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Glucose-independent persistence of PAI-1 gene expression and H3K4 tri-methylation in type 1 diabetic mouse endothelium: Implication in metabolic memory

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ABSTRACT

Clinical trials with type 1 and type 2 diabetes have identified a phenomenon known as "metabolic memory" in which previous periods of hyperglycemia result in the long-lasting deleterious impact on cardiovascular events. Emerging evidence shows that transient hyperglycemic exposure of human endothelial cells induces histone 3 lysine 4 mono-methylation (H3K4me1) on the promoter and persistent mRNA expression of RelA and IL-8 genes, suggesting that epigenetic histone modification and chromatin structure remodeling is a key event underlying metabolic memory. This burgeoning hypothesis, however, critically remains to be tested for relevance in the disease process of diabetes *in vivo*, and for broader applicability to an array of genes involved in endothelial dysfunction. To address this, we used type 1 diabetes mouse model induced by streptozocin to be hyperglycemic for 8 weeks, and isolated endothelial cells that were used either freshly after isolation or after 2 to 3-week cell culture in normoglycemic conditions. mRNA expression profiling in diabetic mouse endothelial cells revealed significant and persistent up-regulation of *Serpine1* encoding PAI-1, the hypo-fibrinolytic mediator leading to thrombotic diseases in diabetes, along with *Rock2*, *Fn1* and *Ccl2*, whereas only *Serpine 1* was persistently elevated in high glucose-treated mouse endothelial cells. Chromosome immunoprecipitation assay in type 1 diabetic mouse endothelial cells showed predominant enrichment of H3K4 tri-methylation on *Serpine1* promoter, suggesting a unique epigenetic regulation in diabetic mice as opposed to high glucose-treated human ECs. Our study demonstrates the importance of combining *in vivo* models of diabetes with high glucose-treated cell culture to better assess the epigenetic mechanisms relevant to disease.

1. Introduction

The DCCT-EDIC and UKPDS epidemiological studies have identified a phenomenon known as metabolic memory or the legacy effect in which early exposure to hyperglycemia predisposes type 1 and type 2 diabetes patients to the development of diabetic complications [1–5]. Hyperglycemia causes endothelial dysfunction, which is thought to mediate cardiovascular complications of diabetes such as myocardial infarction (MI) and stroke [6]. The underlying mechanism of metabolic memory, however, is just beginning to be understood [5].

Recent studies showed that exposure of cultured human and bovine endothelial cells (ECs) to transient hyperglycemia within a day elevates the expression of inflammatory mediators such as NF-κB p65 subunit, VCAM-1, MCP-1 and IL-8, which persist at least several days after re-exposure to normoglycemia [7–9]. Importantly, the sustained up-regulation of p65 subunit is associated with the enrichment of transcription-activating histone H3 lysine 4 mono-methylation (H3K4me1) mark at the gene promoter, suggesting a possible mechanism underlying metabolic memory whereby sustained p65 gene expression initiated by high glucose is mediated by epigenetic mechanisms involving histone modification [7,8]. Increased p65 expression, in turn, causes the persistent up-regulation of VCAM-1 and MCP-1. However, since these studies are based on data from cultured human ECs treated with transient high glucose, the relevance of this mechanism in the *in vivo* disease process of diabetes remains open to question. Furthermore, given

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the many players involved in endothelial dysfunction [6,10], it remains unclear whether such histone modifications occur commonly on other undocumented genes and are responsible for persistence, if any, of their expression in diabetic endothelium.

Diabetic patients show elevated levels of plasminogen activator inhibitor-1 (PAI-1) in plasma [11,12]. PAI-1, a member of the serine protease inhibitor family, is a key negative regulator of fibrinolysis by inhibiting plasminogen activators, and thought to play an important role in the pathogenesis of endothelial dysfunction and thrombosis leading to the incidence of MI and stroke in diabetes [13,14]. In the present study, we used type 1 diabetic mouse primary ECs that were used either freshly after isolation or after 2 to 3-week cell culture in normoglycemic conditions. mRNA expression profiling of a list of genes involved in endothelial dysfunction demonstrated marked and sustained up-regulation of a distinct set of genes that are different from the profile seen in high glucose-treated cells. *Serpine1*, encoding PAI-1, was most prominently elevated in type 1 diabetic ECs. Notably, *Serpine1* promoter showed predominant enrichment in H3K4me3 mark, suggesting a unique epigenetic regulation different from high glucose-treated human ECs.

2. Materials and methods

2.1. Mouse endothelial cells and high glucose challenge

Mouse primary endothelial cells (ECs) were isolated from the lung and the skeletal muscle (gastrocnemius) by an affinity selection method using Dynabeads sheep anti-rat IgG (Life Technologies, Grand Island, NY) and anti-PECAM1 antibody (BD Biosciences, San Jose, CA) as described [15]. The cells were cultivated in DMEM with low glucose (5.5 mmol/l) supplemented with 20% FCS, heparin (100 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and endothelial cell growth factor (ECGF, 50 μg/ml), and grown on dishes coated with 0.1% gelatin. After 8 days, the cells were trypsinized and underwent the second selection using Dynabeads plus anti-ICAM2 antibody (BD Biosciences). Mouse ECs were used for experiments at passage 3–5.

The purity of isolated mouse ECs was assessed through the fraction of cells positive for Alexa488-conjugated acetylated low-density lipoprotein (Ac-LDL) uptake after 4-h incubation in culture media. The cells were fixed with 10% normal buffered formalin for 10 min, followed by extensive wash with PBS and mounting with DAPI-containing media (ProLong Gold anti-fade reagent, Life Technologies). Images were obtained by fluorescence microscopy. The mouse ECs isolated by this method consistently yielded Ac-LDL positivity of 95–100%.

High glucose treatment of the cultured ECs was conducted by changing the glucose concentration in the above-mentioned mouse EC complete media, from 5.5 mmol/l to 25 mmol/l.

2.2. Gene expression assay

The mRNA expression in cells was quantified using quantitative reverse transcription PCR (qRT-PCR). Briefly, the cells were lysed and mRNA was purified with oligo dT using mRNA capture kit according to the supplier's protocol (Roche, Penzberg, Germany). The first-strand cDNA was synthesized using 100 units of ReverTra Ace (Toyobo, Osaka, Japan) in the presence of 22.5 ng/μl random primer (Life Technologies), 1 mM dNTPs and 2 units/μl recombinant RNase inhibitor (Takara, Shiga, Japan) in a 20 μl reaction at 30 °C, 10 min and 42 °C, 60 min. One fortieth of the RT reaction was used for qPCR, with the use of Fast SYBR Green Master Mix (Life Technologies) and 0.5 μM each of forward and reverse primers (see Supplementary Table 1). qPCR was conducted by Step One Plus Real-Time PCR System (Applied Biosystems). The measured value was normalized using an internal control value obtained for 36B4 gene.

2.3. Direct assessment of gene expression in freshly isolated mouse lung ECs

ECs were isolated from the mouse lung through the above-mentioned method, except that the cells captured by Dynabeads-antibody complex were immediately lysed for RNA extraction using the mRNA capture kit.

2.4. Type 1 diabetic mouse model

All animal experiments were approved by Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Approval identification number 0130157A). Type I diabetes was induced in male C57BL6/J mice (8–12 weeks, CLEA, Tokyo, Japan) via intraperitoneal administration of streptozocin (STZ, Sigma, St. Louis, MO) dissolved in 200 μl of 10 mM sodium citrate buffer, pH 4.0 (70 mg/kg × 3 days). The blood glucose level at *ad libitum* condition was assessed consecutively, and the mice whose blood glucose level exceeded 200 mg/dl at day 14 were used for experiments.

2.5. Chromosome immunoprecipitation (ChIP) assay

ChIP assay was performed using an assay kit (MAGnify ChIP Kit, Life Technologies), as described with a slight modification [16,17]. Briefly, the lysate was incubated with protein G-conjugated Dynabeads bound to antibodies for immunoprecipitation: anti-monomethyl-histone H3 (Lys4) (Ab8895, Abcam, Cambridge, MA), anti-dimethyl-histone H3 (Lys4) (ab7766, Abcam), anti-trimethyl-histone H3 (Lys4) (#07-473, Millipore, Billerica, MA), anti-acetyl-histone H3 (Lys9) (#07-352, Millipore) or normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The ChIP-enriched DNA samples were analyzed by qPCR. The qPCR primers for the promoter region (at −3000 to −1) of mouse *Serpine1* gene were designed so that the amplicons encompass the putative binding motifs of important transcription factors reported to regulate *Serpine1* gene transcription (see Supplementary Table 2). The C57BL/6J genome sequence for *Serpine1* gene upstream region (−9999 to −1) was retrieved from NCBI Nucleotide (NCBI Reference Sequence: NC_000071.6) and transcription factor motifs were deduced by TFSEARCH software (Real World Computing Partnership, Japan, http://www.cbrc.jp/research/db/TFSEARCH.html).

2.6. Statistical analysis

Unless otherwise noted, all statistical analysis was carried out by Student *t* test or ANOVA followed by Fisher's test. $P < 0.05$ was considered to be significant.

3. Results

3.1. Glucose-responsive induction of genes involved in endothelial dysfunction and its persistence under normoglycemia in primary mouse endothelial cells

To begin to profile mouse endothelial gene expression in diabetic conditions, we isolated primary ECs from the wild-type mouse lung by an affinity selection method for CD31 and ICAM2 double-positivity (Fig. 1), and cultured for 2–3 weeks with media containing low glucose (5.5 mmol/l). Assessment of the cells for the efficiency in dye-labeled acetylated LDL uptake reproducibly

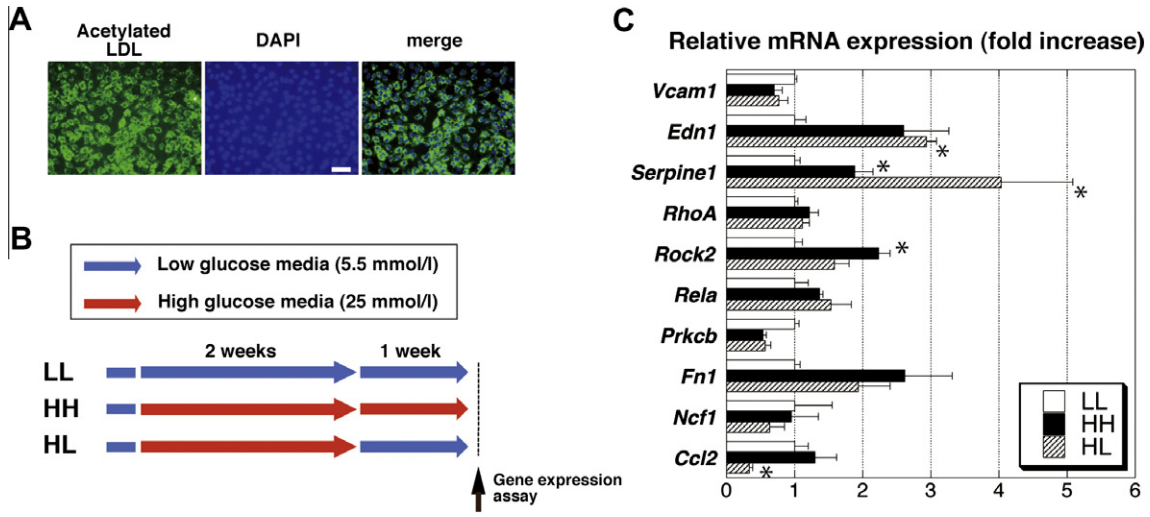

Fig. 1. High glucose-induction and glucose-independent persistence of mouse endothelial cell gene expression related to endothelial dysfunction. (A) Mouse endothelial cell isolation. The purity of isolated endothelial cells was assessed by acetylated LDL uptake and subsequent immunofluorescence. Scale bar, 50 μm. (B) Experimental scheme. (C) The mRNA expression of selected genes involved in endothelial dysfunction was assessed in mouse lung endothelial cells treated according to the scheme in (b). Data are means ± SEM of triplicates. $^{*}P < 0.05$.

showed greater than 95% purity of the isolated ECs (Fig. 1A). The ECs were then divided into 3 groups: (i) 2-week culture under a low glucose condition (5.5 mmol/l) and subsequent 1-week culture with low glucose media (LL); (ii) 2-week high glucose culture (25 mmol/l) followed by 1-week high glucose treatment (HH); (iii) 2-week high glucose culture followed by 1-week low glucose treatment (HL) (Fig. 1B).

We selected a panel of genes that are known to be involved in endothelial dysfunction, activation or inflammation in diabetic or non-diabetic states [6,10,18–20]; *Vcam1* encoding VCAM-1, *Edn1* (endothelin 1), *Serpine1* (PAI-1), *Rhoa* (RhoA), *Rock2* (Rho-associated kinase 2), *Rela* (p65 subunit of NF-κB), *Prkcb* (protein kinase C), *Fn1* (*fibronectin*), *Ncf1* (p47phox), and *Ccl2* (monocyte chemoattractant protein-1, MCP-1). We then compared the mRNA expression of these genes between LL, HH and HL groups of mouse ECs (Fig. 1C). Comparison between LL and HH groups showed that only *Serpine1* and *Rock2* mRNA expression significantly increased in a glucose-sensitive manner, while *Edn1* and *Fn1* also tended to be up-regulated. Comparison between LL and HL showed that exposure of the cells to hyperglycemia and subsequent re-exposure to normoglycemia caused significant up-regulation of *Serpine1* and *Edn1*. These data indicate that, in mouse ECs, *Serpine1* represents a most prominent gene with glucose-inducibility in mRNA abundance that persists after re-exposure to normoglycemia.

3.2. Type 1 diabetic mice exhibit robust induction of Serpine1 expression in endothelium that persists under normoglycemic condition in cell culture

In order to address the impact of the disease process of diabetes on mouse EC gene expression *in vivo*, we used the established conventional model of type 1 diabetes through injection of streptozocin (STZ). The mice with STZ administration immediately developed overt hyperglycemia within 1 week of injection that progressively persisted at least 8 weeks (Fig. 2A, STZ, 577.1 ± 21.4; Control, 124.4 ± 5.5 mg/dl). After the STZ mice were left hyperglycemic for 8 weeks, ECs were affinity-purified from the lung or the gastrocnemius muscle (Fig. 2B). Part of the isolated cells was immediately lysed for mRNA expression analysis using the same panel of genes for Fig. 1C (Fig. 2C), while the remainder was used for 2 to 3-week cell culture under normoglycemia and subsequent mRNA expression assay (Fig. 2D and E).mRNA expression profiling of the lung ECs freshly isolated from the STZ and control mice showed significant up-regulation of *Vcam1*, *Serpine1*, *Rock2*, *Rela*, *Fn1*, *Ncf1* and *Ccl2* (Fig. 2C). It is noteworthy that the genes induced under this condition, except for *Serpine1* and *Rock2*, were not increased by high glucose challenge (Fig. 1C), likely reflecting a difference in the effects of hyperglycemia in cell culture versus type 1 diabetes *in vivo*. STZ mouse-derived lung ECs, after prolonged cell culture under low glucose condition, showed similar but distinct mRNA expression profile compared to the freshly isolated cells from STZ mice (Fig. 2D), which showed significant up-regulation of *Serpine1*, *Rhoa*, *Rock2*, *Fn1* and *Ccl2*, suggesting that the expression of these genes except *Rhoa* is induced by type 1 diabetes and sustained under normoglycemic condition. The mRNA expression of the STZ mouse skeletal muscle-derived ECs that were exposed to normoglycemia showed a similar but distinct profile than the similarly treated lung ECs; STZ muscle ECs showed significant mRNA induction in *Vcam1*, *Edn1*, *Serpine1* and *Fn1*, with most prominent induction by as high as 8 folds seen in *Serpine 1* (Fig. 2E). These results show unique but overlapping response in gene transcription between different vascular bed-derived ECs in type 1 diabetes, with *Serpine 1* commonly upregulated between muscle and lung ECs. The data also suggest that *Serpine1*, along with *Rock2*, *Fn1* and *Ccl2*, is the key gene induced in diabetic endothelium with sustained elevation independent of extracellular glucose level, and thus presage involvement of epigenetic mechanisms in the expression regulation of these genes.

3.3. Type 1 diabetes causes sustained elevation of histone 3 lysine 4 trimethylation associated with Serpine1 promoter in mouse endothelial cells

Emerging evidence implicates histone 3 lysine tail modification as the crucial mechanisms for persistent gene up-regulation in ECs exposed to hyperglycemia [5,21,22]. Because *Serpine1* commonly showed robust and persistent up-regulation in both lung and muscle ECs in type 1 diabetes, we examined the status of enrichment of *Serpine 1* promoter with histone 3 lysine 4 (H3K4) mono-methylation (me1), di-methylation (me2), tri-methylation (me3) and H3K9 acetylation (H3K9Ac), all of which are transcription-activating histone marks (Fig. 3). Few studies have documented the structural

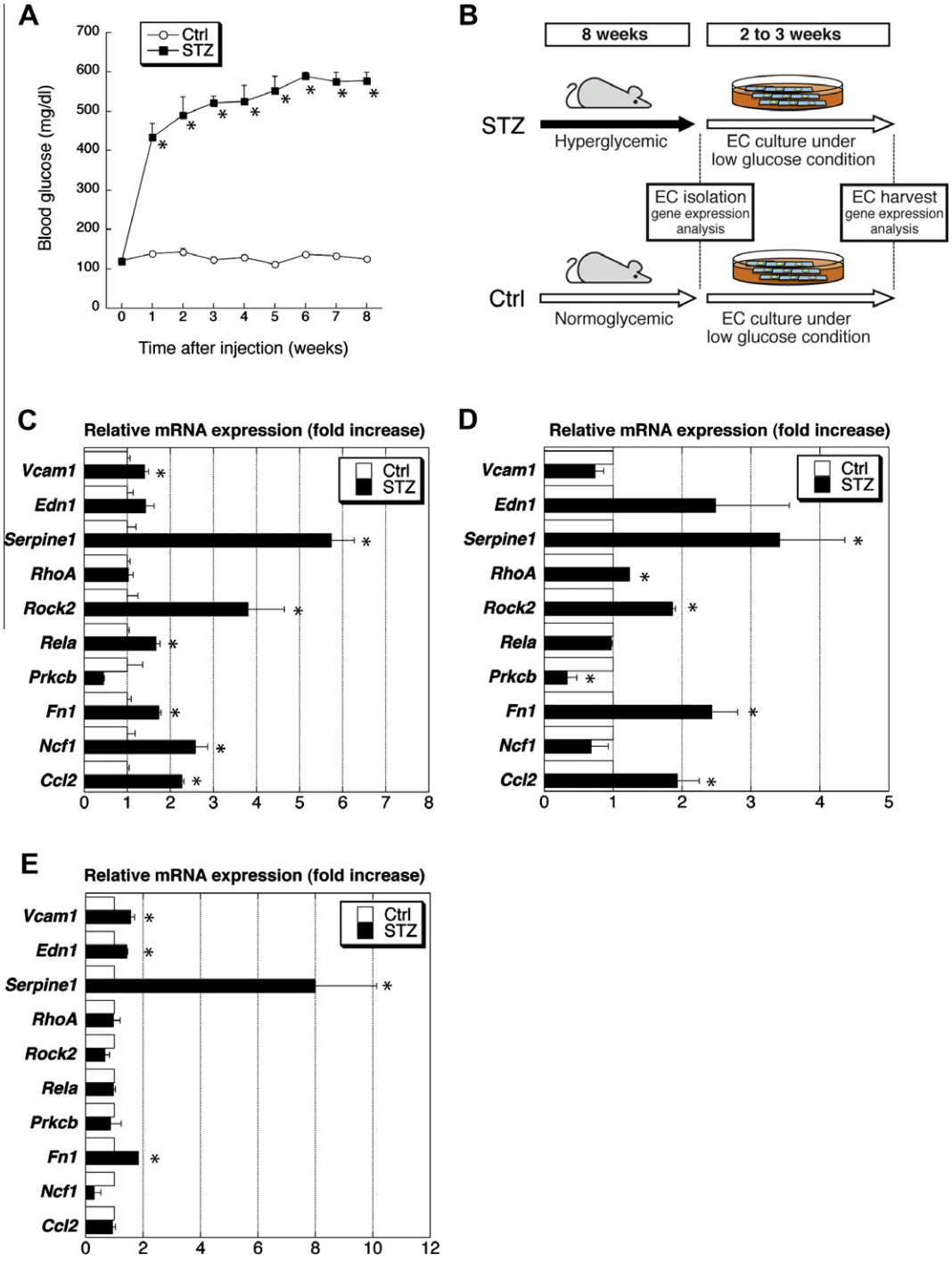

Fig. 2. Augmented gene expression in type 1 diabetic mouse endothelium is partly sustained after prolonged cell culture under low glucose condition. (A) Temporal profile of blood glucose level in control (ctrl) and streptozocin (STZ)-treated mice. (B) Experimental scheme. Mice were euthanized at 8 weeks following injection of STZ or vehicle. Endothelial cells were isolated from the mouse lung and the skeletal muscle, and used for immediate gene expression analysis or 2 to 3-week cell culture under low glucose condition. (C) The mRNA expression of endothelial cells freshly isolated from the control or STZ mouse lung. (D, E) The mRNA expression of mouse lung (D) and muscle (E) endothelial cells after cell culture in low glucose-containing media. Data are means ± SEM ($N = 3–8$). $^*P < 0.05$.

and functional properties of 5′-flanking regulatory region of murine *Serpine1*. Profiling of the nucleotide sequence between −3000 and −1 bp relative to the transcription start site revealed three clusters of transcription factor binding sites; two Sp1 and one NF-κB binding motifs between −2236 and −2144 bp; two C/EBP and one AP1 motifs in −888 and −779; and one C/EBP and one Sp1 motifs in −201 and −138. These transcription factors have been previously implicated in the transactivation of human and mouse *Serpine1* [23–25]. We then designed qPCR primer sets whose amplicons encompass the respective clusters (Fig. 3A). Chromosome immunoprecipitation (ChIP) analysis in the STZ versus control mouse lung ECs with exposure to normoglycemia (Fig. 2D) revealed broad enrichment of H3K4me1, me3, H3K9Ac, but not me2, associated with the three clusters, by as much as five (me3) to ten (K9Ac) folds, in STZ ECs (Fig. 3B). ChIP analysis, in turn, with the mouse muscle ECs with normoglycemic exposure (Fig. 2E) showed increased association in STZ ECs of me1, me2, me3, but not K9Ac, with the three regions, by as much as 6 folds

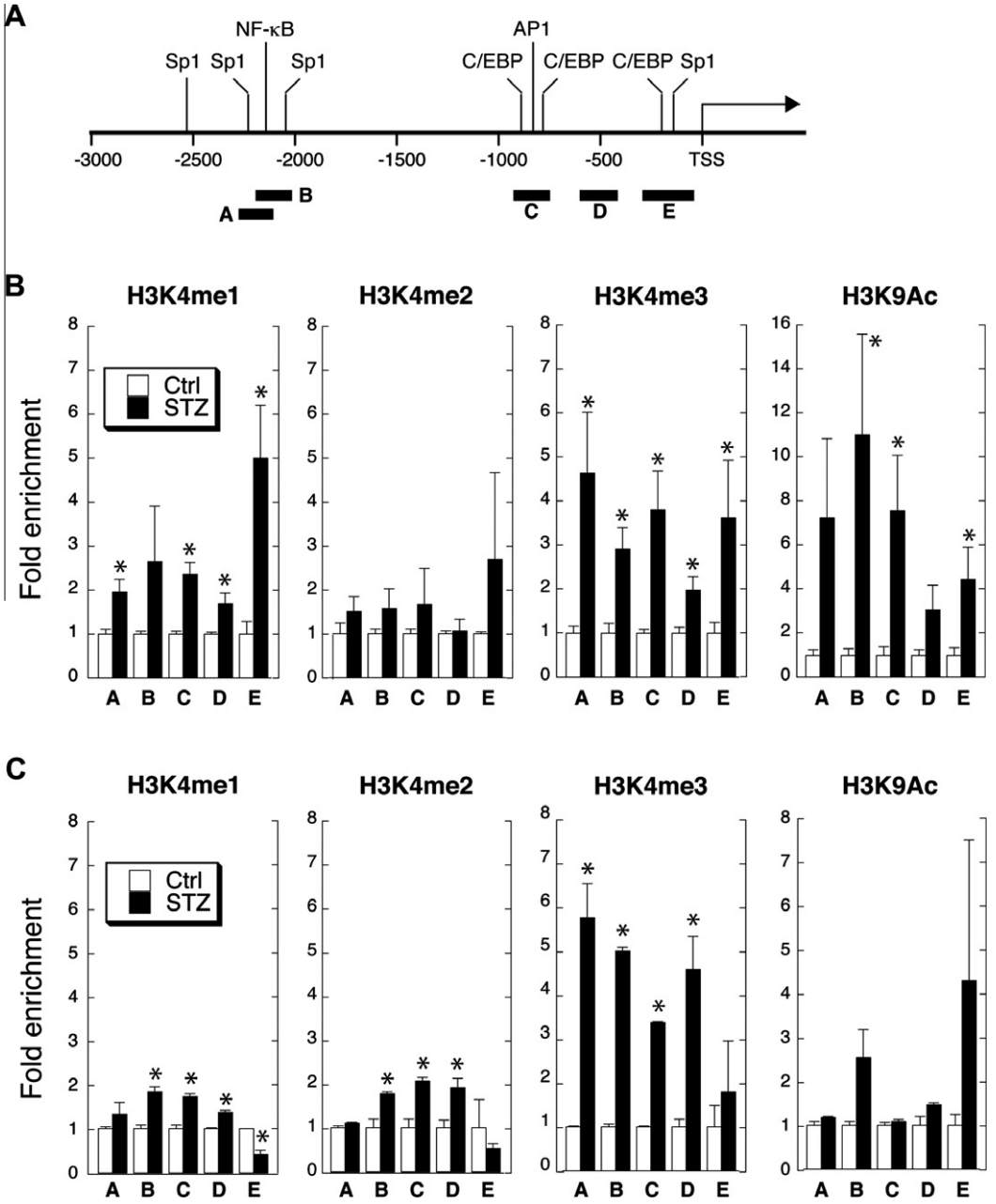

Fig. 3. Prominent enrichment of *Serpine1* gene promoter with H3K4 tri-methylation in type 1 diabetic mouse endothelium after prolonged cell culture under low glucose. (A) Schematic representation of the mouse *Serpine1* gene promoter. Putative transcription factor binding sites are shown. Solid bars, A through D, depict the location and size of the PCR fragments used for ChIP assay in (B) and (C). A, −2274 to −2106; B, −2192 to −2010; C, −922 to −748; D, −601 to −420; E, −288 to −39. TSS, transcription start site. (B, C) ChIP analysis comparing the indicated H3 modification levels in the regions A through D between STZ and control mouse-derived lung (B) and skeletal muscle (C) endothelial cells. Data are means ± SEM of triplicates. $^{*}P < 0.05$.

(Fig. 3C). The data show that increase in H3K4me3 and me1, with me3 being predominant, is the common epigenetic signature on *Serpine1* promoter in type 1 diabetic mouse lung and muscle ECs, which both exhibit persistent expression of *Serpine1* mRNA.

4. Discussion

The present study used primary mouse ECs, a panel of genes reported to mediate endothelial dysfunction, and two distinct models of diabetes: high glucose loading in cell culture, and STZ-induced type 1 diabetes *in vivo*. Using these tools, we sought to explore diabetes-inducible genes in mouse endothelium *in vivo* that are sustained after glucose normalization. Results from DCCT and UKPDS clinical trials unmasked the long-lasting injurious stresses to diabetic vessels as a result of previous hyperglycemia, which gave rise to the concept of metabolic memory [1–5]. The underlying mechanism for metabolic memory is just beginning to be understood, and experimental evidence has been accumulating to suggest the involvement of epigenetic histone 3 lysine tail modification [5,21,22]. Seminal works from El-Osta lab have recently unveiled that transient, 16-h exposure of human aortic and microvascular ECs to hyperglycemia is sufficient to induce sustained mRNA expression of *Rela*, which in turn mediates persistent up-regulation of *Vcam1* and *Ccl2*, under normal glucose [7–9]. Importantly, they demonstrated that H3K4me1 is the predominant

histone mark associated with human *Rela* promoter that is increased in hyperglycemia by Set7 lysine methyltransferase and thereby determines activation and persistence of NF-κB activity, while me2 and me3 level is not altered.

The present study provided data that contrast with El-Osta group's findings in several points. First, neither high glucose-treated cells (Fig. 1C) nor STZ-mouse ECs cultured under normoglycemia (Fig. 2D and E) showed any significant increase in *Rela* expression while both cells indeed showed persistent up-regulation of NF-κB-downstream genes including *Serpine1*, *Vcam1* and *Ccl2*, suggesting that increase in p65 subunit of NF-κB is not requisite for persistent activation of inflammatory gene expression in diabetic mouse ECs. Instead, our assessment of histone modification on *Serpine1* promoter demonstrated that individual downstream genes like *Serpine1*, independent of the status of NF-κB subunit *Rela* expression, are able to undergo direct epigenetic regulation through H3 lysine modification in diabetes. This is in line with a recent report showing that *IL8*, a NF-κB target gene, is induced in human ECs by hyperglycemia via Set7-mediated direct increase in H3K4me1 level [9]. Given the prominent *Serpine1* mRNA up-regulation by 3–8 folds in STZ ECs (Fig. 2D and E), we examined the enrichment of the three transcription factor motif clusters on *Serpine1* promoter with H3 modification (Fig. 3B and C); all regions showed profound enrichment with transcription-activating H3K4me3 by as much as 5 folds. The robust magnitude of induction of *Serpine1* mRNA and H3 modification can be emphasized by comparison to that of *Rela* in high glucose-treated human ECs, which ranges around 2–3 folds [7,8]. Another conflicting data between El-Osta's and ours is that the predominant histone mark associated with *Serpine1* promoter was H3K4me3, but not me1, in STZ mouse lung and muscle-derived ECs showing persistent activation of *Serpine1* gene expression, while weak augmentation of H3K4me1 was also seen. A plausible explanation for this discrepancy is that, as suggested by a recent study [9], different genes may use distinct epigenetic mechanisms in which, for example, human *Rela* and *IL8* use me1 while mouse *Serpine1* use me3. Further clues would be obtained by investigating the H3K4 methylation status on *Serpine1* promoter using the ECs freshly isolated from STZ mice (Fig. 2C).

The discrepancy between our data and El-Osta's may be caused by many factors, but most likely because of the difference in models used. They mostly used transient hyperglycemic challenge, while we used STZ-induced type1 diabetes model with sustained hyperglycemia up to 8 weeks. Comparison of Figs. 1C and 2D clearly show that only part of the genes persistently induced in STZ ECs were increased by high glucose treatment, suggesting that epigenetic changes in *in vivo* diabetic conditions are likely the combined effects of hyperglycemia and associated secondary factors such as inflammation. Since clinical trials show previous uncontrolled hyperglycemia represented by high HbA1c level is the key determinant underlying metabolic memory [1–5], recent knowledge has been mostly obtained through transient high glucose challenge to cells and animals. However, there would be no debate in the importance of *in vivo* animal diabetes models in providing pathophysiologically relevant clues. Thus, our current finding that H3K4me3, and not me1, is the predominant histone mark that accounts for persistent gene expression in type 1 diabetes is of much relevance to human diseases. Interestingly, although the relative contribution between H3K4me1, me2, me3 for chromosomal remodeling and transcription-activating effects remains unclear, a recent paper is in favor of our hypothesis and argues for the existence of functional links between polymerase II occupancy, H3K4me3 enrichment, and enhancer activity in T cell development [26]. Therefore, our data throws out a caveat that the findings obtained from cultured cells treated with transient hyperglycemia requires caution in interpretation, and needs to be combined with the data from animal models of diabetes or diabetic patient-derived specimens to better assess pertinent epigenetic changes.

In conclusion, our study has provided important insights into understanding the epigenetic mechanisms of diabetic metabolic memory in vasculature. Our data suggest that, in animal models of diabetes, important NF-κB target genes including *Serpine1* can be direct targets of H3 modification independent of epigenetic induction of *Rela*. The elevated plasma level of PAI-1, the key fibrinolysis regulator encoded by *Serpine1*, is seen in diabetic subjects and thought to contribute to the increased incidence of myocardial infarction and stroke [11–14]. Our finding that type 1 diabetes causes in mouse ECs marked increase and powerful persistence of *Serpine1* expression, through broad drastic induction of H3K4me3 mark, provides an insight into mechanisms that predispose patients with previous hypergycemia to myocardial infarction and stroke after long euglycemic periods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.064.

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