Molecular Cardiology

Purification of Cardiomyocytes From Differentiating Pluripotent Stem Cells Using Molecular Beacons That Target Cardiomyocyte-Specific mRNA

Kiwon Ban, PhD*; Brian Wile, BSc*; Sangsung Kim, MSc; Hun-Jun Park, MD, PhD; Jaemin Byun, MSc; Kyu-Won Cho, MSc; Talib Saafir, PhD; Ming-Ke Song, PhD; Shan Ping Yu, PhD; Mary Wagner, PhD; Gang Bao, PhD; Young-Sup Yoon, MD, PhD

Background—Although methods for generating cardiomyocytes from pluripotent stem cells have been reported, current methods produce heterogeneous mixtures of cardiomyocytes and noncardiomyocyte cells. Here, we report an entirely novel system in which pluripotent stem cell–derived cardiomyocytes are purified by cardiomyocyte-specific molecular beacons (MBs). MBs are nanoscale probes that emit a fluorescence signal when hybridized to target mRNAs.

Method and Results—Five MBs targeting mRNAs of either cardiac troponin T or myosin heavy chain 6/7 were generated. Among 5 MBs, an MB that targeted myosin heavy chain 6/7 mRNA (MHC1-MB) identified up to 99% of HL-1 cardiomyocytes, a mouse cardiomyocyte cell line, but <3% of 4 noncardiomyocyte cell types in flow cytometry analysis, which indicates that MHC1-MB is specific for identifying cardiomyocytes. We delivered MHC1-MB into cardiomyogenically differentiated pluripotent stem cells through nucleofection. The detection rate of cardiomyocytes was similar to the percentages of cardiac troponin T– or cardiac troponin I–positive cardiomyocytes, which supports the specificity of MBs. Finally, MHC1-MB–positive cells were sorted by fluorescence-activated cell sorter from mouse and human pluripotent stem cell differentiating cultures, and ≈97% cells expressed cardiac troponin T or cardiac troponin I as determined by flow cytometry. These MB-based sorted cells maintained their cardiomyocyte characteristics, which was verified by spontaneous beating, electrophysiological studies, and expression of cardiac proteins. When transplanted in a myocardial infarction model, MB-based purified cardiomyocytes improved cardiac function and demonstrated significant engraftment for 4 weeks without forming tumors.

Conclusions—We developed a novel cardiomyocyte selection system that allows production of highly purified cardiomyocytes. These purified cardiomyocytes and this system can be valuable for cell therapy and drug discovery. (Circulation. 2013;128:1897-1909.)

Key Words: myocytes, cardiac ◆ oligonucleotide probes ◆ pluripotent stem cells ◆ regeneration

Human pluripotent stem cells (hPSCs), which include embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), have emerged as a promising therapeutic option for repairing cardiac damage. Although various methods for differentiating cardiomyocytes from hPSCs have been developed,1–3 without purification, these systems can only generate mixed populations with undifferentiated cells or noncardiomyocytes, which may elicit adverse outcomes.4,5 Hence, one of the major challenges confronting the clinical use of hPSC-derived cardiomyocytes is the development of efficient isolation techniques that allow enrichment of purified cardiomyocytes.

Diverse approaches have been attempted to purify cardiomyocytes from differentiating pluripotent stem cell (PSC) cultures. Genetic approaches using a fluorescent reporter gene driven by a cardiac promoter such as Nkx2-5 (NK2 homeobox 5),6 Isl1 (islet-1),7 or α-MHC (α-myosin heavy chain)8 have been reported to efficiently isolate cardiomyocytes or cardiac progenitor cells but require genetic modification and therefore are incompatible with clinical use. Another approach using a Percoll gradient capitalized on the specific density of cardiomyocytes2–6; because of its crudeness, however, the maximal...
purity of the cells reported was only 53%. The sorting of cells by surface proteins for progenitor cell markers such as KDR (kinase insert domain receptor) or PDGFRα (α-type platelet-derived growth factor) isolated cardiac progenitors but resulted in a mixture of cardiomyocytes together with endothelial and smooth muscle cells. Fluorescence-activated cell sorting (FACS) after application of the fluorescent mitochondrial dye tetramethylrhodamine methyl ester perchlorate (TMRM) allowed cells with high mitochondrial density, such as mature cardiomyocytes, to take up the dye and become amenable for cell sorting. However, because the dye reacts with undifferentiated PSCs and does not detect most of the immature cardiomyocytes, concerns were raised about specificity and sensitivity. Recently, studies identified 2 surface proteins expressed on hPSC-derived cardiomyocytes, SIRPA (signal regulatory protein-α) and VCAM-1 (vascular cell adhesion molecule 1), and used them to enrich populations of cardiomyocytes by cell sorting; however, these proteins are not specifically expressed in cardiomyocytes. For example, SIRPA is more highly expressed in the brain and the lung than the heart and is only useful for a certain stage of cardiomyocytes, which raises concerns about its effectiveness for purification.

Accordingly, we aimed to develop a novel strategy to purify cardiomyocytes from differentiating PSCs by targeting genes that are specifically expressed in cardiomyocytes. We hypothesized that molecular beacons (MBs) hybridized to cardiomyocyte-specific mRNAs could enable isolation of cardiomyocytes from a mixed population. This method can be applied for both basic research and clinical applications. MBs are dual-labeled antisense oligonucleotide nanoscale probes with a fluorophore at one end and a quencher at the other end. (Figure 1A). MBs are designed to form a stem-loop (hairpin) structure in the absence of a complementary target so that fluorescence of the fluorophore is quenched; however, hybridization with the target mRNA opens the hairpin and separates the reporter from the quencher, which allows emission of a fluorescence signal (Figure 1B). MB technology has been tested in a variety of cell types to detect mRNA at various levels of expression and has been demonstrated to not alter the expression level of target genes. We have previously demonstrated the use of MBs for detecting mouse embryonic stem cell (mESCs) by simultaneous targeting of intracellular Oct4 (octamer-binding transcription factor 4) mRNA and surface markers. Although these studies did not attempt to isolate target cells, they suggested that MB technology has unique potential for enriching target cells.

In the present study, we first developed protocols to differentiate mouse and human PSCs to cardiomyocytes and devised a strategy to isolate cardiomyocytes by applying MBs that target cardiomyocyte-specific mRNAs, followed by FACS (Figure 2). Our results suggest the possibility of purifying cardiomyocytes at high efficiency and specificity from hPSC differentiation cultures with this innovative and clinically compatible purification system. These purified cardiomyocytes are functional in vitro and in vivo.

Methods
Details concerning the materials and methods can be found in the online-only Data Supplement.

mESC Culture and Differentiation
mESCs (J1) were maintained as described previously. To differentiate mESCs into cardiac lineage, an embryoid body method was used with some modifications.

hPSC Culture and Differentiation
hESCs (H1) were obtained from WiCell Research Institute (Madison, WI) and hiPSCs (BJ1-iPS10) were provided by George Daley at Harvard University (Cambridge, MA). The use of hPSCs was approved by the Emory University Human Embryonic Stem Cell Oversight Committee. These undifferentiated hPSCs were cultured as described previously. To direct the differentiation of hPSCs to the cardiac lineage, we designed a staged protocol that was divided into 4 distinct phases.

Molecular Beacon Synthesis and Characterization
Five MBs were synthesized by MWG Operon using standard resin-based synthesis methods with HPLC purification (Table 1).

Induction of Myocardial Infarction (MI) and Cell Transplantation
All animal experiments were approved by Emory University IACUC. Myocardial infarction (MI) and cell implantation were performed as we described previously. We randomly assigned the mice into three groups which received saline (PBS) (N=10), 2×10^5 unpurified CMs differentiated from mESCs (N=12), or 2×10^5 purified CMs with MHC1-MB from differentiated mESCs (N=11).

Results
Cardiomyocyte-Specific MB Generation
To determine optimal candidate genes detectable by MBs, we performed quantitative RT-PCR (qRT-PCR) analysis on

![Figure 1. Structure of cardiomyocyte-specific molecular beacons (MBs). A. Molecular dynamics model of an MB in the closed/unbound conformation. The close proximity of the 5′ Cy3 dye and the 3′ Black Hole Quencher allows for direct and FRET (Förster resonance energy transfer) quenching. B. Molecular dynamics model of an MB in the open/bound conformation. The distance between the 5′ Cy3 dye and the 3′ Black Hole Quencher increases greatly to prevent dye quenching. MHC1 MB indicates an MB that targeted myosin heavy chain 6/7 mRNA.](image-url)
known cardiac-specific genes using mRNAs extracted from freshly isolated mouse adult cardiomyocytes (Figure Ia in the online-only Data Supplement), human neonatal heart tissues, and human fetal heart tissues (Figure Ib and Ic in the online-only Data Supplement). The representative cardiac structural genes cardiac troponin T (TNNT2, also known as cTNT) and myosin heavy chain (MYH6/7, also known as α/βMHC) were most highly expressed in all of these samples and thus were determined to be targets for MBs. On the basis of the qRT-PCR results, we designed 5 MBs (Table 1) that targeted unique sites in TNNT2 or MYH6/7 mRNA in both mouse and human and using design rules determined by previous publications30,31 and BLAST (Basic Local Alignment Search Tool; National Library of Medicine, Bethesda, MD) searches to ensure uniqueness. These MBs were synthesized with a Cy3 fluorophore or a FAM fluorophore at the 5′ ends, which allows the probe to fluoresce regardless of its open or closed conformation. We delivered the nonspecific MB into various cell lines, including an immortalized mouse cardiomyocyte cell line [HL-1 cardiomyocytes34] smooth muscle cells, mouse embryonic fibroblasts, and mESCs. Flow cytometry analysis demonstrated that regardless of the cell type, nucleofection consistently delivered MBs into >95% of the cells, showing the highest delivery efficiency (Figure III in the online-only Data Supplement and data not shown).

We systematically determined the sensitivity of MBs designed to target cardiomyocyte-specific mRNAs. As a positive control, we used HL-1 cardiomyocytes. We independently confirmed the cardiomyocyte identity of HL-1 cardiomyocytes by flow cytometry, showing that 97.2% of cells expressed Tnt2 (Figure IV in the online-only Data Supplement). Each of the candidate MBs targeting TNNT2 or MYH mRNA was delivered into HL-1 cardiomyocytes by nucleofection, and efficacy was analyzed by flow cytometry. Among the 5 MBs (TNT1, TNT2, TNT3, MHC1, and MHC2) examined, the MB designated as MHC1-MB yielded the highest rate of MB-signal–positive cells (98.9%; Figure 3A).

To determine the specificity of MHC1-MB, a random-sequence MB (random beacon) that has a 16-base target sequence that has a nonspecific sequence with FAM dyes conjugated to both the 5′ and 3′ ends, which allows the probe to fluoresce regardless of its open or closed conformation. We delivered the nonspecific MB into various cell lines, including an immortalized mouse cardiomyocyte cell line [HL-1 cardiomyocytes34] smooth muscle cells, mouse embryonic fibroblasts, and mESCs. Flow cytometry analysis demonstrated that regardless of the cell type, nucleofection consistently delivered MBs into >95% of the cells, showing the highest delivery efficiency (Figure III in the online-only Data Supplement and data not shown).

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To determine the specificity of MHC1-MB, a random-sequence MB (random beacon) that has a 16-base target sequence that does not match any sequence in the mouse or human genome was delivered as a negative control11 and displayed negligible fluorescence in HL-1 cardiomyocytes (Figure 3B), thus ruling out the possibility that the fluorescence signal from MHC1-MB was caused by nonspecific interactions or probe degradation by endonucleases.

### Table 1. List of Molecular Beacons and Parameters

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<tr>
<th>Beacon Name</th>
<th>Target Sequence</th>
<th>Beacon Sequence</th>
<th>Duplex Melting Temperature, °C</th>
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<tr>
<td>TNT1</td>
<td>CCCAAGATCCCCGATGGAGAGAG</td>
<td>TACCCCTCTCTCCATGGGGATCTCTGGA</td>
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<td>TNT2</td>
<td>AGAACCGCTGCGTCGAAGAGA</td>
<td>CCCTCCTCTAGGCGGTTCTGAGGG</td>
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<tr>
<td>TNT3</td>
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<td>ATCTCAGCGCCCTCTCTGCTGGA5T</td>
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<td>GTCGAGAGAGAGAGATGGAGG</td>
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<td>MHC2</td>
<td>AAGACCGGCGGATGTGGCACA</td>
<td>TTGGCACATTGTCGCCGTCTGCGCA</td>
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In silico description of the molecular beacons synthesized for the study. The duplex melting temperature describes a measure of the predicted affinity between the sequence–specific beacon loop and the target sequence. MHC1 indicates myosin heavy chain 1; MHC2, myosin heavy chain 2; TNT1, troponin T 1; TNT2, troponin T 2; and TNT3, troponin T 3. In Beacon Sequence, italic letters indicate stem sequences.
further verify the specificity of the MHC1-MB, we delivered MHC1-MB into smooth muscle cells, mouse aortic endothelial cells, mouse cardiac fibroblasts, and mESCs, which are the cell types most likely to contaminate in cardiomyogenically differentiated PSC cultures (Figure V in the online-only Data Supplement). Flow cytometry analysis showed that <3% of these cells displayed a detectable fluorescence signal. These results suggest high sensitivity of the MHC1-MB for cardiomyocyte-lineage cells.

Table 2. Primer Sequences Used for Reverse-Transcriptase Polymerase Chain Reaction Analysis

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<th>Reverse 5′-3′</th>
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<td>ATATGCTACGTGAGGCAAT</td>
</tr>
<tr>
<td>Nkx2-5</td>
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<td>CATGAGCCCTCACCACATGCAAA</td>
</tr>
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<td>Myh6</td>
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<tr>
<td>MyoD1</td>
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</tr>
<tr>
<td>Ddr2</td>
<td>CATGATGCCTGAGGCAAT</td>
<td>CATGAGCCCTCACCACATGCAAA</td>
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Primer sequences used (human)

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<th>Gene</th>
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<td>GAPDH</td>
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<td>T</td>
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<tr>
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</tr>
<tr>
<td>TBX5</td>
<td>CCAAGATGAGGCAATGAGGCAAT</td>
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<td>CATGAGCCCTCACCACATGCAAA</td>
</tr>
<tr>
<td>MYH6</td>
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<td>CATGAGCCCTCACCACATGCAAA</td>
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<td>MYH7</td>
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</tr>
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<td>MYO1</td>
<td>GGCAGGCTGAGGCAATGAGGCAAT</td>
<td>CATGAGCCCTCACCACATGCAAA</td>
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<tr>
<td>DRR2</td>
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<td>CATGAGCCCTCACCACATGCAAA</td>
</tr>
<tr>
<td>THY1</td>
<td>ATGGTCTACCAAAATGGAGGCTGCTGAGGCAAT</td>
<td>CATGAGCCCTCACCACATGCAAA</td>
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<tr>
<td>ACTA2</td>
<td>AGGGGTTGAGGTCAGTGAGGCAAT</td>
<td>CATGAGCCCTCACCACATGCAAA</td>
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Purification of mESC-Derived Cardiomyocytes

Next, we investigated whether this MB could be useful for isolating cardiomyocytes from differentiating mouse PSCs. We first established an embryoid body–mediated system to efficiently differentiate mESCs into cardiomyocytes (Figure 4A). In brief, undifferentiated mouse ESCs (J1) maintained on STO feeder cells were enzymatically detached to form embryoid bodies, which were cultured for 5 days and plated on a fibronectin-coated dish for cardiomyocyte differentiation. After 3 to 4 days
of differentiation in the presence of ascorbic acid (50 μg/mL), to spontaneously beating clumps began to appear (Movie I in the online-only Data Supplement). Flow cytometry analysis demonstrated that the percentage of Tnt2-positive cells was 13.4% and 47.1% at days 4 and 9, respectively (Figure 4B). Immunostaining further demonstrated that cells dissociated from beating clumps displayed Tnnt2, Tnni3, and Actn2 (or α-sarcomeric actinin), which confirmed their cardiomyocyte nature (Figure 4C). The results with mouse iPSCs were similar (data not shown). After establishing the differentiation system, we attempted to isolate cardiomyocytes from differentiating mESCs using MBs. The differentiating mESCs at day 9 were subjected to nucleofection with MHC1-MB and subjected to FACS. The percentage of cells positive for fluorescence signal from MHC1-MB was 49.2±4.8% (Figure 4D). There was significant agreement between the detection rate of cardiomyocytes with antibody-based (47.1% Tnt2-positive cells) and MB-based (49.2% MHC1-MB-positive cells) flow cytometry results, which supports the specificity of MHC1-MB. Fluorescent microscopic imaging also confirmed these results (Figure VI in the online-only Data Supplement).

Next, cells were sorted by FACS with MHC1-MB, and 98.4% of the sorted MHC1-MB-positive cells exhibited Tnt2 in flow cytometry (Figure 4E). Immunocytochemistry verified that virtually all sorted MHC1-MB-positive cells expressed Tnt2 and Actn2 (Figure 4F). qRT-PCR analyses demonstrated that these sorted cells expressed 2- to 6-fold higher levels of Tnt2, Myh6, Myh7, and Myl2 than the presorted cells (Figure 4G). Genes representing other lineages were either expressed at negligible levels (Acta2, Ddx2, Gata1, and Sox17) or were undetectable (Pecam1, MyoD, and NeuroD) in the sorted cells (Figure 4G and data not shown). Importantly, these sorted cells showed spontaneous contraction over 2 weeks, which suggests that these MB-based purified cardiomyocytes were functional (Movie II in the online-only Data Supplement). In Movie II, we intentionally selected areas where individual cardiomyocytes were contracting to demonstrate functionality of cardiomyocytes at an individual level.

**Purification of hESC-Derived Cardiomyocytes**

Similarly, we investigated the utility of MB-based cell sorting for hPSC-derived cardiomyocytes. We developed a novel 4-step protocol for differentiating hPSCs into cardiomyocytes (Figure 5A). In phase 1, undifferentiated hESCs (H1) were directly transferred onto Matrigel-coated plates and cultured as a monolayer under mTeSR media (Stemcell Technologies, Vancouver, Canada) for cell expansion. Next, to induce mesodermal differentiation, several combinations of mesodermal inducers were tested and compared by qRT-PCR with mesodermal markers including T (or Brachyury) and KDR. We found that a combination of bone morphogenetic protein 4 (BMP4; 10 ng/mL), activin A (3 ng/mL), and basic fibroblast growth factor (FGF2; 5 ng/mL) was the most efficient for mesodermal differentiation (Figure VIIa in the online-only Data Supplement). To induce cardiac lineage differentiation in phase 3, we tested 4 different methods and found that supplementation with conditioned media produced by the mouse endodermal cell line END-2 induced the highest expression of cardiac lineage markers such as NKX2-5, TNNI3, MYH6, and MYH7 (Figure VIIb in the online-only Data Supplement). Finally, in phase 4, continuous treatment with a β-adrenergic receptor agonist, isoproterenol, for as few as 4 days efficiently generated spontaneously beating cardiomyocytes (Movie III in the online-only Data Supplement).

Flow cytometry analysis demonstrated that the percentage of TNNI3-positive cells was 10.2% and 43.1% at days 9 and 13, respectively (Figure 5B). We delivered the MHC1-MB to the cardiomyogenically differentiated hESCs at day 13 in phase 4. Flow cytometry analysis showed that the percentage of cells positive for MHC1-MB signal was 46.3% (Figure 5C). These MHC1-MB-treated cells were sorted by FACS, and 97.6±1.4% of the sorted MHC1-MB-positive cells exhibited TNNI3 expression in flow cytometry analysis (Figure 5D). Almost all cells showed cardiomyocyte-like morphology and stained positive for TNNI3 and MYH7 by immunocytochemistry (Figure 5E). qRT-PCR analyses showed a significant increase in expression of cardiomyocyte-specific genes (TNNI2, MYH6, MYH7, and MYL2) and a decrease in expression of genes specific for smooth muscle cells (calponin), fibroblasts (THY1), skeletal myocytes (MYOD), neural lineage cells (NEUROD), and endothelial cells (PECAM1 [platelet/endothelial cell adhesion molecule 1]), which suggests enrichment of cardiomyocytes and elimination of other lineage cells by cell sorting with the MB (Figure 5F and 5G).

Stable action potentials were recorded from cardiomyocytes that were purified via MHC1-MB and cultured for 7 to 14 days after FACS. Three major types of action potentials were observed: Nodal-like (6 of 46), atrial-like (11 of 46), and ventricular-like (29 of 46) action potentials (Figure 5H; Table 3). These results indicate that cells purified via a cardiomyocyte-specific MB are electrophysiologically intact, functional cardiomyocytes and can maintain these characteristics in culture.
Figure 4. Purification of cardiomyocytes from differentiating mouse embryonic stem cells (mESCs) with cardiomyocyte-specific molecular beacons (MBs). A, Schematic of the protocol used for differentiating mESCs to the cardiac lineage. EB indicates embryoid body; and ES, embryonic stem cell. B, Percent expression of Tnnt2 at days 4 and 9 during mESC differentiation into cardiomyocytes; n=3. C, Immunocytochemistry results of mESC-derived cardiomyocytes at day 9 for Tnnt2, Tnnt3, and Actn2. Scale bars, 20 µm. D, Flow cytometry analyses of MHC1-MB (an MB that targeted myosin heavy chain 6/7 mRNA) signals in mESC differentiation culture at day 9; n=6. FSC indicates forward scatter. E, Flow cytometry analysis of Tnnt2 expression in mESCs sorted with MHC1-MB and fluorescence-activated cell sorting (FACS) at differentiation day 9; n=3. Numbers represent percentages of MB-positive cells (D, E). F, FACS-sorted MHC1-MB–positive cells exhibited Tnnt2 and Actn2 in immunocytochemistry. Scale bars, 20 µm. G, Quantitative reverse-transcriptase polymerase chain reaction analyses showing difference in gene expression levels between mouse tail tip fibroblasts (MTTF) and presorted (PRE) and postsorbed (POST) mESCs. The cardiomyocyte genes Tnnt2, Myh6, Myh7, and Myl2 were significantly enriched in posted cells with MHC1-MB, and noncardiac lineage genes (Acta2, Ddr2, Gata1, and Sox17) were substantially reduced compared with presorted cells. The y axis represents relative mRNA expression of target genes to GAPDH. A.U. indicates arbitrary units. *P<0.05 compared with presorted group; n=3.
Figure 5. Purification of cardiomyocytes from differentiating human embryonic stem cells (hESCs) by cell sorting via molecular beacon (MB) and fluorescence-activated cell sorting (FACS). A, Schematic of the protocol to differentiate human pluripotent stem cells (hPSCs) to the cardiac lineage. BMP4 indicates bone morphogenetic protein 4; END-2, mouse endoderm-like cells; ES, embryonic stem cell; and FGF2, basic fibroblast growth factor. B, Percent expression of TNNI3 at days 9 and 13 determined by flow cytometry during hESC differentiation into cardiomyocytes; n=3. C, Flow cytometry results of MHC1-MB–positive cells in hESC differentiation culture at day 13; n=6. FSC indicates forward scatter; and MHC1-MB, an MB that targeted myosin heavy chain 6/7 mRNA. D, Flow cytometry results showing TNNI3 expression of FACS-sorted hESCs at day 13 of differentiation after applying MHC1-MB. Numbers represent percentages of MB-positive cells (C, D). n=3.

(continued on next page)
Action potentials were measured in a total of 46 cardiomyocytes that were positive for a molecular beacon that targeted myosin heavy chain 6/7 mRNA; nodal-like (6 of 46), atrial (11 of 46), and ventricular (29 of 46) action potentials were identified. (Figure 6G; Movie V in the online-only Data Supplement). These data suggest that the MHC1-MB–based purified cardiomyocytes were functional cardiomyocytes. Taken together, the present results clearly support the notion that MBs that target cardiomyocyte-specific mRNA in live cells allow isolation of functional cardiomyocytes from differentiating mouse and human PSCs with high specificity and efficiency.

Engraftment and Improvement of Cardiac Function After Implantation of Purified Cardiomyocytes
To determine the behavior and effects of MB-based purified cardiomyocytes in ischemic myocardium, purified or unpurified cardiomyocytes derived from mESCs or the same volume of PBS was injected into the myocardium after induction of MI in mice. Echocardiography was performed weekly to measure cardiac remodeling and function. One week later, however, in the mice that received unpurified cardiomyocytes, a distinct mass grew over 4 weeks (Figure 7A and 7B). Postmortem examination at 3 to 4 weeks revealed tumor masses in 11 of 12 mice. By careful gross examination, tumors invaded internally into myocardium and externally into the pericardium (Figure 7B and 7C). Cardiac tissues were fixed and stained with hematoxylin and eosin. Microscopic examination revealed that all tumors consisted of structures derived from all 3 embryonic germ layers, which indicates teratomas (Figure 7C); however, we did not detect tumors in any of the mice that received MB-based purified cardiomyocytes or PBS over the same follow-up period by echocardiographic or histological examination. Tumor formation in unpurified cardiomyocyte–injected mice did not allow appropriate functional comparison between mice that received unpurified and purified cardiomyocytes; however, purified...
Figure 6. Purification of cardiomyocytes from differentiating human induced pluripotent stem cells (hiPSCs) by cell sorting via molecular beacon (MB) and fluorescence-activated cell sorting (FACS). A, Schematic of the protocol to differentiate hiPSCs to the cardiac lineage. BMP4 indicates bone morphogenetic protein 4; END-2, mouse endoderm-like cells; ES, embryonic stem cells; and FGF2, basic fibroblast growth factor. B, Percent expression of TNNI3 at days 9 and 13 during hiPSC differentiation into cardiomyocytes determined by flow cytometry; n=3. C, Flow cytometric analysis of MHC1-MB signals in hiPSC differentiation culture at day 13; n=6. MHC1-MB indicates an MB that targeted myosin heavy chain 6/7 mRNA. D, Flow cytometric results showing TNNI3 expression of FACS-sorted hiPSCs at day 13 of differentiation after applying MHC1-MB. Numbers represent percentages of MB-positive cells (C, D), n=3. E, Immunocytochemistry for TNNI3 and TNNT2 on MHC1-MB–positive cells sorted from hiPSC cultures. Scale bars, 20 µm. F, mRNA expression levels of cardiac and noncardiac genes measured by quantitative reverse-transcriptase polymerase chain reaction. Comparisons were made among human dermal fibroblast (HDF), presorted hiPSCs at day 13 (PRE), and MB-based FACS-sorted hiPSCs at day 13 (POST). The y axis represents relative mRNA expression of target genes to GAPDH. A.U. indicates arbitrary units. *P<0.05 compared to presorted group; n=3. G, Calcium imaging of MHC1-MB–positive cardiomyocytes sorted from hiPSCs. Left, Confocal scan of representative cells loaded with Fluo-4 AM, with magnification of line-scanned region indicated with red dashed line (white scale bars, 20 µm; legend shows increasing calcium levels, with blue being low calcium). Right, Time course of [Ca²⁺]ᵢ, measured at line-scan region in cell pictured and paced at 0.5 Hz by field stimulation. [Ca²⁺]ᵢ is plotted as fluorescence intensity normalized to baseline (F/F₀).
cardiomyocyte–injected mice showed a higher ejection fraction than PBS-injected mice (Figure 7D), which indicates improved cardiac function. We next conducted immunohistochemistry for cardiomyocytes in cardiac tissues injected with purified cardiomyocytes. Confocal microscopic examination demonstrated that injected cardiomyocytes (DiI-positive) were engrafted as clusters, survived robustly for 4 weeks, and expressed representative cardiomyocyte proteins (Figure 7E). Taken together, these results suggest that injected MB-purified cardiomyocytes are integrated into ischemic myocardium and are functional in vivo.

**Discussion**

In the present study, we have described a highly specific and efficient method for purifying cardiomyocytes from differentiating mouse and human PSCs by directly targeting the mRNA of cardiomyocyte-specific genes. Using an MB that targeted the mRNA of Myh6 and Myh7 followed by FACS, we were able to enrich a population made up of >97% cardiomyocytes from differentiating PSCs. Their identity as cardiomyocytes was verified by a series of experiments that included flow cytometry, immunocytochemistry, and qRT-PCR. Importantly, these purified cardiomyocytes displayed spontaneous beating on further culture and demonstrated stable action potentials and Ca2+ oscillation in electrophysiological studies. When injected into infarcted heart, MB-purified cardiomyocytes were integrated and survived robustly for 4 weeks in post-MI hearts, showing improved cardiac function without forming tumors. On the other hand, mice injected with unpurified cardiomyocytes developed teratomas. These results support the idea that MB-based isolated cardiomyocytes are pure and functionally intact cardiomyocytes and that cardiomyocyte purification is necessary for cell therapy.

Despite continuous improvements in current cardiomyocyte differentiation protocols, the resulting differentiated cell populations still contain a considerable percentage of noncardiomyocytes. Hence, one of the major challenges in recent years has been to develop stable isolation techniques that allow scalable purification of cardiomyocytes. Recently, 3 studies using FACS-based cardiomyocyte purification have attracted attention because of their sound scientific rationale, high purity, and clinical applicability. In the first, a mitochondrial dye, TMRM, was suggested to be useful for cardiomyocyte selection, enriching up 99% of cardiomyocytes as determined by the expression of Acm2 (α-sarcosmin actinin) in immunocytochemistry. This approach was based on findings that cardiomyocytes have high mitochondrial content and can be purified via fluorescent dyes that label mitochondria. However, 2 other subsequent studies demonstrated that TMRM not only failed to identify immature cardiomyocytes early in differentiation culture but also detected noncardiogenic cells or undifferentiated hESCs, because these cells take up significant amounts of TMRM. In addition, 2 independent studies reported the identification of 2 surface marker proteins, SIRPA and VCAM-1, and suggested their utility for isolating cardiomyocytes from differentiating hPSCs. To identify a candidate protein, Dubois et al screened a panel of 370 known commercially available antibodies and identified SIRPA as a specific marker for cardiomyocytes differentiating from hPSCs. Subsequently, isolation via FACS with an antibody against SIRPA enriched cardiac precursors and cardiomyocytes from hPSCs, yielding up to 98% TNNT2+ cells. However, Elliott et al demonstrated that SIRPA+ cells expressed significant amounts of smooth muscle marker genes such as ACTA2 and CNN1 and an endothelial cell gene, CD34, which brings into question the utility of SIRPA as a sole marker for cardiomyocyte isolation. This study also suggested that there are variations in the yield of cardiogenic marker expression (NKX2-5) after further culture of sorted SIRPA+ cells, reaching as low as 2.8% cardiomyocytes depending on the isolation time and duration of culture. Furthermore, NKX2-5+ cells did not express SIRPA, which raised concerns about specificity and sensitivity of SIRPA. Thus, Elliott et al identified another surface protein, VCAM1, through a transcriptome analysis and used it as an additional marker to isolate cardiomyocytes. However, FACS with both SIRPA and VCAM1 antibodies was only able to enrich cardiomyocytes that ranged from 55% to 95% in purity. Moreover, because both proteins are known to be expressed in other cell types, the possibility of contamination with noncardiomyocyte cells even after purification with SIRPA and VCAM1 may be a critical issue.

The MB-based cardiomyocyte purification strategy has a number of unique advantages compared with previously reported cardiomyocyte purification methods. First, by directly targeting specific intracellular mRNA, there is no need to make extensive efforts to identify surface proteins for cell isolation with the corresponding antibodies. Second, MB-based cell purification minimizes contamination of other cells by using MBs designed to hybridize with unique sequences in the mRNAs of interest specifically expressed in the target cell type. Although significant efforts have been made to identify SIRPA and VCAM1 as cell-surface markers for cardiomyocytes, both SIRPA and VCAM1 are expressed in other organs such as brain and lung, which raises questions about the utility of these surface markers in a range of differentiation systems. Another advantage of the MB-based purification method is its application to any species. It allows expansion of the research scope with any desired cells. Because SIRPA is not highly expressed in the mouse, its usage is limited to human cells. In contrast, the MB-based method allows isolation of virtually any cell because of the unlimited accessibility of MBs to intracellular mRNA. Therefore, in addition to cardiomyocytes, the MB-based sorting technique described here can be broadly applied to the isolation of other cell types, such as neural-lineage cells or islet cells, which are critical elements in regenerative medicine but do not have specific surface proteins identified to date.

The present cell transplantation study provides an important insight into the need for purified cardiomyocytes for cardiac cell therapy. An unexpectedly high rate of tumors developed in mice that received unpurified mESC-derived cardiomyocytes. Although unpurified, these cells were differentiated into the cardiac lineage, and ≈50% of cells were cardiomyocytes. To date, a few studies have reported syngeneic or allogeneic transplantation of unpurified mESC-derived cardiomyocytes into infarcted heart, and all demonstrated tumor formation in hearts. The present study clearly demonstrated that purified cardiomyocytes do not form tumors, and this purification process is a prerequisite for cardiac cell therapy with PSCs. Similar cardiac cell therapy studies were conducted with hPSC-derived cardiomyocytes, and many of
them showed functional improvement with no tumor formation, albeit using nonpurified cells.46,47 However, these studies might have underestimated tumorigenicity, because xenogeneic transplantation can induce engrafted cell death by immune reaction, particularly in those noncardiogenic cells that are not aggregated.

To ensure that MBs were safe and effective to use in target cells, we performed several experiments to determine cell viability and functionality immediately and several days after delivery of MBs. MBs were minimally disruptive, because the results did not differ significantly between hPSC cardiomyocytes and control cells in the assays performed. MBs usually degrade within an hour,16–19,31 so cell viability or cell fate change is not a serious concern. In addition, to confirm their specificity, we performed extensive testing to ensure that the MB signal was a robust indicator of cardiomyocytes based on the binding of the probe to MYH6/7. We tested the MBs in solution with synthetic target oligonucleotides that varied from the ideal sequences by 6 bp, the closest that a BLAST search through the human transcriptome predicted that we would see. None of the MBs used in the present study increased their fluorescence by >50% even when incubated with excess target sequence.

The purified hPSC-derived cardiomyocytes sorted via MBs will be enormously useful for clinical applications and preclinical studies. A major obstacle to the use of cells differentiated from hPSCs for clinical applications is potential tumorigenicity or aberrant tissue formation after cell transplantation.41 By eliminating unwanted cells, this technology will advance the use of hPSC-derived cardiomyocytes. Moreover, mechanistic
studies, drug discovery, and disease modeling with nonpure PSC-derived cardiomyocytes, although attempted previously, can be inaccurate because of the effects of noncardiomyogenic cells. This purification technique in combination with cardiomyocytes generated from patient-specific hiPSCs will be of great value for drug screening and disease modeling, as well as cell therapy.

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Disclosures

None.

References

Cardiomyocyte Purification With Molecular Beacons

One of the major obstacles to the clinical use of human pluripotent stem cell–derived cardiomyocytes is their heterogeneity in culture. Although various methods for differentiating cardiomyocytes from human pluripotent stem cells have been developed, these systems generate mixed populations that include undifferentiated cells and noncardiomyocytes, which can induce tumors or aberrant differentiation. Because there is no specific surface protein for cardiomyocytes, it has been a challenge to purify them. Here, we developed an efficient method for cardiomyocyte purification in which fluorescent molecular beacons were hybridized with cardiomyocyte-specific mRNA in target cardiomyocytes, and these molecular beacon–labeled cardiomyocytes were sorted by fluorescence-activated cell sorting. This novel method yielded ≈98% cardiomyocytes from mixed populations. Accordingly, these human pluripotent stem cell–derived purified cardiomyocytes will be enormously useful for cell therapy for cardiac disease, cardiac tissue engineering, drug discovery, mechanistic studies, and disease modeling.

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Purification of Cardiomyocytes From Differentiating Pluripotent Stem Cells Using Molecular Beacons That Target Cardiomyocyte-Specific mRNA
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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. 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) 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 (Amixa 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% CO₂ 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-TNNNT2 (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 ($\Delta CT = CT$ gene of interest$− CT$ GAPDH in experimental samples) were calculated for each target mRNA by subtracting the mean value of GAPDH (relative expression $= 2^{\Delta CT}$). 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% CO₂ 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 (Ionooint 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-81 automated 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\textsuperscript{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\textsuperscript{5} unpurified CMs differentiated from mESCs (N = 12), or 2 x 10\textsuperscript{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-Dil (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 \textsuperscript{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
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


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.