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DESCRIPTION WO2017219232A1

A Cell Reprogramming Method Based on a Three-Dimensional System Technical Field This invention relates to the fields of biotechnology and tissue engineering, to gene delivery

systems, and specifically to the establishment of a cell reprogramming method based on a three-dimensional system.

Background of the Invention

Scientists typically study the self-renewal and multi-directional differentiation potential of pluripotent stem cells in a two-dimensional (2D) system. However, scientific research has found that, compared to 2D environments, three-dimensional (3D) systems are better able to simulate the natural microenvironment of tissues and organs in the body. During embryonic development in vivo, cells not only interact with each other, but also interact with the extracellular matrix (ECM) [Reference: Zheng, W., Zhang, W., Jiang, X. Biomimetic collagen nanofibrous materials for bone tissue engineering[J]. Advanced Engineering Materials, 2010,12(9):B451-B466; Han, J., Chen, L., Luo, G., Dai, B., Wang, X., Dai, J. Three-dimensional culture may promote cell reprogramming[J]. Organogenesis,2013,9(2):118-120.]. The extracellular matrix typically includes collagen and laminin interwoven with heparin sulfate proteoglycans. For example, in mouse embryos, laminin and heparan sulfate proteoglycans are distributed on the cell surface during the two-cell stage; while in the blastocyst inner cell mass stage, the expression of type IV collagen and fibronectin can be detected. These ECMs have multiple functions in cell growth, differentiation, and maintenance of tissue morphology. Cells contact the ECM through surface receptors (such as integrins), which play a role in mechanical anchoring of the cell and transduction of cell membrane signals. More and more scholars are

shifting their interest to the development of biomaterials that simulate the 3D environment in vivo and to the study of ECM in regulating stem cell behavior [Reference: Wang, K., Kievit, F.M., Florczyk, S.J., Stephen, Z.R., Zhang, M. 3d porous chitosan–alginate scaffolds as an in vivo model for evaluating nanoparticle-mediated tumor targeting and gene delivery to prostate cancer[J]. *Biomacromolecules*, 2015,16(10):3362-3372.]. Currently, many studies have focused on the expansion, directed differentiation, and in vivo transplantation of pluripotent stem cells in 3D environments [see: Leisten, I., Kramann, R., Ventura Ferreira, M.S., Bovi, M., Neuss, S., Ziegler, P., Wagner, W., Knüchel, R., Schneider, R.K. 3D co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche[J].

Biomaterials,2012,33(6):1736-1747;Wang,X.,Liu,S.,Zhao,Q.,Li,N.,Zhang,H.,Zhang,X.,Lei,X.,Zhao,H.,Deng,Z.,Qiao ,J.Three-dimensional hydrogel scaffolds facilitate in vitro self-renewal of human skin-derived precursors[J]. *Acta Biomaterialia*,2014,10(7):3177–3187; Elangovan, S., D'Mello,S.R.,Hong,L.,Ross,R.D.,Allamargot,C.,Dawson,D.V.,Stanford,C.M.,Johnson,G.K., Sumner,D.R.,Salem,A.K. The enhancement of bone regeneration by gene activated matrix encoding for platelet derived growth factor[J]. *Biomaterials*,2014,35(2):737-747.]. However, to date, no literature has reported on the induction of pluripotent stem cells in a 3D environment. This invention establishes a novel and efficient non-viral three-dimensional gene delivery system, and for the first time applies it to the induction of iPSCs in a three-dimensional environment.

Summary of the Invention

This invention relates to three components: cationic *Pleurotus eryngii* polysaccharide, calcium phosphate nanoparticles, and a three-dimensional collagen scaffold. The calcium phosphate nanoparticles are modified with cationic *Pleurotus eryngii* polysaccharide using a reverse microemulsion method to further enhance their gene-carrying capacity as a non-viral vector. The polysaccharide-calcium phosphate gene-carrying nanoparticles are fused with the three-dimensional collagen scaffold to form a three-dimensional induction system of iPSCs. This invention applies the three-dimensional system to the reprogramming study of human umbilical cord stem cells, evaluates cell transfection efficiency and cell reprogramming effect, and sets up a two-dimensional gene delivery system under the same conditions as a positive control. The experimental results show that the three-dimensional gene delivery system is significantly better than the two-dimensional gene delivery system in terms of gene transfection efficiency. In addition, human iPSCs induced by the three-dimensional system began to show iPSC cell spheroids on the 4th day after cell seeding, and then gradually formed embryoid-like cell spheroids. Identification using HE staining, laser confocal microscopy, immunohistochemistry, chromosome karyotype analysis, and in vivo teratomas showed that human iPSCs induced by the three-dimensional system have full germ layer differentiation potential similar to embryonic stem cells.

This invention employs a chemical modification method to provide a highly efficient and non-toxic three-dimensional gene delivery system for the directed reprogramming of human umbilical cord stem cells (HUMSCs) into human induced pluripotent stem cells (hiPSCs).

The technical solution of the present invention is as follows:

A cell reprogramming method based on a three-dimensional system specifically includes the following steps:

Step 1. Preparation of collagen scaffolds:

Take an appropriate amount of 2-5% collagen solution into a polytetrafluoroethylene mold ($68 \times 38 \times 5 \text{ mm}^3$) prepared by scaffold preparation, place it at 4°C for 0.5-1h, then quickly place it in a -80°C freezer for more than 3h, and then freeze-dry it; after heat curing in an oven for 8-10h, soak it in anhydrous ethanol for 24h, then wash it with ethanol solutions of 10-100% concentration gradient, and wash it with double-distilled water until there is no alcohol odor; freeze-dry it again, cut it into small pieces of $5 \times 5 \times 3 \text{ mm}^3$, and sterilize it with ultraviolet light in a laminar flow hood for later use;

Step 2. Preparation of polysaccharide calcium phosphate nanoparticles:

A 20–30% (v/v) Igepal CO-520/cyclohexane mixture was prepared and used as a solvent. A 10 μ L mixture of 0.01–0.05 M calcium chloride solution, 0.8–1.5 mg cationic polysaccharide D-Sp, 0.03–0.08 M disodium hydrogen phosphate, and plasmids (containing 2.5 μ g each of Oct4, Sox2, Klf4, and c-Myc) was prepared and subjected to a reverse-phase microemulsion method to obtain a polysaccharide-calcium phosphate hybrid nanoparticle microemulsion. This microemulsion was then subjected to column chromatography, rotary evaporation, and dialysis to obtain a polysaccharide-calcium phosphate hybrid nanoparticle solution.

Step 3. Fabrication of three-dimensional gene-loaded nanoparticle-collagen scaffolds:

A $5 \times 5 \times 3$ mm NER3 scaffold sample was placed into the well of a 24-well plate and thoroughly wetted with serum-free DMEM medium. Excess medium was removed. The concentration of polysaccharide-calcium phosphate hybrid nanoparticles was adjusted so that each 100 μ L nanoparticle solution contained 0.8–1.5 μ g of total plasmid. 100 μ L of polysaccharide-calcium phosphate hybrid nanoparticle solution was added to each wetted $5 \times 5 \times 3$ mm NER4 scaffold and then freeze-dried to obtain a three-dimensional gene-carrying nanoparticle-collagen scaffold.

Step 4. Expression of the Yamanaka factor in a three-dimensional system:

A $5 \times 5 \times 3$ mm NER5 three-dimensional gene-carrying nanoparticle-collagen scaffold was infiltrated with serum-free DMED; 200 μ L of HUMSCs cell suspension ($5-8 \times 10^6$ cells /scaffold) was added to the infiltrated scaffold and incubated at 37°C for 0.5-2h; the scaffold was removed and cultured in DMEM medium with 10% FBS for 2-6h. The expression levels of four exogenous genes were evaluated by RT-PCR. The polysaccharide-calcium phosphate gene nanoparticle transfection group was set as a positive control, and untreated HUMSCs were set as a negative control.

Step 5. Induction and amplification of iPSCs in a three-dimensional system:

Take a $5 \times 5 \times 3$ mm NER7 three-dimensional gene-carrying nanoparticle-collagen scaffold and infiltrate it with serum-free DMED; add 200 μ L of HUMSCs cell suspension ($5 \sim 8 \times 10^4$ cells/scaffold) to the infiltrated scaffold, culture it in complete culture medium for 3~5 days, then change it to human embryonic stem cell culture medium, change the medium every 2~3 days to allow pluripotent stem cells to expand.

Beneficial effects Collagen scaffolds prepared from natural I collagen provide a safe and stable three-dimensional culture environment for HUMSCs. Polysaccharide-calcium phosphate gene-carrying nanoparticles have good gene carrying capacity and are uniformly distributed in the three-dimensional scaffold, providing a sufficient source of exogenous

genes for the targeted reprogramming of HUMSCs to iPSCs and enabling long-term release.

RT-PCR evaluation showed that the expression level of exogenous genes in the three-dimensional system was significantly higher than that in the two-dimensional system.

Identification of iPSCs showed that the human iPSCs formed in the three-dimensional system had the ability to differentiate into all germ layers similar to embryonic stem cells, and could be stably expanded in vitro, passaged to more than 20 generations, and maintained a normal chromosome karyotype. This method provides a novel three-dimensional environment for the induction of iPSCs and provides a theoretical basis for the transition of iPSC applications from the cellular level to the tissue level.

Attached Figure Description Figure 1 is a flowchart of cell reprogramming in a three-dimensional system.

Figure 2 is a scanning electron microscope image of a three-dimensional gene-carrying nanoparticle-collagen scaffold.

Figure 3 shows the morphological changes of HUMSCs in a three-dimensional gene-carrying nanoparticle-collagen scaffold under an optical microscope.

Figure 4 shows HE staining of the three-dimensional gene-carrying nanoparticle-collagen scaffold and its iPSCs.

Figure 5 shows the immunohistochemistry of iPSCs.

Figure 6 shows the karyotype of the cell chromosomes.

Figure 7 shows the immunofluorescence staining used to identify the pluripotency of iPSCs.

Figure 8 shows the methylation levels of the Oct4 and Nanog promoters, where hESCs are human embryonic stem cells.

Figure 9 shows the differentiation of iPSCs cell spheres into the three germ layers in vivo, where: g represents fibrous tissue, which is an endoderm feature; b and c represent bone tissue and cartilage tissue, which are mesodermal differentiation features, respectively; and n represents neural tube, which is an ectoderm marker.

Detailed Implementation The following are the materials and instruments used in the embodiments:

Materials and Reagents: Cationic polysaccharide D-Sp, LB medium (self-made); Type I collagen, Type IV collagenase, agarose, ethidium bromide, L-glutamine, MINI26-1KT, biotinylated IgG (Sigma, USA); trypsin, Tris base, ampicillin, streptomycin, DAB display kit (Beyotime Biotechnology Research Institute); basic fibroblast growth factor (bFGF) (PeproTech, USA); plasmid extraction kit (Promega, USA); calcium chloride (Sinopharm Chemical Reagent Co., Ltd.); MTT (3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazol bromide), PicoGreen dsDNA quantitative kit (Invitrogen, USA); basal medium DMEM, basal medium DMEM/F12, knockout DME, fetal bovine serum (FBS), knockout serum substitutes (GIBCO (USA); Oligo (designed by Nobibio, synthesized by Invitrogen, USA); T-vector kit (D101B, TAKARA); DH5 α competent cells (CD201-02, TransGold); antibiotic plates (prepared by Nobibio); double-distilled water (prepared by Nobibio); plant and animal genomic DNA extraction kit (AP-MN-MS-GDNA-250, Axygen); whole blood genomic DNA extraction kit (AP-MN-BL-GDNA-250, Axygen); methylation kit (D5005, ZYMO); Platinum Taq DNA Polymerase (10966034, Invitrogen, USA); PCR product purification kit (AP-GX-250, Axygen); 100mM dNTPs (18427013, Invitrogen, USA)

. Strains, cell lines, and mice: DH5 α glycerol bacteria (Nanjing Jitian Biotechnology); human umbilical cord mesenchymal stem cells (Jiangsu Beike Biotechnology Co., Ltd.); mouse embryonic fibroblasts (Shanghai Institute of Stem Cell Bank, Chinese Academy of Sciences); non-PEI obese diabetic/severe combined immunodeficiency mice (NOD-SCID) (Yangzhou University Comparative Medicine Center).

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plasmids: non-viral plasmids OCT4, SOX2, KLF4, and CMYC (Guangzhou Funeng Gene Co., Ltd.)

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Antibodies: Anti-OCT4, anti-SSEA-3, anti-SSEA-4, anti-TRA-1-81, anti-NANOG, goat anti-rabbit IgG-Cy3 (the above antibodies were provided by Abcam, Inc., USA).

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primers:

Methylation assay primer sequences:

OCT4 upstream primer: TGGAGGTGAT GGGTTAGG 18

OCT4 downstream primer: CATCAAATA CCCTATCATA ACC 23

NANOG upstream primer: GTGAATGAAA GAGGAAAATG GAG 23

NANOG downstream primer: AATAACCCAC CCCTATAATC C 21

(The primers mentioned above were provided by Shanghai Bioengineering Co., Ltd.)

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. Solution preparation:

(1) 2% collagen solution: Weigh 2.00g of collagen, add 100mL of 1% acetic acid solution, heat in a water bath at 60°C to completely dissolve it, and obtain a translucent solution. Filter through a 0.22μm filter membrane and store at 4°C for later use.

(2) PBS solution: Take 8.00g NaCl, 0.20g KCl, 3.5g NaHPO • 12HO, and 0.2g KHPO • 12HO, add 800mL of double-distilled water, adjust the pH to 7.4, bring the volume to 1000mL, dispense, autoclave, and store at 4°C.

(3) Complete culture medium: low-glucose DMEM, with 10% FBS and 100 U/mL penicillin-streptomycin solution added.

(4) Human embryonic stem cell culture medium: Add 20% serum substitute (Knockout SR), 2 mmol/L L-glutamine, 0.1 mmol/L β -mercaptoethanol, 1% non-essential amino acids, 100 U/mL penicillin-streptomycin solution, and 4 ng/mL human recombinant basic fibroblast growth factor to Knockout DMEM medium in sequence, mix well, and store at 4°C for later use.

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. Instruments: Emission scanning electron microscope (JSM-7001F, NEC); CO_NER13 cell culture incubator (Thermo, USA); Clean bench (SW-CJ-2F, Suzhou Purification Equipment Co., Ltd.); Standard centrifuge (Eppendorf, USA); Ultra-high-speed cryogenic centrifuge (Heraeus, Germany); Fixed-track shaker (Shanghai First Medical College Instrument Factory); Confocal laser scanning microscope (Leica, Germany); Vertical clean bench (SW-CJ-2D, Huijin Purification); High-speed benchtop centrifuge (Neofuge13, Heal Force); Shaking incubator

(THZ-98, Huamei Biochemical); PCR instrument (T100TM, Bio-Rad); Electrophoresis and imaging system (Tanon1600, Tianneng); Sequence analyzer (3730xl, Applied NER14).

Example 1: Fabrication of a three-dimensional collagen scaffold:

(1) Take an appropriate amount of 2% collagen solution into the polytetrafluoroethylene mold ($68 \times 38 \times 5 \text{ mm}^3$) prepared by the scaffold, place it at 4°C for 0.5h, then quickly place it in a -80°C freezer for more than 3h, and then freeze dry.

(2) Heat-cur in an oven at 120°C for 10 hours;

(3) After that, soak in anhydrous ethanol for 24 hours, then wash with ethanol solutions of 95%, 85%, 75%, 65%, 55%, 30%, and 15% concentration gradient, and finally wash with a large amount of double-distilled water until there is no alcohol smell.

(4) Freeze-dry again, cut into small pieces of $5 \times 5 \times 3 \text{ mm}^3$, and sterilize with ultraviolet light for 3 hours in a clean bench for later use.

Aseptic operation is ensured throughout the entire process after heat curing.

Example 2: Preparation of polysaccharide-calcium phosphate hybrid nanoparticles

(1) Igepal CO-520 was dissolved in cyclohexane to prepare an Igepal CO-520/cyclohexane mixture with a volume fraction of 29%;

(2) Preparation of microemulsion A: Take a clean conical flask, add 25 mL of Igepal CO-520 /cyclohexane mixture, then add 650 μ L of 0.01 M calcium chloride solution and 0.8 mg of cationic polysaccharide D-Sp together into the clean conical flask under magnetic stirring, and continue stirring for 2 min to form microemulsion A;

(3) Preparation of microemulsion B: Take another clean conical flask, add 25 mL of Igepal CO-520/cyclohexane mixture, then add 650 μ L of 0.06 M disodium hydrogen phosphate and 10 μ L of plasmid mixture (containing 2.5 μ g each of Oct4, Sox2, Klf4 and c-Myc) to the clean conical flask, and continue stirring for 2 min to form microemulsion B;

(4) At 4°C, microemulsion A was added dropwise to microemulsion B while stirring until the whole system became transparent and clear, thus obtaining polysaccharide-calcium phosphate hybrid nanoparticle microemulsion.

(5) Dilute the polysaccharide-calcium phosphate mixed nanoparticle microemulsion with 5 mL of anhydrous ethanol at pH 7.0;

(6) Then, the column was loaded with silica gel. First, cyclohexane and free DNA were eluted with anhydrous ethanol. Then, polysaccharide-calcium phosphate mixed nanoparticles were eluted with 70% ethanol solution containing 5×10^{-4} mM NaCl. The elution liquids at different time points were collected and their DNA content was determined by HPLC.

(7) The ethanol solution containing polysaccharide-calcium phosphate mixed nanoparticles was removed by rotary evaporation at 37°C for 6 hours. The concentrated solution was placed in a 12kDa dialysis bag and incubated overnight at 4°C in PBS with pH 7.4 to obtain the polysaccharide-calcium phosphate mixed nanoparticle solution.

Example 3: Preparation of three-dimensional gene-loaded nanoparticle-collagen scaffold

(1) Take a 5×5×3mm NER18 scaffold sample and put it into the well of a 24-well plate. Add 0.5mL of serum-free DMEM medium to each well to fully wet the scaffold.

(2) After 0.5 hours, remove the excess culture medium;

(3) Adjust the concentration of polysaccharide-calcium phosphate hybrid nanoparticles so that each 100 µL nanoparticle solution contains 0.8 µg of total plasmids;

(4) Add 100 μL of polysaccharide-calcium phosphate mixed nanoparticle solution to each wetted $5 \times 5 \times 3\text{mm}$ NER19 scaffold. The solution must be added slowly and in small amounts multiple times to ensure that the nanoparticle solution is in full contact with and adsorbed by the scaffold.

(5) Then, freeze-dry to obtain a three-dimensional gene-carrying nanoparticle-collagen scaffold.

(6) Observe the morphology of three-dimensional gene-carrying nanoparticle-collagen scaffold under scanning electron microscopy.

As shown in Figure 2, the three-dimensional gene-carrying nanoparticle-collagen scaffold still exhibits a loose and porous internal structure with pore sizes ranging from 200 to 400 μm .

Moreover, the magnified image on the right clearly shows that the polysaccharide-calcium phosphate hybrid nanoparticles are adsorbed onto the inner surface of the collagen scaffold (as indicated by the arrow).

Example 4: HE staining

- (1) Each $5 \times 5 \times 3 \text{ mm}^3$ three-dimensional gene-carrying nanoparticle-collagen scaffold was moistened with 200 μL of serum-free DMEM medium;
- (2) Prepare a cell suspension of human umbilical cord mesenchymal stem cells by adding 200 μL of cell suspension (5×10^4 cells/scaffold) dropwise onto the moistened scaffold in small amounts and multiple times.
- (3) The scaffold inoculated with cells was placed in an incubator at 37°C and 5% CO_2 for 0.5 h;
- (4) Then, add DMEM medium containing 10% FBS to each well containing the seeded cell scaffold and continue culturing for 4 hours;
- (5) After the cells were implanted, the scaffolds on the 4th, 8th and 12th days were fixed with 4% paraformaldehyde for 24 hours and then dehydrated with ethanol in a gradient from low to high concentration to gradually remove the water from the scaffolds.
- (6) Then place the stent in xylene, a transparent agent that is soluble in both alcohol and paraffin, to replace the ethanol in the stent with xylene;

(7) Place the transparent support in the melted paraffin wax and put it in the wax melting box for heat preservation;

(8)

After the paraffin is completely immersed in the scaffold, embed it: First prepare a container (such as a folded cardboard box), pour in the melted paraffin, quickly pick up the immersed paraffin tissue block and put it in, and let it cool and solidify into a block;

(9)

Fix the embedded wax block on a microtome and cut it into thin slices, generally 5 to 8 μm thick.

The cut slices are often wrinkled, so they need to be flattened in heated water, then attached to a glass slide, and dried in a 45°C constant temperature oven.

(10)

Remove the paraffin from the slices with xylene, then dewax them with alcohol from high concentration to low concentration, and finally hydrate them in distilled water.

(11)

Stain with hematoxylin for 5 min, wash with water; then separate the colors in hydrochloric acid and ammonia water for 30 s each, rinse with running water for 1 h, and then soak in distilled water for a short time.

(12)

Dehydrate in 70% and 90% alcohol for 10 minutes each;

(13)

Stain with alcohol and eosin again for 2-3 minutes, then rinse with water;

(14)

The stained sections were dehydrated with pure alcohol and then made transparent with xylene.

(15)

Apply Canada balsam to the transparent section, cover with a coverslip to seal, and observe the scaffold and cell morphology under a microscope.

Figure 3 shows that the cell spheres in the scaffold are regular spherical or ellipsoidal shapes; Figure 4 shows that the cytoplasm is stained red with eosin, while the cell nucleus is stained blue with hematoxylin, showing a nucleus/cytoplasm ratio similar to that of human embryonic stem cells.

Example 5 Immunohistochemistry:

(1) Paraffin-embedded sections were prepared according to (5-9) in Example 4;

(2) The sections were routinely dewaxed and hydrated, and a portion of the sections were used for alkaline phosphatase (AP) staining;

(3) For the other part of the hydrated slices, add 3% HNER20ONER21 and inactivate the endogenous enzyme at room temperature for 5-10 min, then wash 3 times with distilled water.

(4) Heat repair antigen: Immerse the slides in 0.01M citrate buffer (pH=6.0), heat to boiling in an electric furnace and then turn off the power. Repeat 1-2 times after 5-10 minutes. After cooling, wash 1-2 times with PBS (pH=7.4).

(5) Add 5% BSA blocking solution to the slide, let it stand at room temperature for 20 minutes, shake off the excess liquid, and do not wash.

(6) Add appropriate diluted primary antibodies (SSEA3, Tra-1-81, Oct4 and Nanog) to the slide, incubate overnight at 4°C, and then wash three times with PBS, 2 min each time;

(7) Add biotinylated IgG solution, react at 27°C for 20 min, then wash with PBS 3 times, 2 min each time;

(8) Use the DAB display kit for color development: Take 1 mL of distilled water, add one drop each of reagents A, B and C from the kit, mix well and add to the slide.

Color development was performed at room temperature, and the reaction time was controlled under a microscope. The cells were washed with distilled water, dehydrated, cleared, and mounted. The cells were observed and photographed under a microscope. As can be clearly seen from Figure 5, the cell spheres in the three-dimensional gene-carrying nanoparticle-collagen scaffold showed positive expression of alkaline phosphatase (AP).

Moreover, over time, human embryonic stem cell markers SEA3, TRA-1-81, OCT4, and NANOG gradually showed strong positive expression.

On day 8, all tested pluripotency factors showed positive expression, indicating a transition of cells to a pluripotent state.

In contrast, the cells in the blank collagen scaffold did not show any immunostaining reaction.

The above results demonstrate that the cell spheres in the three-dimensional gene-carrying nanoparticle-collagen scaffold are products of pluripotent cell reprogramming: iPSCs; moreover, compared with the two-dimensional system (the average time required for virus-

induced somatic cell reprogramming is 3 weeks), the reprogramming time required in the three-dimensional non-viral system of this invention is shortened to about 8 days, which greatly accelerates the cell reprogramming process.

Example 6: Karyotype Analysis

Operation process:

(1) Colchicine treatment: 24 μL of colchicine was added to the cell culture flask 3 hours before the cell culture was terminated, so that the final concentration reached 0.8 $\mu\text{g/mL}$, and then the flask was put back into the incubator and cultured for 72 hours.

(2) Collecting cells: Take out the cells, use a pipette to thoroughly blow and blow on the bottle wall to disperse the adhered cells evenly, transfer them into centrifuge tubes, mark them, centrifuge at 1500 rpm for 5 min, and discard the supernatant.

(3) Hypotonic treatment: Add 1 mL of 0.075 mol/L KCl hypotonic solution to the centrifuge tube, gently blow with a pipette to suspend the cells evenly in the hypotonic solution, add 6 mL of 0.075 mol/L KCl hypotonic solution, place in a 37°C constant temperature water bath, and let stand for 20 min to allow the cells to swell and the chromosomes to disperse.

(4) Pre-fixation: Add 1 mL of freshly prepared Carno fixative to the centrifuge tube, gently blow and mix well, let stand at room temperature for 20 min, centrifuge at 1500 rpm for 5 min, and remove the supernatant;

(5) Fixation: Add 6 mL of freshly prepared Carno fixative along the wall of the centrifuge tube, mix well by pipetting, let stand at room temperature for 20 min, centrifuge at 1500 rpm for 5 min, and discard the supernatant;

(6) Dropping: Add about 1 mL of freshly prepared fixative to the precipitate, gently blow the suspended cells with a pipette, aspirate the cell suspension, and drop 2-3 drops vertically onto a clean glass slide pre-soaked in ice water at a certain height. Immediately blow in one direction to disperse the suspension, rinse with alcohol several times, dry, and label each slide.

(7) Staining: Place the slides with cell suspension in the staining jar, making sure there are gaps between the slides, pour the diluted Giemsa stain into the staining jar, stain for 20 minutes, take them out, rinse with tap water, and dry.

(8) Microscopic examination: Locate cells in metaphase under low magnification, then observe them under high magnification and photograph cells with good dispersion.

As shown in Figure 6, the cell spheres released from the three-dimensional scaffold and expanded in the two-dimensional environment showed normal karyotypes in the first and 15th generation cells, which were consistent with the karyotypes of human umbilical cord stem cells. This indicates that the iPSCs induced in the three-dimensional scaffold had normal karyotypes and no chromosomal mutations occurred, demonstrating the good safety of the method.

Example 7: Immunofluorescence

Immunofluorescence staining was performed on iPSCs amplified to different generations (generations 2, 10, 20, and 25) in a two-dimensional environment. The primary antibodies were anti-SSEA3, anti-TRA-1-81, anti-OCT4, and anti-NANOG, and the secondary antibodies were FITC-IgG and Cy3-IgG.

As shown in Figure 7, during the expansion of iPSCs induced in the gene-loaded nanoparticle-collagen scaffold in a two-dimensional environment, cells at different generations (generations 2, 10, 20, and 25) still maintained positive expression of various pluripotency factors, proving that the iPSCs induced in this experiment can be stably passaged.

Example 8: Detection of promoter methylation

(1) Genomic DNA extraction and quality control: Genomic DNA was extracted from three cell samples (human umbilical cord mesenchymal stem cells, iPSCs induced in this experiment, and human embryonic stem cell line HN4) using a specialized gDNA extraction kit, and OD concentration and agarose gel electrophoresis were measured.

(2) Methylation of qualified DNA: Using a specialized methylation kit, strictly follow the instructions to methylate 500 ng - 2 µg of gDNA to obtain 10 µL of methylated DNA.

The specific methylation procedure is as follows: Add 20 µL of gDNA (500 ng to 2 µg) to 130 µL of CT solution (one of the reagents in the kit), mix gently, and proceed as follows: 98 °C for 10 min; 64 °C for 2.5 h; 4 °C for maintenance.

(3) The above reaction products were purified and enriched on a purification column, and methylated DNA was collected by elution with 10 µL of double-distilled water.

(4) PCR amplification and recovery purification: PCR amplification was performed using methylated DNA as a template.

First round PCR system (total volume 25 µL):

The first round of PCR amplification program was as follows: 95°C for 3 min; 95°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec, for 40 cycles; then 72°C for 5 min to finish.

Second round PCR system (total volume 50 µL):

The second round of PCR amplification program was as follows: 95°C for 3 min; 95°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec, for 40 cycles; then 72°C for 5 min to finish.

PCR product recovery and purification: The second round of PCR products were purified by gel extraction, and the concentration was determined by OD measurement.

(5) TA cloning: Using the T vector kit, the purified PCR product was cloned into the T vector, transformed with DH5α competent cells, plated, and cultured overnight at 37°C with the cells inverted (see the T vector kit instructions for details).

(6) Single-clone bacterial culture sequencing: Select effective single clones (avoiding satellite colonies), perform shake culture, and perform PCR bacterial culture testing. Positive single clones are sent for testing.

(7) Sequencing result comparison and analysis: First, sequence alignment software is used to

perform sequencing analysis, and then methylation analysis software is used to perform methylation analysis on the correct sequencing results and to count information such as the number of methylations of CpG islands.

As shown in Figure 8, the OCT4 and NANOG promoter regions of iPSCs showed obvious demethylation, with a degree of methylation similar to that of positive control human embryonic stem cells, but significantly different from that of initial human umbilical cord mesenchymal stem cells.

This result further demonstrates the successful reprogramming of human umbilical cord mesenchymal stem cells in a three-dimensional gene-carrying nanoparticle-collagen scaffold.

Example 9: In vivo teratoma experiment

After digestion and centrifugation, iPSCs were prepared into a cell suspension of 5×10^2 cells/mL, which was then subcutaneously inoculated into the abdomen of 5-week-old male NOD-SCID nude mice, and tumor growth was observed.

Five to eight weeks later, the mice were sacrificed, and the tumors were removed for HE staining analysis.

As shown in Figure 9, iPSCs cell spheres can successfully form teratomas in immunodeficient NOD-SCID nude mice, and the teratomas show obvious differentiation of the three germ layers, represented by endoderm glandular tissue, mesodermal bone and cartilage tissue, and ectoderm neural tube, respectively. These results fully demonstrate that three-dimensional gene-carrying nanoparticle-collagen scaffolds can be successfully applied to cell reprogramming.