

**USE OF A COMPOSITION CONTAINING HUMAN UMBILICAL
CORD BLOOD-DERIVED MESENCHYMAL STEM CELL FOR
INDUCING DIFFERENTIATION AND PROLIFERATION OF NEURAL
PRECURSOR CELLS OR NEURAL STEM CELLS TO NEURAL
CELLS**

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FIELD OF THE INVENTION

The present invention relates to a use of a composition comprising
10 human umbilical cord blood-derived mesenchymal stem cell for inducing
differentiation and proliferation of neural precursor cells or neural stem cells to
neural cells.

BACKGROUND OF THE INVENTION

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Stroke, Parkinson's disease, Alzheimer's disease, Pick's disease,
Huntington's disease, amyotrophic lateral sclerosis, traumatic central nervous
system disease and spinal cord injury disease involve dysneuria caused by
injury of nerve cells, and they have been generally treated by medication or
20 surgical operation which may severely damage normal cells.

Recently a cell replacement therapy in which normal cells are
transplanted to replace destroyed or damaged cells has been recognized to be
effective for such diseases, and stem cells, in particular, which can be
differentiated and proliferated into desired tissues are under intense studies.

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Stem cells are unspecialized cells that can be proliferated unlimitedly in
the undifferentiated stage and can be differentiated into diverse tissues in
response to specific stimuli.

Neural stem cells, from which neurons and/or glia such as astrocytes,
oligodendrocytes and/or Schwann cells form, are also undifferentiated cells

having self-reproduction potency. They differentiate into neural cells, for example neurons or glia via neural precursor cells or glia precursor cells.

Mesenchymal stem cells, which differentiate into bone, cartilage, adipose tissue, muscle, tendon, ligament, neural tissue and others, have been
5 known to be viable for the cell replacement therapy. Mesenchymal stem cells have been obtained mainly from bone marrow, but such mesenchymal stem cells provide only limited applications due to their restrictive potency for differentiation and proliferation. Further, complicated and often painful operations composed of several steps must be conducted for such cell
10 replacement therapy, besides the problem of finding a donor who has histocompatibility antigens identical with that of a patient to exclude graft versus host reaction during bone marrow transplantation.

In recent years, the umbilical cord blood has become a target for researchers because of its high concentrations of stem cells. A number of trials
15 to treat blood diseases by transplanting umbilical cord blood to a patient have been conducted, and umbilical cord blood banks, which preserve umbilical cord blood in a frozen form until use, have been established for the autologous transplantation therapy.

Unlike the bone marrow, the umbilical cord blood can be obtained by a
20 simple operation from an umbilical cord and it causes little graft versus host reaction. For these reasons, worldwide studies for clinical application of the umbilical cord blood have recently been performed.

The present inventors have also extensively studied umbilical cord blood-derived mesenchymal stem cells and found that they are capable of
25 inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells.

SUMMARY OF THE INVENTION

Therefore, it is an object of the present invention to provide a use of a composition comprising umbilical cord blood-derived mesenchymal stem cells for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells.

5 In accordance with another aspect of the present invention, there is provided a method for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells, which comprises co-culturing umbilical cord blood-derived mesenchymal stem cells with the neural precursor cells or the neural stem cells.

10 In accordance with a further aspect of the present invention, there is provided a composition for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells, comprising umbilical cord blood-derived mesenchymal stem cells as an active ingredient.

15 In accordance with a still further aspect of the present invention, there is provided a method for treating a nerve injury disease which comprises administering the composition to a nerve cell injury region of a subject in need of treating the nerve injury disease.

BRIEF DESCRIPTION OF THE DRAWINGS

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The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

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Fig. 1: a diagram of a transwell chamber used for co-culturing umbilical cord blood-derived mesenchymal stem cells and neural precursor cells;

Fig. 2: photographs showing the differentiation and proliferation of NG108-15 (NG108) observed with a phase-contrast microscope ($\times 100$), 4 and 7 days after culturing NG108-15 alone or co-culturing therewith umbilical cord

blood-derived mesenchymal stem cells, respectively;

Fig. 3: photographs showing the immunostaining result for tubulin beta-
III, an early marker for neuronal differentiation, 7 days after culturing NG108-
15 alone or co-culturing therewith umbilical cord blood-derived mesenchymal
5 stem cells, respectively;

Fig. 4: photographs showing the differentiation and proliferation of
NG108-15 observed with a phase-contrast microscope ($\times 100$), 7 days after
culturing the NG108-15 cells alone, supplemented with cAMP or co-culturing
with umbilical cord blood-derived mesenchymal stem cells obtained from two
10 different individuals (hUCB-MSC-1 and hUCB-MSC-2), respectively;

Fig. 5: photographs showing the differentiation and proliferation of
neural stem cells derived from the brain cortex of a fetal mouse observed with a
phase-contrast microscope ($\times 100$), 7 days after co-culturing the neural stem
cells with hUCB-MSC-1 and hUCB-MSC-2 of various concentrations,
15 respectively;

Fig. 6: photographs showing the immunostaining result for tubulin beta-
III and microtubule-associated protein 2 (MAP2), early markers of neuronal
differentiation, 7 days after culturing neural stem cells derived from the brain
cortex of the fetal mouse alone or co-culturing therewith umbilical cord blood-
20 derived mesenchymal stem cells, respectively;

Fig. 7: a graph showing the number of viable cells assessed by the
trypan blue staining, 7 days after co-culturing the NG108-15 with umbilical
cord blood-derived mesenchymal stem cells of various concentrations; and

Fig. 8: a graph showing the number of viable cells assessed by the
25 trypan blue staining, 7 days after co-culturing the neural stem cell, derived from
the brain cortex of the fetal mouse, with umbilical cord blood-derived
mesenchymal stem cells of various concentrations.

DETAILED DESCRIPTION OF THE INVENTION

The inventive composition for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells characteristically
5 comprises umbilical cord blood-derived mesenchymal stem cells as an active ingredient.

As used herein, the term "umbilical cord blood" refers to the blood taken from the umbilical cord vein which links the placenta of a mammal with a newborn baby thereof.

10 The term "umbilical cord blood-derived mesenchymal stem cells" as used herein refers to mesenchymal stem cells which are isolated from the umbilical cord blood of a mammal, preferably human.

The term "a nerve injury disease" as used herein refers to a disease that accompanies, among others, behavior dysfunction due to damaged motor or
15 sensory nerves. Exemplary nerve injury diseases include Stroke, Parkinson's disease, Alzheimer's disease, Pick's disease, Huntington's disease, amyotrophic lateral sclerosis, traumatic central nervous system disease and spinal cord injury disease.

The term "treating" refers to: preventing the manifestation of a not-yet-
20 diagnosed disease or disorder in an animal, preferably a mammal, most preferably human, which is prone to acquire such disease or disorder; or inhibiting the development of a nerve injury disease.

The term "a neural cell" as used herein refers to a neuron of central or peripheral nervous system, and/or glia such as an astrocyte, an oligodendrocyte
25 and/or a Schwann cell.

In isolating a monocyte comprising mesenchymal stem cells from the umbilical cord blood, a common method such as the Ficoll-Hypaque density gradient method can be employed. Specifically, said method comprises the steps of gathering umbilical cord blood from the umbilical vein after parturition

till detachment of placenta; centrifuging the umbilical cord blood with a Ficoll-Hypaque gradient to obtain monocytes; and removing contaminants therefrom. The obtained monocytes may be subjected to isolation of mesenchymal stem cells therefrom, or to ultrafreezing for a long-term safekeeping till use.

5 The isolation of mesenchymal stem cells from the umbilical cord blood-derived monocytes may be performed by the method of Yang SE et al. (Yang SE et al., *Cytotherapy*, 6(5):476-486, 2004). Specifically, monocytes are suspended in a medium containing 5 to 30 weight%, preferably 5 to 15 weight% of fetal bovine serum (FBS), the medium including a conventional one such as
10 DMEM, α -DMEM, Eagle's basal medium or RPMI 1640. Then, the cells in the suspension were divided into media having the same composition as described above and cultured in a 5% CO₂ incubator at 37°C. When the cultured cells form a mono-layer, mesenchymal stem cells having a spindle shape are observed. Then, the mesenchymal stem cells are subcultured
15 repeatedly until the cells are sufficiently amplified.

 According to the present invention, both the differentiation and proliferation of neural precursor cells or neural stem cells to neural cells can be induced by co-culturing umbilical cord blood-derived mesenchymal stem cells with neural precursor cells or neural stem cells. Namely, the umbilical cord
20 blood-derived mesenchymal stem cells are simultaneously effective not only for inducing the differentiation of the neural precursor cells or neural stem cells to neural cells, but also for sustaining and strengthening such effects through increasing the number of the neural cells, to enhance their therapeutic effects.

 Therefore, the present invention provides a use of umbilical cord blood-derived mesenchymal stem cells or a composition comprising the cells for
25 inducing differentiation of neural precursor cells or neural stem cells to neural cells and proliferation of the resulting neural cells.

 Umbilical cord blood-derived mesenchymal stem cells or a composition of comprising the cells can be used for cytotherapy of a patient suffering from a

nerve injury diseases, e.g., stroke, Parkinson's disease, Alzheimer's disease, Pick's diseases, Huntington's disease, amyotrophic lateral sclerosis, traumatic central nervous system diseases and spinal cord injury disease, preferably stroke and spinal cord injury disease.

5 The composition of the present invention may further comprise a pharmaceutically acceptable additive.

A pharmaceutical formulation in a unit dosage form may be prepared employing the composition of the present invention according to the conventional procedures in the art. A formulation for parenteral administration
10 such as an injection or a topical dosage form is preferable. The inventive pharmaceutical formulation may further include a pharmaceutically-acceptable additives, e.g., fillers, expanders, binding agents, wetting agents, disintegrants, diluents such as surfactants and other excipients.

The inventive pharmaceutical formulation can be administered
15 parenterally according to the conventional procedures in the art, for instance, via direct injection into an injury region as well as injection into the cerebrospinal fluid, for example, lumbar puncture and parenchymal injection, vein or artery. Preferably, it can be administered via direct injection into a peripheral or opposite region of brain or spinal cord injury region. Further, the clinical
20 method of Douglas Kondziolka (Douglas Kondziolka, Pittsburgh, 1998) may be employed to administer the inventive pharmaceutical formulations into an injury region. Specifically, a skull of a subject is incised to make a hole having a diameter of 1 cm and a suspension of a mesenchymal stem cells in HBSS (Hank's balanced salt solution) is injected into the hole by employing a long-
25 needle syringe and a stereotactic frame.

A typical dose of the mesenchymal stem cells may range from 1×10^5 to 1×10^7 cells/kg body weight /injection, preferably from 5×10^5 to 5×10^6 cells/kg body weight/injection, which can be administered in a single dose or in divided doses. Further, it should be understood that the amount of the effective

ingredient actually administrated to a certain patient ought to be determined in light of various relevant factors including the amount of neural cells to be differentiated and proliferated, the chosen route of administration, and the body weight, age and sex of an individual patient.

5 The present invention also provides a method for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells, which comprises co-culturing umbilical cord blood-derived mesenchymal stem cells with the neural precursor cells or the neural stem cells. The co-culturing may be carried out by mixing the umbilical cord blood-derived
10 mesenchymal stem cells with the neural precursor cells or the neural stem cells at a ratio of 1:0.1 to 1:10, preferably, 1:1 to 1:2 based on their cell number and cutting the cell mixture in a conventional cell culture medium such as DMEM, α -DMEM, α -MEM, Eagle's basal medium and RPMI 1640. The culture medium may further comprise an antibiotic, e.g., gentamicin, and/or 5 to 15
15 weight% of FBS. The culture period may ranges from 5 to 10 days.

 The present invention also provides a method for treating a nerve injury disease which comprises administering the umbilical cord blood-derived mesenchymal stem cell or a composition comprising the same to the nerve cell injury region of a subject in need of treating the nerve injury disease. The
20 subject may be a mammal including human.

 When administered in a therapeutically effective amount, the umbilical cord blood-derived mesenchymal stem cell induces not only differentiation of neural precursor cells or neural stem cells of central or peripheral nervous system to neural cells, but also the proliferation of the resulting neural cells,
25 thereby resulting in the recovery of the neural functions and treatment of such nerve injury disease. The treating effect of the umbilical cord blood-derived mesenchymal stem cell is greatly enhanced and sustained for a long time by its capability of proliferating the regenerated neural cells.

The following Examples and Test Examples are given for the purpose of illustration only, and are not intended to limit the scope of the invention.

Example 1: Isolation and culture of umbilical cord blood-derived mesenchymal stem cells

(Step 1) Acquisition of umbilical cord blood (UCB)

A UCB sample was obtained from the umbilical vein of a delivering woman under her consent. Specifically, a 16-gauge needle of a UCB collection bag containing 44 ml of CPDA-1 anticoagulant (GREEN CROSS) was inserted into the umbilical vein to allow UCB to flow into the bag. The collected blood was processed within 48 hours and the cell survival rate was over 90%.

(Step 2) Isolation and amplification of mesenchymal stem cells

The UCB obtained in step 1 was subjected to centrifugation using a Ficoll-Hypaque gradient (density: 1.077 g/ml, Sigma) to obtain monocytes. The monocytes were then washed several times to remove impurities and suspended in a minimum basal medium containing 5 to 15 weight% FBS (HyClone) (α -MEM, Gibco BRL). Subsequently, a pre-measured amount of the suspension was added to the same media as above and cultured in a 5% CO₂ incubator at 37°C, while exchanging the media with a fresh batch of medium twice a week. When the cultured cells formed a mono-layer, the generation of mesenchymal stem cells having a spindle shape was confirmed with a microscope. The mesenchymal stem cells thus formed were subcultured repeatedly until the cells were sufficiently amplified (Yang SE et al., *Cytherapy*, 6(5):476-486, 2004).

Example 2: Culture of NG108-15 (NG108)

A mouse brain-derived cell, NG108-15 (Neuroblastoma X glioma hybrid)(ATCC, Cat. No. ATCC-CRL-HB-12317), having similar physiological and morphological characteristics with a neural precursor cell was cultured in
5 DMEM (Dulbecco's modified Eagle's medium)(4 mM/L glutamine, 4.5 g/L glucose, 4.0 mg/L pyridoxin-HCl, 0.1 mM hypoxanthine-guanine, 400 nM aminopterin, 0.016 mM thymidine, 5 to 15 weight% FBS).

Example 3: Culture of neural stem cells

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Neural stem cells derived from a brain cortex of a fetal mouse (Chemicon, Cat. No. SCR029) were cultured in a neural stem cell basal medium (20 ng/ml FGF-2, 20ng/ml EGF and 2mg/ml heparin).

Example 4: Co-culture of umbilical cord blood-derived mesenchymal stem cells and NG108-15 (I)

The human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) of Example 1 were co-cultured with NG108-15 of Example 2
20 (hUCB-MSCs: NG108-15= 1:1) employing a transwell chamber (Fig. 1) and the culture medium of Example 2. As a control group, NG108-15 was cultured alone in the culture medium of Example 2. As shown in Fig. 1, the transwell chamber was composed of lower and upper compartments which were separated from each other by a microporous membrane having 1 μ m-pores. hUCB-
25 MSCs were placed in the upper compartment, and NG108-15, in the lower compartment.

The differentiation of NG108-15 was observed with a phase-contrast microscope ($\times 100$) in 4 and 7 days. As shown in Fig. 2, NG108-15 co-cultured with hUCB-MSCs differentiated to a form of typical matured neuron-

like cells in which the cells spread long branches and differentiated to have a spindle shape.

The differentiated cells were further confirmed to be neuron-like cells in 7 days employing immunostaining for tubulin-beta III, an early marker of neuronal development. Specifically, hUCB-MSCs, NG108-15 and a mixture thereof were respectively cultured in a cover slide, and blocked by adding them to 10% normal goat serum containing 0.3% triton X-100 for 1 hour at a room temperature. The 1st antibody employed in the immunostaining, an anti-tubulin-beta III mouse monoclonal antibody conjugated with phycoerythrin (Chemicon), was diluted by one hundred fold with the goat serum and added thereto. The mixture was then kept overnight at 4 °C, and the resulting mixture was washed three times (each time for 5 minutes) with 0.01 M PBS.

As shown in Fig. 3, NG108-15 co-cultured with the mesenchymal stem cells of Example 1 exhibited a distinct response to the immunostaining for tubulin-beta III, verifying that the differentiated were neuron-like cells.

Example 5: Co-culture of umbilical cord blood-derived mesenchymal stem cells and NG108-15 (II)

The human umbilical cord blood-derived mesenchymal stem cells were obtained by the method of Example 1 from two different individuals (hUCB-MSCs-1 and hUCB-MSCs-2). NG108-15 of Example 2 was cultured alone or co-cultured with hUCB-MSCs-1 or hUCB-MSCs-2 in accordance with the method of Example 4 for 7 days. Differentiation and proliferation of the cells were observed with a phase-contrast microscope (×100).

As a comparative group, 1 mM of cAMP which induces differentiation of NG108-15 to neuron-like cells (*NeuroReport* 9, 1261-1265, 1998) was added to the culture medium of NG108-15.

As shown in Fig. 4, NG108-15 co-cultured with the mesenchymal stem

cells was differentiated to a form of matured neuron-like cells. There was no significant difference between hUCB-MSCs-1 and hUCB-MSCs-2 in their differentiation inducing activities.

5 **Example 6: Co-culture of umbilical cord blood-derived mesenchymal stem cells and neural stem cells (I)**

The neural stem cells of Example 3 were cultured alone (control group) or co-cultured with hUCB-MSCs-1 or hUCB-MSCs-2 of Example 5 in the
10 culture medium of Example 3, employing the transwell chamber. hUCB-MSCs were placed in the upper compartment and the neural stem cells, in the lower compartment.

hUCB-MSCs-1 and hUCB-MSCs-2 were respectively added to the medium in the concentration of 500, 1000, 2000, 4000 and 6000 cells/cm²,
15 respectively, and the neural stem cells were co-cultured with the concentration of 2000 cells/cm². The differentiation and proliferation of the cells were observed with a phase-contrast microscope (×100) (Fig. 5) in 7 days.

As shown in Fig. 5, the neural stem cells co-cultured with the mesenchymal stem cells were differentiated to a form of matured neurons.
20 There was no significant difference between hUCB-MSCs-1 and hUCB-MSCs-2 in their differentiation inducing activities. Further, the extent of differentiation and proliferation of the neural stem cells was directly proportional to the concentration of the mesenchymal stem cells with which co-cultured.

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Example 7: Co-culture of umbilical cord blood-derived mesenchymal stem cells and neural stem cells (II)

The neural stem cells of Example 3 were cultured alone (control group)

or co-cultured with hUCB-MSCs of Example 1 (hUCB-MSCs: neural stem cells = 1:1) in the culture medium of Example 3, employing a transwell chamber. hUCB-MSCs were placed in the upper compartment and the neural stem cells, in the lower compartment.

5 Immunostaining was carried out in 4 and 7 days by employing the method of Example 4 for tubulin-beta III and microtubule-associated protein 2 (MAP2), early markers of neuronal development such that it confirmed that the differentiated were neurons.

As shown in Fig. 6, the neural stem cells co-cultured with the
10 mesenchymal stem cells of Example 1 exhibited a distinct response to immunostaining for tubulin-beta III and MAP2, verifying that the differentiated were neurons.

**Example 8: Co-culture of umbilical cord blood-derived mesenchymal stem
15 cells with neural precursor cells or neural stem cells**

NG108-15 of Example 2 was cultured alone (control group) or co-cultured with hUCB-MSCs of Example 1, and the neural stem cells of Example 3 was cultured alone (control group) or co-cultured with hUCB-MSCs-1 or
20 hUCB-MSCs-2 of Example 5, according to the methods of Examples 4 and 6, respectively. The number of viable cells was counted in 7 days employing the trypan blue staining (Figs. 7 and 8).

As shown in Figs. 7 and 8, the numbers of NG108-15 and the neural stem cells increased in proportion to the concentration of the mesenchymal stem
25 cells with which co-cultured, implying that the umbilical cord blood-derived mesenchymal stem cells can be simultaneously effective not only for inducing the differentiation of the neural precursor cells or neural stem cells to neural cells, but also for sustaining and strengthening such effects through increasing the number of the neural cells.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope
5 of the invention as defined by the appended claims.

What is claimed is:

1. A use of a composition comprising umbilical cord blood-derived mesenchymal stem cells for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells.

2. A method for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells, which comprises co-culturing umbilical cord blood-derived mesenchymal stem cells with the neural precursor cells or the neural stem cells.

3. The method of claim 2, wherein the ratio of umbilical cord blood-derived mesenchymal stem cells to the neural precursor cells or the neural stem cells employed is in the range of 1:0.1 to 1:10.

4. A composition for inducing differentiation and proliferation of neural precursor cells or neural stem cells to neural cells, comprising umbilical cord blood-derived mesenchymal stem cells as an active ingredient.