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Methylglyoxal compromises callus mineralization and impairs fracture healing through suppression of osteoblast terminal differentiation

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ABSTRACT

Impaired fracture healing in diabetic patients leads to prolonged morbidity and increased healthcare costs. Methylglyoxal (MG), a reactive metabolite elevated in diabetes, is implicated in various complications, but its direct impact on bone healing remains unclear. Here, using a non-diabetic murine tibial fracture model, we demonstrate that MG directly impairs fracture healing. Micro-computed tomography revealed decreased volumetric bone mineral density in the callus, while callus volume remained unchanged, resulting in a brittle bone structure. This was accompanied by reduced expression of osteocalcin and bone sialoprotein, both critical for mineralization. Biomechanical analysis indicated that MG reduced the mechanical resilience of the fracture site without altering its elastic strength, suggesting that the impairment was not primarily due to the accumulation of advanced glycation end-products in the bone extracellular matrix. *In vitro* studies confirmed that non-cytotoxic concentrations of MG inhibited osteoblast maturation and mineralization. Transcriptomic analysis identified downregulation of Osterix, a key transcription factor for osteoblast maturation, without altering Runx2 levels, leading to decreased expression of key mineralization-related factors like osteocalcin. These findings align with clinical observations of reduced circulating osteocalcin levels in diabetic patients, suggesting that the detrimental effects of MG on osteoblasts may extend beyond bone metabolism. Our study highlights MG and MG-sensitive pathways as potential therapeutic targets for improving bone repair in individuals with diabetes and other conditions characterized by elevated MG levels.

1. Introduction

Fracture healing is a complex, coordinated process that begins with hematoma formation, triggering inflammation and angiogenesis, followed by anabolic formation of a fibrocartilaginous callus (soft callus) rich in extracellular matrix (ECM) [1–4]. This matrix, produced mainly

Abbreviations: (MG), Methylglyoxal; (ECM), Extracellular matrix; (AGEs), Advanced glycation end-products.

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by fibroblasts and chondrocytes, stabilizes the fracture and serves as a scaffold for pre-osteoblast proliferation and differentiation/maturation of osteoblasts [1–4]. Resultant mature osteoblasts synthesize new bone matrix, depositing type I collagen and mineralization-related factors onto the fibrocartilaginous framework, forming a hardened, mineralized callus [3]. This strengthens the fracture site, facilitating the final remodeling phase where osteoblasts and osteoclasts restore the bone's original architecture [1–4]. Runx2 is the master transcription factor for osteoblast differentiation, governing the expression of numerous genes involved in osteoblast development and function [5,6]. As osteoblasts mature, their secretory phenotype undergoes a dynamic shift, characterized by sustained production of ECM proteins like type I collagen and osteopontin, alongside a progressive increase in the secretion of mineralization-related factors such as osteocalcin and bone sialoprotein [5,7]. This shift, crucial for mineralization phase, is orchestrated by Osterix, another key transcription factor acting downstream of Runx2 [6,8].

Human genetic disorders and animal models have established the essential roles of osteoblasts and their regulators in fracture healing [1,6]. However, this process is often disrupted in diabetic individuals, who exhibit a higher risk of impaired bone repair [1,4,9]. Similarly, diabetic animal models have shown impaired fracture healing [10–12]. Mechanisms may involve microvascular complications that compromise blood flow to the fracture sites, thereby hindering nutrient and oxygen delivery necessary for bone repair [1,4]. Diabetes also promotes systemic inflammation and oxidative stress, favoring osteoclast over osteoblast activity [4,9]. Reactive carbonyl species, particularly methylglyoxal (MG), a glycolysis byproduct, are implicated in diabetic complications [13,14], including impaired bone healing [15,16]. Elevated MG levels have been documented in diabetic patients [14] and animal models [17,18]. MG modifies proteins, lipids, and nucleic acids [14,19], impairing cellular function and tissue integrity by forming advanced glycation end-products (AGEs), advanced lipoxidation end-products (ALEs), and damaging nucleic acids [14,19]. Specifically, elevated MG levels are linked to ECM modification [14,20], mitochondrial dysfunction [14], increased oxidative stress leading to impaired angiogenesis [14], and osteoblast apoptosis [21], all of which could compromise bone metabolism. Therefore, elevated MG levels may contribute to impaired bone healing, but, direct evidence is lacking.

Understanding how diabetes affects bone health may reveal novel targets for medical interventions. In this study, we focused on the direct impact of MG on fracture healing using a tibial shaft fracture model in healthy mice, isolating the effects of MG from the complex diabetic milieu. We also analyzed the effects of MG on osteoblast differentiation and function using an *in vitro* culture system. These approaches aim to provide mechanistic insights into how elevated MG impairs bone matrix construction and repair, potentially paving the way for targeted therapies.

2. Materials and methods

2.1. Animal experiments

Nine-week-old male C57BL/6J mice (Japan SLC, Shizuoka, Japan) were used. All protocols were approved by the Animal Care and Use Committee of Yamaguchi University. Under isoflurane anesthesia, tibial fractures were induced using a No.11 scalpel blade, leaving the fibula intact. A 26-gauge Quincke type spinal needle (UNISIS Corp., Tokyo, Japan) was inserted into the tibial marrow cavity to stabilize the fracture. Following surgery, mice received daily intraperitoneal injections of either MG at 110 mg/kg body weight or phosphate-buffered saline (PBS) for 14 days. Mice were euthanized via CO_2 inhalation, followed by collection of blood and right tibiae.

2.2. Micro-computed tomography (micro-CT)

Tibiae were scanned using CosmoScan GX (Rigaku, Tokyo, Japan) with 2.3 μm voxel size. Callus formation was scored as previously described [22] and analyzed using Analyze v14.0 (AnalyzeDirect, Overland Park, KS, USA). A threshold distinguished callus tissue from cortical bone and soft tissue. Volumetric bone mineral density (vBMD) was calculated using hydroxyapatite phantoms.

2.3. Biomechanical analysis

Biomechanical properties were assessed using a three-point bending test using MZ-500S (Maruto, Tokyo, Japan). Tibiae were placed on support bars (8 mm apart), with fractures centered. A force was applied at 5 mm/min until failure. Load-deformation curves determined stiffness, maximum load, failure time, and energy absorption using CTRwin v1.05 (System Supply, Hokkaido, Japan).

2.4. Primary osteoblast isolation and culture

Primary osteoblasts were isolated from calvariae of postnatal day 2 mice [23]. Calvariae were digested with collagenase type X and Dispase II. Cells were cultured in α-MEM with 10 % FBS and induced to differentiate with 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and varying MG concentrations. Medium was refreshed every 6 h to maintain MG levels.

2.5. Quantitative PCR (qPCR)

Total RNA from tibiae (4-mm segments centered on fractures) was extracted using TissueLyser II and RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). For osteoblasts, RNA was extracted using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA). cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan), and qPCR was performed using THUNDERBIRD Next SYBR qPCR Mix (Toyobo). Relative gene expression was normalized to 18S rRNA. The primers targeting Runx2, Osterix, Bone sialoprotein, Col1a1, Osteopontin, and 18S rRNA were shown in Supplementary Table S1.

2.6. ALP assay and mineralization analyses

ALP activity was assessed using the TRACP & ALP assay kit (Takara Bio, Shiga, Japan). Mineralized nodule formation was assessed by Alizarin Red staining. Calcium content was determined using Metallo Assay CPZ III kit (Metallogenics, Chiba, Japan). Hydroxyproline content, indicating collagen production, was measured after acid hydrolysis (in 1 N HCl for 18 h at 110 °C).

2.7. RNA sequencing and gene expression analysis

Libraries were prepared and sequenced on Illumina NovaSeq 6000. Reads were mapped to GRCm38 reference sequence and assembled into transcripts, using RNA-seq Analysis Portal v2.6 on CLC Genomics Workbench v22.0.1 (Qiagen) and Cufflinks v2.2.1 program, respectively. Gene set enrichment analysis (GSEA) was performed using the GSEA tool v4.3.2.

2.8. Immunoblot analysis

Following primary antibodies were used for immunoblotting; anti-osteocalcin (Cloud-Clone, PAA471Mu01, Katy, TX, USA), anti-osteopontin (Santa Cruz, sc-21742, Dallas, TX, USA), anti-methylglyoxal 5-hydro-5-methylimidazolone (HycultBiotech, HM5017, Wayne, PA, USA), anti-Osterix (Santa Cruz, sc-393325), anti-Runx2 (Santa Cruz, sc-101145), anti-bone sialoprotein (Bioss, bs-4729R, Woburn, MA, USA), and horseradish peroxidase (HRP)-conjugated

anti-β-actin antibody (Proteintech, HRP-60008, Rosemont, IL, USA).

2.9. Glu-osteocalcin quantification in culture supernatants

Undercarboxylated osteocalcin (Glu-osteocalcin) levels in culture supernatants were determined using Mouse Glu-Osteocalcin High Sensitive EIA Kit (Takara Bio).

2.10. Statistical analysis

Data are presented as mean ± standard deviation. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test or unpaired Student's t-test using GraphPad Prism v9.1.1 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

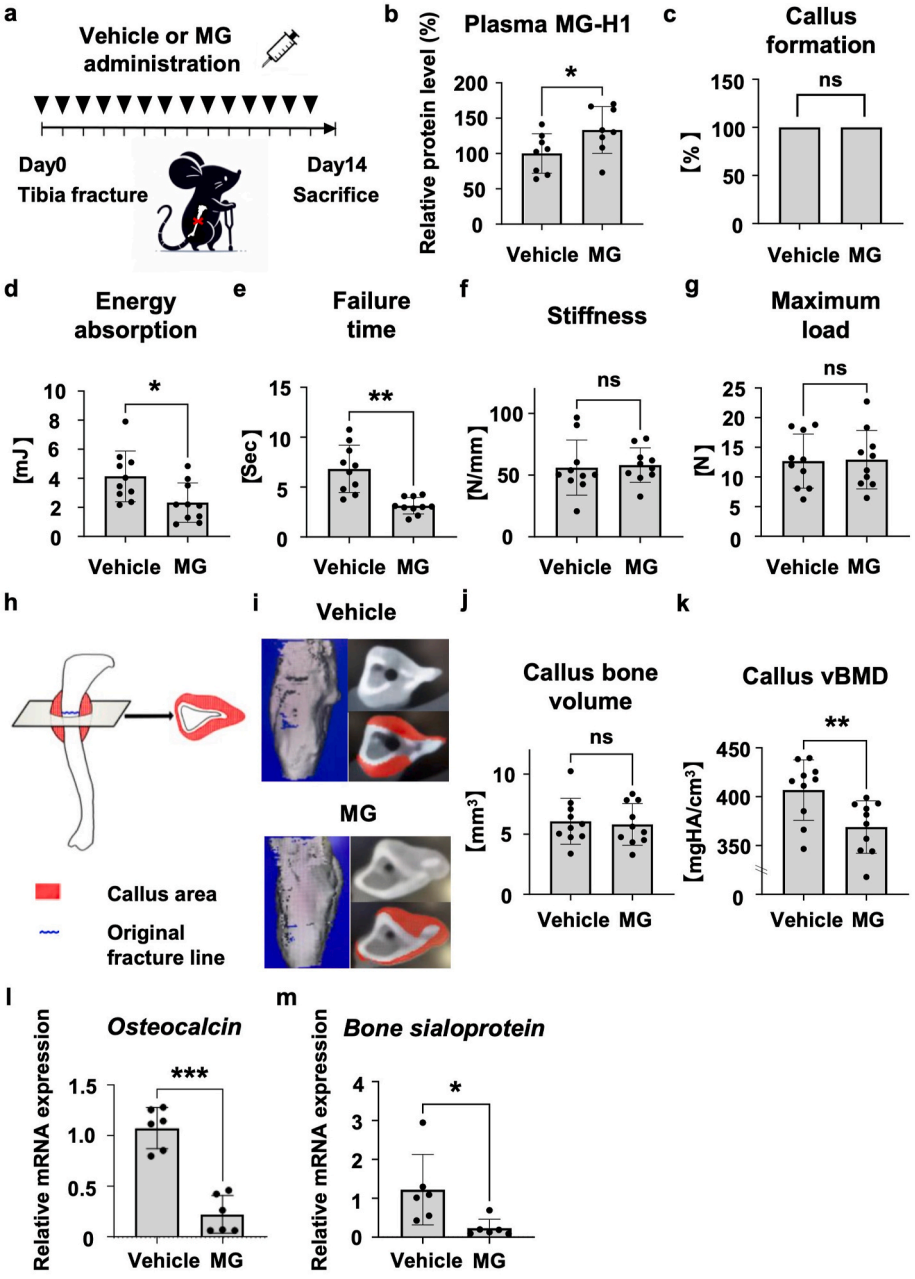

Fig. 1. Methylglyoxal (MG) inhibits callus mineralization and impairs fracture healing.
(**a**) Experimental timeline: Mice with tibial fractures received daily MG (110 mg/kg) or PBS injections for 14 days. (b) Plasma MG-modified proteins quantified by immunoblot densitometry (Supplementary Fig. S1). (c) Radiological assessment of callus formation. (d–g) Biomechanical properties: energy absorption (d), failure time (e), stiffness (f), maximum load (g). (h) Diagram of callus area and fracture line in tibia. (i) Micro-CT images: 3D reconstruction (left), axial images (upper right), highlighted callus area (red, lower right). (j,k) Callus volume (j) and volumetric bone mineral density (vBMD) (k). (l,m) Expression of Osteocalcin (l) and Bone sialoprotein (m) at day 14, normalized to 18S rRNA. Data are mean ± SD. Vehicle vs. MG-treated: $n = 8$ vs. 8 in (**b**); $n = 10$ vs. 10 in (**c-g, j,k**); $n = 6$ vs. 6 in (**l,m**). Statistical significance by unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. MG per se suppresses callus mineralization and impairs fracture healing

To assess the impact of MG on fracture healing in non-diabetic mice, we administered MG intraperitoneally to mice with tibial fractures for 14 days (Fig. 1a). No adverse effects on body weight, liver function, or renal function were observed at the dosage used (Supplementary Table S2). Elevated plasma MG-modified proteins confirmed systemic MG absorption (Fig. 1b and Supplementary Fig. S1). Radiological analysis showed callus formation in all mice at 14 days post-fracture (Fig. 1c). However, biomechanical analysis using a three-point bending test showed MG significantly decreased energy absorption capacity and time to failure under stress (Fig. 1d and e), indicating compromised mechanical resilience and increased susceptibility to damage. Notably, stiffness and maximum load were unaffected by MG (Fig. 1f and g), suggesting initial structural strength and deformation resistance (elastic-strength properties) were maintained.

To elucidate the underlying cause of increased brittleness, we utilized quantitative computed tomography to analyze fracture sites. While callus volume was comparable between MG- and vehicle-treated mice (Fig. 1h–j), vBMD was significantly lower in the MG-treated group (Fig. 1k). This decrease in vBMD, coupled with reduced expression of mineralization-related factors like osteocalcin and bone sialoprotein at the fracture site (Fig. 1l and m), strongly indicates MG impairs osteoblast-mediated callus mineralization.

3.2. MG directly inhibits osteoblast maturation and mineralization in vitro

To investigate the cellular mechanisms behind the reduced callus mineralization in MG-treated mice, we examined the direct effects of MG on osteoblast differentiation and function *in vitro*. Consistent with previous studies [24–26], high doses of MG (≥500 μM) caused significant osteoblast cytotoxicity (Supplementary Fig. S2). On the other hand, at lower, non-cytotoxic concentrations (≤300 μM), MG only exhibited mild cytostatic effects (Supplementary Fig. S2) but significantly decreased alkaline phosphatase (ALP) activity at 7 days post-differentiation induction (Fig. 2a), indicating impaired osteoblast maturation and reduced mineralization capacity. Alizarin Red staining (Fig. 2b and c) and calcium content assays (Fig. 2d) at 21 days post-differentiation induction, confirmed that even low, non-cytotoxic doses of MG significantly inhibited osteoblast mineralization.

Given that cell cycle exit and arrest are prerequisites for terminal differentiation of osteoblasts [27], the observed impairment is unlikely due to MG's cytostatic effects at lower concentrations. This is supported by the finding that MG did not affect the induction of early differentiation markers, *Col1a1* (Fig. 2e and Supplementary Fig. S3a) and *Osteopontin* (Supplementary Fig. S4). These results suggest that low-dose MG does not hinder early osteoblast differentiation but specifically targets later maturation stages, abrogating their mineralization capacity.

3.3. MG-induced molecular changes associated with impaired osteoblast mineralization: transcriptomic and protein analyses reveal downregulation of Osterix

To elucidate the molecular mechanisms underlying MG-mediated

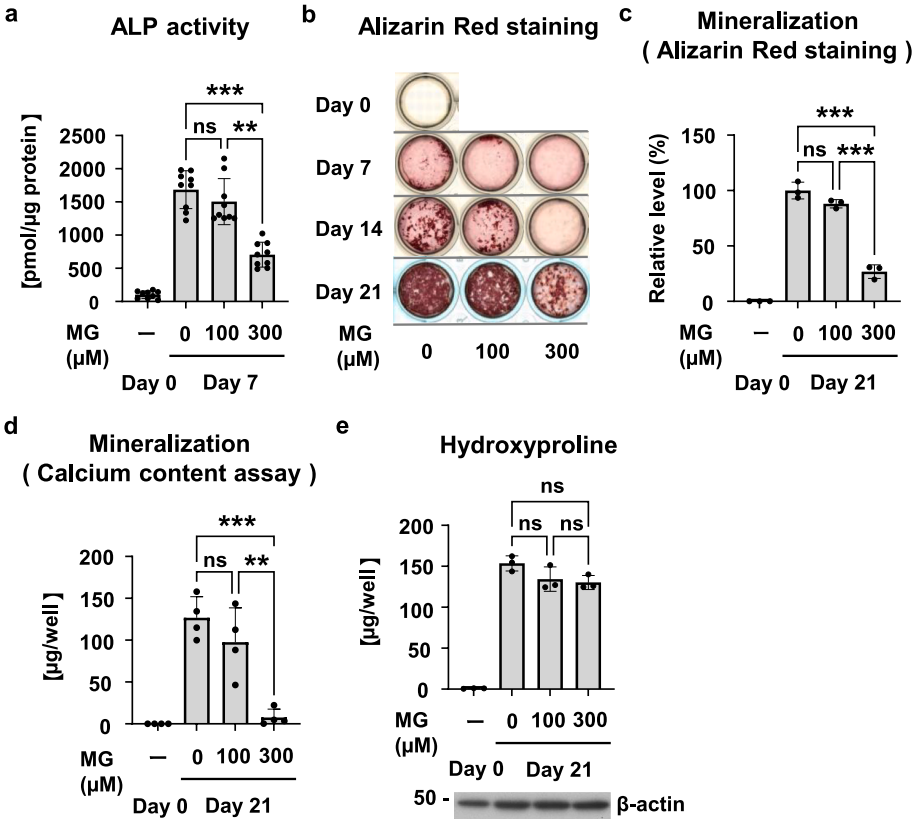

Fig. 2. Methylglyoxal (MG) directly impairs osteoblast maturation and mineralization *in vitro*.
Primary osteoblasts treated with MG for 7 or 21 days, refreshing medium every 6 h. (a) Alkaline phosphatase (ALP) activity normalized to total protein. (b) Alizarin Red staining of mineralized nodules. (c) Quantification of Alizarin Red staining. (d) Total calcium content. (e) Hydroxyproline content indicating collagen production. Data are mean ± SD. $n = 9$ in (**a**), 3 in (**c,e**), and 4 in (**d**). Statistical significance by one-way ANOVA with Tukey's multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inhibition of osteoblast mineralization, we performed transcriptomic analysis using an MG concentration inhibiting osteoblast mineralization (300 μM). MG treatment significantly downregulated genes associated with mineralization (Fig. 3a). Consistent with this finding, we observed decreased protein levels of bone sialoprotein and osteocalcin in MG-treated osteoblasts (Fig. 3b and c and Supplementary Figs. S3b and c). Furthermore, MG treatment also reduced levels of secreted osteocalcin (Glu-osteocalcin) in the culture supernatant (Fig. 3d). These results collectively demonstrate that MG suppresses the expression and secretion of factors critical for osteoblast-mediated mineralization.

Given the pivotal role of transcription factors Runx2 [5,6] and Osterix [6,8] in osteoblast differentiation and mineralization, we examined their expression in MG-treated osteoblasts. Notably, MG specifically suppressed Osterix expression without affecting Runx2 levels (Fig. 3e and f and Supplementary Figs. S3d and e). This strongly suggests that MG disrupts osteoblast terminal differentiation by downregulating Osterix, a key transcriptional regulator for mineralization-related genes [8]. These molecular insights provide a foundation for understanding the detrimental effects of MG on bone health and fracture healing.

4. Discussion

MG, elevated in diabetes [14], has been proposed to delay fracture healing [15,16,28], but direct evidence was lacking. Our study is the first to demonstrate that MG impairs fracture healing in non-diabetic mice by inhibiting callus mineralization, supporting the hypothesis that elevated MG contributes to impaired healing in diabetic patients. MG administration increased the fragility of the newly formed callus/bone complex at the fracture site. This effect was not due to changes in initial callus formation, callus volume, or elastic strength but was primarily due to impaired mineralization and subsequent microstructural alterations. While AGE accumulation in bone has been reported to contribute to bone fragility in diabetes by altering collagen properties [29–32], this may not fully explain our observations. Specifically, unlike AGE-crosslinked collagen in bone, which typically reduces bone deformation capacity [31,32], MG compromises the structural ability of energy absorption and stress resistance without affecting deformation capacity. Further investigation is needed to elucidate the complex interplay between MG, AGEs, and other factors in diabetic bone disease.

Although MG levels vary in diabetic patients [14], they are likely not high enough to cause osteoblast death. This is supported by observations that many diabetic patients, particularly those with type 2 diabetes, have increased bone mass [31], suggesting bone-forming osteoblasts remain viable and functional. Although high MG levels cause osteoblast death (Supplementary Fig. S2) [24–26,33], our study establishes that sub-cytotoxic levels also have a detrimental effect: compromised mineralization. This implies that in diabetes, MG levels may not be cytotoxic for osteoblast-lineage cells but still impair bone healing. Potential mechanisms include impaired proteasomal degradation, oxidative stress, mitochondrial dysfunction, promotion of intracellular and extracellular AGE formation, and signaling via the receptor for AGEs [14]. These multifaceted effects disrupt bone healing, underscoring the importance of MG and its downstream events as therapeutic targets. It should be noted that our experimental model, focusing solely on MG, did not fully capture the complex, multifactorial nature of diabetes. For instance, decreased callus size, common in diabetic models [4,10,11,34,35], was not observed in our model. This discrepancy likely arises due to additional factors present in diabetes, such as other chemical mediators, altered inflammatory responses, and impaired angiogenesis. This highlights the need for further investigation into the intricate interplay of factors contributing to diabetic bone disease.

Our transcriptomic analysis showed MG downregulates genes related to osteoblast mineralization with a corresponding decrease in osteocalcin and bone sialoprotein expression at the fracture site. This suggests a conserved mechanism by which MG impairs mineralized matrix formation both *in vivo* and *in vitro*. We found that MG suppressed Osterix, a master regulator of osteoblast maturation and mineralization [6,8], without affecting Runx2, which is crucial for early osteoblast differentiation [5,6]. This indicates MG disrupts a pathway governing osteoblast terminal differentiation and ECM mineralization, where osteocalcin plays an indispensable role in hydroxyapatite deposition [36,37]. Notably, our finding that MG reduces osteocalcin expression in osteoblasts may provide a mechanistic explanation for the frequently reported clinical observations of decreased circulating osteocalcin levels in diabetic patients [38]. Because uncarboxylated osteocalcin acts as a hormone influencing various organs [39,40], and osteoblast-lineage cells are its primary source [39,40], MG-induced osteocalcin reduction may have broader systemic effects beyond bone metabolism, potentially including dysregulation of glucose metabolism, energy expenditure, and other osteocalcin-mediated processes [39–41].

While other factors like hormones, matrix properties, and availability of nutrition and mineral may also influence the speed and efficiency of ECM mineralization [42,43], our findings emphasize the direct impact of MG on osteoblast function, highlighting its potential role in the pathogenesis of impaired bone healing in diabetes. Interestingly, MG treatment significantly increased osteopontin expression, an early osteoblast marker [5,7] (Supplementary Fig. S4), potentially as a compensatory response to MG-induced cellular stress. However, this possible compensation appears insufficient to overcome the detrimental effects of MG on mineralization and bone formation.

In conclusion, our study provides compelling evidence that sub-cytotoxic MG impairs osteoblast-mediated mineralization, primarily via Osterix suppression. This identifies MG and MG-sensitive pathways as potential therapeutic targets for diabetic bone complications. Further research is warranted to fully elucidate the underlying mechanisms and develop targeted interventions aimed at improving bone repair and regeneration in diabetic patients.

CRediT authorship contribution statement

Tetsuya Seto: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kiminori Yukata:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Shunya Tsuji:** Writing – review & editing, Writing – original draft, Funding acquisition. **Yusuke Takeshima:** Methodology, Data curation. **Takeshi Honda:** Methodology, Data curation, Conceptualization. **Akihiko Sakamoto:** Writing – review & editing. **Kenji Takemoto:** Writing – review & editing. **Hiroki Sakai:** Data curation. **Mayu Matsuo:** Data curation. **Yurika Sasaki:** Data curation. **Mizuki Kaneda:** Data curation. **Mikako Yoshimura:** Data curation. **Atsushi Mihara:** Data curation. **Kazuya Uehara:** Data curation. **Aira Matsugaki:** Data curation. **Takayoshi Nakano:** Data curation. **Koji Harada:** Data curation. **Yoshiro Tahara:** Data curation. **Keiko Iwaisako:** Data curation. **Ryoji Yanai:** Funding acquisition, Data curation. **Norihiko Takeda:** Data curation. **Takashi Sakai:** Supervision, Conceptualization. **Masataka Asagiri:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Data availability statement

The RNA sequencing data are available in the DNA Data Bank of Japan (accession number PRJDB18525). All other data are included within this article and Supplementary Information.

Disclosure

The authors declare no conflicts of interest.

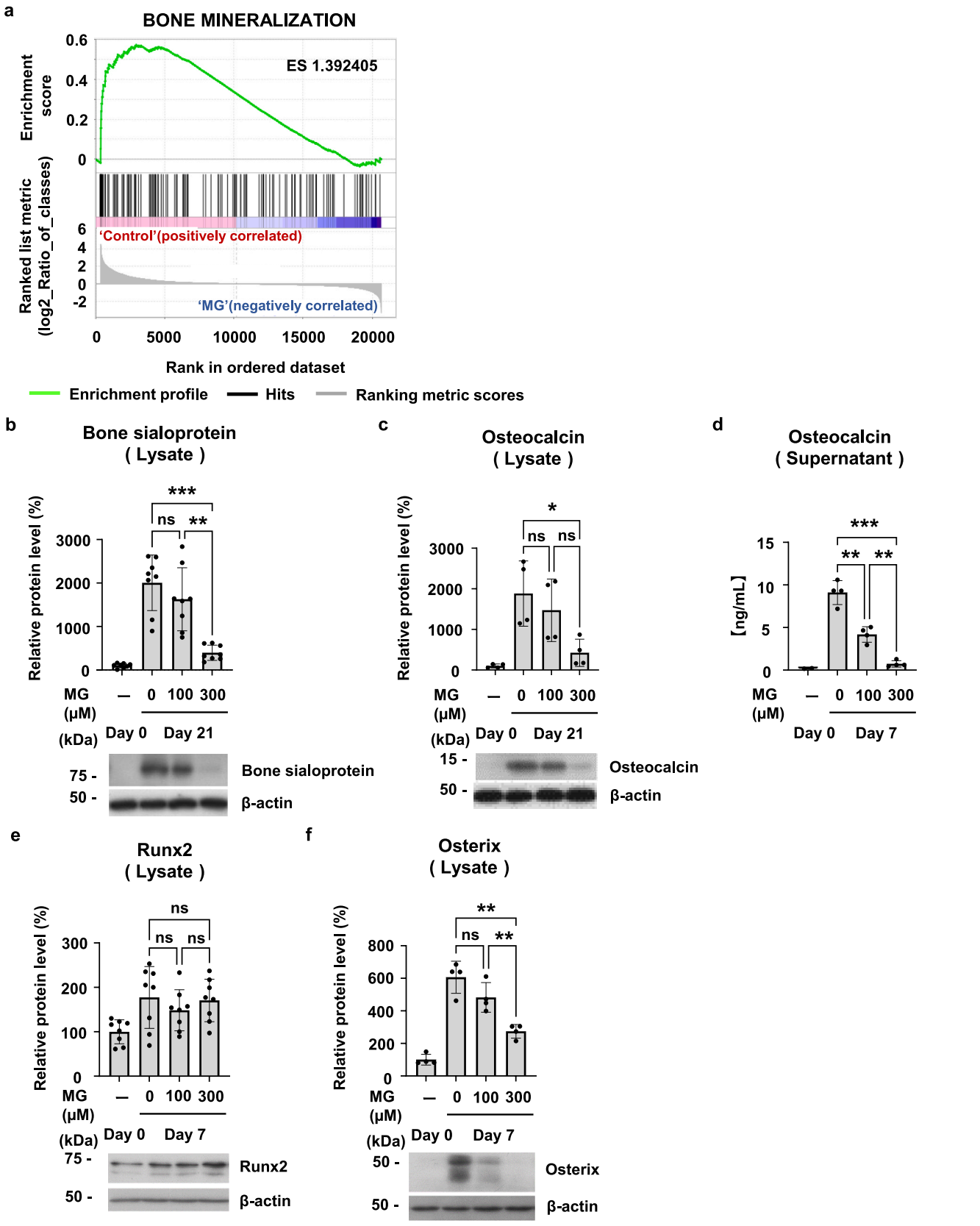

Fig. 3. Methylglyoxal (MG) suppresses Osterix and downstream mineralization-related factors.
(**a**) RNA-seq analysis of osteoblasts treated with MG (300 μM). Gene set enrichment analysis (GSEA) shows downregulation of bone mineralization genes. (b,c) Protein levels of bone sialoprotein (b) and osteocalcin (c) normalized to β-actin. (d) Glu-osteocalcin levels in culture supernatants measured by ELISA. (e,f) Protein levels of Osterix (e) and Runx2 (f) normalized to β-actin. Data are mean ± SD. $n = 8$ in (**b,e**) and 4 in (**c,d,f**). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2025.151312.

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