mitochondrial dysfunction in HCT116 cells. Although $p21^{-/-}$ cells harbor almost twice the normal number of mitochondria, the cellular ATP levels are not increased relative to those in wild-type HCT116 cells (Fig. 4A), suggesting that the mitochondria in $p21^{-/-}$ cells have defects in ATP generation activity. Indeed, $p21^{-/-}$ cells showed a remarkable proliferation impairment in galactose medium (Fig. 4B and C), indicating that the loss of p21 induces defects in mitochondrial respiratory function. Therefore, it is possible that the increase in the mitochondrial mass in $p21^{-/-}$ cells could be a cellular compensatory response to counteract mitochondrial dysfunction.

Taken together, our results indicate that the loss of p21 in HCT116 cells induces mitochondrial biogenesis and mitochondrial dysfunction. Although the precise mechanism by which the lack of p21 induces alterations in the number and function of mitochondria remains to be explored, our results suggest that p21 may play an important role in maintaining proper mitochondrial biogenesis and mitochondrial function.

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