Irisin reduces bone fracture by facilitating osteogenesis and antagonizing TGF-β/Smad signaling in a growing mouse model of osteogenesis imperfecta

Bin Sun, Huiqiao Wu, Jiajia Lu, Rongcheng Zhang, Xiaolong Shen, Yifei Gu, Changgui Shi, Ying Zhang*, Wen Yuan**
Department of Orthopaedics, Changzheng Hospital, Naval Medical University, Shanghai, China

** Three corresponding authors contributed equally to this paper.
*** Corresponding author. Department of Orthopaedics, Changzheng Hospital, Naval Medical University, 415 Fengyang Road, Shanghai, 200003, People's Republic of China.

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Objective: Osteogenesis imperfecta (OI) is a congenital disorder characterized by muscle defect and skeletal fragility, and no cure is yet available. Crosstalk between bone and muscle has become a new coming focus of therapeutic strategy in OI. Irisin, a secreted myokine, was found to be involved in regulating bone metabolism, and may be beneficial for the treatment of OI. However, its effects in OI have yet to be determined. This study sought to determine whether Irisin therapy is capable of reducing fracture risk in OI and to investigate the potential mechanisms of action.

Methods: Fibronectin type III domain containing 5 (FNDC5)/Irisin expression was assessed by enzyme-linked immunosorbent assay (ELISA) and immunohistochemical staining. In vivo, X-ray was used for fracture counting and micro-CT, dynamic histomorphometry analysis, immunohistochemistry, histomorphometry, and biomechanical test were used to evaluate the effects of Irisin on fracture frequency and bone quality in OI mouse model, oim/oim mouse. In vitro, osteogenesis-related gene expressions were determined by quantitative real-time PCR (qRT-PCR), western blot, and osteoblastogenesis assay were assessed by alkaline phosphatase (ALP) staining and alizarin red S (ARS) staining. Mechanistically, cell immunofluorescence staining, co-immunoprecipitation (co-IP), molecular docking, western blot, luciferase reporter assay, and chromatin immunoprecipitation (ChIP) assay were used for elucidating the mechanisms of how Irisin antagonized transforming growth factor-β (TGF-β)/Smad signaling in oim/oim osteoblasts and further attenuated the inhibitory effect of TGF-β1 on osteogenic differentiation.

Results: Musculoskeletal system-related FNDC5/Irisin was decreased in the serum, muscle, and bone in oim/oim mice. Irisin administration reduced bone fracture and attenuated bone abnormalities by improving bone mass and strength and facilitating the expression of osteogenic differentiation markers. In vivo study and in vitro experiments showed that Irisin antagonized TGF-β1/Smad signaling by interfering with TGF-β1-TGF-β receptor II (TβRII) binding. In oim/oim osteoblasts, Irisin alleviated TGF-β1-induced suppression of osteogenic differentiation through both integrin-dependent and integrin-independent mechanisms. Independent of integrin receptors, Irisin affected osteogenesis by activating ERK/p38 signaling and counteracting TGF-β1/Smad2/3 signaling. In particular, Irisin alleviated TGF-β1-induced inhibition of Runx2 function at the osteocalcin promoter through decreasing Smad2/3 signaling and inducing HADC4/5 degeneration.

AEIR SON, HuqiRao Wu, JiJia Lu and Rongcheng Zhang contributed equally to this paper.

E-mail addresses: sunbinspine@smmu.edu.cn (B. Sun), wuhq1028@smmu.edu.cn (H. Wu), edward03218@163.com (J. Lu), zhangrcspine@163.com (R. Zhang), spineshen@163.com (X. Shen), guyifei0001@126.com (Y. Gu), charlieshi@smmu.edu.cn (C. Shi), zy_spine@smmu.edu.cn (Y. Zhang), yuanwencspine@163.com (W. Yuan).

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1. Introduction

Osteogenesis imperfecta (OI) is a genetically heterogeneous connective tissue disorder characterized by skeletal dysplasia, bone fragility and recurrent fractures [1]. Current therapies for OI typically use anti-resorptive or bone anabolic drugs, but various side effects have been reported [2]. Recently, novel approaches have focused on the crosstalk between bone and muscle, and treatments targeting the muscle demonstrated beneficial effects on the bone structure in OI [3]. Although the musculoskeletal system is an ideal research area that can mediate the positive effects of exercise, an increased risk of fracture caused by exercise remains an issue [4]. Therefore, there is a need to explore novel strategies in the muscle-bone unit for the treatment of OI.

Irisin is an N-glycosylated myogenic protein hormone cleaved from fibronectin type III domain 5 (FNDC5) in response to exercise [5]. Growing evidence suggested that Irisin deficiency could weaken exercise-induced increases in bone strength [6], while administration of Irisin had favorable effects through increasing cortical bone mass and restoring bone loss in mouse models [7,8]. Moreover, Irisin administration could mimic some molecular effects of exercise in quadriceps [9]. This finding suggests that Irisin might be a promising therapeutic method for the treatment of musculoskeletal diseases such as OI. However, whether Irisin could improve abnormal bone structures of OI and the underlying mechanism remains unclear.

Disregulation of transforming growth factor-β (TGF-β) signaling is a common pathological mechanism in both dominant and recessive forms of OI [10]. Targeting TGF-β signaling has been confirmed to be a promising treatment strategy for OI [11]. TGF-β signaling in the regulation of bone metabolism is very complex as it can induce initial osteogenic differentiation but inhibit terminal bone formation [12]. Peng et al. reported that Irisin could bind to TGF-β receptor II (TβRII) and then antagonize against the TGF-β/Smad2/3 signaling in primary tubule cells. Therefore, it is possible that Irisin could counter TGF-β1-induced repression of osteoblast differentiation and be a promising target for the treatment of OI.

In this study, we found that the level of FNDC5/Irisin was reduced in serum, muscle, and bone in an oim/oim mouse model of OI. Irisin showed anti-fracture effects and partially reversed impaired bone maturation in oim/oim mice. In vitro study demonstrated that Irisin antagonized TGF-β1/Smad signaling by competitively binding to TβRII. Irisin also promoted osteogenesis and alleviated TGF-β1-induced inhibition of osteogenic differentiation, through both integrin-dependent and integrin-independent mechanisms. Specifically, Irisin ameliorated TGF-β1-induced repression of Runx2 function through attenuating Smad2/3 signaling and increasing HADCA5/4 generation at the osteocalcin promoter. Collectively, these data indicate that Irisin may be effective in preventing bone fractures in OI and thus be used as a potential therapeutic method for the treatment of OI.

2. Materials and methods

2.1. Reagents and antibodies

Irisin (Recombinant-Irisin), Dexamethasone, β-glycerophosphate, L-Ascorbic acid, Calcein, Xylenol orange, Alizarin Red S (ABS), and Hexadecylpyridinium chloride monohydrate were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Recombinant human TGF-β1 protein (Active) was purchased from Abcam (Cambridge, MA, USA). Mouse FNDC5/Irisin enzyme-linked immunosorbent assay (ELISA) Kit was purchased from Novus Biologicals (Littleton, CO, USA). Collagenase Type I was obtained from Gibco (Grand Island, NY, USA). LinKine AbbFuror 488 Labeling Kit and Cell Counting Kit 8 were purchased from Abbkine (Wuhan, HB, China). Fetal bovine serum (FBS), Phosphate-Buffered Saline (PBS), α-Minimum Essential Medium (α-MEM), and penicillin/streptomycin were purchased from HyClone (Logan, Utah, USA). TRizol, Rhodamine-Phalloidin, Lipofectamine 3000 Transfection Reagent, 4, 6-diamidino-2-phenylindole (DAPI), and RIPA lysis buffer were purchased from Thermo Fisher Scientific (Dreieich, Germany). PrimeScript RT Master Mix and TB Green Premix Ex Taq were obtained from Takara Bio, Kusatsu, Japan. Nuclear and Cytoplasmic Protein Extraction Kit, Alkaline phosphatase (ALP) staining kit, ALP assay kit, Mouse IgG, and Mouse IgG (Immunglobulin) Magnetic Beads were obtained from Beyotime (Beiyongmei, Shanghai, China). The 60SE2-luc was designed and synthesized by Transheep BioCo.Ltd (Shanghai, China). Dual-Luciferase Reporter Gene Assay Kit was purchased from Yeasen (Shanghai, China). One Step Mouse Genotyping Kit was purchased from Vazyme (Nanjing, JS, China). COH000, RGDS peptide, U0126, and SB203585 were purchased from Targetmol (Shanghai, China). The following antibodies were used in this study: anti-ALP (ab67228, WB, 1:1000), anti-OCN (ab133612, WB, 1:1000), anti-Runx2 (ab76956, WB, 1:1000). This study demonstrated that Irisin antagonist TGF-β1/Smad signaling by competitively binding to TβRII. Irisin also promoted osteogenesis and alleviated TGF-β1-induced inhibition of osteogenic differentiation, through both integrin-dependent and integrin-independent mechanisms. Specifically, Irisin ameliorated TGF-β1-induced repression of Runx2 function through attenuating Smad2/3 signaling and increasing HADCA5/4 generation at the osteocalcin promoter. Collectively, these data indicate that Irisin may be effective in preventing bone fractures in OI and thus be used as a potential therapeutics method for the treatment of OI.

2.2. Animals and in vivo experiments

The experimental protocol was approved by the Institutional Committee for Animal Care and Use of Naval Medical University. B6C3F1/α/α-ColIa2Gln35 encoding heterozygote oim/wt mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The desired genotype of homozygous oim/oim and littermate wt/wt mice were achieved by crossing heterozygote oim/wt mice, and identified by PCR genotyping and bidirectional Sanger sequencing (Fig. S1). Mice were housed in an air-conditioned room (20-25 °C and 60% relative humidity) with free access to water and food on a 12-h light/dark cycle. Four-week-old female mice of each genotype were randomly assigned into each group and treated with Irisin (100 μg/kg/week) or vehicle via tail vein injection for 8 weeks.

Conclusions: Collectively, Irisin could effectively reduce bone fracture in oim/oim mice through promoting osteogenesis and counteracting TGF-β/Smad signaling.

Translational potential statement: Findings from this study provided evidence for using Irisin as a potential therapeutic reagent to prevent the progression of OI.
2.3. Osteoblast culture

Primary osteoblasts were isolated from the calvaria of 3-5 day neonatal mice by digesting with 0.1% Collagenase Type I. Cells were cultured in α-MEM media with 10% FBS at 37.0 °C in a humidified atmosphere of 5% CO2. Cell culture medium was changed every 2 days and the third to fifth passages were adopted for further experiments. To induce osteogenic differentiation, primary osteoblasts were seeded into a 6-well plate at the density of 1 × 10⁶ cells/well and treated with osteoblast differentiation medium (10 mM β-glycerophosphate, 50 μM L-Ascorbic acid and 100 nM dexamethasone).

2.4. Quantitative real-time PCR (qRT-PCR)

Total mRNA was extracted by Trizol reagent and then subjected to reverse transcription using PrimeScript RT Master Mix. The qRT-PCR was performed using ABI 7500 Real-time PCR System (Applied Biosystems, CA, USA) with TB Green Premix Ex Taq. Primers were synthesized by Sangon Biotech (Shanghai, China). Primer sequences of target genes are summarized in Table S1 in supplementary materials.

2.5. Western blot and Co-immunoprecipitation (Co-IP) assays

The cells were lysed in RIPA buffer with protease inhibitors and phosphatase inhibitors for 30 min at 4 °C. The sample was then boiled with 5 × loading buffer at 100 °C for 10 min. To extract calvaria protein, skull samples were cut into pieces and then lysed in RIPA buffer with protease inhibitors and phosphatase inhibitors for tissue grinding. Nuclear and cytoplasmic proteins were extracted by using Nuclear protease inhibitors and phosphatase inhibitors for tissue grinding. Skull samples were cut into pieces and then lysed in RIPA buffer with β-glycerophosphate, 50 μM L-Ascorbic acid and 100 nM dexamethasone.

2.6. Osteoblastogenesis assay

After inducing osteoblast differentiation, the cells were washed with PBS three times and were fixed with 2% paraformaldehyde solution for 10 min. According to the manufacturer’s protocols, they were stained and structurally optimized with “Protein Preparation Wizard” module in the Schrodinger suite (Maestro 12.5, New York, USA). The prepared proteins were applied for molecular docking with “Protein-Protein docking” module in the Schrodinger suite.

2.7. Cell immunofluorescence staining

For cellular fluorescent staining, the cells were seeded onto 35 mm 4-chamber glass-bottom dishes (Cellvis, Sunnyvale California) at a density of 2 × 10⁴ cells/well. Irisin or TGF-β1 were fluorescently labeled using LinKine AbbFluo 488 Labeling Kit (Abbkine, China). The labeled A488-Irisin or A488-TGF-β1 were used for further experiments. After treatment, the cells were washed with PBS three times and fixed with 2% paraformaldehyde for 15 min. Cells were permeabilized with 0.25% Triton X-100 for 10 min and then blocked with 5% bovine serum albumin for 10 min. According to the manufacturer’s protocol, primary antibodies were applied for overnight incubation at 4 °C. After thorough washing, fluorescent secondary antibodies (Cy3-conjugated goat anti-mouse IgG, ab97035; Cy5-conjugated goat anti-rabbit IgG, ab69393) were added for 1 h and DAPI (Thermo Fisher Scientific, Germany) for 5 min at room temperature. Stained cells were observed and photographed using a confocal microscope (Zeiss, Oberkochen, Germany).

2.8. Luciferase reporter assay

Luciferase reporter containing Runx2-binding site (6OSE2-Luc) with PGL3-basic luciferase vector was constructed and synthesized by Transheep BioCo.Ltd (Shanghai, China). Runx2-overexpression plasmid (NM_001146038.2) and Renilla-expressing plasmid were purchased from Genomeditech Co. Ltd (Shanghai, China). Plasmid transfection was performed using Lipofectamine 3000 transfection reagent and luciferase reporter activity was assessed with Dual-Luciferase Reporter Gene Assay Kit (Yeasen, Shanghai, China) following the manufacturer’s protocol.

2.9. Chromatin immunoprecipitation (ChIP) assay

After treatment, the cells were fixed with 1% formaldehyde for cross-link and lysed using RIPA buffer with protease inhibitors. Soluble cell lysates were prepared by sonication and immunoprecipitated using Acetyl-H4 antibody at 4 °C for one night. Immunoprecipitated DNA was extracted and purified using One Step Mouse Genotyping Kit (Vazyme, China) according to the manufacturer’s instructions. Purified DNA was subjected to semi-quantitative PCR analysis using the sequence of osteocalcin promoter: 5'-GAGCTTGGACGTCCTGGATT-3' (forward) and 5'-ACCTCAGAATGAGC-3' (reverse).

2.10. Molecular docking

Peripheral blood samples were harvested from eye veins, which were let stand at room temperature for 1 h. The serum was prepared following a standard protocol (centrifugation at 12,000 rpm for 15 min, 4 °C). Serum Irisin concentration was measured according to the manufacturer’s instruction using a commercially available ELISA kit (NBP3-08118, Novus Biologicals, USA).

2.11. Radiography analysis and micro-CT analysis

For fracture counting, full-size photographs were obtained using X-ray (Faxitron X-ray, PA, USA). Fracture was defined by any evidence of interruption of continuity, bone deformity, or callus formation. Two independent researchers blinded to the assignment counted fractures in the humerus, radius, femur, tibia, tail bone of each oim/oim mice. After euthanasia, X-ray of the intact femora was obtained. The length was measured as the vertical distance from the tip of the femoral head to the base of the condyles. The left femora of each mouse were scanned and reconstructed by a
high-resolution micro-CT (Skyscan 1172, MA, USA) under a standard condition (50 kV X-ray voltage; 200 μA electric current; 10 μm per pixel). The region of interest (ROI) was selected in the reconstructed 3D image of the femur. The trabecular ROI was obtained as a 1 mm-thick volume of bone tissue (100 ct slices) from the end of the distal growth plate toward the diaphysis (Fig. 3A, Red). The cortical ROI was defined as segments extending from 3 mm (300 ct slices) proximally to the end of the distal growth plate over 1 mm (100 ct slices) toward the diaphysis (Fig. 3A, Green). For quantitative assessment, bone morphological parameters in the trabecular ROIs, such as trabecular bone mineral density (Tb.BMD), trabecular thickness (Tb.Th), trabecular bone volume fraction (Tb.BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp), were calculated. Cortical thickness (Cl.Th), cortical cross-sectional area (Cl.Ar), and marrow area were also quantified for cortical ROIs analysis. For micro-CT analysis, only femora without fractures or obvious deformities in the ROI were included.

2.13. Biomechanical testing

The right femora were harvested and kept hydrated in saline-soaked gauzes. The fresh femora were scanned using a quick micro-CT at the mid-diaphyseal region to obtain the moment of inertia and then immediately subjected to a three-point bending test with a material-testing machine (Instron 5569, MA, USA). To minimize the distortion of results in oim/oim mice, fractured femora with testing position intact were also included. Along the anteroposterior axis, the femora were fixed at the midpoint of 6 mm-spaced apart stage (L), and the mechanical loading was applied at a constant displacement rate of 1 mm/s. Biomechanical data were analyzed based on force-deflection curves by a custom program (MATLAB; MathWorks Inc., MA, USA).

2.14. Immunohistochemistry and histomorphometry

Samples of the tibia and corresponding tibialis anterior (TA) muscle were separated and fixed in 4% paraformaldehyde. After decalcification, the specimens were embedded in paraffin and then dissected into 5 μm thick sections. Hematoxylin and eosin (HE) staining, tartrate-resistant acid phosphatase (TRAP) staining, and immunohistochemical staining were performed according to standard methods. For histomorphometry analysis, pictures of immunohistochemically stained sections were captured under Carl Zeiss microscope (Oberkochen, Germany) and quantitative histomorphometric evaluations were performed using OsteoMetrics software (Georgia, USA). The number of osteoblasts of the bone surface (N.Ob/BS) and the number of osteoclasts of the bone surface (N.Oc/BS) were calculated on HE-stained sections and TRAP-stained sections, respectively. Relative quantified values of N. Oc/BS and N. Ob/BS normalized to Wt/wt + Veh. For quantitative analysis of N. Ob/BS, the selected area was restricted to the subchondral trabecular bone to avoid interference from abnormally elevated immature osteoblasts due to the pathological state ofOI [14].

For dynamic histomorphometry analysis, all mice were injected intraperitoneally with calcine (30 mg/kg) 3 days prior to euthanasia and administered xylene orange (90 mg/kg) 10 days before they were sacrificed. Left tibias were extracted at sacrifice and fixed in 70% ethanol for 24 h. The bone samples were dehydrated through a graded series of ethanol (from 70% to 100%, 10%/grade) and then embedded in methylmethacrylate (MMA; Sigma-Aldrich, St. Louis, MO, USA) with 10% dibutylphthalate (DBP) and 0.05% benzoyl peroxide (BPO). The tibias were cut transversely at the mid-diaphysis and were polished to a thickness of roughly 10 μm-sections. Hard tissue slices were imaged for histomorphometric examination with Zeiss Axioquant microscope (Thornwood, NY, USA). Histomorphometric parameters, including single-labeled surface (SLS), double-labeled surface (dLS), and interlabel thickness (IrLTh), were measured according to the guidelines of the American Society for Bone and Mineral Research [15]. These data were used to calculate the mineral apposition rate (MAR = IrLTh/7 days), mineralizing surface/bone surface ratio (MS/BS = (1/2sLS + dLS)/BS), and bone formation rate (BFR = MAR × MS/BS).

2.15. Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation (SD). The student’s t-test was used for two-group comparisons. One-way ANOVA was used for multi-group comparisons followed by Student-Newman-Keuls or Dunnett’s test wherever appropriate. Mann-Whitney test was used for the number of fractures. Kruskal-Wallis test was used for fracture distribution. P < 0.05 was considered statistically significant.

3. Results

3.1. FNDC5/Irisin was decreased in serum, muscle, and bone in oim/oim mice

At 3 months of age, Irisin level was reduced in the serum of oim/oim mice compared to age-matched wt/wt mice, while no significant difference was detected between male and female mice of the same genotype (Fig. 1A). Previous studies found that oim/oim mice exhibited atrophic muscles with impaired contractile forces, particularly in dorsal flexor and TA muscles [16]. In addition, the strength of healed fractured bones remained weaker than unfractured contralateral ones in oim/oim mice [17]. Therefore, we used TA muscles and the corresponding tibia from the side of the intact bone (femur and tibia) in oim/oim mice for immunohistochemical analyses. Results demonstrated that FNDC5 expression in TA muscles and the corresponding tibial bone were significantly decreased in oim/oim mice as compared with gender-matched wt/wt mice (Fig. 1B-C, E-F, H, J). We further observed lower expressions of FNDC5 in TA muscles and the tibia of the fractured side as compared with the unfractured side from the same oim/oim mice (Fig. 1B, D-E, G, I, K). Collectively, our results indicated that FNDC5/Irisin expression was decreased in the serum, muscle, and bone of oim/oim mice, and bone fracture was a possible cause of decreased FNDC5/Irisin in oim/oim mice.

3.2. Irisin administration increased fracture resistance in oim/oim mice

Continuous monitoring of body weight (Fig. 2A) showed that oim/oim mice were significantly smaller than wt/wt mice. However, weight gains were not significantly different between Irisin and vehicle (veh) administration in oim/oim mice after 8-week treatment. Irisin also showed no significant effect on femoral length at the end of treatment (Fig. 2B and C). Typical X-rays of each group are shown in Fig. 2D. No fracture was detected in wt/wt mice (Fig. 2D). Before the treatment, the number of fractures was similar in oim/oim groups (1.30 ± 0.48 vs. 1.40 ± 0.52, p = 0.66). After the treatment, the number of fractures increased by 1.3 times and 2.6 times for the oim/oim group and oim/oim + Veh group, respectively, with a significant group difference (2.60 ± 0.52 vs. 4.00 ± 1.25, p < 0.01) (Fig. 2E). No group difference was found in fracture site distribution (Fig. 2F). These data indicated that Irisin could significantly decrease the number of fractures in oim/oim mice.

3.3. Irisin boosted the anabolic effect on bone microarchitecture and promoted bone formation in oim/oim mice

We subsequently examined whether the anti-fracture effect of Irisin was due to the remodeling of the bone microarchitecture using micro-CT analysis and double-label staining. Oim/oim mice exhibited evident loss of both trabecular and cortical bone mass, and the loss was partially reversed by Irisin administration (Fig. 3A). After 8 weeks of Irisin injection, an anti-osteoporosis effect was observed in oim/oim mice, as indicated by increased Tb. BMD (Fig. 3B), Tb.Th (Fig. 3C), Tb.N (Fig. 3D), Tb.BV/TV (Fig. 3E), Ct. Th (Fig. 3H), and Ct. Ar (Fig. 3I) and decreased
A significant decrease in marrow area (Fig. 3G) was detected after Irisin treatment, which may be attributed to changes in cortical thickness. Calcein and xylene orange labels were observed by fluorescence light microscopy. Typical dynamic histomorphometry images of the tibia harvested from each group are displayed in Fig. 3J. In the endosteum, Irisin caused an increase in BFR (Fig. 3M) mainly by improving MAR (Fig. 3K) in oim/oim mice, without a significant effect on MS/BS (Fig. 3L). Similarly, an elevation of BFR (Fig. 3P) was observed through a notable increase in MAR (Fig. 3N), without a significant effect on MS/BS (Fig. 3O). These results indicated that Irisin had strong bone anabolic effects on oim/oim mice through improving bone microarchitecture and increasing bone formation.

3.4. Irisin promoted osteogenesis via upregulating the expression of osteogenic markers in oim/oim mice

Irisin could promote bone metabolism, which involves an increase in osteoblast proliferation and differentiation, and a decrease in the formation and resorption of osteoclasts in vitro [18,19]. To further examine the function of Irisin in oim/oim mice, histological staining of TRAP and HE was performed in tibial sections (Fig. 4A). Quantitative analysis showed that oim/oim mice had a significantly higher level of N.Ob/BS and a dramatic lower level of N.Oc/BS than wt/wt mice (Fig. 4B and C). TRAP staining demonstrated that the number of osteoclasts was significantly decreased by Irisin (Fig. 4B). HE staining confirmed that Irisin could significantly increase osteoblast formation in vivo (Fig. 4C). These results indicated that Irisin can improve the imbalance of bone metabolism, which might involve the dual effects of stimulating osteoblast formation and suppressing osteoclast formation. Histomorphometric examination of osteogenic markers further confirmed that oim/oim mice had lower expressions of ALP, OCN, Runx2, and Col1α1 in the distal tibia (Fig. 4D), indicating impaired bone formation. Eight weeks of Irisin treatment significantly upregulated the expression of ALP (Fig. 4E), OCN (Fig. 4F), and Runx2 (Fig. 4G), while few alterations was observed in Col1α1 (p = 0.044, Fig. 4H). Taken together, we found that Irisin could promote osteogenesis via promoting osteogenic markers expression in a mouse model of OI.

3.5. Irisin enhanced the mechanical properties of bone in oim/oim mice

At 12 weeks of age, Veh-treated oim/oim mice showed worse structural mechanical properties and material properties, compared to Veh-treated wt/wt mice (Table 1). The moment of inertia (−48.95%), maximum load (−41.73%), stiffness (−34.81%), total strain (−49.30%),
ultimate stress (-28.59%), energy to failure (-72.70%), elastic energy (-33.18%), plastic energy (-77.87%), post-yield displacement (-61.57%), and post-yield/yield displacement ratio (-58.61%) of femurs from Veh-treated oim/oim were decreased as expected. Irisin injection significantly improved the structural mechanical properties including the moment of inertia (+53.42%), maximum load (+28.53%), stiffness (+40.71%), and energy to failure (+43.68%) in oim/oim mice. Irisin increased the energy to failure through increases in elastic energy (not statistically significant) and plastic energy (+49.40%). In comparison, material properties of Young’s modulus, total strain, and ultimate stress were not significantly affected by Irisin. The post-yield displacement is an indicator of bone brittleness. It decreased dramatically in the femoral cortex of oim/oim mice (-61.57%). However, the inherent bone brittleness in oim/oim mice was partially restored by Irisin injection (post-yield displacement, +59.04%). As a result, the post-yield/yield displacement ratio was significantly increased with the denominator unchanged. The above results indicated that the mechanical properties were improved by Irisin.

3.6. Irisin promoted oim/oim osteoblast differentiation and mineralization in vitro

To analyze the underlying mechanism of Irisin in OI, we used primary osteoblasts from oim/oim mice for the in vitro experiment. The osteogenic properties of primary oim/oim osteoblasts were measured by qRT-PCR, western blot, ALP staining, and ARS staining at different time points during osteogenic differentiation induction. qRT-qPCR demonstrated that the mRNA expression of osteogenesis-related genes (ALP, OCN, Runx2, Col1α1, and Osterix) were increased by Irisin in a dose-response manner (Fig. 5A). Irisin treatment also significantly increased the expression of these osteogenesis-related genes at the protein level after 7 days of osteoblastic differentiation (Fig. 5B and C). Additionally, ALP staining (day 14) and ARS staining (day 21) revealed that Irisin significantly promoted ALP activity and extracellular matrix mineralization (Fig. 5D-H) of osteoblasts from oim/oim mice. Collectively, these results indicated that Irisin could facilitate the differentiation of osteoblasts.
3.7. Irisin attenuated TGF-β1-induced repression of oim/oim osteoblasts differentiation

Excessive TGF-β signaling is a primary mechanism in the pathogenesis of OI, and anti-TGF-β signaling is an effective treatment strategy for OI [10,15,20,21]. Irisin prevented TGF-β1-activated TGF-β signaling in a mouse model of renal injury [13]. We thus focused on the potential effect of Irisin on antagonizing TGF-β signaling in the skeletal system using a mouse model of OI. We first obtained skull samples from wt/wt and oim/oim mice after 8 weeks of Irisin or vehicle injection. Western bolt showed higher expressions of TGF-β signaling in oim/oim mice, characterized by elevated p-Smad2/3. Irisin treatment attenuated the high signal of TGF-β by reducing p-Smad2/3 in oim/oim mice (Fig. 5I). As a founding member of TGF-β superfamily, TGF-β1 is excessively activated in OI due to alterations of collagen in the extracellular matrix [10]. We then investigated whether Irisin could counteract TGF-β1-induced osteogenic inhibition by constructing an in vitro oim/oim osteoblast-based model in the presence of TGF-β1. As shown in Fig. 5J, high concentration of active-TGF-β1 (10 ng/mL) significantly inhibited oim/oim osteoblast differentiation by downregulating the expression of osteogenesis-related genes. ALP staining and ARS staining showed that ALP activity and calcium deposition of osteoblasts from oim/oim mice were markedly inhibited by TGF-β1 (Fig. 5K). In the presence of TGF-β1, adding Irisin increased osteoblast-specifc gene expression in a dose-response manner (Fig. 5L-O). Additionally, Irisin alleviated TGF-β1-induced repression of oim/oim osteoblast differentiation (Fig. 5L-O). Taken together, these results indicated that Irisin counteracted TGF-β1-induced osteogenesis inhibition in osteoblasts.
Fig. 4. Irisin facilitated osteogenesis in a mouse model of OI (A) Representative images of TRAP staining and HE staining of tibial sections from all groups after injecting Irisin (100 μg/kg/week) or vehicle for 8 weeks. Red-stained regions around the trabecular bone in TRAP-staining tibial sections were considered as TRAP-positive osteoclasts. Blue-stained regions around the subchondral trabecular bone in HE-staining tibial sections were considered mature osteoblasts. Scale bar represents 100 μm (B-C) Quantitative measurements of the number of osteoclasts/field of bone tissue (N.Oc/BS) in TRAP-staining sections and the number of osteoblasts/field of bone tissue (N.Ob/BS) in HE-staining sections were analyzed for each group (D) Representative immunohistochemical pictures of ALP, OCN, Runx2, and Collagen I in tibial sections. Scale bar represents 50 μm (E-H) Quantification of ALP, OCN, Runx2, and Collagen I expression in the immunohistochemical staining were analyzed by OsteoMetrics software. Wt/wt + veh group (n = 10), oim/oim + veh group (n = 7) and oim/oim + Irisin group (n = 8). Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Biomechanical Properties of Femoral Cortical Bone

| Parameters                  | Wt/wt + Veh (n=10) | Oim/oim + Veh (n=9) | Oim/oim + Irisin (n=10) |
|-----------------------------|--------------------|---------------------|-------------------------|
| Moment of inertia (mm²)     | 0.143±0.025        | 0.073±0.021***      | 0.112±0.018**           |
| Maximum load (N)            | 19.199±1.559       | 11.186±1.739***     | 14.377±2.283**          |
| Stiffness (N/mm)            | 130.83±8.435       | 85.288±11.811***    | 120.006±12.009***       |
| Young’s modulus (MPa)       | 5151.94±1214.755   | 5012.11±1016.802    | 4997.91±959.686         |
| Total strain                | 0.071±0.005        | 0.036±0.013***      | 0.041±0.014             |
| Ultimate stress (MPa)       | 134.82±28.198      | 96.72±20.817***     | 106.67±21.973           |
| Energy to failure (mj)      | 5.190±0.717        | 1.417±0.369***      | 2.036±0.667*            |
| Elastic energy (mj)         | 0.636±0.127        | 0.425±0.072**       | 0.530±0.175             |
| Plastic energy (mj)         | 4.555±0.619        | 1.096±0.375***      | 1.506±0.557*            |
| Yield displacement (mm)     | 0.085±0.012        | 0.079±0.015         | 0.082±0.011             |
| Post-yield displacement (mm)| 0.216±0.023        | 0.083±0.018**       | 0.132±0.018***          |
| Post-yield/yield displacement ratio | 2.573±0.417  | 1.065±0.182***     | 1.636±0.269***          |

Values are mean±SD,*p < 0.05, **p < 0.01, ***p < 0.001 Oim/oim + Veh versus Wt/wt + Veh, #p<0.05, ##p<0.01, ###p<0.001 Oim/oim + Irisin versus Oim/oim + Veh.
3.8. Irisin competitively antagonized TGF-β1 signaling in oim/oim osteoblasts via binding to TβRII

TGF-β1 first targets homodimeric TβRII with a high affinity for conformational adaptation, and then recruits TGF-β receptor I (TβRI) for activating downstream signaling pathways [22,23]. To elucidate the mechanism by which Irisin counteracted TGF-β1-induced repression of osteoblast differentiation and hyperactive TGF-β signaling, we used an in vitro cell-based model on the background of oim/oim osteoblasts. Double immunofluorescence staining showed co-localization of Irisin and TβRII on the osteoblast membrane (Fig. 6A). Co-IP assay further demonstrated that Irisin could bind to TβRII on the surface of osteoblasts (Fig. 6B). Both Irisin and TGF-β1 can bind to TβRII, the main receptor of TGF-β signaling pathway, we thus hypothesized that Irisin could compete with TGF-β1 to bind with TβRII on the membrane of osteoblasts. To test this hypothesis, we first applied molecular docking to predict specific ligand-receptor binding sites. Cartoon graph of Irisin-TβRII interaction showed that Irisin could bind to TβRII in the cleft between the N and C lobes of TβRII (Fig. 6C-Top left). Structural representation of the Irisin-TβRII complex demonstrated that this interaction was mainly through multiple hydrogen bonds (Y281, R254, Q418 and S432 in TβRII) and salt bridge (R254 in TβRII) (Fig. 6C-Top right). The predictive pose energy of Irisin-TβRII complex was -603.236 kcal/mol. For TGF-β1-TβRII interaction, cartoon illustration showed that TGF-β1 tightly bound to the C lobe...
Fig. 6. Irisin counteracted TGF-β1/Smad signaling via competitively interfering TGF-β1-TβRII binding in oim/oim osteoblasts (A) Oim/oim osteoblasts were firstly cultured with labeled A488-Irisin for 30 min, and followed by incubation with anti-TβRII antibody overnight. Cell fluorescence imaging showed Irisin (green) bound to the membrane surface and co-localized with TβRII (red). Blue indicated DAPI staining of nuclei. Scale bars represent 500 μm and 5 mm (B) Co-IP of Irisin with TβRII in oim/oim osteoblasts (C) Predicted computer modeling of Irisin-TβRII interaction and TGF-β1-TβRII interaction were generated with molecular docking using the Schrodinger suite. Red circle indicated the interfacial region in the binding domain of TβRII (blue) that shared the binding site for Irisin (wheat) and TGF-β1 (pink). Dotted boxes showed details of interaction modes for Irisin-TβRII binding and TGF-β1-TβRII binding. Yellow dotted lines: hydrogen bond interactions; Wine red dotted lines: salt bridge interactions. The predicted pose energy of protein-protein interactions was performed using PIPER program (D) Oim/oim osteoblasts were pretreated with different concentrations of Irisin (10 nM, 50 nM and 100 nM) for 30 min, and followed by adding 10 ng/mL labeled A488-TGF-β1 for 1 h. Representative immunofluorescence images of A488-TGF-β1 in oim/oim osteoblasts with pretreatment with different concentrations of Irisin. Blue indicated DAPI staining of nuclei. Scale bar represents 200 μm (E) Quantitative analysis of A488-TGF-β1 positive immunofluorescence staining and normalized to blank image density (n = 3) (F) Oim/oim osteoblasts were pretreated with Irisin for 30 min and followed by co-culture with TGF-β1 (10 ng/mL) for 1 h. Representative western blot showing Irisin antagonizing TGF-β1-induced Smad2/3 phosphorylation in a dose-response manner but co-activated ERK/p38 signaling (G) Quantitative analysis of protein expression of Smad signaling and MAPK signaling (n = 3) (H) Immunofluorescent staining of p-Smad2/3 (red) in oim/oim osteoblasts after pretreatment with Irisin (100 nM) for 30 min and followed by adding TGF-β1 (10 ng/mL) for 1 h. Blue indicated DAPI staining of nuclei. Scale bar represents 100 μm (I) Quantitative analysis of p-Smad2/3 positive immunofluorescence staining (n = 3) (J) Quantitative analysis of the percentage of p-Smad2/3 nuclear localization (n = 3). Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus Ctrl; #p < 0.05, ##p < 0.01, ###p < 0.001 versus TGF-β1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
of TβRII (Fig. 6C-Bottom left) through multiple hydrogen bonds (D411, E437, E526, L492, D494, and R479 in TβRII) and salt bridge (R479, K15, E526, and D411 in TβRII) (Fig. 6C- Bottom right). The calculated pose energy of TGF-β1-TβRII binding was −904.366 kcal/mol. The red circles in Fig. 6C show the position of the C lobe in TβRII, providing evidence for the possible mechanism by which the TGF-β1-TβRII binding site could be occupied by Irisin. For further validation, a competition experiment was conducted with A488-labeled TGF-β1 in osteoblasts. As shown in Fig. 6D and E, Irisin reduced the fluorescence intensity of A488-labeled TGF-β1 per cell, indicating that Irisin had a competitive inhibitory effect by blocking the interaction between TGF-β1 and TβRII on the cell surface. To ascertain whether Irisin could further affect TGF-β1-induced downstream signaling, we examined the dose-response relationship between Irisin and TGF-β1 in osteoblasts. After treatment with TGF-β1 (10 ng/mL), p-Smad2/3 was significantly increased and Irisin blocked activated Smad signaling in a dose-response manner (Fig. 6F and G). Non-Smad signaling pathways (p-ERK and p-p38) were also activated by TGF-β1 and Irisin, with no antagonistic but synergistic effect of Irisin and TGF-β1 observed (Fig. 6F and G). As the downstream effectors of TGF-β1/Smad signaling, phosphorylated Smads were translocated into the nucleus, interacting with target genes to further mediate functions. In oim/oim osteoblasts, TGF-β1 induced the phosphorylation of Smad2/3 and further lead to a higher proportion of cells exhibiting nuclear localization of p-Smad2/3, as evidenced by immunofluorescence staining (Fig. 6H-J). Irisin significantly decreased the activation of Smad2/3 phosphorylation, and impaired nuclear transfer of p-Smad2/3 in osteoblasts (Fig. 6H-J). Collectively, these results demonstrated that Irisin could compete with TGF-β1 to bind with TβRII on the membrane of osteoblasts, antagonize TGF-β1-induced Smad2/3 signaling, and induce non-Smad mitogen-activated protein kinase (MAPK) signaling of ERK and p38.

3.9. Irisin exerted its actions on osteogenesis in the presence of TGF-β1 through integrin receptor-dependent and integrin receptor-independent mechanisms

As functional receptors of Irisin, αV integrin receptors could contribute to bone remodeling [24]. However, they may also interact with other bone metabolic ligands and interfere with the function of...
Irisin [25]. More importantly, whether Irisin could regulate bone metabolism via other receptors has not been well illustrated. To determine whether Irisin altering the non-Smad signaling pathway depended on αV integrin receptors, we selected RGDS peptide as the antagonist to block Irisin from binding to integrin receptors. Western blot analysis confirmed that RGDS peptide decreased the co-activated p-ERK and p-p38 signaling by Irisin and TGF-β1, without affecting TGF-β1-induced Smad signaling (Fig. 7A and B). Phosphorylated Smads can be translocated into the nucleus, interacting with target genes to further mediate functions. Hence, we further evaluated the role of Irisin in the nuclear localization of p-Smad2 and p-Smad3. Results from nucleocytoplasmic separation showed that Irisin inhibited the translocation of p-Smad2 and p-Smad3 into the nucleus induced by TGF-β1, but this effect cannot be blocked by RGDS peptide (Fig. 7C and D). The results indicated that synergistic activation of Irisin-TGF-β1 on non-Smad pathways was co-triggered by integrin and non-integrin receptors, while the effect of Irisin on antagonizing TGF-β1/Smad signaling did not depend on integrin receptors. ALP staining and ARS staining showed that RGDS peptide resulted in a partial block in Irisin-mediated facilitation effect on osteogenic differentiation in the presence of TGF-β1 (Fig. 7E and F). Additionally, p-ERK signaling blocker U0126 and p-p38 signaling blocker SB203585 further hampered the integrin receptor-independent effect on osteoblastic differentiation of Irisin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Irisin-TGF-β1 co-treatment (Fig. 7E and F). Collectively, these data indicated that Irisin could alleviate TGF-β1-induced inhibition of osteoblasts differentiation, with both integrin and non-integrin receptors involved.

3.10. Irisin ameliorated TGF-β1-induced repression of Runx2 function at the osteocalcin promoter through increasing HADC4/5 degeneration via sumoylation

As the main transcriptional regulator, Runx2 is involved in bone remodeling. Meanwhile, Runx2 functions as a feedback regulator, controlling osteoblast differentiation in response to changes in differentiation state and extracellular environment. A critical event in the TGF-signaling pathway that inhibits osteoblast mineralization has been identified, which occurs through the inhibition of Runx2 function at the Runx2-binding site OSE2 of osteocalcin promoter [12]. We further investigated whether Irisin could attenuate the TGF-β signaling-induced transcription repression of osteocalcin and the exact regulation mechanism. Immunofluorescence results showed that Irisin mediated TGF-β1-induced osteocalcin repression, and this effect was partially dependent on integrin receptors (Fig. 8A-B). To examine whether Irisin could block the inhibition of Runx2 function by TGF-β1, we constructed a 60SE2-Luc plasmid containing the osteocalcin promoter with Runx2-binding site OSE2. As shown in Fig. 8C, dual-luciferase report assay result demonstrated that TGF-β1 inhibited the Runx2 activity at 60SE2-luc and Irisin overcome this repression in response to TGF-β1. More importantly, no difference was observed after the addition of the RGDS peptide (Fig. 8C). It suggested that Irisin, acting independently of integrin receptors, could alleviate TGF-β1-induced inhibition of Runx2 function at the osteocalcin promoter. Since histone H4 deacetylation was involved in TGF-β1-mediated repression of Runx2 function in differentiating osteoblasts, we used a ChIP assay with semi-quantitative PCR to examine chromatin changes at the osteocalcin promoter. Compared to the control group, TGF-β1 treatment resulted in rapid deacetylation of histone H4 at the osteocalcin promoter, and Irisin demonstrated significant antagonism on the deacetylation activity of TGF-β1 (Fig. 8D). HDAC4 and HDAC5, as class IIa HDACs, are required for efficient TGF-β1 mediated inhibition of Runx2 function during osteoblast differentiation.

To elucidate the underlying mechanism, we used qRT-PCR and western blot to examine the effect of Irisin on HDAC4 and HDAC5. As shown in Fig. 8E, the qRT-PCR analysis revealed that the HDAC4 and HDAC5 mRNA did not change significantly after Irisin treatment. However, after 24 hours of Irisin treatment, HDAC4 and HDAC5 protein levels were significantly reduced (Fig. 8F). Sumoylation is a common protein degradation pathway for HDACs, we used Co-IP assay to examine HDAC sumoylation in MC3T3-E1 cells. The sumoylation of HDAC4 and HDAC5 was significantly increased after Irisin treatment (Fig. 8G-H), and the inhibitor of the sumo-activating enzyme COH0000 significantly blocked Irisin's effect on TGF-β1-induced repression of dual luciferase activity of 60SE2-luc (Fig. 8I). Taken together, we found that Irisin could attenuate TGF-β1-induced repression of osteogenesis through integrin and non-integrin receptors. Independent of integrin receptors, Irisin could antagonize TGF-β1-induced repression of Runx2 function at the osteocalcin promoter by promoting HADC4/5 degeneration via sumoylation (Fig. 8J).

4. Discussion

In this study, we demonstrated that the level of FNDC5/Irisin was significantly reduced in the serum, muscle, and bone in a mouse model of OI. Irisin administration effectively restored the incidence of fracture and improved bone microarchitectures. We further revealed the potential mechanism of Irisin in antagonizing TGF-β/Smad2/3 signaling by competitively interacting with TjIRI. In the presence of TGF-β1, Irisin promoted osteogenic differentiation via integrin receptors and non-integrin receptors. Irisin exerted its actions on osteogenesis through activating non-Smad signaling of p-ERK and p-p38 and counteracting TGF-β/Smad2/3 signaling. Specifically, Irisin attenuated TGF-β1-induced suppression of Runx2 function by reducing Smad2/3 signaling and inducing HADC4/5 degeneration at the osteocalcin promoter. These data suggested that Irisin can ameliorate skeletal abnormalities and may be used to prevent the progression of OI. Irisin, a hormone released during exercise, has been closely related to the state of skeletal muscle [26]. Congenital muscle defect and skeletal muscle fatigue are common phenomena in both human and OI mouse models [27]. In the present study, we showed that the overall level of FNDC5/Irisin was reduced in oim/oim mice. This phenomenon suggests that intrinsic muscle defect in OI may contribute to reduced Irisin secretion. Irisin secretion appears to be mediated by various types of exercise and mechanical forces, as well as pathological conditions like diabetes, obesity, and inflammation [28]. Over 6-fold increased expression of FNDC5 protein and Irisin were detected in normal mice after exercise [29]. Using a similar mouse model, we found a lower expression of FNDC5 in fracture-lateral TA muscle and tibia than those from the healthy side. Fractures usually lead to reduced exercise and muscle atrophy, which may explain the low amount of exercise observed among OI patients [30]. Both congenital collagen defect and spontaneous fracture may interfere with the physiological expression of FNDC5/Irisin in OI; however, the underlying mechanism remains to be examined.

Irisin is an active cross-linking molecule between the skeletal muscle and skeleton and plays a significant role during mechanical stress [7,31]. Based on the above evidence, we speculate that impaired FNDC5/Irisin expression is involved in the dysfunctional communication between the muscle and bone, and Irisin may be a therapeutic strategy for the treatment of OI.

To identify whether Irisin administration can influence the occurrence of spontaneous bone fracture in oim/oim mice, we injected 100 µg/kg recombinant Irisin once a week for 8 weeks. We observed the anti-fracture effects of Irisin in oim/oim mice. The enhanced bone anabolic effect was reflected by improved trabecular and cortical bone microarchitectures. Dynamic bone formation indicators and histological analysis confirmed that Irisin significantly promoted bone formation by simultaneously promoting osteoblast activity and inhibiting osteoclast activity. Irisin is suitable for the treatment of OI as it can counteract the pathological features of OI, which is characterized by diminished osteoblast activity and stimulated osteoclast activity [14,32]. The protein expressions of osteogenesis-related markers were also upregulated after the treatment, indicating that Irisin could promote osteogenic differentiation and stimulate a more mature phenotype of osteoblasts in oim/oim mice. Similar to strontium ranelate and alendronate [33], Irisin has few effect on the expression of type 1 collagen as compared with other parameters. It may be due to the inherent genetic defect of type I collagen in oim/oim mice. Dysregulation of bone metabolism leads to bone loss and promotes bone fragility, and fractures usually occur in parts with higher bone fragility [34]. In a mouse model of OI, bone strength of the fractured side is significantly lower than that of the contralateral intact side [17]. Simply selecting the intact femur or replacing the contralateral unfractured femur for testing may create bias. Therefore, we selected ipsilateral intact femur or the middle region of the fractured femur from the same lateral for analyses. Our results showed that Irisin administration effectively improved bone biomechanical properties, which may explain its anti-fracture effects.

An imbalance in osteoblast homeostasis is the main pathological basis of OI, characterized as decreased osteogenic function and impaired bone matrix quality. Our preliminary data confirmed that Irisin could promote osteogenic differentiation in OI, TGF-β/Smad2/3 signaling plays important role in bone metabolism and cartilage homeostasis [12,35]. Elevated TGF-β/Smad2/3 signaling is a common extracellular abnormal signal contributing to the bone pathology in OI, which is resulted from the impaired binding efficiency of TGF-β ligand due to the mutation of type I collagen [10]. Peng [13] et al. reported that Irisin could bind to TjIRI, thereby inhibiting TGF-β/Smad2/3 signaling in primary tubule cells. Similarly, we found that Irisin treatment could attenuate abnormally
elevated TGF-β signaling in a mouse model of OI. In vitro experiments further indicated that Irisin could reverse TGF-β1-induced suppression of osteogenic differentiation in oim/oim osteoblasts. Taken together, our data demonstrated that Irisin can influence bone metabolism via coordinating facilitating osteogenesis, and antagonizing TGF-β1/Smad signaling.

To further clarify the specific molecular mechanism of Irisin on osteogenesis in the presence of TGF-β1, we obtained oim/oim osteoblasts. In line with a previous study [13], we confirmed that Irisin can bind to TGF-β1 specific receptor TβRI on the surface of osteoblasts. Computational docking analysis provided evidence for the potential competitive binding of Irisin and TGF-β1 with TβRI. The overlapping spatial binding site was detected in the C lobe of TβRII compared to the two predicted binding models, Irisin-TβRII and TGF-β1-TβRII. Although relatively weaker, the comparable pose energy of Irisin-TβRII binding (−603.236 kcal/mol) provided a theoretical possibility that Irisin could block TGF-β1-TβRII binding (−904.366 kcal/mol). Fluorescence-based binding assay further confirmed that Irisin blocked the binding of TGF-β1 and TβRII, preventing its downstream Smad pathway from being activated and later nuclear translocation of p-Smad2/3. Nevertheless, unlike its effect on the Smad signaling, Irisin did not antagonize but rather collaborated with TGF-β1 to activate the non-Smad signaling pathway. A possible explanation is that by binding with TβRII, Irisin stimulated the formation of TβRII homotetramers while preventing the assembly of TβRII-TβRII heterotetramers [13]. However, evidence has shown that the effect of Irisin on bone metabolism was mediated by αV integrin receptors [24], Irisin [36] and TGF-β1 [37] mediated skeletal metabolism involves MAPK signaling response. Using RGDS peptide as a functional receptor blocker, we confirmed that Irisin could promote osteogenic differentiation independent of integrin receptors. It may be related to the deactivation of Smad2/3 signaling and activation of MAPK signaling (p-ERK and p-p38) due to the competitive binding of Irisin and TGF-β1 with TβRII. TGF-β1/Smad axis is a key player involved in the TGF-β1-mediated repression of osteoblast differentiation [12,38]. Qi et al. reported that TGF-β1/Smad3 pathway participated in the inhibition of osteoblast mineralization by copper chloride [39]. Huang et al. also confirmed that IncRNA H19/miR-675 promoted osteogenic differentiation via regulating TGF-β1/Smad3/HDAC [40]. In this study, we showed that Irisin inhibited TGF-β1-Smad2/3 signal nuclear transduction and further reversed TGF-β1-induced inhibition of Runx2 activity at the osteocalcin promoter, independent of integrin receptors. Histone H4 acetylation on the 6OSE2 sequence is an important effect phenomenon in TGF-β1-induced repression of osteocalcin, which is exerted by HDAC4/5 [38]. In H9c2 cardiomyoblasts, Irisin treatment facilitated degeneration of HDAC4 via increasing sumoylation [41]. Similarly, Irisin preserved myocardial performance and insulin resistance in db/db mice, which was partially resulted from HDAC4 reduction [42]. Using ChiP assay, we found that the increased deacetylation of histone H4 was attenuated by Irisin treatment when exposed to TGF-β1, which was associated with HDAC4/5 degeneration via sumoylation.

There are several limitations of this study. First, although Irisin has been confirmed to be an antagonist counteracting TGF-β1/Smad signaling and attenuating the inhibitory effect of TGF-β1 on osteogenic differentiation, whether the inhibition of TGF-β1/Smad signaling play a key role in the favorable effects of Irisin in OI model remains for further study. Second, we did not evaluate the effects of Irisin on other organs and its indirect effect on bone. As OI is a systemic connective tissue disease with pathologies existed in various organs, systemic administration of Irisin may have multifactorial role in the regulation of skeletal homeostasis via other factors such as energy metabolism, skeletal muscle mass [28], and angiocrine factors [43]. Third, since OI is a heterogeneous disease with a wide range of phenotypes, it is necessary to further confirm the positive effect of Irisin on bone through different OI mouse models.

In summary, our results demonstrated that the expression of FNDC5/irisin was reduced in the serum, muscle, and bone of a mouse model of OI. In vivo, Irisin injection decreased fracture incidence and skeletal defects. Our data also showed the antagonistic effects of Irisin against TGF-β1/Smad signaling both in vivo and in vitro. Independent of integrin receptors, Irisin regulated the differentiation of osteoblasts by promoting osteogenesis and antagonizing TGF-β1/Smad signaling. We further revealed the mechanism by which Irisin ameliorated TGF-β1-induced repression of Runx2 function through inhibiting Smad signaling and increasing HADC4/5 degeneration. This work was among the first to demonstrate that Irisin could reduce bone fracture by facilitating osteogenesis and antagonizing TGF-β1/Smad signaling in a mouse model of OI.

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Author contributions

Bin Sun: Conceptualization, Methodology, Validation, Writing-Original draft preparation. Huiqiao Wu: Methodology, Validation, Writing-Original draft preparation, Formal analysis. Jiajia Lu: Validation, Writing-Original draft preparation, Formal analysis. Rongcheng Zhang: Validation, Formal analysis, Resources, Data curation. Xiaolong Shen: Resources, Data curation, Yifei Gu: Resources, Data curation, Funding acquisition. Changgui Shi: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision, Project administration, Funding acquisition. Ying Zhang: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision, Project administration, Funding acquisition. Wen Yuan: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

Bin Sun, Huiqiao Wu, Jiajia Lu, Rongcheng Zhang, Xiaolong Shen, Yifei Gu, Changgui Shi, Ying Zhang and Wen Yuan declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.10.012.

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