

Stem Cell–Derived Cardiomyocytes Demonstrate Arrhythmic Potential

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Background—Cardiomyocytes (CMs) derived from pluripotent embryonic stem cells (ESCs) and embryonal carcinoma cells (ECCs) have some but not all characteristics of adult myocytes. ESCs have shown the ability to engraft in areas of myocardial damage, which suggests their use in cell transplantation therapy for cardiomyopathy. We studied the arrhythmogenic properties of CMs differentiated from mouse ESCs and ECCs.

Methods and Results—CMs derived in vitro were studied in the whole-cell patch-clamp mode. CMs from both sources showed action potential (AP) morphology heterogeneity, with reduced maximum upstroke velocities (dV/dt) and prolonged AP durations. CMs demonstrated prolonged, spontaneous electrical activity in culture. Frequent triggered activity was observed with and without pharmacological enhancement. Phase 2 or 3 early afterdepolarizations could be induced easily by Bay K8644 plus tetraethylammonium chloride (TEA) or [TEA]_o after Cs⁺ replacement for [K⁺]_o, respectively. A combination of bradycardic stimulation, hypokalemia, and quinidine resulted in early afterdepolarizations. Delayed afterdepolarizations could be induced easily and reversibly by hypercalcemia or isoproterenol.

Conclusions—ESCs or ECCs differentiated into at least 3 AP phenotypes. CMs showed spontaneous activity, low dV/dt, prolonged AP duration, and easily inducible triggered arrhythmias. These findings raise caution about the use of totipotent ESCs in cell transplantation therapy, because they may act as an unanticipated arrhythmogenic source from any of the 3 classic mechanisms (reentry, automaticity, or triggered activity). (*Circulation*. 2002;106:1294-1299.)

Key Words: cells ■ arrhythmia ■ grafting ■ electrophysiology ■ cardiomyopathy

Approximately 4.7 million Americans suffer from congestive heart failure. The associated mortality and costs are considerable.¹ Cardiac transplantation is limited by the availability of organs. Recently, pluripotent stem cells have shown the ability to engraft in areas of myocardial damage, differentiate into cardiomyocytes (CMs), form intercalated disks, and improve cardiac function.^{2–4} These observations suggest the possibility of cardiac cell transplantation as a treatment for cardiomyopathy. If transplanted myocyte electrophysiology is abnormal, they could contribute to arrhythmias, however. Mechanisms by which they might increase the likelihood of arrhythmias include demonstration of persistent automaticity, slow conduction, or triggered activity, but this potential has not been assessed. Any proclivities would be exacerbated by the ischemic milieu that surrounds the engrafted cells. We evaluated the electrophysiological properties of CMs derived from embryonic stem cells (ESCs) and embryonal carcinoma cells (ECCs) to determine the extent to which they might show arrhythmic proclivity.

Methods

Differentiation of CMs

Mouse ESC R1⁵ and ECC P19⁶ cell lines were used throughout the study. Because of difficulties in the identification and isolation of in

situ differentiated CMs, we used an in vitro differentiation model. Undifferentiated cells were maintained and passaged in high-glucose DMEM (GibcoBRL) supplemented with 15% FBS, 200 mmol/L L-glutamine, 5×10^{-5} mol/L β -mercaptoethanol, 10 mmol/L nonessential amino acids, and 5000 U/mL penicillin/streptomycin. ECCs were expanded on 0.1% gelatin-coated Petri dishes in monolayers to 80% confluence before being split. ESCs were cocultured with mouse embryonic feeder cells. Differentiation was initiated by suspending 400 to 800 ESCs/ECCs in 20 μ L of media supplemented with 20% FBS (for both ESCs and ECCs) and 1% DMSO (for ECCs only) as hanging drops for 2 days. The resulting embryoid body (EB) was cultured for an additional 5 days in suspension. Seven-day-old EBs were plated onto Petri dishes.

Isolation of CMs

CMs were isolated from EBs 12 to 15 days after plating. Spontaneously beating areas of 5 to 10 EBs were dissected and incubated for 30 minutes at 37°C with digesting solution containing (in mmol/L) NaCl 120, KCl 5.4, MgSO₄ 5, Na-pyruvate 5, glucose 20, taurine 20, and HEPES 10, as well as 1 mg/mL collagenase B (Roche) and 30 μ mol/L CaCl₂, pH 6.9. The dissociation process was continued in high K⁺ solution (in mmol/L): KCl 85, K₂HPO₄ 30, MgSO₄ 5, EDTA 1, Na₂ATP 2, pyruvic acid 5, creatine 5, taurine 20, glucose 20, pH 7.4, for 1 hour at room temperature. Single CMs were replated on coverslips coated with 0.1% gelatin and 20 μ g/mL laminin in cultivation medium and incubated at 37°C.

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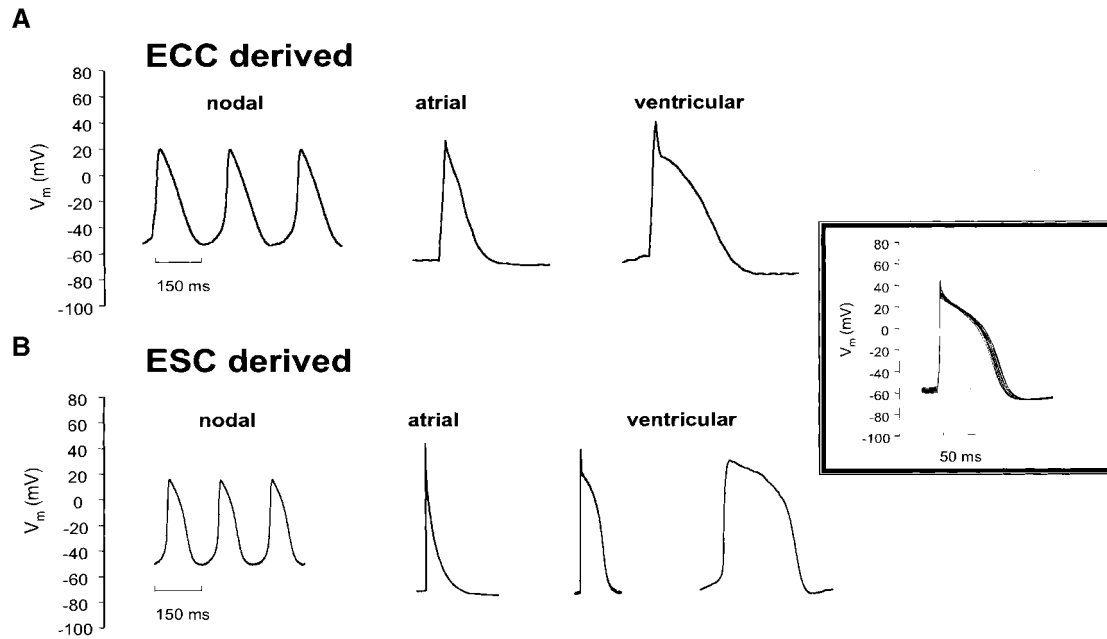


Figure 1. APs obtained from CMs differentiated in vitro from ECCs (A) and ESCs (B). CMs show APs reminiscent of node, atrium, and ventricle. ESC-derived CMs show 2 distinct ventricle-like AP morphologies. Inset shows stability of APs over time obtained from an ESC-derived CM. APs were evoked by pulse current stimulation (200 pA, 10 ms) in current-clamp mode at 37°C.

Electrophysiology of CMs

Electrophysiological studies were performed 1 to 5 days after cell isolation. Before experiments, culture medium was replaced with a solution consisting of (in mmol/L) NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, and glucose 10 (pH 7.4 by NaOH) at 37°C. Patch pipettes were pulled to resistance of 2 to 5 MΩ. The intracellular solution contained (in mmol/L) KCl 120, MgCl₂ 1, MgATP 3, HEPES 10, and EGTA 10 (pH 7.2 by KOH). Spontaneous action potentials (APs) were recorded with an Axopatch 200B amplifier driven by pCLAMP (Axon Instruments). CMs with uniform contractions and beating rates usually <2 Hz were used in the study. For early afterdepolarization (EAD) and delayed afterdepolarization (DAD) studies, cells were paced at 0.125 to 0.5 Hz and 1 to 2 Hz, respectively, with 200-pA, 10-ms pulses. Data were digitized at 10 kHz and filtered at 2 kHz. Recordings were initiated 5 minutes after patch break to allow equilibration of the patch pipette solution with the intracellular milieu. Perforated patch recording utilized pipettes containing 120 μg/mL nystatin. Nystatin stock (30 mg/mL in DMSO) was freshly made before each experiment. Bay K8644 stock was made as 2 mmol/L in DMSO, so that the final concentration of DMSO would be <0.05% in the perfusing solution. Tetrodotoxin (TTX) was diluted in 50 mmol/L acetic acid to make a stock of 3 mmol/L. Quinidine hydrochloride was dissolved in water as a 1-mmol/L stock. All chemicals were purchased from Sigma unless otherwise specified. All data are presented as mean ± SEM. Student *t* tests were used for statistical analysis. In cells that showed more than 1 triggered event, the first was used for analysis.

Results

CMs Showed Spontaneous Electrical Activity and AP Heterogeneity

Demonstrating persistent automaticity, CM clusters in EBs showed spontaneous contractile activity, generally starting on day 7+3 and persisting for up to 7+20 days. Single CMs contracted spontaneously at 0.2 to 5 Hz for 1 to 5 days after isolation. From both sources of cells, CM APs showed heterogeneity and could be divided into at least 3 distinctive

phenotypes based on the maximum diastolic potential (MDP), maximum rate of rise of the AP (dV/dt), and AP duration (APD) at various stages of repolarization (Figure 1). These divisions were reminiscent of those seen with nodal, atrial, and ventricular myocytes. AP morphologies are compared in Table 1. Electrophysiological properties were unassociated with cellular morphology.⁷ Node-like cells from ESCs and ECCs had significantly depolarized MDP and dV/dt values compared with atrium- or ventricle-like cells. A period of hyperpolarization did not alter the node-like AP in these cells. Moreover, preliminary experiments confirm earlier reports⁸ that cells expressing green fluorescent protein under control of the ventricular cell-specific promoter MLC2v show a ventricle-like AP phenotype. Their shorter APD and diminutive AP plateau, reflected in their short APD₃₀, distinguished atrium-like cells.

Both node- and atrium-like APs from ESCs were similar to those of ECCs. The ventricle-like cells from ECCs showed a single, identifiable AP morphology. About 30% of the time, ventricle-like cells from ESCs showed a second morphology with a similar MDP but with a reduced phase 1 repolarization, a prolonged APD, and a larger dV/dt compared with the more common morphology (Figure 1). For all ventricular cells, the APs were considerably longer^{9–11} and the dV/dt values less⁹ than those reported for acutely isolated mouse cells, and this was consistent with reduced Na⁺ channel current in derived CMs (data not shown).

Any effects of patch pipette-induced cytoplasmic dialysis on AP morphology were evaluated with the perforated patch technique. The AP morphologies of ECC-derived ventricle-like CMs were compared with and without the use of a nystatin perforated patch technique. The results showed that there was no statistically significant difference for MDP

TABLE 1. APs of Node-, Atrium-, and Ventricle-Like Cells

Cell Type and AP Morphology	MDP, mV	dV/dt, mV/ms	APD ₃₀ , ms	APD ₅₀ , ms	APD ₉₀ , ms
ECCs					
Node (n=9)	-44.0±7.0*	8.5±5.0*	58.7±8.7	82.3±11.4	161.6±12.8
Atrium (n=8)	-69.5±5.9	59.7±23.5	6.2±2.0*	27.4±4.9*	47.0±34.6*
Ventricle (n=12)	-77.1±3.3	55.4±17.8	55.1±10.0	80.0±15.4	137.2±26.1
Perforated (n=7)	-73.5±2.8	57.3±20.0	46.7±11.0	62.6±28.1	128.8±47.5
ESCs					
Node (n=17)	-47.2±3.4†	5.3±1.1†	71.7±13.1	94.5±15.6	167.4±20.0
Atrium (n=12)	-69.9±5.0	68.3±20.4	5.6±1.7†	20.7±7.2†	60.6±17.0†
Ventricle (n=26)	-74.5±2.4	63.0±10.8	68.8±14.2	99.3±15.9	151.4±22.1

APD₃₀ indicates ADP at 30% repolarization; APD₅₀, APD at 50% repolarization; and APD₉₀, APD at 90% repolarization. Data are mean±SEM. Parameters from node and atrium are compared with their corresponding data from ventricle within ECC and ESC groups, respectively.

* $P<0.05$, † $P<0.05$, ECCs and ESCs, respectively, vs corresponding ventricles.

($P=0.47$), dV/dt ($P=0.95$), APD₃₀ ($P=0.60$), APD₅₀ ($P=0.56$), and APD₉₀ ($P=0.87$) compared with control (Table 1).

CMs Showed EADs

Triggered arrhythmias are the result of oscillatory membrane depolarizations or afterdepolarizations¹² and are favored by ionic and hormonal changes associated with myocyte injury and heart failure. Afterdepolarizations preceding full repolarization, EADs, are thought to cause torsade de pointes, the arrhythmia associated with long-QT syndrome.^{13,14} Triggered activity has not been studied in CMs and is exceedingly rare in normal mouse myocytes.^{10,11,15,16} As expected from their

longer APD, ventricle-like differentiated CMs showed EADs occasionally without and frequently with pharmacological manipulations that prolonged the AP. As shown in Figure 2, in the presence of 20 mmol/L [tetraethylammonium chloride]_o ([TEA]_o) and 1 μmol/L Bay K8644, EADs could be induced in CMs differentiated from either ECCs (Figure 2A) or ESCs (Figure 2B). Under these conditions, 33% and 36% of ECC- and ESC-derived ventricle-like CMs developed EADs. The average MDP of these cells was -70.7±3.8 and -72.0±6.1 mV, respectively, which was not significantly different from the control ventricle-like cells. EAD characteristics are summarized in Table 2. Shortening of the AP

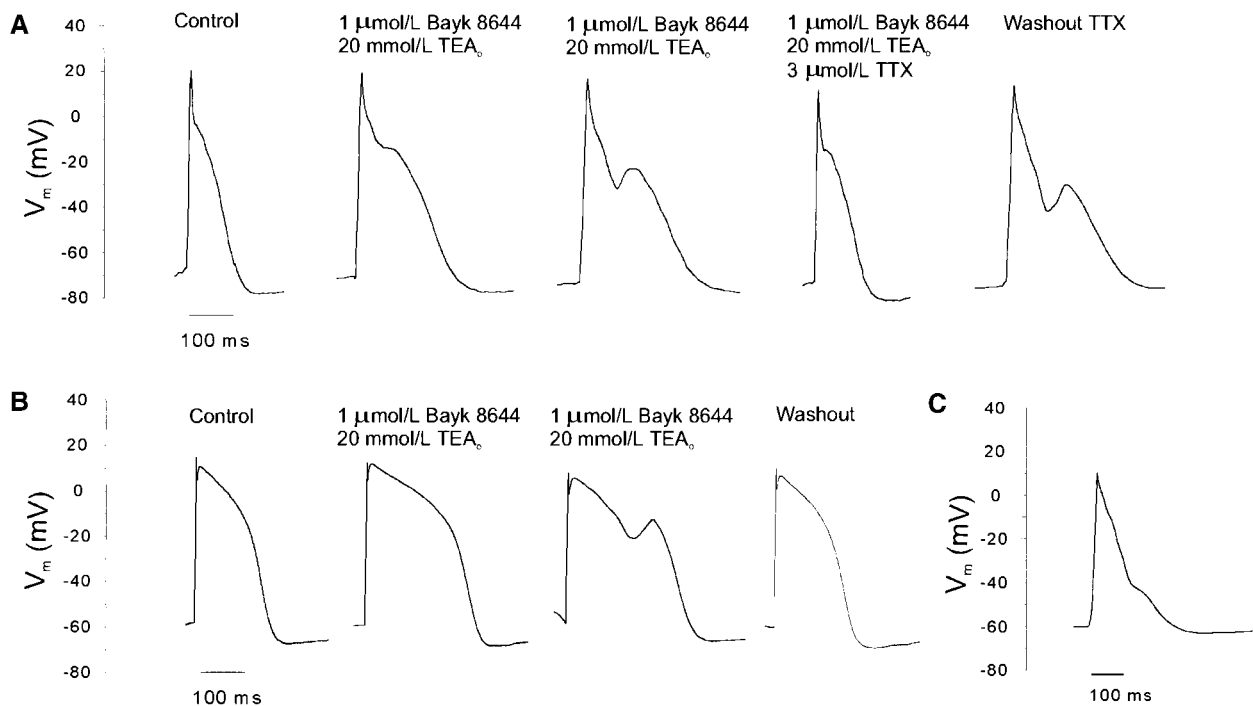


Figure 2. EADs in CMs. A, CM differentiated from ECCs shows AP prolongation and a phase 2 EAD in presence of 20 mmol/L [TEA]_o and 1 μmol/L Bay K8644. Application of 3 μmol/L TTX resulted in shortening of APD and abolished the EAD. With washing out of TTX but in continued presence of TEA and Bay K8644, EADs resumed. B, ESC-derived CM shows similar phase 2 EADs and prolonged AP in the presence of 20 mmol/L [TEA]_o and 1 μmol/L Bay K8644. Effects were reversible. C, ECC-derived CM shows a phase 3 EAD induced by internal Cs⁺ replacement for K⁺ and 20 mmol/L [TEA]_o. In all cases, CMs were paced at 0.2 Hz.

TABLE 2. Characteristics of EADs

	EAD Induction			
	ECCs		ESCs	
	Bay K8644/[TEA] _o (n=4)	Cs ⁺ /[TEA] _o (n=5)	Bay K8644/[TEA] _o (n=5)	Quinidine/ ↓ [K ⁺] _o (n=5)
EAD initiation potential, mV	-34.8±2.7	-53.0±2.0	-30.3±2.5	-47.3±3.1
EAD amplitude, mV	12.4±2.0	3.5±0.1	11.2±1.7	12.1±1.4
Interval between peak of AP and peak of EAD, ms	209.7±29.4	186.3±13.4	283.1±30.3	285.8±25.7

with TTX reversibly abolished the EADs. Two of 4 ESC-derived cells showed multiple EADs in response to a single AP. Rarely, EADs were associated with triggered beats, as demonstrated in Figure 3A.

Both ECC- and ESC-derived CMs showed phase 3 EADs in the presence of 20 mmol/L [TEA]_o, as well as internal Cs⁺ replacement for K⁺ (Figure 2C). Fifty percent of ECC-derived CMs developed EADs under these conditions, and their average MDP was -69.3 ± 6.8 mV, which was not significantly different from the control ventricle-like cells. In Table 2, characteristics of phase 3 EADs are contrasted with those seen arising from phase 2 of the AP. One episode of multiple EADs in association with Cs⁺ and TEA was observed in an ESC-derived CM.

The combination of bradycardia, hypokalemia, and quinidine easily induced EADs in ESC-derived CMs. In the presence of 3.0 mmol/L [K]_o and 1 μmol/L quinidine, a reduction in the pacing rate from 1.0 to 0.125 Hz resulted in AP prolongation and EAD induction (Figure 3B). Of 9 ESC-derived CMs studied, 5 developed EADs under these conditions; the average MDP was -70.6 ± 7.7 mV, which was not significantly different from the control ventricle-like cells. Other characteristics of these EADs are summarized in Table 2. Multiple EADs were seen once with this combination of provocations.

CMs Showed DADs

Afterdepolarizations arising after completion of AP repolarization, DADs, are thought to underlie arrhythmias associated with cocaine use, digoxin toxicity, and ischemia/reperfusion.^{12,17,18} DADs are associated with intracellular Ca²⁺ overload¹⁹ and could be induced easily by exposure of differentiated CMs to increased concentrations of [Ca²⁺]_o (Figure 4). The characteristics of DADs recorded from ECC- and ESC-derived CMs are compared in Table 3. Sixty-three percent of ECC-derived CMs developed DADs, and the average MDP, APD₅₀, and APD₉₀ values were -73.7 ± 4.5 mV, 70.1 ± 18.9 ms, and 121.8 ± 30.1 ms, respectively. Seventy-one percent of ESC-derived CMs developed DADs under the same conditions, and the average MDP, APD₅₀, and APD₉₀ values were -72.1 ± 3.6 mV, 87.6 ± 17.1 ms, and 137.9 ± 11.2 ms, respectively. In both ECCs and ESCs, APs that initiated DADs showed slightly depolarized MDPs and shorter APDs than those that did not elicit DADs, although these changes were not statistically significant. Figure 4 shows that induction of DADs was reversible. Both ECC- and

ESC-derived CMs showed multiple DADs under conditions of elevated [Ca²⁺]_o.

The β-adrenergic agonist isoproterenol, known to facilitate arrhythmias, was able to induce DADs in ESC-derived CMs (Figure 4). The tendency for APs that initiated DADs to start at a more depolarized membrane potential and to have a shortened APD compared with control cells (see Table 1) was similar to the effects of increasing [Ca²⁺]_o alone. The average MDP, APD₅₀, and APD₉₀ of cells that showed DADs in response to isoproterenol were -68.4 ± 10.3 mV, 88.6 ± 19.8 ms, and 145.8 ± 32.3 ms, respectively. Characteristics of induced DADs are summarized in Table 3. The effects of isoproterenol on DADs were consistent with the observation of intact β-adrenergic modulation of the L-type Ca²⁺ current shown in ESC-derived CMs.²⁰

Discussion

Three fundamental arrhythmia mechanisms are automaticity, reentry, and triggered activity. Reentry is facilitated by slow conduction, and one determinate of conduction velocity is the

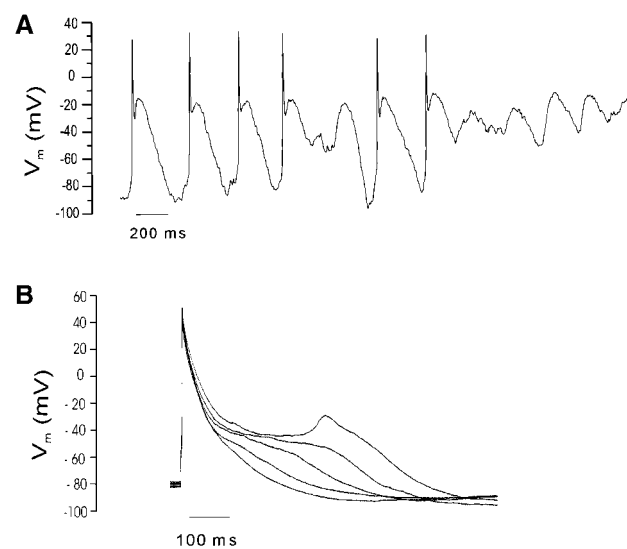


Figure 3. CMs show classic EAD behavior. A, Multiple EADs and repolarization arrest in a CM. ESC-derived CM perfused with solution containing 20 mmol/L [TEA]_o, 1 μmol/L Bay K8644, and 3.6 mmol/L Ca²⁺ showed EADs and subsequent rhythmic activity. B, Induction of EAD in an ESC-derived CM exposed to solution containing 3.0 mmol/L [K⁺]_o and 1 μmol/L quinidine. Shortest AP was evoked at cycle length of 1000 ms. Subsequent APs were recorded at cycle lengths increased to 0.125 Hz. The low driving rate resulted in prolonged APs and an EAD.

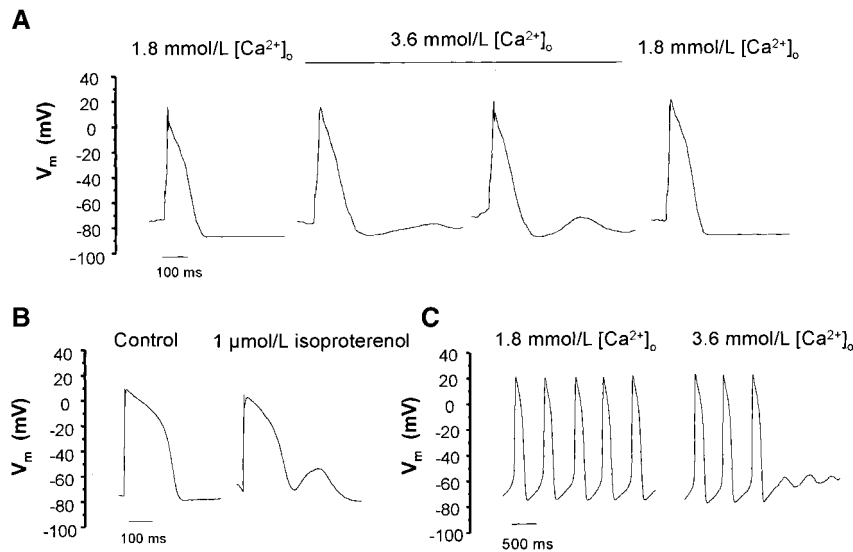


Figure 4. DADs in CMs. A, DADs induced by hypercalcemia. ECC-derived CM was continuously perfused with solution containing 1.8 mmol/L Ca^{2+} . Addition of 3.6 mmol/L Ca^{2+} induced DADs. Returning to 1.8 mmol/L Ca^{2+} abolished DADs, which suggests that the cell was calcium tolerant. B, DADs induced by β -agonist. Isoproterenol (1 $\mu\text{mol/L}$) induced a large DAD in an ESC-derived CM. C, Calcium overload inducing multiple DADs. A train of spontaneous APs from an ESC-derived CM in solution containing 1.8 mmol/L Ca^{2+} . Perfusing the CM with 3.6 mmol/L Ca^{2+} resulted in DADs interrupting the spontaneous train of APs.

dV/dt . In an attempt to study the electrophysiological consequences of cell transplantation therapy, we studied the properties of in vitro differentiated CMs. Our results showed that CMs derived from pluripotent cells showed AP morphology heterogeneity, maintained automaticity for long periods in EBs or as isolated cells, had lower dV/dt and longer APDs than those reported for native cells, and showed easily inducible triggered electrical activity. Results were consistent between 2 pluripotent cell lines.

The presence of 3 AP phenotypes is consistent with the findings of Maltsev et al,⁷ but triggered activity has not been reported previously in CMs. ESC-derived CMs showed 2 ventricle-like AP morphologies. A reduced phase 1 repolarization (ie, notch) and a prolonged APD characterized the more rare morphology. Recently, a report suggested that embryonic myocytes could be induced to form Purkinje cells in the presence of endothelin.²¹ The AP morphology and relative proportion of this second population suggest that they may represent Purkinje cells. Alternatively, these cells could be endocardial or midmyocardial analogs. The fact that these cells were not seen during ECC differentiation suggests that they are unlikely to represent an underdifferentiated form of CM.

APs of differentiated CMs were longer than those of isolated mouse myocytes.^{9–11} The perforated patch results suggested that the prolonged APD seen in derived CMs could not be explained by cytoplasmic dialysis by the pipette

solution. Therefore, differences are more likely the result of channel expression or activity levels. Recently, Doevendans et al²² found reduced levels of delayed rectifier potassium channels in CMs, which is consistent with this idea.

CMs Had Frequent EADs

EADs have been categorized into 2 types: plateau or phase 2 EADs and phase 3 EADs. Our data are the first to show that CMs mature sufficiently to demonstrate both types of EADs, especially with pharmacological provocation. The presence of EADs supports the AP morphology data that the cells studied were ventricle-like, because there are rare reports of EAD induction in nonventricular cells. Because of the relationship of EADs to APD and the longer APDs of CMs, the risk of triggered activity may be higher than that of native mouse cells, which have demonstrated close to no triggered activity, and the rates observed with our interventions were higher than those reported for a variety of genetic interventions.^{10,11,15,16} Any risk would be compounded by conditions experienced in the engraftment area, including hypoxia, acidosis, stretch, and inflammation.

CMs also showed phase 3 EADs that were favored by Cs^+ ,²³ $[\text{Cs}^+]_i$, and $[\text{TEA}]_o$ consistently induced smaller EADs than Bay K8644, and this may result from lower expression of some potassium channels in CMs.²² In addition, this might explain why we observed fewer episodes of multiple phase 3 EADs.

CMs Had Easily Inducible DADs

Circulating catecholamines are elevated in heart failure, and β -adrenergic stimulation is associated with arrhythmias. CMs showed frequent DADs in response to provocations expected in injured myocardium. This DAD frequency is 2- to 3-fold that seen in Ca^{2+} -loaded native cells exposed to ouabain.²⁴ DADs after an early afterhyperpolarization, DADs that occurred without preceding afterhyperpolarization, and DADs that occurred without full repolarization between episodes were seen, which suggests that ESC-derived CMs are sufficiently differentiated to have the complex Ca^{2+} cycling and

TABLE 3. Characteristics of DADs

	DAD Induction		
	ECCs	ESCs	
	3.6 mM $[\text{Ca}^{2+}]_o$ (n=4)	3.6 mM $[\text{Ca}^{2+}]_o$ (n=5)	Isoproterenol (n=5)
DAD initiation potential, mV	-75.9 ± 4.6	-79.8 ± 5.04	-67.9 ± 10.8
DAD amplitude, mV	14.4 ± 1.8	16.2 ± 2.7	18.7 ± 5.6
Interval between peak of AP and peak of DAD, ms	220.1 ± 59.9	218.4 ± 37.1	256 ± 50.2

ionic currents thought to underlie these types of DADs. Our results are consistent with a functional β -adrenergic signaling system in CMs.²⁵

Pluripotent cells able to differentiate into CMs have been proposed as cell replacement therapy in cardiomyopathy. These cells can engraft in areas of injury, partially replacing the damaged myocardium.^{2,3} Although the extent of electrical coupling is unknown, morphologically the applied cells form intercalated disks with host myocytes,⁴ which introduces the possibility that engrafted cells can participate in arrhythmia generation. The present study shows that in addition to AP morphology heterogeneity, in vitro differentiated CMs have slower upstroke velocities, protracted automaticity, prolonged APDs, and easily inducible triggered arrhythmias. Therefore, totipotent stem cells may be the source of unanticipated arrhythmias after cell transplantation therapy by enhancing the likelihood of all 3 basic arrhythmia mechanisms, and stem cell engraftment preferentially in areas of injury is likely to promote any arrhythmic tendency. These findings suggest that cells with restricted developmental potential may be more suitable.^{26,27} Limitations of the present study include that CMs may behave differently in situ than they do when differentiated in vitro. Also, results will need to be confirmed in human-derived CMs.

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