Induction of Cardiomyocyte-like Cells in Infarct Hearts by Gene Transfer of Gata4, Mef2c, and Tbx5

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ABSTRACT

**Rationale:** After myocardial infarction (MI), massive cell death in the myocardium initiates fibrosis and scar formation, leading to heart failure. We recently found that a combination of three cardiac transcription factors, Gata4, Mef2c, and Tbx5 (GMT), reprograms fibroblasts directly into functional cardiomyocytes in vitro.

**Objective:** To investigate whether viral gene transfer of GMT into infarcted hearts induces cardiomyocyte generation.

**Methods and Results:** Coronary artery ligation was employed to generate MI in mouse. In vitro transduction of GMT retrovirus converted cardiac fibroblasts from the infarct region into cardiomyocyte-like cells with cardiac-specific gene expression and sarcomeric structures. Injection of the green fluorescent protein (GFP) retrovirus into mouse hearts, immediately after MI, infected only proliferating non-cardiomyocytes, mainly fibroblasts, in the infarct region. The GFP expression diminished after 2 weeks in immunocompetent mice, but remained stable for 3 months in immunosuppressed mice in which cardiac induction did not happen. In contrast, injection of GMT retrovirus into α-myosin heavy chain (αMHC)-GFP transgenic mouse hearts induced the expression of αMHC-GFP, a marker of cardiomyocytes, in 3% of virus-infected cells after 1 week. A pooled GMT injection into the immunosuppressed mouse hearts induced cardiac marker expression in retrovirus-infected cells within 2 weeks, although few cells showed striated muscle structures. To transduce GMT efficiently in vivo, we generated a polycistronic retrovirus expressing GMT separated by 2A “self-cleaving” peptides (3F2A). The 3F2A-induced cardiomyocyte-like cells in fibrotic tissue expressed sarcomeric α-actinin and cardiac troponin T, and had clear cross striations. Quantitative RT-PCR also demonstrated that FACS-sorted 3F2A-transduced cells expressed cardiac-specific genes.

**Conclusions:** GMT gene transfer induced cardiomyocyte-like cells in infarcted hearts.

**Keywords:** Reprogramming, cardiomyocyte, fibroblast, transcription factor, myocardial infarction, regeneration.

**Non-standard Abbreviations:**
- MI: myocardial infarction
- GMT: Gata4, Mef2c, and Tbx5
- GHMT: Gata4, Hand2, Mef2c, and Tbx5
- iCMs: induced cardiomyocytes
- WT: wild type
- GFP: green fluorescent protein
- MHC: myosin heavy chain
- 3F2A: polycistronic vector expressing GMT
- PBS: phosphate-buffered saline
- CM: cardiomyocytes
- CF: cardiac fibroblasts
- MI-d3 CF: cardiac fibroblasts from 3 days after myocardial infarction
- MI-d7 CF: cardiac fibroblasts from 7 days after myocardial infarction
- col 1: collagen 1
INTRODUCTION

Cardiomyocytes are terminally differentiated cells with limited regenerative capacity in adult heart. Following myocardial infarction (MI), cardiac fibroblasts (CF), which account for more than half of the cells in heart, proliferate, synthesize extracellular matrix, and undergo fibrosis, leading to cardiac dysfunction\(^1,2\). The large population of endogenous CF might be a potential source of cardiomyocytes for regenerative applications if they could be readily and reliably reprogrammed into functional cardiomyocytes.

We recently found that a combination of three cardiac-specific transcription factors, Gata4, Mef2c, and Tbx5 (GMT), could reprogram mouse postnatal CF and dermal fibroblasts directly into functional cardiomyocytes in vitro\(^3\). The induced cardiomyocytes (iCMs) were similar to neonatal cardiomyocytes in global gene expression profile and their ability to contract spontaneously. Zhou et al.\(^4\) also demonstrated that adenoviral gene transfer of the three β-cell specific transcription factors into mouse pancreas reprogrammed pancreatic exocrine cells into functional β cells. Finally, local delivery of MyoD, a master regulator of skeletal myocytes, into cryo-injured hearts induced skeletal muscle differentiation in healing heart lesions\(^5\). In this study, we investigated whether gene transfer of GMT into infarcted hearts could similarly induce cardiomyocyte generation in vivo.

METHODS

Mice.

Transgenic mice overexpressing GFP under the control of an α-myosin heavy chain promoter (αMHC) were generated as described previously\(^3\). Eight-week-old male wild-type ICR and nude mice were obtained from CLEA Japan (Tokyo, Japan). The Keio University Ethics Committee for Animal Experiments approved all experiments in this study.

Construction of retroviruses.

The pMXs retroviral vectors containing GFP, DsRed, Gata4, Mef2c, or Tbx5 were generated as described\(^3\). The 3F2A construct was designed and generated by a custom gene synthesis service with codon optimization (Genscript Japan, Tokyo, Japan). 3F2A was subcloned into the pEnter-D-TOPO vector by PCR and subsequently cloned into pMXs-Gw using the Gateway system (Invitrogen) to generate pMXs-3F2A. The pMXs retroviral vectors were transfected into Plat-E cells with Fugene 6 (Roche) to generate retroviruses. Virus-containing supernatants were collected after 48 h.

In vivo gene transfer.

Pooled virus-containing supernatants were collected, filtered through 0.45-mm pore membranes, concentrated by 10- to 100-fold with centrifugation (8000 G for 16 h), and then resuspended in phosphate-buffered saline (PBS) supplemented with 4 μg/ml polybrene. To generate our MI model, mice were intubated and anesthetized with isoflurane gas. The chest cavity was exposed by cutting the intercostal muscle, and then the left coronary artery was ligated with a 7-0 silk suture, as described previously\(^6\). Immediately after the coronary artery ligation, 30 μl of virus-containing solution was injected into the boundary between the infarct and border zone at one site with a 32-gauge needle. For infection into dermal fibroblasts, mice were intubated and anesthetized with isoflurane gas, the skin was cut, and then 100 μl of virus-containing solution was injected. In some experiments, virus solution was administered after 3 and 7 days of MI. To determine the effect of immunosuppressants, mice were treated with cyclosporin A (Sigma) by intraperitoneal injection for 7 days at dosages of 10-20 mg/kg/day before

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viral delivery and coronary artery ligation. The cyclosporin A treatment was continued until analyses. The mouse surgeon was blinded to the study and mortality after MI was 10%.

**Viral transduction into cultured cardiac fibroblasts.**
The αMHC-GFP mice were subjected to MI by coronary artery ligation and sacrificed after 3 or 7 days. The hearts were excised and the infarcted area was dissected from surrounding normal myocardium under direct visualization. The explants were minced into small pieces less than 1 mm² and cultured for 10 days in explant medium (IMDM/20% FBS) on gelatin-coated dishes. Migrated fibroblastic cells were harvested and filtered with 40–μm cell strainers (BD) to avoid contamination with heart tissue fragments. The αMHC-GFP /Thy1⁺ cells were FACS sorted and plated at a density of 10⁴/cm² for the retrovirus transduction. After 24 h of infection, the medium was replaced with DMEM/M199 medium and changed every 2–3 days.

**Genomic DNA PCR.**
Genomic DNA was extracted from mouse hearts using a standard protocol, and PCR was performed using the following primers: GFP sense, 5'-TGAACCGCATCGAGCTGAAGGG-3'; GFP antisense, 5'-TCCAGCAGGACCATGTGATCGC-3'; GAPDH sense, 5'-AACCTTTGGCATATTGGAAGG-3'; GAPDH antisense 5'-ACACATTGGGGGTAGGAACA-3'.

**FACS analyses and sorting.**
For αMHC-GFP and GFP expression analyses, cells were harvested from cultured dishes and analyzed on a FACS Calibur (BD Biosciences) with FlowJo software. For GFP⁺ cell sorting, mouse cardiac tissues were digested with collagenase and sorted using a FACS Aria (BD Biosciences). For GFP⁺/Thy1⁺ cell expression analyses and αMHC-GFP/Thy1⁺ cell sorting, cells were incubated with APC-conjugated anti-Thy1 antibody (eBioscience) for 30 min before FACS.

**RNA extraction and quantitative RT-PCR.**
Mock- or GMT-transduced cardiac fibroblasts were collected from culture dishes by incubation with trypsin-EDTA, and the GFP- or 3F2A/GFP-transduced cells in hearts were collected by FACS. RNA was extracted from the cells using Pico-Pure RNA Isolation (Arcturus) and quantitative RT-PCR was performed as described previously, using the following TaqMan probes: Actc1 (Mm01333821_m1), Ryr2 (Mm00465877_m1), Nppa (Mm01255748_g1), and Tnnt2 (Mm00441922_m1) (Applied Biosystems). The mRNA levels were normalized by comparison to GAPDH mRNA.

**Western blot analysis.**
Lysates were prepared by homogenization of cells in RIPA buffer and run on SDS-PAGE to separate proteins prior to the immunoblot analyses as described. After transfer to nitrocellulose membranes, immunodetection was performed with antibodies to Gata4, Mef2c, and Tbx5, followed by the appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology). The antibody-bound proteins were visualized by chemiluminescence detection (ECL, Amersham).

**Immunohistochemistry.**
Hearts were fixed in 0.4% paraformaldehyde overnight, and then embedded in OCT compound for freezing in liquid nitrogen. Hearts were cut vertically into 7–μm sections to show both ventricles. Sections were stained with primary antibodies against α–actinin (Sigma Aldrich), vimentin (Progen), GFP (Invitrogen), cTnT (Thermo Scientific), collagen1 (Millipore), CD31 (BD Biosciences), and RFP (Rockland), and then with secondary antibodies conjugated with Alexa 488, 546, or 633 and DAPI (Invitrogen). GFP⁺ cells per area were calculated as the ratio between the number of GFP⁺ cells and the myocardial area, measured by Image J software. The α–actinin⁺ or α–MHC GFP⁺ cells relative to the total number of GFP⁺ or DsRed⁺ cells were counted in three randomly selected fields per section.
data for each mouse were calculated from 20-35 sections and we observed 4-5 mice in each group. The measurements and calculations were conducted in a blinded manner. All confocal microscopy was carried out on an LSM 510 META microscope (Carl Zeiss) and Z-stack images were collected according to the standard protocol.

**Immunocytochemistry.**
Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, blocked, and then incubated with primary antibodies against α-actinin, GFP, cTnT, vimentin, CD31, SMA (Sigma), collagen 1, Mef2c (Aviva Systems Biology), Gata4 (Santa Cruz Biotechnology), ANP (Chemicon), Nkx2.5 (Santa Cruz), or SM-MHC (Biomedical Technologies), with secondary antibodies conjugated to Alexa 488 or 546. Finally, cells were stained with DAPI. The percentage of cells immunopositive for α-actinin, GFP, cTnT, and ANP were counted in six randomly selected fields in triplicate, and 500-1000 cells were counted in total.

**Statistical analyses.**
Differences between groups were examined for statistical significance using Student’s t-test or ANOVA. *P* values < 0.05 were regarded as significant.

**RESULTS**

**Retroviral gene transfer into mouse hearts after myocardial infarction.**

Coronary artery ligation was employed to generate MI in mouse (Figure 1A, Online Figure IA). To determine the appropriate vector for targeting resident CF, we injected a GFP retrovirus or adenovirus into the wild-type (WT) mouse hearts immediately after MI. One week later, the GFP retrovirus efficiently infected the infarct region (Figure 1B, Online Figure IB). Immunohistochemistry demonstrated that retrovirus-transduced GFP+ cells in the infarct-border regions were immunopositive for vimentin and collagen 1, markers of fibroblasts, but not for sarcomeric α-actinin, a marker of cardiomyocytes (Figure 1C-E, Online Figure IC). In contrast, adenovirus preferentially infected cardiomyocytes (Online Figure ID and E).

Immunohistochemistry and genomic DNA PCR analyses for GFP demonstrated that the retrovirus infection efficiency was significantly increased in a dose-dependent manner (Figure 1F-H), and we thereafter used 100-fold concentrated retrovirus for gene transfer. FACS analyses demonstrated that 0.9% of heart cells expressed GFP after 1 week of GFP retrovirus injection and more than half of them expressed Thy-1, a cell surface marker of fibroblasts (Figure 1I)1, 9. We confirmed that vimentin+ fibroblasts, but not α-actinin+ cardiomyocytes, were infected by 100-fold concentrated GFP retrovirus. The z-axis confocal projection demonstrated that vimentin and GFP were expressed in the same cells (Figure 1J and K, Online Figure IF). These results indicated that retrovirus infects non-cardiomyocytes, mainly CF, in infarcted hearts.

Retrovirus-infected cells were reduced in immunocompetent mice, but maintained in immunosuppressed mice after myocardial infarction.

To determine the time course of viral gene expression, we performed immunohistochemistry after GFP retroviral delivery into the WT mouse hearts. GFP+ cells were apparent at 1 week, but reduced 2 weeks after MI and barely detectable after 4 weeks (Figure 2A and B). GFP retrovirus injected into the WT hearts at 3 and 7 days after MI also revealed few GFP+ cells by 2 weeks after the injection in either
case (data not shown). To investigate the effect of a reduced immune response, we injected GFP retrovirus into immunosuppressed nude-mouse hearts immediately after MI. Notably, GFP expression remained stable 2 weeks later, and was present up to 3 months after injection (Figure 2A and B). We also analyzed virus DNA insertion in WT and nude-mouse hearts by genomic DNA PCR at 1, 2, and 4 weeks after injection of GFP retrovirus. The GFP DNA was present at 1 week, but reduced at 2 and 4 weeks after injection into WT mouse hearts, in accordance with the immunohistochemistry data. In contrast, GFP DNA was clearly detected in the nude-mouse hearts at 1, 2, and 4 weeks after the injection (Figure 2C). To also investigate the effect of immunosuppressive treatment on the maintenance of virus-transduced cells, we injected GFP retrovirus into the WT hearts under treatment with an immunosuppressant, cyclosporin A. Despite increasing the dosage of cyclosporin A up to toxic levels, GFP+ cells were still reduced by 2 weeks after the retroviral injection (Figure 2D-F). These results suggested that the retrovirus efficiently infected CF until 1 week after MI, but thereafter the transduced cells and viral gene expression were reduced in immunocompetent mice.

**Cardiac gene induction by Gata4/Mef2c/Tbx5 transduction in vitro.**

To address whether GMT could induce cardiac gene activation in CF, we used αMHC promoter-driven EGFP transgenic mice (αMHC-GFP), in which only cardiomyocytes express GFP (Figure 3A). Immunopositivity for α-actinin, but negative staining for vimentin and CD31, demonstrated that cardiomyocytes expressed αMHC-GFP in the adult mouse hearts (Figure 3B and Online Figure IIA). We also confirmed that dissociated αMHC-GFP+ cells comprised cardiomyocytes, but not smooth muscle cells, endothelial cells, or fibroblasts (Online Figure IIB), as demonstrated in the previous paper.

For the in vitro experiments, we obtained Thy1+/αMHC-GFP+ cells from the infarct region at 3 and 7 days after MI by FACS sorting (Figure 3C). These cells expressed fibroblast markers, vimentin, and collagen 1, but not cardiomyocyte (α-actinin, cTnT, and Nkx2.5), smooth muscle cell (SM-MHC), or endothelial cell (CD31) markers (Figure 3D and Online Figure IIC). The antibody immunoreactivities were confirmed in a positive control (Online Figure IID). The transduction efficiency of CF obtained from the infarcted hearts was reduced to 30-60%, compared to over 90% in CF from intact hearts (Online Figure IIE). We next transduced CF with a pool of GMT and DsRed retrovirus to determine the cardiac induction. αMHC-GFP+ cells were not detected in CF infected with DsRed, but 3-7% of GMT-transduced cells expressed αMHC-GFP after 1 week of culture (Figure 3E). Quantitative RT-PCR demonstrated that cardiomyocyte-specific genes, Actc1 (cardiac α-actin), Nppa (natriuretic peptide precursor type A), Ryr2 (ryanodine receptor 2), and Tnnt2 (cardiac troponin T), were significantly upregulated in GMT-transduced cells (Figure 3F). Immunocytochemistry revealed the expression of cardiac proteins, α-actinin, cardiac troponin T, and atrial natriuretic peptide, after 4 weeks of infection and the induced cardiomyocytes had striated muscle structures (Figure 3G-I). These results indicated that GMT transduction induced cardiac gene expression in fibroblast cells obtained from infarcted hearts.

**Cardiac gene activation by gene transfer of Gata4/Mef2c/Tbx5 in vivo.**

To determine cardiac induction in vivo, we then injected either the mixture of GMT and DsRed or DsRed retrovirus alone directly into the αMHC-GFP mouse hearts after MI. All measurements were performed in a blinded fashion. Cells in fibrotic tissues injected with DsRed did not express αMHC-GFP, whereas approximately 3% of DsRed+ cells coinfectected with GMT and DsRed expressed αMHC-GFP after 1 week of gene transfer (Figure 4A and B). Three-dimensional analyses (Z-stack imaging) confirmed that DsRed and αMHC-GFP were expressed in the same cells (Figure 4C). These results indicated that gene transfer of GMT induced cardiac gene activation in a subset of cells in infarcted hearts.
As retrovirus-transduced cell numbers were reduced after 2 weeks of gene transfer in immunocompetent mice, we used nude mice for further analyses. We injected either a pool of GMT and GFP or GFP alone into the nude mouse hearts after MI. The cells transduced with GFP did not express α-actinin, whereas approximately 1% of GFP+ cells cotransduced with GMT and GFP expressed α-actinin after 2 weeks (Figure 4D, 5G). We found that around 15% of the induced α-actinin+/GFP+ cells had striated muscle structures (Figure 4E, 5H). Three-dimensional analyses confirmed that GFP and α-actinin were expressed in the same cells (Figure 4F). Despite an incubation period extending up to 1 month, most α-actinin+/GFP+ cells were smaller than endogenous ventricular cardiomyocytes and did not have clear cross striations.

Induction of cardiomyocyte-like cells in infarcted hearts by a single polycistronic vector expressing Gata4/Mef2c/Tbx5.

It is possible that cells transduced in vivo by three separate vectors might not receive all three genes at sufficient levels for full cardiac reprogramming. To address this, we generated a polycistronic retrovirus expressing GMT at near equimolar levels from the same promoter using “self-cleaving” 2A peptides (3F2A) (Figure 5A). Western blotting demonstrated that all three genes (Gata4/Mef2c/Tbx5) were expressed in the cells transfected with 3F2A (Figure 5B). In addition, around 70% of fibroblasts transduced with 3F2A retrovirus expressed both Gata4 and Mef2c, suggesting concomitant expression of GMT in fibroblasts (Figure 5C and D). We then injected 3F2A and GFP retroviruses directly into the nude mouse hearts after MI. Four weeks after the gene transfer, we found α-actinin+/GFP+ cells in the infarct area, of which 30% showed cross striations (Figure 5E, G and H). GFP and α-actinin were expressed in the same cells by three-dimensional analyses (Figure 5F). In addition, cTnT, a specific marker of cardiomyocytes, was expressed in the 3F2A-transduced cells (Figure 5I). Next, to determine the cardiac gene induction more quantitatively, we FACS sorted GFP+ cells from infarcted hearts after 1 week of gene transfer with 3F2A/GFP or GFP, and analyzed mRNA expression in the GFP+ cells (Figure 5J). Quantitative RT-PCR demonstrated that cardiac specific genes, Actc1, Nppa, and Tnnt2, were significantly upregulated in 3F2A/GFP-transduced cells compared with GFP alone, suggesting that cardiac genes were induced by the in vivo 3F2A gene transfer (Figure 5K). Importantly, we did not find tumor formation in the transduced mouse hearts. Together, these results demonstrated that a subset of cells in cardiac granulation tissue is differentiated into cardiomyocyte-like cells by GMT, provided that the three genes are appropriately introduced.

DISCUSSION

Our results provide evidence that GMT gene transfer induces cardiomyocyte-like cells in infarcted hearts, and that expression of GMT via a polycistronic vector enhances cardiac differentiation. Several lines of evidence suggest that the αMHC-GFP+/DsRed− cells and α-actinin+/GFP+ cells we describe here are not residual cardiomyocytes, but new cardiomyocyte-like cells induced by the GMT gene mix. First, we found that retrovirus infecting only proliferating cells transduced non-cardiomyocytes, mainly fibroblasts, in the infarcted heart region, consistent with previous reports. Second, control heart showed no αMHC-GFP+/DsRed− cells or α-actinin+/GFP+ cells. Third, high in vitro efficiency of cardiac induction from isolated CF supported the interpretation that endogenous fibroblasts generate iCMs. Finally, cardiac-specific genes were significantly upregulated in FACS-sorted 3F2A/GFP-transduced cells compared with GFP alone by quantitative RT-PCR. Nevertheless, we cannot completely exclude the possibility that rare cardiac progenitors were the origin of iCMs in vivo. To address this, we tried to analyze “fibroblast-lineage tracing mice” obtained by crossing “fibroblast-specific Cre” transgenic mice and indicator mice. However, we could not use the mice due to leaky EGFP expression in the
cardiomyocytes (data not shown). We also injected retroviral GMT or 3F2A with GFP into mouse skin to convert dermal fibroblasts into cardiac cells. However, we did not observe induction of cardiac-specific genes in the transduced skin cells (Online Figure III). There are several possibilities for this result. Although we injected more retrovirus into the skin, infection efficiency was lower in the dermal fibroblasts compared to CF in infarcted hearts. Different cell types, tissue permeabilities, and injury models could account for such a difference. Moreover, skin cells might be more resistant to cardiac conversion than CF, as demonstrated in vitro\textsuperscript{2,13-15}. Given that high transduction efficiency and addition of other factors to GMT would be beneficial for cardiac conversion in vitro, other injury models, modification of the gene delivery system, and optimization of reprogramming factors might be needed for efficient cardiac induction in skin\textsuperscript{11,15,16}.

During the review of this paper, two other studies of in vivo cardiac reprogramming by transcription factors were published by two other independent groups\textsuperscript{10,11}. As concluded in our present study, Qian et al.\textsuperscript{10} found that non-myocytes in infarcted hearts were converted into cardiomyocyte-like cells by GMT retroviral gene transfer. In addition, Song et al.\textsuperscript{11} reported that adding Hand2 to GMT (GHMT) converted CF into functional cardiomyocyte-like cells more efficiently than GMT alone in vitro and in vivo\textsuperscript{11}. Although all three studies (ours and the two others\textsuperscript{10,11}) demonstrated in vivo cardiac reprogramming, the approaches used to address this issue differ. The other two groups used mainly fibroblast-lineage tracing mice to demonstrate cardiac conversion from CF, while we took an alternative approach of co-transduction with GMT plus marker genes into immunosuppressed nude mice, to determine cardiac induction among the retrovirus-infected cells.

Qian et al.\textsuperscript{10} showed that around 35% of cardiomyocytes in the border/infarct zone were newly generated iCMs derived from CF using fibroblast-lineage tracing mice, and half of these cells had well-organized sarcomeric structures. They also transduced a marker gene with GMT and demonstrated that the cardiac conversion rate relative to the total number of GMT-infected cells was 10-15%, which was higher by more than 10-fold compared to our study. Their iCMs also exhibited functional characteristics of adult ventricular CMs, including cellular contraction, electrophysiological properties, and functional coupling to other cardiac cells. Notably, retroviral GMT gene transfer into mouse infarcted hearts significantly improved cardiac function and reduced the fibrotic area at 2 and 3 months after MI. Overall, the reprogramming efficiency and induction of mature iCMs by GMT were higher than those achieved in our study, which is possibly attributable to the different mouse strains, transgene expression levels, and viral titers between studies. We used immunosuppressed nude mice to demonstrate cardiac conversion, as retrovirus-infected cells were dramatically reduced after 2 weeks of MI in immunocompetent mice. Consistent with this, Byun J et al.\textsuperscript{12} also demonstrated that retrovirus lacZ-infected cells were reduced from 14% of heart cells at 1 week to 4% at 4 weeks in immunocompetent rat cryo-injured hearts\textsuperscript{16}. Our results that viral DNA levels were reduced in WT mouse, but maintained in nude mouse hearts, suggest that a T cell-mediated immune response contributed to the loss of retroviral-infected cells in immunocompetent animals. It is possible that Qian et al.\textsuperscript{10} achieved higher infection efficiency of fibrotic cells, as they showed that reprogrammed cells remained after 4 weeks of infection in the lineage tracing immunocompetent mice. However, it is not clear how many transduced cells remained after 4 weeks in their study, and further investigation might be needed to clarify this point.

Song et al.\textsuperscript{11} showed that 2-6% of cardiomyocytes in the border/infarct area were newly generated cardiomyocyte-like cells with clear striations and functional properties similar to endogenous ventricular cardiomyocytes using fibroblast-lineage tracing mice. However, as they did not transduce a marker gene with GHMT, the cardiac conversion rate relative to the total number of GHMT-infected cells remains unclear. Song et al.\textsuperscript{11} also demonstrated that cell fusion events were unlikely for iCMs generated using αMHC-MerCreMer/Rosa26-LacZ mice, in which endogenous cardiomyocytes could be chased by pulse-labeling with tamoxifen. Finally, they demonstrated that cardiac function improved from 2 weeks and was sustained until at last 12 weeks by GHMT gene transfer in MI mouse. The ejection fraction was also...
increased by two-fold in GHMT-treated mice compared to controls by MRI (57% vs. 28%) and the scar size was decreased to half (20% vs. 40%). However, the mechanisms of such functional improvements were undetermined.

We found that separate GMT gene transfer induced mostly immature α–actinin+ cells in vivo with a conversion rate of around 1%. It is possible that the inflammatory environment generated following MI inhibited proper cardiac gene induction. Another possibility is that the three genes were not all sufficiently transduced into cells, due to the low infection efficiency in vivo. High doses of MyoD are required to induce skeletal muscle differentiation in cardiac granulation tissue, while Carey et al. reported that a polycistronic vector encoding Yamanaka’s 4 factors by 2A system reprogrammed fibroblasts into iPSCs with only a single proviral copy insertion. Here, we used a polycistronic retrovirus to transduce all three genes into cells in vivo and generated more mature cardiomyocyte-like cells compared with introducing three separate vectors. However, we did not confirm the functional properties of the induced cardiomyocyte-like cells and improvement of global heart function due to the low cardiac induction efficiency in our study. In this sense, the present data are somewhat preliminary and further investigation and optimizations are needed in future research. Nevertheless, this is the first study demonstrating that the polycistronic 2A system can be used for cellular reprogramming in vivo, which also suggests that this system could be useful for inducing other type of cells.

Although considerable challenges remain, our findings might inform new regenerative strategies for repairing infarcted hearts.

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DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1. Retrovirus infection in mouse hearts. (A) Section of mouse heart 7 days after MI (Azan staining). (B) The retroviral GFP expression was localized to the infarct-border region. The infarcted area is delineated by dots. (C-D) GFP retrovirus was injected into the infarct heart. GFP+ cells were immunopositive for vimentin and collagen 1 (C), but not for α-actinin (D). (E) Ratios of α-actinin+ cardiomyocytes (CM), vimentin+ CF, and others to GFP+ cells (n = 4). (F-G) Dose dependency of retrovirus infection in mouse hearts, with quantitative analyses shown in (G) (n = 4). (H) Genomic DNA PCR analyses for GFP and GAPDH in cardiac tissues infected by 1 to 100-fold concentrated retrovirus solution. (I) FACS analyses for Thy1+/GFP+ cells from mouse hearts after 1 week of GFP retrovirus infection into MI hearts. GFP+ cells were immunopositive for vimentin. (K) Z-stack image of the GFP+/vimentin+ cells in (J). Representative data are shown in each panel. Inset boxes in the first panels are enlarged in the second to fourth panels (C, D, J). All data are presented as means ± SEM. **P < 0.01 vs. relevant control. Scale bars, 1 mm (A, B); 50 μm (C, D, F, J, K).

Figure 2. GFP expression was reduced in immunocompetent mice after myocardial infarction. (A-B) GFP expression was diminished after 2 weeks in WT hearts, but remained up to 1 month in Nude mice. Quantitative analyses are shown in (B) (n = 5). (C) Genomic DNA PCR analyses for GFP and GAPDH in WT and nude mouse hearts injected with GFP retrovirus. Note that GFP DNA was reduced in WT hearts after 2 weeks. (D) Concentration of cyclosporin A in blood samples. The concentration of 20 mg/kg/day was cytotoxic. (E-F) GFP+ cells were reduced after 2 weeks in WT hearts treated with cyclosporin A. Quantitative analyses are shown in (F) (n = 3). Representative data are shown in each panel. All data are presented as means ± SEM. **, P < 0.01 vs. control. Scale bars, 50 μm.

Figure 3. Cardiac Gene Activation by Gata4/Mef2c/Tbx5 in vitro. (A) α–MHC-GFP TG heart after MI. Infarct zone is delineated by dotted lines. (B) α–MHC-GFP was expressed in α–actinin+ cells, but not in vimentin+ cells. White boxes in the first panels are enlarged in the second to fourth panels (n = 3). (C) Thy-1+/GFP+ cells were FACS sorted from explants, obtained from infarct regions. (D) Thy-1+/GFP+ CF were immunopositive for vimentin and collagen 1, but not for αMHC-GFP or α-actinin (n = 3). (E) FACS analyses for α-MHC-GFP. GMT transduction induced αMHC-GFP expression in 7% of CF from 3 days after MI (GMT MI-d3 CF) and 3% of CF from 7 days after MI (GMT MI-d7 CF). (F) GMT-transduced expression of cardiac genes in CF determined by qPCR (n = 3). Note that the order is 10^3. (G-I) GMT induced αMHC-GFP, α-actinin, cTnT, and ANP expression in CF 4 weeks after transduction. High-magnification views show sarcomere-like structures. Quantitative analyses are shown in (I) (n = 3). Representative data are shown in each panel. All data are presented as means ± SEM. **, P < 0.01 vs. control. Scale bars, 1 mm (A); 50 μm (B, D, G, H).

Figure 4. Cardiac gene induction by gene transfer of Gata4/Mef2c/Tbx5 into infarcted hearts. (A-B) DsRed injection did not induce αMHC-GFP expression. GMT induced αMHC-GFP expression in the retrovirus-transduced cells, indicated as αMHC-GFP+/DsRed- cells. Quantitative analyses are shown in (B) (125 sections from 5 mice in Red, 140 sections from 5 mice in GMT/Red). (C) Z-stack image of the αMHC-GFP+/DsRed- cell in (A). (D) A pool of GMT, but not GFP, retrovirus induced α–actinin expression in the transduced cells in Nude mouse hearts (arrows). (E) A pool of GMT retrovirus induced α–actinin expression and sarcomere-like structures. The boxes in the upper panels are enlarged in the lower panels. Insets are enlarged areas of α–actinin+/GFP- cells showing sarcomere-like structures. (F) Z-stack image of the α–actinin+/GFP- cell in (E). Representative data are shown in each panel. White boxes in the first panels are enlarged in the second to fourth panels (A, D). All data are presented as means ± SEM. **, P < 0.01 vs. control. Scale bars, 50 μm.
**Figure 5. Induction of cardiomyocyte-like cells by a polycistronic vector.** (A) Schematic representation of the 3F2A retrovirus containing GMT for polycistronic gene expression. (B) Western blot analysis of 293 cells transfected with 3F2A for 2 days. (C) Immunocytochemistry for Gata4 and vimentin in mouse fibroblasts transduced with 3F2A. (D) Gata4 and Mef2c were expressed in the same cells after transduction of 3F2A. (E) 3F2A retrovirus induced α–actinin expression and cross striations. The cell, indicated by arrows in the first and second panels, is enlarged in the third to fifth panels. White boxes are enlarged in the insets. (F) Z-stack image of the GFP+/α–actinin+ cell in (E). (G-H) Quantitative analyses of the α–actinin+ to GFP+ cell ratios (G) and the proportions of α–actinin+/GFP+ cells showing cross striations (H) (120 sections from 5 mice in control, 140 section from 5 mice in GMT and 110 sections from 5 mice in 3F2A). (I) The 3F2A-expressing cells also showed cTnT expression. White boxes in the first panels are enlarged in the second to fourth panels. (J) Schematic representation to analyze mRNA expression in GFP- or 3F2A/GFP-infected cells. GFP+ cells were sorted by FACS after 1 week. (K) 3F2A/GFP-transduced cells expressed significantly higher cardiac genes compared with controls determined by qPCR (n = 3). Representative data are shown in each panel. All data are presented as means ± SEM. *, P < 0.05; **P < 0.01 vs. control. Scale bars, 50 μm.
Novelty and Significance

What Is Known?

- Gata4, Mef2c, and Tbx5 (GMT) can reprogram cardiac fibroblasts from intact hearts into cardiomyocyte-like cells in vitro.
- Cellular reprogramming is a potentially useful strategy for regenerating damaged organs.
- A polycistronic vector is a useful system to express multiple genes from the same promoter.

What New Information Does This Article Contribute?

- GMT can convert fibroblasts in infarcted hearts into cardiomyocyte-like cells in vitro and vivo.
- Retrovirus-infected cells are reduced in infarcted myocardium by the immune response.
- Gene transfer of a polycistronic vector encoding GMT enhances cardiac differentiation in infarcted hearts.

Following myocardial infarction, cardiac fibroblasts proliferate and synthesize extracellular matrix, leading to fibrosis and heart failure. Reprogramming the large population of endogenous cardiac fibroblasts into cardiomyocytes in situ could provide an ideal basis for regenerative therapy. We found that fibroblasts from infarcted hearts were converted into cardiomyocyte-like cells by GMT in vitro. Retrovirus-transduced cells were reduced in immunocompetent mouse hearts after myocardial infarction by the immune response. We thus used immunosuppressed mice in this study to show that direct gene transfer of GMT induced newly generated cardiomyocyte-like cells in infarcted myocardium and that a polycistronic retrovirus expressing GMT enhanced cardiac maturation. This approach may inform new regenerative strategies for repairing injured hearts.
Figure 2

A

WT day7  WT day14  WT day28

GFP  GFP  GFP

Nude day7  Nude day14  Nude day28

GFP  GFP  GFP

B

GFP+ cells/area

Day7  Day14  Day28

WT  Nude

C

GFP  GAPDH

Day7  Day14  Day28

WT  Nude

D

CyA concentration (µg/ml)

CyA  10  20

(mg/kg/day)

E

WT Day7  WT Day14  WT Day14

CyA 10 mg/kg/day  CyA 10 mg/kg/day  CyA 20 mg/kg/day

GFP  GFP  GFP

F

GFP+ cells/area

Day  7  14  14

CyA  10  20

(mg/kg/day)
Figure 4

A

Red
Merged
αMHC GFP
DsRed
Merged

GMT/Red
Merged
αMHC GFP
DsRed
Merged

B

αMHC GFP* cells / DsRed* cells

(%)

Red GMT/Red

C

GMT/Red
Merged

D

GFP
Merged
GFP
α-actinin
Merged

GMT/GFP
Merged
GFP
α-actinin
Merged

E

GMT/GFP
Merged
GFP
α-actinin
Merged

GMT/GFP
Merged
GFP
α-actinin
Merged

F

GMT/GFP
Merged
Induction of Cardiomyocyte-like Cells in Infarct Hearts by Gene Transfer of Gata4, Mef2c, and Tbx5
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Supplemental Material

Supplemental Figure Legends

Online Figure I. Adenovirus Preferentially Infected Cardiomyocytes

(A) Sections of mouse heart taken at 3 and 28 days after MI (Azan staining).
(B) Representative retroviral GFP expression localized to the infarct-border region. Infarct areas are delineated by dots.
(C) Border zones of infracted heart injected with GFP retrovirus. The expressions of GFP and \( \alpha \)-actinin did not colocalize (n = 4).
(D-E) GFP adenovirus predominantly infected \( \alpha \)-actinin\(^+\) cardiomyocytes (D). Quantitative analyses of the ratios of \( \alpha \)-actinin\(^+\) cells to GFP\(^+\) cells infected by retrovirus or adenovirus (E) (n = 4).
(F) 100-fold concentrated GFP retrovirus did not infect cardiomyocytes. Representative data are shown in each panel. White boxes in the first panels are enlarged in the second to fourth panels (C, D, F). All data are presented as means ± SEM. **, \( P < 0.01 \) vs. relevant control. Scale bars, 1 mm (A, B); 50 \( \mu \)m (C, D, F).

Online Figure II. \( \alpha \)-MHC-GFP Was Specifically Expressed in Cardiomyocytes

(A) \( \alpha \)-MHC-GFP was not expressed in vimentin\(^+\) fibroblastic cells or CD31\(^+\) endothelial cells. The first panels are enlarged in the second to fourth panels.
(B) Dissociated \( \alpha \)-MHC-GFP\(^+\) cardiac cells were immunostained for \( \alpha \)-actinin, cTnT, CD31, collagen 1, SMA, or SM-MHC, and counterstained with DAPI. Note that GFP was expressed only in \( \alpha \)-actinin\(^+\) and cTnT\(^+\) cells.
(C) Thy-1\(^+\)/GFP\(^-\) CF from 3 days after MI (MI-d3 CF) were immunonegative for cTnT, Nkx2.5, CD31, and SM-MHC.
(D) Positive control for immunocytochemistry. \( \alpha \)-actinin, Nkx2.5, and cTnT were expressed in murine cardiac cells. Insets are enlarged areas of cardiomyocytes showing striated structures. The second panel is the merged image of the first panel with DAPI.
(E) FACS analyses of the GFP retrovirus transduction efficiency. GFP\(^+\) cells constituted 60% of CF from 3 days after MI (GFP MI-d3 CF) and 31% of CF from 7 days after MI (GMT MI-d7 CF). Representative data are shown in each panel. Scale bars, 50 \( \mu \)m.
Online Figure III. Gene Transfer of Gata4/Mef2c/Tbx5 did not Convert Skin Cells into Cardiomyocyte-like Cells

Pooled GMT/GFP retrovirus did not induce α-actinin or cTnT expression in the transduced GFP+ cells in nude mouse skin. Representative data are shown in each panel. Scale bars, 50 µm.
Online Figure II

A

Remote Remote Remote Remote
Merged αMHC GFP αMHC GFP Vimentin Merged
Remote Remote Remote Remote
Merged αMHC GFP CD31 Merged

B

GFP α-actinin GFP cTnT GFP CD31
GFP Col1 GFP SMA GFP SM-MHC

C

MI-d3 CF MI-d3 CF MI-d3 CF MI-d3 CF
α-actinin cTnT Nkx2.5 CD31 SM-MHC

D

CM CM CM CM
α-actinin Nkx2.5 Merged cTnT

E

Con MI-d3 CF GFP MI-d3 CF GFP MI-d7 CF
PI 0% 60.1% 31.0%
GFP