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Epigenetic changes caused by diabetes and their potential role in the development of periodontitis

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Abstract

Introduction: Periodontal disease, a chronic inflammation induced by bacteria, is closely linked with diabetes mellitus. Many complications associated with diabetes are related to epigenetic changes. However, the exact epigenetic changes whereby diabetes affects periodontal disease remain largely unknown. Thus, we sought to investigate the role of diabetes dependent epigenetic changes of gingival tissue in the susceptibility to periodontal disease.

Materials and Methods: We studied the effect of streptozotocin induced diabetes in minipigs on gingival morphological and epigenetic tissue changes. Accordingly, we randomly divided 6 minipigs into two groups: streptozotocin induced diabetes group, n = 3; and no-diabetes healthy control group, n = 3. After 85 days, all animals were sacrificed and gingival tissue was collected for histology, DNA methylation analysis, and immunohistochemistry.

Results: A diabetes mellitus model was successfully created, as evidenced by significantly increased blood glucose levels, reduction of pancreatic insulin producing β cells, and histopathological changes in the kidneys. The gingival tissues in the diabetes group presented acanthosis of both gingival squamous epithelium and sulcular/junctional epithelium, and a significant reduction in the number and length of rete pegs. DNA methylation analysis demonstrated a total of 1163 affected genes, of which 599 and 564 were significantly hypermethylated and hypomethylated, respectively. Immunohistochemistry staining showed that the hypomethylated genes; tumor necrosis factor-α, and interleukin 6, were positively expressed under the junctional epithelium area in the diabetes group.

Conclusions: Diabetes mellitus induces morphological and epigenetic changes in periodontal tissue, which may contribute to the increased susceptibility of periodontal diseases in patients with diabetes.
Keywords: diabetes mellitus, DNA methylation, periodontal disease, epigenetics, minipig
Introduction

Diabetes mellitus (DM) represents a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. According to the latest International Diabetes Federation (IDF) data, 1 in every 11 adults worldwide had diabetes in 2019. DM can cause many complications such as retinopathy, nephropathy, peripheral neuropathy, vascular lesions and impaired wound healing. One such complication is periodontitis, a common chronic inflammatory disease caused by bacterial biofilm (dental plaque) colonization of the teeth surface that results in irreversible tissue damage. Susceptibility to the disease is also dependent on factors other than the absolute level of dental plaque, such as genetic polymorphisms, environmental risk factors, lifestyle, and epigenetic changes. DM is a major environmental risk factor for periodontal disease, increasing the prevalence, extent, and severity of periodontitis. Importantly, the relationship between these two complex and chronic diseases is bidirectional.

DM is associated with increased expression of inflammatory related markers such as prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin 1 β (IL-1β), and IL-6, as well as defects in polymorphonuclear leucocyte (PMN) activity, which may induce or accelerate the development of periodontitis. On the other hand, severe periodontitis is also associated with poorer glycemic control and significantly higher glycated hemoglobin A1c (HbA1c). Despite the extensive research conducted over the past years, the exact mechanisms linking these two complex diseases are not entirely understood, thus deserving further investigation.

Epigenetics is defined as the study of heritable and biological changes in gene function that are independent of DNA sequence. One of the most important epigenetic changes is DNA methylation, which mainly occurs at CpG sites where a cytosine nucleotide is followed by a guanine nucleotide from the 5' → 3' direction. Such modification is involved in many biological processes, especially in oncogenesis and inflammation.

The pathogenesis of DM involves both genetic and environmental factors. Life-long environmental exposure to nutritional, endocrine, and chemical perturbation may change gene expression via epigenetic modifications such as DNA methylation, histone modification, and microRNAs, thus affecting cell phenotypic differentiation. Importantly, many of the complications during diabetes are associated with epigenetic modifications. However, the exact diabetes associated pathogenetic changes implicated in the susceptibility to periodontitis...
remain largely unknown. Thus, we sought to understand the epigenetic changes that occur in periodontal tissue during diabetes. To that end, we investigated morphological and epigenetic DNA methylation changes of gingival tissues using a diabetic minipig model. In addition, we discuss the relationship between these changes and the susceptibility of periodontal tissues to inflammation, thus providing a better understanding of the pathophysiology and therapeutic management of periodontitis.

Materials and Methods

Animals

The project was fully compliant with the Guide for Care and Use of Laboratory Animals (8th edition, released by the National Research Council, USA). All animal procedures were approved by the Nanjing University Animal Ethics Committee and the experiments were performed in biosafety level 3 (BSL-3) facilities at the Department of Comparative Medicine at the General Hospital of Eastern Theater Command of PLA (Nanjing, China 210002).

A total of six one-year-old Guangxi Bana minipigs, weighing 60-80 kg, were used in the study. All animals were given clean ordinary pig feed and water ad libitum. The room temperature was 16-18 °C. The animals were randomly divided into 2 groups: diabetes mellitus group (DM, n = 3) and non-diabetes group (NDM, n = 3).

Induction of diabetes

The animals were anesthetized with Ketamine (Hospira, Lake Forest, IL)/Xylazine (Xyla-Ject, Phoenix, St. Josephs, MO) via intramuscular injection (20 mg/kg) and were weighed afterwards. Blood samples were taken from the superior vena cava of each animal before the induction of experimental diabetes, and blood glucose levels were measured as a baseline at the Stomatological Hospital affiliated to Nanjing University. Three pigs were administered with high-dose Streptozotocin (STZ, 150 mg/kg, Sigma, USA) diluted in 9.5 mL/mg sterile saline (0.9% NaCl injection, USP, Baxter) via the auricular vein. Blood samples were obtained at days 1, 45, 65, and 85 after the injection. All pigs were sacrificed after 85 days using a pentobarbital overdose. Then, the gingival, kidney, and pancreatic tissues of each group were collected. A fraction of the fresh
gingival tissue was fixed with TRIzol (Invitrogen, USA) for epigenetic test, and the other was fixed with 4% paraformaldehyde for histology and immunohistochemistry analysis.

Morphological evaluation

Samples fixed in 4% paraformaldehyde were dehydrated through graded ethanol baths (100-70%), cleared in xylene, and then embedded in paraffin wax. The paraffin blocks were serially sectioned at 5μm thickness, using a Leica RM2125 rotary microtome (RM2125 RTS, Leica, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (H&E), scanned using the Pannoramic 250 FLASH scanner, and evaluated using the Pannoramic viewer 1.15.2 software (3D HISTECH, Budapest, Hungary). The length and density of rete pegs were analyzed in 5 random areas per slice using Image J software (National Institutes of Health, USA).

DNA methylation level in the gingival tissues of diabetic and non-diabetic minipigs

Total DNA was extracted and purified using Qiagen DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. The quality of purified DNA was verified with agar gel (1.0%) electrophoresis and the DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). MeDIP-Sequencing library was prepared as described previously with minor modifications25.

For MeDIP-seq, extracted genomic DNA from gingival tissue was sonicated to ~200-500 bp with a bioruptor sonicator (Diagenode, Seraing, Belgium). About 1 μg of sonicated DNA was end-repaired, A-tailed, and ligated to single-end adapters following the standard Illumina Genomic DNA Sample Prep Kit protocol. The unligated adapters were eliminated using AMPure XP beads (Beckman-Coulter, CA, USA), after which the adaptor-ligated DNA was used for immunoprecipitation using a Mouse monoclonal anti-5-methylcytosine antibody (Diagenode, Belgium). For this, DNA was heat-denatured at 94 °C for 10 min, rapidly cooled on ice, and immunoprecipitated with 1μL primary antibody overnight at 4 °C with rocking agitation in 400μL immunoprecipitation buffer (0.5% BSA in PBS). To recover the immunoprecipitated DNA fragments, 100μL of protein G magnetic beads (Life Technologies) were added and incubated for

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an additional 2 hours at 4°C with agitation. Subsequently, five immunoprecipitation washes were performed with ice-cold immunoprecipitation buffer. Nonspecific mouse IgG immunoprecipitation was performed in parallel to methyl DNA immunoprecipitation as a negative control. Washed beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg/mL proteinase K for 2 hours at 65 °C, and then allowed to cool down to room temperature. MeDIP and supernatant DNA were purified using Qiagen MinElute columns and eluted in 16μL EB (Qiagen). Fourteen cycles of PCR were performed on 5μL of the immunoprecipitated DNA using single-end Illumina PCR primers. The resulting reactions were purified with Qiagen MinElute columns, after which a final size selection (~300-600bp) was performed using AMPure XP beads. Libraries were quality controlled with Agilent 2100 Bioanalyzer. An aliquot of each library was diluted in EB to 5ng/μL, and 1μL was used in real-time PCR reactions to confirm the enrichment in methylated regions.

**Sequencing**

The library was denatured with 0.1M NaOH to generate single-stranded DNA molecules, loaded onto channels of the flow cell at 8pM concentration, and amplified in situ using HiSeq 3000/4000 PE Cluster Kit (#PE-410-1001, Illumina, USA). Sequencing was carried out by running 2×150 cycles on Illumina HiSeq 4000 according to the manufacturer’s instructions.

**Data Analysis**

Image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8). After Solexa CHASTITY quality filter, clean reads were aligned to the *Sus scrofa* genome (UCSC version susScr3) using Bowtie2 software (V2.2.7). MeDIP enriched regions (peaks) with statistical significance were identified for each sample, using a q-value threshold of 10-5 by MACS v2. The mRNA associated MeDIP enriched regions (peaks) were annotated by the nearest gene using the newest UCSC RefSeq database. Statistically significantly differentially methylated regions (DMRs) within promoters between the two groups were identified by diffReps (Cut-off: log2FC = 1.0, p-value = 1.0). The mRNA associated DMRs within promoters were annotated by the nearest gene using the UCSC RefSeq and database of multiple databases integration.
Genome wide DNA methylation analysis was performed in both groups. For the genetic function and pathway identification, GO term (http://www.geneontology.org) and KEGG (https://www.genome.jp/kegg) enrichment analyses were performed.

**Immunohistochemistry**

The paraffin embedded gingival tissue blocks were sectioned to a thickness of 4μm and subjected to immunohistochemistry for changes in blood vessels, TNFα and IL-6. Immunohistochemical staining was performed according to a previously reported protocol with some modifications. Briefly, sections were deparaffinized/rehydrated in a xylene-ethanol-PBS serial procedure. Then, antigen retrieval was performed using citrate (pH 6.0) in a microwave oven followed by blocking of endogenous peroxidase with 3% H2O2 to reduce nonspecific background staining. Then, the slices were washed three times in PBS on a rocking device and subsequently pretreated with 3% BSA for 30 min at room temperature. Next, samples were incubated with PBS diluted primary antibodies against α-Smooth muscle actin (α-SMA, 1:500, Abcam, no. ab7817, Cambridge, MA, USA), Tumor necrosis factor-α (TNFα, 1:100, Abcam, no. ab6671), Interleukin 6 (IL-6, 1:500, Abcam, no. ab6672) at 4 ºC overnight. Diluted horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:200, Servicebio, Wuhan, China) was incubated for 50 min at room temperature followed by incubation with DAB (Servicebio, Wuhan, China) as chromogen. Slides were routinely mounted and scanned using the Pannoramic 250 FLASH scanner, and evaluated using the Pannoramic viewer 1.15.2 software (3D HISTECH, Budapest, Hungary). Image J software (National Institutes of Health, USA) was used to count blood vessels and positive stained cells in three different 100× fields for each sample.

**Statistical analyses**

The results were analyzed using SPSS 21.0 statistical software. Data were expressed as mean ± standard deviation. Blood glucose level comparison between the two groups was performed using a two-way ANOVA method, and two tailed Student’s unpaired t-test was used to assess differences in the morphological histology changes. P < 0.05 was considered statistically significant.
Results

Diabetic induction

Before STZ induction, the mean fasting blood glucose level of all animals was $4.43 \pm 1.26$ mmol/L (mean ± SEM). The mean fasting blood glucose level in the DM group was significantly increased 45 days after STZ administration compared to the NDM group and remained elevated throughout the observational period (Fig 1a).

Histological analysis

STZ administration induced structural changes in pancreatic, renal, and gingival tissues of the DM group. While the NDM group showed clear and regular structures, the pancreatic islets were significantly reduced in diabetic pigs, showing atrophied and irregular shapes. Pancreatic beta cells were severely degranulated and unevenly arranged (Fig 1b, c vs d, e). STZ-induced hyperglycemia also resulted in damaged renal tubules with a reduction in their number. Most tubules showed signs of degeneration, presenting vacuolization containing prominent cytoplasmic vacuoles and pyknotic nuclei (Supplementary Figure 1). STZ injection induced gingival epithelium morphological changes. The squamous epithelium of the DM group (Fig 2f, g) showed acanthosis and shortened irregular rete pegs compared with the NDM group (Fig 2b, c). In addition, the subcutaneous connective tissue in the DM group also became looser and thinner compared with the NDM group. DM significantly reduced the number and length of rete pegs compared with the NDM group (Fig 2d, h). The gingival sulcular/junctional epithelium of the DM group was also affected by the injection, with the spinosum layer of the junctional epithelium showing hyperplasia (Fig 2a, e). The immunohistochemical staining showed a significant decrease in gingival tissue blood vessel numbers in the DM group (Fig 2i, j, k). The number of cells positive for TNFα, IL-6 in the DM group was significantly higher than those in the NDM group, respectively (P<0.05).

DNA methylation analysis

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Genome wide DNA methylation analysis showed that STZ-induced diabetic status altered the methylated expression of 1163 genes, of which 599 and 564 genes were significantly hyper- and hypomethylated, respectively, in the DM versus the NDM group (Fig 3a).

To further identify the genetic functions and pathways possibly affected by the methylation changes in the gingival tissue of the DM group, we used GO term and KEGG pathway enrichment analyses. A total of 679 functional gene categories were found in the diabetic group, of which 290 and 389 were hyper- and hypomethylated, respectively. Genes responsible for biological processes comprised the largest portion (534 of 679, hyper 231 vs hypo 312), then, cellular components (69 of 679, hyper 25 vs hypo 44) and molecular functions (67 of 679, hyper 34 vs hypo 33). The top 20 GO terms of differentially expressed genes in biological processes (BP) showed that hypermethylated genes were involved in smooth muscle contraction, lipid transport, hormone secretion, blood vessel size regulation, and immune process, while hypomethylated genes were involved in lipid regulation, inflammation response, mononuclear cell migration, monocyte chemotaxis, regulation of tissue remodeling, and response to external stimuli. (Fig 3b, c). In addition, some differentially methylated GO categories associated with other important signaling pathways possibly related to morphological changes and periodontal tissue status were enriched in the DM group. In the GO hypermethylated BP, these included glucose metabolism, T cell apoptotic process, regulation of cell differentiation, complement activation, stem cell proliferation, regulation of cell fate commitment, lymphocyte apoptotic process, cellular response to chemical stimulus, insulin-like growth factor receptor signaling pathway and regulation of cell maturation; while in hypomethylated BP they included neutrophil chemotaxis, cell growth, cell differentiation, cell adhesion, blood vessel development, T cell regulation, lymphocyte aggregation, monocyte chemotaxis, detection of external stimulus, bone remodeling, toll-like receptor signaling pathway, and mast cell activation involved in immune response. Supplementary Table 1 and Table 2 list the top 20 enrichment differentially expressed genes of both hyper- and hypo-methylated biological processes (BP). Besides the top 20 categories, Supplementary Table 3 lists other interesting GO functional enrichment differentially expressed genes of both hyper- and hypo-methylated BP possibly related to morphological changes and periodontal tissue status in the DM group.

KEGG pathway analysis indicated that different signal pathways, such as cAMP, AMPK and PI3K-Akt, were involved in the hypermethylation process, while PPAR, Jak-STAT, FoxO, TNF, HIF-1, VEGF, and NOD-like receptor signal pathways were involved in the hypomethylation.
process. NF-kappa B was involved in both processes. The immune system was also significantly affected by STZ injection, however, only a small fraction of the signaling pathways were related to hypermethylation, such as complement and coagulation cascades and natural killer cell mediated cytotoxicity, while most signaling pathways were related to hypomethylation, such as T cell and B cell receptor signaling; Th1, Th2, and Th17 cell differentiation; Toll-like receptor; hematopoietic cell lineage; intestinal immune network for IgA production; and NOD-like receptor signaling. Moreover, metabolic and endocrine systems were also affected by STZ injection. All of these processes could contribute to the morphology and structural changes of the gingival tissue (Supplementary Table 4, 5).

Since inflammation regulating cytokines are key factors affecting periodontal tissue status, genes belonging to such systems and related to periodontal disease were selected from GO and KEGG analysis. Hypermethylated genes included complement component 1q (C1QS), mannan-binding lectin-associated serine protease-1 (MASP1), C-C Chemokine Ligand-5 (CCL5), complement component 4 binding protein (C4BPA), interferon-β (INFβ), and transforming growth factor beta (TGFβ); while hypomethylated genes included CXC chemokine ligands 9, 10, 12 (CXCL9, 19 12), CD86, complement C5a receptor 1 (C5AR1), complement component 3 (C3), tumor necrosis factor-related genes (TNFα), interleukin 6 (IL6), and C-C motif chemokine ligand 22 (CCL22). To further investigate the protein expression of such genes in gingival tissue, TNFα and IL-6 were selected for immunohistochemistry staining observation. The results showed that both genes were positively expressed and significantly increased under the junctional epithelium area in the DM versus the NDM group (Fig 4).

Discussion

The pathogenesis of both diabetes and periodontitis is influenced by genetic factors and epigenetic processes. Patients with diabetes show increased prevalence and severity of periodontitis. Although diabetes is associated with important epigenetic changes, the exact changes implicated in the susceptibility to periodontitis remain unknown.

In this study, we investigated the morphological and epigenetic DNA methylation changes of gingival tissues using a diabetic minipig model. Following STZ injection, a sustained significant
increase in blood glucose was maintained throughout the observing period. Histological analysis showed that pancreas and renal tissues presented classic histopathological changes, such as atrophic islets of Langerhans, degranulated pancreatic β-cells, and vacuolization of the renal tubular epithelial cells. The gingival (epithelial and connective) tissues in the lamina propria were also significantly affected by the DM induction, indicating that the periodontal tissue is a sensitive target for diabetes induced changes. Our results are in agreement with previous studies in different DM animal models.

The acanthosis of the gingival epithelium, and the decreased and sparse rete pegs observed upon DM induction in this study could possibly be due to an increase in mitotic activity in the epithelial basal layer, promoting the differentiation of epithelial cells and flattening of rete pegs. Several studies have demonstrated that the onset of diabetes can lead to reduced gingival collagen synthesis, intracellular degradation, and increased solubility, which could explain the irregular connective tissue changes observed in the DM group. In addition, we found that the number of blood vessels in the gingival tissue of the DM group significantly decreased when compared with those in the NDM group, indicating that hyperglycemia affects angiogenesis, which is also consistent with previous reports.

Since DM is closely associated with epigenetic changes, we investigated whether DM dependent epigenetic changes affect the periodontal tissue, and hence may be a potential mechanism for the increased susceptibility to periodontitis. To the best of our knowledge, this is the first study addressing the effect of diabetes on periodontal tissue from an epigenetic perspective. We show that DM significantly changed the DNA methylation level of the periodontal tissue, with 599 genes upregulated and 564 downregulated. Functional analysis of the hypermethylated genes revealed that the changes were closely related to lipid transport, hormone secretion, immune system, inflammatory response, angiogenesis, and metabolic activity, which are vital processes in the development of periodontitis. DNA methylation caused by DM could modify or disrupt the balanced status generally maintained in the ecological interactions between the host and the microbes, thus influencing the host susceptibility to the pathogenic bacteria. For example, the complement system, which can trigger, amplify, and regulate immune and inflammatory processes, is known to be deeply involved in the periodontal disease process. Both GO and KEGG analysis showed that the complement system was affected by DM dependent DNA methylation. The downregulation of C1QS, MASP1, C6, and C8 suggested that the inhibition of the classical and
lectin pathways of the complement cascade, while the hypomethylation of $C3$ and $C5AR1$ indicated that the alternative pathway of the complement system was still in an active state (Fig 5). *Porphyromonas gingivalis* (**P. gingivalis**), which is a key bacterial stimulus for periodontitis, can cause dysbiosis in the local periodontal environment with the help of complement $C5AR1^{46}$. **P. gingivalis** can target $C5AR$ and TLR2 subversive crosstalk, and induce a dysbiotic microbiota community. Once $C3$ is also activated, periodontitis may develop$^{45}$. Importantly, the alternative pathway plays a key role in chronic periodontitis$^{47,48}$. Based on our results, we can conclude that the periodontal tissue in the DM group reflects a compromised state that may be susceptible to the pathogenic mechanisms of **P. gingivalis** and other microorganisms. Consequently, periodontitis is likely to develop and might explain the reason that DM patients have a higher prevalence of periodontitis. DNA methylation changes in the complement system reflect just one of the aspects affected in the DM group.

The alterations observed in inflammation regulating cytokines also indicate that the gingival tissue in the DM group may be in a compromised state that is more susceptible to periodontal disease. Genes associated with inflammatory responses, such as *TNFA*, *IL-6*, and *CCL22*, were hypomethylated. TNFα and IL-6 are proinflammatory cytokines that can facilitate and amplify the inflammatory response through different pathways, and finally induce periodontal tissue degradation and bone resorption$^{49}$. Overexpression of TNFα and IL-6 has been regarded as a major contributor to periodontal disease$^{49-51}$. In this study, we observed high protein expression of the hypomethylated *TNFA* and *IL-6* genes under the junctional epithelium area in the DM group compared with those of the NDM group. CCL22 is a known chemoattractant derived from dendritic cells and macrophages belonging to the CC chemokine family$^{52}$. CC chemokines are crucial for the chemotaxis of monocytes/macrophages and lymphocytes, and regulate the switching of macrophages from M1 to M2 in the inflammation process, which play important roles in the inflammatory-immune response in periodontal disease$^{52}$. Moreover, CCL22 derived microparticles can recruit functional regulatory T cells to the periodontium and alleviate the inflammatory responses$^{53}$. The high expression of CCL22, TNFα and IL-6 all indicated that the periodontal tissue in the DM group was in a balanced, but more susceptible to periodontal disease status.
Overall, our results indicate that DM may cause morphological and epigenetic changes in periodontal tissue, altering the defense status of the tissue and increasing its susceptibility to periodontal disease.

It is worth mentioning that this is a pilot study based on a small sample size. More work needs to be done to further confirm the relationship between the DM and epigenetic changes of gingival tissue. Considering the minipig DM model is created by the injection of STZ, whether the epigenetic changes are induced by the drug itself or the DM model also deserves a further exploration.

Conclusions

The STZ injection induced diabetes mellitus causes significant morphological and DNA methylation changes in gingival tissue. DNA methylation of periodontal tissue could be one of the mechanisms for the increased susceptibility to periodontal disease in patients with diabetes.

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Disclosure

The authors declare that they have no conflict of interests.

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**Figure legends**

Fig 1 Fasting blood glucose levels (Fig 1a) and representative images of pancreas (Fig 1b, c, d, e, HE staining) from the DM and NDM groups. Compared with the NDM group, the glucose level was significantly increased in the DM group from day 45 after intravenous infusion of STZ (Streptozotocin). The islets of Langerhans in the DM group were atrophied and irregular compared with those in the NDM group. The β cells in the islets of the DM group (Fig 1e, yellow arrow) were reduced and severely degranulated compared with those in the NDM group (Fig 1c, red arrows).
Fig 2 Histological (HE staining) and immunohistochemical examination of gingival tissue. Compared to the NDM group (e, f, g), the gingival epithelium of the DM group (a, b, c) was affected by the STZ injection, showing acanthosis and shortened irregular rete pegs (dermal papillae) in the squamous epithelium (b, c vs f, g). The blue line (c,) and yellow line (g) represent the length of rete pegs in the NDM and DM groups, respectively. The gingival connective tissue of the DM group became looser and thinner than that of the NDM group. The STZ injection significantly reduced the number and length of the rete pegs in the DM group compared to the NDM group (d, h, P<0.05). Acanthosis of gingival sulcular/junctional epithelium was also observed in the DM group (e) compared with that of the NDM group (a). Immunohistochemical α-Smooth muscle actin (α-SMA) staining of gingival blood vessels showed that, compared with the NDM group (i), the total number of blood vessels in the DM group (j) was significantly decreased (k) (P<0.05).

Fig 3 Medip-seq analysis and the further Go and KEGG pathway enrichment analyses of the gingival tissues. In total, 1163 genes in the DM group were methylated compared with the NDM group, of which 599 were hypermethylated, and 564 were hypomethylated (Fig 3a). Fig 3b and 3c separately listed the top 20 biological processes (BP) of the differentially expressed genes, including both hyper- and hypo- methylated categories. The hypermethylated BP included smooth muscle contraction, single-organism biosynthetic process, lipid transport, regulation of hormone secretion, immune response etc., while hypomethylated BP included regulation of lipid transport, inflammatory response, regulation of tissue remodeling, response to external stimulus, cell to cell adhesion, leucocyte chemotaxis, monocyte chemotaxis etc.

Fig 4 Immunohistochemical staining of the hypomethylated genes, TNFα and IL-6 in both DM and NDM groups. TNFα and IL-6 were positively expressed and significant increased under the sulcular/junctional epithelium area in the DM group compared with that in the NDM group (red arrow shows positive expression).

Fig 5 Coagulation and complement cascades. Yellow color and red color represent hypermethylated and hypomethylated genes in the DM group, respectively.
**Supplementary data**

Supplementary Fig 1: Histology changes of renal tubules in DM and NDM groups (HE staining). The total number of renal tubules in the DM group was clearly decreased compared with those in the NDM group (a vs c). Vacuolar degeneration in multiple tubules containing prominent cytoplasmic vacuoles and pyknotic nuclei were observed in the DM group.

Supplementary tables:

Table 1: Top 20 categories of enrichment differentially expressed hypermethylated genes

Table 2: Top 20 categories of enrichment differentially expressed hypomethylated genes

Table 3: Interested of enrichment differentially expressed genes in both Hyper- and Hypomethylated BP

Table 4: The interested KEGG Sig pathway of hypermethylated DE genes

Table 5: The interested KEGG Sig pathway of hypomethylated DE genes
