

## Original Article

# Experimental study of super paramagnetic iron oxide labeled synovial mesenchymal stem cells

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**Abstract:** To investigate the feasibility and changes of biological characteristics before and after synovial mesenchymal stem cells (SMSCs) labelled by super paramagnetic iron oxide (SPIO). The rabbit SMSCs were isolated, cultured, purified and identified in vitro. After adding the different concentrations of SPIO-labelled liquid, the cells were incubated 24 h in 37 °C carbon dioxide incubator. The labeled-cell samples were observed by Prussian blue staining, transmission electron microscope (TEM) and the cell biology before and after the labeling was compared. The blue stained particles could be seen in the cytoplasm; the SPIO label was positive in 95% SMSC cells. With the concentration of the label liquid increasing, the blue-stained cytoplasm became darker. A large number of high electron density particles could be seen in the cytoplasm and in the pinocytosis vesicles by TEM, which suggested SPIO label positive. When the SPIO concentration was (12.5~50) µg/mL, the differences in cell proliferation and cell viability between the SMSCs after labelling and the SMSCs before labelling were not significant; when the concentration was over 100 µg/mL, the cell proliferation and cell viability were inhibited. A certain concentration range of SPIO can safely label the rabbit SMSC according to this study, which is important for solving the problem of tracing SMSCs in the joints.

**Keywords:** Super paramagnetic iron oxide nanoparticles, synovial mesenchymal stem cells, cell label

## Introduction

Articular cartilage injury repair transplantation are important and difficult for orthopedic researchers. The findings of pluripotent stem cells provide new ideas and methods for repairing cartilage injury. Currently, bone marrow stem cells (Bone marrow mesenchymal stem cells, BMSC), adipose stem cells (Adipose-derived mesenchymal stem cells, ADSC) [1, 2], because the source is easy, its research is more. The study of synovial mesenchymal stem cells (synovial mesenchymal stem cells, SMSC) is relatively small. The distribution, migration, vesting conditions of SMSC after transplantation require effective means for tracking. Super paramagnetic iron oxide nanoparticles (super paramagnetic iron oxide, SPIO) is a MRI contrast agent approved by the United States FDA [3]. Currently it is successful in the marking BMSC, ADSC and other cells. In order to solve the problem that tracing in SMSC joint cavity after transplantation, in this study through SPIO

labeled SMSC in vitro, joint cavity after transplantation, we discussed its feasibility and safety, and it is as follows.

## Material and methods

### Material

**Animals:** 2-month-old New Zealand rabbits, weighing between 2000 and 3000 g, male, were provided by Experimental Animal Center attached to 302 Hospital of People's Liberation Army.

**The main reagents and instruments:** SPIO (Beijing Deco Gold Technology Limited), DMEM/F12 (1:1) medium, fetal bovine serum, 0.25% trypsin (GIBICO), I collagenase (Sigma, USA), poly-L-lysine (Sigma, USA), double-antibody (GIBCO company), Prussian blue stain (Beijing Reagan biotechnology Co., Ltd.), trypan blue dye (Sigma, USA), CCK-8 (Dojindo), CO<sub>2</sub> incubator (Thermo Co.), the Reader and inverted opti-

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cal phase contrast microscope (Thermo) and the like.

### Methods

*SMSC isolated cultivation, purification and identification in vitro:* The rabbits were anesthetized, and knee skin were disinfected. After the kerchief was paved, they were cut and separated from the subcutaneous tissue and deep fascia layer by layer. Then we open the knee, cut the patellar synovial tissue in the medial capsule of the patellar knee. In the clean table, the separated synovial tissue were cut into small size of 1~2 mm<sup>3</sup> pieces. Adding 0.1% I collagenase, after digested at 37°C 5% CO<sub>2</sub> incubator for 4 h, they were filtered by 120 mesh nylon net (70 um). The filtered was centrifuged and the supernatant was discharged. 10% DMEM culture medium was used for cells resuspended and counted. After 37°C 5% CO<sub>2</sub> incubated for 24 h, the medium was changed in the next day and the non-adherent cells were discarded. When the cells covered about 85% bottom of the plate, cell subculture were proceeded. Synovial stem cells were purified by adherence screening method and limiting dilution method. Dynamic Inverted phase contrast microscope was used to observe cell growth. Take the third generation of well growing synovial mesenchymal stem cells, and then add rabbit anti-mouse CD44, CD90, CD34, CD45 monoclonal monomer. The expression of SMSCs surface antigen were detected by flow cytometry.

*SPIO labeled SMSCs:* SPIO was added to DMEM/F 12 medium (containing 20% FBS, double antibody 100 u/ml). The SPIO was formulated to 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 150 µg/mL and then 0.75 µg/mL concentration of poly-L-lysine was added. After filter sterilized, they were shaking at 37°C labeled solution for 1 h before use. When SMSC grew to about 85% of plate bottom in 12-well culture plate, the original culture medium containing serum were discarded. They were washed 2 times by PBS, and then randomly divided into four groups with three holes, respectively. In each hole of every group, 1 ml different concentrations of SPIO-labeled solution were added. They were cultured for 24 h and then detect the labeling conditions.

*Prussian blue staining:* Take the SPIO-labeled cells and remove the culture medium. They

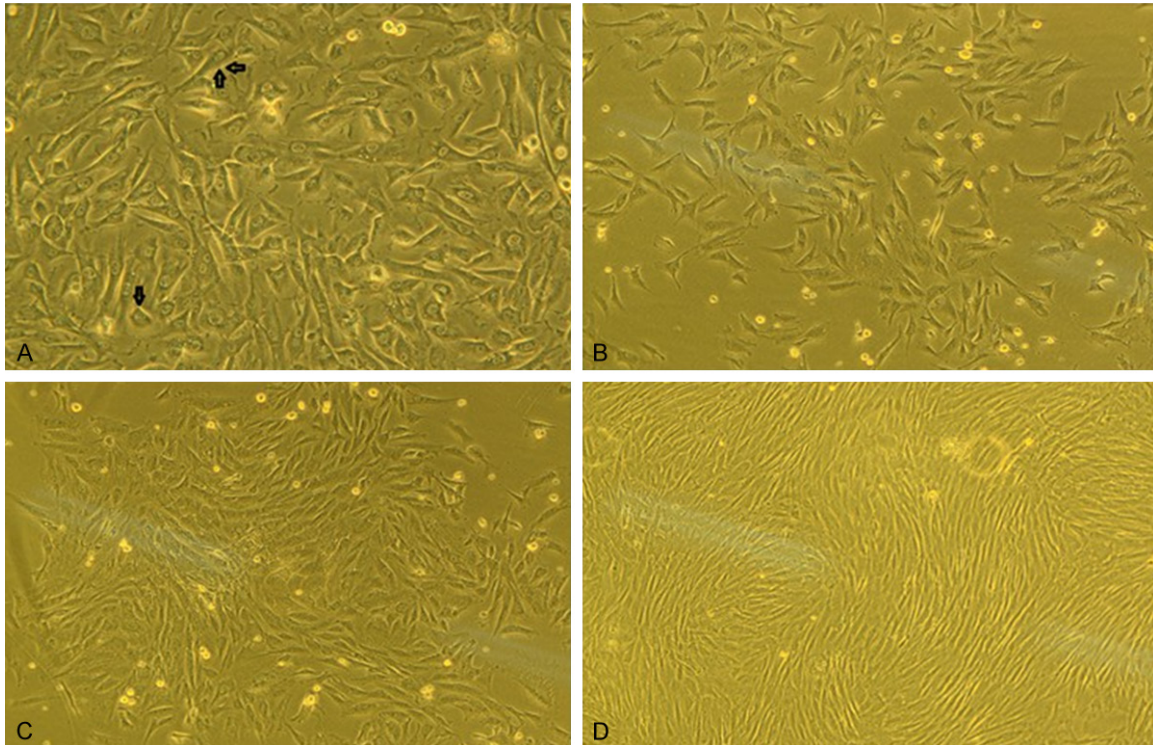
were rinsed with PBS containing heparin to remove the extracellular free iron particles. With 4% paraformaldehyde fixed for 10 min, they were washed twice by distilled water. They were incubated by 2% potassium ferrocyanide and 6% aqueous hydrochloric acid at room temperature for 60 min, and washed three times with distilled water. Nuclear fast red was used to re-stain the nucleus for 5 min. They were washed with distilled water for three times and then observed by inverted phase contrast microscope. Blue particles in the cell indicated success of staining and was referred to as positive cells. We counted the positive cells at 10 magnification for three and the total number of cell. Positive rate SPIO-labeled = positive cells/total cells × 100%.

*Observation of projected electron microscope:* Take 50 µg/mL SPIO labeled SMSCs, and remove supernatant after centrifugation and digestion. They were rinsed 3 times with PBS then the cells were transferred to 1.5 ml Ep tube. Along the wall we added 4% glutaraldehyde solution to fix for 2 h. After rinsed by PBS and double-distilled water, gradient dehydration was performed with different concentrations of ethanol. Then 1% osmium tetroxide was used to fix at 4°C for 30 min. After soaking by embedding agent for 2 h, uranyl acetate was used for staining for 15 min. ultrapure water were used to rinse the lead nitrate staining solution for 5 min. Ultrapure water rinsing, Epon 812 epoxy resin embedding and sectioning were performed. Then transmission electron microscopy was used to observed and photographed.

*Trypan blue staining to detect cell viability:* Trypan blue dye exclusion method was used. Unlabeled SMSC and different concentrations of SPIO-labeled SMSC were incubated in a 37°C carbon dioxide incubator for 24 h, and then the cell suspension in each group was prepared; 100 µL cell suspension was mixed with 10 µL 0.4% trypan blue solution; 1 min later, 10 µL mixture was collected in the cell count panel and observed; 10 horizons were selected, 100 cells per horizon; finally the average was calculated. Cell viability (trypan blue dye exclusion rate) = unstained cells/total cells × 100%.

*Detection of CCK-8 cell proliferation activity:* SMSC with good growth state were digested and suspended before and after mark. Collected and counted in 96-well plates, they

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**Figure 1.** The morphology and growth characteristics of Synovial mesenchymal stem cells (SMSC) under a microscope. A. Primary SMSC was cultured for 4 d; the single arrow showed the cell was round, and the double arrow showed the cell was long fusiform ( $\times 200$ ). B. When the second-generation SMSC was cultured for 2B, cells were substantially long fusiform ( $\times 200$ ). C. Purified monoclonal SMSC showed sunflower-like growth ( $\times 200$ ). D. SMSC showed typical spiral-like growth ( $\times 200$ ).

were seeded in cell suspension (100  $\mu$ L/hole about 2000 cells). When the cells were adherent to the wall, 10  $\mu$ L CCK-8 solution were added to each well. After 4 h incubation, measured with a microplate reader was used to detect the OD value at 450 nm and made a record.

### Results

#### *SMSC's culture, passage and identification*

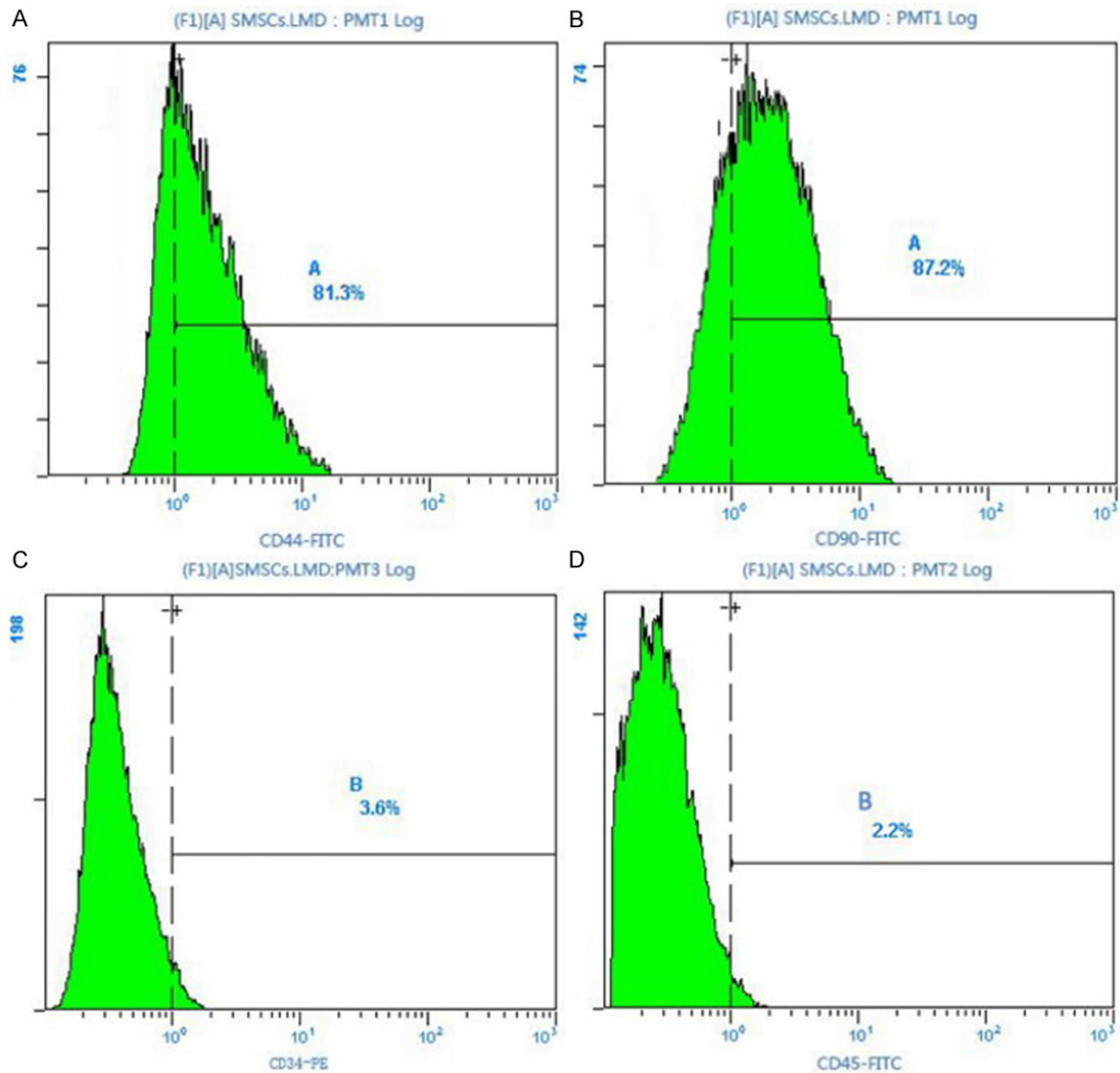
After 4 h of incubation, the primary SMSC can be adherent within 4 h, and 3~4 d later cell proliferated significantly; adherent cells were mainly round and long fusiform (**Figure 1A**); spindle cells showed dominant growth; generally passage was performed after 3 days; after passage, cells were basically long spindle synovial stem cells (**Figure 1B**); after monoclonal purification, SMSC showed sunflower-like cell population growth (**Figure 1C**); When SMSC covered 100% of dish bottom, it showed a typical spiral-like growth (**Figure 1D**). SMSCs of the

3rd generation were detected by flow cytometry; the results showed that mesenchymal stem cell markers CD44 and CD90 were positively expressed; the positive rates of CD44 and CD90 were respectively 81.3% and 87.2%; cells did not express hematopoietic stem cell markers CD34 and CD45; CD34 expression rate was 3.6%; CD45 expression was 2.2% (**Figure 2**).

#### *Detection of SPIO-labeled SMSC*

Prussian blue staining showed that in the cytoplasm of SPIO-labeled SMSC, a lot of dense blue-stained iron particles could be observed, positive; cell labeling rate was more than 95%, and with the increasing concentration of labeling liquid, cytoplasmic blue-stained iron particles increased and color deepened; while in the cytoplasm of the control group, no blue-stained iron particles was found, negative (**Figure 3**). Under the transmission electron microscope, no high-electron-dense particles were observed in the cytoplasm and endocytic vesicles of unlabeled stem cells in blank control group,

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**Figure 2.** Molecular phenotypical identification of Synovial mesenchymal stem cell. The positive rates of CD44 and CD90 were respectively 81.3% and 87.2%; CD34 expression rate was 3.6%; CD45 expression was 2.2%. A: CD44; B: CD 90; C: CD34; D: CD45.

negative (**Figure 4**); while in the cytoplasm and endocytic vesicles of SPIO-labeled SMSC, there were a lot of high-electron-dense particles, positive (**Figure 5**), indicating that preliminary study of SPIO-labeled synovial mesenchymal stem cells is feasible.

### *Effect of SPIO labeling on cell viability*

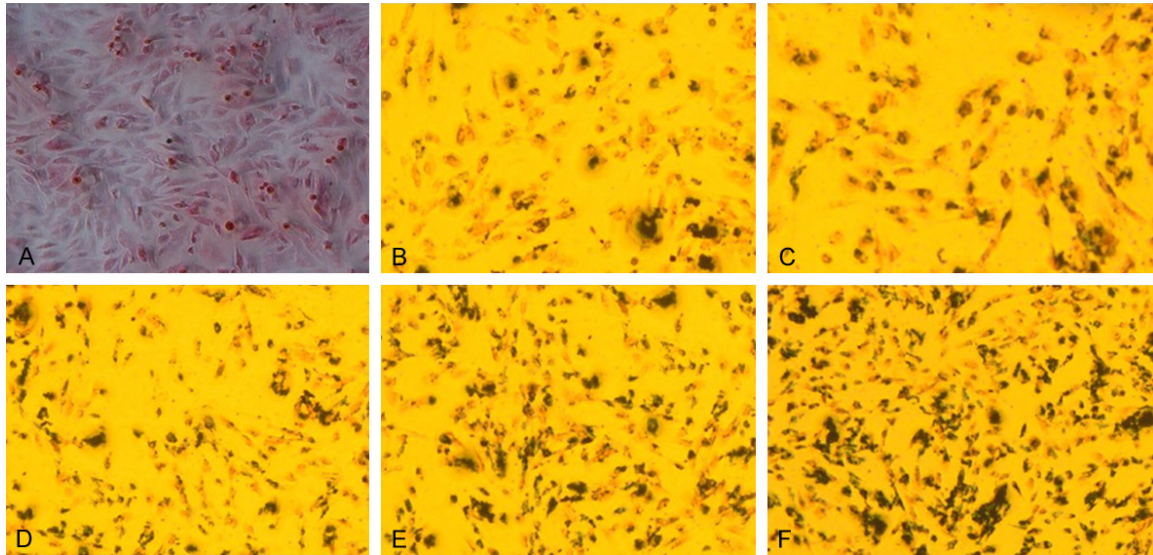
The average trypan blue dye exclusion rate of unlabeled SMSC was 98.7%; After being labeled by 12.5  $\mu\text{g}/\text{mL}$ , 25  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$ , 100  $\mu\text{g}/\text{mL}$  and 150  $\mu\text{g}/\text{mL}$  SPIO-labeled liquid for 24 h, trypan blue dye exclusion rates were 98.6%, 98.5%, 98.6%, 91.6% and 88.5% respectively; at concentrations of 25  $\mu\text{g}/\text{mL}$  and 50  $\mu\text{g}/\text{mL}$ ,

there was no significant difference compared with the trypan blue dye exclusion rate of unlabeled SMSC; when the concentration were 100  $\mu\text{g}/\text{mL}$  and 150  $\mu\text{g}/\text{mL}$ , compared with trypan blue dye exclusion rate of unlabeled SMSC, there were obvious differences, indicating that High concentrations of SPIO affected SMSC vitality.

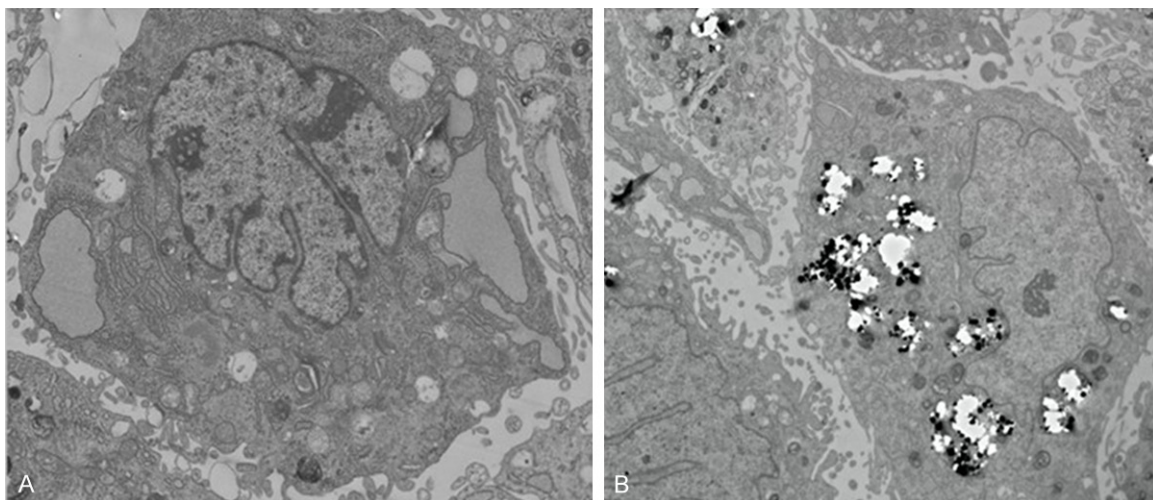
### *The effect of SPIO labeling on cell proliferation*

OD values of SPIO-labeled SMSC and unlabeled SMSC were measured by CCK-8 in 1~8 at a wavelength of 450 nm to plot growth curve; when the concentration of the marker was 12.5  $\mu\text{g}/\text{mL}$ , 25  $\mu\text{g}/\text{mL}$  and 50  $\mu\text{g}/\text{mL}$ , the cell

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**Figure 3.** Prussian blue staining for different concentrations of SPIO-labeled SMSCs ( $\times 100$ ). A. In the control group, there were no blue-stained iron particles in cytoplasm, negative. B-F. Blue iron particles can be seen in the cytoplasm, and with the increased concentration of the marker, color deepened.



**Figure 4.** TEM observations of SPIO-labeled SMSCs ( $\times 5000$ ). A. The transmission electron microscopy results for unlabeled SMSCs, negative. B. Transmission electron microscopy of  $50 \mu\text{g/mL}$  of SPIO-labeled SMSCs. In the cell and endocytic vesicles, a lot of high-electron-dense particles can be found, positive.

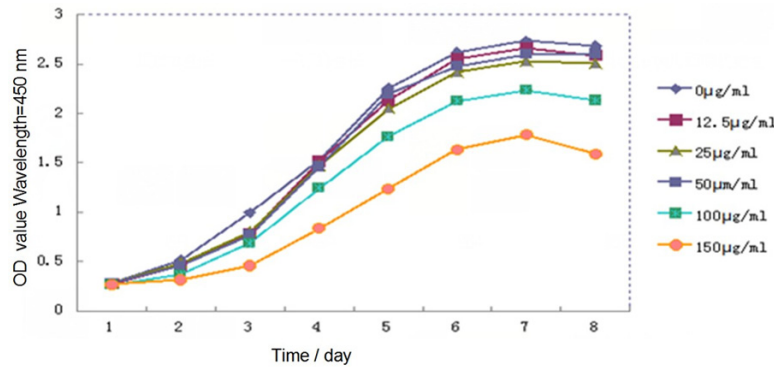
growth curve had no significant difference compared with that of unlabeled SMSC; when the concentration of marker was  $100 \mu\text{g/mL}$  and  $150 \mu\text{g/mL}$ , SMSC growth curve became horizontal and cell growth was inhibited, indicating that High concentrations of SPIO could inhibit the growth of SMSC.

### Discussion

SMSC, like other stem cell, has the potential to differentiate to a variety of tissue cells, such as

bone, cartilage, fat, muscle and cardiac cells, which has become an important source of cells to repair articular structures. Horie et al. [4] and Mizuno et al. [5] injected synovial stem cells into rat joint cavity and found that stem cells promoted the meniscal repair, and stem cell migrated to meniscus injury area rather than other areas. Resolving the identification and tracing the migration and outcome of internal SMSC within the region of joint injury plays an important role in conforming the role and out-

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**Figure 5.** The growth curves of different concentrations of SPIO-labeled SMSCs determined by a microplate reader.

come of exogenous SMSC in defect repair. Superparamagnetic iron oxide particles are by far the most widely used contrast agent in the body; the principle is as follows: cells are magnetically labeled by cellular uptake of the magnetic particles; when labeled cells are implanted in the body, real-time tracing the labeled cells can be achieved by magnetic resonance imaging (MRI) technology [6]. Superparamagnetic iron oxide nanoparticles have higher biocompatibility and biodegradability than other cell markers; SPIO-labeling-MRI has become a particular target cell-probing technology [7]; currently, SPIO labeling has been used in a variety of stem cells. Jing et al. [8] injected SPIO-labeled BMSC into rabbit joint cavity and used MRI to trace articular cartilage defect repair; Tang [9] transplanted SPIO-labeled neural tissue-derived stem cells into the nervous system injury of mice and monkeys and used MRI to trace and observe the repair of neural stem cells; Liu et al. [10] used magnetic resonance imaging to study the feasibility of SPIO-labeled ADSCs, but related reports on SPIO-labeled SMSC are rare.

In this study, SPIO and poly-L-lysine (PLL) were added into serum-containing medium to formulate different concentrations of SPIO labeling liquid; SPIO was transformed into the SMSC by nonspecific surface absorption process. Because SPIO and SMSC surfaces are both negatively charged, mutual repulsion will hinder SPIO entering into the SMSC; PLL is a common cationic polymer with positive charge on the surface, which can eliminate the charge repulsion between SPIO and SMSC to promote SPIO to smoothly entry into the cell. Since PLL can be obtained conveniently and is inexpensive, and

no toxic side effect has been found, it is now used widely [11]. After 24 h of labeling, Prussian blue staining showed that: a large number of dense blue-stained iron particles had been found in the labeled-SMSC cytoplasm; the cell labeling rate was more than 95%, and with the increasing concentration of labeling liquid, cytoplasm blue-stained iron particles increased and color deepened (**Figure 3**); under the transmission electron microscope,

a lot of high electron density particles had been observed in the cytoplasm and endocytic vesicles, positive (**Figures 4, 5**), indicating that the preliminary study of SPIO-labeled synovial mesenchymal stem cells is entirely feasible. Before and after the labeling, the cell proliferation was detected; we found that the growth curve of SPIO-labeled SMSC had no obvious morphological changes in certain concentration range, but high concentrations of SPIO could inhibit cell proliferation; Similarly, trypan blue staining showed that before and after the labeling, dye exclusion rate of SPIO-labeled SMSC had no significant difference in certain concentration range, but high concentrations of SPIO decreased the cell activity (**Figure 3**). Therefore, SPIO-labeling-SMSC in vitro is feasible and safe in a certain concentration range after a preliminary study, which would not significantly affect the cell proliferation and cell viability of SMSC, but high concentrations of SPIO had greater impact. Indeed, SPIO can cause changes in intracellular iron content. There are many studies suggest that, SPIO within a certain range of concentrations does not affect the viability, proliferation and differentiation potential of stem cells after labeling, [12, 13]. There are even reports indicate that, SPIO has inherent peroxidase-like activity, which can promote the growth of labeled cells [14]; but it also has been reported that iron particles or ionic iron in the stem cells can affect the signaling transduction between cells and affect the differentiation of stem cells into chondrocytes cells [15], and the free iron from lysosomal-degraded SPIO can inhibit the differentiation of stem cells into osteoblast-like cells [16]. Therefore, the effect of SPIO labeling on cell differentiation potential is varied in clinical;

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we will deeply study its effect on the SMSC differentiation potential.

### Disclosure of conflict of interest

None.

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