Development of a tree shrew metabolic syndrome model and use of umbilical cord mesenchymal stem cell transplantation for treatment

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Abstract The aim of this study was to establish a tree shrew metabolic syndrome model and demonstrate the utility of MSCs in treating metabolic syndrome. We used tree shrew umbilical cord mesenchymal stem cell (TS-UC-MSC) transplantation for the treatment of metabolic syndrome to demonstrate the clinical application of these stem cells and to provide a theoretical basis and reference methods for this treatment. Tree shrew metabolic syndrome model showed significant insulin resistance, high blood sugar, lipid metabolism disorders, and hypertension, consistent with the diagnostic criteria. TS-UC-MSC transplantation at 16 weeks significantly reduced blood sugar and lipid levels, improved insulin resistance and the regulation of insulin secretion, and reduced the expression levels of the pro-inflammatory

cytokines IL-1 and IL-6 ($P < 0.05$). The transplanted TS-UC-MSCs targeted the liver, kidney and pancreas; reduced liver cell degeneration, necrosis, and inflammatory exudation; mitigated bleeding congestion and inflammatory cell infiltration in the kidney; and reduced islet cell degeneration and necrosis. We successfully developed a tree shrew metabolic syndrome model and showed that MSC migrate in diseased organs and can attenuate metabolic syndrome severity in a tree shrew model.

Keywords Cell transplantation · Metabolic syndrome model · Treatment · Tree shrew · Umbilical cord mesenchymal stem cell

Introduction

Metabolic syndrome is caused by insulin resistance and hyperinsulinemia. These conditions lead to atherosclerotic diseases, including central obesity, hyperglycemia, hypertension, and dyslipidemia, which are due to an accumulation of cardiovascular disease risk factors (Thomas et al. 2015). Metabolic syndrome was discovered in 1988 by Reaven and is also known as syndrome X or insulin resistance syndrome (Reaven and Chen 1988). Recent studies have shown that there is a high prevalence of metabolic syndrome and that it has a close relationship with diabetes and cardiovascular disease (Acton and Barton 2015). In particular, when the various anomalies of metabolic syndrome are combined, the risk

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of cardiovascular disease is high. The pathogenesis of metabolic syndrome is very complex and is not entirely clear (Baptista et al. 2015). For example, the precise interactions between the related genetic factors, dietary factors, environmental factors and immune factors remain unknown. Increased abdominal fat and insulin resistance are the core elements of metabolic syndrome, and an abundance of free fatty acids (FFAs) that accumulate in the liver and blood contribute to the insulin resistance (Asrih and Jornayvaz 2015). Additionally, the excess FFAs further decompose into triglycerides (TGs) that are deposited into the insulin-sensitive parts of the pancreas, producing lipid toxicity among the islet β cells. Furthermore, the release of a large amount of cytokines involved in mediating metabolic syndrome or insulin resistance, dyslipidemia, and hypertension also occurs (Ding et al. 2015). In insulin resistance, the secretion of insulin is normal, but the capacity of target cells to utilize glucose diminishes; therefore, more than the normal amount of insulin is needed to maintain the target cells' normal physiology (Kingwell 2002). The combination of insulin resistance and FFAs interferes with normal fat metabolism, resulting in lipid metabolism disorders and ultimately affecting glucose metabolism, which increases the occurrence of hyperglycemia. An abundance of FFAs and a high level of insulin in turn cause an increase in cardiac output and in renal uptake of sodium, leading to high blood pressure, which promotes the occurrence of atherosclerosis (Kingwell 2002). Currently, metabolic syndrome patients require long-term regular treatment, which has several side effects. Additionally, this treatment does not improve function, instead only slowing the development of metabolic syndrome.

Current animal models of metabolic syndrome include four types: genetic, transgenotype, chemical drug-induced and high-calorie-diet-induced. The model animals are mainly rats, mice, rabbits and primates (Ende et al. 2004; Zhang et al. 2010). It has been reported that a high-calorie diet alone could generate models of high cholesterol, but no obvious high blood pressure was observed (Santos-Gallego et al. 2011). In another study, after 1 month, rats fed a fructose diet had elevated blood pressure (Wen et al. 2011). Studies have also used a high-fat and high-salt diet to induce hypertension in animals (Ende et al. 2005). However, food-induced models only have limited effects. Therefore, models of metabolic syndrome increasingly involve a combination of chemical drugs and a high-

calorie diet. Previous studies (Liew and Andrews 2008) have shown that a high-calorie diet for 4 weeks combined with an injection of streptozotocin (STZ) can be used to successfully establish a metabolic syndrome model in primates. STZ can specifically induce destruction of β cells, severe leukopenia, and hepatic and kidney damage (Muller et al. 2011). However, primates are expensive and large, and they are inconvenient to manage, which limits their applicability. Meanwhile, herbivore animals, such as rabbits, rats and mice, differ greatly from humans in their physical structure and characteristics, so these animals do not accurately mimic the human disease process. The tree shrew is closer to primates than to rodents in terms of evolution. This animal is low cost, has a short gestation and a small size, and is easy to manage, and its physical characteristics are similar to those of humans (Fan et al. 2013). These characteristics have increased the use of tree shrews as animal models for research on hepatitis B viral diseases, eye diseases and diabetes. Additionally, the World Health Organization (WHO) has proposed the use of tree shrews instead of large primates for scientific research (Fan et al. 2013). The most recent whole-genome and proteome sequencing studies have verified the similar genetic characteristics of tree shrews and primates and have further supported the increased use of tree shrews in disease research.

Mesenchymal stem cells have unlimited proliferative capacities. Under specific conditions, they differentiate into a variety of cells that can be directly involved in repairing damage by promoting angiogenesis and by acting via anti-inflammatory, cytoprotective, anti-apoptotic, and immunomodulatory mechanisms (Meier et al. 2013). Furthermore, these cells play a therapeutic role in metabolic syndrome, lipid metabolism disorders, insulin resistance, hypertension, hyperglycemia, atherosclerosis, and low-grade inflammation (Liew and Andrews 2008).

Stem cells can be divided into embryonic stem cells and adult stem cells. Because of ethical issues, embryonic stem cells are limited in their application (Maitra et al. 2004). Umbilical cord mesenchymal stem cells (UC-MSCs) are a more widely available resource, have low immunogenicity, are not associated with ethical issues, have a small chance of viral infection, and have no adverse effects on the donor. These cells also have a better differentiation capacity than bone marrow MSCs (BMSCs) do and thus may replace BMSCs and become a major source of MSCs (Kiss et al. 2008). A number of

studies have shown that UC-MSCs additionally have a multi-differentiation capacity in vitro (Gao et al. 2008; Liu and Han 2008). Furthermore, it has been shown that these cells can be induced to differentiate into islet-like cells capable of both expressing the surface antigen markers of islet β cells and secreting insulin. These islet-like cells can be transplanted into diabetic mice to exert a hypoglycemic effect, which improves the renal pathological changes. Additionally, UC-MSCs can be induced to differentiate into cardiomyocyte-like cells and endothelial cells, which participate in or promote angiogenesis and inhibit the development of atherosclerosis (Kingwell 2002). The use of cell transplantation in treating metabolic syndrome in tree shrews, however, has not been reported. Therefore, using the diagnostic criteria of the Chinese Diabetes Society (CDS) of the Chinese Medical Association (Reyes et al. 2002), we explored methods for generating a tree shrew metabolic syndrome model and examined the efficacy of and the mechanisms involved in using tree shrew UC-MSC (TS-UC-MSC) transplantation for treatment.

Materials and methods

Experimental materials

STZ, superparamagnetic iron oxide (SPIO), poly-lysine, dimethylsulfoxide (DMSO), anti-CD34-FITC, anti-CD105-PE, anti-CD90-FITC, and anti-CD31-FITC antibodies were purchased from Sigma (St. Louis, MO, USA). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Carlsbad, CA, USA). DIR dye was purchased from Beijing Fan Bo Biotechnology Co., Ltd. (Beijing, China). A Prussian Blue staining kit was purchased from Shanghai Yuan Ye Biotechnology Co., Ltd. (Shanghai, China). DMEM/F-12 medium, fetal calf serum, and trypsin were purchased from Gibco (Grand Island, NY, USA). Cholesterol was purchased from Beijing DingGuo Biotechnology Co., Ltd. (Beijing, China). Human Inflammation Antibody Array 3 was purchased from RayBiotech (Norcross, GA, USA) (Item: AAH-INF-3-4). All other reagents were analytical grade.

Experimental methods

Culture, identification and labeling of TS-UC-MSCs

Umbilical cord collection Full-term pregnant tree shrews were anesthetized using intraperitoneal

pentobarbital sodium (50 mg/kg body weight) before caesarean section. The uterus was first surgically opened to fully expose the placenta and the fetal tree shrews. The umbilical cords were then cut at both ends and soaked in DMEM/F-12 medium containing 100 U/ml penicillin and streptomycin. The umbilical cords were obtained at the time of birth, and neither the mothers nor the babies needed to be killed. The experimental protocols were approved by the Experimental Animal Ethics Committee of Kunming General Hospital of Chengdu Military Command.

TS-UC-MSC culture Each umbilical cord was placed in a sterile petri dish, and 100 U/ml penicillin and streptomycin were added. The artery, vein and film tissue were removed using ophthalmic scissors, and the umbilical cord was cut into pieces less than $1 \times 1 \times 1 \text{ mm}^3$ in size. Next, the tissue was placed into a centrifuge tube and centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. The cells were resuspended in 1 ml complete medium, seeded in 25 cm^2 culture flasks, and then placed in a 5 % CO_2 and 37°C incubator. After 5–6 h, when most of the cells had adhered to the flasks, 3–4 ml complete medium was slowly added. After that, every 3–4 days, the medium was changed. After 6–7 days, when the cells reached 80 % confluence, they were digested using 0.25 % trypsin. The cell concentration was then adjusted to 1×10^6 cells/ml in a 75 cm^2 culture bottle. Cell growth was observed under an inverted microscope. The MSC lines we used in the experiments were derived from the same parents. We have established many batches of MSC lines. But only one MSC line was used for transplantation experiments.

Identification of TS-UC-MSCs Morphological analysis: After 24 h of cell culture, the cell growth and cell morphology were evaluated under an inverted microscope.

Flow cytometric analysis: Fourth-passage TS-UC-MSCs were collected using 0.25 % trypsin digestion. To prepare four EP tubes, approximately 5×10^5 cells resuspended in 100 μl PBS were added to each tube. A total of 10 μl anti-CD34-FITC and 5 μl anti-CD105-PE were added to one tube, 10 μl anti-CD90-FITC was added to the second tube, and 10 μl anti-CD31-FITC was added to the third tube. Additionally, an isotype control was added to the fourth tube. The cells were stained for

30 min in the dark, and then 400 µl PBS was added to each tube, followed by flow cytometric analysis.

In vitro differentiation into fat cells: When the 4th passage of cells reached 80–90 % confluence, the cells were digested with 0.25 % trypsin. A total of 2×10^4 cells/cm² was inoculated onto six-well plates in place of a coverslip, and 2 ml complete medium was added to each well. Every 3 days, the complete medium was changed, until the cells reached 100 % confluence. The complete medium was then removed, and after a saline wash, solution A (containing 20 ml FBS, 2 ml glutamine, 400 µl insulin, 200 µl IBMX, 200 µl indole magnesium zinc and 200 µl dexamethasone) was added to the cells. The cells were subsequently cultured for 3 days. The cells were then cultured in solution B (containing 20 ml FBS, 2 ml glutamine, and 400 µl insulin) for 24 h, after which they were returned to solution A. This cycle between solution A and B was repeated 3–5 times, and then the cells were cultured in solution B for 7 days. Negative-control cells were cultured in complete medium without induction. After the cells were cultured, they were fixed with 4 % polyformaldehyde and stained by oil red O staining.

Osteogenic differentiation in vitro: When the 4th passage of cells reached 80–90 % confluence, the cells were digested with 0.25 % trypsin and inoculated at 3×10^3 cells/cm² onto six-well plates in place of a coverslip, and 2 ml complete medium was added to each well. After incubation for 24 h, the fluid was aspirated, the cells were washed with saline, and inducing solution (containing 20 ml FBS, 2 ml glutamine, 20 µl dexamethasone, 2 ml β-glycerol phosphate and 400 µl ascorbic acid) was added to the cells. The cells were continuously cultured, and the medium was changed once every 2–3 days. Negative-control cells were cultured in complete medium without induction. All cells were fixed with 4 % paraformaldehyde, stained using Alizarin red and photographed under a microscope.

DAPI labeling The substance DAPI, which is a specific DNA-binding fluorescent dye, can quickly pass through intact cell membranes and enter living cells to stain the nuclei. After binding, DAPI–DNA complexes exhibit blue fluorescence under UV excitation.

DAPI was added to the 4th-passage TS-UC-MSCs at a concentration of 1 µg/mL. After 12 h of incubation,

the medium was discarded, and the cells were washed six times with PBS to remove the unbound DAPI. After DAPI labeling, the nuclei emitted blue fluorescence. Under a fluorescence microscope, the number of positively labeled cells was determined.

DIR labeling DIR is a lipophilic fluorescent dye that can be used to stain the cell membrane and other fat-soluble biological structures. The DIR emission wavelength is invisible, but after cell staining, DIR emission is deep red fluorescence at a wavelength of 748 nm. DIR can specifically cross the cell membrane to penetrate cells and tissues and is used as a tracer for in vivo imaging. In the present study, a total of 10 mg DIR was added to 3.3 ml DMSO to prepare a 3 mM DIR storage solution. Meanwhile, the 4th-passage TS-UC-MSCs were digested with 0.25 % trypsin, and the cell concentration was adjusted to 1×10^6 cells/ml in 15 ml centrifuge tubes. Next, 1 ml of the cell suspension was added to 5 µl 3 mM DIR solution, with a final DIR staining concentration of 15 µM, followed by incubation at 37 °C for 10 min. The cells were then washed with pre-warmed serum-free medium 3 times and centrifuged at 1500 rpm for 5 min.

For identification, 100 µl cell suspension (containing 1×10^5 cells) was added to a dish that was placed within a living body imager to observe deep red fluorescence.

SPIO labeling and electron microscopy analysis SPIO is a nanosized particle. After modification with positively charged polylysine, SPIO can interact with the negatively charged cell membrane to effectively label cells.

A 0.3 mg/ml stock solution of polylysine and a 1 mol/l PBS solution were mixed at a 1:4 volume ratio to generate a 0.06 mg/ml working solution. A 5 mg/ml SPIO stock solution and 1 mol/l PBS solution were also mixed at a 1:4 volume ratio to generate a 1 mg/ml working solution. A total of 500 µl 1 mg/ml SPIO working solution and 500 µl 0.06 mg/ml polylysine working solution were then mixed and incubated at room temperature for 30 min to neutralize the negative charge of the SPIO. This solution was subsequently added to 20 ml cells to generate a final SPIO concentration of 25 µg/ml and a final polylysine concentration of 6 µg/ml. The cells were then labeled in a 5 % CO₂ and 37 °C incubator for 12 h.

After 12 h, the cells were thoroughly rinsed with PBS three times to remove the free iron particles and

were then fixed with 4 % paraformaldehyde for 5 min. Next, the cells were incubated in 10 % $K_3Fe(CN)_6$ + 20 % HCl in Perls' reaction solution for 20 min, washed with PBS 3 times, stained with neutral red for 40 s, and washed under running water. The positive cells were observed under a microscope and photographed.

For SEM analysis, after the 12 h of labeling, the SPIO-labeled TS-UC-MSCs were digested with 0.25 % trypsin, centrifuged at 2000 rpm for 5 min, and collected. After discarding the supernatant, 2.5 % glutaraldehyde solution was added for 30 min to allow fixation. After rinsing in electron microscopy rinsing liquid, the cells were fixed with 1 % osmium tetroxide for 2 h, dehydrated in graded ethanol, and saturated with uranyl acetate electron stain at 4 °C overnight. The samples were then embedded in epoxy resin. Ultrathin sections were again stained with uranyl acetate, and the samples were observed by transmission electron microscopy (TEM).

Generation of a tree shrew metabolic syndrome model

In this study, a high-sugar, high-cholesterol, high-salt diet combined with a sugar-water diet and STZ induced a metabolic syndrome model in tree shrews. The model was based on the definition of metabolic syndrome, including increased fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TGs), and low-density lipoprotein cholesterol (LDL-C); impaired glucose tolerance; the emergence of insulin resistance; and observed pathological changes in the target organ. There were significant differences in the metabolic syndrome model animals compared with before induction and with the normal control group.

Tree shrew grouping The chosen 48 tree shrews were Chinese *Tupaia belangeri* domesticated by the Institute of Medical Biology, Chinese Academy of Medical Sciences Germplasm Resource Center. These shrews included both males and females and weighed 120–140 g. The animals were housed in 3600 × 3600 × 2000 mm stainless steel cages. The animals were randomly divided into two groups: a normal control group (n = 8), with no change in diet, and a metabolic syndrome model group (n = 40), with a high-sugar, high-cholesterol, high-salt diet combined with a sugar-water diet for 16 weeks and with STZ. During the course of establishing the model, 8 tree shrews died, 32 tree

shrews became models. The tree shrews in the metabolic syndrome model group were randomly divided into a model group (n = 10) and a TS-UC-MSC treatment group (n = 22). The TS-UC-MSC treatment group (n = 22) was in turn divided into four groups: DAPI- (n = 5), DIR- (n = 5), and SPIO-labeled cell treatment groups (n = 5) and an unlabeled cell treatment group (n = 7). The treatment with MSC begins at 16 weeks.

Diet of the model group The high-sugar, high-cholesterol, high-salt diet recipe was freshly prepared every morning and comprised the following: 20 % sucrose, 2.5 % cholesterol, 3 % salt, and 74.5 % basic feed (made by the Chinese Academy of Medical Sciences), which were steamed after mixing. The 10 % sugar water, provided once every morning and every afternoon, consisted of the following: 10 % sucrose and 90 % water (1 L drinking water added to 100 g sucrose). The model group diet was administered for 16 weeks.

Experimental animal treatment The model groups were fed the homemade high-sugar, high-cholesterol, high-salt diet and 10 % sugar water for 16 weeks, and the control group was fed basic feed and normal water. The experimental animals were given a set daily amount of fruit and water. After 8 weeks, the model group was fasted overnight, and the next morning, the animals were administered 100 mg/kg freshly prepared STZ (100 g/l in 0.1 mmol/l; pH value of 4.3 in citrate buffer; filter sterilized) by intraperitoneal injection. After 7 days, there are 10 tree shrews with the FBG did not reach 11.1 mmol/l or more, they were again injected with STZ (80 mg/kg). The control group was injected intraperitoneally with an equal volume of saline. The tree shrews' blood was tested every 2 weeks for FBG, TC, TGs, LDL-C, and insulin, and the insulin resistance index (HOMA-IR) was calculated. Afterward, the arterial blood pressure of the model group was measured according to the method described below.

Model evaluation methods *General observations* The experimental animals were regularly observed in terms of their coat, mental state, diet, excretion, activity, and weight, among other parameters.

Biochemical blood tests Every 4 weeks, the tree shrews were fasted for 12 h. The next morning, a

Roche blood glucose meter was used to measure blood glucose and the TC, TG, LDL-C and insulin levels were determined.

Oral glucose tolerance test (OGTT) The experimental animals were fasted for 12 h, and their FBG levels were tested. After being weighed, the animals were orally administered a 50 % glucose solution at 3.59 ml/kg. Afterward, the blood sugar level was measured at 0, 5, 7, 15, 30, 60, 90, and 120 min, and the area under the curve (AUC) was calculated. Glucose tolerance was considered abnormal if the glucose level significantly increased at each time point.

HOMA-IR The HOMA-IR was used to evaluate individual indicators of insulin resistance levels. The calculation method was as follows: insulin resistance index (HOMA-IR) = fasting blood glucose (FBG, mmol/l) * fasting insulin (FINS, mIU/l)/22.5.

TS-UC-MSC transplantation in the treatment group

TS-UC-MSC transplantation Using the methods described above, DAPI-, DIR-, and SPIO-labeled cells were digested with 0.25 % trypsin, after which the digestion was terminated with complete medium and the cells were centrifuged at 2000 rpm for 5 min. The supernatant was discarded after counting the cells. The cells were then resuspended in saline, adjusted to a cell concentration of 7×10^5 cells/ml (a dose of 5×10^6 cells/kg in a total volume of 1 ml) and transferred to a 1 ml syringe. The treatment groups were injected with labeled or unlabeled TS-UC-MSCs into the tail vein at 16 weeks. The model groups were injected with an equal volume of saline at the same time.

Main outcome measures after transplantation *General observations* The experimental animals were regularly observed in terms of their coat, mental state, diet, excretion, activity, and weight, among other parameters.

Detection of biochemical markers At approximately 18 and 20 weeks (2 and 4 weeks after transplantation, respectively), the tree shrews were fasted for 12 h. The next morning, a 1 ml syringe was used to collect blood from the tail. A Roche blood glucose meter was used to measure the blood glucose. The TC, TG, LDL-C, and FINS levels were also determined.

HOMA-IR $\text{HOMA-IR} = \text{fasting blood glucose (FBG, mmol/l)} * \text{fasting insulin (FINS, mIU/l)} / 22.5$.

Arterial blood pressure (Zhu et al. 2009) Measurements were taken using a non-invasive sphygmomanometer (Model BP-98A) provided by the Institute of Medical Biology. The operator wore double gloves. At approximately 20 weeks (4 weeks after treatment), each tree shrew was placed head first into a network with its tail exposed. The root of the tail was exposed by retracting approximately 4 cm of skin. The tree shrew's tail was then placed into the pressure sensor, which had a rubber membrane. The animal had to remain quiet for 5 min before the start button was pressed. Each animal was measured again after an interval of 5 min. The animals were measured three times, and the average value was considered as the final measured value.

Histopathological observation At approximately 20 weeks (4 weeks after treatment), three tree shrews were selected from each group and were euthanized by spinal cord transection. The liver, kidneys and pancreas were then fixed with 4 % formaldehyde solution and subjected to routine HE staining. Ten sections of each organ in each group and ten visual fields from each section were analyzed. In total, therefore, one hundred visual fields from each group were analyzed. The results are summarized in Table 1.

In vivo distribution of DIR-labeled TS-UC-MSCs On day 3 after cell transplantation, 3 DIR groups and one model group were euthanized by spinal cord transection. The pancreas, kidney, and liver were then fixed with 4 % formaldehyde solution. The samples were placed in a small-animal in vivo imager, and the distribution of the red fluorescence signal was observed at an excitation wavelength of 748 nm.

In vivo distribution of SPIO-labeled TS-UC-MSCs On day 7 after cell transplantation, 3 SPIO groups and one model group were euthanized by spinal cord transection. The pancreas, kidney, and liver were then fixed with 4 % formaldehyde solution and processed for Prussian Blue staining. The sections were placed under an optical microscope, and images were collected.

In vivo tracing of DAPI-labeled TS-UC-MSCs On day 14 after cell transplantation, 3 DAPI groups and

Table 1 Analysis of one hundred visual fields from each group for pathological changes

Organ	Pathological change	Normal control group	Model group*	Treatment group [#]
Liver	Cell degeneration	1	35	9
	Cell necrosis	1	34	10
	Destroyed lobular structure	0	36	15
	Hepatic cord disorder	0	31	13
	Inflammatory cell infiltration	1	33	12
Kidney	Completely occluded glomerular cysts	0	38	10
	Basement membrane thickening	1	37	9
	Bleeding congestion	2	32	8
	Inflammatory cell infiltration	2	34	6
	Renal interstitial fat vacuoles	1	39	9
Pancreas	Reduced islet number	1	38	10
	Decreased islet volume	1	39	12
	Destroyed pancreatic structure	0	40	11
	Hydropic degeneration of islet cells	2	32	8
	Reduced number of cells	2	33	7

Two-way ANOVA revealed significant differences between the groups (* $P < 0.05$ for the model group compared with the normal control group, [#] $P < 0.05$ for the treatment group compared with the model group, $n = 100$)

one model group were euthanized by spinal cord transection. The pancreas, kidney, and liver were then fixed with 4 % formaldehyde solution, and 4 μm -thick serial frozen sections were cut, mounted with glycerin and placed under a fluorescence microscope to observe the distribution of blue fluorescent cells.

Inflammatory cytokine antibody microarray After approximately 20 weeks (4 weeks after transplantation), serum was collected from each group of tree shrews and was sent to Shanghai Kangcheng Biological Engineering Co. for inflammatory factor antibody microarray experiments and screening for differences in the expression of inflammatory factors.

Ethics statement

The experimental protocols were approved by the Experimental Animal Ethics Committee of Kunming General Hospital of Chengdu Military Command.

Statistical analysis

All data were analyzed using the SPSS 17.0 statistical package for statistical analysis. Measurement data are expressed as the mean $\pm$ standard deviation. Differences between the groups were analyzed using

one-way ANOVA or two-way ANOVA. $P < 0.05$ indicates a statistically significant difference.

Results

Culture and identification of TS-UC-MSCs

TS-UC-MSC formation

For the TS-UC-MSCs, 24 h after primary culture, a small number of long spindle-shaped cells surrounding the tissue was observed (Fig. 1a). After 72 h, the adherent cells increased in number, and the cells appeared to be a long shuttle type and exhibited multiple scattered colonies (Fig. 1b). After 5 days, the non-adherent cells had been cleared, and the adherent cells were approximately 80 % confluent and could be passaged (Fig. 1c). The fourth-passage cells appeared as elongated spindle-shaped cells with a swirling growth pattern, and their morphology was similar to that of skin fibroblasts (Fig. 1d).

TS-UC-MSC surface markers

Flow cytometric analysis was used to determine whether the cells expressed MSC surface antigens.

Fig. 1 TS-UC-MSC morphological ($\times 200$) and flow cytometric analyses. **a–d** TS-UC-MSC morphological. **A** Primary culture of TS-UC-MSCs at 24 h; **b** primary culture of TS-UC-MSCs at 72 h; **c** primary culture of TS-UC-MSCs at 5 days; **d** the fourth passage of TS-UC-MSCs. **e–h** flow cytometric analyses. **e** the CD90 expression rate was 99.9 %; **f** the CD31 expression rate was 0.7 %; **g** the CD105 expression rate was 99.8 %, and the CD34 expression rate was 0.0 %; **h** the expression rate of the isotype control was 0.0 %. **i–n** TS-UC-MSC differentiation into osteoblasts and fat cells ($\times 200$): **i** non-induced TS-UC-MSCs after Alizarin red staining; **j** induced TS-UC-MSCs without staining, calcium nodules shown by the *black arrow*; **k** induced TS-UC-MSCs after Alizarin red staining, red dye-labeled calcium nodules shown by the *black arrow*; **l** non-induced TS-UC-MSCs after oil red O staining; **m** induced TS-UC-MSCs without staining, fat droplets shown by the *black arrow*; **n** induced TS-UC-MSCs after oil red O staining, red dye-labeled fat droplets shown by the *black arrow*

Specifically, 4th-passage TS-UC-MSCs were examined by flow cytometry. The results showed that the CD90 and CD105 expression rates were 99.9 and 99.8 %, respectively. The expression rate of the hematopoietic stem cell marker CD34 was 0.0 %, and the expression rate of the endothelial cell marker CD31 was 0.7 %. For reference, the expression rate of the isotype control was 0.0 %. These results showed that the cells had MSC-like phenotypic characteristics (Fig. 1e–h).

In vitro differentiation of TS-UC-MSCs

TS-UC-MSC differentiation into bone cells Before staining, the TS-UC-MSCs after induction (Fig. 1j) were significantly different from the non-induced TS-UC-MSCs (Fig. 1i). After Alizarin red staining, the induced cells were found to have red dye-labeled calcium nodules (Fig. 1k). These results showed that the TS-UC-MSCs had osteoblastic potential.

TS-UC-MSC differentiation into fat cells Before staining, fat droplets were visible in the induced TS-UC-MSCs (Fig. 1m). After oil red O staining, the lipid was significantly dyed red (Fig. 1n). In contrast, the non-induced TS-UC-MSCs had no fat droplets (Fig. 1l). These findings showed that the TS-UC-MSCs had the potential to differentiate into fat cells.

DAPI-labeled TS-UC-MSCs

The 4th-passage TS-UC-MSCs were labeled with DAPI for 12 h (Fig. 2a). Under a fluorescence microscope, blue fluorescent nuclei were visible (Fig. 2b). The numbers of positive and negative cells were used to calculate a rate of DAPI positivity of 98 %.

DIR-labeled TS-UC-MSCs

An imaging device was used to detect the fluorescence signal emitted by the DIR-labeled TS-UC-MSCs, and these cells appeared as deep red cells. The non-labeled cells had no deep red fluorescence signal (Fig. 2c).

SPIO-labeled TS-UC-MSCs

The 4th-passage TS-UC-MSCs were labeled with SPIO for 12 h. Before Prussian Blue staining, the SPIO-labeled cells contained blue particles (Fig. 2e), whereas the unlabeled cells did not contain blue dye particles (Fig. 2d). After Prussian Blue staining, a large number of dense blue particles were observed in the cytoplasm (Fig. 2f). The rate of positively SPIO-labeled TS-UC-MSCs was calculated to be 97 %. Using electron microscopy, a large number of high-density iron ion deposits were observed in the SPIO-labeled cytoplasm (Fig. 2g, h).

Model establishment and TS-UC-MSC treatment

General observations

The model group began with 40 tree shrews but ultimately included 32 model animals because 8 tree shrews died. Compared with the control group, the model group appeared to have a dark coat, tail ulceration and necrosis, polydipsia, and diarrhea. The group also appeared to be unresponsive and to have poor activity, whereas the control group was active and responsive. At 18 weeks (2 weeks after treatment), however, the tail ulcers began to heal, the diarrhea decreased, there was increased activity, and the reaction was more sensitive.

FBG levels

After 4 weeks of being fed the high-sugar, high-cholesterol, high-salt diet, the model group had an

elevated FBG level compared with the control group. At 8 weeks, the mean FBG concentrations in the model group were 10 mmol/l or higher. The FBG levels were 11.1 mmol/l or higher after STZ injection, and these high levels were maintained during the whole study (model group: 25.50 ± 4.50 mmol/l vs control group: 4.19 ± 0.77 mmol/l; $P < 0.01$). Compared with the model group, the treatment group had decreased FBG levels, and these levels continued to decrease over time (Fig. 3a, b).

FINS levels

From the beginning of being fed the high-sugar, high-cholesterol, high-salt diet, the FINS levels in the model group increased, and after 8 weeks, the model group's insulin levels peaked. After the injection of STZ, the STZ further damaged the islet β cells. Cell degeneration and necrosis occurred, at which point the secretory function began to decline; insulin secretion dropped below the level of the control group (model group: 0.96 ± 0.11 μ IU/l vs control group 2.37 ± 1.98 μ IU/l; $P < 0.01$) (Fig. 3c). At 20 weeks (4 weeks after treatment), the treatment group demonstrated significantly increased insulin secretion (treatment group: 1.34 ± 0.33 μ IU/l vs model group: 0.85 ± 0.10 μ IU/l; $P < 0.05$) (Fig. 3d).

HOMA-IR changes

The HOMA-IR was defined as the glucose and insulin product divided by 22.5. At 8 weeks, the model group demonstrated significant insulin resistance (model group: $(4.50 \pm 0.51) \times 10^{-3}$ vs control group: $(0.40 \pm 0.10) \times 10^{-3}$; $P < 0.01$). After the STZ injection, due to the continued high FBG level, causing a decline in islet function and decreased insulin, the HOMA-IR was lower than its previous value in the model group but was still significantly higher than its value in the control group (model group: $(1.01 \pm 0.02) \times 10^{-3}$ vs control group: $(0.44 \pm 0.07) \times 10^{-3}$; $P < 0.01$) (Fig. 3e). At 20 weeks (4 weeks after treatment), the treatment group's HOMA-IR decreased from $(1.10 \pm 0.10) \times 10^{-3}$ to $(0.74 \pm 0.02) \times 10^{-3}$ ($P < 0.05$) (Fig. 3f).

Ogtt

At 8 and 16 weeks, the model group's glucose level peaked at 5 min, and the control group's blood glucose

◀ **Fig. 2** DAPI-, DIR-, and SPIO-labeled TS-UC-MSCs ($\times 200$) and TEM. **a** Unlabeled TS-UC-MSCs; **b** DAPI-labeled TS-UC-MSCs with bright blue fluorescent nuclei shown by the *white arrow*; **c** unlabeled TS-UC-MSCs without fluorescence and DIR-labeled TS-UC-MSCs with deep red fluorescence; **d** unlabeled TS-UC-MSCs; **e** SPIO-labeled TS-UC-MSCs with iron particles shown by the *black arrow*; **f** SPIO-labeled TS-UC-MSCs after Prussian Blue staining, with blue dense iron particles shown by the *black arrow*; **g** SPIO-labeled TS-UC-MSCs ($\times 10,000$) (TEM); **h** SPIO-labeled TS-UC-MSCs ($\times 25,000$) (TEM). The *black arrows* indicate iron particles in the cytoplasm

peaked at 7 min. Compared with the control group, at 8 weeks, after the model group's level peaked at approximately 5 min, the blood glucose level declined rapidly. However, the blood glucose level at 90 min was still 10 mmol/l or higher and was not reduced to the normal level. At 16 weeks, after the model group's first glucose peak, there was no immediate decrease, and a relatively constant blood glucose level, with a plateau between 5 and 15 min, was observed. The AUC of the model group was significantly higher than that of the control group at 8 weeks, showing significantly impaired glucose tolerance (Fig. 3g).

Plasma TG, TC and LDL levels

After being fed the high-sugar, high-cholesterol, high-salt diet for 1 weeks, the model group's TG, TC and LDL levels began to increase significantly during the next 4 weeks. Thereafter, the model demonstrated a trend of increasing instability, with TGs increasing from (0.63 ± 0.17) to (2.11 ± 0.23) mmol/l (Fig. 3h) and TC increasing from (2.31 ± 0.29) to (4.99 ± 0.41) mmol/l (Fig. 3j). At 1 weeks, the LDL appeared to maintain an increasing trend, increasing from (0.20 ± 0.14) to (0.77 ± 0.18) mmol/l, whereas the lipid levels in the control group exhibited no changes (Fig. 3l). These findings indicate that the model group had dyslipidemia. After the treatment, there were significant reductions in the blood lipid levels: the TG levels decreased from (1.49 ± 0.23) to (0.71 ± 0.09) mmol/l (Fig. 3i), the TC decreased from (4.97 ± 0.48) to (2.51 ± 0.73) mmol/l (Fig. 3k), and the LDL levels decreased from (0.79 ± 0.16) to (0.51 ± 0.17) mmol/l. There were significant differences in the treatment group compared with the model group and compared with the treatment group before treatment ($P < 0.01$) (Fig. 3m).

Arterial blood pressure changes

At 20 weeks (4 weeks after treatment), the model group's systolic (SP) and diastolic (DP) blood pressures and mean arterial pressure ($\text{MAP} = (\text{SP} + 2\text{DP})/3$) were slightly elevated compared with those of the control group and the treatment group, and the difference was statistically significant ($P < 0.05$) (Fig. 3n).

Histopathological changes

Ten sections of each organ in each group and ten visual fields from each section were analyzed. In total, therefore, one hundred visual fields from each group were analyzed. The results are summarized in Table 1. Two-way ANOVA revealed significant differences between the groups ($*P < 0.05$ for the model group compared with the control group, $^{\#}P < 0.05$ for the treatment group compared with the model group, $n = 100$). Representative images of each group at 20 weeks (4 weeks after treatment) are shown in Fig. 4.

Liver The tree shrew liver tissue HE staining results for each group were as follows. The normal control group's livers demonstrated normal cell morphology, were radially arranged, and had an intact lobular structure (Fig. 4a). The model group's livers exhibited cell degeneration, with parts of the livers showing cell necrosis; a destroyed lobular structure; hepatic cord disorder; and inflammatory cell infiltration (Fig. 4b). The treatment group's livers exhibited reduced cell degeneration and necrosis compared with the model group's livers. Additionally, the hepatic cord was neatly arranged, and inflammatory exudation was reduced (Fig. 4c).

Kidney The tree shrew kidney tissue HE staining results for each group were as follows. In the normal control group, the glomerular capillary endothelial cells were arranged in neat rows, the glomerular cysts were normal, and the basement membrane did not change (Fig. 4d). In the model group, the glomerular cysts were completely occluded, the basement membrane demonstrated thickening, there was bleeding congestion, and inflammatory cell infiltration and renal interstitial fat vacuoles were observed (Fig. 4e). In the treatment group, the

◀ **Fig. 3** Model establishment and TS-UC-MSC treatment ($n = 5$). **a, b** Changes in the FBG levels; **c, d** changes in the FINS levels; **e, f** changes in the HOMA-IR, where $\text{HOMA-IR} = \text{fasting blood glucose (FBG, mmol/l)} \times \text{fasting insulin (FINS, mIU/l)} / 22.5$; **g** the results of the OGTT (representing the means of measurements); **h–m** changes in the peripheral blood lipid content. ($*P < 0.05$ for the model group compared with the control group, $^{\#}P < 0.05$ for the treatment group compared with the model group); **n** comparison of the blood pressure of the tree shrews in each group at 20 weeks (4 weeks after transplantation) ($n = 5$). At 20 weeks (4 weeks after treatment), the model group's SP and DP and MAP ($\text{MAP} = (\text{SP} + 2\text{DP})/3$) were slightly elevated compared with those of the control group ($*P < 0.05$) and the treatment group ($^{\#}P < 0.05$) ($n = 5$)

glomerular cysts were not completely occluded, bleeding congestion and inflammatory cell infiltration were reduced compared with those in the

model group, and no obvious interstitial fat droplets were observed (Fig. 4f).

Pancreas The tree shrew pancreatic tissue HE staining results were as follows. In the normal control group, the islet structure was complete, there were clear boundaries that were round or oval, and the islet cell nuclei were rounded (Fig. 4g). In the model group, the islet number was reduced, the volume was decreased, the normal structure of the pancreas was destroyed, the surrounding tissue was ill defined, hydropic degeneration of the islet cells was observed, and the number of cells was significantly reduced (Fig. 4h). In the treatment group, compared with the model group, a more complete pancreatic structure

Fig. 4 HE staining of tree shrew liver, kidney and pancreatic tissues ($\times 200$). **a–c** Liver tissue from the control group, the model group and the treatment group, liver cell degeneration, necrosis and inflammatory exudation in the model group (shown by the black arrow); **d–f** kidney tissue from the control group, the model group and the treatment group, kidney bleeding congestion and inflammatory cell infiltration in the model group (shown by the black arrow); **g–i** pancreatic tissue from the

control group, the model group and the treatment group, islet cell degeneration and necrosis in the model group (shown by the black arrow). The images are representative of each group at 20 weeks (4 weeks after treatment). The treatment group showed that liver cell degeneration, necrosis, and inflammatory exudation was reduced; in the kidney, bleeding, congestion, and inflammatory cell infiltration was mitigated; and islet cell degeneration and necrosis was reduced

Fig. 5 In vivo tracing of DIR-, SPIO-, and DAPI-labeled TS-UC-MSCs ($\times 200$). **a–c:** DIR-labeled TS-UC-MSCs. **a, b** liver, kidney and pancreas from the treatment group, with deep red fluorescence shown by the *white arrows*; **c** liver, kidney and pancreas from the model group, without deep red fluorescence. **d–f** SPIO-labeled TS-UC-MSCs; **d–f** liver tissue, renal tissue, and pancreatic tissue from the treatment group, with blue iron particles in all three tissues shown by the *black arrows*; **g–i** liver tissue, renal tissue, and pancreatic tissue from the model group, without blue iron particles; **j–o** DAPI-labeled TS-UC-MSCs; **j–l** liver tissue, renal tissue, and pancreatic tissue from the treatment group, with blue fluorescence in all three tissues shown by the *white arrows*; **m–o** liver tissue, renal tissue, and pancreatic tissue from the model group, without blue fluorescence

was noted, and the surrounding tissue boundaries were more clear (Fig. 4i).

In vitro imaging of DIR-labeled TS-UC-MSCs

On day 3 after the DIR-labeled TS-UC-MSC transplantation, the model and treatment groups' liver, pancreas, and kidneys were collected and placed in an imaging device. In the treatment group's liver, kidney and pancreatic tissues, a deep red fluorescence signal was visible (Fig. 5a, b). In the liver, the deep red fluorescence signal area was largest, suggesting that TS-UC-MSCs injected via the tail vein may mostly colonize the liver. Without cell transplantation, the various organs in the model group showed no deep red fluorescence signal (Fig. 5c).

In vivo tracing of SPIO-labeled TS-UC-MSCs

On day 7 after the SPIO-labeled cell transplantation, the tree shrews' lobules, renal interstitial regions and pancreas were observed to contain dense blue particle deposits (Fig. 5d–f). In the tree shrews that did not undergo cell transplantation, no blue-stained SPIO-positive cells were observed within the various organs (Fig. 5g–i). These results showed that TS-UC-MSCs could colonize the injured liver, kidney and pancreas.

In vivo tracing of DAPI-labeled TS-UC-MSCs

On day 14 after DAPI-labeled TS-UC-MSC transplantation, in the liver and renal interstitial regions and around the islets, small blue nuclei were observed (Fig. 5j–l), which suggested that the TS-UC-MSCs targeted the damaged liver, kidney and pancreas. No DAPI-positive cells were observed in the organs without cell transplantation (Fig. 5m–o).

Inflammatory cytokine antibody array analysis

Using inflammatory cytokine antibody array analysis, we found that at 16 weeks (before treatment), the pro-inflammatory cytokines IL-1 and IL-12 were upregulated and the anti-inflammatory cytokines IL-4, IL-10, tumor necrosis factor (TNF)- β , GM-CSF, and M-CSF were downregulated in the model group compared with the control group (Fig. 6a). These findings suggested that there was an imbalance in the in vivo expression of the pro-inflammatory cytokines and anti-inflammatory factors in the model tree shrews. These findings indicated that the model group's anti-inflammatory capacity and immune function were weakened, resulting in the maintenance of a low-grade inflammation state.

At 20 weeks (4 weeks after transplantation), the pro-inflammatory cytokines IL-1, IL-6, IL-8, IL-12, and TNF- α were downregulated in the treatment group (Fig. 6b). MSCs were shown to reduce IL-1-related inflammation through the production of IL-1Ra and further reduce fibrosis in the liver and in the lungs. This result is consistent with MSCs' suppression of immune function in vivo. Three different serum samples ($n = 3$) were analyzed for cytokine expression in each group.

Discussion

Because of their low immunogenicity and easily accessible source, UC-MSCs have been increasingly used in place of BMSCs to treat various diseases (Ende et al. 2004, 2005; Morigi et al. 2004; Togel et al. 2005; Wu et al. 2007). In the present study, TS-UC-MSCs were isolated, identified and labeled successfully.

Tree shrews are more closely related to non-human primates, and in recent years, they have attracted increasing attention as an animal model for the study of human diseases (Zhang et al. 2013). To date, tree shrew metabolic syndrome models have not been reported because of a lack of a unified standard; therefore, this study used the 2004 CDS diagnostic criteria for metabolic syndrome. The following inclusion criteria were based on model group comparison with the control group; more than three criteria met was considered to indicate a successful model: (1) weight gain; (2) elevated blood glucose, impaired glucose tolerance, and an increased HOMA-IR; (3)

Fig. 6 Inflammatory cytokine antibody array analysis. **a** The model group compared with the control group at 16 weeks (before treatment), **b** the treatment group compared with the model group at 20 weeks (4 weeks after transplantation). A ratio value ≥ 2.0 indicates upregulated expression, and a ratio value ≤ 0.5 or >0 indicated downregulated expression ($P < 0.05$) (the results represent the means of measurements, $n = 3$)

Expression upregulated	IL-1	IL-12 p70	IL-13
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Expression downregulated	IL-10	IL-4	GM-CSF	M-CSF	ICAM-1	IL-15	TNF-beta 1
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Expression upregulated	IL-15	IL-2
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Expression downregulated	IL-1	IL-12p70	IL-12p40	IFN-gamma	IL-6	TNF-α	IL-8
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increased arterial blood pressure; and (4) dyslipidemia.

In this study, a tree shrew metabolic syndrome model was generated for the first time, and the results support the use of tree shrews in clinical research on

metabolic syndrome. Among the various animal models available to study metabolic syndrome, mice are most commonly used. There are many different naturally occurring and gene-targeted mutations in mice that lead to obesity and other metabolic defects

associated with human metabolic syndrome (Kennedy et al. 2010). Although there is no perfect animal model of the human disease, each of the mouse models make them useful for studying both the mechanism of development of metabolic syndrome, as well as potential therapies. New approaches, such as genetically modified models, can provide mechanistic information in the metabolic syndrome (Lehnen et al. 2013).

In this study, we first established a tree shrew metabolic syndrome model. Three methods were then used to label the TS-UC-MSCs prior to treatment. Next, in the treatment group, tree shrews were injected with the TS-UC-MSCs through the tail vein. The control group and the model group were injected with saline at the same time. The results showed that at 20 weeks (4 weeks after treatment), the fasting glucose, TG, TC, and LDL levels and the HOMA-IR decreased significantly compared with before treatment and compared with the model group. As the experiment continued, the model group's insulin continued to decrease to a lower level, and the insulin levels were significantly higher in the treatment group, indicating that TS-UC-MSCs promote the secretion of insulin.

Inflammatory cytokines are divided into pro-inflammatory and anti-inflammatory cytokines. The former group includes TNF- α , IL-1, IL-6, IL-12 and IL-8, and the latter group includes IL-4, IL-10, IL-13, and TNF- β . In model group, IL-1 upregulation is related to islet cell destruction. MSC transplantation can improve the inflammatory environment and liver function. In our study, we found that the model group had elevated serum IL-1, IL-12 and IL-13 levels. IL-12 is a key regulatory factor in the cellular immune response; IL-12 causes the T cell apoptosis rate to significantly increase, and enhances the expansion of Th1 cell differentiation (Scott 1993). Therefore, upregulation of IL-12 indicated abnormal T cell apoptosis and reduced immune function in the model group. When the model group produced a systemic inflammatory response, the expression of inhibitory inflammatory mediators of IL-13 increased. These findings indicated that the model group's anti-inflammatory capacity and immune function were weakened, resulting in the maintenance of a low-grade inflammation state. Microarray results have also suggested that an imbalance of pro-inflammatory cytokines and anti-inflammatory factors is an important element in the development of metabolic syndrome. In

the current study, the model group had disrupted anti-inflammatory and immune functions.

UC-MSCs affect not only innate immune cells but also adaptive immune cells. UC-MSCs specifically inhibit the secretion of IL-6, TNF- α , adjusting the Th1/Th2 balance. So the pro-inflammatory cytokines IL-1, IL-6, TNF- α and IL-8 were downregulated in the treatment group in the present study. This result is consistent with MSCs' suppression of immune function in vivo. Our microarray experiments showed that IL-15 and IL-2 expression was upregulated in the treatment group. It showed TS-UC-MSCs do not have a single immunosuppressive effect in vivo, but rather a wide range of immunoregulatory effects. The mode of cytokine action is similar to that of hormones, can be paracrine, autocrine, or endocrine. Therefore, to clarify these complex cytokine expression patterns in metabolic syndrome, more accurate experimental studies are needed.

In summary, the TS-UC-MSCs used to treat metabolic syndrome symptoms, such as insulin resistance, glucose and lipid metabolism disorders, and an inflammatory state, mediated significant improvement and repaired target organ damage. This study laid the foundation for the use of stem cell transplantation in the treatment of metabolic syndrome.

Conclusions

In this study, TS-UC-MSCs were isolated and cultured, and DAPI, DIR and SPIO labeling of the TS-UC-MSCs was successful. In addition, a high-sugar, high-cholesterol, high-salt diet and sugar water combined with intraperitoneal injection of STZ successfully generated a tree shrew metabolic syndrome model that appeared to have significant insulin resistance, high blood sugar, lipid metabolism disorders, and hypertension. TS-UC-MSC transplantation significantly reduced the blood sugar and lipid content, improved insulin resistance and regulation of insulin secretion, and reduced the expression of the pro-inflammatory cytokines IL-1 and IL-6. The transplanted TS-UC-MSCs also targeted the liver, kidney and pancreas, reducing liver cell degeneration and necrosis, kidney bleeding and inflammatory cell infiltration, and islet cell degeneration and necrosis. These findings demonstrate that TS-UC-MSCs play roles in treating metabolic syndrome.

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Author contributions Lu Zhu and Xiang Yao: performed the cell studies, participated in the cell culture and drafted the manuscript. Ju-fen Liu, Zi-an Li and Jin-xiang Wang: performed the immunoassays and HE staining. Jian-yong Yang and Rong-qing Pang: performed the cell differentiation and labeling. Xing-hua Pan: participated in the design of the study and performed the statistical analysis. Conception and design, financial support. Guang-ping Ruan: conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

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