ORIGINAL ARTICLE

Blockade of receptors of advanced glycation end products ameliorates diabetic osteogenesis of adipose-derived stem cells through DNA methylation and Wnt signalling pathway

Maorui Zhang^{1,2,3} | Yong Li^{1,2,3} | Pengcheng Rao³ | Kui Huang³ | Daowen Luo³ | Xiaoxiao Cai² | Jingang Xiao^{1,3,4}

¹Department of Oral Implantology, The Affiliated Stomatology Hospital of Southwest Medical University, Luzhou, China

²State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China

³Orofacial Reconstruction and Regeneration Laboratory, The Affiliated Stomatology Hospital of Southwest Medical University, Luzhou, China

⁴Department of Oral and Maxillofacial Surgery, The Affiliated Stomatology Hospital of Southwest Medical University, Luzhou, China

Correspondence

Jingang Xiao, Department of Oral and Maxillofacial Surgery, The Affiliated Stomatology Hospital of Southwest Medical University, Luzhou, China.
Email: drxiaojingang@163.com

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Abstract

Objectives: Diabetes mellitus-related osteoporosis is caused by the imbalance between bone absorption and bone formation. Advanced glycation end products (AGEs) are considered a cause of diabetic osteoporosis. Although adipose-derived stem cells (ASCs) are promising adult stem cells in bone tissue regeneration, the ability of osteogenesis of ASCs in diabetic environment needs to explore. This study aimed to investigate the influence of AGEs on the osteogenic potential of ASCs and to explore the signalling pathways involved in its effect.

Materials and methods: ASCs were isolated from inguinal fat and cultured in osteogenic media with or without AGEs and FPS-ZM1, an inhibitor of receptor for AGEs (RAGE). Alizarin red-S, Oil Red-O and Alcian blue staining were used to confirm osteogenic, adipogenic and chondrogenic potential of ASCs, respectively. Immunofluorescence, western blotting and real-time PCR were used to measure changes in markers of osteogenic differentiation, DNA methylation and Wnt signalling.

Results: The multipotentiality of ASCs was confirmed. Treated with AGEs, OPN and RUNX2 expressions of ASCs were reduced and there was a noticeable loss of mineralization, concomitant with an increase in the expression of RAGE, 5-MC, DNMT1 and DNMT3a. AGEs treatment also led to a loss of Wnt signalling pathway markers, including β -Catenin and LEF1, with an increase in GSK-3 β . Treatment with the RAGE inhibitor, FPS-ZM1, rescued AGEs-induced loss of osteogenic potential, modulated DNA methylation and upregulated Wnt signalling in ASCs.

Conclusions: Our results demonstrate that AGEs-RAGE signalling inhibits the osteogenic potential of ASCs under osteoinductive conditions by modulating DNA methylation and Wnt signalling. FPS-ZM1 can rescue the negative effects of AGEs and provide a possible treatment for bone tissue regeneration in patients with diabetic osteoporosis.

1 | INTRODUCTION

Diabetes mellitus is a chronic, systemic disease characterized by high blood glucose levels.¹ Such glucose metabolic disorders can have detrimental effects on the skeletal system. Considerable evidence has shown that diabetes mellitus decreases bone mineral density, increases the risk of fracture and affects normal osteoblast metabolism, thereby altering normal bone regeneration and healing.²⁻⁴

Adult stem cells can be used as seed cells in bone tissue engineering. In recent years, adipose-derived stem cells (ASCs) have been revealed as promising candidates for bone tissue engineering, as they can be induced along the osteogenic lineage in a manner similar to that of bone marrow-derived stem cells (BMSCs) both *in vitro* and *in vivo*.⁵⁻⁸ The potential use of ASCs for bone regeneration in diabetic patients, therefore, deserves our attention.

Advanced glycation end products (AGEs) are formed by the non-enzymatic reaction of glucose with other proteins arising from long-term hyperglycaemia.⁹ Their interaction can modify and alter the function of intracellular proteins and other extracellular matrix components.^{9,10} Receptor for AGEs (RAGE) is a cell membrane-specific receptor that interacts with AGEs to affect cell function. Numerous studies have shown that the AGE-RAGE interaction can induce osteoblast apoptosis, inhibit the proliferation and migration of cells, decrease bone mass and promote osteoporosis in diabetic patients.¹¹⁻¹³ However, it remains unclear how AGEs induce their negative effects on stem cell osteogenic differentiation.

DNA methylation is a form of epigenetic modification that can impact a range of cellular processes, including proliferation and differentiation, by inhibiting gene expression.¹⁴⁻¹⁷ Katarzyna et al¹⁶ reported that aged-related degenerative changes in MSCs could be slowed-down by down-regulating DNA methylation levels. Previously, we showed that DNA methylation levels were higher in diabetic rats than in wild-type rats, with diabetic rats also exhibiting reduced bone mass and density.¹⁵ DNA methylation is mediated by DNA methyltransferases (DNMT1, DNMT3a, DNMT3b), which usually modify cytosine at position 5 in CpG dinucleotides to create 5-methylcytosine (5-MC).^{18,19} Therefore, changes in the expression of 5-MC and DNA methyltransferases can be used to measure whether DNA methylation affects osteogenic differentiation of AGE-treated ASCs. Thus, exploring the epigenetic regulation of ASC osteogenic differentiation may provide insight into the mechanism of diabetes-related bone disease.

In the present study, we isolated multipotent ASCs from Sprague-Dawley rats and treated cells with AGEs while undergoing osteogenic potentiation. Moreover, we tested whether AGE-RAGE signalling inhibits the osteogenic differentiation potential by modulating DNA methylation and explored the signalling pathways involved.

2 | MATERIALS AND METHODS

2.1 | Isolation and multipotential differentiation capacity of ASCs

All animal procedures were reviewed and approved by the Southwest Medical University Ethical Committee. The animal care and anaesthesia were conducted in accordance with the guidelines of the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). The inguinal fat was collected for the production of ASCs from 1-week-old female Sprague-Dawley rats under sterile conditions. Fat was cut finely and immersed in 0.075% collagenase I (Sigma-Aldrich, St Louis, MO, USA) at 37°C in a water bath for 1 h. Digestion was terminated with the addition of 10% foetal bovine serum (FBS, Schaumburg, USA) diluted in α -MEM (Hyclone, USA) and the suspension was centrifuged at 1000 rpm for 5 min. The supernatant was removed and cells were resuspended with α -MEM/10% FBS. The suspension was inoculated into culture flasks and incubated at 37°C and 5% CO₂.

ASCs were passaged to the third generation to obtain relatively pure ASC cultures. For osteogenic induction, ASCs (5×10^4 cells) were seeded into the wells of 6-well plates and cultured in Osteogenic Medium (Cyagen, Santa Clara, USA). For adipogenic induction, ASCs (1×10^5 cells) were seeded into the wells of 6-well plates and cultured in Adipogenic Medium (Cyagen). After 21 days, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 1 h and then incubated with Alizarin red-S (osteogenic) or 0.3% Oil Red-O (adipogenic) stains for 30 min. Cells were observed and imaged using an inverted phase contrast microscope (Nikon, Tokyo, Japan). For cartilage induction, ASCs (2.5×10^5 cells) were centrifuged in centrifuge tubes without resuspending and cultured in Cartilage Medium (Cyagen). After 21 days, cell aggregates were washed twice with PBS, fixed in 4% paraformaldehyde for 1 h and imaged using a stereo fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany). The cells balls were embedded in paraffin and stained with Alcian blue staining. Sections of the cell balls were observed and imaged using an optical microscope (Nikon).

2.2 | Cell proliferation analysis treatment with reagents

We used a Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich) to assess cell proliferation after treatment with advanced glycation end-product bovine serum albumin (AGEs, BioVision, USA) and 4-Chloro-N-Cyclohexyl-N-(Phenylmethyl)-Benzamide (FPS-ZM1, inhibitor of RAGE) (MedChemExpress, Monmouth Junction, NJ 08852 USA). Cells were seeded in 96-well plates at a density of 3000 cells per well and cultured in α -MEM containing 10% FBS for 7 days. Cells were treated with different concentrations of AGEs (40 μ g, 80 μ g, 120 μ g, 160 μ g/mL) and FPS-ZM1 (10 nM, 100 nM, 1 μ M, 10 μ M, 100 μ M/mL) to select the optimum concentration. CCK-8 assay was performed on days 1, 4 and 7 after treatment, according to manufacturer's instructions. A BioTek ELX800 (Bio-Tek, Minneapolis, Minnesota, USA) was to measure the absorbance at 450 nm.

2.3 | Alizarin Red-S staining

Mineralized nodule formation in ASC cultures was assessed using Alizarin red-S staining. ASCs (5×10^4 cells) in 6-well plates were treated with 80 $\mu\text{g/mL}$ AGEs and 10 $\mu\text{M/mL}$ FPS-ZM1 in Osteogenic Medium for 21 days. Cells were then washed twice with PBS, fixed in 4% paraformaldehyde for 30 min and then stained with Alizarin red-S, according to standard procedures.

2.4 | Western blot assay

Cells treated with AGEs and FPS-ZM1 were lysed for total protein using a Total Protein Extraction Kit (Keygen Biotech, China). Protein concentration was determined using the BCA protein assay kit (Thermo Scientific, MA, USA). Proteins were separated on 10% (v/v) SDS-PAGE, and the gels were transferred to polyvinylidene difluoride membranes at 200 mA for 1 h. Membranes were blocked with 5% bovine serum albumin (BSA) diluted in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) for 1 h, and then incubated with antibodies specific to GAPDH (ab181602), OPN (ab91655), DNMT3a (ab188470) and RAGE (ab3611) (Abcam, UK); and RUNX2 (12556s), DNMT1 (5032S), LEF1 (2230p), β -Catenin (D10A8) and GSK-3 β (9315s) (Cell Signaling Technology, USA) overnight at 4°C. Membranes were washed with TBST thrice and then incubated with appropriate secondary antibodies (Beyotime, Shanghai, China) for 1 h. Membranes were again washed thrice with TBST and developed using a Luminol Reagent Kit (Santa Cruz, Dallas, TX, USA) with an enhanced chemiluminescence detection system (Bio-Rad, USA).

2.5 | Immunofluorescence staining

ASCs were cultured for 4 days with different treatments, washed with PBS, fixed with 4% paraformaldehyde for 30 min and permeabilized using 0.5% Triton X-100 for 5 min. Cells were then blocked with 5% goat serum for 1 h and incubated at 4°C overnight with antibodies (RUNX2, OPN, DNMT1, DNMT3a, 5-MC). The next day, the cells were rewarmed at room temperature for 30 min and then incubated with an appropriate fluorescence-conjugated anti-rabbit secondary antibody (Beyotime Shanghai, China.) for 1 h. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Between each step, samples were washed with PBS. Images were captured with an upright fluorescence microscope (Olympus, Japan).

2.6 | Extraction of RNA and real-time polymerase chain reaction (RT-PCR)

RT-PCR was used to measure changes in the expression of a range of markers: osteopontin (*Opn*) and runt-related transcription factor 2 (*Runx2*), DNA methyltransferases 1/3a (*Dnmt1/3a*), receptor of advanced glycation end products (*Rage*), β -catenin, glycogen synthase kinase 3-beta (*Gsk-3 β*) and lymphoid enhancer-binding factor-1 (*Lef1*).

TABLE 1 Primer sequences for amplification of genes for RT-PCR

Genes	Sequence (5'→3')	Length (bp)
<i>Gapdh</i>	Forward: ACAGCAACAGGGTGGTGGAC	233
	Reverse: TTTGAGGGTGCAGCGAACTT	
<i>Runx2</i>	Forward: AGGGACTATGGCGTCAAACA	137
	Reverse: GGCTCACGTCGCTCATCTT	
<i>Opn</i>	Forward: CACTCCAATCGTCCCTACA	160
	Reverse: CTTAGACTCACCGCTCTTCAT	
<i>Dnmt1</i>	Forward: AGCAGCTTGGCCCAGACCTA	190
	Reverse: TGAGGCTCCAAGGTAGCGCC	
<i>Dnmt3a</i>	Forward: GGAAAGATCATGTACGTCGGG	178
	Reverse: GGAGGCGGTAGAACTCAAAGA	
<i>Rage</i>	Forward: ACAGAAACCGGTGATGAAGG	207
	Reverse: ATTCAGCTCTGCACGTTCCCT	
β - <i>Catenin</i>	Forward: AAGTTCTTGGCTATTACGACA	281
	Reverse: ACAGCACCTTCAGCACTCT	
<i>Gsk-3β</i>	Forward: AACTCCACCAGAGGCAATCG	366
	Reverse: CGTTGCACTCTTAGCCCTGT	

Total RNA was extracted from ASCs using the total RNA extraction kit (BioFlux, China). cDNA was synthesized from total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Japan). Primer sequences are presented in Table 1. RT-PCR was performed using the SYBR Premix ExTaq kit (Takara Bio) and an ABI 7300 system (Applied Biosystems, USA) as follows: 95°C for 30 s; and 40 cycles of 95°C for 5 s and 60°C for 31 s. PCR products were verified by melting curve analyses between 60°C and 95°C. Gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, where the values from different samples were averaged and calibrated in relation to *Gapdh* CT values.

2.7 | Statistical analysis

All experiments were repeated at least 3 times independently, and representative data are provided as the mean $\pm$ SD. *P* values were calculated using a one-way ANOVA with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences were accepted as statistically significant if *P* < .05.

3 | RESULTS

3.1 | Multipotential differentiation of ASCs

ASCs from inguinal fat were cultured and passaged up to the third generation (Figure 1A). Treatment with osteogenic and adipogenic media noticeably changed the morphology of ASCs, differentiating into osteogenic-like cells in osteogenic medium, as shown by the mineralized-like extracellular matrix (Figure 1B,E); and adipose-like

FIGURE 1 Multipotential differentiation of ASCs. A, Normal appearance of ASCs after 3 generations of passaging, visualized using an inverted phase contrast microscope. B, ASCs differentiate into osteogenic-like precursor cells in Osteogenic Media, with evidence of a mineralized-like extracellular matrix. C, ASCs differentiate into adipose-like precursor cells in Adipogenic Media, as shown by the presence of oil droplets. D, ASCs agglomerate into a cell ball in the presence of Chondrogenic Media, as viewed using a stereo fluorescence microscope. Alcian blue staining shows the presence of a cartilaginous extracellular matrix (blue; optical microscope). E, Alizarin red-S staining depicts mineralized nodules (red granules). F, Oil red-O staining shows the formation of oil droplets within the cells (bright orange granules)

cells in adipogenic medium demonstrated by the presence of several oil droplets (Figure 1C,F). Similarly, ASCs grown in cartilage medium suspension for 21 days were grouped into cell aggregates and stained positively with Alcian blue, indicative of cartilage-like cells (Figure 1D). Overall, these findings show the multipotentiality of isolated ASCs.

3.2 | Cell proliferation

We treated ASCs with different concentrations of AGEs (40 μ g, 80 μ g, 120 μ g, 160 μ g/mL; or BSA as a negative control) or FPS-ZM1 (10 nM, 100 nM, 1 μ M, 10 μ M or DMSO as a negative control) for 1,

FIGURE 2 Cell proliferation. A-C, Cell viability was significantly inhibited by AGEs in a dose-dependent and time-dependent fashion. D-F, FPS-ZM1 inhibitor is non-toxic to cells at 10 µM. Data are presented as the mean $\pm$ SD ($n = 3$), * $P < .05$, ** $P < .01$, *** $P < .001$

4 and 7 days, and then tested for changes in cell proliferation using the CCK-8 assay. We found that AGEs inhibited ASC proliferation, whereas FPS-ZM1 was non-toxic (Figure 2). To maintain cell viability above 80%, we chose 80 µg/mL AGEs and 10 µM/mL FPS-ZM1 for subsequent experiments.

3.3 | AGEs/RAGE suppress ASCs osteogenic differentiation capacity

To investigate whether AGEs inhibit the osteogenic potential of ASCs through RAGE, we treated ASCs with 80 µg/mL AGEs for 21 days and tested for changes in mineralized nodule formation and gene and protein expression of OPN, RUNX2 and RAGE; the control cells were treated with 80 µg/mL BSA. Using Alizarin red-S staining, we found that AGEs reduced the degree of mineralized nodule formation in the cultures as compared with the control group (Figure 3A).

OPN and RUNX2 protein levels were analysed using immunofluorescence and western blotting. We found AGEs reduced the fluorescence signals (Figure 3B) and band intensity on western blots (Figure 3C) for both proteins as compared with the control treatment in a time-dependent manner. The mRNA levels for *Opn* and *Runx2* in the AGE group were also significantly lower than in the control group at each time point (Figure 3D). As for RAGE, both the

mRNA and protein levels of RAGE were increased at days 1, 4 and 7 (Figure 3E,F).

3.4 | AGEs upregulate the DNA methylation level in ASCs

DNMT1, DNMT3a and DNMT3b are key enzymes in the DNA methylation process, and 5-MC is the product of DNA methylation. We analysed how AGE treatment would affect the expression of these markers using immunofluorescence staining, western blotting and RT-PCR. Using immunofluorescence staining, we found an increase in 5-MC, DNMT1 and DNMT3a after AGE treatment at 4 days (Figure 4A), but no difference in DNMT3b expression (data not shown). These increases in DNMT1 and DNMT3a were confirmed by western blotting (Figure 4B) and RT-PCR (Figure 4C) at days 1, 4 and 7.

3.5 | Inhibitor of RAGE (FPS-ZM1) rescues AGE-induced loss of osteogenic potential and modulates DNA methylation in ASCs

AGE treatment reduced the osteogenic differentiation capacity of ASCs and increased the expression of DNMT1, DNMT3a and

FIGURE 3 AGEs-RAGE signalling suppresses ASCs osteogenic differentiation under conditions of osteogenic induction. A, Alizarin red-S staining shows significantly fewer and smaller mineralized nodules in cells treated with AGEs as compared with the control group. Immunofluorescence staining (B) and western blot (C) for RUNX2 and OPN in ASCs. GAPDH serves as an internal control. Quantitative analysis for western blotting was performed by measuring the OD. D, Runx2 and Opn mRNA levels were quantified by RT-PCR analysis ($n = 3$). GAPDH served as internal control. RT-PCR (E) and Western blot (F) results show an increase in the expression of RAGE in a time-dependent manner. Data are the mean $\pm$ SD ($n = 3$), * $P < .05$, ** $P < .01$, *** $P < .001$

5-MC. Therefore, in our next experiment, we explored the relationship among AGEs, DNA methylation and the osteogenic differentiation potential of ASCs. We used a RAGE inhibitor, 10 µM/

mL FPS-ZM1 (10 µM/mL DMSO as a negative control) to test whether inhibiting AGE function could rescue ASC capacity for osteogenic differentiation. Using western blotting, we found that

FIGURE 4 AGEs upregulate DNA methylation in ASCs. A, Fluorescence staining, (B) western blotting and (C) RT-PCR for 5-MC (green) and DNMT 1/3a (red) at day 4. 5-MC, DNMT1 and DNMT 3a were increased following AGEs treatment. Data are the mean $\pm$ SD ($n = 3$), * $P < .05$, ** $P < .01$, *** $P < .001$

FPS-ZM1 treatment led to a decrease in RAGE protein and mRNA levels (Figures 5A,B). After 21 days, we found that FPS-ZM1 treatment increased Alizarin red-S staining, with and without AGEs (Figure 5C). Similarly, *Opn* and *Runx2* mRNA levels were increased in both the FPS-ZM1 and AGEs+FPS-ZM1 groups as compared with AGE-treated group and control group (Figure 5D). The results of the western blot assay were consistent with those of RT-PCR (Figure 5E).

To further assess the effect of DNA methylation on ASC osteogenic differentiation, we measured changes in the expression of DNMT1, DNMT3a and 5-MC in the presence and absence of FPS-ZM1. 5-MC staining was strongest in the AGE group, with FPS-ZM1 co-treatment decreasing 5-MC expression by day 4 (Figure 6A). At day 7, FPS-ZM1 and AGE co-treatment led to a decrease in *Dnmt1* and *Dnmt3a* mRNA levels as compared with AGE treatment alone (Figure 6B), with consistent findings shown with western blotting for protein levels (Figure 6C). Together, these results suggest that FPS-ZM1 can rescue the loss of osteogenic capacity after AGE treatment, presumably by modulating DNA methylation levels in ASCs.

3.6 | FPS-ZM1 regulates Wnt signalling pathway to ameliorate the AGE-induced loss of osteogenic capacity in ASCs

The Wnt/ β -catenin signalling pathway is crucial in osteogenic differentiation. Therefore, we sought to detect changes in key players within the Wnt/ β -Catenin pathway, including β -Catenin, GSK-3 β and LEF1, in response to AGEs and FPS-ZM1. We show that AGE treatment decreased the expression of β -Catenin and LEF1 but increased the expression of GSK-3 β at days 1, 4 and 7 as compared with the control group (Figure 7A,C). These changes were recovered by co-treatment with FPS-ZM1 at day 7 (Figure 7B,C), suggesting that AGEs inhibit the activity of the WNT signal pathway, which can be recovered by blocking RAGE signalling.

4 | DISCUSSION

Diabetes mellitus causes glucose metabolic dysfunction and is responsible for bone loss, changes in bone quantity and quality, and

FIGURE 5 The effect of FPS-ZM1 treatment on osteogenic potential of ASCs. A-B, FPS-ZM1 blocks RAGE in the FPS and AGEs + FPS groups. C, FPS-ZM1 rescues AGEs-induced osteogenic capacity of ASCs in the AGEs+FPS group, as determined by Alizarin red-S staining. D, E, RUNX2 and OPN gene and protein levels were upregulated following FPS-ZM1 treatment. Data are the mean $\pm$ SD ($n = 3$), * $P < .05$, ** $P < .01$, *** $P < .001$

impaired fracture healing.^{2,4,20} An increase in the concentration of AGEs may contribute to this diabetes-associated loss of bone health. Indeed, others have shown that AGEs can interfere with osteoblast differentiation and induce apoptosis in bone-forming cells in patients with diabetes.^{21,22} In our study, we showed a concentration- and time-dependent reduction in ASC viability with AGE treatment. Therefore, we investigated how AGEs affect the osteogenic potential of ASCs and the molecular mechanisms driving these changes.

Diverse studies have shown that AGEs are involved in various tissue pathologies arising from diabetic complications, including vessels, skin, bone, kidney and periodontal tissues.²³⁻²⁶ Kume et al²² reported that fat, cartilage and bone differentiation from MSCs were reduced following artificially supplemented AGEs. Yamamoto et al²⁴ reported that AGEs caused a significant inhibition of osteocalcin synthesis in human osteoblasts. Others have shown that RAGE, the most studied

receptor of AGEs, was expressed higher in cells from diabetic tissues, rendering the cells more sensitive to the effects of AGEs.²⁷ Treatment with FPS-ZM1, a high-affinity, novel, multimodal, RAGE-specific inhibitor, can rescue the negative effects of AGEs/RAGE in various cell types.²⁸⁻³⁰ We found that normal ASC proliferation, osteogenic differentiation and mineralized nodule formation were reduced by AGEs, along with an upregulation in RAGE expression. When we blocked RAGE using FPS-ZM1, we could rescue these expression levels.

DNA methylation affects a wide range of genetic molecular changes and is often associated with bone diseases.^{31,32} As adult stem cells are important tools for bone regeneration, the potential role of epigenetic modification in osteogenic differentiation has attracted much attention. In a recent study, transplantation of DNA methylation-modified BMSCs could rescue osteopenia in a mouse lupus model, restoring recipient BMSCs function.³³ Liu et al¹⁵ reported a high expression

FIGURE 6 FPS-ZM1 modulates DNA methylation levels in ASCs. A, Immunofluorescence shows that FPS-ZM1 counteracts the high expression of 5-MC induced by AGEs. B, C, FPS-ZM1 and AGEs co-treatment decreases DNMT 1/3a expression as compared with AGE treatment alone. Data are the mean $\pm$ SD ($n = 3$), * $P < .05$, ** $P < .01$, *** $P < .001$

of 5-MC in diabetic rat periodontium and found that DNA demethylation can rescue the impaired osteogenic differentiation ability of human periodontal ligament stem cells in the presence of high glucose. Work by Li et al³⁴ showed that dexamethasone treatment can shift bone marrow stromal cells from an osteoblast fate towards the adipocytic lineage through methylation of the C/EBP α promoter. In our study, we detected a high expression of 5-MC, DNMT1 and DNMT3a in AGE-treated ASCs, suggestive of an upregulation in DNA methylation following AGE treatment. We surmise that the interaction between AGEs and RAGE increased the degree of DNA hypermethylation in the cells, and thereby reduced their potential for osteogenic differentiation.

The Wnt signalling pathway is a highly conserved signalling pathway in cell evolution, playing an important role in several biological

processes.³⁵⁻³⁷ Indeed, abnormal changes in Wnt signalling pathway are linked to a loss of bone mass, altered bone metabolism and osteoporosis.^{38,39} DNA methylation changes have also been previously linked with activation of the Wnt signalling pathway. For example, Cho et al⁴⁰ reported that drug-induced epigenetic activation of *Bmp2* contributes to Wnt3a-mediated transdifferentiation of pre-adipocytes or fibroblasts into osteoblasts. Liang et al⁴¹ showed that *Dkk-1* and *Dkk-3* methylation had marked effects on gene expression of Wnt/ β -catenin signalling pathway in hepatocellular carcinoma. In our study, we found a time-dependent reduction in the expression of β -catenin and Lef1 and an increase in GSK-3 β following AGE treatment. With FPS-ZM1 co-treatment, we were able to upregulate the osteogenic potential of ASCs through the activation of canonical Wnt signalling pathway. Future experiments should include cell microarray and bisulphite genomic sequencing to identify differential

FIGURE 7 Protein and gene expression of Wnt signalling pathway factors in ASCs treated with AGEs and the FPS-ZM1 inhibitor. A, AGEs alone decreased β-Catenin and LEF1 expression over time and increased GSK-3β expression. B, C, At day 7, β-Catenin, LEF1 and GSK-3β expression levels were recovered by AGEs and FPS-ZM1 co-treatment as compared with AGEs treatment alone. Data are the mean ± SD (n = 3), *P < .05, **P < .01, ***P < .001

genes and DNA methylation-variable positions involved in these AGE-mediated changes in osteogenic potential.

Although there has been a significant progress in the field of bone tissue regeneration,⁴²⁻⁴⁴ numerous concerns remain for patients with diabetes mellitus, and further exploration of the detailed mechanisms required to restore the damaged osteogenic potential of ASCs. In this study, we show that FPS-ZM1 treatment can rescue the loss of osteogenic differentiation of ASCs by inhibiting AGE-induced DNA hypermethylation. Furthermore, we show that the Wnt signalling pathway plays an important role in this process. Overall, our findings provide insight into the importance of DNA methylation regulation for improving ASC osteogenic induction in the presence of AGEs, and our findings may also offer a solution for bone tissue regeneration in patients with diabetes-associated osteoporosis.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

ORCID

Jingang Xiao <http://orcid.org/0000-0003-1300-7191>

REFERENCES

1. Bell GI, Polonsky KS. Diabetes mellitus and genetically programmed defects in b-cell function. *Nature*. 2001;414:788-791.
2. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes-a meta-analysis. *Osteoporos Int*. 2007;18:427-444.
3. Forsen L, Meyer HE, Midthjell K, et al. Diabetes mellitus and the incidence of hip fracture: results from the Nord-Trøndelag Health Survey. *Diabetologia*. 1999;42:920-925.
4. Yamamoto M, Sugimoto T. Advanced glycation end products, diabetes, and bone strength. *Curr Osteoporos Rep*. 2016;14:320-326.
5. Lin S, Zhang Q, Shao X, et al. IGF-1 promotes angiogenesis in endothelial cells/adipose-derived stem cells co-culture system with activation of PI3K/Akt signal pathway. *Cell Prolif*. 2017;50:e12390-e12399.

6. Cai X, Xie J, Yao Y, et al. Angiogenesis in a 3D model containing adipose tissue stem cells and endothelial cells is mediated by canonical Wnt signaling. *Bone Res.* 2017;5:17048.
7. Xie Q, Xie J, Zhong J, et al. Hypoxia enhances angiogenesis in an adipose-derived stromal cell/endothelial cell co-culture 3D gel model. *Cell Prolif.* 2016;49:236-245.
8. Zhang T, Lin S, Shao X, et al. Regulating osteogenesis and Adipogenesis in adipose-derived stem cells by controlling underlying substrate stiffness. *J Cell Physiol.* 2017;233:3418-3428.
9. Yang DH, Chiang TI, Chang IC, et al. Increased levels of circulating advanced glycation end-products in menopausal women with osteoporosis. *Int J Med Sci.* 2014;11:453-460.
10. Wu YY, Xiao E, Graves DT. Diabetes mellitus related bone metabolism and periodontal disease. *Int J Oral Sci.* 2015;7:63-72.
11. Ding KH, Wang ZZ, Hamrick MW, et al. Disordered osteoclast formation in RAGE-deficient mouse establishes an essential role for RAGE in diabetes related bone loss. *Biochem Biophys Res Commun.* 2006;340:1091-1097.
12. Yang K, Wang XQ, He YS, et al. Advanced glycation end products induce chemokine/cytokine production via activation of p38 pathway and inhibit proliferation and migration of bone marrow mesenchymal stem cells. *Cardiovasc Diabetol.* 2010;9:66-75.
13. Taylor JJ, Preshaw PM, Lalla E. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. *J Periodontol.* 2013;84:S113-S134.
14. Nishikawa K, Iwamoto Y, Kobayashi Y, et al. DNA methyltransferase 3a regulates osteoclast differentiation by coupling to an S-adenosylmethionine-producing metabolic pathway. *Nat Med.* 2015;21:281-287.
15. Liu Z, Chen T, Sun W, et al. DNA demethylation rescues the impaired osteogenic differentiation ability of human periodontal ligament stem cells in high glucose. *Sci Rep.* 2016;6:27447-27458.
16. Kornicka K, Marycz K, Marekziak M, et al. The effects of the DNA methyltransferases inhibitor 5-Azacytidine on ageing, oxidative stress and DNA methylation of adipose derived stem cells. *J Cell Mol Med.* 2017;21:387-401.
17. Zhou C, Liu Y, Li X, et al. DNA N(6)-methyladenine demethylase ALKBH1 enhances osteogenic differentiation of human MSCs. *Bone Res.* 2016;4:16033-16041.
18. Okano M, Bell DW, Haber DA, et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell.* 1999;99:247-257.
19. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci.* 2006;31:89-97.
20. Lozano D, de Castro LF, Dapia S, et al. Role of parathyroid hormone-related protein in the decreased osteoblast function in diabetes-related osteopenia. *Endocrinology.* 2009;150:2027-2035.
21. Alikhani M, Alikhani Z, Boyd C, et al. Advanced glycation end products stimulate osteoblast apoptosis via the MAP kinase and cytosolic apoptotic pathways. *Bone.* 2007;40:345-353.
22. Kume S, Kato S, Yamagishi S, et al. Advanced glycation end-products attenuate human mesenchymal stem cells and prevent cognate differentiation into adipose tissue, cartilage, and bone. *J Bone Miner Res.* 2005;20:1647-1658.
23. Zhou L, Wang W, Yang C, et al. GADD45a promotes active DNA demethylation of the MMP-9 promoter via base excision repair pathway in AGEs-treated keratinocytes and in diabetic male rat skin. *Endocrinology.* 2017;159:1172-1186.
24. Yamamoto T, Ozono K, Miyauchi A, et al. Role of advanced glycation end products in adynamic bone disease in patients with diabetic nephropathy. *Am J Kidney Dis.* 2001;38:S161-S164.
25. Verzijl N, Bank RA, Tekoppele JM, et al. AGEing and osteoarthritis: a different perspective. *Curr Opin Rheumatol.* 2003;15:616-622.
26. Bohlender JM, Franke S, Stein G, et al. Advanced glycation end products and the kidney. *Am J Physiol Renal Physiol.* 2005;289:F645-F659.
27. Santana RB, Xu L, Chase HB, et al. A role for advanced glycation end products in diminished bone healing in type 1 diabetes. *Diabetes.* 2003;52:1502-1510.
28. Deane R, Singh I, Sagare AP, et al. A multimodal RAGE-specific inhibitor reduces amyloid beta-mediated brain disorder in a mouse model of Alzheimer disease. *J Clin Invest.* 2012;122:1377-1392.
29. Lee H, Park JR, Kim WJ, et al. Blockade of RAGE ameliorates elastase-induced emphysema development and progression via RAGE-DAMP signaling. *Faseb J.* 2017;31:2076-2089.
30. Zhou H, Jin C, Cui L, et al. HMGB1 contributes to the irradiation-induced endothelial barrier injury through receptor for advanced glycation endproducts (RAGE). *J Cell Physiol.* 2017 <https://doi.org/10.1002/jcp.26341>
31. Portela A, Esteller M. Epigenetic modifications and human disease. *Nat Biotechnol.* 2010;28:1057-1068.
32. Kelly TK, de Carvalho DD, Jones PA. Epigenetic modifications as therapeutic targets. *Nat Biotechnol.* 2010;28:1069-1078.
33. Liu S, Liu D, Chen C, et al. MSC transplantation improves osteopenia via epigenetic regulation of Notch Signaling in Lupus. *Cell Metab.* 2015;22:1-13.
34. Li J, Zhang N, Huang X, et al. Dexamethasone shifts bone marrow stromal cells from osteoblasts to adipocytes by C/EBPalpha promoter methylation. *Cell Death Dis.* 2013;4:e832-e843.
35. Macsai CE, Foster BK, Xian CJ. Roles of Wnt signalling in bone growth, remodelling, skeletal disorders and fracture repair. *J Cell Physiol.* 2008;215:578-587.
36. Liu N, Zhou M, Zhang Q, et al. Stiffness regulates the proliferation and osteogenic/odontogenic differentiation of human dental pulp stem cells via the WNT signalling pathway. *Cell Prolif.* 2018;51:e12435.
37. Ma W, Shao X, Zhao D, et al. Self-assembled tetrahedral DNA nanostructures promote neural stem cell proliferation and neuronal differentiation. *ACS Appl Mater Interfaces.* 2018;10:7892-7900.
38. Albers J, Keller J, Baranowsky A, et al. Canonical Wnt signaling inhibits osteoclastogenesis independent of osteoprotegerin. *J Cell Biol.* 2013;200:537-549.
39. Zhang WB, Zhong WJ, Wang L. A signal-amplification circuit between miR-218 and Wnt/beta-catenin signal promotes human adipose tissue-derived stem cells osteogenic differentiation. *Bone.* 2014;58:59-66.
40. Cho YD, Yoon WJ, Kim WJ, et al. Epigenetic modifications and canonical WNT signaling enable trans-differentiation of non-osteogenic cells into osteoblasts. *J Biol Chem.* 2014;289:20120-20128.
41. Liang L, He H, Lv R, et al. Preliminary mechanism on the methylation modification of Dkk-1 and Dkk-3 in hepatocellular carcinoma. *Tumour Biol.* 2015;36:1245-1250.
42. He G, Shi Y, Lim J, et al. Differential involvement of Wnt signaling in Bmp regulation of cancellous versus periosteal bone growth. *Bone Res.* 2017;5:17016.
43. Takakura A, Lee JW, Hirano K, et al. Administration frequency as well as dosage of PTH are associated with development of cortical porosity in ovariectomized rats. *Bone Res.* 2017;5:17002.
44. Gao C, Peng S, Feng P, et al. Bone biomaterials and interactions with stem cells. *Bone Res.* 2017;5:17059.

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