Original Article

Combination of rock inhibitor, hypoxia and melatonin improved differentiation of rabbit induced pluripotent stem cells into cardiac progenitor cells

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Abstract

Cardiac progenitors are a promising cell source for treating myocardial infarction. In this study, we improved the protocol for cardiac differentiation of rabbit induced pluripotent stem cells (iPSC) using Rho-associated protein kinase (ROCK) inhibitor (Y-27632), low O_2 tension (hypoxia) and melatonin treatments. In experiment 1, the rabbit iPSCs were differentiated into cardiac cell fate via embryoid body (EB) formation with or without ROCK inhibitor. EB diameters were measured on day 3 of differentiation. In experiment 2, the EBs were plated on gelatin-coated dishes and further cultured in different oxygen tensions, hypoxia (5% oxygen) and normoxia (20% oxygen). The plated EBs were examined for proliferative activity and the production of reactive oxygen species (ROS). Experiment 3 studied the effects of oxygen tensions and melatonin on the differentiation of cardiac cell fate in terms of cardiac progenitor gene expression (*NKX2.5*) and FLK1 positive cells. ROCK inhibitor significantly improved EB formation by mean of increased EB diameter ($P<0.05$) compared with the control. Hypoxia also significantly increased the numbers of newly DNA synthetic cells indicating greater proliferative activity when compared with normoxia ($P<0.05$). The melanin treatment during iPSCs differentiation significantly decreased ROS production only in hypoxia ($P<0.05$). In addition, the combination of hypoxic condition and melatonin treatment significantly upregulated a *NKX2.5* cardiac progenitor gene and FLK1 positive cells compared with the controls and normoxia-melatonin treatment ($P<0.05$). It is concluded that an optimizing condition using a combination of ROCK inhibitor, hypoxic condition and melatonin improved differentiation of rabbit iPSCs towards cardiac progenitor cells.

Keywords: rabbit, cardiac differentiation, ROCK inhibitor (Y-27632), hypoxia, melatonin

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Introduction

Pluripotent stem cells (PSC) are capable of differentiation into their derivative cells, such as cardiomyocytes, neural cell and intestinal cells (Takahashi et al., 2007; Denham and Dottori, 2011; Iwao et al., 2014). *In vitro* cardiac differentiation requires sequential steps similar to that of cardiac development during early embryogenesis (Rajala et al., 2011; Dierickx et al., 2012). Cardiac progenitors derived from PSC are potential cell sources for treating cardiac infarction with favorable advantages including neovascularization and remodeling of cardiac scars (Le and Chong, 2016). The rabbit has a cardiovascular physiology (Fan and Watanabe, 2003) and phylogeny (Graur et al., 1996) close to human. The rabbit's size is more suitable for surgical interventions and monitoring physiological changes compared with other laboratory rodents (Wei et al., 2016). However, the success of cardiac differentiation from rabbit induced PSC (iPSC) is currently poor (Wei et al., 2016; Phakdeedindan et al., 2018). To induce cardiac differentiation, cellular aggregation into the embryo-like structure so-called EB is commonly used (Kehat et al., 2001; Schell, 2012). This technique is frequently combined with the addition of cardiac promoting agents, such as activin, Bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor (VEGF) and melatonin (Kattman et al., 2011; Ye et al., 2013; Kudova et al., 2016b). However, these factors are species-specific and require optimal timing for activation and/or inhibition of the main cardiac pathways (Dierickx et al., 2012). From a previous study, the rabbit iPSCs were found to be highly sensitive to enzymatic treatment resulting in poor cell viability and EB formation (Phakdeedindan et al., 2018). Rho kinase (ROCK) is a member of the serine/threonine kinases family that plays several roles in fundamental cellular functions, such as contraction, adhesion, migration, proliferation and apoptosis in various cell types (Van Aelst and D'Souza-Schorey, 1997; Aznar and Lacal, 2001; Etienne-Manneville and Hall, 2002; Debidda et al., 2005; Shi and Wei, 2007; Koyanagi et al., 2008). Small molecule Y-27632 is a specific ROCK inhibitor that has been used to decrease apoptosis and cell death during the enzymatic dissociation of several cell types including neuronal progenitor cells and human embryonic stem cells (Watanabe et al., 2007; Koyanagi et al., 2008; Beers et al., 2012). The effect of this small molecule, however, has yet to be tested in rabbit iPSC.

Melatonin, a regulator of circadian rhythms, is a secretary product of the pineal gland (Acikel et al., 2003). It has been reported to protect against heart injury and arrhythmia related to free radicals (Tan et al., 1998; Veneroso et al., 2009). Melatonin also plays a role as free radical scavenger against reactive oxygen species (ROS) by inducing antioxidant enzymes and inhibiting the activation of pro-oxidant enzymes (Zang et al., 1998; Reiter, 2000; Loren et al., 2017). Recent study has demonstrated that melatonin supplementation during differentiation of iPSCs via EB formation significantly upregulated cardiac gene expression such as *NKX2.5*, *MYH6* and *MYH7* and promoted well-developed sarcomeres in mouse ESC by inhibiting hypoxia-inducible factor 1α (Kudova et al., 2016b). Wei and colleagues (2016) provided evidence that cardiomyocyte-like cells could be differentiated from rabbit embryonic stem cells using medium containing melatonin (Wei et al., 2016). However, improving cardiac differentiation of rabbit iPSCs using melatonin remains to be elucidated especially the interaction between melatonin treatment and oxygen tensions. Transient hypoxia in pluripotent cells promoted mesoderm development (Medley et al., 2013) and improved mature cardiomyocytes from pluripotent cells (Bianco et al., 2009). A key transcription factor in cellular adaptation to low oxygen conditions is hypoxia-inducible factors (HIFs). HIFs are sensitive element to oxygen availability and activate genes to acclimatize hypoxia (Görlach, 2014). HIFs are composed of an oxygen-labile α subunit and a stable β subunit. The three isoforms of HIFα are HIF1α, HIF2α and HIF3. Previous studies have suggested that melatonin affects the levels of HIF1α in the cells (Cho et al., 2011; Kim et al., 2013; Zhang et al., 2013). HIF1α involved in the mechanism of Reactive oxygen species (ROS) production and cardiac maturation (Ziello et al., 2007; Kudova et al., 2016a). The effect of hypoxia on cardiac differentiation, however, remain controversial (Medley et al., 2013). In this study, we investigated the effect of ROCK Inhibitor (Y-27632), hypoxia and melatonin on rabbit cardiac differentiation.

Materials and Methods

Experimental design: This study is divided into 3 experiments. In experiment 1, the differentiation of rabbit iPSCs was performed via EB formation with or without ROCK inhibitor. The effects of differentiation concentrations of ROCK inhibitor (0, 5 and 10 μM) and cell seeding densities (1,000, 2,000 and 3,000 cells per EB) on successful EB formation and EB's diameters were examined on day 3 of differentiation. In experiment 2, the EBs were plated and cultured in different oxygen tensions, hypoxia (5% oxygen) and normoxia (20% oxygen). The plated EBs were examined for proliferative activity and the production of reactive oxygen species (ROS). In experiment 3, the effects of melatonin combined with different oxygen tensions were assessed in terms of cardiac progenitor gene expression (*NKX2.5*) and FLK1 positive cells.

Reagents and animals: Cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless otherwise stated. Animal maintenance, care, and use procedures were approved by the Animal Ethics Approval of Chulalongkorn University (N0.1673036) and Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 1531001).

Cell culture: Mitomycin mouse embryonic fibroblasts (MEF) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1 mmol mM L-glutamine and 1% (v/v) antibiotic-antimycotic. The iPSCs were cultured using iPSCs medium containing DMEM/F12, 20% (v/v) knockout serum replacement

(KSR), 1% (v/v) non-essential amino acids (NEAA), 1 mmol mM L-glutamine, 1% (v/v) antibiotic-antimycotic, 0.1 mM Beta-mercaptoethanol (β-ME), 10 ng/ml bFGF and 1000IU LIF. The culture condition was set at 37°C 5% CO_2. The medium was changed every other day. The iPSCs were enzymatically subcultured using 1X TrypLE™ Select.

Characterization of rabbit induced pluripotent stem cells: Rabbit iPSCs were conducted and fully characterized as previously reported (Phakdeedindan et al., 2018). Three lines of rabbit iPSCs (referred as R1, R2 and R3) were used in the current study. The rabbit iPSCs were characterized prior to use by means of alkaline phosphatase (AP) and immunofluorescent staining. In brief, the iPSCs colonies were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and maintained in phosphate-buffered saline (PBS). They were incubated with a mixture of naphthol AS-BI alkaline solution with fast red violet LB (Alkaline Phosphatase Kit, Sigma Aldrich, WI, USA) for AP staining or with primary antibody against OCT protein (SC8628, Santa Cruz Biotechnology, TX, USA, 1:100) at 4 °C overnight. The FITC conjugated secondary antibody was used to detect the expression of the OCT4 protein. The 4', 6'-diamidino-2-phenylindole (DAPI) in mounting medium (Vectashield® Mounting Medium, Vector Laboratories, CA, USA) was used as counter staining. A fluorescent microscope (BX51, Olympus) and DP2-BSW software were used for visualization and recording the samples. The negative control was performed as described above without primary antibody.

Cellular differentiation via EB formation: EB formation was performed via hanging drop technique as described elsewhere with minor modifications. Briefly, iPSCs lines R1, R2 and R3 (passage 29-32) were dissociated and allowed to aggregate into three-dimensions in EB medium containing DMEM-F12, 15% FBS, 1 mM L-glutamine, 1% antibiotic-antimycotic, 0.1 mM NEAA and 0.1 mM β-ME with or without 10 μM Rock inhibitor for 3 days. The EBs (day 3) were then plated onto 0.1% gelatin-coated dishes or performed suspension culture on poly 2-hydroxyethyl methacrylate dishes. The medium was changed every other day. The EBs were maintained at 37°C and 5% CO_2.

To detect the pluripotency of the iPSCs by mean of three-germ layer differentiation, the EBs were further cultured for 14 days and examined by immunohistochemistry (Leica Microsystems BOND-MAX System). The EBs were then fixed with 4% (w/v) PFA, embedded in paraffin and cut (4 μm). In short, the slides were incubated with Bond Dewax Solution (Leica Microsystems) for 60 min at 60°C. The antigen retrieval was conducted with Bond Epitope Retrieval Solution 2 (Leica Microsystems) for 30 min at 100° C. The samples were separately incubated with primary antibodies at 25° C for 40 min. They were rinsed with Bond Wash Solution (Leica Microsystems). Hydrogen peroxide (3%) was then used for 5 min and rinsed. Post primary polymer (Leica Microsystems) was applied on slides for 8 min. The slides were washed, followed with Poly-HRP IgG (Leica Microsystems) for 8 min, and rinsed. The diaminobenzidine chromogen was conducted for 4 min followed by rinsing with deionized water. Slides were counterstained with hematoxylin for 5 min. Primary antibodies in this study were mouse monoclonal anti- glial fibrillary acidic protein (anti- GFAP, 6F2, DAKO, 1:2400), anti-Vimentin (V9, CellMarque, CA, USA, 1:400) and anti-ß-catenin (14, CellMarque, CA, USA, 1:500). Isotype Mouse IgG1, kappa monoclonal (ab91353, Abcam, Cambridge, UK) was used for the negative controls. Brain tumor, appendix and tonsil were utilized as positive controls for GFAP, vimentin and ß-catenin, respectively.

Determination of cellular proliferative activity: The proliferative activity was investigated using Thymidine analogue EdU staining (Molecular probe Click-iT™ Plus EdU Alexa Fluor™ 555 Imaging Kit, C10638) according to the manufacturer's protocol. In brief, the EBs (Day 3) were randomly cultured with either 20% oxygen (O_2, normoxia) or 5% O_2 (hypoxia) condition. The EBs thereafter were assessed for proliferative activity and ROS. For proliferative activity, the plated EBs were cultured for 18 h in a medium supplemented with thymidine analogue EdU (5-ethynyl-2'-deoxyuridine, a nucleoside analog of thymidine. Later, the plated EBs were digested with TrypLE™ into single cells and then fixed in 4%PF. The fluorescent Alexa Fluor® analog EdU dye was visualized using a fluorescent microscope (BX51, Olympus) and DP2-BSW software. The EdU positive cells were counted and calculated in relation to the total cell number.

Cardiac differentiation and melatonin treatment: The cardiac differentiation of induced iPSCs into cardiac progenitor cells was performed in experiment 3. The cardiac differentiation protocol is described in figure 1. The plated EBs were treated with 100 μmol/ L melatonin (M5250, Sigma Aldrich, WI, USA) following previous study with minor modifications (Kudova et al., 2016b). The EBs treated with an equivalent concentration of ethanol (without melatonin) served as a control group. The cells treated with melatonin for 24 and 48 h (referred to day 1 and day 2 of melatonin treatment) were assessed for ROS production which was measured by CM-H_2DCFDA (C6827, Invitrogen) according to the manufacturer's instructions. Briefly, the EBs were washed with PBS twice and then incubated with CM-H_2DCFDA (5 μM) in PBS for 10 min in the dark. The samples were photographed under an inverted fluorescent microscope (IX71, Olympus) and DP2-BSW software. Image J analysis software (https://imagej.nih.gov/ij/) was used to measure the intensity of mean gray scale which was converted into ROS levels.

Determination efficiency of cardiac progenitor differentiation: To determine the cardiac progenitor stage among different conditions, the levels of mRNA expression of cardiac progenitor marker *NKX2. 5* expression were investigated on day 8 using relative real- time polymerase chain reaction (qPCR) in hypoxia melatonin treated group (HM), hypoxia control group (HC), normoxia melatonin treated group

(NM) and normoxia control group (NC). In brief, the extraction of RNA was conducted by RNeasy mini kit (Qiagen, Hilden, Germany). The amount of RNA and purity was measured by Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, DE, USA). The contaminated DNA was eliminated using DNase I (Promega, WI, USA) . The cDNA was synthesized with the Superscript III first-strand synthesis system according to the manufacturer's instructions. RT-PCR was performed using PCR cycles as follows: initialization at 95 °C for 2 min, followed by 30 PCR cycles of denaturation at 95 °C for 30 s, annealing step at 55- 64 °C for 30 s and extension step at 72 °C for 30 s. To determine the expression, qPCR was performed for *NKX2.5* in a standard 40-cycle with KAPA SYBR® FAST qPCR Kits (KK4600) following the manufacturer's protocol using ABI7300 machine. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was conducted for normalization. All primer sequences are shown in Table 1. In addition to gene expression, specific cardiac progenitor protein (FLK1) was also detected using the immunofluorescent technique. The cardiac differentiated cells (HM and HC groups) were dissociated into single cells and then fixed with 4% PF. The cells were then labelled at 4 °C with FLK1 (SC393163, Santa Cruz Biotechnology, 1: 100) overnight. The goat anti-mouse TRITC secondary (1:100 dilution) was used. The Immunofluorescence-labeled cells were finally stained with DAPI and visualized using a fluorescent microscope. The numbers of FLK1 positive cells were counted and expressed as percentage in relation to the total cell numbers.

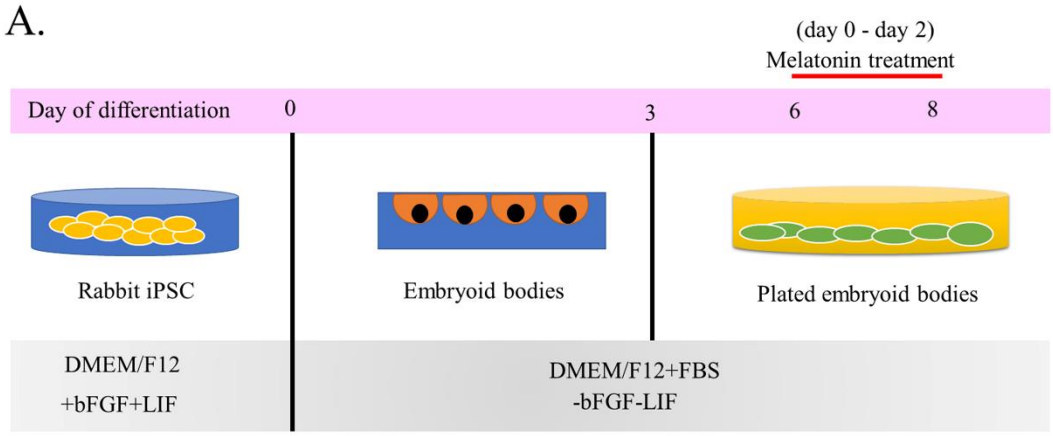

Figure 1 A Diagram demonstrating technique for *in vitro* differentiation of rabbit induced pluripotent stem cells with melatonin. The iPSCs were used to generate EB. The EBs were transferred onto gelatin-coated dish and treated with or without 100 µmol/L melatonin during days 6 to 8 of differentiation.

Table 1 Primers used in polymerase chain reaction (PCR)

	Forward (5′–3′)	Reverse (5′–3′)	Product size (bps)	Tm	Accession number or reference
BT	GCAAAGGAAAGAAGCGACCAC	TGAGCGGAAGGCAGAGAGAGG	164	55	Thieme et al.,2012
NKX2.5	GCAGATAAGAAAGAGCTGTGCG	GTACCGCTGCTGCTTGAAAC	165	55	XM_002710385.3
GATA4	CGGCCTCTACCACAAGATGA	AGGTTCTTGGGCTTCCGTTT	252	55	XM_002709438.3
GAPDH	GGAGCCAAAAGGGTCATCATCTC	GAGGGCCCGTCCACGGTCTTCT	233	60	Lo et al.,2015

Statistical analysis: All data is expressed as mean ± SEM. All experiments were performed as at least 3 independent experiments. The cross-sectional diameters of EBs were measured by ImageJ. Statistical analysis was conducted using unpaired t-test or one-way ANOVA and Tukey's Multiple Comparison Test analysis using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The difference with $P < 0.05$ was considered statistically significant.

Results

All rabbit iPSCs lines demonstrated flat, compact morphology (fig. 2A). They strongly expressed alkaline phosphatase (fig. 2B) and pluripotent marker OCT4 (fig. 2C). The expressions of protein markers for three-germ layer differentiation, including ectoderm (GFAP), mesoderm (Vimentin) and endoderm (ß-catenin) (fig. 2D), indicated the differentiation properties of rabbit iPSCs used in the present study. In experiment 1, the rock inhibitor treatment significantly improved the viability and EB's size (fig. 3A). However, the effects were dependent on the concentrations used (fig. 3B). In general, cell seeding density and cell lines influenced the formation of EB and EB's size. The cell line R1 could not form the EB structure, while cell lines R2 and R3 could be used to generate the EBs. However, this was dependent on the cell seeding density used. The cell line R2 could form the EBs with diameters of 88.94±2.39, 95.78±3.19 and 117.79±3.59 µm for cell densities 1,000, 2,000 and 3,000 cells per EB, respectively. The R3 cell line could aggregate into EB structure only when cell densities of 2,000 and 3,000 cells per EB were used (EB diameters: (127.41±5.52 and 191.84±10.24 µm), respectively. ROCK inhibitor significantly improved the efficiency of EB formation in terms of successful EB generation

and their diameters. The concentrations of ROCK inhibitor at 5 and 10 µM were found to be effective for increasing EB sizes (188.40 ± 11.27 and 320.28 ± 15.36 µm) compared with 0 µM ROCK inhibitor control (95.06 ± 8.21 µm) ($P<0.05$). Cell line R3 could form the largest EB size when compared to other cell lines (fig. 3C). In addition, the EB from all cell lines with ROCK inhibitor treatment also expressed mesoderm marker genes *BT* and cardiac progenitor marker *NKX2.5* and *GATA4* (fig. 3D). Experiment 2 indicated that hypoxia condition enhanced proliferative activity and reduced ROS as demonstrated by the significant increase in numbers of cells positive to the EdU proliferation marker (93.21 ± 1.57, fig. 4A). This was significantly greater than the normoxia control group (77.94 ± 1.58, $P<0.05$, fig. 4B). Furthermore, the level of ROS as detected by CM-H_2DCFDA in hypoxia (6.75±1.28) was also significantly lower than the normoxia control (8.63±1.5, $P<0.05$) (fig. 4C).Within time points, ROS levels were significantly decreased in HM (7.575±1.35, 5.842±0.6) compared with HC (8.7±0.83, 7.228±1.29; $P<0.05$) after melatonin treatment for 24 h and 48 h, respectively. However, there was no significant difference among HM, NM and NC for ROS levels at 24 (fig. 5A) and 48 h (fig. 5B) of the treatment ($P>0.05$). The combination of melatonin and low O_2 tension (HM) demonstrated significant upregulation of cardiac progenitor mRNA (*NKX2.5*) when compared with other groups (fig. 5C). In hypoxic condition, melatonin treatment (HM) significantly improved the numbers of cardiac progenitor cells (73.19 ± 2.29, FLK1 positive cells) when compared with the control group (58.71 ± 1.83, HC; fig. 5D).

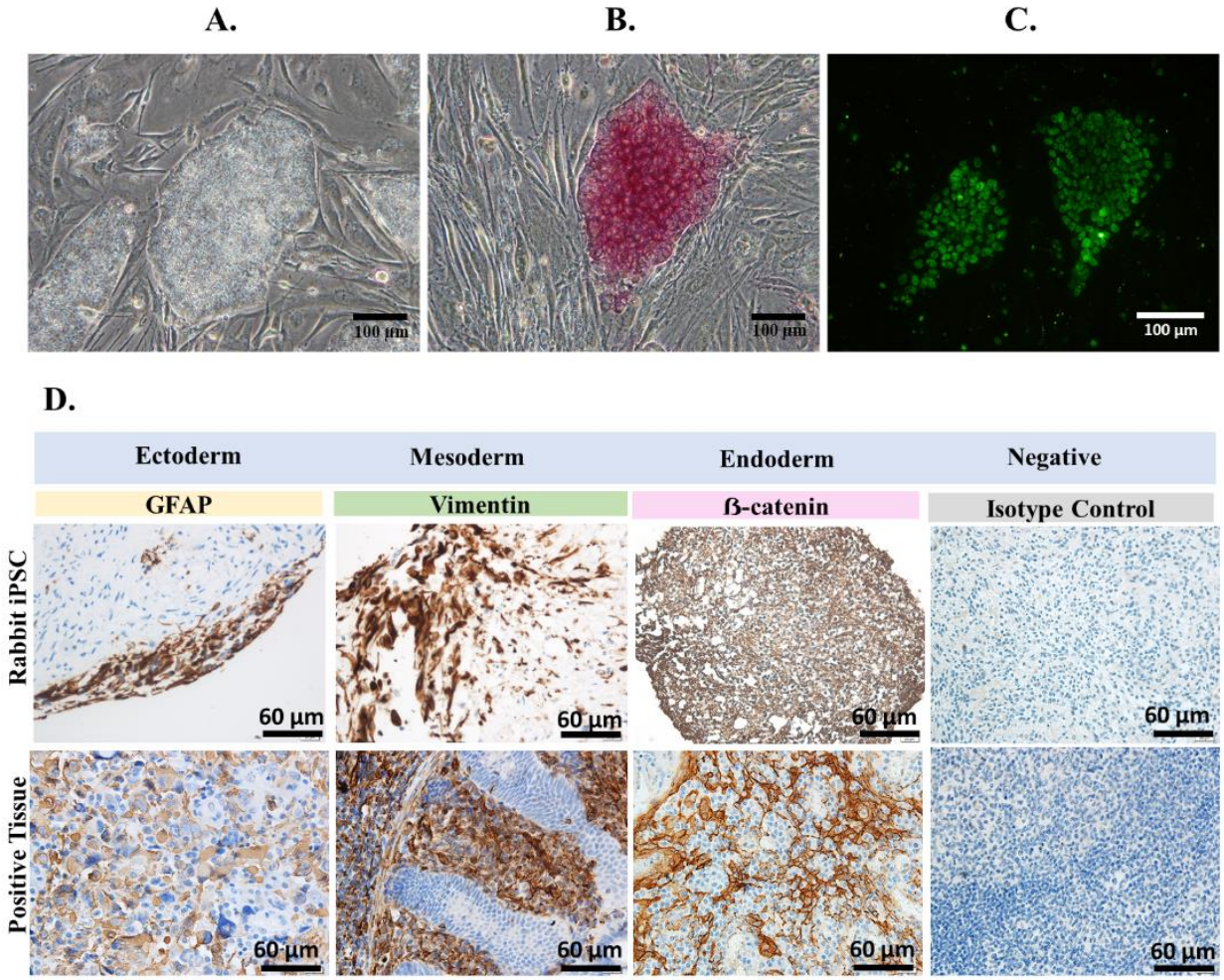

Figure 2 Rabbit iPSCs (A) Typical morphology of iPSCs demonstrating flat colony containing high nuclear/cytoplasmic ratio cells (B) Pink color of AP activity. (C) OCT4 expression (green) in rabbit iPSC. Scale bars represent 100 µm. (D) Day 14-30 EBs were fixed and stained with antibodies against GFAP, vimentin and ß-catenin to identify ectoderm, mesoderm and endoderm respectively. Scale bar represents 60 µm.

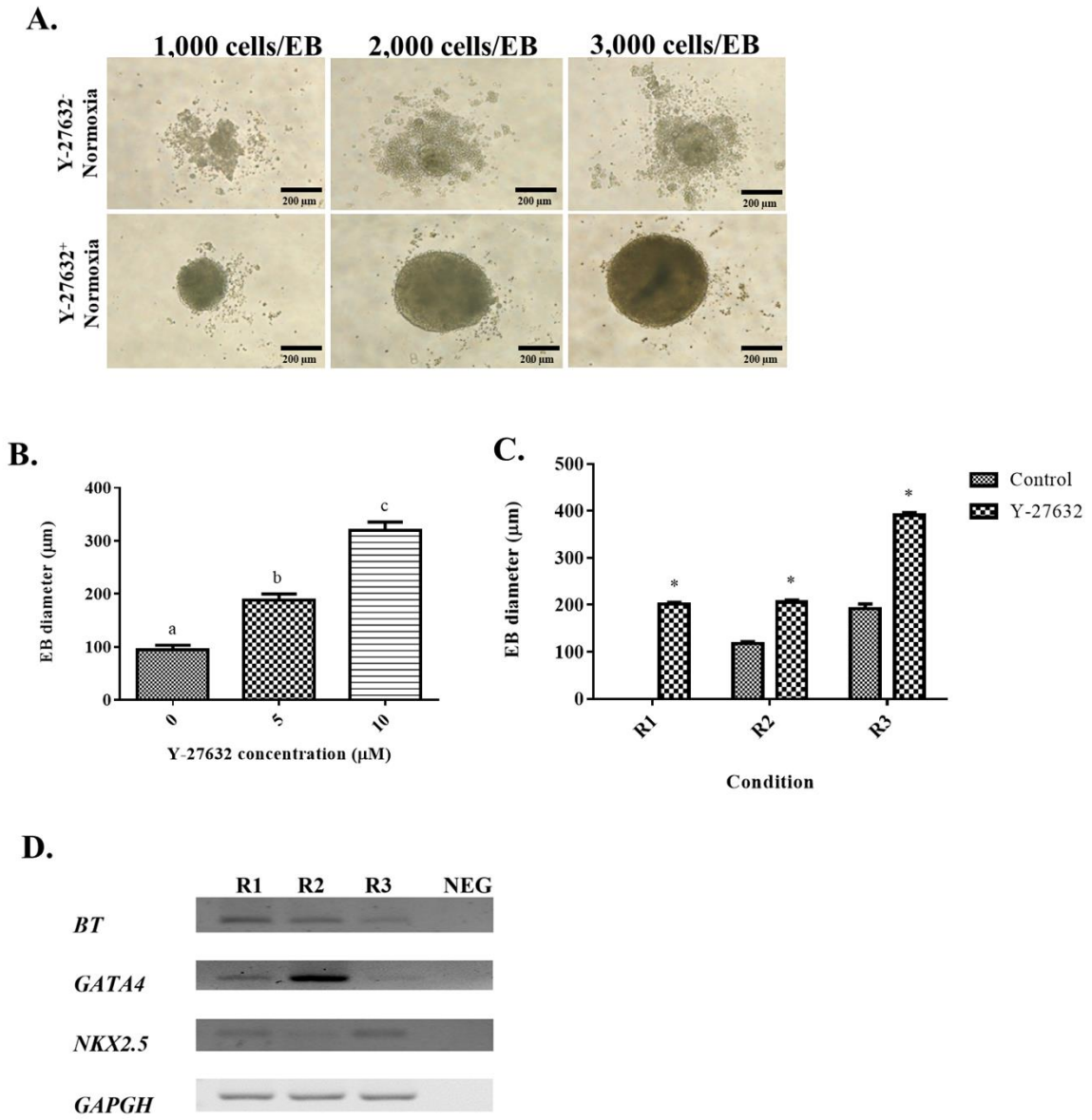

Figure 3 The effect of rock inhibitor on rabbit EB formation (A) Representative EB figures on day 3 of EB formation using 1,000, 2,000 and 3,000 seeding cells seeding per EB. Poor EB formation was found when rock inhibitor (Y-27632) was not used (upper image panel). Scale bars represent 200 µm. (B) EB diameters derived from iPSCs R3 were influenced by concentrations of rock inhibitor. Values with different superscripts indicate significant difference (P≤0.05, One-way ANOVA). The graphs were plotted with letter-coded significant differences (a, b, c). (C) EB diameter derived from 3,000 seeding cells per EB with (Y-27632) and without rock inhibitor (control). Asterisks indicate significant difference (P≤ 0.05) between ROCK treatment group and control group in the same cell lines . (D) EB formation with rock inhibitor promotes cardiac fates in rabbit iPSCs cell line (R1 R2 and R3) on day 7 of differentiation. Mesodern marker *BT* and cardiac progenitor marker *GATA4* and *NKX2.5* were detected using RT-PCR. *GAPDH* was used as internal gene control.

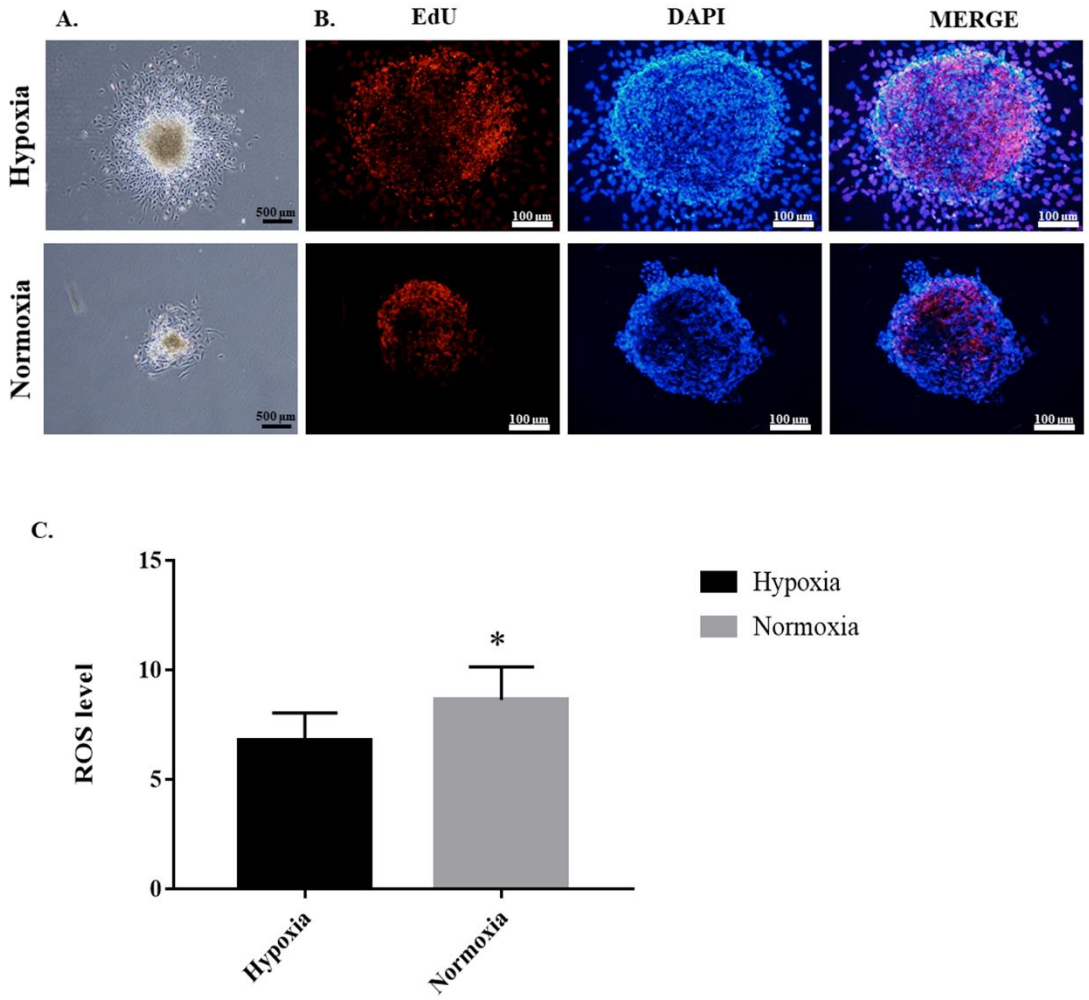

Figure 4 Hypoxia enhanced proliferative activity and decreased ROS levels (A) Plated EB at day 4 under hypoxia and normoxia (upper panel, bright filed). Scale bar represents 500 µm. (B) The cellular proliferation was indicated using EdU (red) and counterstained with DAPI (blue). Scale bar represents 100 µm. (C) The levels of ROS production was significantly reduced in hypoxic condition.

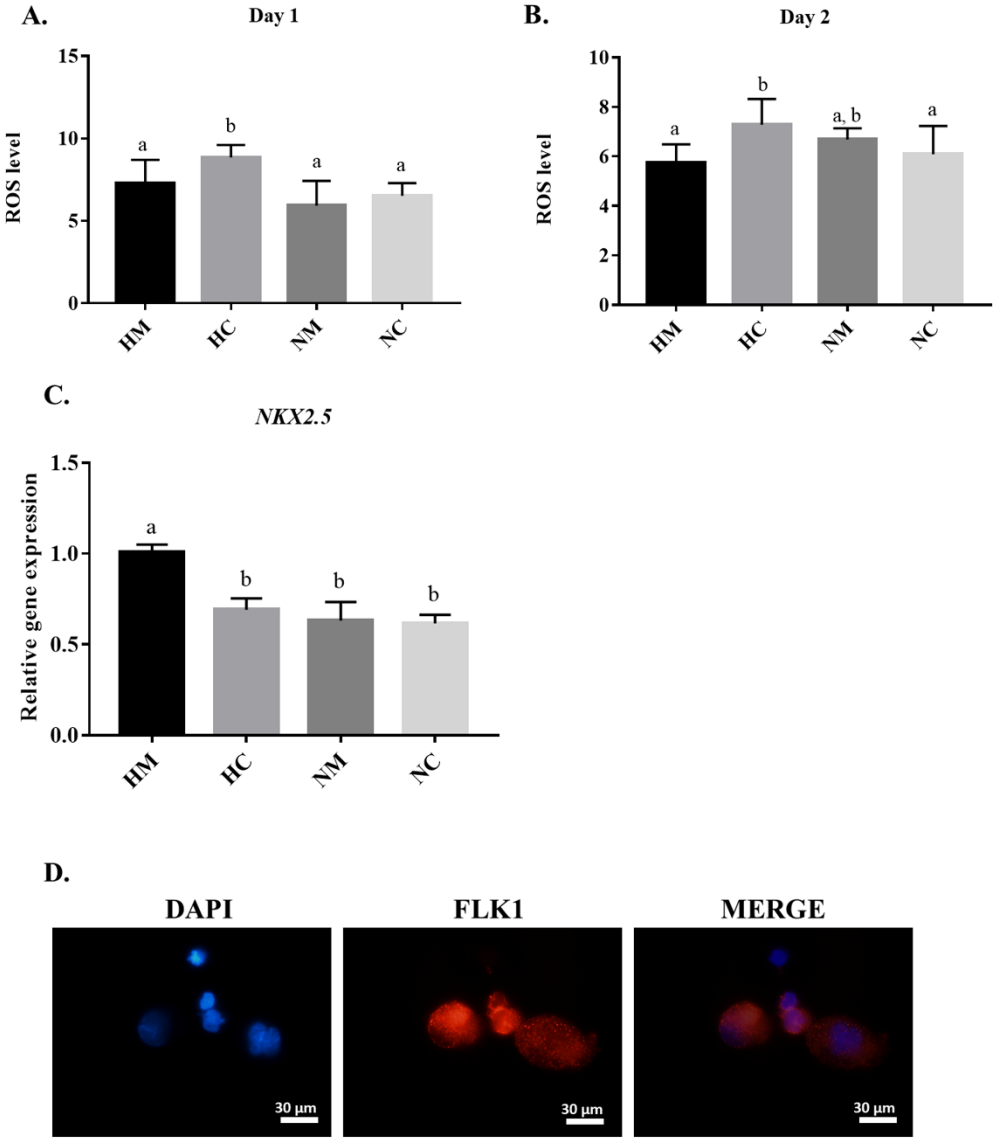

Figure 5 **Improvement of cardiac differentiation using low oxygen condition and melatonin** (A) The levels of ROS production in different conditions after 24 h (A, day 1) and 48 h (B, day 2) of melatonin treatment. (C) Up-regulation of cardiac progenitor *NKX2.5* in HM. (D) Differentiating cells in HM on day 8 were positively stained with surface marker of cardiac progenitor FLK1. Scale bar represents 30 µm HM: hypoxia melatonin treatment group, HC: hypoxia control group, NM: normoxia melatonin treatment group and NC: normoxia control group.

Discussion

In the present study, we found that the rabbit iPSCs partially resemble human iPSCs in terms of colony morphology and factors required for maintaining pluripotency. More importantly, it has been demonstrated in our previous results that indicate that a large number of cells (up to 20,000 cells) would be required for attending optimal EB size (around 300-450 µm) for cardiac differentiation (Hwang et al., 2009; Mohr et al., 2010; Phakdeedindan et al., 2018). It has been hypothesized that the rabbit iPSCs are highly sensitive to enzymatic dissociation similar to that of human iPSCs (Watanabe et al., 2007; Beers et al., 2012). In experiment 1, supplementation of Y-27632, a specific ROCK inhibitor, significantly improved EB size. The poor viability of cells after dissociation has been proposed by the enzymatic treatment induced apoptosis and cell death. ROCK inhibitor has therefore been demonstrated to reduce apoptosis and to enhance survival of human and rabbit embryonic stem cells after enzymatic digestion (Watanabe et al., 2007; Beers et al., 2012; Du et al., 2015). In rabbit iPSC, the information for enzymatic treatment on cellular cascades of apoptosis and cell death is limited. However, the improved efficiency of EB formation in the current study suggests that the apoptosis and cell death of the rabbit iPSCs may relate to the ROCK-dependent hyperactivation of actomyosin similar to human PSC (Ohgushi et al., 2010). ROCK substrates involved the regulation of apoptosis by increasing myosin light chain (MLC) phosphorylation or by inhibiting MLC phosphatase (MYPT1). The increase of MLC phosphorylation via caspase 3-mediated ROCK stimulates actin contraction and regulates morphological apoptosis including plasma membrane blebbing, nuclear disintegration

and fragmentation of apoptotic cells (Shi and Wei, 2007). PTEN mediated ROCK reduces AKT phosphorylation, and thereby inhibits the cell survival process and increases apoptotic events (Li et al., 2005; Chang et al., 2006). The loss of ROCK signaling enhanced both cell survival and antiapoptotic effect. In experiment 2, hypoxia enhanced the proliferating activity of plating EB compared with normoxic condition. Reduced oxygen levels enhanced the proliferation of multiple cell types such as stem cells and progenitor cells (Grayson et al., 2007; Yoshida et al., 2009; Fotia et al., 2015; Kakudo et al., 2015). These proliferative cells prefer glycolysis while mature and low proliferative cells primarily rely on oxidative phosphorylation (Vander Heiden et al., 2009; Singh et al., 2017). The anaerobic condition leads to a drastic drop of energy production and under the regulation of HIF, the glycolysis in these proliferative cells is suitable for anaerobic condition (Hubbi and Semenza, 2015). In addition, previous studies found that HIF1α enhanced proliferating activity in human adipose-derived stem cells and mid-gestational cardiogenesis (Fotia et al., 2015; Guimaraes-Camboa et al., 2015). The relationship between ROS and hypoxia is paradoxical (Guzy et al., 2005; Guzy and Schumacker, 2006; Jung et al., 2008; Tafani et al., 2016). Before melatonin treatment, the ROS level was significantly lower in the hypoxia group. However, the ROS level was later on significantly higher in HC compared with the NC. These results can be explained as ROS is overproduced from mitochondria to stabilize and activate HIF1α which in turn sustains cell survival under low oxygen tension (Chandel et al., 2000; Guzy and Schumacker, 2006; Tafani et al., 2016). The significant decline of ROS in HM compared with the HC on day 1 and day 2 may cause by the antioxidant effect of melatonin. In addition, there was insignificant difference between NM and NC groups. It is therefore hypothesized that melatonin possibly reduces ROS mediated via HIF1α which is present only under low oxygen tension (Gordan and Simon, 2007). A previous study in mice demonstrated that melatonin also improved cardiac gene expression only in the presence of HIF1α (Kudova et al., 2016a; Kudova et al., 2016b). The relationship of the HIF1α and other cardiac related markers such as cardiac troponin, cardiac ryanodine receptor and functional cardiomyocytes should be further examined. Experiment 3 revealed the significant upregulation of *NKX2.5* mRNA and FLK1 protein in the melatonin treatment group under hypoxia. These results supported the combination of melatonin and hypoxia enhanced differentiation of rabbit iPSCs into cardiac progenitors. The positive effects of the melatonin on cardiac differentiation for rabbit iPSCs seem to be time dependent since the benefits of melatonin were found only when the melatonin was used during 6 to 8 day of differentiation. However, melatonin supplementation during day 0 to day 5 of the differentiation of mouse iPSCs did not significantly improve cardiac differentiation (Kudova et al., 2016b). To our knowledge, this is the first study to demonstrate melatonin promoted cardiac differentiation of rabbit iPSC. However, the functions of these cardiac progenitors should be further examined by means of cell transplantation into a rabbit model having acute myocardial injury.

Conclusion

The study demonstrated that a specific ROCK inhibitor improved the efficiency of rabbit EB formation. Low O_2 tension promoted cellular proliferation and reduced the generation of reactive oxygen species. The combination of hypoxic condition and melatonin treatment enhanced differentiation of rabbit iPSCs into cardiac progenitor cells. The findings in this study provided knowledge to improve cardiac induction from rabbit iPSCs for further use.

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