Culturing and differentiation of murine embryonic stem cells in a three-dimensional fibrous matrix

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Abstract

Embryonic stem (ES) cells have indefinite self-renewal ability and pluripotency, and can provide a novel cell source for tissue engineering applications. In this study, a murine CCE ES cell line was used to derive hematopoietic cells in a 3-D fibrous matrix. The 3-D matrix was found to maintain the phenotypes of undifferentiated ES cells as indicated by alkaline phosphatase (ALP) activity and stage specific embryonic antigen-1 (SSEA-1) expression. In hematopoietic differentiation, cells from 3-D culture exhibited similar cell cycle distribution and SSEA-1 expression to those in the initial cell population. The Oct-4 expression was significantly down-regulated, which indicated the occurrence of differentiation, although the level was slightly higher than that in Petri dish culture. The expression of c-kit, cell surface marker for hematopoietic progenitor, was higher in the 3-D culture, suggesting a better-directed hematopoietic differentiation. Cells in the 3-D matrix tended to form large aggregates associated with fibers. For large-scale processes, a perfusion bioreactor can be used for both maintenance and differentiation cultures. As compared to the static culture, a higher growth rate and final cell density were resulted from the perfusion bioreactor due to better control of the reactor environment. At the same time, the differentiation capacity of ES cells was preserved in the perfusion culture. The ES cell culture in the fibrous matrix thus can be used as a 3-D model system to study effects of extracellular environment and associated physico-chemical parameters on ES cell maintenance and differentiation.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst stage of the mammalian embryo, and are able to self-renew and to differentiate, theoretically, into all tissue types (Wobus 2001). ES cell differentiation is also a good model system to study the function of various genes in the early stage of development by 'gain of function' and 'loss of function' analysis (Guan et al. 1999; Filippi et al. 2000). Since human embryonic stem cells were first isolated (Thomson et al. 1998), the potential of ES cells for applications in tissue engineering and cell therapy has become promising (Thomson and

Odorico 2000). Although adult stem cells can be isolated from various types of tissues such as adipose tissue and bone marrow (Zuk et al. 2001; Martin et al. 2001), these cells have a finite capacity for self-renewal and low telomerase activity, indicating early senescence (Zandstra and Nagy 2001). For example, hematopoietic stem cells (HSC) are scarce in bone marrow and cord blood, and their self-renewal ability is limited in *ex vivo* expansion culture (McAdams et al. 1996). In contrast, ES cell-derived hematopoietic stem/progenitor cells (HSC/HPC) could be, in principle, created in unlimited amounts, thus increasing the availability of HSC for the treatment of hematologic disorders. The derivation of various

types of hematopoietic cells and HSC capable of long-term hematopoietic reconstitution can be achieved using mouse ES cells (Hole 1999). Most recently, hematopoietic colony-forming cells were derived from human ES cells, suggesting their potential utility in producing universal hematopoietic cells for transplantation (Kaufman et al. 2001).

Currently, interest is high in using ES cells for tissue engineering, cell therapy, and other clinical applications. Culturing of ES cells for their maintenance, expansion, and directed differentiation is the first critical step in the development of ES cell lines for these clinical applications. Most work to date, however, has employed non-adhesive Petri dishes or conventional two-dimensional (2-D) tissue culture surfaces. However, tissue engineering usually uses a three-dimensional (3-D) culture environment, which has been found to play a key role in regulating cell proliferation, differentiation and long-term tissue development (Ma et al. 2000; Li et al. 2001a). The 3-D tissue scaffold also provides a larger specific surface area for cell adhesion and growth, and reduces contact inhibition normally found in 2-D monolayer culture. The concept of '3D-matrix adhesion' was introduced recently, demonstrating the importance of three-dimensionality in tissue culture (Cukierman et al. 2001). Three-dimensional scaffolds can provide the template for the selective differentiation into a specific lineage. Using mesenchymal progenitors from bone marrow, chondrogenic and osteogenic differentiation was regulated on a 3-D poly(lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) foam (Martin et al. 2001). In general, more *in vivo*-like culture environments can facilitate the desired differentiation from embryonic stem cells. The co-culture of mouse ES cells with OP9 stromal cell line (derived from mice) induced hematopoietic differentiation (Nakano 1996; Kaufman et al. 2001). The co-culture of rat ES cells with differentiated 3-D brain cell aggregates enhanced neuronal cell differentiation (Pardo and Honegger 2000). Finally, the co-culture of murine ES cells with murine fetal osteoblasts improved the derivation of osteoblast lineage progenitors (Buttery et al. 2001). These studies demonstrated that the extracellular environment has critical effect on stem cell differentiation; however, very little has been done for ES cell culture in a 3-D tissue scaffold. A scalable bioprocess also needs to be developed for the controlled differentiation from ES cells and for future cell manufacturing (Zandstra and Nagy 2001).

The objectives of this work were to develop a perfusion bioreactor for 3-D culturing of ES cells and to study the effects of 3-D fibrous matrix on ES cell maintenance, expansion, and hematopoietic differentiation. In this study, a 3-D culture system was employed to maintain undifferentiated ES cells and to induce differentiation along a hematopoietic lineage. We hypothesized that the extracellular environment created by 3-D fibrous scaffolds would affect ES cell growth and differentiation. The analysis of marker expression along the developmental pathway of ES cells is required to investigate phenotypically definable cell lineages (Ling and Neben 1997; Shambloott et al. 2001). Murine ES cells express stage-specific embryonic antigen-1 (SSEA-1), alkaline phosphatase (ALP) and the transcription factor Oct-4 when they are maintained in the undifferentiated state. Thus, ALP, SSEA-1, and Oct-4 can be used as cell markers for the state of culture differentiation or undifferentiation. The expression of these proteins is down-regulated when differentiation is induced biochemically. The expression of c-kit and the case of a functional colony assay can be used to define early hematopoietic lineage differentiation. c-kit is the receptor for stem cell factor (SCF) and expressed on hematopoietic stem cells and progenitors (Ling and Neben 1997). In this study, the expression of these markers under 3-D culturing conditions was studied and compared with 2-D cultures. The effects of culturing conditions on growth kinetics and cell cycle distribution were also studied. Finally, a perfusion fibrous bioreactor was used for ES cell maintenance and differentiation.

Materials and methods

Culture and medium

Murine CCE ES cell line was obtained from StemCell Technologies, Inc. (Vancouver, Canada) (Robertson et al. 1986; Keller et al. 1993). ES cells were maintained on gelatin-coated T25-flasks. Maintenance medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine (StemCell Technologies, Inc.), 100 µM monothioglycerol (Sigma) and 10 ng/ml leukemia inhibitory factor (LIF, StemCell Technologies, Inc.). For hematopoietic differentiation, the

medium was composed of Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% FBS for differentiation, 5 ng/ml of thrombopoietin (Tpo), 25 ng/ml of flt-3/flk-2 ligand (FL) (Tpo and FL were from R&D Systems, Minneapolis, MN), 2 mM L-glutamine and 100 μ M monothioglycerol.

Static cell culture

Seeding cultures were prepared by trypsinizing ES cells grown in a gelatin-coated T25-flask before 15 passages to make a cell suspension containing $0.5\text{--}5 \times 10^6$ cells/ml. Each well containing sterile non-woven polyethylene terephthalate (PET) matrices (prepared as described previously, Li et al. 2001b) was inoculated with a known amount of cells ($\sim 3 \times 10^5$) and then incubated in a CO₂ incubator for ~ 60 min to allow for cell attachment to the fibrous matrix. The PET matrices containing attached cells were then transferred to a new 24-well plate (each well contained 2 ml of the maintenance medium) to begin the cell culture kinetics study. Seeding efficiencies of $\sim 30\%$ were attained for fibrous matrices studied by this seeding procedure. For hematopoietic differentiation, 35 mm non-adherent Petri dish was used as the standard 2-D culture since it was commonly used in previous studies (Zandstra et al. 2000). A graded series of 600–10,000 cells was inoculated into dishes

with 2 ml of the differentiation medium. For 3-D differentiation, 600–30,000 cells were inoculated into the tissue culture wells containing PET matrices and the maintenance medium overnight to allow cell attachment or retention in the matrix. Matrices containing cells were then transferred to new wells with 2 ml of the differentiation medium. All cultures were incubated in a humidified CO₂-incubator (37 °C, 100% humidity, 5% CO₂, 95% air) for 6–7 days. For maintenance culture, medium was completely changed at day 3, 4 and 5. For differentiation culture, 1 ml of the medium was changed at day 6 to minimize nutrient exhaustion. Media samples were taken at various intervals for glucose and lactate determination to monitor the metabolic activity of cells. Parallel control experiments with 2-D PET films were also conducted in the maintenance culture. Unless otherwise noted, all experiments were repeated 2–3 times and the representative data are reported herein.

Cell culture in a perfusion bioreactor

Figure 1 shows the bioreactor (dimension: 2.5 cm in inner diameter and 9.8 cm in length) system used in this study, which is similar to the one reported previously (Ma et al. 1999). The bioreactor was connected to a medium recirculating tank (400 ml), which housed pH and dissolved oxygen (DO) probes. Cells

Figure 1. Schematic diagram of the perfusion bioreactor.

were seeded into PET fibrous matrix (dimension: $7 \times 3.5 \times 0.1$ cm) and incubated for 24 hours in a CO₂-incubator as described above. Scaffolds containing adherent cells were then placed horizontally in the bioreactor chamber. The culture medium (working volume: 150 ml) was pumped through the chamber in the direction parallel to the cell matrix at the flow rate of ~ 3 ml/min. The bioreactor temperature was maintained at 37 °C and the pH was controlled in the physiological range of 7.2–7.4 by adjusting the CO₂ rate into the culture medium recirculating tank, which was agitated with a magnetic stirrer at an appropriate speed (50 – 100 rpm). The air was fed into the headspace of the medium-recirculating tank to adjust the dissolved oxygen level at 20% oxygen tension. Before each run, the oxygen probe was calibrated at 0% and 20% O₂ by saturating media with pure nitrogen gas or air, respectively. To start the cell culture in the bioreactor, the matrix pre-seeded with ES cells was put in the bioreactor. The bioreactor system containing either the maintenance or differentiation medium was then operated in a batch mode for one week. About half or one-third of the medium were removed and replaced with fresh medium aseptically after approximately 4–5 days. Medium samples for glucose and lactate measurements were taken aseptically from sampling ports and stored in a –80 °C freezer until analysis. Cellular samples for cytological assessment, colony-forming unit assay and cell number determination were taken from the bioreactor that was removed from the system at the end of each culturing period.

Analytical methods

Concentrations of glucose, lactate and cell number: Glucose and lactate concentrations in the medium samples were analyzed using YSI Biochemistry Select Analyzer (Yellow Spring, OH). The cell number was estimated from either direct cell counts using a hemocytometer after trypsinizing the cells from the surfaces of the culture flasks or matrices, or total protein content determined by Bio-Rad assay. Calibration between the cell number and the total protein content was obtained using bovine serum albumin as the standard, and it was found that each ES cell contained about 165 pg protein.

Alkaline phosphatase (ALP) assay: Cells on 2-D films and 3-D matrices were lysed with 0.1% Triton X-100. The supernatants and ALP standard solution were mixed with *p*-nitrophenyl phosphate buffer

(Sigma) in 96-well plates. The optical density was measured at 405 nm in a micro-plate reader (Biotek 312E). The protein content of samples was determined using Bio-Rad protein assay to estimate the specific ALP activity.

Cell cycle analysis: Cells were fixed with 70% cold ethanol for more than 2 hours. They were then stained with propidium iodide (PI) (0.02 mg/ml, in PBS) staining solution containing 0.1% Triton X-100 and RNase A (0.2 mg/ml). Cell fluorescence was measured using FACS^{scan} (Becton Dickinson, San Jose, CA) and the DNA content frequency histogram was analyzed using software ModFit (Verity Software House Inc., Topsham, ME).

Cytological assessment

Matrix with cells was fixed in 70% cold ethanol for 30 min and embedded in paraffin. Cross sections (7 µm thick) were cut through the center of the scaffolds. Sections were stained with hematoxylin and eosin (H&E), and examined under a light microscope (ECLIPSE TE200, Nikon, Japan).

Colony-forming unit assay

About 1.5×10^5 cells were added to 4 ml of methylcellulose-containing medium. This medium was supplemented with human growth factors, IL-3 (20 ng/ml), SCF (50 ng/ml), Epo (3 IU/ml) and GM-CSF (30 ng/ml) in 0.5 ml of IMDM containing 2% FBS, 10 IU/ml penicillin, 10 µg/ml streptomycin, and 1 mM L-glutamine (StemCell Technologies, Inc.). The supplemented medium (1.1 ml) was added to 35 mm scored Petri dishes. Triplicate dishes were prepared for each cell concentration. The Petri dishes were placed in a 5% CO₂ incubator at 37 °C for 10 days after which the number of colonies was determined by manual counting.

Immunophenotyping of SSEA-1 and c-kit

Adherent cells in 3-D matrices were examined using immunocytochemistry and with confocal microscopy. Briefly, cell-matrix constructs were rinsed in cold PBS and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. After being rinsed in the blocking buffer (PBS + 5% Horse Serum), samples were incubated with anti-mouse SSEA-1 (1 : 50, Development Studies Hybridoma Bank, Iowa City, IA) for 1 hour. The samples were rinsed in PBS, and

incubated with goat anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. Samples were then completely rinsed in PBS and examined under a confocal laser scanning microscope (CLSM) (BioRad MRC-600, Hercules, CA) using dual excitation filters – 488 nm for FITC labeling and 568 nm for PI labeling. Negative controls were prepared following the same fixation procedures staining with secondary antibody only.

Cells were also analyzed using flow cytometry. Briefly, $2-5 \times 10^5$ cells were washed in PBS containing 0.5% BSA and 2 mM EDTA. One microliter of human FcR blocking reagent was added to prevent nonspecific binding. Various antibodies including anti-mouse SSEA-1 and c-kit (Pharmingen, San Diego, CA) were also added at $20 \mu\text{l}/10^6$ cells, followed by 60 min incubation at 4°C . After washing, the samples were incubated with goat anti-mouse IgG-FITC secondary antibody for another 30 min. The isotype control was prepared in the same procedure incubated with IgG-FITC only (Pharmingen). After washing, cells were fixed in 2% PFA and then analyzed on a FACS^{scan} (Becton Dickinson) with CellQuest software.

Western blotting for Oct-4

Culture samples were rinsed with PBS and then lysed in a solution containing 1% Triton X-100, 120 mM sodium chloride, 25 mM HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ antipain. Cell extracts were incubated at 4°C for 30 min and then centrifuged at 12,000 rpm for 10 min. An appropriate amount of supernatant determined by protein assay was solubilized in 5 X SDS-PAGE buffer (1 X = 68 mM Tris-HCl, pH 6.8, 5% β -mercaptoethanol, 2% SDS, and 10% glycerol) and boiled for 5 min. Protein samples (30 μg per 5–10 μl) were fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose filters, and incubated in Tris-buffered saline, 0.2% Tween-20, and 5% nonfat milk (BLOTTO). Nitrocellulose filters were then incubated with anti-rabbit Oct-4 or anti-mouse GAPDH polyclonal antibody (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibody incubation with peroxidase-conjugated bovine anti-rabbit or mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) was in BLOTTO for 1 hour. Immunoreactive proteins were visualized by development in LumiGLOTM (Kirkegaard & Perry Laboratories, Gaithersburg, MD)

and exposure to X-ray films. Densitometric analysis was performed using Personal Densitometer SI and ImageQualNTTM software (Molecular Dynamics Inc., Sunnyvale, CA).

Statistical analysis

Triplicate or duplicate samples were collected in each measurement, and the results were expressed as means $\pm$ standard deviation. Statistical analysis was carried out using Student's *t* test for the comparison of two groups with a minimal significance of $P < 0.05$.

Figure 2. Kinetics of ES cells grown in 2-D and 3-D systems in the maintenance medium. (A) Glucose and lactate concentration; (B) Glucose consumption and lactate production rates; (C) ALP activity. The data reported here were from one experiment that was representative of three independent experiments performed, all of which showed similar trends.

Figure 3. Organization of ES cells grown in 3-D matrix from static culture in the maintenance medium. (A) Cross section stained with H&E; (B) Confocal images stained with SSEA-1; the magnification is 200.

Results and discussion

Maintenance culture of undifferentiated ES cells

ES cells were cultured on 2-D films or in 3-D matrices in maintenance medium to keep cells in the undifferentiated state. The metabolic activity as indicated by changes in the glucose and lactate concentrations in the culture medium are shown in Figure 2. The glucose concentration quickly dropped to ~ 2.0 g/L and lactate concentration increased to ~ 2.0 g/L (Figure 2A). ES cells have a high rate of metabolism and glucose consumption increased rapidly, especially after 3 days, from ~ 0.01 g/L/h to ~ 0.1 g/L/h (Figure 2B). The metabolic rate was higher in 2-D culture before day 4, but was surpassed in 3-D culture by day 6. Cell associated ALP activity was measured to examine the maintenance of undifferentiated cells as shown in Figure 2C. The specific ALP activity was

maintained at high levels in both the 3-D and 2-D cultures. In contrast, cells lost their ALP activity upon culturing in the differentiation medium, which also can be seen in Figure 2C.

The cell-matrix constructs were processed using H&E staining to study cell morphology and organization in the 3-D PET matrix. As can be seen in Figure 3, ES cells formed large aggregates associated with the fibers. Also, almost all cells in the aggregates were SSEA-1⁺, reflecting the undifferentiated phenotype (Figure 3B). It was also observed that the 3-D culture yielded a higher final cell number than the 2-D culture resulting from a larger surface area for growth. This also permitted larger inocula to be used. The specific growth rate and cell doubling time were estimated from the initial and final cell numbers in each of the culturing system, and the results are given in Table 1. In maintenance medium, undifferentiated ES cells grew at about the same rate with similar lactate yields from glucose (~ 2.0 mol/mol) in both the 2-D and 3-D culture systems. The average doubling time for the maintenance cultures was ~ 35 hours.

Hematopoietic differentiation

Differentiation of ES cells in conventional 35-mm Petri dishes was examined over 6 days under different initial plating cell numbers from 600 to 9600 cells per well. As shown in Figure 4A, the final cell number increased almost linearly with the inoculation number, but this increase reached a plateau when the inoculation number was > 6000 cells. Consequently, the fold of expansion decreased with the inoculation number. Colony-forming units were evaluated to monitor hematopoietic differentiation potential of the murine ES cells. The number of CFU per 10^6 cells decreased with the inoculation number. The total CFU increased initially up to a maximum and then decreased (Figure 4B), indicating that there might be an optimum plating number for total CFU production. For differentiation in 3-D culture, the final cell number increased with inoculation number (Figure 4C). The number of CFU per 10^6 cells decreased slightly with the inoculation number. Total CFU increased initially and then reached a plateau (Figure 4D). The 3-D culture system also gave a similar final cell number, but a higher number of CFU per 10^6 cells than the 2-D culture method. The specific growth rate and cell doubling time were estimated from the initial and final cell numbers in each of the culturing system. As shown in Table 1, ES cells grew at about the same

Table 1. Comparisons of growth kinetics under various culture conditions.

Culture Conditions	Initial cell number ($\times 10^6$)	Cell number after 1 wk ($\times 10^6$)	Specific growth rate (day^{-1}) ^a	Doubling time (h)	CFU per 10^6 cells after 1 wk ^b	Lactate yield (mol/mol) ^c
Maintenance Culture						
2-D Film	0.09	2.55 ± 0.19	0.475 ± 0.011	35.1 ± 0.8	ND	1.85 ± 0.16
3-D Static	0.156	3.65 ± 0.38	0.450 ± 0.021	36.9 ± 1.8	2308 ± 1058	2.06 ± 0.12
	0.09	2.90 ± 0.12	0.498 ± 0.008	33.4 ± 0.6	ND	ND
Perfusion Bioreactor	3.92	210.7 ± 4.6	0.569 ± 0.004	29.2 ± 0.2	1787 ± 420	1.68
Differentiation Culture						
2-D Petri Dish	0.01	3.05 ± 0.47	0.817 ± 0.032	20.4 ± 0.8	526 ± 95	2.86 ± 0.16
3-D Static	0.01	2.98 ± 0.10	0.820 ± 0.007	20.3 ± 0.2	1756 ± 84	2.01 ± 0.05
Perfusion Bioreactor	0.25	97.7 ± 4.1	0.851 ± 0.008	19.5 ± 0.2	1474 ± 121	1.48

Note: The matrix volume for the static culture was 0.12 cm^3 and the final cell density in the matrix was 30.4×10^6 per cm^3 for the maintenance culture and 24.8×10^6 per cm^3 for the differentiation culture. For the perfusion bioreactor, the matrix volume used was 2.45 cm^3 and the final cell density was 86.0×10^6 per cm^3 for the maintenance culture, and 2.10 cm^3 and 46.5×10^6 per cm^3 for the differentiation culture. The initial cell number was estimated from direct cell counting; the final cell number was estimated from the protein assay of duplicate well samples. ND: not determined. ^a Statistical analysis of the data indicated the difference between 3-D static culture and perfusion culture was significant (t-test, $P < 0.05$), but not significant between 2-D and 3-D static cultures. ^b Statistical analysis of the data indicated that the difference between 2-D and 3-D differentiation cultures was significant (t-test, $P < 0.05$), but not significant between 3-D static culture and perfusion bioreactor culture. ^c Statistical analysis of the data indicated that the difference between 2-D and 3-D differentiation cultures was significant (t-test, $P < 0.05$), but not significant between 2-D and 3-D maintenance culture.

Figure 4. Hematopoietic differentiation of ES cells in 2-D and 3-D systems. (A) Cell growth of 2-D suspension culture; (B) CFU content of 2-D suspension culture; (C) Cell growth of 3-D culture in fibrous matrix; (D) CFU content of 3-D culture in the fibrous matrix. Two independent experiments were performed and both showed similar trends. The representative data from one experiment were reported here.

Figure 5. Oct-4 expression in ES cell hematopoietic differentiation as determined by Western Blotting.

rate in both the 2-D and 3-D culture systems, with an average doubling time of ~20.3 hours. Compared to the maintenance culture, the apparently faster growth rate for the differentiation culture could be attributed to the lower initial cell density and different growth factors used in the culture.

Expression of cell markers after 7 days of culture in differentiation medium were studied (Table 2). The inoculum number was 10^4 cells. For the 2-D culture, both ALP and Oct-4 expression decreased quickly (Table 2), indicating rapid *in vitro* differentiation. The expression of Oct-4 was normalized to glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) expression. As can be seen in Figure 5, a high level of specific Oct-4 expression was observed in the initial population, but it decreased to a barely detectable level after 7 days in differentiation medium. SSEA-1 expression, measured by flow cytometry, decreased from 89% in the initial cell population to 60% at day 7. Cell cycle distribution also changed. Initially, about 68% of the cell population was in the S-phase and about 20% in the G1/G0 phase. After 7 days of culture in differentiation medium, the percentage of cells in the S-phase decreased to about 41%, while the cells in the G1/G0 phase increased to about 48%. The remaining 12% of cells was in G2/M phase. Undifferentiated ES cells lack a G1 checkpoint and they spend most of their time in S phase (Smith 2001). The decrease in S phase cells thus indicated the exit from cell cycle and possible differentiation. However, the expression of c-kit, a marker of hematopoietic progenitor, remained essentially unchanged at ~34%. Apparently, the current culture procedure in Petri dish induced the spontaneous differentiation but not or little hematopoietic differentiation.

The hematopoietic differentiation in the 3-D culture exhibited different expression levels of various markers as compared with the 2-D suspension culture. For the static culture in the 3-D fibrous matrix, changes in Oct-4 expression and cell cycle distribution after 7-day culturing indicated occurrence of cell differentiation, but the changes were not as dramatic as in the 2-D culture. The cell cycle distribution for the 3-D culture was similar to the initial cell population, although the 3-D culture exhibited a significantly down-regulated level of Oct-4 expression. Surprising-

Table 2. Comparisons of cell marker expression and cell cycle distribution in 2-D and 3-D static cultures in the differentiation medium after 7 days.

	Initial Conditions	2-D culture	3-D culture
Cell marker expression			
ALP (U/mg Protein)	0.737	0.13 ± 0.06	ND
Oct-4 (density/GAPDH density)	0.70 ± 0.05	0.07 ± 0.01	0.20 ± 0.03
SSEA-1 (%) ^a	89.5 ± 2.0	60.0 ± 3.3	98.2 ± 0.7
	ND	60.2 ± 3.5	94.0 ± 0.8
c-kit (%) ^a	36.2 ± 7.1	33.8 ± 3.5	80.3 ± 3.7
	ND	14.4 ± 2.8	18.6 ± 0.8
Cell cycle distribution			
S phase (%)	67.9 ± 0.4	41.3 ± 2.1	62.8 ± 0.8
G1/G0 phase (%)	19.9 ± 0.4	48.5 ± 1.3	28.5 ± 0.1

^a Two separate sets of experiments were conducted in the determination of SSEA-1 and c-kit expressions by flow cytometry. The large variation in the results for c-kit was attributed to difference in the initial cell population as well as experimental errors. ND: not determined. Statistical analysis of the data indicated significant difference (t-test, $P < 0.05$) between 2-D and 3-D cultures in Oct-4, SSEA-1 and c-kit expressions and in cell cycle distribution.

ly, SSEA-1 and c-kit expressions in the 3-D culture were at unexpectedly high levels, about 94–98% and 80%, respectively. Both were much higher than those in the 2-D culture. It was reported that SSEA-1 expression showed kinetic decrease with differentiation time up to 11 days in suspension culture (Ling and Neben 1997). The lack of decrease in SSEA-1 expression in the 3-D system was unexpected, and the function of SSEA-1 was not totally clear. SSEA-1 is a lactoseries oligosaccharide antigen (Draper et al. 2002). Although it is highly expressed in undifferentiated murine ES cells, it is negative for undifferentiated human ES cells and its expression is upregulated when human ES cells become differentiated (Draper et al. 2002). Nevertheless, the significant decrease in Oct-4 expression revealed the differentiation in the 3-D system. Oct-4 is a germline transcription factor and serves as a key regulator of totipotency (Pesce et al. 1999). The expression of Oct-4 prevents the differentiation of ES cells and the events that down regulate Oct-4 trigger ES cells into differentia-

tion. The higher expression of c-kit in the 3-D culture suggested a better-directed hematopoietic differentiation. Other markers such as telomerase also can be used to determine the differentiation status of embryonic stem cells. It has been reported that the activity of telomerase was high in undifferentiated embryonic stem cells but was low in fully differentiated adult cells (Xu et al. 2001).

It should be noted that the flow cytometry for c-kit expression assay was subjected to a relatively large variation because the intensity of c-kit staining was close to the background. In repeating the same differentiation culture experiments, consistent SSEA-1 expression levels were obtained while the c-kit expression levels were recorded at lower levels, from 12–17% for the 2-D culture and 17.5% to 19% for the 3-D culture. The lower c-kit level could be a result of the low level in the initial cell population, although this was not determined in this set of experiments. Assuming the initial c-kit level was close to the lowest value recorded (~12%), the c-kit expression level in the 3-D system increased by about 50%, while there was a minimum increase for the 2-D culture. A longer differentiation time may be required to reveal the developmental process. The results observed in this study clearly indicate different differentiation mechanisms in the 3-D system and the 2-D suspension culture.

ES cell culture in a perfusion bioreactor

The accelerated metabolic rate of ES cells in long-term culture limits the use of conventional static cultures. It has been reported that improved supply of oxygen and nutrients in a spinner flask culture system significantly improved the endothelial differentiation as compared to the Petri dish suspension culture (Wartenberg et al. 1998). To study the maintenance and expansion of undifferentiated ES cells in the bioreactor, the matrix was seeded at the density of 1.6×10^6 cells/cm³ in the bioreactor and cultured in the maintenance medium. The glucose consumption and lactate production were monitored (Figure 6A). The glucose concentration decreased continuously and the consumption rate increased from ~0.0128 to ~0.0181 g/L/h. The lactate was produced continuously and the production rate increased from ~0.006 to ~0.014 g/L/h. The lactate production yield was constant at 1.68 mol/mol, lower than that in the static culture (2.06 mol/mol). The final cell density at one week was determined and compared with the static culture

Figure 6. Kinetics of metabolic activity of ES cell culture in the perfusion bioreactor. (A) culture in the maintenance medium; (B) culture in the differentiation medium.

Figure 7. Organization of ES cells grown in 3-D matrix from the bioreactor. (A) and (B) are PI-stained confocal images. (A) maintenance culture, the picture is a projection from 11 sections with 5- μ m increment. (B) differentiation culture, the picture is one section; (C) and (D) are cross sections stained with H&E. (C) maintenance culture; (D) differentiation culture.

(Table 1). A higher final cell density was obtained in the perfusion bioreactor (8.6×10^7 vs. 3.0×10^7 cells/cm³). The specific growth rate was ~ 0.57 /day, which was higher than that in the static culture at similar initial cell density (0.45/day). The higher growth rate in the perfusion bioreactor was attributed to the better controls on pH, DO, and nutrient supplies in the system.

To study ES cell differentiation in the bioreactor, the matrix was seeded at a low cell density of 1.2×10^5 cells/cm³ and cultured in the differentiation medium. Similarly, the glucose consumption and lactate production were examined (Figure 6B). The metabolic rate was low at the early stage of the culture but accelerated in the late stage of the culture. The glucose consumption rate increased 4-fold from the initial 0.005 g/L/h to 0.023 g/L/h. The lactate yield from glucose was kept constant at 1.48 mol/mol, which was much lower than that in the static culture (2.01 mol/mol). Compared to the static culture, a

higher final cell density was also resulted in the perfusion bioreactor (4.65×10^7 vs. 2.48×10^7 cells/cm³) (Table 1). The specific growth rate was also higher in the bioreactor than in the static culture. Again, the large difference in the growth rate between the maintenance and differentiation cultures could be attributed to the different growth factors and different initial cell density used in the studies.

CFU assay was used to detect the differentiation potential of the cultured ES cells after one week. A similar number of CFU per 10^6 cells was found for both bioreactor and static cultures (Table 1). It should be mentioned that the colony assay itself cannot differentiate the nonhematopoietic cells that are able to grow in clusters resembling hematopoietic colonies (Kaufman et al. 2001). The evidence of hematopoietic differentiation in this study was not strong, and further studies of different cell marker expressions, including c-kit, are needed. The ES cell organization within the matrix after one-week culturing in both

Figure 8. Morphology of ES cells grown on 2-D surface in static cultures. (A) Undifferentiated ES cell colony cultured on PET film in the maintenance medium at day 6; (B) Embryonic body formation of ES cells on non-adherent Petri dish in the differentiation medium at day 2.

maintenance and differentiation media was examined. From both propidium iodide (PI)-stained confocal images and H&E-stained histological images shown in Figure 7. It is clear that ES cells tend to form large aggregates among the fibers, similar to the ES cell colonies or embryonic bodies in 2-D culture (Figure 8). However, the spatial distribution of different phenotypes of cells in the aggregates needs to be further investigated to differentiate those aggregates as ES colony or structured embryonic body. Also, the size distribution under different culture conditions worth future investigation.

Factors affecting ES cell differentiation

There are many factors known to affect ES cell differentiation, including the surface property of the tissue scaffold and growth factors in the medium. The differentiation process used in this study has not been

Figure 9. Effects of culture surfaces on ES cell differentiation. (A) Oct-4 expression; (B) SSEA-1 expression. UDG: undifferentiated medium with gelatin-coating; DG: differentiated medium with gelatin-coating; Dsus: differentiated medium without gelatin-coating in suspension dish.

optimized. In the 3-D scaffold design, the surface property should be considered. We compared the spontaneous differentiation (without LIF, TPO and FL) on a tissue culture treated polystyrene (PS) surface with a gelatin coating (DG) and a non-tissue culture treated PS surface without gelatin coating (Dsus), using undifferentiated culture on gelatin coated surface as control (UDG). The Oct-4 expression was down regulated upon differentiation and it decreased more quickly under Dsus condition (Figure 9A). Consistently, the percentage of SSEA-1 also decreased upon differentiation (from 88% to 75%), and it decreased more under Dsus condition (70%) (Figure 9B). Thus, the surface characteristics affected differentiation. It has been reported that culturing human ES cells on a laminin coated surface with a feeder layer conditioned medium removed the requirement of the feeder layer by undifferentiated cells (Xu et al. 2001). Clearly, both integrin and ECM played important roles in the ES cell culture.

It is known that the components in the differentiation medium might affect embryonic stem cell development (Wiles and Johansson 1999). In this study, we achieved only partial differentiation as indicated by more than 60% SSEA-1⁺ cells still present in the 7-day cultures. It has been reported that the culture with simple removal of LIF is not sufficient to optimize the differentiation process, although it can alter the possibility of a cell choosing a self-renewal or a

differentiation division (Zandstra et al. 2000; Smith 2001). The cocktail of growth factors thus needs to be optimized. In addition to TPO and FL, the effect of other cytokines such as SCF (stem cell factor) on hematopoietic differentiation also needs to be evaluated. Other medium components present in the hematopoietic differentiation medium used in this study also need to be further optimized.

In studying hematopoietic differentiation, we used a liquid mass culture instead of 'hanging drop' (Wobus 2001) or methylcellulose culture (Hole 1999) method in order to adapt the culture procedure to the 3-D system. It was reported that these three methods reached similar maximum cell number per EB and had similar kinetics of hematopoietic development (Dang et al. 2002). Alternatively, the culture could be inoculated with the dissociated cells from the pre-formed embryonic body, rather than the undifferentiated cells, onto the 3-D matrix. The effects of medium composition, exchange rate, hydrodynamic force, oxygen tension and pH on ES cell differentiation also need to be studied. These physio-chemical parameters should be studied with the perfusion bioreactor before the process can be scaled up.

Conclusions

ES cells have high proliferation potential and are theoretically able to differentiate into all types of cells except trophectoderm. The challenge of using ES cells is to control the differentiation into desired lineages rather than a mixture of various cell types. The parameters affecting the early commitment of ES cells need to be identified and investigated. Driving ES cell differentiation using different growth factors was tested in several studies toward lineage-specific differentiation, including hepatocyte, cardiac muscle cells, neuron cells (Schuldiner et al. 2000), adipocyte (Dani 1999) and insulin-secreting cells (Lumelsky et al. 2001). Besides the differentiation inducers in medium, the plating number and culture duration also affected the directed differentiation (Manuilova et al. 2001). This study investigated the potential of a 3-D fibrous matrix in ES cell maintenance and differentiation, both in static culture and in a perfusion bioreactor. Our results suggest that the 3-D system can maintain the undifferentiated ES cells and promote directed differentiation. The possible effects of physical environment on ES cell differentiation, particularly in the 3-D fibrous matrix, were also studied. The

phenotypes of undifferentiated ES cells were maintained as indicated by ALP activity and SSEA-1 expression in both 2-D and 3-D culturing environments. ES cell differentiation was also induced in the differentiation medium; however, 3-D culture seemed to give a better-directed hematopoietic differentiation, as indicated by the higher level of c-kit expression. The final cell density was also improved using a perfusion bioreactor with the maintenance of differentiation potential. Therefore, the ES cell culture in the 3-D matrix can be used as a model system to study effects of extracellular environment and associated physico-chemical parameters on ES cell maintenance and differentiation. Theoretically, hematopoietic stem cells can be derived from ES cells in an unlimited amount using the perfusion bioreactor with a 3-D matrix; however, further studies to optimize the process are necessary.

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