Adverse Epigenetic Signatures by Histone Methyltransferase Set7 Contribute to Vascular Dysfunction in Patients With Type 2 Diabetes Mellitus

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Background—Cellular studies showed that histone methyltransferase Set7 mediates high glucose–induced inflammation via epigenetic regulation of the transcription factor NF-kB. However, the link between Set7 and vascular dysfunction in patients with diabetes mellitus remains unknown. This study was designed to investigate whether Set7 contributes to vascular dysfunction in patients with type 2 diabetes mellitus (T2DM).

Methods and Results—Set7-driven epigenetic changes on NF-kB p65 promoter and expression of NF-kB-dependent genes, cyclooxygenase 2 and inducible endothelial nitric oxide synthase, were assessed in peripheral blood mononuclear cells isolated from 68 subjects (44 patients with T2DM and 24 age-matched controls). Brachial artery flow–mediated dilation, 24-hour urinary levels of 8-isoprostaglandin F2α, and plasma adhesion molecules, intercellular cell adhesion molecule-1 and monocyte chemoattractant protein-1, were also determined. Experiments in human aortic endothelial cells exposed to high glucose were performed to elucidate the mechanisms of Set7-driven inflammation and oxidative stress. Set7 expression increased in peripheral blood mononuclear cells from patients with T2DM when compared with controls. Patients with T2DM showed Set7-dependent monomethylation of lysine 4 of histone 3 on NF-kB p65 promoter. This epigenetic signature was associated with upregulation of NF-kB, subsequent transcription of oxidant/inflammatory genes, and increased plasma levels of intercellular cell adhesion molecule-1 and monocyte chemoattractant protein-1. Interestingly, we found that Set7 expression significantly correlated with oxidative marker 8-isoprostaglandin F2α (r=0.38; P=0.01) and flow-mediated dilation (r=−0.34; P=0.04). In human aortic endothelial cells, silencing of Set7 prevented monomethylation of lysine 4 of histone 3 and abolished NF-kB–dependent oxidant and inflammatory signaling.

Conclusions—Set7-induced epigenetic changes contribute to vascular dysfunction in patients with T2DM. Targeting this chromatin-modifying enzyme may represent a novel therapeutic approach to prevent atherosclerotic vascular disease in this setting. (Circ Cardiovasc Genet. 2015;8:150-158. DOI: 10.1161/CIRCGENETICS.114.000671.)

Key Words: diabetes mellitus ■ epigenomics ■ inflammation ■ oxidative stress

Type 2 diabetes mellitus (T2DM) is associated with increased risk of micro and macrovascular complications and 2-fold greater risk of mortality when compared with the general population.1,2 Advances in therapy have reduced morbidity and mortality in patients with T2DM. However, cardiovascular risk is far to be eradicated, and mechanism-based therapeutic approaches are needed.3,4 In patients with diabetes mellitus, high-glucose levels trigger endothelial inflammation, mitochondrial oxidative stress, and reduced availability of nitric oxide, a key effector of vascular health.3,5 This chain of events favors the development of coronary atherosclerotic lesions, as well as microvascular disease.6 Although the link between diabetes mellitus and atherosclerosis is well established, a better comprehension of the underlying mechanisms is of utmost importance to identify novel molecular targets. Epigenetic modifications are emerging as key players in cardiovascular disease.6 Acetylation and methylation occurring at DNA/histone complexes significantly alter gene transcription

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by modulating chromatin accessibility.\textsuperscript{7} A growing body of evidence suggests that the mammalian methyltransferase Set7, involved in methylation of histones, may represent an important mechanism of vascular damage under hyperglycemic conditions.\textsuperscript{8−11} In bovine and human endothelial cells exposed to high glucose, Set7 induces monomethylation of lysine 4 of histone 3 (H3K4m1) on the promoter of the RelA gene encoding for the transcription factor NF-κB p65. This epigenetic modification by Set7 favors NF-kB p65 expression and overexpression of inflammatory adhesion molecules.\textsuperscript{8,9} Interestingly, suppression of Set7-dependent epigenetic changes prevented hyperglycemia-induced inflammation.\textsuperscript{9} Despite these data provided important mechanistic insights, the role of Set7 in patients with diabetes mellitus remains unknown. This study was designed to investigate the link between Set7-induced chromatin changes and vascular phenotype in patients with T2DM. Our findings demonstrate that a specific epigenetic signature induced by Set7 regulates NF-kB p65 expression and, hence, contributes to dysregulation of oxidant/inflammatory genes and endothelial dysfunction. These results suggest that targeting Set7 may represent a promising approach to reduce oxidative and inflammatory burden in patients with T2DM.

**Methods**

**Study Population**

A total of 68 subjects (44 patients with T2DM and 24 age-matched healthy controls) between 30 and 70 years of age were consecutively recruited at the Cardiology Units of Santi’Andrea Hospital, Sapienza University, and Catholic University (Rome, Italy). Patients with diabetes mellitus were free from overt cardiovascular disease. Diagnosis of diabetes mellitus was made according to European Society of Cardiology/European Association for the Study of Diabetes recommendations.\textsuperscript{12} On the day of the study, subjects were on a fasting state, refrained from caffeine containing beverages, and avoided exercise for ≥12 hours before the experiment. All participants underwent a full medical history and physical examination. The study protocol was approved by local Ethics Committee, and in accordance with institutional guidelines, all participants were aware of the investigational nature of the study and gave written consent for their participation.

**Isolation of Peripheral Blood Mononuclear Cells**

Blood was collected in Ficoll tubes (Vacutainer CPT, BD Diagnostics) and centrifuged for 20 minutes at 1800 g and room temperature. The turbid white layer above the Ficoll containing the mononuclear blood cells was transferred to a clean tube and washed twice with PBS. Subsequently, monocytes were isolated using magnetic CD14-coated beads and magnetic-activated cell sorting (Miltenyi Biotec).\textsuperscript{13}

**Real-Time Polymerase Chain Reaction**

Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamers (Amersham Bioscience, Piscataway, NJ) in a final volume of 33 µL using 1 µg of cDNA according to the manufacturer's recommendations. Real-time polymerase chain reaction (PCR) amplification was performed in an MX3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma Aldrich, St Louis, MO) in 25 µL of final reaction volume containing 2 µL cDNA, 10 pmol of each primer, 0.25 µL of internal reference dye, and 12.5 µL of JumpStart Taq ReadyMix (buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). TATA binding protein was used as an endogenous control for normalizing RNA concentration. The amplification program consisted of 1 cycle at 95°C for 10 minutes, followed by 40 cycles with a denaturing phase at 95°C for 30 s, an annealing phase at 60°C for 30 s, and an elongation phase at 72°C for 30 s. Differences in C\textsubscript{t} values between test gene and endogenous control (TATA binding protein, ΔC\textsubscript{t}) were calculated and used for statistical analysis.

**Western Blotting**

Human aortic endothelial cells (HAECs) and peripheral blood mononuclear cells (PBMCs) were lysed for immunoblotting (150 mmol/L sodium chloride, 50 mmol/L Tris, 1 mmol/L sodium fluoride, 1 mmol/L DTT, 1 mmol/L EDTA, 10 µg/mL leupeptin, 10 µg/mL aprotonin, 0.1 mmol/L sodium vanadate, 1 mmol/L PMSF, and 0.5% NP-40). Equal amounts (20 µg) of samples were subjected to SDS-PAGE gel for electrophoresis followed by semidry transfer onto Immobilon-P filter papers (Millipore, Billerica, MA). The membranes were blocked with 5% bovine serum albumin in PBS–TWEEN buffer (1% Tween 20, pH 7.5) for 60 minutes and incubated with anti-Set7 antibody (Cell Signaling Technology). Antirabbit secondary antibody was bought from GE Healthcare (Buckinghamshire, United Kingdom). The immunoreactive bands were detected by an enhanced chemiluminescence system (Millipore, Billerica, MA). Related signals were quantified using Scion image software (Scion Corp, Frederick, MD).

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation (ChIP) assay was performed by using a Magna ChiP Assay Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. HAECs and PBMCs were fixed for 10 minutes with 37% formaldehyde. After stopping cross-linking by addition of 0.1 mol/L of glycine, cells were sonicated and centrifuged. ChIP was performed by using 10 µg of anti-H3K4m1 antibody (Millipore, Billerica, MA) and equivalent amount of mouse IgG as a negative control. Washing and elution of immunoprecipitated DNA were performed according to the Magna ChiP protocol (Millipore, Billerica, MA). ChiP quantifications to human NF-kB p65 promoter were performed by Real-Time PCR. Specifically, human NF-kB p65 promoter was divided into 7 regions (region 1: −1381 to −1201; region 2: −1200 to −1021; region 3: −1022 to −841; region 4: −840 to −661; region 5: −660 to −441; region 6: −480 to −241; and region 7: −240 to −1), and real-time PCR reactions were performed for each region by using specific primers synthesized by Microsynth. Quantifications were performed using the comparative cycle threshold method and are reported as the n fold difference in antibody-bound chromatin against the input DNA.

**NF-kB p65 Binding Activity**

The DNA binding reaction was carried out with 10 µg of nuclear protein in a 96-well plate coated with consensus sequences for NF-kB (GGGACTTTCC) for 1 hour at room temperature. After washing, NF-kB p65 antibody (active motif) was added and incubated for 1 hour, followed by incubation with a horseradish peroxidase-conjugated secondary antibody.

**Assessment of Urinary 8-Isoprostaglandin PGF\textsubscript{α} \textsubscript{α} Levels**

Urinary levels of 8-isoprostaglandin F\textsubscript{α} \textsubscript{α} were assessed by using a commercially available kit (Cell Biolabs, San Diego, CA). Purification and extraction of urine samples were performed before assay. The pH of urine (1 mL) was adjusted to 2.2 with hydrochloride, diluted in water to 10 mL, and added to 3H-8-iso PGF\textsubscript{α} \textsubscript{α} as an internal standard. Isoprostane concentrations are expressed as picograms per milligram of urinary creatinine.

**Plasma Levels of Intercellular Cell Adhesion Molecule-1 and Monocyte Chemotactic Protein-1**

Plasma levels of adhesion molecules, intercellular cell adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1
(MCP-1), were determined by using commercially available kits (Abcam, Cambridge, MA).

**Vascular Function**

Vascular function was assessed in the morning at rest with subjects in the supine position from 0:15 minutes, in a quiet air-conditioned room (22–24°C), to minimize the possible negative effect of environmental and physiological influences, including stress. Endothelial-dependent vasodilation was assessed as dilation of the brachial artery in response to increased blood flow in accordance with current guidelines, as previously described.14 The examinations were carried out by 2 sonographers (L.C. and G.S.) by using an echo-Doppler machine (Esaote, Philips, General Electric, and Siemens). The right brachial artery was located and scanned longitudinally between 5 and 10 cm above the elbow using a linear array transducer with a frequency ranging from 7.5 to 10 MHz. The transducer was held in the same position throughout the scan by an adjustable stereotactic clamp to ensure greater image stability. A sphygmomanometer blood pressure cuff was positioned on the right forearm 2 cm below the elbow. Right brachial artery was scanned longitudinally between 5 and 10 cm above the elbow, capturing images starting 1 minute before cuff inflation. The cuff was inflated for 5 minutes at 250 mm Hg and then deflated to induce reactive hyperemia. Endothelium-independent vasodilation was elicited by the administration of low dose (25 µg) of sublingual glyceryl-trinitrate. Recording time frames were 10 minutes for flow-mediated dilation studies (1 minute for baseline, 5 minutes of ischemic period, and 4 minutes for assessing changes in diameter after reactive hyperemia) and 6 minutes for glycerol-trinitrate-mediated dilation (1 minute for baseline and 5 minutes for assessing changes in diameter after glycerol-trinitrate administration).

**Cell Culture**

Primary HAECs purchased from Clonetics® were obtained from healthy donors (men; age, 51±2 years; n=3), free from overt cardiovascular disease and other relevant comorbidities. HAECs (passages 5–7) were grown in fibronectin-coated 75 cm² flasks in optimized endothelial growth medium-2 supplemented with 10% fetal bovine serum. The cells were detached by using trypsin/EDTA for 2 minutes and reseded in fibronectin-coated 10 cm-cell culture dishes or 6 multiwell plates. HAECs were cultured in endothelial growth medium-2 containing 2% fetal bovine serum and exposed for 3 days either to normal-glucose (5 mmol/L) or to high-glucose concentration (25 mmol/L). Mannitol was purchased from Clontech and used at the final concentration of 25 mmol/L as an osmotic control.

**Small Interfering RNA Transfection**

Transient transfection with commercially available human Set7 siRNAs (Santa Cruz Biotechnology) was performed using Lipofectamine Reagent (Invitrogen). As a control, predesigned scrambled siRNA was used. Cytotoxicity was assessed by a colorimetric assay to detect lactate dehydrogenase (Roche).

**Statistical Analysis**

The normality of continuous variables was assessed by the Kolmogorov–Smirnov test. All normally distributed variables are expressed as mean (SD), unless otherwise stated. Data not normally distributed are expressed as median (interquartile range). Comparisons between 2 groups were performed with Student t test or Mann–Whitney test, where appropriate. For multiple comparisons, 1-way ANOVA was used, followed by post hoc testing (Bonferroni correction). Correlations between variables were assessed by Spearman analysis in patients with T2DM. Linear regression analysis (Enter method), adjusted for age, sex, waist circumference, and fasting plasma glucose (FPG), was constructed to determine whether Set7 expression in PBM was independently associated with changes in endothelial function (flow-mediated dilation). For univariate and multivariate analyses, non-Gaussian variables were normalized by log transformation. All analyses were performed with GraphPad Prism (version 5.0) and SPSS (version 20) softwares.

**Results**

**Methyltransferase Set7 Is Upregulated in T2DM**

Set7 expression was assessed in PBM isolated from 44 patients with T2DM and 24 age-matched healthy controls. Clinical characteristics of the study population are delineated in Table. Overall, we found no significant differences in age, sex, blood pressure, total cholesterol, and high-density lipoprotein cholesterol between the 2 groups (Table). However, diabetic patients had higher waist circumference, body mass index, FPG, and glycated hemoglobin (Table). We found that gene and protein expression of methyltransferase Set7 is significantly increased in patients with T2DM when compared with controls (Figure 1A and 1B).

Of interest, FPG and glycated hemoglobin showed significant correlations with Set7 expression in patients with T2DM, suggesting a link between hyperglycemia and upregulation of Set7 in humans (Table I in the Data Supplement).

**Set7-Induced Epigenetic Changes on Human NF-kB p65 Promoter**

Set7 regulates NF-kB transcription by specific H3K4m1.8,9 Whether this epigenetic signature can be found in patients with diabetes mellitus remains unknown. To this aim, PBM-derived chromatin from patients with T2DM and controls was immunoprecipitated with a specific antibody against H3K4m1. We found that histone monomethylation of the NF-kB promoter region (−480/−240) is increased only in patients with T2DM (Figure 2A). In line with this finding, NF-kB p65 expression and binding activity were significantly higher in this group (Figure 2B) and positively correlated with Set7 gene expression, indicating that Set7 may regulate NF-kB signaling in diabetic individuals (Figure 2C).

**Set7 Correlates With NF-kB-Dependent Genes**

NF-kB is required for the transcription of oxidant and inflammatory genes involved in diabetic atherosclerotic disease.5 Here, we tested the hypothesis that Set7 may orchestrate oxidative stress and inflammation via epigenetic regulation of NF-kB in diabetic patients. Expression of NF-kB-dependent genes, such as cyclooxygenase 2 (COX-2), inducible endothelial nitric oxide synthase (iNOS), and adhesion molecules, ICAM-1 and MCP-1, were increased in patients with T2DM when compared with controls (Figure 1 in the Data Supplement). Interestingly enough, Set7 expression significantly correlated with NF-kB downstream genes (Figure 3A and 3B). Moreover, we further investigated whether Set7 may directly regulate transcription of oxidant/inflammatory genes by inducing H3K4m1 signature on their promoter. Real-time PCR performed on H3K4m1-ChIP extracts did not reveal Set7-dependent signatures on the promoter of ICAM-1, MCP-1, iNOS, and COX-2 (data not shown). Collectively, this set of findings suggests that in patients with T2DM, Set7 drives inflammation and oxidative stress indirectly via epigenetic regulation of NF-kB p65.
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We then investigated whether Set7 levels may represent a marker of oxidative stress and endothelial dysfunction, key early steps of the atherosclerotic process. Diabetic patients showed higher urinary excretion rates of free 8-isoprostaglandin F$_2$α and impaired endothelial function when compared with controls (Figure IIA and IIB in the Data Supplement). Nitroglycerine-mediated dilation was similar in the 2 groups (Figure 2B), and no differences were observed in arterial diameter, as well as resting or hyperemic flow (data not shown). We found that Set7 expression significantly correlates with oxidative stress and endothelial dysfunction (Figure 4A and 4B). Linear regression models adjusted for age, sex, body mass index, and FPG showed that Set7 expression was independently associated with log flow-mediated dilation% ($\beta$=−0.40; $P$=0.04).

Targeting Methyltransferase Set7 Blunts NF-κB-Driven Inflammation in Human Endothelial Cells

To further elucidate the relevance of Set7-driven epigenetic signature on hyperglycemia-induced vascular dysfunction, silencing of Set7 was performed in human endothelial cells (HAECs) exposed to high-glucose concentrations. Mannitol, used as an osmotic control, did not affect Set7 expression (data not shown). In this setting, we confirmed Set7 upregulation and subsequent H3K4m1 on NF-κB p65 promoter (Figure 5A and 5B). Interestingly, siRNA-mediated knockdown of Set7 prevented H3K4m1 and abolished NF-κB p65 upregulation (Figure 5B and 5C). Furthermore, targeting Set7 blunted NF-κB-dependent oxidant and inflammatory genes (Figure 5D and 5E).

**Discussion**

This study demonstrates for the first time that histone methyltransferase Set7 contributes to endothelial dysfunction and inflammation in patients with T2DM via epigenetic regulation of the transcription factor NF-κB. Several lines of evidence support our conclusion. First, expression of Set7 was increased in PBM isolated from diabetic patients. Second, Set7 induced H3K4m1 on NF-κB p65 promoter, leading to upregulation of transcription factor and overexpression of oxidant/

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**Table.** Demographics, Laboratory Parameters, and Medications in the Study Population

<table>
<thead>
<tr>
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<th>Controls, n=24</th>
<th>T2DM, n=44</th>
<th>$P$ Value</th>
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<tbody>
<tr>
<td><strong>Age, y, mean (SD)</strong></td>
<td>45±13</td>
<td>51±11</td>
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<td><strong>Sex (F:M)</strong></td>
<td>14:10</td>
<td>20:24</td>
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<td><strong>BMI, kg/m$^2$, mean (SD)</strong></td>
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<td><strong>Waist circumference, cm, mean (SD)</strong></td>
<td>82.5±13</td>
<td>92.6±14</td>
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<td><strong>Systolic blood pressure, mm Hg, mean (SD)</strong></td>
<td>124±14</td>
<td>130±15</td>
<td>0.09*</td>
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<tr>
<td><strong>Diastolic blood pressure, mm Hg, (IQR)</strong></td>
<td>80 (70–85)</td>
<td>80 (74–80)</td>
<td>0.99†</td>
</tr>
<tr>
<td><strong>FPG, mg/dL, mean (SD)</strong></td>
<td>83±9</td>
<td>180±65</td>
<td>&lt;0.001*</td>
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<td><strong>HbA1c, %, (IQR)</strong></td>
<td>5.1 (4.9–5.3)</td>
<td>7.2 (6.6–8.5)</td>
<td>&lt;0.001‡</td>
</tr>
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<td><strong>Triglycerides, mg/dL, (IQR)</strong></td>
<td>95 (73–120)</td>
<td>102 (83–152)</td>
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<td><strong>HDL cholesterol, mg/dL, (IQR)</strong></td>
<td>49 (44–69)</td>
<td>51 (42–56)</td>
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<td><strong>LDL cholesterol, mg/dL, mean (SD)</strong></td>
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<td><strong>Total cholesterol, mg/dL, mean (SD)</strong></td>
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<td><strong>ACE-I/ARBs, %</strong></td>
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<td><strong>Insulin, %</strong></td>
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<td><strong>Hypoglycemic agents, %</strong></td>
<td>—/—</td>
<td>35 (76.0)</td>
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</table>

ACE-I indicates angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycohemoglobin; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; N/A, not applicable; and T2DM, type 2 diabetes mellitus.

*Unpaired 2-sample t test.
†x$^2$ test.
‡Mann–Whitney test.

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**Figure 1.** Box plots indicate gene and protein expression of methyltransferase Set7 in controls and subjects with type 2 diabetes mellitus (T2DM). Real-time polymerase chain reaction (PCR; controls=24; subjects with T2DM=44) and Western blot (WB; controls=10, subjects with T2DM=10) showing Set7 expression in peripheral blood monocytes from subjects with T2DM and age-matched controls. TATA binding protein (TBP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as loading controls for real-time PCR and WB, respectively.
inflammatory genes. Indeed, Set7 expression was positively correlated with NF-kB-dependent genes, COX-2 and iNOS, as well as inflammatory adhesion molecules, ICAM-1 and MCP-1. Moreover, Set7 was associated with oxidative stress and endothelial dysfunction. Third, siRNA-mediated knockdown of Set7 blunted hyperglycemia-induced oxidant and inflammatory pathways. These findings suggest that Set7 is a methyl-writing enzyme critically implicated in the epigenetic regulation of atherosclerotic genes in patients with T2DM.

NF-kB is a pleiotropic transcription factor involved in multiple biological functions, including inflammation, immunity, cell proliferation, and apoptosis.\textsuperscript{15} It is a master regulator of genes implicated in atherosclerosis and diabetes mellitus.\textsuperscript{15,16} NF-kB consists of homo and heterodimers of different subunits, such as p50, p52, p65/RelA, RelB, and c-Rel. p65/RelA and p50 are crucial in NF-kB signaling.\textsuperscript{17} Indeed, nuclear translocation of p65–p50 is the final event enabling transcription of NF-kB-dependent genes. Activation of NF-kB

**Figure 2.** Set7 induces epigenetic changes on NF-kB p65 promoter of patients with type 2 diabetes mellitus (T2DM). A, Chromatin immunoprecipitation assay showing monomethylation of lysine 4 of histone 3 on NF-kB p65 promoter (region, −480/−240) in peripheral blood monocytes (controls=12; patients with T2DM=38). B, Gene expression (controls=12; patients with T2DM=38) and binding activity (controls=8; patients with T2DM=16) of NF-kB p65. TATA binding protein (TBP) was used as a loading control for real-time polymerase chain reaction (PCR). C, Set7 levels positively correlate with NF-kB expression (n=38) and binding activity (n=16). Real-time PCR data are expressed as arbitrary units. \( r \) indicates Spearman correlation coefficient. H3K4m1, monomethylation of histone 3 at lysine 4. n indicates the number of subjects per experiment; and qPCR, quantitative polymerase chain reaction.

**Figure 3.** Set7 expression correlates with NF-kB-dependent oxidant genes and inflammatory adhesion molecules. A and B, Linear correlation of Set7 expression with NF-kB-driven oxidant (cyclooxygenase 2 [COX-2, n=38]; inducible endothelial nitric oxide synthase [iNOS, n=38]) and inflammatory genes (intercellular cell adhesion molecule-1 [ICAM-1, n=38]; monocyte chemoattractant protein-1 [MCP-1, n=37]). Real-time polymerase chain reaction data are expressed as arbitrary units. \( r \) indicates Spearman correlation coefficient. n indicates the number of subjects per experiment.
in diabetes mellitus is linked to an array of molecular events precipitating in endothelial dysfunction, vascular inflammation, and insulin resistance. Understanding the molecular mechanisms that regulate NF-κB expression and downstream signaling is important to prevent diabetes mellitus–related oxidative and inflammatory burst. This may have important clinical implications because cardiovascular risk remains high in diabetic patients despite multifactorial intervention.

It is emerging that acquired modifications of the epigenome may alter gene expression. Gene transcription is a dynamic process involving the conversion of compact heterochromatin into accessible euchromatin. Post-translational modifications

Figure 4. Upregulation of Set7 is associated with oxidative stress and endothelial dysfunction. Linear correlation of Set7 expression with urinary excretion rates of free 8-isoprostaglandin F₂α (8-isoPGF₂α) levels (n=38) and flow-mediated dilation (FMD) (n=36). Real-time polymerase chain reaction data are expressed as arbitrary units. Non-Gaussian variables 8-isoPGF₂α and flow-mediated dilation (FMD) were normalized by log transformation. r is the Spearman correlation coefficient. n indicates the number of subjects per experiment.

Figure 5. Targeting Set7 suppresses NF-κB-dependent oxidant and inflammatory genes in human endothelial cells. A, Set7 gene and protein expression in human aortic endothelial cells (HAECs) exposed to high (25 mmol/L) and normal (5 mmol/L) glucose concentrations. TATA binding protein (TBP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as loading controls for real-time polymerase chain reaction and western blot, respectively. B, Chromatin immunoprecipitation assay showing monomethylation of lysine 4 of histone 3 on NF-κB p65 promoter in HAECs exposed to high glucose in the presence or in the absence of Set7 siRNA. Scrambled siRNA (scr.siRNA) was used as a control. C and D, Gene expression of cyclooxygenase 2 (COX-2), inducible endothelial nitric oxide synthase (iNOS), intercellular cell adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) in glucose-treated HAECs with or without Set7 siRNA. Values are expressed as means±SEM. n=5 for all the experiments. All P values refer to Bonferroni correction for multiple comparisons. HG indicates high glucose; and NG, normal glucose.
of histone tails, namely acetylation and methylation, induce chromatin remodeling that may either enable or repress gene transcription.\textsuperscript{8} In vitro studies have recently showed that Set7 is involved in epigenetic regulation of NF-kB.\textsuperscript{6,9} Furthermore, microarray profiling revealed that the induction of 25% of NF-kB downstream genes is suppressed by Set7 depletion in tumor necrosis factor α–stimulated human immortalized monocytes.\textsuperscript{11} In addition, Set7 induces MCP-1 upregulation and endoplasmic reticulum stress in the kidney of diabetic mice.\textsuperscript{20} Although these studies provide important mechanistic insights on the role of Set7 in hyperglycemia-induced inflammation, it remains unknown whether these mechanisms can be translated to the clinical setting. This issue deserves attention because early markers of diabetes mellitus–related vascular damage remain elusive.\textsuperscript{21,22}

In this study, we have reported that Set7 expression is increased in PBM from patients with T2DM and correlates with NF-kB activation, oxidative stress, and endothelial dysfunction.\textsuperscript{6} The analysis of Set7-driven chromatin modification unmasks H3K4m1 on NF-kB promoter in patients with T2DM but not in controls. Such epigenetic signature was associated with increased NF-kB p65 expression and subsequent transcription of downstream genes, COX-2 and iNOS, as well as inflammatory adhesion molecules, ICAM-1 and MCP-1. By contrast, we could not find any H3K4m1 signature on the promoter of oxidant/inflammatory genes, suggesting that Set7 indirectly affects their transcription by modulating NF-kB p65 expression. Induction of COX-2 and iNOS by inflammatory and apoptotic stimuli causes generation of reactive oxygen species and reduced nitric oxide availability.\textsuperscript{23,24} Furthermore, adhesion molecules are important mediators of the inflammatory milieu triggering monocyte infiltration and foam cell formation.\textsuperscript{15,25} Our findings strongly suggest that histone methyltransferase Set7 is critically involved in the pathophysiology of vascular dysfunction in patients with diabetes mellitus (Figure 6).

In this study, we have also confirmed the mechanistic link between Set7 and oxidant/inflammatory genes in the human endothelium. Reprogramming Set7 expression in primary human endothelial cells deletes H3K4m1 signature and abolishes expression of COX-2, iNOS, and inflammatory adhesion molecules. Our in vitro experiments in HAECs recapitulate previous seminal work showing that Set7 is a critical mediator of hyperglycemia-induced upregulation of ICAM-1 and MCP-1.\textsuperscript{8,9} Similar findings have been also obtained in human immortalized monocytes where Set7 knockdown abolished the expression of tumor necrosis factor α–induced inflammatory genes.\textsuperscript{11} In this work, we have translated previous results to the clinical setting by showing that the Set7/ NF-kB axis may represent a precursor of endothelial dysfunction and inflammation in patients with T2DM. The strength of our study is the observation that a selective chromatin alteration induced by Set7 significantly contributes to key features of vascular disease in patients with diabetes mellitus. This novel concept has important clinical implications because chromatin-modifying agents are being intensively studied to reprogram epigenetic changes in patients with cancer and neurological disease.\textsuperscript{26} In this regard, recent evidence suggests that Food and Drug Administration–approved suberoylanilide hydroxamic acid, an histone deacetylase inhibitor, protects against cardiac ischemic damage by increasing the autophagic flux.\textsuperscript{27} Pharmacological activation of the deacetylase sirtuin 1 by resveratrol and metformin has also shown to improve endothelial function in obese and diabetic individuals.\textsuperscript{28–30} Hence, the relevance of our study is supported by the emerging concept that adverse epigenetic remodeling is reversible on pharmacological reprogramming of chromatin-modifying enzymes.\textsuperscript{26}

The potential of our findings is also highlighted by the observation that epigenetic changes may contribute to explain why vascular complications persist in diabetic patients despite an optimal glycemic control. We and others have recently shown that epigenetic marks induced by hyperglycemia persist even after restoration of normal glucose levels and account for ongoing inflammation and oxidative stress.\textsuperscript{9,29,31,32} On the basis of these findings, the removal of acquired epigenetic modifications may interrupt detrimental signaling cascades responsible for persistent vascular risk in patients with diabetes mellitus.\textsuperscript{28} Our work also confirms that among different risk factors, clustering with T2DM, hyperglycemia remains the most potent driver of Set7 upregulation. Indeed, Set7 expression significantly correlated with FPG and glycated hemoglobin, whereas no significant associations were found for age, blood pressure, and lipids. This aspect may have important implications for patients with type 1 diabetes mellitus where hyperglycemia represents the main pathological alteration causing microvascular pathology.

**Figure 6.** Role of histone methyltransferase Set7 in diabetes mellitus–induced vascular dysfunction. In patients with type 2 diabetes mellitus (T2DM), hyperglycemia induces upregulation of Set7, leading to monomethylation of histone 3 of lysine 4 on NF-kB p65 promoter. This epigenetic signature triggers NF-kB p65 transcription and subsequent upregulation of oxidant and inflammatory genes, thus contributing to vascular dysfunction in this setting. COX-2 indicates cyclooxygenase 2; H3K4m1, monomethylation of histone 3 at lysine 4; ICAM-1, intercellular cell adhesion molecule-1; iNOS, inducible endothelial nitric oxide synthase; and MCP-1, monocyte chemoattractant protein-1.
and macrovascular damage. In this regard, further research is needed to elucidate whether Set7 inhibition may rescue vascular phenotype in patients with type 1 diabetes mellitus. In our study, Set7 expression was assessed in PBM cells from diabetic patients. An increasing body of evidence is supporting the concept that molecular changes in circulating mononuclear cells mirror early alterations in endothelial vasomotor function.\(^3\)\(^4\) In this regard, a strong correlation between oxidative stress in mononuclear cells and endothelial-dependent vasorelaxation in patients with T2DM has been reported recently.\(^3\)\(^4\) These findings support the concept that epigenetic changes observed in circulating cells may represent a reliable indicator of endothelial dysfunction and inflammation. Moreover, we have provided molecular insight to explain the role of Set7 in the endothelium. Indeed, our in vitro experiments suggest that targeting Set7 may prevent endothelial dysfunction and vascular inflammation, thus protecting against atherosclerotic disease. In this study, we have observed that gene silencing of Set7 is sufficient to blunt NF-kB-dependent transcription of inflammatory genes. However, it should be noted that Set7-induced overexpression of NF-kB p65 might not necessarily translate into increased transcriptional activity. The biology of NF-kB signaling is indeed more complex and characterized by further regulatory systems (degradation of inhibitory subunit IkB), thus warranting cytosolic sequestration of RelA. Although we found that Set7 expression correlates with NF-kB binding activity, this aspect remains poorly understood and should be clarified by future mechanistic studies. A possible explanation for this phenomenon is that Set7 might regulate other NF-kB components responsible for RelA nuclear translocation. Furthermore, our data need to be confirmed in larger cohorts to assess whether Set7 represents a putative marker of subclinical atherosclerosis in patients with T2DM.

In conclusion, this study demonstrates that chromatin-modifying enzyme Set7 is upregulated in diabetic patients and involved in the regulation of transcription factor NF-kB, inflammation, oxidative stress, and endothelial dysfunction. Our findings indicate that expression of Set7 in circulating monocytes may represent a potential marker of vascular damage. Furthermore, the development of pharmacological approaches targeting Set7 is a promising option to dampen oxidative stress and vascular inflammation in the setting of diabetes mellitus.

Sources of Funding

This study was supported by grants from Karolinska Institute, Swiss Heart Foundation, and Italian Ministry of Education, University and Research, Projects of national interest and an EFSD/Novo Nordisk award (to Dr Cosentino). Dr Paneni was the recipient of a PhD program in Experimental Medicine at Sapienza University of Rome.

Disclosures

None.

References

23. Wong WT, Tian XY, Huang Y. Endothelial dysfunction in diabetes and hypertension: cross talk in RAS, BMP4, and ROS-dependent COX-2-derived...

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**CLINICAL PERSPECTIVE**

Albeit advances in therapy have reduced morbidity and mortality in patients with diabetes mellitus, cardiovascular risk is far to be eradicated and mechanism-based therapeutic approaches are in high demand. In this perspective, deciphering novel molecular networks of vascular disease will be instrumental to develop novel diagnostic and therapeutic strategies in people affected by diabetes mellitus. There is, therefore, a need to address current knowledge gaps in disease etiology to support innovation in diagnosis and treatment. In this regard, epigenetic signatures of DNA–histone complexes are emerging as important determinants of gene dysregulation and altered cellular homeostasis. This study demonstrates that the histone methyltransferase Set7 may contribute to endothelial dysfunction and inflammation in diabetic patients via epigenetic regulation of the transcription factor NF-κB p65. Set7-induced NF-κB upregulation favors transcription of proatherosclerotic genes, such as cyclooxygenase 2, inducible endothelial nitric oxide synthase, intercellular cell adhesion molecule-1, and monocyte chemoattractant protein-1. Our findings add knowledge to the pathophysiology of diabetic vascular disease and indicate that Set7 expression may serve as a potential marker of vascular damage in patients with type 2 diabetes mellitus. The development of pharmacological approaches targeting Set7 may be a promising option to dampen oxidative stress and vascular inflammation and thus prevent diabetes mellitus–related vascular complications.
Adverse Epigenetic Signatures by Histone Methyltransferase Set7 Contribute to Vascular Dysfunction in Patients With Type 2 Diabetes Mellitus

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_Circ Cardiovasc Genet._ 2015;8:150-158; originally published online December 3, 2014; doi: 10.1161/CIRCGENETICS.114.000671

_Circulation: Cardiovascular Genetics_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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## Supplemental Tables

**Table S1.** Correlations between Set7 gene expression and diabetes-related cardiovascular risk factors. $r$ indicates Spearman’s correlation coefficient. *These variables were normalized by log transformation because of their non-Gaussian distribution.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Set7 mRNA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI ($\text{Kg/m}^2$)</td>
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<td>Systolic blood pressure (mmHg)</td>
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<td>HbA1c* (%)</td>
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<tr>
<td>LDL cholesterol (mg/dL)</td>
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</tbody>
</table>
Supplemental Figures

**Figure S1.** (A-B) Expression of oxidant (COX-2, iNOS) and inflammatory (ICAM-1 and MCP-1) genes in T2DM patients and controls. Values are expressed as mean±SEM. P-value refers to Student t tests. COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase; ICAM-1, intracellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1.

**Figure S2.** Box plots show (A) 24-hour urinary 8-isoPGF\(_2\alpha\) levels in T2DM patients and controls. (B) Flow-mediated dilation and nitroglycerin-mediated dilation. P value refers to Mann-Whitney test. 8-isoPGF\(_2\alpha\), 8-iso-prostaglandin F\(_2\alpha\); FMD, flow-mediated dilation; NMD, nitroglycerin-mediated dilation.