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Lessons from basic pancreatic beta cell research in type-2 diabetes and vascular complications

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Abstract

The changes in life-style with increased access of food and reduced physical activity have resulted in the global epidemic of obesity. Consequently, individuals with type 2 diabetes and cardiovascular disease have also escalated. A central organ in the development of diabetes is the pancreas, and more specifically the pancreatic beta cells within the islets of Langerhans. Beta cells have been assigned the important task of secreting insulin when blood glucose is increased to lower the glucose level. An early sign of diabetes pathogenesis is lack of first phase insulin response and reduced second phase secretion. In this review, which is based on the Foreign investigator award lecture given at the JSDC meeting in Sendai in October 2016, we discuss a possible cellular explanation for the reduced first phase insulin response and how this can be influenced by lipids. Moreover, since patients with cardiovascular disease and high levels of cholesterol are often treated with statins, we summarize recent data regarding effects on statins on glucose homeostasis and insulin secretion. Finally, we suggest microRNAs (miRNAs) as central players in the adjustment of beta cell function during the development of diabetes. We specifically discuss miRNAs regarding their involvement in insulin secretion regulation, differential expression in type 2 diabetes and potential as biomarkers for prediction of diabetes and cardiovascular complications.

Introduction

Excess food intake and reduced physical activity are among the major causes of the worldwide increase in obesity. With increased obesity follows increased number of individuals with type 2 diabetes (T2D) and cardiovascular disease. T2D is increasing exponentially and cardiovascular disease is the leading cause of death worldwide [1]. Moreover, patients with diabetes often develop cardiovascular complications.
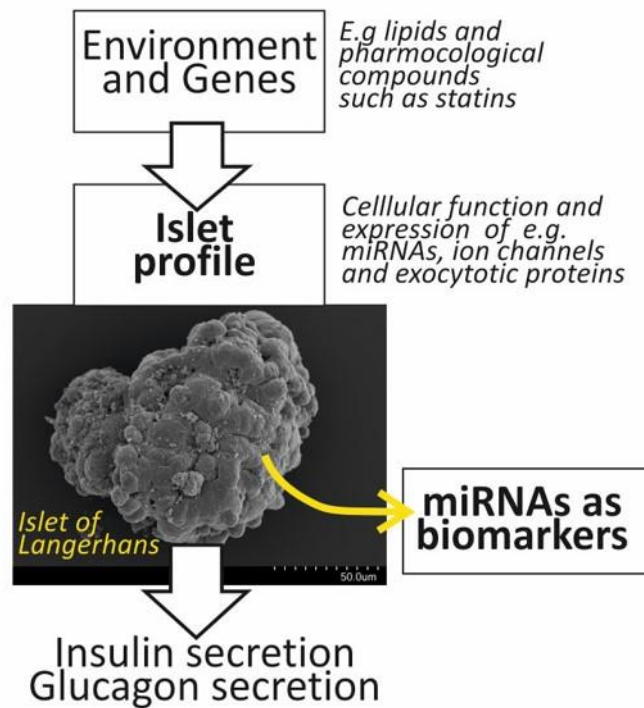
Fig. 1 Impact of genes and environment on islet cell secretion. Model illustrating how environmental factors and genes impact the islet profile and thereby insulin and glucagon secretion. The model also shows how miRNAs from the islets are suggested as biomarkers of beta cell function. The image is a scannining electron micrograph of a human islets.

Diabetes is defined by increased levels of blood glucose. A central organ in the control of blood glucose is the islets of Langerhans. The islets constitute the endocrine part of the pancreas and are clusters of ~2000 cells spread out throughout the exocrine pancreas. Majority of the cells within the islets are beta and alpha cells. The beta cells secrete insulin to lower blood glucose after energy intake and the alpha cells secrete glucagon to increase blood glucose in times of exercise and fasting. In diabetes, the secretion of insulin from the beta cell is impaired and fails to compensate for increased insulin resistance in liver, muscle and adipose tissue [2]. The pancreatic beta cell is an electrically active cell and the release of insulin from the beta cell upon glucose uptake involves several ion channels and ends with an increased intracellular calcium concentration triggering the fusion of insulin-containing granules with the plasma

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membrane. The whole process from glucose uptake to the release of insulin is often referred to as beta cell stimulus secretion coupling, and the final stage is referred to as calcium-dependent exocytosis of insulin granules (See e.g. [3, 4]). Prior to exocytosis the insulin granules, after their release from the Golgi apparatus, go through several stages: 1) transport and mobilization to the plasma membrane, 2) docking at the plasma membrane and 3) priming [5, 6]. The fusion of the pool of primed granules on acellular level have been suggested to account for first phase insulin secretion [7]. A reduced first phase insulin response is often observed in patients with T2D at an early stage [8] and in islets from T2D donors the expression of several proteins involved in priming is reduced [9].

In this review, we will focus on the cellular mechanism involved in exocytosis of insulin granules and put forward the hypothesis that defective exocytosis can contribute to the development of T2D. Environmental factors such as obesity is often associated with increased cholesterol, why cholesterol lowering drugs such as statins are subscribed. We will therefore discuss recent data concerning how cholesterol and statins effect the insulin secretion process. Finally, we will discuss the role of the small non-coding RNAs called miRNAs in insulin secretion and how these can be utilized as future novel prediction of T2D and cardiovascular complications (Fig. 1).

Beta cell insulin secretion and exocytosis

Insulin secretion

Insulin is released from the pancreatic beta cells in response to increased blood glucose levels following food intake. The process by which glucose induce the secretion of insulin is regulated and glucose is the main initiator or trigger of insulin [10]. The consensus model of beta cell stimulus-secretion coupling has been extensively described [3, 4] and illustrated to the right in Fig. 2. In short, the increase in blood glucose results in uptake of glucose through insulin-independent glucose transporters. Glucose is, mediated by glucokinase, turned into glucose-6-phospate and metabolized to ATP. The increase in ATP and decrease in ADP is important for closure of the ATP-dependent K^+ channel (K_{ATP} channel). The K_{ATP} channel consist of an inward rectifying K^+ channel KiR6.2 and the sulphonylurea receptor SUR1. Sulphonylurea binds to SUR1 and binding leads to closure of the channel [11]. When open, the K_{ATP} channel is a major factor determining the resting membrane potential of the beta cell. Once the channel is closed, the beta cell membrane potential depolarize and initiates beta cell electrical activity, Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and Ca^{2+} dependent exocytosis of insulin containing granules [12]. The beta cells are, as the neurons, electrically active and phasic oscillations in plasma membrane potential depend on the opening of voltage dependent Na^+ and Ca^{2+} channels for the depolarizing phase and the opening of voltage sensitive K^+ channels for the repolarizing phase [13].

Insulin secretion can be potentiated by hormones and neurotransmitters. Glucagon and the incretin hormones glucagon-like-peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) bind to different G-protein coupled receptors and generate increased levels of intracellular cAMP. Much focus has recently been put on GLP-1, which amplifies insulin secretion by both PKA-dependent and PKA-independent mechanisms that promote K_{ATP}-channel closure, β cell

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electrical activity, calcium release from intracellular stores, and primarily insulin granule exocytosis [5, 14, 15]. Acetylcholine enhances insulin secretion through binding to muscarine receptors on the beta cell and activation of PKC. Recent studies have also suggested nicotinic acetylcholine receptors to be present on beta cells and important in stimulated insulin secretion [16].

Inhibitors of insulin secretion include somatostatin and adrenalin. Somatostatin is secreted from pancreatic delta cells acting on G protein-coupled somatostatin receptors (SSTRs) [17]. The hormone and neurotransmitter noradrenaline is released from the adrenal medulla along with adrenaline, and by the sympathetic nervous systems. Noradrenaline and adrenaline bind to alpha2A-adrenergic receptors in the beta cells [18]. Recent data have demonstrated a single-nucleotide polymorphism in the human ADRA2A gene that associates with increased risk of T2D. Islets from risk allele carriers showed overexpression of alpha2A-adrenergic receptors and reduced insulin secretion [19]. A clinical follow-up study has demonstrated improved insulin secretion in risk carriers after treatment with pharmacological alpha2A-adrenergic receptor antagonists [20]. The human data on polymorphism in ADRA2A originate from work in a congenic strain of the diabetic Goto-Kakizaki rat model [21, 22], where a genetic locus was linked to reduced exocytosis, impaired insulin secretion and increased expression of the alpha2A-adrenergic receptor [19].

First phase insulin secretion and priming of insulin granules

Insulin secretion is biphasic in response to a square-wave increase in glucose given to either the *in vitro* perfused pancreas or islet, or the *in vivo* pancreas. Upon the immediate glucose increase, insulin secretion in the perforate or plasma rapidly increase and peak within a few minutes, decrease to a nadir after ~15 min, and then steadily increase to a pseudo-steady state after ~3h. The early rapid peak is referred to as the first-phase insulin release, and the following gradual increase is commonly called second-phase insulin release [23]. Much attention has been on the mechanisms behind phasic insulin secretion since patients with T2D often have a loss of first-phase insulin secretion and a reduced second phase, even before the development of the disease when they have impaired glucose tolerance (IGT) [8, 24]. Interestingly, first phase insulin secretion can occur in the absence of metabolic stimulus in the form of ATP. Hence, first phase insulin secretion occur by mere membrane depolarization using K^+ or arginine, whereas the second phase requires glucose or another generator of ATP to arise. On a cellular level, biphasic insulin secretion has been suggested to reflect the presence of different functional pools within the beta cell [7]. Prior to fusion at the release site, the insulin granules undergo a series of maturations steps. Once departed from the Golgi apparatus the granules need to be translocated along microtubule to the plasma membrane, where they dock and undergo a process called priming (Fig. 2). Our group has earlier demonstrated that priming is a Ca^{2+}, ATP, and temperature dependent process [7, 25, 26]. Primed granules belong to a pool of readily releasable granules that can fuse with the plasma membrane upon increase in local Ca^{2+} in the absence of ATP. We have electron microscopic data from mouse pancreatic beta cells showing that each beta cell has ~700 granules docked at the plasma membrane of which one third is gone after depolarization of the islets with high K^+ in the absence of ATP, suggesting that the pool of primed granules constitute ~200 granules in each beta cell [27]. The

presence of pools of granules of different functionality is also supported by FRET-based studies. These studies show that the Soluble NSF Adaptor protein REceptor (SNARE) complexes exist in different states of "pre-assembly"[28].

We have an interest in understanding the cell physiological background to the lack of first phase insulin secretion and reduced second phase in T2D individuals. Due to the above suggested model, much research has focused on mechanism involved in facilitation of insulin granular priming. One model that arose from years of scientific work involves mechanisms around priming of insulin granules and is illustrated in Fig. 2. Once primed the insulin granules need to be fully matured. It has since long been known that mature insulin granules are acidic and a low intragranular pH around 5 is essential for processing of proinsulin to insulin [29]. The model suggest that insulin granules becomes release competent upon granular acidification accomplished by influx of H^+ through a v-type H^+-ATPase. This has been proven by capacitance measurements of exocytosis showing that refilling is reduced in presence of the v-type ATPas inhibitor bafilomycin [30]. The flux of positive ions is counteracted by influx of chloride through a CLC-3 Cl^- channel, present at the insulin granules [30]. Knock-out of this channel in mice specifically reduce first phase insulin secretion, exocytosis of primed granules and increase intragranular pH [31]. On the membrane of the insulin granules are also a granular sulphonylurea receptor (gSUR), which has been speculated to form a channel complex with ClC3 [30, 32]. Moreover, ATP enhance granular priming [7, 33]. One suggestion is that this goes through a direct interaction of ATP with the CLC-3 Cl- channel [30].

Previous data have demonstrated that cAMP fascilitates granular priming [26] and that this effect is mainly PKA-independent, in contrast to mobilization that is strongly PKA-dependent [34]. The PKA-independent effect has been suggested to act through the cAMP receptor protein EPAC2A [35]. As shown in the model in Fig 2, cAMP most likely promotes granular priming through binding to EPAC2A. It has also been suggested that the pharmacological drug sulphonylureas has a direct effect on exocytosis [36]. The function by which sulphonylureas can enhance the exocytotic response is not clear and there are several suggestions. First it was presented that sulphonylureas activate PKC to promote exocytosis in the beta cell [36]. Later others have shown that sulphonylureas can directly bind to EPAC2A and thereby contribute to granular priming [37, 38]. Moreover, data from our group demonstrated that EPAC2A forms a tight interaction with CLC3, gSUR and also SUR1, which suggest that the role of SUR1 stretches beyond being a part of the K_{ATP} channel to also include a major function in granular priming [34]. Altogether, the presence of both cAMP and ATP enhance granular priming through interaction with a network of proteins associated with the acidification of the insulin granules, a process that can be further enhanced in the presence of sulphonylureas.

Recent published data from our group suggest the presence of CFTR in the pancreatic beta cells. CFTR is activated in the presence of ATP and cAMP and we show by capacitance measurements of exocytosis that CFTR has a role in granular priming [39]. The hypothesis is that CFTR forms a complex with another Cl^- channel the anoctamin1 (ANO1) in a similar manner as the Kir6.2 and the SUR1 forms the K_{ATP} channel. CFTR then act as a regulator of ANO1 in the beta cell. Binding of cAMP to CFTR activate ANO1 that then promote granular priming though delivery of Cl^- ions to the CLC3 granular channel essential for priming (Fig.

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2). This cellular data agrees with clinical work showing that patients with mutations in CFTR having cystic fibrosis related diabetes (CFRD) lack first phase insulin secretion [40, 41].

As summary of this part it can be concluded that ATP, generated from glucose, and cAMP, made by binding of e.g. the incretin GLP1 to G-protein coupled receptors, has central roles in beta cell insulin secretion. ATP not only act as the molecule that triggers the insulin secretion cascade by closing the K_{ATP} channels, but are also involved in the amplification of insulin secretion through effects on granular priming. For cAMP, the main amplifying effects on insulin secretion are on PKA-dependent granular mobilization and cAMP-dependent enhancement of granular priming through interaction with EPAC2A.
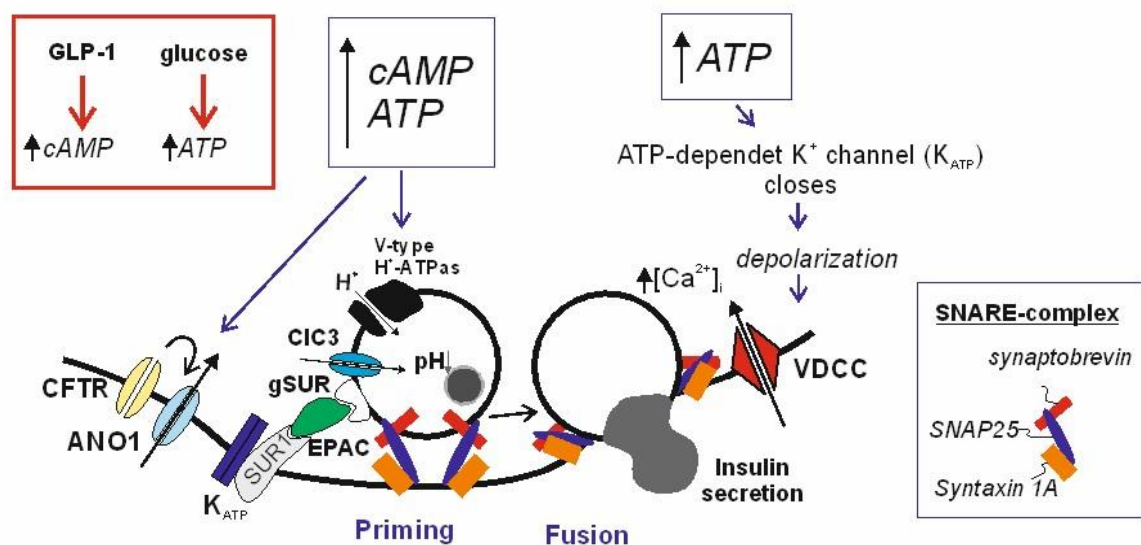

Fig. 2 Regulation of priming and fusion of insulin granules by glucose and the incretin GLP-1. Uptake of glucose into the beta cell leads increased intracellular ATP through metabolism. GLP-1 binds to G-protein coupled receptors and increase intracellular cAMP. The model is valid also for other compounds able to increase intracellular ATP and cAMP. ATP initiate the triggering pathway of beta cell stimulus-secretion coupling (right) leading to the increase in intracellular Ca^{2+} needed for fusion. Both cAMP and ATP facilitate granular priming and are therefore involved in amplification of the secretory response. Many different protins and ion channels are involved as described in detail in the text. VDCC - voltage dependent Ca^{2+} channel; SUR1 - Sulphonylurea receptor; EPAC - Exchange protein directly activated by cAMP; CLC3 - Cl$^-$ voltage gated channel 3; gSUR - granular sulphonylureas receptor; CFTR - Cystic fibrosis transmembrane regulator; ANO1 - Anoctamin 1 Ca^{2+} activated Cl$^-$ channel.

Exocytotic proteins essential for beta cell insulin secretion

The process whereby insulin granules dock to the plasma membrane and fuse with the plasma membrane through Ca^{2+} dependent exocytosis at the release site requires the presence of several exocytotic proteins. Involved in the final fusion of insulin granules is the formation of the SNARE-complex (Soluble NSF Adaptor protein REceptor) (Fig 2), consisting of the vesicular-bound protein VAMP2/synaptobrevin and the plasma membrane proteins, syntaxin1 together

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with SNAP25 associated to the membrane through palmitoylation [5, 6]. It has been shown that prior to exocytosis SNARE-complexes exist in different states of "preassembly" [28]. The assembly of the SNARE complex is modulated by several other components, such as Rab3a and/or Rab27 and Munc18 [42]. Granuphilin/Slp4 is known as a negative regulator of insulin granular exocytosis. Granulphilin interacts with Munc18 and synatxin1, and during glucose stimulation granuphlin is released allowing formation of the SNARE-complex a process facilitated by Munc18 [42, 43]. Recent data have described that docking occur at the same time as formation of synatxin1/Munc18 clusters at the release site [44]. Formation of these clusters hinders undocking [44] and agree with that the pool of docked granules is larger than the readily releasable pool [27]. Later, the granules at the release site becomes release ready and primed, a process that involves the recruitment of SNAP25 and munc13 [44]. A role for SNAP25 in priming of the insulin granules is supported by data that show that truncated SNAP25 reduces the stimulatory action on rapid exocytosis by cAMP [45], and as described above cAMP is central for insulin granule priming. The importance of the exocytotic proteins for functional insulin secretion is reinforced by the fact that islets from T2D human donors or the diabetic GK-rat have reduced expression of these genes [9, 46, 47]. Our own work on human islets [9] have shown that gene expression of *STX1A, SYT4, SYT7, SYT11, SYT13* is lower in islets of T2D donors and that the expression of these genes together with *SNAP25* and *STXBP1* correlates negatively to in vivo measurements of HbA1c levels and positively to in vitro glucose stimulated insulin secretion in human islets. The reduced expression of the exocytotic genes could not be explained by genetic polymorphism and we hypothesize that instead transcription factors, miRNAs or epigenetic factor are involved. Indeed, miRNAs has been shown to control several exocytotic genes as we will discuss below.

Impact of obesity on first phase insulin secretion and beta cell exocytosis

Obesity is a key risk factor in T2D and several models have been suggested to explain the reason why. One such model is the "twin vicious cycle of T2D" [48]. In this model, increased caloric intake will lead to accumulation of fat in the liver. As this process is stimulated by insulin individuals with insulin resistance will gain accumulation of fat much faster due to increased fasting insulin levels. The increased liver fat will increase the release of triglycerides and since the pancreatic beta cell is more susceptible to the increase in triglycerides the cells will take up triglycerides. Presence of triglycerides inside the beta cell will lead to reduced glucose stimulated insulin secretion, mostly first phase insulin secretion. Indeed, fat droplet accumulation inside human pancreatic beta cells measured by transmission electron microscopy is positively correlated with increased BMI [19].

The role of lipids in beta cell function is complex as they could be both good and bad in terms of increasing beta cell insulin secretion. In the short term, free fatty acids (FFA) increase insulin secretion through increased Ca^{2+}-influx and augmentation of granular priming [49]. Also, acute accumulation of the active intracellular form of FFA, acyl-CoA (LC-CoA), increase exocytosis and thereby insulin secretion [50]. However, long term exposure to FFA impairs insulin secretion. Mice treated with high fat diet (HFD) have increased insulin content and reduced *in vitro* insulin secretion when compensated for the increased insulin content indicative of a defective secretion machinery by HFD [51]. Experiments in which islets have been exposed to

FFA palmitate for 72 h indicate that the negative effect on insulin secretion by HFD is not due to changes in the global intracellular Ca^{2+} [52] but rather to imperfect fusion of the insulin granules. Indeed, the influx of Ca^{2+} per see is not changed by palmitate but the Ca^{2+} channels are dissociated away from the release site resulting in reduced local Ca^{2+} concentrations and reduced exocytosis [53]. This is due to that the Ca^{2+} concentration needed for exocytosis is high (~17 µM) and under normal conditions the Ca^{2+} channels needs to be clustered to the release site and the secretory granules for rapid exocytosis [54].

Another necessary compound is cholesterol, - an essential component of the plasma membrane and needed for exocytosis. Membrane rafts are small domains in the membrane that are highly dynamic areas enriched in cholesterol and sphingolipids and suggested to spatially organize ion channels and exocytotic proteins. Cluster formation of SNARE proteins is thought to be associated to such microdomains rich in cholesterol [55]. E.g. SNAP25, known to be attached to the plasma membrane through palmytoilation by 4 cystein residues [56], is dislocated from the plasma membrane upon cholesterol desorption with a resulting reduced exocytosis [31]. Interestingly, the membrane rafts are significantly reduced in islets from T2D donors and the diabetic model the GK-rat [57]. As such cholesterol seems to be important and necessary for insulin secretion. However, too much cholesterol is detrimental for the cell and to high levels of cholesterol inside the beta cell due to impaired outflow of cholesterol will reduce insulin secretion [58].

Although as described above HFD or long-term exposure of FFA to islets reduce insulin secretion, not all obese individuals develop diabetes. Thus, there are individual differences to the susceptibility to develop T2D by HFD. To investigate this question Nagao and co-workers have developed mouse lines with distinctively different susceptibilities (prone and resistant) to HFD-induced glucose intolerance [59]. The two mice strains are called "selectively bred diet-induced glucose intolerance-prone (SDG-P)" and "-resistant (SDG-R)". SDG-P mice have reduced acute insulin response during OGTT and decreased in vitro insulin secretion already before given HFD. Interestingly, SDG-P mice have reduced expression of the exocytotic genes coding for SNAP25 and syntaxin1 [60], suggesting defects in the exocytotic machninery. If and how the innate predisposition in pancreatic islets of this model effects the exocytotic machinery will be the topic of future studies.

In all, the islets are sensitive to changes in lipids and excess of calories will increase the uptake of fatty acids into the beta cell and harm their function by dissociation of Ca^{2+} channels from the release site. In individuals with an innate hereditary predisposition in the pancreatic islets this may be the main determinate of the susceptibility to develop obesity induced T2D.

Impact on insulin secretion and glucose homeostasis by statins

In obesity cholesterol levels increase, and a major risk factor for the development of cardiovascular disease is high cholesterol levels in the blood. It follows that cholesterol lowering drugs, most notably statins, are among the highest selling prescription drugs

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worldwide. Although clearly lifesaving, statins are not without side effects. Almost 10 years ago it was noted in the JUPITER study that in patients treated with rosuvastatin, one of the more potent statins, T2D was reported significantly more often than in the control group [61]. Several other groups have since then confirmed and expanded on these findings [62, 63]. It is now commonly agreed on that statins have diabetogenic effects and accordingly the American FDA (Food and Drug Administration) have added this side effects to the drug labels of statin products. In this context, it is important to point out that patients are not advised against statins because of this. It is general consensus that the benefits of statin treatment, at least for moderate to high risk patients, clearly outweighs any risk of diabetes development [63, 64].

Cholesterol is mainly produced in the liver through the mevalonate pathway. The rate limiting step in the mevalonate pathway is the conversion of 3-hydroxy-3-methylglutaryl-Co-A to mevalonate, a reaction mediated by the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase). Statins acts by inhibiting HMG-CoA reductase and thereby reducing the production of cholesterol. The mevalonate pathway also produce several other bioactive molecules including isoprenoids, farnesylpyrophosphate, and coenzyme Q10. The production of these molecules is also affected by statins [65].
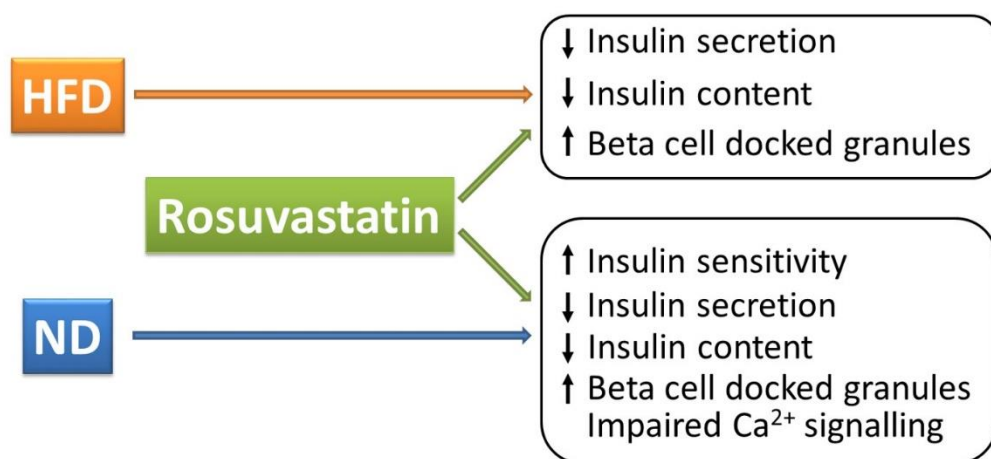

Fig. 3 Effects of rosuvastatin on insulin sensitivity and insulin secretion in mice. Mice were kept on normal diet (ND) and high fat diet (HFD) for four weeks before treatment of rosuvastatin for eight additional weeks with continued diet [51]. The diagram summarize the main effects measured.

Statins and insulin sensitivity

What causes the diabetogenic effects of statins remains unresolved. T2D comprises elements of both insulin resistance and insufficient insulin secretion. With regards to insulin resistance it was reported in the Metabolic Syndrome In Men (METSIM) cohort that statin treatments was associated with decreased insulin sensitivity [62]. Along the same lines it was recently reported that type-1 diabetics on statin treatment showed decreased insulin sensitivity [66]. There are however reports of increased insulin sensitivity with statins, especially with pravastatin [67, 68]. Different statins seem to have different effects on glucose metabolism and pravastatin in particular has been associated with less diabetogenic effects than the other statins [69]. Also

animal studies have indicated that the different statins have different effects on insulin sensitivity [70]. The molecular mechanisms involved are unclear and might be obscured by species-specific differences in the response to statins. Using rodent models several groups, including ours (Fig. 3), have reported improved insulin sensitivity with various statins [51, 71] although reports of reduced insulin sensitivity also exist [72].

One of the mechanisms that have been put forward is that statin exerts an effect on the glucose transporter GLUT-4. It has been reported that the statins atorvastatin and lovastatin reduce the surface expression of GLUT-4 in 3T3L1 adipocytes [73, 74] and atorvastatin reduce the surface expression of GLUT-4 in white adipose tissue from mouse [75]. It was suggested that this was because the statins, by inhibiting the mevalonate pathway, hamper the process of isoprenylation. Several proteins involved in the translocation of GLUT-4 into the plasma membrane are dependent on isoprenylation. For instance, it was shown by Takaguri *et. al.,* that the isoprenoid-dependent proteins Rab-4 and RhoA, both of which are involved in the insulin-induced translocation of GLUT-4, was dislocated from the plasma membrane to the cytosol upon atorvastatin treatment. Curiously in this study the same was not true for pravastatin treatment, possibly because this statin does not readily penetrate the adipocytes due to its hydrophilic character [73]. However, recently it was shown that in human skeletal muscle cells glucose uptake as well as GLUT-4 expression was reduced by several statins including both atorvastatin and pravastatin and to a lesser extent rosuvastatin [76].

To further complicate the picture Naples *et.al.,* have showed that in hamster rosuvastatin *increases* insulin sensitivity most likely by improving phosphorylation of the insulin receptor and IRS-1 which in turn aids insulin signal transduction [77]. Similarly, we have published that rosuvastatin treated mice have a higher glucose uptake in isolated adipocytes [51]. Moreover we have found that rosuvastatin increases the expression of GLUT-4 in soleus muscle in rosuvastatin treated mice on a normal diet while it decreases the expression of GLUT-4 in rosuvastatin treated mice on a high fat diet [51]. Altogether we favour the hypothesis that insulin sensitivity is improved by statin treatment and thereby statins would be favourable acting against the development of diabetes. However, to find out more of the molecular effects involved further investigations are needed. Indeed, the important factor in diabetes development is the level of glucose homeostasis which depends on both insulin sensitivity in target tissues and insulin secretion from the pancreas.

Statins and insulin secretion

What about statins and insulin secretion? We know that the beta cell has an inbuilt high adaptive capacity to increase insulin secretion in times of demand. However, when beta cells fail to compensate, T2D develops. Therefore, factors that perturbe insulin secretion increase the risk of T2D development. In the METSIM cohort [62] statins was reported to reduce insulin secretion. Moreover we and others have reported that statins affects the insulin secretory machinery[51, 78-80]. Reports from a study in human islets showed that statins (atorvastatin, pravastatin, rosuvastatin, and pitavastatin) decreased glucose-induced insulin secretion [76]. The molecular mechanisms behind the negative effect are still elusive. In our investigation, we observed impaired Ca^{2+} signaling, reduced insulin content and secretion and increased number

of docked granules in islets from mice given rosuvastatin for 8 weeks [51], suggesting impaired beta cell function after rosuvastatin treatment (Fig. 3).

It has been reported that acute addition of simvastatin to rat islets [80] and 48h incubation with rosuvastatin in INS-1 832/13 cells [79] reduce the depolarization induced Ca^{2+} current that flows through voltage gated Ca^{2+} channels. Statins have also been reported to reduce ATP levels in the beta cells [64, 81]. This will impact several steps in the stimulus secretion coupling leading to insulin release including the closure of K_{ATP} channels and priming of the insulin vesicles [7, 82], and thus lead to reduced glucose stimulated insulin secretion. Some, including our group, report that the effects of statins on insulin secretion goes through their effects on the mevalonate pathway including the non-cholesterol producing arms [79, 83] while others find effects outside the mevalonate pathway to be more important [84]. The mevalonate pathway is most known to produce cholesterol, but mevalonate is also the precursor of the prenylation enzyme substrates, farnesylpyrophosphate and geranylgeranyl pyrophosphate and other isoprenoid groups. Pyrophosphate and geranylgeranyl-pyrophosphate is added to small G-protein superfamily and thereby effect their function. Isoprenylated proteins have key roles in several cellular functions and their binding to the small G-proteins is required for attachment of these proteins to the plasma membrane [85, 86]. Indeed, lovastatin has been suggested to alter the subcellular localization of the small G-proteins, and rosuvastatin reduce exocytosis in insulin-secreting cells [78, 79]. Thus, it can be suggested that statins interfere with the small GTPases important for beta cell exocytosis such as Rab27 and Rab3a [87], but also the Rap1 for which EPAC2 functions as a guanine nucleotide exchange factor upon cAMP stimulation[37]. Another possibility is that statins act through inhibition of other non-cholesterol mevalonate pathway products such as co-enzyme Q10, which is an essential component of the Krebs cycle and therefore most likely will affect metabolism and the production of ATP essential for beta cell function [88] (. Thus, there are increasing evidence on a cellular mechanistic level that statins interfere with the exocytotic machinery. This is noted not only through the possibility that statins effect the small GTPases important for translocation of granules and exocytosis, but also noted in mice data showing increased number of docked beta cell granules after treatment with rosuvastatin as a sign of impaired exocytosis. Moreover, statins affect Ca^{2+} signaling in the cells that in the long run will impair exocytosis and insulin secretion [51].

Recently, Swerdlow *et. al.,* investigated individuals with a single nucleotide polymorphism (rs 17238484 and rs12916) in the gene encoding for HMG-CoA reductase (the target of statin treatment). The authors found an increase in body weight and waist circumference accompanied by increased plasma insulin and glucose levels with both SNPs [89]. This article points to that, at least some of the diabetogenic effects of statins goes through the inhibition of HMG-CoA reductase and the mevalonate pathway. It also opens for the possibility that inhibition of HMG-CoA reductase could affect body composition. Indeed, it has been reported that statin users increase more in bodyweight over a 10 year span than non-statin users [90]. The increase in bodyweight with statins or in genetic studies does not seem to be enough to explain the diabetogenic effects of the drug [89]. However, it could be relevant when administering lifestyle advice to statin users.

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To conclude; the molecular mechanisms causing the diabetogenic effects of statins remain elusive. While the search continues, good advice on healthy lifestyle choices for statin users can hopefully reduce the number of additional risk factors for these patients.

Role of microRNAs in insulin secretion

In our attempt to better understand how decreased insulin secretion contribute to T2D development we have come across the function of the small non-coding RNAs called microRNAs (miRNAS). Much of the focus on understanding the mechanisms controlling islet function in the pathogenesis of T2D concerns the demand on beta cells to produce and secrete more insulin, which involves complex beta cell adaptations. The fate of the beta cell, when exposed to environmental triggers of the disease, is determined by the possibility to adapt to the new situation by regulation of gene expression. Functionally active miRNAs have the capacity to take part in such adaptations as they have been shown to be key regulatory molecules in various biological processes mediating post-transcriptional fine-tuned regulation of key genes. A working model of a possible scenario is described in Fig. 4.
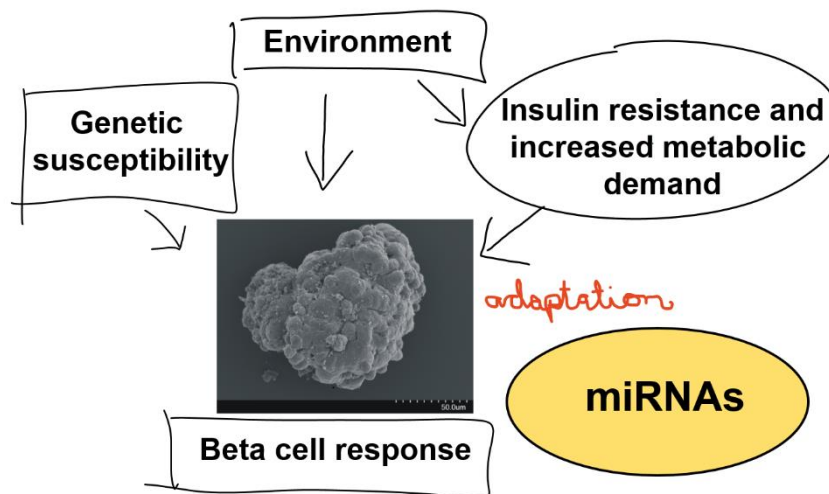
Fig. 4 MicroRNAs are involved in beta cell adaptation during development of T2D. The increased insulin resistance in target tissues lead to increased metabolic demand and the beta cell need to adapt to the situation. Failure for the beta cell to adapt result in T2D. We suggest miRNAs to be central in beta cell adaptation. The image is a scanning electron micrograph of a human islet.

miRNAs

MicroRNAs are short non-coding RNAs that guide the RNA-induced silencing complex to specific sequences within mRNA targets by non-complimentary base pairing. The regulation of miRNA biogenesis and targeting mechanisms are covered more in-depth elsewhere [91, 92].

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In brief, the mature microRNA is ≈22 nucleotide (nt) single-stranded RNA, and is initially processed in the nucleus from double-stranded hairpin pri-miRNA precursor of about ≈1000 nt by two RNAse III enzymes called Drosha and DGCR8. The resulting hairpin pre-miRNA of ≈65 nt is then exported into the cytoplasm and is finally processed into short RNA duplexes by the endonuclease Dicer. The RNA duplex is then loaded into the RNA-induced silencing complex (RISC) wherein one of the strands, called the "guide strand" (mature miRNA) is retained while the "passenger strand" is discarded. The RISC which contains the Argonaute protein (AGO) and facilitates mRNA decay or translational repression, is guided by the mature miRNA generally into the 3'UTR region of target mRNA. The specificity of mRNA targeting is mostly determined by complete base-pairing of only 6-8 nt so called seed sequences located at the 5'-end of the miRNAs into the mRNA target. Hence, a single miRNA may have hundreds of mRNA targets, while a mRNA could be targeted by multiple miRNAs [92]. Consequently, there is considerable impact of miRNAs in animal development and disease pathophysiology [93]. For instance, in diabetes and cardiovascular diseases, the emerging functional roles of miRNAs are now being widely-recognized [94-96].

miRNAs in T2D

Many microRNAs and other non-coding RNAs have been implicated in beta cell dysfunction in rodent models and patients of T2D [95]. In mice, the global effect of miRNAs in the development of T2D has primarily been investigated by deleting/disrupting the gene coding for the enzyme Dicer which is responsible in the final step of miRNA maturation.

We and others have shown that beta-cell specific deletion of Dicer1 in mice leads to overt diabetes due to impaired pancreas development, reduced beta cell mass and/or functional effects on insulin secretion. When Dicer1 was deleted under the promotor of the transcription factor Pdx1, pancreas development was impaired and beta cell mass was reduced already before birth and the mice died early in life [97]. If instead Dicer1 is deleted in adult beta cells using mutant mice, in which the deletion of a Dicer1 conditional allele is directed temporally by a tamoxifen inducible Cre recombinase the islet show reduced insulin content and upregulation of key insulin transcriptional repressors [98, 99]. Moreover, glucose stimulated insulin secretion was reduced mainly due to a defective insulin secretion process with reduced exocytosis that temporally preceded the reduced insulin content [98]. We have in our work deleted Dicer1 under the rat insulin promoter (RIP; Rip-Cre Dicer1$^{\Delta/\Delta}$) [100]. In this model beta cell mass decreased with increasing age from being normal in neonates. Also, the number of insulin granules inside each beta cell was normal in 2-week old Rip-cre Dicer1flox/flox mice, but significantly decreased in older mice. As in the tamoxifen induced model insulin content and insulin secretion was reduced. Moreover, Rip-Cre Dicer1$^{\Delta/\Delta}$ mice showed defects in insulin granular docking and new data measuring exocytosis as changes in membrane capacitance in single beta cells reveal a reduced exocytosis of insulin granules (Fig. 5). Hence, the work on Dicer knockout models reveal the importance of global miRNA expression in the development and function of the beta cell from early development to important regulatory function in beta cell insulin secretion and exocytosis.
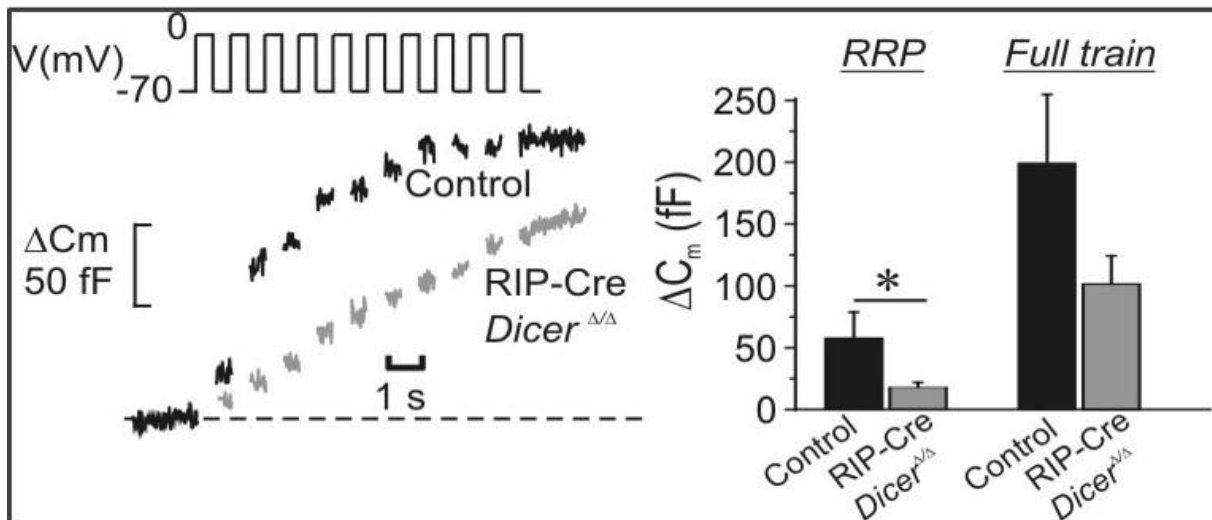
Fig. 5 Exocytosis is reduced in beta cell specific RIP-Cre Dicer $^{\Delta/\Delta}$. Exocytosis was initiated by changes in membrane potential (V) through a train of ten 500-ms depolarizations from -70 mV to 0 mV and measured as increase in membrane capacitance (ΔC_m). Typical traces from control and RIP-Cre Dicer $^{\Delta/\Delta}$ beta cells are shown to the left and the mean increase during the first two depolarizations (RRP) and the total train (Full train) are shown to the right. Data are mean±SEM of n=11-17 experiments in each group; *p<0.05 Students t-test. Other phenotypes of this mice model is described in Kalis et al 2011[100].

To resolve the role of specific miRNAs in pancreatic cell function, it was first important to identify which miRNAs are enriched in the pancreatic beta cell. The pioneering study in 2004 of Poy and colleagues revealed miR-375 to be highly-enriched in pancreatic islet cells, and was shown to target myotrophin (*Mtpn1*) leading to reduced insulin secretion [101]. A follow-up study in which miR-375 was deleted in mice resulted in reduced number of beta cells and at the same time increased number of alpha cells in the animals leading to abnormal glucose homeostasis. Several genes involved in the control of cellular growth and proliferation were identified to be potential targets of miR-375 [102]. Recently, the function of miR-375 in beta-cell proliferation has been demonstrated to be controlled by Argonaute2 (AGO2), which is in turn regulated by another miRNA, miR-184 [103] .

In addition to miR-375, miR-7 and miR-200 families are among the highly-abundant miRNAs in pancreatic islet cells demonstrated to be involved in critical regulation of beta cell functions. Specifically, miR-7a was shown to target alpha-synuclein alpha (*Snca*) influencing SNARE oligomerization [104]. Thus, transgenic mice overexpressing miR-7a developed diabetes due to impaired insulin exocytosis. Additionally the transgenic animals also exhibited beta cell dedifferentiation due to decreased levels of key transcription factors involved in pancreatic endocrine differentiation such as Pdx1, Pax6, and Gata6 [104]. Regarding the miR-200 family, the beta cell specific overexpression of miR141/200c on the other hand, lead to rapid beta cell apoptosis and diabetes due to specific targeting of the chaperone Dnajc3 (also known as p58IPK) and the caspase inhibitor Xiap involved in anti-apoptotic and stress-resistance network in the beta cell [105].

miRNAs in glucose-stimulated insulin secretion

Specific miRNAs have been shown to regulate the expression level, and hence the function of many enzymes and proteins involved in glucose-stimulated insulin secretion. For a more comprehensive review of this topic we refer to a separate review where we have described the different miRNAs involved in glucose stimulated insulin secretion, from insulin biosynthesis, to glucose metabolism, and finally in the release of insulin granules [106].

We began our quest to identify miRNAs involved in the stimulus-secretion coupling in the beta cell by using the Goto-Kakizaki rat T2D model which is primarily characterized by impaired glucose-stimulated insulin secretion [107]. We performed global miRNA profiling in the GK islets, and were able to identify dysregulated miRNAs whose targets were enriched for exocytosis-related functions [108]. Among the upregulated miRNAs was miR-335 which we showed to directly modulate Stxbp1 (syntaxin-binding protein 1)/Munc18-1 involved in first-phase insulin release [109]. Additionally, we identified the upregulation of miR-132/212 cluster from GK islets. We and others later showed cAMP-dependent regulation of the miR-132/212 cluster through a PKA-dependent mechanism [110] involving cAMP-response element (CRE)-binding proteins and CRTC1 [111]. Interestingly, this miRNA cluster was also found to be upregulated in the islets of obese phenotypes of both the diabetes-resistant (B6) and diabetes-susceptible (BTBR) mouse models [112]. Recently, we have also indications that the downregulated exocytotic proteins such as SNAP25 and Syntaxin1 in the islets of selectively bred diet-induced glucose intolerance-resistant prone (SDG-P) mice [60] may also be targeted by miRNAs. The presence of deregulated miRNAs in the islets of different metabolically-perturbed animal models underscores the importance of these regulatory molecules in pathophysiological processes contributing to the development of metabolic diseases.

miRNA biomarkers in T2D and vascular complications

The progression of T2D and its plethora of vascular complications occur gradually over time. Frequently the pathophysiological processes are already transpiring on the cellular level long before overt symptoms of the complication become manifested. Identifying easily accessible and quantifiable biomarkers of T2D and its associated complications holds enormous value in monitoring disease progression, potentially aiding personalized therapeutic intervention programs (Fig 1).

The identification of stable miRNAs in circulation brought about the possibility of blood-based RNA biomarkers capable of predicting the onset of diabetes and monitoring its development. The circulating miRNAs are protected from RNAse degradation by being contained in exosomal vesicles [113] or present in ribonucleoprotein complexes [114].

It has been shown that circulating miRNAs are involved in signaling between different cell-types during pathophysiological condition, as was recently shown in the apparent cross-talk between insulin-resistant skeletal muscle tissues and beta cells facilitated by miRNAs contained within exosome-like vesicles [115]. More importantly, stable miRNAs have been detected in the blood as a consequence of tissue injuries, as in myocardial injury [116] or drug-induced

liver injury [117]. These findings have important implication in detecting vascular injuries which are hallmark feature of diabetic complications.

In one of the earliest studies, Zampetaki *et al.* have shown that reduced plasma levels of miR-126 in a longitudinal cohort may be predictive of future diabetes mellitus [118]. A follow up study showed a positive association between miR-126 plasma levels and future myocardial infarction [119]. A separate study, T2D by Zhang et al ,also validated miR-126 to be predictive for [120]. In children recently diagnosed with type-1 diabetes, miR-197-3p was identified as a strong predictor of residual beta cell function [121]. In cardiovascular-related complications, screening for cardiac miR-21 and miR-150 in plasma showed that reduced levels of the miRNAs associated with atrial fibrillation [122].

Different cohorts have reported changes in blood miRNA profiles in trying to predict diabetes onset and development, but so far, no clear consensus on which set of miRNAs is truly predictive has been shown [123]. Similarly for diabetes complications such as in acute and chronic heart failure, the clinical use of circulating miRNA as biomarkers is still unrealized due to inconsistent results from published reports [124]. The main limitation of many studies has been the lack of statistical power with regards to the number of samples screened. There have also been many confounding technical issues with global profiling of circulating miRNAs when it comes to preparation of sample (plasma or serum) [125], miRNA isolation protocol [126], choice of platform (qPCR, array-based or RNA-seq) and modes of normalizing the read-outs [127]. As quantitative techniques and analytical procedures mature, it is expected that the diagnostic and prognostic value of circulating miRNAs in monitoring T2D and associated complications will be realized in the future.

Concluding remarks

The recent genetic breakthrough in diabetes has put much focus on the pancreatic beta cell and it has long been known that stimulated insulin secretion is impaired in T2D. The impairment could be due to many factor; ER-stress and mitochondrial dysfunction in the beta cell are among the suggested causes [2]. Here we put forward the hypothesis that defective exocytosis can contribute to reduced insulin secretion and the pathogenesis of the disease. Reduced expression of key components of the exocytotic machinery together with reduced functionality of these components result in reduced exocytosis and thereby lack of first phase and reduced second phase insulin response, observed in patients with T2D. Increased accumulation of FFA inside the beta cells with increased BMI can have multiple negative effects, one being disturbed exocytosis. Here we have described how FFA can reduce exocytosis, but future work needs to investigate the uptake of lipids and cholesterol into the beta cell in more detail. Associated with lipid metabolism are levels of cholesterol in the circulation, which often are increased in obese individuals. One would assume that statin treatment would be beneficial for insulin secretion, but our data show the opposite and suggest negative effects [51]. Indeed, statin treatment has been associated with increased reports of diabetes. However, it should be noted that the positive effects of the statins in reducing blood cholesterol and preventing cardiovascular disease overweigh the negative diabetogenic effects. MiRNAs has emerged as novel regulators of insulin secretion and to be differentially expressed in T2D. These non-coding RNAs regulate

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many of the key proteins in exocytosis in the beta cell [95, 106] and have also been shown to regulate expression of HMG-CoA reductase [128], the key enzyme in cholesterol synthesis. Thus, miRNAs are potential pharmacological targets in the treatment of T2D and complications. Moreover, as described above differential expression of certain miRNAs can be measured in the circulation and can thus be used as biomarkers in disease prediction. We still have much to learn and understand regarding how we can utilize this knowledge in the clinic. The future years with rapidly evolving technologies will certainly bring clarity in this area.

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Complience with etichal standards

The procedures used in the in vitro animal experiments presented in Fig 5 were approved by the ethical committee of Lund/Malmö. M255-12. Otherwise there are no ethical issues with human or animal subjects.

Conflict of Interest

The authors have no conflict of interest.

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