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Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension

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Undifferentiated human embryonic stem cells (hESCs) are currently propagated on a relatively small scale as monolayer colonies[1–7]. Culture of hESCs as floating aggregates is widely used for induction of differentiation into embryoid bodies[8]. Here we show that hESC lines can be derived from floating inner cell masses in suspension culture conditions that do not involve feeder cells or microcarriers. This culture system supports prolonged propagation of the pluripotent stem cells as floating clusters without their differentiation into embryoid bodies. HESCs cultivated as aggregates in suspension maintain the expression of pluripotency markers and can differentiate into progeny of the three germ layers both *in vitro* and *in vivo*. We further show the controlled differentiation of hESC clusters in suspension into neural spheres. These results pave the way for large-scale expansion and controlled differentiation of hESCs in suspension, which would be valuable in basic and applied research.

HESCs hold great promise as a renewable source of cells for basic and applied research[1–7]. hESC lines have so far been derived in monolayer cultures from blastocysts, inner cell masses or single blastomeres that were plated on supporting cell layers[9]. In one instance, two hESC lines were derived in a feeder-free monolayer system on extracellular matrix (ECM) proteins[3]. hESC lines are most commonly expanded as adherent colonies, whereas the current notion is that detachment into free-floating clusters induces differentiation. However, adherent culture is a major limitation for large-scale expansion of the cells, that could be overcome by propagation in suspension. Recently, hESCs were expanded either attached to coated microcarriers or unattached in spinner flasks for short periods[10–13]. Here we show the derivation, prolonged propagation and controlled differentiation of hESCs in a defined microcarrier-free suspension culture system. Performing the whole process in a defined suspension system sets the stage for future generation of transplantable cells with minimal labor, in controlled, automated and reproducible bioreactor large-scale culture systems. Such systems will be useful as hESC technology moves toward industrial and clinical applications.

In the course of our studies on the differentiation of hESC clusters in suspension into neural spheres[14], we observed that in Neurobasal medium hESCs remained undifferentiated in a considerable number of the floating clusters. We therefore sought to develop the Neurobasal medium into a suspension culture system for hESCs. We supplemented Neurobasal medium with Knockout serum replacement (KO-SR), commonly used in hESC culture media, and with Nutridoma-CS, a serum replacement designed to promote the proliferation of cells in suspension[15]. The unique component in Nutridoma-CS is beta-D xylopyranose, a ring-shaped sugar originating from xylose. Supplementation of serum replacement with this sugar allows the culture of cells in suspension without serum[16]. In addition, we supplemented the medium with ECM components previously shown to support the cultivation of undifferentiated hESCs in feeder-free culture systems[3,17,18] and with neurotrophic factors (NT-3, NT-4 and brain-derived neurotrophic factor (BDNF)), which were reported to promote hESC survival[19]. Finally, we included fibroblast growth factor (FGF2) and activin A, as accumulating evidence suggests that FGF and activin signaling play a central role in maintaining the pluripotent state of hESCs[18,20,21] (see Online Methods for complete medium composition).

We evaluated the ability of the key components in the culture system to promote proliferation of undifferentiated hESC clusters in suspension. HESC colonies were dissociated from the feeders and cultured for 3 weeks as floating aggregates in either KO medium or Neurobasal medium, each of which was supplemented with a combination of KO-SR, ECM components, FGF2 and activin A. The KO medium poorly supported the proliferation of hESCs, and the cells tended to differentiate (**Supplementary Fig. 1a**,**b**). In contrast, the Neurobasal medium was more effective for expansion of undifferentiated hESCs. Further supplementation of the Neurobasal medium with Nutridoma-CS significantly ($P < 0.01$) increased the total number of cells obtained after 3 weeks of suspension culture without affecting the percentage of undifferentiated cells (**Supplementary Fig. 1a**,**b**).

Analysis of the effect of ECM components in the culture system showed that they significantly ($P < 0.005$) increased the number of cells without affecting the level of differentiation (**Supplementary Fig. 1c**). Fibronectin and laminin each had a significant ($P < 0.05$) effect on the number of cells, as did the combination of laminin, fibronectin and gelatin ($P < 0.005$). Immunostaining showed increased immunoreactivity with anti-laminin and anti-fibronectin antibodies of clusters that were cultured with the corresponding ECM components

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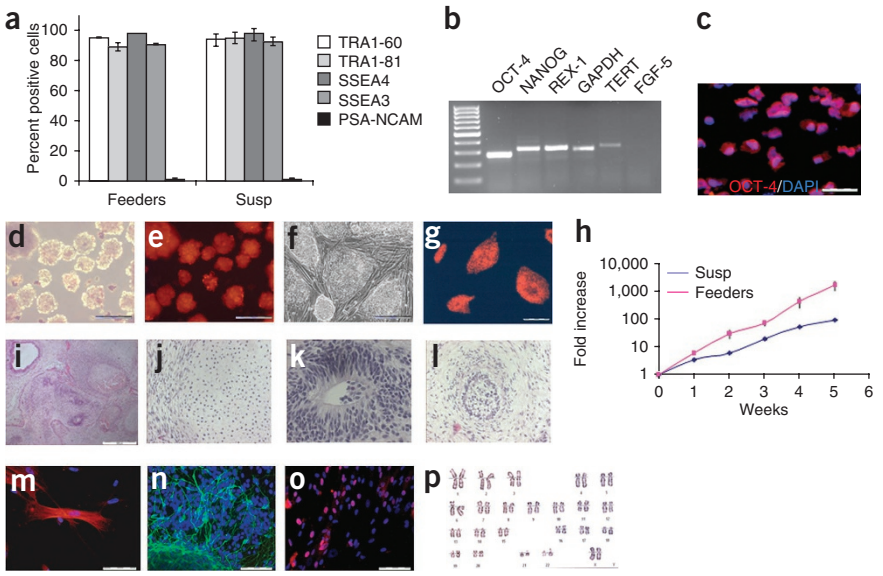

Figure 1 Human ESCs remain pluripotent after 10 weeks propagation in suspension. (**a**) FACS analysis of HES1 cells after cultivation in suspension and on feeders showing that in both culture conditions >90% of the cells express markers of pluripotent stem cells, whereas <2% express PSA-NCAM, which is a marker of early neural differentiation ($n = 3$). Data are presented as mean ± s.d. (**b**) RT-PCR analysis of free-floating clusters of hESCs confirming the expression of transcripts of markers of pluripotency, whereas the expression of the primitive ectoderm marker FGF5 is not detected. (**c**) Immunostaining of cells dissociated from the clusters and plated for 24 h, demonstrating that the majority of cells express OCT-4. (**d**) Darkfield micrograph of the clusters of hESCs in suspension. (**e**) Alkaline phosphatase activity within the hESC aggregates is demonstrated (fluorescence image). (**f**,**g**) After plating of the clusters on feeders, they give rise to colonies with morphological characteristics of colonies of undifferentiated hESCs (**f**, phase contrast image), which are comprised of cells harboring alkaline phosphatase activity (**g**, fluorescence image). (**h**) Proliferation curve showing the fold increase in the number of cells cultivated in suspension or as colonies on feeders. Cell number was monitored each week and the cumulative fold increase in cell number from the starting population was calculated in three independent experiments. (**i**–**l**) Histological analysis of H&E-stained sections of teratoma tumors that developed after inoculation of the hESC-clusters under the testes capsule of NOD/SCID mice showing differentiated tissues representing the three germ layers (low magnitude image (**i**), cartilage (**j**), neural rosette (**k**) and glandular structure (**l**)). (**m**–**o**) Immunostaining of *in vitro*–differentiated progeny, representing the three embryonic germ layers, within the outgrowth of plated embryoid bodies (human muscle actin (**m**), and SOX-17, (**o**)) and neural spheres (β-III Tubulin, (**n**)). (**p**) G-banding analysis showing a normal karyotype. Nuclei are counterstained by DAPI in (**c**) and (**m**–**o**). Scale bars, 20 μm (**c**,**k**,**m**–**o**); 50 μm (**d**,**e**,**j**,**l**); 100 μm (**f**,**g**); 500 μm (**i**).

(**Supplementary Fig. 1d**), suggesting binding of the dissolved ECM components by the hESC clusters. We used the combination of all three ECM components in all subsequent experiments, although it is possible that a less complex ECM composition (e.g., laminin only) would have been sufficient. We also continued to supplement with neurotrophic factors, although they were probably dispensable (**Supplementary Fig. 1e**,**f**).

Next, we used the suspension culture conditions to propagate three hESC lines (HES1, HES2 and H7 (refs. 1,2)) for 10 weeks. The clusters of hESCs formed spheroid aggregates that gradually grew in size and were weekly triturated into small clusters during passaging. During the last 3 weeks of propagation, the hESCs were characterized (**Fig. 1** and **Supplementary Figs. 2** and **3**). Fluorescence-activated cell sorting (FACS) analysis showed that ≥90% of the cells in all three lines expressed markers of pluripotent stem cells, including SSEA-4, TRA-1-60, TRA-1-81 (**Fig. 1a** and **Supplementary Figs. 2a**, **3a** and **4**) and SSEA-3 (**Fig. 1a**; HES1). Immunostaining showed that most of the cells expressed OCT-4 (**Fig. 1c** and **Supplementary Fig. 2b**). The floating clusters expressed alkaline phosphatase (**Fig. 1e**). The percentage of differentiating cells expressing PSA-NCAM (a marker of early neural differentiation) was ≤2% in all three lines (**Fig. 1a** and **Supplementary Figs. 2a**, **3a** and **4**). RT-PCR analysis showed the expression of pluripotent stem cell markers, including OCT-4, NANOG, REX-1 and TERT, lack of expression of FGF5, a marker of primitive ectoderm (**Fig. 1b**), and lack of or low expression of markers of endoderm, mesoderm and extra-embryonic endoderm (**Supplementary Fig. 5a**). Real-time PCR analysis confirmed the high expression levels of OCT-4, NANOG and REX-1 in hESCs cultured as floating clusters similar to monolayer colonies on feeders. It also confirmed the low levels of expression of markers of differentiation (**Supplementary Fig. 5b**). When replated on human feeders, the cell clusters gave rise to colonies comprised of cells with the morphology of undifferentiated hESCs and that expressed alkaline phosphatase (**Fig. 1f**,**g**).

We next demonstrated that hESCs cultivated in suspension maintain their pluripotent potential. Clusters of hESCs from the three lines that were inoculated under the testicular capsule of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice gave rise to teratomas. Histological analysis of the tumors demonstrated differentiated progeny of the three germ layers (**Fig. 1i–l** and **Supplementary Figs. 2c–f** and **3b–e**). To confirm the pluripotent potential *in vitro*, we cultured the clusters from the three lines under conditions that promote neural spheres[14] or embryoid body[22] formation. After plating, immunostaining showed differentiated progeny representing the three germ layers (**Fig. 1m–o** and **Supplementary Figs. 2g–i** and **3f–h**).

The karyotypes of the three lines were normal after 8 weeks in suspension (**Fig. 1p** and **Supplementary Figs. 2j** and **3i**).

We examined the potential of the suspension culture system to promote undifferentiated cultivation (H7 and HES1 lines) for prolonged periods (20 weeks). FACS analysis showed that the percentage of cells expressing markers of pluripotent stem cells remained ≥90%, and the level of background differentiation was low (≤1% PSA-NCAM$^+$ cells; **Supplementary Fig. 6a**). The karyotype of H7 was normal, whereas an abnormal karyotype was identified in HES1 (**Supplementary Fig. 6b**). The potential of hESCs to develop karyotypic abnormalities (in chromosome 1 of HES1 (ref. 23)) in a variety of culture systems has been described[24,25]. It is unclear whether these abnormalities are related to suboptimal culture conditions, the method of passaging, or a generic property of any type of cell that is propagated for prolonged periods with varying susceptibility to karyotypic changes in different cell lines. It is possible that with further development of the culture system, genetic stability could be improved.

The suspension culture system supported the expansion of the hESCs, though at a considerably lower rate compared with feeder-dependent monolayer hESC cultures. (**Fig. 1h**). We therefore characterized the proliferation, doubling time and cell death in the suspension culture system. FACS

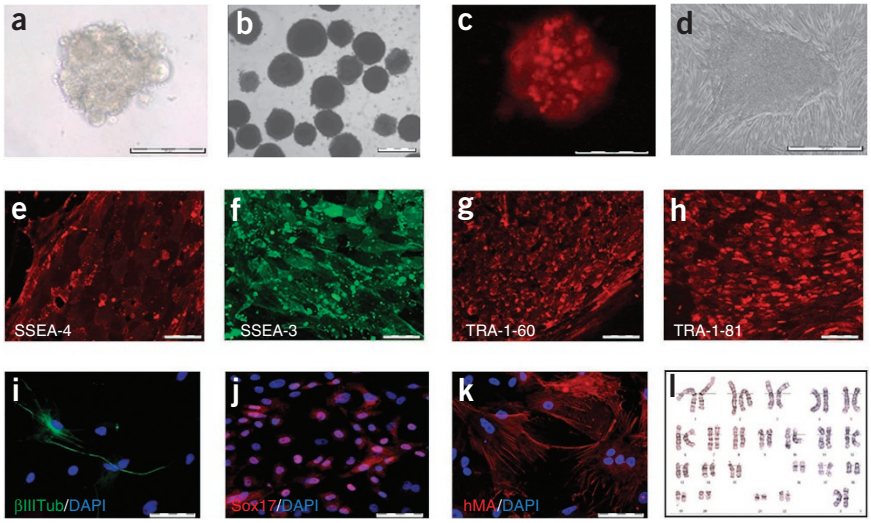

Figure 2 Derivation of hESCs in suspension. (**a**,**b**) Darkfield micrograph of an inner cell mass after transfer to suspension culture conditions (**a**), and of the clusters of cells that were derived from the inner cell mass after 10 weeks of cultivation (**b**). (**c**) Fluorescence image showing alkaline phosphatase activity within a cluster. (**d**–**h**) After plating on feeders, the clusters gave rise to colonies with morphological characteristics of colonies of undifferentiated hESCs (**d**, phase contrast image), which were comprised of cells immunoreactive with anti-SSEA-4 (**e**), SSEA-3 (**f**), TRA-1-60 (**g**) and TRA-1-81 (**h**) (fluorescence images). (**i**–**k**) Immunostaining of *in vitro*–differentiated progeny, representing the three embryonic germ layers, within the outgrowth of plated embryoid bodies (β-III tubulin, (**i**); SOX-17, (**j**); human muscle actin, (**k**)). (**l**) G-banding analysis showing a normal karyotype after 10 weeks of cultivation in suspension. Nuclei are counterstained by DAPI in **i**–**k**. Scale bars, 20 μm (**a**, **e**–**k**); 50 μm (**c**); 100 μm (**b**,**d**). HAD17 hESC line.

analysis of BrdU incorporation showed that 47.8% ± 6.3 (mean ± s.d.) of the cells in suspension were $BrdU^+$ compared to 40.18% ± 3.1 of cells in monolayer cultures ($n = 3$). The doubling time, determined by analyzing the decay of carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling[26] was ~24 h (**Supplementary Fig. 7b**), similar to the doubling time of these hESCs cultured on feeders (23.5 h). The percentage of annexin-V^+ apoptotic cells was 4.6% ± 2.9 in suspension compared to 5.8% ± 2 among hESCs cultivated on feeders, and the percentage of propidium iodide (PI)-positive necrotic cells was 11.3% ± 2.4 and 10.1% ± 5.9, respectively (**Supplementary Fig. 7c**). Thus, the levels of proliferation, cell death and apoptosis in suspension cultures were similar to those in standard feeder-dependent culture conditions.

Given the similar doubling time and cell death rate in our suspension and monolayer cultures, we explored the contribution of cell loss during passaging to the lower expansion rate in suspension. Passaging of the suspension cultures was performed weekly by mechanical trituration into smaller aggregates and further culture for 48 h in the presence of Rho-associated kinase (ROCK) inhibitor[27] to reduce cell death. After mechanical passaging, cell loss was 58.3% ± 5.6 with the suspension culture system, compared to 21.4 ± 7.7 with monolayer cultures ($P < 0.001$). Hence, cell loss during passaging was the major cause of the lower expansion rate in suspension. This problem might be alleviated by refinement of the passaging method.

We evaluated whether the suspension culture conditions can support the derivation of new hESC lines. Sixteen human blastocysts, diagnosed as carriers of genetic abnormalities and destined to be discarded, were obtained. The inner cell masses were isolated with the assistance of a laser[22] from 15 blastocysts. The inner cell masses and an additional intact blastocyst were cultured in the suspension culture conditions. The cells of two of the inner cell masses from embryos with neurofibromatosis, and of the intact embryo, affected by spastic paraplegia 4, proliferated. Each of these three cell clusters were mechanically dissociated into multiple cell aggregates. The three newly derived cell lines (HAD17–19) were further expanded for an additional 10 weeks (**Fig. 2** and **Supplementary Figs. 8** and **9**). The morphological characteristics of the clusters of the three cell lines were similar to those of clusters of established cell lines. The cells within the newly derived clusters expressed alkaline phosphatase and markers of pluripotent

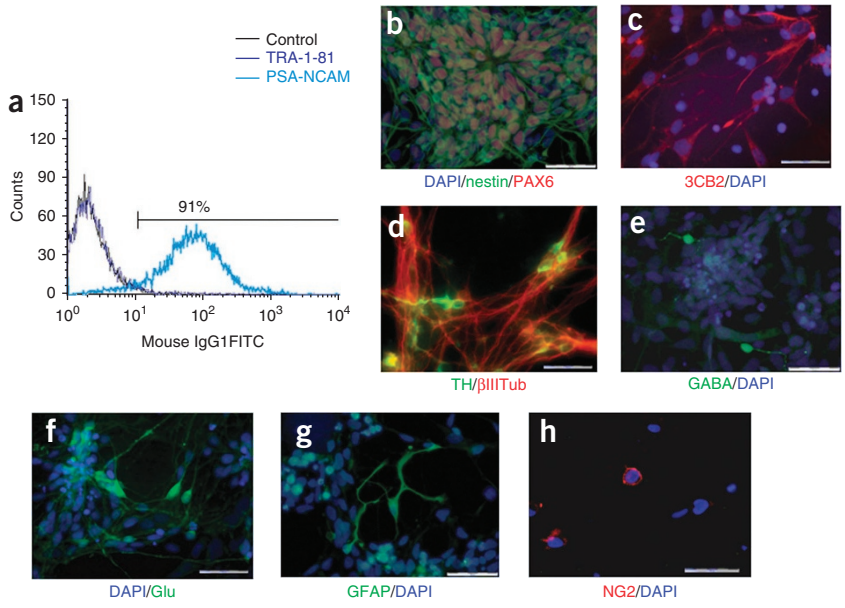

Figure 3 Controlled conversion of the hESC clusters in suspension into neural precursor spheres. Clusters of H7 cells, cultivated in suspension for 7 weeks, were transferred and further cultured 4 weeks in a chemically defined medium supplemented with noggin and FGF2. (**a**) FACS analysis of one representative experiment showing that 91% of the cells expressed PSA-NCAM, whereas 1.2% expressed TRA-1-81. (**b**–**h**) After plating and culturing for 1 week on laminin, indirect immunofluorescence staining showed cells within rosettes expressing markers of neural precursors such as nestin and Pax6 (**b**), the neural stem/radial glial cell marker 3CB2 (**c**), subtypes of neurons expressing β-III tubulin and tyrosine hydroxylase (TH) (**d**), GABA (**e**) and glutamate (**f**), as well as cells expressing the astrocyte marker GFAP (**g**) and the marker of oligodendroglial progenitors NG2 (**h**). Nuclei are counterstained by DAPI. Scale bars, 20 μm.

stem cells. Upon plating on human feeders, they gave rise to colonies and cells with morphological characteristics of undifferentiated hESCs, which expressed markers of pluripotent stem cells, including TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4. Upon differentiation of the free-floating clusters *in vitro* into embryoid bodies and plating, all three lines differentiated into progeny representing the three germ layers. The karyotype at passage 10 was normal in all lines. The clusters were frozen and successfully thawed by vitrification[22]. Although the suspension culture system could promote the derivation of hESC lines, it did not allow clonal expansion. The successful derivation of new hESC lines in suspension demonstrated the robustness of the culture system in promoting proliferation of undifferentiated hESCs. These results showed that feeders or attachment to an ECM layer are not required to develop hESC lines and that new lines can be derived in suspension conditions.

Lastly, we determined whether the hESC free-floating clusters could be directed to differentiate in suspension into clusters enriched for cells of a specific lineage. For this purpose, clusters of HES1 and H7 hESC lines (after 10 weeks cultivation) were cultured for 4 weeks in a chemically defined medium supplemented with FGF2 and noggin, as described[14]. The clusters gradually acquired the morphology of neural spheres, and FACS analysis showed that ≥90% of the cells expressed the neural marker PSA-NCAM, whereas the expression of TRA-1-81 was downregulated (**Fig. 3a**). We next demonstrated the potential of the neural precursors within the spheres to differentiate into progeny representing the three neural lineages. After plating the neural precursors on laminin and culturing for a week in the presence of survival factors, they differentiated into neurons expressing β-III tubulin, tyrosine hydroxylase (TH), GABA and glutamate (**Fig. 3b–h** and **Supplementary Fig. 10**). The neural precursors also gave rise to glial fibrillary acidic protein (GFAP)-expressing astrocytes and NG2-expressing oligodendrocyte progenitors (**Fig. 3g,j**). After 1 week of differentiation, the cultures also included remnants of neural precursors in rosettes co-expressing PAX-6 and nestin, and neural stem/radial glial cells expressing 3CB2 (**Fig. 3b,c** and **Supplementary Fig. 10a,b**). When the plated spheres were differentiated in the presence of FGF8 and sonic hedgehog (SHH) (which promote midbrain dopamine neuronal differentiation[28]), multiple neurons co-expressing engrailed-1 and TH were observed (**Supplementary Fig. 10f**). Co-expression of these markers is characteristic of midbrain dopaminergic neurons[29]. Thus, the clusters of hESCs could be directed to differentiate in suspension into a population highly enriched for precursors of a specific lineage.

In conclusion, we have demonstrated that pluripotent hESCs can be derived, propagated and directed to differentiate into neural precursors in a feeder-free suspension culture system of floating clusters. This is an important step toward developing controlled suspension systems for large-scale expansion followed by directed differentiation in bulk of hESCs.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

D.S. designed and performed the experiments, analyzed the data and wrote the manuscript; H.K. and M.C. performed the neural differentiation study; S.E.-R. conducted immunostainings and confocal analysis; Y.G. performed the teratoma studies; P.I. contributed to developing the concept of suspension culture; T.T. contributed to the experiments; M.I. performed PCR analysis; E.A. contributed to embryo recruitment, culture and isolation of inner cell masses; R.R. and Y.B.-Z. conducted karyotype analysis. B.R. conceived the study and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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1. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* **18**, 399–404 (2000).
2. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
3. Ludwig, T.E. *et al.* Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* **24**, 185–187 (2006).
4. Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C. & Bongso, A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat. Biotechnol.* **20**, 933–936 (2002).
5. Xu, C. *et al.* Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* **19**, 971–974 (2001).
6. Braam, S.R. *et al.* Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification. *Nat. Protoc.* **3**, 1435–1443 (2008).
7. Wang, L. *et al.* Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. *Blood* **110**, 4111–4119 (2007).
8. Kurosawa, H. Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. *J. Biosci. Bioeng.* **103**, 389–398 (2007).
9. McDevitt, T.C. & Palecek, S.P. Innovation in the culture and derivation of pluripotent human stem cells. *Curr. Opin. Biotechnol.* **19**, 527–533 (2008).
10. Lock, L.T. & Tzanakakis, E.S. Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture. *Tissue Eng. Part A* **15**, 2051–2063 (2009).
11. Nie, Y., Bergendahl, V., Hei, D.J., Jones, J.M. & Palecek, S.P. Scalable culture and cryopreservation of human embryonic stem cells on microcarriers. *Biotechnol. Prog.* **25**, 20–31 (2009).
12. Oh, S.K. *et al.* Long-term microcarrier suspension cultures of human embryonic stem cells. *Stem Cell Res. (Amst.)* **4**, 4 (2009).
13. Krawetz, R. *et al.* Large-scale expansion of pluripotent human embryonic stem cells in stirred suspension bioreactors. *Tissue Eng. Part C Methods* **8**, 8 (2009).
14. Itsykson, P. *et al.* Derivation of neural precursors from human embryonic stem cells in the presence of noggin. *Mol. Cell. Neurosci.* **30**, 24–36 (2005).
15. Hoover, C.S. & Martin, R.L. Antibody production and growth of mouse hybridoma cells in Nutridoma media supplements. *Biotechniques* **8**, 76–82 (1990).
16. Stockinger, H. Serum-free medium for mammalian cells. US patent 5,063,157 (1991).
17. Amit, M., Shariki, C., Margulets, V. & Itskovitz-Eldor, J. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol. Reprod.* **70**, 837–845 (2004).
18. Xu, C. *et al.* Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells* **23**, 315–323 (2005).
19. Pyle, A.D., Lock, L.F. & Donovan, P.J. Neurotrophins mediate human embryonic stem cell survival. *Nat. Biotechnol.* **24**, 344–350 (2006).
20. Beattie, G.M. *et al.* Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells* **23**, 489–495 (2005).
21. Furue, M.K. *et al.* Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc. Natl. Acad. Sci. USA* **105**, 13409–13414 (2008).
22. Turetsky, T. *et al.* Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. *Hum. Reprod.* **23**, 46–53 (2008).
23. Herszfeld, D. *et al.* CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. *Nat. Biotechnol.* **24**, 351–357 (2006).
24. Baker, D.E. *et al.* Adaptation to culture of human embryonic stem cells and oncogenesis *in vivo*. *Nat. Biotechnol.* **25**, 207–215 (2007).
25. Mitalipova, M.M. *et al.* Preserving the genetic integrity of human embryonic stem cells. *Nat. Biotechnol.* **23**, 19–20 (2005).
26. Lyons, A.B. & Parish, C.R. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* **171**, 131–137 (1994).
27. Watanabe, K. *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat. Biotechnol.* **25**, 681–686 (2007).
28. Lee, S.H., Lumelsky, N., Studer, L., Auerbach, J.M. & McKay, R.D. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat. Biotechnol.* **18**, 675–679 (2000).
29. Yan, Y. *et al.* Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* **23**, 781–790 (2005).

ONLINE METHODS

HESC feeder-dependent culture system. Human ESCs of HES1, HES2 (ref. 1) and H7 (ref. 2) lines at passages 24–33, 24–27 and 38–45, respectively, with normal karyotypes, were cultured on foreskin human feeders in KO medium consisting of 85% KO-DMEM, 15% KO-SR, 1 mM glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen) and 4 ng/ml FGF2 (Peprotech), as previously described[30]. HES1 and HES2 cells were passaged weekly with Ca/Mg^{2+}-free PBS supplemented with 0.05% EDTA (Biological Industries) or type IV collagenase (1 mg/ml; Invitrogen), whereas mechanical dissociation was used for passaging H7. To determine expansion rate, in each passage, colonies from one well were mechanically removed, dissociated by trituration, and replated on feeders in four wells. To determine average cell number per well, 7 d after passage, the colonies in each of three wells were disaggregated and counted and the average number of cells per well was calculated. The fourth well was further passaged as above. To determine cell loss during passaging, half of the colonies that were removed from each well were triturated as during cell passaging. Cell number was compared between the triturated and nontriturated clusters 2 h after plating.

Suspension culture system for propagating hESC. hESC colonies were dissociated with collagenase IV (1 mg/ml, 1.5–2 h at 37 °C) and by agitation of the culture plate. The colonies were triturated into small cell clusters, which were suspended within nonadherent 12-well tissue culture dishes (Costar, Corning) at a density of ~$0.7–1.2 \times 10^6$ cells/ml in Neurobasal medium, 14% KO-SR, L-glutamine 2 mM, 50 units/ml penicillin, 50 μg/ml streptomycin, 1% nonessential amino acids (all from Invitrogen). The medium was supplemented with FGF2 20 ng/ml, activin A 25 ng/ml (both from Peprotech), fibronectin 1 μg/ml (BD Biosciences), laminin 0.5 μg/ml, gelatin 0.001% (both from Sigma), the neurotrophins BDNF, NT3 and NT4, 10 ng/ml each (Peprotech) and 1× Nutridoma-CS (Roche). Aggregation and overgrowth of clusters was occasionally prevented by trituration with a 1,000 μl pipettor tip as required. Once a week, the clusters from each well were partially disaggregated by gentle trituration and split into two wells for further culture in fresh medium supplemented with ROCK inhibitor (Sigma)[27] 10 μM, which was removed after 48 h. Trituration could be replaced with similar results by using stem cell passaging tool (Invitrogen). The medium was changed every other day. For medium replacement, every other day, the tissue culture dishes were tilted under binocular microscope until cell clusters settled at the lowest part of each well and 80% of the medium was gently replaced. To determine average cell number per well, 7 d after passage the clusters in each of three wells were disaggregated and counted and the average number of cells per well was calculated. To determine cell loss during passaging, half of the clusters in each well were triturated as during cell passaging. Cell number was compared between the triturated and nontriturated clusters after 2 h.

FACS and alkaline phosphatase activity analysis. The hESC clusters were dissociated with PBS solution containing 2.25 mM EDTA and 0.06% trypsin, for 10 min at 37 °C, followed by gentle trituration to a single-cell suspension. The hESCs were then washed with PBS supplemented with 1% BSA and 0.05% sodium azide (both from Sigma). The cells were incubated with anti-SSEA4 (1:100, mouse monoclonal IgG3; Developmental Studies Hybridoma Bank (DHSB)), anti-TRA-1-60 (1:100, monoclonal mouse IgM; Chemicon), anti-TRA-1-81 (1:100, monoclonal mouse IgM; Chemicon), anti-SSEA3 (1:100, monoclonal rat IgM; Chemicon), and anti-PSA-NCAM (1:100, monoclonal mouse IgM; Chemicon). Control hESCs were stained with respective isotype control antibodies. Primary antibodies were detected using fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (1:100, Dako) or Alexa Fluor-labeled goat anti-rat IgM (1:100, Invitrogen). PI was added (final concentration of 4 μg/ml) for better gating of viable cells. FACS analysis was performed using the FACSCalibur system (Becton, Dickinson). Alkaline Phosphatase Substrate kit I (Vector) was used to analyze alkaline phosphatase activity within clusters according to the manufacturer's instructions.

Replating of hESCs cultivated in suspension on feeders. Floating aggregates of hESCs were triturated with a 1,000 μl pipettor tip into small clusters that were plated on fresh feeders, cultured in KO-SR medium supplemented with 4 ng/ml FGF2, and the resulting colonies were passaged routinely as described above. Alkaline phosphatase activity within colonies was analyzed as for clusters.

Differentiation *in vitro*. For embryoid body formation, clusters of undifferentiated hESCs were transferred and cultured for 3–4 weeks in DMEM medium (Invitrogen), supplemented with 20% FBS (Biological Industries), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acid stock, 50 units/ml penicillin, 50 μg/ml streptomycin. For further differentiation, the embryoid bodies were dissociated using trypsin (0.025%, 3 mM EDTA in PBS) and plated on poly-D-lysine (30–70 kDa, 10 μg/ml; Sigma) and laminin (4 μg/ml; Sigma) precoated glass coverslips for an additional 1–2 weeks culture in the same medium.

For controlled differentiation into neural spheres, the hESC clusters were transferred and cultured for 3 weeks in DMEM/F12 medium (Invitrogen) supplemented with B27 (1%, Invitrogen), FGF2 (20 ng/ml) and noggin (600 ng/ml, R&D Systems), followed by 1 week in the presence of FGF2 without noggin.

For further differentiation, the neural spheres were triturated to small clusters and plated on poly-D-lysine and laminin-coated glass coverslips and cultured for an additional week with DMEM/F12/B27 medium in the presence of survival factors (ascorbic acid (200 μM, Sigma), NT-4 and BDNF (10 ng/ml each, Peprotech)). For midbrain differentiation, the plated partially disaggregated spheres were cultured in the presence of 100 ng/ml of FGF8 and 200 ng/ml SHH (both from R&D Systems) for a week followed by further differentiation for a week in the presence of survival factors as above.

Immunostaining. Cells were fixed with 4% paraformaldehyde for 20 min at 23 °C. For immunostaining with anti-intracellular marker antibodies, cell membranes were permeabilized with 0.2% Triton X100 (Sigma) in PBS for 5 min. The cells were incubated with the following primary antibodies: anti-OCT-3/4 (mouse IgG, 1:100, Santa Cruz Biotechnology), anti-β-III-tubulin (mouse monoclonal IgG2b, 1:2,000, Sigma), anti-TH (1:200, Pel Freeze), anti-GABA (1:1,000, Sigma), anti-glutamate (1:2,000, Sigma), anti-3CB2 (1:100, DHSB), anti-GFAP (1:100, Dako), anti-NG2 (1:50, Chemicon), anti-human muscle actin (1:50, Dako), anti-SOX-17 (1:50, R&D Systems). Primary antibody localization was performed by using FITC-conjugated goat anti-rabbit immunoglobulins (1:20-50, Dako), or goat anti-mouse immunoglobulin conjugated with Cy3 (1:500, Jackson ImmunoResearch). Mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector) was used for nuclei counterstaining and the specimens were visualized with a Nikon E600 fluorescent microscope.

For immunostaining of ECM components, clusters of hESCs cultivated in the presence or absence of ECM components were permeabilized in 0.5% Triton X-100/4% paraformaldehyde and fixed in a 4% paraformaldehyde/PBS-5% sucrose solution. The clusters were stained in suspension with anti-fibronectin (rabbit polyclonal antibody, a gift from K.M. Yamada) and anti-human laminin (rat monoclonal antibody, 1:100, Chemicon). Primary antibodies were detected using RhodamineX-conjugated donkey anti-Rabbit IgG and Cy2-conjugated donkey anti-Mouse IgG (Jackson Immunoresearch Labs). Imaging performed by quadruple laser-assisted confocal microscopy (FluoView FV100, Olympus), with a 40× objective UPLAPO40xOI2.

Teratoma formation. The potential of the hESCs to form teratoma tumors was evaluated in NOD/SCID mice. These experiments were approved by the Institutional Committee for animal research of the Hebrew University/Hadassah Medical School. Cluster of hESCs were injected under the testicular capsule of 6-week-old NOD/SCID mice (Harlan) (30–40 clumps per testis). Eight to twelve weeks later, the resulting tumors were removed, embedded in paraffin and sections were stained with H&E. The specimens were visualized with a Nikon TE300 microscope with a 20× objective.

Derivation of hESCs in suspension. *In vitro*–fertilized human embryos that were not diagnosed in the preimplantation period as genetically normal were recruited for the study subject by informed consent of the couples. The study was approved by the ethics committee at the Hadassah Medical Center as well as the Israeli Ministry of Health National Helsinki Committee for Genetic Research in Humans. Fifteen inner cell masses of abnormal blastocysts were isolated by a laser-assisted system[22] and transferred to the suspension culture

conditions as above. The medium was supplemented with ROCK inhibitor 10 μM during the initial 7 d. One whole embryo was also plated in the same conditions. Proliferating clusters were mechanically dissected into smaller aggregates for further propagation in suspension.

Analysis of apoptosis necrosis and proliferation. Proliferation was determined by FACS analysis of BrdU incorporation using the BrdU Flow Kit (BD Biosciences) after 4 h incubation with BrdU according to the manufacturer's instructions. Cell division was monitored according to the technique described by Lyons and Parish (1994) with slight modifications. Clusters of hESCs were incubated in PBS supplemented with CFSE at a final concentration of 5 μM at 23 °C for 15 min. CFSE-labeled cells were washed three times with Neurobasal medium and cultured for up to 72 h. At 24 h intervals, samples of clusters were harvested for FACS analysis (as above) of CFSE fluorescence intensity. Cell apoptosis was measured with the Phosphatidyl Serine Detection kit (IQ Products) according to the manufacturer's instructions. Cells were washed in calcium buffer and incubated with FITC-conjugated anti-annexin V antibodies for 20 min. Propidium iodide (PI) was added to label nonviable cells and FACS analysis was performed as above.

Karyotype. For karyotype analysis, hESC clusters were reseeded on feeders and expanded with collagenase type IV for 3–4 passages. The colonies were incubated for 40 min with 0.2–0.3 μg/ml demecolcine (Sigma). The hESC colonies were then removed from the feeders, dissociated with 0.05% EDTA, centrifuged at 170*g* for 5 min, resuspended in 0.075 M KCl (Sigma) and incubated for 10 min in 37 °C, followed by fixation with 3:1 methanol/acetic acid. The karyotype of 10–20 metaphases was analyzed using the G-banding method.

RT-PCR. Total RNA was isolated using TRI-Reagent (Sigma). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and random primers, according to the manufacturer's instructions (Promega). PCR was carried out using Taq DNA Polymerase (Gibco-BRL) with denaturation, 94 °C; 30 s, annealing, 55 °C; 30 s, extension, 72 °C; 45 s for 40 cycles. Primer sequences (forward and reverse 5′–3′) and the length of the amplified products were as follows:

hOCT4 (AGTGAGAGGCAACCTGGAGA, GTGAAGTGAGGGCTCCCATA; 273 bp);
hNANOG (CGAAGAATAGCAATGGTGTGACG, TTCCAAAGCAGCCTCCA AGTC ; 328 bp);
hREX-1 (GAGCCTGTGTGAACAGAAC, CATAGCACACATAGCCATC; 322 bp);
hTERT (CTGCAGCTCCCATTTCAT, GGATGGTCTTGAAGTCTG; 306 bp);
hFGF5 (GATCCCACGAAGCCAATA, GCTCCGACTGCTTGAATC; 338 bp);
hCG (GTCAACACCACCATCTGTGC; GGCCTTTGAGGAAGAGGAGT; 285 bp);
αFP (CCATGTACATGAGCACTGTTG;CTCCAATAACTCCTGGTATCC; 338 bp);
FOXA2 (ACTGTGTAGACTCCTGCTTCTTC;GCACGCAGAAACCATAAAT; 305 bp);
SOX17 (CGCACGGAATTTGAACAGTA;GGATCAGGGACCTGTCACAC; 180 bp);
Brachyury (CTTCCCTGAGACCCAGTTCA;CAGGGTTGGGTACCTGTCAC; 289 bp);
Goosecoid (TTCCAGGAGACCAAGTACCC,TCGTCTGTCTGTGCAAGTCC; 298 bp);
hGAPDH (GACAACAGCCTCAAGATC, GTCCACCACTGACACGTT; 311 bp)

For Q-PCR TaqMan Assays-on-Demand Gene Expression Products (OCT4, Hs01895061_μ1; NANOG, Hs02387400_g1; REX1, Hs00399279_m1; FGF5, Hs00738132_m1; Brachyury, Hs00610080_m1; Goosecoid, Hs00418279_m1; SOX17, Hs00751752_s1; FOXA2, Hs00232764_m1; hCG, Hs00361224_gH; alphaFP, Hs00173490_m1), TaqMan Universal PCR Master Mix and ABI Prism 7900HT Sequence Detection System (Applied Biosystems) were used. β-glucuronidase (GusB, Hs99999908_m1) was an internal reference for normalization.

Statistical analysis. Data are presented as mean ± s.d. The significance of differences between treatments was calculated using one-tailed *t*-test.

30. Gropp, M. & Reubinoff, B. Lentiviral vector-mediated gene delivery into human embryonic stem cells. *Methods Enzymol.* **420**, 64–81 (2006).

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