

Cell Heterogeneity, Rather Than Cell Solvents Affects the Behavior of Mesenchymal Stem Cells in Vitro and Vivo

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Research

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Abstract

Background: Mesenchymal stem cells (MSCs) have been approved to treat various diseases, but they have to be expanded in vitro to gain sufficient cell doses. During the process of expansion, some obstacles remain to be addressed before MSCs translation to clinic. The purpose of this study was to investigate the effects of cell solvents and cell heterogeneity on the behavior of MSCs in vitro and vivo.

Methods: Human umbilical cord MSCs (UC-MSCs) were dissolved in three different solvents: phosphate buffer solution (PBS), normal saline (NS) and dulbecco's modified eagle medium (DMEM). Their ultrastructure, viability and safety were explored and compared. MSCs from other two separate donors were grouped based on their mean diameters. The ultrastructure, proliferative and hepatic differentiation potential, senescent cell ratio and safety of the two UC-MSC aggregates were investigated and compared. The reason for mice death after UC-MSCs injection was further investigated.

Results: The apoptosis rates, ultrastructure analysis and survival rates of mice among UC-MSCs in DMEM, NS and PBS were similar, and no significant differences were observed. The diameters of UC-MSCs of different sizes were measured. Cells with diameter of $15.58 \pm 3.813 \mu\text{m}$ were renamed as larger UC-MSC aggregates and cells with diameter of $19.14 \pm 4.885 \mu\text{m}$ were smaller aggregates. The mean diameter of larger MSC aggregates was significantly longer than that of smaller aggregates ($p < 0.01$). Smaller MSCs had more potent proliferation potential and higher nucleus/ cytoplasm ratio than large ones. The number of cells positive for senescence-associated β -galactosidase staining was higher in larger UC-MSC aggregates. The survival rates of mice receiving 1×10^6 or 2×10^6 smaller MSCs were 100%, both higher than that receiving larger UC-MSCs sharing same amount. Meanwhile, the reason for mice death was explored and it revealed that larger UC-MSC aggregates were accumulated and evident in the pulmonary capillary lumen in dead mice.

Conclusion: Solvents showed no significant effects on cell behavior, whereas, heterogeneity is quite prevalent in MSCs populations and may limit cell application, but it is easily overlooked. Hence it is necessary to establish a more precise standardization for culture-expanded MSCs and to improve MSCs manufacturing strategies.

Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells with the potential of differentiating into a variety of lineages, representing a novel promising candidate for clinical challenges. MSCs can be harvested from multiple tissues, including bone marrow, adipose tissue, skin, menstrual blood and umbilical cord blood [1]. MSCs are being developed as drugs and have been approved to treat a broad range of diseases, including chondral defect of the knee [2], steroid-resistant graft-versus-host disease [3] and complex perianal fistulas in Crohn's disease [4].

The quality of pharmaceutical production should be strictly tested before being introduced into the clinic, so is the MSC product. In most cases, administration of MSCs entails large scale of cells and MSCs have

to be expanded in vitro to gain sufficient cell doses. Therefore, it is an essential prerequisite to guarantee the generation of cell products conforming to the unified quality control during the course of expansion. However, large-scale expansion of MSCs introduces bias into the culture process that is difficult to control. In the actual experiment studies, we found some MSC populations derived from human umbilical cord blood were safe for animal experiments, while some were not, and the reason remains unclear. These are real-world problems changing for MSCs potent and congruent therapeutic efficacy and needing to be addressed before MSCs translating to clinic.

MSCs are collected and mainly dissolved in three different solvents: phosphate buffer solution (PBS), normal saline (NS) and dulbecco's modified eagle medium (DMEM). Whether MSCs dissolved in three different solvents may lead to different cell behavior and have unexpected harmful effects on mice survival remains indeterminate.

Cell heterogeneity between different MSCs aggregates may be another contributor for the different outcomes of mice. MSCs may display morphological and functional heterogeneity during in vitro culture expansion. When it comes to MSCs characterization, "a minimal standard" has been commonly implemented, that is, plastic adherence, differentiation capacity in vitro (adipogenic, osteogenic, chondrogenic) and a minimalistic panel of special surface markers including CD90, CD29, CD44 and CD105 [5]. However, the "minimal standard" is far from adequate for MSCs definition, because it neither can recognize cell heterogeneity among MSC populations as it is half-baked in terms of cell morphology and function nor can accurately predict cell functions in vivo as the ability of MSCs in vitro and vivo may be inconsistent.

In order to better understand the biology and the safety of MSCs, we endeavored to investigate the effects of cell solvents and cell heterogeneity on the behavior of MSCs in vitro and vivo, hoping to provide a cue for MSCs clinical application.

Materials And Methods

1. Culture and identification of UC-MSCs

Human umbilical cord MSCs (UC-MSCs) were obtained from HuiRong TongChuang Biological Technology Co.LTD. Cells were cultured in DMEM containing 20% fetal bovine serum (Gibco, USA) and 1% penicillin/ streptomycin under standard culture conditions with a change to fresh medium every 3 days, and they were identified by their morphology, the expression of cellular markers and the differentiation potential.

1.1 Morphological observation of UC-MSCs

UC-MSCs were grown to confluence and serially passaged. The adherent cells were observed under an optical microscope Olympus CKX53.

UC-MSCs (about 1×10^6) were collected and resuspended in three different solvents, respectively. The morphological change of UC-MSCs in different solvents (PBS, NS and DMEM) was visualized under an optical microscope.

UC-MSCs from other two separate donors were respectively thawed, cultured and identified, followed by measurement of their diameters. The two groups of UC-MSCs were renamed based on their size for further study.

1.2 Expression of specific markers on UC-MSCs

The phenotype profile of UC-MSCs was assessed by flow cytometry analysis (BD Accuri™ C6 flow cytometer) using PE-labeled CD34, CD90, CD45, CD105 antibody. The PE-labeled IgG1 was used as isotype control. The harvested cells were washed twice with PBS, and then resuspended in the ice-cold PBS. About 100 μ L of the suspension was treated with conjugated antibodies for CD34, CD45, CD90 and CD105, at dilutions recommended by the manufacturer for 45 min at 4 °C. Isotype IgG was used as a negative control. All antibodies were purchased from BD.

1.3 Differentiation potential of UC-MSCs

The induction of adipogenic differentiation was performed using adipogenic differentiation medium kit (Cyagen Biosciences Inc, China) as per manufacturer's protocol. UC-MSCs were seeded in six-wall plate and treated with adipogenic medium for 21 days with medium change 3 times weekly. Adipogenesis was assessed by oil red O staining (Sigma).

To induce osteogenic differentiation, UC-MSCs were seeded in gelatin coated six-wall plate and treated with osteogenic medium (Cyagen Biosciences Inc, China) for 21 days with medium change 3 times weekly. Osteogenesis was assessed by alizarin red dye (Sigma). All photos were taken using microscope Olympus CKX53.

2. UC-MSCs ultrastructural analysis

UC-MSCs ultrastructure analysis was performed using transmission electron microscope (TEM). UC-MSCs dissolved in different solvents and cells of different sizes were all collected and immersed in 2.5% glutaraldehyde for 48 hours at 4 °C, followed by fixation with 1% osmium tetroxide for 30 min 4 °C. The specimens were dehydrated using a graded ethanol series (30, 50, 70, 80, 90 and 100 %) for about 15 min at each step, and then incubated in pure acetone for 20 min. Subsequently, the specimens were embedded in BEEM capsules at 60 °C for 24 h. Ultrathin sections were obtained using ultramicrotome, and then stained with uranyl acetate and lead citrate. The specimens were examined with JEOL-JEM 1010 (Tokyo, Japan).

3. Apoptosis assay of UC-MSCs in different solvents

Apoptotic assay was detected using Annexin V- Fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma) as per manufacturer's protocol. Cells were collected and washed twice with PBS, and then were suspended in 500 μL $1\times$ binding buffer, and were stained with 5 μL of annexin V-FITC conjugate and 10 μL of PI solution. After incubation for 15 min in the dark condition at room temperature, stained cells were analyzed by flow cytometry (BD Accuri™ C6 flow cytometer).

4. Animal experiments

The 6-8 weeks old male C57BL/6 mice were maintained in a controlled environment (24 °C, 55% humidity and 12h day/night rhythm) and were free access to chow and water. About 1×10^6 UC-MSCs dissolved in different solvents (PBS, NS and DMEM) were intravenously injected into mice through the tail vein. And the survival rates of the mice with cells dissolved in different solvents were monitored for 4h after administration using Kaplan-Meier curve.

About 1×10^6 and 2×10^6 larger and smaller UC-MSCs aggregates were intravenously injected into mice through the tail vein, respectively. And the survival rates of the mice with UC-MSCs of different sizes were monitored for 4h after administration.

5. UC-MSCs viability and proliferation assay

Cell viability and proliferation were measured according to the protocol of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit (Beyotime Biotechnology). UC-MSCs of passage 3 or 4 were seeded in 96-well plates and incubated for 2d, 3d, 4d and 5d, respectively. DMSO-treated cells were used as vehicle controls. MTT was added to each well, and MSCs were incubated at 37 °C in the dark for 4 h. MTT was then removed and 150 μL of DMSO was added to each well. The absorbance at 570 nm was measured using a Model ELX800 microplate reader (Bio-Tek Instruments). All experiments were performed in triplicate.

6. Hepatic Differentiation of UC-MSCs

A three-step differentiation protocol using Hepatogenic Differentiation kit (Cyagen) was used to induce hepatic lineage of UC-MSCs according to the manufacturer's protocol. Hepatic induction was performed over a period of 3 weeks. In the first differentiation step, after reaching 80% of confluency, UC-MSCs were submitted to Hepatogenic Differentiation Basal Medium A supplemented with epidermal growth factor (EGF) and basal fibroblast growth factor (bFGF) and cultured for 2 days. Thereafter, cells were incubated with 10mL Hepatogenic Differentiation Basal Medium B supplemented with 2 μL hepatocyte Growth Factor (HGF), 1 μL bFGF and 10 μL Nicotinamide during the hepatic induction stage. The cell medium was changed every 3 days for 7 days. In the last step, that is, hepatocyte mature stage, cells were culture with 10mL Hepatogenic Differentiation Basal Medium C added with 2 μL Oncostatin M, 5 μL Dexamethasone, and 100 μL ITS+Premix. Change the cell medium every 3 days for 7-14 days. After 7 to 14 days of maturation, cells were collected for various evaluation tests

7. Function evaluation and comparison of HLCs

Indocyanine green (ICG, Sigma) was added to culture at a final concentration of 1 mg/mL. Cells were incubated at 37°C for 1h and then washed three times with PBS. The uptake of ICG by HLCs was examined using an inverted microscope.

Glycogen storage of HLCs derived from size-based UC-MSC aggregates was detected using a Periodic acid-Schiff (PAS) kit (Solarbio, China) according to the manufacturer's instructions. Briefly, cells were fixed in 3.7% formaldehyde for 30 min and then oxidized in 1% periodic acid for 10 min and rinsed twice with water. Subsequently, cells were treated with Schiff's reagent for 15 minutes and then rinsed with water. Glycogen storage was assessed under a light microscope.

8. Detection of senescence

Cells senescence were determined using β -Galactosidase (β -gal) Staining kit (Beyotime, China). UC-MSCs were fixed and stained in solution at 37 °C for 24 h. Afterwards, cells positive for β -gal activity were observed under a microscope.

9. Immunofluorescence

Tissue samples were fixed in 10% formalin overnight, cryopreserved in 30% sucrose overnight, followed by frozen in OCT compound (Thermo Scientific) and stored at -80°C. Then cryopreserved samples were cut into 5 μ m sections. Slides were washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma) for 20 min. Samples were washed three times with PBS and then blocked with 2% goat serum in 0.1% Tween™ 20 in PBS for 1h at room temperature. Antibodies were diluted as follows: CD31 (Sigma) and CD90 (Sigma). Slides were incubated at 4°C overnight. Subsequently, slides were washed three times with PBS and incubated with fluorescent secondary antibody (Invitrogen) diluted 1:500 in antibody diluent for two hours at room temperature. Nuclei were stained with DAPI (Life Technologies) for 10 min. Samples were washed three times with PBS and mounted with ProLong™ Gold Antifade Mountant (Invitrogen, P10144). Images were taken on a Nikon Eclipse Ti epifluorescence microscope.

10. Statistics analysis

Statistical analysis was performed using SPSS 20.0. Data were presented using mean plus standard deviation (SD). Student's t test or non-parametric one-way analysis was used to analyze differences between groups, and the results were considered statistically significant if $p < 0.05$.

Results

1. Identification of UC-MSCs

UC-MSCs Morphology UC-MSCs were isolated from umbilical cord of healthy donors with their consent. Cells cultured in vitro were adherent to tissue culture plastic and showed a spindle-like morphology

(Fig. 1A). The cells were variable and not homogeneous amongst UC-MSCs.

Phenotypic characterization of UCMSCs Cell surface markers were analyzed by flow cytometry. The results showed that cells were positive for CD105 (96.3%) and CD90 (99.7%) and negative for CD34 (1.0%) and CD45 (0.8%) (Fig. 1C), demonstrating that cultured cells expressed characteristic stem cell-associated surface markers.

Differentiation capacity of UCMSCs With standard cell induction conditions, the adipogenic and osteogenic potential of UCMSCs was evaluated at passage P3.

Positive staining of Oil red O or Alizarin Red was observed after induction differentiation for 21 days (Fig. 1B).

2. Effect of different solvents on UC-MSCs vitality and function

UC-MSCs dissolved in DMEM, NS and PBS showed similar round appearance (Fig. 2A). About 1×10^6 UC-MSCs dissolved in DMEM, NS and PBS were respectively intravenously injected into mice and the survival rates of mice were monitored and compared. It seemed that no mice died and no differences were observed among UC-MSCs in different solvents (Fig. 2B). The apoptosis rates of UC-MSCs in DMEM, NS and PBS were calculated, about 2.94%, 2.55% and 2.84% for 0 h, 3.23%, 8.33% and 6.03% for 3 h and 4.96%, 8.98% and 6.91% for 6 h, respectively. And no significant difference was observed (Fig. 2C). The effect of different solvents on UC-MSCs ultrastructure was further investigated using TEM. The main organelles, nucleus, mitochondrion, Golgi body and endoplasmic reticulum, were similar among UC-MSCs in different solvents at the same time point, whereas, all UC-MSCs in different solvents swelled as time went by, accompanied with dropsy of cytoplasm and dissolution of some organelles (Fig. 2D). It seemed that solvents showed little effect on UC-MSCs nature.

3. Effect of different morphologies on UC-MSCs vitality and behavior

We measured cell diameters in other two UC-MSC groups under an optical microscope, respectively, and renamed the cells with diameter of $15.58 \pm 3.813 \mu\text{m}$ as larger MSC aggregates and the cells with diameter of $19.14 \pm 4.885 \mu\text{m}$ as smaller MSC aggregates. As showed in Fig. 3A, although all MSCs were spindle-like, the mean diameter of larger MSC aggregates was significantly longer than that of smaller aggregates ($p < 0.01$). Besides, smaller MSCs showed more potent proliferation potential than large ones using MTT assay (Fig. 3B). By TEM, we observed the difference of ultrastructure in UC-MSCs of larger and smaller aggregates and smaller MSC aggregates had higher nucleus/ cytoplasm ratio (Fig. 3C).

Assays were performed to investigate the function of HLCs derived from UC-MSCs of different sizes. Significant positive staining of ICG and glycogen granules were observed in the cytoplasm of HLCs derived from smaller UC-MSC aggregates, whereas weaker ICG and PAS staining signals were detected in HLCs derived from larger UC-MSC aggregates (Fig. 3D). The results indicated that UC-MSCs can successfully differentiate to HLCs, and the synthetic and storage function of HLCs derived from smaller UC-MSC aggregates may be more potent than that derived from larger UC-MSCs.

About 1×10^6 and 2×10^6 UC-MSCs of different sizes were respectively intravenously injected into mice and the survival rates of mice were monitored and compared. The survival rates of mice receiving 1×10^6 or 2×10^6 smaller MSCs were 100%, both higher than that receiving larger UC-MSCs sharing same amount (Fig. 4A).

Senescence was detected by β -gal assay and cells positive for β -gal have the potential for senescence. The results showed that the number of cells positive for β -gal staining was higher in larger UC-MSC aggregates (Fig. 4B).

4. Accumulation of large UC-MSC aggregates in lung tissue

The reason for mice death after UC-MSCs injection was further investigated by tissue immunofluorescence analysis (Fig. 4C). Heart, liver and lung tissues were harvested from mice which were either dead immediately after injection or were sacrificed by spinal cord dislocation. The result revealed that larger UC-MSC aggregates were accumulated and evident in the pulmonary capillary lumen, which may be the cause of mice death.

Discussion

Although great success has been achieved from MSCs pre-clinical and clinical trials, there are some obstacles remaining to be addressed, such as cell solvent and cell heterogeneity, which may affect cell property and behavior and limit their clinical application. In present study, we verified UC-MSCs shared certain common properties, including fibroblastoid morphology, special marker expression and differentiation capacity in vitro. UC-MSCs solved in different solvents (DMEM, NS, PBS) showed comparable morphology, survival rate and ultrastructure, indicating that these common solvents may have little effect on cell behavior.

Cell heterogeneity may be another contributing factor for the different mice survival rates. Cell heterogeneity mainly refers to the difference in the aspects of morphology and function among cell populations. Our study defined cells as larger and smaller UC-MSC aggregates based on their mean diameter, and revealed intergroup size-based morphological difference between the two UC-MSC aggregates though these UC-MSCs were all isolated from human umbilical cord. Morphological heterogeneity may ultimately affect cell function as a previous study reported the chondrogenic differentiation capacity of UC-MSCs was strongly correlated with morphological data based on the size and shape features [6]. In present study, we found smaller UC-MSCs aggregates showed more powerful hepatic differentiation potential than larger cells.

It has well documented that senescent cells are always accompanied with enlarged morphology and they can be maintained in the nonproliferative state for months [7]. It was indeed that in our study more cells positive for senescence-associated β -galactosidase were observed in larger UC-MSC aggregates and these larger cells were less proliferative. Asynchronous cell senescence between the two UC-MSC aggregates may drive different cell activity and behavior. Besides, smaller UC-MSCs aggregates displayed

higher nucleus/ cytoplasm ratio. Other study proved that, the larger cells, to put it more precise, may represent a collection of cells that have ceased the normal cell cycle, whereas smaller cells are mitotically active [8]. Collectively, smaller UC-MSCs aggregates seemed to be more “naïve” and more active in mitosis, whereas larger cells may be restricted in proliferation and hepatic differentiation potential.

Previous studies have reported that morphological feature may strongly affected MSC activity [6] and functional heterogeneity among UC-MSCs can lead to diminished therapeutic efficacy [9], we thus investigated whether the difference in size and function may affect UC-MSC behavior in animal experiment after administration. Our finding presented that larger UC-MSCs were trapped in lung, leading to higher mice mortality than smaller cells. Hence, the different mice outcomes can be attributed to the variability between UC-MSC aggregates, and morphological and functional heterogeneity of UC-MSCs may limit their application.

It is anecdotally established the size of cultured UC-MSCs may increase over the time and more cells with enlarged morphology, reduced expression of surface markers, and decreased differentiation potential may be observed with increasing passage number [7, 8], meanwhile, more senescent UC-MSCs will be detected during long-term culture. This may be the reason why cells at low cell passages are required in the field of MSCs studies. However, in present study, all UC-MSCs are from the same cell line passage and cultured under standard conditions, hence we speculated that the heterogeneity between two UC-MSC aggregates may be attributed to host source, or that is, donor-to-donor heterogeneity. Heterogeneity exists in MSCs from donors living in various backgrounds [10]. MSCs biology is strongly affected by donor age. More senescent cells were found in MSCs isolated from elderly people even at the initial cell passage, and these MSCs had decreased proliferation and differentiation potential compared those from younger donors [11, 12]. Except for age, obesity and health status may also affect MSCs action [13]. MSCs derived from donors of obesity [14] and/ or donors with systemic diseases [15] are dysfunctional.

Heterogeneity also emerges in cells obtained from a single donor, from different tissues and even from the different portions of a same tissue [16]. The conventional assays usually measure UC-MSCs in bulk, and thus may mask cell-to-cell variation, whereas UC-MSCs are comprised of a diverse of subpopulations and these subpopulations may be not homogeneous [17, 18]. Lee et al [19] proved that distinct biophysical characteristics may occur in UC-MSC subpopulations during culture expansion. In present study, intragroup mild morphological differences within UC-MSC populations derived from a single donor and expanded in the same vessel were observed. Additionally, it is generally believed that cell clusters derived from a single cell should be functionally homogeneous stem cells, but this is not the always case. Single-cell colonies are not necessarily homogeneous subpopulation, and colonies cannot accurately represent their entire putative stem cell subpopulation [20]. Heterogeneity is so pervasive that it is reasonable to infer MSC populations are intrinsically heterogeneous [21].

The reasons for heterogeneity within a typically expanded UC-MSC population are complex. One may be that UC-MSCs are composed of multi-cell-derived cells. These multi-cell-derived cells are initially different both in gene and protein expressions, leading to heterogenetic progenies. Defects accumulation and

mutations during long-term culture may be other reasons accounting for UC-MSCs heterogeneity [7]. Cell-to-cell contact may contribute to the change of cell size and morphology [22], but it may be not the fateful factor as Haack-Sorensen et al [23] reported that cell density had no significant influence on MSC phenotype. Besides, conventional isolation and culturing protocols may be not conducive to maintain MSCs homogeneity. Whatever the reasons, measures have to be taken into account to avoid cell heterogeneity and to ensure quality control.

It is a pity that we neither interrogate when and how the heterogeneity are initiated amongst a MSC population nor explore the transcriptome difference between two MSC aggregates, which may help to illustrate the underlying molecular mechanism of heterogeneity and guide cues to regulate MSCs morphology and function.

Conclusion

Taken together, solvents showed no significant effects on cell behavior, whereas, heterogeneity is quite prevalent in MSCs populations and may limit cell application, but it is easily overlooked. The findings in this study may lay a foundation for better understanding of MSC heterogeneity, emphasizing the need to establish a more precise standardization of culture-expanded MSCs and the necessary to improve MSCs manufacturing strategies.

Abbreviations

UC-MSCs: human umbilical cord MSCs; PBS: phosphate buffer solution; NS: normal saline; DMEM: Dulbecco's Modified Eagle Medium; ICG: Indocyanine green; PAS: periodic acid-schiff; EGF: epidermal growth factor; B-FGF: basal fibroblast growth factor; HGF: hepatocyte Growth Factor; TEM: transmission electron microscope.

Declarations

Acknowledgements

None

Authors' contributions

Hong Tang and En-qiang Chen contributed to the study concept and design. Yong-Hong Wang and Ya-Chao Tao contributed to the acquisition of data and drafting. All authors contributed to the analysis and interpretation of data and approved the final version.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All mice received human care under the Institutional Review Board in accordance with the Animal Protection Act of Sichuan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

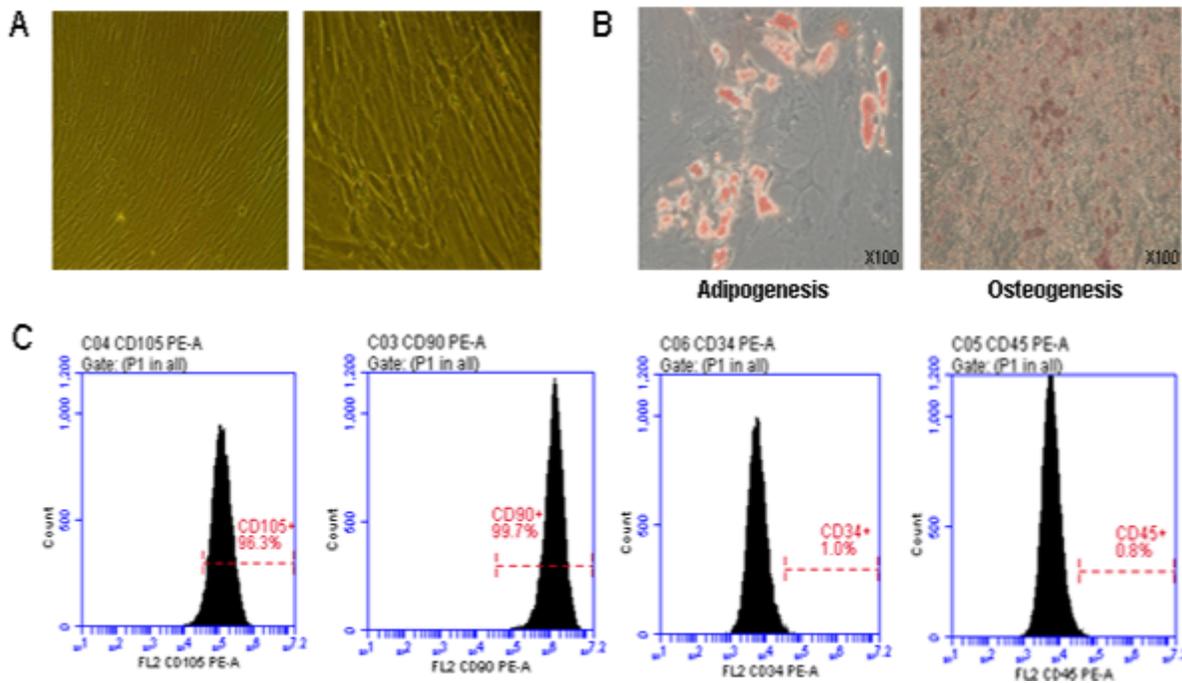


Figure 1

Identification of UC-MSCs. The UC MSCs were identified by cell morphology (A), differentiation capacity (B) and surface markers (C).

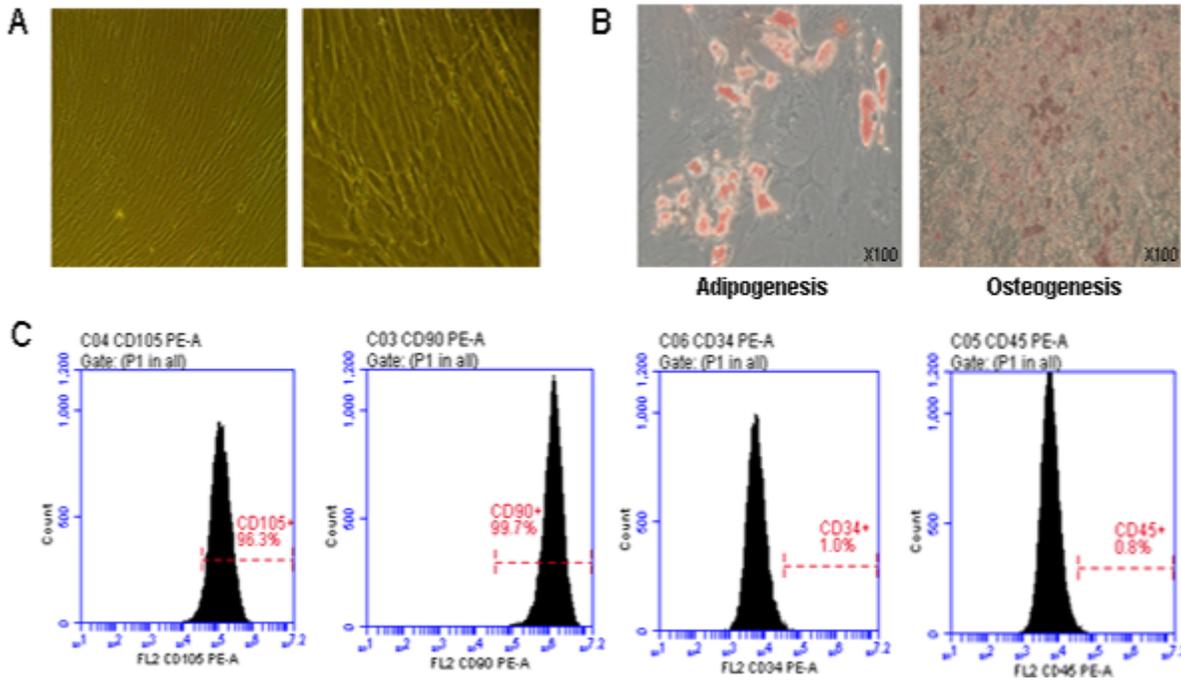


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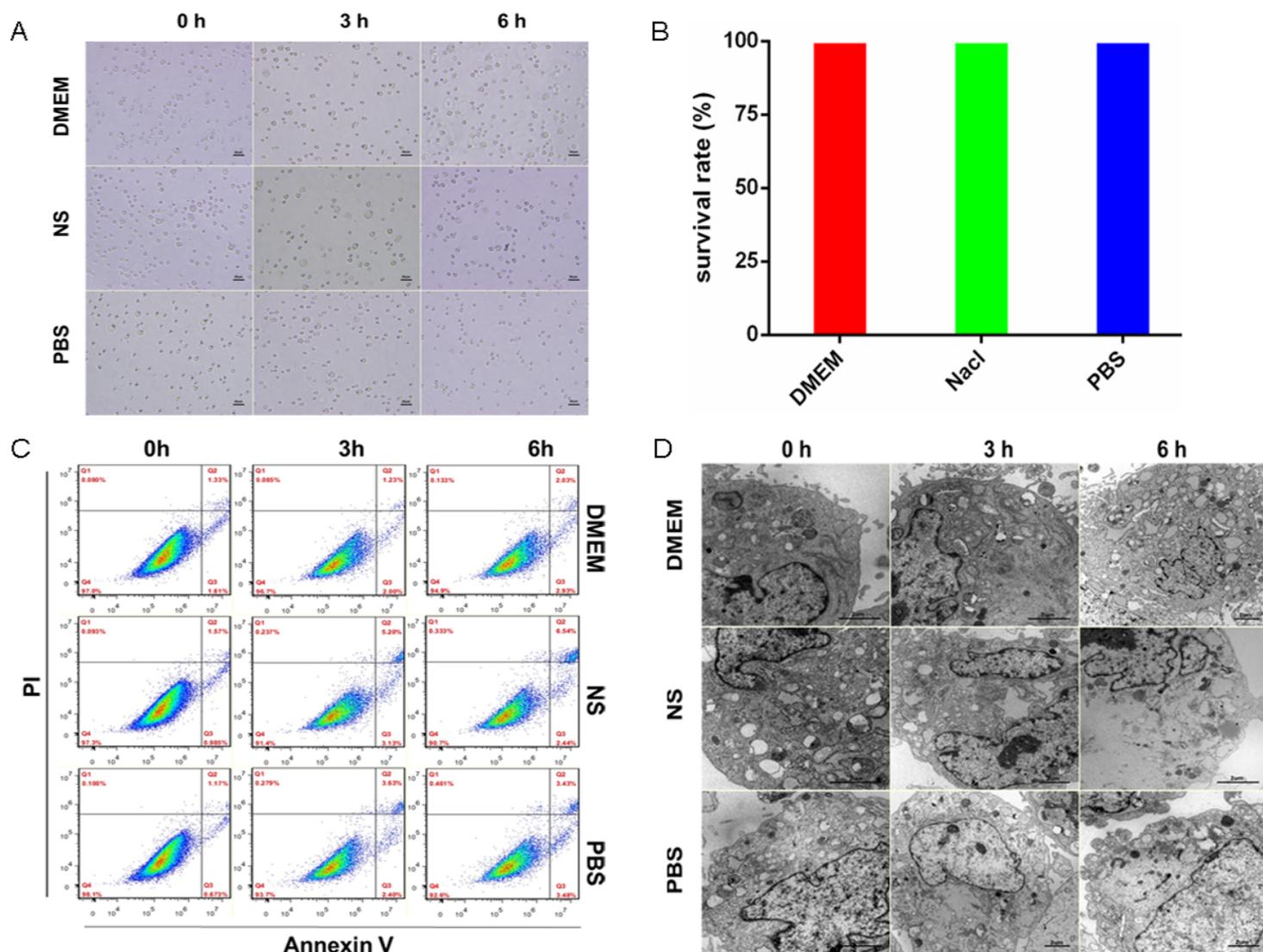


Figure 2

Effect of solvents on UC-MSCs action. (A) Morphology observation of UC-MSCs dissolved in DMEM, NS and PBS as time went by. (B) Comparison of survival rates of mice injected with UC-MSCs dissolved in DMEM, NS and PBS. (C) Calculation of apoptosis rates of UC-MSCs dissolved in DMEM, NS and PBS. (D) Ultrastructure analysis of UC-MSCs dissolved in DMEM, NS and PBS.

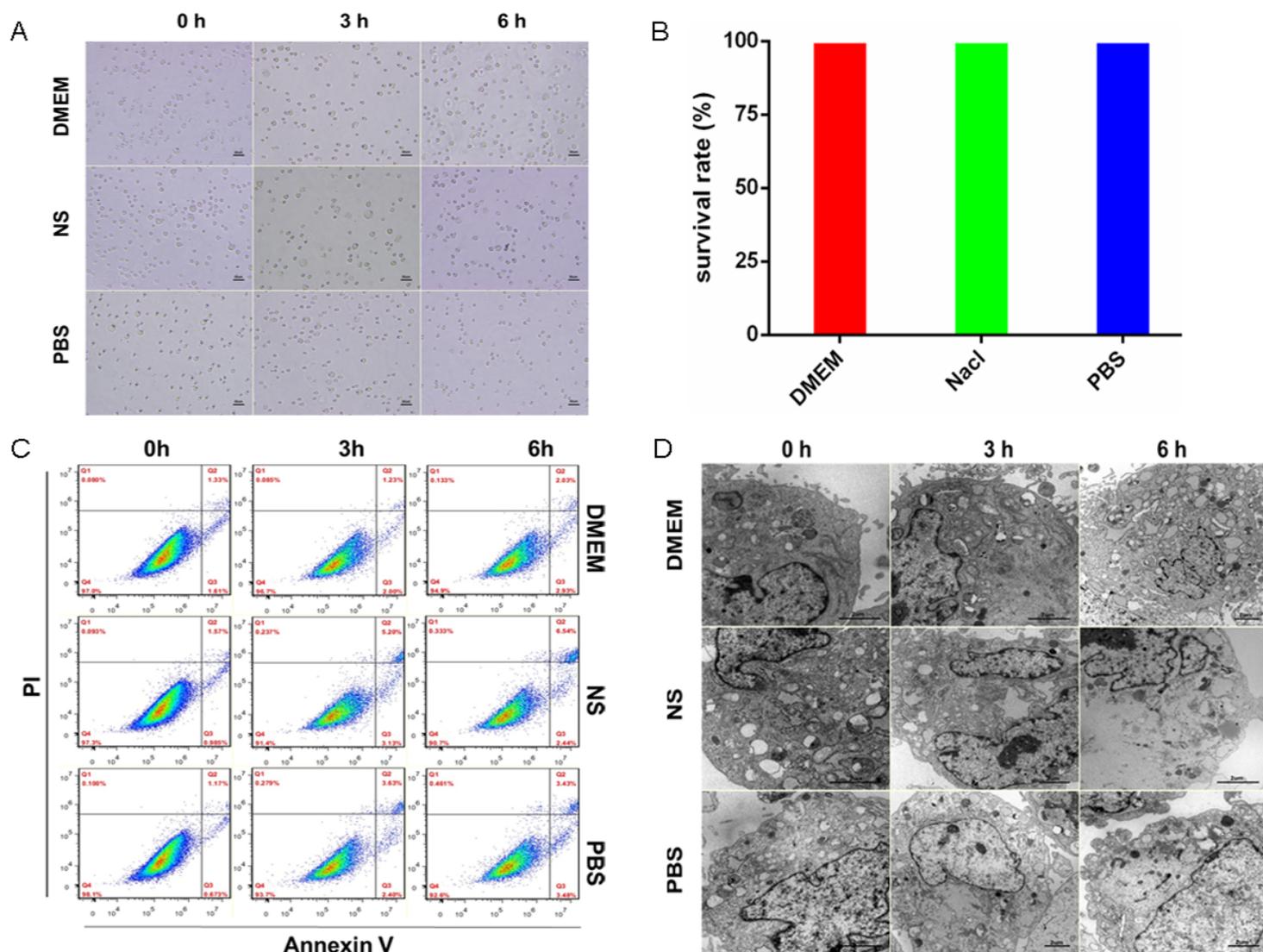


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Effect of solvents on UC-MSCs action. (A) Morphology observation of UC-MSCs dissolved in DMEM, NS and PBS as time went by. (B) Comparison of survival rates of mice injected with UC-MSCs dissolved in DMEM, NS and PBS. (C) Calculation of apoptosis rates of UC-MSCs dissolved in DMEM, NS and PBS. (D) Ultrastructure analysis of UC-MSCs dissolved in DMEM, NS and PBS.

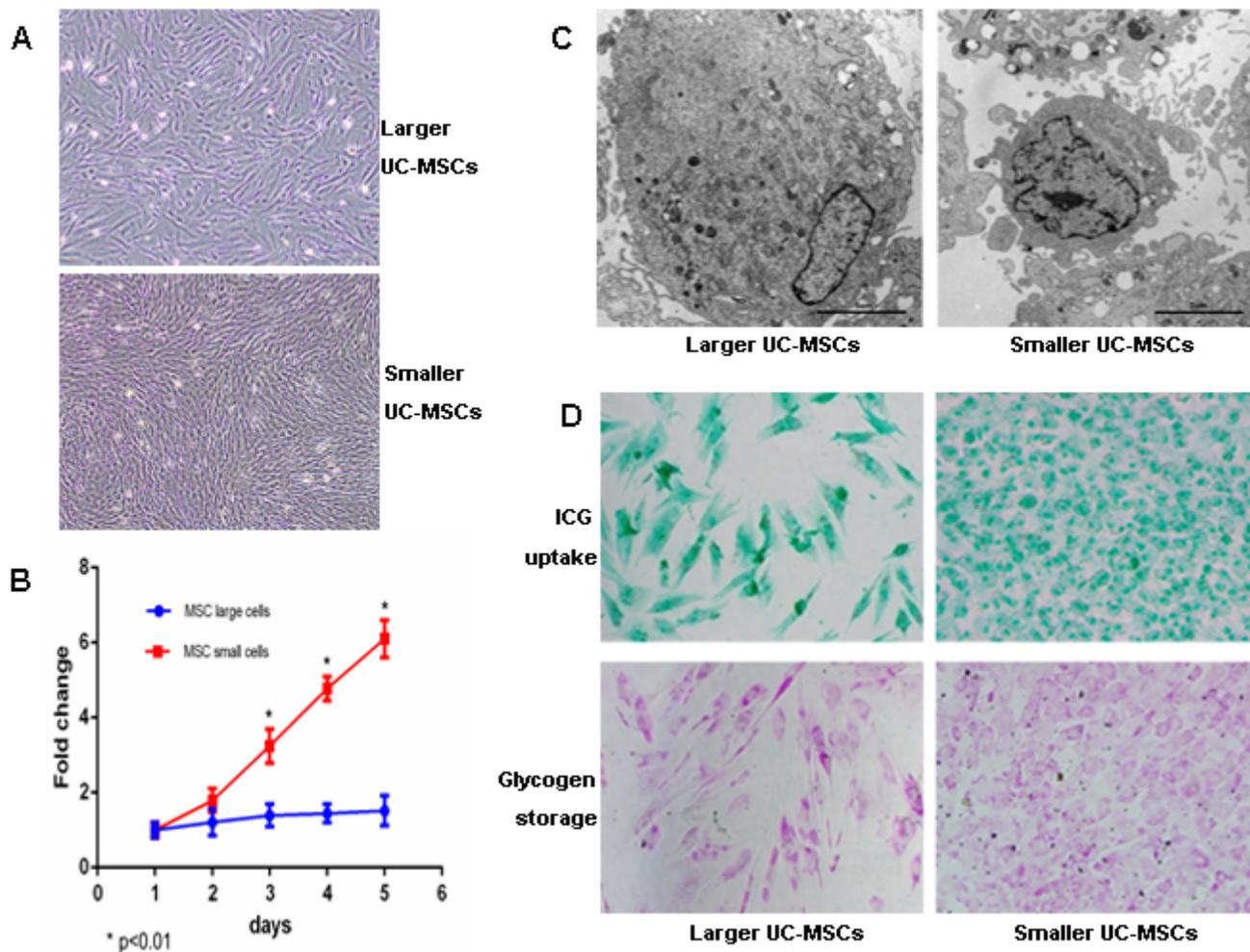


Figure 3

Effect of morphology on UC-MSCs characteristics. (A) Observation of size-based morphology difference in two UC-MSC groups. (B) Comparison of proliferation potential between the two UC-MSC aggregates. (C) Ultrastructure analysis of UC-MSCs of larger and smaller aggregates. (D) Functional comparison of HLCs derived from UC-MSCs of larger and smaller aggregates.

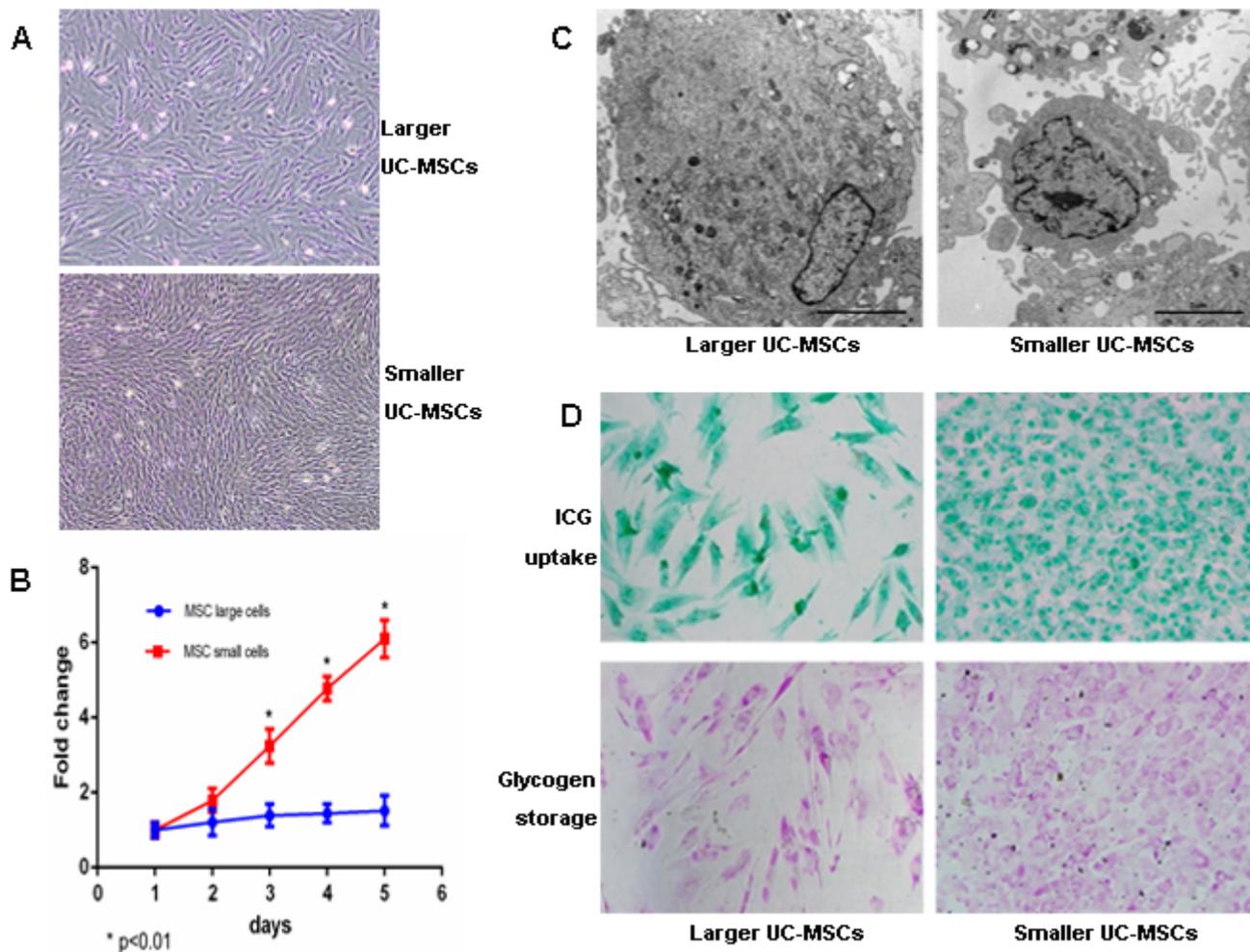


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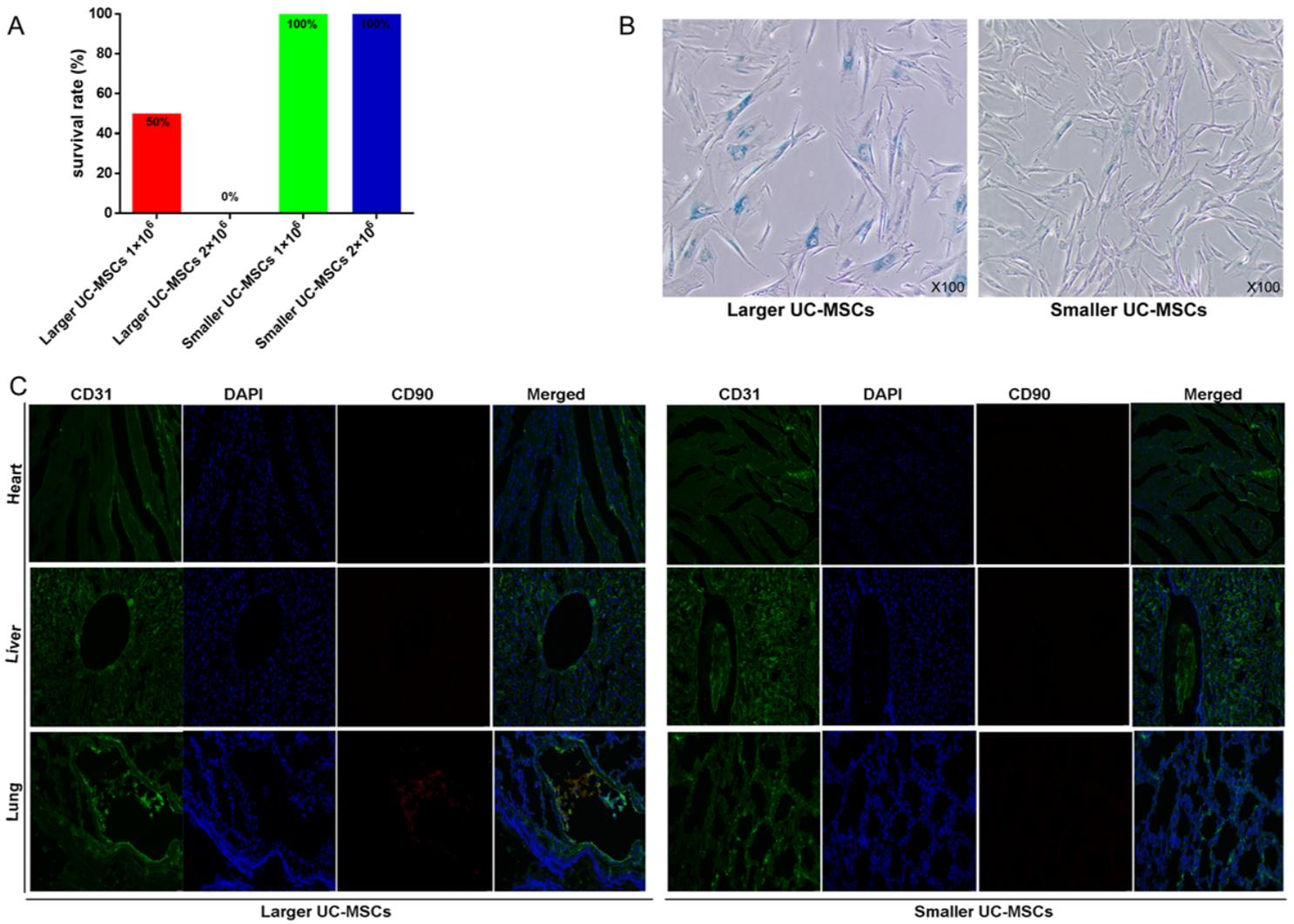


Figure 4

Effect of size-based morphological difference on UC-MSCs behavior. (A) Comparison of survival rates of mice injected with UC-MSCs of different sizes. (B) Observation of senescent cells in UC-MSCs of different sizes. (C) Reason for mice death after injection of UC-MSCs of different sizes.

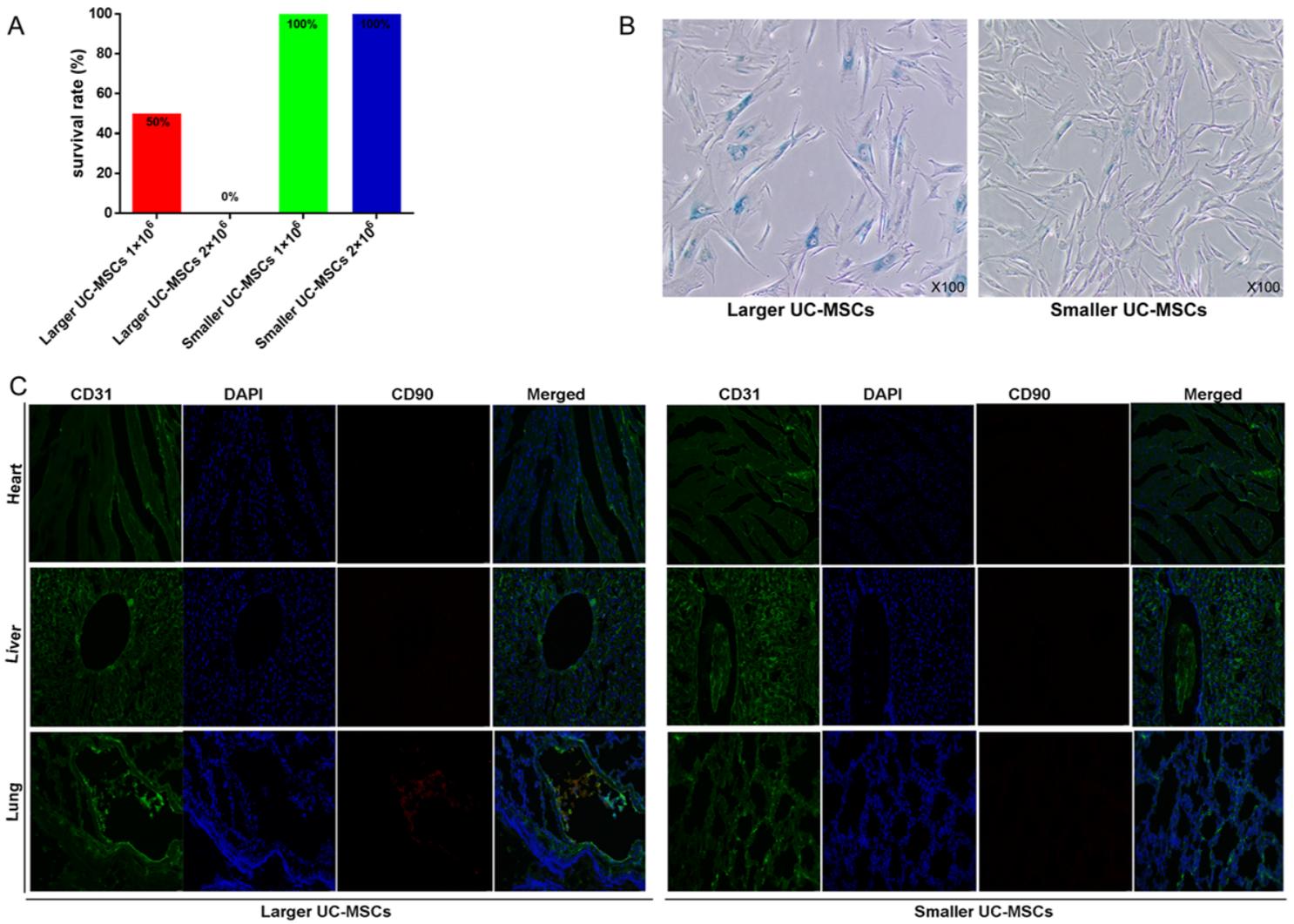


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