Production and preservation of serum-free human umbilical cord-derived mesenchymal stem cell sheets

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Abstract

Background

Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) have attracted much attention because of their fast self-renewal, multipotent nature, painless collection, and compliance for standard amplification. Several types of culture media and systems have been used for large-scale expansion of UC-MSCs and preparation of UC-MSC sheets; however, most rely heavily on fetal bovine serum (FBS) and exogenous growth factors for optimal cell proliferation. Residual bovine serum albumin (BSA), a macromolecular protein in FBS, and exogenous growth factors in final products pose the potential threat of allergic reactions or tumorigenic risks in humans.

Methods

In this study, the production process of UC-MSC sheets was standardized, including donor screening, cell bank establishment and quality control, and cell sheet production and quality control. Serum-free culture medium and pharmaceutical grade coating matrix were used to form UC-MSC sheets to reduce the risk of BSA and exogenous growth factors in clinical application. Additionally, the preservation solution for and method of the UC-MSC sheet were developed for preservation and transportation before cell sheet transplantation. Then, quality standards of the UC-MSC sheet involving product characteristics, safety and functionality were formulated according to national and international regulations. Finally, the UC-MSC banks and the UC-MSC sheet products were identified according to the established quality standards.

Results

This technology enables UC-MSC sheets to be produced safely and stably while preserving the cell viability of UC-MSC sheets above 70% in 24 hours. The quality standards for the UC-MSC sheets were established in this study. Both the fresh and 24-hour preserved UC-MSC sheets met the established quality standards.

Conclusion

The preparation and preservation of clinical grade serum-free UC-MSC sheets provides the possibility for the clinical application of cell therapy.

Introduction

Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) are considered a promising cell source for autologous and allogeneic cell therapy because of their relatively low immunogenicity

compared to MSCs from adult counterparts, in which HLA-ABC is expressed very weakly (1, 2). UC-MSCs have strong biological activity and differentiation capacity even after repeated amplification (3). UC-MSCs are also compliant with standard amplification in potentially large quantities due to their fast self-renewing capacity(4, 5), and UC-MSC banks can be established to cater to market demand after drug approval. Therefore, UC-MSCs are considered a promising cell source for clinical cell therapy (6, 7).

In cell therapy, MSC transplantation is usually carried out by local or transcatheter injection of cell suspension. However, generally only 10–20% of injected cells are available at the injured area within a few hours or days after delivery, and only a few cells actively engraft in the affected tissue (8). In addition, attached cells are digested by enzymes to produce a cell suspension, which destroys cellular connexins and greatly reduces cell activity and function. Therefore, cell suspension injection causes significant loss and death of cells and uneven local distribution, which greatly reduces the expected therapeutic effects (8). Cell sheet technology, developed by Prof. Okano's team [9, 10], eliminates the problem of retention, helps to retain cells, and provides the appropriate lifespan of the transplanted MSCs. Using this technology, Cell sheets were prepared and improved cardiac function in mouse (9) and porcine (10) myocardial infarction models.

For extensive clinical promotion, robust cell sheet production, preservation and quality control systems are essential elements of successful cell therapy. In this study, combining hUC-MSCs with cell sheet technology, the preparation and preservation technology for serum-free UC-MSC sheets was developed for use in clinical trials and preclinical studies. First, a master cell bank (MCB) and several working cell banks (WCB) were established from each umbilical cord to ensure cell source quality for UC-MSC sheet production. Second, a serum-free UC-MSC sheet production technology was successfully developed to ensure drug safety and efficacy, from which the residue of risk substances such as bovine serum albumin (BSA) are qualified to be used in clinical applications. Third, an effective UC-MSC sheet preservation technology was developed to ensure storage and transportation, which can ensure that UC-MSC sheets are preserved for 24 hours with cell viability greater than 70%. Fourth, quality standards involving product characteristics, safety and functionality were established according to the "ICH Q5A: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin", "ICH Q6B: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products". "Guiding Principles for Quality Control of Stem Cell Preparations and Preclinical Research", "Guidelines for Cell Therapy Products and Evaluation Technology" and our research results.

UC-MSC sheets with tight cell connections and intact extracellular matrix express surface markers of mesenchymal stem cells, secrete a variety of growth factors, reduce the immune response, promote the formation of microvasculature and inhibit apoptosis of cardiomyocytes in vitro. Both the freshly prepared and the 24-hour stored cell sheets maintained these features and functions.

Methods

Ethics statements

Umbilical cord sample collection was approved by the OASIS International Hospital local ethics committee (No: LLPJ2018[001]). Written informed consent was obtained from the puerperas. Before parturition, puerperas should test negative for human immunodeficiency virus (HIV), human hepatitis B virus (HBV), human hepatitis C virus (HCV), *Treponema pallidum* (TP), human T-cell leukemia virus (HTLV), cytomegalovirus (CMV), Epstein–Barr virus (EBV). Umbilical cord samples were preserved in sterile saline at 4 °C after collection and transferred to the laboratory within 24 h.

Cell isolation and culture

The human umbilical cord was washed with PBS to remove blood. Then, blood vessels and tunica externa were removed, and Wharton's jelly was separated. After being cut into small pieces, Wharton's jelly was seeded into 100 mm petri dishes coated with FBS. Then, the Petri dishes were cultured at 37 °C, 5% CO_2, and 95% humidity with UC-MSC medium consisting of minimum essential medium-α (α-MEM, Corning) supplemented with 10% FBS (Gibco), 1% L-glutamine (Corning), 1% nonessential amino acid (NEAA, Gibco) and 20 IU/mL bFGF (Langtai). UC-MSCs that migrated from the explants were defined as P0 UC-MSCs.

When reaching 90% confluence, UC-MSCs were detached using TrypLE (Gibco) and passaged to new flasks at a cell density of 2×10^4 cells/cm^2. P1 UC-MSCs cryopreserved at a density of 2×10^6 cells/mL in cell freezing medium (FBS with 10% DMSO (Sigma)) were defined as MCBs. UC-MSCs recovered from MCB and were passaged to P4 as mentioned above. Then, UC-MSCs in P4 were cryopreserved and defined as WCBs.

Cell sheet production

UC-MSCs from the WCB were recovered and cultured to 100% confluence. After being treated with TrypLE, UC-MSCs were washed three times with PBS to remove exogenous FBS and bFGF. Then, the UC-MSCs were suspended in cell sheet forming medium, which consisted of α-MEM supplemented with HSA (Shandong Taibang). A total of 6×10^7 cells were seeded into 100 mm temperature-responsive culture dishes (ThermoFisher) and cultured at 37 °C with 5% CO_2 and 95% humidityovernight. Then, the UC-MSC sheet was detached from the temperature-responsive culture dishes at room temperature (20-25 °C).

Cell number, cell viability and cell apoptosis assays

Cell number and viability were evaluated by AOPI staining and automatic fluorescent cell counter (Countstar S2) detection according to the manufacturers' instructions. Cell apoptosis was tested with the Annexin V-FITC Apoptosis Detection Kit (eBioscience) according to the manufacturer's instructions.

Samples were prepared as follows. For MCB and WCB cells, UC-MSCs were recovered and suspended in MSC medium for detection. For UC-MSC sheets, the cell sheet was digested by TrypLE into a single cell suspension for detection.

Cell growth curve

UC-MSCs in MCB and WCB were recovered and seeded into 96-well plates at a concentration of 6,000 cells/well in MSC medium. Cell growth was evaluated each day by CCK-8 assay (TransGen) according to the manufacturer's instructions.

Cell cycle analysis

UC-MSCs in MCB and WCB were recovered and seeded in T75 flasks at a cell density of 2×10^4 cells/cm^2. When reaching 60%-70% confluence, UC-MSCs were harvested, and the cell cycle was tested with a Cell Cycle Detection Kit (KeyGEN Biotech) according to the manufacturer's instructions.

CFU assay

UC-MSCs in MCB and WCB were recovered and seeded in 96-well plates at a concentration of 1 cell/well. After culturing for 14 days, wells containing 50 or more cells were counted with a microscope (CKX41, OLYMPUS, Japan).

Cell surface marker detection

UC-MSCs in MCB and WCB were recovered and seeded in T75 flasks at a cell density of 2×10^4 cells/cm^2. When they reached 90-100% confluence, UC-MSCs were harvested for cell surface marker detection. For UC-MSC sheets, the cell sheet was digested using TrypLE into a single-cell suspension for detection. UC-MSCs were aliquoted into 1 $\times10^6$ cells/tube. Then, anti-CD73-FITC (BD), anti-CD90-FITC (BD), anti-CD105-APC (BioLegend), anti-CD11b-FITC (BD), anti-CD19-FITC (BioLegend), anti-CD34-PE (BioLegend), anti-CD45-FITC (BD), anti-HLA-DR-FITC (BD), anti-IgG-FITC (BD), anti-IgG-PE (BD) and anti-IgG-APC (BD) were added to the tubes separately. After staining for 30 minutes at room temperature in the dark, the cells were washed twice with PBS and resuspended in staining buffer for flow cytometry analysis.

Differentiation assays

UC-MSCs in MCB and WCB were recovered and resuspended in MSC medium for differentiation. For UC-MSC sheets, the cell sheet was digested by TrypLE into a single cell suspension for differentiation.

For adipogenic differentiation, cells were seeded in 24-well plates at a density of 8×10^4 cells/well and cultured at 37 °C, 5% CO_2, and 95% humidity. When the cells reached 100% confluence, the culture medium was changed to adipogenic differentiation medium (BI) to induce differentiation.
After culturing for 14-21 days with medium change every three days, the cells were fixed and stained with an MSC Adipo-Staining Kit (VivaCell, Shanghai, China).

For osteogenic differentiation, cells were seeded in 24-well plates at a density of 4×10^4 cells/well and cultured at 37 °C, 5% CO_2, and 95% humidity. When the cells reached 70% confluence, the culture medium was changed to osteogenic differentiation medium (BI) to induce differentiation. After culturing for 14-21 days with medium change every three days, the cells were fixed and stained with an MSC Osteo-Staining Kit (VivaCell, Shanghai, China).

For chondrogenic differentiation, cells were seeded in 15 mL tubes at a density of 4×10^5 cells/tube in chondrogenic differentiation medium (BI) as pellets and cultured at 37 °C, 5% CO_2, and 95% humidity for 14-21 days with medium change every three days. The cells were fixed and stained with an MSC Chondro-Staining Kit (VivaCell, Shanghai, China).

Growth factor detection assays

Cell banks: Cells in MCB and WCB were recovered and seeded in T75 flasks at a cell density of 2×10^4 cells/cm^2. Twenty-four hours before cell passage, the culture medium was replaced with 10 mL of fresh MSC medium. When passaging cells, the culture medium was collected and centrifuged at 300×g for 5 min to remove dead cells for growth factor detection. The number of viable cells was monitored using AOPI in the automatic fluorescent cell counter to calculate the amount of factor secretion per 1×10^6 cells in 24 hours.

Cell sheet forming medium: Cell sheet forming medium was collected and centrifuged at 300×g for 5 min to remove dead cells for growth factor detection.

Cell sheet reattachment medium: The freshly produced and 24-hour preserved cell sheets were attached to 100 mm petri dishes in MSC medium and cultured for 24 hours. The medium was collected and centrifuged at 300×g for 5 min to remove dead cells for growth factor detection.

HGF (Invitrogen), VEGF (NOVUS), IL-8 (R&D), and IL-6 (NOVUS) were quantified using commercial ELISA kits following the manufacturers' instructions.

High-risk substance residue detection assays.

The final cell washing solution before cell sheet fabrication was used for bFGF residue detection following the manufacturer's instructions of the Human bFGF ELISA Kit (Life Technologies).

Cell sheets were digested with 3 mL TrypLE, and the digestion was quenched with 7 mL PBS. The suspension was centrifuged at 300×g for 5 min, and the supernatant was harvested for HSA residue detection following the manufacturer's instructions of the Human Albumin ELISA Kit (BETHYL).

Cell sheets were digested with 1 mL TrypLE supplemented with 50 μL 20% HSA, and the digestion was quenched with 1 mL cell sheet forming medium. The suspension was centrifuged at 300×g for 5 min, and the supernatant was harvested for BSA and gentamicin residue detection following the manufacturer's instructions of the Bovine Albumin ELISA Kit (BETHYL) and GENTAMINCIN ELISA Kit (REAGEN), respectively.

Cell sheets were harvested and lysed with 5 mL lysis buffer, which consisted of normal saline supplemented with protease inhibitor (Roche), by a liquid nitrogen repeated freeze–thaw method. The lysate was centrifuged at 12000×g for 20 min at 4 °C, and the supernatant was harvested for TrypLE and

fibrinogen residue detection following the manufacturers' instructions of the TrypLE ELISA Kit (JunYan) and Human Fibrinogen ELISA Kit (Novus), respectively.

The residual amount of bFGF is presented in pg/mL. The residual amounts of BSA, HSA, gentamicin, TrypLE and fibrinogen are presented in ng per cell sheet.

Immunoregulatory test assay

UC-MSCs in MCB and WCB were recovered and seeded in 6-well plates at a density of 5×10^5 cells/well in MSC medium. Cell sheets were digested as described in the "cell number, cell viability and cell apoptosis assays" and seeded in 6-well plates at a density of 5×10^5 cells/well in MSC medium. After overnight culture, the cells were treated with 10 μg/mL mitomycin to inhibit division. Then, human peripheral blood mononuclear cells (PBMCs) were recovered and seeded into wells with or without MSC cells in PBMC medium, which consisted of RPMI 1640 (Gibco) supplemented with 10% FBS, 1% L-glutamine and 1% NEAA, at a density of 1×10^6 cells/well.

For Th1 lymphocyte detection, PBMCs were cocultured with UC-MSCs overnight. Cell Stimulation Cocktail (plus protein transport inhibitors) (500X) (eBioscience) was added to the wells for Th1 lymphocyte activation according to the manufacturer's descriptions. PBMCs cocultured with UC-MSCs without activation were used as a negative control. PBMCs cultured alone with activation were used as a positive control. Then, the proportions of Th1 lymphocytes were detected by anti-CD3-APC (BD), anti-CD8-FITC (BD) and anti-IFNγ-PE (BD) staining. A minimum of 10,000 events were acquired on a BD FACS Canto II flow cytometer, and the proportions of $CD3^+CD8^-IFN\gamma^+$ Th1 lymphocytes were analyzed using FlowJo software.

For lymphocyte proliferation detection, 10 μg/mL PHA-M (Sigma) was added to activate PBMCs cocultured with UC-MSCs. PBMCs cocultured with UC-MSCs without activation were used as a negative control. PBMCs cultured alone with PHA-M activation were used as a positive control. After 3 days of coculturing, 10 μM BrdU was added to each well overnight. Then, the PBMCs were harvested and stained with a BrdU Staining Kit (eBioscience) according to the manufacturer's instructions. A minimum of 10,000 events were acquired on a BD FACS Canto II flow cytometer, and the proportions of BrdU-positive proliferating lymphocytes were analyzed using FlowJo software.

For TNFα detection, the culture medium in the lymphocyte proliferation detection before BrdU staining was harvested for TNFα detection by the Human TNFα ELISA Kit (Invitrogen) according to the manufacturers' descriptions.

Angiogenesis assay

Human umbilical vein epithelial cells were seeded in 48-well plates precoated with 100 μL Matrigel (BD) at a density of 1,000 cells/well. Conditioned cell sheet-forming medium was added to the wells

and cultured for 12 hours. Fresh cell sheet forming medium was used as a negative control. Images were taken using an inverted microscope and analyzed using an angiogenesis analyzer.

Cardiomyocyte apoptosis inhibition assay

H9C2 rat cardiomyocytes were seeded in 6-well plates at a density of 15000 cells/cm^2. When the cells reached 80% confluence, 300 μM $CoCl_2$ was added to the culture medium for 24 hours to induce H9C2 apoptosis. Conditioned cell sheet-forming medium was added to the wells and cultures for 24 hours. Fresh cell sheet forming medium was used as a negative control. Cell apoptosis was tested with the Annexin V-FITC Apoptosis Detection Kit (eBioscience) according to the manufacturer's instructions.

Immunofluorescence staining

UC-MSC sheets were fixed with 4% paraformaldehyde and embedded in the optimal cutting temperature compound (Sakura Finetek). After being cut into 10 μm sections using a cryostat (Leica), the cell sheets were blocked for 1 hour at room temperature with blocking buffer, which consisted of PBS supplemented with 2% BSA, and then labeled with primary antibodies against fibronectin (Abcam) or integrin β1 (Abcam) at 4 °C overnight, followed by incubation with FITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) for visualization at room temperature for 2 hours. Nuclei were counterstained with Hoechst (Life Technologies) at room temperature for 10 min. Then, the sections were assessed by confocal laser-scanning microscopy.

STR Authentication

UC-MSCs in MCB and WCB and cell sheets were prepared as described in the "cell number, cell viability and cell apoptosis assay". One million cells were collected and used for STR authentication by the fluorescence STR method. The detected gene loci included D5S818, D13S317, D7S820, D16S539, VWA, TH01, TPOX, CSF1PO, D3S1358, Penta E, D2S441, D2S1338, Penta D, D10S1248, D19S433, D21S11, D18S51, D6S1043, D8S1179, D12S391 and FGA.

Telomerase activity detection

UC-MSCs in MCB and WCB and cell sheets were prepared as described in the "Cell number, cell viability and cell apoptosis assay" section. One million cells were collected and used for telomerase activity detection with the fluorescence real-time quantitative PCR detection kit for telomerase activity (human) (KeyGEN BioTECH) according to the manufacturer's instructions.

Sterility testing

The culture method was used for sterility testing. Briefly, cell sheets were digested with 3 mL TrypLE (Gibco) at 37 °C for 3 minutes. After quenching of the digestion with 7 mL PBS, 3 mL of the cell suspension was used for sterility testing. For MCB and WCB cells, UC-MSCs were thawed, and 3 mL of the

cell suspension was used for sterility testing. After membrane filtration and culture for 14 days, the results were evaluated.

Mycoplasma detection

Cell culture supernatant before cryopreservation and cell sheet-forming supernatant before cell sheet detachment were tested by the culture method and indicated cell culture method.

For the Q-PCR method, cells in MCB, WCB and cell sheets were prepared as described in the “Cell number, cell viability and cell apoptosis assay” section. One million cells were collected and used for mycoplasma detection with a Mycoplasma DNA Extraction and Purification Kit (Magnetic bead method) and Mycoplasma DNA Detection Kit (PCR-Fluorescent Probe Method), both from Houzhou Shenke, according to the manufacturers’ instructions.

Endotoxin test

Cell sheets were digested as described in the “Sterility testing”, and the digestion supernatant was used for the endotoxin test by the Gel Clot LAL Assay.

Exogenous Virus Detection

Exogenous virus was tested both in vitro and in vivo.

For in vitro different indicator cell inoculation and culture methods, monkey-derived Vero cells, human-derived MRC-5 cells and human MSC cells were used as indicator cells.

For in vivo methods, mouse intraperitoneal and intracerebral vaccination, suckling mouse intraperitoneal and intracerebral vaccination, 5-6 days of chicken embryo yolk sac vaccination and 9-11 days of chicken embryo allantois vaccination were conducted.

Bovine virus detection

The cell culture and fluorescent antibody detection methodswere used for bovine virus detection.

Human virus detection

Either cell culture supernatant or one million cells were used for HIV, HBV, HCV, CMV, EBV, TP, HB19, and HHV-6 detection using the following kits according to the manufacturers’ instructions: Human Immunodeficiency Virus Type I (HIV-1) Nucleic Acid Quantitative Determination Kit (PCR-Fluorescence Probe Method), Hepatitis B virus nucleic acid determination kit (PCR fluorescent probe method), Hepatitis C virus nucleic acid determination kit (PCR fluorescent probe method), Human cytomegalovirus nucleic acid quantitative detection kit (PCR fluorescence method), EB virus nucleic acid amplification (PCR) fluorescence quantitative detection kit, Treponema pallidum nucleic acid detection kit (fluorescence PCR method), Human cell virus B19 nucleic acid detection kit (PCR-fluorescent probe method) and Human

spore virus type 6 nucleic acid detection kit (PCR-fluorescent probe method). The HIV, HBV, HCV, CMV, EBV, HB19 and HHV-6 kits were from Zhongshan Da'an, and the TP kit was from Suzhou Tianlong.

Retroviral detection

TM-PERT method was used for retroviral detection

Results

Standardize the process of large-scale production of UC-MSC sheets

All processes, including the acquisition of umbilical cords, the establishment of the MCBs, the establishment of WCBs and the production of cell sheets, were managed in accordance with Good Manufacturing Practice (GMP). Establishedstandard operation procedures (SOPs) and standard record procedures (SRPs) were strictly implemented in all processes. Intermediate products should meet the established quality standards before theycanenter the next production stage.

For the acquisition of umbilicalcords, donors with informedconsentshould have no family historyandno serious infectious diseases,includinghuman immunodeficiency virus (HIV), human hepatitis B virus (HBV), human hepatitis C virus(HCV), *Treponema pallidum* (TP),humanT-cellleukemia virus(HTLV),cytomegalovirus(CMV), Epstein–Barrvirus(EBV) and SARS-CoV-2 infection.Theumbilical cord should be tested according to our established quality control items and standards (Table 1) before primary cell isolation.

For P0 cell cultivation, Wharton's jelly was separated from the umbilical cord and cultured bythe explantmethod to obtain mesenchymal stem cells. Whentheyreachedconfluence, the P0 cells were all passaged to P1 cells for MCB establishment. When qualified, several tubes of cells from the MCB were recovered andsubculturedto P4 cells for WCB establishment.ThequalifiedWCBswere the final cell sources for cell sheet production. For the quality standards of MCB and WCB, please refer to Table 2.

According to the established processes, one umbilical cord could be used to establish one batch of MCB. One batch of MCB could be used to establish several batches of WCBs.Onebatch of WCBs could be used to produce several batches of cell sheet products. (Figure 1). Therefore, human umbilical cord mesenchymal stem cell sheet products can be mass-produced to ensure safety, effectiveness, and quality.

Serum-free UC-MSC sheet production

In the process of cell sheet formation,theculture medium and petri dish coating matrix are two key factors.

Due to the high density and multilayer cells in the cell sheet, the residual culture medium cannot be eliminated thoroughly by the cell sheet postharvest washing procedures. The safety and efficacy of UC-

MSC sheets may be significantly influenced by the media and petri dish coating matrix chosen.

To date, the reported cell sheet-forming mediumwas eitherFBS-containing media or serum-free mesenchymal stem cell culture media. As a kind of exogenous macromoleculeprotein, bovine serum albumin (BSA) in FBS-containing media can cause allergic reactions. Exogenous growth factors, additives for supportingcellgrowth, in serum-free mesenchymal stem cell culture media could also cause safety risks in humans.In addition, research-grade reagents used inthe manufacturingprocess do not meet the Good Manufacturing Process (GMP) standard and have no safety evaluation in humans.

After large-scale screening, we have developed a safe cell-sheet-forming medium with simple composition, containing only basic cell culturing medium (α-MEM) and a certain concentration of human albumin solution (parenteral drug).For thecoating matrix, fibrinogen, a component ofthehuman fibrin sealant kit (drug), was chosen. Furthermore, the concentration of human albumin solution in the cell sheet forming medium and fibrinogen in the coating buffer were further optimized for stable and highly efficient cell sheet production (datanot shown).

Serum-free UC-MSC sheet preservation

Owing to the three-dimensional structure andrelativelyhigh cell density, traditional cell suspension preservation methods are not suitable for cell sheet preservation to maintain its structure and cell viability. Through theoretical analysis and large-scale screening, a cell sheet preservation solution was chosen, which can maintain the structure and cell viability of the cell sheet for up to 24 hours.

Establishment of quality standards of the UC-MSC sheet.

Based on our research foundations and the “ICH Q5A: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin”, “ICH Q6B: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products”. "Guiding Principles for Quality Control of Stem Cell Preparations and Preclinical Research", “Guidelines for Cell Therapy Products and Evaluation Technology” and the regulations for MSC of the International Society for Cell Therapy (ISCT)(11), quality standards of the cell sheet involving product characteristics, safety and functionality were formulated.Bycomprehensively considering the necessity, representativeness and effectiveness of the testing items, the release quality standards were selected from the total quality standards. Please refer to Table 3 for details.

Identification of MCB and WCB

Both the MCB and the WCB were tested according to items listed in Table 2.The cellmorphology of all UC-MSCs intheMCB and WCB thatadheredto plastic culture dishes wasaspindleshape (Figure 2A). The cell growth curves showed that all UC-MSCshadhigh proliferation activity (Figure 2B). Cell density, cell viability and cell apoptosis were all tested and shownto meet the quality standards (Figure 2C-E). The cells were positive forthecell surfacemarkersCD73, CD90 and CD105 (positive cell proportion ≥95%)andnegative forthecell surfacemarkersCD11b, CD19, CD34, CD45 and HLA-DR (positive cell

proportion≤2%) (Table 4). CycleassaysshowedthatUC-MSCs hadatypical diploid cell cycle (Figure 2F).TheCFUresultsshowedthatUC-MSCs could form single-cell colonies (Figure 2G).In addition, UC-MSCs could differentiateintoosteoblasts, adipocytes and chondroblasts (Figure 2H) under appropriateinduction conditions.

To confirm functionality, UC-MSCs in MCBs and WCBs secreted HGF, IL-6 and IL-8 (Supplementary Figure 1). HGF and IL-6 can promote angiogenesis, and IL-6 can reduce immunogenicity(12,13). UC-MSCs in both the MCB and WCBinhibitedlymphocyte proliferation,downregulated the secretionof TNFα, andinhibitedlymphocyte differentiation intotheTh1 subtype (Supplementary Figure 2). All theseresultsshowed that cells have immunomodulatory effects.

Forthesafety assay, three batches of MCBs and WCBs were tested for bacteria, fungi, and mycoplasma, andtheresultswereall negative. No telomerase activity, which is high in fast proliferating cells such as pluripotent stem cells and cancer cells,wasdetected in the three batches of MCBs and WCBs.TheSTR authentication results showed that the three batches of MCBs and WCBs all hadasingle peak at the21tested sites, and the peaks of the corresponding MCBs and WCBs were the same, indicatingthatno cell cross-contamination occurred in the cell bank construction. Because the MCBs were not directly used for cell sheet product production, some safety tests were only conducted in WCBs. No pathogenic microorganisms, including bovine virus, which may be introduced by FBS usage, retrovirus and human HIV, HBV, HCV, CMV, EBV, TP, HB19, and HHV-6, which may be introduced by the raw material umbilical cord and throughoutthe entireproduction process, were detected in the three tested WCBs. No colony formation in soft agarwasdetected in three batches of WCBs,indicating thatthe cells had no tumorigenicity.Additionally, the three batches of WCBs all had normalkaryotypes, further provingthatthe cells had no risk of tumorigenicity. The methods of the safety test itemsarelisted in Table 2,and the resultsarelisted inTable5.

Identification of cell sheets

Three batches of UC-MSCsheets weretestedaccording to the items listed in Table 3.

For physical and chemical properties, the three batches of UC-MSCsheets tested were all white round sheetstructureswith smoothsurfacesand neat edges (Figure 3A). Morphological observation under the microscope showed tight cell connections in theUC-MSCsheets (Figure 3B). Immunofluorescence results showed that the cell sheet reserved intact extracellular matrix fibronectin and integrin β1 (Figure 3C).

Thediametersof the freshly produced and 24-hour preserved UC-MSCsheets were between 35and50 mm (Figure 4A). The number of UC-MSC sheets wasbetween 30and60 million (Figure 4B).TheAOPI staining results showed thatthecell viability of freshly produced and 24-hour preserved UC-MSCsheetswasmore than 70% (Figure 4C). The cell apoptosis analysis showedresultssimilarto thoseof AOPI staining (Figure 4D).

UC-MSCs in both freshly produced and 24-hour stored cell sheets were positive (positive cell proportion ≥ 95%) forthecell surfacemarkersCD73, CD90 andCD105 andnegative (positive cell proportion ≤ 2%) forthecell surfacemarkersCD11b, CD19, CD34, CD45 and HLA-DR (Figure 5A and Table 6) and could be inducedto differentiate intoosteoblasts, adipocytes and chondroblasts (Figure 5B).

Residues of high-risk substances, including BSA, human serum albumin (HSA), gentamicin, fibrinogen, TrypLE and bFGF, used in the production process were detected and controlled. The quality standardsarelisted in Table 3. The test results showedthatall three batches of cell sheetssatisfiedthe quality standards (Figure 6).

UC-MSC sheets took effect by secreting growth factors, which could promotemicrovesselformation, promote tissue repair, and perform immune regulation. HGF, VEGF and IL-8 can promotemicrovesselformation; IL-6 can reduce immunogenicity,andIL-8 and IL-6 can promote immunoregulation(12,13). The cell sheet could be reattached totheculture dish in culture medium in vitro to imitate its state in the body. All three batches of cell sheet couldsecreteHGF, VEGF, IL-6 and IL-8 in the cell sheet forming process,andthe freshly produced and 24-hour preserved cell sheet reattachment process (Figure 7).

Whencoculturedwith human PBMCs, UC-MSCsheets inhibitedlymphocyte proliferation, Th1 subtype differentiation and inflammatory factor TNFαsecretion, demonstratingtheirimmunomodulatory effect invitro(Figure 8).

Conditionedcell sheet-forming mediumincreased thetotal length of microtubules andthenumber of microtubule junctions in the microtubule formation model in vitro, demonstrating its microtubule formation-promoting ability (Figure 9).

Additionally, the conditioned cell sheet-forming mediuminhibitedcobalt chloride-induced apoptosis ofrat cardiomyocytes in vitro, demonstrating its cell repair ability invitro(Figure 10).

Three batches of UC-MSC sheets were tested, and the results were negative for bacteria, fungi, and mycoplasma. The STR authentication results showed that the three batches of cell sheets all had a single peak at the 21 tested sites, and the peaks were the same as the corresponding MCBs and WCBs, indicating that no cell cross-contamination occurred in the cell sheet production process. Gel Clot LAL Assays were used for the endotoxin test. The results showed that all three batches of cell sheet products were less than and equal to 6.6 EU per cell sheet, which satisfied the quality standard. Please refer to Table 7 for details.

Discussion

With allogeneic cell therapies moving into clinical trials, the safety, stability, and effectiveness of cell product production have become important. In the present study, we explored the production, preparation, and preservation technology of cell sheets and established a quality inspection and control system.

FBS, a nutrient supplement for optimal cell proliferation, is a strong allergen to the human body. Before cells are used, FBS is usually removed by constant washing. Although several serum-free media have been investigated for human MSC expansion (14–17), these media contain a variety of exogenous growth factors, which would also cause safety risks for clinical applications. Due to the high density and multilayer cells in the cell sheet, the residual culture medium cannot be eliminated thoroughly by the cell sheet postharvest washing procedures. Therefore, the serum-containing and serum-free media commonly used are not suitable for cell sheet preparation. The serum-free cell sheet production medium developed in this study does not contain animal sera or exogenous growth factors, which can be safer in clinical use.

Ideally, we should use a serum-free culture system in the whole process of cell sheet manufacture, including cell culture. The preparation of cell sheets requires large numbers of cells, in the range of 20–100 million MSCs/sheet. To meet the huge demand for expanded cells, expansion processes need to be in place. In this study, we found that cells expanded in commercial serum-free media could not form cell sheets successfully. This is also our next challenge. We will further optimize the cell sheet manufacturing process to realize serum-free culture in the whole manufacturing process, including cell preparation and cell sheet formation. In addition, it is necessary to further optimize the process of UC-MSC preparation.

To test the safety of UC-MSC sheets, our unpublished study showed that there was no tumor formation, and the cell sheets only existed in the transplanted tissues or organs in Severe Combined Immunodeficient (SCID) mice.

In the functional study of UC-MSC sheets, the research results showed that UC-MSC sheets could promote microtubule formation and inhibit cell apoptosis in vitro (Figs. 9 and 10). In addition, animal studies show that UC-MSC sheets could obviously improve left ventricular heart function, and ejection fraction (EF) improved from 30–50% in mouse infarction models (9) and from 40–67% in porcine infarction models (unpublished data).

The UC-MSC sheets showed safety and effectiveness in preclinical studies, and the cell sheet preservation technology developed in this study confirmed the safety of the cell sheets in the process of transportation and preservation before clinical use.

Limitations still existed in this study. The validity period of the UC-MSC sheets was only 24 hours, which limits the time for cell sheet transplantation for clinical application. Therefore, further study is needed to develop long-term preservation or freezing technology for cell sheets, including cryopreservation in liquid nitrogen technology (18, 19).

Conclusions

This study has developed a preparation and preservation technology for clinical grade serum-free UC-MSC sheets that is safe and effective and provides application prospects for cell therapy.

Abbreviations

UC-MSCs: umbilical cord-derived mesenchymal stem cells;

FBS: fetal bovine serum;

BSA: bovine serum albumin;

MCB: master cell bank;

WCB: working cell bank;

GMP: Good Manufacturing Practice;

α-MEM: minimum essential medium-α;

bFGF: basic fibroblast growth factor;

HSA: human serum albumin;

HGF: hepatocyte growth factor;

VEGF: vascular endothelial growth factor;

IL-6: interleukin-6;

IL-8: interleukin-8;

IFNγ: interferon γ;

TNFα: tumor necrosis factor-α;

STR: short tandem repeat;

CFU: cell forming unit;

HIV: human immunodeficiency virus;

HBV: human hepatitis B virus;

HCV; human hepatitis C virus;

HTLV: human T-cell leukemia virus;

EBV: Epstein–Barr virus;

CMV: cytomegalovirus;

TP: *Treponema pallidum*;

HB19: human parvovirus B19;

HHV-6: human herpesvirus 6;

ICH: international conference on harmonization;

EF: ejection fraction;

SCID: severe combined immunodeficient.

Declarations

Ethics approval and consent to participate

Umbilical cord sample collection was approved by the OASIS International Hospital local ethics committee (File number: LLPJ2018[001]). Written informed consent was obtained from the puerperas.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Dehua Chang , Juan Wang, Taibin Fan and Mingkui Zhang designed the concept. Juan Wang, Shuang Gao and Yufei Zhao performed the experiments. Juan Wang and Dehua Chang wrote the manuscript and developed the figures. All the authors read and approved the final manuscript.

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Tables

Table 1. Umbilical cord quality standards

Categories	Items	Methods	Quality Standards
Character	Appearance	Naked eye observation	Complete packaging without leakage
	Temperature	Thermometry	2-8°C
	Umbilical cord collection information	Naked eye observation	Complete
	Within the warranty period	Naked eye observation	Less than 24 hours from umbilical cord collection
Safety	HIV	Colloidal gold method	Negative
	HBV	Colloidal gold method	Negative
	HCV	Colloidal gold method	Negative
	TP	Colloidal gold method	Negative
	HTLV	ELISA	Negative
	EBV	ELISA	Negative
	CMV	Colloidal gold method	Negative
	ConA	Q-PCR	Negative

Table 2. MCB and WCB quality standards

Categories	Items	Methods	Quality Standards
Physical and chemical properties	Cell morphology	Microscopic observation	Adherent and spindle-shaped cells
	Cell count	AOPI staining and automatic fluorescent cell counter detection	1.6-2.4×10^6/mL
	Cell survival rate	AOPI staining and automatic fluorescent cell counter detection	≥80%
	Cell apoptosis detection	Annexin V and PI staining and flow cytometry detection	Normal cells ≥80%
Biological characteristics	Cell growth curve	CCK staining	Report Result
	Cell cycle detection	PI staining and flow cytometry detection	Report Result
	Colony-forming unit (CFU)	Single cell culture in 96-well plates	Report Result
	Cell surface marker CD73 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≥95%
	Cell surface marker CD90 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≥95%
	Cell surface marker CD105 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≥95%
	Cell surface marker CD11b detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≤2%
	Cell surface marker CD19 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≤2%
	Cell surface marker CD34 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≤2%
	Cell surface marker CD45 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≤2%
	Cell surface marker HLA-DR detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion ≤2%
	Induced osteoblast differentiation	Induce differentiation and alizarin red staining	Positive for alizarin red staining
	Induced adipocyte differentiation	Induce differentiation and Oil Red O staining	Positive for Oil Red O staining
	Induced chondroblast differentiation	Induce differentiation and alcian blue staining	Positive for alcian blue staining

Categories	Items	Methods	Quality Standards
Biological functions	Lymphocyte proliferation inhibition test	Coculture with peripheral blood mononuclear cells, BrdU staining and flow cytometry detection	Inhibit lymphocyte proliferation
	Th1 Lymphocyte inhibition test	Coculture with peripheral blood mononuclear cells, fluorescent antibody staining and flow cytometry detection	Inhibit Th1 lymphocyte differentiation and proliferation
	Inflammatory factor-TNFα secretion inhibition test	Coculture with peripheral blood mononuclear cells and ELISA detection	Inhibit lymphocyte secrete TNFα
	Secretory factor HGF detection	ELISA	Report Result
	Secretory factor IL-6 detection	ELISA	Report Result
	Secretory factor IL-8 detection	ELISA	Report Result
Safety	STR authentication	Fluorescence STR method	Single peak at detection sites
	Telomerase activity detection	Q-PCR	Negative
	Sterility testing	Membrane filtration method	Negative
	Mycoplasma detection	Culture Method	Negative
	Mycoplasma detection	Indicated cell culture method	Negative
	Mycoplasma detection	Q-PCR	Negative
	*Human Immunodeficiency Virus Type I Test (HIV-1) detection	Q-PCR	Negative
	*Human Hepatitis B Virus detection (HBV)	Q-PCR	Negative
	*Human Hepatitis C Virus detection (HCV)	Q-PCR	Negative
	*Human Cytomegalovirus detection (HCMV)	Q-PCR	Negative

Categories	Items	Methods	Quality Standards
	*Human Epstein–Barr detection (EBV)	Q-PCR	Negative
	*Treponema pallidum virus detection (TP)	Q-PCR	Negative
	*Human parvovirus B19 detection (HB19)	Q-PCR	Negative
	*Human herpes virus type 6 detection (HHV-6)	Q-PCR	Negative
	*Retroviral detection	TM-PERT method	Negative
	*Bovine virus detection	Cytopathic observation method	Negative
	*Bovine virus detection	Hemoabsorption test	Negative
	*Bovine virus detection	Fluorescent antibody test	Negative
	*Exogenous Virus Detection	Vero cell culture test	Normal cell morphology
	*Exogenous Virus Detection	MRC-5 cell culture test	Normal cell morphology
	*Exogenous Virus Detection	Human MSC culture test	Normal cell morphology
	*Exogenous Virus Detection	Vero cell erythrocyte adsorption and blood coagulation test	Negative
	*Exogenous Virus Detection	MRC-5 cell erythrocyte adsorption and blood coagulation test	Negative
	*Exogenous Virus Detection	MSC erythrocyte adsorption and blood coagulation test	Negative
	*Exogenous Virus Detection	Mouse intraperitoneal and intracerebral vaccination	Negative
	*Exogenous Virus Detection	Suckling mouse intraperitoneal and intracerebral vaccination Chinese Pharmacopoeia	Negative
	*Exogenous Virus Detection	5-6 day chicken embryo yolk sac vaccination	Negative
	*Exogenous Virus Detection	9-11 day chicken embryo allantoic vaccination	Negative

Categories	Items	Methods	Quality Standards
	*Exogenous Virus Detection	Erythrocyte adsorption test of 9-11 days chicken embryo allantoic fluid	Negative
	*Karyotype analysis	G-band method	46XX or 46XY
	*Tumorigenicity test	Soft Agar Clone Formation Experiment	Negative

*Only tested in WCB.

Table 3. Cell sheet quality standards

Categories	Items	Methods	Quality Standards
Physical and chemical properties	*Cell sheet appearance	Visual observation	White round sheet structure with smooth surface and neat edges.
	*Cell sheet diameter	Ruler method	35-50 mm
	*Number of cells per cell sheet	AOPI staining and automatic fluorescent cell counter detection	3-6×10^7 cells/cell sheet
	*Cell survival rate	AOPI staining and automatic fluorescent cell counter detection	≥70%
	*Cell apoptosis detection	Annexin V and PI staining and flow cytometry detection	Normal cells ≥70%
Biological characteristics	*Cell surface marker CD73 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≥95%
	*Cell surface marker CD90 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≥95%
	*Cell surface marker CD105 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≥95%
	*Cell surface marker CD11b detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≤2%
	*Cell surface marker CD19 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≤2%
	*Cell surface marker CD34 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≤2%
	*Cell surface marker CD45 detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≤2%
	*Cell surface marker HLA-DR detection	Fluorescent antibody staining and flow cytometry detection	Positive cell proportion≤2%
	Induced osteoblast differentiation	Induce differentiation and alizarin red staining	Positive for alizarin red staining

Categories	Items	Methods	Quality Standards
	Induced adipocyte differentiation	Induce differentiation and Oil Red O staining	Positive for Oil Red O staining
	Induced chondroblast differentiation	Induce differentiation and alcian blue staining	Positive for alcian blue staining
Biological functions	Lymphocyte proliferation inhibition test	Coculture with peripheral blood mononuclear cells, BrdU staining and flow cytometry detection	Inhibit lymphocyte proliferation
	Th1 Lymphocyte inhibition test	Coculture with peripheral blood mononuclear cells, fluorescent antibody staining and flow cytometry detection	Inhibit Th1 lymphocyte differentiation and proliferation
	Inflammatory factor-TNFα secretion inhibition test	Coculture with peripheral blood mononuclear cells and ELISA detection	Inhibit lymphocyte secrete TNFα
	*Secretory factor VEGF detection	ELISA	Report Result
	*Secretory factor HGF detection	ELISA	Report Result
	Secretory factor IL-6 detection	ELISA	Report Result
	Secretory factor IL-8 detection	ELISA	Report Result
safety	*STR Authentication	Fluorescence STR method	Single peak at detection sites
	*Gentamicin residue detection	ELISA	≤0.5 ng/cell sheet
	*Bovine serum albumin residue detection	ELISA	≤16.6 ng/cell sheet
	*Human serum albumin residue detection	ELISA	Report Result
	*bFGF residue detection	ELISA	≤100 pg/mL

Categories	Items	Methods	Quality Standards
	Fibrinogen residue detection	ELISA	Report Result
	*TrypLE residue detection	ELISA	≤16.6 ng/cell sheet
	*Sterility testing	Membrane filtration method	Negative
	*Mycoplasma detection	Culture Method	Negative
	*Mycoplasma detection	Indicated cell culture method	Negative
	*Mycoplasma detection	Q-PCR	Negative
	*Endotoxin test	Gel Clot LAL Assay	≤6.6 EU/cell sheet

Table 4. Cell surface marker test of cell banks

Markers	Positive cell proportion					
	Sample 1		Sample 2		Sample 3	
	MCB	WCB	MCB	WCB	MCB	WCB
CD73	99.7%	99.7%	99.8%	99.9%	99.3%	99.9%
CD90	99.9%	99.8%	99.8%	99.9%	99.8%	99.8%
CD105	99.8%	99.9%	99.8%	99.5%	99.9%	99.9%
CD11b	0.191%	0.020%	0.050%	0.080%	0.060%	0.210%
CD19	0.142%	0.070%	0.120%	0.220%	0.190%	0.240%
CD34	0.122%	0.020%	0.040%	0.060%	0.140%	0.140%
CD45	0.145%	0.050%	0.050%	0.050%	0.070%	0.070%
HLA-DR	0.997%	0.080%	0.050%	0.290%	0.260%	0.280%

Table 5. Safety test of cell banks

Items	Results					
	Sample 1		Sample 2		Sample 3	
	MCB	WCB	MCB	WCB	MCB	WCB
Telomerase activity detection	Negative	Negative	Negative	Negative	Negative	Negative
Sterility testing	Negative	Negative	Negative	Negative	Negative	Negative
Mycoplasma detection	Negative	Negative	Negative	Negative	Negative	Negative
HIV detection	N/A	Negative	N/A	Negative	N/A	Negative
HBV detection	N/A	Negative	N/A	Negative	N/A	Negative
HCV detection	N/A	Negative	N/A	Negative	N/A	Negative
CMV detection	N/A	Negative	N/A	Negative	N/A	Negative
EBV detection	N/A	Negative	N/A	Negative	N/A	Negative
TP detection	N/A	Negative	N/A	Negative	N/A	Negative
HB19 detection	N/A	Negative	N/A	Negative	N/A	Negative
HHV-6 detection	N/A	Negative	N/A	Negative	N/A	Negative
Retrovirus detection	N/A	Negative	N/A	Negative	N/A	Negative
Bovine virus detection	N/A	Negative	N/A	Negative	N/A	Negative
Exogenous virus detection	N/A	Negative	N/A	Negative	N/A	Negative
STR authentication	Single peak at detection sites	Single peak at detection sites	Single peak at detection sites	Single peak at detection sites	Single peak at detection sites	Single peak at detection sites
Karyotype analysis	N/A	46 XX	N/A	46 XY	N/A	46 XY
Tumorigenicity test	N/A	Negative	N/A	Negative	N/A	Negative

Table 6. Cell surface marker test of the cell sheets

Markers	Positive cell proportion					
	Sample 1 CS		Sample 2 CS		Sample 3 CS	
	0 h	24 h	0 h	24 h	0 h	24 h
CD73	100.0%	100.0%	99.8%	99.9%	99.9%	99.9%
CD90	99.9%	99.9%	99.9%	99.9%	99.9%	99.8%
CD105	99.9%	99.9%	99.9%	99.5%	99.6%	99.9%
CD11b	0.612%	0.559%	0.485%	0.916%	0.027%	0.031%
CD19	0.413%	0.259%	0.273%	0.516%	0.074%	0.031%
CD34	0.357%	0.258%	0.231%	0.131%	0.170%	0.015%
CD45	0.402%	0.327%	0.172%	0.538%	0.009%	0.016%
HLA-DR	0.570%	0.314%	0.252%	0.324%	0.064%	0.008%

Table 7. Safety test of the cell sheets

Items	Methods	Results		
		Sample 1 CS	Sample 2 CS	Sample 3 CS
Sterility test	Membrane Filtration Method	Negative	Negative	Negative
Mycoplasma test	Q-PCR Method	Negative	Negative	Negative
Mycoplasma test	Culture Method	Negative	Negative	Negative
STR Authentication	Fluorescence STR Method	Single peak at detection sites	Single peak at detection sites	Single peak at detection sites
Endotoxin Test	Gel Clot LAL Assay	≤6.6EU/CS	≤6.6EU/CS	≤6.6EU/CS

Figures

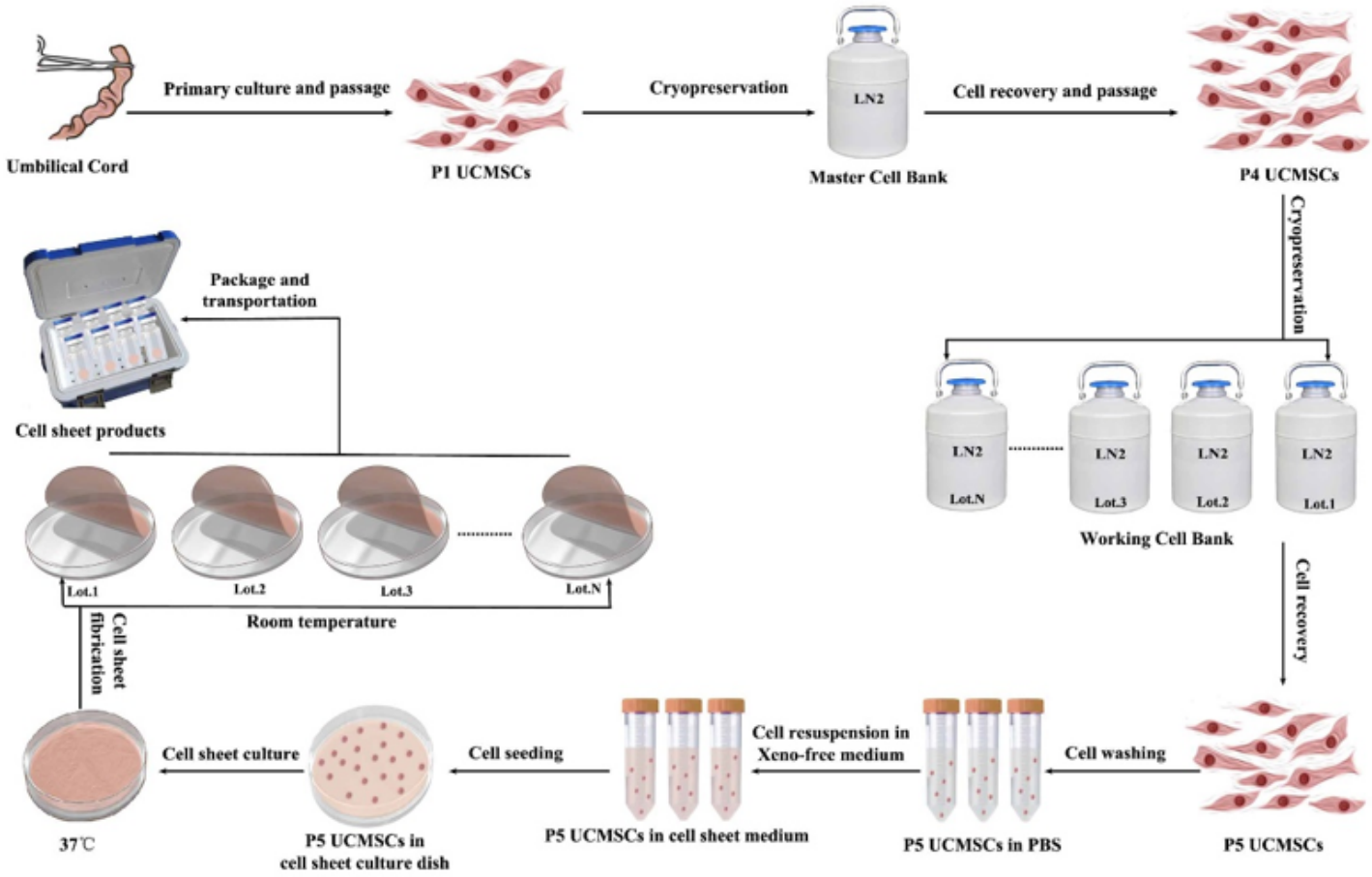

Figure 1

Schematic diagram of the large-scale production of cell sheets.

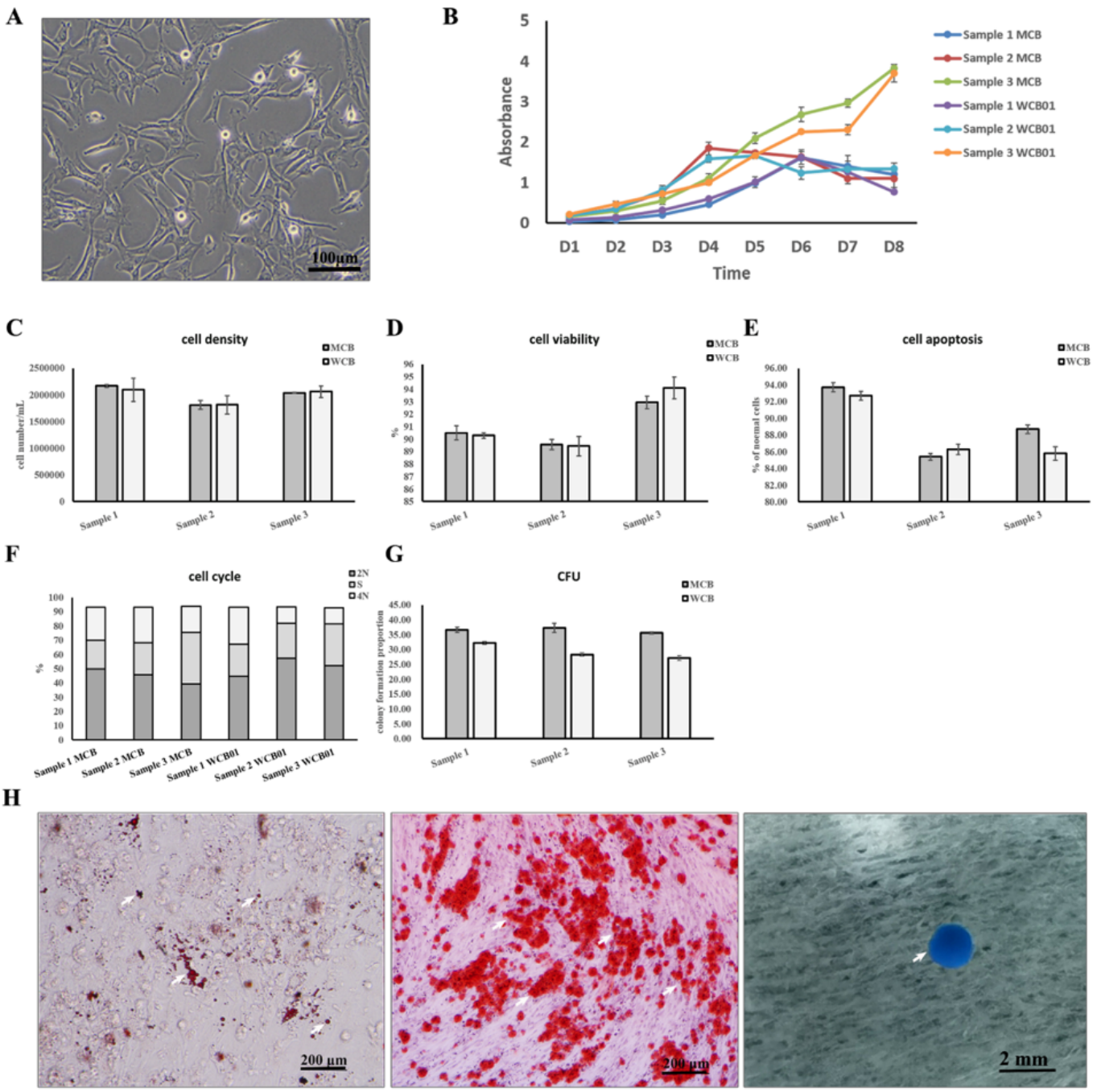

Figure 2

Cell characteristic test of the MCBs and WCBs.

A, Cell morphology of HUC-MSCs.

B, Cell growth curve of the MCBs and WCBs.

C, Cell density of the MCBs and WCBs.

D, Cell viability of the MCBs and WCBs.

E, Cell apoptosis of MCBs and WCBs.

F, Cell cycle of the MCBs and WCBs.

G, Colony formation proportions of the MCB and WCBs.

H, Representative results of induced adipogenic, osteogenic and chondrogenic differentiation of the MCBs and WCBs.

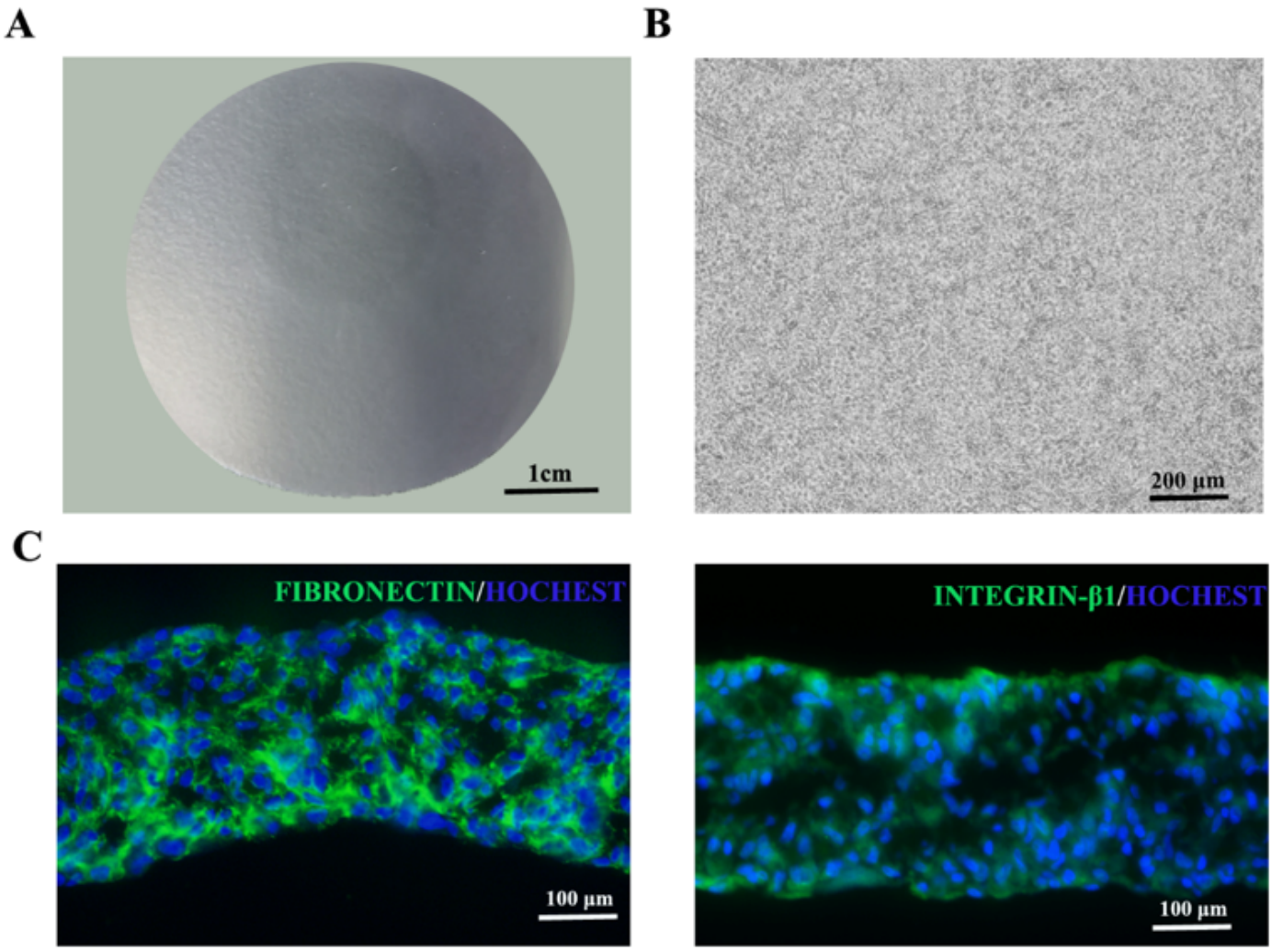

Figure 3

Structure of the cell sheet.

A, Morphology of the cell sheet.

B, Enlarged morphology of the cell sheet.

C, Fibronectin and Integrin β1 staining of the cell sheet.

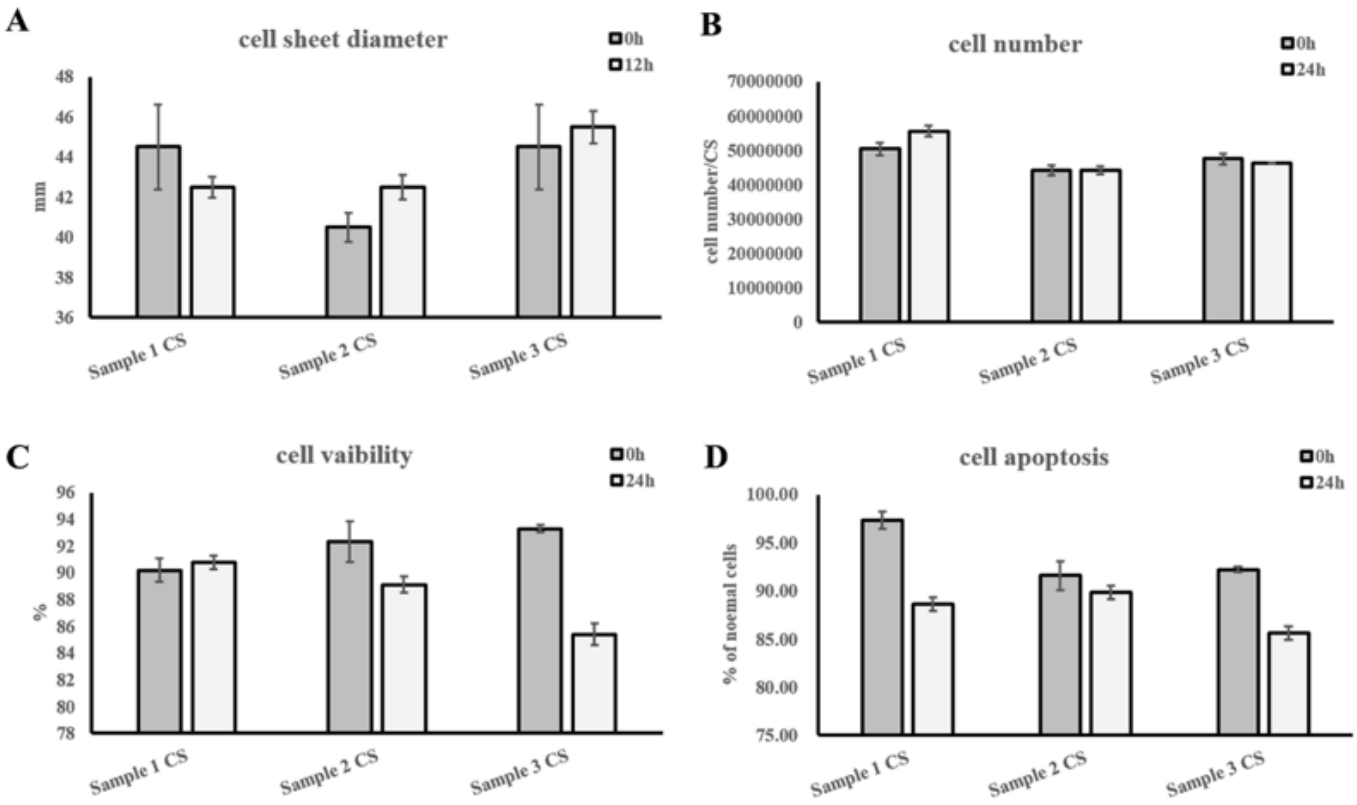

Figure 4

Characteristic tests of cell sheets.

A, Statistics of cell sheet diameters.

B, Statistical analysis of the number of cells per sheet.

C, Cell viability statistics of three batches of cell sheets.

D, Statistical analysis of cell apoptosis in three batches of cell sheets.

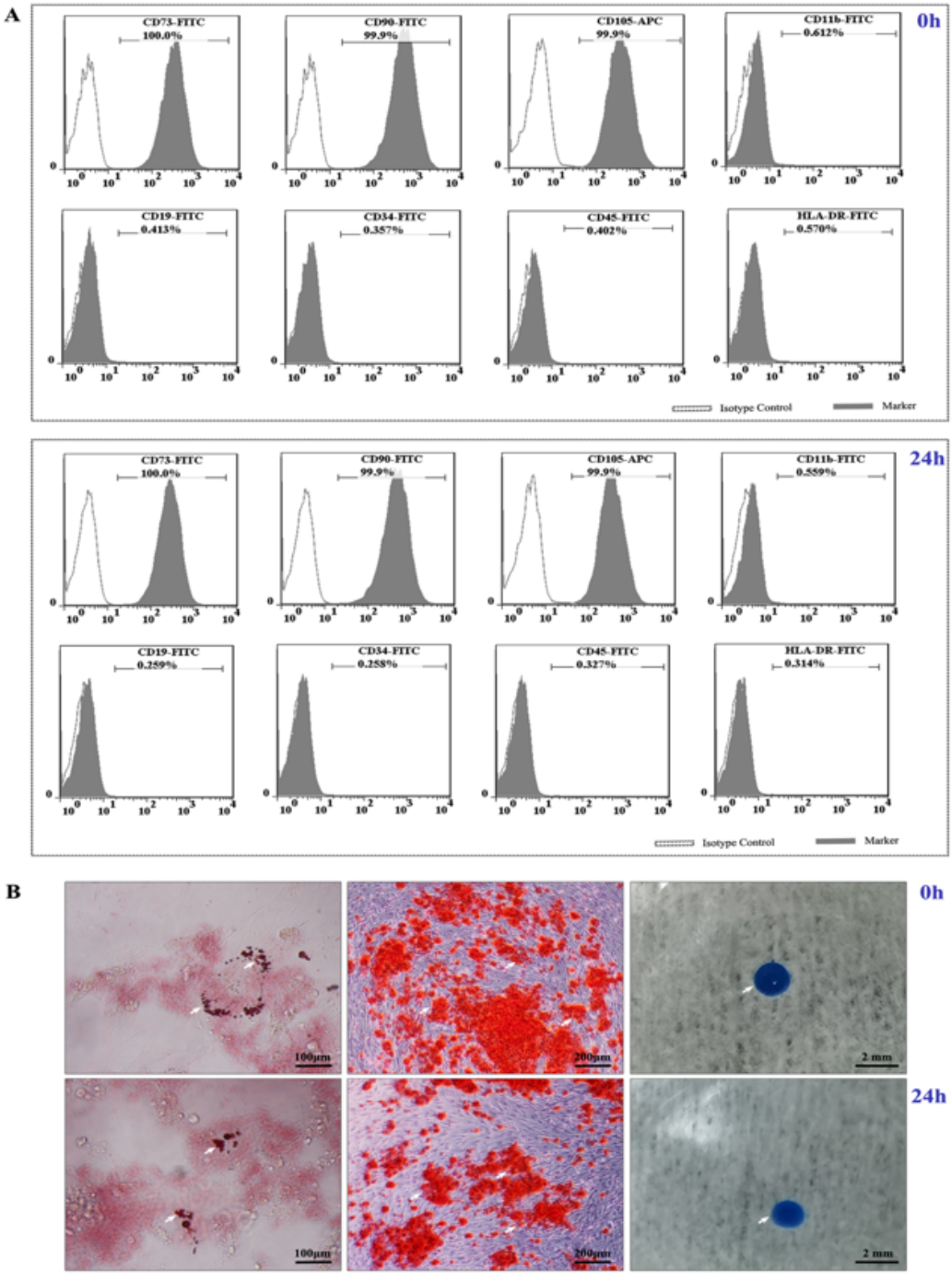

Figure 5

Cell characteristic tests of cell sheets.

A, Cell surface marker expression of freshly and 24-hour preserved cell sheets.

B, Representative results of induced adipogenic, osteogenic and chondrogenic differentiation of freshly and 24-hour preserved cell sheets.

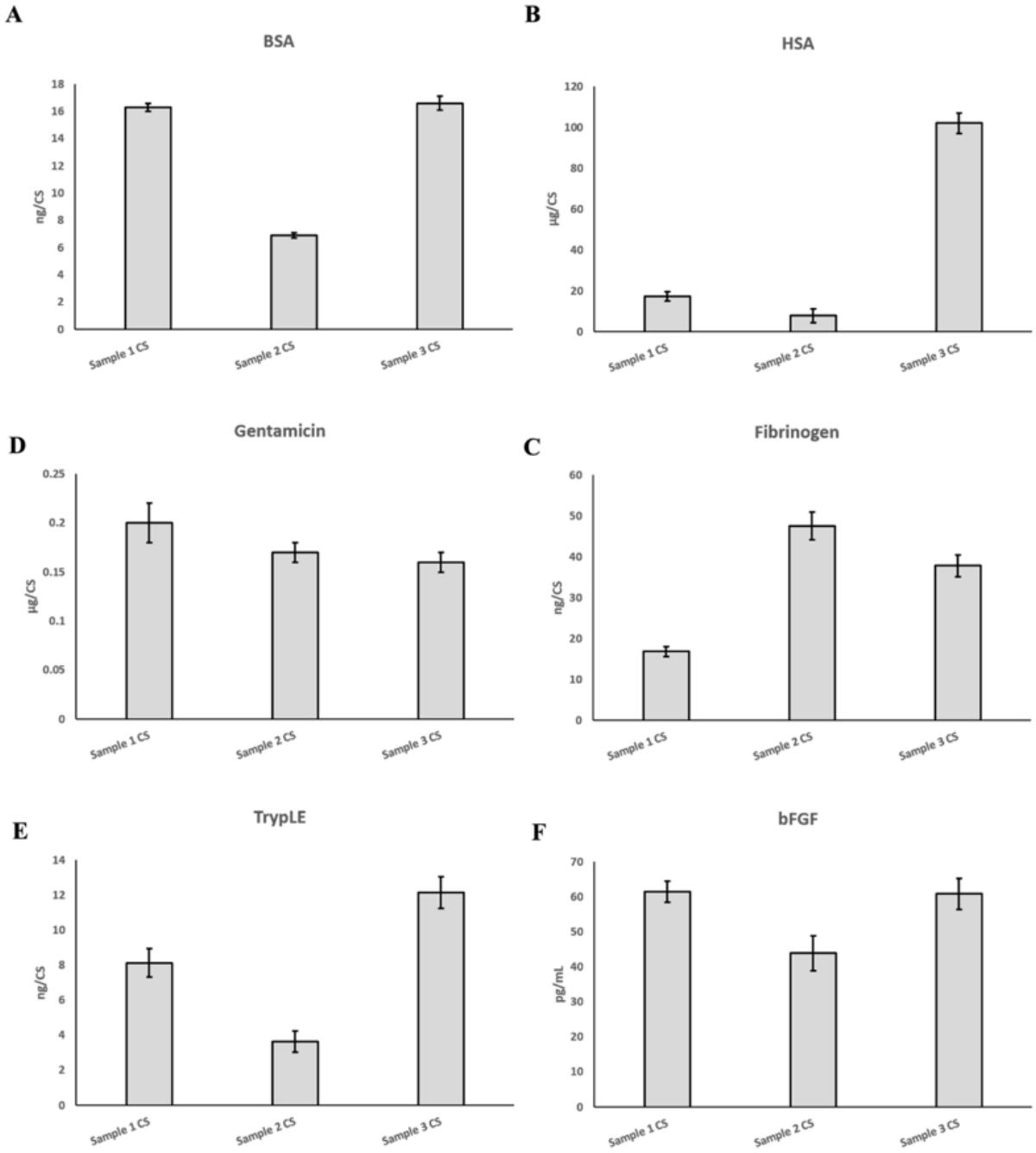

Figure 6

Detection of residues of high-risk substances

A, BSA.

B, HSA.

C, gentamicin.

D, fibrinogen.

E, TrypLE.

F, bFGF.

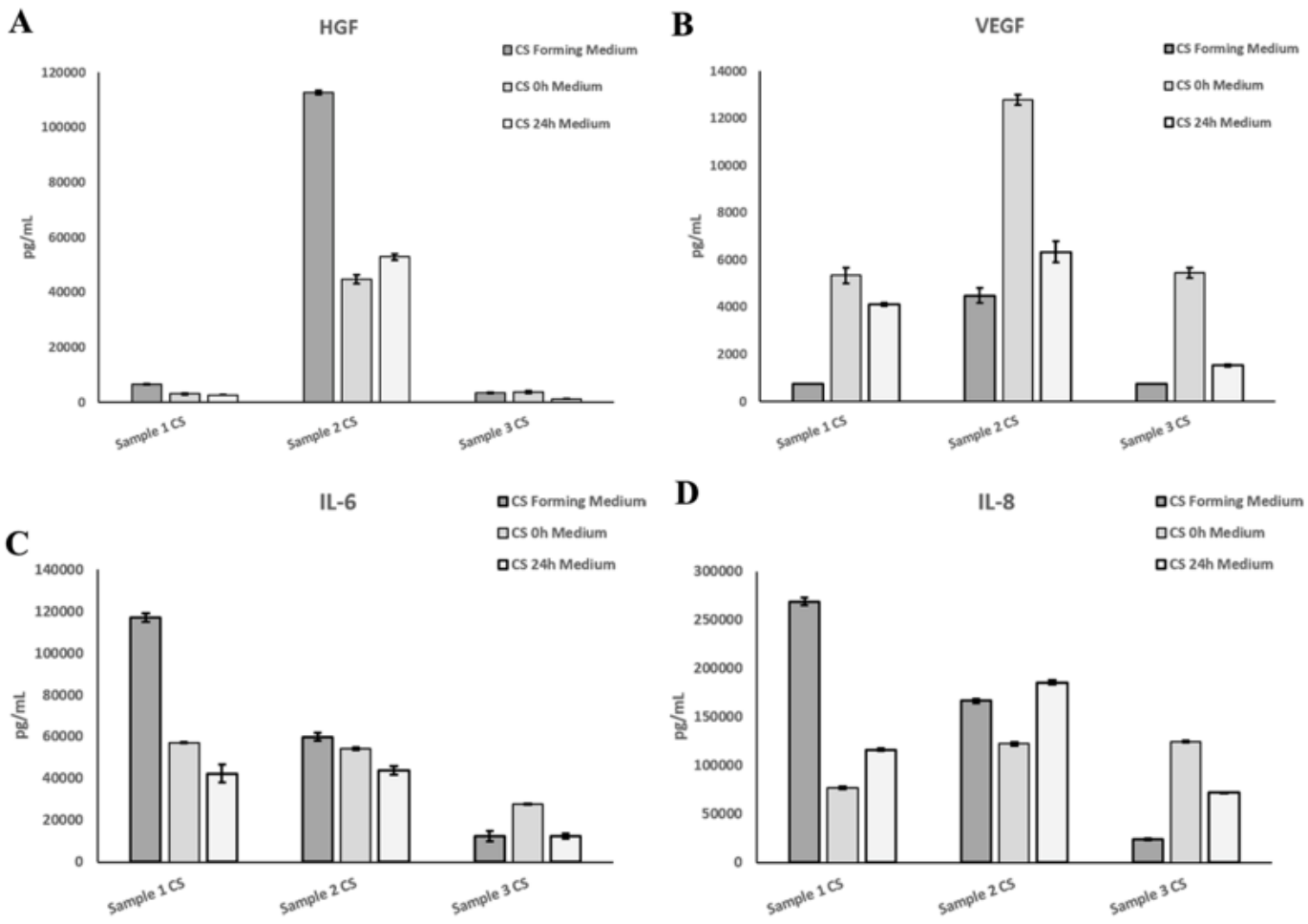

Figure 7

Growth factor detection of cell sheets.

A, HGF

B, VEGF

C, IL-8

D, IL-6

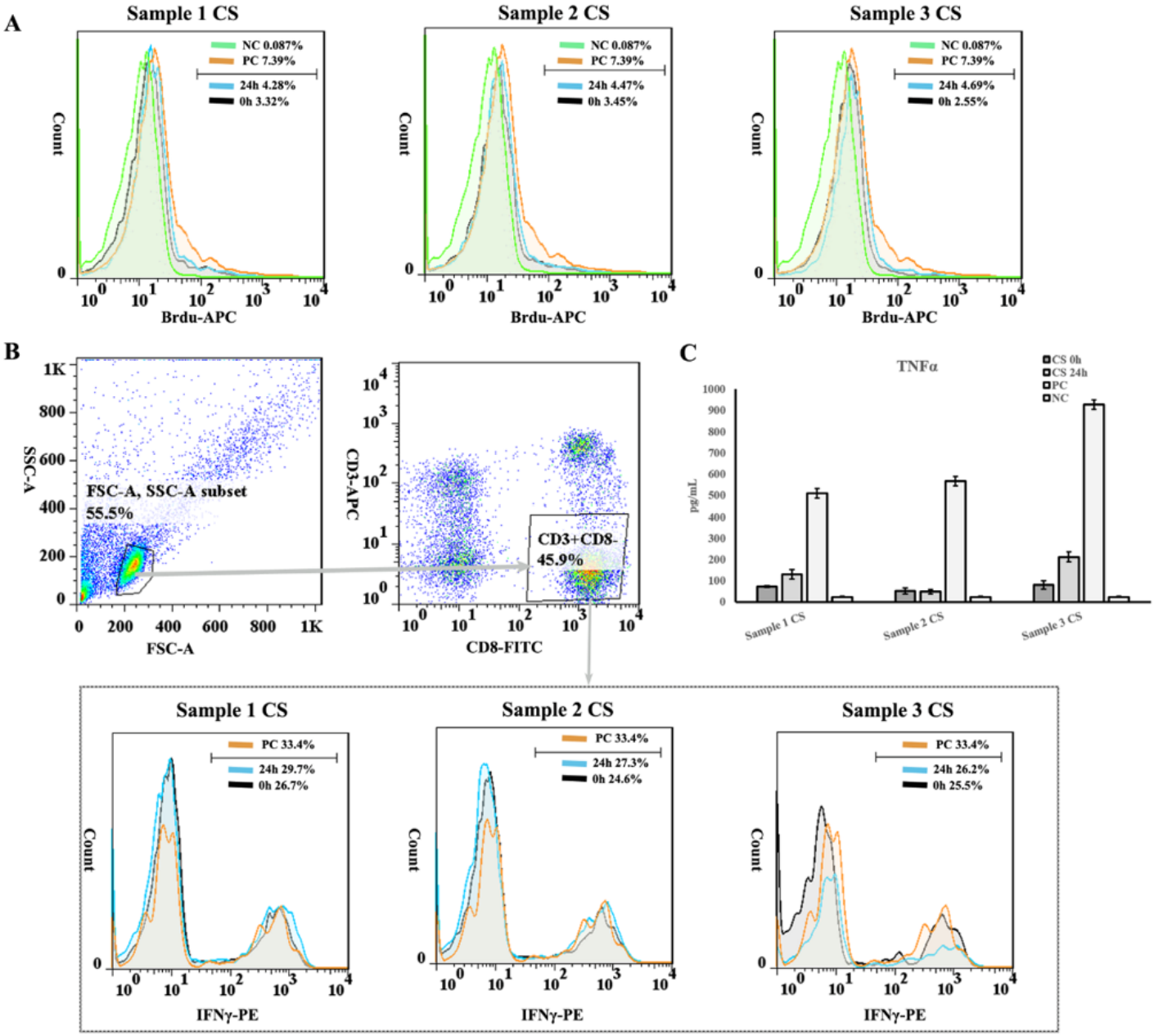

Figure 8

Immune modulating function test of the cell sheet.

A, Detection of lymphocyte proliferation inhibition ability of cell sheets.

B, Detection of lymphocyte TNFα secretion inhibition ability of cell sheets.

C, Detection of the Th1 lymphocyte inhibition ability of cell sheets.

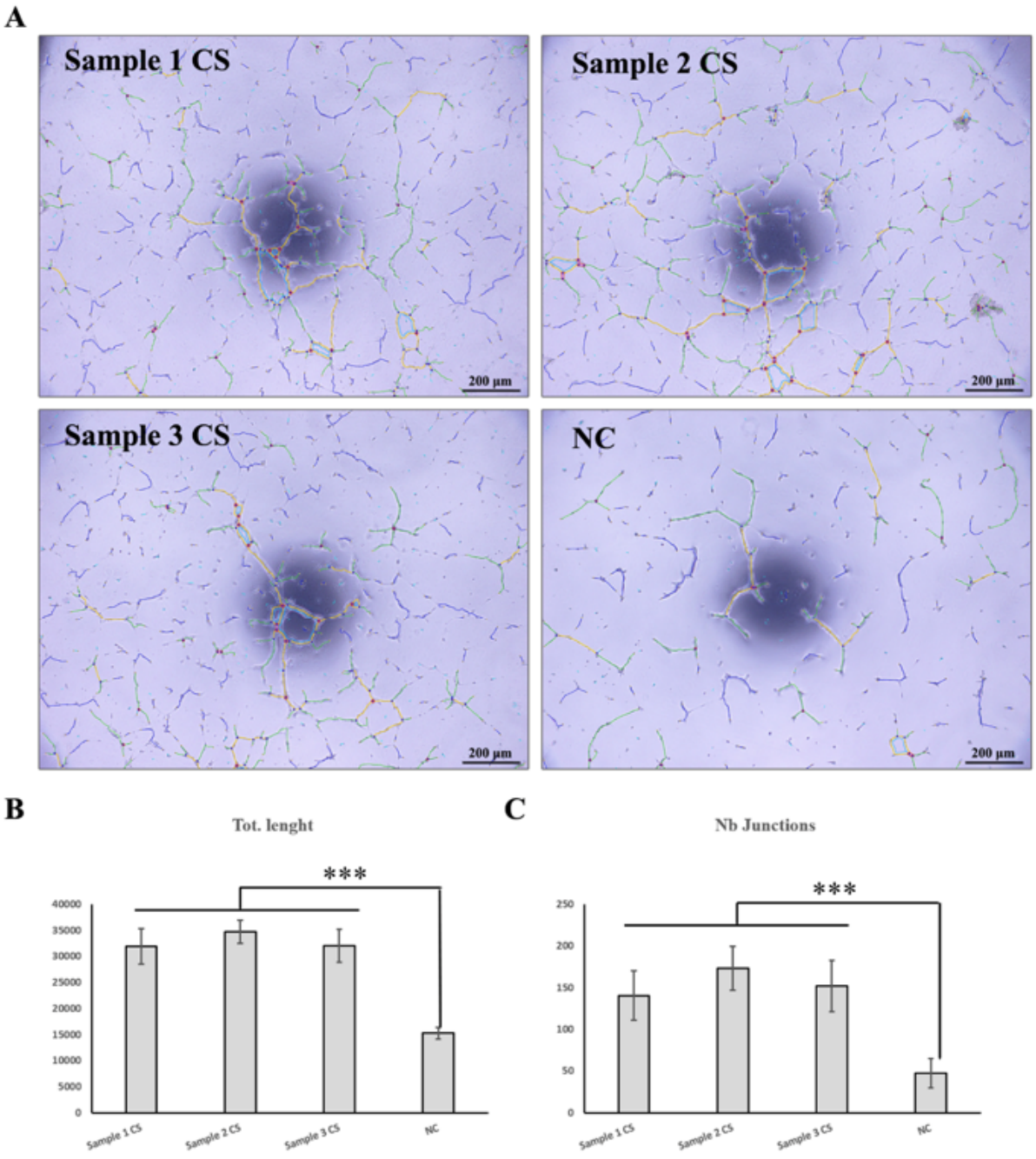

Figure 9

Proangiogenic function test of the cell sheet.

A, ImageJ Angiogenesis Analyzer was used to quantify endothelial tube formation. Image represents HUVECs incubated in cell sheet-forming conditioned medium. Images were taken using an inverted microscope and analyzed using an angiogenesis analyzer. Yellow lines represent the master segments,

tubes that connect together to different junctions; green lines represent branches; light blue surrounding the master segment is the mesh; dark blue represents isolated tubes; red circles represent the master junction points.

B, Statistical results of Nb junctions.

C, Statistical results of total length.

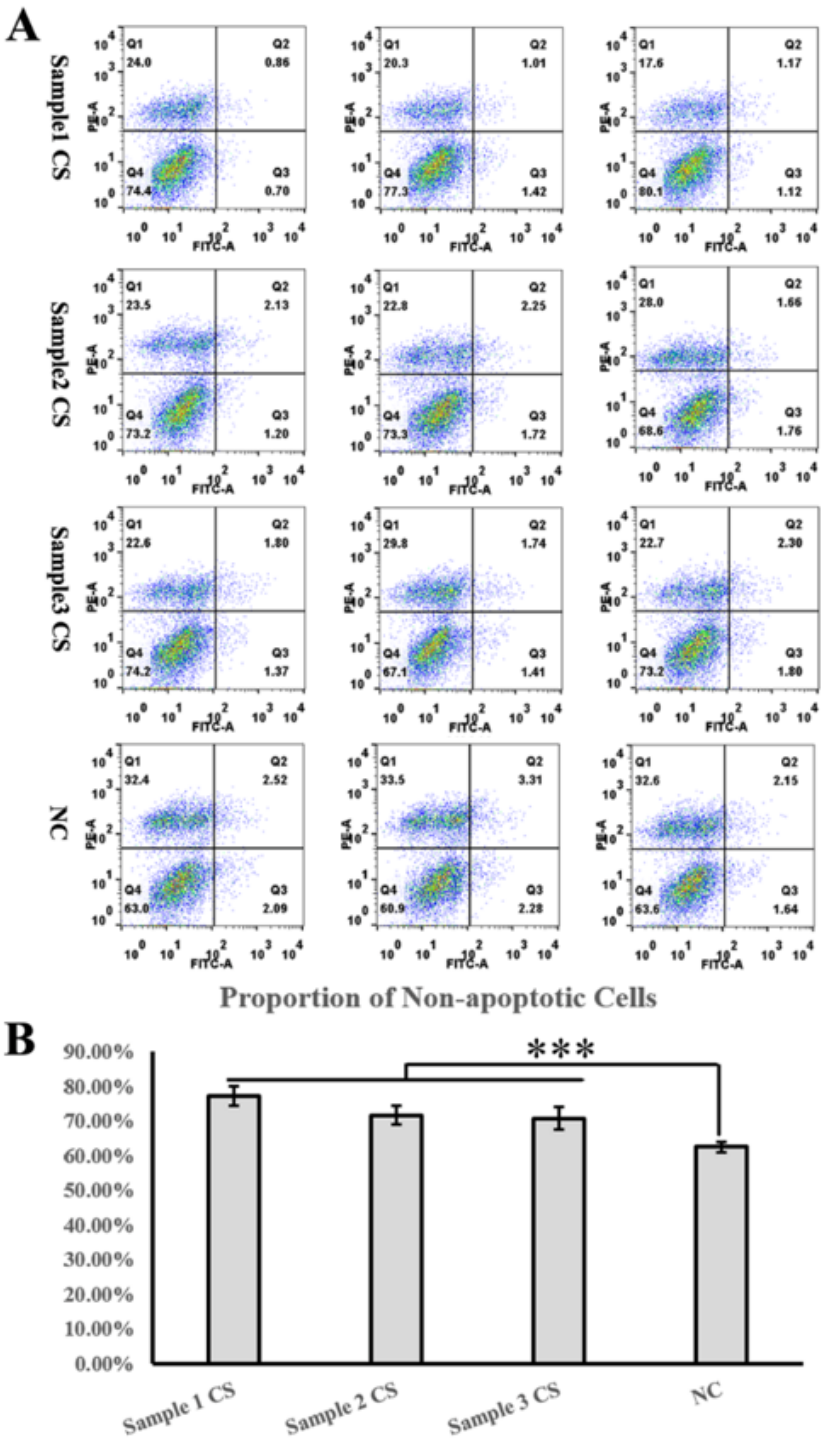

Figure 10

Anti-apoptotic function test of the cell sheet.

A, Apoptosis results of H9C2 cells cultured in cell sheet-forming conditioned medium or negative control medium after induction of apoptosis by cobalt chloride.

B, Statistical results of apoptosis results of H9C2 cells cultured in cell sheet-forming conditioned medium or negative control medium after apoptosis was induced by cobalt chloride.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- sup1.png
- sup2.png