Dissecting the microRNA pathway in the pancreatic β-cell during insulin resistance

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Abbreviations

ADP	Adenosine diphosphate
Ago	Argonaute
ATP	Adenosine triphosphate
BIRKO	β-cell specific insulin receptor knockout
Cadm1	Cell adhesion molecule 1
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
Gck	Glucokinase
Glut	Glucose transporter
Gphn	Gephyrin
GSIS	Glucose stimulated insulin secretion
GWAS	Genome wide association studies
IDDM	Insulin dependent diabetes mellitus
Igf1R	Insulin-like growth factor 1-receptor
IR	Insulin receptor
ISG	Insulin containing secretory granule
K_{ATP}	ATP sensitive potassium channel
KO	Knockout
LIRKO	Liver specific insulin receptor knockout
lncRNA	Long non-coding RNA
mRNA	messenger RNA
MIN6	Mouse insulinoma-6 β-cell line
miRNP	miRNA containing ribonucleoprotein particle
miRISC	miRNA-induced silencing complex
MIRKO	Muscle specific insulin receptor knockout
miRNA	microRNA
MODY	Maturity onset diabetes of the young
mTOR	mammalian target of Rapamycin
Mtpn	Myotrophin
ncRNA	Non-coding RNA

NIDDM	Non-insulin dependent diabetes mellitus
NIRKO	Neuronal specific insulin receptor knockout
PCR	Polymerase chain reaction
PTGS	Posttranscriptional gene silencing
Rasd1	Dexamethasone-induced Ras-related protein 1
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
SG	Stress granule
siRNA	Short interfering RNA
SILAC	Stable isotope labeling of aminoacids in cell culture
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCA	Tricarboxylic acid cycle
UTR	Untranslated region
qRT-PCR	Quantitative Real time PCR

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that fine tune protein output *via* mRNA degradation or inhibition of its translation. Recent studies indicate that miRNAs and their components respond to cellular stress to maintain their steady state physiology. Their functional characterization in the pancreatic β-cell during stress states such as insulin resistance and Type 2 Diabetes (T2D) however is scant. In this thesis, important aspects of β-cell function such as proliferation and insulin release and their regulation by the miRNA pathway were addressed. By combining high-throughput miRNA deep sequencing, proteomics, and mouse genetic approaches, we have shed light on the novel roles of *Argonaute2* (*Ago2*), a critical component of the miRNA-induced silencing complex (miRISC), in regulating β-cell secretome and β-cell mass during insulin resistance. We demonstrated that inhibition of *Ago2* potentiated the release of β-cell secretome in response to high glucose by de-repressing the suppression of *miR-375*-targeted genes such as *Gephyrin* (*Gphn*). Moreover, inhibition of *Gphn* blocked β-cell secretome indicating that *Ago2* functionally regulates *miR-375* and its targets to impact β-cell secretome. Additionally, we demonstrated that the highly conserved miRNA, *miR-184*, was uniquely downregulated in the islets of mouse models of insulin resistance and in human subjects with T2D. Furthermore, we could show that silencing of *miR-184* promoted the expression of its conserved target *Ago2*, which in turn potentiated the function of *miR-375* thereby regulating the levels of growth suppressors such as *Cadm1*. Most importantly, *miR-184* and *Ago2* were inversely correlated in the islets across a cohort of human subjects indicating the existence of feedback mechanisms within the miRNA pathway. Finally, restoration of insulin sensitivity in leptin-deficient *ob/ob* mice returned the expression of *miR-184* that in turn regulated *Ago2* and ultimately β-cell mass. These studies uncovered novel functional roles of the miRNA pathway in mediating stress responses by the β-cell during insulin resistance. Further dissection of this pathway shall uncover several mechanisms by which the β-cells undertake to maximize their efficiency during disease states such as T2D.

Chapter 1
Introduction

1.1 A bird's-eye view into the 3500 years of Diabetes research

Long before terming *diabainein*, meaning a 'siphon' or to 'pass through' by Areteaus and the re-discovery of 'honey-like urine' (glycosuria) by Thomas Willis, who added the term *mellitus*, *Diabetes* has found its first mentioning around 1500 B.C. by the ancient Egyptians. It was recognized as a disease associated with 'excessive emptying of urine' (polyuria) in humans. Subsequent landmark findings include Matthew Dobson's first experimental evidence of increased urine and blood glucose levels (hyperglycemia) in humans with diabetes (Dobson, 1776). In 1889, Joseph von Mering and Oskar Minkowski were the first to provide evidence that removal of pancreas in dogs caused diabetes, suggesting that pancreas regulates glucose levels. Later, Edward Albert Sharpey-Schafer hypothesized that diabetes could be caused as a result of loss of a single pancreatic chemical, which he termed as *insulin* in 1910 (Polonsky, 2012). Taken together, diabetes is now well recognized as a group of heterogeneous disorders characterized by hyperglycemia due to loss of insulin, or its effectiveness, or both. Current global trends show a staggering 382 million people have diabetes and this is predicted to rise to 592 million by the year 2035 (IDF Diabetes Atlas, 2013), suggesting a worldwide diabetes epidemic. Moreover, several disease complications arising as a result of diabetes such as cardiovascular disease, renal failure, diabetic retinopathy, and osteoporosis are constantly on the rise and the number of deaths due to diabetes is worsening year by year (IDF Diabetes Atlas, 2013).

Around the mid-nineteenth century, Claude Bernard's integration of scientific approaches in medicine led to the identification of liver as a major site of glucose production. He was also the first to describe *milieu intérieur*, a term later used and expanded by Walter Bradford Cannon as *homeostasis*, to define the existence of possible regulatory mechanisms within cells to maintain their steady-state physiology (Robin, 1979). This formed the notion that dysregulation of glucose homeostasis is one of the key events central to the progression of diabetes. In light of the proposed involvement of insulin and its source, the pancreas, in the maintenance of glucose homeostasis during the early 1900s, Frederick Banting and Charles Best (with the assistance of J.J.R. Macleod and James Collip), devised a series of

experiments to isolate and purify pancreatic insulin. Furthermore, they were successful in treating patients, who were diagnosed of diabetes mellitus, with the purified insulin (Banting & Best, 1922; Banting *et al.,* 1922). This landmark medical advancement instigated several important discoveries, most notably the delineation of signaling cascades triggered by insulin, setting the stage for a life saving molecule for severe diabetics over almost a century (Cohen, 2006). Consisting of 51 aminoacids across two peptide chains, A and B that are held together by disulfide bonds, insulin is a small peptide hormone derived from a single polypeptide chain called *proinsulin* (Steiner & Oyer, 1967). It is essentially secreted from the islets of Langerhans, specialized endocrine structures within the pancreas first discovered by Paul Langerhans at the Berlin Pathological Institute as early as 1869. Almost a century now from the time insulin was discovered, diabetes still remains an enigmatic disease to cure owing to its complexity.

Although historically, diabetes had been classified as two major types as early as 100-200 B.C. by ancient Indian physicians (Kahn, 1994), the first experimental evidence comes from the pioneering works of Wilhelm Falta and Harold Himsworth. They were the first to develop standardized insulin-glucose tolerance tests in humans to distinguish 'insulin sensitive' from 'insulin insensitive' patients (Himsworth, 1936). These forms of diabetes mellitus are now widely known as "Type 1 Diabetes" (T1D) or insulin dependent diabetes mellitus (IDDM) and "Type 2 Diabetes" (T2D) or non-insulin dependent diabetes mellitus (NIDDM). In general, T1D most commonly affects the youth due to development of an autoimmune response against the insulin producing β-cells. On the other hand, T2D is more common in adults and is mostly associated with the emergence of insulin resistance, dyslipidemia, and obesity- collectively referred to as the "metabolic syndrome" (Moller, 2001). Claude Bernard's classical experimentation paved way for a possible involvement of brain in the maintenance of blood glucose and development of diabetes. However, the discovery of insulin and its miraculous ability to cure diabetes established an islet-centered control of glucose homeostasis (Schwartz et al*.,* 2013). But with several recent investigations, the involvement of brain in the progression of T2D is strongly re-established (Schwartz & Porte Jr., 2005; Schwartz et al*.,* 2013). These observations re-emphasize a complex network of several peripheral insulin responding tissues along with brain central to the maintenance of glucose homeostasis (Saltiel & Kahn, 2001) (Fig. 1). Moreover, besides interactions among these metabolically relevant tissues, gene-environment interactions have played vital roles in the predisposition of obesity, insulin resistance, and T2D (Doria et al., 2008).

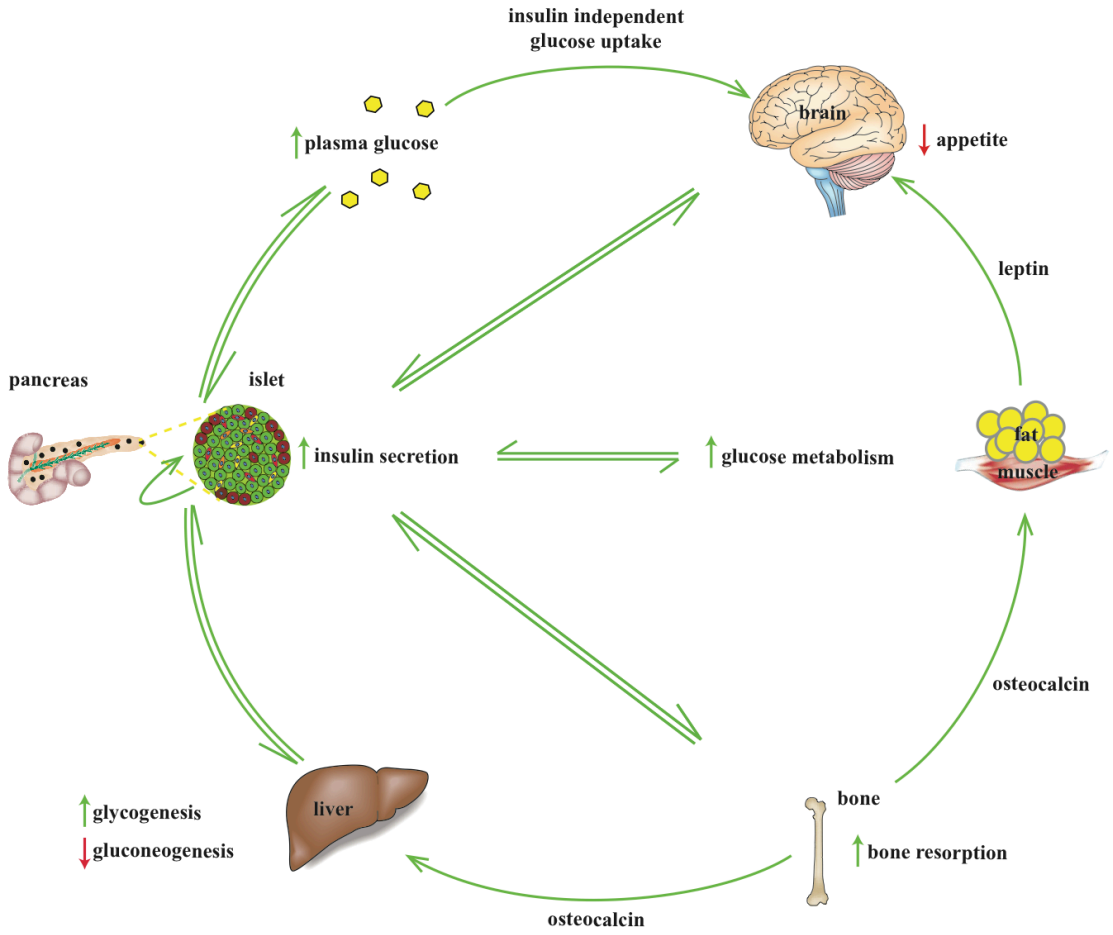


Fig. 1: ***Maintenance of glucose homeostasis by insulin.*** Elevated plasma blood glucose levels stimulate pancreatic islets to secrete insulin into the blood stream. Insulin is known to crosstalk with tissues such as liver, muscle and the fat to uptake glucose for subsequent glucose metabolism. In the liver, insulin binds to insulin receptor (IR) and activates rapid insulin signaling for the uptake of glucose and its subsequent metabolism *via* increasing glycogenesis and decreasing gluconeogenesis. The liver in turn is known to secrete *Betatrophin*, a hormone that maintains β-cell mass in mice. Additionally, insulin has been shown to stimulate bone resorption and osteoblast cells in turn release *Osteocalcin*, a hormone that was shown to crosstalk to β-cells, liver, and fat so as to maintain their functions. Furthermore, insulin stimulates the uptake of glucose by muscle and fat. These tissues in turn can release hormones *Irisin* and *Adipsin*, respectively, that were demonstrated to maintain β-cell function. In addition, *Leptin*, a satiety hormone secreted by fat cells (adipocytes) is known to crosstalk to the brain to regulate food intake. Although the brain is known to uptake glucose via insulin independent manner, insulin is known to bind to brain IR to control appetite. Finally, insulin can mediate β-cell proliferation and insulin secretion in an autocrine way. β-cells in turn were also shown to secrete factors such as *Vgf* that maintain β-cell function in mice. Reversible arrows represent crosstalk from β-cell to respective insulin responsive tissues and *vice versa* (Adapted from Saltiel & Kahn, 2001; Tiano & Mauvais-Jarvis, 2012).

1.2 The complex genetic architecture of T2D

For many years, it has been noted that the emergence of T2D in humans is primarily a result of the body's inability to utilize available circulating glucose. This is either due to the inefficient action of insulin on peripheral tissues such as liver, muscle, and fat (broadly termed insulin resistance), the inability of pancreatic β-cells to secrete sufficient insulin in response to glucose, or both of these combined (Kahn, 1994). Although, much thought was conceived about the involvement of environmental factors together with genetic background in the development and progression of T2D, there was a major deficit in our knowledge about the underlying genetics making T2D a more complex disorder to understand. But now, decades of extensive research into the genetics revealed that T2D develops more commonly due to the involvement of multiple genes (polygenic) rather than the less frequent involvement of single genes (monogenic). Moreover, this is together with a strong association to environmental factors such as over-nutrition and physical inactivity among several others (Doria et al., 2008).

Soon after the successful sequencing of human insulin gene (Bell et al., 1980), several groups discovered various mutations affecting either its processing, proper folding or its structure that resulted in a decreased bioactivity of insulin causing permanent neonatal diabetes in humans (Glaser, 2008). Additionally, mutations have also been successfully discovered in the gene encoding the receptor for insulin (called *INSR* or simply *IR*) in patients with severe insulin resistance directly implicating its role in NIDDM (Kadowaki et al., 1988; Taylor, 1992). These studies highlighted the obvious importance of insulin mediated signaling in bringing glucose homeostasis under control. Moreover, several mutations were identified in at least six additional genes namely *glucokinase*, *hepatocyte nuclear factor* (*HNF*)-*4α, HNF-1α*, *insulin promoter factor 1* (*IPF-1*), *HNF-1β* and *neurogenic differentiation 1/ β-cell E-box transactivator 2* (*NeuroD1/BETA2*) causing an early-onset form of T2D referred to as maturity onset diabetes of the young (MODY) in humans (Taylor, 1999; Bell & Polonsky, 2001; Doria et al., 2008).

This complex genetic architecture expanded further with the development of several systematic high-throughput genetic approaches such as genome-wide association studies (GWAS) (Thomsen & Gloyn, 2014). Such studies allowed for a comprehensive understanding and exploration of several risk factors previously unrecognized as T2D genes that include *Tcf7l2, Slc30a8, Igf2bp2,* and *Fto* to name a few (Doria et al., 2008).

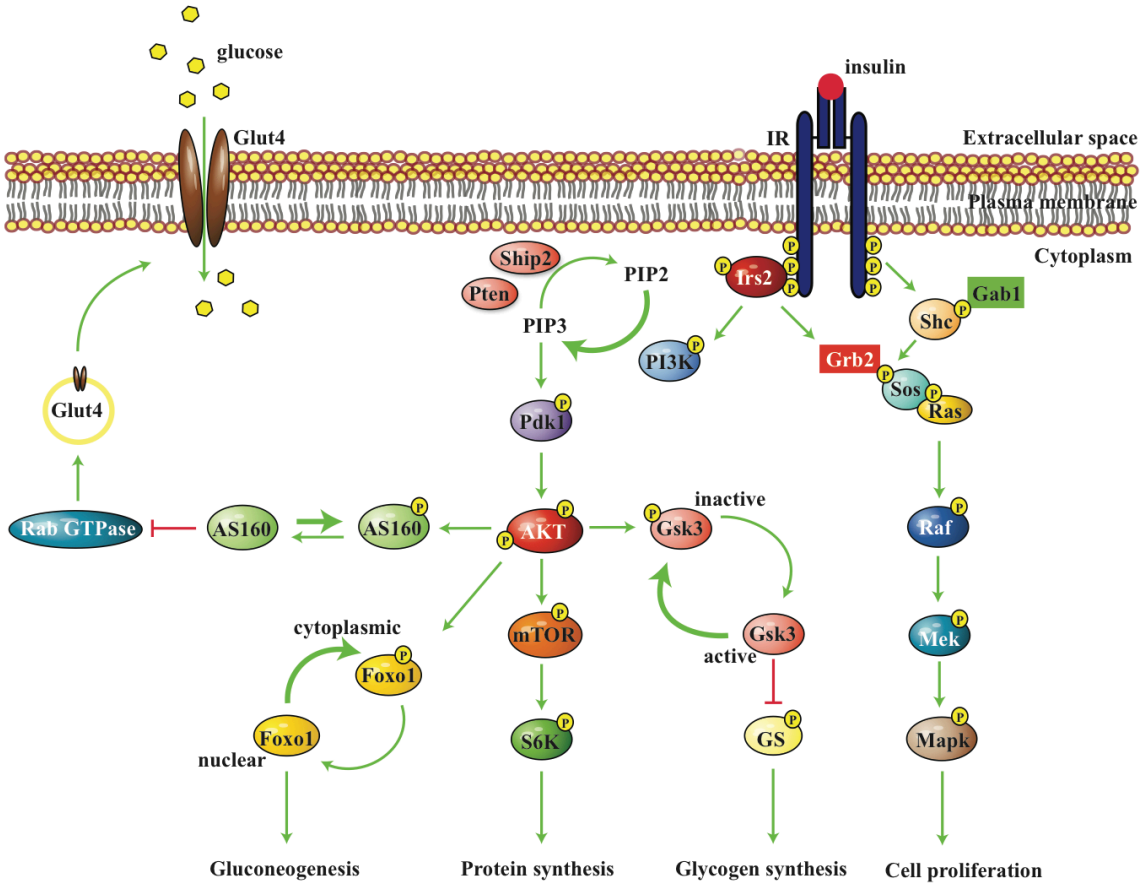


Fig. 2: ***The insulin signaling pathway.*** The insulin released in response to glucose by the β-cells effectively binds to its receptor (*IR*) expressed by insulin responsive tissues such as the liver. *IR* is a known receptor tyrosine kinase (RTK) that gets phosphorylated upon binding to its ligand *insulin*. *Insulin receptor substrate* (*Irs*) is one of the first proteins to subsequently get phosphorylated and mediates the activation of the *phosphoinositol-3 kinase* (*PI3K*) pathway. Once avtivated, *PI3K* phosphorylates the *phosphoinositol-bis-phosphate* (PIP2) to *phosphoinositol-tri-phosphate* (PIP3) that subsequently phosphorylates *Pdk1*. The phosphatases such as *Pten* and *Ship2* are known to inhibit the insulin pathway *via* inactivating the *PI3K* pathway. Activated *Pdk1* phosphorylates the serine/threonine kinase *Akt*, which is recognized as one of critical nodes central to the insulin signaling pathway. *Akt* phosphorylates several proteins and inhibits their activities. For instance, *Akt* can phosphorylate its known substrate, *AS160*, which in an unphosphorylated state inactivates Rab GTPases that are required for the translocation of the glucose transporters such as *Glut4* to the plasma membrane for glucose uptake. *Akt* retains the transcription factor *Foxo1*, which is involved in gluconeogenesis, in the cytoplasm for its subsequent ubiquitination. On the other hand, *Akt* inactivates the glycogen synthase kinase, *Gsk3*, which can inhibit *glycogen synthase* (*GS*) required for glycogen synthesis. Other important downstream functions of *Akt* include the activation of mTOR pathway, which is required for protein synthesis. In addition to *Akt*, the *IR* can mediate the activation of the mitogen activated protein kinase (*MAPK*) pathway *via* the *Ras-Raf-Mek-Mapk* axis, which is critical for cell proliferation. Interruptions along any of these signaling circuits as a result of genetic or environmental factors are known to cause T2D. Thick arrows represent favorable reactions.

In a couple of very recent studies, these risk factors, such as *Slc30a8*, identified through GWAS were successfully validated using mouse models indicating the power and confidence in such high throughput studies (Flannick et al., 2014). Also, additional novel factors can be identified based on existing knowledge of T2D genes that were identified by GWAS. For instance, the obesity-associated *Fto* gene was recently discovered to functionally form long-range connections with the homeobox transcription factor *Irx3* thereby delineating the mechanism by which *Fto* causes obesity and T2D in humans (Smemo et al., 2014).

Besides identification of genes implicated in T2D, several efforts were undertaken to delineate the functional roles of these genes in mice, with a special focus on the components of insulin receptor signaling pathway (Fig. 2). Mice with genetic deletions (knockout) of genes essential for insulin signaling and insulin release were generated so as to dissect the mechanisms behind insulin resistance, β-cell dysfunction and the subsequent progression of T2D. This has led to a comprehensive understanding of the role of insulin signaling in the progression of insulin resistance and T2D, an association suggested in the past in humans (Kadowaki et al., 1988). Several groups were successful in the systematic generation of numerous total and tissue specific knockout (KO) models of genes important for insulin signaling such as *IR*, *Insulin-like growth factor 1 receptor* (*Igf1r*), *Glucose transporter 4* (*Glut-4*), *Insulin receptor substrates* (*Irs*) *1-4*, and downstream targets such as *Akt* (Nandi *et al*., 2004) (Fig. 2). Of note, genetic ablation of IR from tissues such as muscle (MIRKO, Brüning et al., 1998), β-cell (βIRKO, Kulkarni et al., 1999), brain (NIRKO, Brüning et al., 2000), liver (LIRKO, Michael et al., 2000), and osteoblasts (Ferron et al., 2010; Fulzele et al., 2010) have rendered mice with mild to severe forms of insulin resistance and glucose intolerance. This suggests that individual tissue specific insulin signaling is critical for the maintenance of whole body insulin sensitivity and glucose homeostasis. Taken together, these mouse models have furthered our understanding of the involvement of a complex network of insulin receptor pathway genes in the development of insulin resistance and ultimate progression of T2D (Nandi *et al*., 2004).

The association of physical inactivity, overnutrition and obesity as contributing factors in the development of T2D in humans has been observed for several decades. The first experimental evidence to link obesity to the development of T2D in mice, however, came soon after the identification of an obese mouse designated *ob/ob*, which displayed severe obesity, as a result of overeating or hyperphagia. This mouse progressively developed insulin resistance and diabetes, quite similar to the association of morbid obesity and T2D in humans

(Ingalls et al., 1950; Mayer et al., 1951; Batt & Mialhe, 1966; Coleman, 1978; Friedman & Halaas, 1998). Friedman's group subsequently identified the gene responsible for obesity, *ob* (short for *obese*) (Zhang et al., 1994) and its product *leptin*, which is a satiety hormone secreted mainly by adipocytes and known to control food intake and body weight *via* the brain (Friedman, 2000).

Subsequent quest for the identification of the gene encoding the receptor for leptin designated as *Lepr* or *db* (short for *diabetes*) soon followed and *db* mutant mice (*db/db*) were discovered to be similar in phenotype when compared to the leptin deficient *ob/ob* mice (Coleman, 1978; Tartaglia et al., 1995; Chen et al., 1996; Friedman & Halaas, 1998). Importantly, mutations in *ob* as well as *db* were successfully identified in humans with severe early-onset obesity (Montague et al., 1997; Clément et al., 1998). The discoveries of mutations in these genes in humans and their associations to obesity and T2D thus make *ob/ob* and *db/db* mice excellent genetic models to thoroughly explore the mechanisms underlying obesity and diabetes. Additionally, mutations in other genes such as *prohormone convertase 1* (PC1) (Jackson et al., 1997) or more recently *Fto* (Loos & Yeo, 2013; Smemo et al., 2014) were also identified from various obese patients, suggesting the involvement of a wide spectrum of genes causing childhood and severe adult-onset obesity. Being the sole sources for the production and release of insulin, the pancreatic islets respond dynamically to such increased metabolic load due to obesity even without diabetes and often display volumetric islet mass expansion (Bonner-Weir & O'Brien, 2008).

1.3 The islet architecture and β-cell fate determination

The islets of Langerhans are specialized endocrine structures within the pancreas, which is a glandular organ of endodermal origin. Pancreas is derived from the embryonic foregut and is known to possess both endocrine and exocrine functions (Edlund, 2002). The islets represent the endocrine part of the pancreas and are the only source for secreting hormones such as insulin and glucagon that maintain glucose homeostasis. They essentially consist of different cell types designated α, β, δ, PP, and ε that secrete the islet hormones glucagon, insulin, somatostatin, polypeptide Y, and ghrelin respectively. Moreover, islets are known to possess dynamic inter-species plasticity of architecture that is proposed to be adapted over time during the course of evolution (Steiner et al., 2010). During pathological states of insulin resistance, pregnancy, or T2D, the islets expand in size to compensate for increased insulin

demands (Weir & Bonner-Weir, 2004; Kim et al., 2009). Over time, there is a substantial loss of β-cell mass due to genetic and environmental factors such as stress, leading to severe hyperglycemia as a result of loss of insulin (Fig. 3).

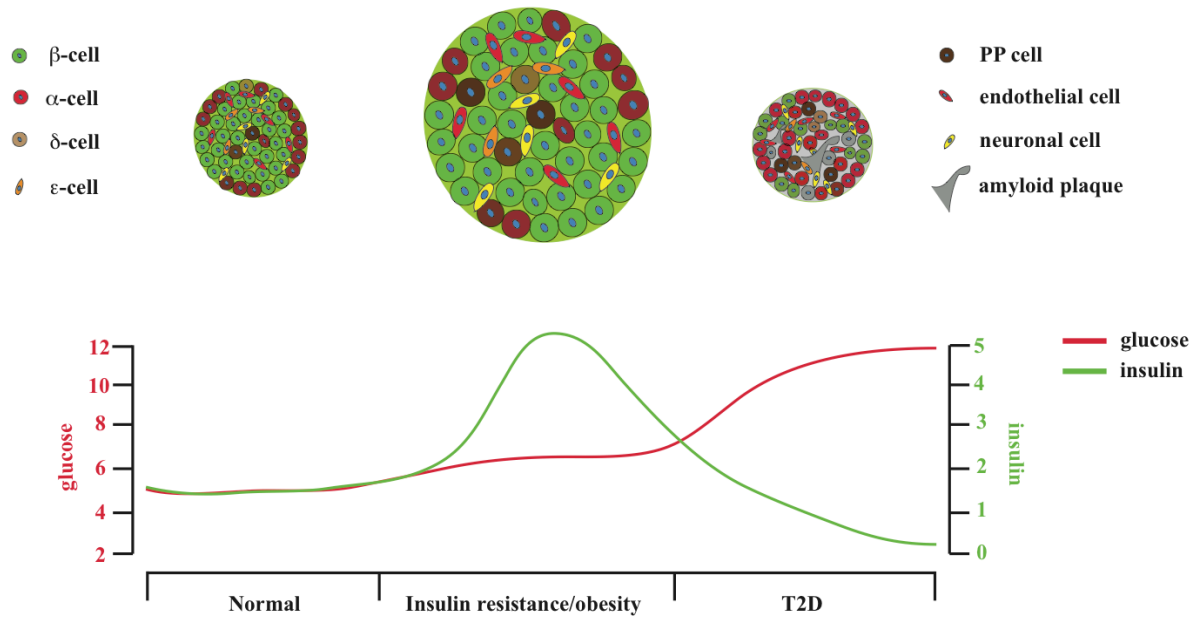


Fig. 3: ***Islet mass during the progression of T2D.*** The pancreatic islets of Langerhans are composed of several cell types designated *α*, *β*, *δ*, *ε*, and *PP* that secrete the hormones glucagon, insulin, somatostatin, ghrelin, and polypeptide Y respectively. These hormones are required to maintain normal glucose homeostasis at steady or stress states. The islets are dynamic in nature and respond to stresses such as insulin resistance during obesity by increasing their rate of proliferation. This compensatory expansion (upper panel) is necessary to meet the metabolic demands of higher insulin production during insulin resistance. As a result, systemic insulin levels substantially rise so as to maintain normoglycemia (lower panel). The manifestation of T2D follows when the β-cells fail to meet such demands due to genetic and/or environmental factors. During such a scenario, there is a massive loss of β-cell mass due to apoptosis or dedifferentiation to progenitor-like cells. The diminished β-cell mass is often replaced by accompanying amyloid plaques (upper panel). The circulating insulin levels thus decline over time thereby rendering the subjects severely hyperglycemic (lower panel). The glucose and insulin concentrations are represented as arbitrary values.

Besides the hormone secreting cell types, the islets are richly vascularized with blood vessels that were shown to be essential for islet survival and function (Lammert et al., 2001; Konstantinova et al., 2007). Also, in a very recent study, it has been postulated that bone marrow derived macrophages together with endothelial cells create an islet microenvironment crucial for β-cell proliferation (Brissova et al., 2014). Furthermore, since

Claude Bernard's observation that a punctured region of the brain led to hyperglycemia, many groups have investigated the potential role of deep innervation of the islets by the sympathetic nervous system. It is now widely accepted that the neuronal system has a crucial role in the maintenance of islet architecture (Woods & Porte, 1974; Borden et al., 2013).

Whilst islets represent only ~1-2% of the total pancreas, the insulin secreting β-cells occupy at least ~65-80% of the islet mass, constituting to approximately 2% of pancreatic weight, and the rest by the other cell types collectively referred to as non β-cells of the islets (Weir & Bonner-Weir, 2013). The maintenance of well-preserved interactions between these different cell types is essential to regulate basal secretion of insulin by the β-cells. This suggests that islet architecture is a key aspect to maintain β-cell function (Halban et al., 1982). During development, these different cell types are known to arise from a single progenitor cell that expresses *Neurogenin3* or *Ngn3*, a transcription factor that determines endocrine cell fate (Edlund, 2002). Eventually, transcription factors such as *Pdx1*, *Pax4*, *Nkx2.2*, *Nkx6.1*, *MafA*, and *Foxo1* maintain the β-cell specification (Ziv et al., 2013). However, the existence of β-cell progenitors in the adult pancreas still remains controversial and unclear.

While it had been considered that β-cells proliferate by self-duplication of pre-existing β-cells rather than stem cell differentiation (Dor et al., 2004), other reports suggested that multipotent cells within adult pancreas could differentiate into β-cells (Xu et al., 2008). The latter study is further supported by investigations that showed how forced expression of β-cell specific transcription factors in non-β cells can trigger a β-cell lineage in mice (Collombat et al., 2009; Al-Hasani et al., 2013). Recent studies also demonstrated that non-β cells such as α- and δ-cells could undergo "transdifferentiation" into β-like cells during conditions of extreme β-cell loss in mice (Thorel et al., 2010; Chera et al., 2014). On the other hand, several studies demonstrated that the β-cells can also lose their identity or "dedifferentiate" into non-β or progenitor cells upon loss of any of the aforementioned β-cell specific transcription factors (Ziv et al., 2013). In addition, a recent study showed that primary human β-cells are capable of converting into α-cells without any genetic alteration (Spijker et al., 2013). Together, these studies have established the dynamic plasticity of islet cells: a genetic and/or stress induced perturbation of which is known to trigger substantial loss of islet mass.

1.4 Glucose stimulated insulin secretion (GSIS)

The unique ability of the β-cells to sense changes in glucose concentrations and secrete insulin into extracellular milieu in order to maintain circulating plasma glucose levels within the narrow range of 4-8mM, is one of the hallmarks of this cell type (Weir & Bonner-Weir, 2013). This is principally achieved by the uptake of extracellular glucose by the glucose transporter 2 (*Glut2*; Bell et al., 1990) at the plasma membrane. Upon uptake, the intracellular glucose sensor, glucokinase (*Gck*), a gene previously implicated in MODY (Vionnet et al., 1992), subjects glucose moieties to rapid metabolism *via* glycolysis (Matschinsky & Ellerman, 1968). This subsequently results in the generation of a three-carbon end product: *pyruvate*, which participates in the tricarboxylic acid (TCA) cycle within the mitochondrion to ultimately generate adenosine triphosphates (ATP) *via* the electron transport chain system. The ATP thus generated elevates the ATP/ADP ratio in the cytoplasm triggering closure of the membrane associated ATP sensitive potassium (K_{ATP}) channel. Importantly, activating mutations in the *kir6.2* subunit of this channel were identified to cause permanent neonatal diabetes in both mice and humans due to loss of insulin secretion as a result of constitutively open K_{ATP} channel (Koster et al., 2000; Gloyn et al., 2004). It has long been known that glucose stimulates the closure of these K_{ATP} channels thereby causing slow membrane depolarization (Ashcroft et al., 1984). This subsequently promotes extracellular calcium influx *via* the voltage dependent calcium channels and potentiates the release of insulin from insulin containing secretory granules (ISGs) (Rajan et al., 1990; Matschinsky et al., 1993; German, 1993; Matschinsky et al., 1998) (Fig. 4). What is striking is the speed in which glucose is uniquely sensed by the β-cells and not by the other pancreatic cell types, reflecting the special feature of β-cells as a "glucostat" (Matschinsky & Ellerman, 1968). Insulin on the other hand is released in an oscillatory fashion in response to the elevated blood glucose and triggers cascades of signaling events in peripheral insulin-responsive tissues for the subsequent uptake of glucose. It has well been demonstrated that islets can easily be entrained to subtle changes in glucose concentrations and thus show high frequency plasma insulin oscillations. Additionally, this unique ability of entrainment by the islets is disrupted in patients with T2D (Mao et al., 1999). This demonstrates that loss of β-cell function as a result of defective insulin secretion is a fundamental problem during the clinical manifestation of T2D.

1.5 Composition of the insulin containing secretory granules (ISGs)

Quite convincingly thus far, it is reminiscent that T2D involves a defective production and/or efficient release of insulin (Rorsman, 2003; Seino et al., 2011; Ashcroft & Rorsman, 2012). Interactions between ISGs and numerous factors that facilitate the trafficking and docking of these granules to the plasma membrane for a subsequent release of insulin in response to glucose has been fairly well documented (Suckale & Solimena, 2010; Seino et al., 2011).

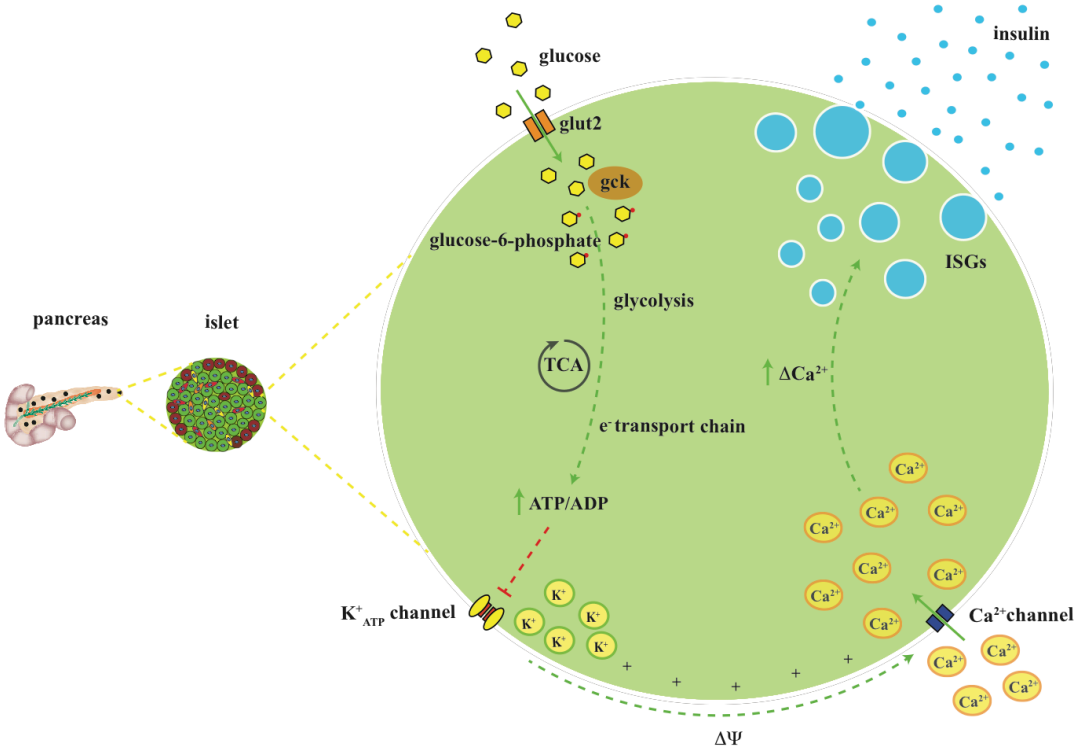


Fig. 4: ***Glucose stimulated insulin secretion (GSIS).*** The β-cells of the islets residing in the pancreas are specialized cell types that are capable of sensing subtle changes in plasma glucose levels and efficiently secrete insulin so as to maintain normoglycemia. Extracellular glucose enters the β-cell *via* the glucose transporter *Glut2* and gets phosphorylated to glucose-6-phosphate by the intracellular glucose sensor glucokinase (*gck*). After subsequent glycolysis, pyruvate enters the tricarboxylic acid cycle (TCA) in the mitochondrion to generate enough electrons for the electron transport chain (ETC). This increases the ratio of ATP to ADP, which is known to inhibit the ATP sensitive potassium (K^+_{ATP}) channel. As a result, depolarization (ΔΨ) across the membrane activates voltage dependent calcium channels (VDCC) to open, allowing an influx of extracellular calcium (Ca^{2+}) ions. The elevated Ca^{2+} ions trigger the insulin containing secretory granules (ISGs) to dock to the membrane and subsequently release a variety of hormones including insulin. Diabetes is known to manifest as a result of inefficient or complete loss of insulin secretion from the β-cells. Several mutations have been uncovered in key genes involved in glucose sensing and insulin exocytotic pathways including *Gck* and the K^+_{ATP} channel.

Rather than being mere insulin carrying vesicles, the ISGs have long been considered as dynamic structures that participate in exocytosis-endocytosis events during insulin release (Orci et al., 1973; Rorsman & Renström, 2003). Moreover, these dynamic structures are thought to harbor several proteins including peptide hormones other than insulin suggesting diverse functions of the β-cell (Suckale & Solimena, 2010). With the recent advancement of mass spectrometry based proteomics that opened new gateways into protein identification (Aebersold & Mann, 2003), close to 150 proteins with distinct functions including energy homeostasis have been described to be associated with the ISGs (Brunner et al., 2007). Additionally, the advent of stable isotope labeling of aminoacids in cell culture (SILAC) based quantitative proteomics (Ong & Mann, 2005; Mann, 2006) allowed for an improved ISG proteome (Schvartz et al., 2012). As yet, evidence is scant as to which of the granule proteins are actually released (broadly defined as "secretome") into the extracellular milieu in response to glucose.

1.6 Compensatory islet expansion during insulin resistance

During states of insulin resistance and obesity, presence of elevated circulating plasma insulin levels (termed hyperinsulinemia) has been well established in polygenic mouse models displaying insulin resistance and in obese human subjects (Yalow & Berson, 1960; Polonsky et al., 1988; Brüning et al., 1997) (Fig. 3, lower panel). This was presumed to be due to an enhanced secretion of insulin. It has subsequently been reported, both in rodents and humans, that this enhanced release of insulin is presumably due to an increase in β-cell mass (commonly referred to as "adaptive β-cell compensation") (Fig. 3, upper panel). Furthermore, this compensatory islet expansion is thought to be a result of β-cell proliferation (Steil et al., 2001) or due to β-cell hypertrophy (Weir & Bonner-Weir, 2004). In stark contrast, "β-cell failure" due to various genetic and environmental factors, is known to cause declined circulating plasma insulin levels in overt diabetics (Maclean & Ogilvie, 1955) (Fig. 3, lower panel). It has been observed that reduced levels of insulin are often associated with a significant loss of β-cell mass due to β-cell death by apoptosis (Butler et al., 2003; Rhodes, 2005) (Fig. 3, upper panel). On the contrary, an alternative mechanism to the established β-cell death due to apoptosis hypothesis suggests that loss of β-cell mass is more likely due to "dedifferentiation" into progenitor-like cell types during diabetes (Talchai et al., 2012; Wang et al., 2014; Latreille et al., 2014).

Besides the diabetes point of view, it has been shown that aged adult β-cells are long-lived and possess very low proliferative rates at steady state. This is due to a potential restriction of the entry of aged β-cells into cell cycle (Teta et al., 2005; Kushner, 2013). Despite this observation, recent evidence suggested that adult β-cells do possess the capacity to proliferate (Stolovich-Rain et al., 2012). In light of these observations on β-cell proliferation, several groups have uncovered numerous proteins essential for promoting β-cell proliferation with the help of knockout and transgenic mouse technologies. Known cell cycle regulators including *Cyclins D1* and *D2*, *Cyclin dependent kinase 4* (*Cdk4*), Cdk inhibitors (CKIs) such as *Cip/Kip* and *INK4*, transcription factors *E2F-1*, *-2*, *Retinoblastoma* (*Rb*) and *p53*, have been shown with genetic mouse models as contributors of β-cell proliferation and survival (reviewed in detail by Heit et al., 2006). These investigations have revealed that the cell cycle components are indispensable for β-cell proliferation.

Although considered as a consequence of loss of insulin action or release, transient high glucose has been shown to promote compensatory increase in β-cell mass, suggesting that glucose possesses "mitogenic" properties (Bonner-Weir et al., 1989). In the recent past, this observation was supported by the fact that metabolism of glucose rather than glucose *per se* is what drives compensatory β-cell proliferation *in vivo* (Porat et al., 2011). Considerable interest has been additionally focused on the mitogenic effects of insulin in driving β-cell proliferation *via* activating the insulin/IRS2 pathway. Several components of the pathway including *IRS2* (Withers et al., 1998) and *AKT* (Bernal-Mizrachi et al., 2001) were proven to be essential for β-cell survival. Additionally, studies have shown that the effect of insulin in β-cell proliferation is potentiated in combination with hyperglycemia (Paris et al., 2003). A more recent study suggests that insulin therapy helps in re-differentiating the de-differentiated β-cells back to insulin positive β-cells, reinforcing the beneficial effects of insulin as a potent β-cell survival factor (Wang et al., 2014).

It is well known that during severe insulin resistance, the pancreatic islets compensate to meet the increasing demands for producing and secreting more insulin by increasing their β-cell mass, resembling the stage 1 compensation phase seen in humans (Tomita et al., 1992; Weir & Bonner-Weir, 2004) (Fig. 3). Additionally, several other polygenic models of severe insulin resistance displayed similar increases in β-cell mass in response to elevated metabolic load (Brüning et al., 1997). Besides involvement of protein coding genes, several non-coding RNAs (ncRNAs), most notably microRNAs and long ncRNAs (lncRNAs) (Ponting et al.,

2009), have been implicated in the ever-growing list of "diabetogenes" adding on to the complex genetic landscape of human diabetes (Morán et al., 2012; Kameswaran et al., 2014; Tattikota et al., 2014).

1.7 microRNAs in β-cell function

1.7.1 A brief history of small RNAs

Decades of intensive research helped dissect the genetic architecture of insulin resistance and T2D. The last decade has seen tremendous attention to a special class of small ncRNAs such as microRNAs (miRNAs) in the maintenance of structure and function of β-cells. With the first discovery of the miRNA *lin-4* in *C. elegans,* two groups demonstrated how the product of a gene encodes two small RNAs, rather than a protein. Moreover, they showed that these small RNAs potentially "bind" to complementary sites residing within the 3' end of untranslated region (UTR) of *lin-14*, a developmentally regulated heterochronic gene. This interaction is shown to negatively regulate the expression of *lin-14* by inhibiting its translation (Lee et al., 1993; Wightman et al., 1993), suggesting miRNAs as negative regulators of gene expression.

Ever since this discovery, a new wave of exciting research gained momentum to delineate novel mechanisms of gene regulation. miRNAs are now classified as small ncRNAs of ~21-22 nucleotides (nt) in length that can partially base-pair within the mRNA of protein coding genes thereby controlling their expression *via* posttranscriptional mechanisms (Bartel, 2004 & 2009). Soon after the initial discovery of *lin-4*, another miRNA called *let-7* was identified whose biogenesis and function were similar to *lin-4*. Several homologs of *let-7* existed and were conserved across the animal phylogeny including flies and humans (Reinhart et al., 2000; Pasquinelli et al., 2000). Soon, an explosion in the discovery of miRNAs occurred mostly through high throughput deep sequencing methods, which resulted in an identification of almost thirty thousand mature miRNA sequences across ~200 species. This includes a staggering number of ~2500 mature human miRNAs (Kozomara & Griffiths-Jones, 2014). Several computational methods based on mathematical modeling of miRNA-target mRNA stable conformations, have been generated that can efficiently predict thousands of potential genes targeted by myriad miRNAs across several species (Lewis et al., 2003; Krek et al., 2005).

1.7.2 miRNA-mediated gene silencing

The mechanism of gene silencing, broadly defined as RNA interference (RNAi) or post transcriptional gene silencing (PTGS) (Hannon, 2002), is now well understood to be mediated by divergent small RNAs such as short interfering RNA (siRNA), piwi interacting RNA (piRNA), or the miRNAs. Principally, their mode of action is similar and the functional difference among these mediators is in their mode of biogenesis within cells (Carthew & Sontheimer, 2009; Ender & Meister, 2010). In light of an additional 61 nt small RNA for the *lin-4* miRNA with a proposed stem-loop based hairpin secondary structure (Lee et al., 1993), mature miRNA sequences are now shown to result from relatively longer miRNA "primary" transcripts (pri-miRNA). It is well established that pri-miRNAs are transcribed from DNA by RNA polymerase (pol) II (Lee et al., 2004; Cai et al., 2004).

Once transcribed, the pri-miRNA sequences are further processed to result in miRNA "precursor" (pre-miRNA) sequences of ~61 nt in length by the RNAse III enzyme *Drosha* (Lee et al., 2003) and its protein partner *DiGeorge syndrome Critical Region 8* (*Dgcr8*) (Wang et al., 2007). Together, these proteins function as a "microprocessor" complex to initiate pri-miRNA processing (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). In contrast, certain miRNAs have also been reported to be originating from introns (called "mirtrons") of primary mRNA sequences. These mirtrons mimic pre-miRNAs and thus escape microprocessing by *Drosha* (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). In general, pre-miRNAs are generated within the nucleus and their export is critically dependent on the nuclear protein *Exportin 5* that mediates the export of correctly processed pre-miRNA to the cytoplasm in a Ran-GTP dependent manner (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004) (Fig. 5).

1.7.3 Argonaute family of proteins mediate miRNA function

Once exported to the cytoplasm, the processed pre-miRNAs are recognized and further processed to mature ~22 nt miRNA duplexes by a Type III RNase enzyme *Dicer*, another key component of the RNAi machinery which is critical for mouse development (Bernstein et al., 2003). Mediated by the *transactivating response RNA-binding protein*, *Trbp* (Chendrimada et al., 2005), *Dicer* transfers one of the strands of the duplex to the miRNA-induced silencing complex (miRISC) that typically comprises *Argonaute* (*Ago*) family of proteins. Thus, miRISC is otherwise termed miRNA-containing ribonucleoprotein particle (miRNP) that forms the catalytic driving force guided by miRNAs to their destined target mRNAs (Fig. 5).

The unfavored second strand of the duplex termed miRNA* (read as miRNA star) sequence is often thought to be degraded (Grishok, et al., 2001; Hutvagner, et al., 2001; Ketting et al., 2001; Khvorova et al., 2003; Schwarz et al., 2003; Ender & Meister, 2010).

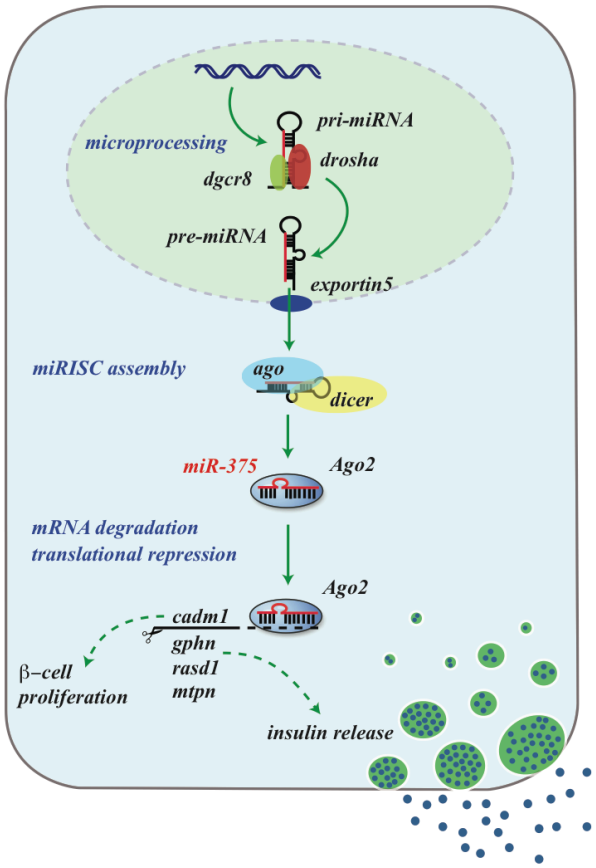


Fig. 5: *miRNA biogenesis and function*. The miRNA genes are transcribed from DNA by RNA polymerase II as primary miRNA transcripts (*pri-miRNA*) that are recognized by the microprocessors *Drosha* and *Dgcr8*. These proteins generate miRNA precursors (*pre-miRNA*) that are subsequently transported to cytoplasm *via Exportin5*. In the cytoplasm, *Dicer,* together with other proteins, further process *pre-miRNA* into mature miRNAs that are subsequently loaded onto the miRNA-induced silencing complex (*miRISC*) comprising the *Argonaute* (*Ago*) family of proteins such as *Ago2*. The mature miRNAs guide *Ago2* to target mRNAs where the miRNA binds to comple- mentary sequences within their 3' UTRs. The miRNAs perturb gene expression *via* mRNA degradation or translational repression. In the β-cells, *miR-375* is known to target genes such as *Cadm1*, *Gphn*, *Rasd1*, and *Mtpn* that are known to regulate β-cell proliferation and insulin secretion.

Central to the miRNP are the *Ago* family of proteins *Ago1*, *Ago2*, *Ago3*, and *Ago4* that are well characterized and ubiquitously expressed in humans (Peters & Meister, 2007). Interestingly, although all four *Ago* members are shown to possess overlapping functions, *Ago2* is expressed at higher levels than either *Ago1, 3* or *4* (Su et al., 2009; Wang et al., 2012), suggesting a greater role for this member. In fact, a genetic loss of *Ago2* renders mice inviable, demonstrating its critical requirement during mouse development (Liu et al., 2004). Contrastingly, *Ago4* knockout mice are viable and loss of this lesser abundant *Ago* member renders male mice infertile (Modzelewski et al., 2012). All *Ago* proteins contain distinct Piwi-Argonaute-Zwille (PAZ) and PIWI domains that are characteristic of the "Slicer" activity known to miRNP complexes. However, only *Ago2* has been shown to have endonucleolytic or "slicing" activity in mammals (Liu et al., 2004; Meister et al., 2004; Tolia

& Joshua-Tor, 2007). Interestingly, in contrast to most pre-miRNAs that require *Dicer* for their maturation, the processing of *pre-miR-451* is independent of *Dicer* and requires the *Ago2* slicer catalytic activity *in vivo* (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). Besides the slicing functions attributed to *Ago2*, a slicer-independent role was also shown using hematopoiesis as a model system in mice, suggesting multitasking roles for *Ago2* in different cellular contexts (O'Carroll et al., 2007).

Once loaded onto the miRISC containing either of the *Ago* members, miRNAs guide the complexes to their target mRNA sequences. These are either fully or partially complementary to a region of the miRNA sequence (typically from 2-7 nucleotides) termed as the "seed" sequence. Ideally this miRNA-mRNA interaction would require a conserved Watson-Crick base pairing (Bartel, 2004). The degree of complementarity, thermodynamic stability, and cross-species conservation are some of the key factors that determine a true target (Bartel, 2009). As yet, two modes of gene silencing are being considered: mRNA degradation (in cases of absolute complementarity) (Hutvagner & Zamore, 2002; Guo et al., 2010) or translational repression (in cases of incomplete complementarity). However, the mechanism by which the latter is carried out is only speculated to destabilize the mRNA *via* inhibiting the mRNA-ribosome complexes (Pillai et al., 2007; Filipowicz et al., 2008). However, recent studies integrating high-throughput mass spectrometry based proteomics with miRNA function have revealed that in addition to decreasing the mRNA levels, miRNAs can also inhibit protein synthesis *via* translational repression of target mRNAs (Baek et al., 2008; Selbach et al, 2008). In light of mRNA degradation, it has been demonstrated that the miRNP complexes localize at defined cytoplasmic loci referred to as Processing or P-bodies or also otherwise termed GW-bodies (Liu et al., 2005). Additionally, it has been shown that *Ago2* is localized at these P-bodies and can interact with their components such as GW-182 suggesting that *Ago2*/GW-182 interaction is critical for miRNA mediated mRNA degradation within these cytoplasmic structures (Sen & Blau, 2005; Jakymiw et al., 2005; Liu et al., 2005).

1.7.4 miRNA function during cellular stress

In addition to its functional role in P-bodies, *Ago2* was also shown to localize at distinct cytoplasmic structures defined as Stress Granules (SGs) under conditions of cellular stress (Leung et al., 2006). Moreover, this SG localization of *Ago2* is dependent on the presence of

miRNAs suggesting a role for miRNA-mediated control of gene expression in response to cellular stress. It has been discussed over the last decade as to how these small RNAs have evolved to respond to signals of stress there by alleviating the disease phenotypes of cells (Mendell & Olson, 2012). Also, it has been suggested that changes in gene expression programs mediated by miRNAs confer robustness to several biological processes during stress. Over the past years, accumulated evidence indicated that a miRNA function becomes more apparent upon induction of stress suggesting that cellular systems have evolved to combat stress, thereby maintaining their physiologic steady states (Ebert & Sharp, 2012). Several stress modules including nutrient stress and hypoxia are known to induce changes in gene expression and miRNAs act to counter aberrantly expressed genes thereby acting as "buffers" (Spriggs et al., 2010; Ebert & Sharp, 2012). It is strongly believed that cellular stress can induce profound changes in gene expression patterns and miRNA biogenesis together with SG localization of certain components of miRNA machinery, to maximize miRNA activity (Leung & Sharp, 2010). Moreover, recent studies indicate that posttranslational modifications can also control the function of *Ago2 via* phosphorylation and hydroxylation so as maintain its stability during cellular stresses including hypoxia (Qi et al., 2008; Zheng et al., 2008; Horman et al., 2013; Shen et al., 2013).

In the β-cell perspective, insulin resistance is known induce several metabolic changes within cells, ultimately triggering β-cell failure (Muoio & Newgard, 2008). It is now imperative to believe how miRNAs respond to such states so as to maintain β-cell function critical during the compensatory phases prior to developing overt T2D. In fact, genetic ablation of islet abundant *miR-375* in mice during insulin resistance demonstrated that miRNA function is indispensable for maintaining β-cell proliferation (Poy et al., 2009). Many islet miRNAs have been shown to be dysregulated during obesity and insulin resistance including pregnancy (Zhao et al., 2009; Jacovetti et al., 2012). However, evidence is still poor on the *in vivo* roles for several other miRNAs together with their mediators such as *Ago2* in the β-cells during insulin resistance and T2D.

1.7.5 miRNAs in insulin responsive tissues

Several miRNAs have been identified to perform critical functions in important peripheral insulin responsive tissues such as the liver, fat, and muscle. In the liver, an abundant *miR-122*

was successfully silenced using chemically designed "antagomirs", which revealed the role of *miR-122* in the cholesterol biosynthesis pathway (Krutzfeldt et al., 2005). More recently, other miRNAs such as *miR-143*, *miR-145*, and *miR-802* were reported to be upregulated in the livers of obese mice and humans, implicating their roles in dysregulated glucose homeostasis during insulin resistance (Jordan et al., 2011; Kornfeld et al., 2013).

Adipocytes have been long considered crucial cells that control glucose homeostasis in response to insulin and its defective signaling is known to increase the risk of T2D. Accumulating evidence suggests that *miR-103/107* and *miR-133*, which are well expressed in fat, contribute to insulin sensitivity in mice by controlling target genes such as *caveolin-1* and *PR domain containing 16* (*Prdm16*), respectively (Trajkovski et al., 2011; Trajkovski et al., 2012). *Prdm16* was previously shown to upregulate a brown fat enriched *miR-193b-365* cluster (Sun et al., 2011). These miRNAs and their target genes have been shown to be critical in relaying insulin signals and brown fat fate decisions (Liu et al., 2013; Yin et al., 2013). More recently, it has been shown that expression of *Dicer* and several miRNAs declines upon ageing in the adipose tissue and a fat specific loss of *Dicer* renders mice hypersensitive to oxidative stress and is associated with lipodystrophy and insulin resistance (Mori et al., 2012; Mori et al., 2014).

In addition to liver and fat, the muscle has been identified to express several miRNAs such as *miR-1*, *miR-133*, and *miR-181*, which have been implicated in controlling pathways for myogenic differentiation (as reviewed by Krutzfeldt & Stoffel, 2006). Moreover, recent studies employing muscle specific loss of either *Lin28a*, an RNA binding protein known to block *let-7* function (Viswanathan et al., 2008; Viswanathan & Daley, 2010; Mayr & Heinemann 2013), or transgenic overexpression of *let-7* rendered mice insulin resistant and glucose intolerant (Frost & Olson, 2011; Zhu et al., 2011). These studies suggest an important role for the *Lin28/let-7* axis in the control of glucose metabolism. In addition to the aforementioned insulin responsive tissues, miRNA expression has also been demonstrated to be critical in tissues such as the brain in maintaining glucose homeostasis. A genetic ablation of *Dicer* from pro-opiomelanocortin (*pomc*) specific neurons, glucose sensing of which is impaired during obesity (Parton et al., 2007), led to the development of obesity in mice (Schneeberger et al., 2012). Put together, it is widely accepted that the miRNAs contribute significantly in modulating insulin sensitivity and resistance to stress so as to maintain systemic glucose homeostasis (Rottiers & Näär, 2012).

1.7.6 miRNAs maintain β-cell function

In the β-cells, *miR-375* was identified as an islet abundant miRNA that was demonstrated to regulate insulin secretion *via* targeting *Myotrophin* (*Mtpn*), a gene implicated in regulating later stages of insulin release (Poy et al., 2004) (Fig. 5). In addition, morpholino-based silencing and genetic loss of this miRNA in zebrafish and mice, respectively, showed that it is critical for the maintenance of islet mass (Kloosterman et al., 2007; Poy et al., 2009). Besides *miR-375*, genetic and environmental factors that cause obesity, insulin resistance, and T2D have all been to shown to affect the expression of miRNAs including *miR-184* (Zhao et al., 2009; Jacovetti et al., 2012; Nesca et al., 2013). However, none of these studies have investigated in detail the functional roles of these miRNA during obesity and insulin resistance *in vivo*.

Dysregulation of certain miRNAs have implications in activating growth pathways thereby controlling β-cell proliferation. For instance, loss of *miR-7*, another major islet miRNA (Bravo-Egana et al., 2008), has been shown to activate the *mammalian Target of Rapamycin* (*mTOR*) pathway and promote β-cell proliferation (Wang et al., 2013). Furthermore, *miR-7* can also target genes such as *Pax6* and several others critical for insulin granule fusion with the plasma membrane to regulate endocrine cell differentiation and insulin exocytosis, respectively (Kredo-Russo et al., 2012; Latreille et al., 2014). On the other hand, upregulation of *miR-24* in the islets during insulin resistance is implicated in the inhibition of β-cell proliferation by targeting MODY cluster of genes such as *NeuroD1* and *Hnf1a* (Zhu et al., 2013). In light of the fact that various pathways are now known to control insulin release from the β-cells in response to glucose (MacDonald et al., 2005), several studies are now being focused on how the miRNA pathway contributes to this important aspect of the β-cell. Indeed, it is indicated that miRNAs including *miR-30d* and *miR-375* respond to changes in extracellular glucose levels (Tang et al., 2009; El Ouaamari et al., 2008) suggesting their functional role in controlling GSIS. Furthermore, increased levels of *miR-375* were shown to be associated with human T2D (Zhao et al., 2010), suggesting miRNAs fine tune expression of several genes critical for β-cell survival and function during the pathogenesis of T2D.

Diminished GSIS is widely considered as one of the key factors for the development and progression of T2D (Weir & Bonner-Weir, 2004). In light of the essential roles of the miRNA pathway in β-cells, several groups have demonstrated that miRNAs such as *miR-375*

(Poy et al., 2004), *miR-9* (Plaisance et al., 2006), *miR-33a* (Wijesekara et al., 2012), *miR-30a-5p* (Kim et al., 2013), *miR-24* (Zhu et al., 2013), *miR-187* (Locke et al., 2014) and more recently *miR-184* (Morita et al., 2013) and *miR-7* (Latreille et al., 2014) all play critical roles in the release of insulin from the β-cell. Whilst most of these studies suggest that miRNAs act to counter GSIS, a few have indicated that miRNAs such as *let-7* (Zhu et al., 2011), *miR-132* and *miR-212* (Soni et al., 2014), can positively regulate insulin secretion potentially alleviating insulin resistance in mice. Although the reports mentioned above have indicated that miRNAs can affect the pathways leading to insulin exocytosis, a comprehensive understanding of how the components of miRNA pathway such as *Ago2* govern insulin release still remains scant. Moreover, in lieu of the fact that ISGs consist of distinct proteins with diverse functions (Suckale & Solimena, 2010), evidence on how the miRNA pathway coordinates the release of these proteins in response to glucose still remains poor.

1.7.7 Association of *miR-184* with β-cell function

First reported as the most abundant miRNA in the murine eye (Lagos-Quintana, 2003), *miR-184* is an evolutionarily conserved miRNA, found to be frequently dysregulated in cancers (Wong et al., 2008; Foley et al., 2010). Moreover, loss of function of this miRNA has been shown to perturb female germline development in *Drosophila* suggesting a conserved functional role across species for *miR-184* (lovino et al., 2009). Computationally, *miR-184* has been predicted to target fewer genes in humans than in flies (Grün et al., 2005). Besides the target genes possessing a conserved seed sequence for this miRNA, *miR-184* has also been shown to target several other genes that do not maintain seed conservation across species. For instance, *miR-184* has been shown to target *Nfat1* in human T cells (Weitzel et al., 2009), *Akt2* in neuroblastoma cells (Foley et al., 2010), *Numbl* in adult neural stem/progenitor cells (Liu et al., 2010), *Tnfaip2* in squamous cell carcinoma of the head and neck (Liu et al., 2011), and more recently *c-Myc* in non-small cell lung cancer (Liu et al., 2014). With regard to predicted targets possessing a conserved seed sequence, *miR-184* was reportedly shown to regulate genes such as *Ship2* in epithelial cells (Yu et al., 2008), *Ncor2* in spermatogonia (Wu et al., 2011), *Ago2* in human keratinocytes (Roberts et al., 2013), and *Slc25a22* in mouse β-cells (Morita et al., 2013).

In the islets, *miR-184* was reported to be strongly downregulated in genetically obese mice suggesting a role for this miRNA during the onset of T2D (Zhao et al., 2009). Interestingly,

miR-184 in human islets negatively correlated to GSIS, suggesting its role in insulin secretion (Bolmeson et al., 2011). Furthermore, its enrichment in human islets, specifically in β-cells, clearly demonstrates it potential role in β-cell function during T2D (van de Bunt et al., 2013). Additionally, while its inhibition in human islets has been shown to increase β-cell proliferation (Nesca et al., 2013), overexpression of *miR-184* inhibits the release of insulin by targeting *Slc25a22* in MIN6 β-cell line (Casimir et al., 2009; Morita et al., 2013). Although the aforementioned data indicates a strong association between *miR-184* and the β-cell, its *in vivo* role in the maintenance of β-cell function during insulin resistance and T2D in mice and humans is unclear.

The present thesis thus focused on elucidation of the contribution of miRNA pathway in regulating the global release of proteins, called the β-cell secretome, and maintenance of β-cell function during insulin resistance. While Chapter 3 revealed the as yet unknown functions of *Ago2* in impacting the β-cell secretome, Chapter 4 uncovered that *Ago2* is important for β-cell proliferation during insulin resistance and that the silencing of *miR-184* promoted its expression *in vivo*. With a combination of several high-throughput and genetic mouse models, the role of *miR-184* was addressed in detail *in vivo* in the β-cell. These studies established that the *miR-184/Ago2* inverse correlation as an essential component during disease states such as insulin resistance and T2D in mice and humans.

Chapter 2
Experimental Methods

2.1 Animal models and *in vivo* physiology

2.1.1 Generation and maintenance of mice

Mice were maintained on a 12-hour light/dark cycle with ad libitum access to regular chow food, high fat diet (60% kcal fat containing diet) (E15741-347, ssniff Spezialdiäten GmbH), or ketogenic diet (E15149-30, ssniff Spezialdiäten GmbH) in accordance to requirements established by Landesamt für Gesundheit und Soziales (Lageso).

The constitutive *miR-184* overexpressing *RIP-184* transgenic mice were generated using a 292 bp genome fragment encompassing the *miR-184* precursor sequence that was amplified and cloned into pCR2.1 vector (Invitrogen) containing an already cloned *Rat Insulin Promoter* (RIP) and the resulting construct was used for microinjections. Generation of *dox-184* mice was similar to that of *RIP-184* mice except the usage of a conditional tetracycline operator (*tetO*) sequence upstream of *miR-184*. Doxyclicline responsive transgenic mice were ultimately obtained by crossing *tetO-184* mice with *Ins-rtTA* (reverse tet-trans activator under the control of insulin promoter) transgenic mice (Jackson Laboratories). Similarly *dox-Ago2* mice were generated using an *Ago2* cDNA vector that was obtained from the lab of Günter Meister, University of Regensburg.

The total *miR-184*KO mice were originally designed and generated by D. Aberdam and R. Shalom-Feuerstein (Institut Clinique de la Souris). The β-cell specific Ago2 knockout (βAgo2KO) mice were obtained after crossing $Ago2^{flox/flox}$ (floxed) mice (obtained from Donal O'Carrol, EMBL) with transgenic mice carrying the *Cre* gene under the control of insulin promoter (*Ins-Cre*; kindly provided by Pedro Herrera, University of Geneva) and genotyped by PCR. The total *Cadm1*KO mice were kindly provided by L.v.D. Weyden from the Sanger Institute.

2.1.1.1 Primer pairs used for generating overexpression constructs

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
miR-184	CTCCTCCTCACGTCCTGTGGTA	TGCTGAAGAGTGGCCTGCTAGG
tetO-184	CTCCTCCTCACGTCCTGTGGTA	TGCTGAAGAGTGGCCTGCTAGG

RIP	TGGGCTATGGGTTTGTGGAAG G	AGGGCCATGTTGGAACAATGAC

2.1.2 Physiological tests in mice

In vivo insulin release and glucose (GTT) or insulin (ITT) tolerance tests were performed following a 6-hour fast and injected intraperitoneally (i.p.) with either glucose (2 g/kg BW) or insulin (0.75 U/kg BW). For *in vivo* insulin release experiments, fasted mice were i.p. injected with glucose and blood was milked from the tail vein at 2.5, 5, and 15 min time points. So as to measure basal insulin release, blood was collected in a similar fashion at time 0 before the i.p. injection of glucose. The resultant blood was spun at 10, 000 rpm for 5 min and the plasma supernatant was subjected to insulin RIA or ELISA for direct quantification of plasma insulin levels. For GTT and ITT, blood glucose was measured at desired time points after a glucose i.p. injection from the tail vein using a standard glucometer (Bayer).

2.2 Molecular biology

2.2.1 Isolation of genomic DNA from mouse tail-tips

Total genomic DNA was isolated after an overnight digestion of mouse tail tips in lysis buffer (4 M Urea, 10 mM EDTA pH = 8.0, 0.1 M Tris-HCl pH = 8.0, 0.5 % sodium sarcosyl, and 0.2 M NaCl) along with 0.18 mg/mL proteinase K (PAN-Biotech) at 55 °C. The following day, the lysate was spun down to eliminate debris and the DNA was precipitated using 1/10th volume sodium acetate and 2.5 volumes absolute ethanol. The suspension was centrifuged for 5 min at maximal speed and the pellets were washed with 70 % ethanol. The DNA pellets were later dried at room temperature for 30 - 45 min and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) and stored at 4 °C until further usage.

2.2.1.1 Primer pairs used for genotyping knockout and transgenic mice

Mouse line	Forward sequence (5'-3')	Reverse sequence (5'-3')
RIP-184	AGGTCATTGTTCCAACATGGCC	TTAGAGTCACAAAGCTGGCTGG
ob/ob	TGTCCAAGATGGACCAGACTC	ACTGGTCTGAGGCAGGGAGCA
Ago2 $^{flox/flox}$	GTGAGCCACTCACTGTGCAC	TGATCATGGTTGAGGTCTGA
Ins-Cre	CCTGTTTTGCACGTTCACCG	ATGCTTCTGTCCGTTTGCCG

*miR-184*KO	ACTGAACATTATTTCATGGGCCGGG	R1:AACTACAACTGTTTGGCTAGCAGGGTG R2:CGCTGAGACCTTGTGATAAACCGTT
tetO-184	AGGCCTATATAAGCAGAGCTCG	CCTTATCAGTTCTCCGTCCAAC
tetO-Ago2	ATCCACGCTGTTTTGACCTC	GAGCAGGAGAAGCAAGAACG
RIP-rtTA	TAGATGTGCTTTACTAAGTCATCGCG	GAGATCGAGCAGGCCCTCGATGGTAG
*Cadm1*KO	CTCGAGCAGGTGAAGAGGGGACCATTGGG	GATGAAGTACTCTTTCTTTTCTTCGGAGT
MIP-GFP	GAAGACAATAGCAGGCATGCTG	ACTGGGCTTACATGGCGATACTC

2.2.2 Southern blotting analysis

For the *RIP-184* transgenic mice, three independent lines designated *Tg04*, *Tg32*, and *Tg96* were selected for further characterization based on the level of transgene integration in the genome as evidenced by Southern blotting. Briefly, total genomic DNA from the founder lines was isolated using an overnight tail lysis and ethanol-sodium acetate precipitation method. 20 μg of total DNA was digested overnight using the restriction enzyme AflIII (NEB). Next day, the digested DNA was resolved on a 0.8% agarose gel at 60 V for 6 hr. After the run, the gel was incubated in 100 mL of 0.5X TAE buffer containing 10 μl ethidium bromide (Et-Br) for 20-30 min before recording the picture along with a fluorescent ruler under a UV chamber. Later, the gel was fixed with 0.25 M HCl (1:50 dilution) for 10 min and washed with water. Next, the fixed gel was denatured for 30 min at RT and later incubated with 10X SSC for 10 min. Meanwhile the membrane was pre-wet with water and the whatman wick with 10X SSC and gel was transferred onto to a nylon membrane by semi-dry method overnight. The following day, the membrane was UV cross-linked at a power of 0.01 J for 10 sec and additionally baked at 80 °C for 1 hr. After an hour's pre-incubation using hybridization buffer, the membrane was incubated overnight using radiolabeled probe that recognizes a region in between the restriction cut sites within the transgene. The probe was labeled using Prime-It II Random Primer Labeling Kit (Stratagene) and the template for the labeling reaction was obtained using the *RIP-184* genotyping primers. After probing overnight, the membrane was washed at least three times using wash buffers and exposed to films in a cassette for at least 6 hr to detect the expected 1 kb bands.

2.2.2.1 Buffers and reagents used for Southern blotting

20X SSC buffer

350.0 g Sodium chloride (NaCl)

176.4 g Sodium citrate ($Na_3C_6H_5O_7$)

2 l Water (H_2O)

Denaturing solution

40.0 g Sodium hydroxide (NaOH)

175.3 g Sodium chloride (NaCl)

1 l Water (H_2O)

Hybridization buffer

12.5 mL 20X SSC

1.0 mL 1M Na_2HPO_4 (pH=7.2)

35.0 mL 10% SDS

1.0 mL 50X Denhardt's solution

0.5 mL Sonicated salmon sperm DNA (10 mg/mL)

2.2.3 Northern blotting analysis

For checking *miR-184* overexpression in MIN6 cells using alternate methods, Northern blotting was performed to test the RIP-184 overexpressing plasmid. Briefly, MIN6 cells were transfected with either RIP-184 or GFP plasmids using Lipofectamin 2000 transfection reagent (Life Technologies). 36-48 hr later, the cells were harvested and total RNA was isolated using TriZol (Life Technologies) based method. 40 μg of total RNA was loaded along with 2X bromophenolblue loading buffer per lane on a 1.5 mm, 15X17 cm large, 12% polyacrylamide gel (National Diagnostics). Samples were run at 10 W for 10 min and later at 20 W until the dye front has reached at least 2 cm from the end of the gel. The separation of RNA was visualized under UV light after incubating the gel in 100 mL of 0.5X TBE buffer contaninig 10μl of ethidium bromide (Et-Br). Later, the gel was transferred onto Hybond-N+ membrane (Amersham Pharmacia) using 0.5X TBE with a constant amperage (1 hr, 3mA/cm^2 or 1.5 hr, 2mA/cm^2) or 48V for 1.2 hr. After the transfer, the membrane was air dried briefly before UV crosslinking at 1200J for 30 sec and subsequently baked for 1 hr at 80 °C. Later, the blot was pre-hybridized using hybridization buffer for 1hr at 65 °C.

Meanwhile, 1 μl of 20 μM oligonucleotide (22 nt complementary sequence to *miR-184*, *miR-375* or *U6* small RNAs) was radiolabeled using 5 μl of γ-^{32}P-ATP (3000Ci/mmol), 2 μl of

10X T4 PNK buffer (NEB), 0.2 µl of T4 PNK enzyme (10 U/µl), and 11.8 µl of nuclease free water. The reaction mixture was incubated for 15 min at 37 °C. Later, 30 µl of 30 mM EDTA (pH 8.0) was added to stop the reaction and the reaction mixture was applied to an equilibrated, dry Sephadex G-25 spin column (GE). 1 µl of the radiolabeled probe was counted and approximately 3,00,000 cpm per blot was used. The probe was boiled at 90-95 °C for 1 min before it was added to the hybridization buffer already on the pre-hybridized blot at 65 °C and incubated overnight. The following day, the membrane was washed at least three times in wash buffers at 65 °C, air dried, and exposed to films.

2.2.3.1 Probe sequences used for Northern blotting:

miRNA	***Oligonucleotide sequence (5'-3')***
miR-184	ACCCTTATCAGTTCTCCGTCCA
miR-375	TCACGCGAGCCGAACGAACAAA

2.2.4 Chromatin immunoprecipitation (ChIP)

ChIP was performed using MIN6 and isolated islets of either WT or *ob/ob* mice to check for enrichment of *Suz12* binding sites upstream of *miR-184* (Marson et al., 2008). Briefly, the previous day of ChIP, 100 µl of protein-G agarose beads (Roche) were washed and incubated overnight with 5 µg of IgG or *Suz12* antibodies in 2.5 bead volumes of blocking solution (0.5 % BSA in PBS). The next day, $1/10^{th}$ media-volumes of 11 % formaldehyde buffer (50 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% Formaldehyde, and water) was added to ~ 20 million MIN6 cells growing on a 15 cm dish and incubated at RT for 10-20 min with shaking. Later, the fixation was halted using 2 mL of 2 M glycine for 5 min at RT and the cells were washed twice with ice cold PBS, scraped, and pelleted. The pellet was resuspended in 6 mL of Lysis buffer 1 (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5 % NP-40, 0.25 % Triton X-100, 1X Protease inhibitor, and water) and gently vortexed before being incubated for 20 min at 4 °C on a rotor. The suspension was gently centrifuged at 1, 400 rpm at 4 °C for 5 min and the pellet was again resuspended with 6 mL of Lysis buffer 2 (10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.5 % NP-40, 1X Protease inhibitor, and water) and incubated at RT for 10 min on a rotor. The suspension was again pelleted and resuspended in 100 µl of Lysis buffer 3 (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1 % SDS,

1X Protease inhibitor, and water) and incubated on ice for 10 min. Later, the lysate was sonicated with medium power at 30 sec on/off cycling for at least 25 times.

The level of sonication was checked by taking 10 µl of chromatin and incubating with 2-5 µl of proteinase K for 1 hr at 65 °C. Ideally, for ChIP sequencing 150-350 bp and for ChIP-PCR 200-1000 bp fragmentation is expected. Now, $1/10^{th}$ volume of 10 % Triton X-100 was added to the chromatin and the suspension was spun at 16, 000 g for 10 min at 4 °C. The supernatant was collected and 9X volume of IP dilution buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1 % Na-deoxycholate, 140 mM NaCl, 1X Protease inhibitor, and water) was added. The NaCl was adjusted to a final concentration of 140 mM. This chromatin suspension was added to the antibody-bead complex and incubated overnight at 4 °C.

The next day, the beads were collected and washed at least 7 times 4 min each with LiCl RIPA buffer (50 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 0.5 M LiCl, 1 % NP-40, 0.7 % Na-deoxycholate, 140 mM NaCl, 1X Protease inhibitor, and water) at RT. The DNA was recovered from the beads with 100 µl of pre-warmed elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 mM EGTA, 1 % SDS, and water) after a 30 min incubation at 65 °C. The NaCl concentration of the chromatin supernatant was adjusted to 110 mM before being de-crosslinked overnight at 65 °C. Finally the next day, DNA was extracted using phenol-chloroform and precipitated by ethanol-sodium acetate along with 1 µl of glycogen at -20 °C overnight. DNA thus obtained was used for downstream qRT-PCR with promoter specific primers.

2.2.5 Fluorescence activated cell sorting (FACS) of GFP positive β-cells

So as to determine the cellular enrichment of *miR-184*, FACS was employed to separate β-cells from the non β-cell fraction of islets obtained from transgenic mice overexpressing green fluorescence protein (GFP) specifically in the β-cells (MIP-GFP; Hara et al., 2003) were utilized. Approximately 2,000 islets were isolated from 10-12 week old *MIP-GFP* transgenic mice and were trypsinized using 0.5% trypsin for 5 min at 37 °C with occasional pipetting. Once cells started to appear dispersed, the reaction was arrested with ice-cold 10% FBS in PBS and quickly spun at 2000 rpm for 4 min. The dispersed cell pellet was resuspended in 500 µl of PBS and filtered using Cell strainer (BD Falcon). Later, 1 µl of 10 mg/mL propium iodide (PI) was added to the cell suspension before docking the tube containing the cells into the cell sorter (Beckman Coulter). Cells were sorted and GFP

positive β-cells were separated from the non β-cells and both cell fractions were directly collected into Trizol for isolation of total RNA for further downstream gene expression analysis by qRT-PCR.

2.2.6 Quantitative gene expression in islets and MIN6 cells

miRNA expression was quantified from the total RNA derived from mouse and human islets or cell lines using TaqMan based miRNA assay kits (Life Technologies Inc.) specific for *U6*, *RNU6B*, *hsa-miR-184*, *hsa-pri-miR-184*, *hsa-miR-375*, *hsa-miR-7*, and *ath-miR-168a* according to manufacturer's instructions. Briefly, 10 ng of the total islet RNA was reverse transcribed for the specific small RNAs using the miRNA reverse transcription kit (Life Technologies Inc.). The cDNA was later used for TaqMan based quantitative real time PCR (qRT-PCR) and the results were analyzed with delta-delta Ct method of quantitation (Pfaffl, 2001).

For mRNA quantification, total islet or cellular RNA was reverse transcribed using RevertAid First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions. Equal amounts (~10 ng) of the resultant cDNA from different samples were used for SyBr Green (Roche) based qRT-PCR and the resulting Ct values were analyzed using delta-delta Ct method of quantitation (Pfaffl, 2001). All primers were designed using online primer designing tools such as OligoCalc and Primer3. The designed primers were checked for their efficiency of template detection prior to using them for downstream qRT-PCR analyses. In the case of relative quantification of isoform specific *Ago* genes, data was obtained after normalization with standard curves established with mouse *Ago1-4* cDNA constructs (kindly provided by Günter Meister, University of Regensburg).

2.2.6.1 Primer pairs used for qRT-PCR:

Gene	Forward (5'-3')	Reverse (5'-3')
Ago1	GAAGACGCCAGTGTATGCTGAA	ATCTTGAGGCAGAGGTTGGACA
Ago2	GCACTTACCATCCATGAGGTAC	AAAGAGAAGGTCGGACGGACTGAT
Ago3	TTGGAAGAAGCGGCAACATC	GATAGTGTGAAGGACGGCTGGT
Ago4	ACACGCTCCGTCTCCATTCC	GACTGCCTTCCGCACTGTCA
Cadm1	CTCGAGCAGGTGAAGAGGGGACCATTGGG	GATGAAGTACTCTTTCTTTTCTTCGGAGT
HuD	CAGGGATGCTAACCTGTATG	CTTTGATGGCTTCTTCTGCC

Ywhaz	AGAAGATCGAGACGGAGCTG	TTGTCATCACCAGCAGCAAC
Gck	CGTTGACTCTGGTAGAGCAGATC	GAGAAAGTCTCCAACTTCTGAGC
hAgo2	AGCCGTGCTTCTGCAAATAC	TCTGCACGTTCTTCATCTGC
Slc25a22	TGATTGCCTCATCAAGACCA	CCAACTTGATGGCCTTCTCC
36B4	TGC CAG GAC GCG CTT GT	GGC CCT GCA CTC TCG CTT TC
Gapdh	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
Insulin	TCTTCTACACACCCATGTCCC	GGTGCAGCACTGATCCAC
Glucagon	AATGAAGACAAACGCCACTC	GCCCTCCAAGTAAGAACTCA
Somatostatin	TCGCTGCTGCCTGAGGACCT	GCCAAGAAGTACTTGGCCAGT
Egfp	GCAGAAGAACGGCATCAAGGT	ACGAACTCCAGCAGGACCATG
Ppy	AGGAGGAGAACACAGGTGGA	AGACAAGAGAGGCTGCAAGT
β-actin	AGGATGCAGAAGGAGATCACTG	GGGTGTAACGCAACTAAGTCATAG
Hprt	TTAGCGATGATGAACCAG	AGCAGGTCAGCAAAGAAC

All primers are specific to mouse unless mentioned *h* for human.

2.2.7 Illumina gene expression analysis in islets and MIN6 cells

Global gene expression analyses in the MIN6 β-cell line transfected with *miR-184* overexpressing constructs were performed in biological triplicates. Briefly, MIN6 cells were transfected with reverse tet-transactivator (rtTA; Clontech) along with *184-tetO* plasmids. Overexpression of *miR-184* in the transfected MIN6 cells was induced by 1 mg/mL doxycycline (Sigma) over 16, 24, 48, and 72 hrs time-points and as biological triplicates. Cells were harvested for total RNA at each time point and proceeded for cRNA synthesis using Illumina TotalPrep RNA Amplification Kit (AMIL1791, Life Technologies Inc.). The amplified cRNA was then used for gene expression analysis using Illumina mouse WG6v2 arrays.

2.2.8 Ribonucleoprotein immunoprecipitation (RIP) assay

Ribonucleoprotein immunoprecipitation (RIP) experiments using Ago2 (Wako 018-22021) and Ago1 (MBL RN028PW) antibodies were performed as described (Keene et al., 2006). Briefly, ~30 million MIN6 cells were lysed using polysome lysis buffer containing 100 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.0), 0.5 % NP-40, 1 mM DTT, 100 Units/mL RNase inhibitor (Fermentas), 400 μM VRC (NEB), and Protease inhibitor cocktail (Roche). After a 5 min incubation on ice, the lysate was stored at -80 °C to enhance lysis. The

previous day, Protein-G agarose beads (Roche) were washed at least twice with NT2 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl2, and 0.05 % NP-40) supplemented with 5% BSA and the washed beads were resuspended in 250 µl of NT2/BSA buffer. 5 µg each of species matched IgG control or Ago1 or Ago2 antibodies were then added to the beads and incubated overnight at 4 °C in an upside down tumbling manner. Later, the antibody-coated beads were washed with ice-cold NT2 buffer at least 6 times to eliminate any unbound antibody or RNases associated, if any, and resuspended in 900 µl of NT2 buffer containing RNase inhibitors and EDTA. To this, 100 µl of the MIN6 cell lysate was added and incubated for 4hr in an upside down tumbling manner at 4 °C. Later, the beads were spun down, washed at least 6 times, and the beads were either directly boiled along with SDS loading dye for western analysis or bound RNA was isolated using Trizol (Life Technologies Inc.) method for subsequent qRT-PCR analysis. Additionally, the trizol was also supplemented with 5 µl of 5 fM plant *ath-miR-168a* as a spike-in control for normalization.

2.2.9 Luciferase assays

Luciferase assays were performed to determine miRNA mediated gene regulation as previously performed using standard established protocols (Poy et al., 2009). Briefly, 3'UTRs of genes potentially targeted by *miR-184* were PCR amplified using gene specific primers and primarily TA cloned into pCR2.1 vector (Life Technologies Inc.) before being finally sub-cloned into pRL-TK plasmid (Promega), towards the 3'end of renilla luciferase gene that acts as reporter. HEK-293 or MIN6 cells were transfected with 100 ng each of pRL-TK::3'UTR, pGL3 control plasmid that possesses firefly luciferase for internal normalization, along with 20 pmol of either *miR-184* or control (ctrl) mimics. 36-48 hr later, cells were harvested and the lysate was used for subsequent luciferase measurements using the Dual Luciferase Assay kit (Promega) according to the manufacturer's protocol. Briefly, 20 µl of the lysate was plated in a 96 well white flat- bottomed plate (Corning) and each well received 30 µl each of Luciferase Assay Reagent (LAR) II and Stop and Glow stepwise with a 2 sec pause and signal acquisition as programmed by the plate reader (TECAN). The data is finally calculated as relative luciferase units (RLU) after normalizing the renilla with that of firefly luciferase units.

2.2.9.1 Primer pairs used for 3' UTR cloning

3' UTR	Forward (5'-3')	Reverse (5'-3')
mAgo2	AGTTGGATTCACACGAGACCAG	GGCTAAATCTATGGAATGGGGC
hAgo2	GTTTAGCGATTGTGTACCGAGTGG	ATGTCACAGTCCTATAGGAC
Slc25a22	CCTGTGTGGTCCTGAAGGGACAAC	TGTCCAGGAGTAGGGCTCTATCAG
Bin3	TCACAGGCTGCTGCTTCCCTGT	ACCCTCTCTGTTTGCCAGGCCT
Hand2	AGAAGAGGAGAGCAGTGAGC	GCAGTGGTTTATTGAATACTTACAATG
Eln	CAGGACAAGGAAATCAGACAGC	TGATAGTGGGTATGCCCAGCCA
Rab1b	GCACCTTGTCCAGATGATGCCC	GGGTGAGACTGGCAAGAGACAG
Sf1	CCTCACCATATGGAGCCAGACATT	GCAGGAGTGCAGGAAGATGTGAAT
Ubap2	AGGAGGGAACACATGGAGCACC	CTGTCCACCTGGCTCAGGACAA
Nt5c3l	GAAGTAAGAGACTGTGAGACCC	GTGTTACTGGGAGCCTGCAAGT
Prkcb1	AGATCCGTAGACCTCCGTCCTC	CAAGGTGACAGCACCCAAGCTG
Cbx6	GTCTGTTGCCCTCTGCTGCTAG	CCCTCACCCCAACATTGCCACT
Epb4.1l5	ACCTGTAACTGGATTGCCCCAC	GTAAGCTCACGCAGTGTACACC
Ppap2b	TGCAGGAGTTGATCTTGCTGTG	GCCTTGGTTAGTTGTGTTATGAGC
MLec	AGTGCATCTCTTGGGATGGGCTTC	GGTATAGATGGATGGAGACAGCCC
Fzd1	AACCCAGACTCTTACCTTACCC	GGAAGGTGGATCTCAAATAAGC
Sidt2	CCTAGTCTGTCCATCTGTCTTG	TGACTCTGTAAACGCCAAGGCT
Nus1	AGGCTTCGGTTCCTGCTACTTC	CTTCGGTAATACAGGCCTCATC
Pp1	GTGGACAGCTCTGATGATGGTG	CCATTCTCAGCACATGGCATTG
Ago2 mutated	CCTCCGTTTTCCAGAATGCCTCGG GCGGCAGATGTCAGACTTGG	CCAAGTCTGACATCTGCCGCCCGAGG CATTCTGGAAAACGGAGG
hAgo2 mutated	CCGTTTTCCAGAATGCCTCGGGCG GCAGATTTCAAACTTGG	CCAAGTTTGAAATCTGCCGCCCGAGG CATTCTGGAAAACGG

All primers are specific to mouse unless mentioned *h* for human.

2.2.10 Subcellular fractionation

For subcellular fractionation, three 140 mm plates with confluent MIN6 cells were scraped into PBS, pelleted (300 g, 5 min at 4 °C) and resuspended in a hypotonic buffer containing 0.3M sucrose, 4mM HEPES pH 7.4, and 0.5mM EGTA. The cell suspension was gently homogenized and the homogenate was spun at 3, 000 g for 10 min at 4 °C. The postnuclear supernatant (PNS) was loaded on an 8-step sucrose gradient made using HEPES buffered 0.2,

0.4, 0.8, 1.2, 1.4, 1.6, 1.8, and 2.0 M sucrose and spun at 55, 000 g for 2 hr at 4 °C using the MLS50 rotor (Beckman Coulter). Antibodies for western blotting were used as described: Gephyrin (BD 610584), Ywhaz (Abcam ab61129), Grp78 (Assay Designs Stressgen SPA-826), Nsf (BD 612272), Rab3A (Abcam ab3335).

2.2.11 Small RNA deep sequencing

Small RNA sequencing libraries were prepared using Illumina small RNA library preparation kits. In brief, small RNA fractions with size range of 10-40 nucleotides (nt) were separated using flashPAGE Fractionator (Ambion) according to the manufacturer's instructions. The small RNA fractions were ligated sequentially at the 3' and 5' end with synthetic RNA adapters, reverse transcribed and amplified using Illumina sequencing primers. Amplified libraries were purified by polyacrylamide gel electrophoresis (PAGE) according to the expected product size. Libraries were sequenced for 50 cycles (Illumina Hi-seq 2000), and then 3' adapter sequences were removed using a custom Perl script. Reads of length between 17 and 30 nt were retained and mapped to known mouse pre-miRNA sequences deposited in miRBase (http://www.mirbase.org/) without allowing any mismatch using soap1 and soap.short, respectively.

2.3 Imaging and microscopy

2.3.1 Morphometric analyses of pancreatic islets

Islet morphometric analysis after intraperitoneal injections of BrdU on 4 consecutive days (50 mg/g BW, Sigma B5002) was performed on 8-micrometer sections of paraffin-embedded pancreas approximately 150-200 mm apart. Sections were dewaxed, washed, and stained for insulin (Dako A0564), glucagon (Millipore AB932), BrdU (Abcam ab6326), or TUNEL (Roche cat. no.12156792910). Cell numbers from all islets in 3-7 sections were counted with ImageJ software from 20X images obtained using a Zeiss LSM700 (Schneider et al., 2012). β-cell mass was measured as the ratio of insulin-positive cell area to the total tissue area, multiplied by the weight of the pancreas using Imaris software (Bitplane, Switzerland).

2.4 Cell culture, islet isolation and *in vitro* analyses

2.4.1 MIN6 cells and western blotting

MIN6 cells were cultured in DMEM (Life Technologies) containing 4.5 g/L glucose supplemented with 15% v/v heat-inactivated FCS, 50 mM β-mercaptoethanol, and 50 mg/mL

penicillin and 100 mg/mL streptomycin. For protein expression analyses by western blotting, MIN6 cells were transfected with various overexpression constructs, short interfering (si) RNAs, or miRNA mimics using Amaxa Nucleofector (Lonza) and plated usually in a 6-well format. 36-48 hr later, cells were washed once with PBS and lysed in 100 ml of RIPA lysis buffer. The wells were thoroughly scraped and incubated on ice for 10 min for complete lysis and later spun at 16, 000 g for 10-15 min at 4 °C. The supernatant was collected and protein content was measured by the Bicinchoninic acid assay (BCA) method. Equal amounts of total protein were later boiled at 95 °C along with 1X SDS loading dye (310 mM Tris, 10 % sodium dodecyl sulfate, 50 % glycerol, 5 mM EDTA, 0.05 % bromophenol blue, 5 % β-mercaptoethanol). The protein samples were resolved by SDS polyacrylamide gel electrophoresis (PAGE) using 1X SDS running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH = 8.3) and later transferred onto nitrocellulose membranes (Carl Roth) at 4 °C using 1X transfer buffer containing 25 mM Tris-HCl pH = 8.4, 192 mM glycine, and 20 % methanol. The membranes were later blocked for at least 30 min with 2.5 % milk in 1X TBST (20 mM Tris, 137 mM NaCl, pH = 7.6) and probed for desired proteins of interest using antibodies overnight on a roller at 4 °C.

The antibodies used were as described: Gephyrin (BD 610584), Rasd1 (Millipore AB15794), Ago2 (for immunoprecipitation, Wako 018-22021), Ago2 (for western blotting, Cell Signaling C34C6), Ago1 (MBL RN028PW), Ago3 (Abcam ab3593), Slc25a22 (Sigma AV44041), HuD/Elavl4 (Santa Cruz sc-48421), γ-tubulin (Sigma T6557), Cadm1 (Sigma S4945), and β-actin (Sigma).

The siRNAs were designed and obtained from Qiagen, Thermo, or Riboxx. The miRNA mimics (wildtype, *wt* and mutant, *mut*) were manually designed and obtained from Qiagen. ContramiRs against various miRNAs were designed and obtained from Riboxx.

2.4.1.1 miRNA mimic sequences

miRNA	***miRNA mimic sequence (5'-3')***
miR-184 (wt)	TGGACGGAGAACTGATAAGGG
miR-184 (mut)	TCCAAAGAGAACTGATAAGGG

2.4.2 Insulin secretion from MIN6 cells

Insulin secretion from MIN6 β-cells was performed as described previously (Poy et al., 2004). Briefly, approximately eight million MIN6 cells were transfected with 200 pmol of

either control or gene specific siRNAs using kit V and Program G-16 of the Amaxa nucleofector (Lonza) according to manufacturer's instructions, and the transfected cells were plated in a 24-well plate format and incubated in a CO_2 incubator at 37 °C. 36-48 hr later, cells were washed gently with pre-warmed Krebs Ringer Buffer (KRB; secretion buffer) and later the cells were incubated at 37 °C for 30 min in 5.5 mM glucose containing KRB so as to prime the MIN6 cells. Later, 5.5 mM glucose was replaced by 2.2 mM (Low), 25 mM (High) glucose, or 30 mM KCl containing KRB. 1 hr later, 400 µl of the supernatant was collected in eppendorf tubes and spun at 2000 rpm for 4 min so as to pellet floating cells, if any. The clean supernatant was used for Radio Immuno Assay (RIA) for quantifying the insulin released. The remaining supernatant from the cells was discarded and to each well, ice-cold 1 mL of acid-ethanol (1.5% HCl – 70% ethanol) was added and the plate was carefully stored at -20 °C to enable complete lysis of cells for measuring the total insulin content of the cells.

Composition of 1X KRB (secretion buffer)

0.10 g $CaCl_2$

0.20 g KCl

2.16 g $Na_2HPO_4.7H_2O$

0.20 g KH_2PO_4

0.35 g $NaHCO_3$

0.14 g $CaCl_2$

0.20 g $MgCl_2.6H_2O$

8.00 g NaCl

4.76 g HEPES

2.00 g BSA

Dissolve all components in 1 l water and adjust pH to 7.4 using NaOH.

2.4.3 Insulin secretion from isolated islets by perifusion method

Pancreatic islets from mouse models of interest were isolated the day before the perifusion and incubated overnight in RPMI medium. Perifusion experiment was performed using the Biorep Perifusion System (Biorep Technologies Inc.) according to standard procedures of the manufacturer and that available in the literature. Briefly, islet chambers were made ready using P-4 gel (Biorad) along with the tubing concerned. Meanwhile, channels of the entire system were pre-primed using pre-warmed KRB without any glucose and later primed using either pre-warmed 3.3 mM (Low), 5.5 mM, or 16.7 mM (High) glucose, or 30 mM KCl in

KRB solutions using defined program settings. During the priming step, the pre-incubated islets were collected and at least 50 islets were loaded onto to the gel within the islet chamber using a pipette. Once transferred, the islets were overlaid with additional layer of P-4 gel and made sure that the chamber had enough free space above the second layer for buffer to flow through. The chambers with the islets were connected to the perifusion tubings, without creating any air bubbles, soon after the priming was complete. The flow rate was adjusted to 100 µl per minute and the entire system was maintained at 37 °C. The sequence of the buffer flow was set in such a way the islets received 5.5 mM glucose for 15 min, low glucose for 15 min, high glucose for 8 min, low glucose for 5 min, and finally KCl for 8 min. The buffer output was collected in 96-well plates and stored for measuring insulin by RIA. The islets from the chambers were recovered after a brief spin in eppendorf tubes and 1 mL of acid-ethanol was added to the islets in gel and incubated at -20 °C until further use.

2.5 Biochemical analyses

2.5.1 Quantification of insulin or glucagon using Radio Immuno Assay (RIA)

The supernatants and the lysates of islets or total pancreases were recovered and subjected to non-sensitive or sensitive (depending on the requirements) insulin or glucagon RIA (Millipore) with slight modifications of the actual protocol as provided by the manufacturer. Briefly, insulin standards with a range of 0.1 – 10 ng/mL insulin or glucagon standards with a range of 20-400 pg/mL glucagon, was prepared and 50 µl of each insulin or glucagon standard along with assay buffers where necessary was pipetted directly into the RIA tubes. Similarly, 50 µl of secretion supernatants and islet or pancreatic lysates were added to the tubes concerned and 50 µl of insulin or glucagon antibody provided by the kit was added to all the RIA tubes containing the standards and samples except for 2 tubes pertaining to the standard curve that were dedicated for radio labeled insulin or glucagon alone. Later, 50 µl of radio labeled insulin or glucagon (^{125}I isotope labeled insulin or glucagon) was added to all the tubes and tubes in the racks were gently vortexed, sealed, and incubated overnight at 4 °C. The next day, 500 µl of precipitation buffer was added to all tubes except the first two and further incubated at 4 °C for 20 min. Later, the tubes or the entire rack was spun at 3000 g for 20 min at 4 °C. The supernatants were discarded and tubes containing the pellets were set up in the gamma counter (PerkinElmer) with a pre-defined special program for insulin or glucagon. Radioactive counts were directly extrapolated from the standard curve and insulin or glucagon concentrations were calculated automatically.

2.6 Proteomics

2.6.1 SILAC labeling and transfections

For SILAC labeling, MIN6 cells were grown in high glucose DMEM (PAA Laboratories GmbH**,** Pasching, Austria) supplemented with 15% dialyzed FCS (Sigma-Aldrich, St. Louis, MO, USA), 4 mM glutamine (PAA Laboratories GmbH**,** Pasching, Austria), 100 U/mL penicillin, 50 mM β-mercaptoethanol, 100 µg/mL streptomycin (Life Technologies Inc.), 48 µg/mL Lys-$C_6H_{14}N_2O_2$ (Lys-0) and 28 µg/mL Arg-$C_6H_{14}N_4O_2$ (Arg-0) (Sigma-Aldrich, St. Louis, MO, USA) or 48 µg/mL Lys-$^{13}C_6$ $H_{14}{}^{15}N_2O_2$ (Lys-8) and 28 µg/mL Arg-$^{13}C_6H_{14}{}^{15}N_4O_2$ (Arg-10) (Sigma Isotec, Miamisburg, OH, USA). Eight passages in 'heavy DMEM' were conducted to achieve a high degree of incorporation of the heavy isotopes into proteins (95.12% ± 4.3 labeling efficiency).

For secretion experiments using SILAC cells, MIN6 cells were washed 6 times before incubating for one hour in modified Krebs Ringer Buffer containing either low (2.8 mM) or high (25 mM) glucose as previously described. Supernatants of light and heavy MIN6 cell cultures were combined and concentrated with Amicon Ultra-15 Centrifugal Filter Units with 3 kDa cut off (EMD Millipore Corporation, MA, USA). Proteins were precipitated and dissolved in 6 M urea/2 M thiourea. Proteins were reduced and alkylated. Lysyl endopeptidase (LysC) (Wako, Osaka, Japan) and trypsin (Promega, Madison, WI, USA) were used for in-solution digestion. Stop and go extraction (STAGE) tips containing C_{18} empore disks (3M, Minneapolis, MN, USA) were used to purify and store peptide extracts.

2.6.2 Liquid Chromatography Mass Spectrometry (LC – MS/MS)

LC – MS/MS analysis was performed as described previously (20). Peptide mixtures were separated by reversed phase chromatography using the EASY-nLC system (Thermo Fisher, Waltham, MA, USA) on in-house manufactured 20 cm fritless silica microcolumns with an inner diameter of 75 µm. Columns were packed with ReproSil-Pur C_{18}-AQ 3 µm resin (Dr. Maisch GmbH, Ammerbuch, Germany). Peptides were separated on a 8 – 60% ACN gradient (182 min) with 0.5% formic acid at a flow rate of 200 nl/min. Eluting peptides were directly ionized by electrospray ionization and transferred into a Q Exactive mass spectrometer (Thermo Fisher, Waltham, MA, USA). Mass spectrometry was performed in the data dependent positive mode with one full scan (m/z = 300 – 1,700; R = 70,000; target value = 3 x 10^6; maximum injection time = 120 ms). The 10 most intense ions with a charge state

greater than one were selected (R = 35,000, target value = 5 x 10^5; isolation window = 4 m/z; maximum injection time = 120 ms). Dynamic exclusion for selected precursor ions was 30 s.

2.6.3 Processing of MS data

MaxQuant software package (version 1.2.2.5) with default settings was used to identify and quantify proteins. In brief, SILAC duplets were quantified using the following settings: heavy label Lys-8, Arg-10; maximum of three labeled amino acids per peptide; top 10 MS/MS peaks per 100 Da. Carbamidomethylation of cysteine was selected as fixed modification, oxidation of methionine and acetylation of the protein N-terminus were used as variable modifications. Trypsin was selected as protease (full specificity) with a maximum of 2 missed cleavages. MS/MS spectra were searched using the Andromeda search engine against an IPI mouse database (release 3.84) including 248 common contaminants. All protein sequences were also reversed to generate a target-decoy database. A minimum of six amino acids per identified peptide and at least one peptide per protein group was required. False discovery rate was set to 1% at both the peptide and protein level. Protein ratios were calculated from the median of all normalized peptide ratios using only unique peptides or peptides assigned to the protein group with the highest number of peptides (razor peptides). Only protein groups with at least three SILAC counts were considered for further analysis.

2.7 Statistical analyses

All qRT-PCR results are expressed as mean ± Standard Error of Mean (SEM). Comparisons between data sets with two groups were evaluated using an unpaired Student's t-test. ANOVA analysis was performed for comparisons of three or more groups. A p-value of less than or equal to 0.05 was considered statistically significant.

Chapter 3

Publication 1

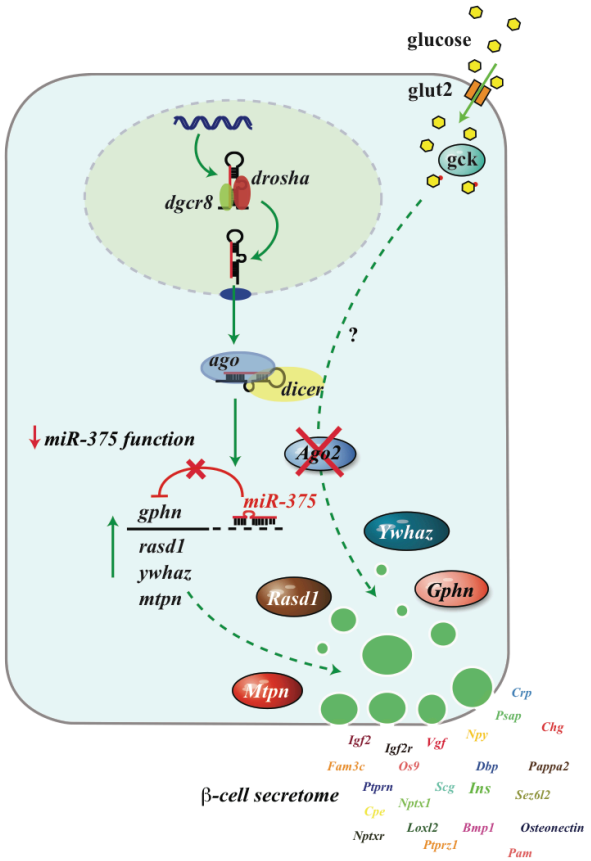
Argonaute2 regulates the pancreatic β-cell secretome

Tattikota SG*, Sury MD*, Rathjen T*, Wessels HH, Pandey AK, You X, Becker C, Chen W, Selbach M, Poy MN.

***Mol. Cell. Proteomics*. 2013; 12(5):1214-25.**

***Highlights*:**

- ***Ago2* and *miR-375* are functionally associated in the β-cell**
- ***Ago2* and *miR-375* regulate the β-cell secretome**
- **Loss of *Ago2* de-represses *miR-375*-targeted genes such as *Gephyrin***
- **Loss of *Gephyrin* blocks the β-cell secretome**



The DOI link to this article is: http://dx.doi.org/10.1074/mcp.M112.024786

Chapter 4

Publication 2

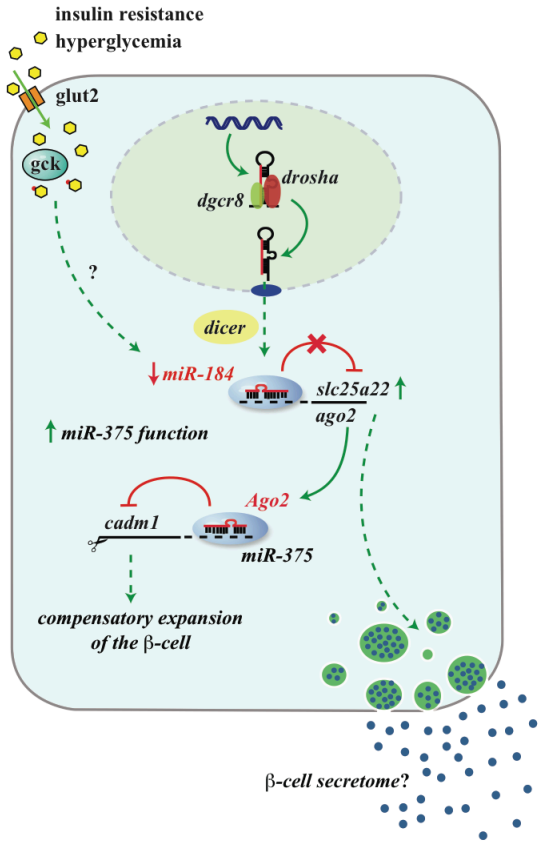
Argonaute2 mediates compensatory expansion of the pancreatic β-cell

Tattikota SG*, Rathjen T*, McAnulty SJ, Wessels HH, Akerman I, van de Bunt M, Hausser J, Esguerra JL, Musahl A, Pandey AK, You X, Chen W, Herrera PL, Johnson PR, O'Carroll D, Eliasson L, Zavolan M, Gloyn AL, Ferrer J, Shalom-Feuerstein R, Aberdam D, Poy MN.

***Cell Metab*. 2014; 19(1):122-34.**

***Highlights*:**

- **Silencing of *miR-184* during insulin resistance promotes its target *Ago2***
- **Loss of *Ago2* during insulin resistance blocks pancreatic β-cell proliferation**
- ***Ago2* mediates the suppression of *Cadm1* by *miR-375* in the β-cell**
- **Administration of the ketogenic diet to *ob/ob* mice rescues *miR-184* in islets**



The DOI link to this article is: http://dx.doi.org/10.1016/j.cmet.2013.11.015

Chapter 5

Publication 3

Micro-managing the pancreatic β-cell

Rathjen T, Tattikota SG, Poy MN.

***Cell Cycle*. 2014; 13(8):1216-7.**

The DOI link to this article is: http://dx.doi.org/10.4161/cc.28513

Chapter 6

Publication 4

Re-dicing the pancreatic β-cell: do microRNAs define cellular identity?

Tattikota SG & Poy MN.

***EMBO J*. 2011; 30(5):797-9.**

The DOI link to this article is: http://dx.doi.org/10.1038/emboj.2011.31

Chapter 7
Discussion

7.1 The miRNA pathway as a key player in the pathogenesis of T2D

We are past thousands of years since the first mentioning of diabetes and its classification into T1D and T2D with a hypothesis driven understanding of the involvement of genetic background with an influence of environmental factors (Kahn, 1994). Considering the present scenario of global outbreak of diabetes epidemic, it seems inevitable for the world to witness an enormous rise in the number of deaths caused as a consequence of T2D that include cardiovascular diseases. Although the genetic architecture has helped reveal the complex nature of diabetes, we are seemingly miles away from a definitive cure (Polonsky, 2012). The development and progression of T2D is known to go through systematic and programmed phases. These start with a stable compensatory phase where islets expand (hyperplasia) and the β-cells secrete more insulin (hypersecretion) so as to meet the demands for higher insulin during severe insulin resistance (Weir & Bonner-Weir, 2004). Several pathways are now known to trigger β-cell proliferation and the release of insulin from this cell type. It has been shown that perturbations along these signaling circuits by genetic or environmental factors could instigate the progression of T2D (Bell & Polonsky, 2001). The miRNA pathway has been well recognized as an important player in both glucose and energy homeostasis, and such the development of overt diabetes (Rottiers & Näär, 2012; Dumortier et al., 2013; Rathjen *et al*., 2014).

The identification of *miR-375* in pancreatic islets laid the first foundation implicating the involvement of a small RNA that potentially controls the release of insulin (Poy et al., 2004). Loss of this miRNA in mice showed that it is essential for β-cell proliferation during stress states such as insulin resistance (Poy et al., 2009). In light of this finding, we envisioned a greater involvement of the miRNA pathway in controlling key events of insulin release and β-cell proliferation. This thesis thus focused on the as yet unknown roles of *Ago2*, a critical component of the miRISC complex, in insulin release and β-cell proliferation (Tattikota et al., 2013; Tattikota et al., 2014). The studies presented in this thesis suggest a larger role for the miRNA pathway in the β-cell than previously thought. Making use of several genetic

mouse models and cell culture based experiments, we demonstrated how feedback mechanisms within the miRNA pathway are essential for the optimal functioning of the β-cell. We have shown that *Ago2* regulates the release of β-cell secretome, besides just controlling the release of insulin (Chapter 3, Tattikota et al., 2013) (Fig. 6A). Also, we have uncovered how insulin resistance mediates the silencing of *miR-184*, which in turn promotes the expression of its conserved target *Ago2*. While increased expression of *Ago2* potentiated β-cell proliferation, a genetic loss specifically in the β-cell blocked growth and proliferation of this cell type during insulin resistance. Moreover, *Ago2* mediated the function of *miR-375* by regulating its target *Cadm1*, a gene previously implicated in growth suppression (Chapter 4, Tattikota et al., 2014) (Fig. 6B). These results highlight that the miRNA pathway maintains the delicate balance between proliferation and the release of β-cell secretome, besides insulin.

7.2 The β-cell secretome

7.2.1 miRNAs act to counter the release of β-cell secretome

Several studies, including the present works herein, indicate that miRNAs act to counter GSIS by targeting key components along the insulin exocytosis pathway (Poy et al., 2004; Plaisance et al., 2006; Wijesekara et al., 2012; Kim et al., 2013; Morita et al., 2013; Zhu et al., 2013; Locke et al., 2014; Tattikota et al., 2014; Latreille et al., 2014). These observations suggest that miRNAs function as 'brakes' on insulin release. Furthermore, decreased expression of *Ago2* from a β-cell line, MIN6, *in vitro* caused hypersecretion of insulin, similar to loss of function of *miR-375*. This observation suggested a close relationship between these two genes. Additionally, in line with other tissue types, β-cells express *Ago2* more abundantly than the other isoforms *Ago1*, *Ago3* or *Ago4*. However, although less in abundance, these isoforms have well been shown to load miRNAs with similar efficiency of *Ago2* (Wang et al., 2012), suggesting a degree of functional overlap among different *Ago* isoforms. It is, therefore, fair enough to suspect the contribution of these isoforms in maintaining β-cell function similar to *Ago2*. But based on the decreased expression levels of *Ago1* (Fig. 2A within Chapter 4) and relatively lower levels of *Ago3* and *Ago4* (unpublished data), it is unlikely that the other isoforms have a significant role in β-cell function. Nevertheless, future studies to elucidate, in detail, the molecular functions of these isoforms in the β-cells *in vivo* may ascribe novel roles for these proteins in the context of diabetes.

The pancreatic islets have long been heralded as "mini" organs possessing different cell types

that secrete hormones in response to changes in extracellular glucose concentrations. With the advent of mass spectrometry based technologies, the dissection of islet proteomes revealed several important proteins required for insulin release during a glucose stimulus (Waanders et al., 2009). Thus far much emphasis has been focused on the composition of the insulin containing secretory granules (ISGs) that harbor many proteins other than just insulin (Brunner et al., 2007; Suckale & Solimena, 2010; Schvartz et al., 2012). Prior to this thesis, evidence was scant on which of these proteins are actually released by the β-cells in response to extracellular glucose concentrations. To date, although miRNAs have been implicated in the release of insulin from this cell type, it had not been established how their mediators such as *Ago2* impact the global release of proteins from the ISGs. In light of this, an unbiased, SILAC based quantitative proteomic approach revealed that several proteins, termed "β-cell secretome", are secreted in response to high glucose. Additionally, inhibition of either *miR-375* or *Ago2* potentiated the release of β-cell secretome, suggesting that the miRNA pathway not only acts to counter insulin release, but also can impact the release of the β-cell secretome in general (Fig. 6A).

In consideration of the important role of *Dicer* in the maintenance of β-cell mass (Lynn et al., 2007; Melkman-Zehavi et al., 2011), there remains a possibility of its role in regulating the release of β-cell secretome, in a mechanism similar to that identified for *Ago2*. It is therefore reasonable to question if a complete loss or down regulation of most miRNAs potentiate insulin release. If we look at the case of β*Dicer*KO mice, which display a strong reduction of total miRNAs in their β-cells, GSIS is shown to be blunted as a result of a severe loss of β-cell mass (Melkman-Zehavi et al., 2011). This observation suggests that loss of most miRNAs does not lead to hypersecretion of insulin unlike loss of miRNA function as a result of *Ago2* inhibition in the β-cells. In addition, our unpublished work on *Dicer* in the β-cells revealed that its knockdown did not perturb the release of β-cell secretome. This observation highlighted the unique role of *Ago2* in impacting the β-cell secretome. *Ago2* in turn can associate with several other miRNAs other than just the islet abundant *miR-375*. Our observation with the other islet enriched miRNA, *miR-7*, revealed a possible involvement of multiple miRNAs in regulating the release of β-cell secretome. These data indicate that miRNAs and their targets converge as a network to maintain the key β-cell functions such as hormone release.

7.2.2 Proteins within the β-cell secretome may contribute to β-cell proliferation

Proteins identified within the β-cell secretome included known hormones and secretory proteins most importantly the insulins, secretogranins, chromogranins, carboxypeptidase E among several others that are established components of the ISG (Suckale & Solimena, 2010; Schvartz et al., 2012). Although insulin and its myriad of extracellular functions including β-cell proliferation are fairly well understood, the precise role of several other factors of the secretome remains to be investigated. However, one of the released factors, *Nerve Growth Factor inducible* (*Vgf*), was recently shown to potentiate insulin release and slow down the onset of diabetes by enhancing β-cell mass *via* suppression of β-cell apoptosis (Stephens et al., 2012). It has also been demonstrated that *Npy*, another β-cell secretome factor and a known satiety hormone, can promote β-cell proliferation (Cho & Kim, 2004). Such studies support the findings presented in this thesis and thus suggest a potential role for the β-cell secretome in maintaining β-cell proliferation.

It should be noted that to assign a protein to be a part of the β-cell secretome, a high level of stringency was applied and hence, there is an ample possibility of the dataset being underrepresented. Such was the case for *Cadm1* and *Manf*; these two proteins were detected to be hypersecreted, but could not be part of the β-cell secretome "signature" repertoire. These proteins could in fact have important functions in β-cell proliferation during disease states of T2D. A recent study demonstrated that whilst a genetic loss of *Manf* resulted in diabetes, recombinant *Manf* enhanced β-cell proliferation and survival in mice (Lindahl et al., 2014). On the other hand, decreased expression of *Cadm1* in human pancreatic islets and its increased shedding correlated with T2D (Inoue et al., 2014). Our studies additionally demonstrated a role for *Cadm1* in β-cell proliferation (Fig. 5H, Chapter 4) and insulin release (Fig. S2B, Chapter 3). It is intriguing to speculate how glucose stimulates its release, as it is a known membrane spanning protein. As yet, the only fragment cleaved as a result of shedding is the intracellular domain (ICD) of *Cadm1*. It thus remains to be seen if shedding causes the release of an extracellular fragment upon a glucose stimulus that may participate in β-cell proliferation. A recent has suggested that β-cell enriched proteases such as BACE 1 and 2 selectively cleave the ectodomain fragments (known as "shedding") of certain proteins (Stützer et al., 2013). Their putative substrates include some of the proteins such as *Cadm1*, *Sez6l*, and *Sez6l2* that were also identified in the β-cell secretome. Future investigations could thus develop methods for a comprehensive understanding of the β-cell "secretome" versus

the “sheddome” in response to glucose in both islets and MIN6 cells. Finally, in lieu of glucose as a β-cell mitogen (Porat et al., 2011), it can thus be concluded that glucose stimulated secretion of proteins such as *Vgf*, *Npy*, *Manf* and *Cadm1*, could potentially mediate β-cell proliferation.

In light of the composition of the pancreatic islet comprising various cell types, it is possible that the “islet secretome” *in vivo* may differ in its composition or regulation. This is due to the opposing functions of different hormones such as *glucagon* and *somatostatin* released by the non β-cell types of the islet. So as to address this issue, future investigations could employ novel labeling techniques and/or use of the SILAC mouse, which was developed for the purpose of *in vivo* quantitation of the proteome (Krüger et al., 2008). Furthermore, in our approach, almost the entire proteome is SILAC labeled in the MIN6 cells and hence secreted proteins that are synthesized *de novo* cannot be distinguished from those that are already synthesized. Therefore, in the future, alternative methods such as pulsed labeling of proteins (pSILAC) (Schwanhäusser et al., 2009; Eichelbaum et al., 2012) should be used in the context of β-cells. These approaches will help in identifying low abundant secreted proteins and also newly synthesized proteins in response to high glucose. Also, in light of the involvement of miRNAs in β-cell proliferation, further studies are warranted as to which of the secretome factors contribute directly or indirectly in changing the miRNA profile. For instance, it is known that incretins can control the expression of certain miRNAs in the islets during pregnancy (Jacovetti et al., 2012). This indicates that β-cell derived factors may participate in modulating miRNA function in the islets during insulin resistance and T2D. All put together, it can be postulated that upon loss of *Ago2*, the β-cell promotes the release of secretome so as to maintain its own survival as a feedback mechanism.

7.2.3 β-cell secretome comprises several proteins involved in bone physiology

Besides known ISG components, it is interesting that numerous other proteins such as *Vitamin D binding protein* (*Dbp*) (Uitterlinden, et al., 1996), *Osteonectin* (Termine et al., 1981), *Bmp1* (Kessler et al., 1996; Safadi et al., 1999), *Fam3c* (Cho et al., 2009), *Igf2* (Chen et al., 2010), and *Pappalysin-2* (*Pappa2*) (Christians et al., 2013) were detected to be hypersecreted upon loss of *Ago2* and in response to high glucose. These secreted proteins were previously implicated in bone physiology including bone repair, regeneration and density. Additionally, reduced serum *Dbp* levels were previously associated with T1D,

indicating that β-cells can secrete proteins related to bone physiology (Blanton et al., 2011; Thrailkill & Fowlkes, 2013). The release of these proteins provokes interesting questions as to why the β-cell secretes proteins that are important for bone physiology. Do β-cells maintain bone homeostasis alongside glucose homeostasis? Or, is signaling between the β-cell and bone also required to maintain glucose homeostasis? And, does loss of β-cell function during diabetes contribute to loss of bone mass?

In fact, *Osteonectin* (also known as *SPARC*), a protein important for bone formation (Termine et al., 1981; Delany et al., 2000), has been previously implicated in diabetes, obesity, insulin resistance, including insulin secretion (Kos & Wilding, 2010; Harries et al., 2013). Moreover, insulin signaling in osteoblast cells has been shown to be crucial in maintaining glucose homeostasis, suggesting that a dynamic interplay between β-cells and the bone is necessary for energy and glucose metabolism (Ferron et al., 2010; Fulzele et al., 2010; Karsenty & Ferron, 2012). Increasing evidence also suggests that loss of function of β-cells during the onset of diabetes instigates a weak bone mineral density thereby increasing the risk of osteoporosis (Hamann et al., 2012). Although these studies partly addressed the questions raised above, evidence is limited as to which protein(s) released by the β-cells specifically mediate β-cell-bone interactions. In addition, there is still a major void in our understanding as to why diabetes is a major risk factor for the predisposition of bone disorders such as osteoporosis. It is, however, provocative to suggest that miRNA mediated control of the β-cell secretome plays a major role in the maintenance of bone homeostasis.

Also, the identification of the β-cell secretome is important given the recent discoveries of how various factors secreted by several tissue types cross talk to the β-cell. Of note, proteins such as bone derived *Osteocalcin* (Fulzele et al., 2010), β-cell derived *Vgf* (Stephens et al., 2012), liver derived *Betatrophin* (Yi et al., 2013), muscle derived myokine *Irisin* (Bostrom et al., 2012; Zhang et al., 2014; Sanchis-Gomar & Perez-Quilis, 2014), and fat derived *Adipsin* (Lo et al., 2014), have been shown to improve β-cell function (see also Fig. 1). Thus, the β-cell secretome is a great resource of secretory proteins that potentially mediate, in autocrine or paracrine ways, interactions with several tissues, including bone, so as to mediate glucose homeostasis. In lieu of this, future approaches to understand which of the factors crosstalk to tissues such as bone will be beneficial. A systematic generation of β-cell specific transgenic mice for most of the factors related to the bone will delineate the mechanisms behind bone disorders that are commonly associated with T2D.

7.2.4 β-cell secretome as a potential signature for β-cell identity

New evidence proposes β-cell dedifferentiation as a potential mechanism to account for the decline in β-cell function and in turn loss of insulin production during T2D (Talchai et al., 2012). What proteins of the β-cell make or break its identity remains to be completely identified and understood. Besides certain β-cell specific transcription factors, the expression of insulin and its secretion is considered as one of the major factors that maintain β-cell identity (Pagliuca & Melton, 2013). As β-cell specific loss of *Dicer* induced partial dedifferentiation (Melkman-Zehavi et al., 2011), it is interesting to consider the contribution of the miRNA pathway in this process. The β-cells in this case maintained most of the molecular characteristics of a β-cell except for insulin, suggesting a set of miRNAs can contribute to the maintenance of β-cell identity (Chapter 6, Tattikota & Poy, 2011; Pagliuca & Melton, 2013; Kaspi et al., 2014). It is, therefore, not far fetched to believe that the β-cell secretome signature contributes to the maintenance of the β-cell identity.

Considering the loss of β-cell mass at the clinical onset of T2D, several approaches are being developed so as to convert various terminally differentiated cell types into β-cells. This includes the use of naïve human embryonic and induced pluripotent stem cells to replace lost β-cells (Pagliuca & Melton, 2013). As yet, insulin is the only prime readout to consider a cell to have successfully differentiated into a β-cell. Future investigations should consider the β-cell secretome signature as an important readout, besides just insulin, for a fully differentiated functional β-cell. The other important aspect to be carefully addressed is how specific are the secretome proteins to β-cells. There have been a considerable number of discoveries made in the field of "secretomics", be it in the context of stem or cancer cells (Bronisz et al., 2011; Korpal et al., 2011; Ranganath et al., 2012). Moreover, metabolically relevant tissues such as the fat and muscle were identified as secretory organs (Hocking et al., 2010; Pedersen & Febbraio, 2012). It thus remains to be seen how specific the cocktail of secreted proteins within the secretome are to the β-cells when compared to other tissue types in the context of obesity and T2D, thus defining the β-cell secretome as a unique signature to β-cells.

7.2.5 Mechanistic role of *Ago2* in regulating the β-cell secretome

It is shown that *Ago2* can mediate the function of hundreds of miRNAs and that each miRNA, although relatively mildly, can potentially tune the expression of several hundreds

of target genes (Baek et al., 2008; Selbach et al., 2008). It is therefore quite possible for *Ago2* to mediate the function of miRNAs such as *miR-375*, which ultimately affects the expression of several target genes relevant to insulin secretion. On similar lines, a recent report involving loss or gain of *miR-7* function in β-cells has demonstrated that multiple genes related to insulin granule fusion and SNARE complex activity are directly targeted by *miR-7* thereby negatively regulating GSIS (Latreille et al., 2014). Likewise, many of the *miR-375*-targeted genes have been shown to interact with components involved in vesicle fusion or membrane organization, events that are essential for insulin secretion (Seino et al., 2011). For instance, the tumor suppressor *Cadm1* has been shown to control actin cytoskeleton assembly (Houshmandi et al., 2006; Masuda et al., 2010; Moiseeva et al., 2014). Additionally, *Gphn* has been known to self-assemble to form scaffolds and interact with the neuronal cytoskeleton (Tyagarajan & Fritschy, 2014). Such dynamics of cytoskeleton are known to be important for insulin secretory granule docking and release (Seino et al., 2011), suggesting similar functions for *Gphn* in the β-cell. Also, while *HuD* (*Elavl4*) has been implicated in the posttranslational control of insulin biosynthesis (Lee et al., 2012), *Rasd1* and *Ywhaz* (14-3-3ζ) have important functions in governing insulin release and β-cell survival during multiple stress states (Lellis-Santos et al., 2012; Lim et al., 2013).

The studies discussed above indicate that the genes targeted by *miR-375* (and mediated by *Ago2*) possess key roles in regulating the β-cell secretome (Fig. 6A). In fact, a siRNA-mediated inhibition of one of the targets, *Gphn*, blocked the release of the β-cell secretome suggesting the functional relevance of this gene in the β-cell. However, it is worth noting that not all of the targets of *miR-375* regulate the release of insulin or β-cell secretome in a similarly positive manner. This is true in the case of *Cadm1* whose depletion from β-cells potentiated the release of both insulin as well as other proteins within the β-cell secretome (Chapter 3; unpublished data). This suggests that some target genes have opposing effects on the release of β-cell secretome. Interestingly, the net effect from the loss of *Ago2* in the β-cell was the potentiation of β-cell secretome, suggesting that *Ago2* orchestrates the expression of several *miR-375*-targeted genes that have potential opposing effects on the β-cell secretome. Future studies to discriminate which target genes potentiate and which perturb the release of secretome are thus warranted. Recent reports have indicated that several components of the RISC, including *Ago2*, interact with *Synaptotagmin-11* (*Syt11*) (Milochau et al., 2014), a member of the synaptotagmin gene family involved in exocytosis and membrane recycling

(Südhof & Jahn, 1991, Gerber & Südhof, 2002). Moreover, a recent study also reported an association of this family of proteins with reduced GSIS in patients with T2D (Andersson et al., 2012). These studies suggest that β-cells rely heavily on the miRNA machinery for insulin exocytosis.

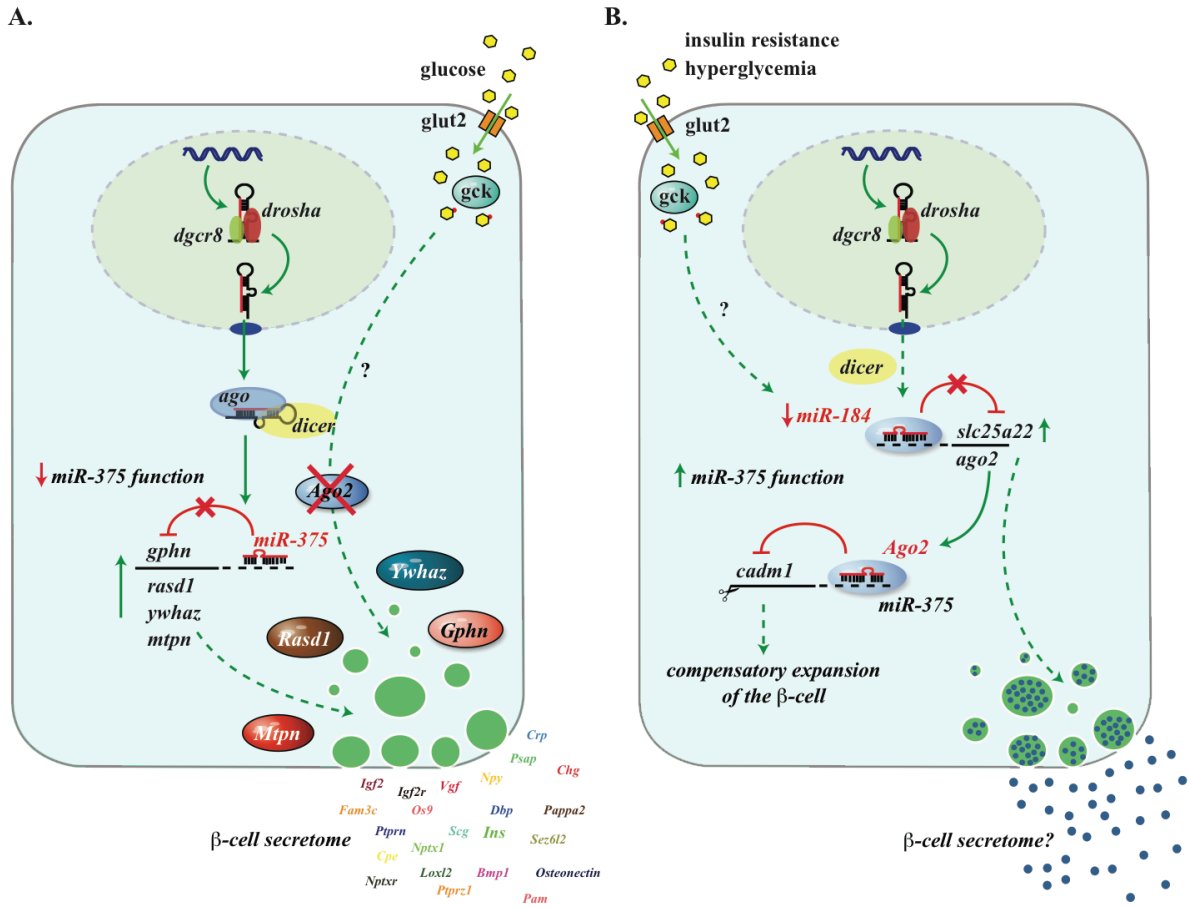


Fig. 6: ***Micromanaging the pancreatic β-cell.*** **A)** *Argonaute2 regulates the β-cell secretome.* Loss of *Ago2* from β-MIN6 cell line potentiates the release of several proteins, termed β-cell secretome, in response to high glucose. *Ago2* mediates the function of islet abundant *miR-375*, which is known to block insulin secretion by inhibiting *Myotrophin* (*Mtpn*). A decrease in *Ago2* expression resulted in de-repression of *miR-375*-targeted genes including *Gephyrin* (*Gphn*), *Rasd1*, *Ywhaz* (*14-3-3ζ*), and *Mtpn* that in turn promote the release of β-cell secretome. **B)** *Ago2 mediates the compensatory expansion of the β-cell.* During states of insulin resistance, the declined expression of *miR-184* promoted the expression of its targets such as *Ago2* and the glutamate transporter, *Slc25a22*. While *Slc25a22* is implicated positively in the regulation of insulin secretion, increased *Ago2* expression enhanced β-cell proliferation. Elevated levels of *Ago2* in turn potentiated the miRNA pathway by increasing the function of *miR-375*, thereby repressing the growth suppressor cell adhesion molecule 1 (*Cadm1*). β-cell specific ablation of *Ago2* during insulin resistance blocks β-cell proliferation. Moreover, administration of ketogenic diet reversed most of the molecular and phenotypic effects of insulin resistance.

7.3 Micro-managing the pancreatic β-cell

Although previous studies established a regulatory role of *miR-375* during insulin resistance, not much was known on the biologic significance of involvement of other miRNAs in the progression of T2D. A thorough investigation in the setting of insulin resistance led to the identification of *miR-184* in this thesis as a unique, and the most downregulated, miRNA in the islets of leptin deficient *ob/ob* mice and T2D human subjects. Moreover, the silencing of *miR-184* was shown to be essential to promote the expression of its target *Ago2*, which in turn enhanced the repression of *miR-375*-targeted genes such as *Cadm1* (Fig. 6B). Previous studies demonstrated that very low carbohydrate, high fat diets such as ketogenic diets (KD) helps restore insulin sensitivity in both mice and humans (Badman et al., 2009; Paoli et al., 2013). The use of KD in leptin deficient *ob/ob* mice restored the islet expression of *miR-184*, *Ago2*, and in turn β-cell mass. This indicates that feedback mechanisms exist within the miRNA pathway to modulate β-cell function according to individual insulin sensitivities (Chapter 4, Tattikota et al., 2014; Chapter 5, Rathjen et al., 2014).

7.3.1 Cross-species conservation of the regulation of *miR-184*

Changes in the expression of miRNAs emphasize the adaptive behavior of cells in response to stress to fine tune their gene expression patterns so as to meet the systemic demands (Leung & Sharp, 2007; Leung & Sharp, 2010; Spriggs et al., 2010). For *miR-184* to uniquely respond to obesity and/or hyperglycemia in the β-cell suggests functional adaptation by this cell type to regulate insulin synthesis and its release during insulin resistance. Decreased expression of *miR-184* was also observed in the islets of pregnant mice at gestational day 18.5 (G18.5), and the expression returned back to normal by post-partum day 5 (P5; unpublished data). These changes are in line with increased β-cell mass in pregnant mice as a result of transient insulin resistance (Rieck & Kaestner, 2010), suggesting a role for *miR-184* in β-cell proliferation. This is supported by other previous observations that several miRNAs are dysregulated in the β-cells during pregnancy (Jacovetti et al., 2012). The neurotransmitter serotonin (5-hydroxytrytamine; 5-HT) is also known to regulate β-cell proliferation during pregnancy (Kim et al., 2010). This observation suggests the possible involvement of similar pathway in controlling the expression of *miR-184* during pregnancy.

The role of serotonin has been previously implicated in reducing the levels of *miR-184* in the central nervous system (CNS) of the sea slug *Aplysia californica*, a known model for learning

and memory (Rajusthupathy et al., 2009). What is most striking is the fact that the expression and regulation of *miR-184* in the CNS region of *Aplysia* is the orthologous site where *Aplysia* insulin producing cells have previously been detected (Floyd et al., 1999). Other lower-order organisms such as *Drosophila* have been previously used as excellent model systems to study insulin-like signaling pathways (Porte Jr., et al., 2005). Additionally, miRNAs such as *dme-miR-278* and *dme-miR-14* have been implicated in insulin resistance and insulin production, respectively, in flies (Teleman et al., 2006; Varghese et al., 2010). Our preliminary data also showed that *dme-miR-184* is dysregulated in flies on a high fat diet (unpublished data), supporting the high degree of conservation in insulin and miRNA pathways. These observations shed light on a possible highly conserved cross-species mechanism of regulation of *miR-184* pertaining to the β-cell function of higher mammals.

7.3.1.1 Transcriptional regulation of *miR-184*

The identification of *Lin28*, an RNA binding protein known to selectively inhibit maturation of *let-7* (Viswanathan et al., 2008; Viswanathan & Daley, 2010; Mayr & Heinemann 2013), opened up novel mechanisms of posttranscriptional regulation of miRNAs (Thomson et al., 2006). It has been suggested that several transcription factors, including the well-known tumor suppressor *p53*, interact with microprocessor components to modulate miRNA processing (Newman & Hammond, 2010). In general, it is known that various transcription factors regulate the expression of several protein and non-protein coding (ncRNA) genes. The decreased expression of *pri-miR-184* in the islets of *ob/ob* mice suggests that the loss of its mature sequence is probably a result of silencing at the transcriptional level. This is supported by a previous study where it was shown that *Methyl-CpG binding protein 1* (*Mbd1*) acts a transcriptional repressor directly affecting the expression of *miR-184* in adult neural stem cells (Liu et al., 2010). Considering a possibility of such regulation in the β-cells, we knocked-down the expression of *Mbd1* in MIN6 β-cell line and observed no changes in the level of *miR-184* (unpublished data), suggesting *Mbd1* does not mediate the transcriptional repression of this miRNA, at least in β-cells.

Key embryonic stem (ES) cell transcription factors such as *Oct4*, *Nanog*, *Sox2*, and *Tcf3* are shown to occupy promoter sites of several miRNA genes in ES cells suggesting a core transcriptional regulation of miRNAs (Marson et al., 2008). Polycomb group (PcG) proteins such as *Suz12*, a known transcriptional repressor, were also shown to co-occupy certain silent

miRNA genes that included *miR-184*. In light of this observation, *Suz12*-chromatin immunoprecipitation (ChIP) from the islets of *ob/ob* mice revealed a substantial enrichment for the promoter region of *miR-184* (unpublished data). These observations indicate the existence of a potentially active transcriptional repression by *Suz12* of *miR-184* expression during insulin resistance. A comprehensive understanding of such epigenetic regulation of genes including those of miRNAs would shed light on the pathogenesis of several human diseases including T2D (Johnstone & Baylin, 2010; Kameswaran et al., 2014). Moreover, specific phenomena termed as miRNA "tailing" and "trimming" and certain ncRNAs are known to mediate miRNA degradation (Ameres et al., 2010; Cazalla et al., 2010). In lieu of these observations, questions remain open if such phenomena do occur in regulating miRNA half-lives in the β-cell during insulin resistance. Thus, future research focusing on miRNA degradation together with their regulation at transcriptional levels during T2D will shed light on novel mechanisms of miRNA mediated gene silencing.

7.3.2 The regulatory role of *Ago2* in the β-cell

Recent studies showed a transcriptome wide increase in the occupancy of *Ago2* majorly at 3' UTRs and coding sequences. This in turn correlated with a stronger translational inhibition during cellular stress (Karginov & Hannon, 2013), suggesting an increased miRNA activity. Identification of genes as potential targets of miRNAs relies on computational bioinformatics approaches. Several target gene prediction tools have been developed to accurately predict miRNA mediated targeting based on thermodynamic modeling of miRNA-mRNA binding (Lewis et al., 2003; John et al., 2004; Rajewsky & Socci, 2004; Krek et al., 2005). Use of such computational tools predicted a transcriptome-wide increase in expression of putative targets of islet abundant miRNAs including *miR-375* due to *miR-184* gain of function in MIN6 cells (unpublished data). This observation suggested that targeting of *Ago2* by *miR-184* perturbs the function of several miRNAs on a global scale. Future studies, therefore, must identify the gene clusters that are governed by *Ago2*, and which have potential functions in the islets. Furthermore, techniques such as high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP; Chi et al., 2009) and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP; Hafner et al., 2010) have been developed to identify transcriptome-wide miRNA-mRNA binding sites. Integration of such cutting-edge technologies will provide greater insights into miRNA targeting mediated by *Ago2* in the islets during insulin resistance and T2D. In addition to its

function in the miRNA pathway, several discoveries uncovered diverse roles for *Ago2* such as interactions with the chromatin in the nucleus (Cernilogar et al., 2011; Moshkovich et al., 2011; Ameyar-Zazoua et al., 2012; Wei et al., 2012; Taliaferro et al., 2013). It is thus plausible that loss of *Ago2* as a result of its targeting by *miR-184* might regulate the expression of β-cell proliferation genes that potentially interact with *Ago2* at the chromatin. Further investigation is thus warranted to demonstrate *Ago2*-chromatin interactions using ChIP from the islets during insulin resistance.

Increased *Ago2* levels were also reported to be associated with posttranslational modification *via* prolyl-hydroxylation during stress states such as hypoxia, potentially to enhance its stability and miRNA activity (Qi et al., 2008; Wu et al., 2011). Intriguingly, cellular hypoxia has been observed with β-cells exposed to high glucose concentrations, as well as in islets of *ob/ob* and *db/db* mice (Sato et al., 2011; Zheng et al., 2012). A potential mechanism may be that increased *Ago2* levels due to silenced *miR-184*, can also be stabilized *via* posttranslational modifications during cellular stress, which then maintains miRNA activity. Additionally, cellular hypoxia has recently been shown to be associated with decreased levels of *Cadm1* expression (Momose et al., 2013). This further reinforces the possibility that the β-cells can enhance their miRNA function during insulin resistance, hyperglycemia, and hypoxia. A recent study also reported that phosphorylation of *Ago2* by epidermal growth factor receptor (EGFR) during hypoxia in a cancer core environment inhibits miRNA maturation (Shen et al., 2013).

EGFR signaling was shown to be essential for β-cell proliferation during pregnancy (Miettinen et al., 2006; Hakonen et al., 2014). It thus remains to be investigated if *Ago2* undergoes such a modification *via* EGFR to promote a negative feedback loop to regulate miRNA pathway in the islets during insulin resistance. Taken together, it is becoming increasingly evident that the β-cells undertake several key measures to maintain the levels of *Ago2* by suppressing its potential regulators such as *miR-184*. In addition to regulation by miRNAs, as yet unexplored posttranslational modifications of *Ago2* in β-cells may be essential for optimal β-cell function during insulin resistance. Also, a recent proteomics-based study identified increased levels of *Dicer* upon high glucose in isolated human pancreatic islets, implicating the miRNA pathway in glucose metabolism (Schrimpe-Rutledge et al., 2012). Likewise, ongoing studies from our lab have identified that increased extracellular glucose concentrations decrease the levels of *miR-184*, which promoted the

increased expression of *Ago2* (unpublished data). In light of the observation that glucose can also act as a β-cell mitogen, future studies should address if glucose regulates the miRNA function by modulating the levels of *Ago2*, thereby mediating β-cell proliferation.

7.3.3 The miRNA pathway modulates β-cell function according to insulin sensitivity

Although *miR-184* was shown to be strongly downregulated in the islets of T2D donors, *Ago2* mRNA levels displayed an increased trend that was not statistically significant. This level of variation in the *Ago2* transcript may be due to a confounding variable, as adequate information with respect to medication or therapy was not available at the time of passing of these human subjects. Also, each person's level of insulin sensitivity might affect the steady state levels of *Ago2*, information for which again, was not available. This is supported by the significant inverse correlation of *miR-184* and *Ago2* across the entire cohort, possibly reflecting the individual levels of insulin sensitivities.

This inverse correlation was also observed in our *in vivo* experimental models. The restoration of circulating glucose levels and insulin sensitivity in *ob/ob* mice by ketogenic diet (KD; *keto/ob*) induced the expression of *miR-184* in islets, thereby restoring the levels of *Ago2* and β-cell mass in *keto/ob* mice. This suggests the miRNA pathway possesses the ability to sense and respond to extracellular changes in glucose concentrations or insulin sensitivity or both. Again, change in the levels of *Ago2* is inversely correlated with the expression of genes such as the growth suppressor *Cadm1* and/or regulators of insulin release such as *Slc25a22*. The decline in both β-cell mass and insulin secretion as seen in *keto/ob* mice could potentially be attributed to miRNA mediated regulation of these genes. During T2D, it remains to be seen if such inverse correlations between the miRNA-targeted genes and *Ago2* hold true in the islets of human.

Consistent with the fact that increased *miR-184* levels inhibit GSIS, our unpublished data also demonstrated a negative regulation of the β-cell secretome. In the *in vivo* setting, there may be a possible inhibition of β-cell secretome upon restoration of insulin sensitivity in *keto/ob* mice due to increased levels of *miR-184*. Does an inhibition of the release of β-cell secretome contribute, at least in part, to increased insulin sensitivity as seen in *keto/ob* mice? This is evidenced by the fact that *ob/ob* mice genetically deleted for one of the β-cell secretome factors *Npy*, have an attenuated obesity syndrome commonly associated with *ob/ob* mice (Erickson et al., 1996). Additionally, mice genetically ablated for another secretome factor

Vgf, displayed improved insulin sensitivity and its loss in *ob/ob* mice rescued both hyperinsulinemia and hyperglycemia quite similar to *keto/ob* mice (Watson, et al., 2005). These data suggest that *miR-184* mediated suppression of β-cell secretome from *keto/ob* islets may contribute, at least in part, to improved insulin sensitivity as observed in *keto/ob* mice. Simply put, modulating the expression of *miR-184* according to nutritional changes affects the miRNA pathway, which then maintains the delicate balance between β-cell mass and insulin release.

In a classical perspective, nutrient sensing by the β-cell has been shown to be critical for the efficient release of insulin based on situational fasting or feeding. For instance, glucose sensing and its metabolism were shown to control the expression and release of insulin from the β-cells (Docherty & Clark, 1994; Schuit et al., 2001; Newsholme et al., 2010). These critical events involve several pathways relaying the signals from extracellular glucose levels to the ultimate exocytosis of insulin to maintain glucose homeostasis (MacDonald et al., 2005). Although efforts have shed light on glucose responsive miRNAs in the β-cell (Tang et al., 2008), much advancement into their biologic function in response to extracellular glucose still largely remains unknown. Also, it should be noted that the relationship between nutrient sensing and the miRNA pathway is very well conserved in organisms outside of the animal kingdom, including plants (Barrio et al., 2014; Chiou, 2007). These observations suggest that the miRNA pathway in the context of nutrient sensing is essential to maintain cellular homeostasis. Therefore, future studies are warranted to further dissect this pathway in metabolically relevant tissues during the progression of T2D in mice and humans.

7.4 General conclusions

The results presented and discussed herein implicate a larger role for the miRNA pathway than previously appreciated in β-cell physiology. Consistent with increased miRNA function observed during cellular stress, we uncovered similar phenomenon in the islets during insulin resistance in mice. Moreover, in lieu of the observation that several miRNAs act to counter insulin release, inhibition of *Ago2* in β-cells promoted hypersecretion of the β-cell secretome, besides insulin upon a glucose stimulus. This was similar to loss of *miR-375* underlining again the functional association between these two genes. This association became more apparent in the β-cell during stress states such as insulin resistance, indicating a prominent role taken up by the miRNA pathway under these conditions. Most importantly, there was a

strong inverse correlation between *miR-184* and its target *Ago2* in the islets of a cohort of healthy and T2D human subjects, indicating the existence of feedback mechanisms within the miRNA pathway in human disease.

It is now apparent that β-cells undertake several measures so as to alleviate cellular stress in order to maintain glucose homeostasis. The observed restoration of insulin sensitivity associated with changes in nutrients reversed the miRNA-target relationship, indicating the "buffering" ability of miRNAs according to extracellular glucose concentrations. Further efforts to dissect this pathway in the β-cell will shed more light on how feedback mechanisms within the miRNA pathway regulate the delicate balance between the release of the β-cell secretome and maintenance of β-cell mass. These two important functions of the β-cell are essential to maintain systemic glucose homeostasis, a distortion of which leads to insulin resistance and T2D in humans.

Chapter 8

Cumulative Summary

In summary, two critical aspects of the β-cell biology have been addressed in this thesis. We have shown that loss of *Ago2* from β-cells potentiated the release of β-cell secretome, besides insulin, as a result of de-repression of *miR-375*-targeted genes. The β-cell secretome led to the identification of several proteins that are important for optimal β-cell function. Interestingly, several proteins within the β-cell secretome were previously shown to be associated with bone physiology. Their release by the β-cells is intriguing and future investigations could shed novel light on the β-cell-bone interactions. On the other hand, we also established that silencing of *miR-184 in vivo* promoted the expression of its target *Ago2* in the islets of several mouse models of insulin resistance. In mouse models that overexpress *Ago2* (*dox-Ago2*) or mice genetically ablated for *miR-184* (*miR-184KO*), increased expression of *Ago2* resulted in increased β-cell proliferation. Elevated levels of *Ago2* in the islets of *ob/ob* mice enhanced miRNA function *via* increased suppression of genes targeted by *miR-375* such as *Cadm1*, *Gphn*, *Rasd1*, *Ywhaz*, and *HuD*. These target genes are implicated in cellular growth and insulin secretion pathways. Importantly, the miRNA pathway in the β-cell is able to effectively sense nutrient changes and adjusts its activity accordingly. This is demonstrated by the fact that restoration of insulin sensitivity by ketogenic diet in *ob/ob* mice promoted the expression of *miR-184* in the islets. This in turn restored the levels of *Ago2*, *Cadm1*, and ultimately the β-cell mass. Lastly, the levels of *miR-184* were found to be downregulated in the islets of T2D human donors, demonstrating the functional relevance of *miR-184* in human disease. In addition, the expression of *Ago2* inversely correlated to that of *miR-184* in islets across the entire cohort of human subjects. These observations clearly indicate that feedback mechanisms exist within the miRNA pathway so as to adjust the optimal release of β-cell secretome, besides insulin, and β-cell proliferation according to insulin sensitivity. Future studies to further dissect this pathway in the β-cell would shed novel light into mechanisms of T2D in humans.

Chapter 9
Zusammenfassung

Die hier vorgelegte Arbeit adressiert zwei kritische funktionelle Aspekte der β-Zellbiologie. Wir konnten zeigen, dass der Verlust von *Ago2* in β-Zellen zur verstärkten Freisetzung von Insulin führt, bedingt durch die Derepression von *miR-375*-Zielgenen. Außerdem konnte gezeigt werden, dass *Ago2* auch das β-Zell-Sekretom reguliert. Durch die erstmalige Identifizierung aller von β-Zellen freigesetzter/sezernierter Proteine, waren wir auch in der Lage, einige Proteine zu identifizieren, die eine wichtige Rolle für die normale Funktion von β-Zellen spielen. Besonders interessant dabei ist, dass eine Reihe von Proteinen, die von β-Zellen freigesetzt werden auch in der Physiologie von Knochenzellen eine Rolle spielen. Dieser Umstand ist überraschend und zusätzliche Untersuchungen könnten bisher unbekannte Verbindungen zwischen Knochen- und β-Zellen aufzeigen.

Zusätzlich haben wir in *in-vivo* Experimenten gezeigt, dass die Expression von *miR-184* in Langerhansschen Inseln bei verschiedenen Mausmodellen der Insulin-Resistenz reduziert ist und zur verstärkten Expression des *miR-184*-Zielgens *Ago2* führt. Eine erhöhte Expression von Ago2, sowohl in transgenen Mäusen, bei denen entweder *Ago2* überexprimiert (*dox-Ago2*) oder *miR-184* (*miR-184KO*) fehlte, führte zur verstärkten β-Zellproliferation. Die verstärkte Expression von *Ago2* in Langerhansschen Inseln von *ob/ob* Mäusen führte zur einer erhöhten Unterdrückung von *miR-375* Zielgenen, wie *Cadm1*, *Gphn*, *Rasd1*, *Ywhaz und HuD*. Diese Zielgene sind an der Regulation von Zellteilung und der Ausschüttung von Insulin beteiligt. Wir konnten außerdem zeigen, dass der miRNA Signalweg massgeblich auf Veränderungen im Nährstoffhaushalt reagiert und die Aktivität entsprechend anpasst. Dies konnte durch die Gabe einer ketogenen Diät bei *ob/ob* Mäusen beobachtet werden, die die Insulin-Resistenz verbessert und zu einer erhöhten Expression von *miR-184* führt. Infolgedessen normalisierte sich die Expression von *Ago2* und *Cadm1* und letztlich die β-Zellmasse. Darüber hinaus konnte eine inverse Korrelation von *miR-184* und *Ago2* Expression auch in humanen Langerhansschen Inseln von gesunden und Typ 2 Diabetes erkrankten Patienten beobachtet werden. Diese Beobachtungen weisen auf Rückkopplungsmechanismen im miRNA Signalweg hin, die eine Anpassung der Insulinsekretion und der Kontrolle der β-Zellteilung in Abhängigkeit von dem Grad der

Insulin-Resistenz ermöglichen. Zukünftige Studien zur Aufklärung dieses Mechanismus in β-Zellen führt möglicherweise zur neuen Erkenntnissen über die Entwicklung von Typ 2-Diabetes beim Menschen.

Appendix

Declaration to the publications

The publications presented in this cumulative thesis are a result of extensive collaborations and equal contributions. All projects and ideas were designed, conceived, and supervised by Dr. Matthew Poy.

Chapter 3: Publication 1

Argonaute2 regulates the pancreatic β-cell secretome

Dr. Matthias Sury, former postdoctoral fellow from the group of Dr. Matthias Selbach, Thomas Rathjen, PhD student from the group of Dr. Matthew Poy, are co-first authors along with myself on this project.

Project execution: I performed all the SILAC labeling of MIN6 cells and all the experiments related to the secretomes. Specifically, I carried out all the RIP *Ago1*, *Ago2* experiments and miRNA association analysis (Figs. **1**F-G), all experiments required for the secretome analysis by proteomics (Figs. **2**B, C, **3**A-D, **5**C, **S1**A-C, **S3**A-C, **S4**A-B), and the sucrose gradient experiments (Fig. **5**B).

Whilst M. Sury performed and analyzed the entire proteomics part, T. Rathjen performed all the *in vitro* insulin secretion experiments in MIN6 cells (Figs. 5A, S2A-B, S4B).

Chapter 4: Publication 2

Argonaute2 mediates compensatory expansion of the pancreatic β-cell

T. Rathjen and I are the co-first authors on this paper.

Project execution: I generated the *RIP-184* and *dox-184* transgenic mice and performed all physiological experiments on these animals. T. Rathjen generated the *dox-Ago2* mice, worked with the *βAgo2KO* and *Cadm1KO* mice, and executed all physiological experiments in these animals.

Specifically, I carried out all qRT-PCR, luciferase assays, and Western blotting analyses pertaining to Figs. **1**A-K. Additionally, Figs. **2**A, B, I, J; **3**A-C, G-K, & P; **4**A-D, F, & I-J; **5**A, B, & I; **6**D-K; **7**A-J, & M; **S1**A-F; **S3**A-Q; **S4**E; **S5**B; **S7**A-B, E, & G, were all executed

and analyzed by me. T. Rathjen executed all the remaining experiments. Dr. Hans-Peter Rahn of the FACS core facility assisted T. Rathjen and myself for the FAC Sorting.

Chapter 5: Publication 3

Micro-managing the pancreatic β-cell

Rathjen T, Tattikota SG, and Poy MN.

Manuscript writing: This was an editorial written to comment on our own (Tattikota et al., 2014) paper at the request of the editors of *Cell Cycle*. While T. Rathjen wrote most of the editorial, I partially contributed to the writing with inputs from Dr. Poy. In this editorial, we discussed how miRNAs respond to nutrients and insulin sensitivity and the possible existence of feedback mechanisms within the miRNA pathway.

Chapter 6: Publication 4

Re-dicing the pancreatic β-cell: Do microRNAs define β-cell identity

Tattikota SG and Poy MN.

Manuscript writing: This was a preview written for the article of Melkman-Zehavi et al., 2011, at the request of the editors of *EMBO J.* While Dr. Poy wrote most of the preview, I partially contributed to writing and literature input. In this preview, we discussed the possibility of miRNAs in defining the β-cell identity, in light of the results described by Melkman-Zehavi et al.

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