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# **Emerging roles of non-coding RNAs in pancreatic $\beta$ -cell function and dysfunction**

Running title: Role of non-coding RNAs in diabetes

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## **Abstract**

Pancreatic  $\beta$ -cells play a central role in glucose homeostasis by tightly regulating insulin release according to the organism demand. Impairment of  $\beta$ -cell function due to hostile environment, such as hyperglycaemia and hyperlipidaemia, or due to autoimmune destruction of  $\beta$ -cells results in diabetes onset. Both environmental factors and genetic predisposition are known to be involved the development of the disease, but the exact mechanisms leading to  $\beta$ -cell dysfunction and death remains to be characterized. Non-coding RNA molecules, such as microRNAs, have been suggested to be necessary for proper  $\beta$ -cell development and function. The present review aims at summarizing the most recent findings about the role of non-coding RNAs in the control of  $\beta$ -cell functions and their involvement in diabetes. We will also provide a perspective view of the future research directions in the field of non-coding RNAs. In particular, we will discuss the implications for diabetes research of the discovery of a new communication mechanism based on cell-to-cell microRNA transfer. Moreover, we will highlight the emerging interconnections between microRNAs and epigenetics and the possible role of long non-coding RNAs in the control of  $\beta$ -cell activities.

**Key words:** non-coding RNAs, microRNAs, pancreatic  $\beta$ -cells, diabetes

## **Introduction**

Pancreatic  $\beta$ -cells are highly specialized endocrine cells located within the islets of Langerhans. Insulin, the hormone released by these cells, plays a central role in the control of carbohydrate and lipid metabolism in the human body. Secretion of inappropriate amounts of insulin, due to  $\beta$ -cell dysfunction and/or loss, leads to different forms of diabetes mellitus. Type 1 diabetes (T1D), representing about 5-10% of the cases, results from the autoimmune destruction of  $\beta$ -cells [1]. Type 2 diabetes (T2D), the most common form of the disease (about 90% of the cases), is initiated by a diminished sensitivity of insulin target tissues often linked to obesity [2]. This insulin resistance state is normally compensated by an increased secretory activity of  $\beta$ -cells and by expansion of the functional  $\beta$ -cell mass. However, in genetically predisposed individuals this adaptive mechanism fails to compensate the increased insulin needs leading to insufficient hormone supply and post-prandial hyperglycemia. The resulting exposure of  $\beta$ -cells to chronically elevated concentrations of glucose, free fatty acids and cytokines has a deleterious impact on their functions, leading to defective insulin secretion and, eventually, partial loss of the cells by apoptosis. This combination of events culminates in the manifestation of T2D.

In the last two decades, a large number of studies attempted to determine the causes of  $\beta$ -cell dysfunction in the initial phases of T1D and T2D focusing mainly on the role of transcription factors. Although activation of specific transcription factors is certainly contributing to gene expression changes associated with  $\beta$ -cell failure, recent studies reported the existence in mammalian cells of an entirely new class of gene regulatory molecules. Indeed, high-sequencing throughput techniques unveiled the

existence of thousands of non-coding RNA molecules generated from independent transcription units located outside of protein-coding genes or produced from intronic regions [3,4]. These non-coding RNAs fall into two major categories: small non-coding RNAs, of which microRNAs (miRNAs) represent the best characterized class, and long non-coding RNAs (LncRNAs). The function of small and long non-coding RNAs is only beginning to emerge but there is already strong evidence of their involvement in the development of several diseases, including diabetes. This review aims to summarize the most recent findings about the role of non-coding RNAs in the control of  $\beta$ -cell functions and their involvement in diabetes, and to present a perspective view of the future research directions.

## **MicroRNAs**

### ***Biogenesis and mode of action***

MiRNAs are small non-coding RNA molecules which function as specific regulators of gene expression [3]. So far, more than 1500 predicted miRNA precursors, leading to up to 2154 mature miRNAs, have been identified in the human genome ([www.miRbase.org](http://www.miRbase.org)). Most mammalian miRNAs are transcribed by RNA polymerase II as primary molecules (pri-miRNAs) containing characteristic stem-loop structures [5]. Primary transcripts are thereafter cleaved by the RNase III-type enzyme Drosha associated with DGCR8/Pasha proteins to produce hairpin-structured precursors of around 70 nucleotides, called pre-miRNAs. Then, pre-miRNAs are transported by the export receptor exportin-5 to the cytoplasm, where they are cleaved by the

endoribonuclease Dicer to generate an imperfect miRNA/miRNA\* duplex of approximately 22 nucleotides. Subsequent to Dicer cleavage, the guide miRNA strand of the duplex is loaded on an Argonaute protein (mainly Ago1 or Ago2) and incorporated into the RNA-induced silencing complex (RISC). The passenger miRNA\* strand (also called, miRNA-3p) can be degraded or also loaded on Ago2 to act as a functional miRNA. Finally, the RISC complex guided by the mature miRNA binds to complementary sites within the 3'untranslated region (3'UTR) of the target messenger RNA (mRNA), leading to translational inhibition and/or transcript degradation [3]. This canonical miRNA biogenesis pathway generates most of the miRNAs expressed in mammals. However, other atypical pathways have been described recently [6,7].

Computational algorithms predict that most of the human protein-coding genes contain miRNA-binding sites within their 3'UTR [8]. A single miRNA has the potential to bind hundreds of mRNA targets while, conversely, a single 3'UTR region can be regulated by several different miRNAs [9]. The ability of miRNAs to associate with the 3'UTR region of target mRNAs and to repress their translation is mainly determined by the so-called seed sequence, which is complementary to residues 2-8 in the 5' region of the miRNA [3,9]. The most popular computational algorithms, predict potential miRNA target genes, based on the complementarities between the sequences of the target and the miRNA with emphasis on perfect base-pairing of the seed region and on sequence conservation between different animal species. Recently, however, alternative target recognition mechanisms involving G-bulge sites at positions 5-6 have been shown to comprise about 15% of the miRNA/mRNA interactions [10].

### ***Subcellular localization***

During their biogenesis, pre-miRNAs are exported into the cytoplasm where they are processed into mature and active single-stranded miRNAs. Therefore, mature miRNAs are generally considered to be cytosolic molecules. Nonetheless, growing evidence suggests that some of the mature miRNAs as well as components of the machinery mediating miRNA function are localized into other cellular compartments such as the nucleus or the mitochondria (Fig.1). The presence of mature miRNAs in isolated nuclei has been confirmed in different cell types using fractionation protocols followed by microarray analysis [11]. Detailed investigations revealed differential expression of miRNAs within the nucleolus, the nucleoplasm or the cytoplasm of rat myoblasts [12]. The homology of some nucleolar miRNAs with small nucleolar-specific RNAs suggests a common biosynthetic route, whereas other nucleolar miRNAs are probably transported there in order to be modified or to play specific roles. Recent studies identified the mitochondria as a new localization site for miRNAs. Both precursor and mature forms of miRNAs were detected in mitochondria isolated from human skeletal muscle cells or HeLa cells [13,14]. However, the origin of these miRNAs, i.e. whether they are produced in the nucleus or directly inside the mitochondria, and if they accomplish specific functions justifying their localization in this organelle remain to be determined. The cellular compartment in which miRNAs are preferentially located may reflect their functions and/or fates. These data suggest the involvement of miRNAs in new regulatory processes, possibly going beyond their known activities as translational inhibitors.

Dynamic structures such as stress granules (SG) or processing bodies (PB) are other compartments in which miRNAs can accumulate (Fig.1). Ago2, which is part of the

RISC machinery, has been shown to translocate to both SG and PB in case of cellular stress. Leung et al., demonstrated that in HeLa cells stress induction by incubation with a translation initiation inhibitor leads to accumulation of miRNAs and Ago2 in SG [15]. Detzer et al. confirmed the translocation of Ago2 to SG in response to stress and the decrease in miRNA activity, but did not observe relocalization of miRNAs [16]. These reports support the hypothesis that miRNA distribution in specific cellular compartments such as cytosol, nucleus and mitochondria might be influenced by environmental cues. During the initial stages of T1D and T2D,  $\beta$ -cells are submitted to diverse types of stress stimuli causing dysfunction and death. It is tempting to hypothesize that these stressful conditions may be associated with modifications in miRNA localization potentially resulting in alterations in  $\beta$ -cell function. However, this appealing scenario remains to be proven because no information is yet available about redistribution of miRNAs in  $\beta$ -cells or in insulin target tissues under physiological or diabetic conditions.

### ***Circulating miRNAs as diabetes biomarkers***

The role of miRNAs may go beyond their function inside the cells, since these non-coding RNAs are present in stable and active forms also in extracellular fluids, such as blood [17]. The analysis of circulating miRNAs revealed that they predominantly originate from neighbouring tissues and from cells lining the vessel walls rather than from blood cells themselves [18]. This discovery boosted the interest in circulating miRNAs as potential disease biomarkers and clinical indicators. In line with this idea, Zampetaki and colleagues performed miRNA profiling in blood samples from a prospective study including 800 individuals and identified a characteristic plasma

signature involving changes in five miRNAs in the samples of T2D subjects [19]. Interestingly, the level of these miRNAs was already modified years before the development of the disease. If confirmed, this miRNA signature might represent a good predictor enabling the identification of individuals at risk of developing T2D [20]. Nonetheless, the use of plasma miRNAs as biomarkers for diabetes risk needs to be evaluated with caution. Indeed, a recent study reported up-regulation of seven diabetes-related miRNAs in blood samples of newly diagnosed T2D patients compared to T2D-susceptible subjects with normal glucose tolerance [21]. However, miRNA profiles of normal glucose tolerant patients were similar to those of pre-diabetic individuals, suggesting that alone this group of miRNAs is not sufficient for the prognosis of diabetes onset. This result is probably due to the fact that the authors tested only miRNAs known to be involved in  $\beta$ -cell dysfunction and in the pathogenesis of T2D. MiRNA changes taking place in dysfunctional  $\beta$ -cells or insulin target tissues may not necessarily be reflected in modifications in the blood miRNA profile and vice-versa. Thus, blood miRNA data can yield different and maybe complementary information to measurements at the cellular/tissue level.

### ***Role of miRNAs in the regulation of pancreatic $\beta$ -cell functions***

Evidence for the role of miRNAs in pancreatic islet development has been provided by the generation of pancreas-specific Dicer1 knockout mice. These mice display impaired formation of the endocrine pancreas, with severe reduction in the amount of  $\beta$ -cells, suggesting that miRNA expression is essential for proper pancreatic development [22]. Similarly, Kalis *et al.* found that  $\beta$ -cell specific deletion of Dicer1 did not affect fetal or

neonatal development of insulin-secreting cells, but resulted in progressive hyperglycemia leading to adulthood-onset diabetes mellitus [23]. Moreover, *in vitro* analysis of pancreatic islets from Dicer1  $\beta$ -cell specific null mice revealed impaired insulin gene expression, with concomitant decrease in insulin secretion, altered islet morphology, and reduced  $\beta$ -cell mass, preceding the hyperglycemic state. Similar results were confirmed in an independent study where Dicer1 expression was diminished in adult  $\beta$ -cells, resulting in a drastic reduction in insulin content and diabetes development but without changes in  $\beta$ -cell architecture [24]. Taken together, these findings point to a central role of the miRNA network in pancreatic development and in the achievement of specialized  $\beta$ -cell functions.

A series of studies provided further clarifications about the role of specific miRNAs in  $\beta$ -cell development (Table 1). A group of four different miRNAs (miR-7, miR-9, miR-375 and miR-376) were reported to be highly expressed during human pancreatic islet development [25]. In addition, miR-375 was shown to be involved in the development of endocrine pancreas and proper islet formation in two different animal models [26,27]. Interestingly, miR-375 is among a subset of miRNAs particularly enriched in pancreatic islet cells [28] and its expression is under the control of Pdx1 and NeuroD1, two critical transcription factors for the development of the endocrine pancreas [29]. Mice lacking miR-375 are hyperglycemic and have elevated plasma glucagon levels in both fed and fasting states. This phenotype correlates with an increase in  $\alpha$ -cell number and a decrease in  $\beta$ -cell mass leading to a profound imbalance in the cellular composition of the endocrine pancreas. In addition, genetic deletion of miR-375 in ob/ob mice, an insulin resistance model characterized by  $\beta$ -cell mass expansion, led to

impairment in  $\beta$ -cell proliferation capacity resulting in a severe diabetic state [27]. Finally, Zhao et al. observed a positive correlation between miR-375 expression in T2D patients with extended amyloid formation and reduced  $\beta$ -cell mass [30]. Other groups investigated the role of miR-375 expression in mature  $\beta$ -cells. In addition to its positive effect on the  $\beta$ -cell mass, miR-375 has a negative impact glucose-induced insulin secretion by reducing the level of myotrophin, a protein thought to be involved in insulin-granule fusion, and to regulate insulin gene expression by targeting PDK1 and the phosphoinositol-3-kinase pathway [28,31]. Therefore, miR-375 is a key regulator of several  $\beta$ -cell features, such as insulin expression and secretion, proliferation and adaptation to insulin resistance.

Other miRNAs have also been investigated for their implication in pancreas development (Table 1). Baroukh et al. observed an up-regulation of miR-124a during pancreatic developmental stages where it modulates diverse intracellular pathways via the regulation, both directly and indirectly, of Foxa2, a transcription factor driving the expression of Pdx-1, which is essential for  $\beta$ -cell differentiation [32]. MiR-7, another miRNA highly expressed in human pancreatic fetal and adult endocrine cells [25] displayed changes in expression during mice pancreatic development. Inhibition of miR-7 at early developmental stages affected the number of  $\beta$ -cells, impaired insulin production and resulted in glucose intolerance in the post-natal period [33].

Taken together, these results indicate that a fine-tuning of the expression of miRNAs takes place already at the early stages of pancreas development and that these variations of expression drive major changes in the level of key genes regulating  $\beta$ -cell generation and differentiation.

Beside their role in endocrine pancreas development, miRNAs have been shown to be involved in the control of adult  $\beta$ -cell mass and function (Table 1). Upon overexpression in the insulin-secreting  $\beta$ -cell line INS-1E, miR-9 reduced insulin secretion triggered by glucose or potassium by targeting Onecut-2. This transcription factor negatively regulates the level of granuphilin, a synaptotagmin-like protein that functions as a brake for insulin-secreting granules [34]. Interestingly, miR-9 expression is increased soon after glucose-induced insulin secretion, leading to direct targeting and inhibition of Sirt1, known to play a role during insulin release [35]. MiR-124a, has been reported to negatively impact on glucose-induced insulin secretion by inducing the expression of SNAP25, Rab3A and synapsin-1A, and by decreasing those of Rab27A and Noc2 [36]. Overexpression of this miRNA enhanced exocytosis under basal conditions while it inhibited glucose-induced hormone release [36]. Two other miRNAs, miR-29a and b, are of particular interest for proper  $\beta$ -cell function because they are involved in the silencing of monocarboxylate transporter Mct1, which is necessary for proper coupling of glucose metabolism with insulin secretion [37]. Glucose-stimulated hormone release is also reduced in human and mouse islets where miR-33a is overexpressed leading to a decrease in the cholesterol transporter ABCA1 expression and a consequent accumulation of cholesterol [38]. Whether miR-33a regulates insulin release via modulation of cholesterol levels and/or by targeting key enzymes implicated in fatty acid metabolism, such as CPT-1, AMPK $\alpha$  and Sirt6 [39] remains to be investigated. Finally, mice specifically lacking *let-7* in the pancreas display an impairment in glucose-induced insulin secretion [40].

So far most of the papers reporting changes in insulin release elicited by miRNAs were carried out *in vitro*. Moreover, they often relied on miRNA overexpression and, although in some cases the level of the non-coding RNAs was calibrated to that observed in other cell types, non-specific off-target effects cannot be excluded. Thus, complementary studies, in particular using *in vivo* animal models, will be necessary to confirm the regulation of insulin secretion in response to glucose and other secretagogues and precisely define their mode of action.

Variations in miRNA expression have been observed upon prolonged exposure of pancreatic  $\beta$ -cells to pathophysiological conditions such as hyperglycemia and/or elevated free fatty acids which lead to  $\beta$ -cell dysfunction and death [2]. Glucose drives important changes in miRNA expression, as observed in Goto-Kakizaki (GK) rats, a model of glucose-impaired insulin secretion characteristic of T2D. A group of miRNAs is indeed differentially expressed upon exposure of GK pancreatic islets to a hyperglycemic environment. Among these, miR-335 has been demonstrated to target the mRNA encoding a member of the SNARE complex implicated in insulin secretion [41]. In another study performed in the  $\beta$ -cell line MIN6B1 high glucose exposure led to changes in the expression of several miRNAs and one of them, miR-30d, was found to modulate insulin gene transcription [42]. Similarly, variation in miR-15a expression in mouse islets in response to acute or long-term incubation at high glucose correlated with insulin mRNA levels. The regulation of insulin biosynthesis by miR-15a was confirmed in MIN6 cells using miR-15a mimics and a miR-15a inhibitor. The exact mechanism remains to be demonstrated but the authors suggested that UCP2, directly targeted by miR-15a, could be involved [43]. In another study performed in human islets, miR-146 was found to be

reduced upon prolonged exposure to elevated glucose, while the expression of miR-133a was enhanced by hyperglycemia, resulting in a decrease in insulin biosynthesis via the targeting of PTB, a protein required for insulin mRNA stability [44]. Also miR-375 was reported to be negatively regulated by high levels of glucose, probably via a feedback loop, since elevated levels of this miRNA attenuate insulin gene transcription by targeting PDK1 [31]. Yet not influenced by glucose, another group of miRNAs including miR-24, miR-26, miR-148 and miR-182 have been recently identified as positive regulators of insulin production through the inhibition of transcriptional repressors [24].

Several miRNAs have also been reported to display expression changes under lipotoxic conditions. The levels of miR-34a and miR-146 increase in presence of palmitate and their overexpression is associated with  $\beta$ -cell apoptosis and, in case of miR-34a, to impaired insulin secretion. Silencing of these two miRNAs was sufficient to attenuate apoptosis induced by palmitate exposure but could not restore normal insulin release [45]. MiR-34a along with miR-146a/b and miR-21 are also up-regulated in  $\beta$ -cells exposed to cytokines [46]. VAMP2 and Rab3a, whose levels are decreased upon IL-1 $\beta$  exposure, are targeted by miR-34a and miR-21, correlating with impaired glucose-induced insulin secretion. MiR-146a overexpression did not affect hormone release but increased  $\beta$ -cell apoptosis as was the case for miR-34a. Down-regulation of miR-21, miR-34a and miR-146a in MIN6 cells using antagomirs was able to restore insulin release in the presence of IL-1 $\beta$  and, in the case of miR-34a and miR-146, to protect the cells from cytokine-induced apoptosis [46]. Concerning miR-21, activation of a NF- $\kappa$ B/miR-21/PDCD4 signaling cascade in NOD mice was proposed to counteract  $\beta$ -cell death observed at early stages of T1D diabetes. Indeed, miR-21 induction is able to

directly target and reduce the level of PDCD4, which is known to sensitize  $\beta$ -cells to cytokine-induced apoptosis [47]. Recently, microarray profiling revealed that the establishment of prediabetic insulinitis in NOD mice is associated with an increase in the expression of miR-29 family members. Overexpression of these miRNAs in primary  $\beta$ -cells led to changes in the expression of key genes involved in exocytosis and cell survival with consequent defects in glucose-induced insulin secretion and sensitization toward apoptosis [48].

The studies described above clearly demonstrate that proper miRNA expression is crucial for  $\beta$ -cell development, survival and function. The miRNA field is still at its infancy and future investigations will probably permit to precisely appreciate the relevance of miRNAs in  $\beta$ -cell biology and in the development of both T1D and T2D.

### **Future research directions in the non-coding RNA field**

#### ***Horizontal miRNA transfer as a novel cell-to-cell communication mechanism***

Beside their potential value as disease biomarkers, plasmatic miRNAs may represent a new class of cell-to-cell communication molecules. Indeed, circulating miRNAs can be taken up by recipient cells where they have the potential to influence gene expression [49]. The precise source of circulating miRNAs is not known yet, but they have been proposed to originate from: passive leakage from broken cells, active secretion via microvesicles or active secretion through an RNA-binding protein-dependant pathway (Fig.2). Moreover, a recent study presented evidence that miRNAs can also associate with high-density lipoproteins (HDL) and to be delivered to recipient cells with

functional targeting capabilities [50]. Interestingly, an important fraction of circulating miRNAs is associated with Ago2 [51,52]. Upon in depth analysis of the miRNAs transported in the human plasma, the majority of the miRNAs was found to co-purify with Ago2 ribonucleoprotein complexes, although some of them, such as for instance miR-142-3p, were predominantly associated with microvesicles. There is strong evidence that miRNAs enclosed in microvesicles, such as exosomes, can be efficiently taken up in active form by recipient cells [53], but the capacity of circulating Ago2/miRNA complexes to enter the cells remains to be demonstrated. More studies will be necessary to elucidate the physiological meaning and the functional differences between circulating miRNAs associated with proteins and those transported in microvesicles. So far, no data are available regarding the physiological impact of circulating miRNAs on the regulation of  $\beta$ -cell functions.

As many other cell types, there is evidence indicating that  $\beta$ -cells are able to release microvesicles with a potential effect on neighbouring cells. The release of miRNA-containing vesicles is a process that can be regulated by different factors including a rise in cytosolic free calcium, sphingomyelinase 2, a protein involved in ceramide biosynthesis and Rab27A, a GTPase belonging to the insulin secretory machinery [53-55]. Therefore, it is tempting to speculate that the release of miRNA-containing vesicles from  $\beta$ -cells may be affected by hormones, nutrients, stress factors or inflammation. In a recent study, Sheng and collaborators investigated the capability of exosomes isolated from MIN6 cells to stimulate an autoimmune response in NOD mice. They found that MIN6-derived exosomes contain diabetes-associated islet autoantigens and stimulate pro-inflammatory cytokine secretion from T cells, leading to islet

inflammation. Furthermore, injection of MIN6-derived exosomes in the diabetes-resistant mouse strain NOR accelerated insulinitis development. This study demonstrates that  $\beta$ -cell exosomes can trigger an auto-immune reaction and favour the attack of pancreatic islets by T cells, resulting in increased diabetes susceptibility [56]. Since exosomes released by cancer cell lines can be different from those secreted by non-transformed cells [55], these findings remain to be confirmed using exosomes derived from primary pancreatic islets.

According to this novel cell-to-cell communication mode, we can assume that exosomes released by  $\beta$ -cells contain miRNAs potentially acting on neighbouring immune cells or endocrine cells to either stimulate adaptation processes or regulate the  $\beta$ -cell fate. Transfer of miRNAs in the opposite direction, i.e. via exosomes secreted by immune cells and taken up by  $\beta$ -cells, may also have relevant physiopathological implications. Future studies should investigate the influence of horizontal miRNA transfer on 1) the interaction between pancreatic islet endocrine cells, including  $\alpha$ -,  $\beta$ -,  $\delta$ -, PP- and  $\epsilon$ -cells, 2) the cross-talk between  $\beta$ -cells, 3) the autoimmune attack of pancreatic  $\beta$ -cells in case of T1D and 4)  $\beta$ -cell failure in case of T2D.

Another potential mechanism of cell-to-cell miRNA transfer so far poorly investigated occurs through gap junctions (Fig.2). This process has been reported to occur between bone marrow cells and breast cancer cells, resulting in cell cycle quiescence of the recipient cells [57]. The potential role of miRNA exchange through gap junctions deserves to be studied also in insulin-secreting cells since gap junction communication is known to be essential for  $\beta$ -cell functionality. Indeed, inhibition of Connexin 36, a major gap junction component in  $\beta$ -cells, induces  $\beta$ -cell death in genetically modified mice, while its overexpression protects them against apoptosis [58]. Hence, the investigation of

gap-junction mediated miRNA transfer could lead to the identification of novel regulatory mechanisms influencing islet cell functions.

### ***Epigenetic regulation of miRNA expression***

It is now well accepted that miRNAs are necessary for proper  $\beta$ -cell development and function. Less understood are the mechanisms regulating miRNA expression under normal and pathophysiological conditions. MiRNAs can be spatially and temporally regulated since they are generally transcribed by Polymerase II [5]. An emerging concept suggests that miRNA expression could be regulated by epigenetic modifications [59]. Epigenetics is defined as heritable changes in gene expression caused by mechanisms that do not affect the DNA sequence itself and is seen as the interface between genetic and environmental factors. The two most widely studied epigenetic mechanisms are DNA methylation and histone modifications which are known to be crucial for proper control of gene expression [59].

DNA methylation occurs mainly in sequences enriched in CpG dinucleotides, so called CpG islands, and is achieved by the addition of a methyl group to the 5 position of a cytosine ring. CpG islands are mainly located in the proximal region of promoters and are underrepresented in the rest of the genome. Methylation of CpG islands is generally associated with gene silencing whereas hypomethylation is linked to hyperactivation of gene expression. Histone modifications refer to post-transcriptional changes of the amino-terminal tails of histone subunits. Some histone modifications, such as acetylation, are labile and associated with gene activation, and others, such as methylation, are stable and lead to gene inactivation [59].

Epigenetics, in the context of diabetes, provides an attractive explanation to reconcile the discordance of monozygotic twins and the interindividual variations in disease severity, and also, to highlight the impact of environmental factors on gene expression. Inappropriate DNA methylation is starting to be investigated in the pathophysiology of diabetes in human. The promoter of *PPARGC1A*, a master regulator of mitochondrial genes, was found to be hypermethylated in islets of T2D donors [60]. Moreover, two recent large-scale studies suggested a link between aberrant DNA methylation and pathological diabetic conditions [61,62]. Zhao et al. [62] observed a positive correlation between insulin resistance and global DNA methylation of leukocytes in blood samples from 84 monozygotic twin donors, and Volkmar et al. [61] profiled several DNA methylation changes in promoter region of different genes in pancreatic islets of T2D patients compared to healthy donors. Further studies are needed to elucidate the role of global and islet-specific DNA methylation in the development of T2D pathogenesis. A genome wide-map of four histone modifications associated with gene activation (H3K4me1, H3K4me2 and H3K4me3) or repression (H3K27me3) in human pancreatic islets was created by Bhandare and colleagues [63]. The pattern of histone modifications observed in human islets is complex and difficult to interpret. The promoter sequence of *Pdx1* and *MafA*, two major transcription factors in  $\beta$ -cells, are highly occupied by modified histone, whereas low levels of histone modifications were present at the insulin and glucagon promoter. Furthermore, developmental genes, like *HOX* genes and neuronal transcription factors, showed bivalent histone marks in concordance with their close regulation during pancreas development and their silencing in adulthood. Further studies are needed to fully understand the purpose of these marks.

However, the work of Bandhare and colleagues provide a starting point to study the impact of environmental factors on histone modifications and gene expression. Finally, another important aspect of epigenetic modifications is that epigenetic marks can occur during embryogenesis or early in life. The intrauterine environment and the maternal nutrition are known to play an important role in the susceptibility of individuals to metabolic disease and diabetes in later life [64,65].

The regulation of miRNA expression by epigenetic mechanisms has been first studied in cancer cells. Several miRNAs have been identified to be epigenetically modified [66]. For instance, the expression levels of miR-9 family, miR-34b/c, miR-124a, miR-127 and miR-148a, have been shown to be deregulated in cancer cells by hypo- or hypermethylation of CpG islands located upstream of the mature microRNA [59,67-69]. Weber et al. estimated that more than 50% of human miRNA genes represent candidate targets to DNA methylation, which is at least one order of magnitude higher than protein-coding genes [70]. Epigenetic regulation of miRNAs had also been studied in human embryonic stem cells (hESC). Using undifferentiated hESC line HS181 and cancer cell lines SIHN-011B and IGR37, Iliou et al. showed that tumor suppressor miRNA genes that undergo *de novo* hypermethylation in cancer cells are enriched in bivalent histone marks in embryonic stem cells [67]. These results emphasize the close relationship between DNA methylation and histone modifications in the regulation of gene expression. They also provide evidences supporting the idea that abnormal regulation of specific genes that are pre-marked during embryonic development, can lead to pathogenesis during adulthood.

To our knowledge, the epigenetic regulation of miRNA genes in  $\beta$ -cells has not been investigated so far, but represents a promising field to uncover new mechanisms leading to  $\beta$ -cell dysfunction in diabetic conditions.

### ***Role of long non-coding RNAs in $\beta$ -cells***

LncRNAs are a new class of transcripts which are commonly grouped based on the fact that their sequence is equal or longer than 200 nucleotides and contrary to protein coding genes, they have poor or no protein-coding potential [71]. LncRNAs are often spliced, may be polyadenylated and can be subdivided into large intergenic non-coding RNAs (lincRNAs) and those that are sense, antisense, intronic or overlapping with protein coding genes [72]. Unlike miRNAs, which act through specific base pair recognition mechanisms to modulate the expression of their target genes [3,9], LncRNAs have been involved in diverse gene-regulatory mechanisms such as transcription, translation, imprinting, genome rearrangement, chromatin modification and other important functions such as nuclear factor trafficking and protein degradation [4,73]. Their molecular mechanisms can be grouped into four major classes: signals, decoys, guides and scaffolds [4]. These groups are not mutually exclusive and most LncRNAs can exert more than one function. As LncRNA expression is under tight transcriptional control, most of them can be used as molecular signatures for specific processes and are therefore classified as signals. LncRNAs acting as decoys directly bind and sequester proteins and presumably act by inhibiting their action. LncRNAs act as guides when they direct the localization of protein complexes to specific targets and finally, LncRNAs can act as scaffolds by serving as platforms upon which multiple molecular components can be assembled.

Another interesting function of LncRNA is the capacity of binding to miRNA and function as competing endogenous RNAs (ceRNA) [74]. ceRNAs compete for miRNAs and regulate their distribution on their target genes. Cesana et al. [75] identified a new LncRNA, linc-MD1, which has the ability of regulating muscle differentiation by targeting miR-133 and miR-135 via a ceRNA mechanism.

Despite their mode of action, which remains partly unknown, LncRNAs have been involved in many different processes such as stem cell differentiation, modulation of apoptosis and invasion and marker of cell fate [76]. Cabili et al.[77] recently showed that LncRNAs exhibit greater tissue specificity than protein coding genes. This group analyzed approximately 8000 lincRNAs in 24 different tissues and cell types and demonstrated that 78% of them were tissue specific, in comparison with only 19% of protein coding genes. This tissue specificity is an integral index/component of their critical regulatory functionality.

LncRNAs are not only tissue specific but they can also localize in different subcellular compartments, including the nucleus, cell body and in specific foci of adult cerebellar purkinje cells [78]. Moreover, their expression can be so specific that the lncRNA Gomafu has been found to localize in regions of the nucleus that are different from any other nuclear compartment identified by known markers [79].

Given their wide variety of functions, LncRNAs and their specific patterns of expression it is not surprising that their dysregulation has been implicated in many human diseases including different forms of cancers and neurodegenerative diseases [73]. In some cases, the mechanism giving rise to the disease has been identified. For example, five different LncRNAs were found to bind and inhibit the action of PSF, a protein involved in the

silencing of the proto-oncogene GAGE6 [27]. For other lncRNAs, specific targets have yet to be identified. This is the case of MALAT1, a lncRNA which expression is three times higher in metastasizing early-stage non-small cell lung cancer versus non-metastasizing tumors [80].

Other lncRNAs involved in diseases are BACE1-AS and ANRIL. BACE1-AS is an antisense lncRNA capable of regulating the expression of BACE1, a gene coding for an enzyme involved in the production of amyloid  $\beta$ -peptide. The accumulation of this protein has been implicated in many neurological disorders and elevated levels of both BACE1 and BACE1-AS have been detected in subjects with Alzheimer's disease [81]. ANRIL was firstly identified by a group studying germ line deletions in melanoma-neural system tumor family [82]. Interestingly, genome wide association studies (GWAS) identified several variants in the intergenic region encompassing ANRIL to be associated with several diseases such as coronary heart disease, intracranial aneurysm, many type of cancers and type 2 diabetes [83]. ANRIL contribution these pathologies has not yet been defined but its action may be mediated through modulation of the expression of the tumor suppressor genes at the INK locus (p14, p15, p16). Indeed, ANRIL has been shown to interact with both components of the polycomb repressive complexes PCR1 and PCR2 [84-85]. Interestingly, we have evidence suggesting that ANRIL may be differentially expressed in *in vitro* models of  $\beta$ -cell dysfunction (our unpublished data).

In recent years, GWAS have identified many other common genetic variants associated with complex diseases such as coronary artery disease, hypertension, T2D and others [86]. Interestingly, the majority of these variations occurs in non-coding and intergenic regions [87] including those associated with T2D. Amongst the 40 variants

associated with the disease, 19 of them map to intergenic regions that lie at great genomic distances from their closest protein coding gene (up to 500kb) [88]. It is becoming clear that the majority of these variants could lie into or in the vicinity of LncRNAs and therefore affect their proper function or expression. A study by Cabili et al. [77], confirmed that a great number of these variants are indeed contained within lincRNAs sequences and that the expression of these lincRNAs is specific to the tissues relevant to the associated disease.

Screenings of LncRNAs in  $\beta$ -cells have yet to be performed but it is likely that many of them will exhibit  $\beta$ -cell specific expression. Given the tissue specificity of LncRNAs and their involvement in multiple cell functions, LncRNAs may be anticipated to play a major effect on the maintenance of  $\beta$ -cell functions and could therefore be implicated in diabetes pathogenesis.

## **Conclusion**

The unexpected discovery of the existence of a multitude of non-coding RNA molecules populating many of the cellular organelles has added a new layer of complexity to the mechanisms governing the biological processes in mammalian cells. We are only beginning to decipher the properties of these newly discovered RNAs but we have already strong indications that they play major regulatory roles in different cellular activities. A better understanding of the mode of action of these fascinating molecules promises to shed new light on the causes of many human diseases, including diabetes.

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## Figure legends

### **Figure 1. Proposed model of cellular localization of microRNAs**

Usually, miRNA biogenesis occurs in the nucleus and is followed by export to the cytoplasm (a) and processing to the mature form acting as translational repressor of target mRNAs. However, mature miRNAs can also localize to other cellular compartments such as nucleus (b), nucleolus (c), p-bodies (d), stress granules (e) and mitochondria (f). In addition, some miRNAs have been suggested to be generated inside the mitochondria.

### **Figure 2. Horizontal transfer of microRNAs as an emerging new form of cell-to-cell communication.**

After being transcribed in the nucleus and exported in the cytoplasm, miRNAs are processed to a mature form. The mature miRNAs can bind to complementary 3'UTR of target mRNAs to inhibit translation (a) or can be packaged and released and transferred to donor cells by several pathways. Secreted miRNAs can be released in the circulation and reach distant cells in a vesicle-associated form or bound to proteins (endocrine transfer) (b), can be transferred to adjacent cells (paracrine transfer) (c), or can enter neighbouring cells through gap junctions (cell junction transfer) (d).

**Table 1:** Overview of all the microRNAs known to have a functional role in pancreatic  $\beta$ -cell development and function. When available, identified and confirmed targets are listed for each miRNA.

<b>MicroRNA</b>	<b>Known functional effect</b>	<b>Targets</b>	<b>References</b>
<b>let-7</b>	insulin secretion		[40]
<b>miR-7</b>	pancreas development & insulin production		[33]
<b>miR-9</b>	insulin secretion	Onecut-2 Sirt1	[34] [35]
<b>miR-15a</b>	regulation of insulin synthesis		[43]
<b>miR-21</b>	insulin secretion protection against cytokines-induced apoptosis	VAMP2, Rab3a PDCD4	[46] [47]
<b>miR-24</b>	insulin-gene transcription	Sox6	[24]
<b>miR-26</b>	insulin-gene transcription	Sox6, Bhlhe22	[24]
<b>miR-29a,b</b>	insulin secretion	Mct11	[37]
<b>miR-30d</b>	insulin-gene transcription		[42]
<b>miR-33a</b>	insulin secretion	ABCA1	[38]
<b>miR-34a</b>	insulin secretion cytokines-induced apoptosis	VAMP2, Rab3a	[45-46] [46]
<b>miR-96</b>	insulin secretion	Noc2	[36]
<b>miR-124a</b>	pancreas development insulin secretion	Foxa2 Rab27a, Noc2	[32] [36]
<b>miR-133</b>	insulin biosynthesis	PTB	[44]
<b>miR-146a,b</b>	cytokines-induced apoptosis		[46]
<b>miR-148a</b>	insulin-gene transcription	Sox6	[24]
<b>miR-182</b>	insulin-gene transcription	Bhlhe22	[24]
<b>miR-200</b>	insulin-gene transcription	Sox6, Bhlhe22	[24]
<b>miR-335</b>	insulin secretion	Stxbp1	[41]
<b>miR-375</b>	insulin-gene transcription insulin secretion pancreatic islets development & morphology	PDK1 Myotrophin	[31] [28] [26-27,30]

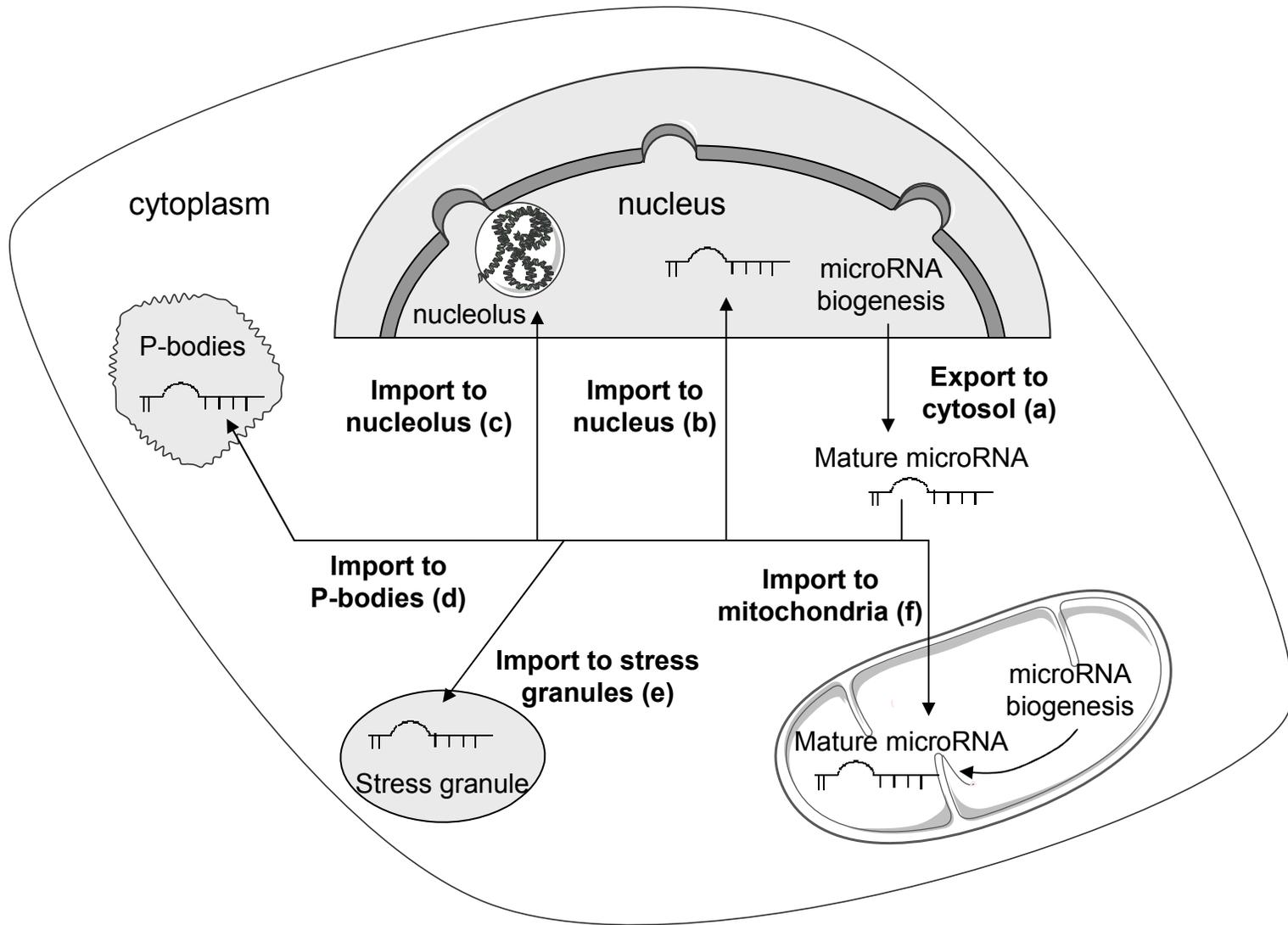


Figure 1 Guay et al.

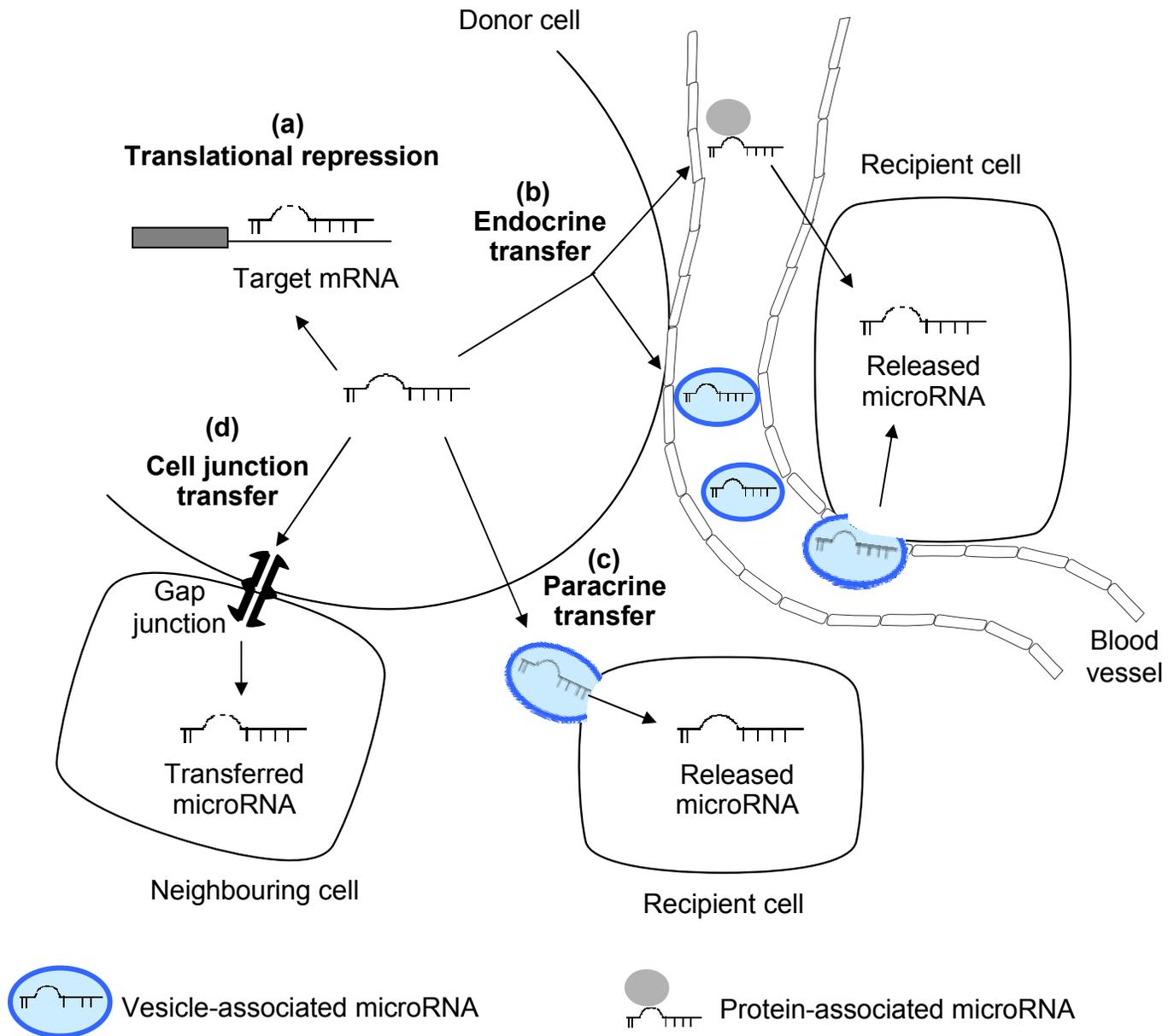


Figure 2 Guay et al.