博士学位論文

アイリシンは糖尿病マウスにおける
骨修復遅延を改善する

近畿大学大学院
医学研究科医学系専攻

木　下　優　子

Doctoral Dissertation

Irisin improves delayed bone repair in diabetic female mice

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Major in Medical Sciences
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課博・論博

同　意　書

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ORIGINAL ARTICLE

Irisin improves delayed bone repair in diabetic female mice

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Abstract

Introduction Irisin is a proteolytic product of fibronectin type II domain-containing 5, which is related to the improvement in glucose metabolism. Numerous studies have suggested that irisin is a crucial myokine linking muscle to bone in physiological and pathophysiological states.

Materials and methods We examined the effects of local irisin administration with gelatin hydrogel sheets and intraperitoneal injection of irisin on the delayed femoral bone repair caused by streptozotocin (STZ)-induced diabetes in female mice. We analyzed the femurs of mice using quantitative computed tomography and histological analyses and then measured the mRNA levels in the damaged mouse tissues.

Results Local irisin administration significantly blunted the delayed bone repair induced by STZ 10 days after a femoral bone defect was generated. Local irisin administration significantly blunted the number of Osterix-positive cells that were suppressed by STZ at the damaged site 4 days after a femoral bone defect was generated, although it did not affect the mRNA levels of chondrogenic and adipogenic genes 4 days after bone injury in the presence or absence of diabetes. On the other hand, intraperitoneal injection of irisin did not affect delayed bone repair induced by STZ 10 days after bone injury. Irisin significantly blunted the decrease in Osterix mRNA levels induced by advanced glycation end products or high-glucose conditions in ST2 cells in the presence of bone morphogenetic protein-2.

Conclusions We first showed that local irisin administration with gelatin hydrogel sheets improves the delayed bone repair induced by diabetic state partially by enhancing osteoblastic differentiation.

Keywords Irisin · Bone repair · Diabetes · Osteoblasts · Gelatin hydrogel

Introduction

Bone and skeletal muscle, through cooperative functions, are the central system controlling body movement and exercise. The interactions between muscle and bone have been recently highlighted, including numerous clinical findings about the relationship between sarcopenia and osteoporosis [1]. The interactions between muscle and bone include the linkages of muscle to bone and bone to muscle. Regarding the local linkage of muscle to bone, local factors produced by skeletal muscle have been shown to enhance fracture healing [1]. On the other hand, abundant evidence indicates that humoral factors secreted by skeletal muscle, namely, myokines, influence bone metabolism by affecting distant bones through the circulation [1, 2]. Among various myokines, irisin, interleukin (IL)-6, IL-7 and IL-15 affect bone and energy metabolism simultaneously.

Irisin is produced by the cleavage of the membrane protein fibronectin type III domain-containing 5 (FNDC5) and is secreted into the circulation [3]. According to a recent study, irisin affects bone and adipose tissues by binding α_V integrin, a putative irisin receptor [4]. Irisin, which is predominantly produced from skeletal muscle, enhances the transdifferentiation of white adipose tissue into brown

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adipose tissue, contributing to thermogenesis during exercise [5]. Several clinical studies have suggested that circulating irisin levels are related to osteoporosis and bone mineral density [6–8]. Regarding the effects of irisin on bone, the intraperitoneal administration of irisin has been shown to increase cortical bone mass in healthy mice [9]. We and another group showed that irisin is involved in unloading-induced osteopenia in mice [10, 11], and we recently reported that irisin is related to a chronic exercise-induced increase in trabecular bone mass in mice subjected to treadmill exercise [12]. Moreover, our study suggested that diabetic state, excess glucocorticoid levels and androgen modulate irisin expression in skeletal muscle [13–15]. Based on these findings, irisin might be a crucial myokine for the mechanical stress, endocrine and metabolic regulation of muscle and bone in vivo. Several in vitro studies indicated that irisin exerts positive effects on osteoblastic phenotypes, such as the proliferation and differentiation of mouse osteoblastic cells [9, 16–18], although controversy exists about the effects of irisin on osteoclasts and osteocytes [4, 9, 11, 19–22].

Bone repair after bone injury occurs in three phases: inflammation, bone restoration and remodeling [23]. Macrophages, chondrogenesis and vessel formation are involved in the bone restoration phase, which is partially related to inflammation [24]. Xin et al. recently reported that the delivery of muscle-derived irisin enhances bone repair after the generation of a skull bone defect through treatment with a silk/calcium silicate/sodium alginate composite scaffold in rats [25], although the mechanisms by which local irisin administration effectively induces bone repair were not shown in that study. Diabetes induces osteoporosis mainly through a decrease in osteoblastic bone formation and delayed wound healing [26]. Delayed bone repair after fractures is observed in diabetes [27]. Previous studies have suggested that impaired mobilization of bone marrow stem cells, osteoblastic bone formation, chondrogenesis, macrophage accumulation and vessel formation might be partially involved in the delayed bone repair associated with diabetes [27, 28]. Moreover, we reported that plasminogen activator inhibitor-1, an adipocytokine, is involved in delayed bone repair induced by diabetic state in female mice [28, 29]. However, effective treatments for impaired bone repair after fractures or bone defects are still limited.

Although irisin modulates thermogenesis and glucose metabolism [5], previous clinical studies have suggested that an elevation in serum irisin levels is associated with an improvement in insulin resistance [30]. However, irisin secretion from adipose tissues contributes to a reduction in serum irisin levels in diabetic obese mice [31]. However, controversy exists about the usefulness of serum irisin measurements for the evaluation of glucose metabolism [2]. Our previous study revealed that diabetes induced by streptozotocin (STZ) treatment reduces irisin expression in the gastrocnemius muscle of female mice [14]. We therefore speculated that irisin might protect against delayed bone repair induced by diabetic state.

In the present study, we investigated the effects of local and systemic irisin administration on delayed bone repair in female mice with STZ-induced diabetes.

Materials and methods

Materials

Recombinant irisin was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). Anti-Osterix and anti-CD31 antibodies were purchased from Abcam (Cambridge, UK). Anti-F4/80, anti-alkaline phosphatase (ALP), anti-SOX9 and anti-perilipin antibodies were purchased from AbD Serotec (Raleigh, NC, USA), Abnova (Taipei, Taiwan), Sigma (St. Louis, MO) and Cell Signaling Technology (Danvers, MA), respectively. Recombinant bone morphogenetic protein-2 (BMP-2) and tumor necrosis factor-α (TNF-α) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animals

C57BL/6J female mice were purchased from CLEA Japan (Tokyo, Japan). Diabetes was randomly induced in 8-week-old female mice by an intraperitoneal injection of the pancreatic β-cell toxin streptozotocin (STZ, Sigma, 200 mg/kg body weight in saline) twice weekly. Control mice were injected with saline alone. After the last injection, we measured blood glucose levels from the tail vein with a blood glucose meter (Glutest Ace, Sanwa Kagaku Kenkyusho, Nagoya, Japan). Mice with blood glucose levels higher than 300 mg/dL were considered diabetic. Two weeks after the induction of diabetes, bone defect surgery was performed on the right femurs of the mice. All animal experiments were performed according to the guidelines of the National Institutes of Health and the institutional rules for the use and care of laboratory animals at Kindai University. The experiments were approved by the Animal Ethics Committee of Kindai University (approval number: KAME-31-051).

Bone defect model and treatment with irisin

A bone defect was induced in the mice using a previously described method with some modifications [28, 32]. Briefly, an incision of 5 mm in length was made in the anterior skin of the central femur of the right leg under 2% isoflurane anesthesia. After splitting the muscle, the surface of the femoral bone was exposed, and a hole was created in the

femur using a drill with a diameter of 0.8 mm. The hole was irrigated with saline to prevent thermal necrosis of the margins. We applied a cationized gelatin hydrogel as a sustained release carrier of irisin in the damaged site of the femur [33]. For local administration of irisin, single disc-shaped gelatin hydrogel sheets with a diameter of 1.5 mm were impregnated with irisin (150 ng) or saline and placed in a femoral defect. We selected the concentration of irisin (150 ng) used in the present study according to a previous report [25] that local administration of irisin (100 or 200 ng) with a silk/calcium silicate/sodium alginate composite scaffold enhances bone repair after the generation of a skull bone defect in rats. We assumed that local administration of irisin in the present study is appropriate based on serum irisin levels (approximately 50–100 ng/mL) in healthy mice reported in a previous study [34]. The incised skin was then sutured in a sterile manner, and anesthesia was discontinued. Systemic administration of irisin was performed using a previously described method with some modifications [11, 15]. Briefly, mice with a femoral defects were treated with saline or 100 μg/kg irisin by intraperitoneal injection on days 0, 2, 4 and 6 after surgery.

Quantitative computed tomography (qCT) analysis

The mice were anesthetized with 2% isoflurane, and the femur was scanned using Cosmo Scan GX II (Rigaku Corporation, Tokyo, Japan) according to the manufacturer's instructions with the following parameters: 90-kV tube voltage, 88-μA tube current, and 25-μm isotropic voxel size. The area of the bone defect in the femur was quantified using an image-processing program (ImageJ, http://rsbweb.nih.gov/ij/download.html). The ratio of bone volume to tissue volume (BV/TV) within the bone defect region was calculated to evaluate new bone formation in the bone defect.

Histological analysis

The mice were anesthetized with 2% isoflurane 2, 4 and 10 days after a femoral bone defect was generated. The femur was removed, fixed with 4% paraformaldehyde, demineralized in a 22.5% formic acid and 340 mM sodium citrate solution for 24 h, and embedded in paraffin. Then, 4-μm-thick sections were obtained.

Immunostaining was performed as described previously [28, 29]. Briefly, the sections were incubated with the anti-Osterix antibody at a dilution of 1:200, anti-ALP antibody at a dilution of 1:200, anti-SOX9 antibody at a dilution of 1:200, anti-perilipin antibody at a dilution of 1:200, anti-F4/80 antibody at a dilution of 1:1000 or anti-CD31 antibody at a dilution of 1:50, followed by an incubation with the appropriate secondary antibody conjugated with horseradish peroxidase. Positive signals were visualized using a tyramide signal amplification system (PerkinElmer, Waltham, MS, USA). These sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Dojindo, Kumamoto, Japan) and photographed with a fluorescence microscope. The numbers of Osterix-, ALP- and F4/80-positive cells per 0.1 mm^2 in the microscopic fields of the damaged site of the femur were quantified. The vessel number was quantified from the number of areas surrounded by CD31-positive cells in the microscopic fields of the damaged site of the femur.

The sections were processed for hematoxylin and eosin (HE), Alcian blue solution (FUJIFILM Wako) and Nuclear Fast Red Solution (ScyTek Laboratories, Logan, UT, USA). The areas of the cartilage matrices that included proteoglycans and glycosaminoglycans around cortical bone near the bone defect were quantified by measuring the Alcian blue-positive areas.

The sections were stained with tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (FUJIFILM Wako). The number of TRAP-positive multinucleated cells (MNCs) on the new bone at the damaged site of the femur was counted.

Cell culture

The mouse bone marrow-derived stromal cell line ST2 was cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, FUJIFILM Wako, containing 5.5 mM glucose) supplemented with 10% FBS and 1% penicillin–streptomycin. ST2 cells were cultured until confluent and then treated with BMP-2, advanced glycation end product 3 (AGE3), TNF-α and high-glucose media (containing 25 mM glucose). AGE3 was prepared as previously described [35].

Quantitative real-time PCR

The mice were anesthetized with 2% isoflurane on days 4 and 10 after surgery. The femur was removed and frozen in liquid nitrogen. Total RNA was isolated from a 5-mm piece of femur containing the damaged site with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was extracted from ST2 cells with Nucleo Spin® RNA Plus (Takara Bio, Shiga, Japan). A reverse transcription reaction with the extracted RNA was performed using a Prime Script RT reagent Kit with gDNA eraser (Takara Bio), and quantitative real-time PCR was performed using a SYBR Premix Ex Taq™ II kit (Takara Bio) for 40 cycles of two-step PCR amplification (95 °C for 3 s and 60 °C for 30 s) in an Applied Biosystems Step One Plus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The PCR primers are listed in Table 1. The specific amplification of the target mRNA was determined as the Ct value

Table 1 Primers used for real-time PCR experiments

Gene		Primer sequence
Runx2	Forward	5'-AAATGCCTCCGCTGTTATGAA-3'
	Reverse	5'-GCTCCGGCCCACAAATCT-3'
Osterix	Forward	5'-AGCGACCACTTGAGCAAACAT-3'
	Reverse	5'-GCGGCTGATTGGCTTCTTCT-3'
ALP	Forward	5'-ATCTTTGGTCTGGCTCCCATG-3'
	Reverse	5'-TTTCCCGTTCACCGTCCAC-3'
Osteocalcin	Forward	5'-CCTGAGTCTGACAAAGCCTTCA-3'
	Reverse	5'-GCCGGAGTCTGTTCACTACCTT-3'
Col 1	Forward	5'-AACCCTGCCCGCACATG-3'
	Reverse	5'-CAGACGGCTGAGTAGGGAACA-3'
Aggrecan	Forward	5'-CCTGCTACTTCATCGACCCC-3'
	Reverse	5'-AGATGCTGTTGACTCGAACCT-3'
Col 2	Forward	5'-CCTCCGTCTACTGTCCACTGA-3'
	Reverse	5'-ATTGGAGCCCTGGATGAGCA-3'
Col 10	Forward	5'-TGGGTAGGCCTGTATAAAGAACGG-3'
	Reverse	5'-CATGGGAGCCACTAGGAATCCTGAGA-3'
PPARγ	Forward	5'-GGAAAGACAACGGACAAATCAC-3'
	Reverse	5'-TACGGATCGAAACTGGCAC-3'
aP-2	Forward	5'-ATCACCGCAGACGACAGGA-3'
	Reverse	5'-CTCATGCCCTTTCATAAACT-3'
Noggin	Forward	5'-GCCAGCACTATCTACACATCC-3'
	Reverse	5'-GCGTCTCGTTCAGATCCTTCTC-3'
Gremlin	Forward	5'-CTGGGGACCCTACTGCCAA-3'
	Reverse	5'-TTTGCACCAATCTCGCTTCAG-3'
Id1	Forward	5'-CCTAGCTGTTCGCTGAAGGC-3'
	Reverse	5'-GTAGAGCAGGACGTTCACCT-3'
β-Actin	Forward	5'-AATCGTGCGTGACATTAAG-3'
	Reverse	5'-GAAGGAAGGCTGGAAGAG-3'
Gapdh	Forward	5′-AGGTCGGTGTGAACGGATTTG-3′
	Reverse	5′-GGGGTCGTTGATGGCAACA-3′

Runx2 Runt-related transcription factor 2, *ALP* alkaline phosphatase, *Col 1* type I collagen, *Col 2* type II collagen, *Col 10* type X collagen, *PPAR-γ* peroxisome proliferator-activated receptor γ, *aP-2* adipocyte protein-2, *Id1* inhibitor of DNA binding 1, *Gapdh* glyceraldehyde- 3-phosphate dehydrogenase

normalized to the β-actin or glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA level.

Statistical analysis

All data are presented as the means ± the standard errors of the means (SEM). One-way ANOVA followed by the Tukey–Kramer post hoc test were used to perform multiple comparisons. The significance level was set to $p < 0.05$. GraphPad PRISM 6 software (La Jolla, CA) was used for all statistical analyses.

Results

Effects of local administration of irisin on bone repair in diabetic mice

STZ treatment significantly decreased the body weight of female mice before a femoral bone defect was generated (Fig. 1A). Blood glucose levels were markedly increased by STZ treatment in mice, indicating that STZ induced diabetic state in mice (Fig. 1B). The femoral bone injury was almost repaired in mice without STZ treatment with or without local administration of irisin 10 days after a femoral bone defect was generated, as assessed using qCT (Fig. 1C). Local administration of irisin significantly blunted the delayed bone repair induced by diabetic states 9 and 10 days after bone defects were generated in mice (Fig. 1C, D). The BV/TV of the new bone in the bone defect region was significantly increased by the local administration of irisin 10 days after bone defects were generated in diabetic mice (Fig. 1E). New bone tissue was generated at the damaged site 10 days after bone injury in control mice (Fig. 1F). Although the new bone tissue was less in diabetic mice, local administration of irisin increased new bone tissue at the damaged site in diabetic mice (Fig. 1F).

Analysis of the expression of osteogenic markers in femurs at the damaged site

Histological analyses of the damaged site in a femoral bone defect were performed to examine the effects of local irisin administration on the induction of osteoblastic cells at the bone injury site during bone repair in mice. STZ treatment significantly decreased the number of Osterix-positive cells at the damaged site of femurs 2 and 4 days after bone injury in mice (Fig. 2A, B). Local administration of irisin significantly blunted the number of Osterix-positive cells that was suppressed by STZ treatment at the damaged site 4 days, but not 2 days, after a femoral bone defect was generated in mice (Fig. 2A, B). Local administration of irisin significantly blunted the number of ALP-positive cells that was suppressed by STZ treatment at the damaged site 10 days after a femoral bone defect was generated in mice (Fig. 2C, D). Next, we examined the effects of local irisin administration on the mRNA levels of osteogenic genes, such as Runx2, Osterix, ALP, osteocalcin and type 1 collagen, at the damaged site 4 and 10 days after bone injury in mice. STZ treatment significantly reduced the Osterix and ALP mRNA levels 4 days after bone injury (Fig. 2E). However, local administration of irisin did not affect the mRNA levels of osteogenic

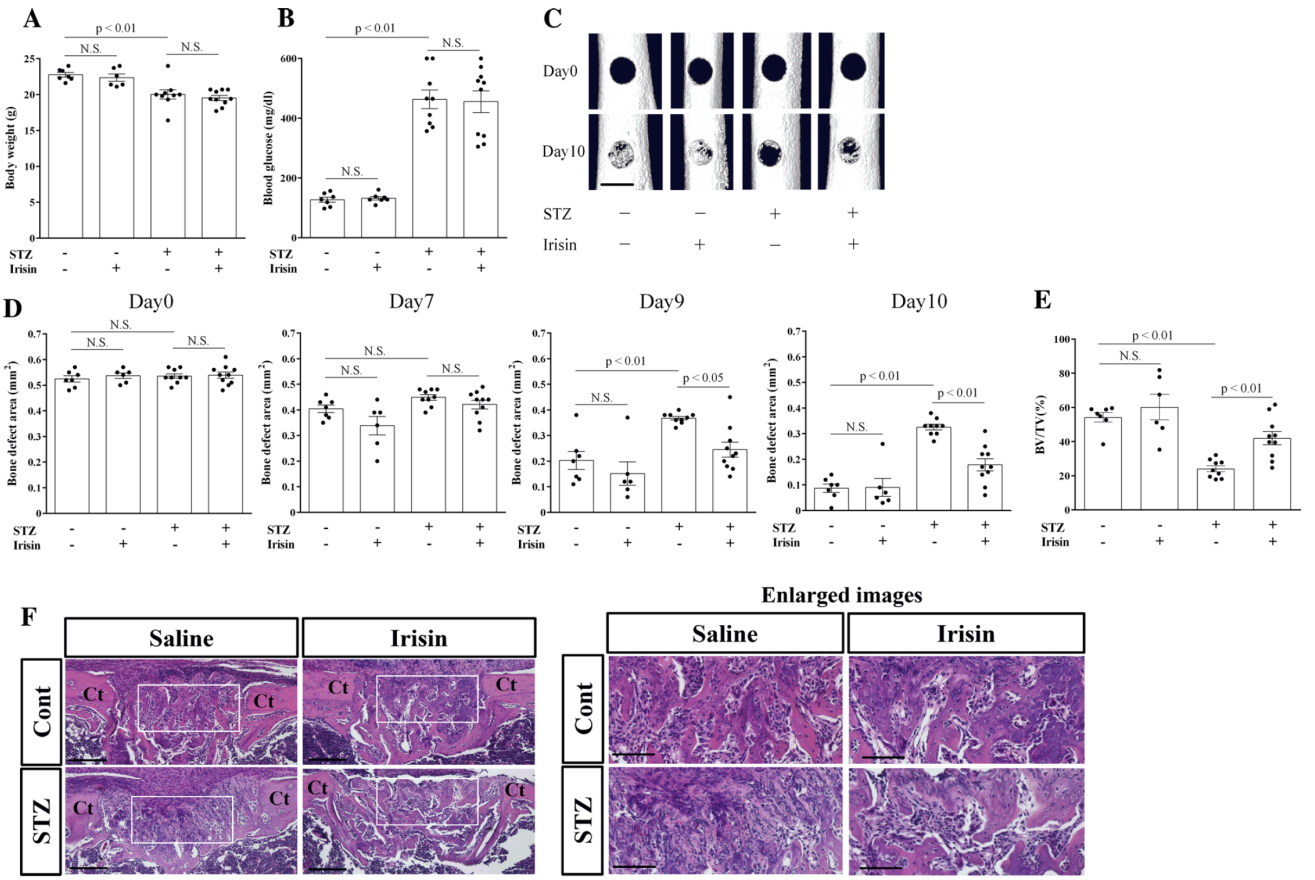

Fig. 1 Effects of local irisin administration on bone repair after a femoral bone defect was generated in female mice. **A** Body weight in the control (Cont) and streptozotocin (STZ)-treated female mice with or without local administration of irisin before a femoral bone defect was generated. **B** Blood glucose levels in control and STZ-treated mice with or without the local administration of irisin before a femoral bone defect was generated. **C** Representative three-dimensional images of the damaged site of femurs in each group 10 days after a femoral bone defect was generated, as assessed using qCT imaging. The scale bar indicates 1 mm. **D** Quantification of the bone defect area assessed at the damaged site in control and STZ-treated mice with or without local administration of irisin at 0, 7, 9 and 10 days after a femoral bone defect was generated. **E** The BV/TV (%) of the mineralized bone formed in the hole region 10 days after a femoral bone defect was generated was analyzed using qCT. **F** Representative microphotographs of HE-stained sections from the damaged site of femur 10 days after a femoral bone defect was generated. Scale bars indicate 200 μm and 100 μm (enlarged images). The enlarged images show the square area in the figure on the left. Data are presented as the means ± SEM of 6–10 mice per group. *N.S.* not significant, *Ct* cortical bone

genes in mice, although it tended to blunt the suppression of the Osterix mRNA level induced by STZ treatment without reaching statistical significance (Fig. 2E, F).

Analysis of the expression of chondrogenic and adipogenic genes at the damaged site

We examined the effects of local irisin administration on chondrogenesis at the damaged site after a femoral bone defect in mice using Alcian blue staining. STZ treatment significantly reduced the formation of cartilage matrix around cortical bone near the bone defect 10 days after bone injury (Fig. 3A, B). Local administration of irisin tended to increase the formation of cartilage matrix around cortical bone near the bone defect, which was decreased by diabetic state in mice without statistically significant differences (Fig. 3B). We next examined the effects of local irisin administration on the expression of chondrogenic genes (aggrecan, type 2 collagen and type 10 collagen) and adipogenic genes (peroxisome proliferator activated receptor γ (PPARγ) and adaptor protein 2 (aP2)) at the damaged site 4 and 10 days after bone injury to assess the effects of irisin on chondrogenesis and adipogenesis during bone repair after a femoral bone defect was generated in mice. Local irisin administration did not alter the aggrecan, type 2 collagen and type 10 collagen mRNA levels at the damaged site 4 and 10 days after bone injury in mice with or without diabetes (Fig. 3C, D). Local administration of irisin did not alter the number of SOX9-positive cells at the damaged site of femur that was suppressed by STZ treatment (Fig. 3E).

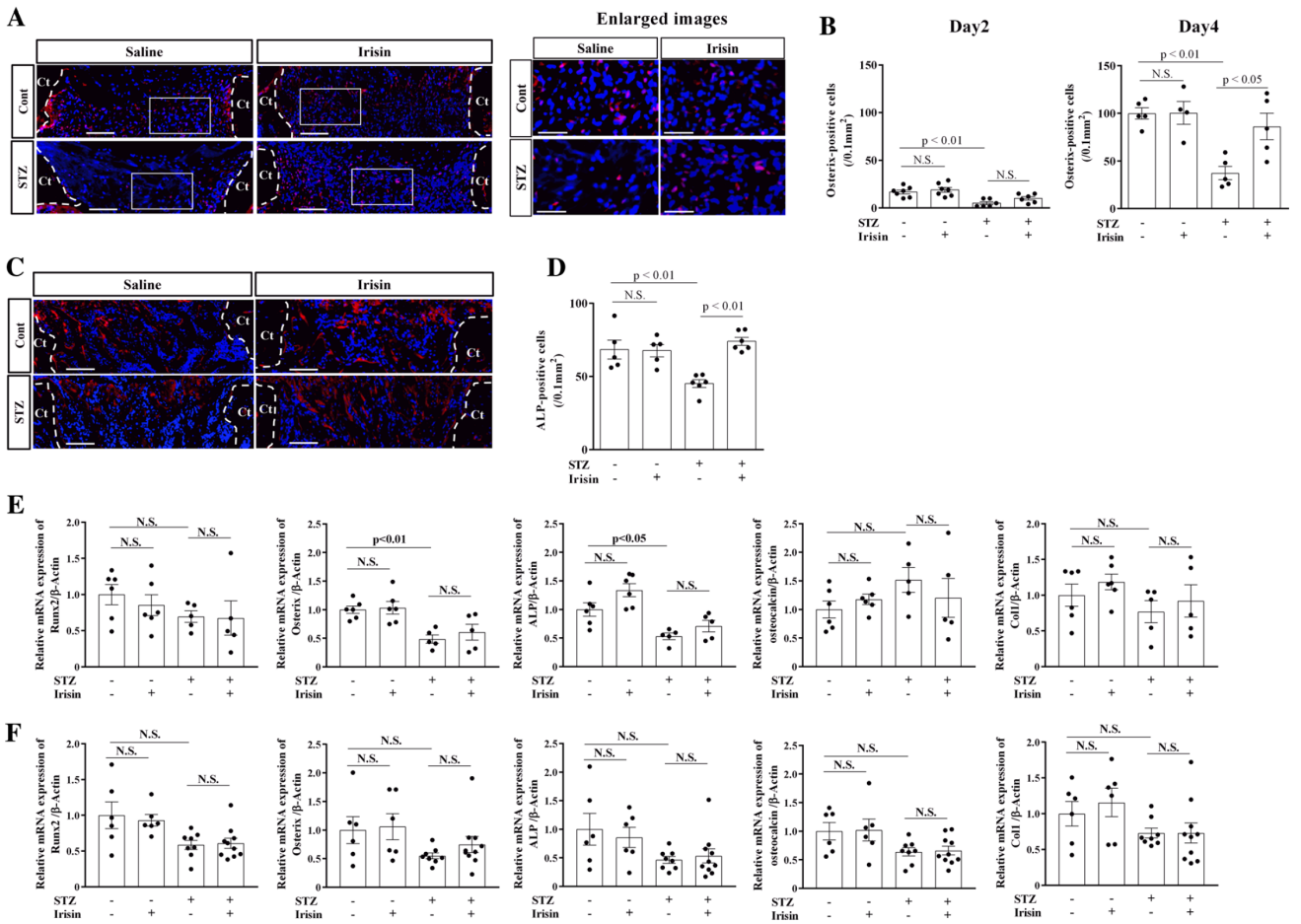

Fig. 2 Effects of local administration of irisin on the expression of osteogenic markers at the damaged site after a femoral bone defect was generated. **A** Representative microphotographs of Osterix-positive cells at the damaged site 4 days after a femoral bone defect was generated in female mice with or without STZ treatment and local administration of irisin. Scale bars indicate 100 μm and 50 μm (enlarged images). The enlarged images show the square area in the figure on the left. Dotted line indicates the boundary with cortical bone. **B** Quantification of the number of Osterix-positive cells per 0.1 mm^2 of microscopic field at the damaged site 2 and 4 days after a femoral bone defect was generated (n = 4–7 mice per group). **C** Representative microphotographs of ALP-positive cells at the damaged site 10 days after a femoral bone defect was generated in female mice with or without STZ treatment and local administration of irisin. Scale bars indicate 100 μm. Dotted line indicates the boundary with cortical bone. **D** Quantification of the number of ALP-positive cells per 0.1 mm^2 of microscopic field at the damaged site 10 days after a femoral bone defect was generated (n = 5–6 mice per group). The mRNA levels of osteogenic genes (Runx2, Osterix, ALP, osteocalcin and type 1 collagen (Col1)) at the damaged site 4 (**E**) and 10 (**F**) days after a femoral bone defect was generated in female mice with or without STZ treatment and local administration of irisin. Data are reported relative to β-actin mRNA values and are presented as the means ± SEM (n = 5–10 mice per group). *Cont* control, *N.S.* not significant, *Ct* cortical bone

Local irisin administration did not alter the PPARγ and aP2 mRNA levels at the damaged site 4 and 10 days after bone injury in mice with or without diabetes (Fig. 3F, G). Perilipin-positive cells were not observed at the damaged site of femur, although they were slightly localized in the bone marrow area (Fig. 3H).

Histological analysis of macrophages, vessels and osteoclasts at the damaged site

STZ treatment significantly decreased the number of F4/80-positive cells at the damaged site of femurs 2 days after bone injury but not 4 days after bone injury in mice (Fig. 4A). Local administration of irisin did not change the number of F4/80-positive cells at the damaged site of femurs 2 and 4 days after bone injury with or without STZ treatment (Fig. 4A). Local administration of irisin did not affect the CD31-positive vessel number at the damaged site of femurs 4 and 10 days after bone injury in mice with or without STZ treatment (Fig. 4B). On the other hand, STZ treatment significantly increased the number of TRAP-positive MNCs at the damaged site of femurs 10 days after bone injury, and local administration of irisin

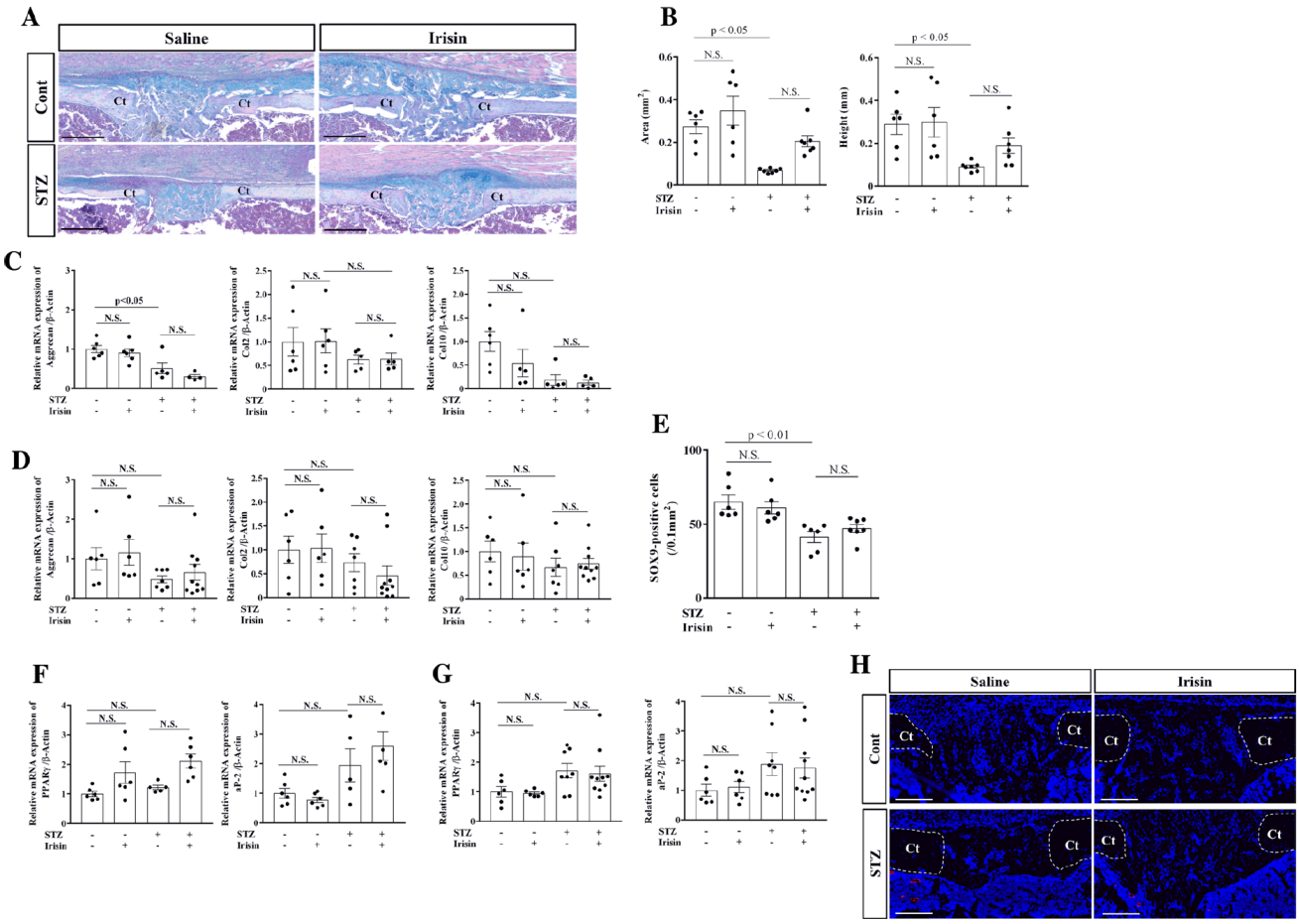

Fig. 3 Effects of local irisin administration on the expression of chondrogenic and adipogenic genes at the damaged site after a femoral bone defect was generated. **A** Representative microphotographs of Alcian blue-stained sections around the damaged site 10 days after a femoral bone defect was generated. Scale bars indicate 500 μm. **B** Quantification of the area and height of the Alcian blue-stained region in the sections 10 days after a femoral bone defect was generated (n = 6–7 mice per group). *Cont* control, *N.S.* not significant. The mRNA levels of chondrogenic genes (aggrecan, type 2 collagen (Col2) and type 10 collagen (Col10)) at the damaged site 4 (**C**) and 10 (**D**) days after a femoral bone defect was generated in female mice with or without STZ treatment and local administration of irisin. **E** Quantification of the number of SOX9-positive cells per 0.1 mm^2 of microscopic field at the damaged site 10 days after a femoral bone defect was generated (n = 6–7 mice per group). The mRNA levels of adipogenic genes (PPARγ and aP2) at the damaged site 4 (**F**) and 10 (**G**) days after a femoral bone defect was generated in female mice with or without STZ treatment and local administration of irisin. **H** Representative microphotographs of perilipin-positive cells at the damaged site 10 days after a femoral bone defect was generated. Scale bars indicate 200 μm. All data are reported relative to β-actin mRNA values and are presented as the means ± SEM (n = 5–10 mice per group). *Cont* control, *N.S.* not significant

did not alter the number of TRAP-positive MNCs in mice with or without STZ treatment (Fig. 4C).

Effects of intraperitoneal irisin treatment on bone repair

We intraperitoneally injected irisin to female mice with a femoral bone defect with or without STZ treatment to investigate the effects of systemic irisin treatment on bone repair. STZ treatment decreased the body weight of the mice beginning 2 weeks after the first injection of STZ, and the intraperitoneal injection of irisin did not affect the body weights of mice with or without STZ treatment (Fig. 5A). Blood glucose levels were markedly increased by STZ treatment immediately before a femoral bone defect was generated and intraperitoneal injections of irisin or saline (Fig. 5B). Intraperitoneal injection of irisin did not affect delayed bone repair induced by STZ treatment 10 days after bone defects were generated in mice (Fig. 5C, D). The BV/TV of the new bone in the bone defect region was not altered by the intraperitoneal injection of irisin 10 days after bone defects were generated in diabetic mice (Fig. 5E).

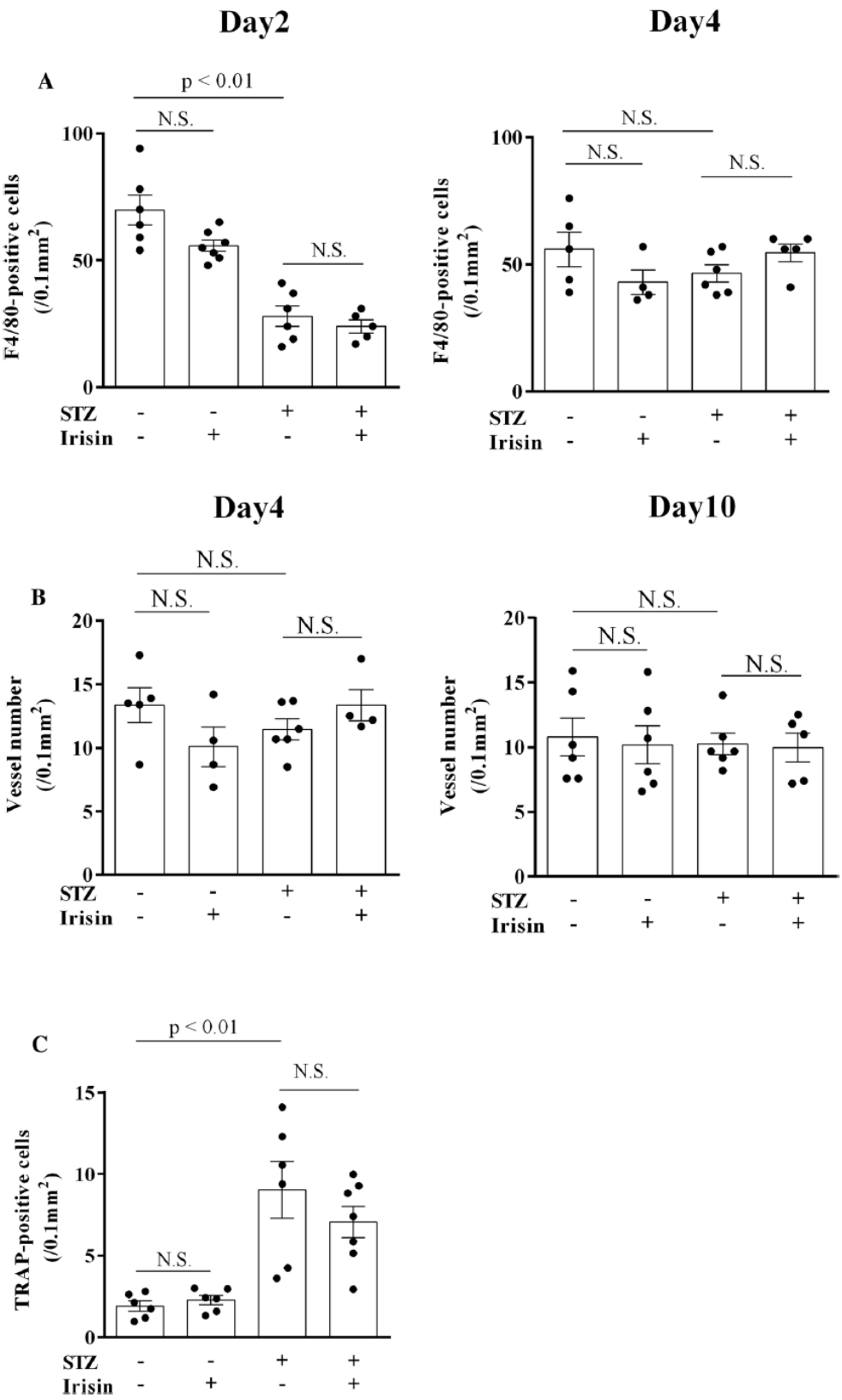

Fig. 4 Effects of local administration of irisin on the numbers of macrophages, vessels and osteoclasts at the damaged site after a femoral bone defect was generated in female mice with or without STZ treatment. **A** Quantification of the number of F4/80-positive cells per 0.1 mm^2 of microscopic field at the damaged site 2 and 4 days after a femoral bone defect was generated (n = 4–7 mice per group). **B** Quantification of the number of blood vessels per 0.1 mm^2 of microscopic field at the damaged site 4 and 10 days after a femoral bone defect was generated (n = 4–6 mice per group). **C** Quantification of the number of TRAP-positive cells per 0.1 mm^2 of microscopic field at the damaged site 10 days after a femoral bone defect was generated (n = 6–7 mice per group). *Cont* control, *N.S.* not significant

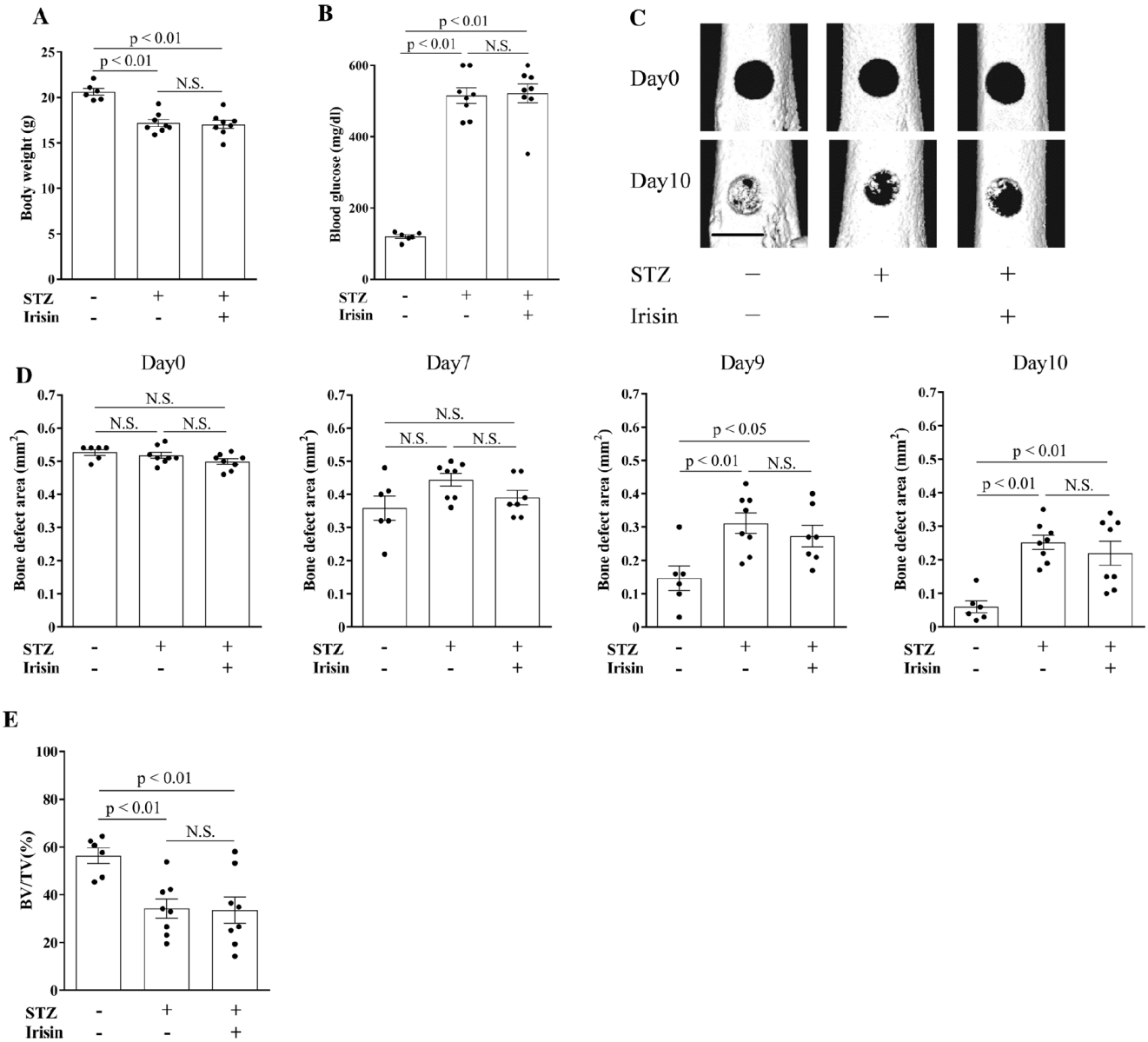

Fig. 5 Effects of systemic irisin administration on bone repair after a femoral bone defect was generated in female mice. **A** Body weights of the control and STZ-treated female mice with or without an intraperitoneal injection of irisin before a femoral bone defect was generated. **B** Blood glucose levels in control and STZ-treated mice with or without the intraperitoneal administration of irisin before a femoral bone defect was generated. **C** Representative three-dimensional images of the damaged site of femurs 10 days after bone injury in each group, as assessed using qCT imaging. The scale bar indicates 1 mm. **D** Quantification of the bone defect area assessed at the damaged site in control and STZ-treated mice with or without an intraperitoneal injection of irisin at 0, 7, 9 and 10 days after a femoral bone defect was generated. **E** The BV/TV (%) of the mineralized bone formed in the hole region 10 days after a femoral bone defect was generated was analyzed using qCT. Data are presented as the means ± SEM of 6–8 mice per group. *Cont* control, *N.S.* not significant

Effects of irisin on osteoblast differentiation in vitro

Finally, we examined the effects of irisin on AGE3, inflammation and high glucose-suppressed osteoblastic differentiation using mouse mesenchymal ST2 cells to investigate the mechanisms by which irisin influenced osteoblast differentiation during bone repair after a femoral bone defect was generated in mice. Irisin treatment significantly increased the Osterix, ALP and osteocalcin mRNA levels that were enhanced by BMP-2 in ST2 cells (Fig. 6). Irisin treatment significantly attenuated the decrease in Osterix mRNA levels induced by AGE3 or high-glucose conditions in the presence of BMP-2 in ST2 cells, although it did not alter the Runx2, ALP, osteocalcin and type 1

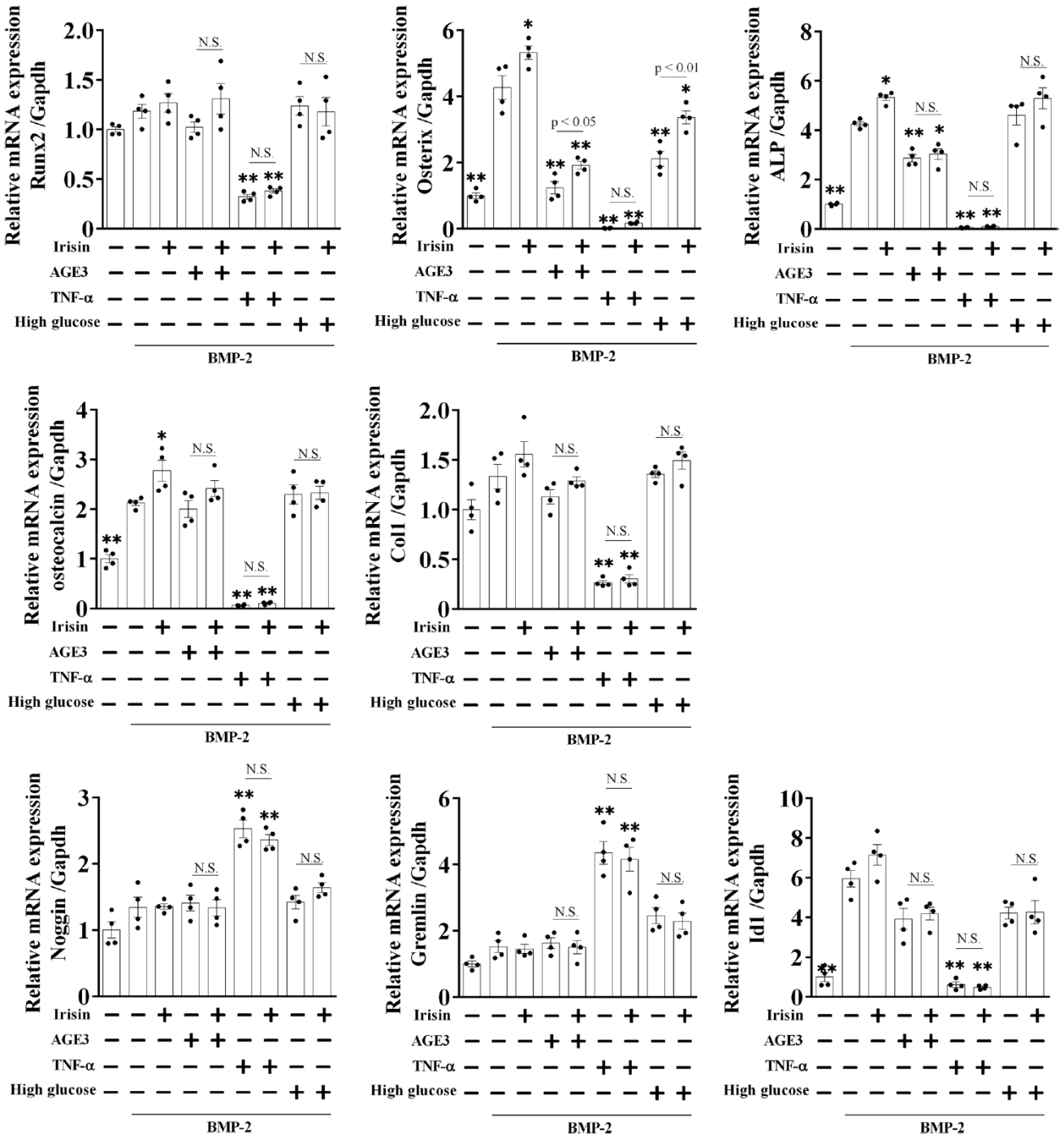

Fig. 6 Effects of irisin on the osteogenic differentiation of ST2 cells. ST2 cells were cultured with or without 200 ng/mL BMP-2, 200 μg/mL AGE3, 10 nM TNF-α or high glucose for 72 h. Total RNA was extracted from ST2 cells for an analysis of Runx2, Osterix, ALP, osteocalcin, type 1 collagen (Col 1), Noggin, Gremlin, Id1 or Gapdh mRNA expression using quantitative real-time PCR. Data are reported relative to Gapdh mRNA levels and are presented as the means ± SEM of 4 experiments in each group. $*p < 0.05$ and $**p < 0.01$ compared with the BMP-2 (+), irisin (−), AGE3 (−), TNF-α (−) and high glucose (−) groups. *N.S.* not significant

collagen mRNA levels in ST2 cells in the presence of AGE3, TNF-α or high glucose (Fig. 6). Irisin treatment did not alter the mRNA levels of BMP-responsive genes, such as Noggin, Gremlin and inhibitor of DNA binding

1 (Id1), in ST2 cells cultured in the presence of AGE3, TNF-α or high glucose (Fig. 6).

Discussion

In the present study, local irisin administration with gelatin hydrogel sheets improved delayed bone repair induced by diabetic state in female mice. Cationized gelatin hydrogels are suitable for the local sustained release of negatively charged molecules due to degradation [33], and transplanted cationized gelatin hydrogels were considered completely degraded 10 days after transplantation in the present study. Thus, irisin is locally sustained and released into the bone defect area, contributing to the improvement in delayed bone repair. Since local irisin administration did not alter serum glucose levels, the effects of local irisin administration on bone repair were presumed to be due to its action on the impairment of the bone repair process induced by diabetic state rather than any changes in hyperglycemia. Previous studies suggested that the effects of diabetic state on the mobilization of bone marrow mesenchymal and hematopoietic stem cells, the accumulation and function of macrophages, the formation of vessels, the process of chondrogenesis and the formation of osteoblastic bone are associated with delayed bone repair induced by diabetes [27, 28]. On the other hand, our study showed that local irisin administration did not affect bone repair after a femoral bone defect was generated in mice in the group without STZ treatment, suggesting that local irisin treatment with gelatin hydrogel sheets is not effective at enhancing bone repair in healthy mice, which was not consistent with the previous preliminary report that the delivery of muscle-derived irisin through a silk/calcium silicate/sodium alginate composite scaffold enhances bone repair after a skull bone defect was generated in rats [25]. This discrepancy might be partially due to the differences in the femur versus the calvaria, scaffolds, species (mice versus rats) and so on. Alternatively, the normal bone repair process is effectively accelerated and active after bone defects and fractures, and it is considered more active in femoral bone than in calvaria after bone defects. Therefore, the effects of local irisin administration on bone repair might not be observed due to the saturation of bone repair activity under physiological conditions.

The mechanisms by which local irisin treatment blunts delayed bone repair induced by diabetic state were not clear in our study. In the present study, we showed that local irisin administration blunted the diabetes-induced decrease in the number of osteoblastic cells at the damaged site, suggesting that the effects of irisin on delayed bone repair induced by diabetic state are partially attributed to a decrease in the number of osteoblasts in mice. This finding is consistent with our data that local irisin administration seemed to blunt the decrease in Osterix mRNA levels induced by diabetic state at the damaged site without significant differences. Several studies have reported that irisin enhances osteoblastic differentiation and proliferation partially through the extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) and canonical Wnt-β-catenin pathways in mouse cells [9, 16–18]. We speculated that local irisin treatment might improve delayed bone repair induced by diabetic state partially by enhancing osteoblastic differentiation, which then stimulates osteoblastic bone formation during the restoration phase of the bone repair process after bone injury. Some discrepancy in Osterix expression was observed between the immunohistochemical staining and analysis of mRNA levels in the bone tissues at the damage site in the present study. We speculated that this discrepancy might be due to the experimental limitation that the bone tissues at the damaged site include various cellular components other than osteoblastic cells.

AGEs and TNF-α are related to most of the pathological effects of diabetic state on various tissues, which might induce diabetic complications, including diabetic osteoporosis [35, 36]. Numerous studies have suggested that AGEs, high-glucose conditions and TNF-α suppress osteoblast differentiation [37–39]. We therefore speculated that irisin might modulate the negative effects of AGEs, high glucose and TNF-α on osteoblast differentiation during bone repair after the establishment of a femoral bone defect. In the present study, irisin treatment blunted the decrease in Osterix expression induced by AGE3 and high glucose in ST2 cells in the presence of BMP-2. Taken together with the effects of local irisin administration on the number of osteoblastic cells at the damaged site, these findings suggest that irisin blunts the diabetes-induced delay in bone repair after a bone defect is generated partially by enhancing early osteoblast differentiation at the restoration phase of bone repair and attenuating the effects of AGEs and high-glucose conditions on osteoblast differentiation.

According to previous studies, diabetic state influences cartilage and adipogenic differentiation [40–42]. In our study, local irisin administration tended to increase the formation of cartilage matrix near the bone defect that was suppressed by diabetic state in mice without a significant change, although it did not alter the expression of chondrogenic genes and number of SOX9-positive cells at the damaged site in mice. These findings are consistent with a recent report suggesting that irisin is related to cartilage development and osteoarthritis in mice [43]. In addition, local irisin administration did not affect the expression of adipogenic genes at the damaged site. Therefore, local irisin treatment in mice affected delayed bone repair induced by diabetic state mainly through osteoblastic bone formation but not through chondrogenesis and adipogenesis.

Accumulating evidence suggests that irisin is a crucial myokine linking muscle to bone [9–12, 15, 16, 20]. Specifically, irisin is physiologically and pathophysiologically related to the effects of exercise, immobilization, microgravity and androgen deficiency on bone mass in mice. Our data suggested that local irisin administration reverses delayed bone repair induced by diabetic state in mice, suggesting that the secretion of the myokine irisin from skeletal muscle might exert protective effects on impaired bone repair in pathological states, such as diabetes. Moreover, systemic irisin administration might be effective for the treatment of delayed bone repair in individuals with diabetes, since systemic irisin administration was an effective treatment for reduced bone mass and osteopenia in previous studies [9, 15, 17, 20]. However, in the present study, systemic irisin administration by intraperitoneal injection during bone repair did not affect the delayed bone repair induced by diabetic state in female mice, although the irisin dose used in the present study was similar to the dose used in the treatment of osteopenia in previous studies. Based on these findings, circulating irisin secreted from skeletal muscle is not physiologically relevant for the compensation of the bone repair process after femoral bone injury in diabetic mice. Moreover, systemic irisin administration, which is an effective treatment for osteoporosis, might not be effective for ameliorating impaired bone repair in diabetes.

This study has a limitation. We selected the concentration of irisin (100 μg/kg) for systemic administration based on a previous report [10]. Moreover, we used the same dose of irisin to determine the effects of systemic irisin administration on the recovery of androgen-deficient or chronic renal failure-induced bone loss in mice [15, 44]. However, we could not exclude the possibility that higher systemic doses or longer term irisin treatment might improve delayed bone repair induced by diabetic state in mice. Alternatively, the resolution of qCT in our study might reduce the sensitivity of the analysis of the effects of systemic irisin treatment on bone repair. Further studies are necessary to clarify the effects of systemic irisin treatment on bone repair.

In conclusion, our study showed that local irisin administration with gelatin hydrogel sheets improves delayed bone repair induced by diabetic state partially by enhancing osteoblastic differentiation. Local irisin treatment might be a potential clinical treatment option for fractures and bone defects in the future.

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Author contributions YK: investigation, formal analysis, and writing—original draft. YT: conceptualization, investigation, supervision, writing—review and editing, and funding acquisition. KO: resources and investigation. YT: investigation. HE: resources and investigation. YM: resources. NK: conceptualization. JJ: resources. YT: resources and writing—review and editing. HK: conceptualization, supervision, writing—review and editing, project administration, and funding acquisition.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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