Biochemical and Biophysical Research Communications 452 (2014) 906–911

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The critical role of UDP-galactose-4-epimerase in osteoarthritis: Modulating proteoglycans synthesis of the articular chondrocytes

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ARTICLE INFO

Article history:
Received 19 August 2014
Available online 6 September 2014

Keywords:
UDP-galactose-4-epimerase
Cartilage homeostasis
Osteoarthritis
Proteoglycan
Interleukin-1beta

ABSTRACT

UDP-galactose-4-epimerase (GALE) is a key enzyme catalyzing the interconversion of UDP-glucose and UDP-galactose, as well as UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine, which are all precursors for the proteoglycans (PGs) synthesis. However, whether GALE is essential in cartilage homeostasis remains unknown. Therefore, we investigated the role of GALE in PGs synthesis of human articular chondrocytes, the GALE expression in OA, and the regulation of GALE expression by interleukin-1beta (IL-1β). Silencing GALE gene with specific siRNAs resulted in a markedly inhibition of PGs synthesis in human articular chondrocytes. GALE protein levels were also decreased in both human and rat OA cartilage, thus leading to losses of PGs contents. Moreover, GALE mRNA expression was stimulated by IL-1β in early phase, but suppressed in late phase, while the suppression of GALE expression induced by IL-1β was mainly mediated by stress-activated protein kinase/c-Jun N-terminal kinase pathway. These data indicated a critical role of GALE in maintaining cartilage homeostasis, and suggested that GALE inhibition might contribute to OA progress.

1. Introduction

Chondrocytes are the only citizen in articular cartilage, which produce the major components of cartilage extracellular matrix, such as the proteoglycans (PGs) and collagens. The PGs and collagens interact with each other and form the skeleton of cartilage, which in turn provide the living environment of chondrocytes and the elasticity of cartilage [1]. Osteoarthritis (OA) is a chronic and degenerative joint disease characterized by progressive and continuous loss of cartilage matrix components [2,3]. Suppressed synthesis and stimulated degeneration of cartilage matrix disturb the cartilage homeostasis and contribute to the consequent loss of articular cartilage in OA [1,4].

UDP-galactose-4-epimerase (GALE), originally known as galactowaldenase, was firstly identified by Leloir in 1951, which catalyzes the interconversion of UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal), as well as UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylgalactosamine (UDP-GalNAc), in the Leloir pathway of galactose metabolism [5–7]. Current data demonstrated that mutations of GALE in human resulted in an inherited metabolic disease, the Type III galactosemia [8,9]. As UDP-glc, UDP-gal, UDP-GlcNAc and UDP-GalNAc are all precursors for the synthesis of PGs [10,11], GALE therefore might play a key role in PGs synthesis and consequently determine the homeostasis of articular cartilage. However, there's hardly any proof suggesting a critical role of GALE in PGs synthesis of human chondrocyte or in OA progress.

Interleukin-1beta (IL-1β) is a key pro-inflammatory cytokine presented in OA progress, which triggers the downstream signaling cascades including stress-activated protein kinase/c-Jun N-terminal kinase (SAP/JNK), p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway [12–14]. It was reported that IL-1β modulated PGs synthesis of articular chondrocytes through modulating gene expression of glycosyltransferases (GTs), namely

Abbreviations: GALE, UDP-galactose-4-epimerase; OA, osteoarthritis; PG, proteoglycan; GAG, glycosaminoglycan; IL-1β, interleukin-1beta; SAP/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.148
0006-291X/

xylosyltransferase 1 (XT-1) and glucuronosyltransferase 1 (GlcAT-1), two key enzymes that catalyze the addition of UDP-xylose and UDP-glucuronic acid to glycosaminoglycan (GAG) chains of PGs, while both SAP/JNK and p38 MAPK signaling pathway mediated the XT-1 gene expression induced by IL-1β [15–18]. However, whether GALE also participates in the IL-1β-modulated PGs synthesis of chondrocyte is still unknown.

So, we hypothesized that GALE might play a critical role in cartilage homeostasis through participating in PGs synthesis of articular chondrocyte, and that IL-1β-modulation of GALE expression might contribute to the continuous PGs loss during OA progress. In the present study, we found for the first time that: (i) GALE gene was essential in PGs synthesis of articular chondrocyte; (ii) suppressed GALE gene expression might contribute to OA progress; (iii) IL-1β suppressed GALE gene expression mainly through SAP/JNK pathway in the processed OA.

2. Materials and methods

2.1. Cartilage collection

Human cartilage samples from the knee joints (14 knees) were collected from patients diagnosed with advanced OA following the criteria of the American College of Rheumatology for OA (8 females, aged 66 ± 10 years) undergoing total knee replacement surgery in the Zhongnan Hospital of Wuhan University. All the donors were fully informed and signed the informed consents. Experiments involving human samples were carried out following the ethical guidelines of the Helsinki Declaration of 1975 (as revised in 2000). Pathogen-free adult Wistar rats weighing 220–280 g were obtained from Experimental Centre of Medical Scientific Academy of Hubei province (No. 2008-0005). The animal study was performed following the Guide for the Care and Use of Laboratory Animals (eighth edition) by the National Research Council of the United States National Academies in the Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, China) accredited by the AAALAC International. Eight male rats were used as control, while another eight rats were induced OA model using papain as previously described [19]. Then, the rats were sacrificed under anesthesia for the knee joints. All the protocols applied in this study were approved by Medical Ethics Committee of the Zhongnan Hospital of Wuhan University (No. 2012030).

2.2. Histopathology assay

Samples from the same tibial plateau or femoral condylar of the same patient were divided into microscopically normal cartilage (MNC) and degenerative cartilage (DC) using a surgical microscope with an 8-fold amplification. Then these MNC and DC samples were paired, numbered and defined as control and OA cartilage, respectively. Rat cartilage samples were also collected. Only cartilage samples (both human and rat) from the weight-bearing area were used in pathological tests following the standard protocols. All the sections were obtained perpendicularly to the cartilage surface. Then, haematoxylin-eosin (HE) and Safranin O staining was performed. Relative GALE protein level was detected using immunohistochemical (IHC) assay with anti-GALE antibody (1:150 v/v, Proteintech, CHI, USA) and analyzed using NIS-elements software (Nikon, Tokyo, Japan) as mean optical density of each chondrocytes.

2.3. Chondrocytes isolation, culture and treatment

Human articular chondrocytes were isolated from cartilage without microscopically visible degeneration. Cartilage samples were dissected and digested with 0.25% trypsin (Sigma–Aldrich, MO, USA) for 30 min and 0.2% collagenase type 2 (Sigma–Aldrich) for 12 h in serum-free DMEM/F-12 (1:1 v/v) (Thermo Fisher, Beijing, China). Then the chondrocytes were collected and cultured as monolayer at 37 °C with 5% CO_2. The components of the culture media were as follows: DMEM/F12 (1:1 v/v), 10% (v/v) fetal bovine serum (Thermo Fisher), 100 IU/ml penicillin (Biyotime, Haimen, China), 100 μg/ml streptomycin (Biyotime), and 2 mM glutamine (Sigma–Aldrich). Hereafter, chondrocytes were treated with GALE specific small interfering RNAs (siRNA, GenePharma, Suzhou, China) and nonsense siRNAs (GenePharma). The details of siRNAs applied in this study were listed in Supplementary Table 1. The transfection was performed using Lipofectamine 2000 reagent (Life Technologies, CA, USA) following the manufacturer's protocol for 48 h. Meanwhile, chondrocytes were also treated with human recombinant IL-1β (10, 20, 40 ng/mL, PeproTech, NJ, USA) for 12, 24 and 48 h, as well as pre-treated with p38 MAPK inhibitor SB203580 (20 μM, Sigma–Aldrich) or SAP/JNK inhibitor SP600125 (10 μM, Sigma–Aldrich) for 30 min and subsequently co-treated with the inhibitors and IL-1β (10 ng/mL) for another 48 h. Total protein, RNA and GAG were harvested. Chondrocytes from at least three individuals were applied in every *in vitro* experiment.

2.4. GAG assay

Total GAG was collected and dyed using 1,9-dimethylmethylene blue (Sigma–Aldrich) color reagent as reported [20]. Absorbance at 570 nm was measured using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was constructed using chondroitin sulfate sodium salt from shark cartilage (Sigma–Aldrich). Total protein of each culture was also quantified using a BCA Protein Assay Kit (Biyotime) to normalize the GAG content.

2.5. Real-time PCR assay

Total RNA was collected using TRIzol reagent (Life Technologies). Single-strand cDNA was obtained using a First Strand cDNA Synthesis Kit (Takara, Dalian, China). The primers were designed using Primer Premier 5.0 (Premier Biosoft, CA, USA) and the NCBI BLAST database. RT-PCR assay was performed on a StepOne thermal cycler (Life Technologies) using a RT-PCR kits (Takara) following the procedure: pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s. The last 2 steps ran for 40 cycles. Relative standard curves were applied in relative quantification. Details of primers and PCR conditions were listed in Supplementary Table 2. Relative expression of all the target genes was obtained using GAPDH expression level to standardize comparison.

2.6. Western blotting assay

Total protein of human cartilage samples and chondrocyte cultures was obtained using RIPA lysis buffer (Biyotime) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride). Then, proteins were size-fractionated by SDS–PAGE and transferred to nitrocellulose membranes (Millipore, MA, USA). Target proteins were probed with anti-GALE (1:1000 v/v, Proteintech) and anti-GAPDH (1:1000 v/v, Proteintech) primary antibodies, and horseradish peroxidase-conjugated secondary antibody (1:5000 v/v, Proteintech). Blots were developed using ECL reagent (Advansta, CA, USA). A Fusion FX system (Vilber Lourmat, Marne-la-Vallée, France) was applied to photograph and quantify the blots. Then, the grayscales from the blots of GALE and GAPDH in each sample were obtained.

The relative GALE protein levels were presented as the fold changes compared with the controls and normalized by the GAPDH protein level.

2.7. Statistical analysis

Results were presented as mean ± SEM. Student's *t*-test or one-way ANOVA followed by Dunnett's test, as appropriate, were applied to analyze the data with SPSS 17.0 (SPSS Science Inc., CHI, USA) and Prism 5.0 software (GraphPad Software, CA, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. GALE was essential in GAG synthesis of articular chondrocytes

Obvious decreases in both the mRNA and protein expression level of GALE were observed in the chondrocytes treated with three different GALE specific siRNAs, respectively (Fig. 1A–C). Meanwhile, total GAG content of chondrocyte culture was also markedly decreased by GALE specific siRNAs by almost 50% (Fig. 1D), which demonstrated that GALE was essential in GAG synthesis of articular chondrocyte.

3.2. GALE expression was down-regulated in human and rat OA cartilage

The staining of GALE protein was much lighter in both human and rat OA cartilage than the corresponding controls (Fig. 2I–N), which was accompanied by a lighter Safranin O staining and the disorganization of the cartilage (Fig. 2A–H). Subsequent quantification of the relative GALE protein level in both human and rat cartilage sections also demonstrated an obvious decrease in GALE protein level in OA, which was further supported by the results from Western blotting analysis of the relative GALE protein level in human cartilage samples (Fig. 2O and P).

3.3. IL-1β modulated gene expression of GALE through SAP/JNK signaling pathway

IL-1β suppressed PGs synthesis in a time- and concentration-dependent manner (Fig. 3A). However, relative GALE mRNA level was up-regulated by 10 ng/ml IL-1β in 24 h, but down-regulated by 40 ng/ml IL-1β after 24 h (Fig. 3B). And both mRNA and protein expression of GALE were suppressed by IL-1β 48 h later, in a concentration-dependent manner (Fig. 3B–D). Moreover, both SAP/JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580 attenuated the suppression of IL-1β on GAG synthesis and GALE mRNA expression of chondrocyte (Fig. 4A and B). However, it was SP600125 but not SB203580 that could better attenuate the inhibition of IL-1β on GALE protein expression (Fig. 4C and D).

4. Discussions

Normal articular cartilage is mainly composed of two major components, namely the chondrocytes and the surrounding extracellular matrix. The chondrocytes maintain the cartilage homeostasis through synthesizing and catabolizing the matrix macromolecules, while the extracellular matrix in turn provides the living environment of chondrocytes [21,22]. In normal conditions, the PGs of articular cartilage undergo a relatively stable and regular metabolic turnover with half-lives that range from days to months according to the age and species [23]. To maintain the homeostasis of cartilage, the articular chondrocyte therefore

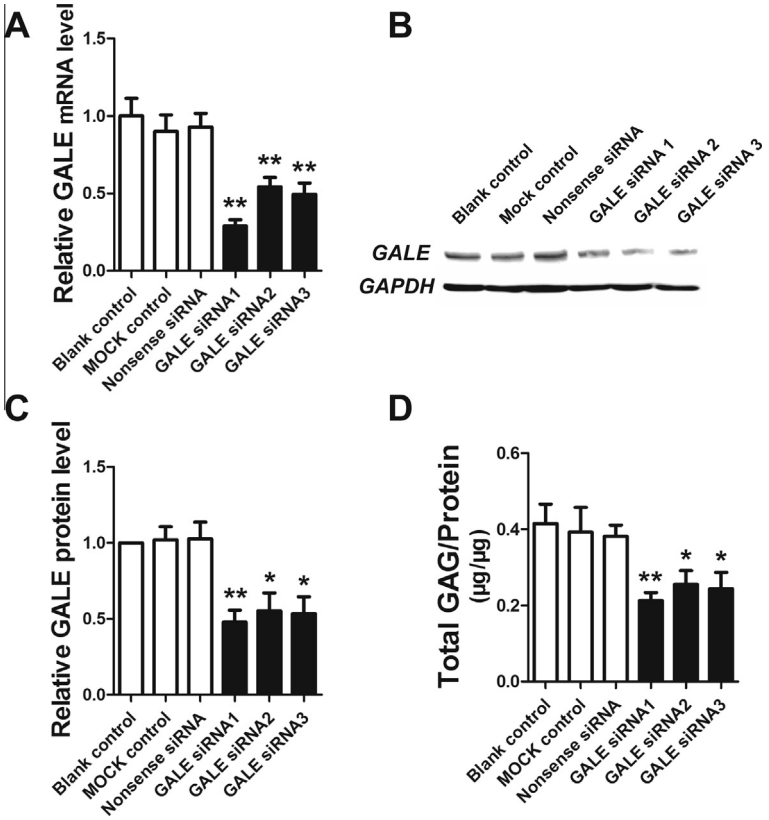

Fig. 1. UDP-galactose-4-epimerase (GALE) was essential in glycosaminoglycan (GAG) synthesis of human articular chondrocyte. GALE gene was silenced by specific siRNAs, while nonsense siRNA was used as control. Then, GALE mRNA (A) and protein level (B and C), as well as total GAG (D), were detected using real-time quantitative PCR, Western blotting and 1,9-dimethylmethylene blue staining, respectively. Values were presented as mean ± SEM. $^{*}P < 0.01$ and $^{**}P < 0.01$ *versus* control.

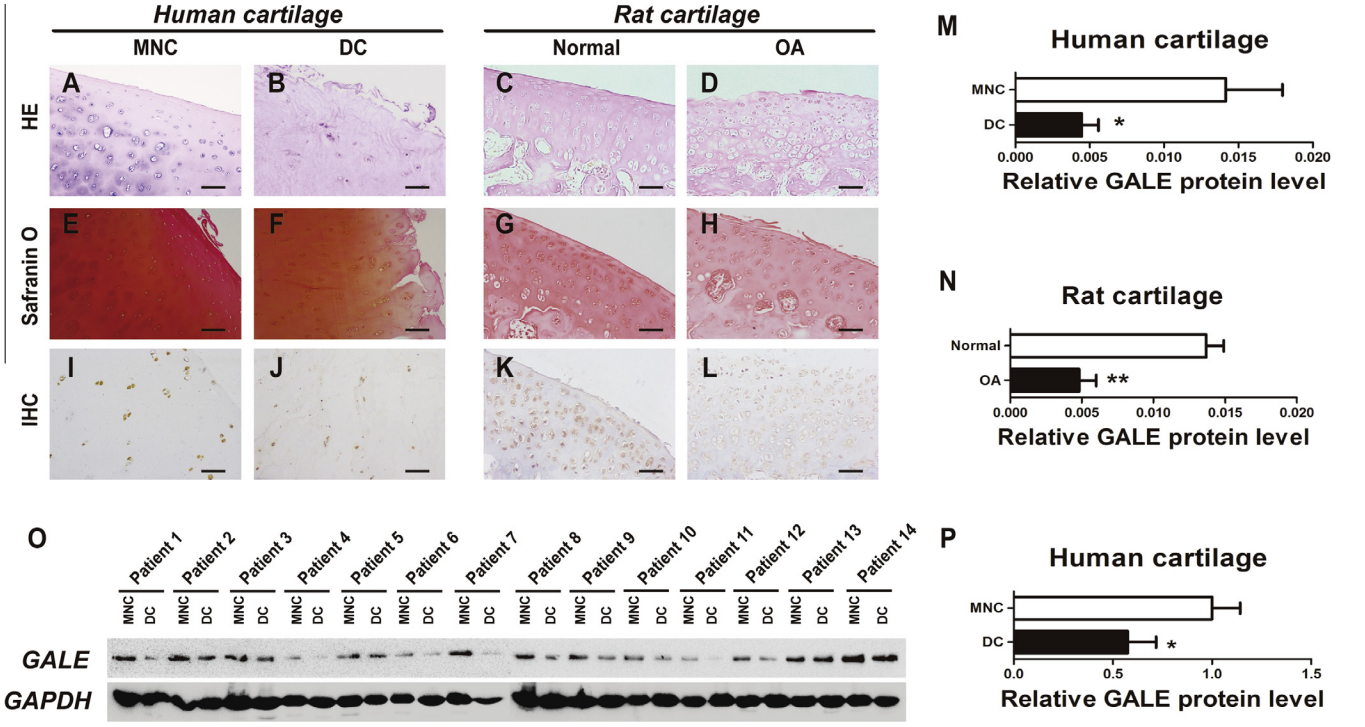

Fig. 2. UDP-galactose-4-epimerase (GALE) expression was suppressed in osteoarthritis (OA) cartilage. Human and rat OA cartilage samples were collected for Haematoxylin-eosin (HE) staining (A–D), Safranin O staining (E–H) and immunohistochemical (IHC) assay (I–L). Relative GALE protein level was presented as mean optical density of the positively stained chondrocyte (M and N). Meanwhile, GALE protein level was further detected using Western blotting assay (O) and presented as the fold change of the grayscale to the control (P). DC, degenerative cartilage; MNC, microscopically normal cartilage; Scale bars, 100 μm. Values were presented as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* control.

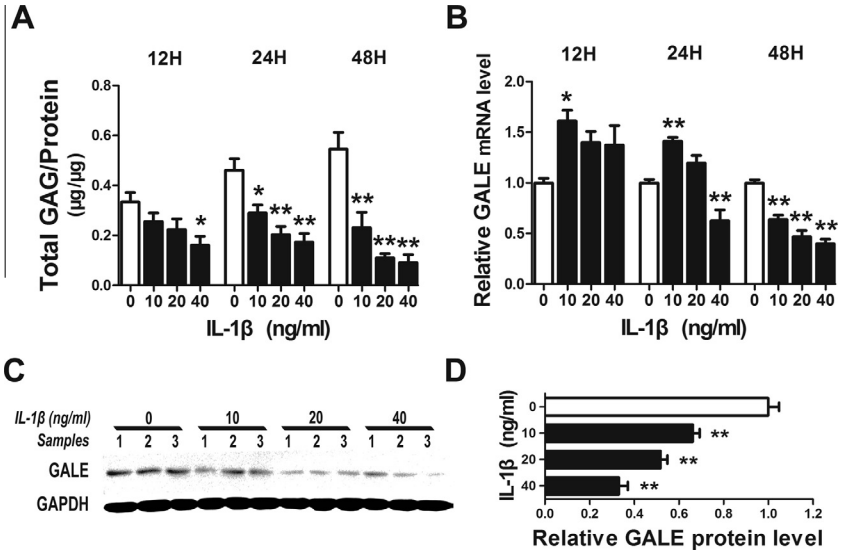

Fig. 3. Interleukin 1 beta (IL-1β) modulated gene expression of UDP-galactose-4-epimerase (GALE) *in vitro*. Human articular chondrocytes from three individuals were treated with 0, 10, 20 and 40 ng/ml human recombinant IL-1β for 12 h, 24 h and 48 h. Total glycosaminoglycan (GAG) content (A) and GALE mRNA expression level (B) was detected using 1,9-dimethylmethylene blue staining and real-time quantitative PCR. Meanwhile, relative GALE protein levels in chondrocytes from three patients (samples 1, 2 and 3) were detected after IL-1β treatment for 48 h (C and D) using Western blotting. Values were presented as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* control.

must keep synthesizing new PGs to replace these lost in the turnover. The biosynthesis of PGs starts with the step-wise addition of carbohydrate moieties from the corresponding high energy donors, the UDP-sugars, onto the serine residues of the core protein, which consequently forms the GAG chain of PGs [10,24,25]. As a key enzyme that catalyzes the interconversion of UDP-Glc and UDP-Gla, as well as UDP-GlcNAc and UDP-GalNAc, GALE has been described essential in modulating GAG biosynthesis at the level of UDP-sugar precursors in species like bacterium and fungus [26–28]. However, there is no direct evidence showing that GALE might also play a critical role in PGs synthesis of chondrocytes. In the present study, GALE specific siRNAs significantly suppressed GAG synthesis of human articular chondrocytes *in vitro*, which indicated a critical role of GALE in restoring the extracellular matrix of chondrocytes, thus in maintaining the homeostasis of articular cartilage.

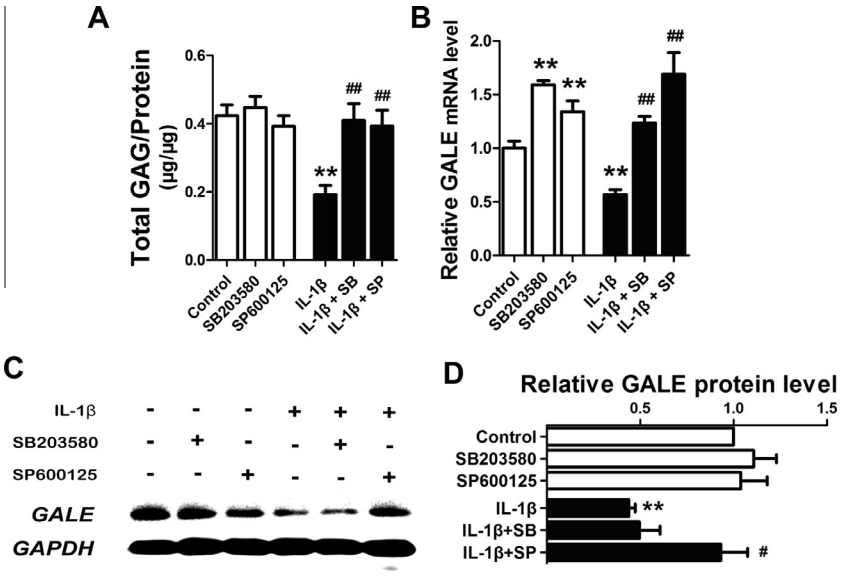

Fig. 4. Pathways that mediated the suppression of interleukin 1 beta (IL-1β) on UDP-galactose-4-epimerase (GALE) expression. Human articular chondrocyte was treated with stress-activated protein kinase/c-Jun N-terminal kinase (SAP/JNK) inhibitor SP600125 (SP, 10 μM) or p38 MAPK inhibitor SB203580 (SB, 20 μM) for 0.5 h and subsequently co-treated with the inhibitors and IL-1β (10 ng/mL) for another 48 h. Then, glycosaminoglycan (GAG) content (A), mRNA (B) and protein (C and D) expression of GALE was detected using 1,9-dimethylmethylene blue staining, real-time quantitative PCR and Western blotting, respectively. Values were presented as mean ± SEM. $^{**}P < 0.01$ *versus* control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* IL-1β group.

OA is a high-prevalence arthritis in the elderly, which is the outcome of a combination of multiple factors including age, obesity and traumas [2,3]. However, the biochemical basis of OA still remains the unbalance between the catabolism and anabolism of chondrocytes and synoviocytes, which disturbs the homeostasis of articular cartilage and consequently causes the continuous loss of the cartilage matrix in OA progress [2–4]. On one hand, the expression of the matrix metalloproteinases (MMPs) and aggrecanases, two major type of factors responsible for the degradation of PGs and collagens of cartilage matrix, are highly stimulated in chondrocytes and synovial cells both in OA patients and OA animal models [29,30]. On the other hand, the synthesis and secretion of matrix components of OA chondrocyte were, instead, suppressed, while the newly synthesized PGs remained less uniform and less functional, which could not restore the structure and biological strength of cartilage [22]. Here, we found that protein level of GALE was obviously decreased in both human and rat OA cartilage, which was accompanied by the loss of PGs and disorganization of cartilage. Combined with the data that GALE is essential in PGs synthesis of articular chondrocyte, our data demonstrated for the first time that suppressed GALE expression might contribute to or even trigger the OA progress due to the suppressed PGs synthesis of articular chondrocytes. Meanwhile, up-regulating GALE gene expression in chondrocytes might possibly help to restore the matrix of articular cartilage in OA.

It is now believed that inflammation is closely involved in the development and progression of OA, even in the early stage [31]. Therefore, pro-inflammatory factors produced by cartilage, synovium and other articular tissues become the critical mediators of the disturbed homeostasis of cartilage [31]. Among all the pro-inflammatory factors involved in OA progress, IL-1β has long been believed to be the key player responsible for the cartilage degeneration, which stimulates the catabolism of articular chondrocyte, namely promotes the release of MMPs, aggrecanases and other degenerative factors, and induces the production of other cytokines like IL-6 and IL-8 as well [30,32–37]. Moreover, numerous studies demonstrated that IL-1β also inhibited the anabolic metabolism of articular chondrocytes, which leaded to the decreased quantity and poor quality of PGs and other major extracellular matrix components of cartilage [38–41]. The suppressive effect of IL-1β on PG synthesis of chondrocytes was mediated by the inhibition of two key GTs involved in the elongation of GAG chains, namely XT-1 and GlcAT-1 [16–18]. In the present study, we found for the first time that IL-1β-induced suppression of PGs synthesis also involved GALE. And IL-1β induced GALE gene expression in the early phase but suppressed its expression in the late phase, which was in accordance with the report that XT-1 mRNA expression was stimulated by IL-1β before 12 h, but suppressed after 24 h [15]. As OA is a chronic disease, there always a long-term exposure of the chondrocytes to IL-1β. So, these data indicated that the change of GALE gene expression from stimulation to suppression by IL-1β might possibly participated in the dynamical alterations from the compensation in the early phase to the decompensation in the late phase of OA progress.

Meanwhile, IL-1β triggers its biological activities through binding to its specific receptor located on the cell surface, namely the IL-1 receptor type 1, and then activates the downstream signaling cascades including SAP/JNK, p38 MAPK and NF-κB signaling pathway [31,42,43]. It is believed that the three pathways are all involved in the metabolic disturbance induced by IL-1β, while NF-κB signaling is mainly responsible for the inflammatory activity of IL-1β [12,32]. Further, both SAP/JNK and p38 MAPK pathway mediated the IL-1β-induced promoter activity of XT-1 gene, which consequently caused the inhibition of XT-1 gene expression and suppression of PGs synthesis in articular chondrocytes [15]. However, the down-regulation of GALE gene expression by IL-1β was mainly mediated by the SAP/JNK pathway, for p38 MAPK pathway just alleviated the suppression of IL-1β on the mRNA expression but not the protein expression of GALE. It is reported that p38 MAPK signaling pathway determines the biogenesis of multiple miRNAs [44,45]. Modulating p38 MAPK pathway might possibly lead to changes in the biogenesis of certain miRNAs, which subsequently caused the discrepancy of the GALE mRNA and protein level after SB203580 treatment.

In conclusion, our present study suggested that GALE gene is essential in maintaining the homeostasis of cartilage matrix due

to its critical role in PGs synthesis. Meanwhile, mainly mediated by the SAP/JNK signaling pathway, the suppressed GALE gene expression induced by IL-1β might contribute to the progress of OA.

Competing interest statement

No potential conflict of interest exists in this study.

Authors' contributions

Y.X.W. designed the study, carried out the experimental work, analyzed and interpreted the data, and drafted the manuscript. J.Q., Y.D., H.W., J.M. and L.B.C. designed the study, interpreted the data, and drafted the manuscript as well. All authors read and approved the final manuscript.

Acknowledgment

This work was supported by Grants from the National Natural Science Foundation of China (Nos. 81371940, 81220108026, 81430089, 81401832).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.148.

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