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P R E S S

Epigenetic Regulation of the DLK1-MEG3 MicroRNA Cluster in Human Type 2 Diabetic Islets

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SUMMARY

Type 2 diabetes mellitus (T2DM) is a complex disease characterized by the inability of the insulin-producing β cells in the endocrine pancreas to overcome insulin resistance in peripheral tissues. To determine if microRNAs are involved in the pathogenesis of human T2DM, we sequenced the small RNAs of human islets from diabetic and nondiabetic organ donors. We identified a cluster of microRNAs in an imprinted locus on human chromosome 14q32 that is highly and specifically expressed in human β cells and dramatically downregulated in islets from T2DM organ donors. The downregulation of this locus strongly correlates with hypermethylation of its promoter. Using HITS-CLIP for the essential RISC-component Argonaute, we identified disease-relevant targets of the chromosome 14q32 microRNAs, such as *IAPP* and *TP53INP1*, that cause increased β cell apoptosis upon overexpression in human islets. Our results support a role for microRNAs and their epigenetic control by DNA methylation in the pathogenesis of T2DM.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex, multifactorial disease, characterized by an insufficient pancreatic β cell response to insulin resistance in peripheral tissues. As of September 2012, the World Health Organization estimated that at least 312 million people worldwide have T2DM. Several studies have indicated that T2DM has a high rate of familial aggregation (Drong et al., 2012; Meigs et al., 2000; Nolan et al., 2011). However, genetic risk loci identified by standard genetic and genome-wide association approaches account for less than 10% of the observed heritability. These results have led to speculation that epigenetic effects may also play a role in the development of T2DM. Indeed,

there is suggestive evidence that diet and intrauterine environment, among other factors, may induce chromatin changes that lead to aberrant gene expression and subsequent disease (Bramswig et al., 2013; Drong et al., 2012).

MicroRNAs (miRNAs), short ncRNAs that posttranscriptionally regulate gene expression, have emerged as a strong molecular candidate in several complex diseases, in part due to their ability to simultaneously regulate the expression of hundreds of target mRNAs (Mendell and Olson, 2012). While several recent studies have suggested a role for miRNAs in human pancreatic islet and β cell function (Klein et al., 2013; van de Bunt et al., 2013), none have profiled the miRNA transcriptome of islets obtained from diabetic donors. To address this knowledge gap, we performed high-throughput sequencing of small RNAs (sRNAs) and have identified several miRNAs as significantly differentially expressed between islets isolated from nondiabetic and T2DM organ donors. Strikingly, included among the miRNAs downregulated in T2DM donors' islets was a cluster of maternally expressed miRNAs mapping to an imprinted locus on human chromosome 14q32. Our results demonstrate that the DLK1-MEG3 miRNA cluster is highly and specifically expressed in human β cells, but strongly repressed in islets from T2DM donors. Furthermore, we identify an epigenetic modification at this locus that correlates with its expression in human diabetic donors' islets. Using high-throughput sequencing of crosslinked and immunoprecipitated RNA (HITS-CLIP), we have identified targets of Chr 14q32 miRNAs, such as *IAPP* and *TP53INP1*, with known association to the pathogenesis of T2DM. Additionally, we identified a subset of sequences within CLIP libraries that are generated by the ligation of miRNAs to their targets while in complex with Argonaute. These reads, called chimeric reads, allow for the direct identification of miRNA:target relationships in vivo.

RESULTS

Differentially Expressed miRNAs in T2DM Human Islets
To determine the miRNA transcriptome of the mature human islet, we isolated the sRNA fraction from islets of three nondiabetic and four T2DM organ donors (donor information available

Table 1. Islet Donor Information

Donor ID	Age	Gender	Blood Type	BMI	Race	Cause of Death
Non T2DM 1	33	Female	A+	31.1	African American	Anoxia, cardiovascular (CVA)
Non T2DM 2	22	Female	O+	24.9	African American	Head trauma
Non T2DM 3	51	Male	O+	26.1	Hispanic/Latino	Head trauma
T2DM 1	61	Female	A−	29.6	Caucasian	Anoxia, CVA
T2DM 2	45	Male	B+	37	Caucasian	Anoxia, CVA
T2DM 3	53	Male	O+	38.4	Caucasian	CVA
T2DM 4	54	Female	A	21.6	Hispanic/Latino	CVA

Characteristics of T2DM and non-T2DM islet donors used in sRNA-sequencing screen are listed. Related to Figure 1 and Table S1.

in Table 1). We employed ultra-high-throughput sequencing and obtained more than 100 million sequence reads, allowing us to identify over 800 miRNAs expressed in the human endocrine pancreas (Table S2 available online). In order to verify that our sequence reads represented miRNAs and not degraded mRNAs, we aligned them to the RefSeq database (Pruitt et al., 2012). As shown in Figure S1A, less than 20% of the reads in the miRNA size range aligned to mRNAs, while more than 85% matched precursor miRNAs, confirming that our sRNA preparation was indeed highly enriched for true miRNAs. To assess the abundance of each mature miRNA, we aligned all sequence reads to known miRNA precursors obtained from miRBase (Kozomara and Griffiths-Jones, 2011). The 15 most highly expressed miRNAs in human islets from nondiabetic and T2DM donors are shown in Figures 1A and 1B. Hsa-miR-375, which was shown to be highly expressed in developing and mature human islets (Bolmeson et al., 2011; Joglekar et al., 2009; Landgraf et al., 2007), is the fourth most abundant miRNA in human islets, with over 100,000 reads per million (RPM). Similarly, miRNAs miR-103 and miR-107, which are important regulators of insulin sensitivity in the livers of obese mouse models, were also identified as highly abundant in human islets (Trajkovski et al., 2011). The abundance of individual miRNAs varied greatly, from 1 to 229,000 RPM; however, 123 of these miRNAs were expressed at more than 100 RPM (Table S2).

Comparative analysis of samples from T2DM donors and nondiabetic donors identified 15 miRNAs with significantly differential expression (Figure 1C). The expression levels of these miRNAs were highly consistent between samples and were sufficient to clearly cluster the samples as T2DM and non-T2DM, as shown in Figure 1D. The differential expression was validated for 9 of these miRNAs by Taqman quantitative RT-PCR (qRT-PCR) on islets from 16 T2DM and 18 nondiabetic donors (Figures S1B and S1C). MiRNAs miR-204 and miR-184, which have previously been reported to be β cell enriched (Klein et al., 2013; van de Bunt et al., 2013), are not differentially expressed between human islets from T2DM and non-T2DM organ donors (Table S2), ruling out the possibility that the observed differential expressions are driven by changed islet composition between the two groups. Expression of these miRNAs did not show a significant correlation with age, sex, or body mass index (BMI) (Figures S1E–S1G).

Of the miRNAs that were identified as differentially expressed between T2DM and nondiabetic donor islets, several have been previously implicated in diabetes and β cell function. For example, miR-7 is a well-characterized islet miRNA that is expressed in the endocrine cells of the developing and adult human pancreas (Correa-Medina et al., 2009). MiR-7 has been shown to regulate insulin expression during the early stages of mouse pancreatic embryogenesis (Nieto et al., 2012) and also negatively affect β cell proliferation in murine and human islets (Wang et al., 2013). Thus, we have identified multiple miRNAs as differentially expressed between T2DM and non-T2DM donors' islets.

Decreased Expression of the Imprinted *MEG3* miRNA Cluster in T2DM Islets

Strikingly, of the ten miRNAs that were significantly downregulated in T2DM islets, seven are derived from the imprinted DLK1-MEG3 locus at human chromosome 14q32. Genomic imprinting refers to the biased expression of genes from either the paternally or maternally inherited chromosome, rather than the more common biallelic expression. Apart from the aforementioned miRNAs, this imprinted locus contains maternally expressed snoRNAs, the ncRNA genes *MEG3*, *MEG8*, and antisense *RTL1*, as well as the paternally expressed genes *DLK1*, *RTL1*, and *DIO3* (Cavaillé et al., 2002; Charlier et al., 2001; Wylie et al., 2000). Decreased expression of *MEG3* and the nearby miRNAs has been associated with numerous diseases, notably hepatocellular carcinoma, acute myeloid leukemia, and ovarian cancer (Benetatos et al., 2012), but not T2DM.

Since the maternally expressed ncRNAs in this locus are likely all processed from the same primary transcript (Seitz et al., 2004) (see additional evidence in Figure 2C below), we asked whether other miRNAs in this cluster were also expressed at lower levels in T2DM donors' islets. As shown in Figure S1D, this was indeed the case, and these results were confirmed by Taqman qRT-PCR in a larger cohort of islet samples (Figure 1E). *MEG3* was also found to be downregulated in islets from T2DM donors compared to nondiabetics (data not shown). Thus, we have identified an imprinted, maternally expressed cluster of ncRNAs to be downregulated in islets obtained from T2DM donors.

The DLK1-MEG3 Cluster of miRNAs Is Specifically Expressed in Human β Cells

To characterize the expression profile of the DLK1-MEG3 cluster of miRNAs in the major human islet cell populations, we applied FACS to sort highly purified human α and β cells (Dorrell et al., 2008) and performed high-throughput sequencing of sRNAs (Figure S2 and Table S3). The expression levels of the miRNAs in the 14q32 cluster were, on average, 16-fold higher in β cells when compared to α cells (Figures 2A and 2B). This is consistent
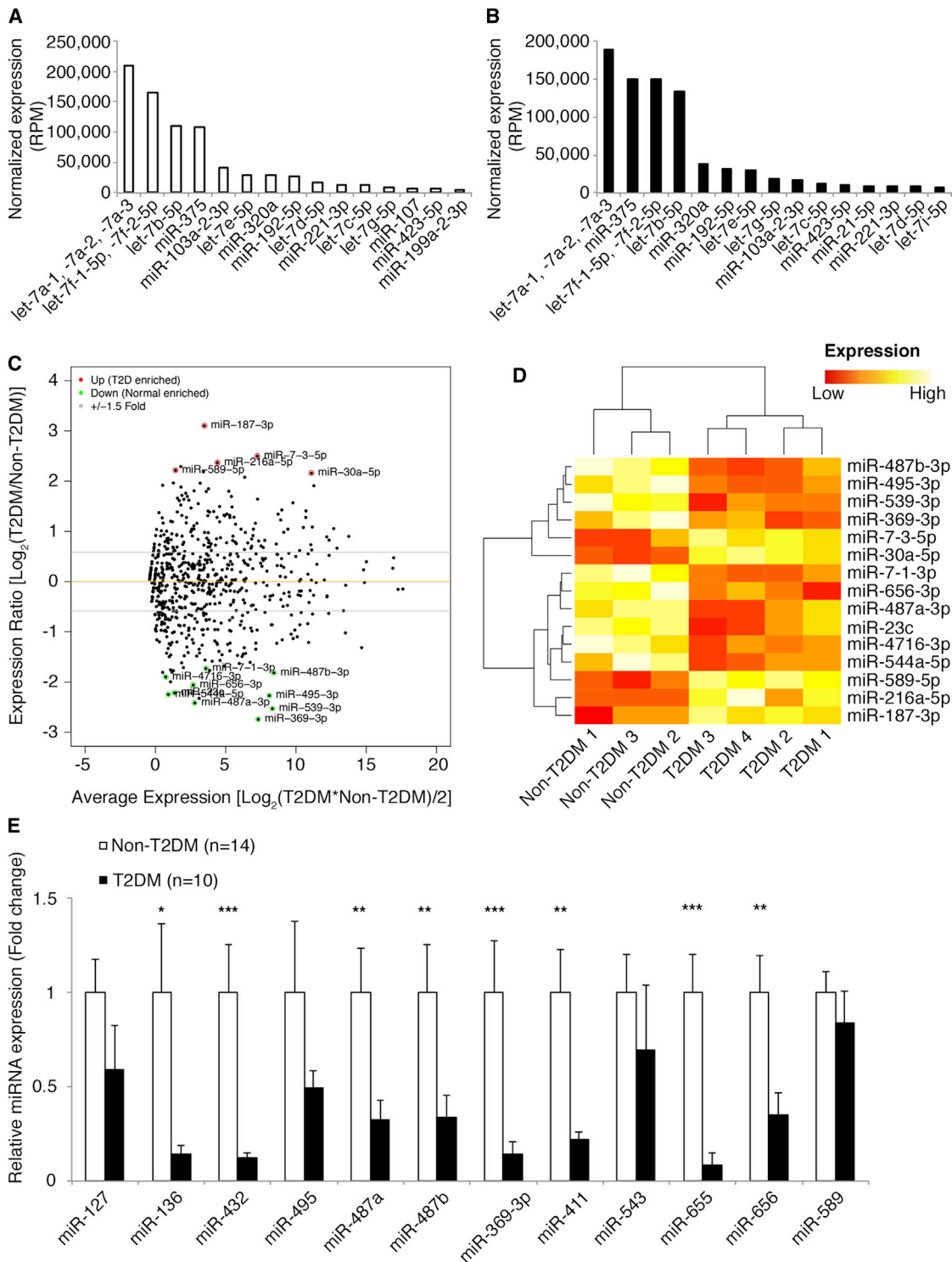
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Figure 1. The Imprinted Chromosome 14q32 miRNA Cluster Is Downregulated in T2DM Islets

(A and B) Expression levels of the 15 most abundant miRNAs in (A) three nondiabetic and (B) four T2DM human islets as identified by sRNA sequencing.

(C) Differentially expressed miRNAs between non-T2DM (n = 3) and T2DM (n = 4) islets, identified by a FDR of 20% and minimum fold change of 1.5×. miRNAs upregulated and downregulated in T2DM islets are highlighted, and miRNAs belonging to the Chr 14q32 cluster are underlined.

(D) Distribution of expression levels of miRNAs identified as significantly differentially expressed by sRNA sequencing across clustered samples.

(E) Relative expression of miRNAs in the Chr 14q32 cluster as determined by Taqman qPCR of 14 non-T2DM and 10 T2DM human islets. p value was calculated using two-tailed Student's t test. *p < 0.05, **p < 0.01, and ***p < 0.005. Error bars indicate SEM. See also Figure S1 and Table S2.
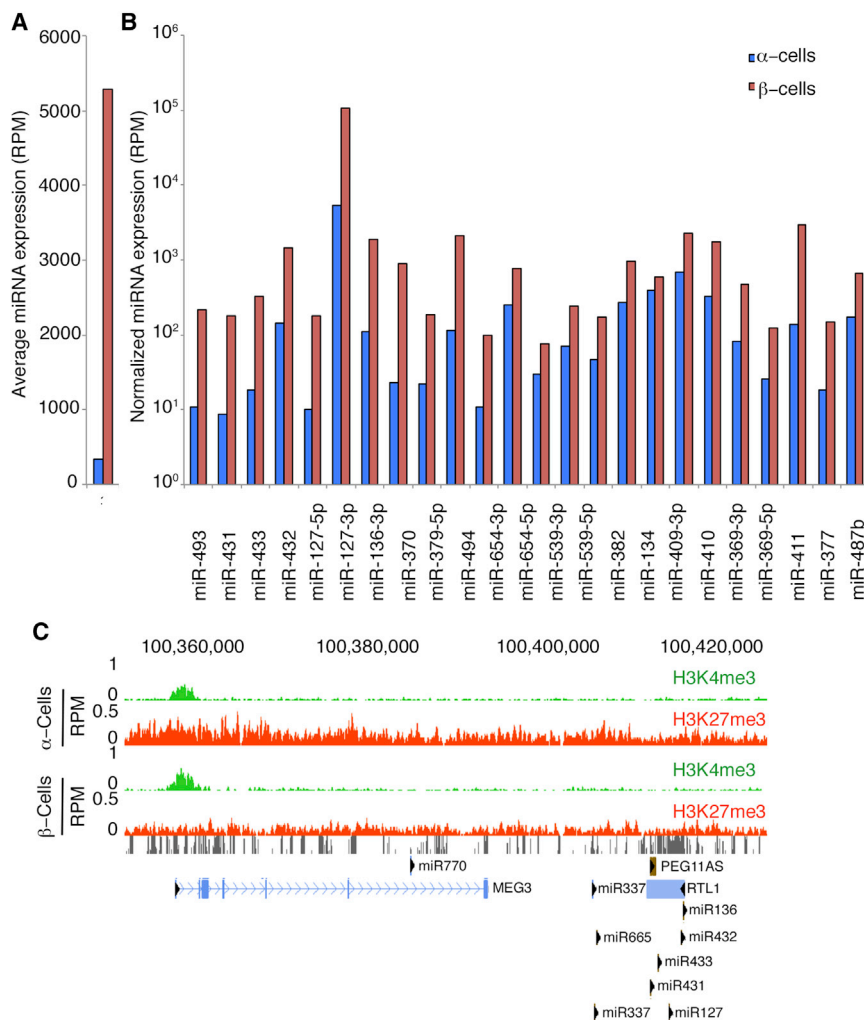
Figure 2. Chr 14q32 miRNAs Are Highly and Specifically Expressed in Human β Cells

(A) Average expression of Chr 14q32 cluster miRNAs in human α and β cells.

(B) Expression of Chr 14q32 miRNAs (minimal expression 50 reads per million [RPM]) in sorted human α and β cells.

(C) Genome browser image of histone modification marks H3K4me3 (n = 4) and H3K27me3 (n = 3) at the *MEG3* promoter (chromosomal location marked on top) of sorted α and β cells from healthy human donors. Gray bars represent sequence conservation. Positions of the miRNA cluster and other nearby transcripts are shown. See also Figure S2 and Table S3.

Epigenetic Regulation of the *MEG3* Promoter in T2DM Islets

To understand the molecular mechanism of the downregulation of the maternal RNAs in the DLK1-MEG3 locus, we considered the possibility that this may be a consequence of the high-glucose conditions that the cells are exposed to in the islets of T2DM organ donors. To test this notion, we cultured islets from nondiabetic donors in different glucose conditions for a prolonged period of time and measured the changes in expression of the miRNAs located in this cluster by high-throughput sequencing of sRNAs from each group. No significant change was detected in the expression of these miRNAs (Figure S3A), suggesting that the expression of this cluster is not regulated acutely by glucose.

with previous reports that utilized different sorting strategies and identified some members of the Chr 14q32 cluster of miRNAs as enriched in human β cells compared to α cells (Klein et al., 2013) and whole islets (van de Bunt et al., 2013). Expression of the long noncoding *MEG3* RNA was also found to be 20-fold higher in β cells compared to α cells (Dorrell et al., 2011), further supporting our results.

To understand the epigenetic landscape that may explain the cell-type-specific expression of this locus in human α and β cells, we performed chromatin immunoprecipitation sequencing (ChIP-Seq) for several histone modification marks in enriched human α and β cell populations (Bramswig et al., 2013). While the *MEG3* promoter was bivalently marked by the activating histone H3 lysine 4 trimethylation (H3K4me3) and the repressive lysine 27 trimethylation (H3K27me3) modifications in glucagon-secreting α cells, insulin-secreting β cells showed a dramatic decrease in H3K27me3 and were only marked by H3K4me3 at this region (Figure 2C). Thus, the observed histone modification marks at the promoter of *MEG3* and its associated miRNAs strongly correlate with its cell-type-specific expression. Taken together, the 14q32 locus is highly and specifically expressed in β cells.

These findings prompted us to consider other genetic and epigenetic explanations for the observed decrease in miRNA levels at the Chr 14q32 locus in T2DM donors' islets. The imprinted status of the maternally expressed RNAs of the DLK1-MEG3 locus is determined by the methylation of two differentially methylated regions (DMRs), the first located 13 kb upstream (termed "IG-DMR") and the second 1.5 kb upstream of the transcription initiation site of *MEG3*, overlapping with the *MEG3* promoter (termed "MEG3-DMR") (Figure 3A) (Kagami et al., 2010; Murphy et al., 2003). Hypermethylation of either of these DMRs has been concomitantly observed with decreased expression of the maternal transcript (Kagami et al., 2010). Using methylation-specific PCR primers designed for the *MEG3-DMR* (Murphy et al., 2003), we tested for differences in DNA methylation between T2DM and non-T2DM donors' islets. While islets from non-T2DM donors showed the predicted equal abundance of the methylated and unmethylated PCR products, we observed a decreased intensity of the unmethylated product in islets from donors with T2DM (Figures 3B and S3B).

To assess this difference at base resolution, we designed quantitative sequence-specific pyrosequencing assays to measure CpG methylation of both DMRs. No difference in
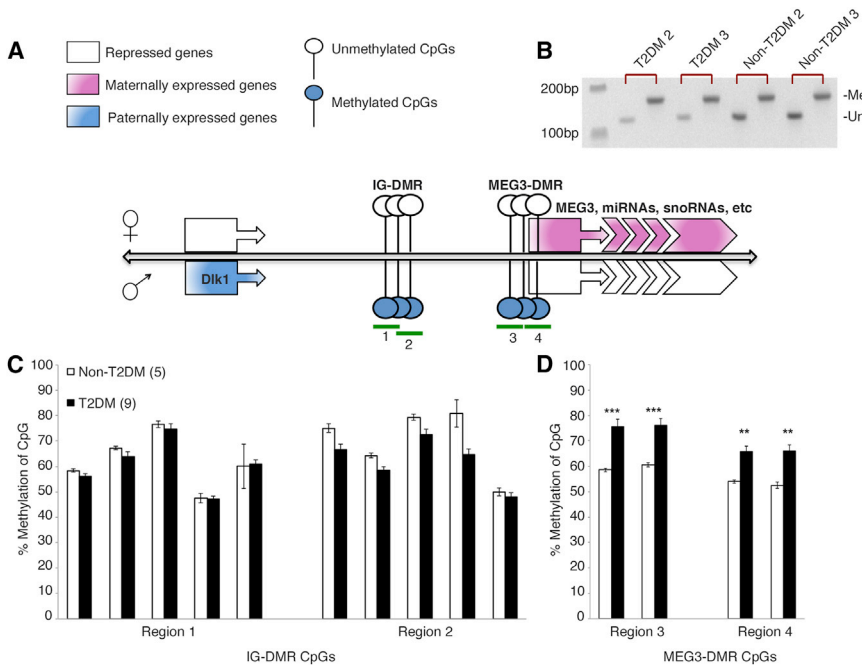
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Figure 3. Increased Methylation of the *MEG3* Differentially Methylated Region in T2DM Islets

(A) Schematic representation of DLK1-MEG3 locus differentially methylated regions (DMRs) with allele-specific gene expression depicted. Regions analyzed for Figures 3C and 3D are marked as green bars.

(B) Methylation-specific PCR for *MEG3* promoter methylation in two T2DM and two nondiabetic donors' islets. The methylated band (Me) is 160 bp and the unmethylated band (Un) is 120 bp.

(C and D) Percent methylation was determined for multiple CpGs in the (C) IG-DMR and (D) *MEG3-DMR* in five nondiabetic and nine T2DM donors' islets by pyrosequencing of bisulfite-converted DNA. Each bar represents an individual CpG, and the regions refer back to schema in part (A). p value calculated using Student's t test. **p < 0.005 and ***p < 0.001. Error bars indicate SEM. See also Figure S3.

methylation levels was detectable at the IG-DMR (Figure 3C, average tested CpG methylation decreased by 4.6% in T2DM islets, p = 0.35). In contrast, we observed significantly increased methylation levels at the *MEG3-DMR* (Figure 3D, average increase of 14.5% across the tested CpGs, p < 0.01), consistent with the decreased expression of the maternally expressed genes under its control. We detected no difference in the methylation levels of α cells sorted from T2DM and non-T2DM donors, suggesting that the observed hypermethylation in T2DM islets is unlikely to arise from this cell population (Figure S3C). These results provide a compelling example of an epigenetic modification that is associated with altered gene expression in islets from T2DM donors.

Targets of Chr 14q32 miRNAs Are Critical to β Cell Health and Function

In order to assess the contribution of specific differentially expressed islet miRNAs to T2DM, an understanding of the mRNAs they target is necessary. Therefore, we performed HITS-CLIP for Argonaute (Chi et al., 2009; McKenna et al., 2010), which forms part of the RNA-induced silencing complex (RISC) that mediates miRNA action. By crosslinking the protein components of the RISC to the paired miRNA and mRNA simultaneously, and isolating these RNA species by immunoprecipitation of Argonaute, we identified miRNA-targeted mRNAs in human islets using high-throughput sequencing (Figure 4A). From these deep-sequencing libraries, we identified 12,492 mRNA footprints and 456 mature human miRNAs as Argonaute associated in human islets. The mRNA footprints were highly enriched (96.85%) for seed sequences of the corresponding miRNAs identified by HITS-CLIP. Although most models of miRNA function propose seed sequence binding preferentially at the 3′ UTR of the target mRNA (Friedman et al., 2009), global analysis of our HITS-CLIP data demonstrated that miRNAs bind their targets in human islets throughout the transcript, with comparable levels at the coding

sequence (Student's t test, p = 7.00 × 10^{-16}) and 3′ UTR (Student's t test, p = 5.26 × 10^{-17}), similar to what has previously been described for other tissues (Chi et al., 2009; Forman et al., 2008; McKenna et al., 2010) (Figures 4B and 4C). Among the mRNAs targeted by the RISC complex in human islets were several encoded by genes known to be essential for islet function.

Of the 54 miRNAs encoded by the 14q32 locus, 38 were detected in our miRNA library of HITS-CLIP in human islets. These 38 miRNAs are predicted to target mRNAs transcribed from 1,784 genes that were detected in the target library (Table S4). Since the 14q32 locus is primarily expressed in β cells, we further filtered these 1,784 potential target mRNAs to those expressed preferentially in human β cells by intersecting our HITS-CLIP data set with β cell RNA sequencing (RNA-seq) expression data (Bramswig et al., 2013). The filtered list contained 717 target mRNAs for the 38 miRNAs detected in our HITS-CLIP library and 996 targets for all 54 mature miRNAs expressed from this locus (Table S5).

Since the expression of the *MEG3*-miRNA locus is downregulated in islets of T2DM donors, we were particularly interested in targets with known detrimental effects to islet function when expressed at higher levels than normal. Several mRNAs identified by our analysis are relevant to diabetes pathogenesis, such as islet amyloid polypeptide (*IAPP*), the major component of the amyloid deposits in pancreatic islets that cause increased β cell apoptosis in T2DM (Butler et al., 2003; Höppener and Lips, 2006; Hull et al., 2004). In order to test if the 14q32 locus miRNAs indeed target the 3′ UTR of the *IAPP* mRNA directly, we performed cotransfection assays of 3′ UTR luciferase reporter constructs with expression plasmids for the relevant miRNAs. The expression of the *IAPP* 3′ UTR luciferase construct was suppressed by 20% upon coexpression of miR-376a and miR-432, but not empty vector, confirming the direct targeting relationship (Figure 4D). This finding suggests that the repression of the 14q32 locus miRNAs in the β cells of T2DM donors results in the misregulation of key biological processes that contribute to the dysfunction of β cells in T2DM.
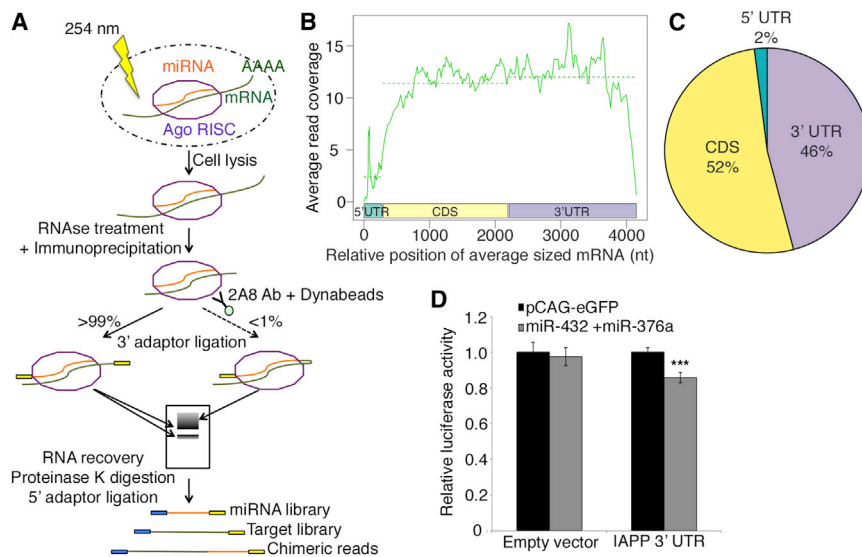
Figure 4. Identification of miRNA Targets in Human Islets by HITS-CLIP

(A) Schema of HITS-CLIP procedure and chimeric reads ligation.

(B) Average read coverage of all HITS-CLIP target mRNA fragments over a standardized mRNA.

(C) Argonaute footprint distribution across target library mRNAs in human islets.

(D) Targeting of human *IAPP* mRNA by miR-432 and miR-376a was validated by luciferase reporter assays. Vectors with or without the 3′ UTR of *IAPP* were cotransfected with either empty pCAG-eGFP vector or miR-432 and miR-376a. Error bars indicate mean ±SEM. ***p value calculated using Student's t test. p = 1.8 × 10^{-5}. See Tables S4 and S5.

Discovery of Chimeric Reads

While performing the alignment of the HITS-CLIP target library to its reference sequences, we discovered a unique class of sequences, termed "chimeric reads," that partially mapped to both miRNAs and target mRNAs simultaneously (Figure S4). Chimeric reads most likely arise from an occasional ligation event of miRNA and mRNA molecules while they are both associated with Argonaute in the RISC complex (Figure 4A). Though few in number (0.27% of all trimmed reads), these reads are an invaluable source for miRNA and target pair information, as the ligation event will only occur between molecules in close proximity to each other. This was confirmed by the fact that the miRanda target prediction algorithm identified base pairing between miRNAs and the fused mRNAs significantly more often than would be expected by chance (p < 1 × 10^{-300}). The 15 most abundant mRNAs and miRNAs found in such chimeras are listed in Figures 5A and 5C, respectively. Many relevant and highly expressed human islet transcripts were found in chimeric reads, such as glucagon, INS-IGF2, chromogranin A, and chromogranin B, among others. We also determined the mRNAs that are highly enriched in chimeric reads, relative to their overall abundance, as these mRNAs are more likely to be highly regulated by miRNAs (Schug et al., 2013). The 15 most highly enriched mRNAs in chimeric reads, relative to abundance, are shown in Figure 5B. A list of chimeric reads found by HITS-CLIP in human islets can be found in Table S6.

Gene ontology analysis of all human islet miRNA targets, identified by both HITS-CLIP (footprint of >150 RPKM) and the chimeric reads analysis (>50 reads), revealed a significant enrichment of biological processes such as "protein localization and transport," "protein ubiquitination," and "regulation of cell death," (Figure 5D) suggesting that mRNAs involved in these processes in human islets are highly regulated by miRNAs. miRNAs were found to form chimeras predominantly with the 3′ UTR regions of the target mRNA compared to the 5′ UTR and coding region (Figures 5E and 5F).

Using this information, we identified several additional targets of miRNAs in the 14q32 locus, including the "p53-induced nuclear protein 1," or *TP53INP1* (Figure 6A). *TP53INP1* is the nearest gene to a T2DM risk-associated SNP in individuals of Caucasian (Voight et al., 2010) and North African Arab descent (Cauchi et al., 2012). *TP53INP1* plays a crucial role in p53-dependent apoptosis (Okamura et al., 2001), and an increase in its expression in pancreatic β cells is associated with increased cell death (Zhou et al., 2012). As expected, we observed an increase in *TP53INP1* mRNA levels in T2DM donors' islets compared to nondiabetic donors by both microarray (data not shown) and qPCR, although these data did not reach statistical significance (Figure 6B). The variability in target mRNA expression is a reflection of the heterogeneity of our donor samples. To address this issue further, we plotted miR-495 and *TP53INP1* mRNA levels for each T2DM islet sample and observed a strong inverse correlation between the two (R^2 = 0.74, Figure 6C). Next, we validated the miR-495 and *TP53INP1* targeting relationship using luciferase reporter assays, as these assays provide a readout of miRNA effects on the mRNA and protein level (Figure 6D). We observed a 20% decrease in luciferase activity in the presence of miR-495 mimic, but not scramble mimic. To further test this targeting relationship in vivo, we constructed tough decoy RNAs (Haraguchi et al., 2009) for miR-495 (TuD495) in a lentiviral backbone to suppress miR-495 activity in human islets. We observed a 1.3-fold increase in *TP53INP1* mRNA levels upon TuD495 transduction relative to control vector, TuDctrl (Figure 6E, p = 0.007), similar to the increase observed in Figure 6B. *ONECUT1*, a previously published target of miR-495 (Simion et al., 2010), also increased to a similar extent (data not significant). In summary, derepression of *TP53INP1* as a consequence of increased miR-495 levels in β cells from T2DM donors is likely to contribute to their increased susceptibility to apoptotic stimuli. These results further underscore the value of the chimeric sequences in identifying miRNA targets.

DISCUSSION

miRNAs have been shown to play a central role in the development and progression of several diseases (Mendell and Olson, 2012). To identify the miRNAs that are key to the pathogenesis of T2DM, we sequenced the sRNAs of islets obtained from healthy and T2DM organ donors. Of the miRNAs that were
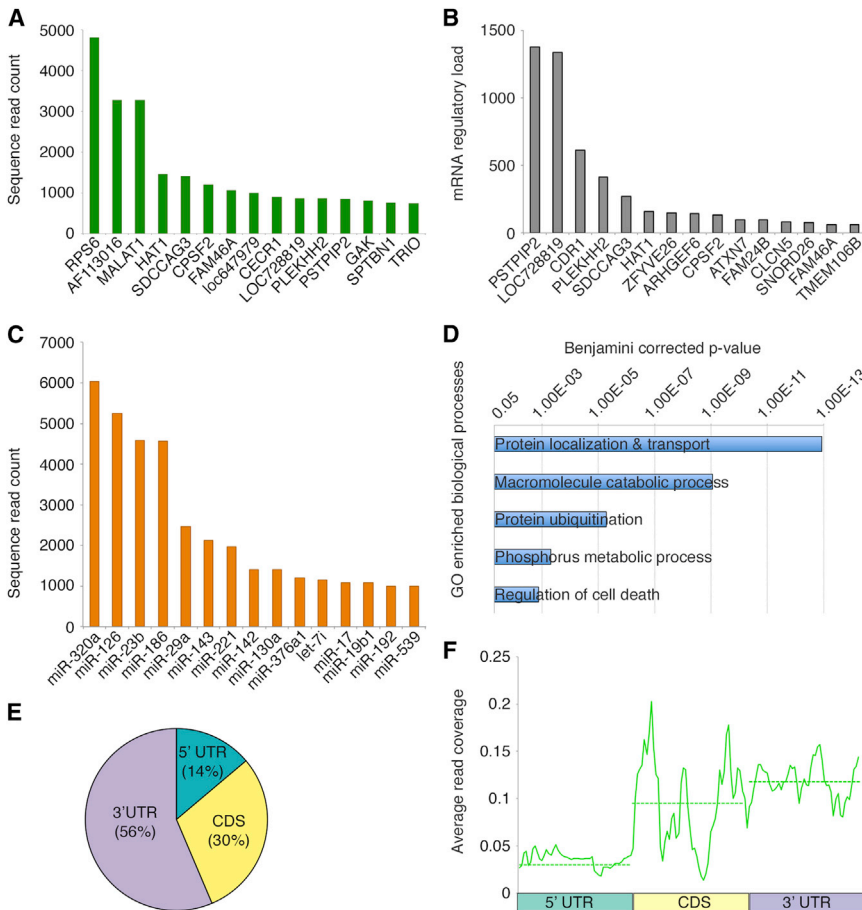
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Figure 5. Determination of Direct miRNA: mRNA Targeting Relationship from Chimeric Reads

Deep sequencing of our Argonaute HITS-CLIP library identified thousands of chimeric reads, consisting of a mature miRNA and a target mRNA fragment.

(A) The 15 most abundant mRNAs found in chimeric reads in human islets.

(B) The 15 most highly miRNA-regulated mRNAs in chimeric reads. The regulatory load ratio is the relative Ago-associated mRNA fraction of the chimeric reads, defined as the ratio of their sequence counts to their normalized abundance in human islets.

(C) The 15 most abundant miRNAs found in chimeric reads in human islets.

(D) Significantly enriched gene ontology biological processes in targets of human islet miRNAs.

(E) Pie chart representation of distribution of mRNA regions found in chimeras with miRNAs.

(F) Average read coverage of chimeric mRNA fragments across an mRNA divided into 150 equal bins. See Figure S4 and Table S6.

differentially expressed in T2DM islets, we identified the maternally expressed, imprinted cluster of ncRNAs on human chromosome 14q32 as downregulated. Our data on the H3K4me3 and H3K27me3 histone modification marks, combined with the miRNA expression data on sorted α and β cells (supported by previous expression studies [Bolmeson et al., 2011; Klein et al., 2013; van de Bunt et al., 2013]) indicate that this complex, maternally expressed gene is primarily transcribed in the insulin secreting β cells, compared to other pancreatic islet cell types.

Repression of this miRNA cluster is strongly correlated with hypermethylation of the *MEG3* differentially methylated region in T2DM islets, demonstrating an epigenetic alteration associated with T2DM. A report from Ling et al. (2008) has shown that a 6% increase in DNA methylation at the *PPARGC1A* promoter was negatively correlated with insulin gene expression and secretion, reaffirming the detrimental functional consequences of aberrant methylation in T2DM islets. Although our results suggest that the change in expression of the miRNAs is unlikely to be induced by high-glucose conditions, we cannot rule out the possibility that the observed hypermethylation at the *MEG3* promoter may be a secondary effect of the diabetic state. Our evidence of loss of imprinting at the differentially methylated region of this locus in T2DM donor islets suggests that modifications at this region markedly increase susceptibility to disease, since imprinted loci are functionally haploid. These results necessitate the study of other imprinted loci, particularly those that are strongly associated with risk for T2DM, such as the maternally expressed genes *KLF14* and *KCNQ1* (Kong et al., 2009; Travers et al., 2012; Voight et al., 2010).

We have integrated high-throughput sequencing of the miRNA transcriptome of the human islet with HITS-CLIP of Argonaute-associated RNAs. Within the CLIP libraries, we identified a unique fraction of sequences, termed chimeric reads, that represent miRNAs fused to their respective targets while in a complex with Argonaute in vivo. Chimeric reads are proposed to result from the ligation of RNA molecules that are stably base paired (Kudla et al., 2011), such as miRNAs and their targets, and were shown to form strong secondary structures with lower mean folding energies than nonchimeric reads of the same length (Kudla et al., 2011). By combining these data sets, we have identified islet-specific miRNAs and their mRNA targets that are misexpressed in T2DM. Several of these targets, such as *IAPP* and *TP53INP1*, have well-established associations with T2DM pathogenesis, and their upregulation is strongly linked to β cell dysfunction and increased cell death. This suggests that upon repression of the Chr 14q32 miRNA cluster, several proapoptotic factors, whose expression is normally tightly regulated, become activated. Derepression of this normally silent genetic locus, together with other risk factors, can result in increased β cell death and T2DM pathogenesis. In sum, our results provide strong evidence for a role of miRNAs and epigenetic modifications, such as DNA methylation, in the pathogenesis of T2DM.

EXPERIMENTAL PROCEDURES

Human Islets

Human islets and relevant donor information including age, gender, diabetes status, hemoglobin A1c, and BMI were obtained from the Islet
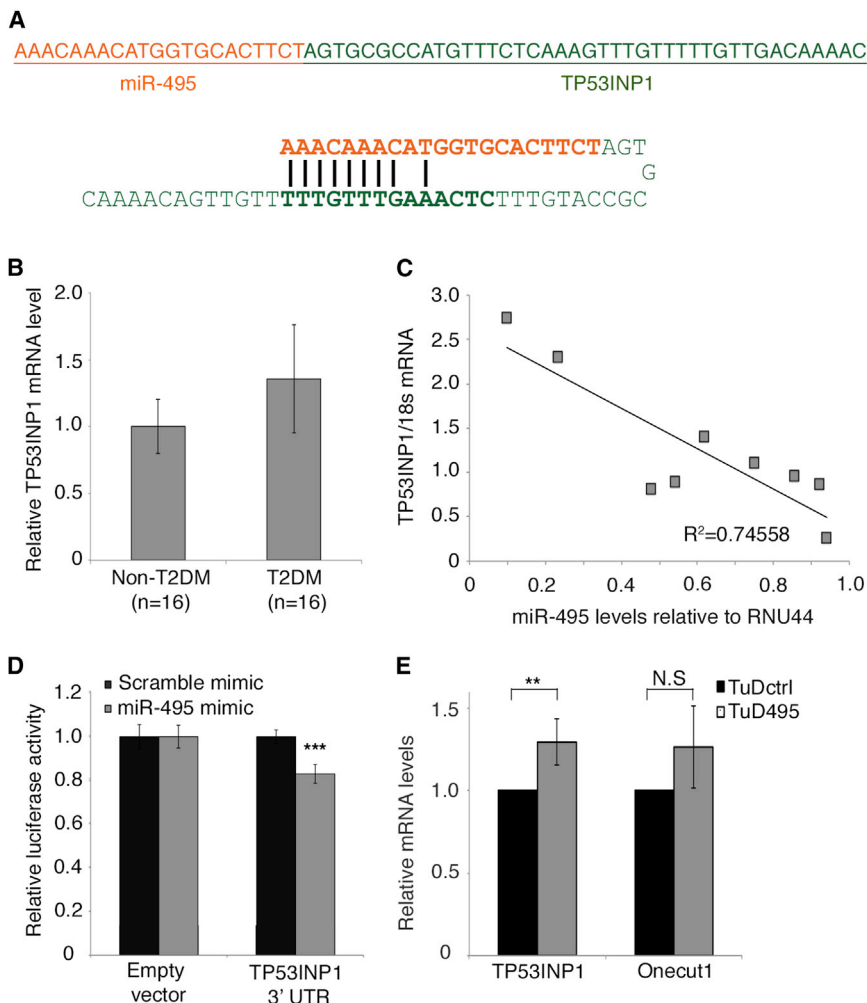
Figure 6. Validation of the miR-495: *TP53INP1* Targeting Relationship

A β cell apoptotic factor, *TP53INP1*, is regulated by miR-495.

(A) The sequence of the miR-495 (orange) and *TP53INP1* 3′ UTR (green) chimera. Folded confirmation with base pairing between the miRNA and 3′ UTR is indicated below.

(B) Relative levels of *TP53INP1* mRNA between T2DM and non-T2DM islet samples. Error bars indicate mean ±SEM.

(C) Anticorrelation between normalized *TP53INP1* and miR-495 in nine T2DM islet donor samples.

(D) Targeting of human *TP53INP1* mRNA by miR-495 was validated by luciferase reporter assays. Vectors with or without the 3′ UTR of *TP53INP1* were cotransfected with either scramble or miR-495 mimics. Error bars indicate mean ±SEM. p value calculated using Student's t test. ***p = 1.94×10^{-5}.

(E) Relative mRNA levels of *TP53INP1* and *ONECUT1* (normalized to the average of HPRT and β-actin transcript levels) in human islets transduced with lentivirus encoding tough decoy constructs for either scramble sequence (TuDctrl) or miRNA-495 (TuD495). Error bars indicate ±SEM. **p = 0.0076, n = 3.

sequenced to 50 bp on an Illumina hiSeq2000. The miRNAs identified as differentially expressed in this study are listed in Table S2.

TaqMan qRT-PCR

qRT-PCR was performed as previously described (Zahm et al., 2012). Briefly, 10 ng of total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4366596) and RT primers from the respective TaqMan MicroRNA Assay kit (Applied Biosystems; probe numbers listed separately in Table S7). qRT-PCR was performed on a Agilent Mx3005P using the TaqMan Universal PCR Master Mix (Applied Biosystems part number 4304437) and the TaqMan probe from the respective TaqMan MicroRNA Assay kit. Tissue miRNA levels were normalized to endogenous snoRNAs RNU44 and RNU48.

Cell Resource Center of the University of Pennsylvania, the NIDDK-supported Integrated Islet Distribution Program (iidp.coh.org), and the National Disease Research Interchange. The donor's diabetes status was defined by the patient's medical record and, when available, the hemoglobin A1c. Donor information is listed in Table S1. sRNA was isolated from the islets by using the mirVana miRNA Isolation Kit (Ambion Cat# AM1560).

Primers

All primers used in this study are listed in Table S7.

miRNA Sequencing and Comparison

The isolated miRNA from seven samples (three from donors without diabetes and four with T2DM) were prepared for sequencing using the Illumina protocol Preparing Samples for Analysis of Small RNA (Illumina FC-102-1009). Sequencing of the amplified libraries was performed on an Illumina Genome Analyzer II (Illumina FC-104-1003). Sequenced libraries were processed as described in the Supplemental Information. We used a false discovery rate of 20% and a minimum fold change of 1.5× to identify differentially expressed miRNAs.

Similarly, sorted cells were obtained by FACS sorting dispersed human islets as described before (Bramswig et al., 2013), and RNA was isolated from the α cell- and β cell-enriched fractions using the miRVana miRNA Isolation kit (Ambion). A total of 3 μg of RNA was used for library preparation using the TruSeq Small RNA sample preparation kit (Illumina) and a Pippin Prep (Sage Science) was used for size selection using a 3% Cassette (CSD3010). sRNA libraries from sorted α and β cells were

DNA Methylation Analysis

Genomic DNA or chromatin was extracted from five nondiabetic donors and nine T2DM donor's islets using All Prep DNA/RNA kit (QIAGEN). A total of 325 ng of extracted DNA (or unsonicated chromatin input) was bisulfite treated with the EpiTect Bisulfite kit (QIAGEN) and eluted in 20 μl of Buffer EB. PCR and sequencing primers were designed using the PyroMark Assay Design software version 2.0 (QIAGEN, sequences listed in Table S7) to cover CpGs at the IG-DMR and *MEG3-DMR*. Bisulfite-converted DNA was amplified by PCR using the PyroMark PCR kit (QIAGEN) at 95°C for 15 min followed by 45 cycles at 95°C for 15 s, 57°C for 30 s, and 72°C for 15 s. Biotinylated PCR products were immobilized onto streptavidin-coated Sepharose beads (GE Healthcare), and DNA strands were separated using PyroMark Denaturation Solution (QIAGEN), washed, and then neutralized using a vacuum prep station (QIAGEN PyroMark Q96 workstation). After annealing the sequencing primer to the immobilized strand, pyrosequencing was performed on the PyroMark Q96 MD (QIAGEN) using PyroMark Gold CDT kit (QIAGEN) according to the manufacturer's instructions. Data were analyzed using the Pyro Q-CpG software program (QIAGEN). Methylation-specific PCR was performed as previously described (Benetatos et al., 2010).

Cell Metabolism

Epigenetic Regulation in Human Diabetic Islets

HITS-CLIP Experiment and Sequencing Data Processing

HITS-CLIP was performed as previously reported, using the monoclonal Argonaute antibody 2A8 (Chi et al., 2009; McKenna et al., 2010). Human islet samples were coarsely homogenized with a Dounce homogenizer and crosslinked three times on ice at 400 mJ/cm^2. Both the miRNA library and mRNA library were sequenced on a Hi-Seq 2000 following standard protocols to a length of 100 nt to yield 120,901,521 and 47,026,559 reads, respectively. Reads from both libraries were preprocessed and mapped to the human genome (USSC hg19 assembly) using a previously described analysis pipeline (Li et al., 2009; Zheng et al., 2010). Potential miRNA-target pairs were predicted between the 456 mature human miRNAs (miRBase 18) detected in our miRNA library and 12,496 Ago footprints using the miRanda program (v3.3a) with non-default parameter as "−en −10." Additionally, we overlaid our mRNA targets of miRNAs encoded by the Chr 14q32 locus to β cell-specific expressed transcripts using published RNA-seq data (Bramswig et al., 2013); in this study, β cell-specific expressed transcripts were defined as those with substantial expression (RPKM ≥ 1) and having higher expression than α cells and exocrine cells. The detailed analysis method is described in the Supplemental Information section.

Chimeric Reads Analysis

A schematic of the chimeric reads detection is shown in Figure S4. Among 47,026,559 total raw reads with 100 bp in length, we found 26,542,918 reads whose length is larger than 15 bp after trimming the adaptor and any reported bases past the adaptor. We checked if a read is a hybrid by mapping the sequence using BLAT (Kent, 2002) to identify if a portion of the sequence is mapped to the genome. From the BLAT results, we selected the result with the maximum number of matched bases among the results with the minimum number of mismatches. We discarded the sequence if the unmapped portion of the sequence has a length smaller than 5, resulting in 3,861,560 reads. As BLAT cannot map sequences less than 20 bp, we applied Bowtie (Langmead et al., 2009) to the remaining portion of the read and collected the reads uniquely mapped to the genome, resulting in 1,233,580 reads (2.6% of the total 47,026,559 reads).

Next, we asked if the identified hybrid reads show miRNA-mRNA sequence match. For this, we used the mature miRNA sequence of the identified miRNA and checked if mRNA sequence has its sequence match pair using miRanda (John et al., 2004). As the mRNA portion of the sequence is short and may not cover the matched sequence, we extended the range of the identified mRNA portion to 10 bp. We found 58,970 sequence matches (out of 127,512 hybrid reads) when we used 10 bp extension.

The Ago-associated regulatory load for mRNAs in chimeric reads was determined by the ratio of sequence read counts to overall mRNA abundance (human islet RNA-seq results obtained from Morán et al. [2012]).

Dual Luciferase Reporter Assay

A total of 6 × 10^4 HEK293FT cells were seeded into 24-well plates. After 24 hr, cells were transfected with either miRNA expression plasmid or mimic along with dual luciferase reporter plasmid. Each construct was transfected in four replicate wells and repeated in three independent experiments. Cells were lysed and processed using Promega Dual-Luciferase Reporter Assay system, as per manufacturer's recommendation. Firefly and renilla luciferase activities were measured on a Synergy HT (KC4 v3.4 software; Bio-Tek Instruments, Inc.) using Stop and Glo reagents (Promega), according to the manufacturer's instructions. Relative light units were calculated as the ratio of renilla to firefly luciferase activity, and the reporters were normalized between the control expression and the empty pMirglo values for a given treatment.

Lentiviral Transduction of Human Islets

Tough decoys were synthesized as described in Haraguchi et al. (2009) and subcloned into a pSlik-Venus lentiviral backbone. Lentivirus was prepared and titrated by the Wistar Protein Expression facility. Viral titers were in the range of 5 × 10^7–1 × 10^9 TU/ml. Groups of 200–250 islets were transduced overnight with 5 × 10^3 TU/islet with 4 μg/ml of polybrene. Media was replaced every day and RNA was extracted 72 hr posttransduction for RT-qPCR.

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