

ORIGINAL RESEARCH

Hippo Pathway Effector Tead1 Induces Cardiac Fibroblast to Cardiomyocyte Reprogramming

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BACKGROUND: The conversion of fibroblasts into induced cardiomyocytes may regenerate myocardial tissue from cardiac scar through in situ cell transdifferentiation. The efficiency transdifferentiation is low, especially for human cells. We explored the leveraging of Hippo pathway intermediates to enhance induced cardiomyocyte generation.

METHODS AND RESULTS: We screened Hippo effectors Yap (yes-associated protein), Taz (transcriptional activator binding domain), and Tead1 (TEA domain transcription factor 1; Td) for their reprogramming efficacy with cardio-differentiating factors Gata4, Mef2C, and Tbx5 (GMT). Td induced nearly 3-fold increased expression of cardiomyocyte marker cTnT (cardiac troponin T) by mouse embryonic and adult rat fibroblasts versus GMT administration alone ($P < 0.0001$), while Yap and Taz failed to enhance cTnT expression. Serial substitution demonstrated that Td replacement of TBX5 induced the greatest cTnT expression enhancement and sarcomere organization in rat fibroblasts treated with all GMT substitutions (GMTd versus GMT: $17 \pm 1.2\%$ versus $5.4 \pm 0.3\%$, $P < 0.0001$). Cell contractility (beating) was seen in 6% of GMTd-treated cells by 4 weeks after treatment, whereas no beating GMT-treated cells were observed. Human cardiac fibroblasts likewise demonstrated increased cTnT expression with GMTd versus GMT treatment ($7.5 \pm 0.3\%$ versus $3.0 \pm 0.3\%$, $P < 0.01$). Mechanistically, GMTd administration increased expression of the trimethylated lysine 4 of histone 3 (H3K4me3) mark at the promoter regions of cardio-differentiation genes and mitochondrial biogenesis regulator genes in rat and human fibroblast, compared with GMT.

CONCLUSIONS: These data suggest that the Hippo pathway intermediate Tead1 is an important regulator of cardiac reprogramming that increases the efficiency of matured induced cardiomyocytes generation and may be a vital component of human cardiodifferentiation strategies.

Key Words: cardiomyocytes ■ direct reprogramming ■ regeneration

Heart failure remains a leading cause of cardiac death worldwide, for which current interventional treatments remain suboptimal.¹ While cardiac regeneration strategies hold great theoretical promise as a treatment for heart failure, they are hindered by the limited innate regenerative capacity of the adult human heart.² The in situ reprogramming of cardiac fibroblasts into induced cardiomyocytes (iCMs) thus represents a promising new myocardial regeneration strategy.^{3,4} We and others have shown that a wide variety of cardiac

reprogramming cocktails based upon the core cardiac transcription factors Gata4, Mef2c and Tbx5 (GMT) can convert fibroblasts into iCMs, albeit with low efficiency in inducing contractile cells in vitro, especially as applied to human cells.^{5–16}

The TEAD (transcriptional enhance associate domain) transcription factor family is a component of the Hippo signaling pathway, the inactivation of which has been implicated in cell replication, growth, and development.^{17–20} The role of TEAD in the Hippo pathway is

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CLINICAL PERSPECTIVE

What Is New?

- Hippo pathway effector Tead1 in combination with Gata4 and Mef2c enhances the efficiency and improves the structural and functional transdifferentiation of reprogrammed rat and human cardiac fibroblasts compared with use of a standard Gata4, Mef2C, and Tbx5 cocktail.
- Tead1 enhance cardiac reprogramming by regulating mitochondrial biogenesis through peroxisome proliferator-activated receptor- γ co-activator PGC (peroxisome proliferator-activated receptor- γ coactivator)-1A/1B.

What Are the Clinical Implications?

- Tead1 may provide an important transcriptional regulatory role in cardiac fibroblast transdifferentiation essential for induced cardiomyocyte generation, providing a cocktail of genetic factors that have potential for further applications in in vivo cardiac regeneration.

Nonstandard Abbreviations and Acronyms

| | |
|---------------|---|
| cTnT | cardiac troponin T |
| GFP | green fluorescent protein |
| GMT | Gata4, Mef2c, and Tbx5 |
| iCMs | induced cardiomyocytes |
| TAZ | transcriptional activator binding domain |
| TEAD | transcriptional enhanced associate domain |
| Tead 1 | TEA domain transcription factor 1 |
| YAP | yes-associated protein |

broadly believed to be regulated by the presence or the absence of nuclear YAP (yes-associated protein) and TAZ (transcriptional activator binding domain).²¹ Recent studies suggest, however, that TEAD itself is actively regulated and may be an active effector in the Hippo pathway.²²

Of the 4 members of the TEAD family, Tead1 (also known as transcriptional enhancer factor 1 [TEF-1]) is the most abundant in the heart, is a transcriptional regulator of myogenic genes, and is required for heart development.^{17,23} A recent report also suggests that Tead1 is an integral component of the cardiac transcriptional regulatory network and that it regulates core cardiomyocyte-specific functions including contraction and metabolism, and shares binding domains with key cardio-differentiation factors such as p300, Gata4, Nkx2.5, and Mef2a.^{24–27}

These observations led us to hypothesize that Tead1 may provide an important transcriptional regulatory role in cardiac fibroblast transdifferentiation essential for iCMs generation. We consequently sought to test Tead1 (Td) as a potential factor that could increase the efficiency of cardiac reprogramming by enhancing cardio-differentiating gene activation.

METHODS

Per the Transparency and Openness Promotion Guidelines, the data, analytic methods, and study materials will be available to other researchers on request to the corresponding author.

Tissue Collection and Isolation of Cardiac Cells

All animal experiments were approved by Institutional Animal Care and Use Committee at Baylor College of Medicine and all methods were carried out in accordance with the NIH guidelines (Guide for the care and use of laboratory animals) and under protocol AN-6223, approved by the Institutional Animal Care and Use Committee. Cardiac fibroblasts were isolated from adult rat cardiac tissues harvested from 6- to 8-week-old male Sprague-Dawley rats (Envigo International Holding Inc., Hackensack, NJ) using standard cell isolation techniques.⁸ Adult human cardiac fibroblasts were harvested from ventricular tissue obtained from heart failure patients undergoing cardiac transplantation at Baylor St. Luke's Medical Center and under a protocol approved by the Baylor College of Medicine Institutional Review Board (IRB H-33421).⁹ Human tissues were manually sharply minced and placed in fibroblast growth medium (Dulbecco modified eagle medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin), as previously described.¹⁰

Cell Reprogramming

Lentivirus vectors encoding Gata4 (G), Mef2c (M), Tbx5 (T), Tead1 (Td), YAP, TAZ, PGC (peroxisome proliferator-activated receptor- γ coactivator)-1A, PGC-1B and GFP (green fluorescent protein) were prepared from relevant plasmids by the Baylor College of Medicine Gene Vector Core, as previously described.^{8,9,15} A combination of 4 factors (GMTTd) was used for reprogramming rat and human cardiac fibroblasts reprogramming. Rat and human cardiac fibroblasts were seeded at a density of 5×10^4 cells per well of a 24-well plate in Iscove modified dulbecco media medium containing 20% FBS, and 1% penicillin/streptomycin. Lentivirus vectors with a multiplicity of infection: 20 was added with polybrene (5 $\mu\text{g}/\mu\text{L}$) after 24 hours. Two days later, medium was replaced with induction medium (iCM media), containing DMEM/Medium-199 (4:1), 10% FBS, 5% horse serum,

1% penicillin/streptomycin, 1% non-essential amino acids, 1% essential amino acids, 1% GlutaMAX, and 1% sodium pyruvate.¹¹ Induction media was changed twice a week until cells were harvested.

For rat contractility studies, iCM medium was changed on day 14 of culture to beating media containing RPMI1640/2% B27/2%FBS/0.05%BSA/50 µg/mL ascorbic acid/0.2 mmol/L GlutaMAX, 1×NEAA/1% insulin-selenium-transferrin/1% vitamin mixture/10% conditioned medium obtained from neonatal rat/mouse cardiomyocyte culture. Medium was changed every 3 to 4 days.

For mitochondrial induction studies, media was replaced with induction media after 48 hours of lentiviral transduction and the compounds PGC-1A activator (ZLN005, MCE, MedChem Express, # HY-17538, 1 µmol/L) PGC-1A inhibitor (SR-18292, MCE, MedChem Express, # HY-101491, 0.1 µmol/L) were added and maintained in culture throughout the duration of the experiment for 14 days.

For human reprogramming studies using co-culture techniques, neonatal rat pups were used to isolate cardiomyocytes under protocol (AN-6223), as previously described.⁹ Human fibroblasts were coinfecting with lentiviral GMT or GMTd. After 1 week of transduction, the infected fibroblasts were harvested and re-plated onto neonatal rat cardiomyocytes at a ratio of 1:10 and cultured in DMEM/M-199/10% FBS medium.¹²

High Throughput Screening

Induced murine embryonic fibroblast cell line were used for high throughput screening for iCMs that overexpressed GMT in response to doxycycline treatment and expressed a GFP reporter gene driven by a promoter sequence responsive to the cardiomyocyte marker alpha muscle heavy chain-GFP.²⁸ In brief, the alpha muscle heavy chain-GFP cardiac reporter in the induced murine embryonic fibroblast cell line indicates reprogramming with GFP reporter expression. Real-time quantification of GFP⁺ events were captured by IncucyteG3 imaging system (Incucyte Live-Cell Analysis System; Sartorius) at 37°C for 7 days. Five images (1.75×1.29 mm FOV) per well at ×10 magnification were acquired every 4 hours using phase contrast and a GFP filter (excitation: 460 nm with passband [440 480 nm]; emission: 524 nm with passband [504, 544 nm]). Images were exported as 8 bit TIFF and 16 bit TIFF for phase and GFP, respectively, for further analysis.

Flow Cytometry

Cells were trypsinized and fixed with fixation buffer (BD Biosciences, San Jose, CA) for 15 minutes at room temperature. Fixed cells were washed with Perm/Wash buffer (BD Biosciences).^{8,9} Then, cells were incubated with primary mouse anti-cTnT (cardiac troponin T) antibody (ab8295) in Perm/Wash buffer for 90 minutes

at room temperature followed by incubation with donkey anti-mouse Alexa Fluor 647 (ab150107). Cells were washed with Perm/Wash buffer, and then analyzed for cTnT expression using an LSR Fortessa cell sorter (BD Biosciences, Franklin Lakes, NJ) with FlowJo software (Flowjo, LLC, Ashland, Ore).

Immunocytochemistry

Immunofluorescence staining was performed as previously described.⁹ Cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton-X solution. Cells were then blocked with 10% goat serum and incubated with primary antibodies against cTnT (1:300 dilution; Thermo Fisher Scientific), Gja 1 (1:500 dilution; Abcam), cTnI (1:200 dilution; Abcam), or α-actinin (1:300 dilution; Sigma-Aldrich, St. Louis, MO), followed by the appropriate Alexa fluorogenic secondary antibodies (Invitrogen), and finally with DAPI (Invitrogen).^{8,9,11} Proliferation assay was performed using anti-Ki-67 (1:250 dilution; Abcam). After being washed with PBS 3 times, cells were incubated with secondary antibodies conjugated with Alexa Fluor 555 (Invitrogen) for 1 hour at room temperature in the dark. DAPI (1:1000; Sigma) was used to stain nuclei. iCM proliferation was calculated by counting Ki-67⁺, cTnT⁺, and DAPI⁺ cells in every image.

Real-Time Polymerase Chain Reaction

Total RNA was extracted using the TRIzol method (Invitrogen), after which relative quantification was performed using SYBR-green detection of polymerase chain reaction (PCR) products in real time with the ABI ViiA 7 (Applied Biosystems Inc., Foster City, CA). The following rat and human primers were used for these studies: CTNT (cardiac troponin T), NKX-2.5 (homeobox protein 2.5), MYL7 (myosin light chain 7), ACTC1 (actin alpha cardiac muscle 1), RYR2 (ryanodine receptor 2), GJA1 (gap junction protein alpha 1), ATP2A2 (sarco-plasmic reticulum calcium-ATPase 2), SCN5A (sodium channel protein 5), HCN4 (hyperpolarization-activated cyclic nucleotide-gated channel), KCNA5 (Potassium Voltage-Gated Channel 5), COL1A1 (Collagen type 1 alpha 1), COL1A2 (Collagen type 1 alpha 2), S100A4 (S100 Calcium-Binding Protein A4), POSTN (periostin), PGC1A/B (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha and beta), and TFAM (mitochondrial transcription factor). Sequences of the primers are listed in Table S1. mRNA levels were normalized by comparison to GAPDH. Briefly, the cycle threshold (Ct) values of each gene of interest and of GAPDH were calculated for each sample and then the normalized value was derived by subtracting the Ct value of GAPDH from that of the gene of interest (ΔCt). Data are shown as mRNA fold change ($2^{-\Delta\Delta Ct}$) relative to the mRNA level of the corresponding transcript in the control samples as indicated.

Western Analysis

To perform Western analyses, cell lysates were prepared by homogenization of cells in cell lysis buffer and run on SDS-PAGE. After separation, the protein bands were transferred to nitrocellulose membrane (Invitrogen Catalog number: IB301001), immune detection was performed with the following primary antibodies: PGC-1A (Abcam: ab106814), PGC-1B (Abcam: ab176328), TFAM (Abcam: ab131607), and actin (Sigma-Aldrich), followed by treatment with appropriate horseradish peroxidase-conjugated secondary antibodies (Millipore, Billerica, MA). Membranes were visualized by chemiluminescence detection (Thermo Scientific catalog no: 34580).

Measurements of Contractility, Calcium Transient, and Beating

To measure cell shortening and calcium transients iCMs were placed to a perfusion chamber mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200). iCMs were continuously perfused with 1.8 mmol/L Ca^{2+} -Tyrode solution containing (in mmol/L): NaCl 140, KCl 5.4, MgCl_2 1, CaCl_2 1.8, HEPES 5, and glucose 10, pH 7.4. When required, cells were field stimulated by means of platinum electrodes within the chamber, with a suprathreshold voltage applied over a range of frequencies. Contractions of iCMs was assessed using a video-based edge detection system (IonOptix, Dublin, Ireland). Ca^{2+} transients measurements was measured with 2 $\mu\text{mol/L}$ Fura-2/AM (Life Technologies) together with 0.1% Pluronic F-127 (Invitrogen) according to the manufacturer's instruction. $[\text{Ca}^{2+}]_i$ was detected by exciting Fura-2 alternately at 340 and 380 nm at 0.5 Hz, and recording emitted fluorescences at 510 nm. Both edge detection and Fura-2 fluorescence measurement were made simultaneously from the same contracting region of the cell monolayer. Data analysis was performed using IonWizard software (IonOptix).^{9,11}

For beating cell counting, we seeded 50 000 fibroblasts per well on 12-well plates, performed cell transductions as detailed above and then analyzed cell contraction by light microscopy performed at room temperature. Spontaneous contracting cells were manually counted in 10 randomly selected high-power fields per well in at least 3 independent experiments.

We identified the individual beating iCMs based on differences in beating frequency, cell-membrane boundary, and GFP fluorescence expression. The cardiac reprogramming efficiency was determined as the number of beating cells per 50 000 starting fibroblasts.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed on murine embryonic fibroblasts (ATCC) using ChromaFlash Chromatin Extraction kits (p-2001, Epigenetek, Farmingdale, NY) as described previously.²⁹ Cells were seeded onto 150 mm dishes at density of 10^6 , treated 24 hours later with lentivirus encoding GFP or Tead1 (20 moi) with 5 $\mu\text{g}/\mu\text{L}$ polybrene. Cells were harvested for chromatin immunoprecipitation assay-quantitative polymerase chain reaction (qPCR) 7 days later. Chromatin immunoprecipitations were conducted with a rabbit polyclonal antibody anti-Histone H3 (tri methyl K4) CHIP Grade (ab8580), and normal rabbit immunoglobulin G (AB-105-C) was obtained from R&D systems. All primers for chromatin immunoprecipitation assay-qPCR assays were synthesized by Sigma. These sequences are listed in Table S2. Fold enrichment of PCR product was calculated after normalization with input of all 3 types of infected cells.

Statistical Analysis

Statistical analysis was performed using SAS, version 9.4. Results are presented as mean \pm SEM. Three independent experiments with triplicate determinations were performed. The non-parametric Mann-Whitney *U* test (Wilcoxon rank-sum test) was used when comparing 2 groups, and the Kruskal-Wallis test, followed by Bonferroni corrections, was used when comparing >2 groups to analyze statistical significance. A $P < 0.05$ was regarded as significant.

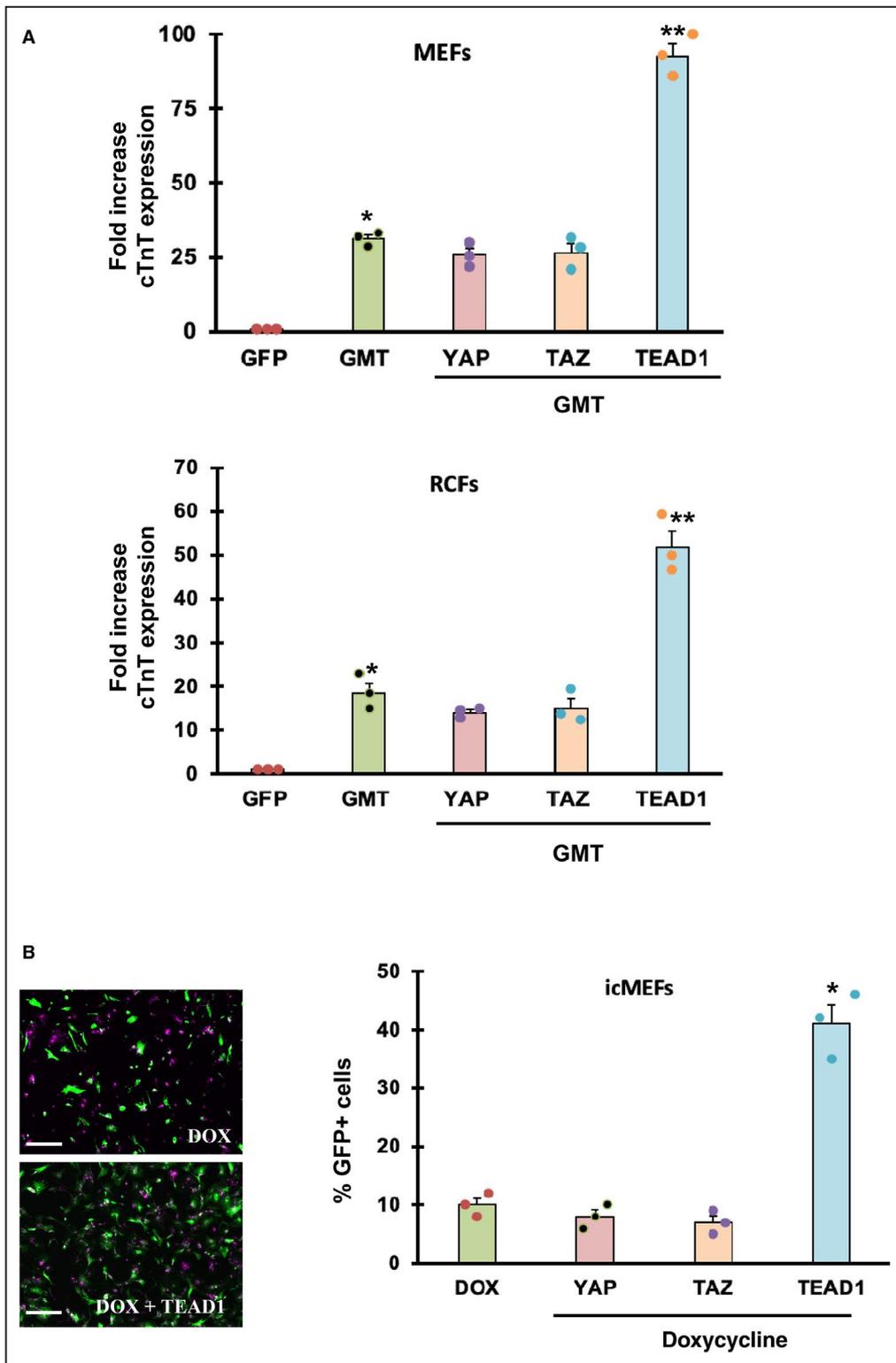
RESULTS

Hippo Pathway Effector Screening Demonstrating That Tead1 Enhances Cardiodifferentiation Marker Expression

We found that Tead1 in combination with GMT administration increased the expression of the

Figure 1. Hippo pathway effector screening in mouse embryonic and adult rat cardiac fibroblasts.

A, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of mouse embryonic fibroblasts (MEFs; top) and adult rat cardiac fibroblasts (RCFs; bottom) demonstrating increased cTnT (cardiac troponin T) gene expression 7 days after combined treatment with Gata4, Mef2c, and Tbx5 (GMT) and Tead1 (TEA domain transcription factor 1) compared with GMT treatment with other indicated effectors ($n=3$ biological replicates, each performed in triplicate; * $P < 0.001$ vs GFP (green fluorescent protein), ** $P < 0.0001$ vs GMT; Wilcoxon rank-sum test). **B**, Real-time high throughput cardiomyocyte marker cell screening using an induced murine embryonic fibroblast cell line and "IncucyteG3" imaging system (see Methods). Left: Representative immunocytochemistry images of GFP-positive cells (indicating activation of α -muscle heavy chain- GFP reporter expression). Right: Quantification of the percentage of GFP-positive cells 7 days after treatment with indicated effectors ($n=3$ biological replicates, each performed in triplicate. cTnT indicates cardiac troponin T; GFP, green fluorescent protein; GMT, Gata4, Mef2C, and Tbx5; icMEF, induced murine embryonic fibroblast cell line; MEF, mouse embryonic fibroblasts; RCFs, rat cardiac fibroblasts; TAZ, transcriptional activator binding domain; and YAP, yes-associated protein. * $P < 0.0001$ vs doxycycline only; Wilcoxon rank-sum test). Scale bar: 400 μm .



cardiomyocyte marker cTnT in mouse embryonic and adult rat cardiac fibroblasts by ≈ 3 -fold compared with GMT administration alone ($P < 0.0001$), whereas 2 other Hippo pathway effectors YAP and TAZ did not increase cTnT expression above GMT treatment levels (Figure 1A). We then used

our GMT- (doxycycline) inducible alpha muscle heavy chain-GFP murine embryonic fibroblast high throughput cardio-differentiated cell assay to demonstrate that lentiviral-mediated administration of Tead1 to GMT overexpressing cells increased cardiac reprogramming efficiency compared with cells

overexpressing GMT without addition of Tead1, as indicated by the percentage of GFP⁺ cells, ($P < 0.0001$). In comparison, we observed that lentiviral-mediated administration of YAP or TAZ failed to enhance cardiac reprogramming efficiency above doxycycline-induced GMT overexpression (Figure 1B).

Tead1 Enhances the Efficiency and Improves the Cardiomyocyte-Like Maturation of Reprogrammed Rat Cardiac Fibroblasts

In serial substitution studies using GMT as our baseline transdifferentiation strategy, we demonstrated that TBX5 was dispensable in the presence of Tead1 and that the GMTd yielded the highest levels of cTnT expression compared with cells treated with GMT alone (GMTd versus GMT: $17 \pm 1.2\%$ versus $5.4 \pm 0.3\%$; $P < 0.0001$; Figure 2A and 2B). Interestingly, replacement of Gata4 and Mef2c with Tead1 in a GMT cocktail did not increase cardiac reprogramming compared with GMT administration alone, suggesting these genes are not dispensable during reprogramming (Figure 2A and 2B).

To assess transdifferentiated cell maturity, we examined the expression of a panel of genes relevant to cardiomyocyte functional structure (CTNT, MYL7, ACTC1, NKX2.5, RYR2), and ion channels (GJA1, ATP2A2, SCN5A, HCN4, KCNA5). We observed by qRT-PCR that GMTd treatment increased the expression of these genes in rat cardiac fibroblasts compared with treatment with GFP alone (Figure 2C). Consistent with these findings, we observed that GMTd also decreased the expression of the fibroblast marker gene COL1A1, COL1A2, S100A4, POSTN versus GFP treatment alone (Figure 2C).

Immunofluorescence analyses likewise demonstrated greater numbers of cells expressing the cardiomyocyte markers cTnT and α -sarcomeric actinin after 2 weeks of reprogramming with GMTd compared with treatment with GMT alone; however, sarcomeric structures was not obvious at this early stage (Figure 3A and 3B). The GMTd-induced iCMs showed clear cardiac

TNT, TNI3, phalloidin and, α -sarcomeric actinin with advanced sarcomere organization after 4 weeks of reprogramming (Figure 3C). In addition, GMTd treatment was seen to induce a ≈ 4 -fold increase in the number of α -sarcomeric actinin⁺ cells with assembled sarcomeres after 4 weeks compared with cells treated with GMT alone (Figure 3D). We also observed formation of gap-junction protein Gja1 channels after GMTd but not after GMT treatment (Figure 3E). In the context of the known proliferative properties of Tead1, Ki67 proliferation assays revealed that the increase in these cardio-differentiated GMTd-treated cells was not attributable to cell proliferation (Figure S1).

Tead1 Enhances the Generation of Functional iCMs

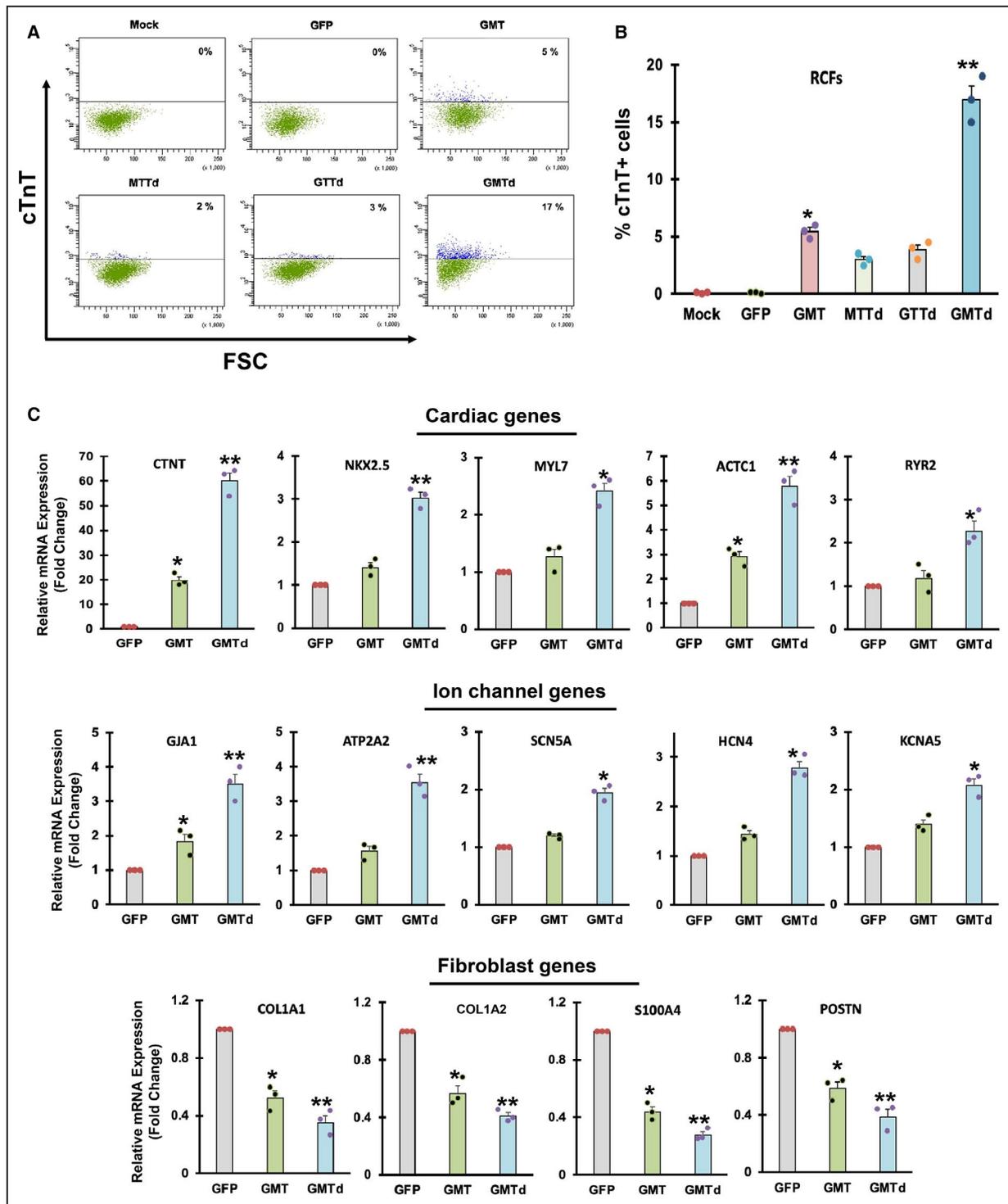
Spontaneous contraction was observed in 6% of rat cardiac fibroblasts as early as 4 weeks after GMTd treatment but was not observed in untreated cells or GMT-treated cells for up 8 weeks after cell transduction. (Figure 4A and Videos S1 through S3). All contracting GMTd-treated cells were also observed to demonstrate calcium transients on electrical stimulation, but calcium transients were not seen in untreated or GMT-treated cells (Figure 4B). The number of these beating GMTd-treated cells increased over time (Figure 4C).

Tead1 Enhances Human Cardiac Fibroblast Reprogramming

Human cardiac fibroblasts treated with GMTd were shown by fluorescence-activated cell sorting to demonstrate increased cTnT expression compared with cells treated with GMT alone ($7.5 \pm 0.3\%$ versus $3 \pm 0.3\%$; $P < 0.01$; Figure 5A and 5B). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis likewise demonstrated increased expression of other cardiomyocyte transdifferentiation genes (CTNT, MYH6, ACTC1) and ion channel genes (SCN5A, HCN4) and downregulation of the fibroblast marker gene (COL1A1, COL1A2, POSTN) after GMTd treatment (Figure 5C). Increased expression of cTnT

Figure 2. Tead1 enhances expression of a portfolio of cardiomyocyte marker genes in adult rat cardiac fibroblasts.

A, Representative flow cytometry plots of rat cardiac fibroblasts 14 days after transduction demonstrating that the combination of Gata4, Mef2c and Tead1 (GMTd) enhances the expression of depicted cardiomyocyte marker genes compared with cell transduction with GFP (green fluorescent protein), GMT, or other Td substitutions of GMT (MTTd, GTTd). **B**, Quantification of the percentage of cTnT⁺ cells treated as above, as assessed by flow cytometry ($n = 3$ biological replicates, each performed in triplicate; $*P < 0.01$ vs GFP, $**P < 0.0001$ vs GMT; Wilcoxon rank-sum test). **C**, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of rat cardiac fibroblasts demonstrating increased expression of depicted cardiomyocyte marker genes (CTNT, MYL7, ACTC1, NKX2.5, RYR2) and ion channel genes (GJA1, ATP2A2, SCN5A, HCN4, KCNA5) and decreased expression of fibroblast marker gene (COL1A1, COL1A2, S100A4, POSTN) 14 days after reprogramming factor administration ($n = 3$ biological replicates, each performed in triplicate. ACTC1, actin alpha cardiac muscle 1; ATP2A2, sarco (endo)plasmic reticulum calcium-ATPase 2; COL1A1, Collagen type 1 alpha 1; COL1A2, Collagen type 1 alpha 2; cTnT indicates cardiac troponin T; FSC, forward scatter; GFP, green fluorescent protein; GMT, Gata4, Mef2C, and Tbx5; GMTd, Gata4, Mef2C, and Tead1; GJA1, gap junction protein alpha 1; HCN4, hyperpolarization-activated cyclic nucleotide-gated channel; KCNA5, Potassium Voltage-Gated Channel 5; MYL7, myosin light chain 7; Nkx-2.5, homeobox protein 2.5; POSTN, periostin; RCFs, rat cardiac fibroblasts; RYR2, ryanodine receptor 2; SCN5A, sodium channel protein 5; and S100A4, S100 Calcium-Binding Protein A4. $*P < 0.01$, $**P < 0.001$ vs GFP; Kruskal-Wallis test).



and α -sarcomeric actinin in GMTd versus GMT-treated human cells was also demonstrated by immunofluorescence studies after 2 weeks of reprogramming; however, sarcomeric structures were not clear at this early stage (Figure 5D and 5E). The GMTd-induced iCMs showed clear cardiac TNT and α -sarcomeric actinin with advanced sarcomere organization after 4 weeks of reprogramming (Figure 5F). GMTd-treated

human cells demonstrated a 4-fold increase in the number of α -sarcomeric actinin⁺ cells with assembled sarcomeres after 4 weeks compared with human cells treated with GMT (Figure 5G).

Although human cardiac fibroblasts treated with GMTd were not observed to contract independently after up to 6 weeks in culture, \approx 5% of human cardiac fibroblasts treated with GMTd, as verified by their GFP

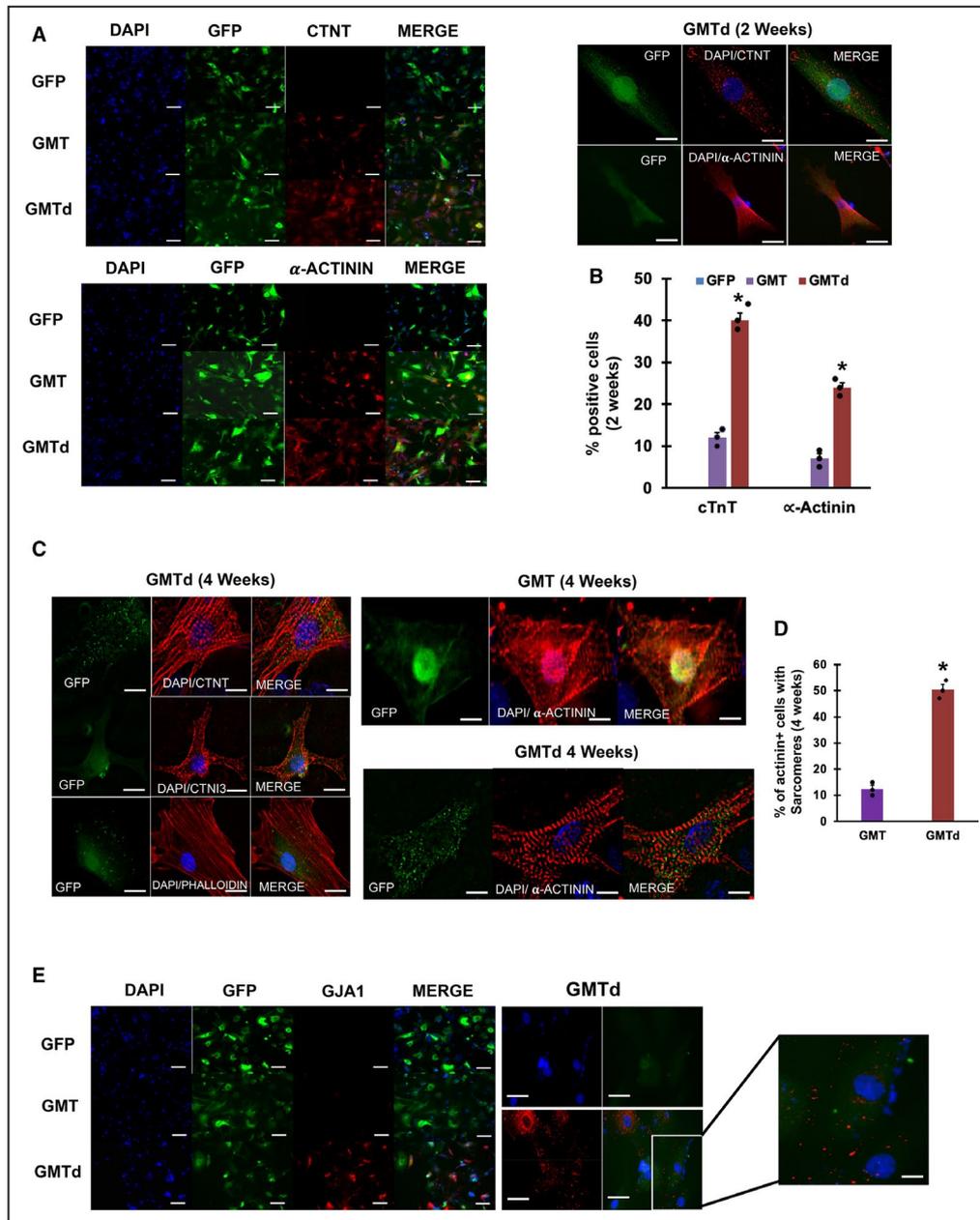


Figure 3. Tead1 enhances cardiomyocyte marker protein expression and ultrastructure.

Immunofluorescence analyses of rat cardiac fibroblasts 14 days after transduction with GFP (green fluorescent protein), GMT, or GMTd ($n=3/\text{group}$). DAPI nuclear marker: blue; GFP: green; cardiomyocyte markers: red. **A**, Representative images of immunofluorescence staining for cTnT (left top) and for α -actinin (left bottom) and high magnification views for cTnT (cardiac troponin T) and α -actinin (right), after 2 weeks. Scale bars: 100 μm (left images) and 25 μm (right [high magnification] images). **B**, Quantification of cells expressing cardiomyocyte markers cTnT and α -actinin after 2 weeks by immunofluorescence labelling ($*P<0.001$ vs GMT; Wilcoxon rank-sum test). **C**, Representative images of immunofluorescence staining for cTnT, CTnI3, and phalloidin (left) and α -actinin (middle) after 4 weeks of GMTd transduction demonstrating sarcomeric structures, most clearly visible in α -actinin labeled cells (middle). Scale bars: 25 μm . **D**, Quantification of cells with well-developed sarcomeres as a percentage of total α -actinin $^{+}$ cells after 4 weeks of GMT or GMTd transduction ($*P<0.001$ vs GMT; Wilcoxon rank-sum test). **E**, Representative images of immunofluorescence staining for GJA1 (gap junction alpha-1) protein demonstrating gap junction channels formed between cells treated with GMTd. GFP indicates green fluorescent protein; and GMT, Gata4, Mef2C, and Tbx5. Scale bars: 100 μm (left images); 25 μm (right [high magnification] images).

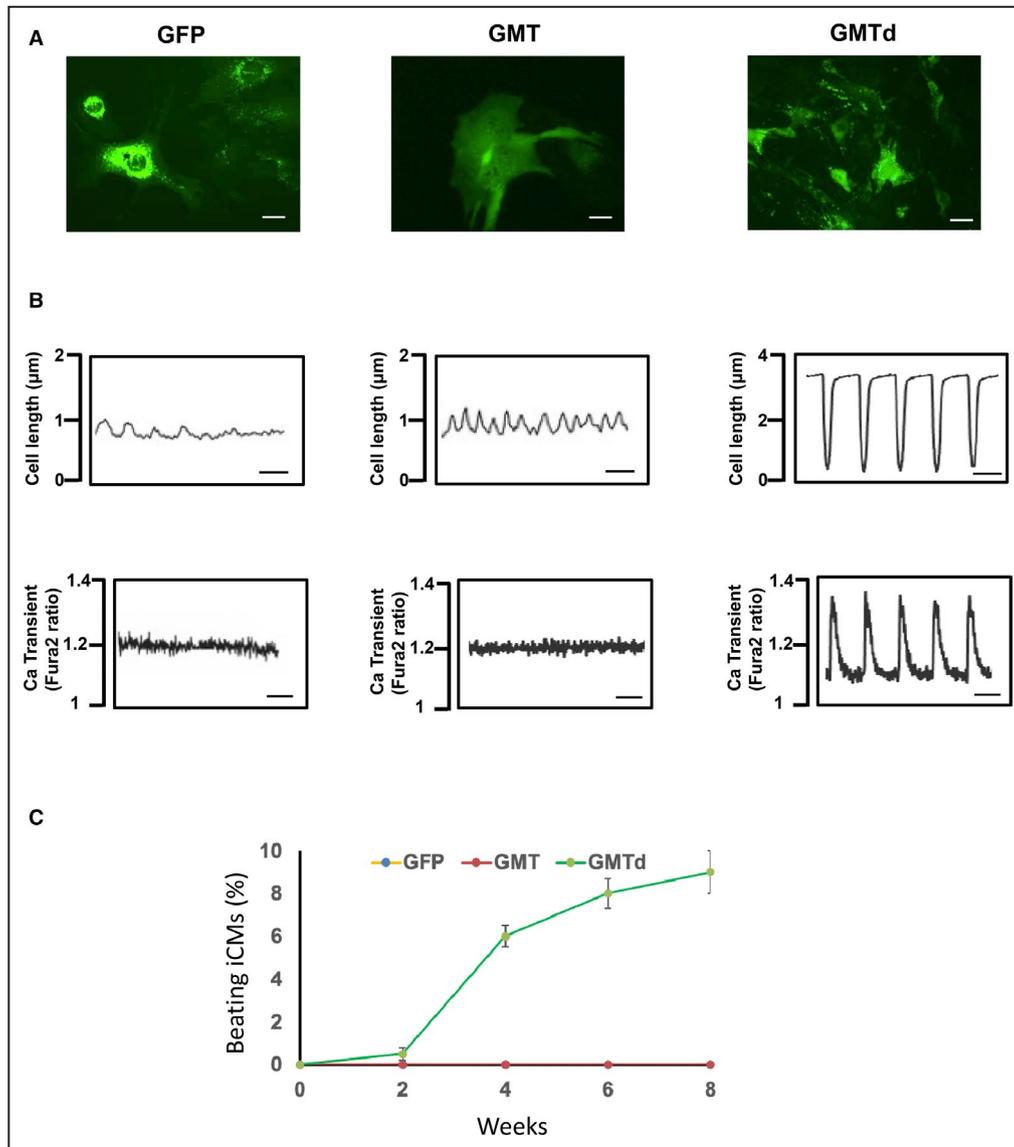


Figure 4. Tead1 enhances contractile function of reprogrammed cells.

A, Representative immunofluorescence staining 4 weeks after rat cardiac fibroblast transfection with lentivirus encoding GFP (green fluorescent protein), Gata4, Mef2C, and Tbx5 (GMT), or GMTd, as depicted by expression of encoded (green) GFP marker protein. Scale bar: 100 μm . **B**, Representative peaks from GFP⁺ rat cells after 4 weeks in culture reflecting cell contraction (top row) and Ca²⁺ transients (bottom row) in cells treated with GMTd, but not in cells treated with GMT or GFP alone. Scale bar: 0.5 seconds. **C**, Quantification of the number of spontaneously beating GMTd-treated cells treated as a function of time (n=3 biological replicates, each performed in triplicate). GFP indicates green fluorescent protein; GMT, Gata4, Mef2C, and Tbx5; GMTd, Gata4, Mef2C, Tead1, and iCMs, induced cardiomyocytes.

expression (Figure 5H), contracted synchronously with surrounding neonatal rat cardiomyocytes after 4 weeks in co-culture. In comparison, cells treated with GMT alone failed to contract in co-culture experiments (Figure 5H and Videos S4, S5). All contracting GMTd-treated human cells were also observed to demonstrate calcium transients on electrical stimulation, but calcium transients were not seen in cells treated with GMT alone (Figure 5I). Verification that the contracting cells were transfected fibroblasts, rather than the

neonatal cardiomyocytes, was provided by the uniform expression of GFP by these contracting cells, reflecting the transfection of the fibroblast culture in isolation before their reseeding into the co-culture plates.

Mechanisms of Action of Tead1 in Cardiac Reprogramming

Enrichment of the transcriptionally active chromatin mark trimethylated lysine 4 of histone 3 (H3K4me3)

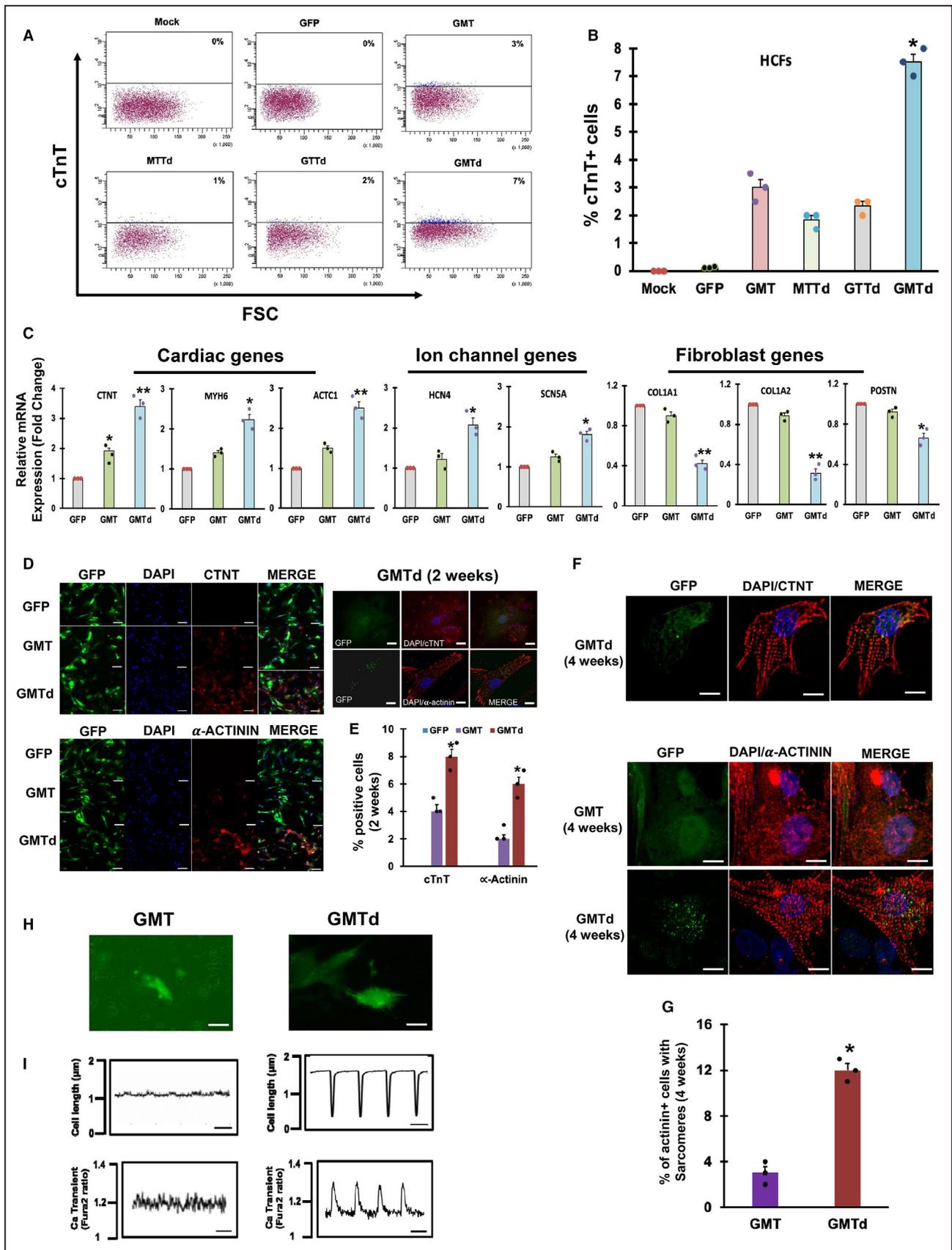


Figure 5. GMTd induces cardio-differentiation of human cardiac fibroblasts.

A, Representative flow cytometry plots of human cardiac fibroblasts 14 days after transduction demonstrating that GMTd enhances the expression of depicted cardiomyocyte marker genes compared with cell transduction with GFP (green fluorescent protein), Gata4, Mef2C, and Tbx5 (GMT), or other Td substitutions of GMT (MTTd, GTTd). **B**, Quantification of the percentage of cTnT⁺ cells treated as above, as assessed by flow cytometry (n=3 biological replicates, each performed in triplicate; **P*<0.01 vs GMT; Wilcoxon rank-sum test). **C**, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of human cardiac fibroblasts demonstrating increased expression of depicted cardiomyocyte marker genes (CTNT, MYH6, ACTC1) and ion channel genes (SCN5A, HCN4) and decreased expression of fibroblast marker gene (COL1A1, COL1A2 and POSTN) 14 days after reprogramming factor administration (n=3 biological replicates, each performed in triplicate; **P*<0.05, ***P*<0.001 vs GFP; Kruskal–Wallis test). Data were normalized against GFP values. Immunofluorescence analyses of human cardiac fibroblasts 14 days after transduction with GFP, GMT, or GMTd (n=3/group). DAPI nuclear marker: blue; GFP: green; cardiomyocyte markers: red. **D**, Representative images of immunofluorescence staining for cTnT (left top) and for α -actinin (left bottom) and high magnification views for cTnT and α -actinin (right) after 2 weeks. Scale bar: 100 μ m (left images) and 25 μ m (right [high magnification] images). **E**, Quantification of cells expressing cardiomyocytes markers cTnT and α -actinin after 2 weeks by immunofluorescence labelling (**P*<0.01 vs GMT; Wilcoxon rank-sum test). **F**, Representative images of immunofluorescence staining for cTnT (top) and α -actinin (bottom) after 4 weeks of GMTd transduction demonstrating sarcomeric structures, most clearly visible in α -actinin labeled cells. Scale bar: 25 μ m. **G**, Quantification of cells with well-developed sarcomeres as a percentage of total α -actinin⁺ cells after 4 weeks of GMT or GMTd transduction (**P*<0.001 vs GMT; Wilcoxon rank-sum test). **H**, Representative immunofluorescence staining demonstrating (green) GFP expression by adult human cardiac fibroblasts treated with GMT (left) or lentivirus expressing GMTd (right) after 4 weeks in co-culture with (non-transduced) neonatal rat cardiomyocytes (negative for GFP). Scale bar: 100 μ m. **I**, Representative peaks from GFP⁺ cells treated with GMTd, reflecting contraction (top row) and Ca²⁺ transients (bottom row; n=3 independent experiment). Contractility parameters were not observed in cells treated with GMT alone. Scale bar: 0.5 seconds. cTnT indicates cardiac troponin T; GMT, Gata4, Mef2C, and Tbx5; FSC, forward scatter; GMTd, Gata4, Mef2C, and Tead1 and HCFs, human cardiac fibroblasts.

was demonstrated in MEFs (mouse embryonic fibroblasts) 1 week after these cells were treated with GMTd compared with GMT-treated cells (Figure 6A). In the GMTd-treated group, H3K4me3 was significantly enriched at the promoters for TNNT2 (2.3x), GATA4 (2.4x), RYR2 (2.6x), and MEF2C (2.5x). These findings suggest that Tead1 may enhance cell cardio-differentiation by reversing chromatin depression of the expression of relevant cardio-differentiation factors.

Increased peroxisome PGC-1A and -1B and TFAM expression in rat and human cardiac fibroblast treated with GMTd was also demonstrated by qRT-PCR and protein expression compared with the treatment of these cells with GMT (Figure 6B and 6C). These findings suggest that Tead1 may enhance cardio-differentiation efficiency by upregulating these energy metabolism and mitochondrial biogenesis regulator factors thought to be relevant to cardiomyocyte viability.³⁰

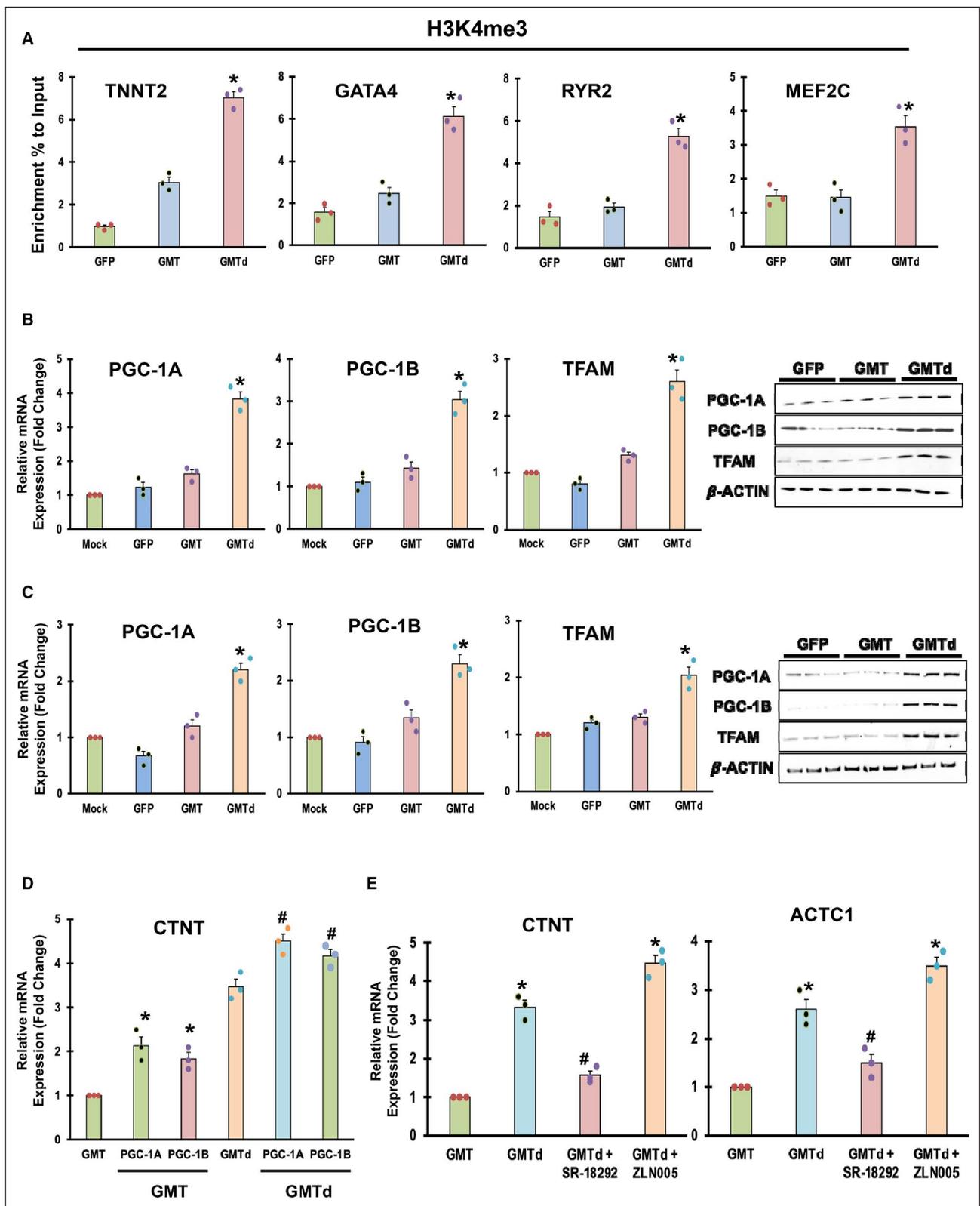
We accordingly investigated whether PGC-1A and -1B could also induce cardiac reprogramming in rat cardiac fibroblasts. In these studies, qRT-PCR analysis demonstrated further increased cTnT expression after overexpression of PGC-1A and PGC-1B together with GMT and GMTd treatment compared with treatment with GMT and GMTd alone (Figure 6D). We then demonstrated that administration of PGC-1A activator (ZLN005) to GMTd-treated cells further increased cTnT and Actc1 expression compared with treatment with GMT or GMTd alone, as detected by qRT-PCR, while PGC-1A inhibitor (SR-18292) administration completely reversed GMTd-mediated upregulation of cTnT and Actc1 expression (Figure 6E). These results suggest that PGC-1A and 1B is necessary for cardiomyocyte reprogramming and that Tead1 may enhance cardiac

reprogramming by regulating mitochondrial biogenesis through PGC-1A.

DISCUSSION

Our present data demonstrate that the Hippo pathway effector Tead1 in combination with Gata4 and Mef2c increases the efficiency of the structural and functional transdifferentiation of rat and human cardiac fibroblasts into mature (contractile) iCMs compared with use of a standard GMT cocktail. We and others have previously demonstrated that other combinations of cardio-differentiating transcription factors, growth factors, kinases, small molecules, microRNAs, and epigenetic factors can also be used to improve GMT-induced cardiac reprogramming *in vitro*.^{9,31–41} Most of these reports, however, used murine or embryonic/juvenile fibroblasts as a substrate for their studies. In comparison our study of adult rat and human cardiac fibroblasts represents the targeting of cells more resistant to reprogramming and less conducive to the generation of functional (contractile) iCMs.^{10,42}

One potential explanation of this enhanced reprogramming efficacy is our finding of increased Tead1-mediated upregulation of the expression of the gene activation H3K4me3 histone mark at the promoter regions of key cardio-differentiation genes. These include cTnT, Gata4, Mef2c, and Ryr2 and the mitochondrial biogenesis regulator genes PGC-1A/1B and TFAM, thought to play an important role in cardiomyocyte development. This latter supposition in particular is supported by our observation that PGC-1A and PGC-1B further increase GMT and GMTd mediated



reprogramming and antagonists of Tead1 effects on mitochondrial biogenesis regulator genes leads to the loss of Tead1-mediated improvements in reprogramming efficacy. These findings of the epigenetic effects of Tead1 is consistent with the observations of the

mechanism of action of other potent reprogramming formulations.^{25,27,30,33,43}

The efficacy of Tead1 in enhancing cardio-differentiation may be reflective of its significant role in enhancing myocyte development through its

Figure 6. Tead 1 mechanisms of action in cardiac fibroblast reprogramming.

A, Chromatin immunoprecipitation assay analysis of mouse embryonic fibroblasts treated with GFP (green fluorescent protein), Gata4, Mef2C, and Tbx5 (GMT), and GMTd demonstrating the trimethylated status of histone H3 of lysine 4 for the promoters of Tnnt2, Gata4, Ryr2, and Mef2c (n=3 biological replicates, each performed in triplicate; * $P < 0.01$ vs GMT; Wilcoxon rank-sum test). **B** and **C**, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) [left] and Western analysis (right) of rat cardiac fibroblasts and human cardiac fibroblasts, respectively, demonstrating increased expression of depicted mitochondrial biogenesis regulator gene mRNA and protein expression 14 days after reprogramming factor administration (n=3 biological replicates, each performed in triplicate; * $P < 0.01$ vs GMT [Wilcoxon rank-sum test] for qRT-PCR, n=3 independent experiments for western blotting. Beta actin is shown as a loading control). **D**, qRT-PCR analysis of cTnT (cardiac troponin T) in rat cardiac fibroblasts transduced with GMT and GMTd administration with or without PGC (peroxisome proliferator-activated receptor- γ coactivator)-1A and PGC-1B, as indicated (n=3 biological replicates, each performed in triplicate; * $P < 0.05$ vs GMT, # $P < 0.05$ vs GMTd; Kruskal–Wallis test). **E**, qRT-PCR analysis of cTnT (left) and Actc1 (right) in rat cardiac fibroblasts after GMT and GMTd administration with or without addition of PGC-1A activator or inhibitor, as indicated (n=3 biological replicates, each performed in triplicate. CTNT indicates cardiac troponin T; GFP, green fluorescent protein; GMTd, Gata4, Mef2C, and Tead1 and GMT, Gata4, Mef2C, and Tbx5. * $P < 0.01$ vs GMT, # $P < 0.05$ vs GMTd; Kruskal–Wallis test).

binding to MCAT (malonyl-coA-acyl carrier protein transacylase) elements and its motif found at muscle gene promoters.⁴⁴ Tead1 and its upstream regulators have likewise been shown to be critical to cardiomyocyte proliferation during embryonic and postnatal periods, with recent studies also demonstrating the role of the mammalian Hippo–Tead1 pathway in cardiomyocyte regeneration after injury.^{45–48} These effects are thought to be mediated via the physical interactions of Tead1 with the cardiac reprogramming factors Nkx2.5, Mef2c, Srf, Gata4, and p300.^{26,27} An earlier study also demonstrated that Tead1 along with Mef2 and FoxO directly target myocardin during cardiovascular development.⁴⁹ Recent studies have also demonstrated that Tead1 is also required for maintenance of adult cardiomyocyte mitochondrial and overall heart function, with its loss associated with lethal dilated cardiomyopathy.^{24,25} While these data support Tead1 as a potentially potent cardio-differentiation factor, the use of Tead1 for this purpose has not to our knowledge been previously reported.

Our observation of significantly enhanced GMTd-mediated reprogramming is particularly noteworthy for its early and robust transdifferentiation of adult rat cell into mature induced cardiomyocytes, as evidenced by our observation of well-organized sarcomeres in 50% of cells within 4 weeks of reprogramming factor administration and onset of cell contractility in 6% of cells within 4 weeks of reprogramming. In comparison, Miyamota et al demonstrated transdifferentiation of 10% of cells into beating iCMs, but used more “plastic” mouse embryonic fibroblasts as their substrate target cell.¹² Likewise, Song et al reported that addition of Hand2, to GMT yielded an “optimized” rate of 0.2% of spontaneously beating adult mouse cardiac fibroblasts, while Addis et al reported that administering HAND2 and NKX2.5 with GMT yielded only rare spontaneous beating cells.^{5,6} The beating percentage reported in the current study is on the other hand consistent with the previous reports of Wang et al³⁵, who demonstrated 1% to 6% beating cells after MGT and shBecn1 transduction using murine cardiac fibroblasts.

Likewise, Mohamed et al⁴⁰; demonstrated that 5% to 40% of cells were beating after treatment with GMT and GMT plus TGF- β /WNT inhibitor using adult mouse cardiac fibroblasts. Recently, Kurotsu et al⁵⁰; likewise reported 6% to 15% beating iCMs after Sev-GMT treatment in mouse cardiac fibroblasts.

Mitochondrial Biogenesis

PGC-1 α is a multi-functional transcriptional coactivator that regulates the activities of multiple nuclear receptors and transcriptional factors involved in mitochondrial biogenesis through TFAM.⁵¹ Our results suggest that Tead1 may enhance cardiac reprogramming by regulating mitochondrial biogenesis through expression of PGC-1A and mitochondrial transcription factor.

Although mitochondria primarily regulate cellular metabolism, they are also key participants in programmed cellular death (apoptosis) since they (1) contain several proapoptotic proteins (ie, cytochrome c) that, on release, can lead to cell death; and (2) produce reactive oxygen species (ROS) that can initiate apoptotic signaling.⁵² Other studies have also correlated reduced PGC-1 α expression levels with decreased mitochondrial function and increased vulnerability to apoptosis.⁵³ In view of mitochondria as an energy center and important for cellular homeostasis, it would be interesting to explore the roles of mitochondrial biogenesis via the PGC-1 α /TFAM pathway which may play crucial roles on apoptotic activity in Tead1 induced cardiac reprogramming.

Study Limitations

Despite our encouraging observations regarding Tead1-mediated cardiomyocyte reprogramming, it is possible that other mechanisms might also be involved in the Tead1-mediated cardiac reprogramming, including alteration of signaling pathways, metabolic switch from glycolysis to fatty acid oxidation and epigenetic changes other than those that we have reported. Identification of such pathways will provide new insights into the mechanism of cardiac reprogramming and help to improve reprogramming efficiency.

Our premise that PGC-1A and PGC-1B are necessary for cardiac reprogramming is supported both by our PGC overexpression and inhibition studies, including those in the presence of GMTd. Additional PGC-1A and -1B gene knock down studies performed in the presence of Tead1 are planned to confirm these findings. Defining the types or size of iCMs that can be obtained from GMTd-induced reprogramming is essential for further research using this model system and for any potential utilization of these cells for gene-based therapies. A variety of techniques can be used to discriminate between different types of CMs including gene expression studies, immunohistochemistry (for the measurement of size and change in sarcomeric length), and perhaps most importantly, functional studies. The cardiac action potential is the result of multiple ion channels and Ca^{2+} cycling proteins interacting in concert, and so the action potential measurement will provide a functional signature for the given type of iCM compared with neonatal/adult heart ventricle, atria, or pacemaker cells. Characterization of these iCMs on the basis of their functional property is the subject of current investigations in our laboratory. Finally, further studies are also needed to clarify the relationship between G, M, and Td binding, epigenetic repatterning, and transcriptional activation of cardiac genes during iCMs reprogramming to further leverage of our current findings into optimized reprogramming strategies.

ARTICLE INFORMATION

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Disclosures

Rosengart is a board member, paid consultant and holds a significant financial interest with XyloCor Therapeutics, LLC and receives royalty payments relevant to this work for intellectual property held by Cornell University. Mathison receives royalty payments relevant to this work for intellectual property held by Cornell University. Martin is a cofounder of and owns shares in Yap Therapeutics. The remaining authors have no disclosures to report.

Supplementary Material

Tables S1–S2

Figure S1

Videos S1–S5

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SUPPLEMENTAL MATERIAL

Table S1. qRT-PCR rat and human primer list**Rat Primers**

| Gene | Forward Primer | Reverse Primer |
|---------------|---------------------------|---------------------------|
| cTnT | AGGCTCACTTCGAGAACAGG | ATTGCGAATACGCTGCTGT |
| Actc1 | GATTATTGCTCCCCCTGAGCG | GTGTAAGGTAGCCGCCTCAGAA |
| Gja1 | GAACAGTCTGCCTTTCGCTG | AAGGACCCAGAAGCGCACGT |
| Nkx2.5 | ACCATGCGGGAAGGCTAT | AGAAGGGCGTGTGTGTGAG |
| Ryr2 | ACATCATGTTTTACCGCCTGAG | TTTGTGGTTATTGAACTCTGGCT |
| Colla1 | GCGAAGGCAACAGTCGATTC | CCCAAGTTCGGTGTGACTC |
| GAPDH | GGCACAGTCAAGGCTGAGAATG | ATGGTGGTGAAGACGCCAGTA |
| Pgc1a | ATCTACTGCCTGGGGACCTT | ATGTGTCGCCTTCTTGCTCT |
| Pgc1 b | ACTATGATCCCACGTCTGAAGAGTC | CCTTGTCTGAGGTATTGAGGTATTC |
| Tfam | GCTAAACACCCAGATGCAAAA | CGAGGTCTTTTTGGTTTTCC |
| Atp2a2 | ATTCCTGGCCCTTTGTGCT | AACAGGAAACTAAGGCTCCGA |
| Scn5a | CCAGATCTCTATGGCAATCCA | GAATCTTCACAGCCGCTCTC |
| Hcn4 | GGGGAATTCGCAACTGAAGC | GGGGAATTCGCAACTGAAGC |
| Kcn5 | CGCAGAGTATTTGAAGTCAC | GGCTACAAATACATCCATCTC |
| Postn | GGCTGAAGACTGCCTTGAATGAC | CGTGGCAGCACCTTCAAAGA |
| Colla2 | GAACGGTCCACGATTGCATG | GGCATGTTGCTAGGCACGAAG |
| My17 | ATCAACTTCACCGTCTTCCTC | ACTCTTCCTTGTTCCACCACC |

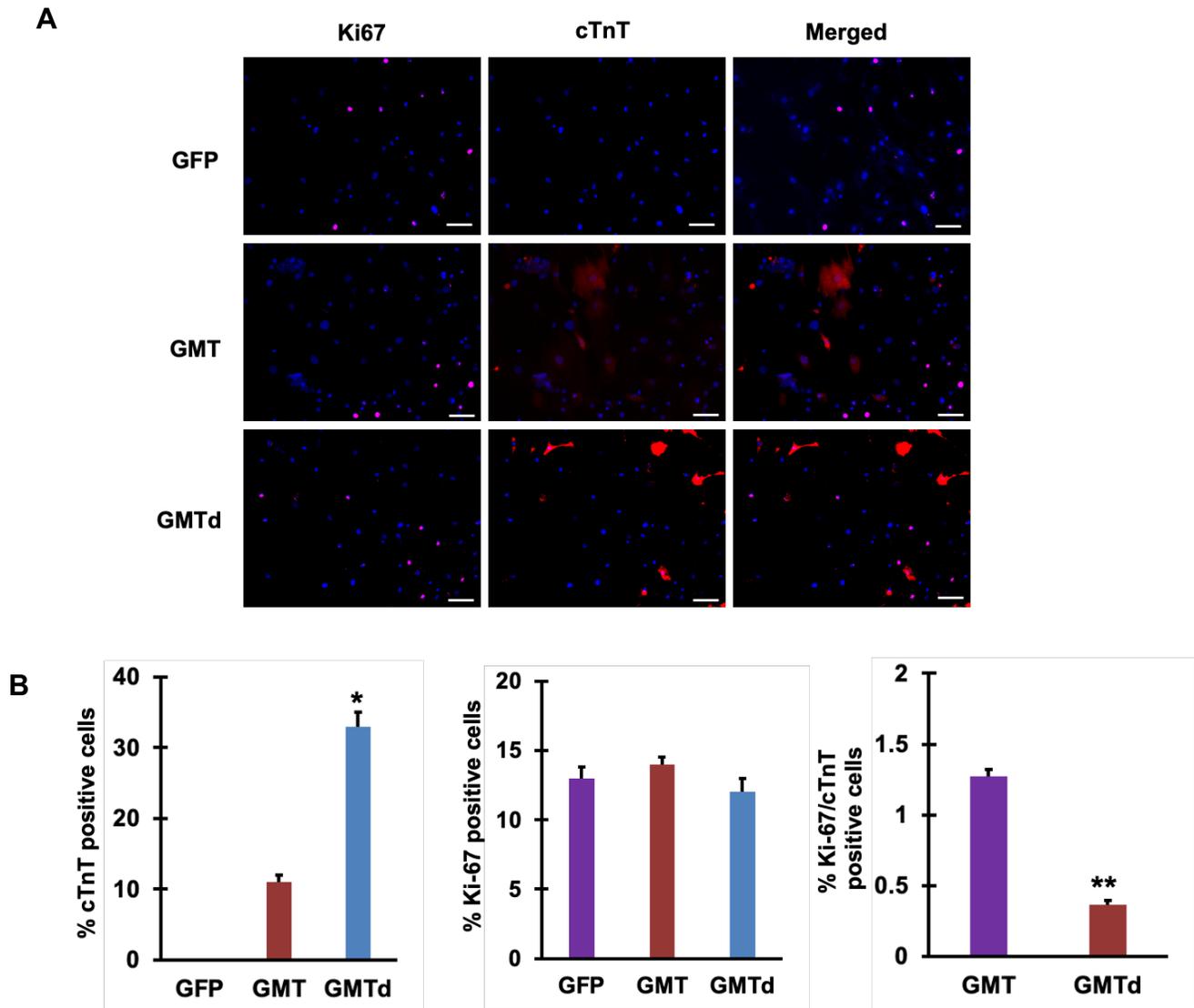
Human Primers

| Gene | Forward Primer | Reverse Primer |
|---------------|-------------------------|------------------------|
| Col1a1 | CATGTTTCAGCTTTGTGGACCT | GCAGCTGACTTCAGGGATGT |
| Myh6 | GCTGGTCACCAACAATCCCTA | CGTCAAAGGCACTATCGGTGG |
| cTnT | AGACGCCTCCAGGATCTGT | TCTCAACAGCTGCTTCTTCC |
| GAPDH | TCACCACCATGGAGAAGGC | GCTAAGCAGTTGGTGGTGCA |
| Pgc1a | CACTCCTCCTCATAAAGCCAAC | GGACTTGCTGAGTTGTGCATA |
| Pgc1 b | GCTGACAAGAAATAGGAGAGGC | TGAATTGGAATCGTAGTCAGTG |
| Tfam | GTGGTTTTTCATCTGTCTTGGC | ACTCCGCCCTATAAGCATCTTG |
| Hcn4 | CTGAGAACTGGAAGGACTTAGC | CAGGACAAGACTGTGGGTTT |
| Scn5A | TCTCTATGGCAATCCACCCCA | TCTCTATGGCAATCCACCCCA |
| Postn | CTCATAGTCGTATCAGGGGTCG | ACACAGTCGTTTTCTGTCCAC |
| Col1a2 | GAGGGCAACAGCAGGTTCACTTA | TCAGCACCACCGATGTCCAA |
| Actc1 | GTACCCTGGTATTGCTGATCG | CCTCATCGTACTCTTGCTTGCT |

Table S2. CHIP-PCR primer list.

| Gene | Forward Primer | Reverse Primer |
|--------------|-----------------------|-----------------------|
| Tnnt2 | GTGTGCAGTCCCTGTTCAGA | AGGGTGGACACAAGCAAAAG |
| Gata4 | GGTTTTCTGGGAAACTGGAG | TCCCAGCTACCTACCTCCCTA |
| Ryr2 | AGCCCCTAGAACATCCTGGT | TATGCTGCTGCTGCTACTGC |
| Mef2c | CCCCTTTTGCAAGAGTGAAA | GCCTTTTCCTTCCGTTTCT |

Figure S1. Immunostaining of rat cardiac fibroblasts for cardiac troponin T and Ki-67 cell proliferation.



A. Representative immunofluorescence staining of rat cardiac fibroblasts 14 days after transduction with lentiviruses expressing GFP or GMT or GMTd. Staining is shown for DAPI (blue) nuclear marker, Ki-67 (pink) DNA proliferation marker, and cTnT (red) cardiomyocyte marker. The majority of cTnT-positive cells were negative for Ki-67. Scale bar 100 μ M. B. Quantification of cells positive for indicated markers from experiment performed as in A (n=3/group; *p < 0.01, **p < 0.001 versus GMT; Wilcoxon rank-sum test).

Supplemental Video Legends:

Video S1. This video shows rat cardiac fibroblasts transduced with a GFP-control vector only did not demonstrate contractions.

Video S2. This video shows rat cardiac fibroblasts transduced with a GMT vector only did not demonstrate contractions.

Video S3. This video shows rat cardiac fibroblasts contracted spontaneously after transduction with GMTd.

Video S4. This video shows adult human cardiac fibroblasts transduced with a GMT vector did not demonstrate contractions in the co-culture experiments.

Video S5. This video shows adult human cardiac fibroblasts contracted synchronously with surrounding rat cardiomyocytes after transduction with GMTd.