

Human embryonic stem cell-derived cardiomyocytes: demonstration of a portion of cardiac cells with fairly mature electrical phenotype

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Abstract

Cardiomyocytes (CMs) derived from human embryonic stem cells (hESC) provide a promising tool for the pharmaceutical industry. In this study the electrical properties and maturation of hESC-CM derived using two differentiation methods were compared and the suitability of hESC-CMs as a cell model for the assessment of drug-induced repolarization delay was evaluated. CMs were differentiated either in END-2 co-culture or by spontaneous differentiation. Action potentials (APs) were recorded from cells in spontaneously beating areas using the whole-cell patch-clamp technique. The hESC-CMs exhibited predominantly a ventricular-like phenotype with heterogeneous properties. Heterogeneity was indicative of the spectrum of hESC-CM maturation from embryonic-like with AP upstroke velocities <30 V/s and maximum diastolic potential (MDP) of close to -60 mV to more mature with values >150 V/s and -80 mV, respectively. The mean MDP was -70 mV and a significant difference was observed between the two differentiation methods (-66 versus -75 mV, $P < 0.001$). The age of the CMs did not correlate with phenotype maturation. The addition of the hERG blocker E-4031 and the sodium channel modulator veratridine significantly prolonged the AP duration. Furthermore, proarrhythmic indices were induced. In conclusion, the main observation was the heterogeneity in electrical properties of the hESC-CMs and this was observed with both differentiation methods. One-third of the hESC-CMs exhibited fairly mature electrophysiological properties, suggesting that mature CMs could be obtained from hESCs. However, improved differentiation methods are needed to produce homogeneous mature human CMs for pharmaceutical and toxicological applications.

Keywords: human embryonic stem cells, cardiomyocytes, differentiation, action potential, cell model, pharmacological testing

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Introduction

Adverse drug reactions, such as syncope, arrhythmia and sudden death, related to torsade de pointes (TdP), a polymorphic ventricular tachycardia, have led to the revision of prescribing information, the refusal of approval or the withdrawal from the market of drugs.¹ In the absence of a complete understanding and direct analysis of TdP, the regulatory authorities have adopted a surrogate biomarker for the possible development of drug-induced TdP, the QT interval of the electrocardiogram (ECG).

Prolongation of the QT interval resulting from a delay in ventricular repolarization, whether drug induced or for

instance congenital arising from mutation of genes (to date LQT1–12), can be associated with TdP,^{1,2} though the relationship is complex.³ The QT interval is the cornerstone of the guidelines for the assessment of new chemical compounds in regard to proarrhythmic potential.^{4,5} Inhibition of the hERG channel ($K_{v}11.1$), which carries the rapid component of the delayed rectifier potassium current (I_{Kr}), is the predominant basis of drug-induced QT prolongation and TdP.^{6,7} Currently a number of preclinical models and assays including *in vivo* QT assays, such as ECG telemetry of conscious dogs, and *in vitro* assays, such as of the hERG channel assay,⁸ have been employed by

pharmaceutical companies. However, current methods give rise to both false-positives and negatives.^{6,9} The use of a battery of non-clinical assays minimizes the risk of clinical QT prolongation but the data are not 100% predictive.⁸

Human embryonic stem cells (hESC) can be induced to differentiate into cardiomyocytes (CMs) by spontaneous differentiation in embryoid bodies (EBs),¹⁰ in co-culture with END-2 cells^{11,12} or with defined growth factors.¹³ Differentiated beating cells express the cardiac complement of proteins and ionic currents, and exhibit spontaneous action potentials (APs) and contractile activity.^{10,11,14} According to electrophysiological studies, the hESC-derived CM (hESC-CM) population is heterogeneous with nodal-, atrial- and ventricular-like cells present.¹⁴ During differentiation and after beating has commenced, the expression of some ion channel genes increases, suggesting that hESC-CM reach a more mature state with time in culture.¹⁵ The hESC-CM may serve as a unique *in vitro* model to bolster drug safety screening but also for target validation and electrophysiological studies.

The aim of this work was to systematically characterize the AP parameters of hESC-CM either by spontaneous differentiation in EBs or with END-2 co-culture and whether these methods would influence the maturation of CMs with time. In addition, the suitability of hESC-CMs for the assessment of drug-induced repolarization delay and as a cellular model of proarrhythmia was evaluated.

Methods

Cell culture

Cell culture was performed as previously described.¹⁶ The hESC-lines Regea 06/015 and Regea 06/040 derived at Regea (Institute for Regenerative Medicine, University of Tampere, Finland), and HS346 and HS360 derived at the Fertility Unit of Karolinska University Hospital, Huddinge (Karolinska Institutet, Stockholm, Sweden) were used. Regea has the approval from the Ethical Committee of Pirkanmaa Hospital District to culture and derive hESC lines.

Differentiation of cardiomyocytes

CM differentiation in co-culture with END-2 cells was done as previously described.¹⁶ Differentiation in EBs was performed by mechanically dissecting the hESC-colonies into pieces and they were grown in EB-medium containing knockout DMEM (Invitrogen, Carlsbad, CA, USA), 20% fetal bovine serum (Invitrogen), 2 mmol/L GlutaMax (Invitrogen), 1% non-essential amino acids (Cambrex Bio Science Inc, Walkersville, MD, USA), and 50 U/mL penicillin/streptomycin (Cambrex). After seven days of suspension culturing, EBs were plated on 0.1% gelatin (Sigma-Aldrich, Munich, Germany)-coated cell culture wells in EB medium. Medium was changed every 2–3 d.

Dissociation of beating cells

The beating areas were dissociated to smaller cell aggregates or single cells after differentiation. The cells were analyzed within

a week after dissociation. The beating areas of the cell colonies were dissociated by collagenase II.¹¹ Dissociated cells were plated on 0.1% gelatin-coated coverslips in EB medium.

Immunocytochemistry

Dissociated beating cells were immunostained with anti-cardiac troponin T (1:2000) (Abcam, Cambridge, UK), anti-myosin (ventricular heavy chain alpha/beta) (1:100) and anti-connexin-43 (1:2000) (Chemicon Temecula, CA, USA). Immunocytochemistry was performed as described.¹⁶ Secondary antibodies used were either Alexa Fluor-488 or -568 (Invitrogen) (1:400) conjugated donkey anti-mouse, anti-rabbit or anti-goat antibodies. Nuclei were Dapi-stained (Vectashield, Mounting Medium with DAPI, Vector Labs, Burlingame, CA, USA).

Electrophysiological measurements

APs were recorded from cells in spontaneously beating areas using the whole-cell configuration of the patch-clamp technique with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). A coverslip with the adhered cells was placed in the recording chamber and perfused with extracellular solution consisting of (in mmol/L): 143 NaCl, 4 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 5 glucose, 10 HEPES (pH 7.4 with NaOH; osmolality adjusted to 301 ± 3 mOsm). The osmolality was measured with an Osmostat OM-6020 osmometer (DIC Kyoto Daiich Kagaku Co Ltd, Kyoto, Japan). Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) and had resistances of 1.5–3 M Ω when filled with a solution consisting of (in mmol/L): 130 KCl, 7 NaCl, 1 MgCl₂, 5 Na₂ATP, 5 EGTA, 5 HEPES (pH 7.2 with KOH; osmolality adjusted to 290 ± 3 mOsm).

To measure calcium currents the extracellular solution consisted of the following (in mmol/L): 137 TEA-Cl, 5.4 CsCl, 2 CaCl₂, 1 MgCl₂, 10 glucose and 5 HEPES (pH 7.4 with TEA-OH; osmolality adjusted to 298 mOsm) and the intracellular solution consisted of the following (in mM): 115 Cs methanesulfonate, 20 CsCl, 2.5 MgCl₂, 2 MgATP, 11 EGTA and 10 HEPES (pH 7.2 with CsOH; osmolality adjusted to 273 mOsm). These recordings were undertaken in voltage-clamp mode with cell capacitance and series resistance compensated the latter by $\geq 70\%$. Calcium currents were elicited from a holding potential of -60 mV by 500 ms voltage steps from -50 to $+40$ mV.

Noradrenaline (Research Biochemicals Inc, Natick, MA, USA) and E-4031 (Alomone Labs, Jerusalem, Israel) were dissolved in water while veratridine (Sigma, St Louis, MO, USA) was dissolved in dimethyl sulfoxide, all to obtain stock solutions of 10 mmol/L from which the final concentrations were prepared daily by dilution with extracellular solution. Experiments were conducted at $35.7 \pm 0.1^\circ\text{C}$. The concentrations of the drugs were: 100 nm noradrenaline, 100 nmol/L E-4031 and 10 $\mu\text{mol/L}$ veratridine.

Statistical analysis

Data acquisition and analysis was performed with pClamp 9.2 software (Molecular Devices). Data are given

as mean $\pm$ standard deviation (SD) of n experiments. Statistical significance of differences was analyzed using one-way analysis of variance test with Bonferroni correction. In the AP figures the dashed line denotes 0 mV.

Results

Differentiation of hESC-CM

Differentiation was performed by two methods: END-2 co-culture and spontaneous differentiation in EBs. Both methods yielded beating cells 12–20 d after differentiation initiation. Differentiated beating cells were cardiac troponin T-positive and most of them ($\sim 80\%$) were also ventricular myosin heavy chain-positive. According to connexin-43 stainings, hESC-CMs had gap junctions between them (Figure 1).

General properties of hESC-CM excitability

Basic electrical properties of CMs obtained from different hESC lines were very similar and thus the results from different lines have been pooled. The spontaneous electrical activity of hESC-CMs exhibited mostly (65/70 cells) a continuous pattern where AP rates were relatively constant throughout the recording period (Figure 2a), with frequencies ranging from 0.4 to 2.6 Hz (mean = 1.2 ± 0.1 Hz). EB-derived CMs were slightly more homogenous since 97% of them beat continuously, while 89% of END2-derived CMs exhibited continuous firing pattern. The remaining hESC-CMs displayed firing patterns either of more than one frequency or episodic in nature (Figure 2b). Exposure of hESC-CMs to noradrenaline increased the frequency of AP firing, concomitant with which was an increase in the diastolic depolarization rate and a decrease in AP duration (APD; Figure 3).

Based on the AP morphology and parameters, such as APD at 90% repolarization (APD₉₀), maximum rise of the

AP upstroke ($dV/dt_{\max}$), AP amplitude (APA) and maximum diastolic potential (MDP), the AP phenotype was classified as nodal-, atrial- or ventricular-like. Atrial-like APs were defined as those with a triangular shape whereas those APs with a significant plateau phase were categorized as ventricular-like.^{14,17} None of the APs (from 69 hESC-CMs patched) fulfilled the criteria adopted for classification as nodal-like, i.e. slow upstroke, prominent phase 4 depolarization, relatively depolarized MDP and small APA. Atrial-like APs were few as ventricular-like predominated (Table 1), distinguished by, respectively, their triangular shape and significant plateau phase (Figure 4a and b). The beating rate of atrial-like CMs was on the average 48/min while the rate of ventricular-like CMs was on the average 78, explaining at least partly the observed longer AP duration in atrial-like CM versus ventricular-like APs (Table 1). All the atrial-type CMs were obtained with the END-2 differentiation method, while none of the EB-derived CMs demonstrated atrial-like AP. The prevalence of ventricular type CMs was 80% (28/35) and 100% (34/34) with the END-2 and EB differentiation methods, respectively.

Within the ventricular-like AP category there was heterogeneity in AP shapes (Figure 4a) and properties. For instance the $dV/dt_{\max}$ ranged from 15.8 to 302.5 V/s. The APs exhibiting a fast upstroke ($dV/dt_{\max} \geq 200$ V/s) were associated with a significantly more hyperpolarized MDP compared with APs with low upstroke ($dV/dt_{\max} \leq 100$ V/s) (Figure 4c, $P < 0.05$). The average MDP was less than -70 mV, ranging from -55 mV to over -80 mV (Figure 4c).

From this the question remains can this diversity in AP characteristics be related to the two methods used to derive the hESC-CMs and/or the time in culture? This is addressed in Figure 4c, 5 and supplementary Figure 1. The $dV/dt_{\max}$ was similar whether differentiation was via END-2 co-culture or spontaneously within the EB and between the various times in culture (Figure 5a). A difference was evident between the differentiation methods in

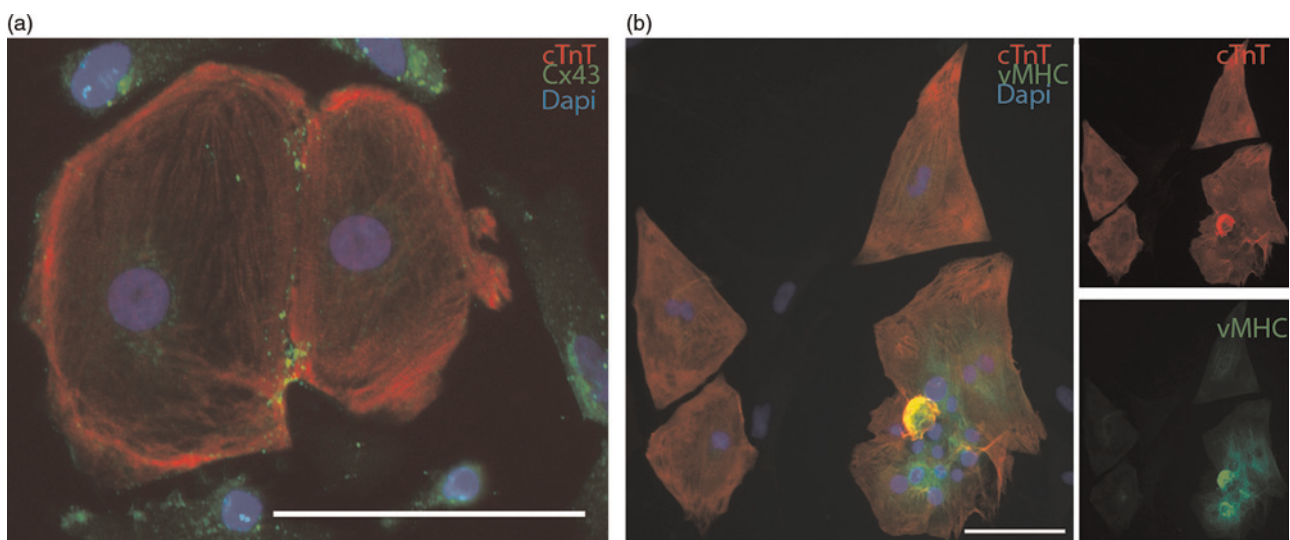


Figure 1 Beating cells were cardiac troponin T-positive (red) with connexin-43 (green) located at the contact surfaces of beating cells (a). Some of the cardiac troponin T-positive cells are also stained positively with ventricular myosin heavy chain (green) (b). Scale bar: 100 μ m

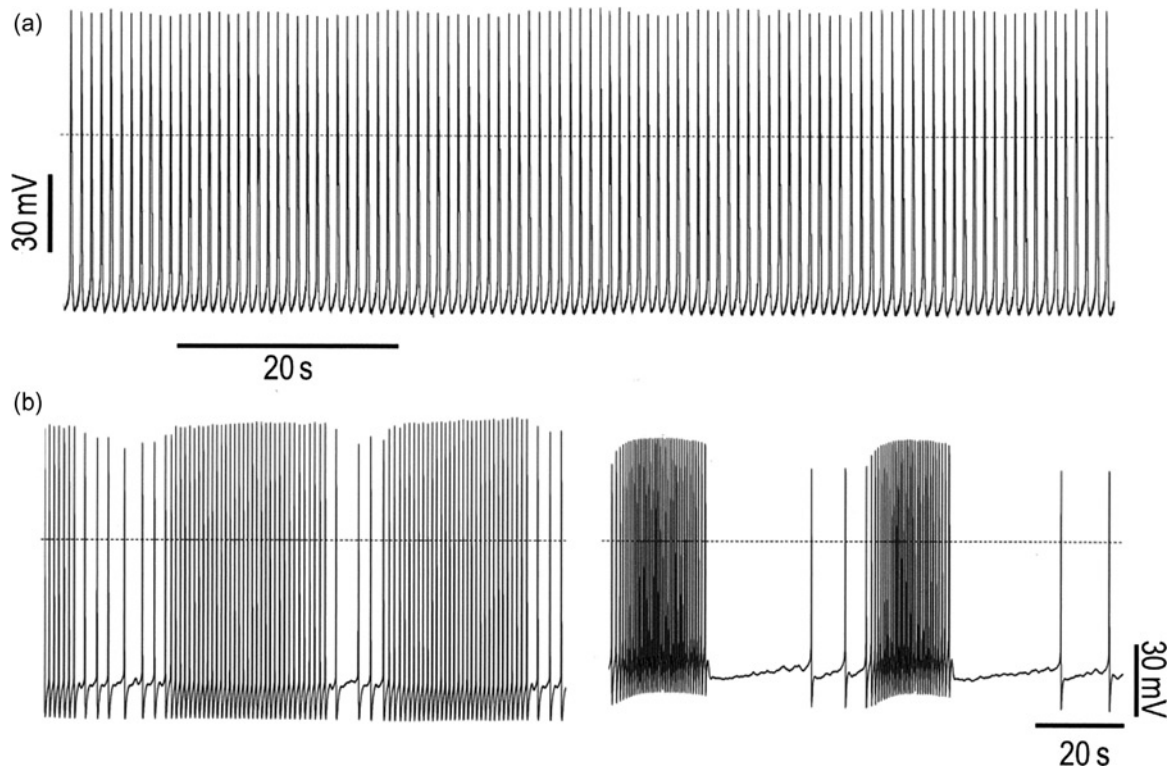


Figure 2 Spontaneous AP firing activity of hESC-CMs. (a) Spontaneous continuous activity and (b) examples of variable (left) and episodic (right) AP firing activity. AP, action potential; hESC-CMs, human embryonic stem cell-derived cardiomyocytes

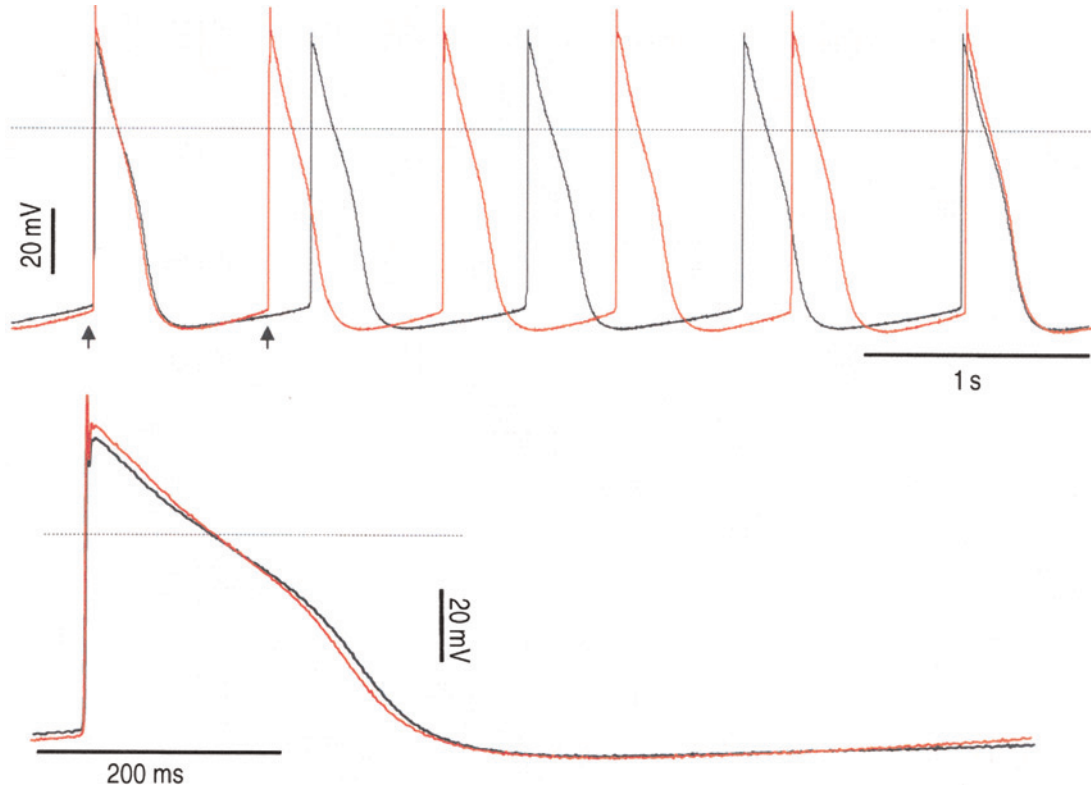


Figure 3 Effect of noradrenaline (100 nmol/L) on hESC-CMs. Overlaid on the APs of the basal state (black) are those of the same cell on later exposure to noradrenaline (red), with the arrows denoting the section enlarged below. Note that with the increase in AP frequency there was an increase in the diastolic depolarization rate (mean from 12.0 to 17.3 mV/s) and a reduction in APD (the mean APD₅₀ and APD₉₀ decreases from 158.1 and 251.0 ms, respectively, to 141.3 and 241.5 ms). AP, action potential; hESC-CMs, human embryonic stem cell-derived cardiomyocytes; APD, AP duration. (A color version of this figure is available in the online journal)

Table 1 Characteristics of the spontaneous APs of hESC-CMs

	APD ₉₀ (ms)	dV/dt _{max} (V/s)	APA (mV)	MDP (mV)
Atrial-like (END-2) (n = 7)	257.6 ± 56.2	29.3 ± 23.4	91.0 ± 8.1	-61.7 ± 4.6
Ventricular-like (END-2) (n = 28)	264.5 ± 55.7	129.4 ± 105.0	113.3 ± 10.7	-67.3 ± 4.5
Ventricular-like (EB) (n = 34)	232.4 ± 89.5	154.6 ± 55.7	120.3 ± 10.0	-74.8 ± 3.9

AP, action potential; APD₉₀, AP duration at 90% repolarization; dV/dt_{max}, maximum rise of the AP upstroke; APA, AP amplitude; MDP, maximum diastolic potential; hESC-CMs, human embryonic stem cell-derived cardiomyocytes

terms of the MDP with the EB groups exhibiting a significantly more hyperpolarized MDP (Figures 4c and 5b). Time of culture did not have an effect on MDP (Figure 4c, 5b and supplementary Figure 1). Older beating cells (>48 d) had APD₉₀ slightly but not significantly shorter than younger cells (<40 d; Figure 5c). This finding also argues against the increased maturation of cardiac cells with time in culture.

hESC-CMs as cellular models of QT prolongation and proarrhythmia

Addition of the selective hERG inhibitor E-4031 resulted in a marked prolongation of the hESC-CM AP (Figure 6a), especially for terminal repolarization (phase 3) as evident from the greater effect on APD₉₀ compared with APD₅₀

(inset in Figure 6a). Such slowing of repolarization leads to triangulation of the AP. An analogous result was observed with inhibition of sodium channel inactivation by veratridine (Figure 6c), which mimics the sodium current (*I*_{Na}) defect of congenital LQT3 and is another mechanism for drug-induced QT prolongation.¹⁸ Furthermore as shown in Figure 6b the APD₉₀ evolved from smooth changes between APs in control (seen as a tight cluster) to, with E-4031 exposure, successive lengthening and later to deviation from unity as successive large changes occur, corresponding to beat-to-beat variability, i.e. APD instability.

Triangulation of the AP, among other effects, accelerates L-type calcium channel recovery from inactivation.¹⁹ The reactivation of these channels during repolarization can generate membrane oscillations or early after depolarizations (EADs).¹⁹ The presence of the L-type Ca²⁺ current

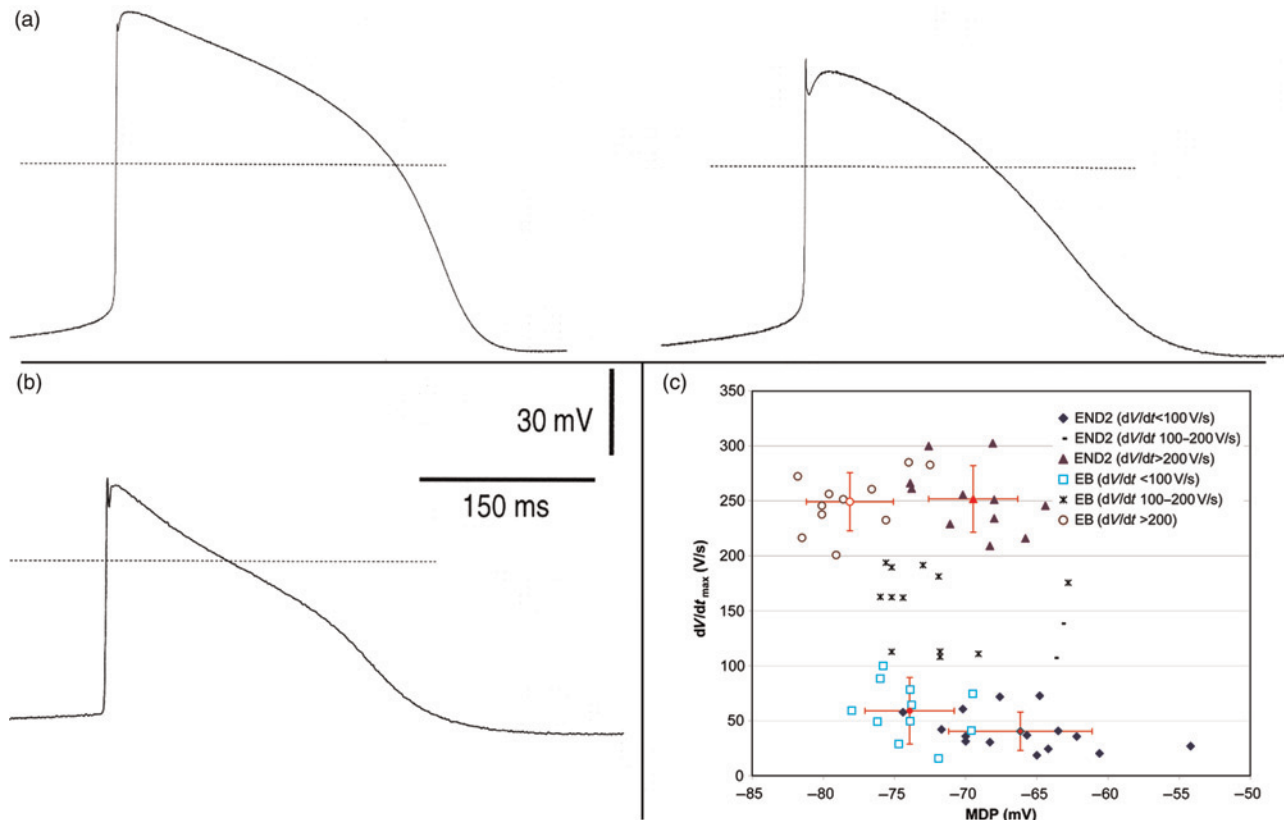


Figure 4 AP morphology and characteristics. hESC-CMs exhibited ventricular-like APs, of diverse morphologies (a) and properties, and atrial-like APs (b). (c) Plot of the maximum upstroke velocity against the maximum diastolic potential of the hESC-CMs with a ventricular-like phenotype (n = 62). There was a significant difference in MDP (*P* < 0.05) between hESC-CM APs with dV/dt_{max} values of ≥200 V/s and those with dV/dt_{max} of ≤100 V/s. Significant difference was also seen when the differentiation method was taken into account; the EB differentiated cells had more polarized MDP compared with END-2 differentiated cells (*P* < 0.05). Means and the SD of the END-2 and EB differentiated cells are indicated in red. AP, action potential; hESC-CMs, human embryonic stem cell-derived cardiomyocytes; MDP, maximum diastolic potential; EB, embryoid body

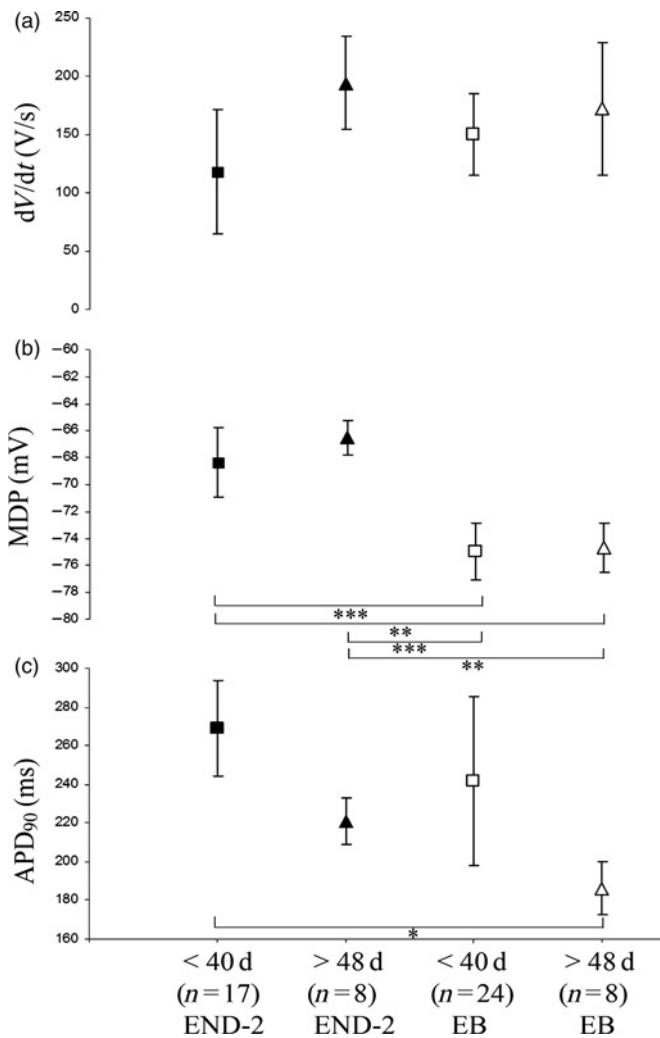


Figure 5 Effect of the hESC-CM differentiation method (END-2 co-cultured, filled symbols or EB, open symbols) and the time (d) in culture on AP characteristics. Only the ventricular type cells were included in the analysis. Means and the SD are presented and *P* values are indicated as **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. AP, action potential; hESC-CMs, human embryonic stem cell-derived cardiomyocytes; EB, embryoid body

(Figure 7a) and EADs (Figure 7b) during prolonged E-4031 exposure in hESC-CMs was readily demonstrable. Another form of triggered activity, delayed after depolarizations, which take place after full repolarization, occurred on occasion spontaneously (Figure 7c).

Discussion

In the present study the hESC-CMs were derived both by spontaneous differentiation and by END-2 co-culture. The main emphasis was to determine whether the differentiation methods influence the AP properties or the maturation of the hESC-CMs. Most of the differentiated CMs were ventricular type with a difference between the differentiation methods (80% and 100% of END-2 and EB-derived CMs, respectively). The differentiation methods had also some effect on AP properties. However, both of the methods resulted in CMs with a great heterogeneity in their electrical properties with as many as about one-third of them

presenting a fairly mature phenotype. Unlike a previous report¹⁵ the AP-phenotype and maturity of the hESC-CM did not correlate with the age of the CMs.

The beating area, in line with previous studies,^{11,20} acted as a functional syncytium with cell-to-cell coupling between the hESC-CMs evident from the homogeneity of the AP frequency within beating areas of cells and the staining of the gap junction protein connexin-43, as well as the synchronous nature of the intracellular calcium oscillations between cells (data not shown). These findings were the same with both differentiation methods. The AP firing was usually constant, but in a few cells different firing patterns were observed confirming earlier findings.¹⁴ The occurrence of other firing patterns may derive from intermittent conduction block as a consequence of structural discontinuities within the cellular network.²⁰ Furthermore, the hESC-CMs exhibited a positive chronotropic response with noradrenaline administration. This effect is already present in human fetal CMs^{10,11,15} and has been reported with hESC-CMs with other inotropic agents.^{16,21}

The ventricular phenotype and its properties were heterogeneous in the current study, consistent with the results of prior reports.^{11,14,17} The heterogeneity was indicative of the spectrum of hESC-CM maturation from embryonic-like with, e.g. AP upstroke velocities <30 V/s and MDP of close to -60 mV to more mature with dV/dt_{max} values >150 V/s (up to ~300 V/s) and MDP values reaching -80 mV. The ventricular-like AP properties previously reported have been mostly comparable to those of cultured human fetal myocytes, with MDPs of ~-50 mV and upstroke velocities of <30 V/s,^{11,15,17} even though more mature cardiac phenotypes in hESC-CMs have also been reported.²² However, even one-third of our hESC-CMs exhibited a more mature phenotype with MDPs of less than -70 mV and upstroke velocities >140 V/s. The age of our beating cells varied between 30 and 60 d, which is in the same range to that used by Cao *et al.*¹⁷ (20–48 d). Slightly older cells were used by He *et al.* (40–95 d)¹⁴ while the widest range in the age of cells was in the study from Caspi *et al.*²³ (15–90 d). However, the phenotype of the cardiac cells was not demonstrated to mature with time in these studies. In one previous study electrical maturation with time has been reported.¹⁵ The expression of channel subunit transcripts and the density of certain currents were shown to change over time during *in vitro* differentiation.^{15,23} These parameters were not analyzed in our study. Furthermore, Sartiani *et al.*¹⁵ reported significant changes in the AP properties of hESC-CMs <40 d post-differentiation versus those >50 d.¹⁵ The APD of the older group was larger with a greater variability, suggested to originate from the appearance of atrial and ventricular-like AP phenotypes, and the dV/dt_{max} significantly increased with time from 4.2 to 6.0 V/s. These observations may reflect the difference in the electrophysiological maturity of the hESC-CMs. Similar results were not, however, observed in our study and, thus, time in culture does not explain the difference in electrical properties in our study. The only major difference between our hESC-CMs and the previously reported ones is that our hESC-lines have been cultured only on human feeder cells. It is possible that

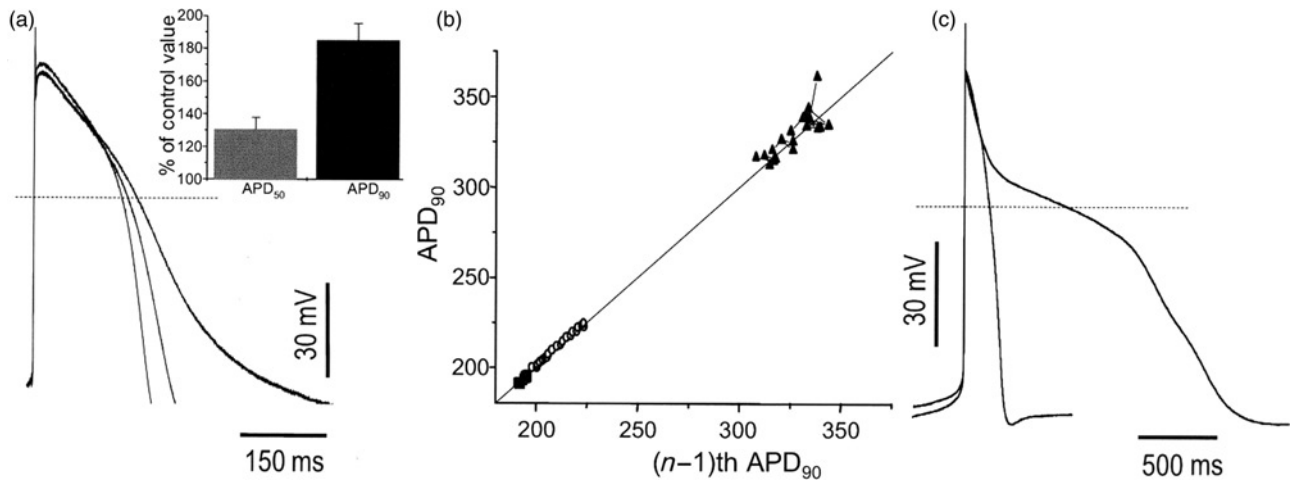


Figure 6 Effect of E-4031 (a and b) and veratridine (c) on hESC-CMs. (a) Exposure to E-4031 (100 nmol/L) progressively prolongs the AP and gives rise to triangulation (APD_{90}/APD_{50} ; for those APs shown the value in control is 1.23 and in E-4031, 1.31 and 1.65 with short and longer exposure). Inset, change in the ventricular-like APD_{50} and APD_{90} with E-4031 from basal ($n = 3$). (b) Plot of APD_{90} against the preceding APD_{90} value showing the instability of APD with exposure. Each group (control -■-, E-4031 -○- and with longer exposure -▲-) contains 20 consecutive data points (including those from the APs in [a]). (c) Veratridine (10 μ mol/L) prolongs the AP. The APD_{50} and APD_{90} values of the APs shown are 156.8 and 227.7 ms (for control) and 728.6 and 1476.7 ms, respectively; thus there was also triangulation. The effect of veratridine, unlike E-4031, was completely reversible. AP, action potential; hESC-CMs, human embryonic stem cell-derived cardiomyocytes; APD_{90} , AP duration at 90% repolarization

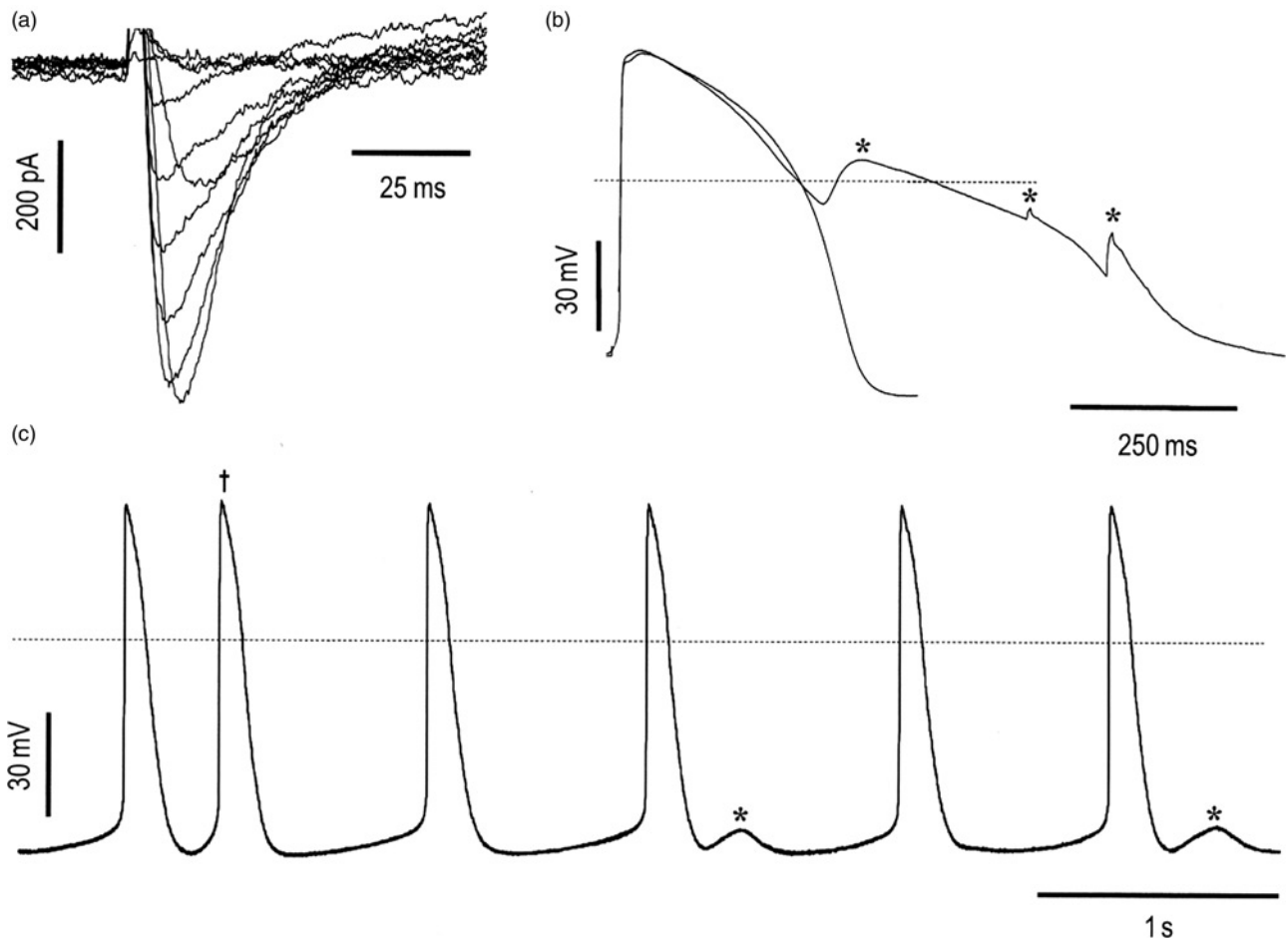


Figure 7 (a) Activation of L-type calcium current by voltage steps from -50 to $+40$ mV. (b) Occurrence of EADs (*) as the AP prolongs in the continued presence of 100 nmol/L E-4031. (c) Occurrence of delayed after depolarizations (*), which are associated with activation of the Na^+/Ca^{2+} exchanger following diastolic sarcoplasmic reticulum Ca^{2+} release, that if reach threshold can initiate premature APs (†) and underlie certain arrhythmias. AP, action potential; EAD, early after depolarization

human feeders still secrete an unknown factor that makes the stem cells capable of obtaining more mature cardiac phenotype. The demonstration of the presence of CMs with $dV/dt_{\max}$ of over 150 and MDP of close to -80 is very important and suggests that mature human cardiac cells can be produced from hESCs, but the optimal conditions are still to be defined.

In earlier studies of AP characteristics, the differentiation method appears to affect little the hESC-CM phenotype, with similar embryonic-like properties obtained whether EB^{14,15,17,23} or END-2 co-culturing¹¹ is employed. We compared the differentiation methods directly and the previous observation is supported for certain AP parameters ($dV/dt_{\max}$ and APD_{90}), but not with all (MDP). MDPs of EB-derived CMs were significantly more negative than those of END-2 co-culture, suggesting a more mature phenotype. All the previous studies have also demonstrated electrical heterogeneity, thus confirming our data that these two differentiation methods do not differ from each other in producing heterogeneous cardiac cells with phenotypes ranging from immature to fairly mature.

In regard to the use of hESC-CMs as a model for proarrhythmia, in the current study the addition of the hERG blocker E-4031 or the sodium channel modulator veratridine significantly prolonged the AP duration. Furthermore, proarrhythmic indices (triangulation and APD instability as well as EADs) were induced. The importance of the hERG channel/IKr in the repolarization of the AP of hESC-CMs, as for the adult human ventricle,^{24,25} is evident here as elsewhere.^{14,15,23} IKr block delays repolarization prolonging the AP which may be either antiarrhythmic, due to the concomitant increase in the refractory period, or proarrhythmic.²⁶ Underlying the latter mechanism are several interacting phenomena including AP triangulation that leads to the occurrence of EADs, which can provide a trigger, and augmentation of the inherent spatial dispersion of repolarization which results in heterogeneity of refractoriness, that creates a substrate for re-entry.^{3,27} As occurs in whole-heart preparations,²⁶ the proarrhythmia indices of triangulation and APD instability could be induced, as illustrated here with E-4031, in hESC-CMs (Figure 6a and b) with EADs subsequently elicited (Figure 7b). Thus the hESC-CM could provide a cellular model sensitive enough to detect such signals and potentially capable of distinguishing proarrhythmic toxicity and antiarrhythmic efficacy.

The repertoire of cardiac ion channels is endogenously expressed by hESC-CMs, unlike heterologous expression in transfected cells currently used in long QT assays.^{6,8} The effects of new chemical compounds could therefore be investigated in cells with native cardiac electrophysiological phenotype. The heterogeneous nature of hESC-CMs, the phenotype and property diversity is, however, an important limitation at the moment in studying the effects of both acute and long-term exposure of new chemicals.

Conclusion

Our hESC-CMs exhibited electrophysiological heterogeneity, but also fairly mature adult cardiac phenotype could

be detected. The two differentiation methods used in this study produced similar cardiac cell heterogeneity. The demonstration of cells with fairly mature electrical phenotype, however, suggests that with more specific and detailed differentiation methods, mature and more homogeneous CM cultures could be obtained. Utilization of these hESC-CMs could in the future fill a niche, enabling an integrated assessment of a drug's antiarrhythmic efficacy (AP prolongation) and proarrhythmic toxicity *in vitro*. Importantly, hESC-CMs would provide a human model, abolishing the problem of interspecies variability in repolarizing currents.^{28,29} Thus, while complementing existing *in vitro* and *in vivo* cardiac safety assays, hESC-CMs may increase the level of predictivity of clinical outcome in regards to QT interval prolongation⁸ and also other cardiac safety aspects, as well as provide cells for future cell replacement therapies.

Author contributions: MP-M performed all tissue culture work with cardiac cells and their analysis, and wrote the manuscript; HC performed all electrical analysis and wrote about them; EK did planning and performed cardiac cell differentiation; RS provided facilities and part of funding; HS provided hESCs; A-PK provided expertise in electrophysiology and designed the electrical experiments; KA-S is leader of the group, planned the experiment, evaluated the results and finalized the manuscript.

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REFERENCES

- 1 Roden DM. Drug-induced prolongation of the QT interval. *N Engl J Med* 2004;**350**:1013–22
- 2 Zareba W, Cygankiewicz I. Long QT syndrome and short QT syndrome. *Prog Cardiovasc Dis* 2008;**51**:264–78
- 3 Shah RR, Hondeghem LM. Refining detection of drug-induced proarrhythmia: QT interval and TRLAD. *Heart Rhythm* 2005;**2**:758–72
- 4 ICH: S7B: The nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. 2005
- 5 ICH: E14: the clinical evaluation of QT/QTc interval prolongation and proarrhythmic potential for non-antiarrhythmic drugs. 2005
- 6 Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, Siegl PK, Strang I, Sullivan AT, Wallis R, Camm AJ, Hammond TG. Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. *Cardiovasc Res* 2003;**58**:32–45
- 7 Hancox JC, McPate MJ, El Harchi A, Zhang YH. The hERG potassium channel and hERG screening for drug-induced torsades de pointes. *Pharmacol Ther* 2008;**119**:118–32
- 8 Pollard CE, Valentin JP, Hammond TG. Strategies to reduce the risk of drug-induced QT interval prolongation: a pharmaceutical company perspective. *Br J Pharmacol* 2008;**154**:1538–43

- 9 Lu HR, Vlamincx E, Hermans AN, Rohrbacher J, Van Ammel K, Towart R, Pugsley M, Gallacher DJ. Predicting drug-induced changes in QT interval and arrhythmias: QT-shortening drugs point to gaps in the ICHS7B guidelines. *Br J Pharmacol* 2008;**154**:1427–38
- 10 Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001;**108**:407–14
- 11 Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere AB, Passier R, Tertoolen L. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 2003;**107**:2733–40
- 12 Passier R, Oostwaard DW, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, de la Riviere AB, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells* 2005;**23**:772–80
- 13 Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* 2007;**25**:1015–24
- 14 He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ Res* 2003;**93**:32–9
- 15 Sartiani L, Bettiol E, Stillitano F, Mugelli A, Cerbai E, Jacon ME. Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach. *Stem Cells* 2007;**25**:1136–44
- 16 Pekkanen-Mattila M, Kerkela E, Tanskanen JM, Pietila M, Pelto-Huikko M, Hyttinen J, Skottman H, Suuronen R, Aalto-Setälä K. Substantial variation in the cardiac differentiation of human embryonic stem cell lines derived and propagated under the same conditions – a comparison of multiple cell lines. *Ann Med* 2009;**41**:360–70
- 17 Cao F, Wagner RA, Wilson KD, Xie X, Fu JD, Drukker M, Lee A, Li RA, Gambhir SS, Weissman IL, Robbins RC, Wu JC. Transcriptional and functional profiling of human embryonic stem cell-derived cardiomyocytes. *PLoS ONE* 2008;**3**:e3474
- 18 Milberg P, Reinsch N, Wasmer K, Monnig G, Stypmann J, Osada N, Breithardt G, Haverkamp W, Eckardt L. Transmural dispersion of repolarization as a key factor of arrhythmogenicity in a novel intact heart model of LQT3. *Cardiovasc Res* 2005;**65**:397–404
- 19 Guo D, Zhao X, Wu Y, Liu T, Kowey PR, Yan GX. L-type calcium current reactivation contributes to arrhythmogenesis associated with action potential triangulation. *J Cardiovasc Electrophysiol* 2007;**18**:196–203
- 20 Kehat I, Gepstein A, Spira A, Itskovitz-Eldor J, Gepstein L. High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: a novel *in vitro* model for the study of conduction. *Circ Res* 2002;**91**:659–61
- 21 Reppel M, Pillekamp F, Lu ZJ, Halbach M, Brockmeier K, Fleischmann BK, Hescheler J. Microelectrode arrays: a new tool to measure embryonic heart activity. *J Electrocardiol* 2004;**37**(Suppl.):104–9
- 22 Satin J, Kehat I, Caspi O, Huber I, Arbel G, Itzhaki I, Magyar J, Schroder EA, Perlman I, Gepstein L. Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes. *J Physiol* 2004;**559**:479–96
- 23 Caspi O, Itzhaki I, Arbel G, Kehat I, Gepstein A, Huber I, Satin J, Gepstein L. *In vitro* electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. *Stem Cells Dev* 2009;**18**:161–72
- 24 Jost N, Virag L, Bitay M, Takacs J, Lengyel C, Biliczki P, Nagy Z, Bogats G, Lathrop DA, Papp JG, Varro A. Restricting excessive cardiac action potential and QT prolongation: a vital role for IKs in human ventricular muscle. *Circulation* 2005;**112**:1392–9
- 25 Li GR, Feng J, Yue L, Carrier M, Nattel S. Evidence for two components of delayed rectifier K⁺ current in human ventricular myocytes. *Circ Res* 1996;**78**:689–96
- 26 Hondeghem LM, Carlsson L, Duker G. Instability and triangulation of the action potential predict serious proarrhythmia, but action potential duration prolongation is antiarrhythmic. *Circulation* 2001;**103**:2004–13
- 27 Belardinelli L, Antzelevitch C, Vos MA. Assessing predictors of drug-induced torsade de pointes. *Trends Pharmacol Sci* 2003;**24**:619–25
- 28 Akar FG, Wu RC, Deschenes I, Armoundas AA, Piacentino V III, Houser SR, Tomaselli GF. Phenotypic differences in transient outward K⁺ current of human and canine ventricular myocytes: insights into molecular composition of ventricular Ito. *Am J Physiol Heart Circ Physiol* 2004;**286**:H602–9
- 29 Dumaine R, Cordeiro JM. Comparison of K⁺ currents in cardiac Purkinje cells isolated from rabbit and dog. *J Mol Cell Cardiol* 2007;**42**:378–89

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