Unravelling Post-transcriptional Regulators of hIAPP translation in Human Pancreatic Islets

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Dedication

To the people whom I love

Acknowledgments

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Abstract

Unravelling Post-transcriptional Regulators of IAPP turnover in Human Pancreatic Islets

A hallmark of late-onset type-2 Diabetes mellitus (T2DM) is the formation of insoluble protein plaques or amyloidosis. Together with other factors, amyloidosis is linked to pancreatic β-cell failure and death, eventually leading to T2DM. Human islet amyloid polypeptide (hIAPP), or amylin is a major constituent of islet amyloid plaques. It is a small peptide hormone that consists of 37 amino acid residues and is secreted by pancreatic β-cells along with insulin in a 1:20 ratio. Insulin and hIAPP regulate blood glucose levels synergistically under normal conditions. Over the past 20 years, a plethora of studies have been done to understand the molecular mechanisms of intracellular and extracellular hIAPP aggregation and its cytotoxic effects on β-cells. These studies revealed that redox stress, inflammation and ionic disbalance are the main culprits in hIAPP-induced β-cell death. Toxic and aggregation prone species of hIAPP are an alarming issue in human health because hIAPP-derived plaques were found in over 90% of type-2 diabetic patients at postmortem together with the progressive β-cell loss. However, the cellular factors and mechanisms regulating hIAPP production under normal and ER stress conditions are still unclear.

If and how cellular stress particularly ER-stress, affects synthesis of hIAPP is less clear, and hence was investigated in this master project.

MicroRNAs (miRNAs) are ~22 nucleotide small non-coding RNA molecules implicated in the regulation of gene expression in eukaryotic cells. At present, there is a lack of studies and understanding in the field regarding a possible contribution and

importance of miRNAs in the regulation of hIAPP translation in β-cells. Hence, the main goal of this master project was to determine the regulatory role of RISC complex and its constituents, micro-RNAs and Ago1/2 proteins, in hIAPP turnover (synthesis and release) in normal and ER-stressed human islets. Recent studies show correlation of pancreatic islet miRNAs with β-cell stress and the development of T2DM, implying the role of miRNAs in T2DM progression and possibly hIAPP-derived islet amyloidosis. Thus, identifying and clarifying regulatory roles of miRNAs in hIAPP translation in pancreatic β-cells can be an important first step in the prevention of islet amyloidosis and T2DM.

In this project, I examined and identified posttranscriptional regulators of hIAPP turnover in normal and ER stressed human islets, notably miRNAs and Ago1/2 proteins. High glucose and ER stress downregulated the miR-335 and several other miRNAs. This downregulation of miRNAs, likely prevents its binding to hIAPP mRNA 3'-UTR, thereby upregulating hIAPP translation that leads to its accumulation, intracellularly and extracellularly. Functional studies confirmed the importance of miR-335 and Ago2 protein in hIAPP synthesis and its release from human islet beta-cells. This regulatory (inhibitory) pathway of hIAPP synthesis by miRNAs/Ago2 complex could potentially serve as a novel, druggable target to limits hIAPP overproduction and aggregation, ultimately preventing hIAPP-induced β-cell demise and progression of T2DM.

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Chapter 1: Introduction

A hallmark of late-onset type-2 Diabetes mellitus (T2DM) is the formation of insoluble protein plaques or amyloidosis (1-6). Together with other factors, amyloidosis is linked to pancreatic β-cell failure and death, eventually leading to T2DM (7-13). Human islet amyloid polypeptide (hIAPP), or amylin is a major constituent of islet amyloid plaques. Amyloid is a small peptide hormone that consists of 37 amino acid residues and is secreted by pancreatic β-cells along with insulin in a 1:20 ratio. Insulin and hIAPP regulate blood glucose levels synergistically under normal conditions (14,15). Over the past 20 years, number of studies have helped our understanding of the molecular mechanisms of intracellular and extracellular hIAPP aggregation, and its cytotoxic effects on β-cells (3,12). These studies revealed that redox stress, inflammation and ionic disbalance are the main culprits in hIAPP-induced β-cell death. However, the cellular factors and mechanisms regulating hIAPP production under normal and ER stress conditions are still unclear. Toxic and aggregation prone species of hIAPP are an alarming issue in human health because hIAPP-derived plaques are found in over 90% of type-2 diabetic patients together with the progressive β-cell loss (1,3).

Over the years, there has been steady progress in our understanding of the impact of hIAPP-derived islet amyloidosis and ER stress, two hallmarks of T2DM on β-cell function and viability. Among other factors, hIAPP and ER stress were linked to β-cell stress and dysfunction leading to islet amyloidosis and T2DM in primates, including humans, and transgenic animals (16). How cellular stress, particularly ER-stress, affects synthesis of hIAPP is less clear, and is central aim of this master project.

MicroRNAs (miRNAs) are ~22 nucleotide small non-coding RNA molecules implicated in the regulation of gene expression in eukaryotic cells (16). At present, there are insufficient studies and understanding in the field regarding possible contribution of miRNAs in the regulation of hIAPP translation in β-cells. Hence, the ***main goal*** of this master project was to determine the regulatory role of RISC complex and its constituents, micro-RNAs and Ago1/2 proteins, in hIAPP turnover (synthesis and release) in normal and ER-stressed human islets. Recent reports show that alterations of several pancreatic islet miRNAs correlate with β-cell stress and the development of T2DM, implying their role in T2DM progression (17). miRNAs regulate gene expression post-transcriptionally by binding to the 3'-UTR sequence of target genes via miRNA 'seed' sequences, resulting in translational repression and/or degradation of mRNA (18). This mechanism allows miRNAs to regulate many important biological processes such as cell differentiation, proliferation, survival, and apoptosis (19,20). According to the genome sequence analysis, most of human genes can be targeted by miRNAs (21). Many studies have reported significant roles of miRNAs in proper functioning and survival of pancreatic β-cells, and established a clear link between miRNA expression and pancreatic β-cell function (16). However, little is known about the miRNA expression and its impact on hIAPP turnover in the pancreas under high glucose and ER stress conditions. In this respect, identifying and clarifying the regulatory roles of miRNAs in hIAPP translation in pancreatic β-cells can be an important first step in the prevention of islet amyloidosis and T2DM. *I have studied* the regulatory role of miRNAs in hIAPP translation under normal and diabetes-mimicking (ER-stress) conditions. Deciphering the role of miRNAs and the RISC complex

in human islet amyloid polypeptide and its turnover is relevant for understanding the etiology of islet amyloidosis and T2DM.

Chapter 2: Literature Review

Type two diabetes mellitus

T2DM is a multifactorial metabolic disease caused by genetic and environmental factors (1-4). In the past several years, the number of people diagnosed with diabetes mellitus worldwide has quadrupled. In 2015, 415 million people aged between 20-79 years were diagnosed with diabetes according to the World Health Organization and The International Diabetes Federation (IDF), approximately 90% of whom have T2DM; and this number was estimated to rise by 2040 (2,4,6). In addition to genetic predisposition, unhealthy lifestyle, diet and being physically inactive contribute to the growth T2DM globally. At molecular level, insulin insensitivity resulting from obesity, and inability of pancreatic β-cells to secrete insulin which increases the need for its secretion, and β-cell apoptosis result hyperglycemia (1,5,6). Several factors are considered as inducers of β-cells apoptosis that involve both intrinsic and extrinsic apoptotic pathways mediated by pro- and antiapoptotic proteins and caspase family, respectively. A well-known factor is chronic hyperglycemia that degranulates β-cells and reduces glucose induced insulin secretion (22). In T2D rodents, the balance of pro-apoptotic genes is exceeded that of anti-apoptotic proteins resulting β-cell apoptosis. Postmortem studies suggest that exposure of β-cells to ER-stress inducers, free fatty acids such as palmitate and oleate induce their apoptosis. Other possible cytotoxic pathway is oxidative stress induced by insulin hypersecretion associated with insulin resistant state in obese patients which in turn leads to the glucotoxicity. β-cell function and viability may also be compromised from ER stress and elevated prolonged Ca^{2+} level in the cytosol (23). In addition to all the given factors, hIAPP-derived toxic oligomers and islet amyloidosis associated β-cell apoptosis are

currently being considered a major focus of many investigators because of their detection in most of T2DM patients at autopsy.

Amyloidosis and T2DM

A hallmark of late-onset type-2 Diabetes mellitus (T2DM) is the formation of insoluble protein plaques or amyloidosis (1-6). hIAPP plaques have been found in the pancreas of ~90% patients with T2DM at postmortem (1-3). Islet amyloid was initially found in diabetics pancreas as proteinaceous deposits, initially named “islet hyalinization” (because of its glassy look), first reported in 1901 (24,25). Since these pancreatic islet amyloids can absorb dyes applied for staining starch, earlier they were thought to be carbohydrates, and the name was changed from “islet hyalinization” to “amyloid” which means “starch-like” (24,25). Biochemical studies identified islet amyloid polypeptide (IAPP), a small peptide hormone consisting of 37 amino acids, as the main component of insoluble protein aggregates in the pancreas harboring amyloid plaques (1,9).

Under normal physiological conditions, hIAPP is co-expressed, co-stored, and co-secreted with insulin that regulates blood glucose levels (1,3,5). In T2DM patients, hIAPP has strong propensity to misfold and form toxic oligomers, and amyloid deposits both intracellularly and extracellularly (5). These toxic oligomers and aggregates largely cause massive β-cells loss in T2DM, thereby accelerating T2DM. Studies reported the formation of islet amyloid plaques, β-cells apoptosis, and progression of diabetes from artificially overexpressed hIAPP in β-cells (5). There is a strong association between hIAPP misfolding and T2DM as many studies observed significant accumulation of - deposits within and surrounding degenerated and apoptotic cells causing loss of β-cells mass (26). In agreement with clinical studies, longitudinal studies done in baboons reported the

presence of hIAPP-derived amyloid deposits not only at the onset of T2DM but even during an initial phase of diabetes when β-cells are able to compensate insulin resistance and hyperinsulinemia (6,7). Complementary studies performed in cats and monkeys also showed preexistence of amyloid deposits prior to the onset of hyperglycemia (3-5). Importantly, the severity and toxicity of islet amyloidosis strongly correlated with the onset and progression of T2DM. Similarly, in hIAPP-expressing transgenic mice severe form of disease often correlated with extensive amyloid deposition in the pancreas and a decrease of islet β-cells (3). Interestingly, in rodents hIAPP does not aggregate, as rodent isoform lacks amyloidogenic region within the polypeptide due to 3 key amino acids (aa) substitutions (6). Exogenous factors may stimulate hIAPP aggregation as islet amyloid plaques often contain hIAPP-associated apolipoprotein E (apoE) and heparan sulphate proteoglycans (5,11).

According to an *in vitro* study, hIAPP has great tendency for rapid aggregation in physiological buffers (unlike rat IAPP). It is reported that only mature insulin can inhibit hIAPP aggregation (1,24). Thus, defects in pro-insulin processing to make fully processed mature form could be one reason for hIAPP aggregation in T2DM. Another reason for hIAPP aggregation could be defective pro-hIAPP processing into 37 aa fully functional mature form since N-terminal intact pro-hIAPP was found in the pancreatic β-cells of diabetic patients (24,25). Even though pro-forms of peptides have characteristics of strong self-association and capability to form amyloid aggregates, they are less amyloidogenic and toxic as compared to mature forms, suggesting protective role of these pro-residues (24). Excessive intracellular and extracellular accumulation of amyloid aggregates induces many pathological events such as calcium overload, cell membrane disruption, ER stress,

mitochondrial dysfunction, defective autophagy, oxidative stress, and activation of JNK and caspase-3 death signaling pathways (9). In addition to the loss of β-cell mass, secretory studies have shown that formation of islet amyloid is associated with the reduction of insulin secretion.

Conformational Changes and Aggregation of hIAPP: A Causal Link

It was reported earlier that the dynamics of hIAPP oligomerization and aggregation are key determinants of amylin toxicity (24). For that reason, the investigators developed the biophysical methods such as fluorescence and circular dichroism (CD) spectroscopy and high-resolution microscopy that track the dynamics in real time and are in frequent use nowadays (24). Advantages of these biophysical approaches are that they allow studying the hIAPP aggregation at molecular level in response to cellular factors (pH, ionic content and temperature) that affect hIAPP oligomerization and development of amyloid deposits. The aggregation kinetics of amyloidogenic peptides *in vitro* is commonly monitored via diagnostic dye Thioflavin (ThT) fluorescence assay (24). The process of fibrillization starts first with Lag (nucleation) phase and follows by sigmoidal (fibril growth) phase, and this conformation or dynamics is common for other amyloidogenic proteins (24).

hIAPP is toxic to pancreatic β-cells

As explained above, some aggregated species of hIAPP show cytotoxic properties, and are believed to be of critical importance for the progression of T2D. While some studies have suggested that smaller non-fibrillar oligomers contribute to the toxic effects of hIAPP (26) in β-cells, *in vitro* and *in vivo* studies identified both hIAPP oligomers and fibrils as

toxic to the pancreatic islets (4). hIAPP oligomers are considered cytotoxic due to their ability to form nonselective membrane ion channels, a common characteristic of other toxic oligomers, resulting in the elevation of cytosolic Ca^{2+}, ER stress and apoptosis (9,17,21,27). Because of its ability to cross the lipid membranes, depending on the lipid-to-peptide ratio, it is believed that locally increased hIAPP concentration may enhance its toxicity (24,25,28).

Moreover, in hIAPP expressing rodents its overexpression led to an accumulation of intracellular reactive oxygen (ROS) such as hydrogen-peroxide (4). This ROS accumulation may impair mitochondrial function, thus leading to hIAPP-mediated oxidative stress and β-cell toxicity (4,5). The overexpressed hIAPP induces unfolded protein response (UPR) with impaired autophagy and detoxification that can also promote the β-cell apoptosis (5). The conclusion is supported by *in vitro* and *in vivo* studies performed in hIAPP-transgenic animals and human islets, which link hIAPP oligomers and aggregates (fibrils) to hIAPP toxicity in pancreatic islets and T2DM progression (28). Thus, hIAPP could be the accelerator of β-cell death by disturbing the functions of essential factors critical to β-cells.

The islet amyloid polypeptide and endoplasmic reticulum (ER) stress

Many studies associate ER stress with β-cell apoptosis in T2D development (29). The state of ER homeostasis can be altered to a stress state via constant overexpression and misfolding of proteins and peptides like hIAPP and insulin that may overwhelm the ER biosynthetic capacity. Consequently, this triggers downstream pathways that support β-cell function and survival. This compensatory response pathway is also known as unfolded

protein response (UPR) which adapt cells to ER stress (5). However, a severe, and chronic ER stress impairs the UPR by activating three apoptotic pathways: CHOP, a key mediator of apoptosis during ER stress, IRE1-JNK pathway, and caspase 12, an inflammatory caspase (29). Generally, apoptotic pathways are there to eliminate damaged cells thereby protecting healthy cells and tissues. Pathological investigations revealed that β-cell apoptosis in human pancreatic islets of Langerhans quantitively correlates with IAPP-derived amyloid deposits (1,29). Excessive accumulation of misfolded insulin and hIAPP along with other pathophysiological conditions impair endoplasmic reticulum (ER) homeostasis in pancreatic β-cells (29). Chang-jiang Huang et al. reported that hIAPP-mediated induction of endoplasmic reticulum (ER) stress causes β-cell apoptosis and increased polyubiquitination of proteins (2,30) indicating possible presence of misfolded or damaged proteins. They also proposed ER stress as a mechanism through which hIAPP induces β-cell apoptosis, and that mechanism is specific for T2D but not T1D. These studies are consistent with the role of misfolded protein mediated β-cell apoptosis in T2D (2,30). Also, as Casas et al. demonstrated extracellularly added hIAPP aggregates induce ER stress in mouse β-cells via inhibition of ubiquitin-proteasome system and activation of pro-apoptotic pathway.

IAPP biology and turnover mechanisms

IAPP is a small peptide (37 amino acid) hormone. Under normal physiological conditions, IAPP is produced, co-stored, and co-secreted with insulin in (human and rodent) pancreatic β-cells (1-3,5). IAPP is also found in rat, mouse, cat, and human gastrointestinal tract, and in rat and mouse sensory neurons (24). However, in chicken IAPP is mainly expressed in the brain and intestine and at lower levels in the pancreas (24).

During early pregnancy, IAPP is primarily expressed in human and rat placenta and is highly transcribed in the placenta in the third trimester of pregnancy in humans, and 16 days of gestational age in rats, and is lower as gestation progresses (24). It was proposed that IAPP could be potential regulator of food intake during the first two trimesters of pregnancy in humans and rats for both the mother and fetus since this time is highly anabolic (24) to synthesize and store nutrients. Although exact hormonal function still needs to be investigated, other studies propose that IAPP controls food intake and energy homeostasis. It regulates food intake by acting as a potent satiation signal that reduces secretion of gastric juices and glucagon hormone and reduces the rate of gastric emptying (10). Peripheral IAPP regulates this satiety by directly binding to area postrema neurons, which are rich for IAPP receptors and subsequently convey this signal to other brain areas (1). Interestingly, the central regulatory pathway by which other gastrointestinal peptides like cholecystokinin, glucagon-like peptide 1 (GLP1) and peptide YY 3–36 suppress eating, widely overlap with that of IAPP (31). In addition, IAPP is also involved in adiposity signaling and, like leptin, in body weight regulation all through adult life. Studies with animal and human subjects showed that combinational application of leptin and IAPP increases leptin responsiveness in anti-obesity treatments, which suggests the synergistic function of these hormones. IAPP regulates energy balance via controlling nutrient flux and energy output of the body; however, more studies are needed to understand the exact mechanism and physiological relevance for this regulation. IAPP is also involved in the development of bone, kidney, and pancreas and in the early postnatal development of hindbrain in mouse (24). In addition to its hormonal role, IAPP imposes important paracrine and autocrine effects in islets by regulating glucagon and insulin release from α-

and β-cells, respectively. IAPP also regulates glucagon and insulin release from α- and β-cells, respectively, by inhibiting their expression thereby imposing its paracrine and autocrine effects (24).

IAPP transcription

IAPP belongs to a calcitonin gene-related peptide family together with calcitonin, adrenomedullin and several other peptides. It is encoded by a single-copy gene on chromosome 12 (1,3). By contrast, other members from a calcitonin family are encoded by genes located on chromosome 11 (3). The promoter that controls IAPP transcription is complex which spans from -2798 to +450 relative to the start site of the transcription (Fig. 1) (31). Human and rodent IAPP gene promoter contains three exons, first of which is a site for IAPP transcription and second for its translation (8). Rat IAPP promoter contains a FLAT-like domain [A3 and A4] and NIR and FAR regions [E1 and E2] (8). Abnormal expression of IAPP is reported with the mutation of these elements (8,9). Rat IAPP promoter also contains a palindromic sequence in its 5' flanking region similar to the promoter sequences of the hIAPP and rat insulin 2 gene (8,9,10). Studies also found that IAPP promoter activity and transcription are stimulated by glucagon and glucagon-like peptide (GLP). Interestingly, this study also found that insulin also stimulates IAPP transcription (31) despite their co-expression. Bhowmick and Jeremic demonstrated the hIAPP promoter activation by its specific transcription factor FoxA2 (will be discussed later), requiring full proteasome activity (23) and intact nucleolar organization. IAPP and insulin promoters contain similar glucose responsive elements with corresponding transcription factors such as PDX1 (Fig. 1) (1, 5, 8).

IAPP transcription occurs via binding of PDX1 and other transcription factors at either the proximal or distal TAAT element thereby activating IAPP promoter (Fig. 1) (8,11). This activation of IAPP promoter is calcium dependent (1). In contrast, PDX1-dependent insulin gene expression does not require calcium (8,12). Additional transcription factors also contribute to IAPP expression such as IEF1 by cooperating with PDX1 (8,12,32). ISL1 is another transcription factor that has binding sites within both hIAPP and insulin promoters. Interestingly, ISL-1 regulates IAPP expression positively when it binds to the FLAT domain of hIAPP promoter and negatively regulates insulin expression through binding to the same element of insulin promoter (32). ISL-1 interaction with carbohydrate responsive element binding protein (CREBP), also activates IAPP expression while the same interaction functions as repressor of insulin expression (32). These contrasting regulations of IAPP and insulin genes are described as a differential regulation of gene expression that happens under pathological conditions such diabetes, obesity, and pancreatic cancer (32) despite their shared biosynthetic pathway. hIAPP and insulin steady state mRNA and protein expression levels also differ in response to high glucose. For example, under normal physiological conditions the steady state level of IAPP is ten times less than that of insulin, while under hyperglycemia (high glucose) conditions, a ten-fold increase is observed in IAPP but not insulin mRNA and protein steady state levels. This differential gene expression probably occurs at the promoter level (differential regulation of their promoters by gene-specific transcription factors such as forkhead box protein A2 (FOXA2) (Fig. 1) (26,33). However, different processing of two peptides at the posttranscriptional level may also contribute to this disproportional rise in IAPP protein levels in high glucose-challenged β-cells (33).

FOXA2, a member of FoxA family proteins, is IAPP-specific transcription factor and has three binding sites on the IAPP promoter. Bhowmick and Jeremic demonstrated that FOXA2 regulates IAPP transcription in proteasome-inhibited human islets. The FOXA2 is also important in glucose induced IAPP transcription. Recent study found the elevated levels of hIAPP expression in ER stress-induced human islets (33). The study found that the conserved proximal FOXA2 (606 bp)-binding site was critical for this promoter activation via increasing the FOXA2 binding by its forkhead box domain by high glucose and ER stress (26,33).Such involvement of FOXA2 in regulation of IAPP transcription is another possibility for IAPP differential expression. In addition to the transcription factors, proteasome complex (5), ISL-1, cAMP, calcium level, and small GTPases are reported as potential regulators of selective expression and secretion of IAPP and insulin (8). For example, FOXA2-dependent and independent proteasome-mediated IAPP transcription was recently reported (33). In addition to the transcriptional regulation of IAPP expression under ER stress conditions, it was proposed that IAPP expression may also be regulated at the posttranscriptional and translational levels (33).

hIAPP translational and posttranslational control mechanisms

It is reported that IAPP is produced predominantly by translational control of pro-hIAPP expression along with pro-PC1/3, pro-PC2, and processing chaperones of them pro-7B2 (34). The hIAPP is initially synthesized in beta-cells as an 89-amino acid (aa) residue preproIAPP (36 kDa) (1,34). The 89-aa preproIAPP consists of a 22-aa signal peptide and two short flanking peptides (1). Similar to insulin, preproIAPP is further processed post-translationally and proteolytically in the endoplasmic reticulum (ER), Golgi, and secretory

vesicles yielding proIAPP (34 kDa) (1). In this processing, the signal peptide cleavage takes place in the ER and two flanking peptides are processed at double basic aa residues and proIAPP conversion to IAPP takes place in the secretory vesicles (3). This processing involves prohormone convertase 1/3 (PC1/3) that starts processing at the COOH-terminal, prohormone convertase 2 (PC2) that cleaves proIAPP at the NH2 terminal, and carboxypeptidase E (CPE) that produces a fully functional 37-aa (6 kDa) small peptide hormone in the late Golgi (1,3). It is known that PC2 can compensate the PC1/3 function, and therefore compromised PC2 function may also promote amyloid deposition in the β-cells of T2DM pathogenesis (34).

The produced IAPP together with insulin then stored in the secretory granules of pancreatic β-cells (1). Under normal physiological conditions, fully processed IAPP becomes functional and controls satiety as a paracrine signaling peptide thereby suppressing gastric emptying, inhibits insulin secretion, and regulates glucose homeostasis by inhibiting glucagon release (1,28). However, when blood glucose level is elevated and other physiological signals are stimulated, (characteristics of), hIAPP misfolds forming toxic oligomers and aggregates (1).

Role of miRNA in protein translation

miRNAs are ~20-22 nucleotide small non-coding RNA molecules that regulate expression of protein coding genes at post-transcriptional level in eukaryotic cells (16). Understanding its biogenesis is important since only mature miRNAs processed through sequential steps are capable of regulating gene expression. miRNA biogenesis takes place in the nucleus and cytoplasm. In the nucleus, miRNAs are initially transcribed via RNA

polymerase II into a long stem loop structure transcript called primary miRNA (pri-miRNA) (Fig. 2) (19,35). The stem loop structure within pri-miRNA contains the final duplex miRNA which is not yet functional (35,36). To become functional, the pri-miRNA is further cleaved by RNase III Drosha in a complex of DGCR8, a double stranded RNA binding protein, producing a small hairpin structure 70 nucleotide precursor miRNA (pre-miRNA), pre-miRNA is then exported from nucleus to the cytoplasm via exportin5 (Fig. 2) (35,36). In the cytoplasm, pre-miRNA undergoes a final step of cleavage by another RNase III enzyme, Dicer in a combination with a transactivation-responsive (TAR) RNA-binding protein (TRBP) that produces a 20-22 base-paired double stranded miRNA, or "miRNA/miRNA*duplex" (Fig. 2) (35). One strand of the duplex that becomes functional is loaded onto AGO2 forming RNA-induced silencing complex (RISC) (35). Functional and mature miRNAs guide AGO2 to the target sequences of mRNAs noncanonically at the 5'UTR and within coding genes, and mostly canonically at the 3'UTR of mRNAs(35). This target sequences are complementary to the mature miRNA "seed sequences" (35). Upon binding to its target gene depending on extensive/perfect or partial/imperfect binding, the RISC complex causes two ways of regulation: mRNA degradation or translational repression or both, respectively (35). miRNAs regulate gene expression in the form of ribonucleoprotein complex (miRISC-miRNA-induced silencing complex) (35). Well studied protein components of mi-RISC are argonaute (Ago) and glycine-tryptophan (GW) repeat-containing 182 kDa (GW182) proteins (35,37).

Generally, mRNA translation is comprised of three steps: initiation, elongation, and termination (35). Many studies showed that miRNA-mediated translation repression occurs at the initiation and post initiation (elongation) steps of translation. mRNA translation is

initiated with 5'cap recognition, including the formation of preinitiation complex of assembled initiation factors with met-tRNA and a 40S ribosome (35,38). It is proposed that the miRISC inhibits the initiation step of translation by preventing the formation of preinitiation and initiation complexes (35). Specifically, it is reported that Ago2 competes with eIF4F for binding to mRNA m7G-cap (by the capability of AGO2 to compete with eIF4E for binding to the cap structure, m7G-cap of mRNA), interferes with recruitment of 40S small ribosomal subunit or preventing the binding of 60S subunit and eventually the formation of 80S ribosomal complex (35). Also, GW182 protein binding to the poly(A)-binding protein (PABP) (106, 156) could prevent the eIF4G-PABP-mediated formation of closed-loop thereby repressing the initiation step of translation (35). The elongation step (post-initiation step) of translation was also reported as a possible target of miRISC by promoting ribosome dissociation or nascent polypeptide break down though the mechanisms are not completely understood at this time (35). Although many studies reported the mechanism of translational repression without reduced mRNA steady-state level, other studies demonstrated miRNA-mediated degradation of mRNAs.

In general, capped and polyadenylated stable mRNA starts degrading with the reduction of the poly(A) tail by 3'-5 exoribonucleases. The deadenylating enzymes are recruited by the protein factors recruited to cis-acting elements within the 3'UTR to stabilize mRNAs (35). One of the examples of these cis-acting elements is AU-rich elements that are the binding sites of the miRNAs (35). Three types of miRNA-mediated mRNA degradation are known: 1.Endonucleolytic cleavage through extensive complementary binding sites of miRNA that lead to the degradation of transcripts or translational repression; 2.Seed sequence (2-8 nucleotides of miRNA) required partially

binding; 3.AU-rich region required mRNA degradation (38). Many studies support the notion that mRNA destabilization occurs through poly(A) tail shortening followed by decapping and degradation of mRNAs by exoribonucleases. These processes, (poly(A) tail shortening and the subsequent mRNA decapping and degradation) require proteins of the Argonaute family (Ago1-Ago4) and GW182, main components of miRISC (35).

Role of Ago2 in protein translation

AGO1-AGO4 proteins comprised of evolutionarily conserved three domains - PAZ, MID, and PIWI (35). Among them, AGO2 is involved in miRNA-mediated repression in mammalian due to its PIWI domain that possesses RNaseH enzyme like activity and cleaves RNAs perfectly binding sites (Fig. 2) (35). The 5' miRNA is loaded in a "deep pocket" like junction of MID and PIWI domains of AGO2 by direct interaction or via a magnesium ion (35). GW182 interacts with AGO2 through GW repeats within GW182 N terminus that binds to the AGO MID/PIWI domain and promotes miRNA-mediated deadenylation of mRNAs by recruiting deadenylase complex, CCR4-NOT1 and direct interaction poly(A)-binding protein (PABP) (35). Moreover, it is reported that GW182 proteins that co similar to those "full-length proteins when tethered to the reporter 3'UTR". The components of miRISC complex (miRNA and Ago and GW182 proteins) were abundantly found in the cytoplasmic processing bodies, some of which believed to play significant role in repressing gene expression effectively by miRISC. Cellular organelles such as multivesicular bodies (MVBs) and endosomes were also found to be involved in miRISC activity. It is reported that competing interaction of GW182-AGO2 by point mutation or GW182 competitor reduces the miRNA-mediated regulation (Fabian et

al). In certain conditions, miRNA-mediated regulation is itself regulated by being prevented or reversed by other types of miRNAs.

In this way, miRNAs regulate many important biological processes such as cell differentiation, proliferation, survival, and apoptosis (17). According to genome sequence analysis, most human genes can be targeted by miRNAs indicating their potential roles in regulation of gene expression (20,21). Many studies report significant roles of miRNAs in proper functioning and survival of pancreatic β-cells and in diabetics, and established a clear link between miRNA expression dynamics and pancreatic β-cell function (16). For example, miR-124a targets IAPP transcription factor FoxA2 thereby downregulating PDX1 which is a transcription factor for both IAPP and insulin (26). It was also shown that glucose increases miR-124 in MIN6 insulinoma cells (36). However, miR-124a is inhibited by glucose induced thioredoxin-interacting protein (TXNIP) in rat INS-1 cells and mouse islets (36). Members of the same family, miR-30a-5p and miR-30d, are upregulated when exposed to high glucose conditions (36). Thus, miRNA levels are important indicators and regulators of normal β-cell function and signaling. However, possible role of RISC complex and its components, Ago proteins and miRNAs, in amylin turnover have not previously been investigated. Thus, main focus of my master project was to determine regulatory roles of Ago proteins and miRNAs in amylin protein synthesis and release from normal and high glucose- or ER-stressed human islets, which is important for understanding the origin of amylin aggregation and islet amyloidosis, and ultimately T2DM.

Chapter 3: Materials and Methods

Cell Culture and treatments

Human islets of non-diabetic donors (85-95% purity and viability) were obtained from the Integrated Islet Distribution Program (iidp.coh.org) for Human Islet Research. Partly dissociated islets were cultured in CMRL media (ATCC) supplemented 2mM glutamine, 1% (v/v) fetal bovine serum (FBS) albumin and 1% penicillin/streptomycin and plated on 96-well non-adherent plastic plates (VWR) at approximately 400-500 islets per well. Islets were cultured with designed treatments in 96-well plate at 37 °C in a humidified incubator with 5% CO_2 for designated period of times.

Quantitative real-time RT-PCR

mRNA analysis: The hIAPP, insulin, and other genes expression was investigated using quantitative real-time PCR (qRT-PCR) analysis. After three days of treatment and/or transfection, total RNA was extracted from human pancreatic islets using TRIzol reagent (Invitrogen) and Direct-zol RNA Mini kit (Zymogen, cat. no R2052) according to the manufacturer's instructions. Total RNA was further purified using in-column DNA digestion with RNase-free DNase to prevent DNA contamination, following manufacturer's instructions (Direct-zol Zymogen, cat. no R2052). cDNA was synthesized from ~250 ng of RNA using multiScribe™ reverse transcriptase and random primers (High-Capacity cDNA Reverse Transcription kit (Applied Biosystem) was then used according to the manufacturer's protocol. qPCR was performed using BioRad CFX 96 Real-time system (C1000 Touch thermal cycler). IAPP, insulin, and other genes levels were amplified using iTaq™ Universal SYBR green supermix (Bio-Rad) using the

following thermal cycling protocol: polymerase activation 95°C, 2 min; DNA denaturation 95°C, 5 sec; annealing and extension 60°C, 30 sec; repeat 40 cycles. All qRT-PCR primer sets are presented in table 1. Gene expression levels of all tested genes were normalized to β-actin expression. Relative mRNA levels of IAPP, insulin and other islet cell transcripts were calculated using the $2^{-\Delta CT}$ method (33).

miRNA analysis: cDNA was synthesized, and individual miRNAs were quantified in a quantitative real-time SYBR Green reverse transcriptase (RT) – PCR reactions using MystiCq miRNA cDNA synthesis kit (Sigma-aldrich, cat. No MIRRT) that includes poly(A) polymerase, ReadyScript reverse transcriptase, specific MystiCq miRNA qPCR assay primers, and MystiCq universal PCR primers available from Sigma (Table 1). miRNAs expression levels were normalized to SNORD44 (a human positive control primer), ubiquitously expressed small nucleolar miRNA. Relative expression levels of miRNAs were calculated using the $2^{-\Delta CT}$ method (1).

Transfections

MiRNA transfection: mission transfection reagent (sigma-aldrich, cat. No S1452) is used for the transfection of miRNA mimics. miRNA transfection complex was prepared by mixing 1 ul of 5 nM miRNA with 1 ul transfection reagent in a 50 ul serum-free islets growth medium followed by 15 min of incubation to allow formation of transfection complex. Transfection complex was then gently poured over the cultured islets, and incubated with human islets in antibiotic-free media at 37 ^{o}C for 24 hours. Following 24h, media was replaced with normal growth islet media, and islets incubated for additional 48 hours.

Ago-2 siRNA transfection: We used transfection reagent (Santa Cruz) to transfect human islets with siRNA complementary to Ago1 and Ago2. siRNA transfection complex was prepared by mixing 0.5 ul of 10 nM siRNA (Santa Cruz) and 0.5 ul transfection reagent in a 50 ul serum-free islet growth medium followed by 15 min of incubation to allow formation of transfection complex. Transfection complex was then gently poured over the cultured islets and incubated at 37^0C for 24 hours in antibiotic-free media. After 24h, media was replaced with normal growth islet media, and islets incubated for additional 48 hours.

Lentiviral Transfection or transduction of rat insulinoma (RIN) cells

mGFP-tagged lentivirus-mediated Ago2 overexpression were carried out with RIN-m5f cells. RIN-m5f cells at the density of 50 000 cells per well (or partially dispersed human islet cells) were plated in 96 well plates. Ready in use lentivirus particles were purchased from Origene. After 24 h of recovery time, lentivirus particles encoding Ago2 were added at the MOI (multiplicity of infection) of 5 and 10 and incubated for 24 h. Polybrene (8 μg/ml) was used during the transduction procedure to enhance transfection efficiency or viral integration. Lentivirus-containing medium was replaced with normal growth medium, and the cells were allowed to grow for an additional 48 h at 37°C. After 72 h of post transduction, proteins were collected for western blot analysis.

Western blot analysis

The regulatory roles of miRNAs and Ago1 and Ago2 on hIAPP and insulin expression were analyzed by western blot using our published procedure (23). Cell extracts of human islets were collected in 1% RIPA buffer (supplemented with protease and phosphatase

inhibitor cocktails, Invitrogen) and BCA assay was performed to identify relative protein concentrations. Equal concentrations of protein samples were separated using 4-15% Tris-Glycine SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) and non-specific IgG binding sites were blocked by incubating with 5% nonfat dry milk (VWR) dissolved in Tris-buffered solution (TBS) that contains 0.1% Tween-20. Membranes were probed with horseradish peroxidase (HRP)-conjugated and non-conjugated primary antibodies, followed by corresponding (rabbit-antirabbit) HRP-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology, Abcam) for 1 hour at room temperature. The following primary antibodies were used: mouse monoclonal anti-amylin-hrp (1:300, Santa Cruz Biotechnology, catalog no. 377530), rabbit monoclonal anti-insulin (1:1000, Abcam, catalog no. 181547), mouse monoclonal anti-insulin (1:300, Santa Cruz Biotechnology, catalog no. 8033), goat polyclonal, mouse monoclonal anti-actin-hrp (1:500, Santa Cruz Biotechnology, catalog no. 47778), rabbit monoclonal anti-eIF2C-hrp (1:500, Abcam, catalog no. 156870), rabbit monoclonal anti-FoxA2 (1:1000, Abcam, catalog no.108422). Blots were developed using Enhanced Chemiluminescence (Thermo Fisher Scientific) and documented using the BIO-RAD Chemi DOC touch gel imaging system. Band intensities were quantified using Bio-Rad Image-Lab software against actin as a loading control.

Statistical Analysis

The Graph Pad Prism 7 Program was used for plotting and statistical analysis. The unpaired Student's t test or one-way ANOVA followed by the Tukey post-hoc test was used for pair wise comparisons among groups when appropriate with significance established at $p< 0.05$.

The number of independent biological replicates per treatment group is listed in the figure legend.

Chapter 4. Results

High glucose and ER stress increase hIAPP but not insulin transcription in human islets

As stated earlier, transcription factors, FOXA2 and PDX1 are major regulators of IAPP transcription in pancreatic β-cells (Fig. 1). FoxA2 regulatory protein, thioredoxin-interacting protein has been implicated in FoxA2-mediated IAPP gene expression in rat and human β-cells cultured under normal and high glucose conditions (26). The authors demonstrated that overexpression of TXNIP increases IAPP transcription by promoting FoxA2 enrichment at the proximal FoxA2 binding site in the IAPP promoter (26). Interestingly, TXNIP is a common target of both ER stress and metabolic pathway in islets and other cell (33). Thus, it is conceivable that ER stress and high glucose conditions associated with T2DM may upregulate hIAPP transcription via shared TXNIP-mediated signaling pathway. To test this, we performed comparative transcriptomic analysis of hIAPP, insulin, and TXNIP gene expression in ER stress induced and high glucose challenged human islets (Fig. 3). The use of selective ER-stress agents, thapsigargin (TG) and tunicamycin (TN) are advantageous to separate metabolic and stress effects on gene expression. The qPCR analysis revealed significant (1.9±0.4, 2.7±0.5 and 3.4±0.3 folds) increase in hIAPP mRNA levels by TG, TN, and HG treatments, respectively compared to control (untreated) islet cells (Fig. 3). By contrast, insulin mRNA levels remained comparable (1.1-1.3 folds) to controls in all treatments (Fig. 3). hIAPP gene expression strongly correlated with the expression pattern of TXNIP evoked by these three agents. HG was the most effective in inducing TXNIP expression (8±0.3 fold vs 1.7±0.4 and 2.6±0.2 folds for TG and TN, respectively) (Fig. 3). Expectedly, synthetic ER-stress inducers were

more potent in inducing ER stress as compared to HG treatment, reflected by an additional 2-3-fold increase in levels of ER-stress marker BIP in TG- and TN-treated cells (3.9±0.4 and 8.9±0.9 folds) as compared to HG-exposed islets (1.6±0.3-fold, Fig. 3). Although overexpression of TXNIP may significantly elevate FoxA2 transcript and protein levels (26), we observed a small and non-significant increase of FoxA2 and PDX1 mRNA levels in stress induced (HG/TG/TN-treated) β-cells respective to controls (1-1.4 folds, Fig. 3) (33). Thus, results of my transcriptome analysis revealed that metabolic (HG-induced) pathways appear to be more potent in activating the TXNIP signaling pathway and its downstream targets such as hIAPP than the synthetic ER-stress inducers. Interestingly, TXNIP/FoxA2-signaling pathway remains operational and stimulates IAPP promoter activity under these adverse (ER stress) conditions (Fig. 3) (33).

A subset of hIAPP miRNAs pool is downregulated in ER stress induced human islets

In addition to transcriptional mechanisms and factors (Figs. 1, 3), posttranscriptional mechanisms are also important for glucose-evoked hIAPP production in β-cells (33). However, no study to date has explained possible mechanism or identified cellular (post-transcriptional) regulators of hIAPP translation under normal or stress conditions. In view of this and considering the importance of miRNAs on 3'-UTR target recognition in eukaryotic gene expression, I *hypothesize* that *miRNAs are critical in the regulation of expression of hIAPP and insulin in normal and ER-stress induced pancreatic β-cells*. To test the hypothesis, we analyzed expression of 13 putative miRNAs (Fig. 5), previously predicted by our lab using bioinformatics tools, to be partially complementary to the 3'UTR of hIAPP mRNA (Fig. 4.). Human islets were treated with either high glucose (HG, 20 mM, 48h) or thapsigargin (TG, 1 μM, 24h) to stimulate

glucose-responsive metabolic pathways (i.e glycolysis/TCA) or cellular stress in β-cells, respectively (Fig. 3, ref). Total RNA was isolated, and miRNAs converted to cDNA by polyadenylating and reverse transcribed (as explained in the method section). A 3-step real-time SYBR Green RT-qPCR method and miRNA-specific primers were used for this analysis (Table 1). RT-qPCR analysis revealed consistent downregulation of expression of mir-183-3p, mir-335-5p, and 495 among miRNAs tested. mir-183-3p and 495 were downregulated consistently at both treatments while mir-335-5p was downregulated only under TG treatment (n=3, Fig. 5). The results support our hypothesis that a subset of putative hIAPP-targeted miRNAs can be consistently downregulated under high glucose and ER stress conditions and regulate hIAPP translation. The above described three most consistently downregulated miRNAs were then used for further *loss of function* analysis described in next chapter.

miRNA mimics downregulates hIAPP protein expression

To determine if and the extent to which the most downregulated miRNAs (miR-182, -335, and -495, Fig. 5) regulate hIAPP translation under diabetes-relevant (high glucose) conditions, we performed transfection studies in HG-treated human islets using the 'mimic' oligonucleotides of these miRNAs (Fig. 6). Freshly isolated human islets were transfected with the transfection complex containing miRNA mimics at a concentration of 5 nM siRNA, transfection reagent and serum free media for 24 h. The islets were further incubated with HG (20mM) for 48 h. After 48 hr HG treatment, islet cell extract was prepared, and equal amounts of protein separated on SDS-PAGE. Resolved proteins were then electroblotted and signal developed with ECL kit, as described in Methods section. Western blot analysis of human islet extracts revealed significant downregulation of hIAPP

expression under mir-335 mimics, by ~2 folds relative to the scrambled oligonucleotides as control (n=3, Fig. 6). hIAPP expression remain unchanged under mir-495 mimics and slightly downregulated by miR-182 mimics (n=3, Fig. 6). Thus, biochemical analysis demonstrated that the miR-335 is critically required in hIAPP translation, as initially hypothesized.

Ago1 and Ago2 silencing increasse hIAPP protein expression and secretion

Argonaute proteins are critical components of RISC complex involved in the regulation of gene expression. In humans, four Ago proteins (Ago1-4) exist, and Ago2 is the only argonaute family member that possesses endo-nucleolytic capability. [Functionally, the difference between Ago1 and Ago2 is not clear]. To gain mechanistic insights into putative miRNAs role in hIAPP translation, we functionally repressed Ago1 and Ago2 proteins using synthetic siRNAs (Fig. 7). For this experiment, we transfected human islets with siAgo1, siAgo2 or scrambled (siSc) oligos (10 nM) to inhibit Ago1 and Ago2 expression/activity under normal and high glucose (HG) conditions. After 72 h of transfection and 24 h HG treatment, protein extracts (from cell lysates) were analyzed by western blot. In agreement with miRNA overexpression experiment, western blot analysis revealed a moderate ~ 20-30% increase in hIAPP protein levels in Ago1 and Ago2 silenced islets as compared to non-transfected HG-treated islets, although this change was not significant (Fig. 7A, B, n=3). This upward trend in hIAPP expression was also observed in human islets treated with siAgo1 and siAgo2 but not in siSc transfected cells (Fig. 7A, B) indicating Ago involvement in translational repression and/or degradation of hIAPP mRNA. Interestingly, siRNA Ago1 and Ago2 did not have any effect on insulin expression ($p>0.05$, n=3, Fig. 7 C, D). This finding is consistent with published data showing that

IAPP and insulin are differentially expressed under metabolic stress (HG) conditions (Bhowmick, 2018, Bhowmick, 2021).

We also tested the effects of Ago1 and Ago2 siRNAs on the secretion of hIAPP and insulin by analyzing their (extracellular) levels in cell media from control and siRNA-treated human islets, by Western blotting (Fig. 7A, B). The data corresponded with the protein expression studies: siAgo2 and to lesser extent siAgo1 but not siRNAsc increased extracellular content of hIAPP (Fig. 7A, B). However, siRNA Ago1 and Ago2 did not have any modulatory effect on the intracellular or extracellular protein levels of other pancreatic hormone, insulin (Fig. 7C, D). The specific effect of siAgo2 on hIAPP but not insulin turnover in human islets rule out non-specific effect of our treatments on general secretion and points out to transcriptional control of hIAPP release from these cells by Ago2. Collectively, transfection studies (Figs. 6-7) support the regulatory (repressor) role of RISC complex in the translation hIAPP.

Ago2 overexpression decreases hIAPP protein expression

To further confirm the role of RISC complex in hIAPP homeostasis, we overexpressed Ago2 by using mGFP-tagged Ago2 lentiviral construct. Instead of human islets, we used RIN cell line stably expressing hIAPP under CMV promoter to avoid cofounding contribution of transcription on hIAPP synthesis. To optimize multiplicity of infection (MOI), which is the number of transducing lentiviral particles per cell, lower (5 MOI) and higher (10 MOI) virus titer was used for transfection of RIN cells. After 72 h of lenti virus transduction, protein extracts were collected for western blot analysis. Initial set of experiments revealed the modest (~30%) decrease in hIAPP intracellular protein levels

in cells with a higher virus load (10 MOI) as compared to control cells (Fig. 8 A, B, n=1). This result is in agreement with siRNA effect on hIAPP protein expression (Fig. 7).

Chapter 5. Discussion

The hIAPP overexpression and accumulation are critical factors in β-cell apoptosis and T2DM progression (33). Therefore, it is important to understand the mechanisms and factors that regulate hIAPP expression under both normal and diabetes-relevant (high glucose and/or ER stress) conditions. Previous studies demonstrated the activation of hIAPP promoter by transcription factor FoxA2, which required proteasome activity (23) and intact nucleolar structure in stressed β-cells (33). Thus, stress-induced hIAPP biosynthesis is likely regulated at both transcriptional and posttranscriptional levels. However, there is no published data on posttranscriptional mechanisms driving hIAPP biosynthesis and turnover. In view of this, I have studied the role of miRNAs and Ago1/2 proteins, main components of the RISC complex, in hIAPP translation under normal and diabetes-mimicking (high glucose and/or ER stress) conditions. miRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level via degradation and/or translational repression of mRNAs (38). Studies demonstrate a pivotal role of miRNAs in cellular response to stress (38). Related to this project, the role of miRNAs in beta-cell apoptosis and T2DM pathogenesis and progression has been previously studied (39).

Here, we report for the first-time, possible involvement of miRNAs in hIAPP translation in beta cells. I *hypothesized* that *miRNAs are critically required for hIAPP synthesis, and that their downregulation caused by high glucose and/or ER stress upregulates hIAPP biosynthesis*. To identify miRNAs that are a key to hIAPP turnover, a comparative bioinformatics approach was used. We identified a set of 13 putative miRNAs complementary to the 3'UTR of hIAPP mRNA (Fig. 4), indicating their possible

involvement in regulation of hIAPP biosynthesis. Induction of metabolic stress (by high glucose) or ER stress (by chemical stressors) substantially (≥ 2 folds) and consistently downregulated miR-182, miR-335-3p, and miR-495, supporting our hypothesis. In line with the above finding, forced overexpression of miRNA mimics, particularly miRNA-335-3p, significantly downregulated hIAPP protein levels in high glucose-exposed human islets compared to negative (scrambled miRNA) control (Fig. 6), indicating its role in the translational repression of hIAPP under metabolic stress. Conversely, downregulation of Ago2 (by Ago2 siRNA) significantly increased hIAPP protein levels based on WB data as compared to the scrambled negative control siRNA, suggesting that Ago2 protein in complex with miRNAs acts as regulatory (limiting) factor in hIAPP synthesis either directly targeting 3'UTR of hIAPP or indirectly by targeting other hIAPP-regulating proteins. In agreement with our miRNA data, intracellular levels of several putative hIAPP miRNAs were observed to be downregulated in stressed human islets isolated from T2DM patents (40), suggesting that our findings may be clinically relevant. For example, replenishment of hIAPP-miRNAs in stressed human islets may halt hIAPP synthesis and accumulation, thereby reducing its aggregation and toxicity. The important contribution of RISC complex in hIAPP synthesis was further suggested in lentiviral experiments in which we overexpressed Ago2 construct in RIN cells stably expressing hIAPP. Initial data point to negative regulation of hIAPP protein levels by Ago2.

Literature further demonstrates that miRNAs directly target gene expression primarily via Ago2 binding at transcripts'(mRNA) 3'-UTRs. However, miRNAs may also regulate gene expression indirectly by downregulating expression of transcription factors. In case of hIAPP, miR-124 was shown to directly target (downregulate) hIAPP's main

transcription factor, FoxA2. Thus, it is possible that mir-124, in addition to targeting Ago2 to hIAPP's 3'UTR, indirectly restricts hIAPP transcription and protein synthesis by limiting the availability of an essential transcription factor FOXA2. However, both types of stressors (HG and TG) had small, non-significant modulatory effect on miR-124 expression (Fig. 5), suggesting this indirect scenario is less likely. Indeed, mRNA (Fig. 3) and proteins levels of FOXA2 are largely unchanged in stress-challenged human islets (33). Additionally, Ago2, a core protein of RISC complex, also competes with eIF4F for binding to mRNA m7G-Cap, preventing protein translation (35,37). This in turn may prevent expression of inducible genes such as hIAPP. In contrast to hIAPP, insulin protein level remained largely unchanged by any of the treatments or expression of transfection constructs. This finding is consistent with many studies in which insulin (biosynthetic) protein levels remain steady under various situations and cellular conditions. This is mostly due to well-known stability, abundance and long half-life of insulin transcripts.

In summary, this project examined and identified posttranscriptional regulators of hIAPP translation in normal and ER stressed human islets, notably miRNAs and Ago1/2 proteins. High glucose and ER stress downregulated the miR-335. The decrease in miR-335 levels in turn may prevent binding of RISC complex on hIAPP 3'-UTR, which requires further verification. This can be done by using hIAPP 3'UTR expression constructs containing mutated miR-335 binding sites. Thus, miRNAs/Ago2 complex could potentially serve as a druggable target to limits hIAPP overproduction and aggregation, ultimately preventing hIAPP-induced β-cell demise and progression of T2DM.

Figures

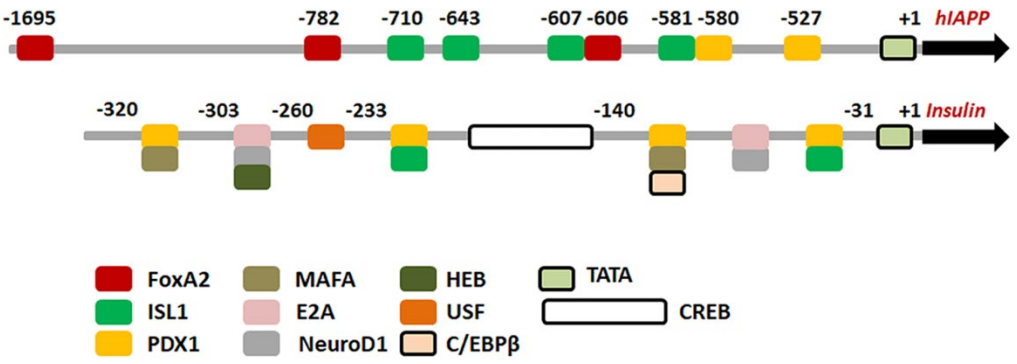
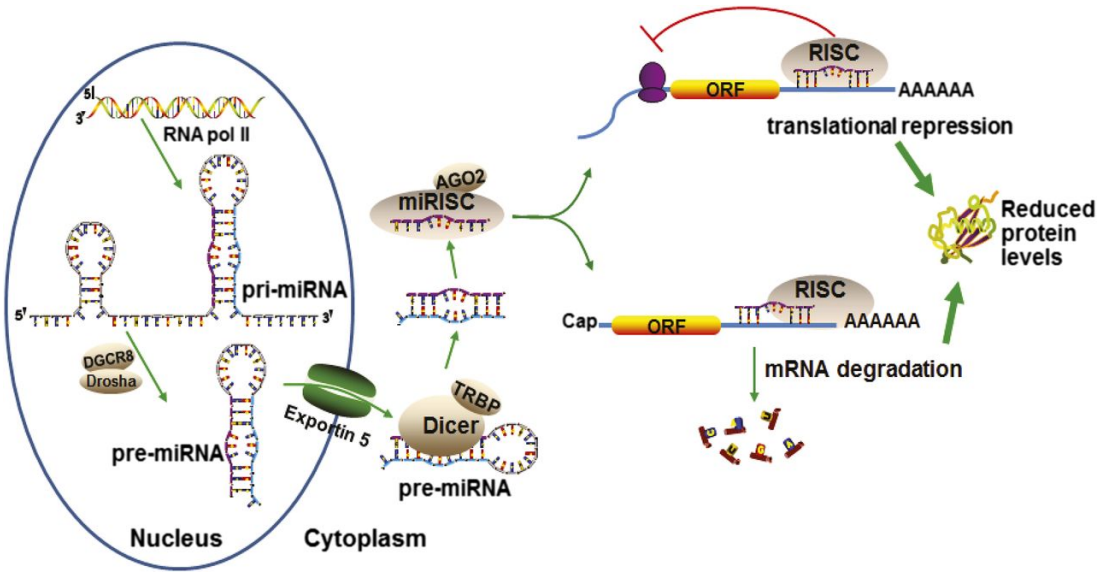


Figure 1. Transcriptional factors involved in the regulation of IAPP and insulin promoters (after Chatterjee Bhowmick et al, 2021).



After A. Gupta et al, 2012

Figure 2. Mechanism of miRNA-mediated protein translation

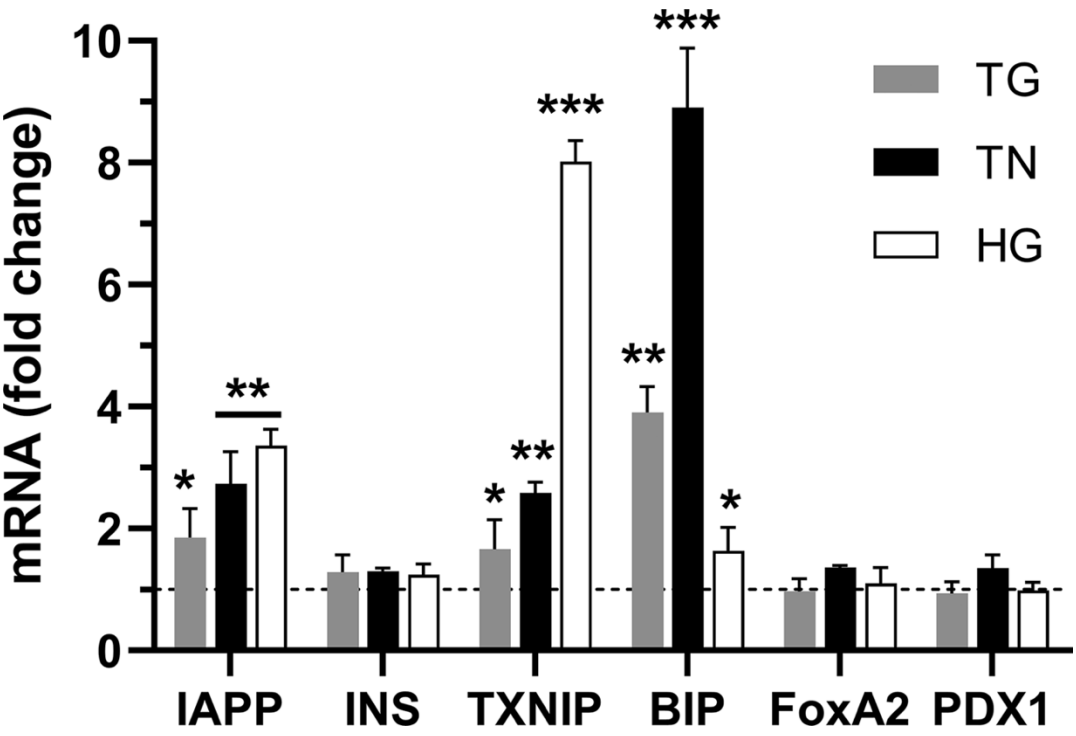


Figure 3. Transcriptomic analysis of normal and stressed cultured human islets. Freshly isolated non-diabetic human islets were cultured in the presence of high (20 mM) glucose (HG), 0.5 μM thapsigargin (TG), or 0.5 μM tunicamycin (TN) for 24 h. Changes in mRNA levels of various genes analyzed by RT-qPCR. Cq values, reflecting mRNA levels, were normalized to a housekeeping gene (actin) and relative gene expression was calculated as described in the method section. Line denotes baseline gene expression in control islets. Significance was established at *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA followed by Tukey's post hoc comparison test. Data represent mean ± SEM of three independent experiments.

1. **has-miR-124**

   5' ...UCCCUUUAUGAAGUGUGCCUUAG... (UTR) **Position 1(1528-1535)**
   ||||||
   3' CGUAAGUGGCGCACGGAAUU (miR)

2. **has-miR-143**

   5' ...UUAACAGUGCCCUUUUCAUCUCC... (UTR) **Position 1(193-199)**
   ||||||| |||||||
   3' CUCGAUGUCACGA----AGUAGAGU (miR)

   5' ...AGCCGAGAUUGCACCACUGCACU... (UTR) **Position 2(731-737)**
   ||||| |||||||
   3' UGGUCUCUACGUCG-UGACGUGG (miR)

3. **has-miR-155**

   5' ...GGUAGCUUUGGAAGUAGCAUUAA... (UTR) **Position 1(1369-1376)**
   |||||||
   3' UGGGGAUAGUGCUAAUCGUAAUU

4. **has-miR-182**

   5' ...CCAGCAACAACUUUGGUGCCAUU... (UTR) **Position 1(22-28)**
   |||||||
   3' UCACUUAAGAUGGUCACGGUAU (miR)

5. **has-miR-330 – overlapped sequences in 3' UTR (highlighted in grey)**

   5' ...UUAAAAGUAUAAAACUGCUUUGU... (UTR) **Position 1 (997-1003)**
   |||||||
   3' AGAGACGUCCGGCACACGAAACG (miR)

   5' ...UUAAAAGUAUAAAACUGCUUUGU... (UTR) **Position 2 (1298-1304)**
   |||||||
   3' AGAGACGUCCGGCACACGAAACG (miR)

   5' ...GAAGAUGAAAUGCUUUGCUUUGA... (UTR) **Position 3 (1303-1310)**
   |||||||
   3' AGAGACGUCCGGCACACGAAACG (miR)

6. **has-miR-335**

   5' ...GGACCAGGCGCGGUGGCUCUUGC... (UTR) **Position 1 (505-511)**
   |||||||
   3' UGUAAAAAGCAAUAACGAGAACU (miR)

7. **has-miR-376**

5' ...AAAAAUAAGCAUUUCAAUCUAAG... (UTR)**Position 1 (1467-1471)**
|||||
3' AUGAGUAUCUUCCUCUUAGAUG (miR)

5' ...AUAUUUAUAAUAAAUUCUAUGUC... (UTR)**Position 2 (1625-1631)**
||| |||||||
3' UGCACCUUAAAGGAGAUACAA (miR)

8. **has-miR-378**

5' ...CAGGCAGAUCACAAGGUCAGGAG... (UTR) **Position 1 (558-564)**
|||||||
3' UGUGUCCUGGACCUCAGUCCUC (miR)

9. **has-miR-495**

5' ...GUCUGUGUGUCUGAUGUUUGUUG... (UTR) **Position 1(230-236)**
|||||||
3' UUCUUCACGUGGUACAAACAAA (miR)

5' ...UUUUUAAAGUCUAUUGUUUGUUG...(UTR) **Position 2 (950-956)**
|||||||
3' UUCUUCACGUGGUACAAACAAA (miR)

10. **has-miR-506**

5' ...UCCCUUUAUGAAGUGUGCCUUAG... (UTR) **Position 1(1529-1535)**
|||| |||||||
3' AGAUGAGUCUUCCCACGGAAUG

11. **has-miR-634**

5' ...AUUGUUUGUUGUGCUUGCUGGUA... (UTR) **Position 1 (962-969)**
|||| |||||||
3' CAGGUUUCAACCCC-ACGACCAA (miR)

12. **has-miR-1185 – overlapped sequences in 3' UTR (highlighted in grey)**

5' ...UCUGGAAACUCUUUG-CUGUAUAA... (UTR) **Position 1(1189-1196)**
||| |||||||
3' UACUCUCAGAGGGGGACAUAUA

13. **has-miR-4789 – overlapped sequences in 3' UTR (highlighted in grey)**

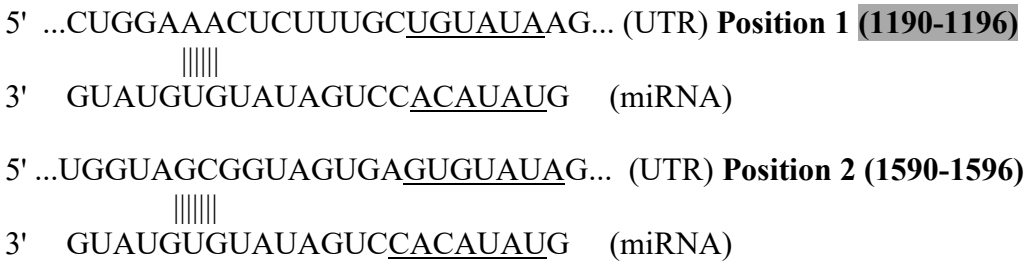


Figure 4. Bioinformatics analysis of IAPP mRNA 3'-UTR. Databases comprising predicted and validated miRNA-target interactions were used. miRanda (http://www.microrna.org/microrna/home.do), DIANA-microT-CDS, mirDB (http://ophid.utoronto.ca/mirDIP) and TargetScan (http://www.targetscan. org/vert_71) collect predicted miRNA targets. Two databases, miRtarbase (http://mirtarbase.mbc.nctu.edu.tw) and DIANATarbase (http://diana. imis.athena-innovation.gr/DianaTools/index.php?r1/4site/index), contained validated miRNA targets. Putative miR's seeding positions (underlined) were predicted using TargetScan (http://www.targetscan. org/vert_71).

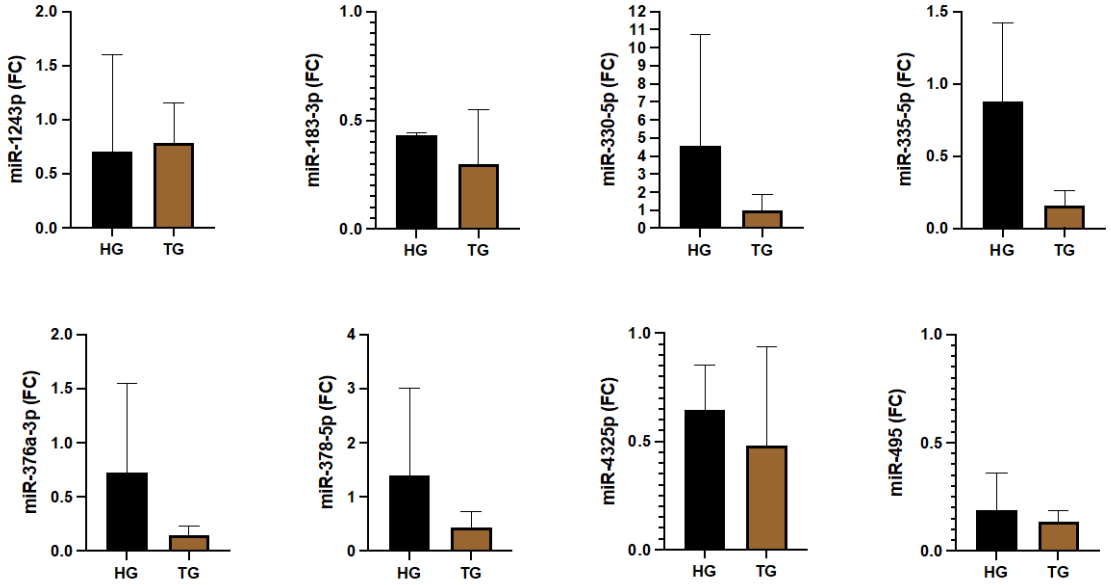


Figure 5. A set of miRNAs are downregulated in ER stress induced human islets. Freshly isolated non-diabetic human islets were cultured in the presence of high (20 mM) glucose (HG), or 0.5 µM thapsigargin (TG) for 24 h. Changes in miRNA levels of various genes were analyzed by RT-qPCR.. Cq values and changes in miRNA expression levels were normalized to a SNORD44 (ubiquitously expressed miRNA) and relative gene expression was calculated as described in the method section.

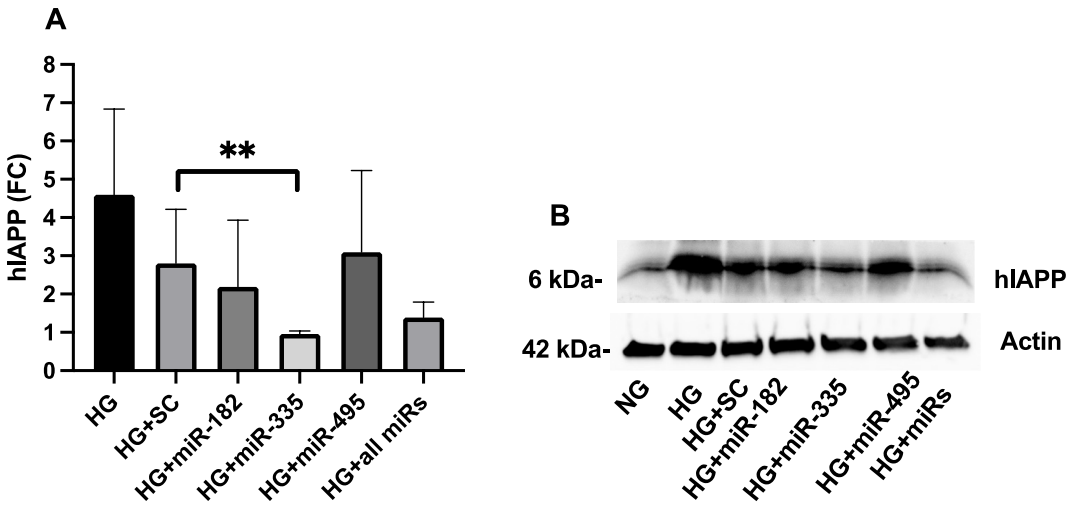


Figure 6. Western blot analysis of hIAPP expression in human islets. Freshly isolated non-diabetic human islets were cultured in the presence of high (20 mM) glucose (HG) or normal (5mM) glucose (NG) supplemented with scrambled (SC) or antisense miRNAs for 72h. hIAPP signal was normalized against actin, and expressed as fold change from control (NG) samples (set to 1). Significance was established at $^{**}P < 0.01$, n=3, ANOVA followed by Tukey's post hoc comparison test. Data represent mean ± SEM of three independent experiments.

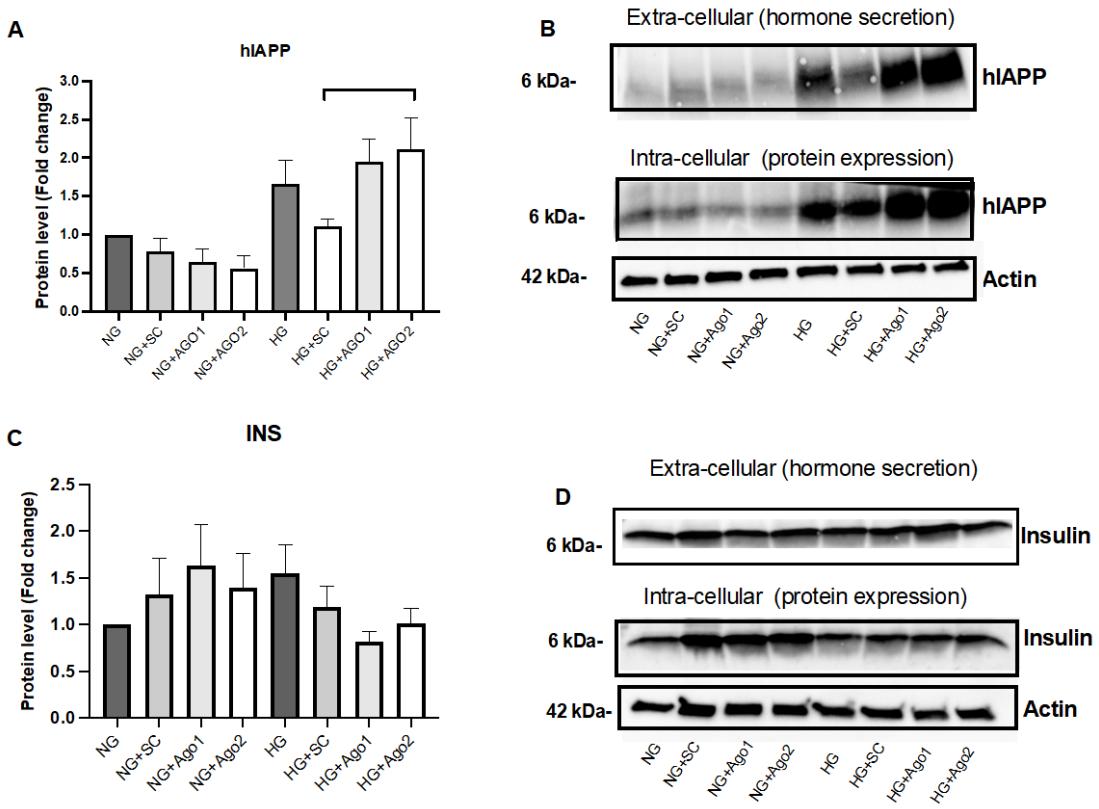


Figure 7. Western blot analysis of hIAPP and insulin protein expression and secretion in human islets. The islets were cultured under normal (NG) and high glucose (HG) conditions. Hormone signal was normalized against actin, and expressed as fold change from control (NG) samples (set to 1). Significance was established at *P < 0.05, n=3, ANOVA followed by Tukey's post hoc comparison test. Data represent mean ± SEM of three independent experiments.

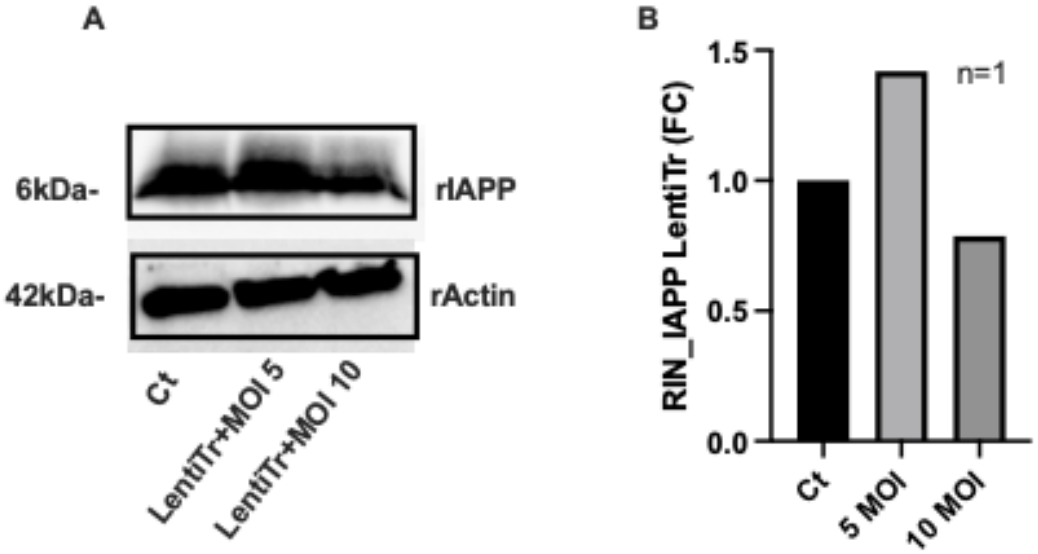


Figure 8. Western blot analysis of hIAPP expression levels in RINm5F cells. Changes in protein levels of hIAPP in lentivirus-transfected cells encoding Ago2 was analyzed by WB. Cells were transfected at 5 and 10 MOIs. hIAPP signal was normalized to a housekeeping gene (actin) and expressed as fold change from control (untransfected) RIN cells (n=1).

Table 1. mRNA primer sequences used for gene expression

Primers	Sequences
hIAPP	F: 5'-AGCTACACCCATTGAAAGTCATC-3' R: 5'-GATGAGAGAATGGCACCAAAGT-3’
Insulin	F: 5'- TGTCCTTCTGCCATGGCCCT-3' R: 5'-TTCACAAAGGCTGCGGCTGG-3'
β-actin	F:5'-AGGCACCAGGGCGTGAT-3' R:5'-GCCCACATAGGAATCCTTCTGAC-3'
TXNIP	F: 5'-ACAGAAAAGGATTCTGTGAAGGTGAT-3' R: 5'-GCCATTGGCAAGGTAAGTGTG-3'
BIP	F: 5’-GAACGTCTGATTGGCGATGC-3’ R: 5’-ACCACCTTGAACGGCAAGAA-3’
FoxA2	F: 5'-TCTCCATCAACAACCTCATGTC-3' R: 5'-GTAGTGCATCACCTGTTCGTAG-3'
PDX1	F: 5'-CCTTGTGCTCGGGTTATGTT-3' R:5'-ATCATCCCACTGCCAGAAAG-3'

Table 2. miRNA primer sequences used for gene expression

Primers	**Sequences (3’ to 5’)**	**Accession No.**	**Position**
hsa-miR- 124-3p	UAAGGCACGCGGUGAAUG CCAA	MIMAT0000422	53–74
hsa-miR-155-3p	CUCCUACAUAUUAGCAUUAACA	MIMAT0004658	43-64
hsa-miR- 182-3p	UGGUUCUAGACUUGCCAA CUA	MIMAT0000260	67-87
hsa-miR-376A-3p	AUCAUAGAGGAAAAUCCACGU	MIMAT0000729	44-64
hsa-miR-330-3p	GCAAAGCACACGGCCUGCAGAGA	MIMAT0000751	57-79
hsa-miR-335-3p	UUUUUCAUUAUUGCUCCUGACC	MIMAT0004703	52-73
hsa-miR-378A-3p	ACUGGACUUGGAGUCAGAAGGC	MI0000732	43-64
hsa-miR- 432-3p	CUGGAUGGCUCCUCCAUGU CU	MIMAT0002815	62-82
hsa-miR-495-3p	AAACAAACAUGGUGCACUUCUU	MIMAT0002817	50-71
hsa-miR-506-3p	UAAGGCACCCUUCUGAGUAGA	MIMAT0002878	71-91

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