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Efficient culture system for human embryonic stem cells using autologous human embryonic stem cell-derived feeder cells

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Abbreviations: AP, alkaline phosphatase; bFGF, basic fibroblast growth factor; Diff$^{\text{Miz-hES6}}$ cells, feeder cells differentiated from Miz-hES6hESCs;EB,embryoidbody;FBS,fetalbovineserum;hESC, humanembryonicstemcell;MEF, mouseembryonicfibroblast;SSEA, stagespecificembryonicantigen

Abstract

Humanembryonicstemcells(hESCs)needfeeder cells for their maintenance in an undifferentiated state. In conventional culture systems, mouse embryonicfibroblasts(MEFs)serveasfeedercells to maintain hESCs. However, the use of MEFs elevatestheriskoftransmittingmousepathogens and thus limits the potential of hESCs in cell replacement therapy. Consequently, the use of human feeder cells would be an important step forwardinthis *invitro* technology.Toaddressthis issue, we used fibroblast-like cells differentiated fromtheMiz-hES6hESCline(Diff$^{\text{Miz-hES6}}$)asfeeder cellstosupportthe *invitro* growthofthreehESC lines. Immunofluorescence microscopy and reversetranscription-PCR assessingtheexpression ofundifferentiatedhESC markersrevealedallthree hESClinesweremaintainedinanundifferentiated state. *Invitro* proliferationproceededasefficiently aswhenthehESCswereculturedonMEFS.Moreover,karyotypeanalysisrevealedthechromosomal normalityofthehESClinesandtheDiff$^{\text{Miz-hES6}}$feeders themselvesaftereven50passages.Furthermore,the hESClinesmaintainedtheir pluripotencysincethey remainedcapableofformingembryoidbodies(EBs) *in vitro* . Thus, hESC-derived fibroblast-like cells successfullysupport *invitro* hESCpropagation.

Keywords:cellculturetechniques;humans;stemcells; stemcelltransplantation

Introduction

Embryonic stem cells (ESCs) can differentiate into all the cells derived from three embryonic germ layers (Choi *et al.,* 2004; Kim *et al.,* 2005a; b). Human embryonic stem cells (hESCs) are derived frominnercellmassesofhumanblastocysts(Thomson *etal.,* 1998). The two fundamental characteristics of hESCs are their pluripotency and capacity for self- renewal. For the long-term culture of hESCs, feeder cell layers are conventionally used. Feeder cells are believed to help maintain hESCs by providing cell-cell contact with hESC colonies and/or by providing many secreting factors, which have not yet beenfullyidentified.

To date, the most commonly used feeder cells are primary MEFs, which are prepared from day 13.5 postcoitumfetusesofpregnantmice.However,MEFs haveseveralseriouslimitationsasfeedercells.First, theyhavealimitedlifespaninculture as the culture can only be passaged seven to nine times before the feeder cells senesce. Second, the use of MEFs introduces the risk of contaminating hESCs with animal pathogens. Due to these considerations, it is desirable to use fresh stocks of feeder cells that originate from human sources in the *in vitro* culture ofhESCs.

Recently,weandothergroupsdemonstratedthatit is possible to culture hESCs on feeder cells that originatefromhumansources(Richards *etal.,* 2002; 2003;Amit *etal.,* 2003a;Cheng *etal.,* 2003;Hovatta

et al., 2003; Lee *et al.,* 2005). In most cases, however, these human feeder cell lines are derived from samples obtained from patients or discarded human fetuses. This may seriously limit the accessibility of these feeder cells to researchers in the field. In addition, it is important to standardize the source of feeder cells used for research, as this is a variable that could hamper comparison between results obtained by different groups.

Feeder-free culture of hESCs has been reported (Xu *et al.,* 2001; Amit *et al.,* 2003b; Rosler *et al.,* 2004). However, this method does require media conditioned by MEFs cultured with fetal bovine serum (FBS). Moreover, the stable and long-term culture of hESCs and the maintenance of their undifferentiated state still requires feeder cells along with the addition of exogenous basic fibroblast growth factor (bFGF) (Kim *et al.,* 2005c).

In the present study, we report the method we developed to supply human feeder cells for hESC culture. These feeder cells are themselves derived from hESCs, namely, the Miz-hES6 hESC line, and were denoted as $Diff^{Miz\text{-}hES6}$. To ensure that this feeder layer fully meets its *in vitro* requirements, we compared the growth rate, undifferentiated phenotype, pluripotency, and genomic integrity of three hESC lines grown over a prolonged period with the human feeders or with MEF feeder cells. The undifferentiated phenotype was assessed by reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence microscopy for undifferentiated markers, while pluripotency was assessed by the capacity of the hESC lines to form embryoid bodies (EBs) *in vitro*, which were analyzed by RT-PCR using primers specific for the three germ layers. We found that hESC-derived feeder cells maintain hESC lines in an undifferentiated, karyotypically-intact state. These autologous hESC-derived fibroblast-like feeders can be easily accessed by researchers and aid the development of a xeno-free hESC culture system, thus promoting the safety of cell replacement therapy.

Materials and Methods

Preparation of $Diff^{Miz\text{-}hES6}$ cells

All cell cultures were incubated at 37°C in 5% CO_2 in air and 95% humidity. The Miz-hES6 hESC line (Kim *et al.,* 2005d) was mechanically dissociated and induced to differentiate spontaneously in DMEM (no pyruvate, high glucose) supplemented with 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin G, 100 g/ml streptomycin, sodium bicarbonate (all from Invitrogen) and 10% FBS (HyClone). The line was subsequently passaged according to the standard 3T3 protocol. The resulting differentiated cells, which are denoted as $Diff^{Miz\text{-}hES6}$, were maintained in DMEM/10% FBS until a consistent morphology was observed. The cells were passaged every 3-5 days with 0.05% trypsin- EDTA (Invitrogen). From the 6th passage, $Diff^{Miz\text{-}hES6}$ cells could be continuously cultured until their 30th passage, at which point the cells senesced (data not shown).

Use of $Diff^{Miz\text{-}hES6}$ cells as feeder cells

When the $Diff^{Miz\text{-}hES6}$ cells reached the subconfluent state after passaging, they were mitotically inactivated by treatment with 10 μg/ml mitomycin C (Sigma) for 1.5 h. The cells were then thoroughly washed in PBS, trypsinized with 0.05% trypsin-EDTA and replated at a concentration of 3.25×10^4 cells/cm^2 in gelatin-coated 4-well culture dishes.

Culture of hESCs

We cultured the Miz-hES1 and Miz-hES4 hESC lines (Kim *et al.,* 2005d) and the HSF-6 hESC line from the University of California at San Francisco. The former two lines are male cell lines. The Miz-hES1 line is registered on the NIH hESC Registry along with the HSF-6 cell line. The three hESC lines were initially maintained on MEF feeder cells and then transferred onto $Diff^{Miz\text{-}hES6}$ cells. They were cultured at 37°C in 5% CO_2 in air and 95% humidity with DMEM/F12 media supplemented with 4 ng/ml bFGF, 20% Knockout Serum Replacement, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin G and 100 g/ml streptomycin (all from Invitrogen). The hESCs were passaged every 3-5 days by mechanically dissociating hESC clumps and transferring them onto newly prepared $Diff^{Miz\text{-}hES6}$ feeder cell layers.

Staining of hESCs for undifferentiated cell markers and immunofluorescence analysis

After the 20th passage of the three hESC lines cultured on $Diff^{Miz\text{-}hES6}$ or MEF feeder cells, the hESCs were stained for undifferentiated hESC markers, namely, alkaline phosphatase (AP), stage-specific embryonic antigen-1 (SSEA-1), SSEA-4, and Tra-1-60 (Chemicon). AP staining was performed by using the NBT/BCIP solution (Roche) with 1:100 in Tris-HCl, pH 9.5. The anti-SSEA-1 and anti-Tra-1-60 antibodies are mouse IgM antibodies, while the anti-SSEA-4 antibody is a mouse IgG antibody. All primary antibodies were diluted at 1:1,000 and incubated with the cells for 1 h at room temperature. After washing, the cells were incubated for 45 min at

room temperature without light exposure with 1:400-diluted FITC-labelled secondary antibodies specific for mouse IgM or IgG. The resulting images were monitored by a fluorescent microscope (Nikon).

In vitro formation and RT-PCR analysis of EBs

To test the pluripotency of hESCs cultured on Diff$^{Miz\text{-}hES6}$ feeder cells, we analyzed their ability to form EBs by transferring dissociated clumps of hESC colonies onto Petri dishes in EB suspension medium (hESC medium lacking bFGF). The EBs were continuously cultured in suspension until days 7 and 14, at which point they were subjected to RT-PCR by using primers specific for the three germ layers, namely, ectoderm (primers specific for Neurofilament 68 and keratin), mesoderm (primers specific for enolase and kallikrein) and endoderm (primers specific for α-fetoprotein [α-FP] and α1-antitrypsin [α1-AT]). The amplified bands were quantified by a densitometer and the data were presented as relative densities.

RT-PCR

The sequences of the primer used are listed in Table 1. The PCRs consisted of an initial denaturation step at 94 °C for 5 min followed by 30 cycles of 30 sec of denaturation at 94 °C, 30 sec of annealing at 62 °C, and 30 sec of extension at 72 °C. A final extension was performed at 72 °C for 10 min. PCR products were visualized by ethidium bromide staining following 1.0% agarose gel electrophoresis.

Karyotype analysis

To analyze the karyotypes of the Diff$^{Miz\text{-}hES6}$ cells and the HSF-6 and Miz-hES4 hESC lines cultured on Diff$^{Miz\text{-}hES6}$ feeder cells, cell division was blocked in metaphase by 0.1 μg/ml colcemid (Gibco/Invitrogen) for 1-2 h. The cells were then trypsinized and resuspended in hypotonic KCl solution (Sigma),

Table 1. Information on RT-PCR primers.

Genes	Primer sequences (Forward, Reverse)	Annealing Tm (°C)	Product size (bp)
Undifferentiated hESC markers			
Nanog [NG_004093]	F:CAAA GGCAAACAACCCACTT	62	426
	R:CTGGATGTTCTGGGTCTGGT		
Oct4 [NM_013633]	F:GACAACAATGAGAACCTTCA	62	218
	R:TTCTGGCGCCGGTTACAGAA		
Rex1 [NM_174900]	F:CTG AAGAAACGGGCAAAGAC	62	344
	R:GAACATTCAAGGGAGCTTGC		
Sox2 [NM_003106]	F:ATGGACAGTTACGCGCACAT	62	268
	R:GACTTGACCACCGAACCCAT		
In vitro differentiation markers			
NF-68 [NM_006158]	F:ACGCTGAGGAATGGTTCAAG	62	561
	R:TAGACGCCTCAATGGTTTCC		
Keratin [NM_173086]	F:A GGCCCAATACGAGGAGATT	62	479
	R:ATAGCCACTGGAGATGGTGG		
Kallikrein [NM_000537]	F:GCTTTCTCAGCCAGGACATC	62	562
	R:TATTCTTTGCCTCCCAGGTG		
Enolase [NM_001428]	F:GTTCAATGTCATCAATGGCG	62	477
	R:GTGAACTTCTGCCAAGCTCC		
α-FP [NM_001134]	F:TGAAAACCCTCTTGAATGCC	62	492
	R:TCTTGCTTCATCGTTTGCAG		
α1-AT [NM_001002236]	F:ACTG TCAACTTCGGGGACAC	62	517
	R:CCCCATTGCTGAAGACCTTA		
Housekeeping gene			
β-actin [BC_013835]	F:AGCAAGCAGGAGTATGACGA	62	260
	R:TGTGAACTTTGGGGGATGGA		
Cell typing markers			
Prolyl 4-hydroxylase β	F:GTCTTTGTGGAGTTCTATGCCC	62	339
(Fibroblast marker)	R:GTCATCGTCTTCCTCCATGTCT		
Cytokeratin 4	F:ACTGGTGTCTCTGTGCTTCCTT	62	359
(Epithelial cell marker)	R:GGGTGTTGGAGAAGTAGTTTGG		

incubated for 20 min at 37°C, and fixed with 3:1 methanol:acetic acid. The chromosomes were visualized by G-band staining. More than 100 cells were analyzed per cell line.

Results

Generation and characterization of fibroblast-like Diff $^{Miz-hES6}$ cells

Diff$^{Miz-hES6}$ cells were spontaneously differentiated from the Miz-hES6 hESC line. To do this, undifferentiated Miz-hES6 cells were mechanically dissociated into clumps and transferred onto a culture dish containing DMEM/10% FBS. After the 3-5th passage, homogeneous fibroblast-like differentiated Diff$^{Miz-hES6}$ cells were obtained (Figure 1A and 1B). To further verify the identity of the feeder cells, we subjected them to RT-PCR using PCR primers for cytokeratin 4 (an epithelial cell marker) and prolyl 4-hydroxylase β (a fibroblast marker). As expected, prolyl 4-hydroxylase β but not cytokeratin 4 is expressed by the Diff$^{Miz-hES6}$ cells, which indicates that Diff$^{Miz-hES6}$ cells are fibroblasts (Figure 1C).

Long-term support of hESC growth on Diff $^{Miz-hES6}$ feeder cells

The three hESC lines Miz-hES1, Miz-hES4 and HSF-6 were initially cultured on MEF feeder cells and then transferred onto Diff$^{Miz-hES6}$ feeder cells. At this point, the Diff$^{Miz-hES6}$ feeder cells had undergone 6 passages, while the individual hESC lines were at passage numbers 34, 107 and 54, respectively. All three hESC lines cultured on Diff$^{Miz-hES6}$ feeder cells grew at a similar rate as those on MEF feeder cells, as they required a 3-5 day passaging interval. Thus, Diff$^{Miz-hES6}$ feeder cells support the proliferation of hESCs as efficiently as MEFs. In contrast, the literature suggests hESCs cultured on other human feeder cells require a 5-7 day passaging interval (data not shown) (Richards *et al.*, 2002; 2003; Amit *et al.*, 2003a; Cheng *et al.*, 2003; Hovatta *et al.*, 2003; Lee *et al.*, 2005). The hESC lines cultured on Diff$^{Miz-hES6}$ feeder cells were relatively circular in shape (Figure 2), which is similar to their morphology when cultured on MEF feeder cells (data not shown). In contrast, the literature suggests hESCs cultured on other human feeder cells become rather irregular in shape (Richards *et al.*, 2002; 2003; Amit *et al.*, 2003a; Cheng *et al.*, 2003; Hovatta *et al.*, 2003; Lee *et al.*, 2005). We could culture the three hESC lines on Diff$^{Miz-hES6}$ cells for over 50 passages without any detecatable impairment of their self-renewal capacity (data not shown).

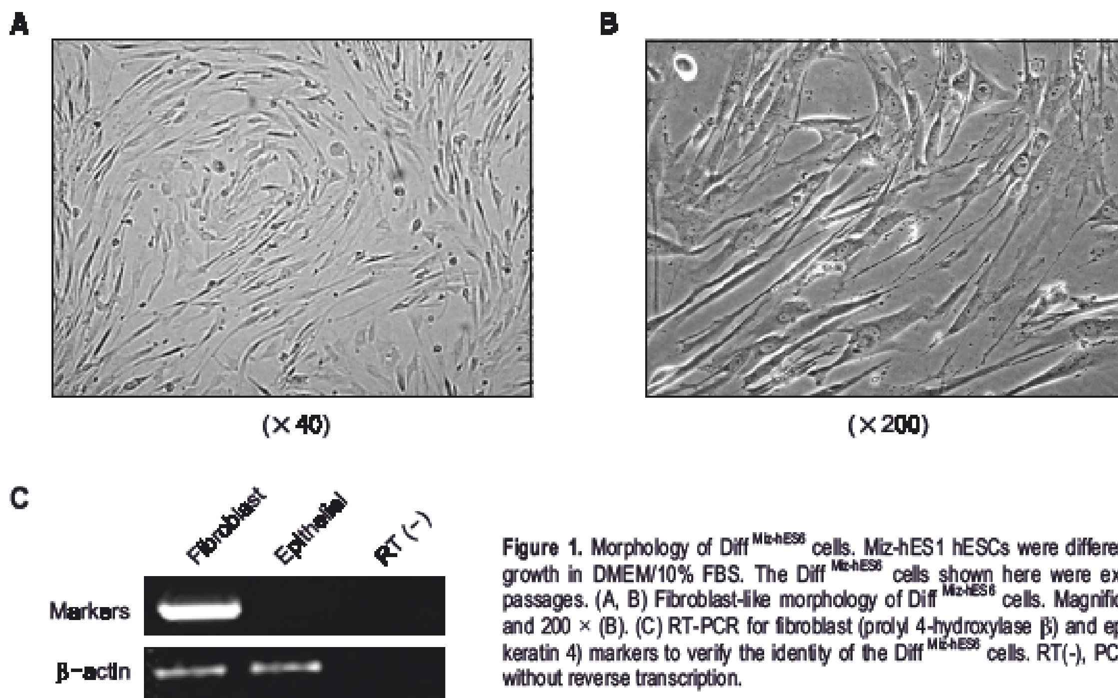

Figure 1. Morphology of Diff $^{Miz-hES6}$ cells. Miz-hES1 hESCs were differentiated *in vitro* by growth in DMEM/10% FBS. The Diff $^{Miz-hES6}$ cells shown here were examined after 3-5 passages. (A, B) Fibroblast-like morphology of Diff $^{Miz-hES6}$ cells. Magnification is 40 × (A) and 200 × (B). (C) RT-PCR for fibroblast (prolyl 4-hydroxylase β) and epithelial cell (cytokeratin 4) markers to verify the identity of the Diff $^{Miz-hES6}$ cells. RT(-), PCR was performed without reverse transcription.

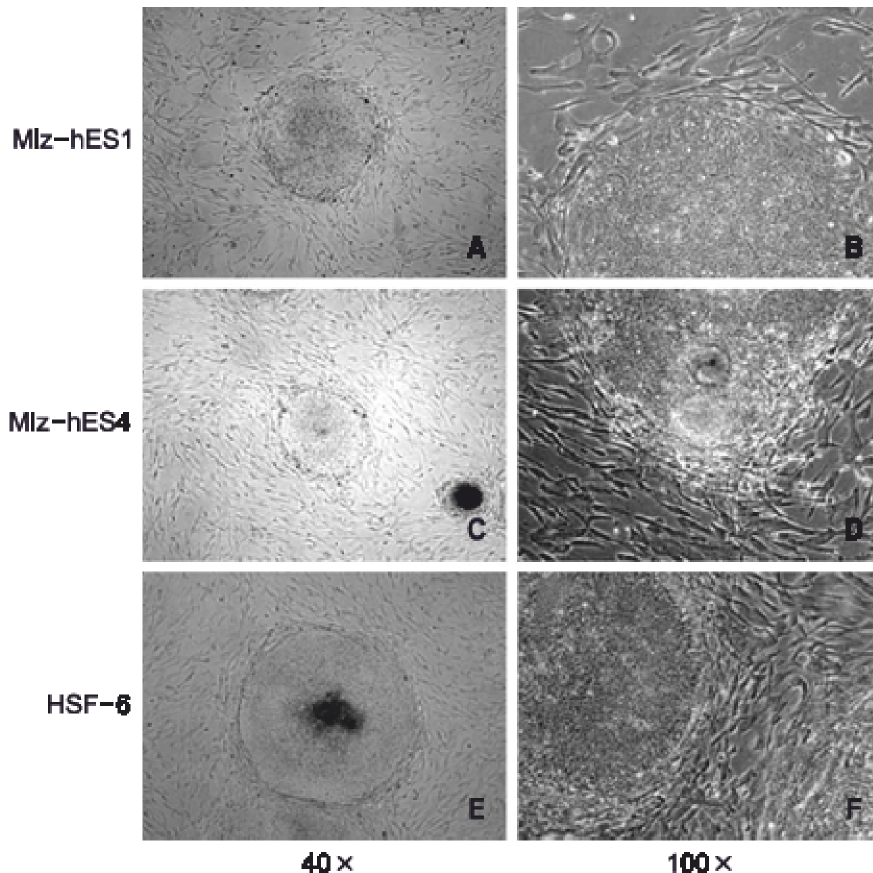

Figure 2. Morphology of the three hESC lines after their culture on the Diff$^{Miz\text{-}hES6}$ feeder layer. The cells were examined at two different magnifications, namely, 40 × (A, C, E). and 200 × (B, D, F). Miz-hES1 (A, B), Miz-hES4 (C, D) and HSF-6 (E, F). The morphology of the three hESC lines was similar to when they were cultured on MEF feeder cells (data not shown).

Expression of hESC markers by hESC lines cultured on Diff$^{Miz\text{-}hES6}$ feeder cells

hESCs express specific markers such as Oct-4, Nanog, Rex-1, Sox-2, TERT, SSEA-1, SSEA-4 and Tra-1-60. To confirm that hESCs are not altered by their culture on Diff$^{Miz\text{-}hES6}$ cells, we subjected them to immunofluorescence microscopy to detect SSEA-1, -4 and Tra-1-60 and AP expression as well as to RT-PCR to detect Oct-4, Nanog, Rex-1, Sox-2 and TERT expression. Of the surface markers, while SSEA-1 was not expressed, SSEA-4 and Tra-1-60 were strongly expressed (Figure 3D-U). The cells were also strongly positive for AP (Figure 3A, 3B and 3C). The RT-PCR data also showed normal expression of Oct-4, Nanog, Rex-1, Sox-2 and TERT (Figure 3V). These data strongly suggest that Diff$^{Miz\text{-}hES6}$ cells successfully support the maintenance of all three hESC lines in an undifferentiated state.

Analysis of the pluripotency of hESCs cultured on Diff$^{Miz\text{-}hES6}$ cells

The pluripotency of the three hESC lines cultured on Diff$^{Miz\text{-}hES6}$ cells was analyzed by inducing them to differentiate *in vitro* into EBs and then analyzing the EBs by RT-PCR with primers specific for three different germ layers (ectoderm, mesoderm and endoderm). EBs were formed by culturing dissociated hESC clumps under suspension in EB media (Figure 4A). RT-PCR was performed on cells harvested before and 7 and 14 days after differentiation. The expression of the hESC-specific transcription factors Oct-4 and Nanog was dramatically downregulated as the days after EB formation increased. Concomitantly, the expression of embryonic germ layer-specific genes (NF-68 and keratin for ectoderm), (enolase and kallikrein for mesoderm) and (α-FP and α1-AT for endoderm) increased (Figure 4B). Thus, hESCs cultured on Diff$^{Miz\text{-}hES6}$ feeder cells maintained their pluripotency.

Karyotypes of Diff$^{Miz\text{-}hES6}$ feeder cells and hESCs cultured on Diff$^{Miz\text{-}hES6}$ cells

Karyotype analysis of the Diff$^{Miz\text{-}hES6}$ feeders and two hESC lines cultured on Diff$^{Miz\text{-}hES6}$ cells showed all have normal karyotypes (HSF-6: 44 + XX, Miz-hES4: 44 + XY, Diff$^{Miz\text{-}hES6}$ feeder cells: 44 + XX) (Figure 5A,

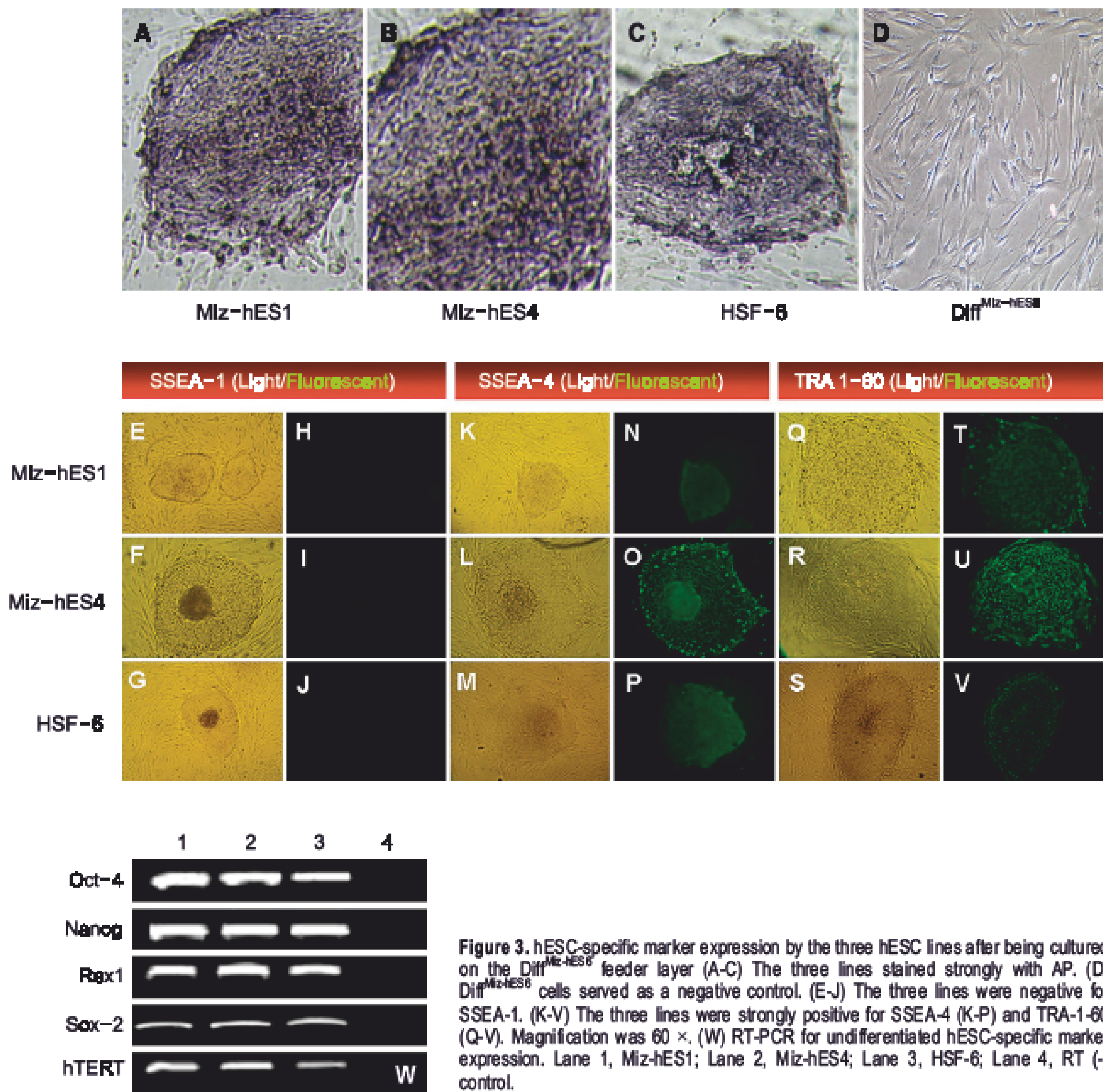

Figure 3. hESC-specific marker expression by the three hESC lines after being cultured on the Diff$^{Miz\text{-}hES6}$ feeder layer (A-C) The three lines stained strongly with AP. (D) Diff$^{Miz\text{-}hES6}$ cells served as a negative control. (E-J) The three lines were negative for SSEA-1. (K-V) The three lines were strongly positive for SSEA-4 (K-P) and TRA-1-60 (Q-V). Magnification was 60 ×. (W) RT-PCR for undifferentiated hESC-specific marker expression. Lane 1, Miz-hES1; Lane 2, Miz-hES4; Lane 3, HSF-6; Lane 4, RT (-) control.

5B and 5C). These results suggest that Diff$^{Miz\text{-}hES6}$ feeder cells successfully maintain hESCs without introducing any genetic abnormalities.

Discussion

hESCs require a feeder layer for their maintenance in an undifferentiated state. In conventional culture protocols for prolonged undifferentiated growth, the feeder layers are comprised of MEF cells. However, the routine propagation of hESCs on MEFs introduces the possibility of the transmission of mouse viruses, which is an impediment for the clincial application of these cells. To address this issue, previous studies have sought to develop a xeno-free support system for hESC culture by using donated human tissues (Richards *et al.*, 2002; 2003). However, the use of human biopsies from fetal tissues raises ethical issues. The use of donated adult and neonatal tissues would be less ethically problematic but still would not be feasible for the mass production of hESCs; moreover, the use of different donors introduces an unwelcome variability into the culture system (Amit *et al.*, 2003a; Hovatta *et al.*, 2003; Lee *et al.*, 2005). Furthermore, the risk of contamination

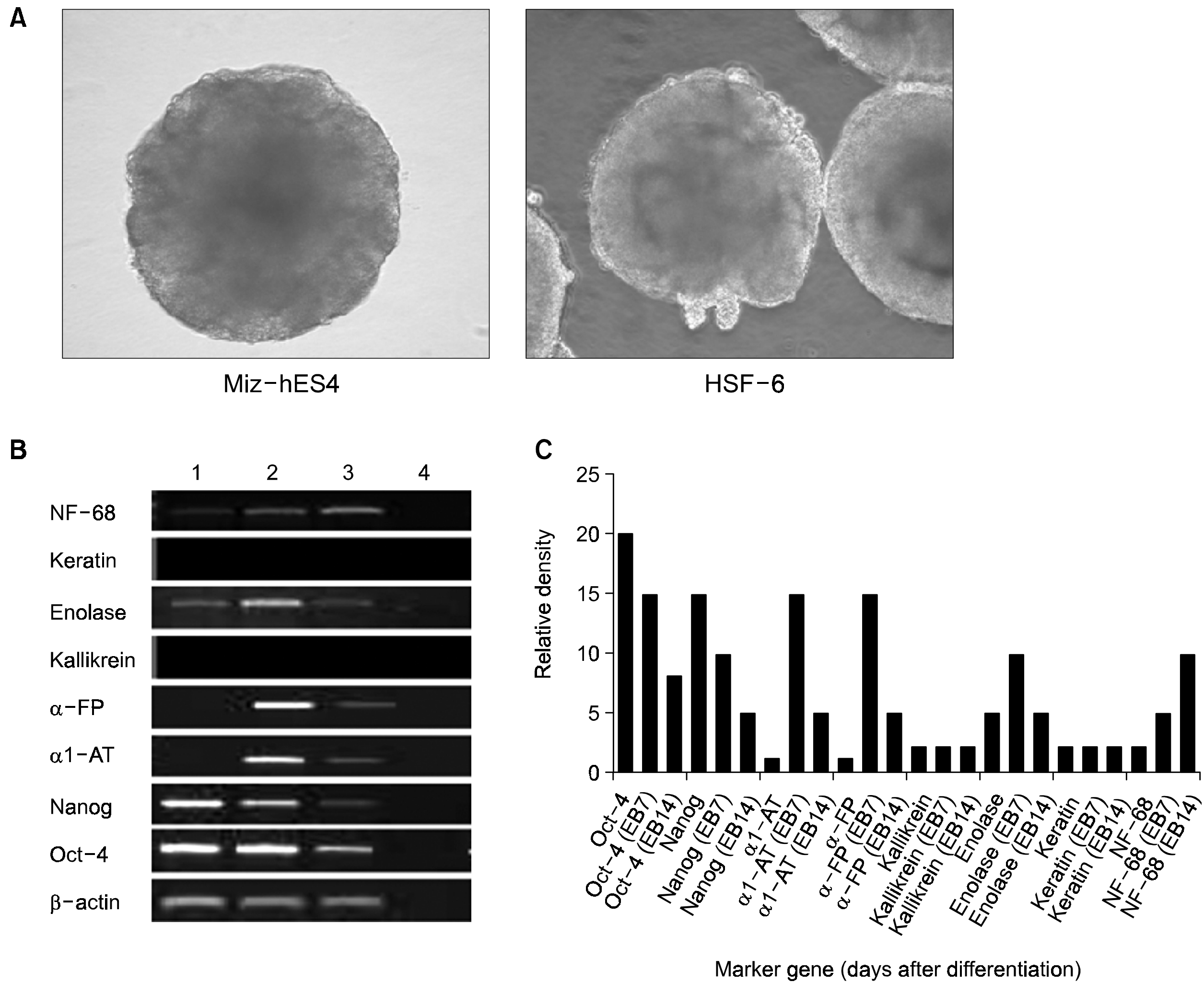

Figure 4. Ability of hESC lines grown on Diff$^{Miz\text{-}hES6}$ cells to differentiate into EBs. (A) Representative images of EBs formed from the Miz-hES4 (14-day-old EB) and HSF-6 (7-day-old EB) hESC lines. (B) Analysis of the undifferentiated hESCs and the EBs 7 and 14 days after their *in vitro* differentiation by RT-PCR using primers specific for three germ layers, namely, ectoderm (NF-68, Keratin), mesoderm (Enolase, Kallikrein) and endoderm (α-FP, α1-AT). Oct-4 and Nanog served as undifferentiated hESC-specific markers. β-actin served as a normalization control. The gel shows representative results from HSF-6 cells. Lane 1, undifferentiated hESCs (HSF-6); Lane 2, EB day 7 (HSF-6); Lane 3, EB day 14 (HSF-6); Lane 4, RT (-) control. The graph depicts these data after band quantification.

with pathogens responsible for newly emerging human diseases, which cannot be screened for yet, remains. All of these concerns constitute serious challenges for the clinical application of hESCs.

In the present study, we developed a simple method by which human feeder cells can be derived from hESCs. In this method, hESCs were continuously subcultured with DMEM/10% FSC until they had a fibroblast-like appearance (about 3-5 passages), which came to be predominant under this culture condition. During their subculture, the Diff$^{Miz\text{-}hES6}$ cells were cryopreserved. This led to sufficient Diff$^{Miz\text{-}hES6}$ cell numbers to meet the demand of long-term hESC culture. Moreover, the quality of the cells was relatively consistent, unlike feeders prepared from primary cultures or biopsies (data not shown). Since the use of hESC-derived feeder cells obviates the need to sacrifice other tissues to supply feeder cells, this method eliminates many of the potential problems associated with other feeder cells, including the ethical issues, the possible transmission of mouse pathogens, and variabilty of the culture system that hampers comparisons between results obtained by using different feeder cells.

Xu *et al.,* have recently reported that hTERT-immortalized fibroblast-like cells derived from hESCs (HEF1-hTERT) can support undifferentiated cell growth, while medium conditioned by HEF1-hTERT cells permit the feeder-free culture of hESCs (Xu *et al.,* 2004). However, hESCs cultured with HEF1-

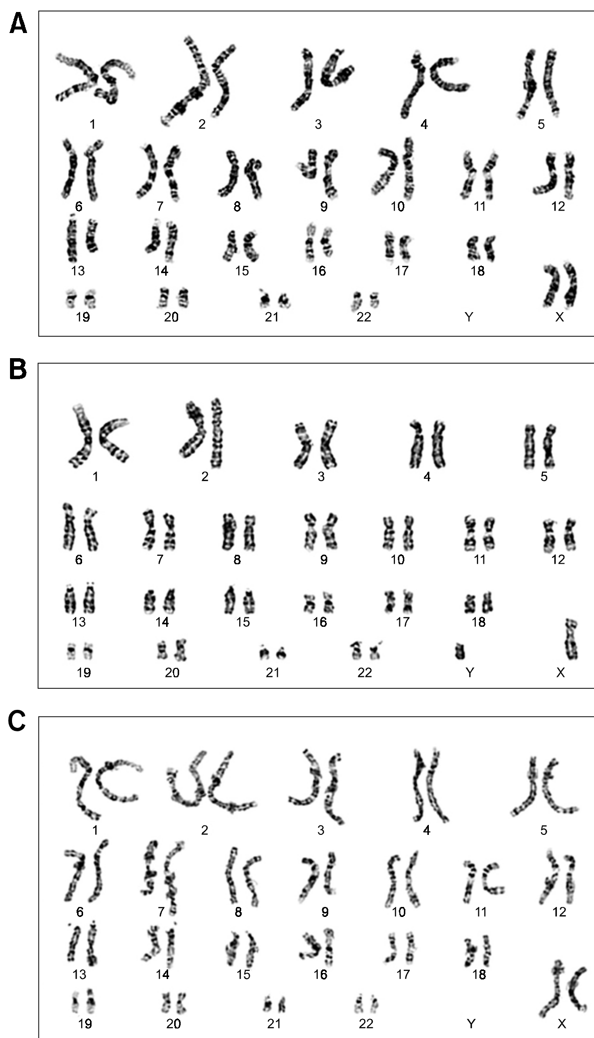

Figure 5. Karyotype analysis of the Diff$^{\text{Miz-hES6}}$ feeder cells and two hESC lines grown on Diff$^{\text{Miz-hES6}}$ feeders. (A) Diff$^{\text{Miz-hES6}}$ feeder cells (44 + XX). (B) HSF-6 (44 + XX). (C) Miz-hES4 (44 + XY).

hTERT feeder cells may have a higher likelihood of carcinogenesis due to the use of hTERT to immortalize the feeders. Consequently, Diff$^{\text{Miz-hES6}}$ feeder cells are safer for use in future cell replacement therapies.

We found that existing hESCs originally established on MEFs were easily adapted to culture on Diff$^{\text{Miz-hES6}}$ feeder cells. We also showed that these hESC-derived fibroblast-like Diff$^{\text{Miz-hES6}}$ feeder cells support the prolonged, undifferentiated maintenance of hESCs as efficiently as MEFs. Support of hESC growth was demonstrated by assessing the morphology of the cells and by comprehensive analyses of hESC marker expression. We found that all three hESC lines we tested could be cultured on Diff$^{\text{Miz-hES6}}$ feeder cells for at least 50 passages without any loss of hESC-specific marker expression (Oct-4, Nanog, Rex-1, Sox-2, TERT, SSEA-1, SSEA-4 and Tra-1-60). In addition, the three hESC lines differentiated successfully into EBs that had all three germ layers. Furthermore, the karyotypes of the Diff$^{\text{Miz-hES6}}$ feeder cells and the hESCs cultured on Diff$^{\text{Miz-hES6}}$ feeder cells remained normal after long-term culture (over 50 passages). To avoid the possibility of feeder cell contamination during the preparation of the undifferentiated hESCs and EBs for the RT-PCR analyses described above, we performed RT-PCR using primers specific for a fibroblast marker (prolyl 4-hydroxylase β); fibroblast marker expression was never detected (data not shown). We have found previously that hESCs cultured on MEFs have a circular shape, whereas hESCs cultured on human feeder cells are relatively irregular in shape (Richards *et al.*, 2002; 2003; Amit *et al.*, 2003a; Cheng *et al.*, 2003; Hovatta *et al.*, 2003; Lee *et al.*, 2005). The reason for this difference is unclear. In this study, however, we found that, like hESCs cultured on MEFs, hESCs cultured on Diff$^{\text{Miz-hES6}}$ feeder cells were circular in shape and showed a prominent nucleus and nucleoli. The circular shape of hESCs cultured on Diff$^{\text{Miz-hES6}}$ feeder cells, along with their equivalent passaging interval relative to hESCs cultured on MEFs (about 5 days), implies that the interaction between hESCs and the Diff$^{\text{Miz-hES6}}$ feeder cells is similar and as efficient as it is with MEF feeder cells.

Our observations show the feasibility of developing a hESC culture system using autologous human feeder cells with minimal exposure to animal materials, which is a highly desirable system with regard to the application of cell replacement therapy. Our system certainly eliminates the risk of contamination with mouse pathogens and unscreened pathogens responsible for newly emerging human diseases. However, our system did use FBS to culture the Diff$^{\text{Miz-hES6}}$ feeder cells. For complete xeno-free culture of hESCs, hESCs should be cultured at all times without any animal materials at all, including animal sera (FBS) and animal feeder cells. To date, while several other hESC culture systems using human feeder cells or conditioned medium with animal serum have been reported, animal serum (FBS)-free culture systems have not been reported. Therefore, we believe that our present hESC culture system employing hESC-derived fibroblast-like feeder cells represents substantial progress with regard to developing the complete xeno-free culture system that would greatly enhance the safety of cell replacement therapy. Further investigations to develop this complete xeno-free hESC culture system are warranted.

In conclusion, we developed a method by which autologous hESC-derived Diff$^{\text{Miz-hES6}}$ feeder cells can

be obtained and used to successfully maintain hESC lines in an undifferentiated state. All three of the hESC lines we cultured on Diff$^{Miz\text{-}hES6}$ feeder cells proliferated efficiently, maintained their undifferentiated state, and retained their pluripotency. Even after long-term culture on Diff$^{Miz\text{-}hES6}$ feeder cells, the feeder cells and the hESCs cultured on these feeders had normal karyotypes. Therefore, we suggest that hESC-derived fibroblast-like Diff$^{Miz\text{-}hES6}$ feeder cells could be an efficient alternative for previously reported human feeder cells and could be useful as autologous feeders to produce stem cells for clinical applications (Heng *et al.,* 2004). However, further investigation is needed to assess whether the hESCs cultured on Diff$^{Miz\text{-}hES6}$ feeder cells maintain their pluripotency *in vivo* with regard to teratoma formation. In addition, further development of this autologous culture system for hESC expansion such that it proceeds without the use of animal serum at any stage is highly desirable, as this complete xeno-free system would promote the clinical applications of cell replacement therapy.

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