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Melatonin modulates dysregulated circadian clocks in mice with diethylnitrosamine-induced hepatocellular carcinoma

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Running head: Circadian clock genes and melatonin in hepatocarcinoma

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Abstract

Disruption of circadian rhythms, which are regulated by the circadian clock machinery, plays an important role in different long-term diseases including hepatocellular carcinoma (HCC). Melatonin has been reported to alleviate promotion and progression of HCC, but the potential contribution of circadian clock modulation is unknown. We investigated the effects of melatonin in mice which received diethylnitrosamine (DEN) (35 mg/kg body weight i.p.) once a week for 8 weeks. Melatonin was given at 5 or 10 mg/kg/day i.p. beginning 4 weeks after the onset of DEN administration and ending at the sacrifice time (10, 20, 30, or 40 weeks). Liver expression of Bmal1, Clock, Npas2, Rorα and Sirt1 increased, whereas Cry1, Per1, Per2, Per3, CK1ε, Rev-erbα and Rev-erbβ decreased following DEN administration. Melatonin treatment prevented changes in the expression of clock genes, and this effect was accompanied by an upregulation of the MT1 receptor and reduced levels of the hypoxia inducible factors Hif-1α and Hif-2α. An increased expression of p21, p53 and PARP1/2 a higher Bax/Bcl-2 ratio, and a lower expression of Cyclin D1, CDK6, HSP70, HSP90 and GRP78 proteins, was also observed in melatonin-treated mice. Melatonin significantly potentiated the suppression of proliferation and cell cycle arrest induced by the synthetic REV-ERB agonist SR9009 in human Hep3B cells, and BMAL1 knocking down attenuated the proapoptotic and antiproliferative effect of melatonin. Results support a contribution of changes in the circadian clock components to the beneficial effects of melatonin in HCC and highlight the usefulness of strategies modulating the circadian machinery in hepatocarcinogenesis.

KEY WORDS

circadian clocks, melatonin, hepatocellular carcinoma, diethylnitrosamine, SR9009, Hep3B cells.

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1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality worldwide, characterised by its high incidence and malignancy, along with an aggressive progression. Moreover, it is well known that HCC develops resistance to chemotherapeutic agents, which complicates patient management.[1] Clinical and experimental animal studies have revealed that a range of genes, proteins and enzymes levels in liver are controlled by circadian rhythms to a great extent.[2] Moreover, disruption of the circadian rhythm leads to increased risk of cancer, and genes that regulate this rhythm, "clock" genes, are reported to influence tumorigenesis related mechanisms as cell proliferation,[3] apoptosis,[4] and cell cycle control,[5] leading to the occurrence and progression of many types of cancer, including HCC.[6]

At cellular and molecular levels, the circadian clock consists on many oscillators that form the feedback timing circuit. Specifically, autoregulatory negative feedback loops which are controlled by the crosstalk between positive and negative clock components.[7] A positive feedback loop includes the brain and muscle arnt-like protein 1 (Bmal1), together with circadian locomotor output cycles protein kaput (Clock) or neuronal PAS domain protein 2 (Npas2). These form heterodimers (BMAL1/CLOCK or BMAL1/NPAS2) which activate transcription of the period genes (Per1, Per2 and Per3), and the cryptochrome genes (Cry1 and Cry2). CRY and PER proteins are transported to the cytoplasm, forming PER/CRY complexes that translocate into the nucleus and associate with BMAL1/CLOCK heterodimer, inhibiting and reducing the transcriptional activation of BMAL1 and CLOCK, and supressing its own gene transcription. The PER/CRY complexes are subsequently phosphorylated by casein kinase 1 epsilon (CK1ε), leading to PER and CRY degradation. Another negative feedback is represented by the nuclear receptor subfamily 1 group D members 1, and 2 (REV-ERBα and β), and the retinoic acid receptor-related orphan receptor alpha (RORα) proteins, which translocate into the nucleus to repress and promote BMAL1 expression,

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respectively.[8] Sirtuin 1 (SIRT1) is reported to be involved in multiple disease processes that include cancer, and also in circadian clock regulation.[9] SIRT1 and PER2 constitute a reciprocal negative regulation loop that plays important roles in modulating hepatic circadian rhythmicity.[10,11]

The pineal hormone melatonin has demonstrated its potential usefulness in the treatment of a number of pathologies, including cancer, where it acts as an effective oncostatic agent.[12-14] Previous research has shown that this indoleamine exerts anti-proliferative, pro-apoptotic, anti-invasiveness and anti-angiogenic effects in HCC cell lines,[1,15-20] and also plays a protective role in murine models of DEN-induced hepatocarcinogenesis.[21,22] Evidence from different studies indicate that melatonin and the core clock genes play a crucial role in breast cancer development,[12] and it has been reported that melatonin administration resynchronizes the circadian rhythm in human prostate cancer cells.[23] The present research aimed to identify if regulation of clock genes may contribute to the beneficial effects of melatonin in a mice model of (DEN)-induced HCC and in human HCC cells. Results obtained support the usefulness of melatonin as an oncostatic agent and the interest of the circadian machinery as an attractive target for therapeutic interventions in HCC.

2 MATERIALS AND METHODS

2.1 Animal experiments and drug treatment

Male ICR mice (Harlan Laboratories, BCN, Spain) weighing 20-25 g were used for this study. The animals were acclimated to the temperature (22±2ºC) and humidity (55±5%) of controlled rooms with a 12 hours light-dark cycle for at least a week prior to experiments. They were allowed free access to food and water *ad libitum* until the time of experiments in the Laboratory Animal Facility of the University of León, Spain. To induce hepatocarcinogenesis 6-week-old mice in treatment groups were given DEN (Sigma Aldrich,

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St. Louis, MO, USA) at a dose of 35 mg/kg body weight (diluted in saline 0,9%) via intraperitoneal injection once a week for 8 weeks. Mice in the control groups received saline from the beginning of the experiments. Melatonin (Sigma Aldrich) was administered 2 hours before lights off via intraperitoneal injection (5 or 10 mg/kg/day), beginning four weeks after the onset of DEN or saline administration and ending at the sacrifice time per each group (10, 20, 30 and 40 weeks). Melatonin was dissolved into absolute ethanol and further dilutions were made in saline; the final concentration of ethanol was 2.5%. The study protocol was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was specifically approved by the Ethics Committee of the University of León. 24 hour after the last injection of melatonin or vehicle, mice were anesthetized with ketamine/xylazine cocktail and sacrificed. The liver was dissected out, weighed and immediately transferred to ice until its storage at -80ºC. Fresh liver samples for further analysis were taken at sacrificed time. Animals were included randomly in 16 groups. Each group consisted of four to eight mice.

2.2 Biochemical analysis

The levels of alanine aminotransferase (ALT) in serum of mice were determined in the Instrumental Techniques Laboratory of the University of León using standard techniques.

2.3 Histology and immunohistochemistry

Tissue samples from sections of three hepatic sublobes (left lateral lobe, left medial lobe, and right medial lobe) were recovered, fixed in 10% buffered formalin for 24 hours. The blocks were dehydrated in a graded ethanol series and embedded in paraffin wax. Serial 3- μm sections were stained with hematoxylin and eosin (H&E), and histologically evaluated. Immunohistochemistry was performed using polyclonal antibodies for BMAL1 and REV-

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ERBα. Tissue samples were recovered, fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were dewaxed and hydrated through graded ethanol, cooked in 25 mM citrate buffer, pH 6.0, in a pressure cooker for 10 minutes, transferred into boiling deionized water and let to cool for 20 minutes. Tissue sections were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidase activity.[24] The slides were incubated with rabbit anti-BMAL1, and rabbit anti-REV-ERBα polyclonal antibodies (ThermoFisher Scientific, Rockford, IL, USA) at a working dilution of 1:200 and 1:100, respectively, overnight at 4°C. Subsequently, the sections were incubated for 30 minutes using the EnVision+ system and developed with a solution of 3-3-diaminobenzidine (DAB) (Vector Lab, Burlingame, CA, USA). The specificity of the technique was evaluated by negative controls (omitting the incubation with the primary antibody and incubating it with non-immune sera). Areas of staining were analyzed by WinRoof version 6.3 software with ten non-consecutive randomly chosen histological fields. Results were expressed as the percentage of stained cells in each field.

2.4 Cell culture

Hep3B cells from the American Type Culture Collection (Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's medium (DMEM)-high glucose, supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/mL) in a humidified 5% CO_2 atmosphere at 37ºC. Cell culture reagents were purchased from Sigma Aldrich. For treatments, regular medium was replaced with fresh medium containing 2% FBS, and 0.5 or 1 mM melatonin with or without SR9009 (Merck KGaA, Darmstadt, Germany) at 10 μM.[25] Both reagents were dissolved in DMSO (0.05% at final concentration).

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2.5 BMAL1 siRNA transfection

Commercial siRNA against BMAL1 (sc-38166), and negative control siRNA (NC-siRNA) encoding a non-targeting sequence (sc-36869) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and introduced into Hep3B cells by reverse transfection using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. BMAL1-siRNA was used with a final concentration of 25 nM. The cells were maintained in transfection medium for 5 hours. Then the medium was replaced with fresh DMEM containing 10% FBS. To assess transfection efficiency, cells transfected with NC-siRNA were analyzed via fluorescent microscopy at 24 hours post-transfection. Then, cells were treated with or without melatonin (0.5 or 1 mM) for 24 hours after transfection assessment. All the transfections were undertaken independently in triplicate. Cells were collected for proliferation assay and examination of protein expression after 48 hours of siRNA transfection.

2.6 Cell proliferation assay

Hep3B cells were seeded into 96-well plates (7.5×10^3 cells/well), and proliferation was determined using a CyQUANT cell proliferation assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The fluorescence intensity was measured at 530 nm spectral wavelength using a fluorescence microplate reader (BioTek Instruments, Inc).

2.7 Quantitative real-time PCR

Total RNA was obtained from frozen mouse liver and Hep3B cells using a Trizol reagent (Life Technologies, Madrid, Spain) and quantified using a Nano Drop1000 spectrophotometer (ThermoFisher Scientific). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA).[26] RNA

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integrity was confirmed by formaldehyde gel electrophoresis. Total RNA (1 μg) was reverse transcribed and mRNA was determined by real-time PCR analysis using SYBER Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and the appropriate primers (Table 1), or Taqman Universal PCR MasterMix (Roche Diagnostics GmbH, Mannheim, Germany). Taqman primers and probes for vascular endothelial growth factor (VEGF) (GenBank accession no. NM_001025250.3 and Mm00437306_m1), endoglin (CD105) (GenBank accession no. NM_001146348.1 and Mm00468256_m1), and β-Actin (GenBank accession no. NM_007393.1 and Mm00607939_s1) genes were derived from the commercially available TaqMan Gene Expression Assay (Applied Biosystems). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta ct}$ method. The cycle number at which the transcripts were detectable (Ct) was normalized to the cycle number of β-Actin gene detection, referred to as ΔCt.[27]

2.8 Western blot assay

Western blot analyses were performed on liver tissue and Hep3B cells. Extracts were homogenized in 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris and a phosphatase and protease inhibitor cocktail (Roche Diagnostics GmbH), maintaining temperature at 4ºC throughout all procedures. Then the homogenate was incubated on ice for 30 minutes, and finally, the samples were centrifuged at 12,000 *g* for 30 minutes at 4ºC. The supernatant fraction was stored at -80ºC in aliquots until use. Protein concentration was measured by Bradford assay. Equal amounts of protein extracts (30 μg) were separated by 9-12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis for 1.5 hours at 100 V and transferred electrically to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 minutes at 37ºC and probed overnight at 4ºC with polyclonal anti-BMAL1,

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CLOCK (ThermoFisher Scientific), heat shock proteins (HSP)70, HSP90, glucose regulated protein 78 (GRP78), REV-ERBα, REV-ERBβ (Abcam, Cambridge, UK), RORα, Cyclin D1, cyclin dependent kinase 6 (CDK6), p21, poly(ADP-ribose) polymerase 1 and 2 (PARP1/2), Bcl-2-associated X protein (Bax), c-Myc (Santa Cruz Biotechnology), p53, B-cell lymphoma 2 (Bcl-2), cleaved caspase 3 (Cell Signaling Technology, Leiden, The Netherlands) antibodies at 1:200-1:1,000 dilution with PBST containing 2.5% non-fat dry milk. Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-β-Actin polyclonal antibody (1:20,000; Sigma Aldrich) for the whole extracts, and with a rabbit anti-Lamin B1 polyclonal antibody (1:200; Santa Cruz Biotechnology) for the nuclear extracts. After washing with TBST, the membranes were incubated for 1 hour at room temperature with secondary HRP conjugated antibody (1:5,000; Dako, Glostrup, Denmark), and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden).[28,29] The density of the specific bands was quantified with an imaging densitometer (Scion Image J Software 1.46a, Bethesda, MD, USA).

2.9 Statistical analysis

Data are expressed as mean values ± standard error of the mean (SEM) and compared by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test when the analysis indicated the presence of a significant difference. Significance was accepted when $P<.05$. Games-Howell's test is used with unequal variances previously determined by Levenne's test. All tests were performed using the statistical package SPSS 22.0 (IBM Corporation, Armonk, NY, USA).

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3 RESULTS

3.1 Effect of melatonin on pathological changes in DEN-treated mice

Results showed that the hepatic-somatic ratio (HSR) and serum ALT activity were significantly higher at 40 weeks in DEN-treated animals compared to melatonin co-administered groups. Both doses of melatonin reduced the increase in the mRNA levels of the angiogenic markers VEGF and CD105 induced by DEN administration. Hepatic lesions showing dysplastic or hyperplastic areas were more frequently in animals treated with melatonin that those receiving DEN. However, DEN-injected animals developed a higher number of nodules categorized as adenomes and carcinomes (Table 2).

3.2 Effect of melatonin on circadian clock genes in DEN-induced hepatocarcinogenesis

In order to investigate whether melatonin ameliorates HCC progression in mice through the regulation of the circadian clock machinery, we examined the mRNA expression of the main clock genes involved in the pathway, Bmal1, Clock, Npas2, Per1, Per2, Per3, Cry1, CK1ε, Rev-erbα, Rev-erbβ, Rorα, and Sirt1, by qRT-PCR (Figure 1A, Figure 2 and Figure 3A). To corroborate the mRNA expression profile data and to assess whether the dysregulation of circadian genes resulted in a differential expression pattern at protein level, BMAL1, CLOCK, REV-ERBα, REV-ERBβ, and RORα were evaluated by Western-blot (Figure 1B and Figure 3B). Since the first experimental periods Bmal1, Clock and Npas2, the main components of the primary circadian loop, showed a significant upregulation at mRNA levels in DEN groups when compared with control group (Figure 1A). BMAL1 and CLOCK protein expression also increased significantly (Figure 1B). These effects were significantly prevented by melatonin administration (Figure 1A,B). To further evaluate the protein expression pattern of the transcription factor BMAL1 immunohistochemistry was performed,

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which revealed a nuclear location with a high expression in DEN groups that was significantly reduced in mice treated with DEN and melatonin (Figure 1C,D). Per1, Per2, Per3, and Cry1, which constitute a regulatory negative feedback loop of BMAL1/CLOCK complex,[2] showed reduced mRNA levels from 20 weeks up to the end of the study in animals receiving DEN. This effect was abolished by melatonin (Figure 2). CK1ε, the casein kinase that phosphorylates PER and CRY proteins thus leading to their degradation, was also downregulated after DEN-induced HCC. However, melatonin upregulated gene expression at the higher dose of the indole, 10 mg/kg (Figure 2).

We also investigated the negative feedback loop comprised by nuclear receptor REV-ERB and RORα. REV-ERBα and REV-ERBβ repress the transcription of BMAL1, in contrast to the role exerted by RORα that activates its transcription.[3] Our results showed, as expected, that both REV-ERBα and REV-ERBβ mRNA and protein expression decreased in DEN groups from 10 weeks. Interestingly, melatonin augmented these expressions in a dose dependent manner, mainly in the two latest time-points of the study (Figure 3A,B). Data were confirmed by immunostaining, which revealed a nuclear location of REV-ERB with a significantly lower expression in DEN groups when compared with control groups since the first periods of the study, in a similar expression pattern of that observed in mRNA and protein assays. Melatonin was able to restore the downregulated expression after DEN administration (Figure 3C,D). In contrast, RORα mRNA and protein were overexpressed in DEN-injected animals and this effect was significantly abrogated by melatonin (Figure 3A,B).

The mRNA expression of Sirt1 was also evaluated owing to its role in modulating the circadian clocks[10] and its influence in cellular responses by regulating cell cycle, DNA repair, cell survival, and apoptosis.[30] Melatonin prevented the Sirt1 upregulated levels from 20 weeks of the onset of the experiment up to the end (Figure 2).

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It is known that melatonin administration, via its MT1 membrane receptor, repress elements of the peripheral oscillator in breast cancer cells.[31] Thus, we evaluated MT1 expression in each of the time-points of the study, finding a significant upregulation from 10 weeks onwards in groups where melatonin was administered (Figure 2). Abnormal expression of clock genes is one of the causes derived from hypoxia in HCC, and it has been reported that overexpression of the hypoxia inducible factors (HIF)-1α and -2α induced in HCC cells after treatment with $CoCl_2$ alters the expression pattern of circadian genes.[32] Results from the present study showed that melatonin is able to reduce the overexpressed levels of these transcription factors at 30 and 40 weeks (Figure 2).

3.3 Effect of melatonin on cell cycle regulatory proteins, apoptosis and unfolded protein response (UPR) in DEN-induced hepatocarcinogenesis

Genes related to cell cycle and apoptosis pathways play critical roles in cancer and their expression may be regulated by the circadian machinery.[5,33,34] In our study, Western blotting showed a progressive increase in Cyclin D1 and CDK6 from 20 weeks after DEN administration. However, in DEN-treated mice receiving melatonin, expressions were significantly lowered, reaching a higher effect with the 10 mg/kg dose of the indole (Figure 4). The expression of the tumor suppressor proteins p21 and p53 decreased with DEN administration at 30 and 40 weeks and increased following melatonin treatment in a dose-dependent manner (Figure 4). We also observed that the expression of the apoptosis marker PARP1/2 decreased in DEN-treated animals from 30 week up to the end, and was significantly augmented by melatonin, showing a restoration of normal levels (Figure 4). Melatonin administration also increased the balance between apoptotic and anti-apoptotic proteins, measured by the Bax/Bcl-2 ratio (Figure 4).

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The endoplasmic reticulum (ER) stress and the UPR play a role in the regulation of cell proliferation and recent data indicate an interesting intersection of the circadian clock with UPR signaling in tumor cell survival.[35] Thus, we evaluated certain chaperones activated by the UPR and the effect of melatonin treatment at the different time-points of the DEN-induced HCC model. Results showed a significant increase in the protein concentration of HSP70, HSP90, and GRP78 from 20 weeks of DEN administration. In contrast, melatonin induced a reduction of overexpressed levels, with a better response when the higher dose (10 mg/kg) was employed (Figure 5).

3.4 Effect of melatonin and the REV-ERB agonist SR9009 on Hep3B cell proliferation

Synthetic ligands of REV-ERB, including SR9009, have been developed as promising molecular clock modulators.[24] In an additional *in vitro* study we sought to examine the effect of SR9009, alone or in combination with melatonin, on Hep3B cell proliferation. Cell treatment with the agonist SR9009 (10 μM) resulted in REV-ERBα and REV-ERBβ overexpression and a decrease of BMAL1 and CLOCK mRNA levels. These effects were potentiated by melatonin treatment (0.5 or 1 mM) (Figure 6A). Coadministration of melatonin and SR9009 induced an additional decrease in the expression of key oncogenes cyclin D1 and c-Myc when compared to SR9009 alone (Figure 6B). A cell proliferation assay was also carried out in order to analyze the melatonin capacity, along with the REV-ERB ligand, to reduce Hep3B cell proliferation. Melatonin induced a significant antiproliferative effect and enhanced that of SR9009 (Figure 6C).

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3.5 Effect of melatonin on apoptosis and proliferation in BMAL1 knockdown Hep3B cells

To further investigate the potential contribution of the circadian system to the phenomena caused by melatonin, additional experiments knocking down BMAL1 were carried out in Hep3B cells. NC-siRNA and BMAL1-siRNA were used, finding ~80% silencing efficiency 48 hours post-transfection (Figure 7A). Data obtained indicate that Hep3B cell proliferation decreased after BMAL1 knockdown, and there was an increased expression of the markers of apoptosis Bax, cleaved caspase 3 and PARP1/2. BMAL1 knocking down attenuated the proapoptotic and antiproliferative effect of melatonin (Figure 7B,C).

4 DISCUSSION

Maintaining the circadian rhythms can be a critical control point for cancer development, and deregulation of circadian clock genes has been implicated in loss of cell cycle control and tumor formation.[36,37] Thus, pharmacological targeting of circadian regulators might be an essential strategy in the fight against cancer and melatonin may play a relevant contribution.[38] The current research was aimed to identify if modulation of dysregulated circadian clocks could contribute to the beneficial effects of melatonin on cell cycle, proliferation, apoptosis and UPR response in an animal model of HCC.

A primary negative feedback loop of the circadian clock involves the genes Clock and its paralog Npas2, Bmal1, Per1, Per2, Per3, Cry1 and Cry2.[36] BMAL1 and CLOCK (or NPAS2) activate the transcription of Per1-3 and Cry1/2. PER and CRY proteins inhibit BMAL1/CLOCK (or NPAS2), and thus their own transcription.[39] In the present study DEN administration resulted in a dysregulation of these clock genes, supporting data from the literature. Thus, a decreased expression of Cry and Per has been reported in human HCC tissue,[40] an association between human circadian rhythms and dysregulated genes has been very recently shown in HepG2 cells,[41] and results from previous research associated low

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levels of PER and high expression of BMAL1 with liver metastasis.[42] We also found that melatonin administration significantly repressed mRNA and protein levels of the transcription factors and significantly increased expression of Per and Cry genes, similarly to a previous report on the effect of the hormone in human prostate cancer cells.[23] These data confirm in a cancer animal model that resynchronization of dysregulated circadian rhythms may contribute to the beneficial effects of melatonin.

A crucial modulator of the circadian clock, SIRT1, is known to regulate several core clocks genes, including BMAL1, PER2 and CRY1.[43] SIRT1 binds BMAL1/CLOCK and promotes the deacetylation and degradation of PER2.[10] The increase in Sirt1 mRNA levels in the DEN-treated mice, along with the changes in Bmal1, Clock, Npas2 and Per2 expression, supports the possible contribution of Sirt1 to the clock mechanisms in HCC. Prevention of these effects by melatonin indicates that SIRT1 may be a focal point through which the indole influences the circadian clock.

In terms of phosphorylation, CK1ε is a critical factor regulating the core circadian protein turnover.[44] In the current study, CK1ε was downregulated in DEN-treated mice, as it occurred with Per and Cry, while Bmal1, Clock and Npas2 genes were upregulated. These data are in agreement with previous research which has shown a changed expression of CK1ε in parallel to the disrupted profile of other circadian clock genes in gastric cancer[45] or human colorectal carcinoma.[46] Melatonin treatment resulted in a CK1ε upregulation that could contribute to its protective effect. Different studies have shown that a high CK1ε expression associates to a better prognosis in cancer patients, including HCC.[47-49]

We also investigated REV-ERBα and REV-ERBβ, which display ligand-dependent transcriptional repression activity and directly regulate the expression of BMAL1.[50] Our results showed how melatonin increased REV-ERBα and β expression, and also repressed RORα, a transcriptional inducer of BMAL1.[12] REV-ERBα and β, apart from their circadian

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function, regulate cell proliferation, and it has been reported that pharmacological activation of REV-ERB leads to reduced proliferation of a range of breast cancer cells.[3] Related to that, we carried out an *in vitro* study in which we analysed the effect of melatonin and the synthetic REV-ERB ligand SR9009 on Hep3B cell proliferation. SR9009 has been developed as a promising molecular clock modulator[24] and has been recently shown to be specifically lethal to cancer cells and oncogene-induced senescent cells.[51] Results obtained demonstrate that SR9009 treatment decreases the expression of key oncogenes Cyclin D1 and c-Myc and cell proliferation, and that coadministration of the synthetic ligand SR9009 and melatonin enhances antiproliferative effects in a dose-dependent manner, suggesting that a combined therapy may be effective to potentiate the REV-ERB agonist effects. These interesting results reinforce the suggestion that melatonin could be a useful coadjuvant in the treatment of different tumor types.[52]

To further investigate the contribution of changes in the expression of clock genes to the beneficial effects of melatonin, BMAL1, the main target of REV-ERB, was knocked down in Hep3B cells. Results obtained support the important role played by BMAL1 in carcinogenesis,[35,42] and show that the proapoptotic and antiproliferative effects of melatonin are attenuated in BMAL1-depleted cells.

Several studies have shown that the anti-cancer actions of melatonin in breast cancer cells lines and xenografts heavily involve MT1-mediated receptor mechanisms contributing to the cytostatic and pro-apoptotic actions of the indole.[12] Moreover, it has been reported that in MCF-7 breast cancer cells melatonin administration significantly represses RORα transactivation, inhibiting its induction of BMAL1 gene expression.[31] In the present research, the early decrease of BMAL1 and RORα expression induced by melatonin was accompanied by a significant upregulation of MT1, thus suggesting that in our animal model of HCC the MT1 receptor may be also involved in the suppression of elements of the clock mechanism.

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Hypoxic microenvironment, a common feature in cancer, can activate HIF-1α and HIF-1β and the overexpression of these transcription factor contribute to the disturbance in the expression of circadian genes in HCC cells.[32] Recent studies have also suggested that PER2 may function as an effector molecule for the recruitment of HIF-1 to promoter regions of its downstream genes.[53] Our results indicate that, in addition to the increase in Per1 and 2 expression, melatonin reduces the overexpressed levels of Hif-1α and Hif-2α in DEN-treated mice, thus further supporting their relationship with the circadian clock.

Many genes involved in cell cycle and survival are under the control of circadian clocks. The CLOCK/BMAL1 heterodimer has been shown to regulate cell cycle genes that are involved in breast cancer progression.[54] Knockdown of NPAS2 significantly represses the expression of several cell cycle and DNA repair genes in breast cancer and lymphoma cells.[55] PER1 influences numerous downstream cell cycle genes including Cyclin D1, CDK6, p21, and p53, thus playing an important role in occurrence and progression, in different tumor types. [56] PER2 acts as a tumor suppressor gene through cell cycle arrest and apoptosis induction in LLC and EMT6 carcinoma cells[4] or K562 leukemia cells,[5] and it has been shown that Per2 mutation in DEN-treated mice severely deregulated cell proliferation.[57] In the present study we observed how melatonin was able to abrogate the dysregulated expression of Cyclin D1, CDK6, p21 and p53 induced by DEN administration. Moreover, downregulation of the apoptotic response after DEN administration was significantly prevented with melatonin treatment. Thus, the anti-proliferative and pro-apoptotic effect of melatonin in DEN-induced HCC could contribute to reduce tumor progression and this effect could be mediated, at least in part, by the modulation of circadian clocks.

The UPR is an ER stress-activated signalling pathway that modifies cell proliferation and survival[58] and plays an important role in HCC development.[59,60] During ER stress different transcription factors regulate the expression of ER chaperones which are often overexpressed

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in tumor tissues.[61] Different studies have shown that the UPR is regulated by the circadian clock.[62,63] In Burkitt´s lymphoma repression of BMAL1 is essential for the UPR-dependent inhibition of protein synthesis,[35] and it has been reported that low PER1 expression and high UPR response associate with poorer prognoses in glioblastoma patients.[64] In the current study, melatonin was able to downregulate the overexpressed chaperones HSP70, HSP90, and GRP78 from 20 weeks of DEN administration, and BMAL1 and CLOCK, the central regulator of the circadian clock, decreased from 10 weeks. Our results reinforce the idea that controlling the interplay between UPR signaling and the circadian clock might be a suitable strategy to slow down cancer progression.[65]

In summary, although further studies using knocking down/overexpression of clock genes or clock mutant mice are still needed, results from experiments here reported suggest that regulation of circadian clocks might contribute to the modulation of cancer progression by melatonin in DEN-induced HCC. This supports effects of melatonin in HCC alleviation and highlights the usefulness of combined strategies involving the circadian machinery modulation in cancer.

CONFLICTS OF INTEREST

None declared.

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FIGURE LEGENDS

FIGURE 1. Effect of melatonin treatment on the expression of Bmal1, Clock and Npas2 in DEN-induced hepatocarcinogenesis. (A): mRNA levels of Bmal1, Clock, and Npas2 analyzed by real-time PCR assay and normalized against β-Actin. (B): Protein concentration of BMAL1 and CLOCK. Representative Western blot photographs (upper panel) and densitometric quantification. Protein from nuclear liver extracts was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by Lamin-B1 bands. (C): Liver BMAL1 immunohistochemistry. Photomicrographs of sections of liver samples taken from (a) Control; (b) Control+Mel; (c) DEN 10wk; (d) DEN 5Mel 10wk; (e) DEN 10Mel 10wk; (f) DEN 20wk; (g) DEN 5Mel 20wk; (h) DEN 10Mel 20wk; (i) DEN 30wk; (j) DEN 5Mel 30wk; (k) DEN 10Mel 30wk; (l) DEN 40wk; (m) DEN 5Mel 40wk; (n) DEN 10Mel 40wk. Paraffin-embedded sections were stained with BMAL1 antibody. Original magnification: 200x. (D): Image analysis of the area of BMAL1 staining was performed using the ImageJ

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software v3.91 (NIH, Bethesda, MD, USA). Melatonin at different doses: 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given for 10, 20, 30 or 40 weeks (wk) to mice receiving DEN or vehicle (Control+Mel). Values are expressed as means ± SEM. [a]$P<.05$, compared with Control. [b]$P<.05$, compared with DEN same period. [c]$P<.05$, compared with DEN+5Mel same period. Data of Control+Mel group are not showed since no significant differences were observed between Control and Control+Mel groups.

FIGURE 2. Effect of melatonin treatment on the expression of circadian rhythm, MT1 and hypoxia-related genes in DEN-induced hepatocarcinogenesis. mRNA levels of Per1, Per2, Per3, Cry1, CK1ε, Sirt1, MT1, Hif-1α and Hif-2α were analyzed by real-time PCR assay and normalized against β-Actin. Melatonin at different doses: 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given for 10, 20, 30 or 40 weeks (wk) to mice receiving DEN or vehicle (Control+Mel). Values are expressed as means±SEM. [a]$P<.05$, compared with Control. [b]$P<.05$, compared with DEN same period. [c]$P<.05$, compared with DEN+5Mel same period. Data of Control+Mel group are not showed since no significant differences were observed between Control and Control+Mel groups.

FIGURE 3. Effect of melatonin treatment on expression of Rev-erbα, Rev-erbβ and Rorα in DEN-induced hepatocarcinogenesis. (A): mRNA levels of Rev-erbα, Rev-erbβ and Rorα analyzed by real-time PCR assay and normalized against β-Actin. (B): Protein concentration of REV-ERBα, REV-ERBβ and RORα. Representative Western blot photographs (upper panel) and densitometric quantification. Protein from nuclear liver extracts was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by Lamin B1 bands. (C): Liver REV-ERBα immunohistochemistry. Photomicrographs of sections of liver samples taken from (a) Control; (b) Control+Mel; (c) DEN 10wk; (d) DEN 5Mel 10wk; (e) DEN 10Mel 10wk; (f)

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DEN 20wk; (g) DEN 5Mel 20wk; (h) DEN 10Mel 20wk; (i) DEN 30wk; (j) DEN 5Mel 30wk; (k) DEN 10Mel 30wk; (l) DEN 40wk; (m) DEN 5Mel 40wk; (n) DEN 10Mel 40wk. Paraffin-embedded sections were stained with REV-ERBα antibody. Original magnification: 200x. (D): Image analysis of the area of REV-ERBα staining was performed using the ImageJ software v3.91 (NIH, Bethesda, MD, USA). Melatonin at different doses: 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given for 10, 20, 30 or 40 weeks (wk) to mice receiving DEN or vehicle (Control+Mel). Values are expressed as means±SEM. [a]$P<.05$, compared with Control. [b]$P<.05$, compared with DEN same period. [c]$P<.05$, compared with DEN+5Mel same period. Data of Control+Mel group are not showed since no significant differences were observed between Control and Control+Mel groups.

FIGURE 4. Effect of melatonin treatment on protein concentration of cell cycle and apoptosis markers in DEN-induced hepatocarcinogenesis. Protein concentration of Cyclin D1, CDK6, p21, p53, PARP1/2, Bcl-2, and Bax. Representative Western blot photographs (upper panel) and densitometric quantification. Protein from liver extracts was isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. Melatonin at different doses: 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given for 10, 20, 30 or 40 weeks (wk) to mice receiving DEN or vehicle (Control+Mel). Values are expressed as means±SEM. [a]$P<.05$, compared with Control. [b]$P<.05$, compared with DEN same period. [c]$P<.05$, compared with DEN+5Mel same period. Data of Control+Mel group are not showed as no significant differences were observed between Control and Control+Mel groups.

FIGURE 5. Effect of melatonin treatment on protein concentration of UPR chaperones HSP70, HSP90 and CRP78 in DEN-induced hepatocarcinogenesis. Representative Western blot photographs (upper panel) and densitometric quantification. Protein from liver extracts

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was isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. Melatonin at different doses: 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given for 10, 20, 30 or 40 weeks (wk) to mice receiving DEN or vehicle (Control+Mel). Values are expressed as means±SEM. [a]*P*<.05, compared with Control. [b]*P*<.05, compared with DEN same period. [c]*P*<.05, compared with DEN+5Mel same period. Data of Control+Mel group are not showed as no significant differences were observed between Control and Control+Mel groups.

FIGURE 6. Effect of SR9009 and melatonin on Hep3B cells proliferation. (A): mRNA levels of REV-ERBα, REV-ERBβ, BMAL1 and CLOCK, analyzed by real-time PCR assay and normalized against β-Actin. (B): Protein concentration of Cyclin D1 and c-Myc analyzed by Western blot. Protein from cell extracts was isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. (C): Proliferative response analyzed by CyQUANT cell proliferation assay. Hep3B cells were treated at 6, 12 or 24 hours with SR9009 (10 μM), and/or melatonin at a dose of 500 μM (500Mel) or 1000 μM (1000Mel) one hour after SR9009. Values are expressed as means±SEM of three independent experiments. [a]*P*<.05, compared with Control at the same time. [b]*P*<.05, compared with SR9009 at the same time. [c]*P*<.05, compared with SR9009+500Mel at the same time.

FIGURE 7. Effect of melatonin on apoptosis and proliferation in BMAL1 knockdown Hep3B cells. Cells were transfected with NC-siRNA and BMAL1-siRNA. Protein samples were collected 48 hours post-transfection. (A): Confirmation of BMAL1 knockdown by Western blot analysis. (B): Protein concentration of Bax, cleaved caspase 3 and PARP1/2 was analyzed by Western blot. Protein from cell extracts was isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of

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proteins is illustrated by β-Actin bands. (C): Proliferative response was analyzed by CyQUANT cell proliferation assay. Hep3B cells were treated at 24 hours post-transfection with or without melatonin at a dose of 500 μM (500Mel) or 1000 μM (1000Mel).Values are expressed as means±SEM of three independent experiments. [a]*P*<.05, compared with Control. [b]*P*<.05, compared with 500Mel.

TABLE 1 Primers used in this study

	Sense primer (5′-3′)	Antisense primer (5′-3′)
Mouse gene		
Clock	TTAGTGACTGCTCCTGTAGCTTGTG	CACCACCTGACCCATAAGCAT
Bmal1 (Arntl)	GATCGAAAAAGCTTCTGCACAA	GGGTGGCCAGCTTTTCAA
Npas2	GCTGATGTTGGAGGCATTAGATG	CATAGATGATGCTGCCGTCTGT
Per1	GCCAGGTGTCGTGATTAAATTAGTC	GGGCTTTTGAGGTCTGGATAAA
Per2	CACGCTGGCAACCTTGAAGT	TGGTAGTACTCCTCATTAGCCTTCAC
Per3	AGCCTCCCGGCCTTGA	GATTGGCTTGGCTTCTTCTGA
Cry1	TCGCCGGCTCTTCCAA	TCAAGACACTGAAGCAAAAATCG
CK1ε (Csnk1e)	GGCAGGGCCTCAAAGCA	CTTCTCGCTAATCCGCTCGTA
Rev-erbα (Nr1d1)	CCCAACGACAACAACCTTTTG	CCCTGGCGTAGACCATTCAG
Rev-erbβ (Nr1d2)	TGATGCCAACGGCAATCC	CTGTGCGGTCACTCTTCAGAAC
Rorα (Rora)	GCGGTTGACCTCGGCATAT	ACGCTGGACTCTGCTGTTACC
MT1 (Mtnr1a)	CATTTCTAATGGGCTTGAGTGTCA	CATAGCGATCCCCGTGATG
Sirt1	CGGCTACCGAGGTCCATATACT	TCGAGGATCGGTGCCAAT
Hif-1α (Hif1a)	GCGGGCACCGATTCG	TTCAGAACTCATCTTTTTCTTCT
Hif-2α (Hif2a)	CCATGAGGAGATCCGTGAGAA	CGGTGGACACGTCTTTGCT
β-Actin (Actb)	AATCGTGCGTGACATCAAAGAG	GCCATCTCCTGCTCGAAGTCT
Human gene		
REV-ERBα (NR1D1)	TTGAGTCAAGGTCCAGTTTGAATG	GGAGTCCAGGGTCGTCATGT
REV-ERBβ (NR1D2)	TTGTGGTAGGAGTTCTGTTGAATGA	CCCGAAGTCTCCAATTACCAGAT
BMAL1 (ARNTL)	AGCTGCCTCGTCGCAATT	CCGTTCACTGGTTGTGGAACT
CLOCK	AAATATGCAAGGCCAAGTTGTTC	TGTGCCAATGTGTCCAGTATTCA
β-Actin (ACTB)	GGACTTCGAGCAAGAGATGG	AGGAAGGAAGGCTGGAAGAG

Abbreviations: Bmal1, brain and muscle arnt-like protein 1; CK1ε, casein kinase 1 epsilon; Clock, circadian locomotor output cycles kaput; Cry1, cryptochrome 1; Hif-1α and 2α, hypoxia inducible factor-1α and 2α; MT1, melatonin receptor 1; Npas2, neuronal PAS domain protein 2; Pers, periods; Rev-erbα and β, nuclear receptor subfamily 1 group D members 1 and 2; Rorα, retinoid acid receptor-related orphan receptor alpha; Sirt1, sirtuin 1.

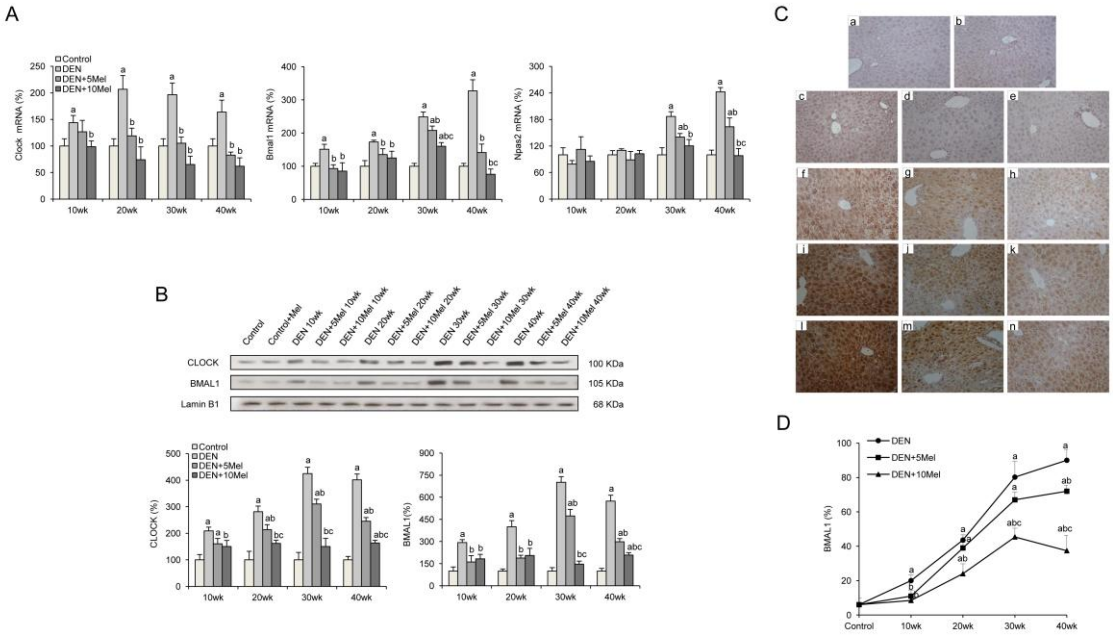
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TABLE 2 Effect of melatonin treatment on hepatic-somatic ratio, serum ALT level, markers for tumor-related angiogenesis and neovascularization, and histological liver lesions at 40 weeks of DEN administration

	HSR (%)	ALT (UI/L)	VEGF mRNA (%)	CD105 mRNA (%)	N. of identified lesions			
					Difuse dysplasia	Hyperplastic foci	Hepatocellular adenoma	Hepatocellular carcinoma
Control	3.5 ± 0.2	30.3 ± 15.5	100 ± 9.1	100 ± 3.5	0	0	0	0
Control+Mel	3.1 ± 1.1	26.5 ± 5.7	109.6 ± 6.8	104 ± 4.3	0	0	0	0
DEN	8.8 ± 0.7[a]	86.3 ± 15.0[a]	225.4 ± 7.9[a]	204.3 ± 11.5[a]	1	0	4	3
DEN+5Mel	7.9 ± 0.4[a]	54 ± 19.0[ab]	173.3 ± 10.8[ab]	138.6 ± 16.7[ab]	5	1	2	0
DEN+10Mel	5.4 ± 0.9[abc]	35 ± 12.6[ab]	142.6 ± 4.9[abc]	107.98 ± 6.3[bc]	1	2	1	0

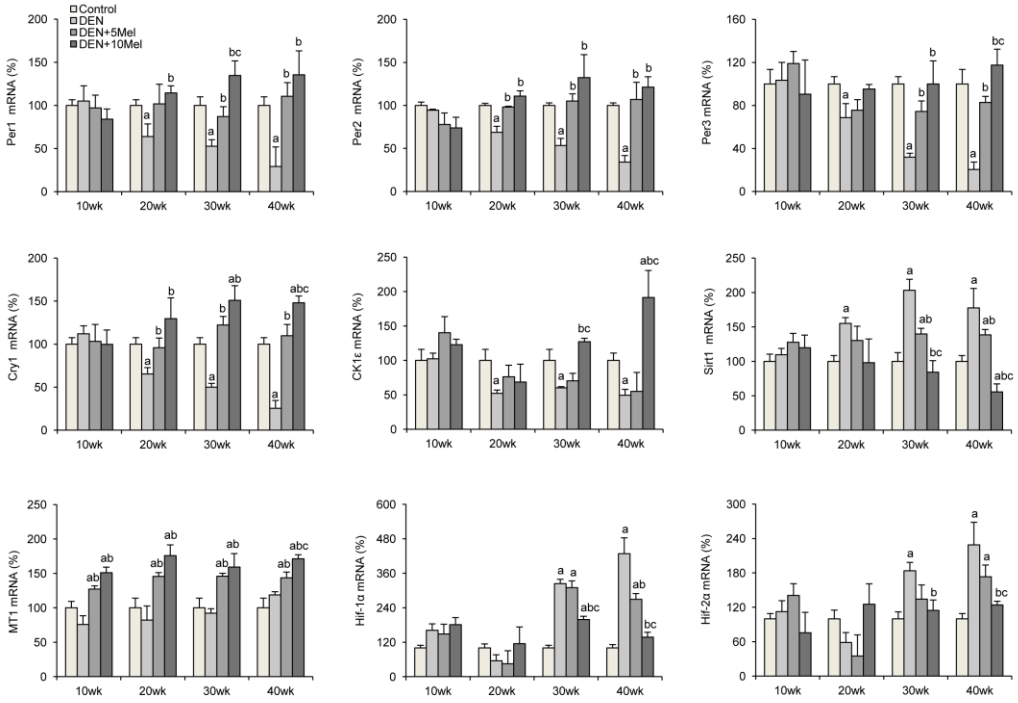
Abbreviations: ALT, alanine aminotransferase; CD105, endoglin; HSR, hepatic-somatic ratio; Mel, melatonin; VEGF, vascular endothelial growth factor.
Values are expressed as means ± standard error of the mean.
[a]$P < 0.05$, compared with Control.
[b]$P < 0.05$, compared with DEN.
[c]$P < 0.05$, compared with DEN+5Mel.



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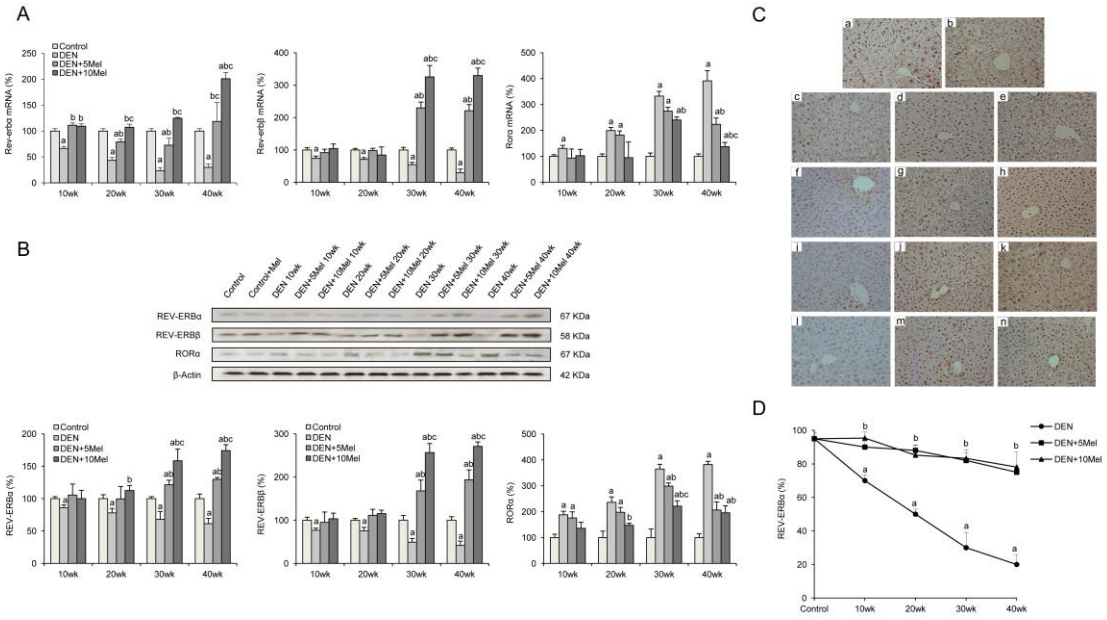
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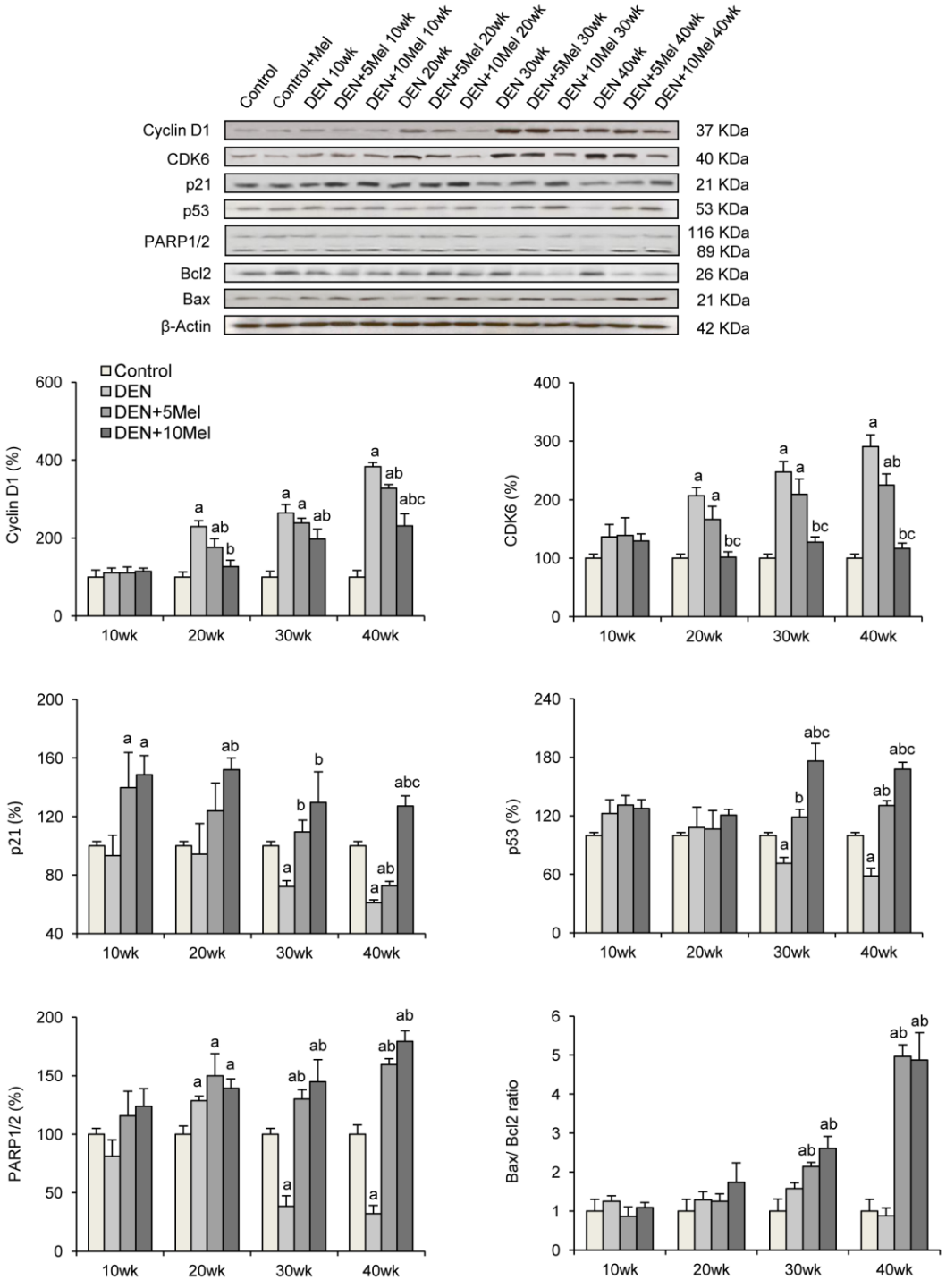
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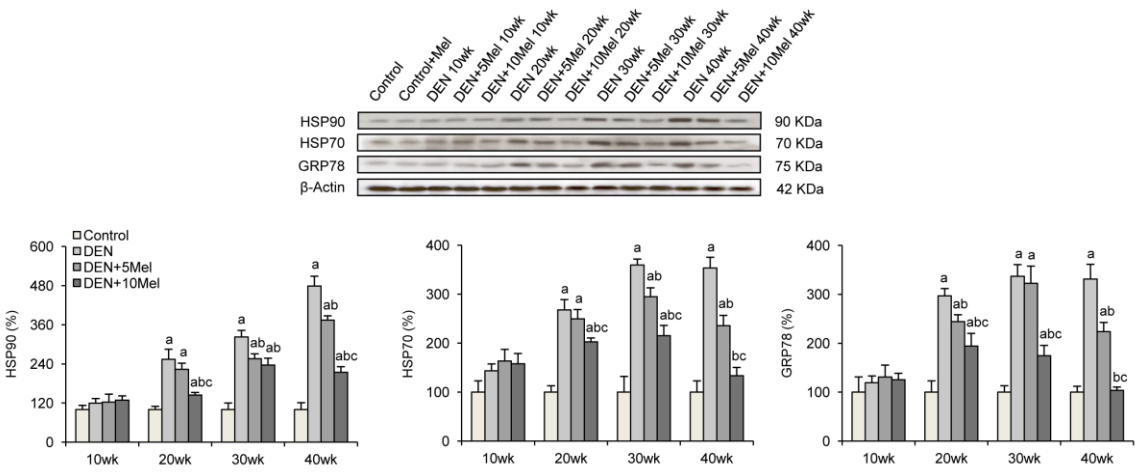
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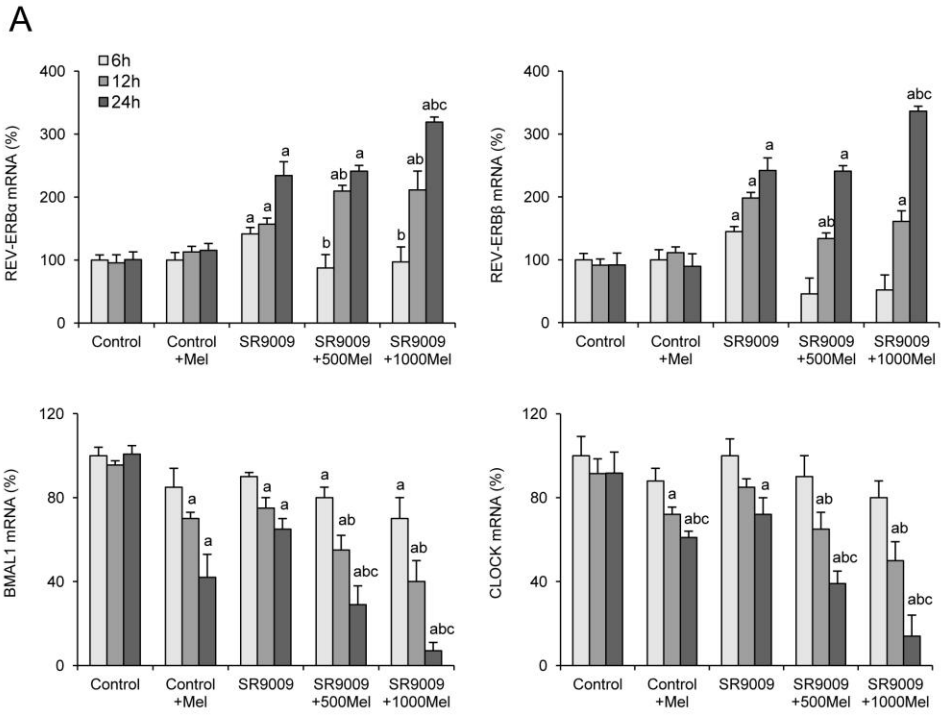
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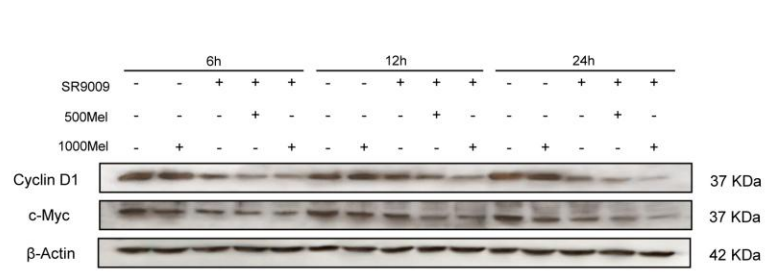
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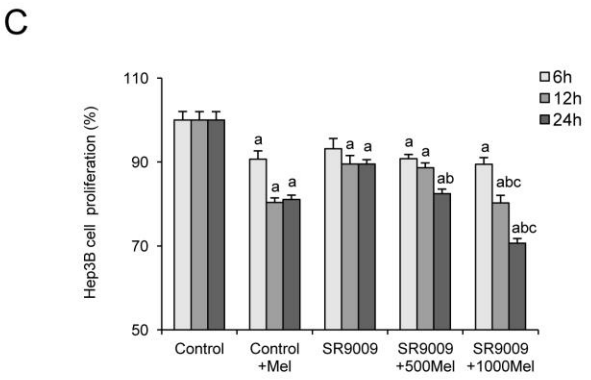


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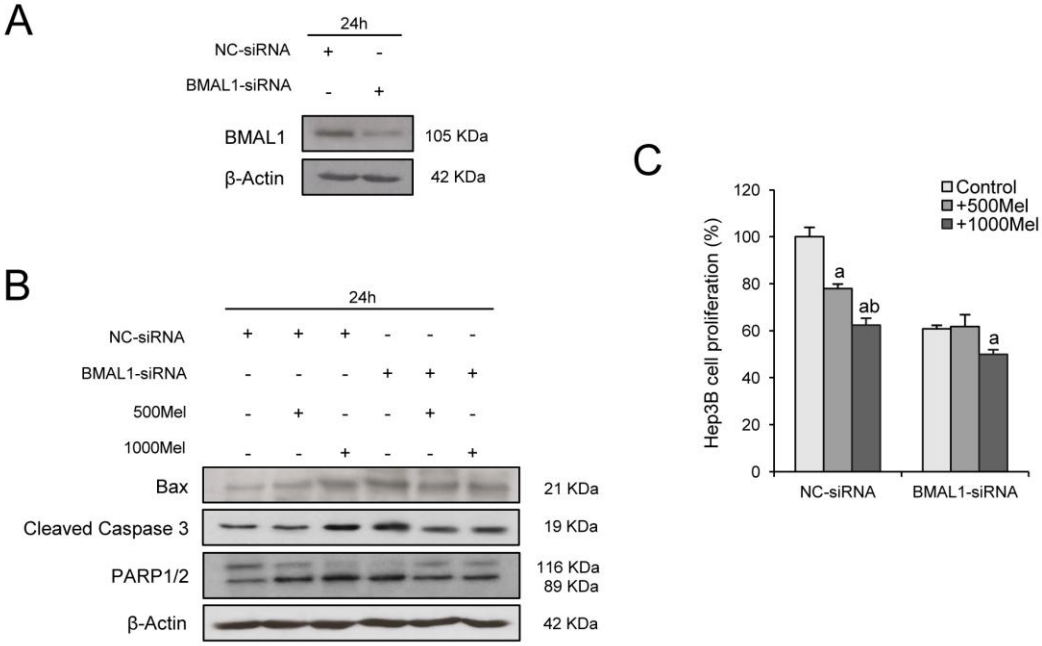






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