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The effect of lactate dehydrogenase B and its mediated histone lactylation on chondrocyte ferroptosis during osteoarthritis

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Abstract

Background Histone lactylation is a novel epigenetic regulator that is reported to participate in gene expression. Ferroptosis is an oxidative form of cell death and chondrocyte ferroptosis crucially impacts the development of osteoarthritis (OA). The study aimed at investigating the effect of lactate dehydrogenase B (LDHB) and its mediated histone lactylation on chondrocyte ferroptosis during OA.

Methods Our study focused on the establishment of in vivo mouse model and in vitro interleukin-1β (IL-1β)-induced chondrocytes model and administrated LDHB knockdown (siLDHB). Histopathological assessment of cartilage was conducted via HE staining, while serum levels of cartilage oligomeric matrix protein (COMP) and crosslinked C-telopeptides of type II collagen (CTX-II) were quantified using ELISA to evaluate OA severity. The matrix degradation was further examined by expression of Collagen II and Aggrecan. Levels of total iron, ferrous iron (Fe2+), and lipid reactive oxygen species (ROS) were considered measurements of ferroptosis. Assessment of cell viability and proliferation relied on cell counting kit 8 (CCK-8) together with colony formation assay. Western blotting assay served for detecting the relative expression of proteins and protein lactylation. The epigenetic regulation of ACSL4 by LDHB was determined by chromatin immunoprecipitation (ChIP) and luciferase reporter gene assay.

Results OA mice presented remarkably elevated protein level of LDHB and H3K18 lactylation in the cartilage versus the sham group. Knockdown of LDHB downregulated the levels of COMP and CTX-II, as well as alleviated chondrocyte ferroptosis in vitro and in vivo. Results from ChIP and luciferase reporter gene assay demonstrated direct histone lactylation of ACSL promoter, and knockdown of LDHB and treatment with LDH inhibitor reduced histone lactylation and expression of ACSL4. ACSL4 overexpression could reverse the impact of LDHB depletion on chondrocyte proliferation and ferroptosis.

Conclusion LDHB promotes ACSL4 by histone lactylation to induce chondrocyte ferroptosis, which further contributes to OA development. The findings in the study assist in understanding the modulating mechanism of LDHB-mediated lactylation against chondrocyte ferroptosis in OA progression.

Keywords Osteoarthritis, Lactylation, Lactate dehydrogenase B, Chondrocyte, Ferroptosis

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Introduction

Osteoarthritis (OA) is a most prevalent degenerative joint disease around the world and the major cause of chronic pain and disability in the elderly population [1]. OA is primarily characterized by degenerated joint tissues resulting from imbalanced synthesis and degradation process of cartilage and other joint tissues, accompanied by subchondral bone sclerosis, synovitis, joint capsule inflammation, tendon involvement (which destabilizes joint mechanics and exacerbates cartilage wear through aberrant biomechanical stress) and other pathological changes [2, 3]. The progression of OA greatly affects the degrading mechanism of matrix-degrading enzymes against the extracellular matrix (ECM) in chondrocytes [4, 5]. Articular cartilage, consisting solely of chondrocytes, is a connective tissue taking charge of producing ECM. The survival of chondrocytes can help to maintain articular cartilage integrity and overall joint homeostasis [6]. According to many studies, cavities exist in cartilage during OA progression, indicating a link between chondrocyte death and OA pathogenesis [7, 8]. Cartilage tissues of OA patients and OA animal models underwent various forms of chondrocyte death, including apoptosis, necroptosis, and autophagy. Despite the improvement of OA animals' prognosis by the inhibition of above three chondrocyte death forms [9, 10], no clinical trials have been carried out to successfully adopt any single cell death inhibitors. All these demonstrate the occurrence of various cell death forms in OA, together benefiting the cartilage degeneration.

Ferroptosis is an oxidative form of cell death featuring iron-dependent lipid hydroperoxides being accumulated to lethal levels [11]. Ferroptosis can serve for the effective prevention of various tumors and degenerative diseases like Alzheimer's disease, Parkinson's disease, and renal degeneration [12–14]. Recent evidence suggests that ferroptosis of chondrocytes contributes to OA progression. Mice with iron overload presented elevated cartilage destruction degree, and intracellular iron uptake benefits chondrocytes that mimic the OA phenotype [15, 16]. Collectively, inhibiting ferroptosis may represent a novel strategy for OA progression prevention.

Lactate is a glycolysis product from pyruvate by lactate dehydrogenase (LDH) and importantly affects the physiological and pathological actions [17, 18]. In a recent study by Zhang et al., histone lactylation is a novel epigenetic modification, which can activate gene transcription [19]. Moreover, histone lactylation acts as an important epigenetic regulator during disease processes. Specifically, histone lactylation increase at reparative macrophage gene loci promotes macrophages to be transferred from an inflammatory to a reparative state to respond to microbial ligands and multiple damaging cues [19]. Additionally, Glis1-induced histone lactylation at pluripotency gene loci facilitates somatic cell reprogramming [20]. Lactate dehydrogenase B (LDHB) is a critical lactate regulator and is involved in the regulation of histone lactylation [17, 18]. However, the role of histone lactylation and LDHB in OA has not been well explored.

Our study focused on the ferroptosis of chondrocytes, and explored the function and underlying mechanism of LDHB and its mediated histone lactylation in OA progression.

Materials and methods

Animal model

The study adopted male C57BL/6 mice aging 8-weeks old to mimic OA in vivo. Mice were randomly divided into four groups ($n = 8$ per group): (1) Sham group (sham surgery + siNC), (2) OA group (DMM surgery + siNC), (3) OA + siLDHB group (DMM surgery + siLDHB), (4) OA + siLDHB + Nala group (DMM surgery + siLDHB + sodium lactate). Briefly, mice were anesthetized by amobarbital sodium injection (25 mg/kg body weight) intraperitoneally, with the right knee joint being exposed through a medial capsular incision. The destabilized medial meniscus (DMM) surgery was conducted, and the incision was stitched. After surgery, mice in the respective groups received articular injection of siNC or siLDHB (20 μmol) and sodium lactate (Nala, 10 mg/kg body weight) two times per week for eight weeks. The mice were killed 8 weeks after surgery. All animal experiments obtained the approval of the Animal Care and Use Committee of Medical School of Yangzhou University.

Histological analysis

After euthanizing the mice, all right knee joints were excised to undergo one day of fixation in 4% paraformaldehyde. Then, the specimens received 2 weeks of decalcification in a 10% EDTA solution, followed by paraffin-embedding. 5 μm thick sections in the sagittal plane were prepared from the specimens and stained with hematoxylin and eosin (HE). Microscopy (Leica, Germany) was utilized to capture images of the stained sections.

Enzyme-linked immunosorbent assay (ELISA)

Cartilage oligomeric matrix protein (COMP) and cross-linked C-telopeptides of type II collagen (CTX-II), as the degradation products of joint tissues, can indicate OA progression biochemically. The collected blood samples underwent centrifugation for acquiring serum. Relevant measurement relied on the ELISA (Thermo, USA) following the manufacturer's protocol.

Isolation and culture of mouse chondrocytes

We isolated primary mouse chondrocytes from the knee joint cartilage of newborn C57BL/6 mice. Initially, cartilage tissue sections underwent 2 h of digestion with 2.5 mg/ml collagenase type II (Gibco) followed by overnight digestion with 0.5 mg/ml collagenase type II at 37 °C. The isolated primary chondrocytes underwent incubation in low glucose DMEM medium (Hyclone, USA) added with 10% FBS (Thermo, USA) and 1% penicillin-streptomycin in a humidified 37 °C incubator filled with 5% CO_2. Cells received the treatment of IL-1β (10 ng/ml), Nala (5 mM), Oxamate (5 mM).

Cell viability and proliferation

Cell Counting Kit-8 (CCK-8) assay (Beyotime, China) was adopted for assessing the chondrocyte viability. Initially, seeded into 96-well plates (5,000 cells/well), chondrocytes underwent 24 h of cell adhesion, followed by IL-1β stimulation and 24 h of Nala treatment. The plates then received 1 h of incubation at 37 °C in the absence of light. At last, we detected the absorbance at 450 nm by virtue of a microplate detector (Thermo Fisher, USA).

Colony formation served for evaluating cell proliferation. In brief, cells were suspended as single cells and seeded into 6-well plate at 1,000 cells/well. Following 14 days of culture, the colonies were visualized by staining in crystal violet solution (Beyotime, China) for 20 min. Images were taken by a digital camera.

Western blotting analysis

Chondrocytes seeded in 6-well plates (1×10^5 cells/well) underwent 24 h of treatment. After that, cells from various groups received 20 min of lysis in RIPA lysis buffer (Beyotime, China) that contained phosphatase inhibitor cocktail on ice. The collected extract underwent half an hour of centrifugation at 12,000 × g and 4 °C. That was followed by the collection of supernatant and the evaluation of protein concentration by a BCA assay kit (Beyotime, China). Same volume of proteins received sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and were moved to PVDF membranes (Millipore, USA). After 1 h of blockage in 5% non-fat milk at room temperature, membranes were hatched for one night by specific primary antibodies for LDHB (Abcam, ab53292), H3K18 lactylation (PTM bio, PTM-1427RM), Collagen (Abcam, ab307674), Aggrecan (Abcam, ab313636), ACSL4 (Santa Cruz, sc-271800), and β-actin (Abcam, ab8227) at 4 °C, followed by 1 h of incubation using anti-mouse or anti-rabbit secondary antibodies at room temperature. After that, protein bands were incubated with chemiluminescence reagent (Millipore, Germany). The last step was the image photographing (Bio-Rad, USA).

Detection of ferroptosis

Ferroptosis could be signaled by abnormal levels of total iron, ferrous iron (Fe2+), and lipid ROS. We detected intracellular total iron and Fe2 + levels using iron assay kit (Abcam, USA) as per the producer's protocol. For lipid peroxidation detection, cells underwent 45 min of incubation using 5 μM of BODIPY581/591 C11 (Invitrogen, USA) at 37 °C and two times of PBS wash, followed by being collected as single-cell suspension. Samples were measured by flow cytometry.

Chromatin Immunoprecipitation (ChIP)

The enrichment of H3K18 lactylation on ACSL4 promoter was measured by CHIP assay kit (Invitrogen, USA) as per the producer's protocol. In brief, chondrocytes were fixed, lysed and sonicated to chromatins with an average size of 500 bp. Then, the chromatins were subjected to 8 h of incubation with the anti-H3K18la antibody or IgG as control at 4 °C with rotation. The immunoprecipitated DNAs were then purified by using RNase A and proteinase K. Purified DNA was evaluated and level of ACSL4 was analyzed by PCR with specific primers.

Quantitative PCR experiment

An Trizol reagent (Invitrogen, USA) was employed for extracting total RNA. Then a Prime Script RT kit (Takara, Japan) served for cDNA synthesis. A SYBR Green system (Takara, Japan) served for the qPCR. The reaction was performed at 95 °C for 2 min, 38 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative gene expression was calculated by virtue of the comparative $2^{-\triangle\triangle Ct}$ method and normalized to the β-actin gene.

Luciferase reporter gene assay

Seeded in 6-well plates 24 h, HEK-293T cells received the transfection of ACSL4 reporter gene vectors with Lipofectamine 2000 reagent (Invitrogen, USA). 48 h of cell incubation was followed by cell lysis. Dual luciferase system (Promega, USA) assisted in detecting the firefly luciferase activity and renilla luciferase activity.

Statistical analysis

The data presentation forms the mean ± SD format, obtained from triplicate samples at a minimum. Statistical analyses relied on GraphPad Prism 7.0, employing unpaired two-tailed Student's t-test, one-way analysis of variance (ANOVA), or Mann-Whitney U test for between-group variance. $p < 0.05$ reported statistical significance.

Results

The expression of LDHB and histone H3K18 lactylation is elevated in OA model

The study focused on the establishment of a mouse OA model to determine the relative expression of LDHB and histone lactylation. The more obvious matrix degradation and inflammation in cartilage from OA mice than those in the sham group (Fig. 1A) suggested the successful establishment of OA model. OA group presented remarkably higher serum levels of CTX-II and COMP (Fig. 1B and C), which confirmed the OA generation. Moreover, the protein level of LDHB and histone H3K18 lactylation were notably elevated in the cartilage of OA mice (Fig. 1D), suggesting that LDHB and H3K18 lactylation are correlated with OA.

LDHB promotes histone H3K18 lactylation and ferroptosis during OA progression in vivo

Subsequently, we used siRNA to downregulate LDHB and sodium lactate (Nala) as suppliers for lactylation. The knockdown of LDHB suppressed the levels of LDHB and H3K18 lactylation in OA mouse model, whereas treatment with Nala abolished the effects of siLDHB (Fig. 2A). Knockdown of LDHB also alleviated matrix degradation in cartilage tissue (Fig. 2B), suppressed serum CTX-II and COMP levels (Fig. 2C and D), and increased expression of matrix protein, such as collagen type II and Aggrecan (Fig. 2E). However, treatment with Nala notably reversed these phenomena. Furthermore, we examined cell ferroptosis during OA and observed that knockdown of LDHB downregulated the OA-induced elevation of total iron, Fe^{2+}, and lipid ROS, whereas treatment with Nala recovered their levels (Fig. 2F-H). These data indicated that LDHB promoted histone H3K18 lactylation and ferroptosis during OA progression *in vivo.*

LDHB suppresses proliferation and induces ferroptosis of chondrocytes

The function of LDHB in the IL-1β-induced chondrocyte model was measured. Similar results with the in vivo

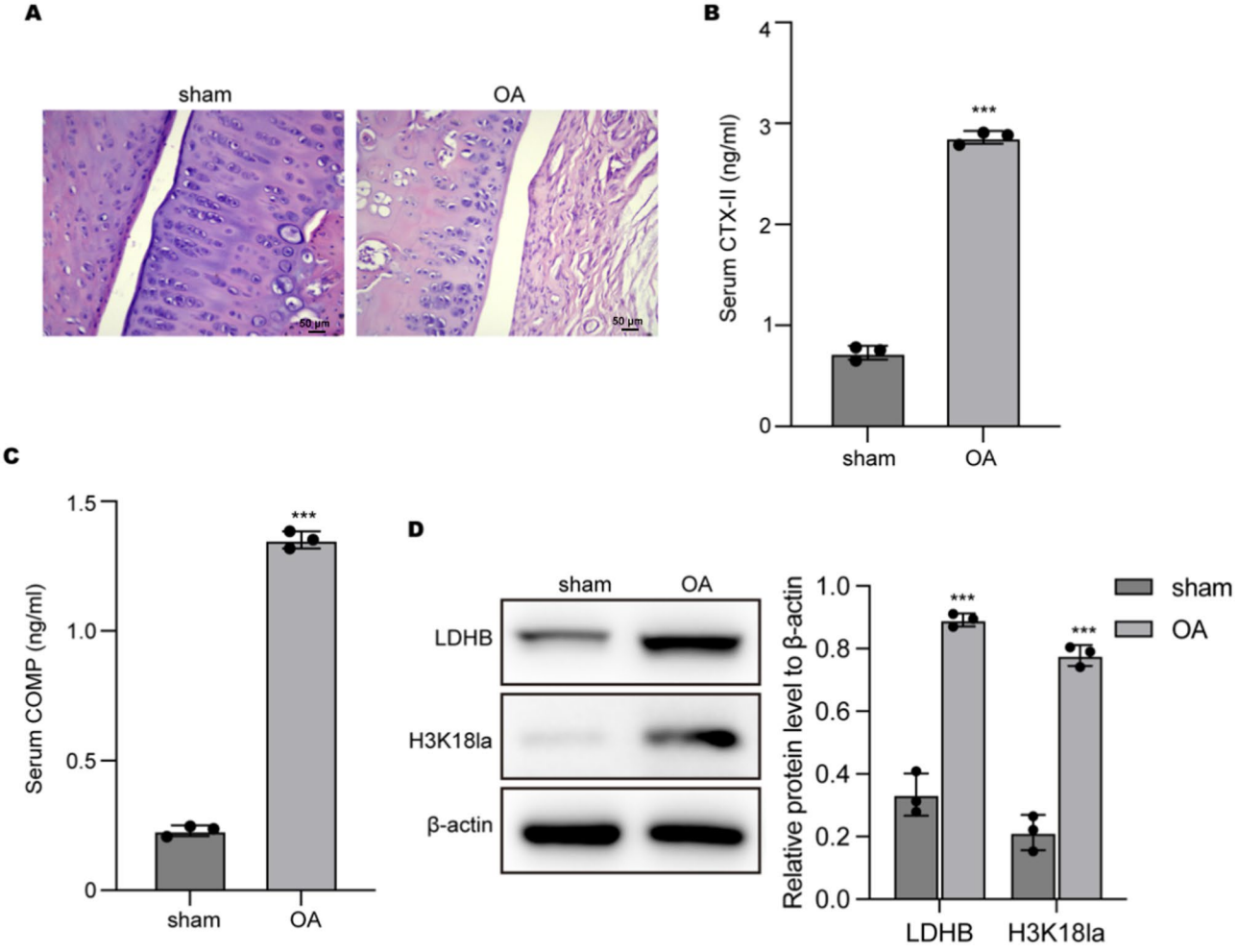

Fig. 1 The expression of LDHB and histone H3K18 lactylation is elevated in OA model. (**A**) HE staining of cartilage tissues. (**B** and **C**) The serum levels of CTX-II and COMP. (D) The protein levels of LDHB and H3K18 lactylation. ***$p < 0.001$

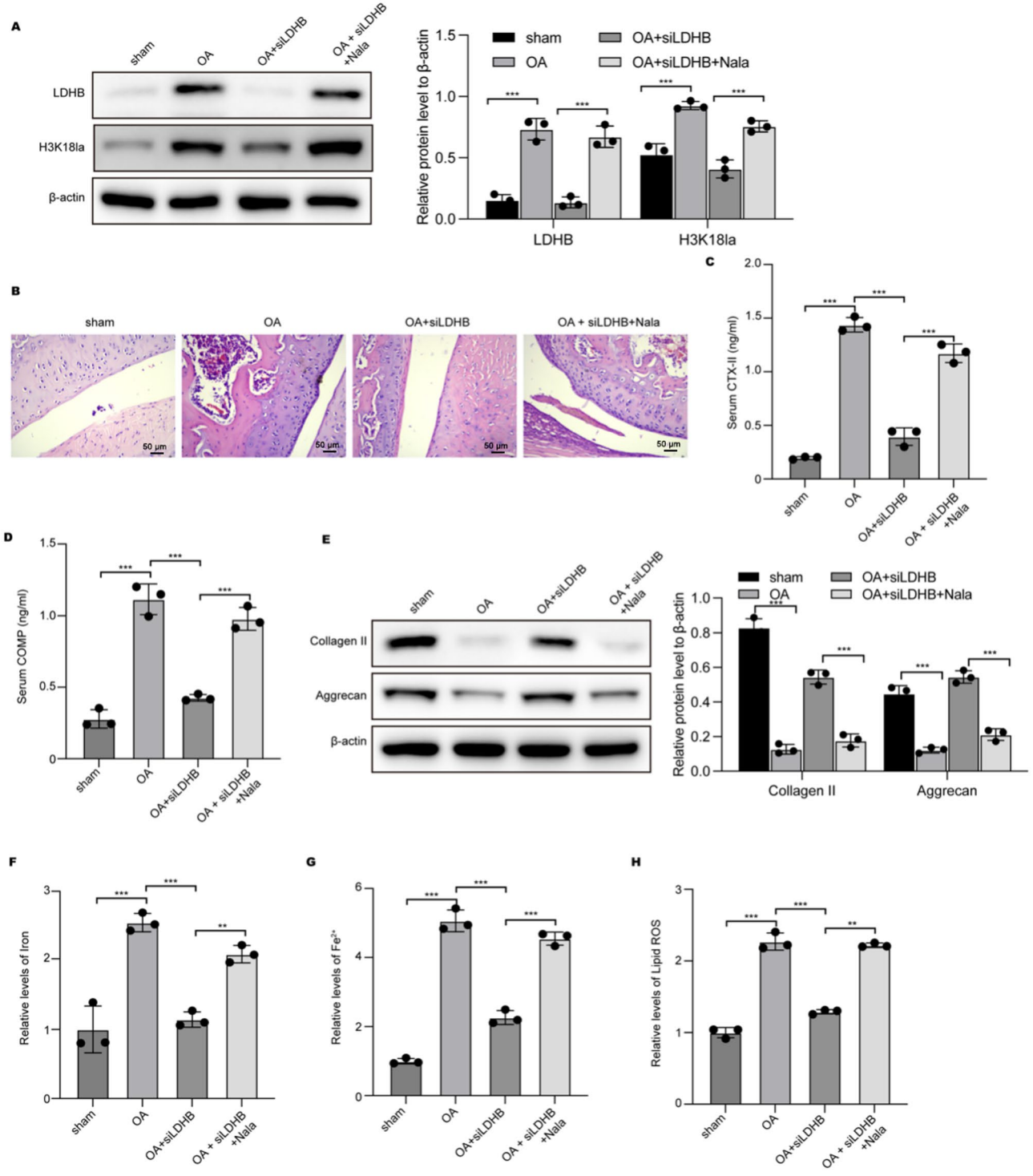

Fig. 2 LDHB promotes histone H3K18 lactylation and ferroptosis during OA progression in vivo. OA model was established and treated with siLDHB and Nala. (**A**) The protein levels of LDHB and H3K18 lactylation. (**B**) HE staining of cartilage tissues. (**C** and **D**) The serum levels of CTX-II and COMP. (**E**) The protein levels of Collage type II and Aggrecan. (**F**-**H**) The levels of total iron, Fe2 + and lipid ROS in cartilage tissues. $^{**}p < 0.01$, $^{***}p < 0.001$

model were observed, as IL-1β stimulation induced elevation of LDHB and histone H3K18 lactylation in chondrocytes (Fig. 3A), suppressed cell proliferation (Fig. 3B and C), reduced expression of Collagen II and Aggrecan (Fig. 3D), whereas LDHB depletion reduced LDHB and H3K18 lactylation, recovered cell proliferation and matrix protein expression. Moreover, the knockdown of LDHB also repressed IL-1β-induced ferroptosis of chondrocytes

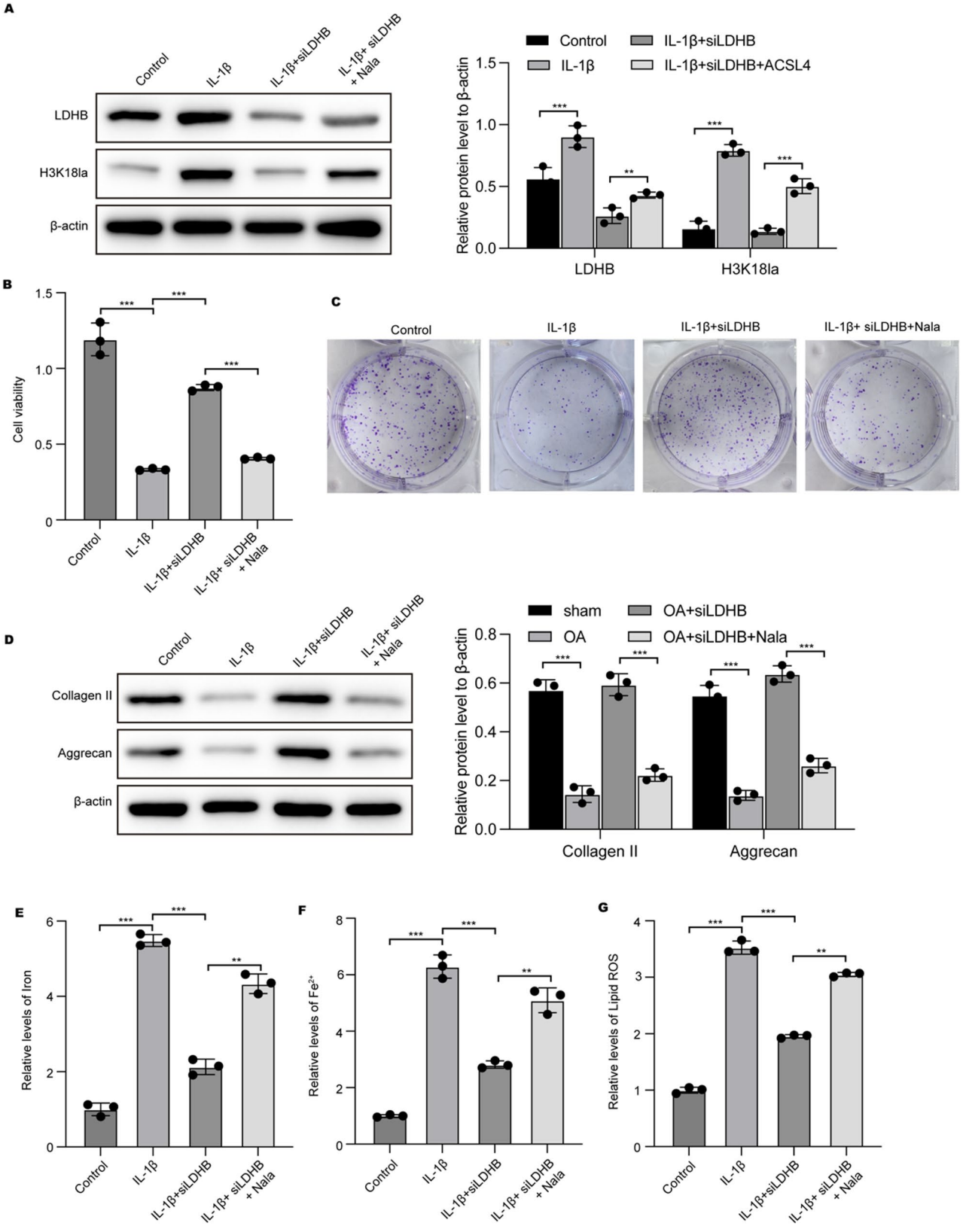

Fig. 3 LDHB suppresses proliferation and induces ferroptosis of chondrocytes. Chondrocytes were induced by IL-1β and treated with siLDHB and Nala. (**A**) The protein levels of LDHB and H3K18 lactylation in chondrocytes. (**B**) Cell viability was measured by CCK-8. (**C**) Cell proliferation was detected by colony formation. (**D**) The protein levels of Collage type II and Aggrecan in chondrocytes. (**E**-**G**) The levels of total iron, Fe2 + and lipid ROS in chondrocytes. $^{**}p < 0.01$, $^{***}p < 0.001$

(Fig. 3E-G). Noteworthy, the treatment with Nala could reverse the effect of siLDHB on chondrocytes.

LDHB epigenetically enhances ACSL4 expression by histone H3K18 lactylation in chondrocytes

To explore the potential mechanisms underlying LDHB-regulated ferroptosis, we analyzed the expression of ACSL4, a known direct regulator of ferroptosis. ACSL4 expression was downregulated by LDHB knockdown and reversed by Nala treatment (Fig. 4A). Similar to siLDHB, treatment with Oxamate, an LDH inhibitor, also suppressed the expression of ACSL4 (Fig. 4B). Moreover, results from the ChIP experiment demonstrated that LDHB depletion reduced the enrichment of H3K18 lactylation of ACSL4 promoter (Fig. 4C) and downregulated the ACSL4 promoter activity (Fig. 4E), which was recovered by Nala treatment. Consistently, Oxamate also reduced the H3K18 lactylation and activity of ACSL4 promoter (Fig. 4D and F).

LDHB induces the ferroptosis of chondrocytes *via* ACSL4

We next administrated ACSL4 overexpression under LDHB depletion in the IL-1β-induced chondrocyte model. As expected, the depletion of LDHB recovered viability, proliferation, and expression of Collagen II and Aggrecan of chondrocytes, and ACSL4 overexpression abolished these effects (Fig. 5A-C). Moreover, LDHB knockdown restricted the IL-1β-induced ferroptosis of chondrocytes, whereas overexpression of ACSL4 recovered the accumulation of total iron, Fe^{2+}, and lipid ROS (Fig. 5D-F). Taken together, LDHB induces the ferroptosis of chondrocytes *via* ACSL4.

LDHB affects chondrocytes ferroptosis through ACSL4 expression regulated by lactation

To further demonstrate that ACSL4 expression is regulated by lactation and that this regulatory process affects chondrocyte proliferation, we used lactate dehydrogenase inhibitor oxamic acid (OXA) to inhibit LDHB activity and overexpressed ACSL4 in IL-1β-induced chondrocyte models. Similar to the results of siLDHB treatment, OXA treatment improved the survival (Fig. 6A) and proliferation (Fig. 6B) of chondrocytes stimulated by IL-1β, and promoted Collagen II and Aggrecan to be expressed (Fig. 6C), while ACSL4 overexpression eliminated these effects. In addition, OXA treatment inhibited IL-1β-induced iron death in chondrocytes, while ACSL4 overexpression made the total iron ion, Fe2, and lipid ROS more accumulated (Fig. 6D and F). These data suggest that LDHB regulates chondrocyte iron death by regulating ACSL4 lactation.

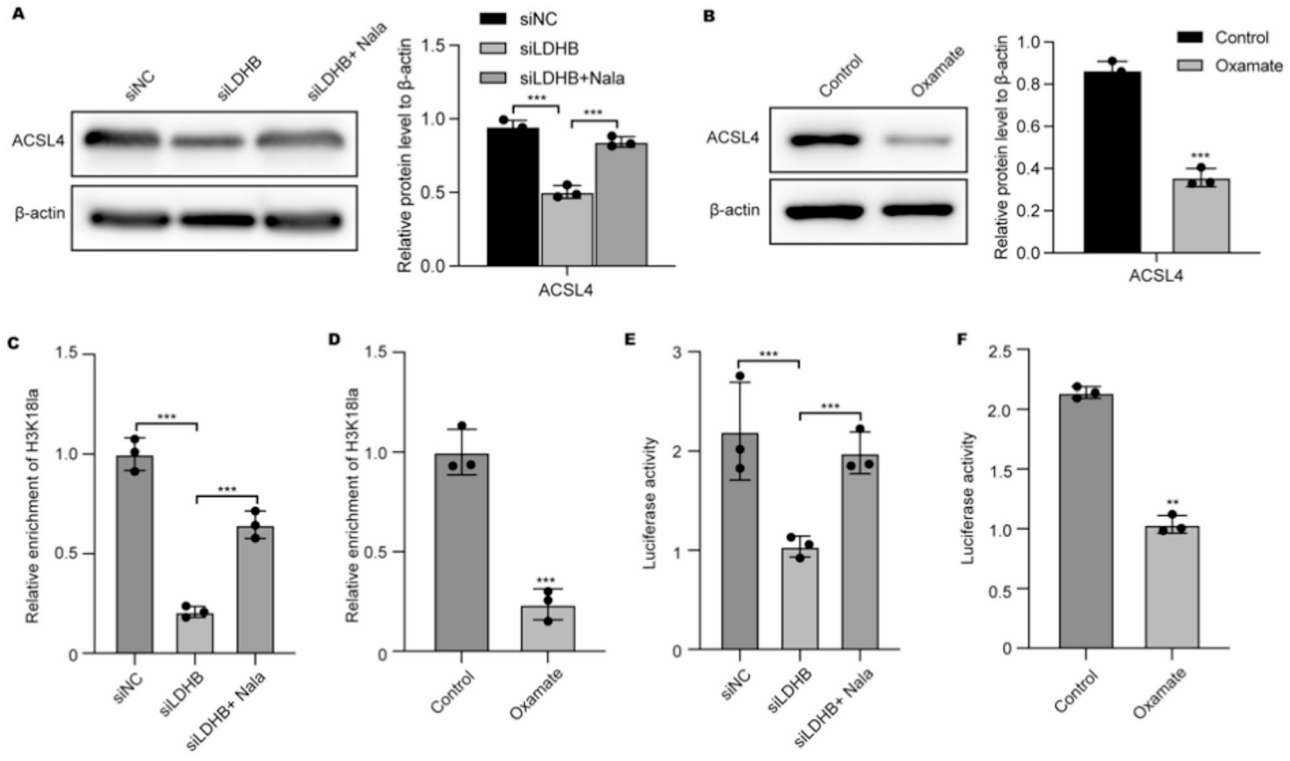

Fig. 4 LDHB epigenetically enhances ACSL4 expression by histone H3K18 lactylation in chondrocytes. (**A** and **B**) Protein expression of ACSL4 in chondrocytes. (**C** and **D**) The enrichment of H3K18 lactylation at ACSL4 promoter was measured by ChIP experiment. (E and F) The activity of ACSL4 promoter was detected by luciferase reporter gene assay. $^{**}p < 0.01$, $^{***}p < 0.001$

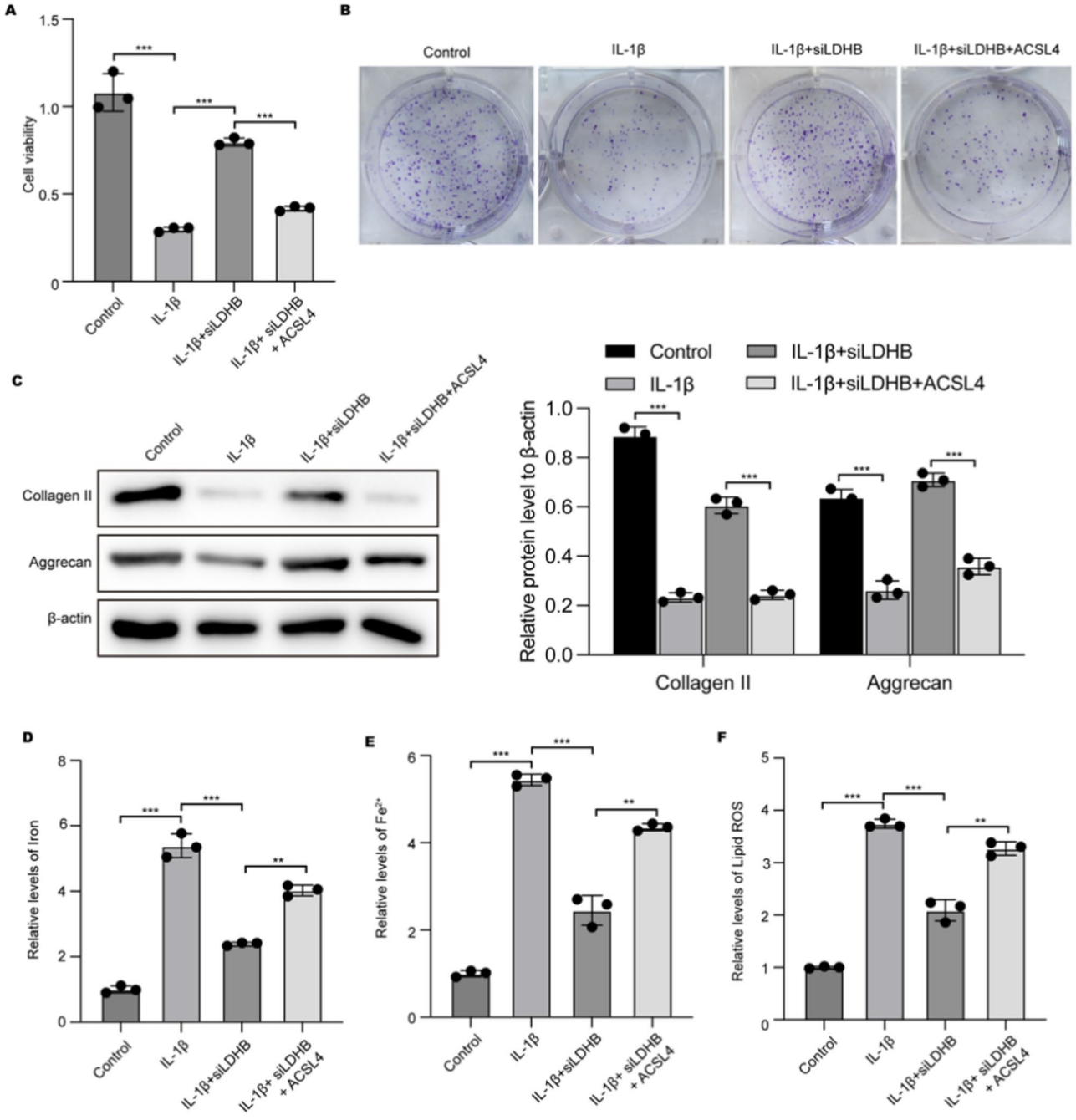

Fig. 5 LDHB induces the ferroptosis of chondrocytes via ACSL4. IL-1β-induced chondrocytes were treated with siLDHB and ACSL4 overexpression. (**A**) Cell viability was measured by CCK-8. (**B**) Cell proliferation was detected by colony formation. (**C**) The protein levels of Collage type II and Aggrecan in chondrocytes. (**D**-**F**) The levels of total iron, Fe2 + and lipid ROS in chondrocytes. $^{**}p < 0.01$, $^{***}p < 0.001$

LDHB affects OA progression through ACSL4 expression regulated by lactation

Subsequently, we validated the function of LDHB-mediated lactate modification in an animal model of OA. We inhibited LDHB function with siLDHB and OXA and compensated with ACSL4 overexpression. As shown in Fig. 7A, in the OA mouse model, siLDHB and OXA treatments inhibited ACSL4 expression levels, while overexpression of ACSL4 restored its protein levels. siLDHB and OXA treatments also mitigated matrix degradation in OA mice' cartilage tissue (Fig. 7B), inhibited serum CTX-II and COMP levels (Fig. 7C and D), and reduced expression of Collagen II and Aggrecan (Fig. 7E). However, overexpression of ACSL4 significantly reversed these conditions. In addition, siLDHB and OXA treatment down-regulate the level of total iron ion, Fe2, and

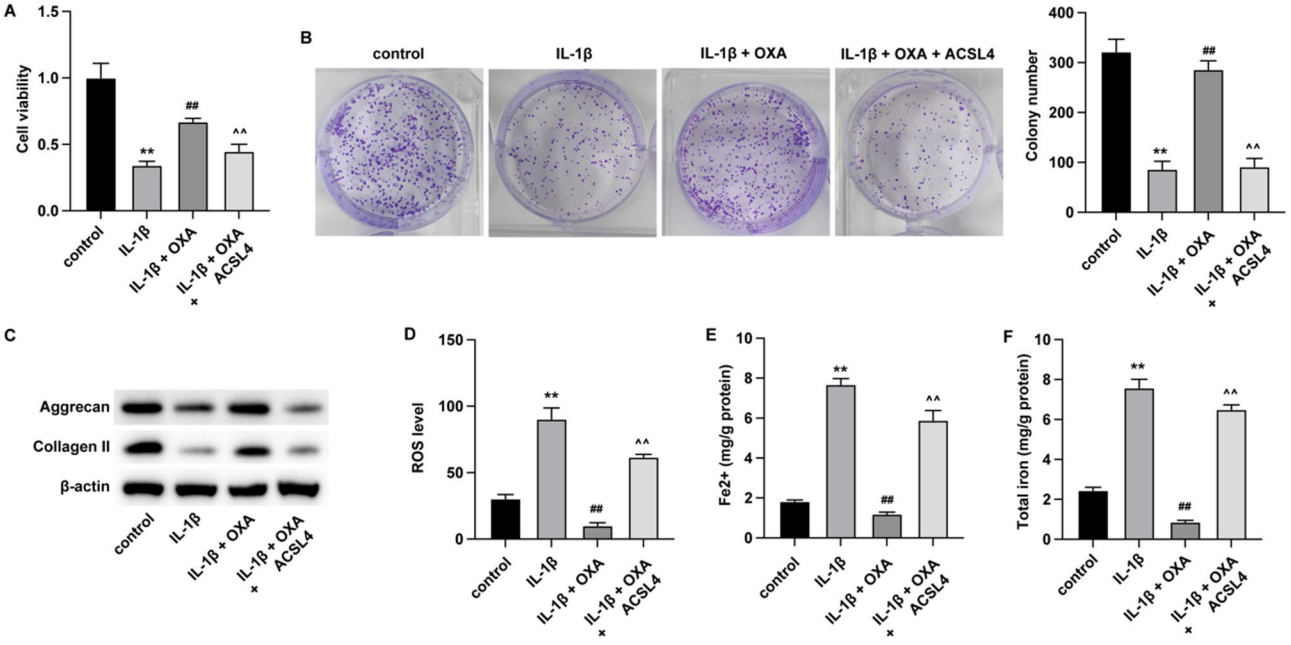

Fig. 6 LDHB affects chondrocytes ferroptosis through ACSL4 expression regulated by lactation. Chondrocytes were induced by IL-1β and treated with siLDHB and Nala. (**A**) Cell viability was measured by CCK-8. (**B**) Cell proliferation was detected by colony formation. (**C**) The protein levels of Collage type II and Aggrecan in chondrocytes. (D-F) The levels of total iron, Fe2 + and lipid ROS in chondrocytes. $^{**}p < 0.01$, $^{***}p < 0.001$

lipid ROS in the joints of OA mice (Fig. 7F and H). Overexpression of ACSL4 can restore the level of these markers of iron death (Fig. 7F and H). These data suggest that LDHB may regulate ACSL4 expression by regulating lactation, thereby inhibiting iron death and alleviating OA progression.

Discussion

Osteoarthritis (OA) is a representative degenerative joint disease, and the major cause of chronic disability in the elderly population, which heavily burdens the society [21]. The corresponding medical cares primarily pay attention to the alleviation of pain symptoms while are incapable of preventing the disease progression [21]. Our study established in vitro and in vivo models and identified that LDHB expression and H3K18 lactylation were notably enhanced in the cartilage tissue of OA mice, and knockdown of LDHB effectively alleviated OA progression. Pathologically, cartilage degeneration is an obvious feature of progressive OA [22].

Evidence suggests that the balance of chondrocyte synthetic metabolism/degradative metabolism and survival crucially impacts articular cartilage homeostasis and OA pathogenesis, emphasizing the importance of controlling chondrocyte fate [23]. Here, according to the results from in vitro and in vivo models, ferroptosis is closely correlated with OA. Consistently, increasing studies have indicated the ferroptosis of chondrocytes as a promising target for OA treatment. According to human cartilage and mouse chondrocyte experiments, mechanical overloading is capable of triggering GPX4-associated ferroptosis. Besides, calcium influx blockage relying on a calcium-free medium greatly eliminated the ferroptosis damage in chondrocytes induced by high-strain mechanical stress [24]. The interleukin-1β (IL-1β)-induced in vitro model and in vivo anterior cruciate ligament transection (ACLT)-induced OA mouse model confirmed that D-mannose could protect chondrocytes through making chondrocytes less sensitive to ferroptosis and inhibiting OA progression, during which HIF-2α crucially mediated the resistance of chondrocytes to ferroptosis induced by D-mannose [25].

LDHB is the critical enzyme for lactate metabolism, hence is probably correlated with histone lactylation [26, 27]. Although the biological significance of H3K18 lactylation is gradually being recognized in cell biology, its specific functions in different biological processes may vary depending on the cell type and physiological state [28–30]. Researching the biological implications of H3K18 lactylation is potentially important for understanding chromatin dynamics, gene regulation, and the development of related diseases. In current study, we observed that LDHB epigenetically modulated the histone lactylation of ACSL4, which mediated the ferroptosis of chondrocytes during OA. In consistent, various studies have revealed the involvement of ACSL4 in ferroptosis-related diseases. For example, targeting ACSL4 significantly inhibited ferroptosis and suppressed the pathological and functional injury of acute kidney injury mice [31]. ACSL4 enhanced lipid peroxidation to

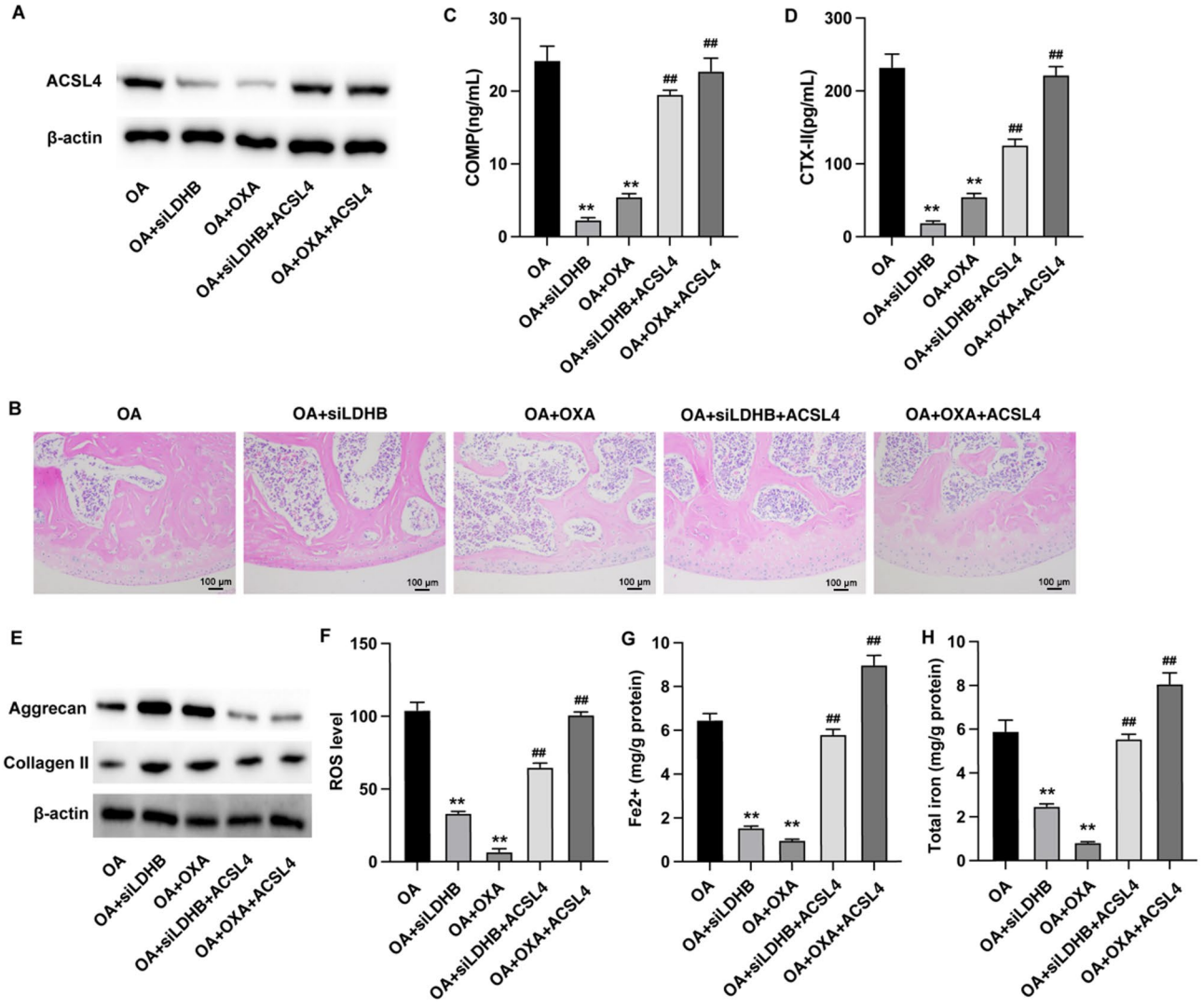

Fig. 7 LDHB affects OA progression through ACSL4 expression regulated by lactation. OA model was established and treated with siLDHB, OXA and ACSL4 overexpression. (**A**) The protein level of ACSL4. (**B**) HE staining of cartilage tissues. (**C** and **D**) The serum levels of CTX-II and COMP. (**E**) The protein levels of Collage II and Aggrecan. (**F**-**H**) The levels of total iron, Fe2 + and lipid ROS in cartilage tissues. $^{**}p < 0.01$, $^{***}p < 0.001$

facilitate neuronal death, and consequently induced neuroinflammation and neuronal death in ischemic stroke [32]. Nevertheless, other potential targets of LDHB-regulation epigenetic modification may be involved in ferroptosis and other potential pathogenesis of OA. Hence, the specific role of LDHB in OA shall be ascertained in future studies.

While our study elucidated the role of LDHB-mediated histone lactylation in chondrocyte ferroptosis and OA progression, certain limitations should be noted. First, proteoglycan content in cartilage was assessed via Aggrecan protein expression and serum biomarkers (COMP/CTX-II), but histochemical staining methods such as Safranin O or Toluidine Blue, which directly visualize proteoglycan distribution, were not employed. Future studies incorporating these techniques could provide additional morphological validation of cartilage degeneration. Second, the precise molecular mechanism linking histone lactylation to ACSL4 transcriptional activation requires further exploration. Nevertheless, our findings establish a foundational link between lactate metabolism, epigenetic regulation, and ferroptosis in OA pathogenesis.

Conclusion

To summarize, we identified that LDHB expression and histone H3K18 lactylation were elevated in OA, and knockdown of LDHB could alleviate OA in vitro and in vivo. Mechanistically, LDHB epigenetically regulated the histone H3K18 lactylation and expression of ACSL4 and consequently induced chondrocyte ferroptosis to promote OA progression.

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Author contributions
Yang Zhang and Chen-Yu Zhao designed the research study. Zheng Zhou and Cheng-Cun Li performed the research. Qiang Wang conducted experiments, analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The protocol was approved by the ethics committee of Dalian Medical University. Informed consent was obtained from all study participants. All the methods were carried out in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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