miRNAs control insulin content in pancreatic β -cells via downregulation of transcriptional repressors

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MicroRNAs (miRNAs) were shown to be important for pancreas development, yet their roles in differentiated β -cells remain unclear. Here, we show that miRNA inactivation in β -cells of adult mice results in a striking diabetic phenotype. While islet architecture is intact and differentiation markers are maintained, Dicer1-deficient β -cells show a dramatic decrease in insulin content and insulin mRNA. As a consequence of the change in insulin content, the animals become diabetic. We provide evidence for involvement of a set of miRNAs in regulating insulin synthesis. The specific knockdown of miR-24, miR-26, miR-182 or miR-148 in cultured β -cells or in isolated primary islets downregulates insulin promoter activity and insulin mRNA levels. Further, miRNA-dependent regulation of insulin expression is associated with upregulation of transcriptional repressors, including *Bhlhe22* and *Sox6*. Thus, miRNAs in the adult pancreas act in a new network that reinforces insulin expression by reducing the expression of insulin transcriptional repressors.

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Introduction

In adult β -cells, insulin expression is tightly regulated by a network of transcriptional activators and repressors that maintain the β -cell fate and activate the gene in response to elevation in plasma glucose (Andrali *et al*, 2008). Transcriptional activators include, for example, Pdx1 (Petersen *et al*, 1994), MafA (Zhao *et al*, 2005; Kaneto *et al*, 2008), NeuroD (Naya *et al*, 1995), Pax6 (Sander *et al*, 1997),

Nkx2.2 (Sussel *et al*, 1998) and Nkx6.1 (Schisler *et al*, 2005; reviewed in Cerf, 2006). In addition, several transcriptional repressors of the insulin gene have been characterized including Hes1, Insm1/IA1, Sox6, Bhlhe22 and Crem (Peyton *et al*, 1996; Inada *et al*, 1998; Jensen *et al*, 2000; Kang *et al*, 2002; Hald *et al*, 2003; Iguchi *et al*, 2005; Gierl *et al*, 2006; Liu *et al*, 2006; Mellitzer *et al*, 2006; Wang *et al*, 2008; Lan and Breslin, 2009). A fine balance between these opposing transcription factors must be kept for effective insulin synthesis.

Genome-encoded miRNAs create an additional regulatory layer that impacts gene expression post-transcriptionally (reviewed in Bartel, 2009). miRNAs are important for β -cell differentiation and function, and specific miRNAs have been proposed to regulate β -cell genes (Joglekar *et al*, 2007; Poy *et al*, 2007; Hennessy and O'Driscoll, 2008). For example, miR-124a was shown to affect the expression of FoxA2 and Pdx1 (Baroukh *et al*, 2007), the secretory pathway proteins SNAP25 and Rab3a, as well as the ion channels Kir6.2 and Sur1 (Baroukh *et al*, 2007; El Ouaamari *et al*, 2008). Likewise, miR-375, one of the most abundant miRNAs in the endocrine pancreas, was suggested to affect insulin secretion through regulation of myotrophin expression in cultured MIN6 cells (Poy *et al*, 2004; Krek *et al*, 2005). Furthermore, genetic loss of miR-375 function in mice causes decreased β -cell proliferation and mass, increased α -cell numbers, increased plasma level of glucagon and increased hepatic gluconeogenesis (Poy *et al*, 2009). Therefore, miR-375 has a pivotal and intriguing endocrine function that encourages further evaluation of the role of miRNAs *in vivo*.

miRNAs are subject to extensive processing, including digestion by Drosha in the nucleus (Lee *et al*, 2003) and by Dicer1 in the cytoplasm (Bernstein *et al*, 2001). Deletion of Dicer1 in the early pancreatic lineage, using a Pdx1-Cre mouse line resulted in inactivation of the entire miRNA pathway in the early pancreatic bud (Lynn *et al*, 2007). This early inactivation of Dicer1 caused pancreas agenesis, demonstrating that miRNAs are important for pancreas organogenesis. However, the severe phenotype precluded analysis of the role of miRNAs in adult β -cells.

We sought to uncover the role of miRNAs in the adult endocrine pancreas and produced mutant mice, in which the deletion of a Dicer1 conditional allele is directed spatially by the rat insulin promoter (RIP) and temporally by a tamoxifen-inducible Cre recombinase. We show that miRNA function is critical for maintenance of the β -cell hormone-producing phenotype, for the proper balance of transcriptional activators and repressors of insulin expression and for normal glucose homeostasis.

Results

β -cell-specific disruption of Dicer1 causes glucose intolerance

As Dicer1 deficiency blocks the output of the entire miRNA repertoire, with just a single described exception

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Figure 1 Tamoxifen treatment induces the deletion of *Dicer1* causing a reduction in *Dicer1* and miRNA levels. (A) *Dicer1* disruption was achieved by deletion of a floxed *Dicer1* allele by a tamoxifen-inducible Cre recombinase under the control of the β -cell-specific rat insulin promoter. Concomitant with *Dicer1* deletion, Cre-mediated recombination activates GFP expression by removal of a stop cassette that prevents its expression. qPCR performed on whole islets from control ($n=4$) and mutant *RIP-CreER;Dicer1^{LoxP/LoxP}* ($n=4$) mice at 3 weeks post-induction reveals (B) a reduction in *Dicer1* mRNA and (C) reduction in the expression of mature miRNA *mir-7*, *mir-375*, *mir-27* and *mir-24* genes. Values shown are mean $\pm$ s.e.m. normalized to control. ** $P < 0.01$.

(Cheloufi *et al*, 2010), it provides an opportunity to assess the overall contribution of this network to β -cell function *in vivo*. We therefore crossed a *Dicer1* conditional allele (Harfe *et al*, 2005) onto an inducible CreER transgene that is driven by the RIP (Dor *et al*, 2004). In the resultant *RIP-CreER;Dicer1^{LoxP/LoxP}* mice, miRNA function is intact until tamoxifen is injected (Figure 1A). Upon tamoxifen injection, CreER translocates into the β -cell nucleus and inactivates the *Dicer1* conditional allele, thereby preventing miRNA processing. Concomitantly, a lacZ/EGFP (Z/EG) reporter transgene (Novak *et al*, 2000) which was crossed into the *RIP-CreER;Dicer1^{LoxP/LoxP}* mouse, is also subject to CreER activity. Thus, cells in which recombination occurred lose *Dicer1* activity and are often additionally labelled with GFP. In control mice, which are heterozygous for the *Dicer1* allele (*RIP-CreER;Dicer1^{LoxP/+}*) and harbour the Z/EG reporter transgene (referred to as 'control' hereafter), tamoxifen induces loss of only one *Dicer1* allele and activates GFP expression.

A quantitative real-time PCR (qPCR) study of *Dicer1* mRNA levels in islets of tamoxifen-treated mice revealed a 50% reduction in *Dicer1* expression (Figure 1B). Taking into account the relative abundance of β -cells in islets (~60%), it is likely that *Dicer1* was deleted in a significant fraction of the β -cells of *RIP-CreER;Dicer1^{LoxP/LoxP}* animals. Consistently, representative miRNA, some of which are characteristic of pancreatic endocrine cells, were downregulated (Figure 1C).

RIP-CreER;Dicer1^{LoxP/LoxP} animals developed fasting and fed hyperglycaemia 2 weeks after tamoxifen induction. The hyperglycaemia rapidly deteriorated by 3 weeks post-induction (Figure 2A). Consistent with this, *RIP-CreER;Dicer1^{LoxP/LoxP}* animals were glucose intolerant (Figure 2B). Taken together,

these results indicate that deletion of *Dicer1* in adult β -cells causes overt diabetes.

Disruption of *Dicer1* causes a decrease in β -cell insulin protein content

Plasma insulin levels were significantly lower in *RIP-CreER;Dicer1^{LoxP/LoxP}* animals than in control animals (Figure 2C), whereas plasma glucagon levels were unaffected by loss of *Dicer1* activity in β -cells (not shown). We next evaluated insulin secretion immediately after an intraperitoneal injection of a glucose bolus. While in control animals, glucose injection resulted in a substantial increase in insulin secretion, in *RIP-CreER;Dicer1^{LoxP/LoxP}* mice insulin secretion was significantly diminished (Figure 2D). Impairment of glucose stimulated insulin secretion may result from a defect in insulin exocytosis, a decrease in pancreas insulin content, or both. Indeed, miRNA were suggested in pathways regulating insulin exocytosis (Poy *et al*, 2004; Plaisance *et al*, 2006; Baroukh *et al*, 2007; Lovis *et al*, 2008). However, measurement of the total pancreatic insulin content revealed an 80% decrease in insulin in *RIP-CreER;Dicer1^{LoxP/LoxP}* animals relative to controls (Figure 2E). Therefore, although defects in insulin exocytosis, resulting from perturbation of miRNA expression, probably also impact insulin secretion, the dramatically low levels of insulin in the mutant pancreata are a key component in the hypoinsulinemic hyperglycaemia as we observed.

Surprisingly, morphometric analysis of *RIP-CreER;Dicer1^{LoxP/LoxP}* pancreata suggests that the β -cell mass calculated from the total area of insulin positive immunoreactivity may be slightly, but not significantly, different from that of control mice. Moreover, analysis of the average islet size showed that the size distribution in mutants was comparable to that of

Figure 2 RIP-CreER;Dicer1^{LoxP/LoxP} mice are hyperglycaemic and display hypoinsulinemia associated with decreased pancreas insulin content. (A) Fed (left) and fasting (right) plasma glucose levels in control and RIP-CreER;Dicer1^{LoxP/LoxP} mutant mice 2 weeks (fed $n \geq 5$, fasting $n \geq 15$) and 3 weeks (both $n \geq 15$) after tamoxifen treatment. (B) A glucose tolerance test performed 2 weeks (left) and 3 weeks (right) after tamoxifen treatment ($n \geq 15$). (C) Fed (left) and fasting (right) plasma insulin levels in control and RIP-CreER;Dicer1^{LoxP/LoxP} mutant mice 2 and 3 weeks after tamoxifen treatment ($n \geq 5$). (D) Plasma insulin levels before a glycemic challenge and 15 min later, reveal poor insulin secretion in the RIP-CreER;Dicer1^{LoxP/LoxP} mutant mice ($n \geq 5$). (E) The pancreatic insulin content of RIP-CreER;Dicer1^{LoxP/LoxP} mice is 1/5 that of the control insulin content, 3 weeks after tamoxifen treatment ($n \geq 4$). Micrographs of insulin immunohistochemical staining of pancreas sections from control (F, G) and RIP-CreER;Dicer1^{LoxP/LoxP} (H, I) 3 weeks after tamoxifen treatment. Panels (G, I) are a magnified view of (F, H). Scale bar = 20 μ m. * $P < 0.05$, ** $P < 0.01$.

controls (Supplementary Figure S1A and B). Accordingly, we did not detect apoptosis using either TUNEL or activated caspase-3 immunostaining (Supplementary Figure S1C).

Because loss of β -cell mass could not account for the dramatic decrease in insulin content in the RIP-CreER;Dicer1^{LoxP/LoxP} pancreata, we evaluated insulin expression in β -cells at the cellular level. While immunohistochemical analysis of insulin expression showed a uniform and strong expression in the controls (Figure 2F and G), the expres-

sion of insulin in RIP-CreER;Dicer1^{LoxP/LoxP} animals was downregulated and varied considerably from cell to cell (Figure 2H and I).

In order to perform our analysis at a single-cell resolution, we took advantage of the incomplete activation of the CreER transgene, resulting in recombination mosaicism within the islet. Thus, in islets of control animals, GFP expression is variable and only a subset of the insulin-positive β -cells co-expressed GFP (Figure 3A–D). The mutant pancreata

Figure 3 RIP-CreER;Dicer1^{LoxP/LoxP} β -cells cease to synthesize insulin. Control (A–D) and RIP-CreER;Dicer1^{LoxP/LoxP} (E–H) mutant mice 3 weeks after tamoxifen treatment were stained for insulin (A, E) and for GFP expression that marks the recombined cells within the islet (B, F). The mosaic expression of GFP reflects incomplete induction by tamoxifen. In the merged view (C, D, G, H), co-localization of insulin and GFP staining yields a yellow colour in control (C, D) but not in RIP-CreER;Dicer1^{LoxP/LoxP} mice (G, H). At least three animals per genotype were examined. Scale bar = 20 μ m. (I) A reduction in the mRNA levels of insulin1 (left), insulin2 (middle), and Cre (right) in RIP-CreER;Dicer1^{LoxP/LoxP} mutant mice relative to control is revealed by qPCR analysis on isolated islets from at least four animals. Values shown are mean $\pm$ s.e.m. * P < 0.05, ** P < 0.01.

exhibit similar mosaicism, which explains the variability in cellular insulin content in Figure 2H and I. Therefore, Dicer1 knockout and wild-type β -cells reside in the same islet, providing the opportunity for molecular analysis at a single-cell resolution. Further, adjacent mutant and wild-type cells are exposed to the same physiological conditions. We compared insulin expression between GFP-positive cells (in which recombination occurred) and GFP-negative cells (in which recombination had not occurred) within the same islet. In control pancreata, which are heterozygous for Dicer1 conditional allele and undergo GFP induction, GFP was co-expressed with insulin, as expected (Figure 3A–D). In contrast, Dicer-null/GFP-positive cells showed reduced or even total absence of insulin expression (Figure 3E–H).

Insulin mRNA levels are downregulated in Dicer1-null β -cells

The observed decrease in insulin at the protein level could result from a defect in any of the steps in insulin production. However, post-translational defects are not very likely as the antibody used to detect insulin recognizes both insulin and pro-insulin. Furthermore, the levels of the insulin processing enzyme, prohormone convertase 1/3, were similar between GFP-positive and GFP-negative cells in RIP-CreER;Dicer1^{LoxP/LoxP}, as well as similar to control pancreata (Supplementary Figure S2). Thus, the effect of Dicer1 loss is

likely due to transcriptional or post-transcriptional regulation of insulin expression.

To address a possible effect of miRNA dysregulation on insulin transcript levels, we measured the expression of the two murine insulin isoforms. qPCR performed on isolated islets from CreER;Dicer1^{LoxP/LoxP} animals revealed 70% decrease in both insulin1 and insulin2 mRNA levels (Figure 3I, left and middle). Thus, loss of insulin expression is a consequence of changes in insulin mRNA levels. While regulation of insulin gene expression occurs primarily at the transcriptional level, mRNA levels may be downregulated due to increased mRNA degradation. To distinguish between these two possibilities, we examined the level of Cre mRNA, a transcript unrelated to insulin, whose expression is artificially driven by the insulin promoter in these transgenic mice. We found Cre mRNA to be significantly downregulated as well (Figure 3I, right), suggesting that the insulin promoter is transcriptionally less active in RIP-CreER;Dicer1^{LoxP/LoxP} β -cells.

Dicer1-null β -cells maintain their differentiation markers

To explain reduced insulin mRNA levels we considered two alternative scenarios. First, RIP-CreER;Dicer1^{LoxP/LoxP} cells might have lost their identity, regressing into an earlier state of differentiation or even assuming a different cell fate altogether. Alternatively, the regulation of the insulin gene might be specifically perturbed in otherwise normal β -cells.

Figure 4 RIP-CreER;Dicer1^{LoxP/LoxP} β -cells maintain β -cell characteristics and do not acquire alternate endocrine markers. Immunofluorescence staining of the endocrine marker: synaptophysin (red, **A, B**) and a mixture of antibodies raised against the hormone markers: glucagon, ghrelin, pancreatic polypeptide (PP) and somatostatin (SS) (red, **C, D**) shows no change in GFP-positive cell marker expression between control (**A, C**) and RIP-CreER;Dicer1^{LoxP/LoxP} mutants (**B, D**). Similarly, characteristic β -cell transcription factors (red) Pdx1 (**E, F**) MafA (**G, H**) Nkx6.1 (**I, J**) Pax6 (**K, L**) are coexpressed with GFP (green) both in controls (**E, G, I, K**), and in RIP-CreER;Dicer1^{LoxP/LoxP} mutants (**F, H, J, L**). At least three animals per genotype were examined. Scale bar = 20 μ m. Yellow colour indicates co-localization of red and green channels.

Analysis of markers for the endocrine identity of β -cells showed that Dicer1 null β -cells (green, Figure 4B and D) were indistinguishable from their control counterparts (green, Figure 4A and C). The RIP-CreER;Dicer1^{LoxP/LoxP} cells maintained the expression of the secretory vesicle protein, synaptophysin (red, Figure 4A and B), and did not express alternative hormone markers including somatostatin, pancreatic polypeptide, glucagon or ghrelin (red, Figure 4C and D). In order to test whether the cells assume a progenitor state, we performed immunostaining for the endocrine progenitor marker, Ngn3, but did not detect any expression (not shown). Therefore, we conclude that Dicer1-null β -cells maintain at least some endocrine features of mature β -cells and do not express alternative fate markers.

In order to further characterize the identity of the Dicer1-null β -cells, we analysed the expression of transcription factors that are typical of mature β -cells, namely Pdx1, MafA, Nkx6.1 and Pax6. The immunostaining of these β -cell markers (red, Figure 4E–L) was similar in GFP-positive/Dicer1-null cells in RIP-CreER;Dicer1^{LoxP/LoxP} mice

(green, Figure 4F, H, J and L) and GFP-positive/Dicer1 heterozygous cells in control mice (green, Figure 4E, G, I and K). Detailed quantification of RIP-CreER;Dicer1^{LoxP/LoxP} pancreata revealed similar expression of Nkx6.1, Pax6 and MafA in GFP-positive/Dicer1-null (mutant) cells, relative to insulin-positive (wild-type) cells (> 1300 individual nuclei/group; Supplementary Figure S3). Likewise, qPCR analysis revealed that the expression level of a set of transcriptional activators—Pdx1, NeuroD/Beta2, Nkx2.2, Nkx6.1 and MafA was comparable between RIP-CreER;Dicer1^{LoxP/LoxP} and control islets (Figure 5A). We conclude that RIP-CreER;Dicer1^{LoxP/LoxP} cells largely maintain their primary β -cell markers, suggesting the inhibition of the insulin promoter activity is likely not due to a decrease in the abundance or altered localization of transcriptional activators. To further analyse the identity of the mutant β -cells, we quantified mRNA levels of genes associated with glucose sensing, including glucokinase (Gck), Glut2 and Kir6.2. These genes were also expressed at comparable levels in mutants and controls (Figure 5A). Hence, RIP-CreER;Dicer1^{LoxP/LoxP}

Figure 5 Transcriptional repressors of insulin expression are upregulated in RIP-CreER;Dicer1^{LoxP/LoxP} islets. **(A)** qPCR analysis of RNA isolated from control and RIP-CreER;Dicer1^{LoxP/LoxP} mutant islets. The expression levels of the transcriptional activators Pdx1, Nkx2.2, Nkx6.1, MafA and NeuroD1 is largely unaffected by the loss of miRNA activity. The expression of Gck, Glut2 and Kir6.2 is unchanged as well. However, the mRNA levels of some of the transcriptional repressors examined, Stx3, Hes1, Crem, Insm1, Sox6, Tle4, Bhlhe22, are significantly increased. Expression levels were normalized to the level in the controls, $n \geq 3$ for all samples. **(B)** Overexpression of transcriptional repressors, Sox6 and Bhlhe22, in HIT cells causes a reduction in luciferase reporter activity that is driven by the rat insulin promoter. $n \geq 3$ for all samples. Values shown are mean $\pm$ s.e.m. ** $P < 0.01$.

β -cells retain most of their mature molecular identity markers including the expression of genes involved in glucose sensing and activator genes upstream of insulin transcription.

Dicer1-null β -cells upregulate the expression of a set of transcriptional repressors

The fact that insulin transcription is downregulated in RIP-CreER;Dicer1^{LoxP/LoxP} β -cells while chief transcriptional activators of the insulin promoter are normally expressed is intriguing. This could be explained by an abnormal upregulation of transcriptional repressors in mutant β -cells. We therefore used qPCR to quantify the expression levels of several transcription factors, previously reported to function as repressors of insulin synthesis (Peyton *et al*, 1996; Inada *et al*, 1998; Jensen *et al*, 2000; Kang *et al*, 2002; Qiu *et al*, 2002; Iguchi *et al*, 2005; Gierl *et al*, 2006; Liu *et al*, 2006; Mellitzer *et al*, 2006; Bar *et al*, 2008; Wang *et al*, 2008). Of the seven repressors that were examined, we noted a four-fold increase in the mRNA levels of Sox6 and a three-fold increase in the expression of Bhlhe22. Other repressors including INSM1, Crem and TLE4 were upregulated to lesser extent (Figure 5A). We therefore considered Sox6 and Bhlhe22 as

prime candidates for controlling insulin expression in RIP-CreER;Dicer1^{LoxP/LoxP} cells.

To directly test the effect of the two repressors on insulin gene transcription, we overexpressed Sox6 and Bhlhe22 along with a construct harbouring luciferase under the control of the RIP in HIT cells. Sox6 and Bhlhe22 were able to significantly repress insulin synthesis, as measured by the reporter, similar to previous reports (Peyton *et al*, 1996; Iguchi *et al*, 2005). These results indicate that Sox6 and Bhlhe22 upregulation in RIP-CreER;Dicer1^{LoxP/LoxP} islets is likely involved in causing the reduced insulin expression (Figure 5B).

Loss of specific miRNA function alters insulin expression

As Dicer1 deletion removes the entire miRNA repertoire in β -cells, we sought to determine specific miRNA genes that may be responsible for the dysregulation of insulin expression.

First, we created a profile of miRNA expression in islets of Langerhans by microarray analysis, identifying the miRNA milieu of the mouse endocrine pancreas (Supplementary Figure S4). We further examined a few miRNAs that were

Figure 6 Individual miRNA genes regulate insulin expression. **(A)** Relative activity of a RIP-luciferase reporter, transfected with various indicated anti-miR oligos. Data normalized to the expression of RIP-luciferase in MIN6. **(B)** Specific miRNA knockdown in isolated islets, using cholesterol-conjugated ZEN-AMOs, attenuated insulin mRNA levels. Specific miRNA knockdown in isolated islets caused concomitant increase in endogenous mRNA of **(C)** Sox6 or **(D)** Bhlhe22. Data shown are mean $\pm$ s.e.m. normalized to the expression in control (NC1-treated) islets. $n \geq 3$ experiments for all samples except miR-182 where $n = 2$. **(E)** A schematic representation of the constructs containing luciferase (Luc) sequences upstream of the 3' UTR sequences of Sox6 or Bhlhe22. Vertical bars mark the location of potential minimal miRNA-binding sites. The reporter constructs were transfected alone into HEK293 cells (black bar), along with various miRNA overexpression constructs (grey bar) or with miRNA overexpression vectors and anti-miRNA inhibitors (white bar). Measurements of luciferase activity controlled by **(F)** the Sox6-3' UTR, or by **(G)** the Bhlhe22-3' UTR. The bars represent the percentage of *renilla* luciferase activity normalized to the activity of firefly luciferase that does not harbour the 3' UTR sequence and then normalized to expression of the 3' UTR alone. Values shown are mean $\pm$ s.e.m. of three samples each. * $P < 0.05$, ** $P < 0.01$.

highly expressed and checked whether they have predicted minimal binding sites within the 3' UTR sequence of Sox6 or Bhlhe22 mRNAs, using *Pita* with maximal sensitivity (Kertesz *et al*, 2007).

Next, we used miRNA inhibitors to knock down specific miRNAs namely, miR-26, miR-148, miR-182, miR-24, miR-

200/141, miR-103 and miR-7 in cultured β -cells and assayed for changes in the expression of a luciferase reporter, driven by the minimal RIP. An anti-miRNA oligo (AMO) directed against miR-7 upregulated insulin, suggesting that this neuroendocrine miRNA is a negative regulator of insulin synthesis. Knockdown of miR-24 and miR-103 did not affect

insulin expression in MIN6 cells. However, the knockdown of miR-26, miR-148, miR-182 and miR-200/141 repressed luciferase synthesis considerably (Figure 6A), suggesting that these miRNAs are positive regulators of insulin transcription.

Based on the data obtained from the study of individual miRNAs in MIN6 cell culture, we carried out a secondary knockdown study in islet organ culture. Thus, we isolated primary murine islets and cultured them in the presence of specific cholesterol-conjugated anti-miR inhibitors oligos (see methods). The cultured islets were harvested after 6 days and endogenous insulin mRNA levels were quantified by qPCR. We discovered that inhibition of miR-26, miR-148, miR-182 and miR-24 downregulated insulin mRNA in primary cultured islets (Figure 6B). Furthermore, Sox6 and Bhlhe22 were upregulated in some of these samples. miR-24 and miR-148a knockdown significantly upregulated Sox6 (Figure 6C) and miR-182 knockdown significantly upregulated Bhlhe22 (Figure 6D), suggesting that miRNA may affect insulin, at least in part, through de-repression of these transcriptional repressors (Figure 6C and D). Finally, in order to functionally assess potential direct interactions of the candidate miRNA with Sox6 and Bhlhe22, we cloned their 3' UTR into vectors expressing a *renilla* luciferase reporter along with a firefly luciferase control. These reporters were transfected into HEK293 cells alone, or in combination with miRNA over-expression with or without the addition of AMOs. Two days later, the luciferase activity of the reporter was measured. We found that indeed the expression of the Bhlhe22 and of the Sox6 3' UTR luciferase reporters responded to changes in the levels of miR-26, miR-148, miR-24 or miR-200, suggesting direct interactions of individual miRNAs and the mRNA encoding for Bhlhe22 and Sox6 (Figure 6E–G).

Collectively, our data suggest that a network of miRNAs act to maintain the natural balance between transcriptional repressors and activators of the insulin 1 and insulin 2 genes in adult β -cells. Dicer1 deletion causes an imbalance between these transcriptional regulators thereby preventing insulin synthesis. Direct interference with the function of specific miRNAs in cultured MIN6 and in primary islets highlighted the involvement of miR-24, miR-26, miR-148 and miR-182 in this process. Thus, loss of specific miRNAs directly impinges on insulin mRNA levels consistent with the observed compromised glucose homeostasis that results from perturbation of miRNA maturation *in vivo*.

Discussion

Our results demonstrate the importance of miRNAs in fully differentiated β -cells. RIP-CreER;Dicer1^{LoxP/LoxP} mice are hyperglycaemic and show severe glucose intolerance because they fail to generate mature miRNAs. Our mouse model provided an opportunity to study the distinctive regulatory functions of miRNAs in the adult organ. We discovered that Dicer1-deficient β -cells mostly retain their identity, including the expression of typical β -cell markers involved in glucose sensing and insulin transcription. However, they show an increase in repressors of insulin transcription. Tissue histomorphology and marker analysis suggested that the effects of miRNA loss of function in adult non-mitotic β -cells are relatively specific to insulin production without affecting the expression of several key transcription factors essential for insulin promoter activity. Nonetheless, previously reported

effects of miRNAs on insulin secretion probably contribute to the low levels of circulating insulin in the Dicer1 model.

Recently, Poy *et al* (2009) generated a mouse loss of function allele of miR-375. This model is very important, as it is the first genetic loss of function model for a miRNA in the endocrine pancreas. The normo-insulinemic hyperglycaemia of miR-375 knockout animals is largely explained by hyperactivation of the glucagon axis, plausibly reflecting critical roles for miR-375 in α -cells (Poy *et al*, 2009). As miR-375 loss of function did not impinge on insulin synthesis and as plasma glucagon levels do not change significantly in our RIP-CreER;Dicer1^{LoxP/LoxP} mice, it is likely that miRNAs other than miR-375 are involved in normal insulin production in adult β -cells.

Our study shows that β -cell miRNAs act to control the tightly regulated network of positive and negative transcription factors that determine insulin production. The reduction in pancreas insulin content following miRNA perturbation is likely related, at least in part, to upregulation of Sox6 and Bhlhe22. However, other negative regulators of insulin synthesis may act downstream of miRNAs alongside these genes.

Intriguingly, Sox6 and Bhlhe22 were implicated previously in repressing essential insulin activators. Sox6 was shown to interfere with Pdx1 function (Iguchi *et al*, 2005) and Bhlhe22 was reported to be a competitive inhibitor of BETA2/NeuroD (Peyton *et al*, 1996). However, the expression levels of Gck, whose transcription is driven also by Pdx1 and NeuroD (Watada *et al*, 1996; Moates *et al*, 2003) was not changed in the Dicer1 model. It will therefore be important to understand if other targets of Pdx1 and Beta2/NeuroD are affected by the upregulation of Bhlhe22 and Sox6 in order to appreciate how these factors control the transcriptional regulatory network in β -cells.

Our individual miRNA knockdown in cultured islets revealed that the global effect of Dicer1 may be deconstructed into the effect of several individual miRNAs, likely acting in concert upstream of insulin expression. These include miR-182, which is encoded by a single copy in the genome, miR-24 and miR-148 with two copies each (miR-24-1, miR-24-2; miR-148a, miR-148b) and miR-26 that has three different genomic copies (miR-26a1, miR-26a2 and miR-26b). The rather limited effect detected for individual miRNA knockdown, compared with the dramatic downregulation of insulin *in vivo* in the Dicer1 model, suggests that a combinatorial function of multiple miRNAs underlies the Dicer1 phenotype. This combined effect allows repression of unwanted genes that would otherwise impair insulin synthesis. Thus, miR-24/26/182/148 act as positive regulator of insulin transcription. Moreover, most of the miRNAs in β -cells must belong to the positive regulator group downstream of Dicer1. In contrast and rather surprisingly, knockdown of the pancreas-specific miR-7 resulted in upregulation of insulin expression, suggesting it acts as a negative regulator.

The significance of miRNAs in β -cells may be related to the physiological response to hyperglycaemia and obesity. For example a few miRNAs, including miR-24 and miR-26, are upregulated in response to glucose stimulation (Tang *et al*, 2009; Hennessy *et al*, 2010). Therefore, insulin transcription may respond to the glycemic state through changes in specific miRNAs upstream of Sox6 and Bhlhe22. Further, in insulin-resistant conditions, Sox6 downregulation leads to increased insulin production (Iguchi *et al*, 2005). In the future, it will be important to check if upregulation of miRNA such as miR-24 and miR-26 or miR-148 is involved in β -cell adaptation to

obesity-related insulin resistance and/or to hyperglycaemia through repression of Sox6 and Bhlhe22 expression.

Regulation of insulin synthesis and its secretion is a fundamental function of β -cells. Studying miRNAs in an adult mammal tissue surprisingly revealed a relatively limited effect, albeit crucial, primarily controlling insulin synthesis. The emerging picture from our study is that in the non-manipulated adult pancreas, miRNAs safeguard against unwanted elevation in the expression of a few transcriptional repressors including Bhlhe22 and Sox6. These miRNAs are intertwined into pivotal cellular pathways in adult β -cells, encouraging future analysis of potential mutations in miRNA genes in diabetes patients. Additionally, studies of specific miRNAs involved in the upkeep of insulin transcription provide promise for improving insulin synthesis thereby advancing towards effective cell-replacement therapy for diabetes.

Materials and methods

Mouse handling and physiology

The RIP-CreER transgene (Dor *et al*, 2004), Dicer1^{flxed} allele (Harfe *et al*, 2005) and Z/EG (Novak *et al*, 2000) mice were crossed and PCR genotyped using primers described in Supplementary data. Mice were housed and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of WIS. Five doses of 8 mg tamoxifen 20 mg/ml dissolved in corn oil were injected subcutaneously, *dieb. alt.* to 1–5 months old animals. Glucose tolerance tests were performed by injecting glucose (2 mg/g BW) intraperitoneally after an overnight fast and measuring blood glucose levels using an *Acsensia elite* glucometer. A glucose stimulated insulin secretion test was performed by retro-orbital bleeding either immediately after an overnight fast or 15 min after an intraperitoneal glucose injection (2 mg/g BW). Pancreatic insulin content and serum insulin levels were determined using an ultrasensitive rat insulin ELISA kit (Crystal Chem).

Pancreatic histology and immunohistochemistry

Dissected pancreata were fixed in 4% paraformaldehyde for 24 h at room temperature and then processed into paraffin blocks. Antigen retrieval in a 2100-Retriever (PickCell Laboratories, The Netherlands) was performed on 3–5 μ m-thick rehydrated sections before immunostaining with antibodies described in Supplementary data. Fluorescence images were captured using a Zeiss LSM510 Laser Scanning confocal microscope system under a magnification of $\times 40$. Insulin immunohistochemistry was conducted with a biotin-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), extravidin-HRP (Sigma) and a DAB substrate kit (Zymed Laboratories). Nuclei were counterstained with Hoechst, 1 μ g/ml (Sigma).

Islet isolation and culture

Islets of Langerhans were isolated by retrograde intra-ductal perfusion of pancreata with 0.166 mg/ml liberase RI or TM (Roche), 1.5 mg/ml DNase I (Roche) following protocol described in Lacy and Kostianovsky (1967). For qPCR experiments, islets were handpicked and frozen in liquid nitrogen. For anti-miRNA treatment, islets were isolated from ICR animals using collagenase P (Roche) at 1.5 mg/ml and cultured over extracellular matrix-coated plates (Novamed), RPMI 1640; 11 mmol/l glucose; 10% fetal bovine serum; 2 mmol/l L-glutamine; 100 U/ml penicillin; 100 μ g/ml streptomycin. AMOs were designed as 2'-O-methyl RNA (2'OMe) reverse complements to the targeted miRNAs, with phosphodiester linkages and a non-nucleotide naphthyl-azo group chemical modifier (dubbed 'ZEN') between the last and next to the last base on both the 5'- and 3'-ends and a 3'-cholesterol-TEG modifier. AMOs used in this study were added to the medium at a final concentration of 500 nM and are listed in Supplementary data.

RNA quantification by qPCR

Islet RNA was extracted using RNeasy or miRNeasy kit (Qiagen) and DNase I treated on columns. cDNA was created from 100 to 500 ng islet RNA using an oligo d(T) primer (Promega) and SuperScript II

reverse transcriptase (Invitrogen). qPCR analysis was performed on LightCycler[®] 480 System (Roche) with primers detailed in Supplementary data using DyNAmo[™] SYBR[®] Green qPCR kit (Finnzymes). GAPDH and HPRT were used as reference genes for normalization. Taqman MicroRNA qPCR Assays (Applied Biosystems), were performed on 10 ng total RNA from each sample and normalized to the relative expression of RNU6B.

Cell culture and heterologous luciferase reporter activity

HEK-293T and HIT cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, at 37°C/5% CO₂ incubator. Passage 16–20 MIN6 cells were the gift of Jun-ichi Miyazaki (Osaka University, Japan) and cultured in DMEM; 10 mM glucose; 15% fetal bovine serum; 2 mM L-glutamine; 100 U/ml penicillin/streptomycin. For miRNA targeting assays: the mouse Bhlhe22 3' UTR (Chr3:17955876–17957373) and Sox6 3' UTR (Chr7:122618117–122619352) were subcloned into psiCHECK-2 vector (Promega) and transfected into HEK-293T cells with JET-PEI reagent (Poly Plus) together with miRNA-24/148/26/182/200/141/103 miRvecs, that were a gift from Reuven Agami (NKI, The Netherlands) with or without cholesterol-conjugated anti-miRNA inhibitors (see above, islet culture with AMO) at a final concentration of 100 nM.

For analysis of insulin transcription, firefly luciferase reporter driven by the rat insulin promoter and an A20-Renilla luciferase construct (gift of Michael Walker), were transfected using Lipofectamine[™] 2000 Reagent (Invitrogen). For repressor overexpression HIT cells were transfected with reporters along with a pCMV-Flag-Sox6 cDNA plasmid (gift of Véronique Lefebvre, Cleveland Clinic Lerner Research Institute, OH) or the Hamster Bhlhe22 (insert of pBETA3, gift of Ming Tsai, Baylor College of Medicine, TX). For anti-miRNA inhibitor experiments MIN6 cells were transfected with reporters along with miScript anti-miRNA inhibitors (Qiagen) at a final concentration of 100 nM. Forty-eight to 72 h post-transfection cells were harvested using the Dual luciferase reporter assay system (Promega).

Statistical analysis

All statistical analyses were performed using Student's *t*-test and ANOVA as needed and are displayed as mean $\pm$ s.e.m. of three or more samples/experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: TMZ, RO, YD, SKR and EH designed the experiments. EH and TMZ wrote the manuscript. TMZ conducted and analysed mouse physiology, histological preparation, *in vitro* repressor overexpression and miRNA inhibition studies. RO performed and analysed islet qRT-PCR. RO, TMZ and TS performed and analysed immunostaining. SKR established and analysed the islet culture method. RO, NR and AM performed miRNA overexpression experiments. TN analysed β -cell mass. MAB and KAL designed and developed the ZEN-AMO. YD provided important tools and reagents.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Andrali SS, Sampley ML, Vanderford NL, Ozcan S (2008) Glucose regulation of insulin gene expression in pancreatic beta-cells. *Biochem J* **415**: 1–10
- Bar Y, Russ HA, Knoller S, Ouziel-Yahalom L, Efrat S (2008) HES-1 is involved in adaptation of adult human beta-cells to proliferation *in vitro*. *Diabetes* **57**: 2413–2420
- Baroukh N, Ravier MA, Loder MK, Hill EV, Bounacer A, Scharfmann R, Rutter GA, Van Obberghen E (2007) MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic beta-cell lines. *J Biol Chem* **282**: 19575–19588
- Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* **136**: 215–233
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**: 363–366
- Cerf ME (2006) Transcription factors regulating beta-cell function. *Eur J Endocrinol* **155**: 671–679
- Cheloufi S, Dos Santos CO, Chong MM, Hannon GJ (2010) A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature* **465**: 584–589
- Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **429**: 41–46
- El Ouamari A, Baroukh N, Martens GA, Lebrun P, Pipeleers D, van Obberghen E (2008) miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. *Diabetes* **57**: 2708–2717
- Gierl MS, Karoulis N, Wende H, Strehle M, Birchmeier C (2006) The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev* **20**: 2465–2478
- Hald J, Hjorth JP, German MS, Madsen OD, Serup P, Jensen J (2003) Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. *Dev Biol* **260**: 426–437
- Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ (2005) The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci USA* **102**: 10898–10903
- Hennessey E, Clynes M, Jeppesen PB, O'Driscoll L (2010) Identification of microRNAs with a role in glucose stimulated insulin secretion by expression profiling of MIN6 cells. *Biochem Biophys Res Commun* **396**: 457–462
- Hennessey E, O'Driscoll L (2008) Molecular medicine of microRNAs: structure, function and implications for diabetes. *Expert Rev Mol Med* **10**: e24
- Iguchi H, Ikeda Y, Okamura M, Tanaka T, Urashima Y, Ohguchi H, Takayasu S, Kojima N, Iwasaki S, Ohashi R, Jiang S, Hasegawa G, Ioka RX, Magoori K, Sumi K, Maejima T, Uchida A, Naito M, Osborne TF, Yanagisawa M et al (2005) SOX6 attenuates glucose-stimulated insulin secretion by repressing PDX1 transcriptional activity and is down-regulated in hyperinsulinemic obese mice. *J Biol Chem* **280**: 37669–37680
- Inada A, Yamada Y, Someya Y, Kubota A, Yasuda K, Ihara Y, Kagimoto S, Kuroe A, Tsuda K, Seino Y (1998) CREM transcriptional repressors are increased in pancreatic islets of type 2 diabetic rats. *Biochem Biophys Res Commun* **253**: 712–718
- Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD (2000) Control of endodermal endocrine development by Hes-1. *Nat Genet* **24**: 36–44
- Joglekar MV, Parekh VS, Hardikar AA (2007) New pancreas from old: microregulators of pancreas regeneration. *Trends Endocrinol Metab* **18**: 393–400
- Kaneto H, Miyatsuka T, Kawamori D, Yamamoto K, Kato K, Shiraiwa T, Katakami N, Yamasaki Y, Matsuhisa M, Matsuoka TA (2008) PDX-1 and MafA play a crucial role in pancreatic beta-cell differentiation and maintenance of mature beta-cell function. *Endocr J* **55**: 235–252
- Kang Y, Huang X, Pasyk EA, Ji J, Holz GG, Wheeler MB, Tsushima RG, Gaisano HY (2002) Syntaxin-3 and syntaxin-1A inhibit L-type calcium channel activity, insulin biosynthesis and exocytosis in beta-cell lines. *Diabetologia* **45**: 231–241
- Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E (2007) The role of site accessibility in microRNA target recognition. *Nat Genet* **39**: 1278–1284
- Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N (2005) Combinatorial microRNA target predictions. *Nat Genet* **37**: 495–500
- Lacy PE, Kostianovsky M (1967) Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16**: 35–39
- Lan MS, Breslin MB (2009) Structure, expression, and biological function of INSM1 transcription factor in neuroendocrine differentiation. *FASEB J* **23**: 2024–2033
- Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**: 415–419
- Liu WD, Wang HW, Muguira M, Breslin MB, Lan MS (2006) INSM1 functions as a transcriptional repressor of the neuroD/beta2 gene through the recruitment of cyclin D1 and histone deacetylases. *Biochem J* **397**: 169–177
- Lovis P, Gattesco S, Regazzi R (2008) Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biol Chem* **389**: 305–312
- Lynn FC, Skewes-Cox P, Kosaka Y, McManus MT, Harfe BD, German MS (2007) MicroRNA expression is required for pancreatic islet cell genesis in the mouse. *Diabetes* **56**: 2938–2945
- Mellitzer G, Bonne S, Luco RF, Van De Castele M, Lenne-Samuel N, Collombat P, Mansouri A, Lee J, Lan M, Pipeleers D, Nielsen FC, Ferrer J, Gradwohl G, Heimberg H (2006) IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. *EMBO J* **25**: 1344–1352
- Moates JM, Nanda S, Cissell MA, Tsai M-J, Stein R (2003) BETA2 activates transcription from the upstream glucokinase gene promoter in islet beta-cells and gut endocrine cells. *Diabetes* **52**: 403–408
- Naya FJ, Stellrecht CM, Tsai MJ (1995) Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev* **9**: 1009–1019
- Novak A, Guo C, Yang W, Nagy A, Lobe CG (2000) Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* **28**: 147–155
- Petersen HV, Serup P, Leonard J, Michelsen BK, Madsen OD (1994) Transcriptional regulation of the human insulin gene is dependent on the homeodomain protein STF1/IPF1 acting through the CT boxes. *Proc Natl Acad Sci USA* **91**: 10465–10469
- Peyton M, Stellrecht CM, Naya FJ, Huang HP, Samora PJ, Tsai MJ (1996) BETA3, a novel helix-loop-helix protein, can act as a negative regulator of BETA2 and MyoD-responsive genes. *Mol Cell Biol* **16**: 626–633
- Plaisance V, Abderrahmani A, Perret-Menoud V, Jacquemin P, Lemaigre F, Regazzi R (2006) MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J Biol Chem* **281**: 26932–26942
- Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432**: 226–230
- Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M (2009) miR-375 maintains normal pancreatic α - and β -cell mass. *Proc Natl Acad Sci USA* **106**: 5813–5818
- Poy MN, Spranger M, Stoffel M (2007) microRNAs and the regulation of glucose and lipid metabolism. *Diabetes Obes Metab* **9** (Suppl 2): 67–73
- Qiu Y, Guo M, Huang S, Stein R (2002) Insulin gene transcription is mediated by interactions between the p300 coactivator and PDX-1, BETA2, and E47. *Mol Cell Biol* **22**: 412–420
- Sander M, Neubuser A, Kalamaras J, Ee HC, Martin GR, German MS (1997) Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev* **11**: 1662–1673
- Schisler JC, Jensen PB, Taylor DG, Becker TC, Knop FK, Takekawa S, German M, Weir GC, Lu D, Mirmira RG, Newgard CB (2005) The Nkx6.1 homeodomain transcription factor sup-

- presses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. *Proc Natl Acad Sci USA* **102**: 7297–7302
- Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS (1998) Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* **125**: 2213–2221
- Tang X, Muniappan L, Tang G, Ozcan S (2009) Identification of glucose-regulated miRNAs from pancreatic {beta} cells reveals a role for miR-30d in insulin transcription. *RNA* **15**: 287–293
- Wang HW, Muguirra M, Liu WD, Zhang T, Chen C, Aucoin R, Breslin MB, Lan MS (2008) Identification of an INSM1-binding site in the insulin promoter: negative regulation of the insulin gene transcription. *J Endocrinol* **198**: 29–39
- Watada H, Kajimoto Y, Umayahara Y, Matsuoka T, Kaneto H, Fujitani Y, Kamada T, Kawamori R, Yamasaki Y (1996) The human glucokinase gene beta-cell-type promoter: an essential role of insulin promoter factor 1/PDX-1 in its activation in HIT-T15 cells. *Diabetes* **45**: 1478–1488
- Zhao L, Guo M, Matsuoka TA, Hagman DK, Parazzoli SD, Poitout V, Stein R (2005) The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. *J Biol Chem* **280**: 11887–11894