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Metformin promotes the proliferation and differentiation of murine preosteoblast by regulating the expression of *sirt6* and *oct4*

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ABSTRACT

Osteopenia, osteoporosis and bone salt metabolism disorder are common diseases in the aged and diabetics. From case reports of patients with T2DM, we have observed that metformin can decrease risk of bone fracture and promote bone formation. However, the underlying mechanism of metformin's effect on bone metabolism remains unknown. In our research, we show that metformin can promote proliferation of murine preosteoblast by regulating AMPK-mTORC2 and AKT-mTORC1 signaling axis. Furthermore, we have observed that metformin can promote SIRT6 expression before and during differentiation of murine preosteoblast. The interaction between SIRT6 and NF-κB is highly important in osteoblast differentiation just as the relationship between OPG and RANKL in the process of bone formation. During differentiation, we show that SIRT6 inhibits phosphorylation of NF-κB and that OPG increases while RANKL decrease in HG groups. In addition, ablation of *sirt6* in mice causes phosphorylation of NF-κB at high-levels and RANKL increases slightly in femur bone cells. However, other bone formation marker proteins such as RUNX2, OSTERIX and OPG appear at low-levels in *sirt6* KO mice. It has been confirmed that downregulation of OCT4 is critical incident in the differentiation of embryonic stem cells. Fortunately, we observe that SIRT6 can suppress OCT4 expression in murine preosteoblast and the expression of OCT4 is at high-level in *sirt6* KO mice. Taken together, this study's results illuminate metformin's effect on bone metabolism under HG condition and help to elucidate why metformin can promote bone fracture healing of patients with T2DM.

1. Introduction

Metformin is a first-line anti-diabetics drug for treating patients with T2DM [1]. It was reported that metformin improves glucose metabolism in hyperglycemia via stimulating AMPK complex [2,3]. It has also been confirmed that AMPK complex has three different subunits that are specifically expressed in certain tissues or organs. For example, the AMPKα subunit is highly expressed in bone tissue, primary osteoblasts, and osteoclasts as well as in a number of bone cell lines [4–7]. Thus, activation of AMPKα by metformin influences bone metabolism either directly or indirectly [8,9].

Previous studies showed that AKT played an important role in regulating glucose uptake into muscle and fat cells through stimulating the translocation of GLUT4 to the plasma membrane [10–12]. Use of alanine mutants clearly show that AKT-Thr^{308} and Ser^{473} can be phosphorylated independently of each other [13]. The mechanism of AKT in osteocyte is still unknown. There are notably few reports describing the relationship between metformin and AKT in bone metabolism.

SIRT6, which is a member of *Sirtuins* family and mainly expressed in nucleus, is indispensable in keeping genome stabilization, DNA repair and gene expression programs [14]. On the grounds of previous investigations, we have known that SIRT6 interacts with NF-κB [15] in bone metabolism and that the expression of NF-κB is essential in normal skeletal remodeling and bone homeostasis by controlling the differentiation of osteoprogeni-

Abbreviations: T2DM, Type 2 diabetes mellitus; NG, normal glucose concentration; HG, high glucose concentration; mTORC, mammalian target of rapamycin complex; GLUT, glucose transporters; LKB1, Liver kinase B1; AMPK, AMP-activated protein kinase; AKT, Protein kinase B; ALP, alkaline phosphatase; RUNX2, runt related transcription factor 2; OSX, osterix; OCN, osteocalcin; OPN, osteopontin; BMP2, bone morphogenetic protein 2; NAD+, nicotinamide adenine dinucleotide; RANKL, receptor activator of nuclear factor kappa-B ligand; OPG, osteoprotegerin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Oct4, octamer-binding transcription factor 4; FOXO1, forkhead box protein O1; TSC, tuberous sclerosis; KO, knockout; FCM, flow cytometer machine.

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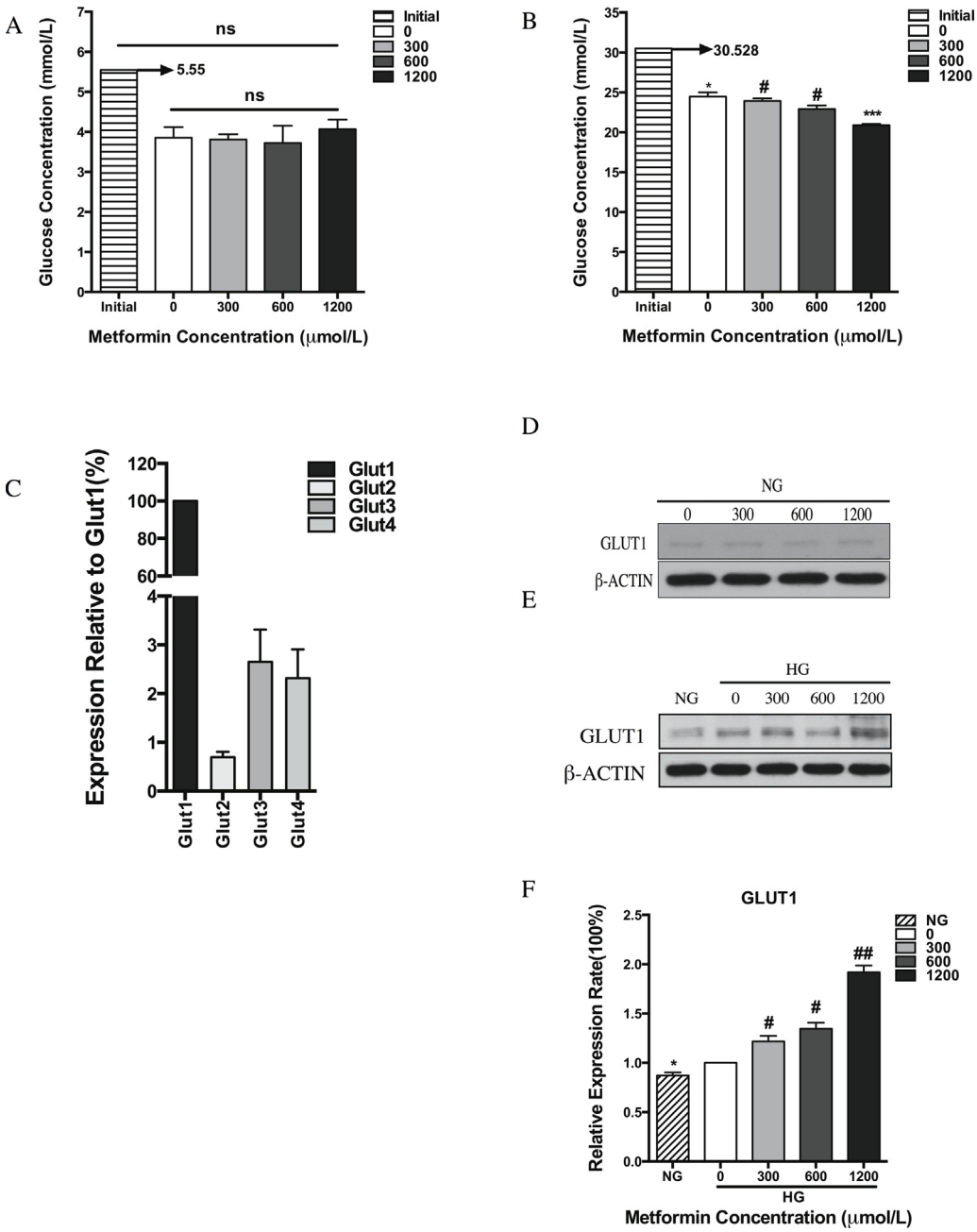

Fig. 1. Metformin promotes proliferation and stimulates insulin-independent glucose uptake in murine preosteoblast.
(A, B) Extracellular glucose concentration changes of MC3T3 E1 under NG and HG conditions measured after culturing for 3 days.
(C) RT-qPCR analysis of *glut1-4* mRNA relative expression on ME3T3 E1 after metformin treatment for 48 h.
(D) GLUT1 expression detected under NG conditions after metformin treatment for 48 h.
(E, F) GLUT1 expression detected under HG conditions after metformin treatment for 48 h and analyzed by Image J software.
All error bars represent SEM. Statistical analysis measured by paired *t*-test (one-tailed) between groups without metformin under NG and HG condition. Statistical analysis measured by one-way ANOVA among groups with different metformin concentration under NG and HG condition. *P < 0.05; #P < 0.01; ***P < 0.005; ##P < 0.001.

tor cells into osteoclasts, osteoblasts, osteocytes and chondrocytes [16]. Phosphorylation of NF-κB at high-levels will result in mature osteoblast function decline, damage production and delay maturation of bone matrix [17].

Furthermore, Etchegaray, J. P et al. reported that SIRT6 was a vital regulators in the differentiation of embryonic stem cells mediated by suppressing expression of *oct4*, *sox2* and *nanog* [18]. However, whether metformin action on SIRT6 precedes inhibition of OCT4 expression is still unclear. Therefore, research on the relationship between metformin and SIRT6 may help to explain the metformin's effect on bone fracture of patients with T2DM and to elucidate molecular action mechanism of metformin.

2. Materials and methods

2.1. Chemicals and regents

D-glucose (dextrose) anhydrous and 4% paraformaldehyde purchased from Amersco Life Science Company. (Amersco, China). β-glycerophosphate disodium and vitamin C purchased from Biodee