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Review

Glycemic memory associated epigenetic changes

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ABSTRACT

It is evident that metabolic memory, whereby diabetic complications continue to develop and progress in individuals who returned to normal glycemic control after a period of transient hyperglycemia, can have long lasting effects. We have primary findings that transient hyperglycemia causes profound transcriptional changes in vascular endothelial cells. We hypothesized that ambient hyperglycemia triggers gene-activating events of the NFκB p65 promoter that are mediated by changes in epigenetic modifications. In a follow-up study we identified two histone-specific writing and erasing enzymes involved in the underlying regulation of gene expression during transient hyperglycemia and subsequent return to normoglycemia. Experimental evidence indicates that previous hyperglycemia is associated with persistent expression of the NFκB p65 gene, which activates NFκB-dependent proteins, such as MCP-1, which are implicated in diabetes-associated vascular injury. Increased gene transcription is correspondent with H3K4m1, but not H3K4m2 and H3K4m3, on the NFκB p65 gene. In vascular endothelial cells the histone methyltransferase Set7 can write the mono-methylation mark H3K4m1 and this methyl-writing enzyme is recruited as a gene co-activator in response to glucose. Furthermore, Set7 knockdown prevents glucose-induced p65 expression. We hypothesize that these molecular events represent an integrated response of the epigenome that lead to changes in the expression of genes and proteins that regulate the development and progression of diabetic vascular complications. Further characterisation of these glucose-induced epigenetic events and the identification of key enzymes involved will improve our understanding of the pathways implicated in diabetic vascular injury.

Contents

Abbreviations: ACCORD, action to control cardiovascular risk in diabetes; ADVANCE, action in diabetes and vascular disease: preterax and diamicron MR controlled evaluation; ATP, adenosine triphosphate; bp, basepairs; CBP, CREB-binding protein; DCCT, diabetes control complications trial; DNA, deoxyribonucleic acid; ECM, extracellular matrix; EDIC, epidemiology of diabetes interventions and complications; Glut2, glucose transporter 2; H, histone; HAT, histone acetyl transferase; HDAC, histone deacetylase; HG, high glucose; HMTase, histone methyltransferase; IL-6, interleukin-6; Ins1/2, insulin1/2; Jhdm2a, JmjC domain-containing histone demethylation protein 2a; K, lysine; kb, kilobase(s); LG, low glucose; LSD1, lysine-specific demethylase 1; m1, mono-methylation; m2, dimethylation; m3, trimethylation; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A; Mcp, 1-monocyte chemoattractant protein-1; miRNA, microRNA; NAD, nicotinamide adenine dinucleotide; ncRNA, non-coding RNA; NFκB, nuclear factor kappa-B; P300, histone acetyltransferase p300; Pdx1, pancreatic and duodenal homeobox 1; PRMT5, protein arginine N-methyltransferase 5; Set7/9, SET domain-containing protein 7/9; siRNA, small interfering RNA; Suv39h1, suppressor of variegation 3–9 homologue 1; TNF-α, tumour necrosis factor-alpha; UKPDS, UK prospective diabetes study.

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1. Glycemic variability and diabetic complications

Cardiovascular complications remain the major cause of morbidity and mortality in the diabetic population [1]. Patients with type 1 or type 2 diabetes have a two- to four-fold higher risk of cardiovascular disease when compared to healthy individuals [2,3], and those with impaired glucose tolerance alone have a cardiovascular disease risk comparable to type 2 diabetics [4]. It is increasingly appreciated that exposure to high glucose is the major factor leading to these complications. Furthermore, there appears to be a "metabolic memory" [5] or "legacy effect" [6] whereby diabetic complications, particularly vascular events, continue to develop and progress even in individuals who have returned to normal glycemic control after a period of transient hyperglycemia. In the latest follow-up from the diabetes control complications Trial (DCCT), the epidemiology of diabetes interventions and complications (EDIC) trial, it is evident that the deleterious effects in the vasculature of both conventional and intensified glycemic control continue to operate more than 5 years after the patients have returned to normoglycemia [5]. Further to this, the results of the action in diabetes and vascular disease: preterax and diamicron MR controlled evaluation (ADVANCE) and action to control cardiovascular risk in diabetes (ACCORD) trials raise the debate about whether tight glucose control is beneficial at all in diabetes [7,8]. Whereas the initial interpretation of the results from the UK Prospective Diabetes Study (UKPDS) showed no significant effect of strict glycemic control on myocardial infarction, meta-analysis of these data show significant reductions in all diabetes-related endpoints [9]. A recent follow-up in type 2 diabetics from this study argues for the utility of long-term hyperglycemic control in preventing cardiovascular disease [10].

Although the underlying molecular explanation for this "metabolic memory" has not yet been clearly defined, this phenomenon has precedence and was first observed more than 20 years ago in the retina of diabetic dogs exposed to high glucose for 2.5 years, then allowed normal glucose control for a further 2.5 years [11]. Those animals that returned to normoglycemia had a similar incidence of retinopathy as their control counterparts who had poor glucose control throughout the 5-year study suggesting that these cells retained a "memory" of the early hyperglycemic episodes [11]. Soon after this study, another group showed that there was a persistent upregulation of extracellular matrix (ECM)-related gene expression in isolated endothelial cells and kidneys of diabetic rats 1 week after glucose normalisation following 2 weeks of hyperglycemia [12]. Recent *in vitro* studies by our laboratory suggest an important role for epigenetic modification as a result of both ambient and prior hyperglycemia, in primary human aortic endothelial cells [13].

Further elucidation of the regulatory mechanisms associated with hyperglycemic memory and the role of histone methyl-transferases, particularly SET domain-containing protein 7 (Set7) was demonstrated in human microvascular endothelial cells [14]. These results indicate that glucose is conferring gene-activating events that are also associated with diabetic complications (see Fig. 1). The ability of epigenetic changes to confer sustained effects on the vasculature, as a result of acute hyperglycemia, emphasises the potential deleterious long-term effects of "metabolic memory". This could have direct clinical implications, emphasising the importance of achieving tight metabolic control. In addition, it may provide a potential mechanism for the adverse outcomes that have been suggested in various studies following strict glycemic control. This includes the adverse effects on retinopathy in the initial phases of the early insulin pump studies in the Kroc Collaborative Study Group or the recent unexpected findings of increased mortality in the ACCORD study [15]. Finally, the delineation of the key epigenetic events *in vivo* that confer glucose-induced gene modulation in blood vessels will assist in identifying appropriate targets for new treatments to reduce, reverse or prevent diabetic complications.

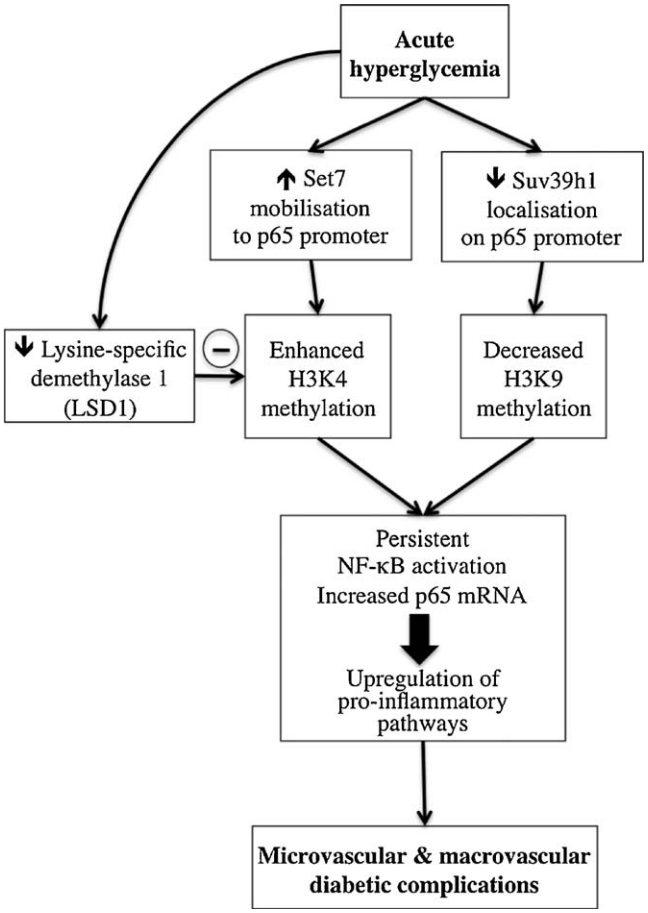

Fig. 1. Proposed mechanism—epigenetic effects of hyperglycemia on vascular cells. Hyperglycemia is associated with epigenetic changes leading to transcriptional activation of the proinflammatory transcription factor NFκB. Recruitment of Set7 to the p65 promoter results in enhanced H3K4m (active mark) leading to transcriptional activation, and this corresponds with reduced Suv39h1 binding on the promoter and decreased H3K9m (repressive mark). Relative levels of the histone demethylase LSD1 are also increased in response to hyperglycemia, therefore blocking its demethylase activity on H3K4. These epigenetic modifications and gene-activating events can persist in the absence of sustained hyperglycemia, establishing a hyperglycemic memory in vascular cells.

2. Studying epigenetic changes

Epigenetics describes the study of heritable changes in gene activity and expression independent of changes in nucleotide sequence [16]. These epigenetic changes are potentially reversible and modulated by the environment, diet or pharmacological intervention (Fig. 2). This in turn can mediate changes in genomic stability and gene expression [17–19]. Essentially the field of epigenetics provides a link between genotype and phenotype, which can help explain how cells carrying identical DNA differentiate into different cell types with distinct functions [20]. The best studied mechanisms of epigenetic changes in mammals DNA methylation and modifications of histone tails, which result mostly in altered chromatin structure [21].

For many years the class of non-protein-coding RNA (ncRNA) including tRNA, rRNA and spliceosomal RNA and this list of ncRNAs has recently expanded to include small nuceolar RNA, microRNA (miRNA), short-interfering RNA (siRNA) and small

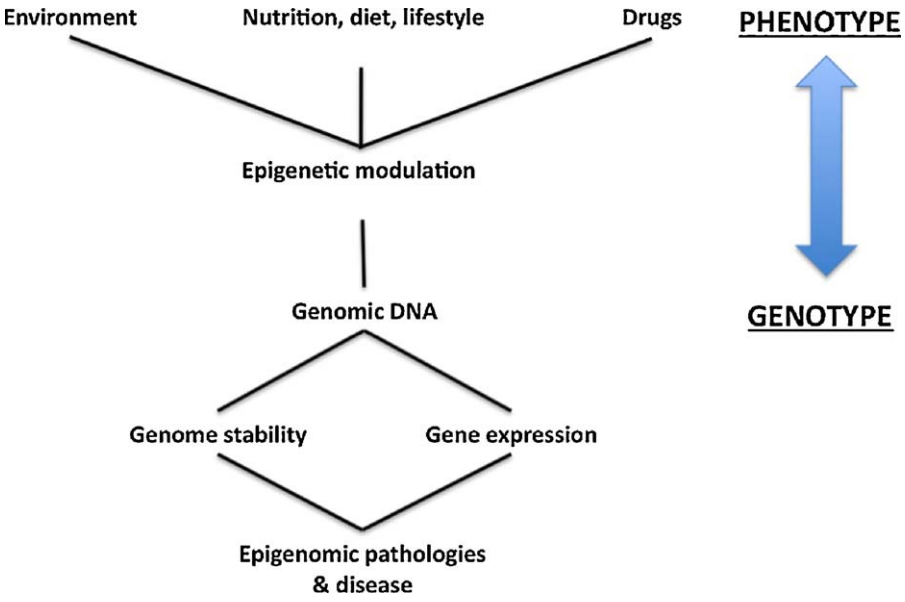

Fig. 2. Schematic of how various stimuli, epigenetic mechanisms and gene-activating events can lead to epigenomic pathologies. Epigenetic changes can be modulated by the environment, diet or pharmacological intervention. As outlined, epigenetics essentially provides a link between genotype and phenotype, which can help explain how cells carrying identical DNA differentiate into different cell types with distinct functions. Nutrient-driven epigenetic changes are also involved in the development of disease.

double-stranded RNA. Recent studies have shown that some of these small RNA molecules (particularly miRNAs) regulate chromatin modifications, imprinting, DNA methylation and transcriptional silencing have been reviewed elsewhere and is outside the context of diabetes and its complications [22].

Genomic DNA is packaged in eukaryotic cells with histone proteins to form a protein/DNA complex known as chromatin. The fundamental unit of chromatin is the nucleosome and is composed of an octamer of the four core histones (H2A, H2B, H3 and H4) around which approximately 146 base pairs (bp) of DNA are wrapped [23] (see Fig. 3). The core histones are predominantly globular except for their amino-terminal tails, which are accessible to histone-modifying enzymes [24]. Histones can be modified at many sites with over 60 amino acid residues currently identified and detected by specific antibodies or by mass spectrometry [17]. The timing of appearance of a particular post-translational modification will depend on the signalling conditions within the nucleus of the cell. Histone modifications of different classes (e.g. acetylation, methylation, ubiquitination and phosphorylation) can define epigenetic regulation of a variety of biological functions [25–27]. Histone modifications help to partition the genome into distinct domains such as active euchromatin, where DNA remains in an open conformation accessible for transcription and in contrast the properties of silent heterochromatin are presented as condensed chromatinized fiber that is inaccessible to transcriptional machinery [25,28]. Gene transcription and activation are dynamic processes involving the conversion of compact heterochromatin into transcription factor-accessible euchromatin [29]. Euchromatin represents a large proportion of the genome allowing DNA flexibility to turn genes on or off and allow DNA repair or replication.

The term "histone code" has been used to describe the role of modifications to enable DNA-related functions [25]. Heterochromatin plays an important role in protecting chromosome ends, regulating genome stability and preventing mutations and translocations [19,30]. In mammals, demarcation between the different environments is set up by boundary elements, which recruit enzymes to modify the chromatin. The regulation of gene expression within euchromatin requires the delivery of chromatin-modifying enzymes by DNA-bound transcription factors [26]. Following an environmental stimulus, such as glucose, and in the presence of essential transcriptional machinery, transcription factors bind to the promoter of gene sequences and initiate regulatory events involved in the activation or suppression of gene activity [26]. Generally, modifications are divided into those that correlate with gene activation and those that correlate with gene repression.

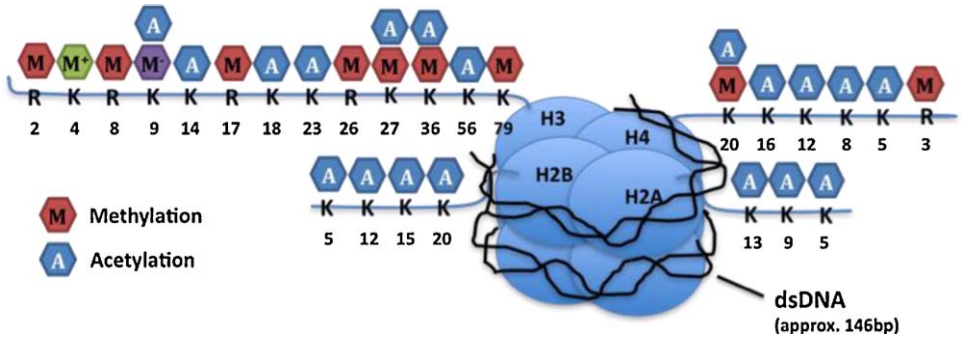

Fig. 3. Nucleosome structure with known histone modifications on specific amino acid residues. A single nucleosome is composed of approximately 146 bp of double-stranded DNA (dsDNA) wrapped around an octamer core of histone proteins from which histone N-terminal tails protrude. Specific amino acids (R – arginine, K – lysine) are subjected to different post-translational modifications. Here only acetylation (A – blue) and methylation (M – red) marks are included, the most relevant to diabetes and hyperglycemic response being: H3K4 methylation (active mark – green +) and H3K9 methylation (repressive mark – purple −).

There are two established mechanisms for the function of modifications. The first is the disruption of contacts between nucleosomes in order to "unravel" chromatin and the second is the recruitment of non-histone proteins [17]. Modifications may affect higher order chromatin structure by affecting the contact between different histones in adjacent nucleosomes or the interaction of histones with DNA. Acetylation has the greatest potential to unfold chromatin, since its major action is to neutralize the basic charge of the lysine residue [25]. The recent development of molecular strategies to design recombinant nucleosomes modified at specific sites has allowed further interrogation of these mechanisms *in vitro*. For example, by chemically ligating modified (tail) peptides onto recombinant histone core preparations, it has been possible to show that acetylation of H4K16 has a negative effect on the formation of the 30-nanometer fiber and consequently higher order chromatin structure [31]. Histone acetyl transferases (HATs), such as p300 and CREB-binding protein have been shown to modify a variety of lysine residues on H3 and H4. The histone deacetylases (HDACs) remove acetyl groups and inhibit transcription factor binding and transcription. Binding proteins are recruited to modifications via specific domains. Unlike histone acetylation, histone methylation can be associated with both gene activation and inactivation, and the effects of methylation are essentially mediated by recruitment of additional positive or negative transacting factors. The recent isolation of several proteins that recognize Histone-3-Lysine-4 methylation (H3K4m) has highlighted the fact that their purpose is to tether enzymatic activities onto chromatin. In mammals, the silent heterochromatic state is associated with low levels of acetylation and high levels of specific methylated sites at H3K9, H3K27 and H4K20 [32]. Further enzymatic activities are required for transcription to take place, which is typically characterised by high levels of acetylation and trimethylation of lysines at H3K4, H3K36 and H3K79 [33]. Additional complexity arises from the fact that methylation occurs not only at lysines but also at arginines, and these may be mono- (m1), di- (m2), or trimethylated (m3) at lysine residues and mono- or dimethylated for arginines (reviewed in [27]).

3. Nutritional intervention and epigenetic modifications

Many studies have examined links between obesity, energy metabolism, nutrient balance and epigenetic modifications [34–38]. Obesity is associated with loss of function of the histone demethylase, Jhdm2a, resulting in decreased expression of the metabolically active gene peroxisome proliferator-activated receptor-alpha (PPAR-alpha) in skeletal muscle and impaired uncoupling protein 1 expression in brown adipose tissue suggesting a relationship between epigenetic mechanisms and obesity [34]. The nicotinamide adenine dinucleotide (NAD+)-dependent sirtuins (class III HDACs) target both histone and non-histone proteins and is another example of epigenetic control of metabolic pathways, including adipogenesis, glucose utilization and insulin secretion [39].

An elegant example of how environmental exposure to nutrients may change gene expression and alter phenotype through epigenetic modifications is the agouti mouse. The agouti gene encodes a paracrine-signalling molecule that promotes melanocytes to produce a yellow pigment rather than black, altering their coat colour and making these yellow mice prone to develop obesity and diabetes [40–42]. The coat colour and subsequent disease susceptibility is essentially controlled by the degree of methylation on the agouti gene. Supplementation of pregnant mice diets with folic acid, a known methyl donor, increases DNA methylation of the agouti gene in offspring, resulted in suppressed gene expression and a brown coat colour [43]. Interestingly, this phenomenon can also be inherited in the next generation via germline transmission [44]. Nutrient-driven epigenetic changes are also involved in the development of disease as outlined in Fig. 2. Indeed, rats exposed to a high-fat diet during pregnancy are associated with impaired glucose tolerance, as well as mitochondrial and cardiovascular dysfunction in adult offspring, and this is thought to be associated with associated with epigenetic change [36–38].

4. Epigenetic regulation of diabetic complications

Oxidative stress, dyslipidemia and hyperglycemia are thought to be associated with the development of diabetic complications. The major event in the progression of diabetic complications is vascular inflammation, triggered by a pathway of mediators to enhance inflammatory signalling. Nuclear factor-κB (NFκB) is one of the predominant transcription factors that are activated under diabetic conditions in the inflammatory pathway, leading to recruitment of monocytes and macrophages to the vessel and macrovasculature atherosclerosis [45]. In fact, poor glycemic control increases NFκB activity in monocytes and in turn up-regulates gene expression of inflammatory cytokines [46,47]. Moreover, recently it has been shown that high glucose levels promote foam cell formation, not only in macrophages but also in human cultured SMC [48]. More recently Set7 was demonstrated in the lysine methylation of NFκB p65 in response to tumour necrosis factor alpha (TNFα) in human kidney cells [49]. This involves an interaction between NFκB and HATs resulting in hyperacetylation of target genes including TNF-α and cyclooxygenase-2 promoters [45]. Hyperglycemia-induced oxidative stress and the formation of advanced glycation end products (AGEs) leads to the release of cytokines, cell adhesion molecules and extracellular matrix modifying genes that facilitate lymphocyte activation and invasion [50–53].

5. The role of histone-modifying enzymes in glycemic variability

Currently there is intensive research into the identification and characterisation of the enzymes that control and direct histone modifications [54,55], of which the methyltransferases are most specific. Lysine methyltransferases have enormous specificity compared to acetyltransferases, usually modifying one single lysine on the histone tail [17,56]. The existence of lysine demethylases remained contentious for many years following the discovery of histone methyltransferases (HMTase). The first of these discovered was lysine-specific demethylase 1 (LSD1), which acts to demethylate H3K4 and repress transcription [57]. This effectively dispelled the myth that histone methylation is a permanent mark [58] (Table 1).

To explore the effects of glycemic variability, we have specifically developed an *in vitro* model to determine gene-activating events that are associated with epigenetic modifications. Primary human aortic endothelial cells were incubated in high glucose (HG, 30 mM) for 16 h then returned to physiological levels (LG, 5 mM) for 6 days. Analyses revealed a persistent increase in expression of the NFκB subunit p65 gene, despite a return to normoglycemia [13]. Parallel experiments were performed in human microvascular endothelial cells, confirming a similar upregulation of p65 as well as proteins linked to ECM accumulation, such as fibronectin [13]. Thus, transient hyperglycemia is capable of inducing persistent gene-activating events associated with epigenetic change. Further studies revealed that this was as a result of histone modifications, specifically H3K4m,

Table 1
Histone-modifying enzymes related to diabetes and glycemic variability.

Enzyme	Target(s)	Effect on transcription	References	Function/family
Set7	H3K4	Activation	[49,54,59,66]	Histone methyltransferase
Suv39h1	H3K9	Repression	[67–69]	Histone methyltransferase
LSD1	H3K4, H3K9	Repression & Activation	[57,70–72]	Lysine demethylase
Jhdm2a	H3K9	Activation	[34,73–75]	Lysine demethylase
p300	Multiple	Activation	[76–78]	Histone acetyltransferase
CBP	Multiple	Activation	[76,79]	Histone acetyltransferase
PRMT5	H4R3, H3R8	Repression	[80,81]	Arginine methyltransferase

Set7: SET domain-containing protein 7, Suv39h1: suppressor of variegation 3–9 homologue 1, LSD1: lysine-specific demethylase 1, Jhdm2a: JmjC domain-containing histone demethylation protein 2a, p300: histone acetyltransferase p300, CBP: CREB-binding protein, PRMT5: protein arginine N-methyltransferase 5.

associated with activation of the p65 gene [13]. Furthermore, increased p65 gene expression was associated with NFκB activation as determined by gel shift analyses and upregulation of the NFκB-dependent chemokine, monocyte chemoattractant protein-1 (Mcp-1), implicated in diabetes-associated vascular injury [13,52].

In our laboratory we are interested in understanding the function of histone methyltransferases (HMTase) and we have recently demonstrated a central role for the HMTase enzyme, Set7, in promoting H3K4 methylation in endothelial cells, both from the macrovasculature and in primary aortic endothelial cells [13,14]. Set7 has also been shown to influence the recruitment of NFκB p65 to gene promoters and thereby its regulation of proinflammatory genes in macrophages from diabetic mice [49]. In order to assess whether Set7 is mobilised to maintain the active transcriptional state, immunopurified chromatin from microvascular endothelial cells exposed to acute hyperglycemia for 16 h was enriched with the Set7 methylase on the p65 promoter [14]. Furthermore, gene silencing of Set7 with small interfering RNA (siRNA) in monocytes significantly inhibited TNF-α induced inflammatory genes and H3K4 methylation on these promoters, as well as monocyte adhesion to endothelial or smooth muscle cells [49].

Further evidence of the involvement of Set7 in glucose-stimulated insulin secretion is that the Set7 gene promoter contains an islet-specific enhancer located 5–6 kb downstream of the transcriptional start site that exhibits pancreatic and duodenal homeobox 1 (Pdx1)-responsive activation in β-cells within the pancreas [59]. Interestingly, siRNA knockdown in insulinoma and mouse islets suppressed genes in glucose-stimulated insulin secretion, including insulin 1/2 (Ins1/2), glucose transporter 2 (Glut2) and v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA). These changes in expression were correlated with reduced H3K4m2 and RNA Polymerase II recruitment on these gene sequences. In fact, Set7 knockdown in primary mouse islets led to defects in glucose-stimulated Ca^{2+} mobilisation and insulin secretion [59].

Hyperglycemia was also associated with reduced localisation of the HMTase Suv39h1 to the p65 promoter and this was indirectly but tightly correlated with reduced H3K9 methylation on gene sequences [14], suggesting that the sustained increase in p65 gene expression is linked to specific epigenetic modifications that are typically associated with increased gene transcription. Furthermore, vascular smooth muscle cells isolated from diabetic mice have reduced levels of H3K9m3 and elevated levels of H3K4m2 at the promoters of inflammatory genes interleukin-6 (IL-6) and Mcp-1 in parallel with decreased levels of methyltransferase Suv39h1 and the histone demethylase LSD-1 [60,61]. Taken together, these studies suggest that hyperglycemia may induce epigenetic modifications on proinflammatory genes, which subsequently regulate gene expression and lead to the development of vascular inflammation. Fig. 1 outlines a proposed mechanism, by which acute hyperglycemia can lead to diabetic complications in specific vascular cells.

6. The current landscape and perspectives—where to next?

Investigators in our laboratory are interested in examining the dynamic state of epigenetic changes in response to environmental stimuli. Specifically, hyperglycemia and the persistence of epigenetic phenomena are a primary focus of some of the research currently investigated. In fact, many researchers are attempting to unravel the molecular determinants associated with recognizing the chromatin template and regulate the histone code. Understanding the exact changes in histone methylation, the enzymes responsible for these post-translational modifications and the direct *in vivo* characterisation of these changes in response to glucose in specific cell types is paramount. Future research will focus on the translation of established *in vitro* findings to an *in vivo* setting, such as the investigation of glucose on the chromatin template in specific vascular cell types, the transcriptional decisions that histone-modifying enzymes mediate and the location of such spatial modifications. The recent advent of massive parallel sequencing now allows investigators to examine in greater depth genome wide associations and unravel the locality of histone modifications. Coupled with the characterisation of changes in the transcriptome, this will allow us to identify gene targets for pharmaceutical or dietary intervention. It may also be possible that drugs which mediate epigenetic changes, such as HDAC inhibitors, will be used in the treatment of diabetic complications [62–64]. In support of this concept are the recent experimental findings indicating that HDAC inhibitors during myocardial infarction can reduce infarct area as well as programmed cell death [65]. Several drugs that currently target epigenetic changes in malignant cells might also be tested for their effects on atherosclerotic plaque formation. The role of epigenetics in the pathogenesis of cardiovascular diseases represents an essentially unexplored territory that may reveal new therapeutic possibilities.

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