Role of site-specific DNA demethylation in TNFα-induced MMP9 expression in keratinocytes

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Abstract

Inappropriately high expression of matrix metalloproteinase 9 (MMP9) in the late stage of diabetic foot ulcers suppresses wound healing. The underlying mechanisms are not completely understood. Site-specific demethylation was reported to function in the regulation of genes, causing persistent high expression of target genes. Therefore, this study was designed to determine whether site-specific DNA demethylation was a key regulatory component of MMP9 expression in diabetic wound healing, and to further verify the crucial CpG site(s). Human keratinocyte cell line (HaCaT) cells were exposed to tumor necrosis factor α (TNFα), and changes in MMP9 expression and DNA methylation status were detected. We found TNFα treatment increased endogenous MMP9 expression in HaCaT cells and decreased the DNA methylation percentage at the −36 bp promoter site in a time-dependent manner. Bisulfite sequencing PCR revealed differentially demethylated CpG sites in the human MMP9 promoter region, but only the change at the −36 bp site was statistically significant. Dual-luciferase reporter assays showed that the promoter with only the −36 bp site demethylated had slightly higher transcriptional activity than the promoter with all other sites except the −36 bp site demethylated. Our results demonstrate that site-specific DNA demethylation plays an important role in MMP9 expression in TNFα-stimulated keratinocytes. The −36 bp site in the MMP9 gene promoter is crucial to this effect, but other CpG sites may exert synergistic effects. Collectively, these data may contribute to the future development of novel therapeutic strategies to treat diabetic foot ulcers and prevent gangrene and amputation.

Key Words
- keratinocytes
- tumor necrosis factor a
- matrix metalloproteinase 9
- site-specific DNA demethylation
- diabetic foot ulcers

Introduction

Diabetic foot ulcers, a common complication of diabetes, have emerged as a significant burden to the healthcare industry (Boulton et al. 2005). Clinical observations have indicated that treatment options for diabetic ulcers are in many cases insufficient, and progression of the condition can ultimately lead to limb amputation. Therefore, a detailed understanding of the pathobiology of diabetic refractory ulcers is necessary.

Abnormal expression of matrix metalloproteinases (MMPs) in diabetic chronic wounds, especially that of MMP9, has been extensively studied (McLennan et al. 2008). Liu et al. (2009) reported that high MMP9 expression in
diabetic wound exudates is predictive of poor wound healing. A subsequent study in a murine model of wound healing further reinforced the concept that MMP9 acts to delay the wound healing process (Reiss et al. 2010). Our previous study comparatively analyzed the dynamic changes of MMP9 in skin wounds of diabetic and nondiabetic rats, and showed that high expression and activity of MMP9 contributed to delayed healing at the late stage of wound healing (after the 7th day) much more so than at the early stage (before the 3rd day) (Yang et al. 2009). MMP9 expression in normal wound healing is complex, with the protein concentration changing according to the phase of healing. The robust upregulation of MMP9 expression in the early phase, also known as the inflammatory phase, acts to promote phagocytosis of harmful substances and keratinocyte migration. In the following proliferative phase, the level of MMP9 decreases rapidly, presumably to protect against excessive degradation of extracellular matrix and growth factors. Imbalance of this regulatory process is believed to lead to the formation of chronic wounds, such as diabetic foot ulcers.

As an inducible gene, the expression of MMP9 was traditionally thought to be transient upon exposure to external stimuli, such as tumor necrosis factor α (TNFα), interleukin-1, interleukin-6, or bacterial endotoxins. TNFα, a known crucial element in the pathogenesis of diabetes (Hotamisligil & Spiegelman 1994) and diabetic macrovasculopathy (Natali et al. 2006), has been proven to be the most significant inflammatory factor in diabetic wounds (Landis et al. 2010). Research has confirmed that TNFα is able to stimulate high expression of MMP9 in keratinocytes (Bahar-Shany et al. 2009), noninflammatory cells that are the key source of MMP9 expression and secretion in diabetic skin wounds (Bahar-Shany et al. 2009), and play a unique role in wound healing through complex mechanisms (Sawicki et al. 2005, Brandner et al. 2012).

In diabetic wounds, the obstinate inflammatory reaction may result in persistent high MMP9 expression by continually produced TNFα. Nonetheless, other mechanisms may also be involved in this process. DNA methylation is one of the best-characterized epigenetic modifications and has been implicated in numerous biological processes. In particular, site-specific demethylation was reported to function in the dynamic regulation of genes that require rapid responses to specific stimuli (Wu & Zhang 2010). Chicoine (2002) indicated, for the first time, that changes in the status of DNA methylation could affect the transcriptional activity of the MMP9 promoter in murine thymic lymphoma cell lines. Roach et al. (2005) demonstrated that perturbed synthesis of MMP9 in osteoarthritic chondrocytes had resulted from demethylation at one particular CpG site in the MMP9 promoter. Based on these existing findings, we hypothesized that DNA demethylation may be a significant regulator of MMP9 expression in the process of diabetic wound healing. If so, abnormally high expression of MMP9 is expected to persist, even though all exogenous stimuli are removed, ultimately resulting in the development of refractory ulcer.

The present study was designed to determine whether DNA demethylation was a key regulatory component of MMP9 expression in diabetic wound healing, and to verify the crucial CpG site related to this mechanism.

Materials and methods

Cell culture and treatment

HaCaT cells, derived from Boukamp’s HaCaT cell line (Boukamp et al. 1988), were generously donated by Prof. Wu Xiaoping of the College of Life Science and Technology at Jinan University (Guangzhou, China). Cells were cultured in a 5% CO2 atmosphere in DMEM containing 10% FCS and 5.56 mM D-glucose (designated as ‘basic’ medium). The medium was changed every 2–3 days, and cell viability was determined using the cell counting kit-8 assay (Dojindo Laboratories, Kumamoto, Japan).

Based on the findings from previous studies (Ahmad et al. 2012, Zubair et al. 2012), a range of TNFα (5, 10, 20, and 50 ng/ml) concentrations were used to stimulate HaCaT cells in our preliminary experiments. Apoptosis was visually observed in cells treated with 50 ng/ml TNFα. Since cells treated with 10 ng/ml TNFα expressed a higher level of MMP9 than those treated with 5 or 20 ng/ml, the 10 ng/ml concentration was selected for the subsequent experiments. In one experiment, HaCaT cells were cultured in basic medium supplemented with 10 ng/ml TNFα (Gibco) for 1, 3, 7, 14, or 21 days. Cells cultured in basic medium alone served as the control group. In another experiment, HaCaT cells were divided into three groups: 5-aza-2’-deoxycytidine (DAC)/trichostatin (TSA)-treated; solvent-treated control; and untreated blank control. In the DAC/TSA-treated group, HaCaT cells were cultured with a combination of 2.5 μM DAC (Sigma–Aldrich) and 300 nM TSA (Alomone Labs, Jerusalem, Israel) for 3 days with media change every 24 h. Afterward, the cells were cultured in basic medium for an additional 2 days and then harvested. In the solvent-treated control group, cells were cultured with DMSO (equivalent volume
to the DAC/TSA treatment). In the untreated blank control group, cells were cultured in basic medium.

In the third experiment, HaCaT cells were stimulated with 10 ng/ml TNFα for 7 days and then changed to basic medium. Cells were harvested at three different time points: day 0, 3, and 7 after TNFα withdrawal. HaCaT cells cultured in basic medium throughout the experiment served as controls.

**Real-time RT-PCR**

Total RNA was isolated from cells using the TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was synthesized with the PrimeScript RT Reagent Kit (Takara, Shiga, Japan), according to the manufacturer’s protocol. The primers used are listed in Table 1. Real-time PCR was performed in a 20-μl reaction mixture that contained 2 μl of RT mixture, 0.2 μM of forward and reverse primers, 10 μM of Premix ExTaq containing SYBR Green I dye (Takara) and 7.2 μl of double-deionized H2O. The thermal cycling reaction was carried out by a LightCycler 480 real-time detection system (Roche Applied Sciences). The hot-start enzyme was activated by incubation at 95°C for 30 s, and cDNA was amplified by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a normalizing control. Relative expression was calculated by the Livak ΔΔCT method (Simon et al. 2011).

**Western blotting**

Treated and untreated HaCaT cells were washed twice with ice-cold PBS and harvested. Total cellular proteins were extracted with lysis buffer (200 mM Tris–HCl, 600 mM NaCl, 1% NaN3, 1% SDS, 5 mg/ml phenylmethylsulphonyl fluoride (PMSF), 100 μg/ml aprotinin, 4% NP-40 and 5% deoxycholic acid) supplemented with a protease inhibitor cocktail (Roche). For western blot analysis, 45 μg of the total cellular proteins were resolved by 10% SDS–PAGE (90 V for 90 min) and electroblotted onto a PVDF membrane using a Mini Trans-Blot Transfer Cell (Bio-Rad). After blocking with 5% skim milk powder in 0.1% Tween-20 in PBS (PBST) and washing with 0.1% PBST, the PVDF membrane was incubated with a primary mouse anti-human MMP9 antibody (1:1000 dilution; Santa Cruz Biotechnology), followed by incubation with a secondary goat anti-mouse IgG-HRP antibody (1:3000; Santa Cruz Biotechnology). Immunoreactive bands were developed with the ECL Plus substrate (Beyotime, Shanghai, China). GAPDH was detected with an anti-human GAPDH antibody (1:1000; Santa Cruz Biotechnology) and

**Table 1** Primes used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Length of PCR product (bp)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9-F</td>
<td>CAACATCACCTATATGGATCC</td>
<td>479</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>MMP9-R</td>
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<tr>
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</tr>
<tr>
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<td>388</td>
<td>BSP</td>
</tr>
<tr>
<td>BSP388-R</td>
<td>AAACCTTCTCCCTACCTCCTA</td>
<td>495</td>
<td></td>
</tr>
<tr>
<td>BSP495-</td>
<td>GATGGGGGATTTTTATGTTATT</td>
<td>89</td>
<td>Ms-HRM</td>
</tr>
<tr>
<td>BSPP9HRM-R</td>
<td>GCCGGCTATCTAAGGACTACT</td>
<td>361^</td>
<td>Cloning different lengths of promoters</td>
</tr>
<tr>
<td>MMP9-342 BamHI-F</td>
<td>cgccgattctTCAAGGAGGATGGGATC</td>
<td>542^</td>
<td></td>
</tr>
<tr>
<td>MMP9-523 BamHI-F</td>
<td>cgccgatcaAGGCCTGAGGAGGAGGAAAGA</td>
<td>559^</td>
<td></td>
</tr>
<tr>
<td>MMP9-540 BamHI-F</td>
<td>cgccgatcaAGGCGAAGCTGATCAAGAAGGC</td>
<td>590^</td>
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</tr>
<tr>
<td>MMP9-571 BamHI-F</td>
<td>cgccgattcATTCTCTCCCGCCCCCAGAT</td>
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<tr>
<td>MMP9-761 BamHI-F</td>
<td>cgccgattagtTATATTGTCTAAGGAGGATGGGATC</td>
<td>1304^</td>
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<tr>
<td>MMP9-1285 BamHI-F</td>
<td>ccaaggtttTGGGATGGGCTAGGAGGAGGAGGATG</td>
<td>45</td>
<td>Mutant GCGC → TAAT</td>
</tr>
<tr>
<td>MMP9mut-F</td>
<td>GGAGGGGTGGGGGTACAGGAGGAGGAGGATG</td>
<td>45</td>
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</tr>
<tr>
<td>MMP9mut-R</td>
<td>GTTGTTGGGTTAGTGGGATGGGAGGAGGATG</td>
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F, forward; R, reverse; BSP, bisulfite sequencing PCR; and Ms-HRM, methylation-sensitive high-resolution melt.

^The expected amplified fragment size is indicated for each forward primer when used with the MMP9HindIII-R reverse primer.
served as the normalizing control. The Alpha Imager 2000 was used to analyze the results (Alpha Innotech Corp., Johannesburg, South Africa).

ELISA

After treatments, the cell culture media were harvested and stored at −80 °C for subsequent evaluation by use of the Human MMP9 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). For all treatment conditions, the medium was changed 48 h prior to the time point of harvesting.

Bisulfite sequencing PCR analysis

Genomic DNA was extracted from HaCaT cells of the control group and the TNFα-treated group on day 14 using the QIAamp DNA Mini Kit (Qiagen). The isolated DNA was modified with the EpiTect Bisulfite Kit (Qiagen), according to the manufacturer’s instructions. PCR primers for detecting the methylation status of the MMP9 promoter were designed by MethPrimer (http://www.urogene.org/methprimer). Twelve CpG sites, located between −920 and +165 bp of the MMP9 promoter, were investigated with two pairs of PCR primers (Table 1).

PCR was performed in a 20-µl reaction solution containing 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 2 µl of 10× PCR buffer II (Applied Biosystems), 2 µl of bisulfite-treated DNA template, 750 nM forward and reverse primers, and 2.5 mM MgCl₂. The thermal cycling conditions included an activation step at 95 °C for 9 min, a two-step amplification program of 40 cycles at 94 °C for 1 min and 60 °C for 1 min, and a final extension at 60 °C for 10 min. The PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen) using the One Shot TOP10 chemically competent Escherichia coli cells provided with the kit. At least eight clones of each DNA specimen were randomly selected for verification. The respective plasmids were purified by the PureLink Quick Plasmid Mini-Prep Kit (Invitrogen) and sent to Invitrogen for sequencing.

DNA methylation percentage was analyzed using BiQ Analyzer software (http://biq-analyzer.bioinf.mpi-inf.mpg.de/). Quality parameters were ‘sequence error’ and ‘bisulfite conversion rate,’ which were set at 80 and 90% respectively.

Methylation-sensitive high-resolution melt analysis

PCR amplification and high-resolution melt (HRM) were performed on the RotorGene 6000 PCR Amplifier (Corbett Life Sciences, Brisbane, Australia). PCR was performed in a 20-µl reaction mixture containing 10 µl of 2× Fast-Plus EvaGreen Master Mix (Biotium, Hayward, CA, USA), 2 µl of bisulfite-treated DNA template, 250 nM forward and reverse primers, and 7 µl of RNase-free water. Each reaction was performed in triplicate. The thermal cycling conditions were as follows: 95 °C for 15 min; 50 cycles at 95 °C for 15 s, 50 °C for 15 s, and 72 °C for 25 s; 95 °C for 1 min and 40 °C for 2 min; continuous acquisition from 65 to 85 °C at 40 acquisitions/s. Each plate included multiple water-only blanks. Unconverted DNA (not treated with the bisulfite reagent) served as a negative control. CpGenome Universal Methylated DNA (Millipore, Bedford, MA, USA) and CpGenome Universal Unmethylated DNA (Millipore) served as the 100 and 0% methylated standards respectively. The standard ranges were constructed by diluting proportionally. The resulting relative signal values (%) reflected the proportion of methylated alleles to non-methylated alleles.

HRM data were analyzed using Gene Scanning software (Roche Applied Sciences). Output plots are presented in the form of normalized temperature-shifted melting curves that show the decrease in fluorescence against increasing temperature. The difference graph analysis function was used to normalize the 0% methylation standard curve to the horizontal axis.

Dual-luciferase reporter assays

To assess the effects of CpG methylation/demethylation on MMP9 expression, the Dual-luciferase Reporter Assay System (Promega) was used. All the reporter constructs were generated with the CpG-free luciferase reporter vector (generously donated by Prof. Rehli of the Department of Hematology and Oncology at the University Hospital in Regensburg, Germany), since the methylated CpGs within the coding region have been shown to suppress transcription activity (Klug & Rehli 2006). First, different lengths of MMP9 promoter sequences were cloned and linked to the pCpGL-basic expression vector. The pCpGL-MMP9 vectors were co-transfected with a phRG-TK vector (Promega) into human embryo kidney T293 cells (No. GNHu17 from the Shanghai Cell Bank, China). The GloMax Bioluminescent Detector (Promega) was used to detect promoter-driven fluorescence expression. The promoter sequence with the strongest transcriptional activity was selected for further analysis. The selected promoter sequence was processed to generate four conditions of different DNA methylation statuses by in vitro site-specific methylation, as follows. The promoter
The promoter sequence treated with M.SssI enzyme (New England Biolabs, Beverly, MA, USA) simulated the condition in which all the CpG sites were methylated. The promoter sequence treated with M.HhaI enzyme (New England Biolabs) simulated the condition in which all the CpG sites, except for the −36 bp site, were demethylated. The promoter sequence carrying a mutation at the −36 bp site was treated with M.SssI enzyme to simulate the condition in which all the CpG sites, except the −36 bp site, were methylated. To eliminate any potential influence on promoter activity by the point mutation at the −36 bp site, a group of mutated promoter sequences without methylase treatment were added. Success of the methylation reaction was verified prior to the reporter assays by digestion with methylation-specific restriction endonuclease. Finally, each of the promoter sequences described above was inserted into the pCpGL-basic vector by way of the standard site-directed PCR method. The primers used are listed in Table 1. The resulting gene fragment was sequenced to verify the integrity of the putative cis-elements that resided in the sequence.

Statistical analysis

Data that were not normally distributed were logarithmically transformed. All statistical analyses were carried out with the SPSS software package, version 13.0 (SPSS). Intergroup differences were analyzed by ANOVA. The least squares difference test was used for comparison between two groups. The data for methylation percentage were analyzed using the Wilcoxon signed-rank test. All data are expressed as mean ± S.D. A P value < 0.05 was considered significant.

Results

TNFα treatment increased MMP9 expression in and secretion from HaCaT cells

Since MMP9 is a secretory protein, we evaluated its mRNA transcription, intracellular and extracellular protein expression by real-time RT-PCR (Fig. 1A), western blot (Fig. 1B and J) and ELISA (Fig. 1C).

Real-time RT-PCR showed that TNFα stimulated MMP9 mRNA expression significantly at all of the time points examined (P < 0.05 vs control group). The maximal level of stimulation occurred at day 7 (41.1 ± 10.5-fold higher than control). The protein levels measured by western blot and ELISA showed the same trend, with 28.6 ± 0.7- and 37.6 ± 3.6-fold increases vs control group at day 7 respectively.

According to the real-time RT-PCR and western blot results, the expression changes between the 7th day and the 14th day were not significantly different (P > 0.05), but the differences between the 7th day and the 21st day were significant (P < 0.05). The concentration of secreted MMP9 in supernatant fluids declined significantly on the 14th day (P < 0.05).

Real-time RT-PCR data showed that a single treatment with TNFα elevated expression of MMP9 to 15.9 ± 2.3-fold higher than control levels by 24 h after the treatment. By 72 h posttreatment, the MMP9 expression had declined to a level that was only 1.7 ± 0.6-fold higher than that observed in control samples and that did not differ significantly from basal levels (P > 0.05; data not shown). By contrast, when expression was induced by repeated TNFα treatments delivered over a 7-day incubation period, the MMP9 expression persisted only briefly after withdrawal and then declined gradually toward normal levels. Immediately after withdrawal of TNFα, expression of MMP9 mRNA was 43.5 ± 2.0-fold higher than that in control samples. By day 3 and 7 after TNFα withdrawal, the MMP9 mRNA expression had declined to 28.4 ± 3.1- and 1.5 ± 0.3-fold respectively (Fig. 1G). The western blotting (Fig. 1H) and ELISA (Fig. 1I) data showed a similar trend.

Increased expression and secretion of MMP9 followed experimental demethylation

To verify the relationship between DNA demethylation and MMP9 expression, we first established an experimental demethylation cell model with the combined treatment of DAC and TSA (Kondo 2004). Indeed, the HaCaT cells cultured with a combination of DAC and TSA showed significantly higher levels of MMP9 mRNA and protein expressions (Fig. 1D, E and F) (P < 0.05 vs control group). To rule out any possible influence on the results by the DMSO solvent, we evaluated the mRNA and protein expressions in a group of cells treated with solvent only, and found no significant differences from the blank control group (P > 0.05).
Differentially demethylated CpG sites exist in the human MMP9 promoter region

The human MMP9 (GenBank Accession No: D10051) promoter region evaluated in this study is illustrated schematically in Fig. 2A. Only ten CpG sites were found in the 1000 bp of the proximal promoter, and two were found after the transcriptional start site. Thus, the MMP9 promoter was classified as a ‘sparse’ CpG promoter according to its lack of CpG islands. Bisulfite sequencing PCR (BSP) results (Fig. 2B) indicated that the overall DNA methylation percentage of the MMP9 promoter was relatively low in cells of the control group (0.43 ± 0.01%). However, the DNA methylation percentage for each particular site was distinctive. Among them, five CpG sites (−764, −712, −233, −223, and −36 bp) had a methylation percentage that was below 50%, such that demethylation prevailed in these sites under normal conditions (without intervention). After TNFα treatment for 14 days, methylation percentages declined significantly at the −36 bp site (P<0.05).
Figure 2
DNA methylation percentage at specific CpG sites, detected by BSP.
(A) Illustration of the MMP9 promoter. The white circles above the solid line represent the CpG dinucleotides. The locations of the two pairs of BSP primers (dotted lines) are presented, and the cis-elements are illustrated in Fig. 4A. (B) Mean (±s.d.) DNA methylation percentages at specific CpG sites are plotted for TNFα-treated and control groups. All the experiments were repeated for at least three times. ‘All ten CpG sites’ refers to the −861, −856, −764, −712, −624, −562, −233, −223, −185, and −36 bp sites.

The ‘#’ symbol indicates significant difference between TNFα-treated cells and control cells at the −36 bp site (P<0.05). (C) DNA methylation percentages at specific CpG sites are plotted for each individual DNA template. Each datum was calculated from sequencing results of eight independent clones. Results are presented as mean ±s.d. of three independent experiments. Full color version of this figure available via http://dx.doi.org/10.1530/JME-12-0172.
Methylation-sensitive-HRM assessment of DNA methylation percentage at the –36 bp site

Methylation-sensitive (Ms)-HRM analysis is a highly sensitive and reproducible method of detecting methylation (Balic et al. 2009). According to our Ms-HRM results (Fig. 3), the methylation percentages in control group cells were relatively stable (13.28 ± 2.51 to 16.18 ± 3.12%) at all the time points examined. After TNFα treatment, the methylation percentages showed a decreasing trend relative to intervention time (day 1: 9.36 ± 1.27%, day 3: 2.46 ± 0.84%, day 7: 0.93 ± 0.17%, day 14: 0.08 ± 0.02%, and day 21: 0.07 ± 0.02%). DAC treatment caused a reduction in methylation percentage (7.51 ± 2.17%), whereas solvent alone had no effect. Upon withdrawal of the TNFα intervention, the DNA methylation percentage was 0.67 ± 0.13%. By day 3 and 7 after TNFα withdrawal, the methylation percentage had changed to 1.98 ± 0.42 and 14.79 ± 2.65% respectively. The percentage at day 7 postwithdrawal did not differ from that observed in the control group (16.87 ± 2.80%).

Spearman’s correlation analysis of MMP9 expression vs DNA methylation percentage at the –36 bp site yielded a correlation coefficient of −0.878 (P<0.01). The demethylation trend observed for the –36 bp site corresponded to an elevation in MMP9 expression.

Dual-luciferase reporter analysis of the effect of DNA demethylation on MMP9 promoter activity

Analysis of a series of sequentially truncated MMP9 promoter sequences (Fig. 4B) showed that the –761 bp sequence had the highest transcriptional activity, but no significant difference from the activities of the –1285 and –571 bp sequences (P>0.05). When various methylation profiles within the MMP9 promoter were evaluated by the dual-luciferase reporter assay, the –36 bp site was the most sensitive regulator of MMP9 expression (Fig. 4D). There was no significant difference in function between the all-methylated promoter construct and the pCpG basic control construct. Methylation of the seven CpG

Figure 3

Ms-HRM results. (A) HRM curves for DNA methylation standards, which are displayed as duplicates. (B) Difference plots for DNA methylation standards. Melting curves were normalized to a 0% standard. The colored lines for the standards are: carmine, 20%; orange, 10%; yellow, 5%; green, 2.5%; blue, 1%; purple, 0.1%; and black, 0%. Each plot represents a representative experiment of HRM. (C) HaCaT cells were cultured in basic medium supplemented with 10 ng/ml TNFα for 1, 3, 7, 14, or 21 days. Cells cultured in basic medium alone served as the control group. The histogram indicates the quantified data of DNA methylation in each experimental group (D). HaCaT cells were cultured with a combination of 2.5 μM DAC and 300 nM TSA for 3 days with media change every 24 h. Afterward, the cells were cultured in basic medium for an additional 2 days and then harvested. Cells cultured with DMSO served as the solvent-treated control, and cells cultured in basic medium served as the untreated blank control. The histogram indicates the quantified data of DNA methylation in each experimental group (E). HaCaT cells were stimulated with 10 ng/ml TNFα for 7 days and then changed to basic medium. Cells were harvested at three different time points: day 0, 3, and 7 after TNFα withdrawal. HaCaT cells cultured in basic medium throughout the experiment served as controls. The histogram indicates the quantified data of DNA methylation in each experimental group. All the experiments were performed in triplicate.
sites suppressed MMP9 transcriptional potency completely. Interestingly, when all CpG sites in the promoter region were demethylated except the $-36$ bp site, transcription potency was suppressed to $0.50 \pm 0.17$-fold of control levels, whereas when all CpG sites in the promoter region were methylated except the $-36$ bp site, transcription potency was suppressed to $0.64 \pm 0.23$-fold of control levels. Hence, a single demethylation at the $-36$ bp site left the promoter with slightly more activity than demethylation of all of the other six sites. The activities, however, were not significantly different from each other ($P > 0.05$). Thus, we concluded that demethylation at the $-36$ bp site plays a key role in MMP9 expression, while other CpG sites may play supportive roles in this regulatory mechanism. The promoter with a point mutation at the $-36$ bp site did not show any alteration in activity, which verified that the mutation itself did not influence the promoter potency.

**Discussion**

Although there is very little doubt that wound healing requires a well-organized regulation of MMP9 expression in different phases and that inappropriately high expression of MMP9 in the late stage of diabetic skin ulcer suppresses wound healing, the underlying
mechanisms remain poorly understood. Previous studies focused on signal transduction pathways, identifying the trans-activators of the MMP9 promoter that promote its transient responses to exogenous stimuli, such as activator protein-1 (AP-1), nuclear factor-kappa B (NF-kB), Sp-1, and polyoma enhancer A-binding protein-3 (Yan & Boyd 2006). Although our current study was based on an in vitro assay system, the data suggest that DNA demethylation may be a key regulatory component of MMP9 expression in keratinocytes. Since DNA methylation status is thought to be heritable to daughter cells, abnormal expression of MMP9 will persist for a longer time span even after stimulating factors are removed, ultimately resulting in progression of refractory ulcer.

Our results show that the −36 bp site of the MMP9 gene promoter plays a key role in TNFα-induced MMP9 expression in keratinocytes. This conclusion is based on three pieces of experimental evidence. First, an approximate inverse correlation was observed between MMP9 expression and DNA methylation percentage at the −36 bp site. Second, DAC successfully induced demethylation at the −36 bp site and increased MMP9 expression. In our study, combined treatment with DAC and TSA was used to establish cell models for investigating DNA demethylation, to overcome the difficulty in using DAC alone. In a previous study, a colon cancer cell line was used to show that histone K9 modification and DNA methylation coexisted at silenced loci (Kondo et al. 2004). It remains unknown whether deacetylation is necessary for DNA demethylation regulation of MMP9 expression, and future studies should address this question. Third, dual-luciferase reporter assays further demonstrated the causal effect between altered DNA methylation status and MMP9 expression. Although more than one CpG site may have contributed to the regulation of MMP9 expression, our data indicate that the −36 bp site is the most sensitive regulatory site for MMP9 expression due to the effects of its demethylation being at least as powerful as (and perhaps even more powerful than) that of all of the other six CpG sites combined. Additional statistically supported studies are warranted to confirm this deduction.

DNA methylation produces transcriptional silencing by repressing the binding of transcription factors. When the CpG locations in the MMP9 promoter were compared with the known transcription factor binding sites (Fig. 2A), we recognized two concentration zones of cis-elements: one around −500 to −600 bp and the other proximal to the transcriptional start site. Our target site, the −36 bp site, is seated in the second concentration zone of cis-elements, which includes AP-1, KER M9 and Sp-1 binding sites. In a previous study, the Sp-1 site has been demonstrated to be critical for MMP9 transcription and heavily hypomethylated in murine lymphoma cells that overexpress MMP9 (Chicoine 2002). The results from Roach et al. (2005) and our current study further confirm the key role of Sp-1 as a regulator of the human MMP9 promoter. TNFα-induced NF-kB expression has been demonstrated to be associated with the demethylation process in other target gene promoters (Hashimoto et al. 2009). However, in our experiment, no significant findings were observed at the two sites closest to the NF-kB binding element (−624 and −562 bp). The synergistic cooperation of AP-1 with either NF-kB or Sp-1 was previously shown to be required for transcription of the MMP9 gene (Chicoine 2002). Therefore, we presume that Sp-1, instead of NF-kB, is more important in the regulation process that was observed in our study. Additionally, although the −36 bp site was shown to play a key role in TNFα-induced MMP9 expression, other CpG sites in the MMP9 promoter were also shown to contribute to this expression and may act to finely tune the response to TNFα in keratinocytes. Further studies are needed, however, to elucidate the underlying mechanism of this complex regulatory interplay.

The MMP9 promoter contains relatively few CpG sites. Accumulating evidence has indicated that methylation status at sparse CpG sites plays important roles in gene expression regulation (Fürst et al. 2012). It was reported that a complete deficiency of gene expression could be driven by promoter CpG island hypermethylation, while differential expression abundance could manifest from DNA methylation/demethylation over various CpG sites adjacent to key cis-elements (Fürst et al. 2012). Keratinocytes express a certain amount of MMP9 in the physiologic state, and this expression level can be induced by exogenous stimuli. Therefore, the expression difference observed in this study may have been achieved by differential methylation status of the −36 bp CpG site that influences the binding capacity of Sp-1. Both methyl-CpG-binding protein 2 and methylated DNA-binding domain-1 have been shown to repress transcription of a sparsely methylated Sp-1-dependent promoter (Fujita et al. 2000), indicating that both of these proteins may play an important role in controlling MMP9 gene transcription in keratinocytes.

In the present study, the percentage of DNA methylation at the −36 bp site was altered in a manner that was related to intervention time. Further demethylation occurred as the time of cell intervention increased. According to our Ms-HRM results, the demethylation
percentage stopped declining only after 14 days of intervention. This effect may be related to the significantly decreased cell viability that occurred after 14 days (data not shown). Since a longer time of exposure to exogenous stimuli leads to progressive demethylation, the time courses of inflammatory reactions in diabetic wounds should be carefully considered. In our cell model system, the methylation status at the −36 bp site was dynamically changed. Intriguingly, DNA methylation status was recently proven to be determined by a dynamic DNA methylation–demethylation bidirectional regulatory process, and the balance of several enzymes was proposed as indispensable to this event, including members of the DNA methyltransferase family and the ten-eleven translocation family (Bhutani et al. 2011). Nevertheless, the precise members that are involved and how the process is spatially and temporally regulated in different cell types and various pathologic contexts (for example, in our cell model) remain to be determined.

It is also noteworthy that our epigenetic data offer potential insights into diabetic metabolic memory in skin wound healing. Epigenetic modifications induced by transient hyperglycemia have been proven to play an important role in diabetic metabolic memory, as has been suggested by epidemiological studies (Ling & Groop 2009). Such regulatory mechanisms have been frequently investigated in diabetic microvascular and macrovascular complications (El-Osta et al. 2008, Villeneuve et al. 2008, Ling et al. 2009). Particularly, in a recent study, DNA hypomethylation and aberrant gene expression were demonstrated to result in heritable transmission of diabetic metabolic memory with the manifestation of impaired wound healing in a zebrafish model of diabetes (Olsen et al. 2012). High-level TNFα expression always accompanies high glucose by hyperglycemia-induced reactive oxygen species and other complex mechanisms, and elevated TNFα concentration is a recognized hallmark of diabetic wounds (Landis et al. 2010). Our study explored whether TNFα could induce heritable DNA demethylation and persistent aberrant MMP9 expression after TNFα withdrawal. We found that high expression of MMP9 and DNA demethylation only persisted for a short time after withdrawal. If this persistent effect is affirmed in future studies, we could infer that TNFα-induced DNA demethylation of MMP9 in keratinocytes may participate in the metabolic memory phenomenon of diabetic refractory ulcer. Therefore, once aberrant DNA demethylation of MMP9 occurs, diabetic ulcers would not be thoroughly resolved by control of blood sugar or elimination of infection, making early intervention particularly important. However, further study is needed.

In summary, our results provide preliminary insights into the regulatory mechanism of DNA demethylation and MMP9 expression. The observations reported here indicate that site-specific DNA demethylation may play an important role in the pathogenesis of diabetic refractory ulcers. Moreover, our epigenetic data offer clues about diabetic metabolic memory in wound healing. Further investigations are required to elucidate the detailed regulatory mechanisms involved in this process in vivo. All together, the present data contribute to the future development of novel therapeutic strategies to treat diabetic foot ulcers and prevent gangrene and amputation.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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