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Effects and mechanisms of melatonin on neural differentiation of induced pluripotent stem cells

Tao Shu [a, 1], Tao Wu [b, 1], Mao Pang [a], Chang Liu [a], Xuan Wang [a], Juan Wang [c], Bin Liu [a], Limin Rong [a, *]

[a] *Department of Spine Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510630, China*
[b] *Department of Emergency, Guangdong Provincial Corps Hospital of Chinese People's Armed Police Forces, Guangzhou Medical University, Guangzhou, Guangdong 510000, China*
[c] *Department of Gynaecology, Common Splendor International Health Management, Guangzhou, Guangdong 510000, China*

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Melatonin, a lipophilic molecule mainly synthesized in the pineal gland, has properties of antioxidation, anti-inflammation, and antiapoptosis to improve neuroprotective functions. Here, we investigate effects and mechanisms of melatonin on neural differentiation of induced pluripotent stem cells (iPSCs). iPSCs were induced into neural stem cells (NSCs), then further differentiated into neurons in medium with or without melatonin, melatonin receptor antagonist (Luzindole) or Phosphatidylinositide 3 kinase (PI3K) inhibitor (LY294002). Melatonin significantly promoted the number of neurospheres and cell viability. In addition, Melatonin markedly up-regulated gene and protein expression of Nestin and MAP2. However, Luzindole or LY294002 attenuated these increase. The expression of pAKT/AKT were increased by Melatonin, while Luzindole or LY294002 declined these melatonin-induced increase. These results suggest that melatonin significantly increased neural differentiation of iPSCs via activating PI3K/AKT signaling pathway through melatonin receptor.

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1. Introduction

Cell transplantation, such as neural stem cells (NSCs), is a method for treating some neurological diseases [1,2]. Induced pluripotent stem cells (iPSCs) generated from fibroblasts have potential similar to embryonic stem cells (ESCs) to differentiate into neural lineages under specific conditions [3,4]. Furthermore, clinical application of iPSCs from any part of humans avoids both immunologic rejection and ethical problems involved in using of human ESCs [5,6]. However, the rate of differentiation of iPSCs into neural lineages is disappointing [7,8]. Thus, efficiently inducing iPSCs to differentiate into NSCs or neurons is of significant study interest.

Melatonin, a lipophilic molecule mainly synthesized in the pineal gland, is a well-known opportunity for the treatment of neurological disease [9]. Previous studies have demonstrated that melatonin possesses the properties of anti-oxidation, anti-inflammatory and anti-apoptosis to protect cells damage, and it improves cells survival, proliferation and differentiation in neural injury or death [10–12]. In addition, melatonin exerts antioxidant effects on neural lineages via G protein-coupled melatonin receptor type 1 (MT1) and type 2 (MT2), MT inhibitor Luzindole prevents these neuroprotective properties of melatonin on cells [13]. However, further mechanism of melatonin through MT on neural lineages is still unclear.

Phosphatidylinositide 3 kinase (PI3K)/AKT pathway, involved in signal transduction from receptor tyrosine kinase, is often linked with cells survival, proliferation and differentiation [14]. Previous studies have showed that activated PI3K/AKT pathway has been associated with neuroprotection during development of nervous system [15,16]. Moreover, melatonin has been confirmed to induce the activation of PI3K/AKT pathway binding to regenerating cells. The pathway is also dependent on MT1/MT2 receptors in this process [17]. However, the effect of melatonin on neurogenic differentiation of iPSCs through PI3K/AKT pathway associated with MT1/MT2 receptors has not yet been reported.

* Corresponding author. Department of Spine Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Tianhe Road 600#, Guangzhou, Guangdong 510630, China.
E-mail address: ronglm21@163.com (L. Rong).
[1] These authors contributed equally to this work.

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Based on the known activities of melatonin, we predict that melatonin may promote differentiation of iPSCs into neuron-like cells via MT1/MT2 receptors and PI3K/Akt pathway, thus paving the way to use of iPSCs-derived NSCs with melatonin for nervous system disorders.

2. Materials and methods

2.1. Materials

Melatonin, retinoic acid (RA), Luzindole and Hoechst 33342 was purchased from Sigma–Aldrich (Saint Louis, MO, USA). LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), N2, B27, β-mercaptoethanol, L-glutamax, Insulin-Trans-Sel-A, nonessential amino acids, Trizol reagent and Triton X-100 were obtained from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Recombinant leukemia inhibitory factor (LIF) and anti-microtubule-associated protein 2 (MAP2) were acquired from Merck Millipore (Darmstadt, Germany). Basic fibroblast growth factor and epidermal growth factor were gotten from PeproTech (Rocky Hill, NJ, USA). Cell Counting Kit-8 (CCK-8) was purchased from KeyGEN Biotech (Nanjing, China). Rabbit anti-nestin was obtained from Abcam (Cambridge, England). pAKT, AKT and rabbit anti-glial fibrillary acidic protein (GFAP) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Revert Aid first-strand cDNA synthesis kit was obtained from Takara Bio (Dalian, China).

2.2. iPSCs culture and neuronal differentiation

Mouse iPSCs, obtained by the Guangzhou Institute of Biological Medicine and Health (Chinese Academy of Science), were co-cultured with mouse embryonic fibroblasts in DMEM consisting of 15% FBS, 0.1% LIF, 1% β-mercaptoethanol, 1% L-glutamax and 1% nonessential amino acids. The cells were then induced in embryoid body (EB) medium without LIF in suspension culture for 2 days. 1 μM RA with or without melatonin, Luzindole, PI3K/Akt inhibitor (LY294002) was added in this medium for a further 2 days. The medium was replaced to N2B27 medium in the presence or absence of 1 μM melatonin, 1 μM Luzindole or 10 μM LY294002 for 7 days. The N2B27 medium contains with 1% N2, 2% B27, 1% L-glutamax, 1% Insulin-Trans-Sel-A, 20 ng/ml basic fibroblast growth factor and 20 ng/ml epidermal growth factor. Neurospheres were plated on poly-L-lysine-coated chamber slides in neurobasal medium supplemented with 5% FBS, 1 μM RA, melatonin (0, 1 μM), Luzindole (0, 1 μM) or LY294002 (0, 10 μM) to induced into neurons for 7 days. Cells were evaluated using the following assays.

2.3. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min at 37 °C. BSA containing 0.1% Triton X-100 were applied to block specimens for 1 h at room temperature. Primary antibody, rabbit anti-nestin (1:1000), anti-MAP2, were added and incubated at 4 °C overnight, followed by secondary antibody labeled with FITC for 60 min at 37 °C. Cells were then treated with 10 μg/ml Hoechst 33342 for nuclear counterstain. Stained cells were visualized by fluorescent microscopy (Leica, Wetzlar, Germany).

2.4. CCK-8 assay for cell viability

The viability of iPSCs-derived NSCs pretreated with melatonin, Luzindole, LY294002 were evaluated by CCK-8 assay. Briefly, all treated cells were seeded in 96-well plates, then incubated with 10 μl CCK-8 for 4 h at 37 °C, the absorbance at 490 nm was obtained using a plate reader (Elx800, Biotek, Winooski, VT, USA). The absorbance of serum-free medium was used for the control group.

2.5. Neurosphere counts

iPSCs-derived NSCs treated with melatonin, Luzindole, LY294002 were seeded on 24-well plates at the density of 1×10^5 cells/ml in N2B27 medium for 7 days. iPSCs-derived NSCs were calculated directly under microscope.

2.6. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent. Then the Revert Aid first-strand cDNA synthesis kit was used for synthesizing cDNA. qRT-PCR thermal cycler conditions consisted of 50 °C for 2 min, 95 °C for 2 min, 40 cycles at 95 °C for 3 s and 60 °C for 32 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. And the $2^{-\Delta\Delta Ct}$ method was used for calculated relative gene level. $2^{-\Delta\Delta Ct} > 2$ or $<1/2$ was considered statistically significant. Primers were listed in Table 1.

2.7. Western blot analysis

Protein was isolated from iPSCs-derived NSCs and late induced neurons after treatments. Specimens were loaded on 10% sodium dodecyl sulfate polyacrylamide gel. The resolved proteins were transferred to polyvinylidene fluoride membranes. The blots were incubated with anti-Nestin antibody (1:1000), anti-MAP2 antibody (1:1000), anti-pAKT antibody (1:1000) and anti-AKT antibody (1:1000), then incubated with secondary antibodies. Bands were observed by densitometry.

2.8. Statistical analysis

All data are presented as mean ± SD. Statistical difference was done using one-way ANOVA. P value < 0.05 was considered statistically significant.

3. Results

3.1. iPSCs induced into NSCs, then differentiated into neurons

iPSCs tended to form packed clones after 3–4 days in culture. Embryoid bodies (EBs) were then formed in suspension culture after 4 days. Neurospheres stepped to form in serum-free after 7 days (Fig. 1A). Immunofluorescence staining showed expression of Nestin in these neurospheres (Fig. 1B). iPSCs-derived NSCs were further induced into neurons, which expressed MAP2 by immunofluorescence (Fig. 1C).

3.2. Melatonin improved the differentiation of iPSCs into NSCs

CCK-8 assay showed that melatonin (0, 0.5, 1, 5 μM) treatment leaded to more proliferation compared with control group (1.59 ± 0.35, 2.19 ± 0.49, 1.42 ± 0.14) ($P < 0.01$, $P < 0.05$; Fig. 2A). And

Table 1
Primers used for qRT-PCR analysis.

Genes	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	CCTCGTCTCATAGACAAGATGGT	GGGTAGAGTCATACTGGAACATG
Nestin	GCAGCCACTGAGGTATCTGG	AGGCCTCAATGAAACCTGGG
MAP2	CTCTGCCTCTAGCAGCCGAA	CACCACTTGCTGCTTCCTCC

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