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Induction of hepatocytes-derived insulin-producing cells using small molecules and identification of microRNA profiles during this procedure

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ABSTRACT

The transplantation of insulin-producing cells (IPCs) or pancreatic progenitor cells is a theoretical therapy for diabetes with insulin insufficiency. Isolated hepatocytes from newborn rats (within 24 h after birth) were progressively induced into IPCs using 5-aza-2′-deoxycytidine, Trichostatin A, retinoic acid, insulin-transferrin-selenium, and nicotinamide. We transplanted Pdx1+ pancreatic progenitors into STZ-induced diabetic mice and found the decreased blood glucose and increased insulin level in comparison with diabetic model. The dynamic expression profiles of microRNAs (miRNAs) were identified using microarray. We found 67 miRNAs were decreasingly expressed; 52 miRNAs were increasingly expressed; 27 miRNAs were specially inhibited in Stage 1 cells (multipotent progenitor cells); and 58 miRNAs were specially inhibited in Pdx1+ cells (Stage 2). Further analysis showed these miRNAs' targets were associated with genetic recombination, stem cell pluripotency maintenance, cellular structure reorganization and insulin secretion. Enrichment analysis using KEGG pathway showed the differentiation of IPCs from hepatocytes was massively more likely not mediated by canonical Wnt/β-catenin signaling. In addition, the BMP/Smad signaling was involved in this progression. We found the dysregulated miRNAs profiles were inconsistent with cell phenotypes and might be responsible for small molecule-mediated cell differentiation during IPCs induction.

1. Introduction

Patients with diabetes suffer from progressive loss of pancreatic β-cell mass and insulin insufficiency [1]. The inhibition and apoptosis of inflammatory β-cell are contributors to insulin insufficiency [2]. Hence, transplantation of pancreatic progenitors or insulin-producing cells (IPCs) has been proposed as strategies to restore insulin production in diabetes [1,3]. IPCs derived from human embryonic stem cells (hESCs) was proposed and recognized as an effective therapy for diabetes [3].

Comparing with hESCs, hepatocytes or liver tissues are easier to obtain and show relative stronger proliferation potential. In addition, both liver and pancreas are derived from embryonic endoderm [4–7]. They share a common bipotential precursor cell within the embryonic endoderm. In comparison with previous incomplete strategies for β-cell phenotypes and insulin secretion function, we had established a complete 4-stage protocol (17-day) to differentiate liver epithelial stem-like WB-F344 cells (WB cells) into IPCs cells using small molecules [1,4,8,9]. WB cells were induced into pancreatic β cells with insulin-secreting function *in vitro*, and the *in vivo* transplantation of Pdx1 positive pancreatic precursors secreted insulin and attenuated blood glucose level in diabetic mice [4]. However, we focused on the identification of differentiation biomarkers rather than the modulation mechanism during IPCs differentiation.

Some regulators including microRNAs (miRNAs) were involved in embryogenesis, cell fate, insulin secretion, pancreas development, pancreatic islet cell genesis and IPCs differentiation [10–14]. For instance, miR-375 was necessary for IPCs induction [12,13,15]. So, the expression profile analysis of miRNAs during the IPCs differentiation was a crucial step for comprehending the mechanism underlying it.

Using mouse hepatocytes, we induced the IPCs using

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combination of small molecules, including 5-aza-2′-deoxycytidine (5-AZA), Trichostatin A (TSA), retinoic acid (RA), insulin-transferrin-selenium (ITS), retinoic acid (RA), and nicotinamide as we previously did to WB cells [4]. Hepatocytes were induced into IPCs in a step-by-step protocol. The miRNA expression profiles during the IPCs differentiation from hepatocytes were monitored using an Affymetrix GeneChip miRNA microarray. MiRNAs showing differentially expression levels during the induction period would be identified and subjected to enrichment analysis. To our knowledge, this was the first report focusing on the dynamic expression profiles of miRNAs during IPCs differentiation from hepatocytes. Moreover, miRNAs directly or indirectly modulated the cell fate during pancreatic β-cell genesis would be discussed.

2. Materials and methods

2.1. Cells and culture conditions

Animal protocols were conducted according to the Guide for the Care and Use of Laboratory Animals. An approval was obtained from the Animal Care Committee of the Second Affiliated Hospital of Nanchang University, Nanchang, China. Mouse hepatocytes were isolated from liver of newborn wild-type C57BL/6 mouse within 24 h after birth. Separated livers tissues were shredded directly using a pair of ophthalmic scissor, followed by digestion (0.25% Trypsin/0.04%EDTA, Sigma, Missouri, USA), filtration, centrifugation, and finally cells (5×10^5/ml-6×10^5/ml) were suspended and cultured in HDMEM supplemented with 10% FBS (PAA, Dresden, Germany) and 1% penicillin-streptomycin (Sigma) at 37 °C in a humidified air of 5% CO_2, with replaced medium every two days. Cells were subcultured for 3–4 days when confluence reached to 70%–80%. Primary cells were digested with 0.25% Trypsin and subcultured under condition as above. The mouse MIN6 pancreatic β cell line which secretes insulin was obtained from ATCC (Manassas, VA, USA) and was maintained in HDMEM under conditions as above.

2.2. In vitro induction of hepatocytes into IPCs

The identification of isolated hepatocytes was performed using periodic acid-Schiff (PAS) staining and flow cytometric analysis with ALB antibody (Fig. 1a). The differentiation of hepatocytes into IPCs followed a three basic step protocol as previously described by Liu et al. [4]. The stepwise protocol of differentiation into IPCs is shown in Fig. 1b. In brief, 2×10^5 hepatocytes/well were seeded into 6-well plates (Corning Inc., Corning, New York, USA) which were supplemented with serum- and FBS-free HDMEM supplemented with the main components, and treated with 5-AZA (5 μM; Sigma) for 48 h, followed by TSA (100 nM; Sigma) for 24 h, 1 × ITS (Invitrogen, Carlsbad, CA, USA) plus RA (2 μM; Sigma) for 7 days, and at last in FGF- and EGF-free DMEM supplemented with nicotinamide (10 mM; Sigma) for 7 days.

2.3. Transmission electron microscopy (TEM)

Cell aggregates were prepared according to the methods described by Liu et al. [4]. Cellular morphology of stage-specific cells were identified using the HitachiH-7650 TEM (Hitachi, Olympus Japan).

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells using TRI reagent (Fermentas, Burlington, ON, Canada), and was reverse-transcribed into cDNA template for PCR amplification using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). PCR amplification was performed on an ABI Realtime RCR machine (ABI Applied Biosystem, California, USA) with a SYBR Green Realtime RCR Master Mix (ToYoBo, Shanghai, China). The qRT-PCR primers were synthesized by Invitrogen (Shanghai, China) and are listed in Table 1.

2.5. In vitro insulin secretion assay

The *in vitro* glucose-stimulated insulin secretion (GSIS) assay was performed as previously described [4]. Before insulin release assay, 2×10^5 cells at each stage were placed into 6-well plates and incubated in serum-free medium supplemented with 0.5% BSA overnight for starvation. Then cells were stimulated with 5.5 mM or 25 mM glucose for 2 h. The secreted insulin content in cellular medium was determined according to the manufacturer's protocol in a commercial mouse insulin ELISA kit (Linco Research, Missouri, USA).

2.6. In vivo Pdx1+ cells transplantation and insulin release assay

Approval for animal procedures was obtained from the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Nanchang University, Nanchang, China. Hyperglycemic mice were induced through single intraperitoneal injection of 200 mg/kg body weight streptozocin (STZ, Sigma, USA). The *in vivo* cell transplantation of 1×10^6 Pdx1+ cells into the left renal subcapsular space of nude mice (8–10 weeks old) was performed on hyperglycemic (diabetes mellitus, DM) mouse [4]. Six days later, Pdx1+ cells or PBS was injected into the left renal subcapsular space of DM ($n = 20$) or control mice ($n = 10$ in control model, DM; and $n = 10$ in blank control, Normal), respectively. The fasting glucose levels were measured every 3 days. Sixty days post Pdx1+ cells transplantation, the tissues (left renal subcapsular space) containing implanted IPCs cells were explanted from half of the Pdx1+ cells transplanted mice ($n = 15$). The introperitoneal glucose tolerance test (IPGTT) and *in vivo* GSIS assay were performed on all animals at 51–60 days post transplantation [4]. The blood glucose levels in tail venous blood were detected at 15, 30, 60, 90, and 120 min post intraperitoneal injection of 2 g/kg body weight glucose. Serum insulin levels were detected at 30, 60, 90, 120, 150, and 180 min post glucose injection. At 75 days post transplantation, all mice were killed, the pancreas and left kidneys were separated for histological immunofluorescence analysis.

2.7. Histological immunofluorescence

The separated pancreas and left kidneys of the Pdx1+ cell-transplanted mice were fixed, embedded, and sectioned for histological immunofluorescence analysis for the glucagon and insulin expression. Hochest 33342 was used for the nuclei observation.

2.8. MiRNA microarray and data procession

The miRNA expression profiling during hepatocytes differentiation into IPCs were identified using the Affymetrix GeneChip miRNA 4.0 Array. Total cellular RNA was isolated using Invitrogen TRIzol reagent (Thermo Fisher Scientific), quantified by the NanoDrop ND-2100 (Thermo Fisher Scientific) and Agilent 2100 (Agilent Technologies). Then, RNA was tailed with Poly A and labeled with Biotin using a 3DNA Array Detection FlashTag™ Biotin HSR kit (Genisphere, Hatfield, PA, U.S.) following the manufacturer's protocol. The labeled RNAs were then hybridized onto the microarray overnight. The slides were then washed and stained using the Affymetrix GeneChip Hybridization Wash and Stain Kit, followed by scanning using the Affymetrix Scanner 3000 7G (Affymetrix, Santa