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Zoledronic acid epigenetically alleviates high-glucose-suppressed osteogenic differentiation of MC3T3-E1 cells

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Abstract. – OBJECTIVE: Due to the impact of excessive glucose on osteogenic differentiation, diabetic osteopathy frequently results in skeletal fragility, osteoporosis, and bone pain. Zoledronic acid, a bisphosphonate (BP) that effectively inhibits osteoclastic bone resorption is given yearly to improve bone mineral density (BMD) in patients with osteoporosis. However, the detailed molecular mechanisms remained unclear. This study investigates the possible pathways by which zoledronic acid regulates osteogenesis when blood glucose levels are high.

MATERIALS AND METHODS: MC3T3-E1 cells were treated with one mM zoledronic acid or not in a standard or high glucose culture medium. A quantitative polymerase chain reaction (qPCR) assay was utilized to assess the expression of the target candidate genes, including *RUNX2, MALAT1, miR-133, miR-20a,* and *miR-204*.

RESULTS: In a high-glucose condition, zoledronic acid treatment significantly lowered *MALAT1* ($p < 0.0001$) and *miR-20a* ($p < 0.0001$) expression. Conversely, in a high-glucose condition, *RUNX2, miR-133,* and *miR-204* expressions were found to be significantly increased in the zoledronic acid treatment group as compared to no treatment (all $p < 0.0001$).

CONCLUSIONS: In conclusion, under a high-glucose environment, zoledronic acid can modulate the expression of the *RUNX2* transcription factor through epigenetic regulation.

Key Words:
Diabetes, Osteogenesis, Zoledronic acid, miRNA, lncRNA, *RUNX2*.

Introduction

Diabetes mellitus (DM) is a multi-metabolic disorder group based on prolonged hyperglycemia[1]. The relationship between diabetic bone, altered bone mineral density (BMD), and elevated bone fracture rates has brought a number of skeletal system issues to the forefront of research[2,3]. Additionally, decreased osteogenesis and reduced bone regeneration may have slowed the recovery of diabetic bones[2,4-7]. Further, osteogenic differentiation is impaired by high glucose levels, which is a critical condition in individuals suffering from hyperglycemia[8]. The molecular and cellular mechanisms behind the physiopathology of diabetic bones are still poorly understood. Therefore, understanding the osteogenesis progress in patients with DM is essential.

Zoledronic acid is a nitrogen-containing bisphosphonate (BP) that effectively inhibits osteoclastic bone resorption. In 2007, the Food and Drug Administration (FDA) of the United States approved using zoledronic acid to treat osteoporosis in postmenopausal women[9]. Having an acceptable safety profile and tolerability, Zoledrone is the only BP currently approved to prevent and treat relevant skeletal events in patients with metastatic bone lesions, particularly bone metastases from advanced renal cell carcinoma, prostate cancer, and breast cancer, due to any solid malignancy[9].

Furthermore, zoledronate has anti-osteosarcoma capabilities and may be used as an adjunctive treatment for high-grade osteosarcoma to improve survival rates and eliminate chemotherapy-related complications. A prior study found that combining zoledronic acid and teriparatide helped increase bone mineral density (BMD) in individuals with type 2 diabetes who had had percutaneous kyphoplasty[10]. According to a recent systematic review[11], zoledronic acid once a year during the peri-operative phase of percutaneous kyphoplasty/vertebroplasty for patients with osteoporotic vertebral compression fractures showed significantly improves in BMD, a decrease in pain scores, and a decreased risk of developing new vertebral fractures.

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RUNX2 is required for osteoblast development as well as chondrocyte maturation. It is only sporadically expressed in uncommitted mesenchymal cells during the differentiation of osteoblast. Its expression is elevated in pre-osteoblasts, peaks in immature osteoblasts, and declines in mature osteoblasts[12]. High glucose suppresses the expression of *RUNX2* and leads to the impairment of bone regeneration, osteogenic differentiation, and proliferation in different mice models[13,14]. Several miRNAs are proven to target *RUNX2* to induce or reduce osteoblast differentiation and are regulated by long non-coding RNA (lncRNAs)[15]. MicroRNA (miR)-204 and its homolog miR-211 were expressed in mesenchymal progenitor cell lines and bone marrow mesenchymal stem cells (BMSCs), but *RUNX2* protein expression was suppressed during adipocyte differentiation[16]. *RUNX2 3'-UTR* reporter activity was increased when *miR-204* binding sites were deleted, indicating that *miR-204/211* binds to *RUNX2*. MiR-204 inhibition, on the other hand, promoted osteogenesis and inhibited adipocyte development in these cells. *MiR-204/211* are endogenous negative regulators of *RUNX2*, inhibiting osteogenesis and promoting adipogenesis in mesenchymal progenitor cells (MPCs) and bone marrow mesenchymal stem cells (BMSCs)[17].

Investigating whether zoledronic acid can promote osteogenesis by regulating miRNA or lncRNAs and increasing *RUNX2* expression would be necessary. This study aimed to examine the relationship between zoledronic acid, miRNAs, and *RUNX2*, as well as potential zoledronic acid regulation mechanisms for osteogenesis under a high-glucose environment.

Materials and Methods

Study Design

Effects of zoledronic acid on MC3T3-E1 cells under standard and high glucose culture conditions were determined. MiRWalk was used to screen for miRNAs targeting *RUNX2* and to create a miRNA-gene network.

Cell Culture and Osteogenic Differentiation

MC3T3-E1 cells from a murine bone calvaria pre-osteoblast were cultured in the base medium containing α-Minimum Essential Medium, ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, but without ascorbic acid (GIBCO, Custom Product, and Catalog No. A1049001); and were supplemented with 10 vol % fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, USA) and antibiotics [100 U/ml penicillin G and 100 mg/ml streptomycin (Sigma Aldrich Corp., St. Louis, USA)]. The culture was carried out at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, with an initial density of 6×10^3 cells per cm^2 (https://www.atcc.org/products/all/CRL-2593.aspx#culturemethod) for two days. For the next 7 days, the medium was replaced with the differentiation medium composed of 10 nM dexamethasone, 25 µg/ml l-ascorbic acid, and 10 mM β-glycerophosphate (Sigma Aldrich Corp., St. Louis, USA), which was changed every 3 to 4 days.

Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Assay

Total RNA was extracted with UNIQ-10 Column Trizol Total RNA Isolation Kit (#B511321, Sangon, China). MiRNA was removed with a miRNA Purification Kit (#CW0627S, CWBio, China). RNA sample (1 µg) was added as a template to RT reactions performed with a miRNA First Strand cDNA Synthesis (Tailing Reaction) kit (#B532451, Sangon Bio., China) or an EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (#AE311, TransGen, China). Data for the RT-qPCR was normalized according to *GAPDH* or *RNU6B* gene. RT-qPCR was carried out with the complementary cDNA in triplicate, using TransStart® Top Green qPCR SuperMix SYBR Green kit purchased from TransGen, Biotech (cat. No.: AQ131), by LightCycler96 (Roche, Basel, Switzerland) PCR machine. The experimental Ct values were normalized to that of *GAPDH* (mRNA) or *RNU6B* (miRNA).

LncRNA Database

RNAcentral is a database of non-coding RNA (ncRNA) sequences aggregating data from specialized ncRNA resources. It assigns unique identifiers to every distinct RNA sequence. Because there is no uniform identity number in the different lncRNA databases, we used identifiers from RNAcentral as unified labels of lncRNAs to ensure the smooth progress of this work.

miRWalk-Based Analysis

We uploaded *RUNX2* to the miRWalk2.0 website to screen for miRNAs that regulate specific genes. We included miRNAs identified as candidate regulatory miRNAs for *RUNX2* using the miRWalk method. (RefSeq: NM_001278478; score > 0.82) (http://mirwalk.umm.uni-heidelberg.de/)[17].

Statistical Analysis

The mean and standard deviation of continuous data are presented. ANOVA with one-way was used to analyze the data. The statistical analysis software GraphPad Prism v6 (Boston, MA, USA) was used for all analyses. A value of $p < 0.05$ is considered to indicate statistical significance.

Results

As summarized in **Supplementary Table I**, the interaction network involved 1,560 miRNAs and *RUNX2*. Considering that *miR-20a, miR-133,* and *miR-204* have been linked to osteogenic differentiation, we chose these three miRNAs for further experiments. The effects of zoledronic acid on the relative expression levels of *RUNX2*, *MALAT1*, *miR133, miR20a,* and *miR-204* through qPCR under standard and high glucose conditions are depicted in Figure 1.

In specific, *RUNX2* expression was significantly lowered in the high-glucose condition without zoledronic acid than in the standard glucose condition ($p < 0.05$). However, in a high-glucose environment, zoledronic acid treatment significantly raised *RUNX2* expression level ($p < 0.0001$) (Figure 1A).

Similarly, in a high glucose environment, *miR-133* and *miR-204* expressions were significantly increased in the zoledronic acid treatment group than in the no zoledronic acid treatment group (all $p < 0.0001$) (Figure 1C-E).

On the contrary, in a high glucose condition, *MALAT1* and *miR-20a* expressions were significantly lowered following zoledronic acid treatment (all $p < 0.0001$) (Figure 1B-D).

A signal transduction mechanism, transcription downstream genes, or epigenetic regulation that follows the high glucose level in the culture environment must be confirmed in order to understand why osteogenic differentiation is decreasing. Since *Runx2* is essential for osteoblast differentiation and chondrocyte maturation, we next searched the RNAcentral database to predict possible lncRNA interaction with *Runx2.* The lncRNAs candidates targeting *RUNX2* were indicated by RNAcentral and listed in Table I.

Discussion

Zoledronic acid functions as an osteoclast apoptosis inducer and thereby modulates bone resorption. However, the effect of zoledronic acid on osteoblast remains unclear. Furthermore, high glucose suppresses osteogenic differentiation in diabetic osteopathy[18]. The present study investigated the expression of several miRNAs and *RUNX2* following zoledronic acid treatment under standard and high-glucose conditions. Also, the lncRNA candidates associated with *RUNX2* were searched. In general, it is indicated that under high-glucose conditions, zoledronic acid treatment results in *RUNX2* upregulation, indicating a recovery of

Table I. The lncRNA candidates potentially for regulating *Runx2* expression.

ID	Length	Databases of Non-coding RNA
R intergenic non-coding RNA isoform 001-008	1,668-6,458	ENA
GeneCards HSALNT0106664	2,510	LncBook
GeneCards lncAB482.1	2,062	ENAGeneCards
HSALNT0106660	1,614	LncBook
GeneCards HSALNT0106667	1,330	LncBook
GeneCards HSALNT0106655	1,059	LncBook
NONCODE GeneCards HSALNT0106656	681	LncBook
NONCODE GeneCards HSALNT0106666	405	LncBook
GeneCards		

ENA, European Nucleotide Archive.

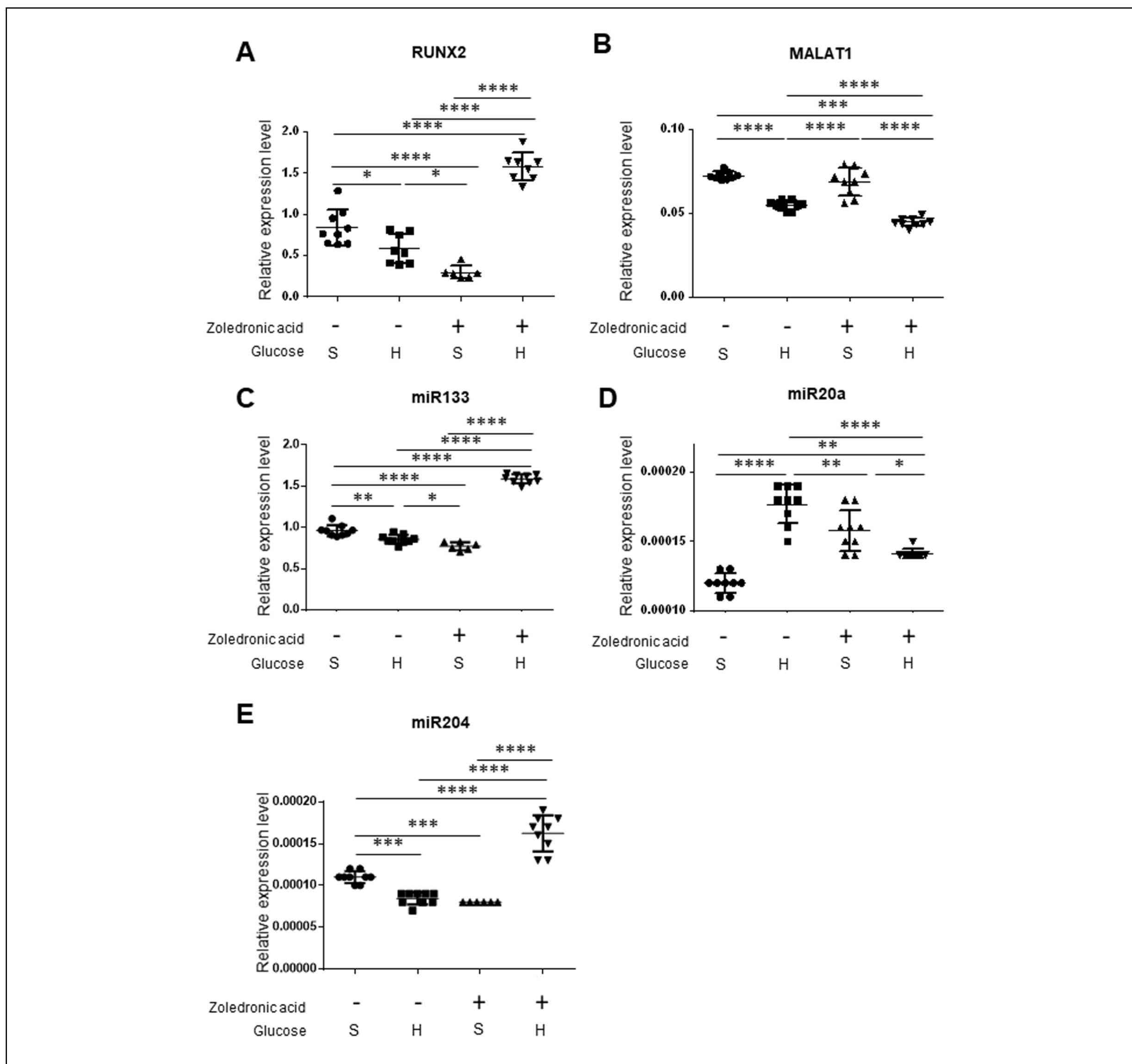

Figure 1. Relative expression level of (**A**) *Runx2*, (**B**) *MALAT1*, (**C**) *miR-133a*, (**D**) *miR-20a*, and (**E**) *miR-204* in groups as indicated. Concentration of zoledronic acid is 1 µM. S: Basal αMEM; H: 25.5 mM glucose in αMEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

osteogenesis. In addition, the upregulation of *miR-133* and *miR-204* observed following zoledronic acid treatment under high-glucose conditions indicates the epigenetic regulation pathways participating in this process.

This study found that, under high-glucose conditions, *RUNX2* mRNA expression levels increased after zoledronic acid treatment. On the other hand, zoledronic acid treatment led to a reduced *RUNX2* expression level under standard glucose conditions, supporting the earlier results[19].

The fundamental processes through which lncRNA functions are carried out are currently poorly understood. BMSCs treated with glucocorticoids showed down-regulation of TCONS_00041960 during adipogenic and osteogenic development. Furthermore, upregulation of TCONS_00041960 promoted expressions of osteogenic genes *RUNX2*, osterix, osteocalcin, and anti-adipogenic glucocorticoid-induced leucine zipper (GILZ). Conversely, the expression of adipocyte-specific markers was decreased in the presence of over-expressed TCONS_00041960. We determined that TCONS_00041960, as a competing endogenous RNA, interacted with *miR-204-5p* and *miR-125a-3p* to regulate *RUNX2* and *GILZ*, respectively[20]. Be-

sides, the enforced lncRNA maternally expressed gene 3 (MEG3) expression positively correlates with the word of *miR-133a-3p* in bone marrow mesenchymal stem cells (BMSCs) derived from PMOP[21]. This regulated expression of *miR-133a-5p* by *MEG3* inhibited BMSC differentiation into osteoblasts[22]. The miRNAs and lncRNAs in the process from BMSCs to osteoblasts have been reviewed and showed several regulation strategies in either migration of BMSCs or osteogenesis[15,22]. In this study, we focused on the possible epigenetic regulations of *RUNX2*. By activating or inactivating specific miRNAs, lncRNA can complicate the epigenetically regulated control of osteogenesis by targeting migration or osteogenesis processes[15]. More studies are required to determine whether the *RUNX2* expression varies according to the lncRNAs and miRNAs impacted by zoledronic acid or whether these changes are caused directly by the drug. Nevertheless, it was first noted in this study that zoledronic acid was associated with increasing *RUNX2* expression at a high glucose condition.

Conclusions

Zoledronic acid has been linked to increased *RUNX2* expression in osteoblast cells under high-glucose conditions for the first time. In the future, it will be investigated whether zoledronic acid can influence downstream signal transmissions and lead to enhanced osteogenesis, slowing the progression of diabetic osteopathy.

Conflict of Interest

The authors declare that they have no competing interests.

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Ethics Approval

This study has been reviewed and approved by the Animal Ethics Committee of Beijing Jishuitan Hospital (registration number: 201905-03).

Availability of Data and Materials

All data supporting this study are included in this manuscript and the supplementary materials.

Authors' Contributions

Hailing Chen: guarantor of integrity of the entire study; study concepts; study design; definition of intellectual content; clinical studies; data analysis; statistical analysis; manuscript preparation; manuscript editing; manuscript review. Jianpeng Hu: literature research; experimental studies; data acquisition; data analysis; statistical analysis; manuscript preparation. Jufen Li: literature research; clinical studies; experimental studies; data analysis; manuscript preparation. Quan Li: clinical studies; data acquisition; data analysis; statistical analysis. Ling Lan: study design; manuscript editing; manuscript review. All authors reviewed and approved the final version of the manuscript.

Informed Consent

Not Applicable.

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