Differentiation of Human Embryonic Stem Cells to Cardiomyocytes
Role of Coculture With Visceral Endoderm-Like Cells

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Background—Cardiomyocytes derived from human embryonic stem (hES) cells could be useful in restoring heart function after myocardial infarction or in heart failure. Here, we induced cardiomyocyte differentiation of hES cells by a novel method and compared their electrophysiological properties and coupling with those of primary human fetal cardiomyocytes.

Methods and Results—hES cells were cocultured with visceral-endoderm (VE)–like cells from the mouse. This initiated differentiation to beating muscle. Sarcomeric marker proteins, chronotropic responses, and ion channel expression and function were typical of cardiomyocytes. Electrophysiology demonstrated that most cells resembled human fetal ventricular cells. Real-time intracellular calcium measurements, Lucifer yellow injection, and connexin 43 expression demonstrated that fetal and hES-derived cardiomyocytes are coupled by gap junctions in culture. Inhibition of electrical responses by verapamil demonstrated the presence of functional $\alpha_\text{h}$-calcium ion channels.

Conclusions—This is the first demonstration of induction of cardiomyocyte differentiation in hES cells that do not undergo spontaneous cardiogenesis. It provides a model for the study of human cardiomyocytes in culture and could be a step forward in the development of cardiomyocyte transplantation therapies. (Circulation. 2002;107:2733-2740.)

Key Words: electrophysiology □ myocytes □ stem cells

Ischemic heart disease is the leading cause of mortality in the western world. Oxygen deprivation and subsequent reperfusion initiate irreversible cell damage, eventually leading to cell death and loss of function. Strategies to regenerate damaged cardiac tissue by cardiomyocyte transplantation may limit postinfarction cardiac failure. We have shown previously that visceral-endoderm (VE)–like cell lines induce mouse P19 embryonal carcinoma (EC) and mouse embryonic stem (ES) cells to aggregate spontaneously in coculture and differentiate to cultures containing beating muscle.1–3 This induction potential was specific for VE-like cells and was also observed when aggregates of P19EC cells were grown in conditioned medium from one VE-like cell line, END-2. Moreover, Dyer et al4 have shown that END-2 cells can induce the differentiation of epiblast cells from the mouse embryo to undergo hematopoiesis and vasculogenesis and respecify prospective neuroectodermal cell fate. These effects were largely attributable to Indian hedgehog secreted by END-2 cells and VE of the mouse embryo.

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Molecular pathways leading to specification and terminal differentiation of cardiomyocytes from embryonic mesoderm during development are still unclear. Data derived from chick and amphibian suggested that cardiac progenitors require interaction with anterior endoderm and possibly the organizer for myocardial differentiation to take place.5–7 More recently, primitive streak and visceral embryonic endoderm were shown to be important for the multistep induction through which cardiac progenitor cells acquired the competence to complete terminal differentiation at day 7.5 of gestation in mice.8

Here, we demonstrate that coculture of pluripotent human ES (hES) cell lines with END-2 cells induces extensive differentiation to 2 distinctive cell types from different lineages. One is epithelial; it forms large cystic structures that stain positively for $\alpha$-fetoprotein and is presumably extraembryonic VE; the others are grouped in areas of high local density and beat spontaneously. We show that these beating
cells are cardiomyocytes. Although differentiation of hES cells to cardiomyocytes has been described previously,1–11 the hES cell lines used differentiate spontaneously to somatic derivatives in embryoid bodies, reminiscent of those formed by mES cells.12 The present work is thus the first describing induction of cardiomyocyte differentiation in hES cells, which do not undergo cardiogenesis spontaneously, even at high local cell densities, and is the first direct electrophysiological comparison of hES-derived cardiomyocytes with primary human fetal cardiomyocytes in culture.

Methods

Cell Culture

END-2 cells and hES2 cells were cultured as described previously.13,13 To initiate cocultures, END-2 cell cultures treated for 3 hours with mitomycin C (mitC; 10 µg/mL)1 replaced mouse embryonic fibroblasts (MEFs) as feeders for hES cells. Cocultures were then grown for up to 6 weeks and scored for the presence of areas of beating muscle from 5 days onward. HepG2 cells, a carcinoma cell line resembling liver parenchymal cells,14 were cultured in DMEM plus 10% FCS. Cocultures were initiated as for END-2 cells. For electrophysiology, beating aggregates were dissociated by use of collagenase and replated on gelatin-coated coverslips.

Immunohistochemistry

Cells were fixed with 3.0% paraformaldehyde, then permeabilized with 0.1% Triton X-100. Undifferentiated hES colonies were stained overnight at 4°C with anti-Oct4 (Sigma) and visualized by use of the avidin-biotin complex/horseradish peroxidase kit (DAKO) and the Fast 3,3′-diaminobenzidine tablet set (Sigma). For immunofluorescence antibodies against α-actinin, tropomyosin, and pan-cadherin (Sigma), myosin light chain (MLC)-2a and -2v (from Dr K. Chien, San Diego School of Medicine, La Jolla, Calif), α1c and Ca1.2a (Alomone Laboratories), connexin 43 (Cx43) (Transduction Laboratories), and phalloidin-Cy3 (Sigma) were used in combination with fluorescein-conjugated secondary antibodies (Jackson Laboratories). Confocal images (Leica Systems) were made (63× objective) from 2D projected z series.

Primary Human Adult and Fetal Cardiomyocytes

Primary tissue was obtained during cardiac surgery or after abortion after individual permission had been obtained by use of standard informed consent procedures and approval of the ethics committee of the University Medical Center, Utrecht. Adult cardiomyocytes were isolated and cultured as reported previously.1,2 Fetal cardiomyocytes were isolated from fetal hearts (16 to 17 weeks) perfused by Langendorff’s method and cultured on glass coverslips. For patch-clamp electrophysiology, cells were collected in Tyrode’s buffer with low Ca2+.

Reverse Transcription–Polymerase Chain Reaction

RNA was isolated by use of Ultraspec (Biotecx Laboratories) and reverse transcribed (RT; 500 ng total RNA) as described previously.14 Primer sequences and conditions for polymerase chain reaction (PCR) are given in Table 1. Products were analyzed on ethidium bromide–stained 1.5% agarose gel. β-Actin or β-tubulin was used as RNA input control.

Electrophysiology

Data were recorded from cells at 33°C in spontaneously beating areas by use of an Axopatch 200B amplifier (Axon Instruments Inc). Cell-attached patches were made in the whole-cell voltage-clamp mode. The pipette offset, series resistance, and transient cancellation were compensated; subsequent action potentials were recorded by switching to the current-clamp mode of the 200B amplifier. Output signals were digitized at 4 kHz by use of a Pentium III equipped with an AD/DAC LAB PC+ acquisition board (National Instruments). Patch pipettes with a resistance between 3 and 3 MΩ were used. Bath medium was (mmol/L) 140 NaCl, 5 KCl, 2 CaCl2, and 10 HEPES, adjusted to pH 7.45 with NaOH. Pipette composition (mmol/L) was 145 KCl, 5 NaCl, 2 CaCl2, 4 EGTA, 2 MgCl2, and 10 HEPES, adjusted to pH 7.30 with KOH. Verapamil was used at 5 µmol/L.

Calcium Measurements

Cells were labeled for 15 minutes at 37°C with 10 µmol/L fura 2-AM. The light from 2 excitation monochromators (SPEX fluorolog, SPEX Industries) was rapidly alternated between 340 (slit width: 8) and 380 (slit width: 8) nm and coupled into a microscope
Dye Coupling
A filtered solution of 3% wt/vol Lucifer yellow lithium salt (Molecular Probes) in 150 mmol/L LiCl was microinjected through Quick-fill glass microelectrodes (Clark Electromedical Instruments). Dye was injected into one of a group of spontaneously beating cells by a 1-Hz square pulse (50% duty cycle), amplitude of $5\times10^{-9}$ A. Directly after injection, confocal laser scanning microscope images were made of the injected areas.

Results
Cardiomyocyte Differentiation of hES Cells
Of the hES cells maintained by coculture with mit.C-treated MEFs in FCS-containing medium\(^3\) (Figure 1A), \(~60\%\) showed nuclear staining for oct-4; flattened cells were negative (Figure 1B). Oct-4 expression thus correlated with phenotypic characteristics of undifferentiated cells. hES cells were subcultured by transferring small clumps of undifferentiated cells onto either new MEFs or END-2 cells. After \(~5\) days, epithelial cells appeared, which gradually become fluid-filled cysts (Figure 1C). These stained for \(\alpha\)-fetoprotein (Figure 1H), suggesting that they represent extraembryonic VE. By 10 days, areas of rhythmically contracting cells in more solid aggregates became evident in the hES-END2 cocultures (Figure 1C, arrow) with a variety of overall morphologies (Figure 1D). In a 12-well plate, 35±10% of the wells (n=30) contained beating areas, each of which could be dissociated and replated to yield up to 12 new colonies of beating cells with a 2D rather than 3D morphology (Figure 1G); this facilitated access to the cells for electrophysiology. Each beating area consisted of 10 to 200 cardiomyocytes. Control cultures on MEFs showed no evidence of beating muscle or extensive cyst formation but had formed very large colonies with many flattened cells at the edges (not shown). Conversely, hES on HepG2 cells did form areas of beating muscle, usually attached to HepG2 cell colonies.

Before and after dissociation, hES-derived cardiomyocytes beat 35 to 90 times per minute (Table 2). Cardiomyocyte

<table>
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*Measured maximum: 20.0 (V/s).
†Measured maximum: 26.7 (V/s).
colonies could be frozen and sometimes resumed beating on thawing. To characterize the cardiomyocytes further, we carried out immunofluorescent staining for sarcomeric proteins, used BIDOPY-ryanodine as a vital stain for ryanodine receptors in the sarcoplasmic reticulum, and analyzed the expression of ion channels by RT-PCR. In each case, we used primary human fetal and adult atrial and ventricular tissue as controls. The data showed that hES-derived cardiomyocytes exhibited sarcomeric striations when stained with α-actinin (Figure 2A), organized in separated bundles. These were reminiscent of the bundles observed in human fetal cardiomyocytes (Figure 2, B and C), although the individual sarcomeres were less well defined. The morphology was different from the highly organized, parallel bundles in cells from biopsies of adult human heart (Figure 2, G and H). hES-derived cardiomyocytes also stained with MLC-2a, MLC-2v (not shown), and tropomyosin (Figure 2D); again, the sarcomeres were less evident than in human fetal and adult cardiomyocytes (Figure 2, E, F, and H).

Expression of Cardiac Ion Channels and Stem Cell/Sarcomere Markers in hES/END-2 Cocultures

Expression of cardiac-specific ion channels was determined in undifferentiated hES cells and at 8 and 15 days after initiation of coculture with END-2 cells (Figure 2K). As shown previously by others,10 areas of beating hES-derived cardiomyocytes express atrial natriuretic factor. Expression of the α-subunits of the cardiac-specific L-type calcium channel (α1c) and the transient outward potassium channel (Kv4.3) was also detected, the expression of Kv4.3 preceding onset of beating by several days. RNA for the delayed rectifier potassium channel KvLQT1 was found in undifferentiated cells, but transcripts disappeared during early differentiation and reappeared later.

Over a similar time course, expression of oct-4 was reduced, whereas transcripts for α-actinin, MLC-2a, and MLC-2v became detectable (Figure 2K), reflecting the results of antibody staining.

Electrophysiology

Patch-clamp electrophysiology on dissociated hES cardiomyocytes showed that different electrical phenotypes were
Ventricular-like action potentials predominated (28 of 33; Table 2), but atrial-like (n = H110052), pacemaker-like (n = H110051), and vascular smooth muscle–like cells (n = H110052) were also found. In areas in which the cells were not beating but had adopted morphologies indistinguishable from those of beating areas (Figure 1F), current injection was sufficient to induce repeated action potentials and sustained synchronous rhythmic contractions. Transcripts for MLC-2v were also detected by RT-PCR in nonbeating, myocyte-like areas (not shown); scoring beating muscle may thus underestimate the number of cardiomyocytes present in culture.

The upstroke velocities (Volts/s) for the ventricular-like cells were low (8 Volts/s) but comparable to those in cultured human fetal ventricular cardiomyocytes, although incidental peak values were found (Table 2). α1-Adrenoceptors, β1-

adrenoceptors (regulated via a cAMP-dependent mechanism), and nicotinic acetylcholine receptors are known to influence cardiac function. Chronotropic responses of dissociated hES cardiomyocytes were also compared with human fetal ventricular cells (Figure 3C). Addition of carbachol decreased the beating rate of hES-derived cardiomyocytes and human fetal ventricular cells, whereas phenylephrine and isoprenaline increased the rate in both cell types. Similar effects were reported in mES-derived cardiomyocytes18 and mouse fetal cells.19

**[Ca2+]i Transients in Differentiated hES Cells**

Calcium oscillations were recorded in dissociated groups of spontaneously beating hES cardiomyocytes (Figure 4). The continuous character of the repetitive line scans in Figure 4B in the left-to-right direction, compared with the vertical lines in 4C, shows that the action potential in Figure 3A propagates in a top-down direction and indicated tightly developed cell-to-cell coupling in this synchronously contracting group of cells. Regular repetitive oscillations in [Ca^{2+}]i, are found in single hES cardiomyocytes (Figure 4E). Coupling between cells was confirmed by Lucifer yellow injection into single cells; the dye spread within minutes to other cells within the group in both hES-derived (Figure 5E) and primary fetal cardiomyocytes (not shown). Cx43 staining (Figure 5, B and D) indicated the presence of gap junctions. Staining with a pan-cadherin antibody also indicated the presence of adhe-


dents junctions between cells in fetal and hES-derived cardio-
mocytes (Figure 5, A and C).

L-type calcium channels compose the predominant route for calcium entry into cardiac myocytes and are key components in excitation-contraction coupling. A specific α1C antibody stained cardiomyocytes in both differentiated hES cultures (Figure 4F) and human fetal ventricular cells (Figure 4G), in agreement with the RT-PCR data (Figure 2K).

**Discussion**

Before hES cells can be applied clinically, it is important to control their growth and differentiation. Both embryonic and adult stem cells from the mouse apparently respond to cues within the mouse embryo to differentiate to (virtually) all somatic tissues (reviewed by Passier and Mummery20 ). If these cues and the signal transduction pathways they activate could be identified, this knowledge could be used to control differentiation of stem cells in culture and in vivo. Here, we have identified visceral endoderm as a cellular source of signals that result in human ES cells differentiating to cardiomyocytes with characteristics of fetal ventricular, atrial, or pacemaker cells. This is the first time that inductive cellular sources of signals have been identified that result in human ES cells forming cardio-
mocytes, although various studies have shown that cells with endoderm-like properties have this effect on mouse ES and EC cells.1,3,21–23 VE (END-2) and liver parenchy-
mal (HepG2) cells share similar protein secretion profiles, so their ability to induce comparable responses in ES cells is not surprising. In contrast to mouse ES cells, in our hands, human ES cells do not easily form embryoid bodies when grown as aggregates and never show “spontaneous”
differentiation to cardiomyocytes, even at high cell densities in overgrowths. This contrasts with other reports in which the hES cells do form embryoid bodies containing cardiomyocytes. Identification of a reproducible source of inductive signals nevertheless represents an important step forward, comparable to a recent report showing differentiation of hES cells to hematopoietic cells after coculture with bone marrow stromal cells or yolk sac endothelial cells. It has been suggested that signals from the endoderm, such as bone morphogenic proteins (BMPs), fibroblast growth factors, and repressors of wnt signaling, may be important for cardiac development. Direct addition of BMP2 to hES cells, however, did not result in cardiomyocyte differentiation; on the contrary, they formed extraembryonic endoderm (data not shown). We therefore think it unlikely that activation of the BMP signaling pathway is the primary event initiated by END-2/hES cell coculture. Likewise, we saw no obvious effect of fibroblast growth factors. These signals could, however, be involved later in differentiation of nascent mesoderm to cardiomyoblasts. Late addition of the demethylating agent 5-azacytidine to developing embryoid bodies has also been shown to be more effective than early addition. Careful stepwise analysis of hES cell differentiation and approaches recapitulating or mimicking endogenous signals in the embryo are the most likely to increase the efficiencies of hES differentiation to specific lineages. In addition, transplantation of committed but immature cells that have retained the capacity to form functional junctions with host cells are likely to have the least chance of introducing arrhythmias.

Staining for junctional proteins showed that the hES-derived cardiomyocytes were very immature, although real-time determination of intracellular Ca\textsuperscript{2+} concentrations clearly showed that the cells were electrically coupled. Kehat et al recently reported similar findings in independently derived hES-cardiomyocytes. It will be of interest to subject these hES-derived cardiomyocytes to oscillating stress to see whether the sarcomeric structure matures to the adult phenotype.

In the adult mammalian myocardium, cellular Ca\textsuperscript{2+} entry is regulated by the sympathetic nervous system. L-type Ca\textsuperscript{2+} channel currents are markedly increased by \(\beta\)-adrenergic agonists, which contribute to changes in rate and contractile activity of the heart. Exactly how this Ca\textsuperscript{2+}...
some features in common with early mouse cardiomyocytes, their calcium channel modulation resembles that in the adult mouse. hES cells may thus represent an excellent system for studying changes in calcium channel function during early human development, which appears to differ significantly from that in mice. Furthermore, the appropriate calcium handling makes the cells more suitable for transplantation. Interesting was the observation of cells with plateau- and nonplateau-type action potentials in the fetal atrial cultures. These have been described dispersed throughout the atrium of intact fetal hearts\textsuperscript{28} and have been considered a possible index of specialization of an atrial fiber, although their significance is not clear. The nonplateau type was not observed among the hES-derived cardiomyocytes.

Finally, vital fluorescent staining with ryanodine or antibodies against cell surface $\alpha_w$ ion channels allowed cardiomyocytes to be identified in mixed cultures. This may provide a means of isolating cells for transplantation without genetic manipulation or compromising viability.

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![Figure 5. Junctional communication in hES-derived and human fetal cardiomyocytes.](image-url)
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