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Direct Cardiac Reprogramming as a Novel Therapeutic Strategy for Treatment of Myocardial Infarction

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Abstract

Direct reprogramming of fibroblasts into induced cardiomyocytes (iCMs) holds great promise as a novel therapy for the treatment of heart failure, a common and morbid disease that is usually caused by irreversible loss of functional cardiomyocytes (CMs). Recently, we and others showed that in a murine model of acute myocardial infarction, delivery of three transcription factors, Gata4, Mef2c, and Tbx5 converted endogenous cardiac fibroblasts into functional iCMs. These iCMs integrated electrically and mechanically with surrounding myocardium, resulting in a reduction in scar size and an improvement in heart function. Our findings suggest that iCM reprogramming may be a means of regenerating functional CMs in vivo for patients with heart disease. However, because relatively little is known about the factors that regulate iCM reprogramming, the applicability of iCM reprogramming is currently limited to the experimental settings in which it has been attempted. Specific hurdles include the relatively low conversion rate of iCMs and the need for reprogramming to occur in the context of acute injury. Therefore, before this treatment can become a viable therapy for human heart disease, the optimal condition for efficient iCM generation must be determined. Here, we provide a detailed protocol for both in vitro and in vivo iCM generation that has been optimized so far in our lab. We hope that this protocol will lay a foundation for future further improvement of iCM generation and provide a platform for mechanistic studies.

Keywords

Cell reprogramming; Induced cardiomyocyte; Fibroblast; Myocardial infarction; Regeneration

1 Introduction

Heart disease is the leading cause of morbidity and mortality in the developed world [1]. In the USA alone, more than 1.5 million people experience an acute myocardial infarction (AMI) each year, and there are five million AMI survivors who suffer from ischemic cardiomyopathy [1]. Because cardiomyocytes (CMs) in the heart have very limited regenerative potential in response to injury, loss of CMs results in impaired pump function and heart failure. Existing treatments are primarily pharmacological and device-based, and do not address the fundamental problem of CM loss. Indeed, the prevalence of cardiomyopathy is steadily increasing worldwide [1], making the identification of novel and effective therapies for this morbid disease an urgent problem in biomedical research.

Unlike the hearts of lower vertebrates [2], the adult human heart has very limited regenerative potential [3, 4]. Although post-natal vertebrate CMs undergo a small amount of cellular renewal, the rate of CM proliferation is very low [3, 5, 6]. One approach to cardiac regeneration has focused on enhancing such proliferative indices genetically or pharmacologically [7–12]. Although whether the magnitude of the beneficial effects would be sufficient to compensate for the functional loss of damaged myocardium awaits confirmation [13], stimulating CM to reenter cell cycle for regenerative purposes is still a vital approach for the treatment of heart disease [7–12]. A second approach to cardiac regeneration has been to derive new CMs for transfer into an injured heart [14–17]. These cells are typically differentiated from multipotent cardiovascular progenitor cells (CPCs) or pluripotent stem cells including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Although the poor cell survival, low maturation efficiency, limited cell–cell interaction, and functional integration of engrafted cells are major hurdles that remain to be overcome [18, 19], efforts to make this a practical therapy are ongoing. A third newly emerging approach to regenerate an injured heart is to directly convert the resident cardiac fibroblasts (CFs) into functional iCMs, which is discussed in more detail below.

Cardiomyocytes comprise 75 % of myocardial mass, but they account for only 30–40 % of the total number of cells in the heart. The majority of the remaining cells are CFs, which are responsible for homeostatic maintenance of extracellular matrix (ECM) in the normal heart [20–22]. CFs also respond to ischemia and other injurious stimuli to coordinate chemical and mechanical signals between cellular components in the heart. Upon injury, CFs migrate to the site of injury, proliferate, and contribute to scar formation through fibrosis [22, 23]. Thus, while the biological importance of CFs has long been recognized, their potential as an endogenous resource for cardiac regeneration has been largely neglected until very recently.

Takahashi and Yamanaka's seminal publication in 2006 [24] demonstrating the creation of iPSCs has ushered in a new era of utilizing cellular reprogramming in regenerative medicine. The idea has been leveraged thus far to directly reprogram fibroblasts into various cell types including pancreatic β -cells [25], blood progenitor cells [26], neurons [27, 28], hepatocytes [29, 30], and CMs [31–36]. Ieda et al. first identified that a cocktail of three cardiac transcription factors, Gata4, Tbx5, Mef2C (GMT), is sufficient to convert cultured neonatal and adult CFs into iCMs that displayed most features of functional CMs [32]. More recently, we showed that transduction of GMT can convert resident CFs into iCMs in vivo in an acutely injured murine heart [35]. Furthermore, this conversion resulted in a reduction in scar size and an improvement in heart function [35]. Subsequently, similar combinations of transcription factors (Tbx5, Mef2c, and Myocardin [34] or GMT plus Hand2 [36]) or microRNAs (miR-1,133,208,499) [33] were used to successfully reprogram CFs into iCMs. The potential of utilizing large pool of endogenous CFs to generate functional iCMs points to an extremely promising avenue to regenerate damaged myocardium in patients. Moreover, this approach circumvents some of the obstacles faced by other approaches including the need to identify a large cellular source and the need for efficient transplantation and integration within the area of injured myocardium.

To further optimize the condition for iCM reprogramming, our lab has recently studied the influence of stoichiometry of G,M,T on iCM reprogramming [37]. We took advantage of the

inherent features of the polycistronic system and generated a complete set of polycistronic constructs to include all possible splicing orders of G,M,T in a single mRNA. With this set of unique tools, we found that varying protein stoichiometry of G,M,T resulted in significant differences in iCM reprogramming efficiency and quality. Moreover, we found the optimal stoichiometry to be a relative high level of Mef2c protein expression and low levels of Gata4 and Tbx5 expression (encoded in MGT construct). By addition of an antibiotic selection marker to the best combination MGT, we were able to further enrich transduced fibroblasts with a homogenous gene expression stoichiometry that resulted in an even higher efficiency in generation of mature iCMs [37]. Furthermore, when delivered into a murine infarcted heart, MGT construct resulted in an increased iCM generation compared to using the traditional mix of separate G,M,T viruses [38]. MGT single triplet introduction also led to a further attenuation of cardiac dysfunction and reduction in scar size [38]. Here we describe in details the step-by-step protocol of in vitro and in vivo iCM reprogramming using both the traditional separate G,M,T and our recently developed single triplet MGT system.

2 Materials

2.1 Generation of Retroviruses

1. pMXs-puro retroviral expression vector.
2. Plat-E cell retroviral packaging cell line.
3. Plat-E cell culture medium: Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1× nonessential amino acids, 1 µg/mL puromycin, 10 µg/mL blasticidin, and 100 U/L penicillin/streptomycin.
4. Plat-E cell transfection medium: DMEM supplemented with 10 % fetal bovine serum, 1× nonessential amino acids.
5. Coating medium: 0.2 % gelatin in PBS.
6. Lipofectamine 2000.
7. Opti-MEM Medium.
8. 0.45 µm syringe filters.
9. Beckman Ultra-Clear centrifuge tube (25 × 89 mm).
10. 1.7 mL microcentrifuge tube.
11. Retro-X qRT-PCR titration kit (Clontech).
12. Polybrene infection reagent.
13. Retrovirus Precipitation Solution (ALSTEM).
14. NIH3T3 cell line.
15. Flow cytometer.

2.2 Generation of Mouse Cardiac Fibroblasts and Cardiac Reprogramming In Vitro

1. α MHC (α -myosin heavy chain)-GFP transgenic mouse.
2. Fluorescent microscope.
3. mFB media: Iscove's Modified Dulbecco's Medium media (IMDM) supplemented with 10 % FBS, and 100 U/L penicillin/streptomycin.
4. 0.05% trypsin-EDTA.
5. 40 μ m cell strainers.
6. collagenase II.
7. HBSS.
8. MACS kit (Miltenyi Biotec).
9. Anti-Biotin MicroBeads (Miltenyi Biotec).
10. iCM media: 400 mL DMEM supplemented with 100 mL M199 media, and 50 mL FBS.
11. B27 medium: 490 mL PRMI1640 medium supplemented with 10 mL B27 supplement.
12. Cardiac troponin T antibody (Cat# MS-295-PO, Thermo Fisher Scientific).
13. GFP antibody (Cat# A11122, Thermo Fisher Scientific).
14. α -actinin antibody (Cat# A7811, Sigma-Aldrich).
15. Connexin43 antibody (Cat# C6219, Sigma-Aldrich).
16. Mef2c antibody (Cat# ab64644, Abcam).
17. Gata4 antibody (Cat# sc-1237, Santa Cruz Biotechnology).
18. Tbx5 antibody (Cat# sc-17866, Santa Cruz Biotechnology).
19. Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Cat# 711-545-152, Jackson ImmunoResearch Inc).
20. Alexa Fluor 647 conjugated donkey anti-mouse IgG (Cat# 715-605-150, Jackson ImmunoResearch Inc).
21. EVOS FL Auto imaging system.
22. SYBR Green Real-Time PCR Master Mixes.
23. Rhod-3 Calcium Imaging Kit (Thermo Fisher Scientific).

2.3 Mouse Myocardial Infarction Model and Gene Transfer In Vivo

1. periostin-Cre mice.
2. Fsp1-Cre mice.
3. R26R-lacZ mice.

4. Ketamine.
5. Xylazine.
6. MiniVent Type 845 mouse ventilator (Hugo Sachs Elektronik-Harvard Apparatus)
7. 8-0 Prolene suture.
8. Mini-Goldstein Retractor.
9. 50 μ L micro syringe.
10. 33 G micro injection needle.
11. 6-0 Prolene suture.

2.4 Evaluation of Transduction Efficiency

1. Cardioplegia buffer: PBS supplemented with 10 mM KCl.
2. Cryosection buffer: PBS supplemented with 0.5 % PFA, and 5% sucrose.
3. 15 mL conical centrifuge tube.
4. OCT.
5. Universal Blocking Buffer (Biogenex).
6. RFP antibody (Cat#3993-100, BioVision).
7. PBST: PBS with 0.1 % Triton 100.
8. Vectashield with DAPI.
9. Wittenberg Isolation Medium (WIM) (pH 7.4): 116 mM NaCl, 5.4 mM KCl, 6.7 mM MgCl₂, 12 mM glucose, 2 mM glutamine, 3.5 mM NaHCO₃, 1.5 mM KH₂PO₄, 1.0 mM Na₂HPO₄, 21 mM HEPES, with 1.5 nM insulin, 1 $\times$ essential vitamins, and 1 $\times$ essential amino acids.
10. Langendorf digestion medium: Wittenberg Isolation Medium (WIM) supplemented with 0.8 mg/mL collagenase II and 10 mM CaCl₂.
11. BD Cytotfix/Cytoperm Solution Kit.
12. Fix buffer: 1 % PFA/PBS.
13. Thy1 antibody (Cat# 553016, BD Biosciences).
14. Flow cytometer.

2.5 Evaluation of Direct Reprogramming In Vivo

1. 2 % Evans blue.
2. Triphenyltetrazolium chloride (TTC).
3. Heart matrices for tissue sampling (Electron Microscopy Sciences).

4. Vevo 2100 High-Resolution Micro-Imaging System with a 40-MHz linear array ultrasound transducer (VisualSonics).
5. Mouse pressure catheter (Millar Instruments).
6. Varian DirectDrive 7 T small-animal scanner.
7. Powerlab system (AD Instruments).
8. Mouse miniature telemetry (Data Sciences International).
9. Mouse ECG.
10. Cx43 antibody (Cat# C6219, Sigma-Aldrich).
11. N-cadherin antibody (Cat# 33-3900, Thermo Fisher Scientific).
12. β -galactosidase antibody (Cat# ab9361, Abcam, Cambridge, MA).
13. Superfusion chamber (Warner Instruments).
14. Ouabain (Sigma-Aldrich).
15. Myocam-S High-Speed Contractility Camera (IonOptix).
16. Video frame grabber (Hauppauge).
17. Dextran-conjugated Cascade Blue (MW 10,000).
18. Calcein.
19. Fluo-4.
20. DMSO.
21. PowerLoad Concentrate (Thermo Fisher Scientific).
22. Tyrode's solution.
23. Motion detector (Crescent Electronics).
24. TRIzol.
25. RIPA buffer supplemented with protease and phosphatase inhibitor cocktail.
26. SuperScript III First-Strand synthesis kit.

3 Methods

3.1 Generation of Retroviruses

1. Clone coding regions of mouse Gata4, Mef2c, and Tbx5 respectively into pMXs based retroviral vector [32]. A single polycistronic vector with optimal ratio of Mef2c, Gata4 and Tbx5 significantly increased reprogramming efficiency both in vitro and in vivo [37, 38]. pMXs-dsRed serves as an indicator for evaluation of transduction efficiency. All these vectors can be requested from the lab of Dr. Li Qian.

2. Maintain Plat-E retroviral packaging cell line in culture medium. Split Plat-E cell to 4×10^6 cells per 10-cm dish 1 day before retroviral packaging (*see* ^{Note 1}).
3. The next day, change with fresh transfection medium at least 1 h prior to transfection. Transfect Plat-E cells with Lipofectamine 2000 when the culture reaches ~80 % confluent. Set up the following two mixtures: (1) dilution of 10 μ g packaging vectors in 500 μ L Opti-MEM Medium. (2) dilution of 20 μ L Lipofectamine 2000 in 500 μ L Opti-MEM Medium. Add vector dilution into diluted Lipofectamine 2000 reagent. Mix transfection solution thoroughly by tapping and incubate the mixture for 15 min at room temperature.
4. Add transfection mixture dropwise to the Plat-E cells. Mix it well by moving the plate in back-and-forth and side-to-side motion. Incubate the plate at 37°C for overnight.
5. After 24 h, replace with fresh prewarmed culture medium without blasticidin and puromycin. Harvest retrovirus containing supernatant at 48 h after transfection. Filter the supernatant with a 0.45 μ m cellulose acetate filter (*see* ^{Note 2}).
6. For virus used for in vitro reprogramming, pellet the virus through incubation with viral precipitation solution. Add 2 mL of the precipitation solution to every 8 mL viral supernatant. Mix gently and precipitate overnight. On the next day, spin the mixture at $1500 \times g$ for 30 min at 4°C. Discard the supernatant and spin again for another 5 min. Gently aspirate the remaining medium. Add 100 μ L cold DMEM to resuspend virus from one 10 cm dish. The retrovirus is ready for use (*see* ^{Note 3}).
7. For virus used for in vivo delivery, pellet the virus through ultracentrifugation. Briefly, transfer the retrovirus containing solution to a Beckman centrifuge tube. Put the tubes into buckets and use SW28 rotor to spin down the virus at $33,000 \times g$ at 4°C for 2 h. Remove the supernatant without disturbing the viral pellet. Wash the pellets once with ice-cold PBS, and combine pellets from 5 to 10 dishes for a higher titer. Resuspend the retroviral pellets with 100 μ L of cold DMEM by gently pipetting up and down. Avoid any air bubbles. Aliquot into 1.7 mL Eppendorf tubes and store them at -80°C.
8. Determine the copy number of the virus by using Retro-X qRT-PCR titration kit.
9. Determine the infectious unit (IFU) of virus. Seed the NIH3T3 cells at 3×10^4 /well in 24-well plate. On the second day, transduce the cells with dsRed retrovirus with 5–10-fold serial dilution. After 48 h culture, analyze fluorescence by flow cytometer to determine the infectious unit (IFU). Assume that the

¹Plat-E should be maintained under antibiotics (puromycin and blasticidin) selection. Plat-E of fewer passages (<30 passages) is highly recommended for viral production. The cells need to be about 70–80 % confluent when starting transfection to produce an efficient packaging.

²The quality and quantity of retrovirus are both critical for direct reprogramming. If most (>80 %) of the control dsRed-transfected Plat-E cells are red, collect all viral particles. Otherwise, improve the transfection first. Usually the best titer and viable virus can achieve at 48 h after transfection. Some other transfection reagents, such as Nanofect, could also be used to produce virus according to manufactures' indications.

³Excessive or sufficient virus can reduce the efficiency of reprogramming [39]. All retroviruses should be used freshly and avoid multiple freeze–thaw cycles. A titration is recommended right before viral delivery to ensure there is enough viable virus.

fluorescent protein-retrovirus and nonfluorescent protein-retrovirus, which are made in the same batch, have the same viability. In other words, their ratio of IFU/copy number should be the same. Determine the IFU of nonfluorescent protein-retrovirus.

10. Dilute the concentrated virus with DMEM into 1×10^{10} IFU/ mL for in vivo gene delivery (*see* Note 3).

3.2 Direct Reprogramming In Vitro

Methods of direct reprogramming in vitro described here provide an easy and fast way for purposes of mechanistic studies, high-throughput screening, and potentially clinical applications. Induced cardiomyocytes (iCMs) generated in vitro could be detected at very early time point (d3 for GFP reporter expression and d14 for sarcomeric gene expression). More importantly, those iCMs start spontaneous contraction from 4 weeks and exhibit cardiomyocytes-like electrophysiological features around 6 weeks.

3.2.1 Generation of Mouse Cardiac Fibroblasts from Explant Culture Method

1. Briefly clean neonatal α MHC (α -myosin heavy chain)-GFP transgenic mouse (P1 to P2) with 75 % ethanol, followed by decapitation. Make a horizontal incision from under one armpit to the other to dissect the heart out. Move one heart into one well of 24-well plate containing ice cold PBS.
2. Check GFP expression in heart by fluorescent microscope. Choose GFP positive hearts and pool them into a 10 cm dish.
3. Cut the hearts into small pieces less than 1 mm^3 in size using a sterile blade and/or scissors.
4. Transfer every 3–4 minced heart pieces in 2 mL mouse fibroblast (mFB) media to one 10 cm dish and incubate it at 37°C for 3 h. Add 8 mL mFB media to the settled tissues and continue to culture for 1 week. Change media every 3 days.
5. On day 7, dissociate the explant cells by incubating the cells with 3 mL 0.05 % trypsin–EDTA at 37°C for 5 min. Inactivate trypsin by adding 5 mL mFB media, followed by gently detaching the cells with cell scraper. Collect cells and pass through $40 \mu\text{m}$ cell strainers to avoid contamination of heart tissue fragments, and then pellet cells by spinning at $200 \times g$ for 5 min.
6. Wash cells once with MACS buffer and cells are ready for sorting.

3.2.2 Generation of Mouse Cardiac Fibroblasts from Enzyme Digestion Method

1. Pool GFP positive hearts into 10 cm dish containing 10 mL ice cold PBS. Squeeze ventricles with sterile forceps to remove blood and rinse once with ice-cold DPBS.
2. Trim the hearts to remove other tissues and fat. Cut the heart into four loosely connected pieces.

3. Incubate the heart tissues (from 20 to 30 hearts) in 50 mL Falcon tube with 15 mL warm 0.05 % trypsin–EDTA, and incubate at 37°C for 15 min.
4. Gently aspirate trypsin, and add 10 mL warm type II collagenase (0.5 mg/mL) in HBSS. Vortex the tube on vortexer for 1 min and incubate the tube in 37°C water bath for 3–5 min. Vortex again for 1 min and let the tissues settle down for 1 min. Collect the supernatant into a new tube containing 10 mL cold mFB medium. Repeat the procedure until all tissue is digested (around 4–5 rounds). At this end, combine all the collections and filter through 40 µm cell strainer to make single cell suspension. Spin down at $200 \times g$ for 5 min, wash once with MACS buffer and finally resuspend in MACS buffer.
5. Optimal: lyse red blood cells. Resuspend cells with 1 mL RBC lysis buffer (150 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA), keep on ice for 1 min, then add 10 mL MACS buffer and spin down at $200 \times g$ for 5 min. Wash one more time with MACS buffer.

3.2.3 Isolation of Thy1.2+ Fibroblasts by MACS (Magnetic-Activated Cell Sorting)

1. Count the viable cell number and add 10 µL Biotin anti-Thy1.2 antibody to every 1×10^7 cells in 90 µL MACS buffer. Incubate the cells with antibody in a refrigerator (2–8 °C) for 30 min.
2. Wash cells once with 10 mL MACS buffer and pellet the cells by spinning at $200 \times g$ for 5 min.
3. Add 10 µL anti-Biotin microbeads to 90 µL MACS buffer and use the mixture to resuspend the cells. Incubate the cells with microbeads in a refrigerator (2–8 °C) for 30 min.
4. Wash cells once with 10 mL MACS buffer and centrifuge again at $200 \times g$ for 5 min. Then resuspend the cells in 2 mL MACS buffer.
5. While washing the cells, set up a MACS Separator in hood. Insert an LS column to the separator. Apply 3 mL MACS buffer to the column to equilibrate it. Pass the cell suspension through the column, and then wash the column three times with 2 mL MACS buffer each time. After that, take the column off the Separator, add 2 mL MACS buffer to the column, and insert the plunger to flush out the beads-binding cells. Pellet the cells by centrifuge at $200 \times g$ for 5 min. Resuspend cells in FB media and they are ready for seeding.

3.2.4 Reprogram Mouse Cardiac Fibroblasts In Vitro

1. Prepare 0.1 % gelatin coated 24-well plates. Seed cells into plates at proper density. For fibroblasts generated from explant culture method, the seeding cells density could be around $2\text{--}3 \times 10^4$ cells/well of 24-well plate. For fibroblasts generated from enzyme digestion method, the cells density could be around $4\text{--}5 \times 10^4$ cells/well of 24-well plate. Culture the cells overnight in mFB media at 37°C (*see* ^{Note 4}).

2. On day 0, change culture media to 0.5 mL pre-warmed iCM media containing 4 µg/mL Polybrene. Add 10 µL retrovirus to each well. Incubate cells with virus for 48 h (see Note 5).
3. On day 2, change virus containing media to 0.5 mL regular iCM media. Change media every 2–3 days.
4. On day 3, change media to iCM media supplemented with 2 µg/mL puromycin for cells transduced with pMx-puro-MGT viruses. Keep it for 3 days. After that, maintain puromycin in the iCM medium at the concentration of 1 µg/mL for additional 7 days.
5. On day 14, change iCM media to B27 media. Change media every 3 days. Spontaneous beating cell loci may be observed from 3 (cells from enzyme digestion method) or 4 (cells from explant culture method) weeks after viral transduction.

3.2.5 Evaluation of Cardiac Reprogramming In Vitro: Immunocytochemistry (ICC)

1. Cardiac fibroblasts have been isolated from aMHC-GFP transgenic mice where the GFP expression is driven by cardiomyocytes-specific aMHC promoter. This allows the tracking of cells that have been converted from fibroblasts (GFP negative) to iCMs (GFP positive). GFP expression could be monitored under microscope 3 days after viral transduction. Cardiac structure genes, like cardiac troponin T (cTnT) and α -actinin exhibit expression from 1 week and sarcomere structures would appear around 2 weeks.
2. To visualize sarcomere structures in reprogrammed cells, stain the cells with antibodies targeting GFP and cTnT. Prepare the cells by washing three times with ice cold PBS. Fix them with 4 % paraformaldehyde/PBS at room temperature for 15–20 min. Wash cells twice with PBS and permeabilized the cells with 0.1 % Triton/PBS, followed by washing twice and blocking for 0.5–1 h with 5 % BSA/PBS. Then incubate the cells with primary antibodies, anti-GFP and anti-cTnT, in 1 % BSA/PBS overnight at 4°C.
3. On the second day, probe the cells with secondary antibodies, Alexa Fluor 488 conjugated donkey anti-rabbit IgG for GFP and Alexa Fluor 647 conjugated donkey anti-mouse IgG for cTnT. Finally, stain the nuclei with DAPI.

⁴The quality and purity of fibroblasts are extremely important. MACS protocol we described here provides a gentle and convenient way to isolate fibroblasts of high-purity and high-viability. The seeding density is also important. Density either too high or too low results in a low percentage of iCMs. If cells are sparsely seeded, they tend to become unhealthy with irregular cell morphology and in large size. It is difficult to convert those senescent-like cells into iCMs. If cells are seeded too densely, the MOI (multiplicity of infection) for viral infection is decreased. Overgrowth of uninfected fibroblasts significantly dilutes the reprogramming events.

⁵The amount of virus should be proportionally increased with increased cell number in different culture plates or dishes. We noticed that co-infection of MGT virus with another pMXs vector based retrovirus will decrease the reprogramming efficiency, which possibly results from the fact that cells preferentially uptake more virus of smaller size than MGT virus. If experiments of overexpression or knockdown some genes are needed for reprogramming, we highly recommend using lentivirus for transduction. In this condition, do MGT infections for 24 h, add the lentivirus directly to MGT-containing media, and incubate cells with both MGT and lentivirus for another 24 h.

4. Place the plate on EVOS FL Auto imaging system and capture images through the EVOS software. Quantification would be performed by counting GFP+ and/or cTnT+ cell number (reprogrammed cell population) and DAPI+ cell number (total cells) in each field. Reprogramming efficiency is calculated by dividing number of total cells with that of reprogrammed cells.

3.2.6 Evaluation of Cardiac Reprogramming In Vitro: Flow Cytometry (FACS)

1. Dissociate the cells with 0.05 % trypsin–EDTA and 96-well deep well plate well to well. Spin at $200 \times g$ for 5 min at 4°C to pellet the cells.
2. Resuspend cells with 100 μ L fixation/permeabilization buffer. Treat the cells for 20 min at 4°C.
3. Wash cell twice with 500 μ L 1 $\times$ wash solution, pellet, and remove supernatant. Thoroughly resuspend each sample with 50 μ L primary antibody solution (anti-GFP and anti-cTnT antibodies) and incubate at 4°C for 30 min.
4. Wash cells once and incubate each sample with 50 μ L secondary antibody solution at 4°C for 30 min.
5. Wash cells once in 1 $\times$ wash solution. Resuspend cells in 400 μ L fixation buffer (DPBS with 1 % PFA). Transfer cell into FACS tube with cell strainer cap. Samples are ready for FACS detection.

3.2.7 Evaluation of Cardiac Reprogramming In Vitro: Gene Regulation

1. Lyse cells in TRIzol according to manufacturer's instructions. Either freeze the lysate at –80°C or proceed immediately to RNA purification.
2. Add 0.2 mL of chloroform, cap sample tubes securely. Shake tube vigorously by hand for 15 s and incubate at RT for 2–3 min.
3. Centrifuge the samples at no more than $12,000 \times g$ for 15 min at 4°C (now the mixture separates into three phases, the upper aqueous phase contains RNAI).
4. Transfer the aqueous phase to a fresh tube and precipitate the RNA by mixing with 0.5 mL of isopropyl alcohol.
5. Incubate samples at RT for 10 min and centrifuge at no more than $12,000 \times g$ for 10 min at 4°C.
6. Remove supernatant, wash RNA pellet with 1 mL of 75 % ethanol, mix the sample by vortexing and centrifuge at no more than $7500 \times g$ for 5 min at 4°C (keep it at –20°C until use).
7. Briefly dry the RNA pellet, dissolve RNA in RNase-free water and incubate for 10 min at 55–60°C. RNA can also be redissolved in 100 % formamide (deionized) and stored at –70°C.
8. Prepare cDNA using First Strand cDNA synthesis kit according to manufacturer's recommendations.

9. Assess for gene expression using SYBR Green Real-Time PCR Master Mixes together with designed primers. Genes involved in sarcomere structures (Myh6, Myh7, Tnnt2, and Actc1), ion channels (Pln, Slc8a1, and Scn5a), and cell junctions (Gja1, Kcna5, and Cacba1c) could be evaluated. Successful cardiac reprogramming shows significant upregulation of those genes and downregulation of genes represent as fibroblast markers including Col1a1 and Col3a1. GAPDH expression should be detected simultaneously as a housekeeping gene for normalization.

3.2.8 Evaluation of Cardiac Reprogramming In Vitro: Ca²⁺ Oscillation at 4 Weeks

1. Calcium oscillation signals could be detected by using Rhod-3 Calcium Imaging Kit according to the manufacturer's instructions. Reconstitute Rhod-3 in 100 μ L of DMSO to yield a stock solution of 10 mM Rhod-3. Store the stock solution desiccated at -20°C and protected from light in single use aliquots.
2. Reconstitute Probenecid in 1 mL of HBSS to prepare 250 mM stock.
3. Wash cells twice with HBSS buffer.
4. Prepare fresh loading buffer containing Rhod-3 and Probenecid. Add loading buffer to cells immediately and incubate cells in dark at room temperature for 30–60 min.
5. Wash cells twice with HBSS.
6. Add 2 mL incubation buffer (2.5 mM probenecid in HBSS) to cells and incubate cells in dark at RT for 30–60 min.
7. Wash cells once with HBSS. Cells are now ready for live-cell imaging (*see* ^{Note 6}).

3.3 Direct Reprogramming In Vivo

Direct cardiac reprogramming opens a new avenue for cardiac regeneration and disease modeling. Establishment of an efficient in vivo reprogramming system would be beneficial for in vivo mechanistic studies and ultimately moving this field further toward clinical applications.

3.3.1 Mouse Myocardial Infarction Model and Delivery of Reprogramming Factors

1. Anesthetize mice with 100 mg/kg ketamine plus 10 mg/kg xylazine. Place mice in a supine position on a heating pad at 37°C .
2. Intubate mice with a 19 G stump needle connected to a MiniVent Type 845 mouse ventilator. Ventilate with 150 μ L of stroke volume at 120 breaths per minute. Ventilation is confirmed by chest movement.

⁶Rhod-3 staining has a high background. Signals start to decrease half hour after final step of washing, especially if incubated at 37°C . We recommend imaging cells immediately and finish the experiments within an hour.

3. Perform anterior thoracotomy by cutting the third rib. Open the chest with a Mini-Goldstein retractor. Remove the pericardium and expose the heart (*see*^{Note 7}).
4. Ligate the left anterior descending artery (LAD) with an 8-0 prolene suture. The ischemia is confirmed by myocardial blanching (*see*^{Note 8}).
5. Mix 10 μ L retroviral solution (1×10^8 IFU virus) containing a pooled viruses (Gata4/Mef2c/Tbx5) or a polycistronic MGT virus or a dsRed virus with 10 μ L of PBS plus 4 μ g/mL polybrene. Inject the mixture into the myocardium through a 50 μ L micro syringe incorporated with 33 G injection needle. Single injection with a full dosage is employed along the boundary between the infarct area and border zone based on the blanched infarct area after LAD ligation (*see*^{Note 9}).
6. After injection, the chest is closed by suturing muscle layer and skin layer separately with a 6-0 Prolene suture. Let the mouse recover with ventilation on the heating pad.
7. Sham-operated animals will be served as surgical controls and will be subjected to the same procedures as the experimental animals with the exception that the LAD is not ligated.

3.3.2 Evaluation of In Vivo Viral Transduction Efficiency

1. Two days after surgery, use cryosection and immunofluorescent staining to visualize transduction. Isolate all the cells from left ventricle and run FACS to quantify transduction efficiency.
2. Anesthetize mice subjected to MI and dsRed virus injection with 100 mg/kg ketamine plus 10 mg/kg xylazine (*see*^{Note 10}).
3. For immunohistochemistry (IHC) on cryosections, open chest to expose the heart. Inject 0.5 mL cardioplegia buffer into left ventricle slowly. Perfuse the heart with 5 mL PBS slowly to remove all the red blood cells. Perfuse the heart with cryosection buffer, then remove the heart and put it into 15 mL conical centrifuge tube containing 10 mL fresh cryosection buffer. Incubate with gentle agitation at 4°C overnight.
4. On the second day, wash the PFA away from the tissue with a serial dilution of sucrose (5 %, 10 %, and 20 % respectively). Embed the tissue with OCT and freeze in liquid nitrogen.

⁷The incision is usually performed in the third intercostal space to avoid extra tissue damage in LAD ligation induced MI model. Instead, we perform the surgery by cutting the third rib which will give a better view for the following viral delivery and is easier to operate [35, 38].

⁸The size of infarct region depends on the ligation site. It is important for the evaluation of cardiac function to keep the LAD ligation procedure consistent among mice, which is determined in Subheading 3.3.4.

⁹The virus is delivered by a single shot along the margin of infarcted area. Make sure the needle isn't advanced into the left ventricle, causing the viral leakage. The viral solution is being injected while the syringe is withdrawn. When injection finishes, hold the syringe in place for a while to let the viral solution diffuse. Also, a concentrated viral solution is used here to minimize the volume of injection.

¹⁰Retrovirus only infect dividing cells, which are mainly the activated cardiac fibroblasts bordering the infarct zone. Most of adult cardiac fibroblasts are regarded as quiescent cells. Therefore infarcted hearts with dsRed infection are used as negative controls.

5. Block sections in Universal Blocking Buffer for 10 min, then stain with RFP primary antibody for 1 h at room temperature. After washing with PBST for three times, incubate section with secondary antibody for 1 h at room temperature followed by washing another three times with PBST. Finally, mount the section with Vectashield with DAPI. Most of dsRed⁺ cells should present in MI border zone.
6. For FACS, left ventricular cells are isolated after removing heart from chest. Cannulate the heart via aorta and perfuse retrograde with Wittenberg Isolation Medium in a Langendorf apparatus with a constant flow of 3 mL/min at 37°C for 5 min. Then switch to digestion medium for another 10 min. Dissect left ventricle and mechanically dissociate all the cells. Fix and incubate the cells for RFP/Thy1 staining with BD Cytofix/Cytoperm Solution Kit. Resuspend the cells with Fix buffer and run FACS. Around 4 % or even more of cells from the left ventricle should be dsRed⁺ Thy1⁺, depending on the viral quantity, quality, and delivery (*see* ^{Note 11}).

3.3.3 Lineage Tracing of In Vivo Reprogrammed iCMs

1. To explore the mechanisms underlying in vivo direct reprogramming, it is critical to trace the lineage of induced cardiomyocyte (iCM) and characterize the iCM based on their origin.
2. Cross periostin-Cre mice or Fsp1-Cre mice and R26R-lacZ mice to obtain Periostin-Cre:R26R-lacZ or Fsp1-Cre:R26R-lacZ mice to trace the fibroblast-origin iCMs using IHC.
3. To evaluate in vivo reprogramming quality and quantity, cohorts of mice (10–12 mice per group) are sacrificed at 4, 8, and 12 weeks after G/M/T or MGT delivery. Hearts are perfusion-fixed and taken out to be further fixed in 4 % PFA or 0.5 % PFA for paraffin sections or cryosections respectively. Cryosections are used for IHC of cardiac markers α Actinin, cTnT, Tropomyocin, α MHC, Connexin43, N-Cadherin, and fibroblast lineage markers Periostin-Cre/Fsp1-Cre; RosaEYFP (anti-GFP) to evaluate the conversion of CFs into iCMs (*also see* Subheading 3.3.6). Paraffin sections are used to determine the scar area using standard Masson-Trichrome staining (Subheading 3.3.4).
4. Cross periostin-Cre mice and R26R-EYFP or R26R-Tomato mice to get Periostin-Cre:R26R-EYFP or Periostin-Cre:R26R-Tomato mice for the following assays such as determining the electrophysiological features (Subheading 3.3.6) and gene expression of iCMs (Subheading 3.3.7) based on EYFP or Tomato labeling.

3.3.4 Evaluation of In Vivo Direct Reprogramming: Determination of Scar Size

1. To evaluate the consistence among different groups, Evans blue/triphenyltetrazolium chloride (TTC) double staining is analyzed 48 h after MI.

¹¹Single cell CM/iCM isolation and the following assays are used to rule out the false positives from overlaying cells.

After anesthetizing and sacrificing the mice, perfuse the hearts retrograde with 2 % Evans blue via aorta. All the heart tissue shows in blue except for the area at risk (AAR). Cut left ventricle into several slices with heart matrices for tissue sampling. Incubate the slices in 1.5 % TTC for 30 min at 37°C. Fix with 4 % PFA overnight at 4°C. The infarcted area shows in white, while viable myocardium is in red. Analyze infarct size/AAR size via planimetry with ImageJ software.

2. To determine the scar size, standard Masson-Trichrome staining is applied on hearts 8 weeks after MI. Use ImageJ software to measure the scar area (blue) and viable area (red) on a serial of transverse sections with the first level right below the ligation.

3.3.5 Evaluation of In Vivo Direct Reprogramming: Cardiac Function

1. Echocardiography, hemodynamics, and MRI are analyzed to evaluate the cardiac function at different time points (4, 8, and 12 weeks) after MI with or without delivery of reprogramming factors.
2. Perform echocardiography on anesthetized mice under 1–1.5% isoflurane at a core temperature of ~37°C with echo-cardiography machine and probes for mice. Capture parasternal long-axis and short-axis views of left ventricle at a frame rate of 400 Hz. Obtain end-systole or end-diastole defined as the phase in which the smallest or largest area of LV, respectively for ejection fraction assessment. Measure left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) from the LV M-mode tracing at the papillary muscle level.
3. Perform hemodynamic assessment by inserting a mouse pressure catheter into the aorta and LV through the right common carotid artery on anesthetized mice under 1–1.5 % isoflurane. Connect the transducer to Powerlab system to record heart rate (HR), blood pressure (BP), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), $+dp/dt$ and $-dp/dt$.
4. Perform MRI by a Varian DirectDrive 7 T small-animal scanner on the mice under anaesthetization by inhalation of 2 % isoflurane/98 % oxygen administered. Insert two ECG leads into the right front and left rear leg for image triggering. After defining the oblique plane of the short axis, use an ECG-triggered two-dimensional gradient echo sequence with an echo time of 2.75 ms, repetition time of 200 ms and a flip angle of 45°C to obtain nine short-axis images at 12 or 13 phases per cardiac cycle. Each scan consists of 8–9 contiguous slices spanning the left ventricle from apex to base with 1-mm thickness, a matrix size of 128×128 , a field of view of 25.6×25.6 mm, and four averages.

3.3.6 Evaluation of In Vivo Direct Reprogramming: Electrophysiology

1. Evaluate the electrophysiology and incorporation of iCMs by miniature telemetry, cell coupling, Ca^{2+} handling/Stimulation, and action potential after direct reprogramming.
2. One of the concerns for cardiac cell therapy is that the cardiomyocyte from exogenous transplant may not incorporate well with the native myocardium, which leads to a potential risk of arrhythmia. To test if direct reprogramming leads to the same concern, ECGs are monitored over the course of iCM conversion. Three days after surgery for a full recovery, implant a miniature telemetry in the abdominal cavity of the mouse. Place and fix two leads to the muscle layer of the neck and xiphoid respectively. Use Dataquest Software to acquire and analyze the ECG data.
3. To determine if iCMs express proteins involved in cell–cell communication, perfuse and fix hearts with 0.5 % PFA for frozen section as described in Subheading 3.3.3. After blocking with universal blocking buffer, stain with primary antibody against Connexin43 or N-cadherin or other gap junction proteins with β -galactosidase antibody to localize iCMs.
4. To assess the functional cell–cell junctions, measure the intercellular transmission of excitable Ca^{2+} wave and molecular probes of gap junctional communication by isolating small groups of cells from infarct/border zone, including tomato-labeling iCMs and nonfluorescent CMs. For doing this, shorten the isolation time from 10 to 4 min as described in Subheading 3.3.4.
5. After isolating myocytes, transfer them to a superfusion chamber. Incubated with 2 mM ouabain for 5–10 min to induce an intracellular Ca^{2+} overload. Then, a Ca^{2+} wave activity is revealed under the videomicroscopy.
6. The whole-cell patch-clamp method is used to assess the interconnectivity between iCMs and endogenous CMs. Add a dye pair, 1 mM immobile dextran-conjugated Cascade Blue (MW 10,000) and 5 mM mobile calcein, in standard intracellular solution with 5 mM EGTA, which help to reseal the sarcolemma of the cell receiving molecular dye, for 2 min cytoplasmic loading. Excite blue fluorescence from the immobile indicator at 365 ± 40 nm, and calcein fluorescence at 470 ± 40 nm. Record fluorescent images using IonOptix Myocam-S via a video frame grabber and process imaging with ImageJ software.
7. Prepare the loading solution. Dissolve Fluo-4 in anhydrous DMSO to 5 mM. Mix Fluo-4/DMSO with PowerLoad Concentrate at 1:10. Dilute Fluo-4/DMSO/PowerLoad 1:100 in Tyrode's solution to get a final loading solution for myocytes.
8. Transfer isolated myocytes in loading solution for 30 min at room temperature. Load the cells to a superfusion chamber and de-esterificate for another 20 min. Evoke contraction and Ca^{2+} transient by pulses of stimuli at 0.33 Hz of frequency, 2 ms of duration and 150 % of threshold voltage. Record Ca^{2+} transients in batches of ten, averaged numbers are used for statistical analyses.

Record resting fluorescence after cessation of pacing, and obtain background light after removing the cells from the field of view at the end of the experiment. Calibrate the Ca^{2+} transients using the pseudo-ratio method, assuming an in situ dissociation constant of 1.1 μM for Fluo-4. Record contractions optically simultaneously with Ca^{2+} transients by illuminating the cell of interest in red light ($>665\text{ nm}$) subsequently directed to a CCD camera. Convert the cell length signals to voltage via a video motion detector.

3.3.7 Evaluation of In Vivo Direct Reprogramming: Gene Regulation

1. As described in Subheading 3.3.4, isolate iCMs from different regions and time points after MI by digestion and FACS (or manual picking if the iCMs are large). Lysate the cells in TRIzol or RIPA buffer to harvest RNA or protein according to manufacturer's recommendations. Prepare cDNA with SuperScript III First-Strand synthesis kit. Assess the gene expression using Taqman system or run western blot to determine protein expression.
2. As described in Subheading 3.3.3, prepare cryosection and evaluate the protein expression of a battery of cardiac or fibroblast markers via immunohistochemistry.

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