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ARTICLE

Differential expression and release of exosomal miRNAs by human islets under inflammatory and hypoxic stress

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Abstract

Aims/hypothesis Pancreatic islets produce non-coding microRNAs (miRNAs) that regulate islet cell function and survival. Our earlier investigations revealed that human islets undergo significant damage due to various types of stresses following transplantation and release miRNAs. Here, we sought to identify and validate exosomal miRNAs (exo-miRNAs) produced by human islets under conditions of cellular stress, preceding loss of cell function and death. We also aimed to identify islet stress signalling pathways targeted by exo-miRNAs to elucidate potential regulatory roles in islet cell stress.

Methods Human islets were subjected to proinflammatory cytokine and hypoxic cell stress and miRNA from exosomes was isolated for RNA sequencing and analysis. Stress-induced exo-miRNAs were evaluated for kinetics of expression and release by intact islets for up to 48 h exposure to cytokines and hypoxia. A subset of stress-induced exo-miRNAs were assessed for recovery and detection as biomarkers of islet cell stress in a diabetic nude mouse xenotransplant model and in patients undergoing total pancreatectomy with islet auto-transplantation (TPIAT). Genes and signalling pathways targeted by stress-induced exo-miRNAs were identified by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and direct interactions of miRNAs with downstream signalling targets were validated in human islet cells using the miRNA Tests for Read Analysis and Prediction (MirTrap) system.

Results Global exo-miRNA sequencing revealed that 879 miRNA species were released from human islets and 190 islet exo-miRNAs were differentially expressed in response to proinflammatory cytokines, hypoxia or both. Release of exo-miRNAs hsa-miR-29b-3p and hsa-miR-216a-5p was detected within 6 h of exposure to cytokines and hypoxia. The remaining subset of stress-induced exo-miRNAs, including hsa-miR-148a-3p and islet cell damage marker hsa-miR-375, showed delayed release at 24–48 h, correlating with apoptosis and cell death. Stress and damage exo-miRNAs were significantly elevated in the circulation in human-to-mouse xenotransplant models and in human transplant recipients. Elevated blood exo-miRNAs negatively correlated with post-transplant islet function based on comparisons of stress and damage exo-miRNA indices with Secretory Unit of Islet Transplant Objects (SUITO) indices. KEGG analysis and further validation of exo-miRNA targets by MirTrap analysis revealed significant enrichment of islet mRNAs involved in phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase signalling pathways.

Conclusions/interpretation The study identifies exo-miRNAs differentially expressed and released by islets in response to damage and stress. These exo-miRNAs could serve as potential biomarkers for assessing islet damage and predicting outcomes in islet transplantation. Notably, exo-miRNAs 29b-3p and 216a-5p could be detected in islets prior to damage-released miRNAs and indicators of cellular apoptosis and death. Thus, these stress-induced exo-miRNAs may have potential diagnostic value for

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Research in context

What is already known about this subject?

- Currently known non-invasive biomarkers of islet beta cell dysfunction, including circulating proinsulin, C-peptide, glucose tolerance tests, SUITO index and HbA_{1c}, do not accurately detect beta cell stress in the prediabetic state or post islet transplantation
- Beta cell-specific miRNA 375 can be used to detect beta cell damage and loss of islet cell mass
- Exosomal miRNAs (exo-miRNAs) play a role in cell communication and adaptation and can be recovered and detected in the circulation at relatively low concentrations

What is the key question?

- What exo-miRNAs are produced by human islets during stress conditions before loss of function and cell death?

What are the new findings?

- One hundred and ninety islet exo-miRNAs are differentially expressed and released in response to proinflammatory cytokines and hypoxic stress
- A subset of exo-miRNAs is released by islets in a stress-specific manner and may provide molecular signatures that can be used as diagnostic biomarkers of islet cell stress preceding islet cell damage and loss of islet function
- Stress-induced islet exo-miRNAs target genes in the PI3K/Akt and mitogen-activated protein kinase (MAPK) signalling pathways

How might this impact on clinical practice in the foreseeable future?

- Stress-selective exo-miRNAs may have potential use as diagnostic biomarkers of islet cell stress in a prediabetic state to allow therapeutic interventions prior to loss of islet cell mass and function

detecting early islet stress prior to progressive loss of islet cell mass and function. Further investigations are warranted to investigate the utility of these exo-miRNAs as early indicators of islet cell stress during prediabetic conditions.

Keywords Cellular stress · Exosomal miRNA · Islet cell damage · Islet transplantation · PI3K–Akt signalling pathway

Abbreviations

BAD	BCL2-associated death promoter
Casp-3	Caspase-3
CC	Cytokine cocktail
c-Casp-3	Cleaved Casp-3
CHOP	CCAAT-enhancer-binding protein homologous protein
DMI	Damage miRNA index
D-miR	Damaged-induced exo-miRNA
ER	Endoplasmic reticulum
exo-miRNA	Exosomal miRNA
FDA	Fluorescein diacetate
FOXO1	Forkhead box protein O1
HIF-1α	Hypoxia inducible factor 1α
Hsp70	Heat-shock protein 70
HYP	Hypoxia (experimental treatment)
IEQ	Islet equivalent
IRE	Inositol-requiring enzyme
KEGG	Kyoto Encyclopedia of Genes and Genomes
miRNA	MicroRNA
MirTrap	miRNA Tests for Read Analysis and Prediction
mTOR	Mammalian target of rapamycin
NTA	Nanoparticle tracking analysis
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative PCR
SMI	Stress miRNA index
S-miR	Stress-induced exo-miRNA
STZ	Streptozotocin
SUITO	Secretory Unit of Islet Transplant Objects
TEM	Transmission electron microscopy
TPIAT	Total pancreatectomy with islet auto-transplantation
XBP1	X-box binding protein 1 isoform

Introduction

Pancreatic islets express inflammatory mediators such as chemokine (C-C motif) ligand 2 (CCL2), C-X-C motif chemokine 10 (CXCL10), IL-1β, TNF-α and IL-6 in response to

metabolic, inflammatory, oxidative and hypoxic stress signals to adapt, repair or restore islet function [1–5]. These inflammatory mediators have been observed in islets under conditions of type 1 and type 2 diabetes as well as during islet transplantation [1, 2, 6]. Currently known non-invasive biomarkers of islet beta cell dysfunction include circulating proinsulin, C-peptide, glucose tolerance tests and HbA_{1c}. However, these biomarkers are limited to detection of beta cell dysfunction at or after diagnosis of diabetes when islet cell function has already been significantly depleted. As yet, there are no reliable methods to detect islet cell stress during transplantation and in the prediabetic state prior to loss of islet cell mass and function. Thus, molecules specifically expressed in islets during cellular stress may qualify as stress-selective biomarkers to track the status of islets during prediabetic conditions. Such investigations would be useful for identifying timely interventions to preserve islet function after transplantation and in the prediabetic state.

Islet microRNAs (miRNAs) are known to play important roles in the regulation of islet function and survival [7–10]. Indeed, multiple miRNAs are associated with type 1 and 2 diabetes [11]. In islet transplantation, islets are subjected to stresses during procurement, isolation and engraftment. Upon transplantation, islets release miRNAs that are detected in the circulation [12, 13]. These miRNA species are packed in exosomes that confer resistance to degradation. Exosomes, ~100–200 nm in diameter, play important roles in cell communication and transfer of molecular species between cells. Thus, plasma-derived exosomal miRNAs (exo-miRNAs) are attractive candidates in the search for non-invasive biomarkers of islet cell stress and may enable tracking early progression of disease by stage-specific molecular signatures.

Several miRNAs correlate with islet damage and transplant outcomes. Notably, elevated hsa-miR-375 in the transplant medium and in sera of islet transplant recipients correlates with lower islet yield and poor transplant outcomes, respectively [12–14]. Elevated hsa-miR-200c is a predictive biomarker of endocrine outcome 1 year after islet autotransplantation [11]. Although hsa-miR-375 is a beta cell-specific biomarker that is sensitive and reliable for monitoring islet cell damage, it is limited to islets that have already been extensively damaged. In this study, we sought to identify and validate miRNAs induced in response to cellular stress before cells enter apoptosis using ex vivo studies and in vivo studies in mouse models of islet transplantation and during islet infusion in patients undergoing total pancreatectomy with islet auto-transplantation (TPIAT). Further analyses of downstream miRNA targets were performed and validated by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and the miRNA Tests for Read Analysis and Prediction (MirTrap) system.

Methods

Human islets For ex vivo studies, human islets were procured from the Integrated Islet Distribution Program (City of Hope, Los Angeles, CA, USA). Blood samples were also tested from six patients admitted for TPIAT at Baylor University Medical Center. Islets were isolated from the pancreas (procured after total pancreatectomy) as described previously (see electronic supplementary material [ESM] Method 1) [12]. Islets were cultured in CMRL 1066 medium containing 10% exosome-depleted FBS, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. For studies using cytokines and hypoxia, the islets were treated with pro-inflammatory cytokine cocktail (CC) containing IL-1β (100 U/ml), TNF-α (1000 U/ml) and IFN-γ (1000 U/ml) and exposed to hypoxia (HYP; 1% O_2, 5% CO_2 and 94% N_2) for 6, 12 and 24 h ex vivo. Viability of islets was determined using propidium iodide (PI)/fluorescein diacetate (FDA) staining. All human islet investigations were approved by the institutional review board.

Animal studies Male nude mice (NU/J-*Foxn1*^{nu}, The Jackson Laboratory, Sacramento, CA, USA) aged 8 weeks, $n = 5$ in streptozotocin (STZ)-induced diabetic control, and kidney capsule and intraportal transplant groups, were housed individually with a 12 h dark–light cycle and fed standard rodent diet ad libitum. Mice were fasted overnight and dosed with STZ (160 mg/kg). Diabetes was established after two consecutive blood glucose measurements >22.2 mmol/l. Human islets (2500 islet equivalents [IEQs], cultured routinely at 37°C, with 5% CO_2 before transplantation) were transplanted under the kidney capsule or intraportally. Blood plasma samples were collected 24 h post transplantation and stored at −80°C until further analyses. The human islet grafts were then resected from the kidney capsule for gene expression analyses using quantitative PCR (qPCR; see ESM Method 2). All animal procedures followed protocols of the institutional animal care and use committee.

Isolation and characterisation of exosomes Exosomes were isolated using the miRCURY exosome isolation kit (Exiqon, Woburn, MA, USA). Briefly, plasma or islet culture supernatant fraction was centrifuged at 10,000 or 3000 *g* for 5 min and exosomes were precipitated using precipitation buffer overnight at 4°C, followed by centrifugation at 10,000 *g* at 20°C. Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) analyses were performed to characterise exosomes (see ESM Method 3).

Exosomal miRNA transcriptome analysis Total RNA was extracted from exosomes using the miRCURY RNA isolation kit (Exiqon) following manufacturer's instructions. Exosomal miRNA (exo-miRNA) expression was analysed using LNA-

based miRNA primers (Exiqon) and miRCURY Universal RT-PCR (Exiqon). For miRNA transcriptome analysis, a multiplex miRNA transcriptome library was constructed using a TruSeq RNA Library Prep Kit (Illumina, San Diego, CA, USA) (see ESM Method 4, 5).

Immunoblotting Expression of inositol-requiring enzyme (IRE-1α), X-box binding protein 1 isoform (XBP1), hypoxia inducible factor 1α (HIF-1α), CCAAT-enhancer-binding protein homologous protein (CHOP), caspase-3 (Casp-3), cleaved Casp-3 (c-Casp-3), CD9 and β-actin in exosomes or islet extracts was determined using standard immunoblotting protocol (see ESM Method 6 for details of methods and antibodies).

Plasma exo-miRNA analyses during TPIAT During TPIAT, blood samples were collected 1 h before islet infusion, during islet infusion, upon completion of islet infusion and at 6 h, 1 day and 7 days after completion of islet infusion. After transplantation, patients were followed up to 1 year to monitor islet graft function using C-peptide, HbA_{1c} and Secretory Unit of Islet Transplant Objects (SUITO) index using established methods (see ESM Method 1).

KEGG analysis and MirTrap system In silico KEGG analysis was performed using DIANA tools mirPath (v.3, http://www.microrna.gr/miRPathv3) to predict the pathways influenced by stress/damage-specific miRNAs. The MirTrap system (Clontech, Mountain View, CA, USA) was used to identify specific miRNA targets and validate KEGG pathway prediction (see ESM Method 7 and Fig. 6b).

Statistical analysis Data were represented as mean ± SEM. Student's *t* test was performed for statistical analysis or one-way ANOVA with Tukey–Kramer post hoc tests for analysis, wherever applicable. A p value <0.05 was considered statistically significant. For correlation studies, Pearson's two-tailed correlation analysis was performed. Randomisation and blinding were not carried out for any analysis. All analyses were carried out using GraphPad Prism (version 7, GraphPad Software, San Diego, CA, USA).

Results

Induction of stress and apoptosis in human islets CC + HYP treatment increased the expression of HIF-1α, IRE-1α, XBP1 and CHOP within 6 h (Fig. 1a–e, $p < 0.01$ to $p < 0.001$). CC + HYP induced apoptotic marker c-Casp-3 within 24 h (Fig. 1f,g, $p < 0.001$). Casp-3 activation correlated with a progressive increase in PI^+/FDA^+ cell frequency/IEQ from 12 h to 48 h (Fig. 1h,i, $p < 0.001$). Thus, CC + HYP effects on islets progressed from activation of endoplasmic reticulum (ER) stress response within 6 h to apoptosis within 12 h, to >50% cell death by 24 h.

Exosome characterisation and exo-miRNA sequencing analyses Approximately 0.8×10^7/ml exosomes per sample were recovered from human islets (2500 IEQ). TEM demonstrated round islet exosomes with cuplike concavity and a diameter of ~100 nm (Fig. 2a–c). NTA confirmed a peak islet exosome size distribution of ~110 nm (Fig. 2d). Islet exosomes expressed exosomal tetraspanin surface protein marker CD9 and heat-shock protein 70 (Hsp70) (Fig. 2d). Biospectral analysis confirmed that exosome RNAs were 18–23 nucleotides in length, with >80% matches, consistent with average size of miRNAs (ESM Fig. 1a). CC and HYP treatments had a significant influence on miRNA expression in islet exosomes compared with control untreated islets and islets only treated with CC (ESM Fig. 1b).

RNA sequencing analysis revealed 879 exo-miRNA species released from human islets; 190 exo-miRNAs were differentially expressed in response to CC and/or HYP compared with control islets (Fig. 2e). Among this group, subgroups of 14, 52 and 33 exo-miRNAs were selectively expressed by human islets under conditions of stress (CC, HYP and CC + HYP, respectively, Fig. 2f).

Release of exo-miRNA from isolated human islets Of the 29 exo-miRNAs differentially expressed with statistical significance ($p < 0.01$, except for hsa-miR-216a-5p and hsa-miR-200c-3p), 11 were expressed under all stress conditions (Fig. 2f, Table 1). Eight of the 29 miRNA species (hsa-miR-375, -216a-5p, -29b-3p, -148a-3p, -92a-3p, -200c-3p, -7-5p and -125b-5p) were validated by qPCR to be differentially released in exosomes from human islets exposed to CC and/or HYP using commercially available primers (Exiqon; ESM Fig. 2).

Ex vivo time course analyses revealed distinction between early and late responses to CC + HYP, with release of exo-miRNAs hsa-miR-29b-3p and hsa-miR-216a-5p within 6 h of CC + HYP exposure (Fig. 3a,b, $p < 0.05$ to $p < 0.001$). Other islet exo-miRNAs were not significantly expressed until 24–48 h (Fig. 3c–h, $p < 0.05$ to $p < 0.001$). Release of exo-miRNAs from stressed islets was suppressed up to 24 h by pre-treatment with GW4869, a neutral sphingomyelinase inhibitor that prevents ceramide-mediated maturation of exosomes. Collectively, these data indicate that hsa-miR-29b-3p and hsa-miR-216a-5p are selectively released in exosomes before apoptosis and cell death. Thus, we classify hsa-miR-29b-3p and hsa-miR-216a-5p as stress-induced exo-miRNA (S-miR) and hsa-miR-375 and hsa-miR-148a-3p as damage-induced exo-miRNA (D-miR) miRNAs.

Exo-miRNA validation in vivo using human islets transplanted into nude mice To validate exo-miRNAs as biomarkers of islet cell stress, we used mouse models of human islet

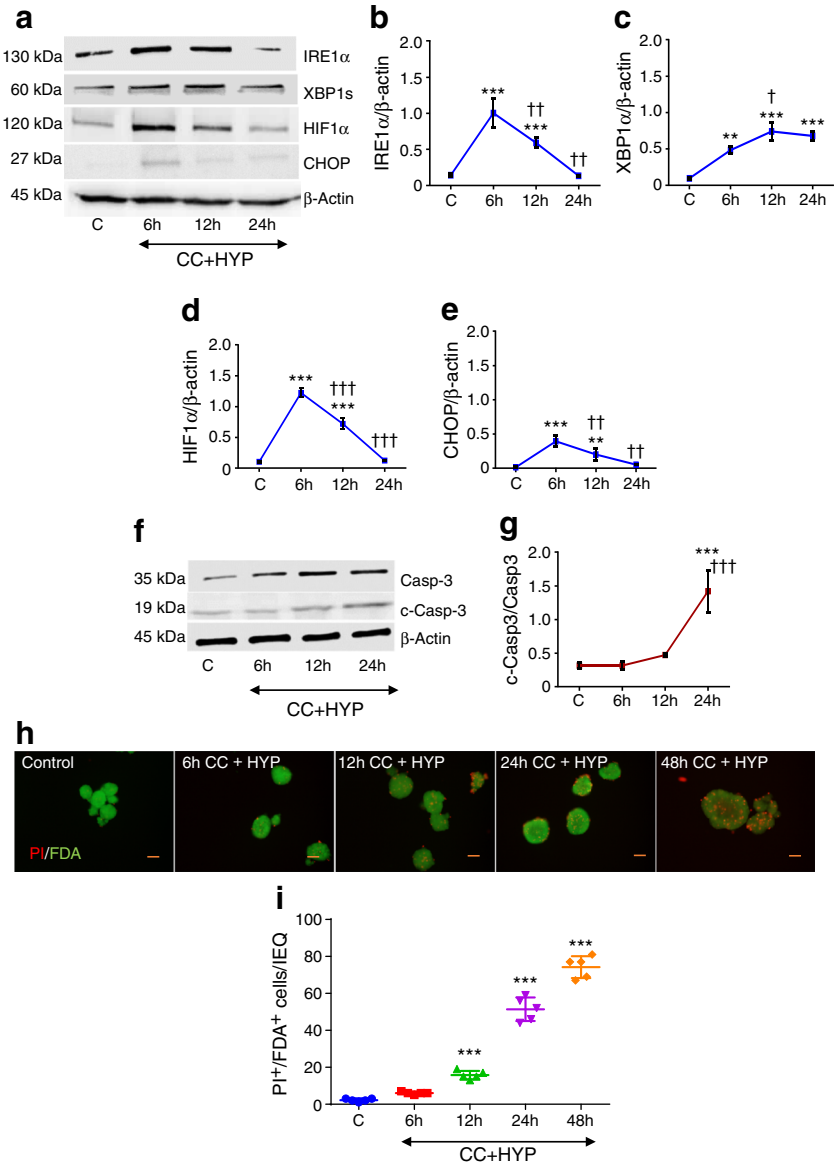

Fig. 1 Proinflammatory cytokines and hypoxia induce ER stress and apoptosis in human islets. Isolated human islets (1000 IEQs) were exposed to proinflammatory cytokines (CC: IL-1β [100 U/ml] + IFN-γ [1000 U/ml] + TNF-α [1000 U/ml]) and hypoxia (HYP: 1% O_2, 5% CO_2 and 94% N_2) for 6, 12 and 24 h for immunoblotting, and for 6, 12, 24 and 48 h for PI/FDA staining. (**a**) Representative blots for expression of IRE-1α, XBP1, HIF-1α, CHOP and β-actin at the indicated time points. (**b**–**e**) Densitometry analysis, with protein expression normalised to β-actin. (**f**) Representative blots for expression of Casp-3, c-Casp-3, and β-actin at the indicated time points. (**g**) Densitometry analysis, with protein expression normalised to Casp-3. (**h**) Representative images showing PI/FDA-stained human islets. Scale bar, 100 μm. (**i**) Quantification of PI^+/FDA^+ cells per IEQ at the indicated time points; approximately 20 IEQs were counted for analyses. Data are presented as mean ± SEM ($n = 3$ samples). One-way ANOVA followed by Tukey's multiple comparison test was performed for statistical analyses. ***$p < 0.001$ compared with untreated control islets at 24 h; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ compared with CC + HYP treatment at 6 h. C denotes untreated control islets cultured routinely for 24 h

transplantation. Diabetes was established in the mice 72 h after STZ administration. Human islets (2500 IEQs) were transplanted under the kidney capsule (Fig. 4a) or intraportally (ESM Fig. 3), and blood samples were collected 24 h post-xenotransplantation. Human islet grafts decreased blood glucose levels 24 h after transplantation (Fig. 4b and ESM Fig. 3a, $p < 0.001$) but did not affect body weight (Fig. 4c and ESM Fig. 3b). Plasma exosomes at 24 h demonstrated similar size distributions and exosomal surface marker CD9 as observed in ex vivo experiments (Fig. 4d). Both S-miR and D-miR were detected in plasma exosomes in the xenotransplantation groups but not in STZ diabetic mice (Fig. 4e–h, $p < 0.001$ and ESM Fig. 3c–g, $p < 0.05$, $p < 0.01$). We normalised S-miRs and D-miRs to a stable exo-miRNA control, hsa-miR-889-3p (high baseMean >500, with no significant changes in expression over time ex vivo [ESM Fig. 2j] or after xenotransplantation [data not shown]), to provide basic quantifiable damage miRNA index (DMI) and stress miRNA index (SMI) (Fig. 4i).

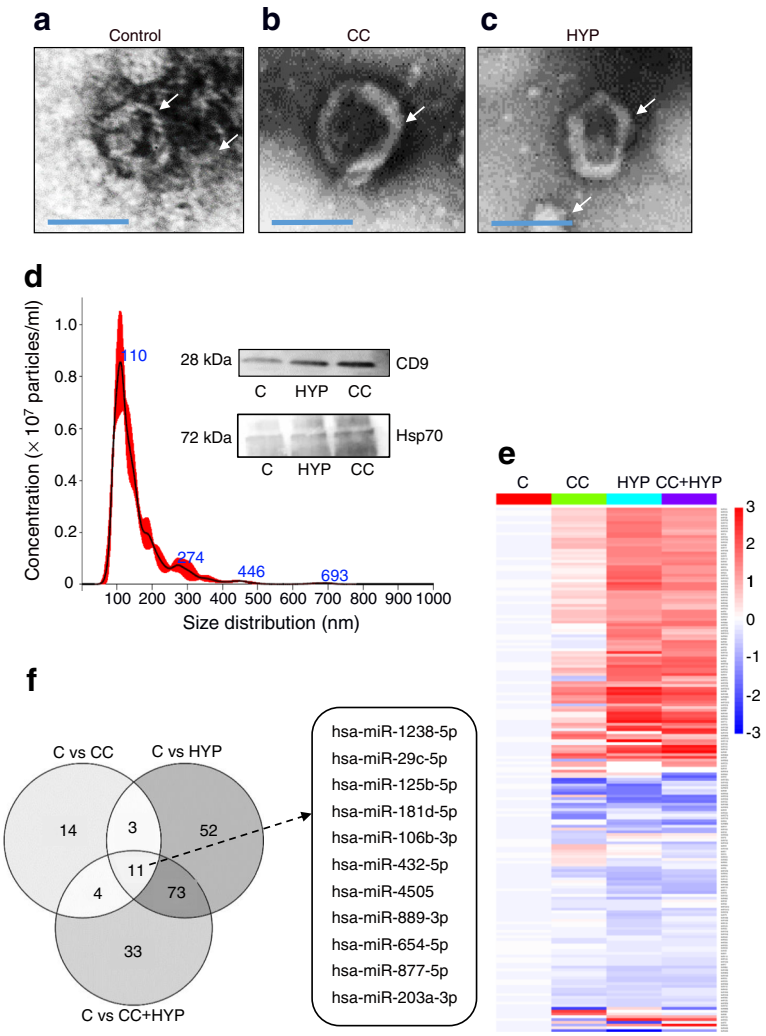

Fig. 2 Exosome characterisation and exo-miRNA sequencing analyses. (**a**–**c**) Representative TEM images of exosomes released from isolated human islets (2500 IEQ) exposed to CC (IL-1β [100 U/ml] + IFN-γ [1000 U/ml] + TNF-α [1000 U/ml]) or HYP (1% O_2, 5% CO_2 and 94% N_2) for 24 h. Scale bar, 100 nm. Arrows indicate exosomes. (**d**) Exosome size distribution (representative data showing control islet exosome distribution), estimated by the dynamic light scattering (DLS) method; inset shows exosomal CD9 and Hsp70 expression, assessed by western blot, from islets exposed to CC or HYP as detailed above. (**e**) Heat map representation of exo-miRNA sequencing data (differential expression analysis based on the negative binomial model) from islets exposed to CC, HYP or CC + HYP as detailed above. Colour codes for fold change are indicated in the heat map. (**f**) Venn diagram showing the number of differentially expressed exo-miRNAs released from human islets during exposure to CC, HYP and CC + HYP (limited to $p < 0.05$ and fold change >1.5 vs control). Data are presented as mean ± SEM ($n = 3$ samples). C denotes untreated control islets cultured routinely for 24 h

The human islet xenografts were resected 24 h after transplantation to assess expression of stress markers and beta cell-enriched genes. Relative mRNA expression of proinflammatory markers *CXCL10*, *TNF*, *IL6*, *NOS2*, *FOS* and *HIF1A* was significantly increased while *INS1* mRNA expression was significantly reduced in the resected grafts compared with normal human islets ($p < 0.05$ and $p < 0.001$, respectively; ESM Fig. 4). *GCG* and *HMGB1* transcription was similar between resected grafts and normal human islets (ESM Fig. 4).

Exo-miRNA profile in patients undergoing TPIAT We measured circulating levels of S-miR and D-miR during islet infusion in TPIAT patients. Details of islet infusions are provided in Table 2. Circulating levels of S-miR and D-miR increased significantly over time, peaking at completion of islet infusion (Fig. 5a, $p < 0.05$ to $p < 0.001$) and returned to normal at 7 days after islet infusion. Circulating levels of other exo-miRNAs (hsa-miR-200c-3p, -92a-3p, -125b-5p, -7f-5p and -889-3p) did not change significantly over time (Fig. 5b). At 1 year post transplantation, DMI ($r = -0.7961$, $p = 0.058$) and SMI ($r = -0.8834$, $p = 0.019$) correlated negatively with SUITO index (Fig. 5c,f). DMI ($r = -0.3053$, $p = 0.556$) did not correlate with C-peptide (Fig. 5d), while SMI ($r = -0.6785$, $p = 0.138$) correlated negatively with C-peptide albeit not significantly (Fig. 5g). DMI ($r = 0.243$, $p = 0.629$) did not correlate with HbA_{1c} (Fig. 5e), while SMI ($r = 0.7709$, $p =$

Table 1 Expression of 29 exo-miRNAs from sequence data with $p < 0.05$[a] or high baseMean

No.	Transcript	baseMean	Log_2FC	*p* value	FDR	Condition
Exo-miRNAs expressed under all stress conditions						
1	hsa-miR-1238-5p	16.6	1.247	0.003	0.030	All
2	hsa-miR-29c-5p	43.3	1.101	0.001	0.014	All
3	hsa-miR-125b-5p	852.1	0.689	0.001	0.021	All
4	hsa-miR-181d-5p	830.3	−0.588	0.017	0.099	All
5	hsa-miR-106b-3p	625.8	−0.663	0.001	0.013	All
6	hsa-miR-432-5p	12841.6	−0.663	0.005	0.048	All
7	hsa-miR-4505	4.5	−0.87	0.034	NA	All
8	hsa-miR-889-3p	10255.3	−0.953	0.0001	0.002	All
9	hsa-miR-654-5p	270.7	−0.985	<0.0001	0.002	All
10	hsa-miR-877-5p	620.6	−1.030	<0.0001	<0.001	All
11	hsa-miR-203a-3p	329.9	−1.791	<0.0001	<0.001	All
Top exo-miRNAs with significant fold change (Log_2FC)						
12	hsa-miR-1200	16.7	1.715	<0.0001	0.002	CC + HYP
13	hsa-miR-29b-3p	607.8	1.646	<0.0001	0.0003	CC + HYP
14	hsa-miR-185-5p	64.0	1.420	<0.001	0.008	CC + HYP
15	hsa-miR-1251-5p	33.1	1.417	<0.001	<0.01	CC + HYP
16	hsa-miR-590-5p	5.2	1.357	<0.001	NA	CC + HYP
17	hsa-miR-3613-3p	14.2	1.279	0.003	0.035	CC + HYP
18	hsa-miR-665	597.8	1.262	<0.0001	0.0003	CC + HYP
19	hsa-miR-7-5p	592.4	1.223	<0.0001	<0.001	CC + HYP
20	hsa-miR-320d	1124.2	1.219	<0.0001	<0.001	CC + HYP
Islet-specific exo-miRNAs with high baseMean (>550)						
21	hsa-miR-375	1083171	−0.610	0.01	0.082	CC + HYP
22	hsa-miR-216a-5p	554.169	0.352	0.075	0.255	CC + HYP
23	hsa-miR-200c-3p	820.160	0.403	0.095	0.288	CC + HYP
24	hsa-miR-148a-3p	397444.2	−1.154	<0.0001	<0.0001	CC + HYP
25	hsa-miR-92a-3p	17655.06	−1.148	<0.0001	<0.0001	CC + HYP
26	hsa-miR-25-3p	11817.34	−0.931	<0.0001	<0.001	CC + HYP
27	hsa-miR-409-3p	22547.35	−0.962	<0.0001	<0.001	CC + HYP
28	hsa-miR-410-3p	25901.78	−0.914	<0.0001	<0.001	CC + HYP
29	hsa-miR-129-5p	12220.80	−0.806	<0.001	<0.01	CC + HYP

[a] Except hsa-miR-216a-5p and hsa-miR-200c-3p

FC, fold change; FDR, false discovery rate

0.069) correlated positively with HbA_{1c} albeit not significantly (Fig. 5h).

Exo-miRNA target analyses using the MirTrap system (hsa-miR-29b-3p; hsa-miR-216a-5p) KEGG analysis revealed phosphoinositide 3-kinase (PI3K)–Akt, forkhead box protein O1 (FOXO1) and mammalian target of rapamycin (mTOR) signalling pathways, extracellular matrix–receptor interaction, biotin metabolism and platelet activation (Table 3, Fig. 6a) as targets of these exo-miRNAs. We validated exo-miRNAs (hsa-miR-216a-5p and hsa-miR-29b-3p) experimentally using the MirTrap system in dissociated human islet cells (Fig. 6b, ESM Method 7). Among 84 genes tested (PI3K–Akt signalling pathway RT^2 profiler PCR array), 21 were significantly enriched after immunoprecipitation in dissociated human islet cells co-transfected with hsa-miR-29b-3p and hsa-miR-216a-5p mimics, and pMirTrap vector (Fig. 6c, Table 4, $p < 0.05$), confirmed by qPCR analyses (15 genes shown, Fig. 6d,e). Positive (*Aequorea coerulescens* GFP [AcGFP1]) and negative (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 [PLOD3]) controls for the experimental control transfection (hsa-miR-132 mimic, pMirTrap vector) validated the specificity of the MirTrap system (Fig. 6d, fold enrichment of AcGFP1 = 25.71, $p < 0.001$). Thus hsa-miR-29b-3p and hsa-miR-216a-5p interact with these signalling pathways in islets.

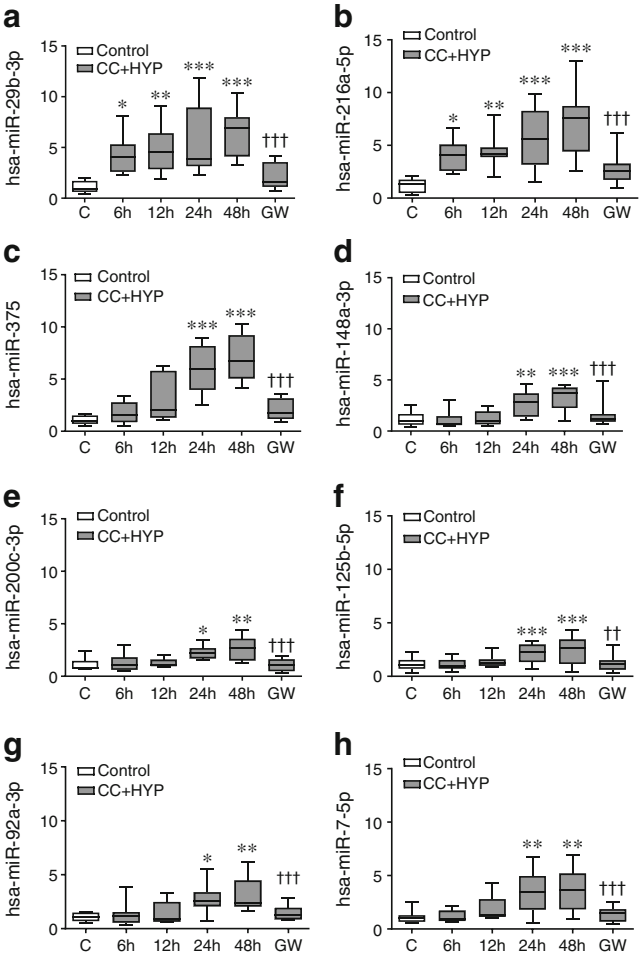

Fig. 3 Proinflammatory cytokines and hypoxia induce release of exosomes containing differentially expressed miRNAs from isolated human islets. Isolated human islets (2000 IEQ) were pre-treated for 2 h with or without the exosome inhibitor GW4869, and then exposed to CC + HYP for the time points indicated in the figure. Exo-miRNA expression was determined using qPCR, normalised to U6 small RNA (spiked in) and plotted as fold change vs control. The following miRNA species were analysed: (**a**) hsa-miR-29b-3p; (**b**) hsa-miR-216a-5p; (**c**) hsa-miR-375; (**d**) hsa-miR-148a-3p; (**e**) hsa-miR-200c-3p; (**f**) hsa-miR-125b-5p; (**g**) hsa-miR-92a-3p; (**h**) hsa-miR-7-5p. Data are presented as mean ± SEM ($n = 3$ samples). One-way ANOVA followed by Tukey's multiple comparison test was performed for statistical analyses. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with untreated control islets cultured routinely for 24 h; $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ compared with CC + HYP treatment at 24 h. C denotes untreated control islets cultured routinely for 24 h; GW, GW4869 (an inhibitor of exosome maturation)

Discussion

Using extensive ex vivo and in vivo mouse and human studies, we have validated two plasma exo-miRNAs, hsa-miR-29b-3p and hsa-miR-216a-5p, specific for islet beta cell stress. In the context of islet transplantation, insulin independence depends on a number of factors including islet engraftment and function. Inflammation and hypoxia account for about 50% of islet cell death within 48–72 h of transplantation [15–20]. Furthermore, stress and damage during islet isolation and in the peri-transplant stages drastically influence the outcome of transplantation [12, 21–23]. Identification of specific circulating biomarkers of the early stages of beta cell stress, common to any islet microenvironments including prediabetes and transplantation, can improve treatment strategies.

Circulating or urinary miRNAs in microvesicles or exosomes are being evaluated as biomarkers of disease progression and diabetes complications [24–26]. Exosomal proteins and nucleic acids are resistant to degradation, as they are protected by a lipid bilayer. Their roles in cell communication, immune regulation, cell adhesion, tissue regeneration and elimination of harmful molecules are well established in multiple contexts [27–29]. Hence, exosome-based biomarkers are reliable and can be used to predict disease progression, predict treatment efficacy and aid in personalised medicine.

Upon exposure to CC + HYP, human islets released exosomes (~110 nm in diameter), as reported earlier [30], with TEM analyses confirming the expected round-shaped morphology. Exosomes expressed CD9 and contained small RNA species (18–23 nucleotides). CC + HYP induced ER stress response within 6 h and apoptosis by 24 h [31–38]. The exo-miRNA sequencing analyses revealed that cytokines and/or hypoxia induced significant changes in exo-miRNA signature (ESM Fig. 1b). Our investigations included >28,000 miRNAs represented in the miRBase database (>80% sequence match). Diabetes environments, including glucotoxic and glucolipotoxic environments, influence alterations in islet miRNA transcription [39–42]. In rat islets, cytokines increased expression of islet miRNAs, including miR-375, miR-29b and miR-200c, after 6 h of exposure [43]. We identified 11 miRNAs that were differentially released in exosomes under all stress conditions. We selected the top nine exo-miRNAs with significant induction ($\log_2$ fold change) and nine with high baseMean (>550) islet-specific exo-miRNAs [10, 12, 13, 44–47] from our global miRNA sequencing (miRNA-seq) data for additional investigations.

Ex vivo qPCR analyses indicated that expression of eight exo-miRNAs was significantly increased after CC + HYP exposure. hsa-miR-29b-3p and hsa-miR-216a-5p were released in exosomes as early as 6 h, coinciding with activation of ER stress response markers IRE-1α, XBP1, HIF-1α and CHOP. Other miRNAs (hsa-miR-375, hsa-miR-148a-3p, hsa-miR-200c-3p, hsa-miR-7-5p, hsa-miR-92a-3p and hsa-miR-125b-5p) were released in exosomes at 24 h, coinciding with Casp-3 activation, cell damage and induction of apoptosis (Fig. 1); GW4869 blocked their release, thus confirming the exosomal origin of these miRNAs. Thus, there is a clear demarcation between release of miRNAs in the early state of cellular stress and the state of cell damage and apoptosis. We classified these two categories of miRNAs as S-miR and D-miR, respectively. These early S-miRs are valuable and promising biomarkers of islet cell stress even before induction of cell damage and death.

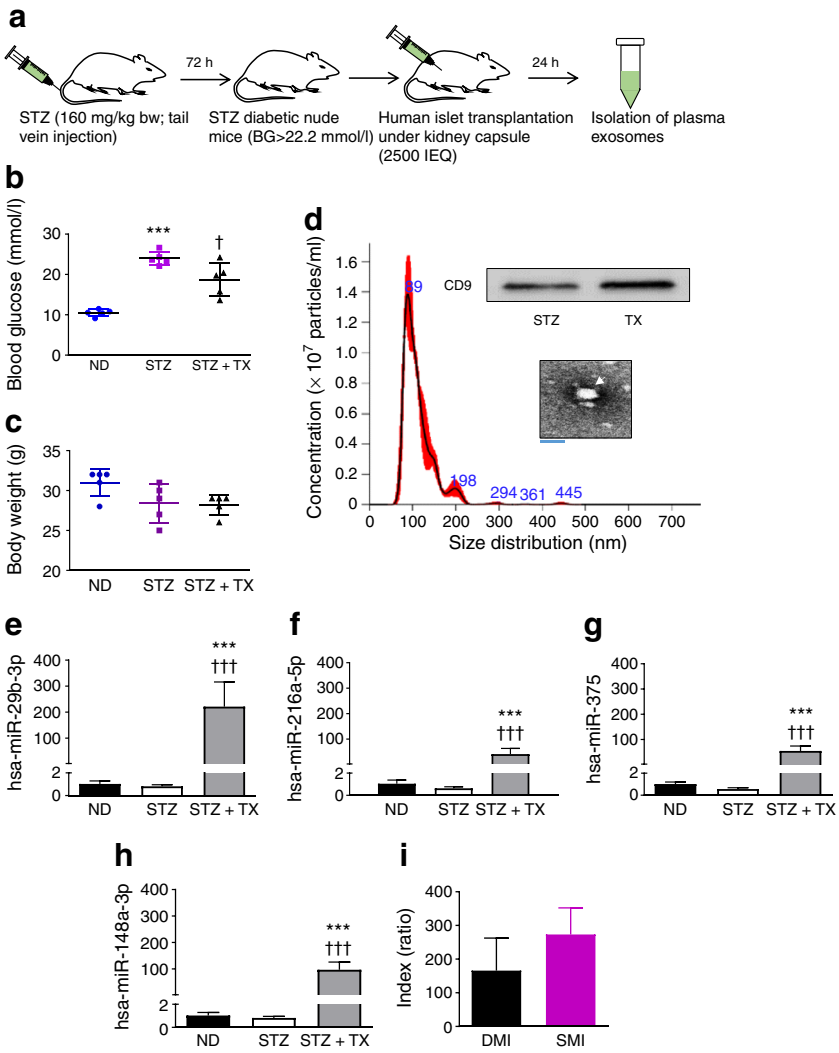

Fig. 4 Exo-miRNA validation in vivo using STZ diabetic nude mice. (**a**) Timeline of xenoislet transplantation under the kidney capsule of STZ diabetic nude mice. Blood glucose level (**b**) and body weight (**c**) of non-diabetic control, STZ diabetic and islet-transplanted STZ diabetic mice. (**d**) Size distribution of plasma exosomes isolated from islet-transplanted STZ diabetic mice, estimated by dynamic light scattering (DLS). Insets show TEM image of plasma exosome from an islet-transplanted STZ diabetic mouse (scale bar, 100 nm) and blot showing exosomal CD9 expression (28 kDa). (**e**–**h**) Plasma exo-miRNA expression. (**i**) DMI and SMI indices. Data are presented as mean ± SEM ($n = 5$ mice). One-way ANOVA followed by Tukey's multiple comparison test was performed for statistical analyses. ***$p < 0.001$ compared with non-diabetic mice; $^{\dagger}p < 0.05$ and $^{\dagger\dagger\dagger}p < 0.001$ compared with STZ diabetic mice. BG, blood glucose; bw, body weight; ND, non-diabetic mice; TX, transplantation

Plasma levels of these S-miRs (miR-29b-3p and miR-216a-5p) and D-miRs (miR-375 and miR-148a-3p) were increased in STZ diabetic nude mice transplanted with isolated human islets under the kidney capsule or intraportally. These human exo-miRNAs were not detected in control STZ diabetic nude mice. Other exo-miRNAs were not detectible in these transplant models except for hsa-miR-200c-3p (fold change 2.95) and hsa-miR-92a-3p (fold change 23.8) in a kidney capsule transplant model, consistent with our previous clinical results [12]. These results clearly suggest that elevated plasma S-miRs and D-miRs correspond to islet stress and damage immediately post transplantation due to inflammation and hypoxia. Islet stress was evident from increased expression of *NOS2*, *HIF1A*, *FOS*, *CXCL10*, *IL6* and *TNF* in islet grafts at 24 h post transplantation (ESM Fig. 4). We calculated stress and damage indices (SMI and DMI) using a stable exo-miRNA, hsa-miR-889, expression of which did not change after cytokine or hypoxia treatment ex vivo (Figs. 4, 5). SMI and DMI were both

Table 2 TPIAT patient information

Patient no.	Transplanted pellet volume (ml)	No. of bags	Islet dose (IEQ)	IEQ/kg body weight	Incubation time before infusion (h)
TPIAT 1	27	3	760334	7558	1
TPIAT 2	16	2	486580	5106	1
TPIAT 3	26	3	375607	6137	1
TPIAT 4	13	2	353000	4095	1
TPIAT 5	15	2	440584	5700	1
TPIAT 6	26	3	419540	5406	1

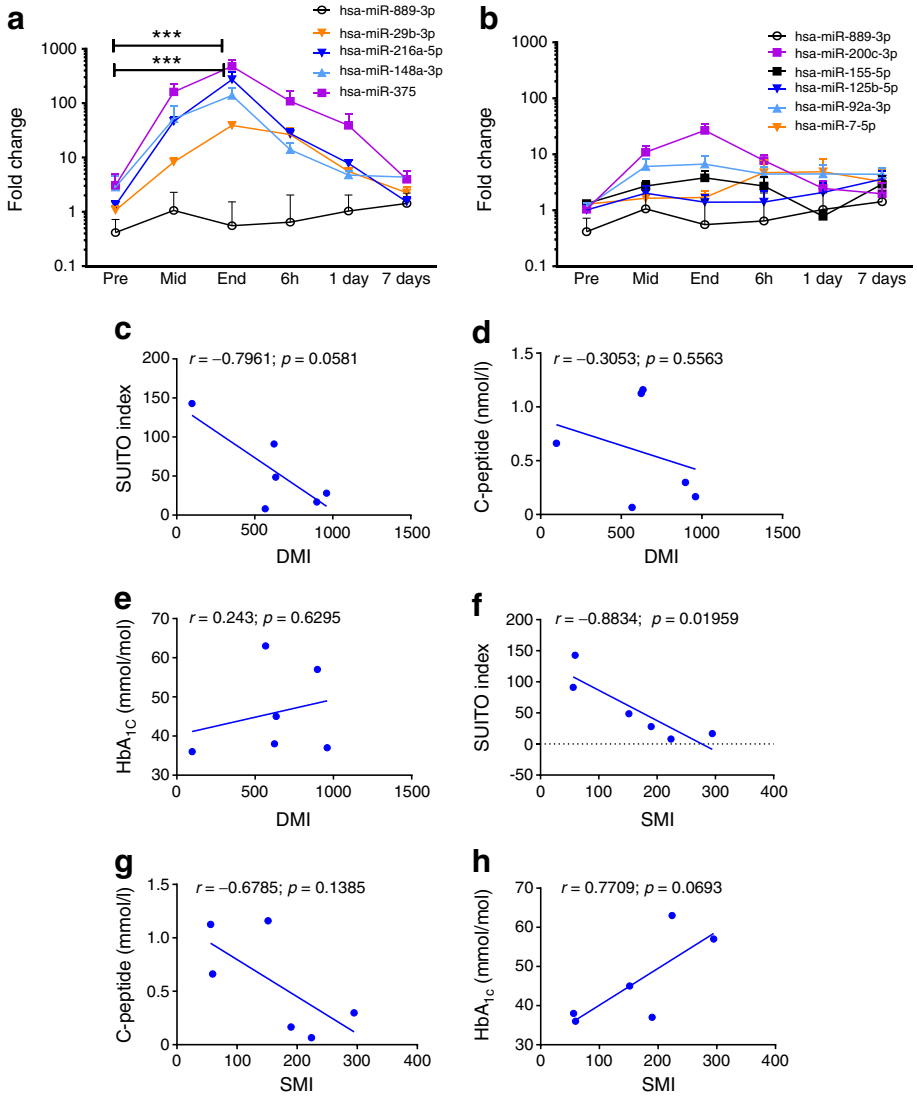

Fig. 5 Exo-miRNA profile during and post TPIAT. (**a**) Time course of change (plotted on a logarithmic scale) in release of S-miR and D-miR miRNAs during (Pre [1 h before islet infusion], Mid [middle of islet infusion] and End [completion of islet infusion]) and post TPIAT (6 h, 1 day and 7 days post islet infusion). (**b**) Time course of change in release of other validated (in vitro) exo-miRNAs during and post TPIAT. (**c**–**h**) Pearson's two-tailed correlation plots of DMI and SMI with SUITO index, C-peptide (mmol/l) and HbA_{1c} (mmol/mol) at 1 year post TPIAT. S-miRs and D-miRs were normalised to hsa-miR-889-3p, a stable exo-miRNA control, and are shown as fold change relative to the Pre islet infusion time point. Data are presented as mean ± SEM ($n = 6$ individuals). One-way ANOVA followed by Tukey's multiple comparison test was performed for analysing time course changes in exo-miRNAs during and post TPIAT (**a**, **b**). ***$p < 0.001$ compared with Pre islet infusion time

increased considerably, with SMI slightly higher than DMI in the kidney capsule transplant model. Islet beta cell stress and dysfunction were also evident from reduced *INS1* mRNA levels in islet grafts at 24 h post transplantation. In a model of human-into-mouse xenoislet transplantation, both islet graft and plasma exosomes contained miR-375 among various other miRNAs, which differed from our list of exo-miRNAs [30]. These differences could be attributed to the reference exo-miRNA and controls used to analyse data. Nevertheless, both studies highlight changes in exosome cargo that can be tapped for identification of non-invasive biomarkers.

In TPIAT patients, time course analyses indicated that plasma levels of hsa-miR-375, hsa-miR-216a-5p, hsa-miR-148a-3p and hsa-miR-29b-3p peaked at completion of islet infusion and normalised to pre-transplant levels after 7 days (Fig. 5). Plasma levels of hsa-miR-200c-3p and hsa-miR-92a-3p were elevated, although not significantly, compared with pre-transplant levels. Our analyses suggested negative correlation between SMI (of peak exo-miRNAs) and the islet SUITO index of islet graft function [48] ($r^2 = -0.8834$; $p = 0.020$) and C-peptide ($r^2 = -0.6785$; $p = 0.139$) and a positive correlation between SMI and HbA_{1c} (%) ($r^2 = 0.7709$; $p = 0.069$). DMI correlated negatively with SUITO index ($r^2 = -0.7961$; $p = 0.058$) but not C-peptide or HbA_{1c}. SUITO index is a valuable tool with which to predict post-transplant insulin independence and islet engraftment [48]. We previously demonstrated that hsa-miR-375 and hsa-miR-200c are reliable biomarkers of islet cell damage and predictors of post-transplant graft function in TPIAT patients [12, 13]. S-miRs provide an opportunity to monitor islet stress and intervene during the transplantation procedure to preserve islet function/survival and enhance efficiency to achieve long-term insulin independence.

We identified the targets of these exo-miRNAs in islets using in silico KEGG analyses and the MirTrap system. hsa-miR-29b-3p, hsa-miR-216a-5p, hsa-miR-375 and hsa-miR-148a-3p targeted mRNAs mainly in PI3K–Akt signalling

Table 3 KEGG analysis to predict the mRNA targets of hsa-miR-216a-5p, hsa-miR-29b-3p, hsa-miR-375 and hsa-miR-148a-3p

KEGG pathway	*p* value	No. of genes
PI3K–Akt signalling pathway	5.03×10^{-5}	63
Focal adhesion	1.36×10^{-5}	47
FOXO signalling pathway	0.0005	30
Proteoglycans in cancer	0.02	30
Protein digestion and absorption	1.36×10^{-5}	27
Amoebiasis	3.72×10^{-9}	26
Extracellular matrix–receptor interaction	1.38×10^{-73}	24
Platelet activation	0.015	24
Thyroid hormone signalling pathway	5.03×10^{-5}	22
Choline metabolism in cancer	0.014	21
Small-cell lung cancer	0.01	20
Prostate cancer	0.04	18
Glioma	0.0004	15
Renal cell carcinoma	0.004	15
mTOR signalling pathway	0.02	15
Non-small-cell lung cancer	0.03	13
Lysine degradation	9.54×10^{-5}	10
Prion diseases	8.91×10^{-21}	5
Biotin metabolism	5.40×10^{-5}	1

pathway. The significance of the PI3K–Akt signalling pathway in regulation of beta cell function/survival is well established [49, 50]. We identified 21 mRNAs in the PI3K–Akt signalling pathway as targets of hsa-miR-29b-3p and hsa-miR-216a-5p using MirTrap–PCR array studies. In addition to PI3K–Akt signalling, these miRNAs targeted myeloid differentiation primary response gene 88 (MyD88), NF-κB1 and BCL2-associated death promoter (BAD). MyD88 plays a crucial role in NF-κB activation and eventually islet dysfunction after clinical islet transplantation [51] and in the progression of type 1 diabetes [52]. Deregulated PI3K–Akt–IRS1 signalling is a hallmark event in beta cell dysfunction in diabetes [53, 54]. PI3K–Akt signalling regulates activity of BAD, a pro-apoptotic protein, during conditions of islet stress [55, 56]. We hypothesise that stress situations induce miRNAs that preserve beta cell function/survival extracellularly in exosomes, in addition to inducing stress response and apoptosis pathways. Exosomes contain various molecules that communicate with other organ systems, possibly to induce damage/repair response to maintain homeostasis. Interestingly, beta cell-specific

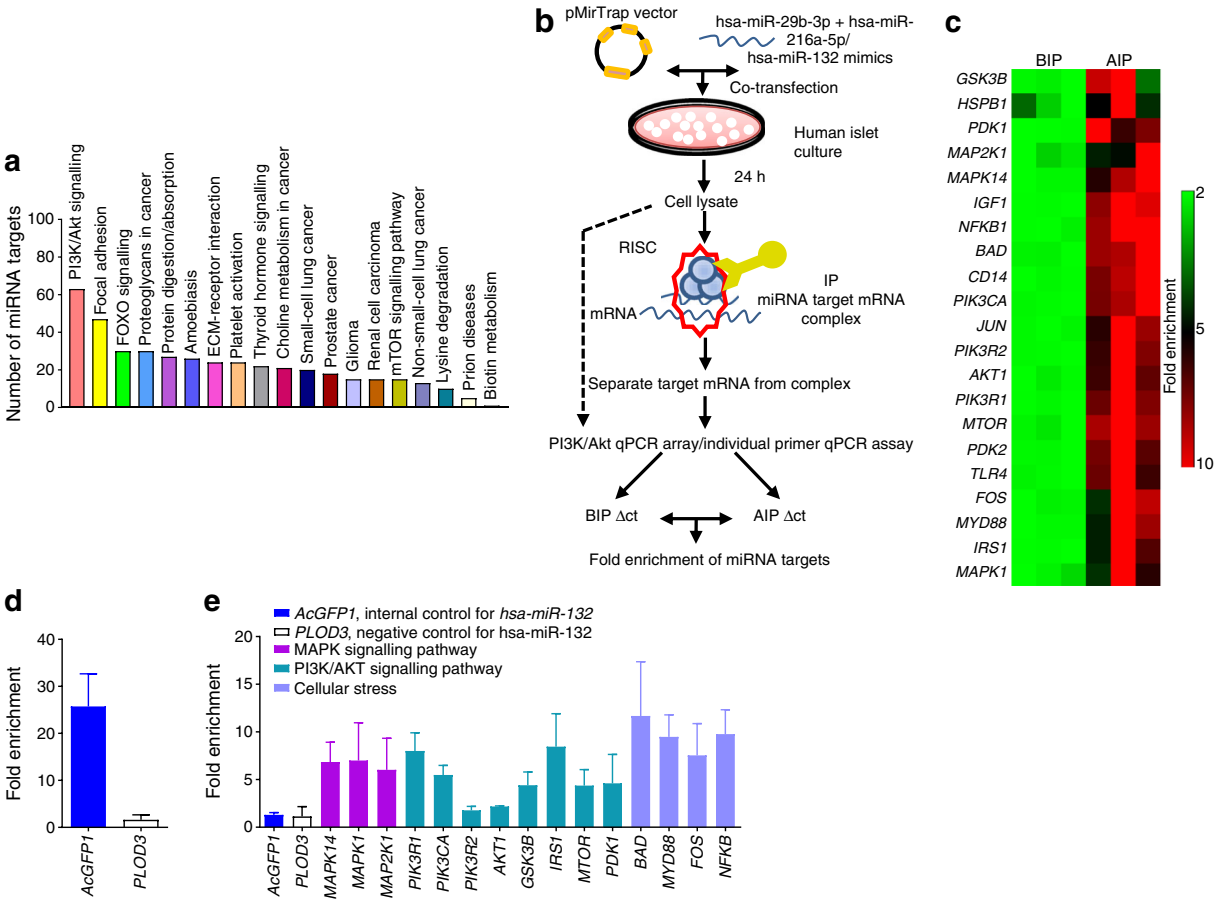

Fig. 6 Exo-miRNA target analyses using the MirTrap system (hsa-miR-29b-3p; hsa-miR-216a-5p). (**a**) KEGG pathway prediction for hsa-miR-29b-3p, hsa-miR-216a-5p, hsa-miR-375 and hsa-miR-148a-3p. (**b**) Design of the MirTrap system. (**c**) Heat map representing fold enrichment before and after immunoprecipitation (BIP/AIP) using the RT^2 profiler PCR array system. (**d**, **e**) Fold enrichment of select mRNAs using qPCR. Data are presented as mean ± SEM ($n = 3$ samples). IP, immunoprecipitate; MAPK, mitogen-activated protein kinase; RISC, RNA-induced silencing complex

Table 4 Genes significantly enriched after immunoprecipitation and PI3K–Akt signalling pathway array after MirTrap

Gene	Average count		SD		*p* value	Fold enrichment
	BIP	AIP	BIP	AIP		
AKT1	34.13	37.22	0.50	0.15	0.05	7.89
BAD	34.75	37.86	0.34	0.29	0.0001	14.50
CD14	35.83	37.55	0.52	0.25	0.0005	9.49
FOS	34.10	36.21	0.79	0.33	0.04	7.10
GSK3B	33.22	37.70	0.18	0.79	0.03	5.64
HSPB1	31.49	37.48	0.38	0.25	0.03	4.98
IGF1	36.29	37.22	0.27	0	0.0009	9.55
IRS1	35.61	36.17	1.25	0.80	0.04	10.60
JUN	34.18	38.22	0.09	0	0.006	3.21
MAP2K1	33.13	37.07	0.73	0.66	0.03	7.14
MAPK1	33.32	36.89	1.74	0.64	0.03	8.46
MAPK14	34.62	36.45	1.06	1.07	0.04	8.82
MTOR	33.19	37.22	0.30	0	0.005	4.12
MYD88	34.59	35.93	0.68	0.67	0.03	12.39
NFKB1	34.17	37.66	0.54	0	0.002	4.41
PDK1	34.57	36.11	0.18	0.71	0.04	5.37
PDK2	31.12	35.24	0.10	0.15	0.001	7.23
PIK3CA	34.10	37.89	0.14	0	0.008	6.10
PIK3R1	35.20	36.12	0.72	0	0.004	4.51
PIK3R2	35.74	36.00	1.01	0	0.002	6.54
TLR4	35.30	38.25	0.28	0	0.002	4.81

AIP, after immunoprecipitation; BIP, before immunoprecipitation

exosomes containing miR-29b-3p exerted immunoregulatory effects through TNF-α, IL-6 and IL-10 cytokine secretion from splenocytes isolated from diabetes-prone NOD mice ex vivo [57]. Further investigations are warranted to understand how stress conditions direct islet miRNAs to exosomes instead of vital biological processes.

Our extensive investigations reported here provide proof of concept, for the first time, that exo-miRNAs in circulation can be utilised for their ability to predict islet beta cell stress even before onset of beta cell failure in the context of prediabetes and islet auto-transplantation. Our reproducible observations, reported here and previously, clearly establish hsa-miR-375 as a beta cell damage marker. While biomarkers of beta cell damage are useful in predicting future insulin dependence, biomarkers of beta cell stress early in the pathological events leading to failure are necessary tools in halting progression of dysfunction. A consistent increase in hsa-miR-29b-3p and hsa-miR-216a-5p as early as 6 h after CC + HYP exposure provides valuable information on the state of beta cell stress. Hormones, hormone mimetics and pharmacological therapies that target beta cell stress and function can be used effectively during states of beta cell stress even before onset of clinical symptoms. These investigations are valuable especially to our clinic and others during the TPIAT procedure to optimise islet preservation strategies and achieve successful engraftment. Overall, we report two S-miRs, hsa-miR-29b-3p and hsa-miR-216a-5p, with great potential as biomarkers of islet cell stress.

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Data availability All data are archived in Institutional Network Drive and are available upon request from the corresponding authors. miRNA-seq data will be uploaded to Gene Expression Omnibus and available once the data are published.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement PBS designed the study, performed experiments, analysed data and wrote the manuscript. SV analysed data and wrote and revised the manuscript. GY and CMD assisted in performing in vivo experiments, data analysis and manuscript preparation. XW and JG analysed microRNA sequencing data and assisted in manuscript preparation. MCL and BN designed and supervised the study, analysed data and wrote the manuscript. All authors approve the final version of the manuscript. BN is responsible for the integrity of the work as a whole.

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