

Melatonin promotes circadian rhythm-induced proliferation through Clock/histone deacetylase 3/c-Myc interaction in mouse adipose tissue

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Abstract

Melatonin is synthesized in the pineal gland and controls circadian rhythm of peripheral adipose tissue, resulting in changes in body weight. Although core regulatory components of clock rhythmicity have been defined, insight into the mechanisms of circadian rhythm-mediated proliferation in adipose tissue is still limited. Here, we showed that melatonin (20 mg/kg/d) promoted circadian and proliferation processes in white adipose tissue. The circadian amplitudes of *brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1* (*Bmal1*, $P < .05$) and *circadian locomotor output cycles kaput* (*Clock*, $P < .05$), *period 2* (*Per2*, $P < .05$), *cyclin E* ($P < .05$), and *c-Myc* ($P < .05$) were directly increased by melatonin in adipose tissue. Melatonin also promoted cell cycle and increased cell numbers ($P < .05$), which was correlated with the *Clock* expression ($P < .05$). Further analysis demonstrated that Clock bound to the E-box elements in the promoter region of *c-Myc* and then directly stimulated *c-Myc* transcription. Moreover, Clock physically interacted with histone deacetylase 3 (HDAC3) and formed a complex with c-Myc to promote adipocyte proliferation. Melatonin also attenuated circadian disruption and promoted adipocyte proliferation in chronic jet-lagged mice and obese mice. Thus, our study found that melatonin promoted adipocyte proliferation by forming a Clock/HDAC3/c-Myc complex and subsequently driving the circadian amplitudes of proliferation genes. Our data reveal a novel mechanism that links circadian rhythm to cell proliferation in adipose tissue. These findings also identify a new potential means for melatonin to prevent and treat sleep deprivation-caused obesity.

KEYWORDS

adipose, circadian clock, c-Myc, melatonin, proliferation

1 | INTRODUCTION

Obesity is a complex chronic disease and an increased risk for human health. Recently, there has been growing evidence that links sleep deprivation with the pathogenesis of obesity.^{1,2} Studies reveal that the disrupted secretion pattern of melatonin

is one of the important consequences.^{3,4} Moreover, patients with metabolic syndrome and type 2 diabetes have lower peak concentrations of melatonin than healthy people during the night.^{5,6}

Melatonin (N-acetyl-5-methoxytryptamine) is an amphiphilic neurohormone synthesized and secreted by the pineal gland mainly during the dark period of the circadian cycle.^{7,8} It regulates a number of neuroendocrine and physiological processes in the central nervous system as well as in

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peripheral tissues in mammals.⁹ Melatonin can efficiently modulate neural stem cell functions such as proliferation, differentiation, and immunomodulation through protecting against oxidative stress in brain tissue.¹⁰⁻¹⁴ Moreover, recent studies show the potential role of melatonin in the prevention of obesity and its complications.¹⁵⁻¹⁷ Several mechanisms of the beneficial actions of melatonin on obesity have been discussed, including its antioxidant properties, the stimulation of the immune system, the activation of brown adipose tissue, and the action of the adipokine synthesis.¹⁸⁻²⁰ However, the regulatory mechanism of melatonin on proliferation of adipocytes in peripheral adipose tissue is still not fully understood.

The number of adipocytes is controlled by the proliferation and differentiation into mature adipocytes.²¹ Cell proliferation is regulated at each phase of the cell cycle by various cell cycle-related proteins.²² The suprachiasmatic nucleus (SCN) is the main central pacemaker. It commands all circadian oscillations sending neural signals to other brain areas and to all peripheral adipose tissues in which the oscillators are semiautonomous or almost autonomous.²³⁻²⁵ Melatonin and its oscillation influence several circadian biological rhythms through controlling the transcription and translation of clock genes in peripheral adipose tissues.²⁶⁻²⁸ There are multiple factors connecting the cell cycle and the circadian clock.²⁹ Circadian locomotor output cycle kaput (Clock) combines with brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (Bmal1) to form a heterodimer. It regulates the clock-controlled genes via the E-box (CACGTG) element.³⁰ Studies show that the Bmal1/Clock activates clock-controlled genes and promotes NIH-3T3 cell proliferation by enhancing β -catenin expression.³¹ However, no direct evidence demonstrates that melatonin regulates proliferation through circadian clock genes in adipocytes.

In this work, we investigated the effects of melatonin on circadian clock and proliferation of adipose tissue. We show that melatonin alleviates cell cycle arrest and promotes proliferation through the interaction with molecular core clock genes with HDAC3/c-Myc complex in mouse adipocytes.

2 | MATERIAL AND METHODS

2.1 | Animal experiment

Six-week-old male C57BL/6J background mice were purchased from the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China). The use of the animals and mouse handling protocols were conducted following the guidelines and regulations approved by the Animal Ethics Committee of Northwest A&F University (Yangling, Shaanxi). Mice were housed as 2-5 per cage and provided ad libitum with water and a standard laboratory chow diet. The animal room was maintained at constant temperature, $25\pm 1^\circ\text{C}$, and humidity, $55\pm 5\%$, and 12-hour light/

dark cycles (24-hour LD cycle). The jet-lagged mice were maintained in the same condition. Zeitgeber time (ZT) zero corresponds to the time of light onset. Chronic jet-lagged mice were induced according to the previous method.³² In brief, mice were transferred between two rooms (room 1: 6:00 AM to 6:00 PM light/6:00 PM to 6:00 AM dark; and room 2: 10:00 AM to 10:00 PM dark/10:00 PM to 10:00 AM light). Mice were transferred once from room 1 to 2 and then returned to room 1. The time of mice transfer occurs between 9:30 AM to 10:00 AM, leading to an 8-hour phase advance from room 1 to 2 (light off at ZT4 instead of ZT12 for jet-lagged mice on the day of transfer) and an 8-hour phase delay from room 2 to 1 (light off at ZT20 instead of ZT12 for jet-lagged mice on the day of transfer) every 2 days for 14 days.

To create diet-induced obesity (DIO), mice were placed on a high-fat diet (HFD, fat provided 60% of the total energy) for 10 weeks, and the control mice were fed a standard diet (chow, fat provided 10% of total energy). Glucose tolerance test (GTT) in mice was carried out after the melatonin treatment (start on the 8 weeks of HFD feeding, melatonin injection at a dose of 20 mg/kg/d for 14 days continually). Mice were injected intraperitoneally 1.2 g glucose/kg body weight after overnight fasting. Blood glucose levels were assessed using Accu-Chek glucose monitor (Roche Diagnostics Crop, Pleasanton, CA, USA) before the injection and then at 15, 30, 60, and 120 minutes postinjection. Serum leptin level was measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA).

The melatonin treatment was further applied to mice fed either a HFD or a chow diet according to a 2 \times 2 factorial design. Half group of mice on either diet received a daily intraperitoneally (IP) injection of a 200 μL solution consisting melatonin (MT, 20 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) before the dark onset for 14 days, whereas the other half group of mice was given a vehicle consisting of a solution of 0.5% ethanol in phosphate-buffered saline (PBS); each group had 10 mice. Jet-lagged mice were pretreated with chronic jet lag for 2 weeks before the melatonin injection experiment. Body weight and food intake of mice were daily recorded. Mice were sacrificed by overdosed ethyl ether within two hours after the last injection of melatonin or the vehicle. Immediately, the epididymal white adipose tissue (eWAT) was dissected and kept for the studies as follows.

2.2 | Primary adipocyte culture and vector infection

The connective fiber and blood vessels in collected eWAT tissues were removed, and the tissue was washed three times with PBS buffer containing 200 U/mL penicillin (Sigma-Aldrich) and 200 U/mL streptomycin (Sigma, St. Louis, MO, USA). The preadipocyte culture was carried out according to our previous publication.²¹ Briefly, adipocytes were seeded onto

35-mm culture dishes at 30% (v/v) confluency and incubated at 37°C under a humidified atmosphere of 5% CO₂ and 95% air for subsequent experiments. Melatonin (MT, Selleck.cn, Shanghai, China) was added into culture medium at a final concentration of 1 μmol/L for 24 hours. Cells were collected at 4, 8, 12, 16, 20, and 24 hours after the addition of MT, separately. For virus vector study, adipocytes were infected with adenovirus or lentiviral recombinant vectors of *Clock* for 48 hours at the titer of 1×10⁹ IFU/mL and then treated with melatonin. The control vectors were pAd-GFP and pGLVU6-GFP. All the vectors were constructed by Gene Pharma (Shanghai, China).

2.3 | Cell proliferation assay

The adipocyte proliferation was measured by 5-ethynyl-20-deoxyuridine (EDU) incorporation assay, using EDU assay kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. Cells were cultured in triplicate in 96-well plates for 12 hours at 37°C; then, 50 μmol/L of EdU was added to each well, and cells were further cultured for 4 hours at 37°C. The cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, and then, 100 μL of 1× Apollo reaction cocktail was added to each well. Then, the cells were stained with Hoechst33342 (Solarbio, Beijing, China) for 30 minutes and visualized under a fluorescent microscope (Nikon TE2000-U, Tokyo, Japan). The EdU-positive cells (red cells) were counted using ImageJ (National Institutes of Health, Bethesda, MD, USA) software. The EdU incorporation rate was expressed as the ratio of EdU-positive cells to total Hoechst33342-positive cells (blue cells).

2.4 | Flow cytometry analysis for cell cycle

Cell cycle analysis was performed using flow cytometry. In brief, cultured cells were harvested using trypsin/EDTA and washed twice with PBS buffer. Aliquots of 2×10⁶ cells were centrifuged at 1,500 g for 5 minutes, and precipitants of cells were fixed in 70% ethanol and stained with 500 μL propidium iodide (PI) solution (100 μg/mL RNase and 50 μg/mL PI in 1× PBS buffer). Percentages of cells within cell cycle compartments (G0/G1, S, and G2/M) were determined by BD FACScan (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using CellQuest software (BD Biosciences).

2.5 | RNA-Seq analysis

Total RNA from the eWAT or adipocytes was prepared with TRIpure Reagent kit (Takara, Dalian, China). Quantification and quality control of the sample libraries were assessed by Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-time PCR System. RNA sequencing was performed using HiSeq 4000 instrument (Illumina,

San Diego, CA, USA). Real-time analysis was used for base calling. Fastq files were mapped to the mouse genome (NCBI37/mm9) using TopHat (version 2.0.4, Johns Hopkins University, Baltimore, MD, USA). Mapped reads were then assembled via Cufflinks (version 2.0.2, University of Washington, Seattle, WA, USA) with the default settings.³³ Assembled transcripts were then merged using the Cuffmerge program with the reference genome. Analysis of mRNA levels was carried out using the Cuffdiff program, with samples being grouped by treatment condition, three replicates per group. Volcano plots comparing log₁₀ (statistical relevance) to log₂ (fold change) were generated using R (version 3.1.1, AT&T Bell Laboratories, New York, NY, USA), using the base plotting system and calibrate library. Gene Ontology (GO) and pathway enrichment analyses were performed to categorize the considerably enriched functional classification or metabolic pathways in which DEGs operated.

2.6 | Plasmid construction and luciferase assay

A 720-bp mouse c-Myc promoter was cloned by PCR amplification of C57BL/6J mouse genomic DNA and inserted in the pGL-3 basic vector. The resulting reporter was named c-Myc₇₂₀-Luc. Further deletion and relegation of the c-Myc₇₂₀-Luc generated c-Myc₄₅₀-Luc and c-Myc₂₁₀-Luc reporters containing 450 bp and 210 bp of c-Myc promoter, respectively. Mutant c-Myc reporter plasmids were generated using the c-Myc₄₅₀-Luc plasmid as a template; a mutagenesis kit (Invitrogen, Carlsbad, CA, USA) was used to create c-Myc₄₅₀-Luc_{E1}, c-Myc₄₅₀-Luc_{E2}, and c-Myc₄₅₀-Luc_{E1},_{E2} with mutation in the two E-boxes of c-Myc promoter. HEK293 cells were cotransfected with luciferase reporter plasmid using X-tremeGENE™ Transfection Reagent (Roche, Basel, Switzerland) and the same amounts of reporter and β-gal expression vectors, variable amounts of expression vector (pcDNA3.1-Clock), and an empty pcDNA3.1 vector to normalize the total amount DNA. After 48-hour transfection, cells were harvested for luciferase assay, and β-gal was used as an internal control.³²

2.7 | Co-immunoprecipitation (co-IP) analysis

HEK293T cells were transfected with plasmids using X-tremeGENE™ Transfection Reagent (Roche, Switzerland). After 24-hour transfection, cells were then snap-frozen in lipid nitrogen. Whole-cell lysate was harvested in lysis buffer with a protease inhibitor. Cells were then sonicated for 10 seconds, and the whole-cell lysate was precleared with protein A for 2 hours and incubated overnight with 2 μg primary antibody at 4°C. Immune complexes were pulled down with protein A-agarose for 2 hours at 4°C with shaking.

Beads were washed once with lysis buffer and three times with wash buffer and then eluted by boiling in SDS sample buffer followed by Western blot detection.

2.8 | Real-time quantitative PCR analysis

Total RNA from eWAT or adipocytes was extracted with TRIpure Reagent kit (Takara). 500 ng of total RNA was reverse-transcribed using M-MLV reverse transcriptase kit (Takara). Primers were synthesized by Invitrogen (Shanghai, China). Quantitative PCR was performed in 25 μ L reaction system containing specific primers and **AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China)**. Amplification was performed in the ABI StepOnePlus™ RT-PCR System (Carlsbad, CA, USA). The levels of mRNA were normalized in relevance to *Gapdh*. The expression of genes was analyzed by the method of $2^{-\Delta\Delta C_t}$.

2.9 | Western blot

Western blot was performed as previously described.²¹ Adipocytes were solubilized in adipocyte lysis buffer. Protein samples (30 μ g) were separated by electrophoresis on 12% and 5% SDS-PAGE gels using slab gel apparatus and transferred to PVDF nitrocellulose membranes (Millipore, Billerica, MA, USA). Antibodies including FLAG-HRP (A-8592, Sigma-Aldrich), anti-HA tag (ab18181; Abcam, Cambridge, UK), anti-His tag (ab18184; Abcam) and the appropriate HRP-conjugated secondary antibody (Baoshen, Beijing, China) were used. Proteins were visualized using chemiluminescent peroxidase substrate (Millipore, Billerica, MA, USA), and then, the blots were quantified using ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

2.10 | Statistical analysis

Statistical analyses were conducted using SAS v8.0 (SAS Institute, Cary, NC, USA). Data were analyzed using two-way ANOVA, and diet and melatonin were fixed factors. The amplitude of circadian gene mRNA was calculated using simple cosinor analysis.^{26,34} Comparisons among individual means were made by Fisher's least significant difference (LSD). Data were presented as mean \pm SEM. $P < .05$ was considered to be significant.

3 | RESULTS

3.1 | Melatonin promotes circadian and proliferation processes in white adipose tissue

To explore the effects of melatonin, we compared white adipose transcriptomes from vehicle- and melatonin-injected mice. Notably, a total of 5348 genes were found to be

significantly altered, and 66% (3535 of 5348) of the genes are increased in melatonin-injected mice, whereas 34% (1813 of 5348) decreased (Figure 1A). Comparative analysis of untreated mice and melatonin-injected animals revealed that signature genes up- or downregulated by melatonin were associated with circadian oscillators or proliferation (Figure 1A). The subsequent Gene Ontology (GO) analysis showed 25% of these genes were located in the nucleus and mainly functioned in circadian rhythmic, cell proliferation, and nucleotide metabolism (Figure 1B). In brief, 21% of these nucleus genes involved in cell proliferation and 22% participated in circadian rhythmicity process (Figure 1C), demonstrating that genes upregulated by melatonin were correlated with circadian and proliferation processes in adipose tissue. Further pathway analysis of the melatonin-regulated genes revealed that lipid metabolism, cell proliferation, and circadian rhythm pathways were all highly enriched (Figure 1D). mRNA expression measurement established that circadian rhythm marker genes *Clock* and *Bmal1* were increased with melatonin injection, but *Dec*, *Per1*, *Per2*, and *Cry1* were decreased; the levels of proliferation genes *p27* and *CD74* were reduced, while *c-Myc* and *cyclin E* were elevated (Figure 1E). Interestingly, melatonin injection significantly stimulated the expression of *HDAC3* (Figure 1E). The genes described in Figure 1E were virtually all shown by the RNA-sequencing (RNA-Seq) analysis in Figure 1A. These findings indicate melatonin affects the circadian rhythm and proliferation process of white adipose tissue.

3.2 | Melatonin drives circadian amplitude of proliferation genes in white adipose tissue

To further analyze the effects of melatonin on circadian clock process, we examined the 24-hour patterns of mRNA of clock marker genes. The 24-hour rhythms of *Per1*, *Per2*, *Bmal1*, and *Cry1* are illustrated in Figure 2A-C. Melatonin injection reduced the circadian amplitude of *Per1* and *Per2*, but increased the circadian amplitude of *Bmal1* and *Clock*, while it did not disrupt the endogenous adipose clock rhythm of these genes (Figure 2A-C). Notably, melatonin injection drove robust circadian rhythms of proliferation genes. Melatonin increased the circadian amplitude of *c-Myc* and *cyclin E* (Figure 2D,E). By contrast, melatonin reduced the circadian amplitude of *p27* (Figure 2F). Together, these data suggest that melatonin enhances proliferation process and drives the circadian amplitude of proliferation genes.

3.3 | Melatonin increases cell proliferation by correlation with *Clock* in adipocytes

We then studied whether melatonin controls circadian process in adipocytes to drive cell proliferation. Heatmap analyses showed melatonin disturbed the expression of 2336 genes

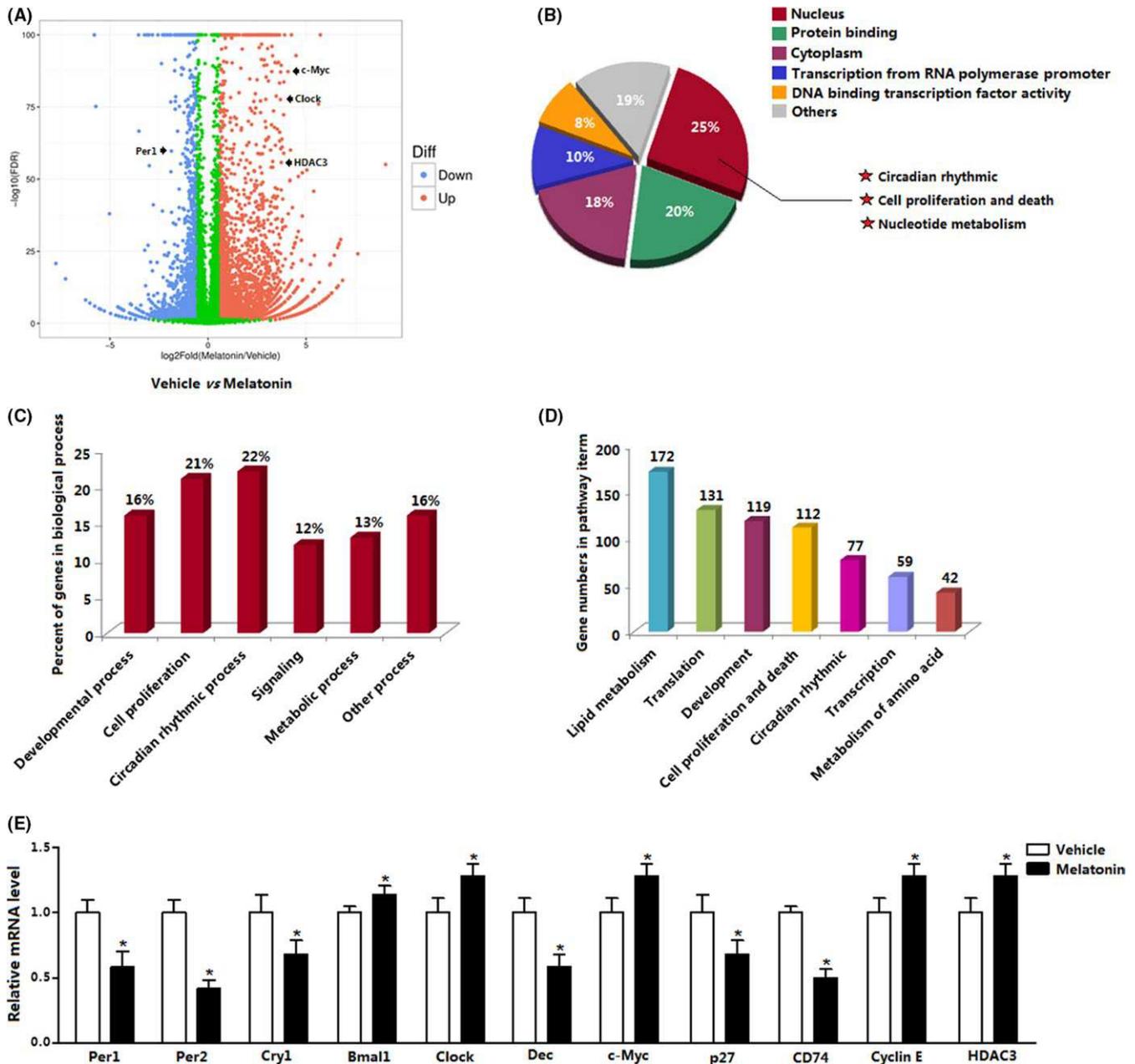


FIGURE 1 Melatonin promotes circadian and proliferation process in white adipose tissue. (A) Volcano plot of transcriptome in the white adipose tissue of mice injected with or without melatonin ($n=3$ each). Among 5348 genes, 3535 were upregulated, while 1813 were downregulated. Individual circadian and proliferation signature genes are highlighted: red: upregulated; blue: downregulated. (B) Gene Ontology (GO) analysis of the gene set in (A). The area of each pie slice represents the number of genes that belong to the indicated GO terms ($n=3$). (C) GO analysis of the target genes in (B), which shows the response of the biology processes to melatonin treatment. (D) Top seven GO terms of pathway analysis enriched in the upregulated genes ($n=3$). (E) mRNA levels of circadian marker genes and proliferation marker genes ($n=3$). Values are means \pm SEM. * $P < .05$ compared with the control group

classified in circadian rhythm and proliferation (Figure 3A). The expression profile of the selected genes from Figure 3A validated the role of melatonin in promoting adipocyte proliferation (Figure 3B). Melatonin also increased the expression of *Clock* and *HDAC3* (Figure 3B). EDU staining further validated the elevation of proliferation of adipocytes with melatonin incubation (Figure 3C). In addition, a higher

percentage of cells underwent the S phase in response to melatonin treatment compared with the control group (Figure 3D). Furthermore, the correlation analysis found that *Clock* had significant positive correlations with the expression of *c-Myc* and *HDAC3* (Figure 3E). These correlations support the notion that melatonin promotes cell proliferation by interaction with circadian clock.

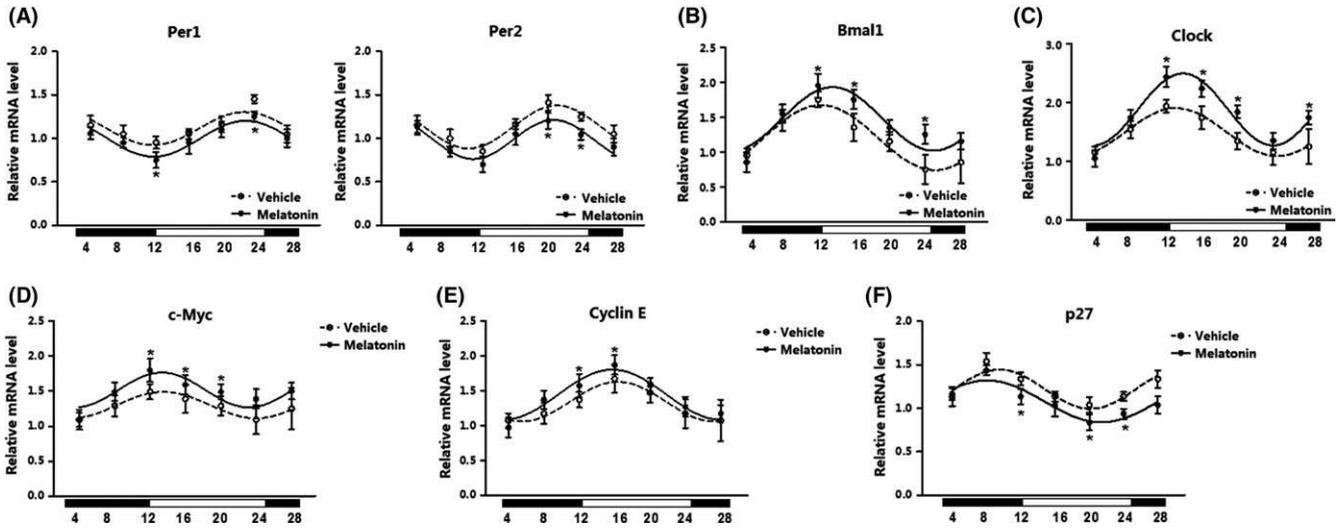


FIGURE 2 Melatonin drives the circadian amplitude of proliferation genes in white adipose tissue injected with melatonin or the PBS (control). The gene expressions at the indicated times were all analyzed by RT-PCR (n=10). (A) *Per1* and *Per2* expression. (B) *Bmal1* expression (n=10). (C) *Clock* expression. (D) *c-Myc* expression. (E) *Cyclin E* expression. (F) *p27* expression. Values are means±SEM. **P*<.05 compared with the control

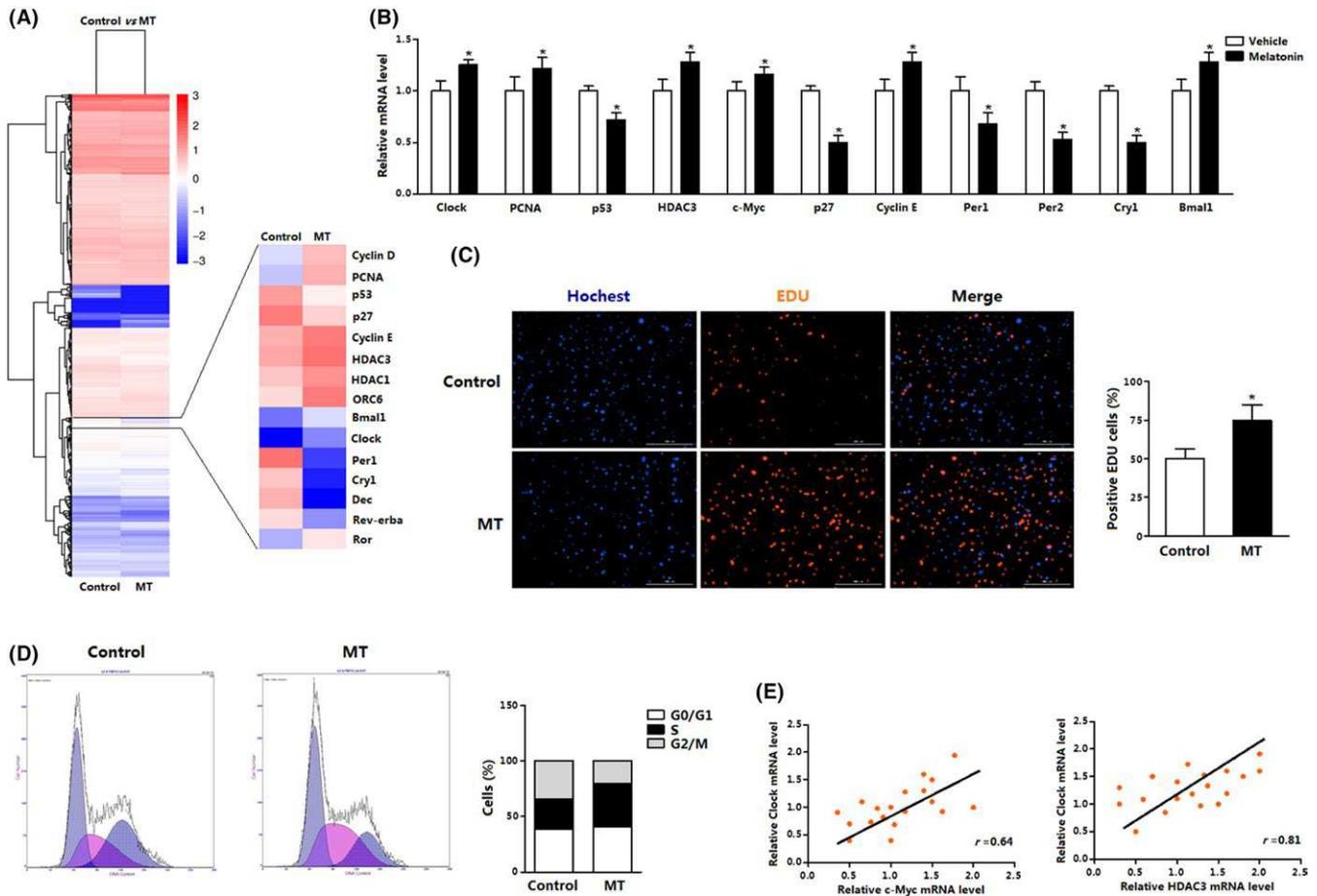


FIGURE 3 Melatonin increases cell proliferation by correlation with *Clock* in adipocytes. (A) Heatmap of genes upregulated or downregulated with melatonin (MT) incubation of adipocytes, along with the top affected genes by MT treatment (n=3). (B) Expression profile of circadian marker genes and proliferation marker genes in adipocytes treated with MT (n=3). (C) EDU staining of adipocytes treated with MT for 24 h (n=3). (D) Flow cytometry analysis of cell cycle in adipocytes treated with MT for 24 h (n=3). (E) Correlation between *Clock* mRNA expression on the x-axis and mRNA expression of *HDAC3* and *c-Myc* on the y-axis (n=3). Values are means±SEM. **P*<.05 compared with the control

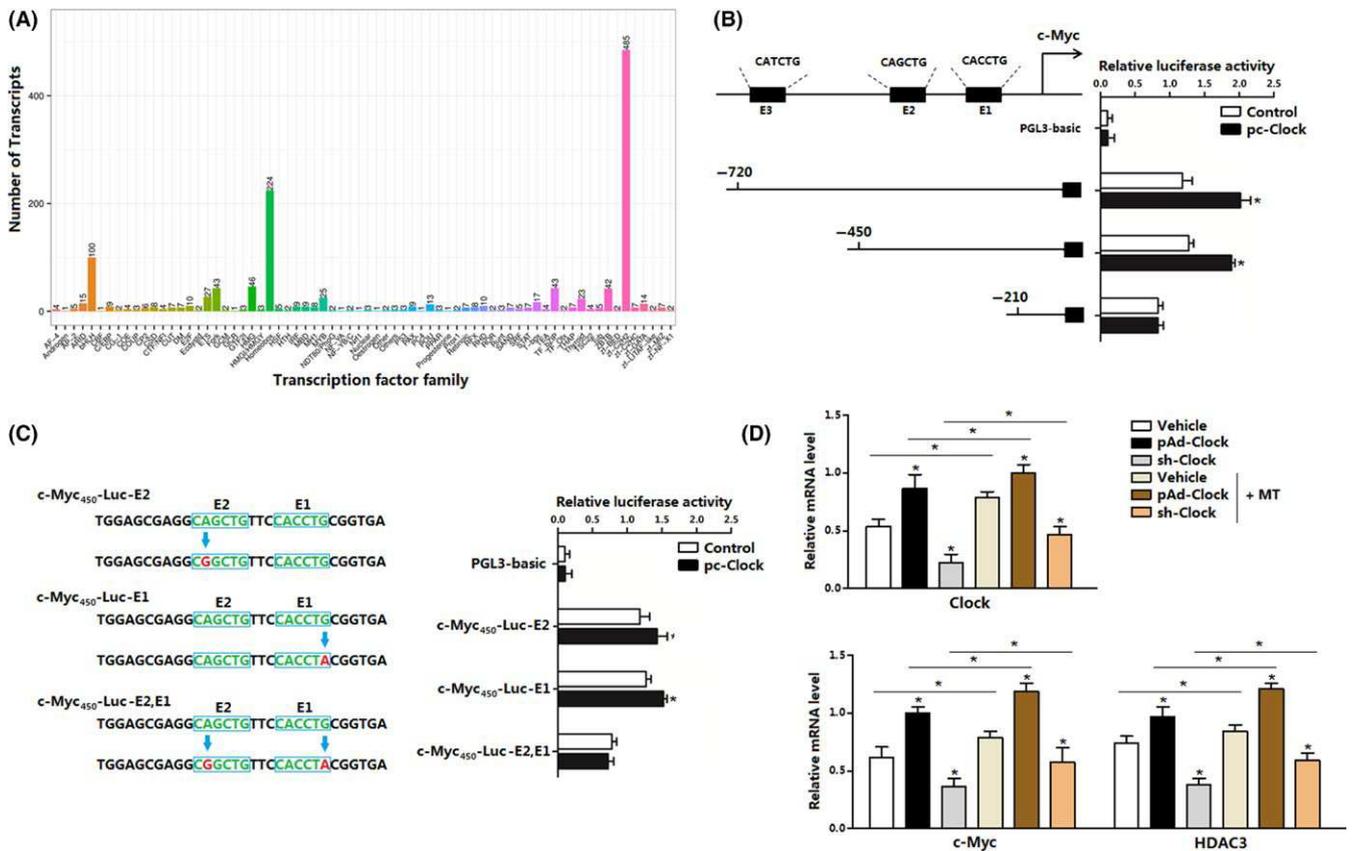


FIGURE 4 Clock promotes proliferation by elevating *c-Myc* transcription in adipocytes. (A) Transcription factor family in genome after melatonin (MT) treatment ($n=3$). (B) Fragments of *c-Myc* promoter fused to a luciferase reporter gene were cotransfected into HEK293 cells together with PGL3-basic (control) or pc-Clock. The binding sites of Clock overlap with consensus E-boxes in *c-Myc* promoter region. Luciferase activity was corrected for Renilla luciferase activity and normalized to the control activity ($n=3$). (C) The strategy for generating mutant *c-Myc* promoter-driven luciferase reporters with mutated bases in blue ($n=3$). (D) mRNA level of *Clock*, *c-Myc*, and *HDAC3* of adipocytes transfected with pAd-Clock or sh-Clock and then treated with or without MT ($n=3$). pAd-Clock: overexpression adenovirus recombinant vectors of *Clock*; sh-Clock: interference lentiviral recombinant vectors of *Clock*. Values are means \pm SEM. * $P < .05$ compared with control

3.4 | Clock promotes proliferation by elevating *c-Myc* transcription in adipocytes

Our data showed the transcription factor cluster of multiple proliferation genes was altered, suggesting the existence of E-box in the promoter region of the genes (Figure 4A). Analysis of our results demonstrated the *c-Myc* promoter contained three consensus E-box elements that were the potential targets of Clock (Figure 4B). And further measurements revealed that two E-box elements, 450–210 bp upstream of the initiation codon of *c-Myc*, functioned (Figure 4B). Mutation of the two E-box elements in the *c-Myc* promoter impaired the effect of Clock on *c-Myc* transcription activity (Figure 4B,C), indicating that both of the two E-box elements regulated the translation of *c-Myc*. We next treated adipocytes with the overexpression recombinant adenovirus vector of *Clock* (pAd-Clock) or interference recombinant lentiviral vector of *Clock* (sh-Clock) with or without melatonin incubation. Figure 4D shows that

cotreatment with melatonin and pAd-Clock increased the mRNA levels of *Clock* and *c-Myc*, along with the elevation of *HDAC3* (Figure 4D). In addition, cell cycle analysis by FACS demonstrated that a higher percentage of cells underwent the S phase after the combined melatonin and pAd-Clock treatment as compared with the control group (Figure 5A). EDU staining confirmed the elevation of cell proliferation in the Clock and melatonin cotreatment group (Figure 5B). Together, the findings strongly suggest that Clock controls proliferation by elevating the *c-Myc* transcription level.

3.5 | Clock forms a complex with HDAC3 and c-Myc

To further dissect the molecular mechanism of Clock on *c-Myc*, we determined whether Clock interacted with *c-Myc*. Firstly, we hypothesized that Clock interacted with HDAC3 and *c-Myc* (Figure 6A) based on the bioinformatics

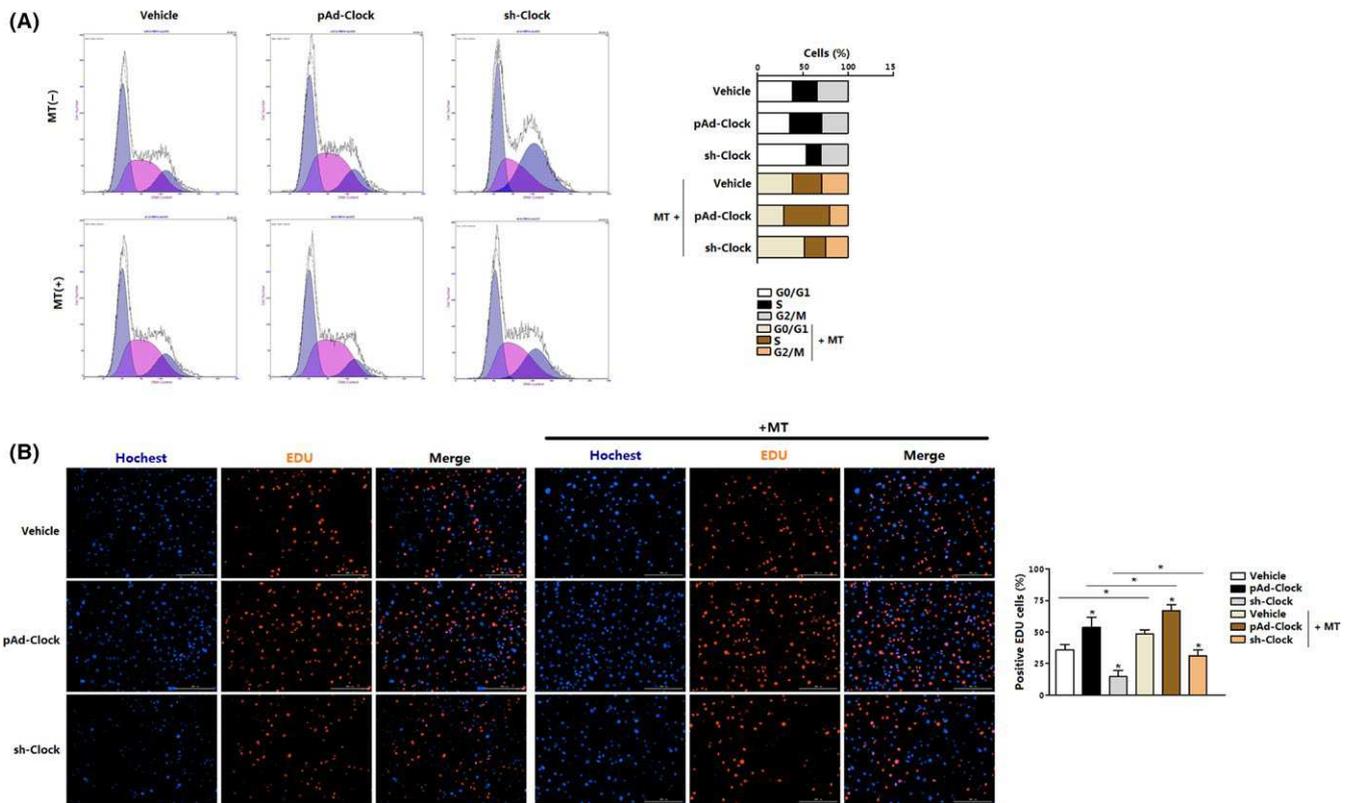


FIGURE 5 Melatonin promotes adipocyte proliferation by Clock. (A) Flow cytometry analysis of cell cycle in adipocytes transfected with pAd-Clock or sh-Clock and then treated with or without melatonin (MT) ($n=3$). (B) EDU staining of adipocytes treated with recombinant Clock vectors or MT ($n=3$). pAd-Clock: overexpression adenovirus recombinant vectors of Clock; sh-Clock: interference lentiviral recombinant vectors of Clock. Values are means \pm SEM. * $P < .05$ compared with control

analysis and previous data sheet. Then by protein-protein measurement, we found that HDAC3 protein interacted strongly with transfected Clock in HEK293T cells and also HDAC3 interacted with c-Myc in transfected HEK293T cells. Thus, these data suggest that Clock, HDAC3, and c-Myc form a complex where HDAC3 helps to stabilize the complex (Figure 6B).

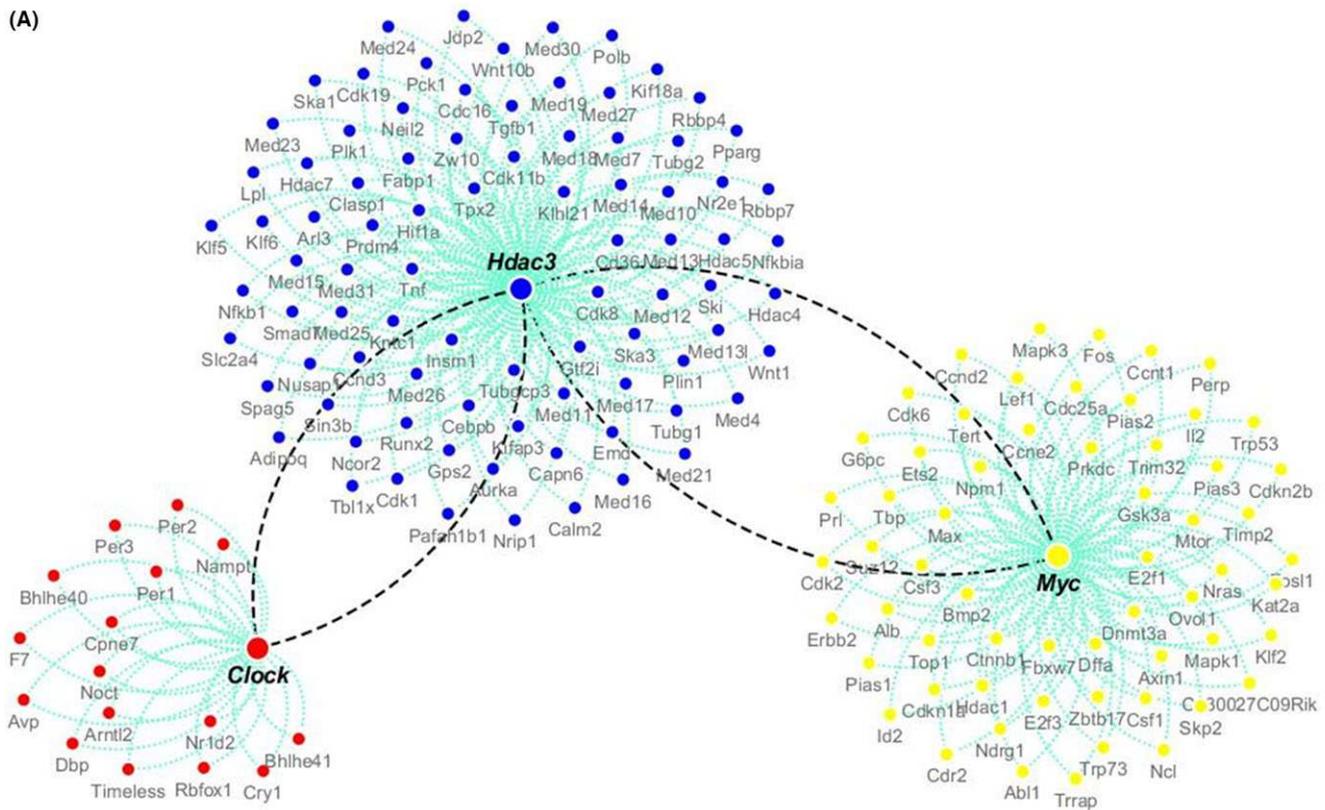
3.6 | Melatonin attenuates jet lagged-reduced proliferation in mouse adipose tissue

To test the effects of circadian misalignment on melatonin and cell proliferation, we used a model of jet lag. Melatonin reduced body weight in the jet-lagged mice (Figure 7A,B). Melatonin promoted circadian rhythm of *Clock* in adipose tissue which was disturbed in jet-lagged mice, and improved the disturbance of *c-Myc* circadian rhythm (Figure 7C,D). As shown in Figure 6E, melatonin increased the mRNA levels of *cyclin D1*, *PCNA*, and *cyclin E*, but reduced *p27* mRNA level in jet-lagged mice. The recovery of circadian rhythm by melatonin also improved adipocyte proliferation and circadian process (Figure 7E). Taken together, melatonin improves adipose proliferation in jet-lagged condition.

3.7 | Melatonin stimulates adipocyte proliferation by elevating circadian clock genes in obese mice

We then studied the function of melatonin in circadian clock and cell proliferation in diet-induced obese mice. The HFD caused the pronounced body weight increases compared with mice on the chow diet, whereas melatonin injection reduced the body weight gain (Figure 8A). The food intake did not differ between the HFD group and the melatonin injection group (Figure 8B). An injection of melatonin elevated blood glucose level in HFD mice (Figure 8C) and reduced serum leptin level (Figure 8D). HFD feeding also decreased the mRNA level of *Clock* and increased the mRNA level of *Per1*, while melatonin treatment restored the expression of these genes to those in the control group, implicating that obesity disrupted the circadian clock and blocked cell proliferation (Figure 8E). Consistently, our results demonstrated that cell proliferation target genes, such as *c-Myc*, were reduced and *p27* was elevated in mice fed HFD, but melatonin inhibited the effect by reducing obesity-induced circadian disorder (Figure 8F). As expected, *HDAC3* had the similar change in the expression profile as *c-Myc* (Figure 8F). Thus, melatonin stimulates cell proliferation by elevating circadian rhythm in obese mice.

(A)



(B)

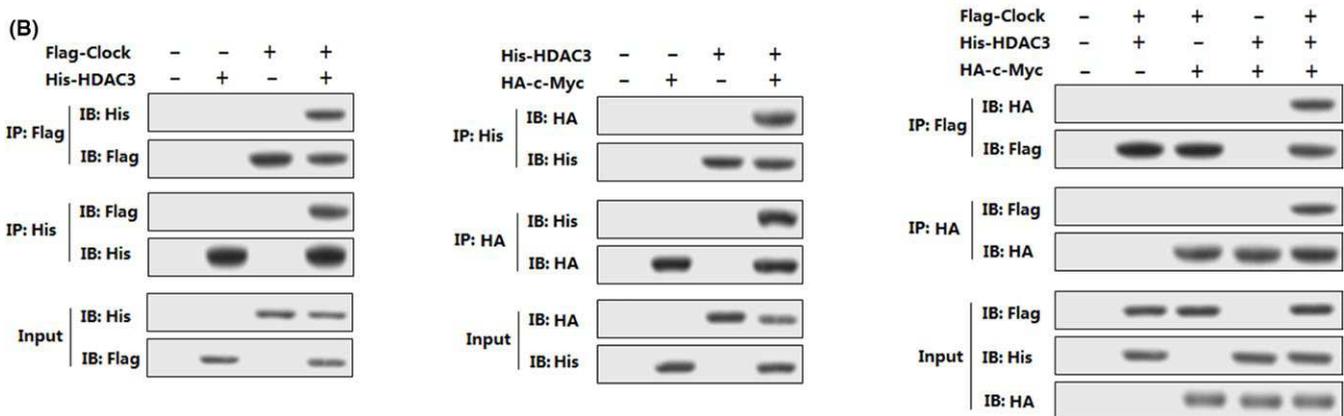


FIGURE 6 Clock forms a complex with HDAC3 and c-Myc. (A) Graphic representation of a network of the target genes. Bioinformatics analysis of the protein-protein interaction. (B) Clock interacted with HDAC3. Co-IP analysis was performed in Flag-Clock- and His-HDAC3-transfected HEK293 cells. HDAC3 interacted with c-Myc. Co-IP analysis in His-HDAC3- and HA-c-Myc-transfected HEK293 cells. HDAC3 interacted with Clock and c-Myc. Co-IP analysis in Flag-Clock-, His-HDAC3-, and HA-c-Myc-transfected HEK293 cells. Values are means \pm SEM

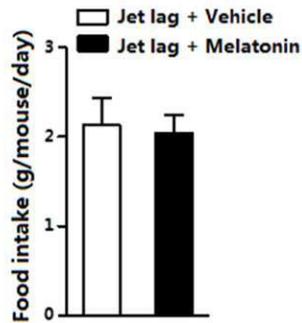
4 | DISCUSSION

Melatonin modulates proliferation of various cell types by sequential activation of numerous cellular pathways,^{35,36} but the effects depend on cell types and culture conditions. Melatonin delays cell proliferation and induces apoptosis by inducing cell cycle arrest in osteoblastic cell lines and breast cancer cells.^{37,38} However, inhibition of cell proliferation was not observed in normal cells.^{4,39} Studies report that melatonin

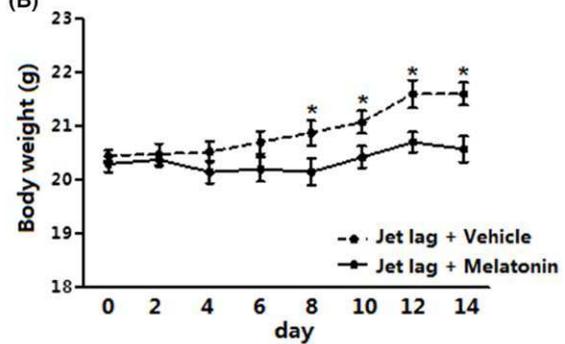
promotes proliferation of neural stem cells and pluripotent stem cells.^{10,40,41} Although melatonin plays a different regulatory role in the proliferation of normal and cancer cells, it still does not determine how adipocyte responds to melatonin during cell proliferation. In this study, we demonstrated that exogenous melatonin stimulated proliferation of adipose tissue. Moreover, melatonin also attenuated the dysfunction of proliferation in obese or jet-lagged mouse models. Thus, our data indicated that melatonin is involved in regulating the proliferation of adipocytes and adipose tissue growth.

(A)

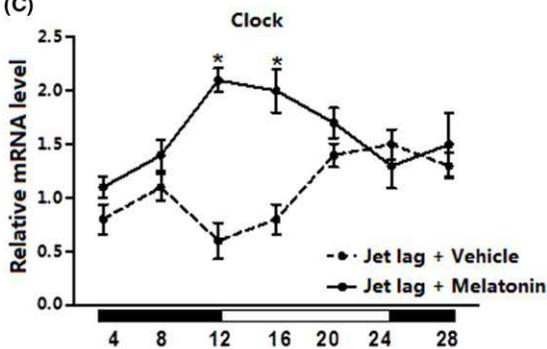
Jet lag + Vehicle Jet lag + Melatonin



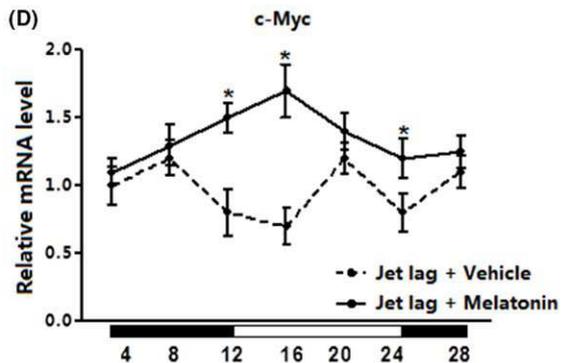
(B)



(C)



(D)



(E)

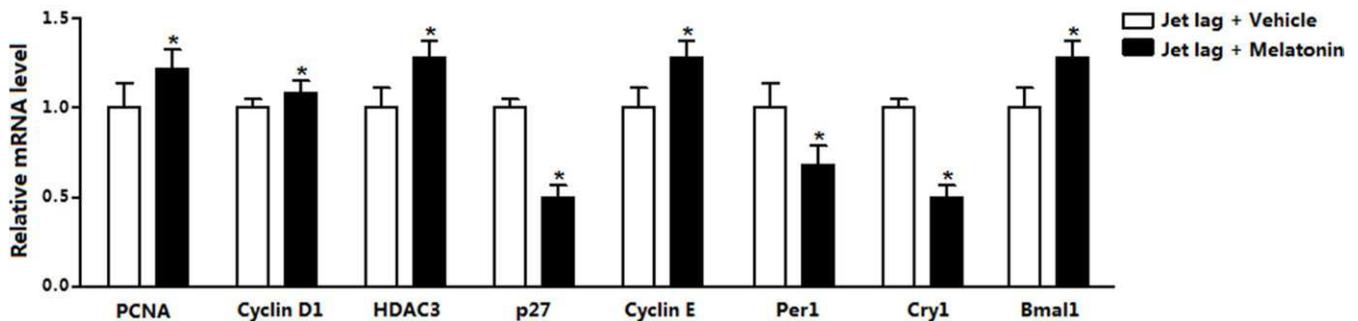


FIGURE 7 Melatonin attenuates jet lagged-reduced proliferation in mouse adipose tissue. The jet-lagged mice were injected with or without melatonin for 14 days; $n=10$ in each group. (A) Representative picture and food intake of the mice. (B) Body weight of the mice. (C) *Clock* expression in the mice at the indicated times, determined by RT-PCR analysis. (D) *c-Myc* expression in the mice at the indicated times, determined by RT-PCR analysis. (E) Relative mRNA levels of circadian markers and proliferation markers of the mice. Values are means \pm SEM. * $P < .05$ compared with the control

Melatonin has also been associated with circadian rhythms by regulating the core circadian genes.⁴² Circadian genes control most of the physiological functions including cell cycle. Studies show that circadian rhythm contributes to tumorigenesis and tumor cell proliferation by regulating clock gene expression.^{43,44} To estimate the effects of circadian rhythms on melatonin-induced proliferation of adipocytes, we investigated the core circadian gene expression profile in adipose tissue and primary adipocytes. We found that melatonin elevated the circadian amplitude of *Clock*, *Bmal1*, *c-Myc*, and *cyclin E1* during the proliferation of adipocytes. Moreover, many biological processes

and pathways which were correlated with cell proliferation are also enriched by the GO analysis. From these findings, we surmise that melatonin participates in the proliferation of adipocytes by modulating the amplitude of circadian clock.

This study pointed out a significant correlation between circadian clock and cell proliferation, which had been reported.⁴⁵ In both liver and skeletal muscle, *Clock* is associated with the cell cycle and cell proliferation by regulating the transcriptional cascade of many genes.⁴⁶ Li et al. report that *Clock* stimulates cell growth and increases the proportion of S phase cells in the cell cycle of cancer cells by interacting

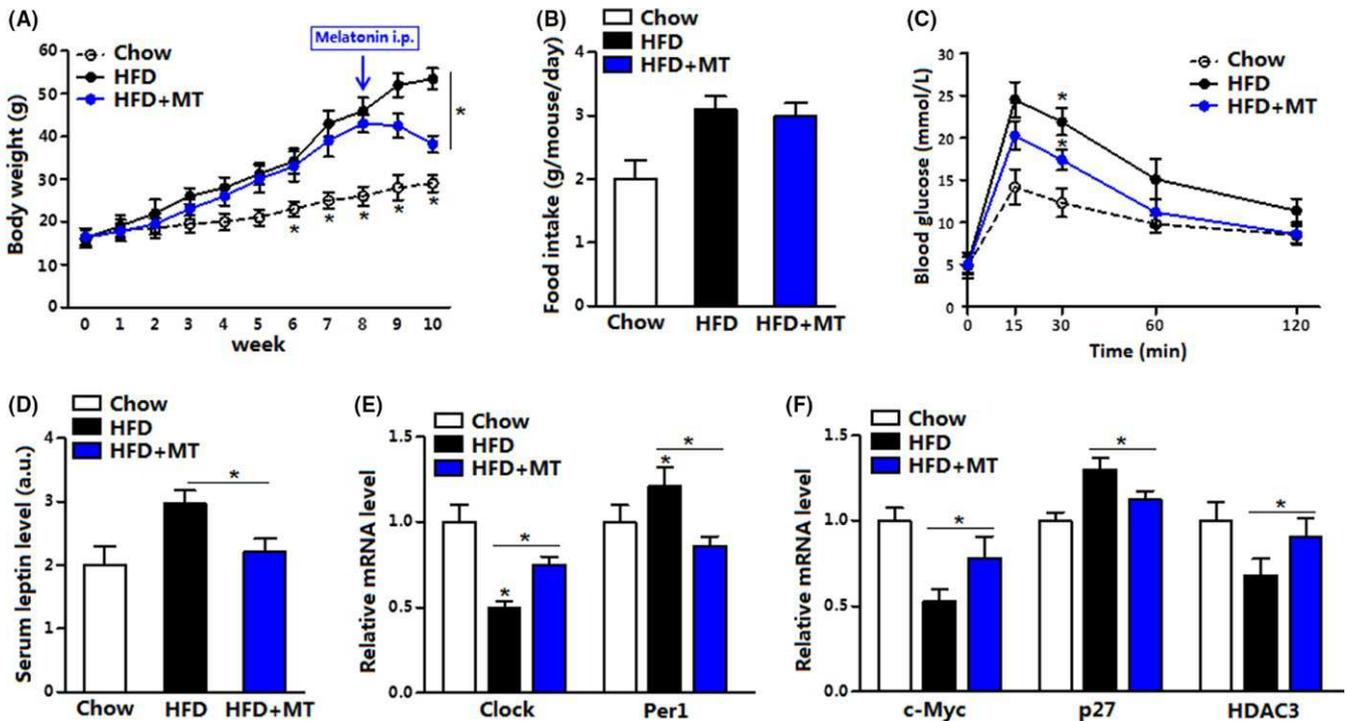


FIGURE 8 Melatonin stimulates adipose proliferation by elevating circadian clock genes in obese mice. Mice were fed on a HFD or on a chow diet. For the HFD group, mice received a dose of melatonin or PBS injection for 14 d continually. Each group had 10 mice. (A) Body weight of the mice in each group. (B) Food intake of the mice in each group. (C) Glucose tolerance test (GTT) of the mice in each group. (D) Serum leptin level of the mice in each group. (E) mRNA level of *Clock* and *Per1* of the mice in each group. (F) mRNA level of *c-Myc*, *p27*, and *HDAC3* of the mice in each group. Values are means \pm SEM. * $P < .05$ compared with the control

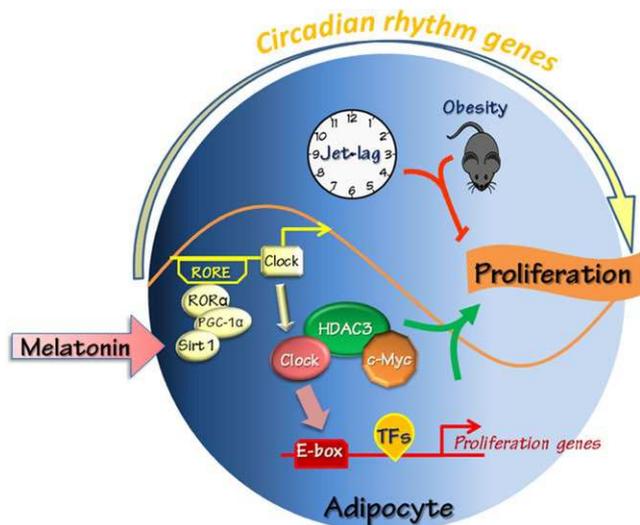


FIGURE 9 Proposed model for melatonin to promote adipocyte proliferation via regulating circadian genes and Clock/HDAC3/c-Myc complex

with estrogen receptor- α .⁴⁷ Although studies showed Clock appeared arrhythmic in various peripheral oscillators,^{48,49} we found a slightly rhythmic mRNA level of *Clock* in adipose tissue and its amplitude was enhanced by melatonin. These findings are consistent with other studies showing that *Clock*

mRNA has weak rhythmicity in peripheral adipose tissue and is an important component of the circadian oscillator in adipose metabolism.^{50,51} Furthermore, we demonstrated that Clock was potent to melatonin-induced proliferation of adipocytes. Recent studies showed that Clock promoted preadipocyte proliferation via the Wnt signal pathway.²⁹ In this study, we found that Clock was associated with c-Myc, a key component of cell proliferation. The profiles of transcription factors which control *Clock* expression were enhanced in melatonin-treated adipocytes. We also confirmed that melatonin stimulated transition of G1/S phase and cell proliferation by elevating Clock expression. These data collectively suggest Clock is the central to melatonin promoting proliferation of adipocytes.

Melatonin exerts many of its physiological effects on adipose tissue not only by the action of sympathetic nervous system and its receptors in adipocyte, but also through the receptor-independent actions via its direct free radical scavenging.^{2,25,29,52-54} Studies have shown that melatonin can regulate amplitude of circadian clock genes by upregulating Sirt1 in which deacetylation of PGC1- α promotes the binding of ROR α to RORE in the promoter of clock genes.^{4,29,55,56} The mechanism of Clock on cell proliferation may depend on E-box, which is a DNA response element.⁵² So we assumed that the promoting role of melatonin in proliferation of

adipocytes may be associated with the binding of Clock to E-box in promoter of the key proliferation genes. *c-Myc* is important in regulating cell cycle and proliferation. Studies show that *c-Myc* activation enhances proliferation of mouse embryonic fibroblasts and 3T3-L1 preadipocytes upon reduction in the NAD(+)-dependent protein deacetylase sirtuin 1.⁵⁷ In this study, we found that Clock elevated *c-Myc* transcription by binding to two proximal E-boxes upstream of the *c-Myc* promoter region. Histone deacetylases (HDACs) are critical regulators of gene expression that enzymatically remove the acetyl group from histones.⁵⁸ Studies show that overexpression of cyclin D1 promotes cellular proliferation of adipocytes by enhancing recruitment of HDAC1 and HDAC3. In this study, we preliminarily determined that the Clock/HDAC3/*c-Myc* complex was formed to mediate the promotion of melatonin on adipocyte proliferation. In addition, the relationship between melatonin and HDAC has been described such that the pharmacological effects of melatonin are closely related to HDAC signaling.⁵⁹ Although the molecular mechanism of complex formation remains elusive, we suspect that it is similar to the Sirt1/Clock interaction by protein deacetylase, and this needs further study.⁶⁰⁻⁶³ Notably, we are the first to demonstrate directly that melatonin enhanced adipocyte proliferation through forming the Clock/HDAC3/*c-Myc* complex to act function. It is noticed that melatonin elevated the mRNA levels of *HDAC3*, *HDAC5*, and *HDAC7* in stem cell differentiation.⁶⁴ In the growth and proliferation of adipocytes, melatonin may regulate the cell cycle by epigenetic modulation of gene transcription, and this will require further investigation.

There are also several limitations in this study. First, the dose of melatonin we used was at a pharmacological level. However, we and others found that this dose did not affect the viability of adipocytes,¹⁵ and had been used in many reports.^{65,66} Second, we did not confirm whether melatonin functions through its specific receptors MT1, MT2, and MT3. However, previous studies have demonstrated that melatonin-mediated proliferation was inhibited by luzindole, an antagonist of melatonin receptors,^{67,68} indicating that melatonin activity requires its receptors.

In summary, the present study demonstrates that melatonin enhances circadian amplitude of circadian clock genes and subsequently promotes adipocyte proliferation. Moreover, melatonin promotes cell cycle progression by elevating Clock and forming Clock/HDAC3/*c-Myc* complex in proliferation of adipocytes (Figure 9). Thus, our results indicate that melatonin has potential as anti-obesity agent to reverse obesity-related circadian disorders.

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CONFLICT OF INTEREST

The authors declare that there is no duality of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS

All the authors contributed to this manuscript. Z.L., L.G., and C.S. planned the experiments; all the authors performed the experiments; L.G. and D.L. analyzed the data; Z.L., L.G., and D.L. contributed reagents or other essential materials; and L.G. and Z.L. wrote the manuscript.

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