REVIEW ARTICLE
Cardiomyocyte differentiation of pluripotent stem cells and their use as cardiac disease models
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More than 10 years after their first isolation, human embryonic stem cells are finally 'coming of age' in research and biotechnology applications as protocols for their differentiation and undifferentiated expansion in culture become robust and scalable, and validated commercial reagents become available. Production of human cardiomyocytes is now feasible on a daily basis for many laboratories with tissue culture expertise. An additional recent surge of interest resulting from the first production of human iPSCs (induced pluripotent stem cells) from somatic cells of patients now makes these technologies of even greater importance since it is likely that (genetic) cardiac disease phenotypes can be captured in the cardiac derivatives of these cells. Although cell therapy based on replacing cardiomyocytes lost or dysfunctional owing to cardiac disease are probably as far away as ever, biotechnology and pharmaceutical applications in safety pharmacology and drug discovery will probably impact this clinical area in the very near future. In the present paper, we review the cutting edge of this exciting area of translational research.

Key words: cardiac disease model, cardiac differentiation, cardiomyocyte, drug discovery, pluripotent stem cell, regenerative medicine.

EMBRYONIC STEM CELLS AND CARDIOMYOCYTE DEVELOPMENT

Embryonic stem cells are derived from early embryos before they implant in the uterus. They are pluripotent, that is they have the unique ability to differentiate into derivatives of the three germ layers of the body while retaining the capacity to self-renew indefinitely. This makes them important scientific tools in biomedical and cellular research since they can, in principal, form ~220 different cell types present in the adult body and do this repeatedly over many years.

After the isolation of the first mESC (mouse embryonic stem cells) in 1981 [1], the potential of these cells in regenerative medicine became evident. Researchers began attempts to isolate hESC (human embryonic stem cells) from surplus embryos used in assisted reproduction procedures (in vitro fertilization). However, owing to differences in growth requirements, the limited availability of surplus human embryos and the nature of hESC themselves, it took another 17 years before the first hESC lines were isolated [2]. Aside from potential clinical applications in cell therapy for the heart, interest in deriving cardiomyocytes from hESC has been driven by curiosity in the differences in the physiology, metabolism and expression of protein markers in the mouse and human heart, and the question of whether these differences would be reflected in cardiac derivatives of stem cells in culture in the absence of a true heart structure. The most obvious difference between the mouse (or any small rodent) and human heart is the rate of beating. The mouse heart beats at 500 beats/min (b/pm) whereas the human heart beats at 70 b/min. Another difference is the expression of MHC (myosin heavy chain) isoforms. βMHC is the isoform predominantly expressed in fetal mouse hearts, whereas αMHC is predominant in the adult; the reverse is seen in humans [3]. Despite these differences, mice are often used as models in cardiac research because it is relatively easy to introduce specific mutations or targeted deletions in mice through mESC. Some of these mutations result in cardiac developmental defects or adult disease, providing models that allow molecular and cellular dissection of the underlying mechanisms causing the abnormality. For studies on isolated cardiomyocytes though, stem cells can provide human alternatives as well as disease models, particularly now that methods for targeting specific genes in hESC have been developed [4–11] and cardiomyocytes can be derived from disease-bearing human iPSC (induced pluripotent stem cells). iPSC are a new kind of pluripotent stem cell that can be generated by reprogramming somatic cells directly rather than using embryos [12,13]. This is particularly attractive since it is difficult to obtain adult or even fetal primary human cardiomyocytes [14] and they cannot be kept in culture for more than a few hours or days.

In the present review, we describe the current state of hESC and human iPSC research with respect to their differentiation into cardiomyocytes and contribution to understanding normal and aberrant cardiomyocyte biology. In addition, we briefly consider the hurdles of using these cells for cell replacement therapy for the heart, but refer the reader to specialized reviews on this subject for more detailed discussion [15,16].

Abbreviations used: b/pm, beats/min; bFGF, basic fibroblast growth factor; BMC, bone marrow-derived cells; BMP, bone morphogenetic protein; CM, cardiomyocyte; EB, embryoid body; ECG, electrocardiogram; END-2, visceral endoderm-like; EpISC, epiblast stem cells; FGF, fibroblast growth factor; Flk1, foetal liver kinase 1; HCM, hypertrophic cardiomyopathy; hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; LIF, leukemia inhibitory factor; LQTS, long-QT syndrome; mESC, mouse embryonic stem cell; MHC, myosin heavy chain; VEGF, vascular endothelial cells growth factor.

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EMBRYONIC STEM CELLS

Embryonic stem cells are derived from the inner-cell mass of blastocyst stage embryos. In humans this is on day 5 post-fertilization [2,17], and in mice on day 3.5 [1]. Most of our prior knowledge on the characteristics of these cells is derived from studies in the 1960s on embryonal carcinoma cells, the tumorigenic counterpart that is derived as either spontaneous or induced testis tumours in mice [18] and from spontaneous germ cell tumours in humans [19]. Although expression of the principle pluripotency transcription factors, like OCT4, SOX2 and NANOG, is conserved between mESCs and hESCs, these cells differ in their expression of surface markers and culture requirements [18]. This has recently been attributed to mESC being in a more naive state than hESCs; mouse EpiSC (epiblast stem cells), isolated from mouse embryos later in development, appeared more like hESC than mESC [20]. For example, mESC require LIF (leukaemia inhibitory factor) in their culture medium to remain undifferentiated and its removal will cause spontaneous differentiation. hESC and mouse EpiSC do not require LIF, but need bFGF (basic fibroblast growth factor) and activin signalling to remain undifferentiated in culture [21,22]. Typically though, hESC and mESC are maintained undifferentiated by co-culture on fibroblast ‘feeder’ cells where they retain the ability to self-renew indefinitely. Removing the cells from feeders and/or growing them in suspension as aggregates [called EBs (embryoid bodies)] causes differentiation to derivatives of the three germ layers: endoderm (pancreas, liver), ectoderm (neurons) and mesoderm (cardiomyocytes, vascular endothelial cells, muscle and blood).

Considerable research effort has gone into simplifying these complex growth and differentiation protocols. In 2001, Xu et al. [23] showed that hESC could be maintained in an undifferentiated state in the absence of feeder cells on MatrigelTM (an extracellular matrix derived from mouse sarcoma; BD Biosciences) or on natural laminin for at least 130 population doublings, although feeder-cell-conditioned medium was required. Human recombinant extracellular matrices, such as laminin-511 [24] or vitronectin [25], in combination with appropriate culture medium, have recently also been shown to maintain hESC in an undifferentiated state, and Villa-Diaz et al. [26] have derived a synthetic polymer which reportedly sustains long-term expansion.

Elimination of feeder cell requirements is not the only improvement that has been made to hESC culture conditions. The first fully defined culture medium was described in 2006 [27] and later marketed as mTeSR®-1 (STEMCELL Technologies). This was shown to be effective in combination with a matrix containing either a mixture of human collagen IV, fibronectin, laminin and vitronectin [27] or Matrigel™. Further development has led to a xenobiotech-free version called TeSR™-2 (STEMCELL Technologies) in which BSA has been replaced by human serum albumin, making it in principle clinically compliant. Various groups have now described defined culture conditions using different basal media and supplements [27–31], although, for most practical purposes, the commercial media are highly reliable for most cell lines used in most laboratories. Furthermore, the addition of a selective inhibitor of p160-ROCK (Rho-associated coiled-coil kinase) to the culture medium has been found to increase the survival of hESC during cryopreservation [32–34], after dissociation [35], passing under serum-free conditions [36,37] and during various differentiation procedures [38]. This has been very helpful when cloning from a single cell, often required during gene targeting or the generation of transgenic hESC lines.

In addition, improvement of hESC culture conditions continues with regards to optimizing methods that allow it to be scaled up while maintaining of karyotypic stability either through enzymatic passaging [39] or suspension cultures [40,41]. Olmer et al. [40] recently extended the suspension culture method to hiPSC. While their procedure is not designed for continuous maintenance, they were successful in scaling up cultures by several orders of magnitude, as would be required for future clinical application as well as biotechnology.

EMBRYONIC STEM CELLS AND iPSC

2006 was a landmark in the history of stem cell biology. The introduction of just four transcription factors into mouse somatic cells turned out to ‘reprogramme’ cells into a pluripotent, embryonic stem cell-like state [42] (Figure 1). A year later, the procedure was repeated with human cells [43,44]. Apart from circumventing the ethical issues surrounding hESC derivation from human embryos, the procedure presented opportunities for deriving pluripotent stem cell lines from specific living individuals without the use of SCNT (somatic cell nuclear transfer; or ‘therapeutic cloning’) [45]. iPSC appear, in general, to be very similar to ESC in terms of morphology, global gene and protein expression and differentiation potential, but epigenetic variations between iPSC and ESC have been described [46–48].

The first iPSC lines were generated from fibroblasts using γ-retroviral vectors to introduce OCT4, SOX2, KLF4 (Kruppel-like factor 4) and c-myc [42,43] or OCT4, SOX2, NANOG and LIN28 [44]. Over a period of several weeks, these transcription factors induced the expression of endogenous pluripotency genes such as OCT4 and SOX2 and then became silenced once the endogenous genes had taken over control of pluripotency. Since the first description of reprogramming by ectopic gene expression in this way, reprogramming of many other somatic cell types using different methods of transcription-factor introduction have been published in quick succession. There are now reports describing mouse iPSC from almost every cell type in the mouse, although creating human iPSC from the equivalent human cell sources is still more difficult. Nevertheless, in addition to adult and fetal dermal fibroblasts, iPSC have been created from multiple human tissues, including lung fibroblasts, fibroblast-like synoviocytes [43], keratinocytes [49], cord blood [50,51], peripheral blood [52,53], mesenchymal stromal cells [54] and more recently oral mucosa fibroblasts [55] and T-cells [56,57]. The use of retroviral vectors to create iPSC has a number of limitations and therefore much effort has gone into finding alternatives. Among these are lentiviral vectors that, in contrast with retroviruses, can infect non-dividing as well as dividing cells and also have a larger insert capacity than retroviral vectors. In principle this allows all of the reprogramming factors to be included in a single vector rather than requiring each to be incorporated into a separate vector. Secondly, other viral vectors which do not integrate into the genome, such as adenoviral vectors, also have been used, although they reprogramme cells with extremely low efficiency [58,59]. Thirdly, viral-free methods are under development. These include the use of transposons [60], and protein- [61] and mRNA- [62] introduction methods. The first two methods also have low efficiency, but the removal or absence of the transgenes has a number of advantages and more efforts into transgene-free or removable transgene methods are ongoing. The mRNA approach, however, has reported efficiencies similar to viral integration so will probably become a preferred method in the future. A fourth area of research investigates the use of small molecules to improve or replace the use of transgenes in reprogramming. For example, the addition of VPA (valproic acid) has been found to improve the overall efficiency of reprogramming [63].

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Differentiation of pluripotent stem cell use as cardiac disease models

There are now multiple sources of pluripotent cells in humans. The most well known are embryonic stem cells isolated from the inner-cell mass of human blastocyst stage embryos on day 5 after fertilization. Among the newest are iPSC generated by introducing key pluripotency-associated transcription factors into somatic cells. In mice there are additionally EpiSC that are isolated from later stages (post-implantation) of embryo development which are similar to hESC in terms of conditions supporting maintenance, growth factor responses and gene expression, although not identical. These three pluripotent cell types are dependent on activin/TGF(β) signalling for self-renewal, but have the ability to differentiate into all three germ layers when these growth factors are removed. Upon directed differentiation towards cardiomyocytes, pluripotent cells will first pass through a progenitor stage which can be identified by the expression of specific transcription factors, for example Nkx2.5. The differentiation can continue further and produce various types of vascular cells.

iPSC have not only expanded the ethical acceptability of pluripotent stem cell research but have also allowed the derivation of cell lines from individual patients, facilitating the creation of genetic disease models in culture [64−66]. Moreover, in the context of clinical applications in cell therapy, once alternatives to viral integration methods for generating iPSC are fully developed, it is possible that tissue matching to the recipient may no longer be an issue; there will still be the same kinds of hESC-associated risks, such as teratoma formation from any residual undifferentiated cells in the transplanted cell populations. These risks aside, the costs of cell production for transplantation would nevertheless need to be greatly reduced for any individualized treatment.

hESCs AND CARDIAC DIFFERENTIATION

Although spontaneous differentiation of ESCs is easily achieved simply by omitting differentiation-repressing factors or growing
The differentiation of pluripotent stem cell to cardiomyocytes is sequential with steps being distinguished by the expression of various (combinations of) transcription factors. There are a number of different differentiation methods, but a specific set of exogenous factors are still needed to force pluripotent stem cells down the cardiac lineage. Although all the steps in the process are not clear, the time of introduction of these factors is very important. In general, initial introduction of BMP4, activin A and bFGF will start the directed differentiation and later introduction of a Wnt signal inhibitor (Dkk1) and VEGF will help to push the cells further to form cardiomyocytes. cTnT, cardiac troponin T; Dkk1, dickkopf homolog 1; FoxC1, forkhead box C1; Gata-4, Gata binding protein 4; MESP1, mesoderm posterior 1 homolog; MHC, major histocompatibility complex; MLC, myosin light chain; Sox2, SRY (sex determining region Y)-box 2.

Ongoing improvements are being made to these methods, often involving the introduction of different growth factors or small molecules at various phases in the differentiation process. In addition, many groups are looking into methods to produce more homogenous cardiac cell populations (i.e. specific atrial-ventricular- and conduction system-type cell populations rather than mixtures) whereas others are addressing the issue of cell maturity. Cardiac cells derived from stem cells generally show immature phenotypes. Recently, Otsuji et al. [87] published a method for inducing maturation involving the replating of cardiomyocytes, derived using the END-2 co-culture method, on to fresh END-2 cells followed by a short three-dimensional culturing step. These cardiomyocytes could be maintained for 1 year, during which time they were found to become more mature in terms of their electrophysiological properties and to contain an increased number of pacemaker cells [87]. Alternatively, allowing the cells to undergo cyclic stretch, or forcing alignment using tissue engineering approaches [88], may enhance maturity.

**PURIFICATION OF hESC DERIVED CARDIOMYOCYTES**

The use of embryonic stem cell derivatives in research, as well as possible clinical therapies, is plagued with many problems including insufficient purity. The inadvertent introduction of any undifferentiated ESC into host tissue will result in teratomas, benign tumours containing derivatives of all three germ layers. In addition, many differentiation techniques produce heterogeneous cell populations with cardiomyocyte numbers varying from 1% to ∼50% of the total cells. In addition, in the END-2 co-culture system, ∼85% of the cardiomyocytes produced are ventricular [84], whereas other EB-based methods produce a mixture of cardiac atrial and ventricular cell types. Probably the most efficient cardiomyocyte differentiation protocols at the present time are based on adaption of undifferentiated hESC to feeder-dependent enzymatic passage in KOSR (knockout serum replacement) followed by spin EBs containing ∼3000 cells and the addition of BMP4 and activin A at concentrations in the range of 20 ng/ml [75]. Re-plating of these EBs after 7 days in regular culture medium can yield up to 50% cardiomyocytes. In attempts to enhance the differentiation of hESC to particular cardiac subtypes, Zhu et al. [89] found that inhibition of the NRG (neuregulin)-1/β/ErbB signalling pathway increased the proportion of nodal-like cells, whereas its activation produced the reverse effect.

Although many researchers are working on improving the efficiencies of differentiation and producing more homogenous populations of cells, others are investigating how best to purify...
the mixture of cells and select the required cell type. There are a number of different approaches being tested to select cardiomyocytes from mixed cell populations. In some cases, fluorescent reporters have been attached to cardiomyocyte-associated promoters and used to produce transgenic reporter lines [90–92]. The yield of cardiomyocytes after selection can be >90%, but requires genetic manipulation that has not been successful in all hESC lines [90] and might not be feasible for routine use in human iPSC. Another approach uses Percoll gradient centrifugation to isolate the cardiomyocytes physically, on the basis of their larger size compared with most other cells [93]. A third method selects cardiomyocyte progenitors using the cell-surface protein Flk1 [fetal liver kinase 1; also called KDR (kinase insert domain-containing receptor) or VEGF receptor] [77]. However, these cells are also progenitors for endothelial and smooth muscle cells making it possible that mixed populations of cells can still result. Furthermore, Flk1 is expressed on undifferentiated cells of some lines. A fourth approach was recently reported by Hattori et al. [94]. They used a non-toxic dye TMRE (tetramethylrhodamine methyl ester perchlorate) that reversibly labelled the mitochondria. Labelling a mixed culture of cells produced three fractions, a high fluorescence fraction with >99% cardiomyocytes, an intermediate fraction with other viable cells and a low fraction which contained dead or blood cells. The ease and reversibility of this method may prove extremely useful in the future, although at present it only works on cardiomyocytes maintained for long periods in culture (>50 days).

**POTENTIAL USES OF hESC-DERIVED CARDIOMYOCYTES**

There are a number of uses envisaged for hESC-derived cardiomyocytes, both as research tools and for clinical applications. These include drug screening and pharmacosafety analysis, disease modelling and gene targeting, and regenerative medicine.

**In vitro drug screening**

Many steps are required before a drug is approved for clinical use and one of these is evidence for low toxic risk on the heart. Drugs may be recalled if significant side effects occur and the heart is one of the most sensitive organs for this. Even after numerous tests in animals, these side effects may remain undetected [95]. This may not be surprising since the difference between animal and human physiology is significant, as mentioned earlier, and the way ion transport is handled differs between species. There is an increasing need for human models of healthy as well as diseased hearts. Braam et al. [95] recently showed that hESC-derived cardiomyocytes respond to specific drugs in much the same way that the human heart responds, as assessed using readouts from microelectrode arrays. Furthermore, the ability of human iPSC to form various cardiac lineages may make them good candidates for some of the drug screening presently carried out using animals.

**Gene targeting in hESC to create reporter lines and disease models**

While there are now multiple descriptions of genetic modification of hESC in the literature, they have been considerably more challenging to modify than mESC. Substantial progress over the last several years has led to multiple cell lines becoming available that have undergone targeted mutation. Genetic modifications can now be introduced through non-homologous, homologous, site-specific or transpositional recombination. Non-homologous recombination is a straightforward and easy method of introducing exogenous DNA but has an extremely low efficiency. In classical homologous recombination, exogenous DNA is introduced in the genome through the natural potential of cells to incorporate identical or similar DNA sequences. The efficiency of this process varies between species and cells types. The efficiency of homologous recombination has also been found to be extremely low. Among the hurdles that have confounded this technology are the difficulty in cloning hESC from single cells [96] and the low efficiency of DNA introduction using conditions that work well with mESC, for example through electroporation [97]. The use of zinc-finger nucleases in homologous recombination has been reported to increase the efficiency significantly. Zinc-finger nucleases introduce double strand breaks at specific sites in the genome using novel DNA binding proteins. Zinc-finger nucleases have been used in targeting a number of human genes including VEGFA (vascular endothelial growth factor A), HoxB13 (homeobox B13) and CFTR (cystic fibrosis transmembrane conductance regulator) [98]. They have been used in combination with defective lentivirus, electroporation and nucleofection in hESC [9–11].

Site-specific recombination allows for more controlled introduction of exogenous DNA sequences. Two types of site-specific recombination are Cre/loxP and phiC31. In the Cre/loxP system a pair of recognition sequences promotes a reciprocal recombination reaction, whereas phiC31 is an irreversible recombination using bacteriophages that attach to attP recognition sites in the genome. Although Cre/loxP has been successfully used with hESC [99], to date there are no reports of genetic modification of hESC using phiC31 [100]. Another technique uses transposons to modify hESC, employing three different systems, Sleeping Beauty [101], PiggyBac [102] and Mu [103]. All of these systems allow the integration of non-targeted DNA along chromosomes. They have different efficiencies, cargo sizes and excisability, but all offer an alternative to viral vector integration of DNA into the genome (reviewed in [104]).

The modification of hESC by introduction of ectopic reporters or by targeting developmentally important loci is becoming a very useful tool in understanding the differentiation of cardiomyocytes. It is of significant interest in the study of heart disease, since it represents an opportunity to introduce different mutations against a single genetic background if using just one hESC line. In this respect, genetic modification of hESC lines can compliment studies with human iPSC since deriving lines from different individual patients automatically implies a different genetic background.

**Which cardiac diseases will be useful to model using human pluripotent stem cells?**

The most important task of the heart is to pump blood throughout the body. To achieve this, highly orchestrated temporal and spatial activities consisting of electrical activation and subsequent mechanical contraction of cardiomyocytes are essential. Disturbances in these processes may lead to serious or even life-threatening conditions. Over the past two decades, our knowledge of the genetic basis of cardiac diseases has increased enormously. In that same period, many different animal models have been generated for studying cardiac diseases and, although these have shed light on our understanding of the onset and progression of cardiac diseases, the emergence of new drugs and improved therapies have lagged behind. Several major breakthroughs in the stem cell field, as described above, have made the academic and industrial biotechnology communities realize that development of human in vitro models for studying disease and drug discovery are within reach. In particular, development of more efficient and robust protocols for the production of
specialized cell types, such as cardiomyocytes, from hESC has strengthened this awareness.

In order to develop predictive human in vitro cardiac disease models, a rational choice would be to avoid complex multigene cardiac diseases involving multiple cell types or secondary aberrations in morphology and/or function. Instead, the initial preference should be in cardiac diseases associated with mutations in single genes that are most likely to have an effect at the single cell level (i.e. autologously on cardiomyocytes). In this regard, many mutations in genes encoding cardiac ion channels and sarcomeric proteins, associated, for instance, with disturbed ion channel function (cardiac channelopathies) or impaired contractility (cardiomyopathies), have been identified. For both channelopathies as well as cardiomyopathies, the majority of mutations associated with the disease are present in a relatively small set of genes (Table 1). Along with further characterization of the hESC and human iPSC models, their value in understanding disease mechanisms needs demonstrating and their use as models for drug discovery remains to be shown. Nevertheless, proof of principle that this approach may be successful is beginning to emerge.

The imbalance in ion currents across the sarcolemma in patients diagnosed with cardiac channelopathies is the cause of a change in the action-potential morphology. It manifests as an abnormal ECG with abnormal peaks and intervals indicating cardiac arrhythmias (Figure 3). Heritable cardiac channelopathies include LQTS (long-QT syndrome), Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, cardiac conduction disease and sinus node dysfunction [105]. LQTS has a prevalence of 1 in 5000 individuals and is the most common cardiac channelopathy. LQTS can be subdivided into multiple types (LQT1 to LQT12) depending on which gene is mutated, but all prolong the duration of the action potential and the QT interval on an ECG.

Cardiomyopathy is a heart disease that may result in inadequate pumping of the heart leading to congestive heart failure. Cardiomyopathy can be further classified into different forms, including HCM (hypertrophic cardiomyopathy), DCM (dilated cardiomyopathy) and RCM (restrictive cardiomyopathy) and ARVD (arrhythmogenic right ventricular dysplasia). Of these, HCM is the most frequent with a reported prevalence of as high as 1 in 500 individuals [106]. It is also the most common cause of sudden cardiac death among young athletes and in people under 30 years of age. HCM is characterized by an increase in the left ventricular wall thickness, in the absence of increased afterload, combined with a disarray of cardiomyocytes and cardiac interstitial fibrosis. It was the first cardiac disease for which a genetic basis was identified. To date, more than 450 mutations, distributed over 13 sarcomere and myofilament-related genes, have been classified (Table 1). Moreover, 50 % of the total number of mutations appear in only two genes, MYH7 (MHC 7 cardiac muscle β) and MYBPC3 (myosin-binding protein C).

The challenge for the coming years is to generate in vitro models using human stem-cell-derived cardiomyocytes harbouring

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An ECG measures the electrical activity of the heart across the chest wall. It is the collection of peaks and intervals which help diagnose diseases. (A) An example of a normal ECG. (B–D) Examples of abnormal ECGs superimposed over a normal ECG. An elongated QT interval (B) is seen in a number of diseases including LQTS and catecholaminergic polymorphic ventricular tachycardia. The elevated ST (seen in ECG C) is often seen with a form of Brugada syndrome and a delta wave (D) is associated with Wolff–Parkinson–White syndrome which accompanies numerous heart defects.
Disease-associated mutations that faithfully recapitulate the cardiac disease phenotype as it manifests in patients. This, in combination with development of robust predictive assay readouts (scalable for high-throughput screening), will provide novel platforms for cardiac safety pharmacology, but, more importantly, also provide opportunities for novel drug discovery.

**Disease modelling in human iPS cells**

The development of iPS cell technology and the possibility of making patient-specific embryonic stem cell-like cells may complement the (rather slow) development of cardiac disease models based on hESC thus far. If human iPS prove to be amenable to cardiac differentiation, which many researchers are beginning to show [107,108], disease-specific human cardiomyocytes will become a very useful tool for studying and perhaps reversing cardiac disease. However, research into human iPS characterization and the effect of reprogramming and (epi)genetic memory on these cells is still ongoing. The uniformity and reliability of differentiation of several well-studied hESC lines may make these the most useful near-term research tool, thus research in targeting and genetic modification of hESC is still vital.

Nevertheless, recent successes using human iPS as models for human cardiac disease have been reported. Two groups have generated human iPS lines from patients with heart disease which show a disease phenotype in culture. Lemischka and co-workers [12] produced cardiomyocytes from two human iPS lines derived from patients with a heterozygous T468M substitution in *PTPN11* (protein tyrosine phosphatase, non-receptor type 11) which leads to LEOPARD syndrome. LEOPARD syndrome affects various parts of the body, but HCM is one of the most common life-threatening symptoms of the disease. The authors estimated hypertrophy based on cell size, sarcomeric organization and nuclear localization of NFATC4 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4), which gave a measure of patient phenotype. Furthermore, the use of human iPS in this research has also lead to several new insights into signalling pathways related to the disease, something which may have eluded researchers if not for human iPS technology.

A second report by Moretti et al. [13] investigated an ion channel mutation in patients with LQT1. These patients have a mutation in the *KCNQ1* gene which results in an elongated QT-interval. The *KCNQ1* gene encodes *IK*, the ion channel controlling the slow component of the delayed rectifier K⁺ current that is partially responsible for the repolarizing phase of the action potential. The authors were able to recapitulate this electrophysiological phenotype in culture using a whole-cell patch-clamp electrophysiology method after the creation human iPS lines from two related patients from a family with an R190Q miss-sense mutation in *KCNQ1*.

These first two ground-breaking articles on cardiac human iPS cell modelling are only the first steps in an area of research with great potential. Both articles bring to light one very important aspect of iPS cell technology: the need for proper assays to determine a cellular phenotype. The heart is not composed of just one cell type but several and therefore recapitulating a phenotype can be difficult. These two examples of human iPS as cardiac disease models for basic characterization of the disease itself are of interest, but the opportunities they present for drug discovery will, in the long run, be of greater interest.

**Direct reprogramming of somatic cells to cardiomyocytes**

In a recent advance in direct reprogramming, Ieda et al. [109] showed the differentiation of mouse fibroblasts directly into cardiomyocytes using just three cardiac transcription factors. Mouse cardiac or tail tip fibroblasts were treated with a cocktail of genes which induced direct cardiac differentiation. After waiting through 14 genes, the researchers found three ‘master regulator genes’, *Gata4*, *Mef2c* and *Tbx5*, which were essential for direct reprogramming of fibroblast to cardiomyocytes. The efficiency of this direct reprogramming was higher than reprogramming to iPSC (20% compared with <0.1%) and it was also more rapid. Using an inducible system, they were also able to show the stability of their procedure. Although this finding remains to be confirmed by others and has not yet been extended into human cells, it could represent another route to studying patient-derived cardiomyocytes for certain purposes. This new development may help eliminate some of the obstacles when using traditional ESC or iPS, including teratoma formation due to residual undifferentiated cells, and may lower the cost and delivery time to patients owing to higher yields and faster production. It has the disadvantage, however, that the emergent cardiomyocytes cannot be expanded in culture. The method thus is probably best seen as a supplement to research with iPSC cells rather than an alternative. It may, however, represent an opportunity to investigate whether direct reprogramming to cardiomyocytes is feasible in the heart in vivo, for example after myocardial infarction.

**Regenerative medicine**

The use of cell therapy to repair the heart is not a new concept. Its foremost aim is to introduce new cardiomyocytes into the heart to replace those that are damaged or dead, for example after an infarct. However, in practice this is rather complex with many aspects of how this could work still unclear. The first attempts at cell therapy for the heart were using a non-cardiomyocyte cell population, like BMC (bone marrow-derived cells). Although BMC initially were reported to be able to trans-differentiate into cardiomyocytes, thereby improving heart function, it was later found that this was not correct [110–112]. Nevertheless, the use of BMC delivery to the heart continued, although with limited long-term benefits regarding cardiac function. Any short-term improvements most probably involved enhanced local angiogenesis and thus blood supply in the border zone of the damaged heart tissue, or paracrine factors of an unknown identity from the transplanted cells. This is also probably the reason for the reported reduction in chest pain symptoms and improved cardiac contractile and relaxation performance [15,16,113].

The inconclusive data from adult stem cell therapy trials to date, and the limited success of sustaining non-cardiomyocyte presence in the heart, may make iPS, cardiac progenitors or hESC a better source for cell therapy, since these cells can form hESC-CM (cardiomyocytes) or hESC-CPC (cardiac progenitor cells) for repair. Studies have shown that hESC-CM introduced into mouse or rat hearts will survive and mature [114–116]. However, the cells are often separated from the animal heart by fibrotic tissue, and these hearts have only shown short-term functional improvements [116]. Whereas most studies have only been performed in small animals, a recent study in pigs showed improved cardiac function after intramyocardial injection of hESC-CM compared with the controls [117], but any long-term follow-up was not described.

Despite these transient improvements in cardiac function following hESC-CM transplantation in animals, there are a number of other obstacles which need to be overcome before transfer to humans may even be considered. In vivo, hESC-CM trigger an immune response. Therefore all of the studies thus far...
have involved immune suppression or used immunocompromised animals. The discovery of iPSC may help overcome this by creating immune-matched cells for transplantation, but a large cell bank would still be necessary to match most of the population since human iPSC lines for individual patients, at present, would not be economically viable. Not only would cheaper reagents be necessary for scaling up the cell production, but the whole procedure would also have to be carried out under conditions of GMP (good manufacturing practice). These practices are by themselves costly and labour intensive. A second obstacle is the removal of all undifferentiated hESC (or human iPSC) before the introduction into the body as described above, since undifferentiated pluripotent stem cells can give rise to teratomas when injected into a living organism, animal or human. The most important consideration though in human patients is the potential risk of arrhythmias, which can be caused by the introduction of immature cardiomyocytes with intrinsic pacemaker-like activity into the human heart, as well as anisotropy and other scar-implanted cell interactions.

CONCLUSIONS AND FUTURE PERSPECTIVES

Even after more than a decade of research, the potential contribution of human embryonic stem cells to understanding cardiac disease and as a cell therapy is far from fully realized. Restricted in some countries by ethical issues, research into these cells was slow to start and often lacked financial support. The discovery of iPSC technology has helped jump-start the field in recent years and has added much to our understanding of pluripotency and manipulation of embryonic stem cells. Even though many questions still remain unanswered, there is already much that has been learned from hESC and this will probably accelerate implementation of pluripotent cells to biomedicine in general as well as to the cardiac arena in particular. The most important and immediate advancement is probably to be the contribution of these cells to drug discovery, since there is a significant unmet need for new drugs as the incidence of myocardial infarction and heart failure increases in the aging population. The number of new drugs entering the market for heart disease is decreasing, whereas investment by the pharmaceutical industry in this area continues to increase. This is perhaps the strongest evidence that the present systems on which drug discovery is based are inadequate for future needs. In the context of safety pharmacology, big pharmaceutical companies generally are conservative and they will not be keen to have additional safety tests imposed if they do not replace those in present use. In addition, replacing present tests may increase their risk of litigation should the new predictors prove wrong.

Although many scientists began research with hESC with a view to their potential in therapy, experiments over the last decade have indicated that this will be very challenging for many types of disease. Possible exceptions probably include macular degeneration (age-related blindness), since it is fairly easy to induce differentiation of hESC to retinal pigment epithelial cells and the eye is fairly insensitive to immune attack; certain types of spinal cord lesion, for which the first transplantation has recently taken place in the U.S.A. [118]; and diabetes, for which the transplantation technique is straightforward and some groups are now able to generate β-islet-like cells with some reproducibility [119]. The heart is certainly among the more challenging organs to repair because integrating cardiomyocytes with host tissue may require more than simple injection. Thus drugs derived from research on stem cell-derived cardiomyocytes may then have the greatest clinical effect.

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Differentiation of pluripotent stem cell use as cardiac disease models


