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MicroRNAs: an adaptive mechanism in the pancreatic β-cell…and beyond?

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Abstract
Recent protocols have been developed to differentiate human stem cells and fibroblasts into insulin-producing cells capable of releasing the hormone in a glucose-stimulated manner. Limitations remain which prevent bringing these protocols to a clinical setting as these models must still undergo complete characterization. Advances in sequencing technologies have driven the identification of several non-coding RNA species including microRNAs (miRNAs). While their diversity and unique expression patterns across different tissues have made deciphering their precise functional role a significant challenge, studies using both cell lines and transgenic mouse models have made substantial progress in understanding their regulatory role on exocytosis and proliferation of the β-cell. These results also indicate miRNAs play an integral role in the fundamental mechanics of how the cell manages the balance between these independent functions. Continued investigation into miRNA function may uncover mechanisms which can be exploited to improve differentiation protocols in producing fully mature β-cells.

Key Words
microRNA, pancreatic islet, metabolic stress, diabetes, beta cell proliferation, insulin secretion, non-coding RNA

Glucose homeostasis and the pancreatic β-cell

The metabolism of glucose as a source of energy is a fundamental property virtually all cell types share from bacteria to humans [1]. As mammals have evolved and their cells increased their diversity over time, so has the expression pattern of genes which regulate the breakdown of glucose as a cellular fuel. Among the most studied mechanisms is the pancreatic β-cell which regulates its output of insulin as part of a complex endocrine network of signaling molecules designed to maintain stable glucose concentrations. In addition to adjusting its secreted load, the β-cell will also adapt its transcriptional programming to promote its proliferation in response to chronic hyperglycemia however it remains unclear how the cell simultaneously balances the energy requirements of both processes [2]. Recent technological breakthroughs have enabled the systematic discovery of several new regulatory molecules which have begun to shed new light on the intracellular mechanisms of the β-cell. The advancement of high-throughput sequencing facilitated the identification of several classes of non-coding RNA species including miRNAs (miRNAs) and a canonical pathway has been established describing the processing and incorporation of these small RNAs into Argonaute-containing complexes [3]. These discoveries have led to a surge in research into exploration of the functional role of post-transcriptional gene regulation within the cell [4]. Here we explore the current state of the art of research into miRNAs and their role in the pancreatic β-cell and how these studies may offer insights into cell types central to the maintenance of energy and glucose homeostasis in the body.

MicroRNA profiling in the pancreatic β-cell and islets

The initial paper reporting the first cloning effort of miRNAs in the pancreatic β-cell identified 67 unique sequences including miR-375 [5]. As current sequencing technologies have long rendered this cloning method obsolete, they have also revealed the potential for hundreds of unique functional sequences to be present in most cell types including the β-cell. To date, 2588 mature human miRNAs have been annotated in the miRBase database V20 which now catalogs 206 different plant and animal species [6]. While several sequences including miR-375, miR-7, and miR-184 appear to be enriched in the endocrine pancreas, profiling results from mouse and human tissues have shown that none of these miRNAs are entirely specific to the islet [7] [8]. In the case of miR-375, expression of this miRNA has been reported in numerous tissue types including the pituitary gland, adrenal gland, intestine, lung, and several types of cancer [9] [10]. Similarly, miR-7 and miR-184 have been shown to be highly enriched in the central nervous system and eye, respectively [11]. Recent studies using mouse knockouts have shown that loss of these three abundant miRNAs will increase β-cell exocytosis however it still remains unclear whether all miRNAs present in the β-cell are committed to an identical functional role and whether they perform the same function in all cell types where they are present [12] [9] [8]. For example, while miR-375 regulates exocytosis and compensatory proliferation of the β-cell, it has not been determined whether it functions in these capacities in the other neuroendocrine cell types where it is expressed [8] [13]. Meanwhile for miRNAs which are abundant in the β-cell however are ubiquitously expressed across many tissues such as the let-7 family, it is not clear whether there is functional overlap to sequences like miR-375. Transgenic over-expression of let-7 resulted in glucose intolerance in mice, however it is not clear if this microRNA regulates β-cell mass or function [14].

In light of the diversity of the miRNA profile of the β-cell in terms of number of sequences and their expression levels, several important questions remain to be addressed for future studies. The issue of combinatorial targeting of genes by multiple miRNAs has not been well studied *in vivo* however the advent of new methodologies including CRISPR/Cas9 site-directed mutagenesis and crosslinking-immunoprecipitation (CLIP) protocols can now expedite the identification of biologically relevant *cis* and *trans* sequences [15] [16]. In addition to improving our understanding miRNA target recognition, these protocols have the potential of establishing the functional importance of low expressed sequences either by loss of function studies or by identifying robust miRNA : target gene interactions.

Elucidating the functional role of microRNAs in the β-cell

Without any doubt, in recent years significant progress has been achieved in understanding miRNAs as regulators of gene expression throughout all areas of biology [3]. As mentioned previously, substantial investment in high throughput sequencing has all but ruled out the possibility of identifying novel abundant sequences and the majority of investigators have since directed their attention on addressing specific biological questions. The chronic hyperglycemia characteristic of diabetes has long been established the result of inadequate insulin action making one of the prime directives of the β-cell field to identify genes and mechanisms which contribute to reduced growth and function of this cell type. Since the first gain and loss of function studies on miR-375, several subsequent reports have identified a number of additional miRNAs as negative regulators of β-cell function including miR-7, miR-184, miR-21, miR-29a and miR-33a [5] [12] [8] [17] [18]. Importantly, the role of several miRNAs as negative regulators has been substantiated using specific genetic mouse knockouts [19]. Likewise, the siRNA-mediated knockdown of Argonaute2 (Ago2), a key mediator of miRNA function in all cell types, also resulted in increased secretory capacity further supporting the notion that several prominent if not the majority of miRNAs act as negative regulators of exocytosis [13]. While it still remains a possibility that some miRNAs target genes that result in an overall positive effect on insulin release, the published data to date points toward an overall inhibitory effect by the pathway on β-cell exocytosis.

With respect to miRNAs and their role in growth and proliferation of the β-cell, a number of significant observations have been made that also affirm a significant functional role for small RNAs in this cell type. The mouse knockout of miR-375 exhibited a decrease in β-cell mass at steady state that was further exacerbated once the model was crossed into the *ob/ob* background [9]. This result provided the first in vivo evidence that miRNAs could play a binary role in regulating both exocytosis and cellular proliferation. β-cell specific knockout mouse of Ago2, a key mediator of the miRNA pathway, exhibited a similar phenotype underlining an important role for small RNAs in the compensatory response of this cell type as the demand for insulin increases [8]. As potent regulators of both secretion and cell proliferation, together these results highlight the potential for studying how this pathway contributes to the most fundamental aspects of β-cell physiology. Future studies can now aim at exploring the precise mechanisms of their function including dissecting their distinct contribution to both exocytosis and proliferation, identifying interacting RNA binding proteins and specific RNA modifications which may impact miRNA function,

expression and localization, and the full extent to which the target genes in the β-cell.

Identification of microRNA targets in the β-cell

As the miRNA field began to gain momentum and the numbers of small RNAs rapidly increased across different species, it became readily apparent that the identification of direct targets required the specialized expertise of computational investigators to develop algorithms that could locate putative binding sites in the genome. Among the first successes in vivo was observed in Drosophila by Cohen and colleagues with the identification of the miRNA bantam targeting the pro-apoptotic gene hid [20]. The first mammalian target myotrophin (Mtpn) was shown to be regulated by miR-375 using a murine insulinoma cell line MIN6 [5]. Based on these first observations, both empirical and computational evidence influenced the development of the first miRNA target algorithms implementing cross-species conservation [3] [21]. Since these early model studies, the TargetScan algorithm has continued to refine its target predictions and remains among the most recognized in the field [22]. Recent reports have implemented biochemical approaches (crosslinking and immunoprecipitation, CLIP) to identify specific miRNA target sites by isolating and sequencing the 3'UTR region bound by Ago proteins [16]. While these protocols are currently dependent upon large quantities of source tissue material in cell culture, this limitation excludes the possibility of studying human islets however the development of the stem cell differentiation protocols may make the CLIP protocol viable for human β-cells in the near future. A recent study profiling miRNAs after the immunoprecipitation of Ago proteins revealed stark differences in the small RNA profile suggesting biochemical approaches to profile miRNAs in FACS-sorted human β-cells may provide insight into distinct populations of miRNAs which interact with specific RNA binding proteins [4] [23]. To date, numerous studies have established specific miRNA:target gene interactions implicating the entire range of β-cell physiology and it appears likely that eventually every functional category (i.e. receptors, signaling, kinases, channels, etc.) and pathway (p53, G-protein coupled receptor, PLC, etc.) will be recognized as being targeted by miRNA [24] [25] [26]. Therefore it is imperative for the field to now put these observations in perspective and understand the implications of miRNA function in the β-cell. What important questions should be addressed and can this mechanism be exploited for the development of future therapeutic strategies?

One perspective on microRNAs in the β-cell

A great deal remains to be described with respect to the collective function of all miRNAs expressed in the β-cell. As computational algorithms have identified putative miRNA-binding sites in the 3'UTRs of the majority of annotated genes, it is still not precisely known why this form of gene regulation exists [22]. Several hypotheses have been proposed on the role of miRNAs and one that is highly plausible suggests that miRNAs anneal to transcripts to inhibit translation as demand for the expression of the mRNA subsides [3] [19]. In the context of the pancreatic β-cell, there are several reasons why this hypothesis is fitting. First, several mechanisms exist in the body beyond the cells of the endocrine pancreas to attenuate and counter insulin action as preventative measures against hypoglycemia [27]. Most notably is the release of glucagon by the α-cells of the pancreas to promote gluconeogenesis in the liver. However during and after food intake, in response to an increase in the concentration of extracellular glucose, hundreds of genes will contribute to the series

of mechanistic events starting with glycolysis, mitochondrial function, the formation and trafficking of insulin granules, and ultimately exocytosis [28]. Therefore, it is reasonable to speculate that the profile of miRNAs present in the β-cell may have diversified to specifically suppress these genes due to their unique functional mandate: to systematically regulate insulin release. It is widely reported that the β-cell releases only a small percentage of its stored granule pool in response to glucose further supporting the notion that exocytosis from the β-cell is a highly regulated process [29] [28]. Therefore the presence of miRNAs may simply serve to manage and synchronize the efficient output of insulin. As observed in the mouse knockouts of miR-375, miR-7, miR-184 and Ago2 in the β-cell, loss of expression of these molecules results in the hypersecretion of insulin and the enrichment of these sequences in the β-cell act to target its distinct profile of genes. Just as cardiocmyocytes, excitatory neurons, hepatocytes, and adipocytes maintain specific functional roles; their microRNA profiles are also distinct to their mRNA expression profiles.

In light of the abundance of literature highlighting the diversity of non-coding RNAs in terms of sequence and function, labeling miRNAs in the β-cell as 'inhibitors of insulin release' is an over-simplification of the matter. To further address how miRNAs meet the role as regulators of gene expression according to metabolic demand, an additional layer to this hypothesis centers on the signals which instigate insulin release. The discussion here began with glucose metabolism and insulin is released by the β-cell primarily to direct glucose uptake in peripheral skeletal muscle and adipose tissue. Therefore, the question is now: how do miRNAs function according to the rise and fall of glucose levels? In the post-prandial state, as extracellular glucose concentrations subside within hours, it is not clear whether these transcripts encoding secretion-mediating genes are quickly degraded or rather stabilized in preparation for subsequent metabolic signals. Therefore, one possible function for miRNAs may be to bind these mRNAs in a sequence specific manner with the assistance of RNA-binding proteins (i.e Argonautes) in order to protect and delay degradation over the course of several hours [30] [31]. In the event that extracellular glucose levels again arise within an acute time frame, the miRNAs can then be 'de-recruited' from these transcripts to facilitate expression of the proteins regulating the breakdown of glucose and insulin release. The annealing of miRNAs to target transcripts may act to preserve these mRNAs which are in continual demand (i.e. when meals are minutes and hours apart). Additional signals may ultimately promote the degradation of these mRNAs once the demand has ceased (i.e. during prolonged fasting or sleep). This idea also coincides with the majority of studies showing the regulation of putative target genes after increasing or decreasing miRNA function in excess of 24 hours or the time to transfect cells in culture [3]. This hypothesis on miRNA function suggests these non-coding RNAs could serve as an energy-efficient means of regulating transcription and gene expression in contrast to continuous de novo synthesis and degradation of mRNAs as the demand for insulin rises and falls. As mentioned previously, several tissues coordinately maintain systemic glucose levels near 5mM and the hypothesis here proposes that miRNAs in the β-cell act at least in part as a safeguard to strictly regulate exocytosis.

The next question with respect to miRNA function extends to chronic increases in glycemia as characterized by individuals with diabetes. How do miRNAs contribute

to β-cell function in the context of metabolic disease? If miRNAs are hypothesized here to strictly regulate secretion, in the presence of hyperglycemia, are these non-coding RNAs then in a state of 'de-recruitment' of their target mRNAs to facilitate increased exocytosis? In short, there are reasons to believe the answer is no, miRNAs still act to strictly regulate secretion even during chronic hyperglycemia. Another important aspect of β-cell function is its concomitant ability to proliferate to increase cellular number and mass in response to increased demand for insulin and glucose has long been known as a potent regulator of β-cell proliferation [32] [33] [34]. The mouse knockouts for miR-375 and Ago2 in the β-cell both showed loss of compensatory proliferation after crossing these models onto the *ob/ob* background indicating miRNA function is essential for this adaptive response. Therefore if miRNA function is necessary to maintain the proliferative capacity of these cells when confronted by hyperglycemia and insulin resistance, is this function exclusive of its role in regulating secretion? Perhaps not. With respect to the *ob/ob* model, pancreatic β-cell mass has been shown to be increased ~8-fold and circulating insulin levels were measured increased over ~30-fold compared to wild-type littermate controls making it clear that proliferation has been increased to accommodate the increased insulin demand [8]. If miRNA function is essential for compensatory proliferation, it is reasonable to speculate that secretion may be compromised in order for the β-cell to devote its energy resources into cellular replication. Can the population of β-cells afford to limit secretion under these circumstances when demand is elevated? In the case of the *ob/ob* model, clearly it can as evidenced by plasma insulin levels of ~30-50 ng/mL [35]. In the presence of hyperglycemia and insulin resistance (at least in the case of *ob/ob* mice), the β-cell appears to deem proliferation and increasing mass to be preferential over increasing exocytosis to achieve demand. It is important to note that the role of miRNAs in inhibiting insulin release has never been shown to equate with complete suppression of the pathway and this is also fitting with its role as a 'modulator' of exocytosis. Therefore to attenuate secretion by 25 or even 50% its normal rate, may be necessary for conserving the resources of the cell after increasing β-cell mass by ~8-fold. Especially under chronic conditions of hyperglycemia and insulin resistance, the reduced secretory output may not only be necessary to maintain proper internal dynamics of the cell but it may also be inconsequential over the course of a 24-hour period. Under chronic conditions, the β-cell may simply operate with reduce output for extended durations of time compared to when the cell is challenged under more acute conditions.

In summary, the hypothesis outlined here proposes a role for miRNAs in the β-cell whereby their function is tightly linked to the energetics of the cell. As stipulated by the first law of thermodynamics, energy in a closed system will always remain conserved [36]. To extrapolate this principle to the β-cell, it is reasonable to presume that in the presence of increased glucose concentrations, the cell does not have the capacity to simultaneously increase secretion and promote proliferation and recent evidence from genetic knockout models support this hypothesis. To substantiate this view, additional studies need to address how precisely miRNAs manage this balance. Do all of the genes targeted by the miRNAs contribute to both cell proliferation and exocytosis? Might RNA-binding proteins respond to metabolic signals and dictate the targeting of genes which drive the cell toward exocytosis and away from proliferation and vice versa [31]? Are miRNAs modified to alter their function [37]? Future studies may also determine that the inability of miRNAs to manage both adaptive

proliferation and the modulation of insulin release are hallmarks of β-cell failure and ultimately lead the cell on a path to either apoptosis or de-differentiation [38].

Perspectives Beyond the β-cell

The views offered here draw from more than a decade of research in the field of miRNAs and the β-cell and at this stage it is prudent to also examine how these hypotheses may extend beyond this one specific cell type. As mentioned in the introduction of this review, the metabolism of glucose extends to virtually all living organisms and as cells in mammalian systems began to diversify, so did the genes which manage the energy resources of the cell. In comparing humans to less complex organisms such as the worm and fly, while insulin-related peptides and their signaling cascades are conserved great distances, the majority of miRNAs are not perhaps owing to several reasons: a decrease in tissue complexity, differences in energy requirements, and lifespan [39] [40] [41]. MiRNAs may have simply evolved as organisms and their cellular make-up transformed with respect to size, complexity, and demand for energy.

Glucokinase in mammals is abundant in the liver hepatocyte, endocrine pancreas, neurons of the brain, and the gut; however its expression becomes more limited in organisms that are more distantly related to humans [42]. Can observations in the b-cell be extended to other key tissues involved in glucose and energy homeostasis? The liver maintains a high regenerative capacity and maintains a central role in both glucose and lipid metabolism and it would be of great interest to determine whether the miRNA pathway is integral to balancing these processes in the hepatocyte [43]. In the steady state, proliferation rates are low, however it is unclear whether its function in gluconeogenesis, glycogen storage, and lipid oxidation are compromised as soon as the cell's demand for growth are increased [44]. The intestine is also known for its high capacity for glucose utilization and proliferation, however it is also unclear whether miRNAs play a role in this tissue to maintain the balance of these functions [45]. Lastly, in humans, the central nervous system maintains the highest demand for energy requiring a continuous supply of glucose from the blood [46]. While proliferation rates throughout the central nervous system are difficult to detect, glucose metabolism is linked with several aspects of cellular physiology of the brain including signaling, plasticity and synapse formation. In light of the high degree of overlap between the β-cell and neurons of the brain in terms of gene expression, it also worthy to address whether miRNAs contribute to the adaptive processes of the nervous system as they relate to glucose metabolism, synaptic transmission and axonal assembly.

Summary

As miRNAs continue to be linked to each aspect of β-cell physiology including insulin expression, vesicle trafficking and exocytosis, mitochondrial function, and proliferation, these observations indicate miRNAs contribute to every fundamental property of this cell type. While many key questions regarding their cellular role remain to be answered, it is clear going forward that miRNAs as well as other non-coding RNAs will ultimately be viewed as important regulators of gene expression. In the entire context of the cellular machinery, miRNAs will presumably soon be viewed as one of the many factors which bind mRNAs to either inhibit their translation or facilitate degradation according to the metabolic context of the cell (i.e. extracellular glucose levels). To bring additional clarity to this picture, future studies should

address the global temporal regulation and stability of β-cell transcripts in response to changes in extracellular glucose concentrations. The crosslinking protocols that have been developed to identify target sites of miRNAs and RNA-binding proteins can be implemented on the stem cell and fibroblast models now available to understand how non-coding RNAs function in as cells differentiate into insulin-producing β-cells [47] [48] [49] [50]. Improving these protocols to optimize the functional properties and viability of these β-cell models remain a top priority for the development of future therapeutic strategies for combating diabetes.

Practice Points

- A greater understanding of the functional role of miRNAs in the β-cell could improve stem cell differentiation protocols to enhance secretory function, insulin production, and viability

Research Agenda

- How do miRNAs precisely contribute to the management of the balance between growth and secretion?
- To what extent and which RNA binding proteins mediate miRNA function?
- In which cellular compartments to miRNAs function and reside?
- To what extent are miRNAs modified and how might this impact their function?
- How does the half-life of a miRNA impact its function?

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