Cbfβ Is a Novel Modulator Against Osteoarthritis by Maintaining Articular Cartilage Homeostasis Through TGF-β Signaling

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Posted Date: 16 February 2023

doi: 10.20944/preprints202302.0284.v1

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Article

Cbfβ is a Novel Modulator against Osteoarthritis by Maintaining Articular Cartilage Homeostasis through TGF-β Signaling

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Abstract: TGF-β signaling is a vital regulator for maintaining articular cartilage homeostasis. Runx transcription factors, downstream targets of TGF-β signaling, have been studied in the context of osteoarthritis (OA). Although Runx partner core binding factor β (Cbfβ) is known to play a pivotal role in chondrocyte and osteoblast differentiation, the role of Cbfβ in maintaining articular cartilage remains obscure. This study investigated Cbfβ as a novel anabolic modulator of TGF-β signaling and determined its role in articular cartilage homeostasis. Cbfβ significantly decreased in aged mouse articular cartilage and human OA cartilage. Articular chondrocyte-specific *Cbfb*-deficient mice (*Cbfb*$^{\triangle ac/\triangle ac}$) exhibited early cartilage degeneration at 20 weeks old and developed OA at 12 months old. *Cbfb*$^{\triangle ac/\triangle ac}$ mice showed enhanced OA progression under the surgical-induced mice OA model. Mechanistically, forced expression of Cbfβ rescued Col2α1 and Runx1 expression in Cbfβ-deficient chondrocytes. TGF-β1-mediated Col2α1 expression failed despite the pSmad3 activation under TGF-β1 treatment in Cbfβ-deficient chondrocytes. Cbfβ protected Runx1 from proteasomal degradation through Cbfβ/Runx1 complex formation. These results indicate that Cbfβ is a novel anabolic regulator for cartilage homeostasis, suggesting that Cbfβ could protect OA development by maintaining the integrity of the TGF-β1 signaling pathway in articular cartilage.

Keywords: articular cartilage; Runx1/Cbfβ complex; Osteoarthritis; TGF-β signaling; proteasomal degradation

1. Introduction

Osteoarthritis (OA), the most common and painful disease of the joints, affects nearly half of the world's elderly population and represents an enormous socioeconomic challenge [1, 2]. OA is a joint disease with structural and functional changes in the articular cartilage, subchondral bone, ligaments, capsule, synovium, sensory nerve endings, meniscus, and periarticular muscles. Among these structural components, many studies focus on cartilage or subchondral bone. Regarding OA initiation, both subchondral bone and articular cartilage changes are critical factors for OA initiation [1, 3-5]. A dynamic equilibrium between synthesis and degradation of the extracellular matrix, such as Col2α1, proteoglycans, and Mmp13, controls articular cartilage homeostasis and integrity [4]. The inactivation of one copy of Col2α1 leads to articular cartilage degeneration and increased OA development [6]. In the early stage of OA, cartilage matrix degradation occurs in the superficial zone

 doi:10.20944/preprints202302.0284.v1

of the cartilage but later extends to deeper zones as OA progresses [7]. Mmp13 is a major enzyme that targets the breakdown of cartilage extracellular matrix such as type II collagen and proteoglycans [8]. Clinical investigation reveals a strong association between articular cartilage destruction with high Mmp13 expression [9].

Tight control of TGF-β signaling plays an essential role in articular cartilage homeostasis [10]. Injection of TGF-β into the knee joint increases proteoglycan, whereas injection of recombinant soluble TGF-β type II receptor, an endogenous inhibitor, markedly worsens OA phenotypes [11, 12]. TGF-β Type I receptor ALK1 (Activin Receptor-like Kinase) correlated well with Mmp13 expression, whereas ALK5 correlates with aggrecan and collagen type II. In senescent and OA articular chondrocytes, ALK5 expression was significantly decreased compared to ALK1, increasing the ALK1/ALK5 ratio, which is associated with the upregulation of Mmp13 in OA [13]. Indeed, chondrocyte-specific loss of Smad3, ALK5, or the overexpression of dominant-negative TGF-β type II receptor in mice results in OA development at an early stage [14, 15]. These findings indicate that abnormal TGF-β signaling increases OA development. Thus, the integrity of the TGF-β signaling pathway is essential for the healthy maintenance of articular cartilage and for preventing OA. Here, we explored the TGF-β signaling integrity regulated by Cbfβ.

Cbfβ is a partner protein of the runt-related transcription factor (Runx) family, which include Runx1, Runx2, and Runx3. It interacts with the Runt domain of Runx to enhance its DNA binding properties and transcription activity [16, 17]. Runx1 is a pivotal transcription factor for chondrogenesis, chondrocyte proliferation, and survival. Runx1 in mouse embryonic mesenchymal cells results in a potent induction of early chondrocyte differentiation markers such as type II collagen but not the hypertrophy marker, type X collagen [18]. Runx1 also acts on the superficial zone of articular chondrocytes to stimulate chondrocyte proliferation and promote anabolic protein expression to maintain articular cartilage integrity [19]. Deletion or carboxyl terminus truncation of Runx2 inhibits endochondral bone formation with the arrest of chondrocyte maturation, resulting in the complete absence of mature osteoblast formation [20-22]. Runx2 and Runx3 are both expressed in cartilaginous condensations of the mouse limb. The cooperative roles of these two proteins in mediating chondrocyte maturation, hypertrophy, and cartilage formation have been established in vivo using double-knockout mice [23]. Homozygous deletion of *Cbfb* in mice causes embryonic lethality during pregnancy due to brain hemorrhage and reduces Runx1 stability and transcriptional activity to block fetal hepatic hematopoiesis [24, 25]. Cbfβ plays an essential role in endochondral bone formation through skeletal tissue cell-type-specific *Cbfb* deletions, including mesenchymal cells [26, 27], chondrocytes [28, 29], and osteoblasts [30]. Since articular chondrocytes remain as non-hypertrophied hyaline cartilage during development, it is interesting to determine how the joint degenerates and progresses to OA as the articular chondrocytes die prematurely or undergo abnormal hypertrophy [7]. However, the role of Cbfβ during articular cartilage development and the evolution of OA is not well understood.

To determine the function of Cbfβ in articular cartilage and its effect on the development of OA, we analyzed the results of the deletion of *Cbfb* from articular chondrocytes using *Gdf5-Cre* and *Cbfb-floxed* mice together with a surgically induced OA model.

2. Materials and Methods

2.1. Antibodies and reagents

We purchased antibodies, including rabbit anti-HA, anti-Runx1, anti-Runx3, monoclonal anti-Actin, and goat anti-Lamin B antibodies from Santa Cruz Biotechnology (CA, USA), rabbit anti-Myc, anti-col2α1, mouse monoclonal anti-Mmp13, anti-Runx2, and anti-type II collagen from Abcam (MA, USA), rabbit anti-Smad3 and anti-phospho-Smad3 from Cell Signaling (MA, USA), monoclonal anti-Flag, an anti-HA-agarose antibody from Sigma-Aldrich (St. Louis, MO, USA). We obtained TGF- β 1, IL-1β, and recombinant TNF- α from R & D Systems (Minneapolis, USA), and hematoxylin, fast green, Safranin-O, human transferrin, and sodium selenite from Sigma-Aldrich.

 doi:10.20944/preprints202302.0284.v1

2.2. Mice and experimental OA

Mice harboring an articular chondrocyte-specific deletion of *Cbfb* (*Cbfb*$^{\Delta ac/\Delta ac}$) obtained by crossing *Gdf5-Cre* transgenic mice (*Cre*$^{Tg/+}$, kindly provided by Dr. David Kingsley from Stanford University, U.S.A) (38) with *Cbfb*$^{fl/fl}$ mice (39), both maintained on C57BL/6N background. The loxP sites of the *Cbfb*$^{fl/fl}$ mice encompass the exon 5 of Cbfβ, resulting in articular chondrocyte-specific deletion of exon 5 in *Cbfb*$^{\Delta ac/\Delta ac}$ mice. *Cbfb*$^{fl/fl}$ served as wild-type control (WT). Twelve-week-old male mice in both knees were conducted destabilization by the medial meniscus (DMM) OA surgery, and mice were sacrificed at 8 weeks after DMM surgery to assess OA progression (each group n=6) (40). We used twelve-month-old male mice for spontaneous OA progression analysis (WT=5, *Cbfb*$^{\Delta ac/\Delta ac}$=5). All animal procedures followed the guidelines issued by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU-201455).

2.3. Human subjects

Human OA joint cartilage tissues were obtained from OA surgery patients through total knee arthroplasty. The Institutional Review Board of Kyungpook National University Hospital approved using human OA cartilage. We obtained written informed consent from all patients prior to the surgical procedure (IRB File No of KNUH 2022-01-010-001).

2.4. β-galactosidase (β-gal) staining

Whole tissues were fixed for 30 min in 4% paraformaldehyde (PFA) in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM MgCl2, 5 mM ethylene glycol tetra-acetic acid (EGTA), then washed for 30 min with 0.1 M potassium phosphate buffer. Tissues were kept in the X-gal staining solution overnight at 37℃ and then washed briefly in phosphate-buffered saline (PBS). We made cryosections of the stained samples, fixed the slides briefly in 4% PFA, washed them in PBS, and counterstained them with Nuclear Fast Red (Sigma). Samples were washed in PBS and analyzed with a Leica microscope [41].

2.5. Assessment of OA severity

Knee joints were fixed with 4% PFA and decalcified with 10% ethylenediaminetetraacetic acid (EDTA; pH 7.4) for 4 weeks. Decalcified tissues were dehydrated by ethanol, embedded in paraffin, and then sectioned with a thickness of 3 μm. We performed Safranin-O staining as in the previous study [5]. Briefly, sections were treated in the following order: deparaffinization, rehydration, and soaking in Weigert's iron hematoxylin solution for 10 min each, followed by fast green solution and 0.1% Safranin-O solution for 5 min each. Assigning OA progression followed the Osteoarthritis Research Society International (OARSI) diagnosis criteria [5].

2.6. Immunohistochemistry

We quenched the sections with 3% H_2O_2 and retrieved antigens by boiling them in TEG buffer (1.211 g of Tris and 0.190 g of EGTA in 1L MilliQ-water, pH 9.0). After blocking with 1% bovine serum albumin for 1 hour at room temperature, sections were incubated with anti-Cbfβ, anti-Runx1, anti-type II collagen, and anti-Mmp13 antibodies overnight at 4°C and incubated with goat anti-rabbit IgG or goat anti-mouse IgG conjugated HRP for 1 hour. Signals developed with a DAB substrate-chromogen system (Dakocytomation, Denmark).

2.7. Cell culture

Primary articular chondrocytes derived from the hindlimb knee joint of wild-type and *Cbfβ*$^{\Delta ac/\Delta ac}$ mice on postnatal day 5, as previously described (42). Chondrocytes were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, ME, USA) containing 10% fetal bovine serum (FBS) (Gibco-BRL, USA) and the appropriate penicillin/streptomycin. Chondrogenic ATDC5 cells were

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cultured in DMEM/F12 (Lonza) medium supplemented with 5% fetal bovine serum (FBS), 10 μg/ml human transferrin, and $3x10^{-8}$ M sodium selenite. Chondrocytes were plated at a density of 2×10^5 cells/well on 6 well plates for drug treatment. Protein or total RNA was isolated from chondrocytes after 24 h treatment with TGF-β1 (1, 10, or 100 ng/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml).

2.8. Western blot analyses

Total protein lysates were isolated from human OA articular cartilage, mouse tibia articular cartilage of 12-month-old mice, and articular chondrocytes derived from articular cartilage by M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford) containing a cocktail of protease inhibitors and phosphatase inhibitors. Proteins were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The western blot was performed using the indicated antibodies.

2.9. qRT-PCR analyses

Total RNA from hind limbs joint cartilage of 12-month-old mice and articular chondrocyte cells were isolated using the easy-BLUE Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea) and cDNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, CA, U.S.A). qRT-PCR was performed using the Power SYBR green master mixture (Applied Biosystems, Foster, CA). The qRT-PCR primers were designed using Primer Express software (Applied Biosystems). Sequences of qRT-PCR primers showed in Table 1.

Target gene	Forward sequence (5′ - 3′)	Reverse sequence (5′ - 3′)
Gapdh	GCATCTCCCTCACAATTTCCA	GTGCAGCGAACTTTATTGATGG
Cbfb	TATGGGTTGCCTGGAGTT TG	AAGGCCTGTTGTGCTAATGC
Col2α1	TTCCACTTCAGCTATGGCGA	GACGTTAGCGGTGTTGGGAG
Aggrecan	GAGAGAGGCGAATCGAACGA	CGTGAAGGGCAGCTGGTAAT
Mmp9	AAACCAGACCCCAGACTCCTC	GAGGACACAGTCTGACCTGAA
Mmp13	GCCAGAACTTCCCAACCATG	TCAGAGCCCAGAATTTTCTCC
Mmp14	GGATGGACACAGAGAACTTCGTG	CGAGAGGTAGTTCTGGGTTGAG
Mmp15	CTGAGCAGCTATGGCACAGACA	TGCTGTGTCTCCTCGTTGAAGC
IL-6	TTGCCTTCTTGGGACTGATG	CTGAAGGACTCTGGCTTTGT
IL-17	CTCAAAGCTCAGCGTGTCCAAACA	TATCAGGGTCTTCATTGCGGTGGA
IL-18	CAGGCCTGACATCTTCTGCAA	TTTGATGTAAGTTAGTGAGAGTGA
IL-22	GGTGACGACCAGAACATCCA	GACGTTAGCTTCTCACTTTCCTT

2.10. Co-immunoprecipitation (Co-IP) assay

Primary articular chondrocytes were plated at a density of 2×10^6 cells/well in 100 mm plates for transfection experiments. Cells were transfected with 10μg DNA, including 5μg of the pcDNA3.1-myc-Cbfβ and 5μg of pCS4-2Flag-Runx1 by using Lipofectamine 2000 (Invitrogen) and grown for 24 h. Cell lysates (400 μg) were bound with anti-Myc at 4^0C overnight with gentle agitation, then incubated with 0.1g of Sepharose A beads (GE Healthcare) in lysis buffer for 2 h at 4 ^{0}C with gentle agitation. Mixtures were washed with lysis buffer five times, and proteins were eluted by boiling with 50μl protein loading buffer. Co-IP was performed by western blot with anti-Myc or anti-Flag antibody.

2.11. Poly-ubiquitination assay

ATDC5 chondrocytes were transfected with 12μg DNA, including 4μg pCS4-Flag-Runx1, 4μg pcDNA3.1-HA-Ubiquitin, and 4μg pCS4-Myc-Cbfβ by using Lipofectamine 2000 (Invitrogen) and

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grown for 24 h. After 24 h from transfection, cells were incubated with 20μm MG132 for 2 h. Cell lysates (400 μg) were allowed to bind with anti-Flag antibody at 4℃ overnight with gentle agitation, then incubated with 0.1g Sepharose A beads (GE Healthcare) in lysis buffer for 2 h at 4℃ with gentle agitation. Proteins were eluted by boiling with 50μl protein loading buffer. Ubiquitination levels were evaluated by western blot with anti-HA antibody.

2.11. Statistical analyses

We used GraphPad Prism 9 (GraphPad, San Diego) for statistical analysis. Paired or unpaired Student's t-test was used to analyze the statistically significant between the two groups comparison. We considered a P <0.05 to be statistically significant (*P < 0.05, **P < 0.01), and ns represents no significance. Data are expressed as mean ± SD or mean ± SE.

3. Results

3.1. Cbfβ was enhanced by anabolism and suppressed by catabolism

To assess the involvement of Cbfβ in articular cartilage metabolism, we analyzed the expression of Cbfβ under TGF-β1, interleukin-1β (IL-1β), or TNF-α activation. Treatment of various concentrations (1, 10, and 100 ng/ml) of TGF-β1, one of the pivotal anabolic factors in the articular cartilage, increased pSmad3 and Cbfβ protein levels in the articular chondrocytes (Fig. 1a). TGF-β1 also increased the mRNA expression of Cbfβ, Type II collagen, and Aggrecan (Fig. 1b). The pro-inflammatory cytokines IL-1β or TNF-α decreased Cbfβ expression along with type II collagen and Aggrecan, while Mmp13 expression increased in primary cultured mouse articular chondrocytes (Fig. 1c). We confirmed that Cbfβ decreased under IL-1β or TNF-α treatment in the fluorescent staining (Fig. 1d). Moreover, Cbfβ silencing in chondrocytes upregulated the expression of Mmps such as Mmp9, Mmp13, Mmp14, and Mmp15, and inflammatory cytokines Il-6, IL-17, 1L-18, and IL-22 in the chondrocytes (Supplementary Fig. 1a and 1b). Taken together, Cbfβ was enhanced by anabolism and suppressed by catabolism in the articular chondrocytes.

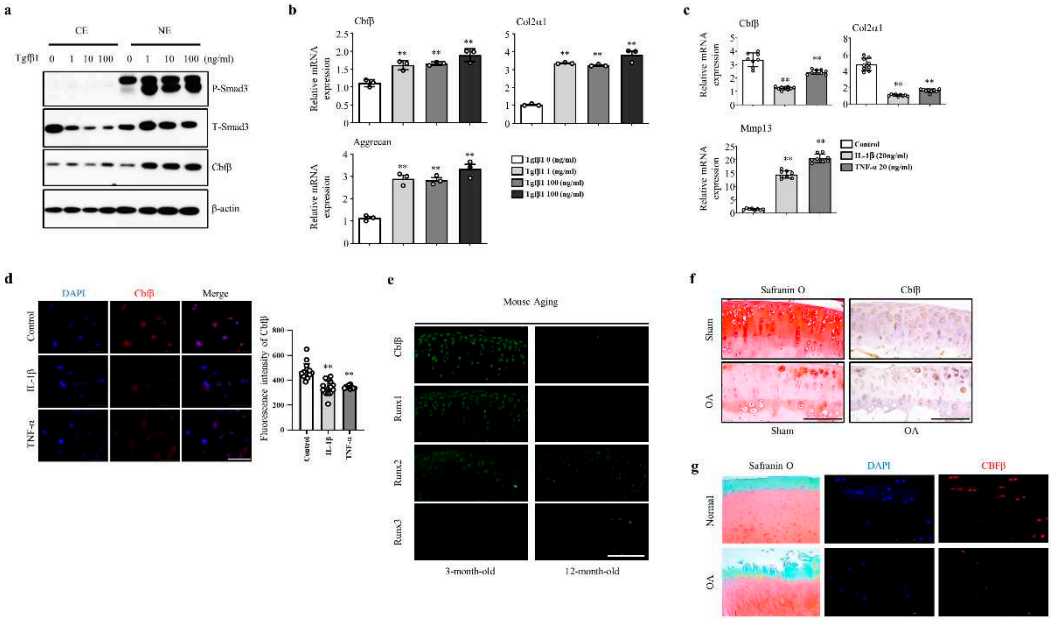

Figure 1. Cbfβ loss is involved in OA pathogenesis. (a) Cbfβ and pSmad 3 protein expression detected by Western blotting in articular chondrocytes under TGF-β1 treatment. NE, nuclear extracts; CE, cytoplasmic extracts (b) Cbfβ, Col2α1, and Aggrecan mRNA expression detected by qRT-PCR in articular chondrocytes under TGF-β1 treatment. (c) Cbfβ, Col2α1, and Mmp13 mRNA expression detected by qRT-PCR in articular chondrocytes under IL-1β or TNF-α treatment. (d) Cbfβ determined by immunofluorescence staining after treatment of IL-1β and TNF-α in articular chondrocytes. Scale bars, 100 μm. (e) Expression of Cbfβ, Runx1, Runx2, and Runx3 at 3 and 12 months of age evaluated

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by immunofluorescence staining (n=3). Scale bars, 100 μm. (f) Mouse joints OA development assessed by Safranin O staining, Cbfβ expression by immunohistochemistry. Scale bars, 100 μm. (g) Human OA development was assessed by Safranin O staining and Cbfβ expression by immunofluorescent (n=6). Scale bars, 100 μm.

3.2. Cbfβ loss is involved in articular cartilage degeneration

We then investigated whether there was a correlation between Cbfβ expression and articular cartilage degeneration *in vivo*. Aged articular cartilage reduced Cbfβ and its partner protein Runx1, but the expression of Runx2 and Runx3 was not significantly different compared to the young articular cartilage (Fig. 1E). Moreover, the expression of Cbfβ decreased in both mouse and human osteoarthritic cartilage (Fig. 1F-G). These results suggest that loss of Cbfβ may be involved in articular cartilage degeneration. Next, we investigated Cbfβ functions in joint development using articular cartilage-specific *Cbfb* deleted *Gdf5-Cre; Cbfβ*$^{fl/fl}$ (*Cbfb*$^{\triangle ac/\triangle ac}$) mice. With *Rosa26* reporter (*R26R*) mice *Gdf5-Cre* activities were specifically observed in the articular cartilage, with around 75% deletion in articular cartilage by qRT- PCR (Supplementary Fig. 2A and 2B) and immunofluorescent staining in E16.5 (Supplementary Fig. 2C and 2D) and 20-week-old (Supplementary Figure 1e). *Cbfb*$^{\triangle ac/\triangle ac}$ mice displayed typical joint and cartilage formation at E14.5 and E16.5 embryonic stages (Fig. 2a and 2b). To further understand the effect of Cbfβ on articular cartilage homeostasis, we performed the articular cartilage integrity assessment with histochemical analysis at 12-month-old mice joints. *Cbfb* $^{\triangle ac/+}$ mice displayed higher OARSI scores than WT mice but without statistical significance. However, *Cbfb*$^{\triangle ac/\triangle ac}$ mice exhibited high OARSI scores with severe cartilage destruction (Fig. 2c and 2d). Cbfβ, Aggrecan, and Col2α1 mRNAs decreased, whereas Mmp13 expression increased in the articular cartilage of 12-month-old *Cbfb*$^{\triangle ac/\triangle ac}$ mice (Fig. 2e). These results indicate that Cbfβ physiologically protects articular cartilage during aging and that deletion of *Cbfb* causes age-dependent spontaneous OA progression.

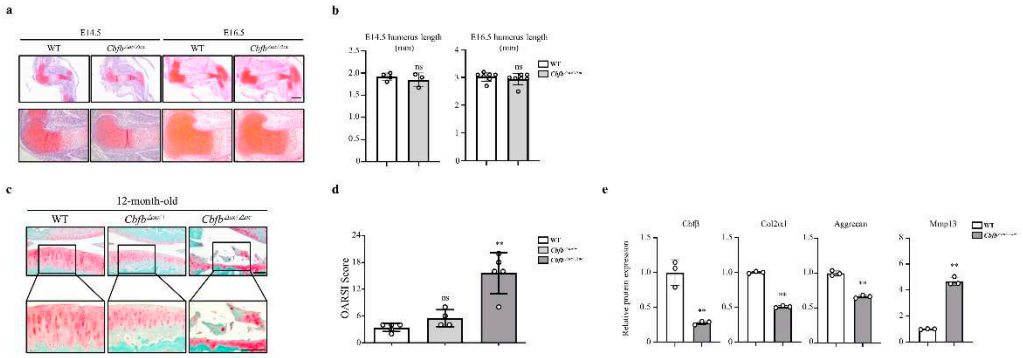

Figure 2. Joint cartilage-specific Cbfb deleted mice exhibit spontaneous cartilage degeneration. (a, b) Evaluation of joint and limb formation by Safranin-O staining at E14.5 (WT=3, *Cbfb*$^{\Delta ac/\Delta ac}$=4) and E16.5 (WT=7, *Cbfb*$^{\Delta ac/\Delta ac}$=7) of *Cbfb*$^{\Delta ac/\Delta ac}$ mice. The humerus length was analyzed with the Leica program. Scale bars, 500 μm & 100 μm. (c) Spontaneous OA development assessed by Safranin O staining in 12-month-old *Cbfb*$^{\Delta ac/\Delta ac}$ mice. (d) OA severity was determined by histological analysis according to OARSI guidelines. Scale bars, 100 μm (WT=5, *Cbfb*$^{\Delta ac/\Delta ac}$=4, *Cbfb*$^{\Delta ac/\Delta ac}$=5). (e) Cbfβ, Col2α1, Aggrecan, and Mmp13 mRNA expression were evaluated by qRT-PCR in 12-month-old *Cbfb*$^{\Delta ac/\Delta ac}$ articular cartilage.

3.3. Genetic deletion of Cbfb accelerated OA progression

Cbfβ deficiency induced spontaneous OA development and presumed accelerated knee joint degradation in a surgically induced OA model. To explore this idea using a DMM surgery-induced mouse OA model, we confirmed the *Cbfb*$^{\triangle ac/\triangle ac}$ mice joints' OA progression. Safranin O staining and scoring by the OARSI method revealed that *Cbfb*$^{\triangle ac/\triangle ac}$ mice joints induced severe damage to articular

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cartilage compared with controls. Indeed, *Cbfb*$^{\triangle ac/\triangle ac}$ mouse joints, even the sham group, showed more cartilage degeneration with a high OARSI score compared to WT mice at 20 weeks (Fig. 3a and 3b). Immunohistochemical staining decreased articular cartilage proteins that provide protection, such as Runx1 and Col2α, were decreased (Fig. 3c). Conversely, the matrix-degrading enzyme Mmp13 increased on the articular surface of *Cbfb*$^{\triangle ac/\triangle ac}$ mice compared to those of WT mice (Fig. 3c). TGF-β1 is one of the most critical anabolic regulators for articular cartilage integrity. Hence, we determined whether Cbfβ could modulate TGF-β1 signaling in the *Cbfb*$^{\triangle ac/\triangle ac}$ mouse articular cartilage. In fluorescence staining, the signal of pSmad3 unexpectedly increased in the *Cbfb*$^{\triangle ac/\triangle ac}$ mouse joint cartilage (Fig. 3d-e). These data raised another question as to why TGF-β1 signaling activation could not increase Type II collagen expression in *Cbfb*$^{\triangle ac/\triangle ac}$ mouse join cartilage.

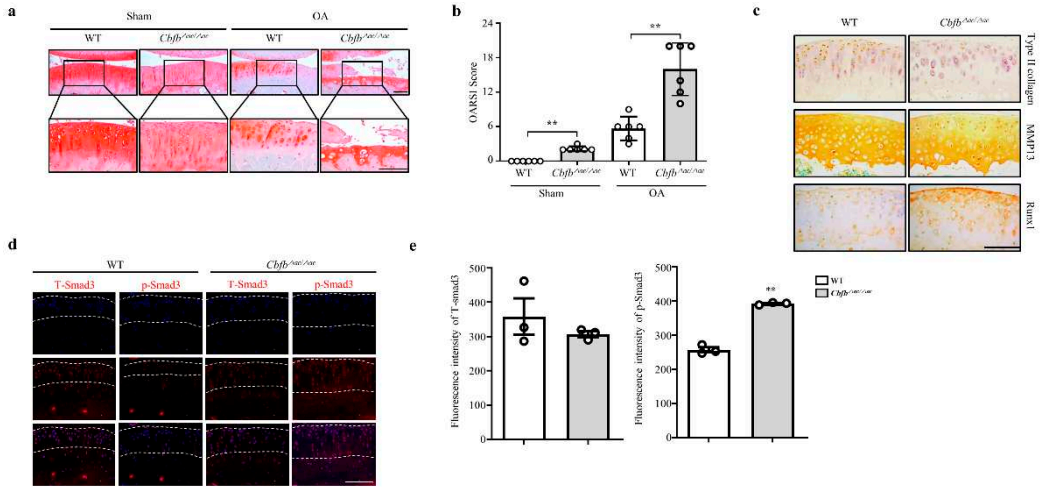

Figure 3. Deletion of *Cbfb* in the articular chondrocytes accelerated OA pathogenesis progression. (a) Articular cartilage destruction was determined by Safranin O staining 8 weeks after DMM surgery. Articular cartilage destructed severely in *Cbfβ*$^{\triangle ac/\triangle ac}$ mice. (b) The severity of OA was analyzed according to OARSI guidelines (n=6). Scale bars, 100 μm. (c) Immunohistochemical staining was performed on paraffin sections using anti-Runx1, -Col2α1, and -Mmp13 antibodies. (d) Expression of pSmad3 (left panel) and total Smad3 (T-Smad, right panel) in articular cartilage (AC) assessed by immunofluorescence staining in WT and *Cbfβ*$^{\triangle ac/\triangle ac}$ 20-week-old mice. (e) The pSmad3 and T-Smad3 fluorescence intensity of (e) were analyzed with the Leica fluorescence analysis program (n=3). Scale bars, 100 μm.

3.4. Cbfβ modulates articular cartilage integrity by modulating TGF-β1 signaling

To clarify how Cbfβ is involved in the TGF-β1 mediated Type II collagen expression, we performed a TGF-β1 responsive assay in the Cbfβ deleted chondrocytes. Interestingly, TGF-β1 treatment increased p-Smad3 in the *Cbfb*$^{\triangle ac/\triangle ac}$ articular chondrocytes compared to WT cells under the (Fig. 4a). Consistently with *in vivo* data, TGF-β1-mediated Cbfβ and Col2α1 expression were reduced in *Cbfb*$^{\triangle ac/\triangle ac}$ articular chondrocytes compared with WT (Fig. 4b-c). These results indicate that Cbfβ plays an essential role in maintaining TGF-β signaling pathway integrity in the articular cartilage.

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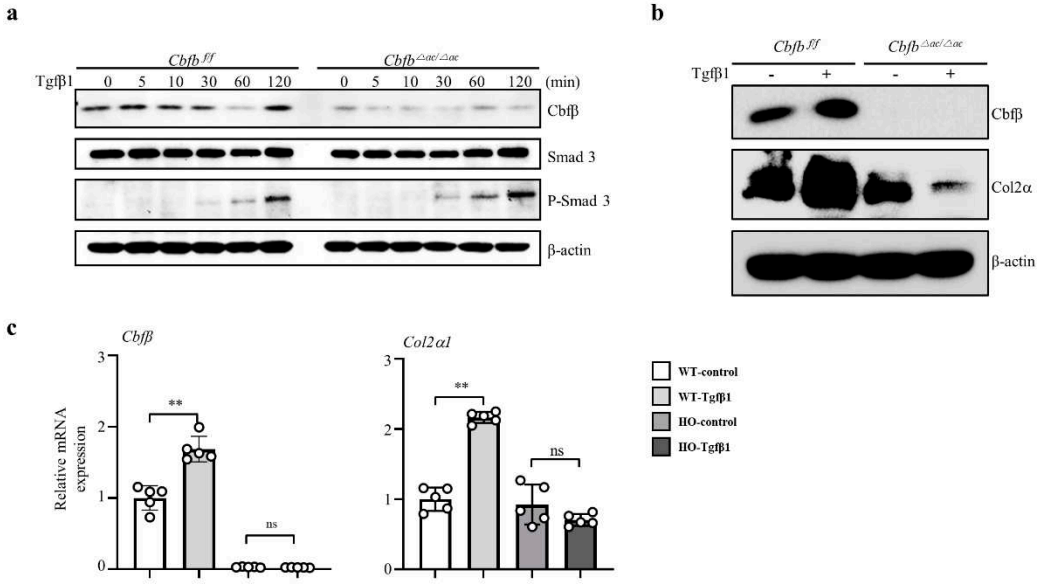

Figure 4. Impaired TGF-β1-mediated Col2α1 transcription in Cbfβ-deficient articular chondrocytes. (a) Cbfβ, pSmad3, and T-smad3 expression were evaluated by western blotting in *Cbfβ*$^{\triangle ac/\triangle ac}$ articular chondrocyte. (b) Effects of TGF-β1 treatment for 24 h on Cbfβ and Col2α1 protein levels by western blotting. (c) The expression of Cbfβ, Runx1, and Col2α1 mRNA was measured by qRT-PCR.

3.4. Cbfβ stabilizes Runx1 in articular chondrocytes

Runx1, Runx2, and Runx3 are crucial targets of TGF-β superfamily signaling [31]. Runx1 is a central regulator of articular cartilage integrity by coordinating YAP, TGF-β, and Wnt signaling in articular cartilage formation and maintenance by enhancing type II collagen and matrix production in collaboration with Sox trios [43]. We, therefore, hypothesized that Runx1 proteolysis resulted in accelerated cartilage degeneration in the Cbfβ deleted articular chondrocytes [32]. To test this hypothesis, we examined the expression of the Runx1 and Col2α1 in the Cbfβ deleted articular chondrocytes. The levels of Runx1 and Col2α1 were reduced in Cbfβ deleted articular chondrocytes from *Cbfb*$^{\triangle ac/\triangle ac}$ mice, whereas rescue of Cbfβ in *Cbfb*-deleted articular chondrocytes restored Runx1 and Col2α1 (Fig. 5a-b). To further investigate the effects of Cbfβ on Runx1stability in articular chondrocytes, we performed co-immunoprecipitation and poly-ubiquitination (Figure 5c). In articular chondrocytes, Cbfβ can endogenously bind Runx1 and protect Runx1 from polyubiquitination-mediated proteasomal degradation (Fig. 5c-d). These results suggest that Cbfβ makes the complex with Runx1, stabilizing Runx1 in articular chondrocytes.

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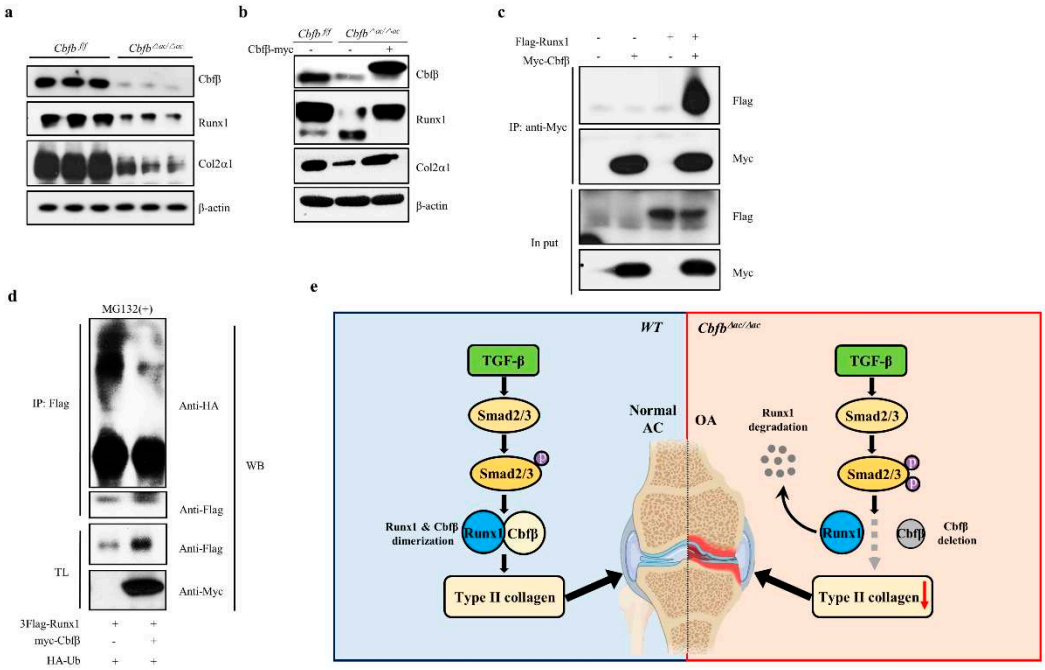

Figure 5. Requirement of Cbfβ for Runx1 stabilization in articular chondrocytes. (a) Total protein was extracted from primary articular chondrocytes derived from WT or *Cbfβ*$^{\triangle ac/\triangle ac}$ mice and western blotting was performed with anti-Cbfβ, -Runx1, -Col2α1, and -β-actin antibodies. β-actin as the loading control. (b) Primary cultured articular chondrocytes derived from *Cbfβ*$^{\triangle ac/\triangle ac}$ mice were transiently transfected with Myc-Cbfβ for 24 h, and the expression levels of Cbfβ, Runx1, Col2α1, Mmp13, and β-actin analyzed by Western blotting. WT was relative control. (c) Primary cultures of articular chondrocytes were co-transfected with vectors expressing Flag-Runx1 and Myc-Cbfβ for 24 h. Total extracts were immunoprecipitated with anti-Myc antibody, and Runx1 was detected with anti-Flag antibody by Western blotting. (d) For polyubiquitination assay, ATDC5 chondrocytes were transfected with HA-Ub, Flag-Runx1, and Myc-Cbfβ for 24 h and treated with 10 μM MG132 for the last 2 h. After immunoprecipitation of Runx1 using anti-Flag antibody, Ub, Runx1, Cbfβ levels were determined by western blotting (WB) using anti-HA, anti-Flag, and anti-Myc antibodies. TL, total lysates. (e) Schematic diagram of how the Cbfβ/Runx1 complex affects TGF-β signaling to regulate articular cartilage homeostasis. Deletion of Cbfβ induces spontaneous OA and surgically induced accelerated OA due to rapid Runx1 degradation.

4. Discussion

In this study, we found that the articular cartilage-specific deletion of Cbfβ resulted in spontaneous OA development and exacerbated OA progression in the surgically induced DMM model. Expression of Cbfβ decreased with aging and OA progression in articular cartilage, and only Runx1 among the Runx transcription factors downregulated in response to a decrease in Cbfβ (Fig. 1c and 1d). The absence or rescue of Cbfβ in *Cbfb*-deleted articular chondrocytes determined the levels of Runx1 and Cbfβ/Runx1 complex as well as Col2α1, indicating that Cbfβ/Runx1 complex formation plays a crucial role in maintaining articular cartilage homeostasis. Moreover, Cbfβ as a component of the Cbfβ/Runx1 complex was a key regulator of TGF-β signaling with modulation of pSmad3 in *Cbfb*-deleted articular chondrocytes. A proper Cbfβ/Runx1 complex formation via TGF-β signaling is vital for maintaining normal articular chondrocyte functional integrity and preventing degenerative cartilage diseases of joints such as OA.

Physiological maintenance of articular cartilage requires tight control of the TGF-β signaling pathway [10]. Our study demonstrates the critical role of Cbfβ and Runx1 as a mediator of TGF-β signaling (Fig. 5e). First, Cbfβ and Runx1, like various components of the TGF-β signaling pathway, such as the ALK1/ALK5 ratio [13], decreased with aging and OA progression. The activation of TGF-β signaling increased Cbfβ and Runx1 expression and Cbfβ/Runx1 complex formation. Deletion of

Cbfβ attenuated the activation of the TGF-β signaling pathway; however, forced expression of Cbfβ recovered Runx1 and Col2α1 expression in Cbfβ-deficient chondrocytes. Moreover, the deletion of Cbfβ increased pSmad3 in the articular cartilage of *Cbfb*$^{\triangle ac/\triangle ac}$ mice. The increase of pSmad3 may result from the regulatory role of the negative feedback loop in the TGF-β signaling pathway. Unknown factors regulated by or involved in the Cbfβ/Runx1 in articular chondrocytes need further studies.

Several studies have suggested their involvement regarding the contribution of Runx transcription factors to OA. For example, Runx1 contributes to articular cartilage maintenance by increasing cartilage matrix production and inhibiting hypertrophic differentiation [32, 33]. Another evidence is that the small molecule KGN binds to filamin A, a cytoplasmic sequestrant of Cbfβ, releasing Cbfβ from filamin A, which translocates Cbfβ to the nucleus and binds to Runx1 but not Runx2 of articular chondrocytes [34]. Runx1 is known to stimulate the differentiation of mesenchymal cells into chondrocytes by regulating the expression of type II collagen rather than type X collagen, a hypertrophy marker [18, 34). This study showed that Cbfβ levels correlated well with Runx1 but not with Runx2 and Runx3. In aged articular cartilage, Cbfβ and Runx1 decreased, but Runx2 and Runx3 were not, which also correlated well with the articular cartilage phenotype of *Cbfb*$^{\triangle ac/\triangle ac}$ mice. We also revealed that disruption of the TGF-β signaling integrity by deletion of Cbfβ in articular chondrocytes increases catabolic cytokines and enzymes like IL-6, IL-17, IL-18, IL-22, Mmp 9, Mmp 13, Mmp14, and Mmp15. Therefore, the positive relationship of the Cbfβ/Runx1 complex with Type II collagen is essential to the TGF-β signaling pathway in articular cartilage integrity.

Regarding Runx2 and Runx3, Runx2 expressed negligibly, while Runx3 was comparable to Runx1 in articular cartilage. Our study showed that diminished Runx1 correlated well with the level of Cbfβ, whereas increased Runx2 and Runx3 in the human OA samples did not. Our previous studies showed the protection of Runx2 by Cbfβ in both chondrocytes and osteoblasts [29, 30], suggesting the presence of differential heterodimerization of Cbfβ with Runx transcription factors in skeletal tissues. Some cases of OA upregulate Runx2 with a transition from articular chondrocytes to hypertrophic chondrocytes (Kawaguchi, 2008). Runx2 haploinsufficiency mice show delayed OA progression after induction of knee joint instability [35]. Additionally, the deletion of Runx2 in articular chondrocytes slows the progression of surgery-induced OA [36]. Moreover, chondrocyte-specific Runx2 overexpression accelerates OA progression [37]. These works of literature indicate a positive relationship between OA progression and aberrant expression of Runx2 in articular chondrocytes. However, the Cbfβ study showed that Runx1, but not Runx2 or Runx3, was primarily involved in the aggravated OA phenotype in the DMM model and Cbfβ-deleted spontaneous OA and human OA samples.

5. Conclusions

In this study, deletion of Cbfβ in articular cartilage decreased Runx1 and type II collagen, which enhanced OA progression spontaneously in *Cbfb*$^{\triangle ac/\triangle ac}$ mice. These results indicate that Cbfβ regulates Runx1 protein stability and subsequently modulates Runx1 target genes such as type II collagen in the articular cartilage. Our findings indicate that Cbfβ prevents articular cartilage destruction by maintaining the integrity of the TGF-β signal pathway through the stabilization of Runx1.

Supplementary Materials: They will be provided when this paper is sent to review.

Author Contributions: Conceptualization, X.C., X.J. and J.Y.C.; methodology, X.C., X.J. and J.Y.C.; software, X.C. and X.J.; validation, X.C., X.J. and J.Y.C.; formal analysis, X.C. and X.J.; investigation, X.C. and X.J.; resources, H.J.K., and H.S.K.; data curation, X.C., X.J., H.J.K., and J.Y.C.; writing—original draft preparation, J.Y.C.; writing—review and editing, X.C., X.J., N.R.P., H.J.K., J.B.L., J.L.S., and G.S.S.; visualization, X.C., X.J.; supervision, H.J.K., J.B.L., J.L.S., and G.S.S.; project administration, X.C., X.J. and J.Y.C.; funding acquisition, X.C., and J.Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07043598 & NRF-2021R1I1A1A01047403), the Ministry of Science & ICT (NRF-

2017R1A5A2015391, 2014M3A9D5A01073658). This study was also supported in part by R01DE029311 NIDCR and R01AR039588 NIAMS.

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU-201455). Human OA joint cartilage tissues were obtained from OA surgery patients through total knee arthroplasty. The Institutional Review Board of Kyungpook National University Hospital approved the use of human OA cartilage and written informed consent was obtained from all patients prior to the surgical procedure (IRB File No of KNUH 2022-01-010-001).

Informed Consent Statement: The Institutional Review Board of Kyungpook National University Hospital approved the use of human OA cartilage, and written informed consent was obtained from all patients prior to the surgical procedure (IRB File No of KNUH 2022-01-010-001).

Data Availability Statement: The data that support the findings of this study are openly available in [repository name "Cells-GDF5-CBFB-Figures" and "Cells-GDF5-CBFB-Figures-Raw data"] at https://drive.google.com/drive/folders/15O7G0gX8ypbl9qFUUkizPEd6v3ycJyXC?usp=sharing and https://drive.google.com/drive/folders/18UjNmtxL8LjM9fcgU0ovSlN2WBioVYEA?usp=sharing.

Conflicts of Interest: The authors declare no conflict of interest.

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