Stoichiometry of Gata4, Mef2c, and Tbx5 Influences the Efficiency and Quality of Induced Cardiac Myocyte Reprogramming

Li Wang, * Ziqing Liu, * Chaoying Yin, Huda Asfour, Olivia Chen, Yanzhen Li, Nenad Bursac, Jiandong Liu, Li Qian

Rationale: Generation of induced cardiac myocytes (iCMs) directly from fibroblasts offers great opportunities for cardiac disease modeling and cardiac regeneration. A major challenge of iCM generation is the low conversion rate of fibroblasts to fully reprogrammed iCMs, which could in part be attributed to unbalanced expression of reprogramming factors Gata4 (G), Mef2c (M), and Tbx5 (T) using the current gene delivery approach. This approach suffers in part from heterogeneous and uncontrollable ratios of G, M, and T the 3 individual reprogramming factors. This approach involves transducing fibroblasts with pooled viruses encoding the 3 individual reprogramming factors. This approach suffers from heterogeneous and uncontrollable ratios of G, M, and T expression among the transduced fibroblasts. Inagawa et al. attempted to address this issue through the use of a polycistronic vector that encoded G, M, and T in a single transgene; however, their study showed only a marginal positive effect on reprogramming efficiency when compared with use of individual viruses.

Methods and Results: We took advantage of the inherent feature of the polycistronic system and generated all possible combinations of G, M, T with identical 2A sequences in a single transgene. We demonstrated that each splicing order of G, M, T gave rise to distinct G, M, T protein expression levels. Combinations that resulted in higher protein level of Mef2c with lower levels of Gata4 and Tbx5 significantly enhanced reprogramming efficiency compared with separate G, M, T transduction. Importantly, after further optimization, the MGT vector resulted in more than a 10-fold increase in the number of mature beating iCM loci. Molecular characterization revealed that more optimal G, M, T stoichiometry correlated with higher expression of mature cardiac myocyte markers.

Conclusions: Our results demonstrate that stoichiometry of G, M, T protein expression influences the efficiency and quality of iCM reprogramming. The established optimal G, M, T expression condition will provide a valuable platform for future iCM studies. (Circ Res. 2015;116:237-244. DOI: 10.1161/CIRCRESAHA.116.305547.)

Key Words: fibroblasts • Gata4 protein • gene expression/regulation • Mef2c protein • myocytes, cardiac • regeneration • Tbx5 protein • transcription factors

The generation of induced cardiac myocyte (iCM) from fibroblast holds great promise for cardiac disease modeling and regenerative medicine. However, the inefficient iCM generation has become a major hurdle for deciphering the mechanism of cardiac reprogramming and in vitro cardiac disease modeling. Although the low conversion rate of fibroblasts to reprogrammed iCMs suggests the existence of major rate-limiting barrier(s), it might also reflect a requirement for a balanced expression of Gata4 (G), Mef2c (M), and Tbx5 (T) to promote successful and complete reprogramming. Current iCM generation involves transducing fibroblasts with pooled viruses encoding the 3 individual reprogramming factors. This approach suffers from heterogeneous and uncontrollable ratios of G, M, and T expression among the transduced fibroblasts. Inagawa et al. attempted to address this issue through the use of a polycistronic vector that encoded G, M, and T in a single transgene; however, their study showed only a marginal positive effect on reprogramming efficiency when compared with use of individual viruses.

G, M, and T are the master regulators residing at the top of the transcriptional hierarchy of the cardiac gene regulatory networks. During heart development, faithful execution of cardiac developmental processes requires a precise dosage and temporal expression of these 3 factors. Disruption of
this delicate balance is likely to compromise cardiac specification and differentiation and cause severe cardiac anomalies.\textsuperscript{24–26,34–36} We therefore postulated that when G, M, and T were delivered in separate viruses, only a subpopulation of cardiac fibroblasts (CFs) would express the optimal amounts of G, M, and T required to trigger cardiac reprogramming. Thus, we surmised that it might be possible to establish a more appropriate balance of G, M, and T expression in non-myocytes through the use of splice-ordered polycistronic vectors.

In this study, we generated a complete set of polycistronic constructs containing G, M, and T in all possible splicing orders with identical 2A sequences in a single mRNA. We found that each splicing order of G, M, and T gave rise to distinct ratios of G, M, and T protein expression and significantly different reprogramming efficiencies. On further optimization, the most desirable combination resulted in a >10-fold increase in generation of beating iCMs. Importantly, at the molecular level, the more optimal G, M, and T stoichiometry, defined by higher protein expression level of Mef2c with lower levels of Gata4 and Tbx5, correlated with higher expression of mature cardiac myocyte markers. Thus, our study demonstrates that stoichiometry of G, M, and T influences both efficiency and quality of iCM induction.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Results**

G, M, and T Protein Levels Differ in Fibroblasts Expressing Each of the 6 Polycistronic Constructs

To manipulate the relative levels of G, M, and T protein expression, we generated 6 polycistronic constructs with identical 2A sequences to include all possible splicing orders of G, M, and T in a single mRNA (Figure 1A). In a first set of experiments, we transduced CFs with G, M, or T retroviruses separately. Western blot analysis showed that G, M, or T proteins were detected at the appropriate molecular weight (Online Figure IA). Next, we performed Western blot on CFs transduced with each of the 6 constructs and observed higher protein expression level when the reprogramming factor was placed at the 5’ end versus 3’ end of the construct (Figure 1B and 1C). After normalization to the loading control, quantification of band intensities revealed that each construct produced distinct ratios of G, M, and T protein expression (Figure 1B and 1C; Online Figure IB). We also determined G, M, and T transcript levels in transduced cells (Online Figure II). As expected, after virus infection, G, M, and T

**Figure 1.** A complete set of polycistronic vectors that result in different Gata4, Mef2c, and Tbx5 (G, M, T) protein levels. A, Diagram of the 6 polycistronic vectors with G, M, T in different splicing orders. Reprogramming factors were cloned in all possible orders (separated by identical P2A and T2A sequences) into the retroviral vector pMX. B, Western blot analysis of cardiac fibroblasts expressing each of the 6 different polycistronic vectors. Cell lysate was collected at 3 days after infection. C, Quantification of G, M, T protein expression levels. Error bars represent SEM. *P<0.05; **P<0.01.
transcript levels were significantly elevated; however, the CFs expressing each of these 6 constructs did not exhibit significant differences in G, M, and T total and endogenous transcripts levels (Online Figure II). These data suggest that our complete set of polycistronic vectors can effectively produce distinct ratios of G, M, and T protein expression in transduced CFs.

**Different iCM Reprogramming Efficiency Using Each of the 6 Polycistronic Constructs**

We next sought to determine iCM reprogramming efficiency using these 6 polycistronic constructs. We isolated CFs from α-muscle heavy chain (αMHC)-green fluorescent protein (GFP) reporter mice, which express GFP in differentiated cardiac myocytes (CMs) but not in CFs. Thus, activation of GFP could allow us to follow the emergence of newly induced iCMs. Furthermore, we used cardiac Troponin T as an additional differentiated CM marker to monitor CM fate induction. We transduced CFs with retroviruses encoding the 6 polycistronic mRNAs. Interestingly, flow analysis and quantification showed that these 6 vectors resulted in significantly different reprogramming efficiencies as indicated by the differences in the percentage of αMHC-GFP+ and cardiac Troponin T+ cells (Figure 2A and 2B). Noticeably, only 2 vectors (MGT and MTG) enhanced reprogramming efficiency compared with separate G, M, and T vectors, whereas the other 4 resulted in a decrease in reprogramming efficiency (Figure 2A and 2B). Similar results were obtained by performing immunocytochemistry with antibody against αMHC-GFP (Figure 2C; Online Figure III). Likewise, Western blot analysis of CFs reprogrammed with the 6 different vectors indicated that reprogramming with MGT resulted in the highest upregulation of αMHC-GFP and α-actinin protein expression (Figure 2D). Based on the analyzed markers, these data suggest that the 6 polycistronic constructs conferred differential effects on iCM induction. Although the experiments were performed with neonatal CFs, these polycistronic vectors exhibited similar relative reprogramming efficiencies (based on the percentage of αMHC-GFP+ iCMs) in neonatal tail tip fibroblasts, adult CFs, and adult tail tip fibroblasts (Online Figure IV). We then focused on the vector with the highest efficiency (MGT) and performed additional experiments to determine and quantify its reprogramming efficiency relative to that of separate G, M, T vectors (Online Figures V and VI). Collectively,
results demonstrate that the 6 polycistronic vectors exhibit significantly different iCM reprogramming capacities, and a more optimal G, M, and T stoichiometry, defined by higher protein level of Mef2c with lower levels of Gata4 and Tbx5, significantly increases iCM reprogramming efficiency.

**Enhancement of the Quality of iCM Reprogramming Using MGT With Antibiotic Selection**

To determine whether a more optimal G, M, and T stoichiometry of the MGT vector could be used to improve functional maturation of iCMs, we added a puromycin resistance gene in the vector (puro-MGT) to enable selection of transduced CFs (Figure 3). Using puro-MGT to reprogram freshly isolated CFs, we observed a 3-fold increase in the percentage of αMHC-GFP+ cells and 5-fold increase in the percentage of cardiac Troponin T+ cells (Figure 3A and 3B). To obtain a global view of iCMs, we took 228 snapshots of 10x immunocytochemistry pictures to cover a full well of a 24-well plate. After stitching all pictures together, we observed a population of cells that were highly enriched with iCMs, which frequently aggregated to form clusters (Figure 3C). These iCMs formed sarcomere structures (stained for cardiac Troponin T and α-actinin) resembling the ones of fetal cardiac myocytes and expressed the gap junction protein Connexin43 at the cell–cell contacts (Figure 3D). We next analyzed intracellular Ca²⁺ flux by Rhod3 dye labeling after 4 weeks of culture. With puromycin selection, ≈35% of transduced cells showed spontaneous Ca²⁺ oscillations and their frequency was variable, as previously reported in reprogrammed iCMs. Figure 3E shows a representative iCM-rich area containing 8 iCMs. Intracellular Ca²⁺ oscillation in this area was recorded in 4 loci, and 3 of them showed periodic Ca²⁺ transients (Nos. 1–3 representative traces) suggestive of advanced reprogramming stages, whereas the fourth trace showed aperiodic oscillations. The number of Ca²⁺ transient-expressing loci was increased by application of puromycin selection (Figure 3E). In addition to periodic Ca²⁺ transients, MGT-transduced iCMs showed spontaneous contractile activity after 3 weeks in culture (Online Movies I and II). With increase in culture time to 6 weeks, the number of beating iCM loci generated using the puro-MGT vector increased to 30 to 40 loci per well of a 24-well plate (Figure 3F), which was >10-fold higher than previously reported numbers of beating loci generated using the same or similar reprogramming factors. Collectively,

Figure 3. Further enhancement of induced cardiac myocyte (iCM) reprogramming efficiency using puro-MGT. A, Representative flow cytometry plots of cardiac Troponin T (cTnT)+, α-muscle heavy chain (αMHC)-green fluorescent protein (GFP)+, and double positive iCMs. B, Quantification of flow cytometry data. **P<0.01. C, Stitched (from 228 individual image) immunocytochemistry (ICC) picture showing iCMs (GFP positive) in 1 well of 24-well plate under optimized condition. Inset showing a representative high-resolution image of an iCM cluster. D, ICC of cardiac markers cTnT, α-actinin, and Connexin43 (Cx43) double labeling with αMHC-GFP. The middle panels are enlarged areas from the left-hand panels as indicated by the white rectangles. Note the striated pattern of sarcomeres in the top 2 middle panels and Cx43 positive spots at the cell–cell contacts in the bottom-middle panel. Scale bar, 200 μm. E, iCM calcium transients measured by Rhod3 dye labeling. Each trace corresponds to the spot numbered in the upper-left panel. Scale bar, 200 μm. Quantification is shown in the lower left histogram. F, Quantification of the number of beating iCM loci over time. Error bars represent SEM. DAPI indicates 4′,6-diamidino-2-phenylindole.
our data suggest that a more optimal stoichiometry of the reprogramming factors affords a more efficient and complete conversion of a CF into a CM fate.

Molecular Characterization of Reprogramming Cells Resulted From Varied G, M, and T Stoichiometry

Next, we sought to determine how G, M, and T protein stoichiometry influenced iCM gene expression profile. We performed real-time quantitative polymerase chain reaction using a set of CM and CF markers that were previously used to characterize iCMs37 (Figure 4). Interestingly, the 6 constructs led to significant differences in CM marker gene expression. The most efficient MGT and MTG vectors resulted in a higher upregulation of sarcomere structure genes Myh6, Myl7, Tntt2, Actc1, muscle contractility genes Pln, Slc8a1, Snc5a, gap junction protein gene Gja1, and ion channel genes Kcnj2, Cacba1c (Figure 4). Noticeably, we observed a reverse correlation of upregulation of cardiac stress genes Nppa and Nppb expression with reprogramming efficiency (Figure 4D and 4E). The 4 constructs that resulted in a decreased reprogramming efficiency upregulated Nppa and Nppb genes expression (Figure 4D and 4E), possibly because of overactivation by high levels of Tbx5 and Gata4. In contrast, fibroblast markers, such as Coll1a1, Col3a1, and Eln, were all significantly downregulated with no significant difference among the 6 constructs, suggesting that they exhibited a similar capacity to repress gene expression of fibroblast markers (Figure 4L–4N). Taken together, these data suggest that the stoichiometry of G, M, and T influences not only the efficiency of iCM induction but also the activation of cardiac myocyte markers in iCMs.

Discussion

In this study, we took advantage of the inherent feature of polycistronic vectors to manipulate the protein expression levels of cardiac reprogramming factors G, M, and T and reveal a critical role of G, M, and T protein stoichiometry in iCM reprogramming. We established that the optimal G, M, and T expression for reprogramming is a relative high level of Mef2c expression and low levels of Gata4 and Tbx5 expression. In addition, our studies suggest that an optimal balance among these 3 reprogramming factors could allow more efficient and complete conversion of CFs into cardiac myocyte–like cells.

To the best of our knowledge, this is the first work that has built a complete set of polycistronic vectors for a reprogramming cocktail. Carey et al37 has previously compared the induced pluripotent stem cell reprogramming between 2 existing polycistronic transgenes (OSKM versus OKSM). They demonstrated that these 2 transgenes conferred differential effects on induced pluripotent stem cell induction, possibly because of the differences in Oct4, Sox2, Klf4, and cMyc protein expression levels as a result of the splicing order of Oct4, Sox2, Klf4, and cMyc. However, the 2 transgenes were generated by different research groups in a way that the backbone of the vectors and the polycistronic cleavage sites were not identical. In this study, we generated a whole series of polycistronic constructs to include all possible splicing orders of cardiac reprogramming factors in the same construct with identical 2A peptides. Our studies demonstrated that difference in protein stoichiometry of G, M, and T alone is sufficient to confer a significantly different effect on cardiac reprogramming outcomes. It will be important to determine whether the inter-regulatory relationships among the 3 factors play a role in controlling and maintaining the optimal balance of G, M, and T expression needed for reprogramming. In addition to the polycistronic system, independent approaches such as those using promoters with different strengths to manipulate the relative levels of G, M, and T protein expression could be undertaken to further determine the effect of stoichiometry on iCM reprogramming. It remains to be determined whether optimized stoichiometry of human G, M, and T could be sufficient to induce cardiac fate in human fibroblasts without the use of additional factors and microRNAs.13–15 Moreover, we found that the unbalanced protein expression of G, M, and T (when using TMG, TGM, GMT, and GTM vectors) led to an inefficient iCM reprogramming. Although these unbalanced G, M, and T combinations were equally capable of suppressing gene expression of fibroblast markers, they failed to robustly activate cardiac program, and instead induced cardiac stress genes Nppa and Nppb. Our results thus could explain some discrepancies among different groups that attempted G, M, and T–mediated iCM induction.7,9,16 For example, similar to G, M, and T expression when using our TGM construct, the inefficient cardiac reprogramming reported by Chen et al27 was likely caused by relatively high expression of Tbx5 and Gata4 as well as the enrichment for potential iCMs based on the expression of Tbx5. The marginal effects of using a polycistronic construct TMG reported by Inagawa et al9 could be similarly attributed to the nonoptimal stoichiometry of reprogramming factors.

In spite of excitement and significant potential of iCM reprogramming in regenerative medicine, the low conversion rate of fibroblasts into iCMs has been a major challenge for future translational efforts. Although this low efficiency is expected, as it has been reported for other reprogramming technologies during their fledgling phase,38 there are ongoing efforts to overcome the hurdles and to identify small molecules to replace transcription factors for therapeutic purposes. All of these attempts would not be fruitful without a consistent and reproducible platform. Our most efficient polycistronic construct that has been further modified by adding antibiotic selection (pMX-puro-MGT) not only eliminates the need for multiple constructs, providing a homogeneous gene expression stoichiometry, but also allows for further enrichment of transduced cells with antibiotic selection. This platform offers a reasonable starting point for future screening and mechanistic studies, especially those that require large amounts of materials to explore genomewide molecular changes that occur during reprogramming.

In addition, our complete set of polycistronic constructs can be further used to study the optimal strategies to convert other nonmyocytes in addition to fibroblasts into cardiac myocytes, offering insights into how stoichiometry of G, M, and T influences iCM induction.
Figure 4. Gene expression analyses of cardiac fibroblasts (CFs) expressing each individual polycistronic vector. Relative expression of a panel of cardiac myocyte or fibroblast marker genes in CFs infected with each of the 6 polycistronic vectors compared with CFs infected with mock (dsRed) or pooled G+M+T at 10 days after infections. Error bars represent SEM.
and T expression can control cardiac fate acquisition under a variety of conditions.

Acknowledgments

We are grateful for the expert technical assistance from the UNC Flow Cytometry Core and UNC Microscopy Core. We thank Dr Taylor and members of the Qian laboratory and the J. Liu laboratory for helpful discussions and critical reviews of the article.

Sources of Funding

This study was supported by National Institutes of Health/National Heart, Lung, and Blood Institute (NIH/NHLBI) R01HL104326 grant to Dr Bursac, NIH/NHLBI R00 HL100797 grant to Dr J. Liu, NIH T35-DK007386 to Dr O. Chen, and American Heart Association 10. We are grateful for the expert technical assistance from the UNC Flow Cytometry Core and UNC Microscopy Core. We thank Dr Taylor and members of the Qian laboratory and the J. Liu laboratory for helpful discussions and critical reviews of the article.

Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Fibroblasts can be directly reprogrammed into cardiac myocyte–like cells (called iCMs).
- A combination of Gata4 (G), Mef2c (M), and Tbx5 (T) is sufficient to convert fibroblasts into iCMs.
- Generation of iCM in vivo has been shown to improve heart function and reduce scar size in a mouse myocardial infarction model.

**What New Information Does This Article Contribute?**

- Stoichiometry of G, M, T protein expression plays important roles in iCM reprogramming.
- The desirable G, M, T expression for reprogramming is a relative high level of Mef2c protein expression and low levels of Gata4 and Tbx5 expression.
- An optimal balance of G, M, T could allow more efficient and complete conversion of fibroblasts into iCMs.

Direct conversion of fibroblasts into cardiac myocyte–like cells (iCMs) using defined factors such as Gata4 (G), Mef2c(M), and Tbx5(T) holds great promise for regenerative medicine. However, the low conversion rate and the considerable variability in iCM generation have hindered further mechanistic studies and optimization for clinical applications. We took advantage of the inherent feature 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. By using this unique tool, we found that varying stoichiometry of G, M, T protein expression resulted in significant differences in iCM reprogramming efficiency and quality. Moreover, we found the optimal stoichiometry for iCM reprogramming to be a relative high level of Mef2c protein expression and low levels of Gata4 and Tbx5 expression. By addition of an antibiotic selection cassette to the optimal G, M, T combination (MGT), we further enriched population of transduced fibroblasts yielding an even higher efficiency of generation of functional iCMs. Our approach provides a valuable platform for further mechanistic studies of direct cardiac reprogramming.
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Circ Res. 2015;116:237-244; originally published online November 21, 2014;
doi: 10.1161/CIRCRESAHA.116.305547
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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METHODS

Mouse Lines
The transgenic mice harboring GFP under control of α-MHC promoter\textsuperscript{1,39} were used for primary cardiac fibroblasts (CFs) and tail-tip fibroblasts (TTFs) preparations. Animal care was performed in accordance with the guidelines established by University of North Carolina, Chapel Hill.

Plasmids
Retroviral vectors encoding mouse Gata4, Mef2c and Tbx5 in pMXs based vectors were described previously\textsuperscript{1,39}. For generating the six polycistronic constructs, a DNA fragment containing oligonucleotides encoding P2A and T2A peptides and desirable restriction enzymes sites was synthesized and cloned into pGEMT-easy vector. Mouse Gata4, Mef2c and Tbx5 were PCR amplified and sequentially cloned into the pGEMT-P2A-T2A vector. The six polycistronic expression cassettes (MGT/MTG/TMG/TGM/GMT/GTM) were then excised from pGEMT-easy vector and cloned into the pMXs vector.

Viral Packaging and Transduction
PlatE packaging cells were maintained in growth media containing DMEM plus10% FBS, 50 units/50 μg/ml penicillin/streptomycin, 1 μg/ml puromycin (Sigma), and 100 μg/ml of blasticidin S (Life Technologies). One day before transfection, 6x10^6 cells were seeded onto 10 cm dish in growth media without puromycin and blasticidin. The next day, pMXs-based retroviral vectors were introduced into Plat-E cells using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's recommendations. Generally, 10 μg of plasmid DNA, diluted with 500 μl Opti-MEM media (Life Technologies) was added to another 500 μl Opti-MEM media containing 20 μl of Lipofectamine. The mixture was incubated at room temperature for 20 min before adding to Plat-E cells. Cells were then incubated overnight at 37°C with 5% CO\textsubscript{2}. Medium was changed the next day and virus containing-supernatant was collected 48 hours after transfection, followed by filtration through a 0.45 μm cellulose acetate filter (Thermo Scientific) and incubation with Retrovirus Precipitation Solution (ALSTEM, CA) overnight. Viruses were then re-suspended by fibroblast media supplemented with 4 μg/ml polybrene (Life Technologies) and added to target cells immediately. Twenty four hours after infection, the virus containing medium was replace with iCM medium (10% FBS of DMEM/M199 (4:1)) and changed every 2-3 days. For positive selection, puromycin at 2 μg/ml was added to target cells three days after viral infection and was maintained in iCM medium at the concentration of 1 μg/ml.

Fibroblasts Isolation
For explant culture, isolated neonatal (day 1.5) hearts or tail tips from αMHC-GFP transgenic mice were minced into 1 mm x 1 mm pieces and placed onto 10 cm\textsuperscript{2} dishes with 2 ml explant medium (IMDM/20% FBS). Three hours later, when tissue pieces settled down, another 10 ml medium was slowly added and was replaced every three days. Migrated cells at day 7 were harvested and filtered through 40 μm cell strainers for cell sorting. For enzyme digestion, hearts were removed from αMHC-GFP transgenic mice and rinsed thoroughly with chilled PBS to remove blood and other tissues. The hearts were then minced roughly into 4-pieces (still connected), transferred to 8 ml warm 0.05% Trypsin-EDTA, and incubated at 37°C for 10 min. The supernatant was discarded and the heart pieces were digested with 5 ml warm 0.2% collagenase type II in HBSS for 3 min at 37°C, followed by vortexing for 1 min. The supernatant was collected and diluted with 5 ml explant culture medium. After five rounds of collagenase digestion and collection, single cell suspension was obtained by passing through 40 μm cell strainer. Cell pellet was surrendered in 1ml of Red cell lysis buffer (150 mM NH\textsubscript{4}Cl, 10 mM KHCO\textsubscript{3}, and 0.1 mM EDTA) for one minute on ice and resuspended in MACS buffer (DPBS with 0.5% BSA and 2 mM EDTA) for sorting.
Flow Cytometry
At 10 days post infections, the reprogrammed cells were washed with PBS and dissociated with 0.05% trypsin (Life Technologies) for 5 min at 37°C. Cells were washed twice with ice-cold FACS buffer (DPBS supplemented with 2% FBS and 2 mM EDTA) and subjected to fixation and staining with Cell Fixation/Permeabilization Kits (BD Biosciences). Incubation with antibodies was done at 4°C for 30 min at concentrations recommended by manufacturers. Cells were then suspended with staining buffer (DPBS with 1% paraformaldehyde) and counted using BD Accuri™ C6 flow cytometer. FACS data were analyzed by FlowJo software (Tree Star). The following antibodies were used: Mouse anti-troponin T, cardiac isoform (Thermo Scientific, 1:200), rabbit anti-GFP IgG (Invitrogen, 1:500), Alexa Fluor 488–conjugated donkey anti-rabbit IgG, Alexa Fluor 647–conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Inc, 1:500).

Magnetic Cell Sorting
Thy1.1 or Thy1.2 positive fibroblast cells, obtained from either explant culture or enzyme digestion, were isolated by Magnetic-activated cell sorting (Miltenyi Biotec) according to manufacturers’ instructions. Briefly, cells (1x10⁷) were suspended in 90 μl MACS buffer and incubated with 10 μl Thy1.1 or Thy1.2 micro-beads in refrigerator for 30 min. After two MACS buffer washes, cells were passed through 30 μm nylon mesh and applied to calibrated LS column. Target cells were flushed out after two washes and resuspended in explant culture medium for further usage.

Immunofluorescence and EVOS AutoScan for Single Well Image
Cells were washes with ice cold PBS for three times and fixed with 4% paraformaldehyde (EMS) at room temperature (RT) for 15 min. After permeabilization with 0.2% Triton/PBS for 15 min and blocking in 5% BSA for 1 hour, cells were treated with primary antibody at 4°C overnight, secondary antibody for 1 hour at RT, and subsequent nuclei staining with DAPI in mounting medium Vectashield (Vector labs). The following primary antibodies were used: cardiac troponin T (Thermo Scientific, 1:400), GFP (Invitrogen, 1:500), α-actinin (Sigma-Aldrich, 1:500), Connexin43 (Sigma-Aldrich, 1:200), Mef2c (Abcam, 1:1000); Gata4 (Santa Cruz Biotechnology 1:200), and Tbx5 (Santa Cruz Biotechnology, 1:200). Images were acquired using EVOS® FL Auto Cell Imaging System (Life Technologies). For whole-well microscopic imaging, a series of single images (228 images totally at 10X magnification) from each monochrome camera were collected and stitched together with the Scan feature on the system and exported as one individual image covering the whole well.

Calcium Imaging
Ca²⁺ signals were imaged with Rhod-3 Calcium Imaging Kit (Life Technologies) according to the manufacturer’s instructions. Briefly, cells were loaded with Rhod-3 for 1 hour at RT, washed and incubated with 2.5 mM probenecid in HBSS for an additional 1 hour to allow de-esterification. Rhod-3 labeled cells were analyzed at room temperature with EVOS. The Ca²⁺ flashing cells were counted in 10 randomly selected fields per well of 24-well plate in triplicates. For counting beating cells, 4-6x10⁴ freshly isolated cardiac fibroblasts were seeded onto one well of 24-well plate, infected with indicated viruses and maintained in iCM medium. The medium was changed every 2 to 3 days. Two weeks later, cells were maintained in PRMI1640 medium with B27 supplement and cells started to spontaneously contract at 3-4 weeks. The number of beating loci was counted in each well from at least three independent experiments at indicated time points.

Quantitative Real-time qPCR
RNA was extracted with Trizol (Invitrogen). Frist strand cDNAs were synthesized by using the Superscript III first-strand synthesis system (Invitrogen). qRT-PCR with Taqman probes was performed using an ABI ViiA 7 real-time PCR system (Applied Biosystems) as per manufacturer’s protocols.

Western Blot
Cells were collected and lysed in 2x SDS loading buffer (Bio-Rad) and subjected to SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes and probed with indicated antibodies. The target proteins were detected by chemiluminescence (ECL, Thermo Scientific). The membranes were stripped with stripping buffer (Sigma) and re-probed with antibody against a second protein or β-Actin for loading control.

**Statistical Analyses**
Differences between groups were examined for statistical significance using two way unpaired student’s t-test or two-way ANOVA. A p-value < 0.05 was regarded as significant. Error bars indicate standard error of the mean (SEM).

**SUPPLEMENTAL FIGURE LEGENDS**

**Online Figure I.** Western blot analysis of CFs expressing G, M, or T individually and MGT, MTG.
(A) Representative western blots on CFs transduced separately with one of G,M, or T. Cell lysates were collected at 3 days post infection (B) Western blot showing protein expression of Tbx5 and Gata4 in CFs transduced with mock (dsRed), MGT and MTG retroviruses. Note that despite of the relative low expression of Tbx5 and Gata4 using MGT and MTG compared to the other 4 G,M,T combinations, overexpression of Tbx5 and Gata4 was achieved compared to the mock control. β-Actin was used as an endogenous loading control.

**Online Figure II.** Analysis of G,M,T total and endogenous transcript levels. qPCR of G,M,T total and endogenous transcript levels in CFs expressing each of the 6 polycistrionic vectors. Error bars represent standard error of mean. The “en” in panels C, D, E stands for endogenous.

**Online Figure III.** Immunocytochemistry of reprogrammed cells. Fluorescent staining for αMHC-GFP, Mef2c and DAPI on CFs transduced with M+G+T, MGT, MTG, TMG, TGM, GMT, GTM at 10 days post infection. (A) Representative images taken with 20X objective lens; (B) Representative images taken with 10X objective lens. Scale bar: 200µm.

**Online Figure IV.** iCM reprogramming using each of the 6 polycistrionic constructs on neonatal tail tip, adult cardiac and tail tip fibroblasts. (A) Flow quantification of the percentage of αMHC-GFP+ cells reprogrammed from neonatal tail tip fibroblasts (TTFs) with each of the 6 polycistrionic vectors. (B) Immunofluorescent staining for αMHC-GFP+ and DAPI on cells reprogrammed from neonatal TTFs with each of the 6 polycistrionic vectors. Scale bar: 200µm. (C) Flow quantification of the percentage of αMHC-GFP+ cells reprogrammed from adult CFs with each of the 6 polycistrionic vectors. (D) Flow quantification of the percentage of αMHC-GFP+ cells reprogrammed from adult TTFs with each of the 6 polycistrionic vectors. Error bars represent standard error of mean.

**Online Figure V.** iCM reprogramming using MGT compared to M+G+T. (A) A representative immunostaining of one well in a 24-well plate (stitched from 228 individual images) showing iCMs generated with MGT labeled by αMHC-GFP, cTnT and DAPI. Inset shows a representative high resolution image of iCMs. The right panels are representative close-ups of iCMs stained with αMHC-GFP, cTnT and DAPI. Scale bar: 200µm. (B) Representative immunostaining for αMHC-GFP and DAPI on CFs (first row) and TTFs (second row) transduced with control (dsRed), M+G+T or MGT. (C) Representative close-ups of αMHC-GFP and DAPI labeled CFs and TTFs transduced with M+G+T or
MGT. Scale bar: 200µm. (D) Quantification of the percentage of αMHC-GFP+, cTnT+ CFs and TTFs transduced with M+G+T or MGT. Error bars represent standard error of mean. *, p<0.05; ** p<0.01.

**Online Figure VI.** Enhanced reprogramming efficiencies in various fibroblast populations transduced by MGT. (A) Representative FACS plots of cTnT+, αMHC-GFP+ and double positive iCMs reprogrammed from Thy1.1+ CFs, Thy1.2+ CFs, Thy1.1+ TTFs, or Thy1.2+ TTFs. (B) Quantification of the percentage of αMHC-GFP+, cTnT+ and double positive cells. Error bars represent standard error of mean. *, p<0.05; ** p<0.01.

**Online Movie I.** Spontaneous contractions of a single iCM.

**Online Movie II.** Spontaneous contractions of iCM clusters.
SUPPLEMENTAL FIGURES

Online Figure I

A

B

Online Figure II

A

B

C

D

E
Online Figure IV

A. Neontal TTF

B. MGT MTG TMG TGM GMT GTM

C. Adult CF

D. Adult TTF
Online Figure V

A

B

C

D

% of cTnT+ cells

% of dMHC-GFP cells