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(54) Title: HUMAN EMBRYONIC STEM CELL CREATED FROM AN OOCYTE AND A SOMATIC CELL DERIVED FROM NON-IDENTICAL INDIVIDUALS AND A METHOD FOR PREPARING THE SAME

(57) Abstract:



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【DESCRIPTION】**【Invention Title】**

HUMAN EMBRYONIC STEM CELL CREATED FROM AN OOCYTE AND A
SOMATIC CELL DERIVED FROM NON-IDENTICAL INDIVIDUALS AND A
5 METHOD FOR PREPARING THE SAME

【Technical Field】

The present invention relates to a method for preparing a human embryonic stem cells and a method for inducing differentiation of the embryonic stem cells into a
10 specific desired type of cells. More specifically, the present invention is about a method for preparing an embryonic stem cell from a somatic cell and an oocyte obtained from different individuals, wherein a somatic cell is isolated from a patient who is expected to receive the specific cell or tissue, and the nucleus of the somatic cell is transferred into an enucleated human oocyte, the resulting nucleus-transferred
15 oocyte is cultured to form a blastocyst, and the inner cell mass isolated from the blastocyst is cultured to form an embryonic stem cell. The present invention also relates to a method for inducing differentiation of the above created stem cell into the desired type of cells.

20 【Background Art】

In general, a stem cell refers to an undifferentiated cell capable of differentiating into all types of mature functional cells constituting a human body. For example, hematopoietic stem cells can differentiate into various types corpuscular cells. An embryonic stem (ES) cell is a pluripotent cell derived from a
25 human embryo and thus can differentiate and develop into all types of organs, tissues and cells that form a human body.

The establishment of a mouse ES cell line in 1981 provided a technique and paradigm for the development of a human ES cell. The development of the ES cell came from a study on mouse teratocarcinoma, a tumor that occurs in the gonad of a

closely bred mouse strain (Evans & Kaufman et al., *Nature*, 292:154-156 (1981)).

Bongso *et al.* reported a method for culturing and maintaining for a short period of time cells isolated from a human embryo created by *in vitro* fertilization (Bongso *et al.*, *Human Reproduction*, 9:2110-2117 (1994)). The cells isolated by
5 Bongso et al. had a morphology expected in a pluripotent stem cell; however, they could not be cultured for a long period of time because a proper feeder layer was not used.

Primate ES cells have been prepared from the blastocyst of a rhesus monkey or a marmoset monkey. The primate ES cells are diploid and very similar to a human
10 ES cell.

The study of ES cells from a monkey and a human has suggested that a pluripotent stem cell might be derived from a human blastocyst, although the ES cells from the monkey and the human are somewhat different from that of a mouse in terms of phenotype (Thomson *et al.*, *Proc. Nat'l Acad. Sci., USA*, 92:7844-7848 (1995)).

15 The characteristic features of human pluripotent ES cells developed by Thomson et al. in 1998 (Thomson *et al.*, *Science*, 282:1145-1147(1998)) are as follows:

- (1) expression of stage-specific embryonic antigen-3 (SSEA-3), stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigen 1-60 (TRA-1-60),
20 GCTM-2(germ cell tumor marker-2), tumor rejection antigen 1-81 (TRA-1-81), alkaline phosphatase and octamer-4(Oct-4);
- (2) high telomerase activity
- (3) differentiation into three types of blastodermal cells when injected into mice;
- (4) dependency on feeder cells; and
- 25 (5) no response to a human leukemia inhibitory factor (hLIF).

Thomson et al. obtained the above ES cells from a blastocyst donated by a couple under medical treatment for sterility. Specifically, a trophectoderm known to inhibit the establishment of an ES cell was removed immunosurgically, an inner cell mass (ICM)

was plated on fibroblast feeder layer derived from a mouse embryo, and the ICM was plated on another feeder layer after a short attachment and expansion period. Thomson's method was not significantly different from the mouse ES cell protocol in terms of medium or culture system; and yet a relatively high success rate was achieved.

5 The isolation of human pluripotent ES cells and breakthroughs in somatic cell nuclear transfer (SCNT) in mammals (Solter, *Nat. Rev. Genet.*, 1:199-207(2000)) have increased the possibility of using human SCNT to generate virtually unlimited sources of undifferentiated cells for research, with potential applications in tissue repair and transplantation medicine. This concept of "therapeutic cloning" refers to the transfer
10 of the nucleus of a somatic cell into an enucleated oocyte (Lanza *et al.*, *Nat. Med.*, 5:975-977(1999)). Previous studies on such therapeutic cloning, dealing with the production of bovine ES-like cells (Cibelli *et al.*, *Nat Biotechnol.*, 16:642-646 (1998)) and mouse ES cells from ICMs of cloned blastocysts (Munsie *et al.*, *Curr. Biol.*, 10:989-992 (2000); Wakayama *et al.*, *Science*, 292:740-743 (2001) and development
15 of cloned human embryos up to 8 to 10 cell stages, opened up the possibility for therapeutic cloning of cells.

Although numerous reports have indicated that an ES cell line can be established by employing a non-human mammalian oocyte, no ES cell line developed from a human oocyte utilizing the nuclear transfer technology has been reported yet.

20 In applying the ES cells, if a somatic cell is derived from the nucleus donor, the patient to whom the cell or tissue are expected to be transferred, side effects such as immunorejection may be prevented. If the patient is a female in her age of fertility, both her oocyte and somatic cell can be used. If, however, the patient is a male, a female child or a female after the time of menopause, then another person's oocyte is
25 required. If the oocyte and the somatic cell are derived from different individuals, the probability of successfully obtaining a stem cell is dramatically lowered. Therefore, overcoming this problem has been a major issue.

【Disclosure】

【Technical Problem】

The inventors, after careful studies, discovered that nucleus-transferred human oocytes can be successfully cultured, from which an embryonic stem cell line can be established.

5 Therefore, the goal of the present invention is to provide embryonic stem cells derived from nucleus-transferred oocyte created by transferring the nucleus of a human somatic cell into an enucleated human oocyte.

 Further, the purpose of the present invention is to provide embryonic stem cells by transferring the nucleus of a somatic cell derived from the patient to whom the
10 stem cell, or the cell or tissue differentiated from the stem cell is expected to be transferred, into another person's enucleated oocyte and culturing such cells.

 Another purpose of the present invention is to provide embryonic stem cells derived from nucleus-transferred oocytes into which the nucleus of the patient's somatic cell is transferred, even when the somatic cell and the oocyte were derived
15 from different individuals, i.e., when the nuclear donor is a male or a female not in her age of fertility and thus cannot produce oocytes.

 A further purpose of the present invention is to provide a culture medium adequate for the *in vitro* culture of the nucleus-transferred oocyte manufactured using the present invention.

20 Yet another purpose of the present invention is to provide desired types of cells such as neuro progenitors, beta cells, cardiomyocytes, etc., or tissue differentiated from an embryonic stem cell line derived from a nucleus-transferred oocyte prepared by transferring a nucleus of a human somatic cell into an enucleated human oocyte.

 Furthermore, the purpose of this invention is to repair damaged tissue or
25 organs by transplanting cell or tissue such as neuro progenitor cell, β -cell, cardiomyocyte, etc., differentiated from an autologous human embryonic stem cell into the patient who donated the nucleus of the nucleus-transferred cell.

【Technical Solution】

The embryonic stem cell prepared in accordance with the present invention, for the purpose of achieving the goal of the present invention, is an embryonic stem cell derived from a nucleus-transferred oocyte created by transferring the nucleus of a human somatic cell into an enucleated human oocyte, with the somatic cell and the
5 oocyte derived from different individuals.

Another embryonic stem cell prepared in accordance with the present invention, is a human embryonic stem cell derived from a nucleus-transferred oocyte created by transferring the nucleus of a somatic cell of a human patient, who is expected to receive the stem cell or the cell or tissue differentiated from the stem cell,
10 into an enucleated human oocyte.

According to the present invention, the method for creating an embryonic stem cell comprises the following steps:

- (1) culturing a human somatic cell derived from an individual to prepare a nuclear donor cell;
- 15 (2) enucleating a human oocyte derived from a different individual from said individual to prepare a recipient oocyte;
- (3) preparing a nucleus-transferred oocyte by transferring the nucleus of a nuclear donor cell into the recipient oocyte and fusing the nucleus of the nuclear donor cell with the recipient oocyte;
- 20 (4) subjecting the nucleus-transferred oocyte to reprogramming, activation and *in vitro* culturing to form a blastocyst; and
- (5) isolating an inner cell mass (ICM) from the blastocyst and culturing the ICM in an undifferentiated state to establish a human embryonic stem cell.

25 Another method for preparing an embryonic stem cell in the present invention comprises the steps of:

- (1) culturing a human somatic cell isolated from the patient to whom the stem cell or the cell or tissue differentiated from the stem cell is expected be transplanted to prepare a nuclear donor cell;

- (2) enucleating a human oocyte to prepare a recipient oocyte;
- (3) preparing a nucleus-transferred oocyte by transferring the nucleus of a nuclear donor cell into the recipient oocyte and fusing the nucleus of the nuclear donor cell with the recipient oocyte;
- 5 (4) subjecting the nucleus-transferred oocyte to reprogramming, activation and *in vitro* culturing to form a blastocyst; and
- (5) isolating an ICM from the blastocyst and culturing the ICM in an undifferentiated state to establish the ES cell line.

10 Meanwhile, the cell differentiated from the embryonic cell prepared in accordance with the present invention is differentiated from the embryonic stem cell derived from the nucleus-transferred cell created by transferring a nucleus of a human somatic cell into an enucleated human oocyte, with the somatic cell and the oocyte derived from different individuals.

15 The cell differentiated from the other embryonic cell prepared in accordance with the present invention is differentiated from the embryonic stem cell derived from the nucleus-transferred cell created by transferring the nucleus of a somatic cell of a human patient to whom the stem cell, or the cell or the tissue differentiated from the stem cell is expected be transplanted into an enucleated human oocyte.

20 The method for inducing differentiation of the embryonic stem cell of the present invention into a specific type of cell comprises the steps of:

- (1) culturing an embryonic stem cell line derived from a nucleus-transferred oocyte prepared by transferring the nucleus of a human somatic cell into an enucleated human oocyte, with the somatic cell and the oocyte derived
25 from different individuals, to form an embryoid body;
- (2) culturing the embryoid body in the presence of an agent suitable for inducing differentiation of the cells of the embryoid body into a desired type of cells;
- (3) selecting the desired type of cells.

Another method for causing differentiation of the human embryonic stem cell of the present invention into a specific type of cell comprises the steps of:

- (1) culturing an embryonic stem cell derived from a nucleus-transferred oocyte prepared by transferring the nucleus of a human somatic cell of a patient to whom the stem cell, or the cell or tissue differentiated from the stem cell, is expected to be transplanted, into an enucleated human oocyte, to form an embryoid body;
- (2) culturing the embryoid body in the presence of an agent suitable for inducing differentiation of the cells of the embryoid body into a desired type of cells.

In each the above inventions regarding an embryonic stem cell, the preparation method thereof, the cell differentiated from the embryonic stem cell and the preparation method thereof, the somatic cell was derived from a female not in her age of fertility or a male.

- In the above method for preparation of an embryonic stem cell, it is preferable that step (3) above be conducted by cell fusion of the nuclear donor cell and the recipient oocyte.

- In the above method for preparation of an embryonic stem cell, the reprogramming in step (4) is conducted for 20 hours or less, preferably 6 hours or less, more preferably 3 hours or less, and most preferably 2 hours or less.

In the above method for preparation of an embryonic stem cell, the activation in step (4) is preferably conducted by first treating the nucleus-transferred oocyte with calcium ionophore and then with 6-dimethylaminopurine.

- In the above method for preparation of an embryonic stem cell, the concentration of the above-mentioned calcium ionophore is preferably within the range of 5 μ M to 15 μ M, more preferably about 10 μ M.

In the above method for preparation of an embryonic stem cell, the concentration of the above-mentioned 6-dimethylaminopurine is preferably within the range of 1.5 mM to 2.5 mM, more preferably about 2.0 mM.

In the above method for preparation of an embryonic stem cell, the *in vitro* culture is sequentially performed using at least two culture media, and it is preferable that each culture medium be different in composition.

In the above method for preparation of an embryonic stem cell, it is preferable that the *in vitro* culture be conducted sequentially using two culture media of different compositions. More preferably, the first culture is performed using the G1 ver.3 culture medium, and the second culture is performed using the mSOFaa culture medium containing human serum albumin (HSA), i.e., SNUnt-2' culture medium. The mSOFaa culture medium containing HSA is preferably the mSOFaa containing 10% HSA.

In the above method for preparation of an embryonic stem cell, it is preferable that the ICM in step (5) be cultured in a feeder layer containing a mixture of mouse-derived and human derived fibroblasts. More preferably, the ICM is cultured in a feeder layer containing somatic cells, preferably fibroblasts, derived from the human patient who is expected to be the recipient of the stem cell line or cells or tissue differentiated from such stem cell line. Most preferably, the ICM is cultured in a feeder layer containing cells, preferably fibroblasts, differentiated from the autologous human embryonic stem cell line.

In the above method for inducing differentiation of the embryonic stem cell or the cell differentiated from the embryonic stem cell, the cell could be one or more of hematopoietic cells, neural progenitor cells, neural cells, β -cells, muscle cells, liver cells, cartilage cells, epithelial cells, and myocardial cells.

In the above method for inducing differentiation of the embryonic stem cell into a desired type of cell, if the desired type of cells is the neural progenitor cell, it is preferable to include after step (2), a step for selecting cells which express a neuro progenitor marker. The agent in step (2) can be one or more selected from the group of supplements comprising insulin, transferrin, sodium selenite and fibronectin mixture, retinoic acid, ascorbic acid, nicotinamide, N-2 supplement and B-27 supplement.

In the above method for inducing differentiation of the embryonic stem cell into a

desired type of cell, if the desired type of cells is the β -cell, it is preferable that the agent in step (2) be one or more selected from the group of supplements comprising insulin, transferrin, sodium selenite and fibronectin mixture, nicotinamide, N-2 supplement and B-27 supplement.

5 In the above method for inducing differentiation of the embryonic stem cell into a desired type of cell, if the desired type of cells is the β -cell, it is preferable that the step (2) above include (i) culturing the embryoid body, by plating the embryoid body on a culture medium containing insulin, transferrin, sodium selenite and fibronectin mixture, as well as N-2 supplement, B-27 supplement and bFGF; and (ii) culturing the above
10 embryoid body after removing bFGF from the culture medium in step (i), adding nicotinamide, lowering the glucose concentration to the range of 500 mg/L to 1500 mg/L. A desirable example is obtained when the glucose concentration is lowered to 901mg/L. The above range of glucose concentration facilitates the differentiation into β -cells.

15 In the above method for causing differentiation of the embryonic stem cell into a desired type of cell, if the desired type of cells is the myocardial cell, it is preferable that the agent in the step (2) above be one or more selected from the group of supplements comprising dimethylsulfoxide (DMSO), retinoic acid and 5-aza-2'-deoxycytidine (5-aza-dC).

20 Meanwhile, the composition for repair of damaged cells, tissue or organ in the present invention is the composition for injection into the individual to induce recovery of the damaged cells, tissue or organ, and contains as its effective ingredient one or more selected from the group comprising the human embryonic stem cell, cells differentiated from such cells, tissue derived from such cells, and organ derived from
25 such tissue.

The kit for transplantation of cells, tissue or organ in the present invention is for the transplantation of cells, tissue or organ into individuals and contains one or more selected from the group comprising the human embryonic stem cell, cells differentiated from such stem cell, tissue derived from such cells, and organ derived

from such tissue.

In addition, the method for repairing the damaged cells, tissue or organ in accordance with the present invention is to repair the damaged cells, tissue or organ by transplanting into the individual who donated the somatic cell for the preparation of the embryonic stem cell line, one or more of the human embryonic stem cell prepared in accordance with the present invention, cells differentiated in accordance with the present invention, tissue derived from such cells and organ derived from such tissue.

In the composition, transplantation kit and method of the present invention the DNA of the above human embryonic stem cell or the above differentiated cells may be transformed to be more suitable for the treatment of the patient's disease.

【Advantageous Effects】

The human autologous embryonic stem cell derived from the human autologous nucleus-transferred oocyte prepared in accordance with the present invention contains the genome of the individual to whom the stem cell is expected to be transplanted, and therefore, the transplantation will not cause any immunorejection. Furthermore, the present invention can be used when the somatic cell and the oocyte are derived from different individuals. In other words, men who cannot produce oocytes and women not in their age of fertility may also obtain an embryonic stem cell derived from a nucleus-transferred oocyte containing the nucleus of his or her somatic cell.

Further, by culturing the embryonic stem cell in an adequate culture medium and under adequate conditions, the embryonic stem cell can be induced to differentiate into a specific type of cells such as hematopoietic cells, nerve cells, β -cells, muscle cells, liver cells, cartilage cells and epithelial cells, etc. The cells or tissue differentiated from the stem cell repairs the damaged cell or tissue and enables the damaged organ to recover its function. The cells or tissues can be used in cell transplantation therapies to cure diseases such as, cell damaging diabetes, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), cerebrum palsy and

cancer and many other diseases.

【Description of Drawings】

Fig. 1 shows photographs of immunofluorescence-stained ES cells derived
5 from autologous nucleus-transferred cells of the present invention. (a): AP (alkaline
phosphatase) (b): SSEA-1 (c): SSEA-3 (d): SSEA-4 (e): TRA-1-60 (f): TRA-1-81 and
(g): Oct-4;

Fig. 2 (a) shows a photograph of fluorescence-stained neuro progenitors
differentiated from undifferentiated colony obtained in accordance with the present
10 invention by adding ITSF (i.e., a mixture of insulin, transferrin, sodium selenite and
fibronectin) (x400); Fig. 2 (b) shows a photograph of fluorescence-stained neuro-
filament further differentiated from the above neuro progenitors (x400);

Fig. 3 shows the incision process of the zona pellucida of an oocyte (3) with a
holding pipette (1) and an incision pipette (2);

15 Fig. 4 shows a photograph showing the removal of the first polar body and the
nucleus of the oocyte (3) with the holding pipette (1) and the incision pipette (2);

Fig. 5 offers a photograph showing the transfer of a nuclear donor cell into an
enucleated recipient oocyte (3) with the holding pipette (1) and a transfer pipette (4);

Fig. 6 shows results of analysis of karyotype of an ES cell line derived from a
20 somatic cell obtained from a male and karyotype of the somatic cell providing the
nucleus used to establish the ES cell line;

Figs. 7 to 10 show the results of DNA fingerprinting.

Figs. 11(a) to (c) show three types of blastodermal cells identified within a
teratoma formed by injecting an undifferentiated cell colony obtained in accordance
25 with the present invention into a testis of an immune deficiency mouse ((a): cartilage
(b): intestinal tract (c): neural tube ((a),(b),(c) x200)

【Best Mode】

The term “nucleus transfer” as used in the present specification refers to the

process of transferring a nucleus of a somatic cell ("nuclear donor cell") into an enucleated oocyte ("recipient oocyte"). The resulting cell obtained by nuclear transfer is referred to as a "nucleus-transferred oocyte" or "nuclear transfer oocyte." The term "somatic cell" as used in the present specification refers to any cell
5 constituting a body that contains two sets of chromosomes (2n), excluding a germ cell that has a single set of chromosomes (n).

The term "autologous nucleus-transferred oocyte" as used in the present specification refers to a nucleus-transferred oocyte prepared by transferring a nucleus of a somatic cell into an enucleated oocyte where the somatic cell is isolated from a
10 human patient, male or female, who will be receiving a stem cell derived from the nucleus-transferred oocyte, or a specific cell or tissue differentiated from the stem cell.

Accordingly, it is expected that the person who receives the specific cell or tissue derived from the autologous nucleus-transferred oocyte would not exhibit
15 immunorejection since such cell or tissue contains the genetic characteristics of the person.

The term "embryonic stem cell (ES cell)" as used in the present specification means an undifferentiated cell derived from an embryo, which has the capability of differentiating into various types of cells. In general, "embryo" means a fertilized
20 egg up to eight (8) weeks after its fertilization or a nucleus-transferred oocyte in the corresponding development stage. An embryo is created by repetitive division of such fertilized egg or nucleus-transferred oocyte, and comprises a blastocyst containing an ICM and an outer trophectoderm.

Therefore, the term "autologous embryonic stem cell" as used in the present
25 specification refers to embryonic stem cell derived from an autologous nucleus-transferred oocyte prepared by transferring into an enucleated oocyte the nucleus of a somatic cell of the patient to whom the stem cell or the cells or tissue differentiated from the stem cell will be transplanted.

The term "autologous feeder layer" as used in the present specification refers

to a feeder layer used to establish ES cell line, containing cells differentiated from an autologous embryonic stem cell derived from an autologous nucleus-transferred oocyte obtained using the somatic cell of the human who is expected to be the recipient.

The term “neuro progenitors” as used in the present specification
5 refers to cells which later differentiate into nerve cells including neurons and glia such as astrocytes, oligodendrocytes, schwann cells, satellite cells, ependymal cells and microglia.

Provided below is a detailed description of the method for preparing an ES
10 cell line in accordance with the present invention.

Step 1: Preparation of a nuclear donor cell

In the present invention, a somatic cell of a human, who is expected to receive the stem cell or cells or tissues derived from such stem cell, is cultured to function as a
15 nuclear donor cell. A somatic cell isolated from a human, regardless of gender, can be used as a nuclear donor cell, and the nucleus of such nuclear donor cell is transferred into an enucleated human oocyte.

Any somatic cell obtained from a human is may be used as a nuclear donor cell. A somatic cell obtained from an institute that stores human cells for commercial
20 purposes may also be used. For example, a skin cell, nerve cell, oviduct epithelial cell, cumulus cell, etc., may be used as a nuclear donor cell.

The nuclear donor cell may be cultured to establish a cell line by applying the Marther and Barnes method (Animal Cell Culture Methods: vol. 57 of Methods in Cell Biology (Marther & Barnes eds., Academic Press, 1998)).

25 According to the preferred embodiment of the present invention, an uterus fluid and a phosphate buffered saline (PBS) containing P/S antibiotic (penicillin 10,000IU, streptomycin 10 mg) are added to a somatic cell. Such somatic cell is centrifuged and washed, and then cultured in a DMEM (Dulbecco's Modified Eagle Medium) containing human serum (HS), nonessential amino acids (NEAAs) and the

P/S antibiotic at, for example, 39°C in 5% CO₂ atmosphere.

In particular, in the case where a cumulus cell is used as a nuclear donor cell, the cumulus cell can be prepared by treating a cumulus-oocyte complex with hyaluronidase to isolate a cumulus cell layer surrounding an oocyte, adding a trypsin-
5 EDTA solution to the cumulus cell layer and placing the resulting solution at, for example, 39°C in 5% CO₂ atmosphere under saturated humidity. After centrifuging and washing, the collected cumulus cells can be cultured under the same condition described above.

10 Step 2: Preparation of recipient oocyte

A recipient oocyte as used in the present invention refers to an oocyte that lacks its own nucleus and receives a foreign nucleus from a human somatic cell.

A mature oocyte may be prepared by collecting a superovulated oocyte from a
15 human ovary or obtaining an oocyte from an institute storing human oocytes for commercial purposes and culturing the oocyte using a method known in the art (Yuzpe et al., J. Reprod. Med., 34:937-942 (1989)). For example, an oocyte may be matured by culturing the oocyte in the G ver.3 medium, marketed by Vitro Life, Goteborg, Sweden, supplemented with 5% human serum albumin (HSA) under the condition of,
20 for example, 5% CO₂ for 4 hours.

Next, an enucleated recipient oocyte is prepared by removing the surrounding cumulus cells from the oocyte, and partially eliminating the zona pellucida and the cytoplasm containing the first polar body.

In a preferred embodiment of the present invention, the enucleation can be
25 performed in the following way.

A mature oocyte is placed in a washing culture solution containing hyaluronidase, and the cumulus cell is physically removed. Next, the mature oocyte is washed with G1 ver.3 medium. Subsequently, the zona pellucida of the oocyte is penetrated to form a small hole therein. The oocyte is enucleated by removing part of

the cytoplasm containing the first polar body corresponding to 10 to 15% of the total cytoplasm through the small hole. The enucleated oocyte is washed with the G1 ver.3 medium and placed in the G1 ver.3 medium for culturing.

The enucleation can be confirmed by, for example, observing the cytoplasm
5 stained with Hoechst 33342 (Sigma Co., St. Louis, MO, U.S. A.) using a UV detector.

Step 3: Preparation of nucleus-transferred oocyte and electrofusion

The nuclear donor cell prepared in Step 1 is transferred into the enucleated
10 recipient oocyte obtained in Step 2, and soon electrofused to form a nucleus-transferred oocyte.

The nuclear transfer of a somatic cell into a recipient oocyte may be done by transferring either the nucleus of the somatic cell or the whole somatic cell into the recipient oocyte.

15 The nuclear transfer and electrofusion in the present invention may be performed as follows:

First, the enucleated oocyte placed in the G1 ver.3 medium obtained in Step 2 above is washed with the G1 ver.3 medium. The nuclear donor cell is injected into the enucleated oocyte in a phytohemagglutinin-P (PHA-P) solution via a small hole
20 formed in the zona pellucida using a transfer pipette to create a nucleus-transferred oocyte. The resulting nucleus-transferred oocyte is then washed with and subsequently placed in the G1 ver.3 medium.

Next, the nucleus-transferred oocyte obtained in Step 2 above is electrofused using a cell manipulator. A mannitol solution is added to the G1 ver.3 medium
25 containing the nucleus-transferred oocyte. The resulting mannitol solution containing the nucleus-transferred oocyte is placed between two electrodes of the cell manipulator and is positioned so that the somatic cell faces the (+) electrode. The nucleus-transferred oocyte is electrofused by treating it with a direct current ranging from 0.75 to 2.00 kV/cm for 10 to 20 μ sec, 1 to 5 times at an interval of 1 second.

Lastly, the fused nucleus-transferred oocyte is washed with a mannitol solution and the G1 ver.3 medium. The mannitol solution used in this step is prepared by dissolving bovine serum albumin (BSA) and mannitol in a 4-(2-hydroxyethyl)-1-perazine ethanesulfonic acid (HEPES) buffer solution at a pH ranging from 7.2 to 7.4.

5

Step 4: Reprogramming, activation and *in vitro* culturing of nucleus-transferred oocyte

In order to induce the nucleus-transferred oocyte prepared in Step 3 to undergo
10 the same developmental process as a normal fertilized oocyte formed as a result of fusion between a sperm and an oocyte, the reprogramming time, activation method and *in vitro* culturing conditions must be carefully selected.

The present invention provides fertilization and development procedures similar to the normal fusion and development process in activating and culturing the
15 nucleus-transferred oocyte. More specifically, the nucleus-transferred oocyte prepared by electrofusion in Step 3 is subjected to reprogramming, activation and *in vitro* culturing to form a blastocyst.

The reprogramming time refers to the time lapsed between the electrofusion and the activation, and the length of the reprogramming time may affect the
20 developmental capacity (in particular, the blastocyst formation rate) of the nucleus-transferred oocyte. This reprogramming time is required to allow the gene expression pattern of the somatic cell to turn into a pattern that is appropriate and necessary for the development of the nucleus-transferred oocyte. Such reprogramming time plays a critical role in chromatin remodeling, and it is known to determine the developmental
25 competence of the nucleus-transferred oocyte *in vivo* and *in vitro*.

The reprogramming time in the present invention may be 20 hours or less, preferably 6 hours or less, more preferably 3 hours or less, and most preferably, about 2 hours.

After the reprogramming, the nucleus-transferred oocyte may be activated by

various chemical or physical stimuli such as calcium ionophore, ionomycin, ethanol, Tyrode's solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) and puromycin, etc. In the present invention, it is preferable to treat the nucleus-transferred oocyte with calcium ionophore to activate it. It is more preferable to treat the nucleus-transferred oocyte with calcium ionophore and then with 6-dimethylaminopurine (6-DMAP). Specifically, the calcium ionophore may be used at a concentration of 5 to 15 μ M, and preferably, about 10 μ M. Said 6-DMAP may be employed at a concentration ranging from 1.5 to 2.5 mM, and preferably, about 2.0 mM.

Both calcium ionophore and 6-DMAP are dissolved in an *in vitro* culture medium. A representative examples of *in vitro* culture medium is the G1 ver.3 medium (Vitro Life, Goteborg, Sweden) comprising alanine, alanyl-glutamine, asparagines, aspartate, CaCl_2 , EDTA, glucose, glutamate, hyaluronan, magnesium sulfate, penicillin G, KCl, proline, serine, NaHCO_3 , NaCl, NaH_2PO_4 , sodium lactate, sodium pyruvate, taurin and WFI (water for injection).

Further, it is preferable to supplement the culture medium with various energy substrates known in the art or employ a sequential culturing system using at least two media with different compositions suitable for each stage of the embryonic development. An example of the sequential culturing system that may be used in the present invention is the G1 ver.3 /mSOFaa (Choi *et al.*, *Theriogenology*, 58;1187-1197, (2002)) medium. A preferable *in vitro* culture medium includes "human modified synthetic oviductal fluid; (hmSOFaa)," which has been established by the inventors of the present invention and designated as "SNUnt-2'-medium." The hmSOFaa medium is prepared by adding human serum albumin (HSA) to the mSOFaa medium (Choi *et al.*, *Theriogenology*, 58; 1187-1197, (2002)). The mSOFaa medium is widely used for culturing bovine embryos.

Specifically, the "SNUnt-2'" medium comprises (1) 95 to 110 mM NaCl, (2) 7.0 to 7.5 mM KCl, (3) 20 to 30 mM NaHCO_3 , (4) 1.0 to 1.5 mM NaH_2PO_4 , (5) 3 to 8 mM Na-lactate, (6) 1.5 to 2.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, (7) 0.3 to 0.8 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, (8) 0.2 to 0.4 mM Na-pyruvate, (9) 1.2 to 1.7 mM glucose, (10) 12 to 20 mg/ml HSA, (11)

0.7 to 0.8 µg/ml kanamycin (12) 1.5 to 3% essential amino acids, (13) 0.5 to 1.5% non-essential amino acids, (14) 0.7 to 1.2 mM L-glutamine and (15) 0.3 to 0.7% insulin, transferrin and sodium selenite mixture. Preferably, the SNUnt-2' medium comprises the ingredients as listed in Table 1.

5

[Table 1]

Ingredient	Concentration
NaCl	99.1~106 mM
KCl	7.2 mM
NaHCO ₃	25 mM
NaH ₂ PO ₄	1.2 mM
Na-lactate	5 mM
CaCl ₂ ·2H ₂ O	1.7 mM
MgCl ₂ ·6H ₂ O	0.5 mM
Na-pyruvate	0.3 mM
Glucose	1.5 mM
HAS	16 mg/ml
Kanamycin	0.75 µg/ml
Essential amino acids	2%
Non-essential amino acids	1%
L-glutamine	1 mM
ITS*	0.5%

*ITS: a mixture of insulin 1.0 g/L, transferrin 0.55 g/L and sodium selenite 0.67 mg/L

10 Meanwhile, the sequential (two-step) culturing system of the present invention may employ any combination of the different media. Preferably, the first culturing is conducted in the G1 ver.3 medium and the second culturing is conducted in SNUnt-2'

medium. Most preferably, the first culturing is conducted in the G1 ver.3 medium and the second culturing is conducted in the SNUnt-2' medium, i.e., the mSOFaa medium containing 10% HSA.

5 Step 5: Removal of all or part of the zona pellucida

In order to obtain an ES cell derived from the blastocyst obtained in Step 4, the zona pellucida must be all or partially removed from the blastocyst. This removal may be carried out by using one of the methods known in the art, e.g., pronase
10 treatment, incubation in acidic Tyrode's solution, or a physical method such as laser dissection.

It is preferable to use pronase dissolved in a suitable medium such as PBS, G2 medium (Vitro Life, Goteborg, Sweden) or S2 medium (Scandinavian IVF Sciences, Goteborg, Sweden). In a preferred embodiment, pronase is dissolved in a mixture of
15 equal volumes of PBS and the S2 medium. The blastocyst is treated with 0.1% pronase for about 1 to 2 minutes, preferably 1 to 1.5 minutes, to remove the zona pellucida.

Step 6: Removal of trophoblast and isolation of ICMs

20

Once the zona pellucida is removed from the blastocyst as described above, the trophoblast is exposed. It is preferable to completely separate the trophoblast from the ICMs. In the present invention, the trophoblast may be separated from the ICM using any one of the methods known in the art.

25 In a preferred embodiment, the trophoblast is removed by an immunosurgical method that treats the trophoblast with an antibody or anti-serum responsive to an epitope located on a surface of the trophoblast or a physical method using a pipette. More preferably, antibody and complement are used together with such method. In this case, an antibody and/or an anti-serum and complement may be used

independently or simultaneously. A preferred combination of antibody and/or anti-serum and complement includes antiplacental alkaline phosphatase antibody (anti-AP) and baby rabbit complement, or anti-human serum antibody and guinea pig complement, etc.

5 Antibody and complement may be diluted with a suitable medium such as SNUnt-2', G2.2(Vitro Life, Goteborg, Sweden) or S2 medium. The G2.2 medium contains alanine, ananyl-glutamine, arginine, asparagines, aspartate, CaCl_2 , calcium pantothenate, choline chlorine, cysteine, folic acid, glucose, glutamate, glycine, hystidine, human serum albumin, inositol, isolucine, lucine, lysine, MgSO_4 ,
10 methionine, nicotine amide, penicillin G, phenylalanine, KCl, proline, pyridoxal HCl, riboflavine, serine, $\text{Na}(\text{HCO}_3)_2$, NaCl, sodium dihydrogen phosphate, sodium lactate, sodium pyruvate, thiamine, threonine, tryptophane, tyrosine, valine and water. Preferably, the anti-AP may be diluted with the S2 medium at the ratio of 1:20; and other antibodies and complements, at the ratio of 1:1.

15 It is preferable to treat the zona pellucida-removed blastocyst with an antibody first and then with a complement. Preferably, the blastocyst is treated with the antibody for about 30 minutes. After exposure to the antibody, the blastocyst is washed with SNUnt-2', G2.2 or S2 medium and then treated with the complement for about 30 minutes.

20 All or part of the trophoblast can be separated from the blastocyst by washing the blastocyst with the SNUnt-2', G2.2 or S2 medium. In such case, the trophoblast may be removed by a mechanical method known in the art, for example, pipetting a solution containing the blastocyst using a pipette having a small bore.

 Through the steps described above, the trophoblast is removed from the
25 blastocyst and the ICMs are obtained.

Step 7: Culturing of ICMs on fibroblast feeder layer

ICMs isolated in Step 6 are cultured on a fibroblast feeder layer in order to

keep them in their undifferentiated state. In some cases, hLIF (leukemia inhibitory factor) has been used to maintain the undifferentiated morphology of ICMs instead of the feeder layer. In the case of human cells, however, it is practically impossible to maintain the cells in their undifferentiated state without using a fibroblast feeder layer, even when a high concentration of LIF is used. Therefore, the condition that does not induce extraembryonic differentiation and apoptosis in the ES cells generally requires culturing on a fibroblast feeder layer.

It is preferable to employ a mouse- and/or human-derived fibroblast for preparing the fibroblast feeder layer. They may be used alone or in a mixture. It is more preferable to use, as a feeder layer, cells differentiated from the ES cells derived from an autologous nucleus-transferred oocyte of a human. This feeder layer has been designated as "autologous feeder layer." It is most preferable to use fibroblasts differentiated from the ES cells derived from an autologous nucleus-transferred oocyte of an individual. The use of such feeder layer can prevent other foreign cells from contaminating the ES cells.

Such human-derived fibroblasts are capable of inducing an optimum growth and inhibiting differentiation of the ES cells when appropriately mixed with mouse-derived fibroblasts.

The cell density in the fibroblast feeder layer may affect its stability and capability. When a mixture of mouse and human fibroblasts are used, it is preferable to maintain the human fibroblasts at a density of, for example, 2.5×10^4 cell/cm² and the mouse fibroblasts at a density of, for example, 7.0×10^4 cells/cm². When solely mouse fibroblasts are used, it is preferable to maintain the density within the range of 3.0×10^4 to 1.0×10^5 cells/cm². It is preferable to establish such feeder layer before 6 to 48 hours before the addition of ES cells.

Further, it is preferable to use mouse or human fibroblasts having a low passage number. The quality of the fibroblasts may affect the ability to support the ES cells. It is especially preferable to use fibroblasts isolated from an embryo. The mouse fibroblasts are preferably obtained from a 13.5-day old fetus, and the human

fibroblasts, from an embryo or a fetal tissue. These fibroblasts can be cultured using a cell culturing method known in the art.

In handling the mouse embryonic fibroblasts, it is important to minimize the use of trypsin and inhibit overcrowding. Otherwise, the mouse embryonic fibroblasts cannot support the growth of undifferentiated ES cells. Each batch of the mouse embryonic fibroblasts so prepared has to be tested first to confirm whether it is suitable for supporting and maintaining the ES cells.

Between fresh primary embryonic fibroblasts and fibroblasts having undergone a freezing-and -thawing treatment, the former is usually considered more suitable for supporting the renewal of the ES cells. It is cumbersome, however, to prepare fresh primary embryonic fibroblasts every time. Certain batches may maintain their potential ability to support the ES cells even after repeated freezing and thawing. It is preferable to test each new batch that proved to be effective in supporting renewal of ES cells once more after freezing and thawing. It is more efficient, in terms of time and costs, to use a batch that maintains its potential after freezing and thawing.

Certain mouse strains can produce embryonic fibroblasts more suitable for supporting ES cells than other strains. For example, it has been demonstrated that fibroblasts obtained from the mice produced by inbreeding of 129/Sv or CBA strain or by crossbreeding of 129/Sv and C57/B16 strains are most suitable for supporting ES cells.

Meanwhile, it is preferable to inhibit the growth of feeder cells by using any one of the methods known in the art, including irradiation and chemical treatment using, for example, mitomycin C. In a preferred method, such cells are treated with mitomycin C.

The fibroblast feeder layer so prepared is cultured on a Petri dish coated with gelatin, preferably 0.1% gelatin.

The fibroblast feeder layer may be maintained in an ES medium. A suitable ES medium is the DMEM/F12 medium supplemented with 20% serum replacement

(Gibco, Grand Island, NY, U.S.A), 0.1mM β -mercaptoethanol, 1% non-essential amino acids, 2mM glutamine and penicillin (100 units/ml), streptomycin (100g/ml), human recombinant fibroblast growth factor (FGF, 4ng/ml).

Such ES medium can be further supplemented with a soluble growth factor
5 capable of stimulating growth or survival of the stem cells or inhibiting differentiation thereof. Representative examples of the growth factor are human pluripotent stem cell factor, ES cell renewal factor, etc.

The isolated ICMs may be cultured for 6 days or longer, and cell colonies are generated therefrom. The colonies typically comprise undifferentiated stem cells.
10 The undifferentiated stem cells may be isolated using a chemical method or a physical method, or both. It is preferable to use a micropipette. Such physical isolation can be conducted simultaneously with the treatment of a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS medium or an enzyme helpful for cell dissociation such as dispase.

15 Step 8: Subculturing of ES cells

The ES cells cultured as described above are detached from the feeder layer and transferred to a fresh feeder layer. Then, the ES cells may be further cultured during sufficient time to propagate in a morphologically undifferentiated state.

20 In this case, it is preferable to culture the ES cells for 5 to 7 days. Undifferentiated stem cell colonies were observed by approximately the second day of culturing. The stem cells are morphologically identified by a high nucleus to cytoplasm ratio, clear nucleoli, condensed colony formation and distinctive cell boundary.

25 The propagation of undifferentiated stem cells is initiated by isolating an undifferentiated stem cell clump from the stem cell colony. Such isolation may be carried out by using either a chemical or a mechanical method. Preferably, the stem cells are isolated from the colony by washing with a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS medium, a mechanical method or a combination thereof. It is more preferable to mechanically

isolate the stem cells from the colony.

In the first method, the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS medium may be used for reducing intercellular adhesive power. After incubation in the above medium for about 15 to 20 minutes, the cells begin to detach themselves gradually from the feeder
5 layer, and finally, are isolated as a clump having a desired size. In case cells are only partially isolated, a mechanical method using a sharp edge of a micropipette may be effectively employed for isolating and cutting the clump.

Alternatively, the chemical method includes the use of an enzyme. The enzyme, preferably dispase, may be used alone or in combination with a mechanical
10 method.

In another preferred embodiment, the clumps are isolated from the colony by treating it with dispase after mechanical cutting of the colony. The cutting of the colony is carried out in a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing PBS medium. The colony can be mechanically cut into clumps, each clump containing about 100 cells, with the aid of a
15 sharp edge of a micropipette. As soon as a clump is isolated, it is picked up with a micropipette having a wider bore, washed with the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing PBS medium, and transferred to a fresh fibroblast feeder layer.

It is necessary to confirm whether the stem cells maintain their undifferentiated state during the culturing process. Undifferentiated stem cells can be
20 identified by examining their typical morphological characteristic features as described above. Such stem cells can be also identified by detecting a cell marker or measuring the gene expression specific for a pluripotent cell.

Examples of genes specific to a pluripotent cell or a typical lineage include, but are not limited to, alkaline phosphatase, Octamer-4 (Oct-4), SSEA-3 and SSEA-4
25 which may be used as stem cell markers. Other examples of genes specific to stem cells include genesis, GDF-3 and crypto. The expression profile of these genes can be analyzed by using one of the methods known in the art, including reverse transcription-polymerase chain reaction (RT-PCR), a differentiation gene expression method, a micro array assay, and the like.

Preferably, the stem cells can be identified by an immunological reaction with a human pluripotent stem cell marker such as SSEA-4, germ cell tumor marker-2 (GCTM-2) antigen, TRA-1-60, etc. In particular, the stem cells may express Oct-4 as a transcription factor and maintain a normal diploid karyotype.

5 The growth progress of the stem cells and maintenance status of their differentiated or undifferentiated state can be monitored by quantitative measurement of the proteins specific to the stem cells excreted into the medium or analysis of fixed cell preparations with enzyme-linked immunosorbent assay (ELISA). Representative examples of the proteins specific to the stem cells are a soluble type of CD antigen and
10 GCTM-2 antigen, and these proteins can be monitored by detecting a cell marker or measuring the gene expression.

It is well known that embryonic stem cells can differentiate into almost any type of cells. Therefore, the embryonic stem cells prepared in accordance with the
15 present invention may be a good source of various types of cells. For example, embryonic stem cells can be induced to differentiate into hematopoietic cells, nerve cells, beta cells, muscle cells, liver cells, cartilage cells, epithelial cells, etc., by culturing them in a medium under conditions suitable for cell differentiation. Such medium and conditions are well known in the art.

20 Accordingly, the ES cell of the present invention may have numerous therapeutic and diagnostic applications. Especially, such ES cell may be used in cell transplantation therapies for the treatment of numerous diseases, e.g., diabetes, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), cerebral palsy and cancer. Further, the ES cell line derived from the autologous nucleus-
25 transferred oocyte can be utilized in cell transplantation therapy since no adverse immunorejection reaction may occur during or after the treatment procedure.

The inventors of the present invention were able to confirm through animal testing that human autologous embryonic stem cell derived from nucleus-transferred

oocyte, prepared by transferring the nucleus of a somatic cell of the patient into an enucleated oocyte, can be induced to differentiated into nerve cells and thus repair the damaged tissue or the function of a damaged organ. In addition, human autologous embryonic stem cells were induced to differentiate into beta cells and cardiomyocytes
5 and their functions were confirmed.

Provided below is a detailed description of the inventive method for preparing specific types of cells from the ES cell.

10 1. Differentiation into neuro progenitors

Step 1: Preparation of an embryoid body

The first step in inducing differentiation of the ntES cells (nuclear transfer
15 embryonic stem cells) derived from the autologous nucleus-transferred oocytes into neuro progenitors is to generate an embryoid body by culturing the ES cells. The embryoid body can be prepared from the ES cells by using one of the methods known in the art (Zhang *et al.*, *Nat. Biotechnol.*, 19:1129-1133 (2001)).

In a preferred embodiment, the embryoid body is obtained by transferring
20 cultured ntES cell colonies into a non-adhesive culture dish containing the DMEM/F12 medium supplemented with a 20% serum replacement (Gibco, Grand Island, NY, U.S.A.) and culturing them for 3 to 5 days. Typically, floating embryoid bodies start to appear about one day after the beginning of the culturing (about 40 to 60 embryoid bodies/dish). At this point, it is preferable to remove any remaining feeder cells while
25 transferring such embryoid bodies to a new dish. Then, the embryoid bodies are plated on an adhesive dish coated with polyornithine/laminin.

Step 2: Inducement of differentiation into neuro progenitors by an agent

Representative agents which may be employed in the present invention to induce differentiation of the embryoid bodies obtained in Step 1 into neuro progenitors include, but are not limited to, ITSF (a mixture of insulin, transferrin, sodium selenite and fibronectin), retinoic acid, ascorbic acid, nicotinamide, N-2 supplement (100X, 5 17502-048; Gibco, Grand Island, NY, U.S.A.) and B-27 supplement (50X, 17504-044, Gibco, Grand Island, NY, U.S.A.). Neuro progenitors differentiated from the ntES cells can be obtained by culturing the embryoid bodies in a medium supplemented with such agents and inducing their expansion and differentiation.

In a preferred embodiment, the embryoid bodies prepared in Step 1 are further 10 cultured for 1 day followed by culturing in the DMEM/F12 medium supplemented with ITSF, i.e., insulin (about 25 $\mu\text{g/ml}$), transferrin (about 100 $\mu\text{g/ml}$), sodium selenite (about 30 nM) and fibronectin (about 5 $\mu\text{g/ml}$) for 5 to 10 days, thereby inducing differentiation of the ntES cells into neuro progenitors.

15 Step 3: Selection and culturing of cells expressing a neuro progenitor marker

The neuro progenitors differentiated from the autologous embryonic stem cells may be obtained by selecting cells expressing a neuro progenitor marker such as nestin among the differentiated cells obtained in Step 2 and culturing them. Further, the 20 obtained neuro progenitors may be differentiated into desired specific type of nerve cells. The differentiation into nerve cells can be carried out through conventional methods such as induction with chemicals, etc.

In a preferred embodiment, cells exhibiting a positive signal for a neuro progenitor marker are selected; their expansion is induced by culturing the selected 25 cells in the DMEM/F12 medium supplemented with the N-2 supplement, laminin and basic fibroblast growth factor (bFGF) for 5 to 7 days; and then, they are further cultured in the DMEM/F12 medium supplemented with only the N-2 supplement and laminin without bFGF for 8 to 14 days.

2. Differentiation into β -cells

The differentiation of the autologous embryonic stem cell of the present invention into β -cells which secrete insulin can be performed by the ordinary method.

5 (Hanna Esgev et al. *Stem Cells*, 22: 265-274, 2004)

Step 1: Formation of an embryoid body

A method similar to the method used in 1. Differentiation into neural
10 progenitor cells can be used for the formation of an embryoid body. (Zhang et al., *Nat. Biotechnol.*, 19: 1129-1133, 2001)

Step 2: Inducement of differentiation into β -cells by the use of an agent

15 In the present invention, examples of the agents that could induce differentiation of the embryoid bodies obtained in Step 1 into β -cells are, but not limited to, ITSF (a mixture of Insulin, Transferrin, Sodium selenite and Fibronectin), nicotinamide, N-2 supplement 100X; 17502-048, Gibco, Grand Island, NY, U.S.A.) and B-27 supplement 50X; 17504-044, Gibco, Grand Island, NY, U.S.A.), etc. β -
20 cells can be obtained from hntES (human nucleus transferred Embryonic Stem) cells by continued culturing of the hntES cells in culture medium containing such supplements and causing expansion and differentiation.

Furthermore, the preferred culture medium inducing differentiation into β -cells is a knockout DMEM containing as supplements, 20% serum replacement (Gibco,
25 Grand Island, NY, U.S.A.), 1% non-essential amino acid (Gibco Invitrogen), 0.1 mmol/L 2-mercaptoethanol (Gibco Invitrogen), 1mmol/L glutamine (Biological Industries, Bet-Haemak, Israel) and 4ng/ml human recombination bFGF (basic fibroblast growth factor)(Pepro Tech, Rocky Hill, NJ).

In a preferred embodiment, differentiation into β -cells is induced by plating

the embryoid bodies obtained in Step 1 on the ITSF culture medium, supplementing the culture medium with N-2 supplements, B-27 supplements and bFGF (basic fibroblast growth factor), lowering the glucose concentration, removing bFGF and adding nicotinamide.

5 The differentiation into β -cells involves the steps of plating the ITSF culture medium with embryoid bodies obtained in Step 1, culturing the embryoid bodies after adding N-2, B-27 and bFGF to the culture medium, lowering the glucose concentration in the medium, eliminating bFGF, adding nicotinamide and suspended fragment culturing in suspension after disintegrating the cell mass using trypsin-EDTA.

10 The cells induced to differentiate through the above-described process secrete insulin, and this can be confirmed by RT-PCR, which detects insulin, PDX1, *ngn3*, glucokinase, GLUT2, etc. that confirms differentiation into β -cells, measurement of insulin concentration in the culture medium, immunofluorescence that confirms insulin, in situ hybridization analysis that confirms expression of the insulin gene, etc.

15

3. Differentiation into cardiomyocytes

In the present invention, the differentiation into cardiomyocytes from ES cells includes the usual method known in the art (Chunhui Xu et al. *Circ Res.* 91: 501-508, 20 2002), and more specifically, the differentiation can be induced by forming embryoid bodies from the embryonic stem cells and culturing them in a culture medium containing agents that induce the differentiation into cardiomyocytes.

In the present invention, the agents that induce the embryoid bodies to differentiate into cardiomyocytes include, but are not limited to, DMSO (dimethyl sulfoxide), RA (retinoic acid), 5-aza-dC (5-aza-2'-deoxycytidine), etc. 25 Cardiomyocytes can be obtained from hntES cells, by continued culturing of the hntES cells in a culture medium containing such supplements, and causing expansion and differentiation.

More specifically, the same method used in the present invention for

differentiation into neuro progenitor cells or β -cells can be used here. For example, the embryoid bodies can be formed by culturing autologous embryonic stem cells on supporting cells whose propagation has been controlled, separating the embryonic stem cells into several masses using 200 U/ml collagenase IV for 5 to 10 minutes at
5 37°C to form circular embryoid bodies. This process can be performed on a culture medium that is used to induce differentiation into cardiomyocytes. For example, the culture medium inducing differentiation contains 80% knock-out DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L beta-mercaptoethanol, 1% human nonessential amino acid and 20% FBS can be used.

10 Differentiation into pulsing cells can be induced by decomposing the cell mass formed through the above culturing using trypsin-EDTA, etc., suspension culturing of the cells in suspension, and transferring them onto a plate coated with gelatin or poly-L-lysine for culturing.

In the present invention, differentiation of autologous embryonic stem cells
15 into cardiomyocytes can be confirmed using a cardiomyocyte marker, for example, cardiac α -myosin heavy chain, cardiac troponin I, atrial natriuretic factor and cardiac transcription factors such as GATA-4, Nkz2.5 and MEF-2. The same can be confirmed using muscle markers such as sMHC, tropomyosin, α -actinin, desmin or cardiac troponin T proteins. In addition, the same can be confirmed using creatin
20 kinase-MB (CK-MB) or myoglobin. In the present invention, the confirmation of the cardiomyocytes derived from autologous embryonic stem cells is possible by detecting the existence of the above markers, and the above markers can be detected using immunofluorescence, RT-PCR or *in vitro* pharmacological agents.

25 The composition for treatment of damaged cells, tissue or organ contain as effective ingredient, the embryonic stem cell according to the present invention, cells differentiated from the embryonic stem cell, tissue derived from such cells or organ derived from such tissue. The composition can be manufactured in the form of a formulation suitable for maintenance of the composition and administration into an

individual, other than in the form of such cells, tissue or organ. The most preferred type of formulation is water-soluble medicine for injection. Examples include, but are not limited to, physiological saline solution, Ringer solution, or other suitable water-soluble formulation. Preferably, the above cells or tissue may be formulated in the form of an injection, by projecting them into distilled water passed through ion exchange resin and distilled for the second time. Water prepared through ultrafiltration or reverse osmosis apparatus may also be used as a solvent for the injection. Additionally, the above composition may partially contain G1. ver 3 culture medium which is used for the culturing of the above cells.

10 The amount of the above mentioned composition to be administered varies depending on the individual. The amount would depend on, for example, the degree of damage to the spinal cord.

The above composition can be administered through many different pathways. Preferred administration pathways are the intravenous injection, intramuscular injection, hypodermic injection, endodermal injection, myelonic injection, instillation, etc. The fact that the preferred administration pathway varies depending on which part of the patient's body is damaged is quite obvious to those skilled in the art.

The kit for transplantation of cells, tissue or organ in the present invention includes the human embryonic stem cell, cells differentiated from such embryonic stem cell, tissue derived from such cells or organ derived from such tissues of the present invention, and further includes equipment, materials, reagents, etc., that are ordinarily required in performing such transplantation.

25 The human embryonic stem cell or cells differentiated from such embryonic stem cell according to the present invention may contain genes transformed through genetic recombination, etc., to provide genetic therapy if the damage to the patient's cell, tissue or organ were caused by genetic reasons. For example, in the case of Alzheimer or Parkinson's disease, the gene causing the disease in the stem cell or cells differentiated from such stem cell derived from the patient who is the somatic cell donor can be replaced by a normal gene, and transplanted back to the patient. This

will cause the damaged cells, tissue or organ to be recovered, and also prevent the recovered cell, tissue or organ from being damaged again due to the genetic reasons. This makes effective gene therapy possible.

Meanwhile, in order to maximize the effect of curing the relevant disease and prevent any relapse, a gene that expresses factors which cure the disease can be inserted into the human embryonic stem cell or cells differentiated from such embryonic stem cell prepared according to the present invention. For example, if a gene encoding the brain growth factor is inserted into the human embryonic stem cell or cells derived from such embryonic stem cell and then transplanted into the patient, the damaged cells, tissue or organ can be recovered and relapse of such disease can be prevented due to the brain growth factors produced as a result of the expression of the inserted gene.

【Mode for Invention】

The following examples are intended to further illustrate the present invention without limiting the scope.

The G1 ver.3 medium (Vitro Life, Goteberg, Sweden) used in these Examples are supplemented with 5% HSA unless indicated otherwise.

Example 1:

(1-1) Preparation of oocyte

Voluntary oocyte donors were screened carefully through physical and mental examinations and were administered with follicle stimulation hormone (FSH) to induce superovulation.

About 36 hours after the administration of human chorionic gonadotropin (hCG) to the donors, cumulus-oocyte complexes (COCs) were recovered and cultured for 40 minutes in the G1 ver.3 medium (Vitro Life, Goteberg, Sweden) using an incubator maintained at 37°C, 5% CO₂ and saturated humidity. Such COC's were

treated with 0.1% (w/v) hyaluronidase (Sigma Co., St. Louis, MO, U.S.A.) for 1 hour to disperse cumulus cells.

The oocytes were obtained by separating such cumulus cells from the COCs using a mouth pipette and were washed with the G1 ver.3 medium (Vitro Life, Goteberg, Sweden).

(1-2) Preparation of nuclear donor cell

A cut about 1cm in length was made near the navel of a male donor, and a tissue in size of $0.5 \times 0.5 \times 0.5\text{cm}^3$ was removed therefrom. The tissue was placed in the DMEM medium supplemented with 10% FBS, washed 3 times with PBS and ground using surgical scissors. The ground tissue was incubated in a medium containing 4.5 ml of the DMEM medium containing 10% FBS and 0.5 ml of the solution containing 2000 unit/ml collagenase type II for 24 hours at 37°C, 5% CO₂. Then the somatic cells were isolated from the ground tissue by repeated pipetting. The isolated somatic cells were washed with the DMEM medium supplemented with 10% FBS and incubated for 2 days. The medium was replaced with a fresh DMEM medium supplemented with 10% FBS when the attachment of the somatic cell was confirmed.

The somatic cells obtained as above were treated with trypsin-EDTA (0.25% (w/v) (1X); Life Technologies, Rockville, MD, U.S.A.) for 3 minutes at room temperature. Those somatic cells having a modal diameter of 18 to 20 μm were selected as nuclear donor cells.

Example 2:

Enucleation of oocyte and cell fusion

The oocyte obtained in Example 1 was cultured in the G1 ver.3 medium for 1 to 2 hours in order to induce the maturation of its nucleus before enucleation. Then

the enucleation, nuclear transfer and electrofusion thereof were performed as follows:

(2-1) Enucleation of oocyte and nuclear transfer from somatic cell

5 The oocyte was washed once with the G1 ver.3 medium. Such oocyte was transferred to a hyaluronidase solution prepared by mixing 1 ml of the G1 ver.3 medium with 111 μ l of a solution in which 0.05 g of hyaluronidase was dissolved in 5 ml of the G1 ver.3 medium and adjusting to a 0.1% (w/v) hyaluronidase concentration. The oocyte was stripped of any remaining cumulus cells, washed three times with the
10 G1 ver.3 medium and placed in the same medium. Then the oocyte was transferred to a cytochalasin B solution prepared by mixing 1 ml of the G1 ver.3 medium supplemented with 10% fetal bovine serum (FBS) with 1 μ l of a solution in which cytochalasin B was dissolved in dimethyl sulfoxide to a concentration of 7.5 μ g/ml. The zona pellucida of the oocyte was incised using a micromanipulator to form a small
15 hole, and the oocyte was enucleated by removing part of the cytoplasm containing the first polar body and corresponding to 10 to 15% of the total cytoplasm through the small hole.

Fig. 3 shows the incision process of the zona pellucida of the oocyte (3) using a holding pipette (1) and an incision pipette (2). Fig. 4 shows the enucleation process
20 removing the first polar body and the nucleus from the oocyte where the oocyte (3) having the small hole vertically positioned was supported by the holding pipette (1) positioned beneath the oocyte and then lightly pressed by the incision pipette (2) to enucleate the same. Such enucleated oocyte was washed three times with the G1 ver.3 medium and placed in the same medium.

25 Subsequently, a nuclear donor cell in a 4 μ l drop of PBS supplemented with 1% FBS was transferred, using a holding pipette and a transfer pipette into an enucleated oocyte in a 4 μ l drop of a solution prepared by mixing 400 μ l of the G1 ver.3 medium with 100 μ l of a PHA-P solution in which 5 mg of PHA-P was dissolved in 10 ml of G1 ver.3 medium. The drops containing the nuclear donor cell and the

enucleated oocyte were coated with a mineral oil to prevent the evaporation of the drops.

Fig.5 describes the process of transferring the nuclear donor cell into the enucleated oocyte. As can be seen from Fig. 5, the enucleated oocyte (3) was fixed to a holding pipette (1), a transfer pipette (4) was inserted through the small hole into the enucleated oocyte (3), and then the nuclear donor cell was injected into the oocyte (3) to obtain a nucleus-transferred oocyte. Such nucleus-transferred oocyte was washed three times with the G1 ver.3 medium and placed in the same medium.

10 (2-2) Preparation of nucleus-transferred oocyte by electrofusion

The nucleus-transferred oocyte was subjected to electrofusion through a BTX-electro cell manipulator (BTX Inc., San Diego, CA, U.S.A.)

15 A 20 μ l drop of a mannitol solution prepared by dissolving 0.1 mM MgSO_4 , 0.05% BSA and 0.28 mM mannitol in a 0.5 mM HEPES buffer (pH 7.2), a 20 μ l drop of a mixing solution containing 10 μ l of the G1 ver.3 medium and 10 μ l of the mannitol solution, and a 20 μ l drop of the G1 ver.3 medium were prepared.

20 First, the nucleus-transferred oocyte obtained in Example (2-1) was incubated in the 20 μ l drop of the mixing solution containing 10 μ l of G1 ver.3 medium and 10 μ l of the mannitol for 1 minute. Next, the nucleus-transferred oocyte was transferred to the 20 μ l drop of the mannitol solution via a mouth pipette and incubated therein for 1 minute. Subsequently, the nucleus-transferred oocyte was transferred to a mannitol solution having the above composition and placed between two electrodes (3.2 mm
25 chamber No. 453) connected to the BTX-electro cell manipulator and was positioned so that the nuclear donor cell would face the (+) electrode. The nucleus-transferred oocyte was electrofused by applying a direct current of 1 kV/cm for 15 μ sec twice, at an interval of 1 second.

The fused nucleus-transferred oocyte was incubated in the 20 μ l drop of the mixing solution containing 10 μ l of G1 ver.3 medium and 10 μ l of the mannitol for 1 minute, transferred to the 20 μ l drop of the G1 ver.3 medium and then washed with the G1 ver.3 medium three times.

5

Example 3: Reprogramming, activating and *in vitro* culturing of nucleus-transferred oocyte

Since the sperm-mediated activation, one of the major factors for the normal embryonic development, was absent in the process of obtaining the nucleus-transferred oocyte in Example 2, an artificial stimulus is necessary. In order to determine the optimum conditions for artificial embryogenesis, therefore, nucleus-transferred oocytes were reprogrammed, activated and *in vitro* cultured under various conditions as shown in Tables 2 to 4.

First, to examine the effect of the reprogramming time on the rate of blastocyst formation, the reprogramming times were set at about 2, 4, 6, and 20 hours, respectively, while applying the same conditions for activation and *in vitro* culturing as shown in Table 2. As a result, the highest rate of blastocyst formation was obtained when the reprogramming time was about 2 hours.

20

[Table 2]

Reprogr- amming time (hr)	Activation condition		In vitro culture condition		No. of oocy- tes	No. of nucleus-transferred oocytes developed		
			1 st medium	2 nd medium		2-cell stage	morula	Blasto- cyst
2	10 μ M ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	16(10 0)	4(25)	4(25)
4	10 μ M ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	15(94)	1(6)	0

6	10 μ M ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	15(94)	1(6)	1(6)
20	10 μ M ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	9(56)	1(6)	0

*calcium ionophore A23187

Next, to find the optimal activation condition for blastocyst formation, nucleus-transferred oocytes subjected to about 2 hour-reprogramming time were treated for 5 minutes with calcium ionophore A23187 (5 or 10 μ M; Sigma Co., St. Louis, MO, U.S.A.) in the G1 ver.3 medium at 37°C as shown in Table 3. Such nucleus transferred oocytes were washed several times with the G1 ver.3 medium, transferred to the G1 ver.3 medium containing 2.0 mM 6-dimethylaminopurine (6-DMAP; Sigma Co., St. Louis, MO, U.S.A.), and activated at 37°C, 5% CO₂, 5% O₂ and 90% N₂ for 4 hours. Then the nucleus-transferred oocytes were in vitro cultured under the same condition. As shown in Table 3, the highest rate of blastocyst formation was observed when the oocyte was sequentially treated with 10 μ M calcium ionophore and 2.0 mM 6-DMAP.

[Table 3]

Reprog- ramming time (hr)	Activation condition		In vitro culture condition		No. of oocy- tes	No. of nucleus- transferred oocytes developed		
			1st medium	2nd medium		2-cell stage	morula	Blast- ocyst
2	5 μ M ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	11(69)	0	0
2	10 μ M ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	16(10 0)	5(31)	3(19)
2	5 μ M	2.0mM	G1 ver.3	SNUnt-2'	16	9(56)	0	0

	ionomycin	6- DMAP						
2	10μM ionomycin	2.0mM 6- DMAP	G1 ver.3	SNUnt-2'	16	12(75)	0	0

*calcium ionophore A23187

Furthermore, in order to confirm the effect of *in vitro* culturing conditions on the rate of blastocyst formation, the nucleus-transferred oocyte, after the above optimum reprogramming time and activation, were washing vigorously with the G1 ver.3 medium and cultured for 48 hours in 10 μl drop of the G1.ver3 medium or mSOFaa at 37°C, 5% CO₂, 5% O₂, 90% N₂. The isolated embryo was transferred to a fresh G2.2 medium or mSOFaa medium containing 5% HSA and cultured for 6 days. The highest rate of blastocyst formation was detected when the oocyte was first cultured in the G1 ver.3 medium and subsequently in the mSOFaa medium.

[Table 4]

Reprogr- amming time (hrs)	Activation condition		1 st medium	2 nd medium	No. of oocy- tes	No. of nucleus- transferred oocytes developed		
						2-cell stage	Mor- ula	Blas- tocyst
2	10μM ionophore*	2.0mM 6- DMAP	G1 ver.3	SNUnt- 2'	16	16 (100)	4(25)	3(19)
2	10μM ionophore*	2.0mM 6- DMAP	G1 ver.3	G2.2	16	16 (100)	0	0
2	10μM	2.0mM	SNUnt-2'		16	16	0	0

	ionophore*	6- DMAP			(100)		
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*calcium ionophore A23187

In conclusion, an optimum embryogenesis of a nucleus-transferred oocyte was achieved by subjecting the oocyte to 2-hour reprogramming, activation through a serial treatment with 10 μ M calcium ionophore and 2.0 mM 6-DMAP, and a sequential culturing in the G1 ver.3 medium and the SNUnt-2' medium (mSOFaa medium supplemented with 10% HSA).

Example 4: Removal of zona pellucida and trophoblast, and isolation of ICMs

The blastocyst obtained in Example 3 above was treated with 0.1% pronase (Sigma Co., St. Louis, MO, U.S.A.) for 1 minute to remove its zona pellucida. Then, it was treated with 100% anti-human serum antibody (Sigma Co., St. Louis, MO, U.S.A.) for 20 minutes, and was exposed to 10 μ l of guinea pig complement (Life Technologies, Rockville, MD, U.S.A.) at 37°C, 5% CO₂ for 30 minutes to remove its trophoblast and isolate ICMs therefrom.

Example 5: Culturing of ICMs

(1) Mouse embryonic fibroblast feeder layer

The ICMs isolated in Example 4 were cultured in a tissue culture dish coated with 0.1% gelatin, which contained a feeder layer (3.75×10^4 cells/cm²) of mitomycin C-inactivated primary mouse (C57BL breed) embryonic fibroblasts, DMEM/F12 medium (Life Technologies, Rockville, MD, U.S.A.) comprising 20% serum replacement (Gibco, Grand Island, NY, U.S.A.), 0.1 mM β -mercaptoethanol (Sigma),

1% NEAAs, 2 mM glutamine, 100 units/ml penicillin, and 100 μ l/ml streptomycin, and 4 ng/ml bFGF (Life Technologies, Rockville, MD, U.S.A.) was used as the culture medium.

At an early stage of culturing the ES cells, the medium was supplemented with
5 a hLIF (100 units/ml; Chemicon, Temecula, CA, U.S.A.). The culturing was continued for more than 6 days until the colonies of undifferentiated ntES cells appeared. The ntES cells were mechanically isolated from the colonies by using a micropipette every five or seven days after such colony formation.

10 (2) A mixed layer of mouse-derived fibroblasts and human-derived fibroblasts

Stem cells were obtained in the same manner as the (1) except for using a feeder layer comprising a mixture of fibroblasts derived from C57BL breed mouse fetus that have been inactivated with mitomycin C at a density of 7.0×10^4 cell/cm³ and human-
15 derived fibroblasts, more specifically, fibroblasts obtained from the skin of the patient who donated the somatic cell used in the preparation of the autologous human embryonic stem cell at a density of 2.5×10^4 cells/cm², instead of a layer feeder comprising fibroblasts derived from mouse fetus only.

20 (3) Autologous fibroblast layer or fibroblast layer derived from autologous embryonic stem cell

Stem cells could be obtained using either a feeder layer comprising fibroblasts from the patient who donated the somatic cell in the present invention, preferably, from
25 the skin of the patient, at a density of 3.75×10^4 cells/cm² or a feeder layer comprising fibroblasts differentiated from autologous embryonic stem cells obtained through the method described in (1) above.

Test Example 1: Identification of human ntES cells obtained in Example 5 by

karyotype analysis

The colonies of undifferentiated ntES cells obtained in Example 5(1) were washed with PBS containing 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+} , fixed with citrate-acetone-
5 formaldehyde (the mixing ratio in volume was 25:65:8) at 4°C for 1 hour, and washed again with PBS containing 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+} . The alkaline phosphatase activity of the ntES cells was determined by a AP kit (Sigma Co., St. Louis, MO, U.S.A.). The monoclonal antibodies employed in this process were, Oct-4 (SC-5279; purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), SSEA-1(MC480),
10 SSEA-3 (MC631) and SSEA-4 (MC-813-70; Developmental Studies Hybridoma Bank, Iowa City, IA), TRA-1-60 and TRA-1-81 (Chemicon, Temecula, CA, U.S.A.). Further, an immunohistochemical assay was performed in order to identify specific surface antigens on the ntES cells, by employing monoclonal antibodies as primary antibodies. Such primary antibodies were detected using a Vectastatin ABC kit
15 (Vector Laboratory, Burlingame, CA, U.S.A.) containing a biotinylated secondary antibody and an avidin-horseradish peroxidase conjugate. As a result, indications specific to human stem cells, AP (alkaline phosphatase) (a), SSEA-1 (b), SSEA-3 (c), SSEA-4 (d), Tra-1-60 (e), Tra-1-81 (f) and Oct-4 (g) were detected, as shown in Fig. 1 (a) to (g).

20 DNA fingerprinting analysis was performed with regard to the genomic DNA and human short tandem repeat (STR) marker using a STR AMP FLSTR PROFILER kit (Applied Biosystems, Foster City, CA, U.S.A.) on the automated ABI 310 Genetic Analyzer (Applied Biosystems, Foster city, CA, U.S.A.). The results ate shown in Figs. 6 to 10.

25 According to Fig. 6, it was confirmed that the karyotype of the ntES cells derived from the nucleus-transferred oocyte prepared in accordance with Examples 1 to 5 above was that of a man, and according to Figs. 7 to 10, DNA fingerprinting analysis shows that the karyotype of the ntES cells were identical to that of the nuclear donor cell. This result demonstrates that the ntES cells of the present invention have been

indeed derived from the autologous nucleus-transferred oocyte.

Test Example 2: Identification of human ntES cells by teratoma analysis

5 100 colonies of the undifferentiated ntES cells obtained in Example 5(1) were isolated from their culture dish, injected into a testis of a SCID mouse (Korea Research Institute of Bioscience and Biotechnology, Korea) using a 1 ml syringe and cultured for 8 weeks. Teratomas thus formed were paraffin-fixed and examined by an immunohistochemical assay to check whether three dermal cells were formed. The
10 result is shown in Fig. 11.

According to Fig.11 (a) to (c), three dermal cells were formed from the ntEL cells obtained in Example 5(1) (cartilage (a): endoderm; intestinal tract (b): mesoderm; neural tube (c): ectoderm) in the testis. This result demonstrates that such ntES cells were pluripotent ES cells having the ability to differentiate into various tissues.

15

Test Example 3: Examination of embryoid body formation through immunohistochemical assay

Colonies of the human ntES cells obtained in Example 5(1) were treated with
20 0.1% trypsin/1 mM EDTA to isolate the ntES cells, which were then transferred to a plastic Petri dish. The human ntES cells were cultured for 14 days in the DMEM/DMEM F12 medium devoid of hLIF and bFGF. For paraffin fixation, such ntES cells were transferred to 1% low-melting temperature agars dissolved in PBS and cooled to 42°C. The resulting solidified agars containing the ntES cells was fixed
25 using 4% paragormaldehyde dissolved in PBS and embedded in paraffin. Each 6-mm section of the paraffin embedded cells was placed on a slide and subjected to an immunohistochemical analysis. As primary antibodies, alpha-1-fetoprotein (18-0003), cytokeratin (18-0234), desmin (18-0016), neurofilament (18-0171) and S-100 (18-0046) purchased from Zemed (South San Francisco, CA, U.S.A.) and HNF-2-

alpha (SC-6556), BMP-4 (SC-6896), Myo D (SC-760) and NCAM (SC-7326) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) were employed. A biotinylated anti-rabbit, anti-mouse or anti-goat antibody was used as a secondary antibody, and the reaction was detected by streptavidin-conjugated horseradish
5 peroxidase and diaminobenzidine chromagen.

As a result, it was confirmed that such ntES cells could form embryoid bodies based on the fact that the marker proteins of endoderm (i.e., alpha-1-fetoprotein, cytokeratin, and HNF-2-alpha), the marker proteins of mesoderm (i.e., BMP-4, Myo D and desmin) the marker proteins of ectoderm (i.e., neurofilament, S-100 and NCAM)
10 were expressed in the ntES cells obtained in Example 5(1). This result demonstrates that the cells obtained in the present invention fall within the scope of an ES cell.

Example 6: Differentiation into neuro progenitors

15 (6-1) Expansion of undifferentiated ES cells

The human undifferentiated ntES cells obtained in Example 5(1) were cultured at 37°C in 5% CO₂ atmosphere on a mouse embryonic fibroblast feeder layer with inactivated cell division, contained in a culture plate coated with 2% gelatin. The
20 culture medium was composed of DMEM/F12 (1:1), 20% knock-out serum replacement (Gibco, Grand Island, NY, U.S.A.), 0.1 mM human NEAAs, 0.1 mM β-mercaptoethanol, 1 mM L-glutamine, 100 U/ml penicillin G, 100 μl/ml streptomycin, and 4 ng/ml bFGF; and was changed everyday.

25 (6-2) Formation of embryoid body

Colonies of the ntES cells cultured as above were collected by lightly scraping the walls with the tip of a pipette and cultured on a non-adhesive culture dish at 37°C in 5% CO₂ atmosphere. The culture medium was identical to that in Example (6-1)

except that 4 ng/ml bFGF was omitted. After one day, such colonies began to grow as floating embryoid bodies (about 50 embryoid bodies/dish). At this point, the embryoid bodies were transferred to a new dish, while completely removing any remaining feeder cells. After further culturing for 4 days, embryoid bodies thus
5 formed were plated on an adhesive dish coated with polyornithine/laminin.

(6-3) Selection of nestin-positive cells

After 1-day culturing on the adhesive dish, embryoid bodies in the process of
10 differentiation were transferred to the DMEM/F12 medium supplemented with insulin (25 µl/ml), transferrin (100 µl/ml), sodium selenite (30 nM) and fibronectin (5 µl/ml) and cultured at 37°C for 6 days. The resulting cells were cultured at 37°C for 40 minutes in a solution wherein anti-nestin antibody (Chemicon, Temecula, CA, U.S.A.) was diluted 1000fold with a solution containing 0.01 M PBS, 1% BSA and 5 mM
15 EDTA. Such cells were washed with the DMEM/F12 medium, treated with phycoerythrin (PE)-conjugated with secondary antibody (Chemicon, Temecula, CA, U.S.A.) for 30 minutes and then washed three times with the DMEM/F12 medium, thereby selecting the nestin-positive cells.

(6-4) Expansion of nestin-positive cells

The nestin-positive cells selected in Example (6-3) were cultured in the DMEM/F12 medium supplemented with the N-2 supplement, laminin (1 ng/ml) and bFGF (10 ng/ml) for 6 days to expand those cells.

25

(6-5) Differentiation into neuro progenitors

The nestin-positive cells expanded in Example (6-4) were cultured at 37°C for 10 days in the DMEM/F12 medium supplemented with the N-2 supplement and

laminin (1 ng/ml) but devoid of bFGF to induce their differentiation into neuro progenitors.

Fig. 2 (a) shows the neuro progenitors differentiated from the nucleus-transferred oocyte prepared according to the present invention, and (b) shows the neuro
5 filaments further differentiated from such neuro progenitors.

Example 7: Treatment of spinal cord injury using neuro progenitors differentiated from stem cells

10 An adult Sprague Dawley (250-350 g, female) was anesthetized by injecting 300 µl of a solution containing a mixture of ketamine (60 mg/kg) and xylazine (5 mg/kg) into its abdominal cavity. The lower part of its back was shaved using a razor, and betadine was spread over the area. A 2 cm incision was made in the center of the 13th position of the thoracic rib and the vertebra, and the muscle and tissue over the
15 thoracic rib was carefully removed. The left L1-L2 area of the Sprague Dawley was cut away in a straight line using the No.11 scalpel and a 1ml syringe (hemicordotomy). After removing the bone in the upper part of the left part of the L1-L2 vertebra, an incision was made using the No.11 scalpel, in a straight line along the central blood vessel. Then the needle of the 1 ml syringe was bent to an "L" shape, and using the
20 end of the needle, the incision made using a No.11 scalpel was repeatedly incised in a straight line, until the left nerve of the spinal cord is disconnected. In doing so, the central blood vessel must not be incised, and the right side of the central blood vessel must not be damaged. Finally, the area from which bone was removed is covered with a slastic sheet cut into an appropriate size, so that the muscle and the tissue would
25 not attach to the spinal cord and lump together. Then incision was sealed using surgic thread and needle, and betadine was spread over the surgic area.

The Sprague Dawleys with spinal cords damaged as above, were divided into 4 groups. The first group was not given any treatment after the damage was inflicted. The second group was subjected to reopening of the L1-L2 area on their backs using a

scalpel, and G1 ver.3 medium, the culture medium for stem cells, was injected using a Hamilton syringe needle at the rate of 5µl/10min, at a point 5 mm towards the head from the L1-L2 area, 8 days after the damage. The third and fourth groups were subjected to reopening of the L1-L2 area on their backs using a scalpel, and the stem
5 cells prepared in Example 5(1) above were injected using a Hamilton syringe needle at the rate of 5 µl/10 min, at a point 5 mm towards the head from the L1-L2 area, 8 days after the damage. After the injection, the damaged area was covered with a slastic sheet cut into an appropriate size, the area of incision was sealed using surgic thread and needle, and betadine was spread over the area.

10 To find out the effect of the stem cells, a behavioral test and an autopsy were conducted.

A modified behavioral test was conducted, based on *Journal of Neurotrauma* Vol.12 1-21p, Number 1, 1995, Michele Basso et al. The behavioral test was
15 conducted every day for two weeks beginning on the day after the surgery, once every 3 days for the third and fourth weeks and once every 7 days for the 5th and 6th weeks. As a result, the Sprague Dawleys of the 3rd and 4th groups did not show any movement in the 1st and 2nd weeks, began to show slight movements after 2 weeks and showed normal movements after 4 weeks. To the contrary, the Sprague Dawleys in the 1st and
20 2nd groups were not able to move even after 6 weeks.

Meanwhile, an autopsy was conducted on the mice after 6 weeks. As a result, it was confirmed that the damaged spinal cords of the Sprague Dawleys in 3rd and 4th groups had almost completely recovered. The damaged spinal cords of Sprague Dawleys in the 1st and 2nd groups, however, were not repaired at all.

25

Example 8: Differentiation into beta-cells

The stem cells prepared in Example 5 (1) were cultured on a inactivated mice embryonic fibroblast feeder layer, with 80% DMEM (Dulbeco's Modified Eagles

Medium), 20% knock-out serum replacement (GIBCO, Grand Island, NY, U.S.A.), 1 mM glutamine, 1% NEAA's, 0.1 mM 2-mercaptoethanol (GIBCO Invitrogen; Paisley, UK), and 4 ng/ml bFGF (GIBCO Invitrogen; Paisley, UK).

The cells were isolated using 1 mg/ml type IV collagenase (GIBCO
5 Invitrogen), and were scraped using a 5 ml pipette after 30 minutes, transferred to a plastic petri dish (Miniplast; Ein-Shemer, Israel) to form colonies (embryoid bodies). The embryoid bodies so formed were cultured using 80% knock-out DMEM (GIBCO Invitrogen), 20% defined FBS (Hyclone; Logan, UT), 1 mM glutamine (GIBCO Invitrogen) and 1% NEAA's (GIBCO Invitrogen) for 7 days, and the medium was
10 changed every 3 days. The 7 days-old embryoid bodies (comprising about 1000 cells on average) were placed on a 6-well plastic culture dish (Nunc; Roskilde, Denmark), about 300 cells in each well, and were cultured in ITS (DMEM/F12 1:1, insulin (10 mg/L)-transferrin (6.7 ng/L)-selenium (5.5 mg/L)) (GIBCO Invitrogen) and 1 mM glutamine (GIBCO Invitrogen) supplemented with 5 µg/ml fibronectin (Roche
15 Diagnostics GmbH; Mannheim, Germany).

After one week, the cells were decomposed with trypsin-EDTA (Biological Industries; Beit Haemek, Israel) into individual cells in ITSF medium (ITS and fibronectin) and spread them on a tissue culture dish in the concentration of 2×10^5 /ml. Also spread on the dish was a medium containing the following: DMEM/F12 1:1
20 supplemented with N-2 supplement in (500 µg/ml progesterone, 1,611 µg/ml putrescine and 0.52 µg/ml selenite) (GIBCO Invitrogen), B-27 supplement medium (GIBCO Invitrogen), 1mM glutamine (GIBCO Invitrogen) and 10ng/ml bFGF (GIBCO Invitrogen). Before they were spread on the dish, the tissue culture dish was coated with 0.1% gelatin or poly-L ornithine (15 ng/ml) (Sigma Chemical Co.; St.
25 Louis, MO). After they were spread on the dish, the cell masses were cultured for one week, and the medium was changed every 2 days. Then bFGF was removed and 10mM of nicotinamide (Sigma Chemical Co.; St. Louis, MO) was added. The glucose concentration in the medium was lowered to 901 mg/L using the DMEM free of glucose. Then the cell masses formed during the 4-day culturing in the medium

containing DMEM/F12 1:1 with 901 mg/L glucose, supplemented with N-2 and B-27 medium, 1 mM glutamine, 10 mM nicotinamide, were disintegrated with trypsin-EDTA and suspension cultured on a petri dish with the above medium.

It was confirmed using microparticle enzyme immunoassay (AXSYM System
5 Insulin Kit Code B2D010; Abbot Laboratories; Chicago, IL) that that the cell masses formed in the above process secreted insulin.

Example 9: Differentiation into cardiomyocytes

10 The stem cells prepared in Example 5(1) were cultured on cell division-inactivated human skin fibroblast feeder layer on a culture plate coated with 2% gelatin, at 37°C, 5% CO₂. For the culturing, a culture medium containing 80% knock-out DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L L-glutamine, 0.1 mmol/L beta-mercaptoethanol, 1% human NEAA's and 20% FBS was used, and the medium was changed every day.

15 The above cultured ES cells colonies were collected by lightly scraping the walls with the tip of a pipette, transferred to a non-adhesive culture dish and cultured at 37°C, 5%, CO₂. The same culture medium as the one described above was used. After one day, ES cell colonies started to grow as floating embryoid bodies (approximately 50/dish). All remaining feeder cells were removed while transferring
20 the embryoid bodies to a new dish. After further culturing for 4 days, the embryoid bodies formed were transferred to an adhesive dish coated with gelatin or poly-L-lysine at the ratio of approximately 1 to 3 EBs/cm² and cultured.

After culturing on the adhesive dish for one day, the cells were suspended in a medium containing 80% knock-out DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L beta-
25 mercaptoethanol, 1% human NEAA's and 20% FBS (Hyclone), and cultured for 4 days. Cell masses formed after the culturing were transferred to a plate coated with gelatine or poly-L-lysine in the ratio of approximately 0.5 to 4 EBs/cm² and were cultured while observing the changes in the cells.

The embryoid bodies going through differentiation were transferred to

medium (containing 80% knock-out DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L beta-mercaptoethanol, 1% human NEAA's and 20% FBS) supplemented with DMSO (dimethyl sulfoxide), RA (retinoic acid), 5-aza-2'-deoxycytidine (5-aza-2'-dC), respectively, and cultured at 37°C for 6 days.

5 The differentiated cultured cells containing sphygmie (throbbing) cells obtained above were washed with PBS or a low-calcium, pH 6.9 solution containing 120 mM NaCl, 5.4 mM KCl, 5 mM MgSO₄, 5mM Na-pyruvate, 20 mM glucose, 20 mM taurin and 10 mM HEPES. The cultured cells were cultured for 1 or 2 hours at 37°C after adding 1 mg/ml collagenase B to a low-calcium solution containing 30 μ M
10 CaCl₂. Then the cultured cells were re-suspended in a pH 7.2 solution containing 85 mM KCl, 30 mM K₂HPO₄, 5 mM MgSO₄, 1 mM EGTA, 2 mM Na₂ATP, 5 mM Na-pyruvate, 5 mM creatin, 20 mM taurin, 20 mM glucose, and were cultured for 15 minutes at 37°C for complete isolation. After the isolation, the cultured cells were applied to the Percoll gradient, and were isolated using a 0.56 units/ml Blendzyme IV
15 for 30 minutes at 37°C.

The differentiated human embryonic stem cells including throbbing cells were isolated, re-suspended in the medium for differentiation and transferred to an interrupted Percoll gradient. Percoll gradient was diluted with a buffer solution containing 20 mmol/L HEPES, 150 mmol/L NaCl, centrifuged at 1500 g for 30
20 minutes, and the cell layers were separated. Cells in other layers were collected, washed and re-suspended in a medium containing 80% knock-out DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L beta-mercaptoethanol, 1% human NEAA's and 20% FBS. Then fragmented cells were spread over the chamber slide, cultured and were subjected to immunostaining. As a result, the existence of cardiomyocytes was
25 confirmed by the detection of markers for cardiomyocytes, i.e., cardiac α -myosin heavy chain, cardiac troponin I, atrial natriuretic factor and cardiac transcription factors GATA-4, Nkz2.5 and MEF-2.

【Industrial Applicability】

The autologous human embryonic stem cells, derived from the human autologous nucleus-transferred oocyte prepared in accordance with the present invention, possess the same genome as that of the recipient. Therefore, the advantages are that the transplant would not cause any immunorejection and that
5 embryonic stem cells derived from a nucleus-transferred oocyte containing the nucleus of the recipient's somatic cell can be obtained even when the somatic cell and the oocytes are derived from difference sources, i.e., from men or women not in their age of fertility who cannot produce oocytes.

Furthermore, by culturing the embryonic stem cells in a medium and under
10 conditions appropriate to induce cell differentiation into a specific type of cells such as hematopoietic cells, nerve cells, beta cells, muscle cells, liver cells, cartilage cells, epithelial cells, etc. Cells or tissue so differentiated causes recovery of damaged cells or tissues and consequently the function of the damaged organ. This can be used in cell transplant for treatment of numerous diseases, for example, cell damage diabetes,
15 Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, cerebrum palsy and cancer.

CLAIMS

[Claim 1]

A human embryonic stem cell derived from a nucleus-transferred oocyte prepared by
5 transferring a nucleus of a human somatic cell into an enucleated human oocyte,
wherein the somatic cell and the oocyte are derived from different individuals.

[Claim 2]

A human embryonic stem cell derived from a nucleus-transferred oocyte prepared by
10 transferring into an enucleated human oocyte a nucleus of a somatic cell of the human
patient who is to receive the stem cell, or cells or tissue differentiated from the stem
cell.

[Claim 3]

15 The human embryonic stem cell according to claim 1 or claim 2, wherein the somatic
cell is derived from a male or a female not in her age of fertility.

[Claim 4]

The human embryonic stem cell according to claim 3, wherein the somatic cell is
20 derived from a male.

[Claim 5]

A method for preparing a human embryonic stem cell comprising

(1) culturing a human somatic cell derived from an individual to prepare a
25 nuclear donor cell;

(2) enucleating a human oocyte derived from a different individual from said
individual, to prepare a recipient oocyte;

(3) preparing a nucleus-transferred oocyte by transferring a nucleus of the
nuclear donor cell into the recipient oocyte and fusing the nucleus of the nuclear donor

cell and the recipient oocyte;

(4) subjecting the nucleus-transferred oocyte to reprogramming, activation and *in vitro* culturing to form a blastocyst; and

(5) isolating an inner cell mass (ICM) from the blastocyst and culturing the
5 ICM in an undifferentiated state to establish a human embryonic stem (ES) cell.

[Claim 6]

A method for preparing a human embryonic stem cell comprising

(1) culturing a somatic cell of the human patient who is expected to receive the
10 stem cells, or cells or tissue differentiated from the stem cells to prepare a nuclear donor cell;

(2) enucleating a human oocyte to prepare a recipient oocyte;

(3) preparing an autologous nucleus-transferred oocyte by transferring a nucleus of the nuclear donor cell into the recipient oocyte and fusing the nucleus of the
15 nuclear donor cell and the recipient oocyte;

(4) subjecting the autologous nucleus-transferred oocyte to reprogramming, activation and *in vitro* culturing to form a blastocyst; and

(5) isolating the inner cell mass from the blastocyst and culturing the inner cell mass in an undifferentiated state to establish an autologous human embryonic stem cell.
20

[Claim 7]

The method according to claim 5 or claim 6, wherein the somatic cell is derived from a male or a female not in her age of fertility.

25 [Claim 8]

The method according to claim 7, wherein the somatic cell is derived from a male.

[Claim 9]

The method according to claim 5 or claim 6, wherein the (3) is carried out by cell

fusion of the nuclear donor cell and the recipient oocyte.

[Claim 10]

The method according to claim 5 or 6, wherein the reprogramming in the (4) is
5 conducted for 20 hours or less.

[Claim 11]

The method according to claim 10, wherein the reprogramming in the (4) is conducted
for 6 hours or less.

10

[Claim 12]

The method according to claim 11, wherein the reprogramming in the (4) is conducted
for 3 hours or less.

15 [Claim 13]

The method according to claim 12, wherein the reprogramming in the (4) is conducted
for 2 hours or less.

[Claim 14]

20 The method according to claim 5 or claim 6, wherein the activation in the (4) is
performed by treating the nucleus-transferred oocyte with a calcium ionophore and
subsequently with 6-dimethylaminopurine.

[Claim 15]

25 The method according to claim 14, wherein the concentration of the calcium ionophore
ranges from 5 μ M to 15 μ M.

[Claim 16]

The method according to claim 15, wherein the concentration of the calcium ionophore

is approximately 10 μ M.

[Claim 17]

The method according to claim 14, wherein the concentration of 6-
5 dimethylaminopurine ranges from 1.5 mM to 2.5 mM.

[Claim 18]

The method according to claim 17, wherein the concentration of 6-
dimethylaminopurine is approximately 2.0 mM.

10

[Claim 19]

The method according to claim 5 or claim 6, wherein the *in vitro* culturing in the (4) is
performed by sequentially using at least two media, each having a different
composition from each other.

15

[Claim 20]

The method according to claim 19, wherein the *in vitro* culturing in the (4) is
performed by sequentially using two media, each having a different composition from
each other.

20

[Claim 21]

The method according to claim 20, the *in vitro* culturing in the (4) is performed by
preliminary culturing in G1 ver.3 medium and secondary culturing in SNUnt-2'
medium.

25

[Claim 22]

The method according to claim 5 or claim 6, wherein the inner cell mass in the (5) is
cultured on a feeder layer containing fibroblast cells derived from the human patient
who is to be the recipient of the stem cell, or cells or tissue derived from the stem cell.

[Claim 23]

The method according to claim 22, wherein the feeder layer is cultured on a feeder layer containing fibroblasts differentiated from the stem cells according to claim 1 or
5 claim 2.

[Claim 24]

The method according to claim 22, wherein the feeder layer further contains mouse-derived fibroblasts.

10

[Claim 25]

A differentiated cell from the embryonic stem cell derived from a nucleus-transferred oocyte prepared by transferring a nucleus of a human somatic cell into an enucleated human oocyte, wherein the somatic cell and the oocyte are derived from different
15 individuals.

[Claim 26]

A differentiated cell from the embryonic stem cell derived from a nucleus-transferred oocyte prepared by transferring a nucleus of a somatic cell of a human patient who is
20 to be the recipient of the stem cell, or cells or tissue differentiated from the stem cell, into an enucleated human oocyte.

[Claim 27]

The differentiated cell according to claim 25 or claim 26, wherein the differentiated
25 cell is a hematopoietic cell, neuro progenitor, nerve cell, beta cell, muscle cell, liver cell, cartilage cell, epithelial cell or cardiomyocytes.

[Claim 28]

A method for inducing differentiation of a human embryonic stem cell into a specific

type of cell comprising

- (1) culturing an embryonic stem cell derived from a nucleus-transferred oocyte prepared by transferring a nucleus of a human somatic cell into an enucleated human oocyte wherein the somatic cell and the oocyte are derived from different individuals
- 5 to form an embryoid body; and

(2) culturing the embryoid body in the presence of an agent suitable for inducing its differentiation into a desired type of cell.

[Claim 29]

- 10 A method for inducing differentiation of the human embryonic stem cell into a desired type of cell comprising

(1) culturing an autologous human embryonic stem cell derived from a nucleus –transferred oocyte prepared by transferring a nucleus of a somatic cell of a human patient who is to be the recipient of the stem cell, or cells or tissue

15 differentiated from the stem cell, into an enucleated human oocyte, to form an embryoid body.

(2) culturing the embryoid body in the presence of a suitable agent for inducing its differentiation into a desired type of cell.

20 [Claim 30]

The method according to claim 28 or claim 29, wherein the desired type of cell is a hematopoietic cell, neuro progenitor, nerve cell, beta cell, muscle cell, liver cell, cartilage cell, epithelial cell or cardiomyocytes.

25 [Claim 31]

The method according to claim 30, wherein the desired type of cell is a neuro progenitor, and after the (2), the method further comprises selecting cells expressing a marker of the neuro progenitor.

[Claim 32]

The method according to claim 31, wherein the agent in the (2) is at least one selected from the group consisting of insulin, transferrin, a mixture of sodium selenite and fibronectin, retinoic acid, ascorbic acid, nicotinamide, N-2 supplement and B-27
5 supplement.

[Claim 33]

The method according to claim 28 or claim 29, wherein the specific type of cell is a beta cell, and the agent in the (2) is at least one selected from the group consisting of
10 insulin, transferrin, a mixture of sodium selenite and fibronectin, nicotinamide, N-2 supplement or B-27 supplement.

[Claim 34]

The method according to Claim 33, wherein the (2) comprises

- 15 (i) spreading the embryoid body on a medium supplemented with insulin, transferring and a mixture of sodium selenite and fibronectin, adding N-2 supplement, B-27 supplement and bFGF into the medium and culturing the embryoid body; and
- (ii) removing bFGF from, and adding nicotinamide to, the medium in the
20 (i) lowering the glucose concentration to be within the range of 500 mg/l to 1500 mg/l, and culturing the embryoid body.

[Claim 35]

The method according to claim 28 or claim 29, wherein the specific type of cell is a
25 cardiomyocyte, and the agent in the (2) is one or more selected from the group consisting of dimethylsulfoxide (DMSO), retinoic acid and 5-aza-2'-deoxycytidine (5-aza-dC).

[Claim 36]

A composition to be injected to an individual patient for treatment of damaged cell, tissue or organ, which contains as an effective ingredient at least one selected from the group consisting of the human embryonic stem cell according to claim 1 or claim 2, the differentiated cell according to claim 25 or claim 26, tissue derived from the cell or
5 organ derived from the tissue.

[Claim 37]

The composition according to claim 36, wherein the human embryonic stem cell or the differentiated cell is genetically transformed to be suitable for treatment of the patient's
10 disease.

[Claim 38]

A transplant kit for transplant of cell, tissue or organ into an individual patient for recovery of a damaged cell, tissue or organ, which contains one or more selected from
15 the group consisting of the human embryonic stem cell according to claim 1 or claim 2, the differentiated cell according to claim 25 or claim 26, tissue derived from the cell or organ derived from the tissue.

[Claim 39]

20 The transplant kit according to claim 38, wherein the human embryonic stem cell or the differentiated cell is genetically transformed to be suitable for treatment of the patient's disease.

[Claim 40]

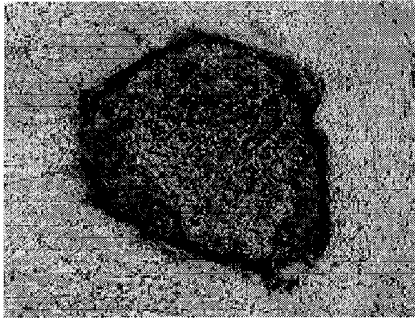
25 The method for treating damaged cell, tissue or organ by transplanting into an individual patient who donated a somatic cell for the preparation of an embryonic stem cell, one or more selected from the group consisting of the human embryonic stem cell according to claim 1 or claim 2, the differentiated cell according to claim 25 or claim 26, tissue derived from the cell or organ derived from the tissue.

[Claim 41]

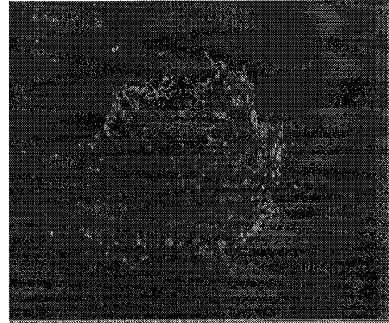
The method according to claim 40, wherein the human embryonic stem cell or the differentiated cell are genetically transformed to be suitable for treatment of the
5 patient's disease.

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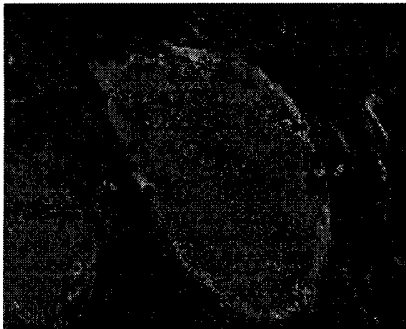
Fig. 1



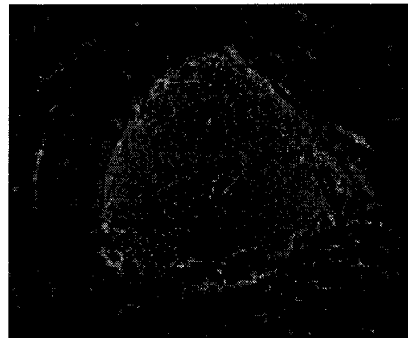
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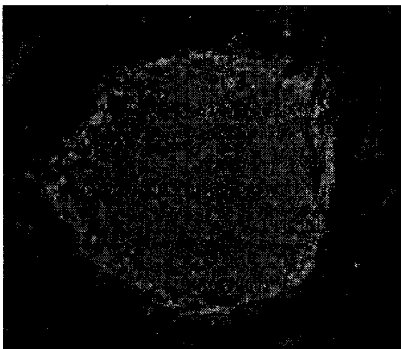
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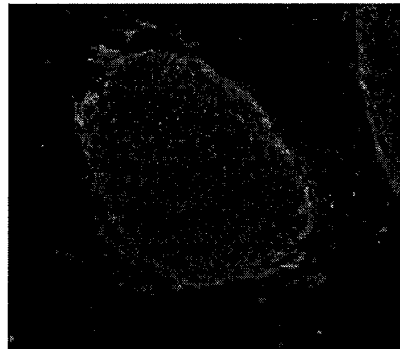
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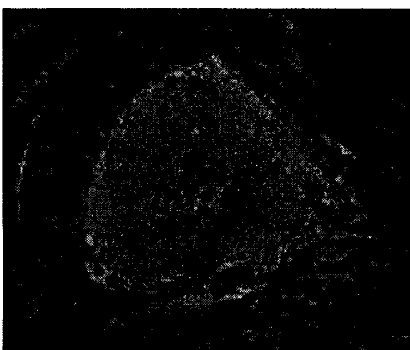
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(e)



(f)



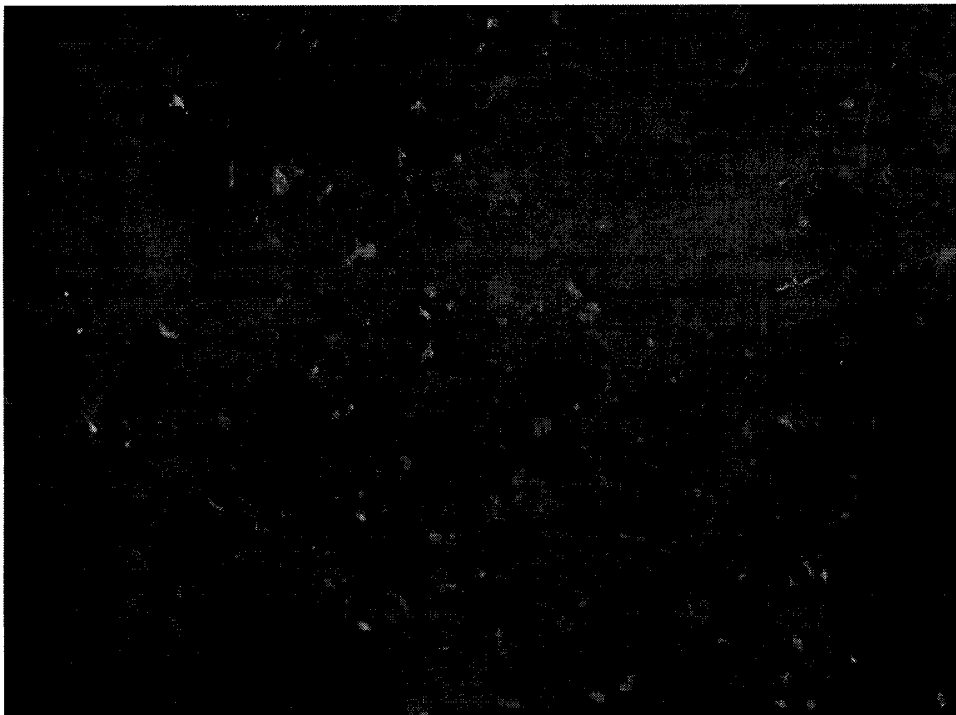
(g)

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Fig. 2



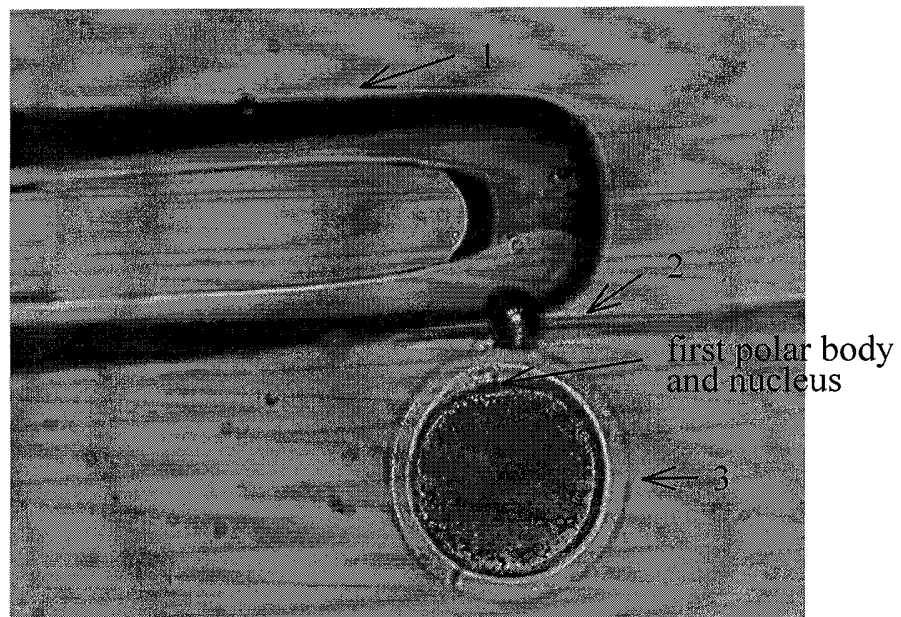
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(b)

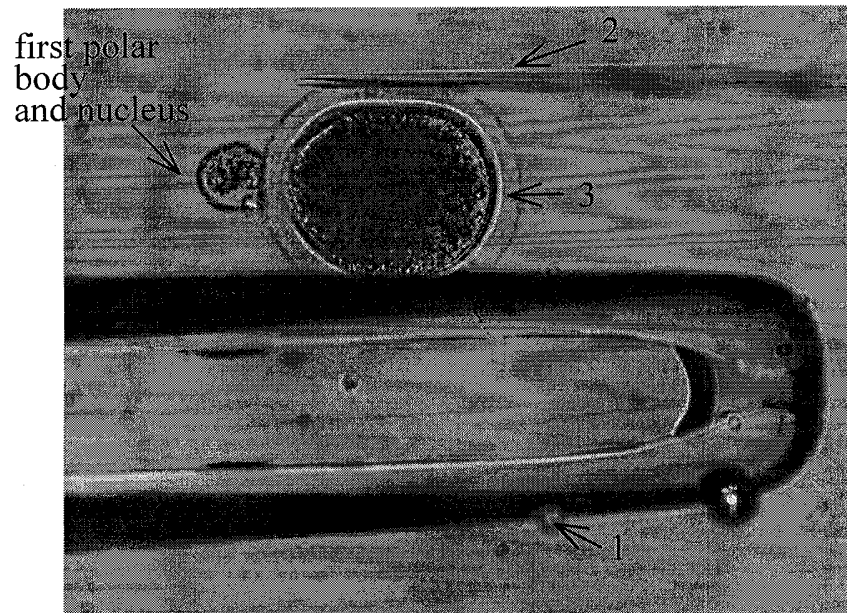
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Fig. 3



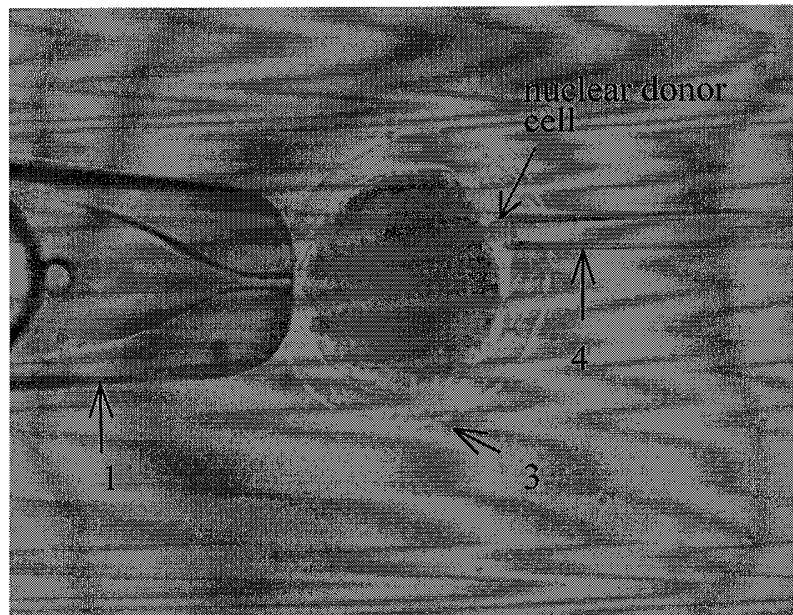
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Fig. 4



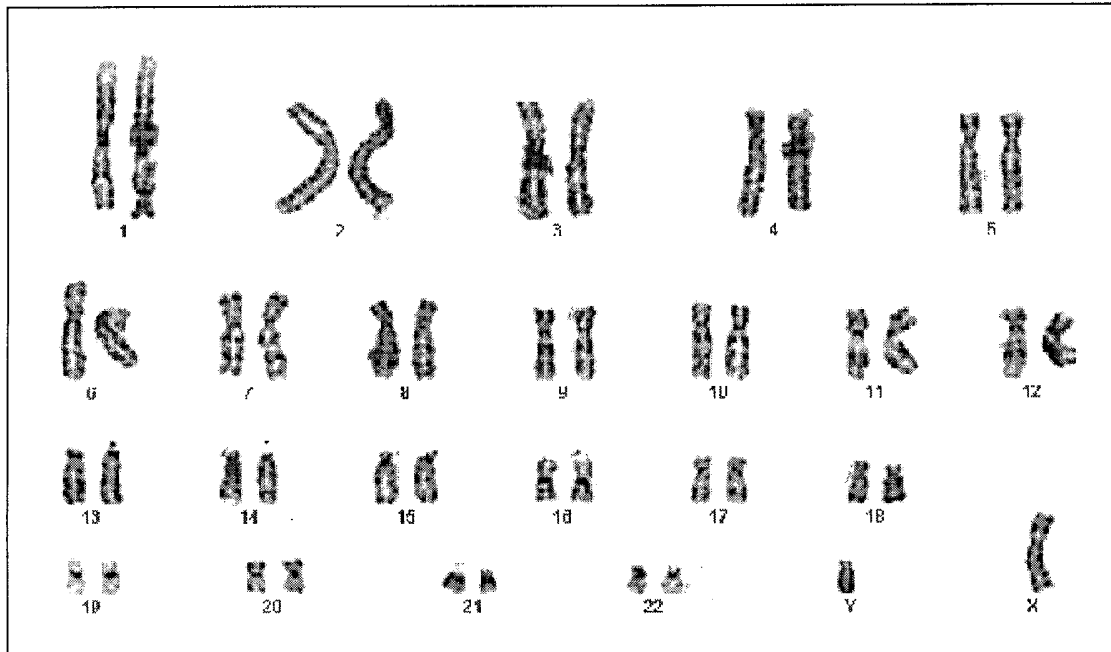
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Fig. 5



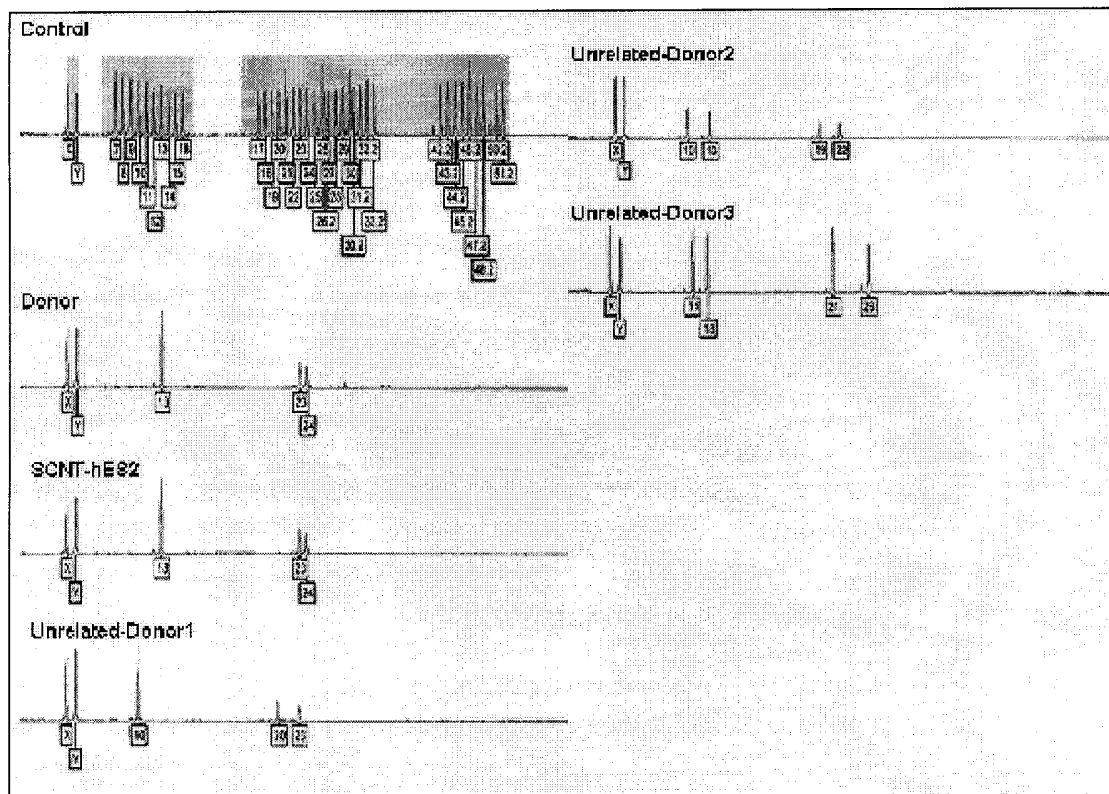
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Fig. 6



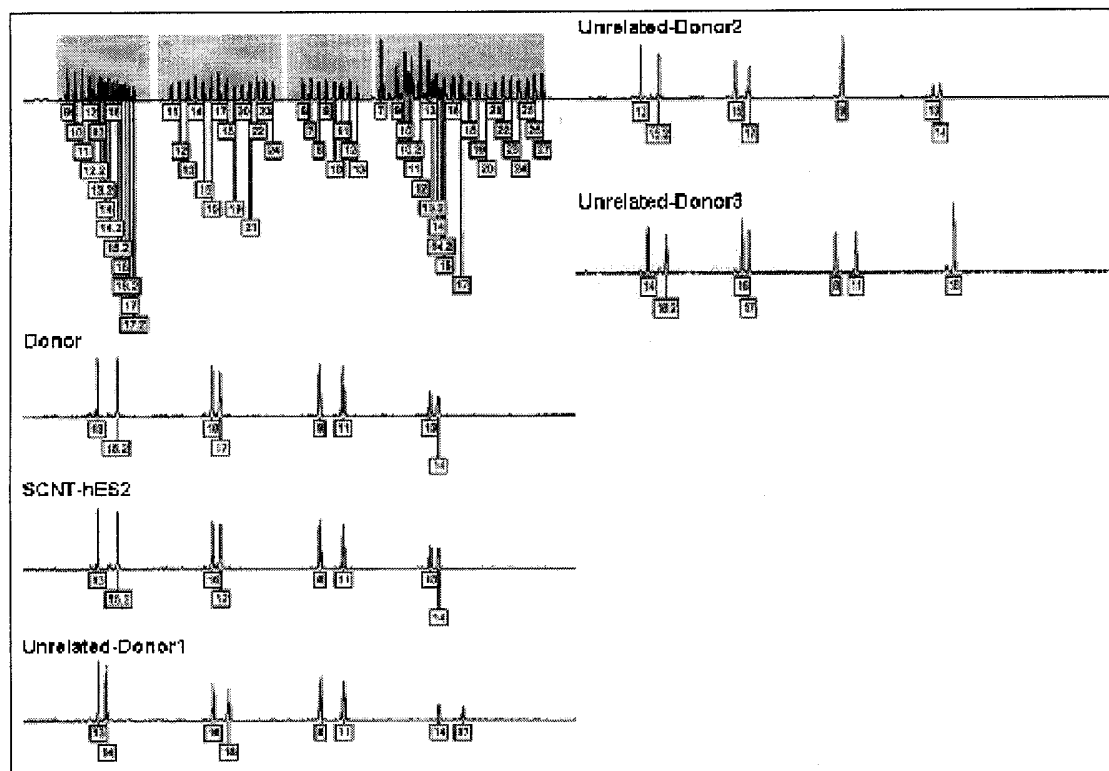
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Fig. 7



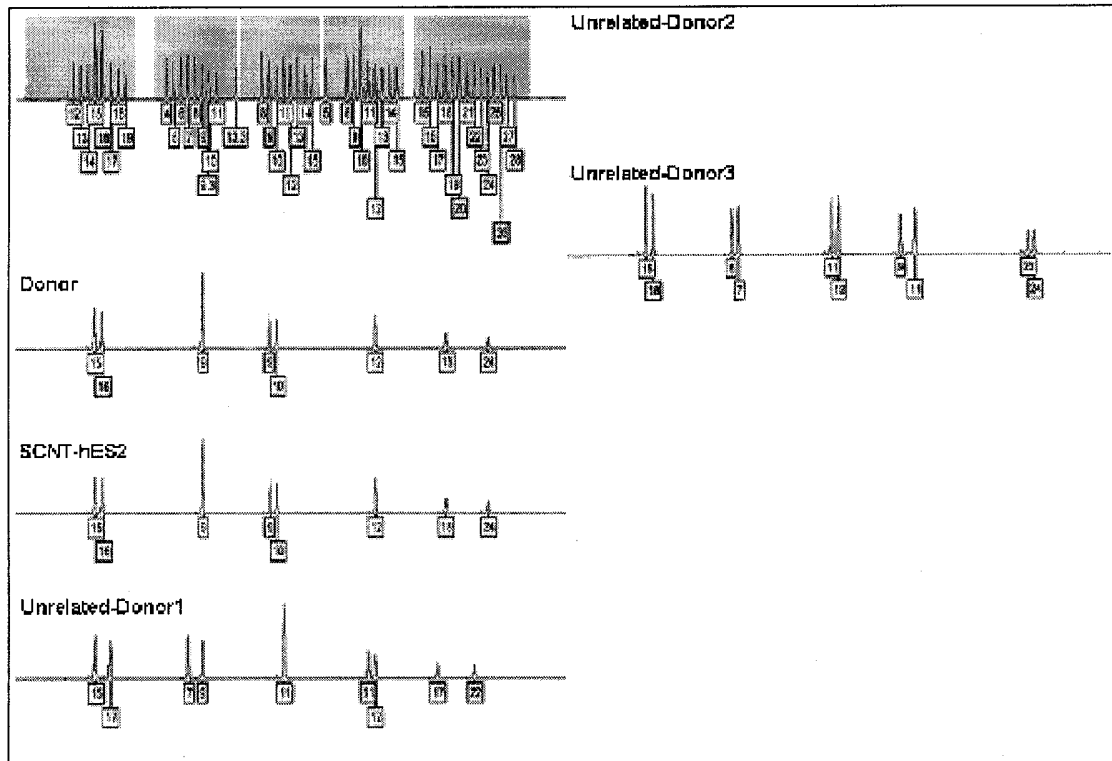
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Fig. 8



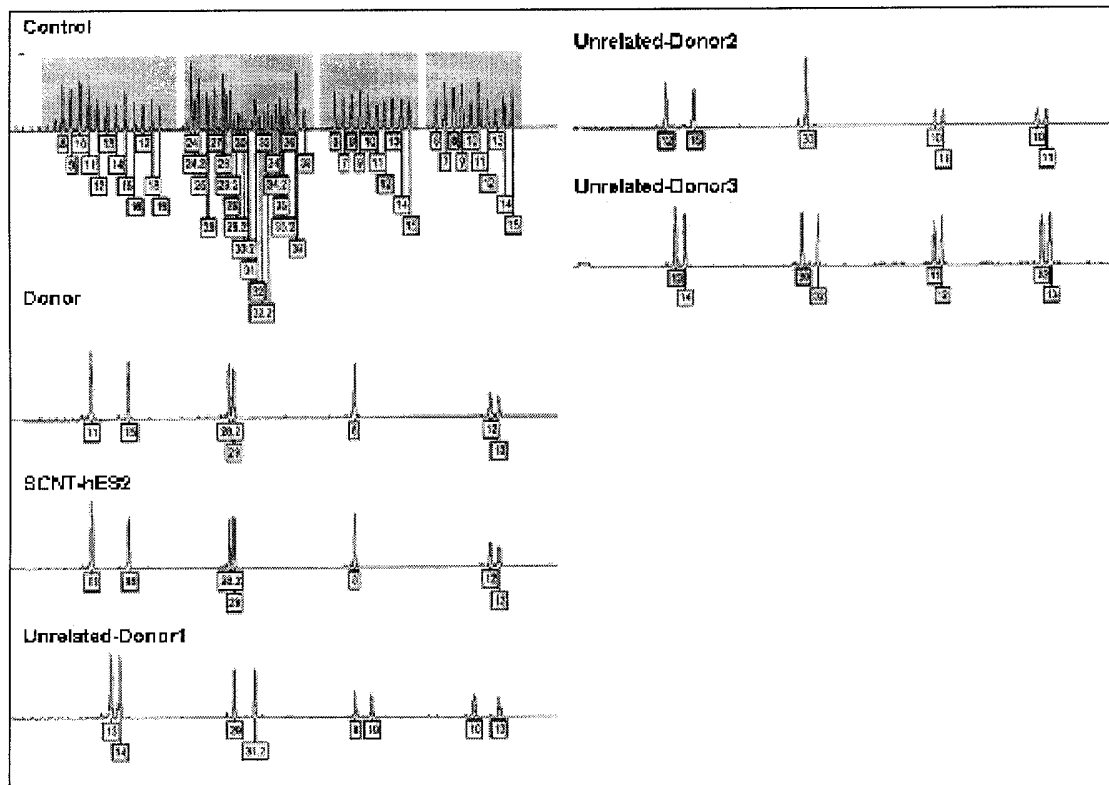
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Fig. 9



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Fig. 10



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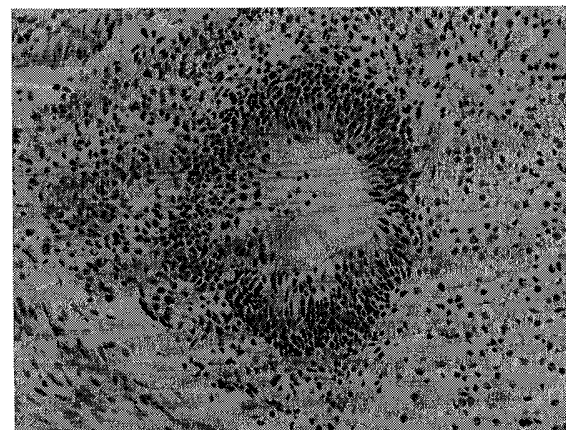
Fig. 11



(a)



(b)



(c)

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13ter.1(c) and (d) and 39)

Applicant's or agent's file reference OF06P001	IMPORTANT DECLARATION	Date of mailing (<i>day/month/year</i>) 17 MAY 2006 (17.05.2006)
International application No. PCT/KR2006/000403	International filing date (<i>day/month/year</i>) 03 FEBRUARY 2006 (03.02.2006)	(Earliest) Priority date (<i>day/month/year</i>) 04 FEBRUARY 2005 (04.02.2005)
International Patent Classification (IPC) or both national classification and IPC <i>C12N 5/08(2006.01)i</i>		
Applicant SEOUL NATIONAL UNIVERSITY INDUSTRY FOUNDATION et al		

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:
 - a. ☐ scientific theories.
 - b. ☐ mathematical theories.
 - c. ☐ plant varieties.
 - d. ☐ animal varieties.
 - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
 - f. ☐ schemes, rules or methods of doing business.
 - g. ☐ schemes, rules or methods of performing purely mental acts.
 - h. ☐ schemes, rules or methods of playing games.
 - i. ☐ methods for treatment of the human body by surgery or therapy.
 - j. ☐ methods for treatment of the animal body by surgery or therapy.
 - k. ☐ diagnostic methods practised on the human or animal body.
 - l. ☐ mere presentation of information.
 - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.
2. ☒ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

☒ the description
 ☒ the claims
 ☐ the drawings
3. ☐ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b)
4. ☐ A meaningful search could not be carried out without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-bis of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.
5. Further comments:

Name and mailing address of ISA/KR  Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LEE, CHUNG HO Telephone No. 82-42-481-8160 
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