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Abstract

Beta cell dysfunction is central to the development and progression of type 2 diabetes (T2D). T2D develops when beta cells are not able to compensate for the increasing demand for insulin caused by insulin resistance. Epigenetic modifications play an important role in establishing and maintaining beta cell identity and function in physiological conditions. On the other hand, epigenetic dysregulation can cause a loss of beta cell identity, which is characterized by reduced expression of genes that are important for beta cell function, ectopic expression of genes that are not supposed to be expressed in beta cells and loss of genetic imprinting. Consequently, this may lead to beta cell dysfunction and impaired insulin secretion. Risk factors that can cause epigenetic dysregulation include parental obesity, an adverse intrauterine environment, hyperglycemia, lipotoxicity, aging, physical inactivity and mitochondrial dysfunction. These risk factors can affect the epigenome at different time points throughout the lifetime of an individual and even before an individual is conceived. The plasticity of the epigenome enables it to change in response to environmental factors such as diet and exercise, and also makes the epigenome a good target for epigenetic drugs that may be used to enhance insulin secretion and potentially treat diabetes.

Introduction

Beta cell failure in type 2 diabetes

Type 2 diabetes (T2D) is characterized by hyperglycemia due to insulin resistance in peripheral tissues and impaired insulin secretion from pancreatic beta cells. Loss of beta cell function is central to the development and progression of T2D (Halban, Polonsky et al. 2014). It has been suggested that beta cell dysfunction goes through different stages during the progression to diabetes, and that each stage is characterized by differences in beta cell mass, phenotype and function (Weir and Bonner-Weir 2004). In addition, T2D develops when beta cells fail to compensate for the increasing demand for insulin caused by insulin resistance (Saisho 2015). T2D is a complex disease that results from interactions of genetic, epigenetic and non-genetic factors (Ling and Groop 2009). The aim of this review is to summarize current research covering the role of epigenetic dysregulation of pancreatic islets in impaired insulin secretion and T2D.

Epigenetics, definition and players

An epigenetic trait is a stable and heritable phenotype that results from changes in a chromosome but without changes in the DNA sequence itself. An epigenator is an extracellular environmental stimulus, such as a change in temperature or nutrients that can send a transient signal to the inside of the cell. This signal is received by an intracellular epigenetic initiator, which translates it by initiating an epigenetic event at a specific chromosomal position via the use of DNA binding proteins and non-coding RNAs that may persist even after the epigenator has been removed. The established chromatin state is then maintained not only in the cell exposed to the epigenator, but also in the daughter cells that originate from it. This is achieved via a number of factors known as epigenetic maintainers,

which include: DNA methylation, histone modifications, histone variants and nucleosome positioning and also by the initiator (Berger, Kouzarides et al. 2009).

DNA methylation

DNA methylation is the covalent attachment of a methyl group to the fifth position of the cytosine ring. In mammalian differentiated cells this modification mainly occurs in a CpG dinucleotide (Bird 2011). However, non-CpG methylation has also been reported in embryonic stem cells; although its function is not fully understood, it seems to play a role in pluripotency, and when present in promoters can regulate the expression of some genes (Ramsahoye, Biniszkiewicz et al. 2000, Patil, Ward et al. 2014, Pinney 2014). DNA methylation is not randomly spread throughout the genome. CpG islands are dense regions of CpG dinucleotides that are often associated with transcription start sites. CpG islands are generally not methylated, in contrast to the rest of the genome, which is less dense in CpG sites but is mostly methylated (Bird 2011). Methylation of promoter CpG islands has been generally associated with gene silencing. Transcriptional silencing can be achieved either directly, by inhibiting the binding of transcription factors, or indirectly, through methyl-binding domain proteins that recruit other chromatin-modifying proteins such as HDACs and other transcriptional co-repressors. Although this mechanism for gene silencing was originally used to repress invading viruses, it has evolved and plays an important role in many biological processes, such as cell-type specific gene expression, imprinting, X-chromosome inactivation, cell differentiation and repression of repeat sequences (Deaton and Bird 2011).

Interestingly, DNA methylation is even more abundant in gene bodies and is often positively associated with gene expression (Jones 1999, Maunakea, Nagarajan et al. 2010, Deaton, Webb et al. 2011, Lev Maor, Yearim et al. 2015). Though the mechanisms behind this positive association are not clearly understood, they were initially thought to involve silencing

of transcription from intragenic spurious promoters, which results in more efficient transcriptional elongation of the gene (Jjingo, Conley et al. 2012). DNA methylation is also found in intergenic and non-coding regions and can play a role in gene expression regulation. DNA methylation of enhancers can regulate transcription of distal promoters by interfering with the binding of transcription factors and chromatin modulating proteins, which will then affect possible physical interactions and DNA looping between the enhancer and promoter (Aran, Sabato et al. 2013).

DNMT1 is the DNA methyl transferase responsible for the maintenance of DNA methylation during mitosis. It performs this maintenance by copying the methylation pattern on the maternal DNA strand to the newly synthesized daughter strand. The *de novo* methyl transferases DNMT3a and DNMT3b are involved in the establishment of DNA methylation patterns during development, and also add methyl groups to DNA during postnatal life in response to environmental stimuli. DNA demethylation can be passive or active. Passive DNA demethylation occurs when the methylation pattern is not copied onto the daughter strand during replication, and can be caused by the lack of methyl donors or DNMT1 absence or inactivity. On the other hand, active DNA demethylation occurs when the methyl groups are actively removed by a number of enzymes involved in DNA repair and by ten-eleven translocation methylcytosine dioxygenase (TET) enzymes (Chen and Riggs 2011). TET enzymes can oxidize 5mC to hydroxymethylcytosine (5hmC), formylcytosine (5fC) and carboxylcytosine (5caC), which, in addition to their role in demethylation, seem to play a role in regulating transcription and chromatin (Iurlaro, Ficiz et al. 2013, Kohli and Zhang 2013, Delatte, Deplus et al. 2014, Huang and Rao 2014).

Histone modifications

In eukaryotic cells, DNA is packed with special proteins known as histones to form chromatin. An octamer consisting of four core histones (H3, H4, H2A and H2B) creates the nucleosome that is then wrapped by 147 bp of DNA. The N terminal tails and globular domains of these histones contain many amino acids that can be modified by certain enzymes, resulting in acetylation, methylation, phosphorylation, etc. These modifications are dynamic and can be added or removed by enzymes, some of which are specific to one or more sites, while others are not. For example, acetyl groups are added by histone acetyl transferases (HATs) and can be removed by histone deacetylases (HDACS). Methyl groups can be added onto the lysine of histone tails via lysine methyl transferases such as G9a and SUV39H1, and can be removed by lysine demethylases such as LSD1 and JHDM1a. A comprehensive list of histone-modifying enzymes can be found in Kouzarides (2007). Histone modifications can regulate gene function by disrupting chromatin contacts or affecting the recruitment of non-histone proteins (Kouzarides 2007). Certain modifications have been associated with distinct genomic elements and levels of gene activity. For example, the promoters of actively transcribed genes are often enriched with H3K4me3 and H3K27ac, while repressed or poised promoters are more enriched with H3K27me3. Strong enhancers are enriched with H3K27ac and H3K4me1, while weak enhancers have less of these modifications. H3K9me2/3 is associated with inactive heterochromatin (Siggens and Ekwall 2014). Actively transcribed gene bodies are enriched with H3K36me3 and H3K79me2 (Consortium 2012). Whether histone modifications have a causal role in forming a certain chromatin state or are the consequence of transcription and nucleosome remodeling is debatable, and may vary for different modifications and cellular functions (Henikoff and Shilatifard 2011).

The genome consists of two types of chromatin environments: silent heterochromatin and active euchromatin. Both heterochromatin and euchromatin domains are characterized by a set of distinct modifications and are separated by boundary elements. The boundary elements

recruit specific chromatin-modifying enzymes via boundary binding transcription factors, such as CTCF (Kouzarides 2007). Thus, the distinct combination of DNA and histone modifications defines which parts of the genome are active or inactive in a specific cell type and at a specific time, and influence a number of biological processes including DNA replication, transcription, DNA repair and chromosome segregation (Siggens and Ekwall 2014). DNA methylation can influence histone modifications in a similar way to that in which histone modifications can influence DNA methylation; for example, H3K9 methylation has been shown to promote DNA methylation, and vice versa (Schubeler, Lorincz et al. 2000). Together, they can establish and maintain a specific chromatin state that is induced by an environmental stimulus (Cedar and Bergman 2009).

Proposed molecular mechanisms for how pancreatic epigenome dysfunction can cause impaired insulin secretion:

Loss of beta cell identity and dedifferentiation

Epigenetic modifications play an important role in establishing cell identity during development and maintaining it throughout life (Law and Jacobsen 2010). Loss of cell identity can have detrimental effects on cells (Fig. 1A). Promoters of genes that are selectively and highly expressed in beta cells, such as pancreatic and duodenal homeobox 1 (*PDX1*), glucagon-like peptide 1 receptor (*GLP1R*), paired box 6 (*PAX6*) and HNF1 homeobox A (*HNF1a*) are often characterized by active chromatin marks (such as H3K4me3) around their promoters, lack of repressive marks (such as H3K27me3) and a low degree of DNA methylation (Yang, Dayeh et al. 2011, Yang, Dayeh et al. 2012, Hall, Dayeh et al. 2013, Pullen and Rutter 2013). In addition, *PDX1*, *PAX6*, NK6 homeobox 1 (*NKX6-1*) and, *GLP1R* and other genes that are important for islet development and are involved in diabetes are located in islet-specific open chromatin (Gaulton, Nammo et al. 2010).

Surprisingly, histone modifications associated with active promoters (H3K4me3, H3K79me2) and DNase I hypersensitive sites (DHS) are absent at some highly expressed islet-specific genes encoding *INS*, glucagon (*GCG*), somatostatin (*SST*) and islet amyloid polypeptide (*IAPP*), suggesting that they have a distinct regulatory mechanism (Stitzel, Sethupathy et al. 2010). Indeed, it has been shown that the human insulin gene is part of a large open chromatin domain specific for human islets. Active modifications are distributed over the entire coding region of the insulin gene and surrounding region. It has also been suggested that non-coding RNAs may play a role in maintaining this open chromatin structure (Mutskov and Felsenfeld 2009).

Arx is a transcription factor that is important for alpha cell differentiation and identity. In alpha cells, *Arx* is expressed and a region 2kb upstream from its transcription start site is hypomethylated. In beta cells, *Arx* expression is silenced via DNA methylation of this locus; this recruits MeCP2, which further recruits PRMT6—an enzyme that methylates histone H3R2 and results in the repression of *Arx* (Dhawan, Georgia et al. 2011). However, upon deletion of *Dnmt1* in mice beta cells, *Arx* DNA methylation is reduced and its expression is derepressed, which makes the beta cells convert to glucagon-producing cells and results in a loss of beta cell identity and abnormal glucose homeostasis (Dhawan, Georgia et al. 2011). This study exemplifies the importance of DNA methylation maintenance in preserving cell identity and function during cell division by recruiting enzymes that can modify histone marks and create a repressed chromatin state in the correct cell type. It is possible that defects in the DNA methylation maintenance machinery caused by the absence of *Dnmt1* or its reduced activity, or by the absence of methyl groups in dividing cells, will eventually lead to passive loss of cytosine methylation and the repressed chromatin state at genes that are supposed to be silenced in beta cells. Consequently, this will cause a loss of beta cell identity and impaired insulin secretion. Beta cell dedifferentiation to progenitor-like cells or alpha

cells that do not secrete insulin has also been suggested as a possible mechanism for diabetic beta cell failure (Weir and Bonner-Weir 2004, Talchai, Xuan et al. 2012).

Derepression of disallowed genes in beta cells

Equally important for beta cell function and identity is the selective repression of a number of specific genes that are expressed in almost all tissues except beta cells. Some of these genes are regulators of cell proliferation, oxidative stress, glucose metabolism and glucose-stimulated insulin secretion (Pullen and Rutter 2013). Among these genes are *LDHA*, which encodes lactate dehydrogenase, and *SLC16A1*, which encodes monocarboxylate/lactate transporter. Expression of both of these genes can regulate insulin secretion inappropriately via pyruvate; thus, their repression is important as it ensures that glucose is efficiently oxidized to CO₂ and H₂O (Pullen and Rutter 2013). Interestingly, the expression of a number of these disallowed genes has been shown to be up-regulated in beta cells from T2D and in ZDF rats, an animal model for T2D (Parton, McMillen et al. 2006, Malmgren, Nicholls et al. 2009, Marselli, Thorne et al. 2010).

Recent evidence suggests that epigenetic mechanisms, such as DNA methylation and histone modifications, may be part of the molecular mechanisms causing this selective repression in beta cells. Promoters of a number of beta-cell-specific repressed genes, including *Ldha*, *Slc16a1*, *Mgst1*, *Cxcl12* and *Acot7*, are enriched for H3K27me3 (van Arensbergen, Garcia-Hurtado et al. 2010, Thorrez, Laudadio et al. 2011, Malmgren, Spegel et al. 2013). *Acot7* is involved in acyl-CoA breakdown and stimulus insulin secretion coupling, *Mgst1* is involved in oxidative stress and *Cxcl12* is involved in down-regulation of proliferative genes (Pullen and Rutter 2013). It is possible that epigenetic dysregulation may play a role in derepression of beta-cell-specific silenced genes, and may contribute to impaired insulin secretion (Malmgren, Spegel et al. 2013) (Fig. 1B).

Impaired beta cell function

Epigenetic dysregulation can affect the expression of genes that are important for insulin synthesis, signaling and secretion (Fig. 1C). Insulin gene expression seems to be regulated by DNA methylation, since DNA methylation of the *INS* promoter is lower in beta cells compared to other tissues that do not express insulin (Kuroda, Rauch et al. 2009, Yang, Dayeh et al. 2011). Methylation of the insulin promoter inhibits the binding of the transcription factor ATF2 *in vitro*, while increasing the binding of MeCP2 (Kuroda, Rauch et al. 2009). Pancreatic islets from T2D patients exhibit increased DNA methylation of the insulin promoter in parallel with both decreased insulin expression and glucose-stimulated insulin secretion (Yang, Dayeh et al. 2011).

The transcription factor PDX1 plays a key role in maintaining beta cell identity and regulating glucose-stimulated insulin secretion in the mature pancreas (Gao, McKenna et al. 2014). In human pancreatic islets, *PDX1* is located in an islet-specific open chromatin structure (Gaulton, Nammo et al. 2010). Epigenetic modifications of *PDX1* play a role in its selective expression in islet beta cells and repression in other cell types. However, aberrant epigenetic modifications, such as increased DNA methylation and altered histone modifications (decreased H3 and H4 acetylation, decreased H3K4me3 and increased H3K9me2), can cause reduced *PDX1* expression in human and rodent islets, and may lead to impaired insulin secretion and diabetes (Simmons, Templeton et al. 2001, Pinney and Simmons 2010, Yang, Dayeh et al. 2012). We have shown that in humans, DNA methylation of the *PDX1* distal promoter and enhancer is increased in pancreatic islets from T2D patients, concomitant with reduced *PDX1* and *INS* expression and impaired insulin secretion (Yang, Dayeh et al. 2012). Furthermore, we have shown that the *GLP1R* promoter, which plays a role in insulin secretion and has been shown to be down-regulated in pancreatic islets from human T2D and

hyperglycemic rats (Xu, Kaneto et al. 2007, Taneera, Lang et al. 2012), exhibits increased DNA methylation in T2D islets (Hall, Dayeh et al. 2013).

Genome-wide DNA methylation analysis in pancreatic islets from T2D and non-diabetic donors have identified a number of epigenetically dysregulated genes that are involved in insulin expression, maturation, signaling and secretion (Volkmar, Dedeurwaerder et al. 2012, Dayeh, Volkov et al. 2014). For example, cyclin-dependent kinase inhibitor 1A (*CDKN1A*), phosphodiesterase 7B (*PDE7B*), septin 9 (*SEPT9*) and exocyst complex component 3-like 2 (*EXOC3L2*) were differentially methylated and expressed in human pancreatic islets from T2D patients. Silencing of *EXOC3L2* in clonal rat beta cells reduced exocytosis while over-expression of *CDKN1A*, *PDE7B* and *SEPT9* perturbed insulin and glucagon secretion in beta and alpha cells, respectively (Dayeh, Volkov et al. 2014). Epigenetically dysregulated genes in T2D islets also include *MAPK1* and *SOX6*, which influence insulin expression; *PPP2R4*, which influences insulin's post-translational maturation; *SLC25A5*, which influences insulin secretion; as well as *GRB10* and *HK1*, which are involved in the signaling pathway in beta cells that control insulin secretion (Volkmar, Dedeurwaerder et al. 2012).

Loss of imprinting and allelic imbalance

Genomic imprinting is a biological process that results in mono-allelic expression of a gene depending on its parental origin. Epigenetic mechanisms play an important role in regulating imprinting and help the transcription machinery identify the parental chromosome that should be expressed (Adalsteinsson and Ferguson-Smith 2014). Loss of imprinting causes allelic expression imbalance and has been implicated in a number of human diseases (Kalish, Jiang et al. 2014) (Fig. 1D).

GRB10, which encodes a negative regulator of insulin signaling, is imprinted in a parent of origin fashion in different tissues. Variants in the *GRB10* gene are associated with reduced

glucose-stimulated insulin secretion and increased risk of T2D if inherited paternally. Surprisingly, these variants are associated with reduced fasting glucose when inherited maternally. A recent study showed that *GRB10* is methylated in human pancreatic islets and that its methylation is associated with decreased expression and allelic imbalance. Furthermore, knock down of *GRB10* in human pancreatic islets reduces insulin and glucagon secretion. This suggests that tissue-specific methylation and possibly imprinting of *GRB10* can influence glucose metabolism and contribute to T2D pathogenesis (Prokopenko, Poon et al. 2014).

KCNQ1 encodes a potassium channel subunit and is located in an imprinted region. Intronic variants of *KCNQ1* only influence susceptibility to diabetes when they are inherited maternally (Kong, Steinthorsdottir et al. 2009). A recent study reported temporal changes in imprinting of *KCNQ1* and *KCNQ1OT1*, along with mono-allelic expression in fetal pancreas and bi-allelic expression in human adult pancreatic islets. In addition, methylation levels of fetal regulatory sequences were influenced by the T2D risk variant rs2237895 (Travers, Mackay et al. 2013). *KCNQ1* was also differentially methylated in human islets from donors with T2D (Dayeh, Volkov et al. 2014).

The paternally expressed gene 3 (*PEG3*) encodes a zinc finger protein that may play a role in proliferation and apoptosis and thus affect beta cell mass (Jiang, Yu et al. 2010). Allelic expression imbalance in *PEG3* is associated with its promoters DNA methylation and T2D status; differential methylation could be the cause of allelic imbalance (Fadista, Vikman et al. 2014).

Differential DNA methylation of imprinted loci can also alter expression of genes via microRNAs. A recent study showed that DNA methylation of the imprinted *DLK1-MEG3* locus on human chromosome 14q32 affects the expression of a cluster of miRNAs. The

imprinted *DLK1-MEG3* miRNA cluster is highly and selectively expressed in human beta cells, while down-regulated in islets from subjects with T2D. Furthermore, down-regulation of maternally expressed (*MEG3*) is strongly correlated with hypermethylation of its promoter. Targets of these microRNAs include *IAPP* and *TP53INP1*, which cause increased beta cell apoptosis upon over-expression in human islets and contribute to the pathogenesis of T2D (Kameswaran, Bramswig et al. 2014).

T2D risk factors that can cause epigenetic dysregulation

Periconceptual adverse environment

Parent obesity is a risk factor for childhood obesity and may impact the reprogramming of imprinted genes during gametogenesis (Fig. 2A), which then affects the offspring's future health. Newborns of obese parents have altered DNA methylation patterns at a number of imprinted genes, such as maternally expressed 3 (*MEG3*), mesoderm-specific transcript (*MEST*), *PEG3*, neuronatin and pleiomorphic adenoma gene-like 1 (Soubry, Murphy et al. 2013). Paternal obesity has been associated with insulin like growth factor 2 (*IGF2*) hypomethylation in newborns, while maternal obesity has been associated with increased DNA methylation at *IGF2* and the imprinted maternally expressed transcript (*H19*) loci (Soubry, Schildkraut et al. 2013). In addition, female rat offspring of fathers exposed to a high-fat diet (SF01-025, SF03-020; 40.7 percent, 43 percent energy as fat; Specialty Feeds) have beta cell dysfunction and early onset of impaired insulin secretion and glucose intolerance. In these offspring, the interleukin 13 receptor, alpha 2 (*Il13ra2*) gene, which is involved in lipid metabolism and molecular transport, was found to be hypomethylated in parallel with its increased expression. The hypomethylated CpG site is a putative transcription factor binding site for T-cell factor-1A and NF-X (Ng, Lin et al. 2010).

Suboptimal intrauterine environment

An adverse intrauterine environment has been associated with an increased risk of metabolic diseases in adulthood. Fetal over- and under-nutrition have an impact on neuroendocrine control systems, energy homeostasis and metabolism. When these environmental stimuli occur in a specific window during development they can cause epigenetic modifications in the form of DNA methylation and histone modifications that can persist until later in life, even after the stimulus is removed (El Hajj, Schneider et al. 2014) (Fig. 2B).

It has been shown that an adverse intrauterine environment can affect fetal development by altering the expression of genes important for beta cell development, such as *PDX1*, through epigenetic modifications. Whether these changes persist during adulthood is highly dependent of whether the cells were undergoing differentiation, proliferation or maturation at the time of exposure (Pinney and Simmons 2010). Rats that are exposed to intrauterine growth retardation exhibit a 50 percent reduction in *Pdx1* expression during fetal life and 80 percent reduction in adulthood. Reduced expression of *Pdx1* is associated with a number of epigenetic changes at its proximal promoter, including a decrease in H3 and H4 acetylation and H3K4me3 concomitant with an increase in DNA methylation and H3K9me2. These changes affect the binding of the upstream transcription factor 1 (USF1). Interestingly, these epigenetic changes persist in adulthood and contribute to impaired insulin secretion and diabetes (Park, Stoffers et al. 2008, Pinney and Simmons 2010).

Furthermore, when pregnant agouti mice are fed a diet supplemented with methyl donors, the majority of their offspring are lean, healthy and have a normal lifespan. This maternal diet contributes to increased DNA methylation of the long terminal repeat controlling the expression of the agouti gene, and hence decreased expression of the agouti gene. In contrast, expression of the agouti gene, when this locus shows a low degree of DNA methylation, causes obesity, cancer and T2D in adulthood (Cooney, Dave et al. 2002). Hepatocyte nuclear factor 4, alpha (Hnf4a) is a transcription factor that is required for beta cell differentiation and

glucose homeostasis, and has been implicated in the etiology of T2D. Expression of *Hnf4a* is regulated by two promoters and an enhancer: P2 is expressed in islets during early development and adulthood, while P1 is expressed in the adult liver, kidney and intestine. In human and rat islets, the active P2 promoter is characterized by low levels of DNA methylation and enrichment with H3ac and H3K4me3, while the enhancer is enriched with H3ac and H3K4me1. On the other hand, P2 is enriched with H3K9me2 and lacks H3K4me3. During *Hnf4a* transcription, the P2 promoter interacts with the enhancer and this interaction is mediated via epigenetic modifications. Suboptimal nutrition caused by a low protein diet during early development leads to epigenetic silencing characterized by reduced H3ac and H3K4me1, and an accumulation of H3K9me2 and H3K27me3 at the enhancer region, which interferes with the P2 promoter–enhancer interaction, resulting in reduced *Hnf4a* expression. *Hnf4a* silencing becomes even worse with aging due to changes at the P2 promoter, such as reduced H3ac and H3K4me3 and accumulation of H3K9me2 and H3K27me3 (Sandovici, Smith et al. 2011).

In humans, offspring of women exposed to the Dutch hunger winter during World War II were born with a low birth weight and had impaired glucose tolerance later in life (Ravelli, van Der Meulen et al. 1999). Furthermore, these individuals had less DNA methylation at the imprinted *IGF2* gene compared to their unexposed siblings (Heijmans, Tobi et al. 2008, Tobi, Goeman et al. 2014).

It is thus possible that some of the epigenetic dysregulations found in pancreatic islets from subjects with T2D occur at an earlier stage during periconception and embryonic development and may cause the disease, while some of them may occur during adult life in response to adverse environmental stimuli and may further worsen the disease phenotype or be a consequence of the disease.

Genetic predisposition

Diabetes is a familial disorder and individuals with a family history of T2D have a two- to six-fold increased risk of developing the disease (Papazafropoulou, Sotiropoulos et al. 2009). Approximately 120 genetic variants have been associated with T2D and its related traits (Prasad and Groop 2015). The majority of these variants are non-coding and are more likely to play a role in gene regulation rather than protein coding (McCarthy 2010). Importantly, the majority of T2D variants are associated with beta cell function (Florez 2008, Rosengren, Braun et al. 2012). However, the molecular mechanisms through which they exert their effects on their target genes, and subsequently beta cell function, are still unknown. Furthermore, genetic variation can influence epigenetic variation and the resulting phenotypes (Fig. 2C). Importantly, we have shown that nearly half of the T2D variants are CpG-SNPs that either introduce or remove a CpG site and are associated with differential DNA methylation in the SNP sites in human pancreatic islets (Dayeh, Olsson et al. 2013). Based on the genomic location, CpG-SNPs may affect splicing, transcription elongation and protein binding. Furthermore, we have found that a number of T2D-associated genes are differentially methylated in T2D compared with non-diabetic islets, including transcription factor 7-like 2 (*TCF7L2* and *KCNQ1*) (Dayeh, Volkov et al. 2014). This could potentially be an indirect mechanism of inheriting epigenetic modifications trans-generationally; that is, by inheriting certain variants that influence an individuals' level of DNA methylation, and possibly histone modifications, so that individuals with a certain genotype have already inherited a level of DNA methylation that will influence their chromatin structure and gene function. More recently, by performing a genome-wide methylation quantitative trait locus (mQTL) analysis in human pancreatic islets, we found that DNA methylation of 11,735 CpG sites in 4504 unique genes was regulated by genetic factors located in cis. Among the significantly identified mQTLs were numerous diabetes loci, such as adenylate cyclase 5 (*ADCY5*),

potassium channel, inwardly rectifying subfamily J, member 11 (*KCNJ11*), major histocompatibility complex (*HLA*), *INS*, *PDX1* and *GRB10*. Some of these mQTLs were also associated with altered gene expression and insulin secretion. Furthermore, via the use of a causal interference test we found that methylation may mediate the genetic impact on gene expression and insulin secretion (Olsson, Volkov et al. 2014).

Additionally, it has been shown that many of the non-coding T2D variants are located in putative regulatory elements and that some of them have enhancer activity including fat mass and obesity associated (*FTO*), *KCNQ1*, *TCF7L2* and Wolfram syndrome 1 (*WFS1*), and some show allele-specific differences in activity such as *TCF7L2* and *WFS1* (Stitzel, Sethupathy et al. 2010). In support of the potential enhancer activity of some of the non-coding T2D SNPs, another study found that 18 of these SNPs were enriched for H3K4me1. In addition, some variants were found in H3K27me3-enriched regions and were suggested to regulate glucose homeostasis in other tissues or cause local disruption of the H3K27me3 distribution in islets (Bhandare, Schug et al. 2010). Furthermore, it has been shown that many of the loci associated with an increased risk of T2D and/or fasting glycemia are situated in pancreatic islet enhancers, and that some of them disrupt DNA binding and islet enhancer activity (Pasquali, Gaulton et al. 2014).

The *TCF7L2* intronic SNP rs7903146 has the most significant association with T2D, and carriers of the risk variant have an increased risk of developing T2D and impaired insulin secretion (Lyssenko, Lupi et al. 2007). *TCF7L2* encodes a transcription factor and is considered a master regulator of a number of genes involved in insulin production and processing (Zhou, Park et al. 2014). The T risk allele of *TCF7L2* is located more in open chromatin, in contrast to the C non-risk allele, and has shown greater enhancer activity, suggesting that accessibility of transcription factors to the DNA may be influenced by genetic

variants (Gaulton, Nammo et al. 2010). This coincides with its increased expression in T2D, especially carriers of the T risk allele (Lyssenko, Lupi et al. 2007).

Aging

T2D is an age-related disease. Insulin resistance increases with age and beta cells struggle to secrete adequate amounts of insulin in response to the increasing demands (De Tata 2014). Previously, we have shown that age influences DNA methylation and expression of genes involved in oxidative phosphorylation in skeletal muscle, DNA methylation of the cytochrome c oxidase subunit VIIa polypeptide 1 *COX7A1* and NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6 (*NDUFB6*) promoters were increased in skeletal muscle from elderly compared with young twins, while gene expression and glucose uptake were reduced (Ling, Poulsen et al. 2007, Ronn, Poulsen et al. 2008). With respect to human pancreatic islets, we have shown that age affects DNA methylation of a number of genes that were also epigenetically dysregulated in T2D islets (Fig. 2C). For example, increased age was associated with decreased methylation of *CDKN1A* and increased methylation of *EXOC3L2*. In line with this, *CDKN1A* shows decreased DNA methylation and reduced mRNA expression, while *EXOC3L2* shows increased DNA methylation and decreased mRNA expression in T2D islets (Dayeh, Volkov et al. 2014).

Physical activity

While physical exercise has been shown to alter the expression of genes involved in glucose and lipid metabolism in human skeletal muscle and adipose tissue (Nitert, Dayeh et al. 2012, Ronn and Ling 2013, Ronn, Volkov et al. 2013, Ronn, Volkov et al. 2014), it is difficult to study the direct impact of exercise interventions on human pancreatic islets. We have previously shown that exercise can alter gene expression in adipose tissue and muscle, and this might be mediated through epigenetic modifications (Nitert, Dayeh et al. 2012, Ronn,

Volkov et al. 2013, Ronn, Volkov et al. 2014) (Fig. 2C). Another study showed that DNA methylation of the peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1A*) increased and gene expression decreased in skeletal muscle in response to bed rest (Alibegovic, Sonne et al. 2010). In line with this, pancreatic islets from subjects with T2D exhibit increased DNA methylation and decreased gene expression of *PPARGC1A* compared with non-diabetic donors (Ling, Del Guerra et al. 2008). Additionally, silencing of *PPARGC1A* in pancreatic islets resulted in decreased insulin secretion (Ling, Del Guerra et al. 2008). It is possible that physical inactivity plays a role in the epigenetic dysregulation that is found in human pancreatic islets from T2D subjects.

Gender

Metabolic differences between females and males have been identified, with females being more insulin-sensitive and secreting more insulin compared to males (Clausen, Borch-Johnsen et al. 1996, Magkos, Wang et al. 2010, Kautzky-Willer, Brazzale et al. 2012). Recently, we have shown that DNA methylation contributes to sex-specific metabolic differences between males and females (Fig. 2C). For example, a number of autosomal and X-chromosome genes involved in insulin secretion are differentially methylated and expressed in human pancreatic islets from females compared with males, including *apelin* (*APLN*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), high-mobility group AT-hook 1 (*HMGAI*), B-cell CLL/lymphoma 11A (*BCL11A*), activating transcription factor 4 (*ATF4*) and angiotensin I converting enzyme 2 (*ACE2*). Both *APLN* and NFkB activating protein (*NKAP*) showed increased DNA methylation and decreased expression in female islets concordant with increased glucose-stimulated insulin secretion (Hall, Volkov et al. 2014). *APLN* encodes a peptide hormone that has been shown to decrease glucose-stimulated insulin secretion (Ringstrom, Nitert et al. 2010). *NKAP* plays a role in regulating the transcription of inflammatory cytokines via activating the transcription factor NFkB, and has also been shown to decrease glucose-

stimulated insulin secretion and to escape X-chromosome inactivation in some cells (Carrel and Willard 2005, Mahdi, Hanzelmann et al. 2012). Silencing of both *APLN* and *NKAP* in clonal rat beta cells resulted in an increase in glucose-stimulated insulin secretion (Hall, Volkov et al. 2014).

Hyperglycemia

It has been shown that transient exposure of aortic endothelial cells to hyperglycemia induces persistent epigenetic changes and altered gene expression of inflammatory genes that remain during normoglycemia (El-Osta, Brasacchio et al. 2008). This so-called hyperglycemic memory is maintained by epigenetic marks including increased H3K4me1 and decreased H3K9me2/3, together with increased recruitment of the lysine-specific histone demethylase LSD1 (Brasacchio, Okabe et al. 2009). Furthermore, hyperglycemia has been shown to increase the recruitment of HATs, and thereby histone acetylation at the promoters of inflammatory genes, which results in chromatin remodeling events that affect transcription (Miao, Gonzalo et al. 2004).

Treating clonal rat beta cells with high glucose for 72 hours increased *Ins* and *Pdx1* promoter DNA methylation and reduced their expression. In addition, DNA methylation of *INS* and *PDX1* in human pancreatic islets correlated positively with HbA1c, which is a long-term measure of circulating glucose levels (Yang, Dayeh et al. 2011, Yang, Dayeh et al. 2012). Furthermore, we found that HbA1c is associated with DNA methylation of genes that are epigenetically dysregulated in islets from donors with T2D (Dayeh, Volkov et al. 2014). Another study cultured clonal rat beta cells for 14 days in normal and high glucose conditions and reported increased DNA methylation of the *Ins* promoter, along with decreased mRNA expression. In addition, the study found an increase in DNA methyl transferase activity, *Dnmt1* expression, and decreased TET activity (Ishikawa, Tsunekawa et al. 2015). It is

therefore possible that hyperglycemia induces epigenetic changes in human pancreatic islets and some of these changes may affect insulin secretion.

Lipotoxicity

T2D patients and obese individuals often have elevated levels of circulating free fatty acids, which have detrimental effects on islet function and insulin secretion (Boden and Shulman 2002, Kahn, Hull et al. 2006). We recently showed that treating human islets with palmitate for 48 hours altered DNA methylation and mRNA expression of 280 genes concomitant with impaired insulin secretion. These genes included some T2D-susceptible genes, such as *TCF7L2* and GLIS family zinc finger 3 (*GLIS3*), as well as genes involved in insulin and *PPAR* signaling and the one carbon pool folate pathway (Hall, Volkov et al. 2014). Altered expression of genes in the carbon pool folate pathway may affect the amount of methyl donors such as S-adenosyl methionine (SAM) in islets exposed to palmitate, and thereby contribute to differential DNA methylation (Locasale 2013). In addition, exposing clonal rat beta cells to palmitate for 48 hours caused coordinated changes in histone modifications, the activity of histone-modifying enzymes, mRNA levels and metabolites, as well as decreased glucose-stimulated insulin secretion (Malmgren, Spegel et al. 2013). Here, the activity of HAT increased while H3K27 methyltransferase activity decreased, and HDAC activity did not change. A number of genes involved in cholesterol synthesis were differentially expressed in parallel with differential enrichment of histone modifications. For example, insulin-induced gene 1 (*Insig1*), lanosterol synthase (*Lss*), peroxisomal D3,D2-enoyl-CoA isomerase (*Peci*), isopentenyl-diphosphate delta isomerase 1 (*Idi1*) and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*Hmgcs1*) exhibited increased mRNA expression in parallel with significantly increased enrichment of promoter H3K9ac and gene body H3K79me2, while the calcium-sensing receptor (*Casr*) exhibited decreased expression in parallel with increased enrichment of H3K27me3, as well as decreased enrichment of H3K9ac and H3K4me3. Furthermore,

significant fold enrichment of H3K9ac, H3K79me2 and H3K4me3 was associated with increased transcriptional activity, while significant fold enrichment of H3K27me3 was associated with transcriptional repression (Malmgren, Spegel et al. 2013). It is likely that these epigenetic changes mediate lipotoxic effects on beta cells in subjects with T2D, and contribute to impaired insulin secretion and loss of glucose responsiveness.

Mitochondrial dysfunction

High-energy intermediates, such as ATP and acetyl-CoA, are required for modification of the epigenome. For example, ATP is required for histone tail phosphorylation, acetyl-CoA for histone acetylation, and SAM for DNA and protein methylation. ATP is generated by glycolysis and oxidative phosphorylation (Wallace 2010). Acetyl-CoA is generated primarily in the mitochondria, but can also be synthesized in the nucleus (Wallace 2010, Sutendra, Kinnaird et al. 2014). In addition, ATP is important for folate metabolism and synthesis of the methyl donor SAM (Wallace 2010). Indeed, it has been shown that altered mitochondrial content results in significant changes in DNA methylation of a number of nuclear genes and may contribute to tumorigenesis (Smiraglia, Kulawiec et al. 2008), suggesting that the mitochondrial function may modulate DNA methylation and histone modifications of the nuclear genome.

Mitochondrial function in pancreatic islets is important for ATP production and insulin secretion (Mulder and Ling 2009, Koeck, Olsson et al. 2011). The down-regulation of genes involved in oxidative phosphorylation has been reported in islets from donors with T2D, and may contribute to decreased ATP production and impaired glucose-stimulated insulin secretion (Olsson, Yang et al. 2011). Additionally, the promoter of the transcriptional coactivator PGC-1 α , which is a master regulator of mitochondrial genes, is hypermethylated in pancreatic islets from patients with T2D concomitant with its reduced mRNA expression

and reduced insulin secretion (Ling, Del Guerra et al. 2008). This is an example of how altered DNA methylation of a nuclear gene may affect mitochondrial function and insulin secretion.

Treatments to improve beta cell function and mass via epigenetic mechanisms

Some epigenetic modifications have been reversed by T2D drugs *in vitro*. Incretin hormones, such as the glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) have been shown to increase global acetylation of H3K9 and H3K18 and phosphorylation of histone H3 serine 10 in INS1 cells. This change in histone modifications has been shown to be associated with increased binding of a number of transcription factors, including phosphorylated cAMP-response element-binding protein (phospho-CREB) and cAMP-responsive CREB coactivator 2 (Kim, Nian et al. 2009).

Furthermore, it has been shown that treating newborn rats that had previously been exposed to intrauterine growth retardation with the GLP-1 analogue Exendin-4 prevented them from developing T2D, potentially by means of epigenetic mechanisms. Indeed, treatment with Exendin-4 increased HAT activity by recruiting transcription factors USF1 and PCAF to the *Pdx1* proximal promoter, which prevented the binding of DNMT1 and hence caused reduced DNA methylation of the *Pdx1* promoter. Altogether, these epigenetic changes restored chromatin structure and *Pdx1* expression (Pinney, Jaeckle Santos et al. 2011).

HDAC inhibitors are promising treatments for T2D and have been shown to promote beta cell development, proliferation, differentiation and function (Christensen, Dahllof et al. 2011) (Fig. 3). Recently, the histone deacetylase inhibitor Trichostatin A was shown to enhance insulin secretion from beta cells and GLP-1 secretion from intestinal endocrine cells (Tiernan, Champion et al. 2015). However, many of the currently available HDAC inhibitors are non-specific and can cause cytotoxic side-effects. Because both histone and non-histone proteins

can be acetylated, using non-specific HDAC inhibitors that target protein acetylation can affect a number of vital regulatory processes including gene expression, mRNA stability, protein interactions, protein stability and enzymatic activity. Thus, attempts are being undertaken to design isoform-selective HDAC inhibitors that may have fewer adverse effects (Micelli and Rastelli 2015).

Epigenetic editing is the use of selective molecules that can target epigenetic enzymes to specific DNA sequences and modulate the expression of specific genes (de Groote, Verschure et al. 2012). Interesting targets for epigenetic editing may include the inhibition of beta cell disallowed genes such as LDHA, which may improve glucose-stimulated insulin secretion and the up-regulation of repressed proliferative genes which may improve beta cell mass (Pullen and Rutter 2013).

Alternative sources of beta cells

Conversion of adult pancreatic alpha cells to beta cells has been reported in mice after extreme beta-cell loss (Thorel, Nepote et al. 2010). Treating human islets with the histone methyl transferase inhibitor ADOX causes partial alpha to beta cell conversion characterized by a decrease in H3K27me3 levels at *ARX*, *MAFA* and *PDX1* in parallel with co-localization of glucagon and insulin in the cells (Bramswig, Everett et al. 2013). It has also been shown that ectopic expression of the beta cell regulator Pax4 in alpha cells can promote their differentiation into insulin producing beta cells (Collombat and Mansouri 2009). Therefore, it is possible that epigenetic manipulation of alpha cells can be used to reprogram them to beta cells that can be used in replacement therapy (Fig. 3).

Biomarkers and prediction

The field of DNA methylation biomarkers is still in its infancy. Obtaining pancreatic islets non-invasively is not possible, hence the need for surrogate tissues/markers. Because DNA methylation is established during early development and maintained through cell division, it is thought that individual and disease-related methylation profiles will probably manifest in most tissues, including accessible tissues such as blood (Dolinoy, Das et al. 2007, Toperoff, Aran et al. 2012). Epigenetic changes in T2D primary tissues, such as pancreatic islets, may be reflected in blood and can potentially be used to monitor beta cell function or death (Fig. 3); however, studies in this area are still lacking. Nevertheless, a recent genome-wide survey found that a CpG site in the *FTO* gene was hypomethylated in blood of T2D cases compared to controls, and could predict T2D. Furthermore, this CpG site was shown to be hypomethylated in blood from young individuals who developed T2D later on in their lives, in comparison to those who remained healthy (Toperoff, Aran et al. 2012). The lack of methylation at the *INS* gene, which is specifically expressed in pancreatic beta cells but not in other tissues, has been used recently to develop an assay that can monitor beta cell death in type 1 diabetes at an early stage by detecting beta-cell-derived un-methylated DNA in blood (Husseiny, Kaye et al. 2014). Although it is debatable whether there is a loss of beta cell mass or functional mass in T2D, using such assays could help in the early detection of this loss.

Conclusion

Impaired insulin secretion observed in T2D may be partly caused by epigenetic dysregulation of pancreatic islets. This epigenetic dysregulation may be caused by a number of risk factors that can affect individuals at a very early point in their lives, as well as throughout their lifetimes. In this review, we have mentioned some of the proposed molecular mechanisms for how epigenetic dysregulation may contribute to impaired beta cell function and impaired

insulin secretion, and also outlined some of the proposed strategies for modifying the pancreatic islet epigenome to enhance insulin secretion and potentially treat T2D.

Draft

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Figure Legends

Fig. 1. Proposed molecular mechanisms for how pancreatic epigenetic dysfunctions may cause impaired insulin secretion. (A) Loss of beta cell identity, (B) Derepression of beta cell disallowed genes, (C) Impaired beta cell function, (D) Loss of genetic imprinting (black circles represent methylated CpG sites and white circles unmethylated CpG sites).

Fig. 2. Time points when T2D risk factors can cause epigenetic dysregulation. (A) Periconception, (B) Intrauterine environment, (C) Throughout life.

Fig. 3. Using epigenetics to enhance insulin secretion and predict T2D. This figure presents some of the potential translational implications of epigenetics in treating and preventing T2D such as: 1. Developing drugs that can epigenetically modify the expression of genes important for insulin secretion and thus enhance insulin secretion 2. Generating beta cells that can be used in replacement therapy by inducing epigenetic changes in progenitor cells that causes their differentiation into beta-like cells 3. Identifying DNA methylation biomarkers in peripheral tissues that can be used to predict future T2D or reflect epigenetic dysregulation in pancreatic islets.

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