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To cite this article: Chunyu Bai, Yuhua Gao, Xiangyang Zhang, Wancai Yang & Weijun Guan (2017): Melatonin promotes self-renewal of nestin-positive pancreatic stem cells through activation of the MT2/ERK/SMAD/nestin axis, *Artificial Cells, Nanomedicine, and Biotechnology*, DOI: [10.1080/21691401.2017.1389747](https://doi.org/10.1080/21691401.2017.1389747)

To link to this article: <http://dx.doi.org/10.1080/21691401.2017.1389747>



Published online: 16 Oct 2017.



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Melatonin promotes self-renewal of nestin-positive pancreatic stem cells through activation of the MT2/ERK/SMAD/nestin axis

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ABSTRACT

Although melatonin has been shown to exhibit a wide variety of biological functions, its effects on promotion of self-renewal in pancreatic stem cells remain unknown. In this study, we incubated murine pancreatic stem cells (PSCs) with various concentrations of melatonin (0.01, 0.1, 1, 10 or 100 μ M) to screen for the optimum culture medium for increasing cell proliferation. We found that 10 μ M melatonin can significantly increase proliferation and enhance expression of a stem cell marker, nestin, in PSCs via melatonin receptor 2 (MT2). Thus, we used 10 μ M melatonin to study the melatonin-mediated molecular mechanisms of cell proliferation in PSCs. We applied extracellular signal-regulated kinase (ERK) pathway inhibitor SCH772984 and transforming growth factor beta (TGF- β) pathway inhibitor SB431542, along with interfering RNAs siERK1, siERK2, siSmad2, siSmad3, siSmad4 and siNestin, to melatonin-treated PSCs to research the roles of these genes in self-renewal. The results revealed a novel molecular mechanism by which melatonin promotes self-renewal of PSCs: a chain reaction in the MT2/ERK/SMAD/nestin axis promoted the aforementioned self-renewal as well as inhibited differentiation. In addition, upregulation of nestin created a positive feedback loop in the regulation of the transforming growth factor beta 1 (TGF- β 1)/SMADs pathway by promoting expression of Smad4. Conversely, knockdown of nestin significantly suppressed the proliferative effect in melatonin-treated PSCs. These are all novel mechanisms through which the ERK pathway cooperatively crosstalks with the SMAD pathway to regulate nestin expression, thereby enhancing self-renewal in PSCs.

ARTICLE HISTORY

Received 3 August 2017
Revised 1 October 2017
Accepted 4 October 2017

KEYWORDS

Pancreatic stem cells; melatonin; cell proliferation; ERK pathway; TGF- β 1/SMAD pathway; nestin

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), an indolamine originally isolated from mammalian pineal tissue, takes part in many important physiological functions. It can scavenge hydroxyl and peroxy radicals [1–4]; reduce myocardial ischemia-reperfusion injury; regulate circadian rhythms; and modulate visual, reproductive, cerebrovascular, neuroendocrine and neuroimmunological activity [5–7]. Nestin is an intermediate filament protein that was initially identified as a marker of neural stem and progenitor cells [8]. Melatonin can modulate nestin-positive neural stem cell (NSC) functions such as proliferation, reprogramming them into NSC-derived pluripotent stem cells as found in our previous research [9]. However, nestin is transiently expressed not only during development of the central nervous system but also in different cell types of embryonic and adult tissues, such as pancreatic cells, developing skeletal muscle, mesenchymal cells, cardiomyocytes and endothelial cells [10]. Nestin also seems to play an important role in regeneration in response to injury in various tissues, and there are reports that nestin regulates cell proliferation in normal stem cells [11] and tumor cells [12]. These findings strongly suggest that nestin plays an important role in controlling cell proliferation.

In pancreatic islets, a small subset of cells express nestin. These cells, called pancreatic stem cells (PSCs), are thought to be precursors of differentiated pancreatic endocrine cells [13] and play an important role in the growth and maintenance of islets [14–16]. Melatonin can modulate cell function in nestin-positive neural stem cells according to previous research, which prompted us to study whether melatonin can also regulate nestin expression to influence cell fate. In addition, melatonin can combine with melatonin receptor 1 (MT1) on cell membranes to promote the progression of breast cancer by influencing nestin expression in nestin-positive breast cancer stem cells [17]. In our research, we found that nestin-positive PSCs and their differentiated beta cells expressed melatonin receptors 1 (MT1) and 2 (MT2), which are G-protein-coupled receptors. These findings prompted us to study whether melatonin also affects proliferation and differentiation of primary cultured PSCs. In the current study, we measured the viability of primary cultured PSCs *in vitro* treated with melatonin to verify optimal PSC amplification, demonstrated that melatonin enhanced the self-renewal of the PSCs via the MT2/extracellular signal-regulated kinase (ERK)/SMAD/nestin axis, and suggested a potential molecular mechanism of melatonin that produces these effects.

Materials and methods

Ethics statement

We conducted this study in strict accordance with recommendations of the Institutional Animal Care and Use Committee of Jining Medical University, China. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jining Medical University (License ID: 2017-JZ-003). All surgery was performed under pentobarbital anesthesia, and all efforts were made to minimize suffering.

Primary cultures of murine PSCs in vitro

We isolated PSCs from the pancreases of suckling mice and cultured them in advanced RPMI 1640 medium (11.1 mM glucose) supplemented with fetal bovine serum (FBS) and cocktail factors according to the method of our previous study [18]. We obtained the fraction containing the majority of nestin-positive PSCs by flow cytometry (FCM) purification. We used immunofluorescence (IF) to analyze the PSCs for specific markers, including nestin, pancreas/duodenum homeobox protein 1 (PDX1), MT1 and MT2.

Differentiation of nestin-positive PSCs toward beta cells

Nicotinamide, a poly (adenosine diphosphate [ADP]-ribose) synthetase inhibitor, has been reported to prevent the development of diabetes in animal models and to promote the proliferation and differentiation of islet beta cells. In this study, we plated nestin-positive PSCs on 24-well cell culture plates. We then cultured them for 7 days in Dulbecco's Modified Eagle's Medium (DMEM)/F12 containing glucose (11.1 mM) and a cocktail of several growth factors, including 10 mM nicotinamide, 100 pmol/L hepatocyte growth factor (HGF) and 2 nmol/L active-A. To test whether insulin release from differentiated beta cells was glucose dependent, we used 2 glucose concentrations (5.5 mM and 20.5 mM). We measured insulin release using an Insulin ELISA Kit. We washed the beta cells with Krebs buffer and then pre-incubated them in low-glucose (5.5 mM) Krebs buffer for 2 h to remove residual insulin. We then washed them 3 times in Krebs buffer, incubated them in low-glucose Krebs buffer for 30 min and collected the supernatant. Finally, we washed the beta cells 3 times in Krebs buffer, incubated them in high-glucose (20.5 mM) Krebs buffer for 30 min and collected the supernatant. We repeated this sequence 3 additional times.

RNA interference

Small interfering RNA (siRNA) of MT1 (Gene ID: 17773), MT2 (Gene ID: 244701), ERK1 (Gene ID: 26417), ERK2 (Gene ID: 26413), Smad2 (Gene ID: 17126), Smad3 (Gene ID: 17127), Smad4 (Gene ID: 17128) and nestin (Gene ID: 18008) were designed, synthesized and cloned into lentiviral vectors. Recombinant lentiviral vectors harbored siRNAs targeting genes, as well as enhanced green fluorescence protein as a marker to detect infection efficiency, and were transfected into 293T cells. Recombinant lentiviruses harvested from

293T cells had titers of 3×10^6 TU/mL. We seeded and infected PSCs with recombinant lentiviruses and used positive cells for subsequent studies (siMT1, siMT2, siERK1, siERK2, siSmad2 [siS2], siSmad3 [siS3], siSmad4 [siS4] and siNestin cells). We used Western blot analysis to measure protein expression of genes targeted by siRNAs.

Proliferation assay

We analyzed PSC proliferation using FCM after Ki67 staining. Ki67 is a nuclear non-histone protein that is preferentially expressed during the late G1, S, G2 and M phases of the cell cycle; while resting, non-cycling cells (G0 phase) lack Ki67 expression. We treated PSCs in different groups and cultured them for 72 h in PSC medium. We then visualized Ki67 expression in PSCs using FCM, counting Ki67-positive stem cells from 3 random culture dishes in each of 3 independent PSC samples.

Immunofluorescent staining

We fixed the cells with 4% paraformaldehyde (PFA) in polybutylene succinate (PBS) for 1 h, then rinsed them 3 times with PBS for 5 min each time. We then permeabilized the cells with 0.01% Triton X-100 for 30 min. After rinsing them 3 times with PBS, we blocked the cells with goat serum for 30 min, then incubated them with primary antibodies in a humidified chamber at 4°C overnight. After three 5-min washes with PBS the following day, we incubated the cells with Cy3 or fluorescein isothiocyanate (FITC)-labeled secondary antibodies at room temperature (RT) for 1 h. Cells were given a final wash with PBS 3 times for 5 min each. We recorded IF data using a confocal optical system.

Western blotting

We lysed the cells with Protein Extraction Reagent (Pierce Biotechnology [Thermo Fisher Scientific], Waltham, MA) supplemented with a protease inhibitor (phenylmethylsulfonyl fluoride). We measured protein concentrations within extracts using a bicinchoninic acid (BCA) assay (Pierce Biotechnology) and equalized them with extraction reagent. We loaded equal amounts of extracts and subjected them to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto nitrocellulose membranes. Primary antibodies (nestin, 1:300; MT1, 1:500; MT2, 1:500; ERK1/2, 1:500; P-ERK1/2, 1:800; Smad3, 1:500; Smad2, 1:500; Smad4, 1:800; and glyceraldehyde 3-phosphate dehydrogenase [GAPDH], 1:10,000) and horseradish peroxidase-coupled secondary antibodies (1:2000) were purchased from Abcam (Cambridge, UK). We probed the membranes using an ultra-enhanced chemiluminescence Western blot reagent. We used GAPDH as an internal control, and we measured protein concentration with Image J software (<http://imagej.nih.gov/ij/>).

Immunohistochemistry (IHC)

We dissected pancreas samples from suckling mice, fixed them in a 4% PFA/PBS solution overnight, and embedded

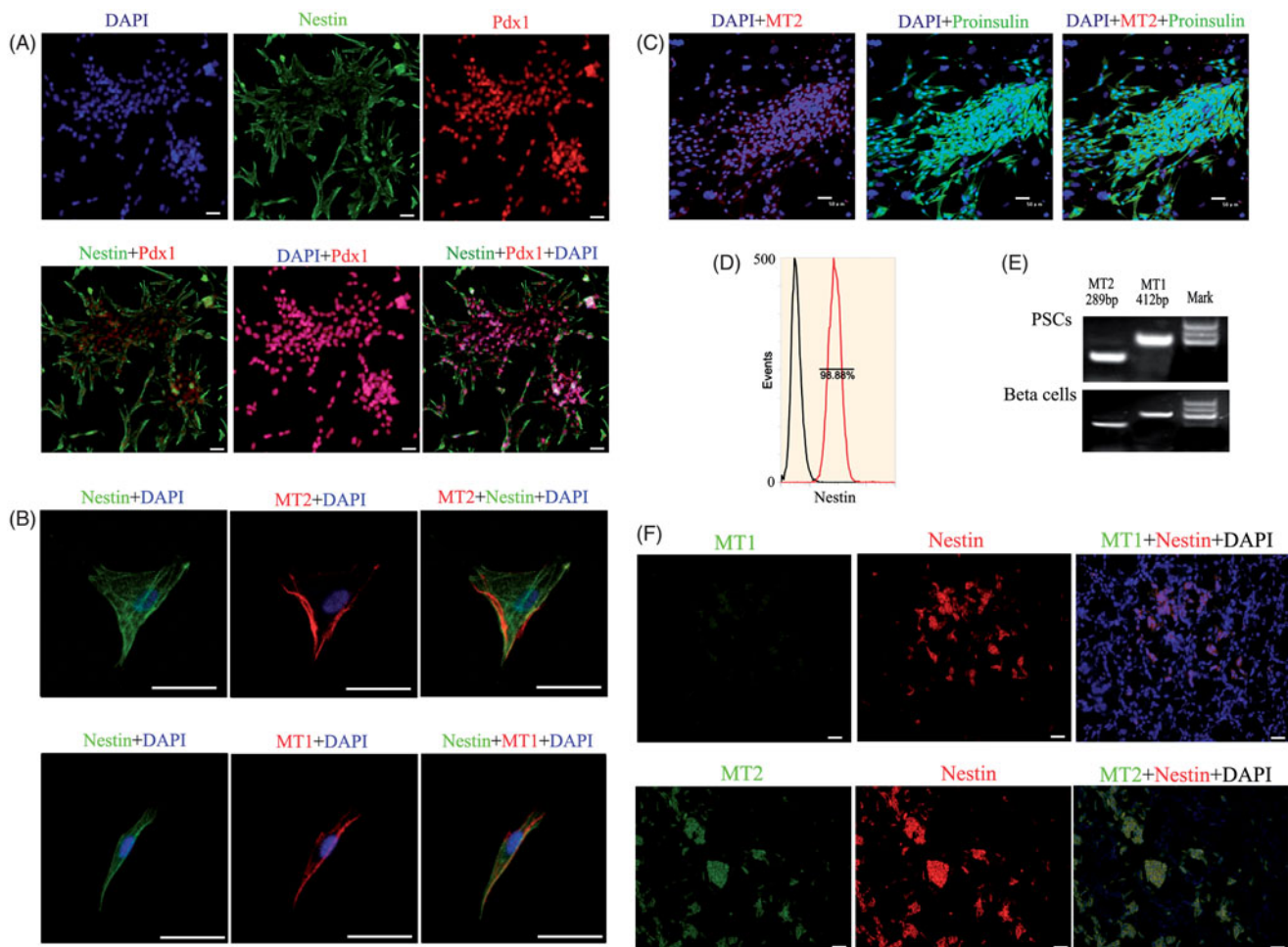


Figure 1. Expression of specific genes in PSCs *in vitro* and *in vivo*. (A) Detection of PSC markers by IF staining. Nestin staining was located in the cytoplasm, while Pdx1 staining was located in the nucleus. The nestin, Pdx1 and DAPI signals appear as garland-like structures in merged images. Pdx1, a transcription factor, is recognized as a marker of PSCs in pancreas development (bar = 50 μ m). (B) Detection of melatonin receptors by IF staining in PSCs *in vitro*. Melatonin receptors include MT1 and MT2, which were co-expressed in PSCs (bar = 200 μ m). (C) IF analysis of sections in differentiated beta cell clusters stained for proinsulin and MT2 (bar = 50 μ m). (D) Purification of PSCs by FCM using nestin antibody. (E) RT-PCR analysis of sections from differentiated beta cells and PSCs tested for MT1 and MT2. (F) Co-expression of nestin, MT1 and MT2 in pancreatic tissue using IHC. The results demonstrated that these genes were co-expressed in pancreatic islet structures (bar = 50 μ m).

them in paraffin. We then cut 5- μ m longitudinal sections from the paraffin blocks, rehydrated them with xylene followed by decreasing concentrations of ethanol, microwaved them in 0.01 M sodium citrate (pH 6.0) for 20 min, and permeabilized them with 1% Triton X-100 in PBS prior to incubation with primary antibodies at 4°C overnight. We used primary antibodies against nestin, MT1 and MT2 at dilutions of 1:100, 1:50 and 1:100, respectively. We then incubated the sections with GARB (1:400) and GAMB (1:100) for 3 h at RT and then with FITC/Cy5-conjugated streptavidin (1:50) for 3 h at RT. After counterstaining the sections with 4',6-diamidino-2-phenylindole (DAPI), we obtained images by confocal microscopy.

Polymerase chain reaction (PCR)

We isolated total RNA from PSCs using TRIzol Reagent (Invitrogen, Carlsbad, CA). We performed cDNA synthesis using the High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA) with 2 ng total RNA as the template. Sequence-specific reverse transcription (RT)-PCR primers for nestin, MT1,

MT2, and the GAPDH endogenous control were synthesized by Sangon Biotech (Shanghai, China).

Statistical analysis

We performed statistical analyses of our data with a 1-way analysis of variance [ANOVA] followed by the Tukey-Kramer honest significant difference (HSD) test for the 3 sets of results ($p < .05$ was considered statistically significant). We assessed our statistical analyses with JMP Statistical Discovery software (SAS Institute, Cary, NC).

Experimental design

We isolated PSCs from the pancreases of suckling mice and cultured them in advanced RPMI 1640 medium, then differentiated beta cells from these PSCs using methods as previously reported [18]. To test co-expression of melatonin receptors and nestin in PSCs and beta cells, we used IF, IHC and PCR to analyze the expression of MT1, MT2 and nestin in these cells and in murine tissue. To study the effect of melatonin on PSC

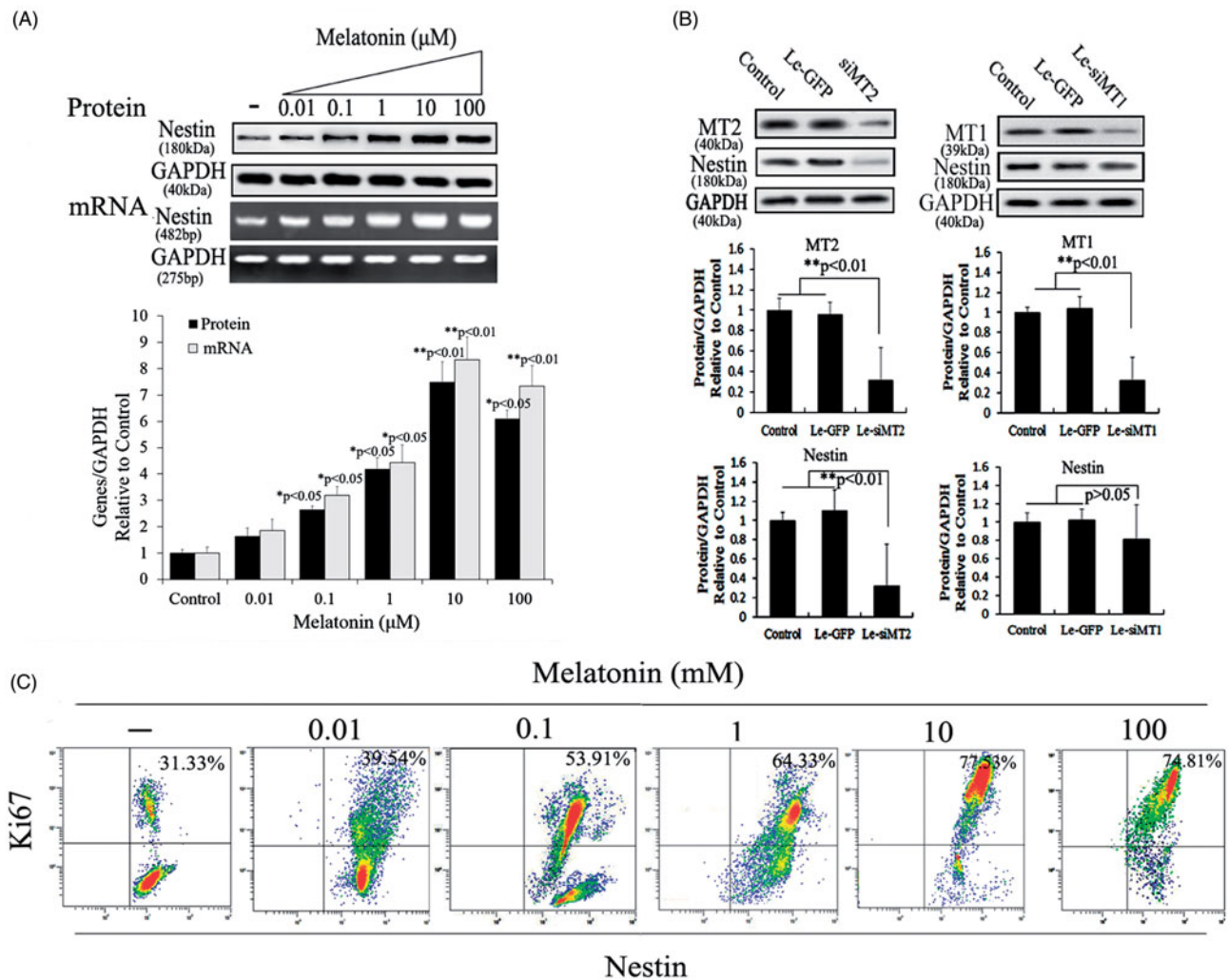


Figure 2. Melatonin increases nestin expression and cell viability in PSCs. (A) Nestin expression was tested using Western blotting and RT-PCR after PSCs were exposed to various melatonin concentrations. Results demonstrated that nestin levels increased in a concentration-dependent manner and that PSCs incubated with 10 μM melatonin had significantly increased expression levels compared with other groups. (B) The role of melatonin receptors in nestin expression. Melatonin receptors, including 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 MT1 or MT2 down when PSCs were exposed to 10 μM melatonin, and protein levels were analyzed using Western blotting. Results indicated that MT2 is an important melatonin receptor for the induction of nestin expression in PSCs. Protein abundance was analyzed using Image J tools and normalized using endogenous control. (C) PSC proliferation was assessed using Ki67-marked methods after treatment with various melatonin concentrations for 72 h.

proliferation, we supplemented melatonin in culture medium at 0.01, 0.1, 1, 10 or 100 μM. We then treated PSCs with melatonin and measured cell proliferation using FCM after Ki67 staining. We selected the optimal melatonin treatment for PSC proliferation for supplementation in differentiation medium of beta cells, and we measured proinsulin expression using FCM. We tested potential mechanisms of melatonin-mediated cell proliferation using ERK pathway inhibitor (SCH772984), TGF-β pathway inhibitor (SB431542) and RNA interference (RNAi). For all experiments, we dissolved melatonin in Dulbecco's Modified Eagle's Medium (DMEM) overnight, and we repeated all experiments independently at least 3 times.

Results

Co-expression of melatonin receptor and nestin in murine PSCs

We isolated PSCs from the pancreases of suckling mice, primarily cultured them in advanced RPMI 1640 medium

supplemented with cocktail factors, and purified them using nestin antibody and FCM. Pdx1, a transcription factor, is recognized as a marker of pancreatic stem cells in pancreatic development, so we determined the phenotype of pancreatic precursors *in vivo*. Figure 1(A) shows nestin and Pdx1 expression using IF; cells in clusters were positive for these proteins. The plasticity of stem cells is their main characteristic. Thus, we used a cocktail of growth factors to induce the formation of beta cells from PSCs, then detected the expression of proinsulin in islet-like structures using IF staining (Figure 1(C)). Melatonin receptors in mammals include 2 high-affinity receptors, MT1 and MT2, which we tested using IF and RT-PCR. Figure 1(B,E) shows that MT1, MT2 and nestin were co-expressed in PSCs. In addition, our IHC results demonstrated that all 3 were also co-expressed in pancreatic islet structures (Figure 1(F)). Taken together, our results demonstrated that nestin, MT1 and MT2 were co-expressed in PSCs both *in vitro* and *in vivo*.

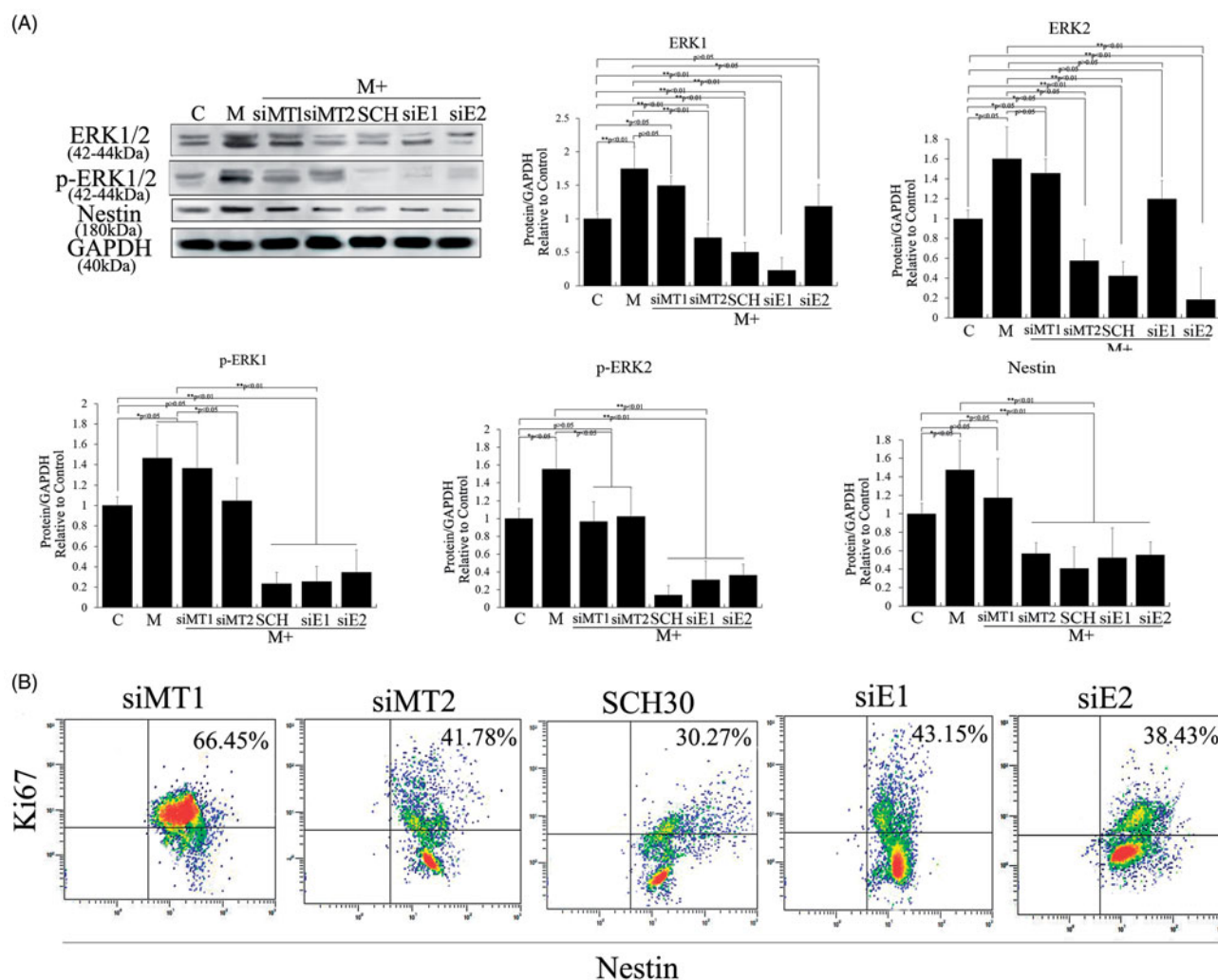


Figure 3. Role of melatonin receptors in melatonin-induced PSC proliferation. Melatonin promoted cell proliferation by activating the ERK pathway. To detect the roles of MT1, MT2 and ERK1/2 in melatonin-treated PSCs, we exposed siMT1, siMT2, siERK1, siERK2 and SCH772984 to melatonin-treated PSCs. (A) Western blotting revealed that melatonin activated the ERK pathway via MT2, and nestin expression levels were positively correlated with activation of the ERK pathway and decreased when siERK1, siERK2 or SCH772984 was exposed to melatonin-treated PSCs. C: control, without melatonin; M: 10 μ M melatonin added to PSC culture; siMT1: siRNA of MT1; siMT2: siRNA of MT2; SCH: SCH772984, ERK pathway inhibitor; siE1: siRNA of ERK1; siE2: siRNA of ERK2. Protein abundance was analyzed using ImageJ tools and normalized using endogenous controls. (B) PSC proliferation was assessed using Ki67-marked methods after treatment with different factors for 72 h.

Melatonin influences nestin expression and cell proliferation in murine PSCs

To determine whether the increased nestin expression in PSCs was due to melatonin treatment, we added various melatonin concentrations (0.01, 0.1, 1, 10 or 100 μ M) to PSC cultures to test for changes in nestin expression. Results indicated that nestin expression levels increased in a concentration-dependent manner in melatonin-treated PSCs. PSCs incubated with 10 μ M melatonin had significantly increased nestin expression levels compared with the other groups ($p < .01$); however, no significant change in expression level occurred between 10 μ M and 100 μ M ($p > .05$) (Figure 2(A)). To detect the role of melatonin receptors in nestin expression, siMT1 and siMT2 were overexpressed in melatonin-induced PSCs to determine which melatonin receptors play a role. The results indicated that MT2 is an important melatonin receptor for the induction of nestin expression in PSCs (Figure 2(B)). After treatment with various melatonin concentrations for 72 h, we measured cell viability data via Ki67 assay; the number of Ki67-positive cells indicated that

proliferation had increased and that melatonin treatment had improved PSC viability in a dose-dependent manner compared with untreated cells. However, PSCs treated with 10 μ M melatonin showed no significant change in cell proliferation compared with PSCs treated with 100 μ M melatonin (Figure 2(C,D)). Therefore, we selected 10 μ M melatonin as the dose to promote cell proliferation of PSCs.

Role of MT, ERK and TGF- β /SMAD pathways in melatonin-treated cell proliferation

Melatonin receptors, which include MT1 and MT2 and which belong to the family of G-protein-coupled receptors and binding sites (putative receptors), have also been found in the nuclei of many cell types [19,20]. To explore the potential mechanisms of action for melatonin, siMT1 and siMT2 were overexpressed in 10 μ M melatonin-treated PSCs to determine which receptor plays a role. Our results indicated that MT2 is an important melatonin receptor for the proliferation of PSCs (Figure 3). The ERK pathway is a chain of proteins in the cell

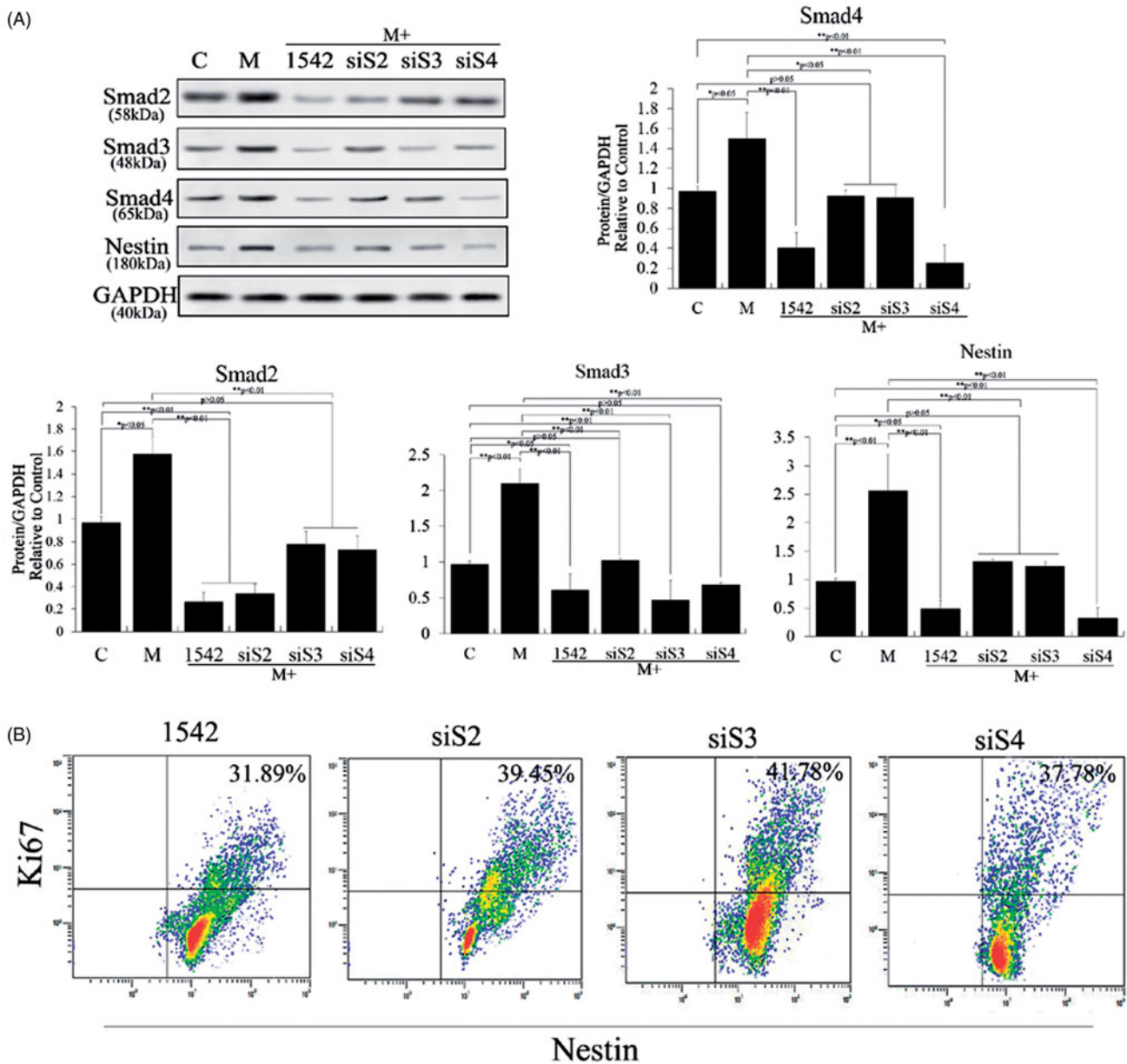


Figure 4. Role of TGF- β /SMAD pathway in melatonin-induced PSC proliferation. TGF- β 1 pathway inhibitor (SB431542), siS2, siS3 and siS4 were used to test the functions of this pathway. (A) Western blotting revealed that melatonin promotes expression of Smad2, Smad3 and Smad4 and that nestin expression levels were positively correlated with expression of Smad2, Smad3 and Smad4. These levels were significantly decreased when siS2, siS3, siS4 or SB431542 was applied to melatonin-treated PSCs. (B) PSC proliferation was assessed using Ki67-marked methods after treatment with different factors for 72 h.

that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell, including ERK1/2, MEK, JNK and P38. Other studies have shown that ERK1/2 is activated by phosphorylation in response to melatonin in cell cultures, it was found that melatonin can promote cell proliferation by activating the ERK pathway. In the current study, we also demonstrated that melatonin regulates ERK pathways that control cell proliferation. As shown in Figure 3, phosphorylation and expression of ERK1/2 were significantly upregulated after 10 μ M melatonin treatment. Phosphorylation and expression of ERK1/2 were also negatively correlated with overexpressed siMT2, and cell proliferation analyses indicated decreased proliferation efficiency after 10 μ M melatonin treatment and siMT2 overexpression, compared with melatonin and siMT1 overexpression. We then

used an EKR pathway inhibitor (SCH772984) and siERK1 (siE1) and siERK2 (siE2) to analyze cell proliferation and nestin expression singly after 10 μ M melatonin treatment to illuminate the functions of ERK pathway members in the self-renewal of PSCs. Our results showed that SCH772984, siERK1 and siERK2 effectively repressed cell proliferation and nestin expression in PSCs ($p < .01$; Figure 3), and demonstrated ERK1/2 played an important role in melatonin-induced cell proliferation and nestin expression.

TGF- β signaling initiates as the ligand binds to the receptors followed by the dimerization of type I and type II receptors. The activation of the type I receptor is subsequently followed by the phosphorylation of Smad proteins including Smad2, Smad3 and Smad4, which then translocates into the nucleus and regulates the transcription of related genes. The

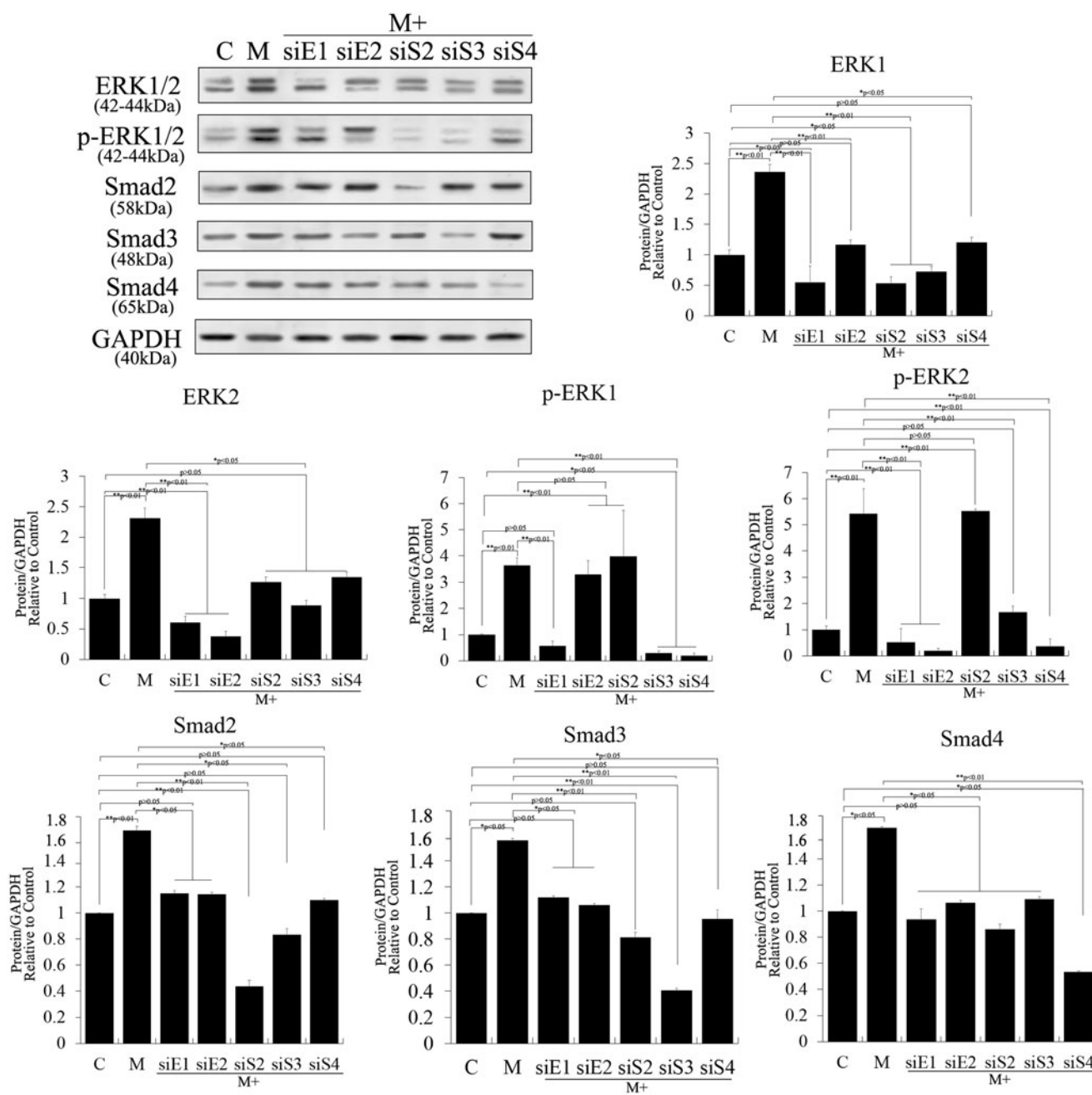


Figure 5. Crosstalk between ERK and TGF- β /SMADs pathways in melatonin-induced PSCs. ERK1, ERK2, Smad2, Smad3 and Smad4 were knocked down using RNAi to analyze their effects in PSCs after melatonin treatment. The results revealed that ERK1 knockdown significantly suppressed melatonin-induced ERK1/2 and Smad2/3 expression ($p < .01$), but ERK2 knockdown did not do so ($p > .05$). Smad2/3 knockdown also significantly suppressed melatonin-induced ERK1/2 and Smad2/3 expression ($p < .01$), but Smad4 knockdown did not ($p > .05$). These results suggest that Smad2/3 may be involved in melatonin-induced phosphorylation and expression of ERK1/2, which is positive feedback in the regulation of Smad2/3 and ERK1/2.

TGF- β /SMAD pathway plays an important role in cell proliferation, which has been extensively explored over recent years; however, there has been no study of TGF- β 1 pathway–altered cell proliferation and nestin expression in PSCs. Smad2, Smad3 and Smad4 are essential components of the intracellular TGF- β /SMAD pathway, and we found that melatonin can promote the expression of all 3 to increase proliferation of PSCs (Figure 4). We also used TGF- β pathway inhibitor SB431542 and siS2, siS3 and siS4 to detect the role of the TGF- β 1 pathway and its members in proliferation and nestin expression of PSCs. Our results showed that SB431542, siS2, siS3 and siS4 effectively repressed cell proliferation and nestin

expression in melatonin-induced PSCs ($p < .01$; Figure 4). These results demonstrated the TGF- β /SMAD pathway played critical role in melatonin-induced cell proliferation and nestin expression.

Crosstalk between the ERK and TGF- β /SMAD pathways in melatonin-treated cell proliferation

Crosstalk between the ERK and TGF- β /SMAD pathways has been demonstrated in many cell types [21,22], but crosstalk between the pathways to promote proliferation of stem cells

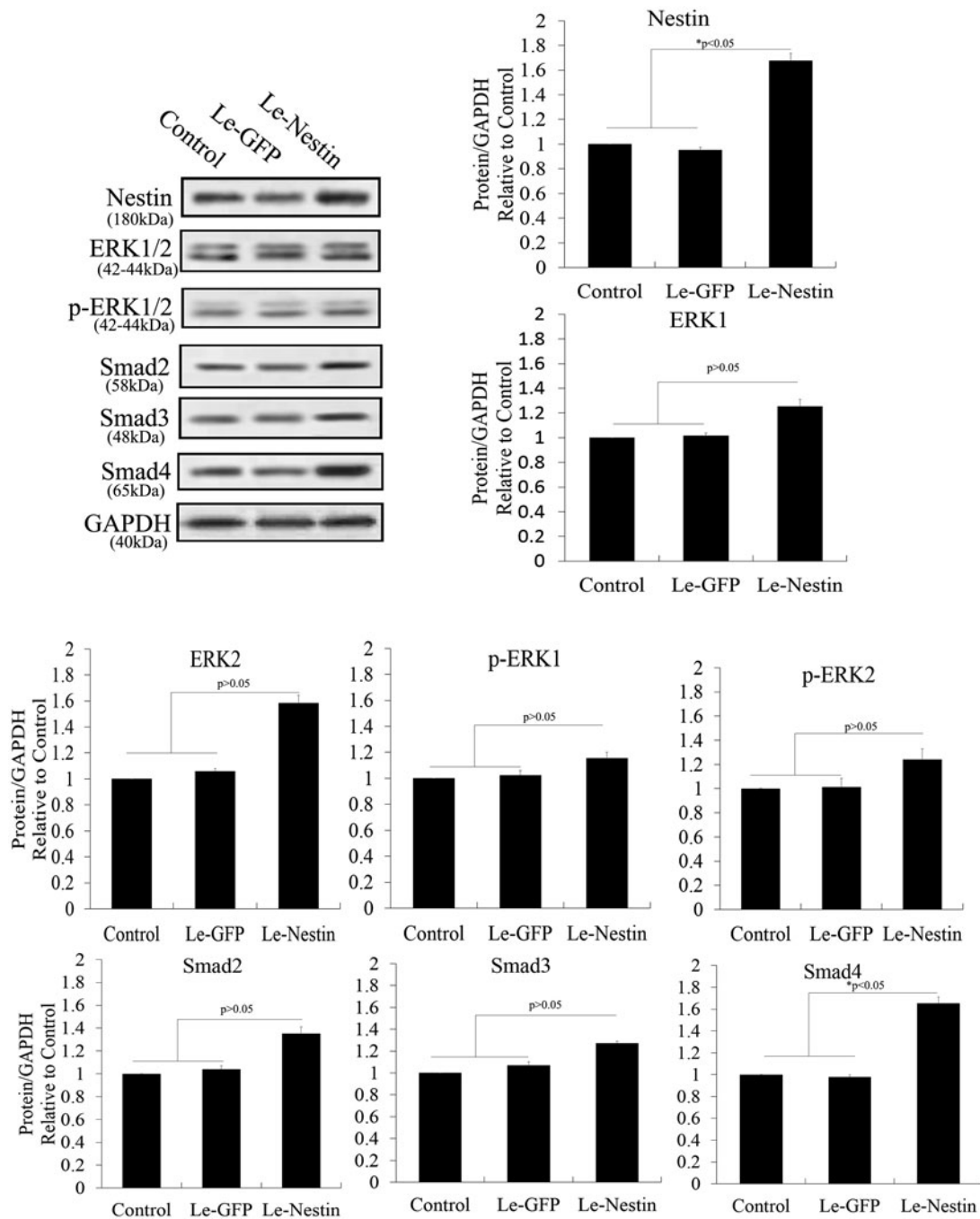


Figure 6. Crosstalk among nestin and the ERK and TGF- β /SMADs pathways in PSCs. Nestin was overexpressed in PSCs to test feedback of the TGF- β /SMADs pathway. Lentivirus expressing nestin significantly increased nestin expression compared with control cells in PSCs. However, nestin overexpression negligibly affected phosphorylation and expression of ERK1/2, Smad2 and Smad3 ($p > .05$). The expression level of Smad4 changed significantly ($p < .05$).

remains poorly understood. In the current study, we knocked ERK1, ERK2, Smad2, Smad3 and Smad4 down respectively using small interfering RNAs in melatonin-induced PSCs to analyze the effects of these factors, and we detected protein levels using Western blotting. As shown in Figure 5, Western blotting revealed that ERK1 knockdown significantly suppressed melatonin-induced ERK1/2 and Smad2/3 expression ($p < .01$), but ERK2 knockdown did not significantly change them ($p > .05$). Meanwhile, Smad2/3 knockdown also significantly suppressed melatonin-induced ERK1/2 and Smad2/3 expression ($p < .01$), but Smad4 knockdown did not do so ($p > .05$). Hence, phosphorylations of ERK1/2 were significantly

decreased in ERK1/2 and Smad3 knockdown experiments ($p < .01$). Together, these results suggest that Smad2/3 may be involved in melatonin-induced phosphorylation and expression of ERK1/2, which is positive feedback in the interaction between Smad2/3 and ERK1/2.

Smad4 plays an important role in the expression of nestin via activation of the TGF- β /SMAD pathway; moreover, increased nestin expression is a positive feedback regulator of the TGF- β pathway [23]. We thus overexpressed nestin in PSCs to test feedback with Smad4. As shown in Figure 6, Western blotting revealed that lentivirus expressing nestin significantly increased that expression compared with control

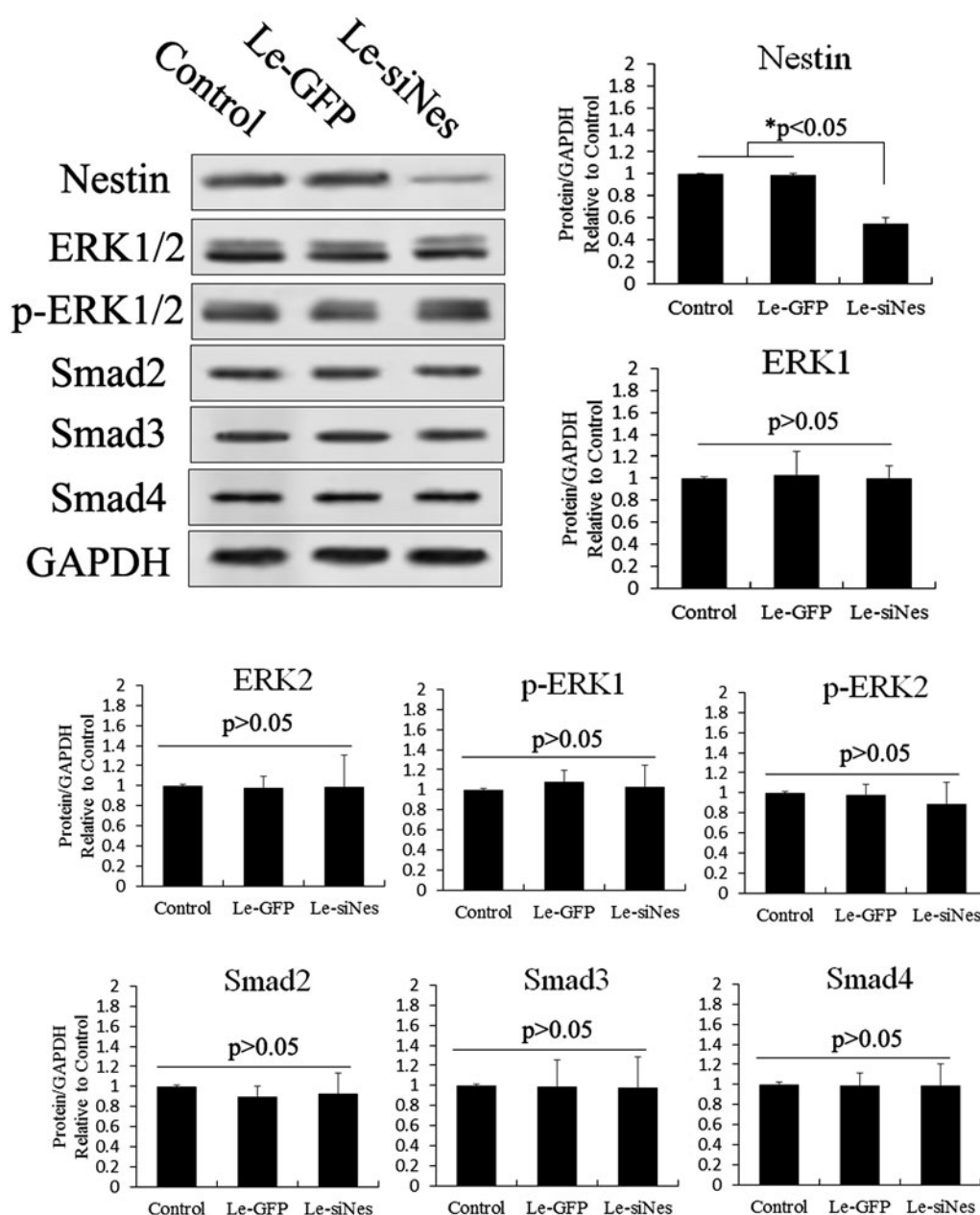


Figure 7. Knockdown of nestin to test feedback of the ERK and TGF- β /SMADs pathways in PSCs. Nestin was knocked down using RNAi. Western blotting revealed that lentivirus expressing siRNA of nestin (siNes) significantly decreased nestin expression in PSCs compared with controls ($p < .05$). However, nestin knockdown negligibly affected the phosphorylation and expression of ERK1/2, Smad2, Smad3 and Smad4 ($p > .05$).

cells. Phosphorylation and expression of ERK1/2, Smad2 and Smad3 were negligibly affected by nestin overexpression ($p > .05$), but the expression of Smad4 changed significantly ($p < .05$). In contrast, knockdown of nestin in PSCs caused no significant changes in the expression of ERK1/2, Smad2, Smad3, or Smad4 (Figure 7). These results suggest that increased nestin expression was a positive feedback regulator of the TGF- β /SMAD pathway via promoting SMAD4 expression. Our studies have indicated that TGF- β /SMAD-induced nestin expression is promoted via activation of the ERK pathway after melatonin treatment in PSCs. Our assessment of cell proliferation after overexpression or knockdown of nestin in melatonin-treated PSCs demonstrated proliferation and inhibition of PSCs, respectively (Figure 8).

Melatonin weakens differentiation of beta cells in PSCs

Proliferation effects of melatonin in PSCs were summarized in above research, and then, to assess the roles of melatonin, MT1, MT2, the TGF- β /SMAD pathways, and the ERK pathway in differentiation of beta cells from PSCs, we used siRNAs of MT1 and MT2 and pathway inhibitors SB431542 and SCH772984. We added each of siMT1, siMT2, SB431542 and SCH772984 to PSCs in combination with melatonin. Effects on the differentiation of beta cells are shown in Figure 9. Single exposures of siMT1, siMT2, SB431542 and SCH772984 in PSCs after 10 μ M melatonin treatment showed that melatonin was an inhibitor, while siMT2, SB431542 and SCH772984 were antagonists, of melatonin in the

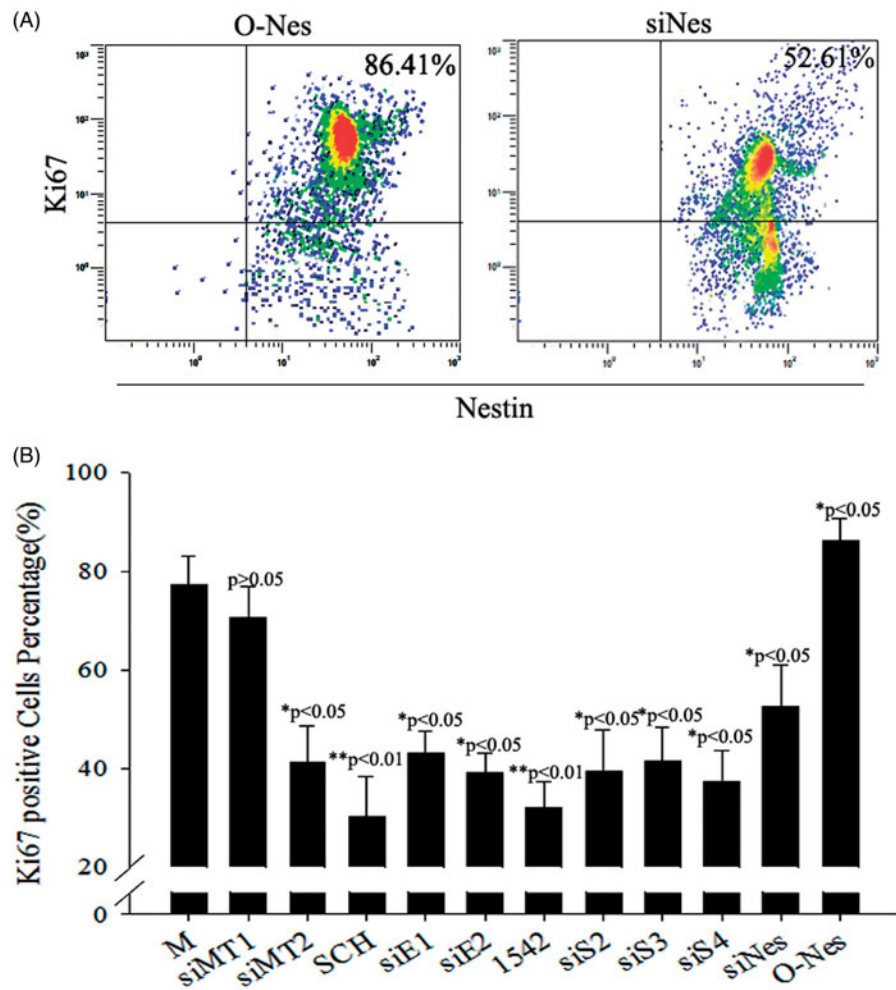


Figure 8. Proliferation assessed after treatment with different siRNAs in melatonin-treated PSCs. (A) Ki67-marked analysis of proliferation after overexpression of nestin in melatonin-treated PSCs, as well as Ki67-marked analysis of proliferation after RNAi-nestin treatment in melatonin-treated PSCs. (B) Statistical analysis of the proliferation effect of gene silencing combined with melatonin treatment in PSCs using Ki67-marked methods. These results showed that melatonin induced cell proliferation by activating the MT2/ERK/SMAD/nestin axis in PSCs. Ki67-positive stem cells were counted from 3 random culture dishes in each of 3 independent PSC samples.

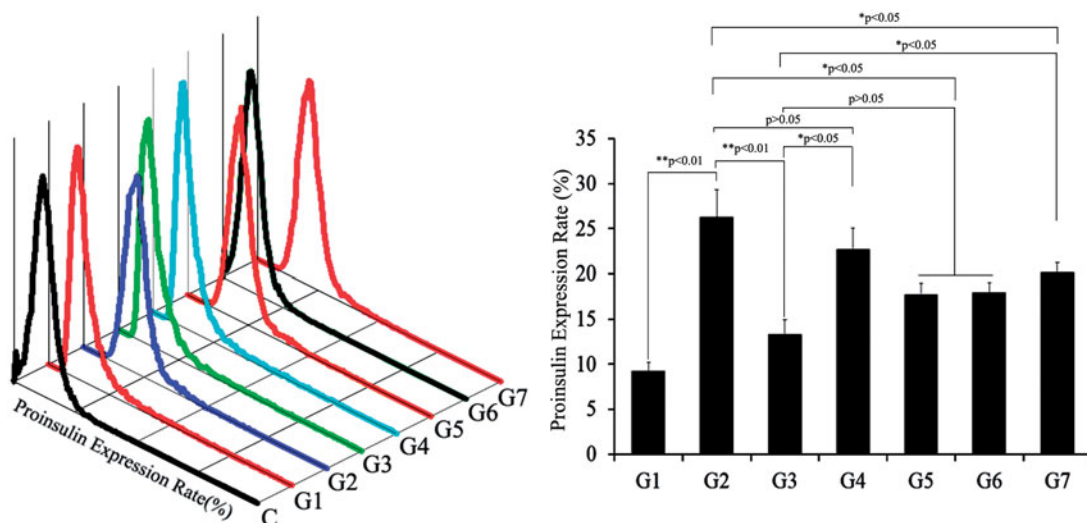


Figure 9. Insulin expression in beta cells derived with different combinations of inducers. Analyses of proinsulin expression using FCM. The percentage of cells expressing insulin was highest in G2. C: control; G1: group 1, PSCs; G2: group 2, PSCs induced with a cocktail of growth factors; G3: group 3, melatonin-treated PSCs induced with growth factors; G4: group 4, melatonin-treated PSCs overexpressing siMT1 and induced with growth factors; G5: group 5, melatonin-treated PSCs overexpressing siMT2 and induced with growth factors; G6: group 6, melatonin plus SCH72984-treated PSCs induced with growth factors; G7: group 7, melatonin plus SB431542-treated PSCs induced with growth factors.

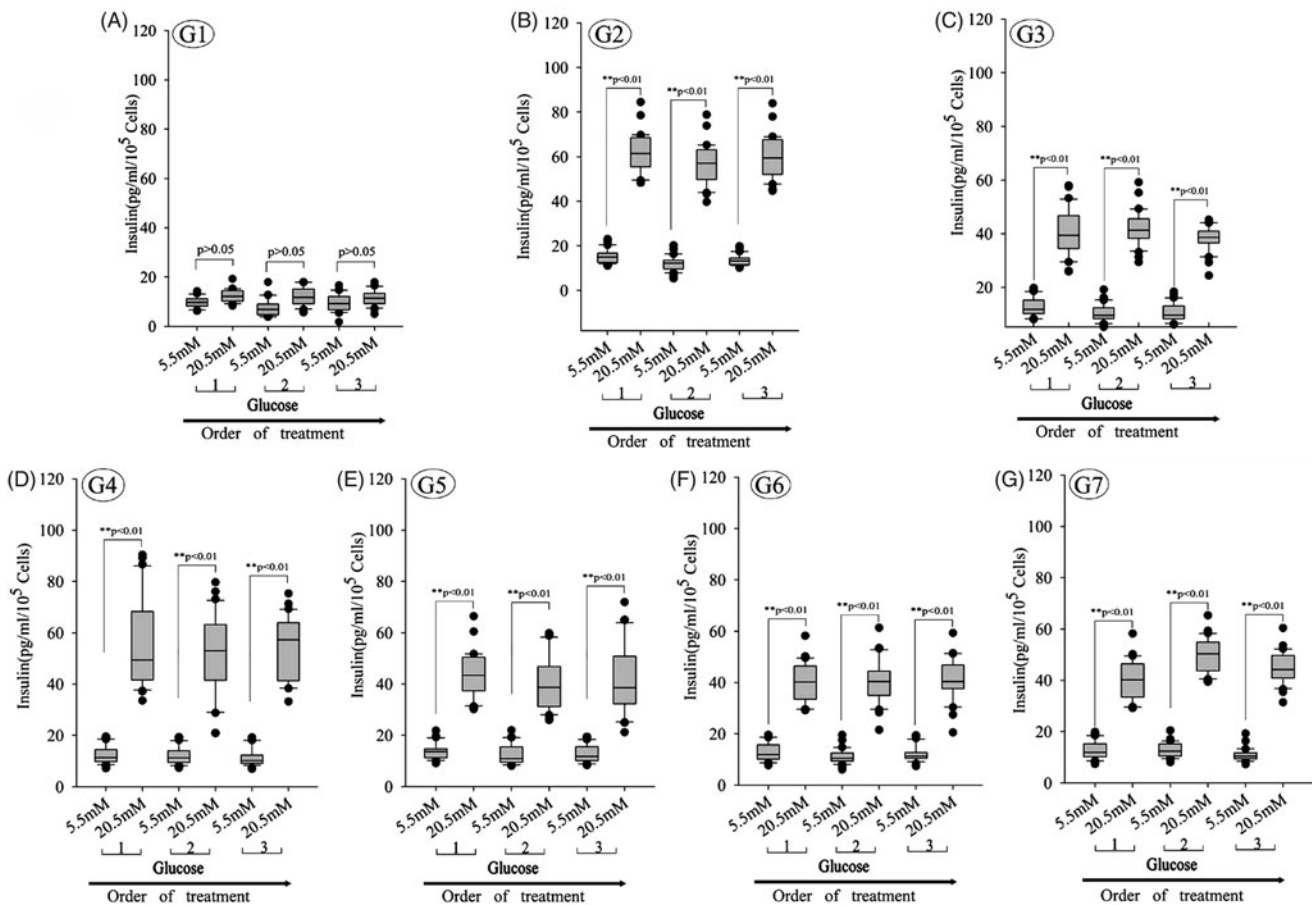


Figure 10. Glucose-induced insulin secretion in beta cells derived with different combinations of inducers. (A–G) Glucose-induced insulin secretion in beta cells derived with different combinations of inducers. The box plots show that insulin secretion increased when the glucose concentration increased in all groups except group 1 ($n = 30$; paired 2-tailed t test). G1: group 1, PSCs; G2: group 2, PSCs induced with a cocktail of growth factors; G3: group 3, melatonin-treated PSCs induced with growth factors; G4: group 4, melatonin-treated PSCs overexpressing siMT1 and induced with growth factors; G5: group 5, melatonin-treated PSCs overexpressing siMT2 and induced with growth factors; G6: group 6, melatonin plus SCH722984-treated PSCs induced with growth factors; G7: group 7, melatonin plus SB431542-treated PSCs induced with growth factors.

differentiation of beta cells from PSCs ($p < .05$). FCM data showed decreased efficiency of beta cells differentiation from PSCs following a combination of melatonin and siMT2 overexpression, compared with melatonin and siMT1 overexpression. These results suggested melatonin weakened differentiation of beta cells via MT2-ERK-TGF- β /SMAD pathways. Taken together, our results suggest that melatonin inhibited the differentiation of beta cells, promoted cell proliferation, and increased expression of the stem cell marker nestin through activation of the ERK and TGF- β 1 pathways.

Functional feature of beta cells from PSCs

A key functional feature of beta cells is its ability to repeatedly perform glucose-stimulated insulin secretion. Beta cells challenged sequentially with 5.5, 20.5, 5.5, 20.5, 5.5 and 20.5 mM glucose, with a 30 min incubation for each concentration. After sequential low/high-glucose challenges, insulin concentration were analyzed in supernatant using ELISA. And glucose-induced insulin secretion demonstrated that PSCs derived from beta cells could release insulin at glucose concentrations of 5.5 mM and 20.5 mM. Analysis of glucose-induced insulin secretion demonstrated that beta cells derived with different combinations of inducers could release

insulin, while undifferentiated progenitor cells (G1) did not release insulin. The box plots show that insulin secretion increased when the glucose concentration increased in G2–G7, when the secreted insulin amounts of IPCs differentiation from pSCs was assessed, the combination of nicotinamide, HGF, and active-A-induced IPCs secretion at a higher efficiency compared with combinations of melatonin (Figure 10). These validation results were largely in agreement with the data of IPCs differentiation.

Discussion

Type I diabetes is caused by the autoimmune destruction of beta cells in pancreatic islets. While replacing pancreatic beta cells would be an ideal therapy [24,25], generation of beta cells from stem or other cells is an attractive alternative [26–28]. In pancreatic islets, only a small subset of cells expresses nestin, and these have been proposed to be precursors of differentiated pancreatic endocrine cells [13]. These nestin-positive cells are called pancreatic stem cells (PSCs), and it has been proposed that they play an important role in the growth and maintenance of pancreatic islets [14–16,29].

Previous studies have indicated that melatonin has multiple important physiological functions within the

neuroimmunoendocrine system and that its wide extracellular and intracellular distribution indicates potentially broad physiological functions. Previous research has reported that persistent activation of melatonin signaling can attenuate pancreatic beta cell loss and dysfunction associated with oxidative and endoplasmic-reticulum stress [30,31]. However, no reports on melatonin promotion of pancreatic progenitor cells are available. Our research on melatonin confirmed it can promote the proliferation of pancreatic progenitor cells. Given that melatonin is a well-known activator of the ERK pathway, Western blotting showed dramatic upregulation of protein expression and phosphorylation levels compared with controls. The ERK1/2 cascade, activated by mitogenic stimuli, is critical for cell proliferation and survival and required for normal progression into mitosis [32,33]. ERK pathways are responsible for melatonin's proliferative actions in various cells, including neural cells, induced pluripotent stem cells and pancreatic beta cells [9,31]. ERK1/2 activation has been well documented as necessary for cell proliferation and is partly involved in G1 and G2/M phase progression [34–36]. As melatonin increased the expression of p-ERK1/2 and ERK1/2, we tested whether SCH772984, a selective inhibitor of the ERK pathway that disrupts activation of ERK1/2, would inhibit the proliferative effect of melatonin in pancreatic progenitor cells. Our results demonstrated that SCH772984 significantly repressed that proliferative effect after melatonin treatment. These findings strongly suggest that ERK1/2 activation plays a central role in the observed melatonin-mediated effects.

TGF- β 1 is a member of the TGF- β superfamily of cytokines, which play roles in growth, development, immunity, wound healing, inflammation, apoptosis and cancer. Previous studies have indicated that the TGF- β 1 pathway acts through SMAD-dependent (canonical) and SMAD-independent (non-canonical) pathways [37,38]. Smad2 and Smad3, the principal receptor-activated proteins involved in the TGF- β pathway, are activated directly by TGF- β type I receptor kinase, then translocated to the nucleus complexed with a common co-SMAD, Smad4, to regulate the transcription of target genes [39,40]. Many signaling pathways interact with the TGF- β 1 pathway. The mitogen-activated protein kinase (MAPK) cascade, composed mainly of ERK, p38 MAPK and c-Jun N-terminal kinases (JNKs), has been shown to represent a major signaling pathway for TGF- β -dependent SMAD activation [41,42]. In crosstalk between the SMAD and MAPK pathways, TGF- β -dependent or -independent induction of activated ERK can occur [40]. We tested crosstalk between the TGF- β /SMAD and ERK pathways in melatonin-induced cell proliferation in our research by transfecting siERK1, siERK2, siS2, siS3 and siS4 into pancreatic progenitor cells treated with melatonin. We demonstrated that knockdown of Smad2 or Smad3 effectively reduced expression levels of ERK1, but knockdown of Smad4 did not. However, knockdown of ERK1 or ERK2 did not result in positive feedback, consistent with data reported previously [22].

Nestin is an intermediate filament protein expressed in dividing cells during the early stages of development in the central nervous system, peripheral nervous system, and myogenic and other tissues. Upon differentiation, nestin becomes downregulated and is replaced by tissue-specific intermediate filament proteins, a theory confirmed in pancreatic progenitor

cells in previous research [18,43]. In the current study, protein levels of nestin in PCs were increased in a concentration-dependent manner after melatonin treatment, suggesting that melatonin is involved in maintaining their self-renewal. However, the molecular mechanisms of nestin expression activation after melatonin treatment remain unknown. We found that proliferation and nestin expression of PCs were enhanced via positive feedback in crosstalk between the ERK and TGF- β /SMAD pathways after melatonin treatment, and siRNA data showed that the TGF- β /SMAD pathway might have induced nestin expression in PCs predominantly mediated by the Smad4-dependent pathway. Meanwhile, Smad4 could be regulated by nestin expression as positive feedback, consistent with data reported previously [44].

In summary, melatonin promotes the cell proliferation of PCs due at least in part to activation of the TGF- β /SMAD and ERK pathways. Melatonin enhances nestin expression of PCs to maintain self-renewal and prevent differentiation, and this may involve crosstalk between the TGF- β /SMAD and ERK pathways. These findings reveal possible novel mechanisms through which the ERK pathway cooperatively interacts with the TGF- β /SMAD pathway to regulate nestin expression.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by the National Natural Science Foundation of China (Grant No. 81700685 to Bai C.), Shandong Provincial Natural Science Foundation, China (ZR2017BH002 to Bai C., ZR2017BH105 to Gao Y.), and Faculty Start-up Funds of Jining Medical University (to Bai C. and Gao Y.).

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