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Directed neuronal differentiation of human embryonic stem cells

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Abstract

Background: We have developed a culture system for the efficient and directed differentiation of human embryonic stem cells (HESCs) to neural precursors and neurons.

HESC were maintained by manual passaging and were differentiated to a morphologically distinct OCT-4$^+$/SSEA-4$^-$ monolayer cell type prior to the derivation of embryoid bodies. Embryoid bodies were grown in suspension in serum free conditions, in the presence of 50% conditioned medium from the human hepatocarcinoma cell line HepG2 (MedII).

Results: A neural precursor population was observed within HESC derived serum free embryoid bodies cultured in MedII conditioned medium, around 7–10 days after derivation. The neural precursors were organized into rosettes comprised of a central cavity surrounded by ring of cells, 4 to 8 cells in width. The central cells within rosettes were proliferating, as indicated by the presence of condensed mitotic chromosomes and by phosphoHistone H3 immunostaining. When plated and maintained in adherent culture, the rosettes of neural precursors were surrounded by large interwoven networks of neurites. Immunostaining demonstrated the expression of nestin in rosettes and associated non-neuronal cell types, and a radial expression of Map-2 in rosettes. Differentiated neurons expressed the markers Map-2 and Neurofilament H, and a subpopulation of the neurons expressed tyrosine hydroxylase, a marker for dopaminergic neurons.

Conclusion: This novel directed differentiation approach led to the efficient derivation of neuronal cultures from HESCs, including the differentiation of tyrosine hydroxylase expressing neurons. HESC were morphologically differentiated to a monolayer OCT-4$^+$ cell type, which was used to derive embryoid bodies directly into serum free conditions. Exposure to the MedII conditioned medium enhanced the derivation of neural precursors, the first example of the effect of this conditioned medium on HESC.

Background

Embryonic stem cells are pluripotent cells that can be derived from the inner cell mass of the preimplantation blastocyst [1,2]. Human ES cells have been isolated by several groups and shown to be able to differentiate to cell types representative of all three germ layers [3,4]. The generation of neural cultures from human embryonic stem cells holds much promise for the investigation of human

neurogenesis, the generation of neurons for pharmacological testing and development of potential cell therapy applications to treat neurological diseases such as Parkinson's Disease [5–7].

There have been several reports of neural differentiation from HESCs, all of which rely on multi-step approaches and stochastic differentiation as embryoid bodies [8,9] or overgrowth of HESC cultures [3,10]. The disadvantages of such approaches include inherent randomness within the differentiation regime, which could lead to variability in the mixed populations that are generated. This could have unforeseen consequences for the proportions and characteristics of specific neuronal subtypes that could be derived. In addition, during the differentiation of ES cells in embryoid bodies a layer of visceral endoderm forms on the outside of the EB [11]. In vivo, the visceral endoderm is known to be an important source of patterning and differentiation signals [12]. A critical step in controlling embryonic stem cell differentiation is overriding or eliminating the early patterning information that is coming from the visceral endoderm in the embryoid bodies.

An approach for directed neural differentiation of mouse embryonic stem cells (MESCs) has recently been described [13]. When mouse ES cells were differentiated as embryoid bodies in the presence of the MedII conditioned medium, an apparently homogeneous stratified epithelium reminiscent of the positionally unspecified neural tube was formed. This differentiation was proposed to occur in the absence of potential signals from extraembryonic endoderm or mesoderm and the neural precursor population generated could therefore represent a naïve population able to be manipulated to differentiate along specific neural pathways.

It is not clear whether differentiation regimes developed for mouse ES cells can be transferred to HESC cultures. One approach that is likely to increase the control over the neural differentiation of HESC cultures is the use of serum free conditions. It is now clear that the emergence of neural lineages during mammalian development requires no, or minimal, cell-extrinsic signalling. There is evidence for an early FGF signal that is required to neuralize the epiblast cells in the chicken and cells within differentiating MESC cultures [14,15]. At a low frequency MESCs can differentiate into a primitive neural stem cell in serum free conditions [16] and MESC-derived neural progenitors thrive in serum free conditions that kill non-neural cell types in differentiating MESC cultures [17]. An additional important factor is FGF2, which has been shown to act as a mitogen for human neural stem cells [18,19], and its activity in enhancing human ES cell proliferation suggests it could also be active in the neural differentiation of these cells.

We have developed an alternate method for the differentiation of neural cultures from HESC. This approach uses an initial controlled differentiation of HESC cell colonies while plated on mouse embryonic fibroblasts, followed by suspension culture of embryoid bodies in serum free conditions, in the presence of 50% MedII and FGF2. Serum free embryoid bodies exposed to MedII (sfEB/MedII) developed a neural precursor population that exhibited characteristic rosette structures 7–10 days after derivation. Parallel cultures not exposed to MedII (sfEB) exhibited much reduced proportions of these neural rosettes. Rosettes were comprised of a central cavity surrounded by ring of cells, 4 to 8 cells in width, striking in their similarity to neurectodermal populations derived from mouse ES cells incubated in MedII. When plated and grown in adherent culture, rosettes grew as circular or ovoid radial structures that were nestin positive, and were surrounded by large interwoven networks of neurons, including presumptive dopaminergic tyrosine hydroxylase positive neurons. This novel directed differentiation approach led to the efficient derivation of neural cultures from HESC and could provide cell populations for the investigation of human neurogenesis, pharmacological studies or cell therapy.

Results

Controlled early differentiation of human ES cells on mouse embryonic fibroblast feeder layers

Human ES cells can be grown on a layer of mouse embryonic fibroblasts (MEFs), where they form domed colonies of undifferentiated cells (Fig. 1A, [3,4]). These cells express high levels of the pluripotent marker protein OCT-4 (Fig. 1C,1G), which is a nuclear-localized POU domain transcription factor. We used the HESC lines BG01 and BG02 [20] in this study, which were indistinguishable in terms of growth and differentiation characteristics in the analysis described here. BG01 and BG02 were maintained on MEFs and passaged by cutting colonies into cubes with drawn out glass needles. HESC culture systems are currently suboptimal and inherent instability is often exhibited, with spontaneous and frequent differentiation occurring within colonies, particularly when the feeder layer is suboptimal or the culture has overgrown. This instability has been used to differentiate HESCs randomly, followed by manual isolation of neural precursors from regions within differentiating colonies [3,10]. In contrast to this essentially uncontrolled differentiation, we observed that the colony morphology of HESCs differed from the optimal, domed colonies when they were passaged onto recently plated feeder cells. When plated on feeder cells that were 0–6 hours old, but not on feeders that were 2 days old or older, typical HESC colonies formed except that in the central region of the colony a "crater" was observed (Fig. 1B). These central cells formed a uniform monolayer, within a ring of stacked

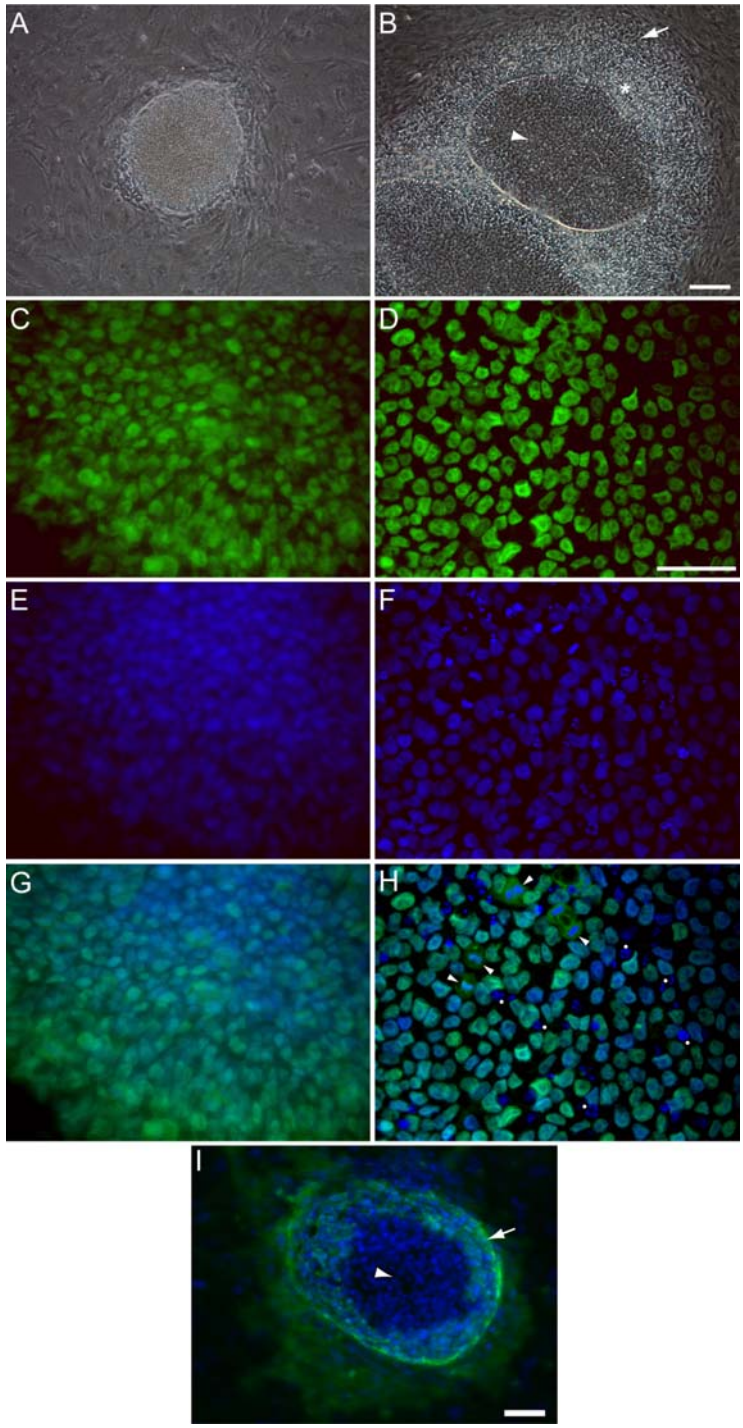

Figure 1
Controlled early differentiation of human ES cell colonies (A) Undifferentiated HESC colony on mouse embryonic fibroblasts. (B) Crater containing colony derived from passaging HESC onto freshly plated mouse embryonic fibroblasts. A central crater of monolayer cells (arrowhead) is surrounded by undifferentiated HESC (*), and differentiating cells in an outer ring adjacent to the feeders (arrow). (C,D) OCT-4 expression in undifferentiated HESC (C) and crater cells (D). (E,F) DAPI staining of the same fields as (C,D). (G,H) merged images of (C-F). Mitotic cells (arrowheads) and some of the apparently apoptotic cells (white dots) are indicated. (I) Anti-SSEA-4 immunostaining of a crater colony. Crater cells (arrowhead) do not stain for the SSEA-4 antigen, a marker of undifferentiated HESC, while the surrounding HESC are SSEA-4$^+$ (arrow). Images and scale bars: (A,B) 10×, 100 μm. (C-H) 40×, 50 μm. (I) 10×, 100 μm.

HESC surrounding the inner monolayer. The monolayer was in direct contact with the tissue culture plastic, or the extracellular matrix that was left behind as the HESC colony had pushed out the underlying feeder layer. HESC colonies typically displace the underlying feeder layer as they proliferate over 3–5 days between passages. The monolayer of cells within the "crater" (crater cells) maintained expression of the OCT-4 protein (Fig. 1D), but were negative for the SSEA-4 antigen (Fig. 1I), a specific marker of undifferentiated HESC [3,4]. Morphological differentiation and loss of the SSEA-4 antigen, coupled with maintenance of OCT-4 suggests that crater cells are an alternate pluripotent cell population derived from HESCs, or an early differentiation intermediate. Domed HESC colonies could not be readily generated by passaging of crater cells (not shown), also suggesting that crater cells were not equivalent to undifferentiated HESC.

The flattened nuclear morphology of crater cells enabled more information on the distribution of OCT-4 within nuclei to be observed (Fig. 1D,1H), compared to undifferentiated HESC (Fig. 1C,1G). For example, OCT-4 appeared to be occluded from some regions of the nucleus. Also, the large majority of OCT-4 protein appeared not to be associated with chromosomal DNA in cells in the metaphase stage of mitosis. OCT-4 immunoreactivity was not observed in fragmented DAPI stained nuclei, a likely indicator of apoptotic cells. Crater cells did not express significant levels of the neural progenitor markers nestin or vimentin (data not shown), which indicated that they were not likely to represent a neural stem cell or progenitor cell population. This data indicated that crater cells could be generated in a controlled manner from HESC within 3–5 days, and that they were essentially morphologically uniform and homogeneous with respect to OCT-4 expression and lacked SSEA-4 expression. This was in contrast to overgrowth of HESCs on mouse feeder layers [3,10], where stochastic differentiation proceeded over several weeks and led to a complex heterogeneous culture from which neural progenitors were manually isolated.

Efficient neural differentiation of HESC "crater cells" in suspension culture

MedII [21], medium conditioned by HepG2 cells, directs the differentiation of mouse ES cells to a neurectodermal population in an apparently homogenous manner [13]. We examined the neuronal differentiation of HESCs and crater cells in response to FGF-2 or FGF-2/MedII, and a summary of our morphological observations of the occurrence of neural precursor rosettes is shown in table 1.

When pieces of HESC colonies were used to generate serum free embryoid bodies, rosettes of neural precursors and differentiated neurons could be generated in suspension and when seeded to a polyornithine and laminin matrix, but the culture was relatively heterogeneous, containing many non-neural cell types (data not shown). In contrast, when HESC derived crater cells were used, far more uniform neural differentiation was observed. Crater cells were purified by peeling the feeder layer and undifferentiated stacked HESC colonies off of the culture dish surface. Watchmaker's forceps were used to hold the feeder layer at the side of the culture dish, and lift this layer and attached HESCs from the dish. This manipulation left the monolayer crater cells attached to the surface. Monolayers of crater cells were cut into 50–200 cell size pieces with glass needles and lifted from the dish. These pieces were transferred to suspension in serum free conditions, where they rounded up into embryoid body-like masses and were referred to as sfEB (serum free embryoid body) or sfEB/MedII (serum free embryoid body exposed to MedII medium). After seven to ten days of suspension culture, sfEB/MedII (Fig. 2A), but generally not sfEB (Fig. 2B), exhibited structured regions consisting of multiple spherical rosette compartments. These were spherical equivalents of the neural rosettes observed during subsequent adherent culture, and were strikingly similar to the morphology exhibited by neurectoderm generated by the differentiation of mouse ES cells in response to MedII [13]. Sectioning and staining with toluidine blue or DAPI demonstrated a characteristic structural organization within sfEB/MedII. Multiple compartments were present in each sfEB/MedII, each with a radial organization of cells often surrounding a central inner cavity (Fig. 2C,2E). The cell layer was typically 4 to 8 cells wide, with nuclei that were elongated and aligned radially. DAPI staining of rosette compartments revealed condensed mitotic chromosomes in dividing cells lining the central core (Fig. 2F,2G), but not elsewhere, indicating that this is likely to be a niche where the proliferative cells of the rosette reside. Toluidine blue staining suggested the presence of extracellular matrix lining the central core, and possibly an additional layer surrounding the rosette unit (Fig. 2E).

Characteristic features of standard embryoid body differentiations using serum were not exhibited by sfEB/MedII:

Table 1: Morphological Assessment of Neural Precursor Differentiation

CELLS	MEDIA	
	FGF-2	FGF-2/MedII
Domed ES cell colonies	+/++	++/+++
Crater cells	+/+++	++++/+++++

+, <10% aggregates exhibit neural precursor rosettes; +++++, >90% aggregates exhibit neural precursor rosettes as the major cell type

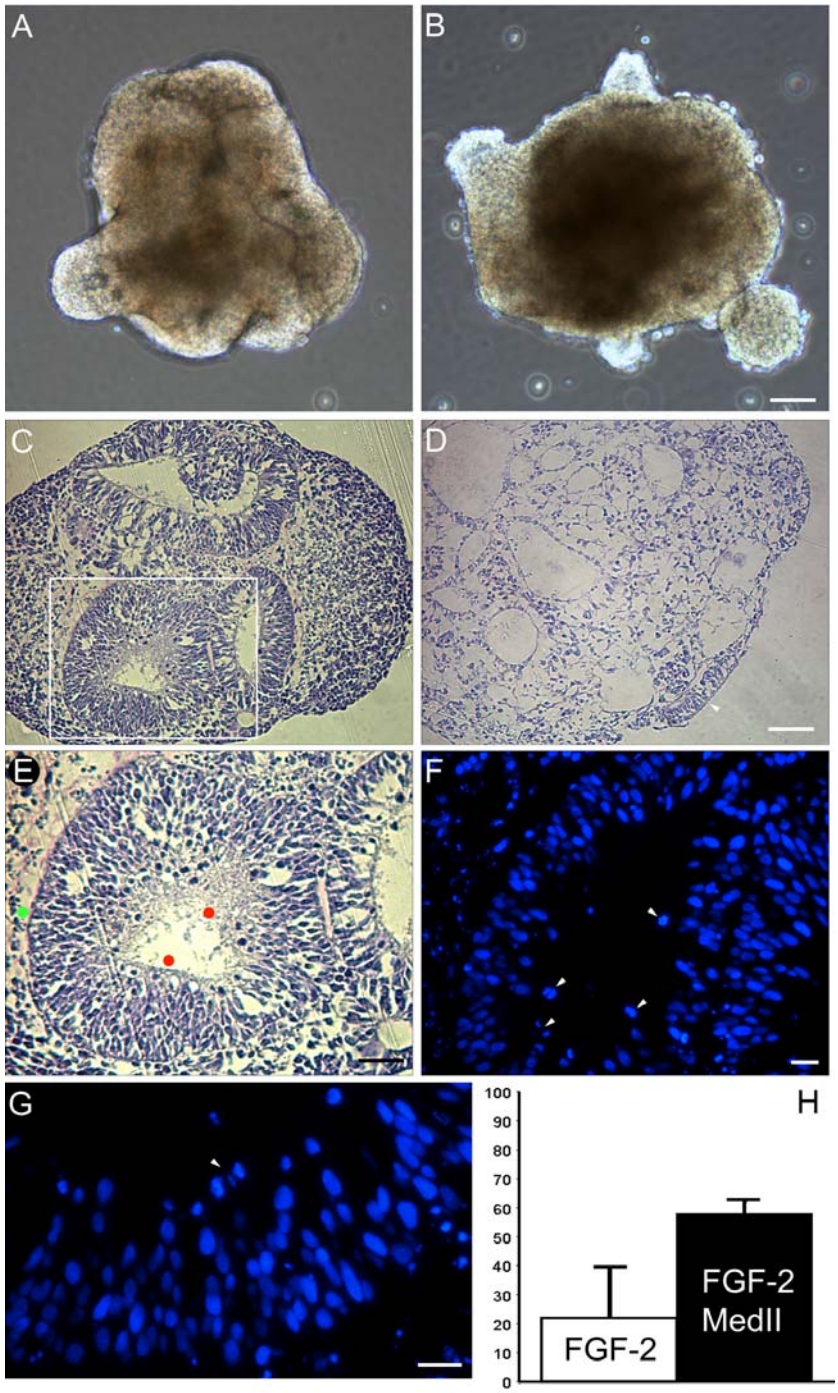

Figure 2

Differentiation of human ES cell derived crater cells in suspension culture in the presence of MedII conditioned medium. Serum free EB/MedII and EB differentiated in suspension in the presence of FGF2/MedII (A) or FGF2 (B). Toluidine blue stained plastic sections of sfEB/MedII (C) and sfEB (D), a partial rosette in sfEB (arrowhead) is indicated. (E) sfEB/MedII rosette, higher magnification of boxed region shown in (C). Presumptive extracellular matrix in central cavity (red dots), and surrounding the rosette (green dot) are indicated. (F) DAPI stained sfEB/MedII rosette indicating the central core of proliferative cells. Condensed mitotic chromosomes are indicated by the arrowheads. (G) DAPI stained sfEB/MedII rosette indicating characteristic elongated radial nuclei and a centralized mitotic figure (arrowhead). (H) Percentage of DAPI stained rosette nuclei in sfEB (white column, 21+/-17%) and in sfEB/MedII (black column, 58.2+/-3.9%) ($P < 0.02$). Images and scale bars: (A,B) 10×, 100 μm. (C,D) 20×, 50 μm. (E) 40×, 25 μm. (F,G) 60×, 10 μm.

a well defined endodermal layer was not observed (Fig. 2A,2C), cysts developed rarely, and beating cardiomyocytes were not observed. A non-rosette cell type(s) was observed in occasional sfEB/MedII derived from crater cells, present as rapidly proliferating regions that were not organized into rosettes (Fig. 2C). Sectioning confirmed the lack of organisation of this cell type, which characteristically had small round nuclei, and could not be found more than approximately 5 cell widths in from the edge of a sfEB/MedII. When further in than this distance, intact nuclei of this cell type(s) were not found, but fragmented and apparently apoptotic/necrotic nuclei were observed. In contrast, rosette cells could survive and proliferate deep within a sfEB/MedII. SfEB/MedII derived from crater cells were often comprised essentially entirely of rosette compartments, or if not, the rosettes could be identified and manually selected for further culture by dissecting them out of the sfEB/MedII.

To quantify the proportions of neural precursors and other cell types in differentiations from crater cells, we counted DAPI stained rosette nuclei and non-rosette nuclei in sections of suspension aggregates (Fig. 2H). In sfEB, the proportion of rosette nuclei was 21.8+/-17% (1470/6715 nuclei), whereas in sfEB/MedII it was 58.2+/-3.9% (3273/5678 nuclei) ($P < 0.02$). Along with reduced proportions of neural precursor rosettes, sections of sfEB demonstrated the presence of heterogeneous, poorly organised cell types and cystic areas (Fig. 2D).

Neuronal differentiation of plated embryoid bodies grown in serum free medium

To test the ability of the rosette cells in the sfEB and sfEB/MedII to differentiate into neurons they were plated on polyornithine and laminin coated cell culture plates. Robust adherent cultures of neurons resulted from plating sfEB and sfEB/MedII, although exposure to MedII enhanced the proportion of neural precursors and neurons significantly (not shown). The embryoid bodies were cut into pieces for plating, where they attached and flattened, with proliferating cells and neuronal extensions migrating out of the seeded pieces over the culture period. SfEB and sfEB/MedII were seeded 3–5 days after derivation, prior to the observation of neural rosettes in suspension, or more than one week after initial derivation, when distinct neural rosettes were formed in sfEB/MedII suspension cultures.

When seeded within one week of derivation, cultures consisting primarily of neural progenitor cell rosettes were generated from sfEB/MedII. By observation of DAPI staining of 10–50 plated pieces we estimated that approximately 50–80% of the outgrowth area was comprised of neural progenitor rosettes in plated sfEB/MedII, compared with only 10–30% in sfEB. Other undefined cell types were usually present in both conditions, but comprised a higher proportion of the culture in the absence of MedII. Rosettes exhibited essentially the same characteristics as seen in suspension culture, except that they generally formed essentially 2-dimensional arrays within or surrounding the seeded embryoid body piece (Fig. 3A). Rosettes could be observed as discrete units, or tightly packed, with circular or ovoid appearance. Larger rosettes typically contained a central depression or space, resembling the cavity seen in rosettes in embryoid bodies. Rosette cells were tightly packed and arrayed in a radial pattern around the center. These cells had characteristic elongated nuclei. The rosette radius was typically 4 to 8 cells wide, and cells could stack several cells high, without being organized into distinct layers. Cells with morphologies consistent with early post-mitotic neurons were also observed, most often closely associated with the outer regions of rosettes. These cells exhibited a domed ovoid cell body with a single neurite extending from each end and dense networks of neurites could be observed (Fig. 3B). SfEB/MedII also contained variable levels of non-rosette cells. These cells expressed nestin (Fig. 4C) and had a high proliferative capacity. Unlike rosette cells, they could be passaged by enzymatic digestion but were not associated with a capacity for neuronal differentiation (data not shown).

To determine the location of proliferating cells within the neural rosettes, phospho-histone H3 immunostaining was used to visualize mitotic cells in seeded cultures. This modified form of Histone H3 is only found in cells undergoing mitosis [22,23]. In rosettes, phospho-H3 was detected in cells lining or close to the center (Fig. 3C). As expected, phospho-H3 staining was closely associated with DAPI stained DNA and in many cases DAPI staining also revealed condensed mitotic chromosomes. This is consistent with our observation of mitotic figures in the centre of rosettes in sfEB/MedII grown in suspension. This demonstrated that the central region of rosettes is where mitosis occurs and could be indicative of a neural stem cell niche. In this scenario, neural stem cells in the center of rosettes would be capable of proliferation to maintain the stem cell state, or asymmetric division to derive daughter cells where one is a stem cell and the other a differentiating cell.

We also plated cells derived from sfEB and sfEB/MedII that had been generated from the crater cells and cultured for longer periods in suspension. These sfEB and sfEB/MedII were plated after one week of suspension culture or after culturing rosettes that had been dissected out from sfEB/MedII and passaged in culture. In these cases extensive networks of neurons were observed emanating from the sfEB and sfEB/MedII pieces that had adhered to the plate (not shown). The presence of MedII enhanced the

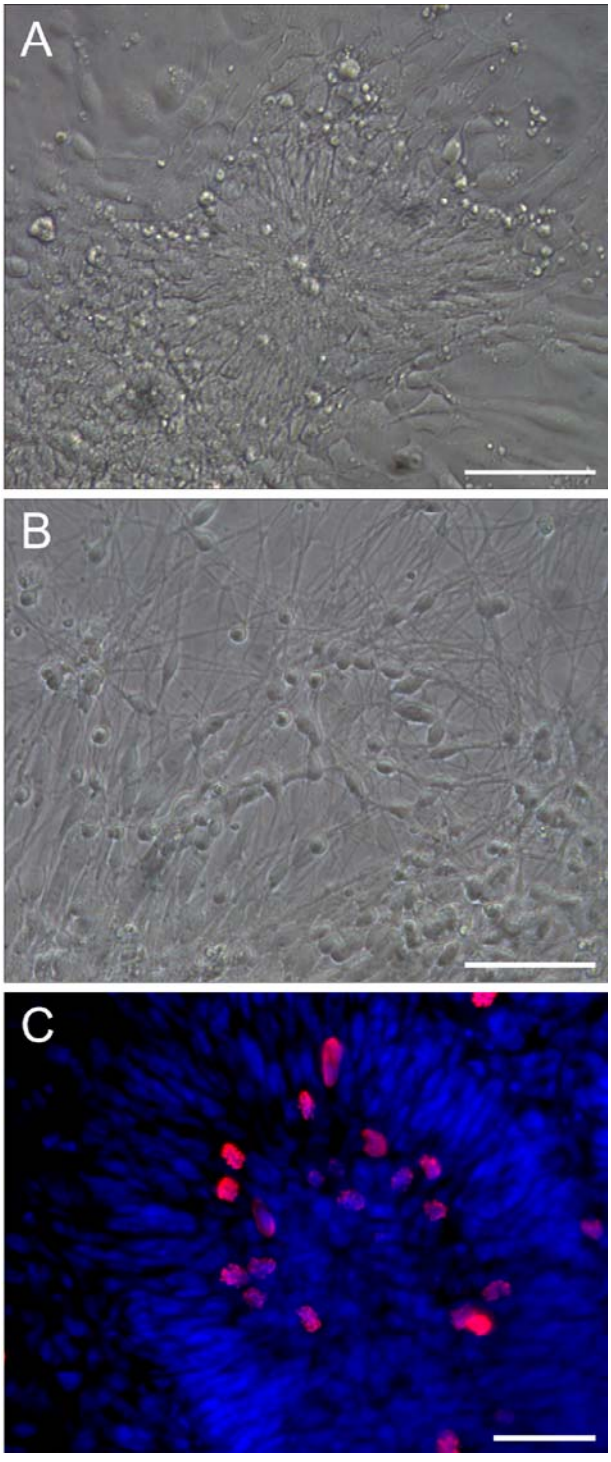

Figure 3
Phospho-Histone H3 expression in cultures derived from plated sfEB/MedII. (A,B) Morphologies of a neural progenitor rosette (A) and of neurite networks (B). (C) PhosphoHistone H3 immunostaining (red) and DAPI overlay of a neural precursor rosette. Mitotic cells within rosettes are present in the central core region. Images and scale bars: (A,B) 40×, 50 μm. (C) 60×, 25 μm.

generation of rosettes and associated neurons to around the same extent as seen when pieces were seeded within one week of derivation. Long neural extensions migrated out from seeded pieces onto the polyornithine and laminin matrix, or on top of other cell types that had proliferated or migrated out from the seeded piece.

Immunofluorescent staining was used to examine the neuronal differentiation of sfEB and sfEB/MedII cultures (Fig. 4). Nestin is an intermediate filament protein expressed in the developing nervous system and is a marker of neural stem/progenitor and stem cell populations [24,25]. Characteristic perinuclear patterns of nestin expression were observed extensively in seeded sfEB and sfEB/MedII cultures, including rosette cells and other undefined cells outside of the rosettes (Fig. 4C). Vimentin, another neural stem/progenitor marker [26] was expressed in a similar profile (data not shown). Differentiating neurons were identified by their expression of the protein Map-2 (Fig. 4D,4H,4I,4J). Map-2$^+$ neurons were observed closely associated with the outer regions of rosettes (Fig. 4H) and spanning their upper surfaces. Map-2 was also expressed by rosette cells, in a striking radial pattern (Fig. 4D,4H,4I). Co-expression of nestin and MAP-2 has been noted previously in human fetal brain cells [27,28]. The Map-2 expression pattern did not appear to be organized into filaments, but could represent a cytoplasmic stain in the tightly packed and elongated rosette cells. That neurites exhibiting strong Map-2 expression were integrated with the outer regions of rosettes, could indicate a progressive differentiation from a neuronal precursor, to an early differentiated neuron, and to a more highly differentiated neuron. Differentiated neurons also expressed other characteristic neuronal markers such as Neurofilament H (Fig. 5A). To determine if dopaminergic differentiation occurred in plated sfEB/MedII cultures derived from BG01 cells, immunofluorescent staining was performed using a sheep anti-tyrosine hydroxylase (TH) antibody. Isolated TH$^+$ neurons and networks of TH$^+$ neurons were observed in crater cell-derived sfEB/MedII (Fig. 5B). Map2$^+$/TH$^+$ neurons were observed, with a minor subpopulation of differentiated neurons being TH$^+$ (data not shown).

Discussion

In our HESC cultures, we regard undifferentiated HESC colonies as those exhibiting a domed morphology, with tight borders, and without overt organized structure within the colony, which may signify differentiation. These cells exhibit the expected pattern of pluripotent marker expression: SSEA-1$^-$, SSEA-3$^+$, SSEA-4$^+$, Tra-1-60$^+$, Tra-1-81$^+$, OCT-4$^+$ [20]. When plated onto freshly plated fibroblast feeder layers, HESC colonies form as normal, but after approximately 3 days, a central depression is observable. This depression progresses over several days,

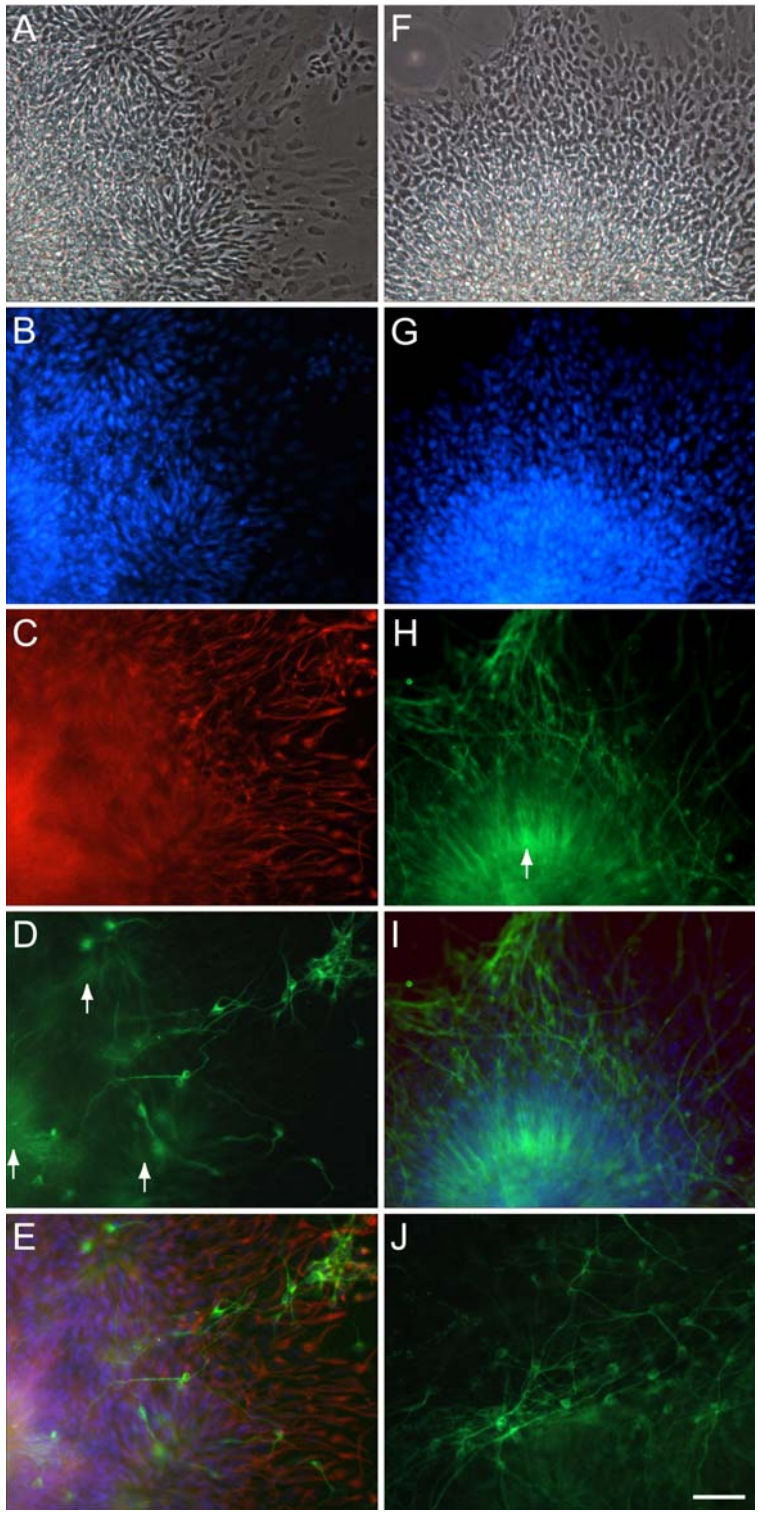

Figure 4
Nestin and MAP-2 expression in cultures derived from plated sfEB/MedII. (A-E) and (F-I) represent images of two different fields. (A,F) Phase contrast image of rosettes, neurons and surrounding cells. (B,G) DAPI stained nuclei. (C) nestin expression in rosettes and surrounding cells. (D,H) Map-2 expression showing individual neurons and radial expression in rosettes (arrows). (I) Overlay of (G,H). (E) Overlay of (B-D). (J) An independent field showing Map-2 expression in a network of associated neurons. Images and scale bars: (A-J) 40×, 25 μm.

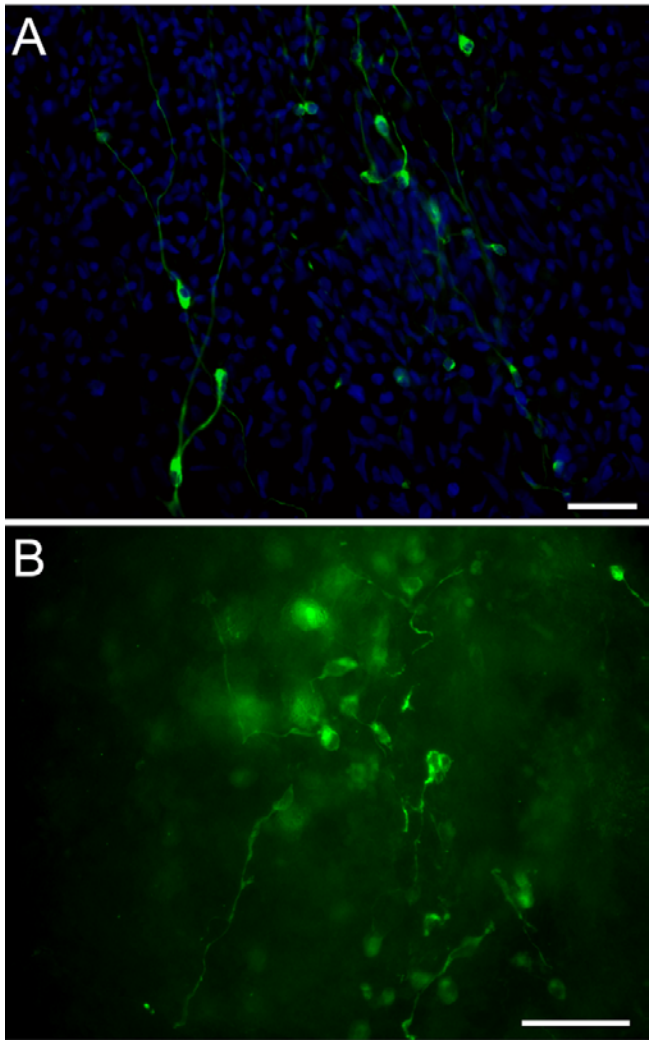

Figure 5
Neurofilament H and tyrosine hydroxylase expression in cultures derived from sfEB/MedII. (A) Neurofilament H expression in neurons merged with DAPI staining of all nuclei. (B) Tyrosine hydroxylase expression in a small network of neurons in plated sfEB(MedII) derived from BG01 cells. The background fluorescence is caused by out of focus TH^+ neurons. Anti-TH immunostaining was not performed on differentiations of BG02 cells. Images and scale bars: (A) 40×, 25 μm. (B) 60×, 25 μm.

to leave a central monolayer of cells. Colonies exhibiting a central depression can be observed in HESC plated on feeders that had been cultured for more than 2 days, but these generally do not progress to a full crater morphology unless the cultures are left to overgrow. Crater cells were morphologically distinct from undifferentiated HESC, retained expression of the pluirpotent marker OCT-4, but did not exhibit the SSEA-4 antigen. This indicated that crater cells may be a partially differentiated, but still pluripotent cell type, that is not distinguishable from undifferentiated HESC on the basis of OCT-4 expression. MESC can be directed to differentiate through pluripotent cell intermediates when exposed to MedII [21], and this has been used as a basis for defining markers that recognise subpopulations within the $OCT\text{-}4^+$ pool in vitro and in vivo [29]. Additional markers are therefore likely to be required to define subpopulations of pluripotent cells derived from HESC.

The monolayer of crater cells could be harvested simply by removing all other cells mechanically, by lifting off the feeder layer. These crater cells could differentiate efficiently to form neurons when cultured in embryoid body-like aggregates in serum-free medium conditioned by HepG2 cells (MedII conditioned medium). When placed in suspension in the presence of MedII, morphologically distinct rosette compartments formed, which, when seeded to culture dishes, were shown to contain neuronal precursor populations and associated differentiated neurons. These cell types could form in the absence of MedII, or from domed HESC colonies, but were formed at a significantly higher frequency from crater cells exposed to MedII. This may indicate that MedII does not contain neural inducing agents, but rather survival or proliferation factors for neural precursor cells, and that crater cells have a higher capacity to form neural precursors than domed HESCs. It is possible that crater cells represent a more homogeneous starting population than domed HESC colonies, which may explain their uniform neural differentiation in response to MedII. However, crater cells were not likely to represent a neural precursor population *per se*, as they did not express appreciable levels of nestin or vimentin, neural progenitor markers which were expressed by rosette cells.

Our approach appears to be a more regulated method for the generation of human neural precursors than the uncontrolled differentiation of HESC cultures [3,10], or the isolation of neural progenitors from seeded embryoid bodies [4]. However, we have not yet been able to dissociate rosettes to single cells to enable viable bulk passaging of neural rosettes. This indicates that cell-cell interaction within the rosettes is critical for cell survival, proliferation and differentiation. This is not unexpected given their highly organized architecture and suggests that sfEB/MedII culture may provide a model to investigate human neural development. Human ES cells may be amenable to precise genetic manipulation [30] and this sfEB/MedII approach may enable the examination of the genesis of neural precursors and rosettes. Furthermore, these approaches could be coupled with lineage tracing of individual cells within rosettes to analyze presumed cell fate

decisions during self-renewal or progressive differentiation to neurons.

It is not yet clear if sfEB/MedII rosettes represent equivalent cell types to neural precursors that have been derived previously from ES cells. Rosette compartments in sfEB/MedII appeared very similar morphologically to the neurectoderm population found in mouse EB/MedII [13], which represent a positionally unspecified neuronal precursor population. If our neural precursor cells are also positionally unspecified, variation of culture conditions, exposure to soluble neuralizing factors or introduction of transgenes could be used to direct differentiation of rosette cells to alternate neuronal fates. Other described neural precursors that may be similar include the appearance of rosettes in seeded embryoid bodies [8], and presumptive rosettes in the spontaneous uncontrolled differentiation of HESCs [3,10]. The differentiation of HESCs to neural rosettes has also been demonstrated following transplantation near the neural tube in a chick chimera model [31]. These previous examples do not describe approaches for the efficient derivation of neural progenitor rosettes, which was a characteristic of sfEB/MedII. One interpretation of our results suggests that neuronal precursors are derived from HESCs and reside in the central core of rosettes. Symmetric cell division may be used for progenitor proliferation and asymmetric cell division may generate an identical daughter neuronal progenitor and a differentiating cell that expresses a low level of Map-2. This cell would be capable of migration away from the core of the rosette and differentiation to a post-mitotic neuron. In this scenario, the majority of the rosette would consist of differentiating cells. Given the structural organization of rosettes and their differentiation capacity, it would be interesting to define their relationship to structured neural progenitor niches in the embryo, such as neuroepithilia, neurectoderm or the neural tube.

During the derivation and culture of sfEB/MedII, significant cell death was not observed, indicating a direct conversion of crater cells to neural precursors. Previously, a default pathway of neural differentiation was suggested for mouse ES cells [16], however only about 0.2% of single ES cells could form neural stem cell colonies directly. Cell-cell communication was not disrupted by dissociation at any time during our culture, which may have enabled a community effect to enhance the proportion of cells that could develop to neural fates. Conversely, the development of crater colonies could represent a similar significant selective step for undifferentiated human ES cells. The generation of neural progenitors directly from mouse ES cells required LIF, which was not an additive to our culture medium. Rathjen et al have reported activity in MedII equivalent to 50–100 U/ml human LIF [13,21] in an assay for the conversion of mouse ES cells to EPL cells, an alternate pluripotent cell state. Therefore it is possible that our differentiation system relies on low levels of human LIF, but the components of MedII that influence neural differentiation have not been determined.

We are yet to examine the electrophysiological characteristics of the neurons generated by this approach. However, expression of neuronal markers Map-2 and Neurofilament H [32] indicates that post mitotic neurons have been generated. Their morphology and marker expression is generally consistent with neurons derived from primate ES cells in the presence of MedII, which exhibit the expected electrophysiological properties [33].

Presumed dopaminergic neurons expressing the tyrosine hydroxylase marker were generated from HESCs using this approach. This demonstrated that exposure to MedII did not restrict HESCs from dopaminergic differentiation and suggests that further development of this approach may enable the generation of populations suitable for cell therapy applications in Parkinson's Disease. Such improvements could possibly involve the enrichment of DA precursors or neurons from our sfEB/MedII system by selection or cell sorting, and the adaptation of HESCs or rosette cells to bulk passaging methodologies to enable large scale differentiation of neurons under the influence of MedII.

Conclusions

We have developed a novel, controlled, neural differentiation regime for human ES cells. HESC cultures were morphologically differentiated to a novel OCT-4$^+$ cell type, "crater cells", which were differentiated to neural precursors and neurons in a serum-free suspension culture system. Neural progenitor populations that were organised into rosette structures developed in the presence of the conditioned medium MedII. These rosette cells expressed nestin and could differentiate to neurons expressing the markers Map-2, Neurofilament H or TH. This demonstrated the efficient derivation of neural cultures from HESC, for the examination of human neural development and potential for using such differentiation approaches to generate cell populations for cell therapy applications.

Methods

Human ES cell culture

The human embryonic stem cell lines BG01 and BG02 [20] were used in this work and were grown in DMEM/F12 (50/50) supplemented with 15% FCS (HyClone), 5% knockout serum replacer, 1× non-essential amino acids, 20 mM L-Glutamine, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 10 ng/ml human LIF (Chemicon) and 4 ng/ml FGF-2 (Sigma), (all from Gibco Invitrogen unless otherwise labelled). HESC were grown on feeder layers of mouse primary embryonic fibroblasts that were

mitotically inactivated by treatment with mitomycin-C. Tissue culture dishes were supplied by Falcon.

Mouse embryonic fibroblasts (MEFs) were prepared from the ICR strain as described previously [34]. Explanted cells were termed P0 (passage 0) and were frozen for storage. Feeder cells were thawed, mitotically inactivated by treatment with mitomycin-C, and re-plated at 1.2×10^6 cells per 35 mm dish to generate P1 feeders. P1 MEFs were cultured for at least 2 days prior to the plating of HESC for routine culture. Human ES cells were grown as domed colonies and were manually passaged on to subsequent fibroblast feeder layers every 3–4 days using a fire-pulled Pasteur pipette. Briefly, the barrel of the Pasteur pipette was melted solid and drawn out to a solid needle, which was sequentially pressed through HESC colonies to form a uniform grid of cuts, then under the colonies to lift them from the feeder layer. Entire plates of HESC were harvested, then the colonies were broken into individual pieces defined by the grid by gentle pipetting using a 5 ml serological pipette. The pieces from a single plate were split between 2 or 3 new plates that were coated with feeder layers of mitotically inactivated mouse primary embryonic fibroblasts.

Controlled differentiation of HESC and serum free embryoid bodies

For the controlled differentiation of HESC, pieces from domed colonies were passaged to mitotically inactivated mouse primary embryonic P1 fibroblasts within 6 hours of when the feeder layer had been plated at 1.2×10^6 cells per 35 mm dish. Under this condition, HESC colonies grew as normal, but after ~4 days formed a central depression in the colony, which developed to become a crater of a monolayer cell type surrounded by morphologically normal HESC. This monolayer cell type was a presumed early differentiation intermediate of HESC, and was used to derive serum free embryoid bodies (sfEB, or "FGF-2" conditions) or serum free embryoid bodies exposed to 50% MedII (sfEB/MedII, or "FGF-2/MedII" conditions). MedII was generated from HepG2 cultures essentially as described previously [21], using the serum free medium DMEM/F12 (50/50) supplemented with 1 × N2 (Gibco Invitrogen), 20 mM L-Glutamine, 0.5 U/ml penicillin, and 0.5 U/ml streptomycin. FGF2 was included in all differentiations as a mitogen and because of its known proliferative effects on neural precursors [18,19]. Crater cells were purified by removing the feeder layer and the HESCs growing on the MEFs. Human ES cell crater cultures were washed once with DMEM/F12, then watchmakers forceps were used to hold the feeder layer at the side of the culture dish, and lift this layer and attached HESC from the dish. This manipulation peeled the feeder layer and the stacked parts of the HESC colonies off of the dish and left behind the monolayer crater cells which were attached to the dish. Glass needles were used to cut the crater monolayers to 50–200 cell size pieces, and lift them from the dish. These pieces were grown in suspension culture in DMEM/F12 (50/50), 1 × N2 (Gibco Invitrogen), 4 ng/ml FGF-2, 20 mM L-Glutamine, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, with or without 50% serum free MedII. Suspension cultures were incubated for up to six weeks, in bacteriological dishes (Falcon) coated with 0.5% agarose (Bio-Rad) in DMEM (Gibco Invitrogen), with replenishment of the medium every 3–4 days. The serum free embryoid bodies were passaged approximately every 7–10 days by cutting them into pieces with drawn out solid glass needles. At passaging, the embryoid bodies contained approximately 5000–10,000 cells and were divided into 4–10 pieces, discarding non rosette-structured regions and rare cystic embryoid bodies.

Immunostaining

Serum free embryoid bodies (sfEB and sfEB/MedII) were cut into pieces using glass needles and 1–15 pieces were plated into wells of polyornithine (Sigma) and laminin (Sigma) coated permanox chamber slides (Nalge Nunc) in the same medium used for suspension culture. Coated slides were prepared by diluting polyornithine to 20 μg/ml in tissue culture grade water, coating chamber wells at 37°C overnight, washing twice with water and coating the chamber wells with 1 μg/ml laminin at 37°C overnight. The slides were washed with water and 1 × PBS (HyClone) prior to plating the cells. The embryoid bodies were cultured on these slides for 2–7 days.

For immunostaining, the cultures were rinsed with 1 × PBS and fixed in 4% paraformaldehyde (Fisher Scientfic), 4% sucrose (Sigma) in 1 × PBS for 30 minutes at 4°C. The cells were then washed in 1 × PBS and stored at 4°C. The cells were washed in block buffer (3% goat serum (Gibco), 1% polyvinyl Pyrolidone (Sigma), 0.3% Triton X-100 (Sigma) in wash buffer) for 30 minutes, and then incubated with the appropriate dilution of the primary antibody, or combination of antibodies for 4–6 hours at room temperature.

The primary antibodies were: anti-Map2, a mouse monoclonal antibody (Sigma, Catalog # M4403) at a 1/500 dilution; anti-Nestin, a rabbit polyclonal antibody (Chemicon, Catalog # AB5922) at a 1/200 dilution; anti-OCT-4, a mouse monoclonal antibody (Santa Cruz, Catalog # sc-5279) at a 1/100 dilution; anti-Neurofilament H, a mouse monoclonal antibody (Sternberger, Catalog # SMI32) at a 1/500 dilution; anti-phosphoHistone H3, a rabbit polyclonal antibody (Upstate, Catalog # 06-570) at a 1/400 dilution; and anti-Tyrosine Hydroxylase, a sheep polyclonal antibody (Pel-Freez, Catalog # P60101-0), at a 1/100 dilution, and anti-SSEA-4, a mouse monoclonal antibody (Developmental Studies Hybridoma Bank,

Catalog # MC-813-70) at a 1/5 dilution. For SSEA-4 cell surface stainings, Triton X-100 was omitted from the block and wash buffers.

The cells were washed 3 × 5 minutes in wash buffer (50 mM Tris-HCl pH 7.5 (Sigma), and 2.5 mM NaCl (Sigma)). The cells were then incubated for a minimum of 2 hours in secondary antibodies diluted 1:1000, followed by washing in wash buffer. The secondary antibodies were Alexa-350 (blue), 488 (green) or 568 (red) conjugated goat anti-rabbit, anti-sheep, or anti-mouse antibodies, all available from Molecular Probes. The cells were stained with 5 ng/ml DAPI (Sigma) to detect cell nuclei, and were then washed from overnight to 2 days in a large volume of wash buffer. The slides were mounted and visualized using a either a NIKON TS100 inverted microscope or a NIKON TE 2000-S inverted microscope with a Q Imaging digital camera. Individual color channels were captured separately and merged in Adobe Photoshop.

Histochemistry

For histochemical analysis, sfEB and sfEB/MedII were fixed with 4% paraformaldehyde, 4% sucrose in 1 × PBS for 30 minutes at 4°C. The cells were then washed in 1 × PBS and stored at 4°C. The serum free embryoid bodies were embedded using the Immuno-Bed kit (Polysciences, Inc., Catalog # 17324). Briefly, the PBS was removed and the embryoid bodies were dehydrated by incubation in a series of 25%, 50%, 75% Ethanol/1 × PBS for 5 minutes at room temperature, followed by 100% Ethanol. Infiltration solution was made by adding 0.25 g Benzoyl Peroxide to 20 ml Immuno-Bed Solution A. The ethanol was removed from the serum free embryoid bodies and 1 ml infiltration solution was added. After one hour, the infiltration solution was changed for three 20 min incubations. For embedding, 1 ml solution B (accelerator) was added to 25 ml fresh infiltration solution. The infiltration solution was removed from the serum free embryoid bodies and 0.5 ml embedding solution was added. The samples were transferred to a mould, a block holder was added and the mould was placed at 4°C to set. 3 micron sections were cut using a Leica microtome, and were stained with either 5 ng/ml DAPI (Sigma) in 1 × PBS, or 0.1% Toluidine Blue (Aldrich) in 30% Ethanol. Slides were washed with 1 × PBS and visualised on a NIKON TE 2000-S inverted microscope with a Q Imaging digital camera. For counting of DAPI stained rosette and non-rosette nuclei, images of sections were captured on a NIKON Eclipse E1000 compound microscope under a 10× objective and counted using the ImagePro software (Media Cybernetics Inc.). The statistical comparison was a 2-tailed t-test, assuming unequal variance.

Authors' contributions

TS designed the study, cultured HESC and neural differentiations, and performed immunostaining, imaging and manuscript preparation. GP cultured HESC and neural differentiations, performed immunostaining and sectioning. DW prepared feeder layers and performed sectioning. SN collaborated on HESC and crater cell immunostaining. MM observed the fresh/aged feeder effect. BC conceived and supervised the study and participated in manuscript preparation. All authors read and approved the final manuscript.

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