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Original article

Optimization of direct fibroblast reprogramming to cardiomyocytes using calcium activity as a functional measure of success☆

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ABSTRACT

Direct conversion of fibroblasts to induced cardiomyocytes (iCMs) has great potential for regenerative medicine. Recent publications have reported significant progress, but the evaluation of reprogramming has relied upon non-functional measures such as flow cytometry for cardiomyocyte markers or GFP expression driven by a cardiomyocyte-specific promoter. The issue is one of practicality: the most stringent measures – electrophysiology to detect cell excitation and the presence of spontaneously contracting myocytes – are not readily quantifiable in the large numbers of cells screened in reprogramming experiments. However, excitation and contraction are linked by a third functional characteristic of cardiomyocytes: the rhythmic oscillation of intracellular calcium levels. We set out to optimize direct conversion of fibroblasts to iCMs with a quantifiable calcium reporter to rapidly assess functional transdifferentiation. We constructed a reporter system in which the calcium indicator GCaMP is driven by the cardiomyocyte-specific Troponin T promoter. Using calcium activity as our primary outcome measure, we compared several published combinations of transcription factors along with novel combinations in mouse embryonic fibroblasts. The most effective combination consisted of Hand2, Nkx2.5, Gata4, Mef2c, and Tbx5 (HNGMT). This combination is >50-fold more efficient than GMT alone and produces iCMs with cardiomyocyte marker expression, robust calcium oscillation, and spontaneous beating that persist for weeks following inactivation of reprogramming factors. HNGMT is also significantly more effective than previously published factor combinations for the transdifferentiation of adult mouse cardiac fibroblasts to iCMs. Quantification of calcium function is a convenient and effective means for the identification and evaluation of cardiomyocytes generated by direct reprogramming. Using this stringent outcome measure, we conclude that HNGMT produces iCMs more efficiently than previously published methods.

1. Introduction

Generation of cardiomyocytes by direct cellular reprogramming provides a valuable source of cells for use in cardiovascular disease research and treatment. Induced cardiomyocytes, or iCMs, may be used for patient-specific drug toxicology screens, transplantations to replace cells lost via myocardial infarction, and to model cardiac development and disease *in vitro*. Unlike pluripotent cell-based strategies, direct reprogramming also offers the tantalizing prospect of direct *in situ* conversions in the target organ. Great progress toward these goals has been reported by several groups [1–5]; however, the effectiveness of cardiac reprogramming remains controversial [6,7]. Published reports on the *in vitro* generation of iCMs have relied upon some combination of GFP reporters, flow cytometry, and RT-PCR for cardiomyocyte-specific markers to evaluate the effectiveness of reprogramming procedures, with highly variable results. We set out to optimize the conversion of fibroblasts to iCMs using a more stringent outcome measure — the calcium oscillation that links excitation to contraction in functional cardiomyocytes.

Genetically-encoded calcium indicators (GECIs) are powerful tools for the visualization of changes in intracellular calcium levels [8–10]. GCaMP is a well-characterized GECI whose activity is comparable to traditional calcium indicator dyes such as Fura-2 and Rhodamine-3 [11]. Unlike indicator dyes, however, GECIs can be targeted specifically to cells or tissues of interest using lineage-restricted gene promoters. Although most widely used in the field of neuroscience to study the calcium oscillations that underlie neural activity [12–16], transgenic mice have been engineered to specifically express GCaMP in smooth muscle [17] and cardiomyocytes

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[18]. More recently, GCaMP was used to demonstrate that human embryonic stem cell-derived cardiomyocytes can electrically couple with host myocytes upon transplantation to infarcted hearts [19]. In this report, we describe the first use of GCaMP as functional tool for the optimization of cardiac reprogramming.

The present study uses mouse embryonic fibroblasts (MEFs) as the starting cell population for initial reprogramming experiments. MEFs were chosen for their ease of isolation from transgenic mouse lines and their extensive history as a starting material for direct reprogramming to a wide range of targets including pluripotent stem cells [20], skeletal muscle [21], neurons [13,22,23], neural progenitor cells [24], blood cells [25], hepatocytes [26,27], Sertoli cells [28], and cardiomyocyte-like cells [3,29]. After determining the optimal factor combination for MEF transdifferentiation, we extend this method to the conversion of adult mouse cardiac fibroblasts to iCMs.

2. Materials and methods

2.1. Plasmid construction

TroponinT-GCaMP5-Zeo vector: The ubiquitin promoter-rtTA cassette of FUdeltaGW-rtTA (Addgene plasmid 19780) was excised and replaced with the human *TNNT2* gene promoter (PCR-amplified from System Biosciences plasmid SR10012PA-1) and a Gateway cassette (PCR-amplified from pEF-DEST51, Invitrogen) to create TroponinT-Gateway. The GCaMP5-2A-Zeo cassette was built by using recombinant PCR to link the GCaMP5 sequence of pCMV-GCaMP5G [14] (Addgene plasmid 31788) to the T2A-Zeocin sequence of System Biosciences plasmid SR10012PA-1. The GCaMP5-2A-Zeo cassette was cloned into pDONR221 (Invitrogen), and subsequently into TroponinT-Gateway, via Gateway recombination. All lentiviral backbone plasmids were amplified in STBL3 bacteria (Invitrogen). Transcription factor open reading frames (listed in Supplemental Table S1) were cloned into the doxycycline-inducible (Tet-on) destination vector FU-tetO-Gateway [13] using LR Clonase II (Invitrogen).

2.2. Lentivirus production

Second-generation lentiviral vectors were packaged in Lenti-X 293T cells (Clontech) using Lipofectamine 2000 (Invitrogen) to deliver 12 μg of the lentiviral backbone plasmid, 7.7 μg psPAX2 (Addgene plasmid 12260) and 4.3 μg pMD2.G (Addgene plasmid 12259) in 3 mL OPTI-MEM (Invitrogen) to ~90% confluent 100 mm plates of 293T cells with 10 mL fresh MEF medium. Viral supernatant (13 mL) was collected at 24 and 48 h post-transfection, combined, and aliquoted for storage at −80 °C. Viral titer was determined using Lenti-X GoStix (Clontech). Lentiviruses used for all experiments had a minimum titer of 5×10^5 IFU/mL. The TroponinT-GCaMP5-Zeo reporter lentivirus was validated by transducing primary embryonic cardiomyocytes (E14.5) on a poly-D-lysine-coated 35 mm FluoroDish (World Precision Instruments) with 500 μL ($\geq 2.5 \times 10^5$ IFU) of virus. GCaMP activity was recorded 48 h post-transduction.

2.3. Preparation of primary cells

To prepare MEFs, embryos were harvested from mice of mixed background at 14.5 dpc followed by decapitation and removal of internal visceral organs, including the heart. The tissue was minced, then digested with trypsin and trituration, followed by resuspension of the cells in DMEM with 10% fetal bovine serum and 2 mM L-glutamine (MEF medium). The cells were plated on a 100 mm plate at a ratio of one embryo per plate and allowed to expand for 24 h, at which time they were passaged 1:3 for further expansion (passage 1). MEFs were used at passages 3–5 for reprogramming experiments.

Primary embryonic cardiomyocytes were isolated at 14.5 dpc as previously described [30].

Cardiac fibroblasts were isolated from adult mice (8–11 weeks old) using a method adapted from Chen et al. [6]. Hearts were removed and minced in cold PBS and then transferred to 4 mg/mL collagenase IV (Sigma) and 10 U/mL deoxyribonuclease I (Worthington Biochemical Corporation) and agitated at 37 °C for 10 min. Samples were spun down and resuspended in TrypLE (Invitrogen) at 37 °C with agitation. After 5 min, medium (DMEM supplemented with 15% FBS, 1% NEAA) was added and the resulting solution was added to gelatin-coated 6 well plates. After 7 days, any remaining pieces of tissue were removed and the cells were passaged 1:5 and frozen when confluent. Cardiac fibroblasts were used at passage 3 for reprogramming experiments.

2.4. Direct conversion of fibroblasts to iCMs

An outline of the reprogramming protocol is shown in Fig. 2A. MEFs or adult mouse cardiac fibroblasts were plated into poly-L-lysine-coated glass-bottomed 6 well dishes (MatTek P06G-1.0-20-F) at 75,000 cells/well on Day −2, with 500 μL ($\geq 2.5 \times 10^5$ IFU) each of rtTA lentivirus (FUdeltaGW-rtTA, Addgene plasmid 19780) and TroponinT-GCaMP-Zeo reporter lentivirus per well with 2 mL MEF medium. On the following day (Day −1), culture medium was replaced with 2 mL fresh MEF medium and the cells were transduced with 500 μL ($\geq 2.5 \times 10^5$ IFU) of each tetO-transcription factor lentivirus (thus, 2.5 mL total volume of lentiviruses for HNGMT-mediated reprogramming). On the next day (Day 0), the medium was switched to 3 mL/well Reprogramming Medium consisting of AGM (Lonza CC-3186) without EGF, supplemented with 2 μg/mL doxycycline (Sigma). Fibroblasts were transduced on Day 0 with 200 μL ($\geq 1 \times 10^5$ IFU) of PGK-H2B-mCherry [31] virus (Addgene plasmid 21217), which constitutively expresses nuclear-localized mCherry red fluorescent protein. Reprogramming Medium was changed every 2–3 days.

2.5. Live imaging and quantification of functional iCMs

Quantification of GCaMP+ cells was achieved by comparing the number of flashing cells to the total cell number, the latter indicated by the constitutively-expressed nuclear red fluorescent marker, PGK-H2B-mCherry [31]. To facilitate fluorescent imaging of live cells, reprogramming was performed on MEFs grown in glass-bottomed 6 well dishes (MatTek P06G-1.0-20-F). To permit counting of total cell number, MEFs were transduced on Day 0 with 200 μL ($\geq 1 \times 10^5$ IFU) of PGK-H2B-mCherry [31] virus (Addgene plasmid 21217), which constitutively expresses nuclear-localized mCherry red fluorescent protein. For GCaMP and mCherry imaging, the medium was replaced with Tyrode's Salt Solution (Sigma T2397). 10 s movies were made of 10–12 unique fields of view in a single well of a 6 well dish with 10× Apo objective. After collecting video with the GFP filter to view GCaMP, a brief segment of video was recorded with the mCherry filter to visualize all nuclei. GCaMP+ cells were counted in ImageJ and total cell number was obtained using the Nucleus Counter plugin (MacBiophotonics ImageJ). A minimum of 3000 total cells were counted per replicate, per condition. All GCaMP quantification data is the result of a minimum of 3 separate reprogramming experiments.

Imaging was performed on an Olympus iX-81 microscope with Metamorph software. Movies were obtained using HyperCam software (Hyperionics Technology). Statistical analysis (shown in Supplemental Table S2) was conducted using a one-way ANOVA with Tukey's post-hoc testing. The GCaMP intensity traces shown in Figs. 1B, 2B, 4C, and 6B were made using the "Intensity v Time Monitor" plugin in ImageJ.

2.6. Immunocytochemistry

Immunostaining was performed as previously described [13]. Briefly, cells for immunostaining were grown on 12 mm glass coverslips coated with poly-L-lysine (BD Biocoat 354085), with 4–5 coverslips per