Stem Cell Rev and Rep (2010) 6:248–259
DOI 10.1007/s12015-010-9149-y

# Suspension Culture of Undifferentiated Human Embryonic and Induced Pluripotent Stem Cells

**Michal Amit · Judith Chebath · Victoria Margulets · Ilana Laevsky · Yael Miropolsky · Kohava Shariki · Meital Peri · Idit Blais · Guy Slutsky · Michel Revel · Joseph Itskovitz-Eldor**

Published online: 30 April 2010


**Abstract** Alongside their contribution to research, human embryonic stem cells (hESC) may also prove valuable for cell-based therapies. Traditionally, these cells have been grown in adhesion culture either with or without feeder cells, allowing for their continuous growth as undifferentiated cells. However, to be applicable in therapy and industry they must be produced in a scalable and controlled process. Here we present for the first time a suspension culture system for undifferentiated hESC and induced pluripotent stem cells (iPSC), based on medium supplemented with the IL6RIL6 chimera (interleukin-6 receptor fused to interleukin-6), and basic fibroblast growth factor. Four hESC lines cultured in this system maintained all ESC features after 20 passages, including stable karyotype and pluripotency. Similar results were obtained when hESC were replaced with iPSC from two different cell lines. We demonstrate that the IL6RIL6 chimera supports the self-renewal and expansion of undifferentiated hESC and iPSC in suspension, and thus present another efficient system for large-scale propagation of undifferentiated pluripotent cells for clinical and translational applications.

**Keywords** Embryonic stem cells · Induced pluripotent stem cells · IL6-IL6 receptor chimera · gp130 · 3D · Suspension · Bioreactor

## Introduction

Human embryonic stem cells (hESC) are pluripotent cells derived from early stage embryos up to and including the blastocyst stage [1]. These unique cells are traditionally isolated and cultured using the methods developed for mouse embryonic stem cells (ESC), i.e. with medium supplemented with fetal bovine serum (FBS) and feeder-layers consisting of inactivated mouse embryonic fibroblasts (MEF) [1]. Recent research has centered around improving the existing hESC culture systems, with efforts focusing on the ability to grow the cells in serum-free and defined conditions [2, 3], on maintenance of the cells as undifferentiated without the use of supporting feeder layers by employing MEF-conditioned medium (CM) [3–7], and on substituting animal feeder cells with human ones [8–11].

Mouse ESC can be cultured continuously without feeder layers provided that the culture medium is supplemented with leukemia inhibitory factor (LIF), which was found to be involved in the maintenance of mouse ESC self-renewal [12–16]. However, accumulating data indicate that LIF fails to prevent hESC differentiation [1, 17–20]. Activation of some elements of the LIF signaling pathway, such as STAT3 phosphorylation, was found to be weak or variable in hESC [18, 19]. As observed in mouse ESC, maintenance of the undifferentiated ESC phenotype could depend on a threshold

**Electronic supplementary material** The online version of this article (doi:10.1007/s12015-010-9149-y) contains supplementary material, which is available to authorized users.

M. Amit · V. Margulets · I. Laevsky · Y. Miropolsky · K. Shariki · M. Peri · I. Blais · J. Itskovitz-Eldor (✉)
Department of Obstetrics and Gynecology, Rambam Medical Center, Haifa, and the Sohnis and Forman Families Stem Cell Center, the Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel
e-mail: Itskovitz@rambam.health.gov.il

J. Chebath · G. Slutsky · M. Revel
Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

of STAT3 activation, which may not be reached or sustained by LIF in hESC [20]. We therefore sought to use more potent LIF-related cytokines. LIF signaling requires two membrane proteins, namely the LIF-receptor (LIF-R) and the glycoprotein gp130, which have to form a heterodimer [ref [21], for review]. IL6 signals through a gp130 homodimer and requires a specific IL-6 receptor, either present in the membrane or in the extracellular compartment as soluble IL-6 receptor (sIL6-R), the latter acting as a strong agonist of IL-6 on human cells [22]. Fusion of the entire sIL6-R (extracellular domain of IL6-R) with the N-terminus of IL-6 produces the IL6RIL6 chimera [23] whose affinity to gp130 is higher than the combination of IL-6 with sIL6-R [24].

Here we present a novel approach for cultivating undifferentiated hESC using suspension cultures. We show that serum-free medium supplemented with full-length IL6RIL6 chimera and bFGF, supported the culture in suspension of several hESC lines. The cell lines expanded through many passages while maintaining pluripotency, as evidenced by their formation of teratomas. With medium containing IL6RIL6 and bFGF, suspension cultures were initiated from adherent hESC grown on MEF, whereas in the absence of the potent agonist of IL-6 signaling, the transfer of adherent hESC onto suspension (e.g. in Petri dishes) resulted in the typical formation of embryoid bodies (EB) containing differentiating cells [25]. Our report constitutes the first description of a highly efficient method for continuous long-term expansion of undifferentiated hESC in 3D suspension and shaking cultures, which could be adequately applied to large-scale cell production of pluripotent stem cells.

## Results

### Suspension Cultures

Suspension cultures of hESC hold significant advantages over conventional ones, particularly in regard to obtaining large amounts of cells for cell and tissue transplantation. To initiate suspension cultures from hESC grown in feeder-dependent conditions [5], a number of growth factors and cytokines were employed. The combination of bFGF and full-length IL6RIL6 chimera [23] yielded the best results, whereas using bFGF only at high doses of 10–40 ng/ml led to differentiation of the cells 5 passages later. Twenty four hours after being placed in suspension culture with the IL6RIL6 chimera (CH100F), the hESC created spheroid clumps, or disc-like structures (Fig. 1a), which, upon histological examination, revealed a homogenous population of small cells with large nuclei (Fig. 1b and SupFig. 1). These spheroids, originating from four different lines (I3, I4, I6 and H9.2), were cultured successfully and split mechanically every 5-7 days, all the while maintaining their morphology, allowing to expand the suspension cultures. Alternatively, by using trypsin –EDTA and ROCK inhibitor treatment, suspended cells dissociated into single cells still formed spheroids of identical morphology and features, thus allowing efficient cell expansion (SupFig. 3). Some cultures were carried out for over 58 passages (a full year of continuous culture). When re-cultured on MEF or fibronectin after 10 and 25 passages in suspension, all spheroids adhered to the MEF or fibronectin, and 24–48

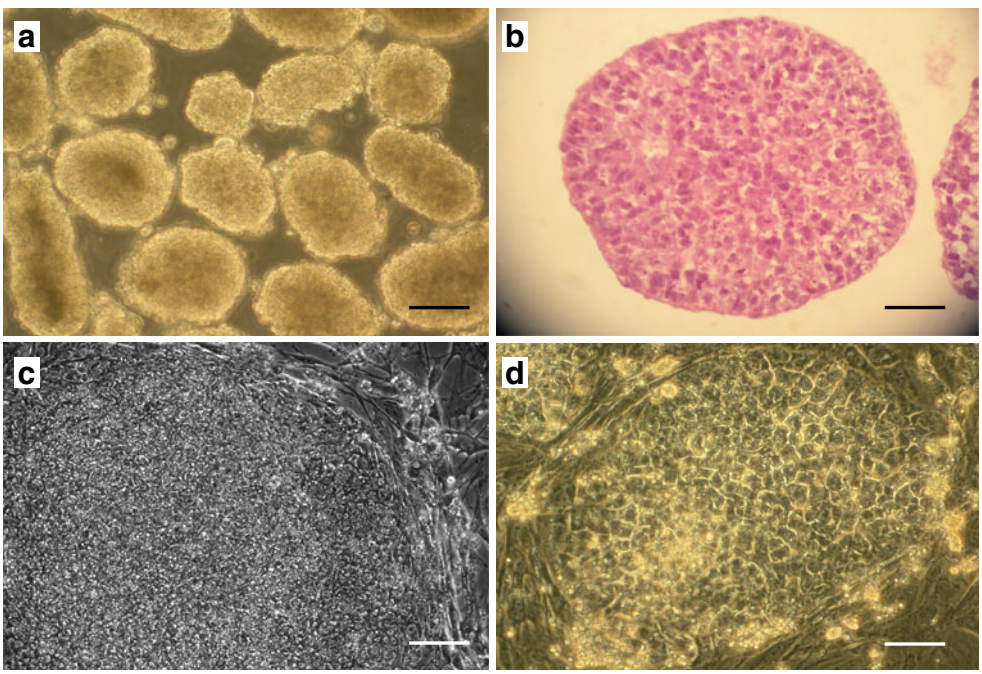


**Fig. 1** Morphology of hESC cultured in suspension in the presence of Il6RIL6 chimera. **a** I3 cells at passage 43 with CH100F medium. Bar 300 μm. **b** Histological section of a spheroid clump of undifferentiated I3 cells at passage 32 . Bar 50 μm. **c** Phase contrast picture of I3 hESC colony cultured for 10 passages in suspension and re-cultured on MEF (passage one on MEF). Bar 200 μm. **d** An I3 hESC colony cultured for 36 passages in suspension and re-cultured on fibronectin (passage 10). Bar 150 μm

hours later exhibited a morphology typical of hESC colonies (Fig. 1c,d).

## Maintenance of Undifferentiated Stem Cell Phenotype

Several surface markers typical of primate undifferentiated ESC were examined using immunofluorescent staining [1, 26, 27]. hESC cultured in suspension with CH100F medium for 40 passages were found to be strongly positive for Stage Specific Embryonic Antigen (SSEA)4, Tumor Recognition Antigen (TRA)-1-60 and TRA-1-81 and *Oct4* (Fig. 2a). As in other primate ESC [26, 27], while cells cultured with MEF displayed weak SSEA3 staining and were negative for SSEA1. The same results were obtained

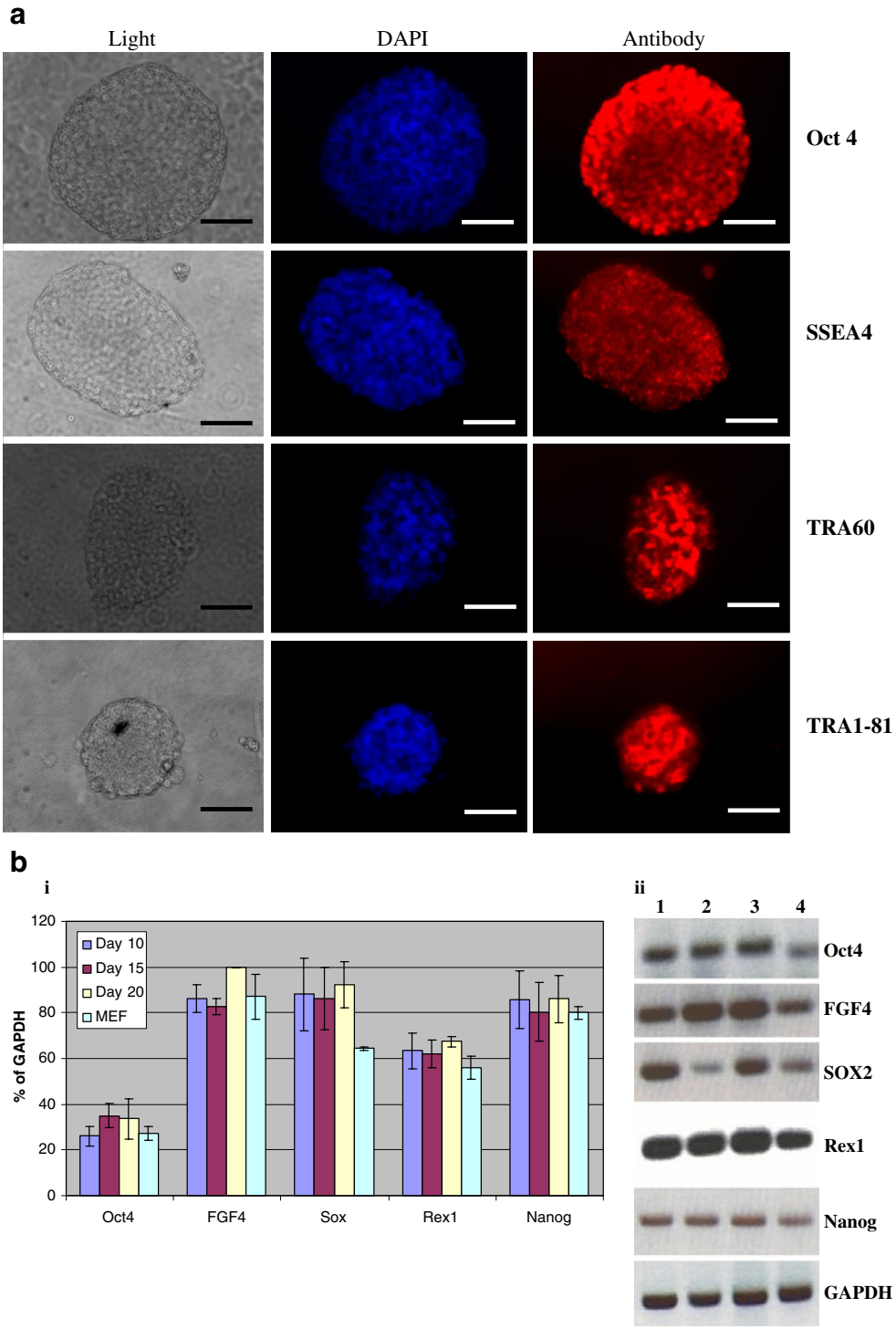


**Fig. 2** Expression of undifferentiation markers by cells cultured in suspension. **a** Fluorescent immunostaining. hESC line I4 cultured for 40 passages in suspension and stained with anti-*Oct4* antibodies, anti-SSEA4 antibodies, anti-TRA-1–60 antibodies, and anti-TRA-1-81 antibodies. Bar 90 μm. **b** *(i)* Relative expression of pluripotency markers as determined by Q-PCR for I3 cells cultured for 10, 15 and 20 passages in suspension and MEF, and representative gels *(ii)*. Similar results were demonstrated for I3 and I6 cells cultured in suspension. Lines were marked 1 to 4 for I3 cells cultured for 10, 15 and 20 passages in suspension and with MEF respectively. RT mix for all genes were negative. **c** Flow cytometry analysis of hESC cultured in suspension. Suspended I4 cells at passage 30 and suspended I6 at passage 20, respectively, were positive for SSEA4. 87% of I3 cellswere found positive at passage 34. The black graph represents the results obtained when using isotype control

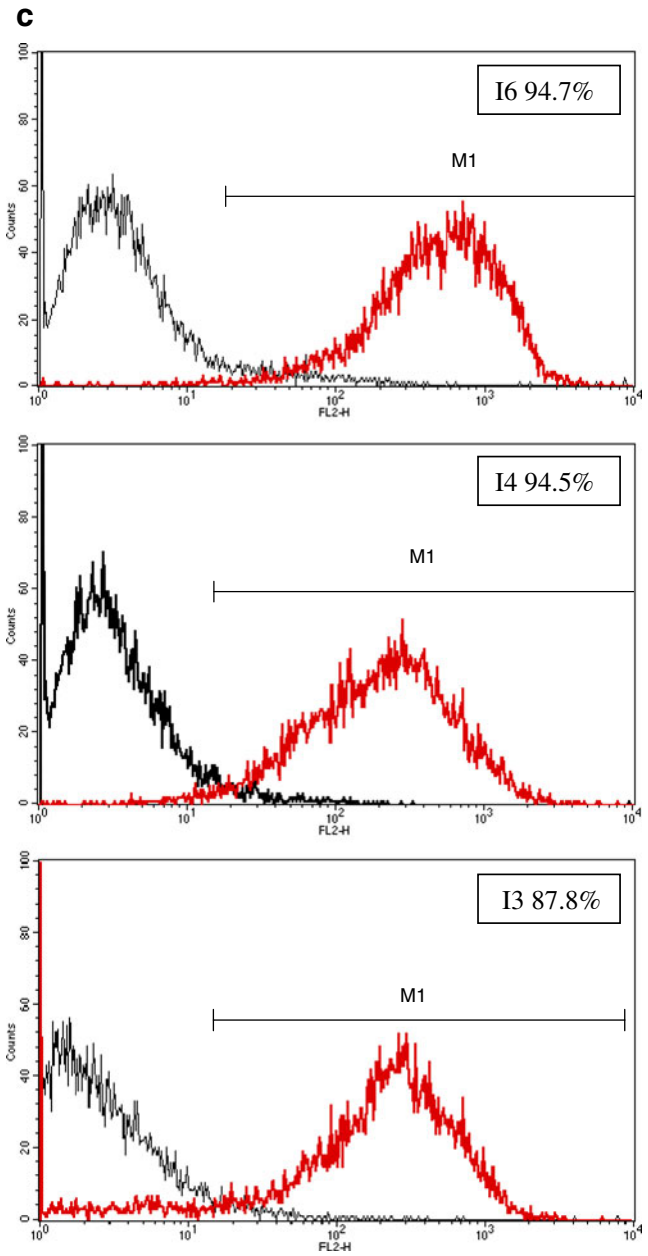


**Fig. 2** (continued)

when I3, I4, I6 and H9.2 cells were used. Staining for stem cell markers remained high when cells cultured in suspension were re-cultured on MEF (not shown). Semi Quantitative-PCR (Q-PCR) analyses showed that, similarly to cells cultured with MEF, hESC cultured in suspension for 10,15 and 20 passages expressed *Oct4*, *Nanog*, *Sox2*, *Rex1*, and *FGF4* (Fig. 2b). No significant difference in gene expression was detected between cells cultured for various lengths of time and passage numbers, nor with cells re-cultured on MEF after continuous culture in suspension. Real time PCR analysis for *Oct4* gene expression levels demonstrated no significant difference between various hESC lines cultured in suspension and those cultured on MEF. The same was observed at different passages of culture in suspension (not shown).

Quantitative analysis by flow cytometry revealed that I4 cells at passage 30 and I6 cells at passage 20, both in suspension, were 94.5% and 94.7% positive for SSEA4, respectively (Fig. 2c). Suspended I3 cells at passage 34 were 87.8% SSEA4 positive (Fig. 2c, lower panel), indicating that these cells have a higher background differentiation. To overcome such increase in background differentiation we removed differentiating spheroids based on their morphology (by the formation of a cyst or detectable epithelial structures) under a dissecting microscope and thereby restored >90% of the expression level of undifferentiation markers such as SSEA4. Differentiated spheroids were routinely removed every 7–10 passages. The same process was applied to cells cultured with MEF, where every 7–10 passages 1–5% of the differentiating colonies were removed. In order to avoid consequent variations, prior to collection for analysis and further experiments, the spheroids were cultured for at least three passages without removal of differentiating clusters.

## Maintenance of Karyotype

Karyotype analysis by Giemsa banding was carried out on each cell line after 58, 23, 36 and 20 passages in suspension, for I3, I6, I4, and H9.2 respectively and the lines were found to be normal. Similar results were obtained when the cells were re-cultured on MEF; all samples but one of I3 (passage 12 on MEF after 10 passages in suspension) demonstrated normal karyotype. The original culture of I3 cells that remained in suspension demonstrated normal karyotype. Similar results were obtained when either mechanical or enzymatic splitting were used. Thus the karyotype of the suspension cell culture remained stable.

## Pluripotency

Following prolonged expansion in suspension, hESC conserved their pluripotent differentiation ability. The developmental potential of the cells was first examined *in vitro* by the formation of EB. When hESC cultured in suspension for over 20 passages were transferred to serum-containing medium without IL6RIL6 chimera, formation of cystic EB was observed after 7–10 days (Fig. 3ia, SupFig. 2), similarly to hESC cultured with MEF where cavitated EB appeared following 10 days in culture, and cystic EB after 14–20 days [25]. Cells harvested from 10 and 20 day-old EB, expressed genes representing the three embryonic germ layers (Fig. 3ii). Although the expression of differentiation markers in EB significantly differed between cells culture with MEF and/or suspension, gene expression pattern was similar (Fig. 3ii).

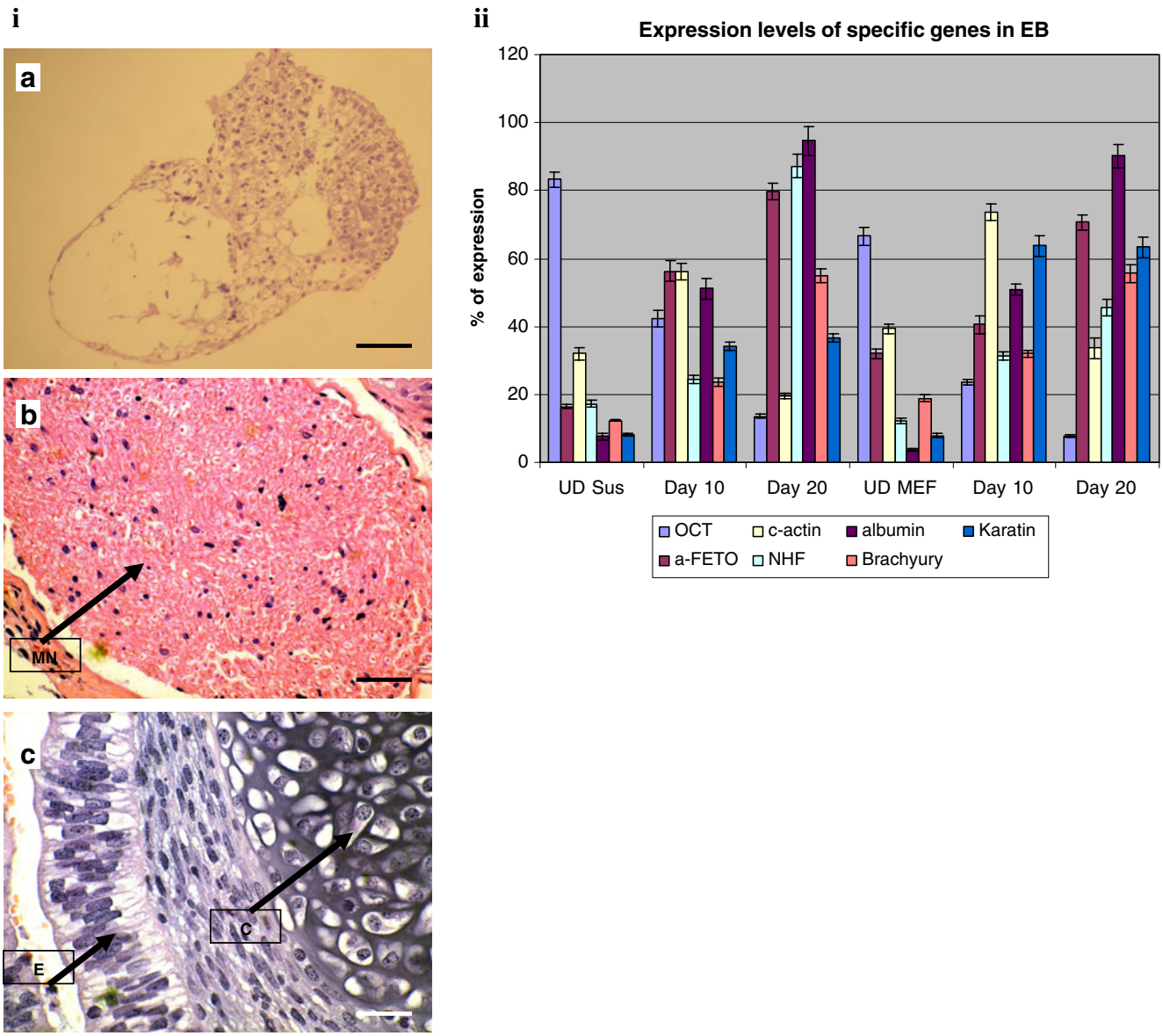


**Fig. 3** Differentiation of cells cultured in suspension. (i) Histological sections of EB and teratomas. **a** 14 day-old cystic EB formed by I4 cells cultured for 8 passages in suspension. Bar 200 μm. (b,c) Teratoma sections formed by I4 cells cultured for 9 passages in suspension prior to injection to SCID-mice include tissues representative of the three embryonic germ layers, **b** myelinated nerve (MN, ectoderm), **c** cartilage tissue (C, mesoderm), and columnar epithelium (E, endoderm). Bar 90 μm. (a–c) Staining with E&H. (ii) Q-PCR of 10 and 20 day-old EB formed by cells cultured in suspension as compared to cells cultured with MEF. The expression levels of *Oct4*, Fetoprotein, cardiac actin, NHF, albumin, brachury and keratin were examined. As control we used cells cultured for 45 passages in suspension and cells cultured with MEF. Densitometry of tested genes was normalized to GAPDH

*Oct4* expression was found by real time Q-PCR to decrease by 38% on day 5 after the suspended hESC were transferred to serum containing medium without IL6RIL6, and by 54% when the same was done with hESC cultured with MEF prior to EB formation (Data not shown). On day 10 the expression of *Oct4* decreased by 50% and 64% for the EB formed by cells cultured in suspension and cells cultured with MEF, respectively. Removing IL6RIL6 while leaving the basic medium with bFGF produced a similar decrease of *Oct4* by 34±6.8% (4 analyses, $p$=0.002), confirming the requirement of IL6RIL6 to maintain the undifferentiated state. Overall, no significant difference was found between the ability of cells cultured in suspension and those cultured with MEF to form EB (not shown).

Pluripotency of the hESC cultured in suspension was further demonstrated *in vivo* by teratoma formation. Cells of three hESC lines cultured in suspension with IL6RIL6 for 9, 10, 14 or 26 passages were injected into SCID Beige mice, and 10 weeks later tumors were formed from cells of all four passages. Within these teratomas, tissues represen-

tative of the three germ layers were observed (Fig. 3b–c). Similar results were obtained when cells cultured for at least 10 passages in suspension were re-cultured on MEF and cultured for additional 5–10 passages before injection into mice for teratoma formation.

The use of iPSC yielded similar results in regard to proliferability, pluripotency and karyotype stability (Supplementary data, SupFig. 6), which were also obtained when relatively high levels of LIF and IL6 were used as substitutes for the chimera in both hESC and iPSC (Supplementary Data, SupFig. 5).

## Cell Expansion

The kinetics of hESC expansion in the suspension cultures was tested by measuring the diameter of the spheroids every second day during a continuous culture of 14 days. By day 7 the average diameter had increased from 150 μm to 300 μm, and on day 14 it reached 500 μm (Fig. 4a,b, SupFig. 4A,B). After dissociation, it was estimated that the spheroids on day 14 contained $9.17\times10^5\pm3937$ viable cells. The calculated doubling time of cells in suspension was 35.17±1.32 h, similarly to a previous report on hESC in 2D cultures [2]. Correspondingly, the cultures were split every 5–7 days. While using trypsin-EDTA, the cells dissociated into single cells and expanded similarly in suspension (SupFig. 3). The spheroids initiated from a single cell grew to approximately 125 μm in diameter within three days and 200 μM by day 9. Cell death after treatment with ROCK inhibitor on the day of splitting with trypsin was 9% as measured by apoptosis levels.

Cell death was investigated at different stages of the suspension cultures. Soon after mechanical passaging, apoptosis level was approximately 16% (Fig. 4c, SupFig. 4C). During the next six days the average level of apoptosis dropped to 4.8%, then increased again on day 10 and peaked at 30% on day 14 of continuous culture (Fig. 4c) where spheroid diameter reached 500 μm. As expected, most of the apoptotic and necrotic cells were located at the center of the sphere, probably due to limited diffusion (Fig. 4d). By trypan blue, the cells' viability was estimated to be around 90% until day 10, and 80% on day 14. The increase in the spheroids' diameter and the low level of apoptosis indicate that days 5–7 are indeed the optimal splitting time where most cell loss by apoptosis is prevented and cell proliferation continues.

## Shaking Suspension Cultures

hESC were cultured in suspension in a shaking Erlenmeyer for 3 months, using the IL6RIL6-containing CH100F medium. An examination after 1 month showed that morphologically the spheroid clumps formed by the cells remained similar to those observed with cells cultured statically using Petri dishes (Fig. 5a). The size of the spheroids seemed more homogenous in the dynamic system, their average diameter being about 112±14 μm, and their average cell content about $3.75\times10^4\pm3\times10^3$ cells per spheroid. When re-cultured on MEF, the cells re-attached, forming again typical colonies of hESC (Fig. 5b). Cells propagated in suspension culture were strongly positive for undifferentiation markers (Fig. 5c–e). Removing of the IL6RIL6 chimera and supplementing the medium with fetal bovine serum resulted in typical EB formation. When re-plated on gelatin, these EB were found to contain cells positively stained for β–III-tubulin, CD31, and α-fetoprotein (Fig. 5f–h). Cells cultured for 1 month in the dynamic system were also demonstrated to form teratomas after their injection into SCID-beige mice (Data not shown). The karyotype of I3 cells cultured for 1 month in the Erlenmeyer was found to be normal.

The proliferation rate of the cells in the shaking Erlenmeyer was measured to evaluate the suitability of this culture system for mass production of hESC. In a typical 25 ml Erlenmeyer, the total number of spheroid clumps increased from $1.33\times10^4\pm461$ on the seeding day to $3.5\times10^5\pm2.8\times10^4$ after 10–11 days, i.e. a 25-fold increase; we estimate that the total cell number increased from $5\times10^8$ to $1.3\times10^{10}$ (the volume being increased through two passages to about 400 ml).

## Gp130 Dependence of IL6RIL6 Effect on hESC Suspension Cultures

The capacity of IL6RIL6 chimera to signal in hESC was first examined by measuring STAT3 phosphorylation (Stat3-P). In hESC line I3 grown on Matrigel in basic medium, addition of IL6RIL6 for 90 min induced STAT3-P (Fig. 6a, left panel). In the same experiment, we did not observe STAT3-P in response to LIF, although others have reported a weak induction by LIF in Matrigel-adherent hESC [19]. In the suspension cultures of hESC maintained with IL6RIL6 for 10 passages, STAT3-P was present at an intensity comparable to that induced by IL6RIL6 in HeLa cells, when compared to either total STAT3 or to ERK1/2 (Fig. 6 middle panel). That the chronic activation of STAT3-P in the suspension hESC cultures is dependent on IL6RIL6 was shown by removal of the cytokine for 24 h followed by a trigger re-addition (Fig. 6, right panel). Quantitative analysis of STAT3-P, normalized for ERK, indicates a decrease when IL6RIL6 is removed (compare first and second column) and an increase upon re-addition of IL6RIL6 for 3 and 24 h (columns 3 and 4). Further, when hESC grown in suspension with IL6RIL6 for 10 passages were re-plated on MEF without IL6RIL6 for 7 passages, phospho-STAT3 disappeared, whereas it was shown to be maintained when IL6RIL6 was added to the cultures on MEF (last two columns).

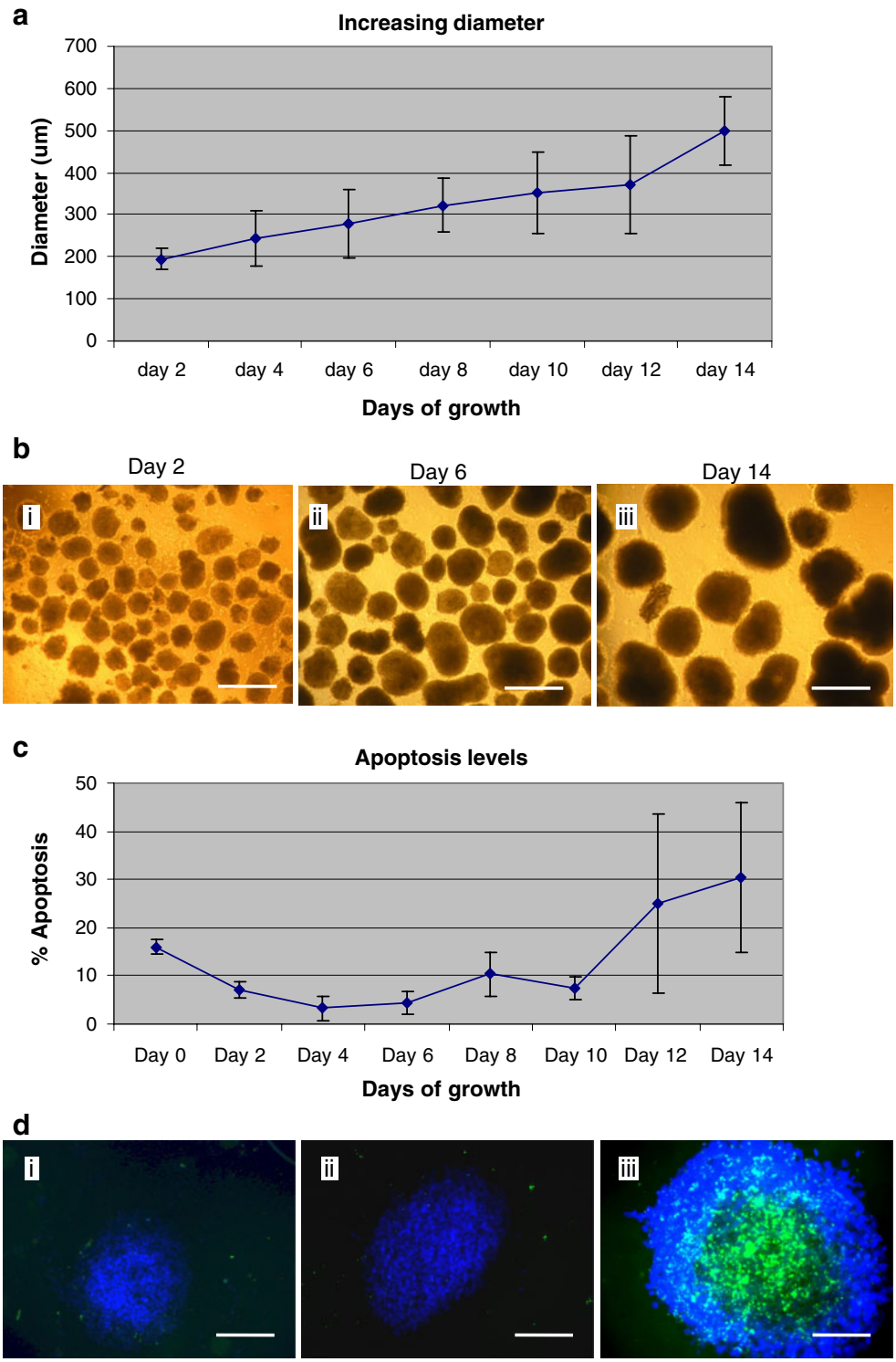


**Fig. 4** Growth curves and apoptosis. I4 cells cultured for more than 20 passages in suspension were used to study the culture system kinetics. The cells were cultured without splitting for 14 days and the following were measured: **a** Increase in spheroid diameter **b** The increasing size of spheroids cultured for 2,6, and 14 days. Bar 300 μm. **c** Apoptosis rate of cells cultured for 14 days in suspension. **d** Apoptotic cells concentrated at the center of the spheroids cultured for 2, 6 and 14 days. Staining was conducted using the Tunnel method. Bar 150 μm

## Discussion

Undifferentiated hESC have so far been traditionally grown in two-dimensional culture, with recent enhancements relating to the use of defined and animal-free medium [3]. This study demonstrates a method by which to culture hESC in free floating cell aggregates for prolonged periods of time, in medium consisting of serum-replacement, bFGF, and IL6RIL6 chimera. We demonstrate the growth of four hESC lines in suspension for over 20 passages, with maintenance of normal hESC features, including the expression of surface markers and genes typical of undifferentiated hESC as detected by FACS, Q-PCR and immunostaining, normal karyotype and teratoma formation. The suspension

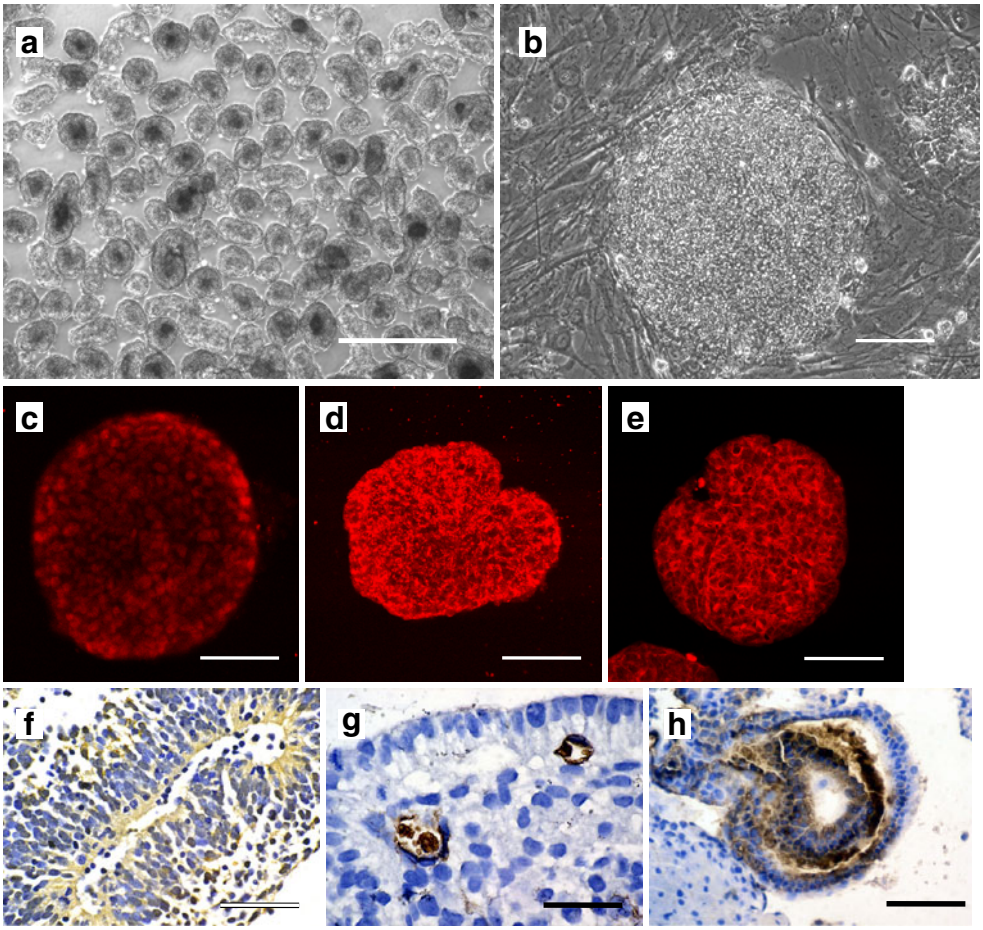


**Fig. 5** Dynamic culture in Erlenmeyer. **a** Spheroids of I3 cells cultured for 1 month in Erlenmeyer (Bar 400 μm) and **b** colony formed by these cells re-cultured on MEF, bar 200 μm. Fluorescent immunostaining of I3 cells cultured for 4 months in Erlenmeyer positively stained for **c** *Oct4*, **d** SSEA4, and **e** TRA-1-60, bars 200 μm. When the cells (1 month of culture in Erlenmeyer) were transferred to medium containing serum they formed EB. EB were fixed and immunostained with horseradish peroxidase for **f** β–tubulin, bar 200 μm, **g** CD31, and **h** α-fetoprotein, bars 100 μm

cells stably retained the undifferentiated hESC phenotype when transferred back onto MEF, even when shuttled four times between MEF and suspension culture. This facilitates the initiation of suspension cultures and makes it possible to study the molecular changes involved in transition from substrate adhesion to floating.

The role of the IL-6 family cytokines in the maintenance of undifferentiated ESC in the absence of feeder cell layers has been studied extensively, with differing results in mouse and hESC. With mouse ESC, prolonged 2D culture of undifferentiated cells is routinely achieved with LIF, which is known to require a heterodimer of LIF-R and gp130 to

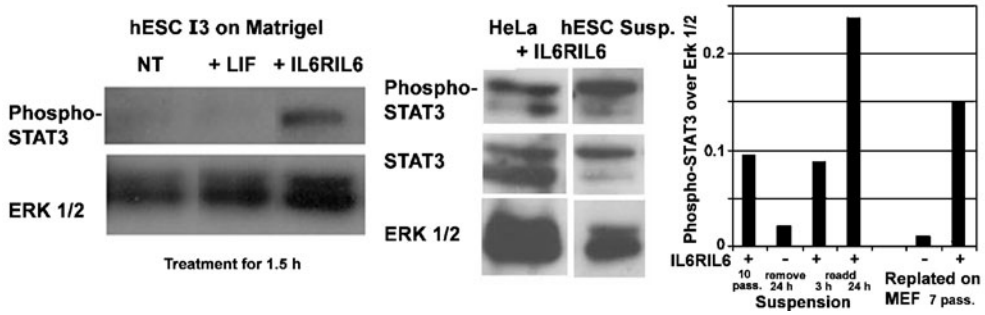


**Fig. 6** Signal transduction by IL6RIL6 and gp130 dependence for maintenance of undifferentiated hESC in suspension cultures. Left panel: I3 cells grown in basic medium with MEF were plated on Matrigel extracellular matrix and treated for 90 min with hLIF (20 ng/ml), or IL6RIL6 (100 pg/ml), or left untreated (NT). Extracted proteins were subjected to immunoblot with antibodies to phosphorylated STAT3 and to control proteins ERK 1/2. Middle panel: Immunoblot with antibodies to STAT3 and to phosphorylated STAT3 with extracts of HeLa cells treated for 90 minutes by IL6RIL6 compared to hESC I3 maintained in suspension cultures with IL6RIL6 (CH100F medium) for 10 passages. Right panel: Graph showing the level of phosphorylated STAT3 in hESC I3 maintained in suspension culture with IL6RIL6 for 10 passages, then 24 h after change to medium without IL6RIL6, and 3 and 24 h after re-adding IL6RIL6 to the medium. A portion of the suspension cells were re-plated on 2D cultures with MEF either without or with IL6RIL6 and analyzed after 7 passages

activate the JAK/STAT3 pathway [12–16]. Other members of the IL6 family, including the combination of IL-6 with its sIL6-R agonist, are also active in the growth of mouse ESC indicating that signaling through gp130 alone is sufficient [28]. Furthermore, it had been demonstrated that LIF plays a role in the preimplantation development of mouse embryos and that it can inhibit EB differentiation [29]. However, neither human LIF nor the combination of IL-6 and sIL6R were able to maintain an undifferentiated state with hESC line H1 cultured on Matrigel instead of MEF feeder cells or MEF-CM [19]. Similarly, LIF failed to maintain other hESC lines on Matrigel or on gelatin, despite the fact that LIF activated STAT3 phosphorylation and translocation in the nucleus [18]. A fusion molecule linking part of the sIL6-R chain to IL-6 (hyper-IL-6), as well as LIF or IL-6, were also unable to maintain the undifferentiated state in hESC HSF6 using laminin as substrate [20].

We repeated these studies and obtained similar results for LIF and IL6 in 2D cultures (not shown). However, we found that using fibronectin as substrate, the combination of bFGF with the full length fusion protein IL6RIL6 supported the cultivation of hESC for over 40 passages (not shown), although "auto-feeders" of differentiating cells formed around the colonies as previously seen with bFGF and TGF-β [5]. The present study demonstrates that when hESC were placed in suspension, the combination of IL6RIL6 and bFGF allowed the undifferentiated cells to proliferate as floating clumps and the cultures could be expanded through many passages without losing stem cell markers and pluripotency. Interestingly, when high levels of LIF or IL6 and its soluble receptor were used, similar results were obtained; hESC were cultured for prolonged periods in suspension as undifferentiated cells maintaining hESC features (Supplementary Data, SupFig. 5). The requirement of gp130 for maintenance of the hESC in suspension cultures was demonstrated by blocking with anti-gp130 antibodies which caused a markedly increased cell differentiation within the spheroid clumps (not shown).

The IL6RIL6 chimera has an affinity for the gp130 protein even higher than that of the combination of its components sIL6-R and IL-6 [27]. It could be able to perform better than other cytokines of this family on cells having a low density of gp130 on their membrane. Our data show that STAT3 phosphorylation was not seen with hLIF whereas it was highly induced by IL6RIL6, to levels comparable to those observed in HeLa cells. Sustained activation of STAT3-P was observed in the hESC suspension cultures, which reduced by removal of the IL6RIL6 and increased again by its re-addition. Removing IL6RIL6 from the suspension culture medium led to a reduction of the level of *Oct4* mRNA expression by over 33%, which is sufficient to induce hESC differentiation [30]. Transfer of the suspended hESC to a medium containing serum but no IL6RIL6 led to the formation of cystic EB containing cells of the three germ layers.

The described suspension culture system clearly allows hESC proliferation. After each passage (5–7 days), the size of each spheroid clump increased 1.5–fold, and the number of cells per spheroid increased 1.66-fold, and displayed low apoptosis levels and high viability rates. Taken together, the kinetic features of this new culture system indicate that the system is at least as proficient as the 2D one. Furthermore, the methodology presented here requires Petri dishes and does not require supportive cells or conditioned media, making it a cost-effective system. Coupled with its simplicity, this approach is an attractive option for the routine culture of hESC. Shifting undifferentiated hESC from adhesion to suspension will facilitate the development of controlled scale-up processes. Our experience with the Erlenmeyer's dynamic system indicate that hESC can be cultured continuously and maintain their typical features, and that our methodology enables a scale-up of 25 folds within 10 days. Although seeding concentrations and medium metabolites should still be optimized, our initial results demonstrate that this system could serve as a basis from which to develop a controlled process for mass production of hESC in bioreactors. The ability to use enzymes such as trypsin in order to split the cells and start the culture as a single cell suspension, may further improve the scalability of this system.

An initial study demonstrated similar results when induced pluripotent stem cells (iPSC) were used, including maintenance of pluripotency, stable karyotype, and similar proliferation rates, after prolonged suspension culture (SupFig. 6).

Future clinical uses of hESC will require a reproducible, defined and xeno-free culture system. The medium used in this study consists of serum replacement which contains "Albumax", a lipid-enriched bovine serum albumin, and can therefore not be considered animal-free. In this respect, further study is needed in aim to eliminate the presence of any animal material all together.

Several agents were reported to support undifferentiated hESC in 2D cultures. These include the combination of $TGF_{\beta 1}$ and bFGF [5], activin [31], high concentrations of Noggin [7] and bFGF [7, 32], the BIO inhibitor GSK3 [19], and a blend of five factors used in defined culture medium [3]. It is possible that some of these agents, acting in different pathways, can support the suspension culture of undifferentiated hESC. Examining bFGF we found that even at high doses of 10-40 ng/ml, it failed to support the culture without the IL6IL6 chimera. Whether other agents are more active remains to be examined.

Cormier et al. reported a successful one-passage culture of mouse ESC in a suspension system which yielded a 31-fold increase in cell number [33]. This system was based on

medium supplemented with calf serum and 1000 u/ml LIF. A more recent publication by the same group demonstrated that the same culture system could also be used for prolonged culture (up to 28 days) by splitting the cells with trypsin [34]. In regard to hESC: indeed two studies described their culture in suspension using microbeads [35, 36], but the methods described exhibited several disadvantages, namely the use of animal- and non-defined materials containing matrices which cover the beads (Matrigel), a decreased ability to culture the cells for prolonged time periods (up to six weeks) and the need to remove the cells from the beads prior to their application [35, 36]. A more recent study discussed suspension culture of hESC without the use of microbeads [37], but following 20 days of culture the expression of pluripotency markers decreased and only 44% of the cells expressed SSEA4 [37].

In summary, the IL6RIL6-based culture conditions described here allow large scale feeder cell-free and serum-free production of hESC and their maintenance and expansion as undifferentiated cells in suspension. This constitutes a dramatic step forward in facilitating the implementation of hESC technologies.

## Materials and Methods

### hESC Culture

hESC lines I3, I4 , I6 and H9.2 [2, 38] were cultured with inactivated MEF for 54–89 passages as previously described [2]. The following culture medium combinations were tested for their ability to support the growth of hESC in adherent (2D) or suspension (3D) cultures:

(i) Basic culture medium consisting of 85% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 15% knockout serum replacement (SR), 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% non-essential amino acid stock, and 4 ng/ml bFGF (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated). This basic culture medium was used as control and for the routine growth of hESC with MEF feeder layers in 2D cultures.

(ii) CH100F medium consisting of the above-mentioned basic culture medium supplemented with 100 pg/ml IL6RIL6 chimera [23]. The 85-Kda IL6RIL6 was produced and purified as described [38] and was provided by Merck–Serono (Geneva, Switzerland).

(iii) For testing bFGF ability to support hESC maintenance in suspension, we used basic medium (without IL6RIL6) with bFGF concentrations of 10, 20, 30 or 40 ng/ml. Three cell lines I3, I6 and H9.2 were tested in three separate experiments for each bFGF concentration.

(iv) Additional medium combinations based on LIF and IL6 are described in the Supplementary Data.

To initiate suspension cultures, hESC were removed from their culture dishes using 1.5 mg/ml type IV collagenase (Worthington biochemical corporation, Lakewood, NJ, USA), broken into small clumps using 200 µl Gilson pipette tips, and cultured in suspension in 58 mm Petri dishes (Greiner, Frickenhausen, Germany) at a cell density of $1\times10^6$–$5\times10^6$ cells/dish. The Petri dishes were kept static in an incubator at 37°C in 5% CO2. When required, differentiating clumps were removed from the culture during the first three passages, allowing the cells to adapt to their new culture conditions. Medium in the suspension culture was changed daily, and the cells were passaged every 5–7 days either by manual cutting of clumps using 27 g needles (only at passages 1–3) or by gentle pipetting using 200 µl Gilson pipette tips. Alternatively, cells were passaged using trypsin EDTA (0.25%, Biological Industries, Beit Haemek, Israel) combined with one hour treatment with 10 M ROCK inhibitor (EMD Biosciences, Inc. La Jolla, CA, USA) before the incubation with trypsin. For calculating cells' doubling time, I3, I4 and H9.2 cells were counted and grown in suspension for 8 days with CH100F medium. Cells were counted every other day. Average doubling time of four biological repeats was calculated.

### Culture in Erlenmeyer

Cell clumps cultured in Petri dishes for at least one passage were transferred to a 125 ml Erlenmeyer (Corning Incorporated, Corning NY, USA) in 25 ml CH100F medium, shaken continuously at 90 rpm using an orbital shaker (S3.02.10L, ELMI ltd, Riga, Latvia), and placed in the incubator. Medium was changed daily. Every 5–7 days the clumps were broken by gentle pipetting and split in a ratio of 1:2.

### Karyotype Analysis

Karyotype analysis (G-banding) was performed on at least 10 cells from each sample, two samples per test (total of 20 methaphases), as previously described [9]. Karyotypes were analyzed and reported according to the "International System for Human Cytogenetic Nomenclature" (ISCN).

### EB Formation

For the formation of EB, hESC were passaged as described and transferred onto 58 mm Petri dishes (Greiner, Frickenhausen, Germany). EB were grown and analyzed as previously described [25].

RT PCR

Total RNA was isolated from hESC grown for 10, 15 and 20 passages in suspension and from 10–14 day-old EB (formed from cells grown in suspension or cells cultured on MEF) using Tri-Reagent (Sigma, St. Louis MO, USA), according to the manufacturer's instructions.

Real Time PCR

RNA was isolated from undifferentiated cells cultured on MEF and from cells cultured in suspension for 10, 15 and 20 passages continuously. First-strand cDNA were synthesized as described above (RT-PCR). TaqMan Universal PCR Master Mix and Assay-on-Demand Agene Expression Probes (Applied Biosystems, Foster City, CA, USA) for *Oct4* and *β-actin* were used according to the manufacturer's guidelines. The reaction was performed with Applied Biosystems 7000 DNA Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative expression of *Oct4* was normalized to the expression of *β-actin* for the same sample. The cDNA of cells cultured on MEF were used as calibrators, and the relative expression of *Oct4* was calculated accordingly by using the standard curve method described by the manufacturer. Three biological repeats were conducted for each sample.

Flow Cytometry

Spheres of hESC cultured in suspension were dissociated to single cells using trypLE (Invitrogen Corporation products, Grand Island, NY, USA). The cells were stained with anti-h/mSSEA4 Ab conjugated to Phycoerythrin, Phycoerythrin conjugated Rat IgG2B were used as isotype control (both (R&D systems, Minneapolis,MN, USA). The stained cells were then analyzed with FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software according to the manufacturer's instructions.

Teratoma Formation

Cells from four to six 58 mm dishes were harvested and injected into the hindlimb muscles of four week-old male of severe combined immunodeficiency (SCID)-beige mice. Ten weeks after the injection the resultant teratomas were harvested and prepared for histological analysis using the same method mentioned for EB.

Western Analysis

Proteins were extracted from I3 cells cultured in suspension for 29 passages, from cells cultured on MEF only, from cells that were cultured in suspension for 10 passages and then re-cultured on MEF, and from cells of the trigger group experiment.

Trigger Experiments

Forty eight hours post splitting, cells cultured in suspension were transferred into the basic culture medium (control), without the addition of IL6RIL6 chimera. 24 h later, IL6RIL6 was re-added to the culture medium and cells were harvested immediately, and again after 3 and 24 h. Cells that were continuously cultured with IL6RIL6 were used as control.

Apoptosis Analysis

Apoptosis levels were examined by TUNEL reaction using the In Situ Cell Death Detection Kit, AP (Roche Diagnostics GmbH, Mannheim, Germany) on the 2nd, 4th, 6th, 8th, 10th and 14th days of continuous growth without splitting. Apoptotic cells were counted by inverted Zeiss Axiovert 200 fluorescent microscopy. At the same time, cell samples were harvested from the same culture, dissociated with trypsin using the same method, and stained with trypan-blue to evaluate the number of viable cells. Cells were counted by inverted Zeiss Axiovert 200 microscope. Three repeats were conducted for each cell line.

Further methods for immunohistochemistry, EB formation, RT PCR and Western analysis can be found in the online Supplementary Data.

**Acknowledgments** We thank Mrs. Hadas O'Neill for editing the manuscript. This research was supported by Technion Research and Development Foundation (TRDF). J.I.-E. holds the Sylvia and Stanley Shirvan Chair in Cell and Tissue Regeneration Research at the Technion—Israel Institute of Technology.

**Conflict of interest** The authors declare no potential conflicts of interest.

## References

1. Thomson, J. A., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*, 1145–1147.
2. Amit, M., et al. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Developmental Biology, 227*, 271–278.
3. Ludwig, T. E., et al. (2006). Derivation of human embryonic stem cells in defined conditions. *Nature Biotechnology, 24*, 185–187.
4. Xu, C., Inokuma, M. S., Denham, J., et al. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology, 19*, 971–974.
5. Amit, M., et al. (2004). Feeder and serum-free culture system for human embryonic stem cells. *Biology of Reproduction, 70*, 837–845.

6. Xu, C., et al. (2005). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells, 23*, 315–323.
7. Xu, R. H., et al. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nature Methods, 2*, 185–190.
8. Richards, M., et al. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nature Biotechnology, 20*, 933–936.
9. Amit, M., et al. (2003). Human feeder layers for human embryonic stem cells. *Biology of Reproduction, 68*, 2150–2156.
10. Hovatta, O., et al. (2003). Culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Human Reproduction, 18*, 1404–1409.
11. Cheng, L., et al. (2003). Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells, 21*, 131–142.
12. Smith, A. G., et al. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature, 336*, 688–690.
13. Williams, R. L., et al. (1988). Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature, 336*, 684–687.
14. Rose, T. M., et al. (1994). Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells in vitro. *Cytokine, 6*, 48–54.
15. Conover, J. C., et al. (1993). Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development, 119*, 559–565.
16. Niwa, H., et al. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes and Development, 12*, 2048–2060.
17. Reubinoff, B. E., et al. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nature Biotechnology, 18*, 399–404.
18. Daheron, L., et al. (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells, 22*, 770–778.
19. Sato, N., et al. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature Medicine, 10*, 55–63.
20. Humphrey, R. K., et al. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells, 22*, 522–530.
21. Taga, T., & Kishimoto, T. (1997). Gp130 and the interleukin-6 family of cytokines. *Annual Review of Immunology, 15*, 797–819.
22. Novick, D., et al. (1992). Enhancement of interleukin-6 cytostatic effect on human breast carcinoma cells by soluble IL-6 receptor from urine and reversion by monoclonal antibody. *Cytokine, 4*, 6–11.
23. Chebath, J., et al. (1997). Interleukin-6 receptor-interleukin-6 fusion proteins with enhanced interleukin-6 type pleiotropic activities. *European Cytokine Network, 8*, 359–365.
24. Kollet, O., et al. (1999). The soluble Interleukin-6 (IL6) Receptor/IL6 fusion protein enhances in vitro maintenance and proliferation of human $CD34^{+}CD38^{-/low}$ cells capable of repopulating Severe Combined Immunodeficiency mice. *Blood, 94*, 923–931.
25. Itskovitz-Eldor, J., et al. (2000). Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Molecular Medicine, 6*, 88–95.
26. Thomson, J. A., et al. (1995). Isolation of a primate embryonic stem cell line. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 7844–7848.
27. Thomson, J. A., et al. (1995). Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. *Biology of Reproduction, 55*, 254–259.
28. Yoshida, K., et al. (1994). Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. *Mechanisms of Development, 45*, 163–171.
29. Shen, M. M., & Leder, P. (1992). Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation in vitro. *Proc Natl Acad Sci U S A, 89*(17), 8240–8244.
30. Rodriguez, R. T., et al. (2007). Manipulation of OCT4 levels in human embryonic stem cells results in induction of differential cell types. *Experimental Biology and Medicine, 232*, 1368–1380.
31. Beattie, G. M., et al. (2005). Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells, 23*, 489–495.
32. Wang, L., et al. (2005). Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development. *Blood, 105*, 4598–4603.
33. Cormier, J. T., et al. (2006). Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors. *Tissue Engineering, 12*, 3233–3245.
34. Zur Nieden, N. I., et al. (2007). Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors. *Journal of Biotechnology, 129*, 421–432.
35. Phillips, B. W., et al. (2008). Attachment and growth of human embryonic stem cells on microcarriers. *Journal of Biotechnology, 138*, 24–32.
36. Oh, S. K., et al. (2009). Long-term microcarrier suspension cultures of human embryonic stem cells. Stem Cells Research. Mar 4. [Epub ahead of print].
37. Krawetz, R., et al. (2009). Large-Scale Expansion of Pluripotent Human Embryonic Stem Cells in Stirred Suspension Bioreactors. Tissue Eng Part C Methods. Sep 8. [Epub ahead of print].
38. Amit, M., & Itskovitz-Eldor, J. (2002). Derivation and spontaneous differentiation of human embryonic stem cells. *Journal of Anatomy, 200*, 225–232.