DIFFERENTIATED HUMAN EMBRYOID CELLS AND A METHOD FOR PRODUCING THEM

FIELD OF THE INVENTION

The present invention is in the field of cell biology and more specifically relates to methods of *in vitro* differentiation.

BACKGROUND OF THE INVENTION

Embryonic stem (ES) cells are derived from totipotent cells in an embyro. Murine ES cells have been shown to be pluripotent cells capable *in vitro* of terminal differentiation into cells of the mesoderm, ectoderm and endoderm lineages (Robertson, 1987; Dushnik-Levinson and Benvenisty, 1995). The pluripotency of murine embryonic stem cells has been established on the basis of three criteria:

(1) When undifferentiated murine ES cells are injected into the cavity of a blastocyst and the blastocysts are subsequently implanted into pseudo-pregnant mice, chimeric mice develop. The injected ES cells contribute to all cell types, including the germ layer. Thus, in the next generation, mice with the genotype of the ES cells are born (Capecchi, 1989; Rossant and Joyner, 1989).

(2) When murine ES cells are injected subcutaneously into syngeneic mice, teratoma tumors develop. These tumors comprise cells of all three embryonic origins (endoderm, ectoderm and mesoderm) (Wobus *et al.*, 1984).

(3) When murine ES cells are allowed to aggregate *in vitro* so as to form embryoid bodies (EBs), the cells differentiate in the EBs into various cell types (Robertson, 1987).

During maturation of murine ES cells *in vitro*, in addition to morphological changes, a cell may acquire a molecular marker characteristic of a differentiated cell type, such as ξ-globin (a marker of hematopoietic cells) (Wiles and Keller, 1991; Lindenbaum and Grosveld, 1990), neurofilament-68kd protein (a marker of neuronal cells) (Bain *et al.*, 1995; Levinson-Dushnik and Benvenisty, 1997), and albumin (a marker of hepatic cells) (Levinson-Dushnik and Benvenisty, 1997).

ES cell lines have also been established from primates such as the common marmoset (Thomson, 1996)), and the rhesus monkey (Thomson, 1995)). However, EB formation by marmoset ES cells is inconsistent and asynchronous, and differentiation of the rhesus ES cells is disorganized and vesicular structures do not form (Thomson, 1998a).

Human ES cell lines have been established derived from human embryos produced by *in vitro* fertilization (Thomson, 1998b). The embryos were cultured to the blastocyst stage, and inner cell masses comprising ES cells were isolated and cultured. These human ES cells are only known to be capable of differentiating when in teratomas (Thomson, 1998b). When cultured *in vitro*, the human ES cells have normal karotypes, express telomerase activity, and proliferate. However, the inability to produce EBs from non-human primate ES cells lead to the belief that EBs could also not be formed from human ES cells (Thomson, 1998a).

SUMMARY OF THE INVENTION

The present invention is based on the finding that, contrary to the aforementioned belief that human ES cells do not form EBs, under appropriate conditions, human embryoid bodies (hEBs) may be obtained *in vitro*, from human ES (hES) cells suspended in a liquid medium. These hEBs contain mesoderm, ectoderm and endoderm cell lineages and may be used as a source of cells of the different lineages, e.g. for transplantation or inoculation into human recipients. By

incubating the hEBs. these basic cell lineages can differentiate into a wide variety of different cell types. e.g. cells having characteristics of cardiac cells. neural stem cells. and others.

The hEBs in accordance with the invention may be used as a source of cells for use in transplantation or inoculation into a human recipient, in order to treat various diseases or disorders. to assist in tissue repair. to substitute for degenerated tissue.

Thus. in accordance with one aspect. the present invention provides a human embryoid body (hEB).

The hEB. in accordance with the invention. is preferably obtained by *in vitro* culturing of hES cells in a vessel under conditions in which the cells or aggregates thereof do not adhere to the vessel walls.

In accordance with another aspect, the invention provides a process for obtaining at least one human-derived embryoid body (hEB), comprising:

(a) providing human embryonic stem (hES) cells;

(b) growing the hES cells *in vitro* in a vessel under conditions in which said cells undergo differentiation and the cells or aggregates thereof do not adhere to the vessel wall; and

(c) incubating for a time sufficient to develop hEBs from said cells.

The conditions whereby the hES cells do not adhere to the vessel wall include culturing the cells in a vessel having walls made of a material to which the cells or aggregates thereof are incapable of adhering, adding one or more factors into the medium which prevent adherence of the cells to the vessel walls etc. Conditions whereby the hES undergo differentiation include the absence of inhibitors of differentiation such as leukemia inhibitory factor and fibroblast growth factor.

The invention also provides a process for preparing cells of a defined cell lineage comprising:

(a) providing human embryonic stem (hES) cells;

(b) growing the hES cells *in vitro* in a vessel under conditions under which the cells undergo differentiation and said cells or aggregates thereof do not adhere to the vessel wall;

(c) incubating for a time sufficient to obtain at least one embryoid body (EB) containing cells of said defined cell lineage; and

(d) isolating said cells from said EB.

In accordance with one embodiment, the cells of the defined cell lineage may be inoculated or transplanted into a human recipient to treat a certain human disease or condition, to allow tissue or organ repair, etc. Thus, in accordance with this preferred embodiment, the cells of the defined cell lineage are formulated in a manner to allow their inoculation or transplantation into the human recipient.

The present invention also provides, by another of its aspects, an inoculable or transplantable preparation comprising cells of said defined cell lineage together with a physiologically acceptable carrier which is compatible with said cells. The term *"compatible with said cells"* should be understood as a medium which ensures the viability of said cells until they are inoculated or transplanted into the human recipient.

The cells of the defined cell lineage may be induced to undergo furthr differentiation and transplanted or inoculated into the individual as a cell suspension or alternatively, they may be cultivated to form a cell mass or an *in vitro* tissue, and the mass or the tissue then being transplanted into the individual.

The hES cells from which the hEBs of the invention are derived may be obtained from established lines (e.g. see Thomson *et al.*, 1998b, *supra*) or may be prepared *de novo* from human embryos which were produced by *in vitro* fertilization.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1A shows cystic hEBs from 3 to 14 days after transfer to petri dishes produced in accordance with the invention. I: simple EB; II & III: cavitated EB; IV: cystic hEB. Scale bar. 40 μm.

Fig. 1B shows 5 μm paraffin embedded sections of hEBs strained with hematoxylin and eosin (H&E, I,II and IV) or DAPI (III) I: scale bar 80 μm; II& III scale bar 20 μm; IV: scale bar 4 μm.

Fig. 2 shows the expression of cell specific genes in hES cells and cystic hEBs;

Fig. 3 shows *in situ* hybridization analysis of expression of α-fetoprotein (αFP), ξ-globin (ξ-Glob), neurofilament 68 kd protein (NF) and α-cardiac actin (cAct) in 20 day old Ebs (Con indicates control);

Fig. 4A shows a pulsating hEB in a relaxed state. Scale bar, 40 μm;

Fig. 4B shows the pulsating hEB of Fig. 4A in a subsequent contracted state. Scale bar, 40 μm; and

Fig. 4C shows serial sections from a pulsating hEB including a section stained with H&E, a hybridized section showing α-cardiac actin expression (cActin), and a control section hybridized with non-specific RNA (Control). Scale bar,100 μm.

MATERIALS AND METHODS

Formation of human cystic EBs

Human ES cells (H9 clone 10) were grown on mouse embryo fibroblasts in a culture medium consisting of 80% KnockOut™ DMEM (an optimized Dulbeco's modified Eagle's medium for ES cells, Gibco-BRL), 20% KnockOut™ SR (a serum-free formulation, Gibco-BRL), 1 mM glutamine (Gibco-BRL), 0.1 mM β-mercaptoetanol (Sigma), 1% non-essential amino acids stock (Gibco-BRL), 10^3units/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 are kept in an undifferentiated state. To induce formation of hEBs, the ES cells were transferred to plastic petri dishes to prevent their adherence to the dish and

promote their aggregation. The concentration of the cells was about 10^5 cells per ml. The hEBs were then cultured in the above culture medium not containing leukemia inhibitor factor or basic fibroblast growth factor.

Detection of expression of cell specific genes in cystic hEBs.

Total RNA was extracted from cells as previously described (Chirgwin *et al.*, 1979) and cDNA was synthesized from 1 μg of total RNA, using a random hexamer ($pd(N)_6$) as primer (Pharmacia Biotech) and M-LMV Reverse Transcriptase (Gibco-BRL). cDNA samples were subject to polymerase chain reaction (PCR) amplification with specific DNA primers. PCR was performed under linear conditions in order 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 pairs (bp). 20 cycles at 60°C in 1 mM $MgCl_2$; ξ-globin: GACTGAGAGGACCATCATTG and TCAGGACAGAGGATACGACC – Product: 397 bp. 25 cycles at 60°C in 1 mM $MgCl_2$; GAPDH; AGCCACATCGCTCAGACACCA and GTACTCAGCGGCCAGCATCG – Product: 302 bp. 20 cycles at 60°C in 1 mM $MgCl_2$. PCR products were analyzed by Western Blot hybridization (Southern, 1975). Probes were radiolabeled by random priming (Boehringer Mannheim) using [α-^{32}P]dCTP (3000 ci/mM, NEN – Life Science Products).

***In situ* hybridization analysis of hEBs**

EBs were stained with hematoxylin and eosin (H&E) or hybridized to specific RNA probes labeled with a fluorescent reagent (Grifman *et al.*, 1998b) and 5 μm paraffin embedded serial sections were obtained. Control hybridizations were performed with non-specific RNA. The probes used were 50-mer 2′-O-methyl 5′-biotinylated cRNA of either α-fetoprotein – TTGTCCCTCTTCAGCAAAGC AGACTTCCTGTTCCTGGCCTTGGCAGCATT, ξ-globin – TGATGGTCCTCT CAGTCTTGGTCAGAGACATGGCGGCAGGGTGGGCAGCT, Neurofilament

68 kd protein – CCTGCGTGCGGATGGACTTGAGGTCGTTGCTGATGGCG GCTACCTGGCTC. or α-cardiac actin – CGGTGGACAATGGATGGGCCTG CCTCATCGTACTCTTGCTTGCTAATCCA.

EXAMPLES

Example 1: Formation of cystic hEBs

Fig. 1A shows several hEBs 3 to 14 days after having been transferred to plastic petri dishes and cultured in medium not containing leukemia inhibitor factor or basic fibroblast growth factor. Initially, simple hEBs form (Fig. 1A-I). Subsequently, the center of the bodies became cavitated (Fig. 1A. II and III), and the bodies began to accumulate fluid, turning to cystic hEBs (Fig. 1A, IV). 20 days after initiation of cellular aggregation most of the structures were cystic and they included a variety of epithelial and mesenchymal cells. Fig. 1B shows 5 μm paraffin embedded sections of 20 day old hEBs stained with H&E (I-IV) or DAPI (V). DAPI staining of the nuclei revealed in some of the cells condensed chromatin that probably corresponds to the apoptosis occurring in the center of the hEB (Fig. 1B-III).

Example 2: Expression of cell specific genes in cystic hEBs.

In order to examine the differentiation status of the EBs, RNA was extracted from hES cells grown on mouse embryo fibroblasts, and from 20 day old EBs. cDNA was synthesized using these RNA samples and expression analysis of several genes was performed by RT-PCR with various human specific DNA primers. The RT-PCR analysis was performed under non-saturating linear conditions. The identity of the amplified DNA product in the PCR assay was verified by sequence analysis. The lane marked EB in Fig. 2 shows that the hEBs express α-fetoprotein (αFP), an endodermal marker (Krumlauf *et al.*, 1985), and ξ-globin, a marker of hematpoietic cells (Leder *et al.*, 1985)) . Very low levels of these markers were observed in the hES cells (Lane ES). The house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), served as an internal control. Linearity of the observed signal is demonstrated by the *"1/10 column"*

showing a PCR assay performed with one-tenth the amount of cDNA used in the PCR assay of the hEB sample.

Example 3: *In situ* hybridization analysis of hEBs

To regionally characterize the differentiating cells within the EBs by *in situ* hybridization. expression of four cell-specific molecular markers, all of which transcribe very early during embryonic differentiation, was examined. Serial sections of the hEBs were hybridized with RNA probes specific to α-fetoprotein (αFP) (Krumlauf *et al.*, 1985), ξ-globin (ξ-glob) ((Leder I., 1985), α-cardiac actin (cAct) (Sassoon *et al.*, 1988)) or neurofilament 68kd protein (NF) (Julien *et al.*, 1986)). As shown in Fig. 3, each of these molecular probes specifically labels distinct regions in the EBs. This suggests that the labeled cells in each section were either clonal (derived from a common progenitor cell), or were affected by the same signals and differentiated into the same specific lineage.

Example 4: Characterization of a pulsating hEB

Differentiation of murine ES cells in EBs into the myocardial lineage has been shown to produce pulsating muscle (Sanchez *et al.*, 1991). The existence of cardiac muscle cells in hEBs was demonstrated by *in situ* hybridization of sections from a human EB with α-cardiac actin, a marker of embryonic myocardial cells (Sassoon *et al.*, 1988). Figs. 4A and B shows a large vacuated hEB including cardiac muscle cells that were pulsating at a rate of about 30 beats/min. The hEB was first photographed in a relaxed state (Fig. 4A) and then in a subsequent a contracted state (Fig. 4B). Fig. 4C shows serial sections of a cell including a section stained with hematoxylin and eosin (H&E), a hybridized section showing α-cardiac actin expression (cActin), and a section hybridized with non-specific RNA (Control).

References

Bain, G., Kitchens, D., Yao, M., Huettner, J.E., Gottlieb, D.I., *Developmental Biology*, **168**:342-57 (1995).

Capecchi, M.R., *Science*, **244**:1288-92 (1989).

Chirgwin, J.M., Przybyla, A.E., Macdonald, R.J., Rutter, W.J., *Biochemistry*, **18**:5294-9 (1979).

Dushnik-Levinson, M., Benvenisty, N., *Biology of the Neonate*, **67**:77-83, (1995).

Julien, J.P., Meyer, D., Flavell, D., Hurst, J., Grosveld, *Brain Research*, **387**:243-50 (1986).

Krumlauf, R., Hammer, R.E., Tilghman, S.M., Brinster, R.L., *Molecular and Cellular Biology*, **5**:1639-48 (1985).

Leder, A., Weir, L., Leder, P., *Molecular and Cellular Biology*, **5**:1025-33 (1985).

Levinson-Dushnik, M., Benvenisty, N., *Molecular and Cellular Biology*, **17**:3817-22 (1997).

Lindenbaum, M.H., Grosveld, F., *Genes and Development*, 4:2075-85 (1990).

Robertson, E., in *Teratocarcinoma and Embryonic Stem Cells: A Practical Approach*, R. EJ, Eds. (IRL Press, Oxford, 1987) pp. 71-112.

Rossant, A.L. Joyner, *Trends in Genetics*, **5**:277-83 (1989).

Sanchez, A., Jones, W.K., Gulick, J., Doetschman, T., Robbins, J., *Journal of Biological Chemistry*, **266**:22419-26 (1991).

Sassoon, D.A., Garner, I., Buckingham, M., *Development*, **104**:155-64 (1988).

Southern, E.M., *Journal of Molecular Biology*, **98**:503-17 (1975).

Thomson, J.A., *et al.*, *Biology of Reproduction*, **55**:254-9 (1996).

Thomson, J.A., *et al.*, *Proceedings of the National Academy of Sciences of The United States of America*, **92**:7844-8 (1995).

Thomson, J.A. and Marshall, V.S., *Curr. Top. Dev. Biol.* **38**, 133 (1988a)

Thomson, J.A., *et al.*, *Science*, **282**:1145-7 (1998b).

Wiles. M.V.. Keller. G.. *Development*. **111**:259-67 (1991).

Wobus. A.M.. Holzhausen. H., Jäkel. P., Schöneich, J.. *Experimental Cell Research*. **152**:212-9 (1984).

CLAIMS:

1. A human embryoid body (hEB) derived from a human embryonic stem cell and containing embryonic tissue.

2. An hEB according to Claim 1, obtained by *in vitro* culturing of human embryonic stem (hES) cells in a vessel under conditions in which the cells undergo differentiation and the cells or aggregates thereof do not attach to the vessel walls.

3. An hEB according to Claim 1, comprising mesoderm, ectoderm and endoderm lineage cells.

4. An hEB according to Claim 3, comprising cells displaying characteristics of cardiac cells.

5. An hEB according to Claim 1, comprising cells expressing a marker selected from the group consisting if α-fetaprotein, ξ-globin, α-cardiac actin and neurofilament 68 kd.

6. A process for obtaining at least one human-derived embryoid body (hEB), comprising:

(a) providing human embryonic stem (hES) cells;

(b) growing the hES cells *in vitro* in a liquid growth medium in a vessel under conditions under which said cells undergo differentiation and said cells or aggregates thereof do not adhere to the vessel wall; and

(c) incubating for a time sufficient to develop hEBs from said cells.

7. A process according to Claim 6, wherein said conditions comprises the absence of leukemia inhibitory factor and fibroblast growth factor from the growth medium.

8. A process according to Claim 6, wherein said condition comprises culturing cells in a vessel having walls of a kind to which cells do not adhere.

9. A process for preparing cells of a defined cell lineage, comprising:

(a) providing human embryonic stem (hES) cells;

(b) growing the hES cells *in vitro* in a vessel under conditions in which said cells undergo differentiation and said cells or aggregates thereof do not adhere to the vessel wall;

(c) incubating for a time sufficient to obtain at least one human embryoid body (hEB) containing cells of said defined cell lineage; and

(d) isolating said cells from said hEB.

10. A process according to Claim 9, wherein said cells are formulated for inoculation or implantation into a recipient individual.

11. An injectable or implantable preparation, comprising cells obtained by the process of Claim 9 or 10 together with a physiologically acceptable carrier compatible with said cells.

12. An implantable cell mass or tissue obtained by the process of Claim 9 or 10.

13. Cells produced according to the process according to Claim 9.