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Production of Cardiomyocyte-like cells by Fibroblast Reprogramming with Defined Factors

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Abstract

Over the last decade, great achievements have been made in the field of direct epigenetic reprogramming, which converts one type of adult somatic cells into another type of differentiated cells, such as direct reprograming of fibroblasts into cardiomyocytes, without passage through an undifferentiated pluripotent stage. Discovery of direct cardiac reprogramming offers a promising therapeutic strategy to prevent/attenuate cardiac fibrotic remodeling in a diseased heart. Furthermore, *in vitro* reprogramming of fibroblasts into cardiomyocyte-like cells provides new avenues to conduct basic mechanistic studies, to test drugs, and to model cardiac diseases in a dish. Here, we describe a detailed step-by-step protocol for *in vitro* production of induced cardiomyocyte-like cells (iCMs) from fibroblasts. The related procedures include high-quality fibroblast isolation of different origins (neonatal cardiac, tail-tip, and adult cardiac fibroblasts), retroviral preparation of reprogramming factors, and iCM generation by fibroblast reprogramming via retroviral transduction of Gata4, Mef2c and Tbx5. A detailed written protocol will help many other laboratories, inexperienced in this area, to use and further improve this technology in their studies of cardiac regenerative medicine.

Keywords

Cardiomyocytes; Fibroblasts; Epigenetic reprogramming; Transdifferentiation; Transcription factors; Heart regeneration; Cell therapy

1. Introduction

Cardiovascular diseases remain the leading cause of death worldwide [1]. Coronary artery disease is the most common form of cardiovascular diseases, resulting in the loss of cardiomyocytes (CMs) at the site of ischemic injury. To compensate for the loss of CMs, cardiac fibroblasts quickly respond to injury and initiate cardiac remodeling in an injured heart [2–4], which leads to dysfunction of the heart and eventually a heart failure. Heart transplantation remains the final solution for patients with an end-stage heart failure, but is limited by the shortage of donor organs. Cellular therapy offers more accessible options for a

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broader group of coronary heart patients and prevents a diseased heart from end-stage failure, and has been investigated with different strategies, including transplantation of autologous adult stem cells [5, 6] or CMs derived from embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) [7, 8], activation of endogenous progenitors [9–11], cell-cycle reentry of adult CMs [12–14], and direct epigenetic reprogramming [15–17].

Since lost CMs in an injured heart are replaced by cardiac fibroblasts it will be a promising therapy for cardiac regenerative medicine if the proliferated cardiac fibroblasts can be transdifferentiated into functional CMs. Transdifferentiation was initially reported in the early 1990s that MyoD alone converts fibroblasts and epithelial cells into skeletal muscle cells [18]. After decades of efforts, the discovery of iPSCs [19] demonstrated that, rather than a single transcription factor, a combination of several defined transcription factors might be required to directly convert a type of terminally-differentiated somatic cells into another type of cells. Indeed, in 2010, Ieda et al. [15] successfully identified a combination of three transcription factors (GMT: *Gata4*, *Mef2c*, and *Tbx5*) that can convert cardiac fibroblasts directly into induced cardiomyocyte-like cells (iCMs) without going through an intermediate pluripotency or progenitor state. Soon after the first discovery, *in vitro* mouse cardiac reprogramming by different combinations of defined factors [17, 20–22], *in vivo* mouse iCM-reprogramming [16, 17, 23], and *in vitro* human iCM-reprogramming [24–27] had been achieved in different laboratories around the world. Since then, many efforts have been invested to improve the efficiency and efficacy of reprogramming by manipulations of signaling pathways [28–30] epigenetic barriers [31], cell cycle regulation [32, 33], chemokine signaling [34], and inflammatory immune signaling [35]. Those progresses have been reviewed in recent publications [36, 37].

Despite the success of multiple groups, it remains challenging to achieve a high efficiency of *in vitro* reprogramming, which requires high quality of cultured fibroblasts and robust expression of all reprogramming factors in individual fibroblasts [38]. *In vitro* iCM reprogramming has significant advantages to study mechanisms of epigenetic reprogramming, to test chemicals for drug development, and to model cardiac diseases in a dish in the future; therefore, the detailed written protocol is important to help many research laboratories master it as well as to inspire further refinement of this technology.

This chapter provides step-by-step protocols for: 1) isolation and culture of high-quality starting cells for reprogramming (i.e. neonatal cardiac, neonatal tail tip, and adult cardiac fibroblasts); 2) preparation of high-titer retroviruses encoding the reprogramming factors; and 3) generation of iCMs from fibroblasts transduced with retroviruses encoding GMT.

2. Material

1. αMHC-GFP transgenic mice (Gladstone Institutes, Dr. Deepak Srivastava laboratory) (*see* Note 1).
2. Anti-Mouse CD90.1 (Thy-1.1) APC.
3. Blasticidin.
4. Fetal bovine serum (FBS) (Hyclone, Cat.# SH30910).

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5. FuGENE® 6 Transfection Reagent.
6. FACS buffer: 2% FBS in PBS with 2 mM EDTA, store at 4 °C.
7. Fibroblast Explant Medium: Combine 395 mL of Iscove's Modified Dulbecco's Medium (IMDM), 100 mL FBS, and 5 mL Penicillin/Streptomycin. Store at 4 °C. Warm up before use.
8. Gelatin 0.1% (wt/vol) solution
9. iCM-Reprogramming Medium: combine 355 mL DMEM, 85 mL Medium 199 (M199), 50 mL FBS, 5 mL non-essential amino acids, and 5 mL Penicillin/Streptomycin. Store at 4 °C. Warm up before use.
10. Nalgene syringe filter, 0.45-μm pore-size, SFCA-membrane.
11. Opti-MEM I reduced-serum medium.
12. PBS without Ca^{2+} and Mg^{2+}.
13. Plat-E Medium: combine 445 mL Dulbecco's Modified Eagle Medium (DMEM) with L-Glutamine, 5 mL Non-essential amino acid solution (NEAA, 100X), and 50 mL FBS. Store at 4°C. Warm up before use.
14. Hexadimethrine bromide (Polybrene).
15. Propidium iodide.
16. Puromycin.
17. 0.05% (wt/vol) Trypsin/EDTA.
18. Plasmids: pMX-Gata4, -Mef2c, -Tbx5 and -dsRed (Gladstone Institutes, Dr. Deepak Srivastava laboratory)

3. Method

To achieve a high efficiency of iCM-reprogramming, it requires freshly-purified fibroblasts with high quality and fresh retroviruses with high titers; therefore, all experiments should be coordinated and started as planned (Figure 1).

3.1 Fibroblast isolation by explant-culture method

Contamination could occur in primary cell culture from freshly-harvested tissues; therefore, all surgical tools (i.e., scissors and forceps) must be sterilized before experiments. Cell isolation should be carried out in a sterile biosafety level II tissue culture hood.

1. Coat cell-culture dishes with 0.1% gelatin solution at 37 °C for >1 hour (*see* Note 2).
2. Dissect hearts and/or collect tail-tips from neonatal pups (between postnatal 0.5 to 1.5 days) or adults (age: >8weeks) of the αMHC-GFP transgenic mice. Put the harvested hearts/tail tips in cold PBS and keep them on ice before fibroblast isolation (*see* Note 3).

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3. Use a fluorescent dissecting microscope to examine for GFP fluorescence in the hearts and identify αMHC-GFP+ animals. After identification of the αMHC-GFP+ animals, use the tissues of heart or tail-tip from the αMHC-GFP+ mice for fibroblast isolation (*see* Note 4).
4. Right before cell isolation, use a curved forceps to transfer the tissues (i.e., hearts and/or tail-tips) of αMHC-GFP+ mice into a dish that contains 70% ethanol. After 3 to 5 seconds in the 70% ethanol, quickly transfer the tissues into another dish containing cold PBS to wash out the residual ethanol, and then transfer the tissues into a third dish containing cold PBS. These wash steps could reduce the chance of bacterial contamination of the cell culture.
5. Transfer hearts or tail tips into a 35-mm dish by a curved forceps and mince tissues into small pieces with a curved scissors (*see* Note 5).
6. Add proper amount of the fibroblast explant medium to the minced tissues (*see* Note 6), and then dissociate the tissue pieces by pipetting up and down gently.
7. Aspirate the coating gelatin solution from the dishes, and then plate the suspended minced tissues evenly on the coated dishes (*see* Note 7).
8. Carefully transfer the dishes into a cell-culture incubator (37 °C, 5% CO_2) and culture for 2–4 hours to allow the minced tissue to settle (*see* Note 8).
9. Carefully take out the dishes from the incubator, attach the pipette on the wall of the dish and add 9 mL of the fresh fibroblast explant medium VERY GENTLY into a 100-mm dish, or 3 mL media for a 60-mm dish (*see* Note 9).
10. Carefully and slowly transfer dishes into the incubator and avoid any turbulence. Culture the tissues for 3 days WITHOUT any disturbance.
11. Afterward, take out the dishes and move dishes back and forth gently to suspend the unattached tissues (*see* Note 10). Aspirate media along with those unattached tissues. Attach the pipette on the wall of the dish and gently add fresh fibroblast explant medium (10 mL per 100-mm dish or 3 mL per 60-mm dish).
12. Culture for 3 to 4 days to allow more fibroblasts to migrate out from the attached tissues (*see* Note 11). Figure 2 shows representative images of fibroblasts from the neonatal hearts, neonatal tail-tip tissues, and adult hearts 7 days after explanation.

3.2 Fibroblast purification by fluorescence-activated cell sorting (FACS)

The health and freshness of the fibroblasts are critical to achieve a high efficiency of iCM reprogramming; therefore, explant-cultured fibroblasts are generally purified by FACS at Day 7 and could be also performed at Day 6 if there are plenty of fibroblasts from cultured tissues. Fibroblasts from the explanted tissue dishes (P0 fibroblasts) should be used for reprogramming (*see* Note 12).

1. Aspirate culture media, and add 5 mL PBS into one 100-mm dish of cultured cells to wash out the residual culture media. Aspirate PBS and repeat PBS wash twice.

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2. Aspirate PBS, and add 2 mL Trypsin (0.05%) into the 100-mm dish. Digest for 5 minutes at 37 °C. Take out the dishes from the incubator, and gently pat the wall of the dishes. Examine the cells under a microscope to check if over 80% of the cells become round in shape and are detached from or loosely attached to the dishes.

3. Quench the digestion with 8 mL of fibroblast explant medium, and pipette up and down to break up clumps into single cells.

4. Harvest the digested cells, and pass the cells through a 40-μm cell strainer to remove undigested cell clumps and collect the filtered single cells into a 15-ml tube. Centrifuge the fibroblasts for 3 minutes at 300× g.

5. Aspirate off the media, and resuspend the cell pellet in 5 mL PBS. Centrifuge the cells for 3 minutes at 300× g.

6. Resuspend the cell pellet in 1 mL FACS buffer (*see* Note 13). Take an aliquot of the resuspended cells (25 μL or 50 μL). Add this aliquot into 0.5 mL FACS solution and keep it on ice without staining of Thy1, which will be used as the negative control in FACS assay.

7. Add 20 μL anti-mouse Thy1-APC (1:50 dilution) in the resuspended cells, and mix gently by finger tapping. Protect the tube from light, and incubate for 30 minutes at room temperature.

8. Add 9 mL PBS, and centrifuge the cells for 3 minutes at 300× g. Repeat PBS wash twice.

9. Resuspend the cells in 1 mL FACS buffer, and add propidium iodide (50 μg/mL) into the Thy1-stained fibroblasts and the aliquoted unstained cells. Keep the stained cells on ice and protect them from light until FACS sorting. In general, more than 70% of the cells should be stained as Thy1$^+$/αMHC-GFP$^-$ fibroblasts (Figure 3), which will be sorted and collected into a tube with 0.5 mL of the fibroblast explant medium.

10. Centrifuge the purified Thy1$^+$/αMHC-GFP$^-$ fibroblasts for 3 minutes at 300× g, and then resuspend the cells with the fibroblast explant media; plate the fibroblasts in a gelatin-coated 6-well plate at a density of 10^4/cm^2, at around 1–1.2×10^5/per well of 6-well plate in 2 mL media. Those sorted fibroblasts will be used for iCM reprogramming the next day by retroviral transduction of GMT.

3.3 Retrovirus preparation

The platinum-E (Plat-E) cells, a derivative of the HEK293T cell line, was established by using the packaging constructs with the EF1α promoter, which ensures stable and robust expression of the retroviral structure proteins (gag, pol, and ecotropic env). In order to maintain robust expression of those retroviral structure proteins, the Plat-E cells are cultured with the Plat-E cell medium in the presence of puromycin (1 μg/mL) and blasticidin (10 μg/mL), which will selectively kill cells that have lost the expression of the packaging

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constructs. The Plat-E cell line is designed for rapid and transient production of high-titer ecotropic retroviruses (*see* Note 14).

1. Aspirate culture media and add 5 mL PBS into the cultured Plat-E cells to wash out the residual culture media; aspirate PBS and repeat PBS wash once again.
2. Aspirate PBS and add 2 mL Trypsin (0.05%) into the 100-mm dish; digest the Plat-E cells for 5 minutes at 37 °C. Quench the digestion with 8 mL of the Plat-E cell medium, and pipette up and down to break up clumps into single cells.
3. Collect the digested Plat-E cells into a 15-ml tube, and centrifuge for 3 minutes at 300× g.
4. Resuspend the Plate-E cells in the Plat-E cell medium, and count cell numbers.
5. Plate 8×10^6 cells in one gelatin-coated 100-mm dish in the Plat-E cell medium without any antibiotics (*see* Note 15).
6. Transfect Plat-E cells in the next day (*see* Note 16) by using FuGENE® 6 system (*see* Note 17). Here is an example for transfection of a 100-mm dish, which produces 10 mL of retroviral supernatants of one reprogramming factor (*see* Note 18).
   a. For each retroviral vector of GMT factors, add 300 μL of Opti-MEM I into a 1.5-ml Eppendorf tube; add 27 μL Fugene® 6 in Opti-Mem, and mix gently by finger tapping. Incubate for 5 minutes at room temperature.
   b. Add 9 μg of one retroviral vector DNA (i.e., pMXs-Gata4, -Mef2c, -Tbx5 or -dsRed), and mix thoroughly by finger tapping. Incubate for 15 minutes at room temperature.
   c. Add the DNA-FuGENE complex into the culture media dropwise, and move the dishes back and forth gently to mix the DNA-FuGENE complex with the media in the dishes. Culture the transfected cells overnight.
7. In the morning of the next day after transfection, remove the spent medium (*see* Note 19), and add 10 mL of fresh pre-warmed Plat-E cell medium. Culture the transfected Plat-E cells for additional 24 hours to produce high-titer viruses.
8. Harvest and filter retroviral supernatants through a Nalgene syringe filter (0.45-μm pore-size, SFCA-membrane) using a 10-mL sterile disposable syringe to remove the cell debris.
9. Add polybrene (final concentration 5 μg/mL) into the filtered virus-containing supernatant, and mix gently by pipetting up and down. To make a retroviral reprogramming cocktail, add equal amount of retroviral supernatant of each factor (i.e. *Gata4*, *Mef2c,* and *Tbx5* in our study), and mix.

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3.4 Production of iCMs from mouse fibroblasts by retroviral transduction of GMT

1. Aspirate the medium from the cultured FACS-purified fibroblasts from Step 10 of Section 3.2. Add 0.5 mL of the fresh iCM reprogramming medium with polybrene (5 μg/mL) into each well of fibroblasts.
2. Add 1.5 mL of the retroviral cocktail of the three factors into each well of fibroblasts, i.e., 0.5 mL retrovirus of each factor (Gata4, Mef2c, and Tbx5) (*see* Note 20).
3. Culture the cells overnight. After 24 hours, remove the transduction medium (*see* Note 19), and add 2 mL fresh iCM reprogramming medium into one well of a 6-well plate.
4. Change medium every 2–3 days. Examine the progress of iCM reprogramming by checking for αMHC-GFP$^+$ cells under a fluorescence microscope (Figure 4A) (*see* Note 21).
5. Determine the reprogramming efficiency by FACS assay to quantify the number of αMHC-GFP$^+$ cells, or by quantification of cells positive for cardiac troponin-T (cTnT) via immunostaining (Figure 4B).
6. Evaluate the reprogramming by examining the expression of the cardiac enriched genes (e.g., α-actinin, cTnT) and the fibroblast-enriched genes at different time points after retroviral transduction. For examples, αMHC-GFP$^+$ and GFP$^-$ cells can be purified by FACS sorting and used to profile gene expression of the reprogrammed iCMs by RNA-microarray or RNA-sequencing; activation of the cardiac genes and inactivation of the fibroblast-enriched genes (*see* Note 22) can be validated by qRT-PCR.
7. Conduct functional characterization of iCMs, such as Ca^{2+} transients, action potential and cell contraction, at 2 weeks to 8 weeks after retroviral transduction (*see* Note 23).

4. Notes

1. Cardiac epigenetic reprogramming could be studied by using other transgenic mouse of cardiac lineage reporter, such as αMHC-mCherry transgenic mice (Stock No: 021577) from The Jackson laboratory.
2. In our experience, tissues from one heart of a neonatal mouse should be plated into one 60-mm dish and three hearts into one 100-mm dish; two tail-tip tissues, harvested at 0.5–0.75 cm in length, should be plated in one 100-mm dish; tissues from one ventricle of an adult mouse should be plated in two or three 100-mm dishes.
3. Keep tissues cold to minimize cell death or senescence. The harvested tissues should be used as soon as possible, but can be stored on ice for 2 hours without obvious negative effect on fibroblast isolation.

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4. Place the heart in one well of 24-well plate and the tail tip from the same animal into the next well of the 24-well plate, so that αMHC-GFP-harboring tail tips can be determined based on GFP expression of the heart from the same animal and will be used for fibroblast isolation.

5. It is critical to mince the tissues into small pieces so that the tissues can attach to cell culture vessels. Attachment is required for fibroblasts to emigrate from the isolated tissues. It is more efficient to mince tissues without addition of media.

6. The amount of media depends on the number of collected αMHC-GFP$^+$ tissues that are used for cell culture. For examples, we use 0.5 mL media for tissues of one neonatal heart that will be plated into one 60-mm dish, and 1.5 mL media for tissues of three neonatal hearts that will be plated into one 100-mm dish.

7. It is important to use a proper amount of media so that the media barely cover the surface of dish. Too much medium prevents attachment of the minced tissues onto the bottom of the culture dishes.

8. Tissue attachment requires a minimum of 2 hours. A period of four hours of attachment is recommended.

9. It is very important to avoid disturbing the attached tissues. Don't add media directly upon tissue.

10. It is a good sign if the media become yellow after 3 days of culture, suggesting that many fibroblasts have emigrated out from those attached tissues. Under a microscope, emigrated fibroblasts can be observed around those attached tissues.

11. Adult cardiac fibroblasts can be cultured for another extra 3 days if needed.

12. It is not recommended to use frozen or passaged fibroblasts for iCM reprogramming.

13. To save the amount of antibody used in Thy1-staining of Step 7, it is fine to resuspend cells in 0.5 ml FACS solution if there are not huge number of cells.

14. The ecotropic retroviruses, prepared from Plat-E cells, infect mouse cells only, and do not infect human cells.

15. It is important that the media of retrovirus preparation shouldn't include any residual puromycin and blasticidin, which will kill fibroblasts when they are infected by the retroviral supernatants.

16. After overnight culture (~16 hours), the culture of Plat-E cells should become >80% confluent for transfection. To improve the virus titer, it is recommended to refresh the Plat-E Cell Medium 1 hour before transfection.

17. Other transfection reagents might be used for transfection. In our experience, FuGENE® 6 gives us the best yield of retrovirus.

18. For each batch of retrovirus preparation, dsRed virus should be included as a control of red fluorescence to monitor the efficiency of transfection and virus preparation. For example, to determine the transfection efficiency, dsRed

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fluorescence should be observed in most of the Plat-E cells (>80%) in the pMX-dsRed control. dsRed retroviruses could be used for virus titration as needed. To save transfection reagents, retrovirus of pMX-dsRed can be prepared in a 60-mm dish.

19. Dishes, pipettes, tips and media, all of which possibly contain virus, must be treated by 10% bleach for >1 hour before discard. After overnight transfection, it is expected that Plat-E cells have produced retroviruses.

20. We routinely use 0.5 mL of viruses for each factor, but 0.3 mL of each viruses gave similar results in our experience. Our FACS assay showed that both 0.3 mL and 0.5 mL dsRed retroviruses could transduce >90% of fibroblasts without significant difference.

21. In our experience, αMHC-GFP^{+} cells can be observed at day 3 after transduction, suggesting a success of reprogramming; 1 week later, many αMHC-GFP^{+} cells should be observed. iCM reprogramming failed if there are very few or no αMHC-GFP^{+} cells 7 days after retroviral transduction.

22. Cardiac muscle genes include *Actc1, Myh6, Ryr2, Myl7, Scn5a, Slc8a1, Myl2, Tnnt2, Pln, Kcna5, Kcnj2, Cacba1c, Gja1, Atp2a2*; fibroblast-enriched genes include *Col1a1, Col1a2, Col3a1, Vim, Posn, Fsp1, Fn.*

23. Fibroblasts infected by dsRed-retroviruses should be included as a negative control in assays of αMHC-GFP^{+} iCM characterization.

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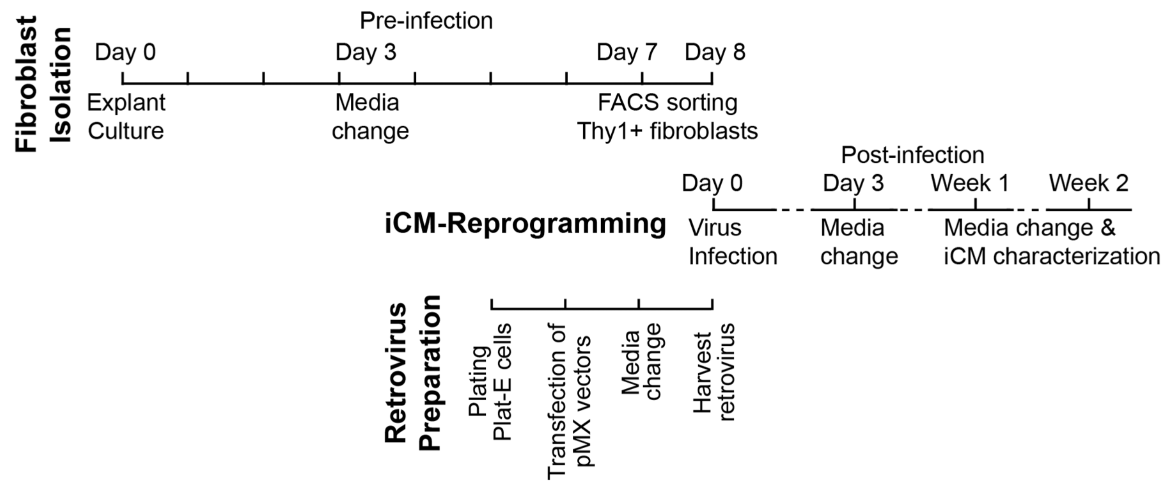

Figure 1.

Scheme of producing cardiomyocytes by epigenetic reprogramming of fibroblasts, including fibroblast isolation, retrovirus preparation and reprogramming of induced cardiomyocytes (iCMs).

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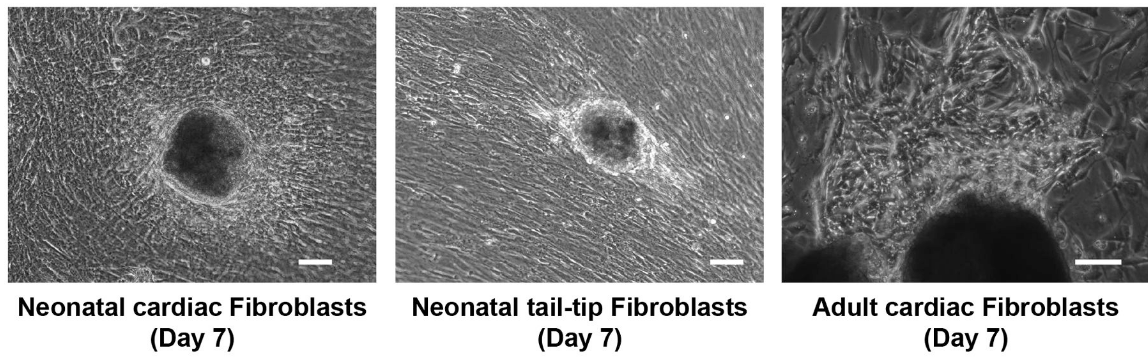

Figure 2.

Representative pictures of cultured neonatal cardiac fibroblasts and tail-tip fibroblasts, and adult cardiac fibroblasts after 7 days of explant culture. Bars indicate 50 μm.

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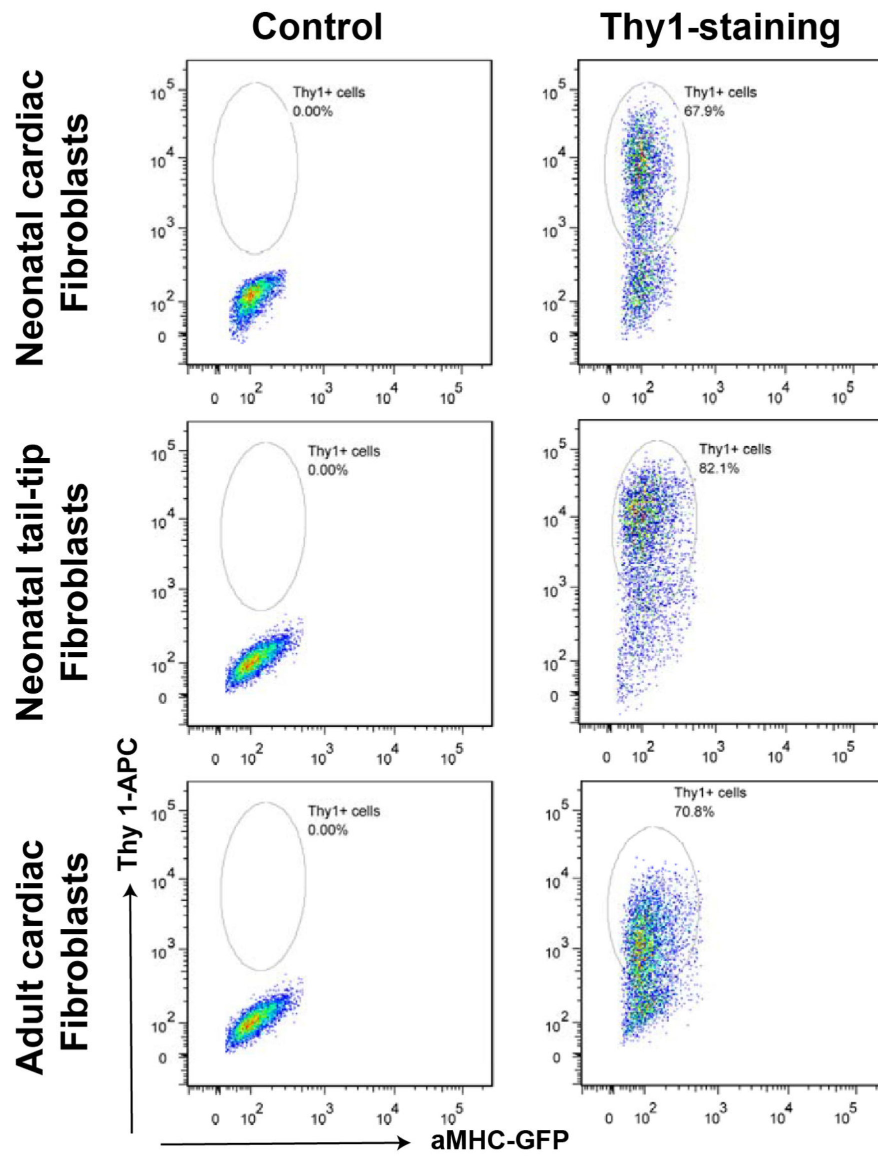

Figure 3.

Representative plots of FACS assays after Thy1-APC staining to purify fibroblasts.

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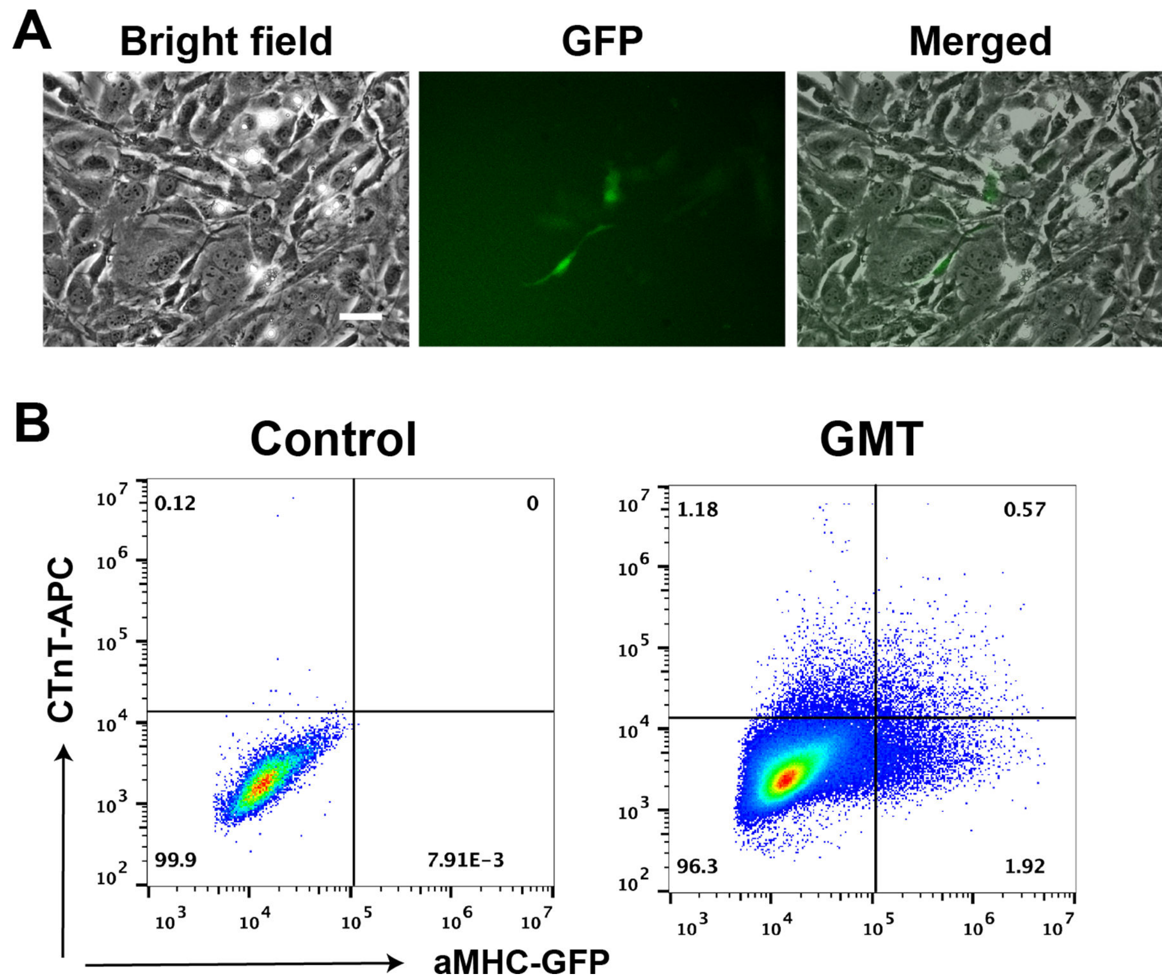

Figure 4.

A) Representative pictures of reprogrammed αMHC-GFP+ iCMs from neonatal cardiac fibroblasts three days after retrovirus infection of Gata4, Meft2c and Tbx5 (GMT). Bar indicates 50 μm. B) FACS assay of cardiac troponin T (cTnT) and αMHC-GFP staining of reprogrammed neonatal cardiac fibroblasts three days after GMT retrovirus infection.