Dissection of microrna-30d's function roles

DISSECTION OF MICRORNA-30D'S FUNCTION ROLES IN MAMMALIAN PANCREATIC-BETA CELLS

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ABSTRACT

MicroRNAs (miRNAs) are a group of small non-coding RNAs (about 21-22 nucleotides long) that fine tune target protein output through messenger RNA degradation or inhibition of its translation. Recent studies showing that miRNAs and their function components respond to cellular stress to maintain steady state physiology of the cells. Since there are thousands of microRNAs existing in all kind of cells, their functional characterization during the normal states or stress conditions is not fully addressed yet.

In this thesis, important aspects of pancreatic Î²-cell function under normal or stress condition such as apoptosis, proliferation, insulin production and release and their regulation by the miRNA were explored. Pancreatic Î²-cell is a group of insulin producing cells and plays critical role in maintaining glucose hemostasis. By combining mouse and cell line genetic approaches, high-throughput deep sequencing, a list of cell assays and molecular techniques, we have shed light on the novel roles of miR-30d, one highly expressed miRNA when Î²-cell responding to high glucose stimulus, in regulating Î²-cell mass on the middle aged mice. We demonstrated that overexpress of miR-30d deteriorated glucose tolerance ability of the mice with or without high fat diet treatment by significantly reducing the Î²-cell mass with less insulin production. Additionally, we demonstrated that the reduced Î²-cell mass is because both apoptosis pathway and proliferation pathway have been effected by miR-30d by targeting variety of protein

factors’ expression. BCL2 interacting protein 3 (BNIP3) and cyclin E2 (CCNE2) have been respectively confirmed as miR-30d’s targets and the effect of their regulation by miR-30d in pancreatic Î²-cell proliferation or apoptosis aspects have been addressed as well. Furthermore, we could show that silencing of miR-30d in MIN6 cell (Î²-cell mimicking cell line) by CRISPR-CAS9 gene editing system promotes the insulin secretion, which is through potentiated expression of MAFA, an insulin transcription factor. These studies uncovered novel functional roles of the miR-30d pathway in mediating Î²-cell function and fate. Further dissection of these pathways shall uncover several mechanisms by which the Î²-cells undertake to maximize their efficiency during disease states such as T2D.

CHAPTER 1: INTRODUCTION AND BACKGROUND

1. 1 The Brief History of Diabetes and Research

Long before terming “ *diabetes* “, which means “ to pass through” by Greek Apollonius of Memphis in 250 BCE and the re-discovery of ‘ honey-like-urine’ (glycosuria) by Thomas Willis, who included the term “ *mellitus* “, *Diabetes* has discovered its first saying around 1500 B. C. in an Egyptian manuscript. It was perceived as a disease related with ‘ too great emptying of the urine’ (polyuria). Later, important discoveries includes Matthew Dobson’s first proof of elevated urine and blood glucose levels (hyperglycemia) in people with diabetes (Dobson, 1776). In 1889, Joseph von Mering and Oskar Minkowski were the first to give evidence that pancreas removal in dogs induced diabetes, proposing that pancreas functional related to glucose levels. Afterward, Edward Albert Sharpey-Schafer proposed that diabetes could be brought on by losing a pancreatic chemical, which he named as *insulin* in

1910 (Polonsky, 2012). Taken together, diabetes is currently well recognized as a gathering of heterogeneous disorders characterized by hyperglycemia because of loss of insulin or its effectiveness. Current worldwide trends demonstrate a surprising 382 million individuals have diabetes and this is predicted to ascend to 592 million by the year 2035 (IDF Diabetes Atlas, 2013). The cases of diabetes complications, including diabetic retinopathy, cardiovascular disease and renal failure are constantly rising and the death rate because of those are worsen every year.

Claude Bernard’s identification of liver as the major glucose production organ led to the first concept of “ *homeostasis* ” which has been termed and expanded by Walter Bradford in the mid-nineteenth century, to describe the maintaining of steady-state physiology of the cells (Robin, 1979). This gave the notion that actually the disturbed glucose homeostasis is one of the important events of the diabetes progression. Given the evidences of insulin is involved in maintaining the glucose homeostasis, Frederick Banting and Charles Best integrated a series of scientific approaches, and were able to purify the insulin from the pancreas. Moreover, they successfully treated the patients who suffer from the diabetes, with their purified insulin (Banting & Best, 1922; Banting et al., 1922). This landmark finding set the stage for treating the severe diabetes with insulin. However, it has been almost a century now since the first time insulin was discovered and purified, diabetes remains the incurable disease, requires life-time attention and treatment because of its complexity.

Diabetes has been classified as a couple different types nowadays, the major two types are known as the “ Type I Diabetes” (T1D) or “ Insulin Dependent

Diabetes Mellitus” (IDDM) and “ Type II Diabetes” (T2D) or “ Non-Insulin Dependent Diabetes Mellitus” (NIDDM). Surprisingly, these two major types has been notified as early as 100-200 B. C by Indian physicians (Kahn, 1994). However, the first scientific evidence was brought by Wilhelm Falta and Harold Himsworth after they set the standardized glucose/insulin tolerance test in human to distinguish the insulin sensitive from non-sensitive patients (Himsworth, 1936). Insulin dependent T1D is featured in insufficient insulin production due to autoimmune response to the pancreatic beta-cells and it mostly affects young kids. T2D is more described as a “ metabolic syndrome” with emergence of insulin resistance and obesity etc, and it is more common among adults (Moller, 2001). There is a complex network of several insulin responding tissues contributes to the maintenance of glucose homeostasis, and of course any abnormality in this network induces the progression of T2D. Besides the metabolic relevant tissues, the gene-interactions also play critical roles in the development of T2D and obesity such kind of metabolic diseases (Doria et al., 2008).

1. 2 The Islet Architecture, Î²-cell Fate determination

The islets of Langerhans are specialized endocrine part of pancreas and are the only part producing the secreting hormones. They basically comprise of various cell sorts named Î±, Î², Î´, PP, and Îµ that secret the islet hormones glucagon, insulin, somatostatin, polypeptide Y, and ghrelin individually, and these hormones are required to maintain the glucose homeostasis at normal or stress state. Furthermore, the islets are known to have dynamic and plastic architecture that is proposed to be adjusted over the time of development (Steiner et al., 2010). During the progression of insulin

resistance, pregnancy or T2D, the islets extend in size to make up for expanded insulin requirement (Weir and Bonner-Weir, 2004; Kim et al., 2009). But afterwards, there is a considerable loss of Î²-cell mass because of environmental or genetic factors, inducing the serious hyperglycemia in cause of insulin deficiency.

While islets count to just ~1-2% of the whole pancreas, the insulin producing Î²-cells represent ~65-80% of the islet mass, constituting to roughly 2% of pancreatic weight. Furthermore, the rest other cell types are considered as non Î²-cells of the islets (Weir and Bonner-Weir, 2013). During the development, these distinctive cell types are known to emerge from a single progenitor cell that producing Neurogenin3 or *Ngn3* , a transcription factor that decides endocrine cell destiny (Edlund, 2002). Afterwards, other transcription factors, for example, *Pdx1, Pax4, Nkx2. 2, Nkx6. 1, MafA* , and *Foxo1* help the Î²-cell fate determination (Ziv et al., 2013). While it had been learned as that Î²-cells proliferate by self-duplication from old Î²-cells instead of differentiation from stem cell (Dor et al., 2004), some other study suggested that multipotent cells inside the pancreas could differentiate into Î²-cells as well (Xu et al., 2008). The latter study is also confirmed by researches showing how expressing the Î²-cell specific transcription factors in non Î²-cell could trigger a Î²-cell lineage in mice (Collombat et al., 2009; Al-Hasani et al., 2013). Recent studies additionally showing that non-Î² cells, for example, Î±-and Î´-cells could experience “ transdifferentiation” into Î²-like cells when the mice is suffering from significant Î²-cell loss (Thorel et al., 2010; Chera et al., 2014). On the other hand, a few researches demonstrate that the Î²-cells can likewise lose their fate or “ dedifferentiate” into non-Î² or

progenitor cells when losing any of the previously mentioned Î²-cell particular transcription factor (Ziv et al., 2013). Moreover, a current study demonstrated that human Î²-cells are capable of converting into Î±-cells with no genetic modification (Spijker et al., 2013). Taken together, all these researches have showed that the dynamic plasticity of islet cells.

1. 3 Glucose Stimulate Insulin Secretion

One unique and significant feature of Î²-cells is to detect the blood glucose changes and secrete insulin into extracellular milieu in response to keeping the glucose levels within the range of 4-8mM (Weir and Bonner-Weir, 2013). This is primarily accomplished by the take-up 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*), subjects glucose moieties to quick metabolism system by glycolysis (Matschinsky and Ellerman, 1968). This brings about the producing of three carbon products: pyruvate, which takes part in the tricarboxylic corrosive (TCA) cycle inside the mitochondrion to eventually create adenosine triphosphates (ATP) by means of the electron transport chain system. The ATP therefore leads to the increase of ATP/ADP proportion in the cytoplasm, activating the closure of the ATP sensitive potassium (K_{ATP}) channel. Vitally, mutation in the *kir6. 2* subunit of this channel were demonstrated to induce neonatal diabetes in both mice and human because of loss of insulin secretion as a consequence of constitutively open K_{ATP} channel (Koster et al., 2000; Gloyn et al., 2004). It has long been realized that glucose stimulates the closure of these K_{ATP} channels thus leading the slow menbrane depolarization (Ashcroft et al., 1984). This promotes extracellular calcium influx by voltage dependent

calcium channels and potentiates the releasing of insulin (Matschinsky et al., 1998). Insulin is secreted in an oscillatory manner because of the blood glucose level and triggers downstream insulin signaling cascade in insulin-responsive tissues for the taking up glucose. It has well been shown that islets can be entrained to small changes in glucose and thus the plasma insulin has high frequency of oscillation. However, this capacity of entrainment of the islets is disturbed in patients with T2D (Mao et al., 1999). It exhibits Î²-cell malfunction because of loss of insulin secretion is a major issue during the clinical indication of T2D.

1. 4 Compensatory Islet Expansion During Insulin Resistance

During the state of insulin resistance or over-weight, elevated plasma insulin levels (named hyperinsulinemia) has been found in polygenic mouse models showing insulin resistance and human subjects because of increasing of insulin secretion (Yalow and Berson, 1960; Polonsky et al., 1988; Brüning et al., 1997). It has been suggested later that both in rodents and people, this improved insulin secretion is apparently because of an expansion in Î²-cell mass by either Î²-cell proliferation (Steil et al., 2001) or Î²-cell hypertrophy (Weir and Bonner-Weir, 2004). On the other hand, “ Î²-cell failure” because of different genetic or environmental variables, is known to cause declined plasma insulin levels in diabetics (Maclean and Ogilvie, 1955). It has been showed that lessened levels of insulin are frequently associated with a noteworthy loss of Î²-cell mass because of Î²-cell apoptosis (Butler et al., 2003; Rhodes, 2005).

Other than the diabetes perspective, it has been demonstrated that matured Î²-cells have long life-span and low proliferative rates at steady state. This is

because of a potential limitation of the entry of matured Î²-cells into cell cycle (Teta et al., 2005; Kushner, 2013). Other than this perception, later study suggested that adult Î²-cells do have the ability to proliferate (Stolovich-Rain et al., 2012). In light of these findings on Î²-cell proliferation, a few research groups have revealed various proteins essential for assisting Î²-cell proliferation on knockout or transgenic mouse. Known cell cycle controllers including *Cyclins D1* and *D2* , *Cyclin subordinate kinase 4 (Cdk4)* , Cdk inhibitors (CKIs) such as *Cip/Kip and INK4* , transcription factors *Retinoblastoma (Rb)* and *p53* , have been proved on genetic mouse models as regulators of Î²-cell proliferation and survival (Heit et al., 2006).

Although transient high glucose has been considered as the result of insufficient insulin secretion or insulin resistance, it has also been revealed to promote the compensate Î²-cell mass expansion (Bonner-Weir et al., 1989). This hypothesis was further supported recently by another observation, that it is the glucose metabolism, instead the glucose itself that triggers compensatory Î²-cell proliferation *in vivo* (Porat et al., 2011). Some other attentions have been centered on the effect of activation of insulin/ *IRS2* pathway on driving Î²-cell proliferation. The components of the pathway including *IRS2* (Withers et al., 1998) and *AKT* (Bernal-Mizrachi et al., 2001) were demonstrated to be fundamental for Î²-cell survival. Moreover, study has shown that the impact of insulin in Î²-cell proliferation is even stronger when with hyperglycemia (Paris et al., 2003). It is notable that when in the state of severe insulin resistance, the pancreatic islets adjust themselves to meet the expanding requirement for producing and secretion more insulin by increasing their Î²-cell mass (Weir and Bonner-Weir, 2004). Other than the

involvement of protein coding genes, a few non-coding RNAs (ncRNAs), mostly microRNAs and long ncRNAs (lncRNAs), have been shown in the “ diabetogenes” list, adding on to the complex genetic architecture of human diabetes.

2. 1 The Brief History of MicroRNAs

The most recent decade has seen huge attention regarding a new and special class of little ncRNAs such as microRNAs (miRNAs) in regulating the structure and function of Î²-cells. With the first observation of a miRNA, *lin-4* in *C. elegans* , researchers showed how a gene product encodes two little RNAs, instead of a protein. Besides, they demonstrated that these small RNAs binds to the compensatory sites at the 3′ end of untranslated region (UTR) of *lin-14* , a development related heterochronic gene. This interaction is appeared to negatively regulate the expression of *lin-14* by blocking its translation (Lee et al., 1993; Wightman et al., 1993), proposing miRNAs as negative regulators of gene expression.

Even since the discovery of microRNAs, a lot of related research results have updated the mechanism of gene regulation to a novel level. miRNAs now are known as a group of small ncRNAs of ~21-22 nucleotides long that can complementary or non-complementary base-pairing the mRNA of protein coding genes, thus to regulate their expression at post-transcriptional level (Bartel, 2004 and 2009). Actually after the identification of *lin-4* , another miRNA called *let-7* was revealed like *lin-4* in both biogenesis and function level. Soon after, there starts a prevail in discovering new microRNASs mostly by high throughput sequencing technologies in research area and surprisingly, about 30, 000 miRNA over about 200 species have been

identified, which includes about 2, 500 mature human miRNAs. (Kozomara and Griffiths-Jones, 2014). Many computational methods have also been produced to predict the potential targets of miRNAs based on the stable miRNA-target mRNA binding model (Lewis et al., 2003; Krek et al., 2005).

2. 2 MicroRNA Induced Gene Silencing

The intercellular gene silencing mechanism, termed as RNA silencing (RNAi) or post-transcriptional gene silencing (PTGS) is currently well known to be led by a group of small RNAs, for example, short interfering RNA (siRNA), piwi interacting RNA (piRNA), or the miRNAs. Basically, their working mechanisms are similar and the difference exist mostly in their biogenesis inside the cells (Ender and Meister, 2010). Mature miRNA producing has been through several steps: transcribed from DNA by RNA polymerase II, primary miRNA (pri-miRNA) has much longer sequence. Once transcribed, the pri-miRNA is further processed to precursor miRNA (pre-miRNA) about 60 nucleotides long by enzyme *Dorsa* and *DGCR8* protein complex (Lee et al., 2003). Once pre-miRNAs are produced inside the nucleus, it will be export out of the nucleus by protein Exportin 5 to the cytoplasm in a Ran-GTP dependent manner (Lund et al., 2004). Another important enzyme, which is also critical for mouse development, *Dicer* would recognize the pre-miRNA and process it to about 22 nucleotides long mature miRNA duplex form (Bernstein et al., 2003). Only one of stands of the duplex will be transported to miRNA-induced silencing complex (RISC) by *Dicer* , and the other strand, termed miRNA* is usually degraded in the end (Schwarz et al., 2003; Ender & Meister, 2010).

There are several components on the RISC, and one key protein component is *AGO* family. There are four well characterized members of *AGO* family in human *AGO1* , *AGO2, AGO3 and AGO4* . And *AGO2* is expressed more often than other forms (Su et al., 2009; Wang et al., 2012). All the *AGO* proteins have the ability to slice mRNA because of their PAZ and PIWI " cleavage" domains under the guidance of miRNA sequence. Each miRNA has an important " seed" region, typically from 2 nd -7 th nucleotides, that could fully or partially bind to the mRNA 3′ UTR sequence. The base pairing condition between the miRNA and target mRNA determines the target recognition and binding of miRISC, but also the fate of the mRNA- to be cleaved or to be repressed in translation. In animal system, the miRNA does not fully complementary bind to the 3′ UTR of the target mRNA, through blocking the translation machinery, miRNA silencing the gene expression, without interference of the target stability. However, recent studies on the miRNA mediated gene silencing in mammalian cells reveals that miRNA may act through two step modes: at first, repressing the translation, then deadenylation and destabilization of target mRNA [27qualifier]. The deadenylation has been suggested to induce mRNA degradation [48] and translation inhibition is probably the requisite of mRNA degradation in mammalian cells [49].