Embryonic stem cell-derived cardiomyocytes

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Cardiomyocytes Purified from Differentiated Embryonic Stem Cells Exhibit Characteristics of Early Chamber Myocardium

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ABSTRACT

Mouse embryonic stem cells easily differentiate towards the cardiac lineage making them suitable as an *in vitro* model to study cardiogenesis and as a potential source of transplantable cells. In this study we show by in situ hybridisation that about 30% of the volume of cultures of differentiating embryonic stem cells consists of cardiomyocytes. RT-PCR analyses showed that the transcription factors *Nkx2.5, Gata4, Mef2c* and *Irx4* were expressed at levels in the same order of magnitude as the levels observed in embryonic, neonatal and adult hearts. *Atrial natriuretic factor* and *Connexin40*, associated with chamber formation *in vivo*, are expressed at relatively low levels, similar to those observed at early heart development *in vivo*. To facilitate the isolation of embryonic stem cell-derived cardiomyocytes, a cell line was constructed by stable transfection of the aminoglycoside phosphotransferase cDNA driven by the cardiac-specific distant upstream part of the Na\(^+\)/Ca\(^{2+}\) exchanger promoter. To accomplish single copy integration, the construct was inserted into the hypoxanthine phosphoribosyltransferase locus of HM1 embryonic stem cells by homologous recombination. Cardiac-specific resistance to G418-sulphate (neomycin) allowed isolation of a pure population of cardiomyocytes. Genetically selected and unselected cell populations were characterised electrophysiologically using patch clamp. To explore whether clusters of cells have a similar differentiation profile, action potentials were measured in aggregates of differentiating embryonic stem cells, using a new method based on the voltage dependent fluorescent dye di-4-ANEPPS. Both whole-cell recordings using patch clamp and optical measurements with di-4-ANEPPS of the action potential showed that upstroke velocity increases and action potential duration decreases with differentiation time, accompanied by a decrease in action potential interval, suggesting the initiation of the developmental program underlying the formation of chamber myocardium.
INTRODUCTION

Embryonic stem (ES) cell lines have a demonstrated ability to differentiate in vitro into cardiomyocytes that recapitulate, at least to a certain extent, cardiogenesis. Mouse ES cell-derived cardiomyocytes express genes for Gata4 and Nkx2.5 transcription factors prior to those for atrial natriuretic factor (Anf), myosin light chain 2v (Mlc2v), α- and β- myosin heavy chain (Mhc), Na\(^{+}\)/Ca\(^{2+}\) exchanger (Ncx1) and phospholamban (Plb). ES cell-derived cardiomyocytes with electrophysiological characteristics that are typical of ventricular, atrial, His-purkinje or pacemaker cells have been reported to appear in ES cell cultures (see for review Boheler et al. (2002)). This system therefore represents a useful model system to study mechanisms underlying cardiomyocyte development.

During mammalian cardiac development, distinct regions in the heart tube differentiate from primary myocardium. Areas at the dorsal and ventral site of this heart tube specialize to form atrial and ventricular chamber myocardium. Further development results in the four-chambered heart. This developmental process can be mapped in situ by distinct spatiotemporal changes in gene expression (Moorman et al. 2000; Christoffels et al. 2000; Moorman and Christoffels, 2003). Initiation of cardiogenesis in vivo is accompanied by the expression of transcription factors like Gata4, Nkx2.5, Mef2c, and Irx4 (Harvey, 2002), followed by the expression of cardiac-restricted gene products (α- and β-Mhc). Cardiac troponin I (cTnl), a sarcomeric protein, and Ncx1, a sarcolemmal protein, are expressed in all cardiomyocytes regardless of location, whereas genes like Anf and connexin (Cx) 40, are associated with development of working myocardium of the chambers in vivo (Christoffels et al. 2000). The expression patterns of these genes have proven invaluable in the elucidation of developmental paradigms associated with cardiac development (Christoffels et al. 2000; Moorman and Christoffels, 2003).

Postnatally, cardiomyocytes almost completely lose their ability to divide, which makes their loss after trauma irreversible. The recent description of the successful differentiation of human ES cell lines to cardiomyocytes in vitro has led to the hope that differentiated ES cells will be useful for cell replacement therapies in human myocardium. A recent study has, however, shown that ES cell-derived cardiomyocytes may act as a source of arrhythmias, which might preclude their potential therapeutic use in the heart (Zhang et al. 2002).

For therapeutic purposes, we hypothesize that the optimal ES cell-derived cardiomyocyte population would be one that expresses a fully differentiated chamber-specific phenotype i.e., which has limited spontaneous beating activity and expresses markers specific to chamber myocardium. To gain insight into the developmental status of ES cell-derived cardiomyocytes, we have evaluated the expression profiles of transcription factors (Nkx2.5, Gata4, Mef2c, Irx4) and cardiac genes (Anf, Cx40, cTnl and Ncx1), using quantitative real-time reverse transcription (RT)-PCR and non-radioactive in
Purification of ES cell-derived cardiomyocytes

Furthermore, we have employed the cardiac restricted Ncx1 promoter to express aminoglycoside phosphotransferase cDNA in cardiomyocytes, to confer resistance to the antibiotic G418-sulphate (neomycin). This early cardiac-restricted promoter has allowed us to isolate a pure population of ES cell-derived cardiomyocytes. Conventional patch clamp techniques and non-invasive electrophysiological methods, based on the voltage dependent fluorescent dye di-4-ANEPPS (ANEPPS), were then employed to study the action potential characteristics of these cells. We find that both the expression profiles and electrophysiological characteristics of these cells are consistent with a developmental program that leads to formation of early chamber myocardial-like cells from about mouse E8.5 in vivo.

EXPERIMENTAL PROCEDURES

Cell Culturing and Cardiac Differentiation
The mouse ES cell line HM-1 (Magin et al. 1992) was cultured in undifferentiated state as described (Fijnvandraat et al. 2002). Cardiac differentiation was evoked by formation of EBs, essentially as described earlier (Fijnvandraat et al. 2002).

FVB embryos were obtained from timed-pregnant mice (Fijnvandraat et al. 2003) and used for RNA isolation and ISH. Animal handling was in accordance with the ‘Dutch Law on Animal Experimentation (WOD)’ and the ‘European Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (European Union Directive #86/609/CEE)’. The experiments were approved by The Committee for Experiments on Animals (DEC) of the University of Amsterdam, The Netherlands.

Cloning and Transfection
An EcoRl-Sall-fragment containing the aminoglycoside phosphotransferase (Neo) cDNA followed by a SV40 poly-A fragment was coupled to an Ncx1-promoter fragment containing the 2730 bp upstream region and 45 bp of the cardiac-specific exon (Koban et al. 2001). The resulting Ncx1pr-Neo-pA fragment was cloned in the pMP83KB vector, which contains HPRT gene sequences for homologous recombination and restoring HPRT function (Bronson et al. 1996) (kind gift of Dr. Sarah Bronson, Pennsylvania State University, College of Medicine). Transfection was performed in HM-1 ES cells that contain a 55 kb deletion in the X-linked HPRT gene and therefore lacks the promoter and exons 1 and 2 of the HPRT gene. The deletion is restored after successful homologous recombination. 1.3x10^7 ES cells and 25-30 μg purified plasmid in PBS were electroporated at 0.8 kV and 1μF (Gene Pulser Apparatus; Biorad) and plated onto 2 gelatin-coated 8.5-cm petridishes covered with a layer of mouse embryonic fibroblasts. After recovery overnight, selection medium containing culture medium (Fijnvandraat et al. 2002) enriched with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine
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(1x HAT; GIBCO BRL; Life Technologies, The Netherlands). After 8-10 days of selection individual undifferentiated colonies, which should contain the construct in their HPRT locus, were picked, propagated, and tested for their cardiac differentiation potential.

**Myocyte Isolation for Electrophysiology and ANEPPS**

For whole cell and ANEPPS measurements, beating EBs were rinsed twice in low Ca\(^{2+}\)-buffer, containing in mM: NaCl 140, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, glucose 5.5, pH was adjusted to 7.4 by NaOH. Dissociation into single cells and clusters was performed by incubation in low Ca\(^{2+}\)-buffer containing 1 mg/ml collagenase B (Roche; Mannheim, Germany) at 37°C for 30 minutes and gentle trituration at regular intervals. Cells were allowed to adhere to 3-aminopropyltriethoxysilane- or poly-D-lysine-coated coverslips overnight.

**Neomycin Selection**

For cardiomyocyte selection, EBs were dissociated as described above and after overnight-attachment to 3-aminopropyltriethoxysilane-coated coverslips cultured for 6-8 days in the presence of 400 μg/ml G-418-sulphate (Gibco). The method is more specified in the result section.

**RNA isolation, cDNA synthesis and RT-PCR**

RNA was isolated for gene expression studies from undifferentiated ES cells, from pools of 7-10 beating EBs cultured in floating culture and from hearts of developing and adult FVB mice. For each time point at least 2 independent RNA samples were used. Total RNA was isolated as described (Lekanne Deprez et al. 2002; Fijnvandraat et al. 2003). First-strand complementary DNA was synthesised from 1 μg total RNA as described (Lekanne Deprez et al. 2002). For each RNA sample 2 separate cDNA synthesis reactions were performed. The primer sets used had a calculated annealing temperature of 58°C (nearest neighbour method). The sense primer for Ncx1 was designed in a heart-specific part of the transcript (Kofuji et al. 1994). Primers were obtained from Biolegio (The Netherlands) and are given in Table 1.

Quantitative RT-PCR was performed as described before (Lekanne Deprez et al. 2002; Fijnvandraat et al. 2003). Absolute copy numbers were estimated using standard curves made from dilution series of amplicon sequence solutions of known concentration (Lekanne Deprez et al. 2002). Approximate average mRNA copy numbers per cell were calculated assuming that a single mammalian cell contains about 45pg total RNA (Alberts et al. 1994) and during reverse transcription a 1:1 relationship is realised between RNA and cDNA molecules. 18S expression levels were used to correct for variations in RNA input. Data are expressed as mean ± SD.
Observations in EBs were based on two independent differentiation experiments. Both time course experiments showed a significant time dependent effect on gene expression profiles with a similar trend, although peaks in expression varied a few days between the independent experiments \( (P<0.01) \).

**Electrophysiology**

Action potentials (APs) from ES cell-derived cardiomyocytes were recorded using whole-cell patch clamp as described (Fijnvandraat et al. 2003). From spontaneous APs and APs stimulated by 2 ms current pulses applied via the patch clamp pipet, the following parameters were measured: upstroke velocity \( (V_{\text{max}}) \) and AP duration (APD) at 20, 50 and 80% repolarisation and spontaneous beating interval. Of each recording the parameters of at least 5 APs were averaged. Data were expressed as mean \( \pm \) SEM.

**ANEPPS**

A 30 \( \mu \)l perfusion chamber, having two needles at opposite sides for superfusion purposes and two thin parallel platinum electrodes for field stimulation (distance of 8 mm, pulses of 40 V/cm and 0.5 ms duration), was tightly positioned onto the coverslip. Microscope stage and perfusion chamber were temperature controlled at 37°C. A representative part of a beating cluster from partially dissociated EBs was selected with a rectangular diaphragm. Fragments were superfused during 2 minutes with solution 1, containing in mmol/l: NaCl 142.6, KCl 4.7, CaCl\(_2\) 1.3, MgCl\(_2\) 2.0, NaHCO\(_3\) 4.3, Na\(_2\)HPO\(_4\) 1.4, HEPES 17, Glucose 11 (pH adjusted to 7.3 with NaOH) with 1\( \mu \)mol/l di-4-ANEPPS (Molecular Probes) and subsequently with ANEPPS-free solution 1 to remove extracellular ANEPPS. Clusters were preconditioned by 2 minutes of field stimulation at 2 Hz before making measurements. ANEPPS was excited at 516 nm with a 100 W Xenon lamp. Emission was measured at 583 nm with a photomultiplier tube. Fluorescence signals (F) were filtered at 1 kHz, digitised at 10 kHz and stored for later analysis. Signal averaging was performed on 10 action potentials. \( V_{\text{max}} \) was calculated as \( \frac{(\Delta F \text{ per ms})_{\text{max}}}{\Delta F \text{ amplitude}} \). Data were expressed as mean \( \pm \) SEM.
Immunocytochemistry
Differentiated cells attached to coverslips were fixed and stained essentially as described before (Fijnvandraat et al. 2003). A rabbit polyclonal antibody against Serca2a (Eggermont et al. 1990) (kind gift of Dr. F. Wuytack, Department of Physiology, K.U. Leuven, Leuven B-3000 Belgium) or a mouse monoclonal antibody MF20 against myosin heavy chain isoforms (Hybridomabank, Iowa City, IA) was applied 1:3000 or 1:50 respectively. To detect specific labelling, cells were incubated with goat anti-rabbit or goat anti-mouse antibodies conjugated to alkaline phosphatase (DAKO) 1:200. NBT/BCIP (Roche) diluted 1:50 in NTM-T was used as a chromogenic substrate. For detection of fluorescence, secondary antibodies were used conjugated to an ALEXA fluorescent group (Nordic). Nuclei were stained with SYTOX-green (Molecular Probes), 1:40,000 in PBS. Labelling was visualised by confocal laser scanning microscopy (Biorad MRC1024).

Non-Radioactive In Situ Hybridisation
Non-radioactive ISH on sections of EBs and embryos was performed as described, using digoxigenin-labeled probes and tyramid-mediated amplification (NEN, Boston) (Fijnvandraat et al. 2002; Fijnvandraat et al. 2003; Moorman et al. 2001). Isoform-specific probes were used complementary to the mRNA coding for cardiac troponin I (cTnl), and Collagen III (Ausoni et al. 1991). Expression patterns were studied in EBs derived from several independent differentiation experiments that showed similar patterns.

Image Acquisition and Quantification
Digital images were acquired using a Zeiss Axiophot microscope and stored. The volume fraction of cells expressing cTnl can be determined by area measurement according to the principle of Delcesse: 

\[ \frac{V(cTnl)}{V(total)} = \frac{\sum A(cTnl)}{\sum A(total)} \]

in which V indicates volume and A indicates area. This volume fraction falls in the category of ratio measurements as defined by Howard and Reed (Howard and Reed, 1998). Based on the variation in expression area of cTnl mRNA and the variation between EBs observed in a pilot study, it was calculated that a series of 22 EBs in which at least 5 sections per EB had to be measured to accurately quantify (with a coefficient of error less than 5%) the cTnl positive fraction per EB and per group. Expression of cTnl mRNA was visualised by non-radioactive ISH on sections of EBs at day 3+7. cTnl mRNA positive areas were determined in triplicate on randomised images by manual signal-background thresholding using the density slice procedure in NIH Image. Data are expressed as mean ± SD.
Statistics
Differences in gene expression level between time points and between culturing experiments were tested with one- and two-way ANOVA and the Student Newman Keuls post hoc test (P=0.05). Time-dependent changes in electrophysiology parameters were tested with regression analysis. Differences between selected and unselected cells and between cells analysed using patch clamp or ANEPPS were tested using ANOVA. P<0.05 was considered statistically significant. Statistics was performed using SPSS (SPSS Inc., version 11.0.1).

RESULTS
Estimation of the Volume Fraction of Cardiomyocytes in EBs
After 2 days in floating culture (day 3+2), approximately 90% of the aggregates of differentiating ES cells, called embryoid bodies (EBs), showed beating activity, encompassing over 50% of their total volume. To determine the cardiac content of EBs, at day 3+7 the volume fraction was calculated from cTnl mRNA expressing cells. In embryonic hearts, cTnl mRNA was expressed exclusively in the myocardium (Figs. 1A and B) and can therefore be regarded as a marker for cardiomyocytes. Collagen III mRNA positive cells were almost exclusively found in the epicardial, endocardial and cardiac cushion tissue of the heart (Figs. 1D and E). Using non-radioactive ISH, we have determined that the area of cTnl mRNA-positive cells was on average 31±3.7% (SD) of the EB volume. The cTnl mRNA-expressing area did not vary significantly between EBs. To examine the homogeneity of the cardiac areas for cardiomyocytes, Collagen III and cTnl mRNA expression was also visualised on consecutive sections. Collagen III and cTnl mRNA displayed a mutually exclusive pattern of expression (Figs. 1C and F) suggesting that the cTnl-positive areas are almost exclusively populated by cardiomyocytes.

Analysis of Levels of Gene Expression
We examined the expression profiles of the transcription factors Nkx2.5, Gata4, Mef2c and Irx4, involved in the specification and differentiation of cardiomyocytes in vivo. In vivo, we observed cardiac expression of Nkx2.5, Gata4, and Irx4 at the onset of cardiac development. Expression reached a plateau after E9 to adulthood (P=0.0001; one-way ANOVA). Mef2c mRNA showed a several fold higher expression level than the other transcription factors. Data are shown in Figure 2A and Table 2. In vitro, expression of these transcription factors increased significantly from the onset of cardiac differentiation and, after a peak around day 3+10, decreased again, except for the more abundant Mef2c mRNA transcripts, which remained elevated throughout the time frame analysed. Data are shown in Figure 2B and Table 2.
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Figure 1. Expression patterns of cTnl (A-C) and Collagen III (D-F) as revealed by non-radioactive ISH on serial sections of E11.5 mouse embryos (A,B,D,E) and on serial EB sections after 3+7 days of differentiation (C,F). cTnl mRNA (A) is expressed in a pattern mutually exclusive to that of Collagen III (D) in the heart and in the EB (C and F). The dotted frame indicates the part magnified in panels B and E. Bars indicate 0.1mm (A,B,D,E), and 0.01mm (C,F). Panel C shows a representative example of an EB section used to calculate the cardiac volume fraction. LA: left atrium; RA: right atrium; oft: outflow tract; RV: right ventricle; MC: myocardium; ecc: endocardial cushions; EN: endocardium; EP: epicardium.

Next, we analysed the expression of several cardiac-restricted gene transcripts. As reported earlier (Fijnvandraat et al. 2003), in vivo, cTnl, Anf and, as reported here, also Cx40 gene expression gradually rose from E7.5 when the heart is formed (P<0.0001; ANOVA) onward. cTnl mRNA expression showed a perinatal increase in expression but was relatively low again at adulthood. Cx40 mRNA expression increased from E8.5 onwards and peaked at E15, after which Cx40 mRNA expression decreased again to adulthood (Table 2).

In vitro, expression of cardiac genes was not detected in undifferentiated cells. Cardiac gene expression was generally upregulated from the initiation of cardiac differentiation onwards and peaked around day 3+14. Expression of cTnl, Anf and Cx40 decreased with continued differentiation after day 3+17 (not shown). The highest expression level of the separate genes observed during ES cell differentiation has been given in Table 2 for comparison with the in vivo data.

In general, transcription factors are expressed at considerably lower levels than the cardiac-restricted genes, but their expression is equal to or even higher in differentiating ES cells compared to the developing heart. Anf and Cx40 mRNAs are expressed at levels similar to those observed at early embryonic stages.
TABLE 2.

<table>
<thead>
<tr>
<th>Development (days)</th>
<th>E7.5</th>
<th>E8.5</th>
<th>E9</th>
<th>E10</th>
<th>E11</th>
<th>E12</th>
<th>E13</th>
<th>N7</th>
<th>Adult</th>
<th>EBs</th>
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<tr>
<td>Nkx2.5</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>2.9 ± 1.1</td>
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<tr>
<td>Gata4</td>
<td>0.7 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>3.3 ± 0.4</td>
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<tr>
<td>Mef2c</td>
<td>7.0 ± 1.7</td>
<td>7.6 ± 2.7</td>
<td>7.6 ± 1.6</td>
<td>6.5 ± 0.9</td>
<td>6.1 ± 1.4</td>
<td>8.4 ± 0.5</td>
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<tr>
<td>Irx4</td>
<td>0</td>
<td>0.04 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.02</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.03</td>
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<td>Ncx1</td>
<td>42.5 ± 10.5</td>
<td>148.5 ± 30.4</td>
<td>173.7 ± 15.3</td>
<td>78.1 ± 11.7</td>
<td>64.4 ± 30.4</td>
<td>112.3 ± 11.1</td>
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<tr>
<td>cTnl</td>
<td>1.1 ± 0.5</td>
<td>20.4 ± 4.9</td>
<td>37.5 ± 7.7</td>
<td>176.5 ± 21.7</td>
<td>89.8 ± 68.4</td>
<td>66.7 ± 9.6</td>
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<tr>
<td>Anf</td>
<td>12.8 ± 4.9</td>
<td>454.2 ± 161.4</td>
<td>321.2 ± 48.9</td>
<td>370.5 ± 35.7</td>
<td>649.3 ± 259.6</td>
<td>20.6 ± 2.6</td>
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<td>Cct40</td>
<td>6.0 ± 2.9</td>
<td>32.9 ± 5.6</td>
<td>34.2 ± 5.1</td>
<td>18.6 ± 2.2</td>
<td>11.8 ± 2.4</td>
<td>22.7 ± 5.2</td>
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</table>

Absolute expression levels of transcription factors involved in cardiogenesis and of several cardiac genes determined at several days during in vivo development. The highest level of expression reached during in vitro differentiation has been indicated in the last column (averaged levels of 2 experiments). Data are indicated as absolute mRNA copy numbers ± SD of two individual differentiation experiments. E, embryonic day; N, neonatal day; EB, embryoid body.

Figure 2. Expression profiles of the transcription factors Nkx2.5, Gata4, Mef2c and Irx4 during cardiac development in vivo (A) and during in vitro differentiation of ES cells (B). Expression levels have been depicted as molecules per cell (see materials and methods section). E: embryonic day, N: neonatal day, A: adult (3 months). '3+' denotes the 3-day hanging drop period.

Ncx1 mRNA Expression and Selection of Cardiomyocytes

Expression of Ncx1 mRNA increased significantly just after formation of the heart, levels off at E9 and decreased after birth (P<0.0001; ANOVA). The developmental profiles of Ncx1 mRNA expression in vivo are given in Figure 3. Similar to the in vivo situation, Ncx1 transcripts are abundant at early time points in vitro, indicating that the endogenous promoter is already active at very early stages of differentiation.

Recently, we described a cardiac-restricted Ncx1 promoter region that might be useful for the isolation of a pure population of cardiomyocytes (Koban et al. 2001). The distant upstream region of the Ncx1 promoter is strongly expressed from E7.5 onwards, and as seen in Figure 4, exclusively and homogenously in all cardiomyocytes (Koban et al. 2001). To acquire a relatively pure population of ES cell-derived cardiomyocytes, a genetic selection system was established that employed the cardiac-restricted Ncx1 promoter. To this end, the hypoxanthine phosphoribosyl transferase (HPRT) approach was employed. HPRT is a ubiquitously expressed gene and the locus is transcriptionally active and
accessible in all tissues at all developmental stages of development (Bronson et al. 1996).

Strong and specific transgene expression has been reported, solely depending on the regulatory DNA sequences of the integrated transgene construct (Evans et al. 2000; Cvetkovic et al. 2000; Guillot et al. 2000). Direct integration in this genomic environment excludes ectopic expression and mosaicism that would result in selection of non-cardiomyocytes and to lower yields. After transfection of the Ncx1 promoter region connected to the neomycin resistance gene in the HPRT locus, 4 cell lines were compared for their cardiogenic potential. All clones gave rise to cardiomyocytes that exhibited vigorous beating encompassing more than 50% of the volume of the EB after 2 days in floating culture. After testing different working concentrations of neomycin, a concentration of 400 µg/ml resulted in an optimal selection of pure populations of beating cardiomyocytes. Highest yields were observed when selection was started around day 3+3, just after the appearance of beating myocytes. After 6-8 days of selection, cardiomyocytes were visibly contracting with a rhythm of approximately 1 Hz. In every selected cell beating activity was observed, indicating a relatively pure population of cardiomyocytes. 5-10% of the initial number of cells plated before selection was left after 7-8 days of neomycin selection.

Cardiac identity of the cells and homogeneity of the population was verified by immunocytochemistry with antibodies against myosin heavy chain (Figs. 5B and C) and Serca2a (Figs. 5D-H), a sarcoplasmic protein expressed in all types of cardiomyocytes (Anger et al. 1994; Moorman et al. 1995). Skeletal muscle-specific MyoD was not detectable using RT-PCR (not shown). Although a difference in labelling intensity was observed among cells, hardly any observed cells were negative for antibody labelling, indicating an efficient selection of cardiomyocytes. A variety of cellular morphologies was present after selection (Figs. 5F-H). No relation was observed between cellular morphology and electrophysiological characteristics (data not shown).
Purification of ES cell-derived cardiomyocytes

Figure 4. Whole-mount staining of a E9.5 mouse embryo (lateral view) to detect β-galactosidase activity as described (Koban et al. 2001). NexI promoter activity is visible in the developing atrium (A) and ventricle (V) and in the inflow tract (IFT) atrioventricular canal (AVC) and the outflow tract (OFT).

Figure 5. Immunocytochemical analysis of differentiated HM1 ES NexI-Neo' cells. Unselected cells after 7 days floating culture, plated overnight and labelled with MF20 showing cardiac areas (A; bar=1 mm). Cluster of cells after an additional 8 day-selection period, before (B) and after (C) antibody staining with MF20 showing staining of all cells in the cluster with varying intensity (bar=0.1mm). Labelling with Serca2a antibody (D-H): Cluster of cells co-labeled with SYTOX-green, showing all cells in the cluster positive for Serca2a (D; bar=0.1 mm). Lawn of cardiomyocytes cultured for 3+14 days followed by a 8 day selection period (E; bar=1 mm). Several morphologies can be distinguished (F-G; bar=0.1 mm).

Electrophysiology

Electrophysiology of ES cell-derived cardiomyocytes was performed after enzymatic dissociation of EBs or after dissociation followed by neomycin selection. The degree of differentiation was determined from the following action potential characteristics: Action
Figure 6. Electrophysiological comparison of neomycin-selected myocytes with unselected myocytes. Open symbols: unselected, closed symbols neomycin-selected. X-axis: differentiation time in days, including the 7-day selection period for the selected myocytes. ’3+’ denotes the 3-day hanging drop period.

APD measured at 20, 50 and 80% depolarisation. APD decays continuously at all levels (regression analysis, \(P<0.01\), both selected and unselected cells). APDs at 3+11 and 3+14 days of unselected cells were not different from those of selected cells (\(P=0.45\) resp 0.88; ANOVA) (A). During differentiation \(V_{\text{max}}\) increases in the unselected cells (regression analysis, \(P<0.01\)). This increase was not significant in selected cells (\(P=0.17\)). \(V_{\text{max}}\) at 3+11 and 3+14 days of unselected cells was not different from those of selected cells (\(P=0.98\) resp 0.74; ANOVA) (B). Spontaneous beating interval decreases during differentiation (\(P<0.01\) for both selected and unselected cells). Interval of unselected cells at 3+11 days was different from that of selected cells (\(P<0.01\); ANOVA) (C).

Potential (AP) duration (APD_{50}), upstroke velocity (\(V_{\text{max}}\)) and spontaneous beating interval. Cells were measured at succeeding times of differentiation. Because cardiomyocytes were selected for at least 7 days, APs of purified cells were quantified from 3+10 days onwards. Selected and unselected cells were largely similar. Figure 6 shows that the APD significantly decreased with differentiation both in cardiomyocytes derived from selected and unselected cultures. During differentiation, \(V_{\text{max}}\) increased. This increase was significant for the unselected cardiomyocytes but not for the selected myocytes. It is likely however, that the highest increase in \(V_{\text{max}}\) has already taken place during the selection period, which is to be expected since in unselected cells \(V_{\text{max}}\) increased particularly at the earlier time points. With differentiation, the spontaneous beating interval decreases from 1s to 0.25s. This trend is observed in both selected and unselected cultures, however, the decrease started later in selected cells. \(V_{\text{max}}\) and APD did not differ at overlapping time points for selected and unselected cardiomyocytes, suggesting only minimal influence of the selection procedure on developmental progress and indicating the Ncx1 promoter as a feasible driver of the selection method.

It can be envisioned that the initiation of chamber myocardium differentiation might be inhibited by the selection procedure and that prolonged culture in the context of the intact EB would result in more mature cells. To investigate this possibility, we measured APs in fragments of EBs using a newly developed non-invasive method based on ANEPPS fluorescence.
We therefore compared whole-cell AP data, recorded from cardiomyocytes with data obtained from optical measurements, recorded from large cell clusters of EBs, using ANEPPS. With this latter technique, the three dimensional EB context is preserved. Figure 7A shows representative action potentials derived from ANEPPS fluorescence (upper trace) and from whole cell recordings (lower trace). Results obtained by both techniques indicated that with differentiation APD50 decreased (Fig. 7B), whereas V\text{max} did not increase significantly (Fig. 7C). The ANEPPS method verified the average patterns as acquired by patch clamp, as depicted in Figures 7A-C. Furthermore, the electrophysiological patterns proved the ANEPPS method as a valid method to assess the average electrical activity in cell clusters. We conclude that based on both electrophysiological methods differentiating ES cell cultures contain cells that show the propensity to develop characteristics reminiscent of developing chamber myocardium in vivo.

DISCUSSION

During cardiac development in vivo, distinct regions of the heart tube differentiate from primary myocardium towards chamber myocardium. This process is reflected by changing patterns of gene expression and electrophysiological characteristics. In vitro, cardiac myocytes are formed with relative ease during differentiation of embryonic stem cells; however, the mechanisms that underlie the differentiation of stem cells along specific myocardial cell-type lineages must be elucidated, before any potential therapeutic
applications. In this study, we focused on the formation of chamber myocardial-like cells. We used both molecular and electrophysiological approaches to examine developmental changes in differentiating ES cells and we successfully isolated a pure culture of cardiomyocytes in which the formation of early chamber myocardium has occurred.

Profiles of Gene Expression
We examined the expression of the transcription factors Nkx2.5, Gata4, Mef2c and Irx4 that are involved in cardiogenesis, during in vitro differentiation of ES cells and during in vivo development. The presence of Nkx2.5 and Gata4 mRNA in EBs was shown before (Leahy et al. 1999; Wobus and Guan, 1998), however, it had not been quantified and related to in vivo expression levels. Although none of the transcription factors examined here are heart-specific, their expression will be predominantly confined to the cardiac fraction of the EB.

The expression patterns of the cardiac-specific genes examined here peaked around day 3+10-14 of differentiation. cTnl mRNA is expressed in vivo in all cardiomyocytes (Fijnvandraat et al. 2003) and is already present in the mouse heart at E8.5 (unpublished data from the author’s lab). The expression level of cTnl mRNA was of the same order of magnitude as that observed in the embryonic heart.

Expression of Anf mRNA in EBs was considerably lower compared to embryonic and adult muscle cells. This difference is even stronger, realizing that during in vivo development Anf expression retracts from the ventricular chamber myocardium and becomes restricted to the peripheral conduction system and the atria (Moorman and Christoffels, 2003), the latter comprising only 10% of the total cardiac mass (van den Hoff et al. 1997). Cx40 is upregulated in vivo in the developing chamber myocardium. The relative content however decreases with continued development, since its expression becomes confined to the conduction system (reviewed in (Moorman et al. 1998)) and the endothelium of the vessels (Gros et al. 1994; van Kempen and Jongsma, 1999). In vivo, Cx40 mRNA expression is not restricted to the heart, since it is also present in the developing lung (Henneman et al. 1992) (and data not shown). The expression profiles of Anf and Cx40 mRNA are most similar to those observed in the early stages of heart development in vivo, where both Anf and Cx40 mRNA levels are relatively low and concomitant formation of chamber myocardium is just initiated.

Selection Clone
During ES cell differentiation, the cardiac-specific Ncx1 mRNA is present at a relatively high level as soon as cardiac differentiation is initiated. This is consistent with the observation that Ncx1 mRNA expression is relatively high in the developing heart and underscores its predominant role in Ca^{2+}-extrusion from the cytoplasm at this developmental stage. (Koban et al. 1998; Boerth et al. 1994; Studer et al. 1997).
The strong and cardiac-restricted expression of the distant upstream part of the \textit{Ncx1} promoter at early stages of cardiac development \textit{in vivo} and \textit{in vitro} allowed us to isolate and purify cardiomyocytes from the very earliest to latest points of differentiation. This has permitted the molecular analysis of early cardiac development without the influence of unknown cells.

Several alternative approaches have been reported to acquire an enrichment or pure population of a desired cell population. ES cell-derived cardiomyocytes have been enriched using a Percoll density gradient (Doevendans \textit{et al.} 2000) or by FACS analysis on the Mlc2v-promoter driven fluorescent gene expression (Müller \textit{et al.} 2001). Only a subset of cardiomyocytes is selected in this way, because Mlc2v mRNA is only expressed in the anterior part of the heart tube and its expression pattern changes during development (Franco \textit{et al.} 1998). A comparable problem is met when cells are selected on the presence of \textit{\alphaMhc} promoter activity. A pure population of cardiomyocytes was elegantly acquired by genetic selection using the \textit{\alphaMhc} promoter to drive the expression of the neomycin resistance gene. \textit{In vivo}, \textit{\alphaMhc} mRNA is expressed in a gradient over the entire heart tube. However, at E9.5 the \textit{\alphaMhc} promoter is downregulated in the downstream region were the ventricles develop (Palermo \textit{et al.} 1996). Therefore, cells that resemble the latter stage of cardiac development might be lost after genetic selection, a complication that might not be met when the \textit{Ncx1} promoter is used to drive the neomycin-resistance gene. A FACS selection method has been described based on GFP expression driven by the cardiac-specific \textit{\alpha}-cardiac actin promoter (Kolossov \textit{et al.} 1998). The study reports 5% ectopic expression that might be due to random integration of the construct, which might also lead to mosaicism, obviated by the \textit{HPRT}-approach in the current study.

In this study we found that before selection about one third of the EB volume is populated by cardiomyocytes. The selection procedure described here proved relatively efficient since a substantial percentage of cells (5-10\%) remained after treatment with neomycin when compared to the cardiomyocyte contents of EBs ranging from 0.5-5\% reported in other studies (Klug \textit{et al.} 1996; Boheler \textit{et al.} 2002; Müller \textit{et al.} 2001).

\textbf{Electrophysiology}

The development of chamber myocardium \textit{in vivo} is electrophysiologically reflected in an increase in \(V_{\text{max}}\) of the action potential and a decreased automaticity of the cells. \textit{In vitro}-derived cardiomyocytes initially develop a phenotype that is most similar to the myocytes of the primary heart tube as observed from the relatively low \(V_{\text{max}}\). We observed an increased \(V_{\text{max}}\) with differentiation time, a property shared with chamber myocytes during \textit{in vivo} development. However, simultaneously a shortening of the action potential and an increase in automaticity was observed during differentiation, which contrasts \textit{in vivo} development (Liu \textit{et al.} 1999; Eisenberg, 2002; Wang \textit{et al.} 1996). The observed increase
in automaticity with differentiation is in agreement with previous reports (Zhang et al. 2002; Fijnvandraat et al. 2003; Banach et al. 2003). High automaticity is regarded as a relatively primitive feature as observed in the heart tube and in nodal cells (Moorman et al. 1998). However, some spontaneous contraction can persist in chamber myocardial cells as known from isolated neonatal and adult rat cells. Notwithstanding progression of maturation, the automatic rate of the primary heart tube increases with development (Satin et al. 1988) and the heart rate of nodal cells, pacing the rate of the chamber myocardium increases even more with further development, but plateaus and decreases with age. Experiments with continued culture time (longer than 3 weeks) suggest differentiation of more mature and electrically stable cardiomyocytes (K. Boheler, personal observations).

No significant difference could be observed between action potential parameters tested in ES cell-derived cardiomyocytes that were genetically selected and those that remained in floating culture. Selected cells, however, showed a delayed decrease in action potential interval. This might suggest that the selection period slightly retarded differentiation but did not influence the differentiation tendency towards chamber myocardium.

The increase in $V_{\text{max}}$ and decrease in APD was confirmed by an independent electrophysiological approach using ANEPPS. This method allowed investigating electrophysiological parameters in a potential favourable condition, since clusters of cells resemble more the in vivo situation in which cells are coupled. Since cardiomyocytes are connected by gap junctions, clusters of ES cell-derived cardiomyocytes will respond to the electrical stimulus of the cell that shows most dominant pacemaker activity. In this way cells with a slower pulse rate are overruled. Variation in phenotypes is only to be detected by patch clamp on single cells. Both ANEPPS and conventional whole cell measurements showed a similar trend in the development of $V_{\text{max}}$ and APD, validating the ANEPPS method to determine the overall electrical signal of cell clusters.

**Conclusion**

Several transcription factors involved in cardiogenesis in vivo are expressed in ES cell-derived cardiomyocytes at levels comparable to those in the embryonic, neonatal and adult heart. Gene expression profiling and electrophysiology suggest that the in vivo expression program underlying formation of chamber myocardium has initiated at least partly in ES cell-derived cardiomyocytes. We demonstrate that the cardiac-restricted Ncx1 promoter can be employed to isolate and purify early cardiomyocytes differentiated in vitro from ES cells. This model system permits investigation of early cardiogenesis, and opens the way to explore known and novel factors responsible for cardiac chamber formation, which may give inroads into clinical application.
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Purification of ES cell-derived cardiomyocytes


