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Development of a model of ischemic heart disease using cardiomyocytes differentiated from human induced pluripotent stem cells

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ABSTRACT

Ischemic heart disease remains the largest cause of death worldwide. Accordingly, many researchers have sought curative options, often using laboratory animal models such as rodents. However, the physiology of the human heart differs significantly from that of the rodent heart. In this study, we developed a model of ischemic heart disease using cardiomyocytes differentiated from human induced pluripotent stem cells (hiPS-CMs). After optimizing the conditions of ischemia, including the concentration of oxygen and duration of application, we evaluated the consequent damage to hiPS-CMs. Notably, exposure to 2% oxygen, 0 mg/ml glucose, and 0% fetal bovine serum increased the percentage of nuclei stained with propidium iodide, an indicator of membrane damage, and decreased cellular viability. These conditions also decreased the contractility of hiPS-CMs. Furthermore, ischemic conditioning increased the mRNA expression of IL-8, consistent with observed conditions in the *in vivo* heart. Taken together, these findings suggest that our hiPS-CM-based model can provide a useful platform for human ischemic heart disease research.

1. Introduction

Ischemic heart disease, defined as the occlusion of the coronary artery by intravascular plaque that has accumulated over time or acute embolization with thrombus, remains the single largest cause of death worldwide despite decreasing rates in most countries [1]. Accordingly, several basic studies of laboratory animal models have been conducted to address this problem. However, animal model-based approaches are associated with ethical, technical, and academic problems. Particularly, animal ethics requires the minimization of animal sacrifice. Moreover, such studies often use occlusion of the anterior descending branch of left coronary artery as a model of ischemic heart disease [2], which requires a high level of skill with microsurgery to produce reproducible data, particularly in rodents. Moreover, various physiological aspects of the human heart, such as the heart rate and action potential formation [3], are different from those in rodents.

The above-mentioned problems can be resolved by using cardiomyocytes derived from human stem cells, such as induced pluripotent stem (iPS) cells. Such an approach avoids the sacrifice of animals and performance of technically difficult surgical procedures. Furthermore, cardiomyocytes differentiated from human iPS cells not only recapitulate the physiology of the human heart, but are also expected to reflect the sensitivity of the donor cells to various drugs. In this study, a pilot experiment with the rat cardiomyocyte cell line, H9c2, led us to establish a model of ischemic heart disease using cardiomyocytes derived from human iPS cells (hiPS-CMs). In our model, oxygen and glucose deprivation led to decreases in hiPS-CM contractility and viability. Our findings suggest that our new model will provide a useful platform for human ischemic heart disease research.

2. Materials and methods

2.1. H9c2 culture

H9c2 rat cardiomyocytes (ATCC, Manassas, VA, USA) were

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cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. The cells were plated in 96-well plates at a density of 3×10^4 cells/well.

2.2. iPS culture

The procedure used to maintain iPS cells has been described elsewhere [4]. We used this procedure with slight modifications. Briefly, human iPS cells (201B7 cell; RIKEN, Tsukuba, Japan) were cultured in StemFit AK02 N medium (Ajinomoto, Tokyo, Japan) on 6-well culture plates coated with laminin (iMatrix-511, Nippi, Tokyo, Japan). Prior to coating, iMatrix-511 was diluted to 0.5 μg/ml in phosphate-buffered saline (PBS) and added to the plates at a total volume of 2.0 ml/well, followed by incubation at 37 °C for 30 min. After coating and removal of the iMatrix-511 solution, the iPS cells were seeded at a density of 3×10^4 cells/well. Human iPS cells were passaged in accordance with the protocol supplied by the manufacturer of the medium.

2.3. Induction of cardiac differentiation of iPS cells

Cells were differentiated in 96-well plates. First, iMatrix-511 was diluted to 1.675 μg/ml with PBS and added to wells in aliquots of 0.1 ml/well, followed by an incubation at 37 °C for 30 min. Subsequently, iPS cells were seeded onto feederless 96-well plates at a density of 3.0×10^4 cells/well. One day later, the cells were cultured in Essential 8 Medium (Thermo Fisher Scientific, Waltham, MA, USA) for 3 days, and the medium was changed every day (Fig. 1A). Subsequently, iPS cell differentiation was initiated using PSC Cardiomyocyte Differentiation Kits (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Briefly, the spent medium was aspirated and slowly replaced with pre-warmed Cardiomyocyte Differentiation Medium A. Two days later, the medium was aspirated and slowly replaced with pre-warmed Cardiomyocyte Differentiation Medium B. After another 2-day incubation, the medium was replaced with pre-warmed Cardiomyocyte Maintenance Medium. Subsequently, the cells were incubated in a CO_2 incubator at 37 °C, and Cardiomyocyte Maintenance Medium was replaced every other day up to Day 30.

2.4. Exposure to hypoxia

Cells were exposed to an incubator infused with nitrogen gas to control the internal oxygen concentration at 2%.

2.5. Immunostaining

Immunocytochemistry was used to evaluate the expression of a cardiac marker, TNNT2. Prior to immunofluorescence staining, the cells were fixed with 4% paraformaldehyde in PBS (Wako, Osaka, Japan) for 10 min at room temperature and washed three times with PBS. Subsequently, the cells were permeabilized with 0.2% Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 15 min and blocked with 3% bovine serum albumin (BSA; A9418, Sigma Aldrich, St. Louis, MO, USA) for 30 min. Subsequently, the cells were incubated with primary antibodies overnight at 4 °C, washed three times with PBS, and subsequently incubated with secondary antibodies for 30 min at room temperature in the dark. Primary anti-cardiac troponin T (TNNT2) mouse monoclonal antibodies (13-11, Thermo Scientific, Rockford, IL, USA) were used at a dilution of 1:750 in 3% BSA. The Alexa Fluor 488-conjugated secondary goat anti-mouse antibody (Life Technologies, Warrington, UK) was diluted to 1:1000 in 3% BSA. Finally, the cells were incubated in NucBlue Fixed Cell Reagent (Thermo Fisher Scientific) and ActinRed 555 ReadyProbes Reagent (Thermo Fisher Scientific) in PBS for 30 min to simultaneously stain the nuclei and actin fibers followed by three

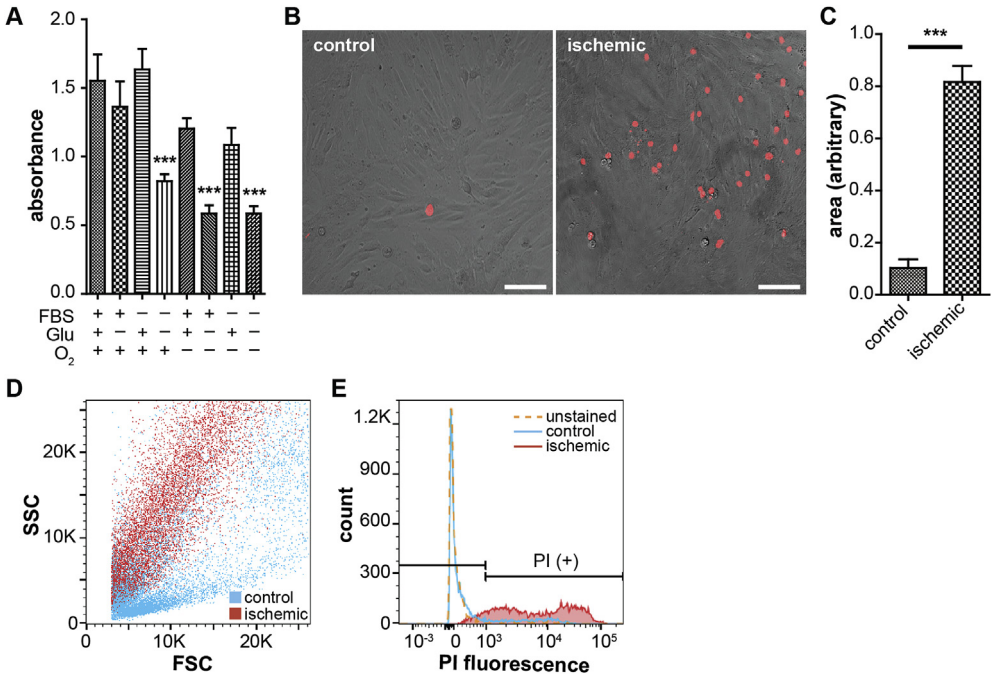

Fig. 1. Nutrients and oxygen deprivation induce damage in H9c2 rat cardiomyocytes. (A) Cellular viability (MTT assay) in the presence or absence of fetal bovine albumin (FBS, 10%), glucose (Glu, 2 mg/ml), and/or oxygen (O_2, 21%). The statistical comparison relative to the control (FBS+, Glu+, and O_2+) was performed using a one-way ANOVA with the Bonferroni post-hoc test. (B) Microscopic image of propidium iodide (PI)-stained nuclei (red) overlaid with a bright-field image. Cells exposed to the control (FBS+, Glu+, and O_2+) and ischemic (FBS−, Glu−, and O_2−) conditions were compared. Scale bar: 100 μm. (C) Measurement of the PI-stained area. (D, E) Flow cytometry analysis of PI-stained cells. Ischemic cells exhibited a decrease in forward scatter intensity (D) and increase in PI staining intensity (E) relative to the control. ***: $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

washes with PBS. The fluorescence images were acquired using a fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan).

2.6. Propidium iodide staining

The cells were subjected to propidium iodide staining to assess the extent of cellular damage. A 1-mg/ml solution of propidium iodide was diluted to 1:3000 in PBS. After two washes with PBS, cells were incubated with 200 μl of diluted propidium iodide solution for 30 min in the dark. After a single wash with PBS, fluorescence images of the cells were acquired using a fluorescence microscope (BZ-X710; KEYENCE).

2.7. MTT reagent-based cellular viability assay

The MTT Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to quantitatively assess the viability of H9c2 cells. The cells were incubated with 10 μl of MTT (37 °C; 3 h) in accordance with the manufacturer's instruction. After removing the supernatant, the insoluble formazan crystals were dissolved in 100 μL of dimethyl sulfoxide solution. The absorbance in each well was measured with a microplate reader at a wavelength of 570 nm, and regarded as cellular viability.

2.8. Quantitative real-time PCR

Total RNA was extracted from cells using the High Pure RNA Isolation Kit (Roche, Indianapolis, IN, USA) and reverse-transcribed into cDNA using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific). The expression levels of the target mRNAs were quantified using real-time PCR with SYBR Green reagents (Life Technologies) and primers specific for TNF-α, IL-1β, IL-8, and caspase 3 mRNAs and 18S rRNA. The nucleotide sequences of primers are shown in Table 1. The expression levels of target genes were normalized to that of 18S rRNA and calculated using the ΔΔCt method. All measurements were performed in triplicate, and three independent samples were measured per treatment group.

2.9. Contractility of iPS-CMs

Details of the method used to evaluate iPS-CM contractility were described elsewhere [4]. Briefly, phase-contrast video microscopic images of contractions of iPS-CM-derived cardiomyocytes were recorded using a microscope (BZ-X710; KEYENCE). Discrete two-dimensional vector fields of cellular displacement were analyzed by calculating the displacement vectors D(x,y) in arbitrary units for every 16-pixel × 16-pixel section between the reference frame (i.e., first frame) and all subsequent frames, using the particle image velocimetry plugin of Fiji software. A maximum displacement vector, M(x, y), was defined for every (x, y) pair, and the contractility value, C, was calculated in arbitrary units as the sum of the absolute values of the maximum displacement vectors.

Table 1
Primers used for quantitative RT-PCR.

Primer	Direction	Sequence	Ref
TNF-α	Forward	5′-TGTGAGGCGGTTGTGGAAGAGT-3′	[5]
	Reverse	5′-AATGGGGGAAGAGGCAGGTGCA-3′	
IL-1β	Forward	5′-AGGCACAAGGCACAACAGGCT-3′	[6]
	Reverse	5′-GGTCCTGGAAGGAGCACTTCATCTG-3′	
IL-8	Forward	5′-AGAAACCACCGGAAGGAACCATCT-3′	[7]
	Reverse	5′-AGAGCTGCAGAAATCAGGAAGGCT-3′	
Caspase 3	Forward	5′-TGTGAGGCGGTTGTGGAAGAGT-3′	[8]
	Reverse	5′-AATGGGGGAAGAGGCAGGTGCA-3′	

2.10. Flow cytometry

A 1-mg/ml solution of propidium iodide was diluted to 1:1000 in PBS. After two washes with PBS, the cells were incubated with the diluted solution for 15 min in the dark. Next, the cells were detached using 0.25% trypsin. After centrifugation, the supernatant was aspirated, and the cells were reconstituted in 1 ml of PBS and passed through a 30 μm filter. The samples were then analyzed using a FACS Aria III system (BD Biosciences, San Jose, CA, USA). The data were analyzed using Flowing Software (Turku Centre for Biotechnology, Turku, Finland).

2.11. Statistical analysis

Data from the MTT assay, areas of propidium iodide (PI) staining, percentages of damaged cells determined by flow cytometry, and the contractility of iPS-CMs are expressed as means ± standard errors of the means. Comparisons between the two groups were conducted using Student's t-test. Comparisons between multiple groups were conducted using a one-way analysis of variance (ANOVA) with the Bonferroni post-hoc test. PRISM software (version 5.01; GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis. Differences were considered significant at a p value < 0.05.

3. Results

3.1. Cellular viability of H9c2 cells under ischemic conditions

We performed an MTT assay to measure cellular viability in the presence or absence of FBS (10% or 0%), glucose (2 or 0 mg/ml), and oxygen (21% or 2%; Fig. 1A). The mean absorbance in each well, an indicator of cell viability, was 1.55 ± 0.20 in the presence of FBS, glucose, and oxygen (control condition). Conditions of FBS/glucose, glucose/oxygen, and FBS/glucose/oxygen deprivation reduced the cell viability to 0.82 ± 0.05 ($p < 0.001$ relative to control), 0.59 ± 0.06 ($p < 0.001$ relative to control), and 0.59 ± 0.05 ($p < 0.001$ relative to control), respectively. Hereafter, the condition with FBS, glucose, and oxygen was defined as "healthy", whereas the condition lacking all three factors was defined as "ischemic."

3.2. H9c2 cell damage induced by the ischemic condition

Next, we performed PI staining to assess the extent of cell damage. Although almost no damage was observed under the control condition, evident damage was observed in the ischemic group (Fig. 1B and C). The area of PI staining was significantly larger in the ischemic group than in the control group (0.82 ± 0.06 vs 0.10 ± 0.03, $p < 0.0001$).

Flow cytometry also revealed an increase in PI-stained cells indicative of cell damage. The forward scatter intensity was lower in the ischemic group than that in the control group (Fig. 1D), suggesting a decrease in cell size. Moreover, the ratio of PI-positive cells was higher in the ischemic group (89.0%) than in the control group (12.4%) (Fig. 1E).

3.3. Induction of cardiomyocyte differentiation from hiPS cells

Next, we differentiated human iPS cells into cardiomyocytes to develop a model of myocardial infarction. Typically, the hiPS-CMs exhibited spontaneous contractions at approximately day 14 (supplementary video). Cardiac troponin T (TNNT2) staining to assess the cardiac differentiation of iPS revealed a typical pattern of TNNT2 striation in hiPS-CMs, with an approximate sarcomere length of 1.54 μm (Fig. 2B).

3.4. Induction of hiPS-CM damage by the ischemic condition

The viability of hiPS-CMs was measured using the MTT assay. Notably, higher absorbance was observed in the control group than in the ischemic group (1.35 ± 0.07 vs 0.73 ± 0.03, $p < 0.0001$; Fig. 3A). Consistent with the findings in H9c2 cells, flow cytometry revealed a reduction in the forward scatter intensity in hiPS-CMs in the ischemic group, compared with those in the control group (Fig. 3B). Moreover, the ratio of PI-positive cells was higher in the ischemic group than in the control group ($68.0 \pm 8.7\%$ vs $20.9 \pm 7.5\%$, $p < 0.02$; Fig. 3C and D). We further evaluated the contractility of hiPS-CMs using a displacement vector analysis and revealed reduced contractility in cells exposed to hypoxia than in cells exposed to the non-hypoxic control condition (0.23 ± 0.08 vs 0.77 ± 0.08, $p < 0.005$; Fig. 3E and F).

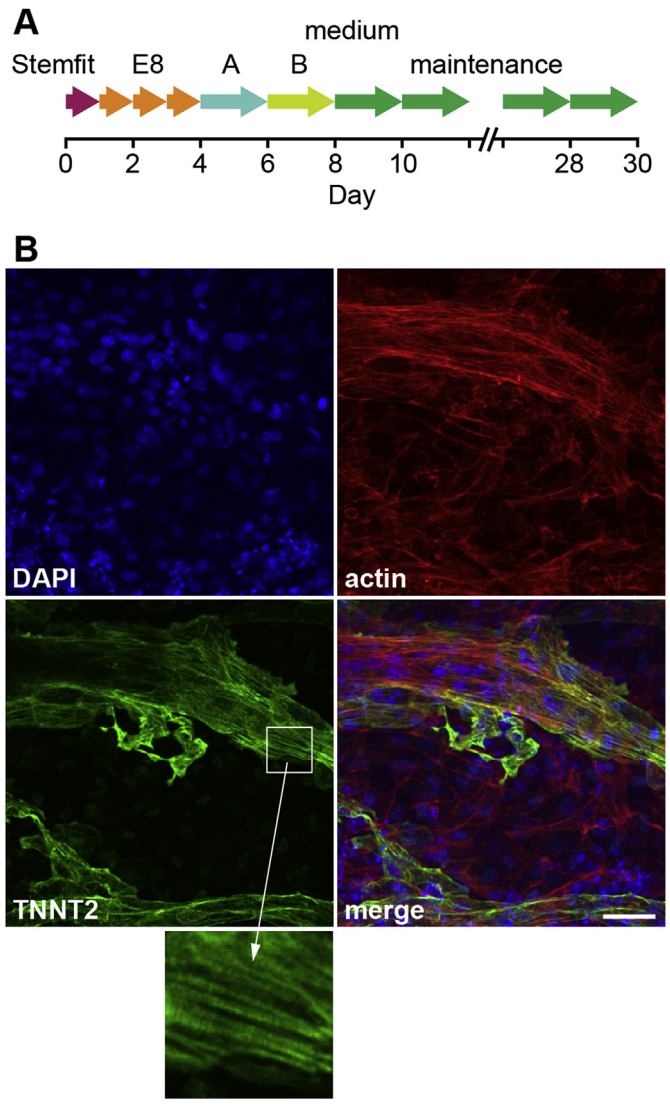

Fig. 2. Cardiac differentiation of human induced pluripotent stem (hiPS) cells. (A) Schematic of the hiPS cell differentiation protocol. StemFit, E8, A, B, and maintenance indicate the types of media used at each stage. (B) Expression of the cardiac marker protein cardiac troponin T (TNNT2). Blue: 4′,6-diamidino-2-phenylindole (DAPI), red: actin, green: TNNT2. Inset: striated expression of TNNT2, which corresponds to the sarcomere structure. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Gene expression analysis of inflammation-related genes

Finally, we used qPCR to evaluate the expression of genes related to inflammation in hiPSC-CM. Exposure to ischemic conditions increased the expression of *TNFa*, *IL1b*, *IL6*, and *IL8* mRNAs relative to controls (1.28 ± 0.35, 3.58 ± 2.14, 1.99 ± 1.50, and 16.02 ± 6.25, respectively; Fig. 4A). The ΔCt values were calculated to compare gene expression between the control and ischemic conditions and compared using Student's t-test in accordance with the method of Yuan et al. [9]. Although the differences in ΔCt values corresponding to *TNFa*, *IL1b*, and *IL6* mRNAs did not differ significantly between the control and ischemic groups, a significant difference was observed in the ΔCt values corresponding to IL-8 mRNA (Fig. 4B).

4. Discussion

We developed a model of human ischemic heart disease using contracting cardiomyocytes differentiated from hiPS cells. This model is physiologically more similar to the human heart than models based on conventional cell lines. Currently, two major cell culture methods are available for mimicking ischemic heart diseases, namely reduced exposure to oxygen and nutrients [10] and exposure to hydrogen peroxide, which induces cell damage [11]. In this study, we exposed rat H9c2 cardiomyocytes to oxygen and glucose concentrations that had been reduced to 2% and 0 mg/ml, respectively, to mimic the conditions of ischemic heart disease. Subsequently, we found that the same ischemic conditions induced damage in hiPS-CMs, as indicated by the observed decrease in cellular viability (Fig. 3A), increase in PI staining (Fig. 3B–D), and decrease in spontaneous contractility (Fig. 3E and F). In summary, our results suggest that our low-oxygen/low-glucose protocol induces cardiomyocyte damage that mimics the effects of ischemic heart disease.

An existing ischemic heart disease model based on mouse iPS-derived cardiomyocytes is useful due to the more rapid cardiomyocyte differentiation process relative to that required for human cells [12]. However, mouse and human cardiomyocytes exhibit several differences, such as the firing pattern of the action potential [3] and expression of ion channel genes in the sinus node [13]. In this study, our use of hiPS-CMs was advantageous because these cells enabled the development of a model of ischemic heart disease based on hiPS cells from individual subjects. In a similar previous study, Mo et al. developed an ischemic heart disease model based on human iPS-CMs. However, while Mo and colleagues focused on the inhibitory effects of microRNA 30e-5p on the development of hypoxia-induced apoptosis in cardiac cells, we focused on the pathophysiological conditions associated with ischemic damage on hiPS-CMs, particularly the changes in contractility and gene expression.

Acute myocardial infarction, a major cause of death, is partially attributable to the effects of ischemia–reperfusion injury [14]. Ischemia–reperfusion injury-induced cell damage is caused by several factors, including peroxidation of the plasma membrane, opening of the mitochondrial permeability transition pore, activation of caspases, and subsequent induction of inflammation [15]. Kukielka et al. reported that ischemia–reperfusion induced *IL8* expression in canine cardiomyocytes, which induced the adhesion of inflammatory neutrophils to the cardiomyocytes [16]. In this study, we observed increased *IL8* mRNA expression in hiPS-CMs after exposure to the ischemic condition. Although we did not intentionally include a reperfusion phase in our experimental procedure, the time lag between the cessation of oxygen/glucose deprivation and extraction of RNA might have enabled the reoxygenation of hiPS-CMs, a main step in the ischemia–reperfusion

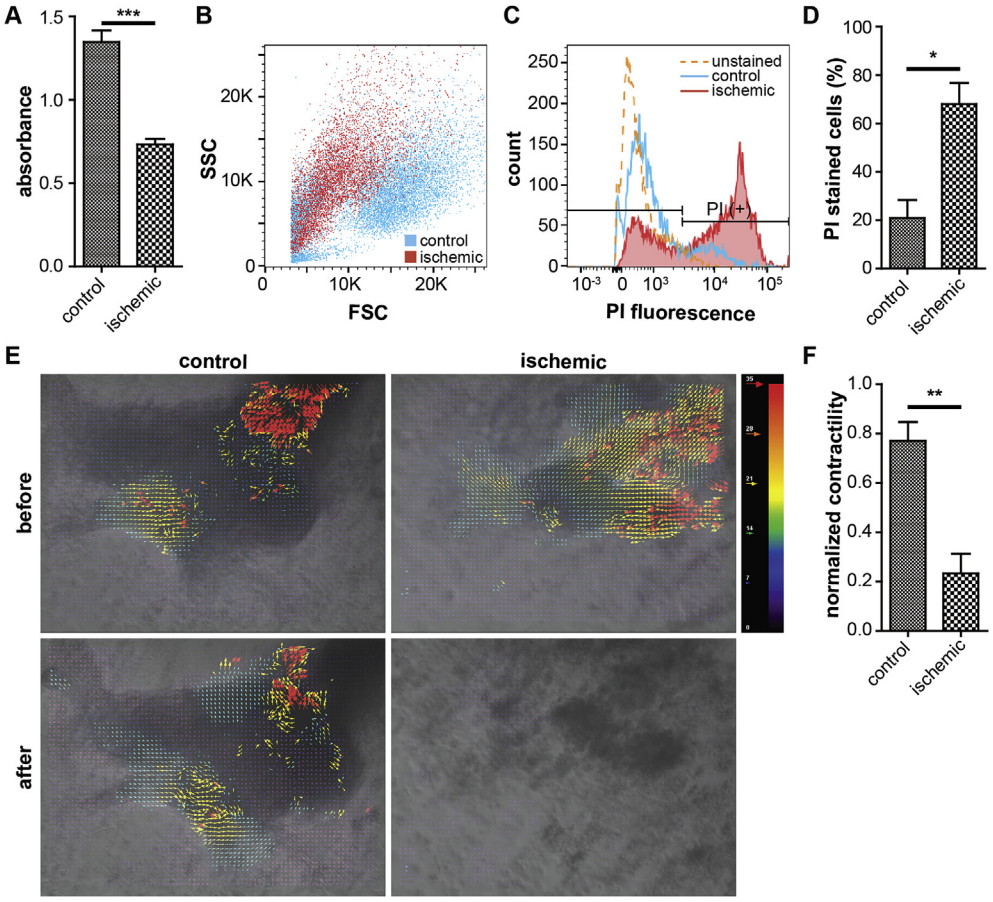

Fig. 3. Ischemic conditions damage cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CMs). (A) Comparison of cellular viability (MTT assay). (B, C, and D) Flow cytometry analysis of propidium iodide (PI)-stained cells. As in Fig. 1D and E, ischemic cells exhibited a reduced forward scatter intensity and increased PI fluorescence, compared with the control. (D) Percentage of PI-stained cells in the flow cytometry analysis. (E) Analysis of iPS-CMs contractility using ImageJ software. The red and blue vectors indicate the largest and smallest contractions, respectively. (F) Quantitative analysis of the iPS-CM contractility. *: $p < 0.05$, **: $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

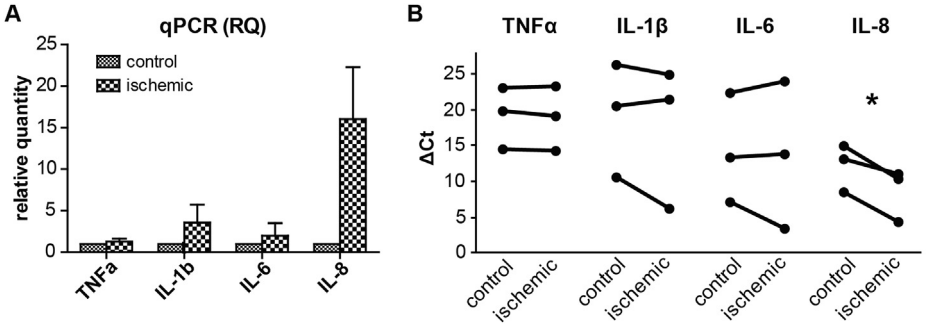

Fig. 4. Quantitative real-time PCR-based analysis of inflammation marker gene expression. (A) Comparison of mRNA expression levels in cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CMs) exposed to control and ischemic conditions. TNFa: tumor necrosis factor-alpha. Bars indicate the standard errors of the means. (B) Statistical analysis of the gene expression ΔCt values from (A). *: $p < 0.05$.

process. The observation that our model mimicked the *in vivo* increase in *IL8* expression is an important outcome of our study.

Although our hiPS-CM model advantageously mimicked human ischemic heart disease, some limitations should be noted. For example, our model did not include important factors such as leukocyte-induced tissue damage and complement system activation. Moreover, the percentage of differentiated cardiomyocytes in the model requires optimization. Nevertheless, our newly developed model of human ischemic heart disease is relatively simple and easy to reproduce and can be used in applications, such as drug screening using patient-derived iPS cells. Our model can also further elucidate the mechanism underlying ischemic heart diseases.

Author contributions

H.W. and K.T. wrote the main manuscript text. H.W. performed the experiments. C.W. and R.G. supervised the experimental

framework. K.N. supervised the analyses. All the authors discussed the analyses and results.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.119.

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