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Feeder Layer- and Serum-Free Culture of Human Embryonic Stem Cells[1]

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ABSTRACT

In addition to their contribution to the research on early human development, human embryonic stem (hES) cells may also be used for cell-based therapies. Traditionally, these cells have been cultured on mouse embryonic fibroblast feeder layers, which allow their continuous growth in an undifferentiated state. However, the use of hES cells in human therapy requires an animal-free culture system, in which exposure to mouse retroviruses is avoided. In this study we present a novel feeder layer-free culture system for hES cells, based on medium supplemented with 15% serum replacement, a combination of growth factors including transforming growth factor β1 (TGFβ1), leukemia inhibitory factor, basic fibroblast growth factor, and fibronectin matrix. Human ES cells grown in these conditions maintain all ES cell features after prolonged culture, including the developmental potential to differentiate into representative tissues of the three embryonic germ layers, unlimited and undifferentiated proliferative ability, and maintenance of normal karyotype. The culture system presented here has two major advantages: 1) application of a well-defined culture system for hES cells and 2) reduced exposure of hES cells to animal pathogens. The feeder layer-free culture system reported here aims at facilitating research practices and providing a safer alternative for future clinical applications of hES cells.

cytokines, developmental biology, early development, embryo, growth factors

INTRODUCTION

In recent years, extensive investigation into improving culture systems for hES cells has yielded three main advances: 1) the ability to grow cells under serum-free conditions [1]; 2) the maintenance of the cells in an undifferentiated state on Matrigel matrix with 100% mouse embryonic fibroblast (MEF)-conditioned medium [2]; and 3) the use of either human embryonic fibroblasts, adult Fallopian tube epithelium [3], or foreskin fibroblasts [4] as feeder layers.

In spite of this progress, several significant disadvantages still exist. Exposure to animal pathogens through MEF-conditioned medium or Matrigel matrix is still a possibility; human feeder layer-based culture systems still require the simultaneous growth of both feeder layers and hES cells; and the culture system cannot be accurately defined due to differences between the various feeder layer lines or the use of conditioned medium. To ensure a feeder layer-free environment for the growth of hES cells, we present a substitute system based on medium supplemented with 20% serum replacement (SR), transforming growth factor β1 (TGFβ1), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and a fibronectin matrix.

Human ES cells have previously been shown to maintain all ES cell characteristics when cultured in medium supplemented with SR and bFGF, which provide a serum-free environment [1]. In this work, two other candidates tested were LIF, which is known to support self-maintenance of mouse ES cells [5] but has no effect on preventing hES cell differentiation [6, 7], and TGFβ1, which is a member of the TGFβ superfamily, and which in the hematopoietic system is known to have either a positive or a negative effect on cellular proliferation, differentiation, or death, depending on the culture environment or stage of development of the cells [8]. Furthermore, TGFβ1 is found in Matrigel matrix, which supports hES cell growth in feeder layer-free conditions [2]. Schuldiner and colleagues reported a comparative study in which the effects of eight different growth factors were evaluated by cell-specific gene expression [9]. In that study, TGFβ1 was assumed to repress cell differentiation because it led to the production of relatively reduced cell-specific gene expression [9].

Fibronectin, a basal lamina component, is often used to increase cell adhesion to the culture dishes, and is effective in differentiation systems of hES cells [9]. It acts through the integrin receptors, which are important mediators of cell adhesion to extracellular matrix proteins. The integrins trigger a variety of intracellular signal transduction cascades, which in turn modulate cell features, such as proliferation, apoptosis, shape, polarity, motility, gene expression, and differentiation [10].

In the present study, the ability of a serum-free medium supplemented with LIF, TGFβ1, and bFGF to support prolonged undifferentiated culture of hES cells was examined.

MATERIALS AND METHODS

ES Cell Culture

Human ES cell lines I-6, I-3 [11], and H-9 [6] were cultured with MEF for 46, 39, and 25 passages respectively, and were then transferred to 50 μg per 10 cm^2 fibronectin-covered plates. Three types of fibronectin were examined: bovine fibronectin, human plasma fibronectin, and human cellular fibronectin (Biological Industries, Beit Haemek, Israel, or Sigma-Aldrich, St. Louis, MO). The cells were grown on MEFs in a culture medium consisting of 85% Knockout Dulbecco modified Eagle medium (Ko-DMEM) supplemented with 15% SR, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acid stock, and 4 ng/ml bFGF (all from Gibco Invitrogen Corporation, Grand Island, NY). When transferred into the feeder layer-free culture system, six combinations of growth factors were tested: 0.12 ng/ml TGFβ1 without bFGF (T) (R&D Systems, Minneapolis, MN), TGFβ1 with 4 ng/ml bFGF (TF), 1000 u/ml LIF with

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TABLE 1. PCR reaction conditions.

Gene product	Forward (F) and reverse (R) primers (5′→3′)	Reaction condition	Size (base pairs)
Oct-4	F: GAGAACAATGAGAACCTTCAGGA R: TTCTGGCGCCGGTTACAGAACCA	30 cycles at 60°C in 1.5 mM $MgCl_2$	219
Albumin	F: TGCTTGAATGTGCTGATGACAGGG R: AAGGCAAGTCAGCAGCCATCTCAT	35 cycles at 60°C in 1.5 mM $MgCl_2$	302
α-fetoprotein	F: GCTGGATTGTCTGCAGGATGGGGAA R: TCCCCTGAAGAAAATTGGTTAAAAT	30 cycles at 60°C in 1.5 mM $MgCl_2$	216
NF-68KD	F: GAGTGAAATGGCACGATACCTA R: TTTCCTCTCCTTCTTCACCTTC	30 cycles at 60°C in 2 mM $MgCl_2$	473
α-cardiac actin	F: GGAGTTATGGTGGGTATGGGTC R: AGTGGTGACAAAGGAGTAGCCA	35 cycles at 65°C in 2 mM $MgCl_2$	486
LIF receptor	F: CAAAAGAGTGTCTGTGAG R: CCATGTATTTACATTGGC	35 cycles at 61°C in 1.5 mM $MgCl_2$	459
β-actin	F: ATCTGGCACCACACCTTCTACAATGAGCTGCG R: CGTCATACTCCTGCTTGCTGATCCACATCTGC	35 cycles at 62°C in 1.5 mM $MgCl_2$	838

bFGF (LF) (Esgro, Minneapolis, MN; R&D Systems; and rhLIF, Chemicon International, Temecula, CA), TGFβ1, LIF, and bFGF (TLF), TGFβ1 and LIF without bFGF (TL), or bFGF only (F). Cells were passaged every 4 to 6 days using 1 mg/ml type IV collagenase (Gibco Invitrogen Corporation). According to the freezing protocol, cells were then frozen in liquid nitrogen using a freezing solution consisting of 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich), 15% SR (Chemicon International), and 75% Ko-DMEM (Gibco Invitrogen Corporation).

Immunohistochemistry

Human ES cells grown in the feeder layer-free culture system and cells dissociated using trypsin-EDTA from 14-day-old embryoid bodies (EBs) were fixed with 4% paraformaldehyde and exposed to the primary antibodies (1:50) overnight at 4°C. Cys 3 conjugated antibodies (Chemicon International) were used as secondary antibodies (1:100); stage-specific embryonic antigen (SSEA) 1, 3, and 4 (Hybridoma Bank, Iowa City, IA), and tumor recognition antigen (TRA) 1-60 and TRA1-81, which were kindly provided by Professor P. Andrews of the University of Sheffield, UK, were used as primary antibodies. Additional primary antibodies were of antitubulin beta II isoform, smooth muscle actin (both from Chemicon International), CD31 (DakoCytomation, Glostrup, Denmark), and anti-$\alpha_5\beta_1$ integrin (1:100; Convance, Berkeley, CA).

Karyotype Analysis

Karyotype analysis (G-banding) was performed on at least 20 cells from each sample as previously described [4]. Karyotypes were analyzed and reported according to the International System for Human Cytogenetic Nomenclature.

Differentiation Experiments

Undifferentiated colonies of hES cell lines I-3, I-6, and H-9 were divided mechanically into 6–10 parts and cultured with fibronectin using the six different culture media, i.e., TLF, TF, LF, T, TL, F, and with MEFs as control. Two groups consisting of TLF and TF medium (from each line) were cultured with gelatin. Each experiment consisted of 10 pieces of ES cell colonies per well with four wells per line. The colonies were examined for morphology-based differentiation every second day. Three separate experiments were conducted.

Growth Curves

Cells from the three ES cell lines were passaged using 1 mg/ml type IV collagenase (Gibco Invitrogen Corporation), and then mechanically dissociated into single cells using Gilson pipette 200-μL tips. One hundred thousand cells per well were plated on fibronectin using culture medium T, F, TLF, or TF. Cells cultured on MEFs served as a control. Cells from three separate wells were harvested every second day, trypsinized (trypsin-EDTA, Gibco Invitrogen Corporation) and counted. Three separate experiments were performed.

Analysis of Cloning Efficiency

The experiments were conducted similarly to the cloning efficiency experiments described earlier [1]. In brief, hES cells from the three ES cell lines were dissociated into single cells as described for the growth curve experiments. 10000 cells per well were plated on fibronectin using TLF or TF. Cells were also plated on MEF as a control. The resulting colonies were counted 6 days after plating. The experiment was repeated three times.

EB Formation

For the formation of EBs, four to six confluent wells were used in a six-well plate (40–60 cm^2). ES cells were removed from their culture dish using 1 mg/ml type IV collagenase, further broken into small clumps using 1000-μL Gilson pipette tips, and cultured in suspension in 58-mm petri dishes (Greiner, Frickenhausen, Germany). EBs were grown in medium consisting of 80% Ko-DMEM, supplemented with 20% defined fetal bovine serum (FBSd; HyClone, Logan, UT), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acid stock (all but FBSd are from Gibco Invitrogen Corporation).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from cells grown for 17–25 passages in feeder layer-free conditions as undifferentiated cells, or from 14-day-old EBs created from cells grown in the feeder layer-free system using Tri-Reagent (Sigma-Aldrich), according to the manufacturer's recommended protocol. Complementary DNA was synthesized from 1 μg total RNA using MMLV reverse transcriptase RNase H minus (Promega, Madison, WI). Polymerase chain reaction (PCR) included denaturation for 5 min at 94°C followed by repeated cycles of 94°C for 30 sec, annealing temperature (as in Table 1) for 30 sec, and extension at 72°C for 30 sec. PCR primers and reaction conditions used are described in Table 1. PCR products were size-fractionated using 2% agarose gel electrophoresis. DNA markers were used to confirm the size of the resultant fragments.

Teratoma Formation

Cells from six confluent wells in a six-well plate (60 cm^2) were harvested and injected into the hindlimb muscle of 4-wk-old male SCID-beige mice. Four mice were injected, representing the two types of media, TF and TLF, following at least 19 passages of continuous culture in the feeder layer-free culture system. All four mice formed teratomas. Twelve weeks postinjection, the resultant teratomas were fixed in 10% neutral-buffered formalin, dehydrated in graduated alcohols (70%–100%) and embedded in paraffin. For histological examination, 5-μm sections were deparaffinized and stained with hematoxylin-eosin. All animal experiments were conducted in accordance with the Guide for the Care and Use of Animals for research purposes, and were approved by the Technion animal ethics committee.

RESULTS

Several possible combinations of growth factors were tested for their ability to support the maintenance of undifferentiated hES cells. Initially, two measures were used to estimate the ability of these cells to grow in the feeder layer-free culture system: percentage of differentiation and

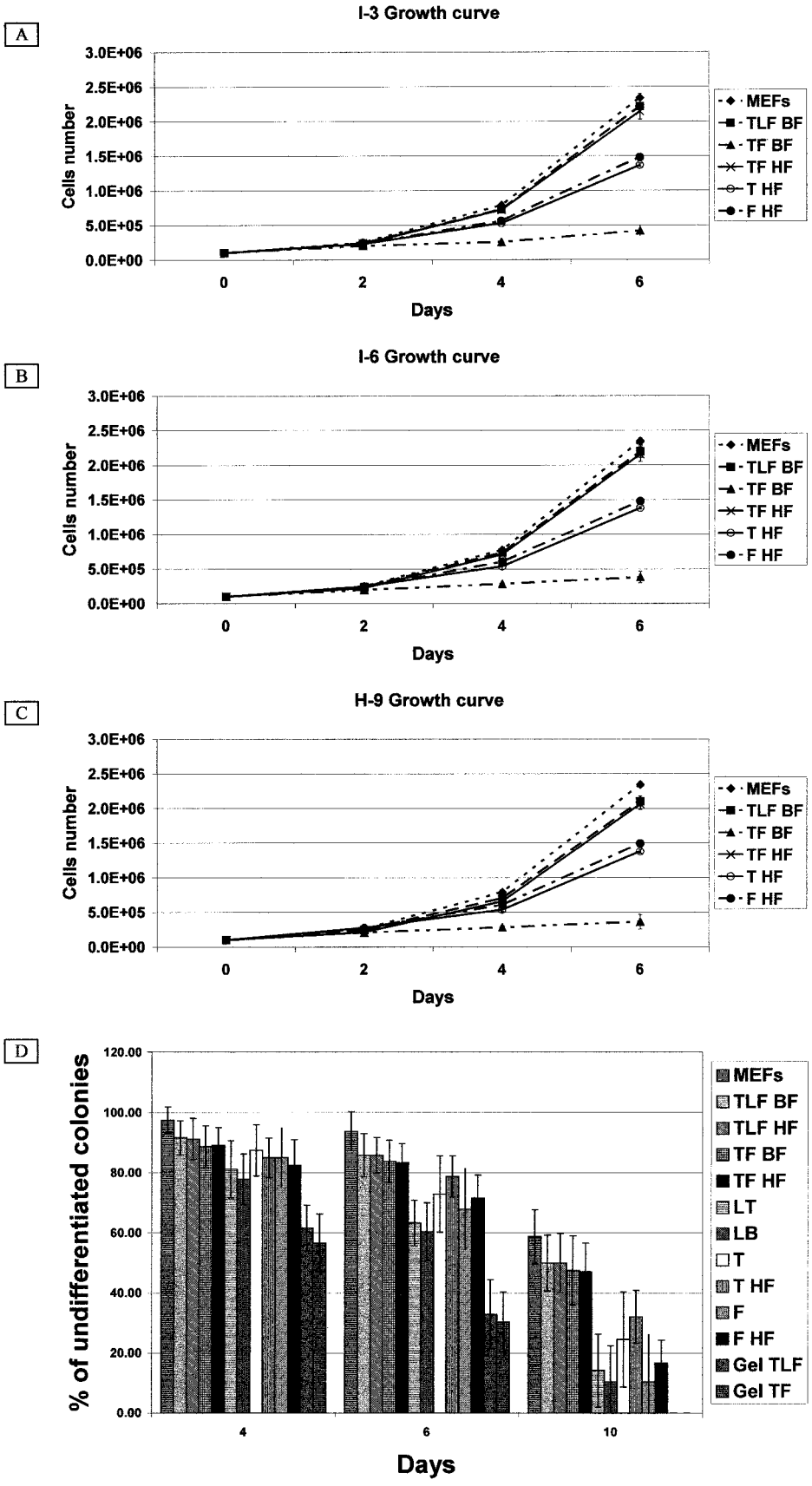

FIG. 1. Growth curves of I-3 (**A**), I-6 (**B**), and H-9 (**C**). **D**) Percentage of undifferentiated colonies in the various medium supplements during continuous culture of hES cells. The histogram summarizes the results from lines I-3, I-6, and H-9. BF, Bovine fibronectin; HF, human fibronectin; Gel, gelatin. The results are presented as mean ± SD.

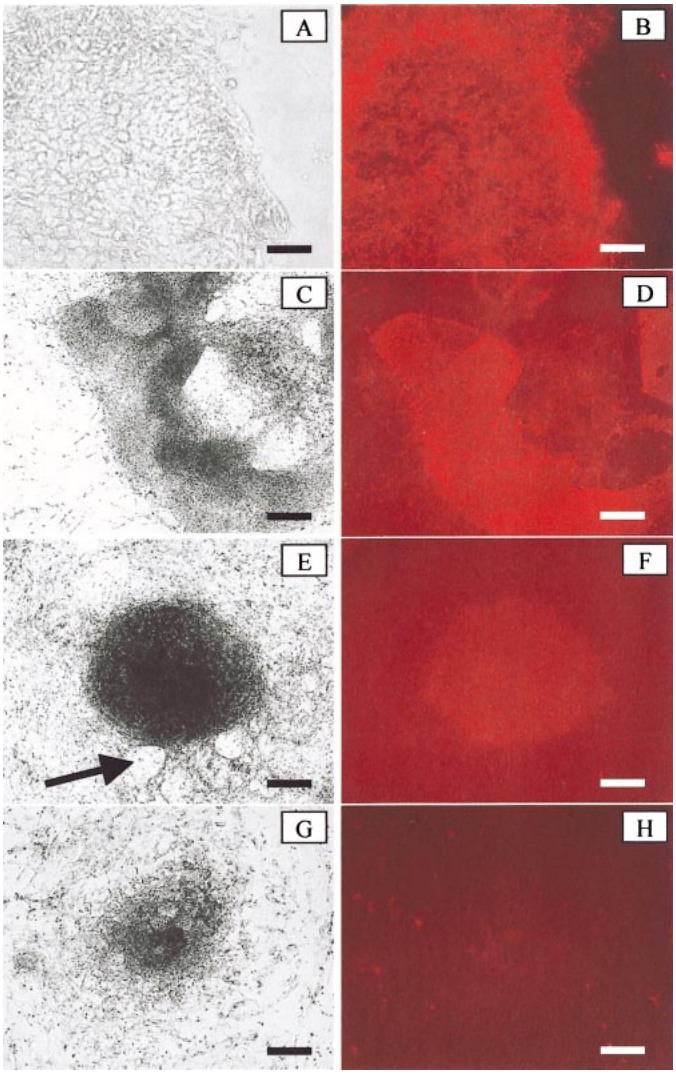

FIG. 2. Example of differentiated and undifferentiated colonies on day 10. **A** and **B**) I-3 undifferentiated colony cultured in TF with fibronectin stained with SSEA4 using light microscopy (**A**) and fluorescent microscopy (**B**). **C** and **D**) Partially differentiated I-3 colony cultured in F with MEFs stained with TRA-1-81 using light microscopy (**C**) and fluorescent microscopy (**D**). **E** and **F**) Partially differentiated I-3 colony cultured in F with MEFs stained with SSEA4 using light microscopy (**E**) and fluorescent microscopy (**F**). **G** and **H**) Differentiated I-6 colony cultured in TLF with fibronectin stained with TRA-1-81 using light microscopy (**G**) and fluorescent microscopy (**H**). Arrow indicates differentiating area. Bar = 50μm.

rate of growth. When grown in supplement F, LT, or LF, cells proliferated, but the differentiation rates were high. As can be seen in Figure 1D, after 6 days of culture, differentiation rates reached 35%–40% of the total culture, leading to a completely differentiated culture after several passages of culture in the feeder layer-free culture system. When grown in T, cells remained at the undifferentiated stage at high rates for more than 10 passages, but proliferated poorly, until no cells remained in the culture at passage 15. When cells were grown in human fibronectin, proliferation rates improved, but cells could not be cultured continuously to a higher passage (Fig. 1, A–C). All lines transferred to TF or TLF continued to proliferate and maintained normal hES cell features. As can be seen in Figure 1D, the differentiation rates in these supplements at Day 6 were less than 20%, which enabled the continuous culture

TABLE 2. Cloning efficiency experiments data.*

Cell line	MEFs	TLF BF	TF BF	TF HF
I-3	0.86 ± 0.06	0.76 ± 0.07	0.32 ± 0.07	0.8 ± 0.11
I-6	0.84 ± 0.06	0.76 ± 0.07	0.32 ± 0.04	0.74 ± 0.04
H-9	0.9 ± 0.08	0.76 ± 0.09	0.29 ± 0.06	0.75 ± 0.08

* Each row represents a diferent cell line. The numbers are the percentages of resulting undifferentiated colonies ± SD in the different medium supplements tested. BF, Bovine fibronectin; HF, human fibronectin.

of the cells. The cells' growth rates in TLF were similar to those seen when cells were cultured on MEFs (Fig. 1, A–C). While using TF, however, differences were found in the growth rates depending on the type of fibronectin used. When cultured with human fibronectin and TF, growth rates resembled the ones obtained when MEFs were used, but use of bovine fibronectin resulted in poor proliferation (Fig. 1, A–C). The percentage of differentiation in the different culture conditions was examined every other day until all colonies were differentiated (16 days). As shown in Figure 1D for Day 10, all experiments revealed a lower differentiation ratio in TF and TLF compared with F, TL, T, and LF and a similar ratio to that shown with MEFs. When gelatin was used instead of fibronectin, the differentiation rates in TF and TLF increased to 70% on Day 6, and led to complete differentiation of the cells on Day 8. Examples of undifferentiated colonies and differentiated colonies from Day 10 of the experiment are demonstrated in Figure 2. Among the culture combinations tested, TF and TLF were found to be the most suitable for the culture of undifferentiated hES cells.

To examine the plating efficiency of the cells in those supplements which were found to support hES cell growth, their clonality was tested. The results are summarized in Table 2. One-way ANOVA using the post hoc Tukey honestly significant difference test revealed that all four supplement groups differed from one another significantly ($P < 0.0001$). As with the growth rates, the cloning efficiency obtained in TLF and TF on human fibronectin was somewhat lower but similar overall to the cloning efficiency on MEFs as demonstrated in the three cell lines tested; the cloning efficiency of lines cultured in TF on bovine fibronectin was dramatically lower than those cultured in MEFs and in the TLF or TF on human fibronectin.

No morphological differences could be observed between colonies grown in the feeder layer-free culture system and those grown on MEF, even after more than 50 passages (more than 200 days) in TLF and 47 passages (more than 188 days) in TF (Fig. 3, A–F). Correspondingly, morphological features remained unchanged on a single-cell level; cells were small and round and exhibited a high nucleus to cytoplasm ratio, with a notable presence of one to three nucleoli and typical spacing between the cells (Fig. 3A). Similarly to cells grown on MEFs, cells were passaged routinely every 4 to 6 days, at the same ratio of 1:2 or 1:3, indicating a similar population doubling time. The similar growth curves observed further support this assumption. The cells were passaged at the same seeding efficiency of about 1.5–2 million cells per 10 cm^2, with the same viability rate of over 90%. Using 15% SR and 10% DMSO, cells were successfully frozen and thawed.

Karyotype analysis by Giemsa banding was carried out on nine separate cultures, representing the two medium conditions, TF and TLF, and the three hES cell lines at

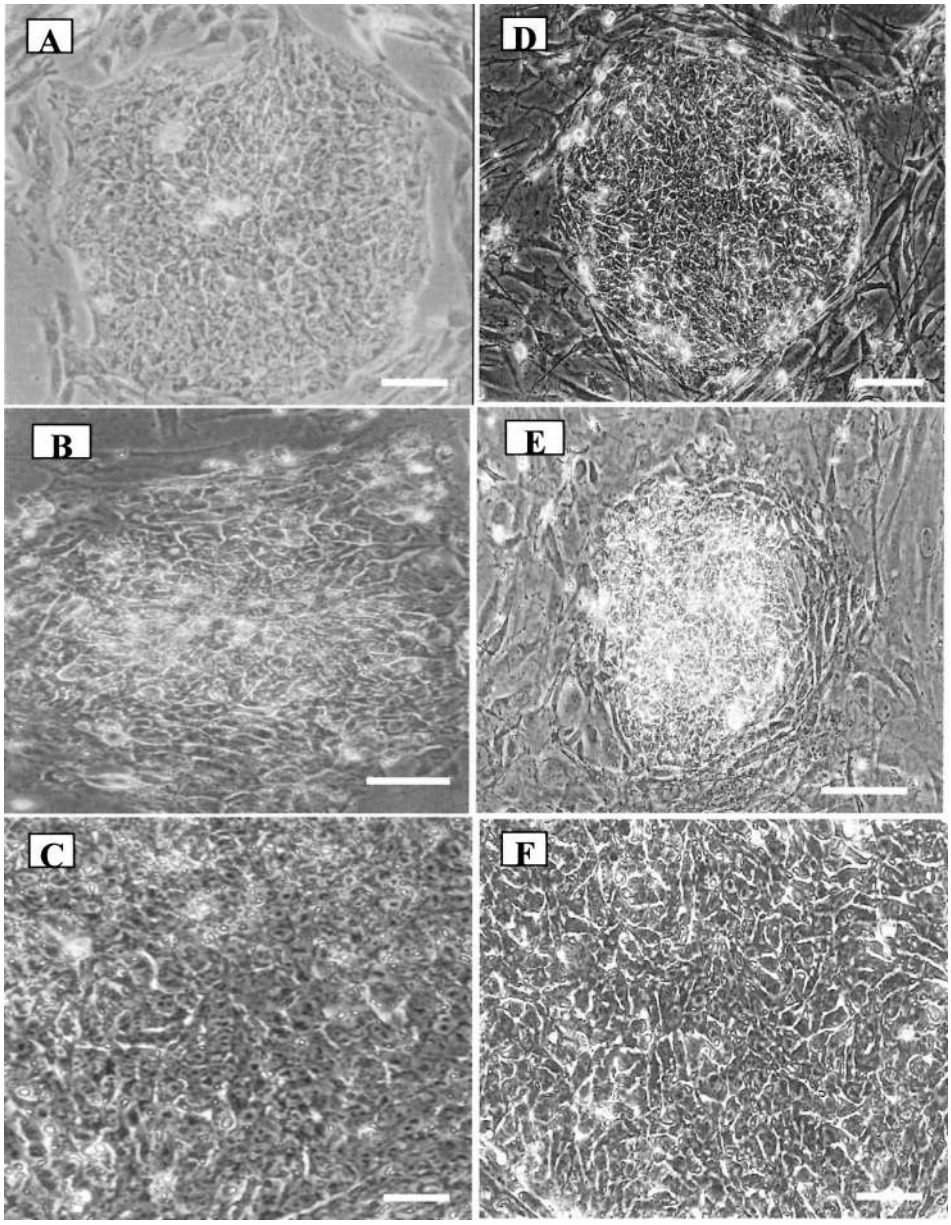

FIG. 3. Examples of the morphology of undifferentiated ES cell colonies and ES single cells grown in the feeder layer-free culture system (**A–C**). **A**) Colony I-6 grown in TLF for 31 passages. Bar = 100 μm. **B**) Colony I-3 grown in TF for 21 passages. Bar = 50 μm. **C**) Cells from cell line I-3 grown in TF for 20 passages. Bar = 38 μm. **D**) Colony from H-9 at passage 50 cultured on MEFs. Bar = 50 μm. **E**) Colony from I-3 at passage 92 cultured on MEFs. Bar = 50 μm. **F**) Cells from H-9 at passage 50. Bar = 38 μm. Note the spaces between the cells (**A** and **B**) and the high nucleus:cytoplasm ratio typical of hES cells (**C** and **F**).

different passages (from 6 to 32 passages in the feeder layer-free environment). Of all cells examined, in only one group were 40% of the cells found to express abnormal karyotype of 47,XXX. These four cells were of the group grown in TLF for 20 passages. The remaining cells demonstrated normal karyotype. A previous report on karyotype stability showed that 4 of 20 H-9 cells belonging to one of four sample groups exhibited abnormal karyotype after 8 mo of continuous growth on MEFs [1]. The abnormal karyotyped cells in the feeder layer-free culture system were at passage 71 postderivation (almost 1 yr of continuous culture), implying that karyotype changes represent a casual event which occurs in prolonged culture. Overall, these results suggest that the cells' karyotype remains stable in these conditions similarly to ES cells grown with MEFs.

Several surface markers typical of primate undifferentiated ES cells were examined using immunofluorescent staining [6, 12, 13]. Human ES cells cultured in TF and TLF for 17 and 38 passages respectively, were found to be strongly positive to surface markers SSEA4, TRA-1-60 and TRA-1-81 (Fig 4. A–C). As in other primate ES cells, staining with SSEA3 was weak and negative for SSEA1.

The developmental potential of the cells after prolonged culture in feeder layer-free conditions was examined in vitro by the formation of EBs. When cultured in suspension, after 22 to 30 passages in TLF and 28 passages in TF, hES cells formed EBs similar to those created by ES cells grown on MEFs (Fig. 5, A–C). Within these EBs, stem cells differentiated into cell types representative of the three embryonic germ layers [14]. In addition, cells harvested from these 2-wk-old EBs expressed ectoderm marker antitubulin beta II isoform and mesoderm markers smooth muscle actin and CD31, as demonstrated by immunostaining (Fig. 5, D–F).

While undifferentiated cells cultured on fibronectin using medium supplemented with TLF or TF expressed Oct4 (Fig. 6), cells harvested from 2-wk-old EBs expressed genes such as α-fetoprotein and albumin (endoderm), α-cardiac actin (mesoderm), and neurofilament (ectoderm) as demonstrated by reverse transcription (RT)-PCR (Fig. 6).

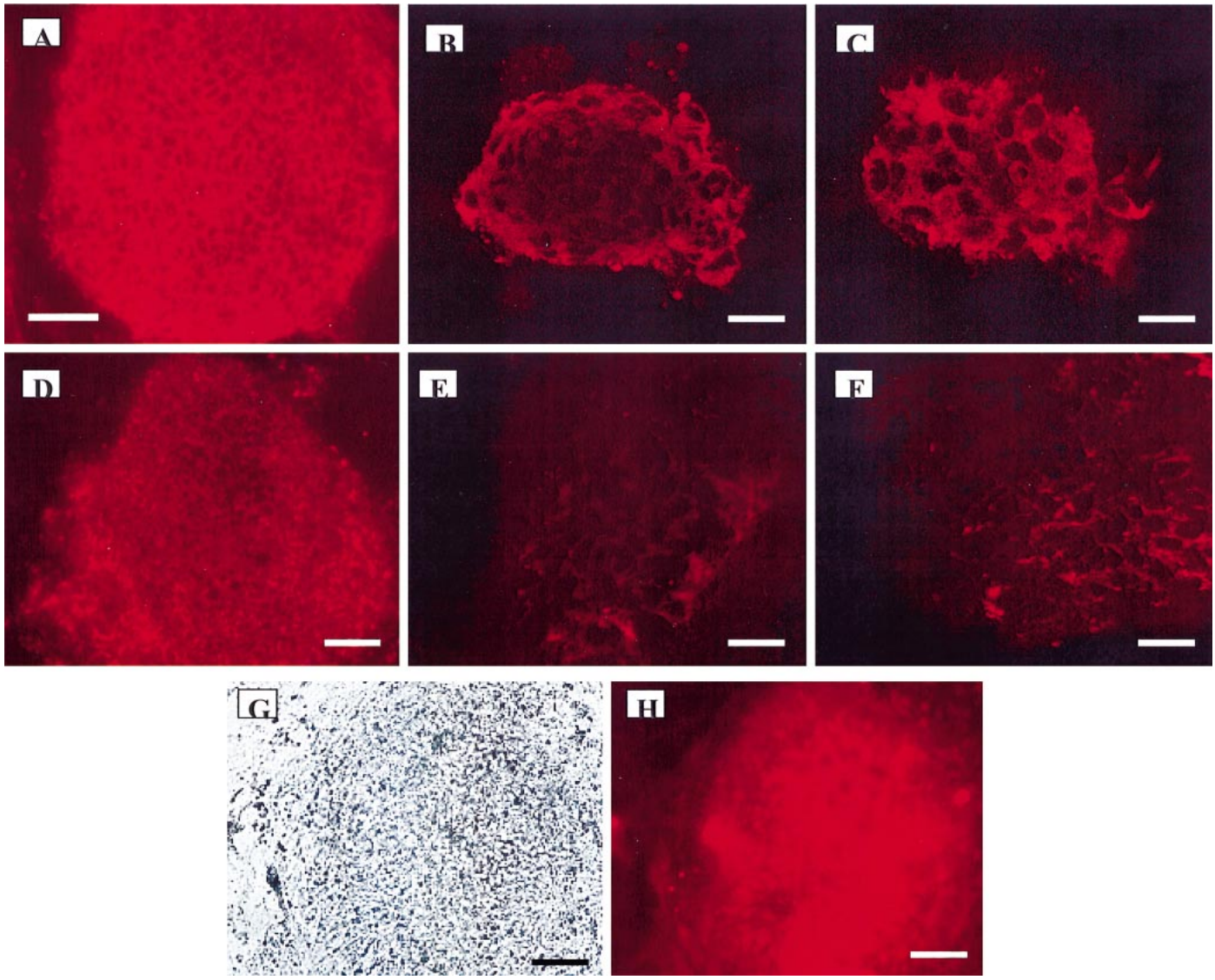

FIG. 4. Fluorescent immunostaining of hES cells grown in the feeder layer-free culture system with surface markers typical of undifferentiated cells (**A**–**C**). **A**) Immunostaining of colony I-3 grown in TF for 17 passages, with anti-SSEA4 antibodies. Bar = 50 μm. **B**) Immunostaining of colony I-3 grown in TLF for 38 passages, with anti-TRA-60 antibodies. Bar = 6 μm. **C**) Immunostaining of colony I-3 grown in TF for 21 passages, with anti-TRA-81 antibodies. Bar = 6 μm. **D**) Immunostaining of colony I-3 cultured with MEFs with SSEA4 antibodies. Bar = 50 μm. **E** and **F**) Colonies of H-9 cultured with MEFs stained with TRA-160 and TRA-181, respectively. Bar = 50 μm. **G** and **H**) Colony of H-9 cultured with MEFs stained with antihuman-$\alpha_5\beta_1$ receptor using light (**G**) and fluorescent microscopy **(H)**. Bar = 50 μm. **A**, **D**, **G**, and **H** were analyzed using an inverted fluorescent microscope; **B**, **C**, **E**, and **F** were analyzed using a confocal microscope.

The developmental potential of the cells was also examined in vivo. Following injection into a hindlimb muscle of SCID-beige mice, I-3 and I-6 cells cultured in TLF for 26 and 19 passages respectively, and I-3 cells cultured for 38 and 36 passages with TF and TLF respectively, formed teratomas. These teratomas contained tissues representative of the three embryonic germ layers, thus providing additional evidence of the pluripotency of the cells (Fig. 7).

DISCUSSION

Human ES cells, like mouse ES cells, are traditionally cultured with MEF, which may expose them to animal pathogens. We have demonstrated a defined serum- and feeder layer-free culture system based on the use of SR, TGFβ1, LIF, bFGF, and human or bovine fibronectin matrix as substitute.

Several possible combinations of growth factors were tested for their ability to maintain hES cells in an undifferentiated state. The most suitable ones were found to be TF or TLF, in which all transferred hES cell lines continued to proliferate while retaining normal hES cell features. Three different types of fibronectin were tested; bovine fibronectin, human plasma fibronectin, and human cellular fibronectin. Human fibronectin will be important for developing a xeno-free culture system, and bovine fibronectin will reduce the costs of research. All types of fibronectin tested were found suitable for the continuous culture of hES cells. On the human fibronectin, no difference could be detected between TF and TLF; on the bovine fibronectin, the TF combination was found inferior to the TLF with regard to rates of growth and cloning efficiency. This difference may be indicative of the role fibronectin plays in maintaining the proliferative ability of hES cells. The increased dif-

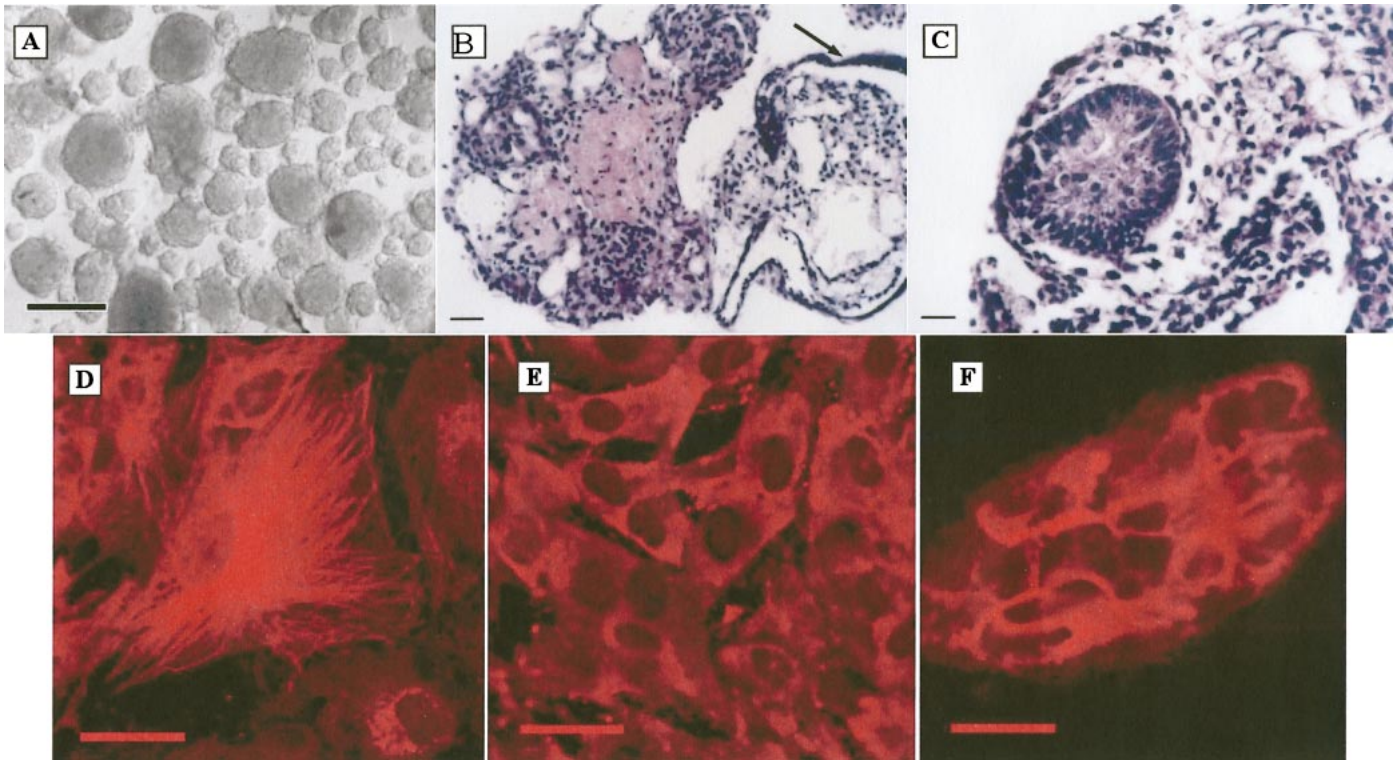

FIG. 5. In vitro differentiation of hES cells grown in the feeder layer-free culture system. **A**–**C**) Histological sections of 14-day-old EBs derived from cells grown in the feeder layer-free culture system. **A**) 24-h-old simple EBs derived from I-3 cell line after being grown for 28 passages in TF. Bar = 100 μm. **B**) Histological section of an EB derived from I-3 cell line grown for 28 passages in TF. Note the external protective epithelium (arrow) surrounding part of the EB (ectodermal origin). Bar = 50 μm. **C**) Histological section of EB derived from cell line I-3 grown for 30 passages in TLF. There is a ball-like structure consisting of columnar epithelium surrounded by mesenchymal tissue (mesodermal origin). Bar = 25 μm. **D**–**F**) Fluorescent immunostaining for representative markers of mesoderm and ectoderm in cells derived from 14-day-old EBs formed by cells grown in various media. **D**) Cells from line I-6 grown in TLF for 22 passages positively stained with neural specific tubulin (ectoderm). Bar = 6 μm. **E**) Cells from line I-3 grown in TLF for 30 passages positively stained with smooth muscle actin (mesoderm). Bar = 6 μm. **F**) Cells from line I-3 grown in TF for 28 passages positively stained with CD-31 (mesoderm). Bar = 6 μm.

ferentiation rates obtained by the use of gelatin rather than fibronectin further support this assumption.

All three parameters proved to be somewhat inferior to the traditional MEF culture, but this difference was marginal, i.e., growth rates and cloning efficiency were lower and the differentiation rates slightly higher, with each passage yielding lower numbers of undifferentiated hES cells compared with hES cells cultured on MEF. The cells proliferated in TLF and TF; the cloning efficiency was high enough to assure cell survival, and the differentiation rates were low enough to enable prolonged undifferentiated culture without the need for continuous removal of the differentiated colonies from the culture.

Cells cultured in these conditions maintained all the characteristics of ES cells. After prolonged culture of more than 50 passages the cells remained undifferentiated, as demonstrated by the colony and single cell morphology and by the expression of surface markers typical of undifferentiated primate ES cells [6, 7, 12, 13]. Karyotype analysis carried out on representative cell samples of the three lines tested, the three fibronectin types used, and the two medium combinations demonstrated that the hES cell karyotype remained stable overall in the proposed conditions.

The cells' pluripotency was examined both in vivo and in vitro. Cells cultured in TF and TLF for more than 19 passages formed teratomas containing tissues representative of the three embryonic germ layers. When cultured in suspension, after continuous culture in the feeder layer-free culture system, hES cells formed EBs similar to those created when grown on MEFs [14]. RT-PCR analysis and immunostaining demonstrated that cells within these EBs differentiated into different cell types representative of the three germ layers.

These results demonstrate that hES cells can be maintained as undifferentiated cells under defined feeder layer- and serum-free conditions while exhibiting hES cell features.

A recent report by Ramalho-Santos et al. described the core stem cell properties, named ''stemness,'' which underline both self-renewal and developmental potential of adult and embryonic stem cells [15]. Interestingly, the essential attributes of ''stemness'' include active JAK/STAT (LIF pathway), active TGFβ1, and interaction with the extracellular matrix via integrin receptors. This is consistent with our finding that these three attributes are sufficient for prolonged undifferentiated differentiation of hES cells. Additional support for these results was reported by Sato et al. who added ligand-receptor pairs and secreted inhibitor factors from the FGF and TGFβ1 pathways [16] to the essential factors involved in the state of ''stemness.'' The mechanism by which hES cells self-maintain is still not clear. Receptors for the three growth factors used in this report are expressed in hES cells as demonstrated by RT-PCR; FGF type I receptors were found to be highly expressed in undifferentiated hES cells, but TGFβ type II receptors were weakly expressed [9]. LIF receptors are also

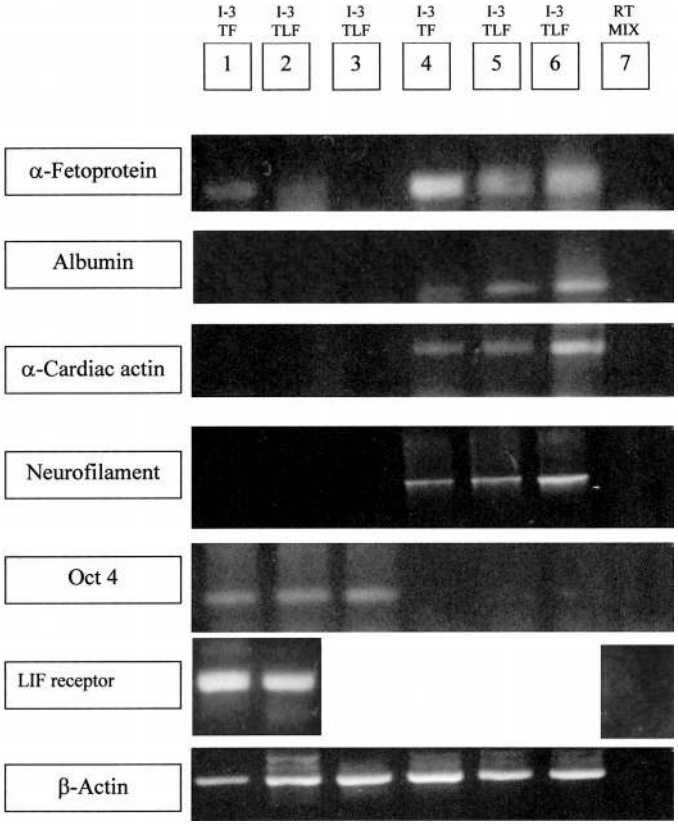

FIG. 6. RT-PCR analysis for the expression of Oct4 and representative genes of the three embryonic germ layers of cells grown on human fibronectin. Cell line I-3 grown in TF for 22 passages (1). Cell line I-3 grown in TLF for 18 passages (2). Cell line I-3 grown in TLF for 17 passages (3). 14-day-old EBs derived from I-3 cells grown in TF for 17 passages (4). 14-day-old EBs derived from I-3 cells grown in TLF for 17 passages (5). 14-day-old EBs derived from I-3 cells grown in TLF for 16 passages (6). Reaction mix as negative control (7).

highly expressed in undifferentiated hES cells (Fig. 6). The fibronectin-specific integrin receptor, $\alpha_5\beta_1$, was demonstrated by immunostaining on undifferentiated hES cells (Fig. 4). Further complementary research is required to explain the mechanism underlying the mechanisms of action of these growth factors at the level of signal transduction, and the possible role of fibronectin.

Future clinical uses of hES cells will require a reproducible, well-defined and xeno-free culture system. Although the SR used in the present study is considered such a system, it contains Albumax, which is a lipid-enriched bovine serum albumin, and therefore is not entirely animal free. The well-defined conditioned media demonstrated in the present study are suitable for culturing hES cells and may be advantageous for undertaking research on the mechanisms of embryonic stem cell self-maintenance, especially of the possible roles of the growth factors and integrin receptors used. Other studies using hES cells, such as research on differentiation pathways and mechanisms, will benefit from the availability of a well-defined and reproducible culture system.

The data presented here clearly show that hES cells grown in a feeder layer-free and serum-free environment maintain hES cell characteristics after prolonged culture (more than 6 mo as of today), thus providing well-defined culture conditions for both research purposes and future clinical applications of hES cells. This culture system is a further step forward toward completely defined culture conditions for hES cells, and will promote further development of a xeno-free culture system for hES cells.

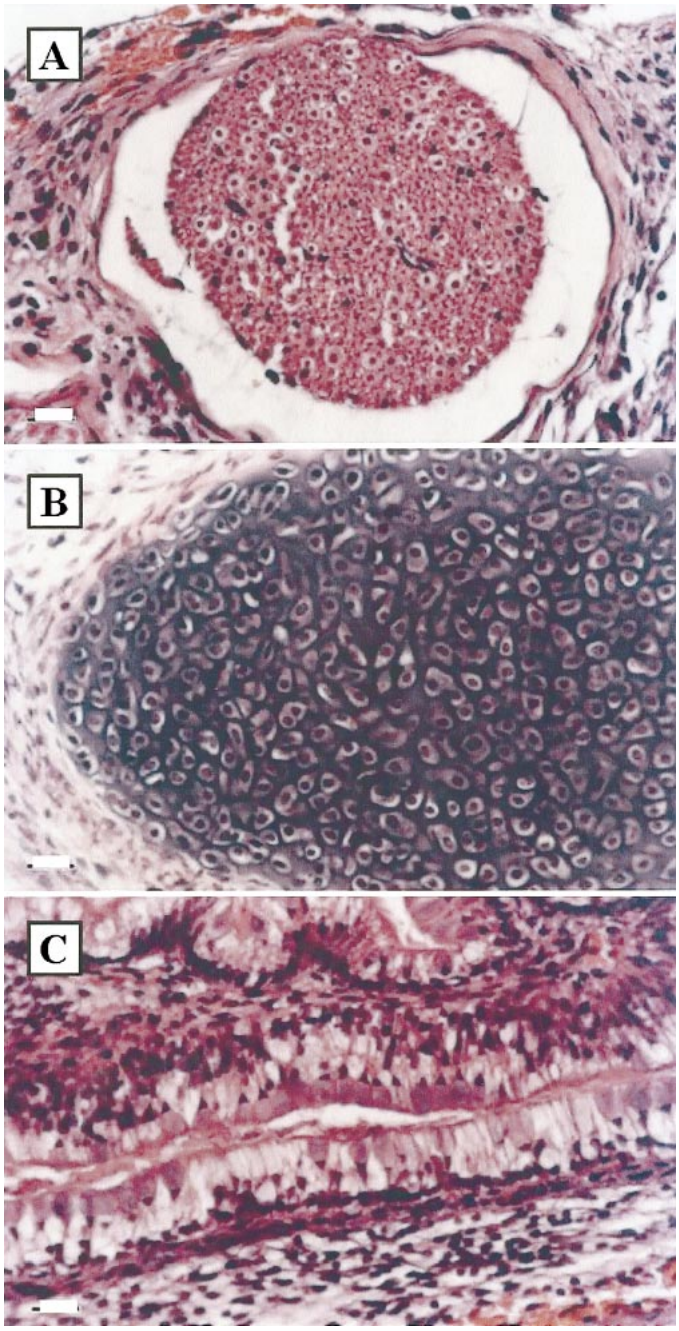

FIG. 7. In vivo differentiation of ES cell lines I-3 and I-6 grown for 26 and 19 passages respectively in TLF in teratomas. **A**) Myelinated nerve (ectoderm). **B**) Details of hyaline cartilage (mesoderm). **C**) Secretory epithelium rich in goblet cells (endoderm). Bar = 25 μm.

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