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Human Pluripotent Stem Cells in Cardiovascular Research and Regenerative Medicine

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1. Introduction

Heart disease is one of the leading causes of mortality worldwide. Because adult cardiomyocytes (CMs) lack the ability to regenerate, malfunctions or significant loss of CMs due to disease or aging can lead to cardiac arrhythmias, heart failure, and subsequently death. Heart transplantation for patients with end stage heat failure is limited by the number of donor organs available. Cell-based therapies offer a promising alternative for myocardial repair, but there are significant challenges involved. The transplantation of human CMs, eg fetal CMs, is difficult for practical and ethical reasons, thus cells of non-cardiac lineage, such as skeletal myoblasts (Murry et al., 1996; Menasche et al., 2003) and mesenchymal stem cells (Shake et al., 2002; Toma et al., 2002), have been considered as alternatives. Animal studies and clinical trials involving these cells have yielded conflicting results. Transplanted non-cardiac cells such as bone marrow-derived hematopoietic cells do not transdifferentiate into the cardiac lineage (Balsam et al., 2004; Murry et al., 2004). They also do not integrate into the host myocardium. For instance, the lack of electrical integration of skeletal myoblasts after their autologous transplantation into the myocardium resulted in the generation of malignant ventricular arrhythmias, which led to the premature termination of clinical trials involving skeletal myoblasts (Menasche et al., 2003; Smits et al., 2003). Therefore, an alternative cell source is needed.

2. Human embryonic stem cells and induced pluripotent stem cells

Human embryonic stem cells (hESCs), isolated from the inner cell mass of blastocysts, can self-renew while maintaining their pluripotency to differentiate into all cell types (Thomson et al., 1998), including CMs (Kehat et al., 2001; Mummery, C. et al., 2002; Xu, C. et al., 2002; Xue et al., 2004; Mummery, C. et al., 2003). Therefore, hESCs may provide an unlimited *ex vivo* source of CMs for cell-based heart therapies. The laboratories of Yamanaka (Takahashi et al., 2007) and Thomson (Yu et al., 2007) showed that adult somatic cells can be

reprogrammed to become pluripotent hES-like cells (a.k.a. induced pluripotent stem cells or iPSCs) via the forced expression of four pluripotency genes (Oct4, Sox2, c-Myc, and Klf4 *or* Oct4, Sox2, Nanog, and Lin28) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Aasen et al., 2008). More recent studies have further demonstrated the successful use of less pluripotency factors (Huangfu et al., 2008; Kim, J. B. et al., 2008; Nakagawa et al., 2008) and non-viral methods (e.g., with synthetic modified RNA (Warren et al., 2010)) to reprogram somatic cells into iPSCs. Although concerns such as induced somatic coding mutations (Gore et al., 2011) have yet to be fully addressed, hiPSCs have morphology, gene expression profile, epigenetic status, *in vitro* and *in vivo* differentiation capacities similar to hESCs (Takahashi et al., 2007; Yu et al., 2007).

3. Differentiation of hESC into CMs

Previous studies studies have demonstrated that hESCs and hiPSCs can spontaneously differentiate into CMs when they aggregate in suspension to form embryoid bodies (Xu, C. et al., 2002; Zwi et al., 2009). Recent studies have focused on improving the yield and purity of CM differentiation. For instance, hESC differentiation into the CM lineage can be enhanced by coculture with visceral endoderm-like cells (Mummery, C. et al., 2003; Mummery, C. L. et al., 2007). Recently, an effective protocol for cardiac differentiation has been successfully developed by the Keller laboratory involving the stage-specific addition of growth factors, including BMP4, Activin-A, DKK, bFGF etc, to drive sequential differentiation into the epiblast, mesoderm and CMs, resulting in greatly increased yield of up to 50%, as gauged by the proportion of cells that express cardiac troponin T (Yang et al., 2008). Other approaches have also been pursued, utilizing different extracellular matrices, serum (Passier et al., 2005) and insulin elimination (Xu, X. Q. et al., 2008). Besides improving the yield of CM differentiation, the isolation of a pure population of CMs is also important in order to prevent malignancy and arrhythmias. Various purification methods have been developed including Percoll gradient centrifugation (Xu, C. et al., 2002), optical signatures (Chan, J. W. et al., 2009) and genetic selection based on the expression of a reporter protein under the transcriptional control of a cardiac-restricted promoter (e.g., α-myosin heavy chain (α-MHC) (Anderson et al., 2007), ventricular myosin light chain (MLC-2v) (Huber et al., 2007; Fu et al., 2010)).

4. Properties of hESC-derived CMs (hESC-CMs)

4.1 Human ESC-CMs have molecular and structural properties of CMs

Gene expression profiles have been examined for hESC-CMs cultured in different laboratories using different differentiation protocols and from different hESC lines (Kehat et al., 2001; Xu, C. et al., 2002; Snir et al., 2003; Norstrom et al., 2006). There is now consensus that hESC-CMs express transcription factors and structural proteins specific to human cardiomyocytes (Kehat et al., 2001; Xu, C. et al., 2002; Snir et al., 2003; Norstrom et al., 2006). Human ESC-CMs express cardiac-specific transcription factors such as NKx2.5, GATA4 and Mef-2 (Kehat et al., 2001; Xu, C. et al., 2002), which are expressed in the precardiac mesoderm but persist in the heart during development. Structural components of the myofibers can also be detected in hESC-CMs. These include α–, β- and sarcomeric-myosin heavy chain (MHC), atrial and ventricular forms of myosin light chain (MLC-2a and –2v), tropomyosin, α-actinin and desmin (Kehat et al., 2001; Xu, C. et al., 2002; Norstrom et al.,

2006). Two members of the troponin complex, cardiac tropinin T, which binds to tropomyosin, and cardiac tropinin I, which regulates Ca^{2+}-sensitive muscle contraction, are also present in hESC-CMs (Kehat et al., 2001; Xu, C. et al., 2002; Norstrom et al., 2006). At the ultrastructural level, hESC-CMs show clearly identifiable sarcomeres and intercalated discs (Kehat et al., 2001; Snir et al., 2003). Morphologically, single hESC-CMs show spindle, round, and tri- or multiangular morphologies, rather than the more defined rod shape of mature cells (Xu, C. et al., 2002). Sarcomeric striations are organized in separated bundles, reminiscent of the pattern seen in human fetal CMs, and rather than the highly organized parallel bundles seen in human adult CMs (Mummery, C. et al., 2003). These data suggest that hESC-CMs display molecular and structural properties consistent with immature human CMs.

4.2 Human ESC-CMs have immature Ca^{2+} handling properties

4.2.1 Mechanism of Ca^{2+}-induced Ca^{2+}-release and Ca^{2+} transient

The contractile apparatus of CMs is dependent on the rise and decay of intracellular Ca^{2+}, known as the Ca^{2+} transient. During an action potential (AP) of adult CMs, Ca^{2+} entry into the cytosol through sarcolemmal L-type Ca^{2+} channels triggers the release of Ca^{2+} from the intracellular Ca^{2+} stores (sarcoplasmic reticulum or SR) via the ryanodine receptors (RyR). This process, the so-called Ca^{2+}-induced Ca^{2+}-release (Bers, 2002), escalates the cytosolic Ca^{2+} ($[Ca^{2+}]i$) to activate the contractile apparatus for contraction. For relaxation, elevated $[Ca^{2+}]i$ gets pumped back into the SR by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and extruded by the Na^{+}-Ca^{2+} exchanger (NCX) to return to the resting $[Ca^{2+}]i$ level. Such a rise and subsequent decay of $[Ca^{2+}]i$ is known as Ca^{2+} transient. Given the central importance of Ca^{2+}-induced Ca^{2+}-release in cardiac excitation-contraction coupling, proper Ca^{2+} handling properties of hESC-CMs are crucial for their successful functional integration with the recipient heart after transplantation. Indeed, abnormal Ca^{2+} handling, as in the case of heart failure, can even be arrhythmogenic.

4.2.2 Human ESC-CMs have functional SRs

Dolnikov et al (2006) were the first to study the Ca^{2+}-handling properties of hESC-CMs in detail (Dolnikov et al., 2006). They reported that Ca^{2+} transients recorded from spontaneously beating or electrically stimulated hESC-CMs respond to neither caffeine nor ryanodine; hESC-CMs recorded as beating clusters also displayed a negative force-frequency relationship that is different from adult CMs. Based on these observations, the authors concluded that hESC-CMs are immature, do not express functional SRs, and that their contractions result from trans-sarcolemmal Ca^{2+} influx (rather than Ca^{2+} release from the SR). Given the paucity of related data, our laboratory performed a comprehensive analysis to better define the Ca^{2+} handling properties of hESC-CMs by comparing Ca^{2+} transients from hESC-CMs and human fetal left ventricular (LV) CMs (16–18 weeks) (Figure 1, adopted with permission from Liu et al 2007 *Stem Cells* Vol. 25, No. 12: pp.3038-3044). Upon electrical stimulation, all of hESC-CMs and fetal LV-CMs generated similar Ca^{2+} transients. However, caffeine induced Ca^{2+} release in 65% of fetal LVCMs and 38% of hESC-CMs. Ryanodine significantly reduced the electrically evoked Ca^{2+} transient amplitudes of caffeine-responsive but not -insensitive hESC-CMs and slowed their upstroke; thapsigargin, which inhibits SERCA, reduced the amplitude of only caffeine-responsive hESC-CMs and slowed the decay (Liu et al., 2007). The discrepancy between our findings and those of Dolnikov et al can be largely attributed to the newly identified caffeine-responsive population.

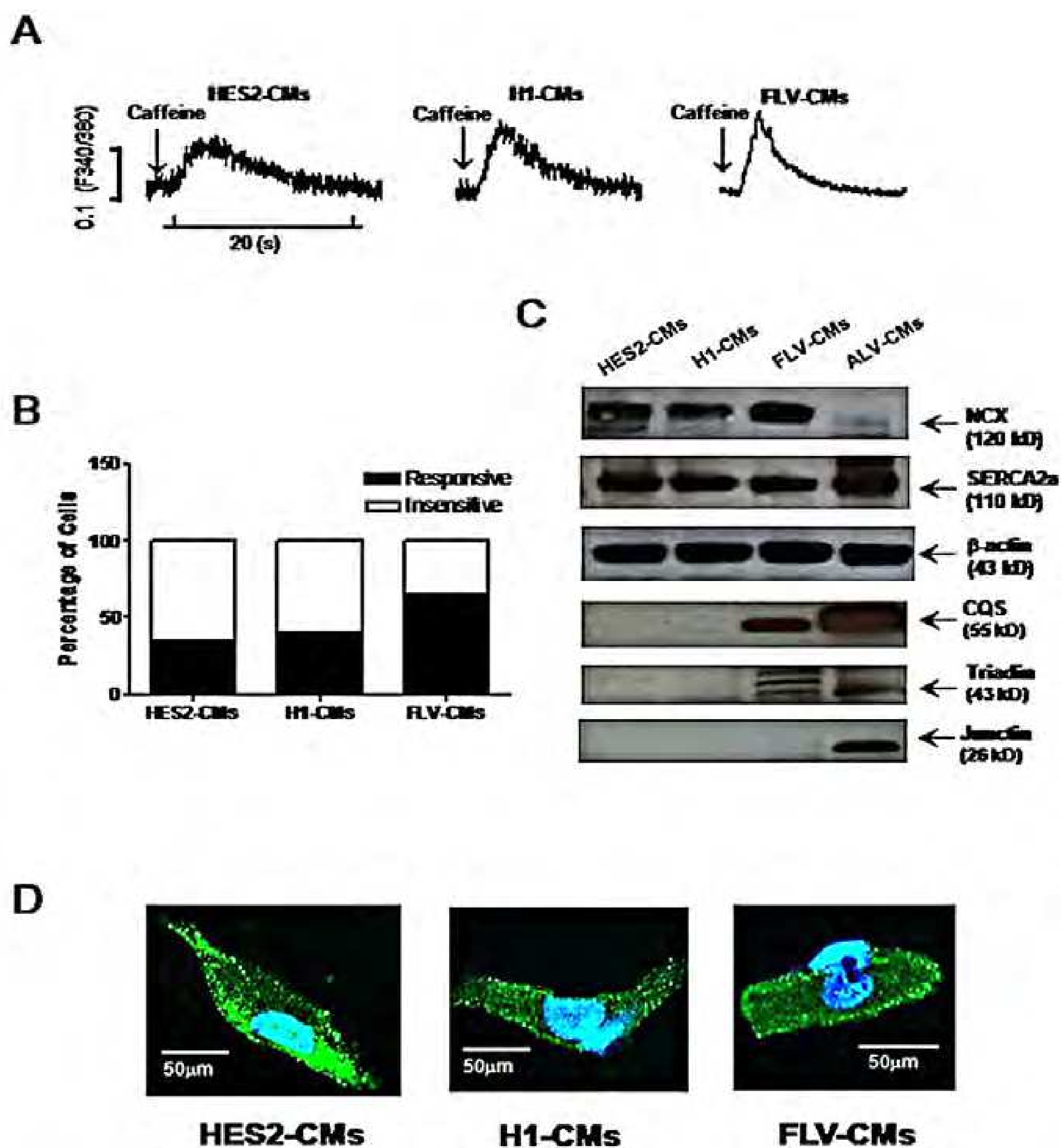

Fig. 1. Ca^{2+} handling properties of hESC-CMs
A) Representative tracings caffeine-induced Ca^{2+} transients of hESC-CMs (HES2-, H1-CMs) and fetal (F) LV-CMs. B) % of caffeine-responsive and -insensitive cells. C) Expression of various Ca^{2+} handling proteins. β-actin was used as the loading control. D) Immunostaining of RyRs. Adopted with permission from Liu et al 2007 *Stem Cells* Vol. 25, No. 12: pp.3038-3044.

4.2.3 Differential expression of Ca^{2+} handling proteins

While hESC-CMs express functional SRs, their Ca^{2+} handling properties are immature and are similar to those of fetal CMs. The functional immaturity of hESC-CMs may be attributed to the expression pattern of Ca^{2+} handling proteins (Figure 1C) (summarized in Table 1, adopted with permission from Kong et al 2010 *Thromb Haemost* Vol. 104, No. 1: pp.30-38). Compared to adult CMs, hESC-CMs express significantly lower levels of RyR, SERCA, phospholamban, calsequestrin and higher levels of calreticulin and NCX (Liu et al., 2007). The regulatory proteins junctin, triadin, and calsequestrin (CSQ) are expressed in adult LV-CMs but are completely absent in hESC-CMs (Liu et al., 2007).

		hESC-CMs	Fetal LVCMs	Adult LVCMs
Expression levels of Ca^{2+}-handling proteins.	RyR	++	++	++++
	SERCA	+++	+++	++++
	Phospholamban	-	++	++++
	CSQ/Triadin/Junctin	-	+	++++
	Calreticulin	++++	++++	+
	NCX	+++	++++	+
Ca^{2+} transient properties	Basal $[Ca^{2+}]_i$	++	+++	++++
	Amplitude	++	++	++++
	Decay	++	++	++++
	Upstroke	++	++	++++

Table 1. Ca^{2+} handling properties of hESC-CMs, fetal and adult LVCMs.
Adopted with permission from Kong et al 2010 *Thromb Haemost* Vol. 104, No. 1: pp.30-38.

4.2.4 T-Tubules are absent in hESC-CMs

Transverse (t) tubules are invaginations in the sarcolemmal membrane that concentrate dihydropyridine receptors and bring them spatially close to RyRs residing on the SR membrane located deeper in the cytoplasm (Brette and Orchard, 2003; Brette and Orchard, 2007). By physically minimizing the diffusion distance, RyRs in CMs can participate in Ca^{2+}-induced Ca^{2+}-release without a lag. The result is a synchronized, faster, and greater transient $[Ca^{2+}]i$ increase from the peripheries to the center, creating a uniform Ca^{2+} wavefront across the transverse section with simultaneous recruitment of all SR. Fast and synchronized activation of RyRs translates into a greater Ca^{2+} transient amplitude, recruitment of more actin–myosin cross-bridge cycling, and generation of greater contractile force. The presence of t-tubules is therefore crucial to the mature Ca^{2+} handling of CMs. Lieu et al used fluorescent staining (Figure 2A and C, adopted with permission from Lieu et al 2009 *Stem Cells Dev* Vol. 18, No. 10: pp.1493-1500) and atomic force microscopy (Figure 2B and D) to detect the presence of t-tubules and showed that the latter is absent in hESC-CMs. Consistent with CMs deficient of t-tubules, hESC-CMs also exhibit a U-shaped Ca^{2+} wavefront that is caused by a delayed Ca^{2+} increase in the central region of the cell relative to the peripheral region (Lieu et al., 2009) (Figure 2E).

4.2.5 Attempts to improve Ca^{2+}-handling properties by genetic modification

We hypothesize that the differential expression of key CM proteins underpins the immaturity of hESC-CMs relative to adult CMs. We tested this idea by overexpressing CSQ in hESC-CMs. CSQ is the most abundant, high-capacity but low-affinity, Ca^{2+}-binding protein in the SR that is anchored to the RyR. The cardiac isoform CSQ2 can store up to 20 mM Ca^{2+} while buffering the free SR $[Ca^{2+}]$ at ~1 mM. This allows repetitive muscle contractions without rundown. While CSQ is robustly expressed in adult CMs, it is completely absent in hESC-CMs (Liu et al., 2007). We hypothesized that forced expression of CSQ in hESC-CMs would induce functional improvement of SR. We tested this hypothesis by transduction of hESC-CMs with the recombinant adenovirus Ad-CMV-CSQ-IRES-GFP (Ad-CSQ) and demonstrated that Ad-CSQ significantly increased the transient amplitude, upstroke velocity, and transient decay compared with the control and a truncated mutant (Liu et al., 2009) (Figure 3, adopted with permission from Liu et al 2009 *Am J Physiol Cell*

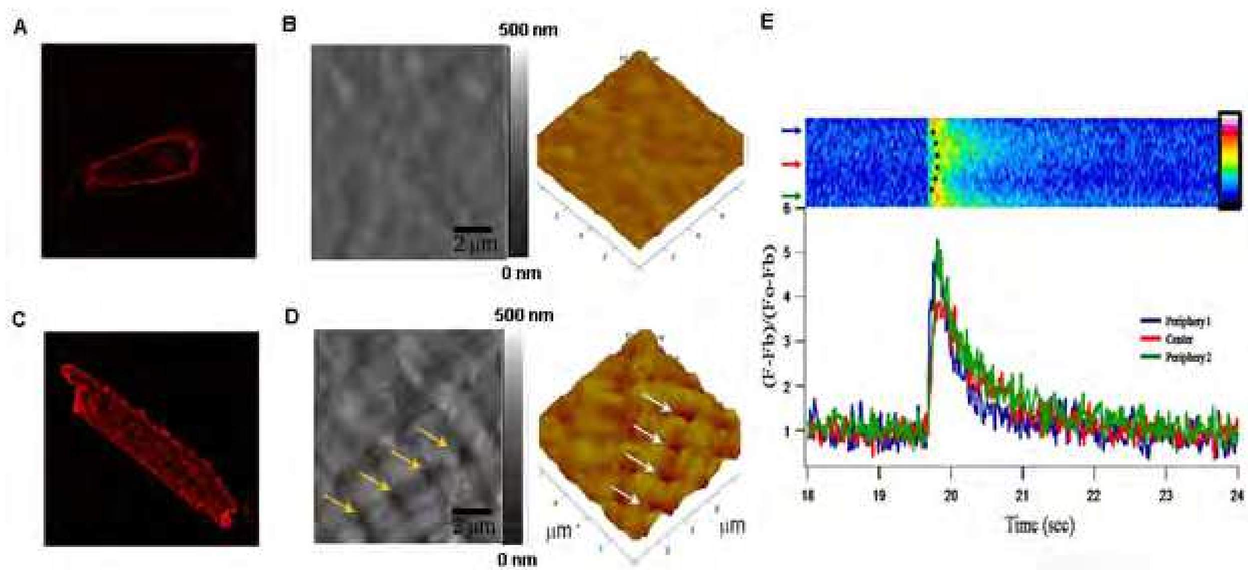

Fig. 2. T-tubule imaging of a hESC-CM and a mature ventricular CM.
Confocal microscopic images of a hESC-CM (A) did not show intracellular fluorescent spots like those in an adult guinea pig ventricular CM (C) suggesting the absence of t-tubules. The absence of t-tubules in ESC-CMs was further confirmed by atomic force microscopy imaging of an adult ventricular cardiomyocyte (D) showing regularly spaced pores in the sarcolemma that coincide with the Z-lines, while hESC-CM (B surface showed comparatively smoother topology with no presence of invaginations that are indicative of t-tubules. E) Electrically induced Ca^{2+} transient in hESC-CMs. Top: Time progression linescans of pseudo-colored transient increase in intracellular Ca^{2+} across the mid-plane of a hESC-CM showed a U-shaped wavefront. Bottom: Quantified Ca^{2+} transient of linescans of the top panel. Adopted with permission from Lieu et al 2009 *Stem Cells Dev* Vol. 18, No. 10: pp.1493-1500.

Physiol Vol. 297, No. 1: pp.C152-159). These results showed that immature Ca^{2+}-handling properties of hESC-CMs can be rescued by genetic modification and improved our understanding of CM maturation.

4.3 Human ESC-CMs demonstrate immature electrophysiological properties similar to 'embryonic' CMs

He et al (2003) were the first to study the electrophysiological properties of hESC-CMs (He et al., 2003). They characterized the contractions and action potentials (APs) from beating EB outgrowths cultured for 40 to 95 days and showed that hESC can differentiate into a heterogeneous mixture of CMs, with APs classified as 'nodal-like', 'embryonic ventricular-like' and 'embryonic atrial-like', analogous to CM specification into pacemaker, ventricular and atrial CMs. The latter two classes are considered 'embryonic' based on their Maximum diastolic potential and more depolarized resting membrane potential, and "slow" type APs based on low dV/dt_{max}. Unlike adult CMs, which are normally electrically silent yet are excitable upon stimulation, the majority of hESC-CMs fire spontaneously, exhibiting a high degree of automaticity. Our laboratory examined triggered activity and found that 'embryonic ventricular-like' CMs exhibit delayed after depolarization, suggesting that hESC-CMs can be arrhythmogenic.

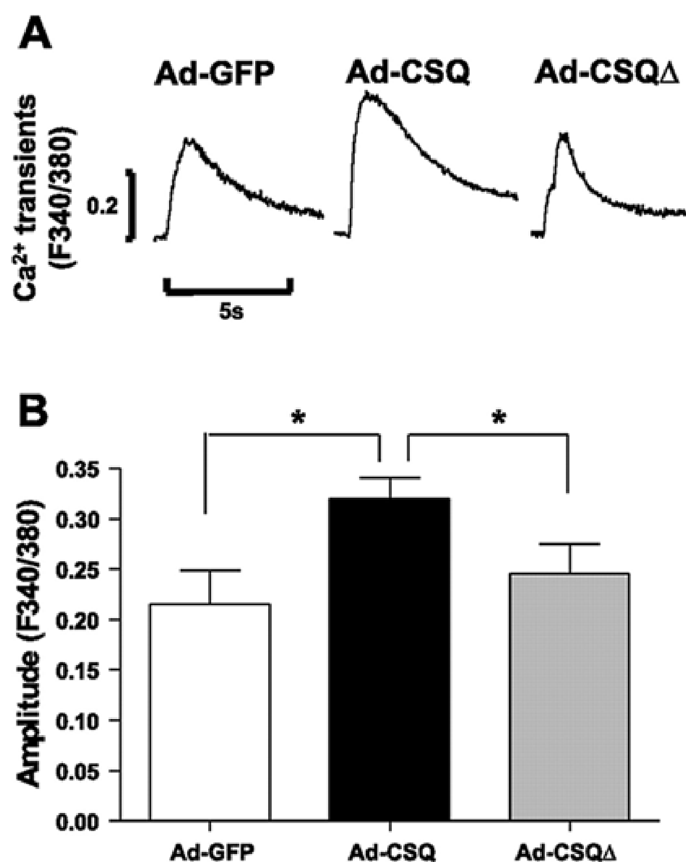

Fig. 3. Effect of CSQ overexpression on hESC-CMs.
A) Representative electrically-induced Ca^{2+} transient tracings for Ad-GFP (n=12) and Ad-CSQ (n=29) and Ad-CSQΔ (truncated mutant) (n=14) transduced hESC-CMs. B) Bar graphs of amplitude.* $P < 0.05$, ** $P < 0.01$. Adopted with permission from Liu et al 2009 *Am J Physiol Cell Physiol Vol. 297, No. 1*: pp.C152-159.

Subsequent studies were done to assess if hESC-CMs mature over time to acquire electrophysiological properties that are comparable to those of adult CMs and results are conflicting. Sartiani et al (2007) examined the AP of CMs over 3 months of culture and concluded that the molecular and functional expression of ion channels in hESC-CMs change over time, although they still do not reach the phenotype typical of adult VCMs (Sartiani et al., 2007). This is in contrast to findings by Pekkanen-Mattila et al (2010), which show that although one third of hESC-CMs exhibit a more mature phenotype, these changes are not correlated with time in culture (Pekkanen-Mattila et al., 2010). Taken together, these data suggest that hESC-CMs are functionally immature and present an arrhythmogenic risk. Therefore, facilitated *in vitro* maturation is important for the translation of hESC-CMs to the clinic and other applications (such as disease modeling, drug discovery and cardiotoxicity screening).

Our group sought to define the immature proarrhythmic electrophysiological properties observed in hESC-CMs by examining the role of different currents in automaticity (Azene et al., 2005; Siu et al., 2006; Xue et al., 2007; Lieu et al., 2008; Chan, Y. C. et al., 2009). I_{K1} (the inward-rectifier K^+ current encoded by Kir2.1), which stabilises a negative resting membrane potential, is important for suppressing automaticity and we hypothesize that its

absence in hESC-CMs may underlie their immature phenotype. Consistent with this, forced Kir2.1 expression alone sufficed to render the electrical phenotype indistinguishable from that of primary adult ventricular cells (Lieu DK, Fu JD and Li RA, unpublished data). These proof-of-concept experiments show that developmentally arrested Ca^{2+} and electrophysiological phenotypes of hESC-CMs can be rescued. We are currently developing a non-genetic, non-pharmacologic method to drive global maturation, by targeting the microenvironmental niches and other non-cell autonomous means.

5. The use of hESC-CMs for myocardial repair and bioartificial pacemakers

Myocardial infarction is the major worldwide cardiovascular disorder in humans and is the leading cause of death in many parts of the world. Immediately after a heart attack, oxygen starvation of myocardial tissues leads to cell death, often resulting in irreversible and permanent damage to the heart. Despite some improvements in short term management of acute myocardial infarction, long term prognosis remains poor. Sudden cardiac death due to ventricular arrhythmias remains a leading cause of morbidity and mortality in the industrialised world, claiming well over 300,000 lives annually in the United States alone. After myocardial infarction, the heart undergoes hypertrophy in an attempt to compensate for loss of CMs, and cardiac fibroblasts secrete collagen and other extracellular matrix proteins during scar formation, leading to impaired cardiac function. Since terminally differentiated CMs have very limited potential for regeneration, transplantation is the only treatment for end-stage heart failure. However, this is hampered by the lack of suitable donor organs and tissues. Cell-based therapy is thus a promising option for myocardial repair. A range of cell sources have been considered, including bone marrow cells, skeletal myoblasts and smooth muscle cells, but their non-cardiac identity has presented major problems. They either do not differentiate into the cardiac lineage or they do not integrate well into the host myocardium (Menasche et al., 2003; Smits et al., 2003; Balsam et al., 2004; Murry et al., 2004). As discussed in previous sections, hESC-CMs have functional and structural properties very similar to human embryonic/fetal CMs and is therefore a very promising cell source. Transplantation of hESC-CMs to mouse/rat hearts showed that the cells survived, formed myocardial tissue and promoted functional improvement in rat models of myocardial infarction (Laflamme et al., 2007; Mignone et al., 2010). More detailed studies into the maturation of hESC-CMs, as well as the electropysiological consequences of hESC-CM transplantation are required before these early successes can be translated into clinical therapies.

Normal rhythms originate in the SA node (SAN), a specialized cardiac tissue consisting of only a few thousands pacemaker cells. Malfunction of cardiac pacemaker cells due to disease or aging can cause rhythm generation disorders. Current treatments include pharmacological intervention and/or implantation of electronic pacemakers, but they are associated with significant shortcomings (e.g. increased susceptibility to infection, haemorrhage, lung collapse and death infection, finite battery life, patient discomfort related to the permanent implantation of a foreign device, and lack of intrinsic responsiveness to neural and hormonal regulation). Therefore 'bioartificial-pacemakers', made up of transplanted cells with pacemaker properties, may be a desirable alternative.

There are two major strategies for creating bioartificial-pacemakers. The first is to confer pacemaker ability on cells that are normally silent. For instance, adult atrial and ventricular

CMs are electrically silent unless they are stimulated by signals transmitted from the SAN. This is due to the absence of I_f, encoded by the HCN channel family, and the presence of I_{K1}. Several gene-based approaches have been pursued to induce pacemaker activity in these normally silent cells. Our group took a protein engineering approach to define criteria important for pacing and created a bioengineered construct of HCN (Lesso and Li, 2003; Tsang et al., 2004; Tsang et al., 2004; Tse et al., 2006; Xue et al., 2007). This engineered-construct was shown to produce pacing *in vitro* and *in vivo* (Tse et al., 2006; Xue et al., 2007). In a sick sinus syndrome porcine model, pacing of the heart was restored and originated from the site of focal transduction in the left atrium with HCN-construct injection (Tse et al., 2006). Somatic gene transfer to create such a gene-based bioartificial pacemaker significantly reduces the dependence on device-supported pacing by electronic pacemaker from 85% to 15%. Alternatively, hESCs can be differentiated into pacemaker-like derivatives for transplantation to recreate a cell-based bioartificial pacemaker (Kehat et al., 2004; Xue et al., 2005). Of note, the construction of cell-based bioartificial-pacemakers requires much fewer cells (several thousands) than myocardial transplantation (hundreds of millions). Furthermore, the spherical SAN is structurally less complex than the left ventricular myocardium. Our group is currently exploring the possibility of using nodal progenitors. We are also testing non-invasive catheter-based delivery techniques for implantation as well as long-term safety and efficacy.

6. Creation of engineered cardiac tissue constructs

The ventricular myocardium is a highly complex structure consisting of aligned, connected CMs, stromal cells and a vascular network systematically embedded in a mesh of extracellular matrix. Indeed, hESC-CMs differentiated *in vitro* lack the sub-cellular organization and higher order structural 2- or 3-dimensionality seen in adult heart. To more closely recapitulate the *in vivo* environment of the heart, various groups have used different approaches to manipulate the surface and geometry of the culture platform, cell and matrix composition. For instance, Luna et al used a tunable culture platform comprised of biomimetic wrinkles to simulate the heart's complex anisotropic and multiscale architecture and showed that the hESC-CMs cultured on these 'microgrooved' substrates display the typical tropomyosin banding pattern consistent with organized sarcomeric structure patterns (Luna et al., 2011) (Figure 4, adopted with permission from Luna et al 2011 *Tissue Eng Part C Methods* Vol. 17, No. 5: pp.579-588). Quantitative assessment based on nuclei shape and actin organization show that the hESC-CMs exhibit increased alignment on microgrooved substrates compared with controls. Functionally, aligned monolayers of hESC-CMs display anisotropic conduction properties with distinct longitudinal and transverse velocities, a signature characteristic of the native heart, not seen in control randomly organized monolayers (Lieu, Wang, Khine and Li, unpublished data). In another approach to mimic the structure of the heart, the Costa lab was among the first to construct 3-D engineered cardiac tissue constructs including cardiac papillary-like muscle strips as well as ventricle-like "organoid" chambers that exhibit key characteristics of cardiac physiology by ejecting fluid and displaying force-frequency and pressure-volume relationships (Kim, Do Eun et al., 2006; Lee et al., 2008). These studies were originally performed using rat cardiac myocytes but are now being applied to hESC/iPSC-CMs. Further optimization of hESC/iPSC-based cardiac tissue constructs will not only provide powerful tools for disease modeling, drug/cardiotoxicity screening and clinical translations,

but physiologic 3D environment also promises to reveal novel insights not possible with conventional rigid 2D culture systems.

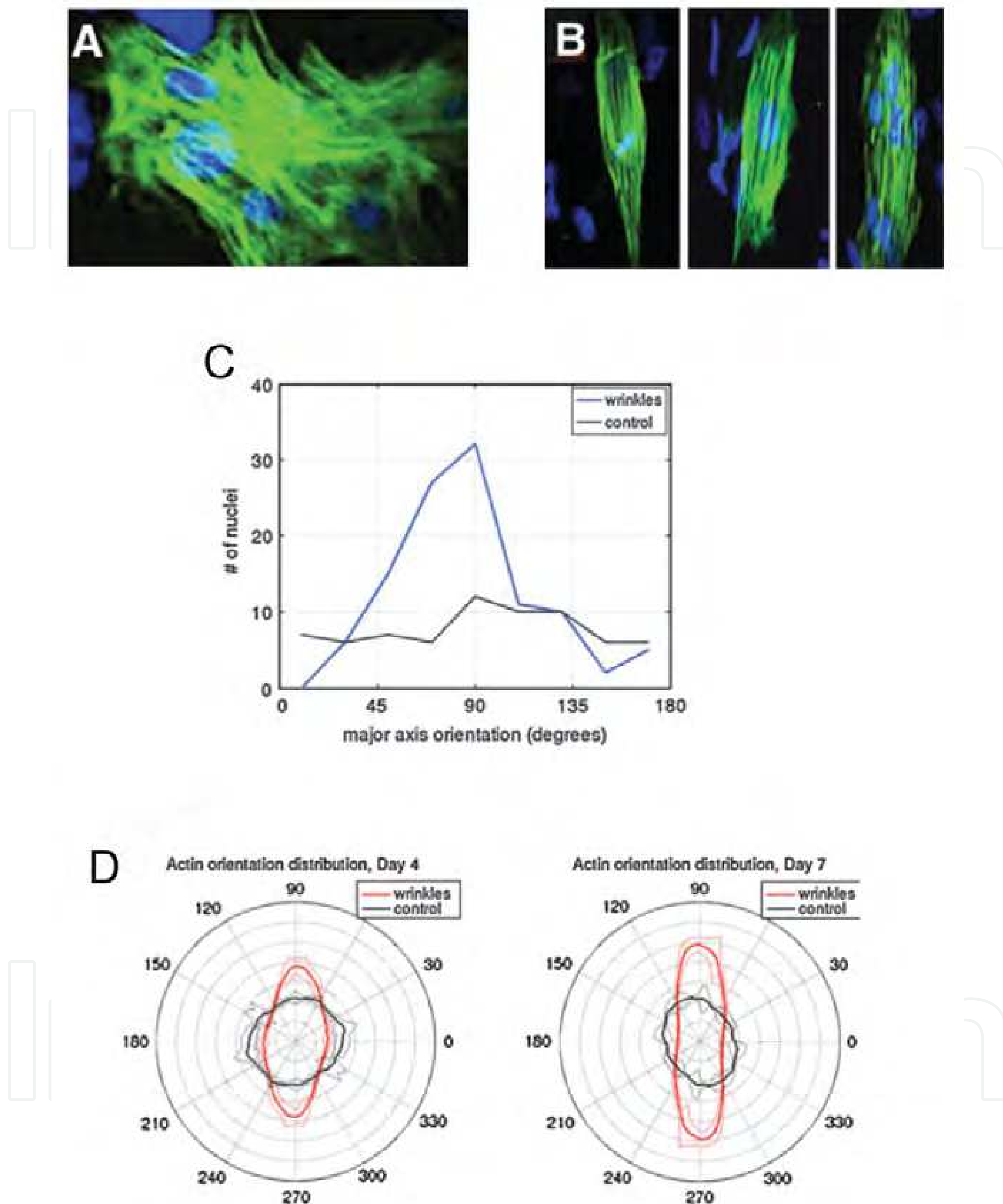

Fig. 4. Confocal micrographs of hESC-derived CMs alignment on wrinkles.
Human ESC-CMs were isolated and cultured on flat substrate (A) and wrinkle substrates (B) for 8 days. Green indicates tropomyosin staining, blue nuclear staining DAPI.
C) Image processing was used to detect the orientation of the DAPI-labeled nuclei. D) Anisotropy analysis of control (black) versus green (on wrinkles) showing that 90° is direction of wrinkles. The thinner lines indicate the standard deviations. Adopted with permission from Luna et al 2011 *Tissue Eng Part C Methods* Vol. 17, No. 5: pp.579-588.

7. Pharmacological testing using hESC-CMs

Adverse cardiac side effects is one of the most frequent reasons that cause drugs to be removed from the market. For instance, Vioxx, a once widely used COX-2 inhibitor prescribed to patients with arthritis and other conditions causing acute and chronic pain, was withdrawn from the market due to unexpected cardiotoxicity. Therefore, cardiotoxicity screening is necessary to test the efficacy and safety of new drug treatments. Cardiotoxicity arises from various mechanisms, including the modulation of signaling pathways and/or interference with the I_{Kr} current. The hERG channel, which produces the I_{Kr} current, is robustly blocked by a large class of drugs. This current has a major role in cardiac repolarization, so it affects the length of the action potential and the QT interval (the duration of ventricular depolarization and subsequent repolarization). QT prolongation may lead to arrhythmias, e.g. torsade de pointes, with potentially lethal consequences. Since adult CMs do not proliferate, animal CMs, isolated hearts or non-cardiac cells, which express cardiac ion channels are used although their non-human origin and instability in culture greatly limit their usefulness. Human ESC and hiPSC may provide an unlimited cell source for cardiotoxicity screening. Preliminary studies have already established that hESC-CMs/hiPSC-CMs do express the hERG mRNA (Sartiani et al., 2007; Tanaka et al., 2009), and display an outward 'I_{Kr}-like' current that is sensitive to selective blockers of I_{Kr} and canonical long QT-inducing inhibitors including E4031, sotalol, and cisapride (Sartiani et al., 2007; Caspi et al., 2009). When treated with a range of cardiac and non-cardiac drugs, hESC-CM also exhibit dose responses predictive of clinical effects (Braam et al., 2010). These suggest that hESC-CMs may be a suitable model for cardiotoxicity testing, although issues of CM purity and maturation should be considered in the design of future experiments, as already discussed elsewhere. It is hoped that high-throughput pharmacological systems involving hESC/hiPSC-CMs will soon be developed.

8. Human ESC-CMs/hiPSC-CMs as models of cardiovascular diseases

The use of hESC-/hiPSC-CMs as models of cardiac disorders is an exciting area of research. Previously, transgenic mouse models were used to study human cardiac diseases, but these mouse models do not always fully recapitulate the same phenotypes as those seen in humans. For instance, electrophysiologically, mice also have shorter action potential duration and higher heart rate compared to humans (Danik et al., 2002), limiting their usefulness as models of some disorders such as arrhythmias. Human pluripotent stem cell derived CMs are therefore logical suitable alternative. Reprogramming technology pioneered by Yamanaka and Thomson has led to the creation of disease- or patient-specific iPSCs. The derivation of hiPSC from patients with a range of diseases including adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Parkinson disease, Huntington disease, juvenile-onset, type 1 diabetes mellitus, Down Syndrome/trisomy 21, and the carrier state of Lesch-Nyhan syndrome has been reported (Park et al., 2008). More recently, Ebert et al (2009) reported the generation of hiPSC line from a patient of spinal muscular atrophy and these cells maintained the disease genotype and generated motor neurons that showed selective deficits compared to those derived from the patient's unaffected mother (Ebert et al., 2009). Some advances have also been made in the area of cardiovascular research. For instance, hiPSC models of long-QT syndrome type 1

and type 2 were generated (Moretti et al., 2010; Itzhaki et al., 2011). CMs differentiated from these hiPSC recapitulated the electrophysiological features of the disorders such as prolongation of AP and arrhythmogenicity and enabled the groups to study the pathogenesis of the diseases. Itzhaki et al (2011) also used the hiPSC-CMs to evaluate the potency of existing and novel pharmacological agents that may either aggravate or ameliorate the long-QT syndrome type 2 disease phenotype. These studies illustrate the potential of human iPSC technology to model the abnormal functional phenotype of inherited cardiac disorders and to identify potential new therapeutic agents.

9. Conclusion

Adult CMs lack the potential to regenerate. Human ESC and hiPSCs, with their potential for unlimited self-renewal and differentiation, offer an exciting means of generating human CMs for research and regenerative medicine. Concentrated effort by research groups worldwide has resulted in higher efficiency of cardiogenic differentiation and better characterization of hESC-/hiPSC-CMs. Experiments using animal models have demonstrated functional improvement after hESC-CM transplantation. However, substantial hurdles have to be overcome before hESC-/hiPSC-CMs can be translated into clinical applications. For instance, hESC-CMs are functionally immature, limiting their use for transplantation and as disease models. More studies are required to evaluate the long term effect of hESC-/hiPSC-CMs transplantation. Nonetheless, there is much reason to believe that hESC and hiPSC technology will bring significant benefit to cardiac research and treatment.

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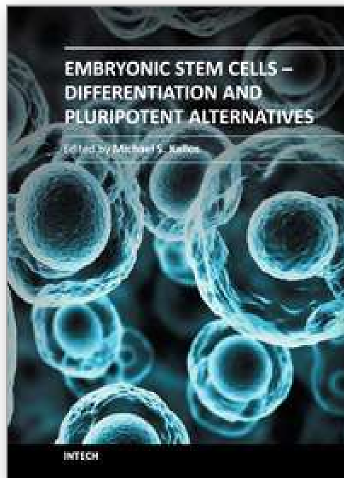

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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