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MicroRNAs 106b and 222 Improve Hyperglycemia in a Mouse Model of Insulin-Deficient Diabetes via Pancreatic β-Cell Proliferation

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ABSTRACT

Major symptoms of diabetes mellitus manifest, once pancreatic β-cell numbers have become inadequate. Although natural regeneration of β-cells after injury is very limited, bone marrow (BM) transplantation (BMT) promotes their regeneration through undetermined mechanism(s) involving inter-cellular (BM cell-to-β-cell) crosstalk. We found that two microRNAs (miRNAs) contribute to BMT-induced β-cell regeneration. Screening murine miRNAs in serum exosomes after BMT revealed 42 miRNAs to be increased. Two of these miRNAs (miR-106b-5p and miR-222-3p) were shown to be secreted by BM cells and increased in pancreatic islet cells after BMT. Treatment with the corresponding anti-miRNAs inhibited BMT-induced β-cell regeneration. Furthermore, intravenous administration of the corresponding miRNA mimics promoted post-injury β-cell proliferation through Cip/Kip family down-regulation, thereby ameliorating hyperglycemia in mice with insulin-deficient diabetes. Thus, these identified miRNAs may lead to the development of therapeutic strategies for diabetes.

1. Introduction

Type 1 diabetes mellitus is characterized by progressive loss of pancreatic β-cells, leading to a life-long dependence on exogenous insulin. In addition, β-cells are reportedly decreased in patients with type 2 diabetes (Butler et al., 2003). In this context, regeneration of pancreatic β-cells is a promising therapeutic strategy for not only type 1 but also some forms of type 2 diabetes. We (Hasegawa et al., 2007) and other groups (Hess et al., 2003, Nakayama et al., 2009) previously reported bone marrow (BM) transplantation (BMT) to promote pancreatic β-cell proliferation after pharmacological β-cell injury, such as that caused by streptozotocin (STZ) treatment. Bone marrow-derived cells were found to have infiltrated sites around regenerating islets (Hess et al., 2003, Hasegawa et al., 2007), suggesting a secretory factor(s) from bone marrow cells to be involved in the underlying mechanism. However, despite intensive research, the specific secretory protein(s)/peptide(s) involved in pancreatic β-cell regeneration after BMT has not as yet been identified.

In addition to secretory proteins, microRNAs (miRNAs) which are transported in exosomes have recently attracted considerable attention for their roles in inter-cellular communication. Exosomes are lipid nano-vesicles, and are secreted by many types of cells and contain a variety of molecules including miRNAs (Turchinovich et al., 2013, Hirsch et al., 2013). miRNAs are a class of endogenous, small, non-coding RNAs that negatively regulate gene expression via translational inhibition or degradation of their target mRNAs (Bartel, 2009). miRNAs have been shown to regulate basic cellular functions including cell proliferation, differentiation, and death (Shenoy and Blelloch, 2014). Therefore, we hypothesized that miRNAs transferred into exosomes mediate the mechanism underlying β-cell regeneration in response to BMT, and comprehensively examined miRNA levels in serum exosomes in BMT-mice. We found that two microRNAs, miR-106b-5p and miR-222-3p, contribute to BMT-induced β-cell regeneration. Furthermore, intravenous administration of the corresponding miRNA mimics promoted post-injury β-cell proliferation, thereby ameliorating hyperglycemia of insulin-deficient diabetes.

Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; CHOP, C/EBP homologous protein; Cip/Kip, CDK interacting protein/kinase inhibitory protein; DAPI, 4′,6-Diamidino-2-phenylindole; GFP, green fluorescent protein; GNZ, a nuclear-localized GFP/LacZ fusion protein; miRNAs, microRNAs; PBS, phosphate-buffered saline; Pri-miR, primary microRNA; STZ, streptozotocin; TM, tamoxifen; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

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2. Materials and Methods

2.1. Animals

C57BL/6J mice (Clea Japan, Tokyo, Japan), RIP-CreER mice (Stock Number: 008122, The Jackson Laboratory, ME, USA) and Rosa26-GNZ mice (Stock Number: 008516, The Jackson Laboratory) were obtained, housed in an air-conditioned environment, with a 12/12-hour light-dark cycle, and ad lib access to a regular unrestricted diet. Hyperglycemia was induced by intraperitoneal infusion of 50 mg/kg body weight STZ (Sigma, MO, USA) for 5 consecutive days. STZ was dissolved in 0.05 M citrate sodium buffer (pH 4.5) and injected into 6-week-old mice. Tamoxifen (TM) (Sigma) was dissolved at 20 mg/ml in corn oil (Sigma) by sonication at room temperature. TM was injected intraperitoneally at 80 mg/kg body weight daily for 5 consecutive days. This study was approved by the ethics committees of Tohoku University. All animal experiments were conducted in accordance with Tohoku University institutional guidelines.

2.2. Measurements

Blood glucose was measured after a 9-hour daytime fast and assayed using Glutest Mint (Sanwa Chemical, Aichi, Japan). Plasma insulin concentrations were determined with an ELISA kit (Morinaga, Kanagawa, Japan). For measurement of the pancreatic insulin content, samples of the pancreas were suspended in ice-cold acid–ethanol (0.18 M HCl in 75% ethanol) and minced with scissors, and left at −20 °C for 48 h, with sonication every 24 h. The supernatant of each sample was diluted with a solution of 1 mM EDTA/1% bovine serum albumin in phosphate-buffered saline (PBS) and subjected to the ELISA assay (Kondo et al., 2013).

2.3. Bone Marrow Transplantation (BMT)

BM cells were collected by dissecting the femurs and tibias, removing excess tissue, cutting the ends of the bones and flushing out the BM with PBS. Then, BM cells were filtrated through a Nylon cell strainer (BD Biosciences, CA, USA). The BM donors were 6-week-old C57BL/6J mice. Recipient mice were lethally irradiated with 10 Gy and reconstituted with a single injection of 4×10^6 donor BM cells through the tail vein (Hasegawa et al., 2007).

2.4. Preparation of Synthetic miRNAs/Atelocollagen Complex

miRNA mimics (miR-106b, miR-222 and non-targeting control) (mirVana miRNA mimics, Ambion, TX, USA), anti-miRNAs (anti-miR-106b-5p, anti-miR-222-3p and non-targeting control) (mirVana miRNA inhibitors, Ambion) or fluorescein-labeled miRNA mimics (miR-106b and miR-222) (Cosmo Bio, Tokyo, Japan) were used in the experiments. For preparation of the synthetic miRNAs/atelocollagen complex (AteloGene; Koken, Tokyo, Japan), equal volumes of atelocollagen and miRNA solution were combined and mixed, following the manufacturer's protocol. Each mouse was restrained and slowly administered 200 μl of mixtures, containing 4 nmol of miRNA mimics (2 nmol of miR-106b and 2 nmol of miR-222), 4 nmol of anti-miRNAs (2 nmol of anti-miR-106b-5p and 2 nmol of anti-miR-222-3p) or 4 nmol of the non-targeting control, via the tail-vein using a disposable insulin syringe (TERUMO Myjector 29G × 1/2″, TERUMO, Tokyo, Japan). The injection speed was <5 μl/s.

2.5. Analyses of Pancreases after Intravenous Administration of Fluorescein-Labeled miRNAs with Atelocollagen

Fluorescein-labeled miRNA mimics (2 nmol of miR-106b and 2 nmol of miR-222) mixed with atelocollagen were injected into the tail veins of STZ-treated mice. After 24 and 48 h, pancreases were removed and embedded in OCT compound and frozen. Cryostat pancreatic sections (8 μm thick) were placed on microscope slides, stained with DAPI (SouthernBiotech, AL, USA) and observed using a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

2.6. Exosome Isolation

Exosomes from mouse serum were isolated using ExoQuick solutions (System Biosciences., CA, USA) following the manufacturer's protocol. Briefly, 1/4 volume of ExoQuick was added to serum and the samples were refrigerated at 4 °C for 30 min. Next, exosomes were precipitated by centrifugation at 1500 ×*g* for 30 min and the supernatant was removed by aspiration. Exosomes from cell culture media were isolated using ExoQuick-TC solutions (System Biosciences) following the manufacturer's protocol. Briefly, cell culture media were centrifuged at 3000 ×*g* for 15 min to remove cells and cell debris. After filtration through 0.22 μm pore size filters (Millex-GV Filter, Merck Millipore, Darmstadt, Germany), 10 ml of cell culture supernatant were mixed with 2 ml of Exoquick-TC and refrigerated overnight. Next, exosomes were precipitated by centrifugation at 1500 ×*g* for 30 min and the supernatant was removed by aspiration.

2.7. Isolation and Culture of BM Cells

Six days after BMT, BM cells were harvested from femurs and tibias and cultured in Dulbecco modified Eagle medium containing 10% exosome-depleted fetal bovine serum media (System Biosciences) and penicillin-streptomycin at 37 °C and 5% CO_2. BM cells were plated in 12 ml of cell culture medium on collagen type-1 coated 10 cm dishes (Iwaki, Tokyo, Japan). After 10 h of cell culture, media were collected and exosomes were isolated as described above.

2.8. Primary Islet Cell Culture and Transfection

Pancreatic islets were isolated from 10- to 11-week-old C57BL/6J mice by retrograde injection of collagenase (Sigma) into the pancreatic duct according to the standard procedure, as described previously (Imai et al., 2008, Gotoh et al., 1985). The freshly isolated islets were dissociated into dispersed islet cells by trypsinization and distributed into 96-well plates (40 islets per well) and maintained in RPMI1640 medium containing 10% fetal bovine serum, penicillin-streptomycin and gentamicin at 37 °C and 5% CO_2 for 2 days. Then, islet cells were co-transfected with 10 pmoles of miR-106b mimics and 10 pmoles of miR-222 mimics (Ambion), or transfected with 20 pmoles of non-targeting control (Ambion) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Three days after transfection, the cells were collected and analyzed for Ki-67 mRNA expression.

2.9. Immunohistochemistry

The pancreases were excised, fixed overnight in 10% paraformaldehyde and embedded in paraffin. Samples were sectioned at 3 μm and stained with hematoxylin-eosin or incubated with primary antibodies: p21Cip1(ab2961, Abcam, Cambridge, UK), p27Kip1(ab7961, Abcam), p57Kip1(ab75974, Abcam), p53 (NCL-p53-CM5p, Leica Biosystems, Wetzlar, Germany), CHOP (sc-575, Santa Cruz Biotechnology, CA, USA), GFP (sc-8334, Santa Cruz), Ki-67 (#12202, Cell Signaling Technology, MA, USA), insulin (I2018, Sigma) or glucagon (A0565, Dako, CA, USA). The immune complexes were visualized with DAB (Histofine Simple Stain Mouse MAX-PO (R) or Histofine Mouse Stain Kit; Nichirei, Tokyo, Japan). Alexa Fluor 488 goat anti-mouse IgG (Sigma) or Alexa Fluor 546 goat anti-rabbit IgG (Dako) was used as the fluorescent secondary antibody. Dapi-Fluoromount-G™ (Southern Biotech, AL, USA) was used to stain nuclei in the final step. At least 20 islets with >1000 islet cells were counted per mouse for evaluation of p21, p27, p53, CHOP, GNZ and Ki-67 expression by IHC staining. GNZ protein was

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