



Melatonin Rescues Mesenchymal Stem Cells from Senescence Induced by the Uremic Toxin *p*-Cresol via Inhibiting mTOR-Dependent Autophagy

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Abstract

p-Cresol, found at high concentrations in the serum of chronic kidney failure patients, is known to cause cell senescence and other complications in different parts of the body. *p*-Cresol is thought to mediate cytotoxic effects through the induction of autophagy response. However, toxic effects of *p*-cresol on mesenchymal stem cells have not been elucidated. Thus, we aimed to investigate whether *p*-cresol induces senescence of mesenchymal stem cells, and whether melatonin can ameliorate abnormal autophagy response caused by *p*-cresol. We found that *p*-cresol concentration-dependently reduced proliferation of mesenchymal stem cells. Pretreatment with melatonin prevented pro-senescence effects of *p*-cresol on mesenchymal stem cells. We found that by inducing phosphorylation of Akt and activating the Akt signaling pathway, melatonin enhanced catalase activity and thereby inhibited the accumulation of reactive oxygen species induced by *p*-cresol in mesenchymal stem cells, ultimately preventing abnormal activation of autophagy. Furthermore, preincubation with melatonin counteracted other pro-senescence changes caused by *p*-cresol, such as the increase in total 5'-AMP-activated protein kinase expression and decrease in the level of phosphorylated mechanistic target of rapamycin. Ultimately, we discovered that melatonin restored the expression of senescence marker protein 30, which is normally suppressed because of the induction of the autophagy pathway in chronic kidney failure patients by *p*-cresol. Our findings suggest that stem cell senescence in patients with chronic kidney failure could be potentially rescued by the administration of melatonin, which grants this hormone a novel therapeutic role.

Key Words: Chronic kidney disease, *p*-Cresol, Melatonin, Senescence, Autophagy

INTRODUCTION

Patients with chronic kidney disease (CKD) experience a progressive loss of kidney function over a period of months or years. Consequently, the diseased kidneys fail to metabolize and excrete toxic metabolites and organic waste solutes, which are normally removed by healthy kidneys. It is known that such retained toxic products, commonly referred to as "uremic toxins," are transported by the blood to other organs, causing adverse effects throughout the body (D'Hooge *et al.*, 2003; Meijers *et al.*, 2010). In fact, toxic metabolites or organic solutes accumulated owing to CKD are known to be directly responsible for various complications, such as cardiovascular disease, anemia, pericarditis, renal osteodystrophy, and

neurological disorders (Meijers *et al.*, 2010; Gabriele *et al.*, 2016). To date, more than 100 different types of uremic toxins have been found. These uremic toxins can be divided into two major categories: 1) free, water-soluble substances that reversibly bind serum proteins and can be removed by dialysis; and 2) protein-bound solutes and peptides that are difficult to remove by conventional dialysis, so they can stay in the body for a long period of time (Neiryneck *et al.*, 2013). *p*-Cresol (PC) belongs to the second type of uremic toxins, as it binds to proteins. PC is frequently detected in the serum of CKD patients (Faure *et al.*, 2006). PC induces cell senescence, apoptosis and immunodeficiencies in the cardiovascular system, brain, and kidneys of affected patients (Azevedo *et al.*, 2016). However, effects of PC on adult stem cells in patients with CKD

Open Access <https://doi.org/10.4062/biomolther.2017.071>

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Received Mar 21, 2017 Revised Apr 22, 2017 Accepted Apr 26, 2017

Published Online Jun 27, 2017

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have not yet been elucidated.

When cells age or become damaged by external stimuli, they are replaced by new cells that differentiate from adult stem cells into corresponding tissue-specific cell types to maintain tissue stability (Krause *et al.*, 2001). Such adult stem cells are divided into various types: hematopoietic stem cells (Till and Mc, 1961), mesenchymal stem cells (Phinney and Prockop, 2007), and neural stem cells (Altman and Das, 1965). Among them, mesenchymal stem cells (MSCs) have a special self-renewal capacity with the potential to differentiate into multiple cell types, including osteoblasts, chondrocytes, and adipocytes, in multiple organs (Yun *et al.*, 2009). MSCs are distributed all over the body, including bone marrow, cord blood, and adipose tissue; they are also present in large numbers in the bloodstream (Qiao *et al.*, 2008). Owing to their ubiquitous nature, MSCs are easily exposed to uremic toxins that circulate in the blood of CKD patients. As a result, they may be vulnerable to toxin effects that can potentially complicate tissue regeneration process. Thus, it is important to evaluate possible harmful effects of uremic toxins, such as PC, on MSCs and find a way of controlling uremic toxin-induced MSC senescence.

Individuals with CKD experience disturbances of sleep, which may be linked to impaired endogenous melatonin secretion during nighttime (Maung *et al.*, 2016). In light of known tissue regeneration problems in CKD patients, it is of interest to note that high blood melatonin levels may alleviate tissue regeneration disorders and other complications caused by CKD (Maung *et al.*, 2016). Melatonin (*N*-acetyl-5-methoxy tryptamine), secreted by the pineal gland, is a hormone that regulates sleep and wakefulness (Hardeland *et al.*, 2006). Recently, melatonin has been used clinically, and it is available off the counter as a sleeping aid that has a strong action and few side effects. The vast potential of melatonin as a therapeutic agent is explained by its ubiquitous nature. Melatonin acts not only on the brain, but also on various locations in the whole body, such as pineal gland, reproductive organs, skin, lymphocytes, and gastrointestinal tract (Acuna-Castroviejo *et al.*, 2014). Specifically, melatonin is known to have many beneficial effects on the body, as it improves motility, induces proliferation of MSCs, and increases the therapeutic effect of MSC transplantation (Lee *et al.*, 2014). Thus, in our study, we sought to investigate possible effects of PC on MSCs, and consequently evaluate the potential of melatonin as a therapeutic agent with protective properties against PC-induced stem cell senescence.

MATERIALS AND METHODS

Cell culture

Human adipose tissue-derived MSCs were obtained from the American Type Culture Collection (Manassas, VA, USA). MSCs were confirmed to be pathogen- and mycoplasma-free. MSCs expressed specific cell surface markers [cluster of differentiation (CD) 73 and CD105, but not CD31]. MSCs were cultured in α -minimum essential medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μ g/mL streptomycin. MSC cultures were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cell proliferation assay

MSCs were exposed to PC (0, 50, 100, 500 μ M; Sigma-Aldrich, St. Louis, MO, USA) for 72 h or PC (500 μ M; for 72 h) with preincubation melatonin (0, 1, 10, 100 μ M; for 30 min; Sigma-Aldrich) or phosphate-buffered saline (PBS; vehicle). Cell proliferation parameters were assessed using a cell proliferation 5-bromo-2'-deoxyuridine (BrdU) ELISA colorimetric kit (Roche, Mannheim, Germany). To perform ELISA, 100 μ g/mL BrdU was added to cultured MSCs and incubated at 37°C for 6 h. Then, 1 M H₂SO₄ was added to stop the reaction. Light absorbance of the samples was measured by an ELISA reader (BMG labtech, Ortenberg, Germany) at 450 nm.

Beta-galactosidase staining assay

Senescence was assessed by measuring the percentage of cultured cells that stained positively for senescence-associated beta-galactosidase (SA- β -gal) activity as described previously (Cong *et al.*, 2006). Cells were washed with phosphate-buffered saline (PBS) twice and fixed with 2% formaldehyde/0.2% glutaraldehyde (Sigma-Aldrich). Fixed cells were incubated at 37°C (without CO₂) for 12 h with a β -gal staining solution (1 mg/mL X-Gal, 40 mM citric acid-sodium phosphate buffer, 150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide; pH 6.0; Sigma-Aldrich). Stained (blue, positive) and non-stained (negative) cells were counted by using phase contrast microscopy (Nikon, Tokyo, Japan) in five independent cultures.

Catalase activity assay

MSCs were harvested from the culture dish by scraping with a rubber policeman on ice. To measure catalase activity, cell lysate protein extracts (40 μ g) were incubated with 20 mM H₂O₂ (Sigma-Aldrich) in 0.1 M Tris-HCl (Sigma-Aldrich) for 30 min. Then, 50 mM Amplex Red reagent (Thermo Scientific, Waltham, MA, USA) and 0.2 U/mL of horseradish peroxidase (Sigma-Aldrich) was added and incubated for 30 min at 37°C. Changes in absorbance values associated with H₂O₂ degradation were measured by an ELISA reader (BMG labtech) at 563 nm.

Western blot assay

MSCs homogenates were extracted by using RIPA lysis buffer (Thermo Scientific). Cell lysates (20 μ g of total protein) were separated by using 6-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membrane (Thermo Scientific). After the blots had been washed with a solution comprising 10 mM Tris-HCl (pH 7.6; Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), and 0.05% Tween-20 (Sigma-Aldrich), the membranes were incubated with 5% bovine serum albumin for 1 h at room temperature and then, incubated with appropriate primary antibodies against phosphoinositide 3-kinase (PI3K), phosphorylated PI3K (p-PI3K), Akt, phosphorylated Akt (p-Akt), phosphorylated 5'-AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR), phosphorylated mTOR (p-mTOR), LC3, beclin 1, ATG7, p62/Sequestosome 1 (p62/SQSTM1), senescence marker protein 30 (SMP30), and β -actin (all from Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were then washed more than twice, and the primary antibodies were detected using a goat anti-rabbit IgG or a goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The bands were visualized

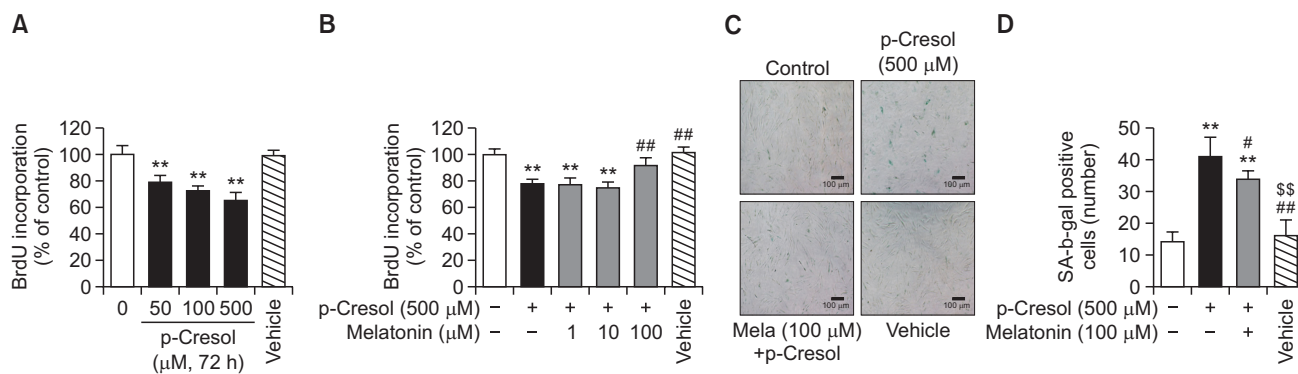


Fig. 1. Effect of melatonin on p-cresol-induced cell senescence. (A) Mesenchymal stem cells (MSCs) were exposed to 0-500 μM p-cresol (PC) or PBS (Vehicle) for 72 h, and 5-bromo-2'-deoxyuridine (BrdU) incorporation was measured by absorbance detection. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** $p < 0.01$ vs. control (untreated MSCs). (B) MSCs were cultured in the absence of modulators or were exposed to 500 μM PC for 72 h with or without pretreatment with 1-100 μM melatonin, or PBS (Vehicle), and BrdU incorporation was measured by absorbance detection. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** $p < 0.01$ vs. control (untreated MSCs); ### $p < 0.01$ vs. PC alone. (C) A representative image of senescence-associated beta-galactosidase (SA- β -gal) activity staining is illustrated. Scale bar=100 μm . (D) Numbers of SA- β -gal-positive cells in different treatment groups are illustrated. The number of SA- β -gal-positive cells was significantly lower in PC-treated MSCs exposed to melatonin than in MSCs treated with PC only. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** $p < 0.01$ vs. control (untreated MSCs); ### $p < 0.01$ vs. PC alone, \$\$\$ $p < 0.01$ vs. melatonin+PC.

by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

Dihydroethidium staining

To measure superoxide anion levels in cultured MSCs, the cells were incubated with 10 μM dihydroethidium (DHE; Sigma-Aldrich) for 30 min at 37°C. After washing with PBS three times, samples were visualized by fluorescence microscopy (Zeiss, Oberkochen, Germany) or analyzed by flow cytometry (Cyflow Cube 8 FACS; Sysmex Partec, Görlitz, Germany). Data were analyzed using standard FSC Express software (De Novo Software, Los Angeles, USA).

Statistical analysis

All data are expressed as the mean \pm standard error of the mean (SEM). Results of all experiments were analyzed by one-way analysis of variance (ANOVA). Comparisons of more than three groups were made by using the Bonferroni-Dunn test. Differences were considered to be statistically significant if $p < 0.05$.

RESULTS

Melatonin prevents the reduction in MSC proliferation rate and MSC senescence induced by PC

Exposure of MSCs to a range of PC concentrations (0-500 μM) for 72 h induced cell senescence as was indicated by decreased BrdU incorporation levels (Fig. 1A). At 500 μM PC, the lowest BrdU incorporation in MSCs was observed (Fig. 1A). It has been shown previously that treatment with 500 μM PC increased reactive oxygen species (ROS) production and inhibited cell viability, proliferation, and migration (Chang *et al.*, 2014). To investigate the protective effect of melatonin, MSCs were pretreated with 0-100 μM melatonin before the

exposure to PC for 72 h. We found that pretreatment with 100 μM melatonin reversed PC-induced decrease in BrdU incorporation (Fig. 1B), whereas incubation with lower concentrations of melatonin (1 and 10 μM) was without effect. Thus, 500 μM PC and 100 μM melatonin were used in the rest of the experiments to maximize the characteristic effects of these substances. To determine the effect of melatonin on cell senescence conferred by PC, cytochemical detection of SA- β -gal activity was performed. Melatonin was found to decrease PC-induced SA β -gal activity (Fig. 1C, 1D). These results support the notion that melatonin can counteract the reduction of MSC proliferation and induction of cell senescence caused by PC.

Melatonin inhibits PC-induced reactive oxygen species generation via PI3K/Akt-dependent catalase activation

The cytoprotective effect of melatonin is thought to be mediated via its binding to melatonin receptors and subsequent activation of various signaling pathways that promote cell survival (Lee *et al.*, 2014; Yu *et al.*, 2017). Thus, we sought to specifically examine whether melatonin affected PI3K/Akt-dependent catalase activity and inhibited ROS generation. As shown in Fig. 2A and 2B, melatonin time-dependently increased levels of phosphorylated PI3K and Akt. Based on these results, we hypothesized that activation of the Akt signal transduction pathway by melatonin could affect catalase activity and ROS generation. Indeed, melatonin increased catalase activity, and this effect was sensitive to an Akt inhibitor (Fig. 2C). Next, to establish whether melatonin could suppress ROS generation induced by PC, we incubated MSCs with DHE, a widely used small-molecule fluorescent probe specific for O_2^- (Zhao *et al.*, 2003). The reaction between O_2^- and DHE generates 2-hydroxyethidium, a highly specific red fluorescent product, that shifts excitation and emission peaks from 350 and 400 to 518 and 605 nm, respectively (Zielonka *et al.*, 2008). MSCs were pretreated with melatonin in the presence or absence of an

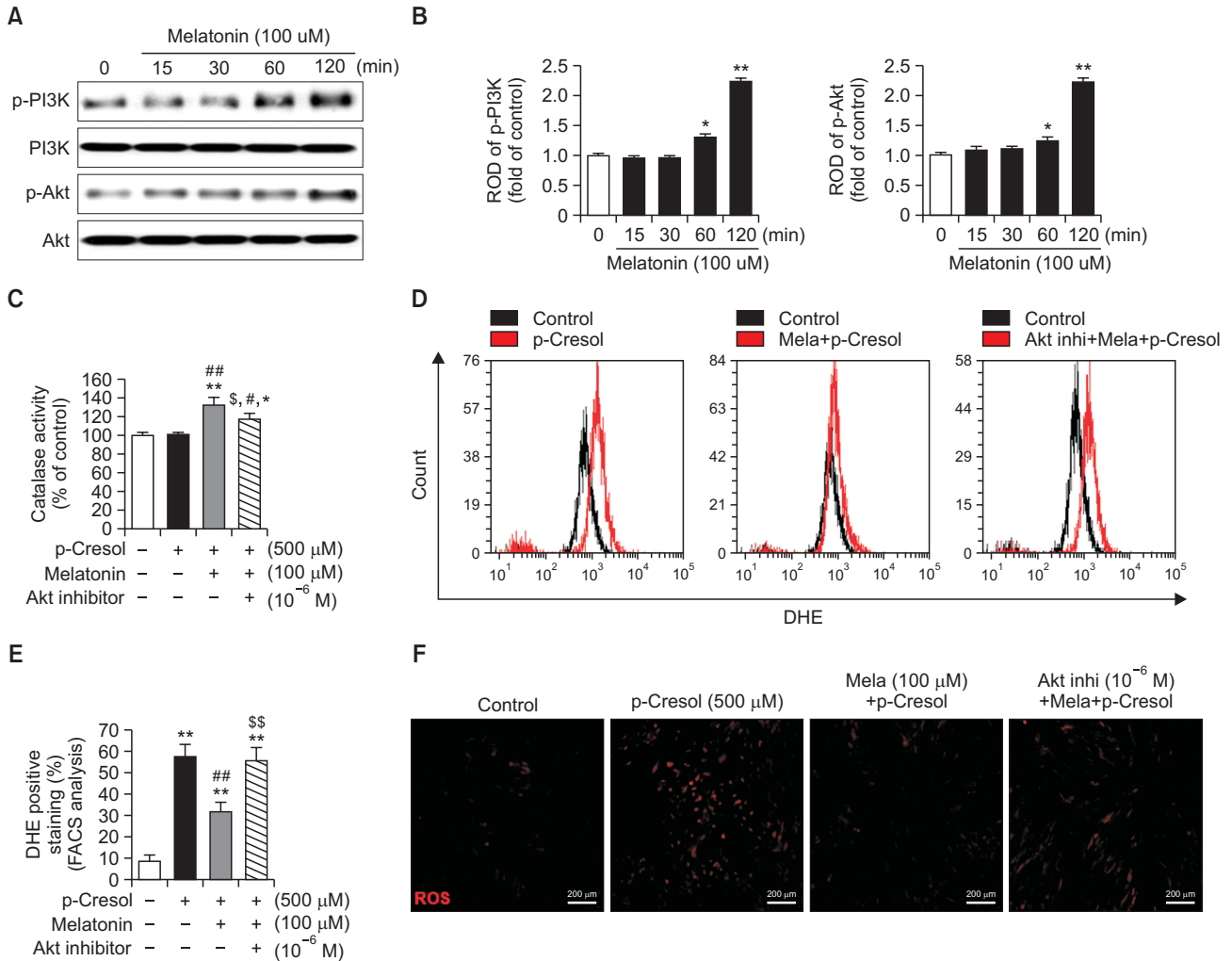


Fig. 2. Protective effect of melatonin on *p*-cresol-induced changes in phospho-PI3K and phospho-Akt, and reactive oxygen species generation via Akt pathway-dependent catalase activation. (A) Mesenchymal stem cells (MSCs) were treated with 100 μ M melatonin for 0-120 min. At 120 min post melatonin, total protein extracts were prepared and immunoblotted with primary antibodies to phospho-PI3K and phospho-Akt. For normalization of band intensities, total PI3K and total Akt amounts were used as internal loading control. (B) Mean normalized levels of phospho-PI3K and phospho-Akt in the indicated samples are illustrated. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was used for multiple group comparisons: * p <0.05, ** p <0.01 vs. control (untreated MSCs). (C) MSCs were pretreated with 1 μ M Akt inhibitor for 2 h before melatonin treatment. After pretreatment with the Akt inhibitor, MSCs were exposed to 100 μ M melatonin for 2 h, and then, incubated for 72 h with *p*-cresol (PC). Next, catalase activity assays were carried out. Catalase activity values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. ANOVA followed by the *post hoc* Bonferroni test was used for multiple group comparisons: * p <0.05, ** p <0.01 vs. control (untreated MSCs); # p <0.05, ## p <0.01 vs. PC alone; $\$$ p <0.05 vs. melatonin+PC. (D) Reactive oxygen species (ROS) generation was measured by using dihydroethidium (DHE) staining and flow cytometry. (E) Quantitative analysis of the percentage of DHE-positive cells by fluorescence-activated cell sorting analysis. ANOVA followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** p <0.01 vs. control (untreated MSCs); ## p <0.01 vs. PC alone; $\$$ p <0.05 vs. melatonin+PC. (F) Representative images of DHE fluorescence staining that indicated amounts of generated ROS in different treatment groups. Scale bar=200 μ m.

Akt inhibitor for 2 h and then exposed to PC for 12 h. Changes in DHE fluorescence excitation and emission peak intensities were quantified by using confocal microscopy and flow cytometry. We found that incubation with PC increased DHE intensity and the number of DHE-positive cells. These changes were inhibited by melatonin (Fig. 2D-2F). These results suggest that melatonin reduced PC-induced ROS generation through increased catalase activity via Akt pathway activation.

Involvement of AMPK/mTOR pathway in the protective effect of melatonin

The increase in ROS is closely related to the activity of AMP-activated protein kinase (AMPK) (Li *et al.*, 2015). AMPK regulates various cellular processes, such as cell growth, apoptosis, and autophagy by modulating mTOR activity (Li *et al.*, 2015). To investigate the involvement of AMPK and mTOR in melatonin-induced cytoprotective effect, we determined changes in AMPK expression and phosphorylation of mTOR induced by the exposure to PC or combined incubation

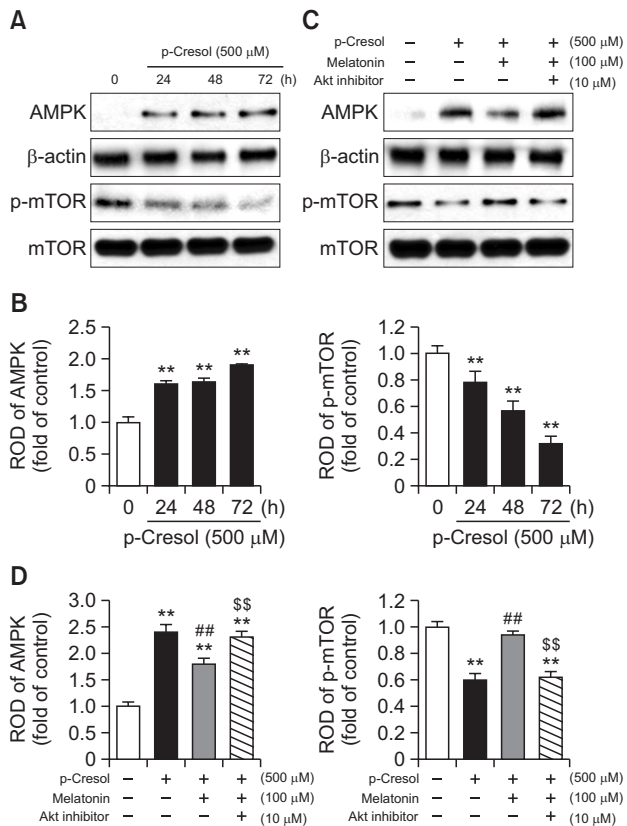


Fig. 3. Effect of melatonin on *p*-cresol-induced AMPK expression and reduction of mTOR phosphorylation. (A) Mesenchymal stem cells (MSCs) were treated with 500 μ M *p*-cresol (PC) for 0–72 h. At 72 h after treatment with PC, total protein was extracted and immunoblotted with primary antibodies against total AMPK and phosphorylated mTOR. For normalization of band intensities, β -actin and total mTOR were used as internal loading control. (B) Mean normalized levels of AMPK and phospho-mTOR in the samples shown in (A) are illustrated. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** p <0.01 vs. control (untreated MSCs). (C) MSCs were pretreated with 1 μ M Akt inhibitor for 2 h before the treatment with 500 μ M for 2 h, and then incubated for 72 h with PC. Total protein extracts were then prepared and immunoblotted with primary antibodies against total AMPK and phosphorylated mTOR. For normalization of band intensities, β -actin and total mTOR were used as internal loading control. (D) Mean normalized levels of AMPK and phospho-mTOR levels in the samples shown in (C) are illustrated. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. ANOVA followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** p <0.01 vs. control (untreated MSCs); ## p <0.01 vs. PC alone; \$\$ p <0.05 vs. melatonin+PC.

of PC with melatonin. We observed that 500 μ M PC increased total AMPK expression (Fig. 3A, 3B), but decreased mTOR phosphorylation (Fig. 3A, 3B) in MSCs in a time-dependent manner. Melatonin completely reversed these effects of PC, whereas pretreatment with an Akt inhibitor, in turn, counteracted the changes caused by melatonin (Fig. 3C, 3D). These results suggest that the protective effect of melatonin on PC-induced MSC senescence is mediated by AMPK and mTOR signaling pathways.

Melatonin inhibits PC-induced autophagy via Akt signaling

Excessive autophagy is associated with the inhibition of cell proliferation and increase in the number of senescent cells (Yang *et al.*, 2011). To examine the effects of melatonin on PC-induced autophagy, we first determined how PC modulated the expression of autophagy-related proteins, such as LC3, beclin 1, ATG7, and p62/SQSTM1, which are key molecules in autophagosome formation. PC time-dependently increased the expression levels of LC3, beclin 1, and ATG7, but decreased the expression of p62/SQSTM1 (Fig. 4A, 4B). Correspondingly, pretreatment with melatonin counteracted PC-induced changes in LC3, beclin 1, and ATG7 expression levels, and restored p62/SQSTM1 expression reduced by PC (Fig. 4C, 4D). Finally, as in the experiments on the modulation of total AMPK and *p*-mTOR levels, co-application of an Akt inhibitor neutralized the effects of melatonin (Fig. 4C, 4D). Taken together, these results suggest that melatonin potentially affects expression levels of autophagy-related proteins via activation of the Akt pathway, ultimately preventing the induction of autophagy by PC.

Protective effect of melatonin on PC-induced MSC senescence

Expression of senescence marker protein 30 (SMP30) decreases with age (Maruyama *et al.*, 2010). In our experiments, PC decreased SMP30 expression in a time-dependent manner (Fig. 5A), which was in line with other senescence-related changes induced by PC. We found that pretreatment with melatonin rescued the reduction of SMP30 caused by PC, whereas protective effects of melatonin were, in turn, sensitive to the Akt inhibitor (Fig. 5B). As was shown above (Fig. 1C, 1D), melatonin prevented pro-senescence effects of PC. That effect of melatonin was also sensitive to the inhibition of the Akt pathway (Fig. 5C, 5D). These results suggest that melatonin diminished PC-induced reduction in the expression level of SMP30, which is involved in cell growth and anti-aging processes. This activity of melatonin was another manifestation of its potent restoring effects against PC-induced cell senescence.

DISCUSSION

In our study, we for the first time demonstrated that the uremic toxin PC, found in individuals with CKD, caused MSC senescence. Furthermore, we demonstrated that pretreatment with melatonin prevented PC-induced senescence of MSCs by activating the PI3K and Akt pathways and by inhibiting ROS-dependent, AMPK pathway-mediated autophagy.

PC, a metabolite of environmental toxins, is a uremic toxic solute normally excreted by the kidneys. In conditions such as CKD, PC accumulates in the body (D'Hooge *et al.*, 2003). In CKD, uremic toxins chronically stimulate renal endothelial cells. PC decreases the expression of endothelial adhesion molecules of endothelial cells and promotes monocyte adhesion to endothelial cells, thereby increasing endothelial monolayer permeability and inflammation (Dou *et al.*, 2002; Cerini *et al.*, 2004). Actual concentration of PC and the mechanism of its formation in several kidney diseases, including glomerulopathy, tubulointerstitial nephropathy, and acute kidney injury, still remain unclear. PC has been shown to activate caspase

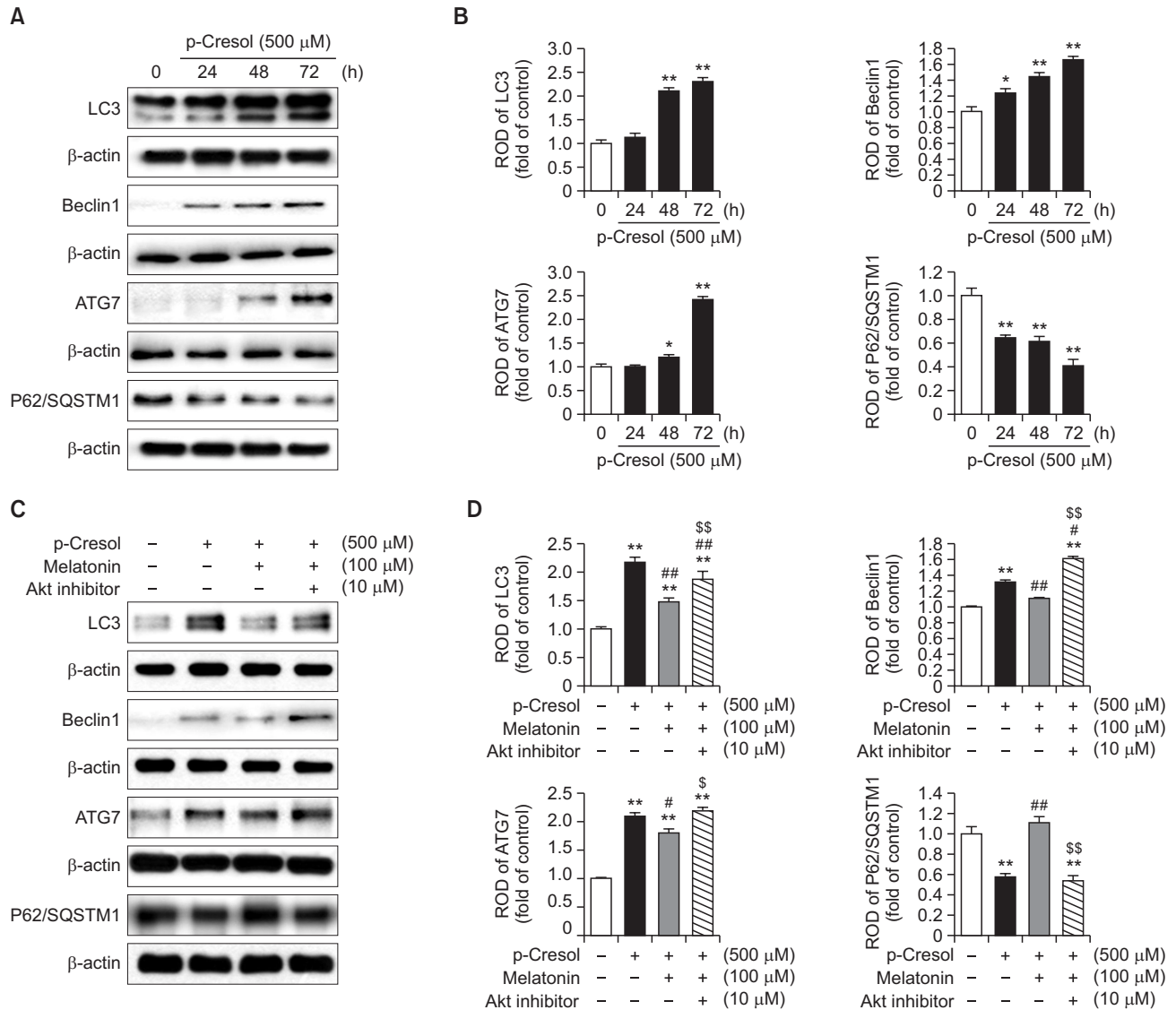


Fig. 4. Effect of melatonin on *p*-cresol-induced autophagy. (A) Mesenchymal stem cells (MSCs) were treated with 500 μM *p*-cresol (PC) for 0-72 h. At 72 h after treatment with PC, total protein was extracted and immunoblotted with primary antibodies against LC3, beclin 1, ATG7, and p62/SQSTM1. For normalization of band intensities, β-actin was used as internal loading control. (B) Mean normalized levels of LC3, beclin 1, ATG7, and p62/SQSTM1 in the samples shown in (A) are illustrated. The values are reported as the mean ± SEM of three independent experiments with triplicate dishes. Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was used for multiple group comparisons: **p*<0.05, ***p*<0.01 vs. control (untreated MSCs). (C) MSCs were pretreated with 1 μM Akt inhibitor for 2 h before the treatment with 500 μM melatonin for 2 h and then incubated for 72 h with PC. Total protein extracts were prepared and immunoblotted with primary antibodies against LC3, beclin 1, ATG7, and p62/SQSTM1. β-actin was used as an internal loading control. (D) Mean normalized levels of LC3, beclin 1, ATG7, and p62/SQSTM1 levels in the samples shown in (C) are illustrated. The values are reported as the mean ± SEM of three independent experiments with triplicate dishes. ANOVA followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ***p*<0.01 vs. control (untreated MSCs); #*p*<0.05, ##*p*<0.01 vs. PC alone; \$*p*<0.05 \$\$*p*<0.01 vs. melatonin+PC.

signaling and induce apoptosis and necrosis of renal epithelial tubular cells, producing a characteristic phenotype of CDK-related damage (Brocca *et al.*, 2013). In addition, PC induces renal proximal tubular cell death by triggering autophagy and apoptosis (Lin *et al.*, 2015). When not excreted properly, uremic toxic solutes adversely affect not only the kidneys, which are their primary target, but also other organs and systems (Meijers *et al.*, 2010; Gabriele *et al.*, 2016). Oxidative stress, DNA/mitochondrial damage, apoptosis, and the number of senescent cells were reported to increase in cardiomyocytes

of mice with modeled cardiorenal syndrome *in vivo* (Chua *et al.*, 2016). In addition, treatment of H9c2 cardiomyocyte cells with PC resulted in a decrease in mitochondrial ATP levels and enhanced senescence (Chua *et al.*, 2016). With regard to physiological functions of stem cells, it has been shown that insulin promoted the differentiation of bone-marrow derived mouse MSCs into target cells *in vivo* by upregulating expression levels of HIF-1α, VEGF, and stromal cell-derived factor 1α via the PI3K/Akt-dependent pathway (Noh *et al.*, 2014). PC has been previously shown to inhibit this signaling pathway

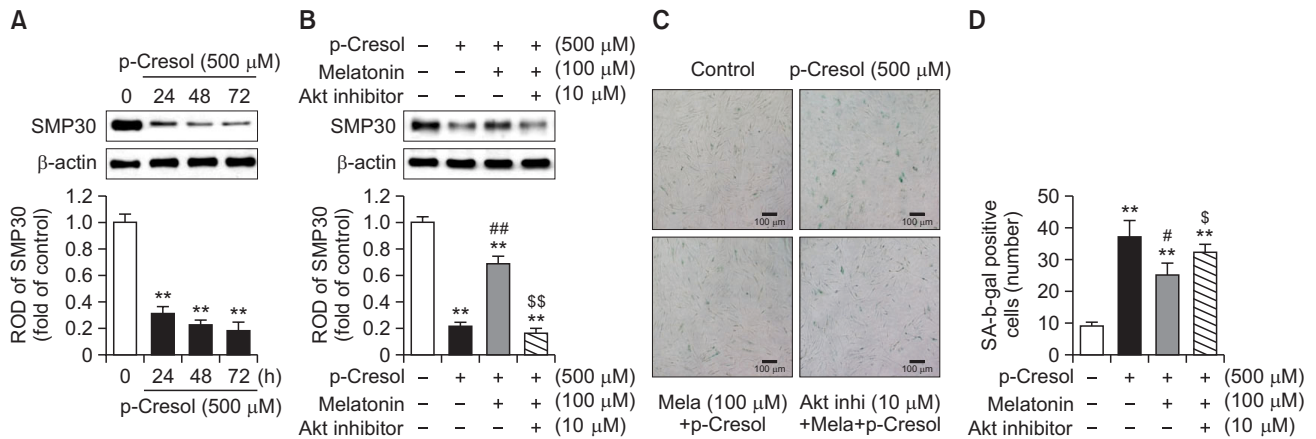


Fig. 5. Effect of melatonin on *p*-cresol-induced reduction of SMP30 expression. (A) Mesenchymal stem cells (MSCs) were treated with 500 μ M *p*-Cresol (PC) for 0-72 h. Upper panel – at 72 h after the treatment with PC, total protein extracts were prepared and immunoblotted with a primary antibody against SMP30. For normalization of band intensities, β -actin was used as internal loading control. Lower panel – mean normalized levels of SMP30 in samples shown in the upper panel are illustrated. The values are reported as mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** p <0.01 vs. control (untreated MSCs). (B) MSCs were pretreated with 1 μ M Akt inhibitor for 2 h before the treatment with 500 μ M melatonin and subsequently incubated with PC for 72 h. Upper panel – total protein extracts were prepared and immunoblotted with an anti-SMP30 antibody. For normalization of band intensities, β -actin was used as internal loading control. Lower panel – mean normalized levels of SMP30 in the samples shown in the upper panel are illustrated. The values are reported as mean \pm SEM of three independent experiments with triplicate dishes. ANOVA followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** p <0.01 vs. control (untreated MSCs); ## p <0.01 vs. PC alone; \$\$ p <0.01 vs. melatonin+PC. (C) A representative image of senescence-associated beta-galactosidase (SA- β -gal) activity staining. Scale bar = 100 μ m. (D) Numbers of SA- β -gal-positive cells in different treatment groups are illustrated. The values are reported as the mean \pm SEM of three independent experiments with triplicate dishes. Analysis of variance followed by the *post hoc* Bonferroni test was used for multiple group comparisons: ** p <0.01 vs. control (untreated MSCs); # p <0.05 vs. PC alone; \$ p <0.05 vs. melatonin+PC.

and suppress mouse MSC differentiation (Noh *et al.*, 2014). Thus, we initiated our study assuming that PC effect on mouse cells could be translated to a human model, so that normal physiological functions, such as proliferation and senescence, would be similarly affected in human MSCs. Indeed, our results showed that incubation with PC inhibited BrdU incorporation and increased the number of β -galactosidase-positive cells (Fig. 1), indicating inhibition of proliferation and induction of senescence. In an attempt to prevent MSC senescence induced by PC, we chose to pretreat MSCs with melatonin, which is an easily applied, inexpensively synthesized, natural protector with few side effects. Melatonin is an endogenous hormone secreted by the pineal gland (Hardeland *et al.*, 2006). Its physiological blood concentrations are maintained within pM or low nM range (Reiter and Tan, 2003). In some tissues, melatonin is present at considerably higher concentrations because of tissue specificity (Reiter and Tan, 2003), and it has a precise concentration-dependent effect on stem cells. Specifically, it has been reported that at low concentrations, proliferation, differentiation, and survival of stem cells was increased by melatonin (Fu *et al.*, 2011), whereas at higher melatonin concentrations, stem cell pluripotency became notably lower (Zhang *et al.*, 2010). Because of this variable concentration-dependent effect on target cells, it was extremely important to find a precise concentration of melatonin that could prevent PC-induced senescence. In our study, we revealed that 100 μ M melatonin restored MSC proliferation reduced by PC and partially counteracted the increase in the number of β -galactosidase positive cells, whereas at concentrations of 1 μ M and 10 μ M, melatonin did not have any significant effect (Fig. 1). Thus, we concluded 100 μ M was the optimal concen-

tration of melatonin for our experiments that sought to address possible signaling mechanisms of the protection against PC-induced MSC senescence.

Previous studies suggested that PC induced oxidative stress in CKD patients by activating catalase and NADPH oxidase (Watanabe *et al.*, 2013; Chang *et al.*, 2014), leading to cell cycle arrest and cytotoxicity. Melatonin, in turn, has been ascribed anti-oxidant properties and demonstrated to alleviate cell damage (Yu *et al.*, 2017) via activation of pro-survival and proliferation-related signaling, such the Akt pathway (Mehrzadi *et al.*, 2016). Consistent with these findings, we found that catalase activity and ROS generation were significantly increased in MSCs exposed to PC and that pretreatment with melatonin prevented these changes. In addition, pretreatment with an Akt inhibitor blocked melatonin rescuing effect against PC action, suggesting that melatonin-induced PI3K/Akt pathway activity is essential in the prevention of PC-induced catalase activation, ROS generation, and, ultimately, MSC senescence. Nevertheless, low concentrations of ROS are known to be effective for cell protection and intracellular debris elimination (Diehn *et al.*, 2009; Park *et al.*, 2016a). In addition, we found that melatonin counteracted increased expression of total AMPK and reduced phosphorylation of mTOR induced by PC (Fig. 3). As in the experiments that examined catalase activity, inhibition of Akt neutralized melatonin inhibitory effects on PC-induced changes in the expression of AMPK and phosphorylated mTOR (Fig. 3C, 3D). Consistent with our results, ROS-mediated AMPK up-regulation has been shown to inhibit mTOR phosphorylation, and to suppress stem cell differentiation and self-renewal (Adams *et al.*, 2016; Riva *et al.*, 2016). In addition, evidence suggests that the AMPK/mTOR

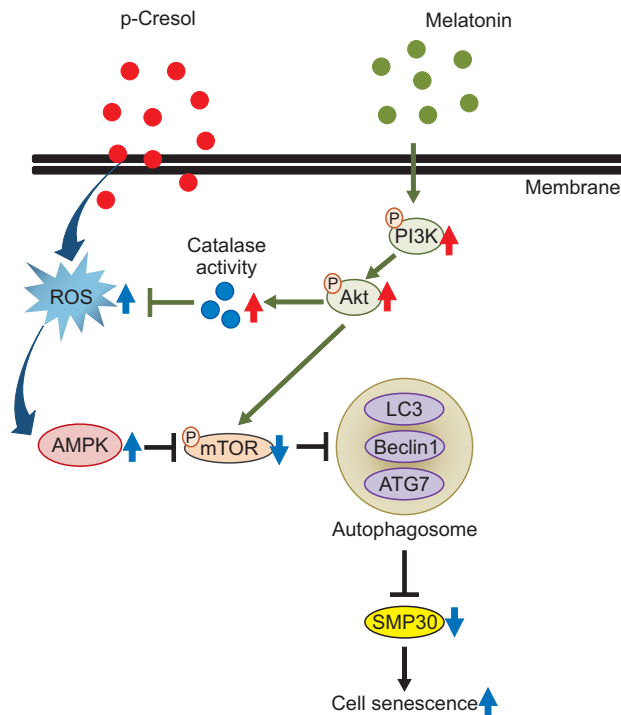


Fig. 6. Hypothetical model for protection effect of melatonin in PC-induced MSC senescence. PC induced MSC senescence through ROS generation, which elicited the AMPK expression but decreased mTOR phosphorylation level. Subsequently, PC decreased SMP30 expression via upregulation of mTOR dependent autophagy. Melatonin blocked the autophagy activation through Akt mediated catalase activation and scavenging the PC-induced mTOR mediated autophagy.

signaling axis is important for autophagy-mediated cell senescence and aging (Michalik and Jarzyna, 2016; Ganesan *et al.*, 2017). Despite that knowledge, PC has not been previously shown to modulate AMPK expression through the generation of ROS, and the possibility that this signaling can regulate mTOR phosphorylation has not been elucidated. Therefore, our results provide a new perspective on the mechanism of cell senescence induction by PC, implicating AMPK/mTOR signaling in this effect. Moreover, we showed that effects of PC on MSCs could be ameliorated by melatonin in an Akt pathway-dependent manner.

Next, we attempted to uncover consequences of mTOR dysregulation for other cellular responses that are important in cell senescence. PC has been shown to induce autophagy in human renal proximal tubular cells (Lin *et al.*, 2015), whereas mTOR signaling is known to regulate autophagy during stem cell senescence and disease (Menendez *et al.*, 2011; Liang *et al.*, 2016; Maiese, 2016). However, whether PC can induce autophagy in MSCs and whether melatonin can prevent that effect, has not been clearly elucidated. It has been shown that PC could participate in autophagosome formation and regulate expression of autophagy-related proteins, such as ATG4, beclin 1, and LC3B-II, ultimately increasing autophagy response (Lin *et al.*, 2015). In contrast, melatonin directly inhibited mTOR-dependent autophagy and ER stress-dependent autophagy, preventing autophagic cell death and senescence (Kang *et al.*, 2014; Fernandez *et al.*, 2015). In the present

study, we observed that PC increased the expression levels of the autophagy marker proteins LC3, beclin-1, and ATG7 in MSCs, but decreased the expression of p62/SQSTM1. In addition, we found that preincubation with melatonin partially or completely restored changes in the expression of autophagy-related proteins altered by PC. Thus, it is plausible that the protective effects of melatonin are partially mediated via the suppression of PC-induced autophagy. Furthermore, we found that pretreatment with an Akt inhibitor prevented the effect of melatonin, which highlighted the critical nature of Akt activation in downstream melatonin signaling. Melatonin has been previously characterized as an autophagy inhibitor that down-regulates expression levels of autophagic proteins in myoblast and glioma cells (Kim *et al.*, 2011, 2012; Martin *et al.*, 2014). Therefore, we suggest that melatonin reduces autophagy and prevents MSC senescence induced by PC via activation of the Akt pathway. In support of this notion, we demonstrated that melatonin affected the expression of SMP30 (Fig. 5). SMP30 expression levels decrease with age and therefore, it is a critical marker of biological maturation and premature aging caused by pathological processes (Abbas *et al.*, 2017; Lesniewski *et al.*, 2017). In our experiments, PC-treated MSCs showed increased numbers of SA- β -gal-positive cells and lower levels of SMP30 expression (Fig. 5). Pretreatment with melatonin suppressed these changes, by decreasing the number of SA- β -gal-positive cells and increasing SMP30 levels (Fig. 5B-5D). Although our results do not directly provide a link between autophagy and SMP30 levels, a previous report showed that SMP30 expression was reduced by autophagy (Park *et al.*, 2016b). Thus, our results strongly support the notion that melatonin-dependent activation of the Akt pathway counteracts PC-induced ROS/AMPK pathway-mediated autophagy and reduction in SMP30 expression, ultimately ameliorating MSC senescence caused by PC.

Overall, our experimental results highlight a considerable therapeutic potential of melatonin anti-autophagic effects that reverse PC-induced MSC senescence. By showing that the protective action of melatonin against PC-induced MSC senescence is sensitive to the incubation with an Akt inhibitor, we demonstrated key significance of the Akt signaling pathway activation in melatonin effects. Therefore, our findings reveal a novel role of melatonin and Akt pathway in autophagy and in MSC senescence associated with CKD. Although our study was performed *in vitro*, the results obtained suggest a way to rescue developing MSC senescence in patients with CKD. Further studies are required to examine the therapeutic effects of melatonin in detail by using a mouse model of CKD.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

This work was supported by National Research Foundation grant funded by the Korean government (NRF-2016R1D-1A3B01007727, NRF-2017M3A9B4032528). The funders had no role in the study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

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