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Original Article

Metformin alleviates genetic and traumatic heterotopic ossification by inhibiting infiltration and mitochondrial metabolism of myeloid cells

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Abstract: Objectives: Heterotopic ossification (HO), whether hereditary or traumatic, refers to the abnormal formation of bone in extraskeletal sites, often triggered by inflammation or flare-ups. Unfortunately, there are currently no effective treatments for HO. Metformin is well-known for its anti-diabetic, anti-inflammatory, anti-aging, and anti-cancer effects. However, its potential role in treating HO remains uncertain. Methods: Metformin was dissolved into water and given to mice. All the mice in this study were examined by microCT and myeloid cell quantification using flow cytometry. Complex activity kit was used to examine the activity of mitochondrial complexes of myeloid cells. Results: In this study, we discovered that metformin effectively inhibits genetic and traumatic HO formation and progression. Additionally, we observed a significant increase in myeloid cells in the genetic and traumatic HO mouse model compared to uninjured mice. Notably, metformin specifically reduced the infiltration of myeloid cells into the injured sites of the genetic and traumatic HO model mice. Further investigations revealed that metformin targets mitochondrial complex I and suppresses mitochondrial metabolism in myeloid cells. Conclusion: These findings suggest that metformin suppresses HO development by potentially downregulating the mitochondrial metabolism of myeloid cells, offering a promising therapeutic option for HO treatment.

Keywords: Heterotopic ossification, metformin, myeloid cells

Introduction

Heterotopic ossification (HO) is identifiable by ectopic bone formation in soft tissue, such as the muscle, tendons, and skin [1]. Two forms of HO are commonly diagnosed: hereditary and acquired. Progressive osseous heteroplasia (POH) and fibrodysplasia ossificans progressive (FOP) are the most common forms of hereditary HO [2, 3]. POH results from loss-of-function (LOF) mutations in the guanine nucleotide-binding protein, alpha stimulating activity polypeptide (*GNAS*), while FOP is caused by gain-of-function (GOF) mutations of Activin A receptor, type I (*ACVR1*). Moreover, HO occurs after trauma, severe burns, fractures, and surgery and is therefore often named acquired HO [4]. Bone morphogenetic proteins and hedgehog signaling pathways are responsible for regulating the osteochondral differentiation of mesenchymal stem cells (MSCs), eventually promoting the pathogenesis of genetic and traumatic HO [5, 6]. However, the role of HO-supportive cells, including immune cells, in HO remains unclear.

Both hereditary and acquired HO are triggered by inflammation [7]. Following injury, an abundance of immune cells infiltrate the local tissue to amplify the inflammatory response [8]. The innate immune system is closely tied to HO [9]. The expression level of complement factor H positively correlates with the probability of HO occurrence [10]. Moreover, the density of mast cells in HO tissue is 40-150 times higher than in normal skeletal muscle, and about 10-40-fold higher than in other inflammatory myopathies [8]. Knockdown of μ-opioid receptor (MOR) expression can lower mast cell activation and prevent HO formation [11]. Further studies have

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suggested that substance P regulates mast cells and participates in HO formation [12]. Monocytes and macrophages are indispensable cellular components in the early stages of HO development [13]. Numerous mononuclear macrophages are located in the area surrounding HO. Elimination of macrophages by chemical or genetic methods can inhibit HO occurrence [14, 15]. Activation of the adaptive immune system promotes the development of HO. The acquired immune system is also essential for the formation of HO [16]. There are more T cells and B cells during the early stages of an inflammatory response. Removal of mature T cells and B cells does not prevent the occurrence of HO but does reduce the rate of HO development [16]. However, the underlying mechanisms driving hyperactivation of immune cells in HO remain largely unknown.

Neutrophils comprise 40% to 70% of all white blood cells (WBCs) and are the most abundant granulocyte [17]. Following infection or injury occurs, neutrophils respond acutely to inflammation and are rapidly recruited to the injured site. Neutrophils can ingest microorganisms or particles [18] and internalize and kill many microbes, with each phagocytic event resulting in phagosome formation into which reactive oxygen species and hydrolytic enzymes are secreted. The consumption of oxygen during reactive oxygen species generation has been named a “respiratory burst”, although the process is unrelated to respiration or energy production. Neutrophils distribute themselves throughout HO lesions [19]. Further studies revealed that neutrophil-derived prostaglandin E2 (PEG2) contributed to HO formation [20]. Neutrophils have been reported not to be associated with acquired neurogenic HO as granulocyte colony-stimulating factor (G-CSF) enhanced the number of neutrophils in the injury site but did not enhance HO development [21]. Therefore, there is an urgent requirement to investigate the role of neutrophils in HO.

Metabolism is responsible for generating energy for cellular processes, and immunometabolism is the study of the interface between metabolism and immunology [22]. Immunometabolism focuses on molecular and biochemical underpinnings of i) the metabolic regulation of immune function and ii) the regulation of metabolism by molecules and cells of the immune system. Neutrophil reprogramming is the switch in metabolic pathways caused by modified biochemical reaction rates, resulting in extensive alterations in cellular behavior [23]. HO progression is closely linked to metabolic dysfunction [13], but the role of metabolism, especially metabolic reprogramming of neutrophils in HO, remains unclear. Additionally, metformin is able to target mitochondrial complex I and lower blood glucose in patients with type 2 diabetes and as a second-line agent for infertility in those with polycystic ovary syndrome. However, metformin’s role in HO and the metabolism of myeloid cells is unclear.

In our study, we determined that metformin can effectively inhibit HO formation in three HO mouse models, including BMP4-dependent, *Acvr1*$^{R206H/+}$-mutant, and traumatic HO model mice. Crucially, we demonstrated that metformin can inhibit HO propagation. Prevention of OXPHOS using metformin restricts myeloid activation and infiltration. These findings show that myeloid cells play a crucial role in the formation of HO. The study suggests that targeting the metabolism of myeloid cells, particularly those with mitochondrial dysfunction, by using metformin could be a new approach for treating HO.

Materials and methods

Mice

Nse-Bmp4 transgenic mice were provided by Dr. Lixin Kan (Northwestern University, USA). As described previously, the *Nse-Bmp4* transgene was developed by cloning a 1246-bp fragment containing the murine *Bmp4* cDNA downstream of the rat neuron-specific enolase (*Nse*) promoter and upstream of an SV40 polyadenylation signal [24]. The R206H mutant sequence was inserted following exon 13 of the WT *Acvr1* to establish conditional knock-in mice. *Tie2-cre* mice were purchased from Jackson laboratory and were then crossed with *Acvr1*$^{R206H/+}$ mice to generate *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice for our experiments. Adult mice, both males and females, were used in this study. All animals were randomly allocated to groups that each contained at least five mice at each time point. All experiments involving mice were conducted as prescribed by the National Guidelines for Animal Usage in

Research (China) and were approved by the Ethics Committee of Anhui Medical University (reference: LLSC20210807; Hefei, China).

HO induction

To establish the Tenotomy HO model, mice were anesthetized using 1% pentobarbital sodium. The skin was incised to expose the Achilles tendon, which received 20 repeated clamps by using hemostatic forceps and was cut with scissors. Finally, the skin was closed using sutures [25]. BMP4-dependent and *Acvr1*$^{R206H/+}$-dependent HO was induced by a pinch injury of the tibial muscle in *Nse-Bmp4* and *Tie2-cre; Acvr1*$^{R206H/+}$ mice, respectively. Specifically, the gastrocnemius and covering skin were held at the approximate mid-belly of the muscle using 2-mm wide tissue forceps, and pressure was applied for 5 seconds. Care was taken to avoid incidental contact with the tibia. Injections were performed while mice were anesthetized with isoflurane.

Flow cytometry

Myeloid cells were quantified by flow cytometry. Briefly, cells from injured muscle sites were harvested, washed, and incubated for 20 minutes at 4°C in phosphatase-buffered saline (PBS) containing the following antibodies: CD11b-FITC (101206, Biolegend), Ly6C-PE (128007, Biolegend), and Ly6G-APC (127613, Biolegend). Neutrophils were defined as $CD11b^+/Ly6G^+/Ly6C^-$, and monocytes were defined as $CD11b^+/Ly6G^-/Ly6C^+$.

MicroCT scanning and HO volume calculation

Mouse hindlimbs were harvested and imaged after injury at various time points. To measure the HO volume and bone parameters, microCT (Skyscan 1176, Bruker) was utilized with parameters set to 180° rotation, constant 90-kV voltage 440, and a voxel size of 72 μm. 3D images were reconstructed using SkyScan software. The HO region was outlined by the ROI module and quantified by individual 3D object analysis.

Metformin treatment

Nse-Bmp4 mice, after receiving the tibial muscle injury, were treated with metformin (HY-B0627, 0.2 g/300 mL or 0.4 g/300 mL, MCE). The drugs were dissolved in the drinking water of the mice and were supplemented every three days. The control *Nse-Bmp4* mice were treated with an equal volume of drinking water and administered identically.

Statistical analysis

Unless otherwise stated, data are presented as mean ± s.d. of biological replicates (independent animals/independent experiments; the numbers (n) are specified in each figure legend). Unpaired two-tailed Student's t-tests were employed to determine the significance of differences between the two groups. $P < 0.05$ was the threshold for statistical significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$).

Results

Metformin inhibits BMP4-dependent HO initiation and propagation

HO is often caused by inflammation [26] and is identified as a segmental progeroid syndrome [27]. Metformin exhibits a robust anti-inflammation and anti-aging effect [28, 29]. Therefore, we examined the effect of metformin on HO formation and development. *Nse-Bmp4* transgenic mice were established to investigate BMP4 signaling in nervous system development, and we found that this mouse model could recapitulate the FOP phenotype, exemplified by HO formation in the muscle of *Nse-Bmp4* mice spontaneously occurring around at six-months-old and/or develop HO in muscle 14 days after injury [24, 30, 31]. To evaluate the therapeutic effect of metformin on BMP4-dependent HO formation, we injured the tibial muscle of *Nse-Bmp4* mice and administered either water or metformin to the mice. After 14 days post-injury (dpi), *Nse-Bmp4* mice were sacrificed, and microCT scanning was conducted (**Figure 1A**). Uninjured *Nse-Bmp4* mice did not develop HO. However, vehicle-treated and metformin-treated *Nse-Bmp4* both exhibited the formation of HO (**Figure 1B**). Moreover, compared to injured *Nse-Bmp4* mice receiving water treatment, metformin-treated NSE-BMP4 mice exhibited significantly decreased HO volume (**Figure 1B** and **1C**). This data revealed that metformin treatment could effectively alleviate the initiation of HO.

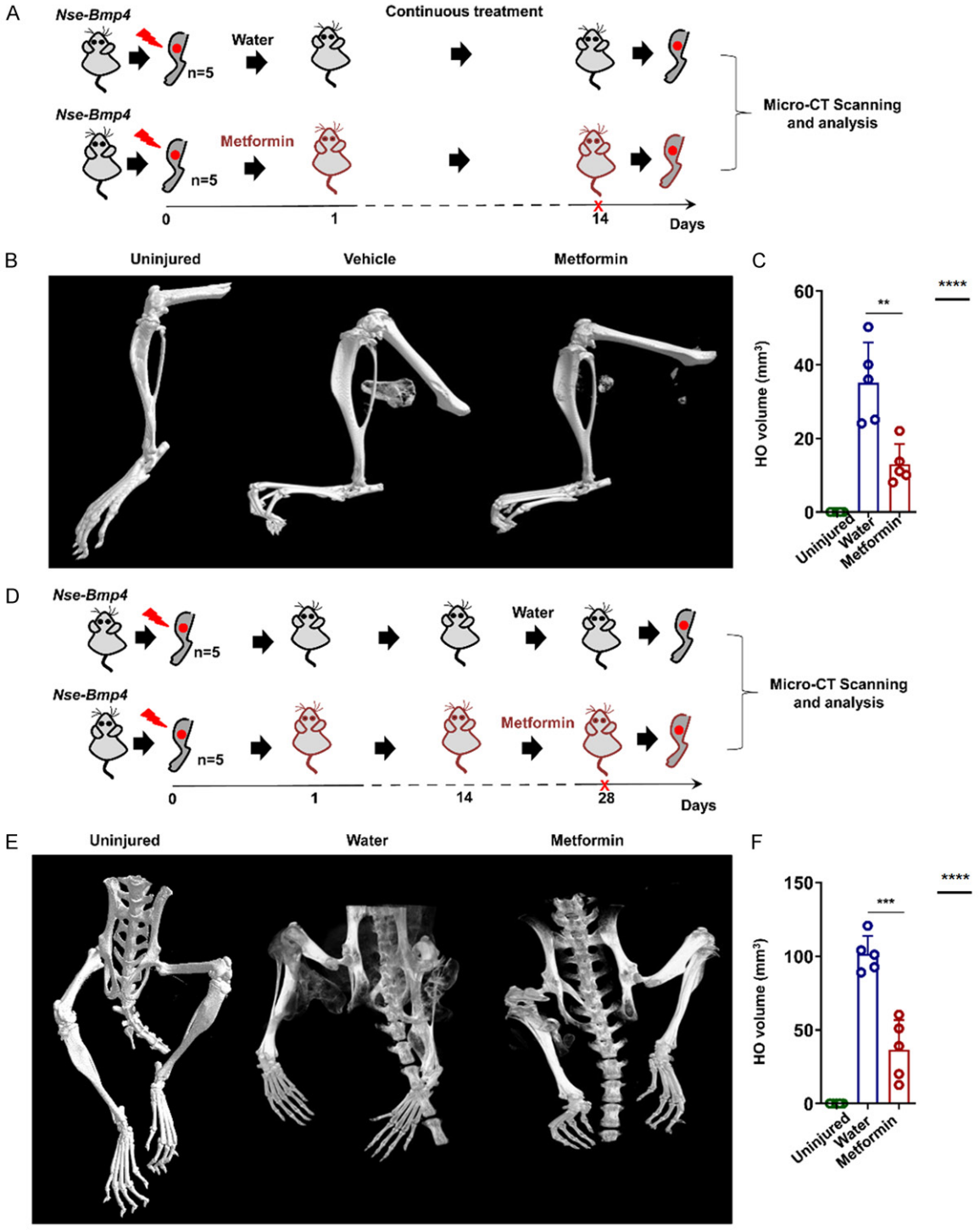

Figure 1. Metformin inhibits HO formation in *Nse-Bmp4* mice. A. Schematic of the study design, highlighting the systematic process of target tissue collection at specified time intervals for microCT analysis. B. Representative microCT images from uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment. C. Statistical analysis of the HO volume in uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). D. Visual representation of our study design, highlighting the systematic process of collecting target tissue at specific time intervals for microCT analysis. E. Representative microCT images from uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment. F. Statistical analysis of the HO volume in uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

HO is often diagnosed after it has progressed and propagated to an adjacent site. Therefore, we next examined whether metformin could alleviate HO propagation in *Nse-Bmp4* mice. After executing a pinch injury of the tibial muscle, *Nse-Bmp4* mice were treated with either water or metformin from 14 to 28 dpi (**Figure 1D**). At 28 dpi, vehicle-treated and metformin-treated *Nse-Bmp4* mice developed HO, while uninjured *Nse-Bmp4* mice did not develop HO. Moreover, vehicle-treated *Nse-Bmp4* mice developed bilateral tibial HO and abdominal HO. However, metformin restricted the HO formation to only the left hind limb (**Figure 1E**). The total HO volume in metformin-treated *Nse-Bmp4* mice was significantly lower than vehicle-treated NSE-BMP4 mice (**Figure 1F**). Together, these results demonstrated that metformin effectively inhibited BMP4-dependent HO initiation and propagation.

Metformin inhibits HO initiation and propagation in FOP mouse model

To further assess the therapeutic potential of metformin on HO, we established a mouse model harboring an *Acvr1*$^{R206H/+}$ mutation which is common in FOP patients [32]. Mutant coding sequences containing the p.R206H site were inserted into the murine *Acvr1* transcript (ENSMUST00000090935.9) using CRISPR-Cas9 technology (**Figure 2A**, **2B**). After being mated with *Tie2-cre* mice, *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice were obtained to evaluate the effect of metformin on HO formation. The tibial muscle of *Tie2-cre*; *Acvr1*$^{R206H/+}$ mouse was injured, and the animals were treated with either water or metformin. After 14 days post-injury (dpi), the *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice were sacrificed, and microCT scanning was conducted (**Figure 3A**). HE staining confirmed that HO was developed in the injured leision of *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice (Supplementary Figure 1). Uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice did not develop HO. However, vehicle-treated, and metformin-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice both exhibited the formation of HO (**Figure 3B**). Moreover, compared to injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving water treatment, metformin-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice had significantly reduced HO volume (**Figure 3B**, **3C**). This data demonstrated that metformin treatment could effectively alleviate HO initiation in FOP model mice.

We next assessed whether metformin could alleviate HO propagation in *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice. After undergoing pinch injury of the tibial muscle, *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice were treated with either water or metformin from 14 to 28 dpi (**Figure 3D**). At 28 dpi, vehicle-treated and metformin-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice developed HO, while uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice did not develop HO. Vehicle-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice developed bilateral tibial HO and abdominal HO. However, metformin restricted HO formation to only the left hind limb (**Figure 3E**). The total HO volume in metformin-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice was significantly lower than in vehicle-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice (**Figure 3F**). Together, these results demonstrated that metformin effectively inhibited HO initiation and propagation in FOP model mice.

Metformin inhibits traumatic HO formation in a tenotomy mouse model

Traumatic HO is a frequent, destructive complication caused by traumatic events, including tendon injury [33, 34], burns, spinal cord injury (SCI), traumatic brain injury (TBI), and orthopedic surgery. The incidence of HO is relatively high (about 11% in TBI, 20% in SCI, 14-35% after significant elbow trauma, and 4.7% after hip arthroscopy) [16, 35, 36]. Patients with HO commonly develop chronic pain, unhealed wounds, restricted joint motion, nerve entrapment, and reduced quality of life, with no effective treatment. Therefore, we examined the effect of metformin on traumatic HO formation. Initially, we performed tenotomy surgery on WT mice (C57BL/6 background). After the injury, metformin was employed to treat injured mice after receiving the injury. After continuous treatment with metformin or water for 42 days, the vehicle-treated and metformin-treated mice were sacrificed, and microCT scan analysis was performed (**Figure 4A**). Uninjured WT mice did not develop HO, while vehicle-treated and metformin-treated mice having undergone tenotomy surgery both did develop HO (**Figure 4B**). Moreover, compared to tenotomy mice receiving water treatment, metformin-treated mice following tenotomy surgery showed significantly reduced HO volume (**Figure 4B**, **4C**). This data revealed that metformin treatment effectively alleviated the initiation of traumatic HO.

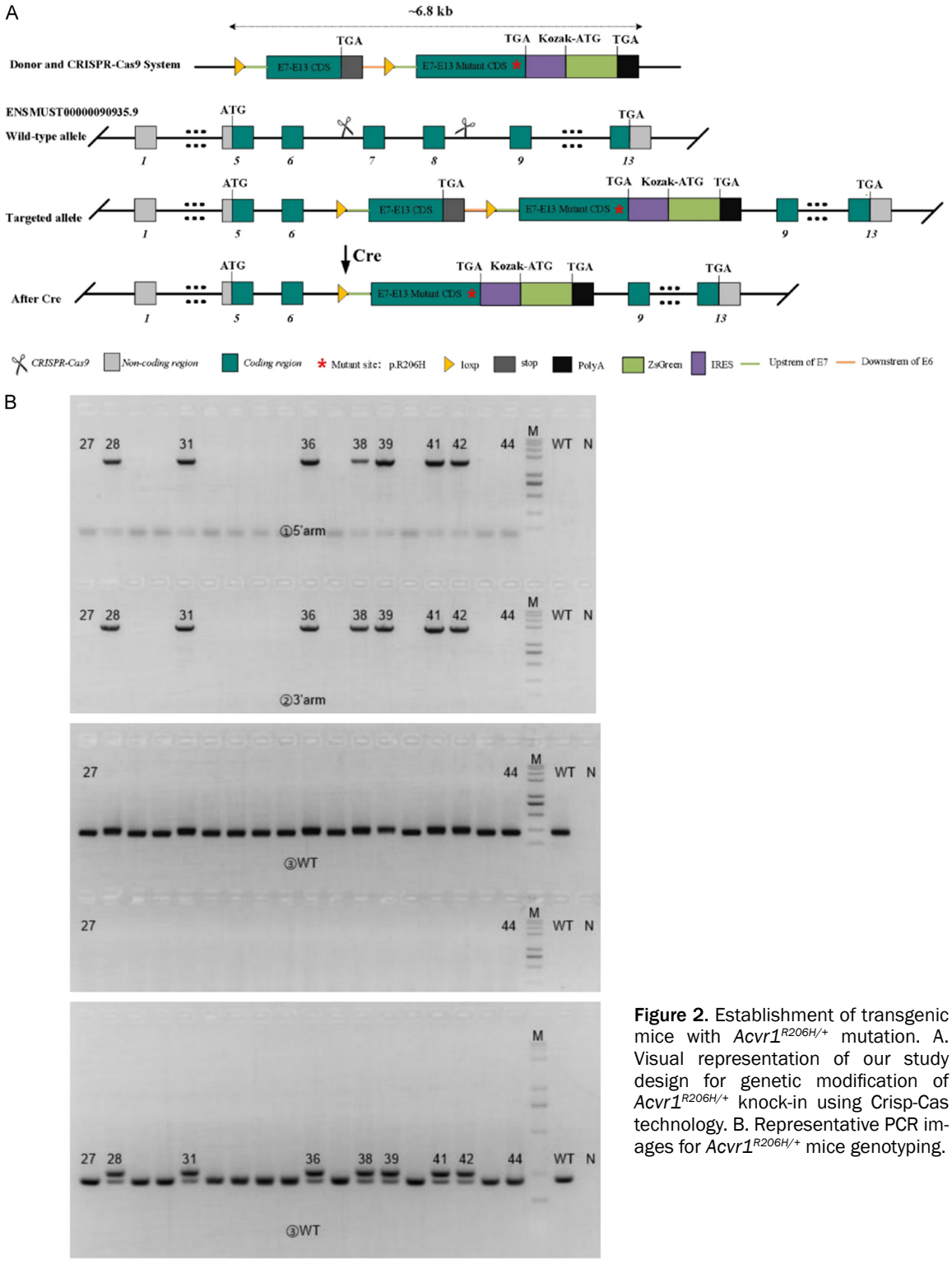

Figure 2. Establishment of transgenic mice with *Acvr1*$^{R206H/+}$ mutation. A. Visual representation of our study design for genetic modification of *Acvr1*$^{R206H/+}$ knock-in using Crisp-Cas technology. B. Representative PCR images for *Acvr1*$^{R206H/+}$ mice genotyping.

Traumatic HO is often advanced at diagnosis and has progressed to adjacent sites. Therefore, we investigated whether metformin could alleviate HO propagation in mice after tenotomy surgery. After tenotomy surgery, mice were treated with either water or metformin from 42 to 56 dpi (**Figure 4D**). At 56 dpi, the vehicle-treated and metformin-treated tenoto-

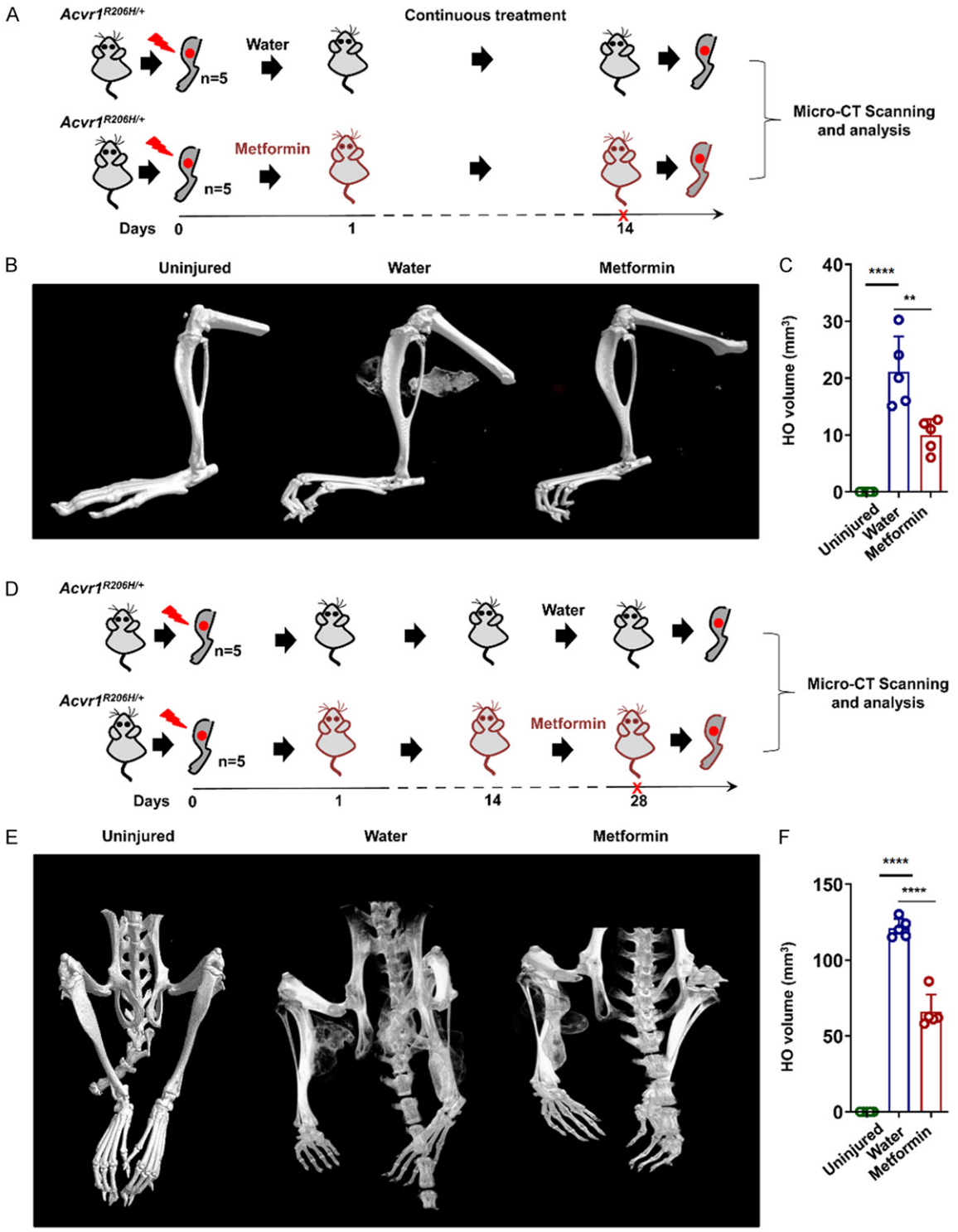

Figure 3. Metformin inhibits HO formation in *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice. A. Visual representation of our study design, highlighting the systematic process of collecting target tissue at specific time intervals for microCT analysis. B. Representative microCT images from uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice and injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving either water or metformin treatment. C. Statistical analysis of the HO volume in uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice and injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). D. Visual representation of our study design, highlighting the systematic process of collecting target tissue at specific time intervals for microCT analysis. E. Representative microCT images from uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice and injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving either water or metformin treatment. F. Statistical analysis of the HO volume in uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice and injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

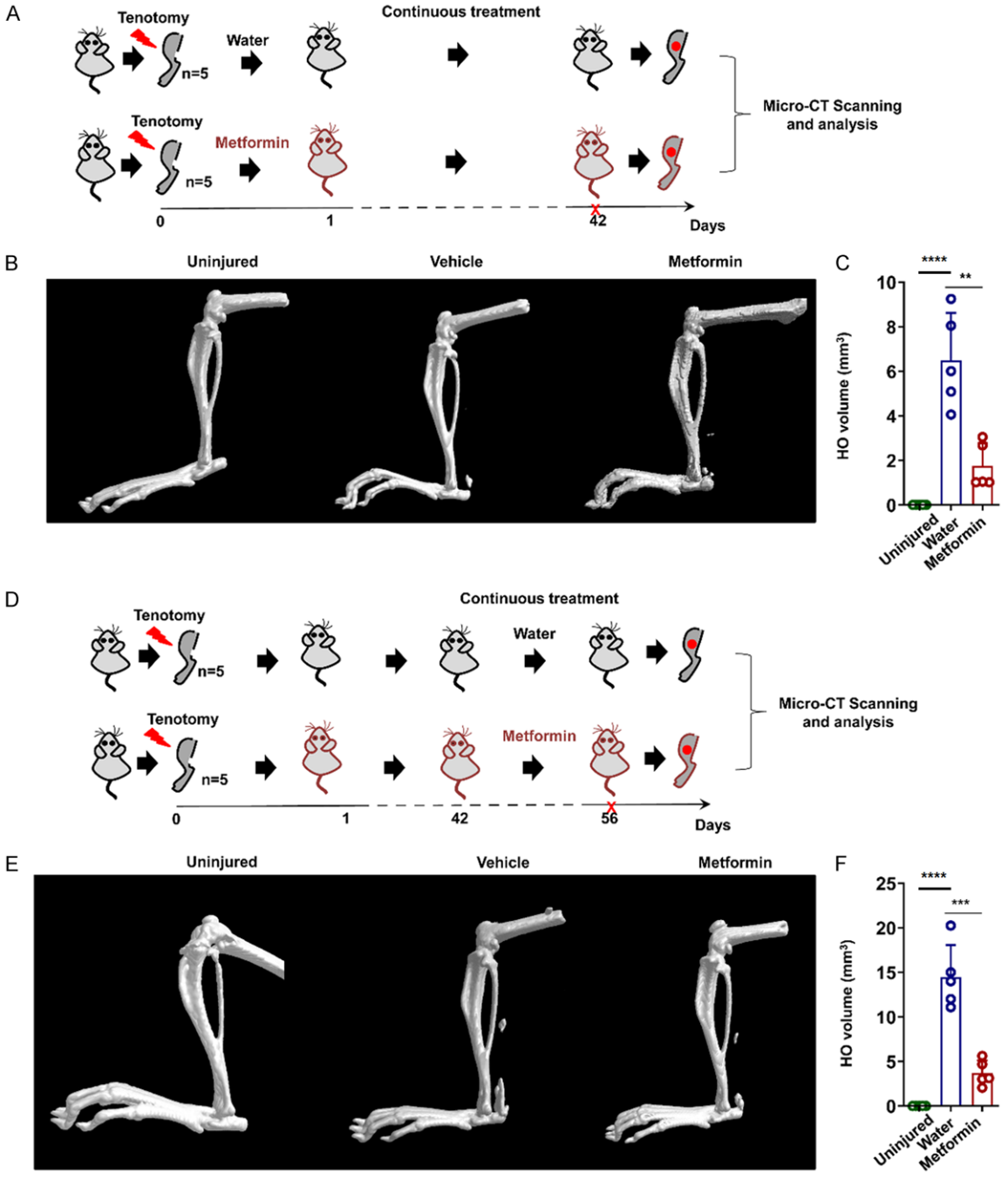

Figure 4. Metformin inhibits HO formation in tenotomy-induced HO model mice. A. Visual representation of our study design, highlighting the systematic process of collecting target tissue at specific time intervals for microCT analysis. B. Representative microCT images from uninjured mice and tenotomy mice receiving either water or metformin treatment. C. Statistical analysis of the HO volume in uninjured mice and tenotomy mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). D. Visual representation of our study design, highlighting the systematic process of collecting target tissue at specific time intervals for microCT analysis. E. Representative microCT images from uninjured mice and tenotomy mice receiving either water or metformin treatment. F. Statistical analysis of the HO volume in uninjured mice and tenotomy mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

my mice developed HO, while uninjured mice did not develop HO. Moreover, vehicle-treated tenotomy mice exhibited bilateral tibial HO and abdominal HO. However, metformin restricted

the formation of HO formation to only the left hind limb (**Figure 4E**). The total HO volume in the metformin-treated tenotomy mice was significantly lower than vehicle-treated tenotomy mice (**Figure 4F**). Together, these findings demonstrated that metformin effectively inhibited traumatic HO initiation and propagation.

Metformin inhibits myeloid cell migration to injured sites in BMP4-dependent HO modeled mice

Inflammatory response underscores the initiation and propagation of HO [37]. Myeloid cells, including neutrophils, monocytes, and macrophages, mediate inflammation and promote HO formation [37, 38]. Hence, we investigated the potential of metformin to inhibit the infiltration of myeloid cells in HO lesions. To achieve this, mice underwent muscle pinch injury, and then we administered metformin or water treatment for 3 days. At 3 dpi, both uninjured and injured *Nse-Bmp4* mice were sacrificed, and flow cytometry analysis was conducted on their collected peripheral blood and tibial muscle. The results revealed that compared to uninjured *Nse-Bmp4* mice, the injured *Nse-Bmp4* mice treated with water showed a significant increase in the percentage of myeloid cells in the peripheral blood (**Figure 5A**, **5B**). Indeed, we observed a consistent trend in the data. The proportion of myeloid cells in the blood of injured NSE-BMP4 mice treated with metformin was significantly higher than that in uninjured *Nse-Bmp4* mice (**Figure 5A**, **5B**). Interestingly, the percentage of myeloid cells in the blood of injured *Nse-Bmp4* mice treated with metformin was markedly lower than that in injured *Nse-Bmp4* mice treated with water (**Figure 5A**, **5B**).

Having observed a reduction in myeloid cells in the blood of *Nse-Bmp4* mice treated with metformin compared to those treated with the vehicle, we proceeded to investigate whether metformin could also hinder the infiltration of myeloid cells into the injured tibial muscle of *Nse-Bmp4* mice. Notably, both water-treated and metformin-treated *Nse-Bmp4* mice exhibited increased accumulation of myeloid cells at the injured site when compared to the uninjured tibial muscle of *Nse-Bmp4* mice (**Figure 5C-F**). Interestingly, mice treated with metformin displayed a significant decrease in the frequency of myeloid cells at the injured site, as compared to those treated with water (**Figure 5C-F**). These compelling findings strongly suggested that metformin effectively inhibits the migration of myeloid cells into the injured site of *Nse-Bmp4* mice.

Metformin inhibits myeloid cell migration into injured sites in FOP modeled mice

To investigate the potential impact of metformin on myeloid cell infiltration in HO lesions, we conducted experiments using *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice, as myeloid cells play a role in FOP pathogenesis. Following muscle pinch injury in these mice, we administered either metformin or water treatment for a period of 3 days. At 3 dpi, we sacrificed uninjured and injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice. We collected peripheral blood and tibial muscle samples for flow cytometry analysis from these mice. Our results revealed a significant increase in the percentage of myeloid cells in the peripheral blood of injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice treated with water, as compared to uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice (**Figure 6A**, **6B**). Intriguingly, a consistent trend was observed, where the percentage of myeloid cells in the blood of injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice with metformin treatment was significantly elevated compared to uninjured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice (**Figure 6A**, **6B**). However, it is worth noting that the percentage of myeloid cells in the blood of injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice with metformin treatment was substantially lower than in injured *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving treatment with water (**Figure 6A**, **6B**). Additionally, we compared the changes in lymphocytes between *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice treated with metformin and those treated with water.

Given the reduced presence of myeloid cells in the blood of *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice undergoing metformin treatment, as compared to those treated with the vehicle, we proceeded to investigate whether metformin could also impede the infiltration of myeloid cells into the injured tibial muscle of *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice. We compared the injured tibial muscle of *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice that received either water or metformin with the uninjured tibial muscle of the same mice. Our findings revealed a notable accumulation of myeloid cells in

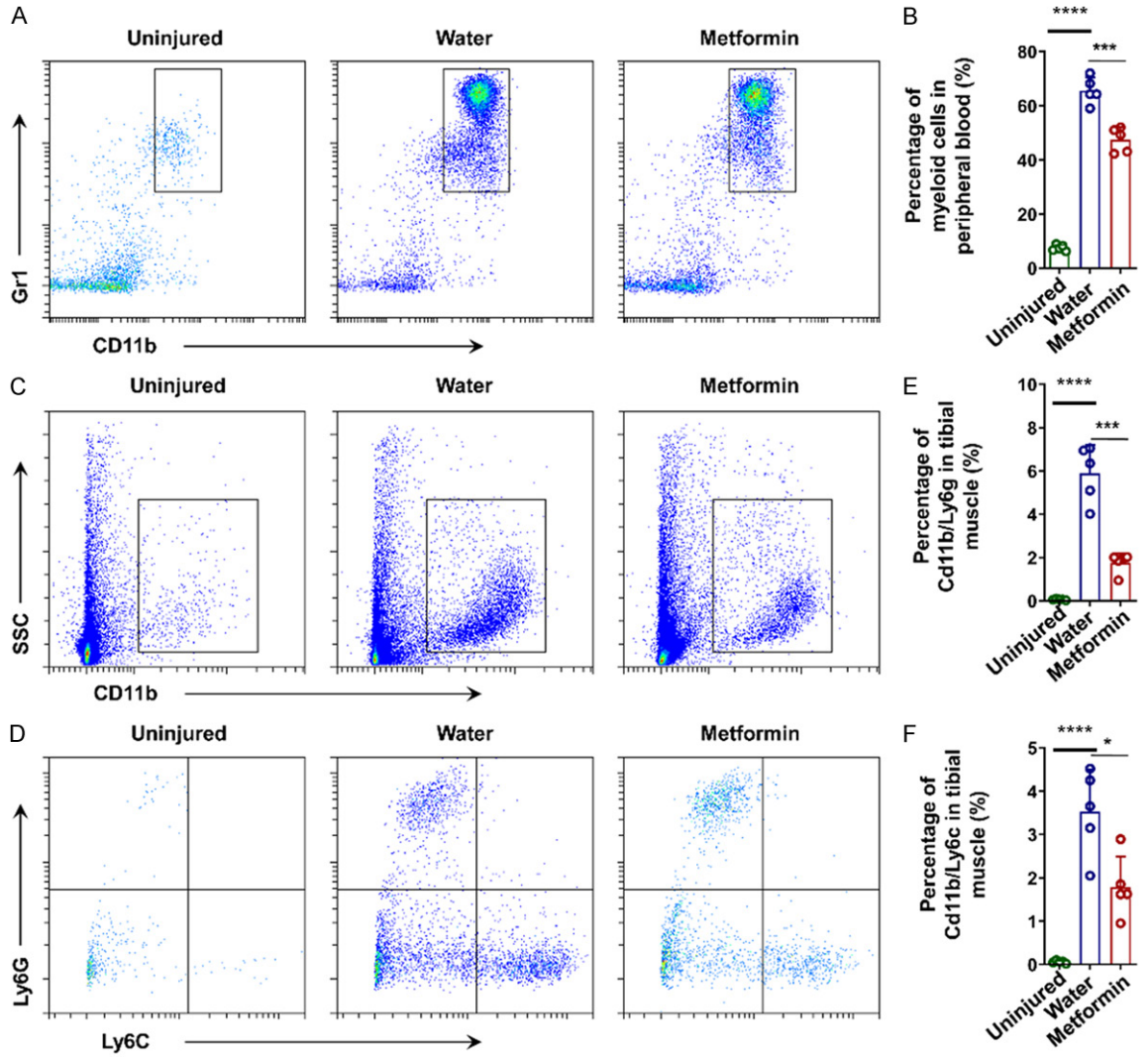

Figure 5. Metformin inhibits the infiltration of myeloid cells in BMP4-dependent HO model mice. A. Representative flow cytometry analysis of myeloid cells in uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment. B. Statistical analysis of the myeloid cell frequency in uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). C, D. Representative flow cytometry analysis of myeloid cells in uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment. E, F. Statistical analysis of the neutrophil and monocyte frequencies in uninjured *Nse-Bmp4* mice and injured *Nse-Bmp4* mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

both water-treated and metformin-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice at the injured site, as opposed to the uninjured tibial muscle (**Figure 6C-F**). However, intriguingly, the frequency of myeloid cells at the injured area was significantly reduced in metformin-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice, compared to those treated with water (**Figure 6C-F**). These compelling findings indicated that metformin effectively inhibited myeloid cell migration into the injured site of *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice.

Metformin inhibits myeloid cell migration into injured sites in traumatic HO modeled mice

We next examined the impact of metformin on myeloid cell infiltration during traumatic HO. After tenotomy injury to WT mice, mice were administered metformin or water for 3 days. At 3 dpi, uninjured WT and tenotomy WT mice were sacrificed, and peripheral blood and tibial muscle from these mice were collected for flow cytometry analysis. Compared to

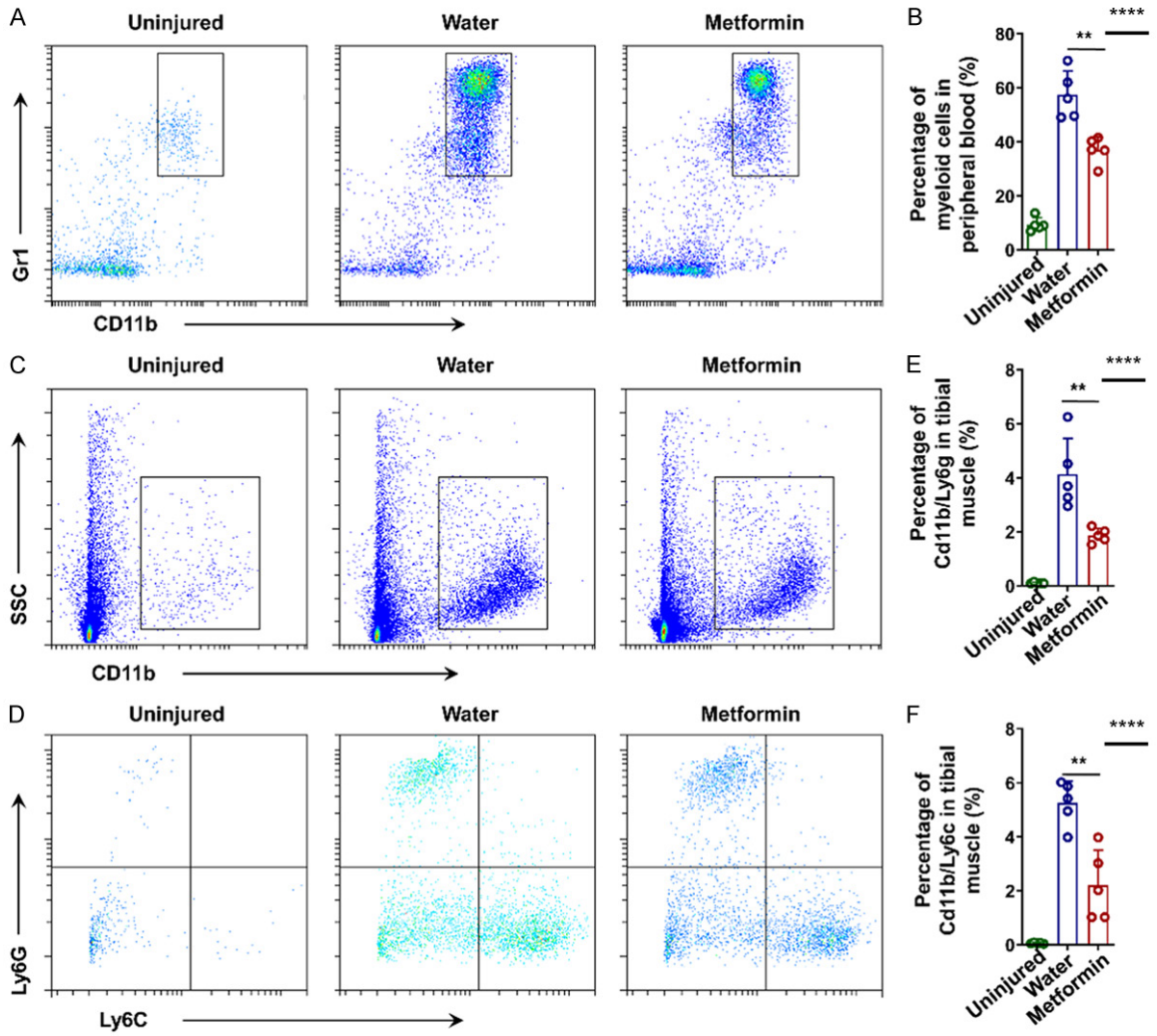

Figure 6. Metformin inhibits the infiltration of myeloid cells in FOP modeled mice. A. Representative flow cytometry analysis of myeloid cells in uninjured FOP model mice and injured FOP model mice receiving either water or metformin treatment. B. Statistical analysis of the myeloid cell frequency in uninjured FOP model mice and injured FOP model mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). C, D. Representative flow cytometry analysis of myeloid cells in uninjured FOP model mice and injured FOP model mice receiving either water or metformin treatment. E, F. Statistical analysis of neutrophil and monocyte frequencies in uninjured FOP model mice and injured FOP modeled mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

uninjured WT mice, WT tenotomy mice receiving water treatment exhibited a significant increase in peripheral blood myeloid cell percentage (**Figure 7A**, **7B**). Consistently, the myeloid cell percentage in the blood of the WT tenotomy mice receiving metformin treatment was significantly higher than that in uninjured WT mice (**Figure 7A**, **7B**). Additionally, the percentage of myeloid cells in the blood of WT tenotomy mice receiving metformin treatment was significantly lower than that of WT tenotomy mice receiving water treatment (**Figure 7A**, **7B**).

Following metformin treatment in WT tenotomy mice, a notable reduction in myeloid cells was observed in their blood compared to vehicle-treated WT tenotomy mice. This prompted us to investigate whether metformin could exert an inhibitory effect on the infiltration of myeloid cells at the injured site in a traumatic HO mouse model. In comparison to the uninjured ten-

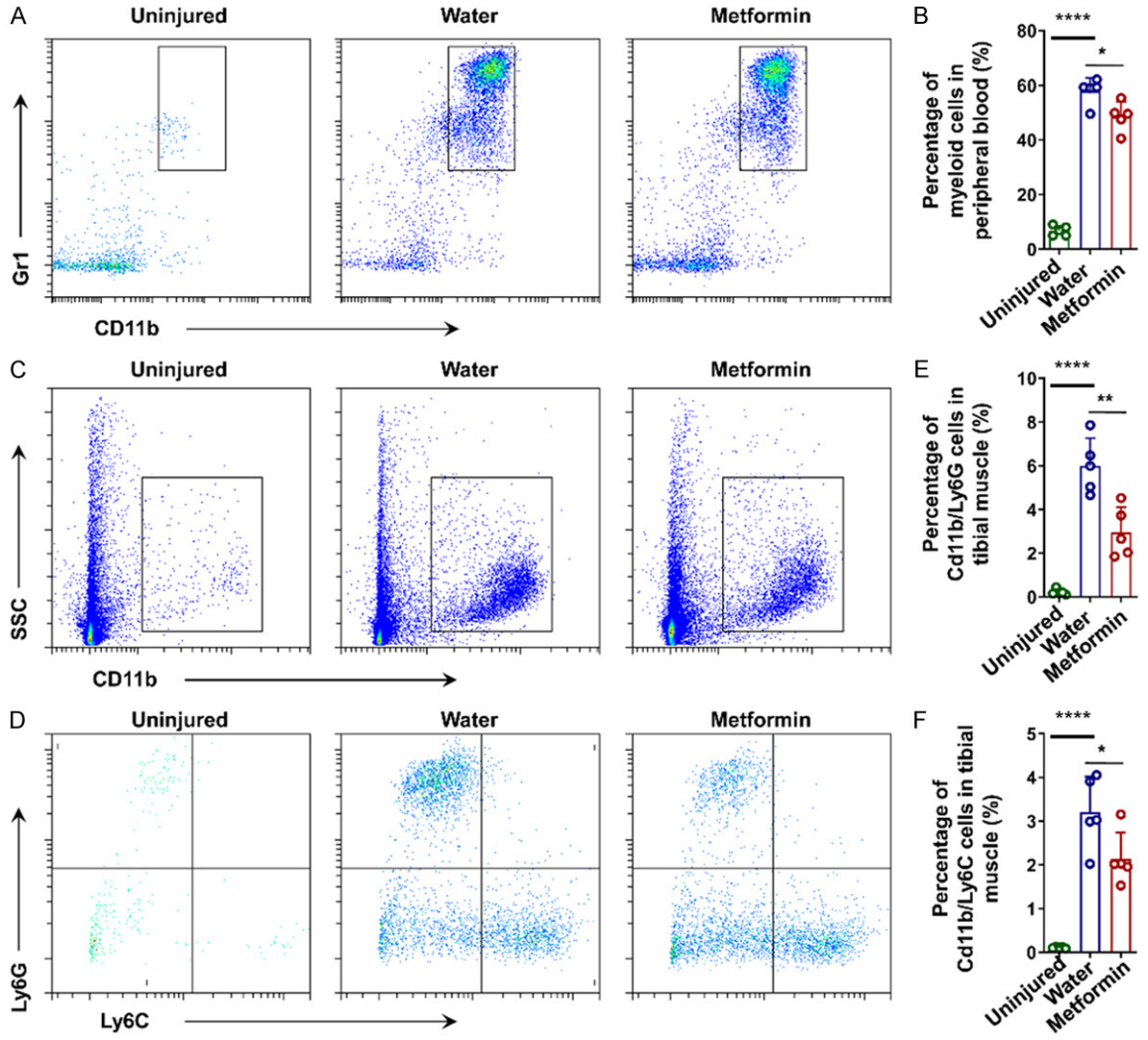

Figure 7. Metformin inhibits the infiltration of myeloid cells in traumatic HO modeled mice. A. Representative flow cytometry analysis of myeloid cells in uninjured WT mice and WT tenotomy mice receiving either water or metformin treatment. B. Statistical analysis of the myeloid cell frequency in uninjured WT mice and WT tenotomy mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). C, D. Representative flow cytometry analysis of myeloid cells in uninjured WT mice and WT tenotomy mice receiving either water or metformin treatment. E, F. Statistical analysis of neutrophil and monocyte frequencies in uninjured WT mice and WT tenotomy mice receiving either water or metformin treatment (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

dons of uninjured WT mice, both water-treated and metformin-treated traumatic HO model mice exhibited a higher accumulation of myeloid cells at the injured site (**Figure 7C-F**). Furthermore, the metformin-treated mice displayed a significantly lower frequency of myeloid cells in the injured area compared to the water-treated traumatic HO model mice (**Figure 7C-F**). These findings suggest that metformin effectively impedes the migration of myeloid cells into the injured site of the traumatic HO model mice.

Metformin inhibits the activity of complex I as well as associated myeloid mitochondrial metabolism of in hereditary and traumatic HO modeled mice

As metformin targets complex I of the mitochondrial respiration chain, we hypothesized that metformin could inhibit complex I activity and the associated mitochondrial metabolism of myeloid cells. Therefore, we examined the activity of myeloid cells from injured sites in water-treated and metformin-treated mice. As

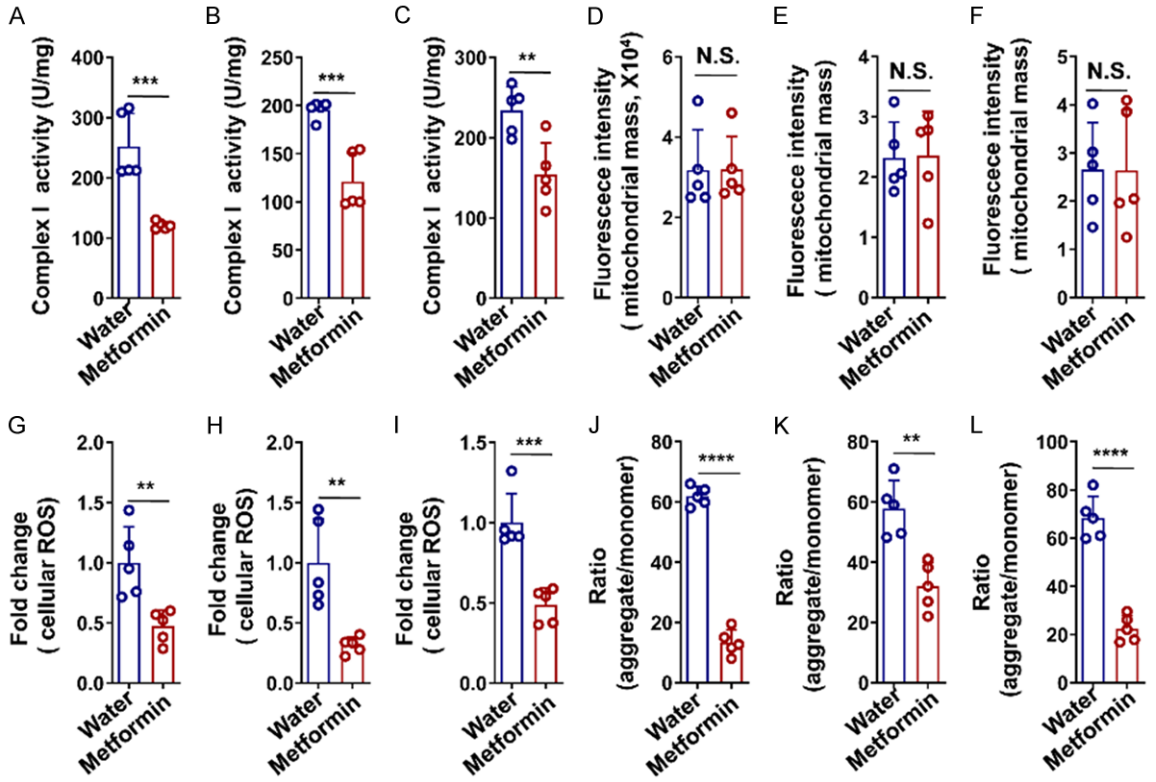

Figure 8. Metformin inhibits the activity of complex I and mitochondrial metabolism in three HO model mice. (A-C) Statistical analysis of complex I activity in myeloid cells from BMP4-dependent HO model mice (A), FOP model mice (B), and traumatic HO model (C) mice (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). (D-F) Statistical analysis of mitochondrial mass in myeloid cells from BMP4-dependent HO model mice (D), FOP model mice (E), and traumatic HO model (F) mice (n=5 per group). Data are presented as mean ± SD of biological replicates. n.s. indicates no significance (unpaired two-tailed t-test). (G-I) Statistical analysis of ROS production in myeloid cells from BMP4-dependent HO model mice (G), FOP model mice (H), and traumatic HO model (I) mice (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test). (J-L) Statistical analysis of MMP in myeloid cells from BMP4-dependent HO model mice (J), FOP model mice (K), and traumatic HO model (L) mice (n=5 per group). Data are presented as mean ± SD of biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired two-tailed t-test).

anticipated, the complex I activity in myeloid cells in HO lesions from *Nse-Bmp4* mice was reduced by metformin (**Figure 8A**). Consistently, the complex I activity in myeloid cells from *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice receiving metformin treatment was substantially lower than that in water-treated *Tie2-cre*; *Acvr1*$^{R206H/+}$ mice (**Figure 8B**). Metformin also inhibited the complex I activity of myeloid cells in traumatic HO model mice (**Figure 8C**).

Furthermore, we investigated the effect of metformin on mitochondrial metabolism, including mitochondrial mass, ROS production, and mitochondrial membrane potential. No significant difference in myeloid cells was detected between metformin-treated and water-treated NSE-BMP4 mice (**Figure 8D** and Supplementary Figure 2A, 2B). Metformin did not impact the mitochondrial mass of myeloid cells in *Tie2-cre*; *Acvr1*$^{R206H/+}$, and traumatic HO model mice (**Figure 8E**, **8F**). Next, we conducted a comparison of the ROS production of myeloid cells between metformin-treated and water-treated *Nse-Bmp4* mice (**Figure 8G** and Supplementary Figure 2C, 2D). The ROS levels of myeloid cells in metformin-treated *Nse-Bmp4* mice were significantly reduced compared to water-treated *Nse-Bmp4* mice (**Figure 8H**). Metformin also significantly reduced the ROS level of myeloid cells in the two other HO model mice (**Figure 8H**, **8I**). Additionally, we examined the mitochondrial membrane potential of myeloid cells, and metformin significantly inhibited the mitochondrial membrane potential of myeloid cells

across three HO model mice (**Figure 8J-L** and Supplementary Figure 2E, 2F).

Discussion

Heterotopic ossification commonly occurs after injury-induced hyperinflammation, whereas there are no effective therapeutic methods to treat HO. This study establishes a crucial connection between myeloid cells and the formation of HO. Moreover, it highlights the potential of metformin to target the mitochondrial metabolism of myeloid cells, making it a promising candidate for treating both traumatic and hereditary HO.

Inflammation triggers both hereditary (FOP) and acquired HO [39]. Myeloid cells are the first cell type involved in the inflammatory response. It was found that neutrophil abundance in the blood and injured muscle was increased at 1, 3, and 7 dpi. Elevation of neutrophil levels indicated inflammatory responses. Within 1 hour of muscle injury, neutrophil invasion began, and the increased concentration of neutrophils remained for at least 5 days [40]. Indeed, after muscle injury in HO model mice, the number of neutrophils persistently increased at 1 and 3 dpi. At 7 dpi, neutrophils were still elevated at a level higher than that in uninjured muscle but drastically decreased compared to the level observed at 3 dpi. Therefore, we cannot conclude that neutrophils participate in the pathogenesis of HO. Rather, HO may be associated with muscle injury. Consequently, we conducted the same analysis to examine neutrophil alteration at the injured muscle lacking HO development. The neutrophil levels in the blood and injured muscle from the HO modeled mice were significantly higher than those from non-HO mice. Thus, the hyperactivation of neutrophils is unique to HO formation. In a burn/tenotomy-injured HO model, neutrophils were persistently increased over 14 days and returned to normal at 21 dpi [41]. There was an increase in neutrophil levels in *Acvr1*$^{R206H/+}$ lesions [14]. However, in neurogenic HO model mice, granulocyte colony-stimulating factor (G-CSF) treatment significantly enhanced the neutrophil levels in the blood, bone marrow, and injured muscle, but did not promote HO formation. We speculated that 1) different types of HO could depend on neutrophils, 2) neutrophils govern HO initiation but not progression, and 3) neutrophil diversity, but not abundance governs HO.

To date, no intensive studies examining the function of myeloid cells on HO have been conducted until now. In this study, we found that metformin could inhibit the infiltration of myeloid cells in injured lesion of HO model mice. Metformin can target the complex I; we next examined the activity of complex I in myeloid cells with either water and metformin treatment. Interestingly, the activity of complex I was significantly decreased after metformin consumption. Moreover, the mitochondrial metabolism of myeloid cells in HO model mice was also inhibited by metformin. We speculated dysfunction of mitochondrial metabolism restricted the mobility of myeloid cells and thus alleviated the inflammatory response [42, 43]. Neutrophils employ diverse metabolic pathways in response to immunological challenges, utilizing specific metabolic pathways for modulating their effector functions [44]. These cells use different metabolic pathways to both fulfill energy requirements and support specialized effector functions, including neutrophil extracellular trap formation, ROS generation, chemotaxis, and degranulation. This is contrasted with other immune cells, which undergo metabolic reprogramming to confirm differentiation into distinct cell subtypes. Further study is required to examine the complexity of the hematopoietic system regulating HO initiation and progression.

Bone remodeling is strictly controlled by metabolism [45]. Similarly, HO formation requires support from metabolites, as HO patients gradually grow thin and visibly waste away [46], suggesting systemic metabolic dysfunction during HO formation. Metformin, an effective drug for the treatment of type 2 diabetes mellitus, has been reported to prevent injury-induced HO [47]. In our study, we applied metformin to inhibit metabolic dysfunction during genetic and traumatic HO formation. As expected, HO was effectively prevented. Importantly, HO propagation can be inhibited, indicating that metformin is an ideal candidate for use by FOP patients, as these patients cannot undergo surgery to remove HO. Interestingly, infiltration of myeloid cells, inducing neutrophils and monocytes, was inhibited, implying that the

metabolic requirement of myeloid cells is indispensable for HO formation. Overall, we uncovered that the mitochondrial metabolism of myeloid cells was closely linked to HO initiation and progression. Inhibition of mitochondrial metabolism in myeloid cells suppressed complex I activity and HO development, serving as a therapeutic option in HO treatment.

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Disclosure of conflict of interest

None.

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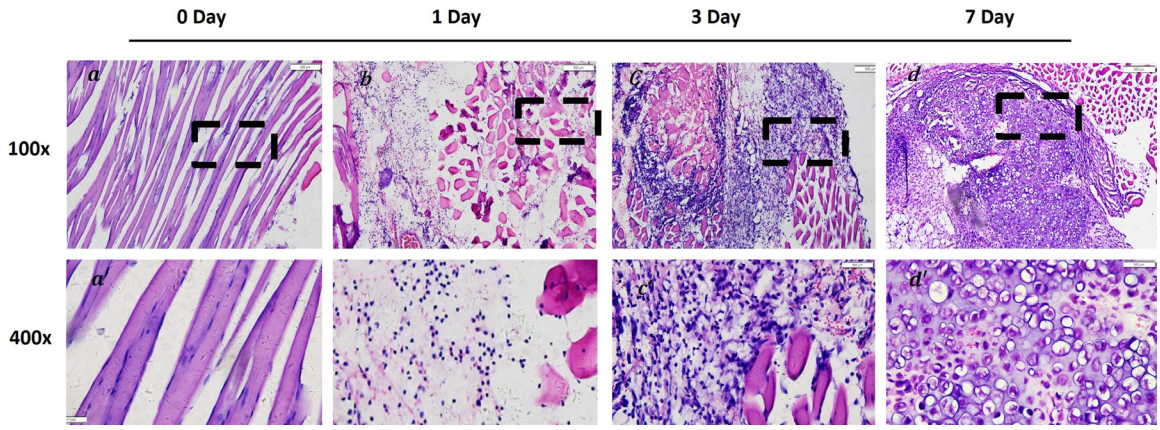
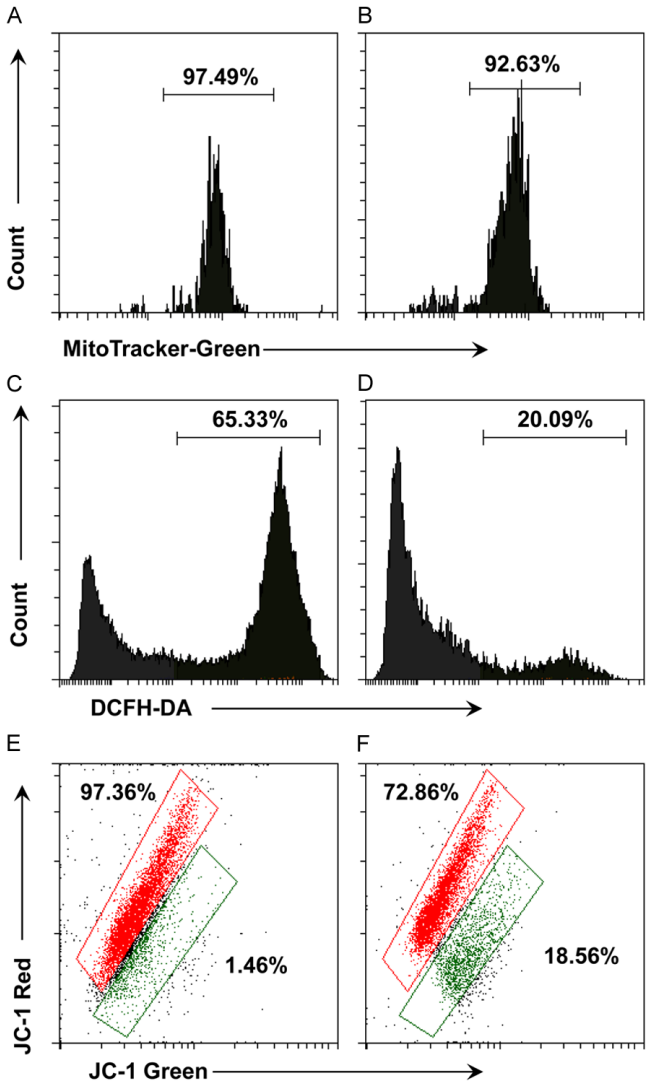


Supplementary Figure 1. Histology analysis of HO from FOP model mice.



Supplementary Figure 2. Metformin inhibits the ROS and MMP, rather than mitochondrial mass of myeloid cells in HO model mice. (A, B) Representative flow cytometry analysis of mitoTracker in myeloid cells of HO model mice with either water (A) and metformin (B) treatment. (C, D) Representative flow cytometry analysis of ROS in myeloid cells of HO model mice with either water (C) and metformin (D) treatment. (E, F) Representative flow cytometry analysis of JC1 staining in myeloid cells of HO model mice with either water (E) and metformin (F) treatment.