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Original Articles

Differentiation of Human Embryonic Stem Cells into Embryoid Bodies Comprising the Three Embryonic Germ Layers

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Abstract

Background: Embryonic stem (ES) cells are lines of cells that are isolated from blastocysts. The murine ES cells were demonstrated to be true pluripotent cells as they differentiate into all embryonic lineages. Yet, in vitro differentiation of rhesus ES cells was somewhat inconsistent and disorganized. The recent isolation of human ES cells calls for exploring their pluripotential nature.

Materials and Methods: Human ES cells were grown in suspension to induce their differentiation into embryoid bodies (EBs). The differentiation status of the human ES cells and EBs was analyzed by following the expression pattern of several lineage-specific molecular markers using reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization.

Results: Here we report the induction in vitro of cystic embryoid bodies from human ES cells. Our findings demonstrate induction of expression of cell-specific genes during differentiation of the human ES cells into EBs. In the human EBs, we could show a characteristic regional expression of embryonic markers specific to different cellular lineages, namely, ζ-globin (mesoderm), neurofilament 68Kd (ectoderm), and α-fetoprotein (endoderm). Moreover, we present a synchronously pulsing embryoid body that expresses the myocardium marker α-cardiac actin. In addition, dissociating the embryoid bodies and plating the cells as monolayers results in multiple morphologies, among them cells with neuronal appearance that express neurofilament 68Kd chain.

Conclusion: Human ES cells can reproducibly differentiate in vitro into EBs comprising the three embryonic germ layers. The ability to induce formation of human embryoid bodies that contain cells of neuronal, hematopoietic and cardiac origins will be useful in studying early human embryonic development as well as in transplantation medicine.

Introduction

The creation of cell sources that may be used for tissue replacement is a major goal of biomedical engineering. Embryonic stem (ES) cells are derived from totipotent cells of the embryo and therefore, can serve as a putative source of numerous types of differentiated cells. Of the various ES cell lines, murine (mES) cells have been investigated extensively and were shown to be genuinely pluripotent, being able to differentiate into all embryonic cell types (1,2). The multipotency of embryonic stem cells is evident from three main features: (A) These undifferentiated cells can be injected into the blastocyst cavity

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and the resultant embryos implanted in pseudopregnant mice. The injected ES cells contribute to all cell types in the chimeric progeny mice, including the germ layer. Thus, in the next generation, mice with the genotype of the ES cells are born (3,4). (B) Subcutaneous injection of embryonic stem cells into syngeneic mice induces teratomas, which may include cells of endodermal, ectodermal or mesodermal origin (5). (C) In vitro aggregation of ES cells results in formation of embryoid bodies (EBs), with regional differentiation into embryonically distinct cell types. During their in vitro maturation, mES cells undergo massive morphological changes, and acquire various molecular markers of differentiated cell types. These include ζ-globin, a marker of hematopoietic cells (6,7), neurofilament-68Kd, a marker of neuronal cells (8,9), and albumin, which is a marker of hepatic cells (9). Thus, the capacity of mES cells to undergo terminal differentiation in vitro to cells of the mesoderm, ectoderm and endoderm lineages has been well documented. Most recently, ES cell lines, derived from human embryos produced by in vitro fertilization, were established (10). The embryos were cultured to the blastocyst stage and inner cell masses were isolated and grown as ES cells. The established cell lines have normal karyotypes, proliferate in culture with an undifferentiated phenotype and differentiate into the various embryonic lineages in teratomas (10). Clearly, any potential future use of human ES (hES) cells will depend on their capacity to undergo embryonic differentiation in vitro. To this end, we initiated a study aimed at inducing hES cell differentiation in vitro into EBs.

Unlike the murine ES cell lines, EBs formation from primate ES cells presented considerable difficulties. Thus, EBs differentiation from ES cells derived from the common marmoset (11) were well organized, but appeared inconsistent and asynchronous, and differentiation of ES cells from the rhesus monkey (12) was disorganized, so that the resultant EBs failed to form vesicular structures (13). To overcome these obstacles, we explored the potential of the hES cells to aggregate in suspension and form EBs.

Materials and Methods

Cell Culture

hES cells (H9 clone; 10) were grown on mouse embryo fibroblasts in culture medium that consisted of 80% KnockOut® DMEM (an optimized Dulbeco's modified Eagle's medium for ES cells; Gibco-BRL, Gaithersburg, MD), 20% KnockOut® SR (a serum-free formulation; Gibco-BRL), 1 mM glutamine (Gibco-BRL), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO), 1% nonessential amino acids stock (Gibco-BRL), 10^3 units/ml leukemia inhibitor factor (LIF) (Gibco-BRL), and 4 ng/ml basic fibroblast growth factor (bFGF; Gibco-BRL). Under these conditions, most of the cells were kept in an undifferentiated state. To induce formation of EBs, ES cells were transferred using either colagenase (1 mg/ml; Gibco-BRL) or trypsin/EDTA (0.1%/ 1 mM) to plastic Petri dishes (Miniplast, Ein-Shemer, Israel) to allow their aggregation and prevent adherence to the plate. Usually about 10^6 ES cells were incubated in each 50 mm Petri plate. The hEBs were grown in the same culture medium, except that it lacked LIF and bFGF.

RNA and RT-PCR

Total RNA was extracted as described (14) and cDNA was synthesized from 1 μg total RNA, using random hexamer ($pd(N)_6$) as primer (Pharmacia Biotech, Uppsala, Sweden) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco-BRL). cDNA samples were subjected to polymerase chain reaction (PCR) amplification with DNA primers selective for the human genes. For each gene, the two DNA primers derived from different exons, so as to ensure that the PCR product represented the specific mRNA species and not the genomic DNA. PCR was performed under linear conditions, to reflect the original amount of the specific transcript. The PCR primers used and the reaction conditions were:

- α-fetoprotein; AGAACCTGTCACAAGCTGTG and GACAGCAAGCTGAGGATGTC; Product: 676 base pair (bp); 20 cycles at 60°C in 1 mM $MgCl_2$.
- ζ-globin; GACTGAGAGGACCATCATTG and TCAGGACAGAGGATACGACC; Product: 397 bp. 25 cycles at 60°C in 1 mM $MgCl_2$.
- GAPDH; AGCCACATCGCTCAGACACC and GTACTCAGCGGCCAGCATCG; Product: 302 bp. 20 cycles at 60°C in 1 mM $MgCl_2$.

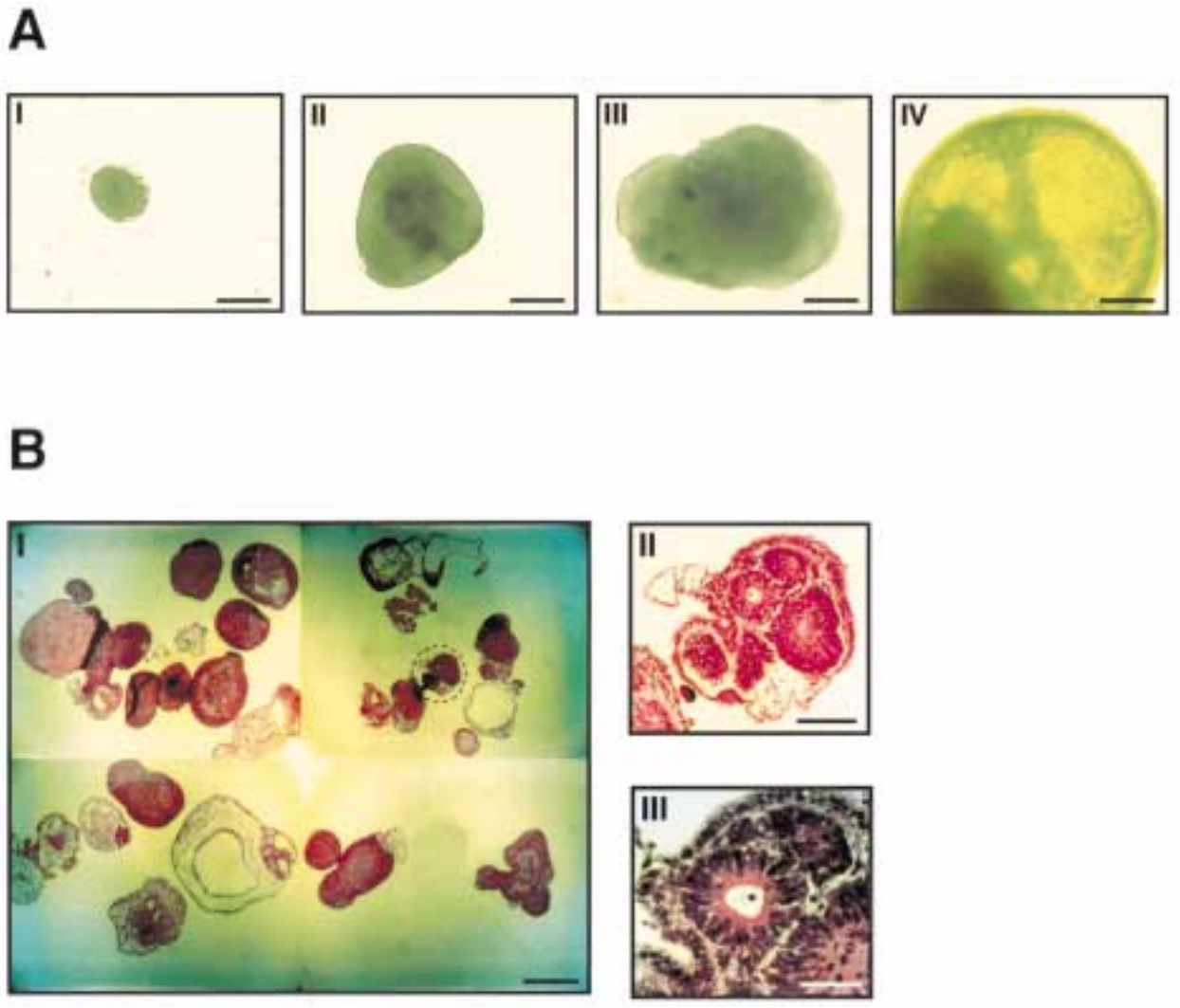

Fig. 1. Formation of human cystic embryoid bodies. (A) Time scale of hEBs development. Shown from left to right is a gradual growth and transformation with time of simple, densely packed hEBs into cystic hEBs. To induce formation of EBs, ES cells were transferred to plastic Petri dishes to allow their aggregation and grown in culture medium lacking leukemia inhibitor factor (LIF) and basic fibroblast growth factor (bFGF). Shown from I to IV are EBs from 3, 7, 10 and 14 days, respectively, after their transfer to Petri dishes. I: simple EB; II & III: cavitated EB; IV: cystic EB. Scale bar, 200 μm. (B) Morphological differentiation. Shown are 5 μm paraffin–embedded sections of 20-day-old EBs stained with hematoxylin and eosin (H&E; I-III), in different magnifications. I: $\times$5; scale bar, 400 μm. II: $\times$20; scale bar, 100 μm. III: $\times$40; scale bar, 50 μm. Note in the enlarged EB, a single cell layer of endodermal–like cells in the periphery and epithelial cells surrounding a lumen in the center.

PCR products were analyzed by Southern blot hybridization (15). Probes were radiolabelled by random priming (Boehringer Mannheim, Indianapolis, IN) using [α-^{32}P]dCTP (3000 Ci/mM; NEN - Life Science Products, Boston, MA).

In Situ Hybridization

The EBs were fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm paraffin–embedded serial sections of EBs were hybridized to specific 5′-biotinilated RNA probes and labeled with a fluorogenic product of streptavidin-conjugated alkaline phosphatase (16). When in situ hybridization was performed on cells from dissociated EBs, the cells were grown on glass cover slides coated with fibronectin (50 μg/ml in phosphate-buffered saline (PBS; Boehringer Mannheim). The probes used were 50-mer 2′-O-methyl 5′-biotinylated cRNA of either α-fetopro-

tein–TTGTCCCTCTTCAGCAAAGCGAGTTC-CTGGCCTTGGCAGCATT, ζ-globin–TGATG-GTCCTCTCAGTCTTGGTCAGAGACATG-GCGGCAGGGTGGGCAGCT, neurofilament 68Kd–CCTGCGTGCGGATGGACTTGAG-GTCGTTGCTGATGGCGGCTACCTGGCTC, or α-cardiacactin–CGGTGGACAATGGATGGGC-CTGCCTCATCGTACTCTTGCTTGTAATCCA.

Results

To explore the potential of the hES cells to differentiate in vitro, we tried to aggregate the hES in suspension in order to form EBs. In suspension, the hES cells were cultured without LIF and bFGF. In addition, we grew the cells on Petri dishes to prevent their adherence to the plate. Under these conditions, hES cells consistently aggregated and formed EBs. Figure 1A shows EBs photographed from 3 to 14 d after initiation of cellular aggregation of the hES cells. Initially, these bodies were largely composed of densely packed hES cells, creating simple EBs (Fig. 1A-I). Soon after, the center of the bodies became cavitated (Fig. 1A-II and III) and the bodies began to accumulate fluid and turn into cystic EBs (Fig. 1A-IV). Twenty days after initiation of cellular aggregation 20–90% of the structures were cystic (Fig. 1B-I). In parallel, we noted the development of a variety of cells with epithelial and endodermal morphology (Fig. 1B-II and III).

To examine the differentiation status of the cultured hEBs, we extracted RNA from 20-day-old hEBs and from ES cells grown on mouse embryo fibroblasts (as feeder cells). cDNA was reverse-transcribed from this RNA and the expression of several marker genes was tested by PCR under nonsaturating linear conditions using several human-specific DNA primers. For each RNA transcript, the DNA primers derived from separate exons to allow verification that the PCR product represented the cDNA and not the genomic DNA. In addition, in each set of reactions, a sample of RNA (and not cDNA) was used as a template to control for any DNA contamination. The identity of each of the amplified PCR products was verified by sequence analysis, using an ABI 377 sequencer (Perkin Elmer, Foster City, CA). Robust expression of α-fetoprotein, an endodermal marker (17) and ζ-globin, a marker of early hematopoietic cells (18; Fig. 2), demonstrated that the EBs had begun differentiating. The house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), served as an internal control. Some significant differentiation also occurred in the ES cells grown on feeders, as demonstrated by low levels of α-fetoprotein and ζ-globin in the ES samples. By comparison with the EB samples, the significantly more intense PCR products from the hEB RNA supported and extended the conclusion

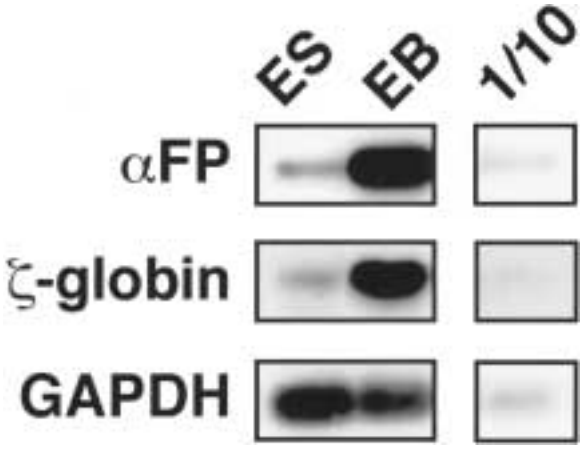

Fig. 2. Expression of cell-specific genes in human cystic embryoid bodies. Shown is an RT-PCR analysis of expression of α-fetoprotein (αFP), ζ-globin and glyceraldehyde–3–phosphate dehydrogenase (GAPDH) in human embryonic stem (ES) cells grown on feeder cells, or in 20 day-old embryoid bodies (EB). PCR was performed under linear conditions in order to reflect the original amount of the specific transcript. Linearity is demonstrated by the 1/10 column showing a PCR assay performed with one-tenth of the amount of cDNA used in the PCR assay of the EB sample.

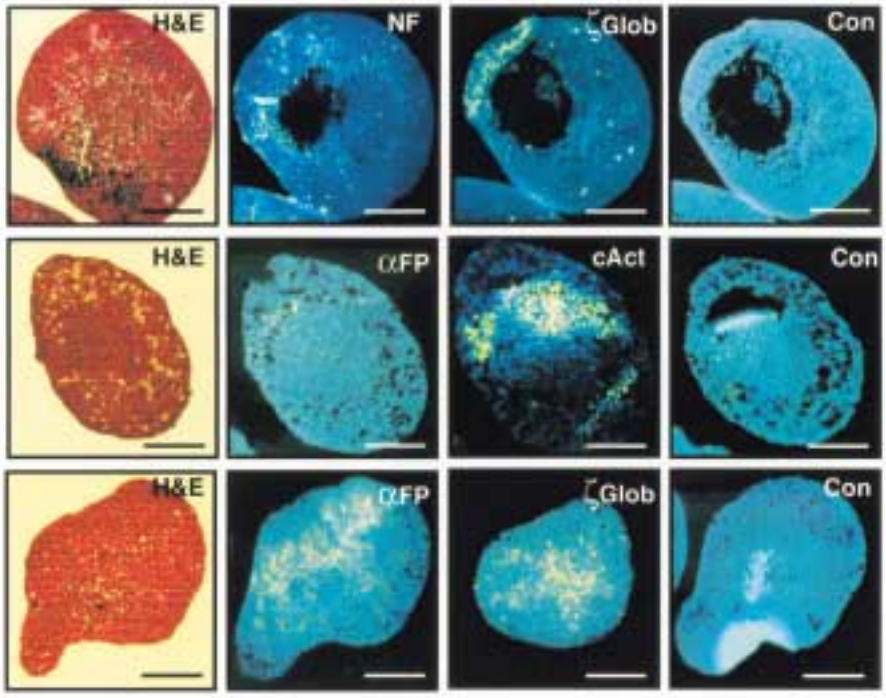

Fig. 3. In situ hybridization analysis of embryoid bodies. Shown is in situ hybridization detection of expression of neurofilament 68Kd subunit (NF), ζ-globin (ζGlob), α-fetoprotein (αFP) and α-cardiac actin (cAct) in 20-day-old EBs. Shown are 5 μm paraffin–embedded serial sections of three different EBs stained with hematoxylin and eosin (H&E) or hybridized to specific 5′-biotinilated RNA probes and labeled with a fluorogenic product of streptavidin-conjugated alkaline phosphatase (16). Con-hybridization with nonspecific RNA. Scale bar, 100 μm.

from microscopic examination that the hEB structures were more differentiated.

To regionally characterize the differentiating cells within the EBs we examined by in situ hybridization the expression of four cell-specific molecular markers, all of which were transcribed very early during embryonic differentiation. Thus, serial sections of the EBs were hybridized with 5′-biotinylated 2′-O-methyl cRNA probes specific to α-fetoprotein (17), ζ-globin (18), α-cardiac actin (19) or neurofilament 68Kd (20). As shown in Fig. 3, each of these probes reproducibly labeled distinct cell layers in the hEBs. The endodermal marker α-fetoprotein was primarily expressed in the interior part (Fig. 3) and in some hEBs it was also expressed in the exterior layer. A similar pattern of expression of α-fetoprotein was observed by others in EBs derived from human embryonic germ cells (21). In each of the sections, the labeled cells were localized in a specific area, suggesting that they were either clonal, and derived from the same progenitor cell, or that the different cells were all affected by similar signals and, thus, differentiated to the same specific lineage.

Differentiation into the myocardial lineage was reported to induce development of pulsing muscle in mEBs (22). In a minority of the cystic hEBs, rhythmic pulsing was observed. In Fig. 4, we demonstrate a large vacuolated embryoid body, including cardiac muscle cell layers that were pulsing in a synchronous rhythm of about 30 pulses/minute (Fig. 4A and B). In situ hybridization of sections from this EB with a probe for α-cardiac actin, a marker of embryonic myocardial cells (19), revealed that the central cavity was indeed surrounded by cardiac muscle cells (Fig. 4C).

To further characterize the differentiated ES cells we dissociated embryoid bodies with trypsin and plated the cells as a monolayer. Cells of various morphologies were recognized, among them neuron-like cells. These cells expressed the neuronal marker neurofilament 68Kd, as evident by an in situ hybridization assay (Fig. 5).

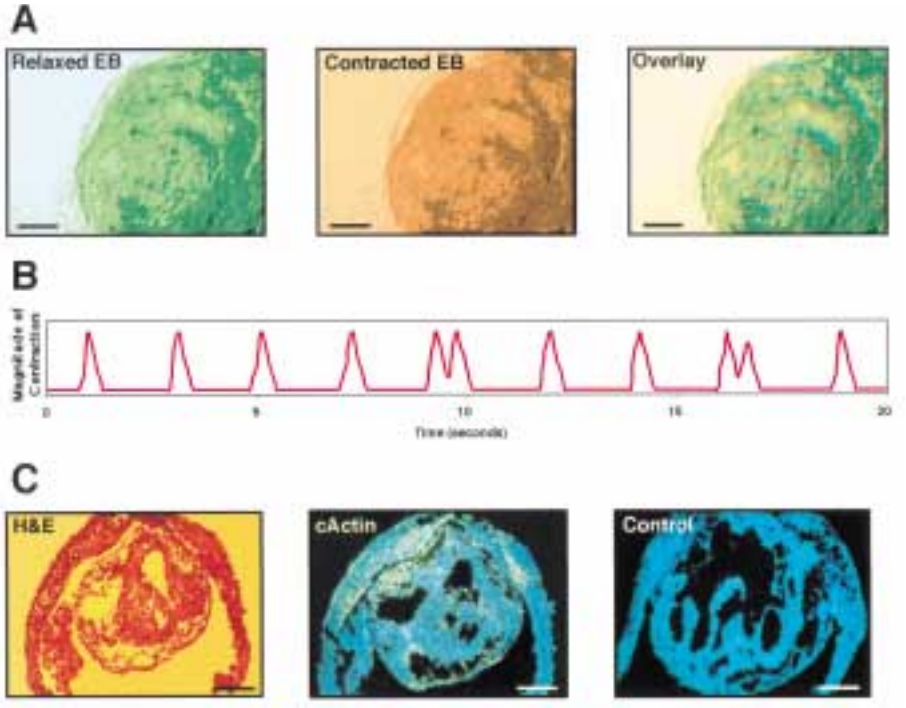

Fig. 4. Cardiac muscle differentiation depicted in a pulsing embryoid body. (A) Contracting embryoid body. Shown is a video micrograph of a pulsing EB in a relaxed or contracted state. Light absorption in the video micrographs was color coded in either green or red; the two photos were overlaid to demonstrate the changes in opacity, reflecting contraction during the pulsation of the EB. Scale bar, 200 μm. (B) Rhythmic contraction. Presented is the magnitude of contraction recorded from the pulsing EB over 20 sec. (C) Layered cardiac muscle cells. Shown are serial sections from the pulsing EB, including in situ hybridization depicting expression of α-cardiac actin (cActin) in it. Also shown is a section stained with hematoxylin and eosin (H&E) and a section hybridized with nonspecific RNA (control). Scale bar, 100 μm.

Discussion

Our findings demonstrate that hES cells readily differentiate to cystic EBs in a similar manner and time scale to those reported for mES cells. In ES cells derived from the primate rhesus monkey, it was reported that differentiation was somewhat disorganized and the cells did not develop cystic embryoid bodies (13). However, human embryonic germ cells also developed into embryoid bodies similar to our ES cells (21). Until now, the hES cells were shown to differentiate into the various embryonic lineages only in teratomas. Here, we demonstrate that human ES cells, when differentiating in suspension in vitro, acquire molecular markers specific to the three embryonic germ layers. The different markers used reflect development into hematopoietic cells (ζ-globin), myocardial cells (α-cardiac actin), neuronal cells (neurofilament 68Kd) and endodermal cells (α-fetoprotein). The differentiating cells also acquired characteristic morphologies, distinct for the hEB regions expressing different markers. They further developed new functions, as evident from the appearance of pulsing muscle cells. As in the mouse, the embryoid bodies displayed the potential to differentiate into various lineages, but no pattern formation or organized organogenesis was observed.

Our findings open the field to direct in vitro differentiation of human ES cells into specific lineages. These can serve as a source of mature cells, which may be used in cell transplantation and offer an opportunity to study in vitro processes involved in early human embryogenesis.

Acknowledgments

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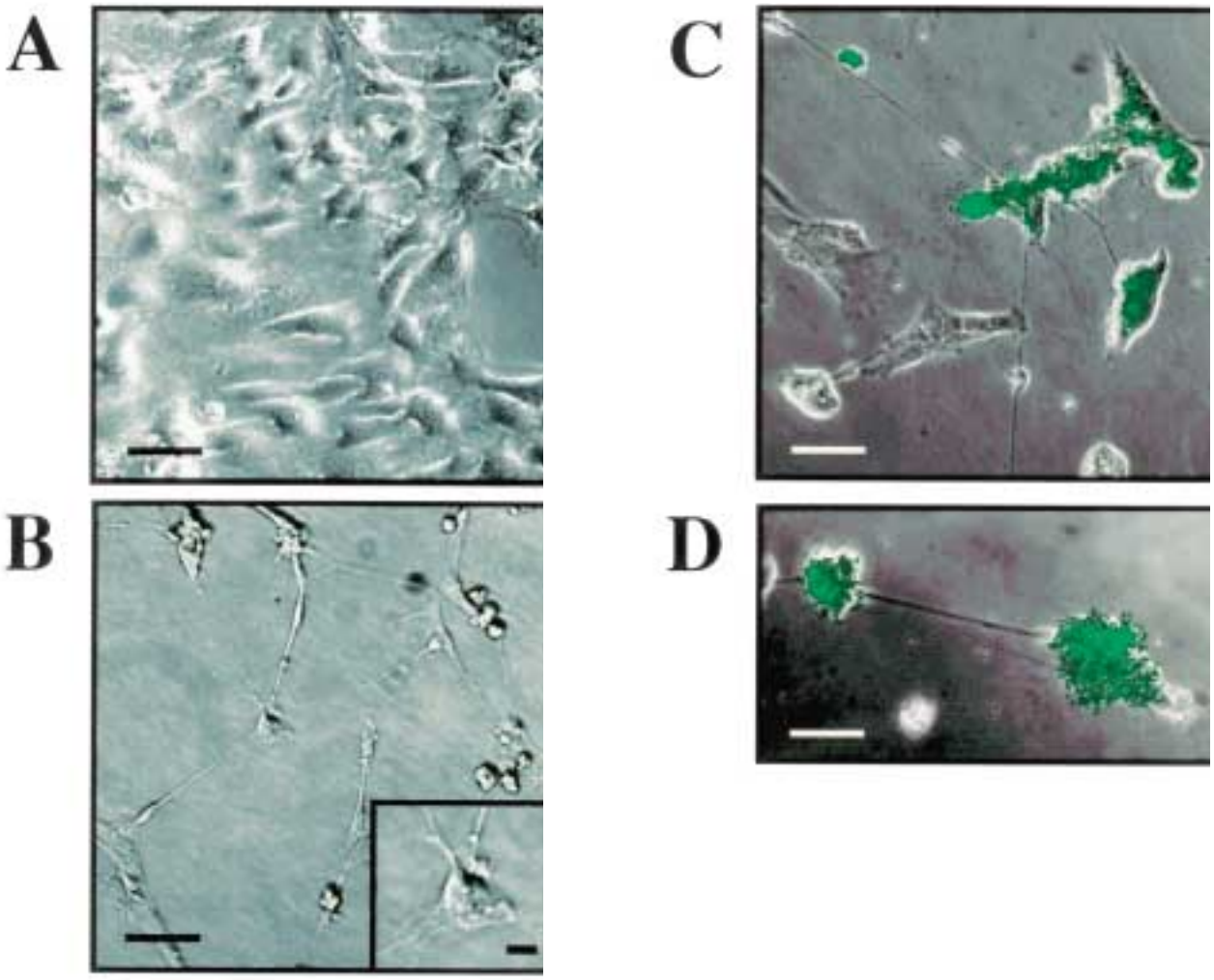

Fig. 5. Dispersed cell cultures derived from hEBs. hEBs were grown for 5 d in suspension and then were dissociated with trypsin and plated as a monolayer on cover slides coated with fibronectin. Shown are cells with fibroblast (A) or neuronal (B) appearance. Insert in (B) is a higher magnification of neurotic outgrowth. The cells with neuritic outgrowth express neurofilament 68Kd as evident by a fluorogenic product in the cells after in situ hybridization with the neuronal marker (C and D). Scale bar, 100 μm.

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