biomolecules

Article

Gene Delivery of Manf to Beta-Cells of the Pancreatic Islets Protects NOD Mice from Type 1 Diabetes Development

Kailash Singh [1,2,*], Orian Bricard [1], Jeason Haughton [3,4], Mikaela Björkqvist [2], Moa Thorstensson [2], Zhengkang Luo [2], Loriana Mascali [3,4,5], Emanuela Pasciuto [3,4,5], Chantal Mathieu [6], James Dooley [1,*,†] and Adrian Liston [1,3,4,*,†]

[1] Immunology Programme, The Babraham Institute, Cambridge CB22 3AT, UK
[2] Department of Medical Cell Biology, Uppsala University, 751 05 Uppsala, Sweden

check for

Citation: Singh, K.; Bricard, O.; Haughton, J.; Björkqvist, M.; Thorstensson, M.; Luo, Z.; Mascali, L.; Pasciuto, E.; Mathieu, C.; Dooley, J.; et al. Gene Delivery of Manf to Beta-Cells of the Pancreatic Islets Protects NOD Mice from Type 1 Diabetes Development. *Biomolecules* **2022**, *12*, 1493. https://doi.org/10.3390/biom12101493

Academic Editor: Alberto Bartolomé

Received: 30 August 2022
Accepted: 14 October 2022
Published: 16 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

resistant to insulin, with reduced signalling through the insulin receptor. The transition between pre-diabetes and early-stage T2D can be difficult to catch in a standard clinical setting and is, at this early stage, reversible [3], with pancreatic beta-cells initially able to compensate for insulin resistance by increasing the amount and duration of insulin secretion. Many patients remain in a grey area of diagnosis at this stage, where diet

2. Materials and Methods

2.1. Mice

NOD mice were inbred and housed under semibarrier conditions in our animal facility, and fed a standard chow diet. Ten-week-old female NOD mice were used. Allocation to treatment group was made randomly at weaning, at the cage level. All experiments were performed in accordance with the University of Leuven Animal Ethics Committee guidelines. Sample sizes for mouse experiments were chosen in conjunction with the Animal Ethics Committee to allow for robust sensitivity without excessive use.

2.2. Diabetes Incidence Study

Mice were kept until 30 weeks of age and tested twice per week for glucose dysregulation by blood glucose and urine assessment with Diastix Reagent Strips (Bayer, Basel, Switzerland). Mice were diagnosed as diabetic when having glucosuria and a blood glucose (FreeStyle Freedom Lite, Abbott, Chicago, IL, USA) level over 250 mg/dL (13.9 mmol/L) for two consecutive readings. Glucose testing was performed on a blinded basis, with mice being coded by number until experimental end.

2.3. AAV Vector Production and Purification

AAV production was performed by VectorBuilder (Neu-Isenburg, Germany), using the classical tri-transfection method, with subsequent vector titration performed using a qPCR-based methodology [16,17]. For AAV8.*ins*-GFP and AAV8.*ins*-Manf, the mouse Manf coding sequence (accession number NM_029103.4) was cloned into a single stranded AAV8-derived expression cassette containing the 705 bp rat Insulin 2 promoter, woodchuck hepatitis post-transcriptional regulatory element (WPRE), and bovine growth hormone

2.5. Immunohistochemical Staining for Insulin

Ten microscope slides were made from each mouse pancreas. Then, three of these slides (slide numbers 1, 6, and 10) were prepared for staining. Two different stainings were chosen: specific staining for insulin and a counterstaining with Haematoxylin. To prepare the samples for the insulin staining, deparaffinization was performed by placing the slides in xylene. Rehydration was then performed by first dipping the slides in the first container with ethanol and then incubating the slides for 5 min in the following one. The dipping and incubation were performed in multiple sets of containers, first in an ethanol series, with a decreasing concentration of ethanol (99.6%, 95%, 80% to 70%), and at last in distilled water. After the deparaffinization and rehydration, the slides were incubated for 10 min at room temperature in a hydrogen peroxide block solution (PBS-Tween and Hydrogen peroxide 30%, EMSURE®, Merck KGaA, Darmstadt, Germany) and then washed in PBS-Tween.

2.8. Confocal Imaging and Mean Fluorescence Intensity Analysis

Confocal imaging was performed using a laser caning confocal microscope Zeiss LSM 780 (Carl Zeiss, Jena, Germany). All images were analysed using Zeiss Zen Blue software. Mean fluorescence intensity was determined using ImajeJ software (NIH, Bethesda, MD, USA).

2.9. Enzyme-Linked Immunesorbent Assay

Serum samples were analysed to determine the insulin levels using Ultra-Sensitive Insulin ELISA kit (Mercodia, Uppsala, Sweden).

2.10. Statistical Analysis

The GraphPad Prism 9.4.0 Software (San Diego, CA, USA) was used for the statistical

Figure 1. AAV8.*ins*-Manf prevented diabetes development in NOD mice. The percentage of non-diabetic NOD mice was controlled after the end of treatment at 30 weeks of age. (**A**) The AAV8.*ins*-Manf group (n = 17) and the AAV8.*ins*-GFP group (n = 14) had 82.4% and 42.8% non-diabetic mice, respectively (p-value of 0.0178). (**B**) The comparison between two groups with glucagon promoters, AAV8.*glu*-Manf (n = 14) and AAV8.*glu*-GFP (n = 14), had a p-value of 0.3045, which show no statistically significant difference. For comparison a Log-rank test was used.

Figure 2. Islets of AAV8.*ins*-Manf- or AAV8.*glu*-Manf-treated mice express Manf. (**A**–**D**) AAV8.ins-Manf-, AAV8.ins-GFP-, AAV8.glu-Manf-, and AAV8.glu-GFP-treated mice were stained with antibodies: Manf (green), insulin (red), or glucagon (red) and DAPI (blue). (**E**,**F**) mean fluorescence intensity (MFI) of Manf in islets of AAV8.ins-Manf-, AAV8.ins-GFP-, AAV8.glu-Manf-, and AAV8.glu-GFP-treated mice. Three to four slides from each mouse (in total 4 mice per group) were stained with antibodies: Manf, insulin, or glucagon and DAPI. Confocal images were captured and analysed using ImageJ software for determining MFI. Results are presented in means SEM (n = 4 per group). Unpaired t-tests were performed for comparison, ** denote $p < 0.05$.

3.2. Beta-Cell-Specific Gene Delivery of Manf Prevents Severe Insulitis in Islets In Vivo

Insulitis grading was performed to determine whether gene delivery of Manf could reduce insulitis in the pancreatic islets. The representative images of different insulitis grades are shown in Figure 3A,C,E,G,I. The mean percentage of islets was calculated for each insulitis grading. The data were analysed and compared between four treatment groups (AAV8.*ins*-Manf, AAV8.*glu*-Manf, AAV8.*ins*-GFP and AAV8.*glu*-GFP). The AAV8.*ins*-Manf group (total number of islets = 333) demonstrated the highest percentages of islets with no

insulitis (15.3%) and with insulitis grade 1 (42.3%) (Figure

Figure 5. Beta-cell-specific gene delivery of Manf increased the serum levels of insulin in islets of NOD mice. Serum samples were analysed using Ultra-Sensitive Insulin ELISA kit. Results are presented in means SEM (n = 11–15 / group). Kruskal–Wallis test followed by Dunn's test was performed for multiple comparisons. * denotes $p < 0.05$.

4. Discussion

The Glis3-Manf pathway appears to be an important fulcrum for diabetes development. There are several lines of evidence indicating that GLIS3 is also a key anti-apoptotic mediator in humans. Using in vitro systems, exposure of human islets to certain dietary fats, such as palmitate and oleate, triggers apoptosis of beta-cells [19–22]. This effect is accompanied by a reduction in GLIS3 expression [23,24], and beta-cell apoptosis (in response to palmitate or inflammatory cytokines) is compounded by GLIS3 knockdown [13]. *GLIS3* polymorphism is linked to susceptibility to both T1D and T2D [25–28] as well as rare mutations also causing neonatal diabetes [29], demonstrating that expression variation can modify diabetes risk. In mouse models of beta-cell stress, decreased expression of Glis3 (from heterozygous status, or downstream of high fat diet exposure) sensitised to beta-cell death following islet stress [9].

Manf is a critical survival factor for pancreatic beta-cells, with Manf-deficient mice developing spontaneous diabetes due to beta-cell apoptosis [15]. Manf demonstrates one of the largest increases in expression following induction of the unfolded protein stress response [9], suggesting that it is a programmed stress-response pathway that enables continued survival. The same process is conserved in humans, with the addition of recombinant MANF protecting human pancreatic beta-cells from stress-induced apoptosis [30]. Evidence suggests that effective Manf upregulation during stress requires Glis3 expression. When Glis3 expression is impeded, either through genetic deficiency or diet-induced deficiency, Manf upregulation in response to stress is stunted [9]. Likewise, in human T2D islets, a positive relationship is observed between GLIS3 expression levels and MANF expression levels [9

in a toxin-mediated model [15]. The results from our study demonstrate the ability of vector modification to impart beta-cell protection following intravenous delivery (at a

29. Senée, V.; Chelala, C.; Duchatelet, S.; Feng, D.; Blanc, H.; Cossec, J.C.; Charon, C.; Nicolino, M.; Boileau, P.; Cavener, D.R.; et al. Mutations in GLIS3 are responsible for a rare syndrome with neonatal diabetes mellitus and congenital hypothyroidism. *Nat. Genet.* **2006**, *38*, 682–687. [CrossRef]
30. Hakonen, E.; Chandra, V.; Fogarty, C.L.; Yu, N.Y.L.; Ustinov, J.; Katayama, S.; Galli, E.; Danilova, T.; Lindholm, P.; Vartiainen, A.; et al. MANF protects human pancreatic beta cells against stress-induced cell death. *Diabetologia* **2018**, *61*, 2202–2214. [CrossRef]
31. Airavaara, M.; Chiocco, M.J.; Howard, D.B.; Zuchowski, K.L.; Peränen, J.; Liu, C.; Fang, S.; Hoffer, B.J.; Wang, Y.; Harvey, B.K. Widespread cortical expression of MANF by AAV serotype 7: Localization and protection against ischemic brain injury. *Exp. Neurol.* **2010**, *225*, 104–113. [CrossRef] [PubMed]
32. Xiao, X.; Guo, P.; Shiota, C.; Zhang, T.; Coudriet, G.M.; Fischbach, S.; Prasadan, K.; Fusco, J.; Ramachandran, S.; Witkowski, P.; et al. Endogenous Reprogramming of Alpha Cells into Beta Cells, Induced by Viral Gene Therapy, Reverses Autoimmune Diabetes. *Cell Stem Cell* **2018**, *22*, 78–90.e4. [CrossRef] [PubMed]
33. Tudurí