Optimized mouse ES cell culture system by suspension growth in a fully defined medium

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Mouse and human embryonic stem (mES and hES) cells have become one of the most intensively studied primary cell types in biomedical research. However, culturing ES cells is notoriously labor intensive. We have optimized current ES cell culture methods by growing mES cells in suspension in a defined medium. This protocol is unsurpassed in time efficiency and typically requires only 20 min of effective hands-on time per week. This protocol maintains a very high degree of pluripotent cells partly by mechanical separation of spontaneously differentiating cells. mES cells can be cultured for extended periods (>6 months) without the loss of pluripotency markers. High passage (>20) adherent mES cultures containing contaminating differentiated cells can be rescued and enriched in undifferentiated ES cells.

INTRODUCTION

Embryonic stem (ES) cells have a unique property in that they can give rise to all cell lineages of the animal including germ cells[1–3]. Mouse ES (mES) cells have been used extensively to generate recombinant mice for two decades, and human ES cells, ever since their first appearance in the literature, have been anticipated to contribute to novel cell-based therapies. However, most current mES cell culture methods[1–6] (**Table 1**) are time-consuming, and the cells are constantly subjected to spontaneous differentiation if not monitored closely. Many existing protocols also have the disadvantage that several components used in culture medium (e.g., FCS, BSA, gelatin and Matrigel) are not fully defined and are derived from animal sources[1–8]. In many laboratories, ES cells are grown on mouse primary embryonic fibroblasts that regularly need to be re-established and are a source of pathogenic contamination. FCS is increasingly substituted with serum-free supplements (often referred to as 'serum replacements'). The contents in commercial serum replacements are typically not disclosed but include high concentrations of proteins, presumably BSA and growth factors. Ideal properties of ES cell culture methods are fully defined culture components obtained from xenobiotic-free sources with minimal batch-to-batch variation.

Consistent with other findings, we have also experienced difficulties in maintaining mES cells using previously described methods without feeder cells for several passages. However, mES cells could be passaged in nondefined medium (FCS) for at least a month without differentiation when forced to grow in suspension using a bioreactor[8].

Defined media have been shown to suppress ES cell differentiation by reducing integrin expression[7]. The use of a defined medium in mES culture was reported by Ying *et al.*[5] This group used DMEM/F12 supplemented with N2 and B27, bone morphogenetic protein 4 (BMP4) and leukemia inhibitory factor (LIF). BMP was found to be essential to suppress neuronal differentiation by induction of Id proteins through the Smad pathway. B27 contains 0.1 mg l^{-1} all-*trans*-retinol, which may be converted into retinoic acid, a strong neural inducer. Although not presenting direct evidence, the authors of this study claimed that BMP was also required in the absence of B27.

With the aim to establish a cell culture platform for high-throughput mES cell research, we re-evaluated current mES culture protocols to develop a novel protocol that is simplified, rapid, robust and highly efficient (**Fig. 1**). We found that mES cells grown in suspension in DMEM/F12 supplemented with N2 (which contains transferrin, insulin, progesterone, putrecine and selenite[9]), LIF and basic fibroblast growth factor (bFGF) (herein called ESN2 medium) obliviated the need for exogenous BMP and sustained mES cell cultures for extended periods. As we found that it was necessary to grow the cells in nonlimiting density conditions, it is possible that endogenous BMP compensates the exclusion of exogenously added BMP. The rate of ES growth in ESN2 medium with and without bFGF as compared with standard culture conditions was not determined, but for short-term culture bFGF was not necessary for mES cell proliferation in ESN2 medium. However, after repeated passages, cells grown without bFGF proliferated slower.

Using the ESN2 medium, we discovered that mES cells readily formed spheres that detached and continued to grow in suspension (**Fig. 2**). This effect allowed for the removal of differentiated cells by filtration and recovery of only fast proliferating colonies. Nonproliferating colonies or slowly proliferating colonies were not retained during filtration and were thus removed. In addition, adherent cells were left in the disposed culture vessel (therefore it is advisable to use standard cell culture-coated vessels as described below, to facilitate this adherence). We observed that suspension growth, in general, appeared to induce less differentiation than adherent growth on various substrates. Filtration provides a simple solution for removing other unwanted cells—when transferring mES from coculture systems with primary mouse embryonic fibroblast-feeder cells, feeder cells will rapidly be phased out from the culture within a few passages as only proliferating and nonadhering cells are recovered during passaging. mES cells grown using our protocol (strains successfully used include D3, E14 with substrains, GSI-1, R1 and EB5) maintain a high proliferation rate as well as a minimal spontaneous differentiation. In this respect, our protocol is comparable with or exceeds current protocols. Indeed our protocol can

TABLE 1 | Examples of mES cell culture methods.

Basal medium	Supplements	Fully defined culture system?	Culture type	Culture vessel surface	Growth factors	Physical separation of differentiated cells?	References
DMEM	FCS	No (contains FCS, MEFs)	Adherent	MEFs	LIF	No	1 and 2
DMEM	SR (contents not disclosed by manufacturer)	No (contains BSA, MEFs)	Adherent	MEFs	LIF	No	4
DMEM/F12	N2 + B27 (contents in B27 not disclosed by manufacturer)	No (contains gelatin)	Adherent	Gelatin	LIF, BMP	No	5
ESF (composition unknown)	N2	No (contains BSA)	Adherent	Various	LIF	No	7
DMEM	FCS	No (contains FCS)	Suspension (bioreactor)	—	LIF	No	6 and 8
DMEM/F12	N2	Yes	Suspension	—	LIF, bFGF	Yes	This protocol

bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; LIF, leukemia inhibitory factor; MEFs, primary mouse embryonic fibroblasts; mES, mouse embryonic stem; SR, serum replacement (Invitrogen).

even be used to rescue and cleanup valuable existing cultures that have deteriorated. We managed to increase the number of cells expressing the stem cell marker SSEA-1 from 17–55% (EB5 ES strain[10], harboring a blasticidin-resistance gene, grown on gelatin in knockout-DMEM, serum replacement, LIF and blasticidin) to >90% (ESN2 without blasticidin) as assayed by flow cytometry[11], after switching to our protocol (see **Fig. 2d**). In mRNA expression microarray experiments, we recently reported that the mES cell transcriptome shows few changes after switching to our protocol and that these changes were reversed after returning the cells to growth on mouse primary embryonic fibroblasts[12]. In addition, the cells efficiently generated neurons[12] and beating cardiomyocytes after differentiation (data not shown). mES cells grown using our protocol were able to generate chimeric mice after injection into blastocysts, which functionally corroborates our data suggesting that they retain pluripotency (**Fig. 2g**). mES cells grown in suspension using this protocol did not appear to acquire an epistem cell (epiSC)[13] fate because expression of Fgf5, a marker for epiSCs, was absent in the cells when analyzed by Taqman RT-PCR (data not shown).

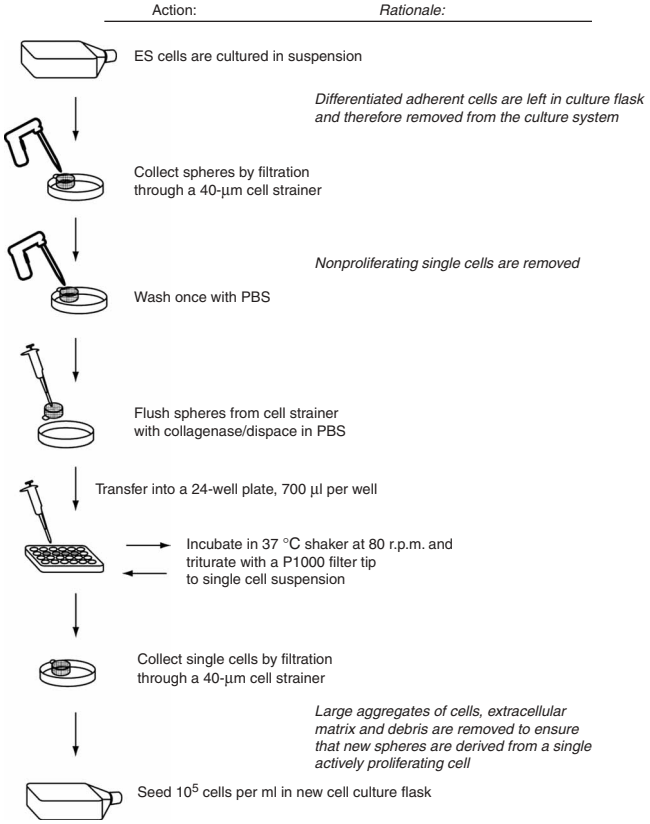

Figure 1 | Schematic drawing outlining the Steps 5–10 of the procedure to passage embryonic stem (ES) cells in suspension.

The DMEM/F12 supplemented with N2 and B27, BMP4 and LIF media used by Ying *et al.*[5] and also commercially available as ESGRO Complete medium (Chemicon) also supported the growth of mES cells in suspension, although the aggregates were often less spherical and appeared to adhere to the cell culture vessel to a larger extent than using the ESN2 medium. Spontaneous necrosis appearing as dark and disintegrating spheres also occurred more often. Hence, mES cells can be grown in suspension with the medium used by Ying *et al.* but this protocol is less robust and requires more attention.

In summary, our protocol significantly increases time and cost efficiency and uses fully defined medium, which ensures minimal variation over time. This protocol provides the following specific advantages:

1. It is markedly less time-consuming than previous methods because it contains fewer steps; for example, filtering replaces centrifugation and medium is not changed between passaging once a week. The use of collagenase/dispase or TrypLE Express, an animal origin-free dissociation enzyme, does not require enzyme inhibition or removal by centrifugation, but only dilution in medium.
2. The medium contains few and defined components. N2 is used alone without B27.
3. A novelty in our protocol is the mechanical enrichment of proliferating stem cells without simultaneous laborious visual inspection under an inverted microscope. Filtration enriches proliferating cells that give rise to new spheres. Nonproliferating as well as proliferating but adherent cells that frequently have a differentiated morphology are removed during filtration.

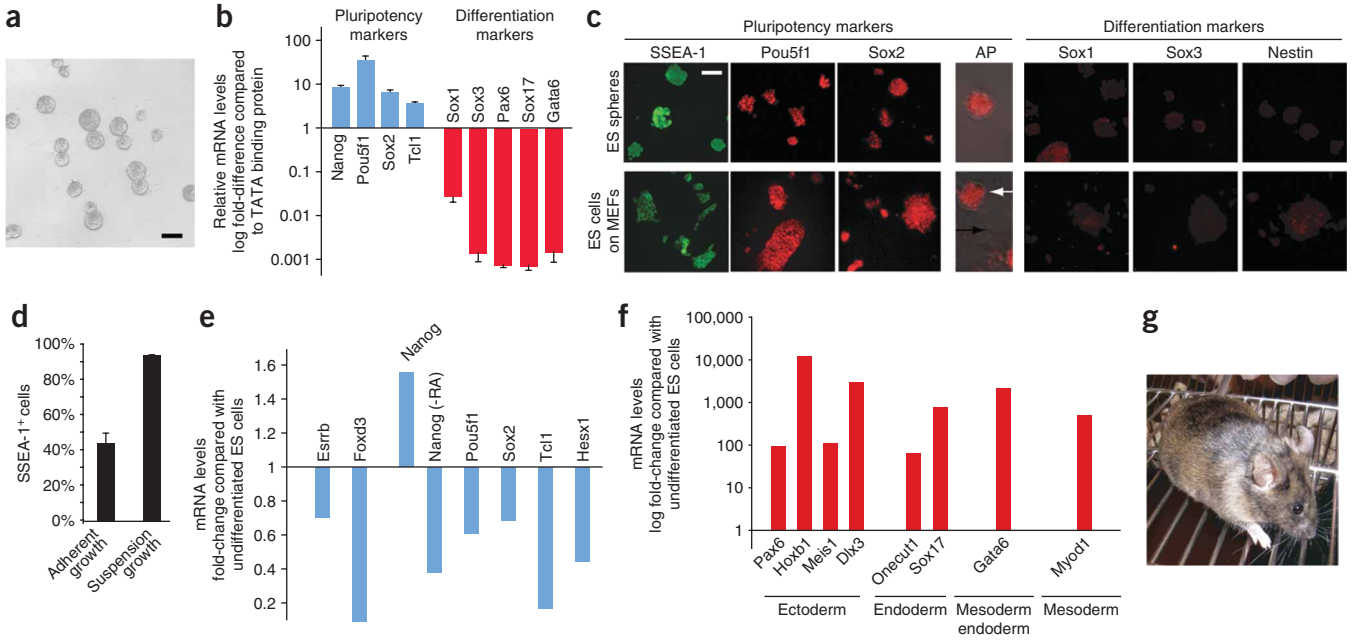

Figure 2 | Analysis of pluripotency of embryonic stem (ES) cells grown in ESN2 medium. (**a**) Photomicrograph of ES cell spheres, 7 d after passaging (the ES cells were grown in suspension in ESN2 medium for 3 months). (**b**) Analysis of pluripotency markers, Nanog, Pou5f1, Sox2 and Tcl1 and differentiation markers Sox1, Sox3, Pax6, Sox17 and Gata6 in ES cells maintained in ESN2 medium for 5–25 passages by Taqman quantitative RT-PCR (qRT-PCR). Shown are expression levels relative to the housekeeping reference gene TATA-binding protein. (**c**) Analysis of pluripotency (SSEA-1, Pou5f1, Sox2) and differentiation (Sox1, Sox3, nestin) markers by immunostaining and alkaline phosphatase (AP) staining of ES cells grown in DMEM with serum replacement adherently on mouse embryonic fibroblasts (MEFs)[4] or as spheres for 8–12 passages (i.e., >3 month). Cells were fixed in 24-well plates with 4% paraformaldehyde for 15 min, washed and stained with antibodies in primary buffer (PBS/1% BSA/0.3% Triton X-100) at 4 °C overnight, followed by staining with secondary antibodies for 2 h in secondary buffer (PBS/1% BSA). White arrow points at an AP$^+$ ES colony and the black arrow at AP$^-$ MEFs. (**d**) Analysis of ES cells grown in suspension in ESN2 medium for more than five passages compared with adherent growth in DMEM with serum replacement on gelatin under blasticidin selection (EB5 strain ES cells[10]). Enrichment of pluripotent cells in suspension culture is visualized by SSEA-1 immunostaining (Alexa Fluor 488-conjugated SSEA-1 antibody in PBS + 1% BSA) followed by flow cytometry. (**e**,**f**) Analysis of pluripotency as determined by differentiation capacity. Taqman qRT-PCR analysis of expression after 48 h of differentiation on laminin in ESN2 with 1 μM retinoic acid (RA) but without leukemia inhibitory factor and basic fibroblast growth factor showing (**e**) a decrease of pluripotency markers (Nanog expression decreased rapidly only when RA was removed) and (**f**) a strong induction of differentiation markers representing all three germ layers (ectoderm, mesoderm and endoderm). (**g**) A chimeric mouse with characteristic patched fur color generated from ES cells grown in suspension in ESN2 medium. Scale bars in (**a**) = 200 μm, (**c**) = 50 μm.

4. This protocol is suitable for upscaling for high-throughput screens. Suspension growth requires no coating (feeder cells or matrix) of cell culture vessels and consequently there is no need for enzymatic removal of cells from surfaces. Centrifugation is substituted by filtration, which enables development of an enclosed culture system using valves and filters.

MATERIALS

REAGENTS

- ES cells
- DMEM/F12 GlutaMAX (Invitrogen, cat. no. 31331028)
- HEPES (Invitrogen, cat. no. 15630056)
- β-Mercaptoethanol (Invitrogen, cat. no. 31350010)
- N2 supplement (Invitrogen, cat. no. 17502048)
- LIF (ESGRO, mouse LIF; Chemicon, cat. no. ESG1107)
- bFGF (human; R & D Systems, cat. no. 233-FB)
- Collagenase/dispase (Roche Diagnostics, cat. no. 11 097 113 001)
- Dulbecco's PBS (D-PBS; Invitrogen, cat. no. 14190094)
- TrypLE Express (Invitrogen, cat. no. 12605010)
- DMSO (Sigma, cat. no. D2650)

EQUIPMENT

- 20-, 200- and 1,000-μl Pipettes
- Filter pipette tips
- 40-μm Cell strainer (BD Falcon, cat. no. 352340)
- Benchtop 37 °C orbital shaker
- Inverted phase contrast microscope with a ×2.5 objective
- Cell culture flasks (175 cm^2; BD Falcon, cat. no. 353028)

REAGENT SETUP

LIF solution (10^6 U ml^{-1}) Dilute LIF to 10^6 U ml^{-1} in DMEM/F12 (1,000×). This diluted stock solution can be stored for more than a year at +4 °C. This gives a final concentration of 1,000 U ml^{-1} when added to ESN2 medium (see below).

bFGF solution (10 μg ml^{-1}) Dissolve bFGF in D-PBS to 10 μg ml^{-1} (1,000×). Make 100 ml aliquots and freeze at –70 °C. The aliquots can be stored for more than a year. Use within 1 week after thawing.

20× Stock collagenase/dispase solution (20 mg ml^{-1}) Dissolve the collagenase/dispase powder in D-PBS to 20 mg ml^{-1} (20× stock solution), sterile filter and make 200 μl aliquots and freeze at –20 °C. The aliquots can be stored for more than a year. This gives a final concentration of 1 mg ml^{-1} when added to ESN2 medium (see below).

ESN2 medium To one 500-ml bottle of DMEM/F12, add 5 ml N2 supplement, 5 ml HEPES and 0.5 ml β-mercaptoethanol. Before use add LIF and bFGF (to give final concentrations of 1,000 U and 10 ng ml^{-1}, respectively).

Freezing medium 10% DMSO in suspension medium.

PROCEDURE

Transfer from adherent culture

1| Prepare a 10-cm diameter cell culture dish with ES cells grown, for example, on mitotically inactivated mouse primary fibroblasts or gelatin.

2| Wash once with PBS and add 3 ml TrypLE Express, incubate at 37 °C for 5–10 min and triturate to single cell suspension. *Note:* TrypLE Express cannot be exchanged to trypsin in combination with the serum-free media used in this protocol as neither FCS nor trypsin inhibitor is added in the next step.

3| Transfer the cells into a 50-ml centrifuge tube and add D-PBS to 50 ml final volume, centrifuge at 160*g* for 5 min, invert rapidly to remove liquid and resuspend in 10 ml ESN2 medium.

4| Filter cell suspension through a 40-μm cell strainer to remove aggregates and transfer the filtrate into a T175 cell culture bottle with 70–100 ml ESN2 medium.

Propagation

5| Collect spheres to passage from medium by filtering through a 40-μm cell strainer. Invert the cell strainer and flush out spheres with 4 ml collagenase/dispase in D-PBS. Alternatively, collect spheres by centrifuging in a 50-ml tube at 160*g* for 5 min, remove supernatant and add 5–10 ml TrypLE, screw the cap tightly and incubate in 37 °C water bath for 5 min. Add 10 ml D-PBS and triturate 5× with a 10-ml pipette. Add D-PBS to 50 ml and centrifuge at 160*g* for 5 min to wash the cells. Remove supernatant by quickly inverting the tube. Finally, add 10 ml warm medium and proceed directly to Step 7.
▲ **CRITICAL STEP** When collecting spheres in a strainer, one has to take precautions not to infect the cells. Use gloves sprayed with 70% ethanol or forceps to hold the strainer when inverting it to flush out the spheres.

6| Incubate cells in a 37 °C orbital shaker for 30 min.
▲ **CRITICAL STEP** Too short incubation time reduces the yield of single cells recovered after filtration. Extending the incubation time has little consequence—we have even recovered live cells after up to 24 h of incubation.

7| After incubation, triturate cells using a P1000 pipette with filter tip to a single cell suspension.

8| Filter the cells again with a 40-μm cell strainer to remove debris and remaining cell aggregates.

9| Determine the cell density with a hematocytometer and seed between 5×10^3 and 1×10^4 cells per ml in a culture flask, typically a T175 bottle with 70–100 ml ESN2 medium. Keep the cells in an incubator at 37 °C/5% CO_2. The medium does need not be changed until the next passage (Step 10).
▲ **CRITICAL STEP** If cells are seeded at a too high density, the culture medium will appear yellow and harm the cells (which may be rescued by passaging them immediately).

10| After 6–8 d of growth, split ES cells again to a 1:4-to-1:8 ratio, depending on the growth rate of the particular ES strain.
▲ **CRITICAL STEP** Spheres must not grow too large (e.g., until they appear to darken when monitored through an inverted microscope). We find it very useful to use a ×2.5 magnification objective to get a good overview.
? TROUBLESHOOTING

Freezing/thawing

11| To freeze, concentrate the spheres grown for 4–8 d after passaging by filtering in a 40-μm cell strainer, inverting the cell strainer and flushing cells out in ESN2 medium supplemented with 10% vol/vol DMSO. Aliquot cells to freezer vials. Freeze cells in a controlled rate isopropanol freezing container in −70 °C before transfering to liquid nitrogen for long-term storage.

12| To thaw, place a frozen vial at 37 °C taking care not to disrupt the spheres which are very fragile. Transfer the content to a 40-μm cell strainer to remove the freezing medium. Flush the spheres out in ESN2 medium and lightly triturate (cells are now sensitive to mechanical insult) and again filter through a 40-μm cell strainer to remove debris and recover single cells.

● TIMING

Steps 1–4, initiation: require <20 min of work
Steps 5–9, propagation: require <20 min of work excluding the 30-min incubation
Step 10, the continued maintenance of an ES line does not require any more work between passaging every 6–8 d
Step 11, freezing: requires <5 min of work
Step 12, thawing: requires <5 min of work

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible reason	Solution
Spheres appear to darken	Too dense culture and/or spheres have become too large	Passage spheres
Spheres cease to grow	Proliferation rate slows when spheres become large	Passage spheres
Spheres proliferate slowly or not at all	Cell density may be too low	Seed the cells at 10^4 cells per ml after passage
	Basic fibroblast growth factor may have reduced potency	Thaw a new aliquot
Appearance of small dark particles resembling bacteria	During clean up of cultures, differentiated cells die after passaging	No action necessary

ANTICIPATED RESULTS

Pluripotency of cultured cells

To assess morphology of ES cells and expression of markers, we analyzed ES cells maintained in ESN2 medium for 5–25 passages for markers of pluripotency and early stages of differentiation. Analyzing gene expression by Taqman quantitative RT-PCR (qRT-PCR), we found that ES markers were highly expressed and differentiation markers absent (**Fig. 2b**). Analyzing protein expression by enzyme and immunostaining, we found that SSEA-1, Pou5f1, alkaline phosphatase and Sox2 were expressed in all cells, while expression of the differentiation markers Sox1, Sox3 and nestin was undetectable (**Fig. 2c**). The pluripotency of cells that had been cultured using our protocol for several passages was also analyzed by production of chimeric mice. Cells were injected into blastocysts, which were implanted into pseudopregnant foster mothers[3]. Chimeric mice were obtained (**Fig. 2g**) showing that pluripotency was maintained in the ES cells during culture, although the potential of the cells to contribute to the germ line was not tested. We furthermore assayed for gene expression by Taqman qRT-PCR after a 48-h differentiation by retinoic acid and found that pluripotency markers rapidly decreased (**Fig. 2e**) and that differentiation markers of all three germ layers were dramatically induced (**Fig. 2f**).

Enrichment of SSEA-1 expressing ES cells

When the relative number of SSEA-1^+ ES cells in ESN2 was analyzed by flow cytometry after staining with Alexa Fluor 488-conjugated SSEA-1 antibody (Chemicon; at 1:400 in PBS with 1% cell culture-tested BSA for 2 h), we found that $>90\%$ of the cells were expressing SSEA-1 (**Fig. 2d**). In contrast, long-term culture of blasticidin-resistant EB5 ES cells kept under selection pressure and in adherent growth conditions on gelatin-coated cell culture dishes with 2,000 U ml^{-1} LIF and commercial undefined serum replacement (Invitrogen) resulted in cells of differentiated morphology and an average of 43% and never $>60\%$ SSEA-1^+ cells (**Fig. 2**).

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1. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
2. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638 (1981).
3. Bradley, A., Evans, M., Kaufman, M.H. & Robertson, E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255–256 (1984).
4. Shimizukawa, R. *et al.* Establishment of a new embryonic stem cell line derived from C57BL/6 mouse expressing EGFP ubiquitously. *Genesis* **42**, 47–52 (2005).
5. Ying, Q.L., Nichols, J., Chambers, I. & Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281–292 (2003).
6. Fok, E.Y. & Zandstra, P.W. Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation. *Stem Cells* **23**, 1333–1342 (2005).
7. Hayashi, Y. *et al.* Integrins regulate mouse embryonic stem cell self-renewal. *Stem Cells* **25**, 3005–3015 (2007).
8. zur Nieden, N.I., Cormier, J.T., Rancourt, D.E. & Kallos, M.S. Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors. *J. Biotechnol.* **129**, 421–432 (2007).
9. Bottenstein, J.E. & Sato, G.H. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA*
10. Niwa, H., Miyazaki, J. & Smith, A.G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372–376 (2000).
11. Andäng, M. *et al.* Histone H2AX-dependent GABAA receptor regulation of stem cell proliferation. *Nature* **451**, 460–464 (2008).
12. Moliner, A., Ibanez, C.F., Ernfors, P. & Andäng, M. Mouse embryonic stem cell-derived spheres with distinct neurogenic potentials. *Stem Cells Dev.* (in the press).
13. Brons, I.G. *et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191–195 (2007).