XBP1S, a BMP2-inducible transcription factor, accelerates endochondral bone growth by activating GEP growth factor

Feng-Jin Guo a, *, Zhangyuan Xiong a, d, Xiaofeng Han a, Chuanju Liu b, Yanna Liu a, Rong Jiang c, Peng Zhang a

a Department of Cell Biology and Genetics, Core Facility of Development Biology, Chongqing Medical University, Chongqing, China
b Departments of Orthopaedic Surgery and Cell Biology, New York University School of Medicine, New York, NY, USA
c Laboratory of Stem Cells and Tissue Engineering, Chongqing Medical University, Chongqing, China
d Present address: The Bashu School of Science, Chongqing, China

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Abstract

We previously reported that transcription factor XBP1S binds to RUNX2 and enhances chondrocyte hypertrophy through acting as a cofactor of RUNX2. Herein, we report that XBP1S is a key downstream molecule of BMP2 and is required for BMP2-mediated chondrocyte differentiation. XBP1S is up-regulated during chondrocyte differentiation and demonstrates the temporal and spatial expression pattern during skeletal development. XBP1S stimulates chondrocyte differentiation from mesenchymal stem cells in vitro and endochondral ossification ex vivo. In addition, XBP1S activates granulin-epithelin precursor (GEP), a growth factor known to stimulate chondrogenesis, and endogenous GEP is required, at least in part, for XBP1S-stimulated chondrocyte hypertrophy, mineralization and endochondral bone formation. Furthermore, XBP1S enhances GEP-stimulated chondrogenesis and endochondral bone formation. Collectively, these findings demonstrate that XBP1S, a BMP2-inducible transcription factor, positively regulates endochondral bone formation by activating GEP chondrogenic growth factor.

Keywords: X-box binding protein 1 spliced (XBP1S) • chondrogenesis • GEP • BMP2 • unfolded protein response

Introduction

During foetal development of the mammalian skeletal system, the majority of bones form through a process of endochondral ossification. Chondrocytes in the primary centre of ossification begin to grow. Elaborate chondrogenesis is controlled exquisitely by cellular interactions with the growth factors, surrounding matrix proteins and other environmental factors that mediate cellular signalling pathways and transcription of specific genes in a temporal-spatial manner [1–3]. Production of and response to different growth factors are observed at all times, such as transforming growth factor-β (TGF-β) superfamily and bone morphogenetic protein (BMP) subfamily. BMP2 is one of the most important cytokines and plays several important roles in a variety of cellular functions ranging from embryogenesis, cell growth, and differentiation to bone development and the repair of bone fractures [4, 5]. Jang et al. [6] reported that BMP2 activates UPR transducers, such as PERK (PKR-like ER-resistant kinase), OAIS and ATF6 (activating transcription factor 6). BMP2 induces osteoblast differentiation through Runx2-dependent ATF6 expression, which directly regulates osteocalcin transcription. OAIS [7], a member of the CREB/ATF family, activates the transcription of Col1a1 through an unfolded protein response element (UPRE)-like sequence in the osteoblast-specific Col1a1 promoter region. The expression of OAIS in osteoblasts is induced by BMP2, the signalling of which is required for bone formation.

Human XBP1 (X-box-binding protein 1) is a signalling molecule downstream of IRE1 in the IRE1-XBP1 pathway of the UPR and participates in IRE1α-mediated UPR signal transmission. In eukaryotic cell, IRE1 is activated by ER stress and subsequently processes XBP1 mRNA to generate the spliced form of XBP1 protein (XBP1S). XBP1 exists in two forms: XBP1S and XBP1U (XBP1 unspliced form) isoforms [8–10]. Tohmonda [11] reported that inositol-requiring protein 1α (IRE1α), one of the most crucial UPR mediators, and its target transcription factor XBP1 is essential for BMP2-induced osteoblast differentiation. Osterix (Osx, a transcription factor that is indispensable for bone formation) is a target gene of XBP1. The IRE1α-XBP1

*Correspondence to: Prof. Feng-Jin GUO, Department of Cell Biology and Genetics, Chongqing Medical University, Chongqing 400016, China. Tel.: 86-23-15310288670 Fax: 86-23-68485555 E-mail: guo.fengjin@gmail.com

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pathway is involved in osteoblast differentiation through promoting Osterix transcription by XBP1. Although there is some evidence that XBP1 plays an important role in the control of cell proliferation and the differentiation of numerous types of cells and tissues, including adipogenesis, myelomapathogenesis, skeletal muscle myotubes and dendritic cells in ER stress [12–15], little is known about the modulation and physiological significance of XBP1S in chondrocyte development and bone formation. Specifically, the molecular mechanism by which XBP1S regulates chondrogenesis also remains unknown.

Granulin-epithelin precursor (GEP), also referred to as pro-granulin, acrogranin, was first purified as a growth factor from conditioned tissue culture media. Granulin-epithelin precursor is a 593 amino acid secreted glycoprotein with an apparent molecular weight of 80 kD [16–18]. Granulin-epithelin precursor is secreted in an intact form and undergoes proteolysis, leading to the release of its constituent peptides, the granulins [19, 20]. Granulin-epithelin precursor contains 7.5 repeats of a cysteine-rich motif (CXC5–6CX4–5CCX6–7CCX0 X7HCPX7CX5) in the order P-G-F-B-A-C-D-E, where A-G are full repeats and P is a half motif. Granulin-epithelin precursor is remarkably expressed in rapidly cycling epithelial cells, in chondrocytes [21–23], in the immune system cells, in neurons and in some human cancers [24–27]. Increasing evidence has implicated GEP in the regulation of differentiation, development and pathological processes. It has been isolated as a differentially expressed gene from macrophage development [28], skeletal muscle differentiation [29] and synovium (morphopathogenesis) in rheumatoid arthritis and osteoarthritis [22, 30]. Granulin-epithelin precursor was also shown to be a critical mediator of wound response and tissue repair [31, 32]. We previously reported that GEP regulates chondrocyte differentiation and endochondral bone formation, and cartilage repair through Erk1/2 signalizing and its target gene, including JunB transcription factor [21].

In this study, we attempted to determine whether XBP1S is essential for skeletal development using both in vitro and in vivo approaches. First, we studied its upstream and downstream molecules during chondrogenesis, as well as its molecular mechanisms by which XBP1S regulates chondrogenesis. Our results support a novel role of XBP1S, a key downstream molecule of BMP2 in the control of chondrogenesis and endochondral bone growth through activating GEP growth factor.

Materials and methods

Plasmids and adenoviruses

To generate pGL3-XBP1-luc reporter plasmid, the corresponding segments were amplified using PCR with the following primers: 5′-GTCACTGGACATCGCCACAC-GG-3′ and 5′-GTGGAGCCCCGGAGAC-GAC-3′ for pGL3-XBP1-luc; PCR products were inserted into the pGL3 vector.

To generate XBP1S smaller interfering RNA (siRNA) expression constructs, siRNA corresponding to the coding sequence of the XBP1S gene (5′-ATGCTAATGCACTCTT-3′) was cloned into a pSUES-HUS vector (an adenoviral shuttle vector expressing siRNA) according to the manufacturer’s instructions. Briefly, equimolar amounts of complementary sense and antisense strands were separately mixed, annealed and slowly cooled to 10°C in a 50-μl reaction buffer (100 mM NaCl and 50 mM HEPES, pH 7.4). The annealed oligonucleotides were inserted into the SfiI sites of pSES-HUS vector. All constructs were verified by nucleic acid sequencing; subsequent analysis was performed with BLAST software (National Institutes of Health, Bethesda, MD, USA).

Adenovirus XBP1S (Ad-XBP1S) siRNA, adenovirus encoding XBP1S and GEP were constructed, respectively, using methods described previously [46, 59, 60].

Mice

All animal studies were performed in accordance with institutional guidelines and approval by the Institutional Animal Care and Use Committee of Chongqing Medical University. The GEP-knockout (GEP−/−) mice were bought from Jackson Laboratories (Bar Harbor, ME, USA), the generation and genotyping of GEP−/− mice on basis of Jackson Laboratory’s protocol were used for these experiments (http://jaxmice.jax.org/query/).

Isolation and culture of mouse bone marrow stromal cells (BMSCs)

Mouse bone marrow was isolated by flushing the femurs and tibiae of 8- to 12-week-old female GEP−/− knockout (GEP KO) mice with 0.6 ml of improved minimal essential medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 20% foetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and 2 mM glutamine (Invitrogen, Carlsbad, CA, USA), and then it was filtered through a cell strainer (Falcon, BD Biosciences, Franklin Lakes, NJ, USA). Cells were centrifuged for 10 min. at 260 × g, washed by the addition of fresh medium, centrifuged again, resuspended and plated out in improved minimal essential medium supplemented with 20% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at a density of 2 × 10⁶ cells/cm² in 25-cm² plastic culture dishes. The cells were incubated at 37°C in 5% CO₂. After 72 hrs, non-adherent cells and debris were removed, and the adherent cells were cultured continuously. Cells were grown to confluence, washed with PBS and lifted by incubation with 0.25% trypsin, 2 mM ethylenediaminetetraacetic acid (Invitrogen) for 5 min. Non-detached cells were discarded, and the remaining cells were regarded as passage 1 of the BMSC culture. Confluent BMSCs were passaged and plated out at 1:2–1:3 dilutions. At passage 3, cells were transferred to DMEM (Invitrogen) supplemented with 10% FBS for differentiation studies.

Cell culture

The micromass culture was performed as described previously [46]. Briefly, trypsinized C3H10T1/2 cells were resuspended in DMEM with 10% FBS at a concentration of 10⁶ cells/ml, and six drops of 100 μl of cells were placed in a 60-mm tissue culture dish (BD Biosciences). After a 2-hr incubation at 37°C, 1 ml of DMEM containing 10% FBS and B2M2 protein (300 ng/ml) was added. The medium was replaced approximately every 2–3 days. To test the effect of overexpression of XBP1S protein on chondrogenesis, C3H10T1/2 cells were infected with XBP1S expression adenovirus or control GFP adenovirus before micromass culture.

To test the effect of knocking down XBP1S on chondrogenesis, C3H10T1/2 cells were infected with Ad-XBP1S siRNA or control RFP
adenovirus before micromass culture. Mouse chondrogenic ATDC5 cells were maintained in a medium consisting of a 1:1 mixture of DMEM and Ham’s F-12 medium (Flow Laboratories, Irvine, UK) containing 5% FBS (Invitrogen), 10 mg/ml of human transferrin (Roche Applied Science, Penzberg, Germany) and 30 nM of sodium selenite (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂ in air. The ATDC5 cells were seeded at a density of 3 × 10⁵ cells/well in 6-well cell culture plates (Corning Life Sciences, Edison, NJ, USA). The medium was replaced every other day. For adenovirus (Ad-XBP1S or Ad-GFP) infection and Ad-XBP1S siRNA and Ad-RFP infection, the same protocol as used with C3H10T1/2 cells was followed.

**Immunohistochemistry**

Sections of post-coital day 12.5, 14.5, 15.5, 17.5 and 18.5 embryos and newborn mice were deparaffinized, rehydrated and placed in Tris buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl]. Serum block was applied for 30 min. at room temperature before incubation of the primary antibody. Antimouse XBP1S (BioLegend, San Diego, CA, USA) was diluted 1:50, and sections were incubated at room temperature for 2 hrs. For detection, biotinylated secondary antibody and horseradish peroxidase (HRP)-streptavidin complex (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used. Horseradish peroxidase substrate was used for visualization, and sections were then counterstained with Mayer’s haematoxylin.

**Immunoblotting analysis**

To examine the expression of XBP1S protein in the course of chondrogenesis, total cell extracts prepared from micromass cultures of ATDC5 cells in the presence of 300 ng/ml recombinant BMP2 protein were mixed with 5 × sample buffer [312.5 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 10% SDS, 0.5% bromphenol blue, 50% glycerol]. Proteins were resolved on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. After blocking in 10% non-fat dry milk in Tris buffer, saline Tween 20 [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Tween 20], blots were incubated with mouse monoclonal anti-XBP1S antibody (diluted 1:500; BioLegend) and pCMV-gal (an internal control for transfection efficiency). Forty-eight hours after transfection, cells were harvested, and luciferase and β-galactosidase activity was measured using the Bioscan Mini-Lum luminometer. Relative transcriptional activity was expressed as a ratio of luciferase reporter gene activity from the experimental vector to that from the internal control vector. The cultures were processed and analysed as described above.

**Chromatin immunoprecipitation**

Micromass culture of ATDC5 cells were plated at a density of 3 × 10⁵ cells/well in 6-well tissue culture plates and transfected with XBP1-specific reporter plasmids (pGL3-XBP1-luc) and pCMV-gal (an internal control for transfection efficiency). Forty-eight hours after transfection, cells were harvested, and luciferase and β-galactosidase activity was measured using the Bioscan Mini-Lum luminometer. Relative transcriptional activity was expressed as a ratio of luciferase reporter gene activity from the experimental vector to that from the internal control vector. The cultures were processed and analysed as described above.

**Culture of foetal mouse bone explants**

Fetal mouse metatarsals were dissected from foetal GEP null mice (GEP-/-, 15-day-old embryos) and cultured in DMEM (Gibco, Carlsbad, CA). 

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whether XBP1S, a vital transcription factor in ER stress, participates in this process. To this end, we sought to determine whether XBP1S, a vital transcription factor in ER stress, participates in cartilage development.

Differential expression of XBP1S in the chondrogenesis of a micromass culture of ATDC5 and C3H10T1/2 cells

It is reported that ER stress signal molecules were associated with chondrogenesis [33–35]. In this study, we sought to determine whether XBP1S, a vital transcription factor in ER stress, participates in cartilage development. We first studied XBP1S expression profiles during chondrocyte differentiation using the ATDC5 cell line and C3H10T1/2 cell line [36–38]. It differentiates specifically to the cartilage lineage at high yields when incubated under high-cell-density micromass cultures as well as when exposed to chondroinductive factors such as a well-documented growth factor BMP2 [5, 39]. Therefore, both ATDC5 and C3H10T1/2 cells have the potential to become chondrocytes, making them a valuable in vitro correlate for studying the mechanisms of chondrogenesis. To obtain XBP1S expression profiles during chondrocyte differentiation, micromass cultures of ATDC5 and C3H10T1/2 cells were incubated in the presence of 300 ng/ml of recombinant BMP2 for induction of chondrocyte differentiation. Cells were harvested at various time-points and then followed by real-time PCR for measures of XBP1S and collagen X (a specific marker for hypertrophic chondrocytes). As shown in Figure 1A and B, the level of XBP1S mRNA was relatively low until day 5; when it had doubled, and thereafter remained at high levels during the differential stage, representing terminal differentiation marked by the increase in collagen X expression. In addition, similar results were also observed in the course of chondrogenesis of C3H10T1/2 cells. It is noteworthy, that the peak level of XBP1S was 2 days earlier than that of collagen X, suggesting that XBP1S may regulate collagen X expression.

We next examined the level of XBP1S protein. Micromass culture of ATDC5 and C3H10T1/2 cells were harvested at various time-points, respectively, followed by Western blotting (Fig. 1C and D). XBP1S protein was markedly elevated at day 5 and thereafter, remained at high levels.

XBP1S expression patterns in chondrocytes during both embryonic and post-natal development stages

Next, we characterized the temporal and spatial expression pattern of XBP1S during skeletal development using an immunostaining assay at multiple time-points, including embryonic day 12.5 (E12.5; onset of chondrogenesis that begins with the proliferation and subsequent condensation of mesenchymal cells), E14.5 (right after cartilage formation but before endochondral bone formation) and E15.5 (onset of skeletal growth), as well as E17.5, E18.5 and newborn. As revealed in Figure 2, XBP1S is detected at E14.5, and its level is increased in the centre of the condensation and around it at E15.5. It demonstrates prominent expression in pre-hypertrophic chondrocytes at E15.5 and E17.5, E18.5 and in newborn mice. A high level of XBP1S throughout the whole growth plate is observed at E17.5, E18.5 and newborn mice, suggesting that the expression profile of XBP1S is closely linked to the entire chondrogenic period.

XBP1S stimulates chondrogenesis in vitro and endochondral bone formation ex vivo

Prominent expression of XBP1S in chondrocytes prompted us to determine whether XBP1S was able to induce chondrocyte differen-
We next sought to determine the role of XBP1S and BMP2 during chondrogenesis in micromass cultures of pre-chondrogenic ATDC5 cells and BMSC cells, which are capable of differentiation into various lineages, including chondrocytes [36–38].

In brief, the high-density culture system was incubated in the absence (CTR) or presence of Ad-XBP1S or 300 ng/ml BMP2 (serving as a positive control) for 3 or 7 days. Chondrogenesis was monitored by analysing the expressions of marker genes specific for chondrocytes (Fig. 3). ATDC5 or BMSC cells were treated with BMP2, adenovirus encoding XBP1S (Ad-XBP1S), Ad-XBP1S+BMP2 and control GFP (Ad-GFP), respectively, then, RNA was extracted every other day for real-time PCR.

As revealed in Figure 3A–F, chondrocyte differentiation was monitored by examining the expression of collagen II, collagen X and RUNX2, three marker genes widely used for chondrocyte maturation and hypertrophy [4, 5]. As for BMP2, XBP1S markedly induced the expression of collagen II, collagen X and RUNX2. Besides, clearly enhanced expressions of collagen II, collagen X and RUNX2 in Ad-XBP1S+BMP2-treated cells were observed compared with those in BMP2-treated or Ad-XBP1S-treated cells, suggesting that XBP1S can enhance BMP2-induced chondrogenesis, thus, XBP1S is a positive mediator for chondrocyte differentiation and hypertrophy.

The effect of XBP1S on endochondral bone formation was then studied in an ex vivo model of 15-day-old foetal mouse metatarsal bones. At the time of explantation, these explants consisted of undifferentiated cartilage. In a 5-day culture period of Ad-XBP1S (MOI 20), these explants underwent all sequential stages of endochondral bone formation. As shown in Figure 4, XBP1S significantly stimulated chondrocyte hypertrophy, mineralization and bone length.

XBP1S is a BMP2-inducible transcription factor and required for BMP2-mediated chondrocyte differentiation

To identify BMP2 downstream molecules, we performed genome-wide DNA chip analysis (Fig. 5A). Total RNA was isolated from human C28I2 chondrocytes treated with 300 ng/ml BMP2 at various time-points and analysed by microarray analysis (Shanghai Kangcheng Biotechnology, Shanghai, China). Approximately 50 genes were up-regulated (twofold) by BMP2, as determined by hierarchical clustering, and some of the BMP2-inducible genes (Fig. 5A), including XBP1S, IRE1α, ATF3, HSPA5, DDIT3 are also known to be activated by ER stress [41–45]. We focused on XBP1S, because BMP2 mediates ER stress, and IRE1α-XBP1 pathway is an important UPR signal pathway.

To testify the result of genome-wide DNA chip analysis, we tested a few cytokines known to be important for chondrogenesis in the primary human chondrocytes. Our results showed that XBP1S mRNA is up-regulated threefold by BMP2 and 1.5-fold by TGF-β. IL-1β had no apparent effects on XBP1S expression (Fig. 5B).

Next, we tested whether XBP1S was required for BMP2-mediated chondrogenesis using the siRNA approach. As shown in Figure 5C and E, infection with siXBP1S adenovirus resulted in 81% and 72%
A reduction in XBP1S mRNA in ATDC5 cells and BMSC cells respectively. Micromass cultures of ATDC5 cells or BMSC cells infected with siXBP1S adenovirus or control adenovirus (CTR) were treated with BMP2 for various time-points. As shown in Figure 5D and F, our real-time PCR assay showed that reductions of the endogenous XBP1S by siXBP1S adenovirus sharply decrease chondrogenic responses induced by BMP2: 77% down of Sox9, 62% down of collagen II, 83% down of collagen X and 75% down of RUNX2 compared with the control group responses in ATDC5 cells (Fig. 5D); 67% down of Sox9, 60% down of collagen II, 78% down of collagen X and 65% down of RUNX2 compared with the control group responses in BMSC cells (Fig. 5F). These results support the concept that XBP1S is a key downstream molecule of BMP2 during chondrocyte development.

BMP2 and Smads activate XBP1S-specific reporter genes

To elucidate the molecular mechanism by which BMP2 activates XBP1S expression, firstly, four XBP1S-specific reporter gene plasmids, −2000→+133XBP1Sluc [labelled p1], −1311→+133 XBP1Sluc [p2], −407→+133XBP1Sluc [p3] and −2000→−407 XBP1Sluc [p4], were generated in which segments of the XBP1S promoter, with or without the NF-Y or NF-Y/ERSE binding site, were inserted upstream of the luciferase coding region of the pGL3 basic vector (Fig. 6). On the other hand, deletion of the region from −407 to +133 leads to the complete loss of the reporter activity, indicating that this region is probably the basic promoter of the XBP1S gene. The core sequence of XBP1S promoter is found from −407 to +133 bp. Applications of BMP2 were able to activate all XBP1S promoter constructs containing the region between −407 and +133. Furthermore, this basic promoter region directly responded to BMP2 (Fig. 6A).

Because BMP2 activates the cellular signalling through Smads, we then tested interaction of Smad4, a coregulatory Smad that binds to Smad1 or Smad5 for transducing BMP2 signalling, with XBP1S promoter regions (in particular, the region of −407 and +133) in vitro using the ChIP assay. As shown in Figure 6B, we observed a clear PCR product using DNA isolated from immunoprecipitated complexes with anti-Smad4 antibodies from BMP2-treated cells, but not from BMP2-untreated cells, suggesting that the Smad4 is recruited into this XBP1S promoter region after exposure to BMP2.

Next, we determined whether Smad transcription factors could directly activate the XBP1S at the transcription level. Cotransfection of the XBP1S luciferase plasmid (−407XBP1Sluc) with an expression plasmid encoding either Smad1, Smad4 and Smad5 (cDNA constructs kindly provided by Dr. Chuanju Liu, Department of Orthopaedic Surgery and Department of Cell Biology, New York University School of Medicine), or a combination of either Smad1/Smad4 or Smad5/Smad4, markedly increased the expression of the XBP1S reporter gene. Both of the combinations of Smad1/Smad4 and combinations of Smad4/Smad5 gave the higher value than the others (Fig. 6C). The above data support the notion that BMP2 controls XBP1S expression through Smad signalling.

XBP1S induces GEP expressions in C3H10T1/2 and ATDC5 cells

We have found that XBP1S is expressed throughout the whole growth plate at E17.5, E18.5 and newborn mice (Fig. 2) and positively
regulates chondrocyte development (Figs 3 and 4). We previously reported that GEP is a key downstream molecule of BMP2, and it is required for BMP2-mediated chondrocyte differentiation. We next used C3H10T1/2 cells and chondroprogenitor ATDC5 cells to examine the relationship between GEP and XBP1S. Micromass cultures of both C3H10T1/2 and ATDC5 cells pre-treated with 300 ng/ml of BMP2 for 1 week were cultured with or without Ad-XBP1S for various time-points, and the level of GEP mRNA was measured by using real-time PCR (Fig. 7A).

Granulin-epithelin precursor mRNA was increased to 2.0-fold at day 1 and to 2.3-fold by day 3 in the XBP1S-untreated control ATDC5 cells. XBP1S markedly enhanced the level of GEP mRNA to 4.2-fold at...
day 1 and to 5.7-fold by day 3 in the XBP1S-treated ATDC5 cells. In the case of C3H10T1/2 cells, GEP mRNA was slightly increased to 1.3-fold at day 1 and to 1.5-fold by day 3 in the XBP1S-untreated cells; And XBP1S significantly induced GEP to 3.6-fold at day 1 and to 3.5-fold by day 3 in the XBP1S-treated C3H10T1/2 cells. GEP mRNA in the XBP1S-treated cells was approximately twofold higher than the mRNA in the control at the same time-point. In addition, induction of the GEP protein level by XBP1S was also visualized by both immunofluorescent cell staining in C28I2 chondrocytes (Fig. 7C) and immunoblotting in ATDC5 cells (Fig. 7B). Taken together, these findings demonstrate that GEP is a XBP1S-inducible gene in the process of chondrogenesis.

Fig. 5 XBP1S is a downstream molecule of BMP2 and is required for BMP2 stimulation of chondrogenesis. (A) Genome-wide DNA chip analysis for isolating BMP2-responsive genes. Total RNA was isolated from human C28I2 chondrocytes treated with 300 ng/ml BMP2 for various time-points, as indicated, and analysed by microarray analysis. Several up-regulated genes after BMP2 treatment were determined by hierarchical clustering. (B) Effects of cytokines on XBP1S mRNA in chondrocytes by real-time PCR. Expression of XBP1S mRNA was normalized against GAPDH (serving as an internal control). **P < 0.01. (C) siRNA against XBP1S mRNA efficiently inhibited expression of endogenous XBP1S in ATDC5 cells. siXBP1S reduces 81% of endogenous XBP1S mRNA in ATDC5 cells. Cells were infected with either siXBP1S adenovirus (MOI 20) or control adenovirus (CTR), and total RNA was collected for real-time PCR. Expression of XBP1S was normalized against the GAPDH endogenous control. The normalized values were then calibrated against the control value, here set as 1. *P < 0.05. (D) Suppression of XBP1S by siRNAs inhibits BMP2-induced chondrogenesis in ATDC5 cells. Micromass cultures of C3H10T1/2 cells infected with either control adenovirus or siXBP1S adenovirus (MOI 20) were used to test whether BMP2 (300 ng/ml)-induced chondrogenesis is XBP1S dependent. Expressions of marker genes, as indicated, were determined by real-time PCR. (E) siRNA against XBP1S mRNA efficiently inhibited expression of endogenous XBP1S in bone marrow stromal cells (BMSC). siXBP1S adenovirus reduces 72% of endogenous XBP1S mRNA in BMSC cells. The method is the same with C. (F) siXBP1S adenovirus inhibits BMP2-induced chondrogenesis in BMSC cells. The method is the same with D.
XBP1S activates chondrogenesis and endochondral bone formation through GEP

We next investigated whether endogenous GEP is required for XBP1S-induced chondrocyte development. Firstly, we isolated GEP null mice (GEP<sup>−/−</sup>) BMSC cells, then performed micromass culture of GEP<sup>−/−</sup> BMSC cells. As shown in Figure 8A–C, Realtime PCR results showed that Ad-XBP1S cannot promote BMP2-induced Col II (Fig. 8A), Col X (Fig. 8B) and RUNX2 (Fig. 8C) expression in GEP<sup>−/−</sup> BMSC cells, however, after infection with Ad-GEP, Ad-XBP1S can increase the expression of Col II (Fig. 8A), Col X (Fig. 8B) and RUNX2 (Fig. 8C) induced by BMP2 in GEP<sup>−/−</sup> BMSC cells. It was indicated that Ad-XBP1S activates BMP2-induced chondrogenesis through GEP growth factor.

In addition, the dependence on GEP of XBP1S-mediated endochondral bone formation was revealed by using cultures of 15-day-old foetal GEP null mice metatarsal bones (Fig. 8D). In line with a previous report [46], XBP1S potently enhanced chondrocyte hypertrophy, and the effect of XBP1S-induced chondrogenesis and endochondral bone formation was largely abolished in GEP<sup>−/−</sup> BMSC cells. These results indicated that XBP1S-mediated chondrocyte differentiation and endochondral bone growth depends, at least in part, on GEP. We next sought to determine whether XBP1S recovered the valid stimulating in growth plates of GEP<sup>−/−</sup> embryos rescued by GEP with safranin O-fast green staining. As shown in Figure 8D, disorganized GEP null growth plates, including reductive chondrocyte hypertrophy, cannot be changed by Ad-XBP1S, however, it can be largely corrected in the presence of Ad-XBP1S+Ad-GEP. XBP1S recovered the potent stimulating effect of chondrocyte differentiation, mineralization and endochondral bone growth in GEP null growth plates rescued by GEP.

Taken together, endogenous GEP is required for XBP1S-stimulated chondrocyte hypertrophy, mineralization and endochondral bone formation.

XBP1S enhances the chondroinductive activity of GEP

Then, we examined whether XBP1S-mediated augment of chondrocyte hypertrophy and endochondral bone growth is exerted by activating GEP’s chondroinductive activity. We previously reported that GEP
is a novel growth factor increasing chondrocyte differentiation and endochondral bone formation, and cartilage repair [21, 22]. For this purpose, we first examined whether XBP1S was able to increase GEP-stimulated chondrocyte hypertrophy using chondroprogenitor ATDC5 cells.

As noted in Figure 9A, XBP1S contains a DNA binding domain (a domain) and a transactivating domain (b domain; Fig. 9A, top scheme). We generated XBP1S derivatives with mutations in the DNA binding domain (XBP1Smt-a), the transactivating domain (XBP1Smt-b) or both domains (XBP1S mt-a/b; Fig. 9A, three lower schemes).

Then, ATDC5 cells pre-treated with BMP2 for 1 week were cultured without Ad-XBP1S (CTR) or with Ad-XBP1S (MOI 20) for various time periods, as indicated. The normalized values against GAPDH were calibrated against controls (day 0), given the value of 1. Asterisk indicates a significant difference from the control at corresponding time-points (P < 0.05). (B) XBP1S induces the expression of GEP protein, assayed by Western blotting. ATDC5 cells pre-treated with recombinant 300 ng/ml of BMP2 for 1 week were cultured without Ad-XBP1S (CTR) or with Ad-XBP1S (MOI 20) for various time periods, as indicated. The cell lysates were detected with either anti-GEP or anti-tubulin (serving as an internal control) antibodies. (C) XBP1S increases the level of GEP protein, assayed by immunofluorescent cell staining. C28I2 cells treated with or without pcDNA3.1(-)-XBP1S for 24 hrs were stained with anti-GEP antibodies (green). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

We next determined whether XBP1S was also able to improve the GEP activity in regulating endochondral bone growth. As expected, GEP growth factor stimulated chondrocyte maturation, mineralization and bone growth; and GEP-mediated endochondral bone growth was clearly increased by the addition of Ad-XBP1S (Fig. 9C). These observations, together with the finding that GEP is required for the XBP1S-induced chondrocyte differentiation and endochondral bone formation, suggested that XBP1S positively regulates chondrocyte hypertrophy and endochondral bone growth through stimulating with GEP and activating its chondrogenic activity.

**Discussion**

Growth and development of endochondral bones is regulated through the well-orchestrated proliferation and differentiation of growth plate chondrocytes. Chondrogenesis is a process that is important for cartilage remodelling both during embryogenesis and in adult life [47, 48]. The IRE1/XBP1 branch of the UPR is known to be essential for normal development. XBP1S is required for the terminal differentiation of B cells, hepatocytes and pancreatic β cells. It is also important for myeloma cells to survive hypoxic stress [49, 50]. Many studies have shown that factors influencing cell fate and/or differentiation are activated in ER stress [51, 52], but how such changes impact differentiation programmes in chondrocytes is poorly understood. Therefore, to test a link between the IRE1/XBP1 branch of the UPR and
chondrocyte differentiation, we focused on the role of XBP1S in chondrogenesis as well as the molecular mechanism involved. Our results showed that XBP1S protein was highly induced in the course of BMP2-stimulated chondrogenesis in vitro (Fig. 1) and also demonstrated prominent expression in the entire growth plate chondrocyte population in vivo (Fig. 2). Real-time PCR for measurements of XBP1S showed that the level of XBP1S mRNA was relatively low until day 5, and at day 7, it tripled and thereafter remained at high levels during the late differential stage (Fig. 1A and B). The different expression between the protein and mRNA of XBP1S during chondrogenesis suggests that post-transcription regulations, such as mRNA stability, translation and protein degradation, might be also important in the control of XBP1S expression during chondrogenesis. The in vitro, ex vivo and in vivo studies support the concept that XBP1S is a potent stimulator of chondrocyte differentiation, mineralization and endochondral bone growth (Figs 3 and 4).

Saito et al. [35] reported that BMP2 induced ER stress in osteoblasts, and ER stress-inducing agents activate the IRE1α/β proteins. IRE1α, a kind of ER type I transmembrane protein containing a serine/threonine kinase module and an endoribonuclease domain, executes site-specific cleavage of XBP1 mRNA to remove a 26-nucleotide intron during UPR. XBP1S is more potent as a transcriptional activator and more stable than XBP1U (unspliced). XBP1S activates the promoters of many genes, including those coding for enzymes necessary for the degradation of improperly folded ER proteins, and participates in cell proliferation and differentiation [53, 54].

Firstly, our work also supports the concept that XBP1S is a key downstream molecule of BMP2 in chondrogenesis and endochondral bone formation.
bone growth based on the following evidence. (i) Both BMP2 and XBP1S are potent in inducing in vitro chondrogenesis and induction of chondrogenic markers such as collagen II, collagen X and RUNX2 (Fig. 3). The effect of XBP1S on chondrogenesis is similar to the effects of BMP2 and differs significantly from many factors that have opposite effects on collagen II and collagen X [40, 47, 48]. (ii) BMP2 induced XBP1S in chondrocytes, as shown in Fig. 5. (iii) Notably, knockdown of XBP1S strongly inhibited BMP2-mediated chondrogenesis, as assayed by collagen II, Sox9, collagen X and Runx2 expression in the course of chondrocyte differentiation (Fig. 5). (iv) Finally, BMP2 activates XBP1S specific reporter genes through Smad transcription factors (Fig. 6).

Granulin-epithelin precursor, as a growth factor, has been linked to development, tissue regeneration, tumourigenesis and inflammation [26, 31, 32, 55, 56]. We previously reported that GEP accelerates chondrocyte hypertrophy, mineralization and endochondral bone growth through Erk1/2 signalling and its target gene, including JunB transcription factor [21]. Herein, we present evidence showing that (i) XBP1S cannot improve chondrocyte differentiation and endochondral bone formation in GEP−/− BMSC cells, and (ii) XBP1S recovered the potent stimulating effect of chondrocyte differentiation, mineralization and endochondral bone growth in GEP null growth plates rescued by GEP (Fig. 8). In addition, XBP1S increases GEP-mediated chondrocyte maturation, mineralization and endochondral bone growth. XBP1S enhances chondrocyte differentiation and endochondral bone formation through activating the chondrogenic activity of GEP (Fig. 9).
Recently, we reported that ADAMTS-7 binds to and degrades COMP and that COMP interacts with GEP and potentiates GEP-stimulated chondrocyte functions, indicating that ADAMTS-7, GEP and COMP form an interaction and interplay network in regulating chondrocyte functions [57–59]. It remains to be determined how the interaction network among ADAMTS-7 metalloproteinase, GEP growth factor and COMP extracellular matrix molecule acts in concert in regulating chondrocyte differentiation and endochondral ossification. Our current study focuses on the relationship between XBP1S transcription factor, a UPR signal molecule, and GEP growth factor in chondrocyte development for the first time.

On the basis of the data in the literature [3, 7, 9, 39], our earlier findings [21, 22, 46] and the results of this study, we propose a model for the role of XBP1S – specifically, its expression and function – in chondrocyte differentiation (Fig. 10). This study provides novel insights into the role of XBP1S, a novel mediator in the BMP2 pathway, in regulating chondrocyte differentiation and endochondral bone formation and sheds light on the molecular mechanism by which XBP1S positively regulates chondrogenesis; i.e. XBP1S, a key down-stream molecule of BMP2, increases chondrocyte differentiation and endochondral bone formation through activating GEP growth factor, and endogenous GEP is required for XBP1S-stimulated chondrocyte hypertrophy, mineralization and endochondral bone growth. Our work supports a hypothesis that XBP1S, a transcription factor induced by BMP2, regulates chondrogenesis and endochondral bone formation through GEP growth factor. The elucidation of XBP1S’s role and molecular events involved in chondrocyte differentiation will better our understanding of normal cartilage development and the pathogenesis of cartilage disease.

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Conflicts of interest

All authors state that they have no conflicts of interest.

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