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In vivo evidence of enhanced di-methylation of histone H3 K4 on upregulated genes in adipose tissue of diabetic *db*/*db* mice

Saki Fujimoto, Toshinao Goda, Kazuki Mochizuki *

The University of Shizuoka, Graduate School of Nutritional and Environmental Sciences and the Global COE, Japan

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ABSTRACT

Di-methylation of histone H3 lysine (K) 4, a component of the epigenetic memory, is associated with gene transactivation. In this study, we examined whether the development of diabetes induces di-methylation of histone H3 K4 on the upregulated genes. We searched for upregulated genes in mesenteric adipose tissue of insulin-resistant/diabetic *db*/*db* mice compared with non-diabetic *db*/*m* mice using microarray analysis. We also performed chromatin immunoprecipitation assays for di-methylation of histone H3 K4 in the upregulated genes in mesenteric adipose tissue of *db*/*m* and *db*/*db* mice. Di-methylation of histone H3 K4 was enhanced at the upstream and/or transcribed regions of upregulated genes including *Atp6v0d2, Mmp12, Trem2* and *Clec4d* genes in mesenteric adipose tissue of *db*/*db* mice, as compared with *db*/*m* mice. These results suggest that di-methylation of histone H3 K4 is involved in the induction of *Atp6v0d2, Mmp12, Trem2* and *Clec4d* in mesenteric adipose tissue in *db*/*db* mice.

1. Introduction

Insulin resistance induces diabetes and abnormalities in lipid metabolism by decreasing insulin sensitivity in various tissues. Diabetes in combination with insulin resistance leads to a severe diabetic condition characterized by a loss of the pancreatic β-cell insulin secretory capacity, and the accumulation of risk factors associated with complications such as cardiovascular disease, hypertension and inflammation of peripheral tissues [1,2]. Changes in adipose tissue are major factors in the development of insulin resistance. It is known that insulin resistant adipocytes secrete greater amounts of cytokines related to insulin resistance such as interleukin 6, tumor necrosis factor α, and resistin, and show reduced expression of genes related to insulin sensitivity, such as lipogenic proteins, and adipocytokines such as adiponectin [3,4]. These results indicate that insulin sensitivity (or resistance) may be determined by the expression levels of genes related to insulin sensitivity, reflecting the stage of adipocyte differentiation. It is believed that the development of insulin resistance in adipose tissue is caused by the accumulation of lifestyle risk factors in daily life, such as excess food intake, rapid eating and little physical activity.

Recent studies have shown that abrupt changes in gene expression, particularly those that frequently occur in differentiating cells, are accompanied by a major chromatin structural change that is triggered by modifications of the histone tail, such as acetylation, methylation and phosphorylation [5,6]. These modifications are known as the epigenetic memory, because the acquired information is imprinted on chromatin. Among several histone modifications that have been identified, acetylation of histone H3 lysines (K) 9 and 14, acetylation of histone H4 K5/8/12/16 and methylation of histone H3 K4 are the most extensively studied, because these histone modifications are frequently involved in the regulation of transactivation of genes [7–9]. Histone H3 K4 methylation is susceptible to three types of modification, namely mono-methylation, di-methylation and tri-methylation. In particular, di- and/or tri-methylation of histone K4 seems to be important for the initial activation of transcription, because di-/tri-methylation of histone H3 K4 is induced before the acetylation of histones and transactivation of genes. In turn, methylation of histone H3 K4 induces the acetylation of histones [10,11]. Several studies have shown that di-/tri-methylation of histone H3 K4 also induces the recruitment of mRNA transcription and elongation complexes onto target genes. This occurs through the binding of proteins containing plant homeodomains (PHD), chromodomains, Tudor domains or WD40 repeat domains to methylated histones [12,13]. Recent cell-based studies have demonstrated that di-methylation of histone H3 K4 on adipocyte-specific genes, such as adiponectin and GLUT4, is closely associated with induction of their expression during adipocyte differentiation [11]. However, methylation of histone H3 K4 on targeted genes in adipose tissue *in vivo* has not yet been reported. Similarly, it is unknown whether histone H3 K4 methylation is associated with changes in gene expression in adipose tissue from insulin resistant and/or diabetic animal models.

* Corresponding author. Address: Laboratory of Nutritional Physiology, School of Food and Nutritional Sciences, The University of Shizuoka, 52-1 Yada, Shizuoka-shi, Shizuoka 422-8526, Japan. Fax: +81 54 264 5565.
E-mail address: kmochi@u-shizuoka-ken.ac.jp (K. Mochizuki).

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In this study, we examined whether the increased expression of genes in mesenteric adipose tissue of *db*/*db* mice, which lack the leptin receptor and show the progression of diabetes from hyperinsulinemia during the insulin resistant state to hypoinsulinemia as a result of the loss of the pancreatic β-cell secretory capacity [14], are associated with enhanced histone H3 K4 methylation.

2. Materials and methods

2.1. Animals

Six *db*/*m* mice and six *db*/*db* mice (6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The mice had free access to standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) and water. They were housed in a temperature (22 ± 2 °C) and humidity-controlled room (55 ± 5%), with a 12-h light/dark cycle (lights on: 07:00–19:00 h) for 1 week. At 7 weeks of age, non-fasted *db*/*db* and *db*/*m* mice were killed by decapitation, and blood and mesenteric adipose tissue were collected. The experimental procedures used in the present study conformed to the guidelines of the Animal Usage Committee of the University of Shizuoka.

2.2. Blood biochemical parameters

Serum levels of glucose, triglycerides and free fatty acids (FFAs) were determined using commercial kits (Wako Pure Chemical Industries, Osaka, Japan). The serum insulin concentration was determined using a mouse insulin enzyme-linked immunosorbent assay (Shibayagi, Gunma, Japan). The body weights of all mice were measured just before decapitation.

2.3. Microarray analysis of mesenteric adipose tissue

Total RNA was extracted from the mesenteric adipose tissue of all mice using an RNeasy kit (Qiagen/BD, Tokyo, Japan). For microarray analyses, we used total RNA extracts from the mesenteric adipose tissue of four *db*/*db* mice and three *db*/*m* mice at the age of 7 weeks, which were randomly selected. Aliquots containing 100 ng of total RNA were individually converted to cRNAs and labeled with biotin using a Whole Transcript (WT) Sense Target Labeling kit (Affymetrix, Tokyo, Japan) according to the manufacturer's instructions. Next, aliquots containing 10 μg of biotin-labeled cRNA were separately hybridized to a mouse Exon 1.0ST array (Affymetrix) according to the manufacturer's protocol. After washing, the microarray plates were analyzed with a GeneChip Scanner 3000 (Affymetrix). Data analysis was performed using the GeneChip operating system (GCOS; Affymetrix), Partec (Ryoka Systems, Tokyo, Japan) and Excel (Microsoft). Variable spots detected by an algorithm in Partec software in both plates were defined as non-expressed genes and removed accordingly. The biotin-labeled signals were normalized by the global median normalization method based on the GCOS algorithm. Biologically variable spots with *P*-values of >0.05, as determined by unpaired Student's *t*-tests, between the *db*/*db* mice and the *db*/*m* mice were also removed. Data are represented by base 2 logarithms.

2.4. Real-time RT-PCR

Real-time RT-PCR was performed in mesenteric adipose tissue of six *db*/*db* mice and six *db*/*m* mice. Total RNA samples (200 ng) were converted to cDNAs by reverse transcription using SuperScript™ III RT (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. PCR amplification was performed using a Light-Cycler instrument (Roche Molecular Biochemicals, Tokyo) using SYBR Premix Ex Taq (Takara, Shiga, Japan), as previously described [15] to quantify the mRNA expression levels of the selected genes. The PCR primer sequences are listed in Supplementary Table 1 The cycle threshold (CT) values of each gene and the *Yy1* gene determined by real-time RT-PCR were converted to signal intensities by the delta–delta method [16], which calculates the difference for one CT value as a 2-fold difference between the signal for each gene and the signal for a normalization gene (i.e., *Yy1*). The formula used was $[2^{(\text{CT each gene-CT } Yy1)}]$.

2.5. Chromatin immunoprecipitation (ChIP) assay

Mesenteric adipose tissue was scraped by a glass slide and incubated in fixation solution (1% formaldehyde, 4.5 mM HEPES pH 8.0, 9 mM NaCl, 0.09 mM EDTA and 0.04 mM EGTA) in 10% FBS/DMEM for 30 min at 37 °C. The reaction was terminated by the addition of glycine to a final concentration of 150 mM. After washing the samples in FACS solution (1× PBS(−), 2% bovine serum, 0.05% NaN_3), they were sonicated in SDS lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) with protease inhibitors (1 tablet/10 mL; Complete Mini, Roche, Tokyo, Japan) in a condition where the DNA size of samples was 200–500 bp. ChIP assays were performed, as previously described [17], using 2 μg of each specific antibody, i.e., anti-mono-methyl-histone H3 K4 antibody (Millipore, Tokyo, Japan), anti-di-methyl-histone H3 K4 antibody (Millipore), anti-tri-methyl-histone H3 K4 antibody (Millipore), or normal rabbit IgG. The precipitated DNA was subjected to real-time PCR using primers corresponding to the indicated sites in the promoter/enhancer and the transcribed sites (Supplementary Table 2). The CT values of the ChIP signals detected by real-time PCR were converted to the percentage of the ChIP signal for the input DNA, which was calculated by the delta–delta method [16], using the formula $100 \times [2^{(\text{CT IP sample}-\text{CT input})}]$.

2.6. Statistical analysis

All data are expressed as means ± SEM. Student's *t*-test was used to determine significant differences between the two groups. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Comparison of blood biochemical parameters between 7-week-old db/m and db/db mice

Body weight and mesenteric adipose tissue weight were significantly greater in *db*/*db* mice than in *db*/*m* mice. Furthermore, the serum glucose and insulin concentrations were greater in *db*/*db* mice than in *db*/*m* mice. No significant differences were observed

Table 1
Body weight and blood biochemical parameters in *db*/*db* mice and *db*/*m* mice.

	db/*m*	*db*/*db*
Body weight (g)	24.3 ± 0.4	33.2 ± 0.8**
Mesenteric adipose tissue (g)	0.31 ± 0.01	0.92 ± 0.04**
Serum glucose (mg/dl)	188 ± 7	492 ± 24**
Serum insulin (ng/ml)	<1.0	7.5 ± 1.4
Serum triglycerides (mg/dl)	158.5 ± 12.2	372.4 ± 50.1
Serum FFA (mEq/l)	2.35 ± 0.26	3.73 ± 0.64

Values are expressed as means ± SEM for 6 animals.
** $P < 0.01$; Significantly different from *db*/*m* mice (Student's *t*-test).

in fasting serum triglyceride or free fatty acid concentrations between *db*/*m* and *db*/*db* mice (Table 1).

3.2. Comparison of gene expression profiles in mesenteric adipose tissue between db/db and db/m mice by microarray analyses

To examine whether gene expression in mesenteric adipose tissue differed between *db*/*m* mice and *db*/*db* mice, we performed microarrays using total RNAs extracted from mesenteric adipose tissues of *db*/*m* mice and *db*/*db* mice. When the differences in signals in the microarray between *db*/*m* mice and *db*/*db* mice were greater than 1 or less than −1 after $\log_2$ transformation, and when they were proved to be significant by unpaired Student's *t*-test ($P < 0.05$), the gene signals were subjected to further analyses. Among the 16,661 gene signals detected in the microarray, 104 were upregulated (1.13%), and 10 were downregulated (0.37%) in the mesenteric adipose tissue of *db*/*db* mice, when compared with those of *db*/*m* mice (Supplementary Tables 3 and 4). The upregulated and downregulated genes identified in the mesenteric adipose tissue of *db*/*db* mice were classified into eight groups based on their functions, namely signal transduction/transcription, metabolism, immune response, structural protein, transcription/chromatin, protein degradation, transporters, and others.

Among the 104 upregulated genes, we selected 13 genes showing $\log_2$ increases >2 on microarray data, and these genes, except for one gene classified in 'others', were tested by real-time RT-PCR. Real-time RT-PCR showed that the mRNA levels of *Atp6v0d2* (ATPase, H+ transporting, lysosomal V0 subunit D2) (7.5-fold, $P < 0.01$), *Mmp12* (Matrix metallopeptidase 12) (59.8-fold, $P < 0.01$), *Gpnmb* [Glycoprotein (transmembrane) nmb] (11.6-fold, $P < 0.01$), *Slc5a7* [Solute carrier family 5 (choline transporter), member 7] (10.4-fold, $P < 0.05$), *Lcn2* (Lipocalin 2) (6.9-fold, $P < 0.05$), *Lep* (Leptin) (4.4-fold, $P < 0.05$), *C3ar1* (Complement component 3a receptor 1) (2.5-fold, $P < 0.01$), *Saa3* (Serum amyloid A 3) (4.1-fold, $P < 0.01$), *Trem2* (Triggering receptor expressed on myeloid cells 2) (11.2-fold, $P < 0.01$) and *Clec4d* (C-type lectin domain family 4, member d) (14.6-fold, $P < 0.01$) were significantly elevated in *db*/*db* mice as compared with *db*/*m* mice. The mRNA levels of *Atf3* (Activating transcription factor 3) and *Serpine1* [Serine (or cysteine) peptidase inhibitor, clade E, member1] were not significantly different between the two groups, but tended to be greater in *db*/*db* mice than in *db*/*m* mice (Fig. 1).

3.3. Di-methylation of histone H3 K4 on upregulated genes in mesenteric adipose tissue of db/db and db/m mice

To investigate whether methylation of histone H3 K4 are associated with increased gene expression in the mesenteric adipose tissue of *db*/*db* mice compared with *db*/*m* mice, ChIP assays were performed for the selected genes using antibodies for mono-, di-, and tri-methylated histone H3 K4. We investigated methylation of histone H3 K4 on the promoter/enhancer and transcribed regions of *Atp6v0d2*, *Mmp12*, *Gpnmb*, *Trem2* and *Clec4d* genes, which showed 5-fold greater expression (all $P < 0.01$) by real-time RT-PCR in *db*/*db* mice than in *db*/*m* mice. The ChIP signals for normal rabbit IgG were <0.2% per input. The ChIP signals for di-methylation of histone H3 K4 at +1000 bp of *Atp6v0d2*, at −2000 bp, −1000 bp, +1000 bp, +2000 bp and over 2000 bp of *Mmp12*, +2000 bp and over 2000 bp of *Trem2* and all regions of *Clec4d*, except for over 2000 bp, were significantly greater in *db*/*db* mice than in *db*/*m* mice. For *Gpnmb*, the ChIP signal at +0 bp was significantly lower in *db*/*db* mice than in *db*/*m* mice (Fig. 2). The ChIP signals for mono- and tri-methylation of histone H3 K4 on these genes were not significantly different from the IgG signals (data not shown).

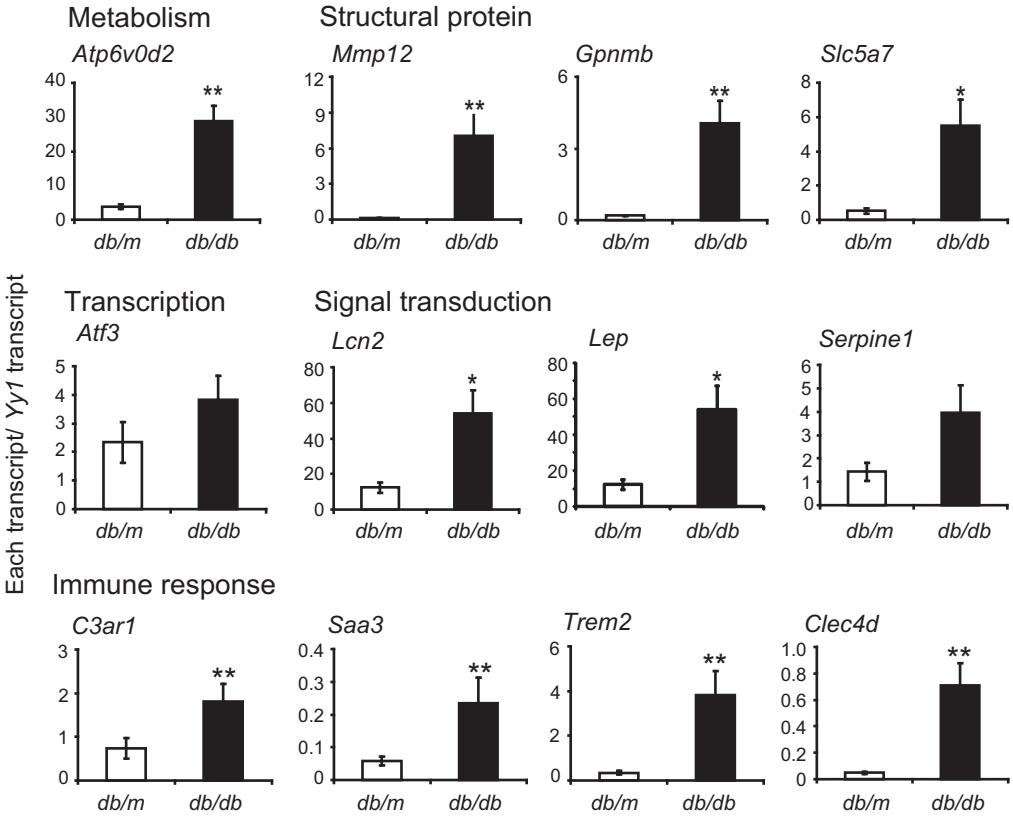

Fig. 1. Quantitative RT-PCR analyses of upregulated genes in *db*/*db* and *db*/*m* mice. The results for each sample were normalized by the corresponding *Yy1* mRNA abundance. The data are means ± SEM for 6 animals. $^{*}P < 0.05$, $^{**}P < 0.01$, versus *db*/*m* mice (Student's *t*-test).

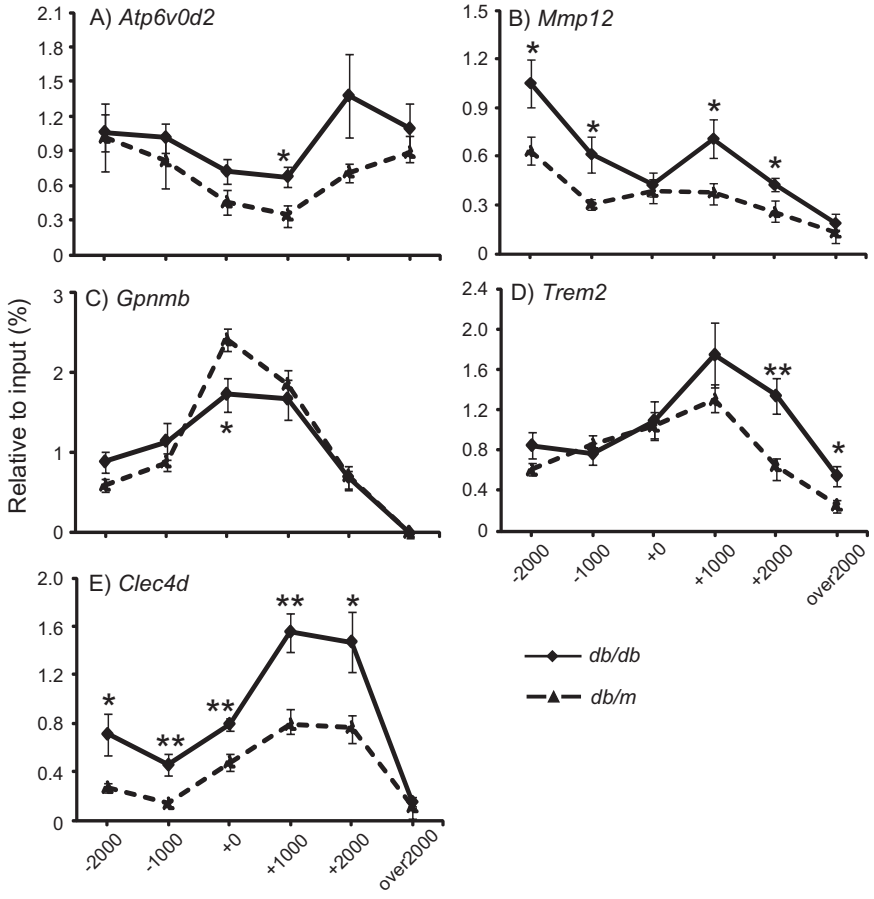

Fig. 2. Di-methylation of histone H3 K4 on upregulated genes in mesenteric adipose tissue of *db*/*db* and *db*/*m* mice. ChIP signals were detected by quantitative real-time PCR following immunoprecipitation of di-methylated histone H3 K4 on the promoter/enhancer and transcribed regions of the *Atp6v0d2*, *Mmp12*, *Gpnmb*, *Trem2* and *Clec4d* genes in the mesenteric adipose tissue of *db*/*db* and *db*/*m* mice. The data are means ± SEM for 6 animals. $^{*}P < 0.05$, $^{**}P < 0.01$, versus *db*/*m* mice (Student's *t*-test).

4. Discussion

In this study, we found that the expression of many genes related to metabolism, structural proteins, signal transduction and the immune response was higher in the mesenteric adipose tissue of *db*/*db* mice than in *db*/*m* mice (Fig. 1 and Supplementary Table 3). In fact, over 40% of the proteins coded by these genes are involved in either signal transduction (20.2%) or immune responses (22.1%). For example, two of the upregulated genes identified in this study, *Clec4d* (14.5-fold by real-time RT-PCR, $P < 0.01$) and *Tlr8* (Toll-like receptor 8) (2.3-fold by real-time RT-PCR, $P < 0.05$), are considered to be localized on the surface or within leukocytes, and may have vital roles in killing bacteria/viruses by binding to the substances produced by bacteria/virus, such as C-type lectin, C-type mannose and nucleic acid [18,19]. The protein coded by *Cd68* (CD68 antigen) (2.2-fold by real-time RT-PCR, $P < 0.05$), which is specifically expressed in monocytes and macrophages, binds to low-density lipoprotein [20]. These results suggest that macrophage infiltration into mesenteric adipose tissue could occur in *db*/*db* mice at 7 weeks of age. These results are consistent with the concept that insulin resistance and diabetes induce macrophage infiltration [21]. Furthermore, we found that the expression of *Atp6v0d2*, which codes an enzyme for the activation of electron transport chain in mitochondria, was upregulated (7.5-fold by real-time RT-PCR, $P < 0.01$) in *db*/*db* mice. It has been reported that upregulation of genes related to the electron transport chain is associated with an increased production of reactive oxygen species, as well as elevated insulin resistance in adipose tissue [22]. In addition, we have demonstrated that many structural proteins were upregulated in *db*/*db* mice. The proteins coded by *Col81a* (procollagen, type VIII, alpha 1), *Fbln* (fibulin)-*2* and *Fbln5* (Supplementary Table 3) are present in the extracellular matrix, while the protein coded by *Acta1* (Actin, alpha 1, skeletal muscle) is a component of the intracellular cytoskeleton. In addition, we have shown that the expression of *Mmp12*, which codes a protease that digests proteins in the extracellular matrix and participates in cell remodeling, needed to change in adipocytes during their transition from smaller size adipocytes (i.e., non-obese adipocytes) to those with a larger size (i.e., obese adipocytes) [23,24]. In the present study, we also found that the expression of this gene was much higher (59.8-fold by real-time RT-PCR, $P < 0.01$) in *db*/*db* mice than in *db*/*m* mice. It is known that the shape of adipocytes changes in the transition from an insulin sensitive phenotype to an insulin resistant phenotype. Indeed, we have demonstrated that the weight of mesenteric adipose tissue was significantly greater in *db*/*db* mice than in *db*/*m* mice (Table 1). Thus, the induction of these structural proteins could be due to adipose tissue remodeling from the non-obese phenotype to the obese phenotype, and/or from the insulin sensitive phenotype to the insulin resistant phenotype.

Interestingly, we found that di-methylation of histone H3 K4 in mesenteric adipose tissues of *db*/*m* mice and *db*/*db* mice was abundant in the promoter/enhancer and/or transcribed regions of *Atp6v0d2*, *Mmp12*, *Trem2* and *Clec4d* genes. To our knowledge this is the first report showing methylation of histone H3 K4 on genes in adipose tissue *in vivo*. Previous studies have suggested that the expression of genes in cultured adipocytes (3T3-L1 adipocytes) is closely associated with di-methylation, but less so with

mono- and tri-methylation, of histone H3 K4 on adipocyte-specific genes [11,25]. Our results, together with these of previous reports, indicate that di-methylation of histone H3 K4 is important for the induction of gene expression in adipocytes. Furthermore, we have demonstrated that di-methylation of histone H3 K4 in the promoter/enhancer and/or transcribed region of *Atp6v0d2, Mmp12*, *Trem2* and *Clec4d* genes in mesenteric adipose tissues was more pronounced in *db*/*db* mice than in *db*/*m* mice. These results suggest that enhanced histone H3 K4 di-methylation on these genes is closely associated with the induction of gene expression in mesenteric adipose tissue of *db*/*db* mice. These results also indicate that information associated with excess eating, development of insulin resistance and diabetes are written on histone H3 K4 of these genes as di-methylated histone H3 K4. It should be noted that di-methylation of histone H3 K4 on the *Gpnmb* gene was not lower in *db*/*db* mice than in *db*/*m* mice. These results indicate that other histone modifications may be associated with changes in the expression of the *Gpnmb* gene. Other modifications on genes upregulated in *db*/*db* mice, including *Gpnmb,* should be examined.

The signals responsible for changes in the di-methylation of histone H3 K4 on the *Atp6v0d2, Mmp12*, *Trem2* and *Clec4d* genes in mesenteric adipose tissue of *db*/*db* mice remain unknown. A recent study showed that transient exposure of vascular cells to high glucose induces mono-methylation of histone H3 K4 on the proximal promoter region of NFκB-target genes, which was followed by the induction of the *P65*, *Mcp1* and *Vcam1* genes, along with recruitment of histone methyltransferase Set 7/9 on the promoter region of these NFκB-target genes [26], although di- and tri-methylation was not clearly detected. Studies need to identify the signals and molecular mechanisms responsible for the di-methylation of histone H3 K4 on the *Atp6v0d2, Mmp12*, *Trem2* and *Clec4d* genes in mesenteric adipose tissue of *db*/*db* mice. In addition, further studies should seek direct evidence for the associations between histone H3 K4 methylation in the upregulated genes in the mesenteric adipose tissue of *db*/*db* mice and the development/progression of diabetes and its complications.

In conclusion, the results of this study suggest that increased expression of the *Atp6v0d2, Mmp12*, *Trem2* and *Clec4d* genes in mesenteric adipose tissue of *db*/*db* mice is closely associated with di-methylation of histone H3 K4 on the genes. To our knowledge, this is the first report showing increased histone modification on genes in adipose tissues of diabetic compared with non-diabetic animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.097.

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