Non-coding RNAs in beta cell insulin secretion - emerging players in Type 2 Diabetes pathogenesis

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Non-coding RNAs in beta cell insulin secretion

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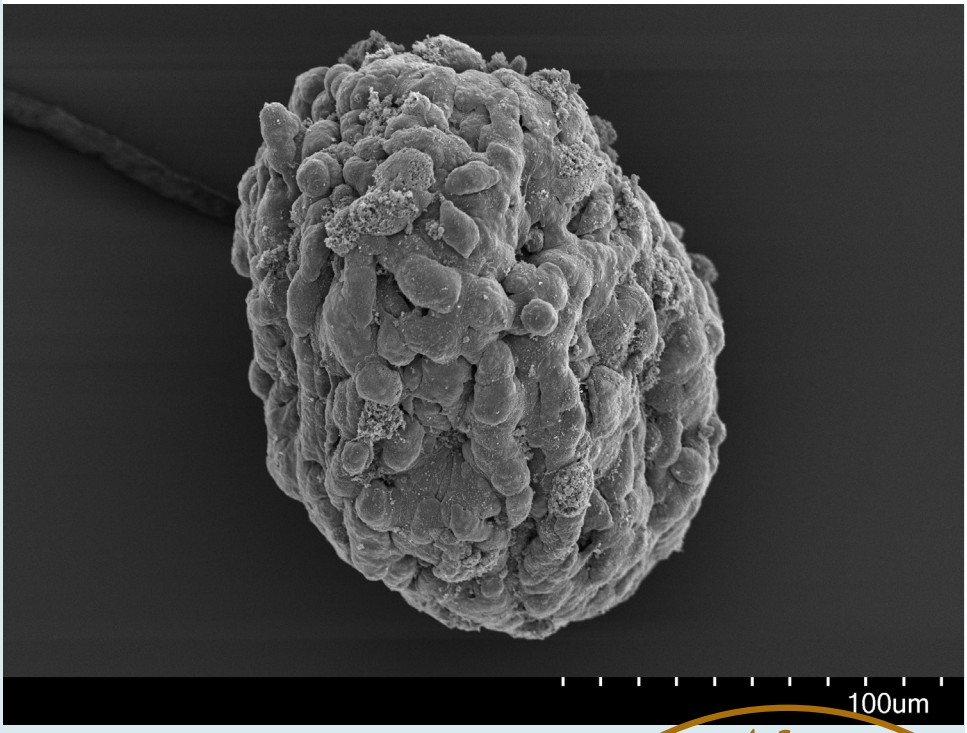

Non-coding RNAs in beta cell insulin secretion

-emerging players in Type 2 Diabetes pathogenesis

Non-coding RNAs

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-emerging players in Type 2 Diabetes pathogenesis

Jones K. Ofori

LUND
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Abstract

Type 2 diabetes (T2D) is a complex polygenic disease influenced by both environmental and genetic factors resulting in impaired insulin release from pancreatic beta cells and insulin resistance in target tissues. The incidence of T2D is escalating and it is projected that over 640 million people will be affected by 2040. Therefore, it is imperative to understand molecular processes that cause T2D to provide novel therapeutic avenues. Impaired insulin secretion is an early sign of T2D prior to insulin resistance. The functional implications of non-coding RNAs (ncRNAs) in glucose –stimulated insulin secretion (GSIS) have been widely recognized but the molecular mechanisms of many dysregulated ncRNAs in these processes has not been fully explored.

Hence, the aim of this thesis was to investigate the cellular mechanism by which non-coding RNAs influence GSIS from pancreatic beta cells. To achieve our aims, we utilized rat INS-1 832/13 cells, human EndoC-βH1 cells, rodent animal models, and pancreatic islets from deceased human donors.

In paper I, we showed elevated levels of miR-130a, miR-130b, and miR-152 expression in islets from hyperglycaemic human donors. Overexpression of miR-152 and miR-130a/b reduced dynamic ATP-levels, GSIS, insulin content, and the secreted proinsulin to insulin ratio in INS-1 832/13 cells. Finally, we confirmed pyruvate dehydrogenase alpha subunit E1 (PDHA1) as a target of miR-152 and knock-down of PDHA1 significantly reduced GSIS, insulin content, and ATP production.

In paper II, we presented data from islets from glucose intolerant donors where expression of miR-335 negativley correlated with insulin secretion index. Overexpression of miR-335 in INS1-832/13 cells reduced GSIS and depolarization-evoked exocytosis, measured as changes in membrane capacitance as well as with TIRF microscopy. The effect was directly on the exocytotic process since the Ca^{2+}-current was not reduced whereas levels of the exocytotic proteins SNAP25, syntaxin-binding protein 1 (STXBP1), and synaptotagmin 11 (SYT11) were reduced.

In paper III, we demonstrated that confluence of cell cultures does not affect expression of miR-375 and its targets *CAV1* and *AIFM1* in rat INS-1 832/13 and human EndoC-βH1cells. This was also true for other miRNAs such as miR-200a,miR-152 and miR-130a whereas expression of miR-212 and miR-132 was to some extent influenced by cell density in INS-1 832/13 cells. In general we showed that miRNAs are not influenced by cell confluence at densities close to 100%.

In paper IV, we showed that treatment of human beta cells with the glucocortocoid dexamethasone reduced expression of the lncRNA GAS5 and impaired GSIS. The negative effect of glucocortocoids on insulin secretion was confirmed *in vivo* from patient material. GAS5 knock-down in EndoC-βH1cells significantly reduced insulin secretion, increased apoptosis and perturbed expression of genes important in beta cell function and glucocorticoid signaling. Overexpression of GAS5 using GAS5 HREM was able to rescue the negative effect of dexamethasone on GSIS. Under hyperglycemic condition, expression of GAS5 was elevated, which partially explained increased expression of GAS5 in the islets from GK rats and T2D individuals.

In conclusion, non-coding RNAs play signifcant role in regulating pancreatic beta cell insulin secretion through effects on metabolism and exocytosis. Understanding their mechanism of action in beta cells will pave way for the development of RNA-based therapeutics to treat T2D.

Key words: T2D, insulin secretion, exocytosis, ncRNAs, GAS5, miR-152, miR-335, miR-130a/b, miR-375, STXBP1, SNAP25, SYT13, PDHA1, PDX1, glucocortiocoid, beta cell function, glucose metabolism

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To

My wonderful and lovely family

... anyone who needs wisdom should ask God, whose very nature is to give to everyone without a second thought, without keeping score. Wisdom will certainly be given to those who ask. James 1:5 (CEM)

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Paper I

Elevated miR-130a/miR130b/miR-152 expression reduces intracellular ATP levels in the pancreatic beta.

Ofori JK, Salunkhe VA, Bagge A, Vishnu N, Nagao M, Mulder H, Wollheim CB, Eliasson L, and Esguerra JL. Scientific Reports. 2017 Mar 23; 7:44986. doi: 10.1038/srep44986

Paper II

MiR-335 overexpression impairs insulin secretion through defective priming of insulin vesicles.

Salunkhe VA, **Ofori JK**, Gandasi NR, Salö SA, Hansson S, Andersson ME, Wendt A, Barg S, Esguerra JL and Eliasson L. *In press Physiological Reports*

Paper III

Confluence does not affect the expression of miR-375 and its direct targets in rat and human insulin-secreting cell lines.

Ofori JK*, Malm HA*, Mollet IG, Eliasson L, and Esguerra JL. PeerJ. 2017 Jun 28 http://dx.doi.org/10.7717/peerj.3503

*Equal contribution

Paper IV

Glucocorticoid induces human beta cell dysfunction and involves GAS5 lincRNA

Ofori JK, Nagao M, Shuto Y, Fadista J, Sugihara H, Groop L, Esguerra JL and Eliasson L. *Manuscript*

Papers not included in this thesis

1. Whole-Genome Bisulfite Sequencing of Human Pancreatic Islets Reveals Novel Differentially Methylated Regions in Type 2 Diabetes Pathogenesis.

   Volkov P, Bacos K, **Ofori JK**, Esguerra JL, Eliasson L, Rönn T, Ling C. Diabetes. 2017 Apr;66(4):1074-1085. doi: 10.2337/db16-0996

2. Dual Effect of Rosuvastatin on Glucose Homeostasis Through Improved Insulin Sensitivity and Reduced Insulin Secretion.

   Salunkhe VA, Mollet IG, **Ofori JK**, Malm HA, Esguerra JL, Reinbothe TM, Stenkula KG, Wendt A, Eliasson L, Vikman J. EBioMedicine. 2016 Aug;10:185-94. doi: 10.1016/j.ebiom.2016.07.007

3. Modulation of microRNA-375 expression alters voltage-gated Na(+) channel properties and exocytosis in insulin-secreting cells.

   Salunkhe VA, Esguerra JL, **Ofori JK**, Mollet IG, Braun M, Stoffel M, Wendt A, Eliasson L. Acta Physiol (Oxf). 2015 Apr;213(4):882-92. doi: 10.1111/apha.12460

4. Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism.

   Fadista J, Vikman P, Laakso EO, Mollet IG, Esguerra JL, Taneera J, Storm P, Osmark P, Ladenvall C, Prasad RB, Hansson KB, Finotello F, Uvebrant K**, Ofori JK**, Di Camillo B, Krus U, Cilio CM, Hansson O, Eliasson L, Rosengren AH, Renström E, Wollheim CB, Groop L. Proc Natl Acad Sci U S A. 2014 Sep 23;111(38):13924-9. doi: 10.1073/pnas.1402665111.

Abbreviations

Ago2	Agonaute-2
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
βlinc1	β-cell long intergenic noncoding RNA 1
cDNA	Complementary DNA
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
GAS5	Growth Arrest-Specific 5
GCK	Glucokinase
GCs	Glucocorticoids
GK-rat	Goto-Kakizaki rat
GLUT	Glucose transporter
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GTP	Guanosine triphosphate
HREM	Hormone response element mimic
IGF	Insulin growth factor
IRS	Insulin receptor substrate
KD	Knockdown
Kir6.2	Inward rectifier potassium channel
LBD	Ligand binding domain
LDHA	Lactate dehydrogenase A

LNA	Locked nucleic acids
lncRNA	long non-coding RNA
Meg3	Maternal expressed gene 3
MCT1	Monocarboxylate Transporter 1
miRNA	MicroRNA
MODY	Maturity-onset diabetes of the young
Mtpn	Myotrophin
NADPH	Nicotinamide adenine dinucleotide phosphate
ncRNA	Non-coding RNA
ND	Neonatal diabetes
NeuroD1	Neurogenic differentiation 1
NKX2-2	NK2 homeobox 2
NKX6-1	NK6 homeobox 1
PAX6	Paired box gene 6
PDX1	Pancreatic and duodenal homeobox-1
PDHA1	Pyruvate dehydrogenase alpha subunit E1
PEPCK	Phosphoenylpyruvate carboxykinase
PIK3R1	Phosphatidylinositol 3-kinases regulatory 1
PI3-K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PVDF	Polyvinylidene difluoride
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rRNAs	Ribosomal RNAs
RT-qPCR	Reverse transcription Real-time quantitative PCR
SGK1	Serum- and glucocorticoid-inducible kinase 1

siRNA	Small interfering RNA
SNAP-25	Synaptosomal-associated protein-25
SNARE	Soluble NSF attachment protein receptor
snoRNAs	Small nucleolar RNAs
SNCA	Synuclein-a
Stxbp1	Syntaxin-binding protein 1
SUR	Sulfonylurea receptor
SYT	Synaptotagmin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCA	Tricarboxylic acid cycle
TUG1	Taurine upregulated gene 1
VAMP	Vesicle-associated membrane proteins

NOTE:

Gene and protein symbols were styled according to HUGO and MGI nomenclature guidelines. For instance,

Human: *PDHA1* (Gene Symbol), PDHA1 (Protein Symbol)

Rat and Mouse: *Pdha1* (Gene Symbol), PDHA1 (Protein Symbol)

Popular science summary

Non-coding RNAs are a class of genetic material that was previously considered not important. The reasons are that they do not produce protein. Recently, non-coding RNAs were shown to play pivotal roles in regulating gene activity that eventually affect the function of a protein.

Non-coding RNAs influence and control many processes in the cell, thus have significant impact on diseases. In type 2 diabetes, where insulin producing beta cells are not able to produce enough insulin to control the blood sugar level, non-coding RNAs have been shown to be involved in regulating the function of beta cells. Beta-cells are situated in the islets of Langerhans within the pancreas and normally these cells release enough insulin upon increase in the blood glucose concentration (e.g. after a meal) to lower blood glucose to normal levels.

In this thesis I have investigated a certain type of small non-coding RNAs known as "microRNAs". We discovered that three specific microRNAs (miR-130a, miR-130b and miR-152) are highly expressed in islet of Langerhans of Type 2 diabetes donors. These three miRNAs affect proteins important for beta cell function. Some of these proteins are involved in regulating the energy (in the form of ATP) produced by the beta cell and needed to make and secrete insulin. When we artificially increased these microRNAs in healthy beta cells, the 'energy state' decreased and the ability to release insulin was reduced.

Secondly, we investigated another microRNA called miR-335. This microRNA affected the expression of proteins involved in the machinery responsible for releasing stored insulin in beta cells; a process called 'exocytosis. These proteins coordinate the processes that ensure the beta cells release their content when needed. Upregulation of this miRNA reduced insulin granule exocytosis and insulin secretion.

The third study in the thesis concerned an important technical aspect. In science on microRNAs we often utilize cell-lines in cell culture. In the cancer field, microRNA expression can change with the confluence of cells in cell culture. Here, we have demonstrated that this was not the case in beta cells for the microRNAs investigated.

Cortisone has a major effect on blood sugar, they act through a receptor on the beta cells and we have discovered that the process that cortisone affect insulin release in beta cell is governed by GAS5, which belongs to the group of non-coding RNAs

called long non-coding RNAs (lncRNAs). This finding highlights the biological process behind steroids influence on beta cells in regulating high blood sugar. Treating beta cells with GAS5, minimize the negative effect of steroids on beta cells, enabling beta cells to release insulin. In principle, controlling the level of GAS5 in beta cells, minimize the negative effect of steroids on the release of insulin.

In conclusion, non-coding RNAs regulate important biological processes in beta cells necessary for insulin release. Understanding the way that non-coding RNAs works in beta cells can help in the future use to diagnose diabetes and furthermore, pave a way to develop RNA-based drugs to treat diabetes patients or prevent the development of the disease.

Populärvetenskaplig Sammanfattning

Icke-kodande RNA är en grupp av genetiskt material som tidigare ansågs oviktiga, eftersom de inte medverkar vid tillverkning av protein. Det har dock på senare tid framkommit att icke-kodande RNA har en viktig roll vid reglering av genuttryck, som i slutändan påverkar uttryck och funktion av olika protein.

Icke-kodande RNA påverkar och kontrollerar flera processer i cellen och det har framkommit att de på detta vis kan ha betydelse vid uppkomst av olika sjukdomar. Typ-2 diabetes är en sjukdom som beror på insulin resistens i muskel och fettväv, samt minskad insulinsekretion från s.k. beta-celler. Beta-celler finns i cellklumpar utspridda i bukspottkörteln som kallas Langerhanska öar. Insulin är det hormon i kroppen som normalt medverkar till att sänka blodglukosnivån i kroppen. Forskning har visat på att icke-kodande RNA medverkar till reglering av beta-celler, och det finns indikationer på att uttrycket av icke-kodande RNA är förändrat i beta-celler hos individer med typ-2 diabetes.

I denna avhandling har jag främst undersökt en viss typ av icke-kodande RNA som kallas ”mikroRNA”. Vi upptäckte att tre specifika mikroRNA (miR-130a, miR-130b och miR-152) är högt uttrycka i de Langerhanska öarna från donatorer med typ-2 diabetes. De tre mikroRNA visade sig vara betydelsefulla för beta-cellens funktion genom att nedreglera uttrycket av specifika proteiner. Vissa av dessa proteiner visade sig viktiga för produktion av energi i form av ATP inne i beta-cellen. ATP är viktigt för både produktion och sekretion av insulin. När vi artificiellt överuttryckte de tre mikroRNA i friska beta-celler minskade ATP och därmed sekretionen av insulin.

I det andra arbetet i denna avhandling har vi undersökt ett annat mikroRNA, miR-335. Detta mikroRNA påverkade uttrycket av protein som reglerar hur insulin tar sig ut ur cellen via en process som kallas ”exocytos”. De tre undersöka proteinerna tillhör en grupp av protein som medverkar till att koordinera så att beta-cellen frisätter insulin när det behövs. Vi fann att överuttryck av miR-335 i insulin-producerade celler ledde till minskat uttryck av dessa tre protein, minskad exocytos och minskad insulinsekretion.

Det tredje arbetet i denna avhandling är av teknisk natur. Vid forskning som berör mikroRNA används ofta cell-linjer i cellkultur. Inom cancerforskning har det visat

sig att uttrycket av vissa mikroRNA påverkas av hur tätt cellerna odlas. Här kunde vi visa att detta inte var fallet för de mikroRNA som vi har studerat.

Kortison och andra sk glukokortikoider verkar blodglukoshöjande och de har en negativ effekt på insulinsekretion. Jag har i det sista arbetet i denna avhandling undersökt hur GAS5, som är ett annan typ av icke-kodande RNA och tillhör gruppen av långa icke-kodande RNA (lncRNA), interfererar med effekten av glukokortikoider på insulinsekretion från beta-cellen. Dessa fynd visar på bakomliggande orsaker till hur steroider påverkar insulinsekretion. Behandling av beta-celler med GAS5, minimerade de negativa effekterna av steroider, så att beta-cellen kunder frisätta tillräckligt med insulin.

Sammanfattningsvis, min avhandling visar på flera exempel som visar att icke-kodande RNA reglerar essentiella processer som medverkar till optimal insulinsekretion. Kunskap om hur icke-kodande RNA fungerar möjliggör för framtida design av verktyg för diagnos och för utveckling av RNA-baserade mediciner för behandling eller prevention av diabetes.

Introduction

Non-coding RNAs

Non-coding RNAs (ncRNAs) are a group of RNAs with important cellular function, even though they do not encode or produce functional proteins. Their role is to modulate or regulate gene expression (Esteller 2011, Eliasson and Esguerra 2014). Over the years, a number of ncRNAs have been identified and these include ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) that play a role in mRNA translation (Schoenmakers, Carlson et al. 2016). Also, small nucleolar RNAs (snoRNAs), which are involved in chemical modification of other RNAs (Huang, Shi et al. 2017), and small nuclear RNAs (snRNAs) that take part in splicing (Kondo, Oubridge et al. 2015) belong to the group of ncRNAs. Among ncRNAs you also find PIWI-interacting RNAs (piRNAs) that protect the genome by repressing transposons (Ishizu, Siomi et al. 2012, Gainetdinov, Skvortsova et al. 2017) and microRNAs (miRNAs) that are involved in gene silencing (Cech 2012). Finally, long non-coding RNAs (lncRNAs), which are longer transcripts involved in a wide range of cellular mechanism (Wang and Chang 2011), are within the group of ncRNAs.

MicroRNAs

MicroRNAs (MiRNAs) are short (≈21-23 nucleotides), small non-coding RNAs that are located within introns of protein-coding genes or intergenic regions (Ozsolak, Poling et al. 2008). They can be transcribed independently or dependently through the promoter of the host gene by RNA polymerase II (Zhou, Ruan et al. 2007).

MiRNAs are processed by two main enzymes, Drosha-DGCR8 and Dicer respectively. This process is known as the canonical pathway and is described in Figure 1, where primary miRNA (pri-miRNA) is cleaved in the nucleus by Drosha-DGCR8 to form precursor miRNA (pre-miRNA). The pre-miRNA is then exported into the cytoplasm by Exportin-5 and cleaved by Dicer to form the mature miRNA (Bartel 2009). Recently, subclasses of miRNAs have been identified that are

independent of either Drosha-DGCR8 (called mitrons) or Dicer (called agotrons). These act in similar function as miRNAs generated via the canonical miRNA pathway (Hansen, Veno et al. 2016). Together with Argonaute (Ago2) proteins, the functional strand of mature miRNA is loaded into the RNA-induced silencing complex (RISC) to direct specific binding to the 3'-untranslated region (3'-UTR) of its target mRNA. The binding is largely determined by the so-called "seed sequence" comprised of 6-7 nt in the 5'end of the miRNA. This results in either mRNA translational repression, cleavage or deadenylation (Winter, Jung et al. 2009, Hansen, Veno et al. 2016, Dalgaard and Eliasson 2017). Due to the short recognition sequence, a single miRNA can target several mRNAs, while one mRNA may be targeted by multiple miRNAs.

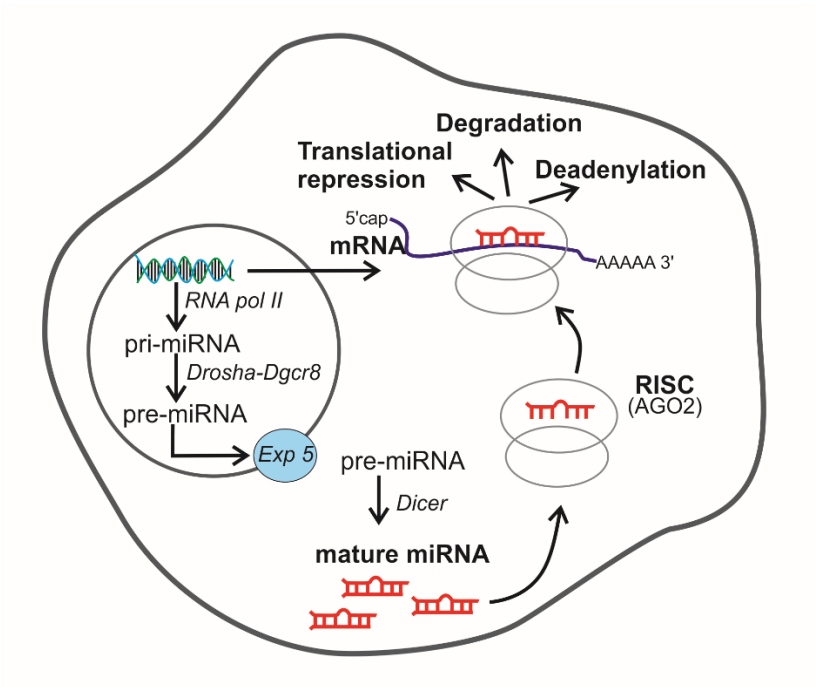

Figure 1: Biogenesis of miRNAs. MiRNAs are processed by two main enzymes Drosha-DGCR8 and Dicer.The mature miRNA is incorporated into the RNA -induced silencing complex (RISC) containg Ago2 protein, where the seed sequence of miRNA base pair with the target mRNA for tranalational repression, deadenylation or degardation.

Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are RNA transcripts that are arbitrarily defined as non-protein coding transcripts greater than 200 nucleotides long. Generally,

lncRNAs are poorly conserved compared to miRNAs, which are highly conserved. Mechanistically, lncRNAs may act within signaling pathways via gene regulation, or as decoys by titrating transcription factors and other proteins away from chromatin (Wang and Chang 2011). Other possibilities is that the lncRNAs work as guides by recruiting chromatin-modifying enzymes to target genes or as scaffolds by bringing together multiple proteins to form complexes (Wang and Chang 2011) (Figure 2) . Sequences of lncRNAs are poorly conserved from one species to another (Derrien, Johnson et al. 2012) and this can have great impact in specifying species-specific differences, for instance in islet cell development and function (Fadista, Vikman et al. 2014).

LncRNAs may be classified as enhancer-derived lncRNAs (Lam, Li et al. 2014), intronic lncRNAs, long intergenic non-coding RNAs (lincRNAs), divergent lncRNAs and antisense lncRNAs in accordance to their genomic location (Knoll, Lodish et al. 2015, Mirza, Kaur et al. 2017). LncRNAs are mostly transcribed by RNA polymerase II and spliced to form RNA transcripts that can be numerous kilobases in length with multiple exons (Guttman, Amit et al. 2009, Knoll, Lodish et al. 2015).

In terms of cellular location, most lncRNAs are located in the nucleus where they modify chromatin structure, and therefore affect gene transcription. Interestingly, there are also other lncRNAs found in the cytosol that influence stability of target mRNA and affect their translation (Knoll, Lodish et al. 2015).

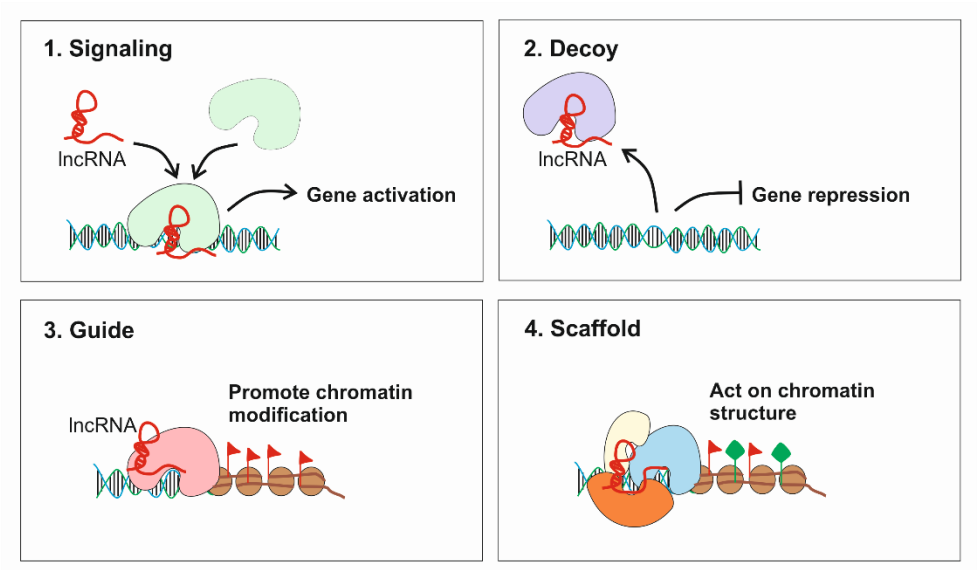

Figure 2: Molecular mechanism of lncRNA. LncRNAs can act as a 1) signal, 2) decoy, 3) guide or 4) a scaffold to combine two or more proteins.

Non-coding RNAs and Diabetes

The traditional classification of diabetes into type 1 diabetes (T1D) and type-2 diabetes (T2D) is typically based on the presence (T1D) or absence (T2D) of autoantibodies against beta cell autoantigens and age of diagnosis (earlier in T1D and late in T2D) (Ahlqvist, Storm et al. 2017). Another subclass which is phenotypically similar to T2D but becomes more T1D-like as the disease progresses is Latent Autoimmune Diabetes in Adults (LADA) and is defined by the presence of autoantibodies against glutamate decarboxylase (GADA), (Tuomi, Groop et al. 1993). In addition, there are monogenic forms such as maturity-onset diabetes of the young (MODY) and neonatal diabetes (ND) (Hattersley and Patel 2017).

Recently, the classification of diabetes has been refined based on five variables: GAD-antibodies, BMI, HbA1c, Homeostasis Model Assessment 2 estimates of beta-cell function (HOMA2-B) and insulin resistance (HOMA2-IR) by colleagues at Lund University Diabetes Centre. Interestingly, they cluster ≈15,000 newly diagnosed diabetic patients from Sweden into five subgroups namely: Severe Autoimmune Diabetes (SAID), Severe Insulin Deficient Diabetes (SIDD), Severe Insulin Resistant Diabetes (SIRD), Mild Obese Diabetes (MOD) and Mild Age-Related Diabetes (MARD). This new stratification will help tailor treatment to individual patients in the different groups, paving a way for precision medicine in diabetes (Ahlqvist, Storm et al. 2017).

For the purpose of this thesis, I will use the traditional classification of diabetes. Over the years, many non-coding RNAs have been implicated in the pathophysiology and pathogenesis of both type 1 diabetes (T1D) and type-2 diabetes (T2D).

T1D is described as autoimmune disease where insulin–producing pancreatic beta cells are selectively destroyed by the immune system. T1D is characterized by islet inflammation (insulitis), eventually leads to lack of beta cells and lifelong insulin treatment of patients (Mathis, Vence et al. 2001, Storling, Overgaard et al. 2013). Interestingly, it has been discovered, in a cohort of 123 children with new-onset T1D, that it is possible to predict residual beta cell function one year after diagnosis through measurements of six circulating miRNAs (hsa-miR-375, hsa-miR-146a-5p, hsa-miR-194-5p, hsa-miR-197-3p, hsa-miR-301a-3p and hsa-miR-24-3p) in the plasma at 3 months after diagnosis (Samandari, Mirza et al. 2017). These miRNAs have potential to be used as biomarkers in T1D. Also, miRNAs have been shown to play important roles in development, activation, and response to antigens in immune cells (Ventriglia, Nigi et al. 2015). For instance, pancreatic beta cells exposed to proinflammatory cytokines such as IL-1β and TNF-α increased the expression of

miR-34a, miR-146a, and miR-21. Blocking these miRNAs with anti-miRs protected MIN6 cells from apoptosis and reduction in glucose-stimulated insulin secretion (GSIS) during IL-1β exposure. Furthermore, over-expression of miR-21 and miR-34a impaired GSIS (Roggli, Britan et al. 2010).

During the progression of insulitis in pancreatic islets from prediabetic NOD mice at different disease stages (4, 8 and 13 weeks of age), the miR-29 family of miRNAs (miR- 29a/b/c) have been shown to increase in expression. Overexpression of miR-29a/b/c in MIN6 cells and mouse islets led to impaired GSIS and increased apoptosis (Roggli, Gattesco et al. 2012). Treatment of human islets with proinflammatory cytokines (IL-1β and IFN-γ) for 48 hours downregulated miR-23a and miR-23b expression. Inhibition of these miRNAs in Human EndoC βH1 cells increased apoptosis by increasing the expression of key proapoptotic Bcl-2 protein family members. These findings give insight into the regulation of cytokine-induced apoptosis observed in early T1D via miRNAs (Grieco, Sebastiani et al. 2017).

T2D is a complex polygenic disease characterized by defective insulin secretion and insulin resistance (Polonsky, Sturis et al. 1996, Del Guerra, Lupi et al. 2005, Prasad and Groop 2015).
A number of miRNAs have been shown to regulate many proteins involved in the insulin-signaling pathway related to insulin resistance. For instance, miR-144 has been confirmed to target insulin receptor substrate 1 (IRS1) and increased circulating levels of miR-144 correlates with decreased expression of IRS1 at both mRNA and protein levels in T2D subjects (Karolina, Armugam et al. 2011, Chakraborty, Doss et al. 2014). The role of insulin growth factors (IGF-1 and IGF-2) in insulin resistance is well established and elevated levels IGF-1 was linked to higher risk of T2D (Yakar, Liu et al. 2001, Sesti, Sciacqua et al. 2005). In adipose tissues, miR-320 regulates IGF-1(Ling, Ou et al. 2009). Also in skeletal muscle, miR-128a regulated the expression of insulin signaling proteins including the insulin receptor (INSR), IRS1, and phosphatidylinositol 3-kinases regulatory 1 (PIK3R1) at both mRNA and protein level (Motohashi, Alexander et al. 2013). GLUT4, which belongs to the GLUT protein family is expressed in the insulin-sensitive tissues fat and muscle. It plays a key role in the whole-body glucose homeostasis (Mueckler 1994). In adipocytes, overexpression of miR-93 significantly reduced GLUT4 gene expression and inhibition of miR-93 led to increased expression of the transporter (Chen, Heneidi et al. 2013). Also, elevated levels of miR-199a in plasma of T2D patients led to reduced GLUT4 expression, contributing to insulin resistance in these patients (Chen, Heneidi et al. 2013). Of note, obesity reduces glucose uptake in response to the insulin signal, which is strongly linked to onset of T2D (Goossens 2008). Certain miRNAs such as miR-335, miR-200a, miR-200b and miR-342 have been shown to contribute to the pathophysiology of obesity and obesity related insulin resistance (Nakanishi,

Nakagawa et al. 2009, Fernandez-Valverde, Taft et al. 2011, Oger, Gheeraert et al. 2014, Zhu, Chen et al. 2014).

Genome wide association studies (GWAS) have revealed that gene variants correlated with increased risk of T2D associated with pancreatic islet cells function (Lawlor, Khetan et al. 2017). Thus, further supporting the idea that beta cell dysfunction, specifically impaired insulin secretion, is the main driver of T2D (Ashcroft and Rorsman 2012). It is therefore important to understand molecular regulation of insulin secretion and mechanisms causing the reduced beta cell function in T2D, to pave way for new therapeutic avenues. MiRNAs are suggested to be part in the adjustment of islet function upon development of T2D (Eliasson and Esguerra 2014) and several miRNAs are indeed differentially expressed in islet from human T2D donors as compared to non-diabetic donors (see E.g. (Bolmeson, Esguerra et al. 2011, Kameswaran, Bramswig et al. 2014)). Moreover, differential miRNA expression is measured in islets from several animal models of diabetes including the GK-rat (Esguerra, Bolmeson et al. 2011) and the db/db mouse (Nesca, Guay et al. 2013). Finally, knockdown of the enzyme *Dicer1* specifically in beta cells resulted in decreased beta cell mass and function, eventually leading to hyperglycemia and diabetes (Kalis, Bolmeson et al. 2011).

Non-coding RNA and insulin secretion

Insulin biosynthesis

The insulin gene encodes 110 amino acids known as preproinsulin. This is cleaved by peptidase to form proinsulin, which is translocated from the endoplasmic reticulum (ER) to the Golgi apparatus. Here, the proinsulin enter into immature secretary vesicles where it is cleaved to produce insulin and C-peptide. Both insulin and C-peptide are packaged and stored in secretory granules ready to be released upon demand. The insulin secreted has a molecular weight of 5.1 kDa and is made up of 51 amino acids (Huang and Arvan 1995, Fu, Gilbert et al. 2013). Insulin biosynthesis is tightly regulated at both transcriptional and translational levels. Pancreatic and duodenal homeobox-1 (PDX1) (Miller, Mcgehee et al. 1994), paired box gene 6 (PAX6) (Sander, Neubuser et al. 1997), B-2/Neurogenic differentiation 1 (NeuroD1) (Naya, Stellrecht et al. 1995) , NK6 homeobox 1 (NKX6-1) (Taylor, Liu et al. 2013) and MafA (Kataoka, Han et al. 2002) regulates insulin transcription.

Recent studies have revealed the involvement of miRNAs in the regulation of insulin biosynthesis. For instance, mice overexpressing miR-7a have reduced insulin expression via downregulation of *Pdx1*, *Pax6*, *MafA*, *Neurod1* and *Nkx6-1*

(Latreille, Hausser et al. 2014, Martinez-Sanchez, Rutter et al. 2016). Also, miR-124a was highly expressed in islets from T2D donors and its overexpression in MIN6 cells led to reduced GSIS. Interestingly, inhibition of miR-124a increased the expression of *Neurod1* in MIN6 cells (Sebastiani, Po et al. 2015). In addition, blocking miR-15 in mice led to increased expression of *Nkx2-2* and *NeuroD1* (Joglekar, Parekh et al. 2007, Martinez-Sanchez, Rutter et al. 2016). Moreover, it has been shown that miR-19b decreases insulin expression by targeting NeuroD1, decreasing its mRNA and protein levels (Zhang, Zhang et al. 2011).

Also, lncRNAs has been shown to regulate important beta cell transcription factors. Knock-down of lncRNA PLUTO in human pancreatic islets and EndoC-βH3 cells reduced the mRNA expression of PDX1 by impairing 3D contacts between the PDX1 promoter and its adjacent enhancer cluster (Akerman, Tu et al. 2017). Knock-down of lncRNA Meg3 (Maternal expressed gene 3) in mice impaired insulin synthesis and secretion by decreasing the expression of PDX1 and MafA (You, Wang et al. 2016). Deletion of lncRNA βlinc1 (β-cell long intergenic noncoding RNA 1) in mice or knock-down of βlinc1 in both MIN6 cells and EndoC βH1 cells caused decreased expression in islet key transcription factors such as *Nkx2.2*, *Mafb* and *Pax6* (Arnes, Akerman et al. 2016, Mirza, Kaur et al. 2017). Also, lncRNA TUG1 (taurine upregulated gene 1) knock-down in mouse beta cells reduced both insulin content and secretion, and decreased *Pdx1*, *NeuroD1* and *MafA* expressions in the process (Yin, Zhang et al. 2015).

Beta cell metabolism

Beta cells respond to nutrients such as glucose, fatty acids, and amino acids but so far glucose has proven to be the main stimuli for insulin secretion release in many animal species (Fu, Gilbert et al. 2013). Glucose enters beta cells via insulin-independent glucose transporters. Upon entering, it is converted to glucose-6-phosphate by the rate-limiting enzyme glucokinase (GCK), a subtype of hexokinase. Through a number of enzymatic reactions, pyruvate is produced as the end product of glycolysis. Finally, via the tricarboxylic acid cycle (TCA), pyruvate is oxidized by mitochondria in the beta cells to generate ATP (Newgard and Mcgarry 1995, Schuit, DeVos et al. 1997). Of note, pyruvate can also be converted to lactate by lactate dehydrogenase (LDH) in other cell types, but this enzyme is very lowly expressed in beta cells. This makes pyruvate oxidization via the TCA cycle the major pathway of glucose metabolism in beta cells (Alcazar, Tiedge et al. 2000).

In beta cells, tight coupling between glucose and mitochondrial metabolism is essential for robust release of insulin (Malmgren, Nicholls et al. 2009) and multi-enzyme complex pyruvate dehydrogenase (PDC) links the glycolytic pathway to

TCA cycle by converting pyruvate to acetyl-CoA and CO_2. The *Pdha1* gene codes for the E1 alpha subunit of PDC (Cullingford, Clark et al. 1994). Deletion of *Pdha1* in beta cells impairs GSIS significantly in mice (Srinivasan, Choi et al. 2010).

Apart from ATP, being the main effector of mitochondrial metabolism on insulin secretion, other products such as glutamate, NADPH and malonyl-CoA can amplify insulin secretion (Corkey, Glennon et al. 1989, Maechler and Wollheim 1999, Lu, Mulder et al. 2002).

Finally, glucose needs to be sufficiently metabolized to generate ATP in beta cells. This is partly arranged for through low levels of monocarboxylate transporter 1 (MCT1, *SLC16A1*) and lactate dehydrogenase A (LDHA). MCT catalyzes the rapid transport across the plasma membrane of many monocarboxylates (such as lactate and pyruvate). LDHA converts pyruvate to lactate. MiRNAs have been suggested to be involved in keeping expression of these genes low. Tamoxifen-induced knockdown of the enzyme *Dicer* increased the expression of *Slc16a* and other beta cell disallowed genes (Martinez-Sanchez, Nguyen-Tu et al. 2015). Interestingly, the in beta cells highly expressed miR-29a and miR-29b target *Mct1*, contributing to specific silencing of this gene (Pullen, Xavier et al. 2011).

Beta cell ion-channels in the stimulus-secretion coupling

The sequence of events from glucose sensing to secretion of insulin is termed "stimulus-secretion coupling". Uptake of glucose by beta cells and glucose metabolism lead to the generation of ATP, closure of ATP-sensitive potassium (K_{ATP}) channels, membrane depolarization, opening of voltage-dependent Ca^{2+} channels, calcium influx and finally exocytosis of insulin from secretory granules. The above processes are indispensable for insulin release and defects in any of the ion channels or proteins involved may contribute to the development of T2D (Ashcroft and Rorsman 2012, Esguerra, Mollet et al. 2014) .

Exocytosis of insulin from secretory granules is directly activated by influx of Ca^{2+} via voltage-dependent Ca^{2+} channels (Ammala, Ashcroft et al. 1993). The main Ca^{2+} channels involved are L-type, P/Q-type and R-type Ca^{2+} channels (Schulla, Renstrom et al. 2003, Jing, Li et al. 2005, Braun, Rantracheya et al. 2008). The opening of voltage-dependent Ca^{2+} channels are triggered by depolarization in the membrane potential initiated by glucose-induced closure of K_{ATP} channels. In human beta cells the depolarization is dependent on T-type Ca^{2+} channels and voltage-gated Na^{+} channels. Inhibition of Na^{+} channels with tetrodotoxin (TTX) in human islets significantly reduces GSIS (Braun, Rantracheya et al. 2008).

Components within the stimulus-secretion pathway are regulated by miRNAs. *Abcc8* and *Kcnj11* genes coding for two subunits of K_{ATP} channel, *Sur1* (sulfonylurea receptor 1) and *Kir6.2* (inward rectifier potassium channel member 6.2), respectively are targeted by miR-124a2 in MIN6 cells. Over-expression of miR-124a2 in MIN6 cells decreased the expression of these genes. (Baroukh, Ravier et al. 2007). We have reported that miR-375 regulate the expression of voltage dependent Na^+ channel subunits, and knockdown of miR-375 shifted the inactivation properties of the Na^+ channel in a species-specific manner. Overexpression of miR-375 resulted in the specific reduction of the Na^+ channel subunits SCN3A and SCN3B expression in INS-1 832/13 cells (Salunkhe, Esguerra et al. 2015).

Exocytosis in beta cells

The process by which insulin secretory granules fuse with the plasma membrane to release their content is known as exocytosis and involve a sequence of events namely; 1) Mobilization or translocation of insulin granules to the plasma membrane, 2) Docking of the granules at the plasma membrane, 3) Priming of the granules and finally, 4). The actual fusion with the plasma membrane (Eliasson, Abdulkader et al. 2008).

The molecular machinery of exocytosis in beta cells is highly coordinated involving soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex (SNARE-complex), which forms a pivotal machinery important for all intracellular membrane fusion events. The SNARE proteins play an essential role in insulin granule membrane fusion and the formed complex bring the vesicle membrane in close contact with the plasma membrane (Marshall, Lund et al. 2017). The SNARE proteins facilitate sorting and targeting of vesicles to different locations of the plasma membrane. The SNARE-proteins include the plasma membrane proteins syntaxin and SNAP25 (synaptosomal-associated protein-25). These interact with the vesicle protein synaptobrevin, (also called vesicle-associated membrane proteins [VAMP]) to form the SNARE-complex (Jewell, Oh et al. 2010, Eliasson 2014). The SNARE proteins exist in multiple isoforms: syntaxin 1, 2, 3, or 4; SNAP 25 and -23 and synaptobrevin 1 and 2 (Gerber and Sudhof 2002, Rorsman and Renstrom 2003). Reduction in SNARE protein expression leads to defective exocytosis (Marshall, Lund et al. 2017); STX1A, SNAP25 and VAMP2 is reduced in islets from T2D donors compared to non-diabetic controls (Ostenson, Gaisano et al. 2006, Andersson, Olsson et al. 2012).

Apart from the SNARE proteins, other important protein families involved in exocytosis are the SM proteins and the different isoforms of Synaptotagmins (SYT). The SM proteins includes munc18-1 (also known as syntaxin-binding protein 1,

STXBP1), munc18-2 (also called munc18b), and munc18c. STXBP1 has been shown to play a role in both docking and priming of granules (Toonen and Verhage 2007, Tomas, Meda et al. 2008) The expression of Stxbp1 is decreased in islets of GK rats and T2D donors (Gaisano, Ostenson et al. 2002, Andersson, Olsson et al. 2012). There exist at least 16 isoforms of synaptotagmins. A majority of the synaptotagmins have a Ca^{2+} sensor and therefore act as such in the exocytotic process (Gauthier and Wollheim 2008). E.g. SYT7 knock-down in mice impairs GSIS as a result of defective Ca^{2+} sensing (Gustavsson, Lao et al. 2008). The expression of SYT4, 7, 11 and 13 are reduced in islets from T2D donors and knock-down of SYT4 and 13 in rat INS1-832/13 cells reduced GSIS (Andersson, Olsson et al. 2012).

Several miRNAs have been reported to be involved in the regulation of exocytosis; miR-7a influenced SNARE activity by targeting the expression of synuclein-a (SNCA) protein. This protein promotes SNARE complex assembly. Inhibition of miR-7a in MIN6 cells, increased SNCA protein levels resulting in elevated exocytosis and GSIS (Burre, Sharma et al. 2010, Latreille, Hausser et al. 2014). *Stxbp1* has been reported by Esguerra *et al* to be a direct target of miR-335 in rat INS-1 832/13 cells (Esguerra, Bolmeson et al. 2011). Also, overexpression of miR-375 in mouse beta cells reduced exocytosis by targeting myotrophin (*Mtpn)* (Poy, Eliasson et al. 2004). In another report, overexpression of miR-29a in INS-1E beta cells decreased mRNA and protein level of Stx1a and impaired GSIS (Bagge, Clausen et al. 2012, Bagge, Dahmcke et al. 2013). Furthermore, overexpression of miR-34a in MIN6B1 cells decreased exocytosis via VAMP2 (Lovis, Roggli et al. 2008).

Glucocorticoids and Diabetes

Glucocorticoids (GCs) are corticosteroid hormones. The most common endogenous GC is cortisol secreted by the adrenal cortex. Exogenous GC includes hydrocortisone, prednisolone, prednisone and dexamethasone (van Raalte, Ouwens et al. 2009, Di Dalmazi, Pagotto et al. 2012).

The effects of GCs on glucose metabolism or homeostasis are complex. Chronic exposure of humans to GC results in Cushing syndrome, obesity, whole-body insulin resistance and beta cell dysfunction (Geer, Shen et al. 2010, Geer, Shen et al. 2012, Geer, Islam et al. 2014). The enzyme phosphoenylpyruvate carboxykinase (PEPCK) regulate glyceroneogenesis in both adipose tissue and liver. In the adipose tissue, PEPCK controls the rate of release of fatty acids into the blood but in the liver, it regulates the production of triacylglycerol from fatty acids and glycerol 3-

phosphate. PEPCK is reciprocally downregulated in adipose tissue and upregulated in liver by glucocorticoids. This results in accumulation of free fatty acids and eventually leads to insulin resistance especially in skeletal muscle (Franckhauser, Antras-Ferry et al. 1995, Cadoudal, Leroyer et al. 2005, Cadoudal, Blouin et al. 2007, Hwang and Weiss 2014). In rat skeletal muscle, dexamethasone directly inhibited insulin signalling by decreasing the expression and phosphorylation of insulin receptor substrate (IRS)-1, phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB)/Akt (Saad, Folli et al. 1993, Long, Barrett et al. 2003, Ruzzin, Wagman et al. 2005, van Raalte, Ouwens et al. 2009)

GCs are used mostly as anti-inflammatory and immunosuppressive drugs to treat many diseases and are implicated in T2D. An unusual increase in blood glucose as results of GC usage is termed as steroid-induced diabetes mellitus (SIDM) (Di Dalmazi, Pagotto et al. 2012, Hwang and Weiss 2014, Suh and Park 2017) and was first reported over 60 years ago (Conn and Fajans 1956). GCs induce hyperglycemia and diabetes. A meta-analysis study on non-diabetic patients undergoing GC treatment suggested that the incidence of developing GC-induced hyperglycemia or diabetes was 32.3% and 18.6%, respectively. (Liu, Zhu et al. 2014) In another study, 64% of the patients undergoing corticosteroid therapy had hyperglycemia (Donihi, Raval et al. 2006). These studies indicate that GC-induced hyperglycemia occurs at a high frequency among patients undergoing GC treatment.

On the cellular level GCs binds to GC receptor (GR). When GCs enter the cell, they activate GR by binding to ligand binding domain (LBD) of the GR in the cytoplasm. This changes the conformation and leads to nuclear translocation of the GR (Beato 1989, Newton 2000). In the nucleus, the GR binds to the GC response elements (GREs) to either activate or repress the transcription of responsive genes via a positive and negative GRE, respectively (Newton 2000, Newton, Leigh et al. 2010). On the other hand, the GR can also bind transcription factors or recruit co-factors, which will lead to either transcriptional activation or repression of target genes (McMaster and Ray 2008, Hapgood, Avenant et al. 2016).

Effect of glucocorticoids on beta cells

Several studies have assessed the effects of GC on beta cells both *in vitro* and *in vivo*. For instance, exposure of INS-1E cells to dexamethasone impairs GSIS and induce apoptosis by lowering *Pdx1* and *Ins* gene expression (Linssen, van Raalte et al. 2011). Also, dexamethasone has been shown to directly inhibit insulin secretion from isolated mouse islets in a concentration- and time-dependent manner by decreasing the efficacy of cytoplasmic Ca^{2+} on the exocytotic process (Lambillotte, Gilon et al. 1997). Treatment of cultured rat islets with dexamethasone impaired GSIS and decreased both mRNA and protein expression of GLUT2. The GC effect

was inhibited by using GR antagonist RU-486 (Gremlich, Roduit et al. 1997). Furthermore, dexamethasone increased the expression of the serum- and GC-inducible kinase 1 (SGK1) in insulin-secreting cells. Upregulation of SGK1 increases the activity of voltage-gated K^+ channels, this in turn leading to decreased Ca^{2+} entry via voltage-gated Ca^{2+} channels and reduced insulin secretion (Ullrich, Berchtold et al. 2005). In both Zucker fatty rats (fa/fa) and ob/ob mice, dexamethasone induces hyperglycemia with a markedly decreased GSIS (Khan, Ostenson et al. 1992, Ogawa, Johnson et al. 1992, Ohneda, Johnson et al. 1993). Moreover, isolated human islets treated with GC for 48 hours resulted in dose-dependent inhibition of GSIS (Lund, Fosby et al. 2008, Miki, Ricordi et al. 2014).

Non-coding RNAs and glucocorticoids

Reports from studies of cancer cells have suggested that Growth Arrest-Specific 5 (GAS5) inhibits GC-mediated induction of several responsive genes during growth arrest. GAS5 is a lncRNA shown to bind to the DNA-binding domain of GR, acting as a decoy GRE (Kino, Hurt et al. 2010). The *GAS5* gene consists of 12 exons hosting small nucleolar RNA (snoRNA) and is encoded at locus 1q25. GAS5 lncRNA, is down-regulated in multiple cancers. It inhibits proliferation and promotes the apoptosis and is currently known as a tumour suppressor (Renganathan, Kresoja et al. 2014, Pickard and Williams 2015). Also, GAS5 suppresses miR-21 expression in breast cancer MCF-7 cells (Zhang, Zhu et al. 2013).

Interestingly, our analysis of RNA sequencing data from human pancreatic islets revealed GAS5 as the most highly expressed lncRNA (Fadista, Vikman et al. 2014). However, the role of GAS5 in pancreatic islet cell function has not been previously investigated.

Aims of this thesis

Impaired insulin secretion is a factor in the progression of T2D, and non-coding RNAs have emerged as important modulators in beta cell compensation upon increased metabolic demand. Measures of miRNA expression in islets from human donors and diabetic animal models have indicated dysregulated expression of non-coding RNAs.

The general objective of this thesis was to identify and elucidate cellular mechanisms affected by specific non-coding RNAs in insulin-secreting beta cells.

Specific aims:

I. Investigate the mechanism by which miR-130a, miR-130b and miR-152 alter insulin secretion in beta cells.

II. Explore the cellular mechanism by which miR-335 affects insulin secretion.

III. Study the effect of beta cell culture density on the expression of miR-375 and other miRNAs.

IV. Examine the influence of the lncRNA GAS5 on insulin secretion and genes potentially regulated by GCs.

Materials and Methods

Cell lines

In this thesis, we used two main beta cell lines namely: rat INS-1 832/13 and human EndoC-βH1. Using cell lines has several advantages in that they are phenotypically stable and provide pure populations of cells. They are easier to maintain, expand for larger studies and easier to manipulate compared to primary cells. Nevertheless, due to their 'engineered nature' to create an artificial system, cell lines are not genotypically or phenotypically identical to primary cells (Kaur and Dufour 2012).

Primary cells on the other hand are limited, exhibit gender differences and have mixed population.

The INS-1 832/13 cell line is one of the most widely used cell lines in studies of biological processes contributing to insulin secretion. They were produced from its parent cell, INS-1 by stable transfections with a plasmid containing human proinsulin gene (Asfari, Janjic et al. 1992, Hohmeier, Mulder et al. 2000). We used INS-1 832/13 cells in Papers I, II and III to study the molecular and cellular function of different miRNAs in beta cells.

Attempts have been made to generate human beta cell lines and this became a reality recently using targeted oncogenesis in human fetal pancreatic tissue. Human fetal pancreases were transduced under the control of the insulin promoter and grafted into mice so they could develop into pancreatic tissues and differentiate to form insulinomas. (Ravassard, Hazhouz et al. 2011). The first generation of this human cell line was called EndoC-βH1. We used human EndoC-βH1 cells in paper II, III and IV.

Primary cells

We used Goto-Kakizaki (GK) rat and human pancreatic islets to confirm our findings from the cell lines. We fully compiled to the ethical permits regarding all experiments involving human and rat pancreatic islets.

The GK rat is a non-obese animal model of T2D produced by selective breeding of glucose-intolerant Wistar rats (Goto, Kakizaki et al. 1976). The GK rat is a commonly used T2D animal model with a well-known phenotype, primarily characterized by impaired GSIS (Portha, Giroix et al. 2012). The defect in GSIS has been attributed to diverse molecular processes regulating insulin release in beta cells such as reduced exocytotic proteins (Nagamatsu, Nakamichi et al. 1999, Gaisano, Ostenson et al. 2002), glucose metabolism (Granhall, Rosengren et al. 2006), and decreased pyruvate dehydrogenase activity in mitochondria (Zhou, Ostenson et al. 1995). We used GK and control Wistar rats in Paper I and Paper IV.

Human islets from deceased donors were obtained from the Human Tissue Lab of EXODIAB/LUDC through the Nordic Network for Islet Transplantation (http://www.nordicislets.org). The donors were grouped based on the glycated haemoglobin (% HbA1c) levels: normal glucose tolerant (NGT) donors (HbA1c < 6%), impaired glucose-tolerant donors (IGT) (6% ≤ HbA1c < 6.5%), and T2D donors (HbA1c ≥ 6.5%). Human islets were used in Paper I, II and IV.

Transfection

We used Lipofectamine RNAiMax reagent to deliver oligonucleotides to either INS-1 832/13 or EndoC βH1 cells. Customized mature miRNAs called PremiR™ miRNA Precursor were used to overexpress a specific miRNA. Knock-down was achieved by using locked nucleic acids (LNA)-based oligonucleotides. LNA is a class of nucleic acids analogue where the furanose ring is chemically 'locked' by introducing a 2'-O,4'-C a methyl bridge. This results in significantly increased melting temperature, better stacking, stability, and enhanced binding to complementary DNA or RNA (Petersen and Wengel 2003, Stenvang, Petri et al. 2012).

We utilized siRNA technology to down-regulate our genes of interest in INS-1 832/13 cells. siRNA are small double strand RNA molecules, typically 20-25 nucleotides long that silence mRNA molecules of complementary nucleotide sequences via RISC. This results in degradation of target homologous mRNA. The biogenesis and function of miRNAs resemble double-stranded RNA-mediated interference (RNAi) activities (Agrawal, Dasaradhi et al. 2003).

For lncRNAs silencing, we used an anti-sense technology called Locked-Nucleic Acid GapmeR (LNA-GapmeR) in which a central DNA stretch of 7-8 nucleotides is flanked at both ends by four stabilizing LNA-modified bases, (LNA-GapmeR). This chimeric LNA-DNA single-stranded molecule can, upon binding to target RNA, activate endogenous RNase H thereby facilitating cleavage of the RNA part

of the RNA/DNA hybrid (Kurreck, Wyszko et al. 2002). This technique has been reported to be more efficient and potent for lncRNAs silencing compared to siRNA mediated gene silencing (Grunweller, Wyszko et al. 2003, Ideue, Hino et al. 2009, Michalik, You et al. 2014).

Reverse transcription Real-time quantitative PCR (RT-qPCR)

Quantitative polymerase chain reaction (qPCR) is the most used technique to measure the expression of genes (mRNA) by detecting a specific DNA sequence and copy number of this sequence relative to a standard. In reverse transcriptase qPCR (RT-qPCR), RNA isolated from a sample is first transformed to cDNA using an enzyme, primers and dNTPs (deoxyribonucleotide triphosphate).

In SYBR Green qPCR the amplification of DNA can be monitored at each cycle of PCR with the help of a fluorescent reporter dye that intercalates with a double stranded DNA. The cycle at which the fluorescent become measureable above the background is called the threshold cycle (CT), therefore using a known amount of standard DNA or internal control, the relative amount of DNA or cDNA in an unknown sample can be calculated from its CT value (Heid, Stevens et al. 1996, Schmittgen and Livak 2008) .

In this thesis, we have mostly used TaqMan assay from Applied Biosystems to relatively quantify the expression (mRNA copy number) of our gene of interest. The TaqMan assay for every gene has a pair of primers and a TaqMan Probe. The TaqMan probe has a fluorescent reporter, FAM™ dye label on the 5' end, and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end (Biosystems 2010). At the start of the qPCR reaction, the signal from the fluorescent dye is inhibited by the NFQ on the 3' end. The fluorescent signal is released and detected at 518 nm only when *Taq* DNA polymerase cleave the reporter dye in the process of synthesizing a new complementary sequence (strand of DNA) using the specific primer (Heid, Stevens et al. 1996). Therefore, increase in fluorescence intensity occurs at each PCR cycle and this is directly proportional to the amount of amplicon produced. The CT value is the measure of relative amount of target product in a PCR cycle and it increases with decreasing amount of template. The advantage of this technique is that two or more target genes can be detected in the same sample (Biosystems 2010). In all experiments, relative expression of genes was normalized to endogenous controls (housekeeping genes) using the ΔΔCt method (Vandesompele, De Preter et al. 2002, Schmittgen and Livak 2008).

Anti-AGO2 RNA immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) is an antibody technique used to detect RNA-protein interactions. An antibody is generated against the protein bound to the RNA complex (RNA binding Protein), and used to pull-down or co-precipitate the protein together with interacting RNA (mRNA or non-coding RNA.). The RNA product can be further identified by sequencing or RT-qPCR (Gagliardi and Matarazzo 2016).

In paper I, we co-purified Argonaute 2 (Ago2)-associated RNAs (miRNAs) in beta cells. In addition to miRNAs, which are expected to associate with Ago2, their target mRNAs should also be recovered from the Ago2 RIP. Based on this knowledge, we could validate *Pdha1* mRNA as a target of miR-152 using an Imprint RNA Immunoprecipitation Kit from Sigma. In short, we overexpressed miR-152 in INS-1 832/13 cells, then the cells were lysed in mild lysis buffer containing protease inhibitor, 1M DTT and ribonuclease inhibitor 40 U/μL. As depicted in Figure 3 we incubated the cell lysate and Protein A magnetic beads coated with either anti-Ago2 or IgG antibody (negative control) overnight at 4 °C. With the help of magnetic beads coated with anti-Ago2 antibody, we pulled down Ago2 protein-RNA binding complex (miR-152 and its associated mRNA targets). Next, we specifically purified the bound-RNA, we performed cDNA and with specific primers for *Pdha* 3'UTR, we determined the enrichment of *Pdha1* 3'UTR using qPCR in Ago2 immunoprecipitation (IP).

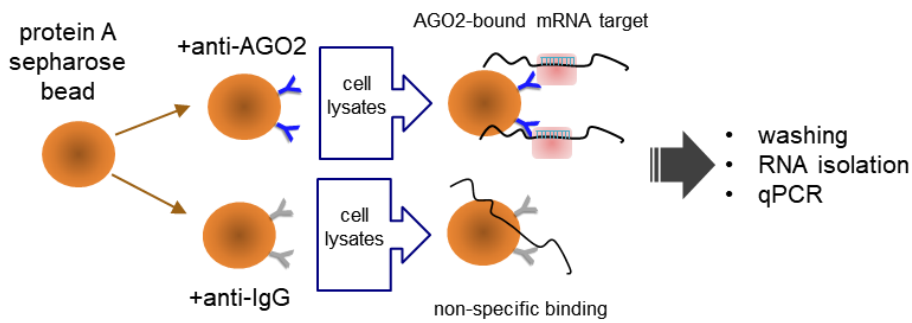

Figure 3: **Workflow of Ago2 RNA immunoprecipitation.** AGO2-bound mRNA target is enriched in the fraction immunoprecipitated by anti-AGO2. The amount of mRNA is determined by qPCR using primers specific to target.

ATP measurement

Adenosine triphosphate (ATP) is key in beta cell metabolism and function. ATP regulate insulin secretion; an increase in intracellular level leads to closure of ATP-dependent K^+-channels (K^+_{ATP}-channels), depolarization of plasma membrane, then opening of voltage-gated Ca^{2+}-channels and exocytosis of insulin granules (Ashcroft and Rorsman 2012, Esguerra, Mollet et al. 2014).

In paper I, we measured total ATP in beta cells using luciferase-based luminescent assay from Bio Thema AB, Sweden. The assay works by monitoring of ATP in the firefly luciferase reaction:

ATP + D-luciferin + O_2 → AMP + PPi (inorganic pyrophosphate) + oxyluciferin + CO_2 + light

A limitation in the above method is that it measures endpoint ATP and therefore accounts for total ATP content without giving any information on metabolic regulation of ATP levels (ATP: ADP ratio) in a cell. We therefore expanded our findings by using PercevalHR technique to monitor the dynamic changes in ATP concentration in beta cells. PercevalHR is a genetically-encoded fluorescence biosensor that detects the changes in ATP:ADP ratio in live single cells. It detects ADP and ATP fluorescence at ≈420 and ≈500 nm, respectively. Hence, the fluorescence signal acquired measure the ATP:ADP ratio rather than changes in fluorescence from only one nucleotide concentration (ATP or ADP) making the system less sensitive to run-down of the signal. (Tantama, Martinez-Francois et al. 2013). We transfected PercevalHR plasmid DNA together with miRNAs or siRNA and with the help of a confocal microscope we could perform live cell measurements of changes in the ATP:ADP ratio in beta cells by measuring the emitted fluorescence.

Western blot

Western blot is the most used technique to detect a specific protein in a biological sample. The workflow of western blot involves treatment of a protein sample with sodium dodecyl sulfate, to unfold and make it negatively charged. The mixture of proteins is then separated according to size by gel electrophoresis. The separated proteins are transferred to a blotting membrane, usually polyvinylidene difluoride (PVDF) or nitrocellulose membrane. The membrane is blocked first to avoid non-specific binding. It is then probed with a primary antibody that binds specifically to the protein of interest. After incubation, the membrane is washed and probed again with a secondary antibody (linked to a reporter enzyme that produce light or colour

for easy detection) that binds to the primary antibody (Renart, Reiser et al. 1979, Towbin, Staehelin et al. 1979, Kurien and Scofield 2015).

In paper I, II and IV, we utilized this method to measure protein expression of potential ncRNA targets.

Insulin secretion measurements

Insulin secretion was performed on rat INS-1 832/13, human EndoC βH1 cells and human islets. In the cell lines experimenst were performed when cells had 100% confluency. Cells were pre-incubated for 2 hours in freshly prepared Secretion Assay Buffer (SAB) supplemented with 2.8 and 1 mM glucose for INS-1832/13 and human EndoC βH1 cells, respectively. Cells were then stimulated for 1 hour with 16.7 mM glucose (INS-1832/13 cells), 20 mM glucose (EndoC βH1 cells) or 50 mM K^+ for 15mins. The latter was used to investigate effects of ATP-independent first phase secretion. For human islets, batches of 5-10 islets were pre-incubated for 30 mins in KREBS buffer (2.5 mM $CaCl_2$, 4.7 mM KCl, 120 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and10 mM HEPES) and stimulated with 16.7 mM glucose in KRB for 1 hour.

We used radioimmunoassay (RIA) and Mercodia insulin ELISA to measure insulin release and insulin content in this thesis. RIA works by this principle, a radioactive ^{125}I-labelled insulin competes with insulin in a sample at a specific site on insulin-specific antibody. ELISA is a plate-based antigen antibody reaction technique used to measure proteins, hormones, antibodies, peptides etc. This principle was invented in 1971 by Peter Perlmann and Eva Engvall at Stockholm University, Sweden (Engvall and Perlmann 1971).

Patch clamp technique

We employed the patch-clamp technique to measure whole-cell ion channel currents and changes in membrane capacitance (to study exocytosis) in single insulin secreting cells in paper II. The patch-clamp technique was invented in 1981 by Hamill and colleagues (Hamill, Marty et al. 1981).

In our study, we used the whole-cell configuration of the patch clamp technique. A high-resistance seal (>1 GΩ, known as ‘‘gigaseal’’) with the plasma membrane was formed using patch pipettes with a tip-size corresponding to 3-6 MΩ when filled with intracellular solution. The plasma membrane enclosed by the pipette was then ruptured to get direct access with the cell interior. Exocytosis was evoked by ten

successive 500-ms depolarizations from -70 mV to 0 mV at 1Hz. Here, exocytosis was measured as a function of changes in cell membrane capacitance (C_m). The technique to measure exocytosis as changes in membrane capacitance using the patch-clamp technique was developed by Neher and Marty in 1982 (Neher and Marty 1982). The relationship between capacitance (C_m) and cell surface area (A) is given by:

$$C_m = \varepsilon * A/d$$

Where ε is a constant (specific membrane capacitance) and *d* is the distance between the two phospholipids layers.

Bioinformatics and Statistical Analysis

We utilized miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/ mirwalk2 /generetsys-self.html) (Dweep and Gretz 2015) and the TargetScan prediction tool (http://www.targetscan.org/) (Agarwal, Bell et al. 2015) to identify putative targets of miRNAs. We used the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 web server (https://david-d.ncifcrf.gov/) (Huang da, Sherman et al. 2009) to determine enriched Gene Ontology categories for genes with unique putative binding sites for certain miRNAs.

In this thesis, significant differences between two groups were determined using Student's t-test or the non-parametric Mann-Whitney test. For multiple groups, one-way ANOVA followed by Dunnett's multiple comparison test was used. Data were presented as mean ± SEM.

Results and discussion

Paper I

Elevated miR-130a/miR130b/miR-152 expression reduces intracellular ATP levels in the pancreatic beta cell

MiR-130a and miR-152 was among 24 miRNAs that our group previously identified to be up-regulated in islets from the GK rat, using a locked nucleic acid (LNA)-based miRNA array profiling approach (Esguerra, Bolmeson et al. 2011). Defective insulin secretion is one of the key characteristics of the GK rat (Portha, Lacraz et al. 2009), and impaired GSIS in pancreatic islets is an early sign of T2D (Ashcroft and Rorsman 2012). We therefore wanted to investigate the contribution of these miRNAs to GSIS, in the context of cellular metabolism.

In this paper, we wanted to answer the following questions:

a) Are there any differences in the expression of miR-130a, miR-130b and miR-152 in pancreatic islets of human T2D donors compared to non-diabetic controls?
b) Do these miRNAs affect GSIS?
c) What are the (putative) targets of miR-130a, miR-130b and miR-152 involved in cellular metabolism of beta cell during GSIS?

Differential islet miRNA expression in T2D donors

We found miR-152, miR-130a and miR-130b to be upregulated in islets from impaired glucose tolerant and T2D donors. Moreover, we identified these miRNAs to be co-expressed even though they are located on different chromosomes (chr11/miR-130a; chr22/miR-130b; chr17/miR-152).

Modulating miRNAs levels affect insulin secretion in INS-1 832/13 cells

To examine whether these miRNAs affect GSIS, we over-expressed (OE) or knocked-down (LNA) miRNAs using miRNA mimics or LNA anti-miRs respectively, in INS-1 832/13 cells. OE152 and OE130a decreased GSIS and insulin content compared to SCR. We also knock-down the micRNAs usign LNA. As expected, LNA152, LNA130a and LNA130b instead increased GSIS and insulin secretion compared to SCR control.

Furthermore, we wanted to know if elevation of miRNAs affects maturation of insulin, therefore we measured the proinsulin to insulin ratio. We observed significant increase in proinsulin to insulin ratio in OE152 cell and OE130a/b cells.

Pdha1 mRNA is a confirmed target of miR-152

We used miRWalk2.0 web-based miRNA-target prediction tool to identify putative targets of miRNAs. We focuse on *Gck* and *Pdha1* genes based on previous findings of decreased *Gck* mRNA expression and pyruvate dehydrogenase activity in the pancreatic islets of GK rats (Zhou, Ostenson et al. 1995, Del Guerra, Lupi et al. 2005, Frese, Bazwinsky et al. 2007).

We observed a reciprocal relationship between the three miRNAs and targets upon one hour incubation of INS-1 832/13 cells at 2.8 mM vs 16.7 mM glucose. At 16.7 mM glucose, decreased expression of the miRNAs resulted in significant increase in GCK and PDHA1. Overexpression of miRNAs resulted in significant decrease in both *Pdha1* mRNA and PDHA1protein level in INS-1 832/13 cells.

Finally, we validated *Pdha1* mRNA as a target of miR-152 by using anti-AGO2 RNA immunoprecipitation (RIP) assay, where we found enrichment of *Pdha1* 3′ UTR fragment in AGO2 RIP compared to the non-specific binding control IgG RIP in response to OE152.

Over-expression of miRNAs decreased ATP in INS-1 832/13 cells

To examine whether upregulated miRNAs have effect on cellular energy metabolism in beta cells we measured ATP content using luciferase-based luminescent assay. Overexpression of miR-130a, miR-130b and miR-152 separately reduced the increased ATP-content, stimulated by 16.7 mM glucose. We expanded our findings by monitoring cytosolic ATP:ADP ratio using genetically-encoded PercevalHR ATP sensor. We observed significant reduction in cytosolic ATP:ADP ratios in cells overexpressing miRNAs at 16.7 mM glucose.

Knock-down of PDHA1 reduced cytosolic ATP levels and GSIS

To confirm that the effect of miRNAs on beta cells is mediated by PDHA1, we knocked-down PDHA1 in INS-1 832/13 cells using siRNA. PDHA1 knock-down resulted in significant reduction in insulin secretion, insulin content, and ATP content. Furthermore, we observed significantly lower levels of cytosolic ATP:ADP ratios during GSIS.

Discussion

In beta cells, the rate of glucose metabolism is proportional to the rate of insulin secretion (Newgard and Mcgarry 1995). GCK, also known as the 'glucose sensor' catalyzes the phosphorylation of glucose to glucose-6-phosphate as the first step of the glycolytic pathway (Schuit, Huypens et al. 2001). Mutations in the *GCK* gene causes MODY. MODY patients have impaired GSIS and need higher glucose levels to evoke GSIS (Byrne, Sturis et al. 1994). These findings highlight the vital role of GCK in normal islet function and regulation of GSIS. Also, we have shown that GCK is reduced in islets from GK rats and T2D donors. Furthermore, overexpression of miR-130a/b and miR-152 significantly reduced GCK in INS-1 832/13 cells.

Metabolic coupling between glucose and mitochondrial metabolism is required for robust insulin secretion in beta cells (Malmgren, Nicholls et al. 2009). The PDH enzyme complex converts pyruvate to acetyl-CoA and CO_2. Thereby this complex links the glycolytic pathway to the TCA cycle. The E1 alpha subunit of this mitochondrial enzyme complex is coded by the *Pdha1* gene. Deletion of the *Pdha1* gene in mice reduced the PDH enzyme activity, insulin content, and impaired GSIS (Srinivasan, Choi et al. 2010). Here, we have confirmed PDHA1 as a target of miR-152. Overexpression of miR-130a/b and miR-152 decreased PDHA1 expression and reduced ATP, insulin secretion, and insulin content. Moreover, knock-down of PDHA1 exhibited the same phenotype as overexpression of these miRNAs.

ATP in central for the insulin secretion process. Elevated mitochondrial metabolism leads to an increase of cytosolic ATP/ADP ratio necessary to trigger insulin exocytosis (Maechler and Wollheim 2000). A mutation in the mitochondrial genome leads to mitochondrial diabetes, a subtype of T2D known as maternally inherited diabetes and deafness (MIDD) (Maassen and Kadowaki 1996). MIDD patients have progressive impairment of insulin release. This illustrate the importance of mitochondrial function and ATP in GSIS (Maechler and Wollheim 2000). Moreover, reduction in mitochondrial ATP production by inhibition of mitochondrial fission blocked GSIS in INS-1E cells (Jhun, Lee et al. 2013).

Insulin maturation and biosynthesis is ATP dependent (Rhodes, Lucas et al. 1987). Also, overexpression of the miRNAs increased the proinsulin to insulin ratio, a sign of defective beta cell insulin granules maturation (Roder, Porte et al. 1998). ATP is required for packaging insulin into secretory granules (Orci, Ravazzola et al. 1987). Furthermore, ATP is also needed for priming of insulin granules (Eliasson, Renstrom et al. 1997).

We have shown that elevated levels of miR-152, miR-130a and miR-130b in beta cells result in decreased PDHA1 and GCK levels contributing to reduction in ATP:ADP ratio in beta cells, eventually leading to reduced insulin content and defective release of insulin from insulin granules as illustrated (Figure 4).

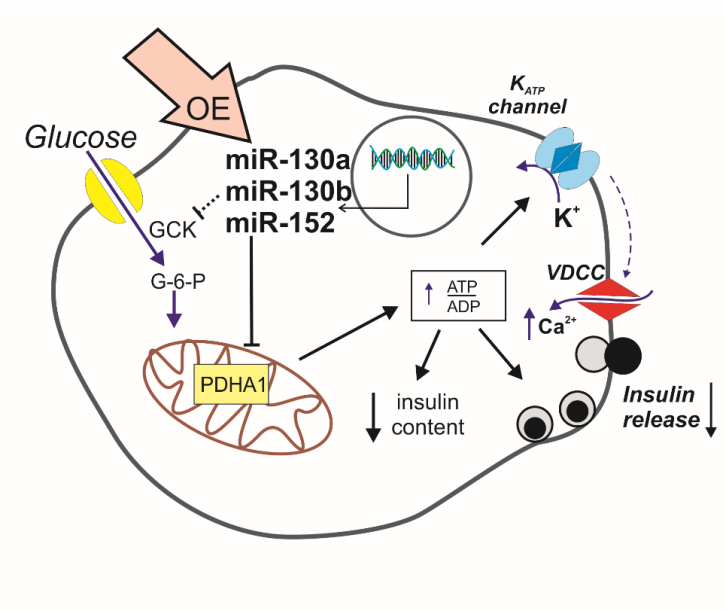

Figure 4: Model figure for Paper 1. Overexpression (OE) of miRNAs decreased the expression of GCK and PDHA1 contributing to reduction in cytosolic ATP:ADP ratio, eventually leading to reduced insulin content and GSIS.

Paper II

MiR-335 overexpression impairs insulin secretion through defective priming of insulin vesicles

As in paper I we utilized data from our previous study in the GK.rat (Esguerra, Bolmeson et al. 2011). Among the 24 miRNAs upregulated we also found miR-335

and validated that the exocytotic gene *Stxbp1* was a direct target of this miRNA (Esguerra, Bolmeson et al. 2011). Interestingly, islets of the GK rat (Gaisano, Ostenson et al. 2002, Zhang, Khan et al. 2002) and human T2D donors have reduced expression of Stxbp1 and other exocytotic genes (Ostenson, Gaisano et al. 2006, Andersson, Olsson et al. 2012). The process of exocytosis of insulin granules and the glucose dependent release of insulin into blood is biphasic. Of note, the first phase insulin secretion is blunted already in pre-diabetic or impaired glucose tolerance (IGT) individuals (Eliasson, Abdulkader et al. 2008, Ashcroft and Rorsman 2012).

Therefore, in this paper, we wanted to know:

a) The specific role of miR-335 during the development of T2D.
b) The effect of miR-335 on insulin secretion and exocytosis.
c) If miR-335 regulates the expression of the validated exocytotic protein, STXBP1 and other putative targets such as SNAP25 and SYT11.

MiR-335 expression negatively correlates with insulin secretion from impaired glucose tolerance donors

The expression of miR-335 negatively correlates with insulin secretion in islets from human islets from impaired glucose tolerant donors, but not in islets from normal glucose tolerant donors. Furthermore, over-expression of miR-335 in human EndoC βH1 cells significantly decreased GSIS.

Decreased expression of STXBP1, SNAP25 and SYT11 in OE335 cells

Using the TargetScan prediction tool (http://www.targetscan.org/), we identified several targets of miR-335 and among them are the exocytotic genes *Snap25*, *Syt11* and *Stxbp1*. We over-expressed miR-335 (OE335) in INS-1 832/13 cells and measured the protein expression of these exocytotic genes using western blot. OE335 cells had significant reduction in the expression of STXPB1, SNAP25 and SYT11.

Reduced exocytosis and GSIS in OE335 cells

In INS-1 832/13 cells, OE335 significantly reduced GSIS and K^+-induced insulin secretion; indicating effects on exocytosis. Using the patch-clamp techinique in combination with capacitance measurements, we examined exocytosis. We

observed significant reduction in membrane capcitance in OE335 cells and an increase in membrane capcitance in LNA335 cells.

We next used TIRF microscopy to image the fluorescence of granular marker neuropeptide-Y (NPY)-mEGFP co-transfected with miR-335 in INS-1 832/13 cells, to validate our findings from the capacitance measuremenst and to investigate granular docking. From the TIRF measurements we observed no difference in number of docked granules between OE335 and SCR cells, but we could confirm a decreased number of exocytotic events in the OE335 cells. Moreover, the release of content from the insulin granules was much faster.

Discussion

The exocytotic proteins SNAP25 and STXBP1 play pivotal roles in Ca^{2+}-triggered exocytosis. Both SNAP25 and STXBP1 are necessary for priming and fusion of insulin granules (Vikman, Ma et al. 2006, Gulyas-Kovacs, de Wit et al. 2007, Vikman, Svensson et al. 2009). Expression of SNAP25 and STXBP1 positively correlated with GSIS in human islets and their expression is reduced in islets from T2D donors (Ostenson, Gaisano et al. 2006, Andersson, Olsson et al. 2012). Although, it is well established that a defective exocytosis machinery can lead to impaired insulin secretion in beta cells (Zhang, Khan et al. 2002), the mechanism by which miRNAs are involved in this process has not been clearly elucidated. Here, we have identified miR-335 as a key miRNA involved in beta cell exocytosis by regulating important exocytotic proteins such as SNAP25, SYT11 and STXBP1 (Figure 5). Using both single-cell capacitance measurements and TIRF microscopy, we have confirmed that miR-335 regulates exocytosis in beta cells via defective priming of already docked granules, eventually leading to impaired GSIS.

In GK rat islets, miR-335 expression is elevated (Esguerra, Bolmeson et al. 2011) and STXBP1 is reduced (Zhang, Khan et al. 2002). Here, overexpression of miR-335 reduced STXBP1, a confirmed target of miR-335 (Esguerra, Bolmeson et al. 2011) and eventually led to defective exocytosis of insulin granules. STXBP1 is known to be involved in granular docking (Gandasi and Barg 2014) but there was no change in number of docked granules upon miR-335 overexpression. The reason could be that ≈25% reduction of STXBP1 is not enough to reduce the docked pool of granules or there is a possibility that miR-335 is targeting other genes that normally prevent granule docking.

Interestingly, miR-335 negatively correlated with insulin secretion from pre-diabetic donors. This is of interest due to that first phase insulin secretion is associated with exocytosis of the pool of primed granules and first pahse insulin secretion is reduced already in pre-diabetic or impaired glucose tolerance individuals (Eliasson, Abdulkader et al. 2008, Ashcroft and Rorsman 2012).

Here, we have demonstrated how a single miRNA, miR-335 regulates multiple targets within the exocytotic cellular process and postulate that under hyperglycaemic conditions miR-335-mediated regulatory effects are enhanced.

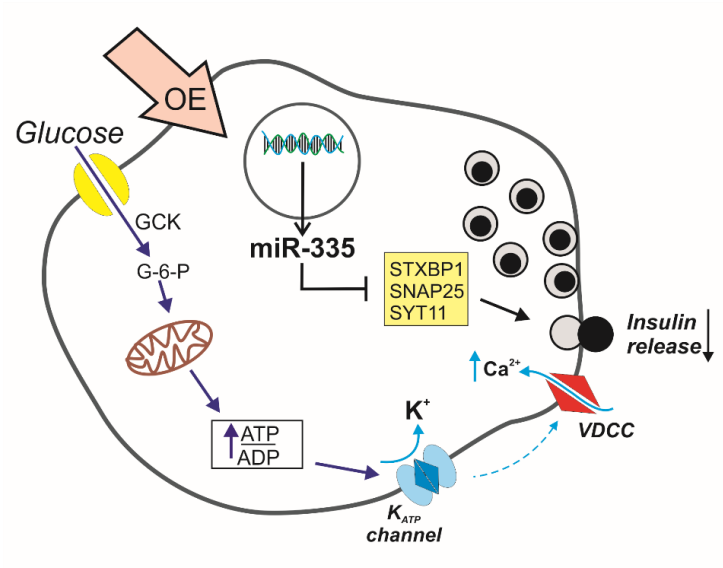

Figure 5: Impact of miR-335 on exocytosis. Overexpression of miR-335 decreases SNAP25, SYT11 and STXBP1 protein resulting in the reduction of insulin exocytosis.

Paper III

Confluence does not affect the expression of miR-375 and its direct targets in rat and human insulin-secreting cell lines

MiRNAs research in beta cells and for that matter T2D is increasing and miRNAs have emerged as important modulators to compensate for metabolic demands in the beta cells. Also, they have been implicated in many cellular processes in the beta cells such as insulin secretion, exocytosis, apoptosis, and proliferation (Poy, Hausser et al. 2009, Esguerra, Bolmeson et al. 2011). MiR-375 is one of the most highly expressed and the first miRNA to be discovered in pancreatic beta cell (Poy, Eliasson et al. 2004, Eliasson 2017). It has been reported in the cancer field that cell density or confluence influences miRNA expression (Hwang, Wentzel et al. 2009, van Rooij 2011), but it is not known whether these findings hold true in the field of diabetes.

We therefore aimed to examine:

a) Whether the expression of miR-375 is influenced by cell density or confluence in rat INS-1 832/13 and human EndoC βH1 cells.
b) If confirmed targets of miR-375 are influenced by cell confluence.
c) If other miRNAs such as miR-152, miR-130a, miR-132, miR-212, and miR-200a with known roles in beta function are dependent on cell density.

Cell-cell distance in EndoC βH1 cells

We measured the average cell to cell distance (μm) in the human EndoC βH1 at different cell density or confluence to be ≈140, ≈80, ≈60 and ≈30 μm for 20%, 60%, 80% and 100% cell confluence, respectively. These differences were statistically significant.

Expression of miR-375 and its targets in EndoC βH1 and INS-1 832/13 cells are not affected by cell density

The expression of miR-375 did not change at different cell confluence in EndoC βH1 cells. Also, expression of two of its confirmed targets, apoptosis-inducing factor, mitochondrion-associated 1 (*AIFM1*) and caveolin1 (*CAV1*) mRNA expression remained unchanged at 60%-100% cell density.

In addition, the expression of miR-152, miR-130a, miR-132, miR-212 and miR-200a are independent of cell confluence in EndoC βH1 cells.

Likewise, cell confluence (20-100%) did not influence the expression of miR-375 and its targets in INS-1 832/13 cells. This result was the same for miR-152, miR-130a and miR-200a.

The situation was different for miR-132 and miR-212. Below 80% confluence the expression was significantly lower as compared to above 80%. Above 80% cell confluence, miR-132 and miR-212 expression remained stable.

Discussion

It has been well established that miRNAs abundance in normal tissues are higher compared to cell lines (Lu, Getz et al. 2005, Gaur, Jewell et al. 2007). Cell confluence has been shown to influence miRNAs expression in a number of cancer cells in *in vitro* systems. Most miRNAs increased with cell density when examined in primary human fibroblasts, NIH 3T3 and Hela cells. Increased miRNA

abundance with increased cell density was attributed to enhanced Drosha processing efficiency that improved RISC formation at high cell confluence (Hwang, Wentzel et al. 2009).

The situation in an *in vitro* system in a beta cell functional assay has not been investigated. MiR-375 is the first miRNA identified and one of the most abundant miRNAs in beta cells (Poy, Eliasson et al. 2004, Eliasson 2017). The expression of miR-375 and miR-375 validated targets (CAV1 and AIFM1) was independent of cell confluence in both rat INS-1 832/13 and human EndoC βH1.

Other known miRNAs such as miR-152, miR-130a, miR-200a, miR-132, and miR-212 (Malm, Mollet et al. 2016, Ofori, Salunkhe et al. 2017) reported to play vital role in normal beta cell function were also not affected by cell density at least within 80-100% cell confluence in which most functional assays are performed in beta cell lines. In Figure 6 the CT-values for all miRNAs measured during the different densities are presented to demonstrate that theire were little changes in their expression.

In conclusion, according to our study miRNA expression is not changed by confluence at least not near 100% where most experimenst are performed. Nevertheless, it is imperative to control for cell densities when studying specific miRNAs in pancreatic beta cells at lower cell densities.

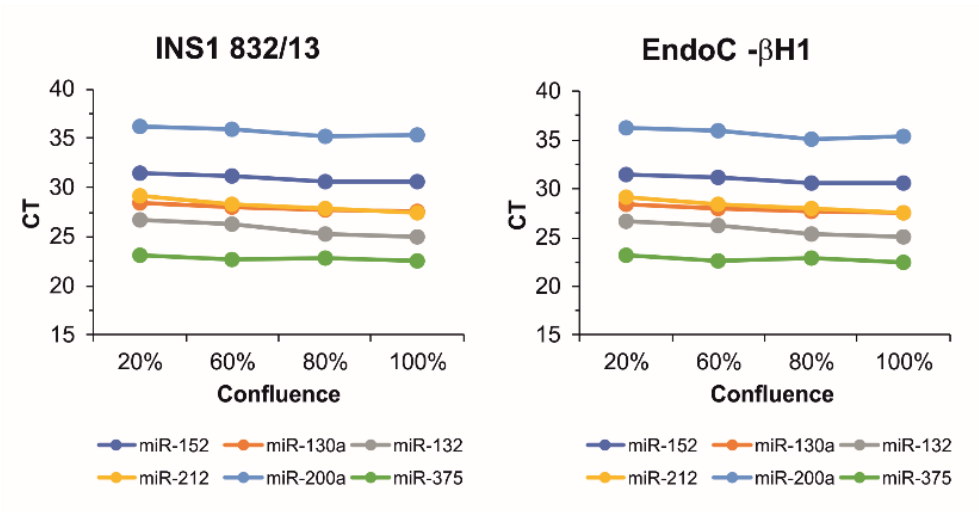

Figure 6: **miRNAs expression independent on cell density.** CT-values **of** miR-375 , miR-152, miR-130a, miR-200a, miR-212 and miR-132 expression in INS-1 832/13 cells (left) and EndoC Hβ1.

Paper IV

Glucocorticoid induces human beta cell dysfunction and involves GAS5 lincRNA

We have recently published RNA-seq data containing lncRNAs from 89 human pancreatic islets from normal and T2D individuals (Fadista, Vikman et al. 2014). Among 493 RefSeq lncRNAs identified, Growth Arrest Specific-5 (GAS5) is the most highly expressed, but its role or function in the beta cell is not known. GAS5 was first isolated in 1988 in search for a novel tumor suppressors (Schneider, King et al. 1988). GAS5 is down-regulated in multiple cancer cells and has been confirmed to be a tumor suppressor (Pickard and Williams 2015). Furthermore, GAS5 has been shown to interact with GC receptor (GR) at its DNA-binding domain (DBD) in a dexamethasone-dependent manner, thereby acting as riborepressor of the GR (Kino, Hurt et al. 2010).

GCs have been implicated in steroid-induced diabetes mellitus for many years (Hwang and Weiss 2014). It has been reported that GCs in combination with exendin-4 improve human islet preparations by reducing pro-inflammatory cytokines (Miki, Ricordi et al. 2014). During pancreatic development, dexamethasone has been shown to reduce expression of important transcription factors in the beta cell such PDX1 and NKX6-1 (Gesina, Tronche et al. 2004). Furthermore, one of the key genes under the regulation of GC, SGK1 has been reported to reduce insulin secretion in the beta cell by increasing the activity of voltage-gated Kv1.5 channels (Ullrich, Berchtold et al. 2005).

In this paper, we aim to:

a) examine the effect of GC on GAS5 expression and insulin secretion,
b) investigate the effect of GAS5 on glucocorticoid signaling and insulin secretion,
c) study GAS5 influence on GC-regulated beta cell genes such as PDX1, NKK6.1, and SYT13,
d) examine the effect of GAS5 expression under glucotoxic conditions, in islets from GK rats and human donors.

Reduced serum C-peptide upon glucocorticoid treatment

First we studied the effect of GC-mediated beta cell dysfunction in *vivo*. We monitored serum C-peptide levels of patients undergoing prednisolone therapy for a period of two weeks. We observed significant reduction in serum C-peptide levels

at the end of the study period, indicating impaired beta cell function. To further examine the direct effect of GC on beta cell function, we treated isolated human islets and EndoC βH1 cells with dexamethasone for 24 or 48 h and observed significant reduction in GSIS. In addition, GAS5 expression was significantly reduced in human islets and EndoC βH1 cells upon dexamethasone treatment.

GAS5 knock-down decreased GSIS in EndoC βH1 cells

We knocked-down GAS5 (≈40-70%) in human EndoC βH1 using LNA gapmeR. This resulted in significant reduction in GSIS and increase in apoptosis. Interestingly, we could recover insulin secretion from dexamethasone-treated cells upon introduction of active segment of GAS5, GAS5 HREM (hormone response element mimic).

Effects of key proteins upon *GAS5* KD and GC treatment

Analysis of RNA-seq data from 195 human islets gave us a hint on potential protein-coding genes co-regulated with GAS5 ncRNA. We observed significant negative correlation of *GAS5* expression with expression of genes coding for *NKX6-1* and *PDX1*, *GR*, and synaptotagmin 13 (*SYT13*) and positive correlation with *SGK1*. Both *GAS5* KD and dexamethasone significantly reduced GAS5 level and this led to significant reduction of NKX6-1, PDX1, GR, and SYT13 with increase in SGK1 protein. Furthermore, the effect of dexamethasone on these genes was reversed in both human islets and EndoC βH1 cells upon addition of GR inhibitor, RU486.

Differentially expression of GAS5

Finally, we measured the expression of GAS5 under hyperglycemic conditions. We treated EndoC βH1 cells with 5 mM and 20 mM glucose for 1, 6 and 24 hours. GAS5 expression was elevated at 20 mM at all-time points. We also measured increased expression of GAS5 in islets from T2D donors, and in islets from the GK-rat.

Discussion

The importance of functional non-coding RNA in various cellular processes are now widely recognized. However, the roles of many lncRNAs such GAS5 are not known in beta cells. In this study, we pursued GAS5 by virtue of its extraordinary high

expression in the human pancreatic islets compared to other expressed lncRNAs (Fadista, Vikman et al. 2014). From our data, we derived to an hypothesis of how GAS5 involve with dexamethasone-induced effects on insulin secretion through interaction with GR (Figure 7).

GAS5 lincRNA is the most abundant ncRNA in the beta cell and we found GAS5 to be co-regulated with important proteins involved in beta cell function such as PDX1, NKX6-1, SYT13 and the GC signaling proteins, GR and SGK1.

Both PDX1 and NKX6-1 are key transcriptional factors necessary for insulin biosynthesis and insulin secretion (Kemmler, Peterson et al. 1972, Taylor, Liu et al. 2013). SYT13 knockdown reduces GSIS in INS-1 832/13 cells and in human islets, its expression negatively correlates with HbA1c; and positively with GSIS (Andersson, Olsson et al. 2012). Here we showed that GAS5 knockdown significantly reduces GSIS.

Dexamethasone, one of the most used synthetic GC activates SGK1 to impair GSIS in INS-1 832/13 cells by upregulating Kv1.5 ion channels (Ullrich, Berchtold et al. 2005). Also, during pancreatic development, dexamethasone has been shown to reduce PDX1 and NKX6-1 (Gesina, Tronche et al. 2004). Interestingly, GAS5 knockdown significantly increased the expression of SGK1, and decreased GR, PDX1, NKX6-1, and SYT13. It is worth mentioning that dexamethasone reduced the expression of GAS5 in both human islets and EndoC βH1 cells and significantly reduced GSIS. We were able to rescue the negative effect of GC on GSIS by introduction of GAS5 HERM, indicating the direct role of GAS5 in mediating the negative effect of GCs in beta cell GSIS.

GAS5 is elevated under hyperglycemic conditions and positively correlates with HbA1c. We therefore hypothesize that upregulation of GAS5 may be an adaptive response to compensate for disrupted GC-GR signaling to prevent beta cell failure.

In conclusion, lncRNA GAS5 is necessary for optimal function of the beta cells. Here we showed that the GC-induced dysfunction in *in vivo* and *in vitro* systems may be acting through GAS5-mediated transcriptional regulation of the GR itself and other important beta cell genes potentially regulated by GCs. Nevertheless, further studies are needed to exploit lncRNA GAS5 as a therapeutic agent in treatment of T2D especially for patients undergoing GC therapy.

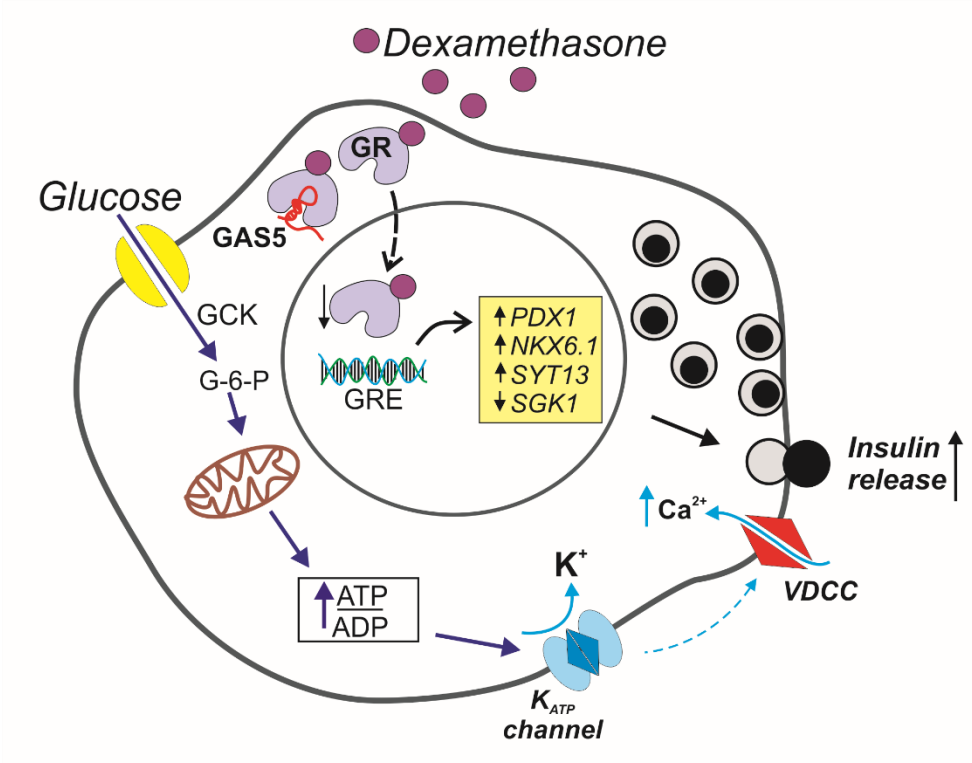

Figure 7: The impact of GAS5 and GC on GSIS. Dexamethasone binds to the GC receptor (GR) and GR is transported into the nucleus. In the nucleus, GR either binds to positive or negative GC response element (GRE/nGRE) of target genes or other transcription factors that will lead to transcriptional activation of SGK1, repression of PDX1,NKX6-1 and SYT13 and reduced GSIS. GAS5 is able to sequester the GR, limiting the amount of GR that gets translocated into the nucleus. This will result in less GR binding and subsequently leading to increased expression of PDX1,NKX6-1 and SYT13 and decreased expression of SGK1; eventually increasing the insulin release.

Concluding Remarks

In this thesis, we examined how ncRNAs affect insulin secretion and exocytosis in pancreatic beta cells in *in vivo* and *in vitro* systems. We provided mechanistic roles for specific miRNAs that have been identified to be dysregulated in T2D. Furthermore, we presented solid evidence on how some miRNAs are either dependent or independent on cell density. Finally, we have demonstrated how GAS5 impairs GSIS, and its potential involvement in GC signaling. These unique findings will pave a way for further studies to help expand the knowledge in this field with the ultimate goal to develop RNA-based drugs to treat T2D.

Specifically, we conclude that:

I. MiR-152, miR-130a and miR-130b reduce ATP production in beta cells via PDHA1 and GCK, which leads to defective GSIS.

II. MiR-335 decreases insulin exocytosis via STXPB1, SNAP25, and SYT11 mainly through defective priming of insulin granules.

III. Expression of miR-375 miR-152, miR-130a, and miR-200a are independent of cell confluence in rat INS-1 832/13 and human EndoC βH1 cells.

IV. GAS5 is the most abundant lncRNA in human beta cells and is involved in GC-mediated inhibition of insulin secretion.

Future Perspective

Impaired insulin secretion is one of the major features of T2D and contributes significantly to the pathogenesis and pathophysiology of this disease. A number of dysregulated ncRNAs have been implicated in this process but molecular mechanisms directly implicating ncRNAs are lacking. It is therefore imperative to establish the molecular role of ncRNAs in pancreatic beta cells in order to elucidate and understand the reason behind defective GSIS in T2D subjects. This will pave way for new insights in the development and treatment of T2D. Moreover, due to its escalating global incidence, there is a need to exploit novel therapeutic avenues such as the use of antisense RNA-based drugs to treat or cure T2D.

In this thesis, we have shown how miR-152, miR-130a and miR-130a influence GSIS and insulin biosynthesis by regulating PDHA1 and GCK which eventually leads to reduction in ATP production in the beta cells. LNA anti-miR *in vitro* against these miRNAs improved insulin secretion and insulin content in INS-1 832/13 cells. Furthermore, we have shown how miR-335 regulates exocytosis via the exocytotic proteins SNP25, STXBP1 and SYT11.

In the near future, it will be very interesting to recapitulate our findings *in vivo* systems. We can do this by generating a conditional knock-out or knock-in of individual miRNA or combined miRNAs in beta cells of rodents. This will enable us to identify phenotypic effect of a single miRNA or combined miRNAs. In this way, we will be able to compare the different phenotypes.

Another line of thought is to treat GK rats with LNA anti-miR to see if we will be able to reverse or restore the diseased state to normal.

Also, we have been able to demonstrate the importance of taking into account cell density when working with miRNAs in pancreatic beta cells.

Furthermore, we have established how GAS5 influence insulin secretion via PDX1, NKX6-1, SYT13 and SGK1. The ability for GAS5 HREM to rescue negative effects of GC on GSIS is a major finding in this thesis. However, further investigation is needed in *in vivo* system to verify this finding. If it holds true, it opens up for the possibility to give patients undergoing steroid therapy GAS5 to alleviate the diabetic effect of steroid treatment.

However, for many years the intracellular delivery of RNA-base therapy has been a challenge. They are either too bulky or too charged to diffuse across lipid bilayer, hence they require a delivery agent to enter into cells. RNA could be trapped in endosomes when taking up by the cells. In addition, RNAs are rapidly degraded by RNases in the blood and tissues. Also, they are rapidly cleared from the blood by the kidney. Furthermore, since miRNAs in particular may have multiple targets in the same tissue or different tissues, specific targeting of miRNAs is an issue (Dowdy 2017).

Coupled with the above challenges, for RNA-based therapy to become reality to treat or cure T2D, in–depth research and knowledge on the role of ncRNAs and molecular mechanisms governing beta cell dysfunction is required. This is our small contribution in bridging this gap.

Regardless of above problem, more than 700 nucleic acid-based (DNA and RNA) therapeutics are in the drug development pipeline (Geall and Ulmer 2015). Currently, RNA therapeutics has become a clinical reality with four antisense oligonucleotides (ASOs) approved by FDA and with a number of RNA candidate drugs in advance stages of human trials (Wang, Zuris et al. 2016, Kaczmarek, Kowalski et al. 2017).

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References

Agarwal, V., G. W. Bell, J. W. Nam and D. P. Bartel (2015). "Predicting effective microRNA target sites in mammalian mRNAs." Elife **4**.

Agrawal, N., P. V. Dasaradhi, A. Mohmmed, P. Malhotra, R. K. Bhatnagar and S. K. Mukherjee (2003). "RNA interference: biology, mechanism, and applications." Microbiol Mol Biol Rev **67**(4): 657-685.

Ahlqvist, E., P. Storm, A. Karajamaki, M. Martinell, M. Dorkhan, A. Carlsson, P. Vikman, R. B. Prasad, D. Mansour Aly, P. Almgren, Y. Wessman, N. Shaat, P. Spegel, H. Mulder, E. Lindholm, O. Melander, O. Hansson, U. Malmqvist, A. Lernmark, K. Lahti, T. Forsen, T. Tuomi, A. H. Rosengren and L. Groop (2017). "Clustering of adult-onset diabetes into novel subgroups guides therapy and improves prediction of outcome." bioRxiv.

Akerman, I., Z. D. Tu, A. Beucher, D. M. Y. Rolando, C. Sauty-Colace, M. Benazra, N. Nakic, J. L. Yang, H. Wang, L. Pasquali, I. Moran, J. Garcia-Hurtado, N. Castro, R. Gonzalez-Franco, A. F. Stewart, C. Bonner, L. Piemonti, T. Berney, L. Groop, J. Kerr-Conte, F. Pattou, C. Argmann, E. Schadt, P. Ravassard and J. Ferrer (2017). "Human Pancreatic beta Cell lncRNAs Control Cell-Specific Regulatory Networks." Cell Metabolism **25**(2): 400-411.

Alcazar, O., M. Tiedge and S. Lenzen (2000). "Importance of lactate dehydrogenase for the regulation of glycolytic flux and insulin secretion in insulin-producing cells." Biochemical Journal **352**: 373-380.

Ammala, C., F. M. Ashcroft and P. Rorsman (1993). "Calcium-independent potentiation of insulin release by cyclic AMP in single beta-cells." Nature **363**(6427): 356-358.

Andersson, S. A., A. H. Olsson, J. L. S. Esguerra, E. Heimann, C. Ladenvall, A. Edlund, A. Salehi, J. Taneera, E. Degerman, L. Groop, C. Ling and L. Eliasson (2012). "Reduced insulin secretion correlates with decreased expression of exocytotic genes in pancreatic islets from patients with type 2 diabetes." Molecular and Cellular Endocrinology **364**(1-2): 36-45.

Arnes, L., I. Akerman, D. A. Balderes, J. Ferrer and L. Sussel (2016). "betalinc1 encodes a long noncoding RNA that regulates islet beta-cell formation and function." Genes Dev **30**(5): 502-507.

Asfari, M., D. Janjic, P. Meda, G. Li, P. A. Halban and C. B. Wollheim (1992). "Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines." Endocrinology **130**(1): 167-178.

Ashcroft, F. M. and P. Rorsman (2012). "Diabetes Mellitus and the beta Cell: The Last Ten Years." Cell **148**(6): 1160-1171.

Bagge, A., T. R. Clausen, S. Larsen, M. Ladefoged, M. W. Rosenstierne, L. Larsen, O. Vang, J. H. Nielsen and L. T. Dalgaard (2012). "MicroRNA-29a is up-regulated in beta-cells by glucose and decreases glucose-stimulated insulin secretion." Biochemical and Biophysical Research Communications **426**(2): 266-272.

Bagge, A., C. M. Dahmcke and L. T. Dalgaard (2013). "Syntaxin-1a is a Direct Target of miR-29a in Insulin-producing beta-Cells." Hormone and Metabolic Research **45**(6): 463-466.

Baroukh, N., M. A. Ravier, M. K. Loder, E. V. Hill, A. Bounacer, R. Scharfmann, G. A. Rutter and E. Van Obberghen (2007). "MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic beta-cell lines." Journal of Biological Chemistry **282**(27): 19575-19588.

Bartel, D. P. (2009). "MicroRNAs: target recognition and regulatory functions." Cell **136**(2): 215-233.

Beato, M. (1989). "Gene-Regulation by Steroid-Hormones." Cell **56**(3): 335-344.

Biosystems, A. (2010). Introduction to Gene Expression Getting Started Guide.

Bolmeson, C., J. L. Esguerra, A. Salehi, D. Speidel, L. Eliasson and C. M. Cilio (2011). "Differences in islet-enriched miRNAs in healthy and glucose intolerant human subjects." Biochem Biophys Res Commun **404**(1): 16-22.

Braun, M., R. Rantracheya, M. Bengtsson, Q. Zhang, J. Karanauskaite, C. Partridge, P. R. Johnson and P. Rorsman (2008). "Voltage-gated ion channels in human pancreatic beta-cells: Electrophysiological characterization and role in insulin secretion." Diabetes **57**(6): 1618-1628.

Burre, J., M. Sharma, T. Tsetsenis, V. Buchman, M. R. Etherton and T. C. Sudhof (2010). "alpha-Synuclein Promotes SNARE-Complex Assembly in Vivo and in Vitro." Science **329**(5999): 1663-1667.

Byrne, M. M., J. Sturis, K. Clement, N. Vionnet, M. E. Pueyo, M. Stoffel, J. Takeda, P. Passa, D. Cohen, G. I. Bell and et al. (1994). "Insulin secretory abnormalities in subjects with hyperglycemia due to glucokinase mutations." J Clin Invest **93**(3): 1120-1130.

Cadoudal, T., J. M. Blouin, M. Collinet, F. Fouque, G. D. Tan, E. Loizon, E. G. Beale, K. N. Frayn, F. Karpe, H. Vidal, C. Benelli and C. Forest (2007). "Acute and selective regulation of glyceroneogenesis and cytosolic phosphoenolpyruvate carboxykinase in adipose tissue by thiazolidinediones in type 2 diabetes." Diabetologia **50**(3): 666-675.

Cadoudal, T., S. Leroyer, A. F. Reis, J. Tordjman, S. Durant, F. Fouque, M. Collinet, J. Quette, G. Chauvet, E. Beale, G. Velho, B. Antoine, C. Benelli and C. Forest (2005). "Proposed involvement of adipocyte glyceroneogenesis and phosphoenolpyruvate carboxykinase in the metabolic syndrome." Biochimie **87**(1): 27-32.

Cech, T. R. (2012). "The RNA Worlds in Context." Cold Spring Harbor Perspectives in Biology **4**(7).

Chakraborty, C., C. G. P. Doss, S. Bandyopadhyay and G. Agoramoorthy (2014). "Influence of miRNA in insulin signaling pathway and insulin resistance: micro-molecules with a major role in type-2 diabetes." Wiley Interdisciplinary Reviews-Rna **5**(5): 697-712.

Chen, Y. H., S. Heneidi, J. M. Lee, L. C. Layman, D. W. Stepp, G. M. Gamboa, B. S. Chen, G. Chazenbalk and R. Azziz (2013). "miRNA-93 Inhibits GLUT4 and Is Overexpressed in Adipose Tissue of Polycystic Ovary Syndrome Patients and Women With Insulin Resistance." Diabetes **62**(7): 2278-2286.

Conn, J. W. and S. S. Fajans (1956). "Influence of adrenal cortical steroids on carbohydrate metabolism in man." Metabolism **5**(2): 114-127.

Corkey, B. E., M. C. Glennon, K. S. Chen, J. T. Deeney, F. M. Matschinsky and M. Prentki (1989). "A Role for Malonyl-Coa in Glucose-Stimulated Insulin-Secretion from Clonal Pancreatic Beta-Cells." Journal of Biological Chemistry **264**(36): 21608-21612.

Cullingford, T. E., J. B. Clark and I. R. Phillips (1994). "The pyruvate dehydrogenase complex: cloning of the rat somatic E1 alpha subunit and its coordinate expression with the mRNAs for the E1 beta, E2, and E3 catalytic subunits in developing rat brain." J Neurochem **62**(5): 1682-1690.

Dalgaard, L. T. and L. Eliasson (2017). "An 'alpha-beta' of pancreatic islet microribonucleotides." Int J Biochem Cell Biol **88**: 208-219.

Del Guerra, S., R. Lupi, L. Marselli, M. Masini, M. Bugliani, S. Sbrana, S. Torri, M. Pollera, U. Boggi, F. Mosca, S. Del Prato and P. Marchetti (2005). "Functional and molecular defects of pancreatic islets in human type 2 diabetes." Diabetes **54**(3): 727-735.

Derrien, T., R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, G. Guernec, D. Martin, A. Merkel, D. G. Knowles, J. Lagarde, L. Veeravalli, X. A. Ruan, Y. J. Ruan, T. Lassmann, P. Carninci, J. B. Brown, L. Lipovich, J. M. Gonzalez, M. Thomas, C. A. Davis, R. Shiekhattar, T. R. Gingeras, T. J. Hubbard, C. Notredame, J. Harrow and R. Guigo (2012).

"The GENCODE v7 catalog of human long noncoding RNAs: Analysis of their gene structure, evolution, and expression." Genome Research **22**(9): 1775-1789.

Di Dalmazi, G., U. Pagotto, R. Pasquali and V. Vicennati (2012). "Glucocorticoids and type 2 diabetes: from physiology to pathology." J Nutr Metab **2012**: 525093.

Donihi, A. C., D. Raval, M. Saul, M. T. Korytkowski and M. A. DeVita (2006). "Prevalence and predictors of corticosteroid-related hyperglycemia in hospitalized patients." Endocr Pract **12**(4): 358-362.

Dowdy, S. F. (2017). "Overcoming cellular barriers for RNA therapeutics." Nat Biotechnol **35**(3): 222-229.

Dweep, H. and N. Gretz (2015). "miRWalk2.0: a comprehensive atlas of microRNA-target interactions." Nat Methods **12**(8): 697.

Eliasson, L. (2014). "The exocytotic machinery." Acta Physiologica **210**(3): 455-457.

Eliasson, L. (2017). "The small RNA miR-375 - a pancreatic islet abundant miRNA with multiple roles in endocrine beta cell function." Mol Cell Endocrinol **456**: 95-101.

Eliasson, L., F. Abdulkader, M. Braun, J. Galvanovskis, M. B. Hoppa and P. Rorsman (2008). "Novel aspects of the molecular mechanisms controlling insulin secretion." Journal of Physiology-London **586**(14): 3313-3324.

Eliasson, L. and J. L. Esguerra (2014). "Role of non-coding RNAs in pancreatic beta-cell development and physiology." Acta Physiol (Oxf) **211**(2): 273-284.

Eliasson, L., E. Renstrom, W. G. Ding, P. Proks and P. Rorsman (1997). "Rapid ATP-dependent priming of secretory granules precedes Ca(2+)-induced exocytosis in mouse pancreatic B-cells." J Physiol **503 (Pt 2)**: 399-412.

Engvall, E. and P. Perlmann (1971). "Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G." Immunochemistry **8**(9): 871-874.

Esguerra, J. L., C. Bolmeson, C. M. Cilio and L. Eliasson (2011). "Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat." PLoS One **6**(4): e18613.

Esguerra, J. L. S., I. G. Mollet, V. A. Salunkhe, A. Wendt and L. Eliasson (2014). "Regulation of Pancreatic Beta Cell Stimulus-Secretion Coupling by microRNAs." Genes **5**(4): 1018-1031.

Esteller, M. (2011). "Non-coding RNAs in human disease." Nat Rev Genet **12**(12): 861-874.

Fadista, J., P. Vikman, E. O. Laakso, I. G. Mollet, J. L. Esguerra, J. Taneera, P. Storm, P. Osmark, C. Ladenvall, R. B. Prasad, K. B. Hansson, F. Finotello, K. Uvebrant, J. K. Ofori, B. Di Camillo, U. Krus, C. M. Cilio, O. Hansson, L. Eliasson, A. H. Rosengren, E. Renstrom, C. B. Wollheim and L. Groop (2014). "Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism." Proceedings of the National Academy of Sciences of the United States of America **111**(38): 13924-13929.

Fernandez-Valverde, S. L., R. J. Taft and J. S. Mattick (2011). "MicroRNAs in beta-Cell Biology, Insulin Resistance, Diabetes and Its Complications." Diabetes **60**(7): 1825-1831.

Franckhauser, S., J. Antras-Ferry, P. Robin, D. Robin, D. K. Granner and C. Forest (1995). "Expression of the phosphoenolpyruvate carboxykinase gene in 3T3-F442A adipose cells: opposite effects of dexamethasone and isoprenaline on transcription." Biochem J **305 (Pt 1)**: 65-71.

Frese, T., I. Bazwinsky, E. Muhlbauer and E. Peschke (2007). "Circadian and age-dependent expression patterns of GLUT2 and glucokinase in the pancreatic beta-cell of diabetic and nondiabetic rats." Horm Metab Res **39**(8): 567-574.

Fu, Z., E. R. Gilbert and D. Liu (2013). "Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes." Curr Diabetes Rev **9**(1): 25-53.

Gagliardi, M. and M. R. Matarazzo (2016). "RIP: RNA Immunoprecipitation." Methods Mol Biol **1480**: 73-86.

Gainetdinov, I., Y. Skvortsova, S. Kondratieva, S. Funikov and T. Azhikina (2017). "Two modes of targeting transposable elements by piRNA pathway in human testis." RNA.

Gaisano, H. Y., C. G. Ostenson, L. Sheu, M. B. Wheeler and S. Efendic (2002). "Abnormal expression of pancreatic islet exocytotic soluble N-ethylmaleimide-sensitive factor attachment protein receptors in Goto-Kakizaki rats is partially restored by phlorizin treatment and accentuated by high glucose treatment." Endocrinology **143**(11): 4218-4226.

Gandasi, N. R. and S. Barg (2014). "Contact-induced clustering of syntaxin and munc18 docks secretory granules at the exocytosis site." Nat Commun **5**: 3914.

Gaur, A., D. A. Jewell, Y. Liang, D. Ridzon, J. H. Moore, C. Chen, V. R. Ambros and M. A. Israel (2007). "Characterization of microRNA expression levels and their biological correlates in human cancer cell lines." Cancer Res **67**(6): 2456-2468.

Gauthier, B. R. and C. B. Wollheim (2008). "Synaptotagmins bind calcium to release insulin." American Journal of Physiology-Endocrinology and Metabolism **295**(6): E1279-E1286.

Geall, A. J. and J. B. Ulmer (2015). "Introduction to RNA-based vaccines and therapeutics Foreword." Expert Review of Vaccines **14**(2): 151-152.

Geer, E. B., J. Islam and C. Buettner (2014). "Mechanisms of glucocorticoid-induced insulin resistance: focus on adipose tissue function and lipid metabolism." Endocrinol Metab Clin North Am **43**(1): 75-102.

Geer, E. B., W. Shen, D. Gallagher, M. Punyanitya, H. C. Looker, K. D. Post and P. U. Freda (2010). "MRI assessment of lean and adipose tissue distribution in female patients with Cushing's disease." Clin Endocrinol (Oxf) **73**(4): 469-475.

Geer, E. B., W. Shen, E. Strohmayer, K. D. Post and P. U. Freda (2012). "Body composition and cardiovascular risk markers after remission of Cushing's disease: a prospective study using whole-body MRI." J Clin Endocrinol Metab **97**(5): 1702-1711.

Gerber, S. H. and T. C. Sudhof (2002). "Section 1: Insulin release: Some molecular requisites - Molecular determinants of regulated exocytosis." Diabetes **51**: S3-S11.

Gesina, E., F. Tronche, P. Herrera, B. Duchene, W. Tales, P. Czernichow and B. Breant (2004). "Dissecting the role of glucocorticoids on pancreas development." Diabetes **53**(9): 2322-2329.

Goossens, G. H. (2008). "The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance." Physiol Behav **94**(2): 206-218.

Goto, Y., M. Kakizaki and N. Masaki (1976). "Production of spontaneous diabetic rats by repetition of selective breeding." Tohoku J Exp Med **119**(1): 85-90.

Granhall, C., A. H. Rosengren, E. Renstrom and H. Luthman (2006). "Separately inherited defects in insulin exocytosis and beta-cell glucose metabolism contribute to type 2 diabetes." Diabetes **55**(12): 3494-3500.

Gremlich, S., R. Roduit and B. Thorens (1997). "Dexamethasone induces posttranslational degradation of GLUT2 and inhibition of insulin secretion in isolated pancreatic beta cells - Comparison with the effects of fatty acids." Journal of Biological Chemistry **272**(6): 3216-3222.

Grieco, F. A., G. Sebastiani, J. Juan-Mateu, O. Villate, L. Marroqui, L. Ladriere, K. Tugay, R. Regazzi, M. Bugliani, P. Marchetti, F. Dotta and D. L. Eizirik (2017). "MicroRNAs miR-23a-3p, miR-23b-3p, and miR-149-5p Regulate the Expression of Proapoptotic BH3-Only Proteins DP5 and PUMA in Human Pancreatic beta-Cells." Diabetes **66**(1): 100-112.

Grunweller, A., E. Wyszko, B. Bieber, R. Jahnel, V. A. Erdmann and J. Kurreck (2003). "Comparison of different antisense strategies in mammalian cells using locked nucleic acids,

2 '-O-methyl RNA, phosphorothioates and small interfering RNA." Nucleic Acids Research **31**(12): 3185-3193.

Gulyas-Kovacs, A., H. de Wit, I. Milosevic, O. Kochubey, R. Toonen, J. Klingauf, M. Verhage and J. B. Sorensen (2007). "Munc18-1: sequential interactions with the fusion machinery stimulate vesicle docking and priming." J Neurosci **27**(32): 8676-8686.

Gustavsson, N., Y. Lao, A. Maximov, J. C. Chuang, E. Kostromina, J. J. Repa, C. Li, G. K. Radda, T. C. Sudhof and W. P. Han (2008). "Impaired insulin secretion and glucose intolerance in synaptotagmin-7 null mutant mice." Proceedings of the National Academy of Sciences of the United States of America **105**(10): 3992-3997.

Guttman, M., I. Amit, M. Garber, C. French, M. F. Lin, D. Feldser, M. Huarte, O. Zuk, B. W. Carey, J. P. Cassady, M. N. Cabili, R. Jaenisch, T. S. Mikkelsen, T. Jacks, N. Hacohen, B. E. Bernstein, M. Kellis, A. Regev, J. L. Rinn and E. S. Lander (2009). "Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals." Nature **458**(7235): 223-227.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann and F. J. Sigworth (1981). "Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches." Pflugers Arch **391**(2): 85-100.

Hansen, T. B., M. T. Veno, T. I. Jensen, A. Schaefer, C. K. Damgaard and J. Kjems (2016). "Argonaute-associated short introns are a novel class of gene regulators." Nat Commun **7**: 11538.

Hapgood, J. P., C. Avenant and J. M. Moliki (2016). "Glucocorticoid-independent modulation of GR activity: Implications for immunotherapy." Pharmacology & Therapeutics **165**: 93-113.

Hattersley, A. T. and K. A. Patel (2017). "Precision diabetes: learning from monogenic diabetes." Diabetologia **60**(5): 769-777.

Heid, C. A., J. Stevens, K. J. Livak and P. M. Williams (1996). "Real time quantitative PCR." Genome Res **6**(10): 986-994.

Hohmeier, H. E., H. Mulder, G. Chen, R. Henkel-Rieger, M. Prentki and C. B. Newgard (2000). "Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion." Diabetes **49**(3): 424-430.

Huang, C., J. Shi, Y. Guo, W. Huang, S. Huang, S. Ming, X. Wu, R. Zhang, J. Ding, W. Zhao, J. Jia, X. Huang, A. P. Xiang, Y. Shi and C. Yao (2017). "A snoRNA modulates mRNA 3' end processing and regulates the expression of a subset of mRNAs." Nucleic Acids Res **45**(15): 8647-8660.

Huang da, W., B. T. Sherman and R. A. Lempicki (2009). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." Nat Protoc **4**(1): 44-57.

Huang, X. F. and P. Arvan (1995). "Intracellular transport of proinsulin in pancreatic beta-cells. Structural maturation probed by disulfide accessibility." J Biol Chem **270**(35): 20417-20423.

Hwang, H. W., E. A. Wentzel and J. T. Mendell (2009). "Cell-cell contact globally activates microRNA biogenesis." Proc Natl Acad Sci U S A **106**(17): 7016-7021.

Hwang, J. L. and R. E. Weiss (2014). "Steroid-induced diabetes: a clinical and molecular approach to understanding and treatment." Diabetes Metab Res Rev **30**(2): 96-102.

Ideue, T., K. Hino, S. Kitao, T. Yokoi and T. Hirose (2009). "Efficient oligonucleotide-mediated degradation of nuclear noncoding RNAs in mammalian cultured cells." Rna-a Publication of the Rna Society **15**(8): 1578-1587.

Ishizu, H., H. Siomi and M. C. Siomi (2012). "Biology of PIWI-interacting RNAs: new insights into biogenesis and function inside and outside of germlines." Genes & Development **26**(21): 2361-2373.

Jewell, J. L., E. Oh and D. C. Thurmond (2010). "Exocytosis mechanisms underlying insulin release and glucose uptake: conserved roles for Munc18c and syntaxin 4." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **298**(3): R517-R531.

Jhun, B. S., H. Lee, Z. G. Jin and Y. Yoon (2013). "Glucose stimulation induces dynamic change of mitochondrial morphology to promote insulin secretion in the insulinoma cell line INS-1E." PLoS One **8**(4): e60810.

Jing, X., D. Q. Li, C. S. Olofsson, A. Salehi, V. V. Surve, J. Caballero, R. Ivarsson, I. Lundquist, A. Pereverzev, T. Schneider, P. Rorsman and E. Renstrom (2005). "CaV2.3 calcium channels control second-phase insulin release." J Clin Invest **115**(1): 146-154.

Joglekar, M. V., V. S. Parekh, S. Mehta, R. R. Bhonde and A. A. Hardikar (2007). "MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3." Dev Biol **311**(2): 603-612.

Kaczmarek, J. C., P. S. Kowalski and D. G. Anderson (2017). "Advances in the delivery of RNA therapeutics: from concept to clinical reality." Genome Medicine **9**.

Kalis, M., C. Bolmeson, J. L. Esguerra, S. Gupta, A. Edlund, N. Tormo-Badia, D. Speidel, D. Holmberg, S. Mayans, N. K. Khoo, A. Wendt, L. Eliasson and C. M. Cilio (2011). "Beta-cell specific deletion of Dicer1 leads to defective insulin secretion and diabetes mellitus." PLoS One **6**(12): e29166.

Kameswaran, V., N. C. Bramswig, L. B. McKenna, M. Penn, J. Schug, N. J. Hand, Y. Chen, I. Choi, A. Vourekas, K. J. Won, C. Liu, K. Vivek, A. Naji, J. R. Friedman and K. H. Kaestner (2014). "Epigenetic regulation of the DLK1-MEG3 microRNA cluster in human type 2 diabetic islets." Cell Metab **19**(1): 135-145.

Karolina, D. S., A. Armugam, S. Tavintharan, M. T. K. Wong, S. C. Lim, C. F. Sum and K. Jeyaseelan (2011). "MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus." Plos One **6**(8).

Kataoka, K., S. I. Han, S. Shioda, M. Hirai, M. Nishizawa and H. Handa (2002). "MafA is a glucose-regulated and pancreatic beta-cell-specific transcriptional activator for the insulin gene." Journal of Biological Chemistry **277**(51): 49903-49910.

Kaur, G. and J. M. Dufour (2012). "Cell lines: Valuable tools or useless artifacts." Spermatogenesis **2**(1): 1-5.

Kemmler, W., J. D. Peterson, A. H. Rubenstein and D. F. Steiner (1972). "On the biosynthesis, intracellular transport and mechanism of conversion of proinsulin to insulin and C-peptide." Diabetes **21**(2 Suppl): 572-581.

Khan, A., C. G. Ostenson, P. O. Berggren and S. Efendic (1992). "Glucocorticoid Increases Glucose Cycling and Inhibits Insulin Release in Pancreatic-Islets of Ob/Ob Mice." American Journal of Physiology **263**(4): E663-E666.

Kino, T., D. E. Hurt, T. Ichijo, N. Nader and G. P. Chrousos (2010). "Noncoding RNA Gas5 Is a Growth Arrest- and Starvation-Associated Repressor of the Glucocorticoid Receptor." Science Signaling **3**(107).

Knoll, M., H. F. Lodish and L. Sun (2015). "Long non-coding RNAs as regulators of the endocrine system." Nat Rev Endocrinol **11**(3): 151-160.

Kondo, Y., C. Oubridge, A. M. van Roon and K. Nagai (2015). "Crystal structure of human U1 snRNP, a small nuclear ribonucleoprotein particle, reveals the mechanism of 5' splice site recognition." Elife **4**.

Kurien, B. T. and R. H. Scofield (2015). "Western blotting: an introduction." Methods Mol Biol **1312**: 17-30.

Kurreck, J., E. Wyszko, C. Gillen and V. A. Erdmann (2002). "Design of antisense oligonucleotides stabilized by locked nucleic acids." Nucleic Acids Res **30**(9): 1911-1918.

Lam, M. T., W. Li, M. G. Rosenfeld and C. K. Glass (2014). "Enhancer RNAs and regulated transcriptional programs." Trends Biochem Sci **39**(4): 170-182.

Lambillotte, C., P. Gilon and J. C. Henquin (1997). "Direct glucocorticoid inhibition of insulin secretion - An in vitro study of dexamethasone effects in mouse islets." Journal of Clinical Investigation **99**(3): 414-423.

Latreille, M., J. Hausser, I. Stutzer, Q. Zhang, B. Hastoy, S. Gargani, J. Kerr-Conte, F. Pattou, M. Zavolan, J. L. S. Esguerra, L. Eliasson, T. Rulicke, P. Rorsman and M. Stoffel (2014). "MicroRNA-7a regulates pancreatic beta cell function." Journal of Clinical Investigation **124**(6): 2722-2735.

Lawlor, N., S. Khetan, D. Ucar and M. L. Stitzel (2017). "Genomics of Islet (Dys)function and Type 2 Diabetes." Trends in Genetics **33**(4): 244-255.

Ling, H. Y., H. S. Ou, S. D. Feng, X. Y. Zhang, Q. H. Tuo, L. X. Chen, B. Y. Zhu, Z. P. Gao, C. K. Tang, W. D. Yin, L. Zhang and D. F. Liao (2009). "CHANGES IN microRNA (miR) profile and effects of miR-320 in insulin-resistant 3T3-L1 adipocytes." Clin Exp Pharmacol Physiol **36**(9): e32-39.

Linssen, M. M. L., D. H. van Raalte, E. J. M. Toonen, W. Alkema, G. C. van der Zon, W. H. Dokter, M. Diamant, B. Guigas and D. M. Ouwens (2011). "Prednisolone-induced beta cell dysfunction is associated with impaired endoplasmic reticulum homeostasis in INS-1E cells." Cellular Signalling **23**(11): 1708-1715.

Liu, X. X., X. M. Zhu, Q. Miao, H. Y. Ye, Z. Y. Zhang and Y. M. Li (2014). "Hyperglycemia induced by glucocorticoids in nondiabetic patients: a meta-analysis." Ann Nutr Metab **65**(4): 324-332.

Long, W., E. J. Barrett, L. P. Wei and Z. Q. Liu (2003). "Adrenalectomy enhances the insulin sensitivity of muscle protein synthesis." American Journal of Physiology-Endocrinology and Metabolism **284**(1): E102-E109.

Lovis, P., E. Roggli, R. Laybutt, S. Gattesco, J. Y. Yang, C. Widmann, A. Abderrahmani and R. Regazzi (2008). "Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction." Diabetes **57**(10): 2728-2736.

Lu, D. H., H. Mulder, P. Y. Zhao, S. C. Burgess, M. V. Jensen, S. Kamzolova, C. B. Newgard and A. D. Sherry (2002). "C-13 NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS)." Proceedings of the National Academy of Sciences of the United States of America **99**(5): 2708-2713.

Lu, J., G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz and T. R. Golub (2005). "MicroRNA expression profiles classify human cancers." Nature **435**(7043): 834-838.

Lund, T., B. Fosby, O. Korsgren, H. Scholz and A. Foss (2008). "Glucocorticoids reduce pro-inflammatory cytokines and tissue factor in vitro and improve function of transplanted human islets in vivo." Transplant International **21**(7): 669-678.

Maassen, J. A. and T. Kadowaki (1996). "Maternally inherited diabetes and deafness: a new diabetes subtype." Diabetologia **39**(4): 375-382.

Maechler, P. and C. B. Wollheim (1999). "Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis." Nature **402**(6762): 685-689.

Maechler, P. and C. B. Wollheim (2000). "Mitochondrial signals in glucose-stimulated insulin secretion in the beta cell." J Physiol **529 Pt 1**: 49-56.

Malm, H. A., I. G. Mollet, C. Berggreen, M. Orho-Melander, J. L. Esguerra, O. Goransson and L. Eliasson (2016). "Transcriptional regulation of the miR-212/miR-132 cluster in insulin-secreting beta-cells by cAMP-regulated transcriptional co-activator 1 and salt-inducible kinases." Mol Cell Endocrinol **424**: 23-33.

Malmgren, S., D. G. Nicholls, J. Taneera, K. Bacos, T. Koeck, A. Tamaddon, R. Wibom, L. Groop, C. Ling, H. Mulder and V. V. Sharoyko (2009). "Tight Coupling between Glucose and Mitochondrial Metabolism in Clonal beta-Cells Is Required for Robust Insulin Secretion." Journal of Biological Chemistry **284**(47): 32395-32404.

Marshall, M., P. E. Lund and S. Barg (2017). "Molecular Mechanisms of V-SNARE Function in Secretory Granule Exocytosis." Biophysical Journal **112**(3): 395a-395a.

Martinez-Sanchez, A., M. S. Nguyen-Tu and G. A. Rutter (2015). "DICER Inactivation Identifies Pancreatic beta-Cell "Disallowed" Genes Targeted by MicroRNAs." Molecular Endocrinology **29**(7): 1067-1079.

Martinez-Sanchez, A., G. A. Rutter and M. Latreille (2016). "MiRNAs in beta-Cell Development, Identity, and Disease." Front Genet **7**: 226.

Mathis, D., L. Vence and C. Benoist (2001). "beta-Cell death during progression to diabetes." Nature **414**(6865): 792-798.

McMaster, A. and D. W. Ray (2008). "Drug insight: selective agonists and antagonists of the glucocorticoid receptor." Nat Clin Pract Endocrinol Metab **4**(2): 91-101.

Michalik, K. M., X. You, Y. Manavski, A. Doddaballapur, M. Zornig, T. Braun, D. John, Y. Ponomareva, W. Chen, S. Uchida, R. A. Boon and S. Dimmeler (2014). "Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth." Circ Res **114**(9): 1389-1397.

Miki, A., C. Ricordi, T. Yamamoto, Y. Sakuma, R. Misawa, A. Mita, L. Inverardi, R. Alejandro and H. Ichii (2014). "Improved Human Islet Preparations Using Glucocorticoid and Exendin-4." Pancreas **43**(8): 1317-1322.

Miller, C. P., R. E. Mcgehee and J. F. Habener (1994). "Idx-1 - a New Homeodomain Transcription Factor Expressed in Rat Pancreatic-Islets and Duodenum That Transactivates the Somatostatin Gene." Embo Journal **13(**5): 1145-1156.

Mirza, A. H., S. Kaur and F. Pociot (2017). "Long non-coding RNAs as novel players in beta cell function and type 1 diabetes." Hum Genomics **11**(1): 17.

Motohashi, N., M. S. Alexander, Y. Shimizu-Motohashi, J. A. Myers, G. Kawahara and L. M. Kunkel (2013). "Regulation of IRS1/Akt insulin signaling by microRNA-128a during myogenesis." J Cell Sci **126**(Pt 12): 2678-2691.

Mueckler, M. (1994). "Facilitative glucose transporters." Eur J Biochem **219**(3): 713-725.

Nagamatsu, S., Y. Nakamichi, C. Yamamura, S. Matsushima, T. Watanabe, S. Ozawa, H. Furukawa and H. Ishida (1999). "Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta-cells is involved in impaired insulin secretion from diabetic GK rat islets - Restoration of decreased t-SNARE proteins improves impaired insulin secretion." Diabetes **48**(12): 2367-2373.

Nakanishi, N., Y. Nakagawa, N. Tokushige, N. Aoki, T. Matsuzaka, K. Ishii, N. Yahagi, K. Kobayashi, S. Yatoh, A. Takahashi, H. Suzuki, O. Urayama, N. Yamada and H. Shimano (2009). "The up-regulation of microRNA-335 is associated with lipid metabolism in liver and white adipose tissue of genetically obese mice." Biochemical and Biophysical Research Communications **385**(4): 492-496.

Naya, F. J., C. M. M. Stellrecht and M. J. Tsai (1995). "Tissue-Specific Regulation of the Insulin Gene by a Novel Basic Helix-Loop-Helix Transcription Factor." Genes & Development **9**(8): 1009-1019.

Neher, E. and A. Marty (1982). "Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells." Proc Natl Acad Sci U S A **79(**21): 6712-6716.

Nesca, V., C. Guay, C. Jacovetti, V. Menoud, M. L. Peyot, D. R. Laybutt, M. Prentki and R. Regazzi (2013). "Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes." Diabetologia **56(**10): 2203-2212.

Newgard, C. B. and J. D. Mcgarry (1995). "Metabolic Coupling Factors in Pancreatic Beta-Cell Signal-Transduction." Annual Review of Biochemistry **64**: 689-719.

Newton, R. (2000). "Molecular mechanisms of glucocorticoid action: what is important?" Thorax **55**(7): 603-613.

Newton, R., R. Leigh and M. A. Giembycz (2010). "Pharmacological strategies for improving the efficacy and therapeutic ratio of glucocorticoids in inflammatory lung diseases." Pharmacol Ther **125**(2): 286-327.

Ofori, J. K., V. A. Salunkhe, A. Bagge, N. Vishnu, M. Nagao, H. Mulder, C. B. Wollheim, L. Eliasson and J. L. Esguerra (2017). "Elevated miR-130a/miR130b/miR-152 expression reduces intracellular ATP levels in the pancreatic beta cell." Sci Rep **7**: 44986.

Ogawa, A., J. H. Johnson, M. Ohneda, C. T. Mcallister, L. Inman, T. Alam and R. H. Unger (1992). "Roles of Insulin Resistance and Beta-Cell Dysfunction in Dexamethasone-Induced Diabetes." Journal of Clinical Investigation **90**(2): 497-504.

Oger, F., C. Gheeraert, D. Mogilenko, Y. Benomar, O. Molendi-Coste, E. Bouchaert, S. Caron, D. Dombrowicz, F. Pattou, H. Duez, J. Eeckhoute, B. Staels and P. Lefebvre (2014). "Cell-Specific Dysregulation of MicroRNA Expression in Obese White Adipose Tissue." Journal of Clinical Endocrinology & Metabolism **99**(8): 2821-2833.

Ohneda, M., J. H. Johnson, L. R. Inman and R. H. Unger (1993). "Glut-2 Function in Glucose-Unresponsive Beta-Cells of Dexamethasone-Induced Diabetes in Rats." Journal of Clinical Investigation **92**(4): 1950-1956.

Orci, L., M. Ravazzola, M. J. Storch, R. G. Anderson, J. D. Vassalli and A. Perrelet (1987). "Proteolytic maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory vesicles." Cell **49**(6): 865-868.

Ostenson, C. G., H. Gaisano, L. Sheu, A. Tibell and T. Bartfai (2006). "Impaired gene and protein expression of exocytotic soluble N-ethylmaleimide attachment protein receptor complex proteins in pancreatic islets of type 2 diabetic patients." Diabetes **55**(2): 435-440.

Ozsolak, F., L. L. Poling, Z. Wang, H. Liu, X. S. Liu, R. G. Roeder, X. Zhang, J. S. Song and D. E. Fisher (2008). "Chromatin structure analyses identify miRNA promoters." Genes Dev **22**(22): 3172-3183.

Petersen, M. and J. Wengel (2003). "LNA: a versatile tool for therapeutics and genomics." Trends Biotechnol **21**(2): 74-81.

Pickard, M. R. and G. T. Williams (2015). "Molecular and Cellular Mechanisms of Action of Tumour Suppressor GAS5 LncRNA." Genes **6**(3): 484-499.

Polonsky, K. S., J. Sturis and G. I. Bell (1996). "Seminars in Medicine of the Beth Israel Hospital, Boston. Non-insulin-dependent diabetes mellitus - a genetically programmed

failure of the beta cell to compensate for insulin resistance." N Engl J Med **334**(12): 777-783.

Portha, B., M. H. Giroix, C. Tourrel-Cuzin, H. Le-Stunff and J. Movassat (2012). "The GK rat: a prototype for the study of non-overweight type 2 diabetes." Methods Mol Biol **933**: 125-159.

Portha, B., G. Lacraz, M. Kergoat, F. Homo-Delarche, M. H. Giroix, D. Bailbe, M. N. Gangnerau, M. Dolz, C. Tourrel-Cuzin and J. Movassat (2009). "The GK rat beta-cell: a prototype for the diseased human beta-cell in type 2 diabetes?" Mol Cell Endocrinol **297**(1-2): 73-85.

Poy, M. N., L. Eliasson, J. Krutzfeldt, S. Kuwajima, X. Ma, P. E. Macdonald, S. Pfeffer, T. Tuschl, N. Rajewsky, P. Rorsman and M. Stoffel (2004). "A pancreatic islet-specific microRNA regulates insulin secretion." Nature **432**(7014): 226-230.

Poy, M. N., J. Hausser, M. Trajkovski, M. Braun, S. Collins, P. Rorsman, M. Zavolan and M. Stoffel (2009). "miR-375 maintains normal pancreatic alpha- and beta-cell mass." Proc Natl Acad Sci U S A **106**(14): 5813-5818.

Prasad, R. B. and L. Groop (2015). "Genetics of Type 2 Diabetes-Pitfalls and Possibilities." Genes **6**(1): 87-123.

Pullen, T. J., G. D. Xavier, G. Kelsey and G. A. Rutter (2011). "miR-29a and miR-29b Contribute to Pancreatic beta-Cell-Specific Silencing of Monocarboxylate Transporter 1 (Mct1)." Molecular and Cellular Biology **31**(15): 3182-3194.

Ravassard, P., Y. Hazhouz, S. Pechberty, E. Bricout-Neveu, M. Armanet, P. Czernichow and R. Scharfmann (2011). "A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion." J Clin Invest **121**(9): 3589-3597.

Renart, J., J. Reiser and G. R. Stark (1979). "Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure." Proc Natl Acad Sci U S A **76**(7): 3116-3120.

Renganathan, A., J. Kresoja, N. Echeverry, G. Ziltener, B. Vrugt, I. Opitz, R. A. Stahel and E. Felley-Bosco (2014). "Gas5 Long Non Coding Rna in Malignant Pleural Mesothelioma." Journal of Thoracic Oncology **9**(4): S51-S51.

Rhodes, C. J., C. A. Lucas, R. L. Mutkoski, L. Orci and P. A. Halban (1987). "Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump." J Biol Chem **262**(22): 10712-10717.

Roder, M. E., D. Porte, Jr., R. S. Schwartz and S. E. Kahn (1998). "Disproportionately elevated proinsulin levels reflect the degree of impaired B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus." J Clin Endocrinol Metab **83**(2): 604-608.

Roggli, E., A. Britan, S. Gattesco, N. Lin-Marq, A. Abderrahmani, P. Meda and R. Regazzi (2010). "Involvement of MicroRNAs in the Cytotoxic Effects Exerted by Proinflammatory Cytokines on Pancreatic beta-Cells." Diabetes **59**(4): 978-986.

Roggli, E., S. Gattesco, D. Caille, C. Briet, C. Boitard, P. Meda and R. Regazzi (2012). "Changes in microRNA expression contribute to pancreatic beta-cell dysfunction in prediabetic NOD mice." Diabetes **61**(7): 1742-1751.

Rorsman, P. and E. Renstrom (2003). "Insulin granule dynamics in pancreatic beta cells." Diabetologia **46**(8): 1029-1045.

Ruzzin, J., A. S. Wagman and J. Jensen (2005). "Glucocorticoid-induced insulin resistance in skeletal muscles: defects in insulin signalling and the effects of a selective glycogen synthase kinase-3 inhibitor." Diabetologia **48**(10): 2119-2130.

Saad, M. J. A., F. Folli, J. A. Kahn and C. R. Kahn (1993). "Modulation of Insulin-Receptor, Insulin-Receptor Substrate-1, and Phosphatidylinositol 3-Kinase in Liver and Muscle of Dexamethasone-Treated Rats." Journal of Clinical Investigation **92**(4): 2065-2072.

Salunkhe, V. A., J. L. S. Esguerra, J. K. Ofori, I. G. Mollet, M. Braun, M. Stoffel, A. Wendt and L. Eliasson (2015). "Modulation of microRNA-375 expression alters voltage-gated Na+ channel properties and exocytosis in insulin-secreting cells." Acta Physiologica **213**(4): 882-892.

Samandari, N., A. H. Mirza, L. B. Nielsen, S. Kaur, P. Hougaard, S. Fredheim, H. B. Mortensen and F. Pociot (2017). "Circulating microRNA levels predict residual beta cell function and glycaemic control in children with type 1 diabetes mellitus." Diabetologia **60**(2): 354-363.

Sander, M., A. Neubuser, J. Kalamaras, H. C. Ee, G. R. Martin and M. S. German (1997). "Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development." Genes & Development **11**(13): 1662-1673.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.

Schneider, C., R. M. King and L. Philipson (1988). "Genes specifically expressed at growth arrest of mammalian cells." Cell **54**(6): 787-793.

Schoenmakers, E., B. Carlson, M. Agostini, C. Moran, O. Rajanayagam, E. Bochukova, R. Tobe, R. Peat, E. Gevers, F. Muntoni, P. Guicheney, N. Schoenmakers, S. Farooqi, G.

Lyons, D. Hatfield and K. Chatterjee (2016). "Mutation in human selenocysteine transfer RNA selectively disrupts selenoprotein synthesis." J Clin Invest **126**(3): 992-996.

Schuit, F., A. DeVos, S. Farfari, K. Moens, D. Pipeleers, T. Brun and M. Prentki (1997). "Metabolic fate of glucose in purified islet cells - Glucose-regulated anaplerosis in beta cells." Journal of Biological Chemistry **272**(30): 18572-18579.

Schuit, F. C., P. Huypens, H. Heimberg and D. G. Pipeleers (2001). "Glucose sensing in pancreatic beta-cells: a model for the study of other glucose-regulated cells in gut, pancreas, and hypothalamus." Diabetes **50**(1): 1-11.

Schulla, V., E. Renstrom, R. Feil, S. Feil, I. Franklin, A. Gjinovci, X. J. Jing, D. Laux, I. Lundquist, M. A. Magnuson, S. Obermuller, C. S. Olofsson, A. Salehi, A. Wendt, N. Klugbauer, C. B. Wollheim, P. Rorsman and F. Hofmann (2003). "Impaired insulin secretion and glucose tolerance in beta cell-selective Ca(v)1.2 Ca2+ channel null mice." EMBO J **22**(15): 3844-3854.

Sebastiani, G., A. Po, E. Miele, G. Ventriglia, E. Ceccarelli, M. Bugliani, L. Marselli, P. Marchetti, A. Gulino, E. Ferretti and F. Dotta (2015). "MicroRNA-124a is hyperexpressed in type 2 diabetic human pancreatic islets and negatively regulates insulin secretion." Acta Diabetologica **52**(3): 523-530.

Sesti, G., A. Sciacqua, M. Cardellini, M. A. Marini, R. Maio, M. Vatrano, E. Succurro, R. Lauro, M. Federici and F. Perticone (2005). "Plasma concentration of IGF-I is independently associated with insulin sensitivity in subjects with different degrees of glucose tolerance." Diabetes Care **28**(1): 120-125.

Srinivasan, M., C. S. Choi, P. Ghoshal, L. Pliss, J. D. Pandya, D. Hill, G. Cline and M. S. Patel (2010). "beta-Cell-specific pyruvate dehydrogenase deficiency impairs glucose-stimulated insulin secretion." American Journal of Physiology-Endocrinology and Metabolism **299**(6): E910-E917.

Stenvang, J., A. Petri, M. Lindow, S. Obad and S. Kauppinen (2012). "Inhibition of microRNA function by antimiR oligonucleotides." Silence **3**(1): 1.

Storling, J., A. J. Overgaard, C. A. Brorsson, F. Piva, C. H. Bang-Berthelsen, C. Haase, J. Nerup and F. Pociot (2013). "Do post-translational beta cell protein modifications trigger type 1 diabetes?" Diabetologia **56**(11): 2347-2354.

Suh, S. and M. K. Park (2017). "Glucocorticoid-Induced Diabetes Mellitus: An Important but Overlooked Problem." Endocrinol Metab (Seoul) **32**(2): 180-189.

Tantama, M., J. R. Martinez-Francois, R. Mongeon and G. Yellen (2013). "Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio." Nat Commun **4**: 2550.

Taylor, B. L., F. F. Liu and M. Sander (2013). "Nkx6.1 Is Essential for Maintaining the Functional State of Pancreatic Beta Cells." Cell Reports **4**(6): 1262-1275.

Tomas, A., P. Meda, R. Regazzi, J. E. Pessin and P. A. Halban (2008). "Munc 18-1 and granuphilin collaborate during insulin granule exocytosis." Traffic **9**(5): 813-832.

Toonen, R. F. G. and M. Verhage (2007). "Munc18=1 in secretion: lonely Munc joins SNARE team and takes control." Trends in Neurosciences **30**(11): 564-572.

Towbin, H., T. Staehelin and J. Gordon (1979). "Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets - Procedure and Some Applications." Proceedings of the National Academy of Sciences of the United States of America **76**(9): 4350-4354.

Tuomi, T., L. C. Groop, P. Z. Zimmet, M. J. Rowley, W. Knowles and I. R. Mackay (1993). "Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease." Diabetes **42**(2): 359-362.

Ullrich, S., S. Berchtold, F. Ranta, G. Seebohm, G. Henke, A. Lupescu, A. F. Mack, C. M. Chao, J. P. Su, R. Nitschke, D. Alexander, B. Friedrich, P. Wulff, D. Kuhl and F. Lang (2005). "Serum- and glucocorticoid-inducible kinase 1 (SGK1) mediates glucocorticoid-induced inhibition of insulin secretion." Diabetes **54**(4): 1090-1099.

van Raalte, D. H., D. M. Ouwens and M. Diamant (2009). "Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options?" European Journal of Clinical Investigation **39**(2): 81-93.

van Rooij, E. (2011). "The art of microRNA research." Circ Res **108**(2): 219-234.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biol **3**(7): RESEARCH0034.

Wang, K. C. and H. Y. Chang (2011). "Molecular Mechanisms of Long Noncoding RNAs." Molecular Cell **43**(6): 904-914.

Wang, M., J. A. Zuris, F. T. Meng, H. Rees, S. Sun, P. Deng, Y. Han, X. Gao, D. Pouli, Q. Wu, I. Georgakoudi, D. R. Liu and Q. B. Xu (2016). "Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles." Proceedings of the National Academy of Sciences of the United States of America **113**(11): 2868-2873.

Ventriglia, G., L. Nigi, G. Sebastiani and F. Dotta (2015). "MicroRNAs: Novel Players in the Dialogue between Pancreatic Islets and Immune System in Autoimmune Diabetes." Biomed Research International.

Vikman, J., X. Ma, G. H. Hockerman, P. Rorsman and L. Eliasson (2006). "Antibody inhibition of synaptosomal protein of 25 kDa (SNAP-25) and syntaxin 1 reduces rapid exocytosis in insulin-secreting cells." J Mol Endocrinol **36**(3): 503-515.

Vikman, J., H. Svensson, Y. C. Huang, Y. Kang, S. A. Andersson, H. Y. Gaisano and L. Eliasson (2009). "Truncation of SNAP-25 reduces the stimulatory action of cAMP on rapid exocytosis in insulin-secreting cells." Am J Physiol Endocrinol Metab **297**(2): E452-461.

Winter, J., S. Jung, S. Keller, R. I. Gregory and S. Diederichs (2009). "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nature Cell Biology **11**(3): 228-234.

Yakar, S., J. L. Liu, A. M. Fernandez, Y. Wu, A. V. Schally, J. Frystyk, S. D. Chernausek, W. Mejia and D. Le Roith (2001). "Liver-specific igf-1 gene deletion leads to muscle insulin insensitivity." Diabetes **50**(5): 1110-1118.

Yin, D. D., E. B. Zhang, L. H. You, N. Wang, L. T. Wang, F. Y. Jin, Y. N. Zhu, L. H. Cao, Q. X. Yuan, W. De and W. Tang (2015). "Downregulation of lncRNA TUG1 affects apoptosis and insulin secretion in mouse pancreatic beta cells." Cell Physiol Biochem **35**(5): 1892-1904.

You, L., N. Wang, D. Yin, L. Wang, F. Jin, Y. Zhu, Q. Yuan and W. De (2016). "Downregulation of Long Noncoding RNA Meg3 Affects Insulin Synthesis and Secretion in Mouse Pancreatic Beta Cells." J Cell Physiol **231**(4): 852-862.

Zhang, W., A. Khan, C. G. Ostenson, P. O. Berggren, S. Efendic and B. Meister (2002). "Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats." Biochem Biophys Res Commun **291**(4): 1038-1044.

Zhang, Z., Z. Zhu, K. Watabe, X. Zhang, C. Bai, M. Xu, F. Wu and Y. Y. Mo (2013). "Negative regulation of lncRNA GAS5 by miR-21." Cell Death and Differentiation **20**(11): 1558-1568.

Zhang, Z. W., L. Q. Zhang, L. Ding, F. Wang, Y. J. Sun, Y. An, Y. Zhao, Y. H. Li and C. B. Teng (2011). "MicroRNA-19b downregulates insulin 1 through targeting transcription factor NeuroD1." Febs Letters **585**(16): 2592-2598.

Zhou, X., J. Ruan, G. Wang and W. Zhang (2007). "Characterization and identification of microRNA core promoters in four model species." PLoS Comput Biol **3**(3): e37.

Zhou, Y. P., C. G. Ostenson, Z. C. Ling and V. Grill (1995). "Deficiency of pyruvate dehydrogenase activity in pancreatic islets of diabetic GK rats." Endocrinology **136**(8): 3546-3551.

Zhu, L., L. Chen, C. M. Shi, G. F. Xu, L. L. Xu, L. L. Zhu, X. R. Guo, Y. H. Ni, Y. Cui and C. B. Ji (2014). "MiR-335, an Adipogenesis-Related MicroRNA, is Involved in Adipose Tissue Inflammation." Cell Biochemistry and Biophysics **68**(2): 283-290.

Paper I

SCIENTIFIC REPORTS

OPEN

Elevated miR-130a/miR130b/miR-152 expression reduces intracellular ATP levels in the pancreatic beta cell

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MicroRNAs have emerged as important players of gene regulation with significant impact in diverse disease processes. In type-2 diabetes, in which impaired insulin secretion is a major factor in disease progression, dysregulated microRNA expression in the insulin-secreting pancreatic beta cell has been widely-implicated. Here, we show that miR-130a-3p, miR-130b-3p, and miR-152-3p levels are elevated in the pancreatic islets of hyperglycaemic donors, corroborating previous findings about their upregulation in the islets of type-2 diabetes model Goto-Kakizaki rats. We demonstrated negative regulatory effects of the three microRNAs on pyruvate dehydrogenase E1 alpha (PDHA1) and on glucokinase (GCK) proteins, which are both involved in ATP production. Consequently, we found both proteins to be downregulated in the Goto-Kakizaki rat islets, while *GCK* mRNA expression showed reduced trend in the islets of type-2 diabetes donors. Overexpression of any of the three microRNAs in the insulin-secreting INS-1 832/13 cell line resulted in altered dynamics of intracellular ATP/ADP ratio ultimately perturbing fundamental ATP-requiring beta cell processes such as glucose-stimulated insulin secretion, insulin biosynthesis and processing. The data further strengthen the wide-ranging influence of microRNAs in pancreatic beta cell function, and hence their potential as therapeutic targets in type-2 diabetes.

Insulin secreted from the pancreatic beta cells is indispensable for maintaining glucose homeostasis in healthy individuals. The molecular events accounting for the insulin secretory response of beta cells to elevated blood glucose are called stimulus-secretion coupling. This process consists of key cellular events: glucose uptake and metabolism to elevate cytosolic ATP/ADP ratios, closure of K_{ATP} channels leading to membrane depolarization, and opening of voltage-sensitive calcium channels causing influx of calcium ions, which ultimately facilitates insulin granule exocytosis[1]. Other nutrients including amino acids and free fatty acids, as well as incretins such as glucagon-like peptide 1 (GLP-1), potentiate insulin secretion. All of these, with the exception of a few amino acids, require the presence of glucose, underlining the central role of mitochondrial glucose metabolism in insulin secretion[2].

The deterioration of glucose-stimulated insulin secretion (GSIS) in the pancreatic beta cell is an early sign of type-2 diabetes (T2D), even preceding insulin resistance in the target tissues[3]. Indeed, genome-wide association studies (GWAS) implicate dozens of genes with important roles in pancreatic beta cell function[4]. Consequently, functional deficiencies in the processes of stimulus-secretion coupling ultimately cause defective insulin secretion. Although there is a canonical understanding of the biochemistry underlying stimulus-secretion coupling in the pancreatic beta cells, the various molecular genetic mechanisms regulating its individual components are incompletely understood.

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The important roles and functional implications of non-coding RNAs in pancreatic beta cell development and physiology are widely recognized[5,6]. For instance, specific microRNAs (miRNAs) have been shown to be involved in the different aspects of GSIS[5]. Mature miRNAs generally bind the 3′UTR region, but may also bind within the coding sequence (CDS) of the target mRNA which leads to degradation, deadenylation and/or translational repression, with the net effect of reduced protein expression of the target[7]. The significance of miRNAs for maintaining beta cell identity is particularly highlighted by the contribution of miR-29a/b in the constitutive repression of the *MCT-1* (monocarboxylate transporter) gene. This gene transcribes the pyruvate/lactate transporter MCT-1, which is "disallowed/forbidden" in the beta cells to prevent muscle-derived pyruvate to stimulate insulin release during exercise[8,9].

We previously showed dysregulated expression of many miRNAs in the pancreatic islets of Goto-Kakizaki (GK) rats[10], a well-studied rodent model of T2D primarily characterized by impaired GSIS[11]. The polygenic effects from at least three *Niddm* (non-insulin dependent diabetes mellitus) loci were discovered to affect insulin release and cause hyperglycaemia[12]. Interestingly, the molecular lesions characterizing impaired GSIS in the GK beta cell were found to be diverse, ranging from decreased expression of certain components of the secretory machinery, *i.e.* exocytotic proteins[13,14], perturbed adrenergic signalling[15] and glucose metabolism[16], to reduced activity of enzymes in specific biochemical pathways, *e.g.* deficient pyruvate dehydrogenase activity in mitochondria[17].

The upregulated miRNAs in the GK islet can down-regulate the expression of exocytotic proteins, thereby leading to reduced insulin secretion and hyperglycaemia in the animals[10]. In addition, we found putative targets of upregulated GK islet miRNAs involved in other aspects of stimulus-secretion coupling. Here, we investigated the effect on GSIS of three upregulated GK islet miRNAs: miR-130a-3p (miR-130a), miR-130b-3p (miR-130b) and miR-152-3p (miR-152), in the context of cellular metabolism by direct measurement of cytosolic ATP in live single insulin beta cells using PercevalHR, a genetically-encoded fluorescent reporter of ATP:ADP ratio[18–20].

We modulated the miRNA levels in the beta cell line, INS-1 832/13 and focused on gene targets relevant for ATP production: (i) the *Pdha1* gene, which codes for the E1 alpha subunit of the multi-enzyme complex pyruvate dehydrogenase (PDH) in the mitochondria, and (ii) the *Gck* (glucokinase) gene, which is the recognized "glucose-sensor" of pancreatic beta cells, a key regulating enzyme catalysing the phosphorylation of glucose as the first step of the glycolytic pathway[21]. We also investigated the effect of elevated levels of the miRNAs in known ATP-requiring processes such as in pro-insulin to insulin conversion[22,23].

Specific deletion of *Pdha1* in mouse beta cells (β-PDHKO) results in deficiency in PDH activity, impaired GSIS and development of hyperglycaemia[24]. Regarding glucokinase, the heterozygote inactivating mutation in this gene is the first reported sub-type of the maturity-onset diabetes of the young (MODY) causing reduced insulin secretion, and hence hyperglycaemia[25,26].

Here, corroborating our previous findings in the T2D model GK rat islets, we report the elevated expression of miR-130a, miR-130b and miR-152 in human islets from donors with impaired glucose tolerance (IGT) and T2D. We consequently found reduced expression in the protein level of both GCK and PDHA1 in the pancreatic islets of GK rats, while there was a trend of decreased *GCK* mRNA expression in islets from T2D donors. We then dissected how the miRNAs influence GSIS via their negative regulatory effects on glucose metabolism in the pancreatic beta cells. Specifically, we show that the negative regulation of *Pdha1* or *Gck* by the miRNAs, can partially account for the reduced cytosolic ATP observed in beta cells with abnormally high levels of miR-130a/b and/or miR-152. Overall, our results support the contribution of dysregulated miRNA expression to the impaired regulation of beta cell stimulus-secretion coupling, a hallmark of beta cell failure in the development of T2D.

Results

Upregulation of miR-152, miR-130a and miR-130b in GK rat islets, and in human islets from donors with impaired glucose tolerance and type-2 diabetes. We previously determined that miR-152 and miR-130a were among the 24 upregulated miRNAs in the islets of the T2D model GK rat, compared to those of Wistar controls using a locked nucleic acid (LNA)-based miRNA array profiling approach[10]. Here, we validated our array findings by qPCR, and showed that miR-130b, which harbours an identical seed sequence as miR-130a and belongs to the miR-130 gene family, was also upregulated in the pancreatic islets of hyperglycaemic GK rats (Fig. 1A).

We hypothesized that the pancreatic islets from hyperglycaemic human donors would likewise exhibit elevated levels of miR-152 and miR-130a/b and therefore grouped the human islets according to the glycated haemoglobin (% HbA1c) levels of the donors, *i.e.*, a long-term measure of glycaemia. The characteristics of human pancreatic islet donors are in Supplementary Table 1. We found that the levels of miR-152, miR-130a and miR-130b were upregulated in the islets of hyperglycaemic donors (IGT/T2D) compared to those of normoglycemic (NGT) donors (Fig. 1B). Moreover, despite different chromosomal locations of the three miRNAs in the human genome (chr11/miR-130a; chr22/miR-130b; chr17/miR-152), there was a notable co-expression among them, indicating highly-coordinated transcriptional regulation of these miRNAs in the human pancreatic islets (Fig. 1C).

Effect of modulating miR-130a, miR-130b, and miR-152 levels on insulin secretion in INS-1 832/13 cells. To dissect the effect of the three miRNAs in pancreatic beta cell stimulus-secretion coupling, we performed transient over-expression or knockdown of the miRNAs using mature miRNA mimics or LNA anti-miRs, respectively, in INS-1 832/13 cells (Supplementary Fig. S1). We hereafter refer to over-expression and knockdown of specific miRNA with the prefix "OE" and "LNA", respectively. The controls were scrambled oligonucleotides referred to as SCR.

OE152 and OE130a resulted in 15–20% reduced GSIS and up to ≈40% reduced insulin content compared to SCR (Fig. 2A,B). When miR-130a and miR-152 in combination were each overexpressed at half the amount of final concentration than when each miRNA was overexpressed separately, similar magnitude of reduction was

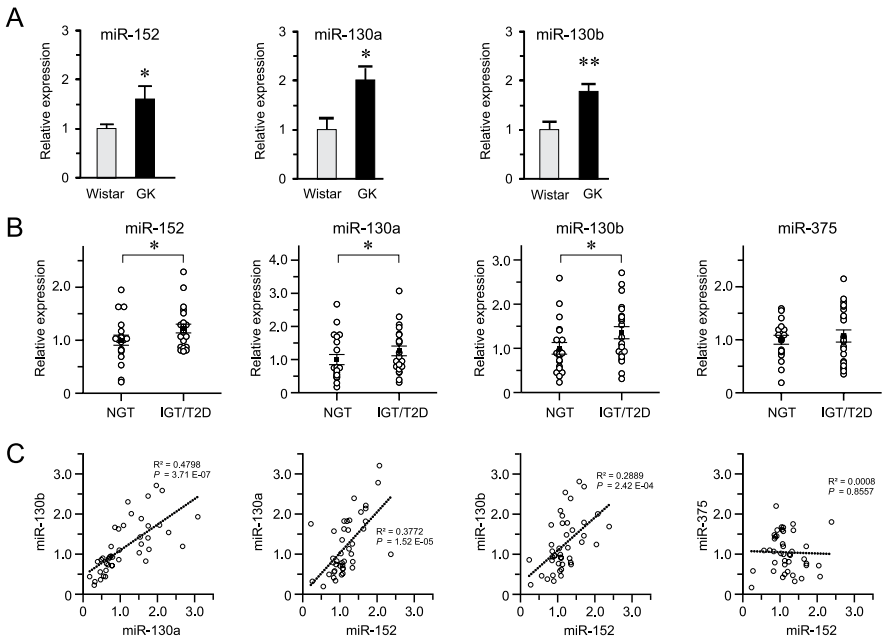

Figure 1. Over-expression and co-expression of miR-152, miR-130a, and miR-130b in pancreatic islets. **(A)** Validation of miR-152, miR-130a, and miR-130b upregulation in GK rat islets by qPCR. Expression of each miRNA was normalized to U87 rat and U6 snRNA expression. Wistar expression level was used as calibrator and relative quantification was done by using the ΔΔCt method. **(B)** Expression in the human pancreatic islets. (NGT = Normal glucose tolerance, IGT = Impaired glucose tolerance, T2D = Type-2 diabetes). RNU48 was used as the normalizer, and relative quantification by ΔΔCt method. **(C)** Co-expression analysis of miRNA expression using simple linear regression with F-test to determine significance at $p < 0.05$. Student's t-test (unpaired, two-sided) was used to determine significance in Wistar (n = 4) vs GK (n = 11) rat islets. For the human qPCR data (NGT, n = 20; IGT/T2D, n = 22), Mann-Whitney U test (unpaired, one-sided) was used to determine the significance. Error bars where present are S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$.

observed for GSIS and insulin content, demonstrating the additive effect of miR-152 and miR-130a. Activation of insulin secretion independent of ATP by addition of 50 mM KCl in the presence of low levels of glucose did not show any reduction in insulin secretion upon miRNA overexpression (Fig. 2C). Interestingly, a significant increase in insulin secretion was seen in OE130b cells, although this increase was not significantly different when compared to OE130a. To determine whether the maturation of insulin is also affected by elevated miRNA levels, we measured the total proinsulin-to-insulin ratio in miRNA overexpressing cells. OE152 cells showed up to ≈50% increased proinsulin-to-insulin ratio, while OE130a and OE130b cells had ≈25% significant increased ratio (Fig. 2D). Taken together, these results suggest that exocytotic processes distal to plasma membrane depolarization are intact[27,28] and that the main negative effect of the upregulated miRNAs under study is on insulin content levels, possibly influenced by upstream insulin maturation processes.

In contrast to the effect of miRNA-overexpressing beta cells, LNA152, LNA130a and LNA130b resulted in 20–30% increase in GSIS and up to ≈45% increased insulin content vs SCR control (Fig. 2E,F). These results further support the direct regulatory effect of the three miRNAs in stimulus-secretion coupling in the beta cells, and imply that insulin secretion may be positively influenced by reducing the levels of specific miRNAs.

Identification of mRNA targets. To determine the putative targets through which the miRNAs under study may regulate insulin secretion in the beta cell, we performed bioinformatics analysis by using multiple target prediction algorithms as implemented in the web-server miRWalk2.0[29]. Previous reports regarding decreased glucokinase (*GCK*) mRNA expression in the pancreatic islets of T2D humans[30] and GK rats[31], and deficiency of pyruvate dehydrogenase activity[17] in the pancreatic islets of diabetic GK rats led us to focus on the potential targeting of miR-130a/b and miR-152 within the 3′UTR and coding sequence (CDS) regions of *GCK/Gck* and *PDHA1/Pdha1* in human/rat (Supplementary Table 2).

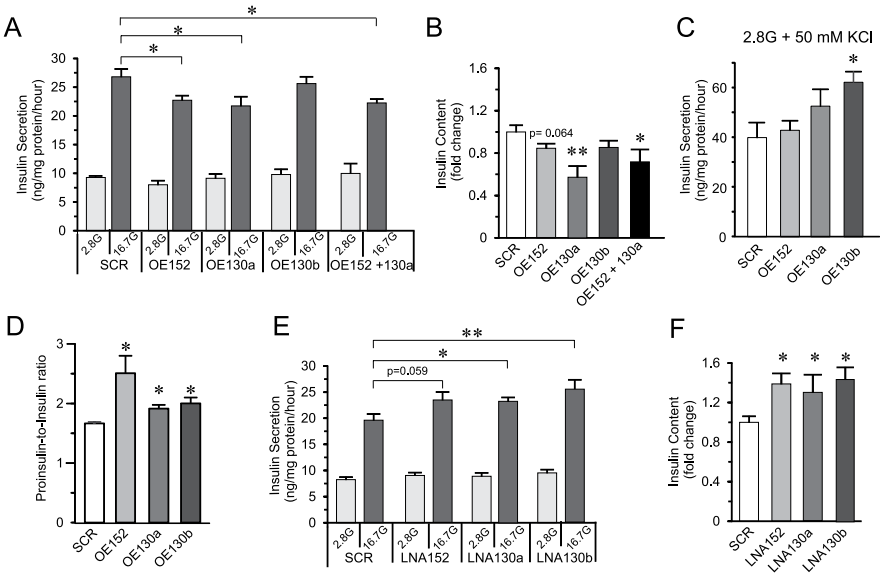

Figure 2. Effect of over-expression or knock-down of miRNAs on GSIS. (A) Insulin secretion measured upon over-expression (OE) of miRNAs (OE152, OE130a and OE130b) or combination of OE152 and OE130a in INS-1 832/13 cells. **(B)** Measurement of insulin content upon miRNA over-expression in INS-1 832/13 cells. **(C)** Insulin secretion of OE-miRNA cells at 2.8 G with 50 mM KCl. **(D)** Levels of pro-insulin to insulin ratio in OE-miRNA cells. **(E,F)** Insulin secretion, and content measurement upon knock-down of miRNAs using LNA (LNA152, LNA130a, LNA130b) in INS-1 832/13 cells, respectively. For all experiments, data are presented as average of n = 3–4 biological replicates each with 3–4 technical replicates. Data are mean ± SEM, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs scramble using Student's two-sided t-test.

To lend support to the hypothesis that the three upregulated miRNAs via downregulation of putative targets, GCK and PDHA1, are contributing to perturbed functions of pancreatic islet cells in the T2D model GK rats and human T2D patients, we measured their expression by qPCR and/or western blot. In the GK rat islets, we observed downregulation of the *Gck* mRNA but no significant change in *Pdha1* mRNA (Fig. 3A). Nonetheless, the protein levels of both GCK and PDHA1 were considerably reduced (Fig. 3B). For the human islets, due to limited amount of sample, we could only measure mRNA levels by qPCR (Fig. 3C). We did not find significant differences in the mRNA expression of *PDHA1* or *GCK* in the islets of controls vs T2D donors, although we saw a clear trend of reduced *GCK* mRNA expression in T2D islets (p = 0.07) which agrees with previous findings regarding the reduced *GCK* mRNA in a larger cohort of T2D islets[30]. Notably in the human islets, we also demonstrated a trend of negative correlation between *GCK* mRNA expression and the long-term indicator of glycaemic control, HbA1c status of the donors (Fig. 3D).

In the rat islet samples, we clearly observed a larger differential regulation between GK and Wistar in the protein level (Fig. 3B) than in the mRNA level (Fig. 3A). This indicates that the miRNAs in this case are acting on the level of translational repression of target genes. As shown in the case of *Pdha1* in rat islets, the mRNA levels were not differentially-regulated between GK and Wistar, but the protein level was ≈75% lower in the GK rat islets. The *Pdha1* and *Gck* mRNA expression patterns exhibited in the rat islets between GK and Wistar (Fig. 3A) were strikingly similar with those exhibited in the human islets between control and T2D donors (Fig. 3C). We therefore hypothesize that the protein levels of PDHA1 and GCK will be similarly reduced in the human islets from T2D donors.

As a first indication of the miRNA-mRNA regulatory interactions in the beta cell, we observed reciprocal expression patterns of the miRNAs and their putative targets after just one-hour incubation of INS-1 832/13 cells in 2.8 mM (low) and 16.7 mM (high) glucose. The expression of all the three miRNAs were 60–80% lower in high glucose compared to low glucose (Supplementary Fig. S2A), whereas the corresponding GCK and PDHA1 targets showed ≈25% and ≈50% increased protein levels, respectively (Supplementary Fig. S2B).

We then demonstrated negative regulatory effect of the miRNAs on the putative targets by over-expression of the miRNAs. We found that miR-130a, miR-130b and miR-152 or combination of miR-130a/miR-152 resulted in significant decrease in the expression of *Pdha1* both in the mRNA and protein levels (Fig. 4A,B). The combined

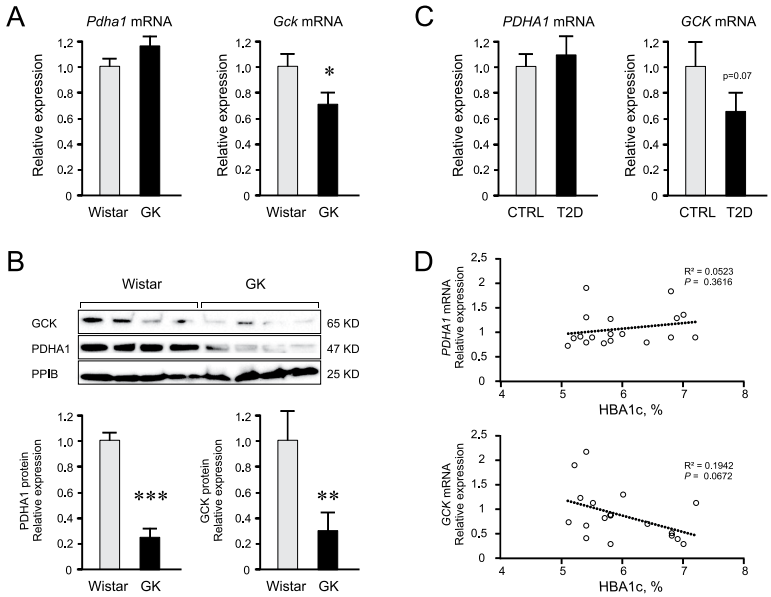

Figure 3. Expression of *Pdha1*/*Gck* in rat or *PDHA1*/*GCK* in human islets, and correlations to donor HbA1c levels. (A) Reduced mRNA levels of *Gck* in the GK (n = 11) islets compared to those of control Wistar (n = 4). **(B)** Reduced protein levels of both PDHA1 and GCK in the GK (n = 9–11) compared to Wistar (n = 4) islet preparations. Blots were cut around specified molecular weights prior to separate probing with specific antibodies. Unedited blots are shown in Supplementary Fig. S4A. **(C)** Reduced trend of *GCK* mRNA expression in the islets from T2D donors (n = 9) compared to controls (n = 10). **(D)** Negative trend of correlation between *GCK* mRNA expression and donor HbA1c levels (n = 18) using simple linear regression with F-test to determine significance at $p < 0.05$. For differential expression of mRNA or protein levels, Student's t-test (unpaired, one-sided) was used to determine significance. Error bars where present are S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

miRNAs were each transfected at half the concentrations, compared to when transfected individually, and although their effect on the level of *Pdha1* expression seemed to be greater, the difference was not found to be statistically significant. For *Gck* targeting, we found reduced GCK protein by ≈35% and ≈25% in OE130a and OE130b cells, respectively (Fig. 4B). Additionally, we also observed reduced *Gck* expression both in the mRNA and protein levels, upon miR-152 overexpression (Fig. 4A,B).

To validate direct biochemical interactions between the miRNAs and target mRNAs, we utilized the anti-AGO2 RNA immunoprecipitation (RIP) assay, using anti-AGO2 to isolate both the miRNA and the interacting target mRNA 3′UTR fragment in INS-1 832/13 cells. We observed a significant increase in the levels of the interacting *Pdha1* 3′UTR in AGO2 RIP compared to the non-specific binding control IgG RIP in response to OE152 (Fig. 4C). However, we found no enrichment of the *Pdha1* 3′UTR fragment nor of the *Gck* 3′UTR fragment predicted to interact with miR-130a (Supplementary Fig. S3). These results provide strong evidence that at least miR-152 via AGO2 association, directly targets *Pdha1* resulting in both reduced transcript and protein expression levels.

Over-expression of miRNAs decreased ATP content and cytosolic ATP:ADP ratios in INS-1 832/13 cells. To resolve the net effect of the upregulated miRNAs on cellular energy metabolism in the beta cells, we analysed the oligomycin-sensitive ATP content by luciferase-based luminescent assay. Compared to controls, the cells overexpressing the miRNAs failed to increase their ATP levels upon incubation from 2.8 mM to 16.7 mM glucose, exhibiting substantial decrease, 40–50%, in ATP content at high glucose concentration (Fig. 5A).

A limitation of measuring ATP content in whole cell extracts is that total, and not metabolically regulated, ATP levels are determined. Furthermore, it is an end-point determination. Therefore, we utilized the genetically-encoded PercevalHR ATP sensor to assess the dynamics of ATP concentration changes as the ATP:ADP ratio in live single cells[20]. We observed profound qualitative differences in the trajectory of cytosolic ATP levels among the different miRNA-overexpressing (miRNA-OE) cells and controls during GSIS (Fig. 5B–D).

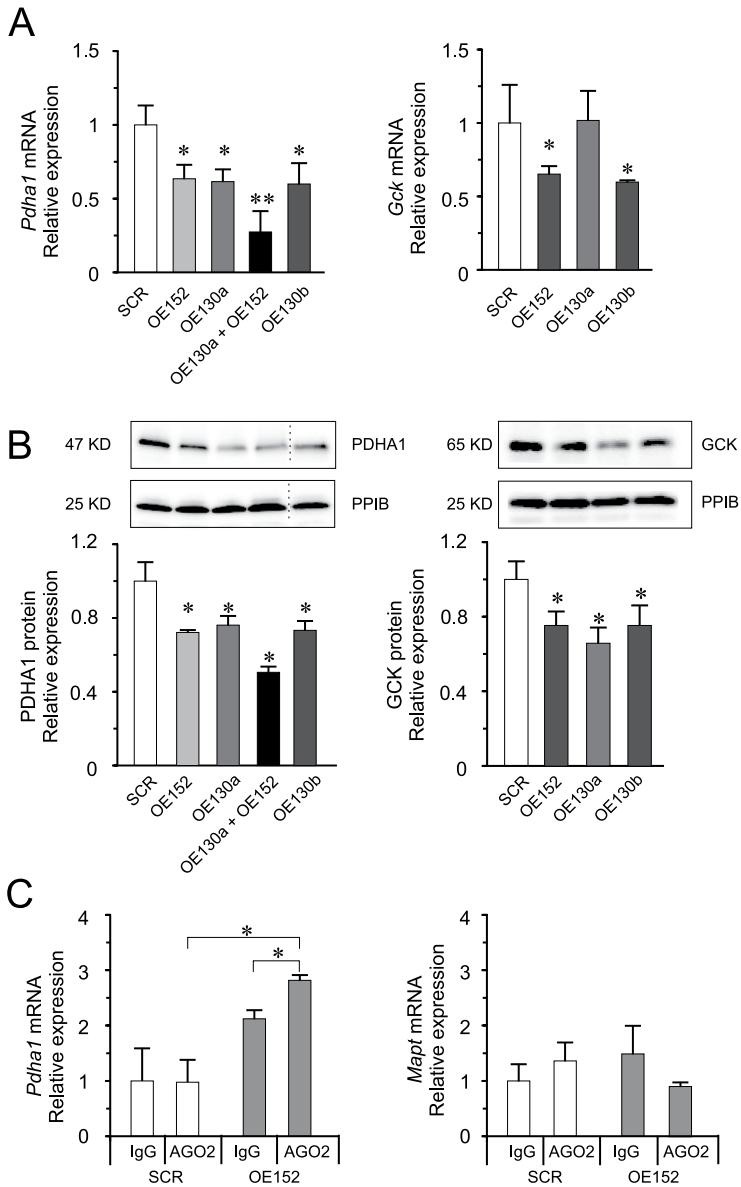

Figure 4. Validation of miRNA-target interactions. (A) Reduced mRNA levels of *Pdha1* and *Gck* upon miRNA overexpression. **(B)** Reduced protein levels of PDHA1 and GCK upon miRNA over-expression. OE130b lane was derived from a separate western blot run. Unedited blots are shown in Supplementary Fig. S4B. **(C)** Expression of 3′UTR target region of *Pdha1* or *Mapt* (non-specific control) after co-immunoprecipitation with anti-AGO2 in OE152 cells. Data are presented as mean ± SEM. n = 3–6, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs SCR using Student's two-sided test.

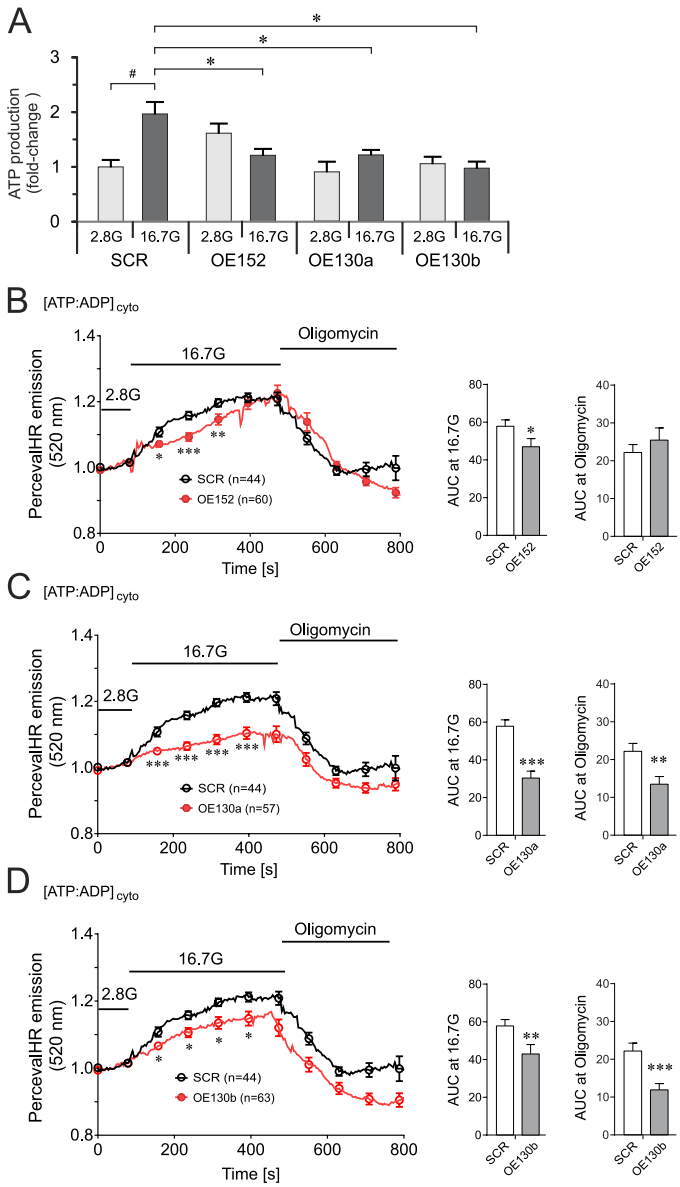

Figure 5. ATP production and cytosolic ATP dynamics in INS-1 832/13 cells upon miRNA-overexpression. **(A)** Luciferase-based luminescent detection of total ATP content in INS-1 832/13 upon over-expression of miRNAs. ATP produced at 2.8 mM glucose in SCR was used as calibrator. Data are presented as mean ± SEM. n = 3, $^{*}p < 0.05$, one-way ANOVA with Dunnett's post-hoc. **(B)** OE152, **(C),** OE130a, and **(D)** OE130b PercevalHR ATP:ADP ratio fluorescent reporter measurement in single-cells during GSIS, and oligomycin treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ using Mann-Whitney U test (unpaired, two-sided).

We then calculated the area under the curve (AUC) during the period of high glucose stimulation, as well as the AUC in the presence of the ATP synthase blocker, oligomycin. In agreement with decreased ATP content in the cells at stimulatory glucose concentration upon miRNA overexpression, we observed reduced cytosolic ATP:ADP ratios in all miRNA-OE cells at 16.7 mM glucose (Fig. 5B–D).

Interestingly, while all the miRNA-OE cells displayed significantly lower ATP:ADP ratios at 16.7 mM glucose, the magnitude of decrease was smaller for OE152 cells. Moreover, as opposed to OE130a or OE130b cells, the decrease in ATP:ADP ratios for OE152 cells upon oligomycin treatment was not significantly reduced compared with the controls (AUC graphs Fig. 5B–D).

Oligomycin treatment effectively blocks ATP synthase within the mitochondria, forcing glycolysis to provide for cellular ATP production. Therefore, one might expect similar defect in cytosolic ATP/ADP ratio between OE152 and OE130a/b since all these miRNAs can negatively regulate the primary glycolytic enzyme, GCK. However, one may also argue that miR-130a/b can have other target enzymes within the glycolytic pathway which contribute to the further reduction of cytosolic ATP/ADP upon oligomycin-mediated inhibition of mitochondrial ATP synthase. We therefore performed more in-depth bioinformatics prediction for miR-130a binding to other glycolysis-related enzymes[32] in the rat beta cell such as in phosphofructokinase (*Pfkm*), pyruvate kinase (*Pklr*), malic enzyme (*Me1*), ATP-citrate lyase (*Alcy*), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). However, using bioinformatics prediction we did not find any of these genes to be potentially targeted by miR-130a/b. Another line of reasoning is that miR-152 may target genes that require cytosolic ATP such that down-regulation of these targets will result in eventual accumulation of cytosolic ATP concentration as observed in the OE152 cells. Indeed, when we performed gene ontology (GO) enrichment on predicted targets unique to miR-152, we saw significant enrichment for the gene ontology molecular function category called "ATP-binding" (Benjamini corrected p-value = 0.013). We did not see enrichment in ATP-binding category for genes containing only miR-130a/miR-130b putative binding sites (Supplementary Table 3). Among the 172 ATP-binding genes with unique putative miR-152 binding sites are the highly expressed adenylyl cyclase in the pancreatic islets, *Adcy5* and *Adcy6*, involved in the conversion of ATP to cAMP[33] and two Na^+/K^+ ATPase transporting subunit genes (*Atp1a2* and *Atp1a3*) involved in establishing and maintaining the electrochemical gradients of Na^+ and K^+ ions across the plasma membrane. For this reason, we hypothesize that overexpression of miR-152, aside from targeting proteins required for ATP-generation such as *Pdha1* and *Gck*, they may also target genes that require ATP in fundamental cellular processes in the beta cell.

Knock-down of PDHA1 in INS-1 832/13 reduces GSIS, ATP production and cytosolic ATP levels. Since miRNAs may have multiple targets, we wanted to confirm whether the effect of miRNA-mediated downregulation of PDHA1 on beta cells can be recapitulated by directly reducing PDHA1 levels by siRNA. We attained ≈70% knockdown of *Pdha1*, both in mRNA and protein levels, in INS-1 832/13 cells (Fig. 6A). This resulted in ≈40% reduced insulin secretion at both 16.7 mM glucose and at 50 mM KCl, and 50% reduction in insulin content (Fig. 6B–C). We saw reduced pro-insulin levels which resulted in no significant change in the proinsulin-to-insulin ratio in the PDHA1 KD cells (Fig. 6D). This indicates that the rate of insulin maturation is intact. The knock-down of *Pdha1* also resulted in reduced ATP content by ≈40% (Fig. 6E), and significantly lower levels of cytosolic ATP:ADP ratios during GSIS (Fig. 6F). To summarize, the similar beta cell functional deficiencies observed between the OE152 cells and PDHA1 KD cells, support the miR-152- mediated control of GSIS at the level of cellular metabolism via the negative regulation of PDHA1.

Discussion

In this study, we showed that islets from hyperglycaemic human donors contain elevated levels of miR-130a, miR-130b and miR-152. We further validated the over-expression of the same miRNAs in the pancreatic islets of the T2D GK rat model. This suggests that similar pathophysiological processes leading to beta cell dysfunction, mediated by the dysregulated miRNAs, may be at play. The remarkable co-expression among the three miRNAs in human pancreatic islets also indicates a convergent transcriptional regulatory response to environmental stimuli.

The mechanism by which the three miRNAs in this study are upregulated in the pancreatic islets of GK rats and T2D humans is under investigation. Of note, the miR-152 and miR-130a are among the 24 miRNAs we previously showed to be upregulated in the pancreatic islets of GK rats[10]. We hypothesized that this was due to a perturbed transcriptional regulation and we therefore performed promoter analysis to find out DNA sequence motifs common to the upregulated miRNAs. However we only found common DNA sequence motif in the promoter region of two other upregulated miRNAs in the GK rat islets, miR-132 and miR-212[34]. In that study our group showed putative transcription factor binding sites for Calmodulin Binding Transcription Activator 1 (Camta1) and NK2 homeobox protein, Nkx2-2. Elsewhere, we and others also demonstrated cAMP-dependent regulation of the miR-132/212 cluster through a PKA-dependent mechanism[35] involving cAMP-response element (CRE)-binding proteins and CRTC1[36]. In *db*/*db* and high-fat diet fed mice, miR-132 and miR-184 in the pancreatic islets were suggested to be induced by hyperglycaemic and hyperlipidaemic conditions typically encountered in prediabetic and diabetic states[37]. Concerning the miRNAs studied here, we previously showed that miR-130a expression is downregulated with increased glucose concentration in Wistar rat islets[10], which was supported by our findings in INS-1 832/13 cells for miR-130a, miR-130b and miR-152 (Supplementary Fig. S2).

Most miRNA profiling studies done so far in relation to diabetes have been exploratory in nature and the exact mechanism of deregulated miRNA expression is largely unexplored. In one miRNA profiling study of human type-2 diabetes islets, a cluster of miRNAs in the so called DLK1-MEG3 locus at chromosome 14q32 was found to be imprinted and containing a promoter region that is hypermethylated leading to downregulation of a dozen miRNAs[38]. However, it was not established whether the aberrant hypermethylation was the cause or consequence of the disease state. Investigating the mechanisms causing dysregulated miRNA expression in pathophysiological conditions is therefore a topic of utmost importance.

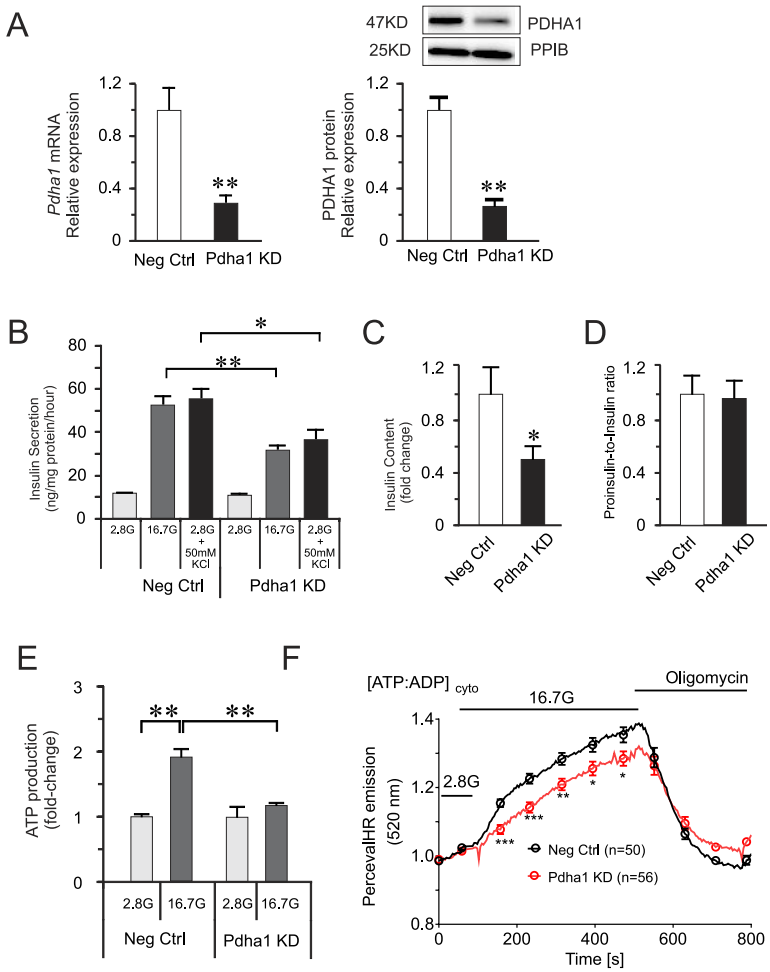

Figure 6. Effect of siRNA knock-down of PDHA1 on GSIS and ATP dynamics. (A) Expression of *Pdha1* in INS-1 832/13 cells after knock-down. Unedited blots are shown in Supplementary Fig. S4C. **(B)** Insulin secretion, **(C)** Insulin content and, **(D)** proinsulin-to-insulin ratio measurement after ≈70% knock-down of PDHA1 in INS-1 832/13. The data are presented as mean ± SEM of n = 3–5 biological replicates, *p < 0.05 and **p < 0.01 vs Neg Ctrl using Students two-tailed test or one-way ANOVA with Dunnett's post-hoc for multiple groups. **(E)** ATP content upon PDHA1 knock-down. **(F)** PercevalHR ATP:ADP ratio fluorescent reporter measurement. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Mann-Whitney U test (unpaired, two-sided).

Impaired GSIS is the hallmark phenotype of the GK rat, reflecting the pancreatic beta cell dysfunction in humans with T2D[11]. In our previous investigation of global miRNA expression in GK rat islets, we focused on the negative impact of the dysregulated miRNAs on the insulin exocytotic process, compatible with previous and recent findings on reduced exocytotic proteins in the pancreatic islets of the GK rats[13,14], and in those of T2D donors[39]. Here, we instead elucidated the contribution of dysregulated miRNAs in beta cell energy metabolism. Prompted by previous findings regarding the deteriorating expression and/or dysfunction of key metabolic enzymes in the beta cell of GK rats and in the islets of T2D individuals, such as glucokinase[30,31] and PDHA1[17], we concentrated our target validation on these two genes which are among the many putative mRNA targets of the

miRNAs under study. Consequently, we found reduced PDHA1 and GCK proteins in the GK islets, and a trend for reduced *GCK* mRNA expression in the islets of human T2D in agreement with previous findings[30].

Subsequently, we could show negative regulatory effects of miR-130a and miR-130b on glucokinase and PDHA1, and the direct biochemical interaction of miR-152, with *Pdha1* mRNA using the AGO2 RIP assay. However, it has been found in mammalian cells that other non-catalytic AGO proteins such as AGO1 and AGO3 may also be equally loaded with distinct[40] or random[41] miRNAs bound to mRNA in the RNA-induced Silencing Complex (RISC) destined for target repression. It is therefore plausible that the direct repression of PDHA1 or GCK by miR-130a may have occurred via AGO1 or AGO3. Taken together, the negative effect of the over-expressed miRNAs on their targets involved in energy metabolism resulted in reduced cytosolic ATP levels, and ultimately in impaired GSIS in the pancreatic beta cell.

PDHA1 is a component of the mitochondrial holoenzyme pyruvate dehydrogenase complex (PDC) which is responsible for the conversion of pyruvate to acetyl-CoA and CO_2, essentially linking glycolysis and the tricarboxylic acid (TCA) cycle. Robust insulin secretion in primary beta cells and insulin-secreting cell lines relies on the normal metabolic output emanating from the TCA cycle[32]. The catalytic components of PDC are known to be tightly-regulated, and maintaining stoichiometric amounts is essential for a functionally-active PDC[42]. Indeed, beta cell-specific deletion of PDHA1 alone in a murine model (β-PDHKO) led to a defect of GSIS both *in vitro* and *in vivo*, as well as reduced islet insulin content resulting in hypoinsulinemia and hyperglycemia in the animals[24]. Recent investigation on the β-PDHKO mice further revealed beta cell development and maturation defects, possibly due to deficient levels of several transcription factors with key roles in beta cell lineage commitment, such as PDX1, Neurogenin3, and NeuroD1[43]. Therefore, the pleiotropic effects of PDC on pancreatic beta cell development and function further highlight the role of glucose metabolism in the pathophysiology of impaired glucose homeostasis.

PDC-deficiency mainly caused by mutations in the *PDHA1* gene in humans are rare, and is mostly manifested as severe neuropathies leading to death at very young age[44]. Nonetheless, a report on a case of a child with PDH deficiency suffering from diabetes due to insufficient insulin[45] should prompt further investigation on the potential co-morbidity of diabetes with the neuropathies in this genetic disease. Indeed, many commonalities between the central nervous system and human islet pathophysiological features have been noted in the literature, e.g. effects of neuronal cell adhesion molecules, EPHs/ephrins, in beta cell GSIS[46].

We showed that the over-expression of the miRNAs under study, or the knockdown of the validated target, PDHA1, in a pancreatic beta cell line with robust insulin secretion capacity, resulted in changes in cellular bioenergetics, in which the net ATP output was substantially reduced. We could further resolve the ATP dynamics during GSIS in single cells using the PercevalHR reporter. The sensitivity of the PercevalHR system to physiological concentration range of ATP, reflecting the ATP:ADP ratio in the beta cells has previously been shown and found to reliably report changes in cellular metabolism[19,20]. This allowed us to see specific effects of the miRNAs on cytosolic ATP levels. For instance, we could observe that miR-152 impacted less on the ATP:ADP ratios during GSIS than either miR-130a or miR-130b. Moreover, the significant reduction of ATP levels after oligomycin treatment was only observed in miR-130a/b over-expressing cells. The similar ATP dynamics profile of miR-130a and miR-130b confirmed the similar regulatory targets of these miRNAs by virtue of their identical seed sequence.

The striking resemblance of ATP concentration changes during GSIS, and after inhibition of ATP synthase (oligomycin treatment) between OE152 cells and PDHA1 KD cells lend strong support to direct miR-152 mediated regulation of PDHA1. However, although both treatments resulted in impaired GSIS, the effect on insulin content was more pronounced when PDHA1 was directly silenced. These phenotypic discrepancies were due to expected differences in the magnitude of PDHA1 downregulation, i.e. miRNA-mediated control of PDHA1, was weaker than directly knocking down the levels of PDHA1 by siRNA. Additionally, the multiple targeting of miRNA may also lead to activation/inactivation of pathways influencing other beta cell functions which may not be sensitive to silencing a single target gene.

The transient modulation of miR-130a, miR-130b, or miR-152 in INS-1 832/13 cells by either over-expression or knock-down resulted in reciprocal effects on GSIS, but not KCl-induced insulin secretion agrees with targets primarily involved in modulating the cytosolic ATP concentration. Interestingly, the results on insulin secretion were related to changes in both insulin content, and proinsulin-to-insulin ratio. The biosynthesis and processing of mature insulin, and its subsequent packaging into granules destined for secretion are known to be ATP-requiring processes[22,23]. More important clinically, elevated proinsulin levels have previously been recognized as indicative of impaired beta cell secretory capacity in non-insulin-dependent diabetes mellitus[47].

A notable result in this study was the further improvement in both insulin secretion and insulin content upon LNA anti-miR treatment of the beta cells. These findings have considerable implication in the development of RNA-based novel therapeutics, which target miRNAs in the diseased beta cell. In this aspect, combinatorial modulation of multiple dysregulated miRNAs could be a more efficacious path in rectifying diseased states than just modulating levels of a single miRNA. Moreover, LNA anti-miRs could most likely be used in combination, if not replace certain drugs currently used to improve insulin secretion *e.g.* sulfonylureas. These compounds have long been utilized to improve insulin secretion through binding to the sulfonylurea subunit SUR1 of the ATP-dependent K^+ channel (K_{ATP} channel) affecting beta cell membrane potential[48]. Hypothetically, LNAs that knock down miR-130a/miR-130b/miR-152 would affect the glucose conversion pathway in the mitochondria and assist in stabilizing the intracellular ATP to an optimal level. ATP acts on the Kir6.2 subunit of the ATP-dependent K^+ channel and increased ATP leads to closure of the channel and membrane depolarization[48]. ATP could also directly amplify exocytosis of insulin granules[49]. Hence, this anti-miR treatment would most likely work in concert with sulfonylureas to improve control of insulin output from the beta cell.

To conclude, we could show that miR-130a, miR-130b and miR-152 influence the metabolic control of GSIS via modulation of ATP levels, partially through targeting of PDHA1 and GCK in the pancreatic beta cell (Fig. 7).

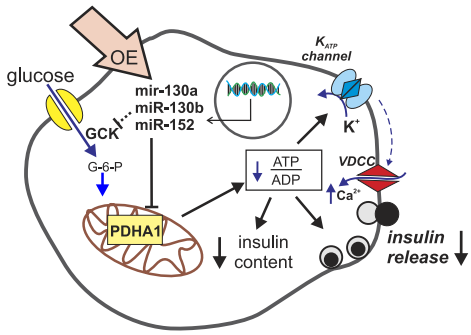

Figure 7. Model of miRNA impact on GSIS via regulation of genes involved in ATP production. In INS-1 832/13 cells, normal levels of miRNAs 130a/b and 152 has no detrimental effect on PDHA1 or GCK expression resulting in robust GSIS. Upon up-regulation of the miRNAs, PDHA1 and GCK levels are reduced contributing to profound reduction in cytosolic ATP:ADP ratio, which ultimately lead to both reduced insulin content and defective insulin release.

Modulating the expression of miRNAs to improve beta cell function in T2D is a promising approach but using this novel therapeutic tool requires further studies.

Methods

Ethical statement. For experiments involving human pancreatic islets, all procedures were approved by Uppsala and Lund University Ethics committees, and fully-complied with the guidelines and regulations as stated in the ethical permit, Dnr 2011/263 issued to Lund University Diabetes Centre (LUDC) and Excellence in Diabetes Research in Sweden (EXODIAB) regarding use of donated human tissues.

Experiments on rodents were performed in full-compliance of guidelines and regulations as stated in the ethical permit, M 105-15 issued by Malmö/Lund Ethical Committee on Animal Research.

Reagents. Unless otherwise stated, all chemicals were from Sigma Aldrich (MO, USA).

Human pancreatic islets. Human pancreatic islets from cadaver donors were procured from the Human Tissue Lab EXODIAB/LUDC through the Nordic Network for Islet Transplantation (http://www.nordicislets.org). Upon receipt, the islets were handpicked under stereomicroscope. The donors were grouped according to Fadista *et al.* based on the glycated haemoglobin (% HbA1c) levels[50]. Here we used pancreatic islets from normal glucose tolerant (NGT) donors (HbA1c $< 6\%$; $n = 20$), impaired glucose-tolerant donors (IGT) ($6\% \leq$ HbA1c $< 6.5\%$; $n = 11$), and T2D donors (HbA1c $\geq 6.5\%$; $n = 11$) (Supplementary Table 1).

Rat pancreatic islets. Pancreatic islets were isolated from male Goto-Kakizaki and control Wistar rats as previously described[10]. All animals were kept in standard 12-hour (h) light-dark cycle and were given standard chow and water *ad libitum*. The animals were used at 8–15 weeks of age, at which point the non-fasting blood glucose of GK rats (22.3 ± 1.2 mmol/L, N = 11) was significantly higher than those of Wistar controls (6.3 ± 0.2 mmol/L, N = 4) (Student's t-test, two-sided, $p < 0.001$).

Cell Culture. Rat INS-1 832/13 cell line[51] was maintained in a complete RPMI 1640 medium with 11.1 mM D-glucose supplemented with 10% Fetal Bovine Serum (FBS), 2% INS-1 supplement, 5 mL Penicillin/Streptomycin (10000 U/10 mg/mL) and 10 mM Hepes (HyClone, UT, USA). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

Over-expression and knock-down of miRNAs. INS-1 832/13 cells were seeded (300,000 cells per well) in a 24-well plate with 1 mL/well complete RPMI 1640 medium without antibiotics a day before transfection. Cells were transfected with mature miRNAs called PremiR™ miRNA Precursor from Life Technologies (CA, USA): PremiR-Scramble (AM17110), Premir-130a (PM105106), Premir-130b (PM10777), Premir-152 (PM12269), or miRcury LNA miRNA inhibitors from Exiqon (Denmark): LNA Scramble (#199005-00), LNA130a (#4102212-001), LNA130b (#4102260-001) and LNA152 (#4103524-001) or after reaching ≈60% confluence using Lipofectamine RNAiMAX. A final transfection volume of 600 μL per well contained 50 nM of Pre-miR or LNA in Opti-MEM reduced serum media and 1.5 μL of Lipofectamine RNAiMAX (Life Technologies, CA, USA). In cells transfected with combined Pre-miR-152 and Pre-miR-130a, the concentration of each Pre-miR was reduced to 25 nM. After 6 h transfection, 500 μL of complete RPMI 1640 medium without antibiotics were added to each well. Medium was changed to complete RPM1 1640 with antibiotics after 24 hours of transfection. Cells were assayed for insulin secretion after reaching ≈100% confluence, while protein and RNA samples were extracted from replicate wells at the same time, 72 h post-transfection.

siRNA knock-down of Pdha1. Using the same transfection protocol as when we overexpressed or knocked-down the miRNAs, we knocked-down Pdha1 in INS-1 832/13 cells in a 24-well plate using Silencer® Select Pre-Designed siPdha1 (#s131695, Life Technologies, CA, USA). Cells were assayed for insulin secretion after reaching ≈100% confluence, while protein and RNA samples were extracted from replicate wells at the same time, 72 h post-transfection.

Insulin Secretion Assay. Confluent plates of INS-1 832/13 cell lines were washed twice carefully with 1 mL pre-warmed Secretion Assay Buffer (SAB), pH 7.2 (1.16 mM $MgSO_4$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 114 mM NaCl, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 20 mM HEPES and 0.2% Bovine Serum Albumin) containing 2.8 mM glucose. Cells were then pre-incubated in fresh 2 mL SAB with 2.8 mM glucose for 2 h. The cells were stimulated for 1 h in 1 mL SAB with 2.8 mM glucose or 16.7 mM glucose or 2.8 mM glucose with 50 mM KCl at 37 °C. Insulin levels were measured using Coat-A-Count radioimmunoassay kit, according to the manufacturer's instructions (Millipore Corporation, MA, USA) and the values were normalized to total protein from each well. Total protein from each well was extracted by using 200 μL RIPA buffer: 0.1% SDS, 150 nM NaCl, 1% Triton X-100, 50 mM Tris-Cl, pH 8 and EDTA-free protease inhibitor (Roche, NJ, USA). The protein content was analyzed by BCA assay (Pierce, IL, USA) on Bio-Rad Model 6870 microplate reader.

Total insulin and Proinsulin measurement. Cells transfected with mature miRNAs or siRNA as described above were lysed in 200 μL RIPA buffer after 72 h post-transfection. Total insulin and proinsulin were determined using Mercodia High Range Rat Insulin ELISA and Rat/Mouse Proinsulin ELISA (Mercodia AB, Sweden) respectively according to the manufacturer's protocol.

RNA Extraction and quality control. Total RNA from INS-1 832/13 cells, GK rat and human islets was extracted by the Qiagen miRNeasy isolation kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The concentration of RNA was measured on a Nanodrop (ND-1000) spectrophotometer. The quality and integrity of RNA were evaluated by both spectrophotometry and electropherogram profiles using Nanodrop (ND-1000) and Experion's automated electrophoresis system (Bio-Rad, CA, USA), respectively.

Quantification of miRNAs and mRNAs by real-time quantitative PCR (qPCR). cDNA was generated by using High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems, CA, USA). qPCR was performed in triplicates on 384-well plate using Applied Biosystems 7900HT standard RT-PCR system under default cycling parameters. Specific primers and probes from TaqMan® MiRNA Assays (Applied Biosystems, CA, USA) were used to measure the expression levels of miR-130a-3p (#TM_000454), miR-130b-3p (#TM_000456), miR-152-3p (#TM_ 000475) and mRNA expression of their targets: Rat Pdha1 (Rn01424346_m1), Rat Gck (Rn00561265_m1), Human PDHA1 (Hs01049345_g1), and Human GCK (Hs01564555_m1). We used these snRNAs: U6 (#TM_ 001973) and U87 (#TM_ 001712) as endogenous controls for rat miRNA quantification or RNU48 (#TM_001006) for human miRNAs, while Rat Hprt1 (Rn_01527840_ml) and Rat Ppia (Rn_00690933_ml) or Human B2M (433766) and Human HPRT (4333768 F) were used for normalizing mRNA expression. All Taqman assays and qPCR reagents were purchased from Thermo Fisher. Relative quantification with multiple controls as applicable was done using the $\Delta\Delta C_t$ method.

Validation of miRNA target using Anti-AGO2 RNA Immunoprecipitation (RIP). Sigma's Imprint® RNA Immunoprecipitation Kit was used. RIP lysates from INS-1 832/13 cells (≈3 million cells per RIP) were immunoprecipitated with 2.5 μg of either rabbit IgG (I5006) or Anti-AGO2 antibody produced in rabbit (SAB4301150) according to the manufacturer's protocol. RT-qPCR analysis of RNAs isolated from RIP was performed using SYBR® Green JumpStart™ Taq ReadyMix™ (S4438) and the following 3' UTR primers to detect mRNA of either Pdha1 or Mapt (non-target negative control): miR-152/*Pdha1* forward primer: 5′-TGT ATT CGA GGC TGG ACT CT-3′, miR-152/*Pdha1* reverse primer: 5′-ACA TAA CGG TCA GTG CCA AA-3′, miR-130ab/*Pdha1* forward primer: 5′-CGA ACA AGG GTC TTT CTG TGT A-3′, miR-130ab/*Pdha1* reverse primer: 5′-CAC ACA CAA ATC CTG CGT TTA C-3′, miR-130ab/*Gck* forward primer: 5′-CTT GCT AGA ATC AAC TAC AGA AA-3′, miR-130ab/*Gck* reverse primer: 5′-GGA AGC AAG AAT CGT GAA AG-3′, *Mapt* forward primer: 5′-TCT GTG AAT GTC CAT ATA GTG TAC TG-3′ and *Mapt* reverse primer: 5′-CAA CAG TCA GTG TAA ATC GTT TGT-3′.

Western Blot Analysis. Total protein (10 μg/mL) extracted 72 h post-transfection was separated by 4–15% Mini-Protean TGX Precast gel from Bio-Rad Laboratories at 80 V. Protein was transferred to PVDF membrane, then blocked with 5% milk and 1% BSA in buffer consisting of 150 mM NaCl, 20 mM Tris -HCl, pH 7.5 and 0.1% (v/w) Tween for 1 h. The blot was probed with PDHA1 (1:500; # ab92696, Abcam, UK), GCK (1:500; #ab37796, Abcam, UK), or Cyclophilin B (1:2000; # ab16045 Abcam, UK) antibodies, and incubated overnight at 4 °C. Horseradish peroxidase conjugated goat anti-rabbit IgG, HRP-linked antibody (1:10 000; #7074; Cell Signaling Technology, Danvers, MA, USA) was used to detect the primary antibodies. Super Signal West Pico Chemiluminescent Substrate or Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, MA, USA) and AlphaImager (ProteinSimple, CA, USA) was used to detect protein and quantification was done by using FluorChem SP software (Protein Simple, CA, USA).

ATP measurement. Luciferase-based luminescent assay (Bio Thema AB, Handen, Sweden) was used to measure total ATP levels after transfecting INS-1 832/13 with mature miRNA mimics or siRNA. Cells were pre-incubated in 2 mL SAB with 2.8 mM glucose for 2 h and stimulated with 1 mL SAB with 16.7 mM glucose for 30 min. The cells were suspended in 100 μL of lysis buffer containing 50 mM Tris (pH 7.5), 2 mM EDTA, 200 mM NaCl and 1% Triton X-100. The cells were quenched on dry ice for 15 min, thawed and sonicated for

5 seconds. An aliquot was diluted 1:50 in a reagent buffer consisting of 187.5 mM sucrose, 18.75 mM KH_2PO_2, 2.5 mM $(CH_3COO)_2$ $Mg.4H_2O$ and 0.625 mM EDTA, pH 7.0. A total volume of 100 µl of diluted sample was incubated with 25 µl of ATP monitoring reagent (Bio Thema AB, Handen, Sweden) at 25 °C for 5 min, and then ATP production was measured. Mitochondrial ATP synthase was inhibited by adding 0.6 mg/mL oligomycin as previously reported[32], and any further ATP produced was measured. The amount of ATP produced during the measurements was calibrated by adding 2 µl of ATP standard diluted 5-fold in Tris-EDTA Buffer (Bio Thema AB, Handen, Sweden). Mitochondrial ATP production was calculated as difference between ATP produced before and after adding oligomycin. We used TECAN Infinite M200 with Magellan software for the ATP luminescence measurements.

ATP measurement in single cells. Approximately 50,000 cells were seeded on poly-D-lysine (1 mg/mL) coated Lab-Tek chambered cover glass (Thermo Scientific, NY, USA). INS-1 832/13 cells were transfected with either Pre-miRs or siRNA together with 1 µg of PercevalHR plasmid DNA (Addgene ID: #21737)[18] per well using Lipofectamine 3000 (Invitrogen) for a period of 48 h. The cells were pre-incubated in experimental buffer (pH 7.4): 3.6 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 10 mM Hepes, 5 mM $NaHCO_3$ and 135 mM NaCl supplemented with 2.8 mM glucose for 90 min at 37 °C. Cover glass with adhered cells was mounted on the stage of Microscope (Zeiss Axiovert 200 M, Carl Zeiss AB, Stockholm, Sweden) equipped with confocal unit. PercevalHR was excited with laser light at 488 nm and emission was detected at 520 nm.

Bioinformatics Analysis. We used the comprehensive atlas of predicted and validated miRNA-target interactions web server miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/generetsys-self.html)[29] to identify putative targets of miR-130a-3p, miR-130b-3p, and miR-152-3p. To retrieve the predicted binding sites in the 3′UTR of *Pdha1* and *Gck* for AGO2-RIP assay, the sequences at TargetScan v7.1 for rat 3′UTRs (http://www.targetscan.org/vert_71/)[52] was used.

To determine enriched Gene Ontology categories for genes with unique putative binding sites for either miR-130a/b or miR-152, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 web server (https://david-d.ncifcrf.gov/)[53].

Statistical Analysis. Significant differences between two groups were determined using Student's t-test. For multiple groups, between scramble and miRNAs in insulin secretion, insulin content and ATP measurements, significant differences were tested using one-way ANOVA followed by Dunnett's multiple comparison test. The human islet qPCR data and AUCs derived from PercevalHR ATP:ADP curves were not normally distributed (Shapiro-Wilk Test) so differences were compared with Mann-Whitney U-test. All statistical tests were performed in IBM SPSS v22. Data were presented as mean ± SEM. Detailed statistical test parameters are indicated in the figure legend for each result.

References

1. Ashcroft, F. M. & Rorsman, P. Diabetes mellitus and the beta cell: the last ten years. *Cell* **148,** 1160–1171, doi: 10.1016/j.cell.2012.02.010 (2012).
2. Wiederkehr, A. & Wollheim, C. B. Mitochondrial signals drive insulin secretion in the pancreatic beta-cell. *Mol Cell Endocrinol* **353,** 128–137, doi: 10.1016/j.mce.2011.07.016 (2012).
3. Gerich, J. E. Is reduced first-phase insulin release the earliest detectable abnormality in individuals destined to develop type 2 diabetes? *Diabetes* **51** Suppl 1, S117–121 (2002).
4. Prasad, R. B. & Groop, L. Genetics of type 2 diabetes-pitfalls and possibilities. *Genes* (*Basel*) 6, 87–123, doi: 10.3390/genes6010087 (2015).
5. Eliasson, L. & Esguerra, J. L. Role of non-coding RNAs in pancreatic beta-cell development and physiology. *Acta Physiol* (*Oxf*) **211,** 273–284, doi: 10.1111/apha.12285 (2014).
6. Esguerra, J. L. & Eliasson, L. Functional implications of long non-coding RNAs in the pancreatic islets of Langerhans. *Front Genet* **5,** 209, doi: 10.3389/fgene.2014.00209 (2014).
7. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136,** 215–233, doi: 10.1016/j.cell.2009.01.002 (2009).
8. Pullen, T. J., da Silva Xavier, G., Kelsey, G. & Rutter, G. A. miR-29a and miR-29b contribute to pancreatic beta-cell-specific silencing of monocarboxylate transporter 1 (Mct1). *Mol Cell Biol* **31,** 3182–3194, doi: 10.1128/MCB.01433-10 (2011).
9. Thorrez, L. *et al.* Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. *Genome Res* **21,** 95–105, doi: 10.1101/gr.109173.110 (2011).
10. Esguerra, J. L., Bolmeson, C., Cilio, C. M. & Eliasson, L. Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLoS One* **6,** e18613, doi: 10.1371/journal.pone.0018613 (2011).
11. Portha, B. *et al.* The GK rat beta-cell: a prototype for the diseased human beta-cell in type 2 diabetes? *Mol Cell Endocrinol* **297,** 73–85, doi: 10.1016/j.mce.2008.06.013 (2009).
12. Galli, J. *et al.* Genetic analysis of non-insulin dependent diabetes mellitus in the GK rat. *Nat Genet* **12,** 31–37, doi: 10.1038/ng0196-31 (1996).
13. Nagamatsu, S. *et al.* Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta-cells is involved in impaired insulin secretion from diabetic GK rat islets: restoration of decreased t-SNARE proteins improves impaired insulin secretion. *Diabetes* **48,** 2367–2373 (1999).
14. Gaisano, H. Y., Ostenson, C. G., Sheu, L., Wheeler, M. B. & Efendic, S. Abnormal expression of pancreatic islet exocytotic soluble N-ethylmaleimide-sensitive factor attachment protein receptors in Goto-Kakizaki rats is partially restored by phlorizin treatment and accentuated by high glucose treatment. *Endocrinology* **143,** 4218–4226, doi: 10.1210/en.2002-220237 (2002).
15. Rosengren, A. H. *et al.* Overexpression of alpha2A-adrenergic receptors contributes to type 2 diabetes. *Science* **327,** 217–220, doi: 10.1126/science.1176827 (2010).
16. Granhall, C., Rosengren, A. H., Renstrom, E. & Luthman, H. Separately inherited defects in insulin exocytosis and beta-cell glucose metabolism contribute to type 2 diabetes. *Diabetes* **55,** 3494–3500, doi: 10.2337/db06-0796 (2006).
17. Zhou, Y. P., Ostenson, C. G., Ling, Z. C. & Grill, V. Deficiency of pyruvate dehydrogenase activity in pancreatic islets of diabetic GK rats. *Endocrinology* **136,** 3546–3551, doi: 10.1210/endo.136.8.7628391 (1995).
18. Berg, J., Hung, Y. P. & Yellen, G. A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nat Methods* **6,** 161–166, doi: 10.1038/nmeth.1288 (2009).

19. Li, J., Shuai, H. Y., Gylfe, E. & Tengholm, A. Oscillations of sub-membrane ATP in glucose-stimulated beta cells depend on negative feedback from Ca(2+). *Diabetologia* **56,** 1577–1586, doi: 10.1007/s00125-013-2894-0 (2013).
20. Tantama, M., Martinez-Francois, J. R., Mongeon, R. & Yellen, G. Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. *Nat Commun* **4,** 2550, doi: 10.1038/ncomms3550 (2013).
21. Schuit, F. C., Huypens, P., Heimberg, H. & Pipeleers, D. G. Glucose sensing in pancreatic beta-cells: a model for the study of other glucose-regulated cells in gut, pancreas, and hypothalamus. *Diabetes* **50,** 1–11 (2001).
22. Rhodes, C. J., Lucas, C. A., Mutkoski, R. L., Orci, L. & Halban, P. A. Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump. *J Biol Chem* **262,** 10712–10717 (1987).
23. Orci, L. *et al.* Proteolytic maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory vesicles. *Cell* **49,** 865–868 (1987).
24. Srinivasan, M. *et al.* Beta-Cell-specific pyruvate dehydrogenase deficiency impairs glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab* **299,** E910–917, doi: 10.1152/ajpendo.00339.2010 (2010).
25. Velho, G. *et al.* Primary pancreatic beta-cell secretory defect caused by mutations in glucokinase gene in kindreds of maturity onset diabetes of the young. *Lancet* **340,** 444–448 (1992).
26. Njolstad, P. R. *et al.* Neonatal diabetes mellitus due to complete glucokinase deficiency. *N Engl J Med* **344,** 1588–1592, doi: 10.1056/NEJM200105243442104 (2001).
27. Eliasson, L. *et al.* Novel aspects of the molecular mechanisms controlling insulin secretion. *J Physiol* **586,** 3313–3324, doi: 10.1113/jphysiol.2008.155317 (2008).
28. Olofsson, C. S. *et al.* Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. *Pflugers Arch* **444,** 43–51, doi: 10.1007/s00424-002-0781-5 (2002).
29. Dweep, H. & Gretz, N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods* **12,** 697, doi: 10.1038/nmeth.3485 (2015).
30. Del Guerra, S. *et al.* Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* **54,** 727–735 (2005).
31. Frese, T., Bazwinsky, I., Muhlbauer, E. & Peschke, E. Circadian and age-dependent expression patterns of GLUT2 and glucokinase in the pancreatic beta-cell of diabetic and nondiabetic rats. *Horm Metab Res* **39,** 567–574, doi: 10.1055/s-2007-984471 (2007).
32. Malmgren, S. *et al.* Tight coupling between glucose and mitochondrial metabolism in clonal beta-cells is required for robust insulin secretion. *J Biol Chem* **284,** 32395–32404, doi: 10.1074/jbc.M109.026708 (2009).
33. Tengholm, A. Cyclic AMP dynamics in the pancreatic beta-cell. *Ups J Med Sci* **117,** 355–369, doi: 10.3109/03009734.2012.724732 (2012).
34. Mollet, I. G., Malm, H. A., Wendt, A., Orho-Melander, M. & Eliasson, L. Integrator of Stress Responses Calmodulin Binding Transcription Activator 1 (Camta1) Regulates miR-212/miR-132 Expression and Insulin Secretion. *J Biol Chem* **291,** 18440–18452, doi: 10.1074/jbc.M116.716860 (2016).
35. Shang, J. *et al.* Induction of miR-132 and miR-212 Expression by Glucagon-Like Peptide 1 (GLP-1) in Rodent and Human Pancreatic beta-Cells. *Mol Endocrinol* **29,** 1243–1253, doi: 10.1210/me.2014-1335 (2015).
36. Malm, H. A. *et al.* Transcriptional regulation of the miR-212/miR-132 cluster in insulin-secreting beta-cells by cAMP-regulated transcriptional co-activator 1 and salt-inducible kinases. *Mol Cell Endocrinol* **424,** 23–33, doi: 10.1016/j.mce.2016.01.010 (2016).
37. Nesca, V. *et al.* Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes. *Diabetologia* **56,** 2203–2212, doi: 10.1007/s00125-013-2993-y (2013).
38. Kameswaran, V. *et al.* Epigenetic regulation of the DLK1-MEG3 microRNA cluster in human type 2 diabetic islets. *Cell Metab* **19,** 135–145, doi: 10.1016/j.cmet.2013.11.016 (2014).
39. Andersson, S. A. *et al.* Reduced insulin secretion correlates with decreased expression of exocytotic genes in pancreatic islets from patients with type 2 diabetes. *Mol Cell Endocrinol* **364,** 36–45, doi: 10.1016/j.mce.2012.08.009 (2012).
40. Burroughs, A. M. *et al.* Deep-sequencing of human Argonaute-associated small RNAs provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin. *RNA Biol* **8,** 158–177 (2011).
41. Dueck, A., Ziegler, C., Eichner, A., Berezikov, E. & Meister, G. microRNAs associated with the different human Argonaute proteins. *Nucleic Acids Res* **40,** 9850–9862, doi: 10.1093/nar/gks705 (2012).
42. Cullingford, T. E., Clark, J. B. & Phillips, I. R. The pyruvate dehydrogenase complex: cloning of the rat somatic E1 alpha subunit and its coordinate expression with the mRNAs for the E1 beta, E2, and E3 catalytic subunits in developing rat brain. *J Neurochem* **62,** 1682–1690 (1994).
43. Patel, M. S., Srinivasan, M., Strutt, B., Mahmood, S. & Hill, D. J. Featured Article: Beta cell specific pyruvate dehydrogenase alpha gene deletion results in a reduced islet number and beta-cell mass postnatally. *Exp Biol Med (Maywood)* **239,** 975–985, doi: 10.1177/1535370214531895 (2014).
44. Patel, K. P., O'Brien, T. W., Subramony, S. H., Shuster, J. & Stacpoole, P. W. The spectrum of pyruvate dehydrogenase complex deficiency: clinical, biochemical and genetic features in 371 patients. *Mol Genet Metab* **105,** 34–43, doi: 10.1016/j.ymgme.2011.09.032 (2012).
45. Henwood, M. J., Thornton, P. S., Preis, C. M., Chee, C. & Grimberg, A. Reconciling diabetes management and the ketogenic diet in a child with pyruvate dehydrogenase deficiency. *J Child Neurol* **21,** 436–439 (2006).
46. Konstantinova, I. *et al.* EphA-Ephrin-A-mediated beta cell communication regulates insulin secretion from pancreatic islets. *Cell* **129,** 359–370, doi: 10.1016/j.cell.2007.02.044 (2007).
47. Roder, M. E., Porte, D. Jr., Schwartz, R. S. & Kahn, S. E. Disproportionately elevated proinsulin levels reflect the degree of impaired B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* **83,** 604–608, doi: 10.1210/jcem.83.2.4544 (1998).
48. Ashcroft, F. M. & Gribble, F. M. Correlating structure and function in ATP-sensitive K+ channels. *Trends Neurosci* **21,** 288–294 (1998).
49. Eliasson, L., Renstrom, E., Ding, W. G., Proks, P. & Rorsman, P. Rapid ATP-dependent priming of secretory granules precedes Ca(2+)-induced exocytosis in mouse pancreatic B-cells. *J Physiol* **503** (Pt 2), 399–412 (1997).
50. Fadista, J. *et al.* Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism. *P Natl Acad Sci USA* **111,** 13924–13929 (2014).
51. Hohmeier, H. E. *et al.* Isolation of INS-1-derived cell lines with robust ATP-sensitive K+channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49,** 424–430 (2000).
52. Agarwal, V., Bell, G. W., Nam, J. W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4,** doi: 10.7554/eLife.05005 (2015).
53. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4,** 44–57, doi: 10.1038/nprot.2008.211 (2009).

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Author Contributions

J.O., H.M., C.W., L.E., and J.L.S.E. designed research; J.O., V.A.S., A.B., N.V., M.N., and J.L.S.E. performed research; J.O., V.A.S., A.B., N.V., M.N., H.M., C.B., L.E., and J.L.S.E. analyzed data; J.O., H.M., C.W., L.E., and J.L.S.E. wrote the paper. All authors reviewed the manuscript.

Additional Information

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SUPPLEMENTARY INFORMATION

Elevated miR-130a/miR130b/miR-152 expression reduces intracellular ATP levels in the pancreatic beta cell

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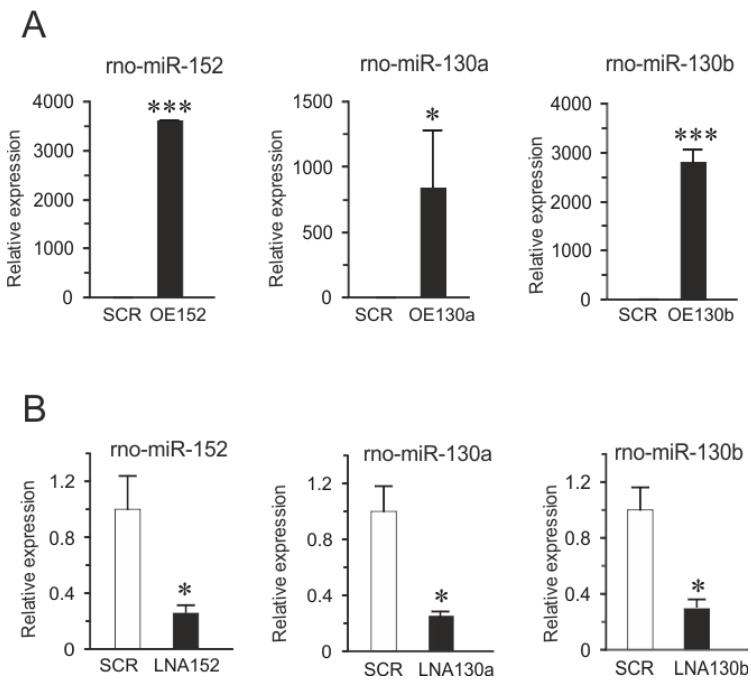
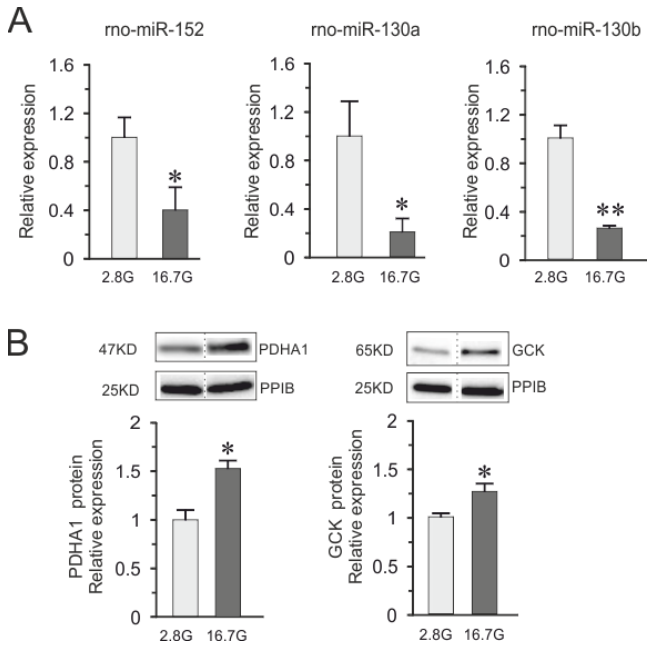
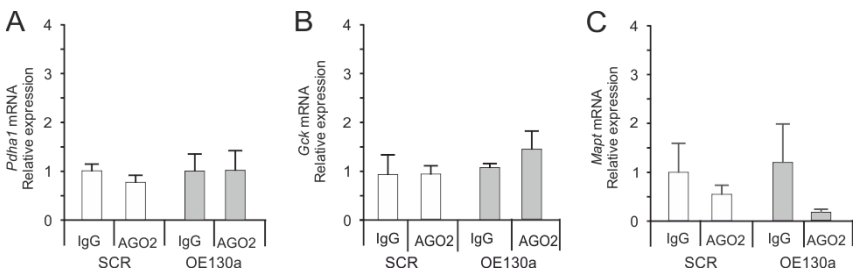
SUPPLEMENTARY FIGURES



Supplementary figure S1. Expression of miRNAs after over-expression or knock-down of miRNAs in INS-1 832/13. (A) Expression of miRNAs after over- expression in INS-1 832/13 cells. **(B)** Knock-down of miRNAs in INS-1 832/13 Data are presented as mean± SEM of n=3. Students t-test, two tailed; (*) $p< 0.05$, (**) $p< 0.01$, and (***) $p< 0.001$.

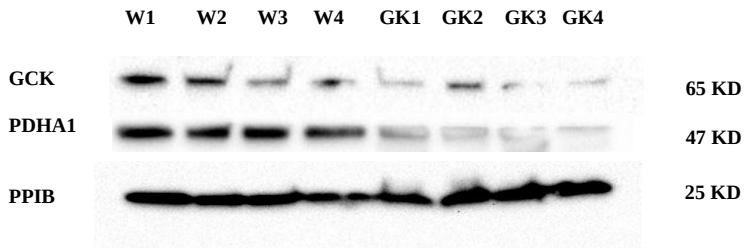


Supplementary figure S2. Glucose-dependence of miRNAs and their putative targets, *Pdha1* and *Gck*. (A) Expression of miRNAs and, **(B)** protein expression levels of PDHA1 and GCK in INS-1 832/13 incubated at 2.8 mM glucose and 16.7mM glucose for 1 hr. In each case the expression at 2.8 mM glucose was used as calibrator. Data are presented as mean± SEM. n=3, (*) $p< 0.05$ and (**) $p< 0.01$ vs 2.8 mM glucose using Student's 2-tailed test.

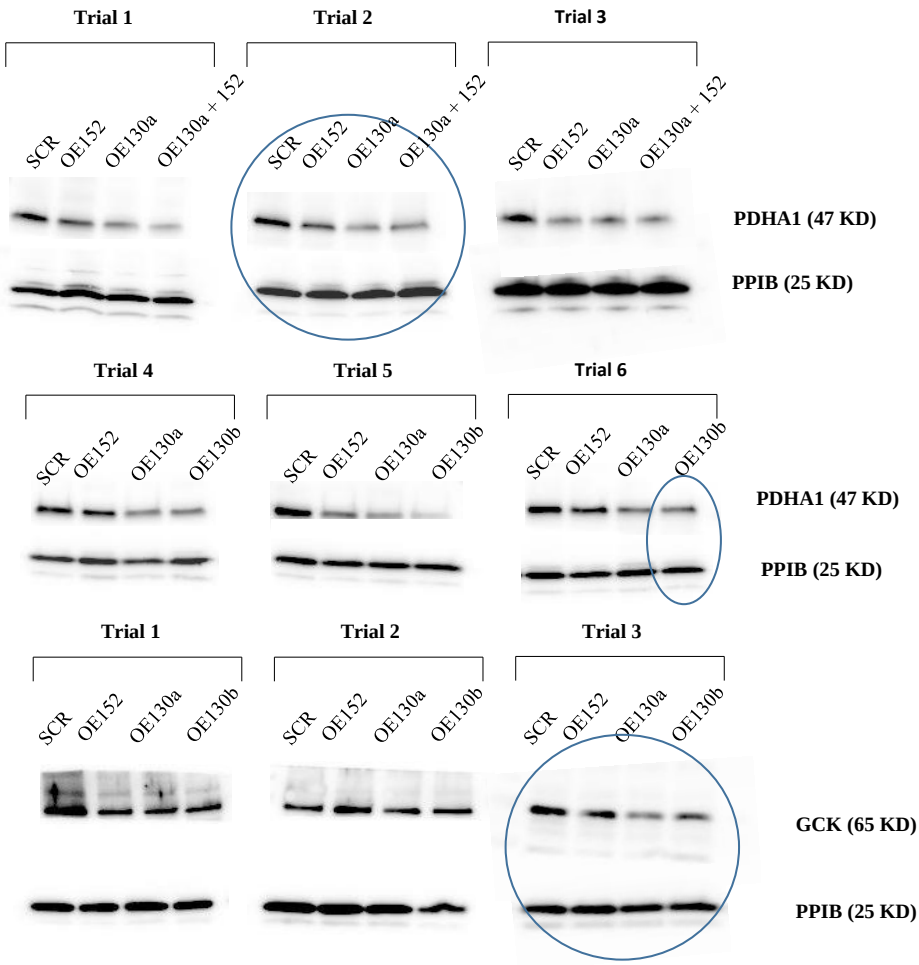


Supplementary figure S3. RIP-AGO2 assay of miR-130a and *Pdha1* or *Gck* targeting. Expression of 3'UTR target region of **(A)** *Pdha1*, **(B)** *Gck*, or **(C)** *Mapt* (non-specific control) after co-immunoprecipitation with anti-AGO2 in OE130a cells. Data are presented as mean ± SEM. n=3, Student's t-test, two tailed.

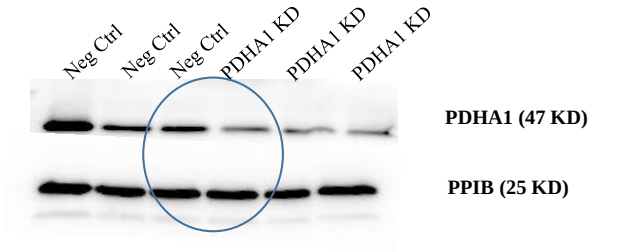
A Unedited blots for Figure 3A.



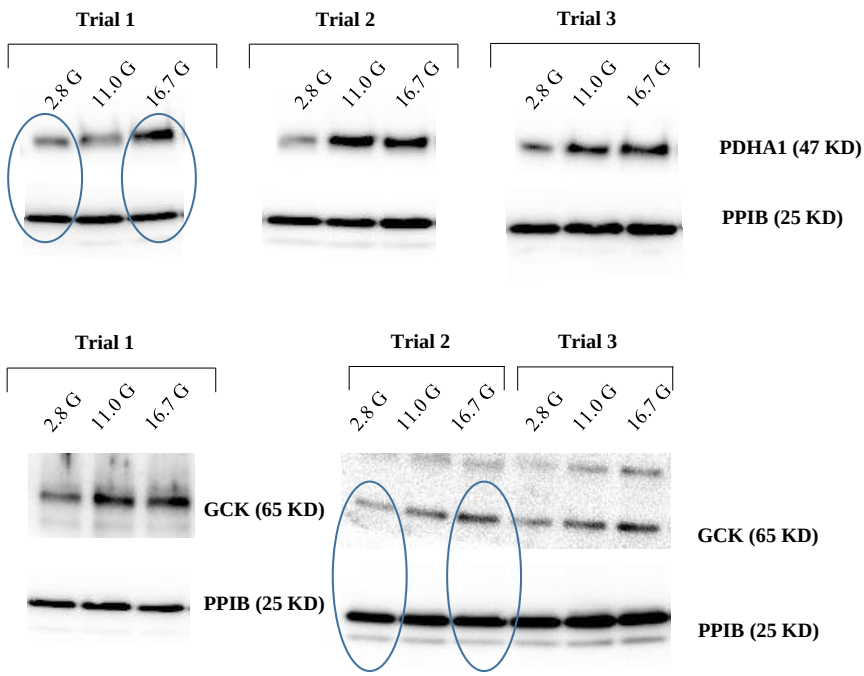
B Unedited blots for Figure 4B. Encircled blots/lanes used as representative in the figure.



C Unedited blots for Figure 6A. Encircled lanes used as representative in the figure.



D Unedited blots for Supplementary figure S2B. Encircled lanes used as representative in the figure.



Supplementary figure S4. Unedited blots used in the figures. Blots were cut around the molecular weight of the protein prior to incubation with specific antibodies. Images of whole blots with molecular weight ladders were not acquired prior to cutting.

SUPPLEMENTARY TABLES

Supplementary Table 1. Characteristics of human pancreatic islet donors.

	NGT (n=20)	IGT/T2D (n=22)	p-value
Gender (Male/Female)	11/9	8/14	
HbA1c (%)	5.4±0.08	6.6±0.14	<0.0001
Age (years)	55.8±2.4	57.7±1.8	0.52
BMI (kg/m2)	26.3±0.7	27.4±0.8	0.33

NGT: normal glucose tolerant; IGT: impaired glucose tolerant; T2D: type-2 diabetes
Data presented as mean ± S.E.M. Mann-Whitney U test (two-tailed).

Supplementary Table 2. Bioinformatics prediction by miRWalk 2.0 for presence of putative miR-130a/miR-130b/miR-152 binding sites within rat Pdha1/Gck and human PDHA1/GCK.

Web address: http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/generetsys-self.html

Date of access: November 21, 2016.

(n.d.: no data)

Human	EntrezID	RefseqID	miRNA	MIMATid	miR Walk	Micro t4	miRanda	mir bridge	miR DB	miR Map	miRNA Map	Pictar2	PITA	RNA22	RNA hybrid	Target scan	SUM	Region
GCK	2645	NM_033508	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_000162	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_033507	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_000162	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_033507	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_033508	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_033507	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_000162	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_033508	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
PDHA1	5160	NM_001173455	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	1	0	0	0	1	0	2	3UTR
PDHA1	5160	NM_001173456	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	1	0	0	0	1	0	2	3UTR
PDHA1	5160	NM_000284	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	1	0	0	0	1	0	2	3UTR
PDHA1	5160	NM_001173454	hsa-miR-130a-3p	MIMAT0000425	0	0	0	0	0	0	1	0	0	0	1	0	2	3UTR
PDHA1	5160	NM_001173454	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	1	1	0	2	3UTR
PDHA1	5160	NM_000284	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
PDHA1	5160	NM_001173455	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
PDHA1	5160	NM_001173456	hsa-miR-130b-3p	MIMAT0000691	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
PDHA1	5160	NM_001173454	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	1	1	0	2	3UTR
PDHA1	5160	NM_001173455	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
PDHA1	5160	NM_001173456	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
PDHA1	5160	NM_000284	hsa-miR-152-3p	MIMAT0000438	0	0	0	0	0	0	0	0	0	0	1	0	1	3UTR
GCK	2645	NM_000162	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	1	1	0	2	CDS
GCK	2645	NM_033507	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
GCK	2645	NM_033508	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
GCK	2645	NM_033507	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS

GCK	2645	NM_000162	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
GCK	2645	NM_033508	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
GCK	2645	NM_000162	hsa-miR-152-3p	MIMAT0000438	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
GCK	2645	NM_033508	hsa-miR-152-3p	MIMAT0000438	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
GCK	2645	NM_033507	hsa-miR-152-3p	MIMAT0000438	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_000284	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173455	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173456	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173454	hsa-miR-130a-3p	MIMAT0000425	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173455	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173456	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173454	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_000284	hsa-miR-130b-3p	MIMAT0000691	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
PDHA1	5160	NM_001173456	hsa-miR-152-3p	MIMAT0000438	1	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	1	3	CDS
PDHA1	5160	NM_001173455	hsa-miR-152-3p	MIMAT0000438	1	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	1	3	CDS
PDHA1	5160	NM_001173454	hsa-miR-152-3p	MIMAT0000438	1	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	1	3	CDS
PDHA1	5160	NM_000284	hsa-miR-152-3p	MIMAT0000438	1	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	1	3	CDS

RAT	EntrezID	RefseqID	miRNA	MIMATid	miR Walk	Micro t4	miRanda	mir bridge	miR DB	miR Map	miRNA Map	Pictar2	PITA	RNA22	RNA hybrid	Target scan	SUM	Region
Gck	24385	NM_012565	rno-miR-130a-3p	MIMAT0000836	1	0	1	0	0	1	0	0	0	0	1	1	5	3UTR
Gck	24385	NM_012565	rno-miR-130b-3p	MIMAT0000837	1	0	1	0	0	1	0	0	0	0	1	1	5	3UTR
Pdha1	29554	NM_001004072	rno-miR-130a-3p	MIMAT0000836	0	0	0	0	0	1	0	0	0	0	1	1	3	3UTR
Pdha1	29554	NM_001004072	rno-miR-130b-3p	MIMAT0000837	1	0	0	0	0	1	0	0	0	0	1	1	4	3UTR
Pdha1	29554	NM_001004072	rno-miR-152-3p	MIMAT0000854	1	0	0	0	0	1	0	0	0	0	1	1	4	3UTR
Gck	24385	NM_012565	rno-miR-130a-3p	MIMAT0000836	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
Gck	24385	NM_012565	rno-miR-130b-3p	MIMAT0000837	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
Gck	24385	NM_012565	rno-miR-152-3p	MIMAT0000854	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
Pdha1	29554	NM_001004072	rno-miR-130a-3p	MIMAT0000836	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
Pdha1	29554	NM_001004072	rno-miR-130b-3p	MIMAT0000837	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS
Pdha1	29554	NM_001004072	rno-miR-152-3p	MIMAT0000854	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	1	0	1	CDS

Supplementary Table 3. Significant Gene ontology (GO)-enriched categories for GOTERM: Metabolic Function of predicted targets unique to (A) miR-152 or (B) miR-130a/b.

GO enrichment performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 web server.
Web address: https://david-d.ncifcrf.gov/
Date of access: 22 November 2016.

(A). Significant GO-enriched categories of targets with unique binding sites for miR-152.

Category	Term	Count	%	PValue	Genes	List Total	Benjamini corrected Pvalue
GOTERM_MF_DIRECT	GO:0005515~protein binding	197	10.3	1.33E-09	NOG, SYT4, PDLIM5, VPS52, VPS53, ADORA1, PGR, HOOK1, BAK1, PICALM, TRAK2, WNK4, CHRNA7, SYK, F11R, SCN2B, ABCB11, SYNJ2BP, PDYN, PPARGC1A, TOX3, HES1, PIAS3, SIPA1L1, MAPK3, RYR2, PRDM2, PIAS2, EIF2AK2, BIN1, CDK5R1, MOB4, ALDOC, CACNB2, RIMS2, STX18, DMD, GIT1, GNAT1, DVL3, KIF3A, SMAD9, NDFIP1, FDXR, SMAD3, YTHDC1, KCNK3, PTPN11, KCNJ8, MAP2, CHRNB2, BEGAIN, NCOR1, SLC9A1, KCNJ16, RTN4, PPARA, LZTS3, TOLLIP, TRPV2, KCNIP3, NR2F2, ERRFI1, FGF2, SCN10A, CEP112, STX1A, STX3, RPH3AL, ARHGEF15, ARHGEF9, SLIT1, SLIT2, MYRIP, IL18BP, GNAQ, SH3KBP1, SIAH2, SNAP29, MYO5A, ATG12, USP2, FHL2, KLC2, ZBTB16, SORBS1, KLC1, KIRREL, ABCD3, APBA2, EXOC3, VMP1, KCNE3, DTNA, UBXN1, DLGAP2, YWHAB, ATP1A3, ATP1A2, CAPN2, ELAVL4, MEF2D, FYN, TENM2, HTR2A, GNA13, CAST, TSPO, SLC6A1, CASK, TBP, MAF1, ANK2, TIAM1, SLC2A3, SLC2A1, TUBG1, DDX20, KCND3, ACTN4, STMN2, RELA, BAIAP2, PRKAB1, PPP1CB, NCAM1, EP300, TIMELESS, RASGRF2, AKAP5, ERC2, CAV1, PANX1, MRAS, KCNA1, CLU, SNX1, SRF, EIF3A, MUC2, MYO1A, EPAS1, KCNB1, PODXL, OMP, FSCN1, ITGA3, PPIF, HDAC4, CDKN1A, PRICKLE1, P2RX1, BBC3, FAAH, DNMT1, IKBKB, PEX3, PAWR, FOS, CASP9, PAK1, LBR, SLC1A1, AKT2, DLG1, SOX11, ARHGAP27, LEF1, UBE2I, ESR2, TIMM23, STOM, KIF1A, KIF1B, CRKL, CARM1, KPNA1, PPP2R2A, DCC, PRKCZ, CABP1, APLP2, HSPA2, TAP1, PPP1R12A, HSPA4, SH2B1, CAMK2A, VPS39, KAT2A, TAB1, PPP1R9B, ATXN3, CYBB, CABIN1, SLC5A7, SMPD3	1514	1.8E-06

GOTERM_MF_DIRECT	GO:0042803~protein homodimerization activity	115	6.0	9.19E-07	ALS2, NOG, HM13, SYT4, GDF6, LHCGR, PRKG2, TPD52, CBX5, KLHL7, BAK1, ABCB10, CHRNA7, NUDT16, PDXK, STK25, ACTN4, RELA, ZHX2, NTSR1, STK4, TOX3, HES1, EIF2AK1, HNF4A, TIMELESS, FGFR1OP, EXT2, DST, CD226, OLFM4, ACHE, SNX6, STK10, HMGCS1, SNX1, GREM2, SRF, TRIM66, RUNX1, SLC30A8, GIT1, MUC2, NADK2, IKZF2, S100A11, SMAD3, FOXP4, FOXP1, KCNK3, TIGIT, ATF3, FAAH, IKBKG, SYT14, PON1, MZF1, TDG, BEGAIN, IKBKB, TCF12, USH1G, MYO7A, DMRTA2, CCDC103, MAX, CRYL1, DGCR8, PSMC3IP, CAT, NR2F2, DPP4, CHUK, ADAM10, PRTFDC1, COL23A1, PFKM, DMRTB1, SLIT2, RAB11FIP4, STOM, RAB11FIP2, RAB11FIP3, SBF2, NPPC, CARM1, CLN6, TYRP1, SLC39A13, CABP1, DCK, RDX, ZBTB16, DROSHA, SH3GLB1, TAP1, ABCD3, HSD17B4, CAMK2A, ACSL6, FLRT3, PLEK, LRRC41, BIRC5, NPR3, STAT1, ADIPOQ, GJB1, MEF2D, TENM2, TENM3, LRP6, DPYD, ZBTB1, RNF40	1514	6.2E-04
GOTERM_MF_DIRECT	GO:0032403~protein complex binding	60	3.1	1.56E-05	RTN4, PPARA, NOG, GNA11, MYO7A, CASK, MMP3, VPS33A, EPCAM, MAX, ANKRD54, BAG3, ATP6V0D1, CIITA, TUB, KIF11, ACTN4, RELA, RPTOR, HES1, NCOA2, EP300, NPTXR, GNAQ, SIPA1L1, PI4K2A, PEX26, AKAP5, RAD18, CPD, ERC2, BIN1, DYNC1I2, PPP2R2A, MED1, CALY, ING2, ABI2, MTHFR, DMD, SH2B3, BRK1, HSPA4, SKIL, GIT1, KIF3A, FBN1, HMBOX1, YWHAB, KCTD2, PARK2, TAB1, PPP1R9B, CDKN1A, SYDE1, FYN, TCEB1, RBPJ, RNF40, HTR2A	1514	7.0E-03
GOTERM_MF_DIRECT	GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding	55	2.9	3.91E-05	PPARA, JDP2, ARNT2, MITF, PAX6, HOXD13, TBP, KCNIP3, PGR, FOS, ZGPAT, MAX, CREB3L2, POU4F2, RBBP4, EGR2, FOXJ2, FOXJ1, RELA, OTX1, LEF1, ESR2, SIX4, MXD1, CARF, EP300, NCOA2, PRDM5, TXK, MED1, DPF2, SRF, MSX2, CHD7, MEIS2, BCL11B, OVOL1, NFAT5, ETV1, SKIL, NFATC2, ETV6, NFATC3, SMAD3, KLF17, STAT1, FOXP4, NKX6-1, GCFC2, FOXP1, MEF2D, ATF3, MZF1, RBPJ, TCF12	1514	1.3E-02

GOTERM_MF_DIRECT	**GO:0005524~ATP binding**	**172**	**9.0**	**5.01E-05**	**RNASEL, ADCY5, STK35, ADCY6, CASK, PRKG2, CCT3, ITPKC, DMPK, DDX17, ATP2B4, WNK4, ABCB10, DDX20, ROS1, MAP2K6, SYK, MAP2K5, CIITA, NMNAT2, PDXK, ROCK1, STK25, ABCB11, ABCC12, CDKL2, STK4, MAP4K3, MYO18B, EIF2AK1, PI4K2A, MAPK3, BMP2K, EIF2AK2, KIF26A, GATC, ERBB3, GNE, STK10, PFKFB2, PEAK1, MAP4K2, MAPKAPK2, MYO9A, UHMK1, ACSBG1, QRSL1, CAMKV, TTBK1, P2RY1, LMTK2, NAT10, RUNX1, STK38L, DNAJA3, DNAJA2, NADK2, MYO1A, KIF3A, TAOK2, ATP11B, TRIO, MYO1F, YTHDC2, OXSR1, DDX5, ATP13A4, ATP7A, GLYCTK, IKBKE, DDX55, ATP2A2, P2RX1, EPHA8, KCNJ8, DYRK1B, DYRK1A, GTF2F2, RAD54L2, SMC1A, IKBKB, UBE2E2, UBE2E1, CDC42BPB, CDK17, NUAK1, ATP5B, MYO7A, PRKAG2, DDR2, CAMKK2, ACVR1C, MTHFD1, MCM9, ACVR1B, HSPH1, PDPK1, PAK2, PAK1, CHUK, AKT3, SIK3, CDK13, AKT2, KIF14, ABCE1, KIF11, CAMK1G, LIMK2, PHKG2, UBE2F, CDK6, UBE2I, UBE2H, PFKM, TTF2, IQCA1, TBCK, MCM6, MAST3, TARS, PDIK1L, ACVR2B, KIF1A, KIF1B, PANK3, HIPK1, SNRNP200, HIPK2, TXK, MYO5A, PRKCZ, BRSK2, DCK, MKNK1, CHD7, MAP3K3, HSPA2, STK40, PARS2, TAP1, TOR1B, DHX15, CHD1, ABCD3, ETNK2, HSPA4, CAMK2A, ACSL6, EHD3, MYO5B, ACSL5, TRIP13, CNNM2, DGKQ, FLT1, NLK, MSH5, PDK4, ATP1A3, STRADB, ATP1A2, FZD7, HSP90B1, FYN, HSPA4L, ABCC4, NLRP14, KATNAL1, CIT, IPPK, ATP8A1**	**1514**	**1.3E-02**
GOTERM_MF_DIRECT	GO:0031625~ubiquitin protein ligase binding	49	2.6	5.04E-05	TRAF1, DERL1, HM13, USP2, TOLLIP, CLU, PAX6, ABI2, CALR, TNFRSF1B, CUL5, KCNQ3, BTBD2, RALA, YOD1, PER3, TRAF6, UBXN1, TXNIP, GABARAPL2, EGR2, SMG5, RELA, NLK, TMBIM6, UBE2F, SMAD3, ERLIN1, UBE2I, CASC3, UBE2H, PARK2, PPARGC1A, MFN2, IKBKE, GPI, ATXN3, CDKN1A, GPR37, SIPA1L1, IKBKG, RAD18, FAF2, PIAS2, FAF1, USP25, MPHOSPH8, RNF40, BTBD11	1514	1.1E-02
GOTERM_MF_DIRECT	GO:0030331~estrogen receptor binding	14	0.7	7.96E-05	MMS19, LEF1, DDX5, PPARGC1A, NRIP1, PAGR1, DDX17, NCOA2, PSMC3IP, DNMT1, PIAS2, NCOR1, NSD1, MED1	1514	1.5E-02
GOTERM_MF_DIRECT	GO:0019901~protein kinase binding	65	3.4	1.33E-04	PRC1, ADCY6, PRKAG2, CSPG4, PAX6, CCNE2, PDPK1, KCNQ3, CDKN2B, ANK2, CASP9, TBC1D14, CHRNA7, MSN, PAK1, CRY1, ERRFI1, MAP2K6, SYK, DLG1, CDK13, KIF14, ELP2, KIF11, ADAM10, RELA, PRKAB1, TPX2, PPP1CB, RPTOR, SPDYA, RAB11FIP2, EP300, RCC2, SIPA1L1, FGFR1OP, AKAP5, RYR2, CD226, MYO5A, PRKCZ, CAV1, CDK5R1, SNAP91, PFKFB2, BRSK2, GCSAM, CACNB2, SORBS1, GYS1, PPP1R12A, TRAF6, DNAJA3, TBL2, SMAD3, RICTOR, PARK2, CDC25B, HDAC4, DUSP3, PRLR, TOM1L1, RAD54L2, FAF1, IKBKB	1514	2.2E-02

GOTERM_MF_DIRECT	GO:0004674~protein serine/threonine kinase activity	52	2.7	1.52E-04	CDK17, FAM20A, NUAK1, STK35, ACVR1C, CAMKK2, DMPK, ACVR1B, PDPK1, WNK4, PAK1, BRD4, AKT3, SIK3, CHUK, AKT2, SYK, LIMK2, ROCK1, CDKL2, STK4, MAST3, MAP4K3, PDIK1L, ACVR2B, HIPK1, HIPK2, MAPK3, BMP2K, EIF2AK2, PRKCZ, STK10, MAP4K2, BRSK2, MKNK1, MAPKAPK2, UHMK1, STK40, TTBK1, LMTK2, STK38L, CAMK2A, TAOK2, NLK, TRIO, OXSR1, FZD7, DYRK1B, DYRK1A, CIT, IKBKB, CDC42BPB	1514	2.3E-02
GOTERM_MF_DIRECT	GO:0042826~histone deacetylase binding	24	1.3	1.99E-04	KAT2A, RBBP4, SMG5, RELA, YWHAB, HR, LEF1, PARK2, UHRF1BP1, SRF, NRIP1, CBX5, HES1, TAL1, MEF2D, HDAC4, DNMT1, AKAP8, DDX20, TRAF6, CRY1, NCOR1, KLF4, PHF6	1514	2.7E-02

(B). Significant GO-enriched categories of targets with unique binding sites for miR-130a/b.

Category	Term	Count	%	PValue	Genes	List Total	Benjamini corrected Pvalue
GOTERM_MF_DIRECT	GO:0003700~transcription factor activity, sequence-specific DNA binding	67	6.4	4.28E-06	CREBRF, MEF2C, BACH1, POU6F1, SOX21, THRA, HNF1A, ELF3, GPBP1, TBX20, PPARG, PAX5, TP63, ZEB1, RORA, FOXO3, DMRTA1, ZBTB38, POU4F1, RARB, MKL2, ALX4, ALX1, AR, YY1, ZHX1, FOXN2, SPEN, TRERF1, GRHL2, MYCL, MYT1L, HOXD4, ST18, NR3C2, SOX4, ELK3, MYT1, HOXA3, TSC22D2, REL, HOXA5, NR1D2, POU2F3, POU3F3, RUNX3, ZBTB7B, IKZF4, IKZF3, KLF7, ERG, TCF7, CREBZF, TBX2, CREB1, RFX6, SMAD5, HOXB3, HOXB2, SP1, ZSCAN30, ZIC5, SP4, HIVEP2, NEUROD1, KLF2, NFIA	825	0.004
GOTERM_MF_DIRECT	GO:0004842~ubiquitin-protein transferase activity	34	3.3	4.09E-05	BACH1, XIAP, RNF217, RNF216, TRIM71, ERCC8, ARIH1, ZFP91, TRIM5, G2E3, UBE2D2, KBTBD3, KLHL28, KBTBD8, HECTD2, ANKIB1, RNF11, KLHL24, KLHL42, TRAF7, RNF34, KLHL20, IPP, PELI1, MUL1, BIRC6, TRIM62, TRIM37, MIB1, KCTD10, RNF6, RNF208, KLHL15, RNF19B	825	0.019
GOTERM_MF_DIRECT	GO:0043565~sequence-specific DNA binding	51	4.9	6.42E-05	MEF2C, CREBRF, POU6F1, THRA, HNF1A, PPARG, MAEL, TP63, FOXO3, RORA, ZEB1, DMRTA1, PAX7, MKX, POU4F1, FOXB1, RARB, ALX4, ALX1, AR, YY1, FOXN2, GRHL2, BPTF, HOXC13, HOXD4, NR3C2, ELK3, ARX, HOXA3, REL, NR1D2, HOXA5, POU2F3, GBX2, POU3F3, IKZF4, IKZF3, ERG, TCF7, CREBZF, TBX2, CREB1, HOXB1, HOXB2, SP1, ZSCAN30, ZBTB4, HIVEP2, NEUROD1, KLF2	825	0.020

GOTERM_MF_DIRECT	GO:0005515~protein binding	102	9.8	1.60E-04	TGOLN2, MEF2C, LDHB, SCN3A, GRIK2, GABRB2, LMO7, RORA, CNOT7, SYP, EPC1, DAB2, MYOCD, SND1, CHRNA5, CHRNA4, RAPGEF4, RARB, RAPGEF3, AKIRIN2, EGFR, MYO6, MAGI2, YY1, WNK1, DLL1, GABRR1, RASGRF1, CLIP1, OPHN1, VAMP3, MAPK8, PTGFRN, SH3GL2, UNC13A, KALRN, DRD1, STX7, ERBB4, UMOD, PFN1, GORASP1, HNRNPD, LURAP1, WDR7, EFEMP1, TGFBR2, SNAPIN, ITGA4, SHANK2, NCKAP1, EPHA5, HDAC5, EPHA7, RAP1A, NUTF2, KAT6A, HNF1A, COPS4, PPARG, GJA1, TP63, KCNJ10, ZEB1, CLTC, GJA3, TRPV4, PLCB1, PRKCA, AR, ARHGEF11, GRM5, BTG1, TPPP, RASD1, TG, PARD3, XIAP, FKBP1A, RGS12, PEX19, TAP2, IPCEF1, PRKAA2, TRIP10, SNAP25, RASA1, TES, NCDN, CREB1, SLC12A5, DPYSL2, APPL1, ITPR1, GCK, SP1, CSNK1D, BAX, GFRA1, CALM2, NFIA, DNM2	825	0.037

Paper II

MiR-335 overexpression impairs insulin secretion through defective priming of insulin vesicles

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Running title: miR-335 and exocytosis in insulin-secreting cells

Abstract

MicroRNAs contribute to the maintenance of optimal cellular functions by fine-tuning protein expression levels. In the pancreatic β-cells, imbalances in the exocytotic machinery components lead to impaired insulin secretion and type 2 diabetes (T2D). We hypothesize that dysregulated miRNA expression exacerbates β-cell dysfunction, and have earlier shown that islets from the diabetic GK-rat model have increased expression of miRNAs, including miR-335-5p (miR-335). Here, we aim to determine the specific role of miR-335 during development of T2D, and the influence of this miRNA on glucose-stimulated insulin secretion and Ca^{2+}-dependent exocytosis. We found that expression of miR-335 negatively correlated to secretion index in human islets of individuals with pre-diabetes. Overexpression of miR-335 in human EndoC-βH1 and in rat INS-1 832/13 cells (OE335) resulted in decreased glucose-stimulated insulin secretion, and OE335 cells showed concomitant reduction of three exocytotic proteins: SNAP25, Syntaxin-binding protein 1 (STXBP1), and synaptotagmin 11 (SYT11). Single-cell capacitance measurements, complemented with TIRF microscopy of the granule marker NPY-mEGFP demonstrated a significant reduction of exocytosis in OE335 cells. The reduction was not associated with defective docking or decreased Ca^{2+} current. More likely, it is a direct consequence of impaired priming of already docked granules. Earlier reports have proposed reduced granular priming as the cause of reduced first-phase insulin secretion during pre-diabetes. Here, we show a specific role of miR-335 in regulating insulin secretion during this transition period. Moreover, we can conclude that miR-335 has the capacity to modulate insulin secretion and Ca^{2+} dependent exocytosis through effects on granular priming.

Keywords: Type 2 Diabetes/ insulin secretion/ beta cell/ microRNA/ exocytosis/SNAP25/STXBP1/ TIRF/patch-clamp

Introduction

Hyperglycaemia and the development of type 2 diabetes (T2D) depend on environmental components together with genetic factors resulting in insulin resistance in the target tissues and reduced capacity of the pancreatic β-cells to secrete enough insulin. The release of insulin is biphasic and evidence have put forward that impaired insulin secretion precedes insulin resistance (Gerich, 2002) and that first phase insulin secretion is lost already when the patient has impaired glucose tolerance (IGT) or pre-diabetes (Del Prato and Tiengo, 2001). In the β-cells exocytosis serves to release insulin from large dense-core vesicles in response to elevated cytosolic $[Ca^{2+}]_i$ (Eliasson et al., 2008b, Wang and Thurmond, 2009, Ammala et al., 1993). Prior to fusion with the plasma membrane, the granules need to dock to the release site and undergo the process of granular priming to make them ready for release of their insulin cargo. Impaired priming has been suggested to underlie the absence of first phase insulin secretion in individuals with pre- and full-blown diabetes (Eliasson et al., 2008a).

As in neuronal cells, the assembly of the SNARE (Soluble NSF Attachment protein Receptor)-proteins, including VAMP2 (vesicle-associated-membrane-protein2), STX1A (Syntaxin1A), and SNAP25 (Synaptosomal-Associated-Protein-of-25-kDa) is a key process in insulin exocytosis (Eliasson et al., 2008b, Pang and Sudhof, 2010, Wang and Thurmond, 2009). In addition, several other proteins are required for the specific targeting of secretory granules to the release sites and the regulation of exocytosis (Pang and Sudhof, 2010, Wang and Thurmond, 2009). These include the Sec1/Munc18 (or syntaxin-binding protein 1 (STXBP1)) proteins, thought to guide SNARE complex assembly (Rizo and Sudhof, 2012, Tomas et al., 2008, Gulyas-Kovacs et al., 2007), and the synaptotagmins (SYTs) the primary Ca^{2+} sensors of exocytosis (Gauthier and Wollheim, 2008, Pang and Sudhof, 2010). The expression of many exocytotic genes is down-regulated in islets from donors with T2D (Ostenson et al., 2006, Andersson et al., 2012) as well as in the spontaneous diabetes model the Goto-Kakizaki (GK) rat (Gaisano et al., 2002, Zhang et al., 2002).

MiRNAs are small (~20-nt), non-protein coding, endogenously expressed RNAs that regulate gene expression (Bartel, 2004). MiRNAs guide the RNA-induced silencing complex (RISC) to target the mRNAs by direct base-pairing, which may include wobble base pairing in mammalian systems, between the miRNA 5' seed region (~7-nt long) and their target mRNA sequence usually in the 3'UTR (Brennecke et al., 2005). The regulation of protein output by miRNAs has been suggested to function either as a classical binary off-switch or as a rheostat, in which miRNAs fine-tune optimal protein output (Bartel, 2009).

In the context of T2D several miRNAs have been shown to directly or indirectly regulate crucial components of glucose-stimulated insulin secretion and exocytosis in β-cells (Eliasson and Esguerra, 2014, Lovis et al., 2008). The exocytotic process involves a plethora of proteins (Eliasson et al., 2008b, Wang and Thurmond, 2009), several of which are regulated by miRNAs (Eliasson and Esguerra, 2014, Lovis et al., 2008, Salunkhe et al., 2015). We have earlier

demonstrated up-regulation of at least 24 miRNAs (Esguerra et al., 2011) in the islets of the GK-rat, whose hallmark phenotype is impaired glucose-stimulated insulin secretion (Portha et al., 2009). We found enrichment of transport and secretory genes among the putative targets of the most upregulated miRNAs in GK rat islets. Specifically, we demonstrated that miR-335 down-regulates *Stxbp1 (*syntaxin-binding protein 1 or Munc18-1) by direct interaction (Esguerra et al., 2011).

Here we investigated if modulation of miR-335 expression correlate to altered insulin secretion output in human islets. In addition, we performed a detailed analysis on how miR-335 overexpression influences the exocytotic process using capacitance measurements, live-TIRF imaging, and western blot analyses of potential targets. The data also highlights the important regulatory role of miRNAs during T2D development.

Methods

Ethical approval

The use of human islets from deceased donors was approved by the ethics committees in Malmö and Uppsala, Sweden.

Cell culture

Rat insulinoma INS-1 832/13 cells (Hohmeier et al., 2000) was maintained in RPMI 1640 medium containing 11.1 mM glucose (HyClone, UT, USA) as previously described (Salunkhe et al., 2015). EndoC-βH1 cells (EndoCells, Paris, France) (Andersson et al., 2015, Ravassard et al., 2011) were maintained in a culture medium containing: DMEM (5.6 mM glucose), 2% BSA fraction V (Roche Diagnostics, Mannheim, Germany), 10 mM nicotinamide (Merck Millipore, Darmstadt, Germany), 50 μM 2-mercaptoethanol, 5.5 μg/mL transferrin, 6.7 ng/mL sodium selenite (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories, Pasching, Austria). Cells were tested for mycoplasma regularly.

Human islets

Islets from 28 human donors (F/M 16/12, age 58.2 ± 1.63, BMI 26.8 ± 0.6 kg/m2, HbA1c 5.82±0.11, days in culture 2.8 ± 0.29) were provided by the Nordic Network for Clinical Islet Transplantation (Uppsala, Sweden). Human islets were hand-picked under microscope to ensure high purity.

Transfection

One day prior to transfection cells were seeded in antibiotic-free RPMI 1640 media in a 24-well-plate. For transient overexpression, a final concentration of 25 nM chemically-modified double-stranded mature miRNA miR-335 Pre-miR™ miRNA Precursor (PM10063, Life Technologies, CA, USA) or Pre-miR™ miRNA Precursor Negative Control #1 (AM17110, Life Technologies, CA, USA) were used. For down-regulation, a final concentration of 50 nM

of the following were used: miRcury anti-miR LNA335 (#410201-00, Exiqon, Denmark), LNA Negative Control B (#199005-00, Exiqon, Denmark) *Silencer® Select Pre-Designed siRNA* against *Syt11* (s133472, Life Technologies CA, USA) and *Silencer*® Select Negative Control No. 2 siRNA (Life Technologies CA, USA) was used. Transfection was performed according to the manufacturer's protocol using lipofectamine® RNAiMAX Reagent (Invitrogen, CA, USA). Cells were transfected 72 hour prior to experiments.

Glucose stimulated insulin secretion

For glucose-stimulated insulin secretion assay, cells were plated in triplicate for each condition and the assay was performed as described (Salunkhe et al., 2015). For K^+-induced insulin secretion the secretion buffer contained 50 mM KCl (adjusted by reducing equimolar amount of NaCl). Insulin secretion measurements were normalized to total protein/well. Protein extraction was performed using RIPA buffer as previously described (Salunkhe et al., 2015). Protein content in cell homogenates was analysed using BCA assay (Pierce®BCA Protein Assay Kit #23227, IL, USA). Secreted and total insulin were measured using Coat-a-Count RIA (Millipore Corporation, MA, USA) and Mercodia insulin ELISA (Mercodia, Uppsala, Sweden).

RNA extraction, RT-PCR and qPCR

RNA extraction, RT-PCR for total RNA, and stem-loop RT-qPCR for microRNA was performed as previously described (Esguerra et al., 2011, Salunkhe et al., 2015) using pooled stem-loop primers from TaqMan®MiRNA Assays: hsa-miR-335-5p (#RT_000546), U6 (#RT_001973) and U87 (#RT_001712). The human miR-335 (miRBase ID: hsa-miR-335-5p /Acc. no. MIMAT0000765) and rat miR-335 (rno-miR-335 /Acc.no. MIMAT0000575) have identical mature sequences and were detected by the same Taqman miRNA assay. qPCR for protein coding genes was performed using primers from TaqMan®Gene Expression Assays; *Syt11* (Rn00581475_m1) and endogenous controls *Hprt1* (Rn01527840_m1) and *Ppia* (Rn00690933_m1). Relative expressions were calculated using the ΔΔCt method.

Western Blot analysis

Protein extraction and measurement of protein content was performed ~72 hrs after transfection as described above. Protein samples were separated on 4-15% precast gradient polyacrylamide gels (Bio-Rad Laboratories, CA, USA) and then transferred to PVDF membranes. The membranes were blocked (at 4°C) with 5% milk and 1% BSA in a buffer consisting of 20 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween-20 (pH 7.5) for 1 h. Proteins were probed with antibodies for SNAP25 (1:500; #111011, Synaptic Systems, Germany), STXBP1 (1:500; #116002, Synaptic Systems, Germany), SYT11 (1:500; #WH0023208M3 Sigma-Aldrich, Germany), Beta-actin (1:1000; #A5441, Sigma-Aldrich, Germany) and Cyclophilin B (1:2000; #ab16045 Abcam, UK), and incubated overnight at 4°C. The primary antibodies were detected using HRP-conjugated goat anti-rabbit/anti-mouse secondary antibody (1:10,000; #7074S, Cell Signaling Techology, USA) and anti-mouse immunoglobulins/HRP antibody (1:1000; #P0448, Dako, Denmark). Bands were visualized using SuperSignal West Femto Maximum Sensitivity

Substrate (#34096; Thermo Scientific, MA, USA) and AlphaImager (ProteinSimple, CA, USA). Quantification was made using FluorChem SP software (ProteinSimple, CA, USA).

Electrophysiology

To measure ion channel currents and exocytosis (as changes in membrane capacitance) whole-cell patch clamp experiments on single cells were performed as previously described (Salunkhe et al., 2015), and with a pipette solution containing (mM): 125 Cs-Glutamate, 10 NaCl, 10 CsCl, 1 MgCl2, 0.05 EGTA, 3 Mg-ATP, 5 HEPES and 0.1 cAMP (pH 7.15 using CsOH) and an extracellular solution with (mM): 118 NaCl, 20 TEA-Cl, 5.6 KCl, 2.6 CaCl2, 1.2 MgCl2, 5 glucose and 5 HEPES (pH 7.4 using NaOH). The recordings were performed using patch master software (version 2-73) and EPC-10 amplifier (Heka Elektronik, Lambrecht, Germany). Exocytosis was measured as changes in cell membrane capacitance, and it was evoked by a train of ten 500-ms depolarisations from -70 mV to 0 mV applied at 1 Hz. Voltage-dependent currents were investigated using an IV-protocol, in which the membrane was depolarized from -70 mV to voltages between -40 mV to +40 mV during 50 ms. All experiments were carried out with constant buffer perfusion at 32 °C. The measured voltage-dependent current consists of Na^+- and Ca^{2+}-current components. The rapid peak-current (Ip) represents the Na^+ current and the sustained current (I_{sus}), measured during the latter 20 ms of the depolarisations, reflects the Ca^{2+}-current. Charge (Q) was measured ~ 2 ms after the onset of the pulse to exclude the Na^+-current and is therefore representative of the Ca^{2+}-influx.

TIRF microscopy

INS-1 832/13 cells were plated on coverslips coated with poly-D-lysine and immediately co-transfected with mature miR-335 and the granule marker NPY-EGFP. Cells were imaged 36 h after plating in a solution containing (in mM) 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 D-glucose, 5 Hepes HEPES (pH 7.4 with NaOH), supplemented with 200 µM Diazoxide and 2µM forskolin. Exocytosis was evoked by timed local application of high K^+ (75 mM KCl equimolarly replacing NaCl) for 1 min through a pressurized glass electrode, similar to those used for patch clamp experiments. All experiments were carried out with constant buffer perfusion at 32 °C.

Cells were imaged using a custom-built lens-type total internal reflection (TIRF) microscope based on an Axiovert 135 microscope with a 100x/1.45 objective (Carl Zeiss). Excitation was from a DPSS laser at 491 (Cobolt, Stockholm, Sweden), controlled with an acousto-optical tunable filter (AA-Opto, France) and using dichroic Di01-R488/561 (Semrock) and emission filter FF01-523/610 (Semrock). Scaling was 160 nm per pixel and exposure time 100 ms per frame at 10 frames/s.

Data analysis

In the TIRF microscopy imaging experiments exocytosis events were found by eye. The moment of exocytosis was defined as the first significant change (2 SD) from the pre-exocytosis baseline. This definition applied both types of event, with or without preceding flash. The decay

time was then defined as the time from exocytosis until the signal reached less than one third of the amplitude of the event. Traces were read out as ΔF, defined as average fluorescence in a 0.5 µm circle minus the average fluorescence in a surrounding annulus of 0.8 µm. The point of exocytosis was calculated by fitting the granule fluorescence during exocytosis with a discontinuous function, which assumes constant fluorescence before fusion, an inverted exponential decay just after fusion, and finally exponential decay during content release.

Eq 1 $c = A1$ for t<t1

$c = A2 + (A1 - A2)e^{-\frac{x-x1}{t1}}$ for t2 > t ≥ t1

$$c = A3 + \left(\left(A2 + (A1 - A2)e^{-\frac{x2}{t1}}\right) - A3\right)e^{-\frac{x-x2}{t2}} \quad \text{for } t \geq t2,$$

where t is time; c is average fluorescence in a 0.48 µm wide circle at the granule site; *A1*, *A2* and *A3* are the fluorescence values at the plateaus; τ1 and τ2 are the decay constants for the fluorescence increase after fusion and content release; and t1 and t2 are the times of fusion and release, respectively.

Statistics

Data are presented as mean ± S.E.M. and statistical differences between groups were tested using two tailed Student's *t*-test unless specified otherwise.

For the human islets, a simple linear regression analysis was performed on the miR-335 expression and stimulatory index data (a measure of insulin secretion capacity in the human islets). F-test was used to determine significance at $p<0.05$ as implemented in IBM SPSS v.22.

Results

Correlation of miR-335 expression and insulin secretion in human islets

We first investigated miR-335 expression in human islets. For this we used miR-335 expression data from 18 donors with HbA1c within the normal glucose tolerance levels (NGT; HbA1c=5.50 ± 0.06), and 10 donors with high HbA1c corresponding to impaired glucose tolerance levels (IGT; HbA1c=6.41 ± 0.15, p=7.69E-06 *vs* NGT). MiR-335 expression negatively correlated with insulin secretion, in islets from donors with IGT, whereas there was no correlation in islets from NGT donors (Fig. 1A-B). In line with these data, the human β-cells EndoC-βH1 overexpressing miR-335 had reduced glucose-stimulated insulin-secretion (Fig 1C). There was also a tendency of reduced K^+-induced secretion in these cells although this did not reach significance (Fig 1D).

Decreased expression of STXBP1, SNAP25 and SYT11 after overexpression of miR-335
To investigate the functional role of miR-335 in β-cells we overexpressed mature miR-335 in the rat INS-1 832/13 β-cell line (Fig. 2A). Hereafter these cells are referred to as OE335 cells and the controls are referred to as SCR (scrambled) cells.

Using TargetScan prediction tool (http://www.targetscan.org/) we found several exocytosis genes to be putative targets of miR-335, both in rodents and/or humans, including the exocytosis genes *Snap25*, *Syt11* and *Stxbp1*, which we decided to investigate further. Overexpression of miR-335 significantly reduced protein levels of all these miR-335 targets, SNAP25, STXBP1 and SYT11 (Fig. 2B-D).

Overexpression of miR-335 leads to reduced glucose-stimulated insulin secretion and exocytosis in INS-1 832/13 cells

As in the human EndoC-βH1 cells, OE335 cells displayed decreased glucose-stimulated insulin secretion compared to SCR cells (Fig. 3A), without any differences in insulin content (Fig 3B). K^+-induced insulin secretion was likewise reduced in OE335 cells (Fig. 3C), indicating effects on exocytosis. We used the patch clamp technique to measure depolarisation-induced exocytosis in OE335 cells (Fig. 3D-E). We found a significantly reduced increase in membrane capacitance elicited by the train of depolarisations ($\sum Depol_{all}$) in OE335 cells as compared to SCR (Fig. 3E). The increase in membrane capacitance evoked by the first two depolarisations ($\sum Depol_{1-2}$), was not affected, but the capacitance increase due to the latter depolarisations ($\sum Depol_{3-10}$) was reduced by ~60% in OE335 cells (Fig. 3E). The average cell size did not differ significantly between OE335 and SCR cells (5.91±0.38 pF *vs* 5.44±0.27 pF; n=13-16). The close link between exocytosis and influx of Ca^{2+} through voltage-gated Ca^{2+}-channels (Ammala et al., 1993) made us investigate the effect of miR-335 modulation on the current-voltage relationships of the voltage-dependent Ca^{2+}-currents in OE335 cells (Fig. 3F-G). We observed no differences in the Ca^{2+}-current at any of the voltages tested.

We also reduced expression of miR-335 using locked nucleotide acid (LNA) against miR-335 (LNA-335; Fig 4A) and investigated the effects on insulin secretion and content (Fig. 4B-D), and on Ca^{2+}-induced exocytosis (Fig. 4E-F). LNA335 reduced insulin content (Fig. 4C), but had no effect on insulin secretion in INS1-832-13 cells (Fig 4B,D). However, LNA-335 significantly increased membrane capacitance elicited by the train of depolarizations ($\sum Depol_{all}$). Reduction of miR-335 expression significantly increased both $\sum Depol_{1-2}$ and $\sum Depol_{3-10}$ as compared to control (Fig. 4E-F).

Overexpression of miR-335 reduces exocytosis and accelerates content release from individual granules in INS-1 832/13 cells
To study in detail the effects of elevated miR-335 levels on insulin granule docking and release, OE335 and SCR cells were co-transfected with granular marker neuropeptide-Y (NPY)-

mEGFP, and imaged by TIRF microscopy. Docked granules were present in both groups and their density was similar to previous estimates in these cells (Gandasi and Barg, 2014), with 0.59±0.02 μm^{-2} (n=31) in OE335 and 0.64±0.02 μm^{-2} (n=53) in SCR (Fig. 5A-B).

We stimulated exocytosis by local application of elevated K^+, which induces rapid exocytosis of docked and primed granules seen as rapid disappearance of granule fluorescence (Fig. 5C). Two thirds of the exocytotic events were preceded by a temporary increase in granule fluorescence (Fig. 5C, I-J) due to unquenching of EGFP-fluorescence that occurs when the granule lumen makes aqueous contact with the extracellular medium. Lack of this feature indicates rapid release and implies instantaneous widening of the fusion pore (Barg et al., 2002). The duration of the transient increase, which reflects fusion pore lifetime, was estimated by non-linear fit of a discontinuous function to the fluorescence time course (Fig. 5F and methods); no significant difference between OE335 cells and SCR cells was detected (Fig. 5G). The kinetics of content release was analysed by fitting a single exponential function to the fluorescence decay of individual events (Fig. 5F-H). The decay of vesicular fluorescence was twice as fast in OE335 cells compared to SCR cells (Fig. 5H). The total number of exocytosis events in OE335 was significantly less than in SCR cells (Fig. 5D-E).

Discussion

The contribution of miRNAs in the regulation of pancreatic β-cell function has been a topic of great interest in diabetes research. Consequently, mechanistic details on how dysregulated miRNA expression in the β-cell contributes to the pathophysiology of T2D are beginning to emerge. We have previously shown that miR-335 is abnormally over-expressed in the pancreatic islets of the GK rat (Esguerra et al., 2011), and we have validated *Stxbp1* as a direct target of miR-335. Here we show a negative correlation between miR-335 expression and insulin secretion in human islets from donors with IGT and provide evidence that overexpression of miR-335 results in 1) down-regulation of three exocytosis protein targets: STXBP1, SNAP25 and SYT11, and 2) impaired exocytosis of insulin granules and decreased insulin secretion.

Although it is known that the defective insulin secretory capacity can be due to defects in the exocytotic machinery, e.g. through reduced expression of exocytosis proteins in the GK-rat (Zhang et al., 2002), it is unclear how β-cell exocytosis in general is influenced by dysregulated expression of specific miRNAs. Our data support the hypothesis that the main function of miR-335 is in the regulation of the final stages of insulin secretion. Indeed, both single-cell capacitance measurements (Fig. 3D-E) and TIRF microscopy data (Fig. 5) confirmed defective priming of already docked granules and deficiencies in post-priming processes of exocytosis after overexpression of miR-335. The expression of miR-335 are >1000 times the endogenous levels, prompting us to perform experiments in which the endogenous levels of miR-335 were silenced (Fig. 4). In these experiments exocytosis was instead increased confirming that rno-

miR-335 indeed are involved in the regulation of β -cell exocytosis. However, while LNA-335 increased exocytosis, it simultaneously reduced insulin content. The reduced insulin content after miR-335 knockdown was to some extent surprising and shows that the knock-down of miR-335 needs to be adjusted if it should be used therapeutically. The summed outcome of reduced insulin content and increased exocytosis being unchanged insulin secretion in LNA-335 cells. Our results demonstrate the inherent complexity by which a single miRNA can influence the regulation of multiple targets and hence the overall targeted cellular process. Classically miRNAs has been thought to act as binary off-switches to repress mRNA expression; models that are more recent suggest that miRNAs can act as rheostats to control protein levels enabling customized expression in different cells and cellular processes (Bartel, 2009). Thus, it seems like miR-335 differentially regulate targets within processes controlling insulin content and insulin exocytosis. Altogether, we can still conclude that overexpression of miR-335 reduce insulin secretion mainly through an effect on exocytosis.

We asked if we could identify potential targets of miR-335 within the exocytosis pathway and if the overexpression reduced the protein level of these targets. Indeed, we found reduced protein expression of SNAP25, STXBP1 and SYT11 in OE335 cells. SYT11 is one of six Ca^{2+}-insensitive isoforms of the synaptotagmin family (Gauthier and Wollheim, 2008, Milochau et al., 2014, Sudhof, 2004). The cellular function of SYT11 is beginning to be understood (Andersson et al., 2012, Arango Duque et al., 2013, Fadista et al., 2014, Milochau et al., 2014), but details regarding its role in exocytosis remain unclear. Recently SYT11 was also shown to interact with components of the RNA-induced silencing complex (RISC) and proteins involved in endoplasmic reticulum/Golgi derived-granule transport in rat beta (INS-1E) cells implicating SYT11 in both gene regulation by miRNAs and membrane trafficking (Milochau et al., 2014). Although less is known about SYT11 our data presented here are in agreement with the established roles of SNAP25 and STXBP1 as essential components in priming and fusion of insulin-containing granules (Tomas et al., 2008, Gulyas-Kovacs et al., 2007, Eliasson et al., 2008b, Kang et al., 2002, Ohara-Imaizumi et al., 2004b, Zhang et al., 2002, Zhang et al., 2000). STXBP1 binds to the folded conformation of STX1A during docking (Han et al., 2011), and subsequently dissociates from STX1A upon Ca^{2+} influx to allow formation of the SNARE complex (Tomas et al., 2008) in the priming step (Gulyas-Kovacs et al., 2007). In addition, studies have implicated STXBP1, as well as SNAP25 and the synaptotagmin isoforms 1 and 7, in the recruitment of granules to the membrane (Ohara-Imaizumi et al., 2007, Tomas et al., 2008, Gulyas-Kovacs et al., 2007, de Wit et al., 2009, Dolai et al., 2016). Indeed, granule docking coincides with the formation of clusters containing STXBP1 (Gandasi and Barg, 2014) and the physiological importance of docking in insulin secretion was shown in the diabetic GK-rat (Ohara-Imaizumi et al., 2004a). Although STXBP1, SNAP25 and SYT11 were down-regulated in the OE335 cells, we did not observe a reduced number of docked granules. This lack of effects on docking might be due to insufficient miR-335-mediated knockdown (Fig. 2B-D) or the possibility that miR-335 has other unidentified targets that normally inhibit granule docking. It is possible that other targets of miR-335 contribute to the regulation of insulin secretion, resulting in a net cumulative negative effect of miR-335 on insulin secretion. For

instance, miR-335 directly targets the transcription factors FOXA2 and SOX17 and miR-335 thereby promotes mesendodermal lineage segregation (Yang et al., 2014). FOXA2 is not only involved in developmental β-cell differentiation but the protein also impact β-cell function. Foxa2 deficient mice have increased insulin secretion and increased number of docked granules (Gao et al., 2007). Nevertheless, previous and current findings link miR-335, SNAP-25, STXBP1 and SYT11 to the exocytotic process where imbalances in any of them could contribute to impaired insulin exocytosis.

We provide data demonstrating that overexpression of miR-335 in the human EndoC-βH1 cells reduce insulin secretion and that miR-335 expression was negatively correlated with insulin secretion in human islets from individuals with pre-diabetes. It is remarkable that miR-335 expression levels negatively correlated with insulin secretion only in islets from high HbA1c donors. We can only speculate that as fine-tuners of cellular processes (Bartel, 2009), miRNAs may be mostly required during non-optimal conditions, perhaps to be able to compensate for the detrimental effects brought about by pathophysiological condition. In our previous study (Esguerra et al., 2011) we showed elevated miR-335 expression in GK-rat as compared to Wistar-rat islets. This was true also when islets were incubated in hypoglycemic conditions (2.8 mM glucose) for 24 h. However, under hyperglycemic conditions (16.7 mM glucose) for the same period we found evidence of compensatory mechanisms bringing the levels of miR-335 in the GK-islets towards that of control. Interestingly, in short-term incubation for 1h miR-335 expression in the GK-islets was instead elevated after incubation in 16.7 mM glucose as compared to 2.8 mM glucose (or the Wistar control). We postulate therefore that under constant hyperglycaemic environment in diabetic patients, miRNA-mediated regulatory effects are more pronounced and hence the negative correlation between miR-335 levels and HbA1c is more apparent in the diseased state.

We find our human data of specific interest since one of the signatures of T2D is reduced first phase insulin secretion prior to full-blown diabetes (Gerich, 2002). On a cellular level first phase insulin secretion has been suggested to be associated with exocytosis and the fusion of primed granules (Eliasson et al., 2008b, Rorsman and Renstrom, 2003). More so, our observation might be essential in a larger metabolic perspective. Expression of miR-335 has been demonstrated to be increased in liver and white adipocyte tissue in ob/ob and db/db mice (Nakanishi et al., 2009, Oger et al., 2014), and in islets of diabetic GK-rats (Esguerra et al., 2011). MiR-335 is suggested to be involved in lipid metabolism, adipogenesis differentiation, and adipose inflammation (Nakanishi et al., 2009, Zhu et al., 2014), and miR-335 expression is proposed to be regulated by adipokines (Zhu et al., 2014). It is well-established that regulation of glucose-transporters in adipose tissue, involves exocytosis proteins (Cheatham, 2000). Hence, our observation that LNA335 and OE335 influence the exocytosis process might not only be valid for pancreatic β-cells but also for other metabolically relevant tissues. In this aspect it is noteworthy that miRNAs, as important players in gene regulation, have the potential to be innovative therapeutic drug targets against diabetes and associated complications

(Eliasson and Esguerra, 2014). To achieve this goal, a necessary first step is to understand the mechanistic principles by which miRNAs participate in cellular processes.

In conclusion, our data demonstrate the cellular mechanisms by which miR-335 influence insulin secretion. Based on our observations we propose that miR-335 over-expression negatively affects insulin secretion via the reduction of multiple exocytosis protein targets and impaired priming of insulin granules.

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information file. Additional access are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no duality of interest associated with this manuscript.

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Author contribution

V.A.S., J.L.S.E, and L.E. designed the study. V. A.S., J.K.O., N.R.G., S.A.S., S.H., M.E.A., A.W., S.B., J.L.S.E and L.E. participated in acquisition and analysis of data. V.A.S., A.W., S.B., J.L.S.E and L.E. participated in interpretation of data. V.A.S., J.L.S.E, S.B. and L.E. drafted the manuscript. V.A.S., J.K.O, N.R.G., S.A.S., A.W., S.B., J.L.S.E, and L.E. revised the manuscript critically for important intellectual content and approved the final version.

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References

AMMALA, C., ELIASSON, L., BOKVIST, K., LARSSON, O., ASHCROFT, F. M. & RORSMAN, P. 1993. Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *J Physiol,* 472**,** 665-88.

ANDERSSON, L. E., VALTAT, B., BAGGE, A., SHAROYKO, V. V., NICHOLLS, D. G., RAVASSARD, P., SCHARFMANN, R., SPEGEL, P. & MULDER, H. 2015. Characterization of stimulus-secretion coupling in the human pancreatic EndoC-betaH1 beta cell line. *PLoS One,* 10**,** e0120879.

ANDERSSON, S. A., OLSSON, A. H., ESGUERRA, J. L., HEIMANN, E., LADENVALL, C., EDLUND, A., SALEHI, A., TANEERA, J., DEGERMAN, E., GROOP, L., LING, C. & ELIASSON, L. 2012. Reduced insulin secretion correlates with decreased expression of exocytotic genes in pancreatic islets from patients with type 2 diabetes. *Mol Cell Endocrinol,* 364**,** 36-45.

ARANGO DUQUE, G., FUKUDA, M. & DESCOTEAUX, A. 2013. Synaptotagmin XI regulates phagocytosis and cytokine secretion in macrophages. *J Immunol,* 190**,** 1737-45.

BARG, S., OLOFSSON, C. S., SCHRIEVER-ABELN, J., WENDT, A., GEBRE-MEDHIN, S., RENSTROM, E. & RORSMAN, P. 2002. Delay between fusion pore opening and peptide release from large dense-core vesicles in neuroendocrine cells. *Neuron,* 33**,** 287-99.

BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* 116**,** 281-97.

BARTEL, D. P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell,* 136**,** 215-33.

BRENNECKE, J., STARK, A., RUSSELL, R. B. & COHEN, S. M. 2005. Principles of microRNA-target recognition. *PLoS biology,* 3**,** e85.

CHEATHAM, B. 2000. GLUT4 and company: SNAREing roles in insulin-regulated glucose uptake. *Trends Endocrinol Metab,* 11**,** 356-61.

DE WIT, H., WALTER, A. M., MILOSEVIC, I., GULYAS-KOVACS, A., RIEDEL, D., SORENSEN, J. B. & VERHAGE, M. 2009. Synaptotagmin-1 docks secretory vesicles to syntaxin-1/SNAP-25 acceptor complexes. *Cell,* 138**,** 935-46.

DEL PRATO, S. & TIENGO, A. 2001. The importance of first-phase insulin secretion: implications for the therapy of type 2 diabetes mellitus. *Diabetes Metab Res Rev,* 17**,** 164-74.

DOLAI, S., XIE, L., ZHU, D., LIANG, T., QIN, T., XIE, H., KANG, Y., CHAPMAN, E. R. & GAISANO, H. Y. 2016. Synaptotagmin-7 Functions to Replenish Insulin Granules for Exocytosis in Human Islet beta-Cells. *Diabetes,* 65**,** 1962-76.

ELIASSON, L., ABDULKADER, F., BRAUN, M., GALVANOVSKIS, J., HOPPA, M. B. & RORSMAN, P. 2008a. Novel aspects of the molecular mechanisms controlling insulin secretion. *J Physiol.,* 586**,** 3313-24.

ELIASSON, L., ABDULKADER, F., BRAUN, M., GALVANOVSKIS, J., HOPPA, M. B. & RORSMAN, P. 2008b. Novel aspects of the molecular mechanisms controlling insulin secretion. *J Physiol,* 586**,** 3313-24.

ELIASSON, L. & ESGUERRA, J. L. 2014. Role of non-coding RNAs in pancreatic beta-cell development and physiology. *Acta Physiol (Oxf).*

ESGUERRA, J. L., BOLMESON, C., CILIO, C. M. & ELIASSON, L. 2011. Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLoS One,* 6**,** e18613.

FADISTA, J., VIKMAN, P., LAAKSO, E. O., MOLLET, I. G., ESGUERRA, J. L., TANEERA, J., STORM, P., OSMARK, P., LADENVALL, C., PRASAD, R. B., HANSSON, K. B., FINOTELLO, F., UVEBRANT, K., OFORI, J. K., DI CAMILLO, B., KRUS, U., CILIO, C. M., HANSSON, O., ELIASSON, L., ROSENGREN, A. H., RENSTROM, E., WOLLHEIM, C. B. & GROOP, L. 2014. Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism. *Proc Natl Acad Sci U S A,* 111**,** 13924-9.

GAISANO, H. Y., OSTENSON, C. G., SHEU, L., WHEELER, M. B. & EFENDIC, S. 2002. Abnormal expression of pancreatic islet exocytotic soluble N-ethylmaleimide-sensitive factor attachment protein receptors in Goto-Kakizaki rats is partially restored by phlorizin treatment and accentuated by high glucose treatment. *Endocrinology,* 143**,** 4218-26.

GANDASI, N. R. & BARG, S. 2014. Contact-induced clustering of syntaxin and munc18 docks secretory granules at the exocytosis site. *Nat Commun,* 5.

GAO, N., WHITE, P., DOLIBA, N., GOLSON, M. L., MATSCHINSKY, F. M. & KAESTNER, K. H. 2007. Foxa2 controls vesicle docking and insulin secretion in mature Beta cells. *Cell Metab,* 6**,** 267-79.

GAUTHIER, B. R. & WOLLHEIM, C. B. 2008. Synaptotagmins bind calcium to release insulin. *Am J Physiol Endocrinol Metab,* 295**,** E1279-86.

GERICH, J. E. 2002. Is reduced first-phase insulin release the earliest detectable abnormality in individuals destined to develop type 2 diabetes? *Diabetes,* 51 Suppl 1**,** S117-21.

GULYAS-KOVACS, A., DE WIT, H., MILOSEVIC, I., KOCHUBEY, O., TOONEN, R., KLINGAUF, J., VERHAGE, M. & SORENSEN, J. B. 2007. Munc18-1: sequential interactions with the fusion machinery stimulate vesicle docking and priming. *J Neurosci,* 27**,** 8676-86.

HAN, G. A., MALINTAN, N. T., SAW, N. M., LI, L., HAN, L., MEUNIER, F. A., COLLINS, B. M. & SUGITA, S. 2011. Munc18-1 domain-1 controls vesicle docking and secretion by interacting with syntaxin-1 and chaperoning it to the plasma membrane. *Molecular biology of the cell,* 22**,** 4134-49.

HOHMEIER, H. E., MULDER, H., CHEN, G., HENKEL-RIEGER, R., PRENTKI, M. & NEWGARD, C. B. 2000. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes,* 49**,** 424-30.

KANG, Y., HUANG, X., PASYK, E. A., JI, J., HOLZ, G. G., WHEELER, M. B., TSUSHIMA, R. G. & GAISANO, H. Y. 2002. Syntaxin-3 and syntaxin-1A inhibit L-type calcium channel activity, insulin biosynthesis and exocytosis in beta-cell lines. *Diabetologia,* 45**,** 231-41.

LOVIS, P., GATTESCO, S. & REGAZZI, R. 2008. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biological chemistry,* 389**,** 305-12.

MILOCHAU, A., LAGREE, V., BENASSY, M. N., CHAIGNEPAIN, S., PAPIN, J., GARCIA-ARCOS, I., LAJOIX, A., MONTERRAT, C., COUDERT, L., SCHMITTER, J. M., OCHOA, B. & LANG, J. 2014. Synaptotagmin 11 interacts with components of the RNA-induced silencing complex RISC in clonal pancreatic beta-cells. *FEBS Lett,* 588**,** 2217-22.

NAKANISHI, N., NAKAGAWA, Y., TOKUSHIGE, N., AOKI, N., MATSUZAKA, T., ISHII, K., YAHAGI, N., KOBAYASHI, K., YATOH, S., TAKAHASHI, A., SUZUKI, H., URAYAMA, O., YAMADA, N. & SHIMANO, H. 2009. The up-regulation of microRNA-335 is associated with lipid metabolism in liver and white adipose tissue of genetically obese mice. *Biochem Biophys Res Commun,* 385**,** 492-6.

OGER, F., GHEERAERT, C., MOGILENKO, D., BENOMAR, Y., MOLENDI-COSTE, O., BOUCHAERT, E., CARON, S., DOMBROWICZ, D., PATTOU, F., DUEZ, H., EECKHOUTE, J., STAELS, B. & LEFEBVRE, P. 2014. Cell-specific dysregulation of microRNA expression in obese white adipose tissue. *J Clin Endocrinol Metab,* 99**,** 2821-33.

OHARA-IMAIZUMI, M., FUJIWARA, T., NAKAMICHI, Y., OKAMURA, T., AKIMOTO, Y., KAWAI, J., MATSUSHIMA, S., KAWAKAMI, H., WATANABE, T., AKAGAWA, K. & NAGAMATSU, S. 2007. Imaging analysis reveals mechanistic differences between first- and second-phase insulin exocytosis. *J Cell Biol,* 177**,** 695-705.

OHARA-IMAIZUMI, M., NISHIWAKI, C., KIKUTA, T., NAGAI, S., NAKAMICHI, Y. & NAGAMATSU, S. 2004a. TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic beta-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat beta-cells. *Biochem J,* 381**,** 13-8.

OHARA-IMAIZUMI, M., NISHIWAKI, C., NAKAMICHI, Y., KIKUTA, T., NAGAI, S. & NAGAMATSU, S. 2004b. Correlation of syntaxin-1 and SNAP-25 clusters with docking and fusion of insulin granules analysed by total internal reflection fluorescence microscopy. *Diabetologia,* 47**,** 2200-7.

OSTENSON, C. G., GAISANO, H., SHEU, L., TIBELL, A. & BARTFAI, T. 2006. Impaired gene and protein expression of exocytotic soluble N-ethylmaleimide attachment protein receptor complex proteins in pancreatic islets of type 2 diabetic patients. *Diabetes,* 55**,** 435-40.

PANG, Z. P. & SUDHOF, T. C. 2010. Cell biology of Ca2+-triggered exocytosis. *Curr Opin Cell Biol,* 22**,** 496-505.

PORTHA, B., LACRAZ, G., KERGOAT, M., HOMO-DELARCHE, F., GIROIX, M. H., BAILBE, D., GANGNERAU, M. N., DOLZ, M., TOURREL-CUZIN, C. & MOVASSAT, J. 2009. The GK rat beta-cell: a prototype for the diseased human beta-cell in type 2 diabetes? *Mol Cell Endocrinol,* 297**,** 73-85.

RAVASSARD, P., HAZHOUZ, Y., PECHBERTY, S., BRICOUT-NEVEU, E., ARMANET, M., CZERNICHOW, P. & SCHARFMANN, R. 2011. A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. *J Clin Invest,* 121**,** 3589-97.

RIZO, J. & SUDHOF, T. C. 2012. The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices--guilty as charged? *Annu Rev Cell Dev Biol,* 28**,** 279-308.

RORSMAN, P. & RENSTROM, E. 2003. Insulin granule dynamics in pancreatic beta cells. *Diabetologia,* 46**,** 1029-45.

SALUNKHE, V. A., ESGUERRA, J. L., OFORI, J. K., MOLLET, I. G., BRAUN, M., STOFFEL, M., WENDT, A. & ELIASSON, L. 2015. Modulation of microRNA-375 expression alters voltage-gated Na channel properties and exocytosis in insulin-secreting cells. *Acta Physiol (Oxf)*.

SUDHOF, T. C. 2004. The synaptic vesicle cycle. *Annu Rev Neurosci,* 27**,** 509-47.

TOMAS, A., MEDA, P., REGAZZI, R., PESSIN, J. E. & HALBAN, P. A. 2008. Munc 18-1 and granuphilin collaborate during insulin granule exocytosis. *Traffic,* 9**,** 813-32.

WANG, Z. & THURMOND, D. C. 2009. Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J Cell Sci,* 122**,** 893-903.

YANG, D., LUTTER, D., BURTSCHER, I., UETZMANN, L., THEIS, F. J. & LICKERT, H. 2014. miR-335 promotes mesendodermal lineage segregation and shapes a transcription factor gradient in the endoderm. *Development,* 141**,** 514-25.

ZHANG, W., EFANOV, A., YANG, S. N., FRIED, G., KOLARE, S., BROWN, H., ZAITSEV, S., BERGGREN, P. O. & MEISTER, B. 2000. Munc-18 associates with syntaxin and serves as a negative regulator of exocytosis in the pancreatic beta -cell. *The Journal of biological chemistry,* 275**,** 41521-7.

ZHANG, W., KHAN, A., OSTENSON, C. G., BERGGREN, P. O., EFENDIC, S. & MEISTER, B. 2002. Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats. *Biochem Biophys Res Commun,* 291**,** 1038-44.

ZHU, L., CHEN, L., SHI, C. M., XU, G. F., XU, L. L., ZHU, L. L., GUO, X. R., NI, Y., CUI, Y. & JI, C. 2014. MiR-335, an adipogenesis-related microRNA, is involved in adipose tissue inflammation. *Cell Biochem Biophys,* 68**,** 283-90.

Figure legends

Figure 1. miR-335 and insulin secretion in human islets and EndoC-βH1 cells

A. miR-335 expression in human islets NGT donors against stimulation index,

B. As in A, but data is measured in human islets from IGT donors.

C. Insulin secretion in EndoC-βH1 cells overexpressing miR-335 (OE335(h); black bars) and in control cells (SCR(h); white bars) cells after stimulation for 1 h in 2.8 mM or 16.7 mM glucose (2.8G and 16.7 G) as indicated. n=3; *P<0.05.

D. As in C, but insulin secretion was measured after 15 min stimulation in 2.8 mM glucose (2.8G) in the absence and presence of 50 mM KCl (K^+).

Figure 2. Overexpression of miR-335 and its effect on the expression of three of its putative protein targets

A. Expression of miR-335 using mature miR-335 mimic (OE335; black bar) relative to its expression using scramble control (SCR; white bar). Mir-335 expression was normalized to endogenous controls U6 and U87. n=4; **P<0.01.

B,C,D. Average protein levels of SNAP25, STXBP1 and SYT11 in OE335 (black bar) compared to SCR (white bar) cells (n=4 each group; *P<0.05). Representative western blots are shown for each protein. Expression levels were normalized to beta-actin or cyclophilin B (PPIB).

Figure 3. Consequence on insulin secretion and exocytosis in INS-1 832/13 cells by miR-335 overexpression

A. Insulin secretion in OE335 (black bars) and SCR (white bars) cells after stimulation for 1 h in 2.8 mM or 16.7 mM glucose as indicated. n=3; **P<0.01. Secreted insulin is measured using human insulin-RIA.

B. Insulin content in OE335 (black bar) and SCR (white bars) cells. n=3

C. Insulin secretion in OE335 (black bars) and SCR (white bars) cells after stimulation for 15 min. 2.8 mM glucose with or without 50 mM K^+ as indicated. n=5; *P<0.05.

D. Representative traces of depolarization-induced increases of membrane capacitance in OE335 (black trace) and SCR (grey trace) cells.

E. Summary of capacitance changes presented as the summed increased in membrane capacitance during all ten depolarisations ($\Sigma Depol_{all}$), increase evoked by the first two depolarisations ($\Sigma Depol_{1-2}$) or the latter eight depolarisations ($\Sigma Depol_{3-10}$). n=13 for OE335 (black bar) and n=16 for SCR (white bar) cells; *P<0.05

F. Representative traces of a voltage-dependent Ca^{2+} currents evoked by a depolarization from -70 mV to 0 mV in a control cell (SCR) and a cell overexpressing miR-335 (OE335), respectively.

G. Summary of the charge (Q)-voltage (V) relationship. n=15 for SCR and n=17 for OE335 cells.

Figure 4. Influence of miR-335 knockdown on insulin secretion and exocytosis in INS-1 832/13 cells

A. Expression of miR-335 in cells after knockdown of miR-335 by LNA against miR-335 (LNA335; black bar) relative to expression in scramble control cells (SCR; white bar). n=4; ***P<0.001.

B. Insulin secretion in LNA335 (black bars) and SCR (white bars) cells after stimulation for 1 h in 2.8 mM or 16.7 mM glucose as indicated (n=4). The amount of released insulin is measured using human/rat insulin-ELISA.

C. Insulin content in in LNA335 (black bars) and SCR (white bars) cells. n=8; *P<0.05

D. Insulin secretion in OE335 (black bars) and SCR (white bars) cells after stimulation for 15 min. in 2.8 mM glucose with 50 mM K^+. n=4.

E. Representative traces of depolarization-induced increases of membrane capacitance in LNA335 (black trace) and SCR (grey trace) cells.

F. Summary of capacitance changes presented as the summed increased in membrane capacitance during all ten depolarisations ($\Sigma Depol_{all}$), increase evoked by the first two depolarisations ($\Sigma Depol_{1-2}$) or the latter eight depolarisations ($\Sigma Depol_{3-10}$). n=10 for LNA335 (black bar) and n=9 for SCR (white bar) cells; *P<0.05

Figure 5. Function of miR-335 on density of granules and exocytosis measured by TIRF microscopy in INS-1 832/13 cells

A. Representative TIRF images of OE335 and SCR cells co-transfected with NPY-mEGFP.

B. Density of granules in OE335 (black bar; n=31) and SCR (white bar; n=53) cells.

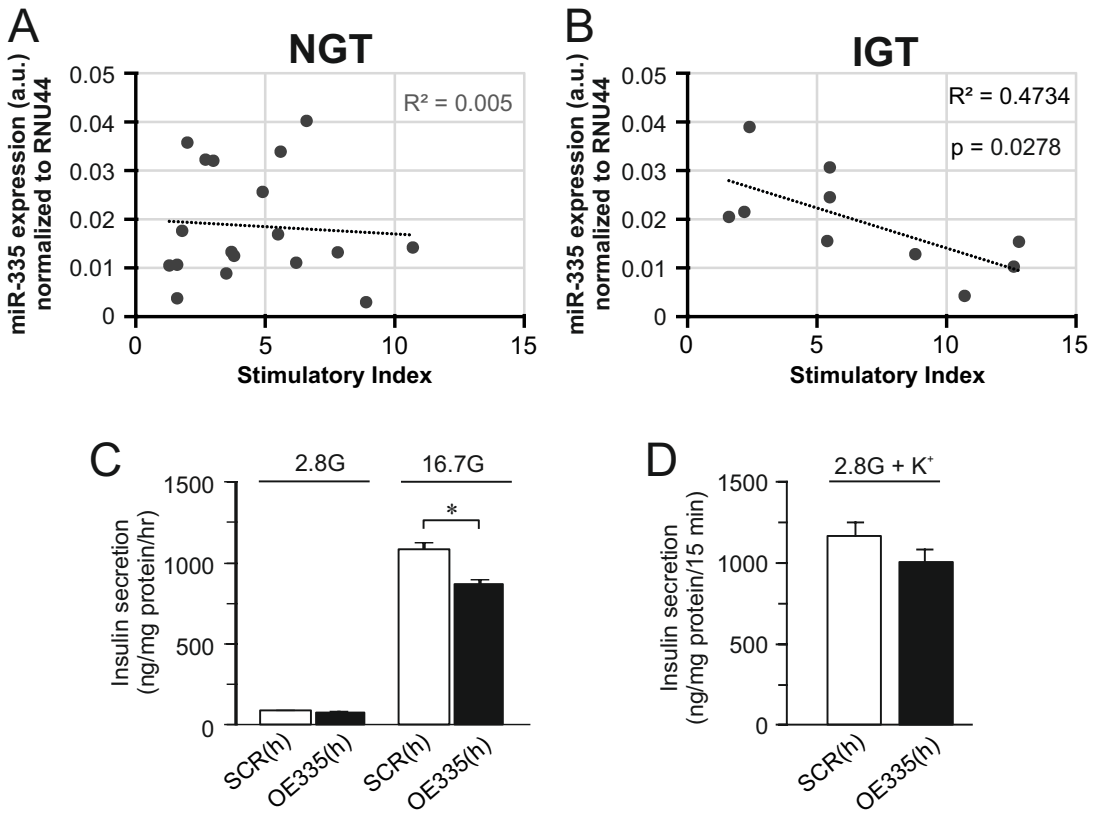
C. Examples of single exocytosis event measured in OE335 and SCR cells, frames are 100 ms apart. Exocytosis was stimulated through depolarization using elevated K^+.

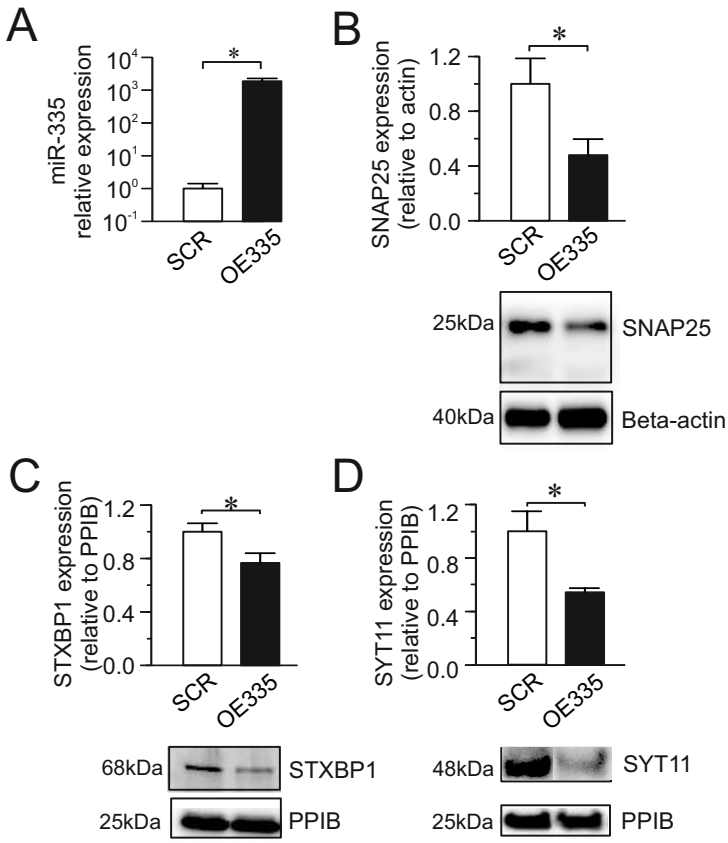
D,E. Cumulative number of exocytosis events per area in OE335 and SCR cells (n=8 each group; ***P<0.001).

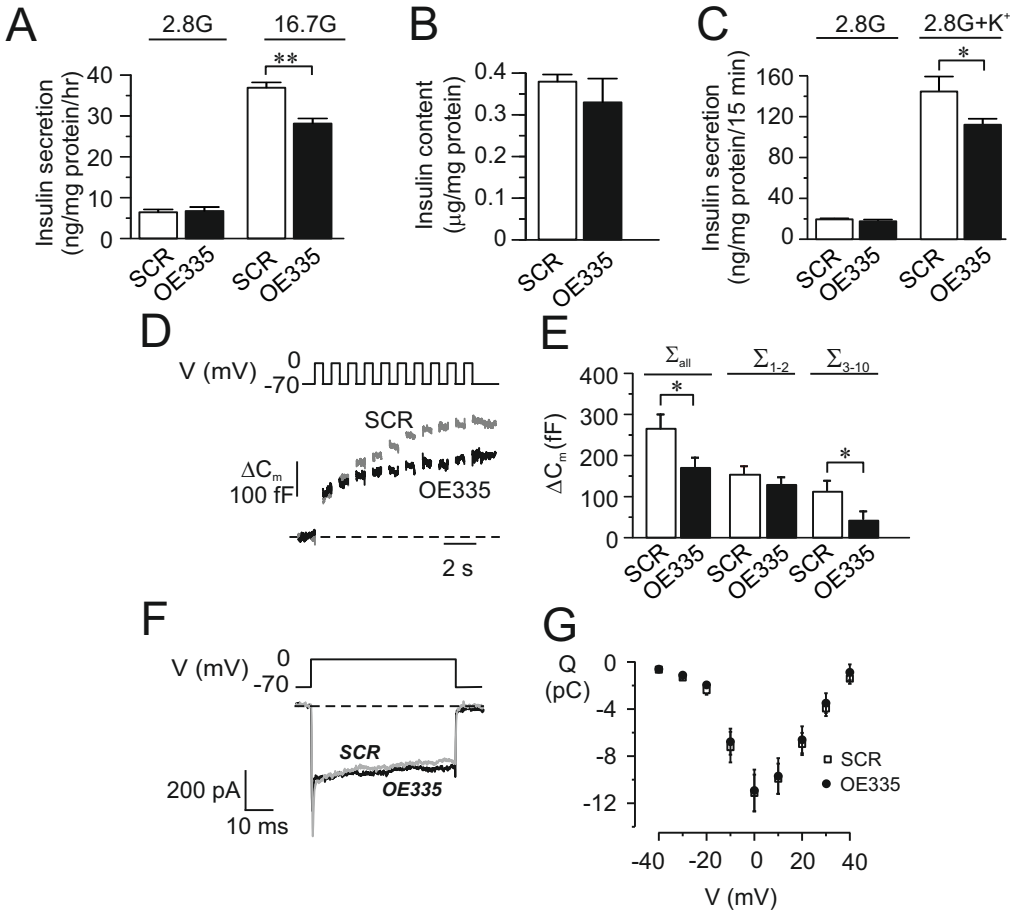
F. Representative image of fluorescence time-course during a single exocytosis event (black) with fit overlaid (red) illustrating the numerical analysis. The interval between the moments of exocytosis/fusion and release is taken as fusion pore lifetime (green).

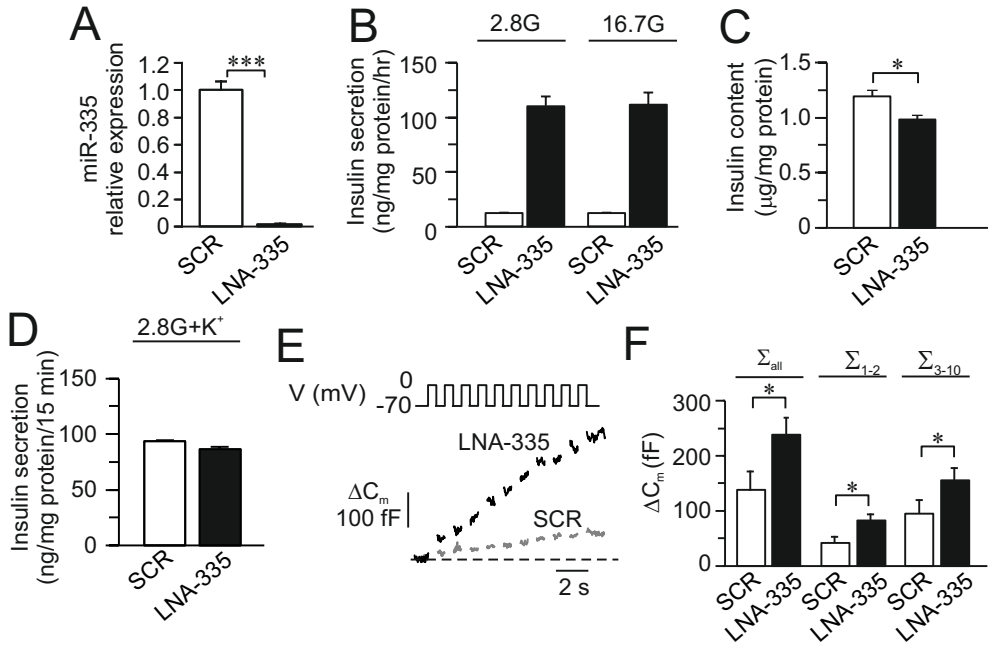
G,H. Summary of fitted exocytosis and decay constants of individual events as described in F. The lifetime of the events (*P=N.S.) and decay constant from individual granules during content release (*P<0.05) was measured in OE335 and SCR cells (n=8 each group).

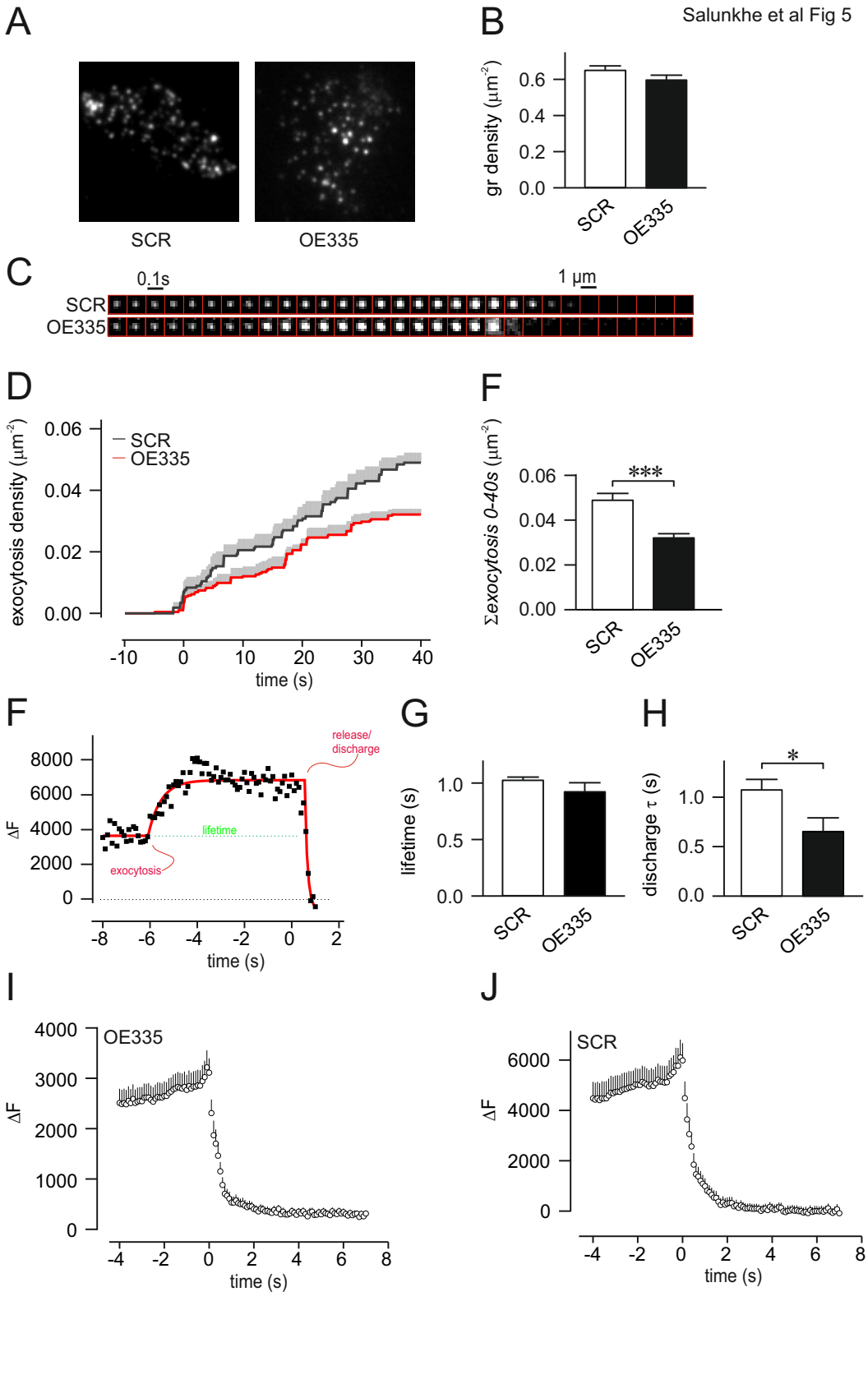
I,J. Average fluorescence signal of granule fluorescence for exocytosis events in OE335 and SCR cells as in C.


A
NGT
R² = 0.005
miR-335 expression (a.u.) normalized to RNU44
Stimulatory Index
B
IGT
R² = 0.4734
p = 0.0278
miR-335 expression (a.u.) normalized to RNU44
Stimulatory Index
C
2.8G
16.7G
*
Insulin secretion (ng/mg protein/hr)
SCR(h)
OE335(h)
SCR(h)
OE335(h)
D
2.8G + K+
Insulin secretion (ng/mg protein/15 min)
SCR(h)
OE335(h)


A
miR-335
relative expression
10^4
10^3
10^2
10^1
10^0
10^{-1}
*
SCR
OE335
B
SNAP25 expression
(relative to actin)
1.2
0.8
0.4
0.0
*
SCR
OE335
25kDa
SNAP25
40kDa
Beta-actin
C
STXBP1 expression
(relative to PPIB)
1.2
0.8
0.4
0.0
*
SCR
OE335
68kDa
STXBP1
25kDa
PPIB
D
SYT11 expression
(relative to PPIB)
1.2
0.8
0.4
0.0
*
SCR
OE335
48kDa
SYT11
25kDa
PPIB


A
2.8G
16.7G
**
Insulin secretion (ng/mg protein/hr)
40
30
20
10
0
SCR
OE335
SCR
OE335
B
Insulin content (µg/mg protein)
0.4
0.3
0.2
0.1
0
SCR
OE335
C
2.8G
2.8G+K+
*
Insulin secretion (ng/mg protein/15 min)
160
120
80
40
0
SCR
OE335
SCR
OE335
D
V (mV)
0
-70
SCR
OE335
ΔCm
100 fF
2 s
E
Σall
Σ1-2
Σ3-10
*
*
ΔCm (fF)
400
300
200
100
0
SCR
OE335
SCR
OE335
SCR
OE335
F
V (mV)
0
-70
SCR
OE335
200 pA
10 ms
G
Q (pC)
0
-4
-8
-12
SCR
OE335
-40
-20
0
20
40
V (mV)


A
miR-335 relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0
***
SCR
LNA-335
B
2.8G
16.7G
Insulin secretion (ng/mg protein/hr)
150
100
50
0
SCR
LNA-335
SCR
LNA-335
C
Insulin content (µg/mg protein)
1.5
1.0
0.5
0
*
SCR
LNA-335
D
2.8G+K+
Insulin secretion (ng/mg protein/15 min)
150
100
50
0
SCR
LNA-335
E
V (mV)
0
-70
LNA-335
ΔCm
100 fF
SCR
2 s
F
Σall
Σ1-2
Σ3-10
ΔCm (fF)
300
200
100
0
*
*
*
SCR
LNA-335
SCR
LNA-335
SCR
LNA-335


A
SCR
OE335
B
gr density (µm-2)
0.6
0.4
0.2
0.0
SCR
OE335
C
0.1s
1 µm
SCR
OE335
D
exocytosis density (µm-2)
0.06
0.04
0.02
0.00
SCR
OE335
-10
0
10
20
30
40
time (s)
F
Σexocytosis 0-40s (µm-2)
0.06
0.04
0.02
0.00
***
SCR
OE335
F
8000
6000
4000
2000
0
ΔF
release/
discharge
lifetime
exocytosis
-8
-6
-4
-2
0
2
time (s)
G
lifetime (s)
1.0
0.5
0.0
SCR
OE335
H
discharge τ (s)
*
1.0
0.5
0.0
SCR
OE335
I
OE335
4000
3000
2000
1000
0
ΔF
-4
-2
0
2
4
6
8
time (s)
J
SCR
6000
4000
2000
0
ΔF
-4
-2
0
2
4
6
8
time (s)

Paper III

Confluence does not affect the expression of miR-375 and its direct targets in rat and human insulin-secreting cell lines

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ABSTRACT

MicroRNAs are small non-coding RNAs, which negatively regulate the expression of target genes. They have emerged as important modulators in beta cell compensation upon increased metabolic demand, failure of which leads to reduced insulin secretion and type 2 diabetes. To elucidate the function of miRNAs in beta cells, insulin-secreting cell lines, such as the rat insulinoma INS-1 832/13 and the human EndoC-βH1, are widely used. Previous studies in the cancer field have suggested that miRNA expression is influenced by confluency of adherent cells. We therefore aimed to investigate whether one of the most enriched miRNAs in the pancreatic endocrine cells, miR-375, and two of its validated targets in mouse, *Cav1* and *Aifm1*, were differentially-expressed in cell cultures with different confluences. Additionally, we measured the expression of other miRNAs, such as miR-152, miR-130a, miR-132, miR-212 and miR-200a, with known roles in beta cell function. We did not see any significant expression changes of miR-375 nor any of the two targets, in both the rat and human beta cell lines at different confluences. Interestingly, among the other miRNAs measured, the expression of miR-132 and miR-212 positively correlated with confluence, but only in the INS-1 832/13 cells. Our results show that the expression of miR-375 and other miRNAs with known roles in beta cell function is independent of, or at least minimally influenced by the density of proliferating adherent cells, especially within the confluence range optimal for functional assays to elucidate miRNA-dependent regulatory mechanisms in the beta cell.

Subjects Molecular Biology, Diabetes and Endocrinology
Keywords microRNA, Pancreatic beta cell, Confluence, Diabetes, miR-375, Cell density

INTRODUCTION

Type-2 diabetes (T2D) is a complex metabolic disease characterized by elevated blood glucose levels due to a combination of insulin resistance and impaired insulin secretion (*Prasad & Groop, 2015*). Western life-style with reduced exercise and unhealthy food-habits result in insulin resistance in target tissues such as in liver, muscle and adipose tissue. To cope with increased metabolic demands, pancreatic beta cells secrete more insulin. Failure to compensate contributes to the development of T2D (*Halban et al., 2014*). By regulating

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various cellular processes within the beta cell, microRNAs (miRNAs) have been suggested to play important roles in rapid compensatory response to changing environments (*Eliasson & Esguerra, 2014*; *Esguerra et al., 2014*).

MiRNAs are small non-coding RNAs involved in the regulation of gene expression. They bind to the 3'UTR of the target mRNA leading to mRNA degradation and/or translational repression (*Bartel, 2009*). In diabetes, several miRNAs have been shown to be differentially expressed and have been shown to be involved in important beta cell functions and in maintaining beta cell mass (*Esguerra et al., 2014*; *Poy et al., 2009*).

The first miRNA discovered in the pancreatic islet cells was miR-375 (*Poy et al., 2004*), which is one of the most highly-enriched miRNAs in the pancreatic islets. Since its discovery, miR-375 has been shown to negatively regulate a plethora of genes involved in pancreatic beta cell function (*Eliasson, 2017*) such as in insulin secretion by regulating myotrophin (*Mtpn*) (*Poy et al., 2004*) and various voltage-gated sodium channels (SCNs) (*Salunkhe et al., 2015*). Knock out of miR-375 in mouse (*375KO*), resulted in hyperglycaemic animals with defective proliferative capacity of endocrine cells leading to decreased beta cell mass (*Poy et al., 2009*). Studies on islets of *375KO* mice reveal direct regulation of multiple genes involved in the negative control of cellular growth and proliferation such as the apoptosis-inducing factor, mitochondrion-associated 1 (*Aifm1*) and caveolin1 (*Cav1*) (*Poy et al., 2009*).

Another highly-enriched beta cell miRNA is miR-200a, demonstrated to be upregulated in islets of the db/db diabetic mouse model and shown to contribute in regulating pancreatic beta cell survival in T2D (*Belgardt et al., 2015*). There are also a number of miRNAs such as miR-132, miR-212, miR-130a and miR-152 shown to be upregulated in the pancreatic islets of the widely-studied T2D model Goto-Kakizaki rats (*Esguerra et al., 2011*) with active roles in beta cell stimulus-secretion coupling (*Malm et al., 2016*; *Ofori et al., 2017*).

Cell lines are commonly utilized to unravel the molecular mechanisms by which miRNAs participate in cellular processes. The ease of handling, maintenance and availability of cell line models make them indispensable tools in molecular biology investigations. Indeed, studying molecular mechanisms underlying fundamental beta cell processes such as the stimulus-secretion coupling have been made possible by tumour-derived rat insulin-secreting cell lines such as INS-1 (*Asfari et al., 1992*), and its more recent derivative sub-line, INS-1 832/13 cells (*Hohmeier et al., 2000*). Recently, the human beta cell line EndoC-βH1 has also been made available which further enabled deeper investigations of molecular mechanisms governing insulin secretion in humans (*Andersson et al., 2015*; *Ravassard et al., 2011*).

It has been noticed that the global abundance of miRNAs is generally lower in cell lines than in primary tissues of cancer (*Lu et al., 2005*). One hypothesis is that the tight cell–cell contacts in primary tissues contribute to the activation of miRNA biogenesis. Indeed, it was shown that higher cellular density or confluence resulted in higher levels of various miRNAs in HeLa and NIH3T3 cells (*Hwang, Wentzel & Mendell, 2009*; *Van Rooij, 2011*). Because many functional assays, e.g., insulin secretion assay, being performed on beta cell lines require optimal culture conditions including cell densities, we therefore set out to investigate whether confluence affects the expression of miR-375 and two of its

validated targets in the mouse beta cell, *Aifm1* and *Cav1*, in the rat INS-1 832/13 cells and in the human EndoC-βH1 cells. We also investigated the influence of confluence on the expression levels of miR-200a, miR-130a, miR-152, miR-132 and miR-212.

MATERIALS & METHODS

Reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

Cell culture, seeding and imaging

EndoC-βH1 cells (EndoCells, Paris, France) (*Andersson et al., 2015*; *Ravassard et al., 2011*) (passages between 76–80) were seeded on 24-well plates coated with Matrigel-fibronectin (100 μg/mL and 2 μg/mL; Sigma-Aldrich, Steinheim, Germany) at the following densities: 390,000 cells/well, 312,000 cells/well, 234,000 cells/well and 78,000 cells/well to reach 100%, 80%, 60% and 20% estimated confluence respectively after 48 h. The cells were maintained in a culture medium containing: DMEM (5.6 mM glucose), 2% BSA fraction V (Roche Diagnostics, Mannheim, Germany), 10 mM nicotinamide (Merck Millipore, Darmstadt, Germany), 50 μM 2-mercaptoethanol, 5.5 μg/mL transferrin, 6.7 ng/mL sodium selenite (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories, Pasching, Austria).

Rat insulinoma INS-1 832/13 cells (passages between 50–55) (*Hohmeier et al., 2000*) were seeded accordingly: 300,000 cells/well, 240,000 cells/well, 180,000 cells/well and 60,000 cells/well to reach 100%, 80%, 60% and 20% estimated confluence respectively after 48 h. The growth area per well in the 24 well plate is 1.9 cm^2. Cells were maintained in RPMI 1640 medium containing 11.1 mM glucose (HyClone, UT, USA) as previously described (*Salunkhe et al., 2015*). Both cell lines were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 48 h. All experiments were performed within culture passages in which the cell lines respond robustly to glucose-stimulated insulin secretion assay.

To measure cell-to-cell contact, 300 uL suspension of EndoC-βH1 cells were seeded on microscope slides with 8 chambered wells (growth area per well: 1.0 cm^2) (Cat. No. 80827; ibidi Gmbh, Planegg, Germany). The corresponding seeding cell densities for each estimated harvest confluency were as follows: 20% confluence: 41,053 cells/cm^2; 60% confluence: 123,158 cells/cm^2; 80% confluence: 1642,10 cells/cm^2; 100% confluence: 205,263 cells/cm^2. The seeded cells were imaged at 40× magnification on a Zeiss LSM 510 microscope. Cell-to-cell distances between randomly selected cells were measured ($n = 40$–90) and averaged. See Fig. S1 for representative image panels with distance measurements.

RNA extraction, RT-PCR and qPCR

RNA extraction, RT-PCR for total RNA and stem-loop RT-qPCR for microRNA was performed as previously described (*Salunkhe et al., 2015*) using the following TaqMan® miRNA Assays: miR-375 (RT_000564), miR-200a (RT_000502), miR-130a (RT_000454), miR-152 (RT_000475), miR-132 (RT_000457), miR-212 (RT_002551), rat U87

(RT_001712), human RNU44 (RT_001094) and human RNU48 (RT_001006) for generating cDNA. The following primers from TaqMan® Gene Expression and TaqMan® miRNA Assays were used for qPCR: Cav1/CAV1 (Rn00755834_m1/Hs00971716_m1), Aifm1/AIFM1 (Rn00442540_m1/ Hs00377585_m1), miR-375 (TM_ 000564), miR-200a (TM_000502), miR-130a (TM_00454), miR-152 (TM_000475), miR-132 (TM_000457) and miR-212 (TM_002551) were used for qPCR. Hprt1/HPRT1 (Rn_01527840/4333768F) and Ppia/PPIA (Rn_ 00690933/4333763F) were used as endogenous controls for mRNA expression, while rat U87 (TM_001712) or human RNU44 (TM_001094) and human RNU48 (TM_001006) were used as endogenous control for miRNA expression. The relative quantities were calculated using the $\Delta\Delta C_t$ method. The average C_t values of qPCR assays for each duplicate or triplicate runs are provided in Table S1.

Statistical analysis

Differences between groups were tested using one-way ANOVA followed by Tukey's multiple comparison test as implemented in GraphPad Prism 7. Data are presented as mean ± SEM.

RESULTS & DISCUSSION

To find out the influence of cell confluence on the expression of selected miRNAs and targets, we utilized the rat (INS-1 832/13) and human (EndoC-βH1) insulin-secreting cell lines seeded at different cell densities, followed by gene expression measurements (Fig. 1A). To quantify the cell-to-cell contact, we also seeded EndoC-βH1 cells in parallel, at different densities corresponding to each confluence harvest point. We measured on average 30 μm between cells in 100% confluent plates, while the lowest 20% confluent plates contained cells with an average of 140 μm cell-to-cell distance (Fig. 1B and Fig. S1).

This study mainly addressed the issue whether confluence affects miR-375 expression, as it is one of the most enriched miRNAs in the pancreatic beta cells influencing diverse molecular processes, from insulin secretion to cellular growth and proliferation (*Eliasson, 2017*; *Poy et al., 2004*; *Poy et al., 2009*; *Salunkhe et al., 2015*). The genes *Aifm1* and *Cav1* are among the many genes shown to be directly targeted by miR-375 in mouse beta cells. The negative effect of miR-375 on both the mRNA and protein levels of the two genes has been demonstrated, and in the islets of 375KO mice, increased expression of these targets was also detected at the mRNA level (*Poy et al., 2009*). *Aifm1* and *Cav1* are involved in signaling mechanisms that negatively regulate cellular growth and proliferation, hence *375KO* mice were found to have reduced beta cell mass and defective proliferative capacity in the pancreatic endocrine cells (*Poy et al., 2009*).

In the rat insulin-secreting cell line, INS-1 832/13, we previously showed the reduction of *Aifm1* and *Cav1* mRNA expression upon miR-375 over-expression delineating the conserved targeting in rodents of these genes by miR-375 (*Salunkhe et al., 2015*). In humans, computational predictions show that miR-375 has two non-conserved target sites in the 3'UTR of *AIFM1* mRNA (Target Scan v.7.1 release June 2016) (*Agarwal et al., 2015*), and one target site in the *CAV1* 3'UTR identified by another miRNA target prediction program (RNA22 algorithm implemented at miRWalk 2.0) (*Dweep & Gretz, 2015*).

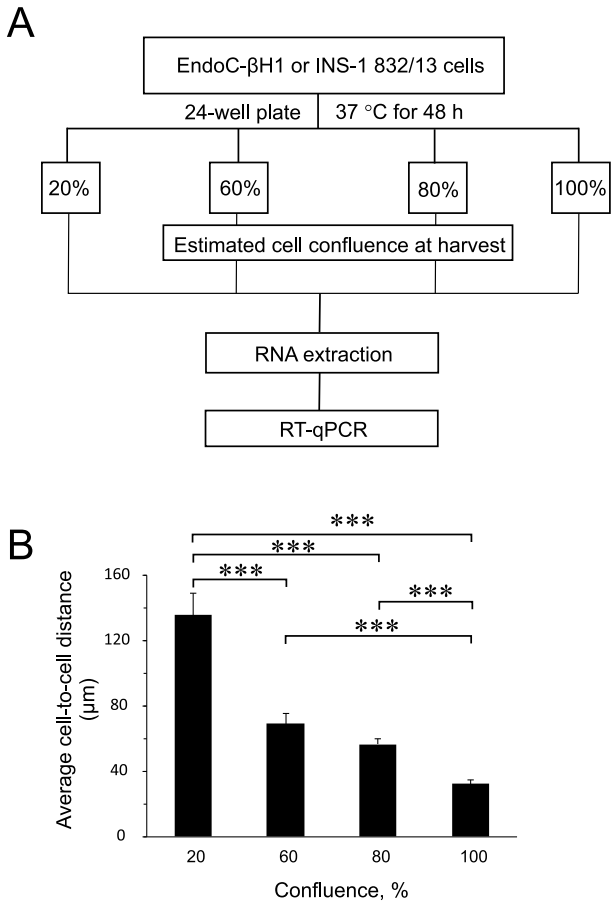

Figure 1 Experimental design and average cell-to-cell distance at harvest. (A) Rat (INS-1832/13) and human (EndoC-βH1) insulin-secreting cell lines were seeded at different cell densities prior to downstream assays as outlined. (B) The average distance between cells at different harvest confluences for EndoC-βH1 cells. Data are presented as mean ± SEM of $N = 40$–90 distance measurements from three independent seedings. (***) $p < 0.001$; one-way ANOVA Tukey's multiple comparison test.

In INS-1 832/13 cells, we did not detect any significantly altered expression of neither miR-375 nor its targets among the different confluences (Figs. 2A–2C). Likewise in the human EndoC-βH1 cells, the expression of miR-375 was similar at all confluences (Fig. 3D). Interestingly, we observed slightly decreased expression of *CAV1* mRNA at 60% and 100% confluence compared to the 20% confluence (Fig. 3F). Although miR-375 is also predicted to target *CAV1* 3'UTR mRNA, miR-375 expression was not elevated at higher confluences, implying that *CAV1* mRNA is potentially regulated by other factors. These results further

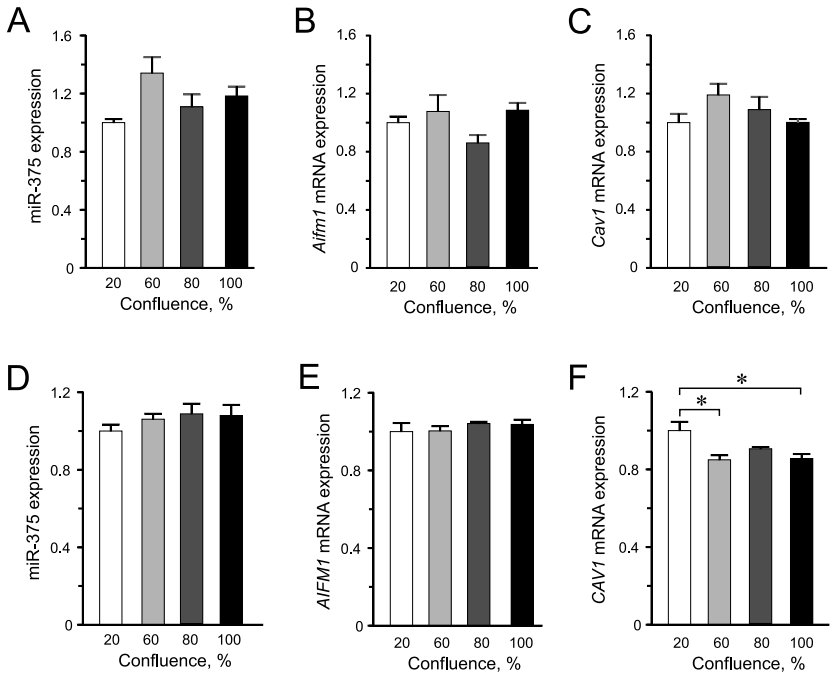

Figure 2 Expression of miR-375 and its targets in INS-1 832/13 cells (A–C) or in EndoC-βH1 cells (D–F). (A) miR-375 expression at different cell confluence of INS-1 832/13 cells. Expression was normalized to rat *U87*. (B and C) *Aifm1* and *Cav1* expression in INS-1 832/13 cells, respectively. Expression was normalized to *Hprt1* and *Ppia*. (D) Expression of miR-375 at different cell density in EndoC-βH1 cells. Expression was normalized to human *RNU44* and *RNU48*. (E and F) *AIFM1* and *CAV1* expression in EndoC-βH1 cells, respectively. Expression were normalized to *HPRT1* and *PPIA*. For all experiments, data are presented as mean of $N = 3$–4 biological replicates, (∗) $p < 0.05$ using one-way ANOVA Tukey's multiple comparison test.

underline the impact of species-specific effects of miRNA-mediated regulation in cellular processes.

Among the other miRNAs included in this study, we observed significantly higher expression levels of miR-132 and miR-212 at higher confluences in INS-1 832/13 cells (Figs. 3A–3B) but only an increasing trend in the human EndoC-βH1 cells (Figs. 3C–3D). For miR-200a, miR-130a and miR-152, the expression levels were found not to be influenced by cellular confluence (Fig. S2). However, although not significant, we observed a trend of increasing miRNA expression from 20% to higher confluences in the EndoC-βH1 cells (Figs. S2D–S2F). Overall, the pattern of increased miRNA expression with increasing confluence in this study, supports previous observation of increased activation of miRNA biogenesis and expression at higher cellular densities (*Hwang, Wentzel & Mendell, 2009*; *Van Rooij, 2011*).

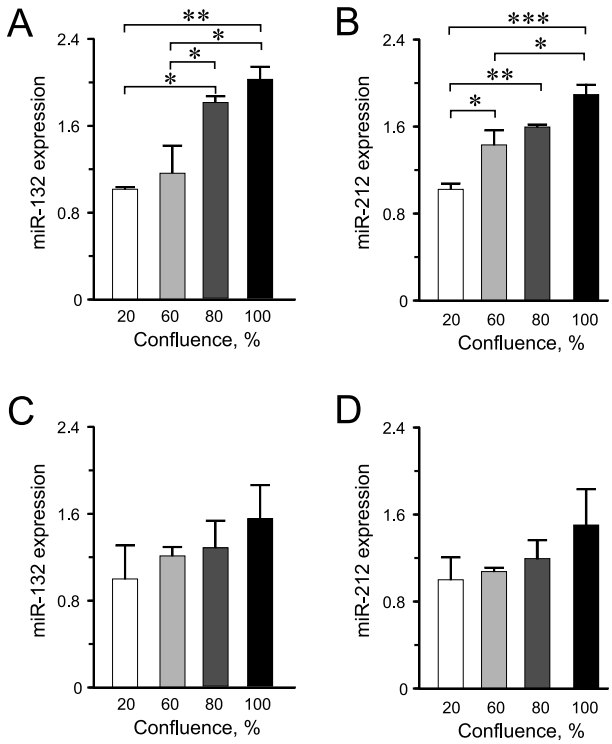

Figure 3 miR-132 and miR-212 expression in INS-1 832/13 cells (A–B) or in EndoC-βH1 cells (C–D) at different confluences. Expression was normalized to rat *U87* or to human *RNU44* and *RNU48*. Data are presented as mean ± SEM of $N = 3$–4 biological replicates. (∗) $p < 0.05$, (∗∗) $p < 0.01$, (∗∗∗) $p < 0.001$; one-way ANOVA Tukey's multiple comparison test.

CONCLUSION

We found virtually no significant differences in the expression levels of miR-375, *CAV1* mRNA and *AIFM1* mRNA at higher confluences, from 60%–100%, either in the rat or human beta cell lines. Moreover, we did not find significant differences in the expression of the other miRNAs tested in either INS-1 832/13 or EndoC-βH1 cells between 80% and 100% confluence. These results are comforting because most functional assays employing pancreatic beta cell lines utilize these confluence levels to attain consistent results. For instance, to ensure optimal insulin secretion in cultured beta cell lines, insulin-secretion assays are commonly performed when the cell culture confluence is at least 90%.

Although we showed that miR-375, which is one of the most enriched beta cell miRNA was not significantly influenced by confluence level in cultured rat and human beta cell lines, we clearly demonstrated that miR-132 and miR-212 are more dependent on cellular densities, as was shown for some miRNAs in other cells types (*Hwang, Wentzel & Mendell,*

2009; *Van Rooij, 2011*). One must therefore be cautious in controlling for cell densities when investigating specific miRNAs in *in vitro* systems.

It has been observed that primary tissues generally exhibit higher global miRNA abundance compared to cell lines in part attributed to tighter, and greater cell-to-cell contact in three-dimensions (*Lu et al., 2005*). Nevertheless, it remains to be seen in the pancreatic endocrine cells how the three-dimensional organization of the cells impacts the global miRNA expression and hence, the regulation of various cellular processes.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jones K. Ofori, Helena A. Malm and Ines G. Mollet conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Lena Eliasson conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Jonathan Lou S. Esguerra conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as a Supplementary File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3503#supplemental-information.

REFERENCES

Agarwal V, Bell GW, Nam JW, Bartel DP. 2015. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4**:e05005 DOI 10.7554/eLife.05005.

Andersson LE, Valtat B, Bagge A, Sharoyko VV, Nicholls DG, Ravassard P, Scharfmann R, Spegel P, Mulder H. 2015. Characterization of stimulus-secretion coupling in the human pancreatic EndoC-betaH1 beta cell line. *PLOS ONE* **10**:e0120879 DOI 10.1371/journal.pone.0120879.

Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. 1992. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* **130**:167–178 DOI 10.1210/endo.130.1.1370150.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**:215–233 DOI 10.1016/j.cell.2009.01.002.

Belgardt BF, Ahmed K, Spranger M, Latreille M, Denzler R, Kondratiuk N, Von Meyenn F, Villena FN, Herrmanns K, Bosco D, Kerr-Conte J, Pattou F, Rulicke T, Stoffel M. 2015. The microRNA-200 family regulates pancreatic beta cell survival in type 2 diabetes. *Nature Medicine* **21**:619–627 DOI 10.1038/nm.3862.

Dweep H, Gretz N. 2015. miRWalk20: a comprehensive atlas of microRNA-target interactions. *Nature Methods* **12**:697 DOI 10.1038/nmeth.3485.

Eliasson L. 2017. The small RNA miR-375—a pancreatic islet abundant miRNA with multiple roles in endocrine beta cell function. *Molecular and Cellular Endocrinology* DOI 10.1016/j.mce.2017.02.043.

Eliasson L, Esguerra JL. 2014. Role of non-coding RNAs in pancreatic beta-cell development and physiology. *Acta Physiologica* **211**:273–284 DOI 10.1111/apha.12285.

Esguerra JL, Bolmeson C, Cilio CM, Eliasson L. 2011. Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLOS ONE* **6**:e18613 DOI 10.1371/journal.pone.0018613.

Esguerra JL, Mollet IG, Salunkhe VA, Wendt A, Eliasson L. 2014. Regulation of Pancreatic Beta Cell Stimulus-Secretion Coupling by microRNAs. *Genes* **5**:1018–1031 DOI 10.3390/genes5041018.

Halban PA, Polonsky KS, Bowden DW, Hawkins MA, Ling C, Mather KJ, Powers AC, Rhodes CJ, Sussel L, Weir GC. 2014. beta-cell failure in type 2 diabetes: postulated mechanisms and prospects for prevention and treatment. *Diabetes Care* **37**:1751–1758 DOI 10.2337/dc14-0396.

Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. 2000. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49**:424–430 DOI 10.2337/diabetes.49.3.424.

Hwang HW, Wentzel EA, Mendell JT. 2009. Cell–cell contact globally activates microRNA biogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106**:7016–7021 DOI 10.1073/pnas.0811523106.

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. 2005. MicroRNA expression profiles classify human cancers. *Nature* **435**:834–838 DOI 10.1038/nature03702.

Malm HA, Mollet IG, Berggreen C, Orho-Melander M, Esguerra JL, Goransson O, Eliasson L. 2016. Transcriptional regulation of the miR-212/miR-132 cluster in insulin-secreting beta-cells by cAMP-regulated transcriptional co-activator 1 and salt-inducible kinases. *Molecular and Cellular Endocrinology* **424**:23–33 DOI 10.1016/j.mce.2016.01.010.

Ofori JK, Salunkhe VA, Bagge A, Vishnu N, Nagao M, Mulder H, Wollheim CB, Eliasson L, Esguerra JL. 2017. Elevated miR-130a/miR130b/miR-152 expression reduces intracellular ATP levels in the pancreatic beta cell. *Scientific Reports* **7**:44986 DOI 10.1038/srep44986.

Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432**:226–230 DOI 10.1038/nature03076.

Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M. 2009. miR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proceedings of the National Academy of Sciences of the United States of America* **106**:5813–5818 DOI 10.1073/pnas.0810550106.

Prasad RB, Groop L. 2015. Genetics of type 2 diabetes-pitfalls and possibilities. *Genes* **6**:87–123 DOI 10.3390/genes6010087.

Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P, Scharfmann R. 2011. A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. *Journal of Clinical Investigation* **121**:3589–3597 DOI 10.1172/JCI58447.

Salunkhe VA, Esguerra JL, Ofori JK, Mollet IG, Braun M, Stoffel M, Wendt A, Eliasson L. 2015. Modulation of microRNA-375 expression alters voltage-gated Na(+) channel properties and exocytosis in insulin-secreting cells. *Acta Physiologica* **213**:882–892 DOI 10.1111/apha.12460.

Van Rooij E. 2011. The art of microRNA research. *Circulation Research* **108**:219–234 DOI 10.1161/CIRCRESAHA.110.227496.

Paper IV

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Non-coding RNAs in beta cell insulin secretion

Photo by Kennet Ruona

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