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Melatonin mitigates the adverse effect of hypoxia during myocardial differentiation in mouse embryonic stem cells

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ABSTRACT

Background: Hypoxia causes oxidative stress and affects cardiovascular function and the programming of cardiovascular disease. Melatonin promotes antioxidant enzymes such as superoxide dismutase, glutathione reductase, glutathione peroxidase, and catalase.
Objectives: This study aims to investigate the correlation between melatonin and hypoxia induction in cardiomyocytes differentiation.
Methods: Mouse embryonic stem cells (mESCs) were induced to myocardial differentiation. To demonstrate the influence of melatonin under hypoxia, mESC was pretreated with melatonin and then cultured in hypoxic condition. The cardiac beating ratio of the mESC-derived cardiomyocytes, mRNA and protein expression levels were investigated.
Results: Under hypoxic condition, the mRNA expression of cardiac-lineage markers (*Brachyury*, *Tbx20*, and *cTn1*) and melatonin receptor (*Mtnr1a*) was reduced. The mRNA expression of *cTn1* and the beating ratio of mESCs increased when melatonin was treated simultaneously with hypoxia, compared to when only exposed to hypoxia. Hypoxia-inducible factor (HIF)-1α protein decreased with melatonin treatment under hypoxia, and *Mtnr1a* mRNA expression increased. When the cells were exposed to hypoxia with melatonin treatment, the protein expressions of phospho-extracellular signal-related kinase (p-ERK) and Bcl-2-associated X proteins (Bax) decreased, however, the levels of phospho-protein kinase B (p-Akt), phosphatidylinositol 3-kinase (PI3K), B-cell lymphoma 2 (Bcl-2) proteins, and antioxidant enzymes including Cu/Zn-SOD, Mn-SOD, and catalase were increased. Competitive melatonin receptor antagonist luzindole blocked the melatonin-induced effects.
Conclusions: This study demonstrates that hypoxia inhibits cardiomyocytes differentiation and melatonin partially mitigates the adverse effect of hypoxia in myocardial differentiation by regulating apoptosis and oxidative stress through the p-AKT and PI3K pathway.

Keywords: Melatonin; hypoxia; cardiomyocytes; mESCs; Apoptosis

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Conflict of Interests

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Lee JH; Data curation: Lee JH, Yoo YM, Jeong S; Formal analysis: Lee JH; Funding acquisition: Jeung EB; Investigation: Yoo YM, Tran DN; Methodology: Yoo YM, Tran DN; Project administration: Tran DN, Jeung EB; Resources: Lee B; Supervision: Jeung EB; Validation: Tran DN; Visualization: Lee B; Writing - original draft: Lee JH; Writing - review & editing: Lee B.

INTRODUCTION

Abnormal heart development and abnormal cardiovascular processes in the fetus can lead to congenital heart disease [1,2]. Studies have shown that chronic hypoxia in fetal stage can cause heart failure in the fetus and increase cardiovascular disease [3-5]. Therefore, early cardiac development of the fetus and the process of cardiovascular formation are significant.

Oxygen is an essential component of cardiac viability and function (heart contraction) [6]. Lack of oxygen can cause cardiac dysfunction (heart failure) or death, so a proper oxygen supply level is necessary. In preeclampsia patient, which is the most common consequence of complex pregnancy, as blood supply to the placenta reduces, the oxygen supplement to the fetus is affected, leading to abnormal growth [7]. Hypoxia is known to associate with myocardial ischemia and cause oxidative damage on cells by changing the redox balance. Hypoxia alters the cytochrome chain activity responsible for mitochondrial oxidative phosphorylation, increasing reactive oxygen species (ROS) production and resulting in improper oxidation of cytoplasm and nucleus, leading to apoptosis, necrosis [8,9].

Embryonic stem cells (ESCs) have pluripotency and differentiate into specific cell types; mouse ESCs can be differentiated into cardiomyocytes *in vitro* [10,11]. The growth of the heart occurs due to the division of cardiomyocytes during the fetal stage. Stem cells have been used in myocardial differentiation studies [10,12].

The pineal gland produces melatonin, a powerful antioxidant that directly removes radicals of cells [13-15]. Melatonin has been known to have a protective effect in the growth and fetal cardiovascular function of the fetus, but it is unclear whether the antioxidant action of melatonin directly protects the heart and circulation functions of the fetus [16,17]. With its antioxidant activity, melatonin can be used to treat cardiovascular diseases [18]. Moreover, melatonin is efficient in limiting the loss of vital cardiac tissue due to abnormal cardiac physiology or ischemia/reperfusion injury; it helps correct heart failure by reducing cardiac hypertrophy [19].

Previous studies have shown that both melatonin and hypoxia are associated with remodeling of the heart [20]. However, during the early stage of myocardial differentiation, the effects of melatonin have not been fully described. Thus, the present study aimed to investigate: i) the effects of hypoxia on the early stages of cardiomyocytes differentiation and ii) the effects of melatonin pretreatment on the hypoxia-induced effects on cardiomyocytes differentiation.

MATERIALS AND METHODS

Mouse embryonic stem cell (mESC) culture

mESCs (ES-E14TG2a) were purchased from the American Type Culture Collection (USA). mESCs were cultured on mitomycin C-treated mouse embryonic fibroblasts (mEFs) at humidified culture incubator (37°C with 5% CO_2). The growth medium was prepared as in previously described [10].

Differentiation into cardiomyocytes

The mESCs were suspended without mouse leukemia inhibitory factor (mLIF) in the differentiation medium containing 15% fetal bovine serum (FBS). Aside from mLIF and 15%

FBS, other ingredients were the same as the growth medium. The mouse embryo bodies (mEBs) were formed (25 μL, 800 cells/drop, 84 drops per plate) on the lid of a Petri dish (SPL Inc., Korea). PBS was added on the bottom of the Petri dish to avoid evaporation of differentiation medium; then the top plate hanging the EBs was inverted and then cultured. After 3 days, mEBs formed on the lid were transferred to an uncoated Petri dish containing 6 mL of differentiation medium. After one day of suspension, the EBs (6–7 EBs per well) were transplanted into a 6-well plate containing 2 mL of differentiation medium.

Hypoxia induction and melatonin and/or luzindole treatment

Differentiation into cardiomyocytes was induced under normoxic (95% O_2, 5% CO_2) and hypoxic conditions (94% N_2, 1% O_2, 5% CO_2) as described in **Fig. 1**. To confirm the effects of melatonin (Sigma-Aldrich, USA) and/or luzindole, melatonin receptor antagonist, (20 μM) (Sigma-Aldrich), it was treated before inducing hypoxia as shown in **Fig. 1B**.

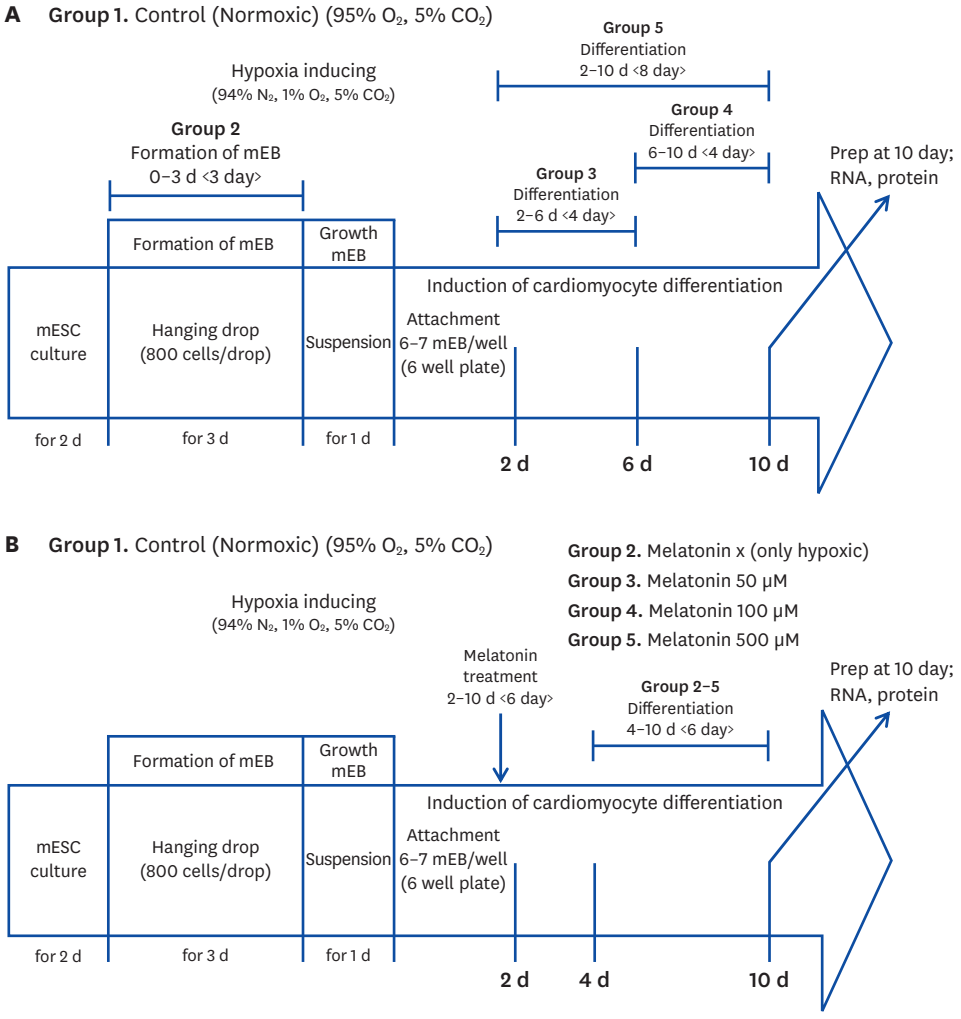

Fig. 1. Schematic diagram of cardiomyocytes differentiation model used to confirm the effects of hypoxic-stress and melatonin. (A) The effects of hypoxia on the early stages of cardiomyocytes differentiation and (B) the action of melatonin treatment on cardiomyocytes differentiation in hypoxic condition.
mEB, mouse embryo body; mESC, mouse embryonic stem cell.

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Table 1. Primer sequences used for real-time qPCR

Gene	Primer sequence (5′→3′)	Accession number
Brachyury	F: TATGAGCCTCGAATCCACATAGT R: CCTCGTTCTGATAAGCAGTCAC	NM 009309.2
Tbx20	F: AAGAAAGACCACACGGCCTC R: GTTCTTCAAAGGTCCCCTCTCA	NM_001205085.1
cTn1	F: AGCCACACGCCAAGAAAAAGT R: CGTGAAGCTGTCGGCATAAG	NM_009406.3
Mtnr1a	F: TGTCAGCGAGCTGCTCAATG R: GGTACACAGACAGGATGACCA	NM_008639
Gapdh	F: AAGGTCATCCCAGAGCTGAA R: AGGAGACAACCTGGTCCTCA	NM_017008.4

qPCR, quantitative polymerase chain reaction; F, forward; R, reverse.

Assessment of beating ratio in mESCs-derived cardiomyocytes

Contraction of mESCs that had differentiated into cardiomyocytes was observed manually via phase-contrast microscopy. The beating ratio was measured by the number of contracted cells relative to the total number of attached embryoid bodies, expressed as a percentage.

Total RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was collected from the cells using TRI reagent, then 1 μg of total RNA was transcribed to produce first-strand cDNA. The real-time qPCR was performed and the fluorescence intensity was measured. The material information and processes for PCR were already described in our previous study [10]. The primer sequences used are shown in **Table 1**. The expression level of each gene is normalized by that of *Gapdh*.

Western blotting analysis

Proteins were extracted using RIPA buffer. Then, protein concentration was measured at 562 nm using a BCA assay. Fifty microgram of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then incubated with primary antibodies for 1 h at room temperature and then overnight at 4°C: hypoxia-inducible factor (HIF)-1α (#14179, 1:1,000 dilution, Cell Signaling Technology, USA), HIF-2α (#7096, 1:1,000 dilution, Cell Signaling Technology), phospho-extracellular signal-related kinase (p-ERK; sc-7383, 1:500 dilution, Santa Cruz Biotechnology), ERK(sc-93, 1:500 dilution, Santa Cruz Biotechnology), phospho-protein kinase B (p-AKT; #4051, 1:1,000 dilution, Cell Signaling Technology), AKT (sc-8321, 1:500 dilution, Santa Cruz Biotechnology), B-cell lymphoma 2 (Bcl-2; sc-7382, 1:500, Santa Cruz Biotechnology), Bcl-2-associated X proteins (Bax; sc-7480, 1:500, Santa Cruz Biotechnology), HSP70 (1:1,000, Cell Signaling Technology), Cu/Zn-SOD (1:1,000, Cell Signaling Technology), Mn-SOD (1:1,000, Cell Signaling Technology), Catalase (1:1,000, Cell Signaling Technology), and GAPDH, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-25778, 1:500 dilution, Santa Cruz Biotechnology). Membranes were incubated with anti-rabbit IgG (#7074, 1:1,000, Cell Signaling Technology) or anti-mouse IgG (#7076, 1:1,000, Cell Signaling Technology)-conjugated horseradish peroxidase secondary antibodies. Other materials and processes were already described in our previous study [10].

Statistical analyses

Significant differences were detected by using analysis of variance. The analyses were performed with the Graph Pad Prism (v.5.0, Graph Pad Software, USA). Each value is

expressed as means ± SD of three separate experiments at least, and a representative result is depicted in the figures. The p values < 0.05 were considered statistically significant.

RESULTS

Hypoxia-induced inhibition on myocardial differentiation

To determine the effect of hypoxia on mESCs, hypoxic states were induced during various differentiation periods. The mRNA expression of mesodermal markers, including *Brachyury* and cardiac-specific markers *Tbx20* and *cTn1,* were investigated with real-time qPCR (**Fig. 2**). When hypoxia was induced during differentiation at 6–10 days and 2–10 days, *Brachyury* mRNA expression was reduced (**Fig. 2A**). *Tbx20* mRNA expression was decreased when hypoxia was induced in differentiation at 6-10 days (**Fig. 2B**). *cTn1* mRNA expression was reduced in all groups, causing hypoxia (**Fig. 2C**). These results show that hypoxia can inhibit cardiogenesis of mESCs.

Hypoxic environment was induced during myocardial differentiation of mESCs; the expression level of HIF-1α, a hypoxia marker, in mECSs was shifted corresponding with the induction of hypoxia. In **Fig. 3A**, the upregulated protein expression of HIF-1α indicated that the mESCs were properly exposed to hypoxia during each differential stages. To identify the effect of melatonin during hypoxic induction, melatonin receptor *Mtnr1a* was investigated in transcriptional level. Under normal oxygen conditions, the expression of *Mtnr1a* mRNA increased during the differentiation of mESCs into cardiomyocytes (**Supplementary Fig. 1**). However, at 6–10 days and 2–10 days of mESC differentiation under hypoxic conditions, *Mtnr1a* mRNA expression was reduced (**Fig. 3B**). These results indicate that hypoxic conditions can influence *Mtnr1a* expression in mESCs during their differentiation into cardiomyocytes.

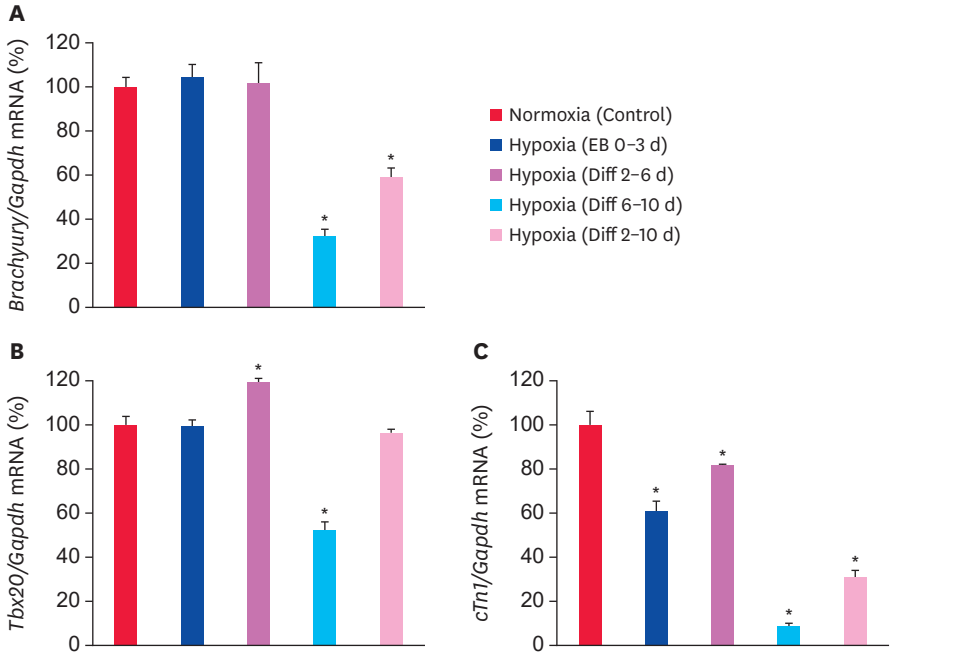

Fig. 2. Confirmation of cardiac marker expression. mRNA transcription of (A) mesodermal marker (*Brachyury*), (B) cardiac progenitor marker (*Tbx20*), and (C) cardiac-specific marker (*cTn1*) on the period of hypoxia treatment. mRNAs were measured by real-time qPCR and standardized to that of *Gapdh*.
*p < 0.05 versus Normoxia (Control).

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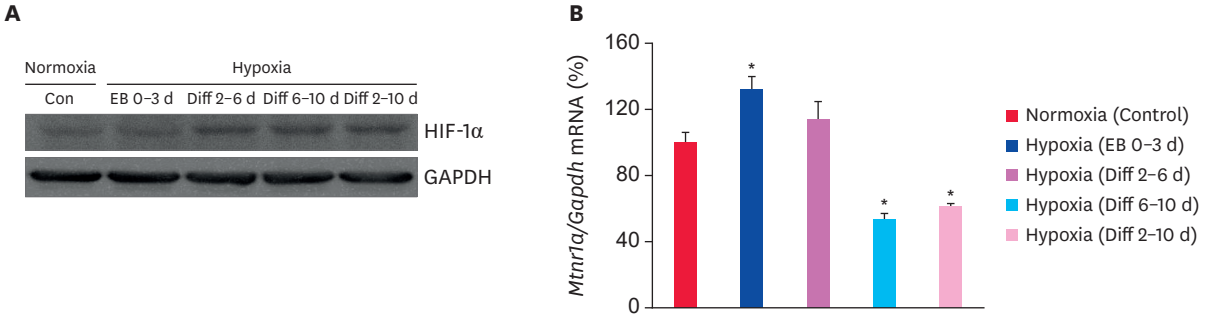

Fig. 3. Hypoxia-induced shift in the mRNA expression of melatonin receptor during cardiomyocytes differentiation. (A) Effect of hypoxia on the expression of HIF-1α proteins by western blotting. (B) Effect of hypoxia on the transcriptional level of *Mtnr1a* (Melatonin receptor). Data was obtained by real-time qPCR and normalized by *Gapdh*.
HIF, hypoxia-inducible factor; qPCR, quantitative polymerase chain reaction; Con, control; EB, embryo body; Diff, differentiation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
*$p < 0.05$ versus Normoxia (Control).

Effect of melatonin pretreatment on cardiomyocytes suppressed by hypoxia

The effects of melatonin on hypoxia-induced cardiomyocytes were examined (**Fig. 4**). Melatonin (50, 100, and 500 μM) was added to the differentiation medium two days before hypoxia induction and cells were continuously maintained in the melatonin-treated media except for the control group. We preemptively performed cell counting kit-8 (CCK-8) assay to screen the potential hazard of our experimental design for the melatonin treatment; from 50 to 500 μM, melatonin per se did not exert inhibition on cell viability in mESCs (**Supplementary Fig. 2**). In all hypoxia-induced groups, the beating ratio of differentiated cardiomyocytes was reduced but recovered when 500 μM of melatonin had been treated before hypoxia induction (**Fig. 4A**). The expression of cardiac progenitor marker *Tbx20* was decreased in all hypoxia induction groups, and there was no significant change in *Tbx20*

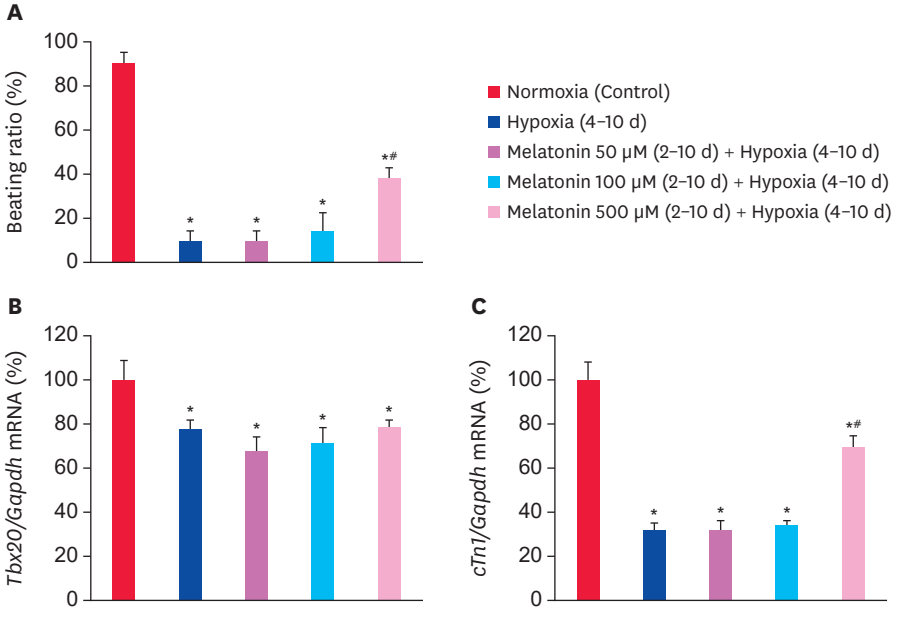

Fig. 4. Effect of melatonin and hypoxia on the differentiation of mESCs-derived cardiomyocytes. (A) Effect of melatonin and hypoxia on beating ratio. Expression levels of (B) cardiac progenitor marker *Tbx20*, (C) cardiac-specific marker *cTn1*. Each value was measured by real-time qPCR and normalized by *Gapdh*.
mESCs, mouse embryonic stem cells; qPCR, quantitative polymerase chain reaction.
*$p < 0.05$ versus Normoxia (Control). #$p < 0.05$ versus Hypoxia.

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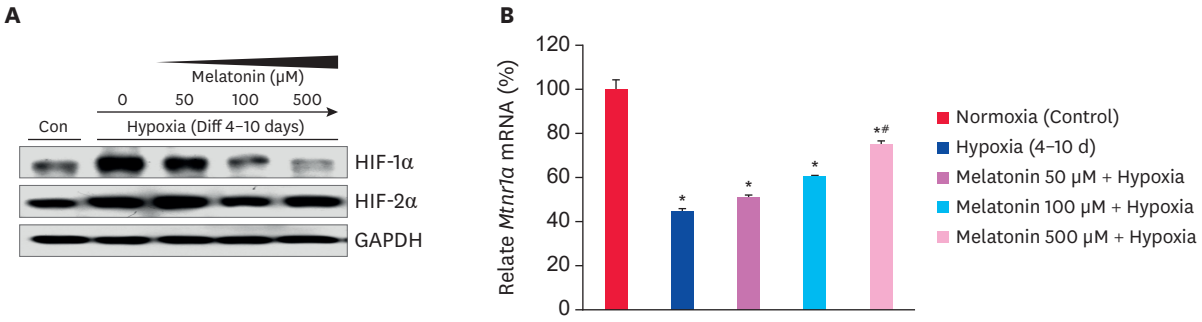

Fig. 5. Effect of melatonin on hypoxia inducible proteins in cardiomyocytes differentiation of mESCs. (A) Effect of melatonin and hypoxia on the protein expression of HIF-1α by western blotting. (B) Effect of hypoxia on the transcriptional level of *Mtnr1a* (Melatonin receptor). mRNA level was measured by real-time qPCR and normalized by *Gapdh*.
mESC, mouse embryonic stem cell; HIF, hypoxia-inducible factor; qPCR, quantitative polymerase chain reaction; Con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
*$p < 0.05$ versus Normoxia (Control). #$p < 0.05$ versus Hypoxia.

when exposed to melatonin (**Fig. 4B**). By contrast, cardiac-specific marker *cTn1* mRNA expression was significantly reduced in hypoxia but was recovered by the 500 μM melatonin administration (**Fig. 4C**). These results indicate that 500 μM of melatonin can alter the hypoxia-induced changes during cardiomyocytes differentiation.

Protein levels of HIF-1α as a hypoxia marker and HIF-2α as a control marker were examined at day 4–10 of differentiation (**Fig. 5**). As shown in **Fig. 5A**, melatonin reduced the expression of HIF-1α protein in a concentration-dependent manner, but the HIF-2α level did not change. Also, under these conditions, the expression of melatonin receptor *Mtnr1a* increased in a concentration-dependent way (**Fig. 5B**). These findings support that melatonin can influence hypoxia during cardiomyocytes differentiation by reducing the HIF-1α protein level and increasing the expression of melatonin receptor *Mtnr1a* mRNA.

Effect of melatonin on ERK/AKT pathway in hypoxia-induced cardiomyocytes differentiation

Melatonin has been known to inhibit ROS and regulate apoptosis [21,22]. To determine that the ERK/AKT pathway is involved by melatonin in hypoxic conditions during myocardial cell differentiation, it was evaluated using Western blot analysis. As depicted in **Fig. 6A**, the induction of hypoxia increased the protein expression of p-ERK, and melatonin suppressed the level of p-ERK in hypoxia-induced cardiomyocytes according to an increase of melatonin concentration. On the other hand, the levels of p-AKT and phosphatidylinositol 3-kinase (PI3K) were reduced during hypoxia induction but increased under melatonin-plus-hypoxia treatment. Apoptosis is a complicated cell signalling process which many genes involved, including Bcl-2, Bax. Bcl-2 protein has an anti-apoptotic and anti-autophagic action, and its expression during cardiomyocytes differentiation decreased while under hypoxic conditions but increased with melatonin pretreatment (**Fig. 6B**). However, the level of the pro-apoptotic protein Bax increased during cardiomyocytes differentiation under hypoxic conditions but decreased with melatonin pretreatment. Overall, the results indicate that melatonin can regulate the inhibition of p-ERK and activation of p-AKT during the differentiation of mESCs into cardiomyocytes.

Melatonin efficiently neutralizes free-radical and indirectly protects against oxidative stress by stimulating the production of antioxidant enzymes [23]. Melatonin stimulates the expression of antioxidants such as superoxide dismutase (SOD), catalase, and glutathione

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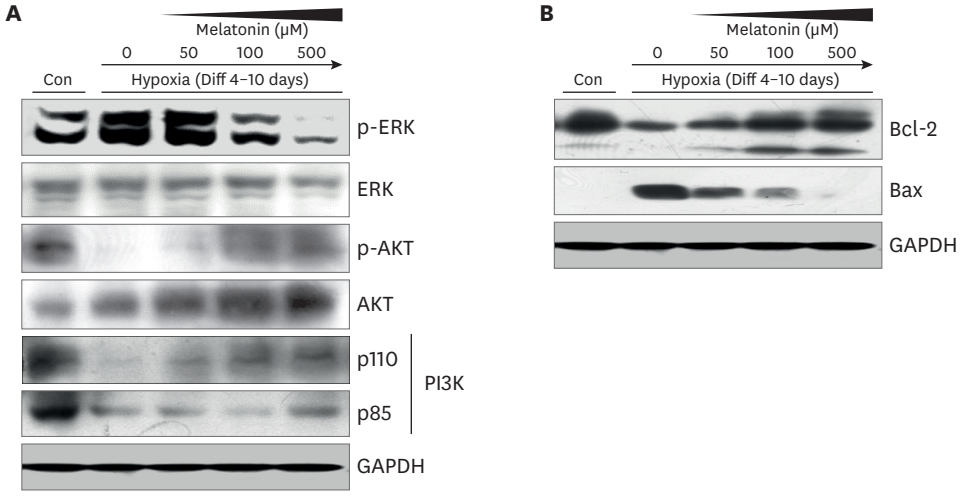

Fig. 6. Melatonin alleviates hypoxia-induced cardiac apoptosis through ERK/AKT pathway. (A) Effect of melatonin and hypoxia on the expression of ERK, AKT, PI3K pathway proteins. (B) The expressions of apoptosis-related proteins were measured by western blotting.
ERK, extracellular signal-related kinase; AKT, protein kinase B; PI3K, phosphatidylinositol 3-kinase; Con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Bcl-2, B-cell lymphoma 2; Bax, cl-2-associated X proteins.

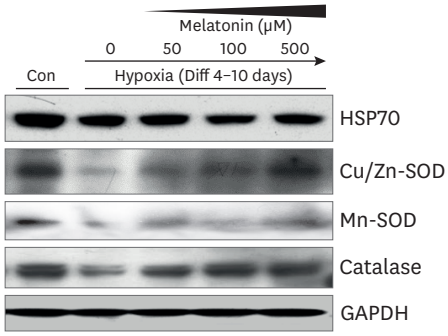

Fig. 7. Melatonin-induced stimulation of antioxidant enzyme. Effect of melatonin and hypoxia on the protein expression of HSP70, Cu/Zn-SOD, Mn-SOD, Catalase by western blotting.
Con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

peroxidase [24]. As shown in **Fig. 7**, protein expression of Cu/Zn-SOD, Mn-SOD, and catalase were reduced in the cardiomyocytes under hypoxic conditions; but the expression of those enzymes were rescued with melatonin pretreatment. These results indicate that melatonin inhibits hypoxia-induced effects on cardiomyocytes differentiation by stimulating the production of Cu/Zn-SOD, Mn-SOD, and catalase enzymes.

To investigate the involvement of melatonin receptors, the melatonin receptor antagonist luzindole was treated simultaneously with melatonin. Luzindole abolished the melatonin-induced effects in the expression of p-AKT, p85 (PI3K), Cu/Zn-SOD, Mn-SOD, and catalase proteins (**Fig. 8**). The results suggest that melatonin pretreatment can mitigate hypoxia effects via the p-Akt and PI3K pathway in the myocardial differentiation of mESCs.

DISCUSSION

In the present study, it was observed that differentiation into cardiomyocytes is impaired under hypoxic conditions. In addition, it was observed that the differentiation of hypoxia-

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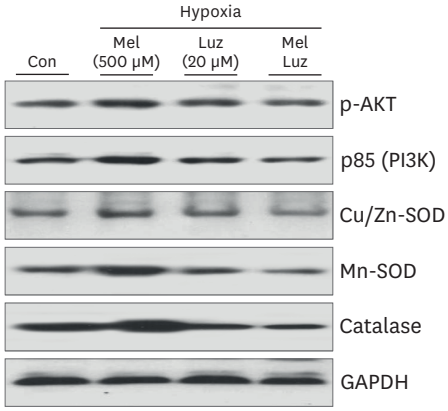

Fig. 8. The expression of p-AKT, p85, Cu/Zn-SOD, Mn-SOD, and catalase proteins with/without melatonin treatment and/or luzindole (20 μM), melatonin receptor antagonist under hypoxia condition.
Con, control; Mel, melatonin; Luz, luzindole; p-AKT, phospho-protein kinase B; PI3K, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

treated cardiomyocytes was recovered by treatment with melatonin. Our results suggest that supraphysiological melatonin treatment can counteract the adverse effect of hypoxia during the myocardial differentiation of mESCs.

mRNA expression of *Mtnr1a* was decreased under hypoxic conditions. It was determined that the adverse effect of hypoxia was altered by melatonin pretreatment, which affected the mRNA expressions of melatonin receptors during myocardial differentiation of mESCs.

Melatonin is reported to have a neuroprotective effect by relieving brain edema and improving nerve function [25,26]. Albeit physiological concentration of melatonin has been known as between 30 to 400 pM in normal plasma; however, supraphysiological dose of melatonin treatment has been reported to hold anti-tumor activity in head and neck squamous cell carcinoma cell lines [27,28]. In addition, Kudová et al. [29] reported that 100 mM promoted myocardial differentiation., while physiological dose of melatonin did not show a protective role against hypoxia. In the present study, three different concentrations (50–100 mM) of melatonin were set. We performed MTT assay and confirmed that melatonin per se did not affect cell viability in every concentration. In hypoxia-treated mESCs, treatment with melatonin 500 μM stabilized the cells' beating ratio. Beating ratio of stem cell-derived cardiomyocyte has been known to be sensitive to environmental stress during differentiation [30]. Our results suggest that 500 μM of melatonin treatment hold a protective effect against hypoxic stress during differentiation. Melatonin significantly reduced protein expression of HIF-1α; the mRNA expression of the melatonin receptors was restored in a concentration-dependent way. These results indicated that melatonin alleviates hypoxia-induced inhibition on cardiomyocytes differentiation.

The mechanism associated with the protective action of melatonin on hypoxia-induced effects in mESCs-derived cardiomyocytes was investigated. Previous studies have shown that activation of the ERK/AKT pathway is involved with melatonin-mediated anti-proliferative effects [31,32]. In the present study, hypoxia increased the expression of p-ERK in cardiomyocytes during differentiation, while melatonin suppressed the expression of p-ERK in hypoxia-treated cardiomyocytes in a concentration-dependent manner. These results provide that melatonin can inhibit phosphorylation of ERK, which is consistent

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with the findings in previous studies; PI3K and AKT regulate various cellular events and are involved in cellular survival and apoptosis [33,34]. In the present study, hypoxia reduced the protein expression of p-AKT and PI3K, while melatonin increased those expressions in hypoxia-treated cardiomyocytes in a concentration-dependent manner, in contrast to the results presented in other studies. Alteration of expression of Bcl-2 family members associated with apoptosis was also identified. Bcl-2 expression was decreased in hypoxia-treated cardiomyocytes during differentiation but was increased by melatonin pretreatment. In contrast, the expression of Bax increased in hypoxia-treated cardiomyocytes but decreased with melatonin pretreatment. Taken together, the results show that melatonin, acting via the AKT and PI3K pathway, can mitigate hypoxia-related effects on the myocardial differentiation of mESCs.

ROS are generated as a byproduct during cellular metabolism and involve in cellular signalling and homeostasis. When ROS significant increase in the cytoplasm, it leads to oxidative stress [35,36]. Melatonin reduces oxidative stress by directly promoting the ROS neutralizers and by increasing the expression of antioxidant enzymes [37,38]. The expression level of Cu/Zn-SOD, Mn-SOD, and catalase in differentiating cardiomyocytes was decreased under hypoxic conditions, but they were increased by pretreatment of melatonin before the induction of hypoxia.

This study demonstrates that melatonin can partially mitigate the adverse effect of hypoxia in hypoxia-treated cardiomyocytes during differentiation by regulating apoptosis and by reducing and oxidative stress of the cells via the actions of the AKT and PI3K pathway. Previous evidence suggests that melatonin hold protective roles in vitro and clinically. Kudová et al. [29] reported that melatonin promoted myocardial differentiation in HIF-1α deficient mESCs by stabilizing HIF-2α. Albeit Kudová et al. [29] did not implement a physical hypoxia environment, their observation and our result both concluded that melatonin exerted a protect effect during myocardial differentiation. In addition, in ischemic heart disease, melatonin can prevent cardiac malfunctions related to ischemic heart disease and myocardial cell death through its antioxidant and anti-inflammatory properties [39]. Overexpression of melatonin receptor 2 can attenuate the hypoxia derived cell injury via the Notch1/HES1/RORa signalling [40]. Furthermore, Guerra-Librero et al. [28] reported that high-dose of melatonin treatment exerted anticancer activity in neck and head squamous cell carcinoma cell line, suggesting the potential possibility for melatonin as clinical use. Our results can support that melatonin hold a regulatory function against hypoxia also in differentiation stage. For a reason, findings from the present study may provide helpful information for understanding the mechanism of melatonin as a mitigator of hypoxic damage.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Expression of melatonin receptor during cardiomyocytes differentiation. Expression levels of *Mtnr1a* (Melatonin receptor 1A) during cardiomyogenesis of mESCs. mRNA level was measured by real-time PCR and normalized by *Gapdh*.

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Supplementary Fig. 2

Cell viability test for melatonin treatment. To investigate any potential chemical toxicity by melatonin treatment, cell counting kit-8 (CCK-8) assay was performed according to the manufacturer's protocol (Dojindo, Japan). Melatonin did non inhibit cell viability in each group (No statistical significance); DMSO, dimethyl sulfoxide.

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