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(54) **CULTURE SYSTEM AND METHOD FOR
MAINTENANCE AND PROLIFERATION OF
UNDIFFERENTIATED HUMAN EMBRYONIC
STEM CELLS**

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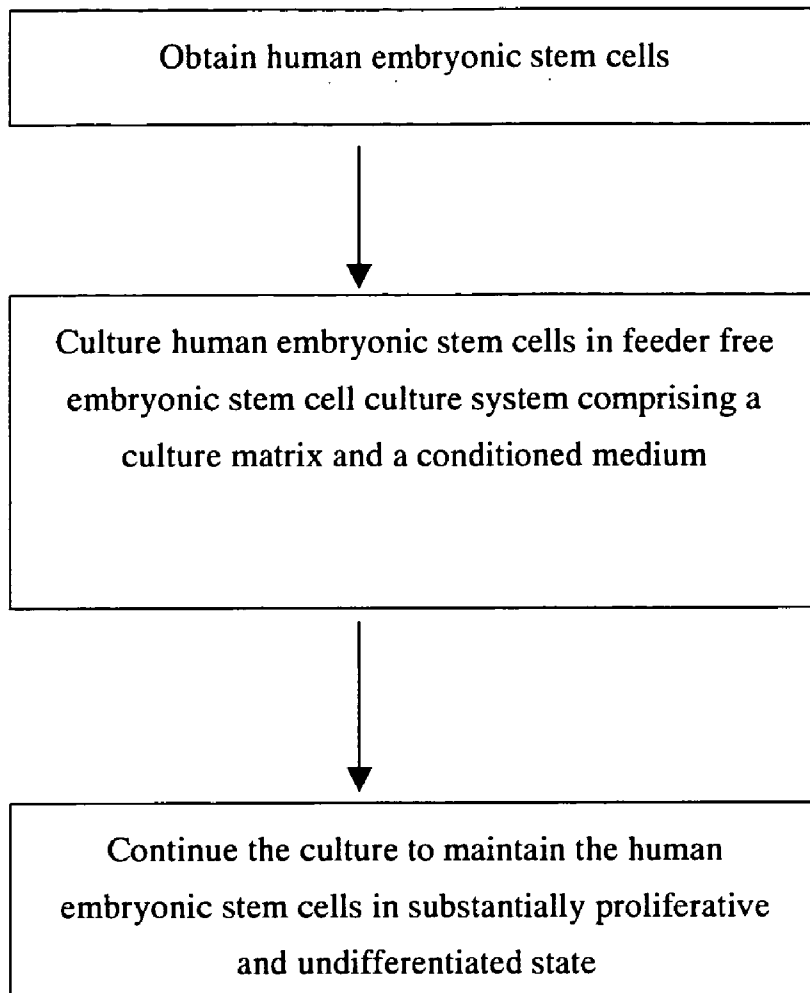
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(57) **ABSTRACT**

The present invention discloses an improved culture system for proliferative and undifferentiated growth of human embryonic stem cells, comprising an extracellular matrix prepared from feeder cells and a conditioned medium pre-conditioned by feeder cells. This invention also relates to a culture method utilizing the foregoing culture system.



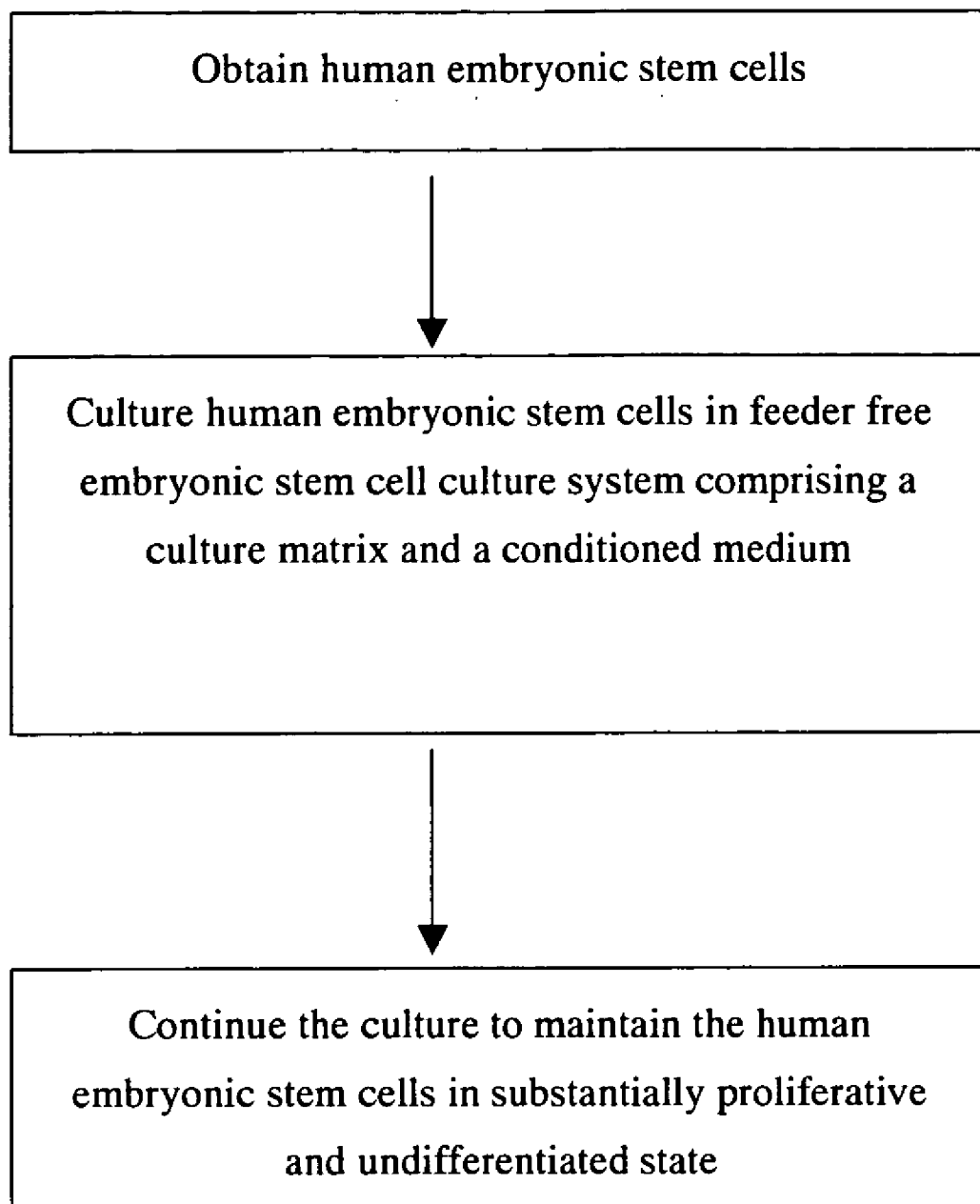


FIG. 1

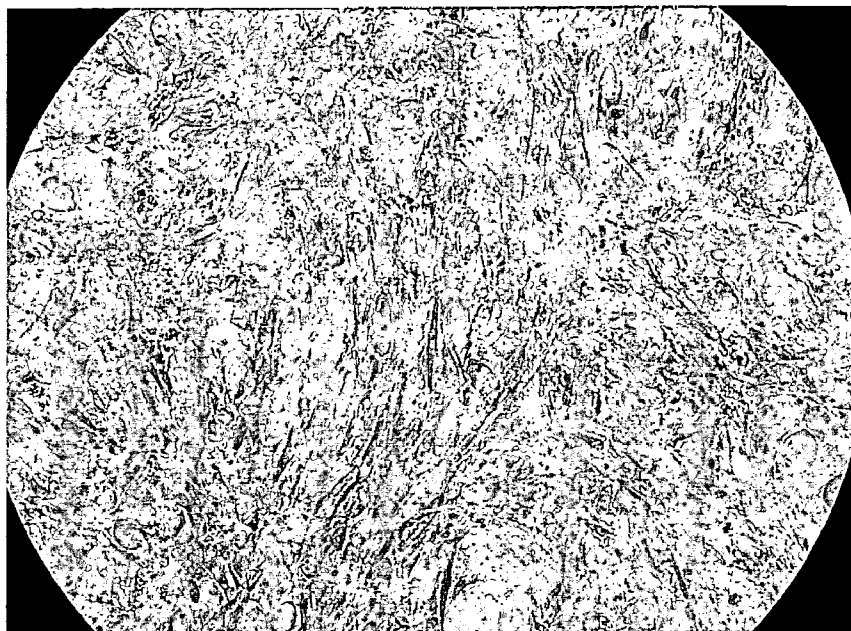


Fig. 2A

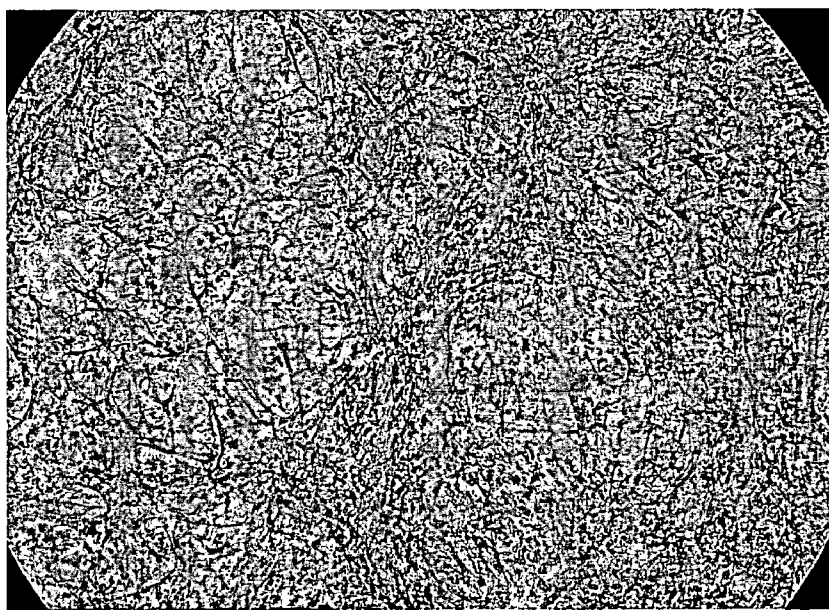


Fig. 2B

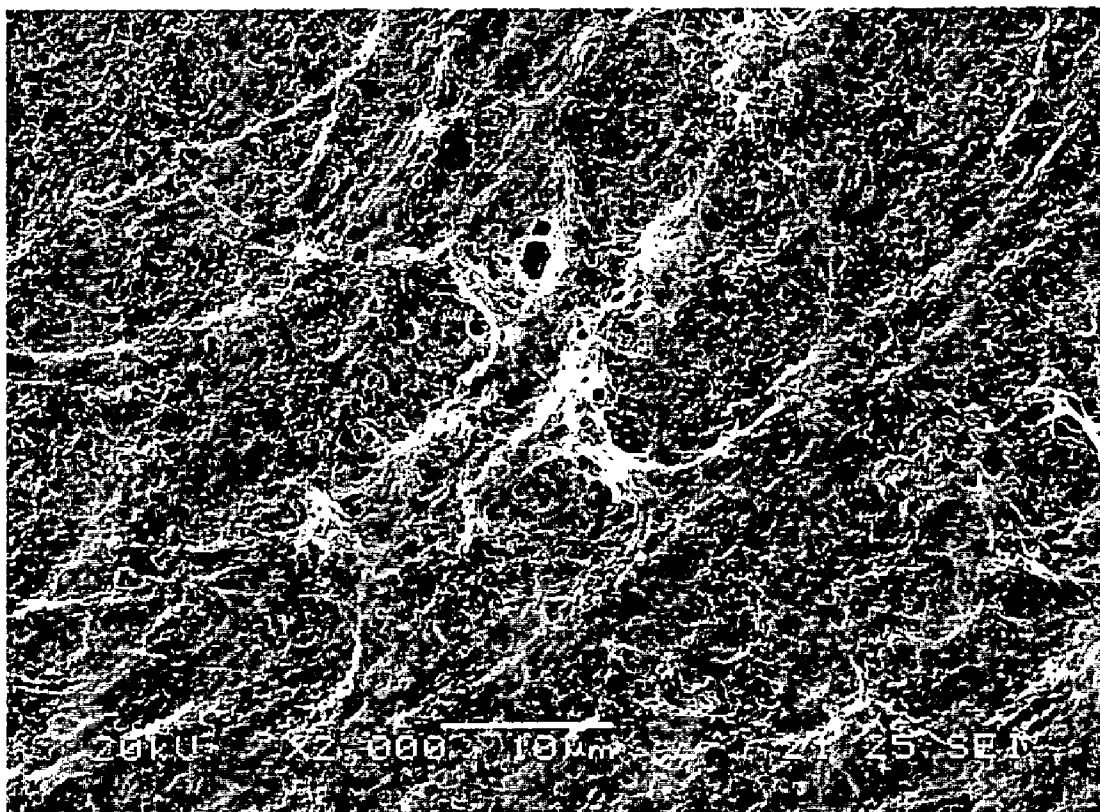


Fig. 2C

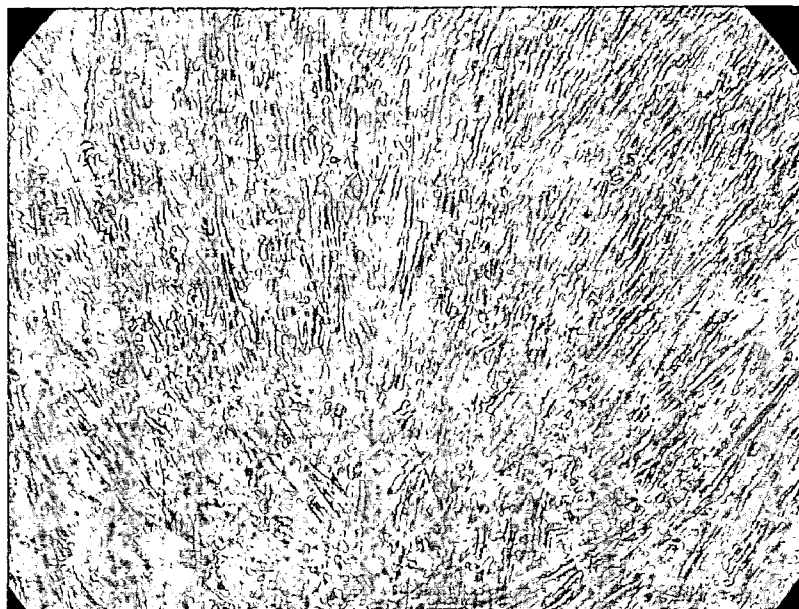


Fig. 3A

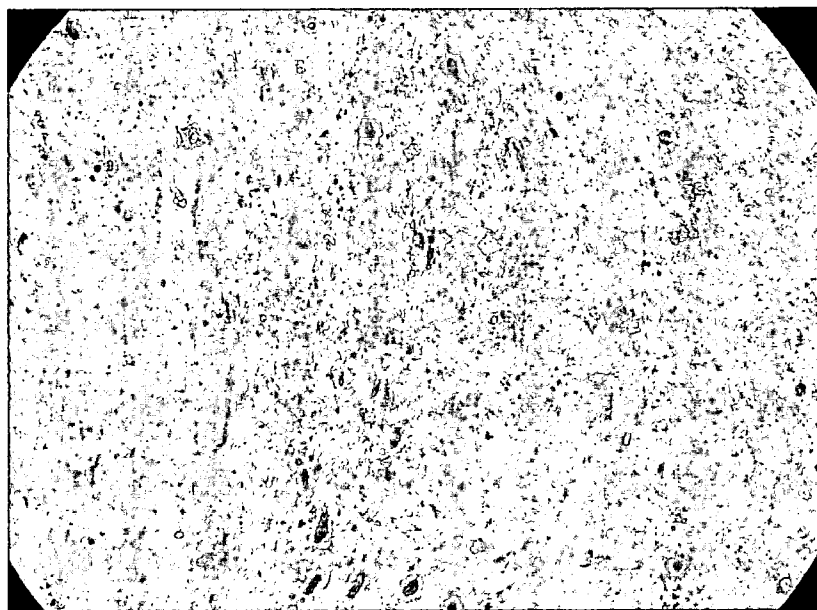


Fig. 3B

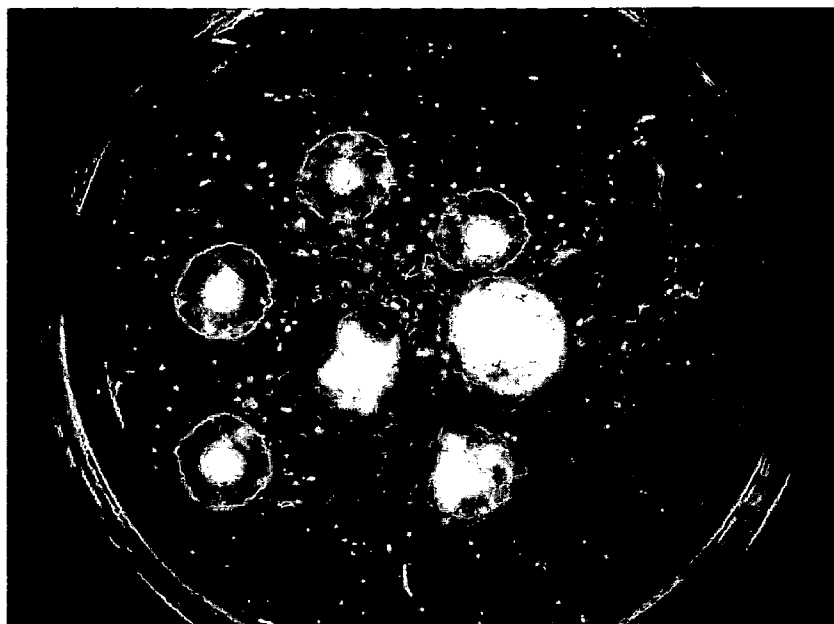


Fig. 4A

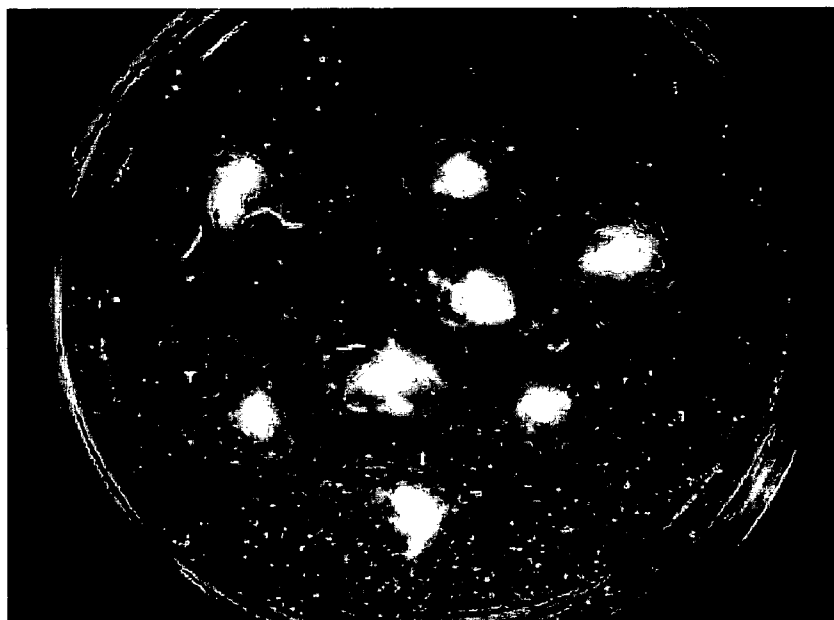


Fig. 4B

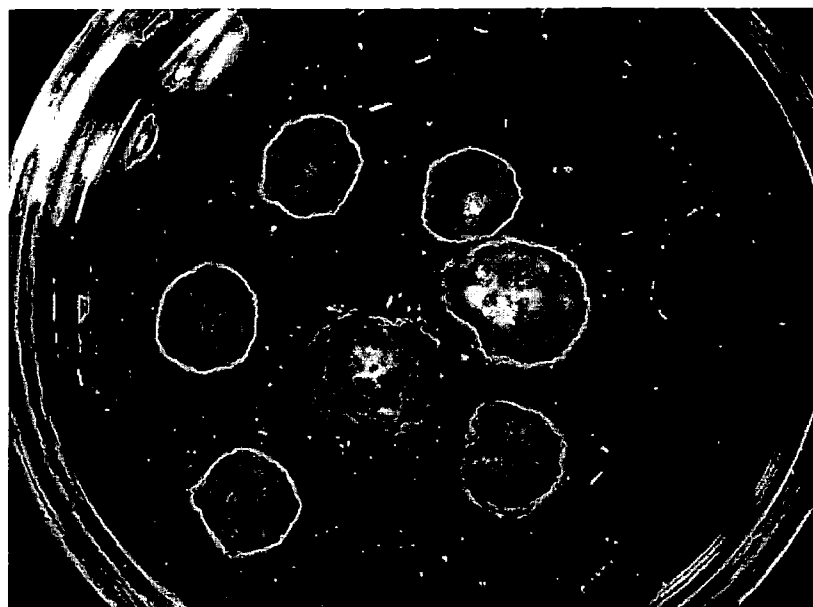


Fig. 5A

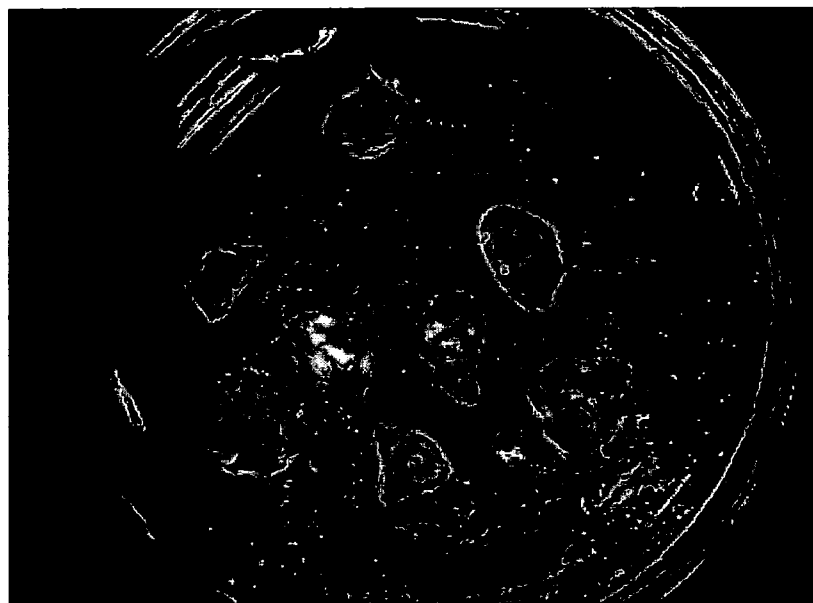


Fig. 5B

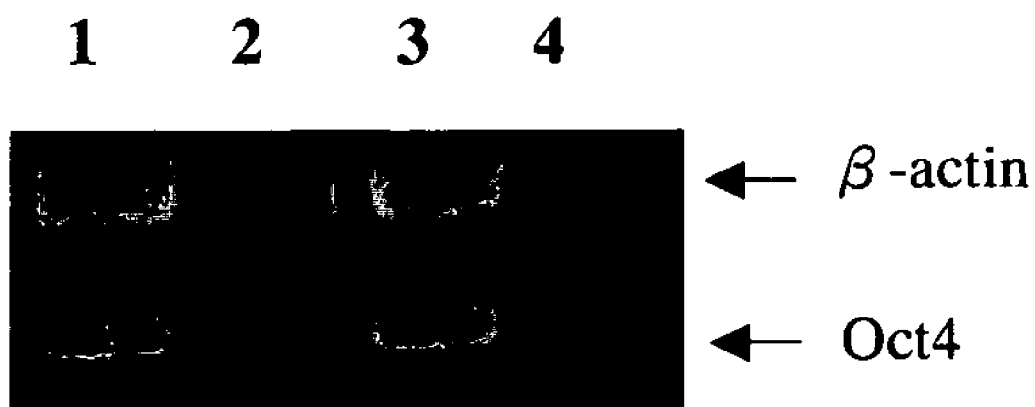


Fig. 6

CULTURE SYSTEM AND METHOD FOR MAINTENANCE AND PROLIFERATION OF UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS

BACKGROUND OF THE INVENTION

[0001] 1. Field of the Invention

[0002] The present invention relates to the field of human embryonic stem (HES) cell culture, in particular undifferentiated HES cell culture, and to methods for maintenance and proliferation of such cells. More particularly, the invention relates to maintenance of HES cells in a substantially proliferative and undifferentiated status in a culture system without feeder layer.

[0003] 2. Description of Related Art

[0004] Human embryonic stem (HES) cells are derived from the inner cell mass of blastocyst. Nowadays, most HES cells can only be maintained in culture in an undifferentiated state when grown on inactivated feeder cells.

[0005] Previous studies have found that the important factors in feeder cells that support the growth and proliferation of ES cells include growth factors secreted by feeder cells in the culture medium and extracellular matrix (ECM) constructed by the feeder cells. The feeder cells can be a primary mouse embryonic fibroblast (PMEF), a mouse embryonic fibroblast cell line (MEF), a murine fetal fibroblast (MFF), a human embryonic fibroblast (HEF), a human fetal muscle cell (HFM), a human fetal skin cell (HFS), a human adult skin cell, a human foreskin fibroblast (HFF), a human adult fallopian tubal epithelial cell (HAFT) and a human marrow stromal cells (hMSCs) (WO 03/02944, WO 03/014313, J. H. Park et al., *Biol Reprod.*, 69: 2007-2017, 2003, M. Amit et al., *Biol Reprod.*, 68 (6): 2150-2156, 2003, Outi Hovattal et al., *Hum. Reprod.*, 18 (7): 1404-1409, 2003, Richards, M. et. Al, *Nat Biotechnol.*, 20 (9): 933-936, 2002, James A. et al., *Science*, 282 (6): 1145-1147, 1998 and Linzhao Cheng et al., *Stem Cells*, 21: 131-142, 2003).

[0006] The extracellular matrix (ECM) is not merely a passive structure. In the past few years, it has emerged that the matrix is a dynamic action zone that functions to instruct cellular phenotype. ECM proteins interact directly with cell surface receptors to initiate signal transduction pathways and to modulate those triggered by growth factors. ECM also controls the activity and presentation of a wide range of growth factors. Thus modulation of the ECM, by remodeling its structure and activity, has profound effects on its function and the consequent behaviour of cells residing on or within it. At present, the interacting mechanisms of the ECM in establishment and maintenance of a ES cell culture is not known. Possible roles for the ECM include the provision of ECM components that provide attachment sites for the ES cells, trigger signaling for cell renewal.

[0007] The feeder layer dependent culture system in scaling up and impedes the mass production and clinical application of HES cells. There are some problems in a feeder layer dependent culture system: (1) the potential risks of transmission of pathogens from the animal feeder cells to the HES cells and the fact that the current system of propagation (human/animal or human/human co-culture) has been construed as a xenotransplant, (2) feeder cells come mainly from primary cells, while primary cells from different

batches offer different effect as feeder cells, rendering the quality control of the cultured HES cells more difficult; (3) the limited sources and numbers of feeder cells hamper the mass production and applications of HES cells. Therefore, the method for the maintenance and proliferation of undifferentiated HES cells without feeder cells is critical for mass product and clinical application of HES cells. (U.S. 2003/143736).

[0008] Xu et al. (*Nat. Biotechnol.*, 19 (10): 971-974, 2001. WO 03/020920 and U.S. 2003/0017589) were the first to successfully maintain undifferentiated HES cells in a feeder-free culture system. In this system, HES cells are cultured on Matrigel from the Engelbreth Holm Swarm (EHS) sarcoma or laminin in medium conditioned by MEF. However, such synthetic matrices and defined-matrix macromolecules are not sufficient to mimic the more complex cell-matrix interactions provided by feeder cells. A study has also indicated that this culture system is only suitable for certain HES cell lines (e.g. H1 and H9), but unsustainable for other HES cell lines (Outi Hovattal et al. *Hum. Reprod.*, 18 (7): 1404-1409, 2003).

[0009] Accordingly, it is an object of the present invention to provide an alternative feeder-free culture system to overcome some of the disadvantages of the prior art.

SUMMARY OF THE INVENTION

[0010] The present invention provides methods and culture system for culturing undifferentiated HES cells. The methods and culture system described herein provide improved culture conditions that allow the maintenance and proliferation of HES cells in a substantially undifferentiated state.

[0011] In one aspect, the present invention provides a cell culture system for growing HES cells in a substantially undifferentiated state. The cell culture system of the invention comprises ECM as culture matrix and conditioned medium.

[0012] The feeder cells to be used in the present invention can be, for example, but not limited to primary mouse embryonic fibroblasts (PMEF), a mouse embryonic fibroblast cell line (MEF), murine fetal fibroblasts (MFF), human embryonic fibroblasts (HEF), human fetal muscle cells (HFM), human fetal skin cells (HFS), human adult skin cells, human foreskin fibroblasts (HFF), human adult fallopian tubal epithelial cells (HAFT) and human marrow stromal cells (hMSCs).

[0013] In another aspect, the present invention provides a culture method for growing HES cells in a substantially undifferentiated state, comprising culturing undifferentiated HES cells in a feeder-free culture system of the invention. The HES cells cultured in such culture system may be maintained in substantially proliferative and undifferentiated state for at least five passages.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] **FIG. 1** shows the flow chart of culturing HES cells in the feeder-free culture system according to this invention.

[0015] **FIG. 2A** shows the morphology of primary mouse embryonic fibroblasts (PMEF) (300x).

[0016] **FIG. 2B** shows the structure of ECM of PMEF (300×).

[0017] **FIG. 2C** shows the macrofibril bundles and network structure of ECM of PMEF observed under scanning electron microscope (2000×).

[0018] **FIG. 3A** shows the morphology of human foreskin fibroblast (HFF) (300×).

[0019] **FIG. 3B** shows the structure of ECM of HFF (300×).

[0020] **FIG. 4A** shows the morphology of HES cells (HES3) cultured on ECM prepared from PMEF according to this invention (10×).

[0021] **FIG. 4B** shows the morphology of HES3 cells cultured on ECM prepared from HFF according to this invention (10×).

[0022] **FIG. 5A** shows high alkaline phosphatase activity in HES3 cells cultured on ECM prepared from PMEF according to this invention (10×).

[0023] **FIG. 5B** shows high alkaline phosphatase activity in HES3 cells cultured on ECM prepared from HFF according to this invention (10×).

[0024] **FIG. 6** shows the OCT-4 expression of HES3 cells cultured in the feeder-free culture system according to this invention.

DETAILED DESCRIPTION OF THE INVENTION

[0025] In one aspect, the present invention relates to a cell culture system for growing HES cells in a substantially undifferentiated state. This invention is characterized by the removal of feeder cells, while retaining the ECM structure and nutrients secreted by the feeder cells, hence developing a feeder-free culture system for HES cells, which breaks the bottleneck facing the large scale production and clinical application of HES cells.

[0026] Specifically, this invention provides a culture system for growing HES cells, comprising a culture matrix consisting of ECM prepared from feeder cells and a conditioned medium being preconditioned by feeder cells.

[0027] The aforesaid culture matrix provides substratum for cell attachment during culture and helps to maintain the HES cells in a substantially undifferentiated state.

[0028] The aforesaid feeder cells may be fibroblasts or other types of cells, which may be inactivated by large-dose radiation, such as y-ray, or by drug, such as mitomycin C, so that the surviving cells lose the capability to proliferate, but retain their physiological functions, such as metabolism and synthesis of growth factors. Specifically, the feeder cell may be selected from the group consisting of primary mouse embryonic fibroblasts (PMEF), a mouse embryonic fibroblast cell line (MEF), murine fetal fibroblasts (MFF), human embryonic fibroblasts (HEF), human fetal muscle cells (HFM), human fetal skin cells (HFS), human adult skin cells, human foreskin fibroblasts (HFF), human adult fallopian tubal epithelial cells (HAFT) and human marrow stromal cells (hMSCs). In the preferred embodiments of the invention, the feeder cells are derived from mouse embryonic fibroblasts (MEF) or human foreskin fibroblasts (HFF).

[0029] In the preferred embodiment of the invention, said HES cells are HES-3 or HES-4 cell lines.

[0030] The preparation of ECM may be performed by those skilled in the art or refer to R. Ian Freshney (2000) *Culture of Animal Cells: A Manual of Basic Technique*, 4th Edition, Wiley-Liss, Inc. Basically, feeder cells were treated with NaOH or trinitrotoluene (Triton) and followed by removing intracellular substances, such as nucleus or organelles, so as to obtain the ECM as culture matrix. The removal of intracellular substances may be achieved by washing with buffered solution or water.

[0031] The aforesaid conditioned medium refers to culture medium in which feeder cells have been cultivated already for a period of time and thus is preconditioned by feeder cells. The preparation of conditioned medium may be performed by those skilled in the art or refer to U.S. Pat. Nos. 5,690,926, 2003/0008392, 2003/0073234, and 2002/0160509 as well as Reubinoff B. E. et al, *Nat. Biotechnol.*, 18 (4): 399-404, 2002. The major ingredients of a conditioned medium are typically amino acids, vitamins, carbohydrates, inorganic ions and some other auxiliary substances. Growth factors that promote cell growth or inhibit differentiation, e.g. leukemia inhibitory factor (ILF), fibroblast growth factor (FGF), stem cell factor (SCF), insulin-transferrin-selenium G supplement (ITS G supplement) may also be added into the culture medium according to WO 03/020920, U.S. 2003/0017589, U.S. Pat. No. 5690926, U.S. Pat. No. 5,453,357, Xu, C. et al., *Nat. Biotechnol.*, 19 (10): 971-974, 2001, and Richards, M. et. Al, *Nat Biotechnol.*, 20 (9): 933-936, 2002.

[0032] It is more specific that the preparation of the aforesaid conditioned medium comprises of the steps of: (a) inactivating the feeder cells; (b) placing the cells obtained in step (a) in a culture solution; and (c) collecting the cell culture solution as conditioned medium. In another aspect, the present invention provides a culture method for growing HES cells in a substantially undifferentiated state, comprising culturing undifferentiated HES cells in the cell culture system of this invention as shown in **FIG. 1**. The method comprises the following steps: obtaining HES cells; culturing the HES cells in the feeder-free culture system described above; and maintaining the HES cells in substantially proliferative and undifferentiated state, wherein HES cells in said feeder-free culture system may be maintained in substantially proliferative and undifferentiated state for at least five passages.

[0033] Definitions

[0034] The following terms will be defined as provided unless otherwise stated. All other terminology used herein will be defined with respect to its usage in the particular art to which it pertains unless otherwise noted.

[0035] "Conditioned Medium"

[0036] Conditioned Medium as used for the purpose of describing the present invention refers to the medium in which feeder cells have been cultivated already for a period of time. The conditioned medium of the present invention can be used for cultivation of HES cells because it contains many mediator substances, such as growth factors and cytokines, that were secreted by the feeder cells cultivated previously and can thus help to promote the growth of HES cells.

[0037] “Extracellular Matrix”

[0038] Extracellular Matrix or ECM or Defined Matrix occupies the space between cells and establishes a complex network of different combinations of collagens, proteoglycans, hyaluronic acid, laminin, fibronectin, and many other glycoproteins including proteolytic enzymes involved in degradation and remodeling of the ECM. ECM plays an important structural and functional role in multicellular organisms and is more than a scaffold that fills extracellular spaces. Many of its components are engaged in processes mediating cell-to-cell interactions. In the present invention, ECM serves as culture matrix prepared from feeder cells so as to provide a support in a feeder-free culture environment for HES cells.

[0039] “Feeder Cells”

[0040] Feeder Cells as used for the purposes of describing the present invention refers to those used as a substratum on which other cells are grown in a culture system. Feeder cells are usually adherent growth-arrested but viable and bioactive cells (primary cells or continuous cell lines) that have been incapacitated, for example by irradiation.

[0041] All other acronyms and abbreviations have the corresponding meaning as published in journals related to the arts of chemistry and biology.

[0042] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention. While the invention is described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

EXAMPLE 1**Preparation of the Compositions of the Culture System****[0043]** 1. Preparation of Conditioned Medium

[0044] Conditioned medium for maintaining HES cells was prepared using the following procedure. Primary mouse embryonic fibroblasts (PMEF) or human foreskin fibroblast (from Animal Technology Research Institute, Taiwan) in the presence of a growth medium prepared from 10% fetal bovine serum (FBS, from HyClone) and 90% Dulbecco's Modified Eagle Medium (DMEM, from Gibco). When the cells reach confluence, mitomycin C was added to inactivate the fibroblasts. These fibroblasts were grown in the presence of a growth medium prepared from 80% Dulbecco's modified eagle medium (DMEM, from Gibco), 20% fetal bovine serum (FBS, from Hyclone), and supplemented with 1 mM β -mercaptoethanol (from Gibco), 1% non-essential amino acids (from Gibco), 1% glutamine (from Gibco), and 1% insulin-transferrin-selenium G supplement (ITS G supplement, from Gibco) (refer to Richards, M. et al., Nat. Biotechnol., 20 (9): 933-936, 2002), in which fetal bovine serum may be substituted by serum replacement to obtain serum-free culture medium. The ES medium was collected and filter-sterilized (0.2 micron filter). This medium was

termed “conditioned ES medium”. The conditioned ES medium was used immediately or frozen at about -20°C . until needed. Based on the requirements of the cultured cells, the growth medium may contain other ingredients without limited to those discussed herein.

[0045] 2. Preparation of Culture Matrix

[0046] Culture matrix for maintaining HES cells was prepared using the following procedure. Primary Mouse embryonic fibroblasts (PMEF) or human foreskin fibroblasts (from Animal Technology Research Institute, Taiwan) were grown to confluence in the presence of a growth medium prepared from 10% fetal bovine serum (FBS, from HyClone) and 90% Dulbecco's Modified Eagle Medium (DMEM, from Gibco). When the cells reached confluence, mitomycin C was added to inactivate the fibroblasts. For harvesting the culture matrix, 0.05N NaOH or 0.1% trinitrotoluene (from Sigma) was used to break the cell membranes and followed by washing with pH 7.4 Dulbecco's Phosphate Buffered Saline (1X, from Gibco) to remove organelle and nucleus so as to harvest the culture matrix.

EXAMPLE 2**Culturing HES Cells using the Culture System of this Invention**

[0047] HES cells (HES-3 or HES-4 cell lines) were plated onto the culture matrix and incubated with conditioned medium mentioned above and cultured in a 5% CO_2 incubator under 37°C . The medium was changed every 1-2 days. After 7 days of culture, HES cells were carried on subculture of cells.

EXAMPLE 3**Observing the Culture Matrix of Culture System**

[0048] **FIG. 2A** shows the morphology of primary mouse embryonic fibroblasts. The culture matrix derived from primary mouse embryonic fibroblasts following the steps in Example 1 is shown in **FIG. 2B**. When observed under scanning electron microscope, the macrofibril bundles and network structure of said culture matrix are visible as shown in **FIG. 2C**.

[0049] **FIG. 3A** and **FIG. 3B** shows respectively the morphology of human foreskin fibroblast and the structure of culture matrix prepared from human foreskin fibroblast according to the steps described above.

EXAMPLE 4**Analyzing the Undifferentiated State of HES Cells**

[0050] The effect of the culture system herein may be further observed using biomarkers specifically expressed in undifferentiated HES cells, e.g. alkaline phosphatase activity, OCT-4, SSEA-3, SEA-4, TRA-1-60, and TRA-1-81 (refer to Thomason J. A. et al, Science, 282 (6): 1145-1147, 1998 or Reubinoff B. E. et al. Nat Biotechnol. 18 (4): 399-404, 2000) to determine the undifferentiated level of ES cells.

[0051] This invention uses the expression of alkaline phosphatase activity and OCT-4 to evaluate the effect of the feeder-free culture system herein. The assay of alkaline

phosphatase activity was employed according to the protocols provided within the Alkaline phosphatase substrate kit (Vector Laboratories, Inc.). The assay of OCT-4 was carried out according to the method described by Richards, M. et. Al, Nat Biotechnol., 20 (9): 933-936, 2002. The results are presented as follows:

[0052] 1. Observation of Cell Morphology:

[0053] **FIG. 4A** and **FIG. 4B** show the morphology of HES cells cultured in the culture system herein using primary mouse embryonic fibroblasts and human foreskin fibroblasts as culture matrix, respectively.

[0054] 2. Assay for Alkaline Phosphatase Activity:

[0055] HES cells show high alkaline phosphatase activity before differentiation, i.e., once they start to differentiate, they lose the alkaline phosphatase activity. Thus the differentiation status of cultured HES cells can be learned from the this enzyme activity. The assay results show that HES cells cultured in this culture system using either primary mouse embryonic fibroblast or human foreskin fibroblasts as culture matrix express high alkaline phosphatase activity (in bright red color as a result of stain), indicating that they were in a substantially undifferentiated state (**FIG. 5A** and **FIG. 5B**). The results also showed that HES cells may be continuously subcultured for at least five passages and maintained the characteristics of substantial proliferation and undifferentiation in the feeder-free culture system of the present invention.

[0056] 3. Assay for Transcription Factor OCT-4:

[0057] As shown in **FIG. 6** which compares the expression of OCT-4 in HES cells cultured in various culture systems, lane 1 is feeder cells derived from primary mouse embryonic fibroblasts; lane 2 is feeder cells derived from human foreskin fibroblasts; lane 3 is feeder-free culture system prepared according to this invention; and lane 4 is a cell-free negative control. Transcription factor OCT-4 can be observed in the ES cells cultured in the feeder-free culture system herein (lane 3), indicating that this culture system is able to maintain HES cells in substantially undifferentiated state. Besides, the expression level of OCT-4 in feeder-free culture system herein (lane 3) was comparable to that in feeder layer culture systems (lane 1 and lane 2).

[0058] The feeder-free culture system for ES cells presented herein offers the following advantages: (1) It prevents the potential risks of transmission of pathogens from the animal/human feeder cells to the human HES cells and the fact that the current system of propagation (human/animal co-culture) has been construed as a xenotransplant; (2) A better quality control of HES cells can be reached; (3) A mass/bulk production of HES cells is feasible; and (4) It sheds light on clinical application of HES cells.

[0059] The examples as disclosed above are used to provide detailed description and are in no way to be considered to limit the scope of the invention in any manner. All modifications and alterations made by those familiar with the skill without departing from the spirits of the invention and appended claims shall remain within the protected scope and claims of the invention.

What is claimed is:

1. A culture system for proliferation and undifferentiated growth of human embryonic stem cells comprising:

- a culture matrix comprising extracellular matrix prepared from feeder cells; and
- a conditioned medium being preconditioned by feeder cells;

wherein said feeder cells are selected from primary mouse embryonic fibroblasts (PMEF), a mouse embryonic fibroblast cell line (MEF), murine fetal fibroblasts (MFF), human embryonic fibroblasts (HEF), human fetal muscle (HFM), human fetal skin cells (HFS), human adult skin cells, human foreskin fibroblasts (HFF), human adult fallopian tubal epithelial cells (HAFT) or human marrow stromal cells (hMSCs).

2. The culture system according to claim 1, wherein said feeder cells are mouse embryonic fibroblasts or human foreskin fibroblasts.

3. The culture system according to claim 1, wherein said culture matrix is prepared by removing intracellular substances by means of treating said feeder cells with NaOH or trinitrotoluene to obtain extracellular matrix as culture matrix.

4. The culture system according to claim 3, wherein the intracellular substances are removed by washing with water or buffered solution.

5. The culture system according to claim 4, wherein said buffered solution is phosphate buffered saline.

6. The culture system according to claim 1, wherein said conditioned medium is prepared by the steps of:

- (a) inactivating the feeder cells;
- (b) placing the cells obtained in step (a) in a culture solution; and
- (c) collecting the cell culture solution as conditioned medium.

7. The culture system according to claim 6, wherein said feeder cells are inactivated by irradiation with gamma ray or treatment with mitomycin C.

8. The culture system according to claim 7, wherein said feeder cells are inactivated by treating with mitomycin C.

9. A method for culturing human embryonic stem cells, comprising the steps of:

- obtaining human embryonic stem cells;
- culturing the human embryonic stem cells in the culture system described according to claims 1; and
- continuing the culture to maintain human embryonic stem cells in a substantially proliferative and undifferentiated state.

10. The method according to claim 9, wherein said human embryonic stem cells are maintained in a substantially undifferentiated and proliferative state over the course of at least five passages in said culture system.

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