

Development of a Perfusion Fed Bioreactor for Embryonic Stem Cell-Derived Cardiomyocyte Generation: Oxygen-Mediated Enhancement of Cardiomyocyte Output

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Abstract: Cell transplantation is emerging as a promising new approach to replace scarred, nonfunctional myocardium in a diseased heart. At present, however, generating the numbers of donor cardiomyocytes required to develop and test animal models is a major limitation. Embryonic stem (ES) cells may be a promising source for therapeutic applications, potentially providing sufficient numbers of functionally relevant cells for transplantation into a variety of organs. We developed a single-step bioprocess for ES cell-derived cardiomyocyte production that enables both medium perfusion and direct monitoring and control of dissolved oxygen. Implementation of the bioprocess required combining methods to prevent ES cell aggregation (hydrogel encapsulation) and to purify for cardiomyocytes from the heterogeneous cell populations (genetic selection), with medium perfusion in a controlled bioreactor environment. We used this bioprocess to investigate the effects of oxygen on cardiomyocyte generation. Parallel vessels (250 mL culture volume) were run under normoxic (20% oxygen tension) or hypoxic (4% oxygen tension) conditions. After 14 days of differentiation (including 5 days of selection), the cardiomyocyte yield per input ES cell achieved in hypoxic vessels was 3.77 ± 0.13 , higher than has previously been reported. We have developed a bioprocess that improves the efficiency of ES cell-derived cardiomyocyte production, and allows the investigation of bioprocess parameters on ES cell-derived cardiomyogenesis. Using this system we have demonstrated that medium oxygen tension is a culture parameter that can be manipulated to improve cardiomyocyte yield. © 2005 Wiley Periodicals, Inc.

Keywords: bioreactor design; stem cells; cardiomyocytes; oxygen; tissue engineering

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INTRODUCTION

Adult cardiomyocytes are terminally differentiated cells with limited capacity for cell division. Currently, the only effective treatment for patients suffering from severe heart failure is organ transplantation. The successful engraftment of several cell types into the heart (Orlic et al., 2001a; Taylor et al., 1998) has given rise to the concept of cell transplantation as a treatment for heart failure. Although a number of contractile cell types have appeared to improve heart function upon transplantation (Orlic et al., 2001a,b; Taylor et al., 1998), cardiomyocytes are the ideal donor cell due to their inherent electrical and physiological properties. However, obtaining adult cardiomyocytes in sufficient quantities for effective transplantation has been hindered by the limited availability and proliferative capacity of these cells (Soonpaa et al., 1996). Embryonic stem (ES) cells, pluripotent cells isolated from the inner cell mass of the developing mammalian blastocyst, self-renew indefinitely while retaining their capacity to differentiate into cell lineages of all three primary germ layers (Evans and Kaufman, 1981), including cardiomyocytes (Maltsev et al., 1993). ES cell culture may be a promising tool for cell therapy, potentially providing a renewable cell source for transplantation into a variety of organs.

In vitro mouse ES cell differentiation typically requires an initial aggregation step to form spherical cell clusters called embryoid bodies (EBs). It has been well established, by our group and others, that cardiomyocytes derived from EB differentiation display properties characteristic of functional (fetal) cardiac cells. Differentiating EBs recapitulate many aspects of cardiogenesis in the embryo, displaying protein expression profiles that parallel the developmental expression pattern exhibited during in vivo

heart formation starting with cardiac transcription factors, followed by chamber-specific proteins, sarcomeric proteins, and ion exchanger proteins (Czyz and Wobus, 2001; Sachinidis et al., 2003). These cells display electrophysiological responses, expressing pacemaker-, atrial-, and ventricular-like action potentials in a developmentally regulated manner (Hescheler et al., 1999; Maltsev et al., 1993).

Herein we report the development of a scalable bioprocess for the culture of ES cell-derived cardiomyocytes in a medium-perfused bioreactor system (Fig. 1). Accomplishing this endpoint required incorporating methods to address specific challenges associated with high-density stirred suspension ES cell differentiation systems. Recognizing that ES cells express surface molecules that promote aggregation between EBs (EB agglomeration), thus inhibiting cell growth and/or differentiation (Dang et al., 2002), we used a hydrogel encapsulation approach (Dang et al., 2004; Magyar et al., 2001) to control EB development during stirred suspension culture. Encapsulation not only permitted direct differentiation of ES cells in stirred suspension, it also significantly improved cell production. Cardiomyocytes typically constitute less than 5% (Klug et al., 1996) of all cells during EB-based differentiation. Furthermore, EB differentiation is often incomplete, resulting in the persistence of undifferentiated ES cells (Erdo et al., 2003; Gulbins et al., 2002; Hilberg and Wagner, 1992). To purify for cardiomyocytes, and to deplete undifferentiated cells, we and others have used a genetic selection technique (Klug et al., 1996; Li et al., 1998; Marchetti

et al., 2002; Zandstra et al., 2003) whereby a transgene, encoding neomycin resistance driven by a myosin heavy chain (MHC) promoter, is stably transfected into ES cells. This technique has been demonstrated to efficiently enrich ES cell-derived cardiomyocytes to greater than 70% (Zandstra et al., 2003). This report additionally begins to address the low cardiomyocyte yield typically achieved in spontaneously differentiating ES cell cultures, demonstrating cardiomyogenesis is enhanced during hypoxic (4% oxygen tension) culture. Taken together, this first demonstration of a one-step controllable perfusion bioreactor system for the generation of embryonic stem cell derivatives should serve as a foundation for ES cell bioprocess development.

MATERIALS AND METHODS

ES Cell Maintenance

The D3 (Doetschman et al., 1985) mouse ES cell line was used in this study, with encapsulation and cardiomyocyte derivation results consistent with the R1 and CM7/1 (Zandstra et al., 2003) (a clone of the J1 ES cell line) ES cell lines, respectively. ES cell maintenance has been previously described (Zandstra et al., 2003). Undifferentiated cells were bulk transfected, via electroporation, with a plasmid carrying both an α -myosin heavy chain promoter in front of the neomycin phosphotransferase gene (MHC-neo^r) and a phosphoglycerate kinase in front of a hygromycin-

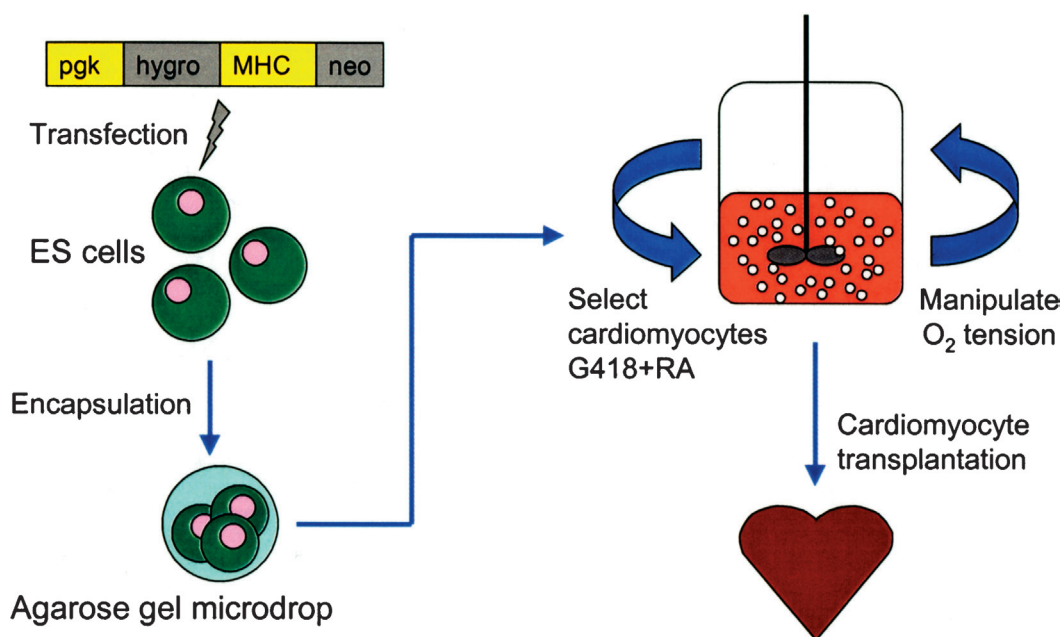


Figure 1. Overview of bioprocess for ES cell-derived cardiomyocytes. Transfected ES cells are encapsulated in hydrogel microcapsules and directly inoculated into spinner cultures. Differentiation proceeds until d9 (oxygen tension can be optimized for improved cardiomyocyte yield). Cultures are treated with G418 and RA to select for cardiomyocytes. On d14, an enriched cardiomyocyte population is harvested that can be used in downstream applications such as tissue engineering and model studies.

resistant (PGK-hygro^r) gene in a common pUCBM20 vector backbone (Boehringer-Mannheim, Germany) (Zandstra et al., 2003).

ES Cell Differentiation Cultures

Unencapsulated Suspension Cultures

Stirred suspension differentiation of ES cells to cardiomyocytes was performed as previously described (Zandstra et al., 2003). This system consists of forming EBs under static conditions, to prevent EB agglomeration, and then transferring the EBs to stirred suspension on day 4 (d4) of differentiation. This system will be referred to as the static/spinner flask (S/SF) system.

Encapsulated Suspension Cultures

Mouse ES cell aggregates were formed by generating a single-cell suspension of 3×10^5 cells/mL in ES cell media and allowing cells to aggregate for 1 day. ES cell aggregates were dispersed in molten 1.5% (weight) low-gelling-temperature agarose (type VII, Sigma, St. Louis, MO) in Dulbecco's phosphate-buffered saline (PBS, GIBCO-BRL, Grand Island, NY) at 2×10^6 cells/mL. The molten agarose mixture was dispensed into 200-centistoke viscosity dimethylpolysiloxane (DMPS, Sigma) at 37°C and subjected to impeller shearing using the CellSys Microdrop Maker (One Cell Systems) to create agarose hydrogel microcapsules (Ryan et al., 1995). Microcapsules were washed twice with Hank's buffered saline solution (HBSS, GIBCO-BRL) and suspended in the appropriate ES cell differentiation media. The microcapsules were inoculated into 250-mL spinner flasks (Bellco Glass, Vineland, NJ) at a density of 4,000 cell/mL, as it was observed that this density resulted in exponential cell growth to our d9 target density (1×10^6 cell/mL), at which point cardiomyocyte selection was initiated by adding Geneticin (G418, GIBCO-BRL) (400 µg/mL) to kill noncardiomyocytes and retinoic acid (RA, Sigma) (10^{-9} M) which has been shown to deplete undifferentiated ES cells and improve cardiomyocyte yield (Zandstra et al., 2003). The encapsulated ES cell aggregates form EBs that differentiate and grow within the capsule until ~d4, at which time most of the EBs have grown large enough to emerge from the capsules (Dang et al., 2004).

Bioreactor Culture

We used DasGip's cellferm-pro culture system (www.dasgip.com), a parallel cultivation system that is capable of monitoring and controlling oxygen tension and pH in four parallel 500-mL vessels. Using an 8-head multipump (inlet and outlet flow to the four vessels), continuous medium perfusion was implemented by attaching a settling tube to the feed outlet. The diameter of the settling tube

(>1.38 mm) was specified so that the velocity of the medium outlet was slower than 150 cm/hr, the settling velocity of the EBs on d9. Iscove's Modified Dulbecco's Medium (IMDM, University of Toronto Media Prep) differentiation medium was fed to the cultures at a rate of one-quarter reactor volume replacement per day.

The bioreactors were maintained under either normoxic (20% O₂ tension) or hypoxic (4% O₂ tension) conditions to d9. On d9, selection was initiated by supplementing the feed with 400 µg/mL G418 and 10^{-9} M RA and the feed rate was increased to three-quarter reactor volume replacement per day to remove the dead cell debris produced by selection, and to maintain G418 and RA concentration. During selection all vessels were controlled at 20% oxygen tension.

C2C12 Cell Culture

Two different culture media were used to induce C2C12 differentiation into myotubes. C2C12 culture medium (nondifferentiating) was used for seeding and consisted of 90% Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL), 10% FBS, 4 mM L-glutamine (Invitrogen, La Jolla, CA), 2 mM L-penicillin (50 IU/mL, Invitrogen), and streptomycin (50 µg/mL, Invitrogen). C2C12 differentiation medium consisted of 98% DMEM, 2% horse serum (HS, GIBCO-BRL), 4 mM L-glutamine, penicillin (50 IU/mL), and streptomycin (50 µg/mL). C2C12 cells were seeded in a 24-well plate at a density of 50,000 cells per well in C2C12 culture media. The following day the cells were washed once with PBS and then cultured in C2C12 differentiation media. Cells were maintained in differentiation media for the next 4 days, replacing the medium every other day.

Immunofluorescence of Whole-Mount Embryoid Bodies and C2C12 Cells

Intracellular antibodies used for immunostaining whole EBs and C2C12 cells required the fixation and permeabilization of the cells within the EBs. EBs were fixed using IntraPrep Fixation Reagent 1 (Immunotech, Westbrook, ME) overnight, then washed by resuspension in 1.5 mL of HBSS containing 2% fetal bovine serum (FBS, Hyclone, Logan, UT) (HF) every hour for a total of five washes. Fixed EBs were permeabilized in IntraPrep Reagent 2 for 1 h and incubated with either anti-sarcomeric myosin heavy chain (MF20, 1:10, DSHB), anti- α -actinin (EA-53, 1:800, Sigma) or anti-nebulin (NB-2, 1:400, Sigma) antibodies overnight. Stained EBs were washed five times in HF and then incubated with secondary antibody and nuclear dye (1:100, FITC-conjugated for MF20 stained EBs, PE-conjugated for NB-2 and EA-53 stained EBs, 1:100, 7-AAD for nuclear dye) overnight and washed the next day. An Olympus inverted IX70 microscope was used in combination with the FV-300 confocal microscope-

scanning unit to observe EBs. The excitation lasers used were 488 nm argon and 543 nm HeNe.

C2C12 cells were washed once in PBS and fixed in 3.7% formaldehyde (EMD Chemicals, San Diego, CA) in PBS for 15 min at 37°C. The cells were subsequently washed three times with PBS and permeabilized in 100% methanol (EMD Chemicals) for 2 min at room temperature. The cells were then washed three times with PBS and blocked overnight with 10% FBS in PBS at 4°C. The next day C2C12 cells were stained with NB-2 for 2 h at room temperature followed by three washes with PBS. The cells were then incubated with Alexaflor 546 goat antimouse TRITC (1:200, Molecular Probes, Eugene, OR) and Hoescht 33342 (1:100, Sigma) at room temperature. The cells were imaged using Arrayscan II high through-put fluorescent microscope (Cellomics).

RT-PCR

Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma). RNA was quantified by a UV spectrophotometer and 0.5 µg RNA was used in each RT-PCR reaction (One-Step RT-PCR kit, Qiagen, Chatsworth, CA). The oligonucleotide primers for MHC and MLC-2v amplification were respectively 5'-CTGATGGCACAGAAG-ATGCT-3' and 5'-GTTCAGGATGCGATACCTCT-3', and 5'-GAACTCTCCAGAGGTGGCAA-3' and 5'-CCTC-TCTGCTTGTGTGGTCA-3'. The PCR amplification conditions were 30 cycles of 30 sec at 94°C, 30 sec at 52°C. The sizes of the anticipated RT-PCR amplification products were 1,058 and 422 bp for MHC and MLC-2v, respectively. Equivalent loading was verified by amplification of α -actin, and primer specificity was verified by RT-free amplification in the second reaction (not shown).

Glucose and Lactate Analysis

Medium samples were analyzed using the BioProfile 200 blood gas analyzer (Nova Biomedical, Waltham, MA). For the semicontinuously fed spinner flasks, samples were collected prior to medium exchange, and then again following medium exchange. For the perfusion fed cultures, medium samples were taken from the feed outlet throughout the course of differentiation.

Flow Cytometry

EBs were dissociated to single cell suspensions as previously described (Zandstra et al., 2003). Cells were double-stained with MF20 and ethidium monoazide (EMA, Molecular Probes), a viability dye that irreversibly binds DNA. Dispersed cells (5×10^5 to 1×10^6) were suspended in 100 µl of HF and 1 µl EMA (0.5 mg/mL EMA) and incubated for 20–30 min exposed to visible light. Samples were washed in HF and the MF20 staining protocol proceeded as previously described (Zandstra et al., 2003).

Statistics

All statistical analyses were performed with Origin 6.1 (OriginLab, Northampton, MA) graphing and data analysis software. All results, generated from at least three independent experiments, were analyzed using a significance level of $P = 0.05$.

RESULTS

Anti-Sarcomeric Myosin Is a Cardiac-Specific Antibody Over the First 14 Days of Differentiation

Sarcomeric myosin heavy chain is a protein expressed in cardiomyocytes as well as skeletal myocytes. To ensure that MF20, an antibody against sarcomeric myosin, could be employed as a marker to screen for cardiomyocytes during the stages of differentiation investigated herein, immunofluorescence of whole-mount EBs was conducted on d9 and d14. EBs were double-stained with either MF20 and EA-53 (skeletal and cardiac myocytes), or MF20 and NB2 (skeletal cells only). Colocalization of MF20 and EA-53 was observed on both d9 and d14, whereas nebulin expression was not observed in either d9 or d14 EBs (Fig. 2A), indicating no differentiation of ES cells into skeletal myocytes during this time frame. Thus, the MF20 antibody is a cardiac-specific marker during the differentiation period in this study.

To further confirm the cardiomyocyte phenotype of these cells we analyzed RNA isolated from cells on d0, d9, and d14. Semiquantitative RT-PCR revealed that the cells cultured in this system exhibit cardiac-specific gene expression (Fig. 2B). Transcripts for MLC-2v, a gene expressed by ventricular cells, were weakly expressed by d9 and strongly expressed by d14, after 5 days of selection. Furthermore, cardiac-specific α -MHC was strongly expressed by d9, when selection was initiated. These characterization studies support other published results (Doevendans et al., 2000; Klug et al., 1995) indicating that sarcomeric myosin heavy chain expression can be used to screen ES cell differentiation to cardiomyocytes in 14-day differentiation cultures.

ES Cell Encapsulation Prevents EB Agglomeration and Does Not Impact Cardiogenic Induction

ES cells express cell adhesion molecules that are down-regulated as they differentiate. Due to the high incidence of cell contact in stirred cultures, previous studies (Zandstra et al., 2003) used the S/SF system, a two-step process that incorporates an initial static differentiation period before transferring the EBs to stirred suspension on d4, at which time cell adhesion molecules are sufficiently down-regulated. In order to eliminate this static culture step and to differentiate ES cells directly in stirred suspension in a one-step process, we evaluated the capacity for encapsulated EBs (Table I, Fig. 3A) to form cardiomyocytes.

Sarcomeric myosin expression on d9 showed that similar cardiomyocyte frequencies were achieved in encapsulated and S/SF differentiation cultures (Fig. 3B), demonstrating that cardiomyocyte formation proceeds normally in encapsulated EBs.

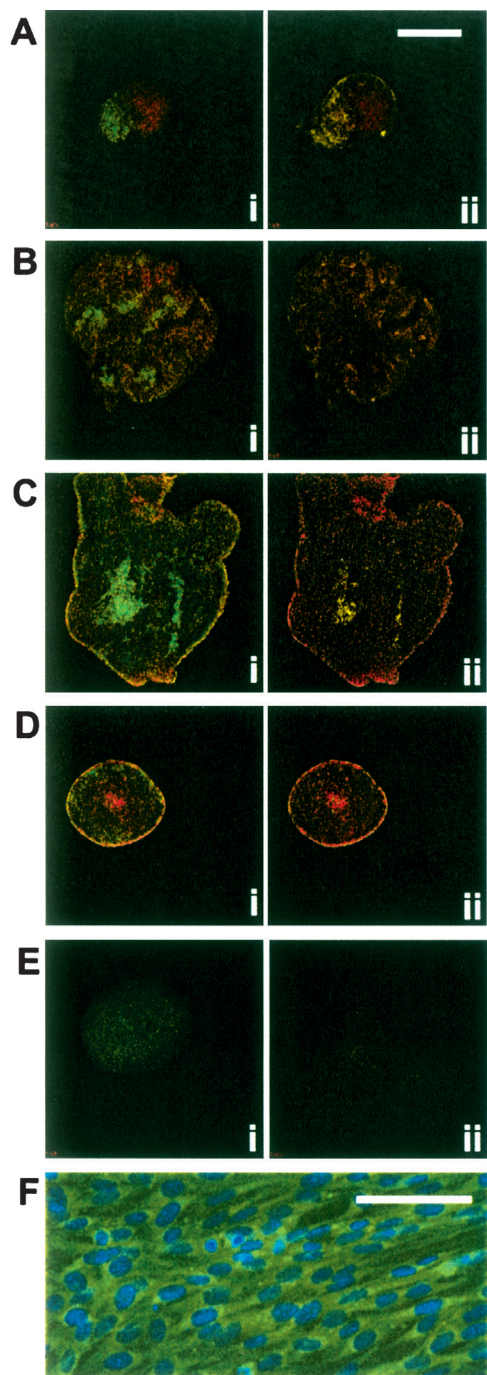


Table I. Cardiomyocyte yield per input ES cell (C/ES) as a function of encapsulation and hypoxic culture.

Study	Condition	C/ES ^a	Fold increase (normalized to control)
Encapsulation	Control (S/SF) ^b	0.16 ± 0.07	
	EC ^c	3.17 ± 0.90	19.8
Oxygen	Control (normoxic)	2.56 ± 0.11	
	Hypoxic	3.77 ± 0.13	1.47

^aC/ES: cardiomyocyte yield per input ES cell.

^bS/SF: 2-step static/spinner flask system.

^cEC: 1-step encapsulation culture system.

Encapsulation Cultures Achieve Higher Cardiomyocyte Yields per Input ES Cell

To reduce undefined interaction effects that may occur at high cell densities, we set a maximum target cell density of 1×10^6 cells/mL, (achieved by d9) in the stirred cultures. In encapsulation cultures, there is an ES cell aggregation step prior to encapsulation and initiating differentiation, whereas in the S/SF system EB formation is initiated with single cells, and thus a higher starting density was required to obtain standard size EBs for cardiomyocyte differentiation. The S/SF cultures required 24×10^6 input ES cells to achieve our target cell density in a 250-mL culture volume. In contrast, only 1×10^6 input ES cells were needed to achieve this target in encapsulated cultures. This represents an improvement in cell production that is over an order of magnitude higher in encapsulated cultures than in the S/SF system (Table I, Fig. 4A). Encapsulation prevented EB agglomeration, suggested by the higher EB numbers (Fig. 4B) despite similar cell number (Fig. 4C), and therefore resulted in more efficient cell growth in differentiating EBs. The greater overall cell expansion in encapsulated cultures improved the cardiomyocyte yield per input ES cell (C/ES) in these studies to 3.17 ± 0.90 vs. 0.16 ± 0.07 in S/SF cultures (Table I, Fig. 4D).

Perfusion Feeding Minimizes Fluctuation of Medium Components

In S/SF cultures, and in typical EB differentiation processes, medium supplementation consists of a half-volume

Figure 2. Immunostaining of EBs revealed that skeletal myocytes were not present in the first 14 days of differentiation. $10\times$ magnification, scale bar = 280 μ m. **A:** A d9 EB demonstrating colocalization of (i) MF20 and (ii) EA-53. **B:** A d9 EB demonstrating (i) presence of MF20 staining and (ii) absence of NB-2 staining. **C:** A d14 EB demonstrating colocalization of (i) MF20 and (ii) EA-53. **D:** A d14 EB demonstrating (i) presence of MF20 staining and (ii) absence of NB-2 staining. **E:** Secondary antibody controls for (i) FITC, and (ii) PE. **F:** NB-2 positive control using C2C12 cells. **G:** RT-PCR analysis of MLC-2v, and α -MHC transcription on d0, d9, and d14 of ES cell differentiation in stirred suspension culture (bands highlighted by boxed region).

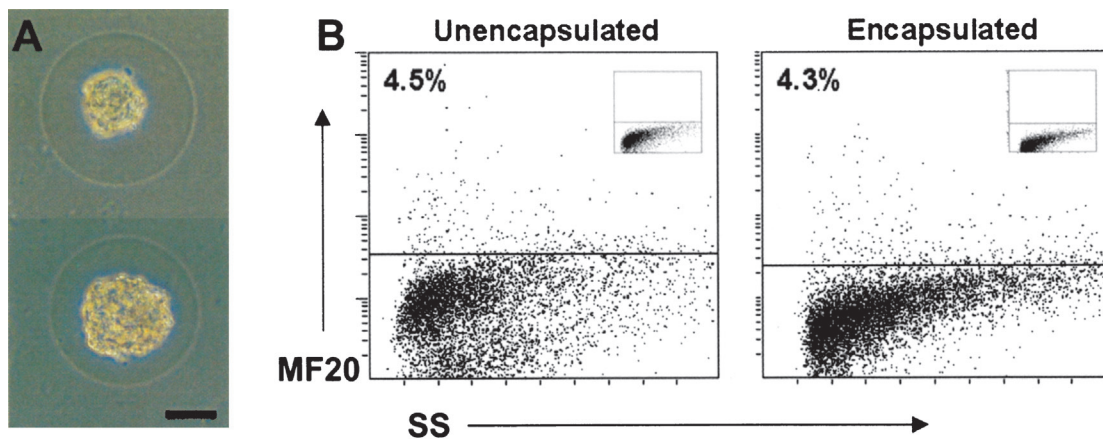


Figure 3. Encapsulated ES cell aggregates form EBs capable of cardiomyocyte differentiation. **A:** Day 0 ES cell aggregates (~150 cells/aggregate) encapsulated in agarose hydrogel microcapsules. 10× magnification, scale bar = 70 μm. **B:** Representative MF20 flow cytometry plots of samples from d9 unencapsulated and encapsulated stirred suspension cultures (negative secondary antibody controls, inset).

medium exchange every second day. In the development of our bioprocess we enabled continuous medium perfusion at the same overall feed rate (half-volume every second day) by incorporating a settling tube to retain cellularity. Auto-

ated continuous perfusion reduced the significant fluctuations in the concentration of medium components, such as glucose (Fig. 5A) and lactate (Fig. 5B), which resulted from the previous semicontinuous method of medium exchange.

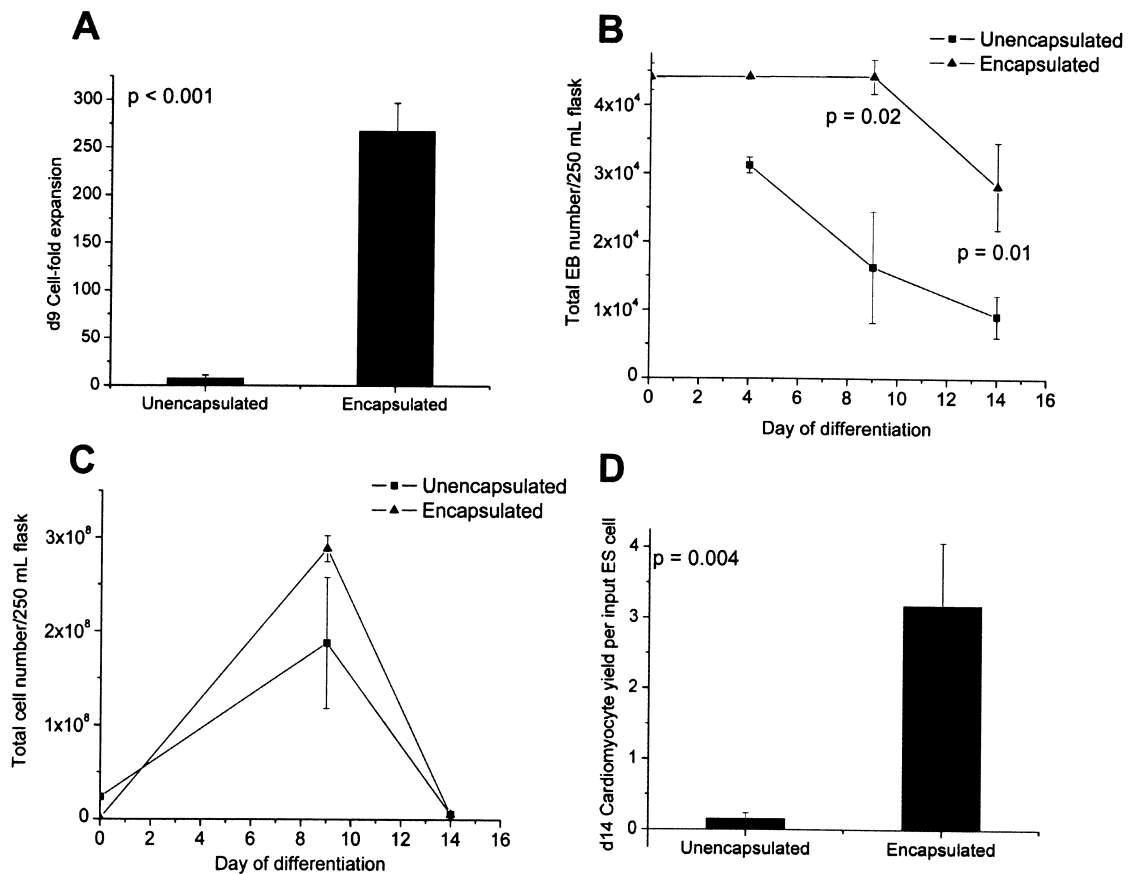


Figure 4. Encapsulating ES cell aggregates resulted in more efficient cell growth leading to higher cardiomyocyte yields per input ES cell. **A:** d9 cell production. **B:** Total EB numbers during the course of differentiation. **C:** Total cell numbers during the course of differentiation. **D:** Cardiomyocyte yield per input ES cell on d14.

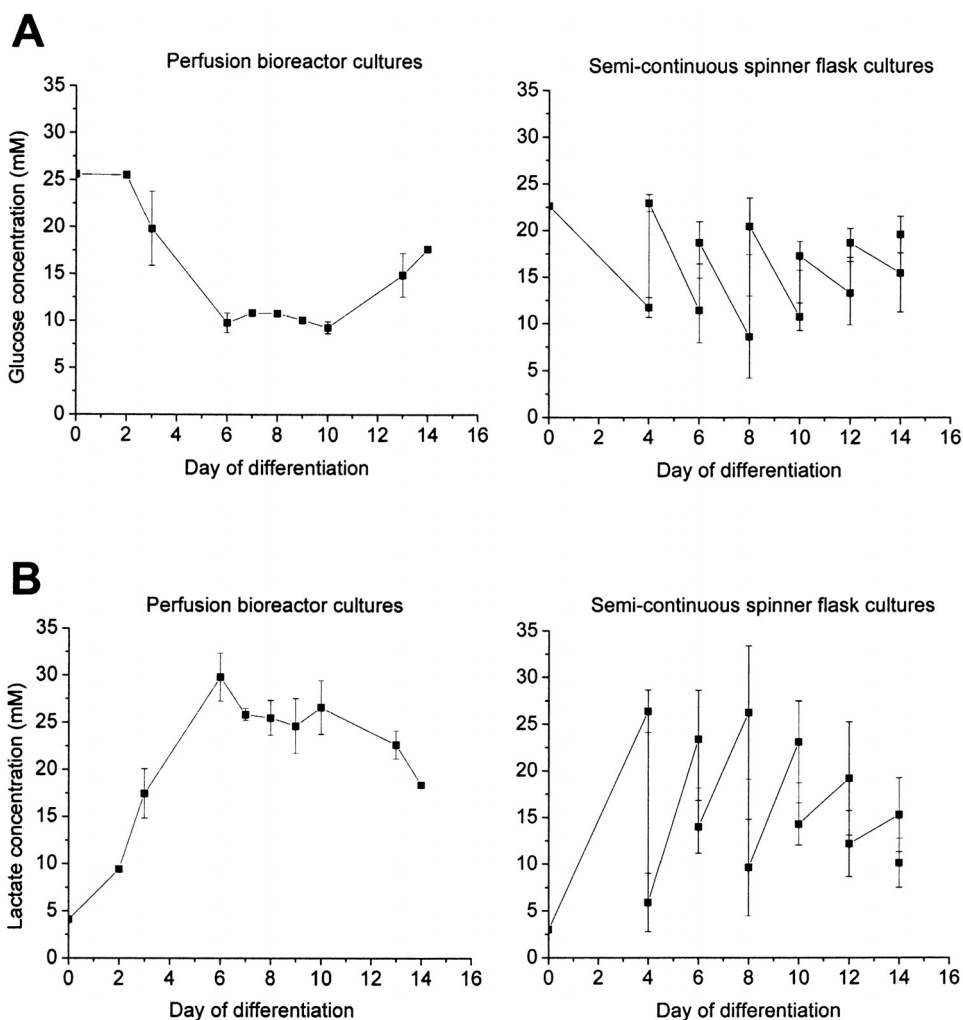


Figure 5. Perfusion feeding reduced fluctuations in the concentrations of medium components. **A:** Glucose concentration over time in perfusion fed and semicontinuous fed culture systems. **B:** Lactate concentration over time in perfusion fed and semicontinuous fed culture systems.

Cardiomyocyte Yield in Differentiating ES Cells Can Be Increased by Manipulating Oxygen Tension

Enabling a single-step bioprocess with automated medium perfusion allowed us to use the DasGip Cellferm-pro bioreactor system's microenvironmental control capacities to investigate the effect of oxygen tension on cardiomyogenesis. Cardiomyocyte yield was compared under normoxic and hypoxic culture conditions. Normoxic cultures were controlled at 20% oxygen tension for the duration of the experiment. Hypoxic conditions were controlled at 4% oxygen based on previous studies suggesting that this level of oxygen is sufficient to activate transcription of hypoxia responsive genes (Adelman et al., 1999; Gassmann et al., 1996). At ~day 7 to day 9 after initiating differentiation (Boheler et al., 2002; Sachinidis et al., 2002) the presence of cardiomyocytes can be observed by the appearance of spontaneous rhythmically contracting areas that increase in number and in area as differentiation proceeds. Therefore, we imposed a hypoxic culture environment, controlled at 4% oxygen tension, until d9 and then maintained oxy-

gen tension at 20% for the remainder of the experiment (Fig. 6A). This design allowed us to focus our investigation on the effects of hypoxia on the initial stages of cardiac development. Both cultures exhibited similar proliferation until d9, indicating that hypoxia does not affect overall cell growth during this stage of differentiation (Fig. 6B). Prior to selection, cardiomyocyte frequency was too low to detect significant differences between the two conditions (data not shown). By d14 (5 days of selection), flow-based MF20 analysis revealed significant enrichment and similar frequencies of cardiomyocytes under both conditions with $66.90 \pm 2.12\%$ and $69.28 \pm 3.34\%$ cardiomyocytes for normoxic and hypoxic conditions, respectively (Fig. 6C). $3.77 \times 10^6 \pm 0.13 \times 10^6$ cardiomyocytes were present in the hypoxic cultures; more than 1.47 times as many as in normoxic cultures (Table I, Fig. 6D). These results suggest that more cardiomyocytes, or cardiomyocyte precursor cells, were formed by d9 under hypoxic conditions, as similar cardiomyocyte frequencies but higher cell yields were observed than under normoxic conditions ($C/ES = 3.77 \pm 0.13$ vs. 2.56 ± 0.11).

DISCUSSION

Effective cardiomyocyte transplantation requires the successful seeding of sufficient cell numbers in an infarcted or diseased heart. It has been estimated that in large myocardial infarctions, which result in heart failure, 10^8 cardiomyocytes are typically lost (Kehat and Gepstein, 2003). Transplantation studies are important to determine the effects of ES cell-derived cardiomyocyte engraftment on parameters such as survival of donor cells, seeding efficiency, tumor formation, etc. It has been shown that ES cell derived cardiomyocyte grafts survive at the transplantation site for up to 30 days in ectopic transplants (Johkura et al., 2003) and 7 weeks injected directly into the heart (Klug et al., 1996). However, such studies on the transplantation potential of ES cell-derived cardiomyocytes have been limited by the number of donor cells available, with the injection of only 10^4 ES cell-derived cardiomyocytes (Klug et al., 1996) into the site of injury. Herein, we demonstrate that more than 3.5×10^6 cardiomyocytes can be produced in a 250-mL culture volume. This system is readily scalable and preliminary results using this bioprocess in a 1-L bioreactor (Applikon Biobundle) have generated greater than 10^7 cardiomyocytes. Scale-up concerns related to enlarging the system to generate clinically relevant numbers of donor cells may be addressed by running multiple smaller reactors in parallel, or increasing bioreactor size. Because of the significant reduction in input ES cell numbers required using encapsulation, generating sufficient numbers of cells to inoculate a 10-L bioreactor would be relatively simple (~ 100 million ES cells or 3 to 5 T-75 flasks). We have taken a process that typically occurs in a 2–5 mL Petri dish and enabled a controlled bioreactor-based single-step bioprocess. Current investigations are focused on adapting the system for higher-density cell cultures ($>5 \times 10^6$ cells per mL, where microenvironmental control will be critical to cell survival).

We have previously reported, using the CM7/1 cell line, a clone selectable for increased cardiomyocyte generation, the production of 1.5×10^7 cardiomyocytes from 2.4×10^7 ES cells ($C/ES = 0.63$) in the S/SF culture system (Zandstra et al., 2003). Another research group, using the same CM7/1 cell line, demonstrated that 2.1×10^6 cardiomyocytes could be generated from 1×10^6 input ES cells ($C/ES = 2.1$) on rotating suspension cultures (Zweigerdt et al., 2003). In the current study, experiments were performed using bulk transfected D3 ES cells, providing evidence that our results were reproducible in other cell lines. A summary of the improvements to cardiomyogenic yield

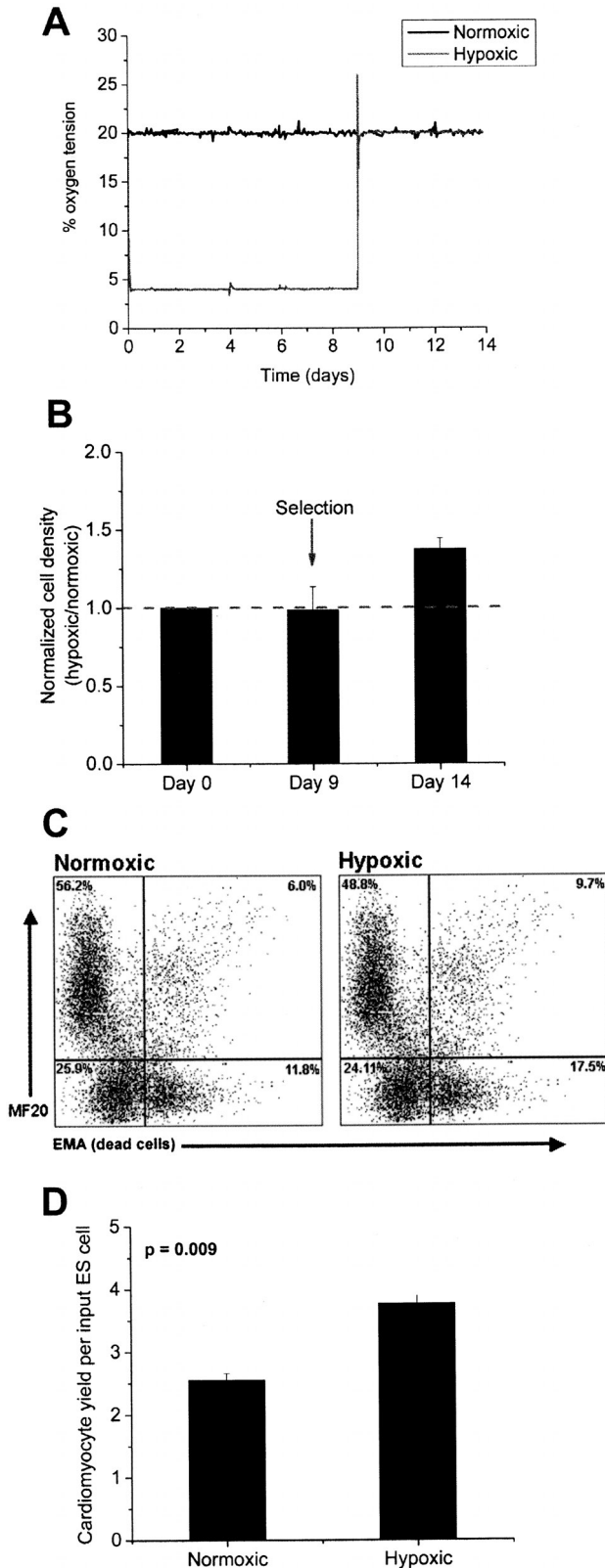


Figure 6. Improved cardiomyocyte yield per input ES cell was observed under hypoxic conditions. **A:** Oxygen tension profiles in controlled hypoxic and normoxic bioreactor cultures. **B:** Cell density in hypoxia normalized to cell density in normoxia in the controlled bioprocess. **C:** Representative flow cytometry plots for MF20 analyses of cells from the hypoxic and normoxic bioreactors on d14. **D:** Cardiomyocyte yield per input ES cell on d14.

enabled by encapsulation and hypoxic culture are outlined in Table I. We report here a C/ES of 0.16 for differentiation of D3 cells using the S/SF culture system, significantly lower than that reported for the CM7/1 cell line (Zandstra et al., 2003). Despite the difference in the magnitude of cardiomyocyte yield achieved, likely due to variability between cell lines (cell growth, cardiomyogenic potential, etc.), the general trends (cell density and cardiomyocyte frequency with respect to time) of the system remain similar between these cell lines. Furthermore, in the bioprocess described here we achieved a C/ES of 3.77, higher than previously reported for any other differentiation system or cell line.

An important aspect of the bioprocess design required implementing a method to prevent EB agglomeration, as this phenomenon inhibits efficient cell growth, thus resulting in lower cardiomyocyte yield. Encapsulation is a technique that provides a barrier between EBs, thereby blocking contact between e-cadherins, adhesion-promoting surface molecules expressed by ES cells, from separate EBs (Dang et al., 2004). Encapsulation prevented EB agglomeration in stirred suspension and contributed to significantly enhanced cardiomyocyte yields. Moreover, encapsulation permits direct differentiation in stirred suspension, thereby allowing us to monitor and control culture conditions from the start of differentiation, and minimizes handling and disturbance of the cultures during early differentiation. Encapsulation will contribute to further development of this bioprocess, specifically for scale-up, as similar cardiomyocyte yields were achieved in encapsulated cultures with 24 times fewer input ES cells.

As we have shown, in perfusion culture systems supplementation is controlled, metabolic wastes do not accumulate, and the severe dilution of cell-secreted factors that occurs in semicontinuous medium exchanges is reduced. However, perfusion may offer additional benefits as this process is further developed. It has been reported that blood stem/progenitor cell growth is enhanced in perfusion controlled bioreactors, likely due to the continuous removal of cell differentiation inducers and metabolites (Koller et al., 1993a,b; Van Zant et al., 1994). It has also been demonstrated in human bone marrow perfusion bioreactors that the types of cells produced can be affected by the consumption and production of a variety of growth factors in the medium (Koller et al., 1995), and that endogenous growth factor production may be stimulated by increased medium exchange (Caldwell et al., 1991). Thus, in future development of the bioprocess, perfusion may be involved in improving cell expansion as well as improving cardiomyocyte production specifically. Ongoing studies are taking advantage of the perfusion control system to reduce the cell density fluctuations (due to initial cell growth followed by significant nontarget cell death upon selection) by employing methods such as metabolic activity-based feeding.

During embryogenesis, and particularly the development of the cardiovascular system, many of the developmental processes that occur involve hypoxia, as diffusion

of oxygen becomes limited by the growth of the embryo (Ramirez-Bergeron and Simon, 2001). We observed that hypoxia had a beneficial effect on ES cell-derived cardiomyocyte yield. Hypoxia activates the expression of hypoxia inducible factor 1 (HIF-1), which may indirectly enhance cardiomyocyte differentiation by the activation of a number of growth factors, including vascular endothelial growth factor (VEGF), erythropoietin (EPO), and basic fibroblast growth factor (bFGF) (Ramirez-Bergeron and Simon, 2001), which have a synergistic effect on mesoderm differentiation processes, including cardiogenesis (Ramirez-Bergeron and Simon, 2001; Semenza, 2001). FGF-4 and VEGF have each been shown to enhance cardiac differentiation in combination with bone morphogenetic protein (BMP-2 or -4) (Lopez-Sanchez et al., 2002; Lough et al., 1996; Nakayama et al., 2000; Peng et al., 2002). BMPs are secreted by the anterior endoderm during embryogenesis and have been shown to promote cardiomyocyte generation (Sachinidis et al., 2002). VEGF may work by enhancing cell survival of BMP-induced mesoderm formation, and thus affect the development of cardiomyocytes. Our system allows, for the first time, the investigation of these mechanisms, by directly measuring and controlling oxygen in differentiating ES cell cultures.

Ultimately, development of this bioprocess leads towards studies utilizing human ES cells (hESC). The mouse system provides a preliminary understanding of the effects of different bioprocess parameters on differentiation to cardiomyocytes. Several differences have already been observed for hESC cardiomyogenic development, some of which may impact bioprocess-related parameters. For instance, beating frequency in differentiating hESC appears to be less frequent than in mouse ES cells (Kehat et al., 2001), suggesting that a lower number of cardiomyocytes may be produced during human EB growth. It has been observed that cardiac differentiation in hESC can be maintained for 260 population doublings (~50 passages), whereas late-passage mES cells may have difficulty differentiating to cardiomyocytes (Xu et al., 2002). Finally, enhancers of cardiomyocyte differentiation for mES cells, such as dimethyl sulfoxide (DMSO) and retinoic acid (RA), do not appear to have the same effect on hESC (Xu et al., 2002). Current investigations are adapting the bioprocess to human cells.

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