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ARTICLE

Minimal contribution of IP_3R2 in cardiac differentiation and derived ventricular-like myocytes from human embryonic stem cells

Peng Zhang[1,2], Ji-jun Huang[1], Kun-fu Ou-yang[3], He Liang[1], Miao-ling Li[4], Yi-jie Wang[1] and Huang-tian Yang[1,2,5]

Type 2 inositol 1,4,5-trisphosphate receptor (IP_3R2) regulates the intracellular Ca^{2+} release from endoplasmic reticulum in human embryonic stem cells (hESCs), cardiovascular progenitor cells (CVPCs), and mammalian cardiomyocytes. However, the role of IP_3R2 in human cardiac development is unknown and its function in mammalian cardiomyocytes is controversial. hESC-derived cardiomyocytes have unique merits in disease modeling, cell therapy, and drug screening. Therefore, understanding the role of IP_3R2 in the generation and function of human cardiomyocytes would be valuable for the application of hESC-derived cardiomyocytes. In the current study, we investigated the role of IP_3R2 in the differentiation of hESCs to cardiomyocytes and in the hESC-derived cardiomyocytes. By using IP_3R2 knockout (IP_3R2KO) hESCs, we showed that IP_3R2KO did not affect the self-renewal of hESCs as well as the differentiation ability of hESCs into CVPCs and cardiomyocytes. Furthermore, we demonstrated the ventricular-like myocyte characteristics of hESC-derived cardiomyocytes. Under the α_1-adrenergic stimulation by phenylephrine (10 μmol/L), the amplitude and maximum rate of depolarization of action potential (AP) were slightly affected in the IP_3R2KO hESC-derived cardiomyocytes at differentiation day 90, whereas the other parameters of APs and the Ca^{2+} transients did not show significant changes compared with these in the wide-type ones. These results demonstrate that IP_3R2 has minimal contribution to the differentiation and function of human cardiomyocytes derived from hESCs, thus provide the new knowledge to the function of IP_3R2 in the generation of human cardiac lineage cells and in the early cardiomyocytes.

Keywords: IP_3R2; human embryonic stem cells; differentiation; cardiovascular progenitor cells; cardiomyocytes; function

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INTRODUCTION

Ca^{2+} signals participate in various aspects of life processes [1, 2], including development [3, 4] and cardiac function [5]. Dysregulated Ca^{2+} signaling correlates with heart diseases [6, 7]. Thus, the elucidation of Ca^{2+} regulatory mechanisms will provide new knowledge in the understanding of heart development, functional maintenance, and disease control.

The cardiac differentiation system of human embryonic stem cells (hESCs) mimics the early developmental process of human hearts [8]. Differentiated cardiomyocytes are structurally and functionally similar to human fetal cardiomyocytes [9, 10]. These properties confer the model of cardiac differentiation of hESCs and derived cardiac lineage cells as a unique model/source for the study of human heart development, heart disease, drug development, and cell therapy [11–15]. However, the contributions of Ca^{2+} signals and Ca^{2+} handling proteins in the cardiac differentiation of hESCs and derived cardiac lineage cells have not yet been fully clarified.

The endoplasmic reticulum (ER) is a major intracellular Ca^{2+} storage site in eukaryotic cells. It plays an important role in balancing intracellular Ca^{2+} homeostasis through Ca^{2+} channels and pumps located in the ER membrane [16–20]. Inositol 1,4,5-trisphosphate receptors (IP_3Rs), which include three subtypes (IP_3R1, IP_3R2, and IP_3R3), and ryanodine receptors, are the two types of Ca^{2+} release channels located on the ER membrane. However, IP_3Rs are the predominant Ca^{2+} release channels expressed in ESCs and early differentiating cells from ESCs, since ryanodine receptors are hardly detected at these stages [17, 20–22]. These properties of IP_3Rs suggest that they might participate in early cell fate decisions. Accordingly, we found that IP_3R3 deficiency inhibits the cardiac differentiation of mouse (m)ESCs by increasing apoptosis in mesoderm cells [23]. However, in vivo studies have revealed that mice with a single deletion of either *Itpr1*, *Itpr2,* or *Itpr3* display normal cardiogenesis [24]. On the other hand, mice with double deletions of *Itpr1* and *Itpr2* die in utero with defects in the ventricles and atrioventricular canal at embryonic day 11.5 [24, 25]. In addition, triple knockout of all three types of *Itpr* genes enhances cardiomyocyte differentiation but suppresses hematopoietic differentiation of mESCs [26]. These findings suggest that

[1]CAS Key Laboratory of Tissue Microenvironment and Tumor, Laboratory of Molecular Cardiology, Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences (CAS) CAS, Shanghai 200031, China; [2]Translational Medical Center for Stem Cell Therapy & Institute for Regenerative Medicine, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200123, China; [3]School of Chemical Biology and Biotechnology, State Key Laboratory of Chemical Oncogenomics, Peking University Shenzhen Graduate School, Shenzhen 518055, China; [4]Key Laboratory of Medical Electrophysiology of Ministry of Education, Medical Electrophysiology Key Lab of Sichuan province, Institute of Cardiovascular Research, Southwest Medical University, Luzhou 646000, China and [5]Institute for Stem Cell and Regenerative, CAS, Beijing 100101, China
Correspondence: Huang-tian Yang (htyang@sibs.ac.cn)

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the role of *ip3rs* in mouse cardiac development is complicated. However, most of our knowledge on the function of IP_3Rs in cardiac development and cardiomyocytes comes from animal models. The precise function of Ca^{2+} signaling and IP_3Rs in early cardiac development and fetal cardiomyocytes in humans remains largely unknown.

The function of IP_3Rs in adult cardiomyocytes is also ambiguous. IP_3R2 has been proposed to be the predominant isoform among the three subtypes of IP_3Rs in working cardiomyocytes [27, 28]. It has also been shown that IP_3R2 regulates the firing rate in rabbit ventricular cardiomyocytes [29] and mouse pacemaker cells [30]. However, deletion of IP_3R2 in mice did not cause obvious changes in baseline cardiac function [24, 31]. The positive inotropic effect of IP_3R2 in mouse cardiomyocytes by activation of G_q protein [32, 33] raises the possibility that IP_3R2 is involved in stress-induced heart disease. This is supported by the upregulation of IP_3R2 in the hypertrophic and failing heart [33–35]. However, deletion of IP_3R2 in mice does not alter the progression of dilated cardiomyopathy or pressure overload-induced hypertrophy [31].

IP_3R2 is expressed in human cardiomyocytes as detected by RNA-seq [36] and Western blot [33], but the roles of IP_3Rs in human cardiac development and heart function are unclear. We recently found that IP_3R2 knockout (IP_3R2KO) significantly inhibits the increase in the intracellular concentration of Ca^{2+} stimulated by ATP or UTP in both hESCs and hESC-derived cardiovascular progenitor cells (hCVPCs) [37], suggesting that IP_3R2 might contribute to the cardiac differentiation of hESCs and the function of hESC-derived cardiomyocytes. In addition, endothelin-1 (ET-1) is highly expressed in $ISL1^+$ cardiac progenitors in human embryos, and the expansion of $ISL1^+$ cardiac progenitors derived from hESCs is dependent on ET-1 [38]. Given the direct activation of IP_3Rs by ET-1 through the G_q protein [39, 40], it is intriguing to determine the role of IP_3R2 in the cardiac differentiation of hESCs and in hESC-derived cardiomyocytes.

In the present study, using the in vitro cardiomyocyte differentiation model of hESCs combined with IP_3R2KO hESCs, we examined (i) the role of IP_3R2 in the generation of CVPCs and cardiomyocytes from hESCs and (ii) the role of IP_3R2 in the function of ventricular-like cardiomyocytes derived from hESCs. These results increase our knowledge of the contribution of Ca^{2+} handling proteins to early cardiac development in humans and to the functional maintenance of early-developing cardiomyocytes.

MATERIALS AND METHODS

hESC culture and in vitro differentiation

hESC culture was carried out as previously described [37, 41, 42]. Briefly, the hESC H7 cell line (WiCell Research Institute, Madison, WI, USA) was maintained in mTeSR1 medium (Stem Cell Technologies, Vancouver, Canada) on Matrigel (Corning, New York, NY, USA)-coated dishes.

For cardiomyocyte differentiation, hESCs were induced following a modified monolayer differentiation protocol as reported previously [43, 44]. Briefly, hESCs were seeded onto Matrigel-coated 12-well plates at a density of 2.5×10^4 cells/cm^2 in mTeSR1 with 10 μmol/L Y-27632 (a ROCK inhibitor, Stem Cell Technologies), and then the medium was changed to one without Y-27632. After the hESCs reached 100% confluence, cardiac differentiation medium (CDM3) containing RPMI-1640 (Gibco, Carlsbad, CA, USA), 213 μg/mL *L*-ascorbic acid 2-phosphate (Sigma-Aldrich, Carlsbad, USA), and 2 mg/mL bovine serum albumin (Sigma-Aldrich) was used to induce cardiomyocyte differentiation. For the first 2 days of cardiac differentiation, CHIR99021 (a glycogen synthase kinase-3β inhibitor, Stem Cell Technologies) at 6 μmol/L was added to CDM3. Then, the medium was changed to CDM3 supplemented with the Wnt signaling inhibitor IWR-1 (Sigma-Aldrich) at 5 μmol/L on day 3 and day 4, followed by CDM3 alone until differentiation day 90 (Fig. S1).

For CVPC induction, hESCs were seeded onto Matrigel-coated 6-well plates at a density of 3.5×10^4 cells/cm^2 in CVPC induction medium (CIM) for 3 days as reported previously [37, 41, 45]. The CIM contained DMEM/F12, 1×B27 supplement without vitamin A, 1% *L*-glutamine, 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), and supplemented with 400 μmol/L *L*-thioglycerol (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sigma-Aldrich), 25 ng/mL bone morphogenetic protein 4 (R&D Systems, Minneapolis, MN, USA), and 3 μmol/L CHIR99021 (Stem Cell Technologies).

Flow cytometry analysis

For the cell cycle analysis, cells were stained with 50 μg/mL propidium iodide (Sigma-Aldrich) before analysis. The cells were then analyzed by flow cytometry (Gallios, Beckman Coulter, Brea, CA, USA). The data were analyzed by ModFit software.

For the characterization of CTNT-positive cells, the cells were digested with 0.05% trypsin (Gibco). Then, the cells were fixed and permeabilized by a Foxp3 Staining Buffer kit (Invitrogen). Unconjugated CTNT antibody (1:200, Abcam, Cambridge, UK) was used, followed by staining with PE-Cy7-conjugated secondary antibody (1:400; eBioscience, San Diego, USA). The cells were then analyzed by flow cytometry (Gallios, Beckman Coulter) and quantified by FlowJo software.

Immunocytochemical staining

Immunocytochemical staining was performed as previously described [42]. Briefly, cells were fixed with 4% PFA, permeabilized with 0.4% Triton X-100 (Sigma-Aldrich), and blocked in 10% goat serum (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used were as follows: OCT4 antibody (1:200, Abcam); SSEA4 antibody (1:200, Millipore, CA, USA); SOX2 antibody (1:200, Abcam); Nkx2.5 antibody (1:200, Santa Cruz Biotechnology, Dallas, TX, USA); ISL1 antibody (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA); CTNT antibody (1:200, Abcam); MLC2V antibody (1:100, Abcam) and α-ACTININ antibody (1:400, Sigma-Aldrich). Alexa 488- or 569-conjugated secondary antibody (Invitrogen) was used for detection. Nuclei were stained with DAPI (Sigma-Aldrich). Images were captured by a Zeiss LSM 710 confocal microscope.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with a RNeasy Mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions and then reverse-transcribed by using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). qRT-PCR was performed using the ViiA 7 Real-Time PCR System (Life Technologies) with SYBR Green qPCR Master Mix (Roche, Mannheim, Germany). The results are presented as fold changes normalized to *GAPDH*. The qRT-PCR primers are listed in Table S1.

Western blot analysis

The cells were harvested and lysed in lysis buffer containing 8 mol/L urea, 2 mol/L thiourea, 3% sodium lauryl sulfate, 75 mmol/L 1,4-dithiothreitol, 50 mmol/L TRIS, and 0.03% bromophenol blue (pH adjusted to 6.8) (Sigma-Aldrich). The antibodies against IP_3R1 and IP_3R2 were generated as previously reported [46]. The membranes were incubated with primary antibodies against IP_3R1 (1:1000), IP_3R2 (1:1000), IP_3R3 (1:1000, BD Biosciences, San Jose, CA, USA), GAPDH (1:20000, Proteintech, Rosemont, IL, USA), and β-actin (1:8000; Sigma-Aldrich) in 3% BSA. IRDye 680LT donkey anti-rabbit IgG or IRDye 800LT donkey anti-mouse IgG (1:8000; Li-COR Biosciences, Lincoln, NE, USA) was used for detection. Images were captured using an Odyssey Infrared Imager (Li-COR Biosciences).

Karyotype analysis

Karyotype analysis was conducted as previously reported [47]. Briefly, hESCs were treated with colchicine (100 μg/mL) for 3 h at 37 °C and then harvested as single cells. The cells were hypotonic in 75 mmol/L KCl at 37 °C for 30 min and then fixed by freshly prepared fixative (glacial acetic acid:methanol = 1:3). After dropping the solution onto clean slides, the samples were stained with Giemsa staining solution. Twenty metaphase cells were counted, of which five cells were analyzed and karyotyped in each cell line.

Recording of Ca^{2+} transients

Cardiomyocytes derived from hESCs at differentiation day 90 were digested using 0.05% trypsin and plated in glass bottom cell culture dishes (Wuxi NEST Biotechnology, Wuxi, China). After 24 to 48 h of plating, the cells were loaded with 2 μmol/L Fluo4-AM (Life Technologies) dissolved in Tyrode's buffer for 15 min at room temperature (RT). Tyrode's buffer contained (in mmol/L) NaCl, 135; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1.0; glucose, 10; and HEPES, 10 (pH adjusted to 7.4). Then, the Ca^{2+} indicator was washed off 3 times, and the cells were incubated at RT for 15 min before use. The Ca^{2+} transients were captured in the line scan model using a Zeiss LSM 710 confocal microscope. A total of 5000 lines with an interval time of 10 ms were scanned. During the recording, the cells were maintained at 35 °C in a heated chamber. Phenylephrine (PE) (an α_1-adrenergic receptor agonist) was used at 10 μmol/L. The Ca^{2+} transients were analyzed with IDL software (ITT Corporation, White Plains, NY, USA).

Recording of action potentials (APs)

APs were recorded as previously described [48]. Briefly, cardiomyocytes derived from hESCs at differentiation day 90 were digested and replated onto coverslips. The cells were then recorded for APs within 24–48 h after plating. The temperature was maintained at 33 °C by perfusion with warm Tyrode's buffer by a peristaltic pump (Cole-Parmer, IL, USA). The internal solution contained (in mmol/L): K^+-aspartate,110; KCl, 20; $MgCl_2$, 1; NaGTP, 0.1; MgATP, 5; Na_2-phospocreatine, 5; EGTA, 1; and HEPES, 10; pH adjusted to 7.3 with KOH. APs were recorded using the EPC-10 amplifier (Heka Electronics, Bellmore, NY, USA) in current-clamp mode. PE was used at 10 μmol/L. The characterization of the cardiomyocyte subtypes was based on previously reported criteria [44]. Briefly, for ventricular-like cardiomyocytes, AP amplitude > 90 mV, AP duration at 90% repolarization/AP duration at 50% repolarization (APD)90/APD50 < 1.4, a rapid AP upstroke, a long plateau phase, and a negative maximum diastolic potential (MDP) (<−48 mV); for atrial-like cardiomyocytes, an absence of a prominent plateau phase, a negative diastolic membrane potential (<−48 mV) and APD90/APD50 > 1.7; and for Nodal-like cardiomyocytes, a more positive MDP, a slower AP upstroke, a prominent phase 4 depolarization and APD90/APD50 between 1.4 and 1.7.

Statistical analysis

The data are presented as the mean ± SEM. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple analysis was used for the qRT-PCR assay of *ITPRs*, cell cycle distribution and flow cytometry analysis of CTNT. Two-way ANOVA with Bonferroni's multiple comparison test was used to analyze the qRT-PCR data for the expression of different genes during cardiomyocyte differentiation and the quantitative properties of APs and Ca^{2+} transients of the three cell lines. A paired *t* test was used to analyze the quantitative properties of APs and Ca^{2+} transients with or without PE. All of the statistical analyses were conducted using GraphPad Prism8. $P < 0.05$ was considered statistically significant.

RESULTS

Differentiation of hESCs to dominant ventricular-like cardiomyocytes

To determine the role of IP_3R2 in the fate of human ventricular cardiomyocytes and the function of hESC-derived cardiomyocytes, we differentiated hESCs into cardiomyocytes following a protocol reported previously [43, 44] with a small modification (Fig. S1a). Spontaneously beating cardiomyocytes appeared on differentiation day 7, and robust beating cardiomyocytes were observed on differentiation day 8 (Movie S1). Flow cytometry analysis confirmed that over 90% of cells were positive for the cardiac-specific marker CTNT at differentiation day 90 (Fig. S1b). The differentiated cardiomyocytes showed a well-arranged sarcomere structure (Fig. S1c). To determine the subtypes of differentiated cardiomyocytes, we recorded the APs of the cells at differentiation day 90. The examined cardiomyocytes derived from wild-type (wt) hESCs showed typical ventricular-like APs (Fig. S1d) with an APD90/APD50 ratio of 1.17 ± 0.01 (Fig. S1e) based on previously reported criteria [44]. These data demonstrate that cardiomyocytes differentiated from hESCs around day 90 are mainly ventricular-like cardiomyocytes.

IP_3R2 is highly expressed in undifferentiated hESCs and downregulated in differentiated cardiomyocytes

Next, we examined the expression profile of IP_3R2 during cardiomyocyte differentiation. qRT-PCR analysis showed that the *ITPR2* gene was highly expressed in undifferentiated hESCs and was quickly downregulated during the first 2 days of differentiation. Then, it was upregulated at differentiation days 3 and 4 and gradually downregulated in the following days, reaching a relatively stable level from differentiation day 6 to day 90 (Fig. 1a). A similar pattern of IP_3R2 protein was observed during cardiac differentiation of hESCs by Western blot analysis (Fig. 1b).

Deficiency in IP_3R2 does not significantly affect the self-renewal of hESCs

To determine the role of IP_3R2 in cardiac differentiation, we used two IP_3R2KO hESC lines (IP_3R2KO-6 and IP_3R2KO-12) generated by using transcription activator-like effector nuclease (TALEN) technology as previously reported [37]. All of the wt, IP_3R2KO-6 and IP_3R2KO-12 hESCs showed the normal karyotype (Fig. S2). The deficiency in IP_3R2 was confirmed in IP_3R2KO-6 and IP_3R2KO-12 lines by Western blot, while the protein levels of other subtypes of IP_3Rs, i.e., IP_3R1 and IP_3R3 (encoded by the *ITPR1* and *ITPR3* gene, respectively), were not affected (Fig. 2a), which was consistent with the qRT-PCR analysis (Fig. 2b).

We previously found that IP_3R2 is a dominantly functional Ca^{2+} channel for mediating Ca^{2+} release from the ER in both mESCs [49] and hESCs [22, 37]. To test whether IP_3R2 deficiency affects the self-renewal of hESCs, we conducted alkaline phosphatase (ALP) staining, immunocytochemical staining and qRT-PCR. All wt, IP_3R2KO-6 and IP_3R2KO-12 hESCs were positive for ALP, OCT4, SSEA4 and SOX2 (Fig. 2c). The mRNA levels of *POU5F1* (encodes OCT4), *NANOG* and *SOX2* were also comparable between these three hESC lines (Fig. 2d). Furthermore, flow cytometry analysis did not detect a difference in the cell cycle distribution among the wt and two IP_3R2KO cell lines (Fig. 2e, f). Therefore, IP_3R2 seems to be dispensable for the maintenance of hESC self-renewal.

IP_3R2 is dispensable for the differentiation of hESCs into CVPCs and cardiomyocytes

The upregulated expression of IP_3R2 from differentiation day 3 to day 4 suggests a possible contribution of IP_3R2 in mediating cardiac progenitor formation, as this is a critical stage for the transition of cardiac mesoderm to cardiac progenitors [44]. To determine the effect of IP_3R2 on the generation of CVPCs from hESCs, we induced wt and IP_3R2KO cells to differentiate into

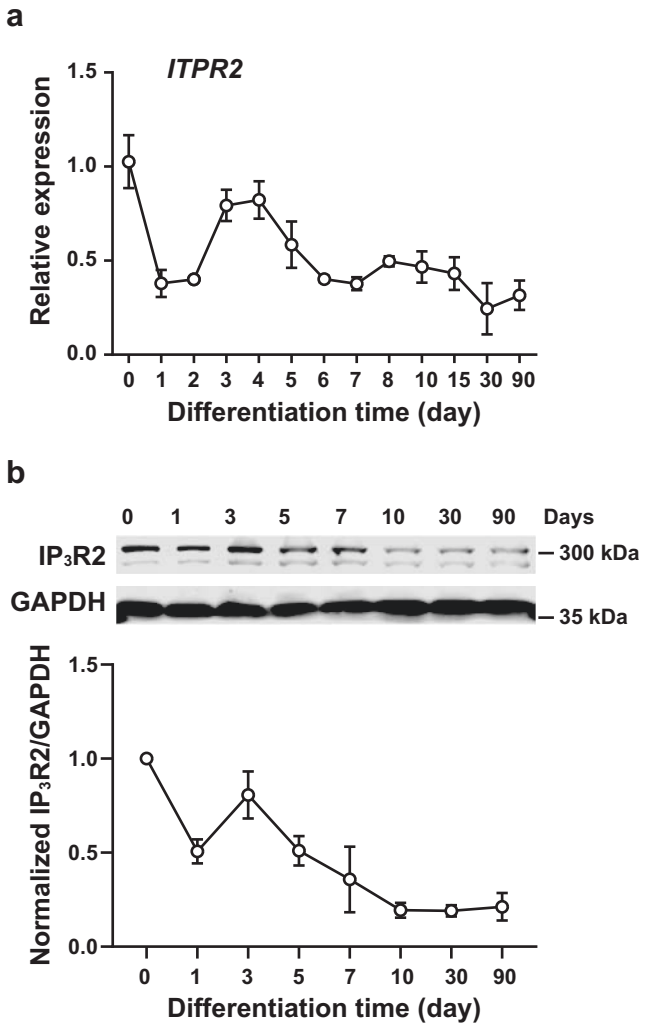

Fig. 1 The expression pattern of IP_3R2 during cardiomyocyte differentiation of hESCs. a qRT-PCR analysis of the expression of the *ITPR2* gene. $n = 3$. **b** Western blot analysis of IP_3R2 protein levels. GAPDH was used as the loading control. $n = 3$.

CVPCs by using a protocol established by our group [41, 45]. Immunocytochemical staining showed that all of the differentiating cells from the wt and IP_3R2KO cells uniformly expressed the primitive CVPC markers NKX2-5 and ISL1 in the nuclei (Fig. 3a). Next, to determine whether IP_3R2 affects cardiomyocyte generation, wt and IP_3R2KO hESCs were further induced to differentiate into cardiomyocytes. We found that the percentages of CTNT-positive cells were comparable between the wt and the two IP_3R2KO cell lines at differentiation day 90 (Fig. 3b). The clear and organized sarcomeric structure of induced cardiomyocytes was similar among the wt and IP_3R2KO cells (Fig. 3c). In addition, the cells were double positive for MLC2V and α-ACTININ (Fig. 3d). Notably, the fiber-like structures observed in the green and red channels were well colocalized (Fig. 3d), indicating that these cells were ventricular-like myocytes. These data are consistent with the observations of the typical ventricular-like myocyte APs shown in Fig. S1d and S1e. qRT-PCR analysis further confirmed that the genes were sequentially expressed following a tandem transition from pluripotency (*POU5F1*), mesoderm (*TBXT*), cardiac mesoderm (*MESP1*), cardiac progenitor (*ISL1* and *NKX2-5*) to cardiomyocytes (*TNNT2*) in the wt and two IP_3R2KO cell lines (Fig. 3e). These data suggest that IP_3R2 deficiency does not significantly affect the induction of CVPCs and cardiomyocytes from hESCs.

IP3R2KO mildly alters APs by stimulating α_1-adrenergic receptors in hESC-derived cardiomyocytes

As the functional contribution of IP_3R2 remains controversial in adult working cardiomyocytes [24, 33] and is unknown in early differentiated human cardiomyocytes, we examined whether IP_3R2 affects APs, a crucial feature of beating cardiomyocytes [50]. The spontaneously beating cardiomyocytes at differentiation day 90 showed typical ventricular-like APs (Fig. 4a), with small variations in the MDP and amplitude but large variations in the beats per minute (Bpm), maximum rate of rise (V_{max}) and depolarization (D_{max}), APD50 and APD90 in the examined cardiomyocytes (Fig. 4b–h). The features of APs in cardiomyocytes derived from IP_3R2KO hESCs were similar to those in the wt hESCs (Fig. 4a). To confirm this, we quantitatively analyzed the parameters of APs in these cells (Fig. 4b–h). The Bpm, amplitude, MDP, V_{max}, D_{max}, APD50, and APD90 in cardiomyocytes derived from IP_3R2KO hESCs were comparable to those in the wt cardiomyocytes. Next, we examined whether IP_3R2 deficiency affects APs under the stimulation of PE, an α_1-adrenergic receptor agonist that activates IP_3Rs through the G_q protein [51, 52]. Spontaneous APs from the same cardiomyocyte with and without PE treatment were recorded. PE stimulation significantly accelerated the beating frequency (Fig. 4a, b), accompanied by increased MDP (Fig. 4d) and decreased amplitude (Fig. 4c), V_{max} (Fig. 4e), APD50 (Fig. 4g), and APD90 (Fig. 4h), while D_{max} was unchanged (Fig. 4f) compared with the corresponding values in wt hESC-derived cardiomyocytes without PE treatment. In the IP_3R2KO hESC-derived cardiomyocytes, the Bpm, MDP, V_{max}, APD50, and APD90 were comparable with those in the wt cardiomyocytes (Fig. 4b, d, e, g, h). However, it is notable that the amplitude in IP_3R2KO-12 cardiomyocytes was decreased by 4.5% compared with that of wt cardiomyocytes, and a similar tendency was observed in IP_3R2KO-6 cardiomyocytes without statistical significance (Fig. 4b). In addition, the D_{max} in IP_3R2KO-6 cardiomyocytes was approximately 17% slower than that in wt cardiomyocytes, and the same tendency was observed in IP_3R2KO-12 cardiomyocytes (Fig. 4f). Overall, IP_3R2KO does not alter spontaneous APs under baseline conditions, but it appears to contribute to the amplitude and D_{max} of APs in hESC-derived cardiomyocytes under the stimulation of α_1-adrenergic receptors.

IP_3R2KO does not significantly affect Ca^{2+} transients in hESC-derived cardiomyocytes

We next examined the contributions of IP_3R2 to Ca^{2+} transients in hESC-derived cardiomyocytes. The rhythmic Ca^{2+} transients were observed in spontaneously beating cardiomyocytes (Fig. 5a). The parameters of Ca^{2+} transients among wt cardiomyocytes, such as the Bpm (Fig. 5b), amplitude (Fig. 5c), time to peak (Fig. 5d), time of decay to 90% (Fig. 5e) and 63% peak (T-90% decay and T-63% decay) (Fig. 5f), varied over a wide range as observed in the APs. The various parameters of Ca^{2+} transients in the cardiomyocytes derived from IP_3R2KO hESCs were similar to those in wt cells (Fig. 5a–f). With PE treatment, the Bpm was significantly increased in wt cardiomyocytes, accompanied by decreases in time to peak, T-90% decay, and T-63% decay, while the amplitude was unchanged (Fig. 5a–f). In the cardiomyocytes derived from the two IP_3R2KO hESC lines, the Bpm, amplitude, time to peak, T-90% decay and T-63% decay were comparable with those in the wt cell lines (Fig. 5b–f). Collectively, these data indicate that the stimulation of α_1-adrenergic receptors significantly increases the speed rising to the peak and recovery of Ca^{2+} transients in hESC-derived cardiomyocytes, although a large variation exists among cardiomyocytes. Moreover, IP_3R2 deficiency does not significantly affect Ca^{2+} transients in hESC-derived cardiomyocytes with or without α_1-adrenergic stimulation.

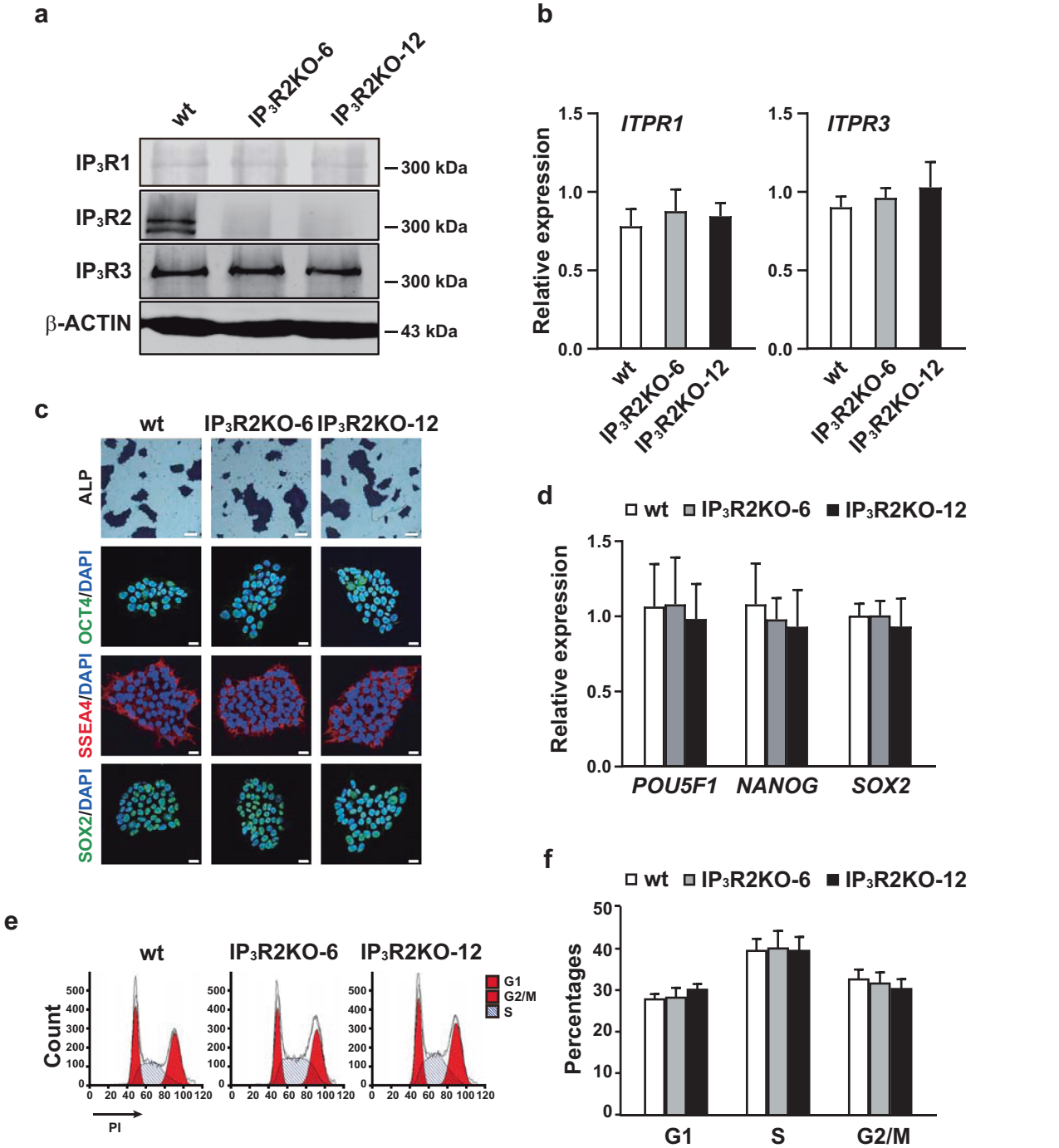

Fig. 2 The comparison of self-renewal properties of undifferentiated wt, IP$_3$R2KO-6, and IP$_3$R2KO-12 hESCs. a Western blot analysis of IP$_3$R1, IP$_3$R2 and IP$_3$R3 proteins. β-actin was used as the loading control. **b** qRT-PCR analysis of *ITPR1* and *ITPR3* gene expression. $n = 3$. **c** The alkaline phosphatase staining and immunocytochemical staining of OCT4, SSEA4 and SOX2. Scale bar = 100 μm (ALP). Scale bar = 10 μm (immunocytochemical staining). **d** qRT-PCR analysis of *POU5F1*, *NANOG* and *SOX2* gene expression. **e** The representative flow cytometry plots of cell cycle analysis. **f** The cell cycle analysis data. $n = 3$.

DISCUSSION

In this study, using the in vitro hESC cardiac differentiation model, combined with IP$_3$R2 knockout, Ca^{2+} imaging, and AP recording, we determined (i) the expression pattern of IP$_3$R2 during cardiomyocyte differentiation of hESCs; (ii) IP$_3$R2 deficiency does not affect the self-renewal of hESCs; (iii) IP$_3$R2 deficiency does not significantly affect CVPC and cardiomyocyte differentiation from hESCs; (iv) hESC-derived cardiomyocytes have ventricular-like APs but with large variations in the parameters of APs and Ca^{2+} transients under both baseline conditions and the stimulation of α_1-adrenergic receptors; (v) IP$_3$R2 deficiency decreases the amplitude and the D_{max} of APs in hESC-derived cardiomyocytes under the stimulation of α_1-adrenergic receptors but not under baseline conditions; and (vi) no obvious changes are observed in the Ca^{2+} transients in IP$_3$R2KO cardiomyocytes with or without the stimulation of α_1-adrenergic receptors. Our results suggest the minimal contribution of IP$_3$R2 in cardiac differentiation and derived ventricular cardiomyocytes from hESCs.

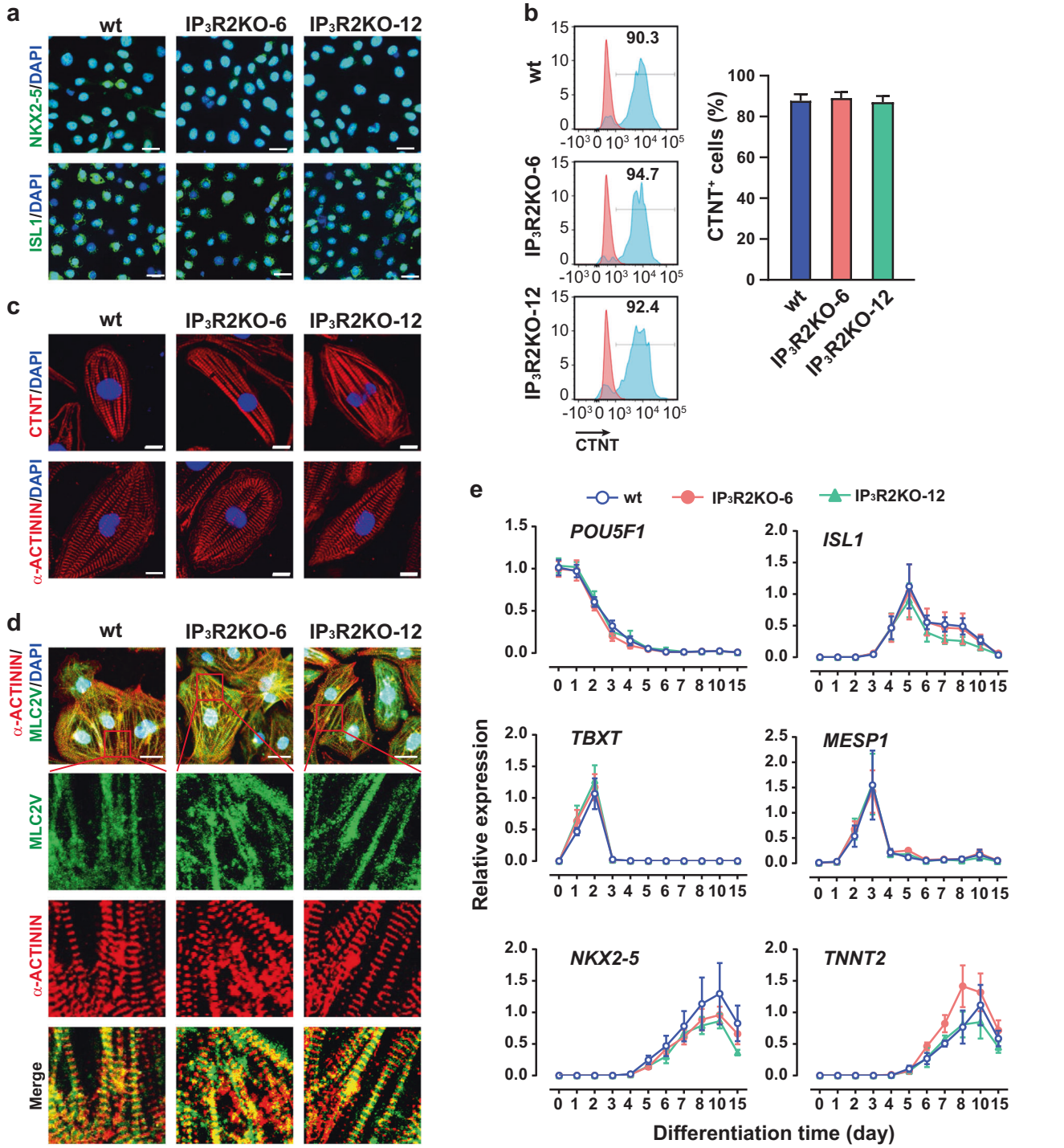

Fig. 3 Effects of IP_3R2 deficiency on the differentiation of hESCs into CVPCs and cardiomyocytes. a Immunocytochemical staining analysis of CVPC marker NKX2-5 and ISL1 in induced CVPCs. Scale bar = 40 μm. **b** Flow cytometry analysis of cardiomyocytes induced from wt, $IP_3R2KO-6$ and $IP_3R2KO-12$ hESCs with cardiomyocyte marker CTNT. $n = 3$. **c** Immunocytochemical staining analysis of cardiomyocyte marker CTNT and α-ACTININ in cardiomyocytes at differentiation day 90. Scale bar = 10 μm. **d** Co-immunocytochemical staining analysis of cardiomyocyte marker α-ACTININ and ventricular myocyte marker MLC2V at cardiac differentiation day 90. Scale bar = 20 μm. **e** The mRNA expression of genes during cardiac differentiation from hESCs. *POU5F1*, pluripotency marker; *TBXT*, early mesoderm marker; *MESP1*, cardiac mesoderm marker; *ISL1* and *NKX2-5*, cardiac progenitor markers; *TNNT2*, cardiomyocyte marker. $n = 3$.

One of the findings here is the determination of the expression patterns of IP_3R2 during the process of cardiomyocyte generation from hESCs. This observation is consistent with previous findings of the presence of IP_3R2 in undifferentiated hESCs, hESC-derived CVPCs [22, 37] and human adult cardiomyocytes [33]. Our findings support previous observations and precisely describe the specific expression patterns of IP_3R2 during the differentiation process of hESCs into cardiomyocytes, especially for ventricular-like myocytes, by revealing the following characteristics. First, the expression of IP_3R2 is downregulated during the formation and maturation of cardiomyocytes from hESCs. This pattern is similar to those previously observed in the generation of CVPCs [37] and

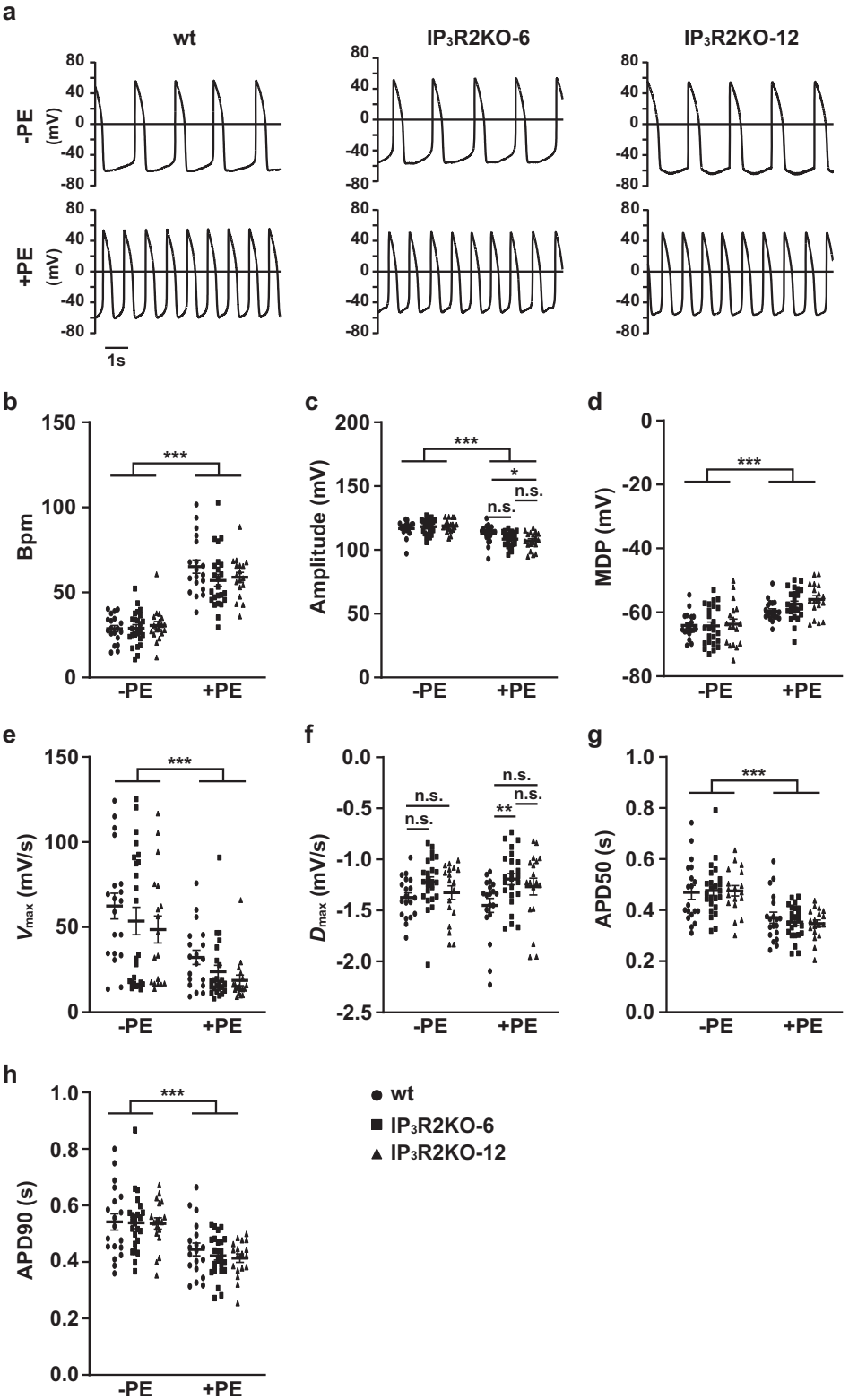

Fig. 4 Effects of IP_3R2 deficiency on the APs of wt and IP_3R2KO hESC-derived cardiomyocytes at differentiation day 90 with or without phenylephrine (PE) treatment. a Representative AP recordings. **b**–**h** Parameters of APs. Bpm, beats per minute (**b**); Amplitude (**c**); MDP, maximum diastolic potential (**d**); V_{max}, the maximum rate of rise of the AP (**e**); D_{max}, the maximum rate of depolarization (**f**); APD50, AP duration at 50% repolarization (**g**); APD90, AP duration at 90% repolarization (**h**). PE, phenylephrine at 10 μmol/L concentration. $n = 19$ for wt cardiomyocytes; $n = 24$ for IP_3R2KO-6 cardiomyocytes; $n = 18$ for IP_3R2KO-12 cardiomyocytes. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n.s., no significant difference.

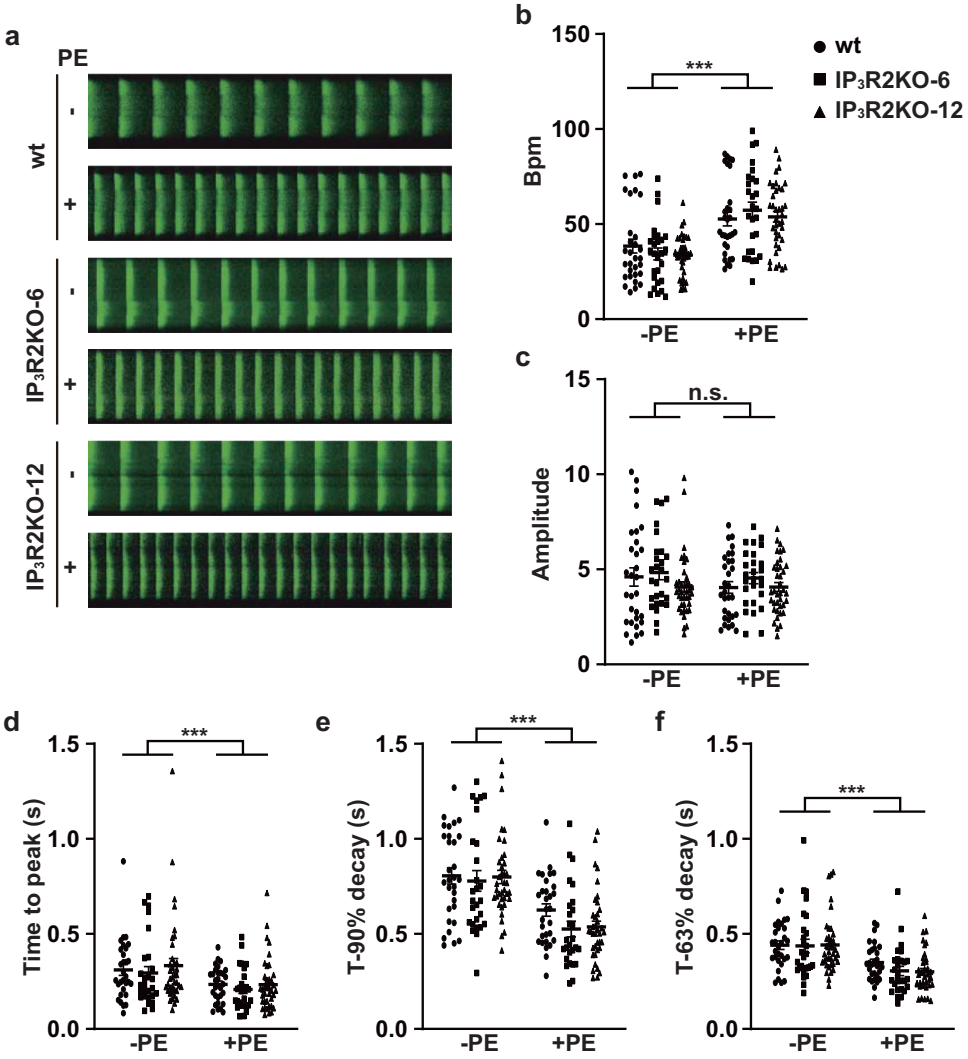

Fig. 5 Effects of IP_3R2 deficiency on Ca^{2+} transients of wt and IP_3R2KO hESC-derived cardiomyocytes at differentiation day 90 with or without phenylephrine (PE) treatment. a Representative images of spontaneous intracellular Ca^{2+}. **b**–**e** Parameters of spontaneous Ca^{2+} transients. The Bpm (**b**), amplitude ($\Delta F/F_0$, **c**), time to peak (**d**), time to 90% decay (**e**), and time to 63% decay (**f**) of the maximum amplitude. $n = 30$ for wt cardiomyocytes; $n = 27$ for IP_3R2KO-6 cardiomyocytes; $n = 38$ for IP_3R2KO-12 cardiomyocytes. $^{***}P < 0.001$ compared with the corresponding control group cells.

cardiomyocytes [53] from hESCs. However, notably, at the early differentiation stage (differentiation day 0 to day 1, representing the transition stage of undifferentiated hESCs to mesoderm cells), the expression of IP_3R2 was sharply downregulated, followed by upregulation at differentiation days 3 and 4 (representing the transition stage from mesoderm to cardiac mesoderm)(Fig. 1). This correlation between the expression of IP_3R2 and cell fate transition suggests the possible role of IP_3R2 in human lineage fate determination. Although it is unknown whether this transient down- and upregulation is involved in the tissue-specific linage fate determination in humans, our previous study showed that the abundance of IP_3R3 is transiently downregulated in the early differentiation stage of mESCs and that knockdown of IP_3R3 in mESCs significantly suppresses the differentiation into mesoderm and cardiomyocytes by specifically increasing the apoptosis of mesodermal cells due to the alternation of Ca^{2+} oscillation [23]. Considering the evidence for the existence of IP_3Rs in both hESCs [22, 37] and mESCs [23, 26, 54] and the functional overlap or negative feedback among these subtypes [1, 23, 25, 26, 55–57], the significance of the transient downregulation of IP_3R2 to the specific cell fate decision during early differentiation of hESCs needs to be elucidated in the future. Moreover, in this study, we demonstrate that the IP_3R2 protein exists in hESC-derived ventricular-like myocytes. We confirmed that the efficiently generated cardiomyocytes from hESCs have ventricular myocyte-like APs (Fig. S1d) and are positive for the ventricular marker MLC2V (Fig. 3d). Therefore, IP_3R2 exists in both differentiated human ventricular-like myocytes (Fig. 1) and human adult left ventricular myocytes [33].

Interestingly, although IP_3R2 is highly expressed in undifferentiated hESCs, it has little contribution to the maintenance of hESC self-renewal. However, the percentage of cells responding to MRS2365, a G_q protein-coupled $P2Y_1$ receptor agonist, is significantly decreased in IP_3R2KO hESCs [37], indicating the important role of IP_3R2 in mediating Ca^{2+} release from intracellular stores in hESCs. A possible interpretation for these observations might be that IP_3R2 deficiency could be compensated by other subtypes of IP_3Rs or Ca^{2+} modulators. Although the robust Ca^{2+} waves are mediated by IP_3R2, the intracellular Ca^{2+} concentration is stable with minor fluctuations in undifferentiated hESCs [22]. Thus, the small fluctuations might be enough for the maintenance of hESCs in the steady state. Moreover, in IP_3R2KO

hESCs, there are still some cells responding to MRS2365, despite a low percentage (<20%) [37], suggesting that IP_3R3 might compensate for the loss of IP_3R2. In addition, the intracellular Ca^{2+} channels and Ca^{2+} modulators located in the plasma membrane might also participate in the Ca^{2+} homeostats maintained after IP_3R2 deficiency. Therefore, the self-renewal property of IP_3R2KO hESCs is maintained. Accordingly, the functional redundancy of IP_3R subtypes is also detected in mice. The *Itpr1*, *Itpr2*, or *Itpr3* single mutants were indistinguishable from the control mice. No contribution of IP_3R2 to CVPC and cardiomyocyte generation could also be caused by the redundancy of other IP_3R subtypes and Ca^{2+} modulators. Indeed, cardiac progenitor and cardiomyocyte differentiation is enhanced, but the hematopoietic mesoderm is reduced in mESCs with triple knockout of *Itpr1*, *Itpr2,* and *Itpr3* [26]. Although it has been reported that ET-1, which can activate IP_3Rs through endothelin receptors [39, 40], is crucial in promoting the expansion of $ISL1^+$ cardiac progenitors from hESCs [38], the underlying mechanisms are not elucidated. Moreover, a recent report [58] argues that the heart defect is caused by double knockout of *Itpr1* and *Itpr2* [24]. Yang and colleagues [58] found that specific deletion of *Itpr1* and *Itpr2* in cardiomyocytes, endothelial/hematopoietic cells or early cardiovascular lineage progenitor cells of mice do not have phenotypes, while the embryonic lethality caused by double knockout of *Itpr1* and *Itpr2* may be due to allantoic-placental defects. Thus, the vascular abnormalities seen in *Itpr1* and *Itpr2* double knockout mice might not be due to the indirect influence but are a secondary effect on allantoic/placental defects. Overall, IP_3R2 deficiency alone does not affect cardiomyocyte differentiation in mice or humans.

Another interesting finding here is that the positive chronotropic effect of PE is not affected by IP_3R2 deficiency, and only mild changes in the amplitude and D_{max} of the APs in IP_3R2KO hESC-derived cardiomyocytes under PE stimulation are observed compared with those in wt cardiomyocytes (Fig. 4c, f). The positive chronotropic effect of PE in immature cardiomyocytes is mediated by α_1-adrenergic receptors [59]; however, the underlying mechanism remains unknown. The positive chronotropic effect induced by β-adrenergic receptor activation is related to the regulation of Ca^{2+} influx through the L-type Ca^{2+} channel [60–63]. This channel is also regulated by the activation of α_1-adrenergic receptors [64]. Thus, the chronotropic effect of PE in immature cardiomyocytes might also be mediated through L-type Ca^{2+} channels, although we could not exclude the participation of other channels, such as sodium channels or potassium channels. In addition, given that IP_3R2 can be located near the plasma membrane [28, 30, 53, 65], IP_3R2 might affect the voltage-gated ion channels on the plasma membrane by regulating the surrounding electrical environment, as hypothesized [66, 67], and this possibility needs to be tested.

Despite the possible role of IP_3R2 in the regulation of ion channels, the contribution of IP_3R2 to the functional properties of hESC-derived cardiomyocytes is weak. Under basal conditions, the APs and Ca^{2+} transients of the cardiomyocytes derived from wt and IP_3R2KO hESCs are similar. It has been shown that IP_3Rs contribute to the regulation of Ca^{2+} transients and/or contraction in mESC- [49] or hESC-derived cardiomyocytes [53, 65], isolated rabbit ventricular myocytes [28, 29], and adult human ventricular cardiomyocytes [33]. However, these conclusions are mainly based on the usage of agonists and antagonists of IP_3Rs, lacking confirmation by using direct gene interference. Therefore, the nonspecific effects of these reagents could not be excluded. For example, the commonly used pan-IP_3R antagonist 2-APB can also inhibit Ca^{2+} release-activated Ca^{2+} channels [68]. Thus, the results collected from studies without direct gene interference should be carefully interpreted. In the present study, the significant chronotropic response to the activation of α_1-adrenergic receptors with PE in wt cardiomyocytes is consistent with a previous report [59, 69], while the amplitude of Ca^{2+} transients remained unchanged under the stimulation of α_1-adrenergic receptors. However, activation of other G_q-coupled receptors in cardiomyocytes, such as ET-1 receptors and purinergic receptors, correlates with enhanced Ca^{2+} transients and increased contractility [33, 70]. Such differences may be due to the diversity of downstream cascades of different receptors. These possibilities need to be tested further. Notably, the PE-induced chronotropic response remained unchanged in IP_3R2KO cardiomyocytes. This may have several interpretations. (i) IP_3R2 minimally contributes to the function of hESC-derived cardiomyocytes. This is supported by the findings that heart function is normal in IP_3R2-deficient mice [24, 31]. (ii) The functional redundancy of other IP_3R subtypes may compensate for the loss of function of IP_3R2. (iii) The large variations in the different parameters of APs and Ca^{2+} transients indicate the heterogeneous maturation of hESC-derived cardiomyocytes, which may conceal the mild involvement of IP_3R2 in the function of early cardiomyocytes. Accordingly, the amplitude and recovery of APs under the activation of α_1-adrenergic receptors were altered in IP_3R2KO cardiomyocytes. (iv) Lastly, IP_3R2 might be involved in the regulation of cardiomyocyte function under long-term stimulation. In the current study, APs were recorded immediately after PE addition, reflecting the acute response of cardiomyocytes. In a mouse model, the overexpression of α_{1A}-adrenergic receptor [71] or IP_3R2 [35] results in cardiac hypertrophy, suggesting that the chronic activation of IP_3R2 might act as a pathological stimulus to the heart. In this circumstance, the relationship of the mild changes of the APs with the pathological outcomes under the chronic activation of IP_3R2 needs to be studied in the future.

In conclusion, we have determined the precise expression pattern of IP_3R2 during cardiomyocyte differentiation of hESCs and demonstrated that IP_3R2 is dispensable in the self-renewal and cardiac differentiation of hESCs. IP_3R2 has minimal contribution to the function of hESC-derived ventricular-like cardiomyocytes. These findings provide new insights into the role of IP_3Rs in human early cardiac development and in human immature ventricular myocytes. This knowledge would also provide references for the use of hESC-derived cardiomyocytes as disease models and drug development.

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AUTHOR CONTRIBUTIONS

HTY and PZ contributed to the concept and experiment design; PZ, JJH, HL and YJW contributed to the data collection; PZ, HTY, KFOY and MLL contributed to the data analysis; HTY and PZ contributed to the data interpretation and manuscript writing; HTY approved the manuscript and provided the funding support.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41401-020-00528-w) contains supplementary material, which is available to authorized users.

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