DESCRIPTION

Lrp4/CORIN DOPAMINERGIC NEURON PROGENITOR CELL MARKERS

Technical Field

The present invention relates to polynucleotide probes and antibodies for detecting and selecting dopaminergic neuron progenitor cells, and methods for detecting and selecting dopaminergic neuron progenitor cells by using them, and kits and therapeutic methods for treating neurodegenerative diseases such as Parkinson's disease using dopaminergic neuron progenitor cells.

Background Art

The dopamine system is an extremely important system for essential motor regulation, hormone secretion regulation, emotion regulation, and such in the mammalian brain. Thus, abnormalities in dopaminergic neural transmission cause various neural disorders. For example, Parkinson's disease is a neurodegenerative disease of the extrapyramidal system that occurs due to specific degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Harrison's Principles of Internal Medicine, Vol. 2, 23rd edition, Isselbacher *et al.*, ed., McGraw-Hill Inc., NY (1994), pp. 2275-7). As a primary therapeutic method for Parkinson's disease, oral administration of L-DOPA (3,4-dihydroxyphenylalanine) is performed to compensate for the decrease in the amount of dopamine produced; however, the duration of the effect is known to be unsatisfactory.

More recently, a therapeutic method for Parkinson's disease was attempted in which the midbrain ventral regions of 6 to 9-week old aborted fetuses containing dopaminergic neuron progenitor cells are transplanted to compensate for the loss of dopaminergic neurons (Patent Document 1; and Non-Patent Documents 1 to 6). However, in addition to cell supply and ethical issues (Rosenstain (1995) Exp. Neurol. 33: 106; Turner *et al.* (1993) Neurosurg. 33: 1031-7), this method is currently under criticism for various other problems, including risk of infection and contamination, immunological rejection of transplants (Lopez-Lozano *et al.* (1997) Transp. Proc. 29: 977-980; Widner and Brudin (1988) Brain Res. Rev. 13: 287-324), and low survival rates due to the primary dependence of fetal tissues on lipid metabolism rather than glycolysis (Rosenstein (1995) Exp. Neurol. 33: 106).

In order to resolve the ethical issues and shortage of supply, methods have been proposed that use, for example, porcine cortex, stria, and midbrain cells (for example, see Patent Documents 2 to 4). In these methods, a complex procedure that involves altering cell surface antigens (MHC class I antigens) is required to suppress rejection. A method

involving local immunosuppression by simultaneously transplanting Sertoli's cells has been proposed as a method for eliminating transplant rejection (Patent Documents 5 and 6; and Non-Patent Document 7). It is possible to obtain transplant cells from relatives that have matching MHCs, bone marrow from other individuals, bone marrow banks, or umbilical cord-blood banks. However, if it were possible to use the patient's own cells, the problem of rejection reactions could be overcome without any laborious procedures or trouble.

Therefore, as transplant materials, the use of dopaminergic neurons differentiated *in vitro* from non-neural cells such as embryonic stem (ES) cells and bone marrow interstitial cells, instead of cells from aborted fetuses, is considered to be promising. In fact, functional dopaminergic neurons were reported to have been formed by transplanting ES cells to lesion stria of a rat Parkinson's disease model (Non-Patent Document 8). It is believed that the importance of regenerative therapy from ES cells or the patient's own nerve stem cells will increase in the future.

In treating damaged nerve tissue, it is necessary to reconstruct brain function, and in order to form a suitable link with surrounding cells (network formation), it is necessary to transplant immature cells, cells capable of differentiating into neurons *in vivo*. In the transplanting of neuron progenitor cells, in addition to the aforementioned problem regarding supply, there is also the possibility that the progenitor cells will differentiate into groups of heterogeneous cells. For example, in treating Parkinson's disease, it is necessary to selectively transplant the catecholamine-containing neurons that produce dopamine. Examples of transplant cells that have previously been proposed for use in the treatment of Parkinson's disease include striatum (Non-Patent Documents 3 and 9), immortalized cell lines derived from human fetal neurons (Patent Documents 7 to 9), human postmitotic neurons derived from NT2Z cells (Patent Document 10), primordial neuron cells (Patent Document 11), cells and bone marrow stroma cells transfected with exogenous genes so as to produce catecholamines such as dopamines (Patent Documents 12 and 13), and genetically engineered ES cells (Non-Patent Document 8). Additionally, the use of dopaminergic neurons formed by contacting nerve progenitor cells derived from fetal midbrain tissue with FGF-8 and Shh (Patent Document 14), and of tyrosine hydroxylase-expressing cells obtained by treating NT2 nerve cells with retinoic acid (Patent Document 15) has been proposed. However, none of these contain only dopaminergic neurons or cells that differentiate into dopaminergic cells.

A method has been proposed for selectively concentrating and isolating dopaminergic neurons from undifferentiated cell populations. In this method, a reporter gene that expresses a fluorescent protein is introduced into each cell of a cell population, under the control of a gene promoter/enhancer such as the tyrosine hydroxylase expressed in dopaminergic neurons (hereinbelow, also referred to as "TH"), and then cells emitting fluorescence are isolated.

The dopaminergic neurons are visualized in their viable state, then concentrated, isolated, and identified (Patent Document 16). This method requires the complicated step of introducing an exogenous gene, and further, the presence of a reporter gene poses problems of toxicity and immunogenicity when used in conjunction with gene therapy.

[Patent Document 1] US Patent No. 5690927
[Patent Document 2] Japanese Patent Kohyo Publication No. (JP-A) H10-508487 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication)
[Patent Document 3] JP-A H10-508488
[Patent Document 4] JP-A H10-509034
[Patent Document 5] JP-A H11-509170
[Patent Document 6] JP-A H11-501818
[Patent Document 7] JP-A H8-509215
[Patent Document 8] JP-A H11-506930
[Patent Document 9] JP-A 2002-522070
[Patent Document 10] JP-A H9-5050554
[Patent Document 11] JP-A H11-509729
[Patent Document 12] JP-A 2002-504503
[Patent Document 13] JP-A 2002-513545
[Patent Document 14] US Patent No. 6277820
[Patent Document 15] International Publication WO 00/06700
[Patent Document 16] Japanese Patent Application Kokai Publication No. (JP-A) 2002-51775 (unexamined, published Japanese patent application)
[Non-Patent Document 1] Spencer *et al.* (1992) N. Engl. J. Med. 327: 1541-8
[Non-Patent Document 2] Freed *et al.* (1992) N. Engl. J. Med. 327: 1549-55
[Non-Patent Document 3] Widner *et al.* (1992) N. Engl. J. Med. 327: 1556-63
[Non-Patent Document 4] Kordower *et al.* (1995) N. Engl. J. Med. 332: 1118-24
[Non-Patent Document 5] Defer *et al.* (1996) Brain 119: 41-50
[Non-Patent Document 6] Lopez-Lozano *et al.* (1997) Transp. Proc. 29: 977-80
[Non-Patent Document 7] Selawry and Cameron (1993) Cell Transplant 2: 123-9
[Non-Patent Document 8] Kim *et al.* (2002) Nature 418: 50-56
[Non-Patent Document 9] Lindvall *et al.* (1989) Arch. Neurol. 46: 615-31

Disclosure of the Invention

Problems to be Solved by the Invention

One of the major problems in Parkinson's disease transplantation therapy at the moment is that both *in vitro* differentiated dopaminergic neuron precursor cells and midbrain ventral region of aborted fetuses are mixtures of a myriad cell types. When considering safety in neural circuit formation, it is preferable to use isolated cells that comprise only the cell type of interest. Furthermore, when considering the risk of tumorigenesis, it is believed better to use isolated postmitotic neurons. Moreover, when considering the survival of cells at their transplantation site in the brain, and their ability to properly form a network, it is expected that therapeutic effects can be further improved by isolating dopaminergic neuron progenitor cells at as early a stage as possible.

Means to Solve the Problems

In order to isolate genes specific to dopaminergic neuron progenitor cells, a gene specifically expressed in the most ventral region of the E12.5 murine midbrain containing dopaminergic neurons was identified using a modification ("Method for Homogenizing the Amounts of DNA Fragments and Subtraction Method", WO 2002/103007 (International Publication Pamphlet)) of the subtraction method (N-RDA: Representational Difference Analysis; RDA (Listsyn N.A. (1995) Trends Genet. 11: 303-7) by additionally dividing the ventral region into two regions in the dorsoventral direction. As a result, the present inventors successfully isolated Lrp4/Corin. Lrp4 encoded a type II transmembrane protein (Fig. 1).

Lrp4 mRNA is specifically expressed in a ventral midline region in the midbrain. The areas where Lrp4 mRNA is expressed match the areas where dopaminergic neuron proliferative progenitor cells are present. Furthermore, when Lrp4 expression is compared to that of TH, which is a marker for dopaminergic neurons, their signals are in identical dorsoventral positions, but they do not overlap (Figs. 4 and 5). This indicated that Lrp4 mRNA is not expressed in these progenitor cells, which had stopped cell division and migrated to the neural tube outer layer. Therefore, by using Lrp4 mRNA as an index, it is possible to specifically detect and select dopaminergic neuron proliferative progenitor cells.

Thus, embodiments of the present invention provide dopaminergic neuron proliferative progenitor cell marker polynucleotide probes capable of specifically detecting Lrp4 mRNA, and methods for selecting dopaminergic neuron proliferative progenitor cells utilizing these probes. Furthermore, embodiments of the present invention relate to dopaminergic neuron proliferative progenitor cells prior to cell cycle exit, selected by using such nucleotide probes (hereinbelow, sometimes referred to simply as "dopaminergic neuron proliferative progenitor cells"); as well as methods for isolating dopaminergic neuron proliferative progenitor cell-specific genes and genes specific for each maturation stage from progenitor cell to dopaminergic neuron, utilizing the

proliferative progenitor cells; and methods of screening for compounds which induce differentiation or proliferation of the progenitor cells, using maturation as an index. It is also possible to culture the proliferative progenitor cells selected by using the nucleotide probes of the present invention, and to obtain dopaminergic neuron lineage cells comprising the postmitotic dopaminergic neuron precursor cells. The term "dopaminergic neuron lineage cells" used herein refers to dopaminergic neuron proliferative progenitor cells, postmitotic dopaminergic neuron precursor cells, and/or dopaminergic neurons. The dopaminergic neuron lineage cells can also be utilized for the methods for isolating genes specific to each stage of maturation to dopaminergic neuron, and the methods of screening for compounds which induce the differentiation or proliferation of progenitor cells, using maturation as an index. Therefore, embodiments of the present invention relate to methods for obtaining the dopaminergic neuron lineage cells by culturing the dopaminergic neuron proliferative progenitor cells selected by using the disclosed nucleotide probes ; cells obtained in this way; methods for isolating genes specific to each stage of maturation to dopaminergic neurons that use the cells; and methods of screening for compounds which induce the differentiation or proliferation of the cells using maturation as an index.

Furthermore, the present inventors produced an anti-Lrp4 antibody, and examined Lrp4 protein expression. First, by analyzing its expression in tissues (Fig. 8), the Lrp4 protein was confirmed to be expressed in the same way as Lrp4 mRNA. In this experiment, Lrp4 protein signals were also detected in TH-expressing areas. However, since the proliferative progenitor cells extend processes toward the outer layer of the neural tube, this signal could not be determined to be caused by detecting proteins on the processes or the TH-expressing cells also expressed the Lrp4 protein. Next, by using the anti-Lrp4 antibody, whether Lrp4 protein was expressed on the cell surface was analyzed by flow cytometry. Cells in which Lrp4 mRNA expression was confirmed were obtained by inducing the differentiation of ES cells into dopaminergic neuron progenitor cells *in vitro* (stromal cell-derived inducing activity (SDIA) method) and used as samples. As a result, it was confirmed that the Lrp4 protein was certainly expressed on the surface of the cells (Fig. 9). Such proteins expressed on the cell surface are particularly preferable to use as separation markers because living cells can be selected (see Fig. 15). In addition, ES cells were induced to differentiate *in vitro* into the dopaminergic neuron progenitor cells by the 5-stage method, and Lrp4 expression was confirmed by RT-PCR and flow cytometry using the anti-Lrp4 monoclonal antibody. As a result, Lrp4 was revealed to also be expressed in dopaminergic neuron progenitor cells differentiated by the 5-stage method (Figs. 17A and 17B).

Next, Lrp4-positive cells were separated from cells induced to differentiate (SDIA method) and from mouse fetal ventral midbrain cells by a cell sorter using the anti-Lrp4

antibody. Gene expression in the separated cells was analyzed by the RT-PCR method. As a result, expression of the neuron proliferative progenitor cell marker Nestin was observed. In addition, cells expressing MAP2, which is a marker for postmitotic neurons, were also revealed to be included (Fig. 10). TH and Nurr1, which is a marker for postmitotic dopaminergic neuron precursor cells, were expressed at higher levels in an Lrp4-positive cell population than in an Lrp4-negative cell population. Therefore, unlike in the case of using Lrp4 mRNA as the index, when cells are selected using antibodies by using Lrp4 protein as an index, it is possible to isolate the dopaminergic neuron progenitor cells, including postmitotic dopaminergic neuron precursor cells. Hereinbelow, the term "dopaminergic neuron progenitor cells" refers to dopaminergic neuron proliferative progenitor cells and postmitotic dopaminergic neuron precursor cells. For cells separated using the anti-Lrp4 antibody from those cells induced to differentiate from the ES cells *in vitro* by the SDIA method, the expression of ERas and Nanog expressed specifically in the ES cells was analyzed. As a result, expression was not observed in Lrp4-positive cells, whereas expression of both genes was observed in Lrp4-negative cells (Fig. 18). Therefore, by selecting cells using the anti-Lrp4 antibody, it is possible to select and remove undifferentiated ES cells after inducing differentiation. Furthermore, Lrp4-positive cells were separated from cell populations including dopaminergic neuron progenitor cells induced to differentiate *in vitro* from ES cells by the 5-stage method. Next, the separated Lrp4-positive cells were cultured *in vitro* to induce TH protein-positive dopaminergic neurons (Fig. 17C). This revealed that Lrp4-positive cells induced by the 5-stage method are dopaminergic neuron progenitor cells and can mature *in vitro*.

Accordingly, embodiments of the present invention provide antibodies which specifically detect the Lrp4 protein and methods that utilize the antibodies to select dopaminergic neuron progenitor cells. Furthermore, embodiments of the present invention relate to dopaminergic neuron progenitor cells selected by using such antibodies; as well as methods that use the progenitor cells to isolate dopaminergic neuron progenitor cell-specific genes and genes specific for each maturation stage from progenitor cell to dopaminergic neuron; and methods of screening for compounds which induce the differentiation or proliferation of the progenitor cells using maturation as an index. It is also possible to obtain dopaminergic neuron lineage cells at other differentiation stages by culturing the progenitor cells selected using the disclosed antibodies.

Such cells can also be utilized in the methods for isolating genes specific to each stage of maturation to dopaminergic neuron, and the methods of screening for compounds which induce the differentiation or proliferation of the progenitor cell using maturation as an index.

Accordingly, embodiments of the present invention also relate to methods for obtaining dopaminergic neuron lineage cells by culturing dopaminergic neuron progenitor cells using the disclosed antibodies;

cells obtained using the methods; methods that use the cells to isolate genes specific to each stage of maturation to dopaminergic neurons; and methods of screening for compounds which induce the differentiation or proliferation of the cells using maturation as an index.

Furthermore, the present inventors transplanted Lrp4-expressing cells separated using the anti-Lrp4 monoclonal antibody into the striatum of Parkinson's disease model mice. As a result, EGFP-positive cells were observed in the striatum of transplanted mice (Table 1). Thus it appears that the transplanted Lrp4 protein-positive cells were successfully engrafted in the striatum of the Parkinson's disease model mice. Most of the successfully engrafted cells were positive for the mature neuron marker MAP2, and EGFP-positive axons were observed to extend into the striatum (Table 1 and Fig. 16). Most of the successfully engrafted cells differentiated into nerve cells and then matured, whereas the transplanted Lrp4 protein-positive cells were neural progenitor cells, and about 20% of the successfully engrafted cells were TH-positive. These findings strongly suggested that at least some of the transplanted Lrp4 protein-positive cells differentiated into dopaminergic neurons. Therefore, the dopaminergic neuron progenitor cells separated according to the embodiments of the present invention can differentiate into dopaminergic neurons upon being transplanted in to the brain, and may be effective for the treatment of Parkinson's disease. That is, embodiments of the present invention also relate to kits for treating neurodegenerative diseases, preferably Parkinson's disease, comprising the dopaminergic neuron progenitor cells separated according to the embodiments of the present invention; and methods for treating neurodegenerative diseases, preferably Parkinson's disease, comprising the step of transplanting dopaminergic neuron progenitor cells into the brains of patients.

In a first aspect, there is provided a method for selecting a dopaminergic neuron proliferative progenitor cell, wherein the method comprises the step of contacting a polynucleotide probe with a cell sample thought to comprise a dopaminergic neuron proliferative progenitor cell, wherein the polynucleotide probe comprises a sequence selected from the following nucleotide sequences (1) to (5):

(1) a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a nucleotide sequence complementary to a nucleotide sequence encoding an amino acid sequence of SEQ ID NO: 3 or 4;

(3) a nucleotide sequence complementary to a nucleotide sequence encoding a sequence lacking a transmembrane domain in an amino acid sequence of SEQ ID NO: 3 or 4;

(4) a nucleotide sequence that hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence of SEQ ID NO: 1 or 2; and

(5) a nucleotide sequence comprising at least 15 contiguous nucleotides selected from sequences of (1) to (4).

In a second aspect, there is provided a method for selecting a dopaminergic neuron lineage cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell using the method of the first aspect for selecting the dopaminergic neuron proliferative progenitor cell;

(2) culturing the proliferative progenitor cell selected in step (1); and

(3) screening the cells cultured in step (2) by using a marker for a postmitotic dopaminergic neuron.

In a third aspect, there is provided a method for isolating a dopaminergic neuron proliferative progenitor cell-specific gene and a gene specific for each maturation stage from the proliferative progenitor cell to a dopaminergic neuron, wherein the method comprises the step of detecting and isolating a gene specifically expressed in a dopaminergic neuron proliferative progenitor cell, which is selected by the method of the first aspect, or a cell which is differentiated, induced, or proliferated from the proliferative progenitor cell isolated from the animal or human body.

In a fourth aspect, there is provided a method of screening for a compound which regulates proliferation and/or differentiation of a dopaminergic neuron lineage cell using maturation as an index, wherein the method comprises the steps of: contacting a test substance with a dopaminergic neuron proliferative progenitor cell, which is selected by the method of the first aspect, or a cell which is differentiated, induced, or proliferated from the proliferative progenitor cell isolated from the animal or human body; and detecting a change of the proliferative progenitor cell or the progenitor cell caused by the contact.

In a fifth aspect, there is provided an antibody against a polypeptide selected from the following (1) to (6), which is produced by the hybridoma FERM BP-10315 or FERM BP-10316.

(1) a polypeptide encoded by a polynucleotide consisting of a nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a polypeptide comprising an amino acid sequence of SEQ ID NO: 3 or 4;

(3) a polypeptide comprising an amino acid sequence lacking a transmembrane domain in an amino acid sequence of SEQ ID NO: 3 or 4;

(4) a polypeptide comprising an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in an amino acid sequence of SEQ ID NO: 3 or 4;

(5) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions with a polynucleotide consisting of a sequence complementary to a nucleotide sequence of SEQ ID NO : 1 or 2; and,

(6) a polypeptide that is a fragment of a polypeptide of (1) to (5) comprising at least eight amino acid residues.

In a sixth aspect, there is provided a method for selecting a dopaminergic neuron progenitor cell, wherein the method comprises the step of contacting the antibody of the fifth aspect with a cell sample isolated from the animal or human body thought to comprise a dopaminergic neuron progenitor cell.

In a seventh aspect, there is provided a method for selecting a dopaminergic neuron lineage cell, wherein the method comprises the step of:

(1) selecting a dopaminergic neuron proliferative progenitor cell using the method of the sixth aspect;

(2) culturing the progenitor cell selected in step (1); and

(3) screening the progenitor cells cultured in step (2) by using a marker for a postmitotic dopaminergic neuron.

In an eighth aspect, there is provided a method for isolating a dopaminergic neuron progenitor cell-specific gene and a gene specific for each maturation stage from the progenitor cell to a dopaminergic neuron, wherein the method comprises the step of detecting and isolating a gene specifically expressed in a dopaminergic neuron progenitor cell, which is selected by the method of the sixth aspect or a cell which is differentiated, induced, or proliferated from the progenitor cell isolated from the animal or human body.

In a ninth aspect, there is provided a method of screening for a compound which regulates proliferation and/or differentiation of a dopaminergic neuron lineage cell using maturation as an index, wherein the method comprises the steps of: contacting a test substance with a dopaminergic neuron progenitor cell, which is selected by the method of the sixth aspect, or a cell which is differentiated, induced, or proliferated from the progenitor cell isolated from the animal or human body: and detecting a differentiated or proliferated progenitor cell caused by the contact.

In a tenth aspect, there is provided a kit for treating Parkinson's disease, which comprises a dopaminergic neuron proliferative progenitor cell, which is selected by the method of the first aspect, or a dopaminergic neuron progenitor cell, which is selected by the method of the sixth aspect.

In an eleventh aspect, there is provided a use of a dopaminergic neuron proliferative progenitor cell, which is selected by the method of the first aspect, or a dopaminergic neuron progenitor cell, which is selected by the method of the sixth aspect, for producing a kit for treating Parkinson's disease.

In a twelfth aspect, there is provided a method for detecting or selecting a dopaminergic neuron proliferative progenitor cell, which comprises the step of contacting a cell sample comprising the dopaminergic neuron proliferative progenitor cell with a second polynucleotide which hybridizes under stringent conditions with a first polynucleotide consisting of any one of:

(1) the nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a nucleotide sequence consisting of a polynucleotide encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 3 or 4;

(3) a nucleotide sequence consisting of a polynucleotide encoding a polypeptide consisting of an amino acid sequence which lacks a transmembrane region in the amino acid sequence of SEQ ID NO: 3 or 4; and

(4) a nucleotide sequence consisting of a polynucleotide which hybridizes with a poly nucleotide consisting of the nucleotide sequence of SEQ ID NO: 1 or 2 under stringent conditions.

In a thirteenth aspect, there is provided a method for producing a postmitotic dopaminergic neuron precursor cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell by the method of the twelfth aspect;

(2), culturing the cell selected in step (1); and

(3) selecting the postmitotic dopaminergic neuron precursor cell from the cells cultured in step (2).

In a fourteenth aspect, there is provided a method for producing a dopaminergic neuron, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell by the method of the twelfth aspect; and

(2) culturing the cell selected in step (1).

In a fifteenth aspect, there is provided a method for detecting or selecting a dopaminergic neuron progenitor cell, which comprises the step of contacting a cell sample comprising the dopaminergic neuron progenitor cell with an antibody which is bound to a polypeptide consisting of the amino acid sequence of any one of:

(1) the amino acid sequence of SEQ ID NO: 3 or 4;

(2) an amino acid sequence which lacks a transmembrane region in the amino acid sequence of SEQ ID NO: 3 or 4;

(3) an amino acid sequence mutated by one or more amino acid deletions, substitutions, or additions, or any combination thereof in the amino acid sequence of SEQ ID NO:3 or 4; and

(4) an amino acid sequence consisting of a polypeptide encoded by a polynucleotide which hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2,
or a partial sequence thereof.

In a sixteenth aspect, there is provided a reagent for discriminating a dopaminergic neuron progenitor cell, which comprises an antibody as an active ingredient which is bound to a polypeptide consisting of the amino acid sequence of any one of:

(1) the amino acid sequence of SEQ ID NO: 3 or 4;

(2) an amino acid sequence which lacks a transmembrane region in the amino acid sequence of SEQ ID NO: 3or 4;

(3) an amino acid sequence mutated by one or more amino acid deletions, substitutions, or additions, or any combination thereof, in the amino acid sequence of SEQ ID NO:3 or 4; and

(4) an amino acid sequence consisting of a polypeptide encoded by a polynucleotide which hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2,
or a partial sequence thereof.

In a seventeenth aspect, there is provided an antibody produced by the hybridoma FERM BP-10315 or FERM BP-10316.

In an eighteenth aspect, there is provided a method for producing a dopaminergic neuron proliferative progenitor cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell by the method of the fifteenth aspect; and

(2) removing a postmitotic dopaminergic neuron precursor cell to select the dopaminergic neuron proliferative progenitor cell.

In a nineteenth aspect, there is provided a method for producing a postmitotic dopaminergic neuron precursor cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell by the method of the fifteenth aspect; and

(2) culturing the cell selected in step (1).

In a twentieth aspect, there is provided a method for producing a dopaminergic neuron, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell by the method of the fifteenth aspect; and

(2) culturing the cell selected in step (1).

Effects of the Invention

There have been no previous reports of genes encoding membrane proteins specifically expressed in dopaminergic neuron proliferative progenitor cells. Antibodies to Lrp4 protein expressed on the cell membrane surface are believed to be extremely effective in isolating dopaminergic neuron progenitor cells. For example, pure dopaminergic neuron progenitor cells can be obtained by isolating Lrp4-expressing cells from the ventral midbrain region or cultured cells containing dopaminergic neuron progenitor cells differentiated *in vitro*, using anti-Lrp4 antibodies (Fig. 15). Lrp4 expression was confirmed on both of the dopaminergic neuron progenitor cells induced by two different differentiation methods (SDIA method and 5-stage method). Accordingly, Lrp4 is useful as a dopaminergic neuron progenitor cell marker, regardless of cell source. In addition, since ERas and Nanog, which are specifically expressed in ES cells, are not expressed in Lrp4-positive cells, it is possible to select undifferentiated ES cells using Lrp4 expression as an index.

Moreover, in the present disclosure, the isolated dopaminergic neuron progenitor cells can also be transplanted directly, or after having been grown in vitro. The disclosed dopaminergic neuron progenitor cells also have the potential to differentiate and mature at the optimum region in the brain, as well as the potential to additionally grow *in vivo*, and can be expected to demonstrate long-term therapeutic effects. In addition, if Lrp4-expressing cells are transplanted after having differentiated and matured *in vitro*, they can be expected to demonstrate therapeutic effects, even if for some reason they do not differentiate into dopaminergic neurons *in vivo*. In consideration of the risks of tumorigenesis and such, an even higher degree of safety can be expected if cells that have been isolated using a postmitotic dopaminergic neuron precursor cell marker such as 65B13 (WO 2004/038018 pamphlet) after differentiating Lrp4-expressing cells grown *in vitro* are transplanted. The use of Lrp4-expressing cells for transplantation therapy after being isolated, regardless of the method, enables a high degree of safety since only the cell type of interest is isolated. In addition, since the earliest dopaminergic neuron progenitor cells can be used, high therapeutic efficacy can be expected in terms of survival rate, network formation ability, and such. Further, even if the best therapeutic effects cannot be achieved by these early progenitor cells immediately after isolation, since progenitor cells isolated using markers of the present invention can mature *in vitro* by culturing or such, materials in the optimum stage of differentiation can be prepared (Fig. 6).

On the other hand, pure dopaminergic neuron progenitor cells may also be useful in the search for therapeutic targets for Parkinson's disease, such as for use in the isolation of genes specific to dopaminergic neurons. In particular, dopaminergic neuron proliferative progenitor cells may be useful for research on the maturation process of dopaminergic neurons, screening systems using maturation as an index, drug screening in which progenitor cells are grown *in vitro* or *in vivo*, screening for drugs that induce differentiation of progenitor cells *in vivo* (*in vivo* regenerative therapy drugs), and the like.

Brief Description of the Drawings

Fig. 1 schematically shows the structure of Lrp4. TM: transmembrane domain, FRI: frizzeled domain, LDLa: LDL receptor domain, SR: scavenger receptor domain, Protease: serine protease domain.

Fig. 2 is a set of photographs showing the results of Lrp4 and Shh mRNA expression analysis in E12.5 mouse ventral hindbrain region and spinal cord by *in situ* hybridization.

Fig. 3 is a set of photographs showing the results of Lrp4, Shh, tyrosine hydroxylase (TH), and NCAM mRNA expression analysis in E12.5 mouse ventral midbrain region by *in situ* hybridization.

Fig. 4 is a schematic diagram of the Lrp4 expression pattern in the midbrain, and photographs indicating the expression of mRNAs of Lrp4, tyrosine hydroxylase (TH), Sim-1, and NCAM in the ventral midbrain of E12.5 mice analyzed by *in situ* hybridization. VZ: ventricular zone; and ML: mantle layer.

Fig. 5 is a set of photographs showing the results of Lrp4 mRNA expression analysis in the E12.5 mouse central nervous system by *in situ* hybridization. A: sagittal cross-section, B: enlarged photograph of the area inside the box in A, C: cross-section at the location of the red line in A, D: Expression of Lrp4, Shh, and tyrosine hydroxylase (TH) mRNA in the E12.5 mouse midbrain ventral region.

Fig. 6 schematically shows the timing of expression of Lrp4, NCAM, TH, and DAT mRNAs from the generation to maturation of dopaminergic neurons.

Fig. 7 (top panel consisting of a drawing and photograph) schematically shows the inhibition of differentiation of ES cells into dopaminergic neurons by the SDIA method. The bottom photograph shows the results of investigating the expression of Lrp4 mRNA in dopaminergic neurons differentiated from ES cells over time, using the SDIA method by RT-PCR.

Fig. 8 is a photograph showing Lrp4 protein expression in the E12.5 mouse midbrain.

Fig. 9 shows Lrp4 protein expression on the SDIA-differentiated cell surface analyzed by flow cytometry using the anti-Lrp4 antibody.

Fig. 10 is a set of photographs showing the expression of various dopaminergic neuron markers in Lrp4-positive cells analyzed by RT-PCR.

Fig 11 schematically shows the expression periods of Lrp4 mRNA and protein, and TH mRNA from the development to maturation of dopaminergic neurons. This shows that both dopaminergic neuron proliferative progenitor cells and postmitotic dopaminergic neuron precursor cells are present in Lrp4-expressing cells.

Fig. 12 is a set of photographs showing the results of examining the differentiation stages of Lrp4-positive cells.

Fig. 13 is a set of photographs showing the *in vitro* proliferation of Lrp4-positive cells.

Fig. 14 is a set of photographs showing that Lrp4-positive cells differentiate into dopaminergic neurons.

Fig. 15 schematically shows the separation and application of dopaminergic neuron progenitor cells using anti-Lrp4 antibody.

Fig. 16 is a set of photographs showing the *in vivo* differentiation of transplanted Lrp4-positive cells.

Fig. 17 shows a diagram and photographs indicating Lrp4 expression in cells

differentiated by the 5-stage method, and differentiation of Lrp4-positive cells into dopaminergic neurons. The arrows in panel C indicate TH protein-positive dopaminergic neurons.

Fig. 18 is a set of photographs showing the results of using RT-PCR to analyze the expression of ES cell-specific genes (ERas and Nanog) in Lrp4-positive cells and Lrp4-negative cells.

Best Mode for Carrying Out the Invention

Hereinbelow, the embodiments of the present invention will be described, but they are exemplified to illustrate the present invention, and not to limit the present invention to these embodiments. The present invention can be carried out in various forms without departing from the scope thereof.

References, publications, patent publications, and patent references cited herein are incorporated by reference.

<Marker Polynucleotide Probes>

The dopaminergic neuron proliferative progenitor cell marker polynucleotide probes of the present invention are used as markers and/or reagents for selecting and/or detecting dopaminergic neuron proliferative progenitor cells. Polynucleotides used for this probe comprise a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or 2 detected in dopaminergic neuron proliferative progenitor cells. SEQ ID NO: 1 is the nucleotide sequence of murine Lrp4 cDNA. SEQ ID NO: 2 is the nucleotide sequence of human Lrp4 cDNA. Both sequences are registered in GenBank (murine: Accession No. NM_016869; human: Accession No. XM_035037).

Here, a "marker polynucleotide probe" refers to a polymer composed of a number of nucleotides, such as deoxyribonucleic acids (DNAs) or ribonucleic acids (RNAs), or nucleotide pairs, where the polymer should be able to detect expression of Lrp4, particularly transcribed mRNA. Double-stranded cDNAs are also known to be able to be used as probes in tissue *in situ* hybridization, and such double-stranded cDNAs are included in the markers of the present invention. RNA probes (riboprobes) are particularly preferable as marker polynucleotide probes for detecting RNAs in tissue. If needed, the marker polynucleotide probes of the present invention can also contain non-naturally-occurring nucleotides such as 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, dihydrouridine, 2'-O-methylpseudouridine, β-D-galactosylqueuosine, 2'-O-methylguanosine, inosine, N6-isopentenyladenosine, 1-methyladenosine, 1-methylpseudouridine, 1-

methylguanosine, 1-methylinosine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, N6-methyladenosine, 7-methylguanosine, 5-methylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, β-D-mannosylqueuosine, 5-methoxycarbonylmethyl-2-thiouridine, 5-methoxycarbonylmethyluridine, 5-methoxyuridine, 2-methylthio-N6- isopentenyladenosine, N-((9-β-D-ribofuranosyl-2- methylthiopurin-6-yl)carbamoyl)threonine, N-((9-β-D-ribofuranosylpurin-6-yl)N-methylcarbamoyl)threonine, uridine-5-oxyacetic acid-methyl ester, uridine-5-oxyacetic acid, wybutoxosine, pseudouridine, queuosine, 2-thiocytidine, 5-methyl-2-thiouridine, 2-thiouridine, 4-thiouridine, 5-methyluridine, N-((9-β-D-ribofuranosylpurin-6-yl)carbamoyl)threonine, 2'-O-methyl-5-methyluridine, 2'-O-methyluridine, wybutosine, and 3-(3-amino-3-carboxy propyl)uridine.

Moreover, marker polynucleotide probes of the present invention comprise nucleotide sequences complementary to nucleotide sequences encoding the amino acid sequence of SEQ ID NO: 3 or 4. The nucleotide sequence that encodes the amino acid sequence of SEQ ID NO: 3 or 4 includes not only nucleotide sequences of SEQ ID NO: 1 or 2, but also nucleotide sequences that differ from the sequences of SEQ ID NO: 1 or 2 due to degeneracy of the genetic code. The marker polynucleotide probes of the present invention also include those which comprise sequences complementary to nucleotide sequences encoding a sequence that lacks the transmembrane domain in the amino acid sequence of SEQ ID NO: 3 or 4. There is no signal sequence in the amino acid sequence of SEQ ID NO: 3 or 4. In murine Lrp4 (SEQ ID NO: 3), amino acid residues 113-135 form a transmembrane domain, while in human Lrp4 (SEQ ID NO: 4), amino acid residues 46-68 form a transmembrane domain. The sequences described in SEQ ID NOs: 3 and 4 are respectively registered in GenBank (human Lrp4, XP_035037; murine Lrp4, NP_058565).

Herein, the phrase "complementary to a nucleotide sequence" encompasses not only cases wherein a nucleotide sequence completely pairs with the template, but also includes those that have at least 70%, preferably 80%, more preferably 90%, and even more preferably 95% or more (for example, 97% or 99%) of the nucleotides paired with the template. Pairing refers to the formation of a chain in which T (U in the case of RNAs) corresponds to A, A corresponds to T or U, G corresponds to C, and C corresponds to G in the nucleotide sequence of the template polynucleotide. Homologies at the nucleotide sequence level between certain polynucleotides can be determined by the BLAST algorithm (Altschul (1990) Proc. Natl. Acad. Sci. USA 87: 2264-8; Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-7). The BLASTN program for nucleotide sequences (Altschul *et al.* (1990) J. Mol. Biol. 215: 403-410) has been developed based on this algorithm, and can be used to determine the homology of marker polynucleotide probe sequences (see http://www.ncbi.nlm.nih.gov for a

specific example of analysis methods).

Moreover, marker polynucleotide probes of the present invention include polynucleotides that contain nucleotide sequences that hybridize under stringent conditions with polynucleotides comprised of the nucleotide sequence of SEQ ID NO: 1 or 2. Although polynucleotides with a nucleotide sequence indicated in SEQ ID NO: 1 or 2 are known with respect to Lrp4, alternative isoforms and allelic variants may also exist. Polynucleotides with a sequence complementary to such isoforms and allelic variants can also be used as marker polynucleotides of the present invention. Such isoforms and allelic variants can be obtained from cDNA libraries or genomic libraries derived from animals such as humans, mice, rats, rabbits, hamsters, chickens, pigs, cows, goats, and sheep, by using a polynucleotide probe comprising a nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2, in known hybridization methods such as colony hybridization, plaque hybridization, or Southern blotting. See "Molecular Cloning, A Laboratory Manual 2nd ed." (Cold Spring Harbor Press (1989)) for methods of cDNA library construction. In addition, commercially available cDNA libraries or genomic libraries may also be used.

More specifically, in constructing a cDNA library, total RNA is first prepared from cells, organs, tissues, or such that express Lrp4, by known techniques such as guanidine ultracentrifugation (Chirwin *et al.* (1979) Biochemistry 18: 5294-5299) or AGPC (Chomczynski and Sacchi (1987) Anal. Biochem. 162: 156-159), followed by purification of mRNA using an mRNA Purification Kit (Pharmacia), or such. A kit for direct mRNA preparation, such as the QuickPrep mRNA Purification Kit (Pharmacia), may also be used. Next, cDNAs are synthesized from the resulting mRNAs using reverse transcriptase. cDNA synthesis kits, such as the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation), are also commercially available. Other methods that use the 5'-RACE method to synthesize and amplify cDNA by PCR may also be used (Frohman *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 8998-9002; Belyavsky *et al.* (1989) Nucleic Acids Res. 17: 2919-32). In addition, in order to construct cDNA libraries containing a high percentage of full-length clones, known techniques such as the oligo-capping method (Maruyama and Sugano (1994) Gene 138: 171-4; Suzuki (1997) Gene 200: 149-56) can also be employed. The cDNA obtained in this manner can be then incorporated into a suitable vector.

Examples of stringent hybridization conditions suitable for use in the present invention include "2x SSC, 0.1% SDS, 50°C", "2x SSC, 0.1% SDS, 42°C" and "1x SSC, 0.1% SDS, 37°C". Examples of conditions of higher stringency include "2x SSC, 0.1% SDS, 65°C", "0.5x SSC, 0.1% SDS, 42°C" and "0.2x SSC, 0.1% SDS, 65°C". More specifically, a method that uses the Rapid-hyb buffer (Amersham Life Science) can be carried out by performing pre-hybridization at 68°C for 30 minutes or more, adding a probe to allow

hybrid formation at 68°C for one hour or more, washing three times in 2x SSC/0.1% SDS at room temperature for 20 minutes each, washing three times in 1x SSC/0.1% SDS at 37°C for 20 minutes each, and finally washing twice in 1x SSC/0.1% SDS at 50°C for 20 minutes each. This can also be carried out using, for example, the Expresshyb Hybridization Solution (CLONTECH), by performing pre-hybridization at 55°C for 30 minutes or more, adding a labeled probe and incubating at 37°C to 55°C for one hour or more, washing three times in 2x SSC/ 0.1% SDS at room temperature for 20 minutes each, and washing once at 37°C for 20 minutes with 1x SSC/0.1% SDS. Herein, conditions of higher stringency can be achieved by increasing the temperature for pre-hybridization, hybridization, or the second wash. For example, pre-hybridization and hybridization temperatures of 60°C can be raised to 68°C for higher stringency. In addition to factors such as salt concentration of the buffer and temperature, those with ordinary skill in the art can also integrate other factors, such as probe concentration, probe length, and reaction time, to obtain Lrp4 isoforms and allelic variants, and corresponding genes derived from other organisms.

References such as Molecular Cloning, A Laboratory Manual 2nd ed. (Cold Spring Harbor Press (1989); Section 9.47-9.58), Current Protocols in Molecular Biology (John Wiley & Sons (1987-1997); Section 6.3-6.4), DNA Cloning 1: Core Techniques, A Practical Approach 2nd ed. (Oxford University (1995); Section2.10 for conditions, in particular), can be referred to for detailed information on hybridization procedures. Examples of hybridizing polynucleotides include polynucleotides containing a nucleotide sequence that has at least 50% or more, preferably 70%, more preferably 80% and even more preferably 90% (for example, 95% or more, or 99%) identity with a nucleotide sequence comprising the nucleotides of SEQ ID NO: 1 or SEQ ID NO: 2. Such identities can be determined by the BLAST algorithm (Altschul (1990) Proc. Natl. Acad. Sci. USA 87: 2264-8; Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-7) as described in the homology determination above. In addition to the above-described BLASTN program for nucleotide sequences, the BLASTX program for determining the identity of amino acid sequences (Altschul *et al.* (1990) J. Mol. Biol. 215: 403-10) and the like has been developed based on this algorithm and can be used (as described above, see http://www.ncbi.nlm.nih.gov. for a specific example of analysis methods).

Lrp4 isoforms or allelic variants, and other genes with an Lrp4-like structure or function, can be obtained from cDNA libraries and genomic libraries of animals such as humans, mice, rats, rabbits, hamsters, chickens, pigs, cows, goats, and sheep, by designing primers based on the nucleotide sequences of SEQ ID NOs: 1 and 2, using gene amplification technology (PCR) (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Sections 6.1-6.4).

The polynucleotide sequences can be confirmed by using conventional sequence determination methods. For example, the dideoxynucleotide chain termination method (Sanger *et al.* (1977) Proc. Natl. Acad. Sci. USA 74: 5463) can be used. In addition, sequences can also be analyzed using a suitable DNA sequencer.

Moreover, marker polynucleotide probes of the present invention include the aforementioned (1) sequences complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, (2) sequences complementary to nucleotide sequences that encode the amino acid sequence described in SEQ ID NO: 3 or 4, (3) sequences complementary to nucleotide sequences that encode a sequence lacking the transmembrane domain portion of the amino acid sequence described in SEQ ID NO: 3 or 4, and (4) polynucleotides comprising nucleotide sequences that contain at least 15 consecutive nucleotides in each of the nucleotide sequences that hybridize under stringent conditions with a polynucleotide comprised of the nucleotide sequence of SEQ ID NO: 1 or 2. Such polynucleotides comprising a nucleotide sequence that contains at least 15 consecutive nucleotides can be used as a probe for detecting, or as a primer for amplifying, the expression of Lrp4 mRNA. The nucleotide chain normally consists of 15 to 100, and is preferably 15 to 35 nucleotides when used as a probe, or at least 15 and preferably 30 nucleotides when used as a primer. A primer can be designed to have a restriction enzyme recognition sequence, a tag or such, added to the 5'-end side thereof, and at the 3' end, a sequence complementary to a target sequence. Such polynucleotides, comprising a nucleotide sequence that contains at lease 15 consecutive nucleotides, can hybridize with an Lrp4 polynucleotide.

Furthermore, the marker polynucleotide probes of the present invention comprise a second polynucleotide which hybridizes under stringent conditions with a first polynucleotide comprising any one of: (1) the nucleotide sequence of SEQ ID NO: 1 or 2; (2) nucleotide sequences comprising polynucleotides encoding polypeptides comprising the amino acid sequence of SEQ ID NO: 3 or 4; (3) nucleotide sequences comprising polynucleotides encoding polypeptides comprising an amino acid sequence which lacks the transmembrane region in the amino acid sequence of SEQ ID NO: 3 or 4; and (4) nucleotide sequences comprising polynucleotides which hybridize under stringent conditions with a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 or 2. Polynucleotides consisting of a nucleotide sequence comprising at least 15 consecutive nucleotides are preferred as the second polynucleotide.

Marker polynucleotide probes of the present invention can be prepared from cells that express Lrp4 by the aforementioned hybridization or PCR or such. In addition, marker polynucleotide probes of the present invention can also be produced by chemical synthesis based on known Lrp4 sequence data. Riboprobes, which are considered to be particularly

preferable for detecting RNA in tissues, can be obtained by, for example, inserting a cloned Lrp4 gene or portion thereof into plasmid vector pSP64 in the reverse direction, followed by run-off transcription of the inserted sequence portion. Although pSP64 contains an SP6 promoter, methods for producing riboprobes by combining phage T3, T7 promoter and RNA polymerase are also known. The marker polynucleotide probes of the present invention may be used as reagents for discriminating the dopaminergic neuron proliferative progenitor cells. In the above-described reagents, the polynucleotides as active ingredients may be mixed as necessary with, for example, sterile water, saline, vegetable oils, surfactants, lipids, solubilizers, buffers, stabilizers, and preservatives.

<Antibodies>

The present invention provides dopaminergic neuron progenitor cell marker antibodies (hereinbelow, sometimes referred to as "antibodies of the present invention") which can be used to select dopaminergic neuron progenitor cells from brain tissue or cultured cells. Unlike Lrp4 mRNA, the Lrp4 polypeptide is expressed not only in the dopaminergic neuron proliferative progenitor cells prior to cell cycle exit, but also in postmitotic dopaminergic neuron precursor cells. Therefore, by using the antibodies of the present invention against the polypeptide, it is possible to select and/or obtain dopaminergic neuron progenitor cells prior to and after cell cycle exit. Antibodies of the present invention include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, single-chain antibodies (scFV) (Huston *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 5879-83; The Pharmacology of Monoclonal Antibody, vol. 113, Rosenburg and Moore ed., Springer Verlag (1994) pp. 269-315), humanized antibodies, multispecific antibodies (LeDoussal *et al.* (1992) Int. J. Cancer Suppl. 7: 58-62; Paulus (1985) Behring Inst. Mitt. 78: 118-32; Millstein and Cuello (1983) Nature 305: 537-9; Zimmermann (1986) Rev. Physiol. Biochem. Pharmacol. 105: 176-260; Van Dijk *et al.* (1989) Int. J. Cancer 43: 944-9), and antibody fragments such as Fab, Fab', F(ab')2, and Fv. Moreover, antibodies of the present invention may also be modified by PEG and such, as necessary. Antibodies of the present invention may also be produced in the form of a fusion protein with β-galactosidase, maltose-binding protein, GST, green fluorescent protein (GFP) and such, to allow detection without the use of a secondary antibody. In addition, antibodies of the present invention may be modified by labeling with biotin or such, to allow recovery using avidin, streptoavidin, or such.

The antibodies of present invention are specific to any of (1) polypeptides encoded by the nucleotide sequence of SEQ ID NO: 1 or 2, (2) polypeptides comprised of the amino acid sequence described in SEQ ID NO: 3 or 4, (3) polypeptides comprised of an amino acid sequence lacking the transmembrane domain in the amino acid sequence described in SEQ ID

NO: 3 or 4, (4) polypeptides comprised of an amino acid sequence wherein one or more amino acids in the amino acid sequence of SEQ ID NO: 3 or 4 are deleted, inserted, substituted, or added, (5) polypeptides encoded by a nucleotide sequence that hybridizes under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, and (6) polypeptides that are fragments of the polypeptides of (1) to (5) above, with at least eight amino acid residues.

Also, the antibodies of the present invention may be antibodies that bind to polypeptides comprising any one of the following amino acid sequences (1) to (4) or parts thereof: (1) the amino acid sequence of SEQ ID NO: 3 or 4; (2) amino acid sequences which lack the transmembrane region in the amino acid sequence of SEQ ID NO: 3 or 4; (3) amino acid sequences mutated by one or more amino acid deletions, substitutions, or additions, or combinations thereof, in the amino acid sequence of SEQ ID NO: 3 or 4; and (4) amino acid sequences comprising polypeptides encoded by polynucleotides which hybridize under stringent conditions with polynucleotides comprising a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or 2. The above-described polypeptides consisting of partial sequences include polypeptides comprising preferably at least six consecutive amino acid residues (e.g., 8, 10, 12, or 15 amino acid residues or more).

The amino acid sequences mutated by one or more amino acid deletions, substitutions, or additions, or combinations thereof in the amino acid sequence of SEQ ID NO: 3 include, for example: (i) amino acid sequences with 1 to 9 (e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2, and still more preferably 1) amino acid deletions in the amino acid sequence of SEQ ID NO: 3; (ii) amino acid sequences with 1 to 9 (e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2, and still more preferably 1) amino acid additions in the amino acid sequence of SEQ ID NO: 3; (iii) amino acid sequences with 1 to 9 (e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2, and still more preferably 1) amino acids substituted by other amino acids in the amino acid sequence of SEQ ID NO: 3; and (iv) an amino acid sequence mutated by any combination of the above (i) to (iii).

The amino acid sequences mutated by one or more amino acid deletions, substitutions, or additions, or any combinations thereof in the amino acid sequence of SEQ ID NO: 4 include, for example: (i) amino acid sequences with 1 to 9 (e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2, and still more preferably 1) amino acid deletions in the amino acid sequence of SEQ ID NO: 4; (ii) amino acid sequences with 1 to 9 (e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2, and still more preferably 1) amino acid additions in the amino acid sequence of SEQ ID NO: 4; (iii) amino acid sequences with 1 to 9 (e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2, and still more preferably 1) amino acids substituted by other amino acids in the amino acid sequence of SEQ ID NO: 4; and (iv) amino acid sequences mutated by

any combination of the above (i) to (iii).

The term "amino acid deletions" herein means variants in which one or more amino acid residues in the sequence are deleted. The term "deletion" includes the deletion of an amino acid at either end of an amino acid sequence, and the deletion of an amino acid in an amino acid sequence.

The term "amino acid additions" herein means variants in which one or more amino acid residues have been added to the sequence. The term "addition" includes the addition of an amino acid to either end of an amino acid sequence and the addition of an amino acid in an amino acid sequence.

The term "amino acid substitutions" herein means variants in which one or more amino acid residues in the sequence are substituted with different amino acid residues. When amino acid sequences are modified by such substitution, conservative substitution is preferably carried out. The term "conservative substitution" means changing the sequence to encode an amino acid with similar properties to the amino acid prior to substitution. Amino acids can be classified according to their properties into, for example: non-polar amino acids (Ala, Ile, Leu, Met, Phe, Pro, Trp, and Val); non-charged amino acids (Asn, Cys, Gln, Gly, Ser, Thr, and Tyr); acidic amino acids (Asp and Glu); basic amino acids (Arg, His, and Lys); neutral amino acids (Ala, Asn, Cys, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val); aliphatic amino acids (Ala and Gly); branched amino acids (Ile, Leu, and Val), hydroxyamino acids (Ser and Thr); amide-type amino acids (Gln and Asn); sulfur-containing amino acids (Cys and Met); aromatic amino acids (His, Phe, Trp, and Tyr); heterocyclic amino acids (His and Trp); and imino acids (Pro and 4Hyp).

Therefore, it is preferable that non-polar amino acids are substituted with each other, or that non-charged amino acids are substituted with each other. Of these, substitutions among Ala, Val, Leu, and Ile, between Ser and Thr, between Asp and Glu, between Asn and Gln, between Lys and Arg, and between Phe and Tyr are preferable for retaining properties of the proteins. The number and sites of mutated amino acids are not particularly limited.

As the antibodies of the present invention, the two anti-Lrp4 antibodies used in Example 4, and modifications comprising fragments thereof, are particularly preferable. The two antibodies have been internationally deposited under the following accession numbers:
(1) Name and address of the depositary institution
Name: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology
Address: Central 6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki Prefecture, Japan 305-8566
(2) Deposit date: July 14, 2004
(3) Accession Nos: FERM BP-10315 and FERM BP-10316

(transferred from FERM P-20120 and FERM P-20121 domestically deposited in Japan)

Antibodies of the present invention can be produced using a sensitizing antigen such as an Lrp4 polypeptide, or fragments thereof, or cells that express Lrp4 polypeptide or Lrp4 polypeptide fragments. In addition, short Lrp4 polypeptide fragments may also be used as immunogens by coupling with a carrier such as bovine serum albumin, Keyhole-limpet hemocyanin, and ovalbumin. In addition, the Lrp4 polypeptide, or fragments thereof, may be used in combination with known adjuvants, such as aluminum adjuvant, Freund's complete (or incomplete) adjuvant, or pertussis adjuvant, to enhance immune response to the antigen.

The "Lrp4 polypeptide" in the present invention is a peptide polymer, a preferred example of which is a protein having the amino acid sequence described in SEQ ID NO: 3 or 4. The amino acid residues that compose an Lrp4 polypeptide may be naturally occurring or modified. Moreover, the Lrp4 polypeptides include proteins lacking a transmembrane domain portion, and fusion proteins modified by other peptide sequences.

In the present invention, the Lrp4 polypeptides should have the antigenicity of the Lrp4 polypeptide, and include polypeptides with an amino acid sequence wherein one or more amino acids in the amino acid sequence of SEQ ID NO: 3 or 4 are deleted, inserted, substituted, or added. It is well known that mutant polypeptides comprising an amino acid sequence in which one or more amino acids are deleted, inserted, substituted, or added, maintain the same biological activity as the original polypeptide (Mark *et al.* (1984) Proc. Natl. Acad. Sci. USA 81: 5662-6; Zoller and Smith (1982) Nucleic Acids Res. 10: 6487-500; Wang *et al.* (1984) Science 224: 1431-3; Dalbadie-McFarland *et al.* (1982) Proc. Natl. Acad. Sci. USA 79: 6409-13). Such polypeptides that maintain the antigenicity of Lrp4 and have an amino acid sequence in which one or more amino acids are deleted, inserted, substituted, or added to the amino acid sequence of SEQ ID NO: 3 or 4, can be obtained by preparing polynucleotides that encode the polypeptides using known methods such as site-directed mutagenesis described in "Molecular Cloning, A Laboratory Manual 2nd ed." (Cold Spring Harbor Press (1989)), "Current Protocols in Molecular Biology" (John Wiley & Sons (1987-1997); especially section 8.1-8.5), Hashimoto-Goto *et al.* (1995) Gene 152: 271-5, Kunkel (1985) Proc. Natl. Acad. Sci. USA 82: 488-92, Kramer and Fritz (1987) Method. Enzymol. 154: 350-67, Kunkel (1988) Method. Enzymol. 85: 2763-6), and others, and then suitably expressing. In addition to the above-described site-directed mutagenesis, methods for subjecting genes to mutagens can be used. Alternatively, methods for selectively cleaving genes; removing, adding, or substituting selected nucleotides; and then ligating the genes can also be used.

Lrp4 polypeptide fragments are identical to a portion of the aforementioned Lrp4 polypeptide, and preferably consists of at least six continuous amino acid residues or more (for

example, 8, 10, 12, or 15 amino acid residues or more). A particularly preferred fragment can be exemplified by a polypeptide fragment lacking an amino terminus, carboxyl terminus, and transmembrane domain. The Lrp4 polypeptide fragments include fragments containing an α-helix and α-helix forming region, α-amphipathic region, β-sheet and β-sheet forming region, β-amphipathic region, substrate binding region, high antigen index region, coil and coil forming region, hydrophilic region, hydrophobic region, turn and turn forming region, and surface forming region. In the context of the present invention, an Lrp4 polypeptide fragment may be any fragment, so long as it has the antigenicity of an Lrp4 polypeptide. The antigen-determining site of a polypeptide can be predicted by using methods for analyzing the hydrophobicity/hydrophilicity of an amino acid sequence of a protein (Kyte-Doolittle (1982) J. Mol. Biol. 157: 105-22), or methods of secondary structure analysis (Chou-Fasman (1978) Ann. Rev. Biochem. 47: 251-76), and can be confirmed using computer programs (Anal. Biochem. 151: 540-6 (1985)), or the PEPSCAN method in which a short peptide is synthesized followed by confirmation of its antigenicity (Published Japanese Translation of International Publication No. Sho 60-500684), or the like.

Lrp4 polypeptides and Lrp4 polypeptide fragments can be isolated from Lrp4-expressing cells, tissues, etc., based on their physical properties and such. In addition, these polypeptides and polypeptide fragments can also be produced using known genetic recombination techniques or chemical synthesis methods. For example, for *in vitro* Lrp4 polypeptide production, Lrp4 polypeptides can be produced in an *in vitro* cell-free system using methods such as *in vitro* translation (Dasso and Jackson (1989) Nucleic Acids Res. 17: 3129-44). In contrast, when producing polypeptides using cells, a polynucleotide that encodes a polypeptide of interest is first incorporated into an appropriate vector, a suitable host cell is selected, and then the cells are transformed by the vector. Subsequently, the transformed cells can be cultured to obtain the polypeptide of interest.

Appropriate vectors include various vectors such as plasmids, cosmids, viruses, bacteriophages, cloning vectors, and expression vectors (Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989); Current Protocols in Molecular Biology, John Wiley & Sons (1987)). The vectors comprise regulatory sequences for the expression of a desired polynucleotide in transfected host cells, and the polynucleotide is incorporated therein so that it will be under the control of the regulatory sequences. Here, the phrase "regulatory sequence" includes promoters, ribosome binding sites, and terminators in the case of a prokaryotic host cell, and promoters and terminators in the case of a eukaryotic host cell, and in some cases, may also contain transactivators, transcription factors, poly A signals which stabilize transcription products, splicing and polyadenylation signals, and others. Such regulatory sequences comprise all the components required for the expression of a

polynucleotide linked thereto. Vectors may further comprise a selection marker. Moreover, a signal peptide required for transferring an intracellularlly expressed polypeptide into the lumen of the endoplasmic reticulum, or the periplasm or extracellular space when the host is a Gram negative microbe, can also be incorporated into an expression vector by linking to a polypeptide of interest. Such signal peptides can be signal peptides derived from heterogeneous proteins. Moreover, a linker may be added, and a start (ATG) or stop codon (TAA, TAG, or TGA) may be inserted as necessary.

Examples of vectors capable of expressing polypeptides *in vitro* include pBEST (Promega). In addition, various vectors are known to be suitable for expression in prokaryotic hosts (see, *e.g.*, "Basic Microbiology Course 8 - Genetic Engineering" (Kyoritsu Publishing)). When selecting prokaryotic cells as the host, a person with ordinary skill in the art can suitably select a vector suitable for the host and a method suitable for introducing the vector into the host. Other examples of hosts that can be used to express Lrp4 polypeptides and their antigenic fragments include fungal cells such as yeasts, higher plants, insects, fish, amphibians, reptiles, birds, mammals, cultured cells (COS, Hela, C127, 3T3, BHK, HEK293, Bowes melanoma cells), myeloma, Vero, Namalwa, Namalwa KJM-1, and HBT5637 (Unexamined Published Japanese Patent Application No. Sho 63-299). Vector systems suitable for each cell and methods for introducing a vector into host cells are also known. Moreover, methods for expressing exogenous proteins in animals *in vivo* (see, *e.g.*, Susumu (1985) Nature 315: 592-4; Lubon (1998) Biotechnol. Annu. Rev. 4: 1-54) and in plant bodies are also known, and can be used to express Lrp4 polynucleotides.

DNAs can be inserted into vectors in a ligase reaction using restriction enzyme sites (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Section 11.4-11.11; Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989) Section 5.61-5.63). In addition, an Lrp4 polypeptide-encoding expression vector can be designed as necessary by selecting a nucleotide sequence that has high expression efficiency in view of the host's codon usage frequency (Grantham *et al.* (1981) Nucleic Acids Res. 9: r43-74). A host that produces an Lrp4 polypeptide comprises in its cells a polynucleotide that encodes an Lrp4 polypeptide. So long as the polynucleotide does not exist at a naturally occurring position in the genome of a host cell, the polynucleotide itself may be regulated by its own promoter, incorporated in the host genome, or maintained as an extrachromosomal structure.

Transduction (transformation/transfection) of host cells with vectors can be performed using well-known methods.

Transformation can be performed, for example, by the method by Cohen *et al.* (Proc. Natl. Acad. Sci. USA 69, 2110 (1972)), protoplast methods (Mol. Gen. Genet., 168, 111 (1979)), and the competent method (J. Mol. Biol., 56, 209 (1971)) for bacteria (*E. coli*,

Bacillus subtilis, etc.); by the method by Hinnen *et al.* (Proc. Natl. Acad. Sci. USA 75, 1927 (1978)) and lithium methods (J. Bacteriol., 153, 163 (1983)) for *Saccharomyces cerevisiae*; by leaf disk methods (Science, 227, 129 (1985)), electroporation methods (Nature, 319, 791 (1986)), and *Agrobacterium* methods (Horsch *et al.*, Science, 227, 129 (1985); and Hiei *et al.*, Plant J., 6, 271-282 (1994)) for plant cells; by the method by Graham (Virology, 52, 456 (1973)) for animal cells; and by the method by Summers *et al.* (Mol. Cell Biol., 3, 2156-2165 (1983)) for insect cells.

Culturing of host cells is carried out using known methods that are appropriate for the host cell selected. For example, when animal cells are selected, culturing can be carried out at a pH of about 6 to 8 and a temperature of 30°C to 40°C for about 15 to 200 hours, using a medium such as DMEM (Virology 8: 396 (1959)), MEM (Science 122: 501 (1952)), RPMI1640 (J. Am. Med. Assoc. 199: 519 (1967)), 199 (Proc. Soc. Biol. Med. 73: 1 (1950)), or IMDM, and adding serum such as fetal calf serum (FCS), as necessary. In addition, the medium may be replaced, aerated, or stirred during the course of culturing, as necessary.

Normally, an Lrp4 polypeptide produced by gene recombination techniques can be recovered from the medium if the polypeptide is secreted outside of a cell, or from the body fluid of a transgenic organism. When a polypeptide is produced inside of a cell, the cells can be dissolved and the polypeptide is recovered from the dissolved product. The polypeptide of interest can be then purified by suitably combining known methods of protein purification, such as salting out, distillation, various types of chromatography, gel electrophoresis, gel filtration, ultrafiltration, recrystallization, acid extraction, dialysis, immunoprecipitation, solvent precipitation, solvent extraction, and ammonium sulfate or ethanol precipitation. Examples of chromatographies include ion exchange chromatography, such as anion or cation exchange chromatography, affinity chromatography, reversed-phase chromatography, adsorption chromatography, gel filtration chromatography, hydrophobic chromatography, hydroxyapatite chromatography, phosphocellulose chromatography, and lectin chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Marshak *et al.* ed., Cold Spring Harbor Laboratory Press (1996)). Chromatography can be carried out using a liquid phase chromatography, such as HPLC or FPLC. In addition, for example, a protein fused with GST can be purified using a glutathione column, and a protein with a histidine tag can be purified using a nickel column. When an Lrp4 polypeptide is produced as a fusion protein, unnecessary portions can be removed using thrombin, factor Xa, or the like, following purification as necessary.

In addition, naturally-occurring polypeptides can also be purified and obtained. For example, polypeptides can be purified by affinity chromatography using antibodies against the Lrp4 polypeptides (Current Protocols in Molecular Biology, John Wiley & Sons (1987)

Section 16.1-16.19). Moreover, purified polypeptides can also be modified using enzymes, such as chymotrypsin, glucosidase, trypsin, protein kinase, and lysyl endopeptidase, as necessary. In addition to the aforementioned synthesis and genetic engineering techniques as used for Lrp4 polypeptides, Lrp4 polypeptide fragments can also be produced by cleaving an Lrp4 polypeptide, using suitable enzymes, such as peptidases.

Polyclonal antibodies for selecting dopaminergic neuron progenitor cells can be obtained from, for example, the serum of an immunized animal after immunizing a mammal with an Lrp4 polypeptide purified as described above, or a fragment thereof, coupled to a desired adjuvant. Although there are no particular limitations on the mammals used, typical examples include rodents, lagomorphs, and primates. Specific examples include rodents such as mice, rats, and hamsters, lagomorphs such as rabbits, and primates such as monkeys, including cynomolgus monkeys, rhesus monkeys, baboons, and chimpanzees. Animal immunization is carried out by suitably diluting and suspending a sensitizing antigen in phosphate-buffered saline (PBS) or physiological saline, mixing with an adjuvant as necessary until emulsified, and injecting into an animal intraperitoneally or subcutaneously. The sensitizing antigen mixed with Freund's incomplete adjuvant is preferably administered several times, every 4 to 21 days. Antibody production can be confirmed by measuring the level of an antibody of interest in the serum using conventional methods. Finally, the serum itself may be used as a polyclonal antibody, or it may be further purified. See, for example, "Current Protocols in Molecular Biology" (John Wiley & Sons (1987) Sections 11.12-11.13), for specific methods.

A monoclonal antibody can be produced by removing the spleen from an animal immunized in the manner described above, separating immunocytes from the spleen, and fusing with a suitable myeloma cell using polyethylene glycol (PEG) or such to establish hybridomas. Cell fusion can be carried out according to the Milstein method (Galfre and Milstein (1981) Methods Enzymol. 73: 3-46). Here, suitable myeloma cells are exemplified particularly by cells that allow chemical selection of fused cells. When using such myeloma cells, fused hybridomas can be selected by culturing in a culture medium (HAT culture medium) that contains hypoxanthine, aminopterin, and thymidine, which destroys cells other than fused cells. Next, clones that produce antibodies against polypeptides of the present invention, or a fragment thereof, can be selected from the established hybridomas. Subsequently, the selected clone is introduced into the abdominal cavity of a mouse or such, and ascites can be collected to obtain a monoclonal antibody. See also "Current Protocols in Molecular Biology" (John Wiley & Sons (1987) Section 11.4-11.11), for information on specific methods. Preferable hybridomas of the present invention can be FERM BP-10315 and FERM BP-10316.

Hybridomas can also be obtained by first sensitizing human lymphocytes that have been infected by EB virus with an immunogen *in vitro*, and fusing the sensitized lymphocytes with human myeloma cells (such as U266) to obtain hybridomas that produce human antibodies (Unexamined Published Japanese Patent Application No. Sho 63-17688). In addition, human antibodies can also be obtained by using antibody-producing cells generated by sensitizing a transgenic animal with a human antibody gene repertoire (WO92/03918; WO93-02227; WO94/02602; WO94/25585; WO96/33735; WO96/34096; Mendez *et al.* (1997) Nat. Genet. 15: 146-156, etc.). Methods that do not use hybridomas can be exemplified by a method in which a cancer gene is introduced to immortalize immunocytes such as antibody-producing lymphocytes.

In addition, antibodies of the present invention can also be produced by genetic recombination techniques (see Borrebaeck and Larrick (1990) Therapeutic Monoclonal Antibodies, MacMillan Publishers Ltd., UK). First, a gene that encodes an antibody is cloned from hybridomas or antibody-producing cells (such as sensitized lymphocytes). The resulting gene is then inserted into a suitable vector, the vector is introduced into a host, and the host is then cultured to produce the antibody. This type of recombinant antibody is also included in the antibodies of the present invention. Typical examples of recombinant antibodies include chimeric antibodies, comprising a non-human antibody-derived variable region and a human antibody-derived constant region, and humanized antibodies, comprising a non-human-derived antibody complementarity determining region (CDR), human antibody-derived framework region (FR), and human antibody constant region (Jones *et al.* (1986) Nature 321: 522-5; Reichmann *et al.* (1988) Nature 332: 323-9; Presta (1992) Curr. Op. Struct. Biol. 2: 593-6; Methods Enzymol. 203: 99-121 (1991)).

Antibody fragments can be produced by treating the aforementioned polyclonal or monoclonal antibodies with enzymes such as papain or pepsin. Alternatively, antibody fragments can be produced by genetic engineering techniques using genes that encode antibody fragments (see Co *et al.*, (1994) J. Immunol. 152: 2968-76; Better and Horwitz (1989) Methods Enzymol. 178: 476-96; Pluckthun and Skerra (1989) Methods Enzymol. 178: 497-515; Lamoyi (1986) Methods Enzymol. 121: 652-63; Rousseaux *et al.* (1986) 121: 663-9; Bird and Walker (1991) Trends Biotechnol. 9: 132-7).

Multispecific antibodies include bispecific antibodies (BsAb), diabodies (Db), and such. Multispecific antibodies can be produced by methods such as (1) chemically coupling antibodies having different specificities with different types of bifunctional linkers (Paulus (1985) Behring Inst. Mill. 78: 118-32), (2) fusing hybridomas that secrete different monoclonal antibodies (Millstein and Cuello (1983) Nature 305: 537-9), or (3) transfecting eukaryotic cell expression systems, such as mouse myeloma cells, with a light chain gene and

a heavy chain gene of different monoclonal antibodies (four types of DNA), followed by the isolation of a bispecific monovalent portion (Zimmermann (1986) Rev. Physio. Biochem. Pharmacol. 105: 176-260; Van Dijk *et al.* (1989) Int. J. Cancer 43: 944-9). Alternatively, diabodies are dimer antibody fragments comprising two bivalent polypeptide chains that can be constructed by gene fusion. These can be produced using known methods (see Holliger *et al.* (1993) Proc. Natl. Acad. Sci. USA 90: 6444-8; EP404097; WO93/11161).

Recovery and purification of antibodies and antibody fragments can be carried out using Protein A and Protein G, or according to the protein purification techniques described above in producing non-antibody polypeptides (Antibodies: A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)). For example, when using Protein A to purify antibodies of the present invention, Protein A columns such as Hyper D, POROS, or Sepharose F.F. (Pharmacia), can be used. The concentration of the resulting antibodies can be determined by measuring absorbance or by enzyme linked immunosorbent assay (ELISA).

The antigen binding activity of an antibody can be determined using absorbance measurements, or by using fluorescent antibody methods, enzyme immunoassay (EIA) methods, radioimmunoassay (RIA) methods, or ELISA. When ELISA is used, for example, antibodies of the present invention are first immobilized onto a support, such as a plate. An Lrp4 polypeptide is added, and then a sample containing the antibody of interest is added. Herein, samples containing an antibody of interest include, for example, culture supernatants of antibody-producing cells, purified antibodies, and such. Next, secondary antibodies that recognize the antibodies of the present invention are added, and the plate is incubated. The plate is then washed and a label attached to the secondary antibody is detected. Namely, if a secondary antibody is labeled with alkaline phosphatase, antigen binding activity can be determined by adding an enzyme substrate such as p-nitrophenyl phosphate, and measuring absorbance. In addition, a commercially available system such as BIAcore (Pharmacia) can also be used to evaluate antibody activities.

The present invention relates to reagents which comprise the anti-Lrp4 antibody as an active ingredient for identifying dopaminergic neuron progenitor cells. In the above-described reagents, the antibodies of the present invention as active ingredients may be mixed as necessary with, for example, sterile water, saline, vegetable oils, surfactants, lipids, solubilizer, buffers, protein stabilizers (such as BSA and gelatin), and preservatives.

<Methods for selecting dopaminergic neuron progenitor cells and such>

The present invention provides methods for selecting dopaminergic neuron proliferative progenitor cells as a selectively uniform population. The dopaminergic neuron proliferative progenitor cells can be selected by using the marker polynucleotide probes of the

present invention. The present invention also provides methods for selecting the dopaminergic neuron progenitor cells as a selectively uniform population. The dopaminergic neuron progenitor cells can suitably be selected using the antibodies of the present invention. As described above, by using the polynucleotide probes or antibodies of the present invention, the dopaminergic neuron lineage cells which eventually differentiate into dopaminergic neurons can be specifically selected.

Here, the term "selected" includes both detecting the presence of cells expressing markers in a sample, and subsequently separating or isolating those progenitor cells after detecting their presence. The present invention provides methods for selecting dopaminergic neuron proliferative progenitor cells, comprising a step of contacting marker polynucleotide probes of the present invention with cell samples containing dopaminergic neuron proliferative progenitor cells. In this method, marker polynucleotide probes are preferably labeled with a radioactive isotope or non-radioactive compound. Examples of the radioactive isotopes used for labeling include ^{35}S and ^{3}H. When using a radiolabeled marker polynucleotide probe, RNA that binds with the marker can be detected by detecting silver particles using emulsion autoradiography. Examples of non-radioactive isotopes for labeling a marker polynucleotide probe include biotin and digoxigenin. A biotin-labeled marker can be detected using, for example, avidin labeled with fluorescence or an enzyme such as alkaline phosphatase or horseradish peroxidase. On the other hand, anti-digoxigenin antibodies labeled with fluorescence or an enzyme, such as alkaline phosphatase or horseradish peroxidase, can be used to detect a digoxigenin-labeled marker. When using enzyme labeling, detection can be carried out by incubating with the enzyme substrate to form a stable pigment at the location of the marker. Fluorescent *in situ* hybridization (FISH) is convenient and particularly preferable.

In addition, the present invention provides methods for selecting dopaminergic neuron progenitor cells comprising the step of contacting antibodies for selecting the dopaminergic neuron progenitor cells of the present invention with cell samples containing dopaminergic neuron progenitor cells. Namely, dopaminergic neuron progenitor cells can be acquired by contacting cell samples containing potential dopaminergic neuron progenitor cells with antibodies of the present invention, and selecting those cells that have bound to the antibody (see Fig. 13). The antibodies of the present invention may also be immobilized on a suitable support, prior to contact with cell samples. Alternatively, cells that bind with the antibodies can be selectively recovered by contacting cell samples with the antibodies of the present invention, allowing them to bind, and then purifying the antibody by affinity chromatography. For example, if the antibodies of the present invention are conjugated to biotin, they can be purified on a plate or column bound with avidin or streptoavidin. In

addition, magnetic particles can be bound to an antibody, for example, and the antibody and cells that express Lrp4 on their surfaces, where the Lrp4 is bound to the antibody, can be recovered using a magnet. Dopaminergic neuron progenitor cells that express Lrp4 can be selected by flow cytometry using a cell sorter and fluorescent-labeled anti-Lrp4 antibodies and such.

The dopaminergic neuron progenitor cell populations obtained as described above are cell populations comprising, for example, 40% or more, preferably 50% or more, more preferably 60% or more, and particularly preferably 70% or more dopaminergic neuron progenitor cells.

The present invention provides methods for selecting and/or producing postmitotic dopaminergic neuron precursor cells, which comprise the step of culturing the dopaminergic neuron progenitor cells selected by using the marker polynucleotide probes or antibodies of the present invention. The present invention also provides methods for selecting and/or producing postmitotic dopaminergic neuron precursorcells, which comprise the steps of contacting cell samples comprising the dopaminergic neuron progenitor cells with the marker polynucleotide probes or antibodies of the present invention; selecting the dopaminergic neuron progenitor cells; and culturing the selected cells.

Furthermore, according to the present invention, the postmitotic dopaminergic neuron precursor cells, which have a low risk of tumorigenesis and are suitable for transplant therapy, can be obtained by culturing the dopaminergic neuron progenitor cells selected by using the marker polynucleotide probes or antibodies of the present invention; and selecting and/or screening by using the postmitotic dopaminergic neuron precursorcell markers. The postmitotic dopaminergic neuron precursor cell markers can include, for example, 65B13, Nurr1, and TH (WO 2004/038018; Kawasaki *et al.*, Neuron 28:31-40 (2000); Wallen *et al.*, Exp. Cell Res., 253:737-46 (1999)). For example, the postmitotic dopaminergic neuron precursor cells can be selected and/or produced by contacting cell samples comprising dopaminergic neuron progenitor cells with the marker polynucleotide probes or antibodies of the present invention; selecting the dopaminergic neuron progenitor cells; and contacting the antibody against 65B13 polypeptide with the dopaminergic neuron progenitor cells further cultured as necessary to select the cells expressing the 65B13 polypeptide.

The present invention provides methods for selecting and/or producing dopaminergic neurons, where the methods comprise the step of culturing the dopaminergic neuron progenitor cells selected by using the marker polynucleotide probes or antibodies of the present invention. The present invention also provides methods for selecting and/or producing dopaminergic neurons which comprise the steps of contacting cell samples containing the dopaminergic neuron progenitor cells with the marker polynucleotide probes or

antibodies of the present invention; selecting the dopaminergic neuron progenitor cells; and culturing the selected cells.

According to the present invention, dopaminergic neurons, which have a low risk of tumorigenesis and are suited to transplant therapy, can also be obtained by culturing the dopaminergic neuron progenitor cells selected by using the marker polynucleotide probes or antibodies of the present invention; and selecting and/or screening by using the dopaminergic neuron markers. The dopaminergic neuron markers can include, for example, DAT (Development 131(5): 1145-55 (2004)). For example, the dopaminergic neurons can be selected and/or produced by contacting cell samples containing the dopaminergic neuron progenitor cells with the marker polynucleotide probes or antibodies of the present invention; selecting the dopaminergic neuron progenitor cells; and contacting an antibody against DAT with the further cultured dopaminergic neuron progenitor cells to select cells expressing DAT.

The present invention provides methods for selecting and/or producing dopaminergic neuron proliferative progenitor cells capable of proliferating when cultivated, where the methods comprise the steps of culturing or not culturing the dopaminergic neuron progenitor cells selected by using the antibodies of the present invention; and removing the postmitotic dopaminergic neuronprecursor cells. The present invention also provides methods for selecting and/or producing the dopaminergic neuron proliferative progenitor cells capable of proliferating when cultivated, where the methods comprise the steps of contacting cell samples containing the dopaminergic neuron progenitor cells with the antibodies of the present invention; selecting the dopaminergic neuron progenitor cells; culturing or not culturing the selected cells; and then removing the postmitotic dopaminergic neuron precursor cells.

In the step of removing the postmitotic dopaminergic neuron precursor cells, dopaminergic neuron proliferative progenitor cells capable of proliferating when cultivated can also be obtained by selection and/or removal using markers for postmitotic dopaminergic neuron precursor cells. Markers for postmitotic dopaminergic neuron precursor cells can include, for example, 65B13, Nurr1, and TH (WO 2004/038018; Kawasaki *et al.*, Neuron 28:31-40 (2000); Wallen *et al.*, Exp. Cell Res., 253:737-46 (1999)). Dopaminergic neuron proliferative progenitor cells can be selected and/or produced by, for example, contacting the antibodies of the present invention with cell samples containing dopaminergic neuron progenitor cells, selecting the dopaminergic neuron progenitor cells, and contacting an antibody against the 65B13 polypeptide with the dopaminergic neuron progenitor cells further cultured as necessary to select cells which do not express the 65B13 polypeptide.

In addition, Lrp4-expressing dopaminergic neuron progenitor cells can also be selected and/or screened using promoters for Lrp4 (see, for example, Unexamined Published Japanese Patent Application No. 2002-51775). For example, a vector harboring a construct

that comprises a gene encoding a detection marker, such as GFP, linked to a promoter region obtained from analyzing the Lrp4 expression regulatory regions to be described later, can be transfected into cells. In addition, a gene encoding a marker can also be knocked in at the Lrp4 gene locus. In either case, specific cells can be selected by detecting the expression of a marker gene specific to dopaminergic neuron progenitor cells.

The cell samples used herein preferably comprise cells of the ventral midbrain region or culture cells containing *in vitro* differentiated dopaminergic neuron progenitor cells. *In vitro* differentiation of dopaminergic neurons can be carried out by known methods using a starting material like cells such as known ES cells, bone marrow interstitial cells, immortalized neuron-derived cell lines (Published Japanese Translation of International Publication No. Hei 8-509215; Published Japanese Translation of International Publication No. Hei 11-506930; Published Japanese Translation of International Publication No. 2002-522070), or primordial neuron cells (Published Japanese Translation of International Publication No. Hei 11-509729). Normally, dopaminergic neurons can be differentiated by co-culturing a tissue obtained from a dopaminergic neuron region of the brain, with a sustentacular cell layer derived from neural tissues. Moreover, methods are also known for deriving dopaminergic neurons from neural tissues that do not normally produce dopamine, such as the striatum and cortex (Published Japanese Translation of International Publication No. Hei 10-509319). In addition, culturing under hypoxic conditions has been reported to produce cells containing a greater number of dopaminergic neurons (Published Japanese Translation of International Publication No. 2002-530068). ES cells (CCE) can also be induced to differentiate to dopaminergic neurons using the 5-stage method (Lee *et al.* (2000) Nat. Biotech. 18: 675-679, mouse dopaminergic neuron differentiation kit (R&D Systems)). Cell samples used in the selection of dopaminergic neuron progenitor cells of the present invention may be cell populations isolated or cultured by any method, including the above-described methods.

In addition, the supports used in immobilizing the antibodies of the present invention are preferably safe for cells. Examples of such supports include synthetic or naturally-occurring organic polymer compounds, inorganic materials such as glass beads, silica gel, alumina, and activated charcoal, and those with surfaces coated with a polysaccharide or synthetic polymer. There are no particular limitations on the form of the support, examples of which include films, fibers, granules, hollow fibers, non-woven fabrics, porous supports, or honeycombed supports. The contact surface area of the supports can be controlled by changing their thickness, surface area, width, length, shape, and size in various ways.

<Kits for treating neurodegenerative diseases comprising dopaminergic neuron progenitor

cells, and methods for treating neurodegenerative diseases using dopaminergic neuron progenitor cells>

The cells acquired using polynucleotide probes and expression of Lrp4 mRNA as an index are dopaminergic neuron proliferative progenitor cells. Cells acquired using the antibodies and expression of Lrp4 polypeptides as an index are dopaminergic neuron progenitor cells. Thus, by using either the mRNAs or the polypeptides as indexes, dopaminergic neuron lineage cell populations can be obtained. The progenitor cells obtained by the methods of the present invention are more preferable for transplant therapy for diseases related to postural reflex, movement, and reward-associated behaviors, particularly neurodegenerative diseases such as Parkinson's disease, schizophrenia, and drug habits (Hynes *et al.*, Cell 80: 95-101 (1995)) in terms of safety, survival rate, and network forming capacity compared to conventional unpurified cell populations or dopaminergic neurons into which exogenous genes are introduced. The cells acquired using Lrp4 expression as an index can be used for transplantation directly or after *in vitro* proliferation (Fig. 13). Such cells are expected to exert therapeutic effects because they are likely to differentiate and mature in an optimal place in the brain. Therefore, the present invention also provides kits (hereinafter, sometimes referred to as "kits of the present invention") for treating neurodegenerative diseases, where the kits comprises dopaminergic neuron proliferative progenitor cells, postmitotice dopaminergic neuron precursor cells, and/or dopaminergic neurons selected and/or produced using the marker polynucleotide probes or antibodies of the present invention, as well as methods for treating neurodegenerative diseases which comprise the step of transplanting the cells into the brains of patients. Furthermore, the present invention also provides uses of dopaminergic neuron proliferative progenitor cells, postmitotic dopaminergic neuron precursorcells, and/or dopaminergic neurons selected and/or produced using the marker polynucleotide probes or antibodies of the present invention for producing the kits for treating neurodegenerative disease. In the present invention, the term "neurodegenerative diseases" preferably includes Parkinson's disease. The kits of the present invention may comprise pharmaceutically acceptable carriers in addition to the cells. The term "carriers" include, for example, salines, phosphate buffers, media, sera, body fluids, carboxymethyl cellulose solutions, scaffolding solids for supporting cells (e.g., cytodex3 (Amersham Bioscience, 17-0485-01)), extracellular matrix components (e.g., collagen, fibronectin, vitronectin, laminin, heparan sulfate, proteoglycan, glucosaminoglycan, chondroitin sulfate, hyaluronate, elastin, or combination of two or more thereof), or gel-like supports. A pH adjuster, buffer, stabilizer, preservative, and the like can be added to the kits of the present invention. The kits of the present invention may be for single or multiple inoculation. It is also possible to appropriately select dosage depending on the body weight and age of the

humans or animals to be inoculated, the administration methods, and so on.

Since the dopaminergic neuron progenitor cells selected using Lrp4 expression as an index are likely to further proliferate *in vivo*, they are expected to exert therapeutic effects for an extended period. Furthermore, the dopaminergic neuron progenitor cells selected using the Lrp4 expression as an index can be differentiated *in vitro* to an appropriate stage by selecting conditions such as the medium, and are preferred as the materials for various neural transplant therapies. As described above, for example, the dopaminergic neuron progenitor cells selected using Lrp4 expression as an index can be subjected to further selection using markers (e.g., 65B13, Nurr1, TH, etc.) for postmitotic dopaminergic neuron precursor cells, to obtain even safer cells for use in transplantation. The present invention relates to methods for culturing the above-described progenitor cells *in vitro* for differentiation and proliferation. The dopaminergic neuron progenitor cells for culturing can be postmitotic precursor cells.

For example, 1×10^2 to 1×10^8 dopaminergic neuron progenitor cells obtained using the methods of the present invention can be transplanted, preferably 1×10^3 to 1×10^6 cells, and more preferably 5×10^4 to 6×10^4 cells. The primary method is stereotaxic surgery, in which a cell suspension is transplanted into the brain. In addition, cells may also be transplanted by microsurgery. For methods of transplanting neuron tissues see Backlund *et al.* (Backlund *et al.* (1985) J. Neurosurg. 62: 169-73), Lindvall *et al.* (Lindvall *et al.* (1987) Ann. Neurol. 22: 457-68), or Madrazo *et al.* (Madrazo *et al.* (1987) New Engl. J. Med. 316: 831-4).

Moreover, the cells of the present invention can also be used to isolate genes specific to dopaminergic neuron proliferative progenitor cells, and genes specific to each stage of maturation from proliferative progenitor cells into dopaminergic neurons. They can also be used to search for therapeutic targets for Parkinson's disease, to elucidate the maturation process of dopaminergic neurons, and in screenings using maturation as an indicator.

<Comparison of Gene Expression Levels>

Dopaminergic neuron progenitor cells, obtained using the polynucleotide probes and antibodies of the present invention, can be used as materials to isolate genes specifically expressed in these cells. They can also be used to investigate and isolate genes specifically expressed in cells that have been differentiated, induced, or proliferated from the dopaminergic neuron progenitor cells of the present invention. In addition, they can also be used to investigate the genes required for *in vivo* differentiation of dopaminergic neurons, by investigating genes that have different expression levels in cells that have differentiated, induced, or proliferated and the original progenitor cells. Since such genes are potential candidates for treating diseases caused by defects in dopaminergic neurons, determining and

isolating them is extremely useful.

Comparison of gene expression levels in the dopaminergic neuron progenitor cells of the present invention with those in cells that have been differentiated, induced, or proliferated therefrom, or other cells; or comparison of gene expression levels of the differentiated, induced, or proliferated cells with those of other cells, can be done using commonly used methods, such as cell *in situ* hybridization, Northern blot hybridization, RNA dot blot hybridization, reverse transcription PCR, RNase protection assay, DNA microarray hybridization, serial analysis of gene expression (SAGE) (Velculescu *et al.* (1995) Science 270: 484-487), subtractive hybridization, and representation difference analysis (RDA) (Lisitsyn (1995) Trends Genet. 11: 303-307).

For cellular *in situ* hybridization, places where RNA processing, transport, and localization into the cytoplasm occur in individual cells can be investigated, by hybridizing total RNA or poly A^+ RNA prepared from cells with a labeling probe specific to a given RNA sequence. In addition, RNA size can be determined from size fractioning using gel electrophoresis. Moreover, RNA transcription products can be visualized *in situ* by using quantitative fluorescent *in situ* hybridization (FISH) and a digital imaging microscope (Femino *et al.* (1998) Science 280: 585-90), which are applicable to the present invention.

When using reverse transcription PCR for gene expression analysis, the expression of specific genes can be roughly quantified. Various isoforms of a single RNA transcription product can also be detected and analyzed using the present methods. For reverse transcription PCR, when the reaction is carried out using exon-specific primers, and amplification products other than the predicted product are detected, mRNA isoforms produced by alternative splicing can be identified by analyzing these products. For more details see, for example, the method described in Pykett *et al.* (1994) Hum. Mol. Genet. 3: 559-64. When a quick and rough analysis of expression pattern is required, the methods which use PCR of the present invention are particularly preferred, in terms of their high speed, high sensitivity, and simplicity.

The efficiency of gene expression screening can be improved by using DNA chips. Herein, a DNA chip refers to a miniature array in which oligonucleotides, DNA clones, or such are immobilized at a high density on a support surface, such as glass. For example, in order to carry out multiple expression screening, cDNA clones for each gene of interest, or oligonucleotides specific to each gene, are immobilized on a chip to produce a microarray. Next, RNAs are prepared from the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom, and treated with reverse transcriptase to yield cDNAs. Next, the resulting cDNA samples are labeled with fluorescent tags or other tags, and then hybridized to the microarray. As a result, genes that are actively

expressed in the cells have a higher percentage of total labeled cDNA, while genes that are not significantly expressed have a lower percentage. Namely, the fluorescent signal intensity, which represents hybridization between a labeled cDNA and a cDNA clone or an oligonucleotide on the chip, reflects the expression level of each sequence in the labeled cDNA, and thereby enables quantification of gene expression.

In addition, multiple genes in the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom, can be simultaneously analyzed by mRNA differential display, which involves reverse transcription PCR using degenerate PCR primers. First, a modified oligo dT primer is prepared, in which one or two nucleotides at the 3' terminus in the poly A tail of a given mRNA have been altered. Then, a reverse transcription reaction is carried out using the total RNAs isolated from the dopaminergic neuron progenitor cells of the present invention, cells differentiated or proliferated therefrom, or control cells to be used for expression comparison (Liang *et al.* (1993) Nucleic Acids Res. 21: 3269-3275). If the altered nucleotide is a "G", then mRNAs with a "C" immediately before the poly A tail can be selectively amplified. If the altered nucleotides are "CA", then mRNAs with "TG" immediately before the poly A tail can be selectively amplified. Next, an arbitrary nucleotide sequence of about 10 nucleotides in length is prepared for use as a second primer, and a PCR amplification reaction is carried out using the modified oligo dT primer and this second primer. The amplification product is subjected to size fractionation by electrophoresis using a long polyacrylamide gel. By using such methods, cDNAs derived from the mRNAs specifically expressed in either of the cells of the present invention or the control cells can be detected as bands only present in the samples that have been electrophoresed. These methods can also be used to analyze expression of unidentified genes.

SAGE analysis does not require a special device for detection, and is one of the preferred analytical methods for simultaneously detecting the expression of a large number of transcription products. First, poly A^+ RNA is extracted from the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom, using standard methods. Next, the RNAs are converted into cDNAs using a biotinylated oligo (dT) primer, and are then treated with a four-base recognizing restriction enzyme (Anchoring Enzyme: AE). Here, the AE-treated fragments contain a biotin group at their 3' terminus. Next, the AE-treated fragments are incubated with streptoavidin for binding. The bound cDNA is divided into two fractions, and each fraction is then linked to a different double-stranded oligonucleotide adapter (linker) A or B. These linkers are composed of: (1) a protruding single strand portion having a sequence complementary to the sequence of the protruding portion formed by the action of the anchoring enzyme, (2) a 5'

nucleotide recognizing sequence of the IIS-type restriction enzyme (cleaves at a predetermined location no more than 20 bp away from the recognition site) serving as a tagging enzyme (TE), and (3) an additional sequence of sufficient length for constructing a PCR-specific primer. Herein, the linker-linked cDNA is cleaved using the tagging enzyme, and only the linker-linked cDNA sequence portion remains, which is present in the form of a short-strand sequence tag. Next, pools of short-strand sequence tags from the two different types of linkers are linked to each other, followed by PCR amplification using primers specific to linkers A and B. As a result, the amplification product is obtained as a mixture comprising myriad sequences of two adjacent sequence tags (ditags) bound to linkers A and B. The amplification product is treated with the anchoring enzyme, and the free ditag portions are linked into strands in a standard linkage reaction. The amplification product is then cloned. The clone's determined nucleotide sequence can be used to obtain a read-out of consecutive ditags of constant length. The presence of mRNA corresponding to each tag can then be identified once from the determination of the clone's nucleotide sequence and information on the sequence tags thus obtained.

Subtraction hybridization is frequently used to clone genes with different expression levels in various tissues or cells, and can also be used to clone genes specifically expressed in the dopaminergic neuron progenitor cells of the present invention, or cells differentiated, induced, or proliferated therefrom. First, from the dopaminergic neuron progenitor cells of the present invention, a DNA sample of a cell to be tested is prepared (hereinafter referred to as "test DNA"). Next, a DNA of a cell to be compared is prepared (hereinafter referred to as "driver DNA"). The test and driver DNAs can also be used interchangeably. In any case, genes present in the test DNA but absent from the driver DNA are detected. Next, the prepared test DNA is mixed with a large excess of driver DNA, denatured to form single-stranded DNA, then annealed. By regulating the annealing conditions, specific sequences absent from the driver DNA can be isolated as double-stranded DNAs comprising only the test DNA sequence. For further detail on this method see, Swaroop *et al.* (1991) Nucleic Acids Res. 19: 1954 and Yasunaga *et al.* (1999) Nature Genet. 21: 363-9.

The RDA method is a method that uses PCR to selectively amplify a sequence of a test DNA that is absent in a driver DNA, and can be used in the present invention similarly to other previously described methods. For more details on the procedure see Lisitsyn (1995) Trends Genet. 11: 303-7 and Schutte *et al.* (1995) Proc. Natl. Acad. Sci. USA 92: 5950-4.

Genes specific to dopaminergic neuron progenitor cells, or cells differentiated, induced, or proliferated therefrom, are detected and isolated as described, and can be inserted into vectors or such, for sequence determination and expression analysis using the various known methods described above.

<Screening Using Dopaminergic Neuron Progenitor Cell Maturation as an Index>

The present invention provides screening methods that comprise the step of contacting test substances with the dopaminergic neuron progenitor cells of the present invention, and the step of detecting the differentiation or proliferation of the progenitor cells that results from that contact. Since compounds obtained by this screening method demonstrate a regulatory function in the differentiation, proliferation, and such, of dopaminergic neurons, they are considered useful as potential therapeutic candidates for diseases caused by defects in dopaminergic neurons. The dopaminergic neuron progenitor cells of the present invention include cells selected by using the polynucleotide probes or antibodies of the present invention, and cells obtained by the proliferation and/or differentiation induction of these cells.

Here, the "test substance" may be any type of compound, examples of which include the expression products of gene libraries, synthetic low molecular weight compound libraries, synthetic peptide libraries, antibodies, substances released by bacteria, cell (microbial, plant, or animal) extracts, cell (microbial, plant, or animal) culture supernatants, purified or partially purified polypeptides, marine organisms, plant or animal extracts, soil, random phage peptide display libraries, and such.

Cell differentiation and proliferation can be detected by comparison with cell status in the absence of the test substance. Cell differentiation and proliferation can be detected by morphological observation under a microscope or by detection or quantification of substances produced in cells, such as dopamine.

<Analysis of Lrp4 Expression Regulatory Region>

Using a sequence of the Lrp4 gene, an expression regulatory region of Lrp4 can be cloned from genomic DNA by known methods. For example, a method for establishing the transcriptional start site, such as the S1 mapping method, is known and can be used (Cell Engineering, Supplement 8, New Cell Engineering Experiment Protocol, Cancer Research Division, The Institute of Medical Science, The University of Tokyo ed., Shujunsha Publishing (1993) pp. 362-374). In general, the expression regulatory region of a gene can be cloned by screening genomic DNA libraries, using probe DNAs comprising a 15-100 bp segment, and preferably a 30-50 bp segment, of the gene's 5' terminus (in the present invention, all or a portion of nucleotides of SEQ ID NO: 1 or SEQ ID NO: 2). A clone obtained in this manner contains a 5' non-coding region of 10 kbp or more, and is shortened or fragmented by exonuclease treatment, or such. Finally, the shortened sequence portion, comprising a potential expression regulatory region, is evaluated for the strength, regulation and such of its

expression using a reporter gene, thereby making it possible to determine the minimum unit required to maintain the activity of the Lrp4 expression regulatory region.

Gene expression regulatory regions can be predicted using a program such as Neural Network (http://www.fruitfly.org./seq_tools/promoter.html; Reese *et al.*, Biocomputing: Proceedings of the 1996 Pacific Symposium, Hunter and Klein ed., World Scientific Publishing Co., Singapore, (1996)). Moreover, a program for predicting the minimum unit required for the activity of an expression regulatory region is also known and can be used (http://biosci.cbs.umn.edu./software/proscan/promoterscan. htm; Prestridge (1995) J. Mol. Biol. 249: 923-932).

The expression regulatory region of the Lrp4 gene isolated in this manner can be used to produce proteins of interest specifically in dopaminergic neuron proliferative progenitor cells *in vivo*.

<Ligand for Lrp4>

The Lrp4 polypeptides have a transmembrane domain, and thus in nature are thought to exist embedded within the cell membrane. Due to its expression in dopaminergic neuron progenitor cells, Lrp4 is believed to be involved in the regulation of dopaminergic neuron progenitor cell proliferation and in neuron differentiation and maturation. Thus, potential ligands that may demonstrate an agonistic or antagonistic function towards Lrp4 may be used to regulate the differentiation of dopaminergic neurons *in vivo*, *ex vivo*, and *in vitro*. In identifying ligands for Lrp4 polypeptides, an Lrp4 polypeptide and a candidate compound are first contacted and tested for the presence of binding. In this case, the Lrp4 polypeptide can be used when immobilized on a support, or embedded in the cell membrane. There are no particular limitations on the candidate compounds, examples of which include expression products of gene libraries, natural substances derived from marine organisms, extracts of various types of cells, known compounds and peptides, natural substances derived from plants, body tissue extracts, microbial culture supernatants and peptide groups randomly produced by the phage display method (J. Mol. Biol. 222: 301-10 (1991)). In addition, the candidate compound may be labeled for detection of binding.

<Inhibition of Lrp4 Expression>

Since the present invention clearly demonstrates that Lrp4 mRNA is transiently expressed in dopaminergic neuron proliferative progenitor cells, Lrp4 may be involved in the control of progenitor cell proliferation as well as neuron differentiation and maturation. Thus, substances that inhibit Lrp4 gene expression may be used to control the differentiation of dopaminergic neurons *in vivo*, *ex vivo*, and *in vitro*. Examples of substances capable of

inhibiting gene expression include antisense nucleic acids, ribozymes, and double-stranded RNAs (small interfering RNA; siRNA). Thus, the present invention provides such antisense nucleic acids, ribozymes, and double-stranded RNAs.

Examples of antisense mechanisms that suppress target gene expression include: (1) inhibition of transcription initiation via triplex formation, (2) transcription suppression through hybrid formation at sites of local open-loop structures formed by RNA polymerase, (3) transcription inhibition through hybrid formation with RNA during synthesis, (4) suppression of splicing through hybrid formation at intron-exon junctions, (5) suppression of splicing through hybrid formation at sites of spliceosome formation, (6) suppression of mRNA migration to the cytoplasm through hybrid formation with mRNA, (7) suppression of splicing through hybrid formation at a capping site or poly A addition site, (8) suppression of translation initiation through hybrid formation at binding sites of translation initiation factors, (9) translation suppression through hybrid formation at ribosome binding sites, (10) suppression of peptide chain elongation through hybrid formation at mRNA coding regions or polysome binding sites, and (11) suppression of gene expression through hybrid formation at sites of nucleic acid/protein interaction (Hirashima and Inoue, "New Biochemistry Experiment Course 2, Nucleic Acids IV, Gene Replication and Expression", Japanese Biochemical Society edit., Tokyo Kagaku Dozin Publishing, pp. 319-347 (1993)).

An Lrp4 antisense nucleic acid of the present invention may be a nucleic acid that inhibits gene expression by any of the mechanisms described in (1) to (11) above. Namely, it may contain an antisense sequence to not only a sequence of a coding region, but also a sequence of a non-coding region of a target gene whose expression is to be inhibited. A DNA that encodes an antisense nucleic acid can be used by linking to a suitable regulatory sequence that allows its expression. The antisense nucleic acid does not need to be completely complementary to the coding region or non-coding region of a target gene, as long as it can effectively inhibit the expression of this gene. Such antisense nucleic acids have a chain length of at least 15 bp or more, preferably 100 bp or more, and more preferably 500 bp or more, and are normally within 3000 bp, preferably within 2000 bp, and more preferably within 1000 bp. It is preferable that such antisense nucleic acids share an identity of 90% or more, and more preferably 95% or more, with the complementary chain of a target gene transcription product. These antisense nucleic acids can be prepared according to phosphorothionate methods (Stein (1988) Nucleic Acids Res. 16: 3209-21) or the like, using Lrp4 polynucleotides.

"Ribozyme" is a generic term referring to catalysts with an RNA component, and ribozymes are broadly classified into large ribozymes and small ribozymes. Large ribozymes cleave the phosphate-ester bonds of a nucleic acid, and after reaction, they leave 5'-phosphoric

acid and 3'-hydroxyl group at the reaction sites. Large ribozymes are further classified into (1) group I intron RNAs, which carry out guanosine-initiated trans-esterification reactions at 5'-splice sites, (2) group II intron RNAs, which perform two-step self-splicing reactions via a lariat structure, and (3) RNA components of ribonuclease P, which cleave precursor tRNAs at their 5' side via hydrolysis reactions. In contrast, small ribozymes are comparatively small structural units (about 40 bp) that cleave RNAs, forming 5'-hydroxyl groups and 2'-3' cyclic phosphoric acids. Small ribozymes include, for example, hammerhead-type ribozymes (Koizumi *et al.* (1988) FEBS Lett. 228: 225) and hairpin-type ribozymes (Buzayan (1986) Nature 323: 349; Kikuchi and Sasaki (1992) Nucleic Acids Res. 19: 6751; Kikuchi (1992) Chemistry and Biology 30: 112). Since ribozymes are easily altered and synthesized, various methods for their modification are known. For example, hammerhead-type ribozymes that recognize and cleave nucleotide sequence UC, UU, or UA within a target RNA can be created, by designing the substrate binding portion of a ribozyme to be complementary to an RNA sequence near the target site (Koizumi *et al.* (1988) FEBS Lett. 228: 225; M. Koizumi and E. Ohtsuka (1990) Protein, Nucleic Acid and Enzyme 35: 2191; Koizumi *et al.* (1989) Nucleic Acids Res. 17: 7059). Hairpin-type ribozymes can also be designed and produced using known methods (Kikuchi and Sasaki (1992) Nucleic Acids Res. 19: 6751; Kikuchi (1992) Chemistry and Biology 30: 112).

Antisense nucleic acids and ribozymes of the present invention can also be used in viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses, and such, or non-viral vectors that use liposomes, or naked DNAs, to control gene expression in cells using *ex vivo* or *in vivo* gene therapy.

RNA interference is a phenomenon in which the introduction of an artificial double-stranded RNA into cells causes RNAs having the same nucleotide sequence to be degraded (Fire *et al.* (1998) Nature 391: 806-11). There are no particular limitations on the siRNAs of the present invention, provided they inhibit transcription of Lrp4 mRNA. Normally, an siRNA is a combination of a sense and antisense chain to the sequence of a target mRNA, and has a nucleotide length of at least 10 to the same number of nucleotides as the target mRNA. These siRNAs preferably have a nucleotide length of 15 to 75, preferably 18 to 50, and more preferably 20 to 25 nucleotides.

In order to suppress Lrp4 expression, siRNAs can be introduced into cells using known methods. For example, a DNA is designed to encode, in a single strand, two RNA chains that compose an siRNA, and this is then incorporated into an expression vector, cells are transformed with the expression vector, and the siRNA can be expressed in the cells in the form of a double-stranded RNA with a hairpin structure. Plasmid expression vectors that continuously produce siRNA by transfection have also been designed (for example, RNAi-

Ready pSIREN Vector, and RNAi-Ready pSIREN-RetroQ Vector (BD Biosciences Clontech)).

The nucleotide sequence of an siRNA can be designed using a computer program such as that disclosed at the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). Kits for screening for functional siRNAs are also commercially available and can be used (for example, BD Knockout RNAi System (BD Biosciences Clontech).

Examples

The present invention will be specifically described using Examples, but it is not be construed as being limited thereto.

[Example 1] Isolation and Sequence Analysis of a Gene Specific to Dopaminergic Neuron Progenitor Cells

To isolate genes specific to dopaminergic neuron progenitor cells, the midbrain ventral region of E12.5 mice was additionally cut into two regions in the dorsoventral direction, and genes specifically expressed in the most ventral region containing dopaminergic neurons were identified by the subtraction (N-RDA) method. One of the isolated cDNA fragments was a fragment encoding Lrp4/Corin. Lrp4 encodes type II transmembrane proteins (Fig. 1).

(1) N-RDA method

(1)-1. Adapter preparation

The following oligonucleotides were annealed to each other, and prepared at 100 μM. (ad2: ad2S+ad2A, ad3: ad3S+ad3A, ad4: ad4S+ad4A, ad5: ad5S+ad5A, ad13: ad13S+ad13A)

ad2S: cagctccacaacctacatcattccgt (SEQ ID NO: 5)
ad2A: acggaatgatgt (SEQ ID NO: 6)
ad3S: gtccatcttctctctgagactctggt (SEQ ID NO: 7)
ad3A: accagagtctca (SEQ ID NO: 8)
ad4S: ctgatgggtgtcttctgtgagtgtgt (SEQ ID NO: 9)
ad4A: acacactcacag (SEQ ID NO: 10)
ad5S: ccagcatcgagaatcagtgtgacagt (SEQ ID NO: 11)
ad5A: actgtcacactg (SEQ ID NO: 12)
ad13S: gtcgatgaacttcgactgtcgatcgt (SEQ ID NO: 13)
ad13A: acgatcgacagt (SEQ ID NO: 14)

(1)-2. cDNA synthesis

Ventral midbrain regions were cut out of E12.5 mouse embryos (Japan SLC), and divided into two sections in the dorsoventral direction. Total RNA was prepared using the RNeasy Mini Kit (Qiagen), and double-stranded cDNA was synthesized using a cDNA Synthesis Kit (Takara). After digestion with restriction enzyme RsaI, ad2 was added. The cDNA was amplified by a 5-minute incubation at 72°C, 15 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C using ad2S as the primer. In all cases, N-RDA PCR was carried out using a reaction solution containing the following components.

10x ExTaq 5 μl
2.5 mM dNTP 4 μl
ExTaq 0.25 μl
100 μM primer 0.5 μl
cDNA 2 μl
Distilled water 38.25 μl

(1)-3. Driver production

The ad2S-amplified cDNA was further amplified by incubating at 94°C for 2 minutes, and then performing five PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C. The cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen), and digested with RsaI. 3 μg was used for each round of subtraction.

(1)-4. Tester production

The ad2S amplified cDNA was further amplified by incubating at 94°C for 2 minutes, and then performing five PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C. The cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen), and digested with RsaI. ad3 was added to 60 ng of the RsaI-digested cDNA.

(1)-5. First round of subtraction

The tester and the driver produced in Sections 1-3 and 1-4 above were mixed, subjected to ethanol precipitation, and then dissolved in 1 μl of 1x PCR buffer. After a 5-minute incubation at 98°C, 1 μl of 1x PCR buffer + 1M NaCl was added. After another 5 minutes of incubation at 98°C, the tester and the driver were hybridized at 68°C for 16 hours.

With ad3S as the primer, the hybridized cDNA was amplified by incubating at 72°C for 5 minutes, and performing ten cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2

minutes at 72°C. Next, the amplified cDNA was digested with the Mung Bean Nuclease (Takara) and purified using a Qiaquick PCR Purification Kit. Then, it was amplified by incubating at 94°C for 2 minutes, and performing 13 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C.

(1)-6. Normalization

1 μl of 2x PCR buffer was added to 8 ng of the cDNA amplified in the first round of subtraction. After incubating at 98°C for 5 minutes, 2 μl of 1x PCR buffer + 1 M NaCl was added. After another 5 minutes of incubation at 98°C, the cDNA was hybridized at 68°C for 16 hours.

The hybridized cDNA was digested with RsaI, and purified using the Qiaquick PCR Purification Kit. Then, it was amplified with ad3S as the primer by incubating at 94°C for 2 minutes, and performing 11 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C, and a final 2-minute incubation at 72°C. The PCR product was then digested with RsaI, followed by the addition of ad4.

(1)-7. Second Round of Subtraction

20 ng of the cDNA to which ad4 was added in Section 1-6 above was used as the tester and mixed with the driver of 1-3 above, and the same subtraction procedure used in Section 1-5 above was performed. Finally, ad5 was added to the cDNA following RsaI digestion.

(1)-8. Third Round of Subtraction

2 ng of the cDNA to which ad5 was added in section 1-7 above was used as the tester and mixed with the driver of 1-3 above, and the same subtraction procedure used in section 1-5 above was carried out. Finally, ad13 was added to the RsaI-digested cDNA.

(1)-9. Fourth Round of Subtraction

2 ng of the cDNA to which ad13 was added in section 1-8 above was used as the tester and mixed with the driver of 1-3 above, and the same subtraction procedure used in Section 1-5 above was carried out. The amplified cDNA was cloned into pCRII vector (Invitrogen), and its nucleotide sequence was analyzed using the ABI3100 sequence analyzer.

[Example 2] Expression Analysis of the Lrp4 Gene

Next, an expression analysis of theLrp4 gene by *in situ* hybridization was carried out according to the following protocol.

First, E12.5 mouse embryos were embedded in O.C.T., and fresh frozen sections of 16 μm thickness were prepared. After drying on a slide glass, the sections were fixed in 4% PFA at room temperature for 30 minutes. After washing with PBS, hybridization was carried out at 65°C for 40 hours (1 μg/ml DIG-labeled RNA probe, 50% formamide, 5x SSC, 1% SDS, 50 μg/ml yeast RNA, 50 μg/ml Heparin). Subsequently, the sections were washed at 65°C (50% formamide, 5x SSC, 1% SDS) and then treated with RNase (5 μg/ml RNase) at room temperature for 5 minutes. After washing with 0.2x SSC at 65°C and washing with 1x TBST at room temperature, blocking was carried out (Blocking reagent: Roche). The sections were then reacted with alkaline phosphatase-labeled anti-DIG antibody (DAKO), washed (1x TBST, 2 mM Levamisole), and color developed using NBT/BCIP (DAKO) as the substrate.

The expression analysis by *in situ* hybridization showed that Lrp4 mRNA is specifically expressed in the ventral midline region from the midbrain to the hindbrain and the spinal cord at the stage E12.5, which corresponds to the time of dopaminergic neuron development. Lrp4 demonstrates a similar expression pattern to Shh mRNA from the hindbrain to the spinal cord, and was clearly determined to be specific to the floor plate, which is the organizer region (Figs. 2 and 5). In the midbrain, Lrp4 expression was observed more centrally than the Shh mRNA expression zone (Figs. 3 and 5).

The results of comparison with the neuron maturation marker NCAM mRNA show that Lrp4 mRNA-expressing cells were proliferative progenitor cells in the NCAM mRNA-negative ventricular zone (VZ). Moreover, when compared with the expression of the dopaminergic neuron marker, TH mRNA, their expression regions completely overlapped along the dorsal-ventral axis (Figs. 3 and 5), although expression of both TH mRNA and Lrp4 mRNA in the same cells was not observed since TH mRNA is only expressed in the mantle layer (ML). In general, neurons present in neural tubes are known to first proliferate in the VZ, exit cell cycle with the commencement of differentiation, and then mature after migrating to the outer ML. Thus, dopaminergic neuron proliferative progenitor cells are believed to proliferate in the VZ which lines the TH expression zone, and express TH mRNA after having migrated to the outside following cell cycle exit. Namely, Lrp4 mRNA is believed to be specifically expressed in the midbrain in dopaminergic neuron proliferative progenitor cells (Figs. 4 and 6).

[Example 3] Expression of Lrp4 in Dopaminergic Neurons Induced to Differentiate from ES Cells

Next, whether Lrp4 is expressed in ES cells that have been induced to differentiate into dopaminergic neurons *in vitro*, was examined.

First, dopaminergic neurons were induced to differentiate from ES cells using the SDIA method (Kawasaki *et al.* (2000) Neuron 28(1): 31-40) (see the upper part of Fig. 7). Cells were recovered 4, 6, 8, 10, and 12 days after induction, and total RNA was recovered using the RNeasy Mini Kit (Qiagen) followed by RT-PCR. In RT-PCR, cDNA was initially synthesized for 1 μg of total RNA using the RNA PCR Kit (TaKaRa). PCR was then carried out in the following reaction system, using as a template cDNA equivalent to 10 ng, 1 ng, and 0.1 ng.

10x ExTaq	2 μl
2.5 mM dNTP	1.6 μl
ExTaq	0.1 μl
100 μM primer	0.2 μl each
cDNA	1 μl
Distilled water	14.9 μl

After incubating for 2 minutes at 94°C, 35 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 2 minutes at 72°C were carried out followed by incubating for 2 minutes at 72°C.

The sequences of the primers used are shown below.

Lrp4: TAGTCTACCACTGCTCGACTGTAACG (SEQ ID NO: 15) /
CAGAGTGAACCCAGTGGACATATCTG (SEQ ID NO: 16)

TH: GTTCCCAAGGAAAGTGTCAGAGTTGG (SEQ ID NO: 17) /
GAAGCTGGAAAGCCTCCAGGTGTTCC (SEQ ID NO: 18)

DAT: CTCCGAGCAGACACCATGACCTTAGC (SEQ ID NO: 19) /
AGGAGTAGGGCTTGTCTCCCAACCTG (SEQ ID NO: 20)

According to the results of expression analysis by RT-PCR, although Lrp4 is not expressed in ES cells (CCE) or stroma cells (PA6), expression was clearly induced starting on day 4 in the same manner as TH as a result of inducing differentiation (Fig. 8). Thus, the marker polynucleotide probes of the present invention are useful as markers not only when isolating dopaminergic neuron proliferative progenitor cells from the fetal midbrain, but also when isolating dopaminergic neuron proliferative progenitor cells that have been induced to differentiate from ES cells *in vitro*.

[Example 4] Analysis of Lrp4 protein expression

The anti-Lrp4 antibody was produced by the protocol described below using the extracellular region encoding sequence in the Lrp4 gene, and expression analyses were performed using immunohistological staining.

First, the extracellular region (161 to 502 amino acids) encoding sequence in the Lrp4

gene was introduced into 293E cells, and the extracellular region of the Lrp4 protein was expressed and collected. Hamsters were immunized with the collected protein, and lymphocytes were removed therefrom and fused with myeloma cells. The fused cells were cultured to obtain a culture supernatant. On day 12.5 mouse embryos were then fixed with 4% PFA/PBS(-) at 4°C for two hours, then the solution was replaced with 20% sucrose/PBS(-) at 4°C overnight, and the embryos were embedded with OTC. 12-μm-thick sections were made, attached on a slide glass, dried at room temperature for 30 minutes, and wetted with PBS(-) again. The sections were blocked (10% normal donkey serum and 10% normal goat serum/Block Ace) at room temperature for 20 minutes, and then reacted with the anti-Lrp4 monoclonal antibody produced as described above (mixture of FERM BP-10315 and FERM BP-10316 [each 1/4-diluted culture supernatants, 10% normal donkey serum, 10% normal goat serum, and 2.5% Block Ace/PBS]) and the anti-TH antibody (Chemicon, 0.7 μg/mL, 10% normal donkey serum, 10% normal goat serum, and 2.5% Block Ace/PBS) at room temperature for one hour, and further reacted at 4°C overnight. The sections were washed four times with 0.1% Triton X-100/PBS(-) at room temperature for 10 minutes. Cy3-labeled anti-hamster IgG antibody or the FITC-labeled anti-mouse IgG antibody (Jackson, 10 μg/mL, 10% normal donkey serum, 10% normal goat serum, and 2.5% Block Ace/PBS) was reacted with the sections at room temperature for one hour. Then, the sections were washed as described above, then washed with PBS(-) at room temperature for 10 minutes, and sealed.

As a result of expression analysis by immunohistological staining using the produced anti-Lrp4 monoclonal antibody, expression of the Lrp4 protein was observed in the ventral midbrain at E12.5, the stage when the dopaminergic neuron developed (Fig. 8) as shown by expression analysis using *in situ* hybridization. Compared with expression of the TH protein, which was the dopaminergic neuron marker, the Lrp4 protein was expressed in the most ventral midbrain (VZ side) where the TH protein was expressed. Accordingly, it appeared that the Lrp4 protein is expressed in the dopaminergic neuron progenitor cells.

Next, using the anti-Lrp4 monoclonal antibody, Lrp4-expressing cells were detected by flow cytometry.

First, ES cells were induced to differentiate to dopaminergic neuron progenitor cells *in vitro* using the SDIA method. A cell population comprising the cells was dispersed using a cell dissociation buffer (Invitrogen), and stained with the anti-Lrp4 monoclonal antibody (mixture of FERM BP-10315 and FERM BP-10316 [each 1/4-diluted culture supernatant, 1% fetal calf serum, and 1 mM EDTA/SDIA differentiation medium]) at 4°C for 20 minutes without fixing and permeabilization. Subsequently, the cells were washed three times with 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium at 4°C for 3 minutes. The cells were stained with biotin-labeled anti-hamster IgG antibody (Jackson, 10 μg/mL, 1%

fetal calf serum and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes, and washed as described above. Then, the cells were stained with PE-labeled streptavidin (Pharmingen, 20 μg/mL, 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes, and washed as described above. After staining, the Lrp4-expressing cells were detected by a flow cytometer.

The results of detecting the Lrp4-expressing cells by flow cytometry using the produced anti-Lrp4 monoclonal antibody detected an Lrp4 protein-expressing population (Fig. 9). Since the Lrp4 protein-expressing cells could be detected without fixing and permeabilization, it appeared that living Lrp4 protein-expressing cells can be separated by using a flow cytometer equipped with a cell sorter. The Lrp4 protein is thought to be expressed in the dopaminergic neuron progenitor cells, and thus the anti-Lrp4 antibody appeared to be useful for the separation of the dopaminergic neuron progenitor cells.

[Example 5] Separation of Lrp4-expresseng cells by antibodies

Lrp4 protein-positive cells separated using the anti-Lrp4 antibody were characterized.

First, a cell population comprising E12.5 mouse fetus ventral midbrain and dopaminergic neuron progenitor cells, induced *in vitro* to differentiate from ES cells by the SDIA method, was stained with the anti-Lrp4 antibody by the method described in Example 4. Lrp4-positive and -negative cells were separated using a cell sorter. Total RNA was collected from the cells immediately after separation using an RNeasy mini kit (Qiagen). Then, cDNA was synthesized, and amplified by the same method as described in Example 1 to use as a template in RT-PCR. The PCR was performed using cDNAs corresponding to the amplified cDNA equivalent to 4 ng, 0.4 ng, and 0.04 ng by the following reaction system.

10 x ExTaq	1 μL
2.5 mM dNTP	0.8 μL
ExTaq	0.05 μL
100 μM primers	0.1 μL each
cDNA	1 μL
Distilled water	6.95 μL

PCR was carried out under conditions of 94°C for 2 minutes, 26 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes, and finally 72°C for 2 minutes.

The sequences of the primers used are shown below.

Lrp4: TAGTCTACCACTGCTCGACTGTAACG (SEQ ID NO: 15) /
CAGAGTGAACCCAGTGGACATATCTG (SEQ ID NO: 16)
TH: GTTCCCAAGGAAAGTGTCAGAGTTGG (SEQ ID NO: 17) /

GAAGCTGGAAAGCCTCCAGGTGTTCC (SEQ ID NO: 18)
Nurr1: CACTCCTGTGTCTAGCTGCCAGATGC (SEQ ID NO: 21) /
AGTGCGAACACCGTAGTGCTGACAGG (SEQ ID NO: 22)
Nestin: GATGAAGAAGAAGGAGCAGAGTCAGG (SEQ ID NO: 23) /
ATTCACTTGCTCTGACTCCAGGTTGG (SEQ ID NO:24)
MAP2: CCATGATCTTTCCCCTCTGGCTTCTG (SEQ ID NO: 25) /
TTTGGCTGGAAAGGGTGACTCTGAGG (SEQ ID NO: 26)

As expected, the results of expression analysis by the RT-PCR indicated expression of the proliferative progenitor cell marker Nestin. It was also revealed that cells expressing MAP2, which was the postmitotic neuron marker, were included in the Lrp4 protein-positive cell population (Fig. 10). Therefore, it was found that Lrp4 protein expression is maintained after stopping mRNA expression, and that Lrp4 protein is useful as a marker for separating not only dopaminergic neuron proliferative progenitor cells but also postmitotic dopaminergic neuron precursor cells (Fig. 11). Furthermore, since Nurr1 and TH, which are markers for postmitotic dopaminergic neuron precursor cells, were expressed at higher levels compared to the Lrp4-negative cell population, Lrp4-positive cells were confirmed to be dopaminergic neuron lineage progenitor cells (Fig. 10).

Next, the present inventors analyzed the ratio of proliferative progenitor cells to postmitotic precursor cells in the Lrp4 protein-positive cell population separated by the anti-Lrp4 antibody.

The separated cells were seeded on a glass slide coated with poly-L-ornithine (Sigma, 0.002% in PBS), laminin (Invitrogen, 5 μg/mL in PBS), and fibronectin (Sigma, 5 μg/mL in PBS), and incubated in N2 (Invitrogen, 1x), B27 (Invitrogen 1x), ascorbic acid (Sigma, 200 μM), and BDNF (Invitrogen, 20 ng/mL)/SDIA differentiation medium at 37°C for 40 minutes to adhere thereon. The adherent cells were fixed in 4% PFA/PBS at 4°C for 20 minutes, and washed twice with PBS at 4°C for 10 minutes. Permeabilization with 0.3% Triton X-100/PBS was performed at room temperature for 15 minutes, and blocking with 10% normal donkey serum/Block Ace was performed at room temperature for 20 minutes. Then, the cells were reacted with anti-Nestin antibody (Chemicon, 2 μg/mL, 10% normal donkey serum, 2.5% Block Ace, 0.1% Triton X-100/PBS) or anti-βIII-tubulin antibody (BABCO, 1/2000, 0.5 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. On the next day, the cells were washed three times with 0.1% Triton X-100/PBS at room temperature for 5 minutes, and reacted with the FITC-labeled anti-mouse IgG antibody or the Cy5-labeled anti-rabbit IgG antibody (both from Jackson, 10 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and

0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed as described above, then washed with PBS at room temperature for 5 minutes, and sealed for observation.

Also, the separated cells were similarly seeded on a glass slide, and cultured in the above-described medium supplemented with BrdU (Roche, 5-Bromo-2'-deoxy-uridine Labeling and Detection kit II, 1x) at 37°C for 18 hours. Then, the above-described procedures were similarly carried out until the blocking step. The cells were reacted in 2 N HCl at 37°C for 20 minutes, washed three times with PBS, and then reacted with anti-BrdU antibody and DNase (Roche, 5-Bromo-2'-deoxy-uridine Labeling and Detection kit II, 1x conc. in incubation buffer) at 37°C for 30 minutes. Furthermore, the cells were reacted with anti-BrdU antibody (Sigma, 44 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. The next day, the cells were washed three times with 0.1% Triton X-100/PBS at room temperature for 5 minutes, and then reacted with the FITC-labeled anti-mouse IgG antibody (Jackson, 10 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed, and sealed for observation as described above.

As a result of the marker staining, it was revealed that a majority of the Lrp4-positive cells are Nestin-positive proliferative progenitor cells and that a part thereof is positive for the postmitotic marker βIII-tubulin (Fig. 12). In addition, the separated cells were confirmed to frequently incorporate BrdU, and to actually proliferate *in vitro* (Fig. 13).

Next, the present inventors confirmed that the separated Lrp4-positive cells differentiate into the dopaminergic neurons.

The separated cells were seeded on a glass slide coated with poly-L-ornithine (Sigma, 0.002% in PBS), laminin (Invitrogen, 5 μg/mL in PBS), and fibronectin (Sigma, 5 μg/mL in PBS), and incubated in N2 (Invitrogen, 1x), B27 (Invitrogen 1x), ascorbic acid (Sigma, 200 μM), BDNF (Invitrogen, 20 ng/mL), and bFGF (R&D, 10 ng/ml)/SDIA differentiation medium at 37°C for 24 hours. The cells were then further cultured in the above medium without bFGF for another six days. The cultured cells were fixed in 4% PFA/PBS at 4°C for 20 minutes, and washed twice with PBS at 4°C for 10 minutes. Permeabilization with 0.3% Triton X-100/PBS was performed at room temperature for 15 minutes, and blocking with 10% normal donkey serum/Block Ace was performed at room temperature for 20 minutes. Then, the cells were reacted with anti-TH antibody (Chemicon, 0.3 μg/mL, 10% normal donkey serum, 2.5% Block Ace, 0.1% Triton X-100/PBS) or anti-βIII-tubulin antibody (BABCO, 1/2000, 0.5 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. On the next

day, the cells were washed three times with 0.1% Triton X-100/PBS at room temperature for 5 minutes, and reacted with the FITC-labeled anti-mouse IgG antibody or the Cy5-labeled anti-rabbit IgG antibody (both from Jackson, 10 μg/mL, 10% normal donkey serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed as described above, then washed with PBS at room temperature for 5 minutes, and sealed for observation.

As a result of culturing the separated cells *in vitro*, it was obvious that more TH protein-positive dopaminergic neurons were induced than with the unseparated control cells. Therefore, it was revealed that the Lrp4-positive cells are certainly dopaminergic neuron lineage progenitor cells and can mature *in vitro* (Fig. 14).

[Example 6] Transplantation of separated Lrp4-positive cells into striatum in Parkinson's disease model mice

Lrp4-expressing cells were separated by flow cytometry using anti-Lrp4 monoclonal antibody, and the cells were transplanted into the striatum of Parkinson's disease model mice.

First, ES cells were induced to differentiate to dopaminergic neuron progenitor cells *in vitro* using the SDIA method. A cell population comprising the cells was dispersed using a cell dissociation buffer (Invitrogen), and stained with the anti-Lrp4 monoclonal antibody prepared in Example 4 (mixture of FERM BP-10315 and FERM BP-10316 [each 1/4-diluted culture supernatant, 1% fetal calf serum, and 1 mM EDTA/SDIA differentiation medium]) at 4°C for 20 minutes without fixing and permeabilization. Subsequently, the cells were washed three times with 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium at 4°C for 3 minutes. The cells were stained with biotin-labeled anti-hamster IgG antibody (Jackson, 10 μg/mL, 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes, and washed as described above. Then, the cells were stained with PE-labeled streptavidin (Pharmingen, 20 μg/mL, 1% fetal calf serum and 1 mM EDTA/SDIA differentiation medium) at 4°C for 20 minutes, and washed as described above. After staining, the Lrp4-expressing cells were separated by a flow cytometer.

Next, the present inventors transplanted the separated Lrp4 protein-positive cells into the striatum of Parkinson's disease model mice, and characterized the Lrp4 protein-positive cells in the brain.

First, the Parkinson's disease model mice were prepared by injecting 1.25 μL of 6-OHDA (Sigma, 2 μg/μL) into one side of the medial forebrain bundle of 12-week-old mice (slc) to deaden the dopaminergic neurons projecting from the midbrain to the striatum. Two weeks after preparing the model mice, 3×10^4 Lrp4-positive cells per mouse were transplanted into the striatum to which 6-OHDA had been injected. The Lrp4 protein-positive cells to be

transplanted were obtained by: transfecting ES cells to express the EGFP gene under the control of CAG promoter (Niwa *et al.*, Gene 108:193-200 (1991)); inducing the ES cells to differentiate into the dopaminergic neuron progenitor cells *in vitro* by the SDIA method; and staining the cell population comprising the dopaminergic neuron progenitor cells with the anti-Lrp4 antibody as described in Example 4 and separating by the cell sorter.

Three weeks after the transplantation, 500 μL of 10% urethan in saline was intraperitoneally injected to anesthetize the mice. Under anesthesia, thoracotomy was performed, 30 mL of saline (Otsuka) was injected from left ventricle for perfusion, and then perfusion fixation was carried out with 30 mL of 4% PFA/PBS(-). After fixation, the brains were removed, and further immersion-fixed in 4% PFA/PBS(-) for another eight hours. The brains were then sliced at a thickness of 2 mm, the solution was replaced with 20 to 40% sucrose/PBS(-) overnight, and the brains were embedded in OCT. 10 to 12 μm-thick sections were made, attached on glass slides, dried at room temperature for 30 minutes, and wetted again with PBS(-). Blocking (10% normal donkey serum/Block Ace) was then performed at room temperature for 20 minutes. The sections were then reacted with anti-GFP antibody (Molecular probes, 20 μg/mL, 10% normal donkey serum, and 10% Block Ace/PBS), anti-MAP2 antibody (Sigma, mouse ascites, diluted 100 times, 10% normal donkey serum, and 10%Block Ace/PBS), or anti-TH antibody (Chemicon, 1 μg/mL, 10% normal donkey serum and 10% Block Ace/PBS) at room temperature for one hour, and again at 4°C overnight. Sections were washed four times with 0.1% Triton X-100/PBS(-) at room temperature for 10 minutes. The sections were then reacted with Alexa Flour 488-labeled anti-rabbit IgG antibody (Molecular Probes, 4 μg/mL, 10% normal donkey serum, and 10% Block Ace/PBS), Cy3-labeled anti-mouse IgG antibody (Jackson, 10 μg/mL, 10% normal donkey serum, and 10% Block Ace/PBS), or Cy5-labeled anti-sheep IgG antibody (Jackson, 10 μg/mL, 10% normal donkey serum, and 10% Block Ace/PBS) at room temperature for one hour. Then, the sections were washed as described above, washed with PBS(-) at room temperature for 10 minutes, and sealed.

The expression of various markers was analyzed by immunohistological staining.

As a result, EGFP-positive cells were observed in the striatum of the transplanted mice (Table 1). This suggests that the transplanted Lrp4 protein-positive cells are successfully engrafted in the striatum of the Parkinson's disease model mice.

Most of the successfully engrafted cells were also observed to be positive for the mature neuron marker MAP2, and EGFP-positive axons were observed to extend far into the striatum (Table 1 and Fig. 16).

Table 1

	EGFP+ CELLS	TH+ CELLS	TH+
#6 SEQ ID NO: 20	95	19	20%
#7 SEQ ID NO: 12	131	21	16%

(Table 1 shows the *in vivo* differentiation of the transplanted Lrp4-positive cells into TH-positive cells. Two weeks after deadening the dopaminergic neurons with 6-OHDA, the Lrp4 protein-positive cells were transplanted into mice #6 and #7. Perfusion was performed three weeks after transplant.

The results indicated that the transplanted Lrp4 protein-positive cells are nerve progenitor cells, whereas most successfully engrafted cells differentiate and mature into mature nerve cells. About 20% of the successfully engrafted cells were TH-positive, strongly suggesting that at least a part of the transplanted Lrp4 protein-positive cells differentiate into dopaminergic neurons.

Therefore, the dopaminergic neuron progenitor cells separated according to the present invention are able to differentiate into the dopaminergic neurons by being transplanted into the brain, and thus the dopaminergic neuron progenitor cells separated according to the present invention are believed useful for therapies.

[Example 7] Lrp4 Expression in dopaminergic neurons induced to differentiate from ES cells using the 5-stage method

The present inventors examined whether or not Lrp4 is expressed when ES cells are induced to differentiate into the dopaminergic neurons *in vitro* using the 5-stage method.

First, ES cells (CCE) were induced to differentiate into dopaminergic neurons using the 5-stage method (Lee *et al.*, Nat. Biotech., 18:675-679 (2000), mouse dopaminergic neuron differentiation kit [R&D Systems]). Cells were collected on day 2 at Stage 1, day 4 at Stage 2, day 6 at Stage 3, days 4 and 6 at Stage 4, and days 4 and 7 at Stage 5. Total RNAs were prepared using RNeasy mini kit (Qiagen) to perform RT-PCR. In the RT-PCR, cDNA was first synthesized from 1 μg of the total RNA using RNA PCR kit (TaKaRa). The PCR was performed by the following reaction system, using cDNAs corresponding to 10 ng, 1 ng, and 0.1 ng as templates.

10 x ExTaq	2 μL
2.5 mM dNTP	1.6μL
ExTaq	0.1 μL
100 μM primers	0.2 μL each
cDNA	1 μL

Distilled water 14.9 μL

PCR was carried out under conditions of 94°C for 2 minutes, 32 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes, and finally 72°C for 2 minutes.

The sequences of the primers used are shown below.

Lrp4: TAGTCTACCACTGCTCGACTGTAACG (SEQ ID NO: 15)/
CAGAGTGAACCCAGTGGACATATCTG (SEQ ID NO: 16)

TH: GTTCCCAAGGAAAGTGTCAGAGTTGG (SEQ ID NO: 17)/
GAAGCTGGAAAGCCTCCAGGTGTTCC (SEQ ID NO: 18)

The results of expression analysis by RT-PCR revealed that Lrp4 is not expressed in Stage 1, at which the cells are undifferentiated ES cells, but is induced to be expressed in Stage 4, at which the dopaminergic neuron progenitor cells are developed (Fig. 17A).

Next, the present inventors detected the Lrp4-expressing cells by flow cytometry using the anti-Lrp4 monoclonal antibody when ES cells were induced to differentiate into the dopaminergic neurons *in vitro* using the 5-stage method.

First, the cell population (day 7 at Stage 4) comprising the dopaminergic neuron progenitor cells induced to differentiate from ES cells *in vitro* using the 5-stage method was dispersed using the cell dispersing solution Accumax (Innovative Cell Technologies, Inc.). Without the steps of fixing and permeabilization, the cells were stained with anti-Lrp4 monoclonal antibody (mixture of FERM BP-10315 and FERM BP-10316 [each 1/2-diluted culture supernatant]) at 4°C for 20 minutes. The cells were then washed three times with 1% fetal calf serum, 1 mM EGTA, 4.5 mg/mL glucose, and 40 ng/mL DNase I/Ca- and Mg-free Hanks' balanced salt solution (HBSS-) at 4°C for 3 minutes. The cells were stained with the PE-labeled anti-hamster IgG antibody (8 μg/mL [BD Biosciences], 1% fetal calf serum, 1 mM EGTA, 4.5 mg/mL glucose, and 40 ng/mL DNase I/ Ca- and Mg-free Hanks' balanced salt solution (HBSS-)) at 4°C for 30 minutes, and washed as described above. After staining, Lrp4-expressing cells were detected using a flow cytometer.

The results of detecting Lrp4-expressing cells by flow cytometry using anti-Lrp4 monoclonal antibody detected an Lrp4 protein-expressing population in the cell group comprising the dopaminergic neuron progenitor cells induced to differentiate from the ES cells *in vitro* using the 5-stage method (Fig. 17B). This suggested that Lrp4 is expressed in the dopaminergic neuron progenitor cells induced to differentiate from the ES cells by not only the SDIA method but also by the 5-stage method, and is useful as a marker for cell separation.

[Example 8] Differentiation and maturation *in vitro* of Lrp4-expressing cells separated by

antibodies

The present inventors examined whether or not Lrp4 protein-positive cells separated using anti-Lrp4 antibody differentiate into dopaminergic neurons *in vitro*.

The cell population comprising the dopaminergic neuron progenitor cells induced to differentiate from ES cells *in vitro* using the 5-stage method was stained using anti-Lrp4 antibody by the method described in Example 4. The Lrp4-positive cells were then separated using a cell sorter. The separated cells were seeded on a glass slide coated with poly-L-ornithine (Sigma, 0.002% in PBS) and fibronectin (Sigma, 5 μg/mL in PBS), and incubated in N2 (Invitrogen, 1x), ascorbic acid (Sigma, 200 μM), and BDNF (R&D Systems, 20 ng/mL)/DMEM/F12 at 37°C for 7 days. The cultured cells were fixed in 2% PFA and 0.15% picric acid/PBS at 4°C for 20 minutes, and washed twice with PBS at 4°C for 10 minutes. Permeabilization with 0.3% Triton X-100/PBS was performed at room temperature for 30 minutes, and blocking with 10% normal donkey serum and 10% normal goat serum/Block Ace was performed at room temperature for 20 minutes. Then, the cells were reacted with anti-TH antibody (Chemicon, 0.4 μg/mL, 10% normal donkey serum, 10% normal goat serum, 2.5% Block Ace, 0.1% Triton X-100/PBS) or anti-MAP2 antibody (Sigma, 1/200 ascites, 0.5 μg/mL, 10% normal donkey serum, 10% normal goat serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for one hour, and subsequently at 4°C overnight. On the next day, the cells were washed four times with 0.1% Triton X-100/PBS at room temperature for 10 minutes, and reacted with the FITC-labeled anti-mouse IgG antibody or the Cy3-labeled anti-rabbit IgG antibody (both from Jackson, 10 μg/mL, 10% normal donkey serum, 10% normal goat serum, 2.5% Block Ace, and 0.1% Triton X-100/PBS) at room temperature for 30 minutes. Subsequently, the cells were washed as described above, then washed with PBS at room temperature for 5 minutes, and sealed for observation.

Culturing the separated Lrp4-positive cells *in vitro* induced many TH protein-positive dopaminergic neurons (Fig. 17C). Therefore, it was revealed that Lrp4-positive cells induced by the 5-stage method are dopaminergic neuron progenitor cells, and can mature *in vitro*. The Lrp4 was expressed in the dopaminergic neuron progenitor cells induced by two different differentiation methods (SDIA method and 5-stage method), and dopaminergic neuron progenitor cells induced by either method could be separated using the anti-Lrp4 antibody. Accordingly, it appeared that Lrp4 is useful as a marker for dopaminergic neuron progenitor cells derived from any cell source. The 5-stage method is capable of inducing differentiation of dopaminergic neuron progenitor cells without contact with animal-derived cells and components, and expected to be applied clinically. Lrp4 is useful as a cell separation marker in the methods, and thus appeared to be most likely applied to transplant therapy for neurodegenerative diseases including Parkinson's disease.

[Example 9] Elimination of undifferentiated ES cells by separating dopaminergic neuron progenitor cells using anti-Lrp4 antibody

When ES cell-derived transplant cells are prepared, it is most important in terms of safety to eliminate the undifferentiated ES cells that result in teratoma. Since Lrp4 is not expressed in undifferentiated ES cells (Example 3), separation using Lrp4 as a marker is expected to eliminate undifferentiated ES cells. To confirm this hypothesis, the present inventors used RT-PCR to examine the expression of ERas (Nature, 423(6939):541-5 (2003)) and Nanog (Cell, 113(5):631-42 (2003)), which are ES cell-specific genes, to investigate whether undifferentiated ES cells were contained in cells separated using anti-Lrp4 antibody from cells induced to differentiate from ES cells *in vitro* using the SDIA method.

First, using the method described in Example 5, Lrp4-positive and -negative cells were separated by a cell sorter from a cell population comprising dopaminergic neuron progenitor cells induced to differentiate from ES cells *in vitro* using the SDIA method. Total RNA was collected immediately after separation, and the cDNA to be amplified was prepared. PCR was performed using cDNAs corresponding to 4 ng, 0.4 ng, and 0.04 ng as templates for the following reaction system:

10x ExTaq	1 μL
2.5 mM dNTP	0.8 μL
ExTaq	0.05 μL
100 μM primers	0.1 μL each
cDNA	1 μL
Distilled water	6.95 μL

PCR was carried out under conditions of 94°C for 2 minutes, 26 cycles (for Lrp4 and Nestin) or 30 cycles (for ERas and Nanog) of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes, and finally 72°C for 2 minutes.

The sequences of the primers used are shown below (the sequences of Lrp4 and Nestin were described in Example 5).

ERas: TGCTCTCACCATCCAGATGACTCACC (SEQ ID NO: 27)/
TGGACCATATCTGCTGCAACTGGTCC (SEQ ID NO: 28)

Nanog: TCCAGCAGATGCAAGAACTCTCCTCC (SEQ ID NO: 29)/
TTATGGAGCGGAGCAGCATTCCAAGG (SEQ ID NO: 30)

As a result, ERas and Nanog, which are expressed specifically in ES cells, were not expressed in the Lrp4-positive cell population; on the other hand, ERas and Nanog expressions

were detected in the Lrp4-negative cell population (Fig. 18). Accordingly, it was revealed that undifferentiated ES cells contained in the cells induced to differentiate by the SDIA method could be eliminated by cell separation using Lrp4.

Industrial Applicability

The present invention identified Lrp4 as a gene expressed specifically and transiently in dopaminergic neuron proliferative progenitor cells. As a result of examining Lrp4 expression in more detail, it was confirmed that Lrp4 mRNA and the Lrp4 protein were expressed specifically in dopaminergic neuron proliferative progenitor cells and in dopaminergic neuron progenitor cells, including cells prior to and after cell cycle exit, respectively. Thus, by using the expression of Lrp4 mRNA or an Lrp4 polypeptide in the cells as an index, it became possible in terms of safety, survival rate, and network formation to select dopaminergic neuron lineage cells suitable for transplant therapy for neurodegenerative diseases including Parkinson's disease. When the cells are obtained using Lrp4 as a marker, as in the present invention, the cells can be easily differentiated into a suitable state *in vitro* even when the therapy requires mature cells. Moreover, dopaminergic neuron progenitor cells obtained by the methods of the present invention can also be used to isolate genes specifically expressed in these cells. The cells are also thought to be useful in developing pharmaceuticals for neurodegenerative diseases such as Parkinson's disease. Since dopaminergic neuron proliferative progenitor cells obtained using Lrp4 mRNA as a marker are involved in early neuron formation, they are useful in elucidating the neuron maturation process, namely, for identifying various factors involved in the maturation process. Elucidation of these factors is expected to contribute greatly to the treatment of neurodegenerative diseases. Moreover, maturation of these cells can be used as an index for screening substances that may regulate (inhibit or promote) the maturation process.

CLAIMS

1. A method for selecting a dopaminergic neuron proliferative progenitor cell, wherein the method comprises the step of contacting a polynucleotide probe with a cell sample thought to comprise a dopaminergic neuron proliferative progenitor cell, wherein the polynucleotide probe comprises a sequence selected from the following nucleotide sequences (1) to (5):

(1) a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a nucleotide sequence complementary to a nucleotide sequence encoding an amino acid sequence of SEQ ID NO: 3 or 4;

(3) a nucleotide sequence complementary to a nucleotide sequence encoding a sequence lacking a transmembrane domain in an amino acid sequence of SEQ ID NO: 3 or 4;

(4) a nucleotide sequence that hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence of SEQ ID NO: 1 or 2; and

(5) a nucleotide sequence comprising at least 15 contiguous nucleotides selected from sequences of (1) to (4).

2. A method for selecting a dopaminergic neuron lineage cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell using the method of claim 1 for selecting the dopaminergic neuron proliferative progenitor cell;

(2) culturing the proliferative progenitor cell selected in step (1); and

(3) screening the cells cultured in step (2) by using a marker for a postmitotic dopaminergic neuron.

3. A method for isolating a dopaminergic neuron proliferative progenitor cell-specific gene and a gene specific for each maturation stage from the proliferative progenitor cell to a dopaminergic neuron, wherein the method comprises the step of detecting and isolating a gene specifically expressed in a dopaminergic neuron proliferative progenitor cell, which is selected by the method of claim 1, or a cell which is differentiated, induced, or proliferated from the proliferative progenitor cell isolated from the animal or human body.

4. A method of screening for a compound which regulates proliferation and/or differentiation of a dopaminergic neuron lineage cell using maturation as an index, wherein the method comprises the steps of: contacting a test substance with a dopaminergic neuron proliferative progenitor cell, which is selected by the method of claim 1, or a cell which is differentiated, induced, or proliferated from the proliferative
progenitor cell isolated from the animal or human body; and detecting a change of the proliferative progenitor cell or the progenitor cell caused by the contact.

5. An antibody against a polypeptide selected from the following (1) to (6), which is produced by the hybridoma FERM BP-10315 or FERM BP-10316.

(1) a polypeptide encoded by a polynucleotide consisting of a nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a polypeptide comprising an amino acid sequence of SEQ ID NO: 3 or 4;

(3) a polypeptide comprising an amino acid sequence lacking a transmembrane domain in an amino acid sequence of SEQ ID NO: 3 or 4;

(4) a polypeptide comprising an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in an amino acid sequence of SEQ ID NO: 3 or 4;

(5) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions with a polynucleotide consisting of a sequence complementary to a nucleotide sequence of SEQ ID NO : 1 or 2; and,

(6) a polypeptide that is a fragment of a polypeptide of (1) to (5) comprising at least eight amino acid residues.

6. A method for selecting a dopaminergic neuron progenitor cell, wherein the method comprises the step of contacting the antibody of claim 5 with a cell sample
isolated from the animal or human body thought to comprise a dopaminergic neuron progenitor cell.

7. A method for selecting a dopaminergic neuron lineage cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell using the method of claim 6;

(2) culturing the progenitor cell selected in step (1); and

(3) screening the progenitor cells cultured in step (2) by using a marker for a postmitotic dopaminergic neuron.

8. A method for isolating a dopaminergic neuron progenitor cell-specific gene and a gene specific for each maturation stage from the progenitor cell to a dopaminergic neuron, wherein the method comprises the step of detecting and isolating a gene specifically expressed in a dopaminergic neuron progenitor cell, which is selected by the method of claim 6 or a cell which is differentiated, induced, or proliferated from the progenitor cell isolated from the animal or human body.

9. A method of screening for a compound which regulates proliferation and/or differentiation of a dopaminergic neuron lineage cell using maturation as an index, wherein the method comprises the steps of: contacting a test substance with a dopaminergic neuron progenitor cell, which is selected by the method of claim 6, or a cell which is differentiated, induced, or proliferated from the progenitor cell isolated from the animal or human body; and detecting a differentiated or proliferated progenitor cell caused by the contact.

10. A kit for treating Parkinson's disease, which comprises a dopaminergic neuron proliferative progenitor cell, which is selected by the method of claim 1, or a dopaminergic neuron progenitor cell, which is selected by the method of claim 6.

11. A use of a dopaminergic neuron proliferative progenitor cell, which is selected by the method of claim 1, or a dopaminergic neuron progenitor cell, which is selected by the method of claim 6, for producing a kit for treating Parkinson's disease.

12. A method for detecting or selecting a dopaminergic neuron proliferative progenitor cell, which comprises the step of contacting a cell sample comprising the dopaminergic neuron proliferative progenitor cell with a second polynucleotide which hybridizes under stringent conditions with a first polynucleotide consisting of any one of:

(1) the nucleotide sequence of SEQ ID NO: 1 or 2;

(2) a nucleotide sequence consisting of a polynucleotide encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 3 or 4;

(3) a nucleotide sequence consisting of a polynucleotide encoding a polypeptide consisting of an amino acid sequence which lacks a transmembrane region in the amino acid sequence of SEQ ID NO: 3 or 4; and

(4) a nucleotide sequence consisting of a polynucleotide which hybridizes with a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 1 or 2 under stringent conditions.

13. The method of claim 12, wherein the second polynucleotide comprises at least 15 nucleotides.

14. A method for producing a postmitotic dopaminergic neuron precursor cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell by the method of claim 12 or 13;

(2) culturing the cell selected in step (1); and

(3) selecting the postmitotic dopaminergic neuron precursor cell from the cells cultured in step (2).

15. A method for producing a dopaminergic neuron, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron proliferative progenitor cell by the method of claim 12 or 13; and

(2) culturing the cell selected in step (1).

16. The method of claim 15, further comprising the step of:

(3) selecting a dopaminergic neuron from the cells cultured in step (2).

17. A method for detecting or selecting a dopaminergic neuron progenitor cell, which comprises the step of contacting a cell sample comprising the dopaminergic neuron progenitor cell with an antibody which is bound to a polypeptide consisting of the amino acid sequence of any one of:

(1) the amino acid sequence of SEQ ID NO: 3 or 4;

(2) an amino acid sequence which lacks a transmembrane region in the amino acid sequence of SEQ ID NO: 3 or 4;

(3) an amino acid sequence mutated by one or more amino acid deletions, substitutions, or additions, or any combination thereof in the amino acid sequence of SEQ ID NO:3 or 4; and

(4) an amino acid sequence consisting of a polypeptide encoded by a polynucleotide which hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2,
or a partial sequence thereof.

18. The method of claim 17, wherein the polypeptide comprising the partial sequence comprises at least six consecutive amino acid residues.

19. A reagent for discriminating a dopaminergic neuron progenitor cell, which comprises an antibody as an active ingredient which is bound to a polypeptide consisting of the amino acid sequence of any one of:

(1) the amino acid sequence of SEQ ID NO: 3 or 4;

(2) an amino acid sequence which lacks a transmembrane region in the amino acid sequence of SEQ ID NO: 3or 4;

(3) an amino acid sequence mutated by one or more amino acid deletions, substitutions, or additions, or any combination thereof, in the amino acid sequence of SEQ ID NO:3 or 4; and

(4) an amino acid sequence consisting of a polypeptide encoded by a polynucleotide which hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to a nucleotide sequence of SEQ ID NO: 1 or 2,
or a partial sequence thereof.

20. The reagent of claim 19, wherein the polypeptide consisting of the partial sequence comprises at least six consecutive amino acid residues.

21. The reagent of claim 19, wherein the antibody is produced by the hybridoma FERM BP-10315 or FERM BP-10316.

22. An antibody produced by the hybridoma FERM BP-10315 or FERM BP-10316.

23. A method for producing a dopaminergic neuron proliferative progenitor cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell by the method of claim 17 or 18; and

(2) removing a postmitotic dopaminergic neuron precursor cell to select the dopaminergic neuron proliferative progenitor cell.

24. A method for producing a postmitotic dopaminergic neuron precursor cell, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell by the method of claim 17 or 18; and

(2) culturing the cell selected in step (1).

25. The method of claim 24, further comprising the step of:

(3) selecting a postmitotic dopaminergic neuron precursor cell from the cells cultured in step (2).

26. A method for producing a dopaminergic neuron, wherein the method comprises the steps of:

(1) selecting a dopaminergic neuron progenitor cell by the method of claim 17 or 18; and

(2) culturing the cell selected in step (1).

27. The method of claim 26, further comprising the step of:

(3) selecting a dopaminergic neuron from the cells cultured in step (2).

ABSTRACT

The present invention relates to polynucleotide probes and antibodies for detecting Lrp4/Corin dopaminergic neuron progenitor cell markers, which enable the efficient separation of dopaminergic neuron progenitor cells; and methods for selecting the progenitor cells by the use thereof.

SEQUENCE LISTING

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Asp Ile Arg Gln Val Leu Pro His Arg Ser Glu Tyr Thr Leu Leu Leu
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Leu Val Phe Trp Ser Asp Val Thr Leu Asp Arg Ile Leu Arg Ala Asn
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Leu Asn Gly Ser Asn Val Glu Glu Val Val Ser Thr Gly Leu Glu Ser
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Pro Gly Gly Leu Ala Val Asp Trp Val His Asp Lys Leu Tyr Trp Thr
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Arg Lys Val Leu Leu Trp Gln Asn Leu Glu Lys Pro Arg Ala Ile Ala
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Leu His Pro Met Glu Gly Thr Ile Tyr Trp Thr Asp Trp Gly Asn Thr
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Pro Arg Ile Glu Ala Ser Ser Met Asp Gly Ser Gly Arg Arg Ile Ile
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Gly Arg Arg Met Tyr Trp Val Asp Ala Lys His His Val Ile Glu Arg
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Ala Asn Leu Asp Gly Ser His Arg Lys Ala Val Ile Ser Gln Val Phe
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Glu Asp Ser Leu Tyr Trp Thr Asp Trp His Thr Lys Ser Ile Asn Ser
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Ala Asn Lys Phe Thr Gly Lys Asn Gln Glu Ile Ile Arg Asn Lys Leu
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His Phe Pro Met Asp Ile His Thr Leu His Pro Gln Arg Gln Pro Ala
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Gly Lys Asn Arg Cys Gly Asp Asn Asn Gly Gly Cys Thr His Leu Cys
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Leu Pro Ser Gly Gln Asn Tyr Thr Cys Ala Cys Pro Thr Gly Phe Arg
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Lys Ile Ser Ser His Ala Cys Ala Gln Ser Leu Asp Lys Phe Leu Leu
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Phe Ala Arg Arg Met Asp Ile Arg Arg Ile Ser Phe Asp Thr Glu Asp
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Leu Ser Asp Asp Val Ile Pro Leu Ala Asp Val Arg Ser Ala Val Ala
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Leu Asp Trp Asp Ser Arg Asp Asp His Val Tyr Trp Thr Asp Val Ser
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Thr Asp Thr Ile Ser Arg Ala Lys Trp Asp Gly Thr Gly Gln Glu Val
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Val Val Asp Thr Ser Leu Glu Ser Pro Ala Gly Leu Ala Ile Asp Trp
755 760 765

Val Thr Asn Lys Leu Tyr Trp Thr Asp Ala Gly Thr Asp Arg Ile Glu
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Val Ala Asn Thr Asp Gly Ser Met Arg Thr Val Leu Ile Trp Glu Asn
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Asp Ala Ser Gly Arg Gln Val Ile Ile Ser Ser Asn Leu Thr Trp Pro
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Asn Gly Leu Ala Ile Asp Tyr Gly Ser Gln Arg Leu Tyr Trp Ala Asp
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Ala Gly Met Lys Thr Ile Glu Phe Ala Gly Leu Asp Gly Ser Lys Arg
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Lys Val Leu Ile Gly Ser Gln Leu Pro His Pro Phe Gly Leu Thr Leu
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Tyr Gly Glu Arg Ile Tyr Trp Thr Asp Trp Gln Thr Lys Ser Ile Gln
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Ser Ala Asp Arg Leu Thr Gly Leu Asp Arg Glu Thr Leu Gln Glu Asn
915 920 925

Leu Glu Asn Leu Met Asp Ile His Val Phe His Arg Arg Arg Pro Pro
930 935 940

Val Ser Thr Pro Cys Ala Met Glu Asn Gly Gly Cys Ser His Leu Cys
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Ile Asn Leu Leu Ser Asp Gly Lys Thr Cys Ser Pro Gly Met Asn Ser
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Ile Pro Tyr Phe Ala Asp Val Val Val Pro Ile Asn Ile Thr Met
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Lys Asn Thr Ile Ala Ile Gly Val Asp Pro Gln Glu Gly Lys Val
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His Thr Leu Val Ser Pro Val Gln His Pro Tyr Gly Leu Thr Leu
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Glu Lys Ser Pro Val Leu Pro Asn Thr Pro Pro Thr Thr Leu Tyr
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Met Tyr Asn Gln Leu Cys Tyr Lys Lys Glu Gly Gly Pro Asp His
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Asn Tyr Thr Lys Glu Lys Ile Lys Ile Val Glu Gly Ile Cys Leu
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Asp Thr Val Ser Ile Gln Ala Ser Ser Gly Ser Leu Asp Asp Thr
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