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Generation of Quiescent Cardiac Fibroblasts Derived from Human Induced Pluripotent Stem Cells

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Abstract

Myocardial fibrosis is a hallmark of cardiac remodeling, which can progressively lead to heart failure, a leading cause of death worldwide. The effector cells of fibrosis in the heart are cardiac fibroblasts (CFs). There is currently no effective therapeutic strategy clinically available to specifically attenuate maladaptive responses of CFs. Large-scale applications such as high-throughput drug screening are difficult due to the limited availability of human primary CFs, thus limiting the development of future treatments. Here, we describe a robust induction protocol that can be used to generate a scalable, consistent, genetically defined source of quiescent CFs from human induced pluripotent stem cells for cardiac fibrosis modeling, drug discovery, and tissue engineering.

Key words Cardiac fibroblast, Fibrosis, Induced pluripotent stem cell

1 Introduction

Fibrotic diseases cause more than 800,000 deaths worldwide annually, of which the majority are cardiac and lung fibrosis [1]. The mechanisms underlying cardiac fibrosis remain poorly understood, and effective anti-fibrosis therapeutic strategies are not yet available. Accumulating evidence suggests that the regulatory networks governing fibrogenesis are tissue specific [2], and fibroblasts in the heart express cardiac-specific genes [3]. Due to the limited availability of primary human cells, as well as the limited translational value of animal models in recapitulating disease phenotype in humans, induced pluripotent stem cell (iPSC)-derived somatic cells have been increasingly used for disease modeling and drug discovery in a patient-specific and/or larger-scale manner [4]. Although research on generating cardiac fibroblasts from iPSCs has recently become available, specific instructions for how to accomplish this method have not yet been published [5]. Here, we described a step-by-step protocol for generating human iPSC-derived cardiac fibroblasts (iPSC-CFs) that closely resemble quiescent primary CFs at the transcriptional, cellular, and functional levels. We thereby offer a powerful in vitro platform to better

understand the mechanisms of cardiac fibrosis and to screen anti-fibrotic drugs at large scale.

2 Materials

1. Accutase (Sigma-Aldrich, cat # A6964)
2. Advanced DMEM/F12 medium (Life Technologies, cat # 12634-028)
3. B27 Supplement Minus Insulin (Life Technologies, cat # A1895601)
4. Essential 8 Medium (Life Technologies, cat # A1517001)
5. Fetal Bovine Serum (Gibco, cat #26140079)
6. Fibroblast Growth Medium 3 (PromoCell, cat # C23025)
7. GlutaMAX (Life Technologies, cat # 35050-061)
8. GSK3 inhibitor, CHIR99021 (Selleckchem, cat # S2924)
9. Matrigel Matrix (Corning, cat #356234)
10. Recombinant Human FGF2 Protein (R&D Systems, cat # 233-FB)
11. Retinoid acid (Sigma, cat # R2625)
12. ROCK inhibitor, Y27632 2 HCl (Selleckchem, cat # s1049)
13. RPMI 1640 medium (Life Technologies, cat # 11875-119)
14. TGFβ inhibitor, SB431542 (Tocris Bioscience, cat # 1614)
15. Wnt inhibitor, IWR-1-endo (Selleckchem, cat # S7086)

3 Methods

By comparing the single-cell transcriptome profiles from various organs (Fig. 1a), we previously identified distinct tissue-specific genes in cardiac fibroblasts, including early cardiac development-related genes (*GATA4*, *TBX20*, *MEF2C*, *HAND2*, and *GATA6*) and epicardial markers such as *TCF21* [5, 6] (Fig. 1b), indicating CFs at large are derived from cardiac and epicardial lineages. Based on that, we are able to show that human iPSC-CFs are successfully generated after the sequential differentiation of intermediate cardiac progenitor cells and epicardial cells (Fig. 1c–e).

3.1 Cardiac Progenitor Differentiation with GSK3 Inhibitor and Wnt Inhibitor

1. Culture human iPSCs on Matrigel-coated 6-well plates in E8 medium as previously reported [7].
2. On day 0, prepare RPMI/B27 minus insulin (RPMI/B27-insulin) medium containing 6 μM of CHIR99021 (CHIR, GSK3 inhibitor). Once the cell confluence reaches 80%–90%, aspirate spent medium and add 3 ml of the newly prepared

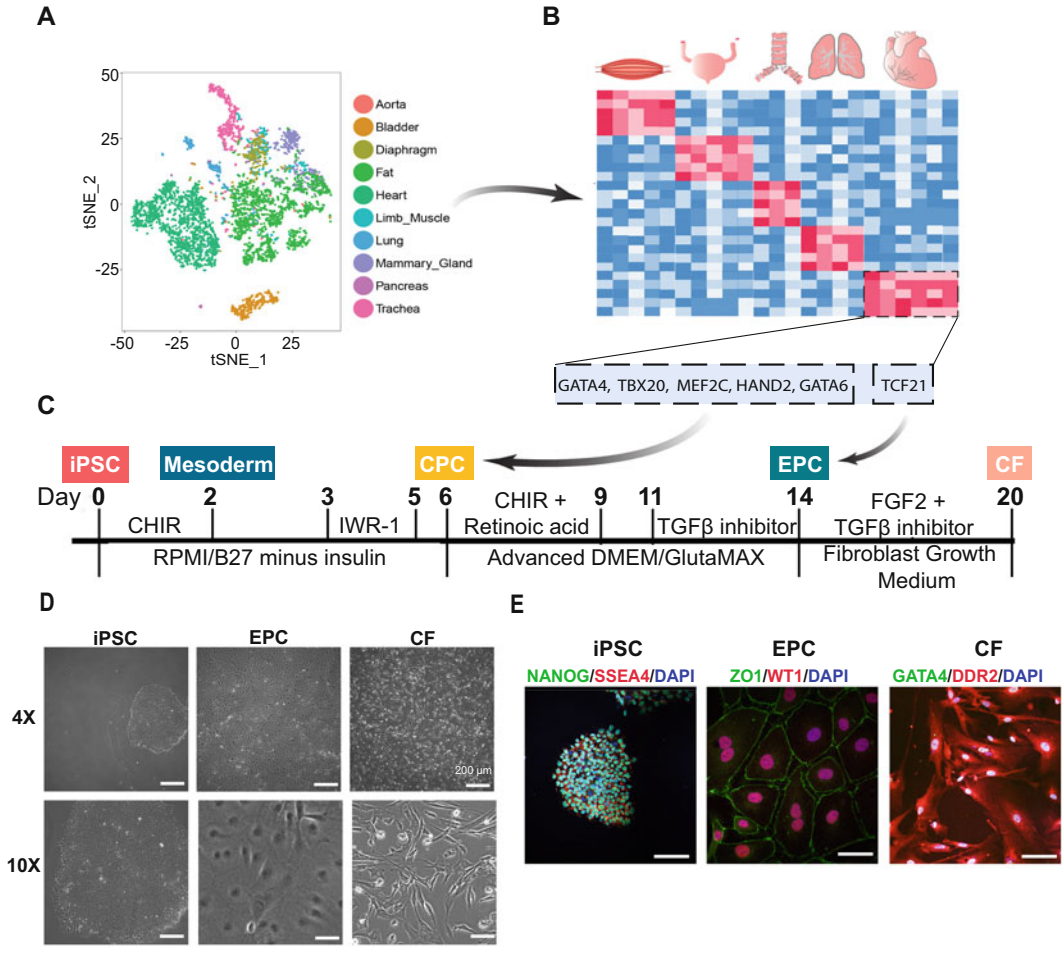

Fig. 1 (**a**) A t-SNE (t-distributed stochastic neighbor embedding) plot showing the distribution patterns of fibroblasts derived from 10 tissues of healthy adult mice. (**b**) A heatmap comparing the most specifically expressed genes in various fibroblast subpopulations, from which GATA4, TBX20, MEF2C, HAND2, GATA6, and TCF21 are highly expressed in cardiac fibroblast. (**c**) A schematic diagram showing the protocol for small molecule-directed differentiation to iPSC-CFs. (**d**) Representative bright-field images showing stage-specific cell morphology changes during the differentiation of human iPSC-CFs. Scale bars, 200 μm. (**e**) Representative immunofluorescence images showing stage-specific cells express pluripotency genes (NANOG and SSEA4), epicardial genes (ZO1 and WT1), and CF genes (GATA4 and DDR2) during differentiation. Scale bars, 100 μm

RPMI/B27-insulin + CHIR medium to each well of 6-well plates. Leave cells undisturbed for 2 days (*see* **Note 1**).

3. On day 2, aspirate the spent medium from each well of 6-well plates and add 3 ml of RPMI/B27-insulin medium.
4. On day 3, prepare RPMI/B27-insulin medium containing 5 μM of IWR-1 (Wnt inhibitor). Aspirate the spent medium

and then add 3 ml of the freshly prepared RPMI/B27-insulin + IWR-1 medium to each well of 6-well plates.

5. On day 5, aspirate the spent medium from each well of 6-well plates and add 3 ml of RPMI/B27-insulin medium (*see* **Note 2**).

3.2 Differentiation of Cardiac Progenitors into Proepicardial Cells

1. On day 6, prepare Advanced DMEM/GlutaMAX medium containing 5 μM of CHIR99021, 2 μM of retinoid acid, 5 μM of Y27632 (ROCK inhibitor), and 1% FBS.
2. Aspirate the spent medium, add 1 ml of Accutase per well in a 6-well plate, and incubate the plate in a 37 °C, 5% CO_2 incubator for 5 min. Gently pipette 5–10 times to singularize the cells, and then transfer the cell mixture to a 15-ml conical tube containing 1 ml of RPMI medium. Count the cell density with a hemocytometer, centrifuge the cells at 200 × *g* for 3 min at room temperature, and aspirate the supernatant.
3. Resuspend the cell pellet in medium prepared in subchapter 3.2, **step 1**, and then seed singularized cells onto a Matrigel-coated cell culture dish at a density of 20,000 per cm^2 (or a 1:12 split ratio). Incubate the plate at 37 °C, 5% CO_2 overnight to allow cell attachment.
4. On day 7, aspirate the spent medium and add 2 ml of Advanced DMEM/GlutaMAX medium containing 5 μM of CHIR99021 and 2 μM of retinoid acid per well.
5. On day 9, aspirate the spent medium and add 2 ml of Advanced DMEM/GlutaMAX medium per well.
6. On day 11, prepare Advanced DMEM/GlutaMAX medium containing 2 μM SB431542 (a TGFβ inhibitor).
7. Aspirate the spent medium, add 1 ml Accutase per well, and incubate the plate in a 37 °C, 5% CO_2 incubator for 5 min. Pipette 5–10 times to singularize the cells, and then transfer the cell mixture to a 15-ml conical tube containing 1 ml of Advanced DMEM/GlutaMAX medium. Centrifuge the cells at 200 × *g* for 3 min and aspirate the supernatant.
8. Resuspend the cell pellet in Advanced DMEM/GlutaMAX medium containing 2 μM SB431542 (prepared in subchapter 3.2, **step 6**), and seed cells onto a Matrigel-coated cell culture dish at a split ratio of 1:3 to 1:6. Incubate the plate at 37 °C, 5% CO_2 overnight to allow cell attachment.
9. On day 12 and every other day thereafter, change the medium with Advanced DMEM/GlutaMAX containing 2 μM of SB431542.
10. Once the cells are confluent, the proepicardial cells can be passaged for long-term maintenance using the same medium prepared in subchapter 3.2, **step 6**, frozen down, or further differentiated into CFs (*see* **Note 3**).

3.3 Differentiation of Quiescent Cardiac Fibroblasts from iPSC-Derived Epicardial Cells

1. On day 14, prepare fibroblast growth medium containing 20 ng/ml of FGF2 and 10 μM of SB431542.
2. Aspirate the spent medium, add 1 ml of Accutase per well in a 6-well plate, and incubate the plate in a 37 °C, 5% CO_2 incubator for 5 min. Pipette 5–10 times to singularize the cells, and then transfer the cell suspension to a 15-ml conical tube containing 1 ml of Advanced DMEM/GlutaMAX medium. Count the cell density with a hemocytometer, centrifuge the cells at 200 × *g* for 3 min at room temperature, and aspirate the supernatant.
3. Resuspend the epicardial cells and seed them onto a Matrigel-coated cell culture dish at a density of 10,000 cells per cm^2 (or a 1:3 split ratio) in the medium prepared in subchapter 3.3, **step 1**. Incubate the plate at 37 °C, 5% CO_2 overnight to allow cell attachment.
4. On day 16, change the spent medium with 2 ml freshly prepared fibroblast growth medium with 20 ng/ml of FGF2 and 10 μM of SB431542.
5. On day 18, resuspend the cells and seed them onto a Matrigel-coated cell culture dish at a density of 10,000 cells per cm^2 (or a 1:3 split ratio) in fibroblast growth medium containing 20 ng/ml of FGF2 and 10 μM of SB431542.
6. On day 20 and each other day thereafter, aspirate the medium from each well of the 6-well plate, and add 2 ml per well of fibroblast growth medium with 10 μM of SB431542. The quiescent CFs (Fig. 2) can be passaged, frozen down for long-term storage, or collected for characterization (*see* **Note 4**).

4 Notes

1. In general, culturing iPSCs in 6 μM of CHIR99021-containing medium for 48 h is sufficient to initiate cardiac progenitor cell differentiation. However, if massive cell death or detachment is observed on day 2, the concentration (4–8 μM) and duration (24–48 h) of CHIR99021 should be titrated for each iPSC line.
2. As both iPSC-derived cardiomyocytes and CFs are generated from the cardiac progenitor cells, differentiation steps during the first 6 days are identical for both cell types. If the cardiac progenitor cells are maintained in original plates on day 6, robust beating sheets of $cTnT^+$ cardiomyocytes will be observed, which suggests that cell density toggles between epicardial vs. cardiomyocyte differentiation.

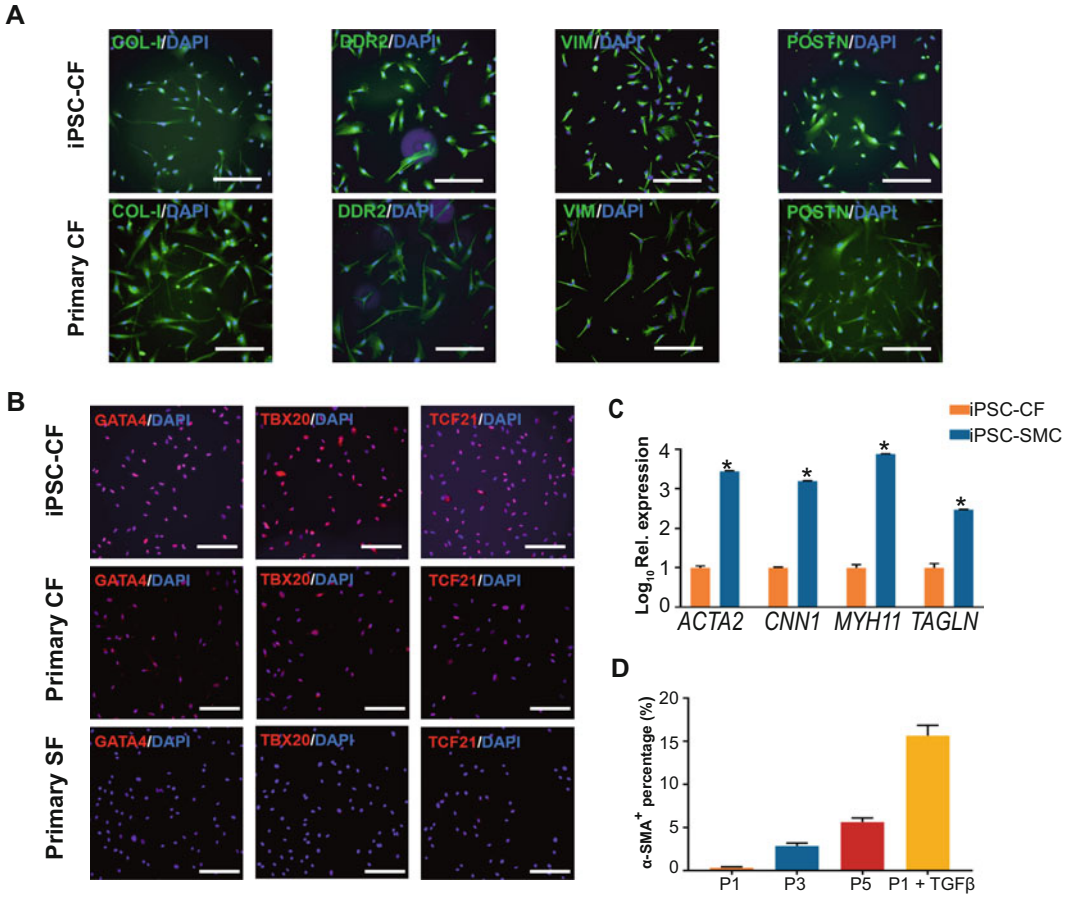

Fig. 2 (**a**) Representative immunofluorescence images showing human iPSC-cardiac fibroblast (CFs) are positive for gene makers highly expressed in primary CFs (COL-I, DDR2, VIM, and POSTN). Scale bars, 100 μm. (**b**) Representative immunofluorescence images showing the expression levels of cardiac-specific genes (GATA4, TBX20, and TCF21) in human iPSC-CFs, primary cardiac fibroblasts, and skin fibroblasts (SFs). Scale bars, 100 μm. (**c**) The expression levels of smooth muscle cell (SMC)-specific genes (ACTA2, CNN1, MYH11, and TAGLN) in human iPSC-CFs vs. iPSC-SMCs of the epicardial lineage. (**d**) The percentage of α-SMA$^+$ myofibroblasts in each indicated passage of iPSC-CFs is measured by flow cytometry

3. This protocol is developed based on an earlier protocol for iPSC-epicardial cell differentiation [8]. A low yield of CFs could be due to poor quality of epicardial cells. Typical epicardial cells should have a cobblestone-like morphology, and the purity of WT1$^+$ cells should be >90% on day 12.
4. Fibroblasts can undergo spontaneous transdifferentiation into myofibroblasts during in vitro culture conditions [9]. The majority of iPSC-derived CFs can preserve a quiescent state (~5% αSMA$^+$ cells) until passage 5 with the presence of TGFβ inhibitor SB431542 in fibroblast growth medium. The TGFβ inhibitor should be removed from the medium at least 1 day prior to transdifferentiation experiments.

Acknowledgments

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