

Direct reprogramming of mouse fibroblasts into cardiomyocytes with chemical cocktails

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The direct conversion, or transdifferentiation, of non-cardiac cells into cardiomyocytes by forced expression of transcription factors and microRNAs provides promising approaches for cardiac regeneration. However, genetic manipulations raise safety concerns and are thus not desirable in most clinical applications. The discovery of full chemically induced pluripotent stem cells suggest the possibility of replacing transcription factors with chemical cocktails. Here, we report the generation of automatically beating cardiomyocyte-like cells from mouse fibroblasts using only chemical cocktails. These chemical-induced cardiomyocyte-like cells (CiCMs) express cardiomyocyte-specific markers, exhibit sarcomeric organization, and possess typical cardiac calcium flux and electrophysiological features. Genetic lineage tracing confirms the fibroblast origin of these CiCMs. Further studies show the generation of CiCMs passes through a cardiac progenitor stage instead of a pluripotent stage. Bypassing the use of viral-derived factors, this proof of concept study lays a foundation for *in vivo* cardiac transdifferentiation with pharmacological agents and possibly safer treatment of heart failure.

Keywords: transdifferentiation; cardiac reprogramming; cardiomyocyte; fibroblast; chemical cocktail; small molecule compounds

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Introduction

Heart failure caused by the loss or dysfunction of cardiomyocytes affects over 15 million people worldwide and is the leading cause of mortality. Although the neonatal mouse heart can regenerate after injury, the adult heart has little or no regenerative capacity [1]. The functional recovery of the heart is further hindered by the formation of fibrotic tissue after injury. So current car-

diac therapies mainly focus on protecting the remaining cardiomyocytes and preventing fibrosis [1, 2].

Transplantation of cardiac stem/progenitor cells to improve cardiac function is theoretically plausible, but limited cell sources and possible rejection in allogeneic transplantation prevent large-scale clinical application [3]. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) both possess cardiogenic potential, but efficiency of cardiac differentiation, functional integration of converted cells, immune compatibility, and risk of tumor formation are among the challenges to be solved [4].

Recent advances in transdifferentiation technology, i.e., reprogramming of one somatic cell type into another cell type without passing through the pluripotent state offer new approaches to generate functional cardiomyocytes [5]. It has been reported that direct reprogramming of

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non-muscle cells into cardiomyocytes can be achieved *in vitro* and *in vivo* by forced expression of cardiac transcription factors, including Gata4, Mef2c, Tbx5, Hand2, etc [6-8]. A combination of microRNAs, including miR-1, -133, -208, and -499 with known roles in regulating broad aspects of cardiac gene expression, was also found effective in inducing cardiomyocytes from fibroblasts [9]. Interestingly, researchers also found conventional reprogramming toward pluripotency by overexpression of Yamanaka factors can be diverted toward cardiogenesis in the presence of a JAK inhibitor and cardiomyocyte-favorable culture condition [10]. The same group recently reported that small molecules can enable cardiac transdifferentiation of mouse fibroblasts with only one transcription factor Oct4 without passing through the pluripotency stage [11].

Although effective in inducing cardiac transdifferentiation, viral vector-carried transcription factors are still not favorable in therapeutic application. The use of small-molecule compounds in reprogramming and transdifferentiation, which might be more amendable in clinical development, has recently been highlighted by the discovery of full chemically induced pluripotent stem cells (CiPSCs) and neural progenitor cells [12-14]. Here, we report the generation and characterization of spontaneously beating cardiomyocyte-like cells from fibroblasts with only chemical cocktails.

Results

Induction of cardiomyocyte-like beating cells from fibroblasts by chemical cocktail

To avoid genetic insertion and carcinogenicity of the iPSCs generated by viral-based methods, Hou *et al.* developed a full chemical induction of CiPSCs from mouse embryonic fibroblasts (MEFs) with a small-molecule combination CRFVPTZ (C, CHIR99021; R, RepSox; F, Forskolin; V, VPA; P, Parnate; T, TTNPB; and Z, DZnep) [12]. In an attempt to repeat the generation of CiPSCs, we unexpectedly observed spontaneously contracting cells or cell clusters resembling cardiomyocytes (Supplementary information, Figure S1A and Movie S1). The beating cells or clusters could be found as early as day 6-8 after the addition of chemical cocktail CRFVPT, which is much earlier than the reported earliest date of the appearance of CiPSCs (day 20). But in this CiPSC induction condition, the beating cells or clusters were scarce, and most of them could no longer be observed after approximately one week.

A two-stage optimization strategy was carried out to improve the induction efficiency and stabilize the cardiomyocyte-like spontaneously contracting cells (Fig-

ure 1A). We first optimized the cardiac reprogramming medium (CRM) used in the first stage of the induction with CRFVPT. We found bFGF, which is essential in CiPSC induction, was dispensable for the generation of contracting cells. In fact, bFGF even reduced vitamin C-stimulated cardiac transdifferentiation (Supplementary information, Figure S1B). The combination of 15% fetal bovine serum (FBS) and 5% knockout serum replacement (KSR) was found to be more effective in inducing beating cells than the original 10% FBS and 10% KSR combination used in CiPSC induction. The addition of N2 and B27 further increased the generation of beating clusters (Supplementary information, Figure S1C). The matrix microstructures have also been reported to play a role in cardiac reprogramming [15]. We tested different extracellular matrix gels and found that BD Matrigel-coated dishes were better than gelatin-coated or uncoated dishes (Supplementary information, Figure S1D). More beating colonies instead of single beating cells could be found after these optimizations. We then optimized the cardiomyocyte-maintaining medium (CMM) used in the second stage (after day 16) of the induction. Exposure to the cocktail CRFVPT beyond day 16 did not further improve the efficiency (Supplementary information, Figure S1E and S1F); we thus removed CRFVPT from the CMM. In contrast, 2i (CHIR99021 and PD0325901), LIF, and insulin, which have been reported to benefit the maintenance of cardiomyocytes *in vitro* [16-18], were found to be very effective in increasing the number of beating clusters (Supplementary information, Figure S1F). BMP4, which has been reported to be beneficial for cardiomyocyte induction [19], did not further enhance the effect of 2i and LIF (Supplementary information, Figure S1F).

The final optimized protocol is described in Materials and Methods section and summarized in Figure 1A. Chemically induced cardiomyocyte-like cells (CiCMs) from MEFs displayed various morphologies: cell patches and single cells with spindle shape, rod shape or round shape (Figure 1B and Supplementary information, Movie S2). A time-dependent increase in the number of beating clusters could be observed (Figure 1C). We next determined the small molecule in the CRFVPT cocktail critical in inducing CiCMs by removing only one compound from the CRFVPT set. Combinations lacking RepSox failed to induce any beating clusters, while removing CHIR99021, or Forskolin, or VPA significantly reduced the number of beating clusters, whereas subtracting Parnate or TTNPB only slightly reduced the beating clusters (Figure 1D). These results suggested that C, R, F and V were most critical for the induction of beating clusters and indeed, the CRFV cocktail was sufficient to generate

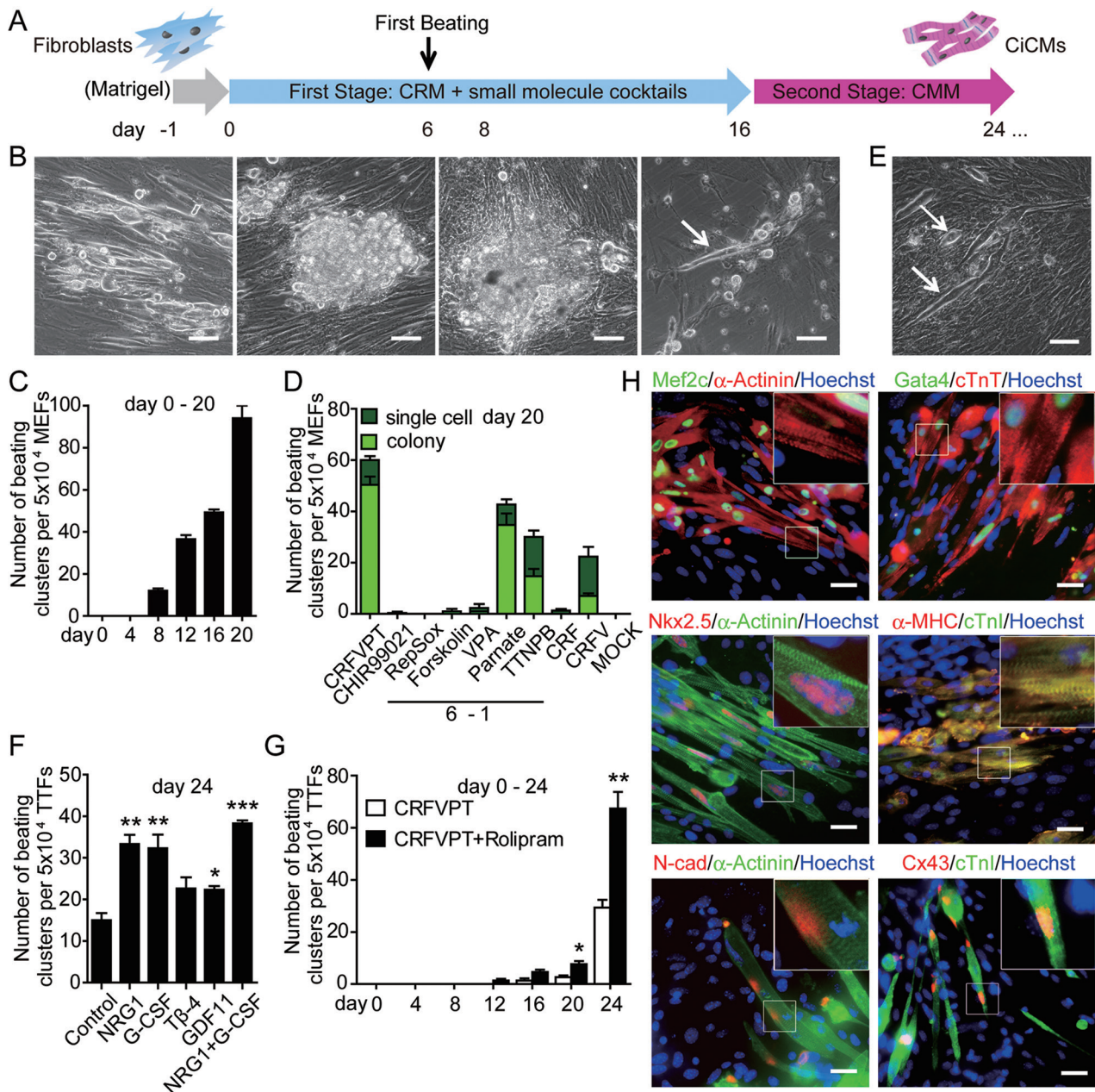


Figure 1 Fibroblasts can be directly reprogrammed to spontaneously contracting patches with chemical cocktails. **(A)** The scheme of direct cardiac reprogramming with small molecule cocktails. Fibroblasts were plated in fibroblast growth medium for one day and then the medium was changed into cardiac reprogramming medium (CRM) containing the small molecule cocktails (first stage). At day 16, the medium was changed into cardiomyocyte maintaining medium (CMM, second stage). The first beating clusters could be observed on day 6-8. **(B)** Representative morphologies of various MEF-derived beating clusters induced by the small molecule cocktail CRFVPT. See also Supplementary information, Movie S2. **(C)** Number of the beating clusters induced from MEFs with CRFVPT at various time points. Results are presented as means \pm SEM, $n = 3$. **(D)** Screening for compounds essential for cardiomyocyte induction. Numbers of beating clusters at day 20 are shown. **(E)** Morphology of TTF-derived beating cells by small molecule cocktail CRFVPT at day 14. See also Supplementary information, Movie S3. **(F)** Induction of TTF-derived beating cells with CMM at the second stage supplemented with various growth factors (NRG1, 100 ng/ml; G-CSF, 20 ng/ml; T β -4, 100 ng/ml; GDF11, 100 ng/ml). **(G)** Induction of TTF-derived beating cells with CRFVPT plus Rolipram (3 μ M) in the first stage, and the growth factors (100 ng/ml NRG-1 and 20 ng/ml G-CSF) in the second stage. Data are means \pm SEM, $n = 3$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **(H)** Immunostaining of cardiac markers Mef2c, Gata4, Nkx2.5, α -MHC, α -actinin, cTnT, cTnI, N-cad, and Cx43 in beating clusters generated from MEFs on day 24. Nuclei were stained with Hoechst. Scale bars represent 50 μ m in **B** and **E**, 20 μ m in **H**.

beating clusters (Figure 1D). A few beating cells could also be found using the CRF cocktail (Figure 1D), although the efficiency was very low. We then used CRFV as the basal induction system and screened a dozen chemicals, including modulators of pathways affecting cardiac development or somatic cell reprogramming, to see whether the generation of CiCMs could be enhanced. Several chemicals, including ICARIIN, PD169316 and Rolipram were found to be effective (Supplementary information, Figure S1G). Rolipram, a phosphodiesterase (PDE) 4 inhibitor, had the best effect, so a few more PDE inhibitors were tested. Another PDE 4 inhibitor, Cilomilast, was also highly effective, while the PDE 1, 2 and 3 inhibitors were less effective, and two PDE 5 inhibitors were ineffective (Supplementary information, Figure S1H).

We next used neonatal mouse tail-tip fibroblasts (TTFs) as the starting cells to induce cardiac transdifferentiation. After a two-week treatment with the CRFVPT cocktail, beating cells were also found. Most TTF-derived CiCMs displayed single-spindle shape morphology (Figure 1E and Supplementary information, Movie S3), and the reprogramming efficiency from TTFs to CiCMs was lower than from MEFs (compare Figure 1F with 1C). A number of growth factors, including neuregulin1 (NRG1), G-CSF, thymosin β 4 (T β -4), and GDF11, have been reported to support the culture and function of cardiomyocytes [20-23]. We thus added these growth factors to the CMM in the second stage of the induction to test if they could facilitate CiCM generation from TTF. Indeed, addition of NRG1 or G-CSF, or both, significantly increased the number of beating cells. However, the recently discovered anti-aging factor GDF11 [23] only had marginal effect (Figure 1F). We also tested the addition of Rolipram to the CRFVPT cocktail in the first stage of the induction, and found it significantly enhanced the reprogramming efficiency of TTFs (Figure 1G).

CiCMs express cardiomyocyte-specific markers and exhibit typical cardiac electrophysiological features

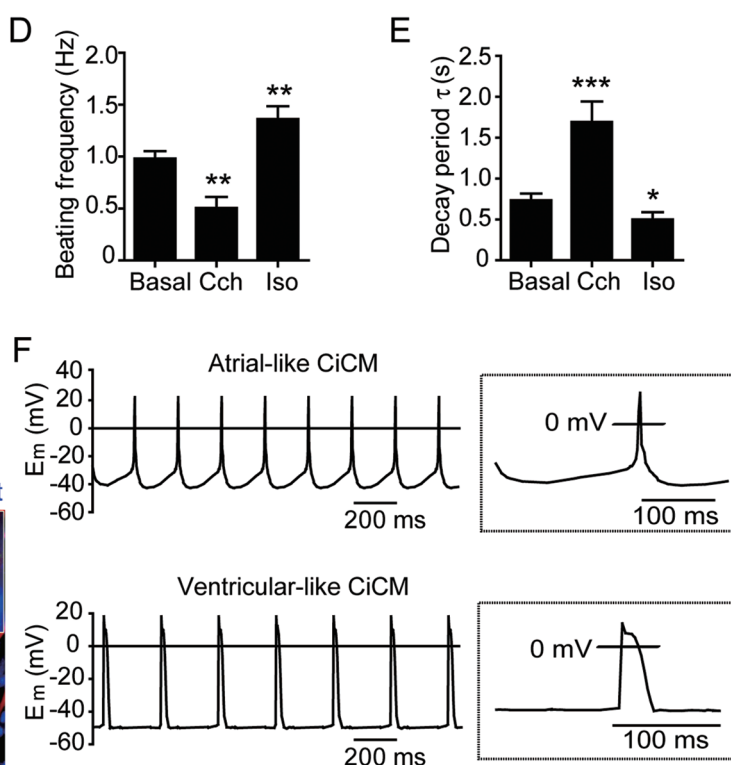
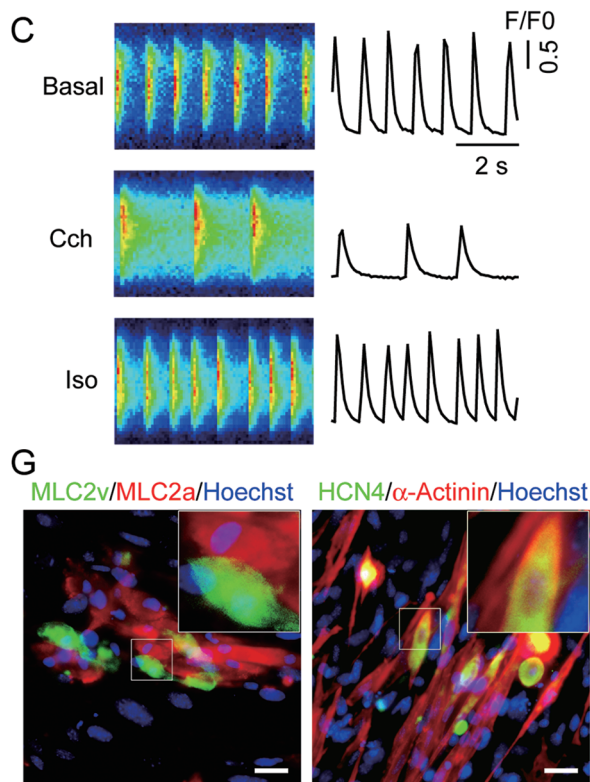
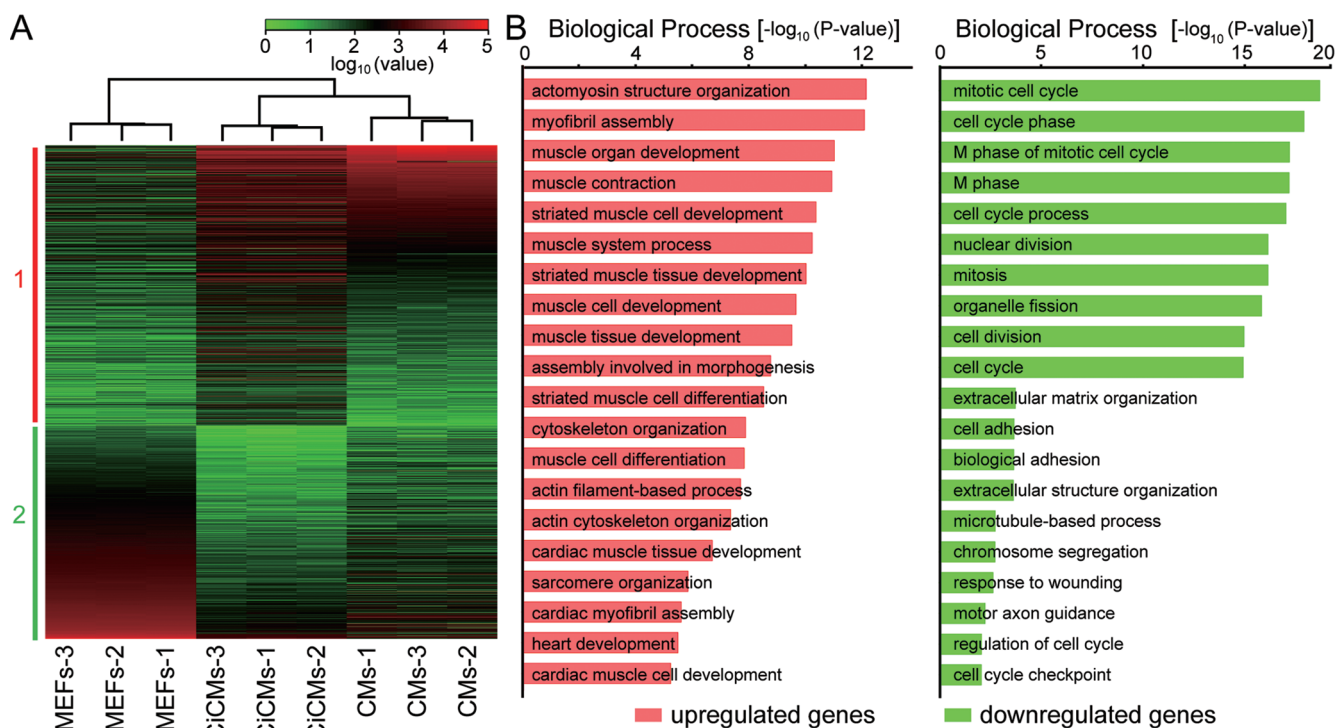
Our characterization focused on MEF- and TTF-derived CiCMs (MEF-CiCMs and TTF-CiCMs) generated with CRFVPT. Immunostaining demonstrated that these MEF-CiCMs (day 24) not only were positive for cardiomyocyte markers, including α -actinin, cardiac troponin-T (cTnT), cardiac troponin-I (cTnI) and α -MHC (Myh6), but also displayed a clear cross-striated pattern (Figure 1H). FACS analysis revealed that approximately 14.5% of cells were α -actinin positive and 9% of cells were α -MHC positive on day 24 (Supplementary information, Figure S2A). Transcription factors important for cardiac development and function, including *Mef2c*, *Gata4*,

and *Nkx2.5*, were also highly expressed in these MEF-CiCMs (Figure 1H). Connexin 43 (Cx43), a component of gap junctions, and N-cadherin (N-cad), an anchor for myofibrils at cell-cell contacts, were also expressed (Figure 1H). In contrast, markers for skeletal myotube development, such as *MyoD* and myogenin, were not found in these MEF-CiCMs (Supplementary information, Figure S2B). The TTF-CiCMs also expressed cardiac-specific markers including *Mef2c*, cTnT, *Gata4*, α -MHC and α -actinin (Supplementary information, Figure S3A).

Quantitative RT-PCR also confirmed the time-dependent increase in the expression of cardiac-specific genes, including *Nkx2.5*, *Mef2c*, *Gata4*, β -MHC, *cTnT*, and *Ryr2*, during the induction (Supplementary information, Figure S2C). The global gene expression patterns of MEFs, MEF-CiCMs, and cardiomyocytes were analyzed with microarrays. Compared with MEFs, 477 genes were upregulated and 276 were downregulated for more than 5 folds in MEF-CiCMs. CiCMs and cardiomyocytes showed very similar expression patterns of these genes and were thus clustered into one group (Figure 2A). Gene ontology (GO) term enrichment analysis of these genes indicated that genes involved in muscle development, myofibril assembly, muscle contraction, and especially cardiac muscle development were significantly upregulated in CiCM samples, while genes involved in cell cycle and mitosis control were significantly downregulated (Figure 2B), indicating a clear transition from dividing MEFs to differentiated cardiomyocyte-like state.

We then used Fluo-4 as an indicator to trace the calcium transients, which underlie the contraction and relaxation of spontaneously beating cardiomyocytes, in these CiCMs. In MEF-CiCMs (Figure 2C and Supplementary information, Movie S4), the peak fluorescence ratio (F/F_0) in these transients was 3.2 ± 0.4 ($n = 11$), comparable to previous measurements made with neonatal or ESC-derived cardiomyocytes [24]. Normal cardiomyocytes can respond to adrenergic and muscarinic signaling and adjust their calcium transient and contractile frequency [25]. Similar phenomenon could be observed in these CiCMs. Addition of the β -adrenergic agonist isoproterenol (Iso, 1 μ M) significantly increased the frequency of the spontaneous calcium transients and shortened the decay period, while treatment of carbachol (Cch, 5 μ M), a muscarinic agonist, significantly reduced the calcium transient frequency and prolonged the decay time (Figure 2C-2E). The TTF-CiCMs also displayed similar spontaneous calcium transients (Supplementary information, Figure S3B).

To further characterize the CiCMs and rule out the possibility that they were myoblasts or smooth muscles, which could also have spontaneous calcium transients



H

Cell Type	dv/dtMax (V/s)	OSP (mV)	MDP (mV)	APA (mV)	Freq (Hz)	APD50 (ms)	APD90 (ms)
A-like (n = 13)	33.7±3.6	22.5±1.9	-49.6±1.4	72.1±1.0	4.1±0.1	33.1±5.3	35.2±5.4
V-like (n = 4)	41.5±5.5	14.1±1.3	-63.1±4.4	77.2±4.1	3.8±0.3	35.9±3.8	49.8±8.3

Figure 2 CiCMs exhibit typical cardiac calcium flux, electrophysiological features, and gene expression profile. **(A)** Heatmap illustration of microarray data from MEFs, MEF-CiCMs (beating clusters picked at day 24) and cardiomyocytes. Groups 1 and 2 contain genes that are upregulated or downregulated for > 5 fold in CiCMs compared with MEFs. **(B)** GO term enrichment analysis of genes that display > 5 fold change in expression in CiCMs and cardiomyocytes compared with MEFs. Left, upregulated genes; right, downregulated genes. **(C)** Calcium flux in MEF-derived CiCMs at day 25 of the induction. Calcium transients were recorded at basal condition, or after 5 μ M carbachol (Cch) or 1 μ M isoproterenol (Iso) treatment. Left, linescan images of calcium transients; right, traces of calcium transients. See also Supplementary information, Movie S4. **(D)** Calcium transient frequency and **(E)** decay rate at basal state ($n = 18$), and after treatment of Cch ($n = 8$) or Iso ($n = 13$). Data are presented as means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **(F)** Representative action potentials (AP) of CiCMs induced with CRFVPT cocktail. E_m , membrane potential in millivolts. **(G)** Immunostaining of MLC2a, MLC2v, α -actinin and HCN4 in MEF-derived CiCMs. Scale bars represent 20 μ m. **(H)** AP parameters of CiCMs including maximum upstroke velocity (dv/dtMax), overshoot potential (OSP), minimum diastolic potential (MDP), AP amplitude (APA), beating frequency (Freq), AP durations (APDs) at the level of 50% (APD50) and 90% repolarization (APD90). Data are means \pm SEM.

and contractility [26], we analyzed the action potentials (APs) of these CiCMs. In single-cell patch clamp assay, APs were recorded from single spontaneously beating cells around day 20 of reprogramming. Most of the MEF-CiCMs ($n = 13$) generated APs that closely resembled atrial-like APs [10, 27], and to a lesser extent earlier pacemaker-like APs [28], with a mean diastolic potential (MDP) of -49.6 mV and a mean overshoot potential (OSP) of 22.5 mV. A small portion of the MEF-CiCMs ($n = 4$) showed ventricular-like AP morphology with a MDP of -63.1 mV and a mean OSP of 14.1 mV (Figure 2F and 2H). Consistent with the electrophysiological study, immunostaining of the MEF-CiCMs confirmed the expression of myosin light chain-2a (MLC2a), an atrial specific marker; MLC2v, a ventricular specific marker, and HCN4, a pacemaker marker (Figure 2G). Both the atrial-like and ventricular-like APs could also be found in TTF-CiCMs (Supplementary information, Figure S3C).

Lineage tracing of chemical-induced transdifferentiation of MEFs toward cardiomyocytes

To ultimately confirm that the CiCMs were indeed transdifferentiated from fibroblasts but not the possible contaminating progenitor cells, we used a lineage-tracing experiment to track the origin of the CiCMs. Transgenic mice that express Cre recombinase under the control of the *fibroblast specific protein-1* (*Fsp1* or *S100A4*) promoter were crossed with the R26R^{tdTomato} mice, in which the expression of tdTomato is prevented by a *loxP*-flanked STOP cassette. Since *Fsp1* is specifically expressed in fibroblasts [29], the progeny of these mice (*Fsp1*-Cre:R26R^{tdTomato}) would have the red fluorescent protein tdTomato expressed specifically in the fibroblasts (Figure 3A).

The *Fsp1*-Cre:R26R^{tdTomato} MEFs, which indeed express tdTomato, were induced for cardiac transdifferentiation with CRFVPT. Spontaneously contracting CiCMs with the expression of tdTomato could be observed in

the culture (Supplementary information, Movie S5). Immunofluorescent analysis on day 24 confirmed that these tdTomato-CiCMs were positive for cardiomyocyte markers, including *Mef2c*, *Nkx2.5*, α -actinin, cTnT, cTnI, and α -MHC, and these cells also displayed a clear cross-striated pattern (Figure 3B). Spontaneous calcium oscillations could also be observed in tdTomato-CiCMs on day 25 (Figure 3C). Single-cell patch clamp assay recorded atrial-like or ventricular-like APs from these tdTomato-CiCMs (Figure 3D-3F). These data clearly demonstrate that the CiCMs generated with chemical cocktail are indeed transdifferentiated from fibroblasts.

CiCMs are generated through a cardiac precursor-like stage but not the iPSC stage

We were intrigued by an observation that many colonies or cell patches formed before the initiation of beating, and examined if the generation of CiCMs went through an iPSC or precursor stage as previously reported in transcription factor-mediated cardiac reprogramming [10]. Quantitative RT-PCR analysis revealed that the expression of pluripotency genes such as *Oct4*, *Sox2*, *Nanog*, and *Rex1* remained at a very low level during the CRFVPT-mediated cardiac reprogramming (Supplementary information, Figure S2D). In contrast, cardiac markers were gradually upregulated (Supplementary information, Figure S2C). We also performed time-lapse imaging of OG2 MEFs (MEFs containing an *Oct4*:GFP reporter) undergoing CRFVPT-induced cardiac reprogramming (Figure 4A). Beating clusters began to appear at day 12 in this experiment, and immunocytochemical staining revealed that these beating clusters expressed α -MHC at day 26 (Figure 4A). However, GFP⁺ cells were never detected throughout the entire reprogramming process. This is in clear contrast to Yamanaka factor (*Oct4*, *Klf4*, *Sox2*, and *cMyc*)-mediated reprogramming, in which GFP⁺ iPSC colonies can be observed at day 12 (Figure 4A). These data indicate that chemical-mediated cardiac

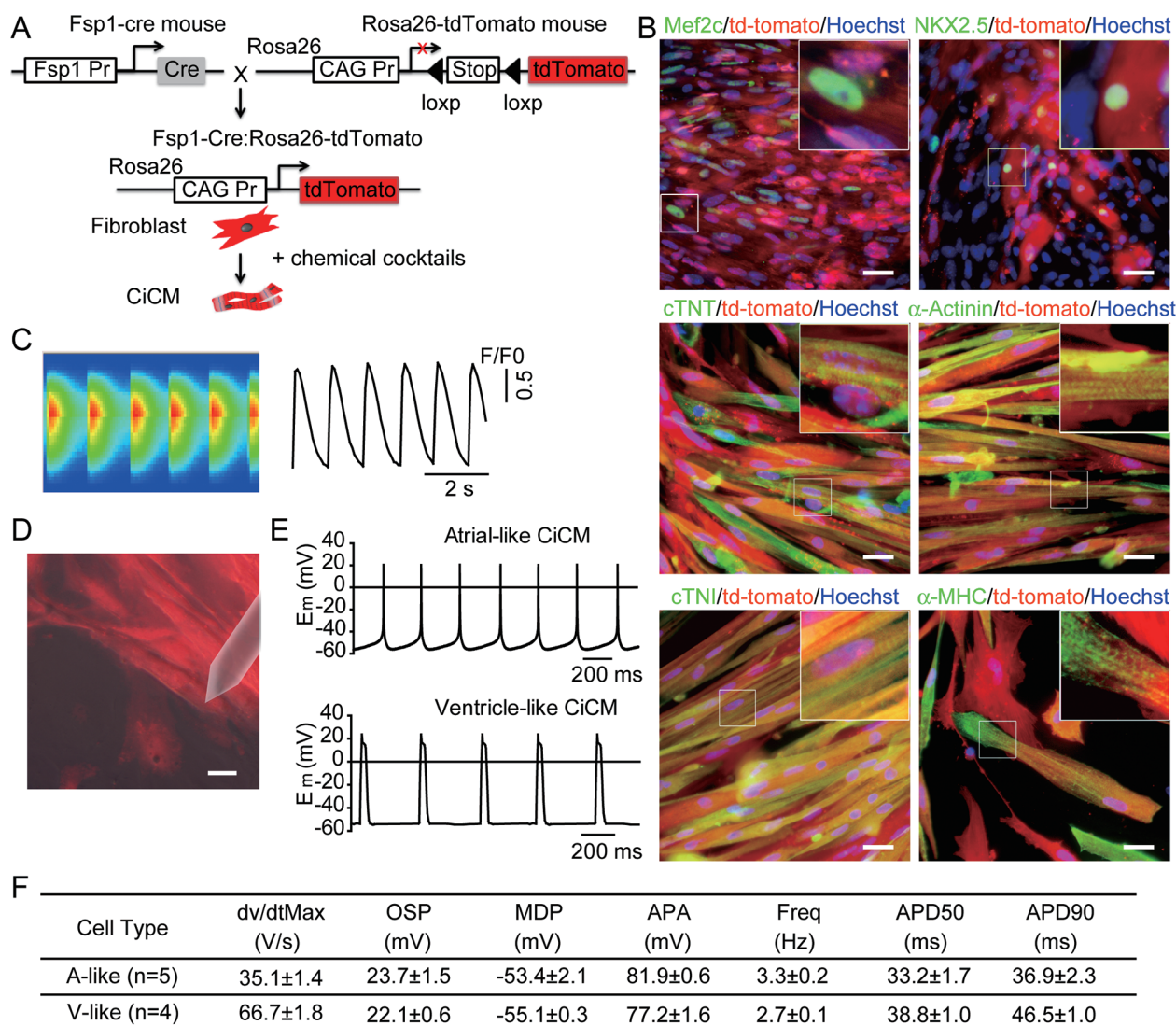


Figure 3 Lineage tracing of chemical induced transdifferentiation of MEFs toward cardiomyocytes. **(A)** Schematic diagram of the genetic fate mapping method used to trace the origin of CiCMs reprogrammed from *Fsp1-Cre:R26R^{tdTomato}* MEFs. **(B)** Co-localization of tdTomato (red) with various cardiac markers, including *Mef2c*, *NKX2.5*, α -actinin, *cTnT*, *cTnI* and α -MHC in beating clusters generated from *Fsp1-Cre:R26R^{tdTomato}* MEFs on day 24 with chemical cocktail CRFVPT. Nuclei are stained with Hoechst. **(C)** Spontaneous calcium transients in the tdTomato-CiCMs at day 25 of induction. Left, linescan images; right, traces of calcium signals. **(D)** A representative tdTomato-CiCM used for electrophysiological characterization. **(E)** Representative action potentials (AP) of tdTomato-CiCM. **(F)** AP parameters of tdTomato-CiCMs. Data are means \pm SEM. Scale bars in **B** and **D** represent 20 μ m.

reprogramming does not transverse an iPSC state.

A cardiac precursor stage is necessary for normal cardiomyocyte development [30]. Previous report also described a cardiac precursor stage in transcription factor-mediated generation of cardiomyocyte-like cells [10]. We also found that the expression of several cardiac precursor markers, including *Sca-1*, *Abcg2*, *Wt1*, *Flk1*, and *Mesp1* [31, 32], was upregulated during the early phase

(typically day 8-20) of chemical-induced cardiac reprogramming, and was gradually downregulated at late stage (after day 24; Figure 4B). *Sca-1⁺* cardiac precursor-like cells could be readily detected in the CRFVPT-induced cardiac reprogramming system at day 20 (Figure 4C). As the cardiac precursors are able to differentiate into smooth muscle cells and endothelial cells during embryonic development [33], we continued to culture the *Sca-*

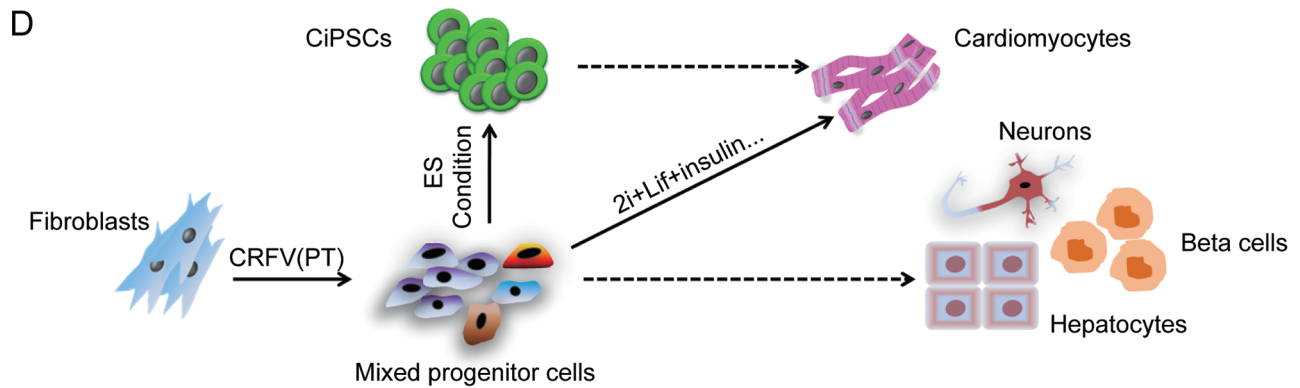
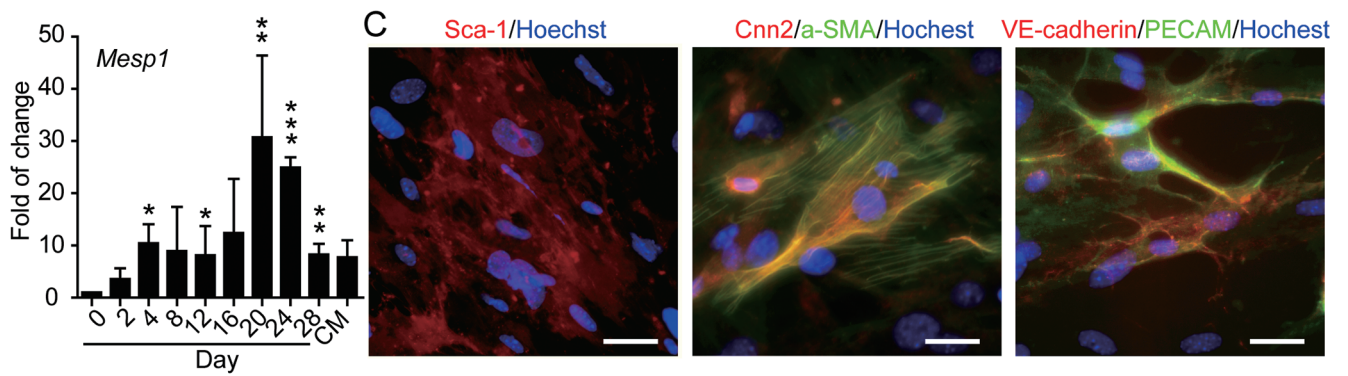
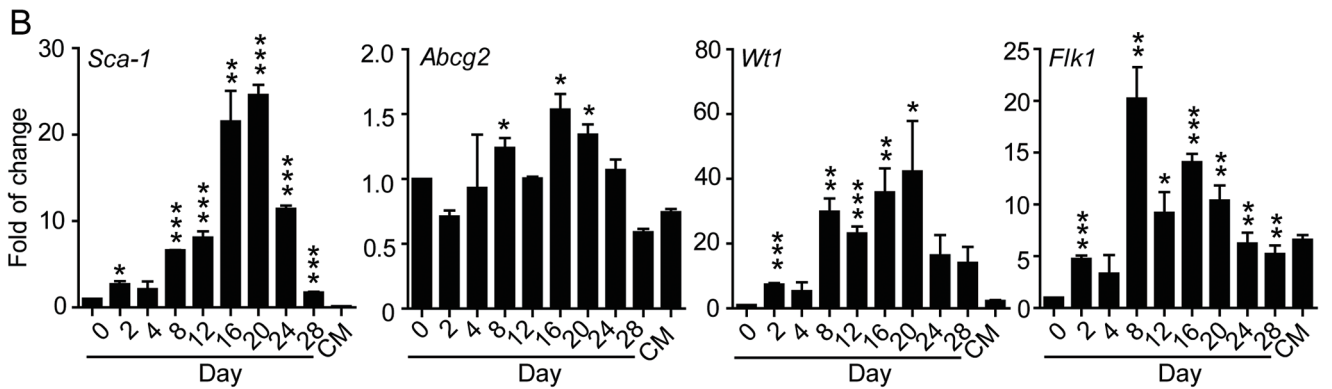
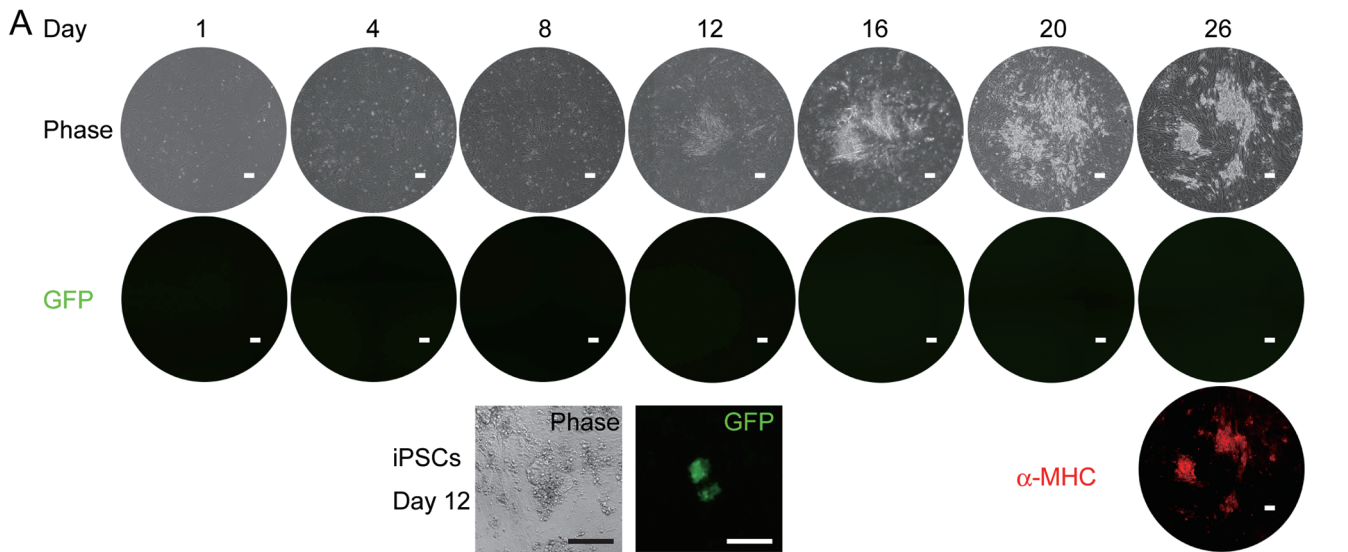


Figure 4 CiCMs were generated through cardiac precursor stage but not via iPSCs. **(A)** Time-lapse images of CiCMs generated from OG2 MEFs carrying an Oct4::GFP reporter (upper panels, phase and GFP). α -MHC (Red) was stained at day 26. Lower panel show iPSCs cells (GFP⁺) at day 12 after induction with Yamanaka factors (Oct4, Sox2, cMyc, and Klf4). **(B)** Expression of cardiac precursor markers: *Sca-1*, *Abcg2*, *Wt1*, *Flk1*, and *Mesp1* by quantitative RT-PCR during CiCMs induction. Data are means \pm SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus MEFs (day 0). **(C)** Sca-1⁺ cells at day 20 of induction (left panel). Continuous culture of Sca-1⁺ cells in either smooth muscle cell differentiation medium or endothelial cell differentiation medium for another two weeks produced α -SMA and Cnn2 positive cells (middle panel), or PECAM and VE-cadherin positive cells (right panel), respectively. Scale bars represent 200 μ m in **A** and 20 μ m in **C**. **(D)** A model for direct reprogramming of MEFs into cardiomyocytes with chemicals. Chemical combination might induce the generation of a mixed progenitor (or unstable intermediate) population. With favorable culture conditions, these progenitor/intermediate cells might be induced to become pluripotent or develop into various functional cells.

1⁺ cardiac precursor-like cells in differentiation conditions that favor the generation and maintenance of either endothelial or smooth muscle cells. As demonstrated in Figure 4C, the α -SMA/Cnn2⁺ and the PECAM/VE-cadherin⁺ cells could be observed in culture conditions favoring smooth muscle cells and endothelial cells, respectively. These data show that the generation of CiCMs goes through a cardiac precursor-like stage, somewhat resembling the natural development of cardiomyocytes.

Discussion

Here, we present the first evidence of chemical-mediated direct conversion of fibroblasts into the cardiomyocyte lineage, and the resulting spontaneously contracting cells are named CiCMs. Previous investigations have demonstrated that cardiomyocyte-like cells can be induced from non-cardiac cells by ectopic expression of transcription factors and microRNAs [6-9], and small molecules can facilitate single genetic factor-mediated cardiac transdifferentiation [11], the unavoidable genetic manipulations are not desirable in clinical applications. Compared with these methods, our full chemical approach is more amendable in clinical use and may induce functional cardiac transdifferentiation *in vivo* and avoid potential complications, including tumor formation, tissue rejection and homing ability of injected cells, in cell transplantation therapy for cardiac diseases [5].

It is interesting to note that the generation of functional CiCMs passes through a cardiac precursor-like stage, and both atrial-like and ventricular-like cells can be found in the culture. This indicates with further manipulation of the chemical cocktails and culture conditions, obtaining specific cell types within a particular lineage could be achieved. The existence of a cardiac precursor-like stage can also be capitalized for *in vivo* application. Cardiac progenitors may be more robust in surviving in the hostile graft environment, and they may not only restore myocardial tissue, but also contribute to revascularization of the heart [34].

The precise mechanisms underlying the chemical induction of cardiac transdifferentiation remain to be elucidated. Notably, the initial beating cells were observed unexpectedly in an attempt to repeat the generation of CiPSCs [12], although the appearance of CiCMs were much earlier (one week after induction) than the reported earliest date of CiPSC appearance (day 20). We do not fully understand why the same chemical combination CRFVPTZ could lead to both CiPSCs and CiCMs, but the later culture condition seem to play a critical role. It has been reported that the pluripotent Yamanaka factors could also be used to generate cardiomyocytes in a cardiomyocyte-favorable culture condition [10]. Similarly, Yamanaka factors have also been used to generate hepatocytes and pancreatic cells from fibroblasts [5]. Hou *et al.* also found that Gata4, a transcription factor used for cardiac transdifferentiation [6], was upregulated at day 12 after the treatment with chemicals [12], and their later culture conditions may favor pluripotency induction. Various morphological changes could be observed at the early stage of chemical induction (data not shown), and we thus propose that the chemical combination might induce the generation of a mixed progenitor (or unstable intermediate) population. When favorable culture conditions are provided, these progenitor/intermediate cells could be induced to become pluripotent or differentiate into various functional cells (Figure 4D).

Our optimized condition for CiCM induction is significantly different from the CiPSC condition. DZnep (Z), which is one of the four core chemicals (CRFZ) in CiPSC induction and crucial for the expression of *Oct4* [12], was dispensable for CiCM generation. bFGF, which is critical in inducing CiPSC [12], did not facilitate CiCM induction. Our study demonstrates that the four core chemicals for CiCM induction are CRFV, with RepSox (R) being the most important, since removing R failed to generate any beating cell. R is a potent and selective inhibitor of TGF β R-1, and CHIR99021 (C) is a GSK3 inhibitor that mimics the activation of Wnt pathway. Both Wnt signaling activation and TGF β inhibition have

been reported to play critical roles in promoting reprogramming, inducing cardiomyocyte differentiation and preventing myocardial fibrosis [35, 36]. Forskolin (F) promotes the generation of intracellular cAMP and may facilitate gene expression via a CREB-dependent mechanism. The increased cAMP may also lead to PKA-mediated phosphorylation of connexins and promote electrical cell-to-cell coupling in cardiac cells [37]. Rolipram and Cilomilast, both PDE 4 inhibitors, can enhance the generation of CiCMs. PDEs are enzymes responsible for the breakdown of the second messengers with different substrate specificities: some are cAMP-selective (PDE 4, 7, and 8); some are cGMP-selective (PDE 5, 6, and 9), and others can hydrolyze both cAMP and cGMP (PDE 1, 2, 3, 10, and 11) [38]. Our results suggest that the inhibitors of cAMP-selective PDEs may be more effective than the inhibitors of cGMP-selective PDEs (although more compounds need to be tested), indicating the importance of the intracellular cAMP level in cardiac reprogramming. VPA (V) as a HADC inhibitor may accelerate the global gene activation during transdifferentiation.

Besides chemicals, several growth factors that favor the survival or function of cardiomyocytes, including NRG1, G-CSF, GDF11, and T β -4 [20-23], can also enhance CiCM induction when added at the second stage. It is most surprising that GDF11, which has recently been reported to rescue cardiac functions in aged mice [23], was not effective in our experiment, possibly reflecting a difference between *in vitro* and *in vivo* conditions.

In conclusion, our chemical cocktail strategy provides a new approach for cardiomyocyte regeneration and lays the foundation for *in vivo* cardiac transdifferentiation using pharmacological agents and possibly safer treatment of heart failure. Future mechanistic studies based on these chemicals will also help us gain insights into the roles of the related signaling pathways in myocardial regeneration.

Materials and Methods

Cell culture

MEFs were isolated from embryonic day 13.5 (E13.5) C57BL/6 mouse embryos. The head, limbs, and internal organs were carefully removed from embryos, and then the rest tissues were cut and trypsinized into single-cell suspensions. MEFs were cultured in fibroblast growth medium consisting of DMEM (Gibco), 15% FBS (Hyclone, 30084), 2 mM Glutamax (Gibco), 0.1 mM non-essential amino acids (NEAA; Gibco), 100 units/ml penicillin and 100 μ g/ml streptomycin. *Fsp1-Cre* mice (JAX) were mated with R26R^{tdTomato} mice (JAX) to generate mice with specific expression of tdTomato in MEFs, which were used for lineage tracing experiments. OG2 mice (JAX) were mated with C57BL/6 to generate OG2 MEFs carrying Oct4::GFP reporter. Mouse TTFs were isolated from C57BL/6 neonate on day 2. Tail tips were cut into pieces

and then dispersed on gelatin-coated 10 cm culture dish containing 2 ml fibroblast growth medium, and additional 8 ml medium was supplemented on the next day.

Generation of CiCMs

MEFs or TTFs were seeded onto six-well plates (coated with 1:100 Matrigel from BD Biosciences for 1 h at room temperature) at a density of 50 000 cells per well and cultured in fibroblast growth medium. After 24 h, the medium was replaced with CRM plus various compound combinations, such as CRFVPT. CRM is composed of knockout DMEM (Gibco), 15% FBS, and 5% KSR (Gibco), 0.5% N2 (Gibco), 2% B27 (Gibco), 1% Glutamax, 1% NEAA, 0.1 mM β -mercaptoethanol (Gibco), 50 μ g/ml 2-phospho-L-ascorbic acid (vitamin C, Sigma), 100 units/ml penicillin and 100 μ g/ml streptomycin. CRFVPT cocktail consists of 10 μ M CHIR99021 (C); 10 μ M RepSox (R); 50 μ M Forskolin (F); 0.5 mM VPA (V); 5 μ M Parnate, (P); 1 μ M TTNPB (T). CRM containing chemical compounds was changed every 4 days. At the second stage of induction (day 16 for MEF, day 20 for TTF), cells were cultured in CMM for various days. CMM is composed of DMEM medium with 15% FBS, 2i (3 μ M CHIR99021 and 1 μ M PD0325901), 1 000 units/ml LIF, 50 μ g/ml vitamin C, and 1 μ g/ml insulin (Sigma). The following growth factors were also tested in CMM for the generation of TTF-derived CiCMs: T β -4 (100 ng/ml, PeproTech), GDF-11 (100 ng/ml, PeproTech), G-CSF (20 ng/ml, R&D systems), NRG1- β 1 (100 ng/ml, R&D systems).

Generation of smooth muscle cells and endothelial cells

Smooth muscle cells and endothelial cells were differentiated following the same protocol for cardiac reprogramming, except after day 20, CMM was replaced with smooth muscle growth medium containing 50% IMDM (Gibco) plus 50% F-12 (Gibco), supplemented with insulin (7 μ g/ml), monothioglycerol (450 μ M, Sigma), bovine serum albumin (BSA; fraction V, 5 mg/ml, Gibco) and PDGF-BB (10 ng/ml, R&D Systems), or the endothelial cell growth medium EGM2 (Lonza) with VEGFA (10 ng/ml, R&D Systems) for another two weeks.

Immunofluorescent staining

Cells were fixed in 4% PFA at room temperature for 30 min and washed with PBS twice. After permeabilizing with 0.3% Triton X-100 for 20 min, the cells were treated with 5% BSA for 1-2 h, then incubated with various primary antibodies at 4 $^{\circ}$ C overnight. After thorough washing, secondary antibodies conjugated with Alexa Fluor 555 or Alexa Fluor 488 were used. Nuclei were visualized with Hoechst 33342 (10 μ g/ml). Images were captured with an Olympus IX71 inverted fluorescent microscope. Antibodies used in this study are as following: cTNT (ab10214, Abcam), GATA4 (MABE477, Millipore and SC-25310 Santa Cruz), NKX2.5 (8792, CST & Ab35842, Abcam), cTNI (ab47003, Abcam), α -actinin (A7811, Sigma), Mef2c (5030S, CST), α -MHC (ab15, Abcam), Sca-1 (17-5981-81, eBioscience), α -SMA (A2547, Sigma), PECAM (SC-1506, Santa Cruz), Cnn2 (SC-16607, Santa Cruz), VE-cadherin (SC-9989, Santa Cruz), MLC2v (ab79935, Abcam), MLC2a (311011, Synaptic systems), and HCN4 (Ab69054, Abcam).

Real-time PCR

Total mRNA was isolated using Trizol (Invitrogen) and 2 μ g

RNA was used to synthesize cDNA using the PrimeScript RTreagent kit (Takara, DRR037A) according to the manufacturer's protocol. Real-time PCR was performed using FastStart Universal Probe Master Mix (Roche) and analyzed with a Stratagene Mx 3000P thermal cycler. Real-Time PCR primer sequences are listed in Supplementary information, Table S1.

FACS Analyses

For FACS analyses, cells were treated with collagenase II (1 mg/ml) and trypsin (0.25%). The single cell suspensions were then treated with fixation/permeabilization diluent (BD bioscience) at 4 °C for 20 min, and stained with anti- α -actinin or anti- α -MHC antibodies, followed by secondary antibodies conjugated with APC or Alexa 488. Cells were then analyzed with a Guava flow cytometer (Millipore) and the data were collected with the FlowJo software.

Intracellular Ca^{2+} measurement

To record calcium transients, the CiCMs were incubated with 2 μ M Fluo-4 AM in HEPES buffered saline solution (140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) at 37 °C for 20 min to allow de-esterification of the dye that had penetrated the cell membrane. After removing excess dye in the buffer, spontaneous Ca^{2+} transients were recorded at 37 °C using an Olympus IX81 motorized inverted fluorescence microscope and a time-lapse recording system (Xcellence). At least 8 fields in each dish were recorded and the recording lasted for at least 1 min for each field. Isoproterenol (Iso, 1 μ M) or carbachol (Cch, 5 μ M) was applied to the cells before the recording for 2 or 5 min, respectively.

Patch clamp recording

Patch clamp recording was performed in a temperature-controlled room at ~25 °C. The Giga-Ohm seal was achieved under the voltage-clamp mode and the APs were recorded under the current-clamp configuration using an Axopatch-200B amplifier (Molecular Devices). The pipette solution contained 145 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, and 10 mM Na_2ATP (pH 7.3 with KOH). During the recording, constant perfusion of extracellular solution was maintained using a BPS perfusion system (ALA scientific Instruments). Extracellular solution contained 140 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.4 with NaOH). Signals were filtered at 1 kHz, and digitized using a DigiData 1440 with pClamp9.2 software (Molecular Devices).

Microarray analysis

Gene expression microarray analyses of MEFs, MEF-CiCMs (beating colonies were picked at day 24), and cardiomyocytes were performed using Affymetrix GeneChip Mouse Genome 430 2.0 arrays in triplicate from independent biological samples. Data were analyzed using the GeneChip Scanner 3000 and Comma Console Software 3.1 with default setting. Raw data were normalized by MAS 5.0 algorithm, Gene Spring Software 11.0. Genes displaying 5-fold or greater changes ($P < 0.05$, t -test) in expression level between MEFs and MEF-CiCMs were selected to generate the heatmap and for GO term enrichment analysis (DAVID 6.7 software) [39]. The microarray data are available at the NCBI Gene Expression Omnibus with accession number GSE69924 (<http://www.ncbi.nlm.nih.gov/geo/info/linking.html>).

Statistic analysis

Values are reported as the means \pm SEM. P -values were calculated by Student's t -test, $P < 0.05$ was considered statistically significant. All graphs were plotted with GraphPad Prism software.

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