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(54) **Title**: CULTURE METHOD OF EMBRYOID BODIES AND/OR NEURAL STEM CELLS DERIVED FROM HUMAN DIFFERENTIATED CELL-DERIVED PLURIPOTENT STEM CELLS

(57) **Abstract**: A method for differentiating a human differentiated cell-derived pluripotent stem cell into a neural stem cell is provided, which includes the steps of: making an embryoid body from the human differentiated cell-derived pluripotent stem cell; and culturing the embryoid body in a medium containing LIF to differentiate into a neural stem cell, so that, when the neural stem cell is allowed to differentiate in vitro after multiple subculturing of the neural stem cell, it differentiate mainly into neurons but substantially not into glial cells.

Description

Title of Invention: CULTURE METHOD OF EMBRYOID BODIES AND/OR NEURAL STEM CELLS DERIVED FROM HUMAN DIFFERENTIATED CELL-DERIVED PLURIPOTENT STEM CELLS

Technical Field

[0001] The present invention relates to culture method of embryoid bodies and/or neural stem cells derived from human differentiated cell-derived pluripotent stem cells.

Background Art

[0002] In recent years, it has become possible to obtain cells having pluripotency similar to embryonic stem cells (hereafter referred to as ES cells) by selecting cells expressing Fbxo15 gene from somatic cells such as fibroblasts in which Oct3/4 gene, Sox2 gene, Klf4 gene, and c-myc gene have been introduced and expressed (International Patent Application Publication No. WO2007/069666; Takahashi K, and Yamanaka S. (2006) Cell 126:663-676). It is considered that if pluripotent stem cells derived from somatic cells thus obtained are used in regenerative medicine, the cells of a patient can become transplanted to the patient himself so that rejection problems would be smaller than when ES cells are used.

[0003] While somatic cell-derived pluripotent stem cells (hereafter referred to as induced pluripotent stem cells, or iPS cells) established by using Fbxo15 gene as a marker were closely similar to embryonic stem cells in terms of cell morphology, proliferation ability, differentiation ability etc., they were different from ES cells in some characteristics such as gene expression and DNA methylation patterns. Then, the cells were selected by using the expression of the Nanog gene as a marker, and iPS cells having pluripotency more similar to ES cells were established (Okita K, Ichisaka T, and Yamanaka S. (2007) Nature 448:313-317).

[0004] Later, iPS cells were isolated using changes in cell morphology as a marker, instead of the expression of Fbxo15 gene or Nanog gene (Meissner A, Wernig M, and Jaenisch R. (2007). Nat Biotechnol 25:1177-1181). iPS cells were also established by using N-myc instead of c-myc (Blelloch R, Venere M, Yen J, Ramalho-Santos M. (2007) Cell Stem Cell 1:245-247). Further, in mice as well as in humans, iPS cells were established by introducing the three genes of Oct3/4, Sox2 and Klf4, without using c-myc gene (Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, and Yamanaka S. (2008). Nat Biotechnol 26:101-106; Wering M, Meissner A, Cassady JP, and Jaenisch R. (2008) Cell Stem Cell 2:10-12).

In addition, iPS cells were established from hepatocytes and gastric epithelial cells, besides fibroblasts (Aoi T, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, and Yamanaka S. (2008) Science (February14, 2008) (published online).).

[0005] Meanwhile, there has also been a growing body of studies using human cells. Human iPS cells were established by introducing into fibroblasts four genes of Oct3/4, Sox2, Nanog, and lin28 (Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, and Thomson JA. (2007) Science 318:1917-1920) the same combination of genes (i.e. Oct3/4 gene, Sox2 gene, Klf4 gene, and c-myc gene) as used for establishment of mouse iPS cells (Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, and Yamanaka S.(2007) Cell 131:861-872).

[0006] Since iPS cells can be produced using cells derived from a patient to be treated, artificial organs and the like which are escaped from rejection are expected to be produced by using iPS cells in the field of regenerative medicine.

Summary of Invention

Technical Problems

[0007] The object of the present invention is to develop culture conditions of embryoid bodies and/or neural stem cells derived from iPS cells, which are appropriate for neuronal differentiation of the neural stem cells.

Solution to Problem

[0008] In one embodiment of the present invention, an agent for culture of an embryoid body derived from a human differentiated cell-derived pluripotent stem cell and/or a neural stem cell derived from the embryoid body contains LIF.

[0009] In another embodiment of the present invention, a method for differentiating an embryoid body derived from a human differentiated cell-derived pluripotent stem cell into a neural stem cell includes the step of culturing the embryoid body in a medium containing LIF to differentiate into a neural stem cell. This method may further include the step of subculturing the neural stem cell in a medium containing LIF.

[0010] In another embodiment of the present invention, a method for culturing a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell includes the step of culturing the neural stem cell in a medium containing LIF.

[0011] In another embodiment of the present invention, a method of preparing a medicine for treating nerve injury, the medicine comprising a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell, includes the steps of: culturing an embryoid body derived from the human differentiated cell-derived pluripotent stem cell in a medium containing LIF to differentiate into a neural stem cell; and preparing the medicine using the neural stem cell. This method may further include the step of

subculturing the neural stem cell in a medium containing LIF.

[0012] In another embodiment of the present invention, a method of preparing a medicine for treating nerve injury, the medicine containing a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell includes the steps of: culturing a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell in a medium containing LIF; and preparing the medicine using the neural stem cell.

[0013] In any of the above embodiments, the LIF concentration is preferably from 10 to 100 ng/ml.

[0014] ==CROSS REFERENCE TO RELATED APPLICATIONS==

This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/206711, filed on February 3, 2009, which is incorporated herein by reference.

Brief Description of Drawings

[0015] [fig.1A]Fig.1A shows the micrographs indicating the morphologies of the neurospheres derived from human iPS cells in one example of the present invention.

[fig.1B]Fig.1B shows the micrographs indicating the differentiation abilities of the neurospheres derived from human iPS cells in one example of the present invention.

[fig.2]Fig.2 shows the results of FACS analyses to examine the presence of undifferentiated cells in the neurospheres derived from human iPS cells in one example of the present invention. Red lines indicate the negative controls.

[fig.3]Fig.3 shows a graph indicating the results of motor function analyses evaluated by the BBB scores of the transplanted mice.

[fig.4]Fig.4 shows the micrographs indicating the differentiation abilities of the primary, secondary and tertiary neurospheres derived from human iPS cells, which have been cultured with or without LIF, in one example of the present invention.

[fig.5]Fig.5 shows the micrographs indicating the morphologies of the primary, secondary and tertiary neurospheres derived from human iPS cells, which have been cultured with or without LIF, in one example of the present invention.

[fig.6]Fig.6 shows the micrographs indicating the subtypes of the neurons differentiated from tertiary neurospheres derived from human iPS 201B7 cells, which have been cultured with LIF, in one example of the present invention.

Description of Embodiments

[0016] == Human differentiated cell-derived pluripotent stem cells ==

A human differentiated cell-derived pluripotent stem cell refers to a human cell having pluripotency and self-reproducing ability, which is artificially induced by reprogramming a differentiated cell other than germline cells such as egg cells, sperm cells and their precursor cells such as oogonia and spermatogonia or undifferentiated

cells derived from embryos at early stages of development such as embryonic stem cells. The differentiated cell may be derived from an embryo, a fetus, or an adult. The characteristics of the differentiated cell is not particularly limited as long as the cell has at least partly lost intrinsic totipotency that a fertilized egg or an ES cell has. Examples of such a differentiated cell include fibroblasts, epithelial cells, hepatocytes, etc.

[0017] The method for reprogramming the differentiated cell is not particularly limited, but it is preferred that introduction of nuclear reprogramming factors into the cell induces the reprogramming so that it possesses pluripotency and self-reproduction ability. For example, the reprogramming method as described in Takahashi et al. (NPL 8) can be used for the reprogramming. This publication is incorporated herein by reference.

[0018] The nuclear reprogramming factor is not particularly limited, but preferred is a combination of products of the genes selected from each one member of the Oct gene family, Klf gene family, and Sox gene family. In terms of efficiency of establishment of iPS cells, more preferred is a combination further containing a gene product of one member of the myc gene family. The genes belonging to the Oct gene family include Oct3/4, Oct1A, Oct6, etc.; the genes belonging to the Klf gene family include Klf1, Klf2, Klf4, Klf5, etc.; the genes belonging to the Sox gene family include Sox1, Sox2, Sox3, Sox7, Sox15, Sox17, Sox18, etc.; and the genes belonging to the myc gene family include c-myc, N-myc, L-myc, etc. In some cases, gene products of the myc gene family may be substituted with a cytokine such as SCF, bFGF, or a chemical compound such as azacitidine and sodium valproate(VPA).

[0019] Examples of the nuclear reprogramming factors other than the above-described combination include a combination containing Nanog gene and lin-28 gene in addition to a gene from the Oct gene family and a gene from the Sox gene family. It should be noted that when introducing such factors into the cells, another type of gene product may be introduced in addition to the genes in the above-described combinations. Examples of such type of gene products include an immortalization-inducing factor such as TERT.

[0020] Since all of the above-mentioned genes are highly conserved among the vertebrates, a gene referred herein includes its homologues and orthologues unless the name of a particular animal is indicated. Moreover, mutated genes including polymorphic genes are also encompassed as long as they have a function comparable to that of the wild-type gene product.

[0021] == The method for preparing human differentiated cell-derived pluripotent stem cells ==

To prepare a human differentiated cell-derived pluripotent cell by using nuclear reprogramming factors, in the case the nuclear reprogramming factor is a protein functioning in a cell, a gene encoding the protein is preferably incorporated into an expression vector, which is introduced into a target differentiated cell such as a somatic

cell, so that the protein is intracellularly expressed (the gene transfer method). The expression vector to be used is not particularly limited, but preferred is a viral vector, particularly preferred is a retroviral vector or a lentiviral vector, and most preferred is a Sendai virus vector. The nuclear reprogramming factor may be introduced into cells by binding a peptide called Protein Transduction Domain (PTD) to the protein, which is added to a culture medium (the protein transduction method). In the case the protein is secreted extracellularly, the factor may be added to the culture medium of the differentiated cell during the preparation of the differentiated cell-derived pluripotent stem cell. If the factor is expressed in the differentiated cell to be reprogrammed, it does not need to be introduced from outside. Also, if a chemical compound capable of substituting for the function of a particular nuclear reprogramming factor is present, it may be used in place of the nuclear reprogramming factor. The chemical compound includes Tranylcypromine, CHIR99021, SB431542, PD0325901, thiazovivin but is not limited thereto.

[0022] Then, in the differentiated cell into which nuclear reprogramming factors have been introduced, a colony of cells maintaining their undifferentiated state, or a colony of cells expressing an undifferentiation marker gene such as Fbxo15 gene or Nanog gene may be selected and isolated while the cells are kept alive. Alternatively, the differentiated cell may have been co-transfected with a retroviral vector to express GFP (green fluorescent protein) or dsRed (red fluorescent protein) as a marker and then a colony of cells in which the expression of the marker is silenced may be selected.

[0023] By using any of the abovementioned markers, the cells being reprogrammed and maintaining undifferentiated state can be selected and isolated from the human differentiated cell into which the nuclear reprogramming factors have been introduced, and the established cell population may be used as the human differentiated cell-derived pluripotent cell.

[0024] == The medicine for treating nerve injury ==

The differentiated cell-derived pluripotent cell can be used to make a medicine for treating nerve injury. The method for making an agent of the medicine for treating nerve injury may be based on a method that has been developed to use embryonic stem cells as an agent for treating nerve injury, as described in Okada et al. (Okada Y, Matsumoto A, Shimazaki T, Enoki R, Koizumi A, Ishii S, Itoyama Y, Sobue G, Okano H. (2008) Stem Cells. vol. 26, pp.3086-98), which is incorporated herein by reference.

[0025] The agent for treating nerve injury may contain another component such as a buffer solution containing salt and/or antibiotics, in addition to the human differentiated cell-derived pluripotent cell. The nervous tissue as a target of the treatment is not particularly limited, being either the central nervous system such as the brain or the spinal cord or the peripheral nervous system. Further, the disease to be treated is not limited

to any specific symptom but includes a traumatic disease such as a spinal cord injury; a neurodegenerative disease such as amyotrophic lateralsclerosis, Alzheimer's disease, Parkinson's disease, progressive supranuclear palsy, Huntington disease, multiple system atrophy, and spino-cerebellar degeneration; necrosis of nerve cells resulting from cerebral infarction, intracerebral hemorrhage, etc.), and not limited to any specific cause but includes the primary cause being associated with injury, cerebral infarction, etc., and the secondary cause being associated with infection, tumor, etc., as long as it is a disease or a pathological condition in which nerve cells are damaged.

[0026] The human differentiated cell-derived pluripotent cell may be administered to a human as it is, but to enhance its ability to differentiate into neural cells, an embryoid body (EB) may be formed and then administered. The EB preferably contains neural stem cells. It is more preferred that the neural stem cells in the EB are amplified in a culture condition for growing the neural stem cells prior to the administration.

[0027] The medium for the culture in which the EB is formed from the human differentiated cell-derived pluripotent cell is not limited but may be a DMEM/F12 medium containing KSR (Knockout Serum Replacement), NEAA(non-essential amino acid), and 2-ME(2-mercaptoethanol). The concentrations of KSR, NEAA and 2-ME are not limited but are preferentially 5% or less, 0.1mM and 0.1mM, respectively. The formed EB may be cultured in a differentiation medium such as a serum-free medium supplemented with FGF-2 (10 to 100 ng/ml) to differentiate into neural stem cells in the form of neurospheres. The culture medium of the neurosphere is not limited but the same serum-free medium supplemented with FGF-2 (10 to 100 ng/ml) can be used to culture the neurospheres. The primary neurospheres containing the primary neural stem cell can be subcultured by dispersing and replating them onto a culture dish so that they proliferate to form secondary neurospheres containing the secondary neural stem cell; and this subculture process can be repeated to make higher-order neurospheres containing higher-order neural stem cells. The neural stem cells are thus formed can be administered to a human, preferably after dispersion of the neurospheres. The neural stem cells to be administered may or may not possess an ability to differentiate into glial cells in vitro. LIF can be added to the medium for either or both of the EB and the neural stem cell and its appropriate concentration can be determined by the artisan but 1 ng/ml or more is preferred, 5 ng/ml or more is more preferred and 10 ng/ml or more is most preferred; 1000 ng/ml or less is preferred, 500 ng/ml or less is more preferred and 100 ng/ml or more is most preferred; and 1-1000 ng/ml is preferred, 5-500 ng/ml is more preferred and 10-100 ng/ml is most preferred.

[0028] When the EB or the neural stem cells is cultured in the medium without LIF, the neural stem cell has obtained a differentiation potential into neuronal cells and glial cells. However, when the EB and the neural stem cells are cultured in the medium with

LIF, the neural stem cells have a differentiation potential mainly into neuronal cells but substantially not into glial cells. In the culture condition with LIF, even after sub-culturing the neural stem cell many times, the neural stem cells still keep the differentiation potential that they can differentiate mainly into neuronal cells but substantially not into glial cells in vitro. It is known that the neural stem cells experience the expansion phase, the neurogenic phase and the gliogenic phase in this order during their development in the CNS (Temple, S., Nature vol.414. p.112-117, 2001). Therefore, the neural stem cells can maintain their differentiation potential of their young stages in vitro by being cultured under the existence of LIF. In addition, the neuronal cells derived from the neural stem cells thus obtained contain early-born neurons such as TH-positive or Isl-positive neurons, which are not generally contained in those derived from the neural stem cells cultured without LIF or those obtained from fetus after the mid-gestation (Nature neurosci. vol.11, p.1014-1023, 2008). This is consistent with the fact that they can keep their potential of their young stage under the existence of LIF. Furthermore, the neural stem cells cultured with LIF form bigger neurospheres in average than those cultured without LIF, probably because the former grows better than the latter.

[0029] The method for in vitro differentiation of the neural stem cells is not particularly limited, and the neurospheres may be cultured in any known differentiation-inducing medium, whose preferred example is a DMEM:F-12 medium supplemented with glucose, glutamine, insulin, transferrin, progesterone, putrescine and selenium chloride (i.e. the medium for proliferating neural stem cells without FGF and heparin). Sonic hedgehog protein may be either present or absent therein. The cells are preferably incubated under the conditions of 5% CO_2 at 35 to 40°C for 5 to 7 days.

[0030] The differentiated cell-derived pluripotent cell, the EB cell or the neural stem cell may be administered either directly or indirectly. For a direct administration, cells may be transplanted to the site of nerve injury, for example. For an indirect administration, cells may be injected intravenously or intraspinally and delivered to the affected site through the circulation of blood or cerebrospinal fluid.

Examples

[0031] == Cells ==

In this example, the differentiated cell-derived pluripotent cells were either the cells (253G4, 253G1) obtained by introducing the combination of Oct3/4, Sox2 and Klf4 as nuclear reprogramming factors to human embryonic fibroblasts, or the cells (201B7, 201B6) obtained by introducing the combination of Oct3/4, Sox2, c-Myc and Klf4 as nuclear reprogramming factors to human embryonic fibroblasts (Yu J et al. (2007). Science 318:1917-1920; Nakagawa M et al., (2008). Nat Biotechnol vol.26,

p.101-106.), all of which were provided by Kyoto University. As for the control, human ES cells (KhES1) (Suemori H et al., (2006), Biochem. and Biophys. Res. Commun. vol.345, p.926-932.) were used.

[0032] <Experiment 1> Production of neural stem cells

To enhance the ability of these cells to differentiate into neural cells, embryoid bodies (EBs) were made by culturing the differentiated cell-derived pluripotent cells in suspension with an embryoid-culturing medium supplemented with 5% KSR in a bacterial-culture dish for 30 days. Then, the EBs formed were dispersed and cultured in a serum-free medium supplemented with FGF-2 (20 ng/ml) and LIF (10 ng/ml). In 12 days, the cells derived from the EBs formed neurospheres, which are called as primary neurospheres or iPS-PNSs. It was possible to dissociate these iPS-PNSs and make the neurospheres again under the same conditions repeatedly. In this specification, the neurospheres subcultured at least one time are collectively called as higher-order neurospheres; specifically, the neurosphere subcultured (N-1) times is called as N-th neurosphere.

[0033] As shown in Fig.1 indicating the morphological images of the neurospheres observed under an optical microscope, the neurospheres were formed as the primary neurospheres, the tertiary neurospheres after being subcultured twice, and the 6th neurospheres after being subcultured five times.

[0034] The primary to tertiary neurospheres thus obtained were dispersed by treatment with TrypLE Select (or a trypsin solution) and pipetting, seeded in culture dishes double-coated with Poly-L-ornitin and fibronectin and filled with the differentiation-inducing medium, and allowed to differentiate by culturing for 7 to 12 days. As for the differentiation-inducing medium, DMEM:F-12 medium supplemented with glucose, glutamine, insulin, transferrin, progesterone, putrescine and selenium chloride to which B27 supplement was added at 2% (i.e. the medium for proliferating neural stem cells without FGF and heparin) was used, and the cells were incubated under the condition of 5% CO_2 at 35 to 40°C for 10 days. Specimens were then immunostained with an antibody against beta III-tubulin (mouse IgG, SIGMA T8660, 1000-fold dilution), a marker for neurons (indicated by green fluorescence), and an antibody against GFAP (rabbit IgG, DAKO ZO334, 4000-fold dilution), a marker for astrocytes (indicated by red fluorescence), and the cellular morphologies and staining were observed under a fluorescent microscope. Hoechst 33258 was used to counterstain cell nuclei (indicated by blue fluorescence).

[0035] As shown in Fig.1B, in the primary to tertiary neurospheres, substantially only neurons differentiated, and glia did not. Such characteristics of human iPS cells are significantly different from those of mouse iPS cells. In the case of mouse cells, when the primary neurospheres are cultured under the same differentiating conditions, only

neurons are differentiated like human cells. However, when the higher-order neurospheres from a mouse which had been subcultured at least once are set under the same differentiating conditions, not only neurons but also glial cells are differentiated.

[0036] <Experiment 2> Presence of undifferentiated cells in neurospheres

It is shown below that no undifferentiated cell was found in the tertiary neurospheres (hereafter called as iPS-TNS) which are derived from human iPS cells.

[0037] The tertiary neurospheres were dispersed by treatment with TrypLE Select (or a trypsin solution) and pipetting, and antibodies against cell surface antigens (TRA-1-60, TRA-1-81, CD56 or CD133) expressed in undifferentiated cells were applied for FACS analysis. The antibodies TRA-1-60-PE, TRA-1-81-PE and CD56-Alexa488 purchased from BD Inc. were used at 5 ul for $1x10^6$ cells in 50 ul, and the antibody CD133-APC purchased from Milteny Biotech inc. was used at 2 ul for $1x10^6$ cells in 50 ul. In a result as shown in Fig.2, neither of expressions of TRA-1-60 and TRA-1-81 was observed in the tertiary neurospheres derived from the human iPS cells like the human ES cells. Furthermore, almost all cells expressed CD56, a marker for the neural stem cells.

[0038] As described so far, the higher-order neurospheres prepared from human iPS cells have no undifferentiated cells at al, or only in quite small number even if a contamination exists, and therefore are useful for cellular transplantation due to the lowered risk of oncogenesis.

[0039] <Experiment 3> Preparation of spinal cord-injured mice, cellular transplantation thereto, and analysis of the transplanted mice

In this example, model mice of spinal cord injury were made by inducing traumatic spinal cord injury of the spinal nerve at the 10th thoracic vertebral level, and used for transplantation of tertiary neurospheres derived from human iPS cells to demonstrate an enhanced recovery, as described below.

[0040] First, 8- to 9-week-old NOD/SCID female mice (weighing 20 to 22 g) were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After laminectomy of the 10th thoracic vertebra, the dorsal surface of the dura mater was exposed, and traumatic spinal cord injury was produced using Infinite Horizon Impactor (60 kdyn; Precision Systems, Kentucky, IL).

[0041] To transplant cells to the injured spinal cord, the injury site was exposed again at 9 days after the injury. The cells of 5×10^5 cells/2ul were introduced into the center of the lesioned area at a rate of 0.5 ul/min using a glass micropipette mounted on a stereotaxic injector (KDS310, Muromachi-kikai, Tokyo, Japan). In this example, clones of 253G1 and 201B7 for the iPS-TNSs and a clone of KhES1 for the ES-SNS were used, and their respective neurospheres were partially dissociated prior to their transplantations. As a control, PBS (vehicle) was injected in the same method as the

cellular transplantation.

[0042] The motor function of hindlimbs was evaluated by the Basso-Beattie-Bresnahan (BBB) score (NPL 12) at every seven days till Day 42. The results are shown in Fig.3.

[0043] In all the four groups, mice were completely paralyzed immediately after the induction of the spinal cord injury, but they all gradually recovered. However, after 3 weeks from the operation, the same degree of recoveries were observed in both the iPS-TNS and the ES-SNS-transplanted groups by comparing the BBB scores, with a significant difference from the group which received only the medium without cells. Also in clinical observations, the iPS-TNS-transplanted mice exhibited marked recovery sufficient for weight-supporting plantar stepping.

[0044] In conclusion, a nerve injury of a spinal cord-injured mouse can be treated by transplanting neurospheres derived from human iPS cells even in the state not to differentiate into glial cells in vitro.

[0045] <Experiment 4> Comparison of the differentiation and proliferation properties between the neurospheres differentiated from the EBs with and without LIF

[0046] Primary, secondary and tertiary neurospheres were formed, allowed to differentiate into neural cells and their cell-types were analyzed according to methods described in Experiment 1.

[0047] As a result shown in Fig. 4, in all of the three clones, neurons indicated by green fluorescence were differentiated with and without LIF, while astrocytes indicated by red fluorescence were differentiated without LIF but not with LIF. Thus, by culturing the EBs and neurospheres in the medium with LIF, the subcultured neurospheres maintain the differentiation property that they can differentiate substantially only into neurons but not into glial cells.

[0048] It should be noted that the neurospheres grew more rapidly in the medium with LIF than in the medium without LIF. An example using 201B7 is shown in Fig.5. It is clear that neurospheres cultured with LIF are generally bigger than those cultured without LIF.

<Experiment 5> Subtypes of neurons that differentiate from the higher-order neurospheres cultured in LIF-containing medium

[0049] Tertiary neurospheres of an human iPS clone 201B7 and an human ES clone KhES1 (control) were formed, allowed to differentiate into neural cells and subtypes were analyzed for the differentiated neurons using marker antibodies according to methods described in Experiment 1. The antibodies used in this experiment are: anti-Islet-1 (39.4D5, mouse IgG2b, 1:200, Developmental Studies of Hybridoma Bank: DSHB), anti- beta III-tubulin (SIGMA T8660 mouse IgG2b, 1:1000), anti-CNPase (SIGMA C5922, mouse IgG1, 1:1000), anti-GFAP (rabbit IgG, DAKO ZO334, rabbit IgG, 1:4000), anti-TH-1 (Chemicon AB152, rabbit IgG, 1:100). CNPase and GFAP are glial

markers for oligodendrocytes and astrocytes, respectively. Islet-1 and TH-1 are markers for early-born neurons.

[0050] As shown in Fig. 6, almost all of the differentiated cells are beta III-tubulin-positive neuronal cells, and CNPase- or GFAP-positive glial cells did not differentiate from the tertiary neurospheres. As for the subtypes of the neuronal cells, Islet-1- or TH-1-positive neurons differentiated, indicating that the differentiated neurons are early-born-type neurons.

Industrial Applicability

[0051] Culture conditions of embryoid bodies and/or neural stem cells derived from human differentiated cell-derived pluripotent stem cells, which are appropriate for neuronal differentiation of the neural stem cells were developed by the present invention.

Claims

[Claim 1] An agent for culture of an embryoid body derived from a human differentiated cell-derived pluripotent stem cell and/or a neural stem cell derived from the embryoid body, comprising LIF.

[Claim 2] The agent of claim 1, wherein the LIF concentration is from 10 to 100 ng/ml.

[Claim 3] A method for differentiating an embryoid body derived from a human differentiated cell-derived pluripotent stem cell into a neural stem cell, comprising the step of culturing the embryoid body in a medium containing LIF to differentiate into a neural stem cell.

[Claim 4] The method of claim 3, further comprising the step of subculturing the neural stem cell in a medium containing LIF.

[Claim 5] A method for culturing a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell, comprising the step of culturing the neural stem cell in a medium containing LIF.

[Claim 6] The method of any one of claims 3-5, wherein the LIF concentration is from 10 to 100 ng/ml.

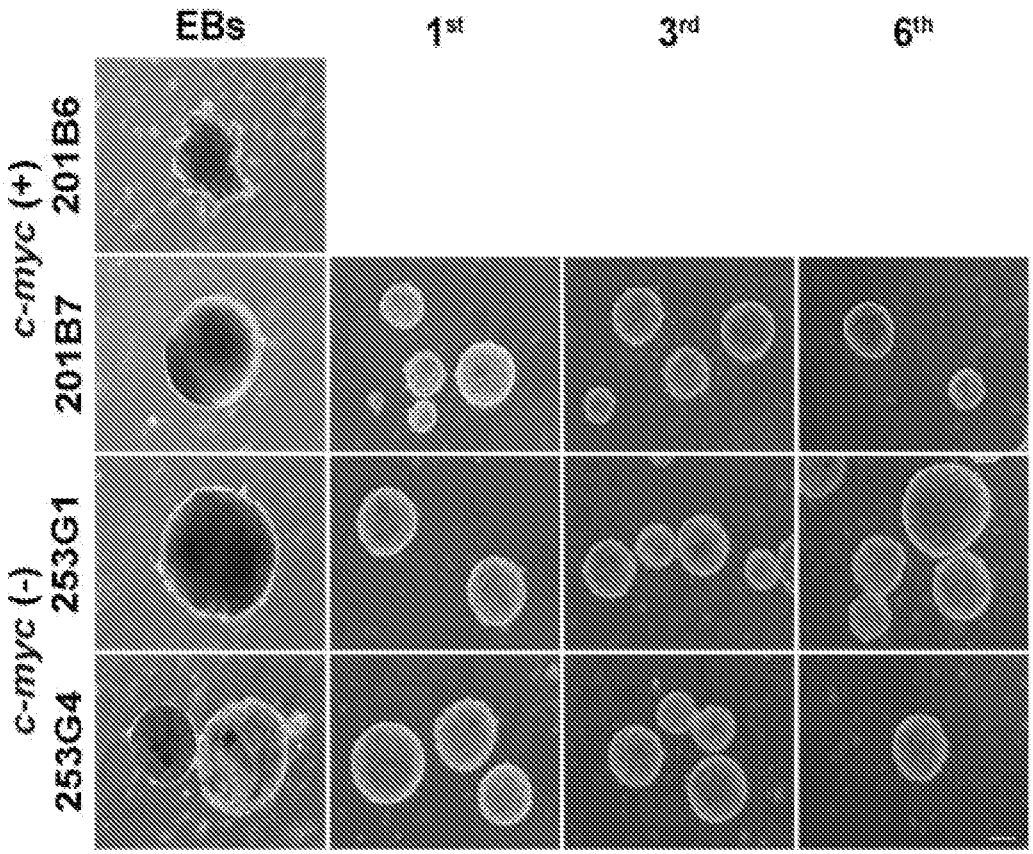
[Claim 7] A method of preparing a medicine for treating nerve injury, the medicine comprising a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell, comprising the steps of:
culturing an embryoid body derived from the human differentiated cell-derived pluripotent stem cell in a medium containing LIF to differentiate into a neural stem cell; and
preparing the medicine using the neural stem cell.

[Claim 8] The method of claim 7, further comprising the step of subculturing the neural stem cell in a medium containing LIF.

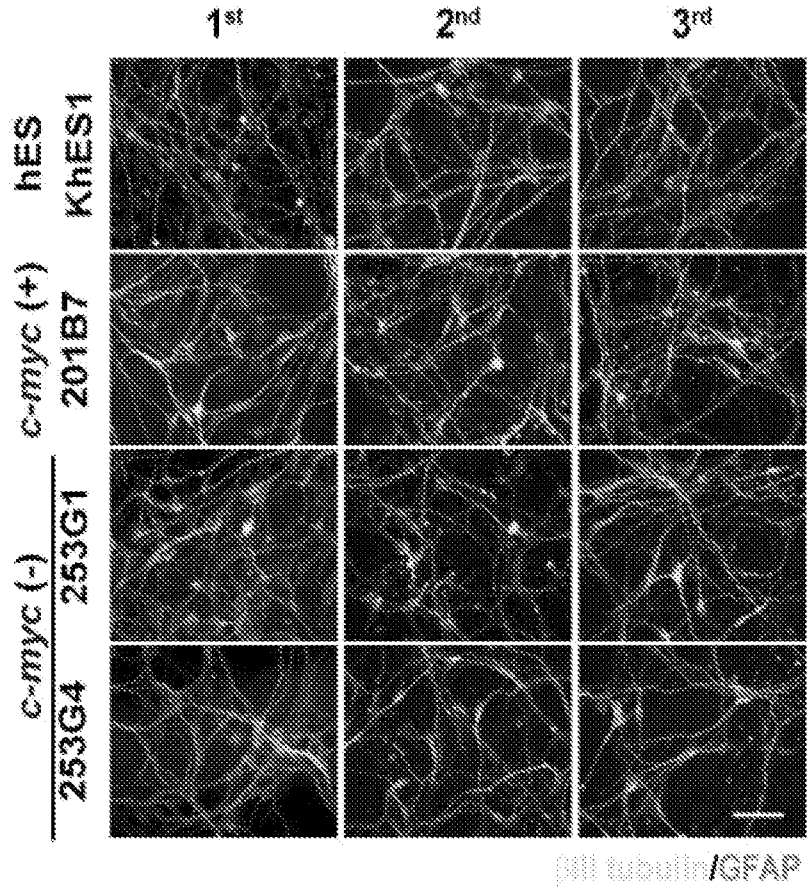
[Claim 9] A method of preparing a medicine for treating nerve injury, the medicine comprising a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell, comprising the steps of:
culturing a neural stem cell derived from a human differentiated cell-derived pluripotent stem cell in a medium containing LIF; and
preparing the medicine using the neural stem cell.

[Claim 10] The method of any one of claims 7-9, wherein the LIF concentration is from 10 to 100 ng/ml.

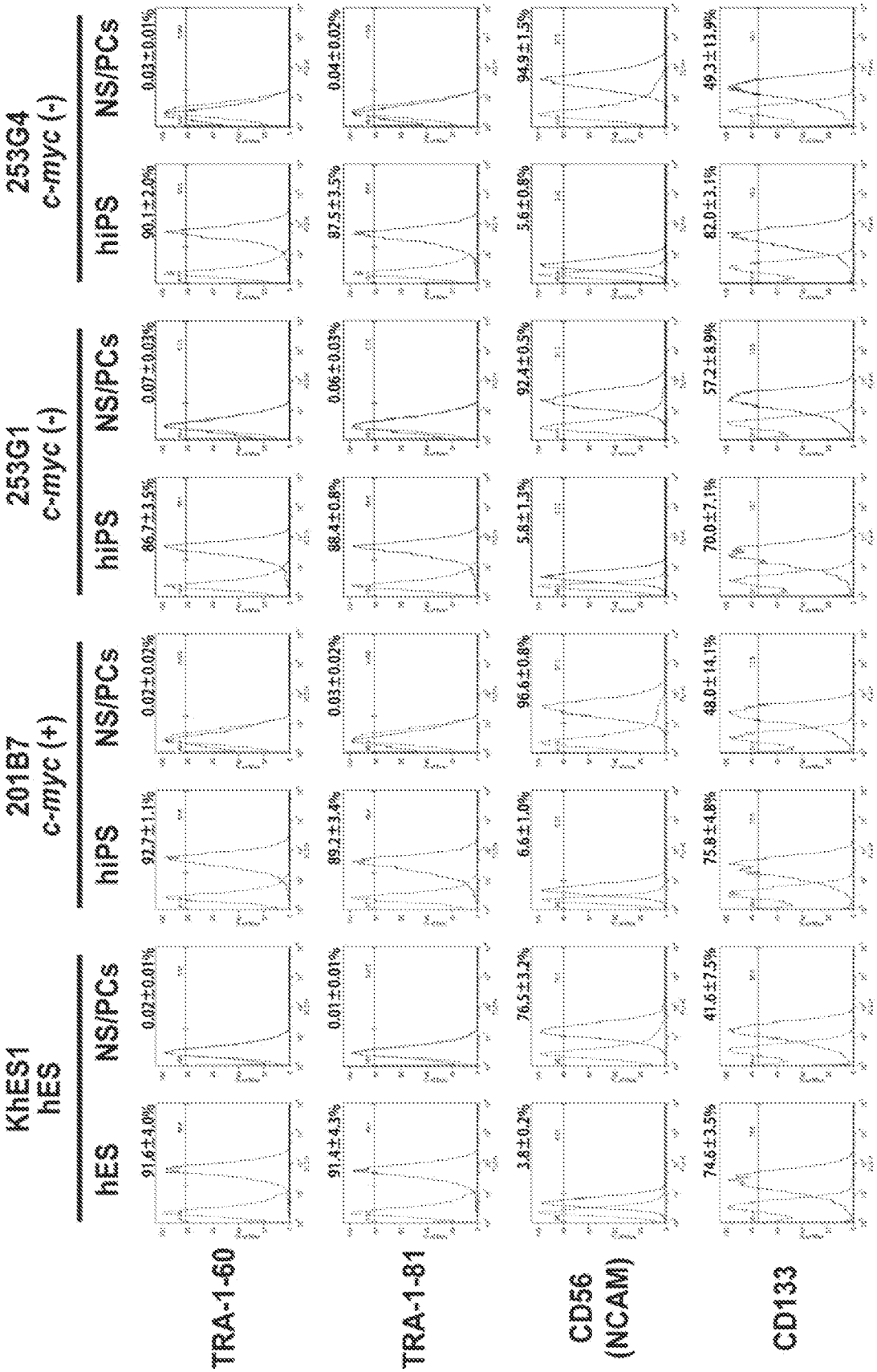
[Fig. 1A]



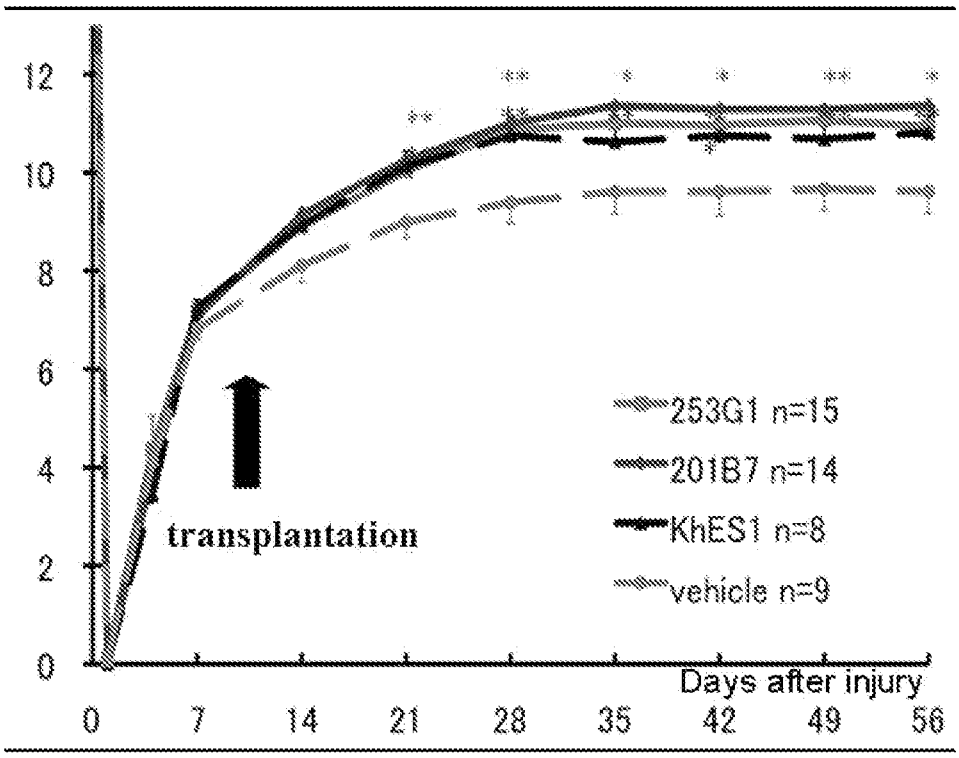
[Fig. 1B]


1st
2nd
3rd
hES
KhES1
c-myc (+)
201B7
c-myc (-)
253G1
253G4
βIII tubulin/GFAP

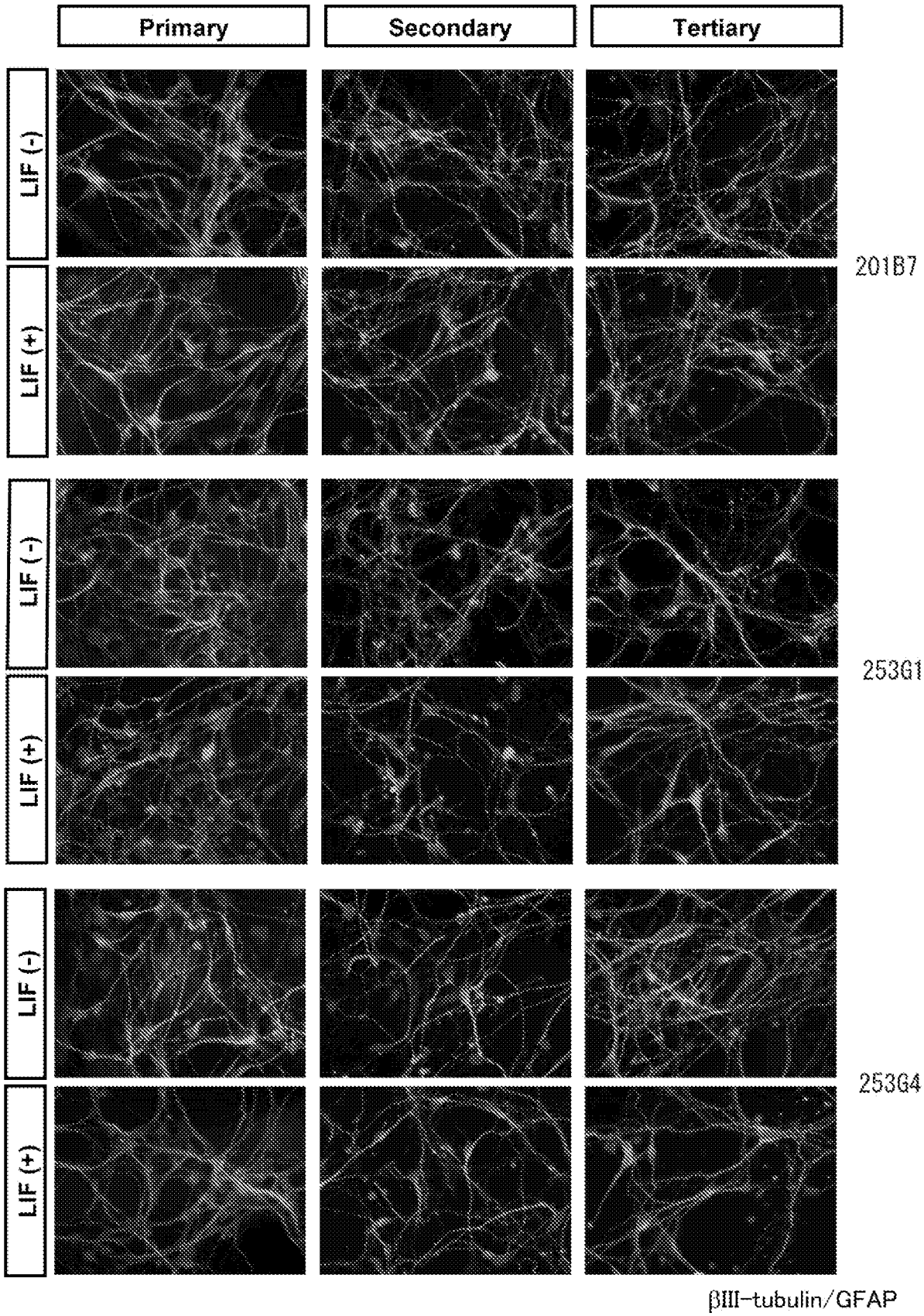
[Fig. 2]


KhES1
hES
201B7
c-myc (+)
253G1
c-myc (-)
253G4
c-myc (-)
hES
NS/PCs
hiPS
NS/PCs
hiPS
NS/PCs
hiPS
NS/PCs
TRA-1-60
91.6±4.0%
0.02±0.01%
92.7±1.1%
0.02±0.02%
86.7±3.5%
0.07±0.03%
90.1±2.0%
0.03±0.01%
TRA-1-81
91.4±4.3%
0.01±0.01%
89.2±3.4%
0.03±0.02%
88.4±0.8%
0.06±0.03%
97.5±3.5%
0.04±0.02%
CD56
(NCAM)
3.6±0.2%
76.5±3.2%
6.6±1.0%
96.6±0.8%
5.8±1.3%
92.4±0.3%
5.6±0.8%
94.9±1.5%
CD133
74.6±3.5%
41.6±7.5%
75.8±4.8%
48.0±14.1%
70.0±7.1%
57.2±8.9%
82.0±3.1%
49.3±13.9%

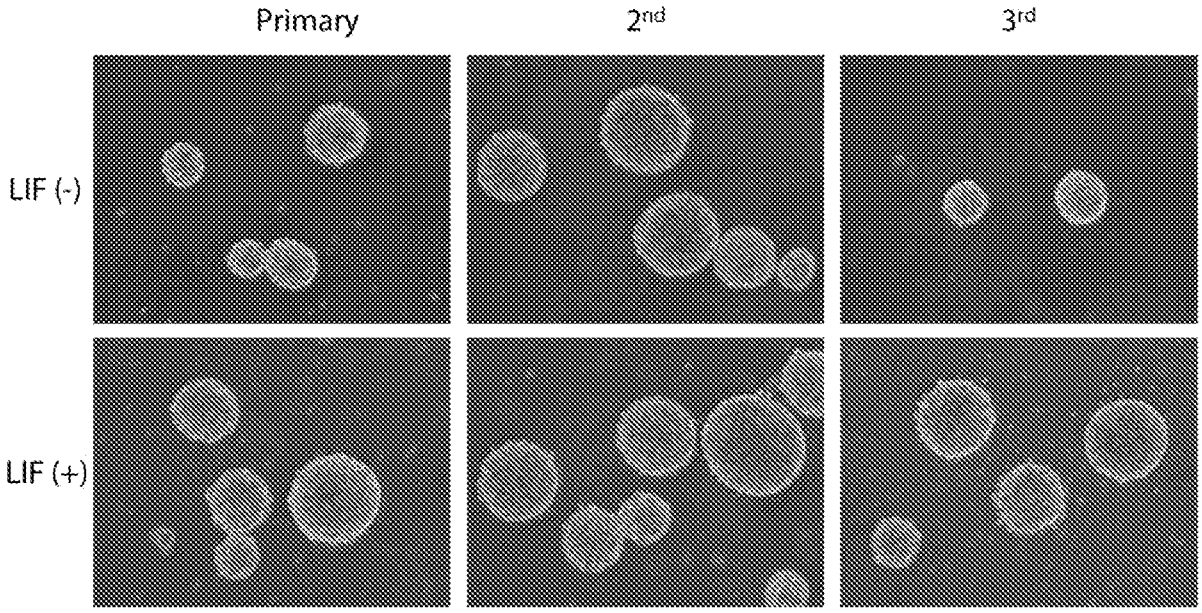
[Fig. 3]


12
10
8
6
4
2
0
0
7
14
21
28
35
42
49
56
Days after injury
transplantation
253G1 n=15
201B7 n=14
KhES1 n=8
vehicle n=9

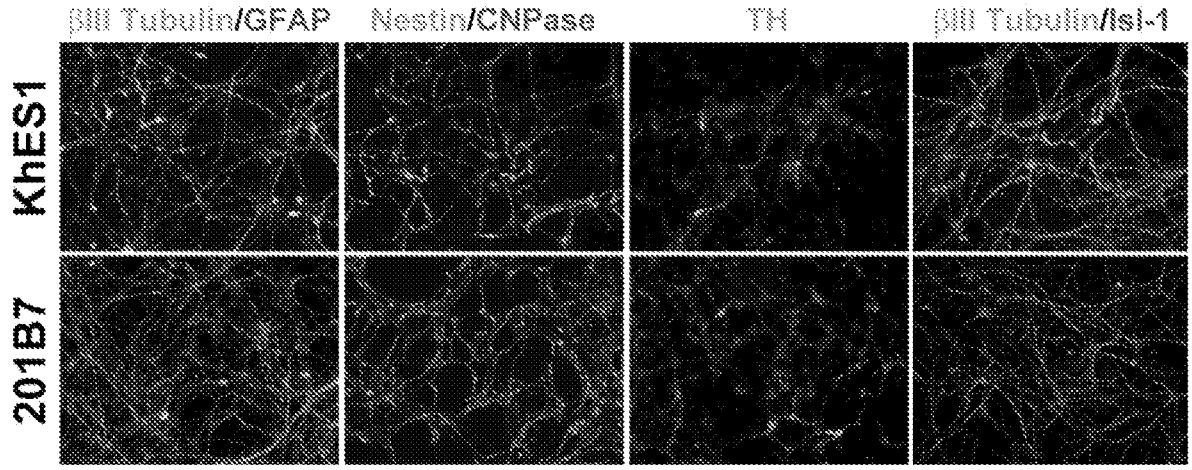
[Fig. 4]


Primary
Secondary
Tertiary
LIF (-)
LIF (+)
201B7
LIF (-)
LIF (+)
253G1
LIF (-)
LIF (+)
253G4
βIII-tubulin/GFAP

[Fig. 5]


Primary
2nd
3rd
LIF (-)
LIF (+)

[Fig. 6]


βIII Tubulin/GFAP
Nestin/CNPase
TH
βIII Tubulin/Isl-1
KhES1
201B7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/000640

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N5/0735(2010.01)i, C12N5/0797(2010.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N5/0735, C12N5/0797

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2010
Registered utility model specifications of Japan 1996-2010
Published registered utility model applications of Japan 1994-2010

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA/BIOSIS/MEDLINE/WPIDS(STN), JSTPlus/JMEDPlus/JST7580(JDreamII), PubMed, CiNii

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y	JP 2002-518990 A (CYTOTHERAPEUTICS,INC) 2002.07.02 especially claim7, [0015] - [0017] & US 5968829 A & US 6103530 A & US 2002/0164309 A1 & WO 1999/011758 A2 & AU 9305998 A & CA 2302484 A & AU 758270 B	1,2/5,6,9,10
X/Y	JP 2006-55069 A (NAT INST OF ADV IND & TECHNOL) 2006.03.02 especially [0034] , [0050] No family	1,2/5,6,9,10
Y	OKANO H ,CNS regeneration using embryonic stem cells and neural stem cells.,Research on diagnosis and therapy of amyotrophic lateral sclerosis.,2008,p.17-20	5-10

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19.04.2010	27.04.2010

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4N 9735

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/000640

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NAKAGAWA M et al, Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts., Nat Biotechnol., 2008,Vol. 26,No. 1,p.101-106	1-10
Y	OKADA Y et al, Spatiotemporal recapitulation of central nervous system development by murine embryonic stem cell-derived neural stem/ progenitor cells., Stem Cells., 2008,Vol. 26,No. 12,p.3086-3098 especially Figure5	1-4,6-8,10
Y	JP 2003-189847 A (KAINOSU KK) 2003.07.08 especially [0030] No family	1-4,6-8,10
Y	ZHAO HE et al,Effect of leukemia inhibitory factor on embryonic stem cell differentiation: implications for supporting neuronal differentiation., Acta Pharmacologica Sinica, 2006, Vol.27,No.1,p.80-90 especially p.83	1-4,6-8,10
Y	US 2003/0036195 A1 (LORENZ STUDER) 2003.02.20 No family especially [0087]	1-4,6-8,10
P,A	OKANO H ,Strategies toward CNS-regeneration using induced pluripotent stem cells.,Genome Inform.,2009 Oct,Vol. 23,No. 1,p.217-220	1-10
P,A	MIURA K et al, Variation in the safety of induced pluripotent stem cell lines., Nat Biotechnol., 2009 Aug,Vol. 27,No. 8,p.743-745	1-10
A	WO 2004/074465 A1 (BIOS RESEARCH INSTITUTE INC) 2004.09.02& US 2007/0054398 A1 & EP 1600502 A1	1-10
A	US 2002/0164791 A1 (DEREK VAN DER KOOY) 2002.11.07 & WO 2002/026941 A2 & AU 9358601 A	1-10