Experimental Diabetes Research

Noncoding RNAs

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Editorial Board

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Editorial
Noncoding RNAs

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Recent years have witnessed many remarkable discoveries in the life sciences that have led to major paradigm shifts. An example is the emerging appreciation of the critical role of noncoding RNAs in a broad range of physiological and pathological processes. One such class of noncoding RNAs, the microRNAs (miRNAs), controls intricate networks of gene expression via post-transcriptional mechanisms. Originally described in animals by V. Ambros and G. Ruvkin in 1993, microRNAs are now recognized to impact human development and health in many ways. Another distinct and rapidly growing class of noncoding RNA, the long ncRNAs (lncRNAs), is also implicated in gene regulation and disease.

Across the phylogenetic kingdoms, bacteria and other prokaryotes have proportionally the least amount of noncoding DNA. Interestingly the total number of protein-coding genes does not consistently increase with increasing complexity of organisms; for example, insects have just twice as many protein-coding genes (~13,500) than yeast (~6,000). Increasing cellularity also does not seem to affect the number of protein-coding genes in the genome. For example *C. elegans*, a worm that is well studied in developmental biology, has around 19,000 protein-coding genes that govern normal development and function of its 959 cells; whereas a similar number of protein coding genes (~25,000) in humans drives development and function of over 10 trillion cells. One major difference between the worm and human genomes is the relative proportion of noncoding DNA, which is seen to increase with complexity of body plan and cellular processes. In recent years it has been demonstrated that much of this "junk DNA" is transcribed as noncoding RNAs (including microRNAs and lncRNAs). Together, these noncoding RNAs have been demonstrated to influence the expression of around 30% of protein-coding genes and have been demonstrated to play important homeostatic roles that act to normalize gene expression and maintain cellular phenotypes during development and disease.

In this special issue focusing on noncoding RNAs, we bring together a collection of articles from various areas of diabetes and islet cell biology. T. Avnit-Sagi et al. and S. Kredo-Russo et al. discuss the regulation of two important pancreas-specific microRNAs, miR-7 and miR-375. Another article by A. D. Mandelbaum et al. describes how miRNAs can affect islet architecture. Research carried out in the past few years from several laboratories has clearly demonstrated that miRNAs and other noncoding RNAs are essential for development and differentiation of pancreatic progenitor cells as well as for regulation of glucose metabolism. An interesting application came through cancer research wherein specific noncoding RNAs were shown to be present at high abundance in the circulation. The possibility that microRNAs can be released in circulation as free microRNAs from dead/dying cells or specifically through exosomes has now been validated. An important area of research that stems from such observations is the possibility to utilize small noncoding RNAs as biomarkers for disease progression. The studies presented by L. B. Nielsen et al. and C. J. Taylor et al. in this issue are important in understanding and

establishing microRNA-based biomarkers that can be used for prediction of diabetes and/or complications. Some of the above studies are reviewed in this issue by M. D. Williams et al. With the emergence of novel technologies, there will likely be many more discoveries linking noncoding RNAs with the development of diabetes. Given the pervasive impact of noncoding RNAs on cellular function, these articles provide a flavour of the wide range of biologies that are controlled by these previously unappreciated RNA molecules.

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Erratum

Erratum to "Circulating Levels of MicroRNA from Children with Newly Diagnosed Type 1 Diabetes and Healthy Controls: Evidence That miR-25 Associates to Residual Beta-Cell Function and Glycaemic Control during Disease Progression"

Lotte B. Nielsen,[1,2] Cheng Wang,[3] Kaspar Sørensen,[4] Claus H. Bang-Berthelsen,[5] Lars Hansen,[1,2] Marie-Louise M. Andersen,[1,2] Philip Hougaard,[6] Anders Juul,[4] Chen-Yu Zhang,[3] Flemming Pociot,[5] and Henrik B. Mortensen[1,2]

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The following sentence: Furthermore, miR-25 one month after diagnosis was positively associated with residual beta-cell function ($P = 0.0037$), and negatively associated with glycaemic control (HbA1c) ($P = 0.0035$) 3 months after disease onset, should replace this sentence in the abstract of the existing paper: Furthermore, we identified miR-25 as negatively associated with residual beta-cell function (est.: -0.12, $P = 0.0037$), and positively associated with glycaemic control (HbA1c) (est.: 0.11, $P = 0.0035$) 3 months after onset.

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Research Article

Dysregulation of Dicer1 in Beta Cells Impairs Islet Architecture and Glucose Metabolism

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microRNAs (miRNAs) play important roles in pancreas development and in regulation of insulin expression in the adult. Here we show that loss of miRNAs activity in beta-cells during embryonic development results in lower beta-cell mass and in impaired glucose tolerance. Dicer1-null cells initially constitute a significant portion of the total beta-cell population. However, during postnatal development, Dicer1-null cells are depleted. Furthermore, wild-type beta cells are repopulating the islets in complex compensatory dynamics. Because loss of Dicer1 is also associated with changes in the distribution of membranous E-cadherin, we hypothesized that E-cadherin activity may play a role in beta cell survival or islet architecture. However, genetic loss of E-cadherin function does not impair islet architecture, suggesting that miRNAs likely function through other or redundant effectors in the endocrine pancreas.

1. Introduction

Genome-encoded miRNA create a regulatory layer that impacts gene expression posttranscriptionally (reviewed in [1]). miRNA are important for beta-cell differentiation and function, and specific miRNAs have been proposed to regulate beta-cell genes [2–7].

miRNA are subject to extensive processing, including digestion by Drosha in the nucleus [8] and by Dicer1 (MGI: 2177178) in the cytoplasm [9]. Deletion of Dicer1 in the early pancreatic lineage, using a Pdx1-Cre mouse line, results in inactivation of the entire miRNA pathway in the early pancreatic bud and causes pancreas agenesis, demonstrating that miRNA are important for pancreas organogenesis [10]. The adult pancreas is also susceptible to loss of Dicer1, as pancreas morphology is distorted in a Dicer1 hypomorph model [11]. Furthermore, we have recently shown that Dicer1 and miRNA function is critical for maintenance of the beta-cell hormone-producing phenotype, by maintaining the proper balance of transcriptional activators and repressors upstream of insulin expression [12].

E-Cadherin is a transmembrane protein encoded by the gene Cdh1 (MGI: 88354), which is involved in homotypic cell-cell interactions [13]. E-Cadherin function was suggested to play a role in endocrine cell clustering and in the establishment of normal islet morphology and function [14–18].

In this work we show the importance of Dicer1 for beta-cell survival and islet architecture. Dicer1-null beta cells are progressively lost within the first few weeks after birth. However, wild-type beta cells, which do not undergo recombination, repopulate the islet. Dicer1-null beta cells also exhibit changes in the distribution of E-Cadherin, reminiscent of previous reports (e.g., [16]). However, genetic loss of Cdh1, which encodes for E-Cadherin, did not exhibit detectable glycemic or tissue phenotype.

2. Results

2.1. Beta-Cell-Specific Disruption of Dicer1 During Embryonic Development Causes Juvenile Glucose Intolerance. Loss of Dicer1 function blocks the maturation of miRNA species, thus providing a platform for assessment of the overall contribution of miRNAs to beta-cell function *in vivo*. With this goal in mind, we created RIP-Cre, Dicer1$^{LoxP/LoxP}$ mice, in which Dicer1 conditional allele is inactivated by Cre thereby preventing miRNA processing. This strain was created by crossing a Dicer1 conditional allele [19] onto a conditional Cre transgene, driven by the rat insulin promoter [20]. Concomitant with Dicer1 inactivation, Cre recombinase activated an enhanced yellow fluorescent protein (YFP) reporter, integrated into the ROSA26 locus [21]. Thus, cells in which recombination occurred lost Dicer1 activity and were labeled with YFP. Control mice were heterozygous for the Dicer1 allele (RIP-Cre; Dicer1$^{LoxP/+}$) and harbored YFP reporter (Figure 1(a)). RIP-Cre; Dicer1$^{LoxP/LoxP}$ animals developed fasting and fed hyperglycemia (Figure 1(b)). Accordingly, when RIP-Cre; Dicer1$^{LoxP/LoxP}$ animals were tested for glucose clearance after intraperitoneal injection of 2 mg/gr glucose they exhibited impaired glucose tolerance at the age of 1-2 months, which was evident also at the age of 8–10 moths (Figure 1(c)).

2.2. Reduced Beta-Cell Mass in Dicer1 Mutants. In order to understand the cause for impaired glucose tolerance, we performed morphometric analysis of RIP-Cre; Dicer1$^{LoxP/LoxP}$ pancreata. Beta-cell mass was calculated from the total insulin-positive immunoreactivity area, relative to the total pancreas area and weight (see methods). Our analysis, performed at the age of 4 months, indicates that RIP-Cre; Dicer1$^{LoxP/LoxP}$ mutant beta-cell mass is reduced compared to control (Figures 1(d) and 1(e)). However, the compromised functionality of the pancreas likely results also from impaired insulin synthesis and defective exocytosis, as these processes were previously shown to be controlled by miRNAs.

To evaluate insulin expression and synthesis, we performed immunoflouresent analysis. Beta cells, that expressed YFP, in which Dicer1 activity was disrupted, did not express insulin (Figures 2(a)–2(d)), suggesting that insulin synthesis is inhibited in Dicer1 knockout beta cells. Nonetheless, cells that lost Dicer1 activity did not express alternative endocrine markers, namely, glucagon and somatostatin (Figure S1). Noteworthy, wild-type beta cells, which did not undergo recombination, within mosaic mutant islets, did not express YFP and maintained the expression of insulin (Figures 2(a)–2(d)). These observations are consistent with our previous study of insulin synthesis in Dicer1 knock-out beta cells in adulthood [12].

2.3. Dicer1 Mutant Cells Are Progressively Lost from Mutant Islets. In early postnatal life, Dicer1 knock-out beta cells were dispersed uniformly throughout the islet tissue. However, the abundance of Dicer1-null cells progressively decreased. Thus, only a minority of the cells were YFP-positive (and presumably Dicer1-negative) at the age of one month (Figures 2(b) and 2(d)).

To rule out a potential artifact, related to the activity of the Rosa-YFP reporter in our model, we sought to detect directly the expression of the Cre transgenic protein, which is controlled by the activity of rat minimal insulin promoter in beta cells. Immunofluorescence studies of pancreata revealed that, at P7, Cre-expressing cells were evenly spread within islets. Examination of older animals revealed a gradual loss of Cre-expressing cells in mutant animals ending at P30, when almost no Cre-positive cells were detected. Furthermore, it appears that a second population of seemingly-normal beta cells, which did not express Cre and maintained insulin expression, gradually compensated for the reduction in Cre-positive cells. Intriguingly, the remaining Cre-expressing cells were progressively and preferentially found at the periphery of the islet (Figure 2(f)). In control islets, however, Cre expressing cells were abundant at all studied time-points and show unaltered distribution (Figure 2(e)).

2.4. Apoptosis Is Not Detected in Dicer1 Mutant Beta Cells. One plausible explanation for the loss of Cre-positive cells may be related to apoptosis, which has been described in some Dicer1 models. However, other cell types do not seem to respond to loss of Dicer1 activity by a programmed cell death response (e.g., [22–24]). In order to evaluate potential apoptosis, we immunostained pancreas sections for activated caspase3 at different time points. Surprisingly, we did not detect apoptosis (Figure S2) in screening 3500 beta cells in 10 different animals by two independent investigators. A third qualitative evaluation of pyknotic nuclei on hematoxylin/eosin staining by a pathologist was also negative (not shown). We conclude that RIP-Cre; Dicer1$^{LoxP/LoxP}$ beta cells do not undergo observable apoptosis. While this study is rather exhaustive and consistent with the work of Kalis et al., [25], we cannot exclude, for example, high clearance dynamics or other reasons for false-negative detection of apoptosis in dying beta cells.

2.5. Abnormal E-Cadherin Expression in Dicer1-Null Cells. Since we noticed that mutant beta cells tend to be positioned at the periphery of the islets (Figure 2), we hypothesized that these cells might have altered adhesion properties. We therefore quantified the expression pattern of E-Cadherin along the beta-cell membrane, based on qualitative observations of Yamagata et al., [16].

This analysis was unbiased, taking a blinded approach. Thus, we first gave each beta-cell a binary value for "normal" or "abnormal" E-cadherin expression and only then, in a secondary step, we have annotated cells as either mutant (YFP-positive) or control (YFP-negative). Then the percentage of cells with abnormal E-Cadherin staining was calculated from the total counted cells.

Initially, we assessed baseline E-cadherin continuity in control beta cells, which did not undergo recombination by comparing the YFP-negative cells in mutant and control pancreata (genotypes: RIP-Cre; Dicer1$^{LoxP/LoxP}$ and RIP-Cre; Dicer1$^{LoxP/+}$, resp. Figures 3(a) and 3(c)). This analysis

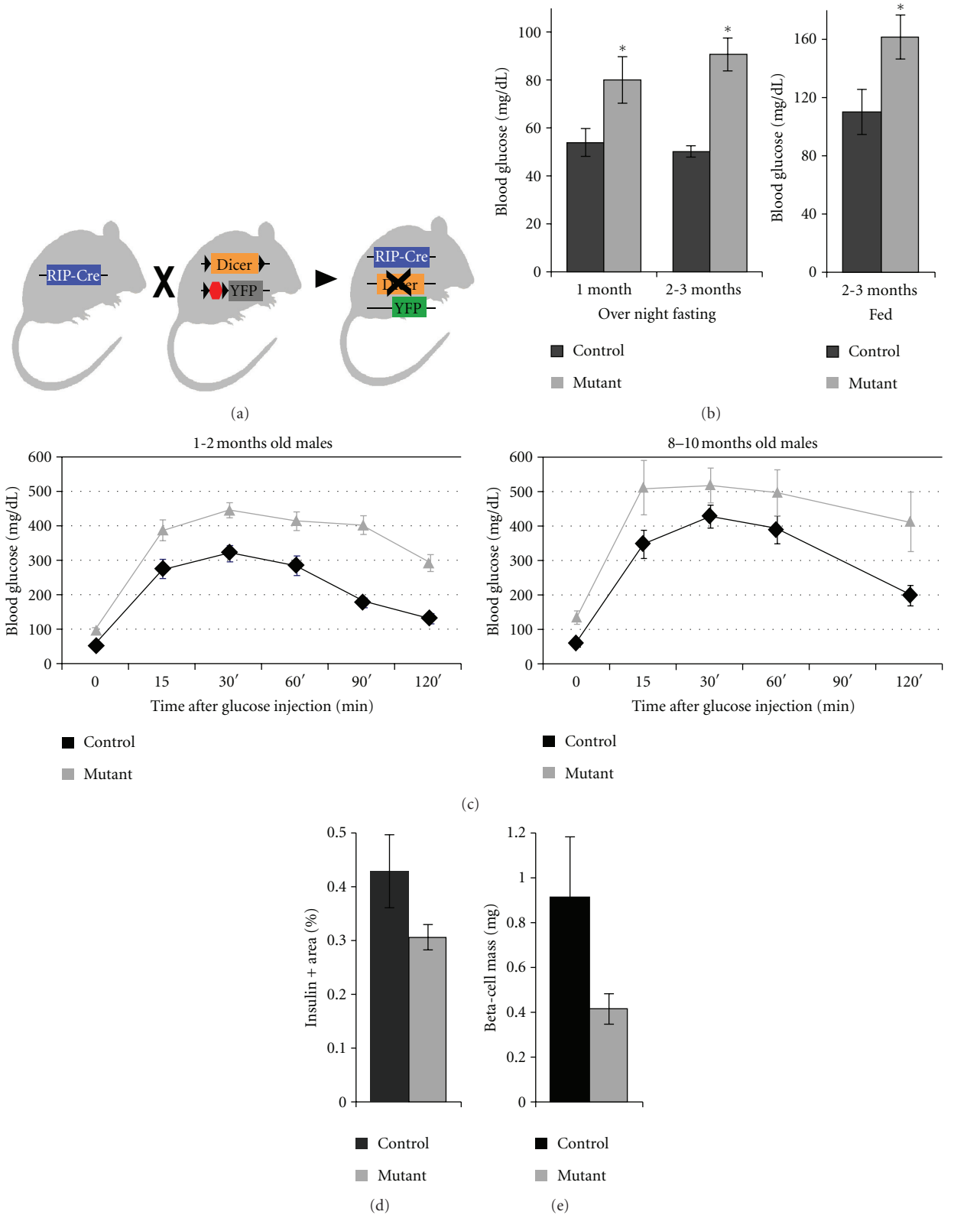

Figure 1: Dicer1 conditional knockout in beta cells, results in impaired glucose tolerance and reduction in beta-cell mass. A diagram of mouse genetics for generation of RIP-Cre; Dicer1$^{LoxP/LoxP}$ mutants that additionally harbor Rosa-EYFP reporter (a). Blood glucose levels of RIP-Cre; Dicer1$^{LoxP/LoxP}$ mutants and RIP-Cre; Dicer1$^{LoxP/+}$ controls, after overnight fasting or when randomly fed (b), and during standard glucose tolerance test (GTT) at multiple time-points after intra-peritoneal injection of a glucose bolus (c). Assessment of the percentage of insulin-positive sectional area relative to the total pancreas area in serial sections of mutant and control pancreata (d)). Calculated beta-cell mass, corrected to pancreas weight (e). $^*P < 0.05$.

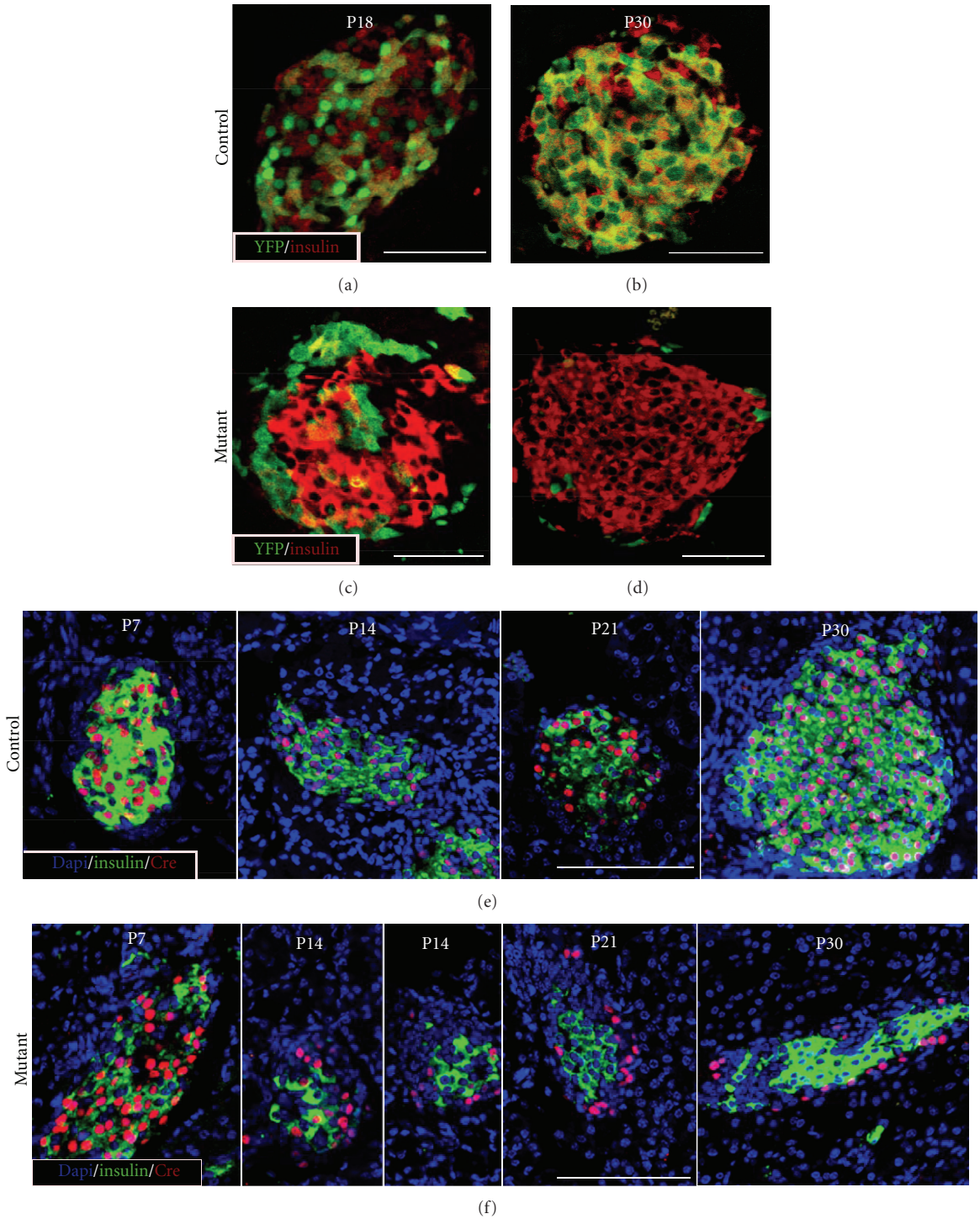

Figure 2: Progressive disappearance of Dicer1 mutant cells during post-embryonic growth of islets of Langerhans. YFP-positive (green) cells are accompanied by wild-type beta cells, which do not express YFP and are immunostained for insulin (red). These two populations of beta cells are comparably abundant in control RIP-Cre; Dicer1$^{LoxP/+}$ islets, at postnatal days 18 (a, P18) and 30 (b, P30). However, "mutant" beta cells in islets of RIP-Cre; Dicer1$^{LoxP/LoxP}$ animals that are detected at P18, are progressively lost and are rarely detected at P30 (c, d). Cre-positive 'control' cells are dispersed throughout islet tissue of Cre; Dicer1$^{LoxP/+}$ at multiple postnatal time points (e). Cre Immunofluorescence reveals the progressive loss of Cre-positive beta-cell from RIP-Cre; Dicer1$^{LoxP/LoxP}$ islets, that are depicted only at the islet periphery at P21 and P30 (f). Bar −50 μm.

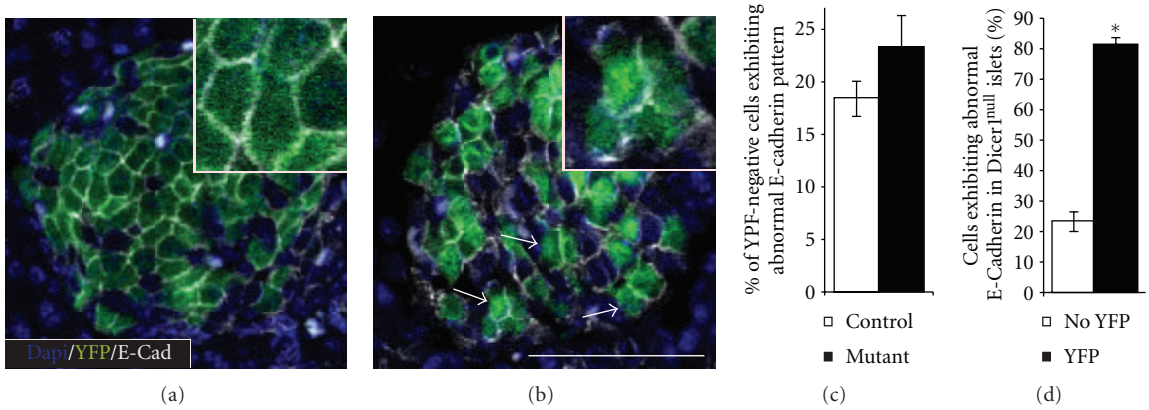

Figure 3: Dysregulated membranal E-Cadherin expression in Dicer1 mutant cells. Representative Control (RIP-Cre; Dicer1$^{LoxP/+}$, (a)) and Mutant (RIP-Cre; Dicer1$^{LoxP/LoxP}$, (b)) P11 islets, immunostained for E-Cadherin (white), and YFP (green) were taken for quantitative analysis. Magnified field in inset. Comparable E-Cadherin expression in YFP-negative cells in Mutant and Control animals (c). The incidence of Dicer1 mutant beta cells exhibiting dysregulated E-Cadherin expression is higher than in control beta cells, which do not express YFP, in the same islet (d). Bar $-50\,\mu$m. $^*P < 0.01$.

suggested a baseline value denoting ~18% of cells as harboring abnormal membranous E-Cadherin pattern. This value is probably sensitive to staining and microscopy biases and is subjective in nature. However, this evaluation method was used in a blinded fashion for controls and mutants and therefore provides the grounds for comparing E-Cadherin expression in mutant cells.

This approach revealed comparable membranous E-cad pattern in control cells of mutant and wild-type animals (Figures 3(a) and 3(c)). However, using the same method for P11 and P18 islets of RIP-Cre; Dicer1$^{LoxP/LoxP}$, we uncovered significant downregulation of E-cadherin continuity in YFP-positive/Dicer1 mutant cells (Figures 3(b) and 3(d)). Thus aberrant distribution of E-Cadherin at the beta-cell membrane was more abundant in beta cells that lost Dicer1 activity.

2.6. Loss of Cdh1 Expression Affects Neither Glucose Homeostasis Nor Islet Architecture. To investigate the hypothesis that E-Cadherin may be involved in islet biology, we took a mouse-genetics approach. We created RIP-Cre; Cdh1$^{LoxP/LoxP}$ mouse line for specific loss of E-Cadherin expression in beta cells, by crossing a Cdh1 conditional allele [26] to an RIP-Cre transgene [20] (Figure 4(a)). Unexpectedly, adult RIP-Cre; Cdh1$^{LoxP/LoxP}$ mice exhibited normal glucose tolerance (Figure 4(b)). Analysis of pancreata from RIP-CRE; Cdh1$^{LoxP/LoxP}$ that also harbor an inducible YFP transgene (i.e., recombined cells lost E-Cadherin expression and were labeled with YFP) by coimmunodetection of E-cad and of YFP showed that tissue composition is unaffected by loss of E-cad (Figure 4(c)). Thus, surprisingly, wild-type and E-Cadherin-null beta cells reside in the same islet. Furthermore, RIP-Cre; Cdh1$^{LoxP/LoxP}$ islet morphology was normal between P7 and the age of five months (Figures 4(d) and 4(e)). From this genetic study we conclude that loss of E-cadherin is not sufficient to interrupt any observable cellular features or endocrine physiology, *in vivo*. Furthermore, although E-cadherin distribution is impaired in Dicer1 mutant beta cells, direct knockout of E-Cadherin in the same cell population did not result in similar phenotypes. We therefore conclude that other elements, downstream of Dicer1 affect islet architecture and the loss of beta cells. However, E-Cadherin may be a marker of attenuated adhesion in Dicer1 mutant beta cells and it may function redundantly with some other factors in the upkeep of beta-cell epithelial features.

3. Discussion

Our results demonstrate the importance of Dicer1 in beta-cell survival and are also consistent with the function of miRNA in maintenance of insulin expression in the adult islet [12]. While this paper was in preparation, Kalis et al. reported a similar model [25]. Together the works of Kalis et al. and ours provide a consistent view of the importance of Dicer1 and miRNAs for juvenile glucose homeostasis. Dicer1 is essential for beta-cell survival. Progressive loss of beta cells in the studies of Kalis et al. [25] and in our study was not depicted by standard apoptotic markers, which might be related to limited assay sensitivity or to nonapoptotic cell death. In addition, mutant beta cells are probably diluted upon the proliferation of wild-type cells in chimeric islets.

When Dicer1 is deleted in adulthood, islet architecture stays intact [12]. This may reveal a role for Dicer1 during the embryonic or early postnatal period, as previously suggested [25]. However, another possibility is that changes in tissue composition are more readily evident in growing islets of the newborn than in older, less dynamic, adult tissue. If this is true, then similar changes in islet architecture may be potentially observed also in the adult model, over longer periods of time.

The positioning of mutant beta cells at islet periphery after postnatal day 14 suggests that differential adhesion properties may preferentially encourage homotypic adhesion

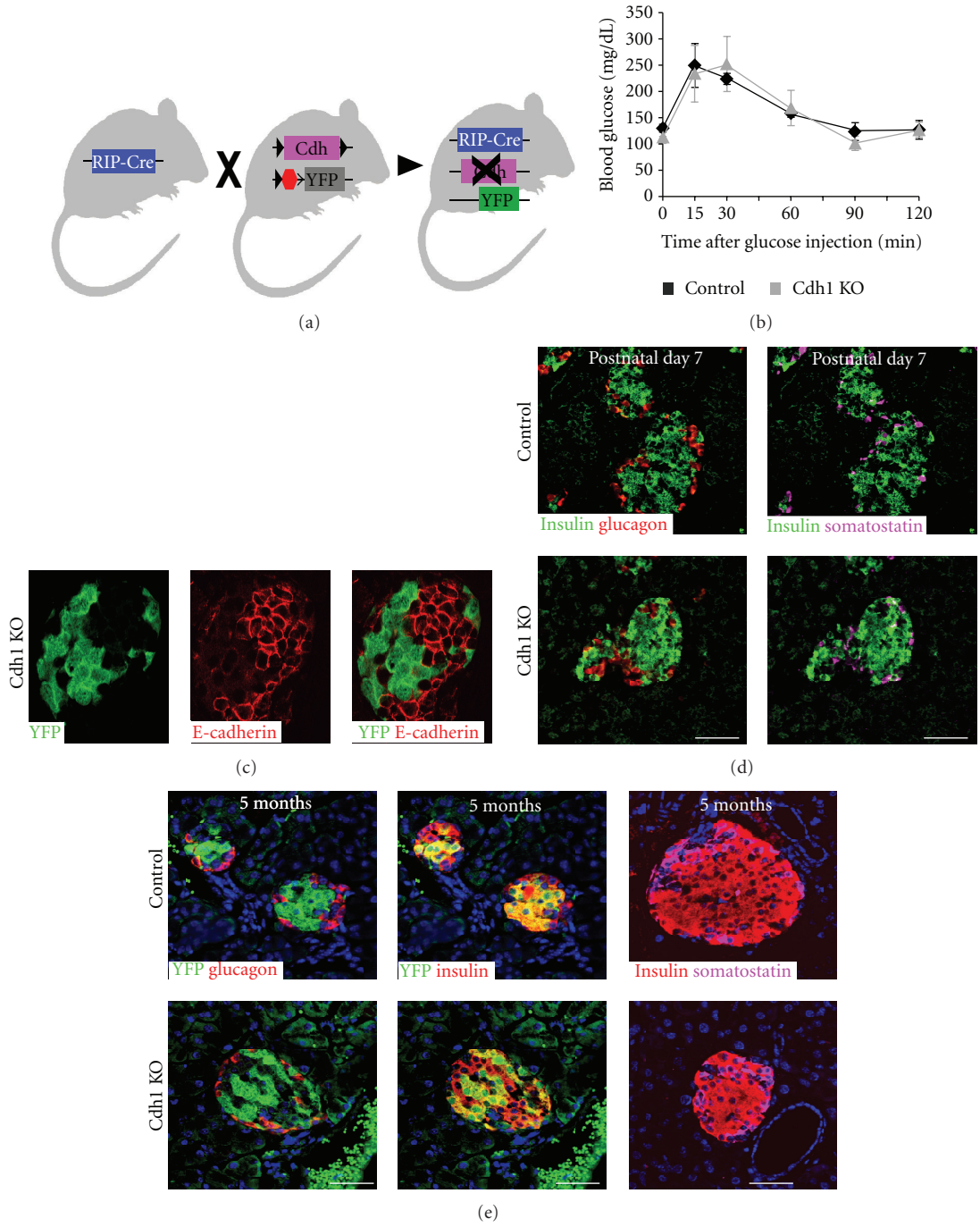

Figure 4: Loss of Cdh1, encoding for E-cadherin, does not attenuate glucose homeostasis or islet architecture. A diagram revealing mouse genetics steps for loss of Cdh1 in beta cells (a). RIP-Cre; $Cdh1^{LoxP/LoxP}$ mice, marked as Cdh1 KO, tolerate at the age of five months intraperitoneal glucose bolus, like control counterparts (b). RIP-Cre; $Cdh1^{LoxP/LoxP}$ mutant cells, which do not express E-Cadherin and are marked by the expression of a YFP reporter, exhibit normal cellular morphology within mature islets, at the age of five months (c). Endocrine markers are comparably expressed in mutant and control islets at the age of 7 days, (d) and of five months, (e). Bar −50 μm.

between wild-type cells. However, rather unexpectedly, direct knockout of E-Cadherin in beta cells did not reveal any physiological phenotype, neither was it associated with changes in islet morphology, or the expression of endocrine markers. This is surprising since past reports suggested a role for E-Cadherin in clustering and in function of endocrine cells [14–18, 27]. Therefore, our *in vivo* results point to the existence of alternative or redundant molecular mechanisms for controlling beta-cell adhesion and islet epithelial properties.

The RIP-Cre; $Dicer1^{LoxP/LoxP}$ model exhibits chimerism denoted by the presence of both mutant and wild-type beta

cells in the same islet. Detailed analysis of a temporal series of mutant pancreata revealed that a wild-type population is replacing Dicer1-null beta cells and eventually repopulates the whole islet. Interestingly, reminiscent tissue dynamics are observed in conditional knock-out model of the insulin receptor substrate 2 (Irs2) gene. In that model, a subset of the beta cells, which evaded Cre-dependent recombination, repopulated the endocrine pancreas [28]. Therefore, extensive compensatory growth of wild-type beta-cell clones may reflect a physiological response to impaired endocrine function, which is imposed by loss of genetic function in subsets of the cells in the organ. This may be observed in other conditional knock-out models, which exhibit chimeric and incomplete recombination, regardless of the preceding genetic insult. Our observations suggest that wild-type clone proliferation capacity is nonetheless limited. Thus, RIP-Cre; Dicer1$^{LoxP/LoxP}$ mice manifest impaired glucose tolerance at the age of two months but also at the late age of 9 ± 1 month, long after Dicer1-null beta cells become an insignificant minority within the organ. This is consistent with the reported finite potential for compensatory proliferation of beta cells and their progenitors [29], even if the required beta-cell mass for euglycemia is not met.

In summary, our study reveals Dicer1 importance for beta-cell survival and the normal function of the insulin axis. The unexpected islet dynamics suggest that Dicer1 mutant cells are outcompeted in time by wild-type beta cells that repopulate the islet, providing an intriguing model that uncovers the limitations of compensatory proliferation in meeting the physiological needs of the animal.

4. Materials and Methods

4.1. Mouse Handling and Physiology. The following mouse alleles were studied: rat insulin promoter-Cre transgene [20], Dicer1^{flox} allele [19], R26R-EYFP [21], and Cdh1 [26]. Mice were housed and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of WIS. Glucose tolerance tests were performed by intra-peritoneal injection of glucose (2 mg/g BW), after an overnight fast and measuring blood glucose levels using an "*Ascensia elite*" glucometer. Primers for PCR genotyping are listed in Supplementary materials available online at doi: 10.1155/2012/470302.

4.2. Pancreatic Histology and Immunohistochemistry. Dissected pancreata were fixed in 4% paraformaldehyde, at room temperature for 2 h (P7), 3.5 h (P14, P21), or 24 h (P30) and then processed into paraffin blocks. Antigen retrieval in a 2100-Retriever (PickCell Laboratories, The Netherlands) was performed on 5 μm thick rehydrated sections prior to immunostaining with antibodies described in the Supplementary Material. Fluorescence images were captured using a Zeiss LSM510 and LSM710 Laser Scanning confocal microscope system under a magnification of $\times 40$. Nuclei counter-stained with Hoechst, 1 μg/mL (Sigma). Beta-cell mass was determined by analysis of consecutive paraffin sections 75 μm apart spanning the entire pancreas (approximately 20 sections/pancreas), stained for insulin and hematoxylin. Digital images of sections at a magnification of $\times 40$ were obtained and stitched using NIS-Elements software (NIKON), and the fraction of tissue covered by insulin staining was determined. The mass of beta cells was calculated as the product of pancreas weight and the fraction of tissue covered by beta cells. Detailed beta-cell mass protocol is described in Nir et al. [30].

4.3. 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.

Authors' Contribution

Amitai D. Mandelbaum and Tal Melkman-Zehavi contributed equally to this work. A. D. Mandelbaum, T. M. Zehavi, R. Oren, S. K. Russo, E. Hornstein designed the study. A. D. Mandelbaum, T. M. Zehavi, R. Oren, S. K. Russo, performed the experiments. T. Nir and Y. Dor provided important reagents and performed beta-cell mass studies. E. Hornstein and A. D. Mandelbaum wrote the paper.

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Review Article

MicroRNAs in Insulin Resistance and Obesity

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MicroRNAs (miRNAs) are a class of short, single-stranded non-protein coding gene products which can regulate the gene expression through post-transcriptional inhibition of messenger RNA (mRNA) translation. They are known to be involved in many essential biological processes including development, insulin secretion, and adipocyte differentiation. miRNAs are involved in complex metabolic processes, such as energy and lipid metabolism, which have been studied in the context of diabetes and obesity. Obesity, hyperlipidemia (elevated levels of blood lipids), and insulin resistance are strongly associated with the onset of type 2 diabetes. These conditions are also associated with aberrant expression of multiple essential miRNAs in pancreatic islets of Langerhans and peripheral tissues, including adipose tissue. A thorough understanding of the physiological role these miRNAs play in these tissues, and changes to their expression under pathological conditions, will allow researchers to develop new therapeutics with the potential to correct the aberrant expression of miRNAs in type 2 diabetes and obesity.

1. Introduction

Type 1 diabetes is characterized by the selective autoimmune-mediated destruction of the β cells in the islets of Langerhans in endocrine pancreas. This pathological loss of β-cell mass in the pancreas results in a failure to produce insulin in response to changes in blood glucose levels. Sufferers of type 1 diabetes require stringent monitoring of blood glucose levels and treatment with exogenous insulin administered through regular injections or through continuous monitoring insulin pumps, to replace the absent insulin. Type 2 diabetes is a less defined condition, culminating in dysregulation of blood glucose levels but caused by the development of insulin resistance and/or relative insulin deficiency. In this context, the body may maintain its ability to secrete insulin; however, the biochemical signalling required for the secretion of insulin or the sensitivity of cells to the released insulin is deficient, resulting in a blunted response and aberrant blood glucose levels. Early detection provides the opportunity for the disease to be managed using life-style changes; however, disease progression and eventual reduction in β-cell mass require insulin supplementation, similar to the pathology of type 1 diabetes. Obesity, hyperlipidemia (elevated levels of blood lipids) and insulin resistance are strongly associated with the onset of type 2 diabetes. Excessive weight strongly correlates with disease progression and increased risk of detrimental complications. Type 2 diabetes and obesity are considered to be so closely related that they are sometimes referred to collectively as "diabesity" [1].

MicroRNAs (miRNAs) are a class of short, single-stranded non-protein coding gene products typically 20–22 nucleotides in length. They are present in the genomes of eukaryotic organisms and known to post-transcriptionally regulate the expression of target genes through interactions with specific mRNAs [2, 3]. miRNAs are known to negatively regulate gene expression at the post-transcriptional level, either by inhibiting translation or by degrading the target mRNA [4, 5]. This is achieved through interactions between the miRNA seed sequence (first 2 to 8 bp of miRNA) and the 3′-untranslated regions (3′-UTRs) of specific mRNAs. The nature of these interactions affords an individual miRNA with the potential to regulate multiple genes across the diverse eukaryote genome. Such interactions have been shown to regulate multiple diverse biological processes including development [6, 7], insulin secretion [8], neurodevelopment [9] and cell differentiation [10].

Following the discovery that miRNAs were involved in many relevant biological processes, including energy and fat metabolism, these molecules have received growing attention in the fields of diabetes and obesity research. Through the understanding of the role miRNAs play in normal physiology and pathological conditions, researchers will be able to unlock a new class of therapeutic targets with the potential to significantly alter multiple aspects of their respective conditions.

2. miRNAs in Pancreatic β Cells

Islets of Langerhans are highly-vascularized microorgans, located within the pancreas, primarily responsible for the maintenance of normal blood glucose concentration [11]. Blood glucose levels are maintained within a narrow reference ranged between 3.6 mM and 5.7 mM ($\text{mmol}\cdot\text{L}^{-1}$) in humans as part of metabolic homeostasis. Glucose homeostasis is primarily achieved through the antagonistic actions of the islet hormones insulin and glucagon, released from islets. Each islet is composed of multiple cell types characterized by the endocrine hormones they produce. These include insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells, ghrelin-producing ε cells, and pancreatic polypeptide-producing (PP) cells [12, 13]. During embryonic development, the pancreatic primordial bud is derived from the posterior foregut endoderm. Endocrine, exocrine, and ductal structures subsequently differentiate through a complex branching morphogenic process [14].

miRNAs have been found to be important for the proper development of the pancreas, in particular the insulin-producing β-cell population. A conditional deletion of Dicer [15], a cleavage protein essential in miRNA biogenesis resulting in total ablation of all miRNA transcripts in pancreatic progenitor cells in a mouse model produced significant defects in all resulting pancreatic cell lineages. Endocrine cells, in particular the β-cell population, were the most dramatically reduced. This effect on endocrine cells was attributed to increased Hes1 expression and consequent reduction in the key pancreatic transcription factor ngn3 (neurogenin-3) during development. The effects of miRNA-mediated regulation of ngn3 activity have been further studied by Joglekar et al. [10], who demonstrated that following 70 percent pancreatectomy, the expression pattern of pancreatic transcription factors upstream of ngn3 was similar to that observed during developing and regenerating pancreas. However, ngn3 (and downstream transcription factors neuroD and nkx2.2) was not detected during regeneration and determined to be post-transcriptionally silenced by miRNAs, including miR-15a, miR-15b, miR-16, and miR-195. The elevated expression of these miRNAs in regenerating pancreas was theorised to facilitate ngn3 independent endocrine regeneration. These studies demonstrate the capacity for miRNAs to significantly alter the behaviour of endocrine cell populations through regulation of key endocrine pancreatic transcription factors under different conditions.

Many genes expressed in pancreatic β cells are differentially regulated in real time with respect to changes in blood glucose levels to optimise the production and secretion of insulin [16]. The functional release of insulin, the transcription and stability of insulin mRNA, insulin translation and insulin processing are all regulated by glucose concentrations in β cells. The abundance of several miRNA transcripts is also known to change in response to altered glucose concentrations. Tang et al. [17] demonstrated that exposing cultured pancreatic β-cell line (MIN6) to simulated prolonged high-glucose conditions could significantly alter the expression of a large number of miRNAs. miR-124a, miR-107 and miR-30d were upregulated and miR-296, miR-484 and miR-690 were downregulated in persistent high glucose conditions. Increased expression of miR-30d in high glucose conditions correlated with increased insulin gene expression but no associated increase in insulin secretion, suggesting that targets of miR-30d are negative regulators of insulin gene expression, independent of insulin secretion. However, a number of other miRNAs have been identified as having direct effects on the various subcellular events involved in glucose-stimulated insulin secretion (GSIS). Hennessy et al. [18] characterized the loss of GSIS in glucose nonresponsive MIN6 cells and discovered that a panel of ten miRNAs (miR-369-5p, miR-130a, miR-27a, miR-410, miR-200a, miR-337, miR-532, miR-320, miR-192 and miR-379) was differentially expressed in non-responsive cultures, implicating these miRNAs in GSIS.

In 2004, Poy et al. [8] cloned 11 novel miRNAs from pancreatic endocrine cell lines. This study revealed that miRNAs were not only involved in regulating pancreas development but were also involved in other vital biological processes including insulin secretion. This study also identified miR-375, an evolutionarily-conserved islet-specific miRNA, which has since become one of the best characterized islet-specific miRNAs implicated in both insulin secretion [8] and glucose homeostasis [19]. miR-375 was initially implicated in insulin release and was able to negatively regulate GSIS with no effect on either ATP production or intracellular calcium levels. miR-375 was experimentally shown to act at a late step of insulin exocytosis to suppress insulin release. Mtpn (myotrophin), a protein implicated in actin depolymerization and vesicular fusion, was predicted to be a target for miR-375. This was experimentally validated, with inhibition of mtpn production recapitulating the effects of miR-375 on insulin secretion. Mtpn also acts in the nucleus as a transcription factor to activate nuclear factor κB (NF-κB), a critical component in maintaining GSIS in β cells [20, 21].

Isolated islets and cell lines exposed to high glucose demonstrate decreased expression of miR-375 which correlated with increases in pdk1 (3′-phosphoinositide-dependent protein kinase 1) and insulin gene expression [22]. Pdk1 is an important component of the pi3k/protein kinase B signal cascade responsible for the growth and developmental effects of insulin [23]. Loss of pdk1 in β cells results in progressive hyperglycemia due to reductions in islet density as well as in the number and size of endocrine cells [24]. Pdk1 was later identified as a direct target of miR-375. Overexpression of miR-375 resulted in reduced β-cell number, viability and reduced sensitivity to glucose-stimulated insulin transcription, all of which are reversed

with inhibition of miR-375. Hyperglycemia is similarly observed in miR-375 knock-out animals, which is also attributable to reduced β-cell mass [19]. In these knock-out animals, the important negative growth regulators modulated by miR-375 were found to be upregulated. The condition was more pronounced in animals subjected to metabolic stress, indicating miR-375 plays a crucial role in β-cell compensation when metabolic demand is increased.

Plaisance et al. [25] demonstrated miR-9, a miRNA expressed in pancreatic β cells in rat and mouse models, was able to regulate insulin release in β cells through interactions with the transcription factor oc2 (onecut-2). Oc2 negatively regulates granuphilin (also known as SLP4/SYTL4), a Rab GTPase effector associated with β cell secretory granules. Basal expression of miR-9 is essential for maintaining appropriate granuphilin levels and optimal insulin secretory capacity in β cells. However, elevated levels of miR-9 results in reduced GSIS in affected cells. Granuphilin-null mice also show impaired GSIS, with β-cells containing fewer insulin granules at the membrane and increased insulin release in response to glucose stimulation [26].

miRNAs have the potential to affect insulin release through other mechanisms, including the expression of the insulin gene. miR-124a exists in three isoforms (miR-124a1, miR-124a2, and miR-124a3). miR-124a2 is significantly upregulated in late pancreas development (e18.5), coinciding with a critical stage in β-cell differentiation. This isoform was found to target foxA2 (forkhead/winged helix transcription factor boxa2) and creb-1 (cAMP-response-element binding protein) mRNA. FoxA2 is an upstream regulator of pdx1 (pancreatic duodenal homeobox 1) [27]. Pdx1 is essential for glucose homeostasis and pancreas development and together with ngn3 and mafA represent the key transcription factors essential for β-cell differentiation. When pdx1, ngn3, and mafA are re-expressed in an experimental context, these three primary factors are capable of inducing differentiation of unrelated cell types into functional insulin-producing, islet-like structures, indistinguishable from endogenous islet β cells capable of ameliorating hyperglycaemia [28, 29]. Foxa2 is an essential activator of genes that function in multiple pathways responsible for insulin secretion. Through the regulation of foxA2, miR-124a can modulate insulin expression indirectly through pdx1 [30]. It can also regulate K_{ATP} channel subunits sur1 (sulphonylurea receptor 1) and kir6.2 (inward rectifier K+ channel member 6.2) [31], both of which are essential for regulated insulin release but have little functional effect on GSIS [30]. However, miR-124a has been shown to target other aspects of the exocytotic machinery responsible for insulin release, including rab27A, snap25, rab3A, syn1 (synapsin 1A) and noc2 (nucleolar complex associated 2) which are involved in GSIS [32].

3. miRNAs in Energy Metabolism

In 2005, Mersey et al. [33] provided the first evidence of the use of a miRNA to exert control on a metabolic pathway in mammals. This discovery provided evidence that miRNAs were involved in complex metabolic processes, such as energy metabolism, which has since been studied in the context of diabetes and adipose tissue biology. Glucose homeostasis requires both the presence of insulin and sensitivity of the target tissues to elicit the necessary response. The aetiology of type 2 diabetes is not fully understood; however, insulin resistance is a persistent fundamental finding in patients, and this usually manifests years before the disease is diagnosed clinically. Insulin resistance is considered the best predictor of type 2 diabetes [34]. Insulin resistance is defined as an inability to properly respond to either endogenously produced or exogenously administered insulin in peripheral tissues, such as adipose, liver and skeletal muscle tissues. This results in impaired insulin-mediated glucose uptake in the skeletal muscle and adipose tissues, and insulin-mediated suppression of hepatic glucose output. The resulting insulin insufficiency and chronic hyperglycemia progress to type 2 diabetes. The role of miRNAs in the physiological and pathological function of adipose tissue has received increased attention, particularly given their roles in glucose and lipid metabolism.

The mechanism responsible for insulin resistance is not fully understood but links between insulin resistance, dyslipidaemia and obesity have been established. Abnormalities of fat metabolism, particularly triglyceride storage and lipolysis are an early manifestation of insulin resistance. Increased nonesterified fatty acid (NEFA) mobilization from adipose tissue leads to plasma NEFA increase and ectopic deposition of triglycerides in nonadipose tissues. Increased lipid deposition in muscle and liver contributes to the metabolic condition seen in type 2 diabetes, with increased lipid depositing in the pancreatic β cells contributing to β-cell dysfunction and accelerated apoptosis [35, 36]. In addition to its role as the principal storage depot of triglycerides, adipose tissue acts as an endocrine organ which contributes to whole-body energy homeostasis. The release of adiponectin, resistin and leptin during adipocyte differentiation regulates many aspects of lipid and glucose metabolism [37]. Adipocytes differentiate from preadipocytes (a progenitor population committed to an adipogenic lineage), in a process which requires multiple transcription factors, including master regulators PPARγ (peroxisome proliferator-activated receptor gamma), C/EBPs (CAAT/enhancer-binding proteins) [38] and extracellular hormones including insulin [39, 40].

Esau et al. [41] were first to describe the role of miRNAs in human adipocyte biology. Using an adipocyte model system, the authors profiled the miRNA signatures of preadipocytes and differentiated adipocytes, which demonstrated that miR-143 was upregulated during differentiation. Inhibition of miR-143, with an antisense oligonucleotide inhibited differentiation, reduced the expression of adipocyte-specific genes (GLUT4, aP2, HSL, and PPAR-γ2) and decreased triglyceride accumulation (up to 75%) in treated cells in a dose-dependent manner. Efforts to further characterize the effects of miRNAs on mouse 3T3-L1 preadipocytes differentiation revealed that both miR-103 and miR-143 were unregulated during adipogenesis in vitro and in vivo [42]. Ectopic expression of miR-103, or miR-143 in preadipocytes, accelerated adipogenesis, increasing both the expression of adipogenesis markers and triglyceride accumulation.

In 2006, Kajimoto et al. [43] studied the expression profile of miRNAs in 3T3-L1 preadipocytes during adipogenic differentiation. In this analysis, miR-10b, miR-15, miR-26a, miR-34c, miR-98, miR-99a, miR-101, miR-101b, miR-143, miR-152, miR-183, miR-185, miR-224, and let-7b were found to be upregulated, and miR-181a and miR-182 were downregulated during differentiation. These changes were not observed at early time points (days 1–5) but were observed at a relatively later stage of differentiation, during which time lipid droplets were visible (day 9). Use of antisense inhibition of differentially expressed miRNAs did not affect adipose differentiation; however, these miRNAs were thought to be involved in modulating adipocyte function.

Several miRNAs have been implicated in the regulation of adipogenesis through actions on key regulatory transcription factor PPARγ. miR-27b is physiologically downregulated in human multipotent adipose-derived cells during adipogenesis. Overexpression of miR-27b was found to suppress differentiation by targeting a predicted miR-27b-binding site on PPARγ mRNA, inhibiting expression of PPARγ and downstream factor C/EBPa [44]. PPARγ has also been found to be targeted and repressed by miR-130, which has a similar effect when overexpressed in human primary preadipocytes [45]. This demonstrates that miR-27b and miR-130 can regulate adipogenesis in these cells through direct activity on PPARγ expression. Incidentally, thiazolidinediones, a class of insulin-sensitizing agents used in the treatment of insulin insensitivity, target PPARγ to elicit their therapeutic effects [46]. PPARα is another member of the PPAR family and important regulator of lipid homeostasis. PPARα has been identified as a target of miR-519d and regulates the accumulation of lipid during adipogenic differentiation [47]. Manipulation of miR-519 expression in human visceral preadipocytes was found to modulate the differentiation of cells through changes in PPARα expression.

The miRNA paralogues miR-103 and miR-107 are thought to be involved in energy metabolism. These paralogues are highly conserved and located within the pantothenate kinase (PANK) gene. miR-103 genes generate two mature miRNAs, miR-103(1) and miR-103(2), while the miR-107 gene generates miR-107. PANK catalyses the rate limiting step of pantothenate phosphorylation during the generation of Coenzyme A (CoA), which is a critical cofactor of several enzymes involved in diverse metabolic pathways. Trajkovski et al. [48] investigated the connection between elevated expression of miR-103/107 and obesity in obese mice. In this study, they identified caveolin-1, a critical regulator of the insulin receptor, as a direct target gene of miR-103/107. Decreased levels of miR-103/107 in adipocytes increased caveolin-1 expression, which led to stabilization of the insulin receptor, enhanced insulin signalling, decreased adipocyte size, and enhanced insulin-stimulated glucose uptake. These findings demonstrate the central importance of miR-103/107 to insulin sensitivity.

The actions of miRNAs on insulin resistance in healthy and Goto-Kakizaki rats (a model of type 2 diabetes) were investigated by He et al. [49]. Insulin responsive tissues were characterized for their miRNA expression, which revealed highly elevated levels of miR-29 family in adipose, liver and skeletal muscle tissue isolated from each animal strain. The expression of miR-29 family genes was upregulated in diabetic tissues. This effect could be reproduced in vitro by incubating 3T3-L1 adipocytes in high glucose (hyperglycemia) and insulin (hyperinsulinemia), simulating a type 2 diabetic microenvironment. The action of these miRNAs on insulin signalling is not clearly understood. However, the authors identified that insulin-activated Akt activity was downregulated by miR-29. Insig1 (insulin-induced gene 1) and Cav2 (caveolin 2) were validated targets of miR-29; however, their role in insulin signalling was not understood. Ling et al. [50] furthered this work and identified 50 upregulated and 29 downregulated miRNAs in insulin-resistant adipocytes, including miR-320 which was expressed 50-fold higher in insulin-resistant cells. The authors found that miR-320 was able to regulate insulin resistance in resistant adipocytes through targeting of a kinase subunit, p85. This increased Akt phosphorylation and levels of Glut4, improving the insulin-PI3-K signalling pathways.

The majority of work characterizing miRNAs involved in insulin signalling and energy metabolism has involved single miRNAs (or paralogues) rather than complete clusters. Xu and Wong [51] completed a bioinformatic analysis screening for mouse signalling pathways targeted by miRNA clusters. The authors identified one miRNA cluster, mmu-mir-183-96-182 as a potential regulator of irs1, rasa1 and grb2, all of which are located in the insulin signalling pathway. It was theorized that a single miRNA cluster could act at multiple levels in a signalling cascade by targeting different components to control the signal transduction process.

4. miRNAs in Obesity

The medical profession and the wider community are very concerned by the increased health risks associated with the growing prevalence of overweight and obesity in developed cultures. Obesity is particularly significant for Australia, having recently overtaken the United States as the world's "fattest" nation. Many factors are contributing to the growing obesity around the world, but this can be primarily attributable to poor dietary habits and sedentary lifestyles conducive to developing the condition.

Obesity, hyperlipidemia (elevated levels of blood lipids) and insulin resistance are strongly associated with the onset of type 2 diabetes. High Body Mass Index (BMI) has a strong correlation with increased (and excessive) adiposity which is principally responsible for a majority of the comorbidities associated with the condition. The activity of adipose tissue is adversely affected in obese patients, resulting in abnormal adipokine release and altered energy metabolism, which causes a state of chronic inflammation that contributes to insulin resistance and elevated free fatty acids in the bloodstream [52]. Obesity is most commonly associated with increased fat mass as a result of adipocyte hypertrophy or hyperplasia [37], which is closely linked to the chronic inflammation seen in patients. The elevated inflammatory cytokine levels typically observed in obese individuals, particularly TNF-α and IL-2 [53], secreted from adipocytes

have been shown to impair adipogenesis, contributing to the detrimental deposition of lipids in other organs [54]. The relationship between obesity and diabetes is strongly related to glucose and lipid metabolism. miRNAs have been shown to regulate the activity of key cellular processes, including insulin release in pancreatic β cells and differentiation of adipocytes and are therefore thought to contribute to these pathologies.

A consistent and counterintuitive observation in obese patients and experimental models of obesity is that the miRNAs normally induced during adipogenesis are downregulated in the obese subjects. Xie et al. [42] demonstrated this inversion in miRNA expression by profiling their expression in 3T3-L1 preadipocytes and adipocytes from leptin deficient ob/ob and diet-induced obese mice. Similar miRNAs were differentially regulated during in vitro and in vivo adipogenesis. miR-422b, miR-148a, miR-107, miR-103, miR-30c, miR-30a-5p, and miR-143 were induced during 3T3-L1 differentiation but downregulated in cells isolated from both models of obesity. This trend was also evident for miR-221 and miR-222, which were found to be downregulated during 3T3-L1 differentiation but upregulated in obese adipocytes. This inverse regulatory pattern has been observed during adipogenesis and obesity across different species, including mice and humans, and validated in different experimental models of obesity.

As described previously, miR-143, miR-103 and miR-107 have been shown to regulate adipocyte differentiation. However, their expression has been found to be downregulated in the mouse model of genetic insulin resistance and obesity (ob/ob mice), possibly through an inflammatory pathway stimulated as part of the model pathology [42]. Visceral adipose tissue isolated from obese mice has been found to contain increased expression of miR-143, which is associated with alterations to PPARγ and aP24 expression [55]. Chronic inflammation is typically seen in obesity and is associated with changes to the miRNA profile of affected tissues. In three murine models of obesity including leptin-deficient ob/ob mice, leptin-receptor-deficient db/db mice, and KKAy44 mice, miR-335 was found to be upregulated in adipose tissue [56], suggesting a role in adipose hyperplasia, despite the lack of experimentally validated targets of miR-335 in adipose tissue.

In a recent study, Zhao et al. [57] profiled 220 miRNAs in pancreatic islets, adipose tissue and liver isolated from diabetes-resistant (B6) and diabetes-susceptible (BTBR) mice. Many miRNAs in these tissues were altered following induction of obesity, with several miRNAs in each tissue responding differently, depending on the animal strain. Approximately 40 miRNAs in liver were downregulated in response to obesity in diabetes-resistant mice but not in diabetes-susceptible animals, indicating that genetic differences between the mouse strains play a critical role in miRNA regulation and illustrating the possible contribution of genetic background to miRNA expression in disease progression.

In 2009, Klöting et al. [58] isolated miRNA from different fat depots of overweight and obese individuals. Paired samples of abdominal subcutaneous and intra-abdominal omental adipose tissue that were obtained from fifteen individuals with either normal glucose tolerance or recently diagnosed type 2 diabetes were analysed. Analysis revealed that of the 106 miRNAs expressed in these tissues, there was no miRNA exclusively expressed in either fat depot. However, 16 miRNAs had an expression pattern dependent on the fat depot. The expression of miRNA-17-5p, miRNA-132, miRNA-99a, miRNA-134, miRNA-181a, miRNA-145 and miRNA-197 were correlated with key metabolic parameters, including, fasting plasma glucose, and circulating leptin, adiponectin and interleukin-6 levels. A negative correlation between miR-99a, miR-325 and IL-6 concentration was determined, as was an inverse correlation between miR-181a expression and adiponectin concentration.

5. miRNAs in Clinical Applications

miRNAs represent a possible diagnostic tool for the early detection of type 1 diabetes. Patients suffering from the disease will have already lost more than 50% of their β-cell mass before presenting with symptoms. There is therefore a need to develop a system for early detection, to allow intervention strategies to be significantly more effective. miRNAs have been detected in blood and other body fluids [59] and miRNAs isolated from serum samples have proven to be stable, reproducible and consistent among individuals [60, 61]. In a recent study, Zampetaki et al. [62] extracted miRNAs from plasma samples of age- and sex-matched type 2 diabetic patients and controls. They determined 13 candidate miRNAs with microarray screening that were differentially expressed in these samples. Quantitative PCR assessment revealed lower plasma levels of miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 and an increase in miR-28-3p in diabetic serum. Interestingly, the expression of miR-15a, miR-28-3p, miR-126, miR-223 and miR-320 was altered before the disease was manifested clinically. Analysis of these miRNAs was sufficient to identify 70% of the type 2 diabetic patients and predict the development of type 2 diabetes in 52% of normoglycemic patients that developed diabetes over the course of the study. This was the first demonstration that a unique miRNA blood signature could exist for diabetes and when combined with other clinical information could distinguish between patients with prevalent or incident diabetes from healthy controls.

The nature of the interactions between miRNAs and the 3′-UTRs of mRNA affords a single miRNA with the potential to post-transcriptionally regulate the expression of a wide pool of target genes. This makes miRNAs a prime target for direct therapeutic intervention. Altering the function of a single miRNA could significantly alter the behaviour of treated cells. In many disease conditions, the expression of miRNAs can be significantly altered. Therefore, pharmacologically returning miRNA expression to normal levels could improve clinical outcomes and improve current therapies. miRNA levels can be experimentally reduced in vivo using antisense oligonucleotide miRNAs (antimiRs). Esau et al. [63] demonstrated the potential therapeutic effects of inhibition of liver miR-122 in mice by using

Table 1: Summary of key biological processes and miRNAs discussed in this paper.

Biological process	Specific miRNAs	References
ngn3-independent endocrine pancreas regeneration	miR-15a, miR-15b, miR-16 and miR-195	[10]
Regulates insulin expression	miR-30d, miR-375, miR-124a2	[17, 22, 30]
Regulates insulin secretion	miR-375, miR-9, miR-124a2	[8, 25, 31]
Regulates glucose-stimulated insulin secretion (GSIS)	miR-369-5p, miR-130a, miR-27a, miR-410, miR-200a, miR-337, miR-532, miR-320, miR-192 and miR-379, miR-375, miR-124a2	[18, 20, 21, 32]
Regulates adipocyte differentiation	miR-143, miR-27b, miR-130, miR-519d	[41, 42, 44, 45, 47]
Regulates insulin sensitivity	miR-103, miR-107, miR-29, miR-320, mmu-mir-183-96-182 (cluster)	[48–51]

2′-*O*methoxyethyl phosphorothioate-modified oligodeoxynucleotides. miR-122 inhibition in a diet-induced obesity mouse model resulted in decreased plasma cholesterol levels, a significant improvement in liver steatosis, and suppression of several lipogenic genes. This study validated this strategy as a novel means to regulate energy metabolism, such as, cholesterol and fatty-acid metabolism in the adult liver. In their study of glucose metabolism in multiple organs, Frost and Olson [64] demonstrated that global knockdown of the Let-7 family with an antimiR was sufficient to prevent and treat impaired glucose tolerance in mice with diet-induced obesity. This effect was thought to be attributable to increased insulin sensitivity in liver and muscle tissue. The antimiR treatment on these mice resulted in increased lean and muscle mass with stable fat mass and prevented ectopic fat deposition in the liver. Their findings demonstrate the potential benefits of miRNA manipulation in the context of insulin resistance, with the Let-7 family representing a potential therapeutic target for the treatment of type 2 diabetes.

The alternative to this technology involves the use of miRNA mimics or mimetics, which can be used to pharmacologically increase the levels of a particular miRNA [65]. As described previously, a consistent and counterintuitive observation in obese patients and experimental models of obesity is that the miRNAs normally induced during adipogenesis are downregulated in the obese subjects [42]. It is therefore unlikely that this technology will be applicable in the context of insulin resistance, as increased expression of disregulated miRNAs would most likely exacerbate, rather than treat the condition. However, this does not mean that this technology is not applicable in other experimental or therapeutic applications. The use of miRNA mimics in a therapeutic context has been demonstrated in a mouse model of nonsmall cell lung cancer [66]. A miR-34a mimic, in a lipid-based vehicle, was able to induce apoptosis and significantly inhibit tumour growth.

Current studies that experimentally alter the activity of miRNAs to elicit a particular biological effect are very limited, with researchers typically confined to altering the expression of a particular family (such as the Let-7 family) or a specific miRNA to monitor its effects. These studies represent fundamental experiments that will determine the clinical potential of therapies utilizing miRNA manipulations. The technology necessary to manipulate multiple miRNAs in a clinical context does not presently exist. However, it is possible that current techniques used to manipulate limited miRNA expression could be adapted for use with conventional treatments. This would represent a class of novel therapies, combining traditional therapeutics with miRNA manipulation technologies to improve clinical outcomes. In the context of insulin resistance, manipulation of only a few specific miRNAs could potentially improve the efficacy of or suppress development of tolerance to pharmaceuticals traditionally used to improve glucose tolerance and insulin sensitivity. In the future, characterization of the miRNA signature in healthy and diseased individuals would facilitate the design of a therapy to restore the healthy expression of an entire repertoire of disregulated miRNAs in the disease state. With the introduction of Personalized Medicine [67], this therapy would also become highly specific, tailored to the miRNA signature of an individual, allowing for very precise and effective treatment.

6. Conclusions

Diabetes and obesity have emerged as one of the greatest challenges faced by contemporary medicine. While motivated patients may overcome type 2 diabetes and obesity through managed life-style changes, current therapies are otherwise limited to restricting disease progression. miRNAs have the potential to regulate the expression of a wide spectrum of target genes, making these small molecules very valuable therapeutic targets (see Table 1). However, it is only through continued investment in the study of miRNAs in metabolic health and disease that their therapeutic potential can be fully realized.

Authors' Contribution

M. D. Williams reviewed the available literature and was responsible for writing the paper. G. M. Mitchell is one of Michael's Ph.D. Supervisors and was responsible for critical reading of the paper.

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Research Article

Circulating Levels of MicroRNA from Children with Newly Diagnosed Type 1 Diabetes and Healthy Controls: Evidence That miR-25 Associates to Residual Beta-Cell Function and Glycaemic Control during Disease Progression

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This study aims to identify key miRNAs in circulation, which predict ongoing beta-cell destruction and regeneration in children with newly diagnosed Type 1 Diabetes (T1D). We compared expression level of sera miRNAs from new onset T1D children and age-matched healthy controls and related the miRNAs expression levels to beta-cell function and glycaemic control. Global miRNA sequencing analyses were performed on sera pools from two T1D cohorts (n = 275 and 129, resp.) and one control group (n = 151). We identified twelve upregulated human miRNAs in T1D patients (miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, miR-200a); several of these miRNAs were linked to apoptosis and beta-cell networks. Furthermore, we identified miR-25 as negatively associated with residual beta-cell function (est.: −0.12, P = 0.0037), and positively associated with glycaemic control (HbA1c) (est.: 0.11, P = 0.0035) 3 months after onset. In conclusion this study demonstrates that miR-25 might be a "tissue-specific" miRNA for glycaemic control 3 months after diagnosis in new onset T1D children and therefore supports the role of circulating miRNAs as predictive biomarkers for tissue physiopathology and potential intervention targets.

1. Introduction

As an autoimmune disease-type 1 diabetes (T1D) results from an immune-mediated destruction of the insulin producing beta-cells reflected by the appearance of the pancreatic autoantibodies. The destruction of these cells implies a progressive, irreversible loss of the endogenous insulin production, leading to daily treatment with exogenous insulin.

At time of diagnosis a child with T1D is estimated to have lost approx. 80–90% of the insulin producing beta-cell function/mass. Shortly after the initial insulin treatment, several children experience a period of increased endogenous insulin production followed by a reduced need

of exogenous insulin, referred to as the remission phase [1]. The regenerative potential that exists within this time interval particularly in children gives a unique possibility of intervention treatment, that is, with beta-cell growth factors to maintain the individual insulin production, leading to improved glycaemic control and fewer complications in eyes, kidneys, and nerves.

The hypothesis of the study is that potential new biomarkers (miRNAs and/or miRNA patterns) in serum from children with newly diagnosed T1D can predict destruction or regeneration of the endogenous residual beta-cell function. miRNAs are small noncoding RNAs involved in posttranscriptional regulation of protein translation either through mRNA destabilization or inhibition [2, 3]. miRNAs are found in solid tissues and cell culture samples, and several studies confirm their presences in body fluids as blood, saliva, urine, and serum [4–7]. The stability of serum miRNAs has been investigated under harsh conditions including boiling, low/high pH, extended storage, and freeze-thaw cycles without any significant difference compared to nontreated serum samples [8]. Taken together these results show that serum miRNAs, are stable and that they may reflect cellular dysfunctions in various chronic diseases. In addition, several miRNAs clearly have a role in metabolic pathways for example, miR-33a/b inhibition in nonhuman primates raises plasma HDL cholesterol and lowers triglycerides [9], and silencing of miR103/107 seems to have beneficial effects on insulin sensitivity in obese mice possibly through its target gene caveolin-1 which is a critical regulator of the insulin receptor [10].

The objective of the current study was therefore to identify new biomarkers (key miRNAs/miRNA patterns), which are predictive for destruction or regeneration of the endogenous residual beta-cell function by investigating miRNAs in serum samples from new-onset T1D patients and age-matched controls.

2. Methods

2.1. Study Cohorts. Serum samples from two unique T1D cohorts and one control group were analysed for miRNA expression.

2.2. International Remission Phase Cohort (The Hvidoere Cohort). The Hvidoere Remission Phase cohort is a longitudinally, observational cohort of newly diagnosed T1D children collected through 18 paediatric centres primarily in Europe [11]. Blood samples for centrally determined HbA1c, stimulated C-peptide, glucagon, incretin hormones, cytokines, immunology, and DNA were collected prospectively at a meal-stimulated test 1, 6, and 12 months after diabetes onset. 275 patients were included in the study.

2.3. The Danish Remission Cohort. The Danish Remission Phase cohort includes new onset T1D children treated at four Danish paediatric centres. The study design is analogous to the Hvidoere cohort, except for the inclusion of a three-month meal-stimulated test. 129 children were included in the study [12].

2.4. Healthy Control Children (The Copenhagen Puberty Study). Healthy controls consist of girls and boys between 6.7 and 13.7 years, recruited from public schools in the Copenhagen area as part of a mixed cross-sectional and longitudinal study, the COPENHAGEN Puberty Study [13, 14]. Information on gender, age, pubertal stage, height, and weight was recorded. Blood samples were analysed for metabolic parameters (glucose, insulin, cholesterol, and triglycerides). 151 children were included in the present study.

2.5. miRNA Purification, Solexa Sequencing, and Quantitative RT-PCR. The work flow was separated into three steps: (i) initial screening by high-throughput Solexa sequencing using pooled serum samples, (ii) qRT-PCR validation in a large number of individual serum samples arranged in multiple training and testing sets, and (iii) statistical evaluation of the diagnostic or prognostic value of the serum miRNA profiling system.

2.5.1. Serum Pools and miRNA Purification. Serum pools were prepared from all three cohorts. From the Hvidoere cohort 100 μL serum from 275 children was pooled, from the Danish cohort 200 μL serum from 129 children was pooled, and finally 200 μL serum from 151 healthy control children was pooled. Total RNA was extracted from serum pools using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Furthermore, total RNA from all individuals in the three cohorts (the Hvidoere, the Danish, and healthy controls) was isolated and stored at -80°C for later testing of candidate miRNAs.

2.5.2. Solexa Sequencing. The pooled samples from the 3 cohorts (a total of 5–10 μg RNA) were sequenced on the Solexa sequencing platform (Illumina). After PAGE purification of small RNA molecules under 30 bases and ligation of a pair of Solexa adaptors to their 5′ and 3′ ends, the small RNA molecules were amplified using the adaptor primers for 17 cycles, and fragments around 90 bp (small RNA+adaptors) were isolated from agarose gel. The purified DNA was used directly for cluster generation and sequencing analysis using the Illumina Genome Analyzer IIx according to the manufacturer's instructions. Image files were generated by the sequencer and were processed to produce digital-quality data. After masking the adaptor sequences and the removal of contaminated reads, clean reads were processed for *in silico* analysis as previously described [8]. After comparing the serum miRNA profile in the control group versus the patients, a panel of differentially (as defined by copy number >100 and 2-fold altered expression) expressed miRNAs was derived.

2.5.3. Quantitative RT-PCR of Mature miRNA. Quantification of mature miRNAs was carried out using Taqman

miRNA probes (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, 2 μL of total RNA is reverse-transcribed to cDNA using AMV reverse transcriptase and a stem-loop RT primer (Applied Biosystems). Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). All reactions, including non-template controls, were run in triplicate. Twenty-four candidate miRNAs were reanalysed by qRT-PCR analyses on individual samples. We examined the expression levels of the candidate miRNAs in a subset of the cohorts consisting of 108 patients (1-month samples from the Danish Remission Phase cohort and 12-month samples from the Hvidoere cohort) and 54 controls.

2.6. Statistics. For the cohort comparisons each miRNA was studied separately as function of cohort, age, and sex on a logarithmic scale. All comparisons were adjusted for multiple testing by Hochberg approach.

Statistical evaluation of miRNA association to disease progression (endpoints: stimulated C-peptide and HbA1c) was done by multiple linear regression analysis adjusting for age and sex. miRNA expression was presented as $2^{(-\Delta Ct)}$ transformed values (the difference in Ct value between a given miRNA and the reference miRNA (a combination of three miRNAs of the let-7 family); all values represent the geometricmean of triplicates measures). Stimulated C-peptide was studied on logarithmic scale to obtain normal distribution, while HbA1c was normally distributed. The results were interpreted as the change in actual HbA1c values (%) and the percentage change in stimulated C-peptide corresponding to the range between the 25 percentile and the 75 percentile (interquartile range) of the ΔCt values of the relevant miRNA. Data were analysed using SAS (version 9.2, SAS Institute; Cary, NC). A P-value of <0.05 was considered statistically significant.

2.7. Ethical Approval. Ethical approval has been obtained for the Hvidoere and the Danish Remission Phase Studies allowing the samples to be analyzed for miRNAs (KA 99063 and KA 04010gm, resp.). The COPENHAGEN Puberty Study (ClinicalTrialsGov ID NCT01411527) including additional genetic and epigenetic studies was approved by the ethical committee (KF 01 282212 and V200.1996/90).

3. Results

3.1. Comparing Serum miRNAs Expression Levels between Two Paediatric Type 1 Diabetes Cohorts and an Age-Matched Control Group. miRNA was purified from a pool of serum from each cohort (serum samples were taken 1 month after diagnosis for both diabetes cohorts), and the presence and levels of miRNAs were identified by global Solexa sequencing. We identified in total 240 different miRNAs from these cohorts; this corresponds to approx. 15% of all known human miRNAs (miRbase18 identifies 1527 human miRNAs). 47 miRNAs fulfilled our criteria for differential expression between the diabetes cohorts and the control group (>100 copy number and 2-fold altered expression). Of these 24 miRNAs (miR-103, miR-10a, miR-125b, miR-134, miR-199a, miR-200c, miR-21, miR-26b, miR-29b, miR-340, miR-320a, miR-222, miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, and miR-200a) were selected for further analyses by qRT-PCR in a subset (n = 54) of samples from each cohort. Consistently we found all 24 miRNAs upregulated by both Solexa sequencing and qPCR analyses. A combination of three miRNAs of the let-7 family was used as reference for normalisation of miRNA expression levels measured by qRT-PCR. By regression analysis adjusting for age, sex, and multiple testing we found 12 miRNAs (miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, and miR-200a) to be significantly differentially expressed between either both diabetes cohorts and the control group or just one of the diabetes cohorts and the controls (Table 1) (P < 0.05). The ΔCt values of these 12 miRNAs are presented in Figure 1.

3.2. Association between Qualified miRNAs and Disease Progression in Children with New Onset Type 1 Diabetes. The qualified miRNAs with differential expression pattern between cases and controls were analysed by qRT-PCR analyses for association with disease progression in the two diabetes cohorts. Disease progression was described by glycaemic control as assessed by HbA1c or residual beta-cell function estimated by stimulated C-peptide. In the Danish cohort miR-25 measured at 1 month after disease onset was negatively associated with the HbA1c level and positively associated with stimulated C-peptide levels 3 months after onset. The regression analyses suggest a 0.22% decrease in HbA1c level (0.08–0.37) (P = 0.0035) and a 22.4% (8.3–34.3) (P = 0.0037) increase in stimulated C-peptide between the 25–75 interquartile range of ΔmiR-25 (Figures 2(a) and 2(b)). miR-25 measured at 12 months after disease onset was not associated with stimulated C-peptide or HbA1c in the Hvidoere cohort (data not shown). Furthermore, we analysed for association between the 12 qualified miRNAs and the pancreatic autoantibodies (GAD, IA, IA-2A and ZnT8Ab) (data not shown). We found no association between these parameters in any of the cohorts.

4. Discussion

This is the first study to compare miRNA levels in serum samples from children with or without T1D. Serum samples from approximately 400 new onset T1D children and 150 healthy age-matched controls were analysed. Twelve differentially expressed miRNAs between cases and controls were identified. Several of these miRNAs are involved in regulation of apoptosis (miR-181a, miR-24, miR-25, miR-210, and miR-26a) [15–19] and beta-cell regulatory networks (miR-24, miR-148a, miR-200a, and miR-29a) [20]. Even more importantly we found quite a few miRNAs with yet unidentified function related to T1D as miR-152 and miR-30a-5p.

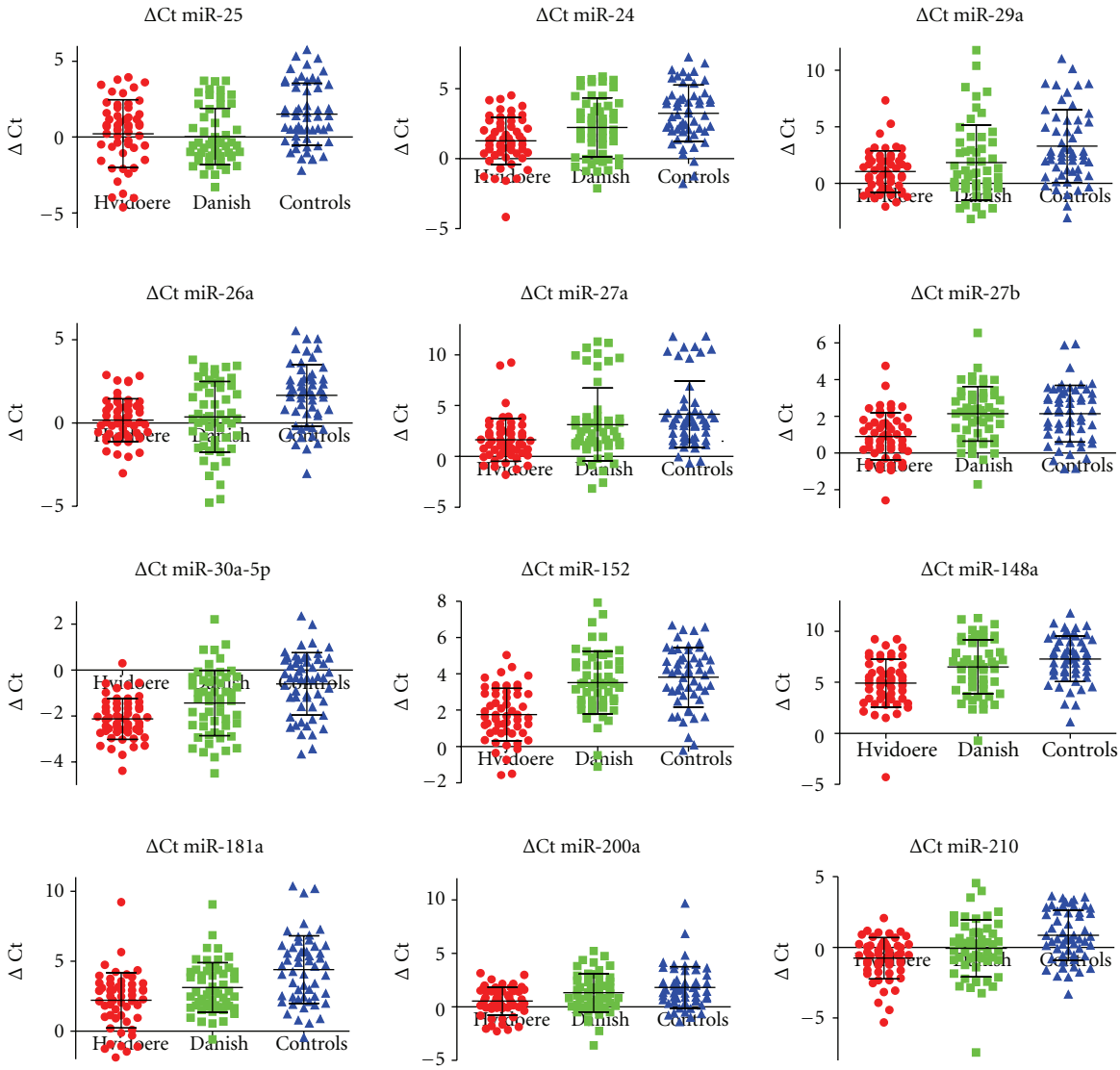


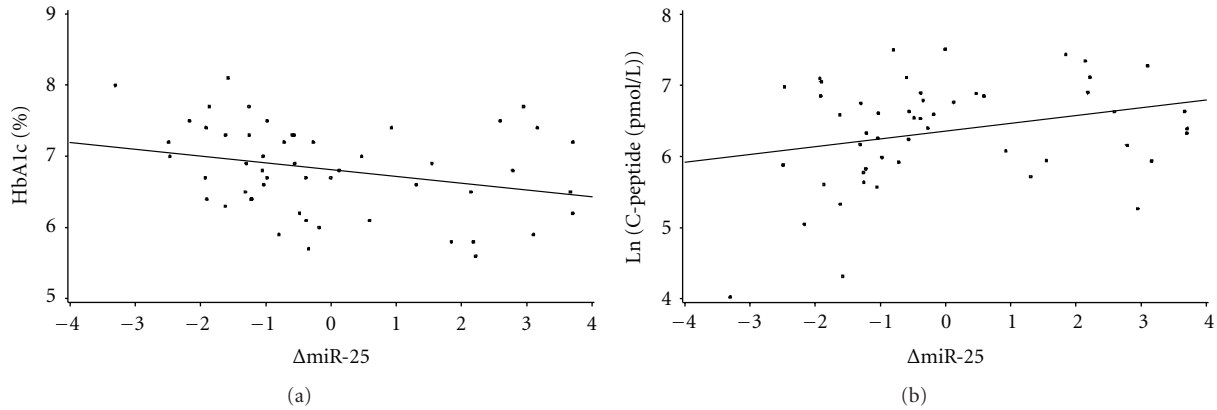
Figure 1: Twelve miRNAs were differentially expressed between the diabetes cohorts and the controls. ΔCt values are plotted for each cohort (Hvidoere (red), Danish (green) and controls (blue)). The bars represent geometric means of the ΔCt values ± SD.

miRNAs are known to play a central role in posttranscriptional gene regulation and are involved in many cellular processes, such as differentiation, proliferation, and apoptosis [20]. miRNAs are detectable in cell-free circulation, that is, plasma and serum, and thus have been investigated as noninvasive biomarkers in several diseases and pathologic processes. In type 2 diabetes (T2D), which is characterized by chronic elevations of blood glucose levels and insulin resistance, one study identified a unique plasma miRNA signature for T2D, which included reduced levels of miR-15a, miR-20b, miR-21, miR-24, miR-126, miR-191, miR-197, miR-223, miR-320, miR-486 and elevated levels of miR-28-3p. Intriguingly, a reduction in the level of some of these miRNAs (miR-126, miR-15a, and miR-223) was already detectable years before the manifestation of diabetes [21]. Of these, only miR-24 overlaps with our observation. miR-24 is a regulator of apoptosis [16] and TGFβ signaling [22] suggesting a role in inflammation which is important in both T1D and T2D development [23]. A study by Kong et al. [24] analyzed seven diabetes-related miRNA candidates in serum from newlydiagnosed T2D patients, prediabetic and healthy subjects and found miR-34a to be the strongest predictor of T2D. No overlap with the markers identified by Zampetaki et al. [21] was observed.

Current circulating biomarkers for T1D and residual beta-cell function are based on specific immunoglobulin (autoantibodies) and C-peptide measurements which are expensive and time consuming in a routine clinical setting. The development of new protein-based biomarkers is often rather cumbersome because of the complexity of protein

Table 1: List of miRNAs differentially expressed in sera from children and adolescents with newly diagnosed T1D compared to sera from age-matched controls.

Systematic miRNA name	ΔCt change	$P_{corrected}$ value	Regulation T1D/Controls	Difference in following cohorts
hsa-miR-152	2.09	<0.0001	Up	Hvidoere/controls
hsa-miR-30a-5p	1.52	<0.0001	Up	Hvidoere/controls Danish/controls
hsa-miR-181a	2.30	<0.0001	Up	Hvidoere/controls Danish/controls
hsa-miR-24	2.25	<0.0001	Up	Hvidoere/controls
hsa-miR-148a	2.25	0.00015	Up	Hvidoere/controls
hsa-miR-210	1.65	0.00078	Up	Hvidoere/controls
hsa-miR-27a	2.79	0.00139	Up	Hvidoere/controls
hsa-miR-29a	2.39	0.00636	Up	Hvidoere/controls
hsa-miR-27b	1.13	0.00953	Up	Hvidoere/controls
hsa-miR-26a	1.31	0.01554	Up	Hvidoere/controls Danish/controls
hsa-miR-25	1.53	0.02013	Up	Hvidoere/controls Danish/controls
hsa-miR-200a	1.23	0.02957	Up	Hvidoere/controls



(a) (b)

Figure 2: (a) A significant negative association between miR-25 one month after diagnosis and HbA1c (%) was shown at 3 months in the Danish Remission Phase Cohort ($P = 0.003$) while (b) stimulated C-peptide (pmol/L) was positively associated to miR-25 at the same time point ($P = 0.0045$).

composition in blood, the diversity of posttranslational modifications, the low abundance of many proteins, and challenges in developing high-sensitivity assays.

The precise cellular release mechanisms of miRNA are largely unknown, and, therefore, the role of circulating miRNAs is not fully understood. However, miRNAs offer many features as attractive biomarkers: stability and evolutionary conservation, and because they can be detected by real-time PCR, assays can be highly sensitive and specific.

Interestingly we found an association between miR-25 and better glycaemic control and residual beta-cell function. There have been several studies linking the miR-25 to different pathological conditions especially in cancer. Several reports associate high serum miR-25 with breast cancer, non-small-cell lung carcinoma and hepatocellular carcinoma [8, 25–27]. Furthermore, miR-25 has been found to be expressed in several malignant cell lines (ovarian cancer cell line and cholangiocarcinoma), where it is involved in regulation of apoptosis and cell proliferation by targeting proapoptotic proteins as Bim and Trail (TNF-related apoptosis inducing ligand) [17, 28]. A previous study in an experimental diabetic nephropathy model investigated the role of miRNAs in the regulation of NADPH expression during hyperglycaemia and found miR-25 was significantly reduced in kidney from these animals [18]. Our present findings of improved residual beta-cell function in patients with high level of miR-25 are in accordance with these results, suggesting a role of miR-25 on cell proliferation of the endocrine cells of the pancreas. This is also supported by the lack of association between miR-25 and the degree of autoimmunity (as assessed by presence of autoantibodies) in our study. The concordance between improved stimulated C-peptide and better HbA1c

levels supports the potential role of this miRNA during disease progression in these children, and that is despite the limited number of individuals included in this study.

This study provides suggestive evidence for the role of miRNAs as clinical applicable biomarkers in T1D. The association of miR-25 with improved glycaemic control and better residual beta-cell function may indicate that this miRNA could be an important biomarker that could be used during early and intensive management of newly diagnosed diabetes to improve blood glucose control and reduce microvascular complications. These findings should be confirmed in an independent study population; however, we are currently investigating the miR-25 levels in all patients from our study cohorts at different time points during disease progression. This study can serve as model for conceptualising the use of miRNAs as clinical relevant biomarkers in which they potentially will be used as beneficial predictors to evaluate clinically meaningful changes in intervention therapies designed to preserve/regenerate beta-cell function in new onset T1D.

5. Conclusions

This study shows that 12 miRNAs have increased expression levels in children with new onset T1D compared to age-matched healthy controls. Furthermore, the residual beta-cell function and glycaemic control after 3 months of clinical disease associate with miR-25 expression level present soon after diagnosis. These findings indicate a potential role for miRNAs in the understanding of disease mechanisms at an early time point; this knowledge may in the future be translated into optimized and individualized diabetes management (bench-to-bedside) to the benefit of the patients.

Abbreviations

T1D: Type 1 diabetes
miRNA: microRNA
HDL : High-density lipoprotein
qRT-PCR: Quantitative real time polymerase chain reaction.

Authors' Contributions

The authors contributed equally to the publication.

Acknowledgments

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Methodology Report

A Protocol for Measurement of Noncoding RNA in Human Serum

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MicroRNAs (miRNAs) are small noncoding RNAs that act as regulators of gene expression by targeting mature messenger RNAs. Following the initial report of the presence of miRNAs in serum and plasma a number of studies have successfully demonstrated the use of these miRNAs as biomarkers of disease. Currently, there are many methods of isolating total RNA from liquid samples. Here, we describe a simple, cost effective method for extraction of RNA from human serum as well as subsequent real time PCR analysis of miRNA levels.

1. Introduction

MicroRNAs (miRNAs) are small noncoding RNAs that act as regulators of gene expression by targeting mature messenger RNAs (mRNAs). Recently, miRNA has been discovered in both serum and plasma, where it appears to be relatively stable [1]. Since this initial report, there have been a number of studies examining miRNA expression in either serum or plasma in diseases including various cancers [2], cardiovascular disease [3], and myopathies such as Duchenne muscular dystrophy [4]. The methods of miRNA isolation, as well as the techniques used to examine expression, vary in these studies. Here, we provide a protocol for the isolation of RNA from serum and subsequent determination of miRNA expression levels using TaqMan-based real-time PCR detection. This procedure, from isolation of RNA to acquisition of real-time PCR data, takes around 8 hours.

2. Materials

2.1. Reagents. The materials used are as follows:

Chloroform (Labserv cat # CL728).

Ethanol (Labserv cat # BSPE6975); *prepare 75% ethanol (v/v) by diluting 100% ethanol in nuclease-free water; we recommend making fresh ethanol solution every time the protocol is carried out.*

Glycogen (molecular biology grade) (Sigma, cat # G1767) (20 μg/μL).

Nuclease-free water (Qiagen, cat # 129117).

Isopropanol.

Taqman Fast Universal Master Mix (2x) (Life Technologies cat #4366073).

Taqman microRNA assays (Life Technologies, various).

Table 1

	μL/reaction	No. of reactions	Volume to add (μL)
10X RT buffer	0.5	12	6.25
100 nM dNTPs	0.05	12	0.625
RNase inhibitor	0.03	12	0.375
Nuclease-free water	1.42	12	17.75
Reverse transcriptase	0.33	12	4.125
Total volume (μL)	2.33		

Table 2

	μL/reaction	No. of reactions	Volume to add
2X Fast PCR mastermix	2.5	12	32.5
20X AOD	0.25	12	3.25
Nuclease-free water	1.45	12	18.85
Total volume	4.2		

The miRNA-specific RT primers and TaqMan probe-primer mix are supplied together as an individual assay for a single miRNA.

TaqMan microRNAs reverse transcription *kit (Life Technologies 4366597)*.

Kit includes 10X RT buffer, 100 mM *dNTP mix, RNase inhibitor (*20 U/μL*), and multiscribe RT enzyme* (50 U/μL)*; the enzyme must be kept at* -20°*C at all times.*

TriReagent (Ambion AM9738).

2.2. Equipment

Optical Adhesive film for PCR (Applied Biosystems, cat # 4311971).

Centrifuge, refrigerated (with rotor for 1.5 mL tubes, e.g., Heraeus Fresco).

Centrifuge, refrigerated (with rotor for 96-well plates and 15 mL tubes, e.g., Heraeus Multifuge X1).

Ice.

Micropipettor and filter tips.

Microplates (96-well half skirt, Axygen Cat # PCR-96-M2-HS-C).

MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems cat #4346906).

Real-time PCR system (7900 HT Fast Real-Time PCR system; Applied Biosystems Cat # 4351405).

Tubes, microcentrifuge (1.5 mL and 2 mL).

Vortex mixer.

8 mL serum separator clot activator vacuette (Greiner Cat # 455078).

21-gauge needle push button blood collection sets (BD Cat # 367344).

3. Method

3.1. Sample Collection. It is important that samples are collected in accordance with the appropriate ethical guidelines.

(1) Collect blood samples using 21-gauge needle push button blood collection sets into 8 mL serum separator clot activator vacuettes.

(2) Invert the tubes five times before allowing clotting for 30 minutes.

(3) Centrifuge the tubes for 10 minutes at 1,000–1,300 g in a swinging bucket rotor.

(4) Remove serum from the tube and aliquot into 500 μL volumes in 1.5 mL microcentrifuge tubes.

Serum can be used immediately or can be frozen at -80°C for future use.

3.2. RNA Isolation

(5) Transfer 400 μL of serum into a 2 mL microcentrifuge tube.

(6) Add 1 mL TRI reagent to each tube. *Following addition of TRI reagent to the serum, yellow globules appear in the solution. These dissipate following vortexing and incubation and do not appear to interfere with downstream processing.*

(7) Add 1 μL of (1 μg/uL) nuclease-free glycogen to each tube. Vortex for 20 seconds. Incubate at room temperature for 10 minutes.

(8) Add 200 μL of chloroform to each tube and shake vigorously for 20 seconds. *This ensures proper mixing of the chloroform and TRI reagent immediately after addition. Tubes should not be vortexed at this stage. Due to the time involved in each stage of processing we recommend that a maximum of 12 tubes be processed at a time.*

(9) Incubate at room temperature for 15 minutes.

(10) Centrifuge at 12,000 g for 15 minutes at 4°C.

(11) Transfer 800 μL of the upper aqueous phase to a fresh 2 mL microcentrifuge tube. *There will be a large amount of white material at the interface of the aqueous and organic layers. Following removal of 800 μL from the aqueous layer, there will be residual aqueous phase remaining.*

(12) Add 1.2 mL isopropanol to each tube as 2×600 μL volumes. Vortex for 5 seconds. *At this stage the tube will be very full.*

(13) Incubate at room temperature for 10 minutes.

(14) Centrifuge at 12,000 g for 8 minutes at 4°C. *Whilst a pellet should be visible, it is still important to orient the tubes before centrifugation so that the hinge of the lid faces the outer rim of the centrifuge.*

(15) Carefully aspirate the supernatant by placing the pipette tip along the wall of the microcentrifuge tube

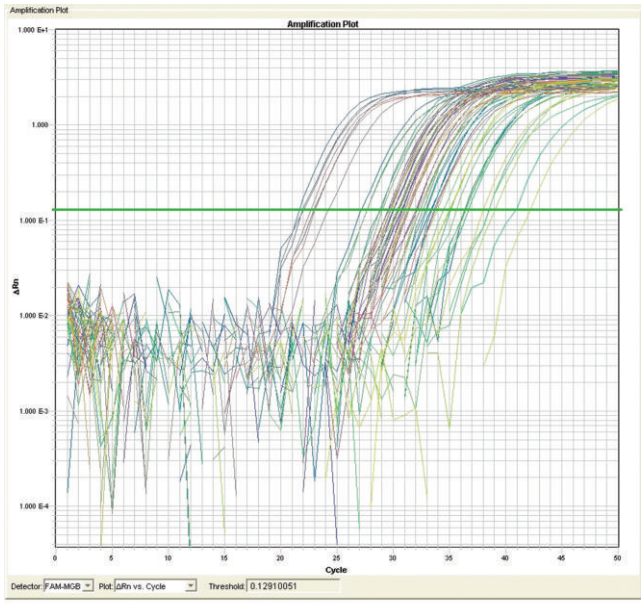

Figure 1: miRNA levels in human serum. RNA was isolated and PCR carried out as described here. Data show the expression of 8 miRNAs from 8 human serum samples.

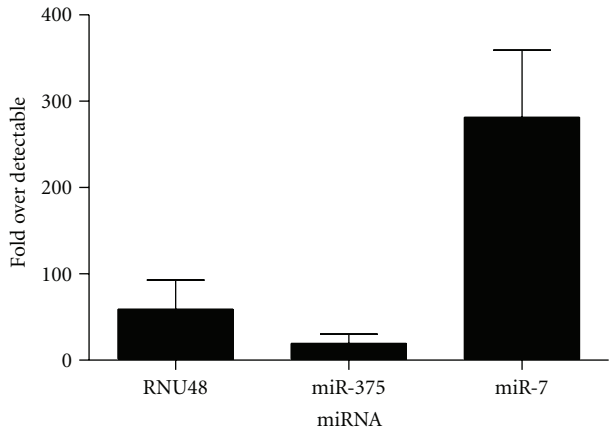

Figure 2: Representative real-time PCR data obtained from human serum. Six human serum samples were processed using the protocol described above and expression of miR-375 and miR-7 as well as the control miRNA RNU48 was examined. Data is expressed as fold over detectable.

opposite from the hinge (and therefore the pellet). *Due to the large volume of liquid needing to be removed it may be possible to carefully tip out the contents of the tube.*

(16) Add 1 mL 75% ethanol to each tube and invert the tube 5 times.

(17) Centrifuge at 7,500 g for 5 minutes at room temperature. Again, place the tubes in the same orientation inside the centrifuge.

(18) Carefully remove the supernatant as described in step (11). In order to ensure that as much of the ethanol is removed as possible it may be necessary to sequentially decrease the size of the pipette tip used.

(19) Allow the tubes to dry out for 5 minutes at room temperature.

(20) Add 20 μL of nuclease-free water to each tube and resuspend pellet thoroughly. *Always store the RNA on ice after this stage.*

(21) Measure the concentration of total RNA using a NanoDrop ND-1000. *We have observed that the 260/280 ratio is often around 1.3. This does not appear to have an effect on downstream processing or data generated.*

RNA obtained using the above protocol is used for miRNA-specific reverse transcription. Remaining RNA can be stored at −80°C.

3.3. miRNA-Specific Reverse Transcription. For ease of handling, we recommend that reverse transcription is carried out in either 8-well 0.2 mL strip tubes or a 96-well thin walled PCR plate.

(22) 10 ng of RNA is reverse transcribed for each miRNA primer. Calculate the volume of RNA required to provide 10 ng final concentration per primer adding an extra reaction in case of error. Add nuclease-free water to make the volume up to 1.67 μL per tube.

(23) Thaw 10x RT buffer, RNase inhibitor, and 100 nM dNTPs (provided in TaqMan microRNAs Reverse Transcription Kit) on ice. *The multiscribe reverse transcriptase must remain at −20 °C until required.*

(24) Prepare master mix by adding the above reagents as per the calculations shown in Table 1. Calculations are shown for each miRNA and 12 serum samples. Total reaction volume is 5 μL. An error of 0.5 reactions is incorporated into the calculations. *Master mix must be mixed by trituration and should remain on ice at all times.*

(25) Add 2.33 μL of RT master mix and 1 μL of miRNA-specific primer to each well, then add 1.67 μL of RNA to all wells. All additions must be made on ice.

(26) Vortex and spin down so that the RNA and master mix are thoroughly mixed.

(27) Reverse transcription is carried out using a BioRad DNA engine with the following program

(1) 16°C for 30 min

(2) 42°C for 30 min

(3) 85°C for 5 min

(4) 4°C on hold.

3.4. Real-Time PCR for miRNAs.

(28) Thaw the assay-on-demand (AOD) on ice. Prepare the master mix for each target miRNA to be detected as per the calculations shown in Table 2. These

calculations are based on 12 samples and have an error of 1 reaction included in the calculation. Total reaction volume is 5 μL/well.

(29) For each target miRNA, add 4.2 μL of the specific master mix to each well of a 0.1 mL optically clear PCR plate, followed by 0.8 μL of the cDNA prepared using miR-specific RT primers in step (27). Cover the plate with an optically clear film. *It is important to ensure that the wells around the edge of the plate are fully sealed as they are more susceptible to evaporation.*

(30) Centrifuge the plate at 3,500 rpm for 5 minutes to ensure that all contents are at the bottom of the well and all air bubbles have been eliminated.

(31) Place the plate in a 7900 HT fast real-time PCR machine and run it in fast mode using the following program
Stage 1: 95°C for 20 sec
Stage 2: 40 cycles of
95°C for 1 sec,
60°C for 20 sec.

The plate is read during the extension step of stage 2. An example of data obtained is shown in Figures 1 and 2.

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Research Article

Transcriptional Mechanisms Controlling miR-375 Gene Expression in the Pancreas

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MicroRNAs (miRNAs) are a class of small non-coding RNAs that play an important role in mediating a broad and expanding range of biological activities. miR-375 is expressed selectively in the pancreas. We have previously shown that selective expression of miR-375 in pancreatic beta cells is controlled by transcriptional mechanisms operating through a TATA box-containing promoter. Expression of miR-375 has been reported in non-beta cells within the endocrine pancreas, and indeed inactivation of miR-375 leads to perturbation in cell mass and number of both alpha and beta cells. Consistent with its expression throughout the endocrine pancreas, we now show that the promoter of the miR-375 gene shows selective activity in pancreatic endocrine alpha cells, comparable to that observed in beta cells. We previously identified a novel negative regulatory element located downstream of the miR-375 gene transcription start site. By generating luciferase reporter genes, we now show that the sequence is functional also when positioned upstream of a heterologous promoter, thus proving that the repressor effect is mediated at least in part at the level of transcription. Further characterization of the transcriptional control mechanism regulating expression of miR-375 and other pancreatic miRNAs will contribute to a better understanding of pancreas development and function.

1. Introduction

In order to accomplish their specialized functions in the controlled secretion of metabolic hormones, pancreatic endocrine cells must strictly regulate expression of a characteristic repertoire of genes. This is mediated through a complex set of transcriptional and post-transcriptional control mechanisms [1, 2]. Recently miRNAs have been shown to play an important role in this process: thus, experiments involving conditional deletion of Dicer, an enzyme required for miRNA maturation, have revealed involvement of miRNAs in the development of both exocrine and endocrine pancreas compartments [3], as well as in mature beta cell function [4].

Although much effort has been devoted to understanding the mechanisms underlying miRNA-mediated inhibition of gene expression [5, 6], less is known concerning the mechanisms controlling expression of miRNA. This is in part due to technical limitations: many miRNA genes are embedded within exons or introns of other genes [7], complicating the analysis of transcription and processing patterns. Furthermore, identification of transcription start sites, a common first step in identification of transcriptional promoters is more challenging for genes encoding miRNAs, since the 5′ terminal sequence of the primary transcript is rapidly processed.

It has been demonstrated that the miR-375 gene is expressed selectively in cells of the endocrine pancreas [8, 9]. Indeed miR-375 is essential for endocrine pancreas function, since inactivation leads to impaired glucose homeostasis involving increased alpha cell mass and decreased beta cell mass [10]. We have examined the mechanisms underlying this selective expression of miR-375. We have characterized the promoter of the miR-375 gene, demonstrated that it shows preferential activity following transfection of beta cell lines, and have identified a number of critical cis elements within the promoter region [11]. We now report that the promoter is also selectively active in alpha cells and have characterized the role of key cis elements for promoter activity. We also have further characterized a negative regulatory element located within the transcribed region of

the miR-375 gene and demonstrate that this element operates at the level of transcription.

2. Materials and Methods

2.1. Cell Culture. The following established cell lines were used in this study: HIT M2.2.2 (hamster β cells) [12], βTC1 (mouse β cells) [13], αTC1 (mouse α cells) [14], NIH-3T3 (mouse fibroblast cells), MIN6 (mouse β cells) [15], Ltk$^-$ (mouse fibroblasts), and AR4-2J (rat exocrine pancreas cells) [16]. αTC1, βTC1, HIT, and NIH-3T3 cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (200 I.U./mL), and streptomycin (100 μg/mL). Ltk$^-$ and AR4-2J were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, penicillin (200 I.U./mL), and streptomycin (100 μg/mL). MIN6 cells were grown on Falcon tissue culture plates in DMEM containing 10 mM D-glucose, supplemented with 15% FCS, penicillin (200 I.U./mL), and streptomycin (100 μg/mL), 2 mM L-glutamine, and 72 mM beta-mercaptoethanol.

2.2. RNA Preparation and Quantitative PCR Reaction. RNA was extracted from αTC1, βTC1, MIN6, AR4-2J, and Ltk$^-$ cells using the miRNeasy kit (QIAGEN) and was treated with Turbo-DNase (Ambion). The treated RNA (1 μg) was reverse-transcribed using miScript kit (QIAGEN). Quantitative PCR reactions were carried out using 2 ng cDNA, and 200 nM oligos corresponding to miR-375, miR-106b or U6, and 7.5 μL Power SYBR-Green PCR master Mix (Applied Biosystems).

miR-375 primers were 5′ TTTGTTCGTTCGGCTCGC 3′, 5′ GATTGAATCGAGCACCAGTTACG 3′; miR-106b primers were: 5′ TAAAGTGCTGACAGTGCAGAT 3′, 5′ GATTGAATCGAGCACCAGTTACG 3′; U6 primers were 5′ GATGACACGCAAATTCGTGAA 3′, 5′ GATTGAATCGAGCACCAGTTACG 3′.

Quantitative PCR reactions were performed in an AB 7300 sequence detection system. Expression of miR-375 and miR-106b was normalized to U6 RNA.

2.3. Plasmid Construction and Transient Transfections. Plasmid manipulations and site-directed mutagenesis were performed as previously described [11]. Transfections were carried out using the transfection reagent jetPEI (Polyplus transfection) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested using passive lysis buffer (Promega) and extracts were subjected to assays to determine the activity of reporter enzymes.

2.4. Luciferase Assays. Firefly luciferase and *Renilla* luciferase assays were carried out as follows: whole cell extracts containing 5–50 μg (1–5 μL) of protein were added to 100 μL of either firefly luciferase assay buffer (20 mM Tricine, 0.1 mM EDTA, 1.07 mM $(MgCO_3)_4Mg(OH)_2{*}5H_2O$, 2.67 mM $MgSO_4$, 3.3 mM DTT, 270 μM Coenzyme A, 470 μM luciferin (Promega) and 530 μM ATP, pH 7.8.) or *Renilla* luciferase assay buffer (0.1 M K_2HPO_4 and 0.1 M KH_2PO_4, pH 7.4, and 0.5 μM coelenterazine (Calbiochem)). The samples were placed in a luminometer (LUMAC Biocounter M2500 or Modulus microplate, Turner Biosystems) and light output was determined over a 10-second interval. Firefly luciferase activity was normalized to the activity of *Renilla* luciferase.

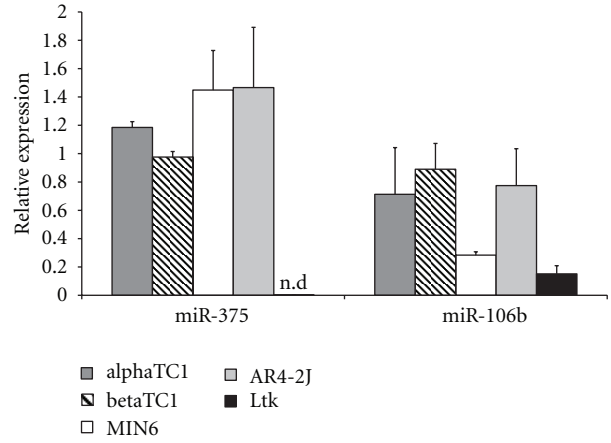

Figure 1: Expression levels of miR-375 and miR-106b in beta cell lines (βTC1 and MIN6), alpha cell line (αTC1), exocrine cell line (AR4-2J), and fibroblasts (Ltk$^-$). Expression of the miRNAs was measured by qRT-PCR and calculated using the absolute quantification method. Results are expressed relative to U6 RNA (mean ± SEM; $n \geq 3$), nd: not detectable.

3. Results

Previous studies have revealed that miR-375 is expressed selectively in pancreatic islets [9, 10]. In order to compare expression in several pancreatic cell lines, we performed quantitative reverse transcriptase PCR (qRT-PCR) analysis on samples of RNA isolated from cell lines derived from pancreatic endocrine alpha cells (αTC1), pancreatic endocrine beta cells (βTC1 and MIN6), pancreatic exocrine cells (AR4-2J), and from non-pancreatic cells (Ltk$^-$). We observed comparable levels of miR-375 in all the pancreatic cells tested, including exocrine cells: on the other hand, levels of miR-375 were at least 100-fold lower in non-pancreatic cells (Ltk$^-$) (Figure 1). For comparison, we determined expression of miR-106b, which is expressed in a broad range of cell types (http://www.microrna.org/). While expression of miR-106b was similar among the pancreatic lines tested, lower yet still significant levels of expression were observed in Ltk$^-$ cells. These results confirm the expression of miR-375 in pancreatic alpha cells and represent the first demonstration of expression of miR-375 in exocrine pancreas cells. Expression may not have been observed previously because high levels of RNase in exocrine pancreas tissue can make detection of RNA technically challenging.

Our previous studies using transfection experiments with luciferase reporter genes revealed that the miR-375 gene promoter shows preferential activity in pancreatic beta cells lines [11]. In order to determine whether the miR-375

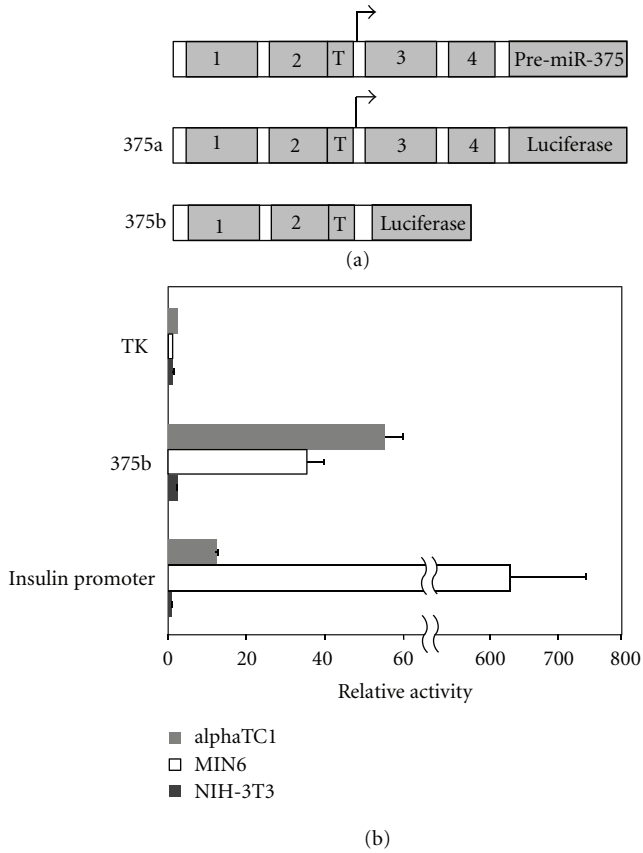

Figure 2: Cell specificity of the miR-375 promoter. (a) Representation of the location of conserved sequence blocks in the miR-375 gene and derived reporter gene constructs. (b) Promoter region of miR-375 (construct 375b) was ligated upstream to the firefly luciferase reporter gene in the promoter-less vector pGL3-basic. The plasmid was compared with luciferase reporter gene plasmids containing the cell-specific insulin gene promoter and the constitutive TK promoter. Promoter activity of each construct was determined following transfection into the alpha cell line αTC1, beta cell line MIN6, and non-pancreatic cell line NIH-3T3. Firefly luciferase activity was measured and normalized for transfection efficiency according to the activity of a cotransfected *Renilla* luciferase plasmid. Values are expressed relative to the activity of promoter-less vector pGL3-basic. Each data point represents mean $\pm$ SEM ($n \geq 3$).

promoter is active also in pancreatic alpha cells, we used the cell line αTC1 which was generated from transgenic mice in a manner analogous to that used for the βTC1 cell line [14]. Indeed, we observed that the promoter shows strong activity in αTC1 cells, similar to that observed in beta cells, but much lower activity in the non-beta cell line NIH-3T3 (Figure 2(b)). In contrast, the TK promoter shows a similar low level of activity in all 3 cell lines tested (2.5 in αTC1, 0.6 in MIN6 and 1.3 in NIH-3T3, Figure 2(b)), whereas the insulin promoter shows strongly preferential activity in beta cells, as compared to alpha cells and non-pancreatic cells (Figure 2(b)). Thus the selective expression of miR-375 observed in alpha cells is at least partly attributable to transcriptional control mediated through the miR-375 promoter. The fact that the relative promoter activity is somewhat higher in αTC1 cells as compared to MIN6, whereas miR-375 levels are similar or slightly lower in αTC1 cells than in MIN6 cells, indicates that post-transcriptional control mechanisms may also play a significant role.

The data presented here showing expression of the miR-375 gene and preferential activity of the miR-375 promoter in alpha cell lines are consistent with previous results based on alpha cells from normal islets: for example, the alpha cell phenotype of mice lacking the miR-375 gene [10] and the activity of the miR-375 promoter in alpha cells of transgenic mice [11].

In our previous studies, we observed that a number of conserved cis elements within the promoter region were essential for full activity in transfected beta cells. These included E boxes, the TATA region, and a short region spanning the consensus binding sites for the transcription factors HNF1/INSM1 [11]. To evaluate the significance of these sequences for expression in alpha cells, we transfected luciferase plasmids containing mutated promoter sequences to αTC1 cells. We observed a pattern quite similar to that seen for the beta cell line HIT [11], namely, that mutations in E box 2, the TATA box, and the Mut2 regions caused a significant reduction in promoter activity (Figure 3(b)). Within the Mut2 region, mutation of the INSM1 site but not the HNF1 site led to reduced promoter activity (Figure 3(b), [11]). Thus a similar combination of transcription factors probably participates in the transcriptional control of the miR-375 promoter. Furthermore, we observed that the presence of conserved blocks 3 and 4 led to a reduction in promoter activity in alpha cells (compare constructs 375a and 375b, Figures 2(a), 3(b)). This is similar to results previously observed in beta cells, and is consistent with the notion that this region functions to inhibit transcription of the promoter. Since the sequences of blocks 3 and 4 are located downstream of the transcription start site, they could conceivably influence gene expression either at a transcriptional or post-transcriptional level. In order to distinguish these possibilities, we built constructs in which blocks 3 and 4 are located upstream of the strong CMV-TK promoter in two orientations. In such a situation, any effect of the block 3-4 region on reporter gene activity can only be mediated transcriptionally. Indeed, we observed that in alpha cells, beta cells and non-pancreatic cells, the block 3-4 region in both orientations was able to reduce significantly reporter gene expression (Figure 4).

4. Discussion

Since miRNAs play a central role in a broad range of biological processes including development, apoptosis, and carcinogenesis, their intracellular concentration must be tightly regulated. Indeed, expression profiles of miRNAs differ substantially among cell types [17]. In principle, this regulation may be controlled at the transcriptional or post-transcriptional level, yet the exact contribution of each of these is unknown. Furthermore, there is relatively little information available regarding the transcription of miRNA

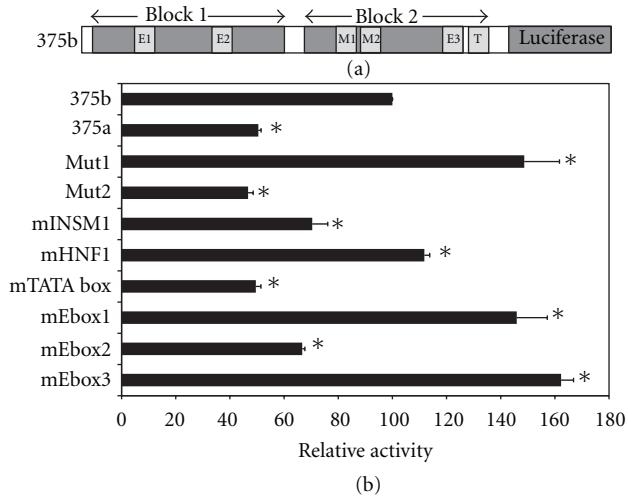

Figure 3: Mutational analysis of miR-375 promoter in alpha cells. (a) Representation of miR-375 gene promoter showing sequence blocks that were subjected to mutational analysis [11]. (b) Luciferase constructs containing promoter mutations were transfected into αTC1 cells. Luciferase activity is expressed relative to wild-type promoter activity (construct 375b). Each data point represents mean ± SEM ($n \geq 3$; $^*P < 0.01$, as compared to luciferase activity of construct 375b). In Mut1 (M1) the sequence TCCATGAG was mutated to TCCTCGAG, in Mut2 (M2) the sequence TATTTGCCCC was mutated to TATGTGCACC, in mE box 1 the sequence AGCCAGGTGGGT was mutated to AGCACTAGTGGT, in mE box 2 the sequence TGACATCTGGTG was mutated to TGAACGCGTGTG, in mE box 3 the sequence GGACAGGTGTG was mutated to GGGACCCGTTG, in mTATA the sequence GCAGTATAAGAG was mutated to GCAGGCGCCGAG.

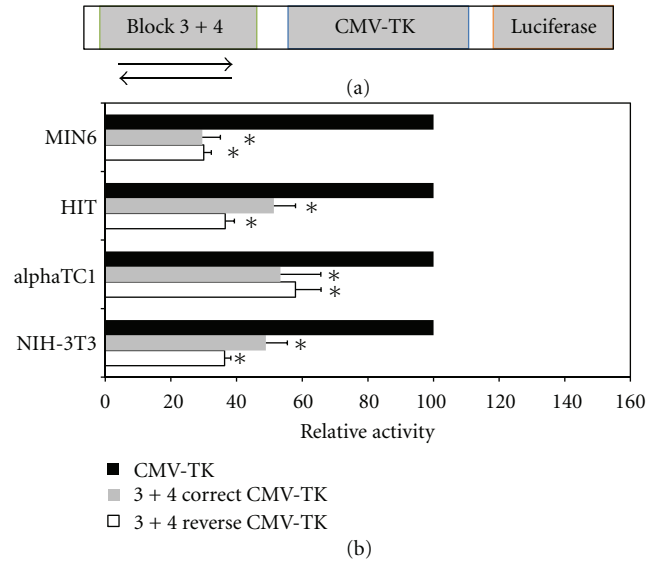

Figure 4: Characterization of the inhibitory effect of blocks 3 and 4. (a) Representation of plasmids containing blocks 3 and 4 inserted in both orientations upstream to the CMV-TK promoter, controlling the firefly luciferase reporter gene. (b) Luciferase activity was measured following transfection into the beta cell lines MIN6 and HIT, the alpha cell line αTC1, and the non-pancreatic cell line NIH-3T3. Activity is expressed relative to luciferase activity of the promoter-less vector pGL3-basic. Each data point represents mean ± SEM ($n \geq 3$; $^*P < 0.05$, as compared to the activity of CMV-TK promoter).

genes and the promoter elements that control the process. According to their location in the genome, miRNA genes can be classified as intragenic and intergenic. Though some intronic miRNAs have been reported to have their own promoters [18], intragenic miRNAs are generally thought to be regulated by their host gene promoter [19]. Transcription of miRNA is believed to be mediated by RNA pol II [20], though examples of involvement of Pol III have been reported [21].

We have focused on miR-375, since it shows a clear pattern of preferential expression in pancreatic cells. miR-375 was originally reported as selectively expressed in pancreatic endocrine alpha and beta cells [8, 9], and expression was subsequently reported in the gastrointestinal tract [22] and pituitary [10]. miR-375 is essential both in developing and adult pancreas: mice lacking miR-375 displayed impaired balance of alpha/beta cells, as well as defective beta cell proliferation in response to increased insulin demand [10]. Our previous results have demonstrated that selective expression in beta cells is controlled at the transcriptional level by an evolutionarily conserved promoter. We have now examined activity of the promoter in alpha cells and find that the promoter exhibits similar transcription selectivity; furthermore mutations in several key cis elements previously demonstrated to be crucial for activity in beta cells, are also important in alpha cells. This indicates that a similar spectrum of transcription factors contributes to selective expression. Based on the cis elements identified, these factors are likely to include bHLH proteins such as BETA2/NeuroD and the transcription factor INSM1. The precise role of miR-375 in alpha cells is unknown: although it may share some target genes with beta cells, loss of the miR-375 gene affects alpha and beta cells differently [10] indicating important target gene differences. The increased alpha cell mass and hyperglucagonemia observed in mice lacking miR-375 are consistent with a role for miR-375 in regulating alpha cell genes that control cell growth and proliferation, and normal glucose sensing.

An intriguing feature that we identified previously in the miR-375 gene promoter is the existence of a negative element located immediately downstream of the transcription start site within the primary transcript. We have found that blocks 3 and 4 repress promoter activity in pancreatic and non-pancreatic cells both in the context of the miR-375 gene promoter, and also when located upstream of a heterologous promoter. This clearly indicates that the repression activity of blocks 3 and 4 operates through transcriptional mechanisms and functions in several cell types. Using bioinformatic tools, we have identified binding sites for several inhibitory transcription factors within blocks 3 and 4, including Krueppel-like transcription factors, ETS1 factors and neuron-restrictive silencer factor (NRSF). Further analysis will be required to verify the transcription factors involved in this interesting repression activity, and to

determine whether the block 3 and 4 regions may also be involved in post-transcriptional regulation.

Acknowledgments

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Research Article

Regulation of Pancreatic microRNA-7 Expression

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Genome-encoded microRNAs (miRNAs) provide a posttranscriptional regulatory layer, which is important for pancreas development. Differentiation of endocrine cells is controlled by a network of pancreatic transcription factors including Ngn3 and NeuroD/Beta2. However, how specific miRNAs are intertwined into this transcriptional network is not well understood. Here, we characterize the regulation of microRNA-7 (miR-7) by endocrine-specific transcription factors. Our data reveal that three independent miR-7 genes are coexpressed in the pancreas. We have identified conserved blocks upstream of pre-miR-7a-2 and pre-miR-7b and demonstrated by functional assays that they possess promoter activity, which is increased by the expression of NeuroD/Beta2. These data suggest that the endocrine specificity of miR-7 expression is governed by transcriptional mechanisms and involves members of the pancreatic endocrine network of transcription factors.

1. Introduction

The development of the endocrine pancreas is governed by a network of transcription factors that specify different endocrine cell types, including insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells, pancreatic polypeptide-producing PP cells, and ghrelin-producing epsilon cells [1–3]. The endocrine differentiation program is initiated by neurogenin3 (Ngn3) [4, 5]. Next, a complex network of transcription factors is activated to differentially specify the endocrine lineages (reviewed in [3, 6]).

Genome-encoded miRNAs act in concert with transcription factors to refine gene expression and confer robustness to developmental transitions [7–10]. Many miRNA genes are nested within introns of protein-coding genes and are subjected to transcriptional control with their host gene [11]. However, other miRNA genes are located in intergenic regions and are expressed autonomously. For example, in a previous study, we characterized the pancreas-enriched miR-375 and demonstrated that cell specificity is controlled transcriptionally through well-defined cis-regulatory elements [12].

Like miR-375, miR-7 is highly and selectively expressed in the endocrine pancreas of zebrafish, mouse, and human [13–16]. miR-7 is an evolutionarily conserved miRNA, encoded by a single gene in flies and by three different genomic loci in mammals. In mice two miR-7 genes are located in intergenic regions of Chr. 7 (mmu-mir-7a-2) and Chr. 17 (mmu-mir-7b), whereas a third miR-7 gene, mmu-mir-7a-1, is embedded within an intron of the gene encoding for the RNA-binding protein, Hnrnpk (MGI: 99894, on Chr. 13).

The two miR-7a genes generate an identical 22nt mature sequence, whereas miR-7b differs by a single nucleotide. However, functionally the three genes are identical, as they harbor the same "seed" sequence. Hence, all miR-7 genes coregulate the very same target set. While a defined set of targets is suggested for miR-7 [17] some of which have been experimentally validated [18], little is known about miR-7 promoter structure or the mechanisms controlling miR-7 expression.

In this study we characterized elements within the mmu-mir-7a-2 and mmu-mir-7b upstream regulatory sequences. We show that miR-7 responds to Ngn3 directly. However, our data suggest that NeuroD/Beta2, the primary effector

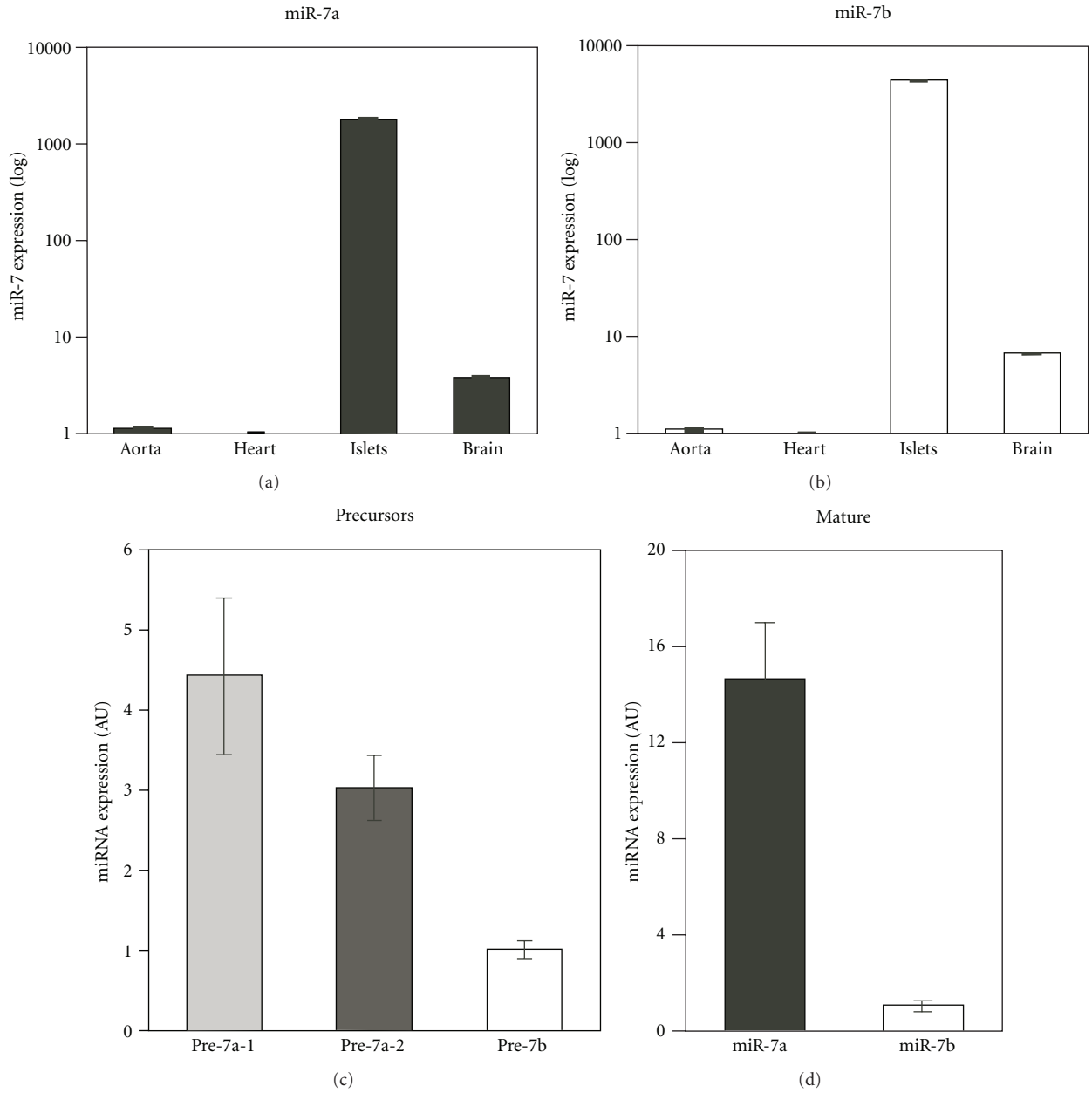

Figure 1: Three miR-7 genes are expressed in beta cells. (a) and (b) Taqman qPCR analysis for mature miR-7a and miR-7b in several tissues: the aorta and pulmonary artery, heart, isolated islets, and brains from three months old mice. Data normalized to sno234. (c) qPCR analysis for miR-7 precursors reveals expression in beta-TC cultured cells. Data normalized to GAPDH and 18S. $n = 4$ independent measurements, duplicates each. (d) Taqman qPCR analysis to mature miR-7a and miR-7b in beta-TC cultured cells. Data normalized to sno234. $n = 4$ independent measurements in duplicates. Error bars represent ± SEM.

of Ngn3, controls miR-7 and is probably responsible for maintenance of miR-7 expression in differentiated endocrine cells.

2. Materials and Methods

2.1. Quantitative PCR for Precursor and Mature miRNA. Extraction of total RNA was carried out by the miRNeasy Mini Kit (Qiagen). For precursor quantification, synthesis of cDNA was performed using miScript system (Qiagen). cDNA was synthesized from miRNAs using Taqman MicroRNA qPCR Assays (Applied Biosystems). qPCR analysis of mRNA was performed on LightCycler 480 System (Roche) using Kapa SYBR Green qPCR kit (Finnzymes). miRNA levels were normalized to the expression of small RNAs (sno234 and U6) and mRNA normalized to GAPDH and HPRT. (primer sequences are described in Supplementary Table 1 available online at doi: 10.1155/2012/695214).

2.2. Cell Culture and Luciferase Reporter Assay. HEK-293T cells (American Type Culture Collection), betaTC-3 (a gift

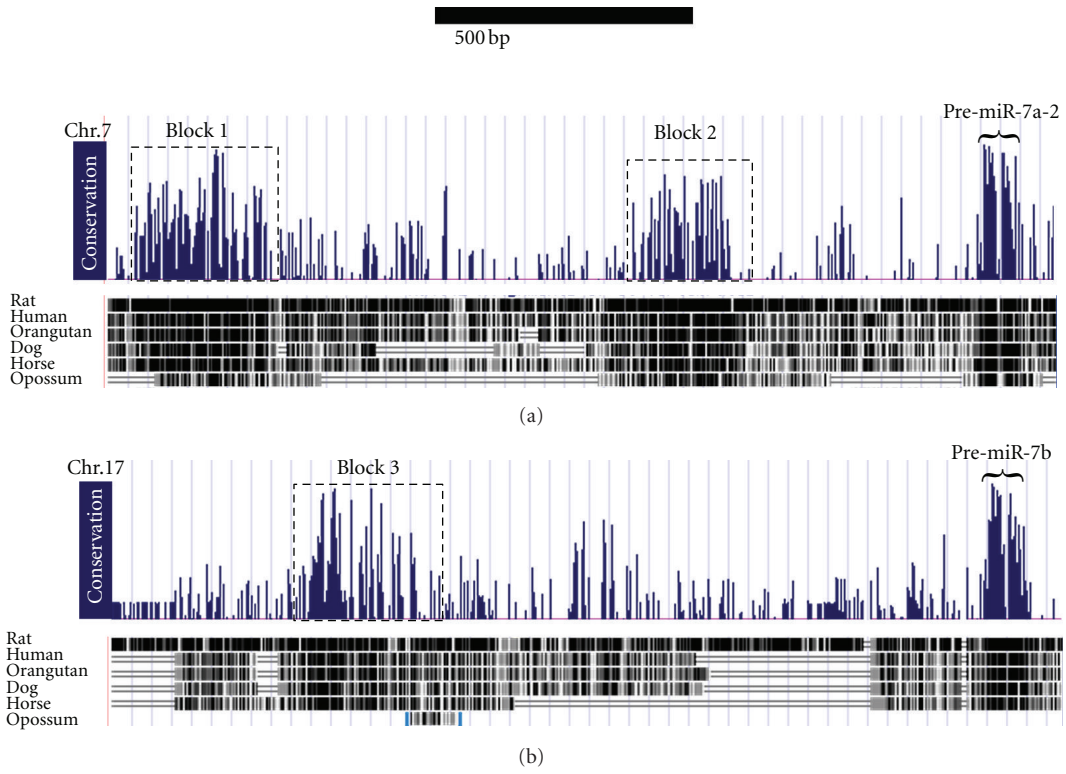

Figure 2: Conserved sequences upstream of miR-7 genes. Screen capture from the UCSC Genome Browser reveals fragments of Mus musculus chromosome 7 (a) and chromosome 17 (b). Tracks for conservation within mammals and the annotation of the pre-miR-7 sequences are depicted. Dashed squares demarcate the sequences that were further investigated. Scale bars represent 500 bp.

from Shimon Efrat), and MIN6 cells (a gift from Jun-ichi Miyazaki) were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin at 37°C at 5% CO_2 in a humidified incubator. Experiments on MIN6 cells were performed between passages 18 to 28.

For miR-7 promoter analyses, fragments of 256 bp, 206 bp, and 156 bp (representing miR-7a-2 "block 1", miR-7a-2 "block 2", and "block 3" of miR-7b, resp.) were subcloned into pGL3-basic, using restriction enzymes BglII and KpnI. Primer sequences are described in Table S1. HEK-293T cells were transfected with 100 ng of the reporter, 20 ng of A20 Renilla reporter, and in addition 50 ng of pcDNA3 empty and/or NeuroD/Beta2 and Ngn3 expression vectors. Reporter activity was measured 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega).

For overexpression analysis, expression vectors for transcription factors, pcDNA3 empty vector, and CMV-GFP vectors were transfected to MIN6 cells using Lipofectamine 2000 reagent (Invitrogen), according to the manufacture's instructions. miR-7 endogenous expression was analyzed by qPCR 48 h later.

2.3. Statistical Analysis. Analysis was performed using either Student's *t*-test or two-way ANOVA by the JMP software. Results are given as mean ± SEM. The null hypothesis was rejected at the 0.05 level (**).

3. Results and Discussion

3.1. Three miR-7 Loci Are Expressed in Endocrine Cells. Since miR-7 gene has three genomic copies in mouse and human, we first determined which of them is expressed in endocrine cells. As previously shown [19], miR-7a and miR-7b were highly and specifically expressed in islets of Langerhans, relative to other organs such as heart and brain (Figures 1(a) and 1(b)). The precursors transcribed from the three different loci can be distinguished by quantitative real-time PCR (qPCR). Therefore, we designed specific primers for each of miR-7 precursors (for oligo sequences, see Table S1) and performed a qPCR study in BetaTC3 cell line. This analysis revealed that all three miR-7 genes are expressed in cultured beta cells (Figure 1(c)). Consistent with these results, qPCR for the mature miR-7a and miR-7b revealed expression of both forms of miR-7 in beta cells (Figure 1(d)). Taken together, the analysis of miR-7 expression suggests that the three miR-7 loci are activated in beta cells and are responsible for the overall high expression of the mature miR-7.

3.2. Sequence Analysis of Potential miR-7 Regulatory Regions. Comparative genomics is commonly used to identify conserved sequences of functional importance [12, 20]. Therefore, we compared the region upstream of the pre-miR-7 sequences among several vertebrate orthologs and identified

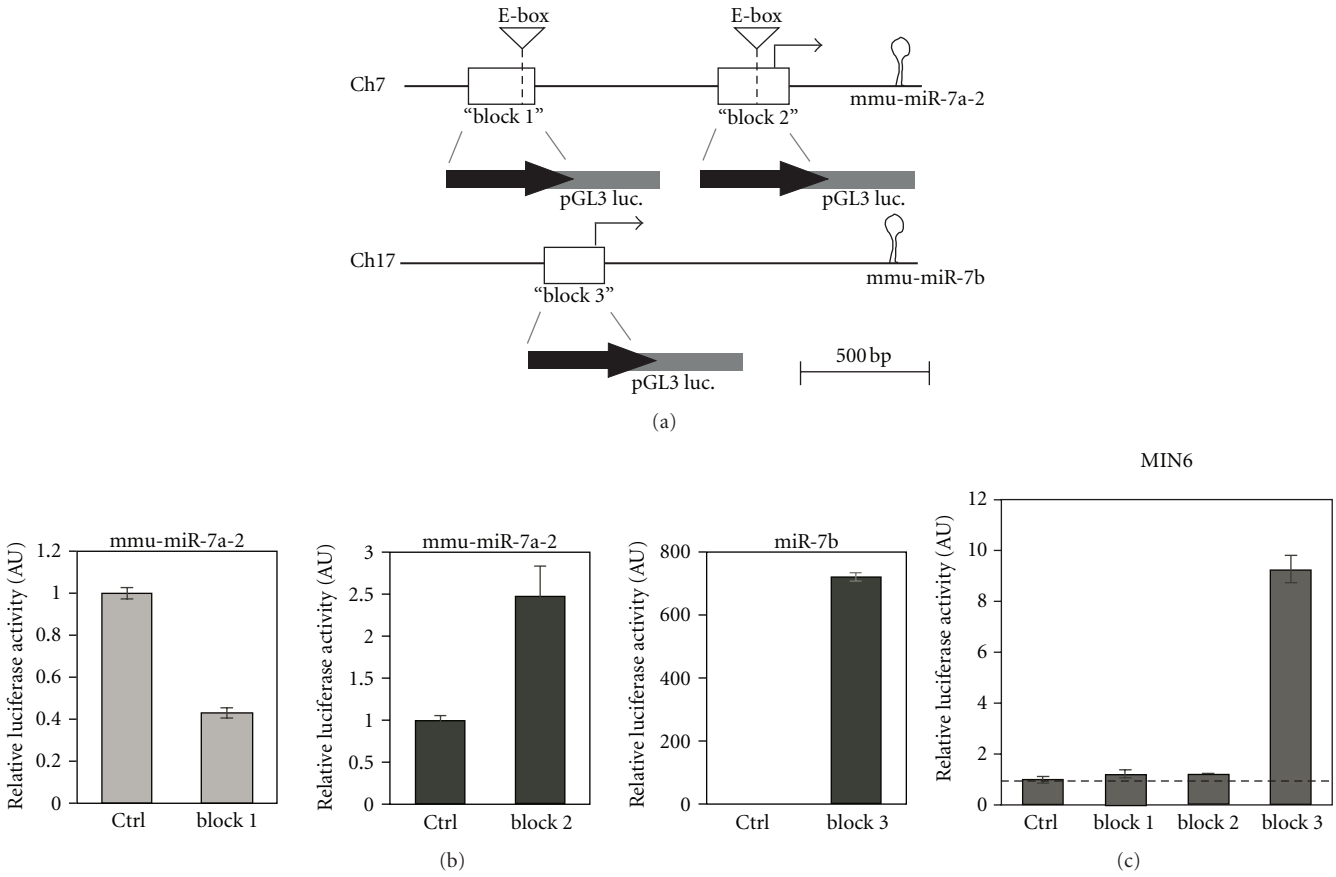

Figure 3: miR-7 putative promoters induce reporter activation. (a) Schematic representation of conserved sequences, upstream of pre-miR-7a-2 hairpins ("block 1" and "block 2") and pre-miR-7b ("block 3") that were subcloned upstream of pGL3 basic luciferase reporter. (b) Relative activation of luciferase firefly to renilla in HEK-239 cells, transfected with various reporters. pGL3-basic serves as control ("Ctrl"). (c) Relative luciferase activation in MIN6 beta cells transfected with the indicated reporters. $n = 3$ experiments, in triplicates. Error bars represent ± SEM.

three highly conserved sequences, which may be potentially functional promoter regions. Trimethylation of lysine 4 of histone 3 (H3K4) is involved in activation of transcription and marks the position of transcriptional start site of many genes, including insulin [21]. Accordingly, we identified typical H3K4 methylation pattern [22, 23], indicating possible transcriptional start sites, at the vicinity of miR-7 genomic regions.

For miR-7a-2 we identified two conserved sequences: the 256 bp long "block 1" and 206 bp "block 2," which are located 1420 bp and 450 bp upstream of the miR-7a-2 hairpin, respectively (Figure 2(a)). For miR-7b, a single 156 bp long "block 3" conserved sequence was identified, positioned 1,235 bp upstream of the miR-7b hairpin (Figure 2(b)). miR-7a-1 was omitted from this analysis since it is embedded in an intron of the Hnrnpk gene and is likely regulated by the promoter of the host gene.

3.3. Activity of the miR-7 Promoters in Cultured Cells. To functionally characterize miR-7 promoters, the fragments consisting of blocks 1–3 were fused separately to a firefly luciferase reporter gene, on a promoterless plasmid (pGL3-basic), and transfected into the pancreatic beta cell line MIN6 and the embryonic kidney line HEK 293.

In HEK-293T cells, "block 2" produced a twofold increase in luciferase activity, relative to pGL3-basic reporter ("Ctrl"), indicating weak promoter activity (Figure 3(b), middle panel). "Block 3", on the other hand, produced a large (200-fold) increase in luciferase activity, indicating the presence of a strong promoter in this region (Figure 3(b), right panel). Surprisingly "block 1" resulted in repression of the luciferase activation, suggesting that this conserved sequence may be involved in repression of gene transcription (Figure 3(b), left panel). We then examined whether the putative promoter sequences could drive luciferase activation in MIN6 beta cells. We observed that only the DNA sequences dubbed "block 3" showed significant promoter activity (Figure 3(c)). Taken together, these results show that "block 2" and "block 3" possess weak and strong promoter activity, respectively. The activity of these fragments is not restricted to pancreatic cells, and presumably these promoter fragments require additional elements to confer selectivity in vivo.

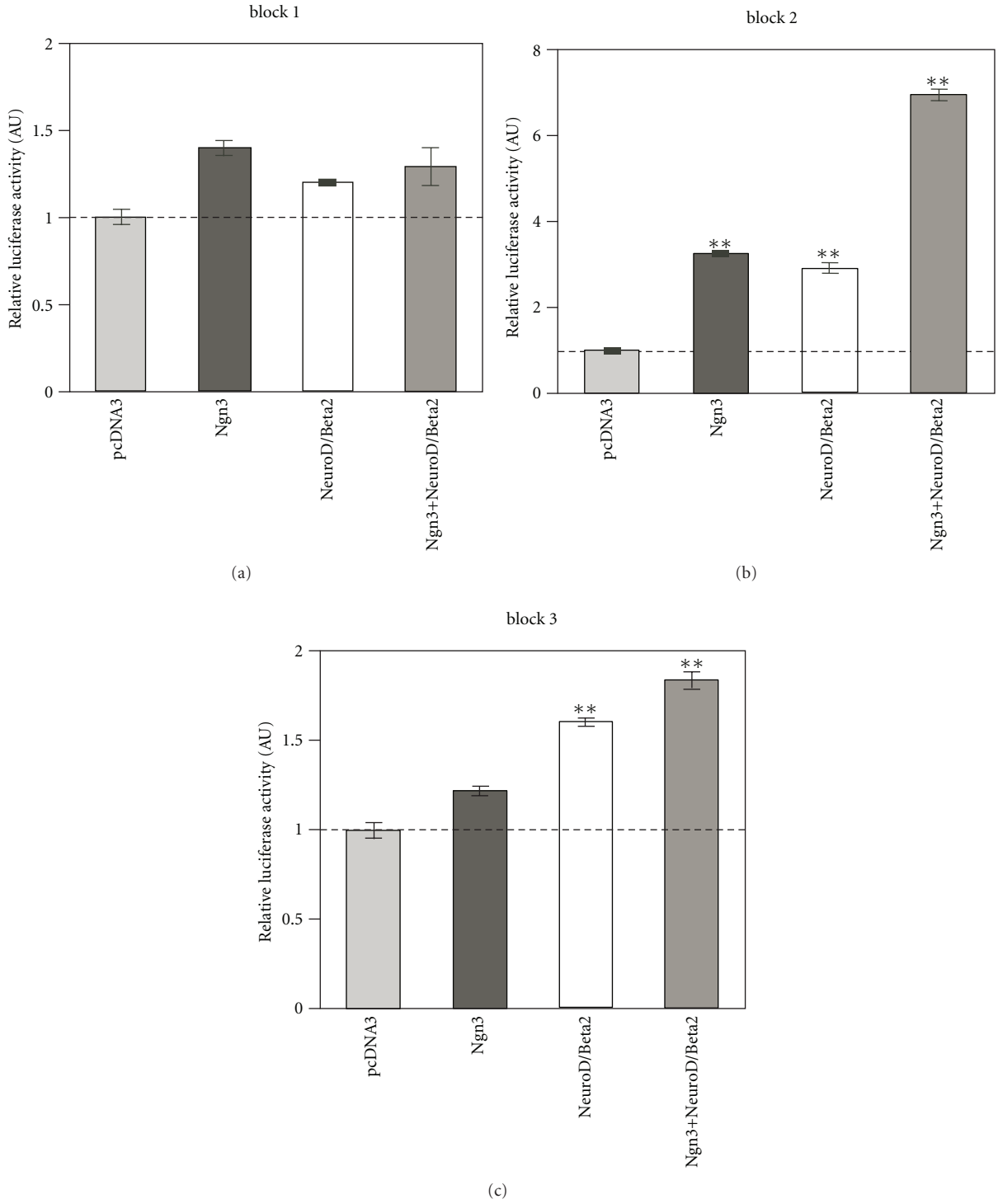

Figure 4: Transactivation of miR-7 reporters in HEK 293T. (a) The luciferase activity of a reporter driven by "block 1" did not demonstrate response to Ngn3 or NeuroD/Beta2. However, reporters driven by "block 2" are induced by both Ngn3 and NeuroD/Beta2 (b). Highest luciferase response is induced by a combination of Ngn3 and NeuroD/Beta2. (c) "Block 3" transcription is transactivated only by NeuroD/Beta2 but not by Ngn3. Luciferase activity normalized to A20-Renilla expression and to transactivation by a control pcDNA3-empty vector. $n = 3$ experiments, in triplicates. Error bars represent ±SEM ($^{**}P < 0.05$).

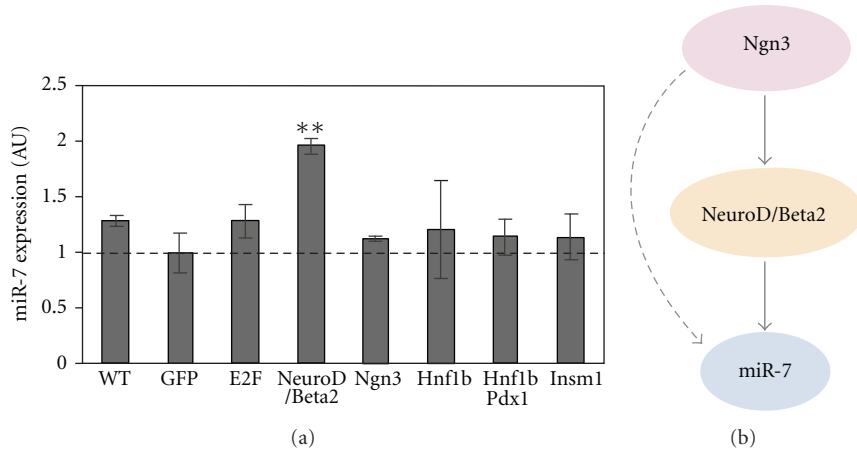

Figure 5: NeuroD/Beta2 regulates miR-7. (a) miR-7 expression in MIN6 cells (passages 22–24), transfected with expression vectors for various transcription factors. Endogenous miR-7 expression is upregulated upon NeuroD/Beta2 introduction, relative to GFP-expressing control vector. (b) A Schema describing miR-7 regulators.

3.4. bHLH Transcription Factors Directly Induce miR-7 Promoter Activity. It has been previously shown that the bHLH transcription factors, Ngn3 and NeuroD1, play a central role in pancreas endocrine development and in mature beta-cell function, respectively. These proteins function through E-boxes (consensus sequence CAxxTG) located in target gene promoters [24]. Since two conserved E-box elements were identified in miR-7 promoter sequences (Figure 3(a)), we tested whether Ngn3 and NeuroD1 expression regulates miR-7 promoter activity.

For this, we performed transactivation experiments, in which luciferase reporter constructs were cotransfected into HEK-293T cells in the presence of expression vectors encoding the endocrine transcription factors. These analyses showed activation of "block 2" promoter in response to both NeuroD/Beta2a and Ngn3. In the presence of both factors, an additive effect was observed (Figure 4(b)). With "block 3", NeuroD/Beta2 produced significant activation, whereas Ngn3 had little or no effect. The expression of block 1-containing promoter was not significantly affected by any of the co-transfected transacting factors.

Our study, therefore, suggests that the sequence upstream of miR-7b is able to activate transcription in cultured beta cells and can be activated by the endocrine transcription factor NeuroD/Beta2. The block 2 region upstream of miR-7a-2 can be activated both by NeuroD/Beta 2 and by Ngn3. However, this sequence shows very low promoter activity in the beta cell line MIN6 and therefore probably works in concert with other elements in order to transcribe the miR-7a-2 locus in beta cells.

A single ancestral miR-7 gene in invertebrates has undergone genomic duplication in the vertebrate clade. While duplication of genes often leads to functional divergence of each locus, our data suggest that miR-7 expression from three independent loci contributes primarily to higher levels of expression. Furthermore, coregulation of miR-7a-2 and miR-7b by NeuroD/Beta2 suggests that these genes respond to similar transacting factors in the endocrine pancreas.

3.5. Endogenous miR-7 Expression Is Activated by NeuroD/Beta2 in Cultured Beta Cells. Finally, we determined whether expression of endogenous miR-7 can be modified by introducing endocrine transcription factors into beta cell lines. To this end, we transfected MIN6 cells with expression vectors for Pdx1, Ngn3, NeuroD/Beta2, Hnf1b, Insm1, and E2A. Of these, miR-7 expression was induced in cultured beta cells only by NeuroD/Beta2 (Figure 5(a)). This is consistent with the results obtained from reporter experiments and suggests that selective expression of miR-7 is controlled by lineage-specific transcription factors, primarily, NeuroD/Beta2. This supports the hypothesis that miR-7 itself may be a component of this cascade with functional roles in controlling endocrine cell development at a posttranscriptional level (see model in Figure 5(b)).

4. Conclusions

During development, Ngn3 induces endocrine cell differentiation by upregulating transcription factors such as NeuroD/Beta2, Pax4, Arx, and Pax6. Indeed, loss of Ngn3 causes blockade in endocrine differentiation [5]. At the same time, Ngn3 also upregulates miR-7 expression through its effector, the transcription factor NeuroD/Beta2, that is likely involved in maintaining miR-7 expression also in mature cells.

In summary, our analysis identifies miR-7 as a novel component downstream of Ngn3 and NeuroD, embedded within the transcription factor network regulating pancreas development. Further dissection of the transcriptional mechanisms controlling expression of miR-7 and other endocrine

miRNAs will contribute substantially to our overall understanding of the role of miRNA in pancreas development and function.

Authors' Contribution

S. K. Russo, M. D. Walker, and E. Hornstein designed the experiments. S. K. Russo, A. Ness and A. D. Mandelbaum conducted all experiments and analyses in this work. S. K. Russo, M. D. Walker, and E. Hornstein, wrote the paper.

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