Histol Histopathol (2019) 34: 645-654

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Melatonin promotes self-renewal and nestin expression in neural stem cells from the retina

Yuhua Gao[1,2,3], Li Ma[4], Chunyu Bai[1,2,3], Xiangyang Zhang[1,2] and Wancai Yang[1,2]

[1]College of Basic Medicine, [2]Institute of Precision Medicine, Jining Medical University, Jining, Shandong Province, [3]Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing and [4]Jining First People's Hospital and the Affiliated Hospital of Jining Medical University, Jining, R.P. China

Gao Yuhua and Ma Li contributed equally to this study

Summary. Although melatonin has been shown to exhibit a wide variety of biological functions, its effects on promoting self-renewal in retinal stem cells remain unknown. We found that melatonin can significantly increase proliferation and enhance expression of a stem cell marker, nestin, in retinal neural stem cells (NSCs) via melatonin receptor 1 (MT1). The ERK pathway inhibitor SCH772984 and TGF-β pathway inhibitor SB431542 were used to study the melatonin-mediated molecular mechanisms of cell proliferation in NSCs. The results revealed a novel molecular mechanism of melatonin promotion of self-renewal of NSCs in which a chain reaction in the ERK and TGF-β/Smad pathways promotes self-renewal and transcription of nestin. In addition, dual-luciferase assays revealed that Smad4 directly regulated nestin transcription after melatonin treatment in NSCs. These findings revealed novel mechanisms through which the ERK pathway cooperates with the Smad pathway to regulate self-renewal in NSCs to enhance nestin expression.

Key words: Retina, Neural stem cells, Melatonin, Cell proliferation, Nestin expression

Offprint requests to: Yuhua Gao and Chunyu Bai, 133 Hehua Road, Jining, Shandong Province, 272067, R.P. China. e-mail: chunyu_bai@hotmail.com and anngyh@126.com

DOI: 10.14670/HH-18-065

Introduction

Melatonin is a small neurohormone that is both lipid and water soluble with diverse physiological functions. It can scavenge hydroxyl and peroxyl radicals (Melchiorri et al., 1995; Garcia et al., 2014; Zhang and Zhang, 2014; Manchester et al., 2015), reduce myocardial ischemia-reperfusion injury, regulate circadian rhythms, and modulate visual, reproductive, cerebrovascular, neuroendocrine, and neuroimmunological activity (Dubocovich, 2007; Hardeland, 2008; Yang et al., 2014). The vertebrate retina is a well-characterized central nervous system, consisting of the outer retinal pigmented epithelium and the inner neural retina, which are arranged in a laminar organization and diaplay no evidence of regeneration in adults. Neural stem cells (NSCs) have been found in a few places in adult mammals, including the subventricular zone, olfactory epithelium and retina, and they can self-renew and differentiate into astrocytes, oligodendrocytes, and neurons (Shi et al., 2010). NSCs from the retina are defined as stem cells with the potential to self-renew and produce all cell types of the nervous system following differentiation, so they are an ideal biomaterial for retinal regeneration. Nestin is initially identified as a marker of pancreatic progenitor cells and neural stem cells, and melatonin can influence functions of nestin-positive pancreatic progenitor cells and neural stem cells, such as proliferation and reprogramming in our previous reports (Bai et al., 2016; Qi, 2016). However, no evidence regarding the promotion of nestin expression by melatonin in retina NSCs is available, and the molecular mechanisms underlying this process are

unclear. In this research, we measured the effects of melatonin in increasing proliferation of retinal NSCs *in vitro*, and demonstrated that melatonin increases the proliferation of retinal NSCs via activation of the ERK1/2 and TGF-β1/Smad pathway, and enhances nestin transcription in retinal NSCs via Smad 4 transcriptional regulation. Finally, we suggest a potential molecular mechanism of melatonin action that maintains stemness of retinal NSCs and promotes its proliferation.

Materials and methods

Ethics statement

This research was executed in strict accordance with recommendations of the Institutional Animal Care and Use Committee of Jining Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jining Medical University (License ID: 2017-JZ-003). Pregnant mice were purchased from the Beijing Laboratory Animal Research Center (Beijing, China).

Immunohistochemistry

Immunohistochemistry (IHC) was performed in accordance with previous reports (Kushner et al., 2002). Briefly, murine retina were obtained from eyes and fixed in 4% paraformaldehyde (PFA) solution overnight, and then the sample was embedded in paraffin. Five-micrometer sections were cut from paraffin blocks and rehydrated with xylene, followed by 95%, 70% and 50% ethanol solution. Sections were microwaved in 0.01 M sodium citrate for 20 min and permeabilized with 1% Triton X-100 in PBS for 15 min at room temperature. Primary antibodies against nestin (Abcam, Cambridge, UK), MT1(Bioss, Beijing, China), and MT2 (Abcam, Cambridge, UK) were used at dilutions of 1:100 to incubate sections at 4°C overnight, and then sections were incubated with FITC/Cy3-conjugated second antibodies for 2 h at room temperature. 4',6-diamidino-2-phenylindole (DAPI) was used to label cell nuclear, and confocal microscopy was used to obtained images after counterstaining.

Cultured NSCs were seeded in poly-L-Lysine coated coverslips and incubated for 48 h, then fixed in PFA for 15 min, and were permeabilized with 0.2% Triton X-100 for 10 min. The cells were blocked with 4% BSA for 30 min and incubated at 4°C overnight in primary antibody (against Musashi 1, 1:100, Abcam, Cambridge, UK). And then the cells were incubated with FITC/Cy3-conjugated second antibodies. DAPI was used to label cell nuclear, and confocal microscopy was used to obtained images after counterstaining.

Primary cultures of murine retinal NSCs in vitro

NSCs were isolated according to the procedure for mammalian retinal stem cell isolation as described previously, with some improvements (Das et al., 2006). Retinal tissue was isolated from suckling mouse, rinsed three times, and then transferred to a plate containing Dulbecco's Modified Eagle's Medium/Ham's F-12 (D/F12, Thermo Fisher, Waltham, MA). Plates were cultivated into monoplast suspension after repetitive mixing with a pipette and mechanical separation, and cells were cultured in a 5% CO_2 incubator at 37°C for 24 h. Then the cell suspension was transferred to untreated T-25 tissue culture flasks, and the culture was continued at 37°C, in 5% CO_2. Epithelial and other cells were attached to the bottom of the plates after a 24-h culture, and NSCs were suspended in the culture medium (Sun et al., 2006). Suspended single cells were plated at a density of 1×10^4 cells/ml in untreated T-25 tissue culture flasks and cultured in complete NSC medium (StemPro NSC SFM, Thermo Fisher), which was half-refreshed every 2 days. The complete NSC medium consisted of D/F12 supplemented with 1% N2 supplement (Gibco, Carlsbad, CA, USA), 20 ng/mL epidermal growth factor (Peprotech, Rocky Hill, TX, USA), 20 ng/mL fibroblast growth factor-basic (Peprotech, Rocky Hill, TX, USA) and 2 μg/mL heparin (Gibco, Carlsbad, CA, USA). After 6 days, cultures were harvested, mechanically dissociated, and replated under the same conditions. After performing this procedure twice to eliminate short-term dividing precursors, bulk cultures were generated by passaging cells at higher density (10^4 cells/cm^2) every 6 days in the same growth medium.

Proliferation assay

To test the function of melatonin in retina NSCs, cells were treated using different doses of melatonin according to previous reports. Briefly, retinal NSCs were seeded in 24-well culture plates as 1×10^4 cells/ml and cultured for 8 days in complete medium at 37°C in 5% CO_2. The different dose of 0.01, 0.1, 1, 10, or 100 μM melatonin were added into NSC cultures at day 0, diameter of neurospheres per well were counted at the end of 8-day proliferation culture period. Neurosphere size was measured using a microscope graticule. Meanwhile, Bromodeoxyuridine (BrdU) was used to analyze retinal NSCs proliferation. BrdU can incorporate into the newly synthesized DNA of replicating cells, cells were treated with 10 μM BrdU (Sigma, Sigma-Aldrich, St. Louis, MO, USA) for 24 h, and then fixed for immunofluorescent antibody assays (Anti-BrdU antibody, 1:50, Abcam, Cambridge, MA, USA), and the BrdU-positive cells were counted and analyzed using flow cytometry. Ki67 as a pan-proliferation marker for NSCs, was also used to analyze retinal NSCs proliferation. The Ki67 antibody (Abcam, Cambridge, MA, USA) was incubated into melatonin-treated retinal NSCs, and then Ki67-positive cells were counted and analyzed using flow cytometry.

RNA interference

Small interfering RNA (siRNA) of melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) were designed

and synthesized, cloned into a lentiviral vector. Recombinant lentiviral vector harbored siRNA targeting the MT1 or MT2, as well as enhanced green fluorescence protein (eGFP) as a marker to detect infection efficiency. The recombinant lentivirus harvested from 293T cells had a titer of 6×10^7 TU/mL. NSCs were infected with the recombinant lentivirus, and positive cells were used for subsequent studies (siMT1 and siMT2 cells). Western blot analysis was used to measure protein expression of genes targeted by siRNAs. siMT1: 5' AAGAACTCGC-TCTGCTACGTG 3', scrambled: 5' ACGUGACACG-UUCGGAGAATT 3', and siMT2: 5' AAGCTGCGG-AACGCAGGTAAT 3', scrambled: 5' UUCUCCGAAC-GUGUCACGUTT 3'.

qPCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, USA). cDNA synthesis was carried out with the High Capacity cDNA synthesis kit (Applied Biosystems, USA) using 2 ng of total RNA as a template. The sequence-specific reverse-transcription PCR primers for MT1, MT2 and endogenous control Gapdh were synthesized by Sangon (Shanghai, China). Real-time PCR analysis was carried out using the Applied Biosystems 7500 real-time PCR system, and was performed in 20 µl mixture containing 10 µl SYBR premix Ex Taq buffer, 0.4µl ROX Reference Dye, 0.8µM each of forward and reverse primers, 1µl template cDNA and 7 µl ddH_2O. The gene expression cycle threshold (CT) values of mRNAs from each sample were calculated by normalization to those of internal control and relative values were plotted. Primers of MT1, F: 5' GCTGACACTCATCGCCATCATG-CCCAACCT 3', R: 5' GCAGAAGATGACAATAATC-ATAGGCACGATGAAATGG 3'. Primers of MT2 , F: 5' ACGCAGGTAATTTGTTTGTGGTGAGTCTGGC 3', R: 5' GTAGCGGTTGATGGCAATGGCTGTGATGTT 3'. Primers of GAPDH F: 5' GAACGGGAAGCTCAC-TGG3', R: 5' GCCTGCTTCACCACCTTCT 3'.

Site-directed mutagenesis

Site-directed mutagenesis was performed with a PCR-based method using the Fast Site-Directed Mutagenesis Kit (Tiangen, Beijing, China). Primers, F: 5' TCTTAGGGTGTTCTGGGCACACTGG 3', R: 5' CCAGTGTGCCCAGAACACCCTAAGA 3' and F: 5' GCTTTGATGTCCGTCGCGCTGCATG 3', R: 5' CATGCAGCGCGACGGACATCAAAGC 3', which contain appropriate base substitutions. PCR was carried out with 1 µl template plasmid DNA, 0.5 µl Prime STAR DNA polymerase, and 1 µl of the primer pairs in a final volume of 50 µl. The products were digested with DpnI at 37°C for 8 h and then transformed into competent *E. coli* cells. The mutations were confirmed by sequencing.

Western blotting

Nestin, ERK1/2, p-ERK1/2, Smad 2, p-Smad2, Smad3, p-Smad3 and Smad4 were purchased from Abcam (Cambridge, UK), and used to detect protein expression following treatment of melatonin or pathway inhibitor. Protein concentrations within extracts were measured using a BCA assay (Pierce, USA) and equalized with extraction reagent. and 15 µg protein lysates were subjected to SDS-PAGE, the electrophoresed proteins were transferred to 0.2 µm PVDF membranes (Millipore, USA), which were blocked in 5% non-fat milk and incubated overnight at 4°C with diluted primary antibodies (nestin, 1:300; ERK1/2, 1:500; p-ERK1/2, 1:800; Smad3, 1:500; Smad2, 1:500; Smad4, 1:800; GAPDH, 1:10,000). PVDF membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000). After washing three times with PBST buffer, the ECL Western Blotting Kit was used to visualize color development on the membranes. GAPDH was used as an internal control.

Statistical analysis

In our research, all studies were performed in three to five separate experiments, each performed in triplicate. Data are expressed as the mean ± SD. Differences between experimental groups were assessed using the two-tailed t-test. Statistical significance was defined as *, $P<0.05$ and **, $P<0.01$.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

Results

Melatonin promotes cell proliferation in retinal NSCs

As shown in Fig. 1A, IHC was used to analyze the expression of MT1, MT2, and nestin in murine retinal tissue, and our results demonstrated that MT1, MT2, and nestin were extensively localized in the stratum nervosum retinae. Retinal NSCs derived from murine were suspended in completed culture medium and tested for cell proliferation after different doses of melatonin treatment. Cultured retinal NSCs revealed neurosphere structures *in vitro*, similar to neural stem cells derived from central nervous system (Fig. 1B,C). Musashi 1, the specific molecular marker for retinal neural stem cells, were detected using immunofluorescence, and is shown in Fig. 1C. To test cell proliferation, we supplemented melatonin in the culture medium at 0.01, 0.1, 1, 10, or 100 µM respectively, retinal NSCs viability measured via neurosphere diameter using a microscope graticule indicated that proliferation stagnated after melatonin treatment. Diameters measured from days 0 to 8 indicated that melatonin treatment improved retinal NSCs viability in a dose-dependent manner compared with untreated cells, and retina NSCs diameter

dramatically increased after 10 μM and 100 μM melatonin treatment but they were not significantly different. Then, BrdU staining was used to confirm retinal NSCs proliferation after melatonin treatment and the results were in agreement with diameter measurement (Fig. 1E,F).

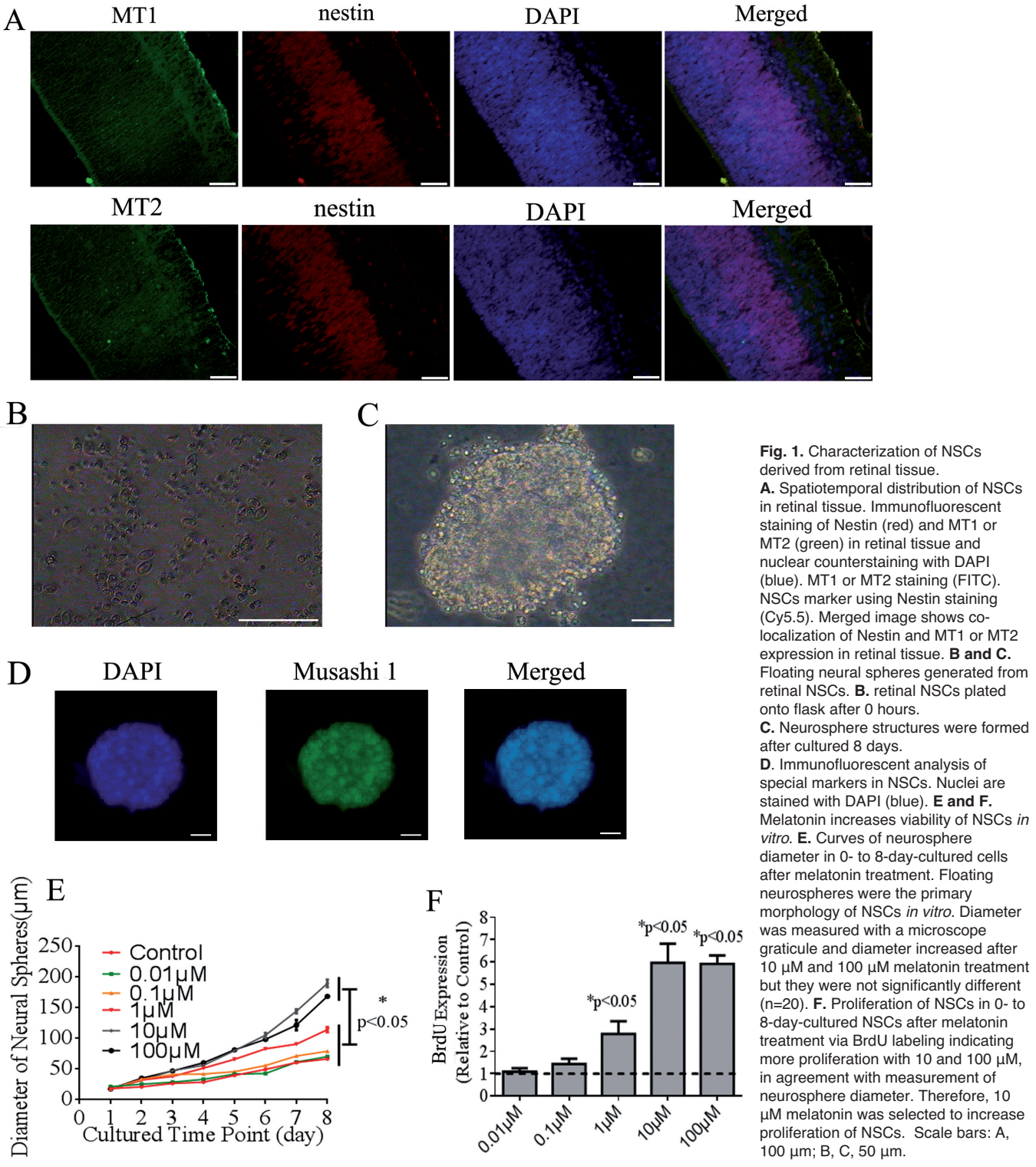

Fig. 1. Characterization of NSCs derived from retinal tissue.
A. Spatiotemporal distribution of NSCs in retinal tissue. Immunofluorescent staining of Nestin (red) and MT1 or MT2 (green) in retinal tissue and nuclear counterstaining with DAPI (blue). MT1 or MT2 staining (FITC). NSCs marker using Nestin staining (Cy5.5). Merged image shows co-localization of Nestin and MT1 or MT2 expression in retinal tissue. **B and C.** Floating neural spheres generated from retinal NSCs. **B.** retinal NSCs plated onto flask after 0 hours.
C. Neurosphere structures were formed after cultured 8 days.
D. Immunofluorescent analysis of special markers in NSCs. Nuclei are stained with DAPI (blue). **E and F.** Melatonin increases viability of NSCs *in vitro*. **E.** Curves of neurosphere diameter in 0- to 8-day-cultured cells after melatonin treatment. Floating neurospheres were the primary morphology of NSCs *in vitro*. Diameter was measured with a microscope graticule and diameter increased after 10 μM and 100 μM melatonin treatment but they were not significantly different (n=20). **F.** Proliferation of NSCs in 0- to 8-day-cultured NSCs after melatonin treatment via BrdU labeling indicating more proliferation with 10 and 100 μM, in agreement with measurement of neurosphere diameter. Therefore, 10 μM melatonin was selected to increase proliferation of NSCs. Scale bars: A, 100 μm; B, C, 50 μm.

Melatonin influences nestin expression in retinal NSCs

Nestin is speculated that when the cells are differentiated, which can be subsequently down-regulated and replaced by tissue specific intermediate filament proteins, a theory confirmed in pancreatic progenitor cells in previous research (Bai et al., 2018 ; Soleimannejad et al., 2018). In this research, to test whether the increased nestin expression in retinal NSCs was due to melatonin treatment, 0.01, 0.1, 1, 10, or 100 μM melatonin were added in NSCs culture medium to detect changes of nestin expression in retinal NSCs. Results indicated that nestin expression levels increased in a dose-dependent manner in melatonin-treated retinal NSCs after melatonin treatment. Retinal NSCs incubated with 10 μM and 100 μM melatonin had dramatically increased expression level of nestin compared with the other groups (0.01 μM, 0.1 μM and 1 μM, $P<0.01$), but no significant changes in expression level occurred between 10 μM and 100 μM ($P>0.05$, Fig. 2A). To assess the role of melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) in melatonin-treated NSCs proliferation, small interfering RNAs (siRNA) for MT1 and MT2 were synthesized and detected effects in down-regulated its targets using western blotting and qPCR, the results demonstrated that MT1 or MT2 was significantly impressed its expression level (Fig. 2B,C). And then, our results indicated that MT1 is an important

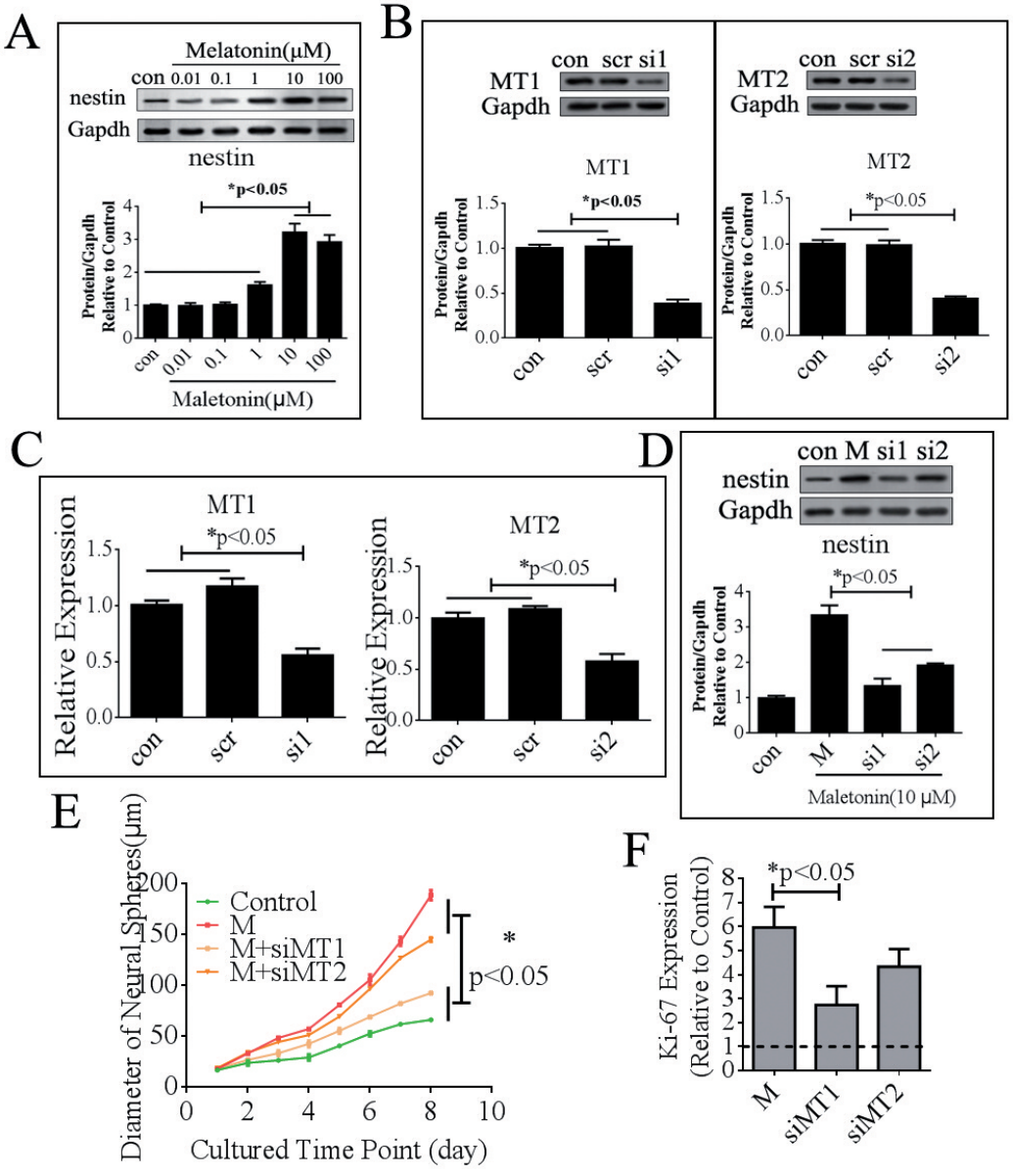

Fig. 2. Role of melatonin receptors in melatonin-induced nestin expression and NSC proliferation. **A.** Nestin expression was tested using western blotting after NSCs were exposed to various melatonin concentrations. These results demonstrated that nestin levels increased in a concentration-dependent manner, and that NSCs incubated with 10 μM melatonin had significantly increased expression levels compared with other groups. **B, C and D.** Role of melatonin receptors in nestin expression. Melatonin receptors, which included MT1 and MT2, belong to the family of G-protein-coupled receptors. For scanning biological functions of MT1 and MT2, siRNAs were used to knock down MT1 or MT2 when NSCs were exposed to 10 μM melatonin, and protein levels were analyzed using western blotting. **B.** Small RNA knocks down MT1 and MT2 expression without melatonin treatment. **C.** Relative expression levels of MT1/2 during the expression of small RNA, data were normalized to control. **D.** Role of MT1 and MT2 receptors in nestin expression under 10 μM melatonin treatment. The results indicated that MT1 is an important melatonin receptor for the induction of nestin expression in NSCs. Protein abundance was analyzed using Image J tools and normalized using endogenous control. Full size WB images are shown in Figure S1. **E and F.** Role of melatonin receptors in melatonin-induced NSC proliferation. The results indicated that MT1 is an important melatonin receptor for cell proliferation in NSCs.

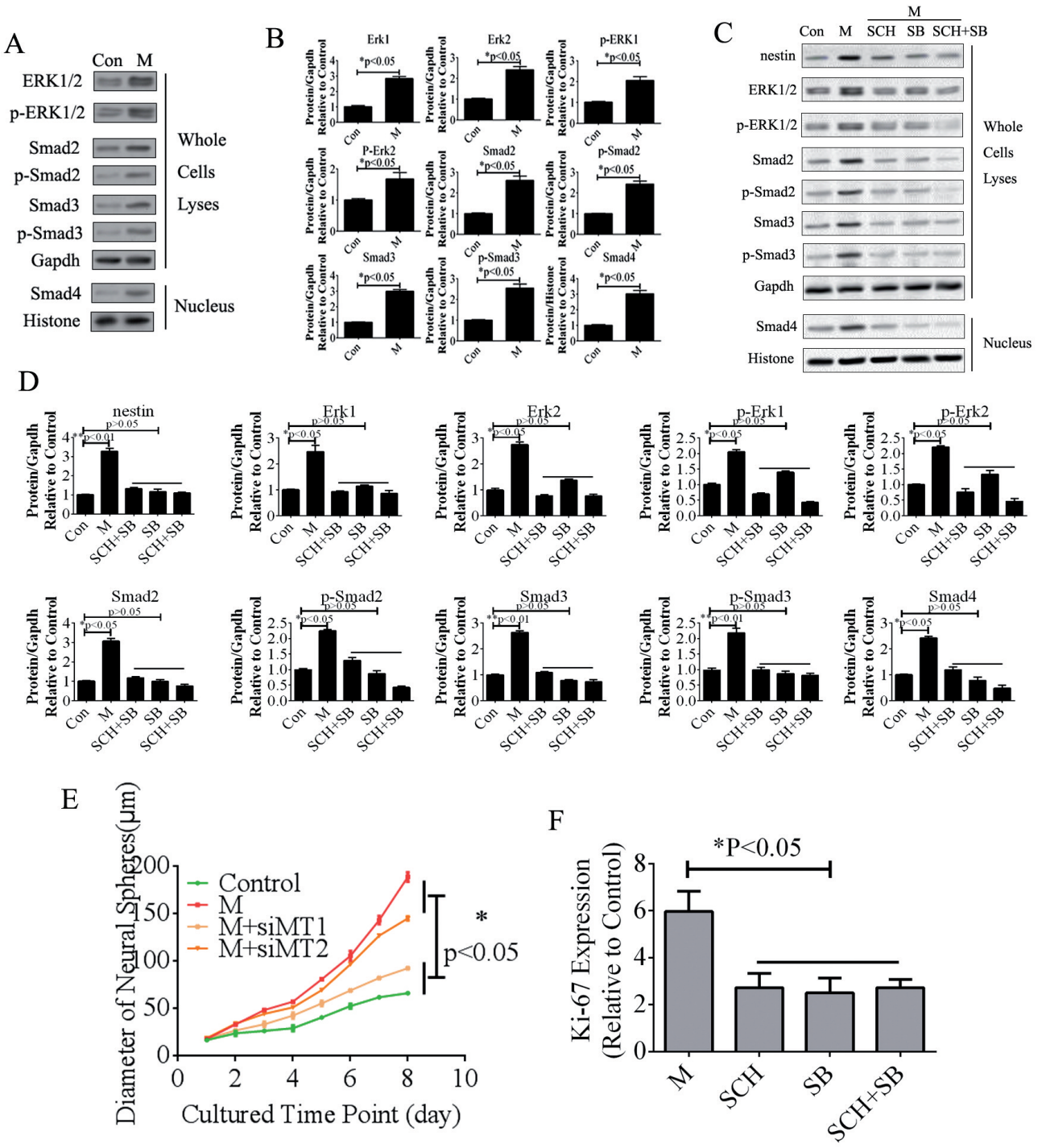

Fig. 3. Role of ERK pathway and TGF-β1/Smad pathway in 10 μM melatonin-induced nestin expression and NSC proliferation. **A.** activation of ERK pathway and TGF-β1/Smad pathway in NSCs after melatonin treatment. The results revealed p-ERK1/2, p-Smad2, p-Smad3 and activated Smad4 were significantly increased after 10 μM melatonin treatment. **B.** Protein abundance of penal A was analyzed and normalized using Image J tools. **C.** Role of ERK pathway and TGF-β1/Smad pathway in melatonin-induced nestin expression. **D.** Protein abundance of penal C was analyzed and normalized using Image J tools. The western blotting revealed that melatonin activated the ERK pathway and TGF-β1/Smad pathway to promote nestin expression, nestin expression levels were positively correlated with activation of the ERK and TGF-β1/Smad pathway, decreased when SB431542 or/and SCH772984 were exposed to melatonin-treated NSCs. **E and F.** Role of ERK pathway and TGF-β1/Smad pathway in 10μM melatonin-induced NSC proliferation. The results indicated that ERK pathway and TGF-β1/Smad pathway play an important role for cell proliferation in NSCs.

receptor for the induction of nestin expression and proliferation in melatonin-treated retinal NSCs (Fig. 2D-F).

Role of ERK and TGF-β1/Smad pathways in melatonin-treated retina NSCs proliferation

The ERK pathway plays an important role in cell survival and proliferation, which can communicate a molecular signal from surface receptor to cellular nucleus, and the members include ERK1/2, MEK, JNK, and P38. Previous research showed that ERK1/2 was activated by phosphorylation after melatonin treatment, and it has been found that melatonin can promote cell proliferation via the ERK pathway (Bai et al., 2018). In this research, the data indicated that melatonin influences phosphorylation of ERK1/2 and promotes cell proliferation. As shown in Fig. 3A, total expression level and phosphorylation of ERK1/2 were dramatically up-regulated after 10 μM melatonin treatment.

TGF-β signaling is involved in many cellular processes, including differentiation, growth, apoptosis, and other cellular functions, which need a ligand to bind TGF-β receptors (type I and type II receptors). The activation of the type I receptor is subsequently followed by the phosphorylation of Smad2 and Smad3, and then Smad4 translocated into the nucleus and regulated the transcription of target genes. The TGF-β1/Smad pathway plays an important role in cell proliferation, and Smad2, Smad3, and Smad4 are essential components of the intracellular TGF-β1/Smad pathway, and we found that melatonin could increase total expression level of Smad2, Smad3, and Smad4, promote the phosphorylation of Smad2 and Smad3, and induce Smad4 to enter the cell nucleus in 10 μM melatonin-treated retinal NSCs.

To test the roles of the ERK1/2 and Smad pathways in retinal NSCs proliferation, SB431542 (TGF-β/SMAD pathway inhibitor) and SCH772984 (ERK pathway inhibitor) were added into culture media of retinal NSCs in combination with 10 μM melatonin. Our data demonstrated that SB431542 or SCH772984 prevented NSC proliferation under melatonin treatment. These results indicated that melatonin promotes NSC proliferation via the ERK and Smad pathways (Fig. 3).

Smad4 promotes transcription of nestin in retinal NSCs

To determine the mechanisms that cause elevated levels of nestin in 10 μM melatonin-treated retinal NSCs, we used ALGGEN-PROMO (version 8.3) and JASPAR (version 7.0) tools to screen potential Smad4-binding sites (S4BSs) in the promotor region of nestin. PROMO is a classical bioinformatics tools for the selection of putative transcription factor-binding sites in promotor sequences of target genes using TRANSFAC database. Transcription factor-binding sites defined in the TRANSFAC database are used to construct specific binding site weight matrices for transcription factor-binding site prediction (Farre et al., 2003). JASPAR is an

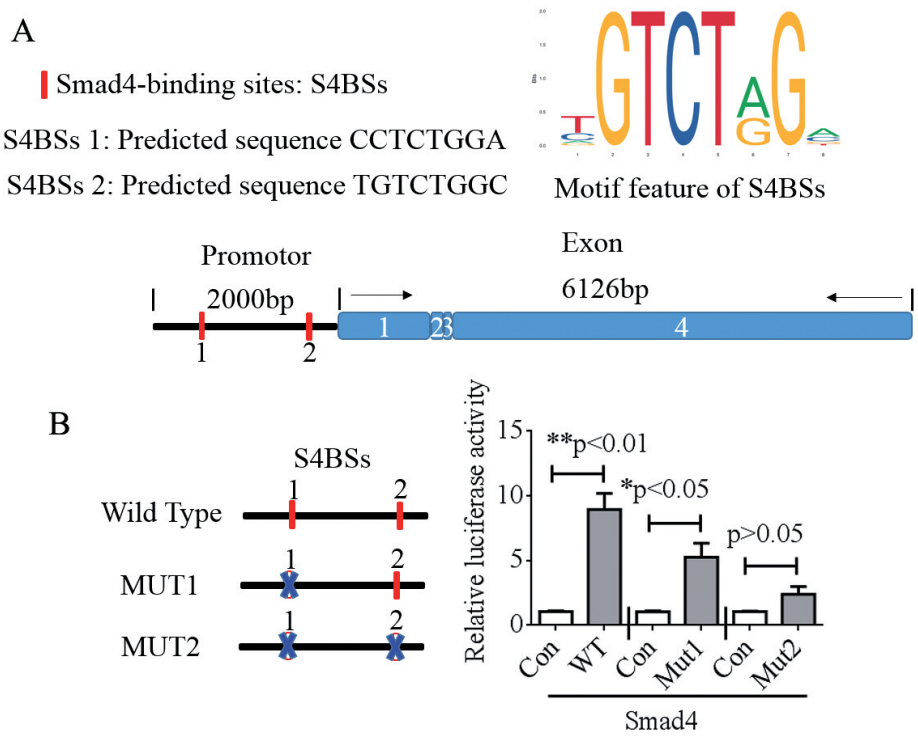

Fig. 4. Smad4 up-regulates expression of nestin through transcriptional activation of its promoter. **A.** Schematic of each Smad4 binding site (S4BSs) in promoter regions of nestin. **B.** Experiments of the full-length nestin promoter (WT) with different Smad4-mutated sites. Co-transfected with pRL-SV40 into NSCs, 48 h later, cells were harvested and lysed with passive lysis buffer. Luciferase activity was measured by using a dual luciferase reporter assay. The pRL-SV40 vector was used as an internal control. The results were expressed as relative luciferase activity. Columns represent the mean of three independent experiments, bar represent s.e.

open-access database of curated, transcription factor binding profiles stored as position frequency matrices and transcription factor flexible models for transcription factors across multiple species in six taxonomic groups (Khan et al., 2018a,b). By combining these tools, we found two putative Smad4-binding motifs within the promotor region of nestin. Next, we constructed a pGL3.0-nestin promoter vector for testing luciferase activity when co-transfected with Smad4 in 293T cells. We separately co-expressed the wild-type (WT) promotor of nestin or its mutated (MUT) counterparts together with an internal control vector in 293T cells with Smad4 overexpression. Forced expression of Smad4 induced activity of the WT but not the MUT promoter of nestin compared with the control (Fig. 4). These results clearly revealed that Smad4 could bind to sequences of the nestin promoter to enhance its transcription.

Discussion

Melatonin is an indolamine originally isolated from pineal tissue, which has many important physiological functions, including sleep-wake timing, blood pressure regulation, seasonal reproduction and many other regulated functions. Previous reports have revealed that melatonin has multiple functions in the neuroimmuno-endocrine system, and a wide extracellular and intracellular distribution, indicating potentially broad functions. Melatonin is also a well-known free radical scavenger, with anti-apoptotic activity and antioxidant (Lezoualc'h et al., 1996; Radogna et al., 2008; Hong et al., 2010; Jou et al., 2010; Espino et al., 2013; Zhang and Zhang, 2014; Manchester et al., 2015; Yang et al., 2015). Previous studies have indicated that melatonin increases the viability of neural stem cells and pancreatic progenitor cells (Kong et al., 2008; Bai et al., 2018), and our study confirms that melatonin can promote the proliferation of retinal NSCs. Previous reports have found that activation of melatonin signaling can attenuate expression of anti-apoptotic genes. Nestin is a structural protein to use a maker for NSCs, when the NSCs are differentiated, which can be subsequently down-regulated and replaced by tissue specific intermediate filament proteins (Bai et al., 2018; Soleimannejad et al., 2018). However, no reports regarding melatonin promotion of nestin for maintaining stemness in retinal NSCs are available.

Our research confirms that melatonin can increase the proliferation of retinal NSCs, and given that it is a well-known activator for the ERK pathway, western blotting showed significant up-regulation of total protein expression and phosphorylation levels of ERK1/2 in melatonin-treated groups. The ERK1/2 acts in a signaling cascade that regulates various cellular processes such as proliferation, differentiation, and cell cycle progression in response to a variety of extracellular signals (Hayne et al., 2000; Johnson and Lapadat, 2002). The ERK pathway plays an important role in melatonin's proliferative actions in neural cells (Kong et al., 2018), induced pluripotent stem cells, and pancreatic beta cells (Costes et al., 2015; Bai et al., 2016). ERK1/2 phosphorylation has been well demonstrated as necessary for cell proliferation, and participates in G1 and G2/M phase of cell cycle (Chambard et al., 2007; Meloche and Pouyssegur, 2007; Liu et al., 2016). Since melatonin increased the phosphorylation of ERK1/2 and expression level of total ERK1/2, we used SCH772984, an inhibitor of the ERK pathway to inhibit phosphorylation of ERK1/2, and found that it disrupted the proliferative effect of melatonin in retinal NSCs. These findings implied that phosphorylation of ERK1/2 plays an important role in the observed melatonin-mediated retinal NSCs proliferation.

TGF-β1 is a polypeptide member of the transforming growth factor beta superfamily of cytokines, which performs many cellular functions, including the control of cell growth, proliferation, differentiation and apoptosis. Previous reports have demonstrated that the TGF-β1 pathway acts through Smad-dependent and -independent pathways (Attisano and Wrana, 2002; Lu et al., 2010). Smad2 and Smad3 are activated by TGF-β type I receptor kinase, then enter the cellular nucleus complex with a common co-Smad, Smad4, to bind to promotor sequence of target genes and regulate its transcription (Shi and Massague, 2003; Akel et al., 2013). We used SB431542, an inhibitor of the TGF-β1/Smad pathway to inhibit the activation of Smads, and found that it disrupted the proliferative effect of melatonin in retinal NSCs. These findings revealed that Smads activation plays an important role in the melatonin-mediated retinal NSCs proliferation. Smad4 is a highly-conserved protein and acts as a mediator of TGF-β signal transduction. Once it enters the cellular nucleus, a complex of SMAD4 and two R-SMADS binds to DNA and regulates the expression of target genes. We demonstrated dramatic elevation of Smad4 expression in the nucleus of NSCs after melatonin treatment in our study. Transcription factors are sequence-specific DNA-binding proteins involved in the transcriptional regulation of gene expression, which bind to DNA through their DNA-binding domains to enhance transcription. In this research, bioinformatics tools, ALGGEN-PROMO (version 8.3) and JASPAR (version 7.0) were used to screen potential Smad4-binding sites in the promotor region of nestin , (Farre et al., 2003, Khan et al., 2018a,b) and bioinformatics analysis showed that Smad4 bound to promoter sequences of nestin. Dual-luciferase assays also revealed that Smad4 directly regulated nestin transcription.

Conclusion

In conclusion, melatonin promotes retinal NSCs proliferation due at least in part to the activation of the ERK and TGF-β1/Smad pathways. Melatonin activates Smad4 to enter the nucleus to enhance the transcription of nestin in retinal NSCs, and this may involve cascade

pathways, including the TGF-β/Smad and ERK pathways. These findings reveal a novel mechanisms melatonin through which the ERK pathway cooperatively interacts with the TGF-β/Smad pathway to enhance nestin transcription in retina NSCs.

Acknowledgements. This research was supported by Shandong Provincial Natural Science Foundation, China (ZR2017BH105 to Gao Y, ZR2017BH002 to Bai C.), Project of Shandong Province Higher Educational Science and Technology Program (J17KA229 to Gao Y.), National Natural Science Foundation cultivation project of Jining medical university (JYP201723 to Gao Y.), Supporting Fund for Teachers' research of Jining Medical University (JY2017KJ027 to Bai C.), and Faculty Start-up Funds of Jining Medical University (to Bai C. and Gao Y.).

Competing financial interests. The authors declare that they have no competing interests.

References

Akel S., Bertolette D. and Ruscetti F.W. (2013). Crosstalk between the smad and the mitogen-activated protein kinase pathways is essential for erythroid differentiation of erythroleukemia cells induced by TGF-beta, activin, hydroxyurea and butyrate. J. Leuk (Los Angel) 1.

Attisano L. and Wrana J.L. (2002). Signal transduction by the TGF-beta superfamily. Science 296, 1646-1647.

Bai C., Gao Y., Zhang X., Yang W. and Guan W. (2018). Melatonin promotes self-renewal of nestin-positive pancreatic stem cells through activation of the mt2/erk/smad/nestin axis. Artif. Cells Nanomed. Biotechnol. 46, 62-74.

Bai C., Li X., Gao Y., Yuan Z., Hu P., Wang H., Liu C., Guan W. and Ma Y. (2016). Melatonin improves reprogramming efficiency and proliferation of bovine-induced pluripotent stem cells. J. Pineal Res. 61, 154-167.

Chambard J.C., Lefloch R., Pouyssegur J. and Lenormand P. (2007). ERK implication in cell cycle regulation. Biochim. Biophys. Acta 1773, 1299-1310.

Costes S., Boss M., Thomas A.P. and Matveyenko A.V. (2015). Activation of melatonin signaling promotes beta-cell survival and function. Mol. Endocrinol. 29, 682-692.

Das A.V., Mallya K.B., Zhao X., Ahmad F., Bhattacharya S., Thoreson W.B., Hegde G.V. and Ahmad I. (2006). Neural stem cell properties of muller glia in the mammalian retina: Regulation by notch and wnt signaling. Dev. Biol. 299, 283-302.

Dubocovich M.L. (2007). Melatonin receptors: Role on sleep and circadian rhythm regulation. Sleep Med. 8 (Suppl. 3), 34-42.

Espino J., Rodriguez A.B. and Pariente J.A. (2013). The inhibition of tnf-alpha-induced leucocyte apoptosis by melatonin involves membrane receptor mt1/mt2 interaction. J. Pineal Res. 54, 442-452.

Farre D., Roset R., Huerta M., Adsuara J.E., Rosello L., Alba M.M. and Messeguer X. (2003). Identification of patterns in biological sequences at the alggen server: Promo and malgen. Nucleic Acids Res. 31, 3651-3653.

Garcia J.J., Lopez-Pingarron L., Almeida-Souza P., Tres A., Escudero P., Garcia-Gil F.A., Tan D.X., Reiter R.J., Ramirez J.M. and Bernal-Perez M. (2014). Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: A review. J. Pineal Res. 56, 225-237.

Hardeland R. (2008). Melatonin, hormone of darkness and more: Occurrence, control mechanisms, actions and bioactive metabolites. Cell. Mol. Life Sci. 65, 2001-2018.

Hayne C., Tzivion G. and Luo Z. (2000). Raf-1/MEk/MAPk pathway is necessary for the G2/M transition induced by nocodazole. J. Biol. Chem. 275, 31876-31882.

Hong Y., Palaksha K.J., Park K., Park S., Kim H.D., Reiter R.J. and Chang K.T. (2010). Melatonin plus exercise-based neuro-rehabilitative therapy for spinal cord injury. J. Pineal Res. 49, 201-209.

Johnson G.L. and Lapadat R. (2002). Mitogen-activated protein kinase pathways mediated by erk, jnk, and p38 protein kinases. Science 298, 1911-1912.

Jou M.J., Peng T.I., Hsu L.F., Jou S.B., Reiter R.J., Yang C.M., Chiao C.C., Lin Y.F. and Chen C.C. (2010). Visualization of melatonin's multiple mitochondrial levels of protection against mitochondrial ca(2+)-mediated permeability transition and beyond in rat brain astrocytes. J. Pineal Res. 48, 20-38.

Khan A., Fornes O., Stigliani A., Gheorghe M., Castro-Mondragon J.A., van der Lee R., Bessy A., Cheneby J., Kulkarni S.R., Tan G., Baranasic D., Arenillas D.J., Sandelin A., Vandepoele K., Lenhard B., Ballester B., Wasserman W.W., Parcy F. and Mathelier A. (2018a). Jaspar 2018: Update of the open-access database of transcription factor binding profiles and its web framework. Nucleic Acids Res. 46, D260-D266.

Khan A., Fornes O., Stigliani A., Gheorghe M., Castro-Mondragon J.A., van der Lee R., Bessy A., Cheneby J., Kulkarni S.R., Tan G., Baranasic D., Arenillas D.J., Sandelin A., Vandepoele K., Lenhard B., Ballester B., Wasserman W.W., Parcy F. and Mathelier A. (2018b). Jaspar 2018: Update of the open-access database of transcription factor binding profiles and its web framework. Nucleic Acids Res. 46, D1284.

Kong X., Li X., Cai Z., Yang N., Liu Y., Shu J., Pan L. and Zuo P. (2008). Melatonin regulates the viability and differentiation of rat midbrain neural stem cells. Cell. Mol. Neurobiol. 28, 569-579.

Kong F.L., Wang X.P., Li Y.N. and Wang H.X. (2018). The role of exosomes derived from cerebrospinal fluid of spinal cord injury in neuron proliferation *in vitro*. Artif. Cells Nanomed Biotechnol. 46, 200-205.

Kushner J.A., Ye J., Schubert M., Burks D.J., Dow M.A., Flint C.L., Dutta S., Wright C.V., Montminy M.R. and White M.F. (2002). Pdx1 restores beta cell function in Irs2 knockout mice. J. Clin. Invest. 109, 1193-1201.

Lezoualc'h F., Skutella T., Widmann M. and Behl C. (1996). Melatonin prevents oxidative stress-induced cell death in hippocampal cells. Neuroreport 7, 2071-2077.

Liu L., Xu Y., Reiter R.J., Pan Y., Chen D., Liu Y., Pu X., Jiang L. and Li Z. (2016). Inhibition of erk1/2 signaling pathway is involved in melatonin's antiproliferative effect on human mg-63 osteosarcoma cells. Cell. Physiol. Biochem. 39, 2297-2307.

Lu L., Wang J., Zhang F., Chai Y., Brand D., Wang X., Horwitz D.A., Shi W. and Zheng S.G. (2010). Role of smad and non-smad signals in the development of th17 and regulatory t cells. J. Immunol. 184, 4295-4306.

Manchester L.C., Coto-Montes A., Boga J.A., Andersen L.P., Zhou Z., Galano A., Vriend J., Tan D.X. and Reiter R.J. (2015). Melatonin: An ancient molecule that makes oxygen metabolically tolerable. J. Pineal Res. 59, 403-419.

Melchiorri D., Reiter R.J., Attia A.M., Hara M., Burgos A. and Nistico G. (1995). Potent protective effect of melatonin on in vivo paraquat-induced oxidative damage in rats. Life Sci. 56, 83-89.

Meloche S. and Pouyssegur J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene 26, 3227-3239.

Qi X. (2016). The role of mir-9 during neuron differentiation of mouse retinal stem cells. Artif. Cells Nanomed. Biotechnol. 44, 1883-1890.

Radogna F., Cristofanon S., Paternoster L., D'Alessio M., De Nicola M., Cerella C., Dicato M., Diederich M. and Ghibelli L. (2008). Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of bcl-2. J. Pineal Res. 44, 316-325.

Shi W., Yao J., Chen X., Lin W., Gu X. and Wang X. (2010). The delayed repair of sciatic nerve defects with tissue-engineered nerve grafts in rats. Artif. Cells Blood Substit. Immobil. Biotechnol. 38, 29-37.

Shi Y. and Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685-700.

Soleimannejad M., Ebrahimi-Barough S., Soleimani M., Nadri S., Tavangar S.M., Roohipoor R., Yazdankhah M., Bayat N., Riazi-Esfahani M. and Ai J. (2018). Fibrin gel as a scaffold for photoreceptor cells differentiation from conjunctiva mesenchymal stem cells in retina tissue engineering. Artif. Cells Nanomed. Biotechnol. 46, 805-814.

Sun G., Asami M., Ohta H., Kosaka J. and Kosaka M. (2006). Retinal stem/progenitor properties of iris pigment epithelial cells. Dev. Biol. 289, 243-252.

Yang Y., Sun Y., Yi W., Li Y., Fan C., Xin Z., Jiang S., Di S., Qu Y., Reiter R.J. and Yi D. (2014). A review of melatonin as a suitable antioxidant against myocardial ischemia-reperfusion injury and clinical heart diseases. J. Pineal Res. 57, 357-366.

Yang Y., Jiang S., Dong Y., Fan C., Zhao L., Yang X., Li J., Di S., Yue L., Liang G., Reiter R.J. and Qu Y. (2015). Melatonin prevents cell death and mitochondrial dysfunction via a sirt1-dependent mechanism during ischemic-stroke in mice. J. Pineal Res. 58, 61-70.

Zhang H.M. and Zhang Y. (2014). Melatonin: A well-documented antioxidant with conditional pro-oxidant actions. J. Pineal Res. 57, 131-146.

Accepted November 16, 2018