

Reg3 β promotes chondrocyte proliferation and ECM metabolism during acetabular roof remodeling in a rat model of DDH-induced residual dysplasia

PENG WANG¹⁻³, MENGXIA LI^{2,3}, WENXUAN WANG^{2,3}, WEIGUO SUN^{2,3}, WEN ZHEN^{2,3},
QIAN SUN^{2,3}, BO NING⁴, GUANGHAO SU^{2,3} and XIAODONG WANG^{2,3}

¹Department of Pediatric Surgery, Jiaying Women and Children's Hospital Affiliated with Wenzhou Medical University, Jiaying, Zhejiang 314000, P.R. China; ²Pediatrics Research Institute, Children's Hospital of Soochow University, Suzhou, Jiangsu 215024, P.R. China; ³Department of Pediatric Orthopedics, Children's Hospital of Soochow University, Suzhou, Jiangsu 215024, P.R. China; ⁴Department of Pediatric Orthopedics, National Children's Medical Center, Children's Hospital of Fudan University, Shanghai 201100, P.R. China

Received June 14, 2024; Accepted February 6, 2025

DOI: 10.3892/mmr.2025.13653

Abstract. Residual acetabular dysplasia (RAD) is a common complication after the successful management of developmental dysplasia of the hip (DDH). RAD remodeling is important for predicting the outcome of the affected hip, and optimal treatment can be chosen accordingly. Regenerating islet-derived protein 3- β (Reg3 β), a multifaceted cytokine, is a prognostic marker for inflammation and cardiac disease. Nevertheless, the roles of Reg3 β in RAD remain unclear. Consequently, the aim of the present study was to assess the role of Reg3 β in RAD and explore its related functions in chondrocytes *in vitro*. First, remodeling of the affected hip after fixation removal was observed in a neonatal rat DDH model, which simulated the process of RAD. Reg3 β expression in RAD was upregulated at weeks 1, 2 and 4, as determined by western blot analysis. The serum concentration of Reg3 β was greater than that of normal rats at 2 weeks and returned to normal levels at 4 weeks. Subsequently, it was found that Reg3 β promoted cell proliferation and extracellular matrix (ECM) metabolism via the Jak2/Stat3/Socs3 signaling pathway through gene knockdown and addition of recombinant Reg3 β protein. These findings suggest that Reg3 β is a

novel potent prognostic biomarker for the remodeling of RAD via regulation of chondrocyte proliferation and metabolism of the ECM.

Introduction

Early ultrasound screening can lead to favorable management of developmental dysplasia of the hip (DDH), one of the most common deformities in children (1-3). However, several complications, such as residual acetabular dysplasia (RAD) and avascular necrosis of the femoral head, are still challenging for pediatric orthopedists, and the underlying mechanism remains unclear (4-6).

To date, imaging morphological methods, such as the acetabulum index, center-edge angle Reimers index and center-head distance discrepancy, are commonly used for the identification of RAD (7,8). However, these parameters only indicate the existing condition of the affected hip and do not reveal the current state of cartilage at the molecular level. Although several risk factors affect the development of the affected hip and are used to predict the outcome of RAD (9,10), the optimal time for secondary surgery remains a challenge (11). Given this situation, several studies have focused on the application of magnetic resonance imaging (MRI) due to its advantages in cartilage imaging (12-16). Articular coverage is presumed to have greater sensitivity in identifying the condition of cartilage (17-21), yet the molecular function of the affected cartilage, which can be impacted prior to the presence of morphological changes, remains unclear. Accordingly, the identification of biomarkers to reflect the biological function of chondrocytes and to predict the outcome of RAD is urgently needed.

In addition to the MRI results of clinical patients, the pathological findings of animal experimental studies have shown that remodeling changes are associated with the thickness of the acetabular roof (11,22-24). Moreover, the remodeling potential is closely related to the risk of residual hip dysplasia. This evidence contributed to the hypothesis that

Correspondence to: Dr Guanghao Su, Pediatrics Research Institute, Children's Hospital of Soochow University, 92 Zhongnan Street, Suzhou, Jiangsu 215024, P.R. China
E-mail: sky2skeleton@163.com

Professor Xiaodong Wang, Department of Pediatric Orthopedics, Children's Hospital of Soochow University, 92 Zhongnan Street, Suzhou, Jiangsu 215024, P.R. China
E-mail: wangxd@suda.edu.cn

Key words: regenerating islet-derived protein 3 β , residual dysplasia of the hip, proliferation, extracellular matrix metabolism, anti-apoptosis, chondrocytes

the pathogenesis of DDH may be caused by delayed endochondral ossification of the acetabular roof (11). However, this hypothesis is limited. Cartilage is composed of corresponding chondrocytes, which form a complex and precise network to balance the internal environment for cartilage developmental remodeling, such as proliferation or apoptosis, along with anabolism and catabolism. Abnormal remodeling can occur if this balance is disrupted at any point (25).

A high-throughput microarray study revealed that regenerating islet-derived protein 3- β (Reg3 β) was significantly upregulated in a DDH rat model (26). Recent studies have shown the potential of growth factors, cytokines and chemokines for tissue repair and regeneration of the liver and neurons (27-29). Moreover, this molecule was also shown to promote the repair of the infarcted myocardium in rats and improve the prognosis of patients with myocardial infarction (30), indicating broad potential for clinical application. However, it is unclear whether Reg3 β is expressed in articular cartilage and affects the remodeling potential and prognosis of patients with RAD. Thus, in the present study, nearly a year was needed to complete the experiment (from January 2022 to February 2023), including establishing a RAD rat model with fixation removal from a 7-day-fixation neonatal rat DDH experimental model, and the expression of Reg3 β in the acetabular roof and serum was determined by western blot (WB) analysis and enzyme-linked immunosorbent assays (ELISAs), respectively, while its function in chondrocytes *in vitro* and the underlying mechanism involved were also investigated.

Materials and methods

Animals and the DDH model. All animal experiments were performed according to the National Research Council's Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Ethics Committee of Jiaxing Women and Children's Hospital Affiliated with Wenzhou Medical University (approval no. 2023-016; Jiaxing, China) and the Ethics Committee of Soochow University (approval no. SUDA20211130A16; Suzhou, China). Newborn Sprague-Dawley rats, purchased from Zhaoyan (Suzhou) New Drug Research Center Co., Ltd., were randomly divided into CON (control) and DDH groups. Each group was evenly distributed by sex. DDH models were generated by fixing the hind limbs via hip adduction and extension as well as knee extension with a medical adhesive tape (3M) to simulate the swaddling position of humans. The tape was released for motion recovery 1 h per day. The RAD model was established by fixation removal as a preplanning time. The housing conditions, including room temperature maintained at 26°C along with a relative humidity of 45-65% and a 12-h light/dark cycle, met the requirements of SPF grade. Both the DDH model rats and control normal rats were fed freely by their mothers together in the same cage. Animal health and behavior were monitored every day. The specific criterion used to determine when the animals should be euthanized was the corresponding age of human developmental stage: i) Toddler (2 weeks) and ii) child (4 weeks). These selected time points are valuable for predicting the outcome of acetabular remodeling. A total of 150 newborn

rats were used in the present study, including 100 for the evaluation of the dislocation ratio, 12 for gross morphology and Reg3 β expression on different fixation days at 2 weeks by WB analysis, and 38 for Reg3 β expression by WB analysis and ELISA from 1 week to 4 weeks after removal. However, during the WB analysis and ELISA procedures, 1 rat each from the CON and DDH groups at 1 week and one rat in the DDH group at 4 weeks succumbed unintentionally, with no obvious signs of infection. Moreover, a total of 10, 2-week-old rats were sacrificed for primary cellular culture and subsequent experiments. Finally, all the rats used in the experiments were euthanized with pentobarbital sodium to minimize suffering and distress at a dose of 200 mg/kg, and the hip was dissected in such a manner that the acetabulum and femoral head were completely isolated for evaluation and subsequent experiments.

Primary chondrocyte culture. The primary cell study protocol was approved by the Ethics Committee of Soochow University (approval no. SUDA20211130A16; Suzhou, China). The 2-week-old rats were sacrificed by overdose anesthesia, and the cartilage from the acetabular roof was dissected after disinfection with 75% ethanol under a clean bench, followed by washing with PBS solution three times. The cartilage was cut into 1-mm³ pieces with scissors and digested with 0.25% trypsin-EDTA for 30 min and 0.2% collagenase II (Worthington Biochemical Corporation) for 3 h at 37°C in a 5% CO₂ incubator. The digestion was terminated with fresh DMEM (BasalMedia) supplemented with 12% fetal bovine serum (FBS) (Dongling) and 1% penicillin-streptomycin (Beyotime). The tissues were passed through a 100- μ m molecular filter, and the resulting cell suspension was centrifuged at 300 g for 5 min at room temperature. The resulting pellet was subsequently resuspended in culture medium and seeded into a 25-cm² vented cap flask with a final cell density of 5x10⁶ cells/ml at 37°C with 5% CO₂ overnight. The medium was replaced with fresh DMEM every 2 days. The cells were then identified via Alcian blue staining and Col2a1 immunostaining. Following fixation with 4% paraformaldehyde for 30 min and permeabilization with 0.2% Triton X-100 for 15 min at room temperature, the samples were either stained with 1% Alcian blue 8GX (cat. no. 801642-25, Macklin) for 2 h at room temperature for Alcian blue staining or processed for Col2a1 immunostaining by blocking with 1% bovine serum albumin (BSA) for 30 min, followed by incubation with a primary antibody against Col2a1 (1:250, cat. no. 28459-1-AP, Proteintech) overnight at 4°C, incubation with a secondary antibody conjugated to Cy3 (1:500, cat. no. abs20024, Absin) for 1 h at room temperature, and nuclear counterstaining with DAPI (cat. no. abs47047616, Absin). Passage 3 chondrocytes were used for subsequent studies.

WB analysis. Total protein from cartilage or chondrocytes was extracted with RIPA lysis buffer (Beyotime) supplemented with phenylmethanesulfonyl fluoride and a phosphatase cocktail. After being shaken for 5 min, the samples were incubated on ice for 30 min, followed by centrifugation at 13,500 g and 4°C for 20 min. The supernatant accompanied by the discarded precipitate was removed for concentration

Table I. List of primary and secondary antibodies.

A, Primary antibodies				
Antibody	Source	Company	Catalogue no.	Concentration
Reg3 β	Mouse	R&D Systems, Inc.	AF1996	1:500
Aggrecan	Rabbit	Abbkine Scientific Co., Ltd.	ABP54013	1:1,000
Col2a1	Rabbit	Proteintech Group, Inc.	28459-1-AP	1:1,000
Adams-5	Rabbit	Abcam	ab41037	1:1,000
Mmp13	Rabbit	Affinity Biosciences	AF5355	1:1,000
Cleaved caspase-3	Rabbit	Proteintech Group, Inc.	25128-1-AP-	1:1,000
Caspase-3	Rabbit	Affinity Biosciences	AF6311	1:1,000
Caspase-9	Rabbit	Proteintech Group, Inc.	10380-1-AP	1:1,000
Bax	Rabbit	Proteintech Group, Inc.	50599-2-Ig	1:1,000
Bcl-2	Rabbit	Proteintech Group, Inc.	60178-1-Ig	1:1,000
Jak2	Rabbit	Affinity Biosciences	AF6022	1:1,000
pJak2	Rabbit	Affinity Biosciences	AF3024	1:1,000
Stat3	Mouse	Affinity Biosciences	BF0354	1:1,000
pStat3	Rabbit	Affinity Biosciences	AF3293	1:1,000
Socs3	Rabbit	Proteintech Group, Inc.	14025-1-AP	1:1,000
Extl3	Rabbit	Abmart Pharmaceutical Technology (Shanghai) Co., Ltd.	JQ007883	1:1,000
GAPDH	Mouse	Abcam	ab8245	1:5,000
α -Tubulin	Rabbit	Proteintech Group, Inc.	11224-1-AP	1:2,500
B, Secondary antibodies				
Antibody	Source	Company	Catalogue no.	Concentration
Anti-Mouse IgG (HRP)	Goat	Abcam	ab6789	1:10,000
Anti-Rabbit IgG (HRP)	Goat	Abcam	ab7090	1:10,000

Reg3b, regenerating islet-derived protein 3-b; Col2a1, collagen type II alpha 1 chain; Adams-5, ADAM metalloproteinase with thrombospondin type 1 motif 5; Mmp13, matrix metalloproteinase 13; Socs3, suppressor of cytokine signaling 3; Extl3, exostosin like glycosyltransferase 3; HRP, horseradish peroxidase.

measurement by OneDrop1000plus Spectrophotometer (Service Card). RIPA buffer was used to balance the concentrations of all the samples. Finally, the samples were mixed with SDS 6X loading buffer and boiled at 95°C for 5 min. The samples (10 μ g loaded per lane) were separated via 12.5% SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% BSA (cat. no. B2064, Sigma-Aldrich) for 2 h at room temperature, followed by incubation with a primary antibody at 4°C overnight. The membranes were subsequently incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Finally, the target bands were visualized by an Enhanced Chemiluminescence Detection Kit (cat. no. 32209, Thermo Fisher Scientific) and detected via AI600 imaging (GE Healthcare) and analyzed via ImageJ analysis software (version 1.46r, National Institutes of Health). The primary and secondary antibodies used are listed in Table I.

ELISA. The serum concentration of Reg3 β was determined with a commercially available ELISA kit cat. no. MB-7526A, Meibiao Biotechnology Co., Ltd) according to the manufacturer's instructions. The absorbance at 450 nm was determined with a microplate reader (Infinite M200; Tecan Group, Ltd.).

Cell Counting Kit-8 (CCK-8) assay. Following the manufacturer's instructions, the CCK-8 assay was used to evaluate the cell proliferation rates. The 96-well plates were seeded with cells at a density of 1×10^4 cells/well for the Reg3 β knockdown experiment and at a density of 2×10^3 cells/well for the addition of recombinant Reg3 β protein. For the gene knockdown experiments, CCK-8 was added after virus transfection for 48 h at 37°C; for the activation of recombinant Reg3 β , CCK-8 was added after the chondrocytes were incubated for 24 h at 37°C. CCK-8 reagent (10 μ l; cat. no. U10014A, UUBIO) was added to the culture for 2 h at 37°C. An auto-microplate reader

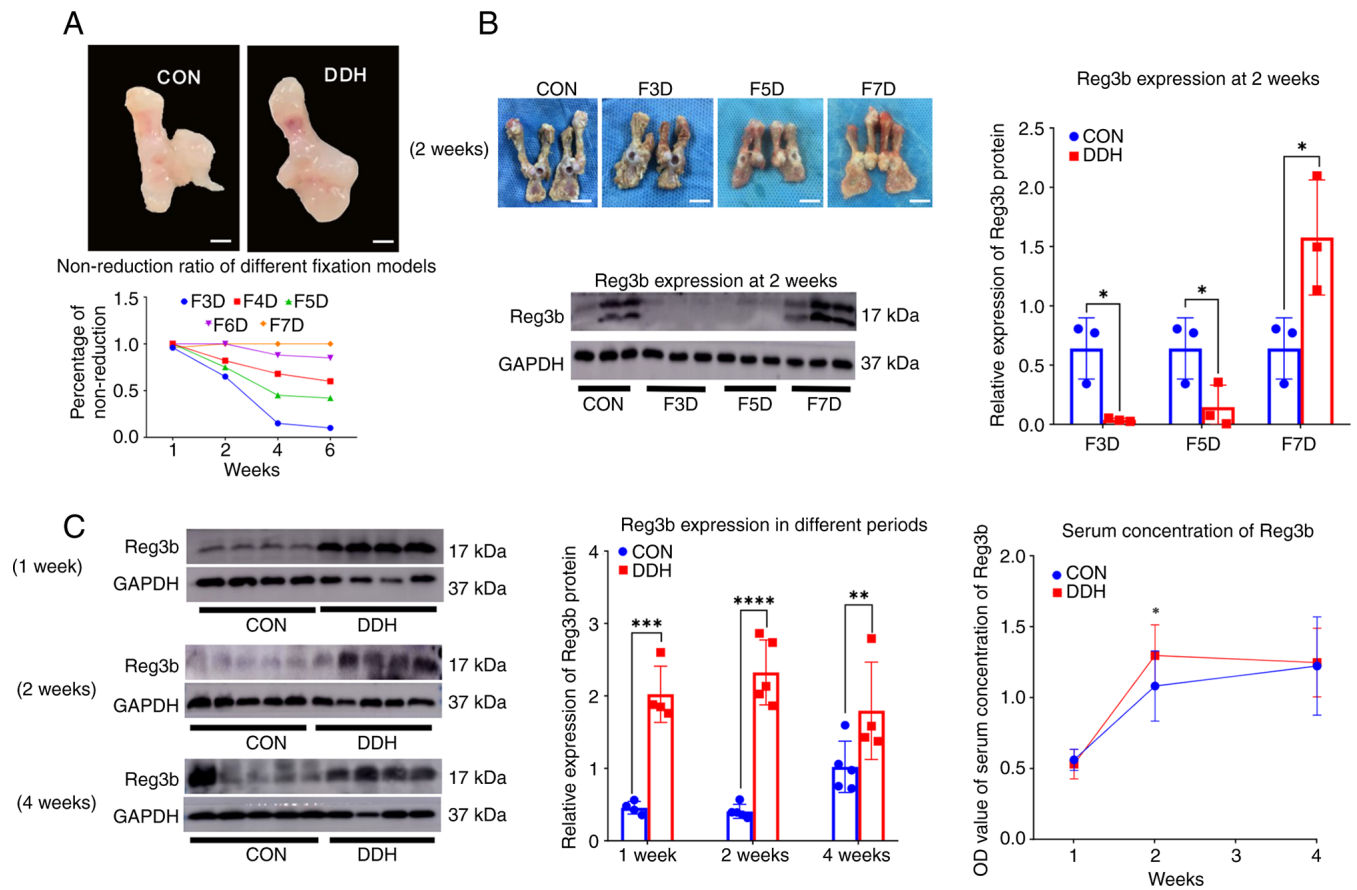


Figure 1. Morphology of a hip with residual dysplasia and the expression of Reg3 β . (A) Success of the DDH model after 2 days of fixation (scale bar, 1 mm) and percentage of failed reduction of the affected hip after removal of fixation (n=20 in each group). (B) Dislocated hip was reduced at 2 weeks with F3D and F5D fixation. By contrast, the affected hip was unreducible along with the thickness of the acetabular roof after F7D, which is consistent with the morphology of RAD (scale bar, 1 cm). In addition, Reg3 β in the acetabular roof was expressed at a higher level in the 7-day fixation group than in the 3-day and 5-day fixation groups and was expressed at lower levels in the 3-day and 5-day fixation groups than in the control group (n=3 in each group). (C) Reg3 β expression was greater in the 7-day fixation group than in the normal group at weeks 1, 2 and 4, and Reg3 β expression was transiently upregulated in the serum at week 2 but returned to the normal level at week 4. Student's t-tests were used to compare the two groups between each time point. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Reg3 β , regenerating islet-derived protein 3- β ; DDH, developmental dysplasia of the hip; F3D, 3-day fixation; F5D, 5-day fixation; F7D, 7-day fixation; RAD, residual acetabular dysplasia; CON, control.

(Infinite M200; Tecan Group, Ltd.) was used to measure the optical density at 450 nm.

Viral transfection and drug treatment in vitro. A third-generation lentiviral vector containing Reg3 β short hairpin RNA was designed using a vector from IGE Bio Biotech. 293T cells (IGEBio Biotech, China) were used for lentivirus packaging. When the 293T cells reached 80% confluence, the medium was changed to serum-free Opti-MEM (BasalMedia) containing a mixture of helper and shuttle plasmids. A total of 5 μ g of lentiviral plasmid was used for transfection, following the protocol for Lipofectamine 2000 (cat. no. 11668-019, Thermo Fisher Scientific). The ratio of lentivirus, packaging, and envelope plasmids was 4:3:2. After 48 h of co-culture at 37°C in a 5% CO₂ incubator, the viral-containing supernatant was harvested and concentrated for transfection into primary chondrocytes and subsequent experimental use. The viral titer was determined using the 50% tissue culture infective dose (TCID₅₀) method. The optimal multiplicity of infection (MOI) was identified by determining the vector concentration (MOI=5). After 24 h of transduction at 37°C in a 5% CO₂ incubator, the viral supernatant was replaced

with complete culture medium containing puromycin (1:100, cat. no. A1113803, Thermo Fisher Scientific). Subsequent experiments were conducted 48 h post-transduction. The chondrocytes were randomly divided into the negative control (NC) group and the S1, S2 and S3 groups. The sequences of all the shRNA sequences, including those of the negative control, were as follows: S1 forward, 5'-TTGCCCTACGTCTGCAAA TTT-3' and reverse, 5'-AAATTTGCAGACGTAGGGCAA-3'; S2 forward, 5'-CTGGAAACAGCTACCAATATA-3' and reverse, 5'-TATATTGGTAGCTGTTTCCAG-3'; S3 forward, 5'-TGGAGTAACAATGACATAATG-3' and reverse, 5'-CAT TATGTCATTGTTACTCCA-3'; and NC forward, 5'-CCT AAGGTTAAGTCGCCCT-3' and reverse, 5'-CGAGGG CGACTTAACCTTAGG-3'. The most effective series was determined via WB analysis. The optimal concentration of recombinant Reg3b protein (cat.no.8288-RG, RD SYSTEMS) was determined via CCK-8 assays.

Colony forming assay. After 24 h, the transfected chondrocytes were digested and replated in 6-well plates at a density of 2x10² cells/well for 1 week. Subsequently, the chondrocytes were fixed with 4% paraformaldehyde for 30 min at room

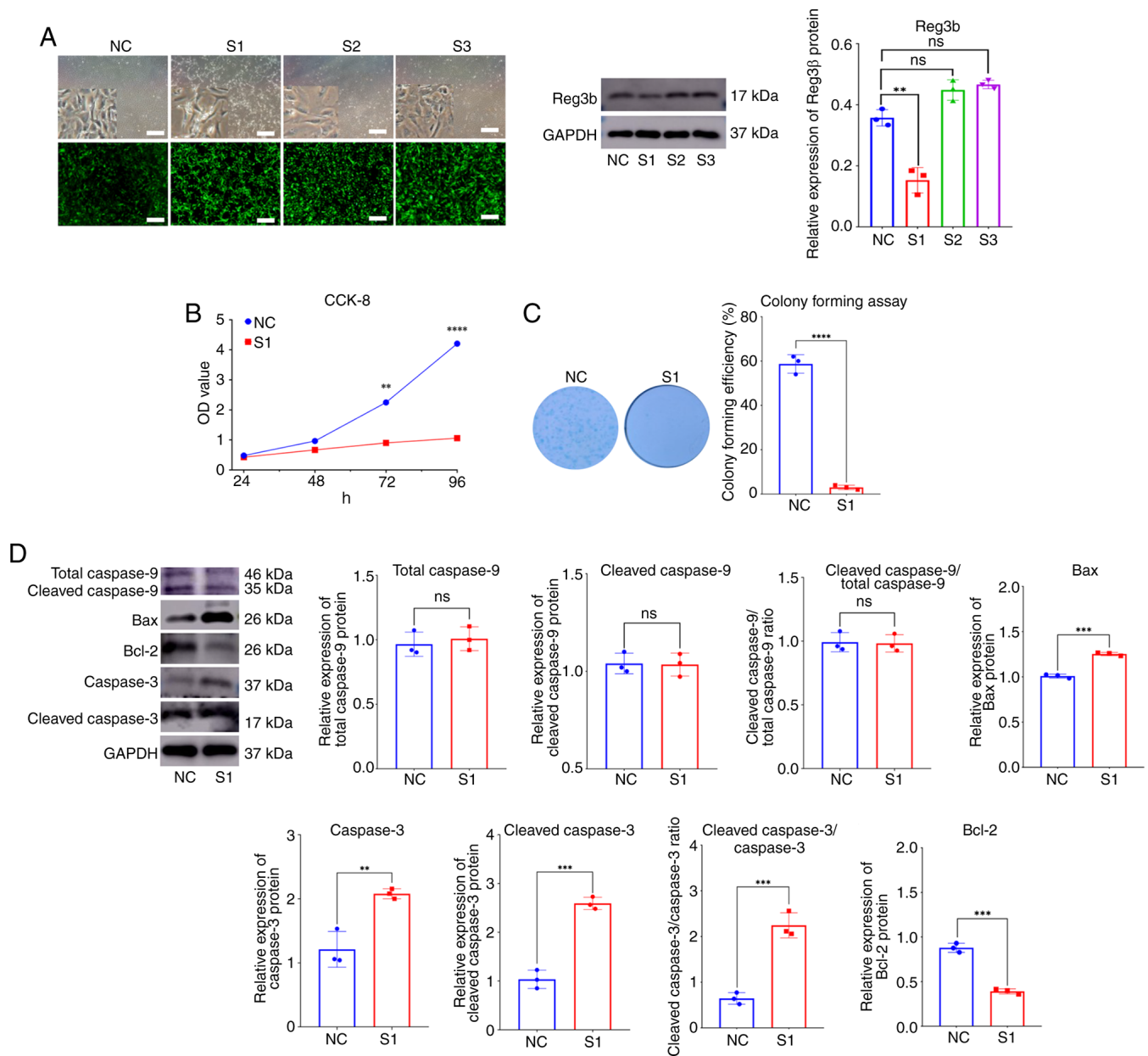


Figure 2. Western blot analysis of Reg3 β expression, proliferation and apoptosis after Reg3 β knockdown. (A) Clear cell death was observed with the S1 sequence, which exhibited the most effective knockdown compared with the other two sequences. One-way analysis of variance was used to compare these groups followed by Dunnett's post hoc test. Scale bar, 100 μ m. (B) CCK-8 assay revealed that the proliferation of chondrocytes was inhibited after 72 h. (C) A colony formation assay with Alcian blue staining revealed that few cells remained after 1 week of S1 infection. (D) Cleaved caspase-3 and caspase-3, along with Bax, were increased after Reg3 β knockdown at 72 h, whereas the level of Bcl-2 was significantly decreased, and the level of caspase-9 remained essentially unchanged. Student's t-tests were conducted to assess the statistical difference between the two groups in Fig. 2B-D. ** $P < 0.01$ and *** $P < 0.001$. Reg3 β , regenerating islet-derived protein 3- β ; CCK-8, Cell Counting Kit-8; NC, negative control; OD, optical density.

temperature and stained with Alcian blue for 2 h at room temperature. The colony formation efficiency was used to determine the proliferation of the test cells, and clones were counted manually (>50 cells, ranging in size from 0.3 to 1.0 mm).

Statistical analysis. The data are presented as the mean $\pm$ SD), and GraphPad Prism 8.0 software (GraphPad; Dotmatics) was used for statistical analysis. At least three technical and biological replicates were used for experiments. To compare 3 or more groups, a one-way analysis of variance followed by Dunnett's post hoc test was employed. For comparisons

between two groups, an unpaired t-test was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Gross morphology of residual dysplasia of the hip, and the expression of Reg3 β is increased after removal of fixation. After 2 days of fixation, the hip samples from the sacrificed rats were dislocated, with an apparent shadow acetabulum and increased labrum thickness (Fig. 1A). Moreover, the longer the fixation continued, the greater the failure of reduction of the dislocated hip. After 7 days of fixation, the dislocated hip

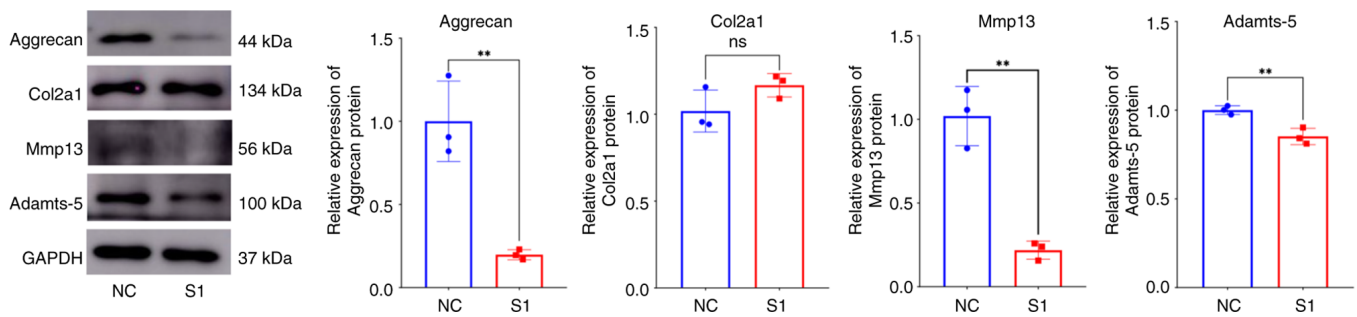


Figure 3. Anabolic and catabolic changes after Reg3 β knockdown. Aggrecan, Mmp13 and Adamts-5 were downregulated, whereas Col2a1 exhibited no obvious change. Student's t-tests were performed to evaluate the statistical difference between the two groups. **P<0.01. Reg3 β , regenerating islet-derived protein 3- β ; NC, negative control; ns, not significant; Mmp13, matrix metalloproteinase 13; Col2a1, collagen type II alpha 1 chain; Adamts-5, ADAM metallopeptidase with thrombospondin type 1 motif 5.

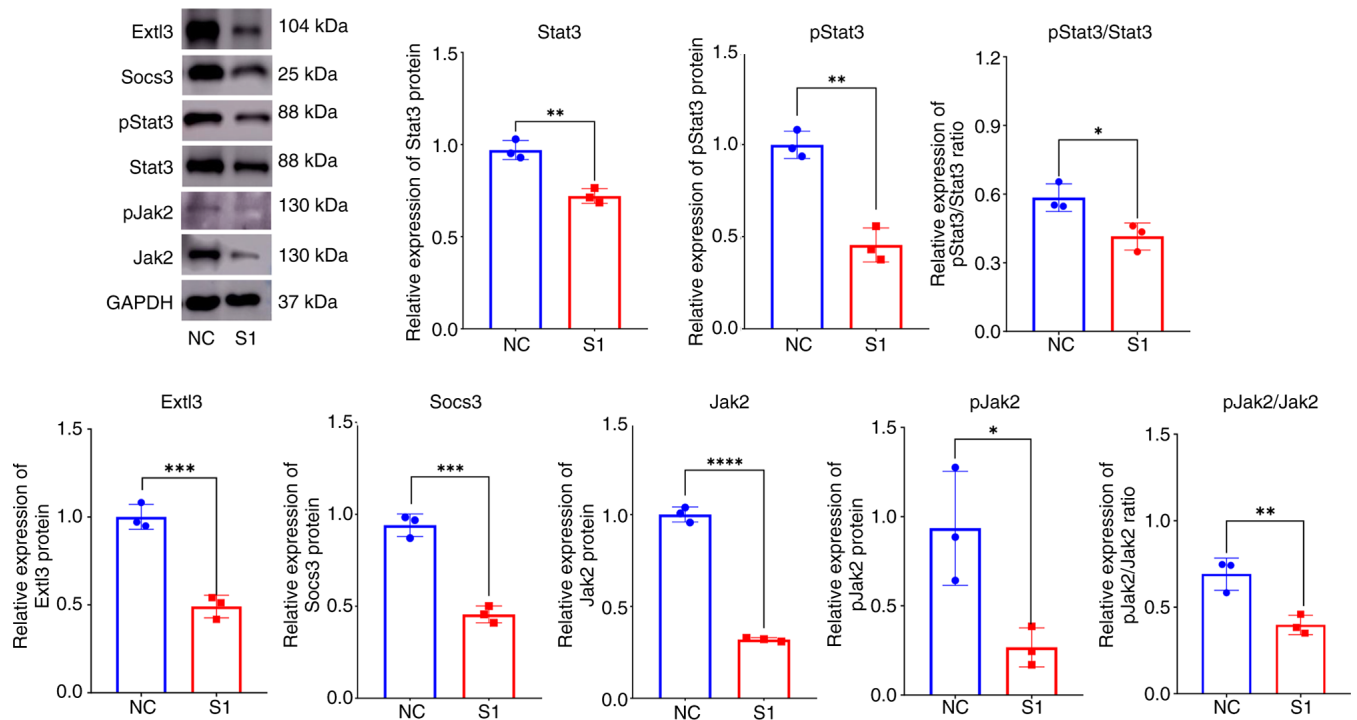


Figure 4. Jak2/Stat3/Socs3 signaling pathway is inhibited after Reg3 β knockdown. Jak2/Stat3 and the corresponding phosphorylated proteins, as well as Extl3 and Socs3, were downregulated. Student's t-tests were performed to evaluate the statistical difference between the two groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Reg3 β , regenerating islet-derived protein 3- β ; Extl3, exostosin like glycosyltransferase 3; Socs3, suppressor of cytokine signaling 3; NC, negative control.

seldom showed a reduction compared with that after 3 days of fixation and 5 days of fixation (Fig. 1B). At 2 weeks, Reg3 β was highly expressed in the acetabular roof after 7 days of fixation, whereas expression of Reg3 β was barely detected after fixation for 3 or 5 days (Fig. 1B). Thus, 7 days of fixation was selected for further RAD experiments. With the removal of fixation, the expression of Reg3 β in the acetabular roof of the RAD group was increased in the 1, 2 and 4-week observation periods, and the serum concentration of Reg3 β transiently increased at week 2 and later returned to a normal level at week 4 (Fig. 1C).

Reg3 β knockdown increases apoptosis and inhibits proliferation in primary chondrocytes. Previous results (Fig. 1) revealed high Reg3 β expression in acetabular cartilage after fixation removal. The next objective was to explore the role of

Reg3 β in chondrocytes. A lentiviral vector containing three Reg3 β shRNA sequences was constructed for the transfection of primary chondrocytes (Fig. 2A). The chondrocytes showed obvious cell death with the addition of the S1 sequence, which was identified as the most effective knockdown sequence via WB analysis (Fig. 2A). The S1 sequence was used for subsequent experiments. First, it was investigated whether Reg3 β influences the proliferative capability of chondrocytes. CCK-8 cell proliferation was evidently inhibited after 72 h of transfection (Fig. 2B). Moreover, the colony formation assay results revealed that few colonies formed after 1 week of transfection (Fig. 2C). To confirm the effect of Reg3 β knockdown on chondrocyte apoptosis, the expression levels of typical apoptotic proteins were measured via WB analyses. The results revealed that the expression of caspase-3 and

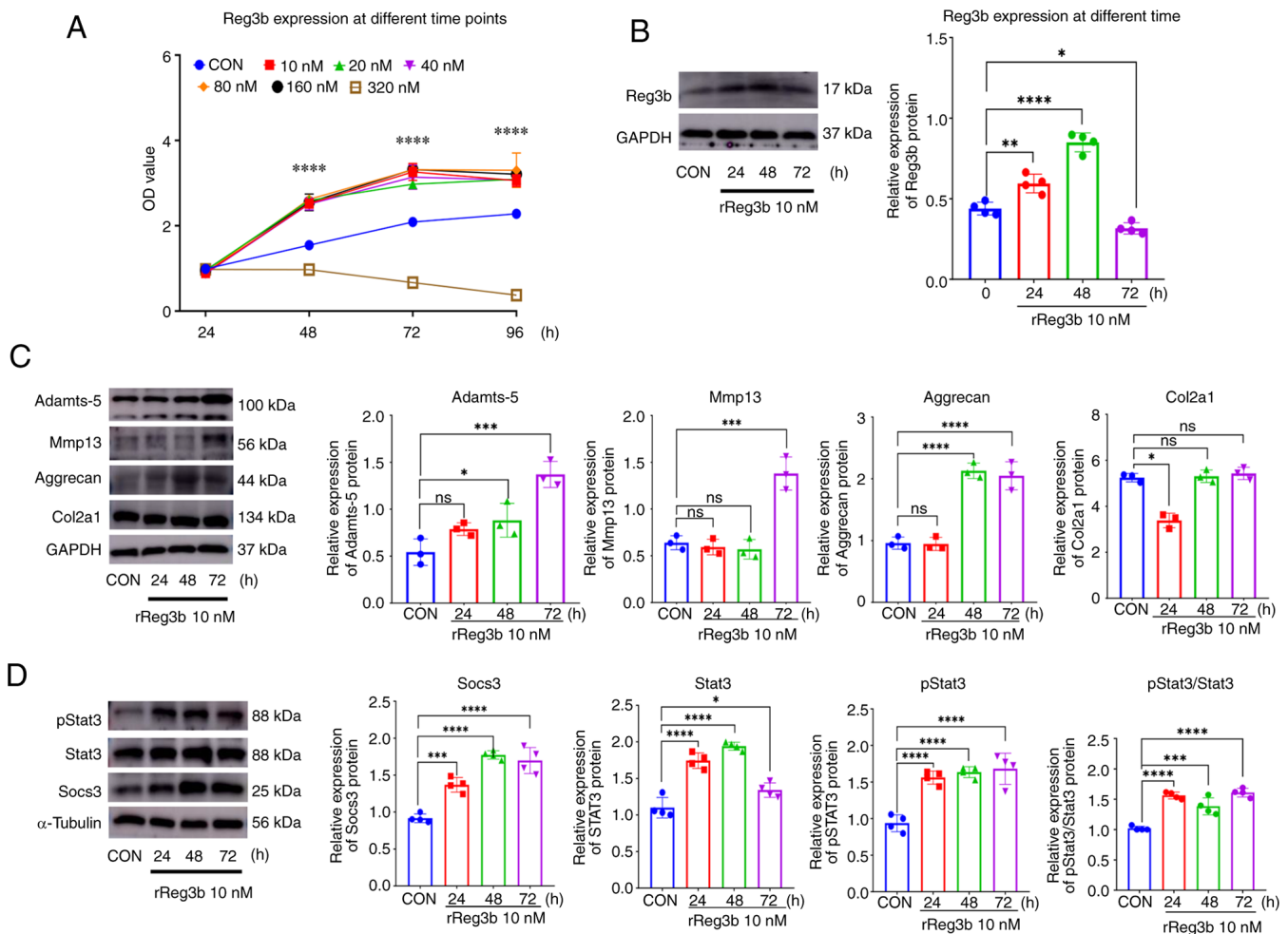


Figure 5. Recombinant Reg3 β protein promotes the activity of chondrocytes and the metabolism of the extracellular matrix. (A) The optimum concentration of recombinant Reg3 β protein for subsequent experiments was determined. (B) Changes in Reg3 β expression over time after the addition of recombinant Reg3 β protein. (C) Aggrecan and corresponding degradative enzymes, such as Adamts-5 and Mmp13, were upregulated significantly after stimulation with the recombinant Reg3 β protein, whereas Col2a1 expression did not change following transient downregulation at 24 h. (D) Stat3 and Socs3 were activated after Reg3 β protein stimulation. ANOVA with Dunnett's post hoc test were performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Reg3 β , regenerating islet-derived protein 3- β ; CCK-8, Cell Counting Kit-8; OD, optical density; CON, control; rReg3 β , recombinant Reg3 β ; Mmp13, matrix metalloproteinase 13; Col2a1, collagen type II alpha 1 chain; Adamts-5, ADAM metalloproteinase with thrombospondin type 1 motif 5; Socs3, suppressor of cytokine signaling 3.

cleaved caspase-3 was upregulated, whereas the expression of caspase-9 was not evidently altered. By contrast, Bcl-2 expression was significantly downregulated, whereas Bax expression was significantly upregulated (Fig. 2D).

Reg3 β knockdown decreases the synthesis and degradation of the extracellular matrix (ECM). After confirming the effects of Reg3 β on the proliferation and apoptosis of chondrocytes *in vitro*, it was further investigated whether Reg3 β affects the synthesis and metabolism of the ECM, which is another important component of articular cartilage (31). Anabolic and catabolic metabolism was assessed via WB analyses. The expression of Aggrecan, ADAM metalloproteinase with thrombospondin type 1 motif 5 (Adamts-5) and matrix metalloproteinase 13 (Mmp13) was significantly decreased, whereas the expression of collagen type II alpha 1 chain (Col2a1) remained unchanged (Fig. 3).

Reg3 β knockdown inhibits the Jak2/Stat3/Socs3 axis signaling pathway. Both Jak2 and Stat3 were inhibited after Reg3 β

knockdown. The corresponding phosphorylated proteins were also significantly downregulated. Moreover, suppressor of cytokine signaling 3 (Socs3) expression was markedly downregulated. In addition, exostosin like glycosyltransferase 3 (Extl3), which is a receptor of Reg3 β (32), was downregulated (Fig. 4).

Recombinant Reg3 β promotes chondrocyte proliferation and ECM metabolism. Subsequently, the promotion of cellular proliferation was verified. A recombinant Reg3 β protein was produced to assess its natural bioactivity. CCK-8 assays were performed to determine the optimal concentration for further experiments. The results showed that recombinant Reg3 β promoted chondrocyte cell proliferation, and 10-160 nM recombinant Reg3 β had a similar effect (Fig. 5A). However, a relatively higher dose of 320 nM had the opposite effect, inducing apparent cell death. Thus, a concentration of 10 nM was selected for the subsequent experiments. To confirm the optimal time for further experiments, the expression of recombinant Reg3 β was evaluated; expression peaked at 48 h after

the addition of recombinant Reg3 β , which was consistent with the results of the CCK-8 assay (Fig. 5B). Further experiments confirmed that recombinant Reg3 β can increase the expression of Aggrecan, Mmp-13 and Adamts-5, whereas Col2a1 exhibited no obvious changes following a transient downregulation at 24 h. Notably, the expression of Adamts-5 and Mmp13 was upregulated at 72 h (Fig. 5C). By contrast, Stat3 was activated by phosphorylation at 24 h, while Socs3 was upregulated after 24 h (Fig. 5D).

Discussion

The present study demonstrated that Reg3 β expression in cartilage and serum is upregulated after fixation removal in a DDH rat model. Further experiments revealed that Reg3 β may have a positive effect on chondrocyte proliferation and metabolism of the ECM, indicating its ability to remodel the acetabulum in RAD.

RAD is a common complication after closed or open reduction of the affected hip. A previous study confirmed that the affected hip may be remodeled after fixation removal in experimental models, which is similar to the results of closed or open reduction treatment in patients with clinical DDH (33). The results of hip remodeling are closely related to the fixation time. The shorter the fixation time of the model is, the greater the possibility of reduction being achieved (23,34,35). Thus, in the present study, the authors created a series of neonatal rat DDH models with different fixation times to identify which model would be optimal for simulating the pathological process of RAD. Compared with those in previous studies, including animal models and clinical imaging, the hip, especially the acetabular roof, in the neonatal rat model showed similar changes after removal of the fixator (23,24). By contrast, when the fixation time reached 7 days, almost no complete reduction was observed. Therefore, 7 days of fixation were selected as the RAD model for subsequent experiments. Notably, Reg3 β expression in acetabular cartilage in the RAD group was significantly upregulated at 1, 2 and 4 weeks after fixation removal. Nevertheless, the serum concentration of Reg3 β transiently increased in the RAD group at 2 weeks, after which it returned to the normal level, strongly indicating a close relationship between Reg3 β and the early remodeling potential of RAD. However, at 2 weeks, Reg3 β expression was greater in the 7-day fixation group than in the 3-day and 5-day fixation groups, which exhibited decreased Reg3 β expression than did the normal cartilage group, whereas, conversely the 3-day fixation and 5-day fixation groups exhibited excellent remodeling. This appeared to be paradoxical and beyond the authors' initial hypothesis, in that it should be at least similar to the normal state. It was presumed that the expression pattern of Reg3 β could be dynamically altered in response to the mechanical internal environment. With short-term fixation, the hip underwent reduction and remodeling, which alleviated the abnormal mechanical stress. On the other hand, the abnormal mechanical stress in the 7-day fixation group was still persistent due to incomplete reduction and remodeling, which resulted in increased expression of Reg3 β . Previous studies have shown that the potential remodeling ability of the acetabulum varies and is observed in individuals ranging

from 2-11 years of age (36-38). Moreover, 2-week-old rats may be equivalent to 4-year-old children. Some evidence suggests that secondary surgery is acceptable if the RAD persists at 4-5 years of age (39-42). Thus, Reg3b may be a potential biomarker for predicting the development and remodeling of the affected hip in patients with RAD.

Previous studies have revealed that Reg3b promotes cell proliferation and differentiation and has anti-inflammatory or antiapoptotic effects on cytokines, growth factors and chemokines (32,43-45). To explore the function of Reg3b, an Reg3b-knockdown vector was created for transfecting primary chondrocytes *in vitro*. As a result, the proliferation of chondrocytes was inhibited, accompanied by an increase in the number of apoptotic cells. Furthermore, caspase-3 and cleaved-caspase-3, key apoptotic proteins, were both significantly upregulated, indicating that the apoptotic signaling pathway, which is based on the caspase family, was activated (46). Moreover, the expression of Bax increased as the corresponding expression of Bcl-2 decreased, which indicated that the induction of chondrocyte apoptosis could occur via the mitochondrial signaling pathway. By contrast, when recombinant Reg3 β protein was added to the culture, cell proliferation was increased. Overall, these results confirm that Reg3 β plays an important positive role in the proliferation of chondrocytes.

Articular cartilage is composed of chondrocytes and the ECM, which is secreted by chondrocytes. Since Reg3 β has a positive effect on chondrocyte proliferation, it was investigated whether Reg3 β affects the metabolism of the ECM. The major components of the ECM are collagens and proteoglycans, which are produced by chondrocytes to acquire a phenotype and form a complex network to support the mechanical characteristics of articular cartilage during skeletal development, especially during endochondral ossification (47,48). By contrast, several types of degradative enzymes such as Mmps, Adamts-4/5 are secreted from chondrocytes or the synovium to maintain the internal environment of the articular cartilage. The dynamic balance of chondrocyte proliferation and apoptosis, as well as the synthesis and degradation of the ECM, promotes the development and remodeling of cartilage under beneficial and harmful mechanical stimuli, such as the process of RAD (23,24,49). In the present study, the findings revealed that Reg3 β primarily regulated the anabolism and catabolism of Aggrecan rather than Col2a1. However, this finding appears paradoxical due to the simultaneous changes in the expression of Aggrecan and corresponding degradative enzymes after Reg3 β intervention. Nevertheless, it is presumed that there may be a more complex regulatory mechanism involved in balancing the ECM.

Previous studies have confirmed that not only Stat3 but also Socs3, which is involved in the negative feedback to Stat3, are implicated in postnatal bone growth (50-52). Thus, the expression of relevant proteins in the signaling pathway was examined. After Reg3b was knocked down, the expression of Jak2/Stat3 was inhibited, and Socs3 was also inhibited. In addition, Extl3, presumed to be a receptor of Reg3 β , was significantly inhibited. Conversely, after the addition of recombinant Reg3 β protein, the phosphorylation of Stat3 and Socs3 was activated. Thus, these findings indicated that Reg3 β affects chondrocyte phenotypes via the Jak2/Stat3/Socs3 signaling pathway. However, this molecule is a negative

feedback regulator of the Jak2/Stat3 signaling pathway, and strategies to precisely regulate these phenotypes and maintain a dynamic remodeling balance are still required.

However, the present study has several limitations. First, our findings are derived from animal models, and it remains to be determined whether similar changes in Reg3b expression occur in children with residual acetabular dysplasia. This necessitates further validation in clinical settings. Moreover, the precise mechanisms through which Reg3b modulates chondrocyte proliferation and extracellular matrix synthesis via the Jak2/Stat3 signaling pathway are not fully understood and warrant additional investigation.

In conclusion, the findings of the present study confirmed that the affected hip of the RAD model can be remodeled after fixation removal. The present study revealed the upregulated expression of Reg3 β in the acetabulum of the RAD model in the early stage, indicating that Reg3 β could be a potent biomarker for predicting hip remodeling. The preliminary remodeling mechanism may be related to Reg3 β -mediated promotion of chondrocyte proliferation and the activation of ECM metabolism.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Project of Jiangsu Province Key Research and Development Plan (grant no. BE2022732), the Project of Zhejiang Provincial Public Welfare Technology Application Research Plan (grant no. 2024KY1693), the National Natural Science Foundation of China (grant no. 82272441) and the Science and Technology Commission of Shanghai Municipality (CN) (grant no. 22Y11912200).

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

PW, GS and XW conceptualized and designed the study. PW, GS, ML, WW, WS, WZ and QS designed the methodology. PW, ML and BN analyzed and interpreted data. PW wrote the manuscript. GS and BN reviewed and edited the manuscript. PW, GS and XW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed according to the National Research Council's Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Ethics Committee of Jiaxing Women and Children's Hospital Affiliated with Wenzhou Medical University (approval no. 2023-016; Jiaxing, China) and the Ethics Committee of Soochow University (approval no. SUDA20211130A16; Suzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Kiani SN, Gornitzky AL, Matheney TH, Schaeffer EK, Mulpuri K, Shah HH, Yihua G, Upasani V, Aroojis A, Krishnamoorthy V, *et al*: A prospective, multicenter study of developmental dysplasia of the hip: What can patients expect after open reduction? *J Pediatr Orthop* 43: 279-285, 2023.
- Håberg Ø, Bremnes T, Foss OA, Angenete O, Lian ØB and Holen KJ: Children treated for developmental dysplasia of the hip at birth and with normal acetabular index at 1 year: How many had residual dysplasia at 5 years? *J Child Orthop* 16: 183-190, 2022.
- Bakti K, Lankinen V, Helminen M, Välipakka J, Laivuori H and Hyvärinen A: Clinical and sonographic improvement of developmental dysplasia of the hip: Analysis of 948 patients. *J Orthop Surg* 17: 538, 2022.
- Dornacher D, Lutz B, Freitag T, Sgroi M, Taurman R and Reichel H: Residual dysplasia of the hip after successful ultrasound-monitored treatment: How does an infant's hip evolve? *J Pediatr Orthop B* 31: 524-531, 2022.
- de Courtivron B, Brulefert K, Portet A and Odent T: Residual acetabular dysplasia in congenital hip dysplasia. *Orthop Traumatol Surg Res* 108: 103172, 2022.
- Tuhanioglu Ü, Cicek H, Ogur HU, Seyfettinoglu F and Kapukaya A: Evaluation of late redislocation in patients who underwent open reduction and pelvic osteotomy as treatment for developmental dysplasia of the hip. *HIP Int* 28: 309-314, 2018.
- McClincy MP, Wylie JD, Yen YM and Novais EN: Mild or borderline hip dysplasia: Are we characterizing hips with a lateral center-edge angle between 18° and 25° appropriately? *Am J Sports Med* 47: 112-122, 2019.
- Barrera CA, Cohen SA, Sankar WN, Ho-Fung VM, Sze RW and Nguyen JC: Imaging of developmental dysplasia of the hip: Ultrasound, radiography and magnetic resonance imaging. *Pediatr Radiol* 49: 1652-1668, 2019.
- Shin CH, Yang E, Lim C, Yoo WJ, Choi IH and Cho TJ: Which Acetabular landmarks are the most useful for measuring the acetabular index and Center-edge angle in developmental dysplasia of the hip? A comparison of two methods. *Clin Orthop* 478: 2120-2131, 2020.
- Miyake T, Tetsunaga T, Endo H, Yamada K, Sanki T, Fujiwara K, Nakata E and Ozaki T: Predicting acetabular growth in developmental dysplasia of the hip following open reduction after walking age. *J Orthop Sci* 24: 326-331, 2019.
- Liu X, Deng X, Ding R, Cheng X and Jia J: Chondrocyte suppression is mediated by miR-129-5p via GDF11/SMAD3 signaling in developmental dysplasia of the hip. *J Orthop Res* 38: 2559-2572, 2020.
- Wakabayashi K, Wada I, Horiuchi O, Mizutani J, Tsuchiya D and Otsuka T: MRI Findings in Residual Hip Dysplasia. *J Pediatr Orthop* 31: 381-387, 2011.
- Kim HT, Kim IB and Lee JS: MR-based parameters as a supplement to radiographs in managing developmental hip dysplasia. *Clin Orthop Surg* 3: 202, 2011.
- Douira-Khomsy W, Smida M, Louati H, Hassine LB, Bouchoucha S, Saied W, Ladeb MF, Ghachem MB and Bellagha I: Magnetic resonance evaluation of acetabular residual dysplasia in developmental dysplasia of the hip: A preliminary study of 27 patients. *J Pediatr Orthop* 30: 37-43, 2010.
- Schmaranzer F, Justo P, Kallini JR, Ferrer MG, Miller PE, Matheney T, Bixby SD and Novais EN: MRI hip morphology is abnormal in unilateral DDH and increased lateral limbus thickness is associated with residual DDH at minimum 10-year follow-up. *J Child Orthop* 17: 86-96, 2023.
- Fu Z, Zhang Z, Deng S, Yang J, Li B, Zhang H and Liu J: MRI assessment of femoral head docking following closed reduction of developmental dysplasia of the hip. *Bone Jt J* 105-B: 140-147, 2023.

17. Johnson MA, Gohel S, Nguyen JC and Sankar WN: MRI predictors of residual dysplasia in developmental dysplasia of the hip following open and closed reduction. *J Pediatr Orthop* 42: 179-185, 2022.
18. Gather KS, Mavrev I, Gantz S, Dreher T, Hagmann S and Beckmann NA: Outcome prognostic factors in MRI during spica cast therapy treating developmental hip dysplasia with midterm follow-up. *Children* 9: 1010, 2022.
19. Tetsunaga T, Tetsunaga T, Akazawa H, Yamada K, Furumatsu T and Ozaki T: Evaluation of the labrum on postoperative magnetic resonance images: A predictor of acetabular development in developmental dysplasia of the hip. *HIP Int* 32: 800-806, 2022.
20. Meng X, Yang J and Wang Z: Magnetic resonance imaging follow-up can screen for soft tissue changes and evaluate the short-term prognosis of patients with developmental dysplasia of the hip after closed reduction. *BMC Pediatr* 21: 115, 2021.
21. Kawamura Y, Tetsunaga T, Akazawa H, Yamada K, Sanki T, Sato Y, Nakata E and Ozaki T: Acetabular depth, an early predictive factor of acetabular development: MRI in patients with developmental dysplasia of the hip after open reduction. *J Pediatr Orthop B* 30: 509-514, 2021.
22. Ding R, Liu X, Zhang J, Yuan J, Zheng S, Cheng X and Jia J: Downregulation of miR-1-3p expression inhibits the hypertrophy and mineralization of chondrocytes in DDH. *J Orthop Surg* 16: 512, 2021.
23. Ning B, Jin R, Wan L and Wang D: Cellular and molecular changes to chondrocytes in an *in vitro* model of developmental dysplasia of the hip-an experimental model of DDH with swaddling position. *Mol Med Rep* 18: 3873-3881, 2018.
24. Li TY and Ma RX: Increasing thickness and fibrosis of the cartilage in acetabular dysplasia: A rabbit model research. *Chin Med J (Engl)* 123: 3061-3066, 2010.
25. Fischer J, Knoch N, Sims T, Rosshirt N and Richter W: Time-dependent contribution of BMP, FGF, IGF, and HH signaling to the proliferation of mesenchymal stroma cells during chondrogenesis. *J Cell Physiol* 233: 8962-8970, 2018.
26. Ji X, Liu T, Zhao S, Li J, Li L and Wang E: WISP-2, an upregulated gene in hip cartilage from the DDH model rats, induces chondrocyte apoptosis through PPAR γ in vitro. *FASEB J* 34: 4904-4917, 2020.
27. Otsuka N, Yoshioka M, Abe Y, Nakagawa Y, Uchinami H and Yamamoto Y: Reg3 α and Reg3 β expressions followed by JAK2/STAT3 activation play a pivotal role in the acceleration of liver hypertrophy in a rat ALPPS model. *Int J Mol Sci* 21: 4077, 2020.
28. Namikawa K, Okamoto T, Suzuki A, Konishi H and Kiyama H: Pancreatitis-associated protein-III is a novel macrophage chemoattractant implicated in nerve regeneration. *J Neurosci* 26: 7460-7467, 2006.
29. Cao Y, Tian Y, Liu Y and Su Z: Reg3 β : A potential therapeutic target for tissue injury and inflammation-associated disorders. *Int Rev Immunol* 41: 160-170, 2022.
30. Lindsey ML, Mouton AJ and Ma Y: Adding Reg3 β to the acute coronary syndrome prognostic marker list. *Int J Cardiol* 258: 24-25, 2018.
31. Chijimatsu R and Saito T: Mechanisms of synovial joint and articular cartilage development. *Cell Mol Life Sci* 76: 3939-3952, 2019.
32. Zhang H, Corredor ALG, Messina-Pacheco J, Li Q, Zogopoulos G, Kaddour N, Wang Y, Shi BY, Gregorieff A, Liu JL and Gao ZH: REG3A/REG3B promotes acinar to ductal metaplasia through binding to EMTL3 and activating the RAS-RAF-MEK-ERK signaling pathway. *Commun Biol* 4: 688, 2021.
33. Raab P, Löhr J and Krauspe R: Remodellierung des Azetabulum nach experimenteller Hüftgelenksdislokation-eine tierexperimentelle Studie an Kaninchen. *Z Für Orthop Ihre Grenzgeb* 136: 519-524, 2008.
34. Yamamoto N: Changes of the acetabular cartilage following experimental subluxation of the hip joint in rabbits. *Nihon Seikeigeka Gakkai Zasshi* 57: 1741-1753, 1983 (In Japanese).
35. Ning B, Sun J, Yuan Y, Yao J, Wang P and Ma R: Early articular cartilage degeneration in a developmental dislocation of the hip model results from activation of β -catenin. *Int J Clin Exp Pathol* 7: 1369-1378, 2014.
36. Brougham D, Broughton N, Cole W and Menelaus M: The predictability of acetabular development after closed reduction for congenital dislocation of the hip. *J Bone Joint Surg Br* 70-B: 733-736, 1988.
37. Bos CF, Bloem JL and Verbout AJ: Magnetic resonance imaging in acetabular residual dysplasia. *Clin Orthop*: 207-217, 1991.
38. Harris NH: Acetabular growth potential in congenital dislocation of the hip and some factors upon which it may depend. *Clin Orthop*: 99-106, 1976.
39. Albinana J, Dolan LA, Spratt KF, Morcuende J, Meyer MD and Weinstein SL: Acetabular dysplasia after treatment for developmental dysplasia of the hip: Implications for secondary procedures. *J Bone Joint Surg Br* 86: 876-886, 2004.
40. Fu M, Liu J, Huang G, Huang Z, Zhang Z, Wu P, Wang B, Yang Z and Liao W: Impaired ossification coupled with accelerated cartilage degeneration in developmental dysplasia of the hip: Evidences from μ CT arthrography in a rat model. *BMC Musculoskelet Disord* 15: 339, 2014.
41. Mansour E, Eid R, Romanos E and Ghanem I: The management of residual acetabular dysplasia: Updates and controversies. *J Pediatr Orthop B* 26: 344-349, 2017.
42. Morris WZ, Hinds S, Worrall H, Jo CH and Kim HKW: Secondary surgery and residual dysplasia following late closed or open reduction of developmental dysplasia of the hip. *J Bone Jt Surg* 103: 235-242, 2021.
43. Chen Z, Huang Z, Xue H, Lin X, Chen R, Chen M and Jin R: REG3A promotes the proliferation, migration, and invasion of gastric cancer cells. *Oncotargets Ther* 10: 2017-2023, 2017.
44. Xu X, Fukui H, Ran Y, Wang X, Inoue Y, Ebisudani N, Nishimura H, Tomita T, Oshima T, Watari J, *et al*: The link between type III Reg and STAT3-associated cytokines in inflamed colonic tissues. *Mediators Inflamm* 2019: 7859460, 2019.
45. Wang L, Quan Y, Zhu Y, Xie X, Wang Z, Wang L, Wei X and Che F: The regenerating protein 3A: A crucial molecular with dual roles in cancer. *Mol Biol Rep* 49: 1491-1500, 2022.
46. Guan M, Yu Q, Zhou G, Wang Y, Yu J, Yang W and Li Z: Mechanisms of chondrocyte cell death in osteoarthritis: Implications for disease progression and treatment. *J Orthop Surg* 19: 550, 2024.
47. Blumer MJF: Bone tissue and histological and molecular events during development of the long bones. *Ann Anat* 235: 151704, 2021.
48. Koosha E, Brenna CTA, Ashique AM, Jain N, Ovens K, Koike T, Kitagawa H and Eames BF: Proteoglycan inhibition of canonical BMP-dependent cartilage maturation delays endochondral ossification. *Development* 151: dev201716, 2024.
49. Noritake K, Yoshihashi Y, Hattori T and Miura T: Acetabular development after closed reduction of congenital dislocation of the hip. *J Bone Joint Surg Br* 75: 737-743, 1993.
50. Sarkar A, Liu NQ, Magallanes J, Tassey J, Lee S, Shkhyan R, Lee Y, Lu J, Ouyang Y, Tang H, *et al*: STAT3 promotes a youthful epigenetic state in articular chondrocytes. *Aging Cell* 22: e13773, 2023.
51. Liu NQ, Lin Y, Li L, Lu J, Geng D, Zhang J, Jashashvili T, Buser Z, Magallanes J, Tassey J, *et al*: gp130/STAT3 signaling is required for homeostatic proliferation and anabolism in post-natal growth plate and articular chondrocytes. *Commun Biol* 5: 64, 2022.
52. Liu X, D'Cruz AA, Hansen J, Croker BA, Lawlor KE, Sims NA and Wicks IP: Deleting suppressor of cytokine Signaling-3 in chondrocytes reduces bone growth by disrupting mitogen-activated protein kinase signaling. *Osteoarthritis Cartilage* 27: 1557-1563, 2019.



Copyright © 2025 Wang et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.