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ORIGINAL ARTICLE

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Abnormal expression of *Pappa2* gene may indirectly affect mouse hip development through the IGF signaling pathway

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Abstract

Introduction Developmental dysplasia of the hip (DDH) is a major cause of disability in children, and the genetic mechanism of this disease remains unclear. In our previous study, we found that pregnancy-associated plasma protein-A2 (PAPP-A2) was associated with DDH significantly.

Objectives The aim of this study was to investigate the insulin-like growth factor (IGF) expression and collagen synthesis as well as cartilage proliferation-related proteins in the case of abnormal expression of *Pappa2* in mice to research the relationship between PAPP-A2 and the pathological changes of DDH.

Methods In vivo animal experiments, the mice were directly injected with 50 µl of Cas9/PAPP-A2 sgRNA lentiviruses around the hip to downregulate the *Pappa2* gene expression and injected with control lentiviruses on the other side, then to observe the expression and localization of related proteins. And in an in vitro experiment, mice fibroblasts and primary chondrocytes were cultured with insulin-like growth factor binding protein-5 (IGFBP-5) protein, PAPP-A2 protein and Cas9/PAPP-A2 sgRNA lentiviruses to detect of related proteins and mRNA expression.

Results Cartilage proliferation-related proteins demonstrated a significant decrease in the PAPP-A2 knockdown hips acetabulum and femoral head cartilage, meanwhile the IGF expression was also downregulated in the soft tissue around the acetabulum compared with the control hips. Furthermore, the role PAPP-A2 played in chondrocytes and fibroblasts was the same as in the in vivo experiments, downregulation of PAPP-A2 expression or upregulation of IGFBP-5 expression can reduce collagen synthesis and cartilage proliferation.

Conclusions PAPP-A2 may be involved in the development of the mouse hip joint by interfering the fibrous and cartilaginous metabolism via IGF pathway-associated proteins pathway.

Keywords Pregnancy-associated plasma protein-A2 · Developmental dysplasia of the hip · Insulin-like growth factor · Insulin-like growth factor binding protein-5

Introduction

Developmental dysplasia of the hip (DDH) is one of the most common causes of disability and premature arthritis in children [1]. DDH represents a range of disease conditions, from mildly dysplastic to irreducible dislocation [2]. Various countries report an incidence of 1.5 to 20 cases of DDH per 1000 births, and the identified risk factors include family history, being first-born, breech presentation, female gender, high birth weight and oligohydramnios [3, 4]. Genetic factors and environmental factors are commonly accepted, but the pathogenesis of DDH is multifactorial and poorly understood [3, 4]. Epidemiological investigations have shown that some forms of DDH exhibit a transmission consistent with autosomal dominant inheritance of several susceptibility genes that are involved in bone or joint biology and in resistance and elasticity of tissues [5, 6]. In our previous study, we found a significant association between pregnancy-associated plasma protein-A2 (PAPP-A2) and DDH in the Chinese Han population [7], but this was not replicated in another study [8].

PAPPA2 is localized on chromosome 1 (1q24) and encodes PAPP-A2, a protein of 1791 residues [9]. PAPP-A2 is a metalloproteinase and shares 45% of its amino acid

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sequence with PAPP-A. It specifically cleaves insulin-like growth factor-binding protein-5 (IGFBP-5) and insulin-like growth factor-binding protein-3 (IGFBP-3). This can cause the dissociation of insulin-like growth factor 1 (IGF1) from IGFBP-containing complexes, which results in an increase in the IGF bioavailability [10]. *PAPPA2* is highly expressed in human placenta during the first trimester and is a candidate gene for the effects of a quantitative trait locus (QTL) that affects skeletal growth in mice [11, 12]. In mice in which *Pappa2* is deleted, Christians et al. observed a decrease in the lengths of some bones and changes in the shapes of some bones, in particular the pelvic girdles, with exhibited a more feminine shape [13]. Furthermore, PAPP-A2 gene associated with cattle daughter calving ease while the mechanism is not clear yet, thus it was assumed that PAPP-A2 might regulate the maternal pelvic size [14]. PAPP-A2 is associated with many human diseases. Specifically, higher levels of PAPP-A2 are found in pregnancies that are complicated by preeclampsia or HELLP syndrome as well as in the placenta and the maternal serum in fetuses with Down syndrome [10, 15]. Loss-of-function variants in *PAPPA2* results in a new syndrome identified by progressive growth failure, moderate microcephaly and thin long bones. These variants also influence bone mass and glucose homeostasis [16, 17]. In this syndrome, impaired proteolysis of IGFBP-5 cause a significant increase in the total IGF-1 concentrations and reduced free IGF-1, which may be the molecular mechanism [16]. Some additional research indicated that recombinant human insulin-like growth factor-1 (rhIGF-1) can be used to treat patients with PAPP-A2 deficiency, in whom it improves short-term growth, whole-body bone mineral density (BMD) and bone mineral content (BMC) with no obvious adverse reactions [18, 19].

IGFBP-5 is one of six insulin-like growth factor-binding proteins (IGFBPs) and is the most abundant form stored in bone [20]. IGFBP-5 stimulates osteoblasts to increase bone formation in an IGF-independent way whereas it enhances the differentiation of growth plate chondrocytes in an IGF-1-dependent way [20, 21]. IGFBP-5 has a high affinity for IGF-1, which competes with the binding of IGF-1 to the type 1 IGF receptor to regulate the amount of available IGF-1 [22, 23]. IGFs play significant roles in postnatal growth by modulating tissue proliferation and differentiation in an autocrine/paracrine manner [24, 25].

We speculate that when the levels of the functional PAPP-A2 protein are reduced due to structural variation or reduced expression of the *PAPPA2* gene, the IGFBP-5 degradation is subsequently reduced, and the bioavailability of IGF1 decreases. PAPP-A2 may have a role in cartilage and fibrous tissue formation through the IGF signaling pathway, thus causing the corresponding pathological changes of DDH. In this study, we performed in vivo animal experiments in which *Pappa2* gene expression was downregulated and evaluated the differences in the mouse hip joint development and IGF signal expression between the control hips and those with PAPP-A2 knockdown hips. Furthermore, we performed in vitro experiments to verify the role that PAPP-A2 played in the IGF signaling pathway in chondrocytes and fibroblasts.

Materials and methods

ICR mice, 6 weeks old and weighing 30–40 g, were obtained from the Animal Experimental Center, Shengjing Hospital of China Medical University. All the animals were housed in an environment with temperature of 22 ± 1 °C, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 h. All animal studies (including the mouse euthanasia procedure) were performed in compliance with the regulations and guidelines of Shengjing Hospital of China Medical University ethics committee and conducted according to the AAALAC and the IACUC guidelines (protocol 2012PS13K).

Sample collection

A total of 25 new-born ICR female mice were directly injected with 50 μl of Cas9/PAPP-A2 sgRNA lentiviruses (Obio Techology Corp., Ltd. China) at a titer of 1E + 8TU/ml around the right hip and control Cas9 sgRNA lentiviruses at a titer of 1E + 8TU/ml around the left hip at 4 days after birth (B4). The injection is completed through positioning trochanter on the surface and penetrating it with an insulin needle. The mice were sacrificed at 14 days after birth (B14), and their hip joints were harvested. The green fluorescent protein (GFP) fluorescence in the hips was observed using a fluorescence stereomicroscope to confirm successful transfection (Fig. 1). Some of the hip samples ($n = 6$ mice) were quickly rinsed in 0.01 M PBS, and then immediately fixed for 2 days in 4% paraformaldehyde in phosphate buffer. These samples were subsequently decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for 15 to 20 days, dehydrated and embedded in paraffin. The rest of the samples were placed in centrifuge tubes and frozen at −80 °C for protein detection ($n = 11$ mice) and RNA isolation ($n = 8$ mice). The sequences of the PAPP-A2 sgRNA and the control sgRNA are shown in Table 1.

Mouse fibroblast (L929 cells) were purchased from KeyGen Biotech Co., Ltd. The cells were selected because their source were from mouse connective tissue fibroblastwas which closer to the soft tissue around the acetabulum. Cells were cultured in DMEM medium (Gibco, American) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in 5% CO_2 and 95% air at 37 °C.

Twenty one-week-old ICR mice were sacrificed, and the knee femoral condyles, tibial plateau cartilage and femoral

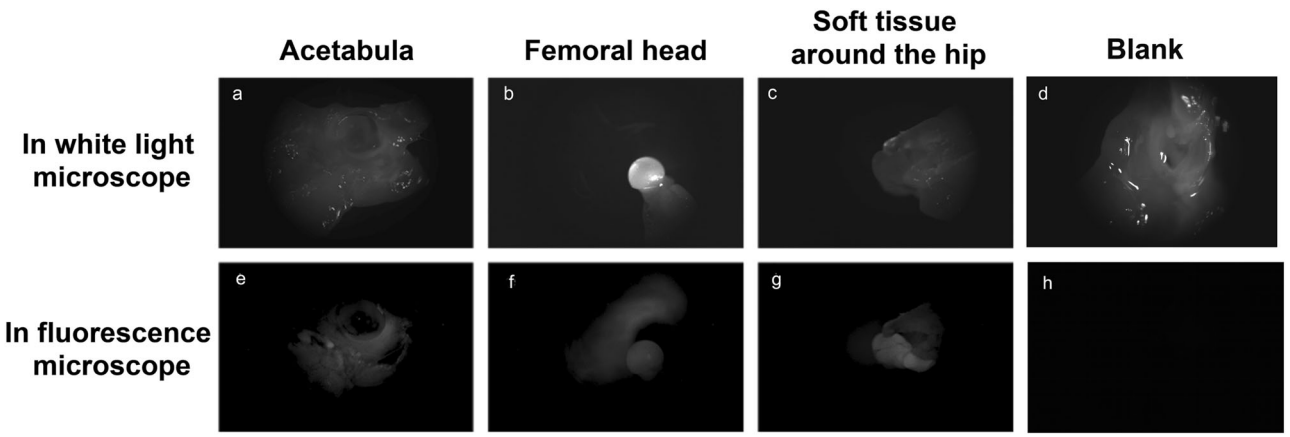

Fig. 1 Fluorescent expression of GFP in hip joint and soft tissue around the hip captured using a black-and-white camera **a**–**d** are the acetabula, femoral head, soft tissue around hip and blank control respectively, observed using white light. **e**–**h** are the fluorescence images of the acetabula, femoral head, soft tissue around hip and blank control, respectively

Table 1 Sequences of sgRNA

Gene	sgRNA Sequences
PAPP-A2	GGTATAAGGTTCGAAGACCC
Control	GCACTACCAGAGCTAACTCA

head cartilage were harvested aseptically. All the cartilage was digested by trypsin and collagenase II to obtain primary chondrocytes. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in 5% CO_2 and 95% air at 37 °C. The cells were identified using type II collagen (1:200 dilution) immunofluorescence (Fig. 2).

L929 cells and primary chondrocytes were plated in 6-well cell culture plates for 12 h, then the old medium was replaced with fresh medium (control plates) or medium that included recombinant human IGFBP-5 protein (R&D Systems, American) or recombinant human PAPP-A2 protein (R&D Systems, American). These plates were cultured for an additional 36 h before collection for RNA isolation or protein analysis, and the culture supernatants of L929 cells were collected for the hydroxyproline assay. The Cas9/PAPP-A2 sgRNA-expressing vector or Cas9 sgRNA-control vector were transfected 4 h after the initiation of the incubation of the cells in 6-well plates (3×10^5 per well). The GFP fluorescence of the cells was observed, and the cells were collected 72 h after transfection for RNA isolation or protein analysis. The culture supernatants of L929 cells were collected for hydroxyproline assay.

L929 cell hydroxyproline assay

Hydroxyproline is present almost exclusively in collagen, therefore, the hydroxyproline assay is a broadly employed method of quantifying collagen [26]. The culture supernatants of L929 cells (1 ml) were collected into 5 ml microcentrifuge tubes, and ultrapure water was used as the blank control. The assay reagents and dilute hydroxyproline standard solution were prepared according to the Hydroxyproline Assay Kit (A030–1, Nanjing Jiancheng Bioengineering Institute, China) instruction manual. The assay reagents were added to the samples, standards and blank wells and incubated according to the instructions. After the incubation, the absorbance at 560 nm was measured in a 96-well plate, and the sample hydroxyproline content was calculated according to the formula.

RNA isolation and real-time PCR

The mouse acetabulae and femoral head samples as well as the soft tissue around the acetabulum were removed from the −80 °C freezer, frozen in liquid nitrogen and subsequently homogenized with pestles. Each tissue homogenate (20 mg) was added to 1 ml of TRIzol (Invitrogen, USA) reagent at room temperature.

The cells in each well of the 6-well plates were combined with 1 ml TRIzol (Invitrogen, USA) reagent at room temperature for 1 min, after which they were triturated and then centrifuged using a high-speed centrifuge (Eppendorf, Germany) at 12,000 rpm at 4 °C for 15 min. Next, 0.2 ml of chloroform was added, and the tubes were shaken vigorously for 1 min, incubated at room temperature for 10 min, and centrifuged at 12,000 rpm at 4 °C for 15 min. Subsequently, the supernatants were removed, and 0.5 ml of isopropanol was added to each supernatant. The samples were mixed thoroughly for 1 min, incubated at room temperature for 10 min and then centrifuged at 12,000 rpm at 4 °C for 15 min. The RNA precipitates were washed with 70% ethanol and then with 100% ethanol, air dried, and dissolved in 20 µl molecular-grade water. A NanoVue Spectrophotometer (Health care Bio-Sciences AB,

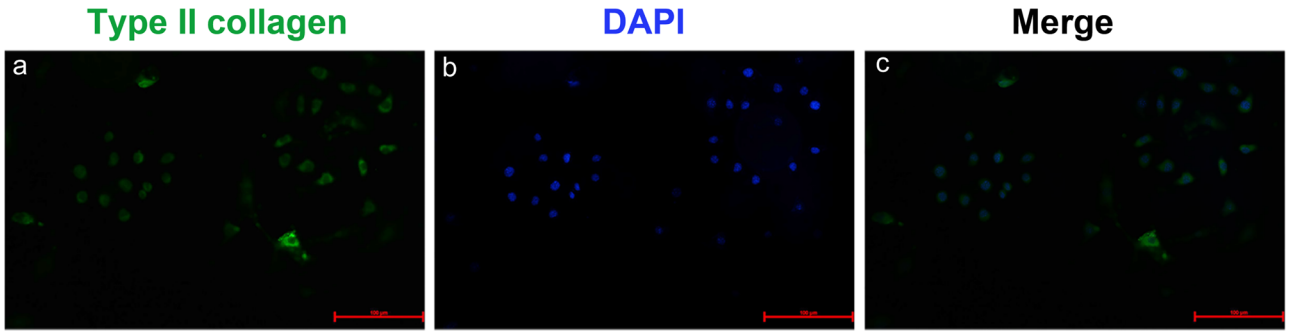

Fig. 2 Primary chondrocytes identified using type II collagen immunofluorescence. **a** Shows the distribution of type II collagen as cellular cytoplasmic green fluorescence staining. **b** Shows the cell nuclei in blue. **c** The cytoplasmic and nuclear overlap proves that most cells are primary chondrocytes

Table 2 Primer sequences used in qRT-PCR

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
Igf1	TCACTGCCCAATTGAAATACGA	TTAGGCCCAGACAGTTTAAACAAAG
Col1a1	GACATGTTCAGCTTTGTGGACCTC	GGGACCCTTAGGCCATTGTGTA
Col2	CATCCAGGGCTCCAATGATGTA	ATGTCCATGGGTGCGATGTC
Acan	AGTGGATCGGTCTGAATGACAGG	AGAAGTTGTCAGGCTGGTTTGGA
Igfbp5	GTGTACCTGCCCAACTGTGACC	GCAGCTTCATTCCGTACTTGTCC
Gapdh	AAATGGTGAAGGTCGGTGTGAAC	CAACAATCTCCACTTTGCCACTG
Pappa2	ATTAATAACCGGGCCTACTGCAAC	GTCACAATCAGCAGCAAATGGAA

Germany) was used to quantify the isolated RNA. cDNA was reverse transcribed from the RNA using the PrimeScript™ RT reagent Kit (RR047A, TAKARA BIO, Japan) and real-time PCR was performed to measure the levels of mRNA. GAPDH was used as the loading control. The primer sequences are shown in Table 2. PCR was carried out using a Lightcycler instrument (D-68298, Roche Molecular Biochemical, Germany) in which all samples were tested in duplicate on 96-well plates. Amplicons were generated in a two-step PCR (95 °C for 30 s for the initial denaturation, then 95 °C for 5 sec and 60 °C for 30 s for 40 cycles) followed by melting curve analysis to exclude contamination with nonspecific products. Each sample was amplified for 40 cycles, and the cycle at which the signal rose above a fixed threshold (Ct) was determined.

Protein extraction and Western blotting

Aliquots of 50 mg of the mouse acetabular and femoral head samples or soft tissue around the acetabulum were homogenized in 100 µl of RIPA (P0013B, Biyuntian Biotechnology Co., Ltd., China) Lysis Buffer containing PMSF (ST506, Biyuntian Biotechnology Co., Ltd., China). The cells in each well of the 6-well plates were combined with 50 µl of RIPA Lysis Buffer containing PMSF, incubated on ice for 30 min and then centrifuged at 12000 rpm at 4 °C for 15 min. The supernatants were then collected. Samples containing 40 µg of protein were mixed with 6 × SDS loading buffer (TransGen Biotech Co., Ltd., China), boiled at 98 °C for 5 min and resolved using a 4% stacking and 8% separating polyacrylamide gel (P0012A, Biyuntian Biotechnology Co., Ltd., China). The gel was equilibrated in transfer buffer, and the proteins were transferred onto PVDF membranes that had been activated by methanol. The membranes were blocked for one and a half hours at room temperature in 5% skim milk powder (BD, USA), and then incubated in a solution containing the primary antibody against PAPP-A2 polyclonal rabbit antibody (1:500 dilution, Absin Bioscience Inc. China), IGFBP-5 polyclonal goat antibody (1:500 dilution, R&D system, USA), IGF1 polyclonal rabbit antibody (1:1000 dilution, Abcam, UK), COL1A1 polyclonal rabbit antibody (1:500 dilution, Abcam, UK), COL2 polyclonal rabbit antibody (1:500 dilution, Abcam, UK), ACAN monoclonal mouse antibody (1:100 dilution, Abcam, UK), or β-actin monoclonal antibody (1:5000 dilution, Proteintech, USA) diluted in 1% BSA antibody buffer at 4 °C overnight. Subsequently, the membranes were washed three times with TBST (0.24% Tris, 0.8% NaCl, 0.1% Tween20) and incubated for 2 h in a solution containing the secondary antibody, which was diluted to the appropriate antibody titer using 1% BSA antibody buffer. The membranes were washed 3 times with TBST then covered with ECL (NCI5079, Thermo Fisher Scientific, USA) fluid for 2 min and observed in Automatic electrophoresis gel imaging analyzer (4/1246, SYOR, USA).

Paraffin sections and immunohistochemistry

The paraffin specimens of the hip were sectioned along the coronal plane at a thickness of 4 μm. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. The samples were then washed in PBS for 15 min, treated with 0.01 M citrate buffer (pH 6.0) and then heated to 98 °C for 10 min in a microwave oven. Subsequently, the sections were washed in PBS for 15 min, and then incubated with peroxidase blockers for 30 min at room temperature. After washing in PBS for an additional 15 min, the samples were incubated with normal non-immunized animal serum at room temperature for 30 min. The sections were then incubated with 30 μl of PAPP-A2 antibody (1:400 dilution), 30 μl of IGFBP-5 antibody (1:20 dilution), 30 μl of IGF1 antibody (1:125 dilution), 30 μl of COL1A1 antibody (1:20 dilution), 30 μl of COL2 antibody (1:100 dilution), or 30 μl of ACAN antibody (1:100 dilution) at 4 °C overnight. Biotinylated secondary antibodies were applied to samples for 20 min followed by streptavidin–HRP incubation (40 min) at room temperature. The signals were visualized using DAB (Maixin Biotechnology Development Co., Ltd., China) under a light microscope (Nikon E800, Japan). The samples were counterstained using Hematoxylin (Maixin Biotechnology Development Co., Ltd., China) diluted 1:5 in sterile water. Cellular cytoplasm that stained brownish yellow indicated positive results. The sections were observed by 400 times visual field, to determine the main distribution of positive cells and the general trend of change. The expression of all the proteins were observed and analyzed using NIS-Elements Basic Research (Ver. 2.1, Nikon, Japan).

Statistical analysis

All data are expressed as the mean ± standard deviation. SPSS statistical software (17.0, IBM, USA) was used to analyze the experimental data using single factor analysis of variance; LSD was used for post hoc test and independent sample t test. $P < 0.05$ was considered statistically significant.

Results

Changes in PAPP-A2 expression affect the expression of IGF pathway-associated proteins in L929 cells and primary chondrocytes

To evaluate the role of PAPP-A2 in regulating the proteolysis of IGFBP-5 in fibroblast and chondrocytes, we performed Cas9 mediated knockdown of Pappa2 in murine L929 fibroblast cells and primary chondrocytes, in addition we also treated those cells with exogenous PAPP-A2 and IGFBP-5 protein, respectively. The hydroxyproline assay was used to determine the hydroxyproline levels, which can be used as an indicator of collagen content. L929 cells in which treated with the exogenous recombinant PAPP-A2 protein showed an increasing trend in the hydroxyproline levels (Fig. 3a) compared with normally cultured L929 cells, whereas exposure to the IGFBP-5 protein produced a significant difference in the opposite direction (Fig. 3b). The hydroxyproline levels were also compared between the Cas9/PAPP-A2 sgRNA-expressing vector-transfected L929 cells and Cas9 sgRNA-control vector-transfected L929 cells and the results showed a significant decrease of hydroxyproline content (Fig. 3c). The outcome indicate that low expression of the *Pappa2* gene can reduce collagen synthesis. Meanwhile, more IGFBP5 presumably reduces IGF availability and is associated with less collagen.

In the L929 cells exposed to the PAPP-A2 protein, the expression levels of *Igf1* mRNA showed a significant increase while *Col1a1* showed very little difference. The same trend was also observed in protein expression (Fig. 4a, c, Tables 3 and 4), whereas *Acan* mRNA and protein showed a significant increase in primary chondrocytes. *Col2*

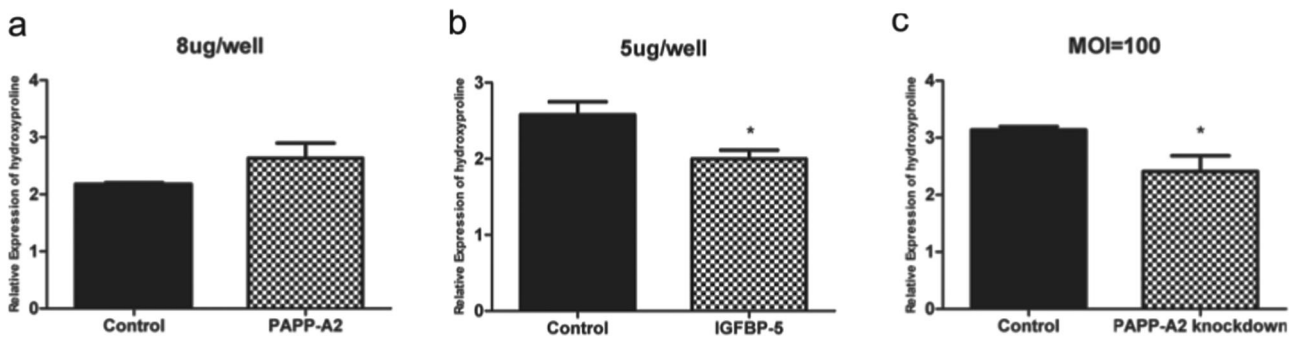

Fig. 3 The hydroxyproline levels in various conditions of L929 cells. $*P < 0.05$. **a** In L929 cells treated with 8 μg/well PAPP-A2 protein, the hydroxyproline levels increased. **b** In L292 cells treated with 5 μg/well IGFBP-5 protein, the hydroxyproline levels decreased significantly. **c** In L929 cells treated with the *PAPP-A2 knockdown* vector at an MOI (multiplicity of infection) = 100, the hydroxyproline level decreased significantly

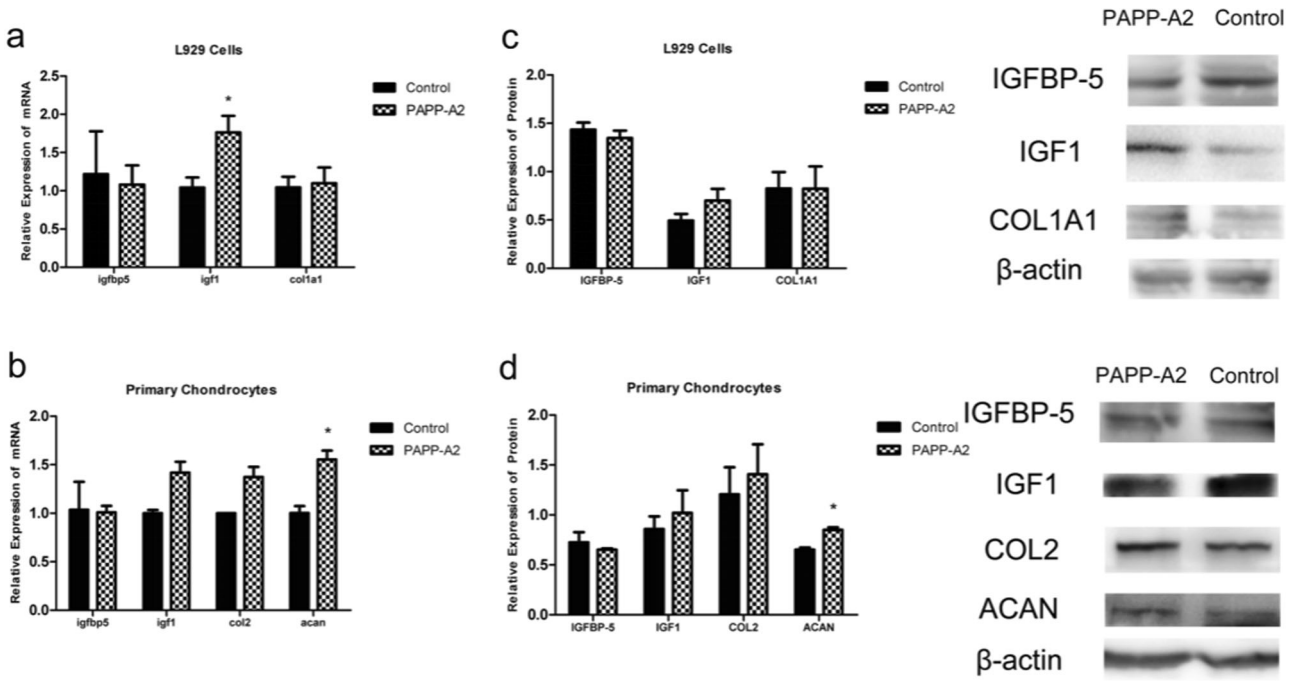

Fig. 4 Expression of related mRNA and protein in L929 cells and primary chondrocytes in the PAPP-A2 protein-exposed group. *$P <$ 0.05. **a**, **c** *Col1a1*, *Igf1*, and *Igfbp5* mRNA and protein expression in L929 cells exposed to 8 μg/well of PAPP-A2 protein. **b**, **d** *Acan*, *Col2*, *Igf1*, and *Igfbp5* mRNA and protein expression in primary chondrocytes exposed to 8 ug/well of PAPP-A2 protein

Table 3 mRNA expression in response to L929 cells and soft tissue in vivo

	PAPP-A2 knockdown L929 cells		PAPP-A2 knockdown soft tissue in vivo		L929 cells treated with PAPP-A2		L929 cells treated with IGFBP-5	
	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
Pappa2	1.00 ± 0.09	0.76 ± 0.18	1.16 ± 0.77	0.60 ± 0.12				
Igfbp5	1.02 ± 0.24	1.64 ± 0.24*	1.05 ± 0.39	1.38 ± 0.40	1.22 ± 0.97	1.08 ± 0.44		
Igf1	1.00 ± 0.09	0.75 ± 0.09*	1.16 ± 0.76	0.53 ± 0.47*	1.04 ± 0.32	1.76 ± 0.53*	1.07 ± 0.38	0.53 ± 0.25*
Col1a1	1.01 ± 0.14	0.90 ± 0.15	1.52 ± 1.34	0.51 ± 0.45	1.04 ± 0.35	1.01 ± 0.46	1.07 ± 0.44	0.46 ± 0.14*

The value in table is means ± standard deviation

Con. control, *Exp*. experimental

*$P < 0.05$

Table 4 Protein expression in response to L929 cells and soft tissue in vivo

	PAPP-A2 knockdown L929 cells		PAPP-A2 knockdown soft tissue in vivo		L929 cells treated with PAPP-A2		L929 cells treated with IGFBP-5	
	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
PAPPA2	0.61 ± 0.07	0.33 ± 0.12*	0.26 ± 0	0.21 ± 0.05				
IGFBP5	0.28 ± 0.02	0.44 ± 0.01*	0.37 ± 0.04	0.44 ± 0.14	1.43 ± 0.10	1.35 ± 0.11		
IGF1	1.10 ± 0.35	0.46 ± 0.22*	0.80 ± 0	0.76 ± 0.14	0.49 ± 0.10	0.70 ± 0.17	1.88 ± 0.48	0.97 ± 0.19*
COL1A1	1.32 ± 0.15	0.64 ± 0.30	0.29 ± 0.06	0.18 ± 0.01	0.83 ± 0.24	0.82 ± 0.32	1.64 ± 0.54	0.88 ± 0.27

The value in table is means ± standard deviation

Con. control, *Exp*. experimental

*$P < 0.05$

Table 5 mRNA expression in response to chondrocytes and cartilages in vivo

	PAPP-A2 knockdown chondrocytes		PAPP-A2 knockdown cartilages in vivo		Chondrocytes treated with PAPP-A2		Chondrocytes treated with IGFBP-5	
	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
Pappa2	1.00 ± 0.09	0.88 ± 0.11	1.01 ± 0.18	0.39 ± 0.04*				
Igfbp5	1.02 ± 0.22	1.66 ± 0.13*	1.24 ± 0.51	1.15 ± 0.40	1.04 ± 0.50	1.01 ± 0.15		
Igf1	1.01 ± 0.15	0.97 ± 0.19	1.05 ± 0.40	0.78 ± 0.16	1.00 ± 0.05	1.42 ± 0.19	1.00 ± 0.03	1.26 ± 0.264
Col2	1.01 ± 0.13	0.71 ± 0.24	1.02 ± 0.28	0.52 ± 0.17*	1.00 ± 0	1.37 ± 0.18	1.00 ± 0	0.75 ± 0.06*
Acan	1.01 ± 0.14	0.54 ± 0.08*	1.01 ± 0.19	0.52 ± 0.08*	1.00 ± 0.10	1.55 ± 0.16*	1.00 ± 0.07	0.58 ± 0.01*

The value in table is means ± standard deviation

Con.control, *Exp*. experimental

*$P < 0.05$

Table 6 Protein expression in response to chondrocytes and cartilages in vivo

	PAPP-A2 knockdown chondrocytes		PAPP-A2 knockdown cartilages in vivo		Chondrocytes treated with PAPP-A2		Chondrocytes treated with IGFBP-5	
	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
PAPPA2	0.81 ± 0.09	0.56 ± 0.15*	0.58 ± 0.19	0.27 ± 0.17*				
IGFBP5	0.92 ± 0.30	0.68 ± 0.23	0.59 ± 0.01	0.77 ± 0.07	0.73 ± 0.14	0.65 ± 0.02		
IGF1	0.82 ± 0.18	0.59 ± 0.16	0.26 ± 0.01	0.14 ± 0.07	0.86 ± 0.22	1.02 ± 0.39	0.86 ± 0.31	0.6 ± 0.09
COL2	0.92 ± 0.09	0.69 ± 0.12*	0.85 ± 0.01	0.56 ± 0.01*	1.21 ± 0.38	1.41 ± 0.42	1.06 ± 0.18	0.64 ± 0.10
ACAN	0.55 ± 0.03	0.42 ± 0.07*	0.78 ± 0.01	0.60 ± 0.02*	0.65 ± 0.03	0.85 ± 0.04*	1.03 ± 0.66	0.79 ± 0.50

The value in table is means ± standard deviation

Con. control, *Exp*. experimental

*$P < 0.05$

mRNA and protein were upregulated, but the difference was not significant (Fig. 4b, d, Tables 5 and 6). *Igfbp5* mRNA and protein did not change significantly in either of the two cell types.

In the IGFBP-5 protein-treated group of L929 cells, the expression levels of *Igf1* and *Col1a1* mRNA showed a significant decrease. The same result was also observed for the expression of the proteins, and the downregulation of IGF1 was significant (Fig. 5a, c, Tables 3 and 4). In primary chondrocytes, *Acan* and *Col2* mRNA showed a significant decrease, and there were same trend in the protein expression (Fig. 5b, d, Tables 5 and 6).

In the *PAPP-A2 knockdown* virus-transfected group, *Igf1* mRNA and protein shown a marked down-regulation whereas *Igfbp5* mRNA and protein showed a significant increasing in L929 cells (Fig. 6a, c, Tables 3 and 4). On the other hand, the expression of *Acan* mRNA and protein showed a significant decrease in the primary chondrocytes whereas the expression of Igfbp5 mRNA was significantly upregulated and COL2 protein demonstrated an opposite (Fig. 6b, d, Tables 5 and 6). All these results indicated that primary chondrocytes and L929 cells shared similar pathway of PAPP-A2 regulation, and affected PAPP-A2 proteins would possibly result in malfunctioned chondrocytes and fibroblasts.

Expression and localization of IGF pathway-associated proteins of PAPP-A2 knockdown virus-injected mouse hip joints.

We next went to examine whether the same phenomenon could be observed in vivo, by locally injecting Cas9/Pappa2 sgRNA expressing lentivirus into mouse hip joints, since fibroblasts were the major constituents of soft tissue. Fristly, we performed immunohistochemistry against PAPP-A2, IGFBP-5, COL1A1 and IGF1 (Fig. 7b). Furthermore, the real-time PCR analysis was undertaken, which indicated trend towards up-regulated on mRNA levels of Igfbp5, whereas that levels of Igf1 were down-regulated (Fig. 7a and Table 3). The western blot results of IGFBP-5 and COL1A1 in PAPP-A2-knockdown soft tissue was similar with their PCR results (Fig. 7c and Table 4). These results suggested that the in vitro L929 cells could act as a model for soft tissue around the hip joint in testing the molecular mechanism underlying PAPP-A2 in mammals.

Next, we analyzed the hip cartilages from the in vivo PAPP-A2-knockdown mice. Similar to PAPP-A2-knockdown primary chondrocytes, immunohistochemistry

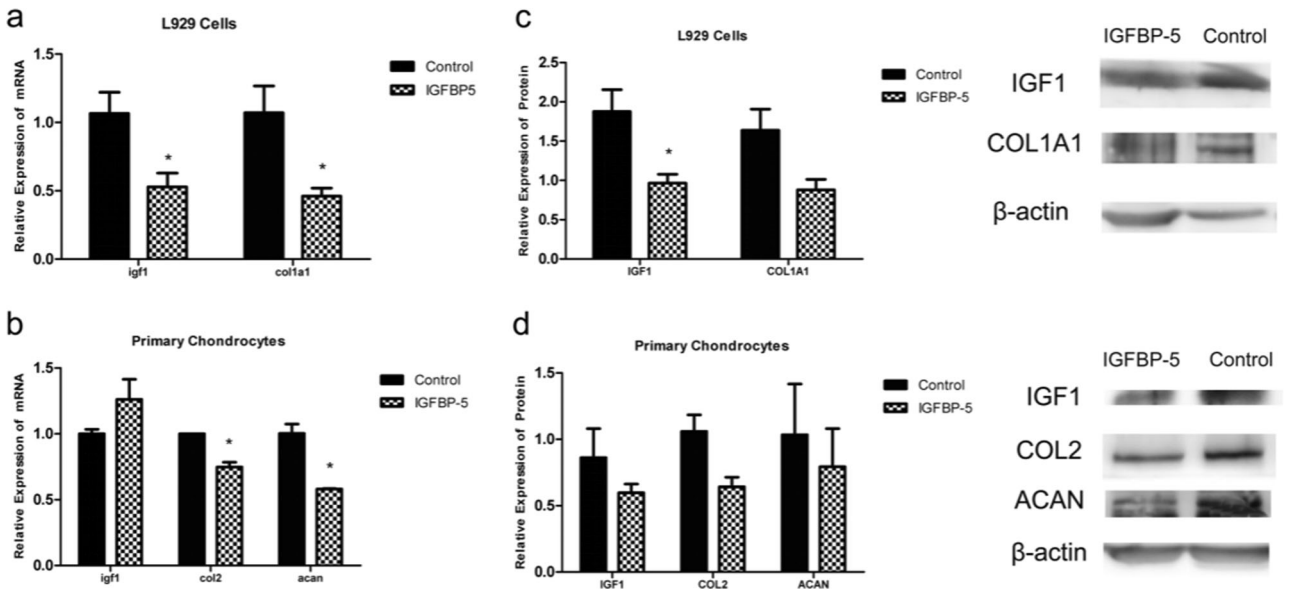

Fig. 5 Expression of related mRNA and protein in L929 cells and primary chondrocytes in the IGFBP-5 protein-exposed group. $*P < 0.05$. **a**, **c** *Col1a1* and *Igf1* mRNA and protein expression in L929 cells treated with 8 μg/well of IGFBP-5 protein. **b**, **d** *Acan*, *Col2*, and *Igf1* mRNA and protein expression in primary chondrocytes exposed to 8 μg/well of IGFBP-5 protein

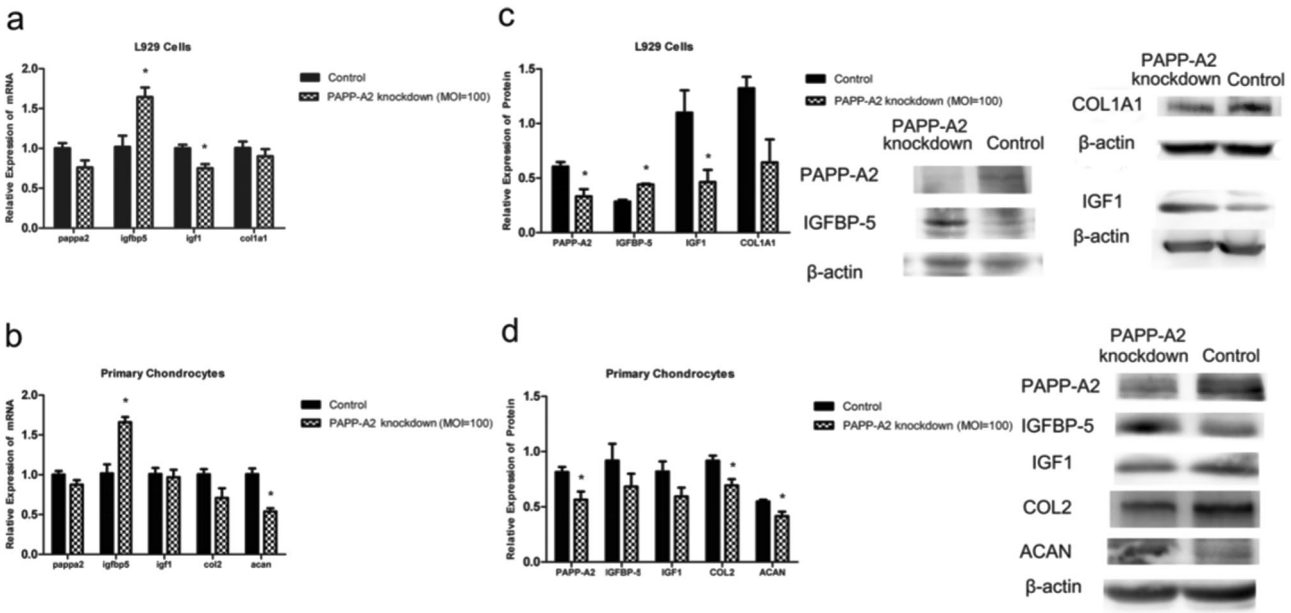

Fig. 6 Expression of related mRNA and protein in L929 cells and primary chondrocytes in the PAPP-A2-knockdown virus-transfected group. $*P < 0.05$. **a**, **c** *Col1a1*, *Igf1*, *Pappa2* and *Igfbp5* mRNA and protein expression in L929 cells treated with the PAPP-A2-knockdown virus. **b**, **d** *Acan*, *Col2*, *Igf1*, *Pappa2*, and *Igfbp5* mRNA and protein expression in primary chondrocytes treated with the PAPP-A2-knockdown virus

was used to investigate PAPP-A2, IGFBP-5, COL2, ACAN, and IGF1 expression (Fig. 8b). Then the results were further validated by western blot analysis, which indicated the expression of PAPP-A2, ACAN, and COL2 were significantly reduced (Fig. 8c and Table 6). The alterations of the mRNA levels were almost the same (Fig. 8a and Table 5).

Discussion

In the present study, we investigated the expression of IGF pathway-associated proteins both in vitro using cell culture and in vivo in mice. We investigated the effect of abnormal *Pappa2* gene expression and the high exogenous IGFBP-5 and PAPP-A2 interference on the IGF pathway.

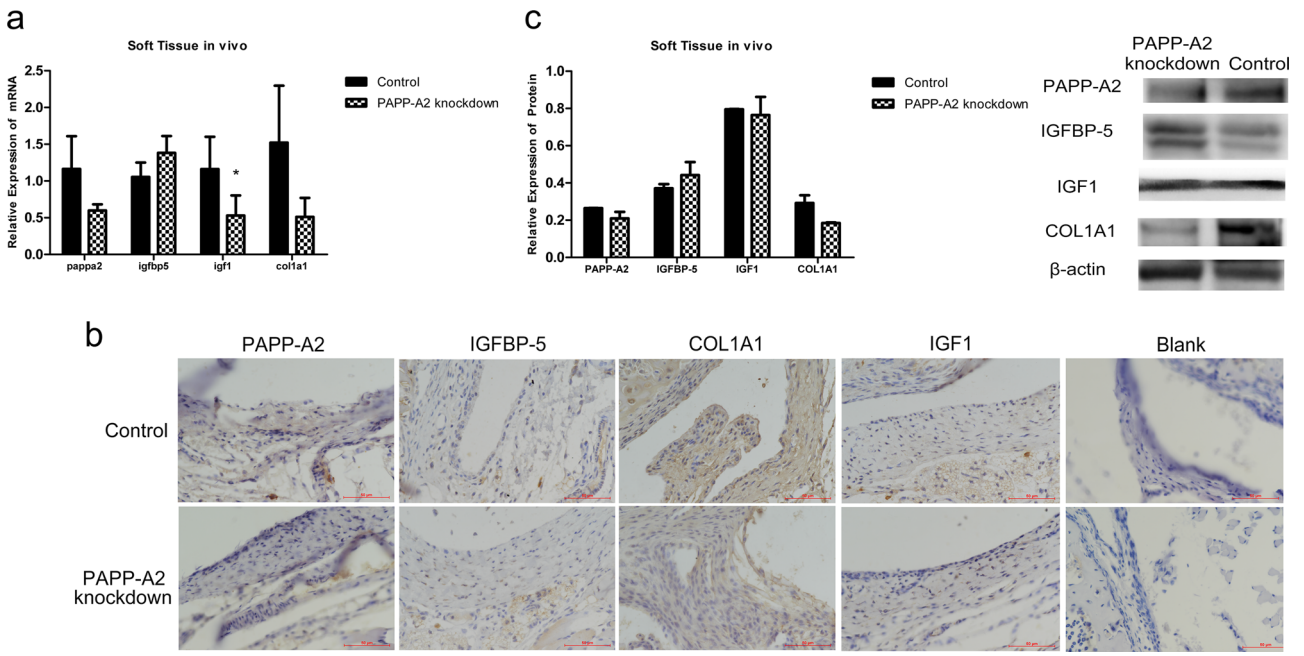

Fig. 7 Expression of related mRNA and protein in the soft tissue around the acetabulum after exposure to the PAPP-A2-knockdown virus. $*P < 0.05$. **a**, **c** *Col1a1*, *Igf1*, *Pappa2*, and *Igfbp5* mRNA and protein expression. **b** localization of PAPP-A2 and IGF pathway-associated proteins in the soft tissue around the acetabulum after exposure to the PAPP-A2-knockdown virus. The tissues were collected at 14 days after birth

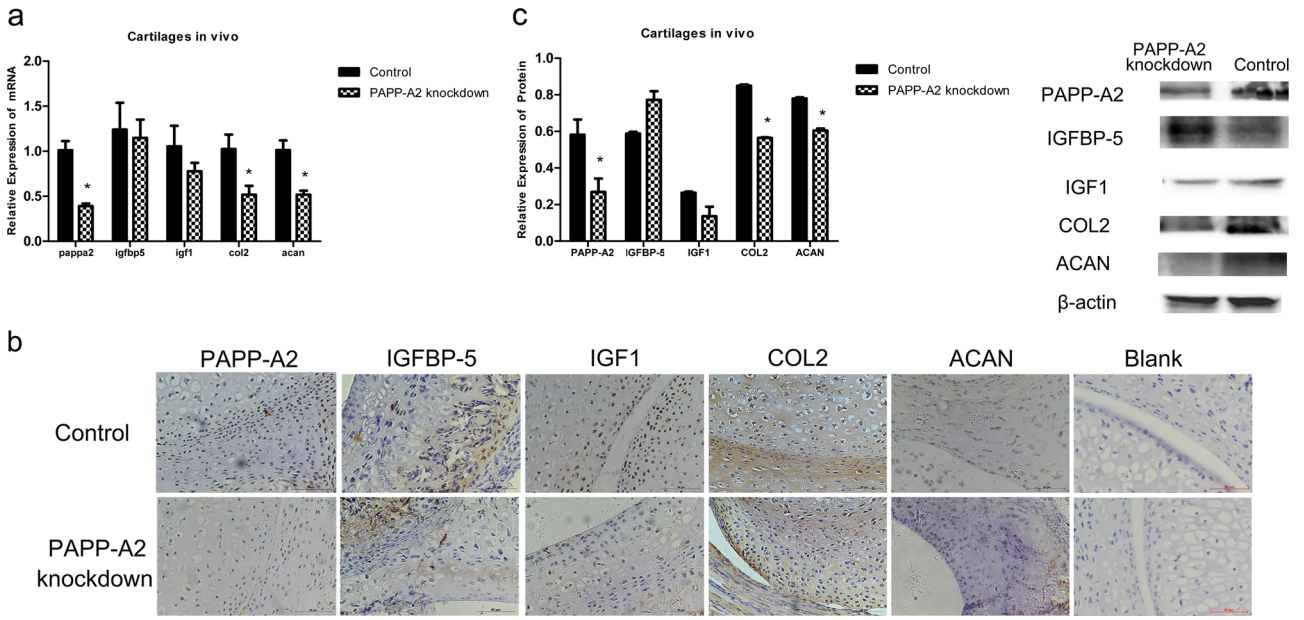

Fig. 8 Expression of related mRNA and protein in the acetabulum and femoral head cartilage after exposure to the PAPP-A2-knockdown virus. $*P < 0.05$. **a** the expression of *Col2*, *Acan*, *Igf1*, *Pappa2*, and *Igfbp5* mRNA. **c** the expression of COL2, ACAN, IGF1, PAPP-A2, and IGFBP-5 protein. **b** localization of PAPP-A2 and IGF pathway-associated proteins in the soft tissue around the acetabulum after exposure to the PAPP-A2-knockdown virus. The tissues were collected at 14 days after birth

In the bone system, IGF-1 is a potent stimulator of chondrocyte proliferation and osteoblastogenesis. This factor exhibits functions such as promotion of collagen secretion, mineralization of the bone matrix and interactions with PTH or sex steroids to induce bone anabolism acting through the IGF-signaling pathway [27, 28]. In connective tissue, IGF-1 is a powerful chemoattractant of fibroblastic cells [28]. Studies have indicated that enhanced production

of IGF-1 can increase fibroblast proliferation and stimulate human lung fibroblast growth [29, 30]. Overall, IGF is involved in the development of fibroblasts and chondrocytes. In addition, the two aspects, acetabular dysplasia or capsular laxity, were also precisely major pathological factors of DDH [31], and PAPP-A2 was identified as a novel modulator of IGF-I bioavailability [32]. In our previous study [8], we performed gene sequencing on four generations of a Chinese family that had five patients with DDH. The results indicated that a SNP associated with the family was localized in the *PAPPA2* gene. These results further suggest an association of PAPP-A2 with sporadic DDH susceptibility in a Chinese Han population.

In our previous research, we first examined the expression of PAPP-A2 and IGF pathway-related proteins in the hip joints of normal rats and DDH models [33]. In the in vitro experiment in our present study, increasing the concentration of IGFBP5 protein in the cell culture medium resulted in a reduction of the collagen synthesis by fibroblasts and retarded the chondrocyte proliferation and differentiation. At the mRNA level, *Igf1* showed a decrease expression, and we observed low expression of IGF1. This meant that increasing the level of IGFBP-5 reduced the expression of IGF1. On other hand, increasing the concentration of PAPP-A2 protein did not produce the exact opposite result, possibly as the result of compensatory effects by other pathways. Cas9/PAPP-A2 sgRNA-expressing vector-transfected cells downregulated the expression of PAPP-A2, thereby increasing the concentration of IGFBP-5 in the cells, which resulted in the downregulation of proteins involved in the IGF signaling pathway. In primary chondrocytes, the expression of the COL2 and ACAN protein and mRNA showed a decreasing trend, which indicated that cartilage proliferation was disturbed when the *Pappa2* gene was downregulated. In animal experiments, we injected the Cas9/PAPP-A2 sgRNA-expressing vector to 4-day-old mice hip. In acetabular and femoral head cartilage, we observed the same results as those in the chondrocytes. Specifically, the COL2 and ACAN protein and mRNA were downregulated in the acetabular and femoral head cartilage. However, there were no significant results in the soft tissue around the acetabulum. We therefore speculate that the transfection of the soft tissue around the acetabulum was unsuccessful because of the dose and angle of the virus injection. On the basis of the account of virus dose and transfection efficiency, most of the changes only showed trends toward downregulation. The next experiment will be to verify these effects in a knockout mouse.

In conclusion, we speculate that decreased expression of the *Pappa2* gene in mice reduced the functional PAPP-A2 protein, which reduced fibroblast collagen synthesis and chondrocyte proliferation. Excess IGFBP-5, which binds specifically to IGF, reduced the content of free IGF thereby further inhibiting the physiological function of the IGF signaling pathway. Furthermore, we speculate that the dysfunction of the *Pappa2* gene would be expected to inhibit the development of the acetabular cartilage and soft tissues around the hip indirectly through the IGF signaling pathway and whether *Pappa2* can improve the susceptibility to DDH need further verification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal studies (including the mouse euthanasia procedure) were performed in compliance with the regulations and guidelines of Shengjing Hospital of China Medical University ethics committee and conducted according to the AAALAC and the IACUC guidelines (protocol 2012PS13K).

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