Methodological Reviews discuss methods that are of broad interest to the community of cardiovascular investigators and that enable a better understanding of cardiovascular biology, particularly recent technologies in which the methods are still in flux and/or not widely known. It is hoped that these articles, written by recognized experts, will be useful to all investigators, but especially to early-career investigators.

Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells to Cardiomyocytes

A Methods Overview

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Abstract: Since human embryonic stem cells were first differentiated to beating cardiomyocytes a decade ago, interest in their potential applications has increased exponentially. This has been further enhanced over recent years by the discovery of methods to induce pluripotency in somatic cells, including those derived from patients with hereditary cardiac diseases. Human pluripotent stem cells have been among the most challenging cell types to grow stably in culture, but advances in reagent development now mean that most laboratories can expand both embryonic and induced pluripotent stem cells robustly using commercially available products. However, differentiation protocols have lagged behind and in many cases only produce the cell types required with low efficiency. Cardiomyocyte differentiation techniques were also initially inefficient and not readily transferable across cell lines, but there are now a number of more robust protocols available. Here, we review the basic biology underlying the differentiation of pluripotent cells to cardiac lineages and describe current state-of-the-art protocols, as well as ongoing refinements. This should provide a useful entry for laboratories new to this area to start their research. Ultimately, efficient and reliable differentiation methodologies are essential to generate desired cardiac lineages to realize the full promise of human pluripotent stem cells for biomedical research, drug development, and clinical applications. (Circ Res. 2012;111:344-358.)

Key Words: embryonic stem cells ■ pluripotent stem cells ■ cardiogenesis ■ cardiac differentiation ■ disease models ■ cardiac development

The successful isolation of human embryonic stem cells (hESCs), and more recently, the generation of induced pluripotent stem cells (hiPSCs), has ushered in a new era of opportunities for cardiovascular research and therapies. These human pluripotent stem cells (hPSCs) can undergo differentiation in vitro to generate derivatives of the 3 primary germ layers and hence potentially all the cell types present in the body. However, to take advantage of the promise of these cell sources, reproducible and efficient differentiation protocols to form the cell types of interest are essential. Protocols for different cell lineages have been described that exhibit variable success. In most cases, the in vitro differentiation recapitulates the stepwise stages of embryological development for the cell type of interest. In this review, we focus on differentiation of hPSCs to cardiomyocytes (CMs).

The generation of hPSC-derived CMs is of growing interest for multiple applications. First, access to an in vitro model of human development permits the study of human heart development in ways not otherwise possible. Second, stem cell–derived CMs serve as a human cardiac model that can be used for diverse basic research studies ranging from cellular electrophysiology to protein biochemistry. Furthermore, the
ability to generate hiPSCs from patients with inherited cardiac diseases provides unprecedented opportunities for studying disease in human CMs. Access to abundant populations of human CMs is of particular interest to the pharmaceutical industry as a tool to develop new cardioactive compounds and, perhaps more importantly, to screen compounds for potential cardiotoxicity, such as drug-induced QT prolongation. Finally, in the long-term, clinical applications using hPSC-derived CMs might provide a powerful approach to repair the injured heart, although the challenges will take time to overcome. Regardless of the use of hPSC-derived CMs, efficient and reproducible differentiation protocols are needed.

Here, we review the current best methods for differentiating hPSC to CMs and describe the underlying biology. There is still room for further improvement, because even the most successful laboratories are continuing to refine their protocols. Compared with just a few years ago, however, it is now possible to determine whether cells have the capacity to differentiate to CMs on the basis of just a few principal protocols. Some of the protocols require that the stem cells have a particular “history” or have been preadapted to a particular starting condition as undifferentiated cells. Some protocols can be scaled up, whereas others are more limited in this respect. We indicate the particular merits and caveats for each protocol discussed.

Lessons From Embryonic Cardiac Development

Because in vitro differentiation of stem cells to CMs mimics the sequential stages of embryonic cardiac development, a brief description of the key steps is provided and factors in cardiac development are highlighted. However, readers are referred to more comprehensive reviews on cardiac development for detailed information. The heart is one of the first identifiable tissues to develop in vertebrate embryos. It forms soon after gastrulation from anterior migrating mesodermal cells that intercalate between the ectoderm and endoderm cells that layer in the primitive streak. Heart-forming or cardiac progenitor cells are primarily localized in the mid-streak. Signals from adjacent cell populations promote induction of cardiac mesoderm, and the endoderm, in particular, appears to have a highly conserved instructive function in cardiogenesis.

Three families of protein growth factors are thought to control these early stages of mesoderm formation and cardiogenesis: Bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β superfamily; the wingless/int proteins (WNTs); and the fibroblast growth factors (FGFs). These factors, or their inhibitors, are expressed in the endoderm. Genetic disruption of their signaling has dramatic effects on cardiac development (reviewed by Olson and Schneider). In vertebrates, BMP signaling generally promotes cardiogenesis, whereas wingless in Drosophila and related Wnt proteins in vertebrates are involved in cardiac specification. Wnt action is complex, however, and may inhibit or promote differentiation depending on spatiotemporal context and whether the canonical signaling pathway (acting via β-catenin/GSK3 to repress cardiogenesis) or the noncanonical pathway (acting via protein kinase C/c-Jun N-terminal kinase to promote cardiogenesis) is activated. Finally, studies in chick and zebrafish have implied a cardioinductive role for FGFs, although in Drosophila, FGFs appear to provide positional cues to cells for specification. BMP2 can upregulate FGF8 ectopically, and BMP2 and FGF8 probably synergize to drive mesodermal cells into myocardial differentiation. Furthermore, conditional knockouts of Fgf8 and Fgfr1 result in impaired outflow tract development caused in part by proliferation defects in cells from the second heart field. Overall, it appears that the timing and relative expression of different growth factor combinations induce then pattern the cardiogenic mesoderm.

Once anteriorly migrated mesoderm cells have received appropriate signals, they switch on a highly conserved heart-specific combination of transcription factors that establish the cardiac transcriptional program. Initially, the mesodermal precursor cells in the primitive streak express transcription factors such as the T-box factor Brachyury (T) and the homeodomain protein Mix1. Before migration from the streak, these cells transiently activate the basic helix-loop-helix transcription factor mesoderm posterior 1 (Mesp1) to enter a “cardiac” mesoderm stage of development. A subset of the Mesp1+ cell population then begins to express the homeodomain transcription factor Nkx2-5, the T-box protein Tbx5, and Isl1, an LIM homeodomain transcription factor, which are early markers of the cardiac lineage that are activated during the formation of the heart fields. In mice, Nkx2-5 is essential for the interpretation of patterning signals within the primitive heart tube and is likely to act in concert with Tbx5 during formation of both the atrial and left
ventricular compartments to positively regulate transcription. Nkx2-5 and Tbx5 associate with members of the GATA family of zinc finger transcription factors (GATA4/5/6) and with serum response factor (SRF) to activate cardiac structural genes, such as actin, myosin light chain, myosin heavy chain (MHC), troponins, and desmin. Tbx5 can also cooperate with Nkx2-5 to activate expression of atrial natriuretic factor and the junctional protein connexin 40. GATA4, which has an early role in heart tube formation through regulation of extraembryonic endoderm and embryo folding, is essential for proepicardium formation and muscle development, because CM-specific ablation of GATA4 results in myocardial hypoplasia. GATA6 also plays a key role in myogenesis, and embryos without both GATA 4 and 6 do not develop heart tissue. Members of the myocyte enhancer factor 2 (MEF2) family of transcription factors also play key roles in CM differentiation by regulating cardiac muscle structural genes. Thus, multiple complex interactions between these highly conserved gene regulatory networks control the initial differentiation, proliferation, and maturation of CMs. Apart from their functional role, many of these factors can be used as markers of emerging CMs in differentiating cultures of hPSCs.

The sequential activation of the transcription factors that drive the formation of the nascent-precardiac mesoderm, and eventually determine cardiac cell fate, is likely to be controlled in hESCs and hiPSCs in much the same way as it is in embryos. As evidence supporting this assumption, many of the successful protocols developed to induce cardiomyogenesis in pluripotent cells are based on activating or inhibiting these known signaling pathways. The same sequence of gene expression in pluripotent cells are based on activating or inhibiting the successful protocols developed to induce cardiomyogenesis, and embryos without both GATA 4 and 6 do not develop heart tissue. Members of the myocyte enhancer factor 2 (MEF2) family of transcription factors also play key roles in CM differentiation by regulating cardiac muscle structural genes. Thus, multiple complex interactions between these highly conserved gene regulatory networks control the initial differentiation, proliferation, and maturation of CMs. Apart from their functional role, many of these factors can be used as markers of emerging CMs in differentiating cultures of hPSCs.

Embryoid Body–Mediated Differentiation
Cultured mouse pluripotent cells, including embryonal carcinoma cells and embryonic stem cells (mESCs), provided the first opportunities to develop methods for in vitro differentiation of pluripotent cells to CMs. When mouse embryonal carcinoma cells and mESC cultures were dissociated to single cells and aggregated, typically in hanging drops, they developed into spheroids with an inner layer of ectoderm-like cells and a single outer layer of endoderm. To reflect the similarity between these spheroids and early postimplantation embryos, they were termed embryoid bodies (EBs). Cells in EBs differentiated to derivatives of the 3 primary germ layers. Unlike normal embryonic development, these EBs were highly variable in structure and composition, but a fraction of the EBs exhibited spontaneously contracting regions that contained CMs. These early studies identified critical parameters for optimizing in vitro cardiogenesis, including the following: (1) The starting number of mESCs used to form aggregates; (2) the specific medium and serum; (3) the mESC line used; and (4) the time of EB plating to allow substrate attachment and outgrowth. More recent studies have attempted to improve the process further by testing a range of different growth factors and small molecules, which are described elsewhere for the mouse system.

The successful isolation of hESCs in 1998 led to efforts to differentiate these cells as EBs. However, it was quickly evident that unlike mESCs, hESCs did not readily tolerate enzymatic isolation to single cells, undergoing apoptosis and failing to form EBs. Therefore, the first successful EB-mediated differentiation of hESCs to CMs used collagenase IV to disperse hESC colonies (from the H9 line) into small clumps of 3 to 20 cells, which were grown as suspension EBs in nonadherent plastic petri dishes. The EBs were transferred to 0.1% gelatin-coated culture or attachment, and beating areas were first observed 4 days after plating. Comparable “spontaneous” EB differentiation protocols soon generated CMs from a variety of other hESC lines, and more recently for hiPSC lines. Furthermore, these early studies provided evidence that CMs were driving the contraction observed, on the basis of gene expression patterns,
immunolabeling for myofilament proteins, and electron microscopy that demonstrated sarcomeric organization typical of developing CMs. In addition, electrophysiological analysis showed that the cells generated spontaneous electric field potentials and action potentials typical of early nodal-, atrial-, and ventricular-like cells. However, these studies were unable to explicitly measure the number of CMs that were actually present in these EBs, and subsequent studies have suggested it was probably just a few percent. Significant line-to-line variability in the efficiency of CM formation was also observed. Reproducibility of these early protocols was problematic for a number of reasons, including the inclusion of undefined components such as serum, the heterogeneous EB sizes, and phenotypic “drift” in the undifferentiated cell population dependent on basal culture conditions. Importantly, in the case of serum, as earlier investigators realized for mouse EB differentiation, testing of serum was essential to identify lots that promote cardiogenesis. Subsequent protocols, therefore, have focused on moving away from serum- and feeder-based culture conditions toward fully defined conditions and generating EBs of uniform and defined sizes (Figure 2).

Media and Growth Factor Optimization for EB Differentiation

Protocols were developed with a variety of serum-free defined media for cardiac differentiation in EBs, including commercially available media, such as APEL (Stem Cell Technologies, Vancouver, BC, Canada) and StemPro-34 (Invitrogen). The APEL (albumin, polyvinyl alcohol, essential lipids) medium is based on the chemically defined medium originally described by Johansson and Wiles, which permitted an objective assessment of the role of exogenously added growth factors in directing differentiation. For both EBs and monolayer approaches, stage-specific application of key growth factors (GFs) in defined media are required for optimal cardiogenesis, although some protocols use fetal bovine serum (FBS) or small molecules to induce cardiogenesis (see text for details of specific protocols). Coculture of mechanically passaged hPSCs with visceral endodermal-like END2 cells takes advantage of cell signaling from END2 cells to promote cardiogenesis. APEL indicates albumin, polyvinyl alcohol, essential lipids; KO, knockout.
growth factor. Optimization of the growth factors for mesendoderm differentiation was assayed with a genetically tagged HES3 cell line (MIXL1/GFP/w) in which the expression of green fluorescent protein (GFP) is linked to the expression of the primitive streak marker MIXL1.\textsuperscript{42} Using this cell line, >90% of cells in day 3 EBs, differentiated in APEL medium containing BMP4, activin A, or combinations of the 2 factors, express MIXL1-GFP. Protocols in which cells are differentiated in BMP4 alone are biased toward posterior mesodermal derivatives such as primitive hematopoietic precursors,\textsuperscript{42} whereas the use of high concentrations of activin A results in endodermal differentiation.\textsuperscript{43}

Cardiac lineages that differentiate from mesodermal progenitors are critically dependent on the concentration of growth factors, including BMP4 and activin A, and the time of their addition and removal. The optimal concentration of these factors for any given hESC or hiPSC line may be determined by the cross titration of these factors.\textsuperscript{39,41} Furthermore, for some growth factors such as Wnts that promote cardiomyogenesis at early stages, it may be important to inhibit signaling later.\textsuperscript{44,45} Cell line variability in growth factor requirements has been attributed in part to variations in endogenous growth factor signaling.\textsuperscript{41} Additional factors in protocols that can improve the efficiency or reproducibility of CM generation from EBs include vascular endothelial growth factor\textsuperscript{18} and the use of a lower concentration of insulin.\textsuperscript{46}

### Spin EBs

The heterogeneity of the EBs formed in the early studies adversely affected the reproducibility and synchronicity of differentiation. To improve the reliability and robustness of hESC differentiation, a protocol was developed in which defined numbers of enzymatically adapted hESCs\textsuperscript{47} were seeded into low-attachment multiwell plates and aggregated by centrifugation.\textsuperscript{48} In this protocol, the centrifugation of undifferentiated cells into U- or V-bottomed wells leads to the formation of aggregates of identical size in each well, and by varying the number of cells in each well, the aggregate size can be controlled precisely.\textsuperscript{48,49} A high-throughput approach for the generation of EBs by forced aggregation has been described with a silicon wafer–based microfabrication technology to generate surfaces that permit the production of hundreds to thousands of EBs per square centimeter, which is commercially available as AggreWells (Stem Cell Technologies).\textsuperscript{50} With this approach, the optimal-sized human EB for cardiogenesis was suggested to be in the range of 250 to 300 \(\mu\)m in diameter on day 3 of differentiation.\textsuperscript{49} The formation of these “spin EBs” hinges on the preadaptation to enzymatic passage combined with the use of APEL medium. The inclusion of polyvinyl alcohol is essential for initial aggregation of the cells to form EBs. The use of spin EBs with optimized concentrations of growth factors is a preferred method for high-efficiency differentiation that can be used without specialized apparatus.\textsuperscript{39}

### Microwell EBs

Engineered microwells provide a way to control hESC colony size and to generate EBs of uniform size. This technology involves the microfabrication of microwells of defined sizes.\textsuperscript{51,52} The bottom of the microwells is coated with Matrigel extracellular matrix (ECM) to promote cell adhesion, and the hESCs seeded into the microwells grow efficiently to fill the microwell and generate colonies of defined size. Growth of hESCs in microwells was found to be able to sustain self-renewal and lead to less background differentiation of hESCs than growth under standard 2-dimensional culture conditions.\textsuperscript{52} By generating cuboidal microwells of different dimensions, different-sized hESC colonies form. Removal of the hESC colonies from the microwells results in cell aggregates of defined sizes that can be used for EB-mediated differentiation. With this approach, it was demonstrated that EBs of 100 to 300 \(\mu\)m X-Y dimension and 120 \(\mu\)m Z dimension were optimal for generating CMs.\textsuperscript{53} This microwell technology has the advantage of generating uniform-sized colonies of hESCs, which have minimal differentiation, and an appropriate substrate for subsequent differentiation. This provides a platform for the standardized input of hESCs for EB formation, similar to the spin EB method. Importantly, such technology also has the potential to be scaled up. However, the microwell technology is not currently commercially available at this time and requires access to the appropriate microengineering technology.

### Micropatterned EBs

An alternate engineering approach to generate EBs of defined sizes uses micropatterning.\textsuperscript{54,55} Using a microcontact printing approach, size-specified hESC colonies are plated from single-cell suspensions onto micropatterned ECM (Matrigel) islands. Such islands of matrix can be considered as localized niches for the hPSCs, and manipulation of the matrix alters the behavior of the cells. Varying the size of the Matrigel islands (200, 400, and 800 \(\mu\)m) varies the size of the hESCs colonies. By mechanically transferring entire colonies into suspension culture, size-controlled EBs are generated. These experiments likewise have demonstrated an optimal size range for maximizing mesoderm formation and cardiac induction. It also produces more homogenously sized EBs than conventional methods. The approach is also amenable to scale-up, but it requires access to microcontact printing equipment.

### Monolayer Differentiation to CMs

An alternative approach for differentiation of hPSCs starts with cells grown as monolayers. Taking advantage of a relatively uniform monolayer of cells without the complex diffusion barriers present in EBs, application of growth factors and other interventions will theoretically be more readily controlled and reproducible. Furthermore, because hESCs maintained in some feeder-free culture systems such as mTeSR (Stemcell Technologies) do not readily form spin EBs (ESN, DAE, and AGE, unpublished results), directed differentiation of hESCs in the monolayer format represents a convenient alternative. The monolayer protocols also do not require the replating steps typical of EB protocols, thus reducing the procedural steps and the use of tissue culture supplies.

Building on monolayer-based protocols for differentiation of endodermal derivatives,\textsuperscript{56} a differentiation protocol has
been described that plates H7 hESCs on Matrigel as a monolayer in the presence of mouse embryonic fibroblast-conditioned media. To direct cardiac differentiation, cells are treated sequentially with activin A for 24 hours followed by BMP4 in serum-free RPMI medium plus a B27 supplement. The protocol for H7 hESCs resulted in >30% CMs, and with a Percoll gradient centrifugation step, the CMs were enriched to >70%, which was a dramatic improvement over the EB method for this cell line. However, this initial protocol has not been applied successfully to many other hESC or hiPSC lines, and so a number of modifications have been tested, such as addition of Wnt3a at the induction of differentiation.

An alternative approach uses APEL medium instead of RPMI/B27 when cytokines are added for induction. In this protocol, enzymatically adapted hESCs grown on mouse embryonic fibroblasts can be dissociated into single cells and replated onto Matrigel in knockout serum replacer (KOSR; Invitrogen, Carlsbad, CA) hESC culture medium. Cells attach and form large numbers of small colonies after 24 to 48 hours, at which time the medium is replaced with APEL medium containing cytokines that induce cardiac mesoderm, specifically BMP4, activin A, and WNT3A. This differentiation process is again critically dependent on the ratio of BMP4 to activin A, the time of addition, and the time of removal of growth factors, because each step in the process of mesoderm formation, specification, and differentiation requires its own growth factor combination and concentration. Furthermore, as for EB-based methods, optimization of the concentrations of BMP4 and activin A for individual specific cell lines has proved important. Although it has been implied that differentiation of CMs in monolayer culture is more efficient than in EBs, results are influenced by the differentiation media used to generate the EBs and the monolayers. For example, when APEL medium is used with both protocols, preparations containing ~30% CMs are obtained.

A recent innovation for monolayer protocols uses application of ECM in combination with cytokines, because ECM proteins play fundamental roles in development and can complement responses to soluble cytokines. For this matrix sandwich approach, hPSCs are seeded as single cells on Matrigel (growth factor reduced)—coated plates and cultured in mTeSR1 media. When cells reach 90% confluence after 2 to 4 days, a matrix overlay is applied on the monolayer of cells by mixing the Matrigel with mTeSR1 media. Cells grow for another 1 to 2 days in mTeSR1 media to reach 100% confluence, then activin A with Matrigel (growth factor reduced) is added in basal media of RPMI/B27 minus insulin supplement to treat the cells for 24 hours, followed by BMP4 and basic FGF for 4 days in the same basal medium. Flow cytometry analysis by cardiac troponin T labeling has demonstrated high purity (40%–90%) of CMs and high yield (4–11 CMs per input hESCIhiPSC) for a range of hESCs and hiPSC lines. Thus, the use of ECM to supplement soluble growth factor signaling can enable reproducible and robust cardiac differentiation across multiple hESC and hiPSC lines. This matrix sandwich monolayer protocol is a preferred approach to generate relatively high purity and yields of CMs.

**Inductive Coculture**

Derivation of CMs from hPSCs by coculture with visceral endoderm-like cells is particularly effective for hESCs and hiPSCs being passaged mechanically and was first described by Mummery et al. with later refinements described by Passier et al. Visceral endoderm plays an important inductive role in the differentiation of cardiogenic precursor cells in the adjacent mesoderm in vivo in developing embryos. Beating areas were observed in hESC colonies during coculture with a mouse visceral endoderm-like cell line (END-2). In the first experiments, END-2 cells were seeded on a 12-well plate, mitotically inactivated with mitomycin C, and cocultured with the hESC line HES2. Beating areas formed in ~35% of the wells after 12 days in coculture; however, only approximately 2% to 3% of the cells were CMs. It was later shown that both serum supplementation of the culture medium and mTeSR, a serum-free culture medium designed for expansion of undifferentiated cells in the absence of feeder cells, reduced the efficiency of differentiation. But if the medium was completely serum- and insulin-free, the number of beating colonies increased 10-fold, and each beating colony contained ~25% CMs based on staining with sarcomeric α-actinin antibodies. Insulin was the most important component of the medium that inhibited mesoderm formation in favor of ectoderm. The protocol is effective not only in hESCs but also in hiPSCs provided they are derived from mouse embryonic fibroblast feeder cells. This protocol is particularly useful for testing the ability of both hESCs and hiPSCs to form cardiac mesoderm cells at an early stage of their derivation, because the cells are generally passed mechanically and the primary goal of the experiment is simply to demonstrate the ability to form functional CMs. Under these circumstances, coculture is convenient because (1) it requires very few cells, (2) it is simple and rapid, and (3) it yields CMs in sufficient numbers and quality to detect visible beating and identify sarcomere structures by immunofluorescent staining. The protocol is described in detail elsewhere. Skeletal myoblasts do not form under these conditions. Another stromal cell line, OP9, has also been reported to induce mesoderm differentiation and cardiogenesis, which illustrates the general concept of coculture with another cell line as supporting directed (cardiac) differentiation.

**Cardiovascular Progenitors Derived From hPSCs**

In the previous section, we presented various methods that result in hPSCs forming cultures that contain beating CMs. This process of hPSC differentiation is thought to proceed through a similar hierarchy of cardiac progenitors as described for cardiac development (Figure 1). This concept is supported by the sequential pattern of gene expression, which suggests the ordered formation of distinct progenitor populations. However, these progenitor populations are only beginning to be isolated and defined in detail. The earliest defined mesodermal progenitors differentiating from hESCs were identified by the expression of neuronal cell adhesion molecule (NCAM/CD56) and loss of expression of epithelial cell adhesion molecule (EpCAM/CD326). These mesoder-
nal progenitors are multipotent and can give rise to all mesodermal lineages, including CMs. Later in differentiation, a population of cells expressing low levels of KDR (kinase insert domain receptor) and no cell surface c-Kit (KDR<sub>low</sub>/c-Kit<sup>−</sup>) was isolated at day 6 of EB-mediated differentiation.38 On the basis of colony-forming assays, the KDR<sub>low</sub>/c-Kit<sup>−</sup> cell population contained cardiovascular colony-forming cells that gave rise to CMs, smooth muscle cells, and endothelial cells. If the KDR<sub>low</sub>/c-Kit<sup>−</sup> cells were plated as monolayers, 50% of the differentiating cells were cardiac troponin T–positive CMs. More recently, the Keller group has shown that KDR and platelet-derived growth factor receptor-α (PDGFRα) are coexpressed in the emerging cardiac mesoderm.41 Furthermore, when the KDR/PDGFRα double-positive population is cultured, >80% of cells differentiate to CMs. However, a disadvantage of using KDR as a cell surface marker to identify cardiac progenitors is that it is also expressed in undifferentiated hESCs.

Another marker that has been proposed to isolate cardiac progenitors from human and nonhuman primate embryonic stem cells (ESCs) is the stage-specific embryonic antigen-1 (SSEA-1).69 Unlike mESCs, primate and human ESCs do not express SSEA-1 in the undifferentiated state; however, on the initiation of differentiation, SSEA-1 is quickly upregulated. In a protocol in which enzymatically passaged hESCs or hiPSCs are cultured on mouse embryonic fibroblasts, a 4- or 6-day treatment of cultures with BMP2 (10 ng/mL) in the presence of the FGF receptor inhibitor SU5402 (1 μmol/L) produced a culture that contained ∼50% SSEA-1<sup>−</sup> cells. The SSEA-1<sup>−</sup> cells were isolated by antibody-conjugated magnetic beads and expressed a variety of markers of cardiac progenitors, including MESP1, MEF2C, NKX2-5, GATA4, TBX5, TBX15, and ISL1. Single-cell clones expanded from these SSEA-1<sup>−</sup> cells appeared to include multipotent cardiac progenitors that could differentiate into CMs, smooth muscle cells, and endothelial cells. In contrast to undifferentiated ESCs and hiPSCs, the purified SSEA-1<sup>−</sup> cells were unable to form teratomas after transplantation into immuno-compromised animals.69 However, SSEA-1 marks all early differentiated derivatives of hPSC and is only appropriate for selection of cells of the cardiac lineage when hPSCs have been exposed to cardiomyogenic growth factors or induction conditions.

An alternative approach for isolating cardiac progenitors involves the use of genetically engineered reporter ESC lines. Typically, these reporter lines express fluorescent proteins such as GFP, either inserted by homologous recombination into the locus of a cardiac-specific gene or randomly inserted as a transgene regulated by cardiac-specific promoters/enhancers to enable the identification and isolation of the cells of interest. This has been a particularly powerful strategy used to generate a variety of reporter mESC lines based on the expression of reporters for Nkx2-5, Isl1, and Mesp1.20,70–72 but this approach is only now beginning to be used for hPSCs. By targeting the ISL1 locus in hESCs, a population of Isl1<sup>+</sup> cardiovascular progenitors was isolated that were derived from the secondary heart field.73 These progenitors were capable of limited proliferation in culture in the presence of Wnt3A and gave rise to clones of smooth muscle cells, with some clones of CM and endothelial cell lineages. The Isl1<sup>+</sup> progenitors can be isolated before the expression of KDR, and subsequently, an Isl1<sup>+/KDR<sup>−</sup></sup> progenitor population was identified that may overlap with the KDR<sup>−</sup>/c-Kit<sup>−</sup> population described above.

An NKX2-5<sup>eGFP<sup>−</sup></sup> targeted hESC line has been described recently that enabled the identification of cardiac-specific progenitors based on eGFP expression.39,74 Characterization of the first cells to express enhanced GFP (eGFP) during cardiac differentiation using both antibody arrays and gene-expression arrays identified the cell surface marker signal regulatory protein-α (SIRPA/CD172a) as specifically present on the eGFP<sup>+</sup> progenitors relative to the eGFP<sup>−</sup> cells. SIRPA expression was not detected in the KDR/PDGFRα double-positive population of cardiac mesoderm cells present earlier in differentiation, but its expression begins in the cardiocommitted progenitors. SIRPA expression persists specifically in CMs. Interestingly, SIRPA is not expressed in the developing mouse heart, in contrast to the human heart, which highlights the need to define markers in the human system. Furthermore, none of the described cell surface markers are exclusively expressed on cardiac cell lineages, and thus, they must be used in conjunction with other markers and cellular characteristics to identify various progenitors specifically. Both new engineered hPSC lines and cell surface markers are anticipated that will provide further definition, as well as isolation of the hierarchy of cardiac progenitors.

**Selection of CMs From Mixed Differentiated Cultures**

Strategies to isolate CMs from mixed differentiating cultures have also been developed, which include physical methods, genetic methods, and nongenetic selection. The ability to isolate relatively pure populations of CMs is essential to multiple applications using the cells. For example, studies of global gene expression are more readily interpretable if a purified population of cells is studied. Cardiac drug-screening assays also require relatively pure populations of CMs so that the observed signals can be attributed to effects on CMs. Finally, clinical applications that propose to use CMs derived from hPSCs will need to be free of contaminating pluripotent stem cells and any other undesired cell type to minimize the risk of tumor formation and other adverse outcomes.

The first described methods for CM selection from hESC EBs relied on physical methods. After manual dissection and dissociation of contracting areas from EBs, between 2% and 70% of the cells stained for cardiac markers, depending on the particular aggregate dissected.34,62 Alternatively, by taking cells isolated from contracting aggregates and using Percoll gradient purification, a further 4-fold enrichment could be obtained (up to 70% of cells stained positively for cardiac markers) in a particular cell fraction compared with the initial differentiated cell suspension.53 Although this method provided enrichment for CMs, it was labor intensive and critically dependent on the successful formation of EBs with well-defined larger contracting outgrowths.

A second method for selection of CMs involves genetically engineering stem cell lines to express a drug resistance gene or reporter protein gene under the control of a cardiac-specific promoter. This strategy is based on the original study by Field...
and colleagues, who engineered mESCs with the cardiac αMHC promoter driving the expression of an antibiotic resistance gene (aminoglycoside phosphotransferase), which enabled selection of relatively pure cultures of CMs in the presence of neomycin. Subsequently, a similar approach was used with a variety of cardiac promoters with different selection or reporter proteins in mESCs. Selection for CMs was achieved either with drug selection to eliminate cells not expressing the drug resistance gene or with fluorescence-activated cell sorting for cells expressing fluorescent reporter proteins. In the case of hESCs, the αMHC promoter has also been expressed ectopically and used to drive fluorescent protein expression (GFP or mCherry) or puromycin resistance to allow selection of hESC-CMs. Likewise, an engineered NkX2-5/GFPknock-in hESC line has been described that enables isolation of GFP CMs. However, because αMHC and NkX2-5 are expressed in all types of CMs, the resulting population of selected CMs represents a mixture of nodal-, atrial-, and ventricular-like CMs. In an effort to select specifically for ventricular-like CMs, Huber and colleagues linked the myosin light chain 2v (MLC2v) promoter to GFP expression, which enabled a highly enriched population of ventricular-like cells to be purified by flow cytometry. Conversely, the proximal promoter/enhancer element from the cGATA6 gene was used to identify nodal-like hESC-derived CMs. Using this approach, it was found that neuregulin-1/β increased the relative abundance of nodal-like CMs, which demonstrates the utility of such markers for lineage enrichment strategies.

Future studies will likely test a variety of additional cardiorelevant promoters and various selection strategies to enhance or refine selection. The subtleties of the promoter/enhancer chosen will be of critical importance, and issues such as species differences between well-characterized mouse regulatory elements and often less well-characterized human elements will need to be considered. Although certain proteins exhibit cardiосpecific expression, and knock-in to the native locus gives similar results to expressing a characterized promoter sequence, in other cases in which genes are more broadly expressed, selected promoter/enhancer elements that give a greater degree of tissue specificity may be more appropriately chosen. The choice of using drug selection or reporter protein cassettes or a combination will depend on the planned application. For enrichment of CMs during culture, drug selection may be the preferred approach, because it does not require cell isolation and sorting, and highly pure populations of CMs have been obtained by this route. The timing of drug selection during differentiation is critical and may affect the ability of CMs to mature in culture. Alternatively, if the goal is to track the time course of differentiation and localize CMs in differentiating cultures, then fluorescent reporter proteins are optimal. Likewise, CMs with fluorescent or other reporters such as luciferase can be tracked in vivo after transplantation in animal models. Finally, it is essential to consider the approach for genetically modifying the cell lines. There are multiple techniques to introduce new reporter constructs, including lentiviral transduction, homologous recombination, random integration, integrase-mediated recombination, zinc finger nuclease assisted recombination, and others. Each genetic engineering approach has advantages and disadvantages, as described elsewhere.

Nongenetic methods for isolating CMs hold appeal that might be more readily applied across various cell lines with specificity and efficiency. Cell surface marker proteins have been used with great success in the hematopoietic system to isolate and define various cell lineages, but cell surface markers on CMs are only just starting to be defined. The cell surface protein SIRPA present on cardiac progenitors is also expressed on differentiated CMs and can be used for selection. Alternatively, an approach that takes advantage of the high density of mitochondria present in mature CMs compared with other cell types has been described recently. Using a fluorescent vital dye that labels mitochondria, tetramethylrhodamine methyl ester perchlorate (TMRE), CMs derived from hESCs could be isolated at >99% purity. The dye rapidly washes out, which suggests limited long-term impact or toxicity on the selected population. The limitation with this method is that it is only effective on relatively late or mature hESC-derived CMs, maintained in culture ≥8 weeks.

In summary, techniques for the separation and purification of CMs from mixed cultures are still being refined. Highly enriched populations of CMs have been obtained by various techniques, but the relative efficiency of these techniques and practical limitations will drive further improvements. In addition, strategies to isolate different types of CMs and different maturities of CMs are required and will be the focus of continued development. Approaches such as different reporters in the same line, as described for mESCs, may be a way forward to improve cell-type specificity.

**Scalable Cardiac Differentiation of hPSC**

For some applications, large-scale production of CMs from hPSCs is of critical importance. Clinical therapies envisioning the use of hPSC-derived CMs may require 10⁶ to 10⁷ cells per patient dose, which reflects the amount of working myocardium lost in a large myocardial infarction. Additionally, high-throughput screening of libraries of small molecules using hPSC-derived CMs for safety or efficacy signals will require large numbers of CMs, although the testing of selected lead compounds is already possible with lesser cell numbers available with current technologies. To enable such applications, both the starting pluripotent stem cells and the differentiation process will need to be scalable. Progress has been made on the scalable culture of hPSCs using defined, humanized culture media and reagents with a variety of bioreactor formats. Current bioreactor technology allows continuous perfusion and automated control of pH, oxygen tension, temperature, and other relevant parameters. This eliminates the large swings in media pH and in the concentrations of metabolites and nutrients that typically occur with medium changes in standard culture formats. Although we will focus only on the scalable generation of hPSC-derived CMs, it is important to acknowledge that limiting background differentiation and maintaining karyotype stability remain critical issues for the large-scale propagation of hPSCs.

Large-scale differentiation of mESCs to CMs was first described with suspension culture of EBs. Suspension
cultures allow a greater cell number--culture volume ratio and hence are more efficient. However, cultures need to be dynamic to prevent the rafting of EBs and to allow adequate exchange of metabolites, nutrients, and growth factors, as well as to maintain homeostasis of physiochemical parameters such as pH and oxygen levels. A variety of dynamic culture systems such as spinner flasks, stirred bioreactors, and rotary culture have been used. In one study, to avoid agglomeration, the EBs were encapsulated in hydrogel, which did not impair cellular viability or differentiation.93 In another study, single-cell suspensions of mESCs were added to a bioreactor and conditions were optimized so that aggregates of appropriate size formed and generated EBs.74 By combining genetic selection with the αMHC promoter with the suspension EB differentiation, as described above, relatively pure populations of mESC-derived CMs could be generated with up to 10⁹ CMs from a 2-L bioreactor.96 Other refinements of the bioprocess, such as differentiation under conditions of low oxygen (4% oxygen tension) or continuous perfusion of the bioreactors, have been suggested to further improve CM yield.95,99–101 To translate this to hESCs cells, micropatterning was used to generate size-specified aggregates of hESCs which, when loaded into a bioreactor, formed EBs that differentiated into CMs.102 Likewise, other dynamic culture systems for human EBs that generate CMs have been described.100,101 However, these initial studies using hESCs did not clearly describe yields of CMs and did not use selection strategies to further purify CMs. Nevertheless, large-scale production of hESC- and hiPSC-derived CMs has been demonstrated in commercial products now available.103

**Basic Characterization of hPSC-Derived CMs**

Determining the success of differentiation protocols requires detailed characterization of the resulting CMs. Typically, the first sign of successful differentiation to CMs is the appearance of contractile foci in the culture. Although early studies using EB-based differentiation protocols usually reported the percentage of contracting EBs as a measure for cardiac differentiation, more recent studies have described the proportion of CMs, estimated by intracellular flow cytometry for a cardiac-specific protein such as cardiac troponin T. This more accurately reflects the efficacy of the protocol. To better characterize and quantify the resulting CMs, a variety of approaches have been used ranging from analyses of gene expression to functional studies. These complementary assays may be considered to span a range of stringencies. The lowest stringency includes reverse transcription–polymerase chain reaction evaluation for expression of a variety of cardiac genes, from transcription factors to myofilament proteins. An assay slightly higher in the hierarchy is provided by immunolabeling of the CMs with antibodies to a variety of myofilament proteins (eg, αMHC, βMHC, ML2a, ML2v, cardiac troponin T, cardiac troponin I, α-actinin) to demonstrate organized myofilaments typical of early CMs (Figure 3). Electron microscopy can also be used to demonstrate the ultrastructure typical of CMs, including bundles of myofilaments and abundant mitochondria.

The highest stringency assays are primarily functional assays that demonstrate the integrated cellular phenotype. A key property of CMs is generation of action potentials (APs) necessary for propagation of the electric signal in the myocardium and for triggering intracellular Ca²⁺ release and ensuing mechanical contraction. Demonstration of cardiac APs by use of microelectrode recordings can provide a high level of evidence that functional CMs are present. Furthermore, properties of the AP such as the AP amplitude, spontaneous phase 4 depolarization, maximum diastolic potential, AD duration, and rate of rise of the AP can provide information regarding the type and maturity of CM.34,36,62 The majority of CMs display ventricular-like APs, but atrial-like and nodal-like APs are also detected (Figure 3). The properties of the APs suggest that the hPSC-derived CMs are relatively immature in phenotype compared with adult CMs, which is consistent with other characteristics of the cells (Table). Furthermore, the CMs typically show evidence of a range of maturities, and for CMs with more depolarized maximum diastolic potentials, discrimination between APs representative of different types of CMs is difficult given overlap between the properties of more mature nodal CMs and immature CMs of all types. The multiple underlying ionic currents responsible for generating the cardiac APs can also be studied in detail by single-cell patch-clamp methodology,103,104 but this is typically beyond the scope of basic characterization studies. However, such detailed electrophysiology is of particular importance in investigations evaluating basic mechanisms of arrhythmia, such as recent studies using patient-specific hiPSCs to study inherited long-QT syndrome.7,105,106 Multielectrode arrays can be used to demonstrate spontaneous electric signals (field potentials) and monitor the electrophysiology of multicellular preparations, providing information on parameters such as conduction velocity among CMs; however, these assays do not have single-cell resolution like microelectrode recordings.107 Nevertheless, multielectrode arrays are more rapid and less technically demanding, and patterns typical of CMs preparations are becoming evident. Intracellular Ca²⁺ can also be monitored with fluorescent Ca²⁺ indicators to determine whether cardiac-typical cardiac Ca²⁺ transients are present. Overall, it is important to note that molecular assays alone are not sufficient to confirm CM identity; functional assays in the highest stringency category are also essential.

**Emerging Strategies for Improving and Troubleshooting CM Differentiation From hPSCs**

Although protocols for cardiac differentiation have advanced dramatically over the past decade, significant limitations remain regarding line-to-line variability in the yield and purity of CMs, reproducibility of protocols, cost of reagents, and the complexity of the protocols. For example, differences in individual hESC and hiPSC lines with respect to their yield of CMs, even when identical protocols are used, have been described.37,49 Various reasons have been given for this interline variability, including differences in the initial state of pluripotency,21,22 conditions used to maintain lines, epigenetic status determined by the tissue of origin in the case of hiPSCs,24,108 and intrinsic differences in endogenous growth factor production between individual lines.41,58,109 More ro-
bust differentiation protocols that are broadly successful across cell lines are needed.

Recent protocols have largely replaced undefined serum with the sequential application of growth factors and, more recently, small molecules to provide more reproducible results. Many of the growth factors are costly to obtain, degrade rapidly, and do not readily diffuse into complex multicellular aggregates. In addition, they exhibit lot-to-lot variation in their bioactivity. Thus, efforts at identifying chemically synthesized small molecules to promote the differentiation process are actively being pursued. A variety of small molecules that promote cardiogenesis of mESCs have been identified by strategies including high-throughput screens with various engineered reporter lines. These mESC studies clearly determined that the timing of application is of critical importance; nevertheless, only a few of these results have been extrapolated to hPSCs at this time. Inhibitors of Wnt signaling, IWR-1, IWP-3, and XAV939, have been shown to promote cardiogenesis when added later in differentiation after mesoderm formation, and Ca2+ -cycling proteins. ES indicates embryonic stem cells; EB, embryoid body; H1 and H9, types of ES; and LV, left ventricle. C, Immunofluorescence microscopy with antibodies specific for myofilament proteins determines whether cells exhibit organized sarcomeres typical of cardiomyocytes. MLC indicates myosin light chain. D, Functional assessment of cardiomyocytes can be provided by cellular electrophysiology measurements to determine whether cardiac action potentials typical of different types of cardiomyocytes are present. E, Extracellular field potential measurements by multielectrode arrays provide another method to detect spontaneous electric activity in the cardiomyocyte preparations. F, Detection of Ca2+ transients typical of cardiomyocytes provides another assessment of the functional integrity of the differentiating cardiomyocytes. Cells loaded with the Ca2+ indicator Fluo-3 were imaged by laser scanning confocal microscopy in the line-scan mode with Ca2+ transients displayed and time versus normalized Ca2+ transient intensity (F/F0) shown below. Panels B and D were modified with permission from Zhang et al.126

Figure 3. Basic characterization of human pluripotent stem cell–derived cardiomyocytes. A, Flow cytometry provides a quantitative method to evaluate the relative yield and purity of cardiomyocytes resulting from differentiation protocols by measuring the number of cells that express cardiac-specific proteins such as cardiac troponin T (cTnT). FSC indicates forward scatter. B, Reverse transcription–polymerase chain reaction can be used as a first assessment for changes in gene expression typical of cardiogenesis by assaying for the expression of cardiac transcription factors, myofilament proteins, and Ca2+ -cycling proteins. ES indicates embryonic stem cells; EB, embryoid body; H1 and H9, types of ES; and LV, left ventricle. C, Immunofluorescence microscopy with antibodies specific for myofilament proteins determines whether cells exhibit organized sarcomeres typical of cardiomyocytes. MLC indicates myosin light chain. D, Functional assessment of cardiomyocytes can be provided by cellular electrophysiology measurements to determine whether cardiac action potentials typical of different types of cardiomyocytes are present. E, Extracellular field potential measurements by multielectrode arrays provide another method to detect spontaneous electric activity in the cardiomyocyte preparations. F, Detection of Ca2+ transients typical of cardiomyocytes provides another assessment of the functional integrity of the differentiating cardiomyocytes. Cells loaded with the Ca2+ indicator Fluo-3 were imaged by laser scanning confocal microscopy in the line-scan mode with Ca2+ transients displayed and time versus normalized Ca2+ transient intensity (F/F0) shown below. Panels B and D were modified with permission from Zhang et al.126
found to increase the effectiveness of hPSC cardiogenesis by some investigators. The role of redox state and reactive oxygen species in cardiogenesis merits further study and may be explored at least 2 times under the indicated conditions before differentiation. Investigators without experience in hPSC culture need training first in this methodology before success can be anticipated with differentiation protocols. Second, high-quality growth factors and small molecules should be obtained that ideally match the protocols regarding source. In some cases, it is necessary to test a range of growth factor concentrations to optimize each individual line. For new investigators, it is also essential to have a validated control hPSC line that has been shown to differentiate well with a given protocol. Ideally, such hPSCs should be obtained from quality-controlled cell banks. Future harmonization of protocols is an important goal, and control transgenic cell lines, in which reporter genes are driven by cardiac-specific promoters, may be useful in benchmarking protocols between laboratories and may provide ways to standardize operator and reagent variability.

Summary

The demonstration that hESCs can give rise to functional CMs has promoted much discussion about the potential utility of hPSC-derived CMs. At the forefront of popular interest has been the promise of cardiomyocyte replacement therapies. However, as experimental data continue to reveal the challenges for clinical application with hPSCs, the goal of hPSC-CM-mediated cardiac repair appears elusive. Nevertheless, for drug safety pharmacology and disease modeling after the advent of hiPSCs, it may transpire that hPSCs can have a much greater impact on cardiovascular health than simply through transplantation to replace CMs lost in disease. Several recent studies that generated hiPSCs from patients with cardiac disease have demonstrated that the phenotype was retained. More importantly, these studies showed that the CMs were suitable for testing the effects of drugs on the disease. Once further explored, this will provide unprecedented opportunities for the development of novel treatment strategies, particularly because robust and efficient methods
for the differentiation and maturation of CMs from hPSCs are well under way.

Acknowledgments

We apologize to those authors whose excellent work we have not cited because of space restrictions. We gratefully acknowledge Randall Loaiza Montoya for providing the Ca^{2+} data shown in Figure 3.

Sources of Funding

C.L.M. receives laboratory funding from the Netherlands Heart Foundation, EU FP7 (“InduStem” PIAP-GA-2008-230675), ZonMW (114000101), The Netherlands Institute of Regenerative Medicine, and the Netherlands Proteomics Consortium (050-040-250). A.G.E. is a Senior Research Fellow of the National Health and Medical Research Council (NHMRC) and receives laboratory funding from the NHMRC, the Australia Research Council, the Australian Stem Cell Centre, the Juvenile Diabetes Research Foundation, and the Queensland Neurosurgery Research Foundation (QNRF). D.A.E. is a Senior Research Fellow of the National Health and Medical Research Council, the Netherlands Institute of Regenerative Medicine, the Netherlands Heart Foundation, the Australia Research Council, the Australian Stem Cell Centre, the Juvenile Diabetes Research Foundation, and the Queensland Neurosurgery Research Foundation (QNRF). T.J.K. receives funding from the National Institutes of Health (R01EB007534, R01HL08416, and U01HL099773).

Disclosures

T.J.K. is a founding shareholder and consultant for Cellular Dynamics International, which produces hPSC-derived CMs for drug discovery, toxicity testing, and research applications. The remaining authors report no conflicts.

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Circ Res. 2012;111:344-358
doi: 10.1161/CIRCRESAHA.110.227512

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