A New Feeder-Free Technique to Expand Human Embryonic Stem Cells and Induced Pluripotent Stem Cells

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Abstract: The optimal maintenance of human embryonic stem cells (hESC) *in vitro* is generally observed in the presence of a feeder-layer of mouse embryonic fibroblasts in a serum-containing medium. Various approaches are now available to remove the feeder requirement. Today, the best feeder-free system for the maintenance of hESC and induced pluripotent stem (iPS) cells is based on a serum-replacement medium on a matrice of Matrigel. Some reports have also shown feeder-free maintenance of hESC in the presence of laminin, fibronectin, vitronectin or collagen IV. However these combinations are expensive. Here we describe an alternative to the current feeder-free short-term amplification of hESC and iPS cells using culture grade plastic dishes pre-coated with 10-20% fetal calf serum. hESC retain expression of various stem cell markers and also retain the ability to differentiate *in vitro.* Similar results were observed with human iPS cells. This feeder-free culture system is reliable, convenient and allows for the rapid amplification of hESC and iPS cells to numbers suitable for many cell biology techniques.

INTRODUCTION

Human embryonic stem cells (hESC) are derived from the inner cell mass of the human blastocyst and are theoretically able to differentiate into each cell type of the body [1, 2] while induced pluripotent stem (iPS) cells are obtained through the introduction of a cocktail of transcription factors (such as Oct-4, Sox2,c-myc and klf4, or Oct-4, Sox2, Nanog and LIN28) into an adult somatic cell resulting in its reprogramming into a pluripotent cell [3-8]. The techniques originally used for hESC derivation and maintenance required the presence of a feeder-layer of mouse embryonic fibroblasts (MEF) in a serum-containing medium, conditions very similar to the ones used to isolate and propagate mouse ESC as well as iPS cells. Although complex and poorly defined, serum culture conditions form the basis for some of the most characterized and reliable hESC information currently available (see [9] for review). Ultimately, researchers would like to achieve fully defined conditions for hESC derivation and propagation in order to work towards clinically acceptable sources of cells, and much work now aims at finding optimal defined animal-free conditions for the *in vitro* maintenance of hESC [9]. Serum-free, feeder-free culture conditions using a matrice of Matrigel and Knockout Serum Replacement (KSR)-based medium in combination with basic fibroblast growth factor (bFGF) dominate the literature and have become accepted practice in most hESC laboratories. Although the majority of publications detailing serum-free propagation of hESC utilizes KSR+bFGF medium, other serum-free and chemically defined media have been reported [10-14]. As Matrigel is an extracellular matrix extracted from the Engelbreth-Holm-Swarm mouse sarcoma, it is a limitation for the development of a chemically defined environment for hESC culture. Some reports have shown feeder-free maintenance in the presence of other substrates than Matrigel, such as laminin (LN), fibronectin (FN), vitronectin (VN) or collagen IV. Since all of these conditions require a matrix of some sort, it is inevitable that there will be variability between cultures due to reagent batches. Ultimately it would be ideal to develop a uniform, well defined and reliable culture system for maintenance of hESC and iPS cells.

In an effort to further develop a chemically-defined environment that robustly maintains hESC, we examined the potential of hESC to grow on alternative matrices than the currently available Matrigel. Our work demonstrates the short term maintenance of hESC on culture grade plastic dishes (Corning® CellBIND®) coated with serum. This protocol allows an efficient method to obtain sufficient feeder-free hESC numbers for most common biochemical techniques. Interestingly, this protocol also allows the short term maintenance of human iPS cells.

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MATERIALS AND METHODOLOGY

All sets of experiments were performed at least three times in triplicates, unless specified (n refers to the number of independent experiments performed on different cell cultures). All data are expressed as mean ± standard error of the mean (SEM).

Cell Culture

HES-3 (WiCell), ENVY-HES-3 (ES Cell international) and MEL-1 (Australian Stem Cell Centre) stocks were cultured as previously described [1, 15]. iPS cell lines MR90 and ESC were cultured as described below. Briefly, hESC and iPS cells were grown in the presence of mitotically inactivated mouse embryonic fibroblasts (MEF), in hESC medium containing Dulbecco's Modified Eagle Medium (DMEM, without sodium pyruvate, glucose 4500 mg/l), supplemented with insulin/transferrin/selenium 1%, β-mercaptoethanol 0.1 mM, NEAA 1%, glutamine 2 mM, penicillin 25 U/ml, streptomycin 25 µg/ml (all from Invitrogen) and fetal calf serum 20% (Hi-Clone). Medium was changed every second day. Alternatively, H1 (WiCell) was maintained on MEF together with medium containing DMEM F12, 20% KSR, 1% NEAA, 2mM glutamine, 0.1mM β-mercaptoethanol and 8ng/ml bFGF (Invitrogen). Medium was changed every day and in some experiments hESC were passaged using collagenase every week. ES4CL1, ES4CL2, ES4CL3, ES4CL4, MR90C2 and MR90C4 iPS cell stocks were obtained from Prof. J. Thomson (University of Wisconsin, USA) and maintained on MEF together with 20% KSR and 100 ng/ml bFGF. iPS cell media was changed daily and cultures were passaged once a week with Collagenase type 1 (Stem Cell Technologies). Cells were kept in a 5% CO_2 incubator at 37°C. For feeder-free cultures, plastic dishes (Corning® CellBIND® Surface) were precoated with 20% fetal calf serum in phosphate buffered saline (PBS) for at least 3 hours at 37°C, unless otherwise stated. For iPS cell experiments, CellBIND 6 well plates were coated overnight at 4°C in hESC medium (containing 20% fetal calf serum). Plates were then washed twice with PBS prior to the addition of hESC/iPS cell fragments. Cells were incubated in the presence of either hESC medium, mTeSR1 basal medium (Stem Cell technologies), KSR and bFGF (R&D), hescgro (Millipore), sphingosine-1-phosphate (S1P, 10 µM, Biomol) and platelet-derived growth factor (PDGF, 20 ng/ml, Peprotech). Medium was changed every 2 days. In some experiments, serum coating was replaced by human FN (1 µg/ml, BD), VN (1 µg/ml, BD) and LN (20 µg/ml, BD) overnight. In some experiments, cells were passaged every week, by either mechanical transfer or by dispase treatment in CELLBIND dishes precoated with serum. Prior to further transfer, eventual dissociated parts of the colonies were scraped off the plates.

Immunofluorescence

hESC were fixed in ethanol, NSC and their derivative cells were cultured on glass chamber slides (seeded with the appropriate matrix) prior to fixation with 4% paraformaldehyde (PFA) or ethanol (for Oct-4), blocked in 10% fetal calf serum-PBT, and immunostained using the following antibodies against: mouse monoclonal anti-Oct3/4 (Santa Cruz), mouse anti-nanog (eBioscience), mouse TG-30 reactive with CD9 (gift from Prof M. Pera), mouse TRA-1-81 (Santa Cruz), S100β protein (Sigma), MAP2ab (Thermo Scientific). Cells were then immunostained with the appropriate conjugated secondary antibodies (Alexa Fluor 568 or 488, Molecular probes-Invitrogen). Nuclei were counter-stained with Hoechst-33342 (Sigma-Aldrich). Specificity of the staining was verified by the absence of staining in negative controls consisting of the appropriate negative control immunoglobulin fraction (Dako, data not shown).

FACS Sorting

For these experiments, there was no scraping of eventual differentiated parts. Colonies were dissociated into a single cell suspension with 0.25% trypsin-EDTA (Gibco), and fixed in 4% PFA for 10 minutes followed by permeabilization in 90% methanol. hESC were immuno-labeled for Oct-4 (Santa Cruz, dilution 1:100) and iPS cells were immuno-labeled for SSEA4 to avoid residual Oct-4 expression (DSHB, 15µg/ml) in blocking solution (PBT +10% FCS) for 60 minutes then were resuspended in anti-mouse Cy5 secondary antibody (Jackson Immunoresearch) for 30 minutes, followed by a wash in blocking solution before being immediately sorted using a FACSCalibur cell sorter. Oct-4 was not used for iPS cells as this marker has been shown not to always be downregulated in iPS clones [8].

Neural Induction of hESC

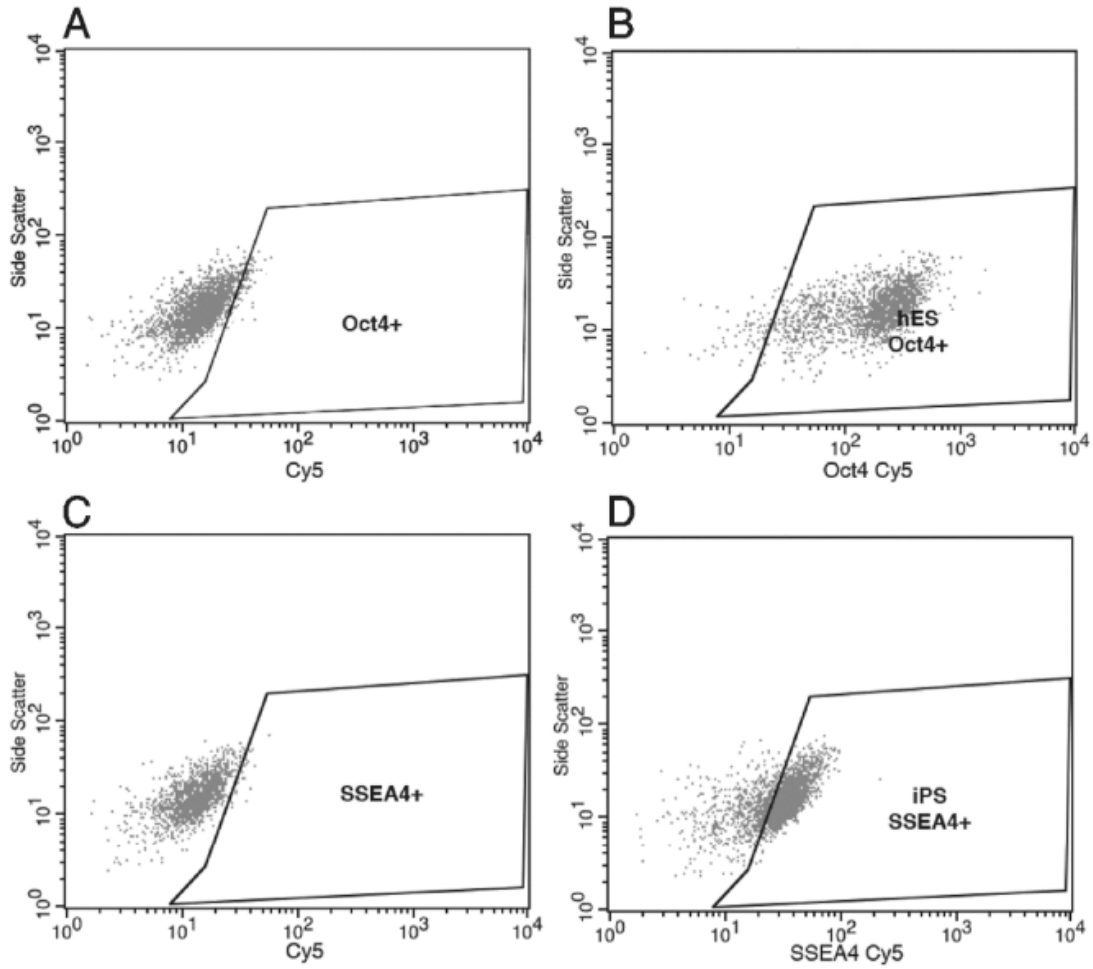
Neuronal induction by noggin (500 ng/ml, R&D) was performed as described [16] in the absence of MEF, on plastic in either hESC-medium, KSR + bFGF 60 ng/ml or mTeSR1. Noggin-treated cells were harvested after 14 days by mechanical dissection and were further subcultured in suspension in low-attachment 96-well plates (Corning) containing neurobasal medium (NBM) with bFGF and EGF (20 ng/ml each, R&D) as neurospheres [17]. After 2 weeks growth in suspension culture, the neurospheres were plated as previously described onto laminin- or fibronectin-coated glass chamber slides in NBM without growth factors [17] and allowed to attach for 5 days. NBM was changed every second day.

RESULTS

hESC were mechanically transferred into dishes pre-coated with either LN, VN, FN or with hESC medium containing 20 % fetal calf serum, in various media that have been demonstrated to maintain hESC undifferentiated: hESC medium (containing 20 % fetal calf serum), 20% knockout serum replacement (KSR) +/- bFGF (4 ng/ml), S1P (10 µM) and PDGF (20 ng/ml), hESCgro and mTeSR1 media. None or little attachment was observed with S1P/PDGF or with hESCgro media on plastic treated with LN, FN, VN while cells attached in hESC medium, KSR or mTeSR1 media, although with variability (data not shown). When plastic dishes were pre-coated with 10-20% serum in culture media or in PBS, cells attached in all conditions. After 6-7 days, immunostaining revealed that Oct-4, nanog, TG-30 and TRA-1-81 were still highly expressed in hESC maintained in mTeSR1 thus suggesting that this medium maintains hESC undifferentiated in the absence of feeder cells and Matrigel

Fig. (1). (**A-F**) Immunostaining of hESC maintained for 7 days on plastic dishes pre-coated with 10% serum in mTeSR1 medium for Oct-4 (**A**), nanog (**C**) and TG-30 (**E**), with Hoechst-33342 counterstains (**B, D, F**). (**G-H**) Bright field pictures of a 7-day colony of hESC plated on CellBIND® (**G**) and on another standard culture grade plastic dishes (Falcon, **H**) coated with serum, magnification x4. Immunostaining of Oct-4 (**I**) and nanog (**K**) of hESC on day 7 following the fourth passage in mTeSR1 medium, with Hoechst-33342 counterstains (**J, L**). Scale bar 100 μm.

matrice (Fig.**1A-F**, supplementary Fig. **1**). Results were retrieved with a variety of fetal calf sera purchased from different companies, suggesting a limited batch to batch variation. Results were not retrieved when plated on other standard culture grade plastic dishes (Falcon) coated with serum as cells did not attach or when attached showed high levels of



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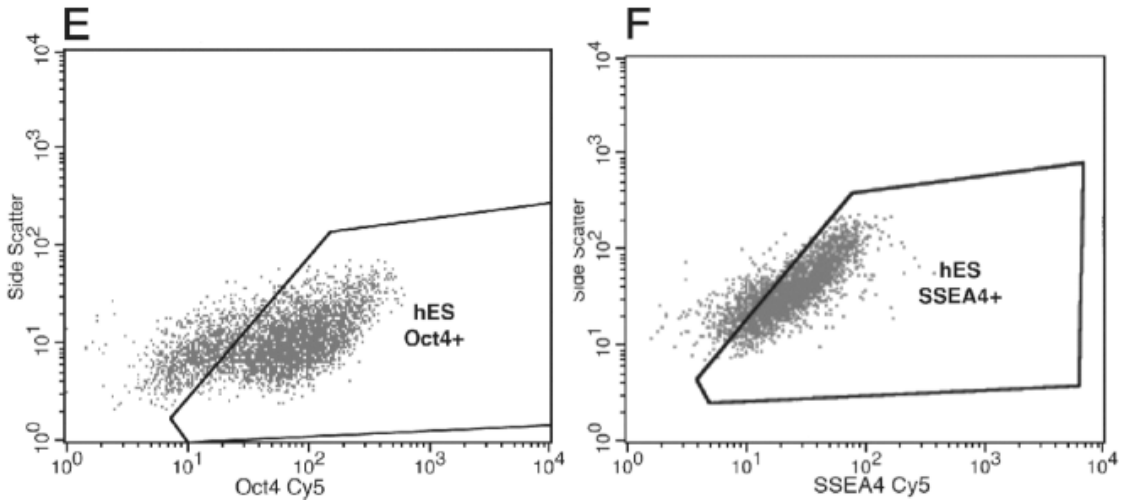

Fig. (2). hESC **(A-B)** and iPS cells **(C-D)** maintained on plastic dishes pre-coated with serum, in mTeSR1 medium or hESC on MEF **(E-F)** were analysed by FACS for Oct-4 and SSEA4 respectively. (**A**) Negative control of hESC with Cy5 secondary. (**B**) hESC labelled with Oct-4 antibody and sorted with Cy5 secondary. (**C**) Negative control of iPS cells with Cy5 secondary. (**D**) iPS cells labelled with SSEA4 antibody and sorted with Cy5 secondary. hESC maintained on MEF labelled with Oct-4 antibody (**E**) or SSEA4 antibody (**F**) and sorted with Cy5 secondary.

spontaneous differentiation (Fig. **1H**). Although parts of colonies cultivated in hESC medium or KSR + 4 ng/ml bFGF retained stem cell marker expression, we observed more spontaneous differentiation in these conditions than in others, rendering these two conditions less suitable for passaging (data not shown). Passaging of cells was thus performed on cells maintained in mTeSR1 media in dishes precoated with serum. On the day of passage (day 7), FACS analysis showed that cells maintained in mTeSR1 medium were mainly Oct-4 positive (63.52 ± 2.623% n=6 *versus* 87.55 ± 3.054% of Oct-4+ve cells in control hESC maintained on MEF n=5; $p<0.001$ by t-test, Fig. **2**) but to maximise the efficiency of transfer, differentiated cells were scraped prior to the mechanical dissection of the hESC colonies. Interestingly, these conditions also allow the culture of iPS cells for 5 days and FACS analysis of the iPS cells showed that 79.28% ± 2.74% were positive for SSEA4 (Fig. **2**). This suggests that the hydrophilicity associated with the CellBIND® dishes enhances hESC and iPS cell attachment and maintenance.

Cells maintained in mTeSR1 medium were passaged every week by enzymatic treatment (dispase, 10 mg/ml, Gibco). On day 4, eventual differentiated parts of the colonies were removed by scraping them off. Seven days following the first passage, colonies mainly express Oct-4 and nanog. Passaging in these conditions was not optimal, as colonies display more differentiation following each passage, as assessed by a decrease in Oct-4 expression. Although cell lines were transferred for up to 4 passages (5 weeks) in both media, we observed limited success in maintenance, with some colonies still positive for Oct-4 and nanog (Fig. **1I-L**) and others showing more differentiation. Similar patterns were observed with mechanical dissociation of hESC colonies (data not shown). Together, these data suggest that culture of hESC on CellBIND® plastic dishes with mTeSR1 is not yet optimal for the passaging of hESC, but allows short term scale-up of cell numbers in feeder-free condition, and thus provides a convenient technique to amplify cell number for experimental purposes. To determine the potential of hESC maintained in feeder-free conditions to undergo directed differentiation, noggin treatment was used as a method for neural induction as previously described [16]. hESC cultured on CellBIND® plastic dishes were treated for 2 weeks with noggin (500 ng/ml) added to either hESC medium as described in [16] or to mTeSR1. Cells were then harvested by mechanical dissec-

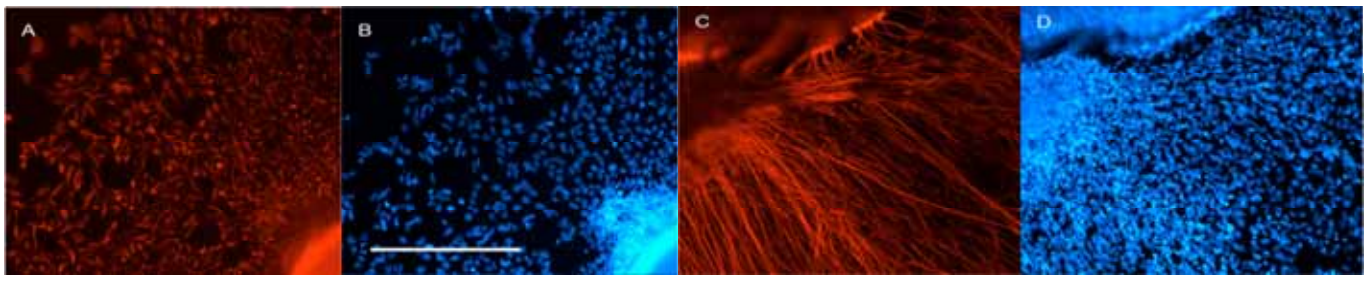

Fig. (3). Neurospheres generated from hESCs grown on Cell Bind dishes were differentiated on fibronectin- and laminin-coated chamber slides. All images were taken at 20x magnification. (**A**) Neurospheres differentiated onto glial and neuronal cells, as revealed by S100β (**A**) and β-tubulin (**C**). (**B, D**) DAPI counterstains. Scale bar = 200 μm.

tion and further subcultured in suspension in NBM with bFGF and EGF (20 ng/ml each). In all culture conditions, cells were able to form neurospheres to the same extent as control cells (noggin-treated hESC on MEF, data not shown), that once plated onto LN or FN gave rise to neural lineages (Fig. **3**). These data were further confirmed by adapting another neural induction protocol [18]. hESC cultured on CellBIND® plastic dishes in mTeSR1 on plastic dishes for 2 and 4 passages were mechanically dissociated, and then subcultured in suspension in NBM supplemented with noggin (700 ng/ml). In these conditions, neurosphere formation was observed and further differentiation of neurospheres to neurons and glia was obtained when plated onto LN or FN substrates, respectively (Fig. **4**). Altogether, these data suggest that the culture of hESC on plastic dishes did not alter the neural differentiation potential of hESC. These data also suggest that the neural induction of hESC by noggin is independent of the feeder layer of MEF, as this effect was retrieved in the absence of MEF.

DISCUSSION

To date, various protocols have been developed for the short and long-term maintenance of hESC in the absence of a feeder-layer of cells. However, these protocols require the use of expensive matrices, such as Matrigel, FN, LN and

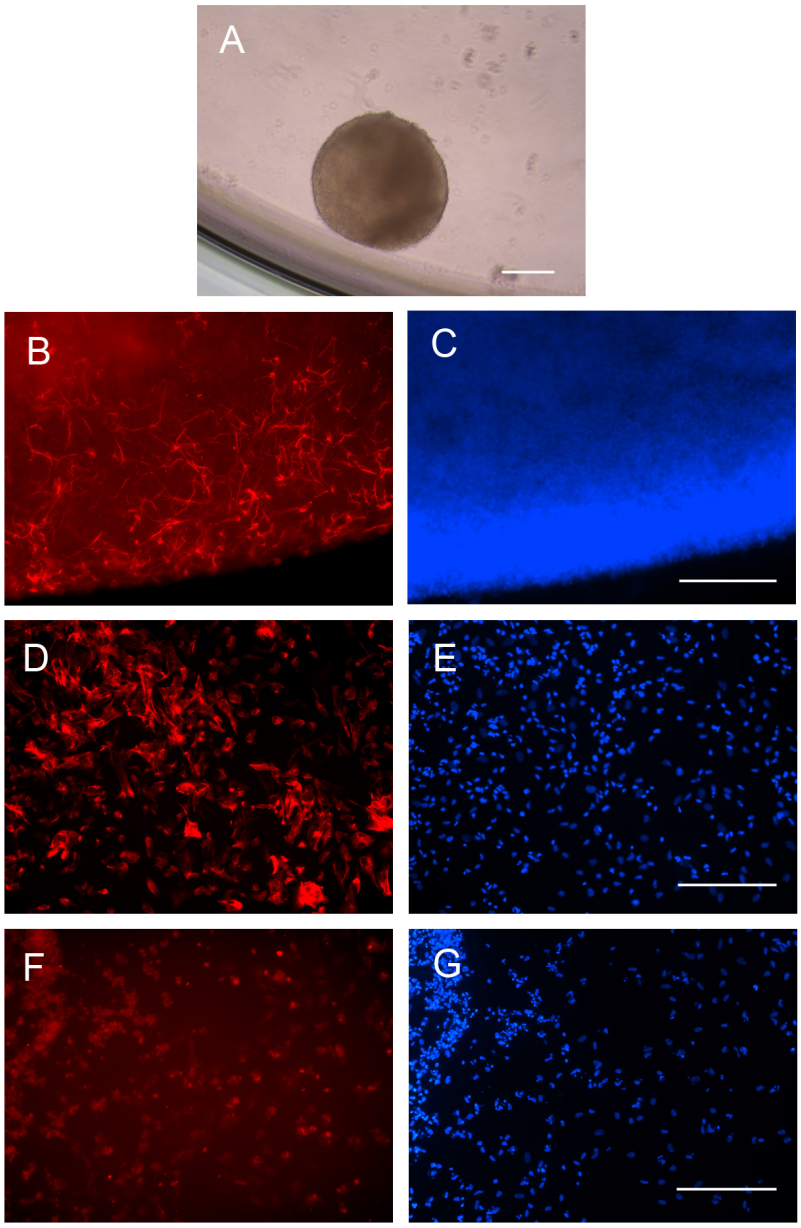

Fig. (4). (**A**) Brightfield images of neurospheres derived from hESC cultured in mTeSR1 following the protocol developed by Itsykson and colleagues (2005). (**B-G**) Neural and glial differentiation of neurospheres derived from hESC cultured on feeder-free substrates in mTeSR1. (**B**) Neurosphere plated onto laminin substrate and immunostained with MAP2ab. (**D**) Neurosphere plated onto fibronectin substrate and immunostained with S100ß. (**F**) Neurospheres plated onto fibronectin substrate and immunostained with mouse IgG (negative control). (**C, E, G**) DAPI counterstains. Scale bar = 200 µm.

collagen or a combination of matrices [19-24]. Apart from Matrigel, extracellular matrix proteins (such as LN, FN, VN) show limited and variable success to maintain hESC. Hence, developing a practical and less expensive protocol for amplifying hESC number for experimental purposes will be a valuable tool for researchers. In this report, we describe an alternative and cost effective method for short-term amplification of hESC in feeder-free and chemically defined media using CellBIND® plastic dishes pre-coated with serum. Noticeably, this protocol also allows the short-term maintenance of human iPS cells.

Previous literature from Vallier *et al.* (2005) reported fetal bovine serum-coating in combination with a chemically defined medium (CDM) for the maintenance of hESC [12] while Bigdeli and colleagues (2008) recently reported the adaptation of two hESC lines to feeder and matrice-free environment [25]. The authors demonstrated that hESC were able to be maintained for prolonged amount of time on culture grade plastic (Primaria® dishes) following a prior adaptation consisting of repetitively passaging cells maintained on Matrigel in neonatal chondrocyte conditioned medium [25]. Although advantageous, this culture system still requires cell conditioned medium and a prior adaptation to the culture condition. Stojkovic and colleagues (2005) reported the maintenance of hESC on tissue culture plates (Nunc) coated with human serum [22]. This protocol has the clear advantage of not requiring the prior adaptation of hESC. However, it still requires the use of conditioned medium from fibroblast-like cells derived from hESC as well as the use of human serum. Lastly, a recent study comparing various matrices and media for the maintenance of hESC showed limited success using human serum but confirmed short-term maintenance of hESC on fetal bovine serum-coating with CDM, yet did not attempt to test this coating with mTeSR1 [26].

The technique described here aims to fulfil a complementary aspect of stem cell biology that is a convenient, flexible and efficient short-term cultivation of hESC that generate sufficient numbers of undifferentiated hESC for experimental procedure, without having to "prime" hESC to a new environment nor to use expensive matrices. Another advantage of the described technique is the non-requirement of high cell number at plating time. Indeed, attachment and short-term maintenance were observed with only 3 hESC colony pieces per well of a standard 6-well dish. As these results were observed on CellBIND® dishes, this suggests that the attachment and short-term maintenance of hESC are linked to the treatment associated to the plastic dishes. Our experiments were performed using various sera, from normal fetal calf serum to hESC-grade serum, and show consistency of results. As we did not observe variation with the various sera tested, this method should not show a modification in attachement due to the serum used for plating. Recently, Harb *et al.* (2008) reported that the ROCK inhibition Y27632 supports hESC culture in matrice-free environment [27], thus suggesting a critical involvement of the Rho/ROCK pathway in hESC maintenance. When assessed on CellBIND® dishes without prior serum coating, we observed that in the presence of Y27632, hESC attached to the surface but did not proliferate to the same extend as in serum-coated conditions (data not shown).

CONCLUSION

This study provides a feeder-free protocol for the neural differentiation of hESC and for the amplification of undifferentiated hESC and iPS cells to numbers suitable for many experimental procedures, such as immunocytochemistry, western-blot, FACS analysis, RT-PCR and various signalling experiments. This protocol has the advantage of being more simplistic, more uniform and less expensive than the use of Matrigel or other extracellular matrix proteins.

ABBREVIATION

bFGF = Basic fibroblast growth factor

FCS = Fetal calf serum

FN = Fibronectin

hESC = Human embryonic stem cells

iPS cells = Induced pluripotent stem cells

LN = Laminin

MEF = Mouse embryonic fibroblasts

NBM = Neural basal medium

KSR = Knockout serum replacement

PBS = Phosphate buffered saline

PDGF = Platelet-derived growth factor

PFA = Paraformaldehyde

S1P = Sphingosine-1-phosphate

VN = Vitronectin.

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