Use of a neonatal rat system as a bioincubator to generate adult-like mature cardiomyocytes from human and mouse pluripotent stem cells

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Pluripotent stem cells (PSCs), including induced PSCs, hold great potential for personalized disease modeling, drug testing and cell-based therapeutics. However, cells differentiated from PSCs remain immature in a dish, and thus there are serious caveats to their use in modeling adult-onset diseases such as cardiomyopathies and Alzheimer's disease. By taking advantage of knowledge gained about mammalian development and from bioinformatics analyses, we recently developed a neonatal rat system that enables maturation of PSC-derived cardiomyocytes into cardiomyocytes analogous to those seen in adult animals. Here we describe a detailed protocol that describes how to initiate the *in vitro* differentiation of mouse and human PSCs into cardiac progenitor cells, followed by intramyocardial delivery of the progenitor cells into neonatal rat hearts, *in vivo* incubation and analysis. The entire process takes ~6 weeks, and the resulting cardiomyocytes can be analyzed for morphology, function and gene expression. The neonatal system provides a valuable tool for understanding the maturation and pathogenesis of adult human heart muscle cells, and this concept may be expanded to maturing other PSC-derived cell types, including those containing mutations that lead to the development of diseases in the adult.

INTRODUCTION

Human induced PSCs (hiPSCs) were first described in 2007 after Takahashi *et al.*[1] reprogrammed somatic cells with certain transcription factors[1]. hiPSCs can differentiate into any cell type of the body and thus hold great promise for disease modeling, drug discovery, repair of nonregenerative organs and the study of human development[2,3]. Since their discovery, numerous hiPSC cell lines from patients with familial diseases have been developed[3,4]. Although iPSCs can differentiate into any type of body cell, they exhibit fetal-like characteristics, remain largely immature and fail to fully integrate into the host organ upon transplantation[5–8]. This means that they are not always suitable for studying diseases that manifest in the adult.

Characteristics of PSC–cardiomyocytes

Heart disease supersedes all other causes of death worldwide[9] and offers tremendous opportunities for modeling genetic cardiomyopathies and treatment of heart failure with regenerative therapies[4,10]. However, nearly all cardiomyopathies develop in adult life, and many PSC-derived cardiomyocytes (PSC-CMs) do not truly recapitulate adult disease phenotypes, probably because of the immaturity of the cells.

Cardiac maturation initiates during early embryonic life and continues to early adulthood. During this process, CMs become rectangular, multinucleated and elongated, and develop more organized sarcomeric structures[5,11]. In addition, myosin heavy-chain subtypes switch, and T-tubule sarcolemma structures and intercalated discs that connect CMs are rapidly formed during the early postnatal period, enabling functional maturation[11,12]. Through analysis of numerous microarray data sets, we demonstrated that even after prolonged culture, PSC-CMs are comparable to CMs from the late embryonic and neonatal stages[7]. In addition, their functional properties, including presence of Ca^{2+} transients and sarcomere shortening, as well as morphological characteristics such as size, shape, nucleation and presence of T-tubules, are all consistent with immature fetal-like myocytes[13,14]. Finally, we have previously demonstrated that a number of transcription regulators are misregulated in long-term cultured PSC-CMs, which may explain the inability of the cells to mature beyond late embryonic/neonatal stages[7].

Methods for PSC-CM maturation

Several groups have recently applied cellular engineering approaches to facilitate differentiation into more mature cardiomyocytes, including electrical stimulation, cell alignment techniques, culturing on different extracellular matrices and mechanical stretching[15–17]. These approaches have resulted in CMs with more mature structural and functional properties, including increased conduction velocity and improved calcium handling properties. In addition, treatment of PSC-CMs with either glucocorticoids or thyroid hormones promoted their maturation by increasing their size and sarcomere length, improving their contractility and so on[18,19]. Therefore, it appears that microenvironmental factors such as paracrine and endocrine signals, physical and electrical forces, and extracellular matrices might promote the maturation of PSC-CMs. Despite all these efforts, the resulting PSC-CMs partially mature and do not form T-tubules, acquire adult membrane potentials or shorten sarcomeres. Recently, Kadota *et al.*[20] used an *in vivo* approach by injecting human induced pluripotent stem cell-derived (hiPSC-derived)-CMs into neonatal and adult rats, but the resulting CMs, determined by heart sections, did not exhibit the size and structure of adult CMs. This might be due to the use of a cell source, incubation time or analysis different from those used in our study[21].

Islet 1 $(Isl1)^+$ cardiac progenitor cells (CPCs) are present in neonatal rodent and human hearts. Unlike PSC-CMs in culture, the vast majority of those neonatal CPCs give rise to fully mature

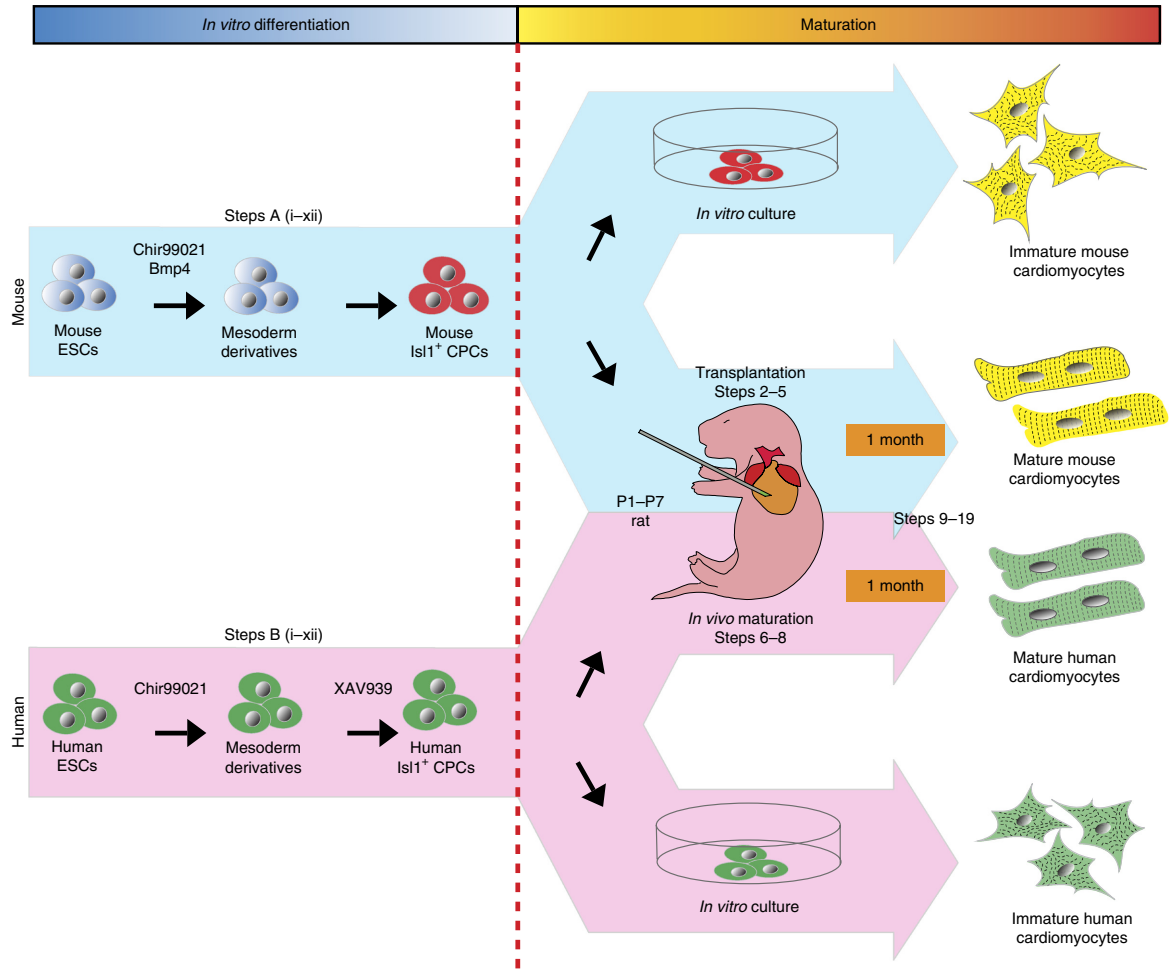

Figure 1 | Experimental protocol for *in vivo* and *in vitro* cardiomyocyte maturation. Left: schematic illustration showing *in vitro* differentiation of mouse Isl1+ CPCs in red and human Isl1+ CPCs in green. Right: *in vitro* versus *in vivo* maturation of cardiomyocytes derived from mouse and human PSCs.

CMs over the next few weeks *in vivo*[5,22,23]. Interestingly, recent comparative microarray data suggest that the maturation of CMs is regulated similarly in mice and humans[24]. On the basis of these findings, we explored the potential of the neonatal heart environment to facilitate maturation of CPCs by injecting mouse and human PSC-derived CPCs into neonatal (postnatal days 3–7) rat hearts[21]. To detect the injected cells in the rat hearts, we used fluorescence-labeled CPCs. We injected FACS-isolated mouse CPCs expressing the Isl1-RFP transgene or unsorted human CPCs expressing high percentages of Isl1. We elected to use rats instead of mice because of their larger heart size and the fact that their heart rate is closer to that of the human heart. To avoid cellular rejection, we used immunosuppressed, athymic animals. We sacrificed the rats at various time points and noted that a relatively small percentage (~1%) of the transplanted cells engrafted[21]. CPCs had a much higher survival and engraftment rate and, within 1 month, *in vivo*-matured PSC-CMs developed the morphological and functional properties of adult CMs, including T-tubule staining, presence of Ca^{2+} transients and sarcomere shortening[21]. Moreover, their gene expression pattern, based on single-cell RNA-sequencing analysis, was similar to that of adult CMs. It is worth noting that PSC-CMs do not mature properly when they are injected into older rat hearts (after postnatal day 14; ref. 21). This suggests the presence of a critical neonatal window that allows PSC-CM maturation.

Applications of the neonatal rat system for the generation of adult-like mature cardiomyocytes from PSCs

In this protocol, we describe how to mature mouse and human PSC-derived CPCs in neonatal rat hearts[21] (**Fig. 1**). Our system can be used to further understand the mechanisms of CM maturation, as well as to study the pathogenesis of several adult cardiac diseases. Furthermore, rats with transplanted matured hiPSC-derived-CMs can be used for the *in vivo* testing of drug therapies and for personalized medicine. Importantly, based on the same principle, our protocol may also be used for the maturation of other cell types, such as neurons, hepatocytes and skeletal muscle cells derived from hiPSCs. In fact, a recent study used the neonatal brain to mature hiPSC-derived neurons *in vivo* for modeling Alzheimer's disease[25]. Finally, considering the late onset of numerous diseases such as certain familial cardiomyopathies and Alzheimer's disease, the neonatal system may be used to uncover the characteristics of cells at the point of disease onset, enabling earlier disease diagnosis, prevention and better disease management.

Limitations

The main limitation of our protocol is the low cell engraftment, which is primarily due to the small size of the host heart and our current cell delivery method. However, it is very likely that the use of larger animals such as pigs and a higher number of cell injections will markedly improve cellular engraftment and the yield of matured PSC-CMs, which will also potentially allow the *in vivo* modeling of human diseases in animal models. In addition, the need to generate a genetically modified reporter PSC line in order to locate the injected cells in the host's heart may make our protocol less suitable for the production of mature cardiomyocytes for clinical applications.

MATERIALS

REAGENTS

- Rats. We used athymic, T-cell-deficient RNU (Charles River Laboratories, strain code 316) rats, between postnatal day (P)1 and P7, as immunosuppressed hosts of injected cells **! CAUTION** Experiments using rodents must conform to all relevant institutional and governmental ethics regulations. This protocol was approved by the Johns Hopkins University Animal Care and Use Committee.
- Embryonic stem cells (ESCs). We used the mouse ESC reporter line (mESC$^{Isl1\text{-}Cre;\ Rosa\text{-}RFP;\ aMHC\text{-}GFP}$), which we have previously generated[26], and the human iPSC line 2016, which was provided by Takahashi *et al.*[1]. **Box 1** describes how to generate a constitutively expressing GFP/RFP hiPSC line **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- Glasgow's MEM (GMEM; Gibco, cat. no. 11710035)
- DMEM (high glucose; Gibco, cat. no. 11965-092)
- Characterized FBS (500 ml; Invitrogen, cat. no. SH30071.03)
- Sodium pyruvate (100 mM; Gibco, cat. no. 11360)
- β-Mercaptoethanol (Sigma-Aldrich, cat. no. M6250)
- ESGRO (LIF; Millipore, cat. no. ESG1106)
- PD0325901 (Selleck Chemicals, cat. no. S1036)
- CHIR99021 (Selleck Chemicals, cat. no. S2924)
- 0.1% (wt/vol) Gelatin (EMD Millipore, cat. no. ES-006-B)
- 1× DPBS without calcium and magnesium (Thermo Fisher Scientific, cat. no. 21-031-CV)
- 1× PBS without calcium and magnesium (Thermo Fisher Scientific, cat. no. 21-040-CV)
- TrypLE (Gibco, cat. no. 12604)
- IMDM (Gibco, cat. no. 12440053)
- Ham's F12 (Gibco, cat. no. 10-080-CV)
- N2 supplement (Gibco, cat. no. 17502-048)
- B27 minus vitamin A (50×; Thermo Fisher Scientific, cat. no. 12587010)
- B27 minus insulin (50×; Thermo Fisher Scientific, cat. no. A1895601)
- BSA (Sigma-Aldrich, cat. no. A2153)

Box 1 | Generation of GFP/RFP-tagged human iPSCs ● TIMING ~3 weeks

Additional reagents

- Lentivirus. We used a CAG-GFP lentivirus (Cellomics Technology, cat. no. PLV-10057-50). Alternatively, a CAR-RFP lentivirus can be used instead (Cellomics Technology, cat. no. PLV-10071-50)

! CAUTION The handling of lentiviral vectors should be carried out using the proper biosafety containment. See guidance on 'Biosafety Considerations for Research with Lentiviral Vectors'. (https://osp.od.nih.gov/wp-content/uploads/Lenti_Containment_Guidance.pdf)

- DMSO (Corning Cellgro, cat. no. 25-950-CQC)

Additional equipment

- Cryogenic vials (Corning, cat. no. CLS431421)

Procedure

1. Coat three wells of a six-well plate with Geltrex as described in Step 1B(i). Dissociate plated hiPSCs as described in Steps 1A(ii) and 1A(iii). Resuspend 6 × 10^5 cells in 6 ml of Essential 8 medium with 10 μM ROCK inhibitor and split over three 15-ml tubes. Quickly thaw one vial of the CAG-GFP/RFP lentivirus and test three different volumes by adding 2, 5 and 10 μl to determine the optimal transduction efficiency of the virus. Mix well and plate the infected hiPSCs in the wells. Incubate in a 5% CO_2 incubator overnight.

▲ **CRITICAL STEP** Split the remaining virus into 2-μl aliquots and store at –80 °C to avoid freeze–thaw cycles, which will decrease the transduction efficiency of the virus. The virus can be maintained without a substantial change in transduction efficiency for up to 1 year. Use bleach to clean all equipment that has come into contact with the virus.

2. The next morning, change the medium to Essential 8 to avoid cell toxicity. Then over the next days, change the medium to Essential 8 daily and determine the transduction efficiency using a fluorescence microscope to check GFP/RFP expression. When the cells reach 60–70% confluence (after ~2–3 d), dissociate the cells in the well with the highest GFP/RFP expression and replate ~5 × 10^4 cells in two Geltrex-coated sterile 10-cm Petri dishes (in Essential 8 with 10 μM ROCK inhibitor).

3. Continue to change Essential 8 medium daily, and then 2–3 d later single cells will form colonies. Pick single GFP/RFP-only colonies using a 20-μl pipette under a fluorescence microscope in a cell culture hood. Transfer the colonies to Geltrex-coated wells of a 24-well plate containing Essential 8, 10 μM ROCK inhibitor and penicillin–streptomycin.

4. Grow the cells for 3–4 d and then replate only the colonies in which all cells have GFP/RFP expression. Replate each colony to a new well of six-well plate and then subsequently expand the cells by growing them in a T25 flask.

5. When GFP-tagged hiPSCs reach 70–80% confluence (after ~3 d), split them into four cryovials with 10% (vol/vol) DMSO in MEF medium and freeze them at 80 C for 2 d and then store them in liquid nitrogen.

▲ **CRITICAL STEP** To improve the survival of the colonies picked in step 3, keep the cells in the same Essential 8 medium with ROCK inhibitor for 48 h.

■ **PAUSE POINT** Cells can be stored in liquid nitrogen for at least 10 years.

6. To thaw the cells, prepare 4 ml of MEF medium prewarmed to 37 °C in a 15-ml tube. Quickly thaw the frozen cells by moving the cryovial from liquid nitrogen directly to a 37 °C water bath, and before the cells are completely thawed, add 1 ml of the prewarmed MEF medium directly to the cells in the cryovial. Mix well with the rest of the MEF medium in the 15-ml tube. Centrifuge for 3 min at 270*g* at room temperature. Aspirate the supernatant and resuspend the cells in Essential 8 medium with 10 μM ROCK inhibitor (Y27632) and replate them in a T25 flask coated with Matrigel as in Step 1B(i).

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- 100× Penicillin–streptomycin (Gibco, cat. no. 15070-063)
- Monothioglycerol (Sigma-Aldrich, cat. no. M-6145)
- Ascorbic acid (Sigma-Aldrich, cat. no. A-4544)
- BMP4 (R&D Systems, cat. no. 314-BP)
- Fluo-3, AM, calcium indicator (Thermo Fisher Scientific, cat. no. F1241)
- Geltrex LDEV-free reduced growth factor basement membrane matrix (Thermo Fisher Scientific, cat. no. A1413202)
- Essential 8 medium (Gibco, cat. no. A1517001)
- RPMI 1640 medium (Gibco, cat. no. 11875119)
- Y-27632 (Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor) (StemCell Technologies, cat. no. 72304)
- XAV939 (Sigma-Aldrich, cat. no. X3004)
- Isoflurane (Forane; Baxter)
- Nonessential amino acid solution (NEAA; Invitrogen, cat. no. 11140-050)
- GlutaMAX (100×; Gibco, cat. no. 35050-061)
- Saponin (Sigma-Aldrich, cat. no. S4521)
- Mouse anti-Islet1 (Developmental Studies Hybridoma Bank)
- Donkey anti-mouse IgG secondary antibody (Alexa Fluor 647 conjugate; Thermo Fisher Scientific, cat. no. A-31571)
- Sodium chloride (Sigma-Aldrich, cat. no. S9888)
- Potassium chloride (Sigma-Aldrich, cat. no. P9333)
- Magnesium sulfate (Sigma-Aldrich, cat. no. M7506)
- Sodium phosphate (monobasic; Sigma-Aldrich, cat. no. S3139)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S5761)
- Glucose (Sigma-Aldrich, cat. no. D9434)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Magnesium chloride (Sigma-Aldrich, cat. no. M8266)
- Calcium chloride (Sigma-Aldrich, cat. no. C1016)
- Collagenase type II (Worthington Biochemical, cat. no. LS004176)
- Protease type XIV (Sigma-Aldrich, cat. no. P5147)
- Fura-2AM (Thermo Fisher Scientific, cat. no. F1221)
- Fluor-594-conjugated WGA antibody (Thermo Fisher Scientific, cat. no. W11262)
- ProLong Diamond antifade mount (Thermo Fisher Scientific, cat. no. P36961)
- RNase inhibitor (Thermo Fisher Scientific, cat. no. N8080119)
- DNase I (RNase-free; New England BioLabs, cat. no. M0303S)
- RNase-free water (Qiagen, cat. no. 129112)
- SMARTScribe reverse transcriptase (Clontech, cat. no. 639536)
- dNTP mix (10 mM each; Thermo Fisher Scientific, cat. no. R0191)
- DTT (1 M; Thermo Fisher Scientific, cat. no. P2325)
- Advantage 2 polymerase mix (Clontech, cat. no. 639201)
- Custom-designed PCR primers (Integrated DNA Technologies)
- AMPure XP PCR purification kit (Beckman Coulter, cat. no. A63880)
- RPMI 1640 without glucose (Thermo Fisher Scientific, cat. no. 11879020)
- Sodium L-lactate (Sigma-Aldrich, cat. no. 71718)
- EDTA (Sigma-Aldrich, cat. no. E6758)
- Betadine swabs (Thermo Fisher Scientific, cat. no. 19-065534)
- Paraformaldehyde (Sigma-Aldrich, cat. no. P6148) ! **CAUTION** Paraformaldehyde is a hazardous solution and a cross-linking agent. Wear gloves and a lab coat when you are handling it. Dispose of it appropriately after use.
- TSO oligo (Integrated DNA Technologies, custom order)
- Advantage 2 Polymerase Mix (Clontech Laboratories, cat. no. 639207)
- Mouse anti-Isl1 antibody (clone 39.4D5-c; Developmental Studies Hybridoma Bank) ▲ **CRITICAL** It is critical that anti-islet1 antibody from this supplier be used, rather than an alternative. We have found that other anti-Isl1 antibodies are not as specific.

EQUIPMENT

- Cell culture plate (six wells; Corning, cat. no. 3506)
- T25 flasks (Corning, cat. no. 353109)
- Falcon 15-ml conical centrifuge tubes (Corning, cat. no. 100150)
- Falcon 50-ml conical centrifuge tubes (Corning, cat. no. 100151)
- Cell culture Petri dish (100 × 20 mm; Corning, cat. no. 430293)
- Suspension culture dish (150 × 25 mm; Corning, cat. no. 430597)
- Scepter 2.0 handheld automated cell counter with pack of 60-µm sensors (Millipore, cat. no. PHCC20060)
- Corning ultra-low-attachment T75 flask (Fisher Scientific, cat. no. 07-200-875)
- Corning BioCoat laminin 60-mm tissue-culture-treated culture dishes (Corning, cat. no. 354405)
- Cell strainer (70 µm; Fisher Scientific, cat. no. 08-771-2)
- Cell strainer (100 µm; Fisher Scientific, cat. no. 08-771-19)
- 5-ml Polystyrene round-bottom tube with a 40-µm cell strainer (BD Falcon, cat. no. 352235)
- FemtoJet microinjector (Eppendorf, cat. no. 10910)
- CO_2 incubator (Thermo Fisher Scientific, cat. no. 51030285)
- Cell sorter (Sony, model no. SH800 or any other fluorescence-activated cell sorter)
- BD Accuri C6 Plus flow cytometer (BD Biosciences)
- EVOSFL microscope (Thermo Fisher Scientific, cat. no. AMF4300)
- Inverted microscope (Nikon, model no. Eclipse TE2000)
- Sorvall Legend XT Centrifuge (Thermo Fisher Scientific, cat. no. 75004508)
- Fine scissors (2.5-mm cutting edge; Fine Science Tools, cat. no. 15000-08)
- Ultra-fine forceps (Fine Science Tools, cat. no. 11370-40)
- Student fine scissors (Fine Science Tools, cat. no. 91460-11)
- Tissue-adhesive glue (3M Vetbond; Fisher Scientific, cat. no. NC9259532)
- Mini rocker (Bio-Rad, cat. no. 166-0710EDU)
- Dissecting stereoscopic microscope (Zeiss, model no. Discovery V8)
- Sure-seal large mouse/rat induction chamber (WPI, cat. no. EZ-1785)
- Stericup 500 ml/1,000 ml Millipore Express (Millipore, cat. no. SCGPU10RE)
- Stericup 500 ml/500 ml Millipore Express (Millipore, cat. no. SCGPU05RE)
- Steriflip 50-ml disposable vacuum filtration system (Millipore, cat. no. SCGP00525)
- IonOptix software (Myocam-S, http://www.ionoptix.com/support/software/)
- pClamp v10 software (Molecular Devices, https://www.moleculardevices.com/systems/conventional-patch-clamp/pclamp-10-software)
- Image J software v1.50e (National Institutes of Health, http://imagej.nih.gov/ij)
- Confocal microscope (Leica, model no. DM2500)
- 5-mm Round cover glass (Warner Instruments, cat. no. W4 64-0700)
- FlowJo software v10.0.8r1 (FlowJo, https://www.flowjo.com/solutions/flowjo/downloads)
- Langendorff system for rats (ADInstruments, cat. no. PL3508B3-V)

REAGENT SETUP

2i medium 1 liter of the medium contains 870 ml of GMEM, 100 ml of FBS, 10 ml of GlutaMAX, 10 ml of NEAA, 10 ml of sodium pyruvate, 3 µl of β-mercaptoethanol, 20 µl of LIF (200 U/ml), 0.3 µM CHIR99021 and 0.1 µM PD0325901. Filter-sterilize it using a 1-liter filter and store it at 4 °C for up to 1 month.

Mouse embryonic fibroblast (MEF) medium 500 ml of this medium contains 435 ml of DMEM, 50 ml of FBS, 5 ml of NEAA, 5 ml of sodium pyruvate and 5 ml of GlutaMAX.
Filter-sterilize using a 500-ml filter and store it at 4 °C for up to 1 month.

Serum-free differentiation (SFD) medium 1 liter of SFD medium contains 715 ml of IMDM, 250 ml of Ham's F12, 5 ml of N2 supplement (0.5% vol/vol), 10 ml of B27 minus vitamin A, 5 ml of 10% (wt/vol) BSA (in PBS), 7.5 ml of GlutaMAX
and 7.5 ml of penicillin–streptomycin. Filter-sterilize using a 500-ml filter and store it at 4 °C for up to 1 month. ▲ **CRITICAL** To differentiate mESCs, add ascorbic acid (50 µg/ml) and 3.9×10^{-3}% (vol/vol) monothioglycerol before use.

FACS sorting solution, 10× This solution contains 1% (vol/vol) FBS, 200 mM HEPES and 10 mM EDTA in PBS. Filter-sterilize the solution using a 50-ml filter and store it at 4 °C for up to 2 months.

RPMI plus B27 minus insulin medium Mix 500 ml of RPMI 1640 medium with 10 ml of B27 minus insulin and store the solution at 4 °C for up to 2 months.

RPMI plus B27 minus vitamin A medium Mix 500 ml of RPMI 1640 medium with 10 ml of B27 minus vitamin A, and store the solution at 4 °C for up to 2 months.

FACS immunostaining solution This solution contains 5% (vol/vol) FBS and 0.75% (wt/vol) saponin in PBS. Filter-sterilize using a 50-ml filter and store the solution at 4 °C for up to 3 months.

Tyrode's solution, 10× This solution contains 1.37 M NaCl, 49 mM KCl, 12 mM $MgSO_4$, 12 mM NaH_2PO_4, 150 mM glucose and 200 mM HEPES in dH_2O. Store this solution at room temperature (23–27 °C) for up to 3 months.

Perfusion buffer, 10× This solution contains 1.2 M NaCl, 54 mM KCl, 12 mM $MgSO_4$, 10 mM NaH_2PO_4, 200 mM $NaHCO_3$ and 56 mM glucose in dH_2O. Filter-sterilize using a 500-ml filter and keep the solution at room temperature for up to 3 months.

Collagenase solution Add 50 mg of collagenase and 1 mg of protease to 50 ml of 1× perfusion buffer before use, and mix well. This solution cannot be stored and must be made fresh each time.

PROCEDURE

Generation of ESC-derived cardiac progenitor cells *in vitro*

1| Generate mouse (option A) or human (option B) progenitor cells.

(A) Generation of mouse ESC-derived cardiac progenitor cells *in vitro* ● **TIMING 5 d**

(i) Grow mESCs (mESCs$^{Isl1\text{-}Cre;\ Rosa\text{-}RFP;\ aMHC\text{-}GFP}$)[26] on 0.1% (wt/vol) gelatin-coated T25 flasks in 2i medium in a 5% CO_2 incubator set at 37 °C. Grow cells under these conditions throughout the experiment. When cells reach 70–80% confluence, proceed to the next step to start cell differentiation. The time to reach 70–80% confluence is ~3 d.

(ii) Rinse the cells once with Ca^{2+}- and Mg^{2+}-free DPBS and then dissociate into single cells by adding 1 ml of TrypLE and incubating at 37 °C for 3 min.

(iii) Inactivate the TrypLE by adding 4 ml of MEF medium. Prepare the cells for counting by diluting the resuspended cells at a 1:10 ratio in MEF medium and mixing well with a pipette. Count the cells. We use the Scepter sensor; to use this, attach the 60-µm Scepter sensor to the Scepter 2.0 handheld automated cell counter and then aspirate the cells. The total cell number equals the number provided by the automated counter multiplied by ten times the total number of milliliters.
▲ **CRITICAL STEP** 10×10^6 cells are needed for cell differentiation. Use the remaining mESCs for cell maintenance.

(iv) Centrifuge 10×10^6 cells for 3 min at 270*g* at room temperature. Then aspirate the supernatant and resuspend the cells in 100 ml of SFD.

(v) Split the resuspended cells into four 150 × 25-mm sterile plates and incubate in the 5% CO_2 incubator for 48 h. The mESCs should form embryoid bodies (EBs) during this 48-h incubation.

(vi) Collect all the EBs in two 50-ml tubes and centrifuge for 3 min at 145*g* at room temperature.
▲ **CRITICAL STEP** Using a low speed (145*g*) enables isolation of almost exclusively EBs and avoids the precipitation of single cells.

(vii) Aspirate the supernatant and resuspend the EBs in 100 ml of SFD medium, 6 µM CHIR99021 and 0.4 ng/ml of BMP4 for differentiation induction. Split the resuspended EBs into four 150 × 25-mm sterile plates and incubate them in the 5% CO_2 incubator for 24 h. Collect all the EBs in two 50-ml tubes and centrifuge at 145*g* for 3 min at room temperature. Aspirate the supernatant and resuspend the EBs in 25 ml of SFD medium. Transfer the resuspended EBs in an ultra-low-attachment 75-cm^2 flask and incubate the EBs in a 5% CO_2 incubator for 48 h.
▲ **CRITICAL STEP** If you wish to also or alternatively mature EBs to mESC-CMs *in vitro*, centrifuge the EBs at 145*g* for 3 min at room temperature and resuspend them in SFD medium. Culture the cells; beating and aMHC-GFP expression should appear 2 d later. Continue to culture the CMs as EBs by changing the medium every other day or dissociating the EBs as in Step A(ii), counting the cells and replating 5–6 × 10^5 cells per well in 0.1% (wt/vol) gelatin-coated 24-well plates. The cells will attach to the bottom and should be incubated in the 5% CO_2 incubator for 1 month while changing the SFD medium every other day. Cells can then be analyzed as described in **Box 2**.

(viii) To dissociate the EBs and collect single CPCs, first centrifuge them at 145*g* for 3 min at room temperature and aspirate the supernatant. Add 1 ml of TrypLE and incubate at 37 °C for 3 min. Mix well by pipetting to dissociate the cells.
▲ **CRITICAL STEP** At this time point, almost all the EBs should express Isl1-RFP; this can be checked under the microscope (**Fig. 2a**).
? TROUBLESHOOTING

(ix) Add 4 ml of MEF medium to inactivate the TrypLE and mix well by pipetting. To remove the nondissociated EBs, filter the mix using a 70-µm strainer and centrifuge the filtered cells for 3 min at 270*g* at room temperature. Aspirate the supernatant and add 1 ml of FACS sorting solution to resuspend.

(x) To remove all cell clusters before sorting, filter the cells once more using a 5-ml polystyrene round-bottom tube with a 40-µm cell strainer. Keep the cells on ice until sorting.

(xi) Sort Isl1-RFP$^+$ cells by FACS (**Fig. 2b**, **Supplementary Fig. 1**) and collect the sorted cells in 1 ml of FBS.
▲ **CRITICAL STEP** Avoid long sorting times and keep the FACS sorting solution, the cell sample and sorted cells at low temperatures (~4–5 °C) to decrease the amount of cell death. Injecting unsorted cells can lead to teratoma formation (**Supplementary Fig. 2**).
? TROUBLESHOOTING

Box 2 | Analysis of *in vitro*-matured PSC-derived cardiomyocytes ● TIMING 1.5–24 h per heart, 30 d after cell culture

1. Various methods can be used to analyze the cells. Follow option A for immunostaining and nucleation analysis; follow option B for a further *in vitro* cell culture and follow option C for sorting.

(A) Immunostaining and nucleation analysis

(i) Fix *in vitro*-plated PSC-CMs directly with 4% (wt/vol) paraformaldehyde in PBS for 30 min.

(ii) Wash the fixed cells twice for 5 min with PBS at room temperature.

(iii) Stain for 5 min with DAPI (1:2,000) in PBS, followed by two washings for 5 min with PBS at room temperature. Keep the cells in PBS.

■ **PAUSE POINT** The cells can either be stored overnight at 4 °C or imaged immediately.

(iv) Image the cells. Save images and manually count and analyze the number of nuclei per cell.

(B) Further culture of cells

(i) Replate the cells by dissociating with TrypLE (as described in Steps 1A(ii) and 1A(iii)) and replating on a gelatin-coated 5-mm round cover glass in either SFD medium for mouse CMs or RPMI plus B27 minus vitamin A with 10% (vol/vol) FBS and 10 μM ROCK inhibitor for human CMs to increase cell survival.

(ii) Transfer the cover glass with plated CMs to the perfusion chamber just before measurement of Ca^{2+} transients and sarcomere shortening.

(iii) Measure whole-cell Ca^{2+} transients and sarcomere shortening as described in Step 19A.

(C) Sorting of cells

(i) Sort cells as single cells using a cell sorter, or pick single cells directly under a microscope (EVOS) and transfer them to a 96-well plate for cDNA preparation as above.

(xii) Centrifuge sorted Isl1-RFP$^+$ CPCs for 3 min at 270*g* at room temperature. Aspirate the supernatant and resuspend the cells in IMDM with Geltrex at a 60:1 ratio to a final concentration of 2 × 10^3 cells per ml. Keep CPCs on ice before transplantation.

▲ **CRITICAL STEP** To avoid the polymerization of Geltrex, which will increase the viscosity of the cell sample and will clog the microneedle during cell injection, keep the sample on ice at all times for no more than 4 h.

■ **PAUSE POINT** Cells can be kept on ice for up to 4 h. Cell death increases with time spent on ice; thus, this should be minimized.

(B) Generation of human iPSC-derived cardiac progenitor cells *in vitro* ● TIMING **5–6 d**

(i) Generate a constitutively expressing GFP/RFP, hiPSC line (**Box 1**). Grow hiPSCs on Geltrex-coated T25 flasks using Essential 8 medium in a 5% CO_2 incubator set at 37 °C until they reach 70–80% confluence (this usually occurs after 3–4 d), at which point you should proceed to the next step to start cell differentiation. Grow cells in a 5% CO_2 incubator set at 37 °C throughout the experiment.

▲ **CRITICAL STEP** Coat each T25 flask with 3 ml of 1:60 Geltrex in DMEM for 30–120 min at room temperature. Determine how many T25 flasks are needed based on the desired cell number. Each T25 flask, when 70–80% confluent, contains ~3–4 × 10^6 cells.

(ii) Coat at least two wells of a six-well plate with Geltrex in DMEM (1:60) using 2 ml per well.

(iii) Rinse the cells from Step B(i) once with Ca^{2+}- and Mg^{2+}-free DPBS and then dissociate into single cells by adding 1 ml of TrypLE and incubating at 37 °C for 3 min.

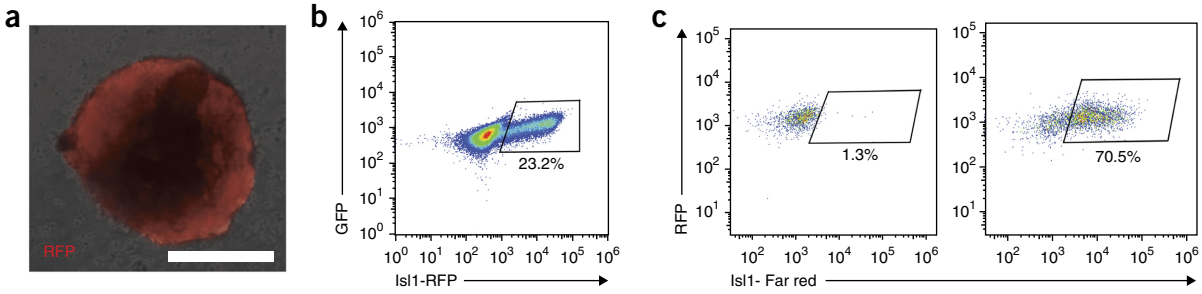

Figure 2 | Isl1$^+$ cardiac progenitor cells. (**a**) Isl1-Cre-driven expression of RFP in a mouse embryoid body. (**b**) RFP$^+$ CPCs are purified by FACS (62,000 live cells were sorted and 14,410 of those were Isl1$^+$ (23.3%)). (**c**) Immunostaining for Isl1 and flow cytometry analysis of fixed human CPCs. Left, negative control with only secondary antibody (2,384 cells were analyzed, and of those 31 were only Isl1$^+$ (1.3%)). Right, immunostaining with Isl1 primary antibody (2,750 cells were analyzed, and of those 1,940 were Isl1$^+$ (70.3%)). Scale bar, 50 μm.

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Box 3 | Immunofluorescence staining of fixed cells for flow cytometry ● TIMING 2–2.5 h

1. Centrifuge the cell mix for 3 min at 270*g* at room temperature. Aspirate the supernatant and resuspend the cells in 4% (wt/vol) paraformaldehyde in PBS. Incubate for 30 min at room temperature to fix the cells.
2. Centrifuge the cell mix for 3 min at 895*g* at room temperature. Aspirate the supernatant and resuspend the cells in PBS to wash the PFA. Repeat this step once more.
3. Aspirate the supernatant and resuspend the cells in FACS immunostaining solution. Split the sample in two and add mouse anti-Isl1 antibody (1:500 dilution) to one sample and use the other as a negative control. Incubate for 30 min at room temperature.
4. Wash the cells twice as described in step 2, using FACS immunostaining solution instead of PBS. Aspirate the supernatant and resuspend both cell samples in FACS immunostaining solution with 1:500 donkey anti-mouse IgG (H + L) secondary antibody, Alexa Fluor 647 conjugate. Incubate for 30 min at room temperature in the dark. Keep the cells in the dark from now on to avoid fluorochrome bleaching.
5. Wash twice with FACS immunostaining solution as in the previous step. Aspirate the supernatant and resuspend the cells in PBS. Use the BD Accuri C6 plus flow cytometer to analyze the cells (**Fig. 2c**, **Supplementary Fig. 1**).

▲ **CRITICAL STEP** Use an anti-mouse secondary antibody with high excitation and emission wavelengths to avoid nonspecific separation of the GFP-tagged CPCs.

(iv) Inactivate the TrypLE by adding 4 ml of MEF medium. Prepare the cells for counting by diluting the resuspended cells at a 1:10 ratio in MEF medium and mixing well with a pipette. Count the cells; we use the Scepter system. To use this, attach the 60-µm Scepter sensor to the Scepter 2.0 handheld automated cell counter and then aspirate the cells. The total cell number equals the number provided by the automated counter multiplied by ten times the total number of milliliters.
▲ **CRITICAL STEP** 3×10^6 cells are needed for cell differentiation.

(v) Centrifuge 3×10^6 hiPSCs for 3 min at 270*g* at room temperature.
▲ **CRITICAL STEP** For cell maintenance, retain a further $3–6 \times 10^5$ hiPSCs and replate them in a T25 flask.

(vi) Aspirate the supernatant and resuspend the cells you are differentiating in Essential 8 medium with 10 µM ROCK inhibitor (Y27632) at a concentration of 6×10^5 hiPSCs per ml and plate 2.5 ml per well of a six-well plate.
▲ **CRITICAL STEP** 100% confluence is critical to efficient CM differentiation. Therefore, after adding the cells, tap the plate from all sides and leave it inside the hood at room temperature for ~15 min. This will enable more homogeneous plating of the cells.

(vii) To initiate cell differentiation the next day, change the medium to RPMI plus B27 minus insulin and 6 µM CHIR99021 and incubate in the 5% CO_2 incubator for 48 h.
▲ **CRITICAL STEP** To maintain consistency and increase differentiation efficiency, all incubation times stated in this PROCEDURE should remain unchanged. In addition, the medium should always be added slowly to avoid mechanical stress, which can affect cell differentiation. Use 2.5 ml of medium per well.

(viii) Change the medium to RPMI plus B27 minus insulin and incubate in the 5% CO_2 incubator for 24 h.

(ix) Change the medium to RPMI plus B27 minus insulin and 10 µM XAV939, and incubate in the 5% CO_2 incubator for 48 h.
▲ **CRITICAL STEP** If you wish to continue to grow cells in culture rather than proceed to transplantation, change the medium of the cells to RPMI plus B27 minus insulin and incubate in the 5% CO_2 incubator for a further 48 h. Then change the medium to RPMI plus B27 without vitamin A and incubate in the 5% CO_2 incubator for 48 h. At this stage, most cells will be contracting CMs. Cells can be cultured for 1 month, during which time continue changing the medium to RPMI plus B27 without vitamin A every other day. Cells can then be analyzed as described in **Box 2**.
? TROUBLESHOOTING

(x) The majority of the cells at this stage should express Isl1. To test the percentage of $Isl1^+$ CPCs, perform immunofluorescence staining and flow cytometry of some of the CPCs (**Box 3**; **Fig. 2c**).
▲ **CRITICAL STEP** To improve the yield of mature CMs, proceed to CPC injection only if ~>60–70% of CPCs are $Isl1^+$. Alternatively, fresh cardiomyocytes appearing at days 8–9 may be used, with the caveat that their engraftment and survival will be substantially lower in comparison with those of cardiac progenitor cells.
? TROUBLESHOOTING

(xi) Add 500 µl of TrypLE per well to dissociate the cells and incubate at 37 °C for 3 min. Pipette and mix the cells gently for ~30 s, to mechanically dissociate the CPCs and generate single cells. Then add 2 ml of MEF medium per well, mix and transfer all the cells to a 15-ml tube. Count the cells as above and centrifuge the CPCs for 3 min at 270*g* at room temperature.

(xii) Aspirate the supernatant and resuspend the cells in cold (4 °C) IMDM with Geltrex at a 60:1 ratio and 10 µM ROCK inhibitor (Y27632). The cell concentration should be 2×10^3 cells per ml.
■ **PAUSE POINT** Keep the CPCs on ice before transplantation for a maximum of 4 h.

Surgery ● TIMING 7–15 min per rat pup

▲ **CRITICAL** Before rat surgery, autoclave all surgical tools.

2| To anesthetize the rat pups, wrap them in foil and place them on an ice bed (**Fig. 3a**). Pups should be anesthetized on the ice bed within 5–10 min.

▲ **CRITICAL STEP** Take one rat pup at a time and use only postnatal day 1–7 rats. Direct contact of pups with ice is associated with higher postprocedure mortality; therefore, keep pups covered with aluminum foil.

▲ **CRITICAL STEP** Hypothermia minimizes blood loss during heart surgery and reduces the pup's heart rate, allowing for better cell engraftment.

▲ **CRITICAL STEP** The time to reach anesthesia varies and depends greatly on the age and weight of the pups. Usually 5 min is enough for P1 (5–7 g) rat pups; however, for P7 (11–13 g) pups, longer times (up to 10 min) are usually needed.

? TROUBLESHOOTING

3| Transfer the pup from the ice bed to the surgical bed. Put the rat pup in a supine position and tape all four limbs for immobilization. To sterilize the skin and prevent infections, wipe the chest with a Betadine swab.

▲ **CRITICAL STEP** For longer anesthesia during surgery, consider cooling the surgical bed using ice or ice packs.

4| Use the fine scissors (2.5-mm cutting edge) to make an incision of ~5–6 mm in the skin of the left upper chest wall, between the third and fourth ribs (**Fig. 3b**). Gently separate the skin from the underlying muscles using fine forceps. Dissect the muscles of the intercostal space and create an opening of ~4–5 mm using the forceps (**Fig. 3b**).

5| Visualize the left ventricular apex and proceed immediately to cell transplantation (**Fig. 3c**).

▲ **CRITICAL STEP** At this stage, the skin and muscles of the chest wall are thin and the heart can usually be located. Entering the anterior mediastinum will require very gentle manipulations and practice to avoid injuring any of the major blood vessels.

▲ **CRITICAL STEP** Although a wider skin incision can help to visualize the heart better, it will decrease the survival of the pup.

? TROUBLESHOOTING

Cell transplantation and recovery ● TIMING 10–20 min per rat pup

6| Immediately load 10 µl of the CPC mix into the glass needle and inject all 10 µl into the left ventricular apex using the Eppendorf FemtoJet microinjector.

▲ **CRITICAL STEP** Use the coarse mode of the Eppendorf FemtoJet microinjector for faster manipulations and overall quicker injections.

▲ **CRITICAL STEP** To avoid penetrating the ventricular wall and to improve the pup survival and cell engraftment, do not advance the needle deep into the ventricular wall. Stop advancing after you feel resistance.

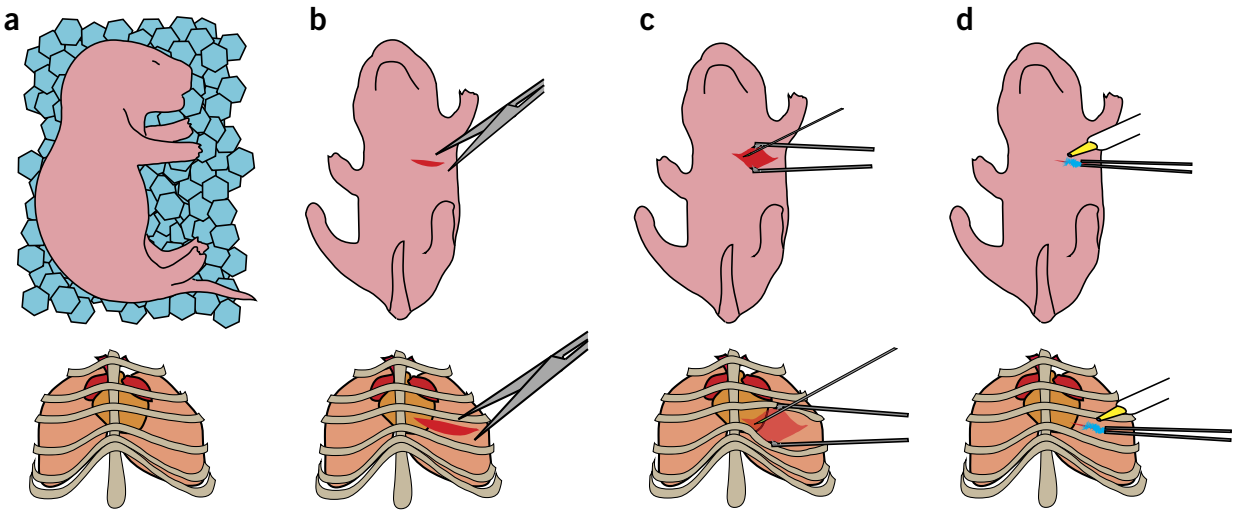

Figure 3 | Surgical transplantation of cardiac progenitor cells for *in vivo* maturation. (**a**) Neonatal rats 1–7 d old are placed on ice to induce anesthesia. (**b**) Use the fine scissors to make an ~5-mm incision in the skin between the third and fourth ribs and gently separate the skin from the underlying muscles using fine forceps. Dissect the muscles of the intercostal space to create an opening of ~4 mm using the forceps. (**c**) Inject CPCs into the left ventricular apex using a microneedle connected to the Eppendorf FemtoJet microinjector. (**d**) Pinch the edges of the incision with the forceps and use no more than 5 µl of tissue-adhesive glue to seal the incision.

7| After the cell injection, pinch the edges of the chest wall incision with the forceps for 10 s and then use no more than 5 µl of tissue-adhesive glue to seal it (**Fig. 3d**).
▲ **CRITICAL STEP** Higher amounts of tissue glue can be toxic to the pup or can increase the probability of cannibalization by the mother not recognizing her own pups.

8| Transfer the pup immediately to a heating pad with the temperature set to 37 °C for 10–15 min and then return to the mother.
▲ **CRITICAL STEP** Ensure that the pup is fully rewarmed and active before returning it to its mother's cage. In addition, rub the pup with cage bedding before returning to its mother to improve survival.
▲ **CRITICAL STEP** As prolonged hypothermia increases pup mortality, surgery should be completed within 5–10 min.
? TROUBLESHOOTING

Isolation of transplanted cardiomyocytes from injected rat heart ● TIMING ~1–1.5 h per heart, 30 d after cell injection
9| Transfer the 1-month-old injected rat to the anesthesia induction chamber and euthanize it by adding isoflurane for 3 min.

10| Quickly remove the heart and place it in perfusion buffer[27]. Cannulate the heart via the aorta and use a Langendorff system to perfuse the heart with perfusion buffer for 3 min at 35–37 °C at a flow of 10 ml per min. Then perfuse for 15 min using collagenase solution at a flow rate of ~3 ml per min. Within 2 min into this perfusion, add 50 µM $CaCl_2$.
▲ **CRITICAL STEP** Decrease the perfusion flow to 2–3 ml per min during collagenase treatment and then increase to 10 ml per min immediately after.

11| Localize the area of transplanted cells by the presence of an obvious myocardial scar or by visualization of the heart under a fluorescence stereoscopic microscope (**Fig. 4a**). Cut the transplanted area using scissors (Student fine scissors) and transfer it into a small plastic container containing 5 ml of collagenase solution, and cut it into smaller pieces (~20 cuts).

12| Add 45 ml of collagenase solution and mix gently up to ten times using a plastic pipette. Transfer 35 ml of supernatant to a 50-ml tube.

13| Centrifuge the supernatant at 46*g* for 1 min at room temperature. Transfer 30 ml of the supernatant to the small plastic container with the rest of the heart pieces from Step 11. Retain the pellet.

14| Place the container on the rocker at low speed for 1 min at room temperature.

15| Transfer the mix to a new 50-ml tube and centrifuge at 46*g* for 1 min at room temperature.

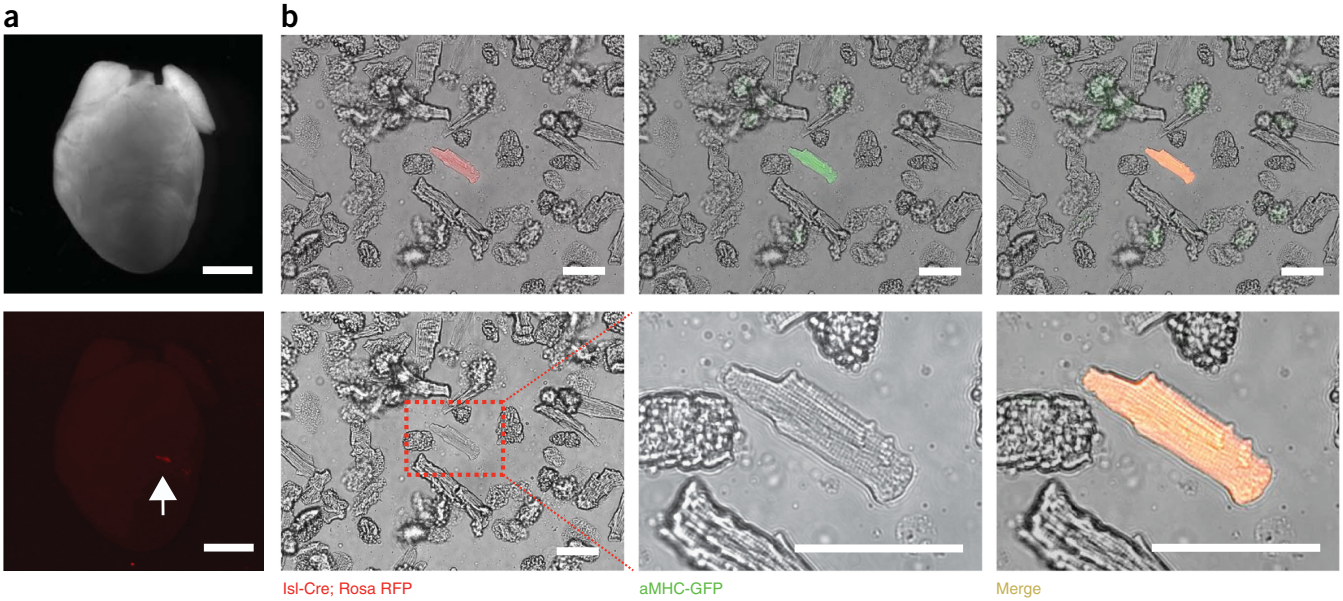

Figure 4 | Isolation of mESC-CMs from injected rat hearts. (**a**) Isolated rat hearts with injected RFP+ mouse CMs (arrow). (**b**) Isolated adult-like mESC-CMs after processing of injected rat heart. The injected mESC-CMs are marked with GFP. Scale bars, 100 µm.

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▲ **CRITICAL STEP** To remove the nondissociated pieces of heart tissue, filter the mix using a 100-µm cell strainer.

16| Remove the supernatant and add 10 ml of Tyrode's solution buffer with 1% (wt/vol) BSA (in PBS) and 250 µM $CaCl_2$ and gently invert to resuspend the CM pellet.

17| Add 10 ml of Tyrode's solution with 0.5% (wt/vol) BSA (in PBS) to the pellet in the 50-ml tube from Step 15. Invert gently to resuspend the CMs.

18| Combine the two mixes from Steps 17 and 13, gently invert to mix and centrifuge for 1 min at 46*g* at room temperature. Cells cannot be stored and must be analyzed immediately after this step (**Fig. 4b**).

Analysis of transplanted cardiomyocytes from injected rat heart ● **TIMING 1.5–5 h per heart**

19| Transplanted cardiomyocytes can be analyzed in various ways. To assess sarcomere shortening and calcium transients, follow option A. To fix cells for T-tubule or other analysis, follow option B. For cDNA generation for single-cell gene expression analysis, follow option C.

(A) Sarcomere shortening and calcium transients

(i) Aspirate the supernatant and add 10 ml of Tyrode's solution with 250 µM $CaCl_2$ and invert gently to resuspend the CM pellet. Leave the tube upright for 10 min at room temperature to let the CMs sink to the bottom. Remove the supernatant and add 10 ml of Tyrode's solution with 500 µM $CaCl_2$.

(ii) Repeat the previous step, but add 10 ml of Tyrode's solution with 1 mM $CaCl_2$.

(iii) Aspirate the supernatant and add 15 ml of Tyrode's solution with 1 mM $CaCl_2$ containing 1 µM of the ratiometric Ca^{2+} indicator dye Fura-2AM and incubate for 10 min at room temperature.

(iv) Transfer the cells to a heated (at 37 °C) perfusion chamber placed on the stage of an inverted microscope. Perfuse the CMs with Tyrode's solution with 1 mM $CaCl_2$ at a flow-through rate of 2 ml per min. Stimulate the cells at 0.5 Hz with electrical pulses from two electrodes placed in the perfusion chamber.

(v) Record CM shortening by video-edge detection using the IonOptix software. Use the same software for data analysis.

(vi) Record intracellular Fluo-3 fluorescence for Ca^{2+} transients and analyze the recorded data using pClamp v10 software[27,28].

(B) Cell fixation for T-tubule staining and analysis

(i) Plate isolated CMs in Tyrode's solution with 1 mM $CaCl_2$ on a laminin-coated 60-mm dish and incubate in a 5% CO_2 incubator at 37 °C for 30 min to let them attach to the dish.

(ii) Aspirate the Tyrode's solution and add 3 ml of 4% (wt/vol) paraformaldehyde in PBS and incubate for 20 min at room temperature for cell fixation. Wash away the paraformaldehyde twice with PBS. The cells can now be used for immunostaining and nucleation assessment with DAPI staining.
▲ **CRITICAL STEP** For confocal microscopy analysis, plate the cells on laminin-coated coverslips.
■ **PAUSE POINT** The cells can be stained immediately or after overnight storage at 4 °C.

(iii) Block the fixed cells with 1% (wt/vol) BSA in PBS by incubating for 1 h at room temperature. For T-tubule staining, add Fluor-594-conjugated WGA antibody at a 1:1,000 dilution and incubate at 4 °C overnight. Wash the cells twice for 5 min with PBS.
▲ **CRITICAL STEP** Avoid shorter incubation times to prevent low intensity of the staining.
▲ **CRITICAL STEP** Alternative antibody combinations can be substituted, and standard immunofluorescence staining performed.

(iv) Add DAPI to stain the nuclei, and incubate for 10 min at room temperature.

(v) Wash the cells twice for 5 min with PBS and mount the cells with ProLong Diamond antifade mount.
■ **PAUSE POINT** The cells can either be imaged immediately or stored overnight at 4 °C and imaged the next day.

(vi) Image the T-tubules using a confocal microscope with a 403 (1.15 numerical aperture) oil-immersion lens.

(vii) Analyze WGA staining using ImageJ. Apply a median filter, create a binary mask of the WGA signal using the Auto Local Threshold plugin and erode the mask once.

(viii) Create a region of interest in each cell. Exclude the boundary membrane to isolate the T-tubule network for segmentation analysis.

(ix) Use the Analyze Particles plugin to measure the T-tubule area (mm^2), which is normalized to the total cell area to calculate the fractional area.
▲ **CRITICAL STEP** Compare two groups using a two-tailed *t* test. Use the same optical field for statistical comparisons between host CMs and *in vivo*-matured CMs to eliminate staining errors.

(C) cDNA generation for single-cell gene expression analysis

(i) Use a 20-µl pipette to manually pick the isolated GFP+/RFP+ CMs (in Tyrode's solution) under a fluorescence microscope (EVOS). Transfer each cell to a single well of a 96-well plate containing 2.4 µl of RNase-free water, 0.2 µl of RNase-free DNase I and 0.25 µl of RNase inhibitor.

(ii) Incubate at 72 °C for 3 min to inactivate DNase I and then quench on ice. Add 1 µl of custom-designed primer (with 30-deoxythymidine anchor) and transfer to 72 °C for 2 min to anneal to polyadenylated RNA and then quench on ice.

(iii) Add a mixture of 1 µl of SMARTScribe reverse transcriptase, 1 µl of custom-designed TSO oligo (12 mM), 0.3 µl of $MgCl_2$ (200 mM), 0.5 µl of RNase inhibitor, 1 µl of dNTP mix and 0.25 µl of DTT (100 mM), and incubate at 42 °C for 90 min, followed by enzyme inactivation at 70 °C for 10 min.

(iv) Add a mixture of 29 µl of water, 5 µl of Advantage 2 taq polymerase buffer, 2 µl of dNTPs, 2 µl of custom-designed amplifying PCR primer (12 mM) and 2 µl of Advantage 2 taq polymerase to the reverse transcription product and amplify for 20 cycles using the following program:

Cycle number	Denature	Anneal	Extend
	95 °C for 1 min		
20 cycles		95 °C for 30 s	68 °C for 3 min
			68 °C for 1 min

(v) Purify the amplification product using Ampure XP beads. Use the cDNA to test gene expression by qPCR or perform RNA sequencing based on previously published protocols[21,29].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
1A(viii)	Usually 25–35% of the cells will be positive for Isl1, but occasionally the percentage of Isl1-RFP cells is <10%	Incubate the EBs for 12–24 h longer to increase the percentage of Isl1+ cells
1A(xi)	High percentage of sorted dead cells	Add DAPI to FACS sorting solution (dilution 1:2,000) to stain and exclude dead cells. In addition, to improve cell survival, consider adding 10 mM Rock inhibitor to the FACS sorting solution
1B(ix)	Low percentage of hiPSC-derived-CMs	Incubate the cells at day 9, in RPMI without glucose and 5 mM sodium lactate. Change the medium every other day for a maximum of 4 d. Then continue culturing the cells in RPMI-B27 without vitamin A
1B(x)	Low percentage of Isl1+ human CPCs	Optimize the cell differentiation protocol by testing different concentrations of CHIR99021 (up to 10 mM, depending on the hiPSC line) and XAV939. Additionally, try analyzing CPCs 12–24 h later
2	Increased postprocedure mortality due to prolonged hypothermia	Monitor the pups closely while they are on ice
5	Pups develop bleeding during surgery	Use hypothermia during surgery and fine forceps with blunt ends
8	CPCs do not engraft or the final number of cells is low	Use a previously published prosurvival cocktail[30] to increase the engraftment and survival of the transplanted cells

● TIMING

Steps 1A(i–xii), generation of mouse ESC-derived cardiac progenitor cells *in vitro*: 5 d

Steps 1B(i–xii), generation of mouse human iPSC-derived cardiac progenitor cells *in vitro*: 5–6 d

Steps 2–5, surgery: 7–15 min per rat pup

Steps 6–8, cell transplantation and recovery: 10–20 min per rat pup

Steps 9–18, isolation of transplanted cardiomyocytes from injected rat heart: ~1–1.5 h per heart, 1 month after cell injection

Step 19, analysis of transplanted cardiomyocytes from injected rat heart: 1.5–5 h per heart

Box 1, generation of GFP/RFP-tagged human iPSCs: ~3 weeks

Box 2, analysis of *in vitro*-matured PSC-derived cardiomyocytes: 1.5–24 h per heart, 30 d after cell culture

Box 3, immunofluorescence staining of fixed cells for flow cytometry: 2–2.5 h

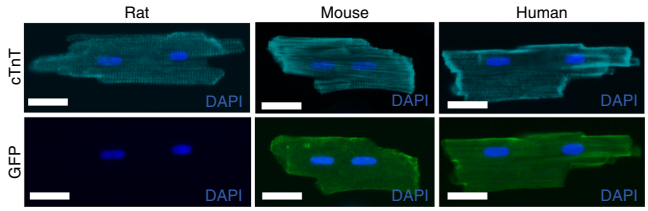

Figure 5 | *In vivo*-matured mouse and human PSC cardiomyocytes. Injected mouse and human cardiomyocytes were identified by GFP expression. *In vivo*-matured CMs had sarcomere structure, size and binucleation very similar to those of adult CMs. For all animal experiments, appropriate permission was obtained from the Johns Hopkins University Animal Care and Use Committee. Scale bars, 50 µm. cTnT, cardiac troponin T.

ANTICIPATED RESULTS

This protocol describes a method to generate mature, adult-like mouse or human PSC-CMs, using neonatal immunosuppressed rats as live bioincubators. It takes advantage of the environmental cues of a neonatal heart to advance CM development, which is otherwise blocked, during their *in vitro* growth, at late embryonic stages.

1 month after CPC transplantation, isolated mouse or human PSC-CMs should acquire the morphologic and functional characteristics of adult CMs (**Fig. 5**). Those cells can be compared with *in vitro*-matured CMs. We have not observed any teratomas after the injection of highly enriched CPCs. Importantly, as we have demonstrated, *in vivo*-matured hiPSCs can recapitulate adult cardiomyopathies and can expedite the modeling of diseases that are otherwise challenging to study *in vitro*[21]. The resulting human–rat chimeras can potentially be used for *in vivo* drug testing, bringing us a step closer to personalized medicine.

More examples of typical morphologic results can be found in Figure 1 of ref. 22. Examples of the functional properties of *in vivo*-matured PSC-CMs can be found in Figures 2 and 4 of ref. 22.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS G.-S.C. and C.K. designed the experiments. G.-S.C., E.T. and P.A. performed the experiments. G.-S.C., E.T. and P.A. analyzed the data. E.T. and P.A. created the figures. E.T., G.-S.C., P.A. and C.K. wrote the manuscript. All authors approved the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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