Molecular and phenotypic analyses of human embryonic stem cell-derived cardiomyocytes

Opportunities and challenges for clinical translation

Gareth Goh1, 2, Tim Self3, Maria D. Barbadillo Muñoz4, 1, Ian P. Hall2, Lorraine Young1, 4, Chris Denning1

1Institute of Genetics, 2Division of Therapeutics and Molecular Medicine, 3Institute of Cell Signalling, *Division of Obstetrics and Gynaecology, University of Nottingham, Queens Medical Centre, Nottingham, UK

Summary
Differentiation of human embryonic stem cells (hESCs) into cardiomyocytes in culture may offer unique opportunities for modeling genetic disorders, screening potentially cardiotoxic pharmaceutical agents or replacing cells of the diseased heart. However, before clinical utility can be realized, numerous hurdles must be overcome. Comprehensive molecular and phenotypic characterization is required but has so far been restricted to cardiomyocytes derived from a limited subset of hESC lines. Thus, we have initiated analysis of cardiomyocyte differentiation and function from a further two independently derived lines, BG01 and HUES-7. The challenge of improving cardiac cell induction, enrichment and maturation must also be addressed to meet the demands of high throughput pharmaceutical screening or to provide sufficient cells to repair an infarcted heart. Transplanted cells must functionally integrate without inducing arrhythmias, while survival and evasion of immune surveillance must be accomplished without tumorigenicity. This review evaluates the opportunities presented by hESC-derived cardiomyocytes and the progress towards surmounting the challenges of clinical translation.

Keywords
Human embryonic stem cells (hESC), characterization, cardiomyocytes, differentiation, clinical translation

Introduction
The heart is the first organ to form in vertebrates and development begins soon after post-gastrulation formation of the three embryonic germ layers (ectoderm, endoderm and mesoderm). The precardiac mesoderm then undergoes a complex series of cellular differentiation steps and morphogenetic changes, which result in initiation of beating by approximately the fifth week of gestation. By midgestation, morphogenesis, growth and integrated function of the heart are essential for survival of the embryo (1–3).

Cardiac function is affected in numerous disease states. Worldwide, the failing heart accounts for ~7.2 million deaths annually and 500 million people with high blood pressure may be at risk of heart attack, stroke and cardiac failure (The World Health Organization, www.who.int/whosis). Disorders can be inherited (e.g. congenital long QT syndrome [LQTS] and dilated / hypertrophic cardiomyopathies) or acquired (e.g. ischemic heart disease and myocardial infarction) (4, 5). Although heart transplantation is currently the most effective solution for some patients, chronic shortage of donor organs is restrictive. Local surgery, implantation of an electronic pacemaker or pharmacological intervention can provide alternative treatments in specific clinical settings.

Heart function is also highly sensitive to many drugs, including certain chemotherapeutic agents (6), and cardiotoxicity is a common dose limiting factor and a major cause of failure in preclinical development of novel compounds (7). One predictor of cardiotoxicity is prolongation of the QT interval, two time interval points that correspond to the duration of the ventricular action potential plus the time associated with transmission across the myocardium (Fig. 1). QT prolongation is associated with a life-threatening type of ventricular arrhythmia known as torsade de pointes (8). Therefore, developing additional methods for therapeutic intervention and in vitro drug screening is becoming a matter of urgency.
Cell-based approaches may help address this need. Recently, progenitor and proliferating cell populations capable of developing into mature cardiac cells have been identified in adult human or animal hearts (9–11). Cardiogenic potential in vitro has also been described from human mesenchymal stem cells from bone marrow (12) and amnion (13), although the ability of these cells to transdifferentiate to cardiomyocytes has recently become highly controversial (14, 15). However, it is the ability to differentiate cardiomyocytes from human embryonic stem cells (hESCs) that will form the focus of this review. We will evaluate the molecular and phenotypic characteristics of hESC-derived cardiomyocytes (hESC-CMs) and discuss the opportunities and challenges they present for clinical translation.

Human embryonic stem cells

Establishment of hESC lines has been achieved by explanting inner cell mass cells from the preimplantation embryo into culture dishes of mitotically inactivated feeder cells (16–19). The source of embryos is mainly from those supernumerary to IVF treatment, although hESC lines have also been established by therapeutic cloning (20) or from embryos discarded after identification of specific genetic lesions by preimplantation genetic diagnosis (21, 22). Under appropriate conditions, hESCs can be grown indefinitely in an undifferentiated state. Generally, hESCs conform to a range of criteria including (i) compact morphology with high nuclear to cytoplasmic ratio and prominent nucleoli, (ii) expression of cell surface stage-specific antigens (SSEA–3 & –4) and keratan sulfate–related antigens (TRA–1–60 and TRA–1–81), (iii) expression of the pluripotency-related genes OCT4, NANOG and TERT, (iv) normal and stable karyotype with high nuclear to cytoplasmic ratio and prominent nucleoli, (v) teratoma formation after injection into severe combined immunodeficient (SCID) mice and (vi) differentiation under in vitro conditions.

In vitro differentiation of hESC into cardiomyocytes

Production of hESC-CMs in vitro had been demonstrated by several groups (23–28). When placed in suspension culture, hESCs agglomerate as three-dimensional structures, termed embryoid bodies (EBs). This initiates differentiation, albeit in a spontaneous and random manner. During subsequent culture in adherent conditions a proportion of the EBs display easily identifiable, rhythmically contracting outgrowths. However, only more detailed interrogation can establish the specific cardiac cell types generated, the degree of maturation they achieve compared to in vivo cardiac development and whether they possess fully functional excitation-contraction coupling machinery that responds appropriately to pharmacological agents. Thus, in considering the suitability of hESC-CMs for clinical translation, comprehensive characterization is required.

Transcriptional profile of hESC-CMs

The gene expression profiles determined for a range of hESC-CMs (Table 1) are reminiscent of both mouse ESC differenti-
### Table 1: Markers for hESC-CM characterization.

<table>
<thead>
<tr>
<th>Marker</th>
<th>hESC line(s)</th>
<th>Method(s) of detection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK2 transcription factor related locus 3 (Nkx2.5)</td>
<td>H1, H7, H9, H9.1, H9.2</td>
<td>RT-PCR</td>
<td>23, 24</td>
</tr>
</tbody>
</table>

| **Structural elements** | | | |
| Cardiac troponin I (cTnI) | H1, H7, H9, H14, H9.1, H9.2 | IF, W, RT-PCR | 23–25, 30 |
| Cardiac troponin T (cTnT) | H1, H7, H9, H9.1, H9.2 | RT-PCR, IF | 23, 24 |
| α-myosin heavy chain (α-MHC) | H1, H7, H9, H9.1, H9.2, BG01 | RT-PCR, IF | 23, 24, u.p. |
| Sarcomeric myosin heavy chain (SMHC) | H1, H7, H9, H9.1, H9.2, H14 | IF | 24, 25 |
| Myosin light chain 2a (MLC-2a) | H9.2, HES-2 | RT-PCR, IF | 23, 26 |
| α-adrenceptors | H1, H7, H9, H9.1/2, H14, HES-2, BG01 | RT-PCR, IF | 23–25, u.p. |
| Desmin | H1, H7, H9, H9.1, H9.2 | IF | 23, 24 |
| Smooth muscle actin (SMA) | H1, H7, H9, H9.1, H9.2 | IF | 24 |

| **Receptors and regulatory elements** | | | |
| Phospholamban (PLN) | HES-2 | RT-PCR | 26 |
| Ryanodine receptor (RyR) | HES-2 | IF | 26 |
| Creatine kinase-MB (CK-MB) | H1, H7, H9, H9.1, H9.2 | IF | 24 |
| Myoglobin | H1, H7, H9, H9.1, H9.2 | IF | 24 |
| α1-adrenceptors | H1, H7, H9, H9.1, H9.2, HES-2 | IF / phenylephrine (ph.) | 23–26 |
| β2-adrenceptors | H1, H7, H9, H9.1, H9.2 | IF / clenbuterol (ph.) | 24 |
| Muscarinic receptors | HES-2, H1, BG01 | Carbachol (ph.) | 26, 27, u.p. |
| Phosphodiesterase | H1, H7, H9, H9.1, H9.2 | IBMX (ph.) | 23, 24 |
| Adenylate cyclase | H9.2 | Forskolin (ph.) | 23 |
| Ki67 (cell division) | H1, H7, H9, H9.1, H9.2 | IF | 24, 30 |

| **Gap junction and adhesion proteins** | | | |
| Connexin 43 | H9.2, HES-2, H1 | IF | 23, 26, 28, 34 |
| Connexin 45 | H9.2 | IF | 23, 34 |
| Connexin 40 | H9.2 | IF | 23 |
| N-cadherin | H1, H7, H9, H9.1, H9.2 | IF | 24 |
| Inhibition of gap junctional intercellular communication | H9.2 | Heptanol (ph.) / 2,3-butanedione monoxime (ph.) | 28, 34 |

| **Ion channels** | | | |
| L-type Ca2+ channel (Cav1.2) | H1, H7, H9, H9.1, H9.2, HES-2 | RT-PCR, IF / diltiazem (ph.) / Nifedipine (ph.) | 24, 26, 38 |
| Calcium channel Cav1.2 | H9.2 | RT-PCR | 38 |
| Cardiac sodium channel (Na+,1.5) | H1 | RT-PCR / Lidocaine (ph.) / Tetrodotoxin (ph.) | 28, 38 |
| Transient outward K+ channel (Kv4.3) | HES-2 | RT-PCR | 26 |
| Slow delayed rectifier K+ channel (Kv7.1/7.2) | HES-2 | RT-PCR | 26 |
| Slow delayed rectifier K+ channel (HERG) | H9, H14 | E4031 (ph.) | 25 |
| Hyperpolarization activated cyclic nucleotide modulated gene family (including HCN2) | H1, H9.2 | ZD7288 (ph.) / Cs+ / RT-PCR | 28, 38 |

Methods of detection are RT-PCR, reverse transcriptase-polymerase chain reaction; IF: immunofluorescence; W: western blot analysis; pharmacological agents are given as agent name (ph.) u.p.: unpublished data (Denning).
fetal, but not adult cardiomyocytes. Correspondingly, individual hESC-CMs, rather than showing the more defined rod shape of mature cells, displayed numerous different morphologies, including spindle, round, tri- or multinuclear (24). Sarcomeric immunostaining revealed sarcomeric striations organized in separated bundles, which parallels the pattern seen in human fetal cardiomyocytes and not the highly organized parallel bundles seen in human adult cardiomyocytes (26). In addition, no developed T tubule system could be detected in hESC-CMs (30).

In order to assess the degree of maturation that does occur in cultured hESC-CMs, Snir et al. (30) used light and electron microscopy to examine the cells at different time points. During early stages of differentiation (day ~10–20) hESC-CMs had a large nucleus to cytoplasm ratio with disoriented myofibrils lacking sarcoplasmic pattern distributed throughout the cytoplasm in a random fashion. However, by day ~20–50 increases in both number and organization of myofibrils were observed. Cells elongated and Z-line assembly from periodically aligned Z bodies was seen. At late stages (>50 days), a high degree of sarcomeric organization developed and discrete A (dark) and I (light) bands could be seen in some sarcomeres. Furthermore, hESC-CMs progressively withdrew from the cell cycle during culture. However, while this clearly indicated that hESC-CM maturation does occur, the level of maturity typical of adult cardiomyocytes is not attained.

**Chronotropic responses and intercellular communication in hESC-CMs**

Several studies now indicate functional excitation-contraction coupling capability in hESC-CMs. Basal contraction rates of hESC-CMs range from ~40 (26, 27) to >130 (23, 25) beats per minute and respond appropriately to chronotropic agents. Phenylephrine and isoprenaline, which act through the α1- and β1-adrenoceptors (ARs) respectively, induced concentration-dependent increases in hESC-CMs derived from the Wisconsin lines H-1, –7, –9 and –14 (24, 25, 27), similar to those seen in human fetal cardiomyocytes (26). Using spontaneously contracting EBs derived from the hESC line HUES-7, we have also found that isoprenaline induced a concentration-dependent increase in beat rate (Fig. 2). He et al. (25) have shown that the β2-AR agonist, clenbuterol, only elicited a response during late stages of in vitro differentiation (days 61 to 72) and not early stages (day 22 and 39), consistent with the observation that sensitivity to β-adrenergic stimulation changes during in vitro development (31). Negative chronotropic responses to the muscarinic agonist, carbachol, have also been observed, again consistent with data from the mouse fetus and mESC-CMs (31, 32).

Beating EBs display continuous and episodic contraction patterns, with the latter being speculatively attributed to conduc- tion block related to tissue geometry, impaired cell to-cell coupling, reduced cellular excitability or immature Ca2+ regulatory system (25). Our initial observations suggest conduction block can be overcome by isoprenaline stimulation, which induced continuous beating from episodic EBs (unpublished observations). Adhesion molecules (N-cadherin) and gap junction proteins (connexins 43 and 45) were detected in hESC-CMs, although connexin 45 is associated with early stages of in vivo development (26, 33). Correspondingly, functional gap junctions formed, as evidenced by visualizing Lucifer Yellow dye spread or Ca2+ movement between connected cells. Co-cultures of hESC-CMs and neonatal rat ventricular myocytes functioned as an electrical syncytium, and during forced electrical pacing synchronous activity was maintained irrespective of whether a human or rat cell was stimulated (34).

**Ion channel expression in hESC-CMs**

During cardiac embryogenesis there are marked chamber- and age-related differences in ion channel expression (35). The main currents involved in the action potential (Fig. 1) are 1) influx of Ca2+ and Na+ during depolarization (phase 0). Inward Ca2+ currents are dominant during early development but Na+ currents dominate later in gestation (35); and 2) efflux or maintenance of K+ during repolarisation and resting potential (phases 1–4) (36, 37). To varying degrees, the activity of each of these ion currents has been investigated in hESC-CMs.

**Ca2+ and Na+ currents**

Recent work by Satin et al. (38) has demonstrated a high membrane density of the cardiac sodium channel (Na1.5). Furthermore, from several lines of analysis (RT-PCR data, inhibition with tetrodotoxin and electrophysiology) these authors concluded that hESC-CMs predominantly expressed a Na+ current and this forms the basis of spontaneous activity. This is fundamentally different from the mouse where the calcium channel ICa-L plays a crucial role in action potential initiation (38). Moreover, ICa-L channels were expressed in hESC-CMs (26) and functionally inhibited by diltiazem in hESC-CMs (24) and verapamil in both human fetal- and hESC-derived cardiomyocytes (26). By contrast, mESC-CMs at early stages of differentiation are non-responsive to these agents despite the presence of ICa-L channels. Thus, Mummery et al. (26) have suggested that although hESC-

![Figure 2: Effect of isoprenaline on beat rate of cardiomyocytes derived from HUES-7.](image-url)
CMs and early human fetal cardiomyocytes show some common features with early mouse cardiomyocytes, their calcium channel modulation resembles that in the adult mouse.

During action potential initiation (phase 0) in the embryonic heart the concentration of cytosolic Ca\(^{2+}\) increases (35, 37). Extracellular Ca\(^{2+}\) is at >1000 times higher concentration than cytosolic. Therefore stimulation of cell surface voltage and / or receptor operated calcium channels allows rapid flux of Ca\(^{2+}\) into the cell, which, in turn, triggers further Ca\(^{2+}\) release from the sarcoplasmic reticulum (39). However, the balance between external and internal Ca\(^{2+}\) sources is markedly altered during development; sarcoplasmic reticulum (SR) is scarce in fetal hearts and thus plays a lesser role (40). It is notable that hESC-CMs have been shown to express the SR proteins ryanodine receptor and phospholamban (26). Surprisingly, we have found that RNA for several receptors and channels important for calcium responsiveness is ubiquitously expressed in undifferentiated FACS sorted SSEA-4 positive and unsorted BG01 hESCs, differentiated EBs (irrespective of whether they display spontaneous contraction) and adult human heart (Fig. 3). This may imply that during cardiomyocyte differentiation it is activation of the machinery to regulate calcium handling activity rather than expression of the component receptors and channels per se that is important. Clearly, future studies to determine the ontogeny of protein expression and function during differentiation will be an important aspect of further hESC-CM characterization. This should also help define the contribution of the calcium from SR and extracellular stores during early development.

### K\(^+\) currents

After depolarization, the membrane potential must be returned to a resting state and this is largely achieved by numerous K\(^+\) channels (37, 41). Among these are transient outward current channels (\(I_{\text{T}}\)) encoded by the genes \(Kv4.2\) and \(Kv4.3\), which cause the early rapid repolarisation seen in phase 1 of the action potential (Fig. 1), and slow activating current (\(I_{\text{Ks}}\), encoded by \(K\text{vLQT1}\) with the \(mink\) subunit), which causes repolarisation associated with phase 3. Mutations in \(K\text{vLQT1}\) are a cause of LQTS (see later) in humans. The \(I_{\text{Ks}}\) current (encoded by human \(eag\)-related gene \(HERG\)) provides the rapid component of delayed rectification. \(I_{\text{Ks}}\) is involved in all phases of repolarisation, but most active during phase 3. Mutations in \(HERG\) have been linked to a congenital form of LQTS and \(I_{\text{Ks}}\) is specifically inhibited by the compound E-4031.

Molecular analysis of hESC-CMs revealed \(Kv4.3\) and \(K\text{vLQT1}\) RNA expression (26). However, while \(Kv4.3\) could be detected in differentiating cells several days before the onset of beating, \(K\text{vLQT1}\) was expressed in undifferentiated hESCs but transcripts disappeared during early differentiation and reappeared later. Functionally, application of E-4031 caused increased duration of phase 3 (terminal repolarisation) and triggered early after depolarisations (EADs) based arrhythmias, providing pharmacological evidence that \(I_{\text{Ks}}\) contributes to repolarisation in hESC-CMs (25). Delayed after depolarisations (DADs) typically occur during Ca\(^{2+}\) overload such as produced by injury or digitoxin toxicity and these were observed to occur spontaneously in hESC-CMs, possibly a result of microelectrode impalement or spontaneous Ca\(^{2+}\) release (25). Interestingly, forced electrical stimulation at increasing frequencies resulted in action potential shortening adaptation in ventricular-like cardiomyocytes. This physiological response leads to systolic shortening at high heart rates thereby maintaining diastolic time for ventricular filling, as shown in the ventricular myocardium of human embryos (25). That hESC-CMs respond in an anticipated manner to physical and pharmacological stimuli is encouraging for their potential use as drug screening tools.

Previously, the electrophysiological characteristics of the action potential, such as resting potential, upstroke, amplitude and duration, have been documented in the intact early human embryonic heart (42). By applying similar electrophysiological analyses to hESC-CMs in culture it is possible to assign cell type (pacemaker, atrial, ventricular, nodal). Initial reports suggested upstroke velocities, which are a measure of the rate of depolarization, were low in hESC-derived ventricular-like cells (average 8 V/s, 25, 26), which is comparable to cultured fetal ventricular cardiomyocytes. Recently however, significantly higher velocities (118 V/s) have been reported (38), close to the expected range of ~150–350 V/s for adult ventricular cells (25). The rea-
sons for these differences are currently unknown but Satin et al. (38) have speculated that their experimental conditions allowed the cardiac sodium channel Na,1.5 to contribute to the upstroke and therefore increased the velocity. Alternatively, differences may relate to the different hESC lines used (H9, H14 [25]; HES-2 [26]; H9.2 [38]). However, the relatively positive resting potential of atrial and ventricular-like hESC-CMs (approx. –40 to –50mV) is comparable to early stages of fetal development (26). Clearly, further research will be required to define the degree to which maturation of hESC-CMs occurs.

On the basis of the electrophysiological studies above, some reports describe up to 85% of hESC-CMs being ventricular-like (Mummery et al. 2003), while others suggest a lesser predominance of this cell type and an increased appearance of atrial- or nodal-like cells (25, 33). This may relate to the time of analysis since the cardiomyocyte composition of mESC-derived embryoid bodies changes during culture duration (43). It may also reflect differences in the hESC lines (see above) or methods of differentiation used, or that the analysis by Mummery et al. (26) used dissociated hESC-CMs rather than EBs.

Application of hESC derived cardiomyocytes

The ability to differentiate hESCs into cardiomyocytes with phenotypic and molecular properties of the developing heart potentially offers a novel, renewable resource for clinical application. Cardiac cell replacement strategies into infarcted myocardium may augment contractile function by replacing those cardiomyocytes lost as a result of injury. Such strategies may also provide an alternative treatment for bradyarrhythmias, which result in abnormally slow heart rate and require surgical implantation of electronic pacemakers.

Standardized sources of cardiomyocytes would also provide new opportunities for in vitro modeling of diseases caused by single genetic lesions, particularly when mice do not show the same pathophysiology as humans. Our laboratory, for example, is developing RNA interference and gene targeting strategies to impede the function of dystrophin in hESCs and derived cardiomyocytes to model Duchenne Muscular dystrophy (DMD). With an incidence of ~1 in 3000 in males, this disorder develops in utero and by the late teens / early twenties death ensues due to cardiac or respiratory failure (44). While extensive mutation mapping has been carried out in the dystrophin gene of DMD patients (45, 46), the molecular and cellular consequences of the absence of dystrophin are poorly understood and additional models would serve to improve knowledge.

Inherited long-QT syndrome (LQTS) results in distortion of transmembrane ion flux during the action potential and is characterized by prolongation of the QT interval (Fig. 1), seizures, and sudden death (5). The molecular basis of Romano-Ward, Jervell and Lange Nielsen, and Brugada syndromes has been associated with mutations in several ion channels, including KCNQ1, HERG, SCN5a, KCNE1, KCNJ2 and KCNE2 (5, 47). In addition, individuals may have ‘silent’ forms of congenital LQTS, which only present in response to pharmacological treatments (48). Thus, by selecting existing hESC lines that harbor ion channel mutations or recapitulating mutations using transgenic techniques, answers on how to prevent or control LQTS may be provided by detailed functional in vitro analyses and drug testing of derived cardiomyocytes. A consistent supply of hESC-CMs would also allow the effect of novel pharmaceutical agents on QT interval to be evaluated to eliminate potential cardiotoxicants and proarrhythmic drugs, prior to animal trials.

Challenges for clinical translation of hESC-CMs

Although hESC-CMs have successfully been produced in small-scale experiments, significant challenges must be overcome to enable routine use in vitro and particularly in vivo (Table 2). Currently a major limitation of the system is the lack of directed differentiation to the cardiomyocyte lineage.

Directed differentiation and enrichment of hESC-CMs

The inherent pluripotentiality of hESCs makes their differentiation into homogeneous populations of cardiomyocytes a major challenge. Beating outgrowths have been observed in ~8% (23) to 70% (24) of EBs but heterogeneity within these structures reduces the actual fraction of cardiomyocytes. For example, Xu et al. (24) found beating areas in 70% of EBs but only 17% of total cells were sMHC-positive and this remains the highest reported yield to date. Therefore the primary goal for clinical translation must be to develop strategies for induction, enrichment and selection of hESC-CMs.

In the developing fetus, endoderm appears to have an instructive function in cardiogenesis (49). Earlier co-culture of END-2 cells, a mouse visceral endoderm-like cell-line, with mouse P19 embryonal carcinoma (EC) cells and mESCs showed that beating areas appeared in aggregated cells (50). Beating areas in 35% of wells were also observed following co-culture of END-2 and hESCs with (26, 51). Alternatively, chemical induction has been assessed. Time and dose dependent treatment of hESCs with the DNA demethylating agent 5-aza-2’-deoxycytidine enhanced cardiomyocyte differentiation, with a 2-fold upregulation seen in α-MHC levels determined by real-time PCR (24). Similar effects on mouse mesenchymal stem cells have also been observed (52).

Factors influencing cardiogenesis in mouse systems, however, do not automatically translate to hESCs. Induction of cardiac differentiation using bone morphogenetic protein (BMP-2), retinoic acid and DMSO has been described for mESCs (53–56) but has not been effective for hESCs (23, 24, 26). Whether this simply adds to the growing list of phenotypic and genetic differences between mouse and human ESCs (57) or that differentiation protocols were not optimal, needs to be examined. Subtle variations in concentration or timing (e.g. 5-aza-2’-deoxycytidine, [24]) and combinatorial effects may alter the potential of cardiogenic factors in hESC differentiation. Alternatively, differences in the differentiation strategy (e.g. END-2 co-culture vs EB induced) or the hESC line used (see below) may influence effect. Thus, systematic analysis of all known physical, chemical and protein-based cardiogenic factors is required (58–60). This will rely in part on consistent production of EBs of uniform sizes; EBs that are too small fail to yield cardiomyocytes, while
oversized EBs develop necrotic centres (61). In the mouse uniform EBs are produced in hanging drop cultures but these are unsuccessful in hESCs (17), which may instead require cultivation in stirring chambers or bioreactors (62).

Even after induction, it is unlikely that pure populations of cardiomyocytes will be produced and methods of enrichment and/or selection will be required. Unfortunately, surface markers entirely specific for cardiomyocytes are not available. Instead, Xu et al. (24) used a discontinuous Percoll gradient that enriched by 4-fold (70% of cells positively stained for cardiac markers). Alternatively, transgenic DNA constructs comprising cardiac specific promoters (e.g. α-MHC, MLC-2v or α-cardiac actin) could be used to drive expression of proteins that confer fluorescence or antibiotic resistance in the cardiomyocytes, a strategy that has achieved purities of close to 100% in the mouse system (63–65).

Characterization of a range of hESC lines

Most hESC lines derived in independent laboratories have been isolated using different methods and culture media. Together with the intrinsic variations in embryos derived from an out-bred human population, each hESC line is likely to display distinct growth and differentiation characteristics (66). Thus, effective clinical translation will require characterization of a range of lines that represent the diverse human genetic pool. This need is highlighted by the significant reliance of published studies on the H1, 7, 9, 13 and 14 lines derived at the University of Wisconsin (16). These lines represent only 23% of those registered with NIH (http://stemcells.nih.gov/research/registry/) but account for more than 70% of experiments reported (Table 1, [66]).

The characteristics of the Wisconsin lines are relatively well defined. Growth in the undifferentiated state is supported by low densities of feeder cells (67, 68) and these hESC lines tolerate a variety of enzymatic bulk passaging methods, including dispase, collagenase, trypsin (16, 69, 70). They grow well on several feeder-free matrices when cultured in feeder cell-conditioned medium (70, 71) and in defined media containing high concentrations of bFGF, bFGF/noggin or bFGF/TGF-b (69, 72, 73).

Finally, efficient differentiation via EBs to a multitude of cell lineages, including cardiomyocytes, has been reported (24). In contrast, other lines require a 3-fold higher feeder density (68) and may display a higher propensity for karyotypic instability with bulk passageing (74, 75), although there have also been recent reports of karyotypic anomalies in the Wisconsin lines (76, 77). Some hESC lines appear not to form EBs (17) and our studies suggest that bFGF alone does not support undifferentiated growth of BG01 and HUES-7 (in preparation). Thus, whether H1, 7, 9, 13 and 14 are representative of the estimated 200 lines worldwide (75) requires further investigation.

Scalability of hESCs and differentiated derivatives

Scalability of hESC culture will be a key aspect for the achievement of clinical translation. Massive cell death occurs during cardiac infarction. Correspondingly, clinical trials testing the efficacy of skeletal muscle satellite cells in myocardial regeneration have injected ~10⁹ cells into the left ventricle (78). For drug

Table 2: Challenges and progress for clinical translation of human embryonic stem cell-derived cardiomyocytes.

<table>
<thead>
<tr>
<th>Current limitation of the hESC system</th>
<th>Challenge</th>
<th>Progress</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inefficient differentiation of hESCs to cardiomyocytes (poor yields)</td>
<td>Cardiogenic induction</td>
<td>END-2/hESC co-culture</td>
<td>26</td>
</tr>
<tr>
<td>Heterogeneity of differentiated hESC-CMs populations</td>
<td>Enrich and select hESC-CMs</td>
<td>Physical enrichment, e.g. percoll gradient for hESC-CMs</td>
<td>24</td>
</tr>
<tr>
<td>Characterization of hESC largely restricted to lines H1, 7, 9, 13 &amp; 14 (University of Wisconsin)</td>
<td>Characterize additional hESC lines</td>
<td>Differentiated derivatives from a range of lines produced</td>
<td>66</td>
</tr>
<tr>
<td>Heterogeneity of undifferentiated hESC cultures</td>
<td>Optimize culture conditions</td>
<td>Analysis of defined media containing FGF, FGF/TGF-β or GSK-3 inhibitor in hESCs</td>
<td>69, 72, 73</td>
</tr>
<tr>
<td>Scalability &amp; automation</td>
<td>Defined culture and differentiation conditions for hESCs</td>
<td>Analysis of defined media as above</td>
<td>81</td>
</tr>
<tr>
<td>Isolation of hESC lines without xenogenic contamination</td>
<td>Isolate GMP lines in defined, animal product-free conditions</td>
<td>Feeder-free culture of hESCs</td>
<td>62, 83</td>
</tr>
<tr>
<td>Delivery, function &amp; survival of hESC-CMs in host myocardium</td>
<td>Develop suitable tissue scaffolds; improve survival</td>
<td>Development of pig and guinea-pig models of cardiac dysfunction</td>
<td>28, 34</td>
</tr>
<tr>
<td>Overcoming immune rejection</td>
<td>Suppress or evade immune system</td>
<td>Development of biodegradable 3-D patches</td>
<td>92, 93</td>
</tr>
<tr>
<td>Embryonic phenotype of hESC-CMs restrictive drug screening and may induce arrhythmias in host myocardium</td>
<td>Induce maturation of hESC-CMs</td>
<td>Heat shock cardiomyocytes prior to transplantation</td>
<td>91</td>
</tr>
<tr>
<td>Tumorigenicity of contaminating hESCs in transplanted hESC-CM populations</td>
<td>Eliminate all undifferentiated hESCs before transplantation; introduce fail-safe system</td>
<td>Hematopoietic chimeraism (human kidney transplant)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hESCs produced by therapeutic cloning</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other suggested strategies: Classic immuno-suppression, HLA matched cell bank, transgenically produced immuno-logically null cell.</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suggested strategies: Mechanical stretching, growth factor treatment (e.g. cardiotrophin)</td>
<td>26, 89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Demonstration of successful hESC ablation with HSV-TK suicide gene system</td>
<td>96</td>
</tr>
</tbody>
</table>
screening and toxicology, most pharmaceutical companies use robotized high-throughput strategies as the primary resource, allowing processing of >100,000 assays per day (79, 80). It is therefore encouraging that methods to improve undifferentiated hESC maintenance utilizing low oxygen tensions (81) and defined media are being investigated (see above; [69, 72, 73]). Pluripotency may also be maintained by the addition of GSK-3 inhibitor, which induces activation of the canonical Wnt pathway (82). Differentiation in bioreactors will facilitate industrially scalable procedures for certain lineages, as already demonstrated for cardiomyocytes from mESCs (82) and hematopoietic cells from hESCs (62). Optimization of culture media and/or conditions will also be required for maintenance of hESC-CMs, since suboptimal culture of cardiomyocytes can lead to cells that are arrhythmic, show signs of cell stress and are predisposed to cell death via a p53 mediated cascade (84).

**Transplantation of hESC-CMs**

With the exception of a limited number of hESC lines that have been isolated on feeder layers of human origin (85–87), most utilize mouse embryonic fibroblasts (MEFs). hESCs grown in coculture with mitotically-inactivated MEFs or in medium containing animal products, such as Knockout Serum Replacer (In-Vitrogen), present non-human sialic acid Neu5Gc residues on their membranes (88). Although these residues diminished in differentiated hESC lineages upon culture with medium containing human serum, the possibility of transfer of xenogenic agents, which presumably include murine retroviruses, requires careful monitoring. Thus, given that current lines may not be suitable for transplantation studies into human recipients, it is imperative that culture and differentiation protocols are transferable to a new range of transplantation grade hESC lines isolated in accordance under good manufacturing practice (GMP).

Transplantation of hESC-CMs has shown functional integration and the ability to pace the rhythm of pig (34) and guinea-pig (28) hearts in which injury had been induced by cell ablation in the bundle of His and atroventricular node, respectively. Furthermore, grafted cells responded appropriately to chronotropic agents (34). Encouragingly, Kehat et al. (34) reported sustained activity of the transplanted cells in half the animals. However, long-term survival and function were not assessed. Given that the degree of hESC-CMs maturation is limited (see previous discussion), post-encephalment arrhythmias could arise from time-dependent electrical remodeling of hESC-CMs such as changes in ion channel expression and action potential duration (28, 38). Suggested strategies to induce *in vitro* maturation prior to transplantation have included the addition of prohypertrophic factors such as cardiacotrin (89) and subjecting the hESC-CMs to oscillating mechanical load (26). Such improvements would also be beneficial for producing cardiomyocytes with adult phenotype for more relevant drug screening and toxicology.

Arrhythmias may also arise from heterogeneous distribution of gap junctions intra-myocardially or between the graft and host myocardium, which has been the cause of serious clinical complications caused by transplantation of skeletal myoblasts in patients (90). Alternatively, ventricular needle puncture caused by direct cell injection or high degree of graft cell death post-transplantation may induce arrhythmias (78). For example, Klug et al. (63) reported that only ~5% mESC-CMs survived after delivery to the myocardium, and the major route of cell death was via apoptosis. One surprising method that improved survival of neonatal rat cardiomyocytes was heat shock treatment prior to transplantation (91). Delivery of cardiomyocytes in a physiologically more relevant format may also improve survival and gap junction intercellular communication while reducing host tissue damage. This could involve biodegradable three-dimensionalgrafable ‘patches’ that readily vascularize (92, 93). However, since major cardiac remodeling occurs after myocardial infarction (93), predicting the behaviour of transplanted hESC-CM will be difficult and suggests caution against premature clinical translation. Instead, controlled studies will be required in carefully chosen animal models, such as the pig, where anatomy, physiology and contraction rate are similar to humans. Long term survival and function of transplanted cells will also require evasion the immune system, with several strategies suggested for hESC derivatives (Table 2 [84]).

Since undifferentiated hESCs form tumors in immunocompromised mice, there is a risk that residual undifferentiated cells within differentiated hESC-CM populations may also display tumorigenicity in patients. While enrichment and genetic selection purification strategies could reduce this risk, it may be prudent to add additional ‘fail-safe’ mechanisms, such as the use of suicide genes. Expression of the Herpes Simplex virus thymidine kinase (*HSV-TK*) suicide gene in mammalian cells sensitizes them to the otherwise non-toxic prodrug ganciclovir (GCV). *HSV-TK* converts GCV into the toxic metabolite GCV-triphosphate, which inhibits DNA synthesis during S-phase of the cell cycle (95). Thus, Schuldiner et al. (96) transfected a hESC line with the *HSV-TK* gene expressed from a constitutive phosphoglycerate kinase (PGK) promoter and demonstrated these cells could be eliminated by GCV *in vitro* and *in vivo* in mice. While encouraging, the concentration of GCV used in the mouse studies was 50 mg/kg, 5–10 times the dose used in humans (97). Furthermore, a strong PGK promoter was used in this study but a weaker pluripotency restricted promoter that is active in undifferentiated hESCs will be needed to ensure ablation of these cells and not the differentiated derivatives. Moreover, GCV cell ablation occurs in a dose-dependent manner and correlates with *HSV-TK* expression level (98). Thus, the efficacy of non-toxic doses on hESCs expressing relatively low levels of the suicide gene will need to be determined. Finally, compared with rodent tumors, a large percentage of cells in human tumors have a long cell cycle or are out of cycle (99), which may reduce the efficiency of a cell cycle-dependent system. Whether the issues of effective GCV dose, level of *HSV-TK* expression levels and kinetics of hESC-derived tumor growth kinetics are real issues of concern will require testing in large animal models, such as the pig. In addition, it will be important to develop in parallel other fail-safe strategies for undifferentiated hESC elimination.

**Conclusions**

Clearly hESCs can be differentiated towards cardiomyocytes that appropriately respond to different stimuli and this offers functional expression of many of the components required for...
excitation-coupling contraction. Several challenges must now be
surmounted to ensure continued progression towards clinical
utility. An immediate goal must be optimization of cardiogenic
induction and selection strategies that are applicable to a range of
hESC lines, including those derived specifically for transplan-
tation purposes. Maturation of hESC-CMs does occur during
prolonged culture but currently these cells fail to attain the char-
acteristics of adult cardiomyocytes. It will be important to assess
novel methods to stimulate maturation so in vitro produced car-
diomyocytes with embryonic or adult characteristics are at
the disposal of the scientific and clinical community. This would be
potentially useful in drug discovery and testing, although issues of
high throughput and scalable cardiomyocyte production must
be addressed. In addition, it might be possible to use hESC-CMs
to develop in vitro models for studying genetic cardiac diseases,
such as channelopathies where specific ion channel mutations
may cause fatal arrhythmias in asymptomatic carriers. Transplan-
tation into the diseased heart raises additional challenges, not
least grafting cells that integrate without inducing arrhythmias
or tumors, and this will require extensive and controlled evalu-
ation. It is encouraging that animal models are being developed
that do not have the high heart rate and adaptive capacity of the
mouse, which can disguise induced arrhythmias. These models will
facilitate in vivo testing. Thus, to varying degrees, each of
the challenges towards clinical translation is being addressed and
continued research could provide a route to producing an addi-
tional, renewable source of human cardiomyocytes.

Acknowledgements

The authors would like to thank Chad Cowan and Doug Melton for Harvard
University (HUES-7) hESCs. In addition, Steve Stice and Christine Mum-
merly for helpful discussions / training in culture of BG01 and HES-2 hESC
lines, respectively.

References

1. Garcia-Martinez V, Schoenwolf GC. Primitive-
 streak origin of the cardiovascular system in avian em-
2. Harvey RP. Patterning the vertebrate heart. Nat Rev.
3. Olson EN. A decade of discoveries in cardiac biology.
4. Caplice NM and Gersh BJ. Stem cells to repair the
5. Splawski I, Shen J, Timothy K et al. Spectrum of
 mutations in long-QT syndrome genes. KVLQT1, HERG, SCN5A, KCNE1, and KCNE2.
6. Schimmel KJ, Richel DJ, van den Brink RB et al.
 Cardiotoxicity of cytokotic drugs. Cancer Treat Rev 2004;
7. Davila JC, Cezar GG, Thiede M et al. Use and ap-
plication of stem cells in toxicology Toxicol Sci. 2004;
8. Cubeddu LX. QT prolongation and fatal arrhyth-
 mias: a review of clinical implications and effects of
 is1+ cardioblasts enter fully differentiated cardio-
10. Matsuura K, Nagai T, Nishigaki N et al. Adult car-
diace Sca-1-positive cells differentiate into beating car-
11. Beltrami AP, Barlucchi L, Torella D et al. Adult car-
diac stem cells are multipotent and support myocardial
12. Xu W, Zhang X, Qian H et al. Mesenchymal stem
 cells from adult human bone marrow differentiate into
 a cardiomyocyte phenotype in vitro. Exp Biol Med
13. Zhao P, Ise H, Hongo M et al. Human amniotic mes-
 enchymal cells have some characteristics of cardio-
14. Murry CE, Soongna MH, Reinecke H et al. Haema-
topoietic stem cells do not transdifferentiate into car-
diace myocytes in myocardial infarcts. Nature 2004;
15. Balsam LB, Wagers AJ et al. Haematopoietic stem
 cells adopt mature haematopoietic fates in ischaemic
 Embryonic stem cell lines derived from human blasto-
17. Reubinoff BE, Pera MF, Fong CY et al. Embryonic
 stem cell lines from human blastocysts: somatic differen-
18. Mitajlova M, Calhoun J, Shin S et al. Human em-
 bryonic stem cell lines derived from discarded em-
19. Cowan CA, Klimanskaya I, McMahon J et al. Deri-
 vation of embryonic stem-cell lines from human blasto-
20. Hwang WS, Ryu YJ, Park JH et al. Evidence of a
 pluripotent human embryonic stem cell line derived from
 of a human embryonic stem cell line encoding the cy-
cistic fibrosis mutation deltaF508, using preimplan-
 Human embryonic stem cell lines with genetic disor-
 embryonic stem cells can differentiate into myocytes
 with structural and functional properties of cardiomyo-
24. Xu C, Police S, Rao N et al. Characterization and
25. He QJ, Ma Y, Lee Y et al. Human embryonic stem
 cells develop into multiple types of cardiac myocytes: ac-
26. Mumenery C, Ward-van Oostwaard D, Doevendans
 CA et al. Cardiomyocytes purified from differentiated
 embryonic stem cells exhibit characteristics of early
 chamber myocardium. J Mol Cell Cardiol 2003; 35:
 1461–72.
27. Srin M, Kehat I, Geppstein A et al. Assessment of the
 ultrastructural and proliferative properties of human em-
 bryonic stem cell-derived cardiomyocytes. Am J Physiol
 myobose embryonic stem cells are able to differentiate
 into cardiomyocytes expressing chronotropic re-
sponses to adrenergic and cholinergic agents and Ca2+
29. An RH, Davies MP, Doevendans PA et al. Develop-
 mental changes in beta-adrenergic modulation of
 L-type Ca2+ channels in embryonic mouse heart. Circ
30. Kehat I, Geppstein A, Spira A et al. Electrophysio-
amical integration of cardiomyocytes derived from
31. Davies MP, An RH, Doevendans P et al. Develop-
 mental changes in ion channel activity in the
32. Shih HT. Anatomy of the action potential in the
33. Nigdy DJ. Structure and function of cardiac po-
34. Satin J, Kehat I, Caspi O et al. Mechanism of spop-
taneous excitability in human embryonic stem cell de-
35. Strehler EE, Treiman M. Calcium pumps of plasma
 membrane and cell interior. Curr Mol Med 2004;
 4: 323–35.
36. Tohse N, Seki S, Kobayashi T et al. Development of
 excitation-contraction coupling in cardiomyocytes. Jpn
37. Anderson ME, Al-Khatib SM, Roden DM et al. Car-
diac repolarization: current knowledge, critical
gaps, and new approaches to drug development and pa-
38. Tuganowski W, Tendera M. Components of the ac-
tion potential of human embryonic auricle. Am J Physi-


52. Fukuda K. Development of regenerative cardiomyocytes. Stem Cells in Cardiovascular Biology and Medicine


62. Denning C, Pitts JD. Bystander effects of different enzyme-prodrug systems for cancer gene therapy depend on different pathways for intercellular transfer of toxic metabolites, a factor that will govern clinical choice of appropriate regimes. Hum Gene Ther 1997; 8: 1825–35.


