

# Histone Deacetylase (HDAC) Inhibition as a Novel Treatment for Diabetes Mellitus

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Both common forms of diabetes have an inflammatory pathogenesis in which immune and metabolic factors converge on interleukin-1 $\beta$  as a key mediator of insulin resistance and  $\beta$ -cell failure. In addition to improving insulin resistance and preventing  $\beta$ -cell inflammatory damage, there is evidence of genetic association between diabetes and histone deacetylases (HDACs); and HDAC inhibitors (HDACi) promote  $\beta$ -cell development, proliferation, differentiation and function and positively affect late diabetic microvascular complications. Here we review this evidence and propose that there is a strong rationale for preclinical studies and clinical trials with the aim of testing the utility of HDACi as a novel therapy for diabetes.

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## INTRODUCTION

Diabetes mellitus designates a group of chronic diseases where absolute or relative lack of insulin leads to aberrancies in substrate metabolism, causing acute and long-term complications. Although the clinical and diagnostic hallmark is elevated blood glucose, the metabolic disturbances in diabetes mellitus are not limited to glucose but encompass most processes of the intermediary metabolism of nutrients, including proteins and lipids. Insulin resistance is a component of most types of diabetes mellitus, but with the exception of rare mutations in the insulin signaling cascade, the disease does not occur if insulin release from the pancreatic  $\beta$  cells in the islets of Langerhans (hereafter islets)

is intact. Preserving a functional  $\beta$ -cell mass is therefore the primary target of novel treatments aimed at curing and preventing diabetes mellitus.

Type 1 diabetes mellitus (T1D) and Type 2 diabetes mellitus (T2D) constitute the two main forms of diabetes. It has been estimated that 250 million individuals are currently afflicted by diabetes worldwide and the prevalence is doubling every 10 years. Whereas T1D is associated with absolute insulin deficiency as a consequence of selective destruction of  $\beta$  cells, T2D is associated with a relative lack of insulin most commonly due to failure of the  $\beta$  cells to compensate for insulin resistance caused by obesity. Of note, T1D and T2D are genetically distinct diseases

(1). Thus, T1D is believed to be an autoimmune disease where variations in mainly immune-regulatory genes predispose individuals to immune-mediated destruction of the  $\beta$  cells by a T-cell-driven chronic inflammatory process in the islets (insulinitis) (2,3). In contrast, genome-wide association scans (GWAS) have suggested T2D to be predominately a disease of the  $\beta$  cell, where variations in genes affecting  $\beta$ -cell function and/or mass impair  $\beta$ -cell compensation to increased insulin demands (4). In both diseases, there are strong gene-environment interactions that trigger the pathogenetic process (in T1D, hitherto unidentified viral and nutritional etiologies, and in T2D, mainly lifestyle-related factors).

With respect to pathogenesis, the strict dichotomy between T1D and T2D is most likely an oversimplification. There is increasing recognition that T1D and T2D may represent extremes of a continuous spectrum with a dominating  $\beta$ -cell defect at one end and dominating insulin resistance at the other (5). However, when disregarding diabetes caused by rare mutations in insulin signaling, insulin resistance is neither necessary nor suffi-

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cient to cause diabetes, whereas  $\beta$ -cell dysfunction is both a necessary and sufficient cause. This notion is supported by studies demonstrating progressive reduction in  $\beta$ -cell function and mass in T2D (6–8). Autoimmune islet inflammation and  $\beta$ -cell destruction are long-recognized causes of T1D, although it is debated if the molecular effector mechanisms involve predominantly classical cytotoxic T-cell-mediated or predominantly inflammatory cytokine-mediated  $\beta$ -cell killing or both. Several mechanisms leading to  $\beta$ -cell destruction in T2D have been proposed: glucolipotoxicity, membrane disruption caused by islet amyloid polypeptide deposition and, more recently, inflammation in the islets (9). Recently, a unifying hypothesis was proposed by the observation that all these stimuli lead to the induction of inflammatory mediators in the pancreatic islets (10,11) that cause  $\beta$ -cell destruction by activating pathways in  $\beta$  cells similar to those in T1D.

Thus, despite their different genetic background, the immune and metabolic pathogenesis of T1D and T2D, respectively, seem to converge on common extracellular inflammatory stressors in the islets and intracellular signaling induced by these stressors (reviewed in [12]). Within the last years, several publications reported that the inflammatory cytokine interleukin (IL)-1 $\beta$  can act as a common extracellular inflammatory stressor. In the mid-1980s, IL-1 $\beta$  secreted from activated mononuclear cells was found to be selectively toxic to  $\beta$  cells and was found to both inhibit  $\beta$ -cell function and induce  $\beta$ -cell death after prolonged exposure (reviewed in [13]). IL-1 $\beta$  has since received much attention as an important mediator of the immune-induced  $\beta$ -cell destruction underlying T1D. Moreover, a number of observations in the last decade argue strongly for an important role of IL-1 $\beta$  in the pathogenesis of T2D as well. Thus, mice deficient in caspase-1 and thereby unable to process pro-IL-1 $\beta$  to mature biologically active IL-1 $\beta$  are more insulin sensitive than wild-type animals (14); IL-1 $\beta$  is secreted by  $\beta$  cells exposed to

high glucose concentrations (15) and the adipocytokine leptin (16) and by macrophages exposed to free fatty acids and islet amyloid polypeptide (10,11); the naturally occurring IL-1 inhibitor, IL-1 receptor antagonist (IL-1Ra), protects against high glucose-induced human  $\beta$ -cell toxicity (glucotoxicity) *in vitro* (15) and diabetes as well as  $\beta$ -cell dysfunction induced by a high-fat diet (lipotoxicity) in an animal model (17); elevated IL-1 $\beta$  levels contribute to the risk of developing T2D (18); and IL-1Ra treatment improves  $\beta$ -cell function in patients with T2D for up to 39 weeks after 13 weeks of treatment (19,20).

In summary, antiinflammatory treatment, and in particular inhibition of IL-1 $\beta$ -induced toxicity, has therapeutic potential in the treatment of both T1D and T2D. However, antiinflammatory biologics are costly and require parenteral administration either via the subcutaneous or intravenous route. There is thus an unmet need to develop safe, inexpensive and patient-convenient (oral) antiinflammatory drugs that mimic the beneficial effects of IL-1 blockade.

#### ANTIINFLAMMATORY PROPERTIES OF HISTONE DEACETYLASE INHIBITORS

As outlined in the current issue of *Molecular Medicine*, histone deacetylase inhibitors (HDACi) show promising antiinflammatory properties, as demonstrated in an increasing number of animal and cellular models of inflammatory diseases (21). As indicated by their name, the molecular function of histone deacetylases (HDACs) was thought to be restricted to histone deacetylation, but recent advances in phylogenetic analysis suggested that HDACs regulate the activity of a wide range of nonhistone proteins (22). This was substantiated in a recent study (23) by the finding of 3,600 acetylation sites (of which only 61 were on histones) on 1,750 proteins including exclusively cytoplasmic proteins. Thus, the impact of acetylation in terms of posttranslational regulation is comparable to that of phosphorylation. A growing number of HDACi are being developed for the treat-

ment of an expanding range of diseases. While transcriptional control over oncogene networks in cancer was the original target of HDAC inhibition, neurodegenerative and other inflammatory diseases are now increasingly being evaluated as novel indications, as illustrated by the reviews in this issue of *Molecular Medicine*.

Acetylation is now recognized to regulate the master transcription factor in the inflammation nuclear factor (NF)- $\kappa$ B (reviewed in [24]). Because the activation of NF $\kappa$ B is a critical event in IL-1 $\beta$ -induced  $\beta$ -cell death (25,26), these findings led to the investigation and demonstration of the protective effects of HDAC inhibition in  $\beta$  cells exposed to toxicity-mediating cytokines (27).

In this article, we review the potential of inhibiting the classical HDACs as a novel treatment for diabetes. This review includes a short overview of genetic associations between HDACs and the etiology of diabetes followed by a discussion of the potential for HDACi as an oral therapy with respect to modulation of the immune system, insulin resistance,  $\beta$ -cell development, differentiation and function, and pathogenetic events relevant for  $\beta$ -cell failure and destruction and islet graft rejection. Of note, HDACi also hold promise with respect to treatment of late diabetic complications such as diabetic nephropathy (28,29) and retinal ischemia playing a central role in diabetic retinopathy (30). HDACi and late diabetic complications will not be discussed further here, and readers are referred to the aforementioned references.

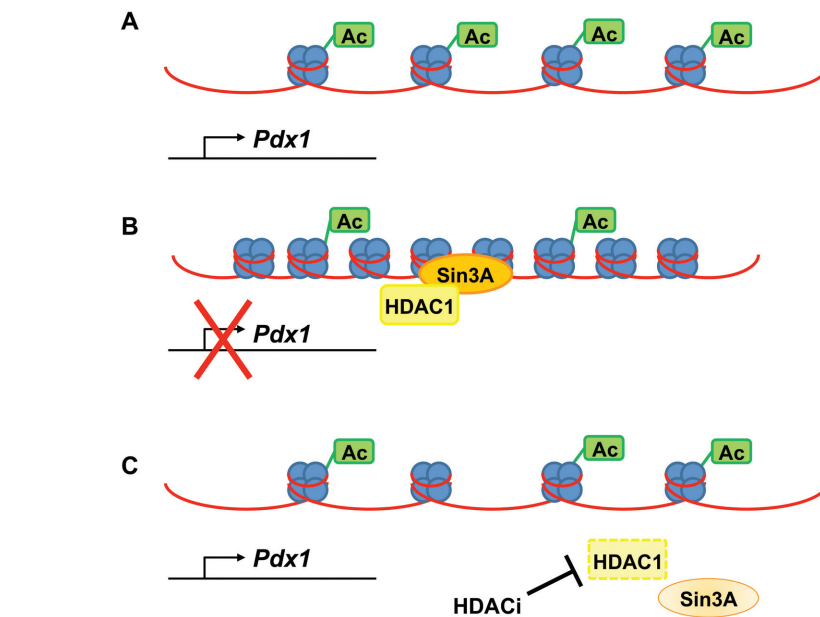
#### HDACs IN THE ETIOLOGY OF DIABETES

As mentioned above, the etiology of diabetes is complex and multifactorial with contributions from many genes and unknown environmental factors. Although GWAS point to T1D and T2D as being genetically distinct (31,32), at least two GWAS studies have found significant linkage between the chromosomal region 6q21, where *HDAC2* is located, and both T1D and T2D (33,34), indicating that *HDAC2* could play a role in both diseases.

Although T1D and T2D are clearly polygenetic disorders, the concordance rate in twin studies is far from 100% (35,36), indicating a significant etiologic contribution from environmental and/or epigenetic factors. Fetal exposure to intrauterine growth retardation (IUGR) contributes to the development of T2D, as reviewed by Pinney and Simmons (37). An adverse fetal milieu affects  $\beta$ -cell development by modifying key regulatory genes such as *pancreatic and duodenal homeobox factor 1 (Pdx1)* (38) as well as muscular glucose transport through *glucose transporter 4 (Glut4)* (39). Interestingly, the reduced expression of *Pdx1* after IUGR is mediated by loss of histone acetylation through the recruitment of HDAC1 in complex with the corepressor Sin3A to the proximal promoter of *Pdx1* (Figure 1). Thereby, a self-propagating epigenetic cycle is induced in which the HDAC1/Sin3 complex recruits a histone demethylase leading to loss of histone 3 lysine 4 trimethylation (H3K4me3), further repressing *Pdx1* transcription. This effect was reversed by HDAC inhibition in the neonatal animal but not in the adult animal, where H3K9 dimethylation and extensive DNA methylation locked the *Pdx1* promoter in its transcriptionally inactive state (38).

Prenatal nutritional restriction leading to IUGR also leads to HDAC1- and HDAC4-mediated loss of histone acetylation of the *Glut4* promoter in adult muscle tissue, thereby inhibiting *Glut4* transcription (39). The effective metabolic repression of this important regulator of peripheral glucose uptake and insulin resistance may contribute importantly to the T2D phenotype. Of note, chromatin remodeling may already be induced by current T2D treatments, since incretin hormones such as glucagon-like peptide 1 and glucose-dependent insulinotropic peptide 1 increase *in vitro* global acetylation of histone H3, leading to increased association with transcription factors (40).

Histone acetylation and HDACs are not only relevant to T1D and T2D but



**Figure 1.** Model of changes in acetylation levels in pancreatic  $\beta$  cells of IUGR. (A) Under normal conditions, the *Pdx1* proximal promoter is associated with acetylated nucleosomes (Ac, green squares). (B) During IUGR, a complex containing HDAC1 and Sin3A induces a progressive loss of histone acetylation in islets from fetal and 2-week-old mice. This then leads to repression of *Pdx1* expression. (C) This effect is reversed by HDAC inhibition. Simplified from Park *et al.* (38).

also to the more infrequent forms of monogenic autosomal diabetes termed “maturity-onset diabetes of the young” (MODY). MODY comprises at least seven distinct subtypes on the basis of the mutated genes in question (31). With the exception of glucokinase and insulin, these genes all encode transcription factors—namely hepatocyte nuclear factor (HNF)-1 $\alpha$ , -1 $\beta$  and -4 $\alpha$ , involved in insulin transcription and hepatic gluconeogenesis, and pancreatic and duodenal homeobox 1 (PDX1) and neurogenic differentiation 1 (NeuroD1), involved in pancreatic development and insulin production. These transcription factors all associate with histone acetyltransferases (HATs) and HDACs, suggesting an important role of histone acetylation in their normal function. Underlining this, some of the MODY mutations directly affect the ability of the transcription factors to interact with HAT/HDACs (41).

In summary, these findings all point to inappropriate chromatin remodeling and

histone acetylation as an important pathogenetic factor in diabetes.

### INNATE AND ADAPTIVE IMMUNE SYSTEMS AND HDACi IN DIABETES

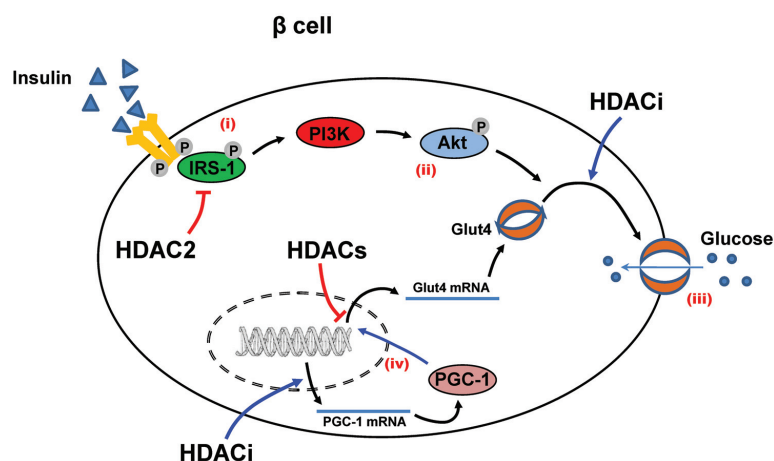
As reviewed in other sections of this issue of *Molecular Medicine*, HDAC inhibition modifies innate and adaptive immune responses (42–45). The specific impact of HDACi on the immune system in relation to T1D and T2D is under-investigated. However, histone H3 is hyperacetylated in the promoters of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and the inflammatory-associated enzyme cyclooxygenase (COX)-2 in monocytes isolated from patients with T1D or T2D (46), suggesting a potential importance of the activity of HATs and HDACs in the expression of proinflammatory genes in monocytes from patients suffering from diabetes (46). *In vitro*, increased histone acetylation is induced by high glucose concentrations and the HDAC inhibitor trichostatin A (TSA) in monocytes from diabetics (46), and the production of the inflammatory

cytokines IL-1 $\beta$  and TNF $\alpha$  was induced by high glucose concentrations through activation of NF $\kappa$ B (47), suggesting that hyperacetylation is a consequence of hyperglycemia or other metabolic aberrancies of diabetes rather than a cause of diabetes. Further, NF $\kappa$ B activity was enhanced by HAT overexpression and TSA and accordingly reversed by overexpression of HDAC1, -2, -3, -4, -5, and -6 (46). Taken together, these data suggest that HDACi treatment of patients suffering from diabetes could have an undesirable effect on cytokine production by monocytes. However, since effects of HDACi are highly concentration dependent, this potential adverse effect may not be seen if lower HDACi concentrations are used, since lower concentrations are generally associated with antiinflammatory responses. In the above-mentioned study by Miao *et al.* (46), TSA was used in a concentration of 300 nmol/L and was found to increase expression of TNF $\alpha$  and COX-2. Similar results were reported from another study using 500 nmol/L TSA (48). Lower concentrations of TSA (1–10 nmol/L) were not reported to have this effect while still causing histone hyperacetylation (48). In contrast to the effects of TSA, the HDAC inhibitor ITF2357 was shown to reduce the inflammatory response of peripheral blood mononuclear cells (PBMCs) by lowering the release of TNF $\alpha$ , secretion of IL-1 $\beta$  and synthesis of interferon  $\gamma$  (IFN $\gamma$ ) (49).

In summary, the effects of HDAC inhibition on the immune system specifically with respect to diabetes are not clarified, and further studies are needed to unravel the dose-response relationships for different HDACi on cytokine production from monocytes. Studies from other inflammatory diseases have to our knowledge not reported monocyte activation as an adverse effect, lending optimism to future safe utility of HDACi in treating diabetes.

### INSULIN RESISTANCE AND HDAC INHIBITION

Insulin action is critical for cellular glucose uptake in most cells. As simplified in



**Figure 2.** Effects of HDACs and HDACi on insulin signaling. For details, see the text.

Figure 2, insulin signals via binding to the insulin receptor leading to receptor autophosphorylation and phosphorylation of IRS family (see *i*, Figure 2). Upon phosphorylation, IRSs bind (and activate) phosphatidylinositol 3-kinase (PI3K), which in turn leads to phosphorylation of the protein kinase Akt (see *ii*, Figure 2). Among other effects, Akt induces translocation of the glucose transporter (GLUT4) from intracellular vesicles to the plasma membrane, mediating glucose uptake (see *iii*, Figure 2) (reviewed in [50]). Obstruction of insulin signaling leading to insulin resistance may occur at several levels in this pathway. As mentioned above, insulin resistance is a feature of both T1D and T2D—in the former case suspected to be secondary to deficient insulin secretion in lean and underweight subjects (51), but also increasingly associated with overweight of T1D subjects. In addition to obesity, aging and genetic predisposition are proposed to enhance risk of developing insulin resistance (52,53).

HDACs have been suggested to play a regulatory role in physiological insulin signaling (see Figure 2). Thus, HDACi increase GLUT4 translocation and augment basal and insulin-induced glucose uptake in skeletal muscle (54). IRS-1 binds to HDAC2 in liver cells from the *ob/ob* mouse, a model of insulin resistance (55). This result was associated with de-

creased acetylation of IRS-1 and reduced insulin receptor-mediated tyrosine phosphorylation of IRS-1. Accordingly, inhibition of HDAC2 with TSA or RNAi-mediated knockdown inhibited deacetylation of IRS-1 and partially restored insulin signaling (55).

Both translocation and expression of GLUT4 are important for glucose uptake. Thus, overexpression of GLUT4 increases basal and insulin-stimulated glucose disposal in mice (56,57). Transcription of *GLUT4* is mainly under the regulation of the GLUT4 enhancer factor (GEF) and the myocyte enhancer factor (MEF)-2, both of which bind to transcriptional elements in the *GLUT4* promoter (58). Through complex formation with GEF and MEF2, HDAC5 functions as a transcriptional repressor of *GLUT4* by histone deacetylation and compacting of the chromatin structure (59–61). The formation of this inhibitory complex is regulated by phosphorylation of HDAC5 by AMPK and CaMK, which induces the release of HDAC5 from the complex (59,62). This allows recruitment of, for example, peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 (PGC-1), which functions as a transcriptional coactivator allowing *GLUT4* transcription (see Figure 2, *iv*) (63,64). The HDAC inhibitor TSA upregulates *PGC-1* expression in skeletal muscle (65). This observation is clinically relevant, since decreased myocyte PGC-1 $\alpha$  expression in patients with

T2D is associated with elevated fasting insulin concentrations (reviewed in [66]). As described above, HDAC1 and HDAC4 are also inhibitors of GLUT4 expression, further underlining an important regulatory role of HDACs in glucose uptake and insulin resistance. HDACs may thus be a target for treatment of insulin resistance in muscular tissue, since compensatory *GLUT4* transcription may reverse the resistant state (56,67).

To summarize, insulin signaling is regulated in a complex and not fully understood manner, and defects causing insulin resistance occur on many levels, including at the level of histone and non-histone protein deacetylation. On the basis of preclinical evidence, inhibition of various HDACs is a promising novel therapeutic principle to correct the insulin-resistant state.

Clinical support for this notion, however, is lacking. Valproate (VPA) used in the long-term treatment of, for example, epilepsy and bipolar disorders is associated with weight gain and hyperinsulinemia. However, the causal interaction and the role of insulin resistance herein are not clarified. The development of insulin resistance is suggested in many studies, mainly on the basis of the occurrence of hyperinsulinemia and on estimations of the homeostasis model assessment–insulin resistance (HOMA-IR) index (reviewed in [68]). In a study that more directly measured insulin resistance using a modified frequently sampled intravenous glucose tolerance test with tolbutamide, Verrotti *et al.* (69) reported increased insulin resistance only in epileptic patients who gained weight and not in those who remained lean following 1 year of VPA treatment. Hyperinsulinemia occurred both in VPA-treated patients with epilepsy who gained weight as well as in VPA-treated patients who remained lean (70), and fasting hyperinsulinemia in VPA-treated patients was not associated with increased fasting serum proinsulin or C-peptide concentrations (71). Together, these data do not imply insulin resistance as the cause of hyperinsulinemia;

rather, inhibition of insulin metabolism in the liver was suggested to be the cause (reviewed in [68]). It remains to be examined if these side effects of VPA are associated with its HDAC inhibitor function, its effects on the central nervous system or other actions of the drug. The fact that VPA is a branched-chain fatty acid may account for these side effects (68). To our knowledge, hyperinsulinemia, insulin resistance and obesity have not been associated with other HDACi in clinical use.

In conclusion, there is a preclinical rationale to conduct clinical trials with HDACi other than VPA to investigate the therapeutic potential of HDAC inhibition in the therapy of insulin resistance.

#### PANCREATIC AND ENDOCRINE CELL DEVELOPMENT AND HDAC INHIBITION

The pancreas consists of mainly two types of tissue: (a) the exocrine tissue composed of acinar cells secreting digestive enzymes into the duodenum and (b) the endocrine tissue that produces hormones such as glucagon ( $\alpha$  cells), insulin ( $\beta$  cells), somatostatin ( $\delta$  cells), pancreatic polypeptide (PP-cells) and ghrelin ( $\epsilon$  cells). The endocrine tissue, representing approximately 1% of the fully developed pancreas is organized into  $\sim 10^6$  islets that develop concomitantly with the ongoing pancreatic morphogenesis.

Characterization of the mechanisms regulating the development of the endocrine pancreas and especially the insulin-producing  $\beta$  cells has undergone an immense development in particular with the mapping of the network of transcription factors that constitute the decision-makers of pancreatic cell fate during morphogenesis, proliferation and differentiation. In nonpancreatic tissues, HDACs are not redundant, and it is generally accepted that individual HDACs are required for specific functions during embryogenesis and postnatal life. For example, HDAC1 is essential for unrestricted cell proliferation by suppressing the expression of cell cycle inhibitors, a function unique to HDAC1 (72); deletion of *Hdac3* was found to lead to early embryonic death and apoptosis due to

DNA damage correlated with inefficient repair of DNA double-strand breaks (73); and HDAC4 inhibits cell cycle progression and has been suggested as a neuroprotective enzyme (74).

A plethora of transcription factors have to be minutely orchestrated at the expressional level to mediate the formation of the fully differentiated tissue. The pancreatic development network of transcription factors, their interaction and temporal control are reviewed elsewhere (75,76). Here, only a few essential transcription factors linked to HDACs will be mentioned. The transcription factor Pdx1 is synthesized in the entire early pancreatic rudiment that comprises the pancreatic buds (77), and *Pdx1* plays a central role in the early development of the pancreas, since deletion of *Pdx1* results in complete pancreatic agenesis (78,79). The *Pdx1*-expressing progenitor cells differentiate into endo- and exocrine cells. It is generally believed that the endocrine differentiation from the *Pdx1*-expressing progenitor cells is initiated by the expression of *neurogenin 3* (*Ngn3*), since *Ngn3*-deficient mice fail to generate endocrine cells (80), and recently, lineage-tracing experiments have provided direct evidence that *Ngn3*-expressing cells are islet progenitors (81). Further, the expression of *Pax4* has been linked to the specific development of the  $\beta/\delta$ -cell lineage in rodents (82,83).

The understanding of the biology of HDACs in pancreatic development is incomplete. HDACs are expressed and developmentally regulated in the pancreas (82). As described above, HDAC1 is involved in silencing of *Pdx1* in a model of IUGR, leading to failure in  $\beta$ -cell development and  $\beta$ -cell dysfunction (38). Additionally, treatment of rat embryonic explants with HDACi *ex vivo* enhances and maintains the expression profile of the proendocrine marker *Ngn3* (82). As *Ngn3* is believed to initiate endocrine differentiation from *Pdx1*-expressing progenitor cells (80), HDACi may lead to an increased pool of endocrine progenitor cells without modifying the proliferation/apoptosis bal-

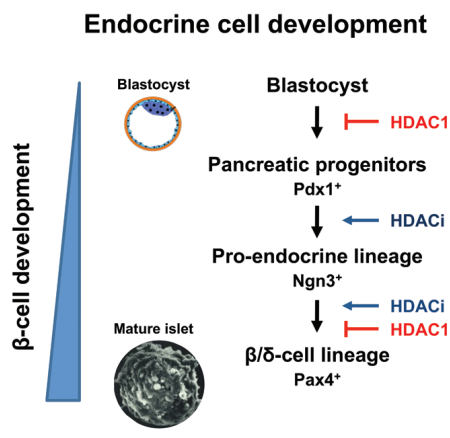
ance (82). Furthermore, HDAC1 associates with the sex-determining region Y-box (SOX)-6, leading to an inhibitory effect of SOX6 on  $\beta$ -cell proliferation (84) (Figure 3).

In zebrafish embryos with HDAC1 loss-of-function or HDAC1 knockdown, the exocrine pancreas failed to form correctly, whereas no marked effects were found on insulin expression (85,86), since ectopic clusters of insulin-expressing cells were observed outside the normal aggregation of endocrine insulin-expressing cells (85). However, the effect of HDAC1 inhibition on endocrine pancreas formation is debated (86).

Different HDACi have distinct effects on endocrine lineage development. Thus, TSA enhances, while VPA suppresses,  $\beta/\delta$ -cell lineage differentiation. In contrast, both inhibitors promote the  $\alpha$ /PP-cell lineage, illustrating the specific series of events that control pancreatic development (82). However, these observations cannot be construed to assign specific functions of certain HDAC subtypes in pancreatic development (87,88), since different HDACi have distinct structures and thereby possibly distinct functions independent of their inhibitory action on HDAC activity and since the action of many HDACi vary with concentration.

HDACi also promote differentiation of embryonic stem (ES) cells into insulin-producing cells, a property of considerable importance for  $\beta$ -cell replacement therapy. TSA inhibits ES cell differentiation, while sodium butyrate (NaB) stimulates early events of pancreatic specification in ES cells (89–91). In concordance with the studies in ES cells, TSA improved the transdifferentiation of bone marrow stem cells into insulin-producing cells (92). The inclusion of NaB in early stages of the differentiation protocol led to differentiation of human ES cells into islet-like clusters expressing insulin as well as glucagon and somatostatin (93).

In summary, HDACi have a potential to differentiate stem cells into insulin-producing cells. However, further studies are needed to clarify the differential importance of various HDAC subtypes



**Figure 3.** Overview of the roles of HDACs in pancreatic endocrine cell development.

and thereby different HDACi and the impact of concentration of HDACi on the effects observed. The use of more specific HDACi along with careful titration studies should allow clarification of these questions.

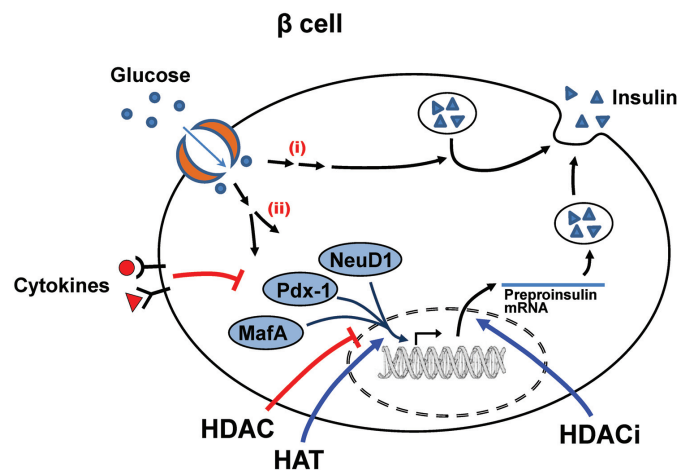
### $\beta$ -CELL FUNCTION AND HDAC INHIBITION

The most important function of the pancreatic  $\beta$  cell is to release insulin in response to nutrients, hormones and other humoral mediators as well as to neuronal signals to maintain glucose homeostasis and lipid and protein metabolism. Insulin is a peptide hormone synthesized as a longer precursor (proinsulin) that con-

sists of three peptide chains (A, B and C). The hormone is processed by prohormone convertases 1 and 2, which excise the central part of the protein (the connecting [C]-peptide), leaving the A and B chains linked by two disulfide bonds. Insulin is finally processed by carboxypeptidase E to produce the mature form that is stored as homohexamers in secretory vesicles and released in response to increased blood glucose and other stimuli (reviewed in [94]). As depicted in Figure 4, glucose induces both release (see *i* in figure) and transcription of insulin (see *ii* in figure), with the latter depending on at least three  $\beta$ -cell-specific transcription factors: Pdx1, NeuroD1 and V-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA) (95).

### *In Vitro* Studies

The expression of insulin from  $\beta$  cells is regulated by acetylation. Thus, at high glucose levels, Pdx1 associates with the histone acetyltransferase p300, leading to increased acetylation of histone H4 in the insulin promoter. These events appear to be necessary for preproinsulin transcription induced by glucose (96–100). Conversely, at low glucose levels where insulin production is shut off, the acetylation of histone H4 at the insulin promoter is abolished, correlating with recruitment of HDAC1 and -2 to the in-



**Figure 4.** Simplified overview of the effect of HDACs and HATs on glucose-induced insulin expression. NeuD1, NeuroD1.

insulin promoter by Pdx1 (97,101). NeuroD1 also interacts with p300 and is acetylated by the p300-associated factor (PCAF). This acetylation increases the binding of the transcription factor to the insulin promoter, leading to enhanced insulin gene expression (102). In  $\beta$  cells, MafA protein is constitutively phosphorylated by glycogen synthase kinase (GSK-3), leading to ubiquitination and proteosomal degradation (103). However, phosphorylation of MafA is also required for binding of the insulin promoter and transactivating properties (104,105). In a non- $\beta$ -cell system, phosphorylated MafA recruits PCAF, the effect of which is not only associated with increased transcriptional activity but also with reduced ubiquitination and degradation of MafA (106). In  $\beta$  cells, the degradation of MafA is delayed by exposure to high concentrations of glucose, even though MafA is still phosphorylated (103). This may suggest that high concentrations of glucose allow interaction between MafA and PCAF (or another HAT), thereby stabilizing MafA and increasing insulin transcription through opening of the chromatin structure in the insulin promoter. However, further studies are needed to clarify the putative effect of PCAF on MafA stability and activity in  $\beta$  cells.

Taken together, the above-mentioned studies suggest that acetylation favors insulin expression and that HDAC activity accordingly decreases insulin expression. The HDACi TSA and NaB increase histone H4 acetylation and enhance insulin expression at low glucose levels (3 mmol/L), supporting a repressive role of HDACs on preproinsulin transcription (97). Of note, TSA and NaB did not potentiate acetylation of H4 after exposure to high concentrations of glucose (30 mmol/L) (97). A stimulatory effect of VPA on insulin release has also been reported in human islets incubated in low glucose concentrations (2.8 mmol/L) (107). In contrast, accumulated insulin release from rat islets incubated in 11 mmol/L glucose was unaffected by suberoylanilide hydroxamic acid

(SAHA) and ITF2357 but was slightly inhibited by TSA (27,108).

### Clinical Studies

As described above, several studies investigated long-term metabolic effects of treatment with VPA. In general, no changes were reported with respect to fasting serum insulin and C-peptide in VPA-treated patients, whereas a single study found increased postprandial C-peptide and proinsulin levels (reviewed in [71]). Although VPA stimulates insulin release from isolated islets *in vitro* (107), a more recent report argues against a direct stimulatory effect of VPA on insulin release *in vivo* and suggests that the elevated insulin concentrations are a consequence of decreased hepatic degradation of insulin, as mentioned above (71). Again, it is unclear whether the effects of VPA are caused by its function as an HDAC inhibitor, its actions as a fatty acid derivative or other putative mechanisms, and more research is needed to shed light on the effects of other HDACi on insulin secretion *in vivo*.

In summary, insulin production depends on acetylation and is inhibited by deacetylation. Taken together, studies examining the effects of HDACi on insulin expression indicate that HDACi treatment increases insulin expression at low glucose levels, whereas insulin release is less affected (see Figure 4). As discussed later, HDACi protects against  $\beta$ -cell inhibitory and cytotoxic effects of cytokines. In addition to a direct induction of apoptosis, cytokines also induce a  $\beta$ -cell dedifferentiation associated with decreased expression and/or activity of *Pdx1*, *NeuroD1* and MafA (109–111). It is likely that a part of the protective effects of HDACi against cytokine-induced toxicity are consequences of a maintained  $\beta$ -cell differentiation.

Recent studies suggest that a 40% reduction of the  $\beta$ -cell mass is sufficient to precipitate clinical symptoms of T1D (112) and that a significant proportion of insulinopenia is likely to be caused by reversible functional inhibition of  $\beta$  cells due to inflammation. If HDACi treat-

ment is able to derepress the functional inhibition of residual  $\beta$ -cell mass, it would have significant therapeutic potential, not only with respect to treatment of patients with T2D, but also in newly diagnosed patients with T1D.

### $\beta$ -CELL DESTRUCTION AND HDACi

To the best of our knowledge, investigations of the role of HDACi on the pathogenetic events that lead to  $\beta$ -cell destruction have been restricted to examinations on cytokine-induced  $\beta$ -cell death. As described above, the proinflammatory cytokine IL-1 $\beta$  causes  $\beta$ -cell apoptosis and is implicated in the pathogenesis of both T1D and T2D. Furthermore, two other proinflammatory cytokines, namely TNF $\alpha$  and IFN $\gamma$ , have been shown to potentiate the toxic effects of IL-1 $\beta$  (113–116). In T1D in particular, there is a pronounced selective destruction of the  $\beta$  cells, and in accordance with a role of proinflammatory cytokines as mediators of the  $\beta$ -cell destruction, the toxic effects of cytokines are selective for  $\beta$  cells (117). This selectivity is further supported by studies showing that maturation of the  $\beta$  cell makes it prone to the toxic effects of IL-1 $\beta$  (118). On the basis of animal studies, IL-1 $\beta$  plays an important role in destruction of transplanted islet grafts (119–123), and blocking cytokines in clinical islet transplantation has also been suggested as a future intervention to prevent graft destruction (124).

We originally reported a protective effect of HDACi treatment against cytokine-induced  $\beta$ -cell death (27), an observation now confirmed by us and others (108,125,126). Accordingly, several HDACi (ITF2357, TSA and SAHA) protect against cytokine-induced reduction in accumulated insulin secretion (27,108). Of note, ITF2357 was not only found to prevent cytokine-mediated reduction of accumulated insulin release but increased insulin release over and above that of control islets (108). Because cytokines at low concentrations stimulate insulin secretion (127), we interpret these observations to suggest that ITF2357 selectively prevents

proapoptotic cytokine signaling while sparing the cytokine-mediated regulatory signaling that stimulates insulin secretion (108). However, a reversal of cytokine-induced reduction of insulin release to levels over and above the control level has not been seen with other HDACi (27), and further studies are needed to substantiate these observations.

Cytokines inhibit both first- and second-phase insulin release through decreased expression of insulin (128) and proteins that are important for insulin secretion (129). Interestingly, the cytokine-mediated reduction in acute glucose-stimulated insulin secretion, which mainly depends on release of preformed insulin vesicles and less on *de novo* insulin transcription, translation and processing, is unaffected by HDACi (27). Taken together, these data indicate that in addition to antiapoptotic effects of HDACi, these compounds preferentially protect preproinsulin transcription, and/or proinsulin translation and processing or expression of genes involved in non-glucose-induced signaling of insulin secretion from the inhibitory effects of cytokines (27), with little effect on insulin granule formation, translocation, docking and exocytosis, although this needs to be investigated in more detail.

The  $\beta$  cell expresses all classical HDACs, albeit at different levels, and they are differently regulated by cytokines (108). On the basis of relative expressions and regulation by cytokines, important roles of especially HDAC1, -2, -6 and -11 have been suggested (108). However, it remains to be experimentally investigated on which specific HDAC family member(s) cytokine-induced proapoptotic signaling depends. Studies that include molecular approaches and/or more selective inhibitors of individual HDAC members are needed to elucidate this question.

#### MECHANISMS OF HDACI-MEDIATED $\beta$ -CELL PROTECTION AGAINST INFLAMMATORY ATTACK

Exposure of islets to the cytokines IL-1 $\beta$  and IFN $\gamma$  modifies the expression

of more than 2,000 genes, many of which are related to pathways signaling apoptosis, cell cycle regulation and endoplasmic reticulum (ER) stress, but also pathways involved in maintenance of differentiation, cell metabolism (for example, through the Krebs cycle) and alternative splicing (130). NF $\kappa$ B has received much attention for its role in cytokine-induced  $\beta$ -cell death and plays an essential role in mediating the proapoptotic effects of cytokines (25,26). HDACi reduce cytokine-induced NF $\kappa$ B activity and decrease expression of NF $\kappa$ B-dependent genes (for example, inducible nitric oxide synthase [iNOS] and inhibitor of kappa B [I $\kappa$ B]) (27,108,125,126). On the basis of results from an electrophoretic mobility shift assay showing no effects of HDACi on cytokine-induced NF $\kappa$ B binding to synthetic oligonucleotides, HDACi were suggested to modulate the chromatin structure of NF $\kappa$ B-dependent genes, resulting in decreased NF $\kappa$ B transactivation by unknown coactivators (27). In non- $\beta$  cells, NF $\kappa$ B interacts with HDAC1, -2 and -3 (131,132), but whether these interactions also take place in  $\beta$  cells and what the impact is of the interplay on the protective effect of HDAC inhibition on cytokine-mediated  $\beta$ -cell toxicity are unknown.

In the  $\beta$  cell, IFN $\gamma$  signal transduction proceeds through the JAK/STAT pathway by activation of STAT1. Purified  $\beta$  cells from STAT1 knockout mice are protected against apoptosis induced by IFN $\gamma$  and IL-1 $\beta$ , suggesting an important role of STAT1 in cytokine-induced  $\beta$ -cell death (133). In non- $\beta$  cells, IFN $\gamma$ -induced JAK activation and STAT1 activity depend on HDAC1, -2 and -3 activity (134). Whether IFN $\gamma$ -induced STAT1 activity is inhibited after HDACi treatment in  $\beta$  cells has to our knowledge not been investigated.

IL-1 $\beta$  in combination with IFN $\gamma$  reduces the expression of the sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA)-2 pump, resulting in depletion of the ER Ca<sup>2+</sup> stores (135,136). Ca<sup>2+</sup> depletion hinders proper protein folding, leading to the unfolded protein response,

ER stress and  $\beta$ -cell death (137). Upon activation of the unfolded protein response, several protective cellular compensatory mechanisms are initiated to stabilize ER homeostasis (for example, production of chaperones, such as Hsp70) (138,139). Accordingly, expression of Hsp70 is also induced by cytokines, and overexpression of Hsp70 protects against cytokine-induced  $\beta$ -cell death (140). In non- $\beta$  cells, Hsp70 forms complexes with HDAC1, -2 and -3 (141), but whether these complexes are also found in  $\beta$  cells and whether HDACs affect Hsp70 activity has not been examined. Furthermore, TSA increases the expression of the chaperone BiP (an Hsp70 class protein) in non- $\beta$  cells (142). In  $\beta$ -cells, overexpression of BiP protects against *in vitro* cytotoxic effects of the fatty acid palmitate (143) but not of cytokines (144). Whether HDACi modulates BiP expression in  $\beta$  cells and whether BiP is part of the protective mechanism require further investigation.

Although the unfolded protein response is a protective ER response, prolonged unfolded protein response leads to  $\beta$ -cell death by mechanisms that are not fully clarified. The transcription factor C/EBP homologous protein (CHOP) is induced upon ER Ca<sup>2+</sup> depletion. CHOP may induce apoptosis through several mechanisms including activation of the intrinsic apoptotic pathway (137). In non- $\beta$  cells, CHOP interacts with HDAC1, -5 and -6, and TSA has been shown to repress degradation of CHOP (145), although other investigators have shown that TSA does not affect the protein level of CHOP (142). Further, the importance of CHOP and ER stress in cytokine-induced  $\beta$ -cell death is debated, since neither knockdown of CHOP nor overexpression of BiP protect against cytokine-induced  $\beta$ -cell death (144). Further, a role of ER stress in the pathogenesis of T1D in humans is also questioned, since CHOP expression was not consistently demonstrated in eight pancreatic autopsies of T1D patients (146).

Another mechanism by which cytokines induce apoptosis is through di-

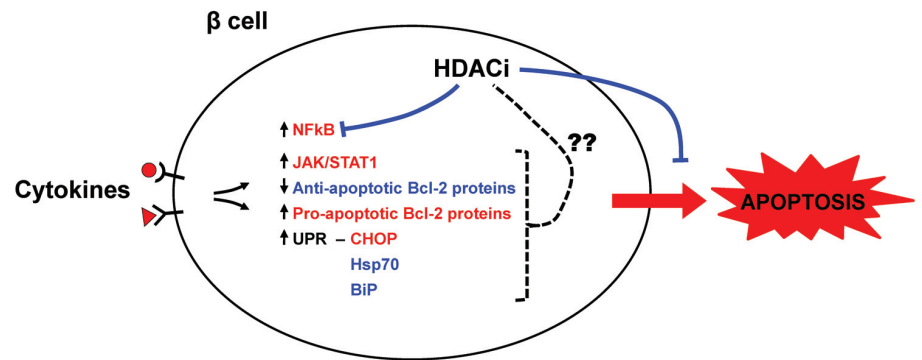


rect activation of the intrinsic apoptotic pathway. Thus, cytokines induce activation of proapoptotic Bcl-2 proteins, and inhibition of antiapoptotic Bcl-2 proteins causes release of cytochrome c from the mitochondria, followed by activation of caspase-9 and subsequently caspase-3 activation (147–152). Overexpression of antiapoptotic Bcl-2 proteins protects against cytokine-induced  $\beta$ -cell death, supporting an important role of the Bcl-2 proteins (153,154). Several links between Bcl-2 proteins and HDACi have been found primary in models of cancer where high concentrations of HDACi are used to induce apoptosis in cancer cells. In transformed cells, HDACi operates via the proapoptotic Bcl-2 proteins Bim (155), Bid (156) and Bax (157), which are upregulated, processed or translocated to the mitochondrial membrane, respectively, while expression of the antiapoptotic Bcl-2 protein Bcl-X<sub>L</sub> is downregulated (158). The effects of lower HDACi concentrations used in inflammatory diseases on the regulation of the Bcl-2 protein family and an effect of HDACi on cytokine-induced activation of the intrinsic apoptotic pathway in  $\beta$  cells are yet to be examined.

As summarized in Figure 5, cytokine-induced  $\beta$ -cell apoptosis depends on HDAC activity, since it is prevented by HDACi treatment. Although NF $\kappa$ B signaling has been identified as an HDACi target, the exact molecular mechanisms by which HDACi prevents cytokine-induced  $\beta$ -cell death are not clarified, and effects of HDACi on other key players in cytokine-induced signaling such as JAK/STAT1, Bcl-2 proteins and proteins involved in ER stress have not yet been investigated. Finally, studies in animal models and phase II clinical trials are needed to shed light on the translational significance of the promising *in vitro* effects of HDACi on cytokine-induced  $\beta$ -cell toxicity.

### CONCLUSIONS AND PERSPECTIVES

The evidence reviewed here indicates that HDACs are involved in several biological pathways relevant for the etiology



**Figure 5.** Proinflammatory cytokines induce  $\beta$ -cell apoptosis through regulation of a range of pro- (red) and antiapoptotic (blue) genes and proteins. The protective effect of HDAC inhibition likely involves NF $\kappa$ B and possibly other death pathways.

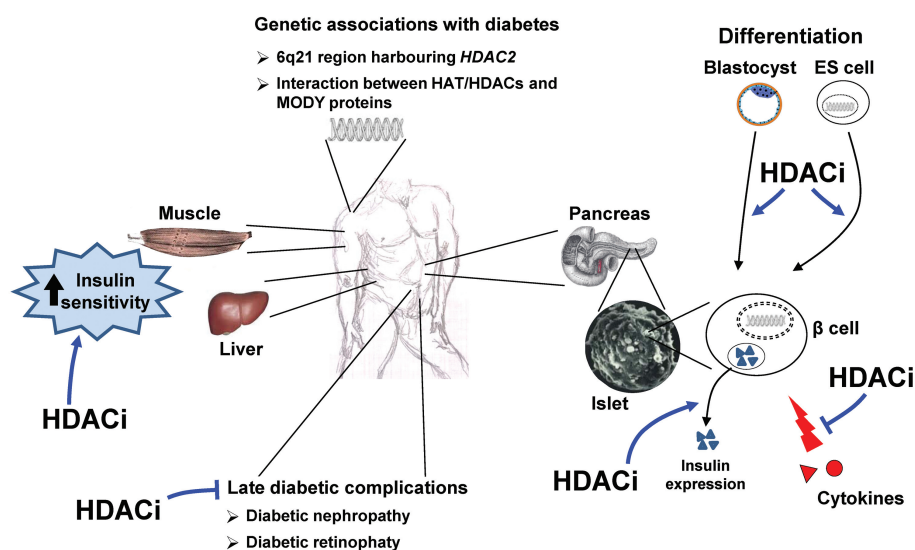
and pathogenesis of T1D and T2D. As summarized in Table 1 and in Figure 6, there is evidence of genetic association between diabetes and HDACs, as there is of HDACi promoting  $\beta$ -cell development, proliferation, differentiation and function; preventing  $\beta$ -cell inflammatory damage; improving insulin resistance; and posi-

tively affecting late diabetic microvascular complications. Taken together, this evidence provides a strong rationale for continuing preclinical studies and initiating clinical trials, with the aim of testing the clinical utility of HDACi in diabetes.

However, there is still much to be learned about the mechanisms of action

**Table 1.** Evidence in favor of HDACs as therapeutic targets in diabetes mellitus.

Genetic associations	Genetic association with the 6q21 region harboring <i>HDAC2</i> with T1D and T2D MODY gene mutations directly affect the ability of the MODY proteins to interact with HAT/HDACs
HDACs and $\beta$ -cell development and function	HDAC1 associates with SOX6 to inhibit $\beta$ -cell proliferation HDAC1 silences <i>Pdx1</i> in IUGR, leading to failure in $\beta$ -cell development and $\beta$ -cell dysfunction and reversible in the neonatal state by HDAC inhibition HDACi enhances and maintains expression of the proendocrine transcription factor <i>Ngn3</i> HDACi stimulates early events of pancreatic specification in embryonic stem cells HDACi improves transdifferentiation of bone marrow-derived stem cells into insulin-producing cells HDACi enhances insulin expression
HDACs and inflammatory pathogenesis of T1D and T2D	HDACi reduces the peripheral blood mononuclear cell production of IL-1 $\beta$ , TNF $\alpha$ and IFN $\gamma$ HDACi protects against cytokine-induced reduction in accumulated insulin secretion HDACi protects $\beta$ cells against cytokine-induced apoptosis by targeting NF $\kappa$ B
HDACs and insulin resistance	IUGR promotes HDAC1- and -4-mediated histone deacetylation of the <i>Glut4</i> promoter in adult muscle tissue, thereby repressing its transcription HDAC5 functions as a transcriptional repressor of <i>GLUT4</i> HDACi upregulates <i>PGC-1</i> expression, GLUT4 translocation and insulin-induced glucose uptake in skeletal muscle HDAC2 inhibition hyperacetylates IRS-1 and partially restores insulin signaling
HDACs and late diabetic complications	HDACi in part protects against diabetic nephropathy and retinopathy



**Figure 6.** Overview of the therapeutic potential of HDACi in diabetes.

and the differential importance of the 11 classic HDACs, the sirtuins and the HATs controlling the acetylation balance in diabetes. An important task will be to define the relative importance of acetylated histones, transcription factors and other nuclear, cytosolic and compartmentalized proteins in the  $\beta$ -cell acetylation, and, eventually, the interaction between acetylation and other posttranslational protein modifications needs to be interpreted into the language now known as the histone or protein code. Lastly, the enigma of how HDAC inhibition, an apparently nonspecific treatment, can exert therapeutic benefit to so many diverse disorders needs to be unraveled. Does HDACi only reset a disturbed protein acetylation balance as a result of cell stress without affecting cell homeostasis? Why does HDACi-mediated hyperacetylation mainly associated with increased gene transcription counteract inflammatory gene expression changes? Is this a consequence of expressional upregulation of genes encoding antiapoptotic proteins or microRNAs? How can this be reconciled with an inhibitory effect of HDACi on NF $\kappa$ B transcriptional activity? These basic research questions and many more will have to be addressed in parallel with further preclinical and clinical de-

velopment to pave the way for future-generation HDACi with higher specificity and safety for the treatment of diabetes and other inflammatory diseases.

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#### DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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