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Diabetes mellitus, a microRNA-related disease?

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Abstract

Diabetes mellitus is a complex disease resulting in altered glucose homeostasis. In both type 1 and type 2 diabetes mellitus, pancreatic β-cells are unable to secrete appropriate amounts of insulin to regulate blood glucose level. Moreover in type 2 diabetes mellitus, altered insulin secretion is combined with a resistance of insulin-target tissues, mainly liver, adipose tissue and skeletal muscle. Both environmental and genetic factors are known to contribute to the development of the disease. There is growing evidence that microRNAs (miRNAs), a class of small non-coding RNA molecules, are involved in the pathogenesis of diabetes. miRNAs function as translational repressors and are emerging as important regulators of key biological processes. Here, we review recent studies reporting changes in miRNA expression in tissues isolated from different diabetic animal models. We also describe the role of several miRNAs in pancreatic β-cells and insulin-target tissues. Finally, we discuss the possible utilisation of miRNAs as blood biomarkers to prevent diabetes development and as tools for gene-based therapy to treat both type 1 and type 2 diabetes mellitus.

Running head: Involvement of microRNAs in diabetes

Abbreviations

miRNA : microRNA

IL-1β : Interleukin-1β

TNF- α : tumor necrosis factor- α

IFN- γ: Interferon- γ

3'UTR: 3'untranslated region

mRNA: messenger RNA

NOD mice: non obese diabetic mice

GK rats: Goto-Kakizaki rats

RNase: ribonuclease

AAV: adeno-associated vector

Introduction

Diabetes mellitus is the most common metabolic disorder worldwide (1). Due to population ageing and increasing trends towards obesity and sedentary lifestyles, the number of affected individuals is rising at worrisome rates and is expected to double within the next twenty years. In industrialized countries, diabetes is already the leading cause of blindness, renal failure and lower limb amputations and is a major risk factor for cardiovascular disease and stroke. The pancreatic β-cell and insulin, its secretory product, play a central role in the pathophysiology of this disease. Indeed, the release of appropriate amounts of insulin in response to circulating levels of glucose and other nutrients is essential to achieve blood glucose homeostasis. Thus, insufficient supply of this hormone leads to hyperglycemia and progression to overt diabetes. Type 1 diabetes mellitus results from the lack of insulin production due to autoimmune destruction of pancreatic β-cells. Proinflammatory cytokines produced by infiltrating leucocytes, including interleukin-1 β (IL-1β), tumor necrosis factor-α (TNF- α) and interferon-γ (IFNγ), play a central role in this process. Type 2 diabetes mellitus (more than 90% of cases) is characterized by the release of inappropriate amounts of insulin to maintain blood glucose concentration within normal physiological ranges. The pathogenesis of type 2 diabetes involves a combination of genetic and environmental/live style factors and is frequently associated with obesity (2). Visceral obesity diminishes insulin sensitivity of target tissues and the β-cell mass needs to expand to compensate for the increase in the metabolic demand. In about one third of obese individuals this adaptive process fails, resulting in relative insulin deficiency and development of type 2 diabetes.

During the last few years, considerable efforts have been devoted to the elucidation of the molecular mechanisms underlying β-cell failure and the development of insulin resistance. Global mRNA profiling has provided valuable information concerning potential changes in gene expression occurring in β-cells and insulin target tissues in the context of diabetes (for review see (3)). It is now becoming clear that microRNAs (miRNAs), a new family of regulatory molecules acting downstream or in conjunction with transcription factors, make an important contribution to alterations in gene expression observed in dysfunctional β-cells and in insulin resistant tissues and are likely to be involved in the development of diabetes.

microRNAs as novel regulators of gene expression

microRNAs are small non-coding RNA molecules of 21-23 nucleotides that regulate gene expression (4). They were first discovered in *Caenorhabditis elegans* in 1993 (5-6) and, later on, in vertebrates and plants. Today, thousands of miRNAs have been identified, making them one of the most abundant classes of gene regulatory molecules in multicellular organisms. These non-coding RNAs behave as specific gene silencers by base pairing to 3'untranslated regions (UTR) of target messenger RNAs (mRNAs). miRNAs are generally assumed to exert their action by inhibiting translation (4) although a recent study, in which the level of miRNAs was artificially modified, suggests that these regulatory molecules may control gene expression mainly by affecting mRNA stability and degradation (7).

Most mammalian miRNAs are transcribed by RNA polymerase II as long precursor molecules containing stem-loop structures. This primary transcript is cleaved

by the Microprocessor complex containing the RNase III-type enzyme Drosha and the DGCR8/Pasha protein yielding a ≈ 70 nucleotide hairpin-structured precursor (pre-miRNA) (8). The pre-miRNA is transported into the cytoplasm and cleaved by another RNase III enzyme called Dicer to generate a ≈ 22 nucleotide RNA duplex (9). Upon separation of the two strands, the guide strand binds to an Argonaute protein and is incorporated into the RISC complex (RNA-induced silencing complex) allowing the identification of complementary sites within the 3' UTRs of target mRNAs (9). Although most miRNAs are generated by this canonical pathway, some of these non-coding RNAs are produced independently from Dicer (10) and others, called mirtrons, are generated via splicing of short hairpin introns (11).

Based on computational algorithms, around 60% of human transcripts contain potential miRNA-binding sites within their 3' UTRs (12). The most important determinant of target recognition is the presence of a "seed sequence" capable of pairing to nucleotides 2-8 of the miRNA (13). However, target pairing to the center of some miRNAs has also been reported (14). A single miRNA can potentially bind more than hundred target mRNAs and multiple miRNAs can cooperate to fine-tune the expression of the same transcript (15-17). Although we are only beginning to appreciate the extraordinary regulatory potential of these small non-coding RNAs, there are no doubts today that they play key roles in a wide variety of physiological processes including cell proliferation, apoptosis and tissue differentiation. Moreover, deregulation in miRNA biogenesis and function have been shown to contribute to the development of human diseases. Thus, it is not surprising that the discovery of an entirely new class of regulators

of gene expression prompted several research teams to investigate the potential involvement of miRNAs in the development of diabetes and its complications.

Approaches to assess an involvement of microRNAs in diabetes

To study the potential involvement of miRNAs in diabetes mellitus a wide range of *in vitro* and *in vivo* strategies can be envisaged. A common and straight forward approach is to expose cell lines or isolated primary cells to pathophysiological conditions mimicking the diabetic milieu, including elevated concentrations of glucose and/or free fatty acids, or cytokines. This permits a detailed analysis of the signaling pathways leading to changes in miRNA expression, the study of the functional impact of individual miRNAs and the elucidation of the molecular mechanisms underlying their action. Another approach is to correlate the alterations in miRNA expression with the development of diabetes in animal models or, whenever possible, in diabetic patients. Several diabetes animal models either issued from inbreeding or produced by molecular biological techniques have specific features (exhaustively described in (18) and (19)), that make them appropriate models of type 1 or type 2 diabetes.

Non-obese (NOD) mice spontaneously develop a form of disease that closely resembles type 1 diabetes in humans. Starting around 6 weeks after birth the pancreatic islets of NOD mice are progressively surrounded by immune cells. At the age of 13-14 weeks immune cells penetrate the islets and selectively kill β-cells, resulting in elevation of blood glucose levels and development of diabetes. Another widely used model of type 1 diabetes consists in injecting multiple doses of streptozotocin, a naturally occurring compound that is particularly toxic to the insulin-producing β-cells. The most often used

models of type 2 diabetes are the *ob/ob* and *db/db* mice lacking leptin and leptin receptor, respectively, and mice or rats fed for several weeks with a high fat diet (also known as diet-induced obesity). These animals become severely obese and insulin resistant, finally leading to β-cell failure and hyperglycemia. Goto-Kakizaki (GK) rats, a non-obese Wistar substrain which develops insulin resistance and mild hyperglycemia early in life, constitutes another popular model of type 2 diabetes mellitus. In the following sections we will review the data obtained in these experimental models (see Table1 for a complete list of miRNA changes reported in the different diabetic animal models) and highlight the characteristics and the functional roles of the miRNAs that were associated with the development of diabetes (summarized in Figure 1).

Role of microRNAs in insulin-secreting cells

miRNAs are necessary for proper pancreatic islet development as demonstrated in a mouse model lacking the miRNA processing enzyme Dicer selectively in the pancreas (20). Pancreatic specific Dicer-null mice display gross defects in all endocrine pancreatic lineages, with insulin-producing β-cells being the most affected. Only a few miRNAs have been identified to be preferentially expressed in islets from mice and human and their roles in islet development are unknown (21-23), except for miR-375 (24). The expression of miR-375, one of the most abundant miRNAs present in islet cells, is under the control Pdx-1 and NeuroD1, two critical transcription factors for the development of the endocrine pancreas (25). In agreement with these observations, targeted inhibition of miR-375 in zebrafish resulted in major defects in pancreatic islet development (24).

In mature β-cells miR-375 plays a negative regulatory role in glucose-induced insulin secretion (26). Overexpression of miR-375 does not affect ATP production, nor the rise in intracellular Ca^{2+} triggered by glucose, but affects a late step in the insulin secretory pathway. This effect was at least partly attributed to a reduction in the expression of myotrophin, a protein involved in insulin-granule fusion (26). Later on, miR-375 was found to be involved also in the control of insulin gene expression (27). The contribution of this miRNA to blood glucose homeostasis has been confirmed in miR-375 knockout mice (28). These mice are hyperglycemic and glucose intolerant despite normal insulin secretion and clearance. This phenotype results from an increase in α-cell number and a consequent elevated plasma glucagon level leading to enhanced gluconeogenesis and hepatic glucose output. In contrast, the β-cell mass of these mice is decreased as a result of impaired proliferation. Interestingly, genetic deletion of miR-375 in *ob/ob* mice profoundly diminished the capacity of the endocrine pancreas to compensate for insulin resistance and resulted in a severely diabetic state (28). Therefore, miR-375 is important for several features of β-cells, such as insulin expression and secretion and β-cell proliferation and adaptation to insulin resistance.

Beside miR-375, other miRNAs participate in the fine tuning of insulin secretion by modulating the level of key components of the exocytotic machinery (see Figure 2). We observed that a rise in miR-9 relieves an inhibitory control on the expression of Granuphilin, a negative regulator of insulin exocytosis, by targeting the transcription factor Onecut 2 resulting in defective insulin release in response to stimuli (29). miR-96 overexpression has a similar impact on Granuphilin expression and insulin secretion, but

in this case the effect is independent from Onecut2 (30). Moreover, miR-96 decreases the expression of Noc2, a Rab GTPase effector that is necessary for insulin exocytosis. In the same study, overexpression of miR-124a was shown to modulate directly or indirectly the expression of several components of the exocytotic machinery. This correlated with an increase in basal insulin release and a decrease in insulin exocytosis in the presence of secretagogues (30). Consistent with these observations, miR-124a was found to induce an elevation of intracellular Ca^{2+} under basal conditions and a reduction in stimulatory conditions (31). In the latter study, Foxa2 was identified as a direct target of miR-124a. Indeed, downstream Foxa2 target genes, including the ATP-sensitive K^+ channel subunits Kir6.2 and Sur-1, and the transcription factor Pdx-1 were down-regulated by miR-124a. More recently, miR-130a, miR-200 and miR-410 were also described to be involved in the regulation of insulin secretion (32).

The studies cited above used molecular tools to enhance or reduce the expression of specific miRNAs in attempt to highlight a potential involvement in the control of pancreatic β-cell functions. With the advent of techniques to perform global miRNA profiling it became possible to systematically search for miRNAs potentially contributing to β-cell dysfunction under physiopathological conditions.

During the initial phases of type 1 diabetes pancreatic islets are infiltrated by immune cells and β-cells become exposed to pro-inflammatory mediators such as IL-1β, TNFα and IFNγ. Chronic exposure to these cytokines has a detrimental impact on pancreatic β-cell functions leading to a reduction in insulin content, defects in insulin secretion and sensitization to apoptosis. Global miRNA profiling of an insulin-secreting cell line treated with pro-inflammatory cytokines revealed a strong induction of miR-21,

miR-34a and miR-146 (33). Similar effects were observed in freshly isolated human pancreatic islets. Moreover, the level of these miRNAs was increased during development of pre-diabetic insulitis in pancreatic islets of NOD mice. Interestingly, blockade of these miRNAs using antisense molecules prevented the reduction in glucose-induced insulin secretion provoked by prolonged exposure to IL-1β and protected the β-cells from cytokine-triggered cell death. The molecular mechanisms involved in the protective action of the anti-miRs were partially elucidated. Indeed, anti-miR34 pretreatment prevented the drop in the expression of the anti-apoptotic protein Bcl2 that is normally observed upon prolonged exposure to cytokines. In contrast, blockade of miR-146a activity permitted to attenuate c-Jun induction triggered by cytokines, partially explaining the amelioration of the survival of β-cells pretreated with anti-miR146.

Chronic exposure to the free fatty acid palmitate mimics the adverse environmental conditions that promote β-cell failure. This results in defective glucose-induced insulin secretion and sensitization to apoptosis. We found that prolonged exposure of insulin-secreting cell lines or pancreatic islets to palmitate leads to an increase of miR-34a and miR-146 (34). Moreover, the level of these two miRNAs is abnormally elevated in islets isolated from diabetic *db/db* mice. The rise in miR-34a expression resulted from the activation of the p53 pathway and led to sensitization of the β-cells to apoptosis and impairment in nutrient-induced insulin secretion (34). These effects were associated with a reduction in the expression of the anti-apoptotic protein Bcl2 and of VAMP-2, one of the central players in insulin exocytosis. Blockade of miR-34a or miR-146 activity partially prevented palmitate-induced β-cell apoptosis but was insufficient to restore normal secretory activity (34).

Hyperglycemia is a hallmark of both type 1 and type 2 diabetes. Prolonged exposure of the pancreatic β-cell line MIN6 to high glucose resulted in changes in the expression of a large set of miRNAs (35). Among them, miR-124a, miR-107 and miR-30d were upregulated in hyperglycemic conditions whereas miR-296, miR-484 and miR-690 were downregulated. Overexpression of miR-30d caused a reduction in insulin gene expression, suggesting a possible involvement of this miRNA in defective insulin biosynthesis under diabetic conditions. Exposure of insulin-secreting cell lines and primary rat islets to elevated glucose concentrations resulted in a reduction in the expression miR-375 and a concomitant rise in the level of phosphoinositide-dependent protein kinase-1 (PDK1), a direct target of the miRNA that plays a central role in the control of insulin gene expression and DNA synthesis (27). Interestingly, the level of miR-375 is diminished in the islets of fed diabetic GK rats, suggesting that this glucose-mediated regulatory mechanism may also operate *in vivo* (27).

Role of microRNAs in insulin target tissues

Blood glucose homeostasis requires an appropriate balance between the amount of insulin released by β-cells and the sensitivity of the target tissues to the action of the hormone. A variety of physiological and pathological events, including obesity, pregnancy and stress can diminish insulin sensitivity of peripheral tissues, like liver, adipose tissue and skeletal muscle, leading to a condition known as insulin resistance. The decreased sensitivity of the tissues to insulin action is normally overcome by an increase in insulin secretion. Failure to compensate for insulin resistance leads to chronic hyperglycemia and progression to type 2 diabetes. The precise molecular mechanisms

underlying insulin resistance is still incompletely understood but a potential involvement of miRNAs has been envisaged (see Figure 1 for an overview of miRNAs implicated in the regulation of insulin-target tissue functions).

Liver

Deregulation of miRNA expression in liver may influence glucose homeostasis and promote the development of diabetes. Selective deletion of the miRNA processing enzyme Dicer in liver early after birth resulted in mild hyperglycemia in the fed state and severe hypoglycemia in fasting state due to depletion of glycogen storage (36). Hepatocytes from Dicer-null mice displayed an increase in apoptosis, compensatory proliferation and elevation of lipid accumulation leading to steatosis. Levels of miR-122, miR-148a, miR-192 and miR-194, four miRNAs highly enriched in liver, were down-regulated in Dicer-null hepatocytes highlighting their possible role in regulating glucose and lipid metabolism.

MiR-122 is the most abundant miRNA in the liver (37) and its role in the control of hepatic functions has been studied by different groups (38-40). Intravenous injection of antagomir-122, a cholesterol-conjugated antisense oligonucleotide, in mice resulted in an almost complete depletion of miR-122 in hepatocytes leading to a decrease in hepatic fatty-acid and cholesterol synthesis rates and a concomitant decrease in plasmatic cholesterol level. Similar results were obtained in African green monkeys (38). Taken together, these results demonstrate an important role for miR-122 in the regulation of circulating cholesterol. Moreover, hepatic miR-122 inhibition in a model of diet-induced obesity in mouse led to a reduction of plasmatic cholesterol and a significant

improvement of liver steatosis, suggesting miR-122 inhibition as a possible therapeutic approach to reduce cholesterol levels.

Global profiling of miRNAs present in liver and adipose tissues revealed a marked upregulation of miR-125a in hyperglycemic GK rats compared to normoglycaemic Brown-Norway (BN) strains (41). Computational predictions suggest that miR-125a can potentially target genes related to glucose and lipid metabolism. Thus, upregulation of miR-125a may contribute to deregulation in gene expression leading to insulin resistance.

Several other miRNAs have been reported to be differently expressed in liver of diabetic animal models, but their precise role in the regulation of liver function remains to be determined (42-44) (see table 1 for a complete list). Among them, miR-335 expression was upregulated in different obese mice models including *ob/ob* and *db/db* mice, which correlates with increased liver and body weight and hepatic triglyceride and cholesterol levels (45). Further studies will be necessary to understand the regulation of miRNAs in the liver during the onset of diabetes and to determine their role on the regulation of glucose homeostasis by the liver.

Adipose tissue

In adipose tissue, insulin stimulates lipogenesis, a process that transforms blood glucose into fatty acids to efficiently favor energy storage. Esau et al. were the first to report involvement of miRNAs in adipose cell biology (46). They identified miR-143 among several miRNAs that were upregulated during human pre-adipocyte differentiation. The role of miR-143 in adipocyte differentiation was then confirmed in a second report (47).

Both miR-103 and miR-143 were up-regulated during *in vitro* and *in vivo* adipogenesis. Overexpression of these two miRNAs in pre-adipocytes increased the expression of adipogenesis markers and led to triglyceride accumulation. Interestingly, miR-103 and miR-143 are downregulated in adipocytes from *ob/ob* mice, a model of insulin resistance and obesity. The mechanism remain to be determined, however, the inflammatory pathway is possibly involved (47).

He et al. discovered that miR-29 family members (miR-29a, b and c) are upregulated in the adipose tissue and skeletal muscles of diabetic GK rats (48). Elevation of miR-29a and miR-29b could be reproduced *in vitro* by incubating 3T3-L1 adipocytes in the presence of high glucose and insulin. These conditions are known to induce insulin resistance, pointing to a possible role for miR-29 in this process in adipocytes. Indeed, overexpression of miR-29 in 3T3-L1 adipocytes decreased insulin-stimulated glucose uptake presumably by inhibiting insulin signaling through AKT phosphorylation. Taken together, these results suggest that miR-29 could mediate part of mechanisms leading to insulin resistance in type 2 diabetes. In a similar study, Herrera et al investigated miRNA profiling in insulin target tissues from hyperglycemic GK rats, intermediate glycemic Wistar Kyoto rats and normoglycemic Brown-Norway rats (43). Among the 29 miRNAs differently expressed between the three rat strains, five of them correlated with the glycemic phenotype of the strain: miR-195 and miR-103 in liver, miR-222 and miR-27a in adipose tissue, and miR-10b in muscle. These results suggest a role for those 5 miRNAs in the pathogenesis of type 2 diabetes, but their function in the regulation of insulin-target tissues remains to be elucidated.

Skeletal muscle

Skeletal muscle is a major site of glucose disposal, where insulin favors glucose storage in glycogen form. So far only few studies analyzed changes in miRNA expression in human tissues in the context of diabetes. Granjon et al. investigated the impact of insulin on skeletal muscle's miRNA expression by analyzing muscle biopsies taken from healthy subjects before and after 3 hours of euglycemic-hyperinsulinemic clamp (49). Insulin down-regulated 39 miRNAs, including miR-1, miR-206 and miR-133a well known for their role in muscle development and growth and miR-29a and miR-29c highly enriched in insulin sensitive-tissues (50). Downregulation of miR-1 and miR-133a by insulin was found to be mediated by the transcription factors SREBP-1c and MEF2C. The effect of insulin on these two miRNAs was altered in skeletal muscle of type 2 diabetic patients, which corroborates the impairment of MEF2C and SREBP-1c stimulation in the same conditions (49, 51). In non insulin-stimulated condition, Granjon et al. did not observe significant differences in miR-1 and miR-133a expression in skeletal muscle of diabetic versus healthy subjects. The results from this study are controversial since other laboratories could not reproduce some of the key findings. Indeed, Nielsen et al. using a larger number of subjects did not detect any changes in miR-1, miR-133a and miR-206 expression in response to a 3 hours euglycemic-hyperinsulinaemic clamp (52), despite measuring a decrease in the level of these miRNAs upon endurance training. A decrease in the expression of several miRNAs upon endurance training improving insulin sensitivity, including miR-1 and miR-133a, was confirmed in an additional study (53). More recently, Gallagher et al. detected alterations in about 60 miRNAs in lean, drug free, type 2 diabetic subjects (54). Interestingly, many of these changes appeared to occur

prior to the onset of diabetes. In the latter study, miR-133a expression was reduced by five-fold in type 2 diabetic patients and altered levels of this miRNA correlated with higher fasting glucose levels and other important clinical parameters including HOMA1 and HbA1c. Gene Ontology profiles of the highest ranked targets of the miRNAs affected in type 2 diabetic patients highlighted a significant enrichment in genes involved in metabolic and developmental processes.

Similar studies were carried out in GK rats, a model of insulin resistance and diabetes. miRNA profiling in skeletal muscle of GK rats was compared to non-diabetic control rats (43, 48, 55). A total of 25 changes were reported by these 3 studies (see Table 1), but only miR-130a upregulation was commonly found in two on them. Also, only miR-29 family was investigated for its role in the insulin resistance, as discuss above (48). Finally, neither miR-1 nor miR-133a expression was reported to be altered in GK rats. More complete studies are now necessary to determine the mechanisms linking hyperglycemia, miRNA deregulation and insulin resistance in the context of diabetes.

The role of miR-1 and miR-133a in the regulation of glucose homeostasis was also investigated *in vitro*. MiR-133a/b has been shown to decrease GLUT4 expression, leading to a reduction of insulin-induced glucose uptake in cardiomyocytes (56). This effect was proposed to be mediated by inhibiting the expression of the Krüppel-like transcription factor KLF15, a direct target of miR-133a/b. Human ether-a-go-go (HERG) and KCNQ1 are two other identified targets of miR-133a/b. Both are involved in the formation of potassium (K^+) current channel in heart and could be involved in the long

QT syndrome in diabetic patients (57-58). miR-1 regulation and function were also first investigated in cardiomyocytes, but results were confirmed in C2C12 myotubes. Exposure of cardiomyocytes to high glucose induces apoptosis with a concomitant elevation of miR-1 expression (59). Two identified targets of miR-1, IGF-1 and IGF-1 receptor (59-60), were involved in this process since glucose-induced mitochondria dysfunction, cytochrome c release and apoptosis were prevented by IGF-1 overexpression. A feedback loop between miR-1 and IGF-1 was observed in both C2C12 myotubes and cardiomyocytes, in which IGF-1 regulated miR-1 expression via the transcription factor Foxo3a (60).

microRNAs as potential biomarkers for diabetes

Recent studies reported detection of miRNAs in blood and other body fluids (61-64). Levels of miRNAs in serum samples from humans and other animals were found to be stable, reproducible, and consistent among individuals (61-62). To verify if plasma miRNAs were coming only from blood cell disruption, plasma miRNAs were compared to blood-cell miRNAs. A majority of miRNAs were found to be common, but several were only found in serum (61). These results suggest that at least part of the circulating miRNAs originate from cells located outside of blood vessels.

The presence of miRNAs in the blood may at first seem surprising since serum contains ribonucleases (RNase). However, plasma miRNAs are resistant to RNase digestion and to several harsh laboratory conditions (61-62). As evidenced by many reports, miRNAs are not present in the blood in native form but are released in microvesicular structures, such as exosomes or apoptotic bodies, and circulate inside

these membrane-delimited vesicles that protect them from degradation. Circulating microvesicles are thought to favour cell-to-cell communication, but also to exchange genetic information between healthy and injured cells and tissues (65-66). In fact, specific tumor miRNAs were identified in serum from cancer patients (67). Therefore, miRNA profiling from microvesicles circulating in the blood could reflect the physio-pathological state of an individual.

Chen et al. (61) compared serum miRNA profiles from diabetic and healthy subjects and identified 65 commonly and 42 differently expressed miRNAs. Since no detail was given on miRNA identity and on the clinical parameters of diabetic patients, it is hard to draw conclusions from these observations. However, these results suggest that plasma miRNAs could vary according to healthy or disease state, making them attractive new biomarkers. In the context of diabetes, biomarkers could be particularly useful to prevent the development of the disease since diabetic patients are generally detected too late. The recent study of Zampetaki et al. (68) highlighted the promising utilisation of plasma miRNAs as biomarkers. The authors determined miRNAs levels in plasma samples obtained from a large prospective population-based study including more than 800 individuals. Among them, 80 subjects were type 2 diabetic patients and 19 individuals that were normoglycemic at the beginning of the study developed type 2 diabetes during the 10 years survey. The comparison of pooled plasma miRNAs from diabetic subjects with age- and sex-matched controls led to the identification of 13 miRNAs differently expressed in diabetic patients. Among them, the expression of 5 miRNAs, miR-15a, miR-28-3p, miR-126, miR-223, and miR-320, was already altered before the manifestation of the disease. The determination of the level of this cluster of 5

miRNAs was sufficient to identify 70% of the type 2 diabetic patients. The diabetic subjects escaping detection had lower fasting glucose levels or were patients will well-controlled diabetes. Interestingly, 52% of the normoglycemic individuals that developed diabetes during the following 10 years were already classified in the diabetic group. Taken together, these results suggest for the first time the existence of a unique miRNA blood signature that could help with other factors distinguishing between individuals with prevalent or incident diabetes from healthy controls.

The validity of this miRNA signature as a tool to diagnose type 2 diabetes remains to be confirmed by other large independent studies. Also, similar investigations should be carried out to determine whether the same or other miRNA signatures can efficiently recognize individuals at risk to develop type 1 diabetes. This would complement the information obtained by the measurement of other biomarkers favouring the identification of subjects that would benefit for treatments aiming at preventing auto-immune degradation of pancreatic β-cells. Nonetheless, blood level of miRNAs is a promising strategy to prevent diabetes development.

MicroRNAs: promising targets for gene-therapy based treatment?

As outlined above, deregulation of miRNA functions has been linked to several human disorders, including diabetes. Whether the disease is a direct cause of altered miRNA expression or this altered expression occurs as a consequence of the pathological state is still unknown. Nonetheless, restoration of miRNA functions to normal levels is an attractive therapeutic strategy.

Different chemically modified oligonucleotides have been used *in vitro* to modulate miRNA expression (for a complete review, see (69)). Among them, miRNA-mimic oligonucleotides increase the expression of a miRNA of interest. Locked nucleic acid (LNA)-antimiRs, antagomirs, and morpholinos are efficient inhibitors of miRNA functions and are effective also *in vivo* (38, 40, 70). The use of oligonucleotides for gene therapy has to face different obstacles because these molecules are relatively unstable and their effects are transient. Therefore, they have to be injected at high doses and necessitate repeated deliveries. Moreover, methods permitting cell-specific delivery of these molecules are not yet available.

The generation of vectors in which miRNA-mimics are placed under the control of an inducible promoter permits to achieve a temporally controlled elevation of miRNA expression *in vivo* (71). Moreover, the use of specific promoters permit the targeting of the miRNA in a particular cell-type. In this context, adeno-associated virus (AAV) vectors have shown promising results for gene therapy. Different capsid serotypes have been tested to improve AAV-gene transduction in specific target tissues. AAV packaged in capsid serotypes 6, 8 and 9 are largely directed to skeletal muscle, liver and heart, respectively, following systemic delivery in small animal models (72). The use of insulin promoter to control gene expression in an AAV8 vector enabled specific delivery of IL-4 to pancreatic β-cells permitting to prevent their destruction and delaying the appearance of diabetes in NOD mice (73). Following promising preclinical studies in small animals, 50 human clinical trials using AAV-mediated gene transfer have been approved (72).

So far AAV vectors have been used mostly for the delivery of protein-coding genes but Kota *et al*. (74) took advantage of this system to overexpress a miRNA. In their

study, miR-26a, which is highly expressed in diverse healthy tissues but decreased in liver cancer cells, was packaged in AAV8 serotypes and injected only once in mouse tail-vein. Animals injected with AAV8.miR26a showed an important protection from hepatic cancer progression with no sign of toxicity. Taken together, these results suggest that AAV-miRNA strategy is a very promising therapeutic approach for miRNAs highly expressed, and therefore tolerated, in normal tissues and underexpressed in the disease state.

Alternative strategies to chemically modified oligonucleotides have also been designed to reduce miRNA functions *in vivo*. Currently, the most promising approach involves the use of "miRNA sponges". MiRNA sponges are artificial miRNA decoys that bind and hold native miRNA, in order to compete with natural targets and then create a loss-of-function of the miRNA of interest. MiRNA sponges contain multiple binding sites directed against one particular miRNA or a miRNA seed family. They are particularly efficient *in vitro* (75). Recent studies have used this strategy *in vivo* to stably decrease the activity of miR-31 or of the miR-15a/16-1 cluster and to investigate their role in cancer development (76-77). Since those two studies used miRNA sponges to favour cancer development, the use of miRNA sponges as a therapeutic treatment still has to be investigated. Their long-term effects and tissue-specific delivery will also have to be determined. Nonetheless, the expression of miRNA sponges in an AAV vector delivery system is an attractive strategy for preclinical gene-therapy studies.

Conclusions

MiRNAs are emerging as key regulators of gene expression. Type 1 and type 2 diabetes are associated with alterations in the level of several miRNAs in insulin-secreting cells as well as in insulin-target tissues. We already have at our disposal molecules capable of efficiently modulating the expression of miRNAs *in vitro* and *in vivo*. Unfortunately, at present approaches to selectively target these molecules in a cell-specific manner are missing. The development of techniques allowing *in vivo* delivery of miRNA mimics or anti-miRs specifically in β-cells and insulin-target tissues could permit to correct the level of key miRNAs under diabetic conditions and open the way to new strategies for treating the disease. However, safety concerns and cost effectiveness will need to be carefully evaluated to determine whether approaches aiming at restoring miRNA levels are appropriate for the treatment of a chronic disease such as diabetes. In addition to alteration in insulin-producing cells and insulin target tissues, diabetes is also associated with distinct changes in the blood miRNA profile. In the future, measurements of the level of specific miRNAs may become useful tools to identify individuals at risk for developing type 1 or type 2 diabetes, hopefully preventing the development of the disease.

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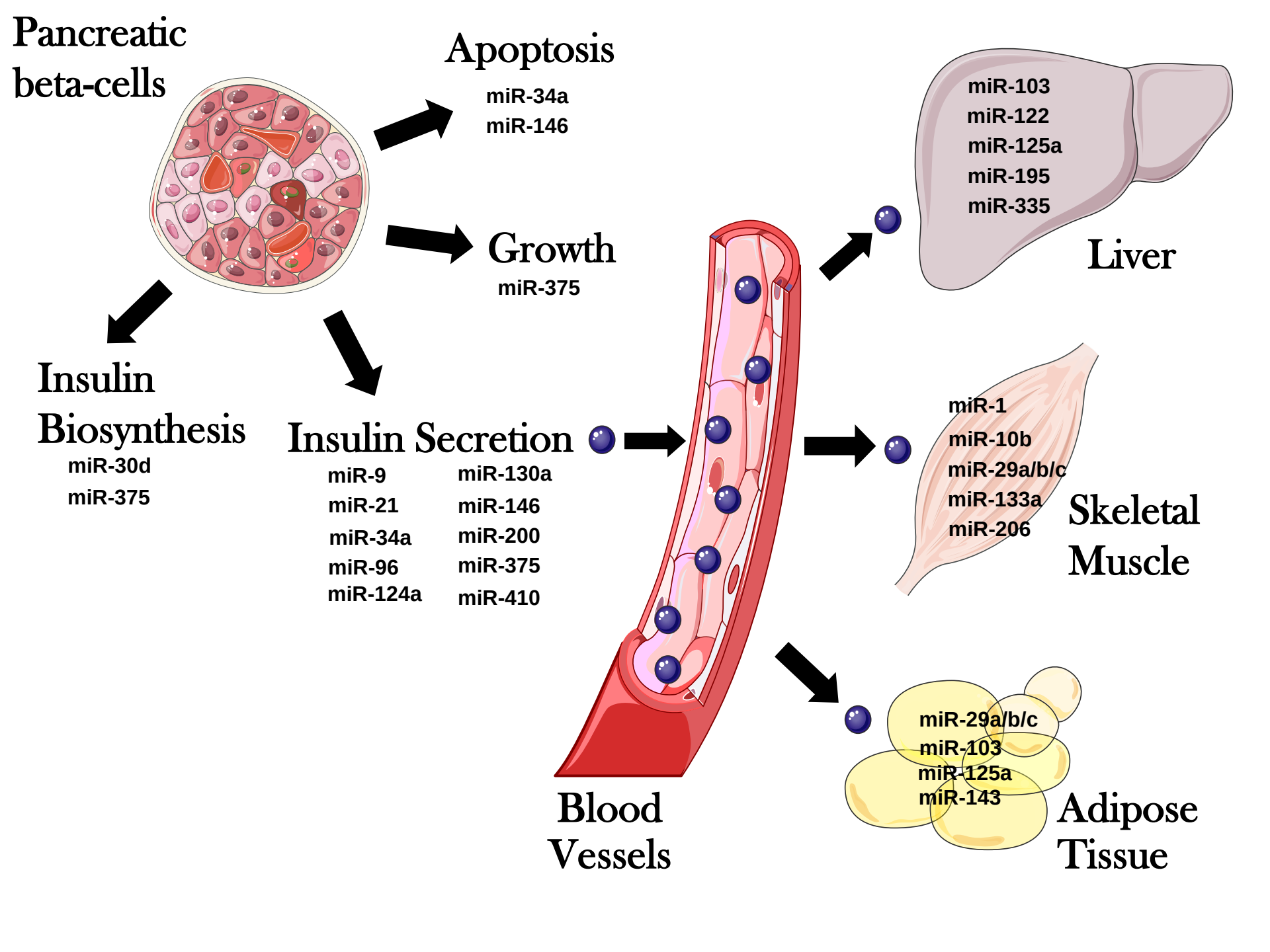
Table legend

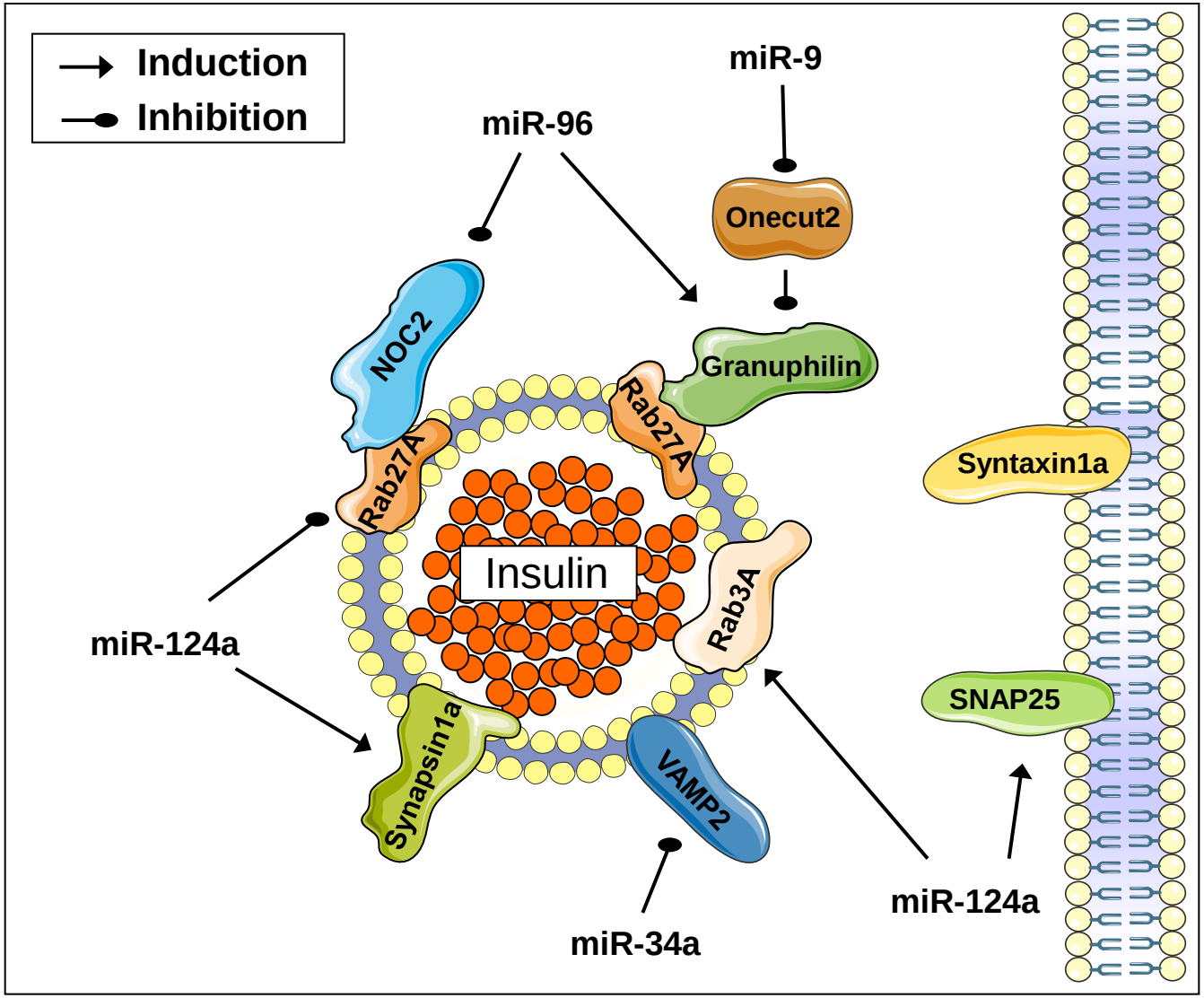
Table 1 : List of miRNAs reported to be differently expressed in pancreatic β-cells, liver, adipose tissue and/or skeletal muscle of animal models of type 1 or type 2 diabetes. a) See text for more details on animal models. b) miRNAs in **bold** are upregulated in the diabetic state whereas miRNAs in *italic* are downregulated.

Figure legends

Figure 1: Schematic overview of the miRNAs involved in the regulation of the functions of pancreatic β-cells or insulin-target tissue in the context of diabetes.

Figure 2 : Regulation of insulin exocytosis by miRNAs. The figure depicts the miRNAs that control directly or indirectly the expression of key components required for β-cell exocytosis associated either with insulin containing secretory granules or with the plasma membrane.


Pancreatic beta-cells
Apoptosis
miR-34a
miR-146
Growth
miR-375
Insulin Biosynthesis
miR-30d
miR-375
Insulin Secretion
miR-9
miR-21
miR-34a
miR-96
miR-124a
miR-130a
miR-146
miR-200
miR-375
miR-410
Blood Vessels
miR-103
miR-122
miR-125a
miR-195
miR-335
Liver
miR-1
miR-10b
miR-29a/b/c
miR-133a
miR-206
Skeletal Muscle
miR-29a/b/c
miR-103
miR-125a
miR-143
Adipose Tissue


Induction
Inhibition
miR-9
miR-96
Onecut2
NOC2
Granuphilin
Rab27A
Rab27A
Syntaxin1a
Insulin
Rab3A
miR-124a
SNAP25
Synapsin1a
VAMP2
miR-124a
miR-34a

Animal models[a]	Characteristics	Tissue	Detection	miR changes[b]	References
TYPE 1 DIABETES ANIMAL MODELS					
Non obese diabetic (NOD) mice	- susceptibility to spontaneous development of T1D	pancreatic islets	qPCR	**miR-21,-34a, -146a**	Roggli E, 2010
streptozotocin-induced T1D mice	- destruction of B-cell by repeated injection of streptozotocin	liver	array	**miR-34a** *miR-122*	Li S, 2009
TYPE 2 DIABETES ANIMAL MODELS					
db/db mice	- leptin receptor gene deficiency	pancreatic islets	qPCR	**miR-34a,-146a**	Lovis P, 2008
	- obese, hyperglycemic, hyperinsulinemic	liver	array	**miR-19a, -183, -207, -212, -326, -328, -335, -409, 434-3P, 467** *miR-33, -34c, -129-3P, -133a, -137, -142-5P, -144, -146, -384, -448*	Nakanishi N, 2009
	- insulin resistant	adipose tissue	qPCR	**miR-335**	Nakanishi N, 2009
ob/ob mice	- leptin gene deficiency	liver	array	**miR-31, -34a, -103, -107, -194, 200a, -221, -335-5P** *miR-2, -29c, -122, -451*	Li S, 2009
	- obese, hyperglycemic, hyperinsulinemic		qPCR	**miR-335**	Nakanishi N, 2009
	- insulin resistant	adipose tissue	array	**miR-146b, -221, -222** *miR-30a-5P, -30c, -99b, -103, -107, -125b, -143, -148a, -422b*	Xie H, 2009
			qPCR	**miR-335**	Nakanishi N, 2009
BTBR-ob & B6-ob mice vs BTBR & B6 mice	- diabetes-susceptible BTBR mice or diabetes-resistant B6 mice were inbreed with ob/ob mice - obese	pancreatic islets	array	**miR-34a, -34b, 132, -133a, -126-5P, -152, -185, -212** *miR-7b, -31, -184, -204*	Zhao E, 2009
		liver	array	**miR-34a, -205** *miR-17-3P, -133a, -151, -201, -298, -328, -329, -330, -380-5p*	Zhao E, 2009
	- mild and transient (B6-ob) or severly (BTBR-ob) hyperglycemic	adipose tissue	array	**miR-221, -222, -342** *miR-1, -34b, -34c, -133b, -135a, -141, -200a, -200b, -200c, -215, -375, -429*	Zhao E, 2009
Goto-Kakizaki (GK) rats	- non-obese Wistar substrain - develop T2D early in life	pancreatic islets	qPCR	**miR-124a** *miR-375*	El Ouaamari, 2008
		liver	Northern	**miR-29a,-29b,-29c**	He A, 2007
			array	**miR-125a**	Herrera BM, 2009
			array	**miR-103, -195**	Herrera BM, 2010
		adipose tissue	Northern	**miR-29a,-29b,-29c**	He A, 2007
			array	**miR-125a**	Herrera BM, 2009
			array	**miR-222, -27a**	Herrera BM, 2010
		muscle	array	**miR-29a,-29b,-29c,-150** *miR-19b, -127, -130a, -148a, -152, -199a, -299-5P, -335, -379, -343-3P, -451*	He A, 2007
			array	**let-7f, miR-301** *miR-23a, -23b, -24,-126,-130a, -424, -450*	Huang B, 2009
			array	*miR-10b*	Herrera BM, 2010