METHODS, NUCLEIC ACID CONSTRUCTS AND CELLS FOR TREATING NEURODEGENERATIVE DISORDERS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to neuronal-like cells capable of controllable synthesis of neurotransmitters and of cell replacement therapy using such cells for treating neurodegenerative disorders such as Parkinson's disease.

Parkinson's disease is an age-related disorder characterized by progressive loss of dopamine producing neurons in the substantia nigra of the midbrain, which in turn leads to progressive loss of motor functions manifested through symptoms such as tremor, rigidity and ataxia. Parkinson's disease can be treated by administration of pharmacological doses of the precursor of dopamine, L-DOPA (Marsden, Trends Neurosci. 9:512, 1986; Vinken *et al.*, in Handbook of Clinical Neurology p. 185, Elsevier, Amsterdam, 1986). Although such treatment is effective in early stage Parkinson's patients, progressive loss of substantia nigra cells eventually leads to an inability of remaining cells to synthesize sufficient dopamine from the administered precursor and to diminishing pharmacogenic effect.

Studies of neurodegenerative diseases suggest that symptoms that arise in afflicted individuals are secondary to defects in local neural circuitry and cannot be treated effectively with systemic drug delivery. Consequently, alternative approaches for treating neurodegenerative diseases have emerged, such as transplantation of cells capable of replacing or supplementing the function of damaged neurons. For such cell replacement therapy to work, implanted cells must survive and integrate, both functionally and structurally, within the damaged tissue.

Parkinson's disease is the first disease of the brain for which intracerebral cell replacement therapy has been used in humans. Several attempts have been made to provide the neurotransmitter dopamine to cells of the diseased basal ganglia of Parkinson's patients by homografting adrenal medullary cells to the brain of patients (Backlund *et al.*, J. Neurosurg. 62:169-173, 1985; Madrazo *et al.*, New Eng. J. Med. 316:831-836, 1987). Transplantation of other donor cells such as fetal brain cells from the substantia nigra, an area of the brain rich in dopamine-containing cell bodies and also the area of the brain most affected in Parkinson's disease, has been shown to be partially effective in reversing the behavioral deficits induced by selective

dopaminergic neurotoxins (Bjorklund *et al.*, Ann. N.Y. Acad. Sci. 457:53-81, 1986; and Dunnett *et al.* (Trends Neurosci. 6:266-270, 1983)

Several cell replacement studies utilizing various non-neuronal cell types from different sources have also been conducted over the past few years. In animal models of Parkinson's disease, researchers have transplanted cells, such as monocytes, bone marrow stem cells, myoblasts, fibrolasts, astrocytes and Sertoli cells (Costantini *et al.*, 2000; Hwan-Wun *et al.*, 1999; Linder *et al.*, 1995; Patridge & Davies 1995; Perry & Gordon 1998; Yadid *et al.*, 1999). In other studies, cells were transplanted after being genetically engineered with growth factor genes (e.g., glial-derived and brain-derived growth factors) to enhance survival rates, or with genes such as tyrosine hydroxylase, aromatic amino acid decarboxylase, or GTP-cyclohydrolase I, which are capable of increasing dopamine synthesis in the transformed cell (Choi-Lundberg *et al.*, 1998; Yoshimoto *et al.*, 1995; Schwarz *et al.*, 1999). However, these cells failed to fully acquire the structural and functional characteristics of the damaged neuronal cells and consequently proved to be therapeutically ineffective (Brundin *et al.*, 2000).

Clinical cell replacement trials for Parkinson's patients have been conducted using fetal cells which comprise just 1-2% are dopaminergic neurons (Freed *et al.*, 1992; Freed *et al.*, 2001; Freeman *et al.*, 1995; Kordower *et al.*, 1995; Kordower *et al.*, 1998; Lindvall O. 1991; and Wenning *et al.*, 1997). Freed *et al* (2001) found that fetal cells transplantation to Parkinson's patients was beneficial only to young patients (<60 years). Furthermore, several patients suffered from severe dyskinesia without levodopa treatment ("runaway dyskinesias") due to an excessive and uncontrolled production and release of dopamine by implanted cells (Freed *et al.*, 2001; Olanow *et al.*, 2003). In addition, the low availability of human fetal tissue substantially limits the number of patients which could benefit from fetal cells transplantation.

Adult bone marrow stromal cells (BMSc) are non-hematopoietic cells, which can differentiate into neuron-like cells demonstrating neuronal markers (Azizi *et al.*, 1998; Deng *et al.*, 2001; Kopen *et al.*, 1999; Sanchez-Ramos *et al.*, 2000; Schwarz *et al.*, 1999; Woodbury *et al.*, 2000), electro-physiological functions (Kohyama *et al.*, 2001) and *in vivo* expression of tyrosine hydroxilase (Schwarz *et al.*, 1999). Moreover, transplantation of BMSc in mouse and rat models of Parkinson's disease resulted in beneficial effects (Li *et al.*, 2001; Schwarz *et al.*, 1999).

Although adult BMSc can be differentiated into neuron-like cells, which are structurally compatible with implantation, engrafted BMSc may release neurotransmitters such as dopamine uncontrollably which in turn may cause severe side effects such as "runaway dyskinesia" and thus rendering the use of BMSc unsuitable for therapy of neurodegenerative disorders.

There is thus a widely recognized need for, and it would be highly advantageous to have, neuronal-like cells which are capable of integration into damaged neuronal tissue and further capable of controllably synthesizing neurotransmitters, such as dopamine, and thus can be utilized to effectively and safely treat neurodegenerative disorders.

SUMMARY OF THE INVENTION

In a first aspect, there is provided use of bone marrow stromal cells transformed with an expression construct including a polynucleotide sequence expressing, in an exogenously regulatable manner, a neurotransmitter, a precursor of said neurotransmitter, or an enzyme participating in said synthesis of said neurotransmitter for the manufacture of a medicament for treating a neurodegenerative disorder.

In a second aspect, there is provided use of an agent capable of regulating synthesis of a neurotransmitter in bone marrow stromal cells capable of exogenously regulatable neurotransmitter synthesis for the manufacture of a medicament for treating a neurodegenerative disorder.

In a third aspect, there is provided a nucleic acid construct, comprising a polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under a control of a regulatory sequence capable of regulating expression of said enzyme in mammalian cells.

In a fourth aspect, there is provided a construct system comprising a first expression construct including a first polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under the transcriptional control of a first regulatory sequence and a second expression construct including transcriptional control of a second regulatory sequence, wherein said transactivator is capable of activating said first regulatory sequence to direct transcription of said first polynucleotide sequence.

In a fifth aspect, there is provided a bone marrow stromal cell comprising the nucleic acid construct as defined above.

In a sixth aspect, there is provided a bone marrow stromal cell comprising the construct system as defined above.

In a seventh aspect, there is provided a method of producing cells for treating neurodegenerative disorders, comprising:

(a) isolating bone marrow cells;

(b) incubating said bone marrow cells in a proliferating medium capable of maintaining arid/or expanding said bone marrow cells;

(c) selecting bone marrow stromal cells from the cells resulting from step (b);

(d) incubating said bone marrow stromal cells in a differentiating medium including at least one polyunsaturated fatty acid and at least one differentiating agent, thereby producing the cells for use in treating neurodegenerative disorders.

In an eighth aspect, there is provided an isolated human cell population, comprising naïve bone marrow stromal cells which synthesize a neurotransmitter.

In a ninth aspect, there is provided an isolated human cell population, comprising naïve bone marrow stromal cells which synthesize at least two types of neurotransmitters.

In a tenth aspect, there is provided use of bone marrow stromal cells which synthesize a neurotransmitter for the manufacture of a medicament for treating a neurodegenerative disorder, wherein said bone marrow stromal cells capable of synthesizing a neurotransmitter are obtained by incubating said bone marrow stromal cells in a differentiation medium which comprises at least one PUFA and at least one differentiation agent.

In an eleventh aspect, there is provided a method of generating cells for treating neurodegenerative disorders, comprising incubating bone marrow stromal cells in a differentiating medium including at least one polyunsaturated fatty acid and at least one differentiating agent, thereby generating the cells for treating neurodegenerative disorders.

In a twelfth aspect, there is provided an isolated human cell population, comprising naïve bone marrow stromal cells which synthesize a neurotransmitter, obtainable by incubating said bone marrow stromal cells in a differentiation medium which comprises at least one PUFA and at least one differentiation agent.

In a thirteen aspect, there is provided an isolated human cell population, comprising naïve bone marrow stromal cells which synthesize at least two types of neurotransmitter obtainable by incubating said bone marrow stromal cells in a differentiation medium which comprises at least one PUFA and at least one differentiation agent.

Disclosed herein is a method of treating a neurodegenerative disorder which includes administering to an individual in need thereof cells capable of exogenously regulatable neurotransmitter synthesis thereby treating the neurodegenerative disorder.

Disclosed herein is a method of treating a neurodegenerative disorder which includes the steps of (a) administering to an individual in need thereof cells capable of exogenously regulatable neurotransmitter synthesis; and (b) periodically exposing the individual to an agent or condition capable of regulating the synthesis of the neurotransmitter in the cells thereby treating the neurodegenerative disorder.

Disclosed herein is a nucleic acid construct which includes a polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under a control of a regulatory sequence capable of regulating expression of the enzyme in mammalian cells.

Disclosed herein is a construct system which includes a first expression construct including a first polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under the transcriptional control of a first regulatory sequence and a second expression construct including a second polynucleotide sequence encoding a transactivator positioned under the transcriptional control of a

second regulatory sequence, wherein the transactivator is capable of activating the first regulatory sequence to direct transcription of the first polynucleotide sequence.

Disclosed herein is a cell comprising a nucleic acid construct which includes a polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under a control of a regulatory sequence capable of regulating expression of the enzyme in the cell.

Disclosed herein is a cell comprising the construct system which includes a first expression construct including a first polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under the transcriptional control of a first regulatory sequence and a second expression construct including a second polynucleotide sequence encoding a transactivator positioned under the transcriptional control of a second regulatory sequence, wherein the transactivator is capable of activating the first regulatory sequence to direct transcription of the first polynucleotide sequence.

Disclosed herein is a method of producing cells for use in treating neurodegenerative disorders. The method includes the steps of: (i) isolating bone marrow cells; (ii) incubating the bone marrow cells in a proliferating medium capable of maintaining and/or expanding the bone marrow cells; (iii) selecting bone marrow stromal cells from the cells resulting from step (ii); and (iv) incubating the bone marrow stromal cells in a differentiating medium including at least one polyunsaturated fatty acid and at least one differentiating agent, thereby producing the cells for use in treating neurodegenerative disorders.

Disclosed herein is a population of cells which includes bone marrow derived stromal cell capable of synthesizing a neurotransmitter.

Disclosed herein is a mixed population of cells which includes bone marrow derived stromal cell capable of synthesizing at least two types neurotransmitters.

According to further features in preferred embodiments described below, the method of treating a neurodegenerative disorder, further includes

exposing the individual to an agent or condition capable of regulating the synthesis of the neurotransmitter in the cells.

According to still further features in the described preferred embodiments the cells are genetically modified so as to enable the exogenously regulatable neurotransmitter synthesis.

According to still further features in the described preferred embodiments the cells are transformed with an expression construct including a polynucleotide sequence encoding an enzyme participating in the synthesis of the neurotransmitter, wherein the expression construct is designed such that expression of the polynucleotide is controllable via the agent.

According to still further features in the described preferred embodiments the agent is capable of downregulating expression of the enzyme participating in the synthesis of the neurotransmitter.

According to still further features in the described preferred embodiments The agent is capable of upregulating expression of the enzyme participating in the synthesis of the neurotransmitter.

According to still further features in the described preferred embodiments the cells are transformed with at least one expression construct including a first polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under the transcriptional control of a first regulatory sequence and a second polynucleotide sequence encoding a transactivator positioned under the transcriptional control of a second regulatory sequence, wherein the transactivator is capable of activating the first regulatory sequence to direct transcription of the first polynucleotide sequence in absence of the agent.

According to still further features in the described preferred embodiments the agent is doxycyline.

According to still further features in the described preferred embodiments the transactivator is a tetracycline-controlled transactivator.

According to still further features in the described preferred embodiments the first regulatory sequence includes a tetracycline response element.

According to still further features in the described preferred embodiments the enzyme is selected from the group consisting of tyrosine hydroxylase, DOPA decarboxylase, GTP cyclohydrolase I, dopamine dopamine β-hydroxylase, glutamate

decarboxylase, tryptophane-5 monooxygenase and choline acetyltransferase.

According to still further features in the described preferred embodiments second regulatory sequence includes a human neuron-specific enolase promoter.

According to still further features in the described preferred embodiments the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, multiple sclerosis, amyatrophic lateral sclerosis, autoimmune encephalomyelitis, Alzhimer's disease and Huntington's disease.

According to still further features in the described preferred embodiments the neurodegenerative disorder is Parkinson's disease.

According to still further features in the described preferred embodiments the neurotransmitter is selected from the group consisting of dopamine, norepinephrine, epinephrine, gamma aminobutyric acid, serotonin, acetylcholine, and glutamic acid.

According to still further features in the described preferred embodiments the neurotransmitter is dopamine.

According to still further features in the described preferred embodiments the cells are bone marrow cells.

According to still further features in the described preferred embodiments the bone marrow cells are bone marrow stromal cells.

According to still further features in the described preferred embodiments the cells are neuron-like cells.

According to still further features in the described preferred embodiments the neuron-like cells express at least one neuronal marker.

According to still further features in the described preferred embodiments the neuronal marker is selected from the group consisting of CD90, neuron-specific nuclear protein, neurofilament heavy, neuron-specific enolase, beta-tubulin 3, tyrosine hydroxylase, microtubule associated protein 2 (MAP-2), nestin and calbindin

According to still further features in the described preferred embodiments administering of cells is effected by transplanting the cells into a brain tissue of the individual.

According to still further features in the described preferred embodiments administering of cells is effected by transplanting the cells into a spinal cord of the individual.

According to still further features in the described preferred embodiments exposing of an individual is effected by oral administration of the agent to the individual.

According to still further features in the described preferred embodiments exposing of an individual is effected by infusion of the agent to the individual.

According to still further features in the described preferred embodiments the cells are genetically modified so as to enable the exogenously regulatable neurotransmitter synthesis.

According to still further features in the described preferred embodiments the cells are transformed with an expression construct including a polynucleotide sequence encoding an enzyme participating in the synthesis of the neurotransmitter, wherein the expression construct is designed such that expression of the polynucleotide is controllable via a regulatory agent.

According to still further features in the described preferred embodiments the agent is capable of downregulating expression of the enzyme participating in the synthesis of the neurotransmitter.

According to still further features in the described preferred embodiments the agent is capable of upregulating expression of the enzyme participating in the synthesis of the neurotransmitter.

According to still further features in the described preferred embodiments the cells are genetically modified to express tyrosine hydroxylase under a regulatory control of the agent, such that when the agent is absent an activator molecule binds a response element thereby upregulating expression of the tyrosine hydroxylase.

According to still further features in the described preferred embodiments the cell is a neuron-like cell devoid of endogenous activity of the enzyme participating in the synthesis of the neurotransmitter.

According to still further features in the described preferred embodiments the cell further includes a polynucleotide encoding an apoptosis inhibiting polypeptide.

According to still further features in the described preferred embodiments the proliferation medium includes DMEM, SPN, L-glutamine, FCS, 2-ß-mercaptoethanol, nonessential amino acids and EGF.

According to still further features in the described preferred embodiments the method of producing cells for treating neurodegenerative disorders includes prior to

step (iv) incubating the cells resulting from step (iii) in a pre-differentiating medium thereby predisposing the cells to differentiate into neuron-like cells.

According to still further features in the described preferred embodiments the pre-differentiating medium includes bFGF.

According to still further features in the described preferred embodiments the pre-differentiation medium further includes DMEM, SPN, L-glutamine, N2 supplement and FCS.

According to still further features in the described preferred embodiments the at least one polyunsaturated fatty acid is docosahexaenoic acid.

According to still further features in the described preferred embodiments the at least one differentiating agent is selected from the group consisting of BHA, dbcAMP and IBMX,

According to still further features in the described preferred embodiments the differentiating medium further includes DMEM, SPN, L-glutamine, N2 supplement and retinoic acid

According to still further features in the described preferred embodiments the method of producing cells for treating neurodegenerative disorders is further effected by prior to step (iv) transforming the cells resulting from step (iii) with the disclosed nucleic acid construct.

According to still further features in the described preferred embodiments step (i) is effected by aspiration.

According to still further features in the described preferred embodiments step (iii) is effected by harvesting surface adhering cells.

According to still further features in the described preferred embodiments the neurotransmitter is dopamine.

According to still further features in the described preferred embodiments the at least two types of neurotransmitters include dopamine..

According to still further features in the described preferred embodiments the least two types of neurotransmitters include serotonin.

The disclosure herein successfully addresses the shortcomings of the presently known methods of treating neurodegenerative diseases by providing neuronal-like cells capable of controllable synthesis of neurotransmitters and of cell replacement

therapy using such cells for treating neurodegenerative disorders such as Parkinson's disease.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the disclosure, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-C illustrate light microscope images of non-differentiated human bone-marrow stromal cells (hBMSc). The hBMSc were cultured in a "proliferation medium" (described in Example 1 of the Examples section below) and were grown to 80-90% confluency over a time period of approximately 15 days. The plastic-adherent hBMSc had a round or spindle body shape (Figure 1A) or a flat body shape (Figures 1B-C).

FIG. 2 illustrates flow cytometer analyses of non-differentiated hBMSc. Fifteen-day-old hBMS cells were stained for the presence of surface markers CD45, CD5, CD20, CD11b and CD34 (characteristic of lymphohematopoietic cells) and with CD90 (Thy-1; a protein which is expressed during synaptogenesis). The cells were analyzed with FACSCalibur™ flow cytometer (Becton Dickinson) equipped with an

argon ion laser (adjusted to an excitation wavelength of 488nm) and the CELLQuest™ software program (Becton Dickinson). The analyses show that the hBMS cells did not express any of the lymphocyte-associated markers but positively expressed the Thy-1 protein.

FIG. 3 illustrates a light microscope image of adult hBMSc differentiated into neurons. Twenty-four hours prior to differentiation, cultured hBMSc were transferred from the "proliferating medium" into the "pre-differentiation" medium (see Examples 1-2 of the Examples section below). Following 24 hr incubation in the "pre-differentiation medium", the hBMSc were transferred into the "differentiation medium" (see Example 2 of the Examples below). The plastic-adherent cells were transformed into neuronal-like cells having a spindle shaped cell body and long branching processes that appeared as early as three hours post differentiation induction and continued to appear 72 h following differentiation induction.

FIG. 4 illustrates ^{3}H-thymidine incorporation (indicative of cell proliferation) in hBMSc that have been cultured in the "differentiating medium" and in hBMSc, which have been cultured in the "pre-differentiating medium" (see Example 2 of the Examples below). The ^{3}H-thymidine incorporation into differentiating cells was reduced by about 45% and 90%, as compared with the non-differentiating cells, following 16 and 39 hr incubation periods, respectively.

FIGs. 5A-D illustrate reverse transcriptase RT-PCR, real-time PCR, and northern blot analyses of RNA extracted from neuronal markers in non-differentiated and in differentiating hBMSc. Figure 5A illustrates an RT-PCR analysis designed for identifying neuronal transcripts. Figure 5B illustrates Northern blot and real-time PCR analyses, which utilized a ^{32}P-labled PCR product of NEGF2 as a probe. Figure 5C illustrates Northern blot analysis, which utilized ^{32}P-labled PCR product of neurofilament 200 (NF-200) as a probe. Figure 5D illustrates northern blot analysis, which utilized a ^{32}P-labled PCR product of neuron specific enolase (NSE) as a probe.

FIGs. 6A-B illustrate fluorescent microscope images of neural markers in hBMSc cultured in "differentiation medium" (see Example 2 of the Examples below) for 12 hr to 5 days. Figure 6A illustrates antibody-labeled neuron nuclei-specific marker (NeuN; expressed after 12 hr), neurofilament heavy (NF-200; expressed after 24 hr), neuron specific enolase (NSE; expressed after 48 hr) and nestin (expressed

after 48 hr). Figure 6B illustrates antibody-labeled glial fibrillary acidic protein (GFAP; expressed after 48 hr) and β-tubulin III (expressed after 5 days).

FIGs. 7A-C illustrate Western blot analyses of differentiated hBMSc indicating elevated expressions of neuron specific enolase (NSE; Figure 7A) neuron nuclei-specific marker (NeuN; Figure 7B) and nestin (Figure 7C).

FIG. 8 illustrates light microscope images of neuron-like differentiated hBMSc following 28 days of incubation in "long-term differentiation medium" (see Example 5 of the Examples below).

FIGs. 9A-C illustrate light and fluorescent microscope images of hBMSc which were incubated in the "proliferation medium" (see Example 1 of the Examples below) or in the "long-term differentiation medium" (see Example 5 of the Examples below). Figure 9A illustrates antibody-labeled expression of the neuronal marker β-tubulin III in the differentiated cells. Figure 9B illustrates antibody-labeled expression of the neuronal marker MAP-2 in the differentiated cells. Figure 9C illustrates antibody-labeled expression of the neuronal marker nestin in the differentiated cells.

FIG. 10 illustrates RT-PCR analyses designed for identifying dopaminogenic markers in hBMSc, which have been cultured for 12-72 hr in the "differentiation medium" (see Example 2 of the Examples below).

FIGs. 11A-C illustrate expression of tyrosine hydroxylase (TH) in differentiated hBMSc. Figure 11A illustrates real-time PCR analysis indicating an elevated TH transcription in the differentiated cells. Figure 11B illustrates Western blot analysis indicating an elevated level of TH in the differentiated cells. Figure 11C illustrates fluorescent microscope images highlighting antibody-labeled TH expression in the differentiated cells.

FIG. 12 illustrates confocal fluorescent microscope images of hBMSc which have been incubated for five days in the "differentiation medium" (see Example 2 of the Examples below) highlighting antibody-labeled vesicular monoamine transporter 2 (VMAT-2) in the differentiated cells.

FIG. 13 illustrates flow cytometer analyses of hBMSc which have been differentiated for 48 hr in the "differentiation medium" (see Example 2 of the Examples below). The cells were stained for the presence of D2 dopamine receptor

and were analyzed with FACSCalibur™ flow cytometer (Becton Dickinson). The analysis shows that a D2 dopamine receptor was present in the differentiated hBMSc.

FIGs. 14A-D illustrate HPLC analyses of differentiating hBMSc. Figure 14A illustrates the levels of dopamine measured in the supernatant of hBMSc which have been incubated for 0-96 hr in the "differentiation medium" (see Example 2 of the Examples below). Figure 14B illustrates the levels of dopamine measured in the supernatant of hBMSc which have been incubated for 0-96 hr in the "differentiation medium" followed by an additional incubation of 10 minutes in 56 mM KCl solution. Figure 14C illustrates the levels of the dopamine precursor DOPA measured in the supernatant of hBMSc which have been incubated in the "differentiation medium" for 0-72 hr. Figure 14D illustrates the levels of dopamine metabolite DOPAC measured in the supernatant of hBMSc which have been cultured in the "differentiation medium" for 0-50 hr.

FIGs. 15A-D illustrate the effect of mouse bone-marrow stromal cells (mBMSc) transplantation on the recovery of amphetamine-induced motor rotation in a rat model for Parkinson's disease. mBMSc were isolated from green fluorescent-protein marked transgenic mice (GFP-Tg). The isolated mBMSc were induced for neural differentiation and transplanted into the nigra of 6-OHDA lesioned rats. The rats were then treated with amphetamine and were examined for rotational response over a period of 45 days post transplantation. Figure 15A illustrates the changes in rotation rates over time followed transplantation indicating diminishing rotations (indicative of improvement of motor function) 45 days post transplantation. Figure 15B illustrates the changes in relative rotations (as compared with non-treated mice) over time followed transplantation indicating 97.9% decrease in relative rotations 45 days post transplantation. Figure 15C illustrates fluorescent microscope images highlighting transplanted mBMSc present in the substania nigra of treated mice 45 days post transplantation. Figure 15D illustrates fluorescent microscope images highlighting transplanted mBMSc present in the striatum of treated mice 45 days post nigral transplantation.

FIGs 16A-C illustrate dopaminergic and serotoninergic activities in differentiated human bone marrow stromal cells (hBMSc). Figure 16A illustrates western blot analysis indicating expression of tryptophane hydroxylase. Figure 16B illustrates RT-PCR analysis indicating tryptophane hydroxylase transcription. Figure

16B illustrates HPLC analysis indicating synthesis of DOPAC (a dopamine metabolite) and 5HIAA (a serotonin metabolite).

FIG. 17 illustrates a construct of an expression vector containing Nurr-1 encoding sequence inserted within pcDNA-3.1A (Invitrogene).

FIG. 18 illustrates a construct designed for a negative selection of dopaminergic cells. The construct includes the human TH (tyrosine hydroxylase) promoter inserted in pMOD (InvivoGene) upstream of the "suicide gene" HSV1-tk (herpes simplex virus type 1 thymidine kinase encoding toxic gancyclovir).

FIGs. 19A-B illustrate the Tet-off Tet-on system for doxycyline-controlled expression of tyrosine hydroxylase (TH). Figure 19A illustrates the regulator and response constructs of the system. Figure 19B illustrates a schematic diagram describing the system mode of action.

FIGs. 20A-B illustrate fluorescent microscope images of differentiated BMSc of a GFP-Tg mouse (mBMSc). Figure 20A illustrates fluorescent microscope image of morphological changes induced by "differentiation medium" (see Example 1). Figure 20B illustrates a fluorescent microscope image highlighting antibody-labeled A2B5 (a marker of oligodendrocyte precursor) expressed in the NT-3 induced mBMSc.

FIG. 21 illustrates changes in the rotational performance of mice expressing SOD1 (an animal model of amyotrophic lateral sclerosis; ALS) over time. The SOD1 mice suffered a substantial reduction in rotational performance as compared with the wild type mice and became completely paralyzed at the age of 4-5 months.

FIG. 22 illustrates a PCR analysis of different tissues of a female mouse sampled one week after male-derived mBMSc had been transplanted into the cisterna magna. The analysis detects Chromosome Y (indicative of the transplanted cells) in the spinal cord of the female mouse but not in other tissues.

FIG. 23 illustrates rotarod performance (indicative of rotational behavior) of wild-type mice which received mBMSc transplantation into their spinal cords at the age of 7 weeks. Mice which were treated with saline injection were use as control. The transplantation of mBMSc did not affect the rotational behavior of the wild type mice.

FIG. 24 illustrates a rotarod performance (indicative of rotational behavior) of SOD1 mice (model of amyotrophic lateral sclerosis) which received mBMSc

transplantation into their spinal cords at the age of 7 weeks. Mice which were treated with saline injection were use as control. The transplantation of mBMSc significantly improved the rotational behavior of the SOD1 mice, as compared with the saline-treated control.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of genetically modified cells capable of controllable synthesis of neurotransmitters and of methods of generating and using such cells in cell replacement therapy of neurodegenerative disorders.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Neurodegenerative disorders which are characterized by loss of neuronal functions, such as Parkinson's disease, cannot be efficiently treated using conventional drug therapy since such drugs have no effect on the underlying disease process which is typically caused by neuronal degeneration. Consequently, drug therapy can not fully compensate for the increasing loss of neuronal cells.

Although prior art cell replacement approaches have been successful when tested in animal models(U.S. Pat. No. 6,528,245; Schwartz *et. al.*, 1999; Li *et al.*, 2001; Costantini *et al.*, 2000; Hwan-Wun *et al.*, 1999; Linder *et al.*, 1995; Patridge & Davies 1995; Perry & Gordon 1998; Yadid *et al.*, 1999; Choi-Lundberg *et al.*, 1998; and Yoshimoto *et al.*, 1995), these approaches suffers from several inherent limitations which may prevent their use in human patients.

While conceiving the present invention, the present inventors realized that in order to provide safe and effective cell-replacement therapy of neurodegenerative diseases, such as Parkinson's disease, one requires cells which can be easily harvested and manipulated and above all be capable of synthesizing neurotransmitters, such as dopamine, in response to an external stimulus.

Although neuron-like bone marrow stromal cells (BMSc) capable of synthesizing neurotransmitters have been described by prior art studies (see, for example, U.S. Pat. No. 6,528,245 and Sanchez-Ramos *et al.* (2000), Woodburry *et al.* (2000), Woodburry *et al.* (J. Nerosci. Res. 96:908-917, 2002), and Deng *et al.* (Biophys. Res. Commun. 282:148-152, 2001), these studies did not demonstrate neurotransmitter production by such differentiated BMSc.

Notwithstanding from the above, even if such prior art cells produced neurotransmitters, use thereof in cell replacement therapy would not be sensible since constitutive neurotransmitter synthesis from implanted cells can lead to formation of severe side effectes such as runaway dyskinesia.

Thus, according to one aspect of the present invention there is provided a method of treating a neurodegenerative disorder. As used herein, the phrase "neurodegenerative disorder" refers to any disorder, disease or condition of the nervous system (preferably CNS) which is characterized by gradual and progressive loss of neural tissue, neurotransmitter, or neural functions. Examples of neurodegenerative disorder include, Parkinson's disease, multiple sclerosis, amyatrophic lateral sclerosis, autoimmune encephalomyelitis, Alzhimer's disease and Huntington's disease.

The term "treating" as used herein refers to refers to reversing, alleviating, inhibiting the progress of, or preventing the disorder, disease or condition to which such term applies, or one or more symptoms of such disorder or condition. The term "treatment" or "therapy" as used herein refer to the act of treating.

The method is effected by administering to an individual in need thereof cells capable of exogenously regulatable neurotransmitter synthesis thereby treating the neurodegenerative disorder.

Cells capable of exogenously regulatable neurotransmitter synthesis (i.e., cells which produce neurotransmitters on demand), such as the cells described in greater detail hereinbelow, are better suited for cell replacement therapy since the undesired side effects associated with cells utilized by prior art approaches described above can be eliminated.

A neurotransmitter according to the teaching of the present invention can be any substances which is released on excitation from the axon terminal of a presynaptic neuron of the central or peripheral nervous system and travel across the

synaptic cleft to either excite or inhibit the target cell. The neurotransmitter can be, for example, dopamine, norepinephrine, epinephrine, gamma aminobutyric acid, serotonin, acetylcholine, or glutamic acid. Preferably the neurotransmitter is dopamine.

Cellular synthesis of such neurotransmitters is directed by a pathway of enzymes which cooperate in converting precursor molecules into the active neurotransmitter. For example, dopamine is synthesized in dopaminergic neurons from L-dihydroxyphenylalanine (L-DOPA) through the action of DOPA decarboxylase, while L-DOPA is produced from tyrosine through the action of tyrosine hydroxylase. Norepinephrine is produced in noreinephrinergic neurons from dopamine through the action of dopamine ß-hydroxylase. Epinephrine is produced in epinephrinergic neurons from norepinephrine through the action of phenylethanolamine N-methyltransferase. Gama aminobutiric acid (GABA) is produced in GABAergic neurons from glutamate through the action of glutamate decarboxylase. Serotonin is produced in serotoninergic neurons from tryptophane through a two-step process by tryptophane-5-monooxygenase (hydroxylation) and by aromatic L-amino acid decarboxylase (decarboxylation). Acetylcholine is produced in cholinergic neurons from choline and acetyl-CoA through the action of choline acetyltransferase (http://www.indstate.edu/thcme/mwking/nerves.html).

The cells utilized by the present invention can be any actively growing cells, preferably bone marrow derived cells, more preferably bone marrow stromal cells (BMSc). The BMSc can be isolated from the iliac crest of an individual by aspiration followed by culturing in a proliferation medium capable of maintaining and/or expanding the isolated cells *ex vivo*. Preferably the proliferation medium includes DMEM, SPN, L-glutamine, FCS, 2-ß-mercaptoethanol, nonessential amino acids and EGF such as described in Example 1 of the Examples section which follows.

The proliferating cells may be directly differentiated to neuron-like cells, or transformed using the constructs and transformation methods described hereinbelow prior to their differentiation to neuron-like cells.

Differentiation to neuron-like cells can be effected by incubating the cells in a differentiating medium such as described in U.S. Pat. No. 6,528,245 and by Sanchez-Ramos *et al.* (2000); Woodburry *et al.* (2000); Woodburry *et al.* (J. Neurisci. Res.

96:908-917, 2001); Black and Woodbury (Blood Cells Mol. Dis. 27:632-635, 2001); Deng *et al.* (2001), Kohyama *et al.* (2001), Reyes and Verfatile (Ann. N. Y. Acad. Sci. 938:231-235, 2001) and Jiang *et al.* (Nature 418:47-49, 2002).

Preferably, differentiation is effected in the presence of at least one type of a long-chain polyunsaturated fatty acids. Long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), are known to be essential for proper neuronal development and function. Such polyunsaturated fatty acids are involved in modulating the biosynthesis and accumulation of the major anionic phospholipids in neuronal membranes [Connor and Neuringer *in:* "Biological Membranes and Aberrations in Membrane Structure and Function", Karnovsky, M., Leaf, A., and Bolis, L., eds., pp. 267–292, Alan R. Liss, New York ,1988; Alan R. L, "The Effect of n-3 Fatty Acid Deficiency and Repletion upon the Fatty Acid Composition and Function of the Brain and Retina", New York, 1988; and Green and Yavin, J. Neurochem. 65: 2555–2560, 1995]. In neuronal cell culture studies ir has been demonstrated that DHA forma a part of phosphatidylethanolamine and phosphatidylserine phospholipids around synapses. (Kim *et al.*, J. Biol. Chem. 275: 35215–35223, 2000; Kim *et al.*, J. Mol. Neurosci. 16: 223–227, 2002; and Akbar and Kim, J. Neurochem. 82: 655–665, 2002).

While reducing the present invention to practice, the inventors uncovered that an addition of 20-100 μM DHA (Sigma) or ethyl-DHA (Nu-Chek-Preo, Elysian, MN) to cultured BMSc substantially promoted formation of neurotransmitter secreting vesicles in the synapses of neuron-like BMSc (data is not shown).

Thus, according to a preferred embodiment of the present invention, differentiation of BMSc to neuron-like cells is effected by incubating the cells in a differentiating medium which includes at least one polyunsaturated fatty acid, such as DHA. Preferably, the differentiating medium also includes at least one neuronal differentiating agent such as BHA, dbcAMP or IBMX. More preferably, the differentiation medium further comprises DMEM, SPN, L-glutamine, N2 supplement and retinoic acid (see Example 2 of the Examples section which follows).

To further increase differentiation efficiency, BMSc are preferably incubated in a "pre-differentiation medium" prior to their incubation in the differentiation medium described above. A suitable pre-differentiation medium may be any growth medium capable of predisposing the cells to neuron-like differentiation, such as a

growth medium supplemented with the mitogen basic fibroblast growth factor (bFGF). Preferably, the pre-differentiating medium further comprises DMEM, SPN, L-glutamine, N2 supplement and FCS, such as described in Example 2 of the Examples section which follows.

The neuronal-like cells resulted from the procedure described hereinabove typically exhibit neuronal cell morphology (illustrated in Figure 3) and express at least one neuronal marker such as, for example, CD90, neuron-specific nuclear protein, neurofilament heavy, neuron-specific enolase, beta-tubulin 3, MAP-2, tyrosine hydroxylase, microtubule associated protein, nestin and calbindin. Expression of neural markers is confirmed using methods, such as immunoassays, flow cytometery, RT-PCR. Real-time PCR, and HPLC methods such as described in Examples 3- 9 and 12 in the Examples section that follows.

The differentiated cells of the present invention are preferably capable of synthesizing a neurotransmitter. A neurotransmitter synthesizing cells can be generated by incubating in a neuron-like differentiation medium such as described above or in a "dopaminergic differentiation medium", such as described in Example 9 of the Examples section which follows.

It will be appreciated that replacement therapy of Parkinson's disease utilizing dopaminergic-only cells may in the long run lead to imbalances in non-dopaminergic transmitter systems and subsequently to side effects such as wearing-off and dyskinesia (Nicholson and Brotchie, Eur J Neurol. 3:1-6, 2002). Accordingly, agents which target non-dopaminergic systems and which are capable of preventing, or limiting, the expression of involuntary movements in Parkinson's disease, have been suggested for use in treating Parkinson's patients (Djaldetti and Melamed, J Neurol. 2:30-5, 2002; Muller, T.,:Expert Opin. Pharmacother. 2:557-72, 2001; and Jenner, P. J. Neurol. 2:43-50, 2000). Furthermore, serotonin receptors have been identified as potential therapeutic targets in Parkinson's disease. (Nicholson and Brotchie, Eur J Neurol. 3:1-6, 2002).

Thus, according to another embodiment of the present invention, the population of cells utilized by the present invention is a mixed population of cells which includes two or more different neurotransmitter producing cells aimed to provide a balanced neurotransmitter production. Preferably, the mixed population of

cells includes dopaminergic as well as serotoninergic cells such as those illustrated in Figures 16A-C.

As is mentioned hereinabove, the cells utilized by this aspect of the present invention are preferably capable of controllable synthesis of the neurotransmitter.

Several approaches can be utilized to generate cells which are capable of such controlled synthesis.

Preferably, cell suitable for neuronal transplantation are harvested or generated as described hereinabove and are genetically modified to enable controllable expression of a neurotransmitter.

Genetic modification is preferably effected by transforming such cells with an expression construct which is designed for controllable expression of an enzyme participating in neurotransmitter synthesis.

The expression construct of the present invention preferably includes a polynucleotide sequence encoding an enzyme participating in synthesis of the neurotransmitter, whereas the expression construct is designed such that the polynucleotide expression is regulated via exposure to an agent or condition. Controllable expression of an enzyme participating in neurotransmitter synthesis can be effected by utilizing an expression construct which includes a polynucleotide sequence encoding the enzyme participating in neurotransmitter synthesis positioned under the transcriptional control of a promoter or regulatory which can be switched "on" (induced) or "off" (suppressed).

The expression construct can be designed as a gene knock-in construct in which case it will lead to genomic integration of construct sequences, or it can be designed as an episomal expression vector.

In any case, the expression construct can be generated using standard ligation and restriction techniques, which are well known in the art (see Maniatis *et al.*, in: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982). Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Polynucleotide sequences which can be utilized in the expression construct of the present invention are described in various databases such as that maintained by the national resource for molecular biology information (http://www.ncbi.nlm.nih.gov/). Examples include sequences set forth in GenBank Accession Nos. NM_000360

(encoding tyrosine hydroxylase); NM_000790 (encoding DOPA decarboxylase); NM_000161 (encoding GTP cyclohydrolase I); NM_000787 (encoding dopamine ß-hydroxylase); NM_002686 (encoding glutamate decarboxylase); NM_003450 (encoding tryptophane-5 monooxygenase) and NM_020549 (encoding choline acetyltransferase).

Promoters suitable for use with the present invention are preferably response elements capable for directing transcription of the polynucleotide sequence so as to confer regulatable synthesis of the neurotransmitter. A suitable response element can be, for example, a tetracycline response element (such as described by Gossen and Bujard (Proc. Natl. Acad. Sci. USA 89:5547-551, 1992); an ectysone-inducible response element (No D *et al.* (Proc Natl Acad Sci U S A. 93:3346-3351, 1996) a metal-ion response element such as described by Mayo *et al.* (Cell. 29:99-108, 1982); Brinster *et al.* (Nature 296:39-42, 1982) and Searle *et al.* (Mol. Cell. Biol. 5:1480-1489, 1985); a heat shock response element such as described by Nouer *et al.* (in: Heat Shock Response, ed. Nouer, L., CRC, Boca Raton, Fla., pp167-220, 1991); or a hormone response element such as described by Lee *et al.* (Nature 294:228-232, 1981); Hynes *et al.* (Proc. Natl. Acad. Sci. USA 78:2038-2042, 1981); Klock *et al.* (Nature 329:734-736, 1987); and Israel and Kaufman (Nucl. Acids Res. 17:2589-2604, 1989). Preferably the response element is an ectysone-inducible response element, more preferably the response element is a tetracycline response element.

The expression construct of the present invention may also include one or more enhancers. Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by reference.

Polyadenylation sequences can also be added to the expression construct in order to increase the translation efficiency of the enzyme expressed from the expression construct of the present invention. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40.

In addition to the elements already described, the expression construct of the present invention may typically contain other specialized elements intended to increase the level of expression of cloned polynucleotides or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The expression construct may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate selectable marker. If the construct does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired polynucleotide.

The expression construct of the present invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide. For example a single expression construct can be designed and co-express two distinct enzymes which participate in a neurotransmitter synthesis, such as the enzymes tyrosine hydroxylase and DOPA decarboxylase which participate in dopamine synthesis.

Examples for mammalian expression constructs include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81,

which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression constructs containing regulatory elements from eukaryotic viruses such as retroviruses can also be used by the present invention. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Viruses are specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. Thus, the type of vector used by the present invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinary skilled artisan and as such no general description of selection consideration is provided herein. For example, bone marrow cells can be targeted using the human T cell leukemia virus type I (HTLV-I).

Recombinant viral vectors are useful for *in vivo* expression of transgenic polynucleotides since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described in the Examples section which follows, the cells of the present invention can also be transformed with an expression construct, or a construct system, which includes a first polynucleotide sequence which is regulated by a transactivator positioned under the transcriptional control of a second regulatory sequence. In such an expression scheme, the transactivator is capable of activating the first regulatory sequence to direct transcription of the first polynucleotide sequence in absence of the agent.

Preferably, the first polynucleotide sequence of the expression construct, or construct system, includes a sequence encoding an enzyme participating in a synthesis of a neurotransmitter which is operably linked to an ecdysone-responsive promoter such as described by No *et al.* (Proc Natl. Acad. Sci. USA. 93:3346-3351, 1996).

More preferably, the first polynucleotide sequence of the expression construct, or construct system, includes a sequence encoding an enzyme participating in a synthesis of a neurotransmitter which is operably linked to a tetracycline control element such as described by Gossen and Bujard (Proc. Natl. Acad. Sci. USA 89:5547-551, 1992). See Example 15 of the Examples section which follows for further details.

The transactivator is preferably a tetracycline controlled transactivator such as described by Gossen and Bujard (Proc. Natl. Acad. Sci. USA 89:5547-551, 1992) operably linked to a human enolase promoter. See Example 15 of the Examples section which follows for further details.

Various methods can be used to introduce the expression construct of the present invention into mammalian cells. Such methods are generally described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang *et al.*, Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega *et al.*, Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Once transformed cells are generated, they are tested (in culture) for their ability to synthesize a functional neurotransmitter in response to an external signal (e.g., presence or absence of an agent). Preferably, the neurotransmitter concentration is comparatively (in presence vs absence of the agent) analyzed using standard chemical analytical methods such as, for example, HPLC or GC-MS. Alternatively the cultures are comparatively analyzed for expression of the recombinant enzyme (e.g., tyrosine hydroxylase), using biochemical analytical methods such as immunoassays, Western blot and Real-time PCR using the procedures such as described in Examples 7 of the Examples section which follows, or by enzyme activity bioassays.

It will be appreciated that in cases where cells capable of endogenously producing a neurotransmitter are selected for use with the present invention (e.g., neuronal-induced BMSc) such cells are preferably genetically manipulated such as to delete or mutate endogenous coding sequences of enzymes participating in the neurotransmitter synthesis (e.g., endogenous tyrosine hydroxylase). Such genetic manipulation can be effected by, for example, by employing gene knock-out or site directed mutation techniques and vectors such as those described by Galli-Taliadoros *et al.* (J Immunol Methods 181:1-15, 1995) and Harris and Ford (Pharmacogenomics. 1:433-43, 2000). Alternatively, cells capable of endogenously producing a neurotransmitter can be eliminated by exposure to nerotoxins (e.g., MPTP) or by transformation with a suicide vector, such as illustrated in Figure 18.

Deletion of endogenous sequences can be combined with knock-in of exogenous enzyme coding sequences (such as those described above) such that cells simultaneously lose the ability to endogenously synthesize neurotransmitters and acquire such an ability (regulatable) through genomic integration of exogenous sequences which encode the enzyme positioned under the transcriptional control of a controllable regulatory sequence.

Alternatively, such cells can also be genetically manipulated such that endogenous enzyme coding sequences are brought under control of a regulatable promoter sequence. Such manipulation can be achieved by replacing the endogenous promoter sequence of the enzyme (e.g., the TH promoter sequence) via gene knock-in of a regulatable promoter sequence.

Optionally, the cells of the present invention are transformed so as to acquire resistance to cell death occurring during brain transplantation. It has been found that cells implanted in brain tissue may undergo apoptosis triggered by hypoxia, hypoglycemia, mechanical trauma, free radicals, growth factor depravation, and excessive extracellular concentrations of excitatory amino acids in the host brain (Brundin *et al.* (Cell Transplant. 9:179-195, 2000). Under circumstances where the risk of apoptosis-induced cell death is high, the cells of the present invention can be transformed with a polynucleotide encoding an apoptosis inhibiting polypeptide such as, for example, the human bcl-2 gene (Adams and Cory, Science 281:1322-1326, 1998). The polypeptide can be expressed under the control of a constitutive promoter such as described hereinabove, or preferably, under a control of a neuronal tissue-specific promoter such as, for example the human neuron-specific enolase (NSE) promoter as described by Levy *et al.* (Journal of Molecular Neuroscience 21:121-132, 2003).

The cells of the present invention can be administered to the treated individual using a variety of transplantation approaches, the nature of which depends on the site of implantation.

The term or phrase "transplantation", "cell replacement" or "grafting" are used interchangeably herein and refer to the introduction of the cells of the present invention to target tissue. The cells can be derived from the recipient or from an allogeneic or xenogeneic donor.

The cells can be grafted into the central nervous system or into the ventricular cavities or subdurally onto the surface of a host brain. Conditions for successful transplantation include: (i) viability of the implant; (ii) retention of the graft at the site of transplantation; and (iii) minimum amount of pathological reaction at the site of transplantation. Methods for transplanting various nerve tissues, for example embryonic brain tissue, into host brains have been described in: "Neural grafting in the mammalian CNS", Bjorklund and Stenevi, eds. (1985) These procedures include intraparenchymal transplantation, i.e. within the host brain (as compared to outside the brain or extraparenchymal transplantation) achieved by injection or deposition of tissue within the host brain so as to be opposed to the brain parenchyma at the time of transplantation

Intraparenchymal transplantation can be effected using two approaches: (i)

injection of cells into the host brain parenchyma or (ii) preparing a cavity by surgical means to expose the host brain parenchyma and then depositing the graft into the cavity. Both methods provide parenchymal deposition between the graft and host brain tissue at the time of grafting, and both facilitate anatomical integration between the graft and host brain tissue. This is of importance if it is required that the graft becomes an integral part of the host brain and survives for the life of the host.

Alternatively, the graft may be placed in a ventricle, e.g. a cerebral ventricle or subdurally, i.e. on the surface of the host brain where it is separated from the host brain parenchyma by the intervening pia mater or arachnoid and pia mater. Grafting to the ventricle may be accomplished by injection of the donor cells or by growing the cells in a substrate such as 3% collagen to form a plug of solid tissue which may then be implanted into the ventricle to prevent dislocation of the graft. For subdural grafting, the cells may be injected around the surface of the brain after making a slit in the dura. Injections into selected regions of the host brain may be made by drilling a hole and piercing the dura to permit the needle of a microsyringe to be inserted. The microsyringe is preferably mounted in a stereotaxic frame and three dimensional stereotaxic coordinates are selected for placing the needle into the desired location of the brain or spinal cord. The cells may also be introduced into the putamen, nucleus basalis, hippocampus cortex, striatum, substantia nigra or caudate regions of the brain, as well as the spinal cord.

For transplanting, the cell suspension is drawn up into the syringe and administered to anesthetized transplantation recipients. Multiple injections may be made using this procedure.

The cellular suspension procedure thus permits grafting of the cells to any predetermined site in the brain or spinal cord, is relatively non-traumatic, allows multiple grafting simultaneously in several different sites or the same site using the same cell suspension, and permits mixtures of cells from different anatomical regions. Multiple grafts may consist of a mixture of cell types, and/or a mixture of transgenes inserted into the cells. Preferably from approximately 10^4 to approximately 10^8 cells are introduced per graft.

For transplantation into cavities, which may be preferred for spinal cord grafting, tissue is removed from regions close to the external surface of the central nerve system (CNS) to form a transplantation cavity, for example as described by

Stenevi *et al.* (Brain Res. 114:1-20., 1976), by removing bone overlying the brain and stopping bleeding with a material such a gelfoam. Suction may be used to create the cavity. The graft is then placed in the cavity. More than one transplant may be placed in the same cavity using injection of cells or solid tissue implants. Preferably, the site of implantation is dictated by the type of neurotransmitter being synthesized by the cells of the present invention. For example, dopaminergic cells are preferably implanted in the sabstantia nigra of a Parkinson's patient.

The cells of the present invention may be co-administered with therapeutic agents useful in treating neurodegenerative disorders, such as growth factors, e.g. nerve growth factor; gangliosides; antibiotics, neurotransmitters, neurohormones, toxins, neurite promoting molecules; and antimetabolites and precursors of these molecules such as L-DOPA.

Following transplantation, the treated individual is carefully and continuously monitored for the level of neurotransmitter released by the implanted cells. The neurotransmitter level is preferably estimated indirectly by using clinical tests suitable for diagnosing the neurodegenerative disorder. For example, the release of dopamine by implanted cells in a Parkinson's disease patient can be estimated using clinical diagnosis tests for Parkinson's disease such as described, for example in Adker, C. H. and Ahlskog, J. E eds. ("Parkinson's Disease and Movement Disorders, Diagnosis and Treatment Guidelines for the Practicing Physician, Humana Press", New Jersey, 2000). Based on monitored indications, the neurotransmitter release rate is adjusted by administering to the individual, or withholding from the individual, an agent capable of regulating synthesis of the neurotransmitter in the implanted cells. The agent may be any molecule capable of upregulating or downregulating the expression of an enzyme participating in the synthesis of the neurotransmitter, such as described hereinabove.

The agent can be administered directly to the individual or as a part (active ingredient) of a pharmaceutical composition.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients or agents described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. Preferably, a dose is formulated in an animal model to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (see e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1). For example, Parkinson's patient can be monitored symptomatically for improved motor functions indicating positive response to treatment, and for runaway diskinesis symptoms indicating an excessive dopamine expression.

The agent can be administered to the patient in various ways, including but not limited to oral administration, parenteral administration, intrathecal administration, intraventricular administration and intranigral application. The exact formulation, route of administration and dosage can be chosen by the individual physician in view

of the patient's condition. (see e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to levels of the active ingredient which are sufficient to effectively regulate the neurotransmitter synthesis by the implanted cells. Dosages necessary to achieve the desired effect will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the individual being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual changing condition. For example, a treated Parkinson's patient will be administered with an amount of agent which is sufficient to promote, or suppress, dopamine synthesis to the level desired, based on the monitoring indications.

Hence, the invention provides novel nucleic acid constructs, construct systems, cells and methods of cell therapy of neurodegenerative diseases which is effective, safe and clinically practical.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984); "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996) and Parfitt *et al.* (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2 (6), 595-610; all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and

are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Isolation and culturing of human bone-marrow stromal cells (hBMSc)

Methods:

Proliferation culture: Bone marrow aspirates (10 ml) were obtained from iliac crest of healthy human donors with informed consent. Mononuclear cells were isolated by centrifugation through a density gradient (Histopaque®-1077), and were plated in a "proliferation medium" [Dulbecco's modified eagle medium (DMEM; Biological Indutries); 100 µg/ml streptomycin, 100 U/ml penicillin, 12.5 units/ml nystatin (SPN; Biological Industries); 2 mM L-glutamine; 5% horse serum; 15% fetal calf serum (FCS; Biological Industries); 0.001% 2-ß-mercaptoethanol (Sigma); 1x non-essential amino acids; 10 ng/ml human epidermal growth factor (EGF)]. The cells were incubated for two days at 37°C in a humidified 5% CO2 incubator, and non-adherent cells were then discarded. The remaining plastic-adherent cells were washed twice with Dulbecco's phosphate-buffered saline (PBS; Biological Industries), and fresh growth medium was added. The medium was replaced every 3 or 4 days. Cells grew to 80-90% confluency within 15 days and appeared round or spindle shaped (Figure 1A) or a flat shaped (Figures 1B-C).

Flow cytometry: Following 15 days under proliferative culturing conditions, the cells were harvested and suspended in 0.05% trypsin and 25 mM EDTA in phosphate-buffered saline (PBS). The cells, in solution at a concentration of 0.5×10^6 cells/ml, were stained with antibodies specific against the cell surface markers CD45, CD5, CD20, CD11b and CD34 (associated with lympho-hematopoietic cells; Becton-Dickinson) for 20 min with an empirically determined amount of each antibody, generally 10 to 20 µl. The antibody-labeled cells were thoroughly washed with two volumes of PBS and fixed in flow buffer (1% paraformaldehyde, 0.1% sodium azide, and 0.5% bovine serum albumin in PBS). The washed cells were analyzed by a FACSCalibur™ flow cytometer (Becton Dickinson), equipped with an argon ion laser, adjusted to an excitation wavelength of 488nm, and by collecting 10,000 events with the CELLQuest™ software program (Becton Dickinson).

Results:

The cultured hBMSc did not express any of the surface markers associated with lympho-hematopoietic cells (i.e., CD45, CD5, CD20, CD11b and CD34), but rather expressed the CD90 surface marker (Thy-1), which is indicative of synaptogenesis (Figure 2).

EXAMPLE 2

In vitro differentiation of human bone-marrow stromal cells (hBMSc)

Methods:

Differentiation cultures: hBMSc were cultured in the "proliferation medium" (described in Example 1 hereinabove) for up to three months prior to differentiation induction. Plastic-adherent cells were then transferred to a "pre-differentiation medium" [DMEM supplemented with SPN, 2 mM glutamine, N2 supplement (insulin 25 µg/ml; progesterone 20 nM; putrescin 100 µM; selenium 30 nM; transferrin 100 µg/ml), 10% FCS, 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, MN), and 10 ng/ml human epidermal growth factor (EGF; R&D Systems)]. Following 24 hr incubation 37°C the cells were transferred to a "differentiating medium" [DMEM supplemented with SPN, 2 mM glutamine, N2 supplement (insulin 25 µg/ml; progesterone 20 nM; putrescin 100 µM; selenium 30 nM; medium to a density of 1 X 10^5 cells/ml. The suspensions were transferrin 100 µg/ml), 200 µM butylated hydroxyanisole (BHA; Sigma), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 0.5 mM isobutylmethlxanthine (IBMX; Sigma), 10 µM docosahexaenoic acid (DHA; Sigma), and 10 µM retinoic acid (RA; Sigma)] and incubated at 37°C for 12-72 hr.

Proliferation assessment: hBMSc were suspended in the "pre-differentiation medium" and in the "differentiation dispensed in 96-well microtiter plates (100 µl/well) and incubated for 16 and 39 hr at 37°C. The cultures were then supplemented with 10 µCi/ml ^{3}H-thymidine and incubated for four additional hours. Cells were then harvested by suspended in 0.05% trypsin and 25 mM EDTA in phosphate-buffered saline (PBS), and analyzed with a liquid scintillation counter to determine the level of ^{3}H-thymidine incorporation in the cells (indicative of proliferation activity).

Results:

Plastic-adherent cells exhibited neuronal-like spindle body shape with long branching processes that appeared as early as three hours post differentiation induction and continued to appear 72 h following differentiation induction (Fig 3A-F).

^{3}H-thymidine incorporation was substantially reduced in the differentiated cells (Figure 4) thus indicating that proliferation was attenuated in the neuronal-like differentiated hBMSc.

EXAMPLE 3

Identification of neuronal transcripts in differentiating hBMSc

Methods

RT-PCR: hBMSc which were incubated in the "proliferation medium" or in the "differentiation medium" (see Examples 1-2 hereinabove) for 3-72 hours at 37°C. Total RNA was extracted from the hBMSc by using the guanidine isothiocyanate method as described by Chomczynski & Sacchi (1987). In addition total RNA was extracted from fresh human lymphocytes (from donor) using the RNA isolated kit (Puregene Gentra, Manneapolis, USA). The RNA samples were separated on 1% agarose formaldehyde–denaturing gel electrophoreses to verify their integrity. For generating cDNA the RNA samples (0.5 µg) were mixed with RT-superscript II enzyme (10 units) contained in a reaction mixture [1.3 µM random primer, 1x Buffer (supplied by InvitroGene), 10 mM DTT, 20 µM dNTPs, and RNase inhibitor] and incubated at 25°C for 10 min, 42°C for 2 hours, 70°C for 15 min and 95°C for 5 min. The resulting cDNA samples were analyzed by PCR using the primers set forth by SEQ ID NOs: 1-2, 11-12, 21-22, 26-26 and 29-30 (see Table 1 below) and amplified under 35 cycles at 94° C for 1 min, 55-58° C for 1 min and 72° C for 1 min.

Table 1

Upstream sense and downstream anti-sense primers from different exons for detection speuronal and dopaminergic transcripts in differentiated hBMSc

Human gene NCBI No.	Cellular Function	5' Primer SEQ ID NO	3' Primer SEQ ID NO	Product size (bp)
CD90 (Thy-1) NM 006288	May play a role during synaptogenesis	1	2	312

Dopamine receptor D2 (D2DR) NM_016574	one of the five types (D1 to D5) of receptors for dopamine	3	4	159
Dopamine transporter (DAT) NM_001044	Amine transporter. Terminates the action of dopamine by its high affinity sodium-dependent reuptake into presynaptic terminals.	5	6	253
Aromatic L-amino acid decarboxylase (AADC) NM_000790	Catalyzes the decarboxylation of L-3,4-dihydroxyphenylalanine (DOPA) to dopamine, L-5-hydroxytryptophan to serotonin and L-tryptophan to tryptamine	7	8	250
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) NM_002046	Catalytic enzyme of glycolysis	9	10	194
Glypican 4 (GPC4) NM_001448	Cell surface proteoglycan may be involved in the development of CNS	11	12	386
GTP cyclohydrolase 1 (GTPCH1) NM_000161	Tetrahydrobiopterin biosynthesis	13	14	153
Necdin NM_002487 (* Only one exone)	Postmitotic neuron-specific growth suppressor	15	16	394
Neurite growth-promoting factor 2 (NEGF2) X55110	Extracellular matrix-associated protein that enhances axonal growth in perinatal cerebral neurons.	17	18	359
Nestin X65964	Intermediate filament protein a predominant marker used to describe stem and progenitor cells in the mammalian CNS	19	20	398
Neuron specific enolase X13120	Isozyme of the glycolytic enzyme enolase is expressed in all neuronal cell types	21	22	356
Neurofilament heavy (NF-H) NM_021076	200kDa filaments slowly transported within the axons towards the synaptic terminals	23	24	400
Neurofilament medium (NF-M) XM_005158	160kDa filaments slowly transported within axons towards the synaptic terminals	25	26	366
Nuclear receptor related 1(Nurr1) NM_006186	Dopaminergic transcription factor	27	28	550
Retinoic acid receptor type α (RA-R) NM_000964	Receptor for retinoic acid	29	30	352

Northern blot analysis: RNA samples extracted from hBMSc were size fractionated on 1% agarose gel supplemented with 3% formaldehyde and MOPS, and transferred to Duralon-UVTM membranes (Stratagene). The membranes were then hybridized overnight with purified ^{32}P-labeled probes for neuronal markers NEGF2 (neurite growth-promoting factor 2), NF-200 (neurofilament heavy), and NSE (neuron specific enolase). The hybridized membranes were washed several times, exposed to storage phosphor screen, autoradiographed by phosphorimager (Cyclone, Packard), stripped and rehybridized with a ^{32}P-labeled probe for GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) to verify equal loading and transfer of RNA.

Real time PCR: Real-time quantitative PCR analysis of the neuronal marker NEGF2 was performed in a "Rotor-Gene DNA sample analysis system" version 4.6 (Corbett Research) using Sybergreen "PCR master mix" and the primers of SEQ ID NOs: 17-18. In addition, Real time PCR analysis of GADPH was performed for providing stimulated conditions for sample normalization using the primers of SEQ ID NOs: 9-10. The amplification protocol was 40 cycles of 95°C for 15 sec, 55°C for 40 sec, 72°C for 40 sec and 77°C for 20 sec. Stimulated conditions for sample normalization were applied by amplification of 18S rRNA. The amplification protocol was 80 cycles of 95°C for 20 and 61°C for 1 min. Quantification of gene expression relative to 18S rRNA was calculated by the protocol's ΔΔCT method and from standard curve method.

Results:

RT-PCR analysis indicates transcriptional expression of neuronal markers nestin, NSE, NF-M, CD90, RA-R and GPC4 in both differentiated and non-differentiated hBMSc (Figure 5A). However, transcriptional expression of the neuronal markers NF-H and necdin occurred only in the differentiated hBMSc (Figure 5A).

Real time PCR analysis shows a seven fold increase of NEGF2 mRNA in the differentiating cells, as compared with non-differentiated cells, following 50 hr incubation (Figure 5B).

Northern blot analyses show that transcriptional expression of the neuronal markers NEGF2, NF-200 and NSE markedly increased in differentiating hBMSc (Figures 5B-D, respectively).

EXAMPLE 4

Identification of neuronal proteins in differentiated hBMSc

Methods:

Western blot: Fifty micrograms of protein extracts obtained from hBMSc were denatured in a sample buffer (62.5 mM Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, 5% 2-ß-mercaptoethanol, 0.0025% bromophenol blue (SIGMA), diluted 1:5 with the sample and boiled for 5 min. Each sample was loaded on a 12.5% SDS-polyacrylamide gel (Bio-Rad Laboratories), according to the manufacturer's instruction. Following electrophoresis, proteins were transferred to polyvinylidene difluride membrane (Bio-Rad Laboratories), followed by blocking with 5% nonfat milk in Tris-buffered saline (TSB 10 mM Tris at 7.5, 150 mM NaCl) with 0.1% Tween-20 (blocking solution). The membranes were probed overnight, at 4°C, with neuron markers-specific antibodies as described in Table 2 hereinbelow. Following incubation, were the membranes were washed twice (15 min each) with blocking solution and once with TBS-T for 15 min, then exposed to horseradish peroxidase-conjugated secondary antibody as described in Table 3 hereinbelow. The membranes were then washed twice (15 min each) with blocking solution and once in TBS-T for 15 min and were stained using the enhanced SuperSignal® chemiluminescent detection kit (Pierce) and exposed to medical X-ray film (Fuji Photo Film). Actin was used to evaluate and quantify the changes during the induction. Densitometry of the specific proteins bands was preformed by using VersaDoc® imaging system (Bio-Rad Laboratories) and Quantity One® software (Bio-Rad).

Table 2

Neuronal and dopaminergic marker-specific antibodies

Specificity	Source	Dilution for IA*	Dilution for WB*	Dilution for FC*	Source
Actin	Mouse		1:1000		Chemicon International
β-tubulin III	Mouse	1:400		1:50	Sigma
D2 Dopamine receptor	Mouse			1:50	Santa-Cruz
Glial fibrillary acidic protein (GFAP)	Rabbit	1:80			Sigma
Glial fibrillary acidic protein (GFAP)	Rabbit			1:50	Dako
Human nestin	Rabbit	1:200			Kindly supplied by Messam CA., NIH
Neurofilament 200 (NF-H)	Mouse	1:200	1:1000		Sigma
Neuron specific enolase (NSE)	Mouse	1:100	1:1000		Cymbus Biotechnology
Neuronal nuclei (NeuN)	Mouse	1:50	1:2500		Chemicon International
Tyrosine hydroxylase (TH)	Rabbit	1:1000			Chemicon International
Tyrosine hydroxylase (TH)	Rabbit			1:50	Calbiochem
Vesicular monoamine transporter 2 (VMAT2)	Rabbit	1:50			Chemicon International

* IA= immunoassay; WB = Western blot; FC = Flow cytometer

Table 3

Anti-mouse and anti-rabbit secondary antibodies

Specificity	Source	Dilution for IA*	Dilution for WB*	Dilution for FC*	Conjugated	Source
Mouse	Goat	1:100		1:100	Cy™2	Jackson ImmunoResearch Laboratories
Mouse	Donkey	1:300			Cy™3	Jackson ImmunoResearch Laboratories
Mouse	Sheep	1:50			FITC	Sigma

Mouse	Goat		1:20000		Peroxidase	Jackson ImmunoResearch Laboratories
Rabbit	Donkey	1:300			Cy™2	Jackson ImmunoResearch Laboratories
Rabbit	Goat	1:400		1:100	Cy™3	Jackson ImmunoResearch Laboratories
Rabbit	Swine	1:20			FITC	Dako
Rabbit	Goat		1:25000		Peroxidase	Jackson ImmunoResearch Laboratories

* IA= immunoassay; WB = Western blot; FC = Flow cytometer

Results:

Immuno-staining revealed the presence of neuronal markers NeuN, NF-200, NSE, in differentiating hBMSc 12, 24, 48 and 48 hr following differentiation induction, respectively (Figure 6A). Antibody-labeled GFAP and β-tubulin III were observed in hBMSc 48 hr and 5 days following differentiation induction, respectively (Figure 6B).

The expression of Neu-N, NSE and nestin proteins (relative to actin), increased substantially following differentiation induction, as indicated by Western blot analysis (Figures 7A-C).

EXAMPLE 5

Long- term survival of differentiating hBMSc

Methods:

Long-term differentiation culture: hBMSCs were incubated in the "pre-differentiation medium" (see Example 2 above) for 24 hr at 37°C then transferred to a "long-term differentiation medium" [DMEM supplemented with SPN, 2mM glutamine, N2 supplement (insulin 25 µg/ml; progesterone 20 nM; putrescin 100 µM; selenium 30 nM; transferrin 100 µg/ml), 1 mM dbcAMP, 0.5 mM IBMX, 10 µM docosahexaenoic acid (DHA; Sigma), 10 ng/ml bFGF, 10 ng/ml human glial cell line-derived neurotrophic factor (GDNF), and 10 ng/ml human beta nerve growth factor

(β-NGF)]. The hMSCs were incubated in the "long-term differentiation medium" for 28 days at 37°C.

Immunoassay: Cells were plated and treated in slide chambers (Nalge Nunc International) previously treated aseptically with poly-L-lysine (Sigma). The cells were fixed with 4% paraformaldehyde in PBS (pH 7.3) for 30 min at 4°C and 30 min a room temperature. The slides were then washed three times with PBS (5 min each) and permeabilized with PBS containing 0.1% Triton X-100 (Sigma) and 10% goat serum (Biological Industries) for 10 min at 4°C and 10 min at room temperature. The slides were then washed three times with PBS (5 min each). The endogenous peroxide was blocked by adding 3% H_2O_2 (Merck) in methanol absolute (Bio-Lab, Israel) for 20 min at room temperature. Following three washes in PBS (5 min each), slides chambers were incubated overnight at 4°C with anti-MAP2, anti-β-tubulin III, or anti-nestin diluted as described in Tables 2-3 in Example 4 hereinabove. On the next day, the slides were washed thoroughly three times in PBS (10 min each) then incubated for 30 minutes at room temperature with Cy™3-conjugated goat anti-rabbit IgG or Cy™2- conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) in 10% goat serum and 0.2% Twenn-20 in PBS. Following incubation, the slides were washed three times with PBS (5 min each), mounted with glycerol vinyl alcohol mounting solution (Zymed Laboratories), covered with glass slips and examined under a florescence microscope.

Results:

hBMSc exhibited typical neuron-like cell morphology following 28 day incubation in the "long-term differentiation medium" (Figure 8).

Antibody-labeled neuronal markers MAP2 (microtubule-associated protein 2), β-tubulin III and nestin were observed in hBMSc following 28 day incubation (Figures 9A-C, respectively).

EXAMPLE 6

Expression of dopaminergic mRNAs in differentiated hBMSc

Methods:

RT-PCR: hBMSc were cultured for 12-72 hr in the "proliferation medium" and in the "differentiation medium" (see Example 1-2 hereinabove). Total RNA was extracted from the hBMSc by using the guanidine isothiocyanate method as described

by Chomczynski & Sacchi (1987). The RT-PCR procedure designed for identifying dopaminergic markers was performed essentially as described in Example 3 hereinabove except for using the primers of SEQ ID NOs: 3-8, 13-14 and 27-28.

Results:

As can be seen in Figure 10, transcripts of several dopaminergic markers were expressed in differentiated and/or non-differentiated hBMSc. These include Nurr1 [nuclear receptor related 1; a transcription factor that has role in the differentiation of midbrain precursors into dopamine neurons], and AADC [aromatic L-amino acid decarboxylase; the enzyme which catalyzes the decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) to dopamine, L-5-hydroxytryptophan to serotonin and L-tryptophan to tryptamine]. Transcriptional expression of GTP [cyclohydrolase 1; the enzyme necessary for production of tetrahydrobiopterin (BH4) cofactor for TH] was markedly higher in the differentiated hBMSc, as compared with the non-differentiated hBMSc, while transcripts of D2 dopamine receptor and DAT dopamine transporter were expressed only in the differentiated hBMSc.

EXAMPLE 7

Induction of tyrosine hydroxylase in differentiated hBMSc

Methods:

Real time PCR: Total RNA was extracted from hBMSc using the guanidine isothiocyanate method as described by Chomczynski & Sacchi (1987). cDNA was generated as described in Example 3 hereinabove by carrying out a RT reaction with random primers. Amplification of cDNA was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems) using TaqMan® universal PCR master mix using specific primers of human TH and 18S rRNA (Applied Biosystems). Stimulated conditions for sample normalization were applied by amplification of 18S rRNA. The amplification protocol was 80 cycles of 95°C for 20 and 61°C for 1 min. Quantification of gene expression relative to 18S rRNA was calculated by the protocol's ΔΔCT method and from standard curve method.

Western blot assay: The assay was performed as described in Example 4 hereinabove except for immunoblotting with anti-TH and anti-actin antibodies.

Immunoassay: The assay was performed as described in Example 5 hereinabove using anti-TH antibody as described in Tables 2-3 in Example 4 hereinabove.

Results

Tyrosine hydroxylase mRNA and protein levels were substantially elevated during neuronal differentiation of hBMSc (Figures 11A-B, respectively). In addition, the presence of antibody-labeled tyrosine hydroxylase was observed in differentiating hBMSc, 6 to 48 hours following differentiation induction (Figure 11C).

EXAMPLE 8

Identification of dopamine-related proteins in hBMSc

Methods:

Immunoassay: hBMSc were incubated for five days in the "differentiation medium" (see Example 2 hereinabove), then harvested and stained with a specific antibody against vesicular monoamine transporter 2 (VMAT-2) using the procedure described in Example 5 above. Antibody binding to VMAT-2 in cells was visualized by using a secondary Cy™3-conjugated antibody. The stained cells were observed under a laser confocal microscope LSM 510 (ZEIZZ, Germany).

Flow cytometry: Following 48 hours incubation in the "differentiation medium" (see Example 2 hereinabove), the cells were harvested and suspended in 0.05% trypsin and 25 mM EDTA in phosphate-buffered saline (PBS). Cell suspensions (0.5 x 10^6 cells/ml), were incubated for 30 minutes with antibodies specific against D2 dopamine receptor for as described in Tables 2-3 in Example 4 above. The antibody-labeled cells were thoroughly washed with two volumes of PBS and fixed in flow buffer (1% paraformaldehyde, 0.1% sodium azide, and 0.5% bovine serum albumin in PBS). The washed cells were analyzed by a FACSCalibur™ flow cytometer (Becton Dickinson).

Results:

Fluorescent microscope images of hBMSc revealed that antibody-labeled VMAT2 domaminogenic marker was present in differentiated hBMSc but not in the non-differentiated cells (Figure 12).

Similarly, flow cytometer analysis shows that the dopaminogenin marker D2 expressed in differentiated hBMSc but not in the non-differentiated cells (Figure 13).

EXAMPLE 9

Dopamine secretion by differentiating hBMSc is induced by neurotrophic factors

Methods:

Cell culture: hBMSc were cultured as described in Table 4 below.

Table 4
Culture media used to induce dopamine secretion by hBMSc

Stage 1: Proliferation medium (weeks)	Dulbecco's modified eagle medium (DMEM; without HEPES); 100 µg/ml streptomycin, 100U/ml penicillin, 12.5 units/ml nystatin (SPN); 2mM L-glutamine; 5% horse serum; 15% fetal calf serum; 0.001% 2-ß-mercaptoethanol; Non-essential amino acids X1; 10 ng/ml human epidermal growth factor (EGF)
Stage 2: Pre-differentiation medium (24-72 hr)	DMEM/F12 (without HEPES); 2mM L-glutamine; SPN; 10ng/ml human basic fibroblast growth factor; 10 ng/ml EGF; *N2 supplement
Stage 3: Dopaminergic differentiation medium (12-96 hr)	DMEM/F12; 2mM L-glutamine; SPN; *N2 supplement; 200µM ascorbic acid; 1 mM dibutyryl cyclic AMP; 0.5 mM isobutylmethlxanthine; 10 µM retinoic acid; 10 µM docosahexaenoic acid; human transforming growth factor β3 (TGF-β3), 2 ng/ml; human galia-derived neurotrophic factor: (GDNF), 2 ng/ml; human neurturin: (hNTN), 20 ng/ml; human brain-derived neurotrophic factor: (BDNF), (10 ng/ml; human neurotrophin: (hNT-3), 20 ng/ml; human interleukin-1β (hIL-1β), 100 pg/ml; fibroblast growth factor-8 (FGF8), 100 ng/ml; and sonic hedgehog (SHH), 200 ng/ml.

*N2 supplement: insulin 25 µg/ml; progesterone 20 nM; putrescin 100 µM; selenium 30 nM; transferrin 100 µg/ml.

HPLC analysis: Samples were stabilized by adding 88 µl of 85% orthophosphoric acid and 4.4 mg of metabisulfite to ml sample. Dopamine was extracted by aluminium adsorption (Alumina, Bioanalytical Systems Inc.). Separation of injected samples (50 µl) was effected by isocratic elution on a HPLC-electron chemical detection (HPLC-ECD) system with a reverse-phase C18 column (125 X 4.6 mm dimension, Hichrom, Inc.) in a monochloroacetate buffer mobile phase. The flow rate was set at 1.2 ml per min, and the oxidative potential of the analytical cell was set at +650 mV. Results were validated by co-elution with dopamine standards under varying buffer conditions and detector settings.

Results:

The amount of dopamine measured in the supernatant of differentiating hBMSc increased from a non-detectable level to about 23 ng/ml (10^5 cells) during the 72 hours incubation period in the "dopaminergic differentiating medium" (Figure 14A). Inducing cell polarization by KCl (supplementing the medium with 56 mM KCl followed by 10 minutes incubation) further enhanced dopamine secretion (Figure 14B). The amount of DOPA (dopamine precursor) synthesized by the differentiating hBMSc increased from about 10 to 300 pg/ml (10^5 cells) during the 72 hours incubation period in the "dopaminergic differentiating medium" (Figure 14C), while the amount of DOPAC (dopamine metabolite) increased from a non-detectable level to about 105 ng/ml (10^5 cells) during the 50 hours incubation period in the "dopaminergic differentiating medium" (Figure 14D).

EXAMPLE 10

Transplantation of mouse BMSc in the striatum of a rat model for Parkinson's disease improves rotational behavior

Methods:

Generating mouse bone marrow stromal cells (mouse BMSc): Mouse BMS cells were obtained from transgenic male mice bearing the enhanced green fluorescent protein (Tg-EGFP; Hadjantonakis *et al.*, 1998). The mice were sacrificed by cervical dislocation and the tibias and femurs were removed and placed in Hank's balanced salt solution (HBSS). Mouse bone marrow cells were collected by flushing out the marrow using a syringe (1 ml) with 25G needle, filled with 0.5 ml sterile HBSS. The collected cells were disaggregated by gentle repeat pipetting until a milky homogenous single-cell suspension was achieved. The single-cell suspension was washed in 5 ml HBSS and centrifuged under 1000g for 20 min at room temperature. Following centrifugation, the supernatant was discarded and the cell pellet was resuspended in 10 ml growth medium.

Cell culture: Isolated mouse bone marrow cells were cultured in the "proliferation medium" (see in Example 1 hereinabove) and incubated for 48 hr at 37°C. The non-adherent layer was then discarded and the tightly adhered cells were washed twice with PBS and cultured in a fresh "proliferation medium". The growth medium was replaced every 3-4 days until cells reached 70%-90% confluency. The

cells were then harvested and mixed in a trypsin-EDTA solution (0.05% trypsin and 25 mM EDTA in PBS), incubated for 5 minutes at 37°C, then transferred to the "pre-differentiation medium" (see in Example 2 hereinabove) for an additional incubation of 72 hr at 37°C. The pre-differentiated cells were washed in PBS and induced for neural-like differentiation by incubation in the "differentiation medium" (see in Example 1 hereinabove) for 12-72 hr at 37°C.

Mouse BMSc transplantation: neural-differentiated bone marrow stromal cells (mBMSc) were injected in the substania nigra of female 6-OHDA lesioned rats using stereotactic frame (as described by Bjorklund *et al.*, 2002). Saline injection was used as a control.

Rotational behavior analysis: 6-OHDA-lesioned rats were treated with amphetamine 5 mg/kg to induce rotational behavior. The rotational response to amphetamine was examined 3, 15, 30 and 45 days post transplantation using a computerized rotameter (San Diego Instruments).

Results:

Transplantation of neuronal-induced mBMSc into amphetamine-induced 6-OHDA rats substantially reduced rotational behavior of the rats, from about 340 to just 25 rotations per 2 hr, 45 days post transplantation (Figure 15A). The relative rotation rate of the treated rats was reduced by 97.9%, as compared with saline-treated rats, 45 days post transplantation (Figure 15B).

EXAMPLE 11

Survival and migration of transplanted mouse BMSc in rat brain

Methods:

Neuronal differentiated Tg-EGEF mouse BMSc were prepared and transplanted in the substania nigra of 6-OHDA rats (both hemispheres), as described in Example 10 hereinabove. Treated and untreated (saline only) rats were sacrificed 45 days post transplantation. Tissue sampled from lesioned and non-lesioned rat hemispheres were sectioned and observed under a fluorescent microscope, for the presence of green fluorescent protein (GFP) marking the transplanted mBMSc.

Results:

mBMSc survived in the treated rats substania nigra (Figure 15C) and immigrated into the treated rats striatum (Figure 15D), 45 days after the nigral

transplantation. In addition, transplanted cells successfully migrated to the cortex and striatum (Figures 15C-D). In rats that were injected with saline the rotational behavior did not changed.

EXAMPLE 12

Mouse BMSc differentiated into oligodendrocytes precursors

Methods:

Cell culture: Mice B5/EGFP (male) were sacrificed by cervical dislocation and were prepared with 70% alcohol solution. After tibias and femurs were removed and placed in Hank's balanced salt solution (HBSS; Biological Industries, Bet-Haemek, Israel), mouse bone marrow cells were collected by flushing out the marrow using a syringe (1 ml) with 25G needle, filled with 0.5 ml sterile HBSS. Cells were disaggregated by gentle pipetting several times until a milky homogenous single-cell suspension was achieved. Bone marrow aspirate was diluted and washed by adding 5 ml HBSS, centrifuged at 1000g for 20 min at room temperature (RT), and removing supernatant. The cell pellet was resuspended in 1 ml growth medium and diluted to 10 ml. The cells were plated in polystyrene plastic tissue cultures 75 cm^2 flask (Corning Incorporated, Corning, NY) in the "proliferation medium" (see Example 1 hereinabove) for one week. The cells were then transferred to polylysin-coated slide-chambers (3200 cells/well), supplemented "proliferation medium" and incubated for 24 hours at 37°C. The growth medium was then replaced with "oligodendrocytes differentiation media" composed of DMEM supplemented with 2mM glutamine, SPN, one or more of the following substances: bFGF (10 ng/ml), EGF (10-20 ng/ml), Interlukin-1b (20-40 ng/ml), dbcAMP (1-2 mM), retinoic acid (0.5 or 1 μM), neurotrophin-3 (50 or 100 ng/ml), human platelet derived growth factor (PDGF-AA; 5-20ng/ml), N2 supplement, triiodothyronien (T3; 40ng/ml) and ciliary neurotrophic factor (20 ng/ml; CNTF).

Immunoassay: The cultures were incubated at 37°C for 1, 2 or 6 days (replacing growth media with fresh media every two days) then fixed in 4% PFA. Cells were blocked in 10% FCS solution then incubated with 5 ug/ml anti-A2B5 monoclonal antibody (1:200; R&D systems; 1:200) overnight at 4°C. Cells were then washed twice in PBS for 10 min and incubated with goat-anti-mouse Cy-3 second antibody (Jackson laboratories; 1:500) at room temperature for 20 min. The incidence

of cells stained positive for A2B5 (an early marker of oligodendrocyte progenitors) was determined by using a fluorescent microscope equipped with Image ProPlus cell-counting program (Cybernetics).

Results:

Neuronal cell morphology was observed in cells cultured with any one of the inducing substances alone (IL-1b, dbcAMP, retinoic acid, or NT-3) following 24 hr incubation. The most pronounced effect on cell-morphology was induced by dbcAMP and NT-3 (Figure 20A) as well as by IL-6 and thyroid factor 3 (data is not shown).

Cell survival was normal following 6 days incubation with any one of the substances alone (at either concentration) cell survival was normal. On the other hand, cell survival decreased when inducing substances were combined.

The incidence of cells stained positive for the oligodendrocyte progenitors marker A2B5 was 8% overall. The highest incidence of antibody- labeled A2B5 was found in the cells treated with NT-3 (Figure 20B).

Thus, BMSc can be induced to differentiate into precursors of oligodendrocytes (myelin producing cells), which may be utilized for treating multiple sclerosis.

EXAMPLE 13

Cell replacement in amyotrophic lateral sclerosis (ALS)

Methods:

Animals: TgN(SOD1-G93A)1Gur transgenic mice, expressing mutated human superoxide dismutase-1 gene (SOD1; Gurney *et al* 1994) were bred in CSJLF1. The transgenic mice were healthy until the age of 3 months then deteriorated with ALS and became completely paralyzed at the age of 4-5 month.

Transplantation: Neuronal-differentiated male mouse BMSc were generated as described in Example 10 hereinabove. The cells were injected into the spinal cord (cisterna magna) of female mutant-SOD1 transgenic mice and of wild-type mice (10^5 cells/injection; 5 animal replications per treatment group). Saline injections were used as control.

Motor function evaluation: rotational behavior of treated and non-treated (saline only) mice was evaluated weekly by using a rotometer (San Diego Instrument Inc.).

Results:

Mice expressing SOD1 suffered from amyotrophic lateral sclerosis (ALS) as indicated by a substantial reduction in rotational performance from week 7 onward, and a complete paralysis after 4-5 months (Figure 21).

PCR analyses detected Y chromosome (indicative of male-derived transplanted cells) present the spinal cord of treated female mice. The Y chromosome was not detected in any other tissue of the treated female mice (Figure 22).

Rotational behavior of 7 week old treated wild-type was not significantly different from non-treated (saline only) wild type mice (Figure 23). On the other hand, treated SOD1 mice exhibited substantial reduction in rotational behavior, indicating motor function improvement (Figure 24).

EXAMPLE 14

Construction of a Nurr1 expression vector

The nuclear receptor-related 1 (Nurr-1) is a transcription factor involved in differentiation of midbrain precursors into dopamine neurons A full-length human Nurr1 cDNA (GeneBank Accession No. NM_173171) was amplified using primers 5' BamHI and 3' XbaI (primers set forth by SEQ ID NOs: 31-32) using high fidelity Taq polymerase (TaKaRa, Japan). The PCR condition of amplification were as follows: 10 cycles of 95°C, 1 min; 56°C, 1 min; 72°C, 1 min; 10 cycles of 95°C, 1 min; 55°C, 1 min; 72°C, 1 min; 10 cycles of 95°C, 1 min; 50°C, 1 min; 72°C, 1 min. The PCR products were digested with BamHI and XbaI restriction enzymes and the resulting fragments were inserted cloned using T4 DNA Ligase (New England BioLabs) into the expression vector pcDNA-3.1A (Invitrogene) as illustrated in Figure 17.

Human bone marrow srtromal cells (hBMSc; 60-80% confluence) were transfected with pcDNANurr1 using FuGENE-6 transfection reagent according to the manufacturer's recommendations (Roche Applied Science). Stably transfected cells were isolated in a growth medium containing 500 µg/mL Neomycin (G418 Sulphate, Clontech, Palo Alto, CA). Total RNA was extracted from the isolated neomycin-resistant hBMSc as described by Chomczynski & Sacchi (1987) and the presence of

Nurr1 transcripts was confirmed using the RT-PCR procedure as described in Example 3 hereinabove.

EXAMPLE 15

Transforming hBMSc for doxycyline- regulated expression of tyrosine hydroxylase

Inducible tyrosine hydroxylase (TH) expression can be effected by transforming hBMSc with a responsive and regulating vectors which can be constructed as follows:

TH responsive vector: The 1.5 kb human tyrosine hydroxylase gene (TH, GenBank Accession No. NM_000360 can be isolated from human cDNA by PCR using high fidelity Taq polymerase (TaKaRa, Japan) the primers set forth by SEQ ID NOs: 39-40. The TH cDNA is inserted in pBI-EGFP (Clontech Tet-Off™ and Tet-On™ Gene Expression Systems), as illustrated in Figure 19A.

TH regulating vector: The promoter of the 1.3 kb human NSE gene (HSENO2, GeneBank Accession No. X51956 is isolated from human cDNA by PCR using the primers of SEQ ID NOs: 37-38. The NSE-promoter cDNA is then inserted upstream of the transcriptional activator gene (tTA; Gossen, M. and Bujard, H. Proc. Natl. Acad. Sci. USA 89:5547-551, 1992) in pRevTet-Off-IN (Clontech), instead of the 5-LTR-Ψ^+ as illustrated in Figure 19A. The positive clones bearing the neo^r gene, are selected using the antibiotic neomycin.

hBMSc can be transformed with both response and regulator vectors (Tet-off/Tet-on system) by using any of the transformation methods described hereinabove. Once introduced into cells, the regulating vector which includes the internal ribosomal entry site (IRES) located between the tetracycline-controlled transactivator (tTA) and the gene encoding neomycin resistance (Neo^r), simultaneously expresses these two elements. The expressed tTA binds the tetracycline response element (TRE) located in the response vector, thereby activating transcription of TH. However, in the presence of doxycyline (a blood brain barrier traversing antibiotic) the binding of tTA to TRE is blocked thereby halting TH transcription. This drug-controlled expression of TH is schematically illustrated in Figure 19B.

Hence, hBMSc can be genetically modified so as to express TH under the control of a negative regulator such as doxycyline which can be orally administered.

Since TH expression results in synthesis of dopamine, the genetically modified hBMSc can be used in cell replacement therapy to provide safe and effective treatment of neurodegenerative diseases such as Parkinson's disease.

It will be appreciated that positive regulation using an agent which induces TH expression can also be effected using for example, the Ecdysone-Inducible Mammalian Expression System (Invitrogen) utilizing the responsive vector pDHSP containing the TH gene and the regulator vector pVgRXR. In the presence of an inducer (e.g., ponasterone A or muristerone A) the functional ecdysone receptor binds upstream of the ecdysone responsive promoter and activates expression of TH.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications and GenBank accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application or GenBank accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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Claims

1. Use of bone marrow stromal cells transformed with an expression construct including a polynucleotide sequence expressing, in an exogenously regulatable manner, a neurotransmitter, a precursor of said neurotransmitter, or an enzyme participating in said synthesis of said neurotransmitter for the manufacture of a medicament for treating a neurodegenerative disorder.

2. The use of claim 1, wherein said agent is capable of downregulating expression of said enzyme participating in said synthesis of said neurotransmitter.

3. The use of claim 1, wherein said agent is capable of upregulating expression of said enzyme participating in said synthesis of said neurotransmitter.

4. The use of claim 1, wherein said bone marrow stromal cells are transformed with at least one expression construct including a first polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under the transcriptional control of a first regulatory sequence and a second polynucleotide sequence encoding a transactivator positioned under the transcriptional control of a second regulatory sequence, wherein said transactivator is capable of activating said first regulatory sequence to direct transcription of said first polynucleotide sequence in absence of said agent.

5. The use of claim 4, wherein said agent is doxycyline.

6. The use of claim 4, wherein said transactivator is a tetracycline controlled transactivator.

7. The use of claim 4, wherein said first regulatory sequence includes a tetracycline response element.

8. The use of claim 4, wherein said enzyme is selected from the group consisting of tyrosine hydroxylase, DOPA decarboxylase, GTP cyclohydrolase I, dopamine ß-hydroxylase, glutamate decarboxylase, tryptophane-5 monooxygenase and choline acetyltransferase.

9. The use of claim 4, wherein said second regulatory sequence includes a human-specific enolase promoter.

10. The use of claim 1, wherein said neurodegenerative disorder is selected from the group consisting of Parkinson's disease, multiple sclerosis, amyatrophic lateral sclerosis, autoimmune encephalomyelitis, Alzheimer's disease and Huntington's disease.

11. The use of claim 10, wherein said neurodegenerative disorder is Parkinson's disease.

12. The use of claim 1, wherein said neurotransmitter is selected from the group consisting of dopamine, norepinephrine, epinephrine, gamma aminobutyric acid, serotonin, acetylcholine and glutamic acid.

13. The use of claim 12, wherein said neurotransmitter is dopamine.

14. The use of claim 1, wherein said medicament is formulated for transplanting said cells into a brain tissue of an individual.

15. The use of claim 1, wherein said medicament is formulated for transplanting said cells into a spinal cord of an individual.

16. Use of an agent capable of regulating synthesis of a neurotransmitter in bone marrow stromal cells capable of exogenously regulatable neurotransmitter synthesis for the manufacture of a medicament for treating a neurodegenerative disorder.

17. The use of claim 16, wherein said bone marrow stromal cells are genetically modified so as to enable said exogenously regulatable neurotransmitter synthesis.

18. The use of claim 17, wherein said bone marrow stromal cells are transformed with an expression construct including a polynucleotide sequence encoding an enzyme participating in said synthesis of said neurotransmitter, wherein said expression construct is designed such that expression of said polynucleotide is controllable via said agent.

19. The use of claim 18, wherein said agent is capable of downregulating expression of said enzyme participating in said synthesis of said neurotransmitter.

20. The use of claim 18, wherein said agent is capable of upregulating expression of said enzyme participating in said synthesis of said neurotransmitter.

21. The use of claim 16, wherein said neurodegenerative disorder is selected from the group consisting of Parkinson's disease, multiple sclerosis, amyatrophic lateral sclerosis, autoimmune encephalomyelitis, Alzheimer's disease and Huntington's disease.

22. The use of claim 21, wherein said neurodegenerative disorder is Parkinson's disease.

23. The use of claim 16, wherein said neurotransmitter is selected from the group consisting of dopamine, norepinephrine, epinephrine, gamma aminobutyric acid, serotonin, acetylcholine and glutamic acid.

24. The use of claim 23, wherein said neurotransmitter is dopamine.

25. The use of claim 18, wherein said enzyme is selected from the group consisting of tyrosine hydroxylase, DOPA decarboxylase, GTP cyclohydrolase I, dopamine ß-hydroxylase, glutamate decarboxylase, tryptophane-5 monooxygenase and choline acetyltransferase.

26. The use of claim 16, wherein said bone marrow stromal cells are genetically modified to express tyrosine hydroxylase under a regulatory control of said agent, such that when said agent is absent an activator molecule binds a response element thereby upregulating expression of said tyrosine hydroxylase.

27. The use of claim 26, wherein said agent is doxycyline.

28. The use of claim 26, wherein said activator molecule is a tetracycline controlled transactivator.

29. The use of claim 26, wherein said response element is a tetracycline response element.

30. A nucleic acid construct, comprising a polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under a control of a regulatory sequence capable of regulating expression of said enzyme in mammalian cells.

31. The nucleic acid construct of claim 30, wherein said regulatory sequence includes a tetracycline response element.

32. The nucleic acid construct of claim 30, wherein said enzyme is selected from the group consisting of tyrosine hydroxylase, DOPA decarboxylase, GTP cyclohydrolase I, dopamine ß-hydroxylase, glutamate decarboxylase, tryptophane-5 monooxygenase and choline acetyltransferase.

33. A construct system comprising a first expression construct including a first polynucleotide sequence encoding an enzyme participating in a synthesis of a neurotransmitter positioned under the transcriptional control of a first regulatory sequence and a second expression construct including transcriptional control of a second regulatory sequence, wherein said transactivator is capable of activating said first regulatory sequence to direct transcription of said first polynucleotide sequence.

34. The construct system of claim 33, wherein said neurotransmitter is dopamine.

35. The construct system of claim 33, wherein said enzyme is tyrosine hydroxylase.

36. The construct system of claim 33, wherein said first regulatory sequence includes a tetracycline response element.

37. The construct system of claim 33, wherein said transactivator is a tetracycline controlled transactivator.

38. A bone marrow stromal cell comprising the nucleic acid construct of claim 30.

39. The bone marrow stromal cell of claim 38, further comprising a polynucleotide encoding an apoptosis inhibiting polypeptide.

40. A bone marrow stromal cell comprising the construct system of claim 33.

41. The cell of claim 40, further comprising a polynucleotide encoding an apoptosis inhibiting polypeptide.

42. A method of producing cells for treating neurodegenerative disorders, comprising:

(a) isolating bone marrow cells;

(b) incubating said bone marrow cells in a proliferating medium capable of maintaining and/or expanding said bone marrow cells;

(c) selecting bone marrow stromal cells from the cells resulting from step (b);

(d) incubating said bone marrow stromal cells in a differentiating medium including at least one polyunsaturated fatty acid and at least one differentiating agent, thereby producing the cells for use in treating neurodegenerative disorders.

43. The method of claim 42, wherein said proliferation medium includes DMEM, SPN, L-glutamine, FCS, 2-ß-mercaptoethanol, nonessential amino acids and EGF.

44. The method of claim 42, further comprising incubating the cells resulting from step (c) in a pre-differentiating medium prior to step (d) thereby predisposing said cells to differentiate into neuron-like cells.

45. The method of claim 44, wherein said pre-differentiating medium includes bFGF.

46. The method of claim 45, wherein said pre-differentiation medium further includes DMEM, SPN, L-glutamine, N2 supplement and FCS.

47. The method of claim 42, wherein said at least one polyunsaturated fatty acid is docosahexaenoic acid.

48. The method of claim 42, wherein said at least one differentiating agent is selected from the group consisting of BHA, dbcAMP and IBMX.

49. The method of claim 42, wherein said differentiating medium further includes DMEM, SPN, L-glutamine, N2 supplement and retinoic acid.

50. The method of claim 42, wherein step (a) is effected by aspiration.

51. The method of claim 42, wherein step (c) is effected by harvesting surface adhering cells.

52. An isolated human cell population, comprising naïve bone marrow stromal cells which synthesize a neurotransmitter.

53. The isolated human cell population of claim 52, wherein said neurotransmitter is dopamine.

54. The isolated human cell population of claim 52, wherein said neurotransmitter is serotonin.

55. An isolated human cell population, comprising naïve bone marrow stromal cells which synthesize at least two types of neurotransmitters.

56. The isolated human cell population of claim 55, wherein said at least two types of neurotransmitters include dopamine.

57. The isolated human cell population of claim 55, wherein said at least two types of neurotransmitters include serotonin.

58. Use of bone marrow stromal cells which synthesize a neurotransmitter for the manufacture of a medicament for treating a neurodegenerative disorder,

wherein said bone marrow stromal cells capable of synthesizing a neurotransmitter are obtained by incubating said bone marrow stromal cells in a differentiation medium which comprises at least one PUFA and at least one differentiation agent.

59. A method of generating cells for treating neurodegenerative disorders, comprising incubating bone marrow stromal cells in a differentiating medium including at least one polyunsaturated fatty acid and at least one differentiating agent, thereby generating the cells for treating neurodegenerative disorders.

60. The method of claim 59 further comprising:
(a) incubating bone marrow stromal cells in a proliferating medium capable of maintaining and/or expanding said bone marrow stromal cells;
(b) selecting bone marrow stromal cells from the cells resulting from step (a) prior to said incubating bone marrow stromal cells.

61. An isolated human cell population, comprising naïve bone marrow stromal cells which synthesize a neurotransmitter, obtainable by incubating said bone marrow stromal cells in a differentiation medium which comprises at least one PUFA and at least one differentiation agent.

62. An isolated human cell population, comprising naïve bone marrow stromal cells which synthesize at least two types of neurotransmitters, obtainable by incubating said bone marrow stromal cells in a differentiation medium which comprises at least one PUFA and at least one differentiation agent.

63. The isolated human cell population of claim 55, capable of inducing behavioral recovery in 6-OHDA-lesioned rats up to 45 days post transplantation.

64. The method of claim 42, wherein the cells express MAP2 and synthesize dopamine and/or serotonin.

65. The isolated human cell population of claim 52, wherein said naïve bone marrow stromal cells express MAP2 and synthesize dopamine and/or serotonin.

66. The use of claim 58, wherein the bone marrow stromal cells express MAP2 and synthesize dopamine and/or serotonin.

67. The method of claim 59, wherein the cell express MAP2 and synthesize dopamine and/or serotonin.

68. The isolated human cell population of claim 61, wherein said naïve bone marrow stromal cells express MAP2 and synthesize dopamine and/or serotonin.

69. The isolated human cell population of claim 62, wherein said naïve bone marrow stromal cells express MAP2 and synthesize dopamine and/or serotonin.

SEQUENCE LISTING

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<120> METHODS, NUCLEIC ACID CONSTRUCTS AND CELLS FOR TREATING NEURODEGENERATIVE DISORDERS

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