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ARTICLE

miRNA-30a-5p-mediated silencing of *Beta2/NeuroD* expression is an important initial event of glucotoxicity-induced beta cell dysfunction in rodent models

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Abstract
Aims/hypothesis The loss of beta cell function is a critical factor in the development of type 2 diabetes. Glucotoxicity plays a major role in the progressive deterioration of beta cell function and development of type 2 diabetes mellitus. Here we demonstrate that microRNA (miR)-30a-5p is a key player in early-stage glucotoxicity-induced beta cell dysfunction.
Methods We performed northern blots, RT-PCR and western blots in glucotoxicity-exposed primary rat islets and INS-1 cells. We also measured glucose-stimulated insulin secretion and insulin content. In vivo approaches were used to evaluate the role of miR-30a-5p in beta cell dysfunction.
Results miR-30a-5p expression was increased in beta cells after exposure to glucotoxic conditions, and exogenous miR-30a-5p overexpression also induced beta cell dysfunction in vitro. miR-30a-5p directly suppressed expression of *Beta2/NeuroD* (also known as *Neurod1*) by binding to a specific binding site in its 3′-untranslated region. After restoration of *Beta2/NeuroD* expression by knockdown miR-30a-5p or transfection of the *Beta2/NeuroD* gene, beta cell dysfunction, including decreased insulin content, gene expression and glucose-stimulated insulin secretion, recovered. Glucose tolerance and beta cell dysfunction improved on direct injection of Ad-si30a-5p into the pancreas of diabetic mice.
Conclusions/interpretation Our data demonstrate that miR-30a-5p-mediated direct suppression of *Beta2/NeuroD* gene expression is an important initiation step of glucotoxicity-induced beta cell dysfunction.

Keywords *Beta2/NeuroD* · Beta cell dysfunction · Glucotoxicity · MicroRNA

Abbreviations

Beta2	Beta cell E-box transcription factor
GFP	Green fluorescent protein
GSIS	Glucose-stimulated insulin secretion
IPGTT	Intraperitoneal glucose tolerance test
LCM	Laser-capture microdissection
miRNA	MicroRNA
MOI	Multiplicity of infection
NeuroD	Neuronal differentiation 1
Pdx-1	Pancreatic and duodenal homeobox 1
3′-UTR	3′-Untranslated region

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Introduction

Glucotoxicity is a critical factor in the development and progression of type 2 diabetes [1, 2]. Prolonged exposure of pancreatic beta cells to elevated levels of glucose is associated with inhibition of glucose-induced insulin secretion, impairment of insulin gene expression, and induction of cell death by apoptosis [3]. Several mechanisms have been implicated in these effects of glucotoxicity, including ceramide formation [4], oxidative stress [5] and inflammation [6]. Alterations in the gene expression of pancreas-specific transcription factors, including pancreatic duodenal homeobox 1 (*Pdx-1*), beta cell E-box transcription factor (*Beta2*)/neuronal differentiation 1 (*NeuroD*; also known as *NEUROD1*) and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (avian) (*Mafa*), contribute to the induction of beta cell dysfunction [7–11]. Our group has reported that the suppression of *Beta2/NeuroD* transcription by overexpression of the gene encoding peroxisome proliferator-activated receptor γ-coactivator-1α (*Pgc-1α*; also known as *Ppargc1a*) is an important initial step in beta cell glucotoxicity [12, 13]. However, suppression of *Pgc-1α* is not sufficient to normalise the repressed *Beta2/NeuroD* and insulin gene expression levels. Therefore, our studies have focused on searching for other factors, such as microRNAs (miRNAs), that could regulate *Beta2/NeuroD* gene expression.

miRNAs are 21–23-nucleotide non-coding RNAs that act as post-translational regulators of gene expression [14]. More than 700 miRNAs have been annotated in the human genome thus far [15]. miRNAs base-pair with the 3′-untranslated regions (UTRs) of cognate mRNAs, leading to translational repression and mRNA degradation. miRNAs play a fundamental role in regulation of gene expression, consequently affecting key biological events. Growing evidence indicates that miRNAs are involved in diverse processes, including development, cell differentiation, cell proliferation, apoptosis and metabolism [16]. Several miRNAs are directly involved in diabetes, and the roles of miRNAs in mouse pancreas development, insulin secretion and insulin gene expression have been demonstrated [17, 18]—for example, knockdown of miR-375, an islet miRNA that negatively controls insulin secretion [19]. Mice lacking miR-375 are hyperglycaemic and exhibit an increase in total pancreatic alpha cell numbers, whereas the pancreatic beta cell mass is decreased [20]. Recent studies indicate that, during human pancreatic development, miR-7, miR-9, miR-375 and miR-376 are specific islet miRNAs expressed at high levels [19, 20]. However, it is not yet known whether miRNAs regulate pancreatic beta cell-specific gene expression via pancreatic beta cell dysfunction such as that induced by glucotoxicity. In this study, we demonstrate that miR-30a-5p is an important mediator of glucotoxicity-induced beta cell dysfunction.

Methods

Isolation of rat islets Rat pancreatic islets were isolated from Sprague–Dawley rats (200–230 g) by digesting the pancreatic duct with collagenase P in PBS as previously described [21]. After digestion, the islets were separated with Histopaque-1077 (Sigma, St Louis, MO, USA). The islets were cultured in RPMI 1640 medium containing 10% FBS.

Cell culture and glucotoxic conditions INS-1 cells were maintained in RPMI 1640 medium supplemented with 5.5 mmol/l glucose, 10% FBS, 2-mercaptoethanol and antibiotics. We used this as the low-glucose condition. Exposure to glucotoxic conditions was followed by treatment for 3 days with 33.3 mmol/l glucose in RPMI 1640 containing 10% FBS.

miRNA array hybridisation and data analysis We used an Ncode multi-species microarray from Invitrogen (Carlsbad, CA, USA). Briefly, small RNAs were isolated from rat islets and directly labelled using the Ncode miRNA labelling system. Labelled RNA was then used as a target for on-chip hybridisation assays under optimised conditions. After overnight hybridisation and washing, the microarrays were incubated with signal-amplifying Alexa Fluor 3 and Alexa Fluor 5 capture reagents. The fluorescent signals were then scanned and analysed. tRNA and 5S rRNA were used as loading controls for normalisation.

Northern blotting Total RNA was obtained from glucotoxicity-exposed INS-1 cells using TRIzol Reagent (Invitrogen). Total RNA extract (2 μg) was separated on 15% denaturing polyacrylamide gels and transferred to Hybond-N^+ membranes (Amersham Bioscience, Uppsala, Sweden) using a Trans-blot SD semi-dry transfer cell (Bio-Rad, Reinach, Switzerland). The synthetic RNA probes were labelled with [α-^{32}P]dATP using T4 polynucleotide kinase. The northern blot probes are shown in electronic supplementary material (ESM) Table 1. Prehybridisation and hybridisation were performed at 37°C using Hybridisation buffer (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The U6 level was used as an internal control.

Real-time PCR quantification Total RNA was obtained from rat islets using TRIzol Reagent. cDNA was synthesised using 1 μg total RNA and SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed with SYBR Green (Invitrogen). The products were detected with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). All primer sequences used for PCR are shown in ESM Table 2. The primers were designed to recognise different exons to eliminate possible DNA contamination.

The PCR signal was detected using the MiniOpticon real-time system (Bio-Rad). The data were analysed using Opticon Monitor software. This software determines the mRNA transcript level using the threshold cycle (C_t) method based on measurements of C_t. Finally, the mRNA level of each target gene, which was normalised to β-actin and relative to a calibrator, was calculated with the $2^{-\Delta\Delta C_t}$ method.

Inhibition and overexpression of miRNAs 2-*O*-Methyl oligonucleotides were synthesised by Samchully Pharm (Seoul, Korea). The 2-*O*-methyl antisense oligonucleotides were transfected into INS-1 cells using Lipofectamine Plus (Invitrogen) (ESM Table 3). The small hairpin RNA structure of miR-30a-5p was inserted into the EcoRI/XhoI sites of the pAdTrack-CMV shuttle vector. The recombinants were amplified in HEK-293 cells and isolated and purified using CsCl (Sigma) gradient centrifugation. The titres were determined using Adeno-X Rapid Titer (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocol. INS-1 cells were infected with Ad-si30a-5p at a multiplicity of infection (MOI) of 30. It was sufficient to infect 80% of the cells as determined by fluorescence. The miR-30a RNA precursor (pre-miR-30a) was purchased from Applied Biosystems (Carlsbad, CA, USA).

Glucose-stimulated insulin secretion (GSIS) and insulin content Isolated rat islets were incubated under normal (5.5 mmol/l glucose) and glucotoxic conditions with or without infection with an MOI of 50 for Ad-green fluorescent protein (GFP) or Ad-si30a-5p for 3 days. Cultured islets were washed in KRB (130 mmol/l NaCl, 3.6 mmol/l KCl, 1.5 mmol/l $CaCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2PO_4, 2.0 mmol/l $NaHCO_3$ and 10 mmol/l Hepes) and incubated in KRB containing 5.5 mmol/l glucose for 1 h. The islets were then stimulated for 1 h in KRB containing 25 mmol/l glucose. Insulin concentrations were measured with an RIA kit (Linco, St Charles, MO, USA). According to the method described by Hamid et al [22], the total intracellular insulin content was extracted by the acid/ethanol method. Briefly, islets were incubated in 1% HCl (ethanol/H_2O_2/HCl, 14:57:3, by vol.) overnight at 4°C. The insulin in the supernatant fraction was detected by RIA and normalised to total protein content by the Bradford assay.

Transfection and western blotting Glucotoxicity-exposed INS-1 cells were transfected with pEGFP-NeuroD (773–2458) and pEGFP-NeuroD (773–1147). The miR-30a-5p-binding site within the *Beta2/NeuroD* 3′-UTR was mutagenised using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). For mutagenesis of the miR-30a-5p-binding site, the forward primer was 5′-TTTCAGTGACTGTCGTATTTATAGAAGGCAGCC TTTTTGC-3′, and the reverse primer was 5′-GCAAAAAGG CTGCCTTCTATAAATACGACAGTCACTGAAA-3′. The INS-1 cells were lysed in radio-immunoprecipitation (RIPA) buffer (Roche, Mannheim, Germany). Usually 40 μg whole protein extract was subjected to 10% SDS-PAGE. The proteins on the gel were electrophoretically transferred to Immobilon-P Teflon membranes (Millipore, Bedford, MA, USA), and western analysis was performed using specific antibodies against GFP and β-actin (Sigma). Proteins were visualised using an enhanced chemiluminescence kit according to the manufacturer's recommendations.

Type 2 diabetes animal model The *db*/*db* mice were 49 days old and ~37 g at the initiation of experiments, and groups were balanced for age and sex. All procedures were pre-approved by the Institutional Animal Care and Use Committee.

In vivo study The *db*/*db* mice were anaesthetised with ketamine and Rompun (5:1, vol./vol.) and treated with 1×10^9 plaque-forming units of Ad-si30a-5p or Ad-GFP adenovirus and 100 μl 90% NaCl by systemic injection into the coeliac artery with a 26 G needle. The suture was removed after 10 min. Their non-fasting glucose concentration and body weight were measured every other day, and they were killed after 21 days.

Intraperitoneal glucose tolerance test (IPGTT) Pancreatectomised mice were bled after overnight fasting to establish basal metabolite concentrations. They were then injected i.p. with a 20% glucose solution at a volume that was calculated to deliver a glucose dose equivalent to 2 g/kg body weight. Blood samples were taken 30, 60, 90 and 120 min after injection. Blood glucose was measured immediately after sampling using a glucometer (Roche), and the area under the glucose curve was calculated.

Laser-capture microdissection (LCM) Immediately before LCM, frozen pancreatic sections were dehydrated and air-dried, as previously described [23]. LCM was performed, using a PixCell II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA, USA), by melting thermoplastic films mounted on transparent LCM caps (Arcturus) on selected cell populations. For each islet, there may have been two to four clumps of bright cells resulting in approximately 20–50 pulses per islet or approximately 200–250 pulses per section. Thus, 20 sections were used to obtain at least 5,000 pulses, which were needed to obtain sufficient RNA for the arrays.

RNA amplification Total RNA was extracted, followed by RNA cleanup using the Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA was extracted from captured cells using the PicoPure RNA Isolation Kit (Arcturus). Total RNA amplification was performed using RiboAmp HS RNA

Amplification Kits (Arcturus) for laser-captured cells [23]. RNA products were purified using MiraCol Purification Columns (Arcturus). cDNA templates were synthesised from total RNA and used for SYBR Green RT-PCR.

Statistical analysis The results are presented as mean±SEM from at least three independent experiments. Analysis of variance was used to compare groups. SAS software (release 8.12; SAS Institute, Cary, NC, USA) was used. A null-hypothesis probability <0.05 was considered significant.

Results

Identification of rat miRNAs that induce glucotoxicity in pancreatic beta cells To investigate the effect of glucotoxicity on beta cell dysfunction, isolated islets were incubated in the presence of a high concentration of glucose (33.3 mmol/l) for 3 days [12]. The glucotoxic condition has been associated with a gradual increase in beta cell death in a time-dependent manner. When the islets were exposed to glucotoxicity for 3 days, insulin gene expression was suppressed (Fig. 1a). We also measured GSIS in a time-dependent manner compared with the low glucose condition. As a result, GSIS was blocked by glucotoxicity (Fig. 1b). Therefore, prolonged exposure to high glucose has a deleterious effect on pancreatic beta cell function.

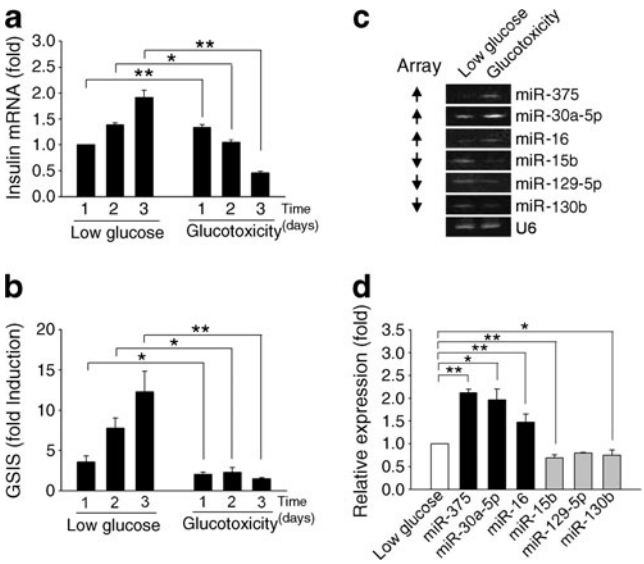

Fig. 1 Identification of rat miRNAs that induce glucotoxicity in pancreatic beta cells. (**a**) Expression of the insulin gene decreased under glucotoxic conditions in a time-dependent manner. The mRNA levels of insulin genes were normalised to glyceraldehyde-3-phosphate dehydrogenase mRNA, and the results were obtained from four independent experiments (mean±SEM). (**b**) Isolated rat islets were incubated under normal and glucotoxic conditions for 3 days. Insulin secretion was quantified by RIA, as described in the Methods section (n=10, *p<0.05, **p<0.01). (**c**) Expression of glucotoxicity-regulated miRNAs from the array data was validated by northern blot. All northern blots were from the INS-1 cell line. (**d**) miRNA band intensities were quantified and normalised to the intensity of U6 (n=3). Northern blot data were summarised as mean±SEM relative to those of the untreated islets (*p<0.05, **p<0.01)

To determine the profile of functional miRNAs in glucotoxicity and to identify novel miRNAs, we used miRNA chip analysis. The miRNA chip analysis data were used to select candidate miRNAs that showed statistically significant changes in expression. We identified 39 different miRNAs: 28 were significantly upregulated and 11 were downregulated during glucotoxicity. We also identified functional miRNAs using the www.targetscan.org and www.pictar.mdc-berlin.de websites (ESM Fig. 1a). Of the 39 selected miRNAs, 16 were potentially related to glucotoxicity. To investigate the effects of changes in miRNA expression in an in vitro system, we used northern blots to measure their expression in the INS-1 cell line. We noticed a discrepancy between the northern blot and chip analyses for some miRNAs (ESM Fig. 1b). Under glucotoxic conditions, expression of miR-129-5p, miR-15b and miR-130b decreased, and expression of miR-375, miR-16 and miR-30a-5p increased (Fig. 1c,d). To investigate which of the glucotoxicity-induced candidate miRNAs regulate the expression of pancreatic beta cell-specific genes, we constructed antisense oligonucleotides against the candidate miRNAs. We transfected the candidate antisense miRNAs into glucotoxicity-stimulated INS-1 cells and performed real-time PCR (Fig. 2a–c). On the basis of our data, we searched for miRNAs that altered *Beta2/NeuroD* and *Pgc-1α* gene expression when knocked down. Glucotoxicity-induced repression of insulin and *Beta2/NeuroD* gene expression was significantly rescued by antisense miR-30a-5p (Fig. 2a,b), whereas *Pgc-1α* expression was not (Fig. 2c). In addition, expressions of insulin, *Beta2/NeuroD* and *Pgc-1α* were similar in the glucolipotoxic condition and in cells transfected with miR-15b, miR-129-5p and miR-130b antisense constructs (ESM Fig. 2). Under normal conditions, induction of miR-30a-5p using an miR-30a precursor (pre-miR-30a) repressed expression of insulin and *Beta2/NeuroD* genes (Fig. 2d,e). However, expression of the *Pgc-1α* gene was increased by glucotoxicity alone. Induction of miR-30a-5p did not increase *Pgc-1α* gene expression (Fig. 2f).

miR-30a-5p directly binds to the Beta2/NeuroD 3′-UTR We considered it unlikely that miR-30a-5p targets both insulin and *Beta2/NeuroD* genes directly because the insulin mRNA 3′-UTR did not contain any TargetScan-predicted miR-30a-5p-binding sites (www.targetscan.org). In contrast, we found that the *Beta2/NeuroD* mRNA had a predicted target site in its 3′-UTR (Fig. 3a). To further demonstrate the role of miR-30a-5p in pancreatic beta cell function, we constructed the vector Ad-si30a-5p using the AdEasy adenoviral vector system to inhibit miR-30a-5p and confirmed

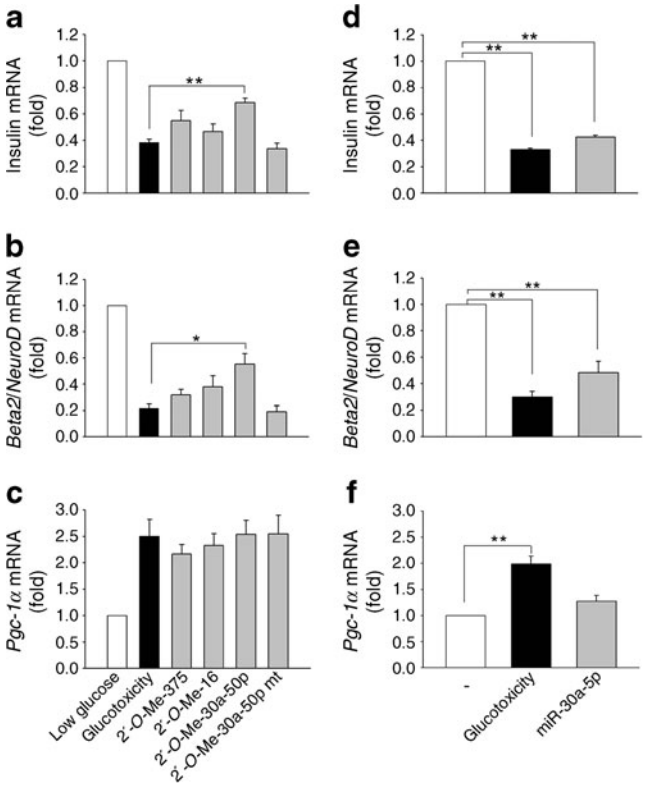

Fig. 2 Expression of beta cell-specific genes induced by miR-30a-5p. (**a**–**c**) Effects of 2′-*O*-methyl (2′-*O*-Me) oligonucleotide complementary miRNAs on insulin, *Beta2/NeuroD* and *Pgc-1α* expression were estimated by real-time quantitative RT-PCR in glucotoxic conditions for 3 days. The C_t values are normalised to β-actin within the same cDNA sample (n=4, mean±SEM, *p<0.05, **p<0.01). (**d**–**f**) Using pre-miR-30a, we observed expression of insulin, *Beta2/NeuroD* and *Pgc-1α* (n=4, mean±SEM, *p<0.05, **p<0.01)

the effect by northern blot (Fig. 3b). Insulin gene transcription is mediated by pancreatic beta cell-specific factors, including Pdx-1, Beta2/NeuroD and Pgc-1α [8, 24]. To investigate whether miR-30a-5p can bind to the 3′-UTR of *Beta2/NeuroD*, we transfected with a GFP-*Beta2/NeuroD* construct and an antisense oligonucleotide against miR-30a-5p in INS-1 cells and observed the effect of miR-30a-5p inhibition on the production of the exogenous GFP-tagged protein (Fig. 3c). In a western blot using an antibody against GFP, we observed that the glucotoxicity-induced repression of *Beta2/NeuroD* level was prevented by the antisense miR-30a-5p. However, the *Beta2/NeuroD* sequence lacking the 3′-UTR and one containing a mutation in the predicted miR-30a-5p target site of the 3′-UTR were not affected (Fig. 3d). The level of endogenous Beta2/NeuroD protein was decreased by glucotoxicity but increased by antisense miR-30a-5p (Fig. 3e).

miR-30a-5p reduces insulin secretion and insulin content We overexpressed or inhibited miR-30a-5p in the islets and measured GSIS and insulin content (Fig. 4a). Under normal conditions, a marked increase in GSIS was observed. However, when the islets were exposed to glucotoxic conditions, the increase in GSIS was blunted. The decreased GSIS was partially recovered by the suppression of miR-30a-5p using Ad-si30a-5p overexpression (Fig. 4a). In contrast, pre-miR-30a overexpression significantly repressed GSIS (Fig. 4b,c).

Effect of miR-30a-5p in db/db mice Next, we examined the function of miR-30a-5p in an in vivo model. Seven-week-old *db*/*db* mice were randomly allocated to control (Ad-GFP-injected) and Ad-si30a-5p-injected groups under non-fasting glucose conditions. At 18 days after injection, we confirmed the miR-30a-5p expression levels in various tissues of the Ad-GFP- and Ad-si30a-5p-injected groups. Interestingly, miR-30a-5p expression in the pancreas was increased in the *db*/*db* compared with the normal *db/dm* mice. Expression of miR-30a-5p was decreased in the pancreas of the Ad-si30a-5p-injected *db*/*db* mice, but its expression was unchanged in other tissues (Fig. 5a). In the Ad-si30a-5p-injected group, insulin and *Beta2/NeuroD* staining was intense and uniform for a large proportion of the islet cells compared with the control group (Fig. 5b). We measured the mRNA levels of insulin and *Beta2/NeuroD* in the Ad-GFP- and Ad-si30a-5p-injected islets using LCM (see Methods for details). Insulin and *Beta2/NeuroD* mRNA expression in the islets of the *db*/*db* mice was increased by Ad-si30a-5p injection (Fig. 5c). We performed an IPGTT and stained the pancreatic tissue for insulin and *Beta2/NeuroD*. Body weight did not differ between the Ad-si30a-5p-injected and control *db*/*db* mice (Fig. 6a). The Ad-si30a-5p-injected group had lower non-fasting glucose than the control mice on days 5 to 18 (Fig. 6b). Moreover, the mean area under the glucose curve during IPGTT was lower in the Ad-si30a-5p-injected group than in the control group (Fig. 6c,d).

Discussion

This study demonstrates that miR-30a-5p is an important mediator of glucotoxicity-induced beta cell dysfunction. It is now clear that miR-30a-5p directly suppresses *Beta2/NeuroD* gene expression, thereby inducing pancreatic beta cell dysfunction.

Pancreatic beta cell-specific insulin gene expression is regulated by a variety of pancreatic transcription factors. *Pdx-1* (also known as *Idx-1/Stf-1/Ipf1*), *Beta2/NeuroD* and *Mafa* are upregulated in tandem under high-glucose conditions. *Pdx-1* plays an important role in pancreas development, beta cell differentiation, and the maintenance of mature beta cell function [25]. *Beta2/NeuroD* binds to the E element of the insulin gene [26] and modulates the expression of genes such as that for SUR1, which forms K^+ channels with Kir6.2, to regulate insulin secretion [27]. MAFA functions as a potent

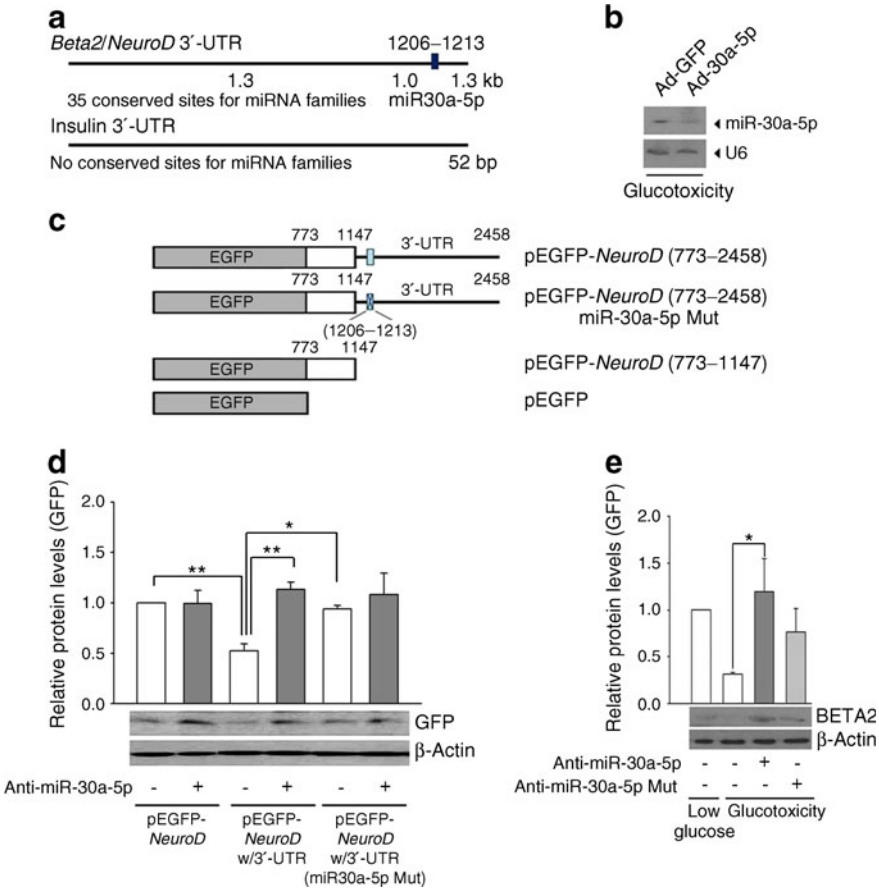

Fig. 3 miR-30a-5p directly targets *Beta2/NeuroD* 3′-UTR. (**a**) The 3′-UTR of *Beta2/NeuroD* has one putative binding site for miR-30a-5p, but insulin does not. (**b**) We constructed Ad-si30a-5p using the AdEasy adenoviral vector system for the inhibition of miR-30a-5p and confirmed this inhibition by a northern blot (n=3, mean±SEM, *p<0.05, **p<0.01). (**c**) Blue indicates a potential binding site (1206–1213) within the *Beta2/NeuroD* 3′-UTR. Glucotoxicity-exposed INS-1 cells were transfected with GFP- *Beta2/NeuroD* or GFP- *Beta2/NeuroD* with a point mutation (Mut) in the putative miR-30a-5p-recognition site and co-transfected with antisense miR-30a-5p. (**d**) We performed western blotting and used the relative expression values detected with the GFP antibody to measure exogenous Beta2/NeuroD production. (**e**) To measure endogenous Beta2/NeuroD production, we used Beta2 antibody (n=3, mean± SEM, *p<0.05, **p<0.01)

transactivator of the insulin gene [28]. Forkhead box (FOX) A2 (hepatocyte nuclear factor 3β) binds to the *Pdx-1* promoter to positively regulate Pdx-1 gene expression [29]. In short, the upregulation of *Mafa*, together with Pdx-1 and *Beta2/NeuroD*, markedly induces insulin biosynthesis and regulates insulin-related gene expression to enhance insulin secretion in response to high glucose levels. Recent studies in pancreatic tissue have demonstrated that miRNA modulates insulin secretion-related genes. A number of miRNAs, including miR-24/26/182/148, are also positive regulators of insulin transcription [30]. Overexpression of miR-30d prevents downregulation of both *Mafa* and *IRS2* [31]. Furthermore, *Foxa2* was identified as a direct target of miR-124a, and target genes downstream of *Foxa2*, including those for the ATP-sensitive K^+ channel subunits Kir6.2 and SUR-1 and the transcription factor Pdx-1, were downregulated by miR-124a. miR-130a, miR-200 and miR-410 were also reported to be involved in the regulation of insulin secretion [32]. miR-29a and miR-29b contributed to the pancreatic beta cell-specific silencing of monocarboxylate transporter 1 [33]. More recently, Bagge et al [34] demonstrated that glucose-induced upregulation of miR-29a reduced GSIS. He et al [35]

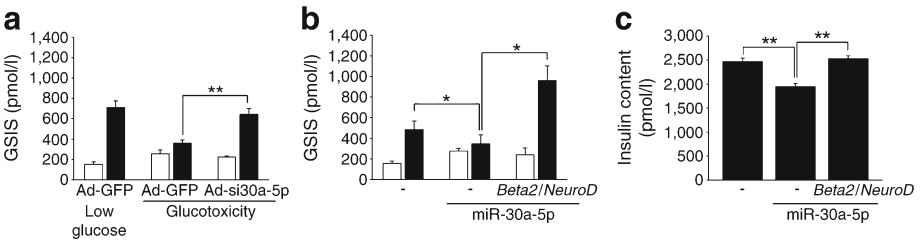

Fig. 4 Supression of miR-30a-5p increased the insulin secretion and insulin content (**a**–**c**). To analyse the function of miR-30a-5p, we measured GSIS and insulin content under conditions of glucotoxicity or suppression of miR-30a-5p using Ad-si30a-5p after overexpression of *Beta2/NeuroD* in primary rat islets. We used 10 islets in each group and obtained material from 10 independent experiments (n=10, mean± SEM, *p<0.05, **p<0.01). White bars, 5.5 mmol/l glucose; black bars, 25 mmol/l glucose

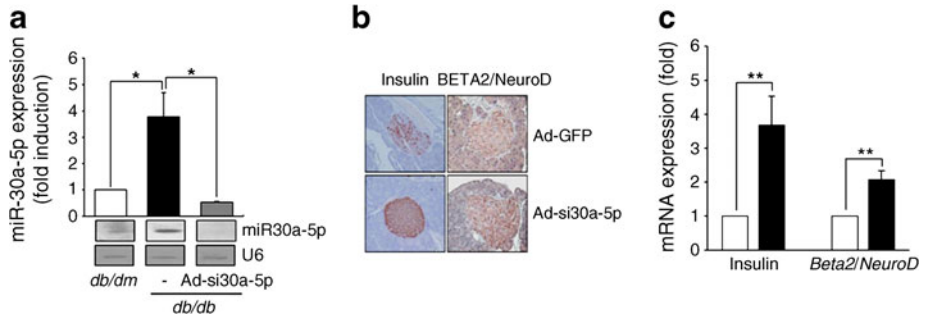

Fig. 5 Levels of insulin and Beta2/NeuroD were increased by Ad-si30a-5p. Seven-week-old *db/db* mice were randomly allocated to an Ad-GFP-injected control group (n=10) or an Ad-si30a-5p-injected group (n=11) according to non-fasting glucose concentration. The *db/db* mice were subjected to systemic injection of Ad-si30a-5p (1×10^9 plaque-forming units) into the coeliac artery. We used the coeliac artery to deliver Ad-si30a-5p directly to the pancreas. (**a**) At 18 days after injection, we performed northern blots on six tissue samples of the *db*/*db* mice. We used wild-type C57BL/6 mice as the control (n=5 tissue samples for each group). (**b**) Immunohistochemical changes in insulin and Beta2/NeuroD production in the pancreatic islets of the Ad-GFP- and Ad-si30a-5p-injected groups. (**c**) To investigate changes in expression of the insulin and *Beta2/NeuroD* genes in an in vivo system, we obtained islets from the Ad-GFP- and Ad-si30a-5p-injected groups using LCM and performed real-time PCR (n=10, mean±SEM, *p< 0.05, **p<0.01). White bars, Ad-GFP injection; black bars, Ad-si30a-5p injection

showed that miR-29a upregulation in several cell types, including adipocytes, caused insulin resistance. It is possible that miR-29a upregulation promotes development of type 2 diabetes by decreasing insulin secretion in beta cells and via peripheral insulin resistance. The studies described above used molecular tools to reduce the expression of specific miRNAs in an attempt to highlight their potential involvement in the control of pancreatic beta cell function.

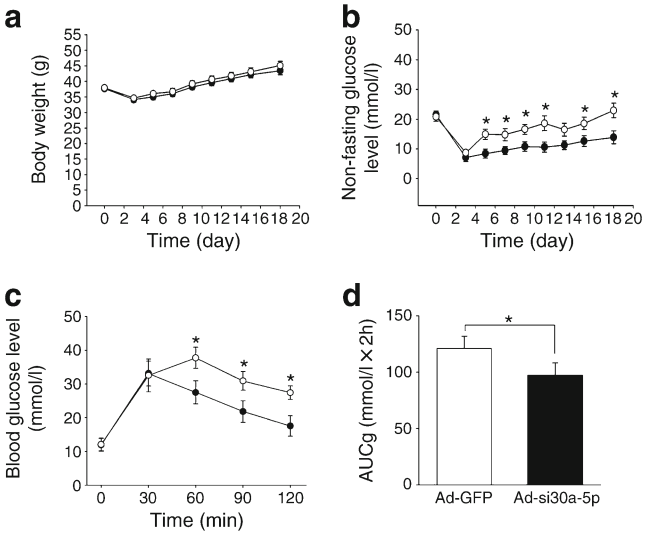

Fig. 6 Suppression of miR-30a-5p protects against beta cell dysfunction in *db/db* mice. Mice were treated as in Fig. 5. (**a**, **b**) Body weights and morning non-fasting glucose values were measured for 18 days. (**c**, **d**) At 12 days after adenovirus injection, the diabetic rats were injected i.p. with 20% glucose. Blood samples were collected at 30, 60, 90 and 120 min after the injection. We then performed an IPGTT and measured the area under the glucose curve (AUCg) (mean±SEM, *p<0.05). White circles, Ad-GFP injection; black circles, Ad-si30a-5p injection

Furthermore, studies investigating the modulation of miRNA, which plays an essential role in diabetes-related gene expression and insulin secretion, are important for establishing successful diabetes management strategies.

Using miRNA chip and northern blot analyses in beta cells, we found six miRNAs (three upregulated and three downregulated) that exhibited changes in expression after 3 days of exposure to glucotoxic conditions. miR-375 downregulates insulin exocytosis by targeting myotrophin and inhibiting insulin secretion, and miR-15b and miR-16 have been proposed to be involved in post-transcriptional regulation of neurogenin 3 [36]. miR-129-5p is involved in the antitumour activity of histone deacetylase inhibitors, highlighting the existence of an miRNA-driven cell death mechanism [37]. Meanwhile, miR-130 strongly affects adipocyte differentiation: its overexpression impairs adipogenesis, whereas its downregulation enhances adipogenesis [38]. miR-30 family proteins function in the regulatory signalling events that are involved in the cellular response of pancreatic epithelial cells during epithelial–mesenchymal transition [39, 40]. However, relationships between *Beta2/NeuroD* gene expression and these miRNAs have never been described. In our study, we focused on miRNAs that may influence *Beta2/NeuroD*, insulin and *Pgc-1α* gene expression. We examined the effects of the three upregulated miRNAs on the expression of insulin and *Beta2/NeuroD* and the effects of the three downregulated miRNAs on the expression of *Pgc-1α* using antisense miRNAs. *Pgc-1α* was expressed at similar levels under glucotoxic conditions and upon transfection with miR-15b, miR-129-5p and miR-130b antisense constructs (ESM Fig. 2). The experiments presented here show that miR-30a-5p specifically regulates the expression of the insulin and *Beta2/NeuroD* genes but not *Pgc-1α* .The glucotoxicity-induced repression of insulin

and *Beta2/NeuroD* gene expression was very weakly increased by antisense miR-375 and miR-16, although this difference was not statistically significant. We hypothesise that the weak induction of expression might be an indirect effect of other endogenous factors in glucotoxicity-induced beta cells. We also confirmed that the insulin and *Beta2/NeuroD* 3′-UTRs did not contain any miR-375 or miR-16 binding sites, as predicted by TargetScan. Thus, miR-375 and miR-16 have no effect on the regulation of insulin or *Beta2/NeuroD* genes in response to glucotoxicity. Moreover, we confirmed that miR-30a-5p was expressed in the pancreas, liver, muscle, brain, spleen and fat of C57BL/6 and *db/db* mice (ESM Fig. 3). Interestingly, expression of miR-30a-5p was altered only in the pancreas of diabetic mice, providing evidence that miR-30a-5p has an important function under diabetic conditions.

We found that the 3′-UTR of *Beta2/NeuroD* contained an miR-30a-5p-binding site and that miR-30a-5p negatively regulated *Beta2/NeuroD* expression through direct base-pairing with the 3′-UTR. Moreover, the decrease in GSIS during glucotoxicity was partially prevented by suppression of miR-30a-5p. Consistent with this finding, the pre-miR-30a-induced decreases in GSIS and insulin content were completely restored by overexpression of *Beta2/NeuroD*. These results indicate that miR-30a-5p is specifically involved in insulin secretion and that *Beta2/NeuroD* plays a key role in the regulation of pancreatic beta cell dysfunction during glucotoxicity.

We then followed up on our in vitro data with an in vivo study. We used Ad-si30a-5p to inhibit miR-30a-5p in *db/db* mice, in which insulin resistance is the principal symptom of diabetes [41]. Ad-GFP or Ad-si30a-5p was delivered directly to the pancreas via the coeliac artery. Trypan blue injection was used to confirm the success of the delivery by staining the affected area; the cranial portion of the pancreas, a portion of the stomach, and a portion of the duodenum, but not the liver, were stained [12]. Induction of miR-30a-5p expression in *db/db* mice increased mRNA and protein levels of *Beta2/NeuroD* and insulin, whereas inhibition of miR-30a-5p by Ad-si30a-5p normalised mRNA and protein levels. Moreover, the observed blood glucose levels were significantly lower in Ad-si30a-5p-injected *db/db* mice than in Ad-GFP-injected *db/db* mice. These results clearly indicate that inhibition of miR-30a-5p by Ad-si30a-5p improves glucose tolerance, normalises insulin and Beta2/NeuroD protein levels, and has a protective effect on pancreatic islets in type 2 diabetes.

In conclusion, these findings suggest that expression of miR-30a-5p and *Pgc-1α*: (1) is an early event in glucotoxicity; and (2) involves two independent pathways that are regulated by *Beta2/NeuroD*. Therefore, miRNAs may constitute novel pharmacological targets for the treatment of diabetes. Moreover, the sequence of the human miR-30a-5p gene is similar to that of rat miR-30a-5p, suggesting that our findings may have important clinical implications. Finally, many miRNAs in pancreatic beta cells are involved in regulating the expression of beta cell-specific transcription factors such as *Pdx-1*, neurogenin 3 and MAFA. Therefore, identification of multiple miRNA-regulating systems is a valuable goal.

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Contribution statement J-WK, Y-HY, SJ, HS-K, I-KL, J-HC and K-HY designed and performed the studies and analysed data. K-HY directed the study, interpreted the data, and wrote the paper. All authors critically revised and approved the final version.

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