PROBE FOR VISUALIZING CELL CYCLE
SONDE ZUR SICHTBARMACHUNG DES ZELLZYKLUS
SONDE UTILISÉE POUR OBSERVER LE CYCLE CELLULAIRE

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• BALLABENI A ET AL: “Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis” EMBO J., vol. 23, no. 15, 1 January 2004 (2004-01-01), pages 3122-3132, XP008117404

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• XOURI GEORGIA ET AL: "Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines." EUROPEAN JOURNAL OF BIOCHEMISTRY / FEBS AUG 2004 LNKD- PUBMED:15291814, vol. 271, no. 16, August 2004 (2004-08), pages 3368-3378, XP002577361 ISSN: 0014-2956

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• ITO S. ET AL.: 'Epstein-Barr virus nuclear antigen-1 is highly colocalized with interphase chromatin and its newly replicated regions in particular' J. GEN. VIROL. vol. 83, no. 10, 2002, pages 2377 - 2383, XP002257661

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The present invention relates to (i) a method for performing phase identification of a cell cycle by visualizing, by using a marker, a gene-expression product whose amount in a cell changes in a cell-cycle dependent manner and (ii) a gene construct for use in the method.

A cell cycle is a process in which a cell produced by a cell division undergoes another cell division to produce a new cell. Of the cell cycle, a phase during which mitosis takes place is called an M phase. Generally, the M phase completes in approximately one hour. An interval between one M phase and another M phase is called an interphase during which cell growth as well as biosynthesis and/or metabolism of a substance occur. The interphase can be further divided into a G1 phase, an S phase, and a G2 phase. In the S phase, DNA replication takes place. The G1 phase is a phase between the M phase and the S phase, and the G2 phase is a phase between the S phase and the M phase.

As a method for analyzing a specific phase of the cell cycle (G1 phase, S phase, G2 phase, or M phase), a method using a BrdU label is known. In specific, the method includes: causing a BrdU to be taken into a cell for a given period; and subsequently, carrying out immunohistochemistry by using an anti-BrdU antibody. However, with the method, it is impossible to carry out observation in real-time. There is also known a method using cell synchronization and a biochemical model. With the method, however, it is impossible to carry out real-time observation.

As a method for visualizing a specific phase of the cell cycle, there is a method using the G2M cell cycle phase marker (G" MCCPM) (Amersham Bioscience K.K). Because the method uses promoter activity of cyclin, there is a problem in that transformation by gene introduction is remarkably influenced depending on how a transgene is integrated into a chromosome. Further, because the G1 phase is not visualized, (i) it is difficult to track a cell cycle, and (ii) a contrast is unclear.

Prior art document Xouri G et al. 2007. Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin. EMBO J. 26:1303-1314 describes that Cdt1 exhibits dynamic interactions with chromatin throughout G1 phase and that the protein domains responsible for chromatin and Geminin interactions are separable. It also describes that Cdt1 simultaneously binds Geminin and chromatin in vivo, thereby recruiting Geminin onto chromatin.

Prior art document Ballabeni et al. 2004. Human Geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. EMBO J. 23:3122-3132 describes that Geminin ensures basal levels of CDT1 during S phase and its accumulation during mitosis and that consistently inhibition of Geminin synthesis during M phase leads to impairment of pre-RC formation and DNA replication during the following cell cycle. In addition, it describes that inhibition of CDR1 during mitosis, and not Geminin depletion, is sufficient for premature formation of pre-RCs, indicating that CDK activity is the major mitotic inhibitor of licensing in human cells.

Peptide binding to Geminin and inhibitory for DNA replication. BBRC. 317: 218-222 describes that delivery of a peptide sequence that binds the 31-111 amino acid residues of Germinin into the nucleus of HCT116 human colon cancer cells resulted in the suppression of BrdU incorporation.


Xouri G et al. 2004. Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines. 271:3368-3378 described the expression levels of Germinin in cancer cells.

An object of the present invention is to provide a method for performing phase identification of a cell cycle, by which method it is possible to easily distinguish a proliferation phase of the cell cycle from a resting phase thereof in real time. The present invention further has an object to provide a gene construct for use in the method of the present invention.

The inventors of the present invention made a diligent study in order to attain the aforementioned objects of
the present invention. As a result, the inventors found it possible to distinguish a proliferation phase of a cell cycle from a resting phase thereof by (i) visualizing, by using a marker, at least one or more gene-expression products whose amounts in a cell change in a cell-cycle dependent manner and (ii) detecting the marker. By this, the inventors of the present invention accomplished the present invention.  

[0012] Namely, the present invention provides the following:

(1) a method for performing phase identification of a cell cycle, the method comprising the steps of:

- visualizing, at least two or more gene-expression products whose amounts in a cell change in a cell-cycle dependent manner; and
- detecting in vitro the at least two or more gene-expression products so as to distinguish a proliferation phase of the cell cycle from a resting phase of the cell cycle, wherein:

  - as the at least two or more gene-expression products, at least (i) a first gene-expression product whose amount increases in a G1 phase and decreases in an S/G2/M phase and (ii) a second gene-expression product whose amount decreases in the G1 phase and increases in the S/G2/M phase are used in one cell;
  - the first gene-expression product is the partial fragment of Cdt1, which partial fragment of Cdt1 is remaining of Cdt1 from which a Geminin binding site is excluded;
  - the second gene-expression product is the partial fragment of Geminin, which partial fragment of Geminin is remaining of Geminin from which a Cdt1 binding site is excluded, and
  - the first gene-expression product and the second gene-expression product being labeled by the markers different from each other, so as to enable the first gene-expression product and the second gene-expression product to be visualized and detected, wherein the first gene-expression product is the partial fragment of Cdt1, which partial fragment of Cdt1 is composed of 30th through 120th amino acids of said Cdt1, and wherein the second gene-expression product is the partial fragment of Geminin, which partial fragment of Geminin is composed of 1st through 110th amino acids of said Geminin.

(2) The method as set forth in aspect 1, wherein the marker is a fluorescent protein or a luminescent protein.

(3) The method as set forth in any one of aspects 1 or 2, wherein the marker is detected over time by carrying out a time-lapse imaging observation on a living cell or a living tissue.

(4) A transformed cell or a transgenic nonhuman animal, coexpressing in a cell, (i) a first gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Cdt1 which is remaining of Cdt1 from which a Geminin binding site is excluded and (ii) a second gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Geminin which is remaining of Geminin from which a Cdt1 binding site is excluded, the markers being different from each other, wherein

- the gene encoding a partial fragment of Cdt1 which is remaining of Cdt1 from which a Geminin binding site is excluded is a gene encoding a partial fragment of Cdt1 composed of 30th through 120th amino acids of said Cdt1, and wherein
- the gene encoding a partial fragment of Geminin which is remaining of Geminin from which a Cdt1 binding site is excluded is a gene encoding a partial fragment of Geminin composed of 1st through 110th amino acids of said Geminin.

(5) The transformed cell or the transgenic nonhuman animal as set forth in aspect 4, wherein:

- the gene encoding the marker is a gene encoding a fluorescent protein or a luminescent protein.

(6) A method for in vitro screening a cell-cycle inhibitor comprising the steps of:

- incubating a cell in the presence of a candidate substance for the cell-cycle inhibitor, and
- performing phase identification of a cell cycle in accordance with a method as set forth in any one of aspects 1 through 3, so as to select a candidate substance for inducing cell cycle arrest.

(7) A method for performing phase identification of a cell cycle as set forth in aspect 1, wherein:

- the marker for the first gene-expression product is a red fluorescent protein, and the marker for the second gene-expression product is a green fluorescent protein.
(8) A transformed cell or a transgenic nonhuman animal as set forth in aspect 4, wherein the one of the markers encoded by the first gene construct is a red fluorescent protein and the other one encoded by the second gene construct is a green fluorescent protein.

(9) A probe for visualising cell cycle comprising:

- a first gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Cdt1 which is remaining of Cdt1 from which a Geminin binding site is excluded; and
- a second gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Geminin which is remaining of Geminin from which a Cdt1 binding site is excluded,

the markers being different from each other.

(10) The probe for visualising cell cycle as set forth in aspect 9, wherein the marker encoded by the first gene construct is a red fluorescent protein and the marker encoded by the second gene construct is a green fluorescent protein.

Advantageous Effects of Invention

[0013] With the present invention, it is possible to easily distinguish a proliferation phase of a cell cycle from a resting phase thereof in real-time. The present invention provides a probe, by use of which G1/S transition (transition from a G1 phase to an S phase, which transition is the most important in considering control of a cell cycle) can be detected as a color conversion from a red color to a green color (a contrast between red and green colors is higher than a contrast between any other colors). In particular, because the method of the present invention does not use cell-cycle dependent gene transcription (promoter), a permanent promoter can be used, thereby making it possible to easily produce a transgenic organism. Furthermore, the present invention is arranged so that a signal for a cell cycle is localized in a nucleus, thereby making it possible that the probe of the present invention be expressed at the same time with various fluorescent probes working in a cytoplasm. The present invention makes it possible to study how the cell cycle is coordinated with various cell functions.

Brief Description of Drawings

[0014] Fig. 1
Fig. 1 is a graph showing a result obtained by comparing mKO and mKO2 in terms of fluorescent brightness.

Fig. 2
Fig. 2 shows images of a HeLa cell stably expressing Fucci, in which images a nucleus of the HeLa cell shows a fluorescent pattern corresponding to cell-cycle progression.

Fig. 3
Fig. 3 shows an experiment in which a mechanism of and performance assessment of a Fucci probe are studied.

Fig. 4
Fig. 4 shows monitoring results of behavioral changes of and cell-cycle dynamics of the cell stably expressing Fucci.

Fig. 5
Fig. 5 shows results obtained by monitoring, by marking with the Fucci probe, cell-cycle progression of a tumor cell in a live mouse.

Fig. 6
Fig. 6 shows results of cell-cycle analysis of generation of a neural tissue in a Fucci transgenic mouse.

Fig. 7
Fig. 7 shows results of a time-lapse imaging experiment in which a slice of brain primordium of an E13 Fucci transgenic mouse embryo is used.

Fig. 8
Fig. 8 shows images of a PC12 cell stably expressing next-generation Fucci (mCherry-Cdt1#10 and mVenus-Geminin#2).

Fig. 9
Fig. 9 shows images of a HeLa cell stably expressing a next generation Fucci (mCherry-Cdt1#10 and mCGFP-Geminin#2).

Fig. 10
Fig. 10 shows results of screening anticancer agents by using Fucci probe.
A method for performing phase identification of a cell cycle includes: visualizing, by marking with a marker, at least one or more gene-expression products whose amounts in a cell change in a cell-cycle dependent manner; and detecting the marker so as to distinguish a proliferation phase of a cell cycle from a resting phase thereof.

In the present specification, a G1 phase is a prereplicative resting-phase (which is simply referred to as a "resting phase" herein), and an S/G2/M phase is a DNA synthesis/cell division phase (which is simply referred to as a "proliferation phase" herein).

It is disclosed but not part of the invention that at least one or more gene-expression products above be at least two or more gene-expression products. Its cell change in cell-cycle dependent manners different from one another. It is particularly preferable that at least one or more gene-expression products above, a gene-expression product whose amount in a cell changes in the G1 phase and the S/G2/M phase be used. In specific, as at least one or more gene-expression products above, (i) a first gene-expression product whose amount increases in the G1 phase and decreases in the S/G2/M phase and (ii) a second gene-expression product whose amount decreases in the G1 phase and increases in the S/G2/M phase can be used.

Concrete examples, not part of the invention, of the first gene-expression product whose amount increases in the G1 phase and decreases in the S/G2/M phase encompass p27, p57, p21, p130, Cyclin A, Cyclin D, Cyclin E, CDK9, MYC, E2F1, ORC1, CDT1, B-MYB, RAG2, SMAD4, FOXO1, UBP43, Viral E7, Notch1, Notch 4, JUN, Presenilin 1/2, SREBP 1/2, β-catenin, Viral Vpu, ATF4, D1G1, INFα-R, PRL-R, Snail, PER 1/2, Claspin, and the like. Among them, Cdt1 or a partial fragment thereof. The invention is limited to a partial fragment of Cdt1 being a partial fragment obtained by removing a Geminin binding site from Cdt1.

Concrete examples, not part of the invention, of the gene-expression product whose amount decreases in the G1 phase and increases in the S/G2/M phase encompass Cyclin A, Cyclin B, CDC20, PLK1, Aurora A/B, NEK2A, mE2-C, Geminin, CDC6, SKP2, SNON, RRR2, TK1, TPX2, CDH1, Securin, KIP, Survivin, Dbf 4, Hsl 1, Sgo 1, Sororin, R2, UBC10/E2-C/Vihar, Cks 1, Ase 1/Prc 1, Cin 8, Anillin, and the like. Among them, Geminin or a fragment thereof. The invention is limited to a partial fragment of Geminin being a partial fragment obtained by removing a Cdt1 binding site from Geminin.

The gene-expression products can be visualized by being labeled with a marker. In a case where two or more gene-expression products are used, they are labeled with different markers, respectively, so as to be visualized. For the marker to be used, a fluorescent protein or a luminescent protein is preferable. It is preferable that the fluorescent protein or the luminescent protein to be used be a protein which (i) quickly acquires fluorescence activity or luminescence activity and (ii) is degraded along with degradation of a protein (the gene-expression product) to which the fluorescent protein or the luminescent protein (marker) is fused, and rapidly loses the fluorescence activity or the luminescent activity.

As the fluorescent protein, a variety of fluorescent proteins are cloned typically from a cnidarian, hydrozoa, Aequorea-victoria derived AequoreaGFP (Green Fluorescent Protein), and also from other organisms such as anthoza (coral and actinia), hydrozoa other than Aequorea Victoria, anthropod and crustacea, and the like. Further, as the fluorescent protein, a variant obtained by inducing a mutation into any of the above fluorescent proteins is reported.


The description of the fluorescent protein and the luminescent protein usable are shown below. However, neither the fluorescent protein nor the luminescent protein usable is limited to the examples.

### Table 1

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<td>Midoriishi-Cyan (MiCy)</td>
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Table 2 (continued to the next page.)

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There is herein described a gene construct which includes (i) a gene for or a partial fragment of a gene for an expression product whose amount in the cell changes in a cell-cycle dependent manner and (ii) a gene encoding the marker. It is possible that the gene construct be used as a probe which uses protein degradation occurring in a cell-cycle dependent manner. The gene construct can be constructed as follows: obtaining, in accordance with normal gene-recombination technologies, (i) the gene for or the partial fragment of the gene for the expression product whose amount in the cell changes in a cell-cycle dependent manner and (ii) the gene encoding the marker; and subsequently linking the gene for or the partial fragment of the gene for the expression product and the gene encoding the marker.

For example, a gene encoding a fluorescent protein can be obtained by: providing (i) a template DNA containing cDNA of the fluorescent protein and (ii) a primer corresponding to a DNA sequence of the fluorescent protein; and carrying out PCR by use of the template DNA and primer. Likewise, a gene for or a fragment of a gene for an expression product whose amount in a cell changes in a cell-cycle dependent manner can be obtained by: providing a primer corresponding to a DNA sequence of the gene; and carrying out PCR by use of the primer. In a case where a restriction enzyme site is introduced into the primer for use in amplifying a gene fragment by PCR, it is possible to insert an amplified product obtained by the PCR into a corresponding restriction enzyme site in a proper vector. By inserting, adjacenty

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<tr>
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<td>HcRed1</td>
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<tr>
<td></td>
<td>HcRed1-tandem</td>
<td>EVRGEN</td>
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<tr>
<td></td>
<td>KFP1 (KFP-Red)</td>
<td>EVRGEN</td>
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<td>asCP / A148S</td>
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<td></td>
<td>AsRed2</td>
<td>Clontech (TAKARA)</td>
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<tr>
<td></td>
<td>AQ143</td>
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<tr>
<td></td>
<td>Keima</td>
<td>MBL (Amalgaam)</td>
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Table 5

<table>
<thead>
<tr>
<th>Name of Luminescent Protein</th>
<th>Biological origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase (Luc)</td>
<td>firefly</td>
</tr>
<tr>
<td></td>
<td>Renilla</td>
</tr>
<tr>
<td></td>
<td>click beetle</td>
</tr>
</tbody>
</table>

[0024] There is herein described a gene construct which includes (i) a gene for or a partial fragment of a gene for an expression product whose amount in the cell changes in a cell-cycle dependent manner and (ii) a gene encoding the marker. It is possible that the gene construct be used as a probe which uses protein degradation occurring in a cell-cycle dependent manner. The gene construct can be constructed as follows: obtaining, in accordance with normal gene-recombination technologies, (i) the gene for or the partial fragment of the gene for the expression product whose amount in the cell changes in a cell-cycle dependent manner and (ii) the gene encoding the marker; and subsequently linking the gene for or the partial fragment of the gene for the expression product and the gene encoding the marker.

[0025] For example, a gene encoding a fluorescent protein can be obtained by: providing (i) a template DNA containing cDNA of the fluorescent protein and (ii) a primer corresponding to a DNA sequence of the fluorescent protein; and carrying out PCR by use of the template DNA and primer. Likewise, a gene for or a fragment of a gene for an expression product whose amount in a cell changes in a cell-cycle dependent manner can be obtained by: providing a primer corresponding to a DNA sequence of the gene; and carrying out PCR by use of the primer. In a case where a restriction enzyme site is introduced into the primer for use in amplifying a gene fragment by PCR, it is possible to insert an amplified product obtained by the PCR into a corresponding restriction enzyme site in a proper vector. By inserting, adjacenty
into a same vector, (i) the gene for or the partial fragment of the gene for the expression product whose amount in the cell changes in the cell-cycle dependent manner and (ii) the gene encoding the marker, it is possible to produce the gene construct including the gene for or the partial fragment of the gene for the expression product above and the gene encoding the marker.

[0026] A vector for use in the method described herein is not limited to a particular kind. For example, the vector can be a vector (e.g., plasmid or the like) which autonomously replicates itself or a vector which is, when being introduced into a host cell, integrated into a genome of the host cell and replicated together with a chromosome of the host cell. Preferably, the vector for use is an expression vector. In the expression vector, a gene is arranged in such a way that elements (e.g., a promoter or the like) necessary for transcription are functionally linked to the gene. The promoter is a DNA sequence showing transcription activity in the host cell, and can be selected as appropriate based on a kind of the host cell.

[0027] The gene construct obtained in the above way is introduced into a cell, and (i) the gene for or the partial fragment of the gene for the expression product above and (ii) the marker, is expressed. By detecting the marker, it is possible to distinguish the proliferation phase of the cell cycle from the resting phase thereof, thereby making it possible to perform phase identification of the cell cycle.

[0028] Construction of the expression vector and introduction (transfection) thereof into a cell can be carried out in accordance with methods well known to a person skilled in the art. The details are described in "Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY" (Sambrook, et al. 1989) and "Current Protocols in Molecular Biology" (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., most recent Supplement) (F.M. Ausubel, et al., eds.).

[0029] For example, transfection can be carried out in accordance with a DNA transfection method such as a calcium phosphate coprecipitation method, microinjection, electroporation, insertion of a plasmid which has been introduced into a liposome or a virus vector, or the like.

[0030] The method for performing phase identification of a cell cycle can be applied in, for example, a method for screening a drug (such as a cell-cycle inhibitor, a drug for a cell-cycle related disease, or the like drug). In the method, phase identification of a cell cycle is performed onto a cell incubated in the presence of a drug candidate. This makes it possible to select a drug candidate having an influence on the cell cycle, thereby making it possible to screen the cell-cycle inhibitor, the drug for the cell-cycle related disease, or the like.

[0031] Furthermore, there is described herein a transformant containing the gene construct. By introducing the gene construct into a proper host cell, it is possible to produce the transformant. The host cell is not particularly limited in a kind, and examples of the host cell can encompass a bacterial cell, a yeast cell, a fungal cell, a higher eukaryotic cell, and the like cell. Examples of the bacterial cell can encompass Gram-positive bacteria, such as bacillus, streptomycyes, and the like, and Gram-negative bacteria, such as Escherichia coli and the like. Examples of a mammalian cell can encompass an HEK293 cell, a HeLa cell, a COS cell, a BHK cell, a CHL cell, a CHO cell, and the like cell. A method for transforming a mammalian cell and expressing a DNA sequence introduced into the mammalian cell is also well known, and usable examples of the method encompass an electroporation method, a calcium phosphate transfection method, a lipofection method, and the like method. Examples of the yeast cell can encompass cells belonging to saccharomyces and those belonging to schizosaccharomyces, and examples of such cells can encompass Saccharomyces cerevisiae, Saccharomyces kluveri, and the like. Examples of a method for introducing a recombinant vector into a yeast host can encompass an electroporation method, a spheroplast method, a lithium acetate method, and the like method. An example of other fungal cells is filamentous fungus, which is a cell belonging to, for example, Aspergillus, Neurospora, Fusarium, or Trichoderma. In a case where the host cell is one of the filamentous fungus, transformation can be performed by integrating a DNA construct into a host chromosome so as to produce a recombinant host cell. Integration of the DNA construct into the host chromosome can be carried out in a well known manner by use of, for example, homologous recombination or heterologous recombination.

[0032] Furthermore, there is described herein a transgenic nonhuman animal having the above gene construct.

[0033] A method for producing the transgenic nonhuman animal is not particularly limited. For example, the transgenic nonhuman animal can be produced by introducing the gene construct into a fertilized egg or the like. It is preferable that the gene construct, which is used as a transgene in producing the transgenic nonhuman animal, be a recombinant gene produced by linking (i) the gene for or the partial fragment of the gene for the expression product whose amount in a cell changes in a cell-cycle dependent manner and (ii) the gene encoding the marker to a downstream of a proper mammalian promoter. The transgenic nonhuman animal can be produced by, for example, (i) introducing the gene construct into a fertilized egg of a nonhuman animal and (ii) implanting the fertilized egg into a pseudopregnant female nonhuman animal, so as to deliver a nonhuman animal in which the gene construct has been introduced. Usable examples of the nonhuman animal are a rodent such as a mouse, a hamster, a guinea pig, a rat, a rabbit, and the like, and an animal such as a dog, a cat, a goat, a sheep, a bovine, a pig, a monkey, a zebrafish, a drosophila, and the like. In view of easiness of production, development, use, or the like, a rodent such as a mouse, a hamster, a guinea pig, a rat, a rabbit, and the like is more preferable, and among them, a mouse is the most preferable.
After it is confirmed that members of the transgenic animal stably possess the gene construct after being produced by breeding, the transgenic animal can be reared, as an animal having the gene, for generations in a normal rearing environment. A homozygous animal having a transgene in both pairs of a homologous chromosome is obtained, and a male and a female member of the homozygous animal are bred with each other. This makes it possible to breed the homozygous animal for generations in such a manner that every descendant excessively possesses the transgene. In order to identify a site where (i) the gene for or the partial fragment of the gene for the expression product whose amount in the cell changes in the cell-cycle dependent manner and (ii) the gene encoding the marker are expressed, expression of the transgene can be observed at each of an individual level, an organ level, a tissue level, and a cellular level.

Examples

Example 1: Construction of Plasmid

(1) Construction of mKO2-Cdt1

(A) Development of Fluorescence Emission Ability Quick-Acquiring Variant of Fluorescent Protein Monomer Kusabira-Orange (mKO)

In a case where a fluorescent protein is used as a reporter in detecting a cell cycle phase and/or a physiological signal in a cell, it is necessary that the fluorescent protein emit fluorescence immediately after being translated. If there is a significant time gap between (i) occurrence of an event intended to be visualized and (ii) acquisition of fluorescence emission ability by the fluorescent protein, a detection model itself may become useless. In view of this, for the fluorescent protein to be used as the reporter, a variant (i.e., fluorescence emission ability quick-acquiring variant) which quickly matures after translation to acquire the fluorescence emission ability was produced. The variant was produced from mKO, which was a monomeric variant of a fluorescent protein Kusabira-Orange (KO) isolated from Fungia scutaria of Scleractinia Corals. mKO is commercially available with product name "mKO1" from Medical & Biological Laboratories Co., Ltd. and Amalgaam, Inc.

(B) Production of Fluorescence Emission Ability Quick-Acquiring Variant By Introduction of Point Mutation

According to a predicted structure of mKO which is predicted based on its primary structure, several sites (points) of an mKO gene, which were seemingly capable of quickly acquiring fluorescence emission, were selected, and introduced with amino-acid substitution point mutation in such a manner that the fluorescent characteristic would be retained. Introduction of the amino-acid substitution point mutation was carried out by using an amino-acid substitution point mutation introduction primer and, as a template, an E. coli expression vector (pRSET B Invitrogen) inserted with the mKO gene. DNA thus introduced with the amino-acid substitution point mutation was treated in accordance with thermal cycling so that a template DNA was dissociated, the primer was annealed, and the primer elongation was performed repeatedly. Thus, the DNA was amplified. The primer used in amplification was phosphorylated at a 5' terminal.

(a) Phosphorylation of Primer at 5' terminal

(b) Point Mutation Introduction PCR

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>100 µM primer</td>
<td>2 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10× T4 polynucleotide kinase buffer</td>
<td>5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM AMP</td>
<td>0.5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterilized water</td>
<td>41.5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 polynucleotide kinase (10 U/µl)</td>
<td>1 µl</td>
<td></td>
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</tr>
<tr>
<td>5' phosphorylated primer mix</td>
<td>final 4 µl</td>
<td></td>
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</tr>
<tr>
<td>template (mKO-pRSET B)</td>
<td>100 ng</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10× polymerase buffer</td>
<td>2.5 µl</td>
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</tbody>
</table>
A sterilized water was provided in an amount so that a total of amounts would be 50 μl.

Program

A thermal cycler was GeneAmp PCR system 9700.

Reaction Condition:

Above 3 steps were carried out for 25 cycles.

75 °C 7 min (final elongation)
4 °C storing

(c) DpnI Treatment

Mixed-Base Notation: w=a/t, r=a/g, s=g/c, v=a/g/c, n=a/t/g/c

(d) Transformation into Escherichia coil

Escherichia coil JM109 (DE3) was transfected with the sample thus processed with the DpnI treatment, so as to produce a transformant. The transformant expressing a fluorescent protein was compared with one another in terms of a fluorescent intensity, so as to pick up a candidate clone. An expression vector plasmid of a picked-up clone was purified by using Wizard Plus SV Minipreps DNA Purification System (Promega). Then, by using the expression vector plasmid as a template, introduction of amino-acid substitution point mutation was repeatedly carried out, so as to evolve mKO. Purification of the expression vector plasmid was carried out in accordance with a protocol for the kit.
(e) Determination of Base Sequence of mKO Variant

A transformant clone which was eventually selected out was incubated. An expression vector plasmid of the transformant clone was purified by using Wizard Plus SV Minipreps DNA Purification System (Promega). Purification of the expression vector plasmid was carried out in accordance with a protocol for the kit. A base sequence of an mKO variant in the purified expression vector plasmid was analyzed, so as to determine an amino acid sequence. In analysis of the base sequence, BigDye Terminator ver.1 Cycle Sequencing Kit (Applied Biosystems) was used. The analysis of the base sequence was carried out in accordance with a protocol for the kit. A result was that lysine (K), which was 49th in an mKO amino acid sequence, was substituted with glutamic acid (E), proline (P), which was 70th in the mKO amino acid sequence, was substituted with valine (V), phenylalanine (F), which was 176th in the mKO amino acid sequence, was substituted with methionine (M), lysine (K), which was 185th in the mKO amino acid sequence, was substituted with glutamic acid (E), lysine (k), which was 188th in the mKO amino acid sequence, was substituted with glutamic acid (E), serine (S), which was 192th in the mKO amino acid sequence, was substituted with aspartic acid (D), serine (S), which was 196th in the mKO amino acid sequence, was substituted with glycine (G), and leucine (L), which was 210th in the mKO amino acid sequence, was substituted with glutamine (Q). A variant of the above sequence was named mKO2. The base sequence of a fluorescent protein mKO2 is shown by SEQ ID 20 herein, and an amino acid sequence thereof is shown by SEQ ID 21 herein.

(f) Analysis of Fluorescent Characteristic

A recombinant protein of mKO2 fused with His-Tag, was expressed by using Escherichia coli, and then purified by using Ni-NTA Agarose (QIAGEN). Purification of the recombinant protein was carried out in accordance with a protocol for the kit. Respective absorbing spectra of solutions of 20 μM fluorescent protein, 150 mM KCl, and 50 mM HEPES-KOH pH7.4 were measured with a spectrophotometer (U-3310 HITACHI), and molar absorption coefficients were worked out from peak values of the respective absorbing spectra. mKO and mKO2 were dissolved into the 150 mM KCl and the 500 mM HEPES-KOH pH7.4 solution in such a manner that an absorbing value at 500 nm would be 0.005. Respective fluorescence spectra of mKO and mKO2 excited by light at 500 nm were measured with a fluorescence spectrophotometer (F-2500 HITACHI), and areas of the respective fluorescence spectra were worked out. Fluorescence quantum yield of mKO was set to 0.6, and fluorescence quantum yield of mKO2 was worked out according to a ratio of the areas. In order to work out pH sensitivity (pKa) of mKO2, 2 μl of an mKO2 protein solution (19.1 mg/ml) was added into 100 μl of the 100 mM buffer liquid, and then measured for an absorbing spectrum.

Buffer solutions of respective pH were as follows:

- pH4, 5: acetic acid buffer solution
- pH6: phosphate buffer solution
- pH7, 8: HEPES buffer solution
- pH9, 10: glycine buffer solution.

Table 6 shows comparison of the fluorescent characteristic of mKO and that of mKO2. Because an absolute brightness of a fluorescent protein molecule was expressed by a formula "molar absorption coefficient × quantum yield", an absolute brightness of mKO was 51600 × 0.6 = 30.9k, and that of mKO2 was 63800 × 0.57 = 36.3k. Thus, the absolute brightness of the fluorescent protein mKO2 was approximately 1.2 times brighter than that of mKO.

<table>
<thead>
<tr>
<th></th>
<th>Excitation Maximum</th>
<th>Fluorescent Maximum</th>
<th>Molar Absorption Coefficient</th>
<th>Quantum yield</th>
<th>pH Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mKO</td>
<td>548 nm</td>
<td>559 nm</td>
<td>51600 (548 nm)</td>
<td>0.60</td>
<td>pKa = 5.0</td>
</tr>
<tr>
<td>mKO2</td>
<td>551 nm</td>
<td>565 nm</td>
<td>63800 (551 nm)</td>
<td>0.57</td>
<td>pKa = 5.5</td>
</tr>
</tbody>
</table>

(g) Evaluation of mKO2 Expressed in Incubated Cell

A fluorescent protein expression vector plasmid pmKO1-MN1 (Medical & Biological Laboratories Co., Ltd.), an mKO (mKO1) gene site was cut out by using restriction enzymes NotI and Xbal. Subsequently, the fluorescent protein expression vector plasmid pmKO1-MN1 was inserted alternatively with an mKO2 gene which was ligated with an NotI site at the 5’ end and an XbaI site at the 3’ end. Ligation of the restriction enzyme site sequences was carried out by a PCR. A thermal cycler was GeneAmp PCR system 9700.
Composition of PCR Reaction Liquid

[0050]

<table>
<thead>
<tr>
<th>5</th>
<th>Template (mKO2-pRSET B) 1 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>×10 pfu buffer 5 µl</td>
</tr>
<tr>
<td>10</td>
<td>2.5 mM dNTPs 3 µl</td>
</tr>
<tr>
<td>10</td>
<td>20 µM forward primer 1 µl</td>
</tr>
<tr>
<td>15</td>
<td>20 µM reverse primer 1 µl</td>
</tr>
<tr>
<td>20</td>
<td>DMSO 5 µl</td>
</tr>
<tr>
<td>20</td>
<td>Mili-Q 33 µl</td>
</tr>
<tr>
<td>25</td>
<td>pfu polymerase (2.5 U/µl) 1 µl</td>
</tr>
</tbody>
</table>

PCR Reaction Condition

[0051]

| 94 °C 1 min (PAD) |
| 94 °C 30 sec (denaturation) |
| 52 °C 30 sec (annealing of a primer to a template) |
| 72 °C 1 min (primer elongation) |

[0052] Above 3 steps were carried out for 30 cycles.

72 °C 7 min (final elongation)

4 °C storing

Forward primer

[0053]

5’- ataagaatgctgcccgggaccatggtgattgtgattaaccagag -3’ (SEQ ID 9)

Reverse primer

[0054]

5’- cgctagattagagtctgtcgcttccttccttacca -3’ (SEQ ID 10)

[0055] Approximately 700 bp of an amplified band obtained by 1 % agarose gel electrophoresis was cut out. The amplified band was then purified and subcloned into pmKO1-MN1 treated with the restriction enzymes Ntol and XbaI, so as to produce pmKO2-MN1. pmKO1-MN1 and a constructed expression vector plasmid pmKO2-MN1 were used in comparing fluorescent emission ability of mKO (mKO1) and that of mKO2 in a HeLa cell.

[0056] It was prepared such that HeLa cells in 35-mm glass bottom dishes would be 40 % confluent. The HeLa cells were then introduced with pmKO1-MN1 or pmKO2-MN1 by use of a gene induction reagent, polyfect (QIAGEN), so as to express fluorescent proteins. Setting of a cell-culture condition and induction of the genes were carried out in accordance with a protocol for polyfect. 1 µg of the respective expression vector plasmids was used per 35-mm glass bottom dish.

[0057] In a time sequential manner, fluorescence images were acquired, and fluorescent brightness was compared to one another. The fluorescent images were acquired by use of an excitation filter 25BP520-540HQ, a fluorescent filter 25BA555-600HQ, and a dichroic mirror DM545HQ. For an excitation light, a xenon light source was used. Light from the xenon light source was irradiated for 0.5 second while 70 % of the light was cut off (30 % transmission). A microscope was an inverted microscope IX-71 (Olympus Co., Ltd), and a lens was 20× Uapo/340 N.A. 0.75 (Olympus Co., Ltd). For image acquisition and analysis, Metamolph (Nippon Roper Co., Ltd) was used in a mode set to binning 2. The fluorescence images were acquired with a cooling CCD camera ORCA-ER (Hamamatsu Photonics K.K).

[0058] After 6, 8, and 10 hours of introduction of the expression vector plasmids, fluorescence images were acquired,
and fluorescence brightness was plotted by working out average brightness per cell (see Fig. 1). Because a model was not capable of controlling the expression, there were unevenness in a time length from intake of the expression vector plasmids to expression of the fluorescent proteins. However, a result was that clearly, (i) mKO2 acquired the fluorescence emission ability in a shorter time period and (ii) mKO2 emitted fluorescence two times or more brighter than did mKO1. Thus, a fluorescence emission ability quick-acquiring variant mKO2 was used in subsequent experiment.

(c) Construction of mKO2-Cdt1

[0059] Fluorescent protein mKO2 was amplified by a PCR in which primers 1 and 2 described below were used. Then, the amplified mKO2 was introduced into an EcoRI-EcoRV site of a pcDNA3 vector. Subsequently, a fragment of Cdt1 (Genbank Accession No.; NM_030928) was amplified by a PCR in which primers 3 and 4 described below were used in combination (by combinational use of the primers 3 and 4, a fragment corresponding to 30th to 100th amino acids of Cdt1 was amplified), or by a PCR in which primers 3 and 5 described below were used in combination (by combinational use of the primers 3 and 5, a fragment corresponding to 30th to 120th amino acids of Cdt1 was amplified). Then, the fragment of Cdt1 was introduced into an Xho I-XbaI site. Transferring into a lentivirus vector (CSII-EF-MCS) was carried out by using the EcoRI-XbaI site. Respective PCRs were carried out in the following condition.

Reaction liquid:

[0060] Template DNA; 1 to 10 ng/1 \( \mu \)l
10× polymerase buffer 10 \( \mu \)l
2.5 mM dNTP mix; 8 \( \mu \)l
forward primer (20 \( \mu \)M); 1 \( \mu \)l
reverse primer (20 \( \mu \)M); 1 \( \mu \)l
DMSO; 5 \( \mu \)l
pfu polymerase 2.5 U/\( \mu \)l 1 \( \mu \)l
Mili Q 73 \( \mu \)l

[0061] A thermal cycle was GeneAmp PCR system 9700.

Reaction Condition:

[0062] 94 °C 2 min
94 °C 1 min
50 °C 30 sec
72 °C 1. 5 min

repeat the above 3 reactions for 28 cycles, and subsequently

[0063] 72 °C 7 min
4 °C storing

(2) Construction of mAG-Geminin

[0064] Fluorescent protein mAG was a monomeric variant of Azami Green (AG) isolated from Galaxea fascicularis of Scleractinia Corals, and commercially available, as a product name mAG1, from Medical and Biological Laboratories Co., Ltd and Amalgaam, Inc.

[0065] mAG was amplified by a PCR in which primers 6 and 7 described below were used. Then, mAG was introduced into an EcoRI-EcoRV site of a pcDNA3 vector. Subsequently, a fragment of Geminin (Genbank Accession No.; NM_015895) was amplified by a PCR in which primers 8 and 9 described below were used (by use of the primer 8 and 9, a fragment corresponding to 1st to 110th amino acids of Geminin). Then, the fragment of Geminin was introduced
into an Xho I-XbaI site. Transferring into a lentivirus vector (CSII-EF-MCS) was carried out by using an EcoRI-XbaI site. Respective PCRs were carried out in the following condition.

Reaction liquid:

- Template DNA: 1 to 10 ng/1 μl
- 10× polymerase buffer: 10 μl
- 2.5 mM dNTP mix: 8 μl
- forward primer (20 μM): 1 μl
- reverse primer (20 μM): 1 μl
- DMSO: 5 μl
- pfu polymerase 2.5 U/μl: 1 μl
- Milli Q: 73 μl

A thermal cycle was GeneAmp PCR system 9700.

Reaction Condition:

- 94 °C 2 min
- 94 °C 1 min
- 50 °C 30 sec
- 72 °C 1.5 min

repeat the above 3 reactions for 28 cycles, and subsequently

- 72 °C 7 min
- 4 °C storing

Primer 1: mKO2 forward primer (M12-EcoN-F)

5'- ggg gaa ttc gcc acc atg gtg agt gtg att aaa cca gag (SEQ ID 11)

Primer 2: mKO2 reverse primer (m11-AGCter-EcoV-R)

5'- atg gat atc cgc cct ggg aag gca aca ttg agt aat gag cta ctg cat ctt cta c (SEQ ID 12)


5'- gcc ctc gag ccc agc ccc gcc agg ccc gca (SEQ ID 13)
Primer 4: Hu.Cdt (100) ter.XbaI (R):

[0071]

5'- gca tct aga tta ttt ctt tat ctt ctg gcc cgg aga (SEQ ID 14)

Primer 5: Hu.Cdt (120) ter.XbaI (R):

[0072]

5'- gca tct aga tta gat ggt gtc ctg gtc ctg cgc (SEQ ID 15)

Primer 6: mAG forward primer (hM12-EcoN-F):

[0073]

5'- ggg gaa ttc gcc acc atg gtg agc gtg atc aag ccc ga (SEQ ID 16)

Primer 7: mAG reverse primer (hM12-EcoV-R):

[0074]

5'- atg gat atc cct tgg cct ggc tgg gca gca t (SEQ ID 17)


[0075]

5'- gcc ctc gag atg aat ccc agt atg aag cag aaa c (SEQ ID 18)

Primer 9: Hu. Geminin (110) ter.XbaI (R):

[0076]

5'- gca tct aga tta cag cgc ctt tct ccg ttt ttc tgc (SEQ ID 19)

Example 2: Transfection and Imaging

Cell Culturing Method

[0077] HeLa cells and COS7 cells were incubated in a DMEM into which a 10 % fetal-bovine serum and penicillin/streptomycin were added. Mouse NMuMG mammary gland epithelial cells were incubated in a DMEM (high glucose) into which a 10 % fetal-bovine serum, penicillin/streptomycin, and a 10 mg/ml insulin (sigma) were added. EGF and TGFβ1 were purchased from R&D Co., Ltd.

Transfection

[0078] The HeLa cells were transfected with a gene construct produced in Example 1, in accordance with a lipofectin method. A specific procedure for transfection of the gene construct was as follows. On a 35-mm glass bottom dish, the HeLa cells were incubated in a phenol red-free Dulbecco's Modified Eagle Medium containing a 10 % bovine serum (FBS). A liquid A (which contained 1 μg of plasmid and 100 μl of an Opti-MEM) and a liquid B (which contained 4 μl of lipofectin and 100 μl of an Opti-MEM) were prepared separately, and mixed with each other, so as to prepare a liquid mixture thereof. The liquid mixture was then left for 15 minutes at room temperature. A culture supernatant of the HeLa cells incubated on the 35-mm glass-bottom dish in advance was replaced with an Opti-MEM. Into a culture medium of the HeLa cells, the liquid mixture of the liquids A and B was added. After 4 hours, a culture supernatant was replaced with a new culture medium.

[0079] After 1 to 2 days, imaging was carried out by using an incubator microscope (Olympus Co., Ltd. LCV 100) for 24 to 60 hours, so as to screen HeLa cells in each of which a fluorescent signal was emitted in a cell cycle-specific
manner in its nucleus. DIC images were acquired at Wavelength 1 (LED620 nm). An mKO2 fluorescent signal was collected at Wavelength 2 (ex: BP520-540HQ, em: BA555-600HQ). An mAG signal was collected at Wavelength 3 (ex: 470DF35, em: 510WB40).

Furthermore, after the transfection, the HeLa cells were incubated in a culture medium into which 500 µg/ml of G418 was added, and assessed for cytotoxicity by testing whether a clone of the HeLa cells could proliferate or not.

As a result, it was confirmed that in the case of transfection with (i) the fragment (hereinafter also referred to as mKO2-Cdt1#10) corresponding to 30th to 120th amino acids of Cdt1 and (ii) the fragment (hereinafter also referred to as mAG-Geminin#2) corresponding to 1 st to 110th amino acids of Geminin, a cell cycle-specific nuclear fluorescent pattern was acquired, and no cytotoxicity was generated.

Respective base sequences of mKO2-Cdt1#10 and mAG-Geminin#2 thus produced have been deposited as AB370332 and AB370333 in DDBJ database.

Example 3: Production of Lentivirus and Transduction into Cell

Lentiviruses of mKO2-Cdt1#10 and those of mAG-Geminin#2 were produced by use of HEK293 cells, and transduced into various cells. A specific procedure for transduction was as follows.

Production of Lentivirus

A procedure for production of the lentiviruses was developed by modifying a method developed by Dr. Miyoshi (Subteam for Manipulation of Cell Fate, Technology and Development for BioSignal Program, BioResource Center, RIKEN). Namely;

Liquid A;

- pCAG-HIVgp plasmid 10 µg
- pCMV-VSV-G-RSV-Rev plasmid 10 µg
- CSII-EF-MCS-mKO-Cdt1#10
- CSII-EF-MCS-mAG-Geminin#2 17 µg

Liquid B;

- Lipofectamine 2000 36 µl
- Opti-MEM 1.5 ml

The liquids A and B were prepared, and mixed with each other to produce a liquid mixture. The liquid mixture was then left at room temperature for 20 minutes. The HEK 293T cells were treated with trypsinization so as to be dissociated, and the number of the HEK 293T cells was counted. It was prepared such that the number of the HEK 293T cells would be $6 \times 10^6$ cells/5 ml. A 10 cm-dish was provided, in which 5 ml of a culture medium was poured and then the liquid mixture was added therein. Into this, 5 ml of the HEK 293T cells were added finally. The HEK 293T cells in the medium were incubated by an incubator under a condition of 5 % CO$_2$ at 37 °C. After 24 hours, the culture medium was replaced. After 2 days, a culture supernatant was collected so as to produce a virus fluid. The culture supernatant was centrifuged at a rotation speed of 3000 rpm for 5 minutes, so as to produce a supernatant which was then collected and dispensed. The supernatant was stored at -80 °C. To the HEK 293T cells, a new culture medium was provided. After 3 days, a culture supernatant was collected in a same manner and stocked as a virus fluid.

Transduction Into Cells

Arbitrary cells were provided. Into a culture supernatant of the cells adhering to or floating in a plastic dish, the virus fluid was added to roughly 30 to 300 µl/dish sized of 3.5 cm. After 2 to 3 days, when the cells were observed with
a fluorescent microscope, it was possible to observe that the cells transduced (the cells being infected by a virus in the virus fluid and thereby having a genome integrated with a gene of the virus) emitted fluorescence. After 1 week of co-transduction with two viruses having mKO-Cdt1#10 and mAG-Geminin#2, respectively, single cell cloning of the cells was started. In approximately 4 weeks, the single cell replicated to form a colony emitting two colors of fluorescence. The colony was then collected so as to produce indicator-expression cells. Fig. 2 shows results obtained by observing clones of HeLa_LV_mKO2-Cdt1#10 and mAG-Geminin#2 with the fluorescent microscope.

[0089] In accordance with the above manner, HeLa cells (cultured cells of human cervical cancer cells), HEK293 cells (cultured cells derived from human embryonic kidney cells), COS cells (cultured cells of monkey cells), CHO cells (cultured cells of a hamster), NMuMG cells (cultured cells of mouse cells), primary cultured cells of rat neuronal cells, primary cultured cells of mouse bone marrow-derived cells, and the like cells were co-transduced with two viruses (mKO2-Cdt1#10 and mAG-Geminin#2). Then, each of the cells thus co-transduced with genes of the respective viruses were imaged. It was observed that in each of the cells, fluorescence by mKO was localized in a cell nucleus in a resting phase (G1 phase), whereas fluorescence by mAG was localized in the cell nucleus in a proliferating phase (S, G2, and M phases). Those members of the PC12, COS cell, NMuMG cell which expressed both indicators were cloned by single cell cloning, so as to obtain several cell lines.

[0090] For concrete examples, Fig. 2 shows the results obtained from imaging the HeLa cells each being co-transduced with two kinds of lentiviruses (mKO2-Cdt1#10 and mAG-Geminin#2) and thus introduced with genes of the respective lentiviruses. A cell cycle period was presumably variable due to differences in cell density and/or serum concentration. Because green fluorescence rapidly disappeared just before the end of the M phase and red fluorescence became detectable in the early G1 phase, a small gap in fluorescence indicated existence of a newborn daughter cell. On the other hand, while the fluorescence was changed from a red color to a green color, red and green fluorescence overlap each other so as to yield a yellow color in the nucleus. In order to examine whether a timing of color change correlated with the onset of the S phase or not, transformants were pulse-labeled with BrdU for 5 minutes, and immunostained for BrdU immediately after pulse-labeling. C of Fig. 3 shows typical confocal images of the HeLa cells at G1/S transition and in the G1 phase, the S phase, the G2 phase and the M phase. Because all the HeLa cells emitting yellow fluorescence in their nuclei showed incorporation of BrdU, emergence of the green fluorescence was indicative of the onset of the S phase. Similar results were obtained from separate experiments in each of which the HeLa cells were immunostained for PCNA. Thus, the HeLa cells emitting yellow fluorescence in their nuclei showed incorporation of BrdU, emergence of the green fluorescence was indicative of the onset of the S phase. Similar results were obtained from separate experiments in each of which the HeLa cells were immunostained for PCNA. Thus, the HeLa cells emitting yellow fluorescence in their nuclei were observed. Such HeLa cells were either in the S phase or the G2 phase, and distinguishable by nuclear BrdU immunostaining or PCNA immunostaining. These results showed that mKO2-Cdt1#10 accumulated in the G1 phase, whereas mAG-Geminin#2 accumulated in the S/G2/M phase. Such fluorescent ubiquitination-based cell cycle indicators were named "Fucci". Analysis of DNA content by flow cytometry revealed that Fucci-expressing HeLa cells and parent HeLa cells show the same distribution (see D of Fig. 3, right). On this regard, differential profiling of cells at G1 phase and S/G2/M phase can be achieved by (i) sorting a population of cells emitting red, yellow, or green fluorescence and (ii) examining various cellular functions, such as gene expression and antigen surface expression.

Example 4: Immunocytochemical Cell Cycle Analysis

Analytical method

[0091] Fucci-expressing HeLa cells were incubated on a cover glass, and treated with BrdU (sigma) at 37 °C for 5 minutes. After being washed with PBS(-), the Fucci-expressing HeLa cells were fixed by 4 % PFA at 4 °C for 10 minutes, and then treated with 0.1 % TritonX-100/PBS(-) at room temperature for 5 minutes.

[0092] Antibodies used were goat anti-mouse IgG conjugated with Alexa Fluor 633 (Molecular Probe), mouse anti-BrdU mAb (Immunological Direct), and mouse anti-PCNA mAb (Dako). Images were acquired with FV 500 (Olympus Co., Ltd) confocal microscope system equipped with a 488-nm laser line (Ar), a 543-nm laser line (He/Ne), and a 633-nm laser line (He/Ne).

Flow Cytometry

[0093] Hoechst 33342 (56 μl of a 1 mg/ml stock) (DOJINDO Laboratories) was added to a 10-cm dish containing the parent HeLa cells or the Fucci-expressing HeLa cells. After incubation for 30 minutes, incubated cells were collected, and analyzed by using a BD™ LSR (Becton, Dickinson and Company Co., Ltd). mKO 2 and mAG were excited by a 488-nm laser line (Ar) and Hoechst 33342 was excited by a 325-nm laser line (He/Cd). Fluorescent signals were collected
Monitoring Result of Structural and Behavioral Changes and Cell-Cycle Dynamics of Cultured Cell

Epithelial-mesenchymal transition (EMT) is a fundamental morphogenetic process by which a mesenchymal cell is formed from an epithelium during embryonic development, wound repair, and tumor progression in a multicellular tissue. In vitro, EMT is characterized by dissolution of a cell-cell junction, cytoskeletal rearrangement, and an increased motility of a cultured cell. A specific stage of a cell cycle may be involved in the process. Actually, a transforming growth factor β (TGFβ) efficiently induces EMT in an AML-12 hepatocyte synchronized at the G1/S phase, but is inactive in the AML-12 hepatocyte synchronized at the G2/M phase. Further, an NMuMG cell undergoes EMT in response to the TGFβ.

In order to study the cell cycle progression during EMT, NMuMG cells which were transformed stably and expressed Fucci were examined.

Cells were dispersed on a cover glass. Subsequently, the cells proliferated and adhered to neighboring ones, so as to form a cluster (Fig. 4A, 1h). It was evidenced that these cells had high proliferation ability, by proliferation images in each of which cells emitting green fluorescence in their nuclei were dominant (Fig. 4A, 25h to 49h). However, at confluence, the cells emitting green fluorescence in their nuclei were not seen anymore, and replaced with those expressing red fluorescence in their nuclei (Fig. 4A, 73h). This indicated that the cells remained in the G1 phase. When a wound was introduced into the confluent monolayer (Fig. 4C, 1h), cells along the wound started emitting green fluorescence (C of Fig. 4, 13h, arrow). This indicated that the NMuMG cells were required to proliferate due to introduction of the wound. After 9 to 13 hours of the introduction of the wound, cells emitting green fluorescence in their nuclei appeared remarkably. Such a time delay of more than 8 hours was reproducibly observed in other similar wound healing experiments and was reminiscent of an 8-hour interval required for an NIH 3T3 cell to re-enter a cell cycle from a state of quiescence (Go) after the onset of a proliferation stimulus. Thus, it might be a case that the confluent NMuMG cells remained in the Go phase (A of Fig. 4, 85h). Next, same experiments were carried out in the presence of 1 ng/ml TGFβ.

Within 1 day of treatment with TGFβ, cells emitting green fluorescence in their nuclei were increased in number. This indicated that this ligand induced a G1/S transition (B of Fig. 4, 1h through 49h). Subsequently, each of the cells began to adopt a spindle-shaped, fibroblast-like morphology and high motility (B of Fig. 4, 49h). After 2 days of the treatment with TGFβ, the cells emitting fluorescence in their nuclei were decreased in number. This reflected a G1 arrest effect of TGFβ (B of Fig. 4, 49h to 85h). Thus, the cells treated with TGFβ spread without proliferation, in contrast with untreated NMuMG cells which were densely packed in a confluent monolayer. In addition, introduction of the wound did not result in proliferation, but rather in a further expansion of the cells (D of Fig. 4).

Example 5: Production of Transgenic Animal

Plasmids each prepared by incorporating mKO-Cdt1#10 and mAG-Geminin#2 into respective pCAGGS vectors were named pCAGGS_mKO-Cdt1#10 and pCAGGS_mAG-Geminin#2. By use of the respective plasmids, a fragment to be injected into a mouse egg cell was produced.

```
pCAGGS_mKO-Cdt1#10
or
pCAGGS_mAG-Geminin#2 20 μg/40 μl
10× H buffer 10 μl
H2O 50 μl
```

Restriction enzymes Sal I, Pst I, Pvu I

Reaction liquids above were mixed with each other, and incubation was carried out at 37 °C for 2 hours. Bands were extracted by electrophoresis, and then 3.2 Kb of a band was purified for mKO-Cdt1#10, while 3.3 kb of a band was purified for mAG-Geminin#2. Production of a transgenic mouse was outsourced to the Research Resource Center, Brain Science Institute, Riken. As a result of genotyping, 16 lines of transgenic mice having genes for mKO-Cdt1#10 and 8 lines of transgenic mice having genes for mAG-Geminin#2 were obtained.
Example 6: Cell-Cycle Progression of Tumor Cell in Live Mouse

Whole-Body Imaging of Mice

[0098] (i) Subcutaneous and intravenous injection of a cultured cell and (ii) whole imaging with OV100 (Olympus Co., Ltd) were carried out as described in a document by Hoffman, Yang, et al. (Nat. Protocol, 3, pp1429 - 1438. 2006). In order to visualize a blood vessel, Angio Sense-IVM750 (VisEn Medical, Inc) was injected, or an endothelial cell was stained by using anti-CD31 mAb (Chemicon, Inc).

[0099] By use of a CAG promoter, transgenic mouse lines expressing mKO2-Cdt1#10 were produced. Of the 16 lines of transgenic mice emitting Red fluorescence, #596 was chosen for further characteristic analysis. Further, 8 lines of green fluorescence mAG-Geminin#2 mice were produced, of which #504 was chosen for further characteristic analysis. Such mouse lines provided an unprecedented model with which to study coordination of a cell cycle and development. #504 was particularly useful because it provided in vivo information on a proliferation pattern. During early development of a mammalian cerebral cortex, neural progenitors in a ventricular zone (VZ) continuously proliferated. In order to determine whether mAG-Geminin#2 green fluorescence was emitted by the neural progenitors, a #504 transgenic mouse embryo aged an embryonic day (E) 14 was treated with immunohistochemistry on telencephalic sections. Because telencephalic cells having green nucleus were immunopositive for Nestin but not MAP 2, these cells could be neural progenitors.

[0100] The #596 transgenic mouse and the #504 transgenic mouse were crossbred with each other so as to generate a mouse line producing Fucci in which every somatic cell nucleus exhibited either red or green fluorescence.

[0101] E13 Fucci (#596/#504) embryos were perfused transcardially with a fixative (4 % PFA), placed in an ice-cold fixative for 2 hours, cryoprotected in a PBS containing 20 % sucrose, and embedded in an OCT compound. Coronal head sections (each of which had a thickness of 15 μm) were imaged by using FV100 equipped with two laser diodes (473 nm and 559 nm). Images were put together so as to create wide-filed pictures. Brain sections from an E14/#504 embryo was fixed, and incubated with mouse antiMAP2 mAb (Chemicon, Inc) or mouse anti-Nestin mAb (PharMingen, Inc). The brain sections from the E14/#504 embryo was then incubated with goat anti-mouse IgG conjugated with Alex-aFluor 546X (Molecular Probes, Inc).

Imaging Method of Cultured Brain Slice

[0102] Brain slices were prepared from Fucci-expressing mice (#596/#504) at E13, and cultured in a collagen gel as described in Miyata et al. (J Neurosci. Res. 69, pp861-868. 2002). The brain slices were exposed to 5 % CO2 and 40 % O2. 3D imaging was carried out in an xyz-t mode by using FV1000 multiposition stage system. A recording interval was 10 minutes. At each time point, 20 confocal images along a z-axis (2 μm step) were acquired. In order to avoid crossdetection of green and red fluorescence signals, the images were sequentially acquired at 488 nm (Ar) and 543 nm (He/Ne). Green and red fluorescence images were merged for each confocal image. Image registration and proper alignment of FV1000 equipped with the two laser lines and a detection channels were verified by using double-labeled fluorescent beads (TetraSpeck Fluorescent Microsphere Standards, diameter of 0.5 μm, Molecular Probes, Inc). Data analysis was carried out by using Volocity software (Improvision, Inc) and METAMORPF software (Universal Imaging, Media, PA).

Monitoring Result of Cell-Cycle Progression of Tumor Cell in Live Mouse

[0103] Whole-body imaging and intravital cellular imaging of mice injected with cultured tumor cells genetically labeled with fluorescent proteins were powerful techniques for investigating tumor development. Fucci-expressing NMuMG cells were subcutaneously injected into mammary glands of nude mice, so as to use Fucci in monitoring tumor development (A of Fig. 5). After 1 day of injection, both cells emitting green fluorescence and cells emitting red fluorescence were observed (B of Fig. 5). After 16 days, however, only the cells emitting red fluorescence were seen (C of Fig. 5). This indicated that NMuMG cells stopped proliferating. Next, Fucci-expressing HeLa cells were injected into nude mice in a similar manner (D of Fig. 5). Each of the injected HeLa cells grew gradually, and stably emitted either green or red fluorescence, thereby indicating tumor progression (E and F of Fig. 5). After 27 days of the injection, expanded mass was observed through a skin under a microscope (Olympus Co., Ltd. IV100, 10x, UplanFL N N.A.=0.30) (G of Fig. 5). Well-developed tumor mass was visualized by being loaded with AngioSense 750, which emitted far-red fluorescence. Although triple-color live imaging identified HeLa cells in the G1 phase and the S/G2 phase, their positions relative to the tumor mass were not clear due to a low spatial resolution. The tumor was fixed, sectioned, and stained with an
Next, cell-cycle progression of tumor cells was examined during initial steps of a classic metastatic cascade, such as (i) adhesion to endothelial cells and (ii) extravasation and metastasis. Fucci-expressing HeLa cells in a gel were injected into a skin vein, and intravital cellular imaging was carried out. Interestingly, at early stages, nearly all of the HeLa cells attached to an inner wall of veins were in the G_1 phase (I and J of Fig. 5). A cell in the process of extravasation and metastasis was imaged (K, L, and M of Fig. 5). Within a cluster of HeLa cells across a vein wall, an elongated cell emitting yellow fluorescence in a fragmented nucleus was observed to pass through the vein wall. After 4 days of injection, HeLa cells were found to invade tissues and proliferate therein (N and O of Fig. 5). This indicated that extravasation and metastasis were repeated plural times.

Previous work showed that cultured cells with differentially labeled cytoplasm and nuclei, which cultured cells were to be injected into mice, could be used in imagining nuclear-cytoplasmic dynamics in order to monitor cancer cell tracking, cell death in live mice, deformation, extravasation and metastasis, and mitosis. In combination with such cytoplasmic labeling techniques, fluorescence imaging of Fucci-expressing cells which are stably transformed and introduced into live animals will provide reliable pharmacodynamic readouts for growth and metastatic behavior of tumors.

Result of Cell-Cycle Analysis of Developing Neural Tissue in Fucci Transgenic Mouse

One major advantage of a genetically encoded probe is that it need not depend on transcriptional regulation; its transcription can be driven by using constitutive promoters. Thus, transgenic organism for cell-cycle analysis can be easily generated. An E 13 Fucci transgenic mouse embryo was fixed, and coronal sections of its brain were prepared. Red or green fluorescence was examined in every section by using confocal scanning microscope. A, E, and I of Fig. 6 show fluorescence images of three representative sections. Red and green signals appeared to be well balanced at an embryonic stage, but an overall ratio of green-to-red signal decreased as the mouse grew.

In a developing cerebral cortex, (B, F, G, and J of Fig. 6), nuclei emitting red mKO2-Cdt1#10 fluorescence were identified in two main cell populations: which were post-mitotic neurons capable of populating different layers in a cortical plate (CP), and mitotic neural progenitors in a VZ. The post-mitotic neurons exhibited much brighter red fluorescence, probably due to accumulation of mKO2-Cdt1#10 after cell-cycle exit. Nuclei in blood vessels exhibiting bright red were visualized in the VZ (B and F of Fig. 6). In a diencephalon, there was a stripe of cells in the G_1 phase, which stripe of cells corresponded to zona limitans intrathalamica (ZLI). A dorsal thalamus contained more green nuclei than did a ventral thalamus (I and J of Fig. 6). This suggested that cells in a ventral region undergo cell-cycle exit for differentiation prior to those in a dorsal region.

A differential intensity of red fluorescence between mitotic cells and post-mitotic cells was observed also in a developing neuroepithelia of an olfactory system and a vomeronasal system (C and D of Fig. 6, respectively) as well as a developing neuroepithelia of a retina (H of Fig. 6). Random distribution of high- and low-intensity fluorescent nuclei might suggest that respective architectures of an olfactory epithelium and a vomeronasal epithelium were not yet established at E13. In contrast, bright red nuclei were observed in a central apical region of a developing retina (H of Fig. 6), whose developing retina ganglion cells were to undergo centrifugal differentiation. Epithelial cells of a lens had also exited the cell cycle by this stage. Other extra-neural tissues with bright red fluorescence included a trigeminal ganglion (k of Fig. 6) and a pituitary gland (L of Fig. 6).

Geminin and Cdt1 are previously shown to be abundantly expressed by neural progenitors during early mouse neurogenesis, but transcriptionally downregulated at late development stages. Fucci signal is not affected by transcriptional regulation in transgenic mice.

In the developing cerebral cortex, some neural progenitors exit the cell cycle and migrate beyond the VZ, where they differentiate into neurons or, at later stages, into ganglion cells. Neural progenitors also undergo a typical migration pattern within the VZ; their nuclei undergo characteristic movements, known as interkinetic nuclear movements. Nuclei in the M phase are localized on the ventricular surface, whereas nuclei in the S phase migrate to the ventricular zone. In order to observe spatial and temporal regulation of proliferation, differentiation, and migration of neural progenitors, a time-lapse imaging experiment was carried out in which slices of a dorsal telencephalon prepared from an E13 Fucci transgenic mouse embryo were used (A of Fig. 7). The time-lapse imaging experiment in which cortical slices are used is usually acquired at 3 hours or longer intervals. With such long intervals, neither nuclear movements nor cell-cycle progression can be monitored adequately. However, the bright Fucci fluorescence enables 3D time-lapse imaging with 10 minutes intervals in an xzy-t mode by using FV1000 multiposition stage system. At each time point, 20 confocal images along a z-axis (2 μm step) were acquired. In addition, exposure of slices to 40 % oxygen (instead of usual exposure to 20 %) had significantly improved cell proliferation, differentiation, and migration during imaging experiment. As mentioned earlier, the red fluorescence of neural progenitors nuclei observed immediately after mitosis was much dimmer than was red fluorescence of differentiated neural cells nuclei. In order to visualize migration of the nuclei in the cell cycle within the VZ, photomultiplier tube (PMT) sensitivity for red fluorescence was increased. While nuclei in the
CP showed saturated red fluorescence, nuclei in the VZ exhibited equivalent levels of either green or red fluorescence (B of Fig. 7). Under such conditions, it was possible that change in a color between green and red during cell cycle progression and migration of cells were clearly followed.

Trajectories of neural progenitor nuclei corresponding to interkinetic nuclear movements were followed. C of Fig. 7 (left) shows a successive trajectory of a migrating cell, in which the cell in the S phase and near a subventricular zone (IZ) underwent the G2 phase, migrated to the ventricular zone (VZ), and underwent the M phase there to divide itself into two G1 cells through cell division. The two G1 cells in the post-mitotic phase emitted red, and started migrating away from the ventricular surface. Other cells exhibited so-called elevator movement (C of Fig. 7, center), in which they rose toward the subventricular zone during the G1 phase, made a hairpin turn in the subventricular zone at timing of the G1/S phase, and migrated to the ventricular surface during the S phase. It was known that many of the cells undergo the mitotic phase in the ventricular surface, whereas several percent of the cells undergo the mitotic phase also in the subventricular zone. Latter ones of the cells were able to be imaged (C of Fig. 7, right). Also, a phenomenon in which red nuclei quickly traveled across the subventricular zone was imaged eventually. Such nuclei were likely to belong to cortical GABA (y-amino-butyrlic-acid) neurons, which are known to be born in subpallial telencephalon of archaic humans and to migrate tangentially to reach their final destination.

Nuclear localization of Fucci is advantageous in the following respects. In order to identify a cell type and observe cell morphology, additional far-red fluorescent proteins (mCherry, mKeima, and the like) spectrally distinct from both mAG and mKO2 are tagged with Nuclear Export Signal (NES) and expressed in cytoplasm. This makes it possible to distinguish the additional far-red fluorescent proteins. A third color of a fluorescence signal can also be provided by a chemical dye. In experiment shown in Fig. 7, clear DiD crystals were placed on a pial surface of a brain slice so as to sparsely label progenitors connecting cranial pia mater. This was an example in which it was possible to identify bipolar morphology of a progenitor, the progenitor having a green nucleus whose movement was tracked (data are not shown).

By expressing a FRET indicator, such as cameleon and Raichu-Ras, in cytoplasm, it is possible to understand a cell cycle in parallel with intracellular events (data are not shown). For example, when a Fucci-expressing COS7 cell was transfected with Raichu-Ras, it was verified that Ras was more active in the G1 phase than in the S/G2 phase in response to an epidermal growth factor (EGF) signal. Thus, cell-cycle dependency of plural intracellular events can be elucidated without using cell-cycle synchronization techniques. Multicolor imaging in combination with such fluorescent probes and proteins will further expand the application of the Fucci technology.

The Fucci technology allows dual-color imaging, thereby making it possible to distinguish actual cells in the G1 phase from those in the S/G2/M phase. The Fucci technology allows in-vivo analysis of spatial and temporal patterns of cell-cycle dynamics, owing to brightness and high contrast of two colors (red and green) of fluorescence. Although Fucci is composed of mKO2-Cdt1#10 and mAG-Geminin#2, transfection of either one of them is sufficient in obtaining a cell-cycle indicator function. For example, the transgenic mouse line #504 produces mAG-Geminin#2, but still provides in vivo information on proliferation patterns. However, coexpression of both constructs is still considerably more useful because it highlights the G1/S transition with a yellow signal, and because it allows continuous tracking of migrating cells or nuclei in the cell cycle. In this regard, reliable gene transfer technique by which stoichiometry of two constructs is controlled is necessary.

Further research were made so as to attain objects including (1) production of developed Fucci (Figs. 8 and 9), which was different from that using mKO2 and mAG in combination, (2) a development of probe capable of visualizing a cell-cycle phase other than the G1/S transition, and (3) development of a Fucci derivative which functioned in a non-mammalian individual. Such research benefited from exploration of molecular mechanisms underlying both cell-cycle progression and ubiquitin-mediated protein degradation. Regarding the objects, it was notable that a primary structure of Cdt1 and Geminin varied among species. By tagging mKO2 or mAG to certain domain of the two proteins (Cdt1 and Geminin) each having a lower homology among eukaryote, a derivative of Fucci which functioned in a fish cell and an insect cell was developed. Further, transgenic zebrafish and a Drosophila line expressing non-mammalian Fucci were developed, so as to investigate spatial and temporal regulation of cell-cycle progression during major morphogenetic events such as gastrulation and metamorphosis, and during basic morphogenetic processes such as invagination, involution, and branching.

Example 7: Screening of Anticancer Agent by Using Fucci Probe

Benign tumor cells (NMuMG cells) stably expressing Fucci and Malignant tumor cells (HeLa cells) stably expressing Fucci were used in observing reaction to an anticancer agent (Fig. 10). Data obtained from cells (control group) treated with DMSO treatment showed mixture of a red color and a green color, thereby indicating normal proliferation. A CDK4 inhibitor (G1-phase inhibitor) sufficiently worked on the benign tumor cell, the NMuMG cells, while being ineffective to the malignant tumor cells, the HeLa cells. Etoposide (inhibitor of topoisomerase 2) sufficiently worked on the malignant tumor cell and thereby caused cell-cycle arrest at the S/G2 phase, while having strong effect to the NMuMG cells and thereby induced apoptosis. By Nocodazole (M-phase inhibitor), all the cells were arrested in a rounded state at
the M phase.

(Distribution Source of Material)

[0118] DNA constructs of mKO2-Cdt1#10 and mAG-Geminin#2, their stable transformant cell lines, and transgenic mouse lines described herein will be distributed with concomitant purchase of cDNA for mKO2 or mAG from MB International Corp (Amalgaam, Ltd).

SEQUENCE LISTING

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Claims

1. A method for performing phase identification of a cell cycle, the method comprising the steps of:

visualizing, at least two or more gene-expression products whose amounts in a cell change in a cell-cycle dependent manner; and

detecting in vitro the at least two or more gene-expression products so as to distinguish a proliferation phase of the cell cycle from a resting phase of the cell cycle, wherein:

as the at least two or more gene-expression products, at least (i) a first gene-expression product whose amount increases in a G1 phase and decreases in an S/G2/M phase and (ii) a second gene-expression product whose amount decreases in the G1 phase and increases in the S/G2/M phase are used in one cell;
the first gene-expression product is the partial fragment of Cdt1, which partial fragment of Cdt1 is remaining of Cdt1 from which a Geminin binding site is excluded;
the second gene-expression product is the partial fragment of Geminin, which partial fragment of Geminin is remaining of Geminin from which a Cdt1 binding site is excluded, and
the first gene-expression product and the second gene-expression product being labeled by the markers different from each other, so as to enable the first gene-expression product and the second gene-expression product to be visualized and detected, wherein the first gene-expression product is the partial fragment of Cdt1, which partial fragment of Cdt1 is composed of 30th through 120th amino acids of said Cdt1, and wherein the second gene-expression product is the partial fragment of Geminin, which partial fragment of Geminin is composed of 1st through 110th amino acids of said Geminin.

2. The method as set forth in claim 1, wherein the marker is a fluorescent protein or a luminescent protein.

3. The method as set forth in any one of Claims 1 or 2, wherein the marker is detected over time by carrying out a time-lapse imaging observation on a living cell or a living tissue.

4. A transformed cell or a transgenic nonhuman animal, coexpressing in a cell, (i) a first gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Cdt1 which is remaining of Cdt1 from which a Geminin binding site is excluded and (ii) a second gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Geminin which is remaining of Geminin from which a Cdt1 binding site is excluded, the markers being different from each other, wherein
the gene encoding the partial fragment of Cdt1 which is remaining of Cdt1 from which a Geminin binding site is excluded is a gene encoding a partial fragment of Cdt1 composed of 30th through 120th amino acids of said Cdt1, and wherein
the gene encoding the partial fragment of Geminin which is remaining of Geminin from which a Cdt1 binding site is excluded is a gene encoding a partial fragment of Geminin composed of 1st through 110th amino acids of said Geminin.

5. The transformed cell or the transgenic nonhuman animal as set forth in Claim 4, wherein:

the gene encoding the marker is a gene encoding a fluorescent protein or a luminescent protein.

6. A method for in vitro screening a cell-cycle inhibitor comprising the steps of:
incubating a cell in the presence of a candidate substance for the cell-cycle inhibitor, and
performing phase identification of a cell cycle in accordance with a method as set forth in any one of Claims 1
through 3, so as to select a candidate substance for inducing cell cycle arrest.

7. A method for performing phase identification of a cell cycle as set forth in claim 1, wherein:

the marker for the first gene-expression product is a red fluorescent protein, and the marker for the second
gene-expression product is a green fluorescent protein.

8. A transformed cell or a transgenic nonhuman animal as set forth in Claim 4, wherein the one of the markers encoded
by the first gene construct is a red fluorescent protein and the other one encoded by the second gene construct is
a green fluorescent protein.

9. A probe for visualising cell cycle comprising:

a first gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Cdt1
which is remaining of Cdt1 from which a Geminin binding site is excluded; and
a second gene construct including a gene encoding a marker, and a gene encoding a partial fragment of Geminin
which is remaining of Geminin from which a Cdt1 binding site is excluded,
the markers being different from each other.

10. The probe for visualising cell cycle as set forth in Claim 9, wherein the marker encoded by the first gene construct
is a red fluorescent protein and the marker encoded by the second gene construct is a green fluorescent protein.

Patentansprüche

1. Verfahren für die Durchführung von Phasenidentifikation eines Zellzyklus, wobei das Verfahren die folgenden Schritte
umfasst:

Visualisieren von mindestens zwei oder mehr Genexpressionsprodukten, deren Mengen sich in einer Zelle in
einer Zellzyklus-abhängigen Weise ändern;
und
Nachweisen in vitro der mindestens zwei oder mehr Genexpressionsprodukte, um eine Proliferationsphase des
Zellzyklus von einer Ruhephase des Zellzyklus zu unterscheiden, wobei:

als die mindestens zwei oder mehr Genexpressionsprodukte mindestens (i) ein erstes Genexpressions-
produkt, dessen Menge sich in einer G1-Phase erhöht und in einer S/G2/M-Phase verringert, und (ii) ein
zweites Genexpressionsprodukt, dessen Menge sich in der G1-Phase verringert und in der S/G2/M-Phase
erhöht, in einer Zelle verwendet werden;

das erste Genexpressionsprodukt das Teilfragment von Cdt1 ist, welches Teilfragment von Cdt1 von Cdt1
verbleibt, von dem eine Geminin-Bindungsstelle herausgenommen ist;
das zweite Genexpressionsprodukt das Teilfragment von Geminin ist, welches Teilfragment von Geminin
von Geminin verbleibt, von dem eine Cdt1-Bindungsstelle herausgenommen ist, und
das erste Genexpressionsprodukt und das zweite Genexpressionsprodukt durch die Marker markiert sind
die sich voneinander unterscheiden, um zu ermöglichen, dass das erste Genexpressionsprodukt und das
zweite Genexpressionsprodukt visualisiert und nachgewiesen werden, wobei das erste Genexpressions-
produkt das Teilfragment von Cdt1 ist, welches Teilfragment von Cdt1 aus der 30. bis 120. Aminosäure
von Cdt1 besteht, und wobei das zweite Genexpressionsprodukt das Teilfragment von Geminin ist, welches
Teilfragment von Geminin aus der 1. bis 110. Aminosäure von Geminin besteht.

2. Verfahren nach Anspruch 1, wobei der Marker ein fluoreszierendes Protein oder ein lumineszierendes Protein ist.

3. Verfahren nach einem der Ansprüche 1 oder 2, wobei der Marker im Zeitverlauf durch Durchführung einer Zeitraffer-
Bildbeobachtung einer lebenden Zelle oder eines lebenden Gewebes nachgewiesen wird.

4. Transformierte Zelle oder ein transgenes nichthumanes Tier, das in einer Zelle Folgendes coexprimiert: (i) ein erstes
Genkonstruk, das ein Gen, das einen Marker kodiert, und ein Gen einschließt, das ein Teilfragment von Cdt1
Transformierte Zelle oder das transgene nichthumane Tier nach Anspruch 4, wobei:

5. das Gen, das den Marker kodiert, ein Gen ist, das ein fluoreszierendes Protein oder ein lumineszierendes Protein kodiert.

6. Verfahren für das in-vitro-Screening eines Zellzyklus-Inhibitors, das die folgenden Schritte umfasst:

   Inkubieren einer Zelle in Gegenwart einer Kandidaten-Substanz für den Zellzyklus-Inhibitor und Durchführen von Phasenidentifikation eines Zellzyklus gemäß einem Verfahren nach einem der Ansprüche 1 bis 3, um eine Kandidaten-Substanz für die Induktion von Zellzyklus-Arrest auszuwählen.

7. Verfahren für die Durchführung von Phasenidentifikation eines Zellzyklus nach Anspruch 1, wobei:

   der Marker für das erste Genexpressionsprodukt ein rot fluoreszierendes Protein ist und der Marker für das zweite Genexpressionsprodukt ein grün fluoreszierendes Protein ist.

8. Transformierte Zelle oder ein transgenes nichthumanes Tier nach Anspruch 4, wobei der eine von den Markern, der von dem ersten Genkonstrukt kodiert wird, ein rot fluoreszierendes Protein ist und der andere, der von dem zweiten Genkonstrukt kodiert wird, ein grün fluoreszierendes Protein ist.

9. Sonde für die Visualisierung eines Zellzyklus, die Folgendes umfasst:

   ein erstes Genkonstrukt, das ein Gen, das einen Marker kodiert, und ein Gen einschließt, das ein Teilfragment von Cdt1 kodiert, das von Cdt1 verbleibt, von dem eine Geminin-Bindungsstelle herausgenommen ist; und ein zweites Genkonstrukt, das ein Gen, das einen Marker kodiert, und ein Gen einschließt, das ein Teilfragment von Geminin kodiert, das von Geminin verbleibt, von dem eine Cdt1-Bindungsstelle herausgenommen ist, wobei die Marker sich voneinander unterscheiden.

10. Sonde für die Visualisierung eines Zellzyklus nach Anspruch 9, wobei der Marker, der von dem ersten Genkonstrukt kodiert wird, ein rot fluoreszierendes Protein ist und der Marker, der von dem zweiten Genkonstrukt kodiert wird, ein grün fluoreszierendes Protein ist.

Revendications

1. Méthode de mise en œuvre de l’identification de phase d’un cycle cellulaire, la méthode comprenant les étapes consistant à :

   visualiser au moins deux, ou plus, produits d’expression génique dont les quantités dans une cellule changent d’une manière dépendante du cycle cellulaire; et détecter in vitro les au moins deux, ou plus, produits d’expression génique de façon à distinguer une phase de prolifération du cycle cellulaire d’une phase de repos du cycle cellulaire, où :

   comme les au moins deux, ou plus, produits d’expression génique, au moins (i) un premier produit d’expression génique dont la quantité augmente dans une phase G1 et diminue dans une phase S/G2/M et (ii) un deuxième produit d’expression génique dont la quantité diminue dans la phase S/G2/M et augmente dans la phase S/G2/M, sont utilisés dans une seule cellule;

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le premier produit d'expression génique est le fragment partiel de Cdt1, lequel fragment partiel de Cdt1 est un reste de Cdt1 à partir duquel un site de fixation de Géminine est exclu;
le deuxième produit d'expression génique est le fragment partiel de Géminine, lequel fragment partiel de Géminine est un reste de Géminine à partir duquel un site de fixation de Cdt1 est exclu; et
le premier produit d'expression génique et le deuxième produit d'expression génique étant marqués par des marqueurs différents l'un de l'autre, de façon à permettre la visualisation et la détection du premier produit d'expression génique et du deuxième produit d'expression génique, où le premier produit d'expression génique est le fragment partiel de Cdt1, lequel fragment partiel de Cdt1 est composé du 30ème jusqu'au 120ème acide aminé de Cdt1, et où le deuxième produit d'expression génique est le fragment partiel de Géminine, lequel fragment partiel de Géminine est composé du 1er jusqu'au 110ème acide aminé de Géminine.

2. Méthode selon la revendication 1, dans laquelle le marqueur est une protéine fluorescente ou une protéine luminescente.

3. Méthode selon l'une quelconque des revendications 1 ou 2, dans laquelle le marqueur est détecté au cours du temps par la mise en œuvre d'une observation d'imagerie accélérée sur une cellule vivante ou un tissu vivant.

4. Cellule transformée ou animal non humain transgénique, co-exprimant dans une cellule (i) une première construction de gène comportant un gène codant pour un marqueur, et un gène codant pour un fragment partiel de Cdt1 qui est un reste de Cdt1 à partir duquel un site de fixation de Géminine est exclu et (ii) une deuxième construction de gène comportant un gène codant pour un marqueur, et un gène codant pour un fragment partiel de Géminine qui est un reste de Géminine à partir duquel un site de fixation de Cdt1 est exclu, les marqueurs étant différents l'un de l'autre, où le gène codant pour un fragment partiel de Cdt1 qui est un reste de Cdt1 à partir duquel un site de fixation de Géminine est exclu est un gène codant pour un fragment partiel de Cdt1 composé du 30ème jusqu'au 120ème acide aminé dudit Cdt1, et où le gène codant pour un fragment partiel de Géminine qui est un reste de Géminine à partir duquel un site de fixation de Cdt1 est exclu est un gène codant pour un fragment partiel de Géminine composé du 1er jusqu'au 110ème acide aminé de ladite Géminine.

5. Cellule transformée ou animal non humain transgénique selon la revendication 4, où le gène codant pour le marqueur est un gène codant pour une protéine fluorescente ou une protéine luminescente.

6. Méthode de criblage in vitro d'un inhibiteur de cycle cellulaire, comprenant les étapes consistant à :
    incuber une cellule en présence d'une substance candidate pour l'inhibiteur de cycle cellulaire, et
    mettre en œuvre l'identification de phase d'un cycle cellulaire conformément à une méthode selon l'une quelconque des revendications 1 à 3, de façon à choisir une substance candidate pour induire un arrêt du cycle cellulaire.

7. Méthode de mise en œuvre de l'identification de phase d'un cycle cellulaire selon la revendication 1, dans laquelle :
    le marqueur pour le premier produit d'expression génique est une protéine fluorescente rouge, et le marqueur pour le deuxième produit d'expression génique est une protéine fluorescente verte.

8. Cellule transformée ou animal non humain transgénique selon la revendication 4, où l'un des marqueurs codé par la première construction de gène est une protéine fluorescente rouge, et l'autre codé par la deuxième construction de gène est une protéine fluorescente verte.

9. Sonde destinée à visualiser un cycle cellulaire, comprenant :
    une première construction de gène comportant un gène codant pour un marqueur, et un gène codant pour un fragment partiel de Cdt1 qui est un reste de Cdt1 à partir duquel un site de fixation de Géminine est exclu; et
    une deuxième construction de gène comportant un gène codant pour un marqueur, et un gène codant pour un fragment partiel de Géminine qui est un reste de Géminine à partir duquel un site de fixation de Cdt1 est exclu; les marqueurs étant différents l'un de l'autre.
10. Sonde destinée à visualiser un cycle cellulaire selon la revendication 9, dans laquelle le marqueur codé par la première construction de gène est une protéine fluorescente rouge, et le marqueur codé par la deuxième construction de gène est une protéine fluorescente verte.
FIG. 1

COMPARISON OF FLUORESCENT BRIGHTNESS

Fluor. Int. (AU) vs. Time (hrs.)

- mKO
- mKO2
FIG. 4

A control  B + TGFβ 1 ng/ml

1 h
17 h
25 h
49 h
73 h
85 h

1 h
13 h
15 h
24 h
36 h
48 h

C control  D + TGFβ 1 ng/ml
FIG. 10

Fucci- BENIGN TUMOR CELL (NMuMG cells)  R: RED  G: GREEN  Y: YELLOW

DMSO 24 h  CDK4 inhibitor 24 h  etoposide 24 h  nocodazole 24 h

Fucci- MALIG NANT TUMOR CELL (HeLa cells)

DMSO 24 h  CDK4 inhibitor 24 h  etoposide 24 h  nocodazole 12 h
REFERENCES CITED IN THE DESCRIPTION

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