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ARTICLE

PIWI-interacting RNAs as novel regulators of pancreatic beta cell function

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Abstract
Aims/hypothesis P-element induced Wimpy testis (PIWI)-interacting RNAs (piRNAs) are small non-coding RNAs that interact with PIWI proteins and guide them to silence transposable elements. They are abundantly expressed in germline cells and play key roles in spermatogenesis. There is mounting evidence that piRNAs are also present in somatic cells, where they may accomplish additional regulatory tasks. The aim of this study was to identify the piRNAs expressed in pancreatic islets and to determine whether they are involved in the control of beta cell activities.
Methods piRNA profiling of rat pancreatic islets was performed by microarray analysis. The functions of piRNAs were investigated by silencing the two main *Piwi* genes or by modulating the level of selected piRNAs in islet cells.
Results We detected about 18,000 piRNAs in rat pancreatic islets, many of which were differentially expressed throughout islet postnatal development. Moreover, we identified changes in the level of several piRNAs in the islets of Goto–Kakizaki rats, a well-established animal model of type 2 diabetes. Silencing of *Piwil2* or *Piwil4* genes in adult rat islets caused a reduction in the level of several piRNAs and resulted in defective insulin secretion and increased resistance of the cells to cytokine-induced cell death. Furthermore, overexpression in the islets of control animals of two piRNAs that are upregulated in diabetic rats led to a selective defect in glucose-induced insulin release.
Conclusions/interpretation Our results provide evidence for a role of PIWI proteins and their associated piRNAs in the control of beta cell functions, and suggest a possible involvement in the development of type 2 diabetes.
Data availability Data have been deposited in Gene Expression Omnibus repository under the accession number GSE93792. Data can be accessed via the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ojklueugdzehpkv&acc=GSE93792

Keywords Diabetes · Insulin secretion · Pancreatic islets · piRNAs · *Piwil* genes

Abbreviations

GK	Goto–Kakizaki
piRNA	PIWI-interacting RNA
PIWI	P-element induced Wimpy testis
qRT-PCR	Quantitative reverse transcription real-time PCR
siRNA	Small interfering RNA

Introduction

P-element induced Wimpy testis (PIWI)-interacting RNAs (piRNAs) are small non-coding RNAs that are very abundant in animal gonads. They were named from their ability to associate with Argonaute proteins of the PIWI subfamily [1–4]. piRNAs share common biochemical features, including the phosphorylation of the 5′ end and the 2-*O*-methylation of the 3′ end. Mature piRNAs are processed via a maturation pathway

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involving the cleavage by PIWI proteins of long single-stranded RNAs transcribed from 'piRNA clusters' present in both intra- and intergenic regions of the genome [5, 6].

piRNAs are believed to maintain genome integrity by guiding PIWI proteins to repress transposon activity, regulate the assembly of the telomere protection complex and be involved in RNA silencing and in the epigenetic control of gene expression [5, 7–9]. Until recently, the piRNA pathway was perceived as germline-specific, but piRNAs have also been detected in stem cells and other cell types, suggesting that these small non-coding RNAs may accomplish additional tasks [10–12]. Despite the poorly understood role of piRNAs in somatic cells, there is increasing evidence indicating that these non-coding RNAs control gene expression by acting at genomic and also at transcriptional and post-transcriptional levels [13–17]. Indeed, recent studies have highlighted the involvement of the PIWI–piRNA pathway in both physiological and pathological processes, including development [18], memory [19], liver regeneration [20] and cancer [21–26]. However, nothing is known about their role in metabolic diseases, such as diabetes.

Pancreatic islets play key roles in the regulation of metabolism and energy homeostasis by secreting hormones in response to changes in nutritional status. Pancreatic beta cell dysfunction and loss are critical determinants for the development of type 2 diabetes. There is strong evidence indicating that altered expression of both protein-coding and non-coding genes is associated with beta cell dysfunction under conditions of impaired glucose tolerance and diabetes [27]. Several studies have implicated miRNAs and long non-coding RNAs as key players in diabetes [28–30]. So far, no information has been available about the presence of PIWI–piRNAs complexes in beta cells and their possible involvement in islet physiology and the development of diabetes. The goal of this study was to analyse the piRNA expression patterns of rat pancreatic islets under both physiological and pathological conditions, and to assess their contribution to the maintenance of beta cell function.

Methods

Animals

Male Sprague Dawley and Wistar rats and pregnant female Sprague Dawley rats were obtained from Janvier Laboratories (Le Genest-Saint-Isle, France) and housed under a 12 h light, 12 h dark cycle in climate-controlled and pathogen-free facilities. The detailed sources of Goto–Kakizaki (GK) rats have previously been described [31]. All animal procedures were performed in accordance with the National Institutes of Health guidelines and were approved by the Swiss Research Councils and Veterinary Offices or by the local ethics committee in Malmö.

Islet isolation, organ collection and cell culture Rat pancreatic islets were isolated by collagenase digestion (Roche Diagnostics, Rotkreuz Switzerland), followed by purification on a Histopaque density gradient (Sigma-Aldrich, St Louis, MO, USA) [32] and final cleaning by hand-picking. The islets were cultured in RPMI 1640 Glutamax medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FCS (Gibco, Zug Switzerland), 100 μg/ml streptomycin, 100 IU/ml penicillin, 1 mmol/l sodium pyruvate and 10 mmol/l HEPES, pH 7.4. Human islets obtained from the Cell Isolation and Transplantation Center (University of Geneva, Geneva, Switzerland) were cultured in CMRL1066 medium (Invitrogen) supplemented with 10% FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin, 2 mmol/l L-glutamine and 250 μmol/l HEPES. The use of human islets was approved by the Geneva local ethical committee. Dissociated islet cells were obtained by digestion with 5 mg/ml of trypsin (Gibco). Adipose tissue, brain, liver and skeletal muscle were collected from Wistar rats aged 12 weeks.

Fluorescence activated cell sorting of islet cells Dissociated islet cells from newborn and adult rats were sorted by FACS based on beta cell autofluorescence [33]. Immunocytochemistry analysis using anti-insulin antibodies (no. A0564, Dako, Basel, Switzerland) revealed that 94 ± 1% of the cells in the purified fraction were insulin-positive.

Cell transfection Dissociated islet cells were transfected with predesigned small interfering RNAs (siRNAs; Life Technologies, Basel Switzerland), using as control an siRNA directed against GFP (Eurogentec, Seraing, Belgium). Synthetic RNA oligonucleotides with 5′ phosphorylation and 3′ 2-*O*-methylation (Integrated DNA Technologies, Leuwen Belgium) were used as piRNA mimics. Transfection was carried out using Lipofectamine RNAiMax (Invitrogen). To overexpress the piRNAs, we used the following sequences: (DQ732700) 5′-UAUGAAGA AUGACUUGGGGUACAUGACC-3′; (DQ746748) 5′-ACUGGAAACGGAAAACUCAGAGCGCCC-3′; control oligonucleotide 5′-GUGUAACACGUCUAUACGCCCA-3′. Functional assays were performed 48 h after transfection.

Insulin secretion Islet cells were preincubated in Krebs buffer containing 25 mmol/l HEPES, pH 7.4, 0.1% BSA (Sigma-Aldrich) and 2 mmol/l glucose for 30 min at 37°C. The medium was then replaced by Krebs buffer containing 0.1% BSA supplemented with 2 mmol/l glucose, 20 mmol/l glucose or 2 mmol/l glucose and 35 mmol/l KCl. After 45 min, the supernatant fractions were collected and centrifuged at 1200*g* for 5 min to remove cell debris. Total cellular insulin contents were recovered in EtOH acid (75% ethanol, 0.55% HCl), and protein contents were collected on ice in a lysis buffer containing 50 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA, 0.5%

Triton X-100 and protease inhibitors (Roche). The amount of insulin in the samples was determined by ELISA (Mercodia, Uppsala, Sweden). Protein content was evaluated using the Bradford protein assay (Bio-Rad, Reinach, Switzerland).

Proliferation assay Dissociated islet cells were cultured on poly-L-lysine-coated glass coverslips and treated, or not, for 48 h with 500 ng/ml prolactin (Sigma-Aldrich) to stimulate proliferation. The cells were fixed in 4% paraformaldehyde and incubated with PBS supplemented with 0.5% saponin (Sigma-Aldrich) for 20 min. The coverslips were incubated for 30 min in PBS supplemented with 0.5% saponin and 1% BSA and exposed for 1 h to the following antibodies: 1:1500 rabbit anti-Ki67 (Abcam, Cambridge, UK) and 1:100 guinea pig anti-insulin (Millipore, Zug, Switzerland). They were then washed and incubated for 1 h with goat anti-rabbit Alexa Fluor 488 or goat anti-guinea pig Alexa Fluor 594 diluted at 1:400 (Invitrogen). Finally, the coverslips were incubated for 10 min with Hoechst 33342 (Invitrogen) and mounted on microscope glass slides. The cells were visualised with a Zeiss AxioVision fluorescence microscope (Zeiss, Feldbach, Switzerland). At least 600 cells were analysed for each condition. The total number of cells was assessed using ImageJ software (https://imagej.net/), while Ki67-positive cells were counted manually.

Cell death assessment Cell death was triggered by exposing the cells to proinflammatory cytokines (30 ng/ml IFN-γ, 10 ng/ml TNF-α and 0.1 ng/ml IL-1β) for 24 h. The cells were incubated for 5 min at 37°C with Hoechst 33342 (Invitrogen). About 400 cells per condition were analysed by fluorescence microscopy (Axio Vert 25; Zeiss) to score the fraction displaying pyknotic nuclei.

RNA extraction and measurement Total RNA from islets or from other rat organs was extracted using the miRNeasy kit (Qiagen, Basel, Switzerland). For microarray analysis and quantitative reverse transcription real-time PCR (qRT-PCR), pancreatic islets from two to five 10-day-old pups (without sex distinction) were pooled, while islets from adult male rats were extracted individually. piRNA levels were measured using specific custom primers and the miScript Plant RT kit (Qiagen). Measurement of mRNA levels was performed by qRT-PCR (SsoAdvanced Universal SYBR Green Supermix; Bio-Rad) using custom primers (Microsynth, Balgach, Switzerland) (see electronic supplementary material [ESM] Methods). All PCR products were validated by sequencing (Microsynth). piRNA expression was normalised to U6, while mRNA expression was normalised to α-tubulin mRNA using the ΔC_t method.

piRNA profiling Global piRNA profiling was carried out using a Rat piRNA Array (Arraystar, Rockville, MD, USA). Rat piRNA sequences from the NCBI database were mapped to the RN4 genome using UCSC Blat (https://genome.ucsc.edu). Probes for about 40,000 piRNAs were spotted on an Agilent array platform (Agilent Technologies, Santa Clara, CA, USA). Samples were labelled using a RNA ligase method and hybridised onto the piRNA Array in Agilent's SureHyb Hybridization Chambers. Quantile normalisation and data processing were performed using the GeneSpring GX v11.5.1 software (Agilent Technologies). Differentially expressed piRNAs with statistical significance between the two groups were identified through volcano plot filtering. Hierarchical clustering was performed to show the distinguishable piRNA expression pattern among samples.

Prediction of piRNA target genes The putative binding sites of DQ732700 and DQ746748 present in the 3′ UTR sequences of all the genes expressed in rat islets [34] were identified with the Probability of Interaction by Target Accessibility (PITA) algorithm using default parameters [35]. For each piRNA, the top 300 putative targets based on the PITA score were annotated with their gene ontology using biomaRt (http://www.ensembl.org/info/data/biomart/biomart_r_package.html) [36]. Pathway enrichment analysis was performed using DAVID (https://david.ncifcrf.gov/) with Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway annotations.

Statistical analysis Statistical differences were tested using Student's *t* test or, for multiple comparisons, one-way ANOVA followed by a post hoc Dunnett's test, with a discriminating *p* value of 0.05 (GraphPad Software, San Diego, CA). Differences in piRNAs expression between purified beta cells of 10-day-old and adult rats were assessed using 1-sample *t* test, with a discriminating *p* value of 0.05 using the Statistical Package for the Social Sciences (IBM SPSS version 23, www.ibm.com/analytics/us/en/technology/spss/).

Results

The PIWI/piRNA pathway is active in rat pancreatic islets There is mounting evidence to suggest that, in addition to their well-established role in germinal cells, piRNAs may also contribute to the regulation of somatic cell activities [11]. To investigate the potential involvement of piRNAs in the control of pancreatic beta cell functions, we first assessed whether insulin-secreting cells expressed some of the key components of the piRNA pathway. Although to a much lower level compared with rat testis (Fig. 1a), qRT-PCR analysis revealed the expression in rat pancreatic islets of the PIWI-like genes *Piwil2* and *Piwil4* but not *Piwil1* (Fig. 1b). The expression of these two PIWI-like genes was confirmed in FACS-sorted beta cells from adult (3-month-old) (Fig. 1c) and newborn

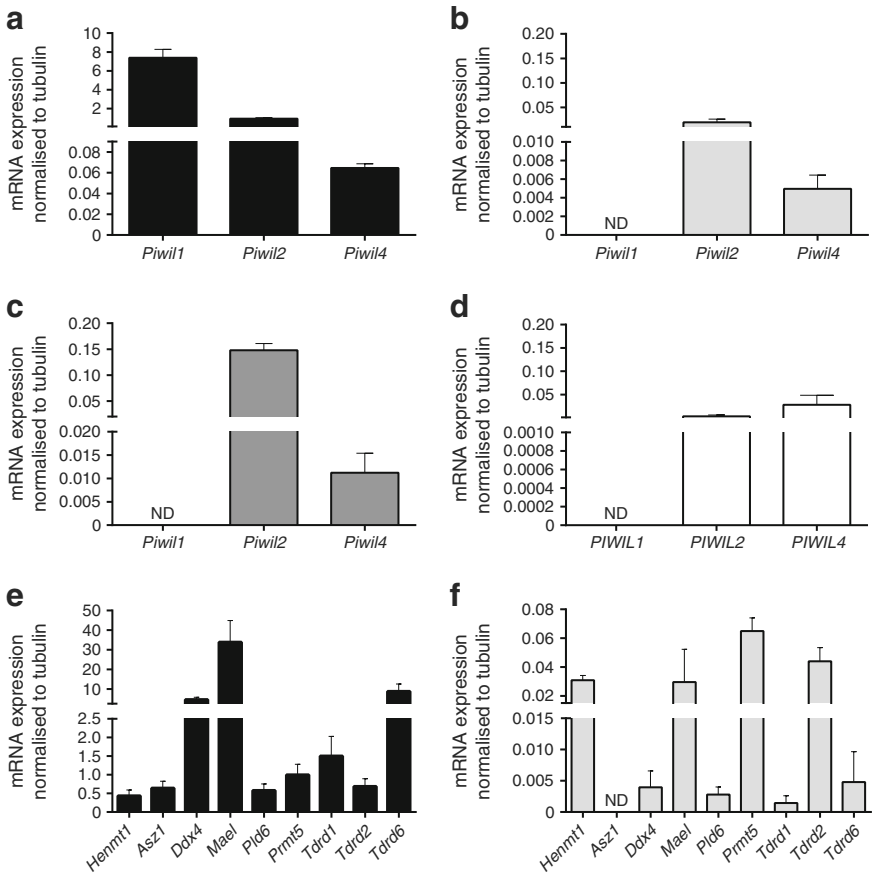

Fig. 1 Pancreatic islets express several components of the *Piwil*–piRNA pathway. qRT-PCR measurement of the level of *Piwil* mRNA in rat testis (**a**), rat pancreatic islets (**b**), FACS-sorted rat beta cells (**c**) and human islets (**d**). qRT-PCR analysis of the mRNAs of selected components of the *Piwil*–piRNA pathway in rat testis (**e**) and rat islets (**f**). Data are mean ± SD, n = 3–4. ND, not detected

(10-day-old) rats (ESM Fig. 1). Similar findings were obtained with human pancreatic islets that were found to express the human orthologues of these genes, *PIWIL2* (also known as *HILI*) and *PIWIL4* (also known as *HIWI2*) (Fig. 1d). The expression of PIWI-like genes in islets was comparable with the level measured in other somatic tissues such as adipose tissue, brain, liver and skeletal muscle (ESM Fig. 1). Beside *Piwil2* and *Piwil4*, qRT-PCR analysis also unveiled the presence in rat pancreatic islets of the mRNAs of several other genes involved in piRNA biogenesis [5, 6, 37], including *Henmt1*, *Mael*, *Ddx4*, *Pld6* and *Prmt5*, which are main players in the pathway, as well as some of the Tudor domain-containing genes [38], *Tdrd1*, *Tdrd2* (also known as *Tdrkh*) and *Tdrd6* (Fig. 1e, f).

The presence of several components of the piRNA pathway prompted us to assess whether pancreatic islets expressed a specific pool of piRNAs. Indeed, analysis of islet RNA isolated from adult and newborn rats using a dedicated microarray allowed the detection of 18,540 of the 40,000 tested piRNAs (GSE93790). Hierarchical clustering summarises the most differentially regulated piRNAs (Fig. 2a).

We then assessed whether islet piRNA expression would be modulated in response to physiological or pathological conditions. Newborn beta cells display major functional differences compared with fully mature beta cells. Neonatal beta cells show a much higher proliferation rate than adult beta cells [34]. Moreover, newborn beta cells are unable to secrete insulin in response to glucose, acquiring this property only after undergoing a postnatal maturation process that involves a major rearrangement in the gene expression profile [34]. We observed that the functional maturation of beta cells was associated with changes in the level of numerous piRNAs. Indeed, by comparing the levels in newborn (10-day-old) and adult (3-month-old) rat islets by microarray analysis, we found that the expression of 735 piRNAs was reduced, whereas that of 1056 piRNAs was increased upon functional beta cell maturation (nominal $p \leq 0.05$, fold change ≥2) (GSE93790). The observed changes in the level of selected piRNAs were confirmed by qRT-PCR in whole islets (Fig. 2b) and in highly purified beta cell fractions (Fig. 2c). These findings suggest that piRNAs may possibly contribute to the functional maturation of beta cells.

We next assessed whether altered piRNA expression could contribute to the beta cell dysfunction observed under diabetic conditions. For this purpose, we compared the piRNA expression profile of the islets of Wistar and GK rats, a well-established animal model of non-obese type 2 diabetes characterised by impaired glucose-stimulated insulin secretion [31, 39]. By microarray analysis, we identified 347 piRNAs that were differentially expressed ($p \leq 0.05$, fold change ≥2) in

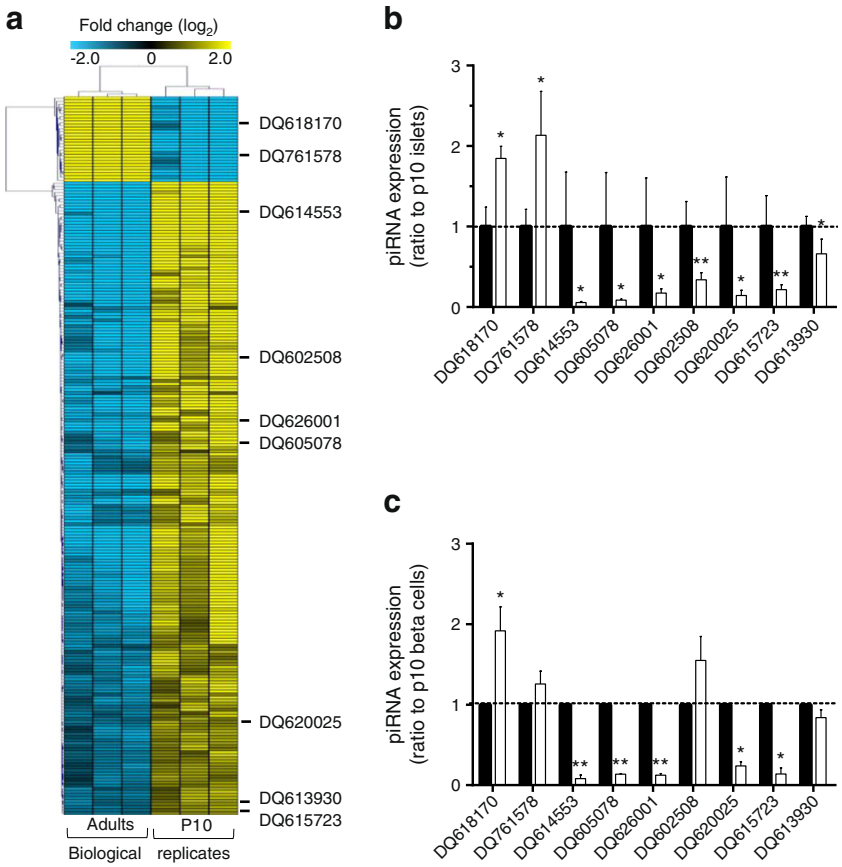

Fig. 2 Changes in the level of piRNAs during beta cell maturation. (**a**) Hierarchical clustering of 236 piRNAs that were differentially regulated ($p < 0.05$, absolute fold change >2, normalised expression >6) between pancreatic islets of adult and newborn rats. piRNAs displaying an upregulation compared with the other group are in yellow, and those displaying a downregulation are in blue. Dark shading represents no significant change. The level of the indicated piRNAs in whole islets (**b**) and purified beta cells (**c**) of newborn (black bars) and adult rats (white bars) was analysed by qRT-PCR. Data are mean ± SD of the ΔC_t values. Statistical differences were assessed by Student's *t* test: $*p \leq 0.05$, $**p \leq 0.01$, $n = 3$

the islets of diabetic GK rats (128 upregulated and 219 downregulated) (GSE93791). Hierarchical clustering summarises the most differentially regulated piRNAs (Fig. 3a). The changes in the level of three of these piRNAs was confirmed by qRT-PCR (Fig. 3b). Thus, our results indicate that the piRNA expression profile is modified under diabetic conditions, potentially contributing to the development of the disease.

PIWI/piRNAs impact on beta cell functions We next investigated whether global changes in piRNA activity would impact on beta cell functions. For this purpose, we used RNA

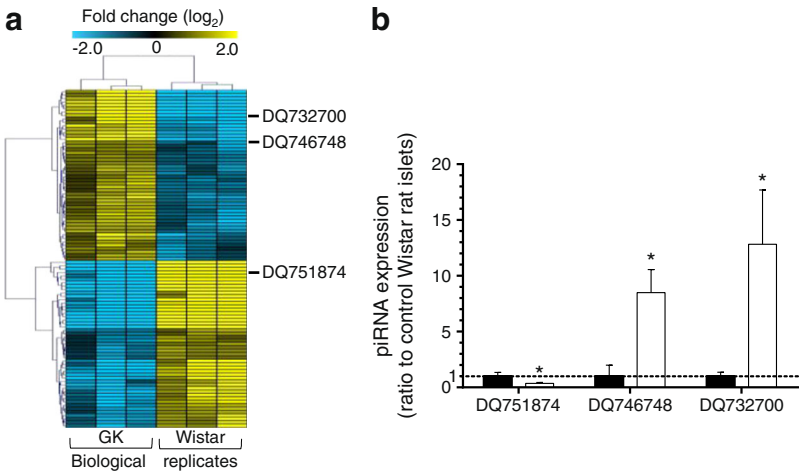

Fig. 3 Changes in piRNA expression in rat models of type 2 diabetes. (**a**) Hierarchical clustering of 99 piRNAs ($p < 0.05$, absolute fold change >2, normalised expression >6) differentially expressed between GK and Wistar rat islets. Dark shading represents no significant change, yellow an upregulation and blue a downregulation. (**b**) The expression of the three indicated piRNAs in the islets of GK (white bars) and Wistar rats (black bars) was measured by qRT-PCR. Data are mean ± SD of the ΔC_t values. Statistical differences were assessed by Student's *t* test: $*p \leq 0.05$, $n = 3$

interference to silence the *Piwil2* and *Piwil4* genes in pancreatic islet cells. si*Piwil2* and si*Piwil4* reduced the expression of their target mRNAs by about 80% and 50%, respectively (Fig. 4a,b). Silencing of *Piwil2* and *Piwil4* genes resulted in a reduction in the level of several piRNAs (Fig. 4c). To investigate whether the impaired piRNA expression would affect the activities of beta cells, we measured insulin secretion in response to glucose or to depolarising KCl concentrations upon silencing of *Piwil2* or *Piwil4* in islet cells. We found that knockdown of *Piwil2* or *Piwil4* did not affect insulin content (Fig. 5a, c). However, in the absence of *Piwil2* or *Piwil4*, both glucose- and KCl-induced insulin secretion were significantly decreased (Fig. 5b, d), suggesting a functional impairment in the secretory machinery that could also involve a defect in glucose metabolism. We then tested cell survival and proliferation. This revealed that, upon silencing of *Piwil2* or *Piwil4*, beta cells became more resistant to cytokine-induced cell death (Fig. 5e). In contrast, beta cell proliferation both in the presence or absence of the mitogenic hormone prolactin was not significantly affected (Fig. 5f).

The observed changes in expression of piRNAs occurring during the acquisition of the mature beta cell phenotype and in diabetic conditions suggest that deregulation of specific piRNAs may potentially contribute to beta cell dysfunction in disease states. To test this hypothesis, we overexpressed DQ732700 and DQ746748 piRNAs in the islet cells of normoglycaemic Wistar rats, in order to mimic the increase

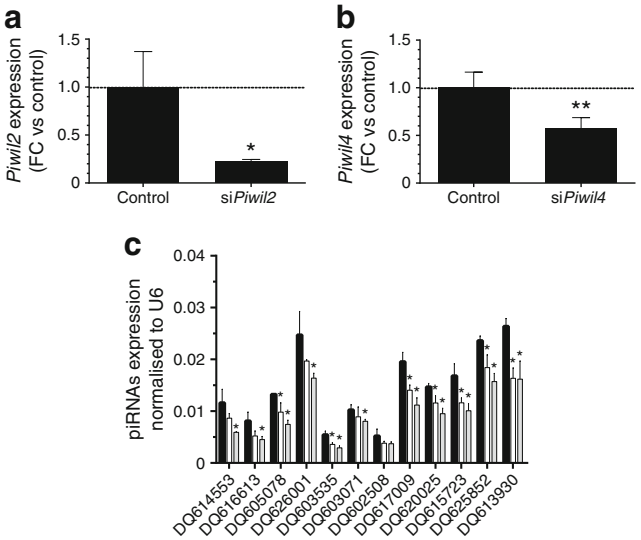

Fig. 4 Downregulation of *Piwil2* and *Piwil4* expression in rat islet cells using siRNAs. (**a**–**c**) Expression of the indicated mRNAs and piRNAs was measured by qRT-PCR. (**a**, **b**) Dissociated rat islet cells were transfected with a control siRNA or with siRNAs directed against *Piwil2* (**a**) or *Piwil4* (**b**). The expression of the indicated mRNAs was measured 48 h after transfection by qRT-PCR (FC, fold change vs control siRNA). (**c**) The level of the indicated piRNAs was measured by qRT-PCR 48 h after transfection with a control siRNA (black bars), si*Piwil2* (white bars) or si*Piwil4* (grey bars). Data are mean ± SD. Statistical differences were assessed by Student's *t* test vs control: $*p \leq 0.05$, $**p \leq 0.01$, $n = 3–5$

observed in the islets of diabetic GK rats, and assessed the impact on insulin secretion. We found that overexpression of DQ732700 or DQ746748 did not affect cellular insulin content (Fig. 6a, c). However, the increase in the level of these two piRNAs resulted in a decrease in insulin release in the presence of elevated glucose concentrations (Fig. 6b). In contrast, insulin secretion in response to depolarising concentrations of KCl was not affected (Fig. 6d). As type 2 diabetes is in some cases associated with beta cell apoptosis, we also assessed whether the overexpression of these two piRNAs would affect the survival of the insulin-secreting cells. As shown in Fig. 6e, the increase in these two piRNAs did not modify the survival of the cells in either the presence or absence of a mix of proinflammatory cytokines including IL-1β, TNF-α and IFN-γ.

piRNAs have been proposed to exert a translational repression through mechanisms similar to those of miRNAs [14]. Thus, we used a computational approach to search for the potential targets of DQ732700 and DQ746748. Interestingly, the putative targets of these piRNAs were significantly enriched for genes involved in insulin secretion and insulin action (GSE93792; ESM Tables 1 and 2).

Discussion

Type 2 diabetes is characterised by a diminished sensitivity of insulin target tissues and defective insulin secretion from beta cells. The mechanisms underlying these phenomena are not fully understood but appear to involve alterations in the expression of mRNAs and non-coding RNAs [28–30]. piRNAs constitute an abundant class of non-coding RNAs and contribute to genome stability. In germ cells, they interact with PIWI proteins during early embryogenesis, enabling the silencing of transposable elements in the genome [5]. In addition to their well-established role in the germline, there is emerging evidence for an involvement of these small RNAs in the regulation of gene expression in somatic cells [10–12, 40]. However, the presence of piRNAs in beta cells and their possible contribution to the regulation of gene expression have so far not been explored. In this study, we used a microarray approach to obtain a comprehensive picture of the piRNAs expressed in rat pancreatic islets and to evaluate the modifications taking place during the acquisition of a fully mature beta cell phenotype. This led to the identification of a large number of piRNAs, many of which display changes in their level occurring in association with the functional maturation of beta cells. These findings suggest that piRNAs may potentially contribute to the development of pancreatic beta cells. Additional studies will be needed to delineate the precise role of piRNAs in the acquisition of specific properties of beta cells, such as the capacity to secrete insulin in response to glucose.

PIWI proteins are essential for the biogenesis and activity of piRNAs and have been suggested to be involved in the

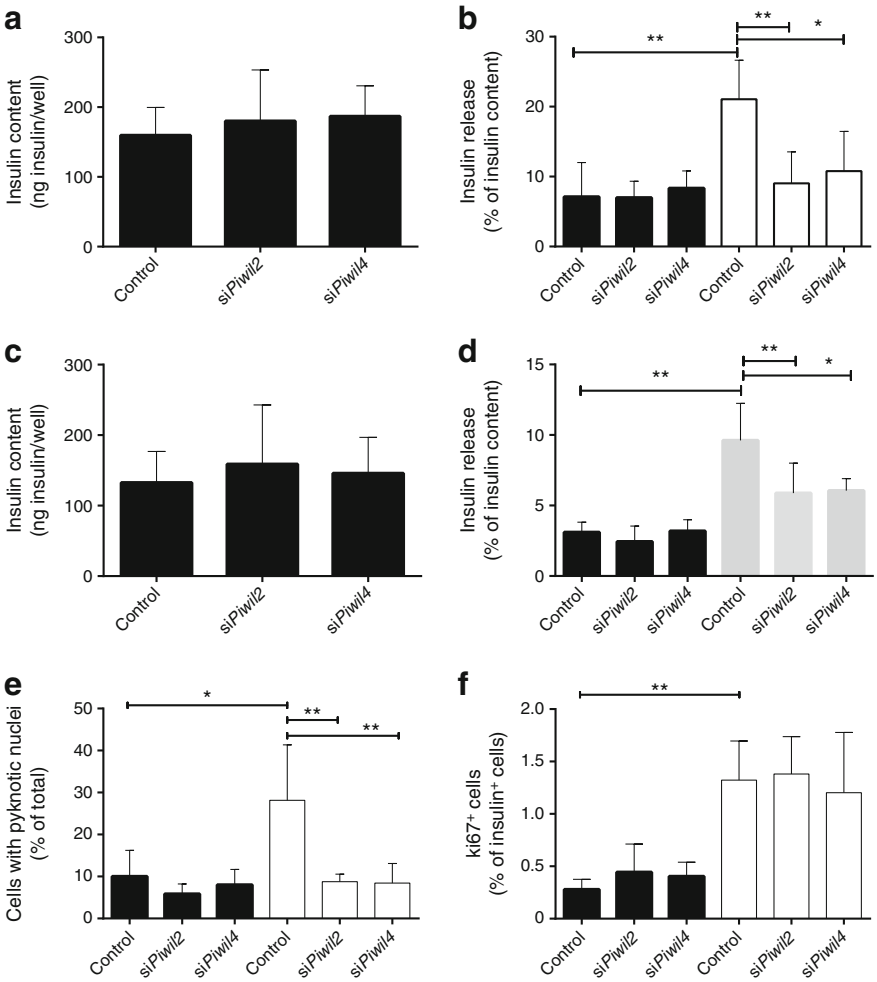

Fig. 5 Functional impact of *Piwil2* and *Piwil4* silencing. Dissociated rat islet cells were transfected with a control siRNA or with siRNAs directed against *Piwil2* or *Piwil4*. The secretory properties of the cells were assessed 48 h later. (**a**) Insulin content and (**b**) insulin secretion in the presence of 2 mmol/l (black bars) or 20 mmol/l glucose (white bars) were measured by ELISA. (**c**) Insulin content and (**d**) insulin secretion at 2 mmol/l glucose in the absence (black bars) or presence (grey bars) of 35 mmol/l KCl. (**e**) Adult rat islet cells were transfected with the indicated siRNAs. One day later, the cells were incubated in the presence (white bars) or absence (black bars) of proinflammatory cytokines (IL-1β, TNF-α and IFN-γ). Cell death was assessed 24 h later by counting the number of cells displaying pyknotic nuclei. (**f**) Proliferation of beta cells transfected with the indicated siRNAs was assessed by counting the fraction of cells positive for insulin and Ki67 in the presence (white bars) or absence (black bars) of 500 ng/ml prolactin. Data are mean ± SD. Statistical differences were assessed by one-way ANOVA: $*p \leq 0.05$, $**p \leq 0.01$, $n = 4–5$

transcriptional, post-transcriptional and epigenetic regulation of gene expression [13–17, 40, 41]. We found that rat and human pancreatic islet cells express two *PIWI* genes. These observations were confirmed in highly purified beta cell fractions, rendering unlikely the possibility that the expression of these genes is confined exclusively to other cells present in the islets. Interestingly, downregulation of *Piwil2* and *Piwil4* mRNAs in rat islets resulted in a significant decrease in insulin secretion upon glucose or KCl stimulation, suggesting that the activity of these PIWI proteins is important to preserve the secretory capacity of beta cells. The silencing of *Piwil2* and *Piwil4* was associated with a decrease in the level of several piRNAs, suggesting that the effect may be linked to changes in the biogenesis, stability and/or activity of these small non-coding RNAs.

The involvement of piRNAs in pathological processes is starting to become clearer. Indeed, the levels of some of these small RNAs have been associated with different clinical conditions and have been proposed as prognostic markers for different types of cancer [42]. However, the understanding of the contribution of these non-coding RNAs to diseases is still rudimentary and necessitates further exploration. *Piwil* mRNA levels have been reported to be deregulated in cancerous tissues and to be correlated with clinicopathological features of the tumours [43–45]. In certain human cancers, *Piwil2* has been reported to affect the survival of the cells by interacting with signal transducer and activator of transcription 3 (STAT3) and by regulating the p53 signalling pathway [46]. Moreover, *Piwil4* has been shown to promote cell

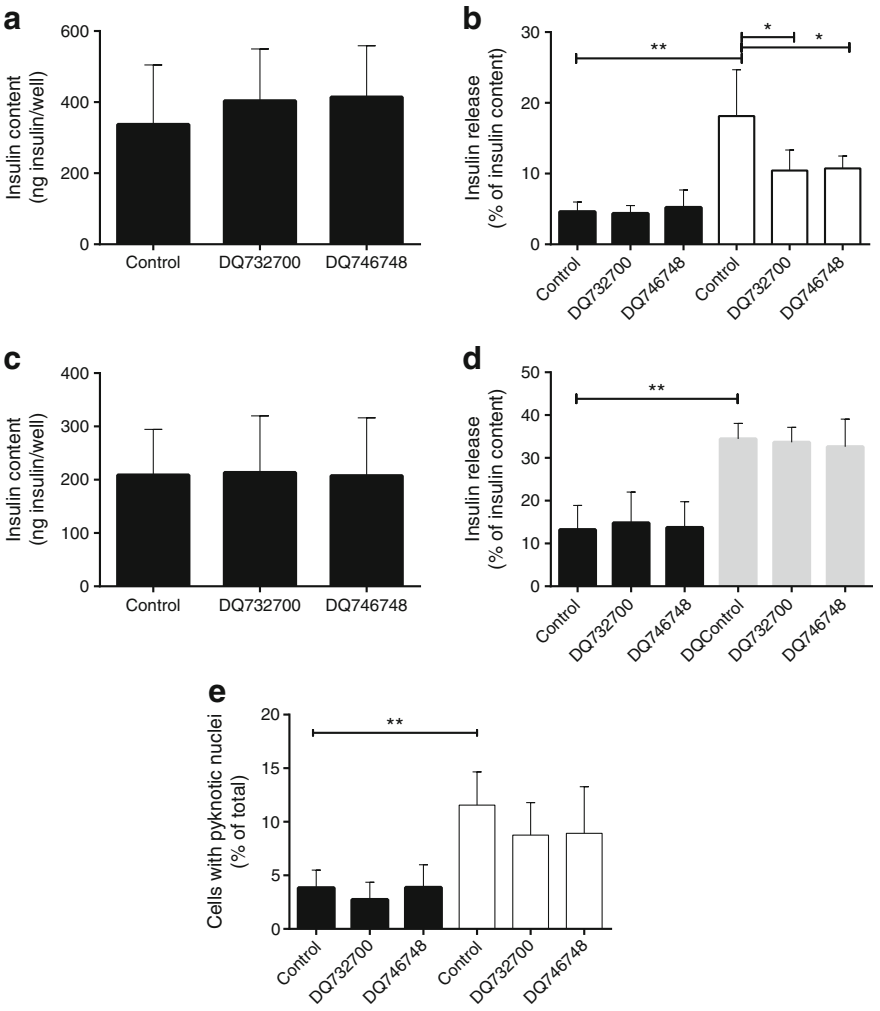

Fig. 6 Effect of the overexpression of DQ732700 and DQ746748 piRNAs. Dissociated rat islet cells were transfected with a control oligonucleotide or with oligonucleotides mimicking the sequence of DQ732700 or DQ746748 piRNAs. Two days later, the cells were incubated for 45 min with 2 mmol/l glucose, 20 mmol/l glucose or 35 mmol/l KCl. (**a**) Insulin content and (**b**) insulin secretion at 2 mmol/l (black bars) and 20 mmol/l (white bars) glucose. (**c**) Insulin content and (**d**) insulin secretion at 2 mmol/l glucose in the absence (black bars) or presence (grey bars) of 35 mmol/l KCl. (**e**) Rat islet cells were transfected with the indicated piRNAs. The following day, the cells were incubated in the absence (black bars) or presence (white bars) of IL-1β, TNF-α and IFN-γ. Cell death was assessed 24 h later by counting the number of cells displaying pyknotic nuclei. Data are mean ± SD. Statistical differences were assessed by one-way ANOVA: $*p \leq 0.05$, $**p \leq 0.01$, $n = 5–6$

proliferation and to inhibit apoptosis in human cervical cancer tissues by downregulating the p14ARF/p53 pathway [47]. A decrease in level of these *Piwil* genes has also been found to be associated with tumour progression and reduced survival of individuals with renal carcinoma [43]. Thus, the role of *Piwil* genes may vary according to the cellular context. In our study, we observed that downregulation of *Piwil2* and *Piwil4* mRNAs in beta cells had a protective effect against cytokine-induced cell death and no significant impact on proliferation. Further investigations will be needed to precisely delineate the signalling pathways through which *Piwil2* and *Piwil4* affect the survival of beta cells.

In this study, we identified several piRNAs that are differentially expressed in the islets of diabetic GK rats, a Wistar substrain obtained by selective breeding of a colony displaying high blood glucose levels. The genetic determinants responsible for the development of diabetes in GK rats are not yet fully established. Thus, the observed changes in piRNA expression may either be due to genetic differences between the GK and Wistar substrains or be the consequence of chronic hyperglycaemia. To investigate the potential role of these piRNAs in beta cell dysfunction and the development of type 2 diabetes, we selected two piRNAs that are upregulated in the islets of GK rats and overexpressed them in the islets of control Wistar rats. Interestingly, this resulted in a selective defect in glucose-induced insulin secretion, providing initial evidence for a contribution of piRNA deregulation to the diabetic phenotype of GK rats. The overexpression of these two piRNAs did not affect cell survival, indicating that the defect in insulin secretion did not result from a toxic effect of these small RNAs. The mechanism through which DQ732700 and DQ746748 affect insulin secretion remains to be determined. As KCl-induced insulin secretion is not impaired, the overexpression of these two piRNAs is unlikely to perturb the expression of general components of the exocytotic machinery.

Our findings would rather suggest that the overexpression of DQ732700 and DQ746748 interferes with the generation of metabolic factors coupling glucose sensing to insulin release. Alternatively, the piRNAs may affect granular recruitment, a process that is dependent on metabolic factors [48, 49]. piRNAs have been suggested to control gene expression via mechanisms analogous to those of microRNAs [14].

Computational prediction of the potential targets of these two piRNAs highlighted an enrichment in genes involved in insulin secretion, insulin action or carbohydrate digestion. Future studies need to experimentally validate these targets and assess whether their silencing can indeed contribute to the effect of the piRNAs.

The unexpected discovery of the existence of thousands of non-coding transcripts with regulatory properties opens new perspectives in the understanding of the mechanisms that govern the activities of mammalian cells. Our data provide initial evidence for an involvement of piRNAs in the control of beta cell functions under both physiological and pathological conditions. These findings add an additional layer of control to the regulation of gene expression in insulin-secreting cells. A better understanding of the role and the mode of action of piRNAs in beta cells will help to elucidate the molecular events driving the acquisition of a mature beta cell phenotype and the causes of beta cell dysfunction in diabetes conditions. This knowledge will be instrumental in engineering beta cell surrogates to replace insulin-secreting cells and in promoting the development of new therapeutic strategies for the treatment of type 2 diabetes.

Data availability Data have been deposited in Gene Expression Omnibus repository under the accession number GSE93792. Data can be accessed via the following link:
https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ojklueugdzehpkv&acc=GSE93792

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

Contribution statement IH, CJ, IGM, CG and JS generated and analysed the data. CJ, IGM, CG and JS critically revised the manuscript and approved its final version. LE contributed to interpreting the data, critically revised the manuscript and approved its final version. RR designed the experiments and interpreted the data. IH and RR wrote the manuscript and approved its final version. RR is the guarantor of this work.

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