Metformin regulates bone marrow stromal cells to accelerate bone healing in diabetic mice

Succinct Title: Metformin protects bone under hyperglycemic condition

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Abstract

Diabetes mellitus is a group of chronic diseases characterized by high blood glucose levels. Diabetic patients have a higher risk of sustaining osteoporotic fractures than non-diabetic people. The fracture healing is usually impaired in diabetics and our understanding of the detrimental effects of hyperglycemia on fracture healing is still inadequate. Metformin is the first-line medicine for type-2 diabetes (T2D). However, its effects on bone in T2D patients remain to be studied. To assess the impacts of metformin on fracture healing, we compared the healing process of closed wound fixed fracture, non-fixed radial fracture, and femoral drill-hole injury models in T2D mice with and without metformin treatment. Our results demonstrated that metformin rescued the delayed bone healing and remodeling in T2D mice in all injury models. In vitro analysis indicated that compromised proliferation, osteogenesis, chondrogenesis of the bone marrow stromal cells (BMSCs) derived from T2D mice were rescued by metformin treatment when compared to WT controls. Furthermore, metformin could effectively rescue the impaired detrimental lineage commitment of BMSCs isolated from T2D mice \textit{in vivo} as assessed by subcutaneous ossicle formation of the BMSC implants in recipient T2D mice. Moreover, the Safranin O staining of cartilage formation in the endochondral ossification under hyperglycemic condition significantly increased at day 14 post-fracture in T2D mice receiving metformin treatment. The chondrocyte transcript factor SOX9 and PGC1α, important to maintain chondrocyte homeostasis, were both significantly upregulated in callus tissue isolated at the fracture site of metformin-treated MKR mice on day 12 post-fracture. Metformin also rescued the chondrocyte disc formation of BMSCs isolated from T2D mice. Taken together, our study demonstrated that metformin
facilitated bone healing, more specifically bone formation and chondrogenesis in T2D mouse models.
Introduction

Diabetes mellitus is a group of chronic diseases characterized by high blood glucose levels. It is estimated that more than 347 million people worldwide currently have diabetes and many more people are estimated to become diabetic soon [1]. Among the diabetic patients, more than 90% are suffering from Type-2 diabetes (T2D), which is caused by insulin resistance in peripheral tissues. Many tissues including the skeleton will be adversely affected by hyperglycemia if not controlled [2]. Both Type 1 diabetes and T2D are associated with an increased risk of osteoporosis and fragility fractures [2]. It is recognized that oral antidiabetic medicines affect bone metabolism and turnover [2]. As an insulin sensitizer, patients with T2D are frequently prescribed with metformin. In T2D patients, metformin treatment was associated with a decreased risk of bone fracture [3]. The osteogenic effects of metformin have been documented in both cellular and rodent models. Metformin promoted osteoblast differentiation and inhibited adipocyte differentiation in rat bone marrow mesenchymal stem cells culture [4]. In rat primary osteoblasts culture, metformin increased trabecular bone nodule formation [5]. In ovariectomized mice, metformin was also shown to improve the compromised bone mass and quality [6]. Furthermore, in streptozotocin-induced Type-1 DM model, metformin stimulated bone lesion regeneration in rats [7]. However, to date, there is no study on the skeletal effects of metformin in a T2D model. A recent study found that the incidence of total knee replacement over four years was 19% lower among patients with type 2 diabetes who were regular metformin users, compared with non-users [8]. In addition to reducing glucose levels, metformin may modulate inflammatory and metabolic factors, leading to reduced inflammation and plasma lipid levels [8]. Better knowledge of how
metformin treatment influences skeletal tissues under T2D condition is of great clinical relevance in view of the fast-growing population of patients with T2D.

MKR mouse model was generated and characterized by LeRoith and colleagues and expresses a dominant negative mutant of human IGFI receptor specifically in skeletal muscles [9]. The expression of this dominant negative mutant human IGFI receptor decreased glucose uptake and causes insulin resistance, the MKR transgenic mouse rapidly develops severe diabetes [9]. This mouse model has been widely used in T2D research. In this study, the effects of metformin on bone cell lineage determination and regeneration were characterized using MKR mouse model.

Results

Metformin promotes healing in fracture models under hyperglycemic condition.

In order to evaluate the fracture healing which involves endochondral ossification in mice, we adapted this well accepted Bonnarens and Einhorn fracture mouse model (Fig 1A). Animals were sacrificed on day 14, 23, or 31 post-fracture representing the inflammatory stage, the endochondral stage, and the remodeling stage during the femur fracture repair process [10]. As shown in Fig 1-Supplement Figure1, WT and MKR mice of 12-week-old were treated with PBS or metformin through daily intraperitoneal injection. Blood glucose level (Fig 1- Figure Supplement 1A) and fasting glucose tolerant test (Fig 1- Figure Supplement 1B) showed that metformin significantly reduced glucose levels in MKR mice as anticipated. Meanwhile, metformin treated MKR mice exhibited similar healing and remodeling speed to that of the WT groups throughout the entire healing
process, and the fractured bones were completely healed on day 31 (Fig 1B). On the other hand, the healing process in MKR-PBS group was much delayed, with visible amount of callus tissue remained at the fracture site on day 31. Additionally, histological analysis of the callus area (Fig. 1C) disclosed a postponed peak callus formation in MKR-PBS group when compared to WT-PBS, WT-met, and MKR-met groups on day 23 post-fracture, as well as a delayed remodeling on day 31 post-fracture. A quantitative analysis of the callus at the fracture area (Fig. 1D) confirmed that metformin significantly enhanced bone healing in T2D mice comparing to PBS treated MKR mice. We also harvested the callus tissue at fracture sites on day 12 and day 21 post fracture to examine the expression of genes that contribute to adipogenesis, osteogenesis and chondrogenesis (Figure 1-Figure Supplement 2). A significant reduction of adipogenesis transcription factor accompanied with a significant induction of chondrogenesis transcript factor SOX9 were detected in the callus harvested on day 12 post-fracture. SOX9 is a master transcription factor that play a key role in chondrogenesis[11]. In addition, at day 21 post fracture, PGC1α which is required for chondrocyte metabolism and cartilage homeostasis [12, 13], was significantly upregulated in metformin treated MKR mice.

A similar effect of metformin was also observed in a non-fixed radial fracture model (Fig. 2). After non-fixed radial fracture was introduced, animal was treated with either PBS or metformin for 14 or 23 days (Fig. 2A). In the WT groups at 14 days post-fracture, both PBS and metformin treated animals started to exhibit sufficient amount of callus with the sign of bridging of the fracture ends (Fig. 2B). In the 14-day post-fracture MKR mice, those treated with metformin exhibited more healing callus than the PBS treated ones.
with significantly greater percentage of callus bridging at the fracture site (Fig. 2C). Bone mineral density was also higher in metformin-treated MKR mice when compared to the PBS-treated MKR mice (Fig. 2D). Bone volume/tissue volume ratio (Fig. 2E) and trabecular thickness (Fig. 2F) were not regulated by metformin in either WT or MKR mice. In the 23 days post-fracture groups, only PBS-treated MKR animals failed to show callus formation and bridging at the fracture site compared to all the other groups (Fig. 2G and 2H), indicating a delayed healing process in MKR PBS group. Bone mineral density levels in metformin-treated MKR mice were similar to the PBS-treated WT mice and were significantly higher than the PBS-treated MKR mice (Fig. 2I). In the 23 days post-fracture groups, the bone volume/tissue volume ratio (Fig. 2J) and trabecular thickness (Fig. 2K) were significantly improved by metformin in MKR mice in comparison to the PBS-treated MKR mice. These data suggest that 23 days of metformin treatment favorably affected bone healing. The beneficial effects of 23 days of metformin treatment on the callus bridging (Fig. 2H), BV/TV% (Fig. 2J), and Tb. Thickness (Fig. 2K) became significant in contrast to the trends of improvement observed in the mice received short term treatment for 14 days (Fig 2C, 2E and 2F).

**Metformin improves healing in the drill hole injury model under hyperglycemic condition.**

To further investigate metformin’s effect on bone repair, a drill-hole model was established (Fig. 3A). After 14 days treatment with PBS or metformin, we examined the injury site using μCT scanning. Exterior and interior general view of the femur indicate a non-filled open wound exhibited only in PBS treated MKR group, while all the other
three groups showed mainly filled holes indicating a faster healing speed (Fig. 3B-D). Tracing of the drill-hole injury sites presented a detailed view of the callus tissues formed within the drill hole (Fig. 3D). In the WT animals, quantitative analysis of the µCT images showed no difference between the PBS and the metformin treatments. Significant lower BMD (Fig. 3E) and BV/TV ratio (Fig. 3F) were observed in PBS treated MKR mice when compared to the metformin treated ones. The PBS treated MKR mice also demonstrated prominently higher bone porosity (Fig. 3G) and total pore space (Fig. 3H) within the callus tissue, suggesting delayed bone healing and remodeling in MKR mice was rescued by metformin.

**Metformin accelerates bone formation under hyperglycemic condition.**

In order to examine metformin’s effects on bone formation *in vivo*, we conducted bone Alizarin Red and Calcein Double Labeling injections on WT and MKR mice. Fig. 4A suggested that the florescent labels in WT mice remained the same between PBS and metformin treated animals. In contrast, the distance between the two labels was greater in metformin treated than the PBS treated MKR mice. This observation was supported by serum levels of amino-terminal propeptide of type 1 procollagen (P1NP). P1NP is considered a sensitive marker of bone formation [14] and the ELISA assay was performed using the serum samples collected at 14, 23, and 31 days post femoral fracture. In WT animals, there is no difference in P1NP levels between metformin treated and PBS-treated mice at all three time points (Fig. 4B). On the contrary, we observed significantly higher P1NP levels at all three time points post-fracture in the MKR mice treated with metformin compared those treated with PBS (Fig 4B). Collectively, the data
indicate that metformin can promote bone formation only under hyperglycemic conditions.

**Metformin regulates proliferation and lineage commitment of the bone marrow stromal cell (BMSC) in MKR mice.**

Considering that the multipotent mesenchymal stem/progenitor cells (BMSCs) in bone are critical in maintaining bone quality, function and regeneration, we then tested whether metformin stimulated BMSCs proliferation in MKR mice. As expected, hyperglycemic condition in MKR mice impaired the proliferation of their BMSCs, as indicated by CFU-F staining when compared to WT controls (Fig. 5A). Administration of metformin in MKR mice successfully salvaged the BMSC proliferation capability and brought it back to the equivalent level as observed in WT group. Under all three seeding densities, compromised CFU-F colony formation observed in MKR group can be rescued by metformin treatment as shown in Fig. 5B. However, metformin did not affect the proliferation of BMSCs in WT group when compared to the PBS vehicle. It is noteworthy that metformin was not administrated to the culture. The daily metformin treatment in MKR mice prior to cell isolation appeared to be sufficient to protect the proliferation potential of BMSCs from the detrimental effects of hyperglycemia. BMSCs lineage differentiation potential was also tested by ALP staining and von Kossa staining. After 14 days under osteogenic [1] differentiation, in contrast to the weak ALP activity observed in MKR-PBS group, while MKR-met group showed compatible ALP activity to the WT groups (Fig. 5C and 5D). ALP played a critical role in calcium crystallization and mineralization during bone formation, therefor we speculated this aberrant ALP activity
of the MKR-PBS group would further lead to an impaired bone mineralization. As expected, bone mineralization was barely detected in MKR-PBS group after 21 days osteoblast differentiation visualized by von Kossa staining and metformin significantly enhanced bone mineralization in MKR mice when compared to the PBS-treated MKR animals (Fig. 5E and 5F). Notably, the BMSCs from metformin treated T2D mice could maintain the improved bone formation feature when re-exposed to the same hyperglycemic levels as in the T2D mice. BMSCs isolated from metformin treated MKR mice and PBS controls were implanted to the recipient MKR mice (Fig. 5G). Masson’s Trichrome staining on the ossicles formed by the BMSCs from metformin treated MKR mice showed greater bone formation than that of PBS treated MKR mice (Fig5 H-I). The result indicated that the previous in vivo treatment of metformin protected the BMSCs from the hyperglycemic and inflammatory conditions in the MKR mice, thus they have better potential and capacity to form ossicles in vivo even in MKR recipient mice. Metformin could effectively rescue the impaired differentiation potential of BMSCs in MKR mice.

Improved chondrogenesis of BMSC from metformin treated MKR mice

Chondrogenesis and endochondral ossification are critical steps during the healing process after a bone injury. In order to examine metformin’s effect on chondrogenesis during bone healing, we compared the cartilage deposition within fracture healing sites throughout the healing process (14 days, 23 days, and 31 days post fracture). The cartilage deposition was significantly lower in PBS-treated MKR mice as compared to the PBS-treated WT mice at 14days post fracture (Fig. 6A-B) which is consistent with the
observation of SOX9 and PGC1α induction in the callus on day 12 and day 21 respectively of metformin treated MKR mice (Fig 1-Figure Supplement 2). As expected, no difference between the PBS or metformin treated animals was observed in WT groups. On the other hand, metformin significantly promoted cartilage formation in MKR mice on day 14 post fracture, and the trend continued till day 23 (Fig. 6A-B). By day 31 post fracture, except in the PBS treated MKR mice, no discernible callus remained in any other groups (Fig. 6A). To further investigate if metformin modulates chondrogenesis of BMSCs. BMSCs were isolated for chondrogenesis culture in vitro. Only BMSCs isolated from PBS treated MKR mice failed to form the chondrocyte disc as shown in Fig. 6C after 3 days chondrogenic culture. BMSCs isolated from metformin treated MKR mice could form the chondrocyte disc as well as the WT controls (Fig. 6C).

Discussion

Being the most commonly prescribed diabetic medication in the world, metformin’s effects on bone healing in T2D patients remain unclear. To assess the impacts of metformin on fracture healing under hyperglycemic condition, we applied several classic bone fracture models in T2D mice. Fracture healing is complex, and it involves the following stages: hematoma formation, granulation tissue formation, bony callus formation, and bone remodeling. During the bone healing, chondrogenesis lays down a collagen-rich fibrocartilaginous network spanning the fracture ends and fibroblasts differentiate into osteoblasts so that cartilaginous callus eventually undergoes endochondral ossification[10]. Our results demonstrated that in all injury models tested, metformin successfully rescued the delayed bone healing and remodeling in T2D mice by
facilitating bone formations. Further cell culture studies demonstrated the mechanism of metformin’s action at cellular level via promoting the proliferation, differentiation, and lineage commitment of primary BMSCs. Taken together, metformin showed its potential as an effective drug for increasing the rate and success of bone healing in diabetic patients that are not taking metformin on regular basis.

In all the bone fracture models studied in this study, metformin significantly enhanced bone-healing parameters in MKR mice. However, in the WT animals, quantitative analysis of images showed no difference between the PBS and the metformin treatment in terms of bone healing. These data suggest that metformin is only beneficial for bone healing under hyperglycemic conditions but does not enhance bone healing in WT animals without diabetes. In all BMSC based essays, metformin was not administered to culture media in vitro but administered to animals in vivo before the BMSC were isolated. Administration of metformin in MKR mice successfully salvaged the BMSC proliferation capability and lineage commitment and brought it back to the equivalent level as observed in WT group. Interestingly, metformin did not affect the proliferation and lineage commitment of BMSCs in WT group when compared to the PBS vehicle. These data are consistent with those obtained from bone fracture models, suggesting that metformin may exert its effects through normalizing hyperglycemia, glucose tolerance and other metabolic disturbance under diabetic conditions and does not enhance bone healing in WT animals. In ovariectomized rats, impaired bone density and quality were significantly improved by the treatment of metformin [6]. Taken together, it seems that metformin does not promote further bone growth under physiological conditions but
helps to maintain bone homeostasis under pathological conditions such as hyperglycemia and lack of estrogen. To our knowledge, there are very scarce amount of research on the direct effects of metformin on BMSCs. In an in vitro study using mouse bone marrow-derived multipotent mesenchymal stromal cells, metformin causes inhibition of proliferation and abnormalities of their morphology and ultrastructure and decreased IGF-2 secretion in the supernatant of the culture media [15]. In another paper, metformin added to culture is shown to promote the osteogenesis of BMSCs isolated from T2D patients and osseointegration when administered in rats via the AMPK/BMP/Smad signalling pathway [16]. This discrepancy might be due the different level of metformin used in the culture system and different species of the experimental model. In one study [15], inhibition of mouse BMSC proliferation was observed with 1, 5 and 10 mM of metformin. However, in another study [16], 125 μM was the optimal concentration to stimulate proliferation of BMSC from human and rats. At concentrations over 200 μM, metformin showed an inhibitory effect on BMSCs isolated from T2DM patients. Another possibility is that metformin does not work directly on BMSCs but requires other tissues, cells and the in vivo context to exert its effect. Whether metformin has direct beneficial effects on BMSCs remains to be investigated.

As the most prescribed antidiabetic medicine and a drug poses benefits beyond the treatment diabetes, the molecular and cellular mechanism of metformin demands a lot of research attention. The AMP-activated protein kinase plays a major role in its mechanism of action [17, 18]; however, the exact signaling cascade and the direct molecular target of metformin remains unknown. Recently, low-dose metformin has been shown to target the
lysosomal AMPK pathway through PEN2, a subunit of gamma-secretase [19]. Clinically relevant concentrations of metformin binds PEN2 to activate AMPK pathways following glucose starvation. Loss of function analysis of PEN2 blunts AMPK activation, abolishes metformin-mediated reduction of hepatic fat content, and metformin’s glucose-lowering effects. These novel findings indicate that metformin binds PEN2 directly and initiates a signaling route that intersects the lysosomal glucose-sensing pathway for AMPK activation. To our knowledge, there is no research done on the role of PEN2 in the beneficial effects of metformin on bone homeostasis and healing. It is very much worth the efforts to investigate whether metformin binds PEN2 and utilizes the same signaling pathways in BMSCs. Another important aspect of the mechanism of metformin is its effects on osteoclasts. Metformin has been shown to inhibit osteoclast differentiation in various studies. Metformin inhibits osteoclastogenesis in ovariectomized mice by downregulating autophagy via E2F1-dependent BECN1 and BCL2 downregulation [20]. In osteoarthritis mouse model, metformin inhibits osteoclast activation and it may exert its effects through AMPK/NF-kappaB/ERK signaling pathway [21]. In a rat osteonecrosis model, metformin inhibits osteoclast activity in the necrotic femoral head. [22]. In an in vitro model of periodontitis, osteoclast formation as well as activity was inhibited by metformin in M-CSF and RANKL stimulated monocyte cultures, probably by reduction of RANK expression [23]. In another study, metformin inhibits osteoclast differentiation at a low concentration and promotes osteoclast apoptosis at a higher concentration [24]. The effects of metformin on osteoclasts in various bone fracture models warrant future research.
As reviewed by Roszer, T. [25], diabetes is accompanied by increased level of pro-inflammatory factors, reactive oxygen species (ROS) generation and accumulation of advanced glycation end products (AGEs). The increased inflammatory state could result in apoptosis of osteoblasts and prolonged survival of osteoclasts, which lead to early destruction of callus tissue and impair bone fracture healing of diabetic patients. Thus, antagonizing inflammatory signal pathways and inhibition of inflammation may deserve greater attention in the management of diabetic fracture healing. There are substantial evidence supporting that metformin not only improves chronic inflammation by attenuating hyperglycemia but also has a direct anti-inflammatory effect. Targeting inflammatory pathways seems to be an important part of the comprehensive mechanisms of action of this drug [26]. In addition to AMPK activation and inhibition of mTOR pathways, metformin acts on mitochondrial function and cellular homeostasis processes such as autophagy [26]. Both dysregulated mitochondria and failure of the autophagy pathways affect cellular health drastically and can trigger the onset of metabolic and age-associated inflammation and diseases. For example, T-helper type 17 (Th17) cells, an important proinflammatory CD4+ T cell subset secreting interleukin 17 (IL-17), has been suggested to play an essential role in development of diabetes mellitus [27]. Metformin can ameliorate the pro-inflammatory profile of Th17 by increasing autophagy and improving mitochondrial bioenergetics [28]. In addition, at day 21 post fracture, the PGC1α in callus tissues isolated from the fracture site was significantly upregulated in metformin treated MKR mice. As reviewed by Halling and Pilegaard [29], PGC-1α not only regulates mitochondrial biogenesis but also its function. PGC-1α-mediated regulation of mitochondrial quality may contribute to many age-related dysfunctions.
including insulin sensitivity. Anti-inflammation and enhancement of mitochondrial function could be very important means that metformin utilizes to facilitate bone formation and healing under hyperglycemic conditions.

Diabetic hyperglycemia has been suggested to play a role in osteoarthritis. The metabolic alterations in body fluid such as hyperglycemia could negatively affect the cartilage through direct effects on chondrocytes by stimulating the production of advanced glycosylation end products (AGEs) accumulation in the synovium [30]. PPARγ is highly expressed in adipocytes and the downregulation of PPARγ expression in the callus of metformin treated MKR mice reflected the shift of mesenchymal cells fate. In T2DM mouse model, differentiation of growth plate chondrocytes is delayed and this delay may result from premature apoptosis of the growth plate chondrocytes [31]. Besides its effects on bone formation, there are also interests in studying the effects of metformin on chondrocytes, especially in the context of osteoarthritis development. Limited reference showing that metformin is protective against development of osteoarthritis by reducing chondrocyte apoptosis and alleviating chondrocyte degeneration [32-34]. Consistent with the above reports, our data suggest that metformin promoted the cartilage formation in the endochondral ossification at day 14 post-fracture in T2D mice. Moreover, metformin rescued the chondrocyte disc formation in BMSCs isolated from T2D mice when compared to the PBS treated control. Metformin also upregulated chondrocyte transcript factor SOX9 and in callus tissue isolated at the fracture site in metformin treated MKR mice. Sox-9 plays an essential role in regulation of cartilage matrix production and cartilage repair [11]. In addition, PGC1α was significantly upregulated in
metformin treated MKR mice when compared to the PBS-treated MKR animals. PGC1alpha is important to maintain chondrocyte metabolic flexibility and tissue homeostasis. The loss of PGC1α in chondrocytes during OA pathogenesis resulted in the activation of mitophagy and stimulated cartilage degradation and apoptotic death of chondrocytes [35]. The activation of PGC1α is a potential strategy to delay or prevent the development of OA. The cellular signaling pathways through which metformin exerts its protective effects in chondrocytes warrant further research.

In conclusion, our study demonstrated that metformin can facilitate bone healing, bone formation and chondrogenesis in T2D mice. The molecular mechanism of metformin’s action demands further research in hope to identify specific therapeutic target to facilitate bone healing and repair in diabetic patients.

**Materials and Methods**

**Animals**

All animal experiments were carried on with the compliance of New York University Institutional Animal Care and Use Committee (IACUC). Hyperglycemic mouse model MKR (available from Jackson Laboratory Strain #:016618) breeders were generously provided by LeRoith and colleagues [9]. Friend Virus B (FVB) background wildtype (WT) breeders were ordered from Jackson Lab (Bar Harbor, ME).

**Bone injury models**

1. Femoral closed fracture model

The gravity-induced Bonnarens and Einhorn fracture model was adapted here as previously described in order to establish a standard closed fracture [36, 37]. Briefly,
male WT and MKR mice of 12-week-old were anesthetized with a Ketamine/ Xylazine cocktail, and a 1 cm sagittal incision was made at the right knee beneath the patella. A 3/8-inch length 27-gauge needle was inserted into bone canal right between medial and lateral condyles. The needle end was cut, and the blunt end was pushed forward and buried between medial and lateral condyles to avoid tissue damage afterwards. With the fixative needle inside of the femur canal, animal was moved to the fracture apparatus. The right femur was placed over the two supports, and the blunt guillotine blade was dropped from a pre-tested height onto the femur, to create sufficient force to cause the fracture. The wound was then closed with suture, and mice were randomly assigned into vehicle (PBS) or metformin (Met, 200mg/kg BW) daily treatment groups. In this study, metformin was dissolved in PBS for administration.

2. Radius non-union fracture model

Male WT and MKR mice of 12-week-old were anesthetized using a Ketamine/ Xylazine cocktail. A 0.5 cm coronal incision was made over the right radius. The brachioradialis and pronator teres were carefully separated with blunt surgical instruments to reveal the radius. A super sharp Stevens Tenotomy Scissor was used to cut at the middle of the radius and created the non-union radial fracture. The wound was then closed, and mice were randomly assigned into PBS or metformin daily treatment groups.

3. Femoral drill hole model

WT and MKR male mice of 12-week-old were anesthetized using a Ketamine/ Xylazine cocktail. A 1 cm coronal incision was made over the right lateral femur. Quadriceps were carefully separated with blunt surgical instruments to reveal the femur. A drill bit (#66)
was used to create a 0.8 mm diameter hole on the femur. After closing the wound, mice were randomly assigned into PBS or metformin daily treatment groups.

### Tissue Collection and Processing

The micro computed tomography (μCT) and bone histomorphometry were utilized to assess static and dynamic indices of bone structure and formation. Briefly, bone injuries were introduced in animals as described above, PBS and metformin treatment was administrated daily for indicated time. After sacrificing the animal, injured bone samples were fixed in 10% buffered formalin for 48 hours, then rinsed with PBS before being analyzed by μCT. Bones were evaluated using a SkyScan 1172 high-resolution scanner (Brucker, Billerica, MA, USA) with 60 kV voltage and 167μA current at a 9.7 μm resolution and reconstructed using NRecon (V.1.6.10.2.). Whole scanned region was included as VOI (volume of interest) [38] from femoral fracture model to generate a general 3D view of the femur fracture cite. In the radius fracture model, a total of 3 mm (311 transverse anatomic slides) radius including the entire injury site was selected as VOI. In order to analyze only the fracture callus bone parameters, two sets of regions of interest [9] were manually drawn within each VOI slides. The first set traced the external fracture callus (inclusion ROI) and the second set traced the cortical bones within the callus (exclusion ROI). In the femur drill hole model, a round shaped ROI with 0.611 mm diameter (63 pixels) was surrounded in the center of injury hole throughout the depth where callus exhibit to generate the VOI consist of callus within the drill cite. VOI from each animals were analyzed with CTan (V.1.13.2.1.) to calculate the following morphometric parameters: bone mineral
density (BMD), relative bone volume (BV/TV), trabecular thickness (Tb.Th),

trabecular separation (Tb. Sp), porosity, and total pore space. CTVox (V.3.3.0.0.) was

used to generate the 3 spatial images of the VOI.

Callus bridging score was ranking from 0 to 3. (0: no bridging; 1: less than 33%

bridging; 2: more than 34% but less than 66% bridging; 3: more than 67% till complete

bridging). Callus bridging ratio was calculated by each animals’ callus bridging score/3.

For double labeling experiment, ten-week-old male WT and MKR mice were

randomly assigned to receive PBS or metformin daily injections for 14 days. Mice

also received intraperitoneal injections of calcein (10 mg/kg) on the 5th day and alizarin

red (15 mg/kg) on the 12th days during the 14-day period. For histomorphometry and

peripheral quantitative computed tomography, femurs were preserved in 70% ethanol

until they were processed for plastic embedding in methyl methacrylate resin or 428
decalcified for paraffin embedding.

Blood was collected by cardiac puncture after euthanasia, left at room temperature for

30 min before centrifuging at 200 g for 10 min to separate sera.

Cell culture and analysis:

As previously described, after 14-day treatment of PBS or metformin in-vivo, WT and

MKR mice were sacrificed and proceeded with bone marrow primary cell culture. 434

Excessive tissue was removed from femur and tibia from each mouse, and quickly

rinsed with 70% ethanol and then followed by triple cold PBS wash to ensure sterility.

Bone marrow cells were flushed out using cold PBS and broken down into individual

cell suspension by passing through a 19-gauge needle for 5 times. Cells were then

cultured in MEM Alpha Modification (α-MEM) medium supplemented with 10% Fetal
Bovine Serum (R&D Systems, USA), 100 μg/ml streptomycin, and 100 units/ml penicillin (Gibco, Grand Island, NY, USA) in a 37 °C, 5% (v/v) CO₂, humidified incubator. After removed non-adherent cells, the adherent cells were cultured as bone marrow stromal cells (BMSCs) for 7 days and sub-cultured for following assays:

1. Colony-forming unit fibroblastic assay (CFU-F)

   Isolated BMSCs were seeded at 100, 500, and 1000 cells/well in 6-well plate, followed by 10 days culture with α-MEM complete medium. Cells were fixed with 10% buffered formalin, washed 3 times with PBS followed by staining with 0.25% wt/vol crystal violet solution. Plate images were captured using ChemiDoc XRS System (BioRad Laboratories, Inc. Hercules, CA, USA) and analyzed with ImageJ.

2. Osteoblast differentiation

   Isolated BMSCs were seeded at 6.4 x 10^4 cells/well into 6-well plates, and cultured with osteogenic medium containing α-MEM complete medium supplement with 50 μg/mL L-Ascorbic acid-2-phosphate and 10mM β-glycerophosphate for 2~3 weeks followed by ALP and von Kossa staining. Briefly, after 2 weeks of osteogenic differentiation, cells were fixed and checked for alkaline phosphatase activity using ALP kit (86R-1KT, Sigma) following manufacture’s protocol. After 3 weeks under osteogenic culture, cells were fixed with 95% ethanol and rehydrated through gradient ethanol to water. Cells were then carefully washed with water after being incubated with 5% silver nitrate solution at 37 °C for one hour, exposed under UV light for 10 minutes. All plates’ images were captured using ChemiDoc XRS System, and ALP positive area and mineralized regions were measured by ImageJ software.

3. Chondrocytes differentiation and disc formation
Isolated BMSCs were further cultured and passaged twice using standard growth media of DMEM+10% FBS in order to enrich the cell number. A cell solution of $1.6 \times 10^7$ cells/mL was generated using StemPro™ Chondrogenesis Differentiation Kit (ThermoFisher), and 5 µL droplets were applied in the center of 48-well plate wells for micro-mass culture. 3 hours later, warmed chondrogenic medium were overlayed over the micro-mass and the formation of osteogenic pellets were observed after 3 days of culture.

**In vivo Ossicle formation assay**

Adapted from previous report [39] and as described above, BMSCs from different treatment groups were obtained and cultured using standard growth media of DMEM+10% FBS with customized glucose level. We measured and calculated mean value of recipient MKR mice blood glucose level (N=4, average glucose level = 436 mg/dL) and prepared the ex-vivo culture medium accordingly to avoid glucose level change from ex-vivo culture to the body fluid of the recipient MKR mice. Briefly, BMSCs from PBS or metformin treated mice were growing to 90% confluent, then $1.0 \times 10^8$ cells/mL cells solution was obtained and 20 µL of this cell solution was soaked in a 4mm x 4mm gel foam and grafted at the flank of the recipient MKR mice subcutaneously. Four weeks after the implantation, the gel foams were dissected and processed for histological assays.

**Histology and image analysis**

Femurs from fracture models and bone ossicles were decalcified using 10% EDTA for 2 weeks. EDTA solution was refreshed every other day for the best decalcification efficacy. Tissues were then processed through automatic tissue processor, followed by paraffin
embedding. Five µm thick sections were cut from the paraffin-embedded tissues and subjected to H&E, safranin O, and Mason’s Trichrome staining respectively. The total callus area and cartilage area within it were traced and analyzed using image J software. Bone area ratio within ossicles were also analyzed using image J.

**Glucose test and Glucose tolerance test**

WT and MKR mice of 12-week-old were treated with PBS or metformin through daily intraperitoneal injection as previously described. Blood glucose level was recorded at indicated time points for each mice. After 14 days PBS or metformin injection, animals were fasting overnight for 16 hours by transferring into a clean new cage supplied with water. 20% (W/V) glucose solution was applied to animals through oral gavage (1.6g of glucose/ KG body weight). Blood glucose was measured at indicated time points till two hours post glucose oral gavage.

**Quantitative PCR**

Femoral closed fracture was performed as previously described. Callus tissue at the fracture cite was collected and lysate in Trizol Reagent. RNA was extracted and purified using the RNeasy Plus kit. cDNA was prepared by using a Reverse Transcriptase Kit, and PCR was then performed with a Bio-Rad CFX384 Real-Time PCR detection System. Primers information as below:

mβ-actin Fr: AGCCATGTACGTAGCCATCC;
mβ-actin Rv: CTCTCAGCTGTGGTGTTGAA;
mPGC1α Fr: GAATCAAGCCACTACAGACCCG;
mPGC1α Rv: CATCCCTCTTGGAGCCTTTCGT;
Statistics

We used ANOVA analysis when the study subjects included more than two groups, followed by post-hoc tests as indicated in the legends. We used the two-tailed Student’s t-test to compare the difference between two experimental groups. A value of P<0.05 was considered to be statistically significant. Bars in figures represent the mean ± SEM unless stated otherwise.
Figure 1. Improved healing in closed transverse fracture. A) Schematic representation of the experimental design (created with BioRender.com). B) Representative µCT images of femurs from each treatment group. C) H&E staining of longitudinal femur sections (scale bar, 1 mm) and D) Histomorphometry analysis was performed on those H&E slides to evaluate the callus area at fracture site from each treatment group. (ANOVA, followed by Tukey’s post-hoc test. Bars show mean ± SEM. N= 3~6, *p<0.05, ** p<0.005).

Figure 2. Improved healing in non-fixed radial fracture at two different time points (14 days and 23 days post fracture). A) Schematic representation of the experimental design. (Created with BioRender.com). B) Representative µCT images of mouse radiuses (top) and fracture sites (bottom) 14 days post-fracture (scale bar, 500 µm). Measured parameters (C-F) by Micro-CT at 14 days post-fracture. C) Percentage of callus bridging (%). D) Bone mineral density (BMD; g/cm³). E) Bone volume/tissue volume (BV/TV; %). F) Trabecular thickness (Tb.Th; mm). G) Representative µCT images of mouse radiuses (top) and fracture sites (bottom) 23 days post-fracture (scale bar, 500 µm). Measured parameters (H-K) by Micro-CT at 23 days post-fracture. H) Percentage of callus bridging (%). I) Bone mineral density (BMD; g/cm³). J) Bone volume/tissue volume (BV/TV; %) - K) Trabecular thickness (Tb.Th; mm). Results of quantitative µCT data analysis (ANOVA, followed by Tukey’s post-hoc test. Bars show mean ± SEM. N=5~6).
Figure 3. Improved healing in drill-hole bone repair model. A) Schematic representation of the experimental design (Created with BioRender.com). B) Representative 3D images of mouse femurs with both exterior and interior general view at 14 days post-surgery. C) Cross plane images at center of the drill site. D) Representative 3D images within the drill site (scale bar, 100 µm) and E) -H) Results of quantitative µCT data analysis (ANOVA, followed by Tukey’s post-hoc test. Bars show mean ± SEM. n=6~9, *p<0.05, **p<0.01, *** p<0.005, **** p<0.0001). E) Bone mineral density (BMD; g/cm3). F) Bone volume/tissue volume ratio (BV/TV; %). G) Porosity (%). H) Total pore space (mm³).

Figure 4. Metformin promotes bone formation. A) Representative images of calcein double-labeling in cortical periosteum of the femur mid-shaft. B) Serum P1NP level (ng/ml) from each treatment group at 14, 23, and 31 days post-femoral fracture. (ANOVA, followed by Sidak’s post-hoc test. Bars show mean ± SEM. N= 5~8, *p<0.05, **** p<0.0001)

Figure 5. Improved osteogenesis of BMSC from metformin treated MKR mice. A) Primary BMSCs were isolated from animals that were treated with PBS or metformin in-vivo for 14 days and were seeded at indicated density for CFU-F culture. B) Results from quantitative analysis of colony counts measured by ImageJ. C) Primary BMSCs from mice received 14-day treatment of PBS or metformin were plated in 6-well plates and cultured using differentiation medium and were tested for ALP activity, D) total ALP positive area per well were measured by ImageJ. E) von Kossa staining to examine
mineralization. F) Calculation of mineralized area. G) Schematic representation of the experimental design. H) Masson's Trichrome staining on the ossicle sections, and I) percentage of bone area (blue) per field was measured using ImageJ. (Bars show mean ± SEM. n= 6~9, *p<0.05, **p<0.01, *** p<0.005, **** p<0.0001).

Figure 6. Improved chondrogenesis of BMSC from metformin treated MKR mice. A) Safranin O staining of longitudinal femur sections from femoral fractures at different time points (scale bar, 1 mm). B) Cartilage area at fracture site were measured using ImageJ. C) Chondrocyte pellet culture of BMSCs from PBS or metformin treated animals. Micromass culture were generated by seeding 5 µL of primary BMSCs (1.6x10^7 cells/mL) in the center of 48-well plate and cultured under chondrogenic condition for 3 days. (ANOVA, followed by Sidak’s post-hoc test. Bars show mean ± SEM. n=4~6, *p<0.05, **p<0.01).
Conflict of interest disclosure

Authors declare no conflict of interest.

ACKNOWLEDGMENTS

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References:


Nikolajczyk, Metformin Enhances Autophagy and Normalizes Mitochondrial Function to Alleviate Aging-Associated Inflammation, Cell Metab, 32 (2020) 44-55 e46.


Legends for supplementary figures:

Figure 1 - Figure supplement 1.
Metformin’s effect on glucose levels in MKR mice. A) Glucose levels in WT and MKR mice throughout the 14 days PBS or metformin treatment. B) Glucose tolerance test (GTT) after fasting post 14 days PBS or metformin treatment. (N=6, SD) If different letters are shown at a time point, they are statistically different from one another (ANOVA, p<0.05 by post-hoc Tukey’s).

Figure 1 - Figure supplement 2.
Transcript factor expressions within the femoral fracture callus tissue. A) adipocyte, B) osteoblast differentiation, and C) chodrogenesis transcript factors expression in the callus tissue at 12 days post-fracture. D) adipocyte, E) osteoblast differentiation, and F) chodrogenesis transcript factors expression in the callus tissue at 21 days post-fracture. G) PGC1α expression in the callus tissue at 12 days H) 21 days post-fracture.
Additional files

The data used for generating the figures in this manuscript has been included in the following source data files (from “Figure 1-source data 1” through “Figure 1- Figure Supplement 2-Source data 1”). Each figure resource file within the provided file represents the raw data for the corresponding figure.

- Figure 1-source data 1: Figure 1D
- Figure 2-source data 1: Figure 2C-F, Figure 2H-K
- Figure 3-source data 1: Figure 3E-H
- Figure 4-source data 1: Figure 4B
- Figure 5-source data 1: Figure 5B, 5D, 5F, and 5I
- Figure 6-source data 1: Figure 6B
- Figure 1- Figure Supplement 1-Source data 1: Figure Supplement 1A-B
- Figure 1- Figure Supplement 2-Source data 1: Figure Supplement 2A-H
Figure 1. Improved healing in closed wound fixed fracture

A

i.p injection with PBS/metformin for 14/23/31 days

B

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<thead>
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C

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D

Callus Area (mm²)

- 14 days
- 23 days
- 31 days

- ns
- ✱✱
Figure 2. Improved healing in non-fixed radial fracture

A

i.p injection with PBS/metformin for 14/23 days

B

WT PBS  WT Met  MKR PBS  MKR Met

3 mm

C

% callus bridging

ns

D

BMD (g/cm³)

ns

E

BV/TV %

ns

F

Tb.Th (mm)

ns

G

WT PBS  WT Met  MKR PBS  MKR Met

3 mm

H

% callus bridging

**

I

BMD (g/cm³)

ns

J

BV/TV %

ns

K

Tb.Th (mm)

ns
Figure 3. Improved healing in drill-hole model

A

B

WT PBS  WT Met  MKR PBS  MKR Met

C

D

E

F

G

H

WT PBSWT MetMKR PBS MKR Met

i.p injection with PBS/metformin for 14 days

ns ✱✱✱

% BV/TV

ns

% Porosity

ns

Total pore space (mm³)

ns

BMD (g/cm³)
Figure 4. Metformin facilitates bone injury healing by promoting bone formation.
Figure 5. Improved osteogenesis of BMSC from metformin treated MKR mice

A. WT Mice

B. METMICE

C. BMSCs

D. METMICE

E. BMSCs

F. METMICE

G. METMICE

H. PBS

I. METMICE

J. METMICE
Figure 6. Improved chondrogenesis of BMSC from metformin treated MKR mice

A.

WT PBS    WT Met    MKR PBS    MKR Met

D14

D23

D31

B.

Cartilage area (mm²)

C.

WT-PBS    WT-Met

MKR-PBS    MKR-Met
Figure 1- Figure Supplement 1. Metformin's effect on glucose levels in MKR mice. A) Glucose levels in WT and MKR mice throughout the 14 days PBS or metformin treatment. B) Glucose tolerance test (GTT) after fasting post 14 days PBS or metformin treatment. (N=6, SD) If different letters are shown at a time point, they are statistically different from one another (ANOVA, p<0.05 by post-hoc Tukey's).
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