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Umbilical cord mesenchymal stem cells are able to undergo differentiation into functional islet-like cells in type 2 diabetic tree shrews

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ABSTRACT

Islet transplantation is arguably one of the most promising strategies to treat patients suffering with diabetes mellitus. However, a combination of a lack of donors and chronic immune rejection limit clinical applications. Here, we evaluated the efficacy of cell therapy using islet-like cells differentiated from umbilical cord mesenchymal stem cells (UC-MSCs) of tree shrews for the treatment of type 2 diabetes. Enhanced green fluorescent protein (eGFP) labeled UC-MSCs were directly injected into type 2 diabetic tree shrews, where UC-MSC differentiated into functional islet-like cells and alleviated disease severity, as evidenced by improved biochemical features and reduced concentrations of inflammatory cytokines. We also demonstrated that *in vitro* culture of UC-MSCs for six days in a high-glucose environment (40 mmol/L or 60 mmol/L glucose) resulted in significant gene methylation. The potency of UC-MSCs differentiated into insulin-secreting cells was attributed to the activation of Notch signal pathways. This study provides evidence that cell therapy of islet-like cells differentiated from UC-MSCs is a feasible, simple and inexpensive approach in the treatment of type 2 diabetes.

1. Introduction

Diabetes mellitus (DM) affects more than 366 million people worldwide, and its global prevalence is expected to double in the next 15 years [1]. In China, the prevalence of type 2 diabetes in adults is estimated at 9.7%, affecting 92.4 million adults [2]. Type 2 diabetes mellitus is characterized by disorder in the metabolism of carbohydrates, accompanied by impaired glucose tolerance and/or defects in insulin secretion [3]. Indeed, beta cell dysfunction in the pancreas contributes significantly to the pathogenesis of type 2 diabetes. Current treatment options are mainly restricted to conservative therapies that aim to relieve symptoms, including oral hypoglycemic drugs, insulin, traditional Chinese medicine and gastric surgery [4,5]. To date, no optimal strategies exist to regenerate impaired pancreatic beta cells. At present, islet transplantation is one of the most promising strategies to treat patients suffering with diabetes mellitus. However, lack of donors, high cost and immune rejection limit clinical application of transplantation. Therefore, there is a significant need for development of novel therapies that compensate for impaired insulin secretion function.

Mesenchymal stem cells (MSCs) are rare multipotent stem cells of mesenchymal origin, and are isolated from a variety of tissues including bone marrow, umbilical cord, cord blood and, adipose tissues. MSCs have been reported to have potential of multilineage differentiation, anti-inflammatory properties and immune modulation [6–8]. MSCs have been widely tested, with their safety and

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efficacy for a variety of congenital and acquired diseases in both animal models and clinical trials analyzed, partially due to their ease of availability and extensive capacity for *in vitro* expansion. Their capacity of multilineage differentiate and ability to form functional insulin-producing cells has been reported and, thus, their use in treating diabetes has been widely suggested [9–11].

In this study, we evaluated the efficacy of cell therapy using islet-like cells differentiated from umbilical cord mesenchymal stem cells (UC-MSCs) of tree shrews for the treatment of type 2 diabetes. In addition, we also used genome-wide methylation analysis to identify any changes in methylation status in high-glucose cultured UC-MSCs.

2. Methods and materials

2.1. Culture of tree shrews umbilical cord mesenchymal stem cells

Primary umbilical cord-derived mesenchymal stem cells (UC-MSCs) from healthy tree shrews after caesarean birth were cultured. The umbilical cord tissues (1×1 cm^2) were digested in DMEM medium (Invitrogen, Carlsbad, CA, USA) containing 200 U/mL collagenase and 300 U/mL hyaluronidase (Sigma, St Louis, MO, USA), followed by digestion with 0.25% trypsin (Invitrogene, Carlsbad, CA, USA). After centrifugation, cells were cultured in DMEM/F12 medium at concentration of 2×10^5/mL and used for cell differentiation [12].

All procedures were performed in accordance with the guidelines set by the Committee on Laboratory Resources, National Institutes of Health and Institutional Animal Care and Use Committee of Hospital of Kunming, China.

2.2. Flow cytometric analysis

Suspended cells (1×10^6) were fixed with 100 μL of 4% paraformaldehyde for 10–15 min and were then incubated with 100 μL 0.5% saponin solution at room temperature for 15 min. After being washed twice with phosphate buffered saline (PBS) containing 0.5% saponin, cells were incubated with anti-human CD31-phycoerythrin (PE), CD44-fluorescein isothiocyanate (FITC), CD34-PE or CD90-FITC antibody (BD Biosciences, San Jose, CA, USA) at room temperature for 30 min. Isotype controls were stained with an isotype-matched control of irrelevant specificity. Quadrant markers based on isotype control and unstained cells were set. Data were acquired with a FACSCalibur flow cytometer and analyzed with CELLQuest software (BD Biosciences, San Jose, CA, USA).

2.3. RNA extraction and real-time qRT-PCR

Total RNA was extracted from cultured cells by TRI-REAGENT (Sigma, MO, USA), and total RNA (10 μg) was reverse-transcribed into complementary DNA (cDNA) using the GoScriptTM reverse transcriptase (Promega Corp, Madison, USA). The sequences of the primers used are shown in Tables 1 and 2. DNA amplification was performed in a PCR system thermocycler (CFX96, Biorad, Hercules, CA, USA) using the following conditions: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension phase at 72 °C for 5 min. Expression was assessed by evaluating CT values. The relative amount of expressed mRNA was calculated by the $2^{-\triangle\triangle CT}$ method [13].

2.4. Differentiation of UC-MSCs into adipocytes, osteocytes and, insulin-secreting cells

For adipocyte differentiation, attached UC-MSCs were treated with 10^{-6} M of all-trans-retinoic acid (ATRA) for 3 d, followed by 10^{-7} M of insulin and 2×10^{-9} M of triiodothyronine (T3) [14]. Differentiated adipocytes were stained with Oil-red-O and counterstained with hematoxylin.

For osteocyte differentiation [15], UC-MSCs were treated with a modified STEMPRO® Osteogenesis Differentiation Kit (Gibco, Grand Island, NY, USA) for 3 weeks *in vitro*. Differentiated osteocytes were stained with alizarin red staining.

A three-step method [16]was used with minor modifications for insulin-secreting cell differentiation. The identification of the insulin-secreted cell clusters were performed by staining with primary antibodies of insulin AB-6 mouse monoclonal antibody (1:200, Lab Vision, Fremont, CA, USA), and anti-*C*-Peptide antibody (1:100, LINCO Research Inc. St Louis, MO, USA). The reaction was visualized using DAB (3,3'-diaminobenzidine). At the same time, total RNA was extracted by TRI-REAGENT (Sigma, St Louis, MO, USA), and cDNA was synthesized with RNA reverse transcriptase. Beta cell differentiation-related genes and primers are shown in Table 2 [17].

2.5. Establishment of tree shrews model of type 2 diabetes

Tree shrews were divided into three groups, healthy tree shrews (control group), vehicle (saline solution)-treated diabetic tree shrews (mode group) and UC-MSC-treated diabetic tree shrews (treated group). Type 2 diabetic model of tree shrews were prepared as previously described [18]. Forty healthy tree shrews, weighed 120–140 g purchased from the Kunming Animal Institute (Yunnan, China), were given high-sugar, high-fat diet for six weeks [19], followed by an intraperitoneal injection of low dose streptozotocin (STZ, 100 mg/kg) [20]. During the last four weeks, rats with fasting blood glucose (FBG) levels $\geq$ 11.1 mmol/L were considered to have type 2 diabetes and were used in subsequent experiments.

2.6. UC-MSC transplantation into type 2 diabetic tree shrews

A low molecular weight heparin calcium injection (125 IU per 5×10^6 cells) was used prior to UC-MSC transplantation. 5×10^6 cells/kg UC-MSCs (approval number: CFDA H20020470) and slowly injected into the caudal vein of the established diabetic tree shrews after sterilization with 75% ethanol, once a week for three weeks.

Cell distribution was examined in the liver, kidney and pancreas three days after the last transplantation. Four weeks after last transplantation, blood samples were collected for the measurement of fasting blood glucose, fasting insulin (FINS), C peptide, homeostasis model assessment of insulin resistance (HOMA IR), total cholesterol, triglycerides and oral glucose tolerance test (OGTT). Six weeks after transplantation, liver, kidney and pancreas tissue samples were collected to evaluate any histological changes.

2.7. Genome-wide methylation analysis of high glucose-cultured UC-MSCs

Third passage exponentially growing UC-MSCs were seperated with trypsin–ethylene diamine tetraacetic acid (EDTA, Invitrogen, Carlsbad, CA, USA). The cells were transferred to 24-well plates at a density of 4×10^5 cells per well, and incubated with DMEM/F12 medium containing 40 mmol/L or 60 mmol/L glucose for six days. The cells were then collected for genome-wide methylation analysis and total RNA extracted.

2.8. DNA methylation analysis and qPCR verification

An amount of 150–300 ng of DNA was isolated from

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