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DESCRIPTION CN113278686A

A method for studying DNA methylation-regulated reprogramming processes

[0001]

Technical Field

[n0001]

This invention relates to a method for studying the DNA methylation-regulated reprogramming process, belonging to the field of biotechnology.

[0003]

Background Technology

[n0002]

Cell reprogramming is a process of dedifferentiation. Through methods such as somatic cell nuclear transfer, transcription factor induction, cell fusion, and cytoplasmic incubation, fully differentiated somatic cells can be reprogrammed into totipotent cells.

Somatic cell nuclear transfer involves collecting somatic cell nuclei and injecting them into enucleated oocytes. The fused cells are then cultured in vitro to form an embryo, which is then implanted into the mother to obtain a cloned animal.

This method is effective and can actually produce offspring, but it violates ethical principles to some extent and cannot be used on humans.

Cell fusion techniques are widely used in plant breeding and the preparation of monoclonal antibodies, but ethical issues still arise when applied to animals. Since Shinya Yamanaka discovered in 2006 that somatic cells could be reprogrammed into induced pluripotent stem cells by introducing four transcription factors (Oct4, Sox2, Klf4, and c-Myc), transcription

factor induction technology has continued to develop. Its advantage of obtaining pluripotent stem cells without using egg cells and embryos, but only using other human cells, avoids ethical controversies and has therefore attracted widespread attention. However, it only studied the factors of somatic cell reprogramming from the perspective of transcription factors, and a large number of regulatory factors have not yet been discovered. Studies have shown that the addition of histone deacetylase inhibitor VPA significantly improves the reprogramming process, and there are still a considerable number of epigenetic factors to be explored.

[0005]

Summary of the Invention

[n0003]

The purpose of this invention is to address the aforementioned problems. DNA methylation, as a type of epigenetic modification, is widely present in various life processes.

In somatic cells, pluripotency genes are often in a hypermethylated state, and their expression is suppressed to maintain somatic cell stability. In contrast, in pluripotent stem cells, DNA methylation levels are mostly in a hypomethylated state, and pluripotency genes are active.

Therefore, DNA methylation undoubtedly determines the fate of somatic cell reprogramming. However, there are currently no reports on the systematic study of the regulation of somatic cell reprogramming by DNA methylation, and further research is needed. This invention provides a method for studying the DNA methylation-regulated reprogramming process.

[n0004]

The technical solution of this invention is: a method for studying the DNA methylation-regulated reprogramming process, characterized by comprising the following steps:

[0008]

Step (1): Based on the induction of Yamanaka factors Oct4, Sox2, Klf4 and c-Myc, add DNA methyltransferase inhibitor 5' azacytosine, and induce somatic cells according to the induction method provided by Shinya Yamanaka. Observe cell morphology at different induction days.

[0009]

Step (2): When cell clones are observed, collect cells for quantitative fluorescence detection, indirect immunofluorescence detection, alkaline phosphatase staining, chromosome karyotype analysis, and edU proliferation detection;

[0010]

Step (3): After determining the effect of 5' aza, the four factors and 5' aza were combined to induce somatic cells. The formation of ips clones was detected according to the method in step (2) to determine the most effective combination.

[0011]

Based on this, 5' aza was used alone for induction, and ips clone formation was detected according to the method in step (2) to further determine the individual induction effect of DNA methylation inhibitor; thus effectively exploring the role of DNA methylation in the reprogramming process.

[n0005]

In step (2), the method of quantitative fluorescence detection is as follows: collect clone-like cells, add Trizol lysis buffer, extract RNA, reverse transcribe it into cDNA, and then use real-time quantitative PCR to detect the expression of Oct4, Sox2, Klf4 and c-Myc, while detecting the expression of pluripotency genes and somatic cell marker genes.

[0013]

The indirect immunofluorescence detection method was as follows: the culture medium in the cell culture plate was removed, 4% paraformaldehyde was added to fix the cells for 30 min, washed with PBS, and then treated with 0.1% Triton permeate for 15 min. After centrifugation and washing, the cells were blocked for 2 h, Oct4, Sox2, Klf4, c-Myc, pluripotent marker protein and somatic cell marker antibody were added, and the cells were incubated overnight. After washing, secondary antibody was added and incubated for 2 h. After washing, DAPI was added and incubated for 10 min. The expression of the marker protein was observed under a fluorescent inverted microscope.

[0014]

The alkaline phosphatase staining method is as follows: remove the culture medium, wash with PBS, add ALP fixative, fix for 3 min, wash with PBS, add ALP incubation solution, incubate in the dark for 15-20 min, wash with PBS; add nuclear solid red staining or methyl green staining for 3-5 min, wash with PBS and examine under a microscope to observe whether the cloned cells show alkaline phosphatase activity.

[0015]

The method for chromosome karyotype analysis was as follows: Cell clones were seeded at a density of 5×10^1 NER1¹ in 100 mm diameter culture dishes. After reaching the logarithmic growth phase, 0.2 ml of 10 μ mol/L colchicine was added, and the cells were cultured for 4 h. The culture medium was then discarded, and trypsin was added for incubation for 3 min.

Digestion was terminated by adding 10% FBS in DMEM. After centrifugation, the cells were resuspended in a hypotonic solution of 40 mmol/L KCl and 25 mmol/L sodium citrate. After standing, an equal volume of freshly prepared, ice-cold 1:3 acetic acid-methanol fixative was added, and the cells were allowed to stand for 10 min. After 10 min of fixative, the cells were resuspended in the acetic acid-methanol solution. A drop of the cell suspension was aspirated using a Pasteur pipette and dropped from a height of 3 meters onto a glass slide cooled with ice. After drying, Giemsa staining was performed, and the staining solution was rinsed off after 5 min. The chromosome karyotype was then observed.

[0016]

The Edu proliferation assay was performed as follows: Log-grown cell clones were seeded at 1×10^4 NER2 cells per well in a 96-well plate and cultured continuously. 50 μ M Edu medium was added to each well and incubated for 2 hours. The medium was discarded, and the cells were washed twice with PBS. 4% paraformaldehyde was added and incubated at room temperature for 30 minutes. The fixative was discarded. Glycine was added and incubated for 5 minutes. After washing, 0.5% Triton X-100 in PBS was added and incubated for 10 minutes. After washing, $1 \times$ Apollo[®] staining solution was added and incubated in the dark for 10

minutes. The reaction solution was discarded, and 0.5% Triton X-10 in PBS was added and washed 2-3 times. Methanol was washed 1-2 times, and Hoechst 33342 reaction solution was used for incubation for 30 minutes. After washing with PBS, the cells were observed.

[n0006]

The method of this invention is advanced and scientific. Through this invention, the operation is simple and convenient, the experimental idea is clear, the feasibility is high, and it has strong applicability and extensibility.

By inducing somatic cell reprogramming through different combinations of Yamanaka factor and the DNA methylation inhibitor 5'aza, the role of DNA methylation in the induction of somatic cell reprogramming was explored.

This study has wide applications and can be used in somatic cells of different species.

[n0007]

Specific implementation methods

[0019]

The present invention will be further described below with reference to specific embodiments.

However, the scope of protection of this invention is not limited thereto:

[0020]

1.

DNA methylation inhibitors combined with Yamanaka factor-induced reprogramming;

[0021]

Somatic cells were induced by combining retrovirus-encapsulated Oct4, Sox2, Klf4 and c-Myc overexpression vectors with the DNA methyltransferase inhibitor 5' aza. Cell status was observed and photographed at different induction days.

[n0008]

2. Identification of iPS cells;

[0023]

When clone-like cells are observed, the cells are collected for identification.

[n0009]

Quantitative real-time PCR was used to detect the expression of Oct4, Sox2, Klf4 and c-Myc, as well as the expression of pluripotency genes and somatic cell marker genes.

[n0010]

Indirect immunofluorescence: Remove the culture medium from the cell culture plate, add 4% paraformaldehyde to fix the cells for 30 min, wash with PBS, permeabilize with 0.1% Triton for 15 min, centrifuge and wash, block for 2 h, add Oct4, Sox2, Klf4, c-Myc, pluripotent marker protein and somatic cell marker antibody, incubate overnight, wash, add secondary antibody and incubate for 2 h, wash again, add DAPI and incubate for 10 min, observe the expression of marker protein under a fluorescence inverted microscope.

[n0011]

Alkaline phosphatase staining: Remove the culture medium, wash with PBS, add ALP fixative, fix for 3 min, and wash with PBS.

Add ALP incubation solution, incubate in the dark for 15-20 min, then wash with PBS.

Add nuclear solid red staining or methyl green staining for 3-5 minutes, wash with PBS, and then examine under a microscope.

Observe whether cloned cells can exhibit alkaline phosphatase activity.

[n0012]

Chromosome karyotype analysis: Cell clones were seeded at a density of 5×10^3 NER³ cells in 100 mm diameter culture dishes. At the logarithmic growth phase, 0.2 ml of 10 μ mol/L colchicine was added, and the cells were cultured for 4 h. The culture medium was then discarded, and trypsin was added for 3 min. Digestion was terminated by adding 10% FBS in DMEM. After centrifugation, the cells were resuspended in a hypotonic solution of 40 mmol/L KCl and 25 mmol/L sodium citrate. After standing, an equal volume of freshly prepared, ice-cold 1:3 acetic acid-methanol fixative was added, and the cells were allowed to stand for 10 min. After 10 min of incubation, the cells were resuspended in the acetic acid-methanol solution.

Use a Pasteur pipette to draw a drop of cell suspension and drop it from about 3 meters onto a glass slide that has been cooled with ice.

After drying, perform Giemsa staining, rinse off the stain after 5 minutes, and observe the chromosome karyotype.

[n0013]

Edu proliferation assay: Logarithmically growing cell clones were seeded at 1×10^4 NER⁴ cells per well in a 96-well plate and cultured continuously.

Add 50 μ M Edu medium to each well and incubate for 2 hours. Discard the medium, wash twice with PBS, add 4% paraformaldehyde and incubate at room temperature for 30 minutes. Discard the fixative.

Add glycine and incubate for 5 min. After washing, add 0.5% Triton X-100 PBS and incubate for 10 min.

After washing, add 1 $\times$ Apollo[®] staining reaction solution, incubate in the dark for 10 min, discard the reaction solution, add 0.5% Triton X-10 PBS to wash 2-3 times, wash 1-2 times

with methanol, incubate with Hoechst 33342 reaction solution for 30 min, wash with PBS, and then observe.

[n0014]

3.

To determine the regulatory role of DNA methylation in cell reprogramming;

[0030]

The four factors were combined with 5' aza to induce somatic cells. Cell clones were collected when cell clones were observed and identified by quantitative fluorescence detection, indirect immunofluorescence detection, alkaline phosphatase staining,

chromosome karyotype analysis and edu proliferation detection to determine the most effective combination.

Based on this, 5' aza was used alone for cell induction, and cells were collected when cell clones were observed for cell identification using quantitative fluorescence detection, indirect immunofluorescence detection, alkaline phosphatase staining, chromosome karyotype analysis, and edu proliferation detection, in order to determine the regulatory role of DNA methylation in cell reprogramming.