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Running Title: Melatonin inhibits glycolysis in HCC cells

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ABSTRACT

Multiple mechanisms have been suggested to explain the chemopreventive and tumor-inhibitory effects of melatonin. Despite growing evidence supporting melatonin-induced mitochondrial dysfunction, it remains largely unknown how this phenomenon modulates metabolic reprogramming in cancer cells. The aim of this study was to identify the mechanism underlying the anti-proliferative and apoptotic effects of melatonin, which is known to inhibit glycolysis. We analyzed the time-dependent effects of melatonin on mitochondrial respiration and glycolysis in liver cancer cells and found that from a cell bioenergetic point of view, melatonin caused an acute reduction in mitochondrial respiration, but increased reactive oxygen species production, thereby inhibiting mTORC1 activity from an early stage post-treatment without affecting glycolysis. However, administration of melatonin for a longer time reduced expression of c-Myc protein, thereby suppressing glycolysis via downregulation of HK2 and LDHA. The data presented herein suggest that melatonin suppresses mitochondrial respiration and glycolysis simultaneously in HCC cells, leading to anti-cancer effects. Thus, melatonin has potential as an adjuvant agent for therapy of liver cancer.

Keywords: Melatonin, Glycolysis, mTORC1, mitochondrial respiration, Hepatocellular carcinoma

INTRODUCTION

Melatonin (N-acetyl-methoxytryptamine) is an endogenous hormone that regulates circadian and seasonal rhythms. However, after Cohen et al first discovered the role of the pineal gland in the etiology of breast cancer, numerous studies have provided a possible link between melatonin and cancer (1). Physiologic and pharmacologic concentrations of melatonin show oncostatic and tumor-inhibitory effects in a variety of *in vitro* and *in vivo* experimental models of neoplasia (2). Multiple mechanisms, including cell cycle arrest, apoptosis, angiogenesis, and anti-cancer immunity, are suggested to underlie the anti-oncogenic effects of melatonin (3).

Accumulating evidence shows that melatonin induces mitochondrial energetic stress by reducing the efficacy of oxidative phosphorylation (4, 5). Moreover, melatonin-induced generation of mitochondrial reactive oxygen species (ROS) enhances the toxicity of cisplatin and radiation against head and neck cancer cells (6), this result is in contrast to previous results showing that melatonin acts as an anti-oxidant (7). Despite abundant reports showing the anti-tumor effects of melatonin, the underlying molecular mechanisms (particularly dynamic changes that are dependent on exposure time) and the effects on cell bioenergetics remain poorly understood. Moreover, it is unclear whether melatonin-induced mitochondrial dysfunction affects metabolic reprogramming of cancer cells.

Cancer cells undergo metabolic reprogramming, including enhancement of aerobic glycolysis, to support their increased energy requirements for biosynthesis of macromolecules during rapid proliferation (8). mTORC1 is a major regulator of cell growth and metabolism in cancer cells, and is implicated in upregulation of transcription factors such as hypoxia-inducible factor-1α and c-Myc (9, 10). A previous study shows that melatonin inhibits glycolysis and the tricarboxylic acid and pentose phosphate pathways in prostate cancer cells, suggesting that reduced glucose uptake is an anti-tumor effect of melatonin (11); however, it is

unclear whether melatonin-induced mitochondrial dysfunction in cancer cells affects metabolic reprogramming of glycolysis via mTORC1 and c-Myc.

Here, we evaluated the bioenergetic state of melatonin-treated cancer cells in parallel with changes in glycolysis. To analyze dynamic changes in melatonin-induced metabolic reprogramming, we focused particularly on temporal changes in mTORC1 activity and glycolysis

RESULTS

Acute effects of melatonin on mitochondrial function and production of reactive oxygen species in HCC cells

To explore the effects of melatonin on the bioenergetic status of cancer cells (7), we first measured mitochondrial respiration and glycolysis in Hep3B and Huh7 HCC cells treated with melatonin. Interestingly, acute treatment with melatonin led to an abrupt decrease in major parameters of mitochondrial function in HCC cells, including basal oxygen consumption rate (OCR) and maximal and ATP-linked respiration, without reducing glucose uptake, suggesting that mitochondrial dysfunction might precede changes in glycolysis in melatonin-treated HCC cells (Figure 1A–E). Indeed, melatonin had no effect on glycolysis, as measured by the extracellular acidification rate (ECAR) (Figure 1F and G). We also found that production of mitochondria-derived superoxide by HCC cells increased upon acute treatment with melatonin (Figure 1H and I). These data suggest that although acute administration of melatonin to HCC cells impairs mitochondrial function, accompanied by increased production of mitochondrial ROS, it does not affect glycolysis.

Temporal effects of melatonin on mTORC1, c-Myc, HK, and LDHA levels in HCC cells

Next, we investigated dynamic changes in mTORC1 activity and expression of glycolysis-related genes over time. Consistent with previous reports showing that mitochondrial ROS reduces mTORC1 (12), we found that after 3 h of treatment with melatonin, mTORC1 activity in HCC cells fell, as measured by detection of phosphorylated mTOR (S2448) and 70 kDa ribosomal protein S6 kinase (T389). However, expression of c-Myc and glycolysis-related genes, including HK2 and LDHA, did not change significantly (Figure 2A). By contrast,

activity of c-Myc and its downstream molecules HK2 and LDHA, as well as that of mTORC1, fell markedly when cells were exposed to melatonin for a longer time (Figure 2B). Consistent with changes in protein levels, expression of mRNA encoding HK2 and LDHA fell significantly and in a dose-dependent manner at 48 h post-melatonin administration; however, these alterations were not observed after 3 h of melatonin administration (Figure 2C and D). Notably, expression of mRNA encoding c-Myc was unchanged, even after 48 h of treatment (Figure 2C and 2D), confirming previous findings that S6K1 increases c-Myc translation initiation efficiency by modulating phosphorylation of eukaryotic initiation factor eIF4B (13). Furthermore, overexpression of c-Myc reversed melatonin-induced downregulation of HK2 and LDHA levels in HCC cells (Figure 2E and F). Given that ROS increases HIF-1α levels and melatonin decreases HIF-1α levels (14-16), we investigated whether melatonin-induced changes in HIF-1α levels are responsible for metabolic reprogramming in HCC cells. Considering the very low level of HIF-1α protein under normoxic condition (Supplementary Figure 1), it seems unlikely that HIF-1α is involved in the regulation of glycolysis-related genes in melatonin-treated HCC cells, although melatonin tended to reduce HIF-1α protein levels. Taken together, the data show that prolonged treatment with melatonin, which inhibits mTORC1 activity, downregulates c-Myc expression and glycolysis in HCC cells.

Chronic effects of melatonin on glycolysis and proliferation of HCC cells

Based on the results showing reduced levels of c-Myc, HK2, and LDHA after 48 h of treatment with melatonin, we next examined the effects of melatonin on glycolysis over a longer time scale. We found that melatonin reduced glycolysis and mitochondrial respiration in HCC cells (Figure 3A–F). Finally, we showed that melatonin reduced HCC cell proliferation and viability in a dose-dependent manner (Figure 4A–C). Overexpression of c-Myc significantly reverted

melatonin-induced suppression of HCC cell proliferation, supporting that c-Myc is a key factor in the regulation of cell proliferation in melatonin-treated HCC cells (Figure 4D). A clonogenic assay confirmed the effects of melatonin on HCC proliferation (Figure 4E). Consistent with these data, caspase 3/7 activity, the levels of cleaved caspase-9, -3, -7 and PARP-1 in melatonin-treated HCC cells increased, further confirming the pro-apoptotic function of melatonin in cancer cells (Figure 4F and G).

DISCUSSION

The data presented herein reveal the bioenergetic status of melatonin-treated HCC cells over time. Melatonin acutely induced mitochondrial energetic stress and ROS accumulation, which attenuated mTORC1 activity. Longer administration of melatonin decreased protein levels of c-Myc, which was responsible for suppressing glycolysis via downregulation of HK2 and LDHA (Figure 4H). Thus, simultaneous attenuation of oxidative phosphorylation and glycolysis contributes to the anti-cancer effects of melatonin against HCC cells.

Although some previous studies report that melatonin improves mitochondrial respiration and ATP production, more recent studies report that melatonin induces mitochondrial depolarization and energetic stress in some cancer cells, while at the same time decreasing oxidative phosphorylation by inhibiting complex IV (4, 7, 17). Furthermore, melatonin evokes a concentration-dependent increase in ROS generation by mitochondria (18). We also observed melatonin-induced mitochondrial ROS accumulation in HCC cells, which was accompanied by melatonin-induced mitochondrial energetic stress, as evidenced by a decrease in basal OCR, maximal respiration, and ATP-linked respiration; these data support previous findings demonstrating that mitochondrial ROS induce damage to the mitochondrial respiratory chain (19).

Mitochondrial ROS can induce dephosphorylation of mTOR and p70 ribosomal protein S6 kinase directly in glioma cells in a Bcl-2/E1B 19 kDa interacting protein 3 (BNIP3)-dependent manner (12, 20). Mitochondrial energy stress is also responsible for suppression of mTORC1 activity (5). Given that dysregulated mTORC1 signaling is implicated in cancer progression (21), melatonin-induced suppression of mTORC1 activity may be an anti-tumor effect of melatonin (22). Consistent with this, we found that acute treatment with melatonin-induced energetic stress and mitochondrial ROS generation, both of which contribute to

attenuation of mTORC1 activity in HCC cells.

mTORC1 promotes glycolysis via HIF-1α and c-Myc (9). Intriguingly, we found that melatonin-induced inhibition of mTORC1 activity occurred at 3 h post-treatment with melatonin. However, glycolysis (as evidenced by measurement of ECAR and altered expression of metabolism-related genes such as those encoding enzymes required for glycolysis, i.e., HK2 and LDHA) was affected only after longer treatment with melatonin. Given that translation of c-Myc protein is increased by activated mTORC1, our findings suggest that melatonin reduces mTORC1 activity by inducing energetic stress, which then leads to further disruption of the bioenergetic needs of the growing cancer cell via downregulation of c-Myc and, subsequently, glycolysis. Thus, the anti-cancer effects of melatonin in HCC are due to simultaneous suppression of mitochondrial OCR and glycolysis.

In summary, we show here that melatonin suppresses HCC proliferation via downregulation of oxidative phosphorylation and glycolysis. We uncovered that melatonin abrogates both oxidative phosphorylation and glycolysis by analyzing changes in mitochondrial ROS and mTORC1, c-Myc, and glycolysis over time. Given its broad clinical utility, melatonin could act as an adjuvant in a potential therapy for liver cancer.

MATERIALS AND METHODS

Cell culture

The liver cancer cell lines Hep3B (ATCC, Manassas, VA, USA) and Huh7 (Korean Cell Line Bank, Seoul, Korea) were cultured in EMEM and DMEM medium, respectively, containing 10% fetal bovine serum and 1% penicillin/streptomycin (P/S).

Measurement of OCR and ECAR

OCR and ECAR were measured in 24-well plates using a Seahorse XF-24 analyzer (Seahorse Bioscience, North Billerica, MA, USA). The short-term XF assay used to assess the effects of melatonin on metabolic function was performed by injecting vehicle or melatonin (2 mM; Sigma, St. Louis, MO, USA) at an indicated time during OCR and ECAR measurement. The long-term effect of melatonin on metabolic function was measured after Hep3B and Huh7 cells were treated with vehicle or melatonin for 48 h. Oligomycin (1 μM; Sigma), carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 5 μM; Sigma), and rotenone (1 μM; Sigma) were added at the indicated times during OCR measurement. For ECAR measurements, glucose (10 mM; Sigma), oligomycin (1 μM; Sigma), and 2-deoxyglucose (100 mM; Sigma) were added at the indicated time points during ECAR measurement. Seahorse datasets were normalized to protein content.

Measurement of glucose uptake

After cells were treated with melatonin for 10 min, glucose uptake was measured using the Glucose Uptake-Glo™ Assay (Promega, Madison, WI, USA).

MitoSOX

Mitochondrial ROS generation was assessed using MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific, Waltham, MA, USA). Hep3B and Huh7 cells were treated with or without melatonin for 30 min and stained with 5 μM MitoSOX reagent for 10 min at 37°C in the dark. The cells were then washed gently three times with warm HBSS buffer. Finally, cells were counterstained with NucBlue Live Cell Stain ReadyProbes (Thermo Fisher Scientific) and mounted in warm buffer for imaging. MitoSOX fluorescence intensity was quantified using Image J software.

Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 5 mM EDTA [pH 8.0], 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40) containing aprotinin, leupeptin, PMSF, and phosphatase inhibitor cocktail 3 (Sigma). Protein samples were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated overnight at 4°C with appropriate primary antibodies against phospho-mTOR (S2448), mTOR, phospho-p70S6K (T389), p70S6K, c-Myc, LDHA, HK2, HIF-1α, cleaved caspase-3, -7, -9, PARP-1 (Cell Signaling Technology, Danvers, MA, USA), or β-actin (Sigma)). Membranes were washed three times with TBST and then incubated with an HRP-conjugated anti-rabbit or anti-mouse secondary antibody (GeneTex, Irvine, CA, USA). HRP was detected using the ECL reagent (BioNote, Suwon, Korea).

Real-time PCR

Total RNA was prepared using QIAzol lysis reagent (Qiagen, Fredrick, MD, USA) and complementary DNA (cDNA) was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). The resultant cDNA was amplified on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated using the $\Delta\Delta C_T$ method; the levels of each mRNA were normalized against the corresponding level of a *36B4* mRNA primer. The primer sequences were as follows: *c-myc* forward, CGAGGAGGAGAACTTCTACCAGC, and reverse, CGAGAAGCCGCTCCACATACAGTCC; *LDHA* forward, CACCCAGTTTCCACCATGATT, and reverse, CAAGGAACACTAAGGAAGACATCATC; *HK2* forward, TGCAACACTTAGGGCTTGAG, and reverse, TTCTCGTATCCTGTCCACCA; *36B4* forward, CCACGCTGCTGAACATGCT, and reverse, TCGAACACCTGCTGGATGAC.

Transfection of cDNA construct

Cells were transfected with the c-Myc expression vector (Korean Human Gene Bank, Daejeon, Korea) or a control vector using TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions.

Cell proliferation and caspase 3/7 activity

Hep3B and Huh7 cells were treated with or without melatonin for 48 h. For cell counting, cells were trypsinized, stained with Trypan blue solution, and counted with a hemocytometer. NADH- or ATP-based cell viability was measured using CCK8 Solution Reagent (CK04; Dojindo, Japan) or a CellTiter-Glo® Luminescent Cell Viability Assay (Promega), respectively. The Caspase-Glo 3/7 assay (Promega) was used to measure caspase 3/7 activity. For the

clonogenic assay, HCC cells were treated with or without melatonin for 10 days, followed by fixation and staining with 0.5% crystal violet (Sigma).

Statistical analyses

All values are presented as the mean ± SEM. Statistical analysis was performed using a two-tailed Student's t-test. A p value <0.05 was considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflicting interests.

FIGURE LEGENDS

Figure 1. Acute effects of melatonin on mitochondrial dysfunction and ROS production in Hep3B and Huh7 cells. (A) Mitochondrial oxygen consumption rate (OCR) in Hep3B and Huh7 cells treated with or without melatonin (2 mM) at the indicated times following exposure to oligomycin (1 μM), CCCP (5 μM), rotenone (1 μM). Data are expressed as a ratio relative to baseline. (B-D) Relative basal OCR (B), relative maximal respiration (C), and relative ATP-linked respiration (D) in the melatonin-treated HCC cells shown in (A). (E) Relative glucose uptake in Hep3B and Huh7 cells treated with or without melatonin for 10 min. (F) Extracellular acidification rate (ECAR) in Hep3B and Huh7 cells treated with or without melatonin (2 mM) at the indicated times with glucose (10 mM), oligomycin (1 μM), and 2-deoxyglucose (2-DG; 100 mM). Data are expressed as ratios relative to the baseline. (G) Relative glycolysis in the HCC cells shown in (F). Data are expressed as the mean ± SEM (n = 4-5). (H) Representative fluorescence micrographs showing mitochondrial superoxide production by Hep3B and Huh7 cells treated with or without melatonin for 30 min. (I) Bar graph showing quantitation of MitoSOX fluorescence intensity. Scale bar, 20 μM. Data are expressed as the mean ± SEM (n = 3). n.s., not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2. Temporal effects of melatonin on mTORC1 and glycolytic proteins in Hep3B and Huh7 cells. (A, B) Levels of phosphorylated mTOR, S6K, c-Myc, LDHA, and HK2 protein in Hep3B and Huh7 cells treated with or without melatonin for 3 h (A) or 48 h (B). (C, D) Levels of *c-Myc*, *LDHA*, and *HK2* mRNA in Hep3B and Huh7 cells treated with or without melatonin for 3 h (C) or 48 h (D). Data are normalized against 36B4 mRNA levels and expressed as the mean ± SEM of three independent experiments. (E and F) Effects of c-Myc on levels of LDHA and HK2 in Hep3B (E) and Huh7 (F) cells treated with melatonin (2mM)

for 48 h. n.s., not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3. Chronic effects of melatonin on mitochondrial dysfunction and glycolysis in Hep3B and Huh7 cells. (A, B) Mitochondrial oxygen consumption rate (A) and extracellular acidification rate (B) in Hep3B and Huh7 cells treated with or without melatonin (2 mM) for 48 h following exposure to oligomycin (1 μM), CCCP (5 μM), rotenone (1 μM), glucose (10 mM), and 2-deoxyglucose (2-DG; 100 mM). (C–E) Basal OCR (C), maximal respiration (D), and ATP-linked respiration (E) in the Hep3B and Huh7 cells shown in (A). (F) Glycolysis in the Hep3B and Huh7 cells shown in (B). Data are expressed as the mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. Melatonin inhibits Hep3B and Huh7 cell proliferation and increases apoptosis. (A–C) Hep3B and Huh7 cells were treated with or without melatonin for 48 h. Relative cell number (A) and measurement of ATP- or NADH&NADPH-based cell viability (B, C) in melatonin-treated Hep3B and Huh7 cells. (D) Effects of c-Myc (as shown in Fig. 2E and F) on relative cell numbers of Hep3B and Huh7 cells treated with melatonin (2mM) for 48 h. (E) Clonogenic assay of Hep3B and Huh7 cells treated with or without melatonin for 10 days (left panel). Quantification of the number of colonies following treatment with the indicated concentrations of melatonin (right panel). (F) Relative caspase 3/7 activity in Hep3B and Huh7 cells treated with melatonin for 48 h. (G) Levels of cleaved caspase-9, -3, -7 and PARP-1 in Hep3B and Huh7 cells treated with melatonin for 48 h. (H) Schematic showing the role of melatonin in inhibition of glycolysis via downregulation of mitochondrial respiration and mTORC1 activity. Data are expressed as the mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

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Figure 1

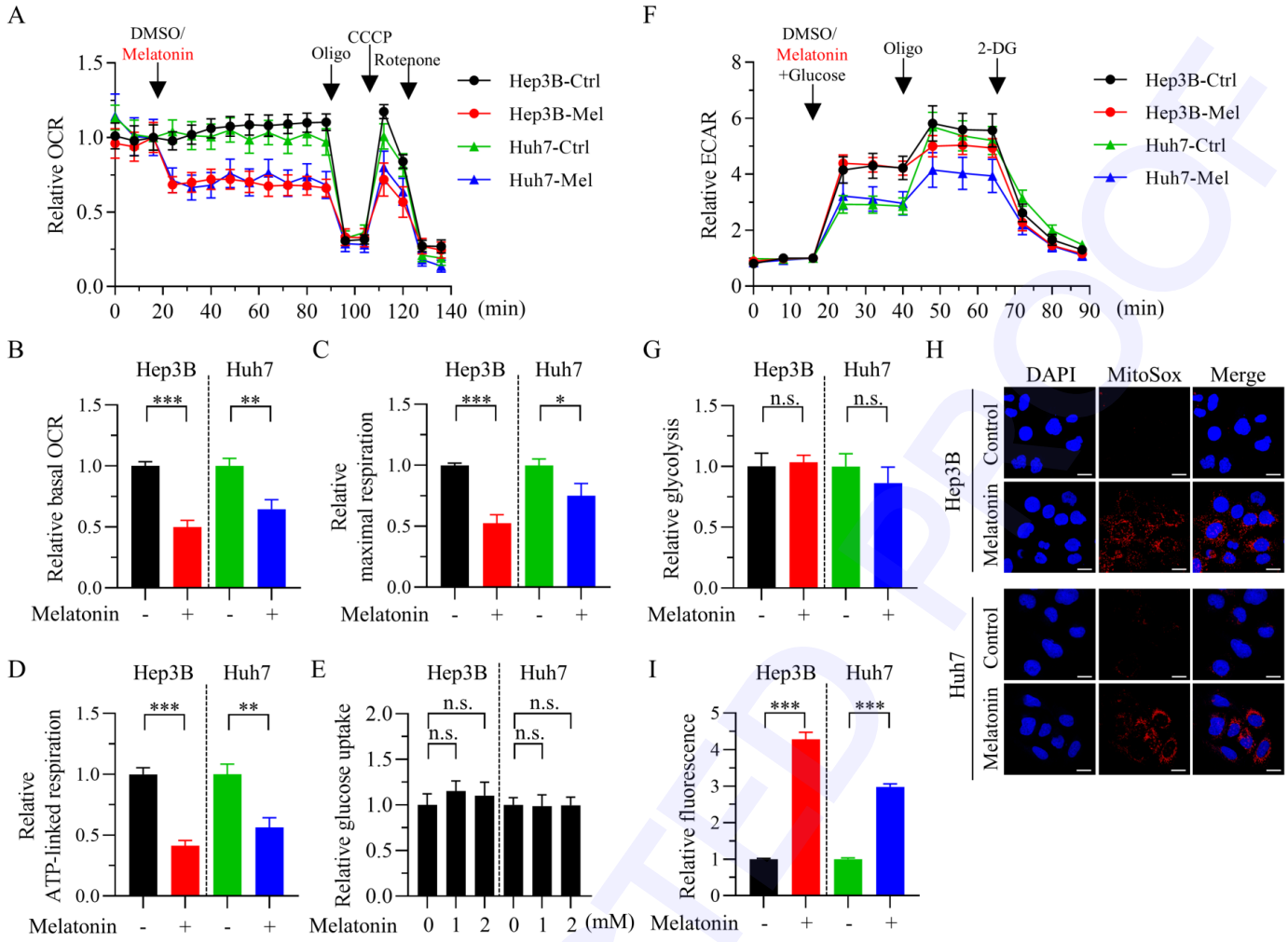

Fig. 1. Revised Figure 1

Figure 2

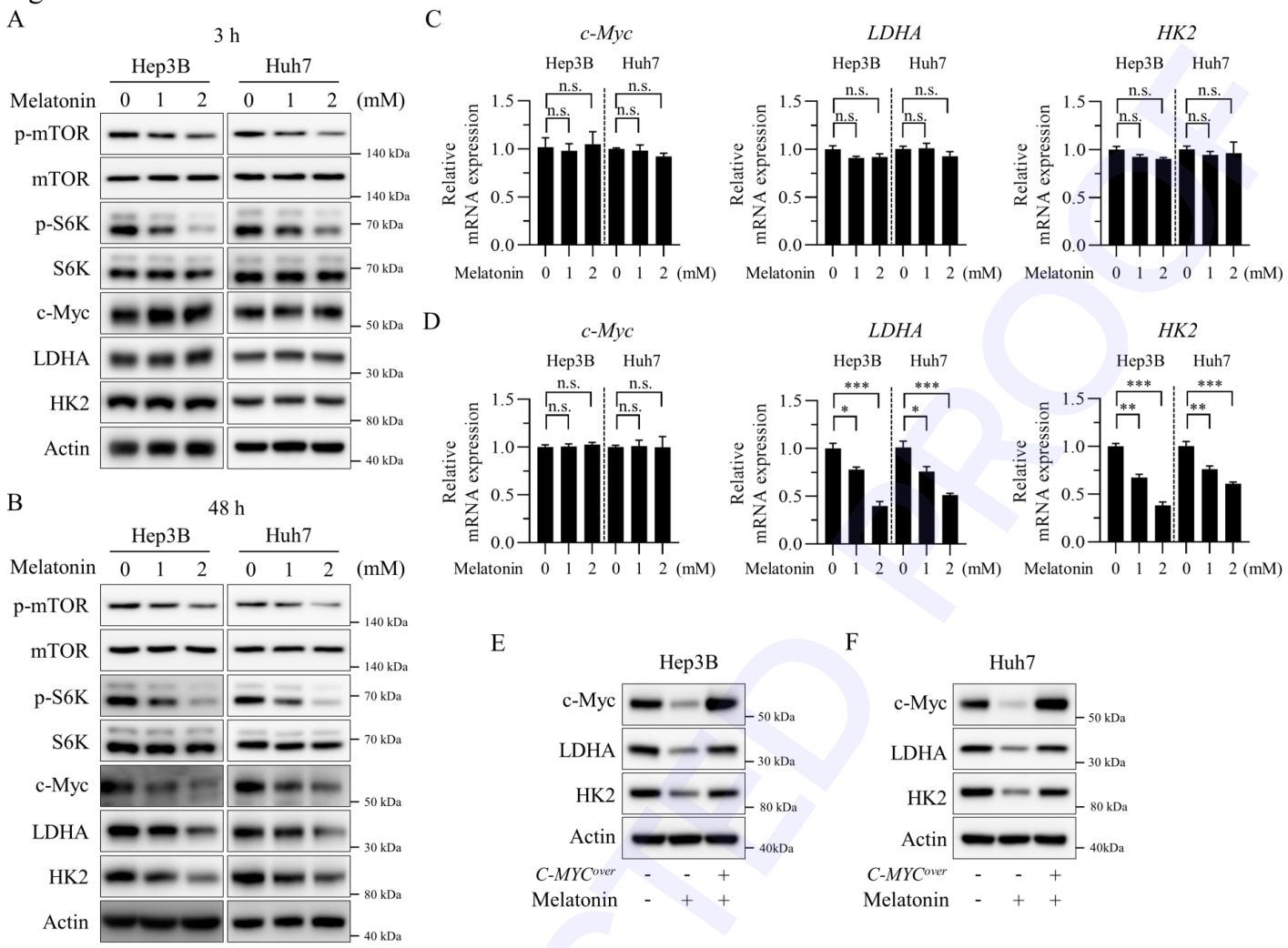

Fig. 2. Revised Figure 2

Figure 3

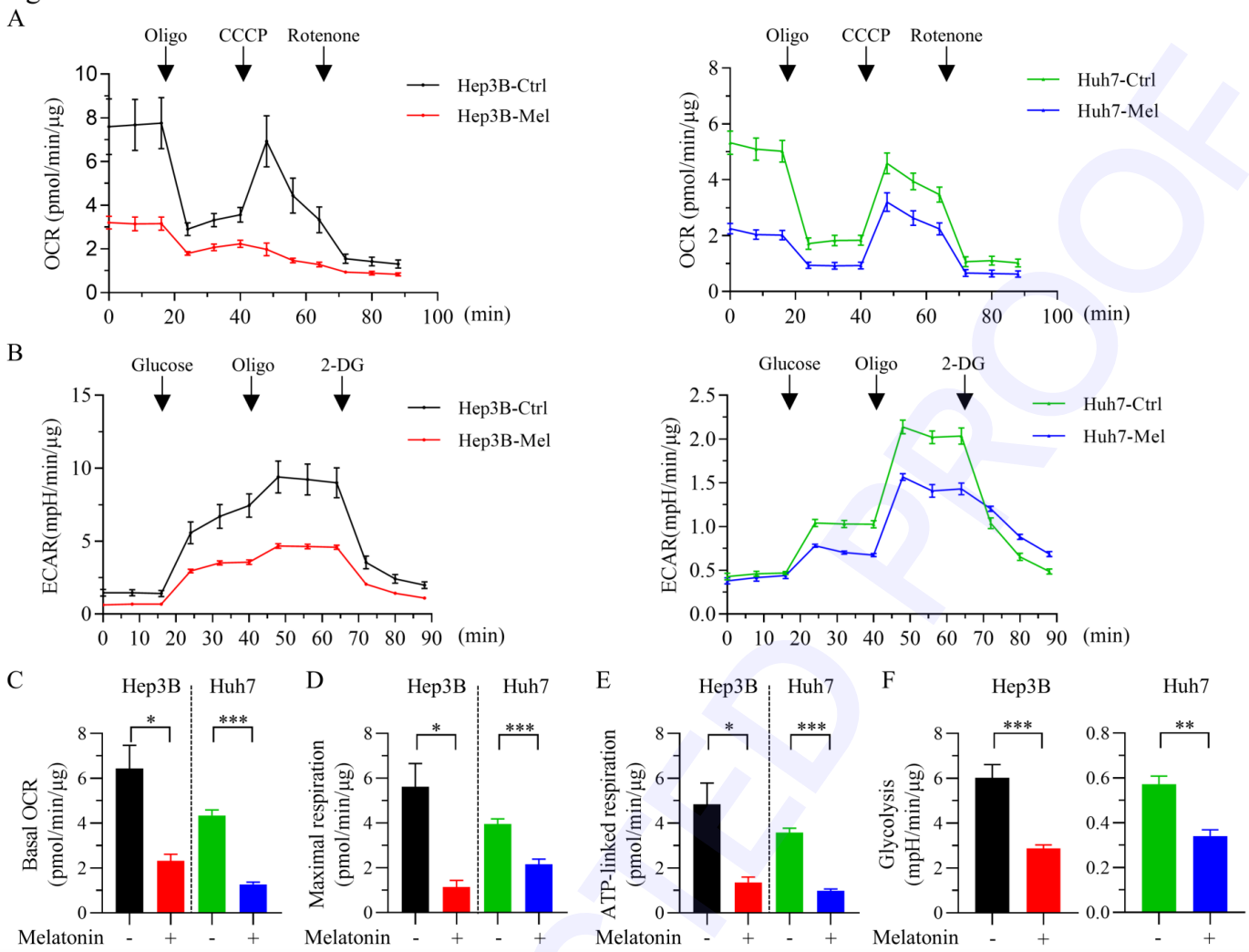

Fig. 3. Revised Figure 3

Figure 4

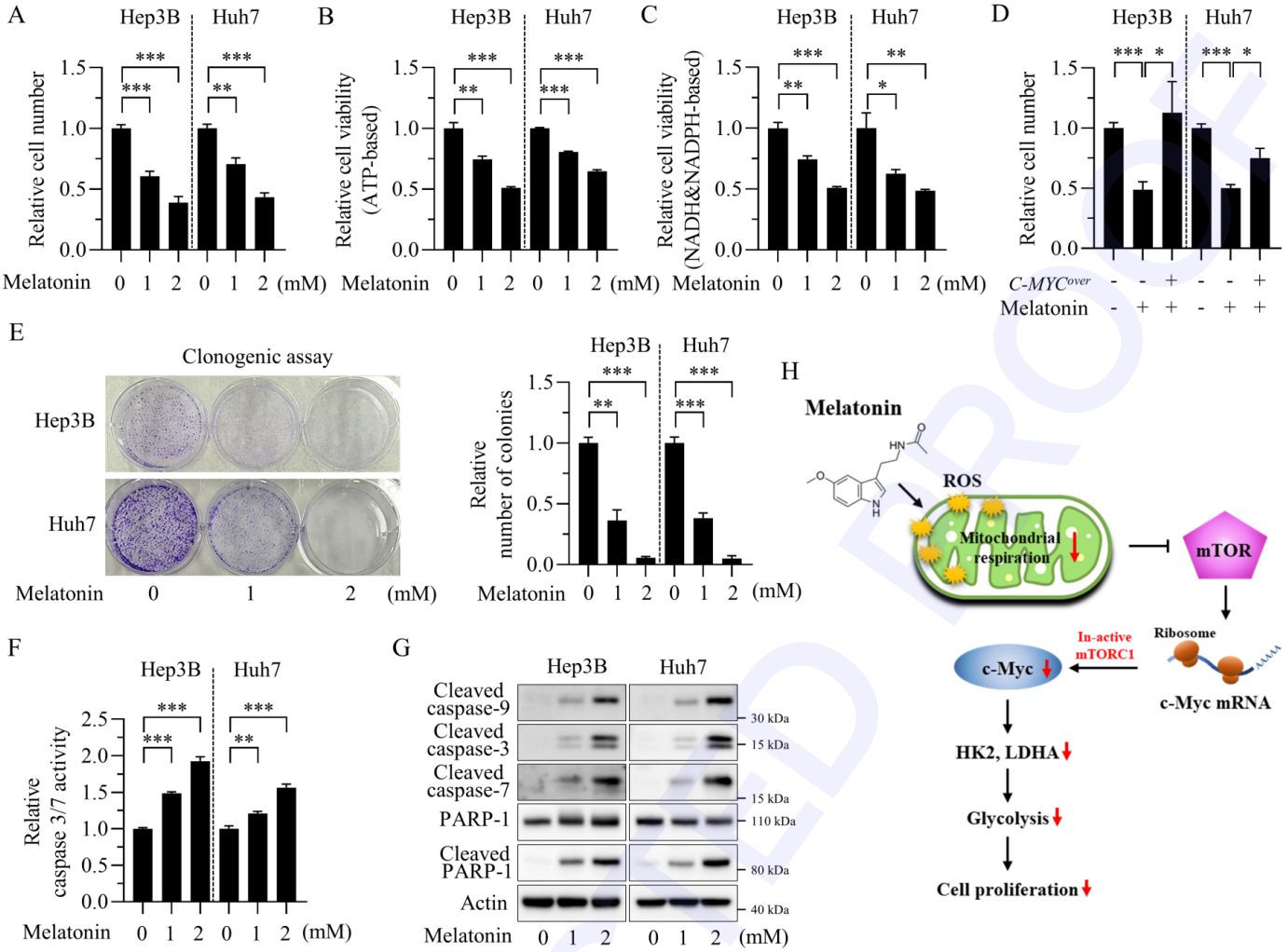

Fig. 4. Revised Figure 4

Manuscript Type: Article

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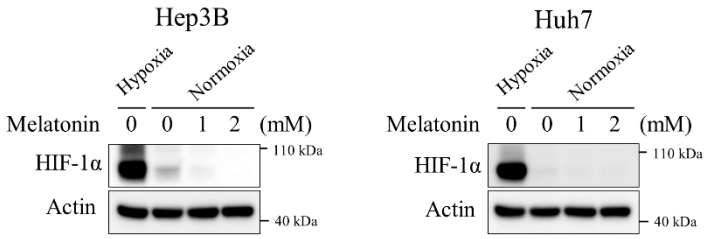
Supplementary figure 1



Supplementary Figure 1. Effects of melatonin on HIF-1α levels. Levels of HIF-1α in Hep3B and Huh7 cells treated with or without melatonin under hypoxia (1%) or normoxia for 3 h.