Stem Cell Reports, Volume *5*

Supplemental Information

Non-genetic Purification of Ventricular Cardiomyocytes from Differentiating Embryonic Stem Cells through Molecular Beacons Targeting IRX-4

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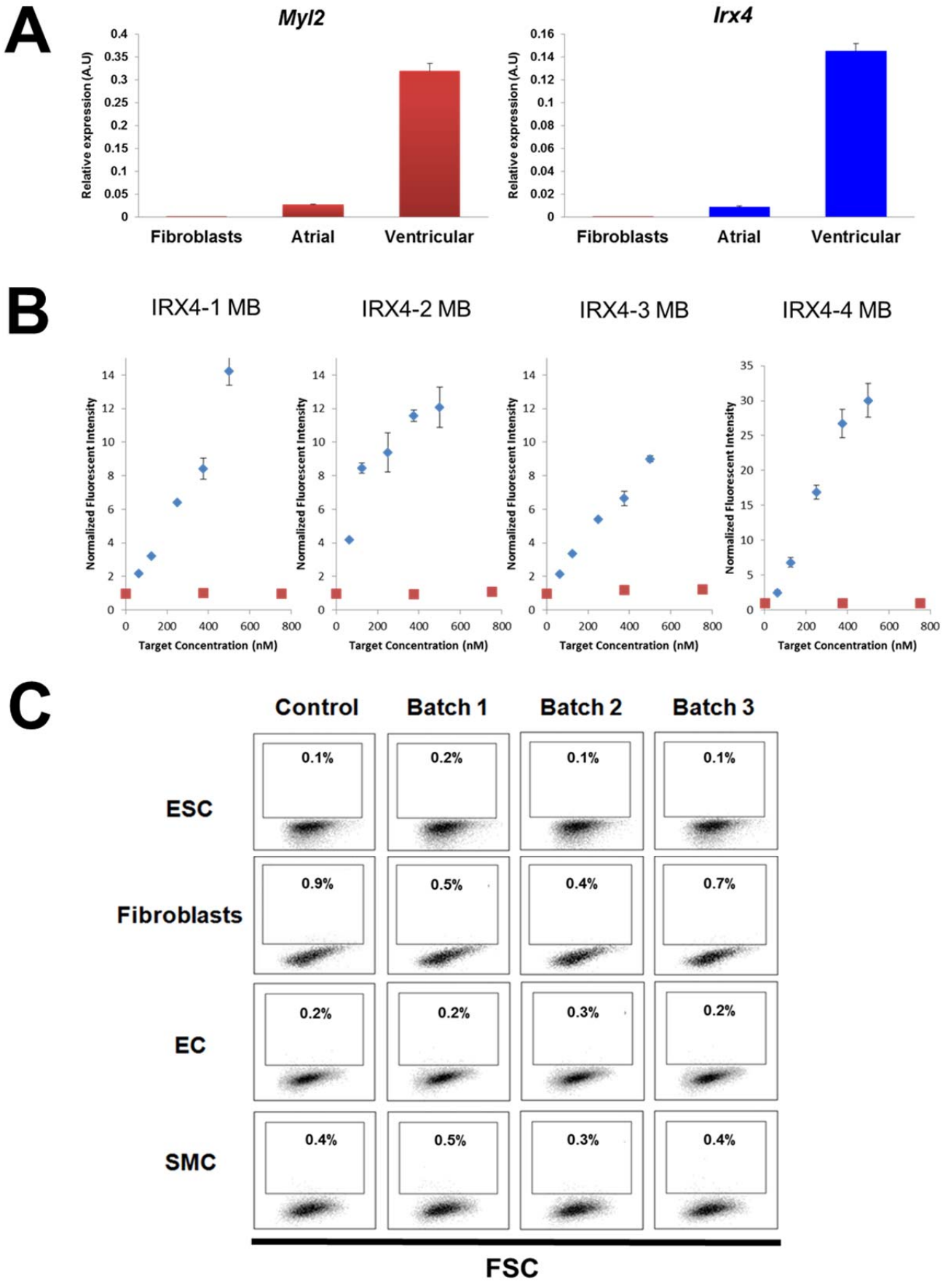
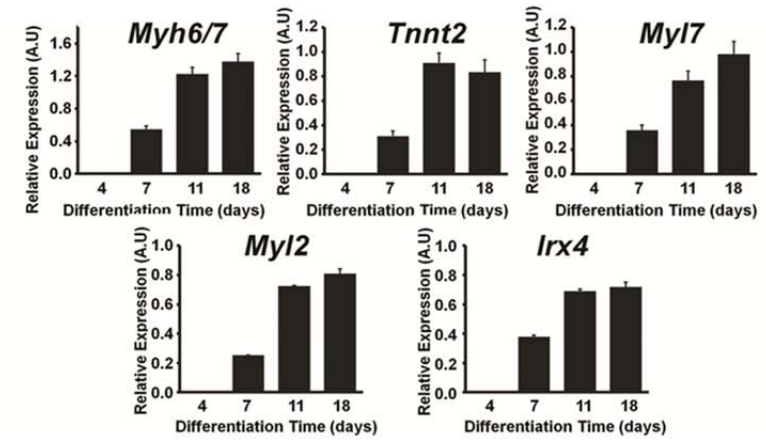
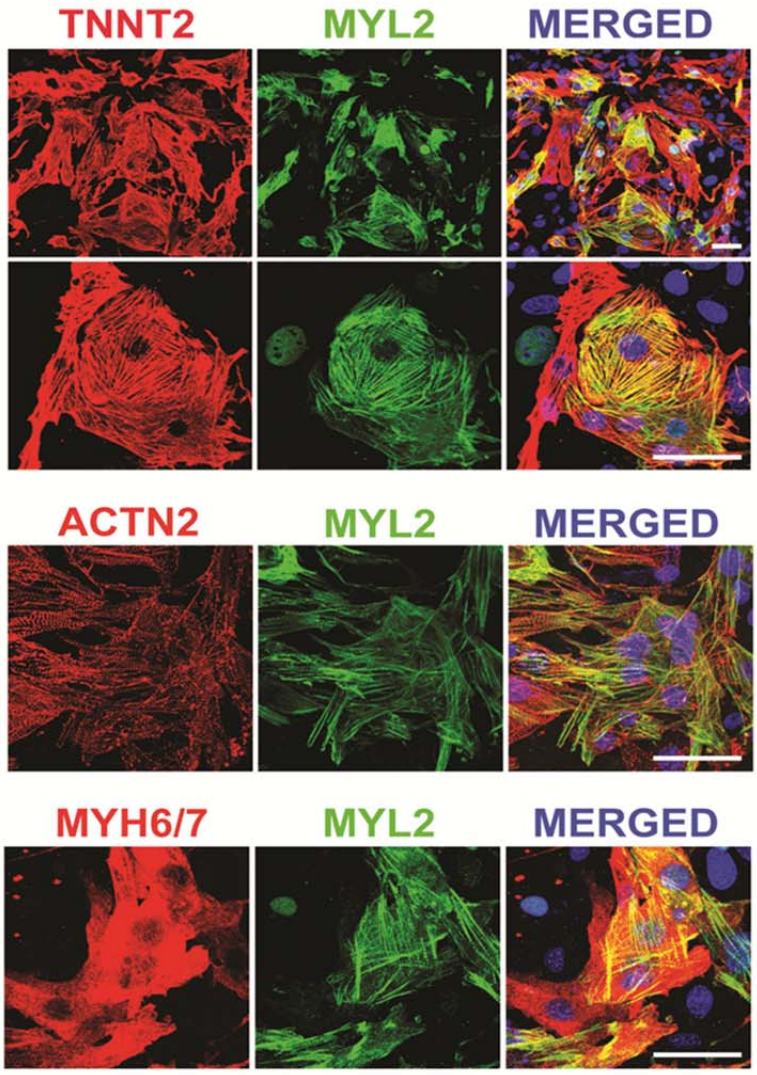
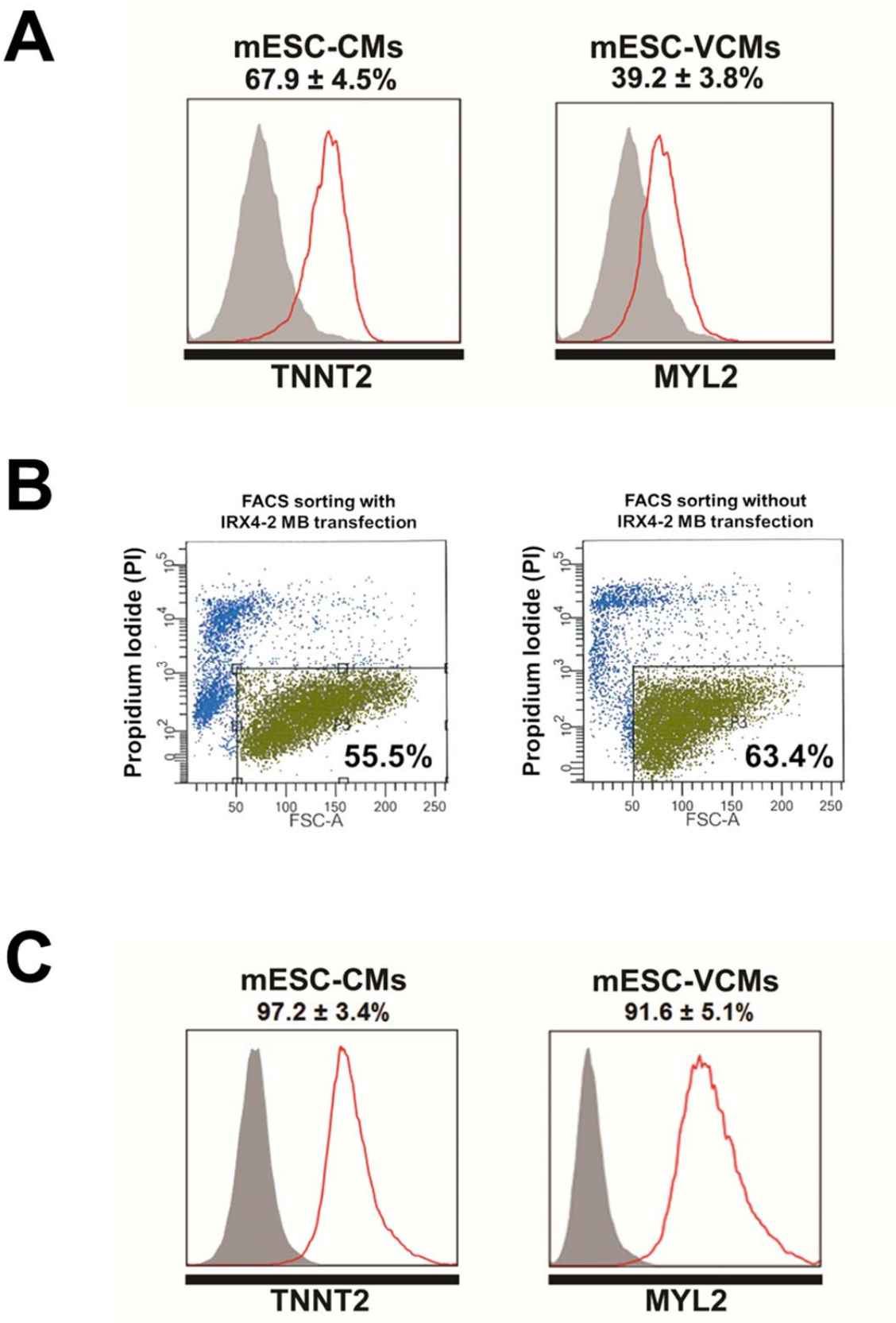

A
Myl2
Relative expression (A.U)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Fibroblasts
Atrial
Ventricular
Irx4
Relative expression (A.U)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Fibroblasts
Atrial
Ventricular
B
IRX4-1 MB
IRX4-2 MB
IRX4-3 MB
IRX4-4 MB
Normalized Fluorescent Intensity
Target Concentration (nM)
0
200
400
600
800
C
Control
Batch 1
Batch 2
Batch 3
ESC
Fibroblasts
EC
SMC
0.1%
0.2%
0.1%
0.1%
0.9%
0.5%
0.4%
0.7%
0.2%
0.2%
0.3%
0.2%
0.4%
0.5%
0.3%
0.4%
FSC

A


Myh6/7
Tnnt2
Myl7
Myl2
Irx4
Relative Expression (A.U)
Differentiation Time (days)
4
7
11
18

B

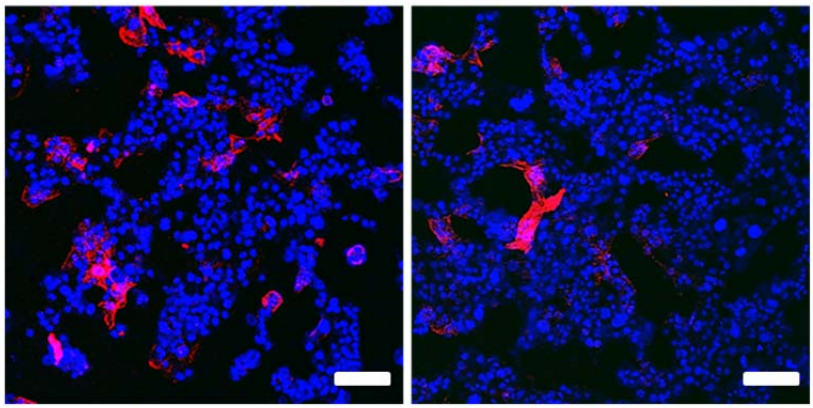

TNNT2
MYL2
MERGED
ACTN2
MYL2
MERGED
MYH6/7
MYL2
MERGED


A
mESC-CMs
67.9 ± 4.5%
TNNT2
mESC-VCMs
39.2 ± 3.8%
MYL2
B
FACS sorting with
IRX4-2 MB transfection
Propidium Iodide (PI)
55.5%
FSC-A
FACS sorting without
IRX4-2 MB transfection
Propidium Iodide (PI)
63.4%
FSC-A
C
mESC-CMs
97.2 ± 3.4%
TNNT2
mESC-VCMs
91.6 ± 5.1%
MYL2

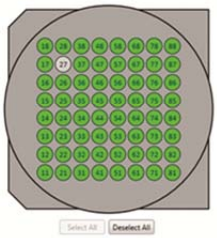
A

IRX4-2 MB⁻ cells

ACTN2, MYL2, DAPI

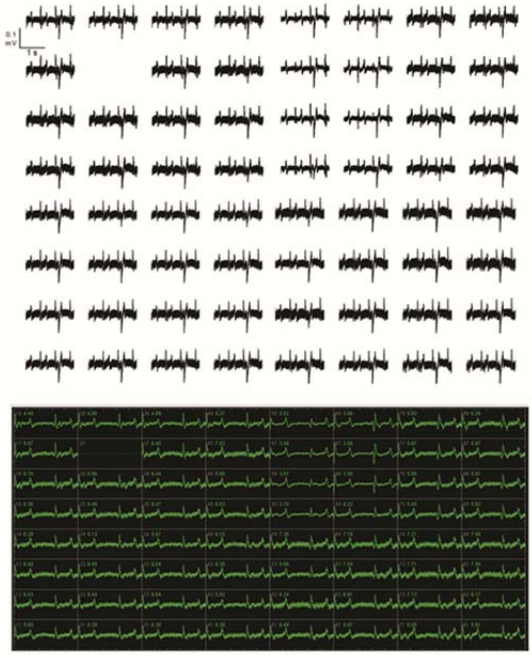


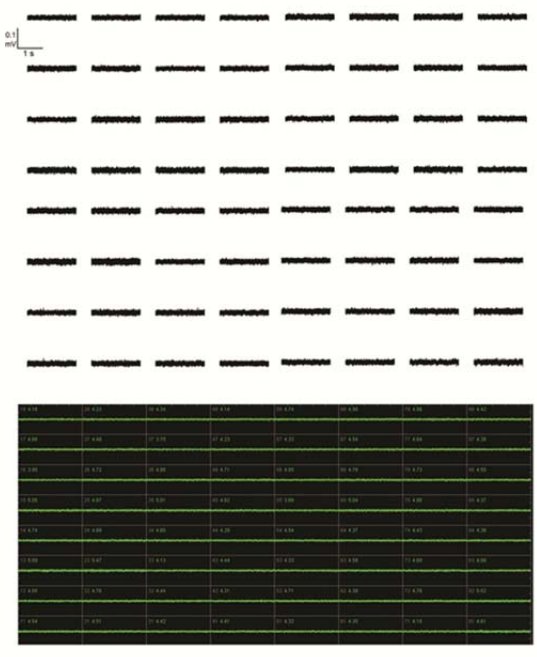
B



IRX4-2 MB⁺

IRX4-2 MB⁻





Supplemental figure legend

Figure S1, Related to Figure 1. Verification of *Irx4* mRNA as a target for ventricular CM specific MB generation. (A) qRT-PCR analysis was performed for *Irx4* and *Myl2* (also known as *Mlc2v*) mRNAs extracted from mouse cardiac fibroblasts and adult mouse atrial or ventricular CMs to determine optimal candidate genes for generating CM-specific MBs. Y axis represents relative mRNA expression of target genes to GAPDH. *P < 0.001. Data are represented as mean ± SEM. All experiments were performed on three independent biological replicates. **(B) Hybridization specificity of IRX4 MBs.** Each IRX4 MB was incubated with its synthetic 20-30 bp complementary sequence (blue) in PBS solution at 37˚C to verify that they would respond to increases in target concentration in a linear fashion. MB fluorescence was measured within 10 minutes to ensure quick signal response and robust signal maintenance. The MBs were also tested against synthetic targets with 6 bp mismatches (red) under identical conditions. All experiments were performed on three independent biological replicates. **(C) Specificity of IRX4-2 MBs on several types of non-cardiomyocyte cells.** Flow cytometry analysis demonstrated that IRX4-2 MB detected non-cardiomyocyte cells such as SMCs, mECs, mCFs and mESCs at a very low rate. FSC indicates forward scatter.

Figure S2, Related to Figure 2. (A) Expression profiles of the CM-specific genes (*Tnnt2 and Myh6/7*) and chamber-specific CM genes (*Myl7, Myl2 and Irx4*) during cardiomyocyte differentiation from mESCs. Y axis represents relative mRNA expression of target genes to GAPDH. Data are represented as mean ± SEM. All experiments were performed on three independent biological replicates. **(B)** Immunocytochemistry results for general (Tnnt2, Actn2) and ventricular (Myh6/7) cardiomyocyte markers for cardiomyogenically differentiated mESCs at day 18. Scale bars, 50 μm.

Figure S3, Related to Figure 2. (A) Flow cytometry plots demonstrating the percentages of the cells expressing TNNT2 or MYL2 at differentiation day 18. **(B)** Viability of cells after FACS-sorting with or without IRX4-2 MB transfection via nucleofection. **(C)** Flow cytometry histograms demonstrating the percentages of the cells expressing TNNT2 or MYL2 after FACS-sorting with IRX4-2-MB at differentiation day 20. All experiments were performed on three independent biological replicates (A-C).

Figure S4, Related to Figure 3. (A) Immunocytochemistry demonstrates that the IRX4-2-MB-negative population comprises ~20% of $ACTN2^+$ cardiomyocytes but no $MYL2^+$ ventricular cardiomyocytes. Scale bars, 50 μm **(B)** Electrical activities recorded from IRX4-2-MB^+ and IRX4-2-MB^- mESC-derived CMs through multielectrode arrays.

Table S1. Primer sequences used for RT qPCR analysis.

GENE	FORWARD 5'-3'	REVERSE 5'-3'
Gapdh	TGTGATGGGTGTGAACCACGAGAA	CATGAGCCCTTCCACAATGCCAAA
Tnnt2	TCACAACCTGGAGGCTGAGAAGTT	TCATCTATTTCCAACGCCCGGTGA
Myh6	AGCTGACAGGGGCCATCAT	ACATACTCGTTCCCCACCTTC
Myh 7	TGCCAATGACGACCTGAAGGAGAA	TCTTCTGGTTGATGAGGCTGGTGT
Myl2	AGATGCTGACCACACAAGCAGAGA	TCCGTGGGTAATGATGTGGACCAA
Myl7	AAGGGAAGGGTCCCATCAACTTCA	AACTTGTCTGCCTGGGTCATGAGA
Irx4	TCC TAC CCG CAG TTT GCA TAC	GCG GCA GAA TTG AGT TCG T
Neurod	GACGGGGTCCCAAAAAGAAAA	GCCAAGCGCAGTGTCTCTATT
Pecam	TCTATGACCTCGCCCTCCACAAA	GAACGGTGTCTTCAGGTTGGTATTTCA
Cdh5	TGGAGAAGTGGCATCAGTCAACAG	TCTACAATCCCTTGCAGTGTGAG
Gata1	TGTCCTCACCATCAGATTCCA	TCCCTCCATACTGTTGAGCAG
Myod1	CCACGACCACCTCTCAGAAC	GACAGGACAGTATGCAGTGGA
Ddr2	ATGATCCCGATTCCCAGAATGC	CATATTTGGCAGCCGTGGATT
Acta2	CATGTACGTCGCCATTCAAGC	TTGATGTCTCGCACAATTTCTCT

Movie S1. Contraction of mESC-derived cardiomyocytes.

Movie S2. Contraction of mouse-ESC derived ventricular CMs purified via IRX4-2 MB and FACS.

Supplemental Experimental Procedures

Isolation and culture of neonatal mouse ventricular cardiomyocytes

All animal experiments were approved by Emory University Institutional Animal Care and Use Committee and were performed in accordance with federal guidelines. The hearts from mice at embryonic day at 17-18 or postnatal day 1 were harvested and digested with 50 U/ml collagenase type II (Worthington Biochemical) in calcium- and bicarbonate-free Hanks' buffer with HEPES at room temperature, followed by overnight digestion with 0.5 mg/ml of trypsin (Invitrogen embryonic day 17-18 mouse) at 4°C. Fibroblast and endothelial cell numbers were minimized by differential plating. CMs were plated in 100mm dishes at a density of 2 x 10^6 cells, and cultured at 37°C in growth media containing Dulbecco's modified Eagle's medium/Ham's F-12 [1:1 (v/v); Invitrogen], 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin (Invitrogen) supplemented with 0.1 mM bromodeoxyuridine (Sigma-Aldrich) and 20 μM arabinosylcytosine (Sigma-Aldrich) to inhibit proliferation of non-CMs. After 24 hrs in culture, the medium was replaced with serum-free medium supplemented with 1% insulin-transferrin-selenium (Invitrogen) in DMEM/F12 [1:1 (v/v)] and cultures were maintained until further use.

HL-1 cell culture

HL-1 CMs, a cell line derived from adult mouse atria, was received from Dr. William Claycomb (Louisiana State University, LA, USA) and cultivated as described in the literature (Claycomb et al., 1998). The HL-1 CMs were plated in a dish coated with 12.5 μg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma), and maintained in complete Claycomb medium (Sigma) supplemented with 10 μM norepinephrine (Sigma), 0.3 mM L-ascorbic acid (Sigma), 4 mM L-glutamine (Gibco) and 10% FBS (Sigma) in a 5% CO_2 atmosphere at 37°C.

Mouse ESC culture and differentiation

mESCs (J1) were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Atlanta Biologicals), 1% non-essential amino acids solution, 1% L-glutamine, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin and 2,000 U ml^{-1} mouse LIF (Millipore) on feeder layers of mitotically inactivated STO cells, a mouse embryonic fibroblast line (ATCC). Prior to differentiation, mESCs were passaged twice on gelatin-coated dishes to remove the STO cells. To differentiate mESCs into cardiac lineage, an embryoid body (EB) method was employed with some modifications (Hescheler et al., 1997). EBs were formed by suspending the cells at 10^7 cells/mL in 10 mL of differentiation media; alpha-modified Eagle medium (αMEM; Invitrogen) supplemented with 10% FBS, 1% non-essential amino acids, 1% L-glutamine, 1% β-mercaptoethanol, L-ascorbic acid (50 μg/ml; Sigma), and 1% penicillin/streptomycin. By day 1, cells aggregated to form EBs. Differentiation medium was changed every day. Four days after initiation of EB formation, floating EBs were collected by centrifugation and transferred to fibronectin (Sigma)-coated plates. These attached EBs were cultured as a monolayer in non-serum culture medium: DMEM/F12 (Invitrogen) supplemented with L-ascorbic acid (50 μg/ml) for further differentiation into CMs. Typically, beating cells appeared on day 7.

Percoll™ separation

To partially enrich the cardiomyocytes from the differentiation cultures, we performed Percoll gradient separation with some modifications (Xu et al., 2002). At differentiation day 11, the cardiomyogenically differentiated mESCs were dissociated with Trypsin (0.05%) and resuspended in 10ml of □MEM medium. The 10 ml of cell suspension was added to the mixture of 10ml of 40.5% Percoll™ (Amersham Biosciences AB, Uppsala, Sweden) and 10ml of 58.5% Percoll™ and centrifuged at 1,500 g for 30 min. Four fractions of layers appeared after centrifugation and the third and fourth layers from the bottom of tubes were collected, combined, washed, and resuspended in □MEM (Invitrogen) supplemented with 0% FBS, 1% non-essential amino acids, 1% L-glutamine, 1% β-mercaptoethanol, 1% penicillin/streptomycin, L-ascorbic acid (50 μg/ml; Sigma), and Cyclosporin A (30 μg/ml; Calbiochem). Finally the cell suspension was plated on fibronectin (Sigma)-coated plates for further culture.

Nucleofection

Target cells were detached by treatment with Accutase (e-Bioscience) and filtered through a 70-μm cell strainer (BD science) immediately before nucleofection. The dissociated cells (0.5 - 2 × 10^6) were carefully suspended in 100 μl of nucleofector Solution V (Lonza) maintained at room temperature, and 0.5 μl of 500 nM MB was added for each reaction. Nucleofection was performed using a Nucleofector II (Amaxa Biosystems) set to the A033 program. After termination of nucleofection, 500 μl of cold DMEM/F12 media was added to the reaction cuvette and the contents were gently transferred into a clean tube with a flexible pipette (Lonza). All procedures for nucleofection were performed inside a biological safety cabinet (Labconco) in the dark to prevent light induced non-specific reaction of

MBs. Subsequently, 1 ml of pre-warmed DMEM/F12 media was added to each tube, which was further incubated in a 5% CO_2 atmosphere at 37°C for 10 min for the MB reaction.

Supplemental References

Claycomb, W.C., Lanson, N.A., Stallworth, B.S., Egeland, D.B., Delcarpio, J.B., Bahinski, A., and Izzo, N.J. (1998). HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proceedings of the National Academy of Sciences *95*, 2979-2984.

Hescheler, J., Fleischmann, B.K., Lentini, S., Maltsev, V.A., Rohwedel, J., Wobus, A.M., and Addicks, K. (1997). Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. Cardiovascular Research *36*, 149-162.

Xu, C., Police, S., Rao, N., and Carpenter, M.K. (2002). Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells. Circulation Research *91*, 501-508.