Melatonin reduces intramuscular fat deposition by promoting lipolysis and increasing mitochondrial function

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Running title: Melatonin Reduces IMF Deposition

Abbreviations: Intramuscular fat (IMF), interleukin (IL6), Fibroblast growth factor 21 (FGF21), CCAAT/enhancer binding protein α (C/EBPα), CCAAT/enhancer binding protein β (C/EBPβ), Cell Counting Kit-8 (CCK8), cytochrome c oxidase subunit 2 (Cox2), 0.25 mM dexamethasone, 1 mM 3-isobutyI-1-methylxanthine, and 5 μg/mL insulin (DMI), fluorescence-activated cell sorting (FACS), fatty acid-binding protein 4 (FABP4), Institute of Cancer Research (ICR), longissimus dorsi muscles (LDM), 3-isobutyI-1-methylxanthine (IBMX), 4-phenyl-2-propionamidotetralin (4-P-PDOT), vastus lateralis (VL), 5-ethyl-2'-deoxyuridine (EdU), optical density (OD), oxygen consumption rate (OCR), peroxisome proliferator activate receptor γ (PPARγ), peroxisome proliferator-activated receptor gamma (PPARG), PPARG coactivator 1α (PGC-1α), polyvinylidene difluoride
(PVDF), transcription factor A mitochondrial (TFAM), carnitine palmitoyltransferase 1β (CPT-1β), uncoupling protein 3 (UCP3), cell death-inducing DFFA-like effector a (CIDEA), penicillin-streptomycin (PS), PR domain containing 16 (PRDM16), carbonyl cyanide-4-( trifluoromethoxy), phenyl-hydrazone (FCCP), hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), protein kinase A (PKA), perilipin 1 (PLIN1), melatonin receptor 1A (MT1), melatonin receptor 1B (MT2), growth medium (GM), standard error of mean (SEM).
ABSTRACT

In obesity and diabetes, intramuscular fat (IMF) content correlates markedly with insulin sensitivity, which makes IMF manipulation an area of therapeutic interest. Melatonin, an important circadian rhythm-regulating hormone, reportedly regulates fat deposition, but its effects on different types of adipose vary. Little is known about the role of melatonin in IMF deposition. Here, using intramuscular preadipocytes in pigs, we investigated whether melatonin affects or regulates IMF deposition. We found that melatonin greatly inhibited porcine intramuscular preadipocyte proliferation. Although melatonin administration significantly upregulated the expression of adipogenic genes, smaller lipid droplets were formed in intramuscular adipocytes. Additional investigation demonstrated that melatonin promoted lipolysis of IMF by activating protein kinase A and the signaling of extracellular signal-regulated kinases 1/2. Moreover, melatonin increased thermogenesis in intramuscular adipocytes by enhancing mitochondrial biogenesis and mitochondrial respiration. A mouse model, in which untreated controls were compared with mice that received 3 weeks of melatonin treatment, verified the effect of melatonin on IMF deposition. In conclusion, melatonin reduces IMF deposition by upregulating lipolysis and mitochondrial bioactivities. These data establish a link between melatonin signaling and lipid metabolism in mammalian models and suggest the potential for melatonin administration to treat or prevent obesity and related diseases.

Key Words: Melatonin, IMF, Cell proliferation, Mitochondrial biogenesis, Lipolysis
INTRODUCTION

Intramuscular fat (IMF) refers to adipose tissue located between skeletal muscle fibers (1). IMF is an important economic trait in farm animals (2)-reducing IMF levels to increase the lean meat percentage of pork or increasing the IMF content to improve the meat flavor is the most important part of the breeding work (3, 4). However, in humans, IMF content has a significant correlation with insulin resistance and type 2 diabetes (5). The reduction of IMF significantly improves the insulin sensitivity of muscle tissue (6). Therefore, finding a mechanism to regulate the proliferation and differentiation of IMF cells has important economic and medical value.

Melatonin, a derivative of tryptophan, is synthesized and secreted by the pineal gland in mammalian animals (7) and mainly mediates circadian rhythms and reproductive cycles (8, 9). Recent researchers found that melatonin has significantly regulated fat metabolism (10). In vitro experiments show melatonin significantly promotes the adipogenic differentiation of 3T3-L1 precursor adipocytes by increasing the expression of both CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator activated receptor γ (PPARγ) (11). Alonso et al. reported melatonin inhibited adipocyte differentiation through regulated CCAAT/enhancer binding protein β (C/EBPβ) transcriptional activity (12). This inconsistency in findings may be due to the differences in the timing melatonin treatment of 3T3-L1 cells.

Other studies reported melatonin reduces fat content by promoting lipid metabolism in porcine oocytes (13). Although recent findings indicate melatonin promotes the differentiation of bovine intramuscular preadipocytes into adipocytes by increasing the expression levels of
*PPARγ, C/EBPβ, and C/EBPα* via melatonin receptor 1B (MT2) (14), laboratory experiments demonstrate chronic supplementation with melatonin leads to the suppression of body weight gain and a reduction of adiposity in laboratory animals by modifying gut microbiota in mice (15). However, in postpubertal heifers, melatonin promotes fat deposition in the rib and longissimus muscle (16). *In vitro* and *in vivo* findings indicate melatonin plays an important regulatory role in the deposition of fat, yet the effect of melatonin on adipose tissue varies by report, and the study of melatonin on IMF is limited.

We describe a potential signaling pathway for melatonin-affected IMF deposition at the cellular level. Melatonin has a dual role in the deposition of IMF, promoting both fat differentiation and lipolysis. Long-term melatonin treatment significantly increases the catabolism of IMF cells and reduces IMF deposition.
MATERIALS AND METHODS

Ethics statement

All experiments were performed in accordance with the guidelines of the regional Animal Ethics Committee and were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Porcine intramuscular preadipocytes isolation and in vitro differentiation.

Three male Erhualian pigs (age, 5 days) were humanely killed for porcine intramuscular preadipocytes isolation and culture (17). Briefly, longissimus dorsi muscles (LDM) were harvested from piglets, minced, and digested with 2 mg/mL collagenase type I (Sigma-Aldrich, USA) in DMEM/F12 (Hyclone, Thermo Fisher Scientific, USA) containing 1% fatty acid-free BSA (Sigma-Aldrich, USA) in a shaking water bath at 40 rpm speeds for 2 hours at 37 °C. The digest samples were collected with a cell strainer (70-μm and 200-μm diameter) and rinsed 3 times with serum-free DMEM/F12 medium, then spun twice in a centrifuge at 1500× g for 10 minutes. Finally, cells were transferred to plates, grown in growth medium (GM; DMEM/F12 [Hyclone] supplemented with 10% FBS [Sigma-Aldrich] and 1% penicillin-streptomycin [PS]) at 37 °C with 5% CO₂. After 2 hours, the non-adherent cells were washed and replaced with fresh medium to continue culturing.

After reaching 90% confluence, the intramuscular preadipocytes were induced to differentiate with the adipogenic agent DMI (0.25 mM dexamethasone, 1 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL insulin) for 4 days. The medium was replaced with
maintenance medium containing 5 μg/mL insulin, 10% FBS-DMEM until day 8, and fresh medium was changed every 2 days.

**Cell proliferation assay**

Preadipocytes were seeded into a 96-well plate and cultured with 10% FBS-DMEM medium. Twenty-four hours later, the medium was replaced with 10% serum medium containing melatonin (Sigma-Alrich, USA) at 0 mM, 0.01 mM, 0.1 mM, 0.5 mM, 1 mM, and 2 mM doses for 12, 24, 36 or 48 hours. After exposure of the cells to melatonin, culture media was changed for serum-free culture media, we added 10 μL Cell Counting Kit-8 (CCK8) reagent dissolved in growth medium (GM), then continue culturing at 37 °C for 2 hours. The absorbance of each well was measured at 450 nm wavelength using an automated microplate reader (Bio-Rad, Japan).

**EdU stain**

Preadipocytes were treated with or without 1 mM of melatonin for 24 hours, and cells were cultured with fresh growth medium containing EdU (5-ethynyl-2'-deoxyuridine, final concentration, 10 mM) for two hours. EdU staining was conducted using Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit (RiboBio, Guangzhou, China) according to the manufacturer’s protocol. The EdU-labeled cells were reviewed with the confocal microscope.

**Apoptosis analysis**

Preadipocytes were treated with or without 1 mM melatonin for 24 hours. Cells were
digested using 0.25% trypsin without EDTA, washed twice with phosphate-buffered saline, fixed in 500-μL binding buffer (100 mM HEPES, 140 mM NaCl, 25 mM CaCl2, pH = 7.4), and stained with 100-μg/mL Annexin V-FITC/100 μg/mL propidium iodide at room temperature for 15 minutes. The apoptosis percentage of porcine intramuscular preadipocytes was measured using an Apoptosis Detection Kit (Vazyme Biotech, China) according to the manufacturer’s protocol. We sorted 1×10⁵ cells by flow cytometry (fluorescence-activated cell sorting [FACS]); Becton-Dickinson, USA). The apoptosis rate was calculated using the following equation: (number of cells in the right upper quadrant + number of cells in the right lower quadrant)/total number of cells.

**Cycle analysis**

After treatment with or without 1 mM of melatonin for 24 hours, the cells were harvested by trypsinization, washed twice with ice-cold PBS and fixed in 75% ethanol for 24 hours at 4 °C. We added 100 μL RNase A and incubated the cells for 30 minutes at 37 °C, then added 400 μL PI staining mix and stored them away from light for 30 minutes at 4 °C. The wavelength of red fluorescence used for excitation on the machine test was 488 nm. For each sample, 10,000 events were acquired with a FACS, and the percentage of cells in G0/G1, S and G2/M phases of the cell cycle were determined by Flowjo analytical software version 9.3.2 (BD FACS Calibur, USA).

**Oil Red O stain**

To induce porcine intramuscular preadipocytes differentiation into mature lipid droplets
in DMI with or without melatonin, cells were washed 3 times with PBS, then fixed with 10% formalin for 15 minutes. After fixation, the cells were washed 3 times with PBS and stained with Oil Red O (0.5% Oil-Red-O in isopropanol, diluted 3:2 in water and filtered with a 0.22-μm filter; Sigma-Aldrich, USA). After 20 minutes, the cells were washed with 60% isopropanol for 20 seconds and photographed using a microscope. To measure triglyceride contents, stained adipocytes were eluted with isopropanol for 20 minutes. The optical density (OD) values were detected with a spectrophotometer at a wavelength of 510 nm, as described previously (18).

**LipiD-QuanT analysis**

Mature adipocytes were treated with maintenance medium containing 1 mM melatonin or not. Lipid droplet images were taken two days later. A total of 803 lipid droplets were used to assess lipid droplet size by the LipiD-QuanT method (19).

**Lipolysis analysis**

Intramuscular preadipocytes were induced to differentiate at 6 days with or without melatonin. Samples were collected on days 0, 2, 4, and 6 to extract total RNA. The expression of lipolysis-related genes was analyzed by RT-qPCR. To further demonstrate the occurrence of lipolysis in the melatonin treatment process, intramuscular preadipocytes were induced to differentiate into mature adipocytes, then incubated for 48 hours in fresh GM with or without 1 mM melatonin. At the end of the incubation, supernatants were collected and analyzed for free glycerol according to the protocol of the Adipolysis Assay Kit (Abcam, Shanghai). Total
protein samples were also collected and analyzed by western blot analysis for the expression of lipolytic proteins.

Bioenergetics profiling

Porcine intramuscular preadipocytes (approximately $2.5 \times 10^5$ cells/well) were seeded into gelatin-coated XF24 culture microplates and grown in GM for 12 hours at 37 °C in an atmosphere of 5% CO$_2$. The cells were treated with DMI, DMI + melatonin (1 mM), and DMI + melatonin (1 mM) + 4-phenyl-2-propionamidotetralin (4-P-PDOT; 10 μM) for an additional 24 hours. The O$_2$ consumption was measured with a Seahorse Bioscience XF24-3 extracellular flux analyzer. We added 1 μM oligomycin to the cells to measure the oxygen consumption rate (OCR) independent of oxidative phosphorylation. Subsequently, 0.5-μM carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone and 2 μM respiratory chain inhibitor rotenone were added to measure the maximal respiration and basal non-mitochondrial respiration rates. Statistical comparisons were made using Student’s t-test.

Intracellular ATP assay

The intracellular ATP concentration was measured using an ATP assay kit (Beyotime Biotechnology, Jiangsu, China) following the manufacturer's protocol. Briefly, the cells were cultured in DMI, as well as DMI + melatonin (1 mM), or DMI + melatonin (1 mM) + 4-P-PDOT (10 μM). After 24 hours, the treated cells were lysed in the lysis buffer and spun in a centrifuge at 4 °C, 12,000 rpm for 5 minutes. The supernatants were used to detect ATP, and the protein concentrations were determined by BCA assay kit (Beyotime Biotechnology,
Jiangsu, China). We then added 20 μl of supernatant into 100 μl of ATP detection solution that had been incubated at room temperature for 5 minutes, mixed it immediately, and determined the luminescence using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, Germany). The concentration of ATP was calculated according to a standard curve and converted into nmol/μg protein.

Mitochondrial Content

After 24 hours of intramuscular preadipocyte treatment with or without 1 mM melatonin, samples were collected, and the total DNA was extracted from cells using a DNA extraction kit (Jiancheng Bioengineering Institute, Nanjing, China). To quantify mitochondrial cytochrome c oxidase subunit 2 (Cox2) and the nuclear gene β-globin copy number, a reference curve was established using serially diluted standard DNA through RT-qPCR. The copy number ratio of mtDNA:nDNA was used to evaluate the relative number of mitochondria as previously described (20). The primers of mtDNA and nDNA are shown in Table 1.

RT-qPCR

After porcine intramuscular preadipocytes were treated with 1 mM melatonin for 24 hours, total RNA was extracted using a TRIzol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. We used a Godenstar™ RT6 cDNA Synthesis Kit (TsingKe Biotech, Nanjing, China) to reverse-transcribe 1.0 μg of each sample to First-strand cDNA. The RT-qPCR was performed using SYBR Green Master Mix (Vazyme Biotech, Nanjing,
China), and relative expression levels were analyzed using the 2^{ΔΔCt} method. The primers used are listed in Table 1. *RPLP0* was used as the endogenous control.

**Western blotting**

Total protein was extracted from intramuscular preadipocytes using RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China), and protein quantities were measured by the BCA Protein Assay kit (Beyotime Biotechnology, Jiangsu, China). We prepared 12% SDS-PAGE gel and loaded 20 μg of protein. After 1 hour of electrophoresis, the total protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% BSA and then incubated with primary antibodies overnight at 4 °C. After washing, the appropriate secondary antibodies were used, and chemiluminescence was detected. Images were captured with the VersaDoc 4000 MP system (Bio-Rad, USA). The antibodies used herein mainly include: MT1 (1:1000. Thermo Fisher, #PA5-19109), MT2 (1:1000. Affinity, #DF4994), PKA (1:800. Affinity, #AF7746), Phospho-PKA (Thr197, 1:500. Affinity, #AF7246), Phospho-ERK1/2 (Thr202/Tyr204, 1:500. Thermo Fisher, #PA5-37828), HSL (1:1000. Affinity, #AF6403), Phospho-HSL (Ser660, 1:500. Affinity, #AF8026), PLIN1 (1:1000, Affinity, #DF7602), Phospho-PLIN1(Ser522, 1:500. Vala sciences, #4856) α-tubulin (1:1000. Affinity, #AF7010), GAPDH (1:1000. Affinity, #AF7021).

**A mouse experiment to verify the effect of melatonin on IMF deposition**

We conducted an *in vivo* experiment to investigate the effect of melatonin on fat
deposition using 3-week-old healthy male Institute of Cancer Research (ICR) mice (n = 8; initial body weight, 19 g ± 0.5 g; Qing Long Shan Co., Animal Breeding Center, Nanjing, China) randomly divided into 2 groups. Melatonin (20 mg/kg) was administered via intravenous injection to the tail every other day for 4 weeks. Meanwhile, the minimum volume of alcohol used to dilute melatonin (20 mg/kg) was injected into another group as a control group. All mice had free access to standard high-fat diet and water. All mice were housed in a temperature-controlled (22 ± 2 °C) room with a 12-hour:12-hour light:dark cycle (lights on from 7:00 A.M. to 7:00 P.M.). During the experimental period, body weights were recorded every 3 days. On day 27, all mice were bled, weighed, and humanely killed. The fat tissues were peeled off, weighed, photographed, and then quick-frozen for the subsequent experiment.

**Histology and imaging**

The vastus lateralis (VL) was released from the mouse hind limb to detect neutral lipid accumulation in muscle tissue. Frozen VL samples were sectioned with a cryostat (thickness, 7 μm). All sections were washed with deionized water, quickly dipped in 60% isopropanol, then stained for 20 minutes in the Oil Red O working solution. They were then rinsed with isopropanol, following by deionized water, and cover-slipped with glycerol jelly. Images were captured using a microscope.

**Statistical analysis**

Data were analyzed using the IBM SPSS Statistics for Windows, Version 20.0 (IBM
Corp., Armonk, NY, USA) and GraphPad Prism (GraphPad Software, Inc., La Jolla, USA).

The statistical significance was calculated using the student's t-test and one-way ANOVA (\(*P < 0.05, **P < 0.01, ***P < 0.001\)). All data are presented as means ± standard error of mean (SEM).

RESULTS

Melatonin inhibits the proliferation of porcine intramuscular preadipocytes

To understand the effect of melatonin on IMF deposition better, different concentrations of melatonin were administered to porcine intramuscular preadipocytes (Fig. 1A). The result shows that melatonin was both dose- and time-dependent on cell proliferation. Melatonin 1 mM significantly inhibited preadipocytes proliferation from 24 to 48 hours (24 hours, \(P < 0.001\); 36 hours, \(P < 0.001\); 48 hours, \(P < 0.001\)). Melatonin 2 mM inhibited cell proliferation and caused death from 12 hours to 48 hours (all \(P < 0.001\)).

EdU staining assay verified the inhibited proliferation of intramuscular preadipocytes related to the 1-mM melatonin dose. After treating intramuscular preadipocytes with 1 mM melatonin for 24 hours, we found the proportion of EdU-positive cells decreased by 80% (Fig. 1B and 1C), and no significant apoptotic cells were found by flow cytometry tests (Fig. 1D and 1E). Cell cycle analysis revealed that 1-mM melatonin significantly increased the fraction of cells in the G0/G1 phase of the cell cycle, while simultaneously reducing the proportion in the G2/M phase, not the S phase after treatment for 24 hours (Fig. 1F and 1G). These results suggest melatonin could suppress preadipocyte proliferation by arresting the cell cycle.
process.

Melatonin significantly reduces fat deposition in intramuscular preadipocytes

After adipogenic differentiation, the lipid droplets appeared on the third day using a medium with DMI+melatonin, while the lipid droplets developed on the fourth day using the normal medium (Fig. 2A). The medium was replaced with maintenance medium on the fifth day, but the treatment group contained 1 mM melatonin and continued to culture for 3 days. Oil Red O staining and triglyceride levels showed that preadipocytes treated with melatonin have smaller lipid droplets and less triglyceride accumulation than those not treated with melatonin (Fig. 2B). However, the expression levels of adipogenic genes C/EBPα, adipocyte fatty acid-binding protein 4 (FABP4), FASN, and PPARγ were significantly up-regulated (P < 0.05) in the melatonin-treated samples compared with the control group in the 2-, 4-, and 6-day time points (Fig. 2C-F). These results indicate melatonin treatment, given time, could promote fat cell differentiation earlier, but long-term melatonin treatment decreases lipid accumulation.

Melatonin promotes lipolysis of intramuscular preadipocytes

To determine whether melatonin is also involved in regulating other lipid metabolism activities, we investigated the expression of genes related to lipolysis and thermogenesis, including hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), perilipin 1 (PLIN1), and uncoupling protein 3 (UCP3). The expression levels of these genes in the melatonin-treated group were significantly higher than those in the control groups at 2 and 4
days. On the 6th day, although the difference of HSL and PLIN1 expression levels between these two groups was slight, the expression of ATGL and UCP3 were still significantly higher in the melatonin-treated group than the control group. (Fig. 3A-D). These results indicate melatonin may promote the hydrolysis of porcine intramuscular preadipocytes. The lipid droplets diameters are mainly distributed between 0-8 px after 2 days of melatonin-treated porcine mature adipocytes. In the control group, the mean of lipid droplets diameters size distributions shifted toward larger lipid droplets, and part of them are distributed in the 8-16 px region (Fig. 3E and F). Furthermore, melatonin significantly increases the amount of free glycerol in the medium (Fig. 3G). These data suggested melatonin promotes lipolysis of porcine intramuscular preadipocytes.

**Melatonin promotes the lipolysis of porcine adipocytes through MT2**

To examine the mechanism of melatonin promoting lipolysis of IMF, mature adipocytes were exposed to 1 mM melatonin for 24 hours. RT-qPCR and western blots analyses showed melatonin significantly promoted the expression of melatonin receptor 1B (MT2) in IMF cells. However, it does not change the expression of melatonin receptor 1A (MT1) (Fig. 4A-C). This suggests MT2 may play an important role in melatonin-mediated lipolysis of intramuscular adipocytes. As expected, 4-P-PDOT, a selective antagonist of MT2 (21), can significantly inhibit the upregulation of lipolysis-related genes (HSL and ATGL) caused by melatonin (Fig. 4D and 4E). Moreover, it rescued the down-regulation of PLIN1 caused by melatonin in mature adipocytes. These results indicate the effect of melatonin on lipolysis in porcine
intramuscular adipocytes may be mainly mediated by MT2.

**Melatonin promotes intramuscular adipocytes lipolysis by activating ERK1/2 and protein kinase A signaling pathways**

The protein kinase A (PKA) and ERK1/2 pathways are two classic pathways for lipolysis signaling, and they can be activated by melatonin. Therefore, we explored whether these pathways are involved in mediating melatonin-induced IMF lipolysis. The PKA (Thr197) and ERK1/2 (Thr202/Tyr204) phosphorylation levels in mature intramuscular adipocytes treated for 24 hours with 1 mM melatonin were detected by western blots, and the protein levels of phosphorylated PKA and ERK1/2 were significantly increased in the melatonin-treated samples compared with the control. However, after blocking the melatonin signal through the MT2 inhibitor 4-P-PDOT, the effect of melatonin was offset (Fig. 5A-D). As downstream lipolysis–associated genes of PKA and ERK1/2 signals, the phosphorylation levels of HSL (Ser660), PLIN1 (Ser522), and the protein expression of ATGL were increased (Fig. 5I and 5J). However, the expression of PLIN1 was significantly decreased compared with the control group (Fig. 5G and 5H). Meanwhile, blocking the melatonin signal by 4-P-PDOT reduced the protein expression related to lipolysis, including HSL, PLIN1, and ATGL (Fig.5E-J). These findings indicate ERK1/2 and PKA might play a role in melatonin-mediated lipolysis.

**The role of PKA and ERK1 / 2 signaling in melatonin-induced lipolysis**

To further explore the contribution of PKA or ERK1/2 in melatonin-induced lipolysis, preadipocytes were treated with 1 mM melatonin, 1 mM melatonin + 10 μM H89 (inhibitor of
PKA, #HY-15979) and 1 mM melatonin + 1 μM SCH772984 (inhibitor of ERK1/2, #HY50846), respectively, for 24 hours. Western bolt showed that p-PKA and p-ERK significantly increased in melatonin group, meanwhile p-PKA expression significantly reduced in melatonin + H89 and p-ERK expression significantly reduced in SCH772984 group (Fig. 6A-C). The presence of H89 or SCH772984 significantly reduced mRNA expression of PLIN1, HSL and ATGL compared with melatonin-treated groups (Fig. 6D-F). Similarly, phosphorylated PLIN1 and HSL also decreased significantly under the influence of H89 or SCH772984 (Fig. 6G-I). More reduction of ATGL appeared after the ERK1/2 signal is suppressed (Fig. 6G and 6J). This indicated that melatonin through PKA signal plays a major role in the regulation of PLIN1 and HSL, while ERK1/2 has a more significant effect on ATGL. Oil Red O staining showed that H89 could be more effective on blocking melatonin to induce lipid droplets lipolysis, resulting in a larger area of Oil Red O in H89+ melatonin group (Fig. 6k and 6L). Although SCH772984 can also inhibit melatonin-induced lipolysis, the effect is slightly weaker than H89.

**Melatonin increases mitochondrial content and function in intramuscular preadipocytes.**

To understand the effect of melatonin on the metabolism of intramuscular preadipocytes, we examined the level of cellular oxygen consumption in different treatment groups. Our data found a similar basal OCR in 3 treatments (Fig. 7A and 7B). However, the addition of the exogenous uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenyl-hydrazone (FCCP)
increased the maximum respiratory rate to a higher level in the melatonin group cells compared with the control group cells. The addition of a melatonin antagonist (4-P-PDOT) can partly reduce the OCR (Fig. 7A and 7C). Meanwhile, melatonin significantly increased the intracellular ATP generation in porcine intramuscular adipocytes (Fig. 7D).

Melatonin treatment significantly increased the mitochondrial copy number compared with control (Fig. 7E). Furthermore, melatonin significantly up-regulated the expression of mitochondrial-related genes including PGC-1α, TFAM, and CPT-1β, thermogenesis gene UCP3, and white adipose browning genes including CIDEA and PRDM16 compared to controls. However, this effect was weakened by the addition of 4-P-PDOT (Fig. 7F-K). Therefore, melatonin increases mitochondrial content and respiratory function, thereby enhances fatty acid oxidation or thermogenesis in intramuscular preadipocytes.

**Effects of PKA and ERK1/2 signaling on mitochondrial function during melatonin treatment of intramuscular preadipocytes**

To study the effects of PKA and ERK1/2 signaling pathways on mitochondrial function of melatonin-treated intramuscular preadipocytes. Preadipocytes were treated with 1 mM melatonin, 1 mM melatonin + 10 μM H89 and 1 mM melatonin + 1 μM SCH772984, respectively, for 24 hours. Both melatonin + H89 and melatonin + SCH772984 treatment significantly inhibited the increase of mitochondrial copy number, compared with the melatonin group (Fig. 8A). The total ATP levels also showed the same trend (Fig. 8B). Moreover, H89 significantly inhibited the effect of melatonin in up-regulating mitochondrial
biogenesis-related genes PGC-1α, TFAM, CPT-1β and CPT-1α, as well as browning genes CIDEA and PRDM16 (Fig. 8C-H). SCH772984 also significantly inhibited the up-regulation of TFAM, CIDEA and PRDM16 by melatonin, but the effects on PGC-1α, CPT-1α and CPT-1β were not significant (Fig. 8C, E and F). In addition, the expression of the thermogenic gene UCP3 is significantly reduced in melatonin + SCH772984 compared with melatonin + h89 (Fig. 8I). These data indicate that melatonin affects the expression of UCP3 mainly through the ERK1/2 signal, and affects mitochondrial biogenesis as well as intramuscular preadipocyte browning through the PKA signal.

**Melatonin lead to a decrease in body weight and IMF deposition**

Furthermore, our study *in vivo* found that injection of melatonin through the tail vein of ICR mice for three weeks was associated with a significantly decreased body weight compared with control group animals (Fig. 9A-C). Meanwhile, melatonin significantly reduced perineal fat and inguinal fat (Fig. 9D and 9E). PLIN1, an ideal marker for adipose tissue in muscle, and its mRNA, protein levels were significantly reduced in the vastus lateralis (VL) muscle tissue of mice treated with melatonin, but the phosphorylation level was significantly increased (Fig. 9F and 9G). The expression of other lipolysis-related genes was also significantly up-regulated in the melatonin-treated group, such as HSL, ATGL, UCP3 (Fig.9H-J), the area of Oil Red O staining in VL muscle was significantly reduced in mice treated with melatonin, (Fig. 9K). Moreover, phosphorylation levels of both PKA and ERK1/2 were significantly elevated (Fig. 9L and 9M). The expression of critical genes of
mitochondrial biogenesis and browning genes were significantly increased in the VL muscle of melatonin-treated group (Fig. 9N). The above data indicated that melatonin can reduce intramuscular fat deposition by promoting lipolysis and fat metabolism in vivo.
DISCUSSION

Diabetes and obesity are becoming the leading cause of cancer and death worldwide. Cancers caused by diabetes and high body-mass index (> 25 kg/m²) account for 5.6% of the world's new cancer cases in 2012 (22). Therefore, how to effectively regulate IMF deposition has become an area of great concern. Melatonin (N-acetyl-5-methoxytryptamine) regulates a variety of important central and peripheral actions related to circadian rhythms, reproduction, and neuroendocrine signaling in mammals (23-25). Melatonin also plays a crucial role in the regulation of adipocyte metabolism by promoting adipocyte proliferation (26), differentiation (11), and white fat browning (27, 28). Our study confirmed melatonin doses of 0.5 mM and 1 mM (especially 1 mM) inhibited intramuscular preadipocytes proliferation. This finding aligns with the previously published report regarding the human osteoblastic cell line (29). However, Linsen Zan reported that 1 mM melatonin did not affect the proliferation of bovine intramuscular preadipocytes (14). This inconsistency may be due to the different contributions of melatonin to different animal-derived cells. Moreover, the G1 and S phases of the cell cycle are an important regulatory period of the metabolic process. In the G0/G1 phase, the key gene for fat synthesis (PPARγ) is activated to promote adipogenesis by phosphorylation (30, 31). We found melatonin arrested intramuscular preadipocytes in the G0/G1 phase and up-regulated C/EBPa, FABP4, FASN, and PPARγ mRNA levels. Moreover, melatonin can promote the early adipogenic differentiation of intramuscular preadipocytes, but long-term melatonin treatment reduces the accumulation of triglycerides.

Adipocyte lipolysis is mediated by a complicated process that depends on lipases and
proteins associated with lipid droplets including HSL, ATGL, and PLIN1 (32), and lipolysis is closely related to thermogenesis (33). Our results showed lipolysis and thermogenic-related genes were significantly elevated by melatonin treatment. With melatonin treatment, lipid droplets became smaller and glycerol levels increased. This result is consistent with studies in the 3T3-L1 cell line (34). Our data indicated melatonin reduces lipid deposition by increasing lipolysis and thermogenesis in intramuscular preadipocytes.

In mammals, activated MT1 and MT2 are important mediators of the physiological function of melatonin. The reactivity of different types of cells to melatonin depends on the expression level of its receptor (35, 36). Melatonin modulates glucose homeostasis and energy balance through the direct activation of MT1 receptors on adipocytes (37). However, in bovine intramuscular preadipocytes, melatonin directly activates MT2, promoting triacylglycerol accumulation (14). Interestingly, our data revealed melatonin significantly promotes lipolysis by increasing the level of MT2 in mature adipocytes.

Melatonin may be closely related to the PKA and ERK1/2 pathways (38-40), which play a key role in lipolysis (41). Melatonin can trigger PKA activation in the rodent malaria parasite Plasmodium chabaudi (42). Melatonin also can activate ERK1/2 in osteoblasts (43). However, no studies indicate if melatonin can activate PKA or ERK1/2 in porcine intramuscular preadipocytes. Our current data suggest that melatonin activates PKA and ERK1/2, leading to reduced PLIN1 expression and increased phosphorylation of PLIN1, which is the main cause of melatonin-induced lipolysis of adipocytes. PLIN1 serves as a lipid droplet protective protein to prevent hydrolysis of triacylglycerol by HSL, while the reduction
in PLIN1 and/or the increase in its phosphorylation would impair the barrier function of the protein (41, 44). HSL is mainly located in the cytoplasm. After phosphorylation, it will translocate from the cytosol to the surface of lipid droplets, and synergize with pPLIN1 and ATGL to further increase the lipolysis reaction (45). More and more HSL are phosphorylated and translocated to the surface of lipid droplets, which are involved in the lipolysis reaction. Part of the HSL enters the small lipid droplets produced by lipolysis (46). This may result in a decrease in intracellular HSL protein, then lead to a compensatory increase in HSL mRNA. In addition, our study also showed that melatonin promoted the phosphorylation of HSL and PLIN1 more efficiently through the PKA signaling pathway. ERK1/2 had a more significant effect on the expression of ATGL.

Lipolysis produces glycerol and fatty acids, where fatty acids are catalyzed by fatty acyl-CoA synthetases located in the endoplasmic reticulum and mitochondrial outer membranes to form acyl-CoA, which enters the mitochondria to participate in β-oxidation and release heat (47). This is the main route of fatty acid degradation and is closely linked to mitochondrial content and respiration capability (48). A previous study showed that an increase of PGC-1α promotes the expression of TFAM, an important regulator in mitochondria biogenesis (40). TFAM translocates into the mitochondria to stimulate DNA replication and gene expression (49, 50), thereby promoting mitochondrial biogenesis and function. Our data showed melatonin treatments significantly increased cellular respiratory capacity, up-regulated the expression of PGC-1α and TFAM, and increased mitochondrial copy number. The addition of melatonin also induced the robust expression of thermogenic genes
in intramuscular preadipocytes including CPT-1β and UCP3 and triggered differentiation toward a beige phenotype gene CIDEA and Prdm16. However, when the melatonin signal was switched off via the MT2 antagonist 4-P-PDOT, the melatonin-promoting effect diminishes or disappears. Further study showed that blocking PKA and ERK1/2 signaling reduced the promotion of melatonin on downstream lipolysis. We found that melatonin-activated PKA signaling is more likely to up-regulated mitochondrial and browning-related genes, whereas activated ERK1/2 signaling primarily affects ATGL and UCP3 expression. These findings suggest melatonin accelerates intramuscular preadipocyte expenditures in vitro by enhancing mitochondrial biogenesis and energy metabolism, thereby reduced porcine intramuscular preadipocytes deposition.

The body of data suggesting melatonin reduces porcine IMF deposition in vitro is growing. An in vivo test was performed on mice to determine if melatonin can reduce IMF deposition. By injecting exogenous melatonin, tissue sections with Oil-Red-O staining indicated melatonin indeed reduces IMF deposition in VL muscle. Melatonin also promotes the expression of lipolytic genes, mitochondria-associated genes, and thermogenic genes in vivo. These results are consistent with the results of in vitro studies. Therefore, melatonin—at least partially—reduces IMF deposition by promoting lipolysis and heat production.

In conclusion, melatonin can inhibit proliferation, promote differentiation, and enhance the mitochondrial function of porcine intramuscular preadipocytes, and the promotion of intramuscular adipocytes lipolysis was mediated by MT2 activating ERK1/2 and PKA signaling. Our data establish a correlation between melatonin signaling and lipid metabolism.
in mammalian intramuscular preadipocytes. These findings further deepened our understanding of the mechanism of melatonin in regulating IMF deposition. Additional work is needed to determine melatonin reduce intramuscular fat deposition in pigs, and to confirm the physiological consequence of melatonin-regulated intramuscular fat deposition to systemic energy balance and metabolism in vivo.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant 31872334). The Natural Science Foundation of Jiangsu Province (Grants No. BK20160727). Fundamental Research Funds for the Central Universities (Grant No. KYZ201639).

REFERENCES

1. Hocquette, J. F., F. Gondret, E. Baeza, F. Medale, C. Jurie, and D. W. Pethick. 2010. Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. Animal 4: 303-319.

2. Malek, M., J. C. M. Dekkers, H. K. Lee, T. J. Baas, K. Prusa, E. Huff-Lonergan, and M. F. Rothschild. 2001. A molecular genome scan analysis to identify chromosomal regions influencing economic traits in the pig. II. Meat and muscle composition. Mamm Genome 12: 637-645.
3. Wood, J. D., G. R. Nute, R. I. Richardson, F. M. Whittington, O. Southwood, G. Plastow, R. Mansbridge, N. da Costa, and K. C. Chang. 2004. Effects of breed, diet and muscle on fat deposition and eating quality in pigs. *Meat Sci* **67**: 651-667.

4. Chen, G. S., and Y. N. Sui. 2018. Production, performance, slaughter characteristics, and meat quality of Ziwuling wild crossbred pigs. *Trop Anim Health Pro* **50**: 365-372.

5. Goodpaster, B. H., F. L. Thaete, and D. E. Kelley. 2000. Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus. *Am J Clin Nutr* **71**: 885-892.

6. Prior, S. J., L. J. Joseph, J. Brandauer, L. I. Katzel, J. M. Hagberg, and A. S. Ryan. 2007. Reduction in mid thigh low-density muscle with aerobic exercise training and weight loss impacts glucose tolerance in older men. *J Clin Endocr Metab* **92**: 880-886.

7. Reiter, R. J. 1991. Pineal Melatonin - Cell Biology of Its Synthesis and of Its Physiological Interactions. *Endocr Rev* **12**: 151-180.

8. Mura, M. C., S. Luridiana, F. Farci, M. V. Di Stefano, C. Daga, L. Pulinas, J. Staric, and V. Carcangiu. 2017. Melatonin treatment in winter and spring and reproductive recovery in Sarda breed sheep. *Anim Reprod Sci* **185**: 104-108.

9. Cassone, V. M. 1990. Effects of melatonin on vertebrate circadian systems. *Trends Neurosci* **13**: 457-464.
10. Amstrup, A. K., T. Sikjaer, S. B. Pedersen, L. Heickendorff, L. Mosekilde, and L. Rejnmark. 2016. Reduced fat mass and increased lean mass in response to 1 year of melatonin treatment in postmenopausal women: A randomized placebo-controlled trial. Clin Endocrinol (Oxf) 84: 342-347.

11. Gonzalez, A., V. Alvarez-Garcia, C. Martinez-Campa, C. Alonso-Gonzalez, and S. Cos. 2012. Melatonin promotes differentiation of 3T3-L1 fibroblasts. Journal of Pineal Research 52: 12-20.

12. Alonso-Vale, M. I. C., S. B. Peres, C. Vernochet, S. R. Farmer, and F. B. Lima. 2009. Adipocyte differentiation is inhibited by melatonin through the regulation of C/EBP beta transcriptional activity. Journal of Pineal Research 47: 221-227.

13. Jin, J. X., S. Lee, A. Taweechaipaisankul, G. A. Kim, and B. C. Lee. 2017. Melatonin regulates lipid metabolism in porcine oocytes. J Pineal Res 62.

14. Yang, W. C., K. Q. Tang, Y. N. Wang, Y. Y. Zhang, and L. S. Zan. 2017. Melatonin promotes triacylglycerol accumulation via MT2 receptor during differentiation in bovine intramuscular preadipocytes. Sci Rep-Uk 7.

15. Xu, P. F., J. L. Wang, F. Hong, S. Wang, X. Jin, T. T. Xue, L. Jia, and Y. G. Zhai. 2017. Melatonin prevents obesity through modulation of gut microbiota in mice. Journal of Pineal Research 62.

16. Zinn, S. A., L. T. Chapin, W. J. Enright, A. L. Schroeder, E. P. Stanisiewski, and H. A. Tucker. 1988. Growth, Carcass Composition and Plasma Melatonin in Postpubertal Beef Heifers Fed Melatonin. J Anim Sci 66: 21-27.
17. Han, H. Y., W. Wei, W. W. Chu, K. Q. Liu, Y. Tian, Z. H. Jiang, and J. Chen. 2017. Muscle Conditional Medium Reduces Intramuscular Adipocyte Differentiation and Lipid Accumulation through Regulating Insulin Signaling. *International Journal of Molecular Sciences* **18**.

18. Che, Y. Y., Q. H. Wang, R. Y. Xiao, J. Y. Zhang, Y. Q. Zhang, W. Gu, G. X. Rao, C. F. Wang, and H. X. Kuang. 2018. Kudinoside-D, a triterpenoid saponin derived from Ilex kudingcha suppresses adipogenesis through modulation of the AMPK pathway in 3T3-L1 adipocytes. *Fitoterapia* **125**: 208-216.

19. Varinli, H., M. J. Osmond-McLeod, P. L. Molloy, and P. Vallotton. 2015. LipiD-QuanT: a novel method to quantify lipid accumulation in live cells. *J Lipid Res* **56**: 2206-2216.

20. Fernandez-Galilea, M., P. Perez-Matute, P. L. Prieto-Hontoria, M. Houssier, M. A. Burrell, D. Langin, J. A. Martinez, and M. J. Moreno-Aliaga. 2015. alpha-Lipoic acid treatment increases mitochondrial biogenesis and promotes beige adipose features in subcutaneous adipocytes from overweight/obese subjects. *Biochim Biophys Acta* **1851**: 273-281.

21. Rhee, S. W., and M. J. Tanga. 2000. Synthesis of tritium labelled 4P-PDOT, a selective melatonin receptor antagonist. *J Labelled Compd Rad* **43**: 925-932.

22. Pearson-Stuttard, J., B. Zhou, V. Kontis, J. Bentham, M. J. Gunter, and M. Ezzati. 2018. Worldwide burden of cancer attributable to diabetes and high body-mass index: a comparative risk assessment. *Lancet Diabetes Endo* **6**: 95-104.
23. Soderquist, F., E. T. Janson, A. J. Rasmusson, A. Alit, M. Stridsberg, and J. L. Cunningham. 2016. Melatonin Immunoreactivity in Malignant Small Intestinal Neuroendocrine Tumours. *Plos One* **11**.

24. Yu, K., S. L. Deng, T. C. Sun, Y. Y. Li, and Y. X. Liu. 2018. Melatonin Regulates the Synthesis of Steroid Hormones on Male Reproduction: A Review. *Molecules* **23**.

25. Pfeffer, M., H. W. Korf, and H. Wicht. 2018. Synchronizing effects of melatonin on diurnal and circadian rhythms. *Gen Comp Endocr* **258**: 215-221.

26. Liu, Z. J., L. Gan, D. Luo, and C. Sun. 2017. Melatonin promotes circadian rhythm-induced proliferation through Clock/histone deacetylase 3/c-Myc interaction in mouse adipose tissue. *Journal of Pineal Research* **62**.

27. Jimenez-Aranda, A., G. Fernandez-Vazquez, D. Campos, M. Tassi, L. Velasco-Perez, D. X. Tan, R. J. Reiter, and A. Agil. 2013. Melatonin induces browning of inguinal white adipose tissue in Zucker diabetic fatty rats. *Journal of Pineal Research* **55**: 416-423.

28. Seron-Ferre, M., H. Reynolds, N. A. Mendez, M. Mondaca, F. Valenzuela, R. Ebensperger, G. J. Valenzuela, E. A. Herrera, A. J. Llanos, and C. Torres-Farfan. 2015. Impact of maternal melatonin suppression on amount and functionality of brown adipose tissue (BAT) in the newborn sheep. *Front Endocrinol* **5**.
29. Liu, L. F., Y. Zhu, Y. Xu, and R. J. Reiter. 2012. Prevention of ERK activation involves melatonin-induced G1 and G2/M phase arrest in the human osteoblastic cell line hFOB 1.19. *Journal of Pineal Research* **53**: 60-66.

30. Sarruf, D. A., I. Iankova, A. Abella, S. Assou, S. Miard, and L. Fajas. 2005. Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma. *Mol Cell Biol* **25**: 9985-9995.

31. Lopez-Mejia, I. C., J. Castillo-Armengol, S. Lagarrigue, and L. Fajas. 2018. Role of cell cycle regulators in adipose tissue and whole body energy homeostasis. *Cell Mol Life Sci* **75**: 975-987.

32. Carmen, G. Y., and S. M. Victor. 2006. Signalling mechanisms regulating lipolysis. *Cell Signal* **18**: 401-408.

33. Dalbo, V. J., M. D. Roberts, J. R. Stout, and C. M. Kerksick. 2008. Acute effects of ingesting a commercial thermogenic drink on changes in energy expenditure and markers of lipolysis. *J Int Soc Sports Nutr* **5**: 6.

34. Kato, H., G. Tanaka, S. Masuda, J. Ogasawara, T. Sakurai, T. Kizaki, H. Ohno, and T. Izawa. 2015. Melatonin promotes adipogenesis and mitochondrial biogenesis in 3T3-L1 preadipocytes. *Journal of Pineal Research* **59**: 267-275.

35. Reppert, S. M., C. Godson, C. D. Mahle, D. R. Weaver, S. A. Slaugenhaupt, and J. F. Gusella. 1995. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. *Proc Natl Acad Sci USA* **92**: 8734-8738.
36. Pandi-Perumal, S. R., I. Trakht, V. Srinivasan, D. W. Spence, G. J. Maestroni, N. Zisapel, and D. P. Cardinali. 2008. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Prog Neurobiol* **85**: 335-353.

37. Alonso-Vale, M. I., S. Andreotti, S. B. Peres, G. F. Anhe, C. das Neves Borges-Silva, J. C. Neto, and F. B. Lima. 2005. Melatonin enhances leptin expression by rat adipocytes in the presence of insulin. *Am J Physiol Endocrinol Metab* **288**: E805-812.

38. Picinato, M. C., E. P. Haber, J. Cipolla-Neto, R. Curi, C. R. D. Carvalho, and A. R. Carpinelli. 2002. Melatonin inhibits insulin secretion and decreases PKA levels without interfering with glucose metabolism in rat pancreatic islets. *Journal of Pineal Research* **33**: 156-160.

39. Tao, L., and Y. Zhu. 2018. Melatonin regulates CRE-dependent gene transcription underlying osteoblast proliferation by activating Src and PKA in parallel. *Am J Transl Res* **10**: 86-100.

40. Lan, L. H., M. M. Guo, Y. Ai, F. H. Chen, Y. Zhang, L. Xia, D. W. Huang, L. L. Niu, Y. Zheng, C. K. Suzuki, Y. H. Zhang, Y. Z. Liu, and B. Lu. 2017. Tetramethylpyrazine blocks TFAM degradation and up-regulates mitochondrial DNA copy number by interacting with TFAM. *Bioscience Rep* **37**.

41. Liu, L. R., S. P. Lin, C. C. Chen, Y. J. Chen, C. C. Tai, S. C. Chang, R. H. Juang, Y. W. Tseng, B. H. Liu, H. J. Mersmann, T. L. Shen, and S. T. Ding. 2011. Serum
Amyloid A Induces Lipolysis by Downregulating Perilipin Through ERK1/2 and PKA Signaling Pathways. *Obesity* **19**: 2301-2309.

42. Gazarini, M. L., F. H. Beraldo, F. M. Almeida, M. Bootman, A. M. da Silva, and C. R. S. Garcia. 2011. Melatonin triggers PKA activation in the rodent malaria parasite Plasmodium chabaudi. *Journal of Pineal Research* **50**: 64-70.

43. Liu, L. F., Y. Xu, R. J. Reiter, Y. T. Pan, D. Chen, Y. Z. Liu, X. Y. Pu, L. G. Jiang, and Z. C. Li. 2016. Inhibition of ERK1/2 Signaling Pathway is in Melatonin's Antiproliferative Effect on Human MG-63 Osteosarcoma Cells. *Cell Physiol Biochem* **39**: 2297-2307.

44. Karbowska, J., and Z. Kochan. 2012. Fat-reducing effects of dehydroepiandrosterone involve upregulation of ATGL and HSL expression, and stimulation of lipolysis in adipose tissue. *Steroids* **77**: 1359-1365.

45. Sztylandr, C., G. H. Xu, H. Dorward, J. T. Tansey, J. A. Contreras, A. R. Kimmel, and C. Londos. 2003. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation (vol 161, pg 1093, 2003). *J Cell Biol* **162**: 353-353.

46. Yeaman, S. J. 2004. Hormone-sensitive lipase--new roles for an old enzyme. *Biochem J* **379**: 11-22.

47. Kunau, W. H., V. Dommes, and H. Schulz. 1995. beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: A century of continued progress. *Prog Lipid Res* **34**: 267-342.
48. Bargut, T. C. L., V. Souza-Mello, M. B. Aguila, and C. A. Mandarim-de-Lacerda. 2017. Browning of white adipose tissue: lessons from experimental models. *Horm Mol Biol Clin Investig* **31**.

49. Liang, H. Y., and W. F. Ward. 2006. PGC-1 alpha: a key regulator of energy metabolism. *Adv Physiol Educ* **30**: 145-151.

50. Huang, C. L., D. R. Chen, Q. H. Xie, Y. Yang, and W. L. Shen. 2013. Nebivolol stimulates mitochondrial biogenesis in 3T3-L1 adipocytes. *Biochem Bioph Res Co* **438**: 211-217.

**AUTHOR CONTRIBUTION**

Jie Chen designed the experiments in this study; KaiQing Liu, Wensai Yu, XinBao Zhang, Xin Liu, and Chao Dong participated in the design and performed the experiments described in the manuscript; Wangjun Wu and Lifan Zhang analyzed the experiments and prepared the figures; Kaiqing Liu, Ye Tian, Melak Sherif a and Wei Wei wrote the final version of the manuscript. All authors reviewed the results and approved the final version of the manuscript.
### Table 1 Primers used in this study

| Gene   | Primer sequences                        | Product size (bp) | Tm (°C) |
|--------|-----------------------------------------|-------------------|---------|
| C/EBPα | 5′ ACACGGTGCGTCTAAGATGAG 5′ TCGGAGCGGTAGTTG | 238               | 59.1    |
|        | 5′ TCAGTGCAGGTAGTTG |                    |         |
| FABP4  | 5′ GGTGCACCGGTACCAGAA 5′ AAATCATTACATCGCCTTA | 203               | 60.3    |
|        | 5′ AAATCATTACATCGCCTTA |                    |         |
| FASN   | 5′ GCCAGCAGCGTGAGGTG 5′ CCAGTGCAGGTACGGGAAT | 196               | 58.6    |
|        | 5′ CCAGTGCAGGTACGGGAAT |                    |         |
| PPARγ  | 5′ GCCATTCGATCTTTTCAGGG | 213               | 59.3    |
|        | 5′ GCCATTCGATCTTTTCAGGG |                    |         |
| HSL    | 5′ TGTCTTTCGCGTGATTCCG 5′ GCCTGTTCATTCGTCTGA | 241               | 59.1    |
|        | 5′ GCCTGTTCATTCGTCTGA |                    |         |
| ATGL   | 5′ TGGATAAAGGAGCAGACAGG 5′ TCGGAGGGAGGCGAGT | 216               | 60      |
|        | 5′ TCGGAGGGAGGCGAGT |                    |         |
| PLIN1  | 5′ TGAAATGCGAGCAGG | 187               | 60.5    |
|        | 5′ TGAAATGCGAGCAGG |                    |         |
| UCP3   | 5′ TCGGAGAATGGAGGAGGAGGAA 5′ TCGGCTGATTTCGAAGGTA | 270               | 58.8    |
|        | 5′ TCGGCTGATTTCGAAGGTA |                    |         |
| Tfam   | 5′ CCTAGGAGCGACTACTGCG 5′ GCAACTTCTCAGACCTGCT | 126               | 59.7    |
|        | 5′ GCAACTTCTCAGACCTGCT |                    |         |
| Gene   | 5' Sequence                      | 3' Sequence                      | Length | Tm  |
|--------|----------------------------------|----------------------------------|--------|-----|
| CPT-1β | 5' TGGGCTGGTCAATCACATC           | 5' GCCGTGCATCTCAAACATCC          | 188    | 59.1|
|        |                                  |                                  |        |     |
| CPT-1α | 5' GTCTGTCAACTTTGTGCTGG          | 5' CAGGTGCTGGTGCTTTTCAC          | 97     | 60  |
|        |                                  |                                  |        |     |
| Pgc1a  | 5' GGACTGACATCGAGTGCTGCT         | 5' GCGTCTCTGTGAGAACTGCT          | 246    | 60.3|
|        |                                  |                                  |        |     |
| Cidea  | 5' GGCTCACAAGTGGATAGGGGG         | 5' AGATGAAGAGGAAGCATGGAAGT       | 250    | 60.1|
|        |                                  |                                  |        |     |
| Prdm16 | 5' CCACAAGTCTACACGCGAGT          | 5' CGGGTAATGGTTCTTGCCCT          | 160    | 60.3|
|        |                                  |                                  |        |     |
| Cox2   | 5' CATACTAACAACAAAACGTA          | 5' TGTCAGTTTTGTTGTAGTATG         | 346    | 59.3|
|        |                                  |                                  |        |     |
| β-globin| 5' CAACCTCAGGGGACCTGGCTA         | 5' GGCCCTCTCTTGACACG             | 150    | 60  |
|        |                                  |                                  |        |     |
| RPLP0  | 5' GTGCTGATGGGCAAGAAC            | 5' CTCCAGGAAGCGAGAATG            | 225    | 59.6|

Fig. 1. Effect of melatonin on cell proliferation in porcine intramuscular preadipocytes.

(A) The cells were treated with various melatonin concentrations (0, 0.01, 0.1, 0.5, 1 and 2
mM) at various times 0, 12, 24, 36 or 48 hours. After treatment, cell proliferation was estimated by CCK8 assay. Results were shown as a relative percentage of untreated cells at 0 hours, (n = 4 per group). (B) Detection of intramuscular preadipocytes cellular activity by EdU staining after treatment with or without 1 mM melatonin for 24 hours. The proliferating nuclei were stained red with EdU while the nuclei of all cells were stained blue with Hoechst for 30 minutes (scale bar = 100 μm). (C) Quantify the proportion of EdU-positive cells. (D) The effect of melatonin on the cell cycles and apoptosis of preadipocytes. Cells were stained with propidium iodide (PI) and Annexin V; then the apoptosis rate was evaluated by flow cytometry and (E) displayed as column charts after quantification. (F) Cell cycle analysis; cells were stained with PI, then detected by flow cytometry and (G) displayed as column charts after quantification, Data represented the mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n ≥ 3).
Fig. 2. Effect of melatonin on adipogenesis of porcine intramuscular preadipocytes. (A) The intramuscular preadipocytes were induced by DMI with or without melatonin and photographed by inverted microscopy in 0, 2, 3 and 4 days. Red scale bar = 100 μm, white scale bar = 200 μm. (B) Oil red O staining for neutral lipids and quantification of Oil Red O analyzed lipid accumulation of intramuscular preadipocytes on day 8 of differentiation, (n = 3 per group). Red scale bar = 100 μm. (C-F) Intramuscular preadipocytes were cultured in adipogenic medium with or without melatonin and samples were collected on 0, 2, 4, and 6 days. The expression levels of the related genes (CEBPα, FABP4, FASN, and PPARγ) were
detected by RT-qPCR. Data represented the mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n = 3).

Figure 3.

**Fig. 3. Melatonin promotes IMF lipolysis.** (A-D) Intramuscular preadipocytes were cultured in DMI with or without 1 mM melatonin and samples were collected on 0, 2, 4, and 6 days. The expression levels of the related genes (HSL, ATGL, PLIN1, and UCP3) were detected by RT-qPCR. (E) After 2 days of melatonin-treated porcine mature adipocytes, they showed smaller lipid droplets (Image for viewing), Red scale bar = 100 μm. (F) Smoothed distribution of lipid droplets diameter sizes obtained using the LipiD-QuanT software during adipogenesis (n = 803 lipid droplets). (G) Free glycerol release was assessed by Glycerol Assay Kit. Data
represented the mean ± S.E.M. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n = 3$).

Figure 4.

Fig. 4. Melatonin receptor (MT2) participates in lipolysis in precursor adipocytes. (A) The mRNA levels of melatonin receptor 1A (MT1) and melatonin receptor 1B (MT2) mRNA were measured in melatonin-treated mature adipocytes by RT-qPCR. (B-C) Western blot analyses were performed to detect the changes of MT1 and MT2. (D-F) RT-qPCR analysis of genes involved in lipolysis in mature adipocytes cultivated in GM, GM + melatonin and GM + melatonin + 4-P-PDOT medium for 48 hours. Data represented the mean ± S.E.M. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n = 3$).
Fig. 5. Melatonin activated PKA and ERK1/2 mediates lipolysis in porcine intramuscular preadipocytes. (A-J) Fully differentiated adipocytes were treated to control, 1
mM melatonin and 1 mM melatonin plus 10 μM 4-P-PDOT for 24 hours. The expression levels of PKA, ERK1/2, HSL, PLIN1, and ATGL and the phosphorylation levels of PKA (pPKA Thr197), ERK1/2 (pERK1/2 Thr202/Tyr204), HSL (pHSL Ser660), PLIN1 (pPLIN1 Ser522), and α-tubulin were evaluated by western blotting. The results were presented as mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n = 3).
Fig. 6. The role of PKA and ERK1/2 signaling in melatonin-induced lipolysis. (A-C)

Intramuscular preadipocytes were treated to 1 mM melatonin, 1 mM melatonin + 10 μM H89 and 1 mM melatonin + 1 μM SCH772984 respectively, for 24 hours. The phosphorylation levels of PKA (pPKA Thr197) and ERK1/2 (pERK1/2 Thr202/Tyr204) were evaluated by
western blotting. (D-F) The expression levels of HSL, PLIN1, and ATGL were measured by RT-qPCR. (G-J) Western blots detected protein expression levels of HSL (pHSL Ser660), PLIN1(pPLIN1 Ser522) and ATGL. (K and L) Oil red O staining for neutral lipids and quantification of Oil Red O analyzed lipid accumulation of intramuscular preadipocytes, black scale bar = 100 μm. The results were presented as mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n = 3).
Fig. 7. Melatonin may accelerate the expenditure of porcine intramuscular preadipocytes by increasing mitochondrial biogenesis. Measurement of oxygen uptake, mitochondrial biogenesis, and gene expression of key regulators of energy metabolism.
consumption rates (OCR) of intramuscular preadipocytes in control, 1 mM melatonin and 1 mM melatonin + 10 μM 4-P-PDOT, (A) Quantitative, (B) basal OCR and (C) maximum respiratory rate. (D) Relative ATP level in porcine intramuscular preadipocytes treated with 1 mM melatonin and 1 mM melatonin + 10 μM 4-P-PDOT. (E) Mitochondrial contents were evaluated by measuring the ratio of mtDNA:nDNA (n = 3 per treatment). (F) RT-qPCR evaluated expression levels of genes related to mitochondrial biogenesis TFAM, (G) thermogenic genes CPT-1B, (H) PGC-1α, (I) UCP3, (J) trans-differentiation related genes CIDEA and (K) PRDM16. The results were presented as mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n = 3).
Fig. 8. Effects of PKA and ERK1/2 signaling on mitochondrial function during melatonin treatment of intramuscular preadipocytes. Intramuscular preadipocytes were treated with 1 mM melatonin, 1 mM melatonin + 10 μM H89 and 1 mM melatonin + 1 μM SCH772984 respectively, for 24 hours. (A) The ratio of mtDNA:nDNA were used to evaluate mitochondrial contents (n = 3 per treatment). (B) Relative ATP level were measured in different treatment of intramuscular preadipocytes. (C-I) The expression levels of mitochondrial biogenesis (PGC-1α, TFAM, CPT-1β, CPT-1α), browning (CIDEA, PRDM16),
and themogenesis genes (UCP3) were measured by RT-qPCR. The results were presented as mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n = 3).

Figure 9.

**Fig. 9. Melatonin reduced IMF deposition in the mice body.** (A) The dosage and frequency of melatonin administration in the mouse model. (B) Morphological appearance of mice
injected with or without melatonin 24 days later. (C) Mouse weight gain curve during injection. (D) Morphological appearance of significant reduced adipose tissue of inguinal and perirenal. (E) Different adipose tissue/body weight ratio. (F and G) The mRNA and protein expression of PLIN1 or p-PLIN1 was tested by RT-qPCR and western blots in vastus lateralis (VL) muscle. (H-J) The mRNA expression levels of HSL, ATGL and UCP3 were detected by RT-qPCR in the VL muscle. (K) Oil-Red-O staining of VL muscle adipose tissue sections. (L and M) Western blots evaluated the expression p-PKA and p-ERK1/2 in VL muscle. (N) RT-qPCR was used to test the expression of mitochondrial biogenesis (PGC-1α, TFAM, CPT-1α, CPT-1β), browning (CIDEA, PRDM16). The results were presented as mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; n = 4 per group).