

## Original Article

# Transplantation of multipotent Isl1+ cardiac progenitor cells preserves infarcted heart function in mice

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**Abstract:** Cell-based cardiac therapy is a promising therapeutic strategy to restore heart function after myocardial infarction (MI). However, the cell type selection and ensuing effects remain controversial. Here, we intramyocardially injected Isl1+ cardiac progenitor cells (CPCs) derived from EGFP/luciferase double-tagged mouse embryonic stem (dt-mES) cells with vehicle (fibrin gel) or phosphate-buffered saline (PBS) into the infarcted area in nude mice to assess the contribution of CPCs to the recovery of cardiac function post-MI. Our results showed that Isl1+ CPCs differentiated normally into three cardiac lineages (cardiomyocytes (CMs), endothelial cells and smooth muscle cells) both on cell culture plates and in fibrin gel. Cell retention was significantly increased when the transplanted cells were injected with vehicle. Importantly, 28 days after injection, CPCs were observed to differentiate into CMs within the infarcted area. Moreover, numerous CD31+ endothelial cells derived from endogenous revascularization and differentiation of the injected CPCs were detected. SMMHC-, Ki67- and CX-43-positive cells were identified in the injected CPC population, further demonstrating the proliferation, differentiation and integration of the transplanted CPCs in host cells. Furthermore, animal hearts injected with CPCs showed increased angiogenesis, decreased infarct size, and improved heart function. In conclusion, our studies showed that Isl1+ CPCs, when combined with a suitable vehicle, can produce notable therapeutic effects in the infarcted heart, suggesting that CPCs might be an ideal cell source for cardiac therapy.

**Keywords:** Heart regeneration, myocardial infarction, cardiac progenitor cell, cardiac function

## Introduction

Cardiovascular disease (CVD) is the leading cause of death globally, and the number of afflicted individuals is predicted to continue to increase [1]. Myocardial infarction (MI) is the most common CVD disease and has high morbidity and mortality, resulting in a heavy economic burden on society [2]. MI normally occurs when the blood supply in the heart is interrupted, leading to myocardial ischemia and necrosis followed by the formation of a large, noncontractile scar [3] and a high risk of sudden death [4]. MI leads to loss of cardiomyocytes (CMs), and due to the very limited regenerative capacity of the human heart (0.5%-1% per year), scarred areas appear to persist indefinitely [5].

Currently, the only definitive treatment for heart failure is heart transplantation, which is limited by a lack of organ donation, immunological rejection, and high risk associated with the surgical procedure [3]. Stem cell-based heart regeneration is a promising alternative method to regenerate the injured heart. The rationale is to repair the damaged tissue by implanting cardiomyogenic or angiogenic cells into the infarcted ventricle, with the expectation that the engrafted cells will contribute to generate new myocardial tissue and vessels [6-10]. However, many challenges must be addressed for cell based therapy, including identifying the most effective cell source, improving cell retention and survival, and reducing immune rejection.

Many cell types have been applied for cardiac regeneration, such as induced pluripotent stem cells, CMs, bone marrow stem cells, and cardiac progenitor cells (CPCs) [11]. Bone marrow stem cells secrete paracrine factors that can stimulate angiogenesis but cannot recreate functional myocardium [12, 13]. Great benefits have been obtained by CM transplantation, but only modest functional recovery has been achieved. A major reason could be that the extracellular matrix (ECM) secreted by the transplanted CMs differ from the ECM of the infarcted heart, preventing coupling of CMs with host cells [14]. Autologous CPCs may produce positive effects on cardiac function and remodeling in animals. However, recent clinical trials failed to recover cardiac function using autologous adult CPCs [15, 16]. In addition, the appropriate markers for selecting CPCs remain a matter of dispute. *c-kit*<sup>+</sup> and *Sca-1*<sup>+</sup> adult CPCs have been isolated and characterized, but their ability to differentiate into CMs is controversial [11]. Embryonic CPCs identified by the specific marker *Isl1* have been isolated from second heart fields [17, 18]. *Isl1*<sup>+</sup> CPCs are bona fide cardiac progenitors that give rise to all cardiac lineages found in the heart and are likely to be a more suitable candidate for use in cell therapy applications [19-22].

In addition to the cell source, optimized delivery strategies can improve the retention and integration of injected cells in injured hearts. Commonly used cell delivery methods include intravenous, intracoronary and intramyocardial injection. The intravenous and intracoronary injection methods result in rapid cell loss due to the blood circulation, with very low rates of homing to the target sites. Intramyocardial injection, which can directly deliver cells into the infarcted region, is more efficient and more widely used at present [23]. Even with intramyocardial injections, more than 90% of injected cells are lost within 24 hours due to immediate leakage from the puncture hole and venous system [24, 25]. Therefore, delivering cells with a vehicle to prevent cell leakage might improve cell retention after cell injection.

In this study, we aimed to investigate the therapeutic potential of mouse *Isl1*<sup>+</sup> CPCs carried by a suitable vehicle and transplanted into infarcted mouse hearts.

## Materials and methods

### *Construction of an EGFP/luciferase double-tagged mouse embryonic stem (dt-mES) cell line*

To track injected cells in vivo, CRISPR/Cas9 was applied to generate a knock-in mES reporter cell line with stable expression of EGFP and luciferase. Targeting vectors were constructed by modifying a published *Rosa26* donor vector [26]. EGFP and luciferase sequences were linked by a T2A fragment. The EGFP-T2A expression cassette flanked by a 1.5 K left homology arm and a 0.9 K right homology arm was inserted into the intron of *Rosa26* loci. The Cas9 expression plasmid JDS246 and sgRNA expression plasmid DR274 obtained from Addgene were used for CRISPR/Cas9-mediated knock-in. A 20 bp sgRNA (GCTCTATAATAACT-AT) was synthesized and cloned into the plasmid DR274. Then, the target vector, JDS246 plasmid and DR274-containing sgRNA sequence were co-transfected into mouse ES cells. EGFP-positive clones were isolated and characterized.

### *Cell culture, differentiation and characterization*

The dt-mES cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM  $\beta$ -mercaptoethanol, 0.1 mg/ml Leukemia Inhibitory Factor (LIF) (Invitrogen, Grand Island, NY, USA), 0.4 mM PDO325901 and 3 mM CHIR99021 (Sigma-Aldrich, St Louis, MO, USA). Differentiation into CPCs was performed according to a previously published protocol with some modifications [27]. In brief, dt-mES cells were digested with TrypLE (Invitrogen) and cultured suspended in differentiation medium 1 (DM1, containing IMDM supplemented with Ham's F12, Bovine Serum Albumin (BSA), B27, N2 (Invitrogen), monothioglycerol and vitamin C (Sigma-Aldrich)) at a density of 75,000 cells/ml in a Petri dish for 48 hours. Then, the embryoid bodies were collected, dissociated and cultured in DM1 supplemented with 5 ng/ml VEGF, 5 ng/ml Activin A and 0.8 ng/ml BMP4 (R&D Systems, Minneapolis, MN, USA) at a density of 100,000 cells/ml in a Petri dish for 40 hours. The EBs were collected again, dissociated and cultured in cell culture dishes coated with 0.1%

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gelatin in differentiation medium 2 (DM2, containing StemPro-34 SFM supplemented with vitamin C, glutamine (Invitrogen), monothio glycerol (Sigma-Aldrich), 5 ng/ml VEGF, 10 ng/ml bFGF and 25 ng/ml FGF10 (R&D Systems)) for another 32 hours to generate Isl1+ CPCs. The CPCs were either collected for identification, injection or random differentiation (CMs) in DM2 medium without growth factors (VEGF, bFGF and FGF10) for 7 days for further immunofluorescence (IF) staining characterization using standard protocols. The primary and secondary antibodies are listed in [Table S1](#).

### *Vehicle biocompatibility assessment*

Fibrin gel (EVICEL® Fibrin Sealant (Human), Ethicon, Somerville, MA, USA) was used as a vehicle for both the biocompatibility assessments described here and cell delivery described below. The two gel components, fibrinogen and thrombin, were diluted in phosphate-buffered saline (PBS) to final concentrations of 20 mg/ml and 100 IU/ml, respectively. Our preliminary data showed that a ratio of 20 mg/100 IU was suitable for obtaining an appropriate gel viscosity and coagulation time. A total of  $1 \times 10^6$  Isl1+ CPCs were stained by Dil (Invitrogen), either seeded directly onto 24-well plates or mixed with 40  $\mu$ l of fibrin gel and then cultured while floating in DM2 medium without growth factors (VEGF, bFGF and FGF10). On days 1, 3 and 7, fluorescence images were captured, cck-8 reagent (Dojindo Molecular Technologies, Rockville, MD, USA) was added to the wells, and the cell culture was continued for another 2 hours. A 200- $\mu$ l volume of culture medium was then transferred to a 96-well plate, and the absorbance was measured at 450 nm using a microplate reader. On day 7, the differentiated cells were fixed by zinc fixative solution (BD Pharmingen, San Jose, CA, USA) for further IF staining characterization.

### *Animals*

This study was approved by the Committee for Animal Research of the University of Michigan and was performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

### *MI and cell injection in mice*

In this study, 60 nude mice ( $25 \pm 5$  g, 6-8 weeks) were used and were divided into 6 groups: MI only (7 mice), fibrin only (5 mice), PBS+CPC (16 mice), GEL+CPC (16 mice), GEL+CM (16 mice).

MI was induced by permanent ligation of the left anterior descending coronary artery (LAD) [28]. A total of  $1 \times 10^6$  cells suspended in 10  $\mu$ l of gel or 10  $\mu$ l of PBS was injected intramyocardially into the infarct zone with a 30-gauge needle, and the animals were euthanized 28 days after cell transplantation.

### *Bioluminescence imaging (BLI)*

BLI was performed 1, 3, 7, 14, 21 and 28 days after cell injection using an IVIS Lumina II (Caliper Lifesciences, Hopkinton, MA, USA). After isoflurane anesthesia, luciferin (Caliper LifeSciences, Hopkinton, MA, USA), a luciferase substrate, was intraperitoneally injected at a concentration of 150 mg/kg 10 min prior to detection. The total flux density was used for quantification.

In addition, BLI was performed to detect luciferase expression in dt-mES cells or dt-mES-derived CPCs and in the cardiomyocyte stage. A 100- $\mu$ l volume of luciferin was added to 1 ml of culture medium prior to detection.

### *Histological assessment*

Histological studies were performed using standard protocols. Briefly, the mice were sacrificed, and the hearts were perfused with 20% KCl. Then, the hearts were fixed with zinc fixative solution (BD Pharmingen) and dehydrated with 30% sucrose. After embedding in OCT compound (BD Pharmingen), the samples were sectioned and processed for immunostaining, including hematoxylin and eosin (HE), trichrome, immunohistochemistry (IHC) and IF. Images were captured by Aperio (Leica Biosystems, Buffalo Grove, IL, USA) and a confocal microscope (Nikon, Melville, NY, USA). Infarct size was measured using Image J software [29].

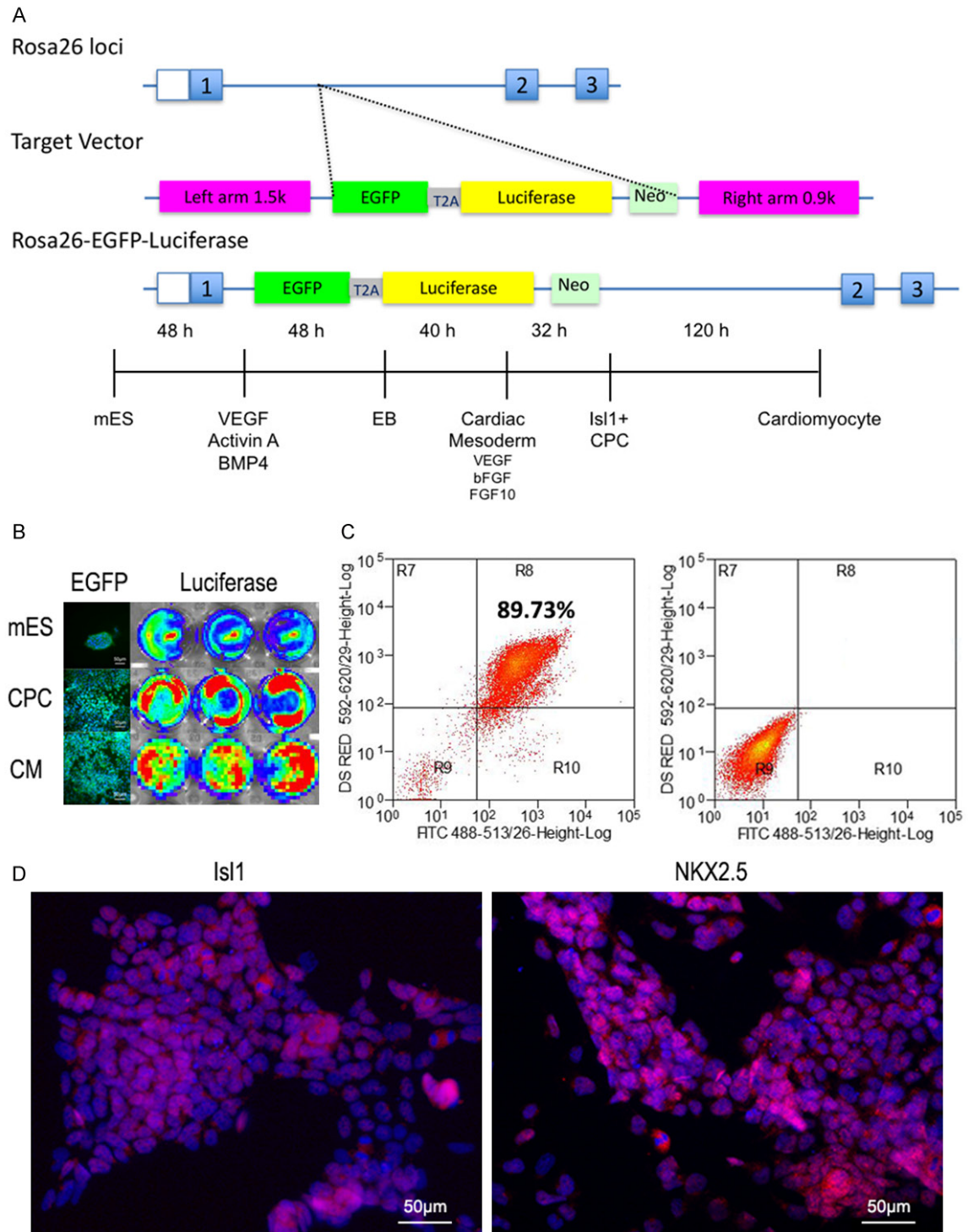
### *Echocardiography*

Echocardiography was performed 28 days post-MI using a Vevo 770 system (Visualsonic, Toronto, Canada). The ejection fraction (EF) was measured by an investigator blinded to the respective treatments.

### *Statistics*

GraphPad Prism software was used for statistical analyses. Data are expressed as the mean  $\pm$  SD. For statistical analyses, we performed

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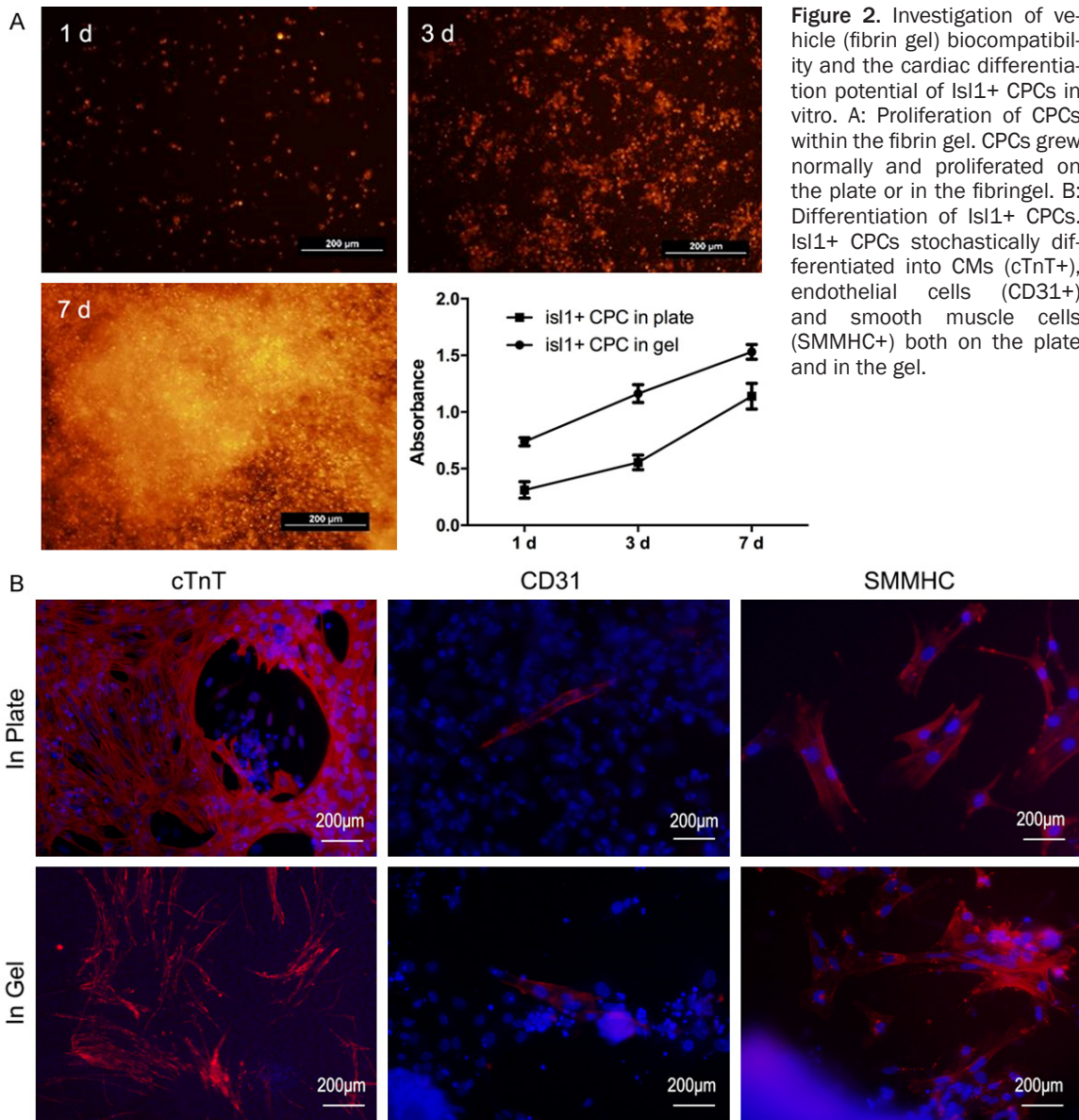


**Figure 1.** Preparation and identification of EGFP/luciferase double-tagged mES cell-derived Isl1+ CPCs. **A:** Schematic of the double-tagged EGFP/luciferase construct and the Isl1+ CPC differentiation protocol. **B:** Characterization of EGFP and luciferase by fluorescent microscopy and IVIS Lumina II during the dt-mES, CPC and CM stages. EGFP and luciferase were expressed stably throughout the entire experiment. **C:** Characterization of Isl1+ CPCs by flow cytometry. The X and Y axes correspond to EGFP and Isl1, respectively. **D:** Characterization of Isl1+ CPCs by IF staining with antibodies against Isl1 and Nkx2.5.

ANOVA followed by Tukey's multiple comparisons test and differences were considered sig-

nificant at  $P < 0.05$ . All reported  $P$ -values are two-sided.

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### Results

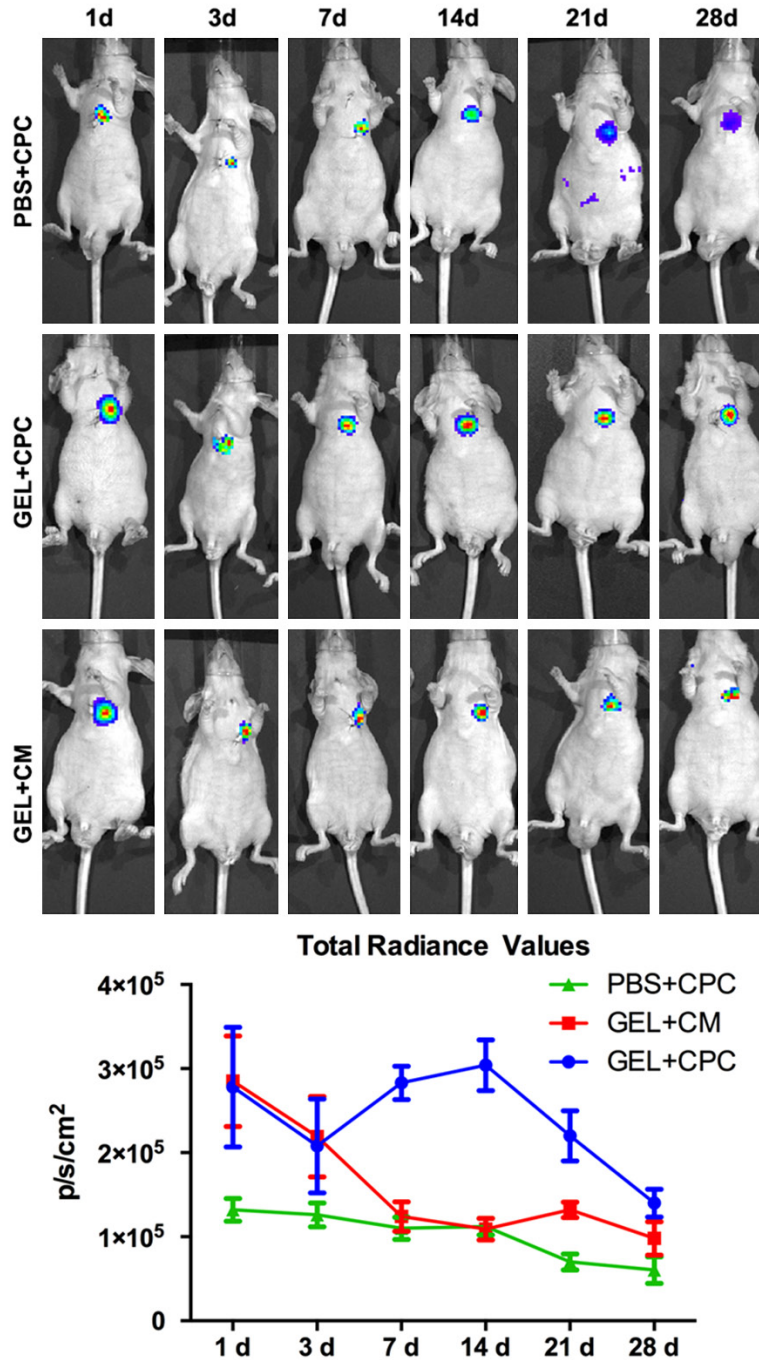
#### *Differentiation of Isl1+ CPCs from mouse ES cells*

The EGFP/luciferase-positive mES cell line was established using CRISPR/Cas9 technology [30, 31] (**Figure 1A**). An EGFP-T2A-luciferase reporter was inserted into the Rosa26 locus by CRISPR/Cas9-mediated homologous recombination. Single cell-derived mouse ES cell clones were isolated and characterized. EGFP fluorescence and luciferase bioluminescence were detected at every stage during dt-mES maintenance and cardiac differentiation (mES cells,

CPCs and CMs). EGFP/luciferase expression was stably maintained throughout the experiment (**Figure 1B**).

To produce Isl1+ CPCs, the dt-mES cells were differentiated using a modified embryoid body-based cardiac differentiation protocol (**Figure 1A**), which was based on a previous published method with some modifications [27]. Similar to Keller's data, our results also showed that the Isl1+ CPC stage was observed at 32 hours after the cardiac mesoderm stage. At this time point, the cells were collected for analysis, and flow cytometry demonstrated that the yield of Isl1+ cells reached 89.73% (**Figure 1C**). IF stain-

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**Figure 3.** Live animal bioluminescence evaluation of Isl1+ CPC retention and survival. At day 1 after cell injection, the fibrin gel group maintained a 2.1-fold greater cell density compared with the PBS group. Over the first 3 days, the cell retention in all groups exhibited a decreasing trend, and the signals in the PBS+CPC and GEL+CM groups continued to decrease until day 28. By contrast, the cell retention in the GEL+CPC group increased from day 3 to day 14, reaching a peak value 3.1-fold greater than those of the other two groups. A decreased signal was also observed in the GEL+CPC group. At the final time point, cell retention in the GEL+CPC group was 2-fold greater than that in the other two groups.

ing for Isl1 and NKX2.5 also identified the specific CPC stage (Figure 1D), and Isl1+ CPCs

were used for subsequent experiments.

### Characterization of Isl1+ CPC differentiation in vitro

To characterize the Isl1+ CPCs and assess the biocompatibility of the vehicle with the Isl1+ CPCs, CPCs were stained with Dil and cultured in the fibrin gel. Cell activity assays revealed that the CPCs proliferated dramatically (Figure 2A).

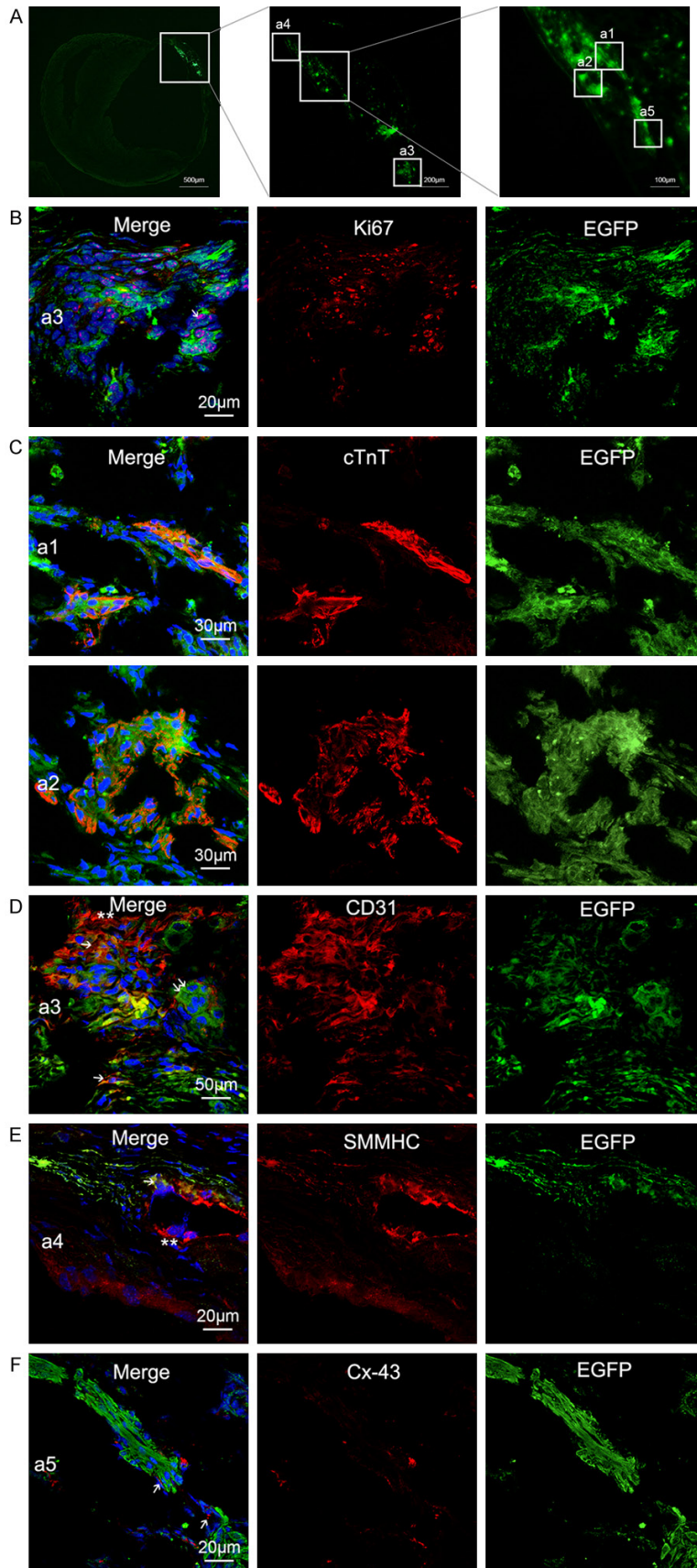
To evaluate the multi-lineage differentiation potential of Isl1+ CPCs, growth factors for CM induction were removed from the DM2 culture medium. The IF results showed that 7 days after the Isl1+ stage, CPCs stochastically differentiated into CMs (Troponin T- and cTnT-positive), endothelial cells (CD31-positive) and smooth muscle cells (SMMHC-positive) in the plate (Figure 2B and Video S1). Fibrin gel is a degradable material and dissolves approximately 5 days after its formation. The cells grew out from the gel and differentiated into the three lineages (CMs, endothelial cells and smooth muscle cells) (Figure 2B). In addition, cell beating was observed in both the plate and after seeding into the gel for 5 days (Video S2).

### Fibrin gel significantly increased cell retention of both Isl1+ CPCs and CMs after transplantation into infarcted mouse hearts

We next used vehicle (fibrin gel) or PBS for our cell transplantation studies to determine if vehicle could significantly improve the retention and integration of the transplanted cells. mES cell-derived

CMs were applied as a control cell source in our experiments. Our results showed that, on

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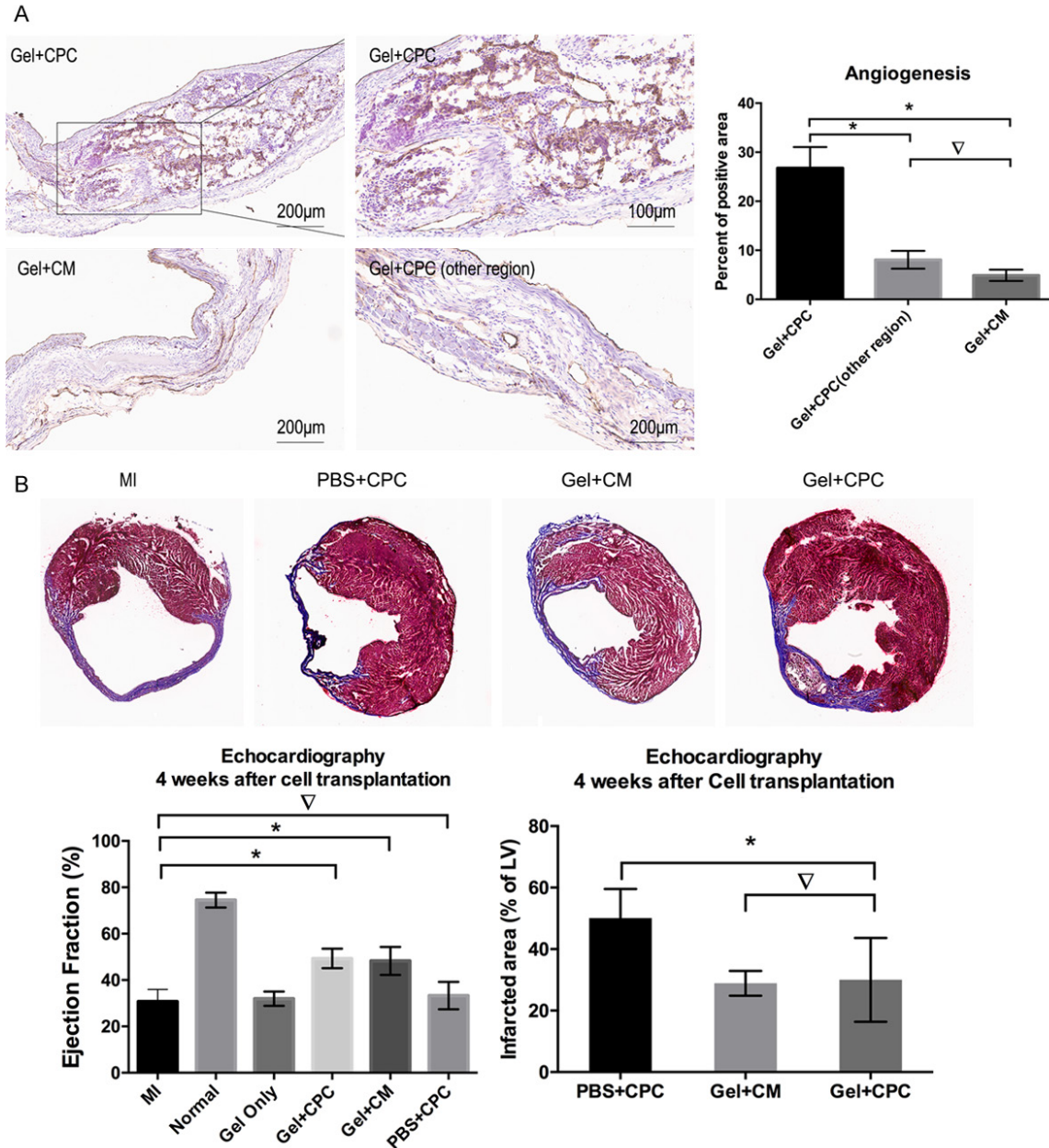
**Figure 4.** Differentiation and integration of Isl1+ CPCs 28 days after transplantation. A: Low-magnification confocal images showing the integration of EGFP-positive CPC progenies in infarcted hearts. B: The proliferation of Isl1+ CPCs in infarcted hearts was detected by Ki67 staining (single white arrow). C: Differentiation of EGFP-positive CPCs into CMs. D: Differentiation of EGFP-positive CPCs into endothelial cells. Note that CD31-positive cells were derived both from CPCs (single white arrow, red and green) and host cells (double white stars, red only). E: Differentiation of EGFP-positive CPCs into smooth muscle cells marked by SMMHC (single white arrow) and host cells (double white stars). F: Integration of Isl1+ CPC-derived CMs with host cells detected by Cx-43 staining (single white arrow). Abbreviations: a 1-5 indicates areas 1-5.

day 1 after injection, the fibrin gel group exhibited 2.1-fold higher cell retention compared with the PBS group. Over the first 3 days, the cell retention in all groups showed a decreasing trend, and the retention in the PBS+CPC and GEL+CM groups continued to decrease until day 28. By contrast, the cell retention in the GEL+CPC group increased from day 3 to day 14, reaching a peak value of 3.1-fold versus the other two groups. We subsequently also observed a decreasing trend in the GEL+PCP group. At the final time point, the cell retention in the GEL+CPC group was approximately two fold higher than in the other two groups (**Figure 3**).

*Transplantation of Isl1+ CPCs led to engraftment of cardiac tissues with both CMs and vasculature*

To investigate the therapeutic effects of the injected cells

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**Figure 5.** Cardiac functional recovery 28 days after cell injection. A: Evaluation of angiogenesis within the Isl1+ CPC-injected area compared with the cardiomyocyte-injected area and other regions. The GEL+CPC group exhibited a much higher vascular density, especially in the cell-injected region. By contrast, the MI-only group (other region) and GEL+CM group did not exhibit significant capillary generation. The semi-quantitative analysis was performed with ImageJ software; \* $P < 0.05$ . B: The infarct size was calculated based on trichrome Masson's staining, and cardiac functional recovery was evaluated by EF. Animals without MI were used as a positive control. The MI-only and GEL-only groups (only gel was injected into the MI region) were used as negative controls. Note that the cardiac function showed some recovery in the GEL+CPC and GEL+CM groups but not in the normal control group; \* $P < 0.05$ .

after transplantation into the infarcted hearts, the animals were euthanized at day 28 after transplantation, and their hearts were harvested for histological analysis (Figure 4). The injected cells were distinguished from host cells by EGFP expression (Figure 4A). One advantage

of CPCs is their proliferative ability, and Ki67 staining confirmed that the Isl1+ CPCs proliferated after transplantation (Figure 4B).

Further analyses indicated that Isl1+ CPCs differentiated into CMs, endothelial cells, and



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smooth muscle cells after transplantation. A subset of EGFP-positive cells expressed cardiac troponin T (**Figure 4C**), demonstrating the in vivo CM differentiation ability of Isl1+ CPCs. Interestingly, a massive increase in endothelial cells stained by CD31 was observed in the injection region of the CPC+GEL group compared with the PBS or GEL+CM group (**Figure 4D** and **Figure S1**). The overlapping image shows that these new cells were generated from both the injected CPCs and the endogenous cells. By contrast, only endogenous CD31+ cells were detected in the GEL+CM group (**Figure S1**). SMMHC staining demonstrated that revascularization occurred via both the injected and host cells (**Figure 4E**). Furthermore, the coupling of injected cells with host cells was observed by Connexin-43 staining (Cx-43) (**Figure 4F**). These results demonstrated that the Isl1+ CPCs were capable of proliferating, differentiating into CMs, smooth muscle and endothelial cells and integrating with host tissue after transplantation into infarcted hearts.

*Transplantation of Isl1+ CPCs decreased infarcted areas, increased vascular density, and improved heart function*

We next examined the effect of Isl1+ CPC transplantation on the overall regeneration of CMs and vasculature in the infarcted area 28 days after injection. The GEL+CPC group exhibited significantly increased vascular density, especially in the regions of cell injection. By contrast, the MI-only area (other region) and the GEL+CM group did not show significant revascularization (**Figure 5A**). Echocardiograph analysis was also performed 28 days after cell transplantation. The increased EF in the GEL+CPC and GEL+CM groups indicated functional recovery after the cell injection therapy. By contrast, we detected only modest therapeutic effects in the PBS+CPC group (**Figure 5B**). Moreover, the GEL+CPC and GEL+CM groups exhibited a significantly reduced infarct size, consistent with our echocardiography results. These data suggested that under the current experimental settings, cell transplantation with an appropriate vehicle substantially improved the healing efficiency. The therapeutic potential of Isl1+ CPCs for cardiac therapy appeared to be superior to that of CMs.

### Discussion

In this study, we investigated whether Isl1+ CPCs are a suitable cell type for heart regener-

ation following MI by simultaneously regenerating myocardium and vasculature. To achieve this goal, we adapted an EB-based differentiation protocol to generate a high percentage of Isl1+ CPCs from dt-mES cells, which expressed EGFP/luciferase steadily throughout the entire experiment. We first characterized the multi-lineage potential of Isl1+ CPCs in vitro. We then intramyocardially injected the Isl1+ CPCs or CMs into infarcted mouse hearts with either a vehicle or PBS as a control. Cell retention was evaluated with a bioluminescence detection system, and multi-lineage differentiation was evaluated by immunostaining. The recovery of cardiac function was evaluated by the size of the infarcted area and EF, which confirmed the therapeutic potential of the Isl1+ CPCs in promoting cardiac regeneration. Our data also indicated significantly increased angiogenesis in the GEL+CPC group, suggesting that the CPCs exert therapeutic effects through cardiac muscle formation and angiogenesis.

The major discovery in this study was that both Isl1+ CPCs and CMs showed prominent therapeutic potential, with CPCs likely being more potent. The CM cell type is beneficial in the functional recovery of MI cardiac tissue [32, 33]. Our EF results indicated greater therapeutic effects in the GEL+CPC and GEL+CM groups compared with the PBS and CPC-only groups. We speculated that cell retention was likely a key factor for the recovery of cardiac function. Increased cell retention could lead to a smaller infarcted area and thicker ventricle wall. Therefore, to evaluate the cell retention of injected cells in a live animal model, we used bioluminescence to observe the total radiance values when the luciferase in the injected cells reacted with its substrate, luciferin. During the healing process, cell retention in the GEL+CPC group initially decreased (d1 to d3), followed by an increase (d3 to d14) and decrease (d14 to d28). By contrast, cell retention continued to decrease in the GEL+CM group, although cell retention was two fold greater in both the GEL+CPC and GEL+CM groups compared with the PBS+CPC group on day 1 post-injection, similar to Nakamuta's result (2.5-fold more transplanted cells remaining) [34]. Encouragingly, the GEL+CPC group reached a peak value of 3.1 times greater than the other two groups at day 14. This observation is consistent with Rojas's data [35], although we observed opposite results in our PBS group. The

difference in cell retention may have been due to the increased proliferative capacity of CPCs within the MI area compared with CMs. Indeed, the presence of Ki67+ and EGFP+ dual-positive cells indicated that the injected CPCs entered into the cell cycle (**Figure 4B**, area a3) after cell transplantation.

The three-lineage differentiation capacity of Isl1+ CPCs was likely a major contributor to the functional recovery of the injured heart. EGFP and cTnT double-positive cells were detected within the injected area, indicating that Isl1+ CPCs differentiated into CMs in the mouse MI heart (**Figure 4C**, areas a1 and a2). We also observed transplanted CPC differentiation into endothelial cells, as indicated by the presence of CD31+ and EGFP+ dual-positive cells. We compared the angiogenesis of the injected area with other regions (i.e., those not receiving cells) and observed that injection of GEL+CPCs led to an increased capillary density within the infarcted heart (**Figure 5A**). SMMHC/EGFP double-positive cells were also observed in the infarct region, further indicating the differentiation of transplanted CPCs into three cardiac lineages (**Figure 4E**, area a4).

CPC transplantation also resulted in other benefits that likely contributed to heart functional recovery. Our data suggested that Isl1+ CPCs stimulated vascularization from endogenous host cells. Consistent with this notion, within the infarcted area, many more cells that were CD31 positive but EGFP negative were observed (**Figure 4D**, area a3). By contrast, our data suggest that transplanted CMs only promoted endogenous vascularization repair (**Figure S1**), similar to a recent report [36]. In addition, improved connection or integration of injected cells with endogenous host cells was observed in the Isl1+ CPC transplantation group (**Figure 4F**, area a5), as indicated by staining of connexin-43, a gap junction protein normally expressed between CMs [37-40].

Our studies demonstrated that engineered cell vehicles greatly improved the efficiency of cell transplantation therapy. One major challenge for the efficacy of cell transplantation in heart regeneration is the low cell retention rate, which may be due to leakage of cells [41-43] or environmental disadvantages for cell growth, such as inflammation, ischemia due to poor vascularization of the injected areas, and apop-

tosis [44]. We chose fibrin gel as the vehicle in our study due to its biocompatibility and bioresorbability [45-50]. We evaluated the use of the vehicle with our specific cell types, Isl1+ CPCs and CMs derived from dt-mES prior to injection. Isl1+ CPCs grew and differentiated normally in fibrin gel. Interestingly, after the Isl1+ CPCs were seeded into the gel and cultured while floating in medium, troponin T structure-like CMs grew out from the gel when the gel began to dissolve, and behaviors of primary cultures were observed. In addition, CD31- and SMMHC-positive cells were observed under this condition, and all cell behaviors in the gel were similar to those observed in the plate. Importantly, the handling time, which is considered a critical factor [51], was improved because the vehicle simultaneously delivered the cells and sealed the needle hole after injection, preventing cell loss, especially from the beating heart, resulting in higher cell retention and a prominent therapeutic effect. We conclude that better-engineered biocompatible cell vehicles will help achieve high cell retention, survival, and integration of transplanted cells.

### Conclusion

Our study showed that Isl1+ CPCs differentiated into three cardiac lineages after transplantation into MI mice. The transplanted Isl1+ CPCs reduced infarct size and improved cardiac function. Compared with CMs, the contributions from Isl1+ CPCs included not only myocardium regeneration but also more significant vascular formation. Our study indicates that Isl1+ CPCs are a promising cell source for heart regeneration. Future studies using more potent cell vehicles and large animal models should provide more insights into the therapeutic efficacy and safety of Isl1+ CPCs in heart therapy.

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### Disclosure of conflict of interest

None.

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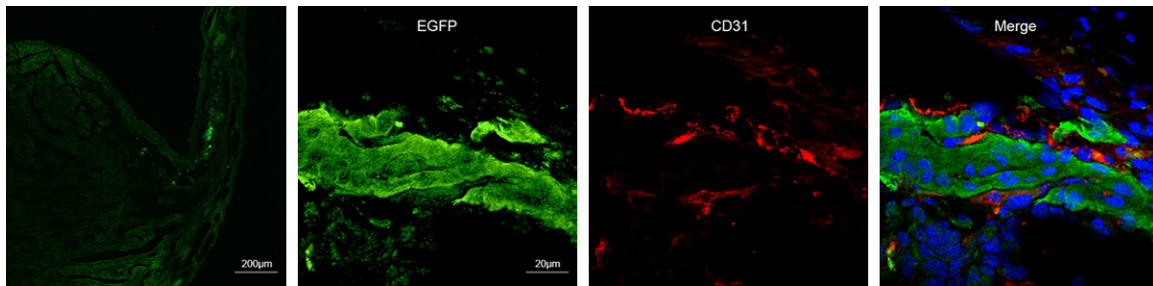
# Isl1+ CPC injection preserves cardiac function after MI

**Table S1.** Primary and secondary antibodies

Name	Concentration	Manufacturer
Isl1	1:100	Abcam
NKX2.5	1:200	Abcam
Troponin T (cTnT)	1:200	Thermo Fisher
CD31	1:50	Abcam
Smooth Muscle Myosin Heavy Chain (SMMHC)	1:200	Abcam
Alexa Fluor® 488 donkey anti-mouse IgG	1:1000	Invitrogen
Alexa Fluor® 488 donkey anti-rabbit IgG	1:1000	Invitrogen
Alexa Fluor® 594 donkey anti-mouse IgG	1:1000	Invitrogen
Alexa Fluor® 594 donkey anti-rabbit IgG	1:1000	Invitrogen

**Video S1.** CMs beating on the plate. A total of  $1 \times 10^6$  Isl1+ CPCs were seeded and cultured in DM2 on 24-well plates without growth factors for stochastic differentiation. After seven days, beating CMs were observed.

**Video S2.** CMs beating in the fibrin gel. A total of  $1 \times 10^6$  Isl1+ CPCs were mixed with 40  $\mu$ l of fibrin gel and cultured floating in DM2 without growth factors in 24-well plates. After seven days, beating CMs were observed in the gel.



**Figure S1.** Confocal imaging of the CM injection area. The CD31-positive cells were derived from the host cells.