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ORIGINAL RESEARCH

Enhanced Generation of Induced Cardiomyocytes Using a Small-Molecule Cocktail to Overcome Barriers to Cardiac Cellular Reprogramming

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BACKGROUND: Given known inefficiencies in reprogramming of fibroblasts into mature induced cardiomyocytes (iCMs), we sought to identify small molecules that would overcome these barriers to cardiac cell transdifferentiation.

METHODS AND RESULTS: We screened alternative combinations of compounds known to impact cell reprogramming using morphologic and functional cell differentiation assays in vitro. After screening 6 putative reprogramming factors, we found that a combination of the histone deacetylase inhibitor sodium butyrate, the WNT inhibitor ICG-001, and the cardiac growth regulator retinoic acid (RA) maximally enhanced iCM generation from primary rat cardiac fibroblasts when combined with administration of the cardiodifferentiating transcription factors Gata4, Mef2C, and Tbx5 (GMT) compared with GMT administration alone (23±1.5% versus 3.3±0.2%; $P<0.0001$). Expression of the cardiac markers cardiac troponin T, Myh6, and Nkx2.5 was upregulated as early as 10 days after GMT–sodium butyrate, ICG-001, and RA treatment. Human iCM generation was likewise enhanced when administration of the human cardiac reprogramming factors GMT, Hand2, and Myocardin plus miR-590 was combined with sodium butyrate, ICG-001, and RA compared with GMT, Hand2, and Myocardin plus miR-590 treatment alone (25±1.3% versus 5.7±0.4%; $P<0.0001$). Rat and human iCMs also more frequently demonstrated spontaneous beating in coculture with neonatal cardiomyocytes with the addition of sodium butyrate, ICG-001, and RA to transcription factor cocktails compared with transcription factor treatment alone.

CONCLUSIONS: The combined administration of histone deacetylase and WNT inhibitors with RA enhances rat and human iCM generation induced by transcription factor administration alone. These findings suggest opportunities for improved translational approaches for cardiac regeneration.

Key Words: cardiomyocytes ■ direct reprogramming ■ small molecules

Heart failure caused by the loss or dysfunction of cardiomyocytes affects almost 25 million individuals worldwide and continues to be a heavy burden to healthcare systems.[1] Because of the lack of intrinsic regenerative capabilities of the adult human heart, the millions of cardiomyocytes lost after injury are typically replaced with fibrotic scar tissue.[2] Transplantation of cardiac stem cells or stem cell–derived cardiomyocytes to improve cardiac function has been tested as a myocardial regeneration strategy but has proved to be relatively inefficient, and generally failed to yield significant results in clinical trials.[3] Thus, the ability to generate new cardiomyocytes from scarred myocardium represents an important therapeutic opportunity for treating heart failure.

Direct reprogramming of "induced" cardiomyocytes (iCMs) from fibroblasts has emerged over the

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CLINICAL PERSPECTIVE

What Is New?

- Screening of candidate cardiac cell reprogramming small molecules in adult rat cardiac fibroblasts and induced murine embryonic fibroblast, we found that the histone deacetylase inhibitor sodium butyrate, the WNT pathway inhibitor ICG-001, and the cardiac growth regulator retinoic acid induce transcription factor–mediated transdifferentiation of fibroblasts into cardiac myocytes.
- A combination of small-molecules sodium butyrate, ICG-001, and retinoic acid enhances the efficiency and functional development of induced cardiomyocytes generated from adult rat and human cardiac fibroblasts.

What Are the Clinical Implications?

- These findings may facilitate combined gene and small-molecule therapy approaches for heart failure and further support the need for developing new strategies that improve translational approaches for cardiac regeneration.

Nonstandard Acronyms and Abbreviations

ALL	sodium butyrate, ICG-001, and retinoic acid
cTnT	cardiac troponin T
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
GMT	Gata4, Mef2c, and Tbx5
GMTHMy	GMT, Hand2, and Myocardin
HDAC	histone deacetylase
iCM	induced cardiomyocyte
RA	retinoic acid
SB	sodium butyrate

past decade as a promising strategy for myocardial regeneration.[4] Following encouraging in vitro studies, forced expression of the cardiodifferentiating transcription factors Gata4, Mef2c, and Tbx5 (GMT) and/or other factors in the myocardium following myocardial infarction was shown to improve cardiac function and diminished adverse myocardial remodeling in mouse and rat myocardial infarction models.[5–10] Despite these successes, the efficiency of direct reprogramming has remained relatively low, especially for adult versus juvenile or neonatal cells, as well as for human versus rodent cells,[11,12] and is thus a barrier for future clinical application of direct cardiac reprogramming.

We tested a variety of combinations of small molecules previously shown to increase the differentiation of pluripotent stem cells into cardiomyocytes or involved in embryonic and postnatal cardiodifferentiation as candidate enhancers of iCM generation from adult rat and human cardiac fibroblasts.[13–18] Of the 6 factors that we selected for testing based on these reports, we demonstrated that those that impacted the epigenetic regulator histone deacetylase (HDAC), the cell fate regulator WNT, and the retinoic acid (RA) cardiac growth signaling pathway most significantly enhanced iCM generation from rat and human fibroblasts when combined with GMT or GMT, Hand2, and Myocardin plus miR-590 (GMTHMy/miR-590) treatments, respectively.

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

Following protocol approval by the Baylor College of Medicine Institutional Animal Care and Use Committee (AN-6223), adult cardiac fibroblasts were harvested from 8- to 10-week-old male Sprague-Dawley rats (Harlan Sprague-Dawley Inc, Indianapolis, IN) using standard cell isolation techniques.[10] Following approval by the Baylor College of Medicine Institutional Review Board (IRB H-33421), standard explantation protocols were likewise used to harvest adult human cardiac fibroblasts from ventricular myocardial tissue obtained from explants of heart failure patients undergoing mechanical assist device placement or cardiac transplantation at Baylor St. Luke's Medical Center.[19] After mincing of these tissues, explants were cultured in DMEM, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin, as previously described.[20] Fibroblasts were allowed to migrate out from these explants over a period of 2 weeks, after which they were passaged 3 times in M106 medium (catalog No. M106500; Thermo Fisher Scientific Inc, Waltham, MA), 10% FBS, and LSGS kit supplements (catalog No. S-003-K; Thermo Fisher Scientific).

Cell Reprogramming

Lentiviral vectors were constructed from plasmids pCDH-Gata4, pCDH-Mef2c, pCDH-Tbx5,

pCDH-Hand2, pCDH-Myocardin, pCDH-miR590, and GFP (green fluorescent protein), as previously described.[9,10,19] HDAC and Wnt3a plasmids were purchased from Addgene (catalog Nos. 13820 and 35908). Rat and human cardiac fibroblasts derived from adult rats and adult human patients, as described above, were seeded onto 6- or 10-cm culture dishes (for fluorescence-activated cell sorting [FACS] analyses), onto 6-well plates (for Quantitative Reverse Transcription [qRT]-polymerase chain reaction [PCR] analyses), or onto 24-well dishes (for immunofluorescence analyses) precoated with Surecoat (Cellutron Life Technologies, Baltimore, MD). Rat and human cardiac fibroblasts were treated with a combination of 3 factors (GMT) or 6 factors (GMTHMy/miR-590) to induce rat or human iCM generation, respectively. Twenty-four hours after cells were 70% to 80% confluent, lentivirus vectors (multiplicity of infection, 20) expressing these genes were added to cell culture plates in a mixture with polybrene at a final concentration of 5 μg/μL. Two days later, the initial transfer medium (DMEM/199 [4:1], 10% FBS, and 1% penicillin/streptomycin) was replaced with induction medium (iCM media), containing DMEM/199 (4:1), 10% FBS, 5% horse serum, 1% penicillin/streptomycin, 1% nonessential amino acids, 1% essential amino acids, 1% B-27, 1% insulin-selenium-transferrin, 1% vitamin mixture, and 1% sodium pyruvate,[19] with or without addition of candidate small molecules (Table S1). Induction medium was replaced every 2 days until cells were harvested.

For reprogramming studies using coculture techniques, neonatal rat cardiomyocytes were isolated from 0- to 3-day-old neonatal rat pups (AN-6223), as previously described.[21] One week after treatment, as described above, adult rat and human cardiac fibroblasts were harvested and replated onto cultures of neonatal rat cardiomyocytes at a ratio of 1:10 in DMEM/M199/10% FBS medium.[22]

High-Throughput Screening

High-throughput screening for iCMs was performed using an induced murine embryonic fibroblast cell line that overexpressed GMT in response to doxycycline treatment and expressed a GFP reporter gene driven by a promoter sequence responsive to the cardiomyocyte marker α-major histocompatibility complex. Real-time quantification of GFP^+ events was captured by IncucyteG3 imaging system.[23]

Flow Cytometry

Adherent fibroblasts, treated as outlined above, were prepared for flow cytometry studies, as previously described.[10,19] These cells were then fixed with fixation buffer (BD Biosciences, San Jose, CA) for 15 minutes at room temperature and then washed with Perm/Wash buffer (BD Biosciences) and incubated with mouse monoclonal anti–cardiac troponin T (cTnT) antibody (Thermo Fisher Scientific) at 1:500 dilution in Perm/Wash buffer for 90 minutes at room temperature. They were then incubated with donkey anti-mouse Alexa Fluor 647 (Invitrogen, Carlsbad, CA) at 1:200 dilution for 1 hour, washed 3× with Perm/Wash buffer again, and then analyzed for cTnT expression using an FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using Diva software (version 6.0).

Immunocytochemistry

Immunocytochemical analyses were performed on cells fixed in 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized with permeabilization buffer (0.5% Triton-X) for 15 minutes. Cells were then blocked with 10% goat serum for 30 minutes and incubated with primary antibodies against cTnT (1:400 dilution; Thermo Fisher Scientific), Gja1 (1:500 dilution; Abcam), or α-actinin (1:300 dilution; Sigma-Aldrich, St. Louis, MO). After washing with Dulbecco's PBS, Alexa fluorogenic secondary antibodies (Invitrogen) were used to detect signal.[10,19,21]

Real-Time PCR

Real-time PCR was performed by first extracting total RNA using the TRIzol method (Invitrogen), after which relative quantification was performed using SYBR green detection of PCR products in real time with the ABI ViiA 7 (Applied Biosystems Inc, Foster City, CA). Primers for qRT-PCR were selected as detailed in Table S2. mRNA levels were normalized by comparison to GAPDH.

Western Analysis

Cell lysates for Western analyses were washed with Dulbecco's PBS and homogenized in cell lysis buffer. Protein (25 μg) was then loaded onto SDS-PAGE. Once separated, the protein bands were transferred to nitrocellulose membrane (Invitrogen catalog No. IB301001). Membranes were then washed in 1× Tris-buffered saline with Tween 20 and treated with blocking buffer for 1 hour at room temperature. Immune detection was performed by incubating blots in primary antibody overnight at 4°C, followed by treatment with appropriate horseradish peroxidase–conjugated secondary antibodies (Millipore, Billerica, MA) for 1 hour at room temperature. The following primary antibodies were used: anti–histone 3 lysine 27 acetylation (ab4729), anti–histone 3 (CST9715), β-catenin (CST9562), and phosphorylated β-catenin (CST9561). Membranes were then washed with 1× Tris-buffered saline with Tween 20 and visualized by chemiluminescence detection (Thermo Scientific catalog No. 34580).

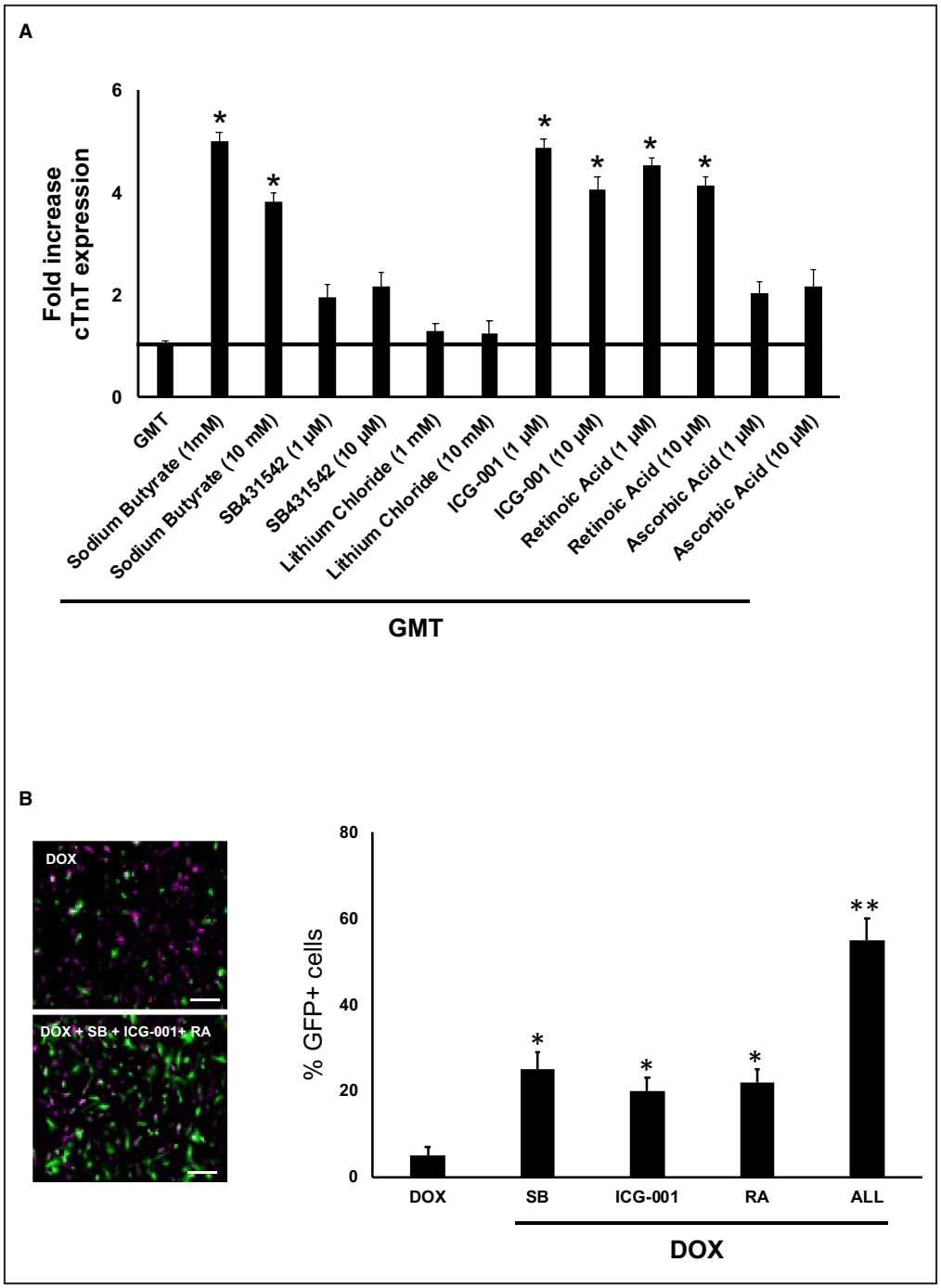


Figure 1. Screening of candidate small molecules for their enhancement of direct cardiac reprogramming.

A, Increase of cardiac troponin T (cTnT) gene expression by rat cardiac fibroblasts, as assessed by qRT-polymerase chain reaction after 10 days of combined treatment with Gata4, Mef2c, and Tbx5 (GMT) or GMT plus indicated compounds (n=5 biological replicates, each performed in triplicate; **P*<0.0001 vs GMT). **B**, Real-time high-throughput cardiomyocyte marker cell screening in induced murine embryonic fibroblast cell line using "IncucyteG3" imaging system (see Methods). Representative immunocytochemistry images for GFP (green fluorescent protein)–positive cells in green (indicating activation of α-major histocompatibility complex–GFP reporter expression). Bar graph shows quantification of the percentage of GFP-positive cells in 10 days after treatment with indicated compounds (n=5 biological replicates, each performed in triplicate; **P*<0.005, ***P*<0.0001 vs doxycycline [DOX] only). All data are presented as mean±SEM. Drug dosages: sodium butyrate (SB; 1 mmol/L), ICG-001 (1 μmol/L), retinoic acid (RA; 1 μmol/L), and DOX (1 mg/mL). Bar=400 μm. ALL indicates SB, ICG-001, and RA.

Measurements of Contractility and Calcium Transient

Cell contractility (cell shortening) and calcium transients were measured in coculture studies at room temperature (22°C–23°C). The room temperature allowed the iCMs to be stable for up to 2 hours. The iCMs were placed in a plexiglass chamber, which was positioned on the stage in an inverted epifluorescence microscope (Nikon Diaphot 200), and perfused with 1.8 mmol/L Ca^{2+}-Tyrode's 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. The iCMs were visually identified by GFP expression and contraction. Field stimulation was provided by a Grass S5 stimulator using platinum electrodes placed alongside a cell culture bath containing 1.8 mmol/L Ca^{2+}, with bipolar pulses at voltages 50% above myocyte stimulation thresholds. Contractions of myocytes from random fields were videotaped and digitized on a computer. For Ca^{2+} signal measurements, cells were loaded with 2 μmol/L of Fura-2/AM (Life Technologies) and alternately excited at 340 and 380 nm at 0.5 Hz by use of a Delta Scan dual-beam spectrophotofluorometer (Photon Technology International, Edison, NJ). Ca^{2+} transients were expressed as the 340/380-nm ratios of the resulting 510-nm emissions. Data were analyzed using Felix software (Photon Technology International).[19,21]

Statistical Analysis

Three independent biological replicates, each measured in technical triplicates, were performed for all studies, unless otherwise indicated. All data are expressed as the mean±SEM. Statistical analysis was performed using SAS, version 9.4. The nonparametric Mann-Whitney *U* test (Wilcoxon rank-sum test) was used when comparing 2 groups, and the Kruskal-Wallis test, followed by Bonferroni correction, was used when comparing multiple groups to analyze statistical significance for data that were not normally distributed. A $P<0.05$ was considered to indicate significance.

RESULTS

Candidate Small-Molecule Screening

Of our 6 screened candidate cardiac cell reprogramming small molecules, we found that the HDAC inhibitor sodium butyrate (SB), the WNT pathway inhibitor ICG-001, and the cardiac growth regulator RA each enhanced expression of the cardiomyocyte marker cTnT in rat cardiac fibroblasts at least 4-fold ($P<0.0001$), even at low dosages, when combined with GMT administration compared with administration of GMT alone (Figure 1A). In comparison, we found that the other compounds we tested (a transforming growth factor-β inhibitor [SB431542], a glycogen synthase kinase inhibitor [LiCl], and a cell senescence pathway regulator [vitamin C]) did not enhance cTnT expression above GMT treatment levels (Figure 1A). We also screened the combination of 2 small molecules to achieve maximum reprogramming efficiency. However, the reprogramming efficiency was not improved compared with single drug treatment (Figure S1).

On the basis of these data, we found using our "real-time" GMT-inducible α-major histocompatibility complex·GFP (induced murine embryonic fibroblast) high-throughput cell assay that a combination of SB, ICG-001, and RA ("ALL") increased cardiac reprogramming efficiency, as indicated by the percentage of GFP^+ cells, compared with reprogramming observed with GMT overexpression alone ($P<0.0001$; Figure 1B).

SB, ICG-001, and RA Enhance iCM Differentiation

FACS studies confirmed that GMT-transduced rat cardiac fibroblasts treated with a combination of SB, ICG-001, and RA (GMT-ALL) yielded significant increases in cTnT expression compared with cells treated with single compounds alone or with no small-molecule treatment (23±1.5% versus 8±0.8% versus 3.3±0.2%; $P<0.0001$; Figure 2A and 2B). The expression of a panel of other genes relevant to cardiomyocyte functional structure (*cTnT*, *Myh6*, and *Gja1*), development (*Nkx2.5*), and differentiation (eg, *Gata4*, *Mef2c*, and *Tbx5*) was also demonstrated by qRT-PCR to be increased by GMT-ALL treatment compared with rat cardiac fibroblast treatment via GMT transduction alone ($P<0.005$; Figure 2C). Consistent with these findings, GMT-ALL was found to decrease the expression of the fibroblast marker gene *Col1a1* compared with treatment via GMT transduction (Figure 2C). These findings were further confirmed by immunofluorescence analyses, which showed greater numbers of cells expressing the cardiomyocyte markers cTnT and α-sarcomeric actinin after reprogramming with GMT-ALL compared with treatment with GMT alone (Figure 3A through 3C). In addition, GMT-ALL resulted in a 3-fold increase in the number of α-sarcomeric actinin+iCMs that assembled sarcomeres compared with GMT alone (Figure 3D). We also observed formation of gap-junction protein Gja1 channels after GMT-ALL infection (Figure 3E).

SB, ICG-001, and RA Enhance the Generation of Functional iCMs

Although rat cardiac fibroblasts treated with GMT-ALL were not observed to contract independently after up to 8 weeks in culture, ≈10% of rat cardiac fibroblasts

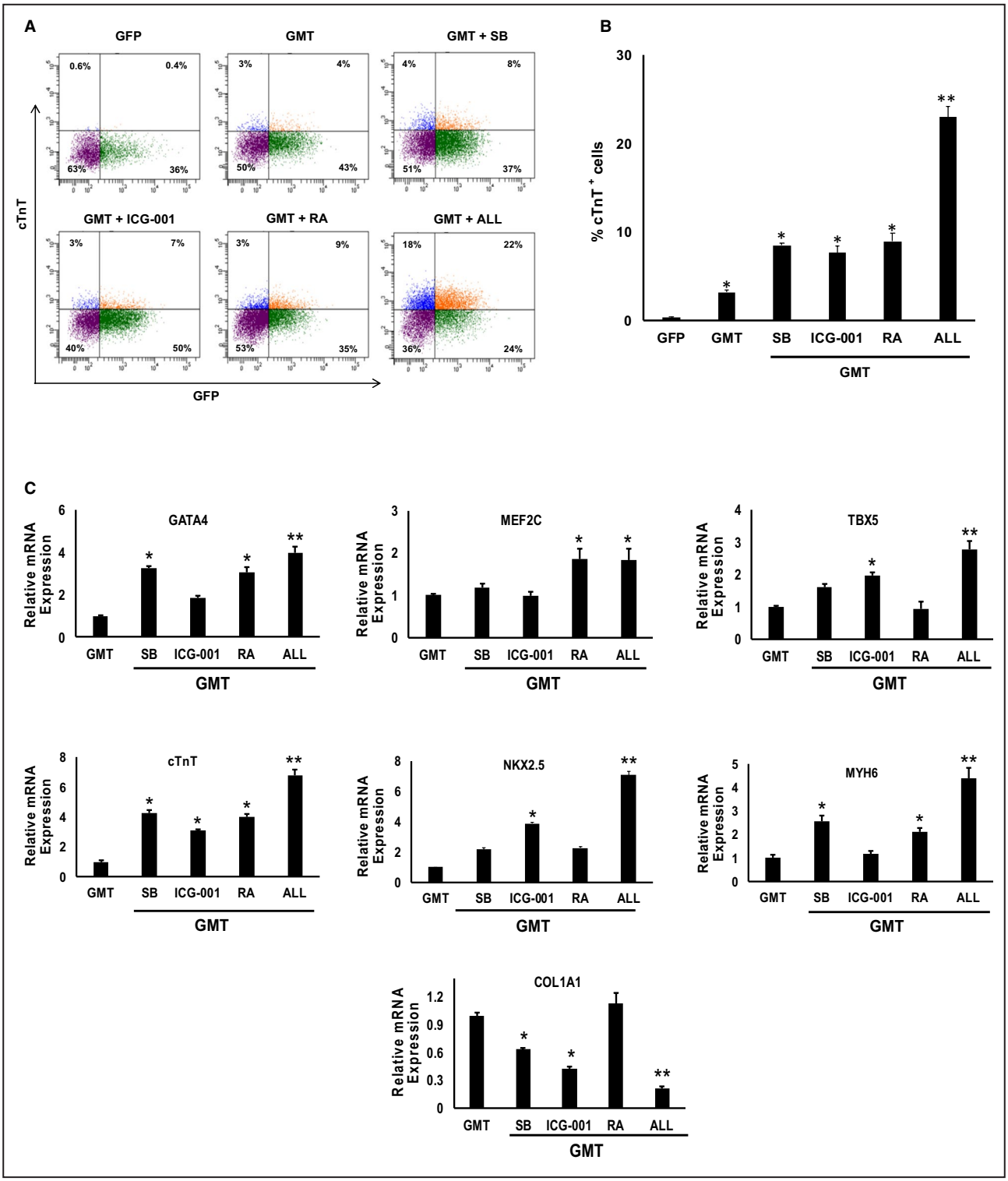


Figure 2. Sodium butyrate (SB), ICG-001, and retinoic acid (RA) (ALL) induce expression of cardiac marker genes in rat cardiac fibroblasts.

A, Representative flow cytometry plots for cardiac troponin T–positive (cTnT+) cells 10 days after transduction of rat cardiac fibroblasts with lentiviruses expressing GFP (green fluorescent protein) or Gata4, Mef2c, and Tbx5 (GMT), with or without addition of small molecules (ALL). **B**, 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). **C**, Cardiac and fibroblast marker gene mRNA expression 10 days after reprogramming factor and small-molecule administration to rat cardiac fibroblasts, as assessed by Quantitative Reverse Transcription (qRT)-polymerase chain reaction (n=3 biological replicates, each performed in triplicate; **P*<0.05, ***P*<0.005 vs GMT). Data are normalized against GMT values. All data are presented as mean±SEM.

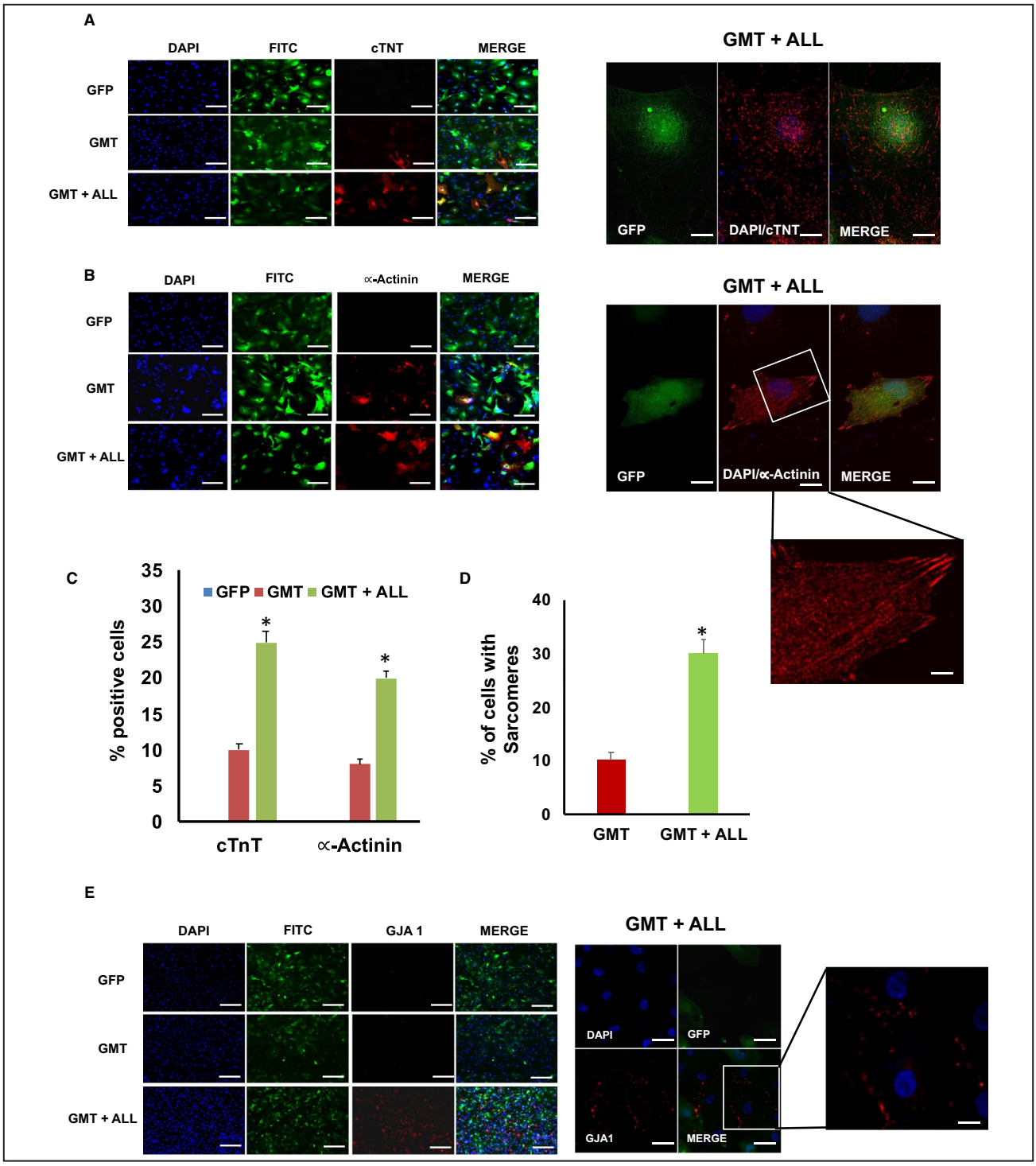


Figure 3. Immunostaining of rat cardiac fibroblasts for cardiomyocyte markers.
Representative immunofluorescence staining of rat cardiac fibroblasts 10 days after transduction with lentiviruses expressing GFP (green fluorescent protein) or Gata4, Mef2c, and Tbx5 (GMT), with or without addition of small molecules (sodium butyrate+ICG-001+retinoic acid; ALL). Staining is shown for 4',6-diamidino-2-phenylindole (DAPI) (blue) nuclear marker, fluorescein isothiocyanate (FITC) (green) lentivirus infection marker, and (red) cardiomyocyte markers. **A**, Cardiac troponin T (cTnT). **B**, α-Actinin. Bar=100 μm. High-magnification views of cTnT (**A**, right panel) and α-actinin (**B**, right panel) staining show sarcomeric structures in cells treated with GMT+ALL. Bar=25 μm. **C**, Quantification of cells positive for indicated markers from experiment performed as in (**A** and **B**) (n=4/group; *P<0.001 vs GMT). **D**, Quantification of percentage of cells demonstrating sarcomeric structures vs total α-actinin cells (n=3/group; *P<0.001 vs GMT). **E**, GJA1 (bar=400 μm). High-magnification views of GJA 1 (right panel) channels formed between induced cardiomyocytes in cells treated with GMT+ALL. All data are presented as mean±SEM. Bar=25 μm.

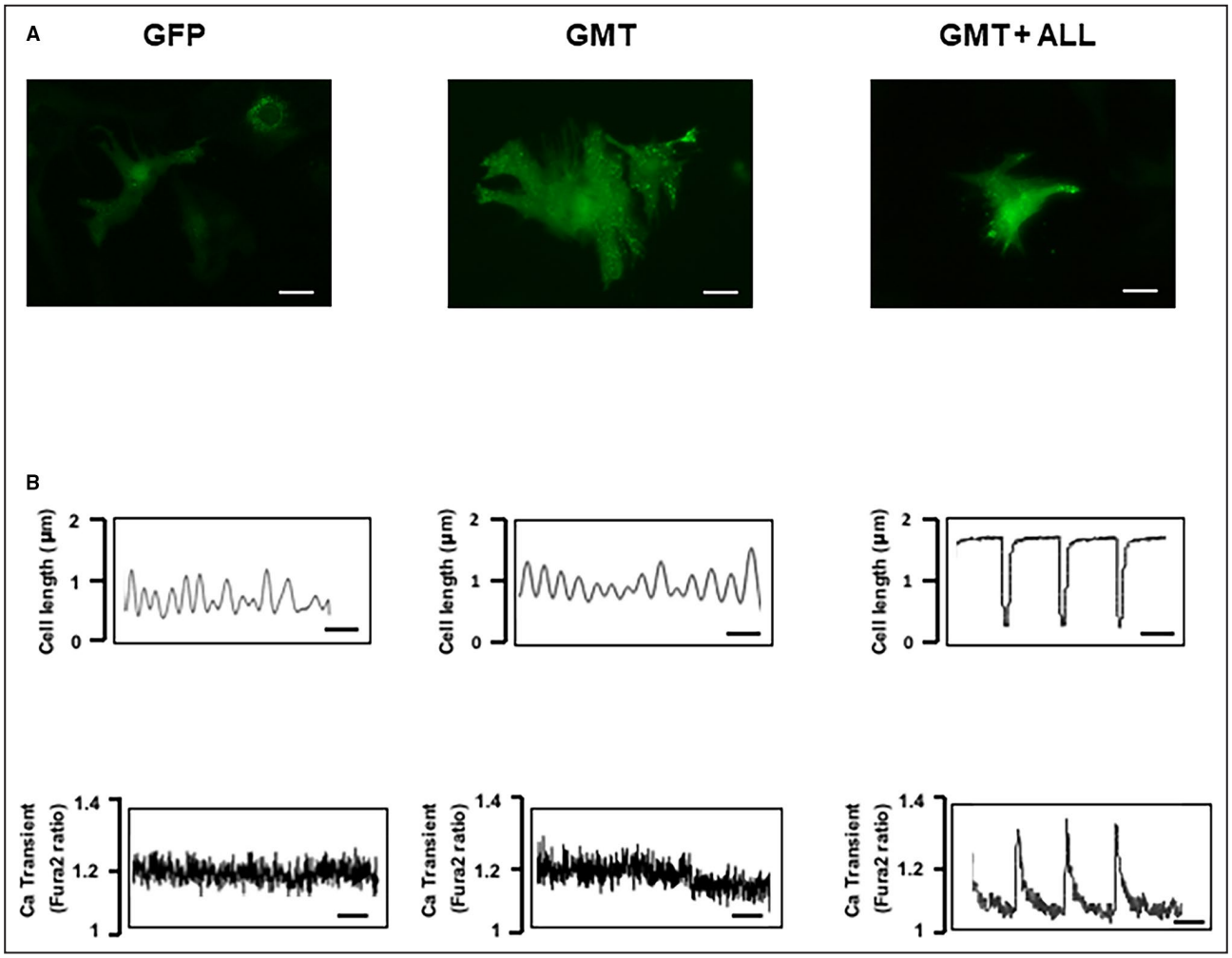


Figure 4. Functional efficacy of reprogramming of rat cardiac fibroblast reprograming after coculture with neonatal rat cardiomyocytes.

Adult rat cardiac fibroblasts were transduced with lentivirus expressing a GFP (green fluorescent marker) marker alone (negative control group; left); lentivirus also expressing Gata4, Mef2c, and Tbx5 (GMT) (middle); or lentivirus expressing GMT treated in combination with small molecules (right). One week after initial transduction, these rat cardiac fibroblasts were cocultured with (untreated) neonatal rat cardiomyocytes (negative for GFP). **A**, Representative immunofluorescence staining demonstrating (green) GFP expression in rat induced cardiomyocytes (iCMs) after 4 weeks in coculture (bar=100 μm). **B**, Representative peaks from GFP^+ rat iCMs after 4 weeks of coculture, reflecting contraction (top row) and Ca^{2+} transients (bottom row) in cells treated with GMT+sodium butyrate, ICG-001, and retinoic acid (ALL) (n=3 independent experiments). Bar=0.5 seconds.

treated with GMT-ALL, as verified by their GFP expression (Figure 4A), spontaneously contracted synchronously after 4 weeks with surrounding cocultured neonatal cardiomyocytes. In comparison, cells treated with GMT alone and untreated cells failed to contract in coculture experiments (Figure 4B, Videos S1 through S3). We observed all of the contracting cells also demonstrated calcium transients on electrical stimulation in the group treated with GMT-ALL (Figure 4B).

Mechanisms of Action of SB, ICG-001, and RA in Cardiac Reprogramming

We observed that acetylation of histone 3 lysine 27 (an indicator of HDAC deactivation) was increased in GMT-transduced fibroblasts treated with SB compared with cells treated with GMT alone (Figure 5A, left). We further observed that HDAC overexpression (induced by HDAC plasmid administration) reversed the effects of SB on cTnT expression, as assessed by FACS and qRT-PCR (Figure 5B and 5C and Figure S1), validating that SB-induced enhancement of cardiodifferentiation is mediated via antagonism of HDAC. We likewise observed that although ICG-001 treatment reduced expression of β-catenin (indicator of WNT activation) compared with treatment with GMT alone (Figure 5A, middle), WNT3a overexpression (induced by WNT3a plasmid administration) completely reversed the effects of ICG-001 on GMT-mediated cTnT expression (Figure 5B and 5C and Figure S2). Similarly, although

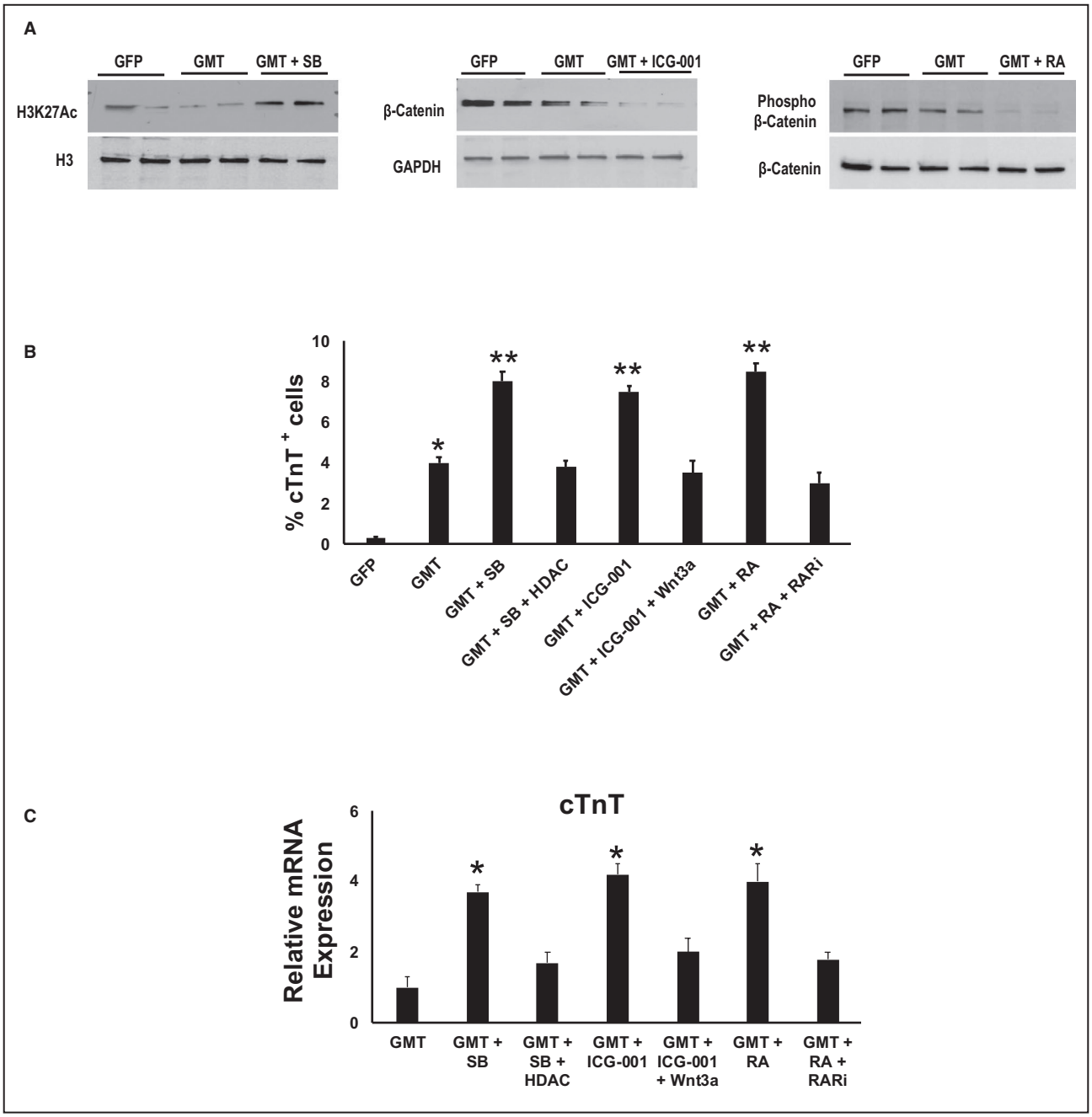


Figure 5. Sodium butyrate (SB), ICG-001, and retinoic acid (RA) mechanisms of action in rat cardiac fibroblast reprogramming. **A**, Western analysis of histone 3 lysine 27 acetylation (H3K27Ac) and a histone-3 standard (left), β-catenin (middle), and phosphorylated (Phospho) β-catenin with a total β-catenin standard (right) 4 days after rat cardiac fibroblasts were transduced with lentivirus expressing Gata4, Mef2c, and Tbx5 (GMT) or GFP (green fluorescent protein), with or without addition of SB (1 mmol/L), ICG-001 (1 μmol/L), or RA (1 μmol/L), respectively. **B**, Percentage of cardiac troponin T–positive (cTnT+) cells, as assessed by flow cytometry, 10 days after infection of rat cardiac fibroblasts with lentiviruses expressing GFP or GMT, with or without addition of small molecules and inhibitors, as indicated (n=3 biological replicates, each performed in triplicate; **P*=0.0003, ***P*<0.0001 vs GFP). **C**, Quantitative Reverse Transcription (qRT)-polymerase chain reaction of mRNA expression of cTnT after GMT administration with or without addition of small molecules and inhibitors, as indicated (n=3 biological replicates, each performed in triplicate; **P*<0.001 vs GMT). All data are presented as mean±SEM. HDAC indicates histone deacetylase; and RARi, RA receptor inhibitor.

we observed that RA decreased phosphorylation of β-catenin in GMT-transduced cells compared with cells not treated with RA (Figure 5A, right), we likewise observed that addition of the RA receptor antagonist AGN 194310 (MedChemExpress [MCE] No. HY-1668) completely reversed the effects of RA on cTnT expression (Figure 5B and 5C and Figure S2). These data suggest that SB, ICG-001, and RA appear to improve

cardiac reprogramming by inhibiting HDAC and WNT signaling and activating RA receptor signaling.

SB, ICG-001, and RA Also Enhance Human iCM Differentiation

Flow cytometry studies demonstrated increased cTnT expression by human cardiac fibroblasts transduced with GMTHMy/miR-590+ALL compared with cells transduced with GMTHMy/miR-590 alone (25±1.3% versus 5.7±0.4%; $P<0.0001$; Figure 6A and 6B). qRT-PCR analysis likewise demonstrated significantly increased expression of other cardiomyocyte transdifferentiation genes and downregulation of *Col1a1* after addition of ALL to GMTHMy/miR-590 treatment of human cardiac fibroblasts compared with treatment with GMTHMy/miR-590 alone ($P<0.001$; Figure 6C). Increased expression of cTnT and α-sarcomeric actinin was likewise demonstrated in immunofluorescence studies to be increased with GMTHMy/miR-590+ALL treatment of human cardiac fibroblasts compared with treatment with GMTHMy/miR-590 alone (Figure 6D and 6E). In addition, GMTHMy/miR-590+ALL resulted in a 2-fold increase in the number of α-sarcomeric actinin+iCMs that assembled sarcomeres compared with GMTHMy/miR-590 alone (Figure 6F).

As with rat cardiac fibroblasts, although no cells treated with GMTHMy/miR-590 alone contracted spontaneously in coculture with neonatal rat cardiomyocytes or on electrical stimulation, ≈5% of human cardiac fibroblasts treated with GMTHMy/miR-590+ALL, as verified by their GFP expression (Figure 7A), contracted synchronously with surrounding neonatal rat cardiomyocytes (Figure 7B, Videos S4 and S5). Furthermore, all of the contracting cells also demonstrated calcium transients on electrical stimulation in the group treated with GMTHMy/miR-590+ALL (Figure 7B). Consistent with murine studies, cTnT expression by human cardiac fibroblasts treated with GMTHMy/miR-590 and SB, ICG-001, or RA was reversed by HDAC overexpression, Wnt3a overexpression, and RA receptor blockade, respectively (Figure 7C), suggesting a consistent mechanism of action in rat and human cells.

DISCUSSION

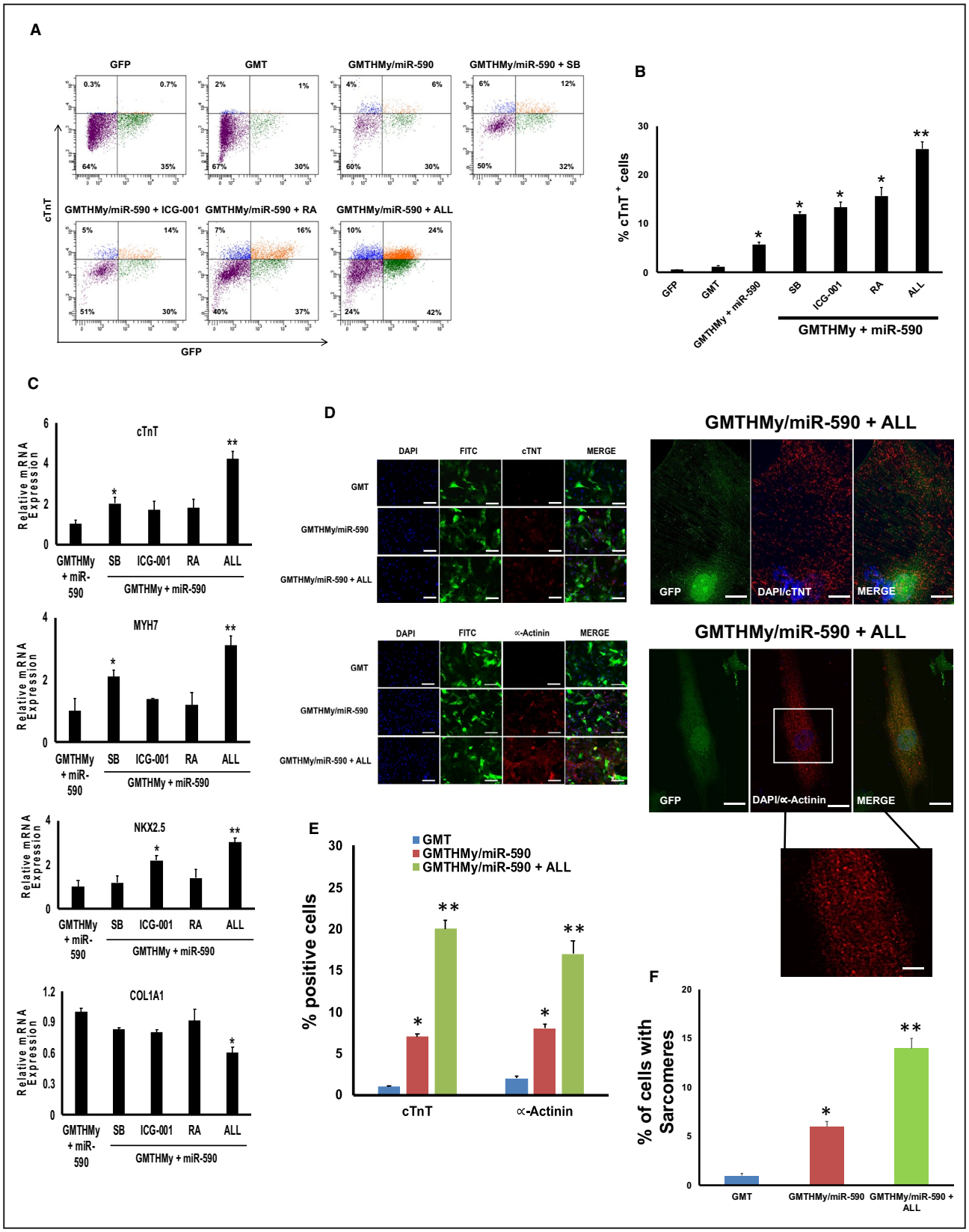
Despite encouraging early indications that various combinations of transcription and other reprogramming factors induce the transdifferentiation of cardiac fibroblasts into iCMs, we and others have shown that generation of functional (contractile) iCMs remains relatively inefficient, especially for adult versus embryonic or other juvenile cells, and for human versus rodent cells.[10,19–21,24–27] The current study demonstrates that effective cardiodifferentiation can be enhanced by a combination of small molecules that includes the HDAC inhibitor SB, the WNT inhibitor ICG-001, and the cardiac growth regulator RA. More important, encouragement to the field can be derived from our observation of enhanced iCM generation compared with previously described reprogramming factor formulas, especially when considering results achieved for adult and human cardiac fibroblast target cells, which have been shown to be more resistant to reprogramming than juvenile and human cells.[20,28]

Other small-molecule cocktails that have also been shown to enhance cell reprogramming prominently include antifibrogenic factors targeting Rho-associated kinase and transforming growth factor-β inhibition, and those targeting the chemokine signaling pathways, the Notch and protein kinase B pathways, which are known to promote differentiation and proliferation.[29–33] Coadministration of nonsteroidal anti-inflammatory agents (diclofenac), glycogen synthase kinase 3, and Janus Kinase (JAK) inhibitor, and use of alternative WNT and transforming growth factor-β inhibitors (eg, XAV939) together with reprogramming transcription factors have likewise been shown to enhance reprogramming.[34–36] Zhou et al likewise demonstrated that innate immunity plays an important role in human cardiodifferentiation, at least in part through changes in DNA methylation, and that silencing of several downstream targets of miR-133 could replace its role in enhancing human cardiodifferentiation.[37]

In comparison to previously reported small-molecule reprogramming strategies, our small-molecule cocktail yielded 7-fold increases in rat fibroblast reprogramming

Figure 6. Sodium butyrate (SB), ICG-001, and retinoic acid (RA) (ALL) induce cardiodifferentiation of human cardiac fibroblasts.

A, Representative flow cytometry plots for cardiac troponin T–positive (cTnT$^+$) cells 10 days after transduction of human cardiac fibroblasts with lentiviruses expressing GFP (green fluorescent protein), Gata4, Mef2c, and Tbx5 (GMT), or GMT, Hand2, and Myocardin (GMTHMy)/miR-590 with or without addition of small molecules, as indicated. **B,** 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, **$P<0.0001$ vs GMTHMy/miR-590). **C**, Cardiac and fibroblast marker gene mRNA expression, as assessed by qRT-polymerase chain reaction (n=3 biological replicates, each performed in triplicate; *$P<0.05$, **$P<0.001$ vs GMTHMy/miR-590). Data were normalized against GMTHMy/miR-590 values. **D**, Representative immunofluorescence staining for 4',6-diamidino-2-phenylindole (DAPI) (blue), fluorescein isothiocyanate (FITC) (green), and (red) cardiomyocyte markers cTnT (left top) and α-actinin (left bottom). Bar=100 μm. High-magnification views of cTnT and α-actinin staining show sarcomeric structures in cells treated with GMTHMy/miR-590+ALL (right panel). Bar=25 μm. **E**, Quantification of cells positive for indicated markers from experiment performed as in (**D**) (n=4/group; *$P<0.05$, **$P<0.001$ vs GMT). **F**, Quantification of percentage of cells demonstrating sarcomeric structures vs total α-actinin cells (n=3/group; *$P<0.05$, **$P<0.01$ vs GMT). All data are presented as mean±SEM.



(versus 4-fold improvements in prior studies) and 4-fold increases in human cell reprogramming (versus 2-fold in prior studies), as assessed by cTnT expression levels versus "standard" transcription factor/miR-based cocktails.[25,29–36] Our outcomes exceed prior reprogramming efficiency results in *absolute* terms

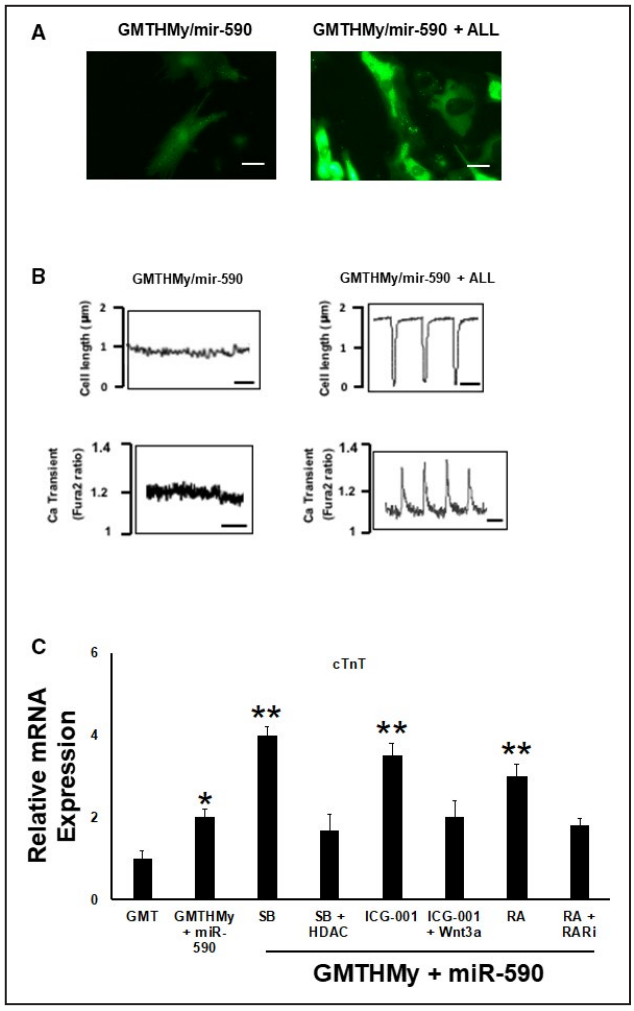


Figure 7. Functional efficacy of reprogramming of human cardiac fibroblast reprograming after coculture with neonatal rat cardiomyocytes.
Adult human cardiac fibroblasts were transduced with lentivirus expressing Gata4, Mef2c, and Tbx5 (GMT), Hand2, and Myocardin (GMTHMy)/miR-590 (left) or lentivirus expressing GMTHMy/miR-590 treated in combination with small molecules (right). One week after initial transduction, these human cardiac fibroblasts were cocultured with (untreated) neonatal rat cardiomyocytes (negative for GFP [green fluorescent protein]). **A**, Representative immunofluorescence staining demonstrating (green) GFP expression in human induced cardiomyocytes (iCMs) after 4 weeks in coculture. Bar=100 μm. **B**, Representative peaks from GFP$^+$ human iCMs treated with GMTHMy/miR-590+sodium butyrate (SB), ICG-001, and retinoic acid (RA) (ALL) after 4 weeks of coculture, reflecting contraction (top row) and Ca^{2+} transients (bottom row; n=3 independent experiment). Bar=0.5 seconds. **C**, ALL mechanisms of action in human cardiac fibroblast reprogramming. Quantitative Reverse Transcription (qRT)-polymerase chain reaction of mRNA expression of cardiac troponin T after GMTHMy/miR-590 and small-molecule administration with or without addition of small molecules and inhibitors, as indicated (n=3 biological replicates, each performed in triplicate; *P<0.05, **P<0.005 vs GMT). All data are presented as mean±SEM. RARi indicates RA receptor inhibitor.

as well as relative "fold" improvement. For example, compared with the only 1 of 3 prior human cell studies that specifically examined human cardiac fibroblast reprogramming, we demonstrated a reprogramming rate of 25% cTnT$^+$ cells (versus 6% cTnT$^+$ cells without small-molecule treatment), whereas Mohamed et al reported 13% cTnT$^+$ cells after small-molecule supplement of standard cocktails (versus 6% without supplementation).[36]

There are several mechanistic reasons justifying the potential advantages of our selected molecules over previously described small-molecule strategies. First, given the demonstrated importance of epigenetic downregulation of potential reprogramming pathways in adult and human cells, our use of SB is supported by the known efficacy of SB as a potent inhibitor of (HDAC-mediated) epigenetic downregulation of gene expression.[38,39] In this regard, SB has been shown to be more potent than other HDAC inhibitors, such as valproic acid and trichostatin A, in reprogramming human embryonic stem cells and other pluripotent cells to a more primitive state.[40,41] On the other hand, additional benefits may be yielded by modifying innate immunity and other epigenetic controls on cardiodifferentiation, as noted by Zhou et al.[37]

We likewise selected ICG-001 because of evidence that its role in modulating WNT signaling through inhibition of β-catenin expression potently increases the expression of genes beneficial for cardiac regeneration (ie, Tbx5 and Nkx2.5).[42] Supportive of the importance of this pathway, WNT signaling likely works to modulate chromatin, such that it facilitates binding of the all-important GMT reprogramming transcription factors to cardiac gene expression sites.[43] Our strategy of inhibiting WNT signaling to promote cardiac reprogramming is consistent with a prior study, where Mohamed et al[36] demonstrated that administration of the WNT inhibitor XAV939 enhanced cardiac reprogramming efficiency (by reducing β-catenin stability) and administration of WNT pathway activator CHIR99021 repressed cardiac reprogramming (by inhibiting glycogen synthase kinase 3β). We similarly demonstrated that the WNT inhibitor ICG-001 promoted cardiac reprogramming (while blocking β-catenin's transcriptional activation activity in a direction similar to the effects of XAV939 in reducing β-catenin stability). Likewise, our demonstration of the reversal of the reprogramming effects of ICG-001 by the activation of WNT signaling (via overexpressing Wnt3a) is directionally similar to the studies of Mohamed et al using CHIR99021. Finally, our selection of RA is supported by observations of its significant relative importance as a vertebrate embryo cardiodifferentiation signaling molecule.[44]

Our finding that the small molecules that we tested did not increase each of our cardio-differentiation factors of interest in a uniform manner (ie, all upregulated or downregulated at equivalent fold differences) is not surprising, and is consistent with prior studies. For example, consistent with our observations, ICG-001 has

been shown to specifically increase the expression of TBX5,[45] RA to increase the expression of MEF2C,[46] and histone deacetylase inhibitors to increase GATA-4 expression.[47] These varied effects are likely caused by the different specific binding and interaction profiles of these small molecules with the transcriptional regulatory pathways governing the expression of each of our tested effector genes. Our selection of the small molecules included in this study was predicated in part on our awareness of these potentially synergistic and complementary effects. The increased expression of GATA4/TBX5/MEF2C in our study after small-molecule treatment combined with lentiviral-mediated GMT transgene delivery compared with GATA4/TBX5/MEF2C expression after lentiviral-mediated GMT transgene delivery alone reflects, by definition, a combination of exogenous transgene and endogenous GATA4/TBX5/MEF2C gene expression.

The use of small molecules such as those included in our cocktail holds other potential advantages over the use of reprogramming transcription factors and miRNAs, which have been the mainstay of cell reprogramming efforts since the inception of this field.[5,19,25] For example, the relative inefficiencies of gene transfer of these factors, and their potential promiscuity of effect and carcinogenicity in their interactions with the host genome, may represent checks on their use in clinical applications. In comparison, small molecules are nonimmunogenic and can be effectively delivered to target cells via standard drug delivery strategies. Likewise, given evidence that cell transdifferentiation is tightly controlled by multiple pathways, the diffuse effects of small molecules offer the potential advantages of their pluripotent effects while at the same time being constrained by their interface with cell processes that typically undergo tight internal self-regulation (ie, chromatin-mediated gene activation, cell differentiation, and cell growth).[48,49]

Despite the encouraging findings of the current study, the use of small molecules also poses risks and limitations. Most important, systemically delivered small molecules could induce promiscuous effects on bystander cells or cell pathways distinct from their reprogramming effects on target cells. These theoretical risks could be distinct or even exceed those posed by standard reprogramming strategies. Although such risks could theoretically be minimized by the requisite need for reprogramming cofactors, the potential for such promiscuous effects would mandate thorough organism-wide study before any clinical applications. The benefit of these small molecules might be alternatively leveraged to identify specific cofactors modulated by these small molecules that could, in turn, be used in a more selective reprogramming strategy. Recent studies in our laboratory have, for example, identified p63 downregulation as an intriguing "next-generation" strategy.[21]

Furthermore, even with the use of small molecules, cell contraction was observed in only a relatively limited number of cells in our study compared with prior studies,[22,50] and only in a coculture environment. These findings may, in part, be explained by our use of adult rat cells and, in particular, clinically derived primary human cells, which offer greater relevance to potential clinical applications compared with the use in most other studies of murine or embryonic cells or an immortalized human cell line, but in which (spontaneous) contractile function is also less inductive.[35,37] These results are thus consistent with other in vitro results and evidence that the in vivo milieu is more conducive to complete cardiac cell transdifferentiation,[20–22] but they nevertheless reflect the remaining challenges to effective reprogramming. Although we used GMT and GMTHMy/miR590 as our base reprogramming cocktails on the basis of prior reports demonstrating their efficacy in rodent and human cell reprogramming, respectively,[9,10,19–22] we also did not demonstrate that the addition of small molecules allowed us to reduce the number of transcription factors in these base formulations.

Finally, we did observe in our FACS analysis that some cTnT$^+$ cells were negative for GFP expression (Figures 2A and 6A). We believe that these could be reprogrammed (cardiodifferentiated) cells that may be demonstrating weak GFP expression not detectable in FACS after lentiviral transduction. We have observed similar findings of heterogeneous GFP expression, especially over time, in some of our other own studies.[10,19,21] It was also interesting to observe that the transduction efficiency of lentiviral-mediated transgene transduction efficiency was higher in groups that were administered small molecules compared with those that were not (eg, GFP expression in GMTHMy/miR590+ALL group versus GMTHMy/miR590 group; Figure 6A). Evidence suggests that this may be related to the ability of small molecules to enhance lentiviral GFP transduction efficiency, as observed with administration of the HDAC inhibitor vorinostat, prostaglandin E2, and rapamycin.[51–53]

In summary, we have found that a combination of the small molecules SB, ICG-001, and RA significantly promotes cardiac reprogramming of adult rat and human fibroblasts when added to already described reprogramming cocktails, and appears to do so to an extent that may exceed prior small-molecule strategies. These findings suggest combined gene and small-molecule therapy as an optimized means to enhance cardiac cell reprogramming. Despite these encouraging observations, it is thus nevertheless possible that other small molecules and transcription factors or other reprogramming genes, either alone or in combination with those that we propose, could yield even greater reprogramming benefits, either alone or

in combination with our proposed reprogramming factors. Examination of these alternative strategies is the subject of current investigations in our laboratory.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Materials

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SUPPLEMENTAL MATERIAL

Table S1. List of chemical compounds and their dosage.

Chemicals	Functional role	Dosage	Source
Sodium butyrate	Epigenetic regulator	1 mM	Sigma Aldrich #B5887
ICG-001	Cell fate regulator	1 μM	Adooq biosciences #A11039
Retinoic acid	Cardiac growth regulator	1 μM	Sigma Aldrich #R2625
Lithium chloride	Glycogen synthase kinase (GSK) 3 signaling regulator	1 mM	Abcam #ab120853
SB431542	TGF-beta signaling	1 μM	Sigma Aldrich #S4317
Ascorbic acid	Cell senescence pathway regulator	1 M	Sigma Aldrich #A4403
Retinoic acid receptor antagonist AGN194310	Retinoic acid activity inhibitor	1 μM	194310; MCE # HY-1668

Table S2. qRT-PCR rat and human primer list.

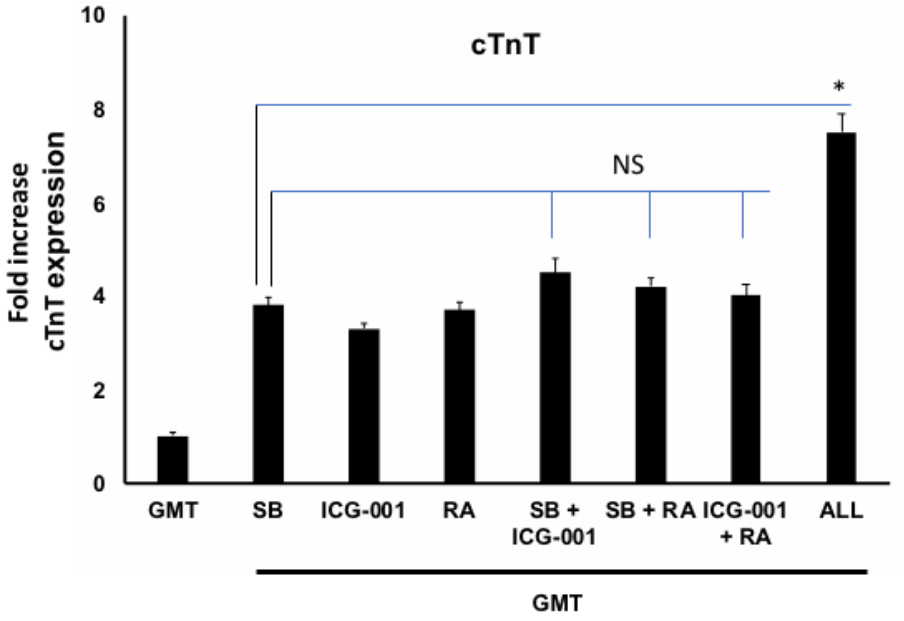
Rat Primers

Gene	Forward Primer	Reverse Primer
Gata4	CCGGGCTGTCATCTCACTAT	GAGAGCTTCAGAGCCGACAG
Mef2c	GGCATGACAGACGAGAAGG	TTCTTGTTCAGGTTACCAGGTG
Tbx5	GCACAGAAATGATCATCACCAA	GGCCAGTCACCTTCACTTTG
Myh6	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
Nkx2.5	ACCATGCGGGAAGGCTAT	AGAAGGGCGTGTGTGTGAG
cTnT	AGGCTCACTTCGAGAACAGG	ATTGCGAATACGCTGCTGT
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

Human Primers

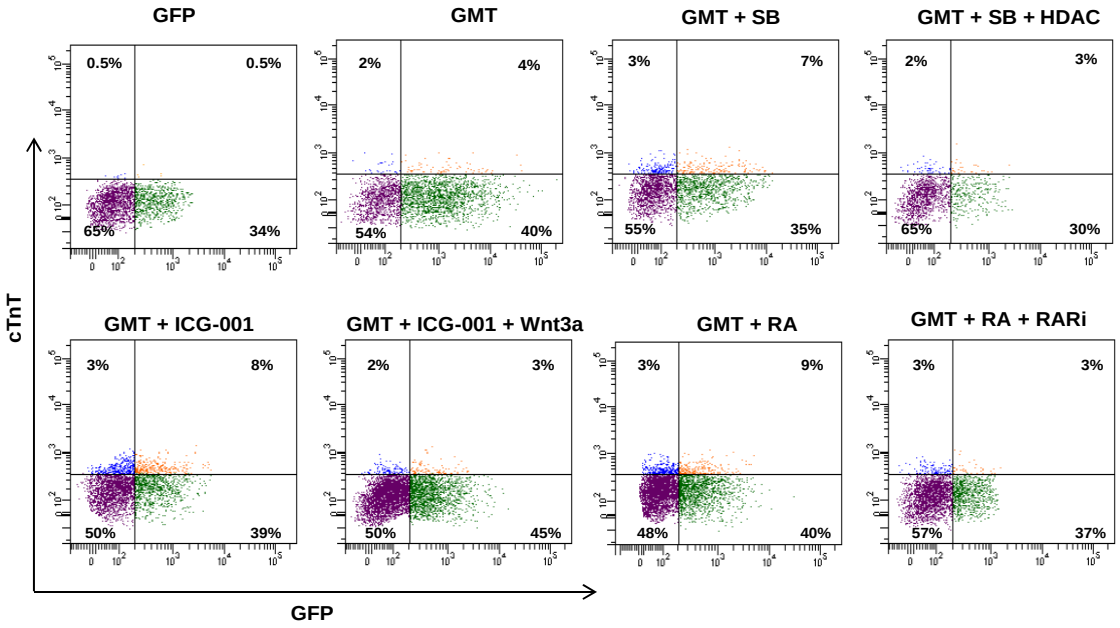
Gene	Forward Primer	Reverse Primer
Col1a1	CATGTTCAGCTTTGTGGACCT	GCAGCTGACTTCAGGGATGT
Myh7	ACACCCTGACTAAGGCCAAA	ATGCGCACCTTCTTCTCTTG
Nkx2.5	AGAAGACAGAGGCGGACAAC	AGGTACCGCTGCTGCTTG
cTnT	AGACGCCTCCAGGATCTGT	TCTTCAACAGCTGCTTCTTCC
GAPDH	CAACGACCACTTTGTCAAGC	GGTGGTCCAGGGGTCTTACT

Figure S1. Screening of candidate small molecules as a single or combination of 2 factors or combination of ALL for their enhancement of direct cardiac reprogramming.



cTnT gene expression in rat cardiac fibroblasts as assessed by qRT-PCR after 10 days of combined treatment with GMT or GMT plus indicated compounds (n=5; *$p < 0.001$ versus GMT+SB).

Figure S2. Activation of WNT signaling, HDAC and inhibition of Retinoic Acid Receptors (RARs) disrupt cardiac reprogramming in RCFs.



Representative flow cytometry plots of cTnT + cells 10 days after infection of rat cardiac fibroblasts with lentiviruses expressing GFP, GMT, GMT + small molecule as indicated.

Supplemental Video Legends:

Video S1. This video shows rat cardiac fibroblasts transduced with a GFP-control vector only did not demonstrate contractions in the co-culture experiments. Best viewed with Windows Media Player.

Video S2. This video shows rat cardiac fibroblasts transduced with a GMT vector only did not demonstrate contractions in the co-culture experiments. Best viewed with Windows Media Player.

Video S3. This video shows rat cardiac fibroblasts contracted synchronously with surrounding rat cardiomyocytes after transduction with GMT plus ALL (SB + ICG-001 + RA). Best viewed with Windows Media Player.

Video S4. This video shows adult human cardiac fibroblasts transduced with a GMTHMy/miR-590 vectors did not demonstrate contractions in the co-culture experiments. Best viewed with Windows Media Player.

Video S5. **This video shows adult human cardiac fibroblasts contracted synchronously with surrounding rat cardiomyocytes after transduction with GMTHMy/miR-590 plus ALL (SB + ICG-001 + RA).** Best viewed with Windows Media Player.