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Cardiac Progenitors Induced from Human Induced Pluripotent Stem Cells with Cardiogenic Small Molecule Effectively Regenerate Infarcted Hearts and Attenuate Fibrosis

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Abstract

Cardiac progenitor cells (CPCs) being multipotent offer a promising source for cardiac repair due to their ability to proliferate and multiply into cardiac lineage cells. Here, we explored a novel strategy for human CPCs generation from human induced pluripotent stem cells (hiPSCs) using a cardiogenic small molecule, isoxazole (ISX-9) and their ability to grow in the scar tissue for functional improvement in the infarcted myocardium. CPCs were induced from hiPSCs with ISX-9. CPCs were characterized by immunocytochemistry and RT-PCR. The CPC survival and differentiation in the infarcted hearts were determined by in vivo transplantation in immunodeficient mice following left anterior descending artery (LAD) ligation and their effects were determined on fibrosis and functional improvement. ISX-9 simultaneously induced expression of cardiac transcription factors, Nkx2.5, ISL1, GATA4, Mef2c in hiPSCs within 3 days of treatment and successfully differentiated into three cardiac lineages in vitro. mRNA and miRNA-sequencing results showed that ISX-9 targeted multiple cardiac differentiation, proliferation signaling pathways and upregulated myogenesis and cardiac hypertrophy related-miRNAs. ISX-9 activated multiple pathways including TGF- β induced epithelial–mesenchymal transition (EMT) signaling, canonical and non-canonical Wnt signaling at different stages of cardiac differentiation. CPCs transplantation promoted myoangiogenesis, attenuated fibrosis and led to functional improvement in treated mice.

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Keywords

Cardiac progenitor cell; Myocardial infarction; Isoxazole; Stem cell; Regeneration

Introduction

Stem cell-based therapies hold great promise and potential for cardiac regeneration. Human induced pluripotent stem cells (hiPSCs) could potentially generate unlimited functional human cell types in vitro for autologous and personalized medicine. Previous studies have reported that induced pluripotent stem cells (iPSCs)-derived cardiomyocytes (CMs) transplantation provided functional benefits in the rodent models of myocardial infarction (MI) [1, 2]. However, poor engraftment of CMs after transplantation to the infarcted heart is a common problem which impacts the final outcome. The beneficial effects of CMs transplantation is mainly due to paracrine mechanism rather than graft cell integration with the host myocardium [2]. These limitations are possibly attributed to poor cell survival and retention under ischemic conditions, limited proliferative capacity of differentiated CMs and lack of vascularization in the graft [3]. In addition, the arrhythmogenic risks after cardiac transplantation due to immaturity and heterogeneity of iPSCs-CMs or embryonic stem cells (ESCs)-CMs remain to be resolved [4, 5] prior to their full scale application.

Cardiac progenitor cells (CPCs) offer a promising avenue for cardiac repair due to their multipotency and ability to proliferate. Previous studies demonstrated that CPCs derived from hiPSCs or ESCs are capable of differentiation into multiple cardiac lineages without teratoma formation [3, 6]. Moreover, transplantation of CPCs derived from hiPSCs and murine iPSCs more effectively improved cardiac function compared with iPSCs-CMs [7, 8]. Two recent studies reported that the CPCs derived from mouse fibroblasts spontaneously differentiated into CMs, ECs and SMCs in infarcted mouse hearts and improved cardiac function following MI [9, 10]. Furthermore, several clinical trials on cell therapies for cardiac diseases are ongoing using adult stem cells [11, 12]. For example, the European Consortium CARE-MI is using human CPCs isolated from the right atria appendage of donors for acute myocardial infarction treatment [13]. It has been reported recently that SSEA-1⁺ CPCs derived from human ESCs were delivered into the infarcted area of a single patient suffering with severe heart failure, and they led to cardiac functional improvement [14]. This clinical study demonstrated a potential feasibility of generating a clinical-grade population of human ESCs-derived CPCs and provided strong encouragement for future clinical application of hiPSCs or ESCs-derived CPCs. Current strategies of human CPCs generation include isolation from atria appendage of donors and expansion in vitro [15], derivation from hiPSCs or ESCs, which have to be converted into embryoid bodies or treated with multiple small molecules and growth factors (activin, BMP4) [7, 16]. These strategies are labor-intensive and time-consuming, with high production costs, which limit clinical application.

Efficient cardiac-lineage priming with small molecules prior to hiPSC or hESC differentiation decreases not only risk of tumorigenicity by reducing numbers of undifferentiated cells, but also limits the risk of immune rejection. Isoxazole compounds

have been shown to induce cardiac lineage priming in adipose-derived stem cells, which can be differentiated into CM that improve heart function when transplanted in the mouse model of myocardial infarction [17]. However, direct injection of ISX-9 failed to regenerate infarcted myocardium and mitigate scar tissue formation after MI but did activate cardiac muscle genes in epicardial derived cells [18]. The improvement in cardiac function in the later study did not persist beyond three days post MI. In addition, ISX-9 induced neuronal differentiation in neural progenitor cell line [19], though the effect of isoxazole varied immensely amongst various brain progenitor cells from differentiation /proliferation to suppression of angiogenesis [20]. Our experimental design was quite different from Russell protocol (18). Therefore, the rationale of our significant findings in this study was that hiPSC induced CPCs by ISX-9 were able to differentiate into cardiac lineage cells in the infarcted myocardium.

Materials and Methods

In vitro cardiac progenitor cells differentiation from hiPSC and characterization of hiPSCs-derived cardiac cells

The hiPSCs cell line (ACS-1021™) induced from human fibroblasts was purchased from ATCC Company. Briefly, hiPSCs maintained on vitronectin coated six-well plate in mTeSR1 were dissociated into single cells using accutase (Invitrogen) at 37 °C for 10 min and then were seeded on to a vitronectin-coated six-well plate at 1×10^6 cell/well in mTeSR1 supplemented with 5μM ROCK inhibitor (Y-27632, Stem Cell Technology) for 24 h. Afterwards cells were cultured in mTeSR1, which was changed daily. At day 0, the medium was changed to RPMI/B27 minus insulin supplemented with ISX-9 (20 uM, dissolved in DMSO) for 7 days. Schematic of protocol for generation of cardiac progenitors is shown in Fig.1A. At day 3 and 7, cardiac genes were analyzed by RT-PCR and immunostaining. The purity of Nkx2.5 positive cells in ISX-9 treated cells was analyzed by FACS. For CMs differentiation, after 7 day of ISX-9 treatment, culture medium was switched to RPMI/B27 with insulin for another 10 and 30 days. For endothelial cells (ECs) differentiation, culture medium was switched to EGM-2V medium (Lonza) for another 10 days. For smooth muscle cells (SMCs) differentiation, culture medium was replaced by DMEM-F12 medium supplemented with TGFβ (2 ng/ml, R&D) and PDGFBB (10 ng/ml, R&D) for 10 days. CMs, ECs and SMCs were characterized and detailed in the supplemental appendix. To determine the potential mechanism of cardiac differentiation induced by ISX-9 in hiPSCs, cells were treated with TGFβ signaling pathway inhibitor, 5μM LY2109761 (Selleck) or Wnt signaling pathway inhibitors, 3 μM XAV939 (Selleck) or 5 μM IWP2 (Selleck) at Day -1 and Day 0, recombinant Human WIF-1 Protein (R&D, 200ng/ml), siWnt5 and siWnt11 at Day 3 and Day 4 respectively.

Cytoprotection and TUNEL staining

To analyze the cytoprotection of CPCs generated by ISX-9, hiPSCs treated with ISX-9 were replated in coverslips and subjected to simulated ischemia in vitro under hypoxic condition (5% CO₂, 94% N₂ and 1% O₂) at 37 °C in hypoxic chamber (INVIVO₂500) for 12h and 24h. hiPSCs were cultured in RPMI/B27 with or without 0.5% DMSO. To localize nuclear DNA fragmentation in cultured cells, in situ terminal deoxynucleotidyl transferase-mediated

dUTP nick-end labeling (TUNEL) was performed using commercial apoptosis detection kit (Roche, USA). All procedures were performed according to the directions of the manufacturer. Cells were counterstained with DAPI to visualize nuclei. The number of TUNEL-positive cells was determined by randomly counting 10 fields (20 ×) from 3 coverslips in the same group and was expressed as a percentage of the cells with normal nuclei. To further determine the cytoprotection by CPCs in ischemic heart, CPCs were transplanted into the infarcted mouse heart 10 minutes after coronary artery ligation. After 3 days, mice were sacrificed and heart tissue was processed for TUNEL staining. CMs were identified by α -sarcomeric actinin staining. Cells were counterstained with DAPI to visualize nuclei. The number of total TUNEL-positive cells and TUNEL-positive CMs was determined by counting 5 fields (20 ×) from border area in each heart (n = 3). DPBS and hiPSCs treated hearts served as control groups.

In vitro analysis of paracrine function

In order to determine whether the CPCs induced by ISX-9 expressed and secreted proangiogenic and prosurvival cytokines under ischemic conditions in vitro, we performed multiple angiogenesis factors and cytokines assays (Millipore) to identify paracrine factors in induced CPCs under normal and ischemic conditions. Briefly, induced CPCs were subjected to simulated ischemia in vitro by hypoxia (5% CO₂, 94% N₂ and 1% O₂) at 37°C or normal culture condition for 12h and 24h, then the supernatants (n = 4/group) were collected and centrifuged at 10,000 g for 10 min at 4°C and stored at -80°C. Analysis of secreted factors was performed using a Luminex-based platform (Bio-Rad Bio Plex-100) as described. hiPSCs cultured in RPMI/B27 with or without 0.5% DMSO served as control groups.

Murine model of myocardial infarction and cell transplantation

NOD/SCID mice were housed in an air-conditioned room with a 12 h light–dark cycle and were given standard chow with free access to tap water. Myocardial Infarction model was induced in 8–9-week-old NOD/SCID mice (The Jackson Laboratory) by left anterior descending artery (LAD) ligation under 2% inhaled isoflurane anesthesia. Briefly, the heart was exposed by left-sided limited thoracotomy and the LAD was ligated with a prolene #8–0 suture. Myocardial ischemia was confirmed by color change of the left ventricular wall. Mice were randomized into 3 groups: (1) Ischemic control group injected with DPBS without cells; (2) Ischemic group injected with 1×10^6 hiPSCs (undifferentiated hiPSCs); (3) Ischemic group injected with 1×10^6 CPCs (ISX-9 induced CPCs). The cells were injected 10 minutes after LAD ligation at 2 sites of border area. For post-engraftment tracking of the transplanted cells and determination of their fate, the cells were labeled with PKH-26 (Sigma, Product # PKH-26-GL) according to manufacturer's instructions. The chest was closed and the mice were allowed to recover. Buprenorphine was used as analgesic. Mice were euthanized by inhalant isoflurane (5%) overdose followed by cervical dislocation. The experimental protocols were approved by the University of Illinois at Chicago Animal Care and Use Committee (ACC NO.16-181) and the procedures were performed in accordance with the guide for the Care and Use of Laboratory Animals by the Institute of Animal Resources.

The cardiac function measurement by echocardiography was performed at different time points over a period of 3 months after MI as previously described. Mice were sacrificed post 3 months MI and hearts were removed for scar tissue measurement and histological analysis.

Vessel density assessment

Vessel density was assessed in 9 animals (3 in each group) sacrificed at 3 M after MI. The number of vessels was counted in a blind fashion on 27 sections (3 sections per heart) in the infarct and border areas of all mice after staining with an antibody, anti-CD31 or α -SMA using a fluorescence microscope at a $400\times$ magnification. Vascular density was determined by counting CD31 positive vascular structures and arteriole density was determined by counting α -SMA positive vascular structures. Five high-power fields in each section were randomly selected, and the number of vessels in each field was averaged and expressed as the number of vessels per high-power field (0.2 mm^2).

RNA-sequencing in CPCs generated by ISX-9 treatment

mRNA-Sequencing transcriptome analysis was performed to reveal differences in gene expression amongst undifferentiated hiPSCs, DMSO or ISX-9 treated hiPSCs ($n = 4$). Global miRNA expression profiles in undifferentiated hiPSCs, DMSO or ISX-9 treated hiPSCs were also determined. mRNA-sequencing and miRNA-sequencing were performed by Core Genomics Facility and DNA services facility at University of Illinois at Chicago. The accession number in GEO for the RNA-sequencing data reported in this paper: GSE95389 and GSE 95390.

Detailed information on immunohistochemistry, infarct size measurement, flow cytometry, ultrastructure analysis by electron microscopy, echocardiography, qPCR and bioinformatics analysis are provided in supplemental appendix.

Statistical Analysis

Data were expressed as mean $\pm$ SEM. Normal distribution of the data sets was always verified. Statistical analysis of differences between two groups was compared by Student's t-test. Statistical analysis of differences was compared by analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. Two-way repeated measures of ANOVA was used in analyzing cardiac function data at different time points. A probability value of $P < 0.05$ was considered statistically significant.

Results

Cardiac Differentiation and Characterization of hiPSCs-derived Cardiac-Lineage CMs, ECS and SMCs

Compared with DMSO treated hiPSCs, ISX-9 treatment promoted formation of clusters of proliferating cells in RPMI/B27 differentiation medium (Fig.1B). Real-time PCR results showed that ISX-9 dramatically induced the expression of multiple CPCs related genes in hiPSC. As shown in Fig.1C, Nkx2.5, GATA4, ISL-1 and Mef2c upregulation was observed after 3-day ISX-9 treatment and further enhanced after 7-day treatment (except GATA4). As compared with DMSO treated cells, ISX-9 increased expression of the above

cardiac transcription factors (Fig 1D). Immunofluorescence staining also showed that transcription factors (Nkx2.5, GATA4 and ISL-1) were highly expressed in hiPSC after 7-day ISX-9 treatment (Fig.1F and Fig.S5) and by FACS $96.5 \pm 2.5\%$ cells were Nkx2.5 positive, suggesting the high purity of CPCs generation (Fig.1E). These Nkx2.5⁺ cells were multipotent and directly differentiated into all three cardiovascular lineages, including CMs, ECs and SMCs in basal differentiation conditions without any specific induction signaling molecules (Fig.2). These cardiac cells expressed CMs-, ECs- and SMCs-specific proteins. By transmission electron microscopy (TEM), the developing CMs were rich in endoplasmic reticulum with ribosomes, developing myofilaments, mitochondria and glycogen particles. Chromatin material was uniformly distributed in the nucleoplasm (Fig.2B). In addition, the differentiated ECs exhibited a similar phenotype and function to primary ECs. Formation of tube-like structures and LDL-uptake were observed in differentiated ECs (Fig.2 D and Fig.2 E). To characterize cells more precisely after CPCs differentiation, we analyzed the percentage of CMs (TnT⁺), ECs (CD31⁺) and SMCs (α -SMA⁺) in CMs, ECs and SMCs differentiation conditions for 10 days by FACS. FACS analysis revealed high efficiency in cardiac lineages differentiation in these induced CPCs. We can obtain about $95.2 \pm 2.1\%$ CMs, $90.3 \pm 2.5\%$ ECs and $92.3 \pm 1.8\%$ SMCs in basal differentiation medium respectively (Fig.2 G). Moreover, ISX-9 acts as a very strong differentiation inducer in hiPSCs even when hiPSCs were cultured in mTeSR1 undifferentiation medium (Fig.S3). In mTeSR1 undifferentiation medium, ISX-9 could still induce Nkx2.5, GATA4 and ISL-1 expression in part of the cultured hiPSCs (Fig.S4). Taken together, these results suggest that successful generation of CPCs by a novel small molecule ISX-9 is remarkable.

Potential key signaling pathways and miRNAs expression mediated by ISX-9 treatment

We performed transcriptome analysis to reveal differences in gene expression in hiPSC by ISX-9 treatment. mRNA-sequencing showed different gene expression profile amongst groups (Fig.3A). Accordingly, we attempted to discover possible related signaling pathways regulated by ISX-9. Signaling pathway enrichment analyses of upregulated and downregulated genes in different groups were shown in Fig.3C. As compared with DMSO treatment and undifferentiated hiPSCs (Fig.3C and Fig.S7), ISX-9 promoted WNT and cytoskeleton remodeling and TGF- β induced EMT signaling, which were involved in cardiac differentiation. In addition, other genes were related to cardiac differentiation signaling pathways, for example, development of PIP3 signaling in cardiomyocytes, muscle contraction and NF-AT hypertrophy signaling. Interestingly, by real-time PCR we found that the expression of Wnt3a, the key activator of canonical Wnt signaling pathway, was dramatically upregulated at day 3 and day 7 compared with undifferentiated hiPSCs. Wnt3a expression was not significantly different at day 3 and day 7. However, the expression of Wnt5a and Wnt11, the trigger molecules of non-canonical Wnt signaling pathway were upregulated at day 7 but not at day 3 (Fig.4A). In comparison to DMSO-treated group, ISX-9 induced upregulation of Wnt3a, Wnt5a and Wnt11 (Fig.4B). Continuous treatment with ISX-9 for 7 days increased expression of Wnt5, Wnt11 and cardiac transcription factors (Nkx2.5, Mef2c, GATA4 and ISL-1) compared with treatment only for 3 days (Fig. 4C and 4D). We further verified the mRNA-sequencing results using TGF β signaling pathway inhibitor, LY2109761, Wnt signaling pathway inhibitors, XAV939 and IWP2. Schematic description of the protocol is shown in Fig.4E. The real-time PCR results showed that

inhibition of TGF β signaling pathway and canonical Wnt signaling pathway in the initial differentiation stage significantly decreased expression of cardiac transcription factors (Nkx2.5, Mef2c, GATA4 and ISL-1) (Fig.4F), suggesting the critical role of TGF- β induced EMT signaling and canonical Wnt signaling in ISX-9 induced cardiac differentiation. Knock down of Wnt5a or Wnt11 using siRNA at late differentiation stages significantly inhibited expression of cardiac transcription factors in ISX-9 treated cells (Fig. 4G). Efficiency of knock down for Wnt5a and Wnt11 is shown in Fig.S8. In addition, treatment with WIF-1 during the late stage, a non-canonical Wnt signaling pathway inhibitor, showed the similar result (Fig.4G), suggesting non-canonical Wnt signaling pathway was involved in ISX-9 induced cardiac differentiation. Moreover, signaling pathway enrichment analyses also revealed other beneficial effects by ISX-9 on cell proliferation, migration, and anti-apoptosis. A series of genes related to migration, proliferation and anti-apoptosis signaling were upregulated by ISX-9, meanwhile genes related to DNA damage and oxidative stress were downregulated. In addition, miRNA-sequencing showed several myogenesis related miRNAs and also upregulation of cardiac hypertrophy related-miRNAs, including miR-335, miR-21, miR-30c, and miR-214 (Fig.3B).

Cytoprotection of hiPSCs-CPCs against ischemia

To determine whether the induced CPCs exhibit cytoprotective effects, we cultured hiPSC with different treatments under hypoxic condition. Culturing of these cells under 1% O₂ for 12h and 24h resulted in cell death with or without DMSO treatment. TUNEL assay revealed dramatic increase in apoptotic cells in hiPSC from mock and DMSO control groups due to hypoxia of 1% O₂, while fewer apoptotic cells were noted in ISX-9 treated cells (Fig.5A and 5B). Approximately 70% $\pm$ 2.5% and 80% $\pm$ 6.5% cells underwent apoptosis in DMSO group after 12h and 24h 1% O₂ exposure, respectively. However, ISX-9 treatment reduced the apoptotic cells to 15% $\pm$ 3.5% and 24% $\pm$ 4.3% after 12h and 24h 1% O₂ exposure, respectively (Fig.5C). Furthermore, CPCs transplantation reduced apoptosis in the border area of infarcted hearts after 3 days post-MI (Fig.5E–5G). We then performed multiple cytokines assays to identify paracrine factors which might be responsible for these cytoprotective effects. Several cytokines were significantly increased with ISX-9 treatment under normoxia or hypoxic stress (1% O₂) for 12h or 24h respectively, compared with DMSO treatment ($P < 0.05$). These protective cytokines included anti-apoptosis factors (angiopoietin-2, IL-6, MMP-1, PDGF-BB, and TIMP-1), cell migration inducing factors (angiopoietin-2, IL-8, MCP-1, MMP-9 and VEGF-A) and angiogenesis factors (angiopoietin-2, PDGF-BB and VEGF-A). Compared with normoxia condition, 24h hypoxic stress further increased concentrations of all these protective cytokines ($P < 0.05$) (Fig.4D and Fig.S9). These results indicate that ISX-9 treatment induced cytoprotective effects on CPCs and the resident cells in ischemic hearts.

Transplantation of hiPSCs-Derived CPCs attenuated cardiac remodeling after MI

Next, we explored whether these CPCs generated by ISX-9 could attenuate cardiac remodeling after MI. Serial echocardiographic examination of DPBS and hiPSC treated mice showed a time-dependent increase in LVEDD and LVESD after MI, as well as a progressive decline in left ventricular fractional shortening (LVFS) and ejection fraction (EF) over the follow-up period of 3 months. In contrast, transplantation of CPCs

significantly slowed the progression of LV enlargement (left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) and LVFS depression in mice with MI, and these differences became more obvious over time (Fig.6A–D). Transplantation of CPCs significantly increased LVEF to $71.95 \pm 1.53\%$ from $43.39 \pm 2.31\%$ and LVFS to $40.14 \pm 1.53\%$ from $21.24 \pm 1.30\%$ in comparison with the DPBS treated mice (n = 13 in DPBS group; n=16 in CPCs and hiPSCs group). The pathological remodeling of LVESD and LVEDD was also significantly reduced in CPCs treated hearts as compared with DPBS and hiPSCs treated mice (Fig.6E). In parallel to functional indices, significant smaller scar size in mice transplanted with CPCs 3 months post-MI was observed in comparison with DPBS and hiPSCs treated mice (Fig.6F–H). These data suggest that transplantation of CPCs attenuates cardiac remodeling after MI.

In vivo differentiation and growth of hiPSC-CPCs after transplantation into infarcted myocardium

We directly injected hiPSC and hiPSC-CPCs along the infarcted area of left ventricle. We observed extensive survival, proliferation, and differentiation of PKH-26 labeled cardiac progenitors 2months and 3months after transplantation in the infarcted region. Immunofluorescence analyses showed co-localization of PKH-26 red fluorescence and α -sarcomeric actinin or human specific cardiac troponin T (cTnT) staining (Green fluorescence) at 2 months (Fig.S10) and 3 months (Fig.7A and 7B) after MI, suggesting that the transplanted CPCs grew into new CMs which replaced scar tissue. The total number of transplanted human cells retained in the peri-infarcted area was significantly higher in CPCs treated mice when compared with hiPSCs treated mice 72h post-MI using human mitochondria antigen tracking ($30.03 \pm 1.69\%$ vs. $6.27 \pm 0.85\%$) (Fig.7E–G). Moreover, we analyzed the number of differentiated CMs from transplanted CPCs or hiPSCs in the peri-infarcted area by staining myocardial sections for human mitochondrial antigen and cardiac troponin I expression. At 3 months post-MI, the number of differentiated CMs from transplanted CPCs were detected in the peri-infarcted area by 8-fold when compared with hiPSCs transplanted hearts (Fig.7H–J). In addition, engrafted CPCs differentiated into ECs and SMCs which formed vascular structures in the border area of infarcted hearts (Fig.7C and 7D). No tumor formation was observed in the hearts from any of the transplanted animals used in this study (n = 16), which were examined by echocardiography and histological sections. These observations support the idea that CPCs generated by ISX-9 successfully engrafted and differentiated into three cardiac lineages, which formed new CMs and blood vessels in the infarcted heart.

Transplantation of hiPSC-CPCs enhanced vasculogenesis in infarcted area

Angiogenesis could contribute to cardiac function improvement after ischemia. Therefore, we further determined whether transplanted CPCs could promote angiogenesis in the infarct and border areas of infarcted heart 3 months after ischemic injury, we found CPCs significantly increased vascular density and arteriole density in the infarct and border areas compared with DPBS and hiPSC treated groups (Fig. S11). The increased neovascularization in border zone might be due to paracrine mechanisms in the host myocardium while CPC differentiation process may also be positively impacted by paracrine mechanism.

Discussion

In the present study, we successfully generated human CPCs from hiPSCs using a simple novel method without using cumbersome intermediate cocktails of small molecules and growth factors. These CPCs protected themselves by releasing cytokines against ischemic environment. After transplantation, they differentiated into multiple cardiac lineage cells and covered the scar area with new myofibers and vessels in the infarcted heart. These CPCs also showed long term beneficial effects on cardiac function improvement reflecting higher FS and EF.

According to our study, as early as 3 days treatment with ISX-9 promoted simultaneous upregulation of multiple cardiac transcription factors in hiPSCs with ISX-9. Therefore, induction of several transcription factors with ISX-9 (ISL1, Nkx2.5, GATA4, and Mef2c) initiated multilineage differentiation program in hiPSC. At the end of 7 days' treatment with ISX-9, these CPCs can be separately converted into myocytes, EC and smooth muscle progenitors.

The primary aim of cell regenerative medicine is the replacement of the dead cells with the new cells in order to restore the cardiac structure and function. The regenerative field at the present is controversial due to conflicting results ranging from no new cell formation to sparse newly formed cells in the infarcted tissue [2, 4, 9]. However, the majority of studies provide alternative explanation of cell therapy due to beneficial effects caused by paracrine factors which reduce cell death and stimulate cell migration and proliferation [21–23]. Our study supports both these concepts which are complimentary to each other. Our results demonstrate that CPCs were tolerant to ischemic condition and bestowed additional protection to resident CMs in ischemic heart by preventing cell death. Furthermore, these CPCs also released angiogenesis factors in a paracrine manner. We found CPCs transplantation increased vessel density in the infarcted heart, which is partly due to these angiogenesis factors released from CPCs. Consistent with cytokine and signaling pathway enrichment analyses, ISX-9 provided additional beneficial effects on cell proliferation, migration, and apoptosis, thus assuring cell survival and successful engraftment of transplanted CPCs. Poor engraftment of transplanted CMs in previous studies might be due to lack of oxygen and nutrition in the infarcted myocardium. Here, our CPCs showed anti-oxidant effects, which might partly overcome the problem of poor cell survival in ischemic heart. Previously it was reported that approximately 10% of cells were retained in the border area [24]. However, in our study the higher rate of CPCs retention and survival suggest that our CPCs were more tolerant to ischemia in the ischemic environment. This was further supported by our data that about $16.93 \pm 1.5\%$ of newly differentiated CMs were observed in the border area of the infarct hearts in CPCs-treated mice 3M post-MI. It is also possible that sustained release of cytokines from the engrafted CPCs and differentiated cells might have mediated protection and promoted regeneration. The engrafted CPCs not only survived but formed new CMs and blood vessels to replace scar tissue in ischemic myocardium as supported by the current data.

In order to understand the possible underlying mechanism of cardiac differentiation induced by ISX-9, we performed RNA-sequencing in hiPSC treated with ISX-9. Interestingly, with

global transcriptome analysis, we discovered that ISX-9 upregulated genes related to multiple cardiac differentiation signaling pathways including WNT and cytoskeleton remodeling and TGF- β induced epithelial–mesenchymal transition (EMT) signaling, VEGF and activin A signaling, which strongly pointed out that ISX-9 targets multiple signaling pathways necessary for cardiac differentiation. Of note, TGF- β family members, BMP and activin, WNT and FGF family members are known to be crucial for cardiac development. For example, TGF- β is expressed early in the cardiac region of the mesoderm [25]. Growth factors and small molecules have been used for induction of cardiac differentiation in stem cells via manipulation of these key developmental pathways. TGF- β has been reported to induce differentiation of bone marrow stem cells into immature CMs and induce cardiac differentiation in ESCs [26]. Inhibition of TGF- β activity inhibited induction of the cardiac transcription factor Nkx2.5 [26]. Blockade of TGF- β signaling with LY2109761 decreased expression of cardiac transcription factors induced by ISX-9, which validated our RNA-sequencing data. WNT proteins have been shown to play multiple roles during cardiac development and differentiation. Wnt/ β -catenin signaling enhanced early cardiac development but later it showed negative influences on cardiac specification [27]. It was demonstrated that temporal modulation of Wnt/ β -catenin signaling using an inhibitor promoted robust cardiomyocyte differentiation [28]. In contrast, non-canonical Wnt signaling promoted cardiac specification. Wnt5a was upregulated by Mesp1 and may play a role in cardiogenesis [27]. Wnt5a/Wnt11 could trigger a cardiogenic program in mesenchymal stem cells or bone marrow stem cells via activation of PKC [27]. Of note, Wnt5a and Wnt11 mainly triggered non-canonical Wnt signaling, which have been shown to inhibit canonical Wnt signaling through multiple mechanisms [27]. Additionally, Wnt5a and Wnt11 were reported to inhibit canonical Wnt pathway and subsequently promoted cardiac progenitor generation via the caspase-dependent degradation of AKT [29]. Wnt5a and Wnt11 were essential for second heart field progenitor development [30]. Additionally, non-canonical Wnt pathways have been linked to cytoskeletal rearrangements [30]. The pathway enrichment analysis confirmed that genes related to WNT and cytoskeleton remodeling and Wnt5a were upregulated by ISX-9. Moreover, the real-time PCR results showed that ISX-9 induced Wnt3a upregulation in the initial stages of CPC differentiation while Wnt5a and Wnt11 were upregulated in the later stages. Inhibition of Wnt pathway both in the early and later stages resulted in decreased expression of cardiac transcription factors. Consistent with findings from a previous study [30], our results showed that knock down of either Wnt5 or Wnt11 led to a dramatic decrease of cardiac transcription factor expression, suggesting their required participation in cardiac progenitor generation. Taken together, these results further supported that ISX-9 might promote cardiac differentiation and specification via temporal and sequential regulation of canonical and non-canonical Wnt signaling. Different cells might have different responses to ISX-9 treatment. It was demonstrated that in neural progenitor cells, ISX-9 treatment promoted them toward neurons [19]. Based on our results, ISX-9 might induce mesoderm and cardiac mesoderm differentiation in hiPSCs via activation of TGF- β induced EMT signaling and canonical Wnt signaling in the initial stage while continuous ISX-9 stimulation led to upregulation of Wnt5 and Wnt11 in the late stage of CPC formation. Blocking activation of canonical Wnt signaling during the later phase of cardiac differentiation was accompanied by significant up-regulation of non-canonical Wnt expression [31]. Interestingly, a loss of Wnt5a and Wnt11 led to a dramatic loss of second

heart field progenitors and was accompanied by an increase in canonical Wnt signaling in the developing heart [30]. These studies suggest a cross-talk between canonical and non-canonical signaling during cardiogenesis. More interestingly, ISX-9 induced time-dependent expression of cardiac transcription factors, and targeted multiple developmental signaling pathways which need further investigation. In addition, the role of ISX-9 in muscle gene development was further strengthened by upregulation of genes related to development of PIP3 signaling in cardiomyocytes, muscle contraction and NF-AT hypertrophy signaling pathways. Thus, our results strongly suggest that ISX-9 is a strong promoter of cardiac differentiation in iPSCs.

ISX-9 in light of its role in cardiac differentiation through diverse signaling pathways also activated myogenesis miRNAs and cardiac hypertrophy related-miRNAs including miR-335, miR-21, miR-30c, and miR-214. These miRs are known to play a positive role in myogenic differentiation or muscle regeneration [32]. The most abundant miRNAs in cardiac muscle are miR-let-7, miR-30c. miR-let-7 family played a key role in normal cardiac maintenance and also maturation of stem cell derived CMs [33]. Moreover, miR-21 was involved in cardiac hypertrophy and was highly expressed in the fetal heart [34]. miR-30c and miR-335 were shown to be upregulated in mouse stem cells and CMs, implying their potential roles during heart development [35]. However, further studies are needed to better understand the underlying mechanism in regulation of miRNAs and signaling pathways important in cardiac differentiation of stem cells.

Conclusion

This study demonstrates a novel technique of producing pure CPCs from hiPSCs using a single cardiogenic small molecule, ISX-9 with robust muscle differentiation potential through diverse signaling pathways. These CPCs were multipotent and differentiated into three cardiac lineages to form new CMs and vessels in the infarcted heart improving functional indices by reducing fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol*. 2007; 50(19):1884–1893. [PubMed: 17980256]
2. Ong SG, Huber BC, Lee WH, Kodo K, Ebert AD, Ma Y, Nguyen PK, Diecke S, Chen WY, Wu JC. Microfluidic Single-Cell Analysis of Transplanted Human Induced Pluripotent Stem Cell-Derived

Cardiomyocytes After Acute Myocardial Infarction. *Circulation*. 2015; 132(8):762–771. [PubMed: 26304668]

3. Lam JT, Moretti A, Laugwitz KL. Multipotent progenitor cells in regenerative cardiovascular medicine. *Pediatr Cardiol*. 2009; 30(5):690–698. [PubMed: 19415155]
4. Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, et al. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature*. 2014; 510(7504):273–277. [PubMed: 24776797]
5. Poon E, Kong CW, Li RA. Human pluripotent stem cell-based approaches for myocardial repair: from the electrophysiological perspective. *Mol Pharm*. 2011; 8(5):1495–1504. [PubMed: 21879736]
6. Bartulos O, Zhuang ZW, Huang Y, Mikush N, Suh C, Bregasi A, Wang L, Chang W, Krause DS, Young LH, et al. ISL1 cardiovascular progenitor cells for cardiac repair after myocardial infarction. *JCI Insight*. 2016; 1(10)
7. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden R, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature*. 2008; 453(7194):524–528. [PubMed: 18432194]
8. Mauritz C, Martens A, Rojas SV, Schnick T, Rathert C, Schecker N, Menke S, Glage S, Zweigerdt R, Haverich A, et al. Induced pluripotent stem cell (iPSC)-derived Flk-1 progenitor cells engraft, differentiate, and improve heart function in a mouse model of acute myocardial infarction. *Eur Heart J*. 2011; 32(21):2634–2641. [PubMed: 21596799]
9. Zhang Y, Cao N, Huang Y, Spencer CI, Fu JD, Yu C, Liu K, Nie B, Xu T, Li K, et al. Expandable Cardiovascular Progenitor Cells Reprogrammed from Fibroblasts. *Cell Stem Cell*. 2016; 18(3):368–381. [PubMed: 26942852]
10. Lalit PA, Salick MR, Nelson DO, Squirrell JM, Shafer CM, Patel NG, Saeed I, Schmuck EG, Markandeya YS, Wong R, et al. Lineage Reprogramming of Fibroblasts into Proliferative Induced Cardiac Progenitor Cells by Defined Factors. *Cell Stem Cell*. 2016; 18(3):354–367. [PubMed: 26877223]
11. Sheridan C. Cardiac stem cell therapies inch toward clinical litmus test. *Nat Biotechnol*. 2013; 31(1):5–6. [PubMed: 23302912]
12. Malliaras K, Marban E. Moving beyond surrogate endpoints in cell therapy trials for heart disease. *Stem Cells Transl Med*. 2014; 3(1):2–6. [PubMed: 24292794]
13. Gomes-Alves P, Serra M, Brito C, Ricardo CP, Cunha R, Sousa MF, Sanchez B, Bernad A, Carrondo MJ, Rodriguez-Borlado L, et al. In vitro expansion of human cardiac progenitor cells: exploring ‘omics tools for characterization of cell-based allogeneic products. *Transl Res*. 2016; 171:96–110. e111–113. [PubMed: 26924043]
14. Menasche P, Vanneaux V, Hagege A, Bel A, Cholley B, Cacciapuoti I, Parouchev A, Benhamouda N, Tachdjian G, Tosca L, et al. Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report. *Eur Heart J*. 2015; 36(30):2011–2017. [PubMed: 25990469]
15. Koninckx R, Daniels A, Windmolders S, Mees U, Macianskiene R, Mubagwa K, Steels P, Jamaer L, Dubois J, Robic B, et al. The cardiac atrial appendage stem cell: a new and promising candidate for myocardial repair. *Cardiovasc Res*. 2013; 97(3):413–423. [PubMed: 23257022]
16. Moretti A, Bellin M, Jung CB, Thies TM, Takashima Y, Bernshausen A, Schiemann M, Fischer S, Moosmang S, Smith AG, et al. Mouse and human induced pluripotent stem cells as a source for multipotent Isl1+ cardiovascular progenitors. *FASEB J*. 2010; 24(3):700–711. [PubMed: 19850773]
17. Burchfield JS, Paul AL, Lanka V, Tan W, Kong Y, McCallister C, Rothermel BA, Schneider JW, Gillette TG, Hill JA. Pharmacological priming of adipose-derived stem cells promotes myocardial repair. *J Invest Med*. 2016; 64(1):50–62.
18. Russell JL, Goetsch SC, Aguilar HR, Frantz DE, Schneider JW. Targeting native adult heart progenitors with cardiogenic small molecules. *ACS Chem Biol*. 2012; 7(6):1067–1076. [PubMed: 22413910]
19. Zhang L, Li P, Hsu T, Aguilar HR, Frantz DE, Schneider JW, Bachoo RM, Hsieh J. Small-molecule blocks malignant astrocyte proliferation and induces neuronal gene expression. *Differentiation*. 2011; 81(4):233–242. [PubMed: 21419563]

20. Koh SH, Liang AC, Takahashi Y, Maki T, Shindo A, Osumi N, Zhao J, Lin H, Holder JC, Chuang TT, et al. Differential Effects of Isoxazole-9 on Neural Stem/Progenitor Cells, Oligodendrocyte Precursor Cells, and Endothelial Progenitor Cells. *PLoS One*. 2015; 10(9):e0138724. [PubMed: 26407349]
21. Wang M, Tsai BM, Crisostomo PR, Meldrum DR. Pretreatment with adult progenitor cells improves recovery and decreases native myocardial proinflammatory signaling after ischemia. *Shock*. 2006; 25(5):454–459. [PubMed: 16680009]
22. Herrmann JL, Wang Y, Abarbanell AM, Weil BR, Tan J, Meldrum DR. Preconditioning mesenchymal stem cells with transforming growth factor- α improves mesenchymal stem cell-mediated cardioprotection. *Shock*. 2010; 33(1):24–30. [PubMed: 19996917]
23. Crisostomo PR, Wang M, Markel TA, Lahm T, Abarbanell AM, Herrmann JL, Meldrum DR. Stem cell mechanisms and paracrine effects: potential in cardiac surgery. *Shock*. 2007; 28(4):375–383. [PubMed: 17577135]
24. Sharma S, Mishra R, Bigham GE, Wehman B, Khan MM, Xu H, Saha P, Goo YA, Datla SR, Chen L, et al. A Deep Proteome Analysis Identifies the Complete Secretome as the Functional Unit of Human Cardiac Progenitor Cells. *Circ Res*. 2017; 120(5):816–834. [PubMed: 27908912]
25. Puceat M. TGF β in the differentiation of embryonic stem cells. *Cardiovasc Res*. 2007; 74(2):256–261. [PubMed: 17250814]
26. Lim JY, Kim WH, Kim J, Park SI. Involvement of TGF- β 1 signaling in cardiomyocyte differentiation from P19CL6 cells. *Mol Cells*. 2007; 24(3):431–436. [PubMed: 18182860]
27. Gessert S, Kuhl M. The multiple phases and faces of wnt signaling during cardiac differentiation and development. *Circ Res*. 2010; 107(2):186–199. [PubMed: 20651295]
28. Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK, Zhang J, Kamp TJ, Palecek SP. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc Natl Acad Sci U S A*. 2012; 109(27):E1848–1857. [PubMed: 22645348]
29. Bisson JA, Mills B, Paul Helt JC, Zwaka TP, Cohen ED. Wnt5a and Wnt11 inhibit the canonical Wnt pathway and promote cardiac progenitor development via the Caspase-dependent degradation of AKT. *Dev Biol*. 2015; 398(1):80–96. [PubMed: 25482987]
30. Cohen ED, Miller MF, Wang Z, Moon RT, Morrissey EE. Wnt5a and Wnt11 are essential for second heart field progenitor development. *Development*. 2012; 139(11):1931–1940. [PubMed: 22569553]
31. Mehta A, Ramachandra CJ, Sequiera GL, Sudibyo Y, Nandihalli M, Yong PJ, Koh CH, Shim W. Phasic modulation of Wnt signaling enhances cardiac differentiation in human pluripotent stem cells by recapitulating developmental ontogeny. *Biochim Biophys Acta*. 2014; 1843(11):2394–2402. [PubMed: 24978297]
32. Wei Y, Tao X, Xu H, Chen Y, Zhu L, Tang G, Li M, Jiang A, Shuai S, Ma J, et al. Role of miR-181a-5p and endoplasmic reticulum stress in the regulation of myogenic differentiation. *Gene*. 2016; 592(1):60–70. [PubMed: 27461948]
33. Kuppasamy KT, Jones DC, Sperber H, Madan A, Fischer KA, Rodriguez ML, Pabon L, Zhu WZ, Tulloch NL, Yang X, et al. Let-7 family of microRNA is required for maturation and adult-like metabolism in stem cell-derived cardiomyocytes. *Proc Natl Acad Sci U S A*. 2015; 112(21):E2785–2794. [PubMed: 25964336]
34. Romaine SP, Tomaszewski M, Condorelli G, Samani NJ. MicroRNAs in cardiovascular disease: an introduction for clinicians. *Heart*. 2015; 101(12):921–928. [PubMed: 25814653]
35. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, et al. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation*. 2007; 116(3):258–267. [PubMed: 17606841]

Figure 1. Generation and characterization of hiPSC derived cardiac progenitor cells (CPCs)
 (A) Schematic outline of generation of hiPSC-CPCs. (B) Bright field of hiPSC treated with DMSO or ISX-9 in RPMI/B27 minus insulin at Day 0, Day 3 and Day 7. (C) Time dependent expression of Nkx2.5, GATA4, ISL-1 and Mef2c.* vs. Day 0, $P < 0.05$; # vs. Day 3, $P < 0.05$, from 3 biological repeated experiments. (D) ISX-9 upregulated expression of Nkx2.5, GATA4, ISL-1 and Mef2c at day 7 compared with DMSO group, * vs. DMSO group, $P < 0.05$, from 3 biological repeated experiments. (E) Fluorescence activated cell sorting analysis (FACS) showing $96.5 \pm 2.3\%$ Nkx2.5 positive cells after ISX-9 treatment.

(F) Immunofluorescence staining showed that transcription factors (Nkx2.5, GATA4, and ISL-1) were highly upregulated in hiPSC treated with ISX-9. Scale bar represents 200 μm .

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Figure 2. In vitro differentiation of CPCs into three cardiac lineage cells
 According to treatment outline, the CPCs expressed CM, EC, SMC specific proteins. (A) CM progenitors expressed α -sarcomeric actinin, cTnI, MLC2v, cTnT and Cx43, scale bar = 50 μ m. (B) Under TEM, these cells were rich in endoplasmic reticulum (ER) studded with ribosomes, developing myofilaments (mf), mitochondria (arrowhead) and glycogen particles. Nuclear chromatin material (N) was uniformly distributed in the nucleoplasm. (C) Endothelial progenitors expressed VE-cadherin and CD31, scale bar = 200 μ m. LDL uptake by these cells was observed (D) and these also formed tube like structures (E). (F) Smooth

muscle progenitors expressed α -SMA and calponin, scale bar = 200 μ m. (G) FACS analysis revealed $95.2 \pm 2.1\%$ CMS, $90.3 \pm 2.5\%$ ECs and $92.3 \pm 1.8\%$ SMCs in basal differentiation medium.

Figure 3. Global mRNA and miRNA expression profile in CPCs

(A) Heat map of differentially expressed mRNA (Q value $< 10^{-15}$) among undifferentiated hiPSCs, DMSO or ISX-9 treated hiPSCs detected by mRNA-seq. n = 4, from 4 biological repeated experiments. (B) Heat map of differentially expressed miRNAs that exhibited significant increase or decrease individually between DMSO and ISX-9 treated hiPSCs. (C) Pathway enrichment analysis of upregulated and downregulated genes in CPCs induced by ISX-9 in comparison with DMSO treated hiPSCs.

Figure 4. Potential key signaling pathways mediated by ISX-9 treatment

(A) Time dependent expression of Wnt5, Wnt11 and Wnt3a with ISX-9 treatment. * vs. Day0, $P < 0.05$; # vs. Day3, $P < 0.05$, $n=6$ from 3 biological repeated experiments. (B) Continuous treatment for 7 days with ISX-9 increased expression of Wnt5, Wnt 1 and Wnt 3a compared with DMSO group. * vs. DMSO group, $P < 0.05$, from 3 biological repeated experiments. Continuous treatment with ISX-9 for 7 days increased expression of Wnt5, Wnt11(C) and cardiac transcription factors (Nkx2.5, Mef2c, GATA4 and ISL-1) (D) compared with treatment only for 3 days. * vs. 3-day treatment group, $P < 0.05$, from 3

biological repeated experiments. (E) Schematic description of the protocol to study the role of TGF β , canonical and non-canonical Wnt signaling in cardiac differentiation induced by ISX-9. Inhibition of TGF β signaling pathway with LY2109761 or canonical Wnt signaling pathway with XAV939 and IWP2 in the initial differentiation stage (F) or blockade of non-canonical Wnt signaling in late differentiation stage (G) with siWnt5, siWnt11 or WIF-1 along treatment with ISX-9 significantly decreased expression of cardiac transcription factors (Nkx2.5, Mef2c, GATA4 and ISL-1). * vs. ISX-9 group, $P < 0.05$, from 3 biological repeated experiments.

Figure 5. Cytoprotective effects of ISX-9 on CPCs

(A) Morphology of hiPSCs in RPMI / B27 medium treated with DMSO or ISX-9 under 1% O₂ for 12h or 24h. (B) Representative images of TUNEL staining in Mock, DMSO and ISX-9 treated groups after 24h hypoxic stresses. (C) Semi quantitative estimate of TUNEL positive cells. * vs. DMSO group, $P < 0.05$. (D) Heat map comparing concentrations of paracrine factors released from cells with different treatments. Each row represents a cytokine and each column represents an independent condition. The heat map color scale corresponds to the absolute concentration (log10, pg/ml) of cytokines with minimum and

maximum of all values is shown on the right. (E) Representative images of TUNEL staining 3 days post-MI. The host cardiomyocytes were identified by α -sarcomeric actinin. (F) Quantitation of total TUNEL positive cells in the border area of infarct with different treatments. * vs. DPBS group, $P < 0.05$; # vs. hiPSC group, $P < 0.05$. (G) Quantitation of TUNEL positive cardiomyocytes in the border area of infarct hearts from mice in different groups. * vs. DPBS group, $P < 0.05$; # vs. hiPSC group, $P < 0.05$. Quantitative analysis and images were studied from 3 biological repeated experiments.

Figure 6. Transplantation of CPCs reversed cardiac remodeling after MI

Temporal Changes in LVESD (A), LVEDD (B), FS(C) and EF (D) in mice post transplantation. * $P < 0.05$ Vs. DPBS treated group at the same time point, # $P < 0.05$ vs. hiPSC treated group at the same time point. DPBS = 13 mice, hiPSC = 16 mice and CPC = 16 mice. (E) Representative M-mode and B- mode echocardiographs following 3M post MI. (F) Cardiac fibrosis was evaluated at seven levels by Masson's trichrome staining at 3M post-MI. (G) Sections of representative hearts are shown. (H) Quantification of scar tissue

size. * $P < 0.05$ Vs. DPBS treated group at the same time point, # $P < 0.05$ vs. hiPSC treated group (n = 5).

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Figure 7. Differentiation of transplanted CPCs into cardiac lineage cells in the infarcted heart. (A-B) Engrafted CPCs were identified by PKH-26 fluorescence (red fluorescence); muscle fibers were visualized via immunostaining for human specific cTnT or α -sarcomeric actinin (Green fluorescence) at 3M post-MI, n = 6. (C-D) Immunofluorescence images of PKH-26 and ECs and SMCs makers in the infarcted heart at 2M post-MI (Rectangular area further magnified). Scale bar = 50 μ m. (E-G) Representative immunofluorescence images and quantification of transplanted CPCs (E) and hiPSCs (F) in infarct and border area 72h post-MI using human mitochondria antigen (human-mit) tracking. * $P < 0.05$ vs. hiPSCs treated

group, n = 3. (H-J) Representative immunofluorescence images and quantification of differentiated CMs in infarct and border area 3M post-MI from CPCs (H) and hiPSCs (I) treated mice using cTnI and human-mit tracking. * $P < 0.05$ vs. hiPSCs treated group, n = 3.