Inefficient Reprogramming of Fibroblasts into Cardiomyocytes Using Gata4, Mef2c, and Tbx5


Rationale: Direct reprogramming of fibroblasts into cardiomyocytes is a novel strategy for cardiac regeneration. However, the key determinants involved in this process are unknown.

Objective: To assess the efficiency of direct fibroblast reprogramming via viral overexpression of GATA4, Mef2c, and Tbx5 (GMT).

Methods and Results: We induced GMT overexpression in murine tail tip fibroblasts (TTFs) and cardiac fibroblasts (CFs) from multiple lines of transgenic mice carrying different cardiomyocyte lineage reporters. We found that the induction of GMT overexpression in TTFs and CFs is inefficient at inducing molecular and electrophysiological phenotypes of mature cardiomyocytes. In addition, transplantation of GMT infected CFs into injured mouse hearts resulted in decreased cell survival with minimal induction of cardiomyocyte genes.

Conclusions: Significant challenges remain in our ability to convert fibroblasts into cardiomyocyte-like cells and a greater understanding of cardiovascular epigenetics is needed to increase the translational potential of this strategy. (Circ Res. 2012;111:00-00.)

Key Words: gene expression ■ Ca++ channels ■ cardiac development ■ myocardial ischemia ■ myocyte regeneration
Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>αMHC</td>
<td>α-myosin heavy chain</td>
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<td>CF</td>
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<td>eGFP</td>
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<td>Gja1</td>
<td>gap junction protein, alpha 1</td>
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<td>TTF</td>
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Materials.

hearts are eGFP from TTFs, we used GMT Overexpression
Reprogramming Tail Tip Fibroblasts by Ieda et al10 were kindly provided by Dr. Deepak Srivastava. To assess the efficiency of cardiomyocyte reprogramming, we found no eGFP+ cells among either αMHC-Cre/Rosa26mTmG or Nkx2.5-Cre/Rosa26mTmG CFs (Figure 1F and 1G). Following GMT Overexpression, we found no eGFP+ cells among either αMHC-Cre/Rosa26mTmG or Nkx2.5-Cre/Rosa26mTmG CFs (Figure 1F and 1G).

Reprogramming Cardiac Fibroblasts by GMT Overexpression

We hypothesized that reprogramming CFs might be more efficient than reprogramming TTFs as CFs share developmental lineage history with cardiomyocytes. We overexpressed GMT factors in CFs from 2- to 3-week-old αMHC-Cre/Rosa26mTmG mice and FACS-purified Thy1.2+/eGFP− CFs from Nkx2.5-Cre/Rosa26mTmG reporter mice, which express eGFP in immature cardiomyocytes (Figure 1A). No eGFP+ cells were detected after 3 weeks among infected fibroblasts (Figure 1E), suggesting a lack of Nkx2.5 upregulation.

Quantitative PCR Analysis of Cardiac Genes Following GMT Overexpression

As neither αMHC− nor Nkx2.5 lineage-reporting fibroblasts conveyed cardiac reprogramming, we suspected that not all cardiac genes are equally induced by GMT overexpression. Quantitative PCR analysis of GMT-infected CFs across a panel of cardiac genes confirmed the induction of some but not all cardiac genes (Figure 1H). Interestingly, while cTnT levels postinfection appeared modest in comparison with the high levels found in E10.5 hearts (Figure 1H), this represented a 250-fold increase in cTnT expression in comparison with uninfected CFs (Online Figure III). In GMT-infected CFs, transcript levels of SERCA2a, Tbx20, and Gata6 were comparable to those in E10.5 cardiomyocytes (Figure 1I). Levels of cTnT, MyBPC, and Gja1 also significantly increased, but a number of important sarcomeric proteins failed to be induced. Since cTnT was robustly upregulated by GMT factors, we overexpressed GMT in freshly isolated TTFs from cTnT-Cre/Rosa26mTmG mice. Remarkably, up to ~35% of the cells became eGFP+ 3 weeks postinfection (Figure 2A). However, although upregulation of MeF2c in infected TTFs remained modest (~10-fold) despite increases in viral titer, further investigation revealed that baseline levels of MeF2c in uninfected TTFs are already significantly elevated (Online Figure ID). Immunocytochemical staining for GMT proteins demonstrated their nuclear localization (Online Figure IB). Luciferase reporter assays using enhancer/promoter elements previously described to report the transcriptional activities of Gata4, MeF2c, and Tbx5 proteins12–14 confirmed that each transcription factor is active in vitro (Online Figure IC). To ensure that the ROSA26mTmG reporter can be efficiently excised by αMHC-Cre in vitro, we generated and differentiated αMHC-Cre/Rosa26mTmG ES cells and found robust expression of eGFP in beating cardiomyocytes (Online Figure II).

As αMHC is a marker of mature cardiomyocytes, we hypothesized that overexpression of developmentally essential genes Gata4/Mef2c/Tbx5 might induce an immature cardiac phenotype. We overexpressed GMT factors in TTFs from Nkx2.5 knock-in Cre/Rosa26mTmG reporter mice, which express eGFP in immature cardiomyocytes (Figure 1A). No eGFP+ cells were detected after 3 weeks among infected fibroblasts (Figure 1E), suggesting a lack of Nkx2.5 upregulation.

Reprogramming Tail Tip Fibroblasts by GMT Overexpression

To assess the efficiency of cardiac myocyte reprogramming from TTFs, we used αMHC-Cre/Rosa26mTmG mice that express membrane-tethered tandem dimerized Tomato (dTomato) at baseline, and switch to membrane-tethered enhanced GFP (eGFP) on Cre-mediated excision in the ROSA locus (Rosa26mTmG−/−) (Figure 1A and 1B).11 αMHC-Cre/Rosa26mTmG hearts are eGFP+ (Figure 1C), but TTFs are dTomato+ prior to GMT overexpression (Figure 1D). We infected freshly isolated TTFs from αMHC-Cre/Rosa26mTmG mice with lentiviruses constitutively expressing rtTA along with doxycycline-inducible lentiviruses expressing Gata4, MeF2c, and Tbx5. Following induction with doxycycline for 3 weeks, we found no eGFP+ cells by immunofluorescence microscopy and flow cytometry with or without GMT lentiviral infection (Figure 1D).

To investigate this unexpected finding, we evaluated the induction of GMT overexpression in infected TTFs and found up to 1000-fold increases in GMT factors (Online Figure IA). Current without a spontaneous action potential, suggesting incomplete electrophysiological reprogramming. Furthermore, GMT-infected fibroblasts exhibited poor survival and minimal cardiac gene expression following transplantation into an injured murine heart in vivo. Together, our data suggest that direct cardiomyocyte reprogramming by GMT factors is inefficient, and a greater understanding of transcription factor-mediated epigenetic change will be need to translate this promising approach into therapy.

Methods

The lentiviral tetracycline-inducible GMT expression vectors reported by Ieda et al10 were kindly provided by Dr. Deepak Srivastava. Detailed methods can be found in the Online Supplemental Materials.

Results

Reprogramming Tail Tip Fibroblasts by GMT Overexpression

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To investigate this unexpected finding, we evaluated the induction of GMT overexpression in infected TTFs and found up to 1000-fold increases in GMT factors (Online Figure IA).
we noted that eGFP+ cells remained morphologically indistinguishable from eGFP− cells and exhibited no spontaneous beating activity (Figure 2B).

**Global Transcriptional Profiles of TTFs and CFs Before and After GMT Overexpression**

To further examine GMT-induced changes to gene expression on a genome-wide scale, we performed microarrays of CFs and TTFs before and after GMT infection. We selected for Tbx5 expressing cells by using a Tbx5-IRES-Puro lentivirus and treating the GMT infected cells with puromycin. Interestingly, we found no significant change in global gene expression profiles of CFs and TTFs after GMT overexpression (Online Figure IV). We noted, however, a subset of cardiac genes shifted toward cardiomyocyte-like expression patterns, but these genes were either experimentally introduced (e.g., Tbx5) or known from the qPCR data above (e.g., cTnT) (data not shown).

**Electrophysiological Assessment of GMT-Overexpressing Fibroblasts**

While the global gene expression data show a low overall efficiency of cardiomyocyte reprogramming by GMT factors, the possibility remains that rare cells are more fully reprogrammed. To investigate this, we performed electrophysiological assessment of GMT-infected TTFs at a single cell level. We compared GMT-overexpressing TTFs (n=32) with uninfected fibroblasts (n=26) and embryonic stem (ES) cell–derived cardiomyocytes (n=20) at 3 weeks postinfection. We found no spontaneous action potentials in GMT-infected (0 out of 32 cells) or uninfected (0 out of 26 cells) TTFs, while ES cell–derived cardiomyocytes were all spontaneously active (20 out of 20 cells) (data not shown). On pacing, ES cell–derived cardiomyocytes displayed typical murine cardiac action potentials, while uninfected fibroblasts demonstrated passive exponential decay of membrane potential consistent with a lack of active

![Figure 1](http://circres.ahajournals.org/lookup/doi/10.1161/CIRCRESAHA.117.311935)
repolarization (26 out of 26 cells) (Figure 2C). Interestingly, 7 out of 32 (21.8%) of GMT-infected cells demonstrated up-sloping pacing induced action potential followed by passive exponential decay (Figure 2C).

We further examined this GMT-induced depolarization response in TTFs by introducing increasing stimulus amplitudes and found a graded response distinct from the "all or none" sodium current–dependent excitation typical of cardiomyocytes15 (Figure 2D). This absence of inward voltage-activated sodium currents in GMT-infected TTFs and their lack of active repolarization is likely responsible for their inability to fire repetitively on high-frequency pacing stimulation (data not shown). The ability of nifedipine, a dihydropyridine calcium channel antagonist, to block pacing induced action potentials (Figure 2E, red curve) revealed that the predominant component of these transient depolarizations was mediated by calcium and not sodium channels.

**Survival and Reprogramming of Transplanted GMT-Overexpressing Fibroblasts**

The low efficiency observed in GMT-overexpressing fibroblasts in vitro could have been explained by the absence of a supportive reprogramming environment. To examine the influence of a myocardial environment on reprogramming, we overexpressed GMT in CFs derived from transgenic mice that constitutively express luciferase and eGFP16 and injected these cells into the hearts of female SCID mice (5×10⁵ cells/heart, n=3) that had just undergone surgical ligation of their left anterior descending (LAD) coronary arteries (Figure 3A). In parallel, uninfected cardiac-derived cells16 (5×10⁵ cells/heart) were injected into the injured hearts of other SCID mice (n=3) as controls. Bioluminescence imaging over 8 days revealed a rapid loss of luciferase activity in hearts transplanted with GMT-infected CFs, while only a modest degree of attrition was observed among uninfected cells (Figure 3B and 3C). To assess whether engrafted GMT overexpressing fibroblasts underwent cardiomyocyte reprogramming, we recovered transplanted single eGFP+ cells by FACS and evaluated their expression of a panel of cardiac genes using a novel Fluidigm® single-cell PCR array. We found that recovered cells predominantly expressed vimentin, a marker of fibroblasts, while rare cells expressed a small number of cardiac genes (Figure 3D).

**Discussion**

Direct cardiomyocyte reprogramming by overexpression of cardiac transcription factors is a conceptually appealing strategy for cardiomyocyte regeneration. Using transgenic mice expressing Cre recombinase under the regulation of αMHC, Nkx2.5, and cTnT promoters, we found that GMT overexpression in
TTFs and CFs only induced expression of a subset of cardiac genes with minimal alteration of the fibroblast phenotype. We detected calcium channel–mediated depolarization currents in a subset of infected cells, suggesting that GMT reprogramming factors induced incomplete electrophysiological reprogramming. Transplantation of GMT-infected CFs into injured hearts resulted in no further improvements in the efficiency of cardiomyocyte phenotype conversion. Altogether, these data support a need for improved efficiency in cardiomyocyte reprogramming. A greater understanding of epigenetic changes associated with transcription factor overexpression will enhance the therapeutic potential of this approach.

Recent reports of direct reprogramming of fibroblast into other tissues such as blood progenitors and neurons by overexpression of lineage-specific transcription factors offer hope that we may apply similar strategies to cardiac regenerative therapies. In our hands, however, the overall efficiency of cardiomyocyte reprogramming with GMT overexpression is extremely low. Potential differences in experimental protocols (eg, the method of fibroblast isolation, the method of virus production) or reagents used (eg, genetic background of mouse strain, the cardiomyocyte-lineage reporters used) can influence the level of GMT overexpression and may account for some of the differences between our findings and those of Ieda et al. As an example, we found significant differences in the interpretations of reprogramming efficiency when different reporters (eg, cTnT versus aMHC or Nkx2.5) are used. It is worth mentioning that the percentage of cTnT-expressing cells in Ieda et al was only 5% of the total infected cell population, and among these, only a fraction of them are likely to express a more complete cardiac gene expression.

Our results highlight many challenges in transcription factor–based cardiac reprogramming. Importantly, we demonstrated the profound influences that choices of lineage reporters, cell types, and methods of evaluating cardiac phenotypes have on assessments of reprogramming efficiency. Moreover, our study raises important caveats for using GMT-reprogrammed fibroblasts as transplantable cardiomyocyte-like cells, because these cells demonstrate poor survival posttransplantation (a common finding in previous cardiac transplantation experiments) and are therefore unlikely to integrate with surrounding cardiomyocytes. Whether by adding different transcription factors and epigenetic modifiers to the GMT mix or by changing the starting cell type, significant improvements in the efficiency
of cardiomyocyte reprogramming are needed before this strategy can be applied therapeutically.

Acknowledgments

We thank Dr. William Pu at Boston Children’s Hospital for cTnt-Cre/ROSA26RtTA reporter fibroblasts; Dr. Robert Swartz at University of Houston for Nkx2.5-Cre knock-in mice; Drs. X.J. Yang and Q. Wang for promoter constructs used in luciferase reporter assays; Drs. Konrad Hochdelinger and Matthias Stadtfeld for the rtTA plasmid vector. Microarray studies were performed by the Molecular Genetics Core Facility at Children’s Hospital Boston supported by NIH-P50-NS40828 and NIH-P30-HD18655. Ms. Laura Prickett-Rice at the MGH-Center for Regenerative Medicine flow cytometry core facility provided assistance with FACS.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Cellular reprogramming is a potentially useful strategy for generating therapeutically important cell types such as cardiomyocytes.
- GATA4, MEF2C, and Tbx5 (GMT) have been reported to reprogram fibroblasts into cardiomyocytes in vitro as well as in vivo.

What New Information Does This Article Contribute?

- The efficiency of direct cardiac reprogramming by GMT overexpression in cardiac fibroblasts (CF) and tail tip fibroblasts (TTF) is very low.
- While GMT overexpression upregulates a subset of cardiac genes and alters the electrophysiological phenotype in fibroblasts, this phenotype does not resemble those of a bona fide cardiomyocyte.
Inefficient Reprogramming of Fibroblasts into Cardiomyocytes Using Gata4, Mef2c, and Tbx5


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Supplemental Methods

Tetracycline-inducible GMT expression constructs
Lentiviral tetracycline-inducible GMT expression vectors were kindly provided by Dr. Deepak Srivastava from the Gladstone Institute and the University of California at San Francisco. The lentiviral tetO-Tbx5-IRES-puromycin vector was generated using a previously described lentiviral expression vector and Tbx5 cDNA from the commercially available Tbx5 pYX-Asc expression vector (Open Biosystems).

Lentivirus production
293T cells were transfected with rtTA or doxycycline-inducible mGata4, mMef2c, or mTbx5 expressing lentiviral plasmids along with VSV-G and D8.9 packaging plasmids using FuGENE HD Transfection Reagent according the manufacturer’s recommended protocol (Roche, NJ). Viral supernatants were harvested for three consecutive days beginning 24h following transfection and then concentrated 100-fold by ultracentrifugation.

Isolation of TTFs and CFs
Alpha-MHC-Cre transgenic, cTnT-Cre, and Nkx2.5-Cre knock-in mice were bred with the ROSA26 Cre reporter mice (Jackson Laboratory, ME) to generate αMHC-Cre/ROSA26 Cre, cTnT-Cre/ ROSA26 Cre, Nkx2.5-Cre/ROSA26 Cre reporter mice, respectively. CFs and TTFs were derived from these mice by collagenase digestion (37°C for 60 min) of minced whole heart and tail biopsies from 3-6 week-old mice. Thy1.2+ CFs from Nkx2.5-Cre/ROSA26 Cre mouse hearts were purified by FACS using an APC-conjugated Thy1.2 antibody (eBioscience, CA). All animal studies described have received prior approval from the Subcommittee on Research Animal Care at Massachusetts General Hospital.

Flow Cytometry
Flow cytometry analysis was performed on a FACSCalibur® flow cytometer (BD Biosciences) using the CellQuest v3.3 software (BD Biosciences, San Jose, CA). The results were analyzed with the FlowJo v7.6 software (Tree Star, Ashland, OR).

Analysis of gene expression by qPCR and microarray
Total RNA was isolated using RNeasy Mini Kits (Qiagen, CA) according to the manufacturer’s protocols. cDNA was made using I-Script cDNA Synthesis Kits (BioRad, CA). Quantitative PCR was performed using gene-specific primers with SYBR Green® substrate (BioRad, CA) for 40 cycles on a Realpex Mastercycler (Eppendorf, Germany). Primers sequences are available upon request. For microarray analysis, total RNA was purified, linearly amplified and hybridized onto an Affymetrix Mouse Gene 1.0 ST Array (Affymetrix, CA) at the Molecular Genetics Core Facility at Children’s Hospital Boston. Gene expression data was background corrected and normalized by rma using Bioconductor. Genes with low expression and low variance across samples were filtered out. Samples were clustered on the remaining 4858 probe sets using the pvclust package.

Eletrophysiological studies
Patch clamping analysis was performed with a standard Tyrode extracellular solution (mM): 140 NaCl, 3.5 KCl, 10 Dextrose, 2 CaCl2, 1 MgCl, 0.3 NaH2PO4, 10 HEPES (PH 7.4 with NaOH). Action potential recordings were recorded using glass micropipettes (6-9 MΩ) filled with an intracellular solution (mM): 130 KCl, 5 Mg-ATP, 5 EGTA, 1 MgCl, 10 HEPES (PH of 7.2 with KOH). Nifedipine (Sigma, MO) was added to the extracellular solution at 5um where indicated. Recordings were taken at room temperature with an Axopatch 200B amplifier (Axon Instruments, CA).

Animal surgery and bioluminescence imaging
8-12 week female SCID mice were used as transplant recipients. Each SCID mouse (n=3) was subjected to permanent LAD ligation, followed immediately by an injection of 5×10^5 GMT-infected luciferase +eGFP+ CFs harvested at one week after GMT infection. Luciferase+eGFP+ CFs were derived from double transgenic luciferase+eGFP+ male mice described previously. As a control, uninfected cardiac cells from luciferase+eGFP+ male mice was injected into LAD ligated SCID mice in parallel (n=3). Bioluminescence imaging was performed on isoflurane anesthetized cell transplanted mice on days 1, 3, 5, and 8 using the Xenogen IVIS 200 System (Caliper, MA). After acquiring baseline images, mice were treated with D-luciferin (200 mg/Kg) by intraperitoneal injection, and images were captured 20 minutes post-injection. Peak signals (photons/s/cm²/sr) from a defined region of interest were recorded.
Single cell PCR array

Eight days after transplantation of luciferase+eGFP+ GMT-infected CFs, the recipient hearts were harvested and enzymatically digested into single cell suspension and sorted for single eGFP+/H-2Kd− (i.e. non-SCID origin) cells. Isolation of total RNA and synthesis of cDNA from each single cell was performed using a one-step PCR kit (Invitrogen, NY). Multi-gene qPCR array was performed according to the manufacturer’s suggested protocol (Fluidigm, CA). Resulting gene expression values were normalized to GAPDH.

Immunocytochemical staining

Cells were fixed using 4% paraformaldehyde, then permeabilized and stained according to the manufacturer’s recommendations (Abcam, MA). Tbx5 primary antibody – ab18531 (Abcam, MA); Gata4 primary antibody – SC 1237 (Santa Cruz, CA); Mef2c primary antibody – SC 13266 (Santa Cruz, CA); Secondary antibodies A11055 and A11034 (AlexaFluor/Invitrogen, CA).

Luciferase assay for mGata4, mMef2c, mTbx5 transcriptional activity

To assess the protein activity of overexpressed Gata4, Mef2c and Tbx5, Nkx2.5 cardiac enhancer-luciferase reporter, myosin light chain kinase-luciferase and ANF-luciferase reporters were used respectively. For each expression cDNA plasmid (100 to 200 ng), SuperFect® transfection reagent (Qiagen) was used to introduce the corresponding luciferase reporter plasmid (200 ng) into H9C2 cells. pBluescript KSII(+) was used to normalize the total quantity of plasmid used in each transfection, and pCMV-β-Gal (50 ng) was cotransfected for normalization of transfection efficiency. After 48 h, cells were lysed in situ, and luciferase reporter activity was determined by using d−(−)-luciferin (Boehringer Mannheim) as the substrate. Galactosidase activity was measured with Galacto-Light Plus (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus was measured on a Luminometer plate reader (Berthold). Each transfection was performed in triplicate in at least three independent experiments.

ES in vitro cardiac differentiation

Mouse ES cells were cultured on gelatin-coated dishes in LIF supplemented media prior to differentiation. ES cells were differentiated in hanging droplet aggregates starting on day 0 in the absence of LIF.

Statistical Analysis

For studies related to the upregulation of gene expression and luciferase activity after GMT overexpression, one-tailed Student’s t-tests were performed. For studies on cardiac gene induction after GMT overexpression, two-tailed Student’s t-tests were performed.

References


**Online Figure I. Confirmation of GMT overexpression in CFs and TTFs**

(A) Expression of mGata4, mMef2c, and mTbx5 in TTFs following lentiviral infection with GMT lentiviruses compared with uninfected TTFs.

(B) Immunocytochemical staining of mGata4, mMef2c and mTbx5 proteins in GMT infected TTFs. Images acquired at 10x magnification.

(C) *In vitro* luciferase reporter assays to assess the transcriptional activity of mGata4, mMef2c and mTbx5.

(D) Comparison of mMef2c transcript levels in uninfected TTFs and CFs with ES cells.

(E) Expression of mGata4, mMef2c, and mTbx5 in CFs following lentiviral infection with GMT lentiviruses compare with uninfected CFs.

(F) Comparison of the level of expression of mGata4 transcripts in uninfected TTFs and CFs with ES cells. Asterisks denote statistically significant increases in gene expression or luciferase activity: * p<0.05, ** p<0.01.

**Online Figure II. In vitro cardiomyocyte differentiation of αMHC-Cre/ROSA26tmTmG ES cells.**

The αMHC-Cre/ROSA26tmTmG ES cell line was derived from blastocyst-stage embryos. The double transgenic ES cells were in vitro differentiated for 15 days and fluorescence microscopy was performed on undifferentiated (top) and differentiated (bottom) ES cells. Note the appearance of eGFP+ cardiomyocytes against a background of dTomato+ non-cardiomyocytes.

**Online Figure III. Relative expression of cardiac Troponin T in TTFs.**

The relative expression of cTnT in uninfected and GMT infected TTFs 3 weeks post-infection compared with E10.5 murine heart cells. Asterisks denote statistically significant increases in gene expression after GMT overexpression: ** p<0.01.

**Online Figure IV. Global analysis of gene expression before and after GMT overexpression**

Heat map of global gene expression patterns in uninfected and GMT overexpressing TTFs and CFs compared to E10.5 heart.
Figure a: Confirmation of GMT overexpression in CFs and TTFs

(A) Expression of mGata4, mMef2c, and mTbx5 in TTFs following lentiviral infection with GMT lentiviruses compared with uninfected TTFs.
(B) Immunocytochemical staining of mGata4, mMef2c and mTbx5 proteins in GMT infected TTFs. Images acquired at 10x magnification.
(C) In vitro luciferase reporter assays to assess the transcriptional activity of mGata4, mMef2c and mTbx5.
(D) Comparison of mMef2c transcript levels in uninfected TTFs and CFs with ES cells.
(E) Expression of mGata4, mMef2c, and mTbx5 in CFs following lentiviral infection with GMT lentiviruses compare with uninfected CFs.
(F) Comparison of the level of expression of mGata4 transcripts in uninfected TTFs and CFs with ES cells.

Asterisks denote statistically significant increases in gene expression or luciferase activity: * p<0.05, ** p<0.01.
**Figure 1:** In vitro cardiomyocyte differentiation of αMHC-Cre/ROSA26<sup>mtmG</sup> ES cells. The αMHC-Cre/ROSA26<sup>mtmG</sup> ES cell line was derived from blastocyst-stage embryos. The double transgenic ES cells were in vitro differentiated for 15 days and fluorescence microscopy was performed on undifferentiated (top) and differentiated (bottom) ES cells. Note the appearance of eGFP+ cardiomyocytes against a background of dTomato+ non-cardiomyocytes.
**Figure** Relative expression of cardiac Troponin T in TTFs.

The relative expression of cTnT in uninfected and GMT infected TTFs 3 weeks post-infection compared with E10.5 murine heart cells. Asterisks denote statistically significant increases in gene expression after GMT overexpression: **p<0.01.**
**Figure 1. Global gene expression**
Heat map of global gene expression patterns in uninfected and GMT overexpressing TTFs and CFs compared to E10.5 heart.