



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 256 625 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
13.11.2002 Bulletin 2002/46

(21) Application number: **01904415.5**

(22) Date of filing: **14.02.2001**

(51) Int Cl.7: **C12N 5/16, C12N 15/18,
G01N 33/50, G01N 33/15,
C12N 15/37, C12N 15/63,
A01K 67/027**

(86) International application number:
PCT/JP01/01016

(87) International publication number:
WO 01/060984 (23.08.2001 Gazette 2001/34)

(84) Designated Contracting States:
CH DE FR GB LI

(30) Priority: **16.02.2000 JP 2000037827**

(83) **Declaration under Rule 28(4) EPC (expert
solution)**

(71) Applicant: **JAPAN SCIENCE AND TECHNOLOGY
CORPORATION**
Kawaguchi-shi, Saitama 332-0012 (JP)

(72) Inventors:
• **IIZASA, Hisashi**
Tokyo 110-0001 (JP)

- **HATTORI, Kenji Room 202, Shenesuhauze
Ashigarakami-gun Kanagawa 258-0021 (JP)**
- **NAKASHIMA, Emi**
Minato-ku, Tokyo 105-0012 (JP)
- **TERASAKI, Tetsuya**
Sendai-shi, Miyagi 981-3101 (JP)
- **OBINATA, Masuo**
Sendai-shi, Miyagi 980-0871 (JP)

(74) Representative: **Harding, Charles Thomas et al**
D. Young & Co.
21 New Fetter Lane
London EC4A 1DA (GB)

(54) **IMMORTALIZED VASCULAR ADVENTITIAL CELL LINE**

(57) To provide an immortalized capillary pericyte line which maintains the original function/property of the cell line-deriving tissue, its establishment method, and the screening method for useful substance using the immortalized capillary pericyte line. Cerebral tissue of a transgenic rat carrying the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 is homogenized and the resultant brain capillaries are treated with protease,

thus obtained brain capillary cells are subcultured to establish an immortalized cell that expresses SV40 thermo-sensitive large T antigen, PDGF receptor β , and Angiopoietin-1. In addition, the immortalized vascular pericyte line has ability to deposit calcium on matrix by dense culture.

EP 1 256 625 A1

Description**Technical Field**

5 [0001] The present invention relates to an immortalized vascular pericyte line, more particularly to an immortalized vascular pericyte line which can be established through subculturings of brain capillary cells of a transgenic rat introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58, and which expresses PDGF (Platelet-Derived Growth Factor) receptor β and Angiopoietin-1.

Prior Arts

10 [0002] Conventionally, animals have mainly been used in experimental studies for testing safety or effectiveness of pharmaceuticals. However, in order to prevent cruelty to animals, techniques using cultured cells etc. instead of using animals to test and study effectiveness or safety of pharmaceuticals in vitro are now being studied for practical application. For example, animal experiments are carried out after preliminarily experimented by methods using primary cultured cells from living tissues or immortalized cell (established cell) lines that proliferate infinitely. Primary cells, however, proliferate well at the early stage, but their proliferation will gradually come to a halt in the course of repeated subculturings and ends up with death (this event is called cell aging). Further, primary cells are said to alter their properties in the course of subculturings, in addition to the risk that their properties differ each time they are extracted from living tissues. To obtain primary cells sufficient for use in experiments is said to be extremely difficult, especially when the proliferation rate is considerably slow or the cells are micro-organ derived

15 [0003] Meanwhile, immortalized cells, that are free from cell aging and have acquired ability for infinite proliferation in the course of subculturings of the primary culture, have stable and equal properties. Still, many of such immortalized cells lose part or whole of morphology or function that the cells originally possessed in the organisms, and therefore in the experiments using such immortalized cells, it has been considered difficult to precisely reflect their original properties that the cells displayed in their deriving tissues. Hence, an attempt has been made to transform a primary cell by introducing cancer-causing genes such as ras gene or c-myc gene, E1A gene from adenovirus, the large T antigen gene from SV40 virus, HPV16 gene from Human Papiloma Virus, and the like, to successively maintain the high proliferation ability of the primary cell; and to establish a cell which will not lose its original property even in the course of subculturings. However, to obtain an immortalized cell in its strict definition, i.e. maintaining its original function has been a hard task, because even such an immortalized cell loses some of its function as early as at the preparation of the primary cell and at the introduction of the foregoing cancer genes or large T antigen gene, depending on the tissue used. It has extremely been difficult, especially when preparing and making a cell line using a primary cell with very low speed of proliferation or such deriving from a microorgan.

20 [0004] Meanwhile, a method of establishing an immortalized cell which utilizes a recently established technique to introduce a gene into an individual animal is reported, wherein a transgenic animal which is stably imported with a cancer gene or the large T antigen gene into its chromosome is generated instead of introducing such gene into each cell, and wherein an immortalized cell is established by subculturing the primary cell prepared from an animal organ having a cancer gene or the large T antigen gene in the cell already at the time of the ontogeny. For instance, Japanese Laid-Open Patent Application No. 1993-292958, inventors of which are the present inventors, discloses a method of establishing an immortalized cell line comprising: introducing thermo-sensitive mutant SV40 large T antigen gene into a totipotent cell of a mammal; collecting tissue cells from various organs such as hepatic cells or bone marrow cells of a transgenic animal which was normally born from said mammal; and subculturing the tissue cells. An immortalized cell from an organ of the transgenic mouse introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 is, especially, said to be very effective in that its proliferation or the expression of differential property can be manipulated by changing temperature (Noble M et al.(1995) Transgenic Research 4, 215-225; Obinata M.(1997) Genes to Cells 2, 235-244).

25 [0005] Capillary pericytes have been considered with great interest in the studies of vascular wall denatured diseases. Methods using primary cells of capillary pericytes instead of using animals have experimentally been tried in view of preventing cruelty to animals. In doing so, sufficient amount of cells for the experiment use is hard to be obtained from small experimental animals where the numbers of capillary pericytes differ among the tissues, so that tissues of large-sized cattle such as cows or the like should be used. However, information obtained from the cultured cells of large-sized cattle such as cows etc. can hardly be reduced back to the organism, therefore, a capillary pericyte line as an effective alternative have been longed for. The subject of the present invention is to provide an immortalized vascular pericyte line maintaining its original function/property in the tissue from which said cell line derived, a method of establishing said cell line, and a method for screening an effective substance using said cell line.

30
35
40
45
50
55

Summary of the Invention

5 [0006] The present inventors have made a keen study to solve the above-mentioned problems and have found that when generating a cell line from a microorganism such as brain capillary pericyte, rats with about tenfold weight of mice is advantageously used to prepare cells for establishing an immortalized cell line. According to the finding, the inventors have found that an immortalized capillary pericyte line can be established by the step comprising: producing a transgenic rat introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 as an immortalized gene; homogenizing the cerebral tissue of said transgenic rat to separate the brain capillary; treating the obtained brain capillary with protease; and subcultivating the obtained cell. The present invention is thus completed.

10 [0007] The present invention relates to an immortalized vascular pericyte line which maintains ability to express PDGF receptor β and Angiopoietin-1 (claim 1), an immortalized vascular pericyte line of claim 1 which derives from a brain capillary pericyte (claim 2), an immortalized vascular pericyte line of claim 1 or 2 which expresses the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 (claim 3), an immortalized vascular pericyte line according to any one of claims 1 to 3, which has ability to deposit calcium on matrix by dense culture (claim 4), an immortalized vascular pericyte line according to any one of claims 1 to 4, which is a rodent-origin (claim 5), an immortalized vascular pericyte line of claim 5 wherein the rodent is a rat (claim 6), an immortalized brain capillary pericyte line TR-PCT1 (FERM BP-7024) (claim 7).

15 [0008] The present invention also relates to a producing method of an immortalized vascular pericyte line comprising: homogenizing the cerebral tissue of a transgenic rat which is introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58; treating the obtained brain capillary with protease; subcultivating said brain capillary cell; and establishing an immortalized cell expressing thermo-sensitive SV 40 large T antigen, PDGF receptor β and Angiopoietin-1 (claim 8), and a producing method of an immortalized vascular pericyte line of claim 8, wherein the immortalized vascular pericyte line has ability to deposit calcium on matrix by dense culture (claim 9).

20 [0009] The present invention further relates to a screening method of a promoting agent or an inhibitory substance for the expression of differential property in an immortalized vascular pericyte, wherein the immortalized vascular pericyte line according to any one of claims 1 to 7 is cultured in the presence of a subject material and the activity and/or the expression level of the marker protein in said cell is measured and evaluated (claim 10), a screening method of a promoting agent or an inhibitory substance for the expression of differential property in the immortalized vascular pericyte of claim 10, wherein the marker protein is PDGF receptor β , Thy-1, ICAM-1, or Angiopoietin-1 (claim 11), a screening method of a promoting agent or an inhibitory substance for the proliferation in an immortalized vascular pericyte, wherein the immortalized vascular pericyte line according to any one of claims 1 to 7 is cultured in the presence of a subject material and the proliferation level of said cell is measured and evaluated (claim 12), and a screening method of a promoting agent or an inhibitory substance for calcium deposition on matrix in an immortalized vascular pericyte, wherein the immortalized vascular pericyte line according to any one of claims 1 to 7 is dense-cultured in the presence of a subject material and the calcium level deposited on matrix in said cell is detected and evaluated (claim 13).

25 [0010] The present invention still further relates to a promoting agent for the expression of differential property in the immortalized vascular pericyte obtained by the screening method of claim 10 or 11 (claim 14), a promoting agent for the proliferation in the immortalized vascular pericyte obtained by the screening method of claim 12 (claim 15), a promoting agent of the calcium deposition on matrix in the immortalized vascular pericyte obtained by the screening method of claim 13 (claim 16), an inhibitory substance for the expression of differential property in the immortalized vascular pericyte obtained by the screening method of claim 10 or 11 (claim 17), an inhibitory substance for the proliferation in the immortalized vascular pericyte obtained by the screening method of claim 12 (claim 18), and an inhibitory substance for the calcium deposition on matrix in the immortalized vascular pericyte obtained by the screening method of claim 13 (claim 19).

The best mode to carry out the Invention

30 [0011] An immortalized vascular pericyte line of the present invention may be any one among established cells that maintain ability to express PDGF receptor β and Angiopoietin-1. The examples include cell lines which derives from the brain capillary pericyte, which expresses the large T antigen gene of SV40 thermo-sensitive mutant line tsA58, and which can deposit calcium on matrix by dense culture. TR-PCT1 line (FERM BP-7024) is a particular example of such immortalized vascular pericyte. An immortalized vascular pericyte line of the present invention can be obtained from rodents such as rats or the like. Among them, rats are preferable for use in preparation of the cell for establishment of a cell line from various organs, especially when generating a line from a microorganism-derived cell such as a brain capillary pericyte due to the easiness to obtain primary cells from rats by separating the organ. The method of producing an immortalized vascular pericyte line will now be explained in the following with examples using rats.

35 [0012] For instance, the cerebral tissue of a transgenic rat introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 is homogenized to separate brain capillaries. The obtained brain capillaries are treated

with protease such as collagenase, after which the brain capillary pericytes are subcultured, which leads to the establishment of the immortalized cells expressing PDGF receptor β which is a surface marker for a perithelial cell and Angiopoietin-1 which is specific for the vascular wall cell.

5 **[0013]** The transgenic rat introduced with the above-mentioned large T antigen gene of SV40 thermo-sensitive mutant line tsA58 can be produced as described below. For example, total DNA of tsA58 ori (-)-2, where the replication origin (ori) of SV40 is deleted, is digested and opened with the restriction enzyme BamHI and is introduced into pBR322, to obtain plasmid pSVtsA58 (-)-2 (Ohno T. et al., Cytotechnology 7, 165-172, 1991), which is then considerably amplified in E. Coli following the usual protocol. These amplified plasmids are then digested with the restriction enzyme BamHI
10 to remove the vector sites, and thus DNA fragment having the large T antigen gene of tsA58 is prepared. When DNA fragment containing the promoting agent of this large T antigen gene is genetically introduced into a totipotent cell of an animal like rodent such as a rat etc. according to an ordinary protocol, a gene-introduced rat, i.e. transgenic rat, can be produced which has the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 in every each of its cells. Every somatic cell of this transgenic rat expresses the large T antigen genes of tsA58. Specific examples of the above totipotent cells include pluripotent ES cells other than fertilized eggs or early embryos. Known methods of gene
15 introduction such as microinjection, electric pulsing, Liposome method, Calcium-Phosphate method, or the like may be exemplified as methods to introduce DNA into totipotent cells.

[0014] The large T antigen gene of SV40 thermo-sensitive mutant line tsA58 can be introduced into the ovum by transplanting the nucleus of totipotent cells (cultured cells) of animals like rodents such as rats as mentioned above to an enucleated unfertilized egg for initializtion (nuclear transplantation). And it is possible to effectively generate a
20 gene-introduced rat, i.e. a transgenic rat, introduced with the large T antigen gene of tsA58 into the chromosomes of cells of each tissue already at the time of the individual development, as follows: the male pronucleus of pronucleus-stage fertilized egg is microinjected with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58; the resulting ovum is transplanted into oviduct of the recipient rat to gain a newborn rat; baby rats having the injected gene are selected; and as a consequence, individuals carrying such gene are constantly obtained.

25 **[0015]** With regard to the immortalized vascular pericyte of the present invention, the pelletes can be obtained as follows: the cerebrum of the transgenic rat generated as the foregoing is extracted and thoroughly washed in buffer solution; the cerebrum is cut into thin strips that are ground with a homogenizer for slurry; and thus obtained slurry is added buffer solution to be centrifuged. The pellets thus obtained are suspended in the enzymic (protease) solution and enzymically treated as the solution is shaken so that capillaries are separated from the unnecessary tissues for
30 centrifuging again, and the pellets obtained through the centrifugation are suspended in DMEM containing 20% fetal bovine serum. This suspension solution is spread on a culture glass plate and is subjected to two subculturings, and the colonies are formed. The colonies with relatively high speed of proliferation are isolated from the surrounding cells with a penicillin cup and the colonies are formed again. Again, the colonies with relatively high speed of proliferation can be isolated from the peripheral cells with a penicillin cup.

35 **[0016]** The immortalized vascular pericyte isolated as described above is characteristic in maintaining infinite proliferation ability at 33°C to 37°C and in controlling the expression of cell-specific differential property to halt the proliferation at 39°C. Further, the isolated immortalized vascular pericytes can be identified by detecting the expression of thermo-sensitive SV40 large T antigen, PDGF receptor β , and Angiopoietin-1 by RT-PCR and/or Western blotting. Said immortalized vascular pericyte line shows high proliferation activity at 33°C even after 50 subculturings and still main-
40 tains the function of the brain capillary pericyte.

[0017] The immortalized vascular pericyte line of the present invention is useful for studying vascular wall denatured diseases, autoimmune diseases originating in the brain, the diseases associated with nutritional metabolism of the brain and with disorder of homeostasis function, neovascularization-involved diseases such as solid tumor, arterio-
45 sclerosis, etc., and for studying diagnosis or the development of the therapy for these diseases at the cell level. In addition, the immortalized vascular pericyte line of the present invention maintains expression of various cell markers and the cultivation on the dense glass plate brings about matrix structure with abundant calcium composition. Therefore, as described below, it may be applied in the screening of useful substances such as pharmaceuticals etc. that target vascular pericytes.

[0018] The screening method of the present invention includes: a screening method of a promoting agent or an inhibitory substance for the differential property expression in an immortalized vascular pericyte, wherein the above
50 immortalized vascular pericyte line is cultured in the presence of a subject material and the activity and/or the expression level of marker proteins such as PDGF receptor β , Thy-1, ICAM-1, Angiopoietin-1 or the like in said cell is measured/evaluated; a screening method of a promoting agent or an inhibitory substance for proliferation in an immortalized vascular pericyte, wherein the above immortalized vascular pericyte line is cultured in the presence of a subject material and the proliferation level of said cell is measured/evaluated; and a screening method of a promoting agent or an
55 inhibitory substance for calcium deposition on matrix in an immortalized vascular pericyte, wherein the above immortalized vascular pericyte line is dense-cultured in the presence of a subject material and the calcium level deposited on matrix of the cell is detected/evaluated.

[0019] The screening of a promoting agent or an inhibitory substance for the differential property expression in the above mentioned immortalized vascular pericyte is carried out by cultivating the immortalized vascular pericyte line in the presence of a subject material of various concentrations respectively, detecting/measuring the level of marker proteins expressed after a certain period of cultivation, and comparing with the control cultured in the absence of a subject material for evaluation. For instance, PDGF receptor β or Thy-1, a surface marker for a vascular pericyte, can be immunochemically detected for measurement by the usual method using a specific antibody for each. In addition, vascular wall-specific Angiopoietin-1 or ICAM-1 is measured by detecting expression of the corresponding mRNA by the usual method. Screening of a promoting agent or a inhibitory substance for proliferation in the above immortalized vascular pericyte is carried out by cultivating the immortalized vascular pericyte line in the presence of a subject material of various concentrations respectively, measuring/analyzing the cell count and cell morphology after a certain period of cultivation, and comparing with the control cultured in the absence of a subject material for evaluation. Further, a promoting agent or an inhibitory substance for calcium deposition on matrix in the above immortalized vascular pericyte is screened by dense culture of the immortalized vascular pericyte line in the presence of a subject material of various concentrations which is followed by, after a certain period of cultivation, detecting calcium deposition on matrix with a microscope etc., and by comparing with the control cultured in the absence of a subject material for evaluation.

[0020] Promoting agents or inhibitory substances for differential property expression in the immortalized vascular pericyte, for proliferation in an immortalized vascular pericyte, and for calcium deposition on matrix in the immortalized vascular pericyte, which are obtainable by these screening methods, can be employed and will be useful as a remedy and as a drug for prevention and/or symptom-amelioration for treating patients suffering vascular wall denatured diseases, neovascular diseases, autoimmune diseases of the brain, diseases associated with nutritional metabolism of the brain and with disorder of homeostasis function.

[0021] The present invention will now be explained in more detail with examples below, but the scope of the invention will by no means be limited by these examples.

Example 1 (production of transgenic rats)

[0022] A transgenic rat introduced with DNA of SV40 thermo-sensitive mutant line tsA58 was produced by the following procedure.

(Preparation of the gene to be introduced)

[0023] Genomic DNA of SV40 thermo-sensitive mutant line tsA58 was used for microinjection. DNA to be introduced was prepared following the usual protocol from DNA clone pSVtsA58ori (-)-2 (Ohno T. et al., Cytotechnology, 165-172, 1991; see Fig.1) in which genomic DNA of tsA58 was digested and opened with restriction enzyme BamHI and was then introduced into BamHI site of pBR322 and in which SfiI sequence is subsequently converted to Sac II to have ori (-) which lacks SV40 replication origin (ori). Briefly, plasmid DNA, pSVtsA58ori(-)-2, obtained by having been considerably amplified within E.coli was digested with restriction enzyme BamHI (Takara), and was then separated by agarose gel electrophoresis (1% gel; Boehringer) after which the gel was lysed. Subsequently, DNA was collected by phenol/chloroform treatment and ethanol precipitation. The purified DNA collected was lysed in TE buffer (10mM Tris-HCl containing 1mM EDTA; pH7.6) and the solution containing 170 μ g/ml purified DNA was obtained. The DNA solution was diluted to 5 μ g/ml with injectable buffer (10mM Tris-HCl containing 0.1mM EDTA; pH7.6) to prepare the injectable DNA solution. The prepared DNA solution was kept at -20°C until performing injection.

(Production of transgenic rats)

[0024] The injectable DNA solution prepared according to the foregoing procedure was microinjected into a rat's fertilized egg at the pronucleus stage as follows. Sexually matured 8-week-old Wister rats were fed under 12-hour bright/dark cycles (bright hours: 4:00-16:00), temperature of 23 \pm 2°C, and moisture of 55 \pm 5%. Sexual cycles of the female rats were monitored with their vaginal smear and the day to administer hormone was selected. First, the female rats were intraperitoneally injected with 150 IU/kg pregnant mare serum gonadotropin (PMSG) (Nippon Zenyaku; PMS Zenyaku), and 48 hours later with 75 IU/kg human chorionic gonadotropin (hCG) (Sankyo Zoki; Puberogen) to induce superovulation, after which the female rats were co-resided with male rats for intercrossing. Fertilized eggs at the pronucleus stage were collected by oviduct perfusion at 32 hours after the hCG injection. mKRB solution (Toyoda Y. and Chang M.C., J. Reprod. Fertil., 36, 9-22, 1974) was used for oviduct perfusion and egg cultivation. The fertilized eggs collected were enzymically treated for 5 min. at 37°C in mKRB solution containing 0.1% Hyaluronidase (Sigma; Hyaluronidase Type1-S) to remove cumulus oophorus cells, after which the eggs were washed three times in mKRB solution to remove enzyme and were kept in a CO₂-incubator (5% CO₂-95% Air, 37°C, saturated moisture) until DNA injection was conducted. The rat's fertilized eggs, thus obtained, were injected with the above DNA solution in the male

pro-nucleus. 228 eggs injected with the DNA solution were transplanted into 9 recipient rats and 80 rats were born. Whether DNA is introduced into the rats by injection was examined by PCR with DNA prepared from tails that are cut off just after weaning [primers used; tsA58-1A, 5'-TCCTAATGTGCAGTCAGGTG-3' (corresponding to the 1365-1384 site), tsA58-1B, 5'-ATGACGAGCTTTGGCACTTG-3' (corresponding to the 1571-1590 site)]. As a result, transgenic rats from 11 lines (male lines: #07-2, #07-5, #09-6, #12-3, #19-5, female lines: #09-7, #11-6, #12-5, #12-7, #18-5, #19-8), that survived up to 12 weeks when sexually maturing period is over, were obtained from among 20 rats that were confirmed to have been gene-transferred (6 males, 8 females, 6 sex-unknown). These G₀-generation transgenic rats and Wistar rats were intercrossed and the gene transmission to the next and following generations were confirmed in 2 lines and 3 lines among male founders (#07-2, #07-5) and female founders (#09-7, #11-6, #19-8), respectively.

Example 2 (Separation of brain capillary pericytes)

[0025] To separate brain capillary pericyte from a cerebrum, a method was employed which is an improvement of the methods of Ichikawa and others (Ichikawa N et al., (1996) J. Pharmacol. Toxicol. Method., 36, 45-52) and Capetandes and others (Capetandes A and Gerristen ME (1990) Invest. Ophthalmol. Vis. Sci., 31, 1738-1744). The brain of the transgenic rat (single) which was introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58 obtained according to Example 1 was extracted. The extracted brain was removed of diencephalon and brain stem etc. except for the cerebrum, and the cerebrum was then thoroughly washed with the buffer for preparation (122mM NaCl, 3mM KCl, 1.4mM CaCl₂, 1.2mM MgSO₄ · 7H₂O, 0.4mM K₂HPO₄, 10mM Glucose, 10mM Hepes; pH7.4) which was ice-cooled in a clean bench. The tissue of the washed cerebrum was cut into small strips of 1 to 2 mm² and the strips were transferred to a 10mL Potter-Elvehjem homogenizer (Tokyo Rikakikai). The sectioned brain was then added the ice-cooled buffer for preparation with the four-fold amount of the brain and was stroked up and down for 10 times for homogenizing the tissue. Then the same amount of PBS containing 32% dextran was added therein and the tissue was stroked up and down for 3 times to obtain the slurry. The pellets obtained by centrifuging the slurry (4°C, 15 min at 4500g) were suspended in 2.4mL enzymic solution [PBS containing 0.066% collagenase/dispase (Boehringer Mannheim), 0.033% BSA (Sigma)], followed by enzymic treatment (37°C, 3h) in order to have unnecessary extracellular matrix separated and the pellets were obtained by centrifugation (4°C, 5 min at 600g). The pellets thus obtained were dispersed in 10mL culture solution (DMEM supplemented with 100U/mL benzylpenicillin potassium, 100 µg/mL streptomycin sulfate, 2.50 µg/mL amphotericin B, 20% FCS) and were seeded onto four 35mm φ culture grass plates (Falcon). The pellets were cultured (primary culture) in a 33°C CO₂ incubator (5% CO₂-95% Air, saturated humidity). The media were replaced twice a week, and subculturings were carried out around every each week with Trypsin solution (0.05% Trypsin, 0.53mM EDTA; Gibco BRL). After undergoing two subculturings, 10⁴ cells were seeded onto a 100mm φ culture plate (Falcon). The media was replaced twice a week. Colonies with relatively high speed of proliferation where colonies were formed on day 7-10 were isolated from the surrounding cells with a penicillin cup. 10³ cells obtained were seeded again onto a 100 mm φ culture plate followed by cultivation in a 33°C CO₂ incubator for colony formation. Two cell lines (TR-PCT1, TR-PCT2) were obtained by isolating colonies with relatively high speed of proliferation from the surrounding cells with a penicillin cup.

[0026] This TR-PCT1 line is adopted to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, METI (1-3, Higashi 1-chome, Tsukuba-city, Ibaragi, 305-8566 Japan) with the adoption No."NIBH FERM BP-7024", under the Budapest Treaty defining international acknowledgement concerning microorganism adoption in the procedure of patent application.

Example 3 (Confirmation of large T antigen protein)

[0027] Large T antigen protein of two cell lines obtained according to Example 2 was detected by western blotting (Jikken-igaku bessatsu manual UP series "Bunshi-seibutsugakuteki approach niyoru gann-kenkyu protocol" (A special number of experimental medicine: manual up series, "Protocols for studying cancer by molecular biological approach") pp108-115, Yodosha, 1995). The two cell lines were washed with PBS respectively, solubilized in 1mL solubilized solution (1% SDS, 10mM Tris, 1mM EDTA, 10% glycerin), and heated at 100°C for 10 min. The cell lines were then centrifuged (10 min at 10000rpm) to remove the insolubilized fractions and the total protein was quantified by Bradford protein assay (BCA protein assay reagent A, PIERCE, was used). Proteins of 10 µg each were separated by SDS polyacrylamid gel electrophoresis and were transcribed onto a nitrocellulose membrane. Anti-SV40 large T antigen mouse antibody (DP02-C; CALBIOCHEM) as the primary antibody and HRP-labeled anti mouse IgG antibody (Amersham) as the secondary antibody were respectively provided to cause reaction on the nitrocellulose membrane blocked with 3% skim milk solution. Subsequently, the reaction specific for large T antigen was detected by using the Amersham's ECL western blotting detection system (RPN2106M1). The result is shown in Table 1. The result confirmed that the large T antigen protein is present in both cell lines obtained.

Table 1

Cell line	TR-PCT1	TR-PCT2
T antigen	+	+

Example 4 (Confirmation of PDGF receptor β and Thy-1)

[0028] The cell lines obtained were monolayer-cultured and PDGF receptor β and Thy-1 expressed on the cell membrane were immuno-stained for microscopical detection. The cell line TR-PCT1 obtained according to Example 2 was cultured on the 24 well dish (Falcon) cover glass. The culture solution was removed and the cells were washed with PBS, after which 0.5mL fixing solution (3.6% paraformaldehyde) was added, left at 4°C for 20 min, and washed thoroughly with PBS. 0.5mL of 3% BSA-PBS was then added and left at room temperature for 2 hours for blocking, and the cells were reacted with the primary antibody (anti PDGF receptor β goat antibody; Santacruz, FITC-labeled anti Thy-1 mouse antibody; Pharmingen) at room temperature for 1 hour. After washing five times in 0.1% BSA-PBS, the secondary antibody (FITC-labeled anti goat IgG; Sigma (used only for detection of PDGF receptor β)) was provided to cause reaction at room temperature for 1 hour, then washed five times with 0.1% BSA-PBS. Finally, the labeled cells were sealed with glycerin sealing solution (90% glycerol, 1mg/mL p-phenyldiamine, 0.01M Na₂HPO₄; pH 8.5), and were microscopically observed. The result revealed the expression of the PDGF receptor β and Thy-1 on the cell membrane of the cell line TR-PCT1. In addition, the PDGF receptor β expression was also confirmed by western blotting and RT-PCR. Similar result was obtained for TR-PCT2.

Example 5 (Confirmation of Angiopoietin-1, Osteopontin, and ICAM-1)

[0029] The cell lines obtained were monolayer-cultured and Angiopoietin-1, Osteopontin, and ICAM-1 expressed on the cells were detected by RT-PCR. The cell line TR-PCT1 obtained in Example 2 was cultured on a 100mm ϕ culture plate (Falcon). The culture solution was removed and the cells were washed with PBS, after which the cells were collected with the cell-scraper (Iwaki) and total RNA was extracted with the RNA extraction reagent (Trizol; Gibco). cDNA was synthesized from 1 μ g of the extracted total RNA using reverse transcriptase (Rev Tra Ace: TOYOBO) and its expression was confirmed by PCR (exTaq; Takara). The PCR amplification product was confirmed by electrophoresis using 5% acrylamide gel. As a result, the expression of Angiopoietin-1, Osteopontin, and ICAM-1 was confirmed in the cell line TR-PCT1. TR-PCT2 displayed the similar result.

Example 6 (Confirmation of calcium deposition on matrix by dense culture)

[0030] The cell lines obtained were monolayer-cultured, and calcium deposited on the matrix was confirmed by vonkossa staining. 10⁶ cell lines of TR-PCT1 obtained in Example 2 were cultured in DMEM on a 100mm ϕ culture plate (Falcon) and a 100mm ϕ collagen type I-coated culture plate (Iwaki). The culture solution for the 100mm ϕ collagen type I-coated culture plate was added 10mM β -glycerophosphate. On day 3, the culture solution was removed and the cells were washed with PBS, after which 8mL fixing solution (PBS containing 0.1% glutaraldehyde) was added, and left at room temperature for 15 min. The cells were then washed twice in distilled water. Subsequently, 8mL of 5% silver nitrate solution (Wako) was added to block light, then the cells were left at room temperature for 30 min, after which time the 5% silver nitrate solution was removed and the cells were washed twice in distilled water. The plate was exposed to light at room temperature for 30 min and was microscopically observed. The result revealed the calcium deposition on the matrix for the cell line TR-PCT1, and the increase in the calcium deposition was confirmed on the collagen-coated culture plate. TR-PCT2 also had the similar result.

Industrial Applicability

[0031] The present invention enables to provide an immortalized vascular pericyte line useful for: studies of vascular wall denatured diseases, nutritional metabolism of the brain, neovascularization, and autoimmune diseases originating in the brain; screening of the drugs that target vascular pericytes; diagnosis of the diseases associated with nutritional metabolism of the brain and with disorder of homeostasis function; and a study at a cell-level for development of the therapy for these diseases.

SEQUENCE LISTING

5 <110> Japan Science and Technology Corporation

<120> Immortalization

10 <130> A081-02PCT

<140>

<141>

15 <150> JP 2000/037827

<151> 2000-02-16

<160> 2

20 <170> PatentIn Ver. 2.1

<210> 1

25 <211> 20

<212> DNA

<213> Artificial Sequence

<220>

30 <223> Description of Artificial Sequence:Primer

<400> 1

35 tcctaattgtg cagtcagggtg 20

<210> 2

<211> 20

40 <212> DNA

<213> Artificial Sequence

<220>

45 <223> Description of Artificial Sequence:Primer

<400> 2

atgacgagct ttggcacttg 20

Claims

- 55 1. An immortalized vascular pericyte line which maintains ability to express PDGF receptor β and Angiopoietin-1.
2. An immortalized vascular pericyte line of claim 1 which derives from a brain capillary pericyte.
3. An immortalized vascular pericyte line of claim 1 or 2 which expresses the large T antigen gene of SV40 thermo-

sensitive mutant line tsA58.

- 5
4. An immortalized vascular pericyte line according to any one of claims 1 to 3, which has ability to deposit calcium on matrix by dense culture.
5. An immortalized vascular pericyte line according to any one of claims 1 to 4, which is a rodent-origin.
6. An immortalized vascular pericyte line of claim 5 wherein the rodent is a rat.
- 10
7. An immortalized brain capillary pericyte line TR-PCT1 (FERM BP-7024).
8. A producing method of an immortalized vascular pericyte line comprising: homogenizing the cerebral tissue of a transgenic rat which is introduced with the large T antigen gene of SV40 thermo-sensitive mutant line tsA58; treating the obtained brain capillary with protease; subcultivating said brain capillary cell; and establishing an immortalized cell expressing thermo-sensitive SV 40 large T antigen, PDGF receptor β and Angiopoietin-1.
- 15
9. A producing method of the immortalized vascular pericyte line of claim 8, wherein the immortalized vascular pericyte line has ability to deposit calcium on matrix by dense culture.
- 20
10. A screening method of a promoting agent or an inhibitory substance for the expression of differential property in an immortalized vascular pericyte, wherein the immortalized vascular pericyte line according to any one of claims 1 to 7 is cultured in the presence of a subject material and the activity and/or the expression level of the marker protein in said cell is measured and evaluated.
- 25
11. A screening method of a promoting agent or an inhibitory substance for the expression of differential property in the immortalized vascular pericyte of claim 10, wherein the marker protein is PDGF receptor β , Thy-1, ICAM-1, or Angiopoietin-1.
- 30
12. A screening method of a promoting agent or an inhibitory substance for the proliferation in an immortalized vascular pericyte, wherein the immortalized vascular pericyte line according to any one of claims 1 to 7 is cultured in the presence of a subject material and the proliferation level of said cell is measured and evaluated.
- 35
13. A screening method of a promoting agent or an inhibitory substance for calcium deposition on matrix in an immortalized vascular pericyte, wherein the immortalized vascular pericyte line according to any one of claims 1 to 7 is dense-cultured in the presence of a subject material and the calcium level deposited on matrix in said cell is detected and evaluated.
- 40
14. A promoting agent for the expression of differential property in the immortalized vascular pericyte obtained by the screening method of claim 10 or 11.
- 45
15. A promoting agent for the proliferation in the immortalized vascular pericyte obtained by the screening method of claim 12.
16. A promoting agent of the calcium deposition on matrix in the immortalized vascular pericyte obtained by the screening method of claim 13.
- 50
17. An inhibitory substance for the expression of differential property in the immortalized vascular pericyte obtained by the screening method of claim 10 or 11.
- 55
18. An inhibitory substance for the proliferation in the immortalized vascular pericyte obtained by the screening method of claim 12.
19. An inhibitory substance for the calcium deposition on matrix in the immortalized vascular pericyte obtained by the screening method of claim 13.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/01016

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ⁷ C12N5/16, 15/18, G01N33/50, 33/15, C12N15/37, 15/63, A01K67/027		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) Int.Cl ⁷ C12N5/16, 15/18, G01N33/50, 33/15, C12N15/37, 15/63, A01K67/027		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA (STN), MEDLINE (STN), Genbank/EMBL/DDBJ/PIR/SwissProt/Genseq, WPI (DIALOG), BIOSIS (DIALOG)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO, 00/20599, A (YS New Technology Kenkyusho K.K.), 13 April, 2000 (13.04.00), Claims; example, etc. (Family: none)	1-9
Y	JP, 5-292958, A (Res. Dev Corp. of Japan.), 09 November, 1993 (09.11.93), Claims; example, etc. (Family: none)	1-13
Y	Kwon, Yunhee Kim et al., "Expression of brain-derived neurotrophic factor mRNA stimulated by basic fibroblast growth factor and platelet-derived growth factor in rat hippocampal cell line" Mol. Cells, Vol.7(3), pp.320-325 (1997)	1-13
Y	Delphime LECHARDEUR et al., "Induction of Blood Brain Barrier Differentiation in a Rat Brain-Derived Endothelial Cell Line" Exp. Cell Res., Vol.220(1), pp.161-170 (1995)	1-13
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 15 May, 2001 (15.05.01)	Date of mailing of the international search report 22 May, 2001 (22.05.01)	
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer	
Facsimile No.	Telephone No.	

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/01016

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. Claims Nos.: 14-19
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Although the statement in the description is taken into consideration, it is unknown what particular compounds are involved and what particular compounds are excluded from the scopes of "differentiation character expression promoters", "proliferation promoters", "calcium matrix mass deposition promoters", "differentiation character expression inhibitors", "proliferation inhibitors" and "calcium matrix mass deposition inhibitors". Namely, these claims are described in an extremely unclear manner. Therefore, no meaningful international search can be practiced on the above claims.
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.