SUPPLEMENTAL MATERIAL

Supplemental Methods

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[1]. 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[2]. 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 15% 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. 5 days after initiation of EB formation, floating EBs were enzymatically dissociated by treatment with Accutase (e-Bioscience) and were transferred to fibronectin-coated plates. These EB-dissociated cells were cultured in non-serum culture medium: DMEM/F12 (Invitrogen)

supplemented with norepinephrine (100 μM) and L-ascorbic acid (50 μg/ml) for further differentiation into CMs. Typically, beating cells appeared on day 7.

Human PSC Culture and Differentiation

hESC (H1) were obtained from WiCell Research Institute (Madison, WI) and hiPSC (BJ1-iPS10) was kindly provided by George Daley at Harvard University[3]. The use of hPSCs was approved by Emory University. These undifferentiated hPSCs were cultured on mitotically inactivated STO cells in DMEM/F12 supplemented with 20% serum replacement (Invitrogen), 1% L-glutamine, 1% nonessential amino acids, 100 mM β-mercaptoethanol, and 4ng/ml basic fibroblast growth factor (bFGF; R & D systems). The medium was changed every day and the hPSCs were transferred to new feeder cells every 5 to 7 days. To direct the differentiation of hPSCs to the cardiac lineage, we designed a staged protocol that is divided into four distinct phases **(Supplementary Figure 7a & b)**. In phase 1, undifferentiated hPSCs were dissociated to small clusters (10–20 cells) by treatment with Dispase (1 mg/ml; Invitrogen) and directly transferred onto growth factor reduced Matrigel (BD Biosciences)-coated plates as a two dimensional culture. These cells were cultured for 24-48 hrs in mTeSR® media (STEMCELL Technologies) for their expansion. Next, in phase 2, to induce the expanded hPSCs into mesodermal lineage, a combination of BMP4 (10ng/ml), Activin A (3ng/ml) and FGF2 (5ng/ml) was added for 2 days. In phase 3, differentiating hPSCs were cultured in END-2 conditioned media for 4 days. To produce END-2 conditioned media, mouse endodermal cell line END-2 cells (gift from Dr Christine Mummery, Leiden University, Netherlands)[4] were cultured in DMEM/F12 media supplemented with 1% Insulin-Transferrin-Selenium (ITS; Invitrogen), 1% penicillin-streptomycin, and 0.1 mM β-mercaptoethanol at a seeding density of 5.0×10^4 cells/cm^2 in 0.1% gelatin coated T-75 flasks (Fisher Scientific). After 3 days of culture, the supernatant was collected, filter-sterilized through a 0.22-μm filter (Nalgene) and stored at –80°C until further use. Finally, in phase 4, continuous

treatment with β–adrenergic receptor agonist isoproterenol (10 μM) for as short as 4 days efficiently generated spontaneously beating CMs.

Human heart tissue

Neonatal human heart tissue was obtained from ventricular tissue that was required to be removed as part of the surgical repair for congenital heart defects. The protocols used in this study were approved by the Institutional Review Board of Emory University and Children's Healthcare of Atlanta. RNA from fetal human heart tissues was purchased from Clontech Laboratories, Inc.

Molecular beacon synthesis and characterization

Five MBs were synthesized by MWG Operon using standard resin-based synthesis methods with HPLC purification. Beacons were re-suspended in nuclease free TE buffer, pH 8.0 to maximize beacon stability. Beacons were tested against synthetic 20-30 bp complementary sequences in PBS solution to verify their activity**.** The specificity of molecular beacons has been well established[5-7]. To confirm beacon synthesis specificity, beacons were also tested against synthetic targets with 6 bp mismatches for 2 hours at 37°C. This was to determine the potential non-specific hybridization with all other endogenous mRNA sequences in mice and humans (MBs were designed to have at least a 6-bp mismatch with any other sequence in the mouse and human genomes). MBs showing a signal to noise ratio lower than 5 were not used for further testing.

Nucleofection

Target cells were dissociated by treatment with Accutase (e-Bioscience) and filtered through a 40-μm cell strainer (BD science) immediately before nucleofection. The dissociated cells (0.5-1 × 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 nucleofector 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 by 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.

Flow Cytometry

After nucleofection, cells were centrifuged at 1500 rpm for 2 min, re-suspended in DMEM/F12 basal media, and maintained on ice for 20 min to recover. Cells were then analyzed by C6 Flow Cytometer (BD Biosciences) or sorted using a BD FACS Aria II cell sorter (BD Biosciences). MB signal was recorded using a 561 nm laser with a 585/15 nm emission filter to optimally excite and detect Cy3. Data were analyzed using FlowJo software (Treestar).

Immunocytochemistry and Immunohistochemistry

Cells or frozen heart sections prepared with OCT compound (Tissue-TeK 4583, Sakura Finetek Inc) were fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.1 or 0.5% Triton X-100 in PBS for 10 min. Samples were then blocked with 1% BSA in PBS for 60 min at room temperature and incubated with anti-ACTN2 (Sigma; 1:100), mouse anti-TNNT2 (NeoMarkers; 1:100), or rabbit anti-cTnI (Abcam; 1:100) at 4°C overnight. The cells were washed three times with 1% Tween 20 in PBS and incubated with anti-mouse IgG– Alexa Fluor 594 (Invitrogen; 1:1000) or anti-rabbit IgG–Alexa Fluor 488 (Invotrogen; 1:1000) in PBS for 1 h at room temperature. DAPI was used for nuclear staining.

The samples were visualized under a fluorescent microscope (Nikon) and a Zeiss LSM 510 Meta confocal laser scanning microscope and LSM 510 Image software (CLSM, Carl Zeiss).

Real-time RT-PCR

Total RNA was prepared with the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. The extracted RNA (100 ng to 1 mg) was reverse transcribed into cDNA (reverse transcription) via Taqman reverse transcription reagents including random hexamers, oligo (dT), and MultiScribe™ MuLV reverse transcriptase (Applied Biosystems). qPCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using Fast SYBR Green master mix (Applied Biosystems). All annealing steps were carried out at 60 °C. Relative mRNA expression of target genes was calculated with the comparative CT method. All target genes were normalized to *GAPDH* in multiplexed reactions performed in triplicate. Differences in CT values (ΔCT = CT gene of interest−CT GAPDH in experimental samples) were calculated for each target mRNA by subtracting the mean value of *GAPDH* (relative expression = $2^{-\Delta CT}$)[8]. Information on primer sets (Eurofins) used in this study is listed in **Table 2**.

Intracellular calcium (Ca^{2+}) imaging

For calcium imaging, MB-based purified cells were plated on 25 mm square glass coverslips (Corning) and incubated in 5% CO_2 at 37°C for 20 min in Tyrode's solution containing calcium dye, Fluo-4AM (7μM, Molecular Probes, Eugene OR). Cells on coverslips were mounted onto an FHD IonOptix (Ionoptix Scientific Instruments, Milton, MA) chamber between two platinum electrodes placed 5 mm apart. Cells were perfused with Tyrodes solution at 37°C for 30 min before imaging for deesterification of the dye. Cells were paced by field stimulation through the platinum electrodes with a 10 ms duration pulse at 0.5 Hz (HSE stimulator P, Hugo Sachs Electronik, F.R. Germany) Confocal imaging was performed using an Olympus FV-1000 system coupled to an Olympus IX-81automated inverted microscope (Olympus) equipped with a 40X

water immersion lens (NA=1.15). Line scan images were taken for determining calcium transients ([Ca^{2+}]) and plotted as F/F_0 where F_0 was the baseline fluorescence measured prior to field stimulation[9].

Action potential measurement

For intracellular action potential (AP) recording, the purified cells via MHC-1 MBs were transferred and cultured on 0.1% fibronectin-coated glass bottom microwell dishes for 7 to 14 days. Next, the 35-mm dishes were mounted on an inverted microscope (Olympus IX71, Japan) and heated by a heating/cooling bath temperature controller (DTC-200, Dagan Corporation, Minneapolis, MN). The cells were perfused with Tyrode's solution containing (mmol/L) 140 NaCl, 5.4 KC1, 1 MgCl2, 10 HEPES, 10 glucose, 1.8 CaCl2, pH 7.4 with NAOH 37 °C. Glass microelectrodes were fabricated from borosilicate glass (PG52151-4, World Precision Instruments, Inc., Sarasota, FL) and pulled on a P-87 Flaming/Brown puller (Sutter Instrument Company, Novato, CA). The tip resistance of the microelectrode was 40–80 MΩ when filled with a 3 mol/L KCl solution. Intracellular recordings of membrane potential were performed using an EPC 7 amplifier (List Medical, Darmstadt, Germany) in current clamp mode at 37 ± 0.5 °C. The junction potential between the microelectrode solution and the bath solution was adjusted to zero and the microelectrodes' capacitance was compensated. Individual cells were impaled with the sharp microelectrodes and the spontaneous APs were filtered at 10 kHz and digitized on a computer at 10 kHz. APs were analyzed using Origin 6.0 software (Microcal Inc., Northampton, MA).

Induction of myocardial infarction (MI) and cell transplantation

All animal experiments were approved by Emory University Institutional Animal Care and Use Committee and were performed in accordance with federal guidelines. Studies were performed using the male athymic nude mice ($Foxn1^{nu}$) (Harlan, USA). Myocardial infarction (MI) and cell

implantation were performed as we described previously[10]. Briefly, we induced MI in athymic nude mice by ligation of the left anterior descending coronary artery and injected cells or PBS with a 30 G needle at two sites in the border zone of myocardium. We randomly assigned the mice into three groups which received phosphate buffered saline (PBS) (N = 10), 2 x 10^5 unpurified CMs differentiated from mESCs (N = 12), or 2 x 10^5 purified CMs with MHC1-MB from differentiated mESCs (N = 11). Both purified and unpurified CMs were obtained from the same differentiation batch and pre-labeled with CM-DiI (red fluorescence) before cell injection for cell tracking in histology.

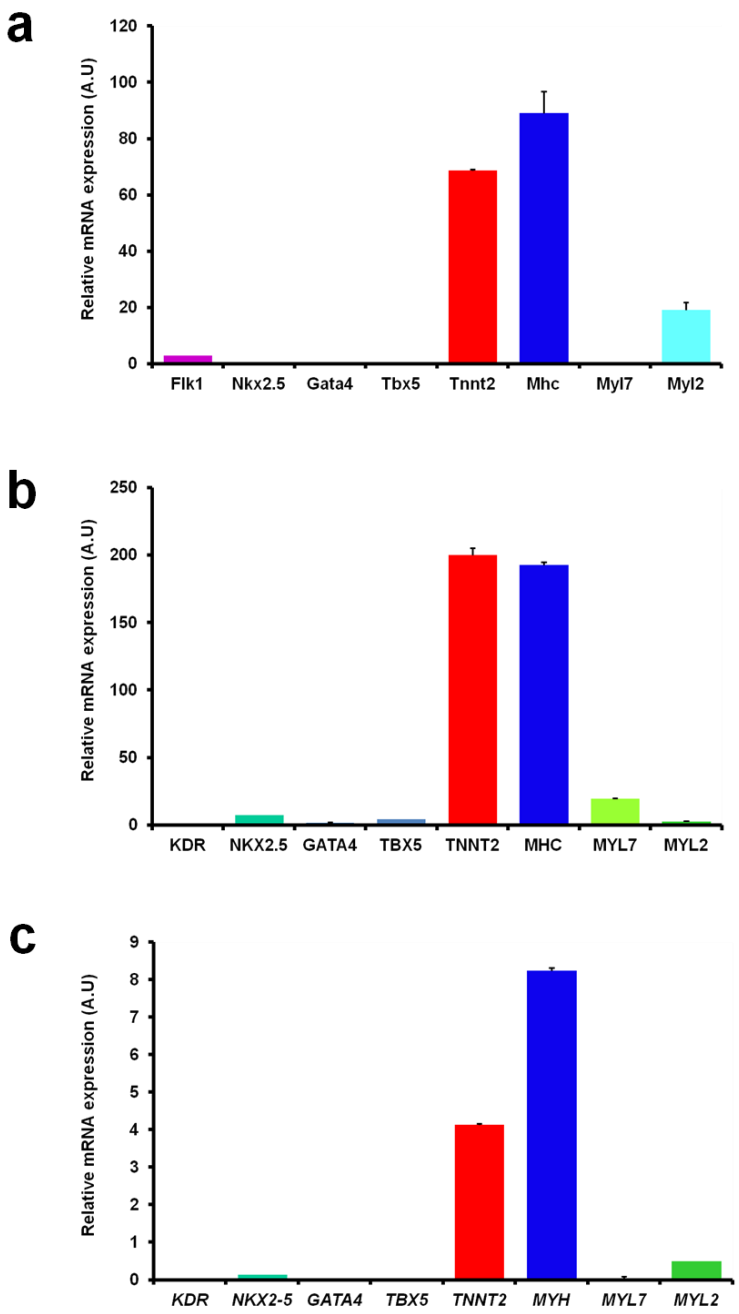
Echocardiographic measurement of cardiac function

Echocardiography was performed at days 7, 14, and 28 after surgery using Vevo 770TM Imaging System (VisualSonics, Inc) as previously described [11]. Ejection fraction (EF) and fractional shortening (FS) were measured using two-dimensional and M-mode images.

Statistical analyses

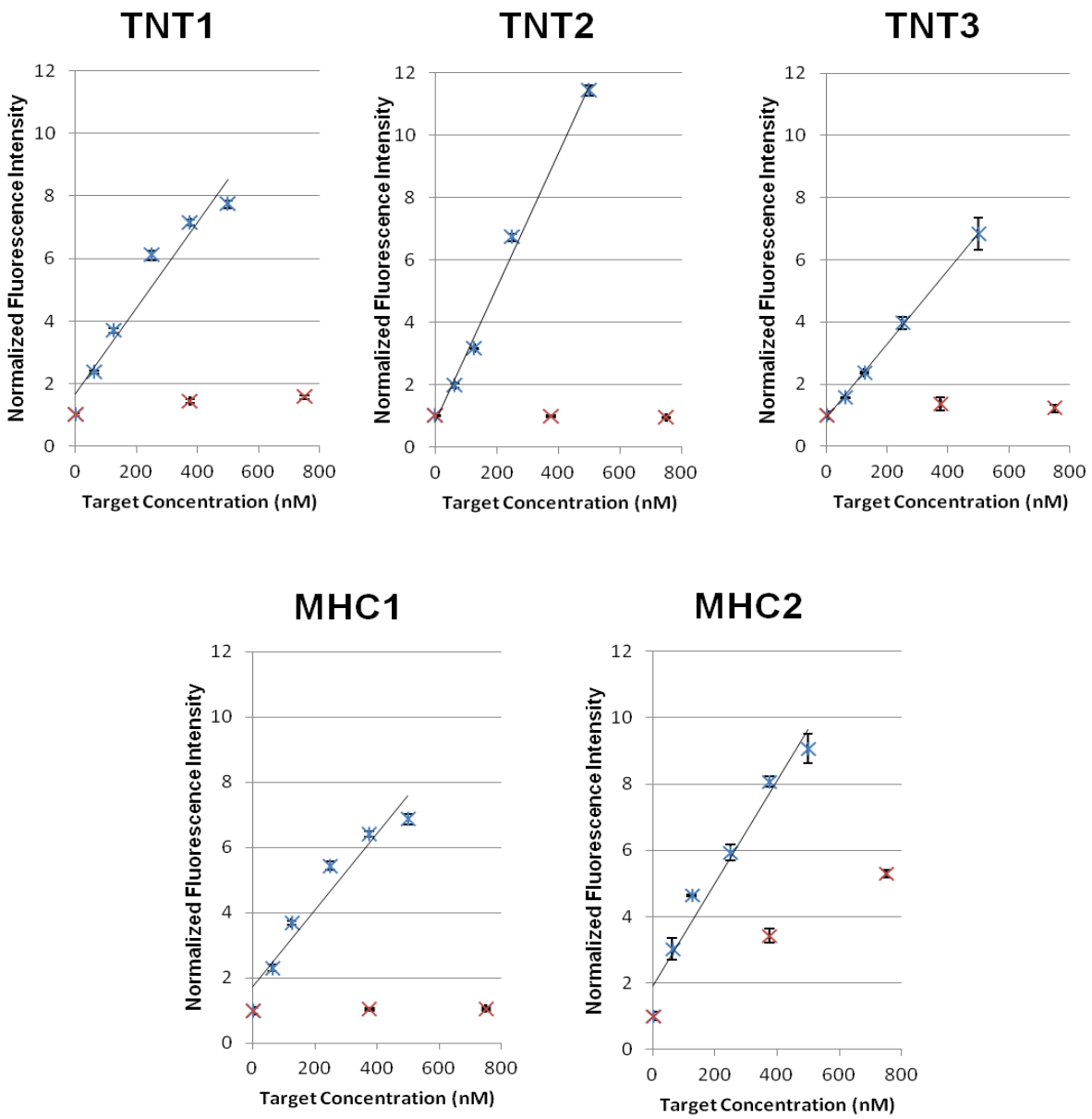
All data were expressed as mean ± SEM. Kruskal-Wallis ANOVA test was used for the statistical analysis for data shown in Figure 4G, 5F and 6F. Repeated measures ANOVA was used for data shown in Figures 7D. Values of $P < 0.05$ were considered to denote statistical significance. All statistical analyses were conducted using SPSS 20.0 (SPSS Inc).

Supplemental Figures

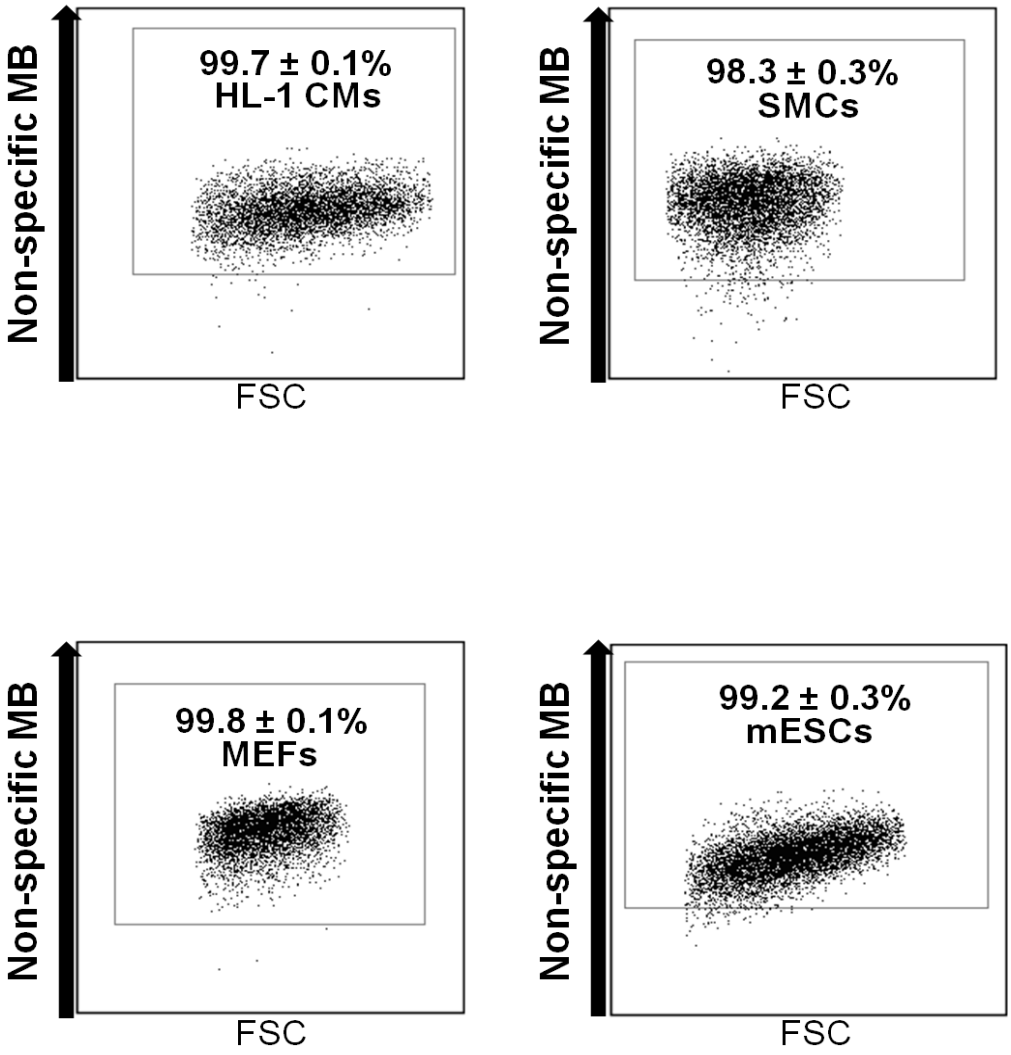


Supplementary figure 1. Determination of optimal target for MB generation.

qRT-PCR analysis was performed against known cardiac-specific genes using mRNAs extracted from (a) freshly isolated adult mouse CMs, (b) human neonatal and (C) fetal heart tissues to determine optimal candidate genes for generating CM-specific MBs. Y axis represents relative mRNA expression of target genes to GAPDH. *P < 0.001. N = 3.

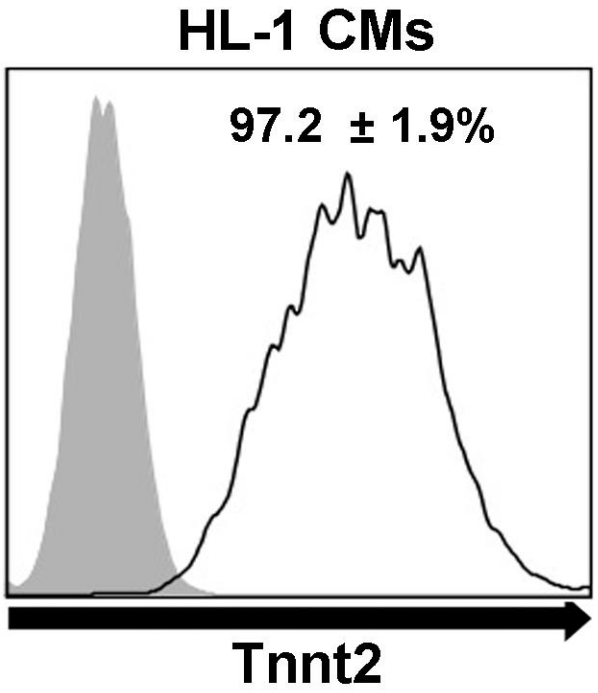


Supplementary figure 2. Hybridization specificity of MB. MBs were incubated with their synthetic 20-30 bp complementary sequences (blue) in PBS solution at 37˚C to verify that they would respond to increases in target concentration in a linear fashion. MB fluorescence was measured every 10 minutes to ensure quick signal response and robust signal maintenance. The MBs were also tested against synthetic targets with 6 bp mismatches (red) under identical conditions. N = 3

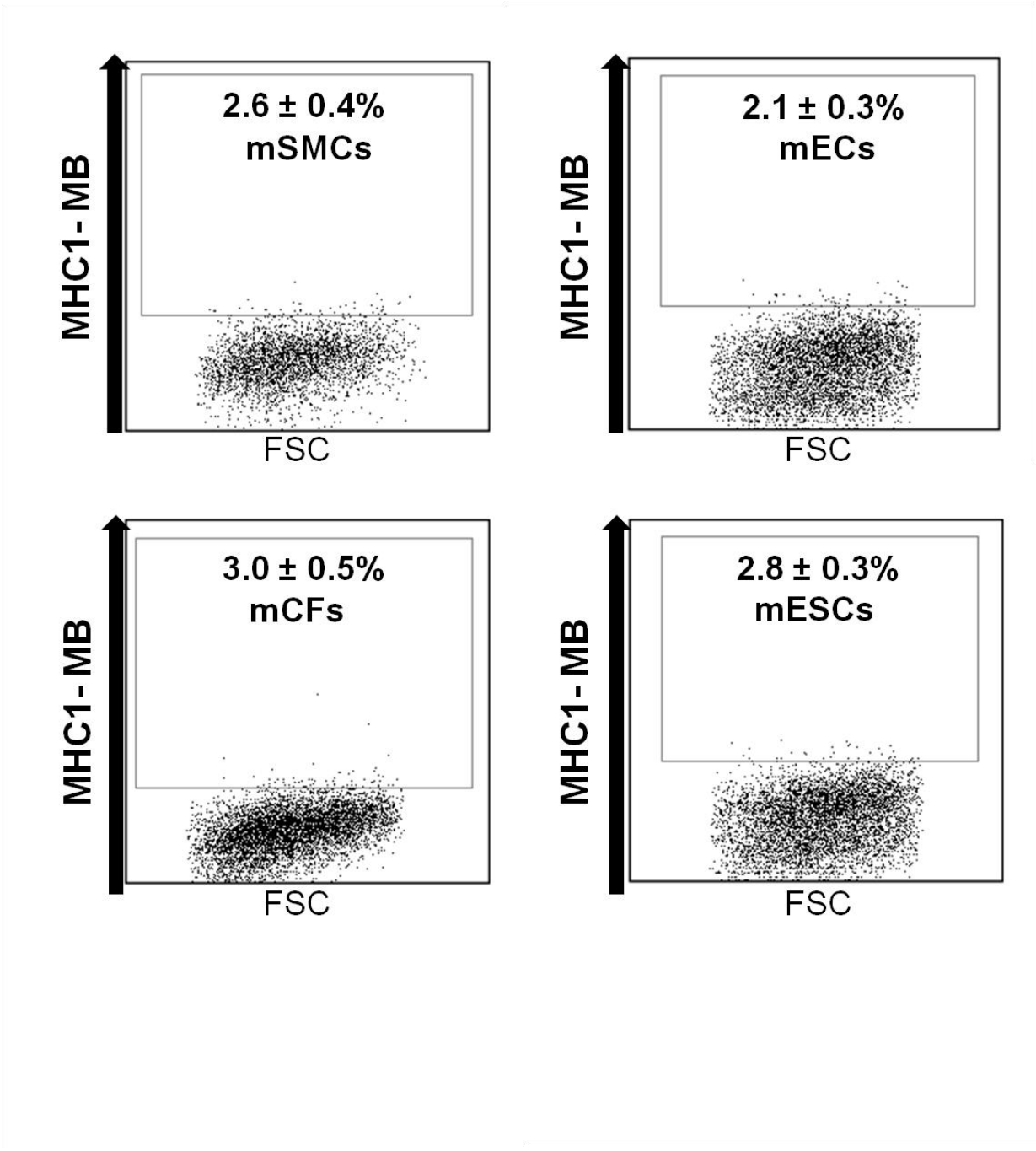


Supplementary figure 3. Efficiency of nucleofection for delivering non-specific MB into live cells.

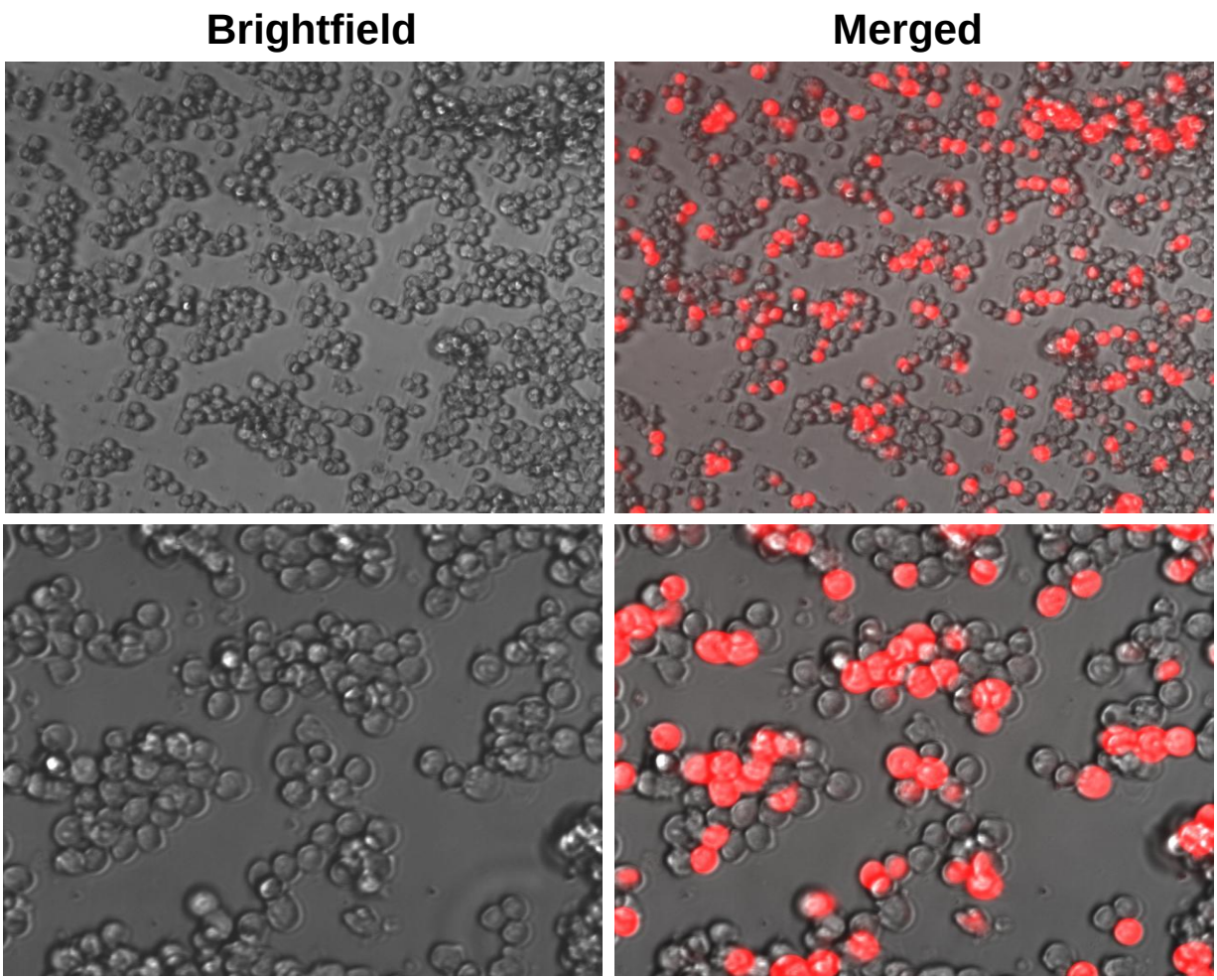
Flow cytometry analysis demonstrated that nucleofection equally transfected the non-specific MB into different types of cells such as HL-1 CMs, smooth muscle cells (SMCs), mouse embryonic fibroblasts (MEF) and mouse embryonic stem cells (mESCs). The non-specific MB was specifically designed to emit the fluorescent signal independent of its open or closed conformation for evaluating the transfection efficiency. N = 3.



Supplementary figure 4. Identity of HL-1 cells as CMs was verified by quantifying Tnnt2 expression via flow cytometry. The gray histogram indicates isotype control. N = 3.

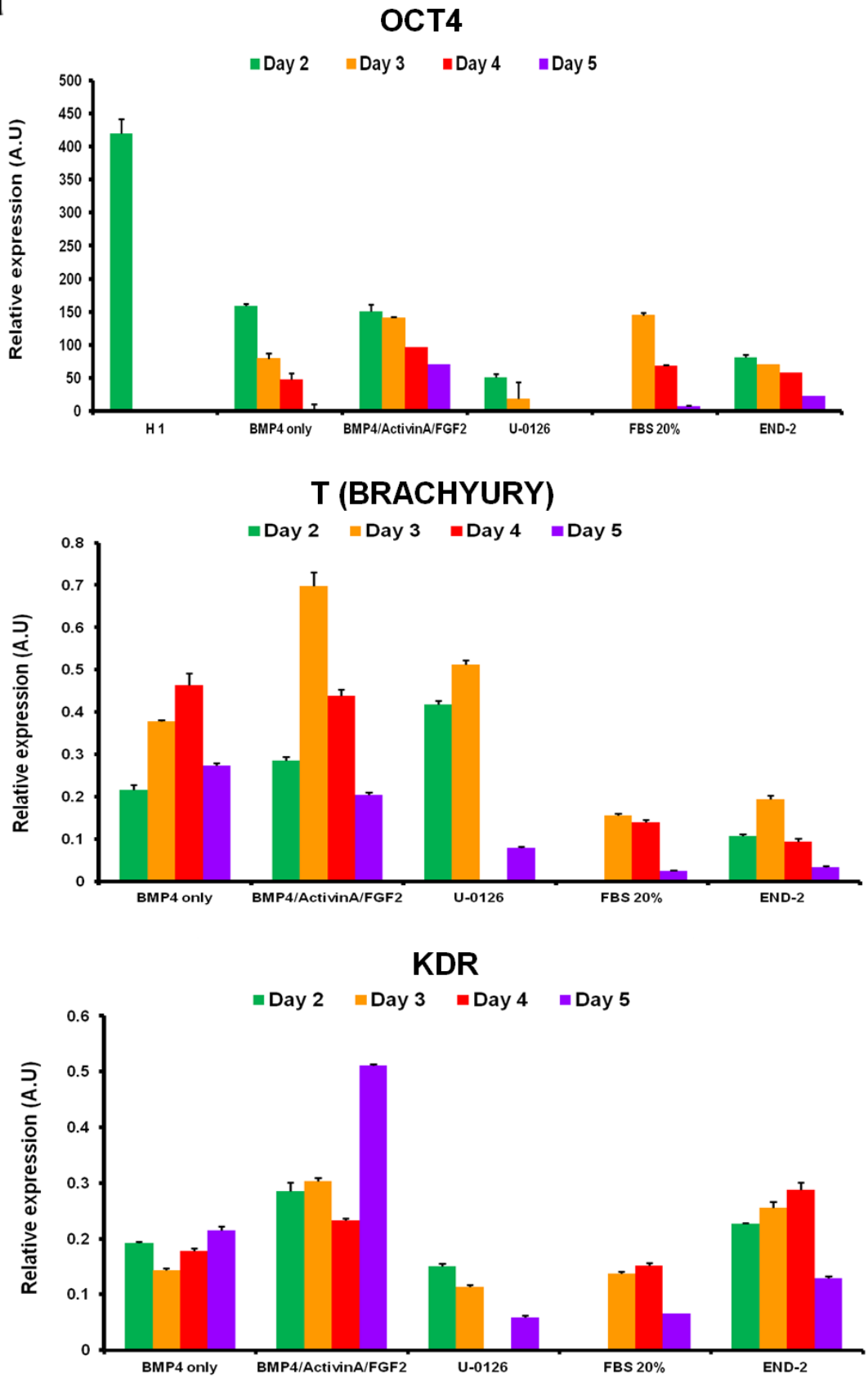


Supplementary figure 5. Specificity of cardiomyocyte-specific MB.
Flow cytometry analysis demonstrated that CM-specific MHC1-MB detected non-cardiomyocyte cells such as SMCs, mECs, mCFs and mESCs at a very low rate. N = 3

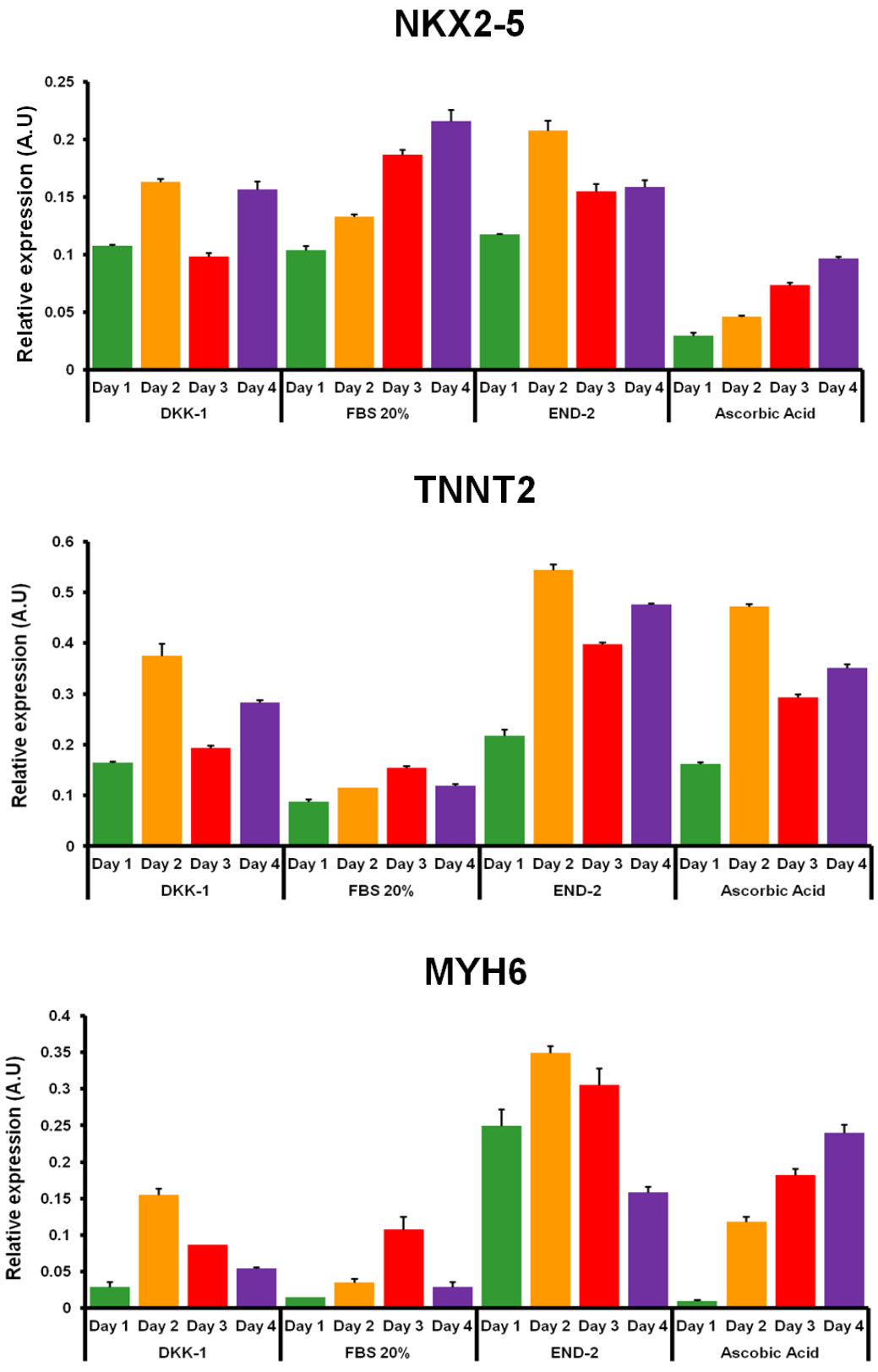


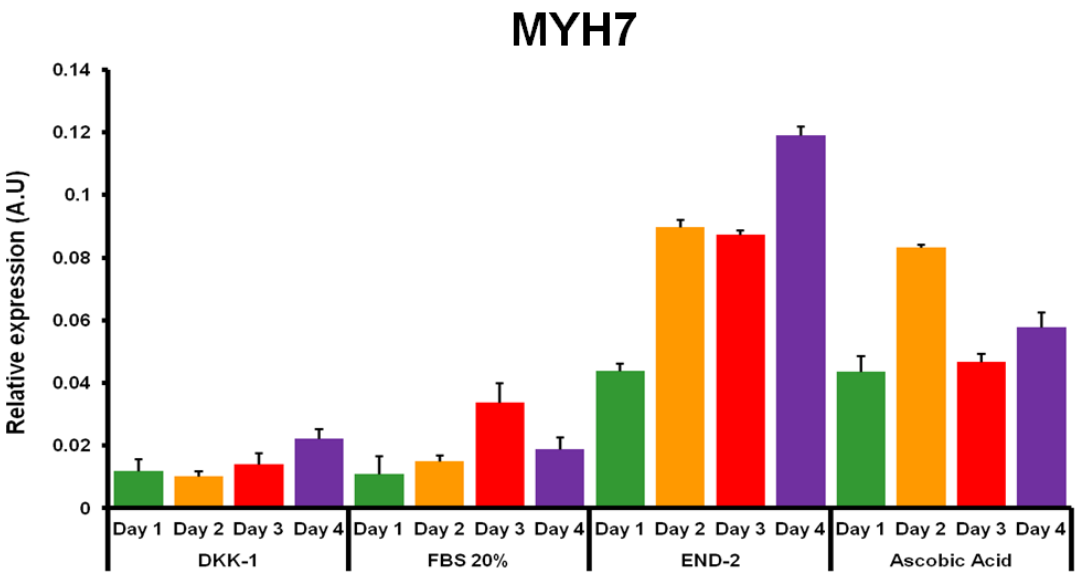
Supplementary figure 6. Microscopic images of differentiating mESCs at day 9 after nucleofection with MHC1-MB

a


OCT4
Day 2
Day 3
Day 4
Day 5
Relative expression (A.U)
500
450
400
350
300
250
200
150
100
50
0
H 1
BMP4 only
BMP4/ActivinA/FGF2
U-0126
FBS 20%
END-2
T (BRACHYURY)
Day 2
Day 3
Day 4
Day 5
Relative expression (A.U)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BMP4 only
BMP4/ActivinA/FGF2
U-0126
FBS 20%
END-2
KDR
Day 2
Day 3
Day 4
Day 5
Relative expression (A.U)
0.6
0.5
0.4
0.3
0.2
0.1
0
BMP4 only
BMP4/ActivinA/FGF2
U-0126
FBS 20%
END-2

b


NKX2-5
Relative expression (A.U)
0.25
0.2
0.15
0.1
0.05
0
Day 1 Day 2 Day 3 Day 4
DKK-1
FBS 20%
END-2
Ascorbic Acid
TNNT2
Relative expression (A.U)
0.6
0.5
0.4
0.3
0.2
0.1
0
Day 1 Day 2 Day 3 Day 4
DKK-1
FBS 20%
END-2
Ascorbic Acid
MYH6
Relative expression (A.U)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Day 1 Day 2 Day 3 Day 4
DKK-1
FBS 20%
END-2
Ascobic Acid



Supplementary figure 7. Expression of mesodermal and cardiac genes during differentiation of hPSCs into cardiomyocytes using four differentiation protocols.
(a) qRT-PCR results showing temporal expression of a pluripotency gene *OCT4* and mesodermal genes T (Brachyury) and KDR during differentiation of hESCs (H1) cultured on Matrigel-coated plates for 5 days under various culture conditions. A combination of BMP4, Activin A and FGF2 was the most efficient for mesodermal induction. U-0126: ERK1/2 inhibitor, FBS 20%: 20% fetal bovine serum, END-2: Conditioned media produced from the cultures of END-2 cells, a mouse endodermal cell line.
(b) qRT-PCR results demonstrating temporal expression of cardiac-lineage genes during differentiation of mesodermally differentiated hESCs under various culture conditions. Days shown are after applying the indicated cardiac inducing conditions following 5 days of culture in stage 2 with BMP4, Activin A and FGF2. Supplementation with conditioned media produced by the END-2 induced the highest expression of cardiac-lineage genes. Y axis represents relative mRNA expression of target genes to GAPDH. N = 3.

Supplemental References

1. Claycomb WC, Lanson NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ. 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*. 1998;95:2979-2984
2. Hescheler J, Fleischmann BK, Lentini S, Maltsev VA, Rohwedel J, Wobus AM, Addicks K. Embryonic stem cells: A model to study structural and functional properties in cardiomyogenesis. *Cardiovascular Research*. 1997;36:149-162
3. Park I-H, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008;451:141-146
4. Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere AB, Passier R, Tertoolen L. Differentiation of human embryonic stem cells to cardiomyocytes: Role of coculture with visceral endoderm-like cells. *Circulation*. 2003;107:2733-2740
5. Rhee WJ, Bao G. Simultaneous detection of mrna and protein stem cell markers in live cells. *BMC Biotechnol*. 2009;9:30
6. Rhee WJ, Santangelo PJ, Jo H, Bao G. Target accessibility and signal specificity in live-cell detection of bmp-4 mrna using molecular beacons. *Nucleic Acids Research*. 2008;36:e30
7. Santangelo P, Nitin N, Bao G. Nanostructured probes for rna detection in living cells. *Ann Biomed Eng*. 2006;34:39-50
8. Kim H, Cho H-J, Kim S-W, Liu B, Choi YJ, Lee J, Sohn Y-D, Lee M-Y, Houge MA, Yoon Y-s. Cd31+ cells represent highly angiogenic and vasculogenic cells in bone marrow / novelty and significance. *Circulation Research*. 2010;107:602-614
9. Wagner MB, Wang Y, Kumar R, Tipparaju SM, Joyner RW. Calcium transients in infant human atrial myocytes. *Pediatr Res*. 2005;57:28-34
10. Jeong J-O, Han JW, Kim J-M, Cho H-J, Park C, Lee N, Kim D-W, Yoon Y-S. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circulation Research*. 2011;108:1340-1347
11. Cho H-J, Lee N, Lee JY, Choi YJ, Ii M, Wecker A, Jeong J-O, Curry C, Qin G, Yoon Y-s. Role of host tissues for sustained humoral effects after endothelial progenitor cell transplantation into the ischemic heart. *The Journal of Experimental Medicine*. 2007;204:3257-3269

Supplementary Movie 1 Contraction of mESC-derived cardiomyocytes.

Supplementary Movie 2 Contraction of mouse-ESC derived CMs purified via MHC1-MB and FACS.

Supplementary Movie 3 Contraction of hESC-derived cardiomyocytes.

Supplementary Movie 4 Contraction of hiPSC-derived cardiomyocytes purified via MHC1-MB and FACS.

Supplementary Movie 5 Ca^{2+} transient of hiPSC-derived cardiomyocytes purified via MHC1-MB and FACS.