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Leukemia Inhibitory Factor-Expressing Human Embryonic Lung Fibroblasts as Feeder Cells for Human Embryonic Germ Cells

Fangfei Li[a] Yonggang Liu[a] Dilong Chen[a] Xuemei Lin[a] Jing Li[a]
Jianwei Wang[a] Yan Peng[a] Shali Wang[b] Yaping Wang[a]

Departments of [a]Histology and Embryology and [b]Physiology, Chongqing Medical University, Chongqing, People's Republic of China

Key Words
Feeder layer · Human embryonic germ cells · Human embryonic stem cells · Leukemia inhibitory factor · Primordial germ cells

Abstract
A robust culture system is critical for maintaining both proliferation and the developmental potential of human embryonic germ (hEG) cells. Here, we use human embryonic lung fibroblasts (hELF) overexpressing leukemia inhibitory factor (LIF) as feeder cells to support the self-renewal of hEG cells. We examine the morphology, gene expression, and developmental potential of hEG cells grown on a feeder layer of LIF-expressing hELF (hELF/lif) cells. hEG cells were positive for alkaline phosphatase (AP), stage-specific embryonic antigen (SSEA)-1, SSEA-4, tumor rejection antigen (TRA)-1–60, and TRA-1–81. In addition, hEG cells maintained on hELF/lif expressed higher levels of pluripotency genes such as Oct4 and Nanog. In addition, hEG cells maintained on hELF/lif cells gave rise to differentiated tissues when grown as embryoid bodies, consistent with the broad developmental potential of the starting population. Our results suggest that a hELF/lif feeder layer can support the proliferation of hEG cells, and that LIF signaling plays an essential role in this process. This human-derived culture system provides an attractive alternative to more commonly used mouse-derived feeder layers for use in clinical applications.

Abbreviations used in this paper

AP	alkaline phosphatase
DMEM	Dulbecco's modified Eagle's medium
EBs	embryoid bodies
FBS	fetal bovine serum
FGF	fibroblast growth factor
hEG	human embryonic germ
hELF	human embryonic lung fibroblasts
hES	human embryonic stem
LIF	leukemia inhibitory factor
PFA	paraformaldehyde
PGCs	primordial germ cells
SSEA	stage-specific embryonic antigen
TRA	tumor rejection antigen

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Prof. Yaping Wang
Department of Histology and Embryology
Chongqing Medical University, 1 Yi Xue Yuan Road
Chongqing 400016 (People's Republic of China)
Tel./Fax +86 23 6848 5968, E-Mail ypwang.cmu@163.com

Introduction

Primordial germ cells (PGCs) are the embryonic precursors of male and female gametes. Similarity to the cells of the inner cell mass can lead to the generation of embryonic stem (ES) cells. PGCs can also give rise to pluripotent stem cells, termed embryonic germ (EG) cells. A human EG (hEG) cell line was first established by Gearhart and his colleagues in 1998 [Shamblott et al., 1998]. EG cells demonstrate unlimited proliferation on mouse STO fibroblast feeder layers in the presence of human recombinant leukemia inhibitory factor (hrLIF), human recombinant basic fibroblast growth factor (hrbFGF), and forskolin [Shamblott et al., 1998]. Importantly, EG cells exhibit key stem cell characteristics, including self-renewal, expression of stem cell markers, and high levels of telomerase activity. Although reports on hEG cells remain limited from 1998 to now, plenty of human ES (hES) cell lines have been derived [Wilmut et al., 2005]. hEG cells share significant similarities with hES cells [Thomson et al., 1998], and thus promise significant therapeutic potential. A suitable and clinically safe culture system for maintaining hEG cells would thus significantly advance the field of stem cells and regenerative medicine.

Several factors are critically required for successful derivation and maintenance of self-renewing, undifferentiated hEG cells, including stem cell factor, LIF, and FGF [Turnpenny et al., 2006]. hEG cells quickly differentiate and cease proliferation upon withdrawal of hrLIF or hrbFGF [Shamblott et al., 1998]. In addition, a feeder layer, usually mouse STO fibroblasts or mouse embryonic fibroblasts, is required to maintain hEG cells in the undifferentiated, self-renewing state. However, the use of animal feeder cells presents certain obstacles for therapeutic applications, such as the risk of pathogen transmission and viral infection [Holden, 2005]. Although several human-derived feeder layers have been tested for their ability to support growth of human PGCs in culture, none have provided results comparable to STO cells [Shamblott et al., 1998].

Here we examine the use human embryonic lung fibroblasts (hELF) overexpressing LIF as an alternative feeder layer to support the growth of undifferentiated, pluripotent hEG cells.

Materials and Methods

Isolation and Culture of hELF

Human embryonic lung tissues were obtained from 9-week-old fetuses following termination of pregnancy. Lung tissues were minced and enzymatically digested with 0.25% trypsin (Hyclone) and 10 U/ml DNase I (Sigma) for 5 min. Tissues were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) with 10% fetal bovine serum (FBS, Hyclone) and were passaged every 3 days.

Vector Construction and Transfection

A cDNA encoding human LIF was PCR amplified from human gravid endometrium and cloned into the pcdna3.1 expression vector (pcdna3.1/lif). hELF cells were cultured in 6-well plates until they reached 85–90% confluence. Each well was then treated with 5 μl Lipofectamine (Invitrogen) and 2 μg LIF expression vector in the absence of serum. Culture medium was replaced with DMEM containing 10% FBS 2 h after transfection. To isolate stable transfectants, cells were harvested with 0.25% trypsin 48 h after transfection, then replated into 75-ml culture flasks, and cultured in G418 selection medium (300 μg/ml) for 14 days. Then, the cells were harvested for RT-PCR and Western blot analysis. PCR was carried out with LIF primer given in table 1. Lysates (20 μg) were separated on a 15% gradient gel by SDS-PAGE, and then transferred to nitrocellulose. Immunoblotting was performed using anti-LIF (1/500, Chemicon) and anti-actin (1/1,000, Sigma) according to the instructions of the manufacturer.

Preparation of Feeder Layers

Both untransfected hELF and hELF/lif cells (passage 6–10) were mitotically inactivated by treating with 12.5 μg/ml mitomycin C (Sigma) for 3 h and then washed three times with phosphate-buffered saline (Gibco/BRL). Cells were harvested using 0.25% trypsin and washed three times in DMEM. Cells were counted and plated onto 0.1% gelatin-coated 4-well plates at a density of 2.0×10^5 cells/well in DMEM with 10% FBS.

hEG Cell Isolation, Culture, and Embryoid Body Formation

Human embryos at 5–9 weeks were collected from the Chongqing Medical University Hospital with the approval of the Chongqing Medical University Local Research Ethics Committee and donors' written consent. Gonadal ridges were dissected and disaggregated in 0.1% type IV collagenase (Sigma), at 37°C for 30 min, followed by trituration. Cells were subsequently plated on untransfected hELF or hELF/lif feeder layers in DMEM or knockout DMEM (DMEM, Gibco/BRL) with 15% FBS or knockout serum replacement (Gibco/BRL), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 ng/ml hrbFGF (Gibco/BRL), and 10 μM forskolin (Sigma). Cultures were maintained at 37°C under 5% CO_2, and were passaged every 4 days onto fresh feeders by harvesting with 0.25% trypsin at 37°C for 5–10 min and mechanical disaggregation. Colonies of presumptive hEG cells were isolated for characterization at each passage. Embryoid bodies (EBs) were formed by transferring hEG to EB culture medium (lacking bFGF and forskolin) in the absence of feeder cells, and culturing for 2 weeks.

Immunolocalization

Alkaline phosphatase (AP) activity was detected by fixing cells in 4% paraformaldehyde (PFA) for 1 min, then staining with naphthol/fast red violet solution (Chemicon). To detect expression of stem cell markers, colonies were fixed in 15% PFA for 15 min, then blocked for 30 min with 4% goat serum. Samples were then incubated with monoclonal antibodies specific to stage-spe-

Table 1. PCR primers for expression analysis of the listed genes

Gene	Primer sequence (5′–3′)	Product size (bp)
GAPDH	Forward: ACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTA	452
LIF	Forward: ATGAAGGTCTTGGCGGCAGG Reverse: AGCACACTTCAAGACCTCCTGCTA	657
Oct4	Forward: ACGACCATCTGCCGCTTGAG Reverse: CCCCCTGTCCCCCATTCCTA	574
Nanog	Forward: CGGCTTCCTCCTCTTCCTCTATAC Reverse: ATCGATTTCACTCATCTTCACACGT	1,026
Rex1	Forward: GCGTACGCAAATTAAAGTCCAGA Reverse: CAGCATCCTAAACAGCTCGCAGAAT	306
hTERT	Forward: GTGTGCTGCAGCTCCCATTTC Reverse: GCTGCGTCTGGGCTGTCC	264
LIFR	Forward: CTGGAACAGGCCGTGGTACT Reverse: ACTCCACTCTTCGAGACCAG	497
c-Myc	Forward: TTCTCTCCGTCCTCGGATTC Reverse: GTAGTTGTGCTGATGTGTGG	282
α-Fetoprotein	Forward: CCATGTACATGAGCACTGTTG Reverse: CTCCAATAACTCCTGGTATCC	357
CD34	Forward: TGAAGCCTAGCCTGTCACCT Reverse: CGCACAGCTGGAGGTCTTAT	200
Enolase	Forward: GTTCAATGTCATCAATGGCG Reverse: GTGAACTTCTGCCAAGCTCC	476
NF68	Forward: ACGCTGAGGAATGGTTCAAG Reverse: TAGACGCCTCAATGGTTTCC	561

cific embryonic antigen (SSEA)-1 (1:25), SSEA-4 (1:50), tumor rejection antigen (TRA)-1–60 (1:50), or TRA-1–81 (1:50) (Chemicon) according to manufacturer's instructions. Samples were then incubated with FITC or TRITC-conjugated secondary antibodies (1:100) and examined by fluorescence microscopy.

EBs were collected and fixed individually in 4% PFA for 2 h and were then frozen sectioned for immunohistochemical or immunofluorescent staining. Sections were then incubated with antibodies specific to desmin (1:100, Dako) or α-fetoprotein (1:50, Dako). Sections were then incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies (SP9000 kit, Zymed), followed by streptavidin-horseradish peroxidase, 3,3′-diaminobenzidine, and H_2O_2 or with FITC-conjugated secondary antibodies.

RT-PCR Analysis

RNA was extracted from hEG cells grown on hELF or hELF/lif cells using Trizol reagent (Sigma). RNA was treated with DNase (Sigma) and then used for oligo(dT)-primed first-strand cDNA synthesis with reverse transcriptase (Takara). PCR amplification was performed using gene-specific primers (table 1). GAPDH was used as an internal control.

Karyotype Analysis

Cell division was blocked by treating hEG cells grown on hELF/lif feeders with 0.1 μg/ml colcemide for 3–4 h. Cells were then harvested, resuspended in 0.075 M KCl, incubated for 20 min at 37°C, then fixed in 3:1 methanol:acetic acid. Chromosomes were stained using a standard G-banding technique. More than 100 cells were examined from each of two hEG lines.

Results

Culture of Transfected hELF

hELFs were isolated and grown for about five passages and then transfected with an LIF expression vector. Five colonies stably expressing hLIF (hELF/lif) were isolated following selection by G418. Cells overexpressing LIF exhibited no obvious difference in morphology compared with untransfected cells (fig. 1a, and not shown). Expression of LIF mRNA was detected by RT-PCR analysis in hELF/lif cells, but not in untransfected hELF cells (fig. 1b). Similarly, LIF protein was detected in hELF/lif cells, but not in untransfected hELF cells by Western blot analysis (fig. 1c).

hEG Cells Grown on hELF/lif Feeders Exhibit Characteristic Morphology

hEG cells maintained on hELF/lif feeder layers exhibited a characteristic morphology. Soon after plating (24–48 h), individual stationary and migratory cells were detected (data not shown). Thereafter, tightly compacted, multicellular colonies were observed. Most hELF/lif-based colonies were elliptical and distinct from feeder cells (fig. 2a). Colony morphology was un-

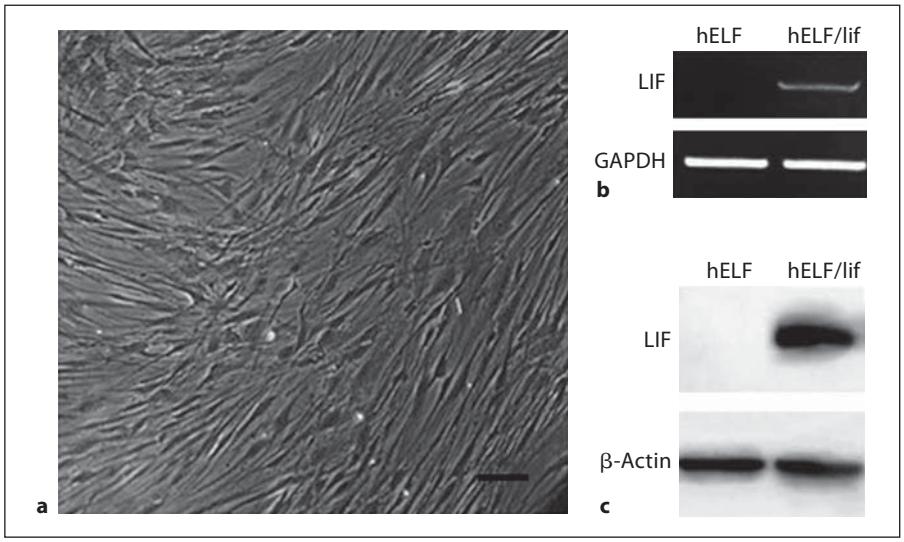

Fig. 1. Establishment of hELF/lif cells. **a** Morphology of hELF/lif cells. Bar = 100 μm. **b**, **c** RT-PCR analysis of LIF mRNA (657 bp) expression (**b**) and Western blot analysis of LIF protein (26 kDa) expression (**c**) in transfected and untransfected hELF cells.

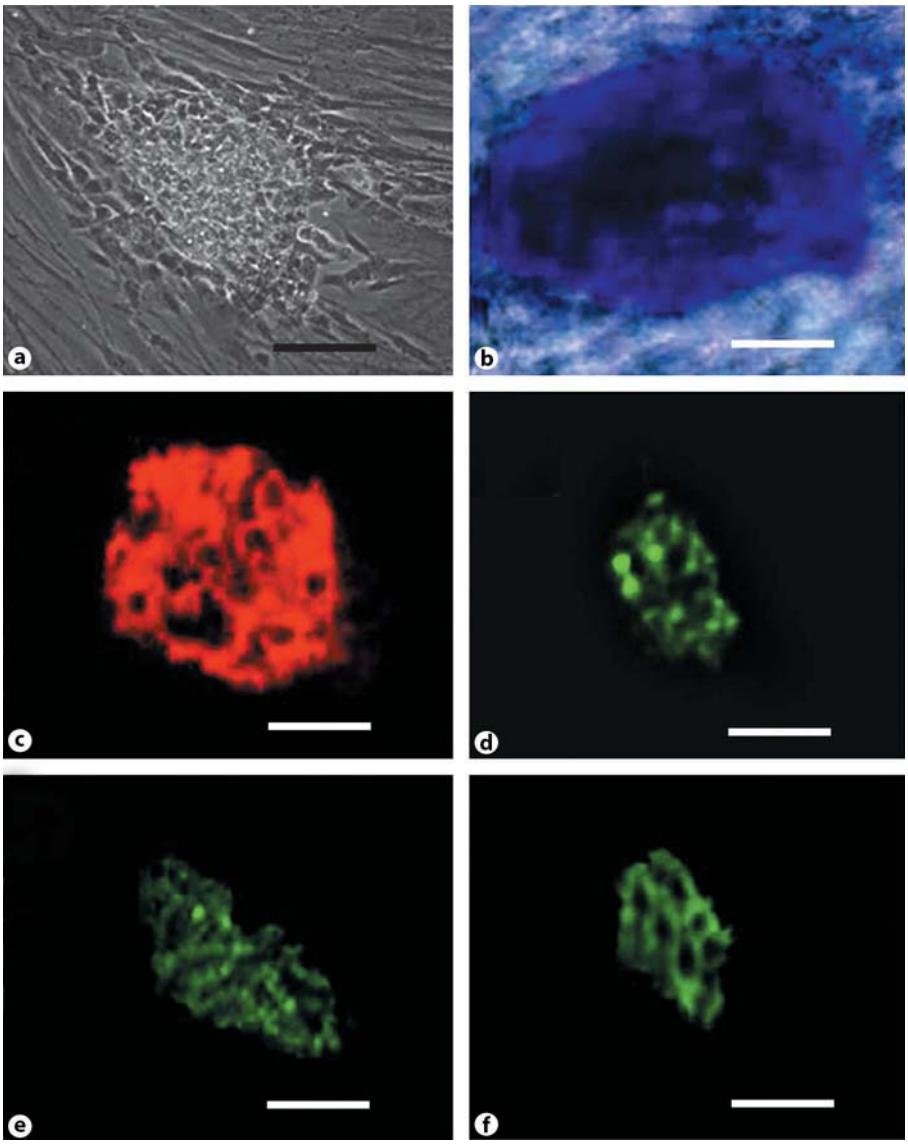

Fig. 2. Characteristics of hEG colonies grown on hELF/lif cells. **a** Morphology of a typical hEG colony grown on a feeder layer of hELF/lif cells 4 days after passage. **b** AP expression (blue) in hEG cells. **c–f** Immunofluorescent localization of SSEA-1 (**c**), SSEA-4 (**d**), TRA-1–60 (**e**) and TRA-1–81 expression (**f**) in hEG cells. Bars = 100 μm.

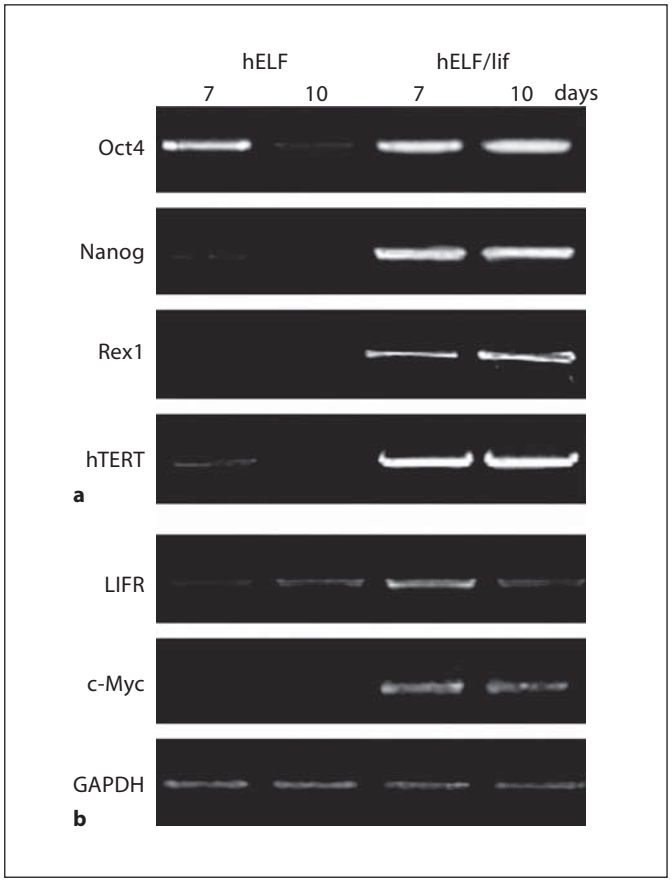

Fig. 3. Expression of pluripotency and LIF signaling pathway genes in hEG cells. **a** RT-PCR analysis of hEG cells cultured on hELF/lif or untransfected hELF cells at 7 and 10 days of culture. Cells grown on hELF/lif expressed relatively higher levels of Oct4, Nanog, Rex1, and hTERT than cells grown on untransfected hELF cells. The difference was most pronounced at 10 days. **b** LIFRβ was detected in hEG cells grown both on hELF and hELF/lif, but the LIF target c-Myc was detectable at much higher levels in hEG cells grown on hELF/lif cells. GAPDH was used as a control.

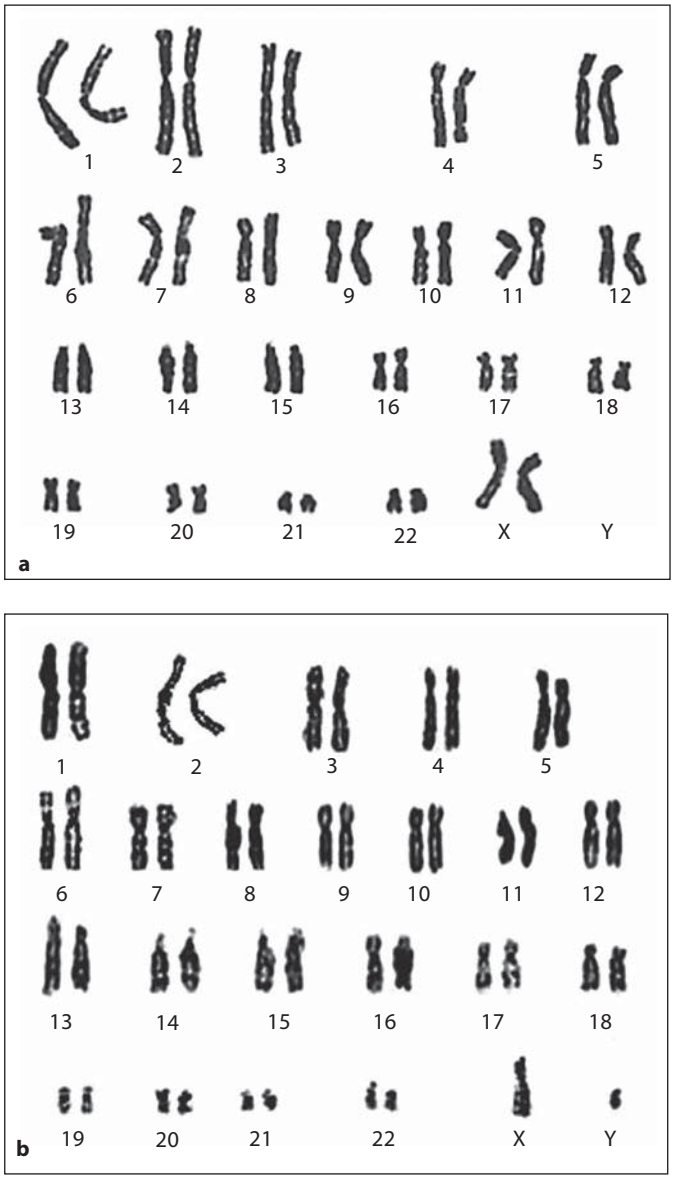

Fig. 4. Karyotype analysis of hEG cells grown on hELF/lif cells. Representative chromosome spread from an hEG cell line exhibiting a normal 46XX (**a**) and a normal 46 XY karyotype (**b**).

changed in the presence of DMEM with FBS or knockout DMEM with knockout serum replacement (data not shown).

AP and Pluripotent Stem Cell Marker Expression of Derived hEG Cells

We examined the expression of pluripotency markers in hEG cells (passage 10) maintained on hELF/lif. The vast majority of hEG cells were found to be AP positive (fig. 2b). In addition, SSEA-1, SSEA-4, TRA-1–60, and TRA-1–81 were detected in hEG cells grown on hELF/lif feeders by immunofluorescence (fig. 2c–f), suggesting that hEG cells are undifferentiated when maintained on hELF/lif cells.

To further characterize the necessity of LIF in maintaining the pluripotency of hEG cells, we examined the expression of pluripotency-associated genes in hEG cells grown on both untransfected hELF and hELF/lif cells by semiquantitative RT-PCR. Interestingly, Oct4, Nanog, Rex1, and hTERT were detected at higher levels

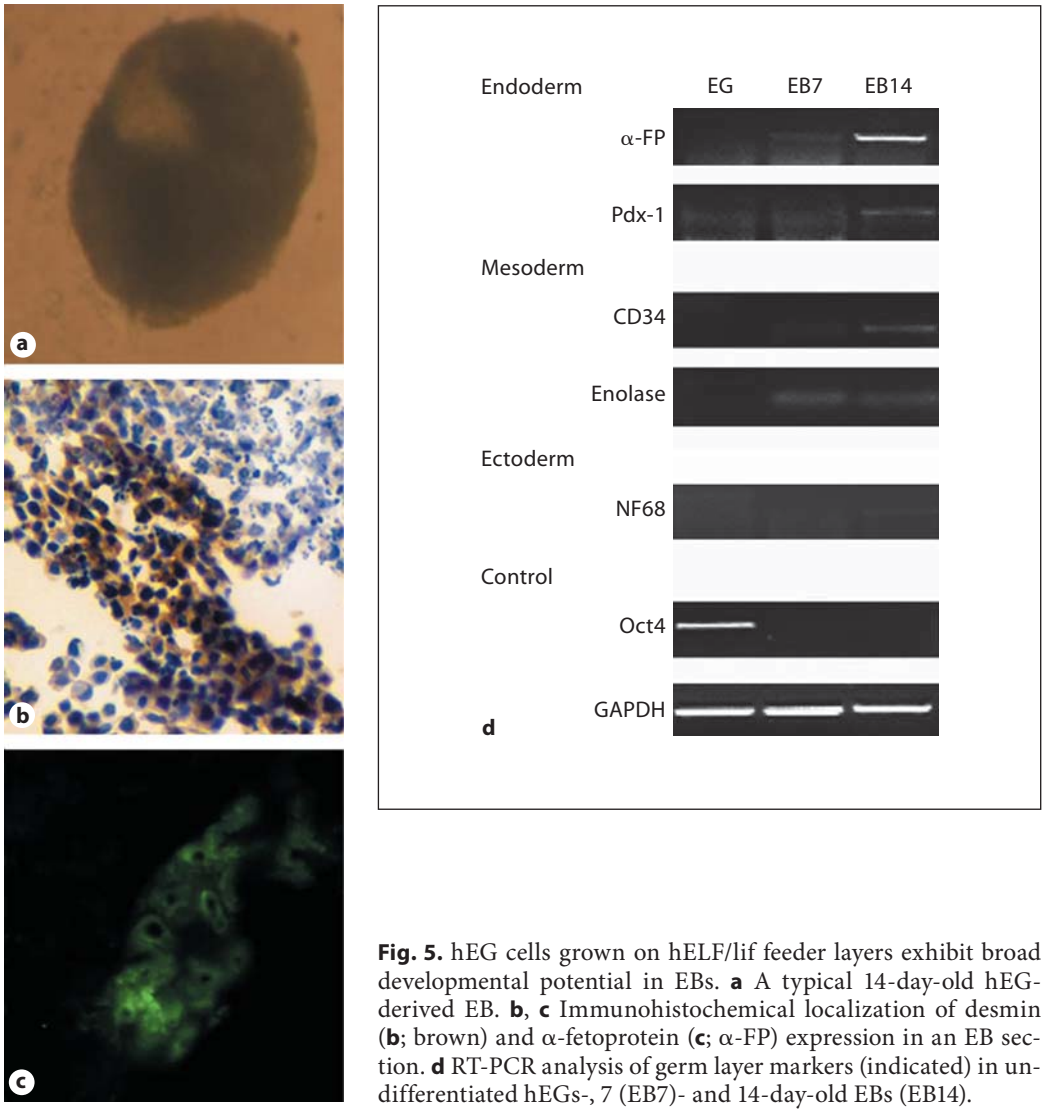

Fig. 5. hEG cells grown on hELF/lif feeder layers exhibit broad developmental potential in EBs. **a** A typical 14-day-old hEG-derived EB. **b**, **c** Immunohistochemical localization of desmin (**b**; brown) and α-fetoprotein (**c**; α-FP) expression in an EB section. **d** RT-PCR analysis of germ layer markers (indicated) in undifferentiated hEGs-, 7 (EB7)- and 14-day-old EBs (EB14).

in hEG cells grown on hELF/lif than those grown on hELF alone (fig. 3). These results suggest that hELF/lif cells prevent differentiation of hEG cells to a greater extent than do hELF cells alone. Importantly, RT-PCR analysis of LIF signaling pathway genes, LIF receptor (LIFR) β and the LIF target gene c-Myc, showed that LIF is capable of signaling in hEG cells and that while LIFRβ was detectable in hEG cells grown on either feeder layer, the c-Myc level was greatly enhanced in cells grown on hELF/lif feeders compared to those grown on untransfected hELF cells (fig. 3 and data not shown). Therefore, hEG cells maintained on hELF/lif feeder layers express markers of pluripotency and LIF pathway activation.

Karyotype Analysis

Karyotype analysis was performed on hEG cells expanded on hELF/lif feeder cells at passages 10–11. These cells exhibited normal human karyotypes (46 XX or 46 XY; n = 100 cells each; fig. 4).

Differentiation Capacity of hEG Cells

To functionally assess the pluripotentiality of hEG cells maintained on hELF/lif feeder layers, cells were harvested and grown in suspension culture medium to form EBs. EBs were observed within 48 h and were cultured for 7 and 14 days (fig. 5a) and subsequently examined for the expression of markers of tissue differentiation. EBs expressed markers associated with three different embry-

onic germ layers, including Pdx-1 and α-fetoprotein (endoderm), CD34 and enolase (mesoderm), and NF68 (ectoderm), and their expression increased during culture (fig. 5b–d). Importantly, the expression of Oct-4 decreased during culture (fig. 5d).

Discussion

The survival and proliferation of mammalian PGCs depends critically on specific growth factors and other unidentified molecules. hEG cells are an analogue of and alternative to hES cells, and a robust and non-animal culture system will significantly improve efforts to isolate and maintain hEG cell lines for both experimental and clinical use.

Feeder cells are thought to produce both soluble and insoluble, unidentified factors that are necessary to enable the self-renewal and proliferation of cocultured stem cells [Kerr et al., 2006]. A variety of primary human cells, including fetal skin fibroblasts, fetal lung fibroblasts, foreskin fibroblasts, and adult skin fibroblasts, have been used as feeder layers for hEG cells in our laboratory and others. However, none of these has surpassed the ability of the mouse-derived STO cells to support the growth and pluripotency of hEG cells. Here we showed that hELF cells overexpressing LIF can robustly support hEG derivation and maintenance. hEG colonies grown on LIF-transfected cells exhibited a typical, tightly packed morphology, expressed high levels of AP, and stem cell-associated markers (SSEA-1, SSEA-4, TRA-1–60, and TRA-1–81), and transcription factors (Oct4 and Nanog), and were karyotypically normal. Furthermore, hEG cells grown in this way maintained their capacity to differentiate into EB and expressed markers of three germ layers.

LIF is a pleiotropic cytokine that belongs to the interleukin-6 family [Williams et al., 1988]. LIFR consists of gp130, which is used by all members of the interleukin-6 family of cytokines, and LIFRβ (gp190), which is specific to the LIF signaling pathway. Activation of gp130 leads to the activation of STAT and Ras/MAPK pathways [Gearing et al., 1992]. The activation of STAT3 appears to be both necessary and sufficient for mouse ES cell self-renewal [Aghajanova et al., 2006].

Although the central role of LIF-STAT3 signaling has been established for mouse ES cell self-renewal, the role of this pathway in hES or hEG cells is unclear. LIF signaling is not required for self-renewal of hES cells [Humphrey et al., 2004]. However, LIF signaling may play a role, at least in mouse PGCs. While PGCs from LIF-deficient mice are normal, LIFR/gp130/Stat3b signaling is involved in the growth of mouse PGCs in vitro [Farini et al., 2005]. In humans, LIF is commonly included during hEG cell derivation and culture [Hwang et al., 2004; Liu et al., 2004; Pan et al., 2005], and is thus considered to be a critical factor for hEG cell proliferation. Interestingly, we were able to detect the expression of LIFRβ in hEG cells and evidence LIF signaling in the presence of LIF, suggesting that LIF plays a role in hEG cells. Consistent with this hypothesis, we found that untransfected hELF cells, which do not express LIF, could not support prolonged culture of hEG cells, while STO feeder cells are known to produce LIF. Thus LIF signaling appears to contribute to hEG cell self-renewal. Surprisingly, our data indicate that exogenous LIF is not sufficient to maintain hEGs grown on hELF feeder layers. One possible explanation for this observation is that hEG cell self-renewal may require a higher concentration or continuous supply of LIF. Another possible explanation involves the fact that three distinct transcripts, LIF-D, LIF-M and LIF-T, are encoded by the LIF gene, and these may have different activities [Haines et al., 1999]. Nonetheless, LIF expressed by feeder cells appears to be sufficient to maintain hEG cells in our system.

The establishment of xeno-free culture conditions is a prominent goal in the field of stem cell biology and regenerative medicine. While our system shows significant promise towards this end, our conditions still rely on the use of FBS and are thus still subject to risks of pathogen transmission. Future studies will focus on the development of effective and safer culture conditions that will enable the study and development of hEG cell-derived therapies for human disease intervention.

Acknowledgments

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