Molecular and Pharmacological Properties of Human Embryonic Stem Cell–Derived Cardiomyocytes

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Human embryonic stem cells (hESCs) can be coaxed to differentiate into specific cell types, including cardiomyocyte-like cells. These cells express cardiac-specific markers and display functional similarities to their adult counterparts. Based on these properties, hESC-derived cardiomyocytes have the potential to be extremely useful in various *in vitro* applications and to provide the opportunity for cardiac cell replacement therapies. However, before this can become a reality, the molecular and functional characteristics of these cells need to be investigated in more detail. In the present study we differentiate hESCs into cardiomyocyte-like cells *via* embryoid bodies (EBs). The fraction of spontaneously beating clusters obtained from the EBs averaged approximately 30% of the total number of EBs used. These cell clusters were isolated, dissociated into single-cell suspensions, and frozen for long-term storage. The cryopreserved cells could be successfully thawed and subcultured. Using electron microscopy, we observed Z discs and tight junctions in the hESC-derived cardiomyocytes, and by immunohistochemical analysis we detected expression of cardiac-specific markers (cTnI and cMHC). Notably, using BrdU labeling we also could demonstrate that some of the hESC-derived cardiomyocytes retain a proliferative capacity. Furthermore, pharmacological stimulation of the cells resulted in responses indicative of functional adrenergic and muscarinic receptor coupling systems. Taken together, these results lend support to the notion that hESCs can be used as a source for the procurement of cardiomyocytes for *in vitro* and *in vivo* applications. Exp Biol Med 231:1753–1762, 2006

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Introduction

Information about the molecular pathways that govern early differentiation and maturation of human cardiomyocytes is very limited. Adult cardiomyocytes do not regenerate due to their permanent withdrawal from the cell cycle, which limits the usefulness of these cells in studies of the cardiomyogenic program. Interestingly, recent studies have demonstrated the presence of cardiac progenitors in the adult heart (1, 2). However, the absolute number of resident precursor cells in the postnatal heart is quite low, creating obstacles for interrogating these cells experimentally. On the other hand, novel opportunities have emerged over the last few years, and recent reports have illustrated the possibility of deriving cardiac progenitors and multiple types of cardiac myocytes from human embryonic stem cells (hESCs; Refs. 3–6).

Populations of pluripotent hESCs can be isolated from the inner cell mass of blastocysts, and these cells have the capacity for indefinite, undifferentiated proliferation *in vitro* (7–11). Besides their importance in basic research, promising future applications of hESCs and their derivatives include cell replacement therapies (12, 13). In addition, the hESC technology platform holds tremendous potential for the development of novel approaches for drug development and *in vitro* toxicology (14, 15). Many of these applications are dependent on the derivation and purification of specialized cells from undifferentiated hESCs. In addition, extensive functional characterization of these cells is required in order to apply them appropriately in various *in vitro* or *in vivo* settings.

Differentiation of hESCs may occur spontaneously *in vitro*, especially during suboptimal culture conditions (7, 8). In addition, hESCs can be coaxed to differentiate in a directed fashion along specific pathways, forming a variety

of specialized cell types, including cardiomyocytes, endothelial cells, neuronal cells, insulin-producing β-cells, and hematopoietic cells (for a review see Ref. 16). However, relatively little is known currently about how to control and manipulate hESC differentiation to produce exclusive populations of specific cell types. In principle, two different procedures have been reported for the derivation of cardiomyocytes from hESCs. The first is through differentiation of hESCs initiated when the cells are cultured in suspension and form embryoid bodies (EBs; Refs. 3, 17). Within these mixed populations of cells, contracting areas with the functional properties of cardiomyocytes can be observed. The second procedure is based on the coculture of hESCs with END-2 cells (a visceral endoderm-like cell line), which results in the formation of beating clusters of cells that also display the characteristics of cardiomyocytes (6, 18). Although the molecular mechanisms responsible for initiating and sustaining cardiogenesis in hESCs remain elusive, taken together, these studies draw attention to the possibility of deriving cardiomyocytes from hESCs. However, much research remains to be done in order to better define these cell populations on functional, structural, and molecular levels.

In the present investigation we derived spontaneously beating clusters of cells from undifferentiated hESCs. These cells were shown to display the morphology and marker expression indicative of cardiomyocytes. Notably, some of these cells demonstrated the capacity to proliferate, as indicated by BrdU labeling. In addition, the contractile properties and pharmacological response to chronotropic agents of the hESC-derived cardiomyocytes were determined.

Materials and Methods

Culture and Differentiation of hESCs. The hESC lines SA002 and SA121 (Cellartis AB, Göteborg, Sweden) were maintained *in vitro* at Cellartis as previously described (11). To induce EB formation, the hESC colonies were manually dissected and transferred to suspension cultures (17). After 4–6 days the EBs were plated onto gelatin-coated culture dishes and were incubated in knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1 m*M* GlutaMAX, 0.1 m*M* β-mercaptoethanol, 1% MEM nonessential amino acids, and 1% penicillin-streptomycin solution (all from Invitrogen, Carlsbad, CA). The medium was changed every 2–3 days.

Dissociation and Cryopreservation of hESC-Derived Cardiomyocytes. To dissociate contracting clusters into single cells, beating areas were dissected with a stem cell cutting tool (Swemed; Vitrolife AB, Kungsbacka, Sweden) and were transferred to *in vitro* fertilization (IVF) cell culture dishes (Falcon 3653; Becton Dickinson, Franklin Lakes, NJ) and washed once with phosphate-buffered saline (PBS; Gibco/Invitrogen Life Technologies, Carlsbad, CA). The cells were incubated with trypsin/EDTA (Gibco/Invitrogen Corp., Grand Island, NY) for 10–15 mins at 37°C, and then FBS (Gibco) was added, and the cell suspension was centrifuged for 5 mins at 400 *g*. Subsequently, the cells were plated onto gelatin-coated IVF cell culture dishes. The day after plating, contracting single cells were observed microscopically.

For cryopreservation, contracting areas were dissociated as described above. The cells were resuspended in culture medium containing 10% dimethylsulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO) and were placed in cryotubes (CryoTube Vials; Nunc, Roskilde, Denmark) in a freezing container (Nalgene 1°C freezing container; Nalge Nunc International, Rochester, NY) and kept at −80°C overnight. The next day the tubes were transferred to liquid nitrogen for storage. Frozen ampoules were thawed quickly in a water bath (37°C), and the suspension was transferred to prewarmed culture medium. The cells were centrifuged for 5 mins at 400 *g*, resuspended in fresh culture medium, and seeded onto gelatin-coated culture dishes. Within a few days after thawing, the cells started to contract spontaneously.

As an alternative to the slow freezing approach, we also used vitrification for cryopreservation of hESC-derived cardiomyocytes. Contracting clusters of cells were mechanically dissected into smaller clumps, and vitrification in closed straws was performed as previously described for undifferentiated hESCs (11, 19).

Electron Microscopy. The contracting areas were dissected free and transferred to cacodylate-buffered 3% glutaraldehyde in plastic Eppendorf tubes. The tissue samples were embedded in 4% agarose and postfixed for 1 hr in 0.1 *M* sodium cacodylate–buffered osmium tetroxid. The samples were dehydrated in graded series of ethanol and propylene oxide and then embedded in plastic. Ultrathin (60 nm) sections were prepared and mounted on single-slot Formvar-coated copper grids. Uranyl acetate and lead citrate were used for contrast staining. Microscopy was performed using a Philips CM 10 electron microscope (FEI, Acht, The Netherlands).

Immunohistochemical Analysis and BrdU Labeling. Cells used for immunohistochemistry were dissociated into single cells and seeded as described above. Immunohistochemical staining was performed as described previously (20). Primary antibodies used were mouse antidesmin (1:100; Chemicon International, Temecula, CA), mouse anticardiac Troponin I (cTnI; 1:1000; Chemicon), mouse antihuman cardiac myosin heavy chain (cMHC; 1:250; Upstate, Lake Placid, NY), rabbit antihuman Nkx2.5 (1:500), and rabbit antihuman GATA-4 (1:250; Santa Cruz Biotechnology, Inc.). Secondary antibodies used were fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgG2b (Southern Biotechnology Associates Inc., Birmingham, AL), Alexa Fluor 488 anti-mouse IgG (Molecular Probes, Eugene, OR), and anti-rabbit IgG (Molecular Probes).

Dissociated contracting cells were subcultured for 4 days with medium containing 10 μ*M* BrdU (Sigma, St.

Louis, MO), and fixed for 10 mins in 4% paraformaldehyde solution. The cells were double stained for cTnI (as described above) and BrdU according to the instructions from the manufacturer (Sigma), with minor modifications.

Pharmacological Experiments. The hESCs were differentiated for at least 3 weeks after plating of the EBs before the chronotropic effects of cardioactive drugs were evaluated. Contractile areas were dissected under the microscope and plated in culture dishes in the same medium used for hESC differentiation as described above. Pharmacological experiments started 48 hrs after transfer. The effects of different chronotropic agents were monitored visually, and the beating rate was registered. In the majority of experiments—forming the basis for the statistical calculations—the drugs were administered, and contractile activity was registered at 30-min intervals. In separate experiments contractile activity was followed at shorter intervals. No washing or change of medium was done between the additions of different concentrations of an agent. At the end of each experiment the medium was changed three times, and the same beating area could be used for another experiment after 48 hrs, provided the endogenous, spontaneous contractile frequency had been reestablished. In a minority of the EBs the contraction frequency was irregular, and these EBs were omitted from the study.

The following pharmacological agents were used: noradrenaline and adrenaline (Apoteket, Umeå, Sweden); phenylephrine and forskolin (Sigma Chemicals, St. Louis, MO); phenoxybenzamine and acetylcholine (KeLab, Göteborg, Sweden); atenolol and atropin (NM Pharma AB, Täby, Sweden); labetalol (GlaxoSmithKline, Mölndal, Sweden); and verapamil (Abbot Scandinavia AB, Solna, Sweden).

Statistics. The stimulatory or inhibitory effect on contraction frequency of an administered substance was expressed in percentage of the basal contraction frequency. Statistical significance between two groups was determined using Wilcoxon matched pairs test. A P value < 0.05 was considered statistically significant.

Results

Undifferentiated hESCs form aggregates commonly referred to as EBs when cultured in suspension. Based on the constituents, in certain aspects the EB formation resembles the early stages of normal embryonic development. In particular, similar to blastocysts, the EBs have an outer layer of primitive endoderm surrounding undifferentiated ESCs and primitive ectoderm. Further differentiation of the EBs in adherent cultures gives rise to spontaneously beating clusters of cardiomyocyte-like cells. The efficiency of EB formation was monitored in 16 independent experiments, and the average yield was about 80% (Fig. 1). The fraction of spontaneously beating clusters obtained from the EBs averaged approximately 30% of the total number of EBs (Fig. 1). These contracting areas were normally visible beginning 4 days after plating of the EBs, and additional areas appeared up to 20 days after plating.

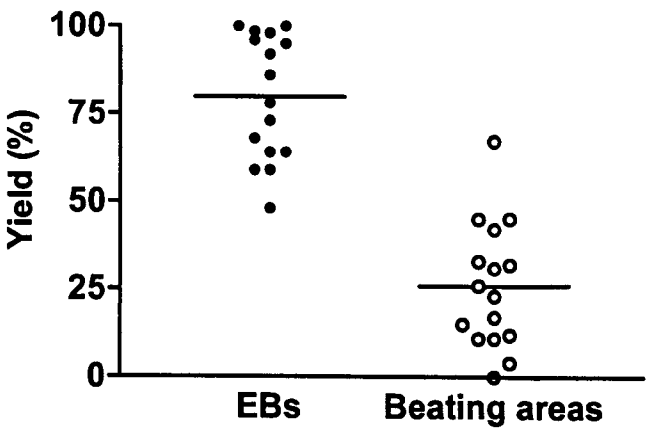


Figure 1. Efficiency of EB formation and derivation of contracting clusters from undifferentiated hESCs. Undifferentiated hESC colonies were manually dissected and transferred from the MEF feeder layers to suspension cultures to allow formation of EBs. After 4–6 days, the EBs were plated and subcultured to further differentiate the hESCs into spontaneously beating cardiomyocyte-like cells. The graph shows the yield in EB formation relative to the total number of undifferentiated hESC colonies used, and the yield in spontaneously beating clusters obtained relative to the total number of EBs. The data are from 16 independent experiments using hESC line SA002 in passages 22–53. In each experiment, 60–80 undifferentiated hESC colonies were evaluated.

The contracting cells mostly emerged in the outgrowths of the EBs as well-demarcated clusters of cells, or sometimes as strands of interconnected contracting cells (Fig. 2A). The isolated contracting areas usually preserved their morphology for up to 8 weeks. Occasionally, signs of devitalization were observed as distending and granulation of the cells. Contracting areas were isolated by mechanical dissection and subsequently dissociated into single-cell suspensions using trypsin. The sizes and shapes of the hESC-derived cardiomyocytes varied, and cells with rod-shaped, round, spindle, triangular, and multiangular morphologies were observed. The cells were either cryopreserved for storage or directly subcultured in new dishes. Examples of the morphologies of contracting single cells and clusters are shown in Figure 2B–D. For cryopreservation of hESC-derived cardiomyocytes, traditional slow freezing was used either with enzymatically dissociated cell suspensions or with mechanically dissected small cell clumps. For single-cell suspensions, the slow freezing technique worked well. However, initial experiments indicated that the cells frozen in clumps were less viable after thawing. The reason for this observation requires further investigations, but one important parameter for cell survival during the freeze–thaw cycle might be the size of cell clusters. Vitrification in closed straws was employed as an alternative approach for cryopreservation of clusters of hESC-derived cardiomyocytes. This approach allowed an efficient recovery of viable contracting clusters of hESC-

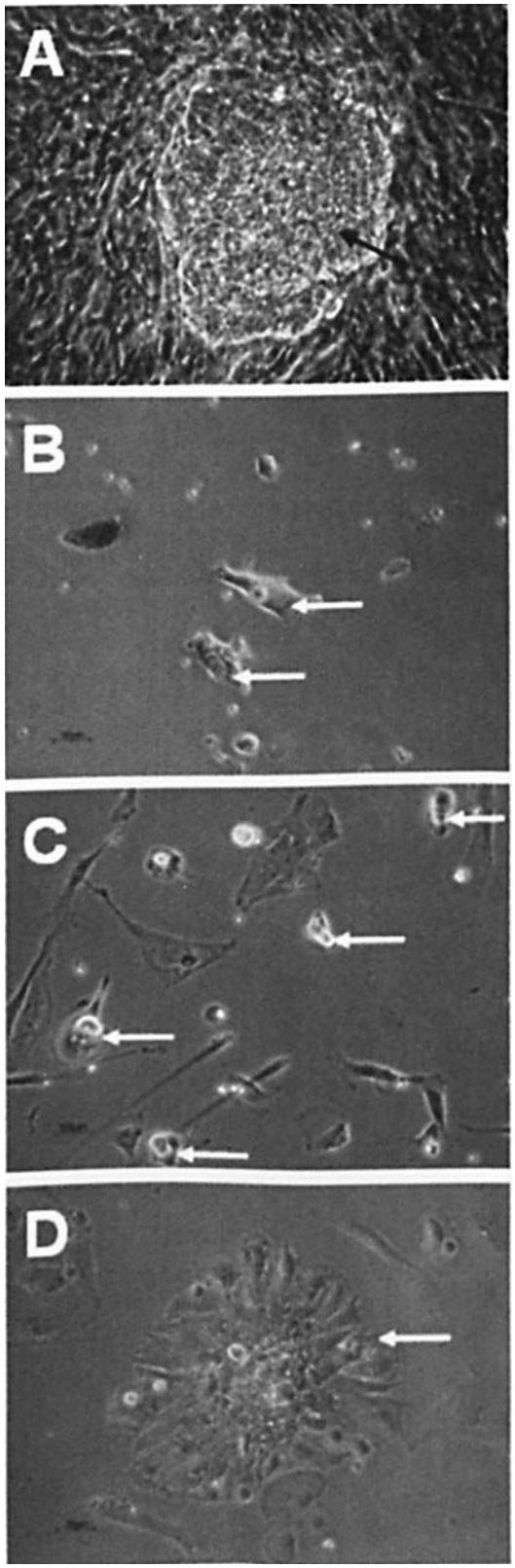


Figure 2. Morphology of spontaneously contracting cells obtained from hESCs. (A) A beating area derived from an EB surrounded by proliferating cells. (B–D) A dissociated and replated beating area that has been subcultured for 3 days (B), 6 days (C), and 15 days (D). The arrows indicate contracting cells and clusters.

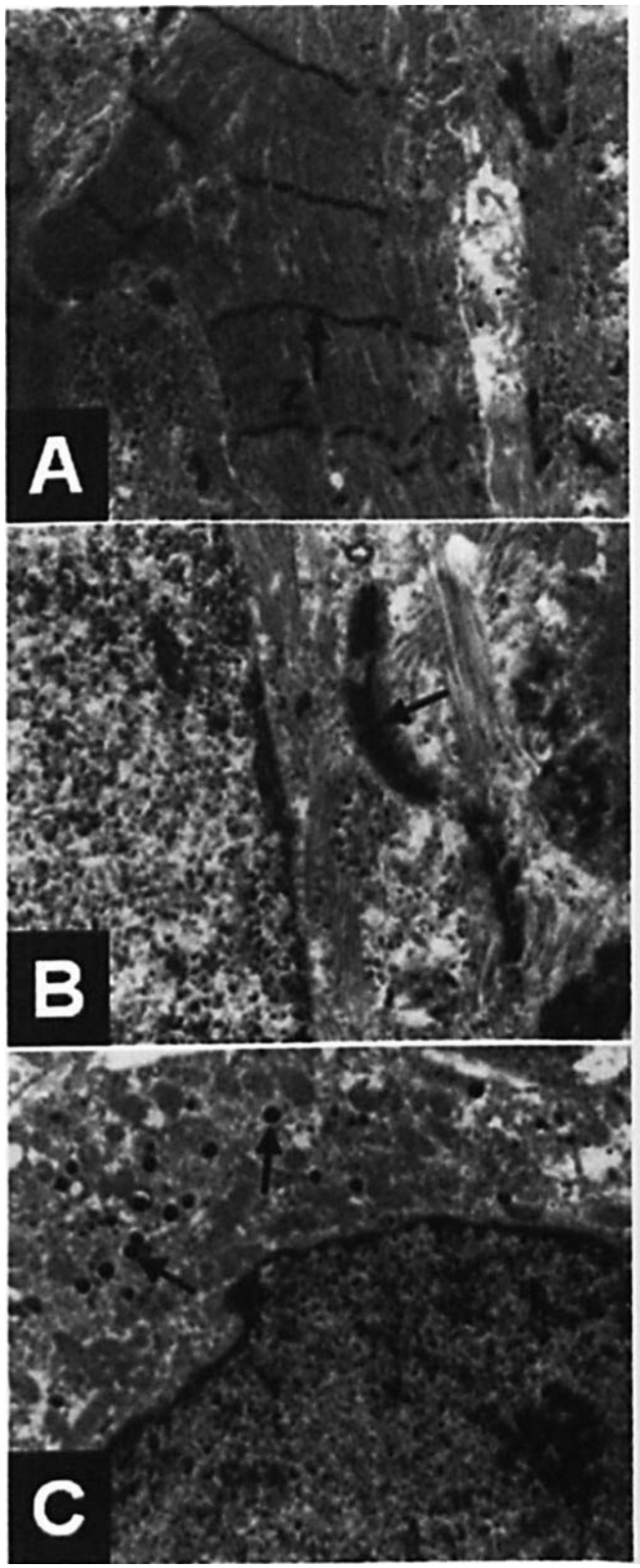
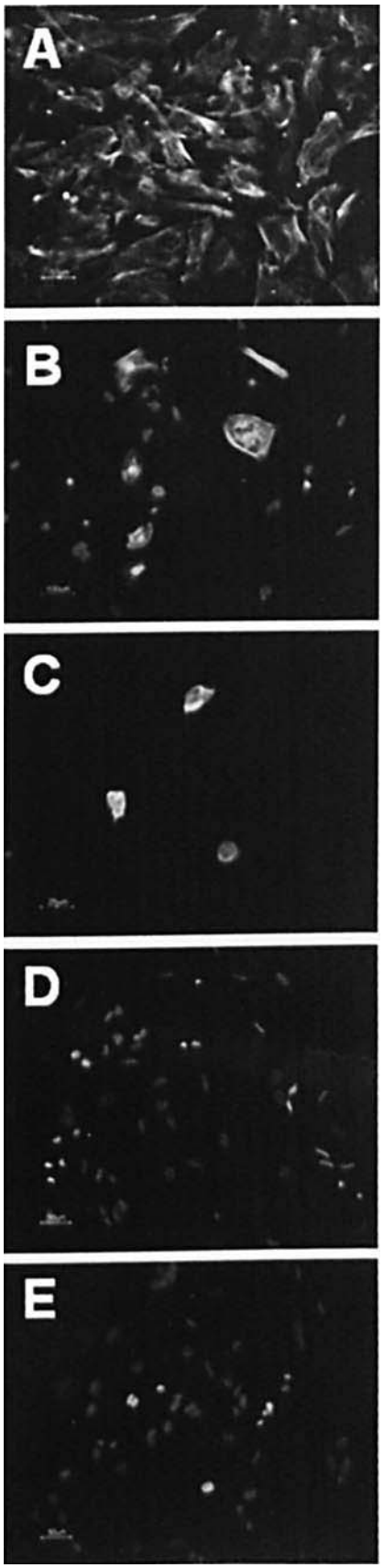


Figure 3. Electron micrographs of a contracting area derived from hESCs. (A) Muscle cells with cardiac muscle–specific intercalated Z discs (arrow). Magnification: ×7750. (B) Adjacent muscle cells with tight junctions (arrow). Magnification: ×22,000. (C) Sparsely distributed cell containing secretory granules (arrows). Magnification: ×7750.



derived cardiomyocytes, which did not appear to be substantially affected by the actual cluster size.

Using electron microscopy it was observed that the predominant cell type of the contracting beating areas was elongated, striated cells, demonstrating intercalated Z discs and tight junctions between adjacent cells (Fig. 3A and B). Sparsely, granulated cells were observed, having a dispersed chromatin, demarcated nucleolus, and dense cytoplasmatic granules, giving them the character of secretory cells (Fig. 3C).

Dissociated and subcultured beating areas of hESC-derived cardiomyocytes stained positively for desmin (Fig. 4A), suggesting a mesodermal lineage commitment of these cells. Furthermore, antibodies directed against specific cardiac markers, cTnI and cMHC, confirmed the cardiomyocyte phenotype of these cells (Fig. 4B and C). Using immunohistochemical analysis, expression of connexin 43, however, was not detected in these cells (data not shown). The reason for this observation currently is unclear but may reflect limitations in the detection level of the assay or destruction of the antigen by the enzymatic dissociation of the cell clusters. Early cardiac progenitors also were present in these cell populations and stained positive for the nuclear proteins Nkx2.5 and GATA-4 (Fig. 4D and E). In addition, initial observations using light microscopy suggested that some of the subcultured beating cells had the potential to replicate. In order to experimentally test this hypothesis, BrdU labeling and cTnI immunostaining were used as ways to detect proliferation of these cells. As shown in Figure 5, proliferating cTnI-positive cells as well as nonproliferating cTnI-positive cells were detected. A proportion of the cells were also BrdU positive but cTnI negative.

The basal spontaneous contraction frequency of the isolated beating areas varied from 12 to 120 bpm. Normally, after dissection of the EBs contractile activity reestablished within a couple of hours at the same frequency as that observed prior to isolation. Contractions were regular in most EBs, but irregular, bigeminal, or trigeminal rhythms were noticed occasionally. In some specimens contractions occurred intermittently, with more or less regular pausing. In a few beating clusters, contractile activity ceased for days but the clusters retained their contractile potency, as evidenced by their response to stimulatory agents. Specimens that did not demonstrate persistent regular contractile activity were omitted from the pharmacological experiments (see below). Changing the culture medium every 2–3 days, contractile activity was registered up to 8 weeks after excision of the contracting EBs. The majority of specimens followed that long exhibited their endogenous basal

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Figure 4. Immunohistochemical analysis of dissociated and replated contracting cells derived from hESCs was performed as described in Materials and Methods. The cell nuclei were visualized using DAPI staining (blue). (A) Desmin. (B) cTnI. (C) cMHC. (D) Nkx2.5. (E) GATA-4. Color figure available in online version of the journal.

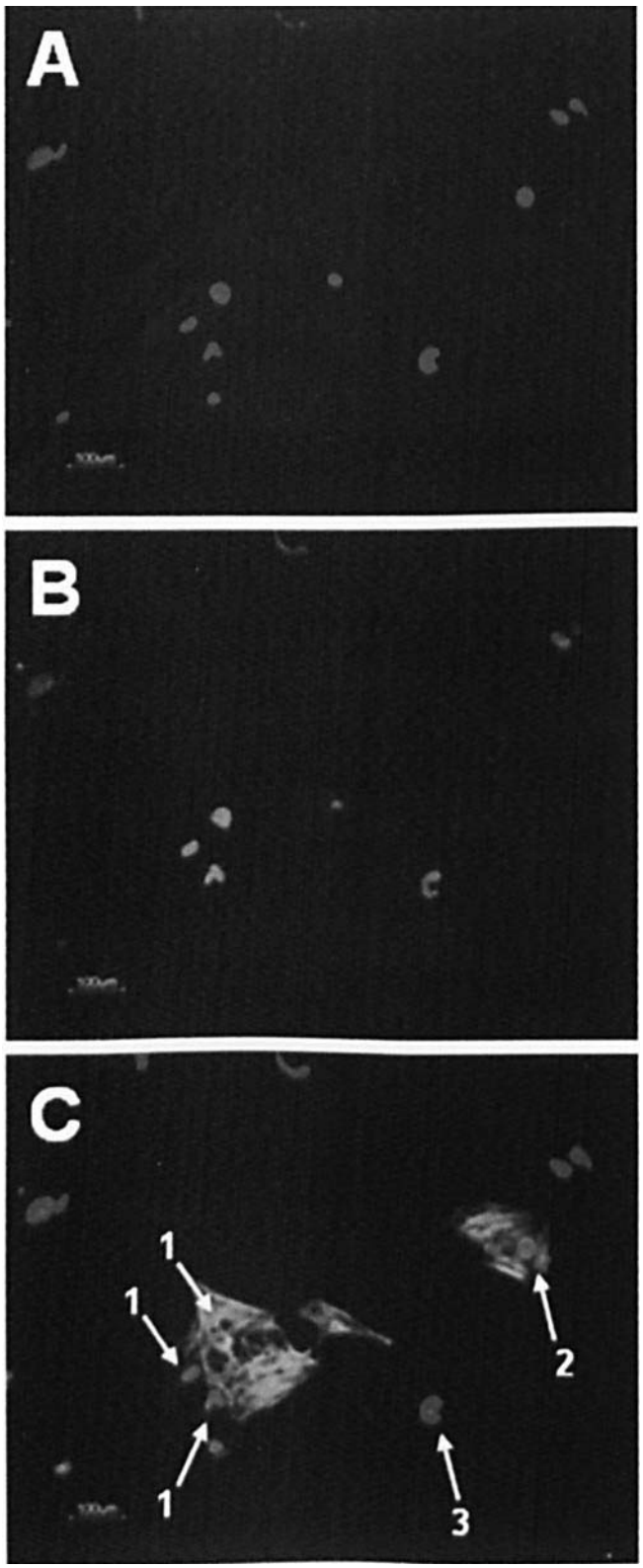

Figure 5. Immunohistochemical analysis and BrdU labeling of dissociated and replated contracting cells derived from hESCs was performed as described in Materials and Methods. DAPI staining (A) and BrdU labeling (B) of proliferating cells. (C) An overlay of DAPI, BrdU, and cTnI immunostaining. The arrows indicate (1) proliferating cTnI-positive cells, (2) nonproliferating cTnI cells, and (3) proliferating cTnI-negative cells. Color figure available in online version of the journal.

Table 1. Contractile Activity (Beats Per Min), Registered Consecutively Every 30 Min, Expressed in Percentage of Basal Activity in Each Experiment (Mean ± SEM)[ab]

Concentration	Beat frequency (%)	Range (%)
PHE/PHBA ($n = 6$)		
PHE		
$10^{-11}M$	119 ± 16.6	108–138
$10^{-10}M$	177 ± 50*	107–212
$10^{-9}M$	202 ± 60	117–250
PHBA		
$10^{-7}M$	170 ± 52	100–235
$10^{-6}M$	146 ± 46*	87–200
$10^{-5}M$	106 ± 18	88–156
ADR/AT ($n = 4$)		
ADR		
$10^{-8}M$	215 ± 30*	185–258
$10^{-7}M$	273 ± 101	188–416
$10^{-6}M$	196 ± 54	242–300
AT		
$10^{-8}M$	186 ± 28*	158–214
$10^{-7}M$	146 ± 37	75–171
$10^{-6}M$	81 ± 37	50–133
NA/PHBA ($n = 6$)		
NA		
$10^{-8}M$	120 ± 13.8	110–156
$10^{-7}M$	132 ± 8.5*	124–140
$10^{-6}M$	153 ± 7.5	144–169
$10^{-5}M$	195 ± 22	168–230
PHBA		
$10^{-5}M$	103 ± 8*	93–115
ACH/ATR ($n = 6$)		
ACH		
$10^{-6}M$	83 ± 23	49–100
$10^{-5}M$	83 ± 22	39–100
$10^{-4}M$	70 ± 26*	0–98
$10^{-3}M$	57 ± 23	0–81
ATR		
$10^{-4}M$	106 ± 5*	100–114
Forskolin ($n = 6$)		
$10^{-11}M$	61 ± 2	59–64
$10^{-10}M$	138 ± 17*	115–167
$10^{-9}M$	162 ± 66	100–291
$10^{-8}M$	174 ± 59	104–273
$10^{-7}M$	180 ± 64	108–300
$10^{-6}M$	198 ± 74	111–309

[a] The lowest concentration giving a significant change of beat frequency in relation to control or maximal stimulation/inhibition is indicated.
[b] PHE, phenylephrine; PHBA, phenoxybenzamine; ADR, adrenaline; AT, atenolol; NA, noradrenaline; ACH, acetylcholine.
* $P < 0.01$.

contraction frequency throughout this period, although in some beating areas demonstrating morphologic degenerative signs, a reduction of the beating frequency could be observed at term. In a few cases contractile activity was observed in newly formed satellite areas exhibiting a contraction frequency similar to that of the originally dissected EB. Various agents known to exert chronotropic effects on cardiac musculature were tested in pharmacological experiments.

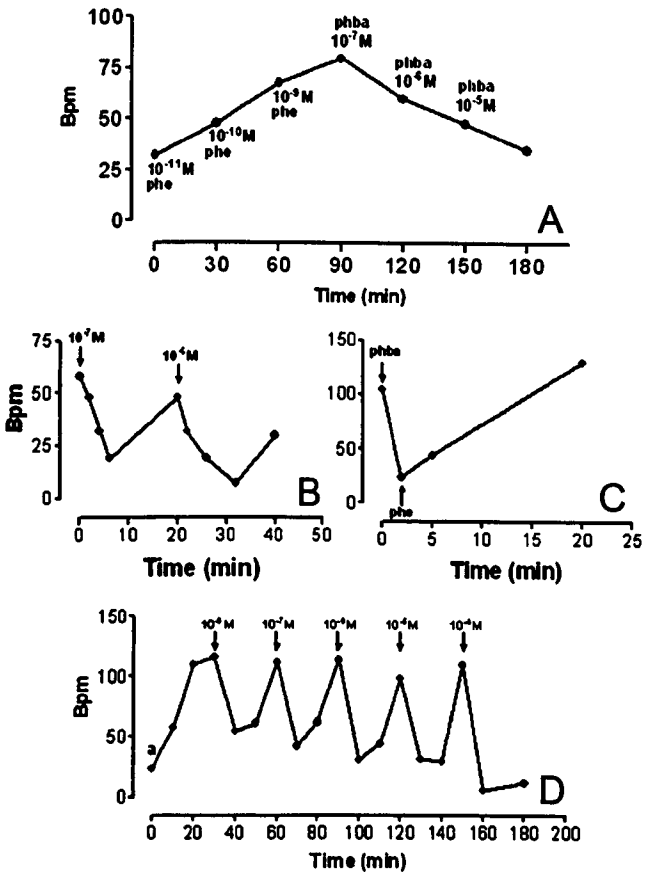

Figure 6. Adrenoceptor response in hESC-derived beating clusters. Beating areas were derived and treated as described in Materials and Methods. (A) The effects of phenylephrine (phe) and phenoxybenzamine (pba) on contractile activity in hESC-derived beating cells recorded 30 mins after administration (representative data from 1 of 6 experiments). (B) The effect of phenoxybenzamine occurring within minutes, with a partial recovery after 20 mins (representative data from 1 of 4 experiments). (C) The effects of phenoxybenzamine (phba) and phenylephrine (phe) (representative data from 1 of 3 experiments). (D) The effect of labetalol on the beating frequency was monitored after administration of 10^{-8} *M* adrenaline (a) (representative data from 1 of 2 experiments).

Phenylephrine, an α_1-adrenoceptor agonist, was administered to contracting areas at increasing concentrations in 30-min intervals. Contractile activity was stimulated in a dose-dependent manner (Table 1). The stimulatory effect was more pronounced in specimens with a low basal contraction frequency. One of twelve beating areas was nonresponsive to phenylephrine. Phenylephrine (10^{-7} *M*) administered to arrested areas initiated contractile activity promptly (data not shown). The stimulatory effect of phenylephrine was counteracted by phenoxybenzamine, a blocker of α_1 and α_2 receptors (Table 1 and Fig. 6A). Irregular contractions were occasionally observed at maximal stimulation by phenylephrine but returned to regular beats following inhibition by phenoxybenzamine. When administrated primarily to contracting clusters of cells, phenoxybenzamine reduced the contraction frequency (Fig. 6B). This effect was immediate, and there was a successive restitution of contraction frequency. The inhibition was reversed by phenylephrine (Fig. 6C).

Adrenaline, exhibiting predominantly β_1-adrenoceptor agonistic effects in the myocardium, stimulated contractile activity in 9 of 10 experiments. The stimulatory effect was registered within minutes, and maximal stimulation occurred at a concentration of 10^{-7} *M* (Table 1). In specimens exhibiting low contraction frequency (<20 bpm), a 3-fold increase of frequency was observed at 10^{-9} *M*, with minimal increase at 10^{-8} to 10^{-6} *M*. The stimulatory effect was reduced within minutes following administration of atenolol, a ß-receptor blocker (Table 1). Likewise, in two experiments, labetolol, a ß- and α_1-receptor blocker, was shown to reduce the stimulation of contraction frequency exerted by adrenaline (Fig. 6D). Atenolol (10^{-7} *M*), primarily given to contracting EBs, reduced spontaneous contractile activity within 2 mins (data not shown). Noradrenaline, an α- and β_1-adrenoceptor agonist, stimulated contractile activity in a concentration–response manner (Table 1). Maximal stimulation was reached within 10 mins, and this response was reversed by phenoxybenzamine. Two of twelve EBs, with spontaneous contraction frequencies of 19 and 69 bpm, respectively, did not respond to 10^{-10} to 10^{-6} *M* noradrenaline. Noradrenaline (10^{-7} *M*) administered to an arrested EB initiated contractile activity within minutes (data not shown).

Acetylcholine, exerting muscarinic effect in the myocardium, reduced the contractile activity in the hESC-derived cardiomyocytes in a concentration-dependent manner (Table 1). Following administration of 10^{-4} to 10^{-3} *M* acetylcholine, total inhibition was sometimes observed, and it persisted for hours. Atropin, which competes with acetylcholine for a common binding site on the muscarinic receptor, counteracted the inhibition by acetylcholine (Table 1). Primarily administered atropin (10^{-6} *M*) stimulated spontaneous contractile activity (data not shown).

Verapamil, a blocker of the transmembranous flow of Ca^{2+} ions and an inhibitor of mobilization of intracellular Ca^{2+}, reduced or abolished the contractile activity, depending on the concentration primarily administered. In three contracting areas, the beating frequency was reduced to approximately 50% of baseline frequency at 10^{-8} *M* and was abolished at 10^{-7} *M* after 30 mins (data not shown). Administrating verapamil at lower concentrations (10^{-12} to 10^{-9} *M*) to three other contracting clusters of cells, a concentration–response effect was observed at 5 and 30 mins (Fig. 7). Spontaneous contractions of basal frequency were reestablished following washing and exchange of medium within 30 mins (data not shown).

Forskolin generally is recognized as a stimulator of adenylate cyclase and formation of cyclic AMP (cAMP). A stimulatory response was registered after 30 mins of treatment with 10^{-10} to 10^{-6} *M* forskolin, compared with baseline frequency (Table 1). Two beating areas were exposed to picomolar concentrations of forskolin. In these areas the contractile activity was inhibited at 10^{-12} *M* during

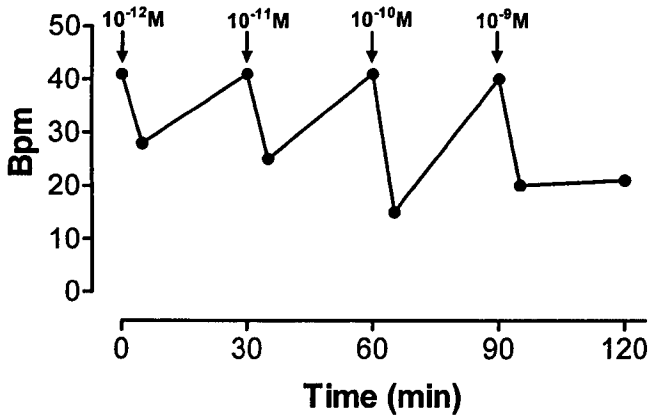

Figure 7. Modulation of intracellular mobilization and flux of Ca^{2+} ions in beating areas derived from hESCs by verapamil. Beating areas were derived and treated as described in Materials and Methods. In dose–response experiments the effect on the contractile activity of verapamil was monitored. At 10^{-9} *M* a persistent reduction of the beating frequency was observed. The graph shows representative data from 1 of 3 experiments.

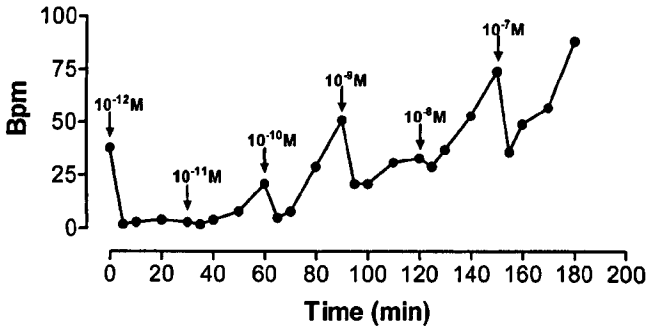

Figure 8. Modulation of intracellular cAMP levels in beating areas derived from hESCs by forskolin. Beating areas were derived and treated as described in Materials and Methods. Forskolin inhibited the contractile activity at low concentrations. At increasing concentrations a transient reduction of beat frequency was followed by stimulation as observed 30 mins after administration. The graph shows representative data from 1 of 3 experiments.

registration between 5 and 30 mins but was stimulated at higher concentrations of forskolin, although an initial but transient inhibition was observed 5 mins after administration (Fig. 8).

Discussion

Based on the pioneering work concerning hESC derivation in the late 1990s, a new field in human biology has developed, forming wide clinical perspectives (7). The pluripotency of hESCs gives them the capacity to differentiate into organ-specific cells, which raises the hope of using them in the regeneration of damaged tissue, including the injured myocardium (13, 21). In addition, specialized functional cells derived from hESCs have a huge potential as investigative tools for the cost-effective development of safer drugs (14). In particular, hESCs can differentiate into spontaneously contracting cells that resemble early-stage cardiomyocytes. For obvious reasons, the availability of primary human cardiomyocytes for pharmaceutical drug discovery is very limited, but it is likely that hESC-derived cardiomyocytes will find considerable use in areas such as target identification and validation studies. Functional as well as molecular evaluations of these cells have been performed by a few independent laboratories, providing support for an embryonic cardiomyocyte phenotype of the cells (3–6). However, much research remains to be done in order to better define the population of cells that may prove useful for regenerative medicine or for various *in vitro* applications. In the present study, we derived and isolated cardiomyocyte-like cells from contracting EBs. We could further manipulate and successfully cryopreserve these cells. Molecular and pharmacological evaluation indicated that the cells display properties of cardiomyocytes, lending support to the ongoing efforts of the development of novel drug discovery tools and disease intervention opportunities based on hESCs.

The efficiency of cardiogenesis in hESCs has been reported to vary, probably due to variations of the culture conditions and inherent differences between hESC lines. Our results (Fig. 1) are within the range previously reported (3–6). It is still unclear which factors may trigger the differentiation of hESCs to cardiomyocytes. However, some data have been published on conditions that may enhance this differentiation. For example, in murine ESCs, members of the transforming growth factor-β (TGF-β) family, fibroblast growth factors, and nitric oxide have been shown to promote differentiation into cardiomyocytes (22–24). Furthermore, among 880 tested compounds, ascorbic acid was shown to enhance the differentiation of mouse ESCs into cardiomyocytes (25), a finding recently reproduced in serum-free cocultures of hESCs and END-2 cells (18). Among compounds shown as inducing cardiomyocyte differentiation in murine ESCs, 5-aza-2′-deoxycytidine, but not dimethyl sulfoxide or retinoic acid, was shown to also enhance cardiomyocyte formation of hESCs (4). It appears that the timing of administration and the concentration of a given exogenous factor are crucial for induction of cardiogenesis, but the molecular mechanisms that regulate this process remain to be determined. In the present study, no efforts were made in order to explore conditions which might enhance cardiomyocyte differentiation from hESCs.

The dissected contracting foci preserved their general morphology up to 8 weeks after isolation, as observed by light microscopy. Cells adjacent to the dissected areas proceeded to proliferate as monolayers and by forming bridges of condensed cells and structures similar to the beating areas. However, only in a few cases did minor satellite areas demonstrate contractile activity at a frequency similar to that of the neighboring originally transferred area. At the present time it cannot be ruled out whether the cells of noncontracting areas possessed some characteristics of

cardiomyocytes, although not exhibition of contractile activity (e.g., quiescent ventricular myocytes). Electron microscopy showed that the predominance of cells in the contracting EBs represented striated cardiac muscle cells, with typical intercalated discs and dense bodies between adjacent cells (Fig. 3). Our observations are similar to those previously reported (3, 26). Cells containing secretory granules were observed sparsely in some specimens. These cells may be atrial natriuretic peptide–secreting cells, as documented previously (3, 4). In our cultures, immunohistochemical analysis showed positive staining in the contracting cells for the sarcomeric proteins cTnI and αMHC, thus further confirming the identity of these cells as cardiomyocytes (Fig. 4B and C). More strikingly, we observed that some of the cTnI-expressing cells proliferated, as evidenced by BrdU labeling (Fig. 5). Similar observations recently have been reported *in vitro* and after transplantation of contracting clusters of cells to immunodeficient rodents (26–28). These findings suggest that under suitable experimental conditions there are possibilities to isolate and expand an early-stage cardiomyocyte-like cell population derived from undifferentiated hESCs. In support of this observation, we also detected cardiac progenitors expressing Nkx2.5 and GATA-4 in our cultures (Fig. 4D and E).

Based on the variation in spontaneous contraction frequency of cell clusters obtained from different EBs, it is likely to assume that the cells represent atrial, ventricular, as well as nodal cardiac muscle cells. Nevertheless, their response to chronotropic agents was principally similar, although in cell clusters exhibiting low beat frequency, the response to adrenoceptor agonists generally was more pronounced. Thus, our results (Table 1) support the notion of the development of functioning both adrenergic and cholinergic mechanisms as an early event in embryonic cardiomyocyte differentiation, as indicated previously (3, 4, 6). The endogenous spontaneous contractile activity and the response to pharmacological agents appeared unaffected by repeated experiments, since the basal contractile activity returned after washing, and the pharmacological response could be repeated after weeks in culture. Exceptionally, contracting clusters did not respond to any chronotropic agent, which may imply an impediment of receptor differentiation. It has previously been shown that the response to adrenoceptor agonists is time dependent and better recognized in more differentiated cardiomyocytes (4).

Cyclic AMP is the main second messenger in adrenoceptor signaling (29). Forskolin is a cell-permeable diterpenoid with antihypertensive and positive inotropic effects. Forskolin exhibits adenocyclase activity and stimulates the formation of intracellular cAMP. The stimulation observed after administration of forskolin may illustrate the existence of a functioning cAMP system in the embryonic cardiomyocytes. Forskolin also may interfere with calcium transport and calcium currents. The persistent inhibition of contractions observed after forskolin administered in picomolar concentration and the initial but transient inhibition observed at higher concentration might possibly be due to immediate calcium ion–related events that are overcome by cAMP-induced stimulation of contractile activity (Table 1, Fig. 8). The existence and dependence of functioning calcium ion channels for the maintenance of contractile activity were further demonstrated by the reduction and eventual arrest of contractions by administration of increasing concentration of the Ca^{2+} channel blocker verapamil (Fig. 7).

Together, the results presented in this study and those from previously published reports broaden our understanding of cardiac differentiation of hESCs and clearly show that the hESC-derived cardiac cluster consists of a mixture of cell types (e.g., atrial, ventricular, nodal, and pacemaker cells) in various developmental stages (4, 6, 26). Major efforts are urgently needed to develop novel and more specific differentiation protocols to successfully produce large numbers of cardiomyocytes from hESCs and to further subject these cells to separation protocols in order to yield purified and defined cell populations. Besides their potential use in regenerative medicine (27, 30), promising future applications for hESC-derived cardiomyocytes include the use of these cells in pharmaceutical safety studies in order to assess the potential cardiac safety risks of novel drug candidates. In particular, the effects on QT interval prolongation or direct arrhythmic effects can be tested (31). It is anticipated that additional basic research in the area of hESC differentiation will contribute to realizing the huge potential of these cells both as therapeutic agents and as a means of providing novel tools for pharmaceutical drug discovery.

1. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433:647–653, 2005.
2. Parmacek MS, Epstein JA. Pursuing cardiac progenitors: regeneration redux. Cell 120:295–298, 2005.
3. 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 108:407–414, 2001.
4. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 91:501–508, 2002.
5. 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 93:32–39, 2003.
6. 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 107:2733–2740, 2003.
7. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147, 1998.
8. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic

stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399–404, 2000.

9. Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350:1353–1356, 2004.
10. Zeng X, Miura T, Luo Y, Bhattacharya B, Condie B, Chen J, Ginis I, Lyons I, Mejido J, Puri RK, Rao MS, Freed WJ. Properties of pluripotent human embryonic stem cells BG01 and BG02. Stem Cells 22:292–312, 2004.
11. Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C, Semb H. Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22:367–376, 2004.
12. Pera MF. Human pluripotent stem cells: a progress report. Curr Opin Genet Dev 11:595–599, 2001.
13. Bjorquist P, Jeppsson A. Cell transplantation for myocardial regeneration: hype or reality? Scand Cardiovasc J 38:259–264, 2004.
14. McNeish J. Embryonic stem cells in drug discovery. Nat Rev Drug Discov 3:70–80, 2004.
15. Davila JC, Cezar GG, Thiede M, Strom S, Miki T, Trosko J. Use and application of stem cells in toxicology. Toxicol Sci 79:214–223, 2004.
16. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 19:1129–1155, 2005.
17. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6:88–95, 2000.
18. 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 23:772–780, 2005.
19. Reubinoff BE, Pera MF, Vajta G, Trounson AO. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod 16:2187–2194, 2001.
20. Noaksson K, Zoric N, Zeng X, Rao MS, Hyllner J, Semb H, Kubista M, Sartipy P. Monitoring differentiation of human embryonic stem cells using real-time PCR. Stem Cells 23:1460–1467, 2005.
21. Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol 23: 845–856, 2005.
22. Kanno S, Kim PK, Sallam K, Lei J, Billiar TR, Shears LL, 2nd. Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. Proc Natl Acad Sci U S A 101:12277–12281, 2004.
23. Dell'Era P, Ronca R, Coco L, Nicoli S, Metra M, Presta M. Fibroblast growth factor receptor-1 is essential for in vitro cardiomyocyte development. Circ Res 93:414–420, 2003.
24. Behfar, A, Zingman, LV, Hodgson, DM, Rauzier, JM, Kane, GC, Terzic, A, and Puceat, M. Stem cell differentiation requires a paracrine pathway in the heart. Faseb J 16:1558–1566, 2002.
25. Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Gullans SR, Lee RT. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 107:1912–1916, 2003.
26. Snir M, Kehat I, Gepstein A, Coleman R, Itskovitz-Eldor J, Livne E, Gepstein L. Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. Am J Physiol Heart Circ Physiol 285:H2355–H2363, 2003.
27. Laflamme MA, Gold J, Xu C, Hassanipour M, Rosler E, Police S, Muskheli V, Murry CE. Formation of human myocardium in the rat heart from human embryonic stem cells. Am J Pathol 167:663–671, 2005.
28. Passier R, Mummery C. Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16:498–502, 2005.
29. Post SR, Hammond HK, Insel PA. Beta-adrenergic receptors and receptor signaling in heart failure. Annu Rev Pharmacol Toxicol 39: 343–360, 1999.
30. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, Huber I, Satin J, Itskovitz-Eldor J, and Gepstein L. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nat Biotechnol 22:1282–1289, 2004.
31. Reppel M, Pillekamp F, Brockmeier K, Matzkies M, Bekcioglu A, Lipke T, Nguemo F, Bonnemeier H, Hescheler J. The electrocardiogram of human embryonic stem cell-derived cardiomyocytes. J Electrocardiol 38:166–170, 2005.