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Review

Production of De Novo Cardiomyocytes: Human Pluripotent Stem Cell Differentiation and Direct Reprogramming

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Cardiovascular disease is a leading cause of death worldwide. The limited capability of heart tissue to regenerate has prompted methodological developments for creating de novo cardiomyocytes, both in vitro and in vivo. Beyond uses in cell replacement therapy, patient-specific cardiomyocytes may find applications in drug testing, drug discovery, and disease modeling. Recently, approaches for generating cardiomyocytes have expanded to encompass three major sources of starting cells: human pluripotent stem cells (hPSCs), adult heart-derived cardiac progenitor cells (CPCs), and reprogrammed fibroblasts. We discuss state-of-the-art methods for generating de novo cardiomyocytes from hPSCs and reprogrammed fibroblasts, highlighting potential applications and future challenges.

Introduction

Heart disease is the most significant cause of morbidity and mortality in the United States, accounting for more than 800,000 deaths per year on average (equivalent to 1 death every 39 s) (Roger et al., 2011). Each year, nearly 6 million patients suffer heart failure and 1.25 million patients suffer a new or recurrent myocardial infarction. Both of these conditions result in cardiomyocyte death by apoptosis and/or necrosis. Dead cardiomyocytes are replaced by fibroblasts that divide and migrate into the damaged area to form scar tissue, leading to the development of a thin ventricular wall that no longer contracts properly. Formation of a fibroblastic scar initiates a series of events that lead to remodeling, hypertrophy, and ultimately heart failure and further cell death.

The persistence of scar tissue following myocardial infarction suggests that the heart has little if any capacity to generate new cardiomyocytes. Shortly after birth, myocardial growth transitions from a hyperplastic to a hypertrophic phase, characterized by the formation of binucleated cardiomyocytes that withdraw from the cell cycle (Pasumarthi and Field, 2002). This transition gave rise to the notion that adult cardiomyocytes are incapable of proliferating; that is, they are terminally differentiated.

Whereas the majority of adult cardiomyocytes do not proliferate, evidence exists indicating that the adult heart has limited regenerative capacity, although insufficient to compensate for the cell death caused by heart disease. Radiocarbon dating of postmortem cardiac tissue has demonstrated that human adult cardiomyocytes have a turnover rate of less than 1% per year and that 40% of the mature adult heart consists of postnatally generated cardiomyocytes (Bergmann et al., 2009). In addition, cardiac regeneration without scar tissue has been demonstrated in model organisms, such as zebrafish, after surgical removal of up to 20% of the ventricle (Poss et al., 2002). This regeneration occurs through the proliferation and dedifferentiation of cardiomyocytes (Jopling et al., 2010), and similar regeneration has been demonstrated in mice, but only within the first week postpartum (Porrello et al., 2011). Work in adult mice provided evidence that limited numbers of new cardiomyocytes may be formed after a myocardial infarction, and lineage tracing experiments suggest these new cells are not derived from existing cardiomyocytes but rather develop from a progenitor population (Loffredo et al., 2011), some of which are derived from the epicardium (Smart et al., 2011). Finally, mononuclear rat cardiomyocytes have been shown to dedifferentiate and proliferate in vitro (Zhang et al., 2010). Growth factors from prenatal cardiac development, such as neuregulin1 (NRG1) (Bersell et al., 2009) and periostin (POSTN) (Kühn et al., 2007), can induce subpopulations of these cardiomyocytes to reenter the cell cycle in vitro. However, it is not clear to what extent this phenomenon occurs in vivo.

In clinical trials, transplantation of noncardiac stem cells such as skeletal muscle progenitors and bone-marrow-derived cells has resulted in minor improvement in left ventricular ejection fraction (LVEF) (Hansson et al., 2009), but also the induction of arrhythmias (Menasché et al., 2008). Because these transplanted populations do not generate new cardiomyocytes, the improvement may be due to paracrine effects that lead to enhanced vascularization (Gnecchi et al., 2005). The modest improvement provided by these nonmyocyte populations coupled with the limited regenerative capacity of the adult heart has led researchers to investigate new methods for the de novo derivation of cardiomyocytes. One potential source of these cells is the adult heart itself, in the form of CPCs, expertly reviewed elsewhere (Perino et al., 2008). With the discovery of human embryonic stem cells (hESCs) (Thomson et al., 1998), and more recently, human induced pluripotent stem cells (hiPSCs)

(Takahashi et al., 2007; Yu et al., 2007), many investigators have focused their efforts on developing strategies to efficiently and reliably direct stem cell differentiation to the cardiovascular lineage. Since the initial demonstration that contracting cardiomyocytes can be generated from both types of human pluripotent stem cells (hPSCs) (Itskovitz-Eldor et al., 2000; Zwi et al., 2009), the possibility of producing unlimited numbers of human cardiomyocytes to rebuild the heart has tantalized researchers. Substantial effort has been made to improve the efficiency and reproducibility of differentiation, while advancing the aims of progressing to defined conditions and producing cells on a clinically relevant scale. Advances in embryology and hPSC differentiation have offered key insights into the mechanisms of cardiopoiesis, providing hope that in the future injured hearts may be repaired through clinical applications of these cells.

A potential alternative source of de novo cardiomyocytes could result from the ''direct reprogramming'' of murine cardiac fibroblasts and other adult cell types into cardiomyocytes using cardiac-specific transcription factors (*Gata4*, *Mef2c*, and *Tbx5*) (Ieda et al., 2010). This approach offers the possibility of reprogramming cardiac fibroblasts in vivo for heart regeneration, using direct delivery of these transcription factors. A variation of the theme of reprogramming fibroblasts into cardiomyocytes has been recently described, in which fibroblasts are first partially reprogrammed using exogenous expression of pluripotency genes (*Oct4*, *Sox2*, and *Klf4*) (OSK) and then differentiated to the cardiac lineage (Efe et al., 2011).

Applications of De Novo Cardiomyocytes

One of the main long-term goals of de novo cardiomyocyte production is to provide a source of donor cardiomyocytes for cell replacement in damaged hearts. Many forms of heart disease, including congenital defects and acquired injuries, are irreversible because they are associated with the loss of nonregenerative, terminally differentiated cardiomyocytes. Current therapeutic regimes are palliative, and in the case of end-stage heart failure, transplantation remains the last resort. However, transplantation is limited by a severe shortage of both donor cells and organs. In cases of myocardial infarction, 1 billion cells would potentially need to be replaced (Laflamme and Murry, 2005), highlighting the need for high-throughput and reproducible methodologies for de novo cardiomyocyte production. A major challenge in this field is to establish the most efficient format for the transplantation of these substantial numbers of cells. Transplantation of single cell suspensions is easiest, but engraftment of 3D engineered constructs may be the best approach for replacing scar tissue with new working myocardium. In addition, concerns over cell survival, immune rejection, electrical maturation, electrical coupling, arrhythmia, and whether autologous hiPSCs possess immune privileges (a question that has recently been raised with murine iPSCs; Zhao et al., 2011) still need to be addressed.

A second application lies in novel cardiac drug discovery, development, and safety testing, a process that is collectively long, arduous, and expensive, and one which is confounded by the lack of economical and reliable methods to accurately mimic the human cardiac physiological response, among other challenges. Many drug discovery programs have failed because targets validated in animal models proved unreliable and non-predictive in humans (Denning and Anderson, 2008). The pharmaceutical industry currently invests approximately $1.5 billion to successfully develop a candidate drug from primary screening to market. Among the drugs that ultimately make it to market, many are later withdrawn due to side effects associated with electrophysiological alterations of the heart (Braam et al., 2010). The use of de novo human cardiomyocytes offers the pharmaceutical industry an invaluable tool for preclinical screening of candidate drugs to treat cardiomyopathy, arrhythmia, and heart failure, as well as therapeutics to combat secondary cardiac toxicities. Studies have already demonstrated that hiPSC-derived cardiomyocytes will react to cardioactive drugs with the expected response, indicating that these cells can be used in the context of larger predictive toxicology screens (Davis et al., 2011). The development of new screens using human cardiomyocytes should reduce the time and cost of bringing new drugs to market.

A third application is in developmental biology, disease modeling, and postgenomic personalized medicine. Deriving hiPSCs from patients with specific cardiac diseases, differentiating them to cardiomyocytes, and then performing electrophysiological and molecular analyses may provide a powerful tool for deciphering the molecular mechanisms of disease (Josowitz et al., 2011). Studies to date have largely concentrated on recapitulating genetic disease phenotypes in vitro, such as long QT syndromes (Itzhaki et al., 2011a; Matsa et al., 2011; Moretti et al., 2010), Timothy syndrome (Yazawa et al., 2011), and LEOPARD syndrome (Carvajal-Vergara et al., 2010). The possibility of modeling cardiac diseases without a known genetic element is another exciting prospect. The combination of novel drug discovery and efficacy testing with cardiomyocytes derived from patient-specific hiPSCs is a potentially groundbreaking option for personalized medicine. Additionally, the utility of pluripotent stem cells as a tool for modeling cardiac development is another important application that has recently been demonstrated for a variety of aneuploid syndromes (Li et al., 2012).

Cardiomyogenesis

To effectively serve the above applications, efficient and reproducible generation of cardiovascular cells in vitro must be developed. Because hPSC differentiation recapitulates embryonic development, understanding how the cardiac lineage is established in the early embryo is essential for differentiation and development strategies. Understanding cardiogenesis also allows access to the feedforward gene regulatory networks that occur during development and ultimately deriving physiologically relevant cells. Cardiomyogenesis begins with the generation of mesoderm via the process of gastrulation, which has been best studied in the mouse (Arnold and Robertson, 2009; Buckingham et al., 2005; Tam and Loebel, 2007) (Figure 1). Mesoderm induction begins with NODAL signaling in the proximal epiblast on mouse embryonic day 5 (E5.0), which maintains BMP4 expression in the extraembryonic ectoderm adjacent to the epiblast. BMP4 acts by inducing WNT3 expression in the proximal epiblast. Around E5.5, expression of the WNT antagonist *Dkk1* and the NODAL antagonists *Lefty1* and *Cer1* moves to the anterior visceral endoderm, restricting NODAL and WNT signaling to the posterior epiblast. At E5.75, WNT induces the

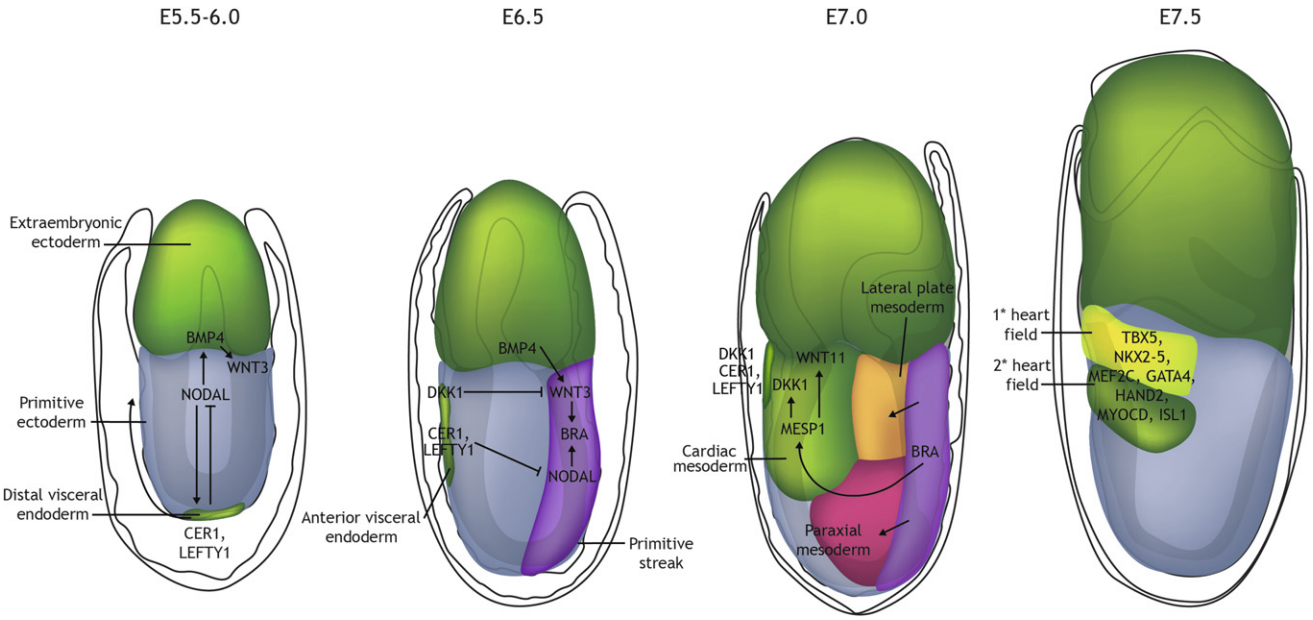

Figure 1. The Mouse as a Model for Human Cardiogenesis

(A) Gastrulation begins with the formation of the primitive streak. Anterior primitive ectoderm cells perform an epithelial to mesenchymal transition (EMT), pass through the primitive streak, and move laterally between the primitive ectoderm and visceral endoderm.

(B) Cells in the most proximal portion of the primitive streak will form the extraembryonic mesoderm, midprimitive streak cells will form the embryonic tissues such as heart and blood, and cells of the distal portion of the primitive streak will form the endoderm.

(C) In the case of cardiac progenitors, this midstreak mesoderm progresses laterally, then ventrally, around both sides of the embryo, becoming the lateral plate mesoderm from which the first heart field (FHF) is derived. The lateral plate mesoderm then delaminates to form the splanchnic mesoderm (on the ventral side), which forms the second heart field (SHF) and somatic mesoderm (on the dorsal side).

(D) The FHF progenitors form the cardiac crescent, whereas SHF progenitors are found medial to the crescent. The cells of both heart fields then move to the midline, where the FHF progenitors form a linear heart tube that later contributes to the left ventricle. Cells of the SHF proliferate, migrate, and join with the cardiomyocytes of the FHF, resulting in the rightward looping of the cardiac tube, a process that culminates in a segmented structure and the formation of cardiac chambers.

expression of mesoendodermal markers such as *T* (*brachyury*) and *Eomes*. Subsequently, genes involved in mesoderm patterning and epithelial-mesenchymal transitions (EMTs), such as *Fgf4* and *Fgf8*, are expressed in the developing primitive streak. T and EOMES then induce the expression of MESP1 (Costello et al., 2011; David et al., 2011), which has been described as the "master regulator" of cardiac progenitor specification (Bondue et al., 2008).

MESP1 can drive cardiac differentiation via the DKK1-mediated inhibition of WNT signaling (David et al., 2008). It is now clear that canonical WNT/β-catenin signaling has a biphasic effect on cardiogenesis (Naito et al., 2006; Ueno et al., 2007). Downstream of MESP1, cardiogenesis relies on a complex web of interacting factors and genes, including *Tbx5*, *Nkx2-5*, *Mef2c*, *Gata4*, *Hand2*, *Myocd*, *Isl1*, and *Foxh1* (Bondue and Blanpain, 2010). Once cardiogenic mesoderm is specified, canonical WNT and NOTCH signaling regulate CPC maintenance and differentiation, respectively (Qyang et al., 2007; Kwon et al., 2009).

The cardiac mesoderm gives rise to the endocardium, the first heart field (FHF, which forms the atria, left ventricle, and the nodal conduction system), the secondary heart field (SHF, which forms the right ventricle, outflow tract, and part of the atria), and the proepicardial mesenchyme (Buckingham et al., 2005). The FHF differentiates at the cardiac crescent stage, whereas the SHF (marked by the expression of *Isl1*) remains in an undifferentiated progenitor state, due to inhibitory WNT signals from the midline, until incorporation into the heart (Kwon et al., 2007). Once the cardiac crescent is formed, it is exposed to BMP signaling from the underlying anterior ectoderm as well as to BMP, FGF, anticanonical WNT, and noncanonical WNT signaling from the overlying anterior endoderm (Solloway and Harvey, 2003). By E8.0, these cells form a primitive heart tube, consisting of an interior layer of endocardial cells and an exterior layer of myocardial cells. Once within the heart, FHF and SHF cells appear to proliferate in response to endocardial-derived signals, such as neuregulin1 (NRG1), which is driven by NOTCH signaling (Grego-Bessa et al., 2007), and epicardial signals, such as retinoic acid, that function via FGF signaling (Lavine et al., 2005). Further study of the possibility of maintaining and proliferating cardiac progenitors, along with the signaling that differentiates atrial from ventricular cells and decoupling this from signaling involved with morphological movement, is of great interest to the further development of reproducible de novo cardiomyocyte generation methodologies.

Cardiac Differentiation of hPSCs

The ability to differentiate hPSCs in a directed manner has progressed considerably in the past 10 years. The most reproducible and efficient strategies involve stage-specific activation and inhibition of different signaling pathways in defined culture conditions, recapitulating key steps in cardiac development

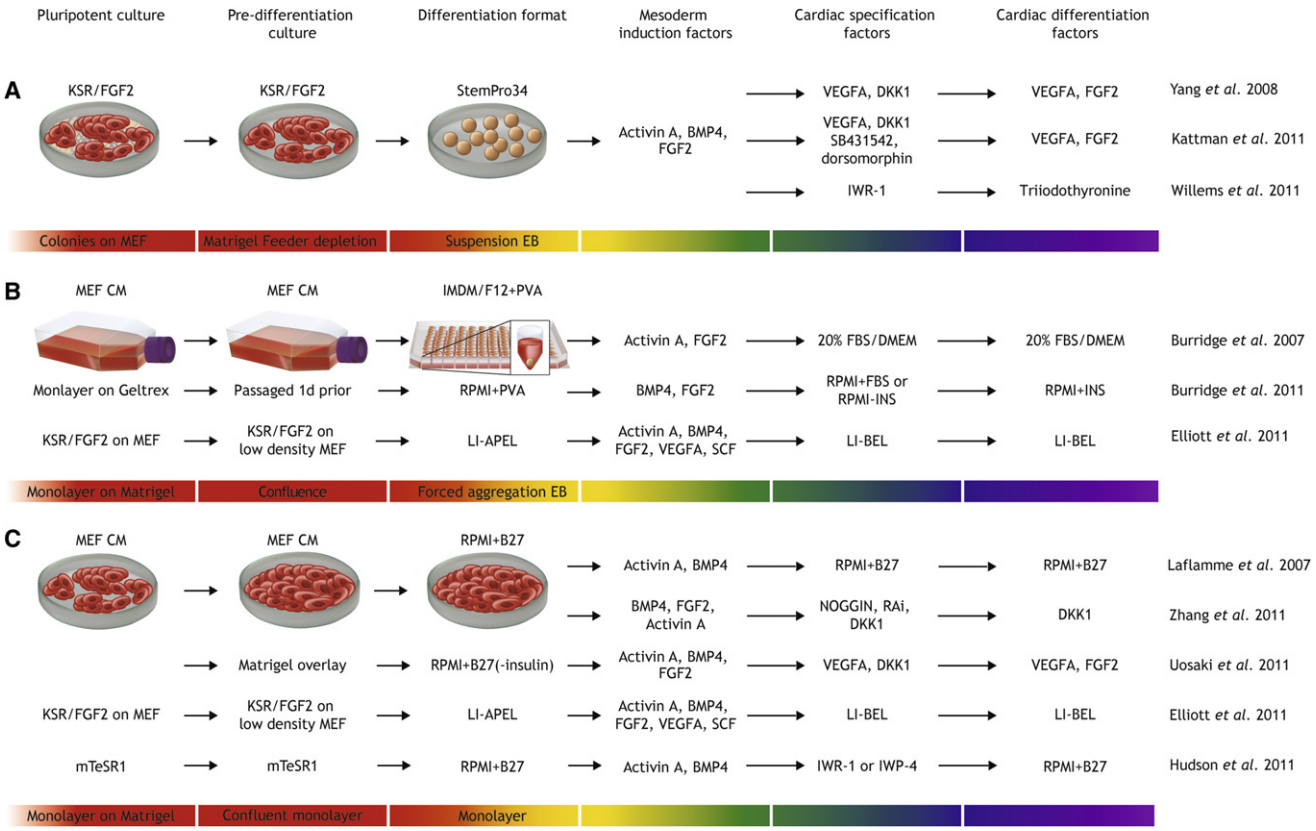

Figure 2. Methods for the Differentiation of Human Pluripotent Stem Cells

Three methods for differentiating hPSCs, highlighting commonalities at each of the six steps: pluripotent culture, predifferentiation culture, differentiation format, and treatment with mesoderm induction factors, cardiac specification factors, and cardiac differentiation factors. (A), Yang et al. (2008) suspension EBs in StemPro34; (B), Burridge et al. (2007) forced aggregation; (C), Laflamme et al. (2007) monolayer differentiation. Abbreviations: KSR, Knockout Serum Replacement; FGF2, fibroblast growth factor 2; StemPro34, proprietary medium from Invitrogen; BMP4, bone morphogenic protein 4; VEGFA, vascular endothelial growth factor A; DKK1, dickkopf homolog 1; SB431542, TGFB/activin/NODAL signaling inhibitor (ALK4,5,7); dorsomorphin, BMP signaling inhibitor (ALK2,3,6); IWR-1, WNT signaling inhibitor; MEF CM, mouse embryonic fibroblast conditioned hESC medium; IMDM/F12+PVA, IMDM/F12-based media supplemented with polyvinyl alcohol; RPMI, Roswell Park Memorial Insitute 1640 basal medium; FBS, fetal bovine serum; DMEM, basal media; RPMI+PVA, RPMI-based media supplemented with polyvinyl alcohol; RPMI-INS, RPMI-based media without insulin; B27, media supplement; NOGGIN, BMP signaling inhibitor; RAi, retinoic acid signaling inhibitor; LI-APEL, low insulin, Albucult, polyvinyl alcohol, essential lipids media; SCF, stem cell factor (KITLG); LI-BEL, low insulin, bovine serum albumin, essential lipids media; IWP-4, WNT signaling inhibitor.

in the early embryo. There is consensus that the cardiac differentiation process is very delicate, and the variability in each individual component of the cardiac differentiation strategy must be carefully optimized to reliably produce cardiomyocytes. One of the first directed differentiation methods involved hESCs cocultured with mouse visceral endoderm-like cells (END-2) (Mummery et al., 2003), which is relatively inefficient but has been shown to generate mostly (~85%) ventricular-like cardiomyocytes (Mummery et al., 2003). This protocol provided early insight into methods for improving differentiation efficiency, such as removing fetal bovine serum (FBS), adding L-ascorbic acid (Passier et al., 2005), and removing insulin between d0 and d4 (Freund et al., 2008). Two basic methods for the cardiac differentiation of hPSCs are now widely in use: the formation of embryoid bodies (EBs), and culturing hPSCs as a monolayer. Mature versions of each method rely on progressive sequential inductive environments using growth factors and/or small molecules. Figure 2 summarizes the principles of the reported strategies used for cardiomyocyte differentiation from hPSCs.

The EB methodology initially involved suspending hPSC colonies in media containing 20% FBS to form spherical aggregates (Kehat et al., 2001). This technique produces differentiated cell types of all three germ layers, including cardiomyocytes, when it is followed by adhesion at a later stage (~d4–d7). Although inefficient, nearly all cell lines (including hiPSCs) form some rhythmically contracting outgrowths in the 5%–15% range (Zwi et al., 2009). This protocol can be enhanced by adding BMP4 during d0–d4 (Takei et al., 2009), by the addition of WNT3A during d0–d2 (Tran et al., 2009), and via small molecule inhibition of WNT signaling by IWR-1 during d4–d6 (Ren et al., 2011). FBS- and insulin-free media can support EB cardiac differentiation, the efficiency of which can be enhanced by adding prostaglandin I2 and the MAP kinase inhibitor SB203580 (Xu et al., 2008), specifically between d4 and d6 (Gaur et al., 2010). Factors such as WNT3A (Bu et al., 2009), G-CSF (Shimoji et al., 2010), and L-ascorbic acid (Cao et al., 2011) can improve cardiac differentiation by enhancing CPC proliferation, whereas IGF1 and IGF2 can enhance hPSC-derived cardiomyocyte proliferation (McDevitt et al., 2005).

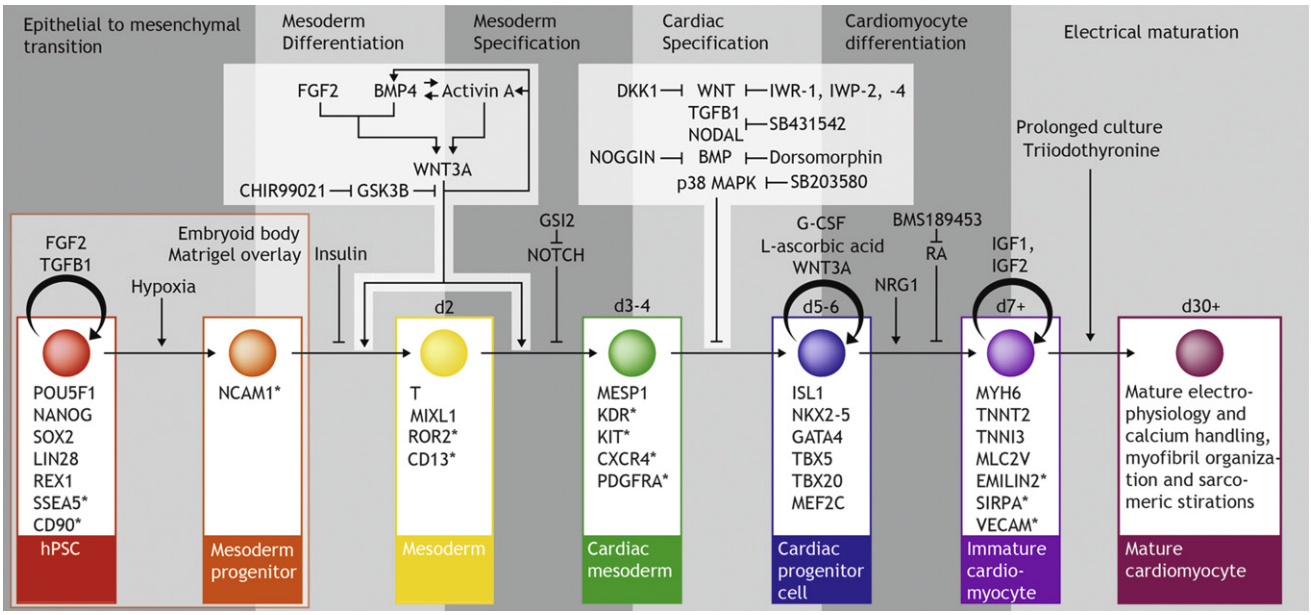

Figure 3. Schematic of Current Knowledge of Factors Involved in hPSC Cardiac Differentiation

Factors that influence the progression through each of the six major steps of hPSC cardiomyogenesis: epithelial to mesenchymal transition, mesoderm differentiation, mesoderm speciation, cardiac specification, cardiomyocyte differentiation, and electrical maturation. Data shown are derived from developmental biology models that have been directly assessed and proved functional in hPSC cardiac differentiation, along with knowledge gained directly from hPSC differentiation. Below are the markers associated with each of the seven cell types during differentiation; surface markers are marked with an asterisk.

A serum-free, EB-based suspension technique developed in the Keller Lab (Figure 2A) has proven highly effective for analyzing growth factor variables and timing during cardiac differentiation (Kattman et al., 2011; Yang et al., 2008). In this protocol, EBs are formed in the presence of a low level of BMP4 and treated with optimized levels of BMP4, fibroblast growth factor 2 (FGF2), and activin A between d1 and d4, VEGFA and DKK1 during d4 to d8, and VEGF and FGF2 from d8 onward. EBs are maintained under hypoxic (5% oxygen) conditions for 12 days. Using this method, approximately 70% of HES2 EBs spontaneously contract (Yang et al., 2008). Inhibition of TGFB/activin/NODAL and BMP signaling using the small molecules SB431542 and dorsomorphin during d3–d5 (Kattman et al., 2011) or the WNT inhibitor IWR-1 (d4 to 10) (Willems et al., 2011) has been shown to enhance this system. To overcome the limitation of EB size heterogeneity, a ''forced aggregation'' protocol using V- or U-shaped 96-well plates has been employed (Figure 2B). Initial versions of this system using activin A and FGF2 produced ~23% beating EBs from four different hESC lines (Burridge et al., 2007). Further developments of this system, using BMP4 and FGF2 as mesoderm inducers (Burridge et al., 2011) under hypoxia, increased differentiation efficiency to ~94% of EB beating in a wide range of hESC and hiPSC lines. Elliot and colleagues (Elliott et al., 2011) demonstrated a similarly high differentiation efficiency by employing a forced aggregation protocol that used BMP4, activin A, WNT3A, KITLG (SCF), and VEGFA for the first 3 days.

The suspension EB and forced aggregation methods are mature and commonly produce >70% cardiomyocytes, but these methods are technically complex and time consuming, which has led to the development of a monolayer-based method (Figure 2C) (Laflamme et al., 2007), where hPSCs are cultured to a high density, then treated with a high dose of activin A, followed by 4 days of treatment with BMP4; crucially, the media is not changed during these 4 days to allow the secreted factors to accumulate. Contracting cells can be seen at day 12, with a purity of approximately 30% cardiomyocytes. Improvements to this system, such as the addition of WNT3A at d0–d1 and DKK1 from d5 to d11, have been demonstrated (Paige et al., 2010). Further improvements have also been made using a Matrigel overlay 1 day prior to differentiation to enhance EMT, along with the removal of insulin and addition of FGF2 from d1 to d5 and DKK1 from d5 to d7 (Uosaki et al., 2011). As with the EB system, the use of the WNT signaling inhibitors IWR-1 or IWP-4 has proven effective, in this case when applied from d3 (Hudson et al., 2011).

One important aspect of these protocols is the required identification of markers to monitor the efficiency and progress of differentiation. Although assays that measure the number of beating EBs and expression of cardiac troponin T (TNNT2) have proven effective for optimizing differentiation efficiency, more rigorous definition of the growth factor regime by analyzing cardiac mesoderm populations, such as the KDR+/PDGFRA− population (Kattman et al., 2011) and a genetically modified *NKX2-5*-GFP cell line, to identify the CPC population (Elliott et al., 2011) has proven effective for optimizing the ratio of activin A and BMP4 during early differentiation. The further identification of specific markers expressed during cardiac differentiation is a high priority.

Collectively, these studies demonstrate that the importance of exposure of hPSCs to various growth factors at specific times and in specific doses is essential for directing differentiation from early mesendoderm via mesoderm toward a more specific cardiac fate (Figure 3). Data collected from experiments using

both the EB and monolayer methodologies show that four major signaling pathways are involved in early cardiac differentiation of hPSCs—BMP, TGFB/activin/NODAL, WNT, and FGF—with highly specific temporal windows for effectiveness. There is also significant evidence that synergistic relationships exist between growth factors such as BMP4 and FGF2 (Yu et al., 2011). As with cardiac development in vivo, it is now clear that inhibition of WNT as well as BMP and TGFB/activin/NODAL during the middifferentiation stage has a major role in hPSC cardiomyogenesis (Ren et al., 2011; Willems et al., 2011; Kattman et al., 2011; Zhang et al., 2011). Although less well studied, Notch inhibition using gamma-secretase inhibitor II from d0 to d8 has also been demonstrated to affect hPSC cardiac mesoderm induction (Jang et al., 2008), indicating possible involvement of a fifth signaling pathway.

The Developmental Status of hPSC-Derived Cardiomyocytes

The cardiomyocytes produced to date from hPSCs are largely immature and most analogous to fetal stages of development; these hPSC-derived cardiomyocytes exhibit automaticity (spontaneous contraction), fetal-type ion channel expression (Beqqali et al., 2006), fetal-type electrophysiological signals (Davis et al., 2011), fetal-type gene expression patterns, and fetal-type physical phenotypes (Cao et al., 2008). Conflicting data exist regarding the maturity of Ca^{2+} handling and sarcoplasmic reticulum status for hPSC-derived cardiomyocytes, although there is evidence that hPSC-derived cardiomyocytes demonstrate some mature properties (Itzhaki et al., 2011b). The lack of maturity of hPSC-derived cardiomyocytes may reduce the suitability of drug testing but may also have benefits in regards to regenerative medicine. For example, rodent fetal cardiomyocytes have been demonstrated to have enhanced cell survival over adult cardiomyocytes after engraftment into the rat heart (Reinecke et al., 1999), although the issue of automaticity still remains. Three major subtypes of hPSC-derived cardiomyocytes can be derived that have atrial-, ventricular-, or nodal-like phenotypes as determined by electrophysiological analysis of action potentials (APs). Common hPSC differentiation methodologies create a mixture of these cell types. An enriched population of nodal-like cells could potentially be used in the formation of a biological pacemaker, whereas ventricular types may be used for recovery from myocardial infarction. Zhu and colleagues demonstrated that GATA6-GFP can mark nodal-like cells (Zhu et al., 2010) and that inhibition of NRG1β/ERBB signaling can enhance the population of nodal-like cardiomyocytes. Zhang and colleagues have similarly demonstrated that retinoic acid can increase the proportion of atrial-like cardiomyocytes and that retinoic acid inhibition can increase the proportion of ventricular-like cells (Zhang et al., 2011).

Overcoming Interline Variability in Cardiac Differentiation of hiPSCs

The diversity in cardiac differentiation capacities between hESC lines has now been well established (Burridge et al., 2007; Kattman et al., 2011), and it is possible that hiPSCs may have more differentiation variability than hESCs. Many factors likely contribute to this line-to-line variation, including the conditions used to establish and maintain lines, the efficiency of reprogramming, the cell source used for reprogramming, and levels of expression of endogenous growth factors. Maintenance of high-quality hPSCs is essential for efficient and reproducible differentiation; the level of expression of pluripotency factors such as *POU5F1* (*OCT4*) and *NANOG* has been demonstrated to have a role in mesoderm induction (Yu et al., 2011). The progress toward a defined culture of hPSCs, although initially focused on eliminating feeder layers and animal serum products to allow future clinical translation, has resulted in higher levels of control over the pluripotent state, which in turn enhances the reproducibility of differentiation and reduces costs for a future large-scale culture. hESCs were first derived on a feeder layer of mouse embryonic fibroblasts (MEFs) in media containing FBS (Thomson et al., 1998). Substantial progress has since been made in controlling hPSC culture, through second-generation media containing the commercial product knockout serum replacement (KSR) and FGF2 (Amit et al., 2000). In addition, KSR/FGF2 media conditioned on MEFs have been shown to support hPSC pluripotency on a Matrigel layer (Xu et al., 2001). Progress in elucidating a role for high levels of FGF2 along with TGF-β1 to stimulate the PI3K, MEK/ERK, and SMAD2/3 pathways has resulted in a third-generation "defined" media in which KSR is replaced with BSA or human serum albumin (HSA). Despite some difficulties in implementation (Akopian et al., 2010), several formulas have achieved widespread use, including XVIVO10 supplemented with FGF2 and TGF-β1 (Li et al., 2005) and the commercially available mTeSR1 (Ludwig et al., 2006) and StemPro hESC (Wang et al., 2007). Most recently, Chen and colleagues achieved a breakthrough in hESC and hiPSC culture by formulating a fourth-generation, chemically defined medium (referred to as E8) that contains the growth factors FGF2 and TGF-β1 while lacking HSA and the antioxidant 2-mercaptoethanol (Chen et al., 2011). This medium, when used in combination with a recombinant truncated variant of vitronectin, has proved successful in deriving new hiPSC lines. Although progress toward a chemically defined culture of hPSCs has been slow and gradual, this simple and elegant solution has improved the reproducibility of pluripotent cell culture and overcomes a significant hurdle in the development of a reproducible cardiac differentiation system.

In addition to culture conditions, the considerable transcript heterogeneity within hiPSCs of a single cell line (Narsinh et al., 2011) and the hot spots of aberrant epigenomic reprogramming (Lister et al., 2011) likely contribute to variability in differentiation potential. Another source of variability for hiPSCs is cell type of origin (Martinez-Fernandez et al., 2011). It has been well documented that certain cell types, such as neural stem cells (Kim et al., 2009), can be more easily reprogrammed, but whether this tendency is due to a more malleable epigenetic state, the existing expression of a subset of pluripotency-associated genes, or the activity of epigenetic-state-modifying genes is still unclear.

The retention of epigenetic memory of cell origin (Kim et al., 2010b) was initially thought to be restricted to low passage (<15) hiPSCs (Polo et al., 2010), but recent data suggest that this effect is maintained in later passages as well (Ohi et al., 2011). This issue becomes more complex given that hPSCs progressively acquire epigenetic heterogeneity after prolonged culture, which affects subsequent differentiation (Tanasijevic et al., 2009), and that different passages of the same cell line have different cardiac potentials (Paige et al., 2010). Another

question that has not been addressed in human cells is whether the method used to induce pluripotency influences subsequent differentiation. It has been demonstrated in mouse iPSCs that *c-Myc*-independent (OSK) conditions favor cardiogenic potential of iPSCs (Martinez-Fernandez et al., 2010), and it has also been shown that hiPSCs generated under different conditions have differing cell cycles and DNA replication gene profiles (Chung et al., 2011). The effects of generating hiPSCs using defined media, matrices, and small-molecule epigenetic modifiers on subsequent differentiation have yet to be fully explored. Differences in levels of expression of endogenous signaling cytokines such as *NODAL*, *BMP4*, and *WNT3A* (Kattman et al., 2011; Paige et al., 2010) clearly contribute to the variability of cardiovascular development. Sustained TGFB/activin/NODAL, BMP, or WNT signaling beyond the cardiac mesoderm step can have profound inhibitory effects on the generation of contracting cardiomyocytes within the culture (Kattman et al., 2011). These differences are not necessarily related to reprogramming, as they are also observed between different hESC lines. While the underlying causes of these differences are not known, the development of quantitative assays to rapidly measure the levels of endogenous signaling would enable one to appropriately modify the induction protocols for each cell line.

Direct Reprogramming and Partial Reprogramming

It is well established that exogenous expression of *Myod* can convert murine fibroblasts directly to skeletal muscle (Davis et al., 1987). This capability was also demonstrated in a variety of other nonfibroblast cell types (Choi et al., 1990). This process is comparatively simple because *Myod* acts as a master regulator of skeletal muscle formation (Wang et al., 2003), whereas it would appear that no such single gene exists for the direct reprogramming of cardiomyocytes. It has been demonstrated that exogenous expression of *Gata4*, *Tbx5*, and *Baf60c* (a subunit of the Swi/Snf-like BAF chromatin remodeling complex) was sufficient to reprogram noncardiogenic mesoderm into beating cardiomyocytes (Takeuchi and Bruneau, 2009). In this case, *Gata4* and *Baf60c* induce *Nkx2-5* expression, which acts with *Gata4* to initiate the cardiac program, with *Tbx5* shown to be required for full cardiomyocyte differentiation. Surprisingly, none of the expected master regulators of cardiac differentiation, including *Mesp1*, *Nkx2-5*, and *Isl1,* were required for this reprogramming.

Work on reprogramming to pluripotency has provided new insight into direct reprogramming, combining high-expression retroviral vectors with the subtractive assessment strategy (Takahashi and Yamanaka, 2006). Implementing these advances, Ieda and colleagues successfully reprogrammed cardiac fibroblasts into cardiomyocyte-like cells (referred to as iCMs) via the exogenous expression of *Gata4*, *Mef2c*, and *Tbx5* (Ieda et al., 2010) (Figure 4). These investigators began with a selection of 14 key genes related to cardiac development, including transcription factors and epigenetic remodeling factors, and expressed them in cardiac fibroblasts isolated from neonatal hearts obtained from αMHC-GFP transgenic mice. Seven days following transduction, 1.7% of cells expressed GFP. By serial reduction of the 14 factors, it was found that the optimal combination of *Gata4*, *Mef2c*, and *Tbx5* resulted in GFP expression in ~20% of the cells. Approximately 6% of cells were positive for both GFP and TNNT2 (a marker of the sarcomere structure of cardiomyocytes), although the percentage of TNNT2+ cells increased over an additional 3 weeks of culture. Ieda and colleagues went on to demonstrate that this reprogramming was not achieved via an ISL1+ CPC or pluripotent intermediate, but was the result of gradual loss of fibroblast identity and progressive upregulation of cardiomyocyte-specific genes. iCMs were induced in vitro within 3 days, although the reprogramming factors were shown to be required for 2 weeks for stable reprogramming. The iCMs produced electrophysiological and gene expression profiles similar to those of fetal cardiomyocytes, although only 30% of the iCMs (approximately 6% of the total cell number) exhibited spontaneous contraction. The GMT cocktail was also shown to be able to reprogram adult tail-tip dermal fibroblasts to iCMs to a similar efficiency, albeit with lower expression of TNNT2 and no demonstration of spontaneous contraction or electrophysiological properties. Transplantation of neonatal cardiac fibroblasts into mouse hearts 1 day after reprogramming showed that reprogramming can be achieved in vivo.

However, the main drawbacks of direct reprogramming are the low efficiency rates (~1 in 20 fibroblasts are successfully reprogrammed) at present, the use of randomly integrating retroviruses (which preclude future clinical application), the experimental complexity (which will make the GMP production process challenging), and the potential for contamination by endogenous cardiomyocytes; most importantly, use of this methodology has not yet been independently verified or demonstrated in human cells to date. Reprogramming cardiac fibroblasts into cardiac cells based on isolation of GFP−/Thy1+ cardiac fibroblasts from the heart may not necessarily be stringent enough; fluorescence-activated cell sorting (FACS) does not remove 100% of cardiac cells, and the retroviruses may still transfect even nondividing cardiac cells. The relationship between the properties of iCMs and genuine cardiomyocytes has also not been fully established using more stringent criteria. Finally, definitive proof of this methodology lies in reprogramming skin fibroblasts (and various other cell types) into cardiac cells, as can be done reproducibly for the reprogramming of various adult somatic cell types to pluripotency (Yu et al., 2007; Takahashi et al., 2007).

Partial Reprogramming

An alternative method to generate differentiated cell types from fibroblasts is to create partially reprogrammed cells that can be differentiated to the desired cell type. The potential advantage of this approach over first establishing iPSCs and then differentiating them into particular cell lineages is one of speed. This approach has been used successfully by Efe and colleagues, who showed that murine fibroblasts can be reprogrammed to cardiomyocytes using the established OSK combination (with or without *c-Myc*) in 11–12 days (Efe et al., 2011) (Figure 4). Using Nebulette-LacZ (a cardiac-muscle-specific marker) in transgenic MEFs, they carried out reprogramming under tissue culture conditions designed for cardiac differentiation rather than iPSC generation. To prevent the cells from reaching a pluripotent state, an inhibitor of Janus kinase/signal transducer and activator of transcription (JAK/STAT) was included in cultures. Cardiomyocytes were efficiently

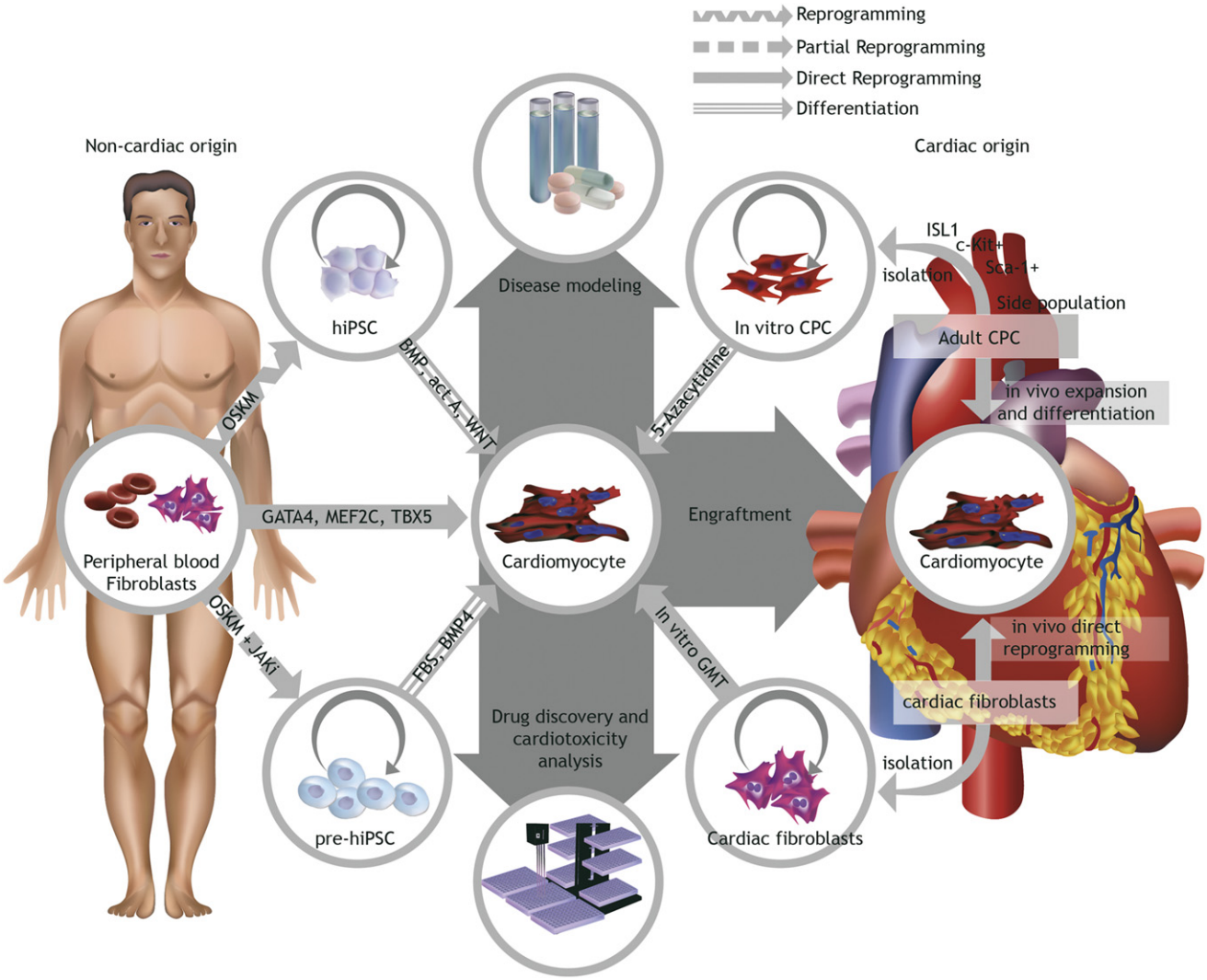

Figure 4. Schematic of De Novo Cardiomyocyte Generation and Applications
Demonstrating peripheral blood and skin fibroblasts as a cell source, hiPSC derivation and differentiation, direct reprogramming using GMT, and partial reprogramming using OSKM and a full reprogramming inhibitor. Applications in disease modeling, engraftment into the heart, drug discovery, and cardiotoxicity analysis are shown. Adult heart sources of cardiomyocytes from cardiac progenitor cells and application for in vivo expansion and differentiation are also shown, as is direct reprogramming of adult cardiac fibroblasts in vivo. Abbreviations: OSKM, *Oct4*, *Sox2*, *Klf4*, *c-Myc*; JAKi, JAK/STAT inhibitor; GMT, *Gata4*, *Mef2c*, *Tbx5*; CPC, cardiac progenitor cell.

produced and demonstrated typical gene expression profiles and calcium transients. A similar partial reprogramming protocol has been used by the same lab to generate neural progenitors (Kim et al., 2011). This technique still needs to be demonstrated in human cells, wherein an inhibitor suitable for replacing the pluripotency prevention function of JAK inhibitor would need to be used due to the differences in mechanisms that control murine and human pluripotency.

Both the direct reprogramming and partial reprogramming approaches offer exciting prospects for the future. Both methods may be entirely suitable to provide the small numbers of cells required for individualized drug screening and disease modeling. The direct reprogramming method could potentially be applied in vivo and would avoid the danger of residual pluripotent cells, thereby eliminating the risk of teratoma formation. However, direct in vivo reprogramming could also lead to ectopic myocardium in scar tissue, which is a known risk factor for arrhythmias after infarction in humans and requires electrical ablation by a cardiac elecrophysiologist. Another major concern is what the in vivo consequence would be for cells that do not receive the required dose of the GMT cocktail and are only partially reprogrammed to iCMs. Finally, in both systems, the problem of viral genomic integration and genomic mutations due to the use of retroviruses, along with issues of epigenetic status and scalability, must be overcome.

Cardiomyocyte Purification

Despite considerable progress in improving the efficiency of cardiac differentiation, the isolation of cardiomyocytes or the removal of unwanted cell populations may be required.

Traditional methods for the purification of cardiomyocytes that involve density gradient centrifugation (Xu et al., 2002) are unsuitable for large-scale practice and routinely result in only a 5- to 10-fold enrichment in cardiomyocyte populations (Murry and Keller, 2008). Genetic selection of cells based on the expression of a selectable marker driven by a lineage-restricted promoter such as NKX2-5 (Elliott et al., 2011), MYH6 (Anderson et al., 2007), MLC2V (Huber et al., 2007), and ISL1 (Bu et al., 2009) offers the possibility of isolating myocardial precursors at high purity; when this process is coupled with antibiotic selection, it is possible to generate cells at greater than 99% purity. Indeed, it has been demonstrated that an MYH6-blastocidin hiPSC line can be differentiated using simple suspension culture and 10% FBS in DMEM to produce hiPSC-derived cardiomyocytes in commercial quantities (Ma et al., 2011). The principal drawback of genetic selection is the necessity (at present) of inserting a selection cassette into the host genome, which may increase the risk of tumorigenesis and is therefore unsuitable for clinical practice.

Antibodies to cell surface markers have the advantage of not requiring genetic modification of stem cell populations, and therefore may be applicable to all hPSC lines. FACS has the ability to analyze multiple surface markers simultaneously, and it has been used to isolate cardiac progenitor populations based on the expression of the receptor tyrosine kinases KDR (FLK1/VEGFR2) and PDGFRA (Kattman et al., 2011). Because this population also contains endothelial and smooth muscle populations, the differentiated progeny represents a mixture of cardiomyocyte and vascular lineages. The recent identification of markers expressed specifically on cardiomyocytes, including EMILIN2 (Van Hoof et al., 2010), SIRPA (Dubois et al., 2011; Elliott et al., 2011), and VCAM (Elliott et al., 2011; Uosaki et al., 2011), has made it possible to isolate highly enriched populations of these cells from hESCs or hiPSCs by FACS or magnetic bead sorting.

Another nongenetic method for isolating hPSC-derived cardiomyocytes is based on the use of the mitochondrial dye tetramethylrhodamine methyl ester perchlorate (TMRM) (Hattori et al., 2010). Because this dye only functions in cardiomyocytes with high mitochondrial density, it does not detect the most immature cells that develop in the cultures (Dubois et al., 2011). Collectively, these recent discoveries have provided tools and reagents for the generation of highly enriched populations of hPSC-derived cardiomyocytes.

Future Directions and Challenges

The field is advancing quickly and we are already witnessing early applications of hPSCs in modeling human disease, understanding human biology, and developing platforms for drug discovery and predictive toxicology. Challenges remain before these applications can be widely used to develop regenerative medicine strategies for treating cardiovascular disease. The following section highlights some of these challenges.

Currently, monolayer differentiation protocols using hESCs can produce 2×10^8 enriched cardiomyocytes per T225 flask (J.D.G., unpublished data). Greater scalability could be achieved using multilayer tissue culture flasks or culture on suspended microcarriers. The EB methodology can also be adapted to high-throughput using flasks with rotating paddles (Amit et al., 2011), or by using a simple suspension culture to produce hiPSC-derived cardiomyocytes in commercial quantities (Ma et al., 2011). The elimination of growth factors from the monolayer protocol will permit a substantial reduction in costs, and along with the application of other low-molecular-weight compounds (Willems et al., 2009), will continue to improve scalability.

Although development toward maturity in the electrophysiological properties of hPSC-cardiomyocytes has been demonstrated over time in culture, better strategies for full development of mature intracellular Ca^{2+} handling machinery and ion channels within cells in vitro must be developed. Techniques that can induce maturation include the use of small molecules such as triiodothyronine binding thyroid hormone receptors (Lee et al., 2010), overexpression or downregulation of specific genes (Yamanaka et al., 2008), and extrinsic cues from other noncardiac cells, including endothelial cells (Kim et al., 2010a). Exercise via mechanical force (Tulloch et al., 2011) (which will likely be required for tissue constructs) has also been demonstrated to enhance maturation. Other important considerations, such classifying the type of cardiomyocyte (ventricular, atrial, or nodal) based on AP (Ma et al., 2011) and selecting relevant populations (either through development of modified differentiation protocols specific for one cell type [Zhu et al., 2010] or via selection based on electrophysiology) will need to be addressed. Indeed, the timing of when subtype fate specification occurs in development, how it is regulated, and by what signals is still largely unknown.

Finally, issues surrounding donor cell death (Nguyen et al., 2011), tumorigenesis (Cao et al., 2009; Lee et al., 2009), and prevention of immune rejection (Pearl et al., 2011; Swijnenburg et al., 2008) remain to be addressed. When engrafted, the new cardiomyocytes must both induce vascularization to keep the graft alive as well as electrically couple with the existing cardiomyocytes and function in synchronization with the existing heart muscle, or risk generating potentially fatal arrhythmias (Boudoulas and Hatzopoulos, 2009). In addition, pluripotent cells remaining in the population might be tumorigenic, and the ability to detect the presence of these cells with suitable markers (Ramirez et al., 2011), such as the recently discovered SSEA5 (Tang et al., 2011), will be crucial. To date, engraftment of hESC-derived cardiomyocytes in a single-cell format has yielded similar engraftment results to those from other cell types, with only a 5%–10% improvement in LVEF (Cao et al., 2008; van Laake et al., 2007), even with the use of ''prosurvival cocktails'' (Laflamme et al., 2007). Optimal engraftment may require applying cells as a 3D engineered tissue. For the successful use of these engineered tissues, the cardiomyocytes are supplemented with endothelial cells and fibroblasts, as insufficient graft vascularization can result in poor graft survival (Caspi et al., 2007). Scaffold-free hESC-derived cardiac patches have been demonstrated to be successful, along with synthetic polymers, hydrogels, and natural polymers such as fibrin and type I collagen (Tulloch et al., 2011). All work thus far with hPSC-derived cardiomyocytes has been performed in animal models (mostly in rodents), and it may be difficult for human cardiomyocytes to keep pace with the ~450 beats/min required in the rodent heart (Murry and Keller, 2008). This high beating rate may also mask arrhythmias. The guinea pig model has a more suitable heart rate (230–380 beats/min), but at present

immunocompromised strains suitable for cardiac engraftment do not exist, requiring the use of immunosuppressants such as cyclosporin A. Finally, assessment of human-sized cardiac patches in larger animals such as pigs will be required before transfer to the clinic is feasible.

In conclusion, our ability to generate cardiomyocytes de novo has progressed rapidly in the past 5 years, and an increasing number of potential sources of cells have become viable. The three major applications of these cardiomyocytes (in regenerative medicine, drug testing, and disease modeling) each have their own specific requirements for number of cells, speed of derivation, characterization, and similarity to adult cardiomyocytes. It is likely that a variety of approaches for making cardiomyocytes will be used in the future, depending on the specific parameters set by the application. In addition to the plethora of differentiation methods that have been devised, the core pathways controlling cardiomyocyte differentiation (BMP, TGFB/activin/NODAL, and WNT) have been identified. There are complex questions regarding the application of newly generated cardiomyocytes still to be answered, including the best methods for cell delivery and how to ensure a normal physiological response of the cells. In addition, many technical issues remain unresolved. For example, what if cells successfully engraft and electrically couple, yet do not respond normally to endogenous stimuli? In addition, the choice of hESCs versus hiPSCs as the starting material depends on the ultimate use for which the cells are intended; where ethical considerations are preeminent, hiPSCs may be the less controversial source. Certainly, for applications in genetic disease modeling or personalized therapies, hiPSCs are the obvious choice. By contrast, the extensive characterization and epigenetic stability of hESCs may make them more suitable for the large-scale generation of allogeneic cells for regenerative medicine. Such questions should not obscure the exciting prospect that, for the first time, it is possible to envisage overcoming the significant hurdles that have blocked the path to successful clinical application of de novo generated cardiomyocytes.

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