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Non-coding RNAs in Endocrinology

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From Cradle to the Grave: Tissue-specific microRNA signatures in detecting clinical progression of diabetes

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Abstract

Ever since the discovery of small non-coding RNAs, microRNAs have been identified to play a critical role in development and function of pancreatic insulin-producing beta cells. Research carried out until now demonstrates that microRNAs can specifically target key pancreatic transcription factors and signalling molecules. This in turn may influence changes in insulin production and secretion. microRNAs are also identified in insulin target organs that are altered as a result of hyperglycemia and insulin resistance. Recent studies demonstrate that microRNAs are not only confined to cells but are also detected in biological fluids including serum, plasma and urine. These data indicate that miRNAs may be looked upon having a dual role, as biomarkers and as regulators of disease. We review the existing literature in understanding the role of microRNAs in development, function and death of pancreatic beta cells as well as in the development of metabolic disease. We discuss the possible mechanisms that contribute to identifying the role of microRNAs as sensitive and efficient biomarkers to predict the progression of diabetes. Understanding tissue-specific microRNA signatures and their role as a cause or effect of diabetes would provide more information on progression of this disease.

Keywords

Circulating microRNAs • Biomarkers • Metabolic disease

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Introduction to microRNAs (miRNA)

Since the discovery of the first non coding RNA by Lee et al in 1993 [1], 21,643 mature miRNAs in 168 species have been described (miRBase release 18, November 2011) [2]. These represent approximately 1% of the genome in different species and, in humans, target approximately 30% of protein coding genes [3].

microRNAs are small non-coding RNAs of around 18-22 nucleotides in length, which are highly conserved across species [4]. Mature miRNAs are processed in 2 stages from primary-miRNA transcripts (pri-miRNAs), which are the longer precursors to the mature miRNA molecules [5]. The first stage occurs in the nucleus where pri-miRNAs are cleaved by the RNase III enzyme Drosha to generate 70 to 100 nucleotide long hairpin miRNA precursors (pre-miRNA) [6]. Pre-miRNA sequences possess a 2 nucleotide overhang at the 3' end. Following transport of pre-miRNA into the cytoplasm [7], the RNase III enzyme Dicer cleaves sequences to generate miRNA duplexes ~ 22 nucleotides in length [8]. One strand of these duplexes is then selected for incorporation into a ribonucleoprotein complex called the RNA-induced silencing complex (RISC) and functions as a mature miRNA. Proteins of the Argonaute family are key components of RISC, with Argonaute 2 (Ago2) shown to play a critical role in miRNA-mediated mRNA silencing [9,10]. Mature miRNA guides the RISC to complementary mRNA sequences, usually in the 3' untranslated region (3'-UTR). Partial complementarity between the miRNA and mRNA leads to inhibition of translation whilst perfect complementarity results in degradation of the target mRNA [3,11,12]. (Summarized in Figure 1)

It has been estimated that each miRNA may be regulating around 200 transcripts [13]. miRNAs play vital roles in all biological processes including proliferation, differentiation, cell growth, cell death and stress resistance (reviewed by [3,12]). Whilst majority of miRNAs are found intracellularly, a number of miRNAs have also been detected outside of cells; in body fluids including serum / plasma [14-16], urine [17,18], faeces [19,20], saliva [21,22] and breast milk [23]. It is now widely accepted that miRNAs in the circulation can act as biomarkers of certain diseases, particularly cancer. This review will examine the role of microRNAs in pancreas biology, in insulin target tissues as well as in prediction of diabetes (Table 1).

microRNAs in pancreas biology

It has been well demonstrated that miRNAs are a requirement for life. In the complete absence of the miRNA processing enzyme Dicer1, mice fail to complete normal embryonic development [24]. Dicer-1 expression levels at 20% of normal were shown to be sufficient for normal development [25] and survival of

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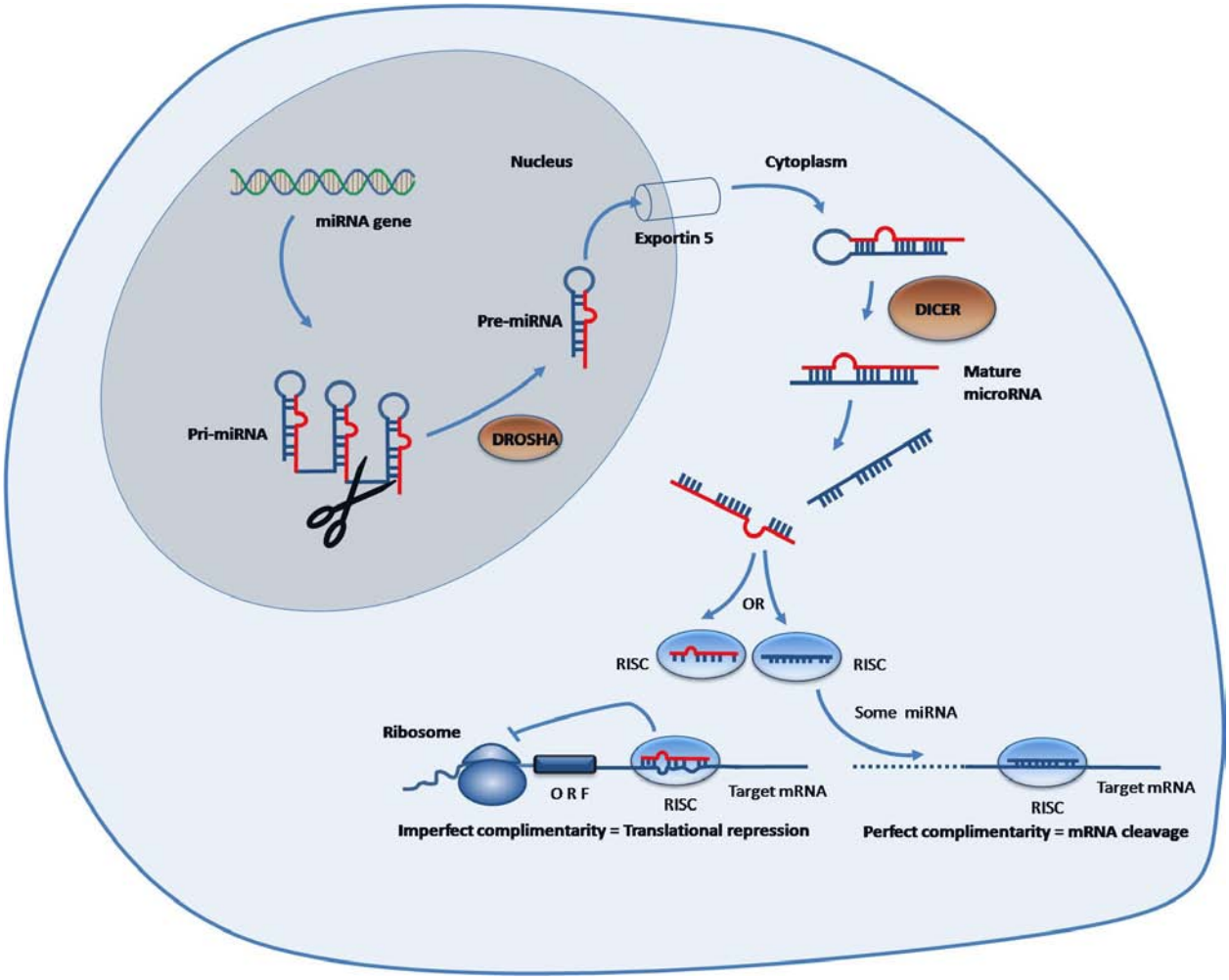

Figure 1. A schematic depicting synthesis and function of miRNA. Mature miRNAs are synthesized in the nucleus as primary miRNAs and processed to precursor miRNAs by Drosha. These precursors are then transported outside the nucleus, where they are further truncated by Dicer into double stranded mature miRNAs. Single strands of mature miRs are incorporated into RISC (RNA Induced Silencing Complex), which then targets the gene transcripts. Based on the complementarity of the mRNA : miRNA, the target mRNA is either destroyed or translationally repressed.

these mice. In 2007, Lynn et al demonstrated using a pancreatic Dicer1 conditional knockout mouse that miRNAs are required for normal development of the pancreas [26]. Animals that survived until birth failed to grow and died by day 3, showing gross pancreatic defects with all endocrine cells particularly β-cells being dramatically reduced. It has been shown that Dicer-1 is required during early development and in maintenance of the adult pancreas [27].

Although microRNAs have been known since they were first described by Victor Ambros and Gary Ruvkin in 1993, their importance in pancreatic biology became evident in 2004. During this time Poy et al [28] cloned miR-375 and miR-376 from MIN6 cells and demonstrated that they were found specifically in mouse pancreatic islets. Several lines of evidence suggest that miRNAs are important in pancreas biology and play a role in insulin secretion [28-32], pancreatic islet development [33-35], beta cell proliferation [36-38] maintenance of beta cell function [39-41], pancreas regeneration, [42,43] and islet cell death [44-46]. All these articles confirm the importance of miRNAs in pancreas biology. These islet-specific miRNAs are believed to fine-tune many of the physiological and pathological events in pancreatic beta cells. Islet specific miRNAs (including miR-375, miR-9, miR-7, miR-30 family, miR-338-3p, miR-29a/b/c, miR-21, miR-124a) have been described in depth in recent reviews and will not be covered here [47-51] (Summarized in Table 1).

microRNAs in insulin target tissues

Blood glucose homeostasis requires an appropriate balance between the release of insulin from pancreatic β cells and the sensitivity of the target tissues to the action of insulin. Diminished insulin sensitivity of peripheral tissues such as adipose tissue, skeletal muscle and liver leads to insulin resistance which, under normal circumstances is overcome by increased insulin secretion. Failure to compensate for insulin resistance leads to chronic hyperglycemia and progression to type 2 diabetes.

Table 1. Important microRNAs involved in pancreatic cell biology and diabetes.

microRNA	Target(s)	Function	Location of Expression	References
miR-1	IGF1 IGF1R	Glucose homeostasis	Skeletal and cardiac muscle	[56,57]
miR-7	Not known	Pancreatic development, β-cell mass, insulin production	Pancreatic islets	[34,124-126]
miR-9	Onecut-2 Sirt1	Insulin exocytosis	Pancreatic β-cells	[31,32,34]
miR-29	INSIG1 CAV2	Insulin signalling pathway, lipid metabolism	Skeletal muscle and adipose tissue	[60,127]
miR-30c/d	Vimentin Snail1	Epithelial-to-mesenchymal transition	Pancreatic islets	[40]
miR-33a/b	IRS-2	Insulin signalling pathway	Hepatocytes	[74]
miR-122	CAT-1	Cholesterol and fatty acid metabolism,	Liver	[69,70]
miR-124a	FOXA2 Rab27A Myotropin	Pancreatic development and insulin secretion	Pancreatic β-cells	[13,39,128]
miR-126	IRS-1	Insulin signalling pathway	Hepatic cancer cell line, SK-Hep-1	[75]
miR-133a	KLF15	Glucose homeostasis	Skeletal muscle	[47,55]
miR-143	ERK5/MAPK7	Adipocyte differentiation	Adipose tissue	[65,66]
miR-208a	MED13	Energy homeostasis	Cardiac muscle	[80]
miR-338-3p	GPR30 GLP1	β-cell proliferation	Pancreatic β-cells	[37]
miR-375	Myotrophin PDX-1	Islet development, insulin secretion, glucose homeostasis	Pancreatic islets	[28,33,34,41]

Representative pancreas-specific and peripheral tissue-specific miRNAs.
There are several miRNAs that are reported to be important in pancreas development and function as well in insulin target organs. Here we summarize some important ones with their target genes, location and function based on present available knowledge. We believe that these miRs can be useful biomarkers for prediction of diabetes and diabetes complications, however more work needs to be done to reach to any conclusion.

Whilst the mechanism of insulin resistance in insulin target tissues is not fully understood, it is now thought that altered miRNA expression may play a role.

Skeletal muscle

Muscle tissue, the primary site of glucose uptake, accounts for approximately 75% of insulin-dependent glucose removal from the plasma [52]. miR-1, miR-133 and miR-206, which are also called as myomiRs due to their muscle-specific expression, regulate muscle proliferation and differentiation [53,54]. Gene targets of miR-1 and miR-133a are involved in glucose homeostasis. miR-133 has been shown to alter expression of insulin like growth factor-1 receptor (IGF-R1) in developing skeletal muscle [47] as well as the glucose transporter GLUT4 by inhibiting expression of the Krüppel-like transcription factor KLF15 [55]. miR-1 also targets insulin like growth factor 1 (IGF-1), blocking the capacity of glucose induced IGF-1 to cause mitochondrial dysfunction, cytochrome-C release and apoptosis in cardiomyocytes [56]. Both IGF-1 and IGF-R1 were shown to be targets of miR-1 in C2C12 myoblasts; interestingly, this study also determined that IGF-1 is able to regulate the expression of miR-1 via the transcription factor FoxO3a [57].

Studies have examined the expression of miRNAs in skeletal muscle from Goto Kakizaki rats [58-60], a model of insulin resistance and diabetes [61]. Different miRNAs including miR-222, miR-27a, miR-195, miR-103 are altered in type 2 diabetes. A recent study also suggests a role of miR-106b in mitochondrial dysfunction and insulin resistance of C2C12 myotubes [62]. miR-106b regulates mitochondrial function by targeting mitofusin-2 and also controls skeletal muscle insulin sensitivity.

A study carried out in 2009 examined the expression levels of miRNA in the skeletal muscle of healthy individuals before and after a 3 hour euglycemic-hyperinsulinemic clamp [63]. Out of 216 microRNAs expressed in skeletal muscle, 39 were found to be down-regulated by insulin including the muscle specific miRNAs miR-1, miR-133a and miR-206. This down-regulation of miR1/miR-133a by insulin was shown to be mediated by the transcription factors sterol regulatory element binding protein (SREBP)-1c and myocyte enhancer factor 2c (MEF2c). The effect of insulin on miR-1 and miR-133a was altered in the skeletal muscle of type 2 diabetic individuals; however, levels of these miRNAs in the absence of insulin were not altered compared to healthy individuals. Another study by Gallagher et al [64] examined mRNA and miRNA changes in skeletal muscle of healthy (non-diabetic) individuals and those with impaired

glucose tolerance or type 2 diabetes. Of 172 miRNAs expressed in skeletal muscle, 61 were altered in individuals with T2D with a subset also found to be altered in individuals who had impaired glucose tolerance [64] suggesting that some expression changes occur before the onset of diabetes.

Adipose tissue

In adipose tissue, insulin stimulates lipogenesis, the conversion of blood glucose into fatty acids for efficient energy storage. microRNAs are demonstrated to be important in adipocyte lineage commitment, differentiation and proliferation. miR-143 has been shown to be involved in adipocyte differentiation both in vivo and in vitro [65,66]. Overexpression of miR-143 and miR-103 in pre-adipocytes leads to an increase in the expression of adipogenesis markers and triglyceride accumulation. Interestingly, both miR-143 and miR-103 are downregulated in adipocytes from ob/ob mice which are insulin resistant and obese [66]. miR-143 overexpressing mice demonstrate impaired insulin sensitivity, while miR-143 knock out animals do not develop obesity symptoms [67]. This indicates that miR-143 is one of the regulators of obesity and diabetes.

The miR-29 family (miR-29a, b and c) have been shown to be upregulated in both the skeletal muscle and adipose tissue of diabetic Goto Kakizaki rats [60]. The upregulation of miR-29a and miR-29b could be reproduced in vitro when 3T3-L1 adipocytes were incubated in the presence of high glucose and insulin. Indeed, overexpresion of miR-29 in the same cell type decreased insulin-stimulated glucose uptake [60]. These results suggest that miR-29 may be involved in mechanisms leading to insulin resistance in type 2 diabetes.

Liver

Selective deletion of the miRNA processing enzyme Dicer1 in the liver early after birth leads to mild hyperglycemia in the fed state and in severe hypoglycaemia in the fasting state due to a depletion of glycogen storage [68]. The expression levels of 4 microRNAs that are highly enriched in the liver, miR-122, miR-148a, miR-192 and miR-194, were found to be decreased in Dicer null hepatocytes indicating that these miRNAs are important for hepatic glucose homeostasis.

miR-122 is the most abundant miRNA in the liver, with copy numbers reaching 50,000-82,000 per average mouse liver cell, and 135,000 per primary human hepatocyte [69]. In mice, inhibition of miR-122 leads to a decrease in hepatic fatty acid and cholesterol synthesis along with a reduction in plasma cholesterol [70]. Inhibition of miR-122 in a mouse model of diet-induced obesity also led to a reduction of plasma cholesterol. These results suggest that miR-122 inhibition may be of therapeutic benefit in lowering plasma cholesterol levels.

The expression levels of miRNAs in liver have been examined in animal models of diabetes [58,71-73], however, their precise role in regulation of liver function are not yet determined. In vitro, a number of microRNAs have been shown to inhibit the expression of insulin receptor substrates (IRS). miR-33a/b inhibit the expression of IRS-2 in cultured hepatic cells reducing the activation of insulin signalling pathways including AKT and ERK [74]. IRS-1 has been shown to be down-regulated by miR-126 in the hepatic cancer cell line SK-Hep-1 [75]. miR-145 has also been shown to down-regulate IRS-1 in human colon cancer cells but it is unclear whether this is also the case in the liver [76]. Recently, the effect of the miR-29 family on the liver of diabetic mice has been explored [77]. Overexpression of these miRNAs, through adenovirus vectors, leads to improved fasting glucose and insulin tolerance in diet-induced obese mice. It is thought that this effect is a direct result of lowering hepatic gluconeogenesis via the decrease of PGC-1α and G6Pase protein levels.

Cardiac Muscle

Cardiac muscle, and muscle tissue in general, is a major site of glucose uptake. Therefore, many of the cardiac-specific miRNAs impact upon this process. miR-133, as discussed under the 'skeletal muscle' section (above), is seen to negatively regulate the expression of GLUT4, a major glucose transporter in cardiac muscle [78]. Indeed, when miR-133 is overexpressed in cardiomyocytes it lowers GLUT4 expression and the associated insulin-induced glucose uptake [55]. Conversely, miR-223 has been shown to increase the levels of GLUT4 within cardiomyocytes [79].

A cardiac-specific miRNA, miR-208a, has recently been demonstrated to impact upon energy homeostasis in mice by down regulating the expression of MED13, a component of the Mediator complex [80]. Inactivation of miR-208a, either by pharmacological means or the generation of miR-208a$^{-/-}$ mice, confers resistance to high-fat diet induced obesity and improves glucose tolerance and insulin sensitivity. Additionally, miR-208a$^{-/-}$ mice are resistant to cardiac stress-induced remodelling [81]. Further miRNAs, such as miR-378 [82], have been theorised to regulate energy homeostasis within cardiac cells, although more work is needed to validate the potential targets.

microRNA as biomarkers of disease

Many of the blood based biomarkers in current clinical practice are specific protein molecules, such as troponin for cardiovascular disease, prostate specific antigen (PSA) for prostate cancer and aminotransferases such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) for liver function. There are a number of challenges involved in the development of new protein biomarkers making the development process both time consuming and expensive. In addition to protein, DNA markers are used for conditions such as systemic lupus erythematosus, β-thalassemia and prenatal diagnosis of Down Syndrome.

The discovery of microRNAs in circulation opened an entire new area of biomarker discovery. Recently, the number of tumor-associated exosomes containing miRNAs was found to be in proportion to the stage of ovarian cancer and to the miRNA abundance in the cancer tissue respectively [83,84]. Exosomes,

which are ~40 nm wide vesicles released from mammalian cells, are used as shuttles to transport genetic information. These also include miRNA transfer to the surrounding cells, where they act as regulatory elements. Skog et al. demonstrated in vitro that RNA-molecules packaged into exosomes can enter human brain microvascular endothelial cells, as a mechanism for tumor propagation and invasion [85]. A recent study has also indicated that activated macrophages release oncogenic miRNAs in exosomes that increase invasiveness of breast cancer cells [86]. There are other reports indicating a role of exosomal miRNAs in immune response modulation [87,88]. All of these and several other reports (not included due to space constraints) have established the importance of exosomes in cell-cell communication by transferring nucleic acids (mRNAs and miRNAs) between cells [89]. However, the exact mechanism for microvesicle formation and nucleic acid incorporation in these exosomes is not well known. Protection in the extracellular milieu is not only conferred via exosomes, but also by binding to protein complexes, such as Argonaute2 [90]. These complexes protect miRNAs from endogenous RNase degradation. Gap junctions are also shown to be a way of miRNA transfer between adjacent cells and hence another means of cell-cell communication [91]. Several such studies [92,93] have clearly indicated that microRNAs can communicate with neighbouring cells via transport through gap junctions or can be released via incorporation into exosomes (Figure 2). On reaching the target cells, they regulate gene expression either via transcriptional repression (preventing the assembly of initiation complex), mRNA degradation or premature translation repression (ribosome dropoff). It is now well known that microRNAs found in serum appear to be released as a result of tissue injury. For example, it was demonstrated that miR-208, exclusively expressed in the heart, was measured in serum after cardiac tissue injury [94]. As demonstrated in Figure 2, a damaged cell will result in release of such tissue-specific microRNA containing vesicles into surrounding space that drains either to vascular / lymphatic systems. Similar mechanism involving the release of cellular contents (including miRNAs) can exist in conditions such as endothelial damage, diabetes and cancer, which involve the death/lysis of specific cell types.

There are a number of criteria that are required for a molecule to be considered as a good biomarker. Overall, microRNAs fulfil most/all of these criteria and can therefore be good biomarkers, if the specificity is identified.

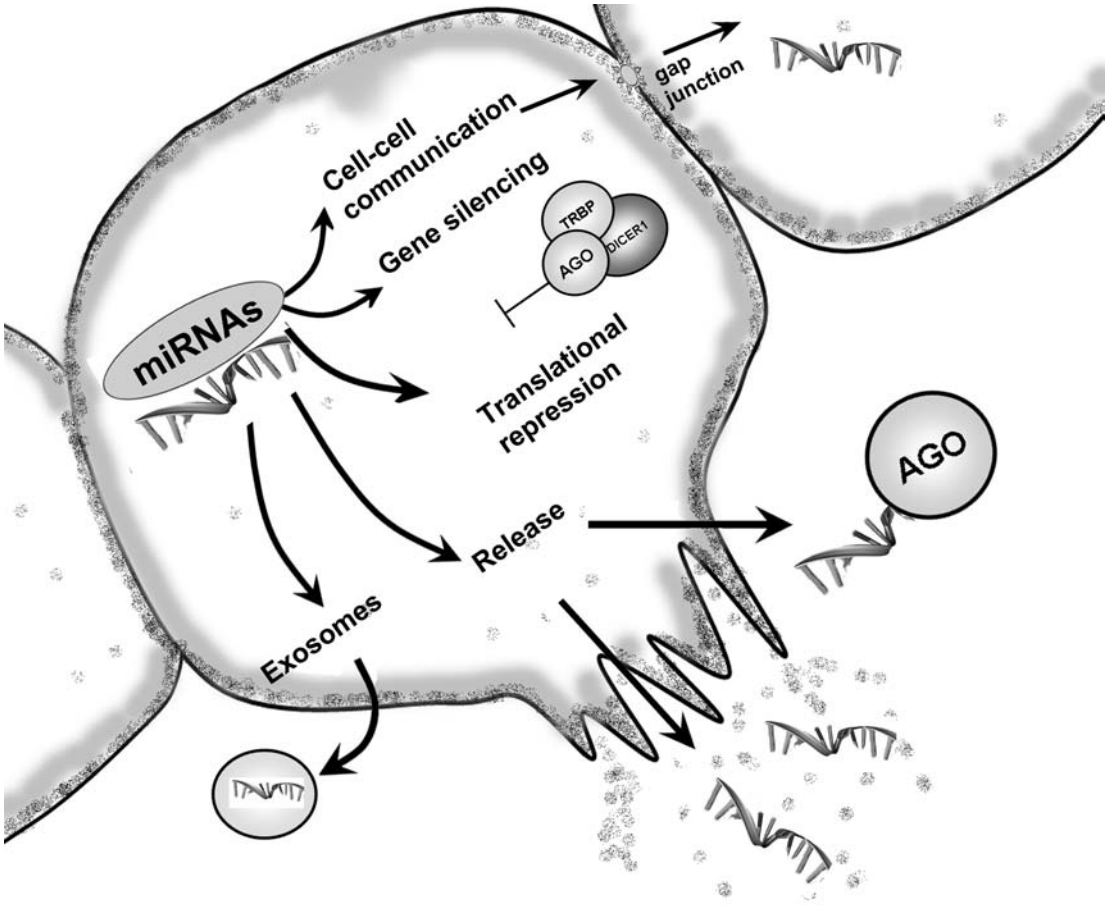

Figure 2. A schematic representing the modes of miRNA transfer. Cellular miRNAs can travel via gap-junctions or exosome-mediated transfer to surrounding cells. They can then bring about gene silencing at multiple levels (see text). microRNAs that are released from damaged cells (ruptured cell membrane above) can be detected in body fluids, including in the peripheral circulation, where they are known to be fairly stable due to their resistance to endogenous nucleases.

1. Specificity – the molecule should be specific to a diseased organ or tissue and able to differentiate pathologies. Additionally, the levels should be proportional to the degree of severity of pathology. There has been extensive research into the miRNA profiles of many different cancer types reviewed in [95] and there are a number of studies suggesting that differential serum miRNA levels are able to determine patient prognosis [96,97]. Such information is lacking for diabetes. Once a signature of microRNAs that offers the highest specificity for detection of beta cell death is available, monitoring of an individual's pancreatic health and clinical prognosis would be attainable.

2. Sensitive – there should be significant release upon the development of pathology in a short time frame. A number of studies have examined the expression of microRNAs in the serum of individuals following myocardial infarction (MI). miR-1 [98] and miR-208 [94] have been shown to be elevated in the hours following MI, returning to baseline levels in the following weeks. Another study was able to detect miR-208 in 20 patients following MI whilst troponin, the most commonly used biomarker of cardiac damage, was only detected in 17 of these individuals [99].

3. Stable – the molecule should be able to be detected in samples which may not have been stored in ideal conditions or have undergone fixation protocols. Serum miRNA has been shown to be resistant to multiple cycles of freeze-thawing as well as high pH [14,15]. miRNA profiles are also able to be determined in formalin fixed tissue [96,100,101].

4. Robust – able to be detected in a rapid, simple, accurate and inexpensive method. This is currently where the use of miRNAs as biomarkers is impeded. Expression of microRNAs is determined either by real-time PCR (qPCR), gene array or sequencing. Whilst all of these methods have their drawbacks, qPCR is currently the favoured approach [102-104].

5. Non-invasive – the molecule should be present in easily accessible fluid samples. To date miRNAs have been detected in a number of body fluids including serum and plasma [14-16], whole blood [105], urine [17,18], faeces [19,20], saliva [21,22] and breast milk [23].

In recent years, there have been reports of using miRNAs for prediction / early detection of diabetes using human samples. Zampetaki [106] and Kong et al [107] were among the first to describe differentially expressed miRNAs in diabetic populations. A study further correlated an increase of miR-27a and miR-320a with high blood glucose and associated these miRNAs with T2D [108]. Another very recent study has examined miR-375, an islet-specific miRNA, in streptozotocin-induced diabetic animal models, implicating a release of this miRNA due to beta cell death [109]. Nielsen et al described a microRNA signature (group of 12 miRNAs) that is differentially expressed between newly diagnosed T1D and control individuals [110]. They also correlated the expression of miR-25 to residual beta cell function as measured by circulating C-peptide levels. Few studies also analysed miRNAs in immune cells of T1D patients to understand if there are any changes associated with the miRNA profiles and initiation of autoimmune attack [111,112]. Though these reports clearly indicate the importance and potential of miRNAs as biomarkers, more detailed studies are needed for making use of these molecules in translational research.

Complications resulting from high glucose concentrations are unavoidable even in individuals with efficient glucose management. Early detection of the events leading to these complications will help in initiating preventive measures early and delay the debilitating renal / retinal / vascular damages. microRNAs are also potential biomarkers for early prediction of diabetic complications. Circulating miRNAs in serum as well as in urine are identified in different renal diseases [113-116]. In one study, circulating miRNAs (miR-16, miR-21, miR-210, miR-638) were found at lower levels in chronic kidney disease and their expression correlated with glomerular filtration rate [113]. Recently, differentially expressed miRNAs are reported in the urine samples from individuals at various stages of diabetic nephropathy [116]. Though there are no studies reporting circulating miRNAs as biomarkers for other diabetic complications, they are predicted to be important in early diagnosis of the disease pathology [117,118].

Many of the complications associated with diabetes are macro- or micro-vascular in origin and place diabetic individuals at risk of stroke, heart disease, blindness, limb amputations and renal failure. Hyperglycaemic damage to endothelial cells is often mediated via the altered expression of numerous factors, including miRNAs. Wang and colleagues were the first to investigate differential miRNA expression in response to the hyperglycaemic conditions of type 2 diabetic rats [119]. They identified that miR-320 was elevated in myocardial microvascular endothelial cells of diabetic rats, and that this miRNA impaired angiogenesis. Numerous miRNAs have since been identified with diabetic endothelial dysfunction, with the most notable being miR-125b [120], miR-221 [121,122], miR-222 [121] and miR-503 [123]. While these miRNAs have their own individual action, together they may provide a useful tool for the early diagnosis of diabetic vascular complications.

Concluding remarks

Whilst serum miRNA represent an attractive option for determining disease status in individuals, more research needs to be focussed on identifying specificity of islet-specific microRNAs before a test / kit for islet cell death measurement can reach the clinics. This would most likely involve taking a path from the cradle to grave by understanding miRs that are necessary for development and function of endocrine pancreas and relating these to the level of beta cell death measured through in vitro, animal models and human clinical studies. Studies performed to date have been largely based on a candidate microRNA profile but it is unclear whether these individual microRNAs can prove to be the most reliable measurement for identification of pancreatic beta cell death in humans. Most current studies involve few patient samples (<50) and are not sufficiently powered to identify any differences. The exception to this was the Bruneck cohort, which considered over 800 individuals, however, data was only

available on a small number who developed diabetes during the course of the study [106]. In order to determine a miRNA profile that is predictive of diabetes, a large number of individuals need to be included in a longitudinal study setting that involves at least 3 time points over several months – years. Another issue that needs to be addressed is the number of miRNA that need to be considered in order to accurately assess an individual's risk of disease. To date, ~ 1 in 3 diabetic individuals were incorrectly assessed on the expression of 5 [106] or 7 [107] miRNA in the serum or plasma, clearly suggesting that the number of miRNA considered needs to be reassessed for their specificity, suitability and stability.

A major drawback in the use of miRNAs as biomarkers is the length of time required for sample processing. In our hands, this process from RNA isolation to data generation takes approximately 8 hours. In most labs, this is labour intensive process but has several potential stages for automation wherein robotics and micro- / nano-fluidics can be integrated to improve the throughput and sensitivity of the assay. However, significant differences can be introduced between labs that use different chemistries (eg: SybrG Vs TaqMan) or platform for detection (conventional plates with 5 to 10ul reaction mix Vs Fluidigm or Open array-based platform with a few nanolitres of total reaction volume). The dynamic range of expression of microRNAs gets severely limited when 10 microlitre reactions are sized down to 33 nanolitres for increasing the throughput. So, an optimal platform for detection of microRNA signatures and cleaner chemistries (TaqMan primer-probes rather than SybrGreen) are amongst the few critical issues that need to be put into practice. Once uniform practices and standard protocols are achieved, the expression levels of less abundant miRNAs will not be missed out. Users should also carefully consider any pre-amplification steps and often use multiple technical replicates, appropriate internal controls and inter-assay positive / negative controls to check for inter-assay and intra-assay variability. It is likely that as technology improves and sequencing technologies become more readily applicable to clinical laboratory settings, detection of new miRNAs through discovery analysis would become more easy to achieve.

Currently, it is difficult to understand the potentially multiple roles that miRNAs play in circulation as well as their origin in the circulation. It is crucial to select a panel of miRNAs that are highly disease specific and should not mislead to other organ damages. As discussed earlier, determining the specificity of miRs towards a particular biology, in this case beta cell death, is important. One approach is to understand the miRNAs that are crucial to development of the endocrine pancreas. Such miRs would be potentially involved in function and maintenance of endocrine pancreatic cells and can therefore be used as potential biomarkers of beta cell death. An example of this is the recent presentation by Erener, S. et al [111] of miR-375 as a biomarker of beta cell death (in mice). Using such a signature of multiple miRNAs involved in beta cell development ("cradle") for identification of beta cell death ("grave") would offer a better handle for assigning a microRNA biomarkers signature in diabetes prediction. It is attractive to suggest that these miRNAs act as intercellular signalling molecules communicating the degree of insulin resistance between target tissues and the pancreas or the pancreas signalling to target tissues to alter the expression levels of components of the insulin signalling pathway. However, there is currently insufficient data to support these hypotheses. It will be also interesting to consider and test circulating miRNAs as a response to treatments for diabetes including different drugs as well as islet transplantation. The coming years would unravel a number of high throughput and systematic clinical studies that will establish the role of microRNAs in prediction and / or induction of diabetes.

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