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SPECIAL REPORTS

Standards of induced pluripotent stem cells derived clinical-grade neural stem cells preparation and quality control (2021 China version)

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ABSTRACT

Induced pluripotent stem cells (iPSCs) have become the leading research object in the clinical application of restorative medicine. They are easily generated from diverse cell sources and functionally indistinguishable from embryonic stem cells without the accompanying ethical issues. To date, the use of iPSC-derived neural stem cells and their progeny in the treatment of neurodegenerative and injurious diseases has achieved good results, with great potential in cell drug development. However, because of some unique biological properties and differences from traditional drug production processes, cell drug research and development has many problems that can hinder clinical applications. Given this situation, the Chinese Association of Neurorestoratology (Preparatory) and China Committee of the International Association of Neurorestoratology have organized relevant professional experts to formulate the standard presented here. Overall, the aim was to promote the clinical application of neural stem cells (NSCs) and their further derived neural cells from iPSC sources and promote cell drugs' production and development. This standard refers to the latest research results, quality evaluation criteria for traditional medicines, and the regulatory framework for cellular treatments. The standard considers general biological properties of cells, including cell morphology, cell cycle, karyotype, and cell viability. The specific biological properties of NSCs, such as cell surface markers and differentiation ability, general drug standards, such as aseptic testing, endotoxins, human virus detection, and cell-related drug standards, such as telomerase activity and tumorigenicity, are also considered. This standard will serve as a reference for physicians and scientists who focus on clinical nervous cell applications and studies related to iPSCs.

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Preface

This standard is made following the rules given in GB/T 1.1-2009. Moreover, it is set up by the Chinese Association of Neurorestoratology (CANR; Preparatory) and China Committee of International Association of Neurorestoratology (IANR-China Committee). Currently, the clinical application of neural stem cells (NSCs) and their further derived neural cells has attracted interests from researchers and clinicians. However, the unified standards of NSCs preparation and quality control have yet to be established, which may hamper the application of NSCs in regenerative medicine.

This document aims to set up unified standards of preparation and quality control of induced pluripotent stem cells (iPSCs) derived NSCs in the development of clinical products for regenerative medicine for the member units of the CANR (Preparatory) and IANR-China Committee. It also serves as a reference for physicians around the world who perform iPSCs derived NSCs clinical applications. Notably, this standard represents the current agreements for the preparation and quality control of clinical-grade NSCs in neurorestorative treatment. These standards may be modified according to progress made in preclinical and clinical studies.

Meng Cai, Fabin Han, Nanxiang Xiong, Yihao Wang, Shiqing Feng, Jiajing Wang, Xiang Li, Jun Wei, and Changkai Sun drafed this standard.

1 Scope

The cells mentioned in this standard refer to the products of human neural stem cells (hereinafter referred to as NSCs) derived from induced pluripotent stem cells (hereinafter referred to as iPSCs)

which transformed from human (cord) blood, umbilical cord, skin, urine or other tissues. These standards also cover the functional neural cells further derived from NSCs except for certain cell type-related items.

2 Scope of application

2.1 This document specifies the standards for iPSC donor evaluation, sample collection, iPSC induction, iPSC banking and quality control of intermediate iPSC cell products. In addition, it also specifies the guidelines for the use and management of materials, equipment and clean environment according to good medical practice (GMP) standards.

2.2 This standard applies to clinical-grade NSCs and NSC further derived neural cell products preparation and quality control using iPSCs as the original source. These standards must be abided by the member units of the Chinese Association of Neurorestoratology (CANR; Preparatory) and the China Committee of the International Association of Neurorestoratology (IANR-China Committee). It also serves as a reference for clinicians, physicians and scientists who focus on iPSC-originated clinical nervous cell applications and studies.

3 Normative references

The following documents were indispensable in the preparation of this standard. For the dated references, only the dated version applies to this document. For undated references, the latest version (including all amendments) applies to this document.

T11/CSSCR 001-2017 General Requirements for Stem Cells
Good Manufacturing Practice Pharmaceutical Products (2010 Edition)
Pharmacopoeia of the People's Republic of China (2020 Edition)
Guiding Principles for Quality Control of Stem Cell Preparations and Preclinical Research (Trial)
Code for Quality Management of Cell Banking
Sterile Drug Appendix to Pharmaceutical Production Quality Management Regulations
Regulations of the People's Republic of China on the Administration of Human Genetic Resources
Guiding Principles for Ethical Review of Drug Clinical Trials
Technical Guidelines for Research and Evaluation of Cell Therapy Products (Trial)
GMP Appendix-Cell Therapy Products (Draft for Comment)
Setting Global Standards for Stem Cell Research and Clinical Translation: The 2016 ISSCR Guidelines

4 Terms and definitions

The following terms and definitions apply to this standard.

4.1 Induced pluripotent stem cells

Induced pluripotent stem cells (iPS cells or iPSCs) are a type of embryonic-like pluripotent stem cell

that can be reversely induced from somatic cells. The iPSCs can self-renew indefinitely in culture, while retaining the capacity to differentiate into any type of cell in the body.

4.2 Neural stem cells and derived neural cells

Neural stem cells are a group of ectodermal progenitor cells, which can give rise to sub-types of neural cell, such as neurons, astrocytes or oligodendrocytes. The NSCs derived neural cells also include the neural progenitor cells that are not fully differentiated into functional neurons, such as dopaminergic progenitor cells (DPCs) and retinal progenitor cells (RPCs).

5 Overview

Induced pluripotent stem cells are a type of stem cells reprogrammed from somatic cells. They can self-renew and have pluripotent differentiation potential [1]. As iPSCs can differentiate into various adult cells that are easy to access and permitted for use, they are potentially the best cell materials for restorative medicine and tissue repair engineering; thus, they have great potential in clinical applications [2, 3]. The methodology of inducing stem cells to differentiate into NSCs and neurons has been well developed [4]. However, due to the lack of uniform standards and quality control of cell products, the clinical application of iPSCs has, to date, been hindered [5].

The products of NSCs from iPSCs for clinical use should conform to GMP standards. According to the GMP appendix related to cell products for therapy use (draft; China Food and Drug Administration, CFDA), the production laboratory of cell products should meet environmental cleanness requirements and be equipped with biological safety protection facilities. The materials used for cell culture should be safe, traceable, and meet the clinical quality standards and ethical requirements. The production process of cell products should follow a strict quality management system, including a product traceability system and full risk assessment. The above standards are the basic requirements to ensure cell preparation of a high standard. More importantly, clinical-grade cell products should have relevant biological characteristics and functions to ensure their therapeutic effects. Therefore, cell products' final quality is closely related to cell preparation and the quality control monitoring of cell biological characteristics. Based on relevant regulations, current research results, expert opinions, and the rules provided in GB/T 1.1-2009, the Chinese Association of Neurorestoratology (Preparatory) and China Committee of International Association of Neurorestoratology have created this standard for iPSC-derived NSC culture and quality control. This standard will be a reference for physicians and scientists who focus on clinical nervous cell application and studies related to iPSCs in the field of Neurorestoratology.

6 Preparation of iPSCs

6.1 Selection of somatic cell donors

6.1.1 Bsic principles

The donors of somatic cells being used to generate iPSCs should follow the relevant provisions of the regulations of the People's Republic of China on human genetic resource management (Order No.

717 of the State Council of the People's Republic of China), with adequate ethical review and informed consent. The ethics committee of relevant institutions should initially review the purpose of somatic cell collection under the *Guiding Principles for Ethical Review of Drug Clinical Trials (2010)*. Samples should not be collected until the ethical requirements are met. The sample collection process must strictly abide by the principle of informed consent to protect somatic cell donors' rights and interests.

6.1.2 Inclusion criteria

The donors of somatic cells being used to produce iPSCs should meet the following requirements.

6.1.2.1 The donors are healthy and under the age of 70. There is no limitation on their gender.

6.1.2.2 The donors do not have any trauma affecting the nervous system or other chronic nervous system lesions.

6.1.2.3 The donors do not have chronic diseases, chronic infections, or systemic lesions that require long-term medication.

6.1.2.4 The donors have no other physiological status that affects sample collection.

6.1.3 Exclusion criteria

The donors with following situations should be excluded.

6.1.3.1 The donors have suffered chronic nervous system disease or injury that has affected their nervous function.

6.1.3.2 The donors have had a urinary disease and/or skin disease.

6.1.3.3 The donors have been diagnosed with a genetic disease and/or hematological disorders.

6.2 Selection of pregnant women donors

The requirements for healthy pregnant women donors are as follows.

6.2.1 The donors have no family history of genetic defects.

6.2.2 The donors have no cancer, tumor, or other malignant diseases.

6.2.3 The donors have no chronic virus infection, such as hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV), Epstein–Barr virus (EBV), and cytomegalovirus (CMV), and *Treponema pallidum* (TP), etc.

6.2.4 The donors have no severe pregnancy-related diseases, such as precursor epilepsy, pregnancy nephropathy syndrome, etc.

6.3 Sample collection

6.3.1 The sources of adult cells for iPSC generation usually include peripheral blood (CD34-positive cells or mononuclear cells), skin (fibroblasts), urine (renal epithelial cells), umbilical cord

(mesenchymal cells) and umbilical cord blood.

6.3.2 For peripheral blood, 5–10 mL is usually required and collected in tubes with heparin. Ficoll density gradient centrifugation is used to isolated the peripheral blood mononuclear cells (PBMCs) with further expanding [6].

6.3.3 Arm skin is typically collected using a 6-mm puncture biopsy. After collection, the skin punch is immediately placed in a 15-mL Falcon tube preloaded with Dulbecco's modified Eagle medium (DMEM; including 1% penicillin-streptomycin), ensuring that the liquid level exceeds the tissue level. The fibroblast cells are then cultured for iPSC induction.

6.3.4 Renal epithelial cells in urine can be isolated from 30–50 mL of urine and then expanded for iPSC generation.

6.3.5 For umbilical cord samples, a cord of 10–15 cm in length is collected by a health professional; the cord's stromal cells are then isolated from the cord tissue and further cultured. It is necessary to collect 5–10 mL of cord blood in tubes with heparin to generate blood-derived iPSCs.

6.3.6 It is worth noting that iPSCs induced by different sources of somatic cells have significant differences in immunogenicity. Because of the low immunogenicity of CD34-positive cells derived from cord-stromal cells or cord blood, iPSCs induced from CD34-positive cells also have low immunogenicity and are suitable for clinical tissue repair [7]. Also, due to the donor variability, significant differences can exist in cell properties, functions, and biological activities of cells and tissues from an autogenous source. Therefore, a particular quality control system should be established for the production process and intermediate products to ensure that the products meet the minimum quality standards and are traceable throughout the process. The donor materials used to produce the cell products should be prepared from heterogeneous sources following clinical standards to establish a cell production system and ensure the cell products' stability and reliability for clinical treatment.

6.4 Methods of generating iPSCs

iPSCs are usually induced from somatic cells by transduction of retroviral or Sendai viral vectors, or transfection of plasmids that express OCT4, SOX2, MYC, KLF4 or OCT4, SOX2, and NANOG [1, 8]. Although these methods have long been used, many problems related to clinical application still exist. First, the method of virus induction remains a safety concern when the product is applied to patients in the clinic, while the random integration of transgenes in the genome may lead to insertional mutagenesis and tumorigenicity.

Furthermore, traditional methods' induction efficiency remains very low (0.01%–0.30%) [9]. In contrast, the induction time of iPSCs is relatively long (sometimes 1–2 months for full preparation), which can be inconvenient for clinical application in patients [9]. Small molecules and nonintegrated expression vectors are used to induce iPSCs and to improve safety. For example, the use of small molecules or recombinant proteins with precise chemical composition and no animal origin, combined with nonintegrated expression vectors to induce iPSCs, avoids the safety problems in traditional methods [8, 10]. Moreover, various physical interventions and small chemical molecules

can effectively improve iPSC induction efficiency and accelerate the induction process. Combining these methods can provide a safer and more efficient approach for inducing clinical-grade iPSCs and accelerating their clinical application.

The process of generating iPSCs is listed below.

(1) The required cells are isolated from the individuals' skin tissue, blood, urine, or umbilical cord. Usually, the skin fibroblasts, blood mononuclear cells, or urine epithelial cells are cultured several days before iPSC generation.

(2) The cultured cells are expanded in a specific medium to favor the tissue cells' growth.

(3) Nonintegrating vectors are used to transfect the appropriate somatic cells, followed by culturing in a human ES cell medium containing small molecules and/or recombinant proteins to induce iPSCs. This process usually takes 2–3 weeks, at which point iPSC colonies are appeared.

(4) After the iPSC clones' appear, an individual colony is selected, and the cells are expanded in an iPSC maintenance medium. After 4–5 passages, the iPSCs are frozen and stored in liquid nitrogen.

(5) The iPSCs with typical ES cell morphology are selected for further characterization, such as the expression of pluripotency markers, alkaline phosphatase staining, karyotype analysis, and directed differentiation.

7 Characterization and quality standards of iPSCs

7.1 iPSC banking

7.1.1 Presently, clinical applications use autogenous or allogeneic iPSCs. Due to a shorter preparation cycle and broader applications, allogeneic cell therapy is more promising than personalized treatment. However, since iPSCs from allogeneic sources are related to the legitimacy of donors and the homogeneity of cell quality, it is necessary to strengthen the effective management and supervision of iPSCs for clinical therapy. Therefore, an iPS cell bank must be established that meets the ethical requirements and quality control standards required for clinical application. This cell bank must comply with the relevant provisions of international guidelines set by the International Society for Stem Cell Research (ISSCR), the regulations for the preparation of animal cell matrix for the production and application of biological products, the relevant contents of the quality management specifications for cell bank, and GMP standards issued by China Pharmaceutical Biotechnology Association.

7.1.2 iPSCs stored in a cell bank should have a legal source and reliable quality. The cell bank should conduct strict quality assurance for iPSC lines from different sample sources and select the strains with the best indicators for undifferentiated proliferation under GMP conditions. iPSC lines in the cell bank should also meet the Pharmacopeia's requirements of the People's Republic of China on the construction of three-level cell banks, such as the establishment of a seed bank, a master cell bank, and a working cell bank. Each cell line in the cell bank should have a strict registration management

system, with the record of cell preservation conditions being regularly checked and periodical testing being performed on cells' biological activity.

7.2 Quality control of iPSCs

7.2.1 iPSCs cannot be directly used for clinical treatment of related diseases until iPSC-derived cells are approved for clinical applications by the regulatory agencies. Except for the international guidelines, each country has its additional requirements. Presently, the functional cells or progenitors/precursors differentiated from iPSCs are being used in clinical trials, including iPSC-derived dopaminergic precursor cells for Parkinson's disease and iPSC-derived retinal pigment epithelial cells for retinal degeneration [11, 12].

7.2.2 The quality characteristics of iPSCs determine the therapeutic effect of the differentiated functional cells. Therefore, iPSCs must meet the quality standards of clinical-grade products [5, 13]. Since iPSCs have special biological properties, the quality controls are different from those of other biological products; they mainly include cell short tandem repeat (STR) identification, karyotype analysis, alkaline phosphatase assay, telomerase activity, immunological response, and cell surface molecular markers, etc. Since the most important feature of iPSCs is the ability to differentiate into various somatic cells, tissues, and organs, the differentiation ability of iPSCs must be tested via a teratoma formation assay in nude mice. Also, the iPSCs' ability to differentiate into NSCs should be determined for the specific purpose of clinical application in neural diseases.

7.2.3 Notably, the cells' *in vitro* culture inevitably cause genetic alterations, such as chromosomal abnormalities and genomic mutations, which may link to carcinogenesis. Chromosomal abnormalities in cells are monitored by karyotype analysis after certain passages. Recently, next-generation sequencing (NGS) has been applied to detect genomic mutations. However, smaller genomic mutations, such as single nucleotide variations, are difficult to evaluate and can be controversial due to data acquisition and interpretation problems. NGS technology still faces difficulties in covering whole-genome sequences. Moreover, the link between genomic mutations and mutations is uncertain, and many mutations in cancer genes are classified as having "unknown significance" [14]. Therefore, genomic mutations in the cells must be carefully monitored and interpreted.

7.3 Quality requirements and testing method of clinical iPSCs

The quality requirements and testing methods for iPSCs are summarized in Table 1.

7.3.1 Morphological characteristics

Similar to embryonic stem cells, the cell colonies of iPSC are flat, closely arranged, and regular in shape; the ratio of the cell nucleus to the cytoplasm is large, and there are apparent nucleoli.

7.3.2 Cell viability

Cell viability is analyzed by Annexin V and propidium iodide (PI) staining using flow cytometry. The percentage of live cells in the whole cell population can be calculated using the ratio of total live/total cells (live and dead).

Table 1 Quality requirements and testing method for iPSCs.

Test item	Specification	Test method
Cell morphology	Consistent with the morphology of iPSC	Microscopy
Cell viability	≥ 85% (live cells) or ≥ 70% (frozen cells)	Annexin V and PI staining by flow cytometry
Bacteria	Negative	Chinese Pharmacopeia 2020, IV, 1101
Fungus	Negative	Chinese Pharmacopeia 2020, IV, 1101
Endotoxin	< 2.0 EU/mL	Chromogenic substrate Limulus amebocyte lysate (LAL) assay; Chinese Pharmacopeia 2020, IV, 1143
Mycoplasma	Negative	Chinese Pharmacopeia 2020, IV, 3301
HCV, HBV, EBV, CMV, HIV, and TP	Negative	ELISA or quantitative PCR
Cell cycle	In the period of G0\S\G2\M and no sub-G1	Flow cytometry
Cell doubling time	Report result	Cell culture
STR (short tandem repeat) analysis	Single source	PCR
Chromosome karyotype analysis	46, XX or 46, XY	Fluorescence *in situ* hybridization
OCT4-positive rate	≥ 70%	Flow cytometry
NANOG-positive rate	≥ 70%	Flow cytometry
alkaline phosphatase (AP)-positive rate	≥ 95%	AP colorimetric assay
Telomerase activity	Report result	Quantitative PCR or immunoassay
Differentiation ability (*in vitro*)	Differentiated NSCs	Cell culture
Differentiation ability (*in vivo*)	Form teratomas with three germ layers: endodermal, mesodermal, and ectodermal	Teratoma formation assay
Genomic mutations	Report result	Next-generation sequencing

7.3.3 Sterility

Contaminated bacteria and fungus are detected by the microbiological examination method outlined in the Part IV of the *Pharmacopoeia of the People's Republic of China (2020 Edition)*, entry 1101. For release tests, a bacterial (fungal) contamination test kit is used in biological products.

7.3.4 Detection of mycoplasma

Mycoplasma contamination is assessed using the mycoplasma test method detailed in the Part IV of the *Pharmacopoeia of the People's Republic of China (2020 Edition)*, entry 3301.

7.3.5 Endotoxin test

Endotoxin levels are measured using the bacterial endotoxin test method described in the Part IV of the *Pharmacopoeia of the People's Republic of China (2020 Edition)*, entry 1143.

7.3.6 Virus detection

Tests for HBV, HCV, HIV, EBV, CMV, and TP are mandatory.

7.3.7 Cell cycle check

Cells are first stained through the infiltration of PI into DNA and then examined by flow cytometry to determine the cell cycle distribution. The process is as follows:

(1) Collect about 1×10^6 cells and the original culture solution, mix and add the mixture to a 15-mL centrifuge tube;

(2) Centrifuge at 1,000 rpm for 5 min (short-time and low-speed centrifugation);

(3) Discard the supernatant and add 1.5 mL of pre-cooled phosphate-buffered saline (PBS), centrifuge at 1,000 rpm for 5 min to remove the cell fragments in PBS and cell suspension;

(4) Add 1.5 mL of pre-cooled PBS and 3.5 mL of absolute ethanol, mix and fix the solution at 4 °C for 30 min or at−20 °C for long-term storage;

(5) Centrifuge at 1,000 rpm for 5 min to absorb ethanol, clean and mix with PBS;

(6) Centrifuge at 1,000 rpm for 5 min to remove the remaining ethanol from the cells;

(7) Remove PBS from the centrifugation tube and add 200 μL of PBS and 2 μL of RNase (0.25 mg/mL, incubated at 37 °C for 30 min);

(8) Add 0.5 mL of PI solution (50 μg/mL) to the centrifuge tube and maintain for 30 min in the dark at room temperature;

(9) Filter the cells (using 300-μm nylon mesh) from the centrifuge tube to an Eppendorf tube containing PBS (PI has strong adhesiveness and can easily cause cells to agglomerate), and mark the Eppendorf tube appropriately;

(10) Use appropriate equipment to detect and analyze the proportion of cells in each cell cycle.

7.3.8 Cell doubling time

Cells at an appropriate density (~100,000 cells/mL) should be placed in 96-well culture plates to determine the cell doubling time. On the 3rd and 5th day of culture, CCK-8 is detected and calculated using the following formula: $DT = t \lg 2 / (\lg N_t - \lg N_o)$, where t is the culture time; N_o is the number of cells recorded for the first time; and N_t is the number of cells at time t [15].

7.3.9 Cell authentication by STR analysis

STRs are formed by core repeat units connected from head to end many times. The structure of the core sequence in each STR is the same, the length of the STR is 2–6 base pairs, but the number of repeat units and the size of the repeat region differ in cells from different donors. Thus, the distribution of STRs in different races and diverse populations is very different, which constitutes the genetic polymorphism of STRs. The repeat times of a homologous STR locus among various individuals also differ; therefore, STR locus analysis can similarly identify individuals with fingerprint recognition. Gene files can be created by detecting specific sequence repeats in an individual genome at specific location points [16].

Detection requirements: Samples for STR detection are human-derived cells from a single source with 21 gene loci that must be examined, which are D5S818, D13S317, D7S820, D16S539, VWA, TH01, TPOX, CSF1PO, D3S1358, D2S441, D2S1338, Penta E, D10S1248, D19S433, D21S11, D18S51, D6S1043, D8S1179, D12S391, FGA, and Amelogenin [17].

7.3.10 Chromosome karyotype analysis

For karyotype analysis, the cultured cells are treated with colchicine at 37 °C for 60 min. After dissociation, the cells are incubated with 0.075 mol/L KCl in a 37 °C water bath for 15 min. Finally, the cells are fixed with 3–5 drops of fresh fixative (3:1, ethanol : acetic acid) for karyotype analysis or stored at 4 °C until analysis.

The cell growth criteria for karyotype analysis are as follows:

(1) Cells are integrated with clear contours, and chromosomes are distributed at the same level;

(2) Chromosome morphology and distribution are in a good state;

(3) Cell-on-cell overlap is not observed, when overlap exists, it should be clearly identified;

(4) Observed cells are in the same mitotic stage, i.e., the degree of chromosome helization or the chromosome's length is roughly the same;

(5) Around the observed cells, there are no discrete single or multiple chromosomes.

The standard of karyotype analysis is: Karyotypes are determined according to the nucleus' characteristics, including the number, shape, size, etc. They are divided into seven groups (A, B, C, D, E, F, and G) and a group of sex chromosomes. An ideogram is required to show the karyotype in the form of a pattern map.

7.3.11 Expression of stemness markers

The rates of OCT4 and NANOG positive cells in the whole population are detected by flow cytometry using antibodies against human OCT4 and NANOG.

7.3.12 Alkaline phosphatase assay

Alkaline phosphatase (AP or ALP) is a type of hydrolase that can remove the phosphate group on the substrate molecule and generate phosphate ions and free hydroxyl by hydrolyzing phosphate monoester. 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) is a typical substrate of alkaline phosphatase. BCIP is hydrolyzed under the action of alkaline phosphatase, and the hydrolysate reacts with NBT to form an insoluble dark blue to blue-purple NBT formazan substance, which can be used to visualize AP-positive iPSCs.

The staining process is as follows:

(1) Cells are fixed in a 24-well plate with 4% PFA at room temperature for 30 min before being washed in the 24-well plate with PBS 3–5 times for 3–5 min per wash;

(2) A proper volume of AP dye working solution is prepared according to the proportion of buffer

solution : dye solution BCIP:dye solution NBT = 3 mL:10 μL:20 μL;

(3) After the last wash, the wash solution is removed, and 200 μL of AP dye working solution is added;

(4) Cells are incubated at room temperature in the dark for 5–30 min or longer until the color development reaches the desired effect;

(5) The working solution of AP staining reagent is removed, and cells are washed with distilled water once or twice to stop the reaction;

(6) If necessary, neutral red staining is used to facilitate iPSC colony observation.

7.3.13 Telomerase activity

The activity of telomerase can be detected by quantitative PCR or an immunoassay. Using SYBR green screen light dye as a marker signal, combined with telomere repeat amplification, all kinds of DNA templates can be amplified conveniently using the specific primers to telomerase or telomere. Also, the double antibody sandwich method can also be used to measure the expression of human telomerase. The human telomerase antibody is coated on a microporous plate to produce a solid-phase antibody. The iPSC lysate is then added to the microporous coated plate and combined with the horseradish peroxidase (HRP)-labeled secondary antibody to form an antigen–primary antibody–secondary antibody complex. After washing, the substrate 3,3′,5,5′-tetramethylbenzidine is added to detect color intensity; this substrate is converted to blue under the catalysis of the HRP enzyme and finally to yellow. The color intensity is positively correlated with the expression and activity of telomerase in the samples. The absorbance is measured at 450 nm (OD) by an enzyme-labeling instrument, and telomerase activity in the sample is calculated using a standard curve.

7.3.14 Differentiation ability test (*in vitro*)

iPSCs are cultured in T25 culture flasks or 6-well culture plates; they are induced for neural differentiation according to the standard NSC-differentiation protocol [18]. After differentiation, a fluorescence immunoassay is conducted to examine the specific markers of NSCs (i.e., NESTIN, SOX1, PAX6, OLIG2, and GFAP) and identify the neural differentiation ability of iPSCs.

7.3.15 Differentiation ability test (*in vivo*)

The differentiation ability of iPSCs is tested according to previously published references. Briefly, the iPSCs are implanted into immunodeficient mice at the following sites: (1) intramuscular, (2) subcutaneous, (3) under the testis capsule, and (4) under the kidney capsule. At 6–12 weeks from implantation, the teratomas formed are removed and analyzed by histopathological staining [19].

8 Preparation of NSCs derived from iPSCs

iPSCs can differentiate into NSCs, which have the potential to differentiate into various types of neurons, astrocytes, and oligodendrocytes while preserving the ability of self-renewal [20]. Protocols

for differentiating NSCs from iPSCs *in vitro* have been published in numerous research papers [21, 22]. However, some problems still exist, such as inconsistent preparation methods and uneven cell quality [23].

First, the iPSCs that are to be differentiated from NSCs should come from strictly identified iPSC lines to ensure reliability. Second, the preparation of NSCs for clinical treatment should also ensure that they meet the preparation conditions of GMP. A perfect and effective quality control system should be established, and the cells must be prepared in accordance with the *Technical Guidelines for Research and Evaluation of Cell Therapy Products* issued by China Food and Drug Administration. Also, to ensure safety in clinical treatment, the NSCs should be of high purity; measures should be taken to remove residual iPSCs that may be contained in NSC products.

Traditionally, the neural sphere formation assay has been used to differentiate iPSCs from NSCs. However, due to the structural limitations of neural spheres, this process has some issues, including uneven differentiation and low differentiation efficiency. To date, a method for achieving neural differentiation of iPSCs via a monolayer cell culture combined with small molecular compounds has been most effective at improving differentiation efficiency; therefore, it provides a feasible protocol for producing high-quality NSCs [24, 25].

The directed differentiation of NSCs from iPSCs includes the following stages:

(1) iPSCs should be spread onto suitable culture plates and cultured in an NSC-differentiation medium. The culture medium is changed every 2–3 days for 18–20 days. Briefly, the iPSCs are resuspended in STEMdiff Neural Induction Medium (Stemcell Technologies) supplemented with 10-μmol/L Y-27632 and seeded onto Matrigel (BD Bioscience) pre-coated culture plates at a density of 10,000–25,000 cells/cm^2. After seeding, the medium is replaced daily with a fresh STEMdiff Neural Induction Medium without Y-27632. On days 6–9, the cell cultures will be confluent and ready for passage using TrypLE Select (Thermo Fisher Scientific) cell dissociation reagent. After the first passage, the cells should be passaged once they reach ~80% confluency and adjusted to a cell density of about 10,000–25,000 cells/cm^2. Y-27632 should be added to the medium at day 1 of each passage to ensure the cell attachment and then removed from the medium at day 2. After the 3rd passage, the cells should be cultured with STEMdiff Neural Progenitor Medium (Stemcell Technologies) to maintain NSC growth.

(2) After culturing for about 20 days, the cultured cells will show a nerve rosette structure, which indicates that the directional differentiation is complete.

(3) The cell samples are cultured on climbing slices, and the specific markers on the cell surface are detected by immunofluorescence. The differentiated cells can also be preserved in a liquid nitrogen tank.

9 Quality control

9.1 Quality standard of clinical-grade NSCs

NSCs derived from different iPSC lines by diverse methods have various cellular attributes that

specifically affect their biological function [26]. Therefore, studies on the biological properties and specific quality standards of NSCs are beneficial to furthering research and applying cell products. Such tasks should include several aspects, i.e., the biological characteristics of NSCs (e.g. cell surface markers, cell electrophysiological activity, cell doubling time, chromosome karyotype analysis, cell STR identification, and cell differentiation ability, etc.), clinical application-related biological attributes (e.g. cell immunogenicity, cell viability, and telomerase activity, etc.), clinical-grade standards for cell products (e.g., aseptic, low endotoxin, virus-free, mycoplasma-free), as well as the tumorigenicity/oncogenicity of cells (Table 2).

For clinical application, the best formulation for a cell drug product should be obtained through relevant research on the final product form, including research into packaging materials, transportation method, validity period, and other aspects of NSCs.

Most of the quality testing items of NSCs are the same as those used for iPSCs, but some specific detection items for NSCs are described below.

9.2 Positive rate of PAX6 and NURR1

The NSCs are cultured and grown on cell climbing slices. After the cell confluency reaches 90%, the cells are examined by immunostaining with PAX6 and NURR1 antibodies after a standard immune-ofluorescence staining protocol. They are then observed and counted under a fluorescence microscope.

Table 2 Quality testing items and standards for clinical-grade NSCs

Test item	Specification	Testing method
Cell morphology	Consistent with the morphology of NSC	Microscopy
Cell viability	≥ 85% (live cells) or ≥ 70% (frozen cells)	Annexin V and PI staining by flow cytometry
Bacteria	Negative	Chinese Pharmacopeia 2020, IV, 1101
Fungus	Negative	Chinese Pharmacopeia 2020, IV, 1101
Mycoplasma	Negative	Chinese Pharmacopeia 2020, IV, 3301
Endotoxin	< 2.0EU/mL	Chromogenic substrate LAL assay; Chinese Pharmacopeia 2020, IV, 1143
HCV, HBV, EBV, CMV, HIV, and TP	Negative	ELISA or quantitative PCR
Cell cycle	In the period of G0\S\G2\M and no sub-G1	Flow cytometry
Cell doubling time	Report result	Cell culture
STR analysis	Single source	PCR
Chromosome karyotype analysis	Expected 46, XX or 46, XY	Fluorescence *in situ* hybridization
PAX6-positive rate	≥ 80%	Flow cytometry
NURR1-positive rate	≥ 80%	Flow cytometry
Differentiation ability	Differentiate into dopamine neurons	Cell culture
Immunological reaction	Report result	PBMC coculture proliferation assay
Telomerase activity	Report result	Quantitative PCR or immunoassay
Tumorigenecity	Soft agar colony formation test and *in vivo* detection show no clone or tumor formed	Chinese Pharmacopeia 2020, III
Genomic mutations	Report result	Next-generation sequencing

9.3 Differentiation ability test

NSCs are placed in culture plates and cultured according to the standard neuron differentiation protocol depending on the purpose of the endpoint application. For example, to treat Parkinson's disease, the NSCs will be tested for dopaminergic neuron differentiation. However, for CNS injury, the NSCs will be tested for motor neuron differentiation ability. Thus, the testing depends on the end application. After differentiation, a fluorescence immunoassay is performed according to the labeling requirements of neurons, astrocytes, and oligodendrocytes to identify the directional differentiation ability of NSCs.

9.3.1 Dopaminergic neuron differentiation test

(1) In terms of reagents and medium, recombinant bFGF is reconstituted in 10 mmol/L Tris solution (pH 7.6) to prepare a 20 μg/mL stock solution. L-ascorbic acid is dissolved in double-distilled water to achieve a 200 mmol/L stock solution. Dopamine-inducing factors (Cat# DIF1) and neural stem cell media (Cat# NN1) are supplied from Neoneuron (USA). Poly-l-ornithine hydrobromide (PLO) is dissolved in double-distilled water at 0.16 mg/mL as a 10×stock solution.

Differentiation medium-A is prepared by mixing 37.5 mL of DIF1 (75%, v/v), 12.5 mL of NN1 medium (25%,, v/v), 25 μL of bFGF stock solution, 50 μL of L-ascorbic acid stock solution, and 500 μL of fetal bovine serum (FBS). Differentiation medium-B is prepared by mixing 12.5 mL of DIF1 (25%, v/v), 37.5 mL of NN1 medium (75%, v/v), 25 μL of bFGF stock solution, 50 μL of L-ascorbic acid stock solution, and 500 μL of FBS.

(2) Culture plates are pre-coated with PLO at a working concentration of 16 μg/mL for at least 2 h. At day 0, the NSCs are seeded onto the pre-coated plates and cultured with differentiation medium-A.

(3) The medium is changed daily, and NSCs are cultured in differentiation medium-A for 3 days.

(4) On day 4 (72 h after seeding), the culture medium is switched to differentiation medium-B. The cells continue to be cultured for another three days with daily medium replacement.

(5) On day 7, the cells are fixed with 4% paraformaldehyde and processed for immunocytochemistry using antibodies against dopaminergic neuron markers.

9.3.2 Motor neuron differentiation test

(1) Differentiation medium-A is prepared in DMEM/F12 basal medium supplemented with 0.2 μmol/L of LDN-193189 (Stemcell Technologies), 10 μmol/L of SB431542 (Stemcell Technologies), 10 ng/mL of brain-derived neurotrophic factor (R&D Systems), 0.4 μg/mL of l-ascorbic acid (Sigma), 2 mmol/L GlutaMAX-I supplement (Thermo Fisher Scientific), 1% N-2 supplement (Thermo Fisher Scientific), and 1% nonessential amino acids (NEAA; Thermo Fisher Scientific).

(2) Differentiation medium-B is prepared in Neurobasal medium (Thermo Fisher Scientific) supplemented with 2 mmol/L GlutaMAX-I, 2% B-27 (Thermo Fisher Scientific), 1% NEAA, 0.4 μg/mL of amino acids solution (Thermo Fisher Scientific), 10 ng/mL of GDNF (R&D Systems),

and 10 ng/mL of CNTF (R&D Systems).

(3) The culture plates are pre-coated with Geltrex (Thermo Fisher Scientific) at a concentration of 50–100 μL of Geltrex/cm^2 for 2 h. On day 0, the NSCs are seeded onto the pre-coated plates and cultured with differentiation medium-A. The medium is then changed daily.

(4) At day 3 (48 h after seeding), 1 μmol/L retinoic acid is added to differentiation medium-A and the cells are cultured for 1 day.

(5) On day 4, LDN-193189 and SB431542 are removed from medium-A and replaced with 1 μmol/L smoothened agonist (Santa Cruz) and 0.5 μmol/L purmorphamine.

(6) The cells are cultured until day 14, and at which point the medium is switch to differentiation medium-B for further culturing.

(7) The medium is then changed every 2–3 days. On day 21–28, the cells are fixed with 4% paraformaldehyde and processed for immunocytochemistry.

9.4 Immunological reaction detection

A PBMC proliferation assay (conducted as follows) is used to detect whether the coculture of NSCs and PBMCs causes the activation and proliferation of PBMCs.

(1) PBMCs are isolated from 5–10 mL of peripheral blood and counted.

(2) 200 NSCs are added to each well of a 96-well plate, and the PBMCs are cocultured at ratios of 1:100, 1:500, and 1:1000. Three replicates are used for each group. The same PBMC settings without NSCs (i.e., PBMCs alone) are also performed to serve as controls.

(3) NSCs and PBMCs are cocultured for 3 days. Subsequently, the cells are collected, and cell precipitation samples are taken by centrifugation to detect the value of MTT. Results are reported as the percentage of the proliferation rate of PBMCs represented by the MTT OD values using the following formula: $(OD_{coculture} - OD_{PBMCs\ alone})/OD_{PBMCs\ alone} \times 100\%$.

9.5 Tumorigenecity detection

9.5.1 Detection by soft agar colony formation

(1) Cells are collected at the logarithmic growth phase, and cell density is adjusted to 1×10^6 cells/mL with DMEM containing 20% FBS. Further dilution is completed according to experimental requirements.

(2) Low-melting point agarose solutions with concentrations of 1.2% and 0.7% are prepared from distilled water. After autoclaving, these are maintained at 40 °C without solidification.

(3) After 1.2% agarose is mixed with 2× DMEM medium (containing 2× antibiotics and 20% FBS) at a 1:1 ratio, 3 mL of the mixture is injected into a plate with a diameter of 6 cm (7–10 mL is injected into a 10-cm plate). The mixture is cooled and solidified; it can be used as bottom agar and stored in a CO_2 incubator for later use.

(4) After 0.7% agarose and 2× DMEM medium are mixed in sterile test tubes at a ratio of 1:1, 0.2 mL of cell suspension is added to the tube. This solution is thoroughly mixed and then injected into the bottom plate covered with 1.2% agar to form a double agar layer. After the upper agar is solidified, it is cultured in a 5% CO_2 incubator at 37 °C for 10–14 days.

(5) The plate is then placed under an inverted microscope to observe the number of cell clones and calculate the colony formation rate. Clinical-grade NSCs should not form any clone in the soft agar.

9.5.2 Detection *in vivo*

A certain number of NSCs are injected into the striatum area of nude mice (SPF-grade NPG mice) via a stereotaxic injection to detect tumorigenicity *in vivo*. Ten weeks after the injection, an anatomical examination is conducted to observe tumor formation in the transplanted sites and other organs. Tissue samples are also taken for pathological sections and stained for specific antibodies. Clinical-grade NSCs should not form any tumor in the nude mice.

10 Summary

Following the current literature and government regulations, standards have been set out that meet the quality requirements for preparing clinical-grade iPSC-derived NSCs to ensure patient safety and appropriate treatment outcomes. These standards cover the sample collection, processing, cell culture, formulation, and quality control of cell products. Higher standards of clinical-grade cell products will be produced with future advancements in this field.

Conflict of interests

The authors declare that they have no competing interests in this work.

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