ATF6a, a Runx2-activable transcription factor, is a novel regulator of chondrocyte hypertrophy

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Abstract

Our previous research testified that XBP1S is a significant downstream mediator of BMP2 and is involved in BMP2-stimulated chondrocyte differentiation. Herein we report that ATF6 and ATF6a are expressed in growth plate chondrocytes. There are differentially induced during BMP2-triggered chondrocyte differentiation. This differential expression is probably resulted from the activation of the ATF6 gene by Runx2 and repression by Sox6 transcription factor. Runx2 and Sox6 combine with their respective binding elements of ATF6 gene. When overexpressed, ATF6 and ATF6a intensify chondrogenesis; our studies demonstrate that under the stimulation of ATF6 and ATF6a, chondrocytes tend to be hypertrophied and mineralized, a process leading to bone formation. Additionally, lowering expression of ATF6a using its specific siRNA suppresses chondrocyte differentiation. Moreover, ATF6a interacts with Runx2 and augments Runx2-mediated hypertrophied chondrocyte. Importantly, overexpression and knockdown of ATF6a in chondrocyte hypertrophy also lead to altered expressions of IHH and PTHrP. Taken together, these findings indicate that ATF6a favorably controls chondrogenesis and bone formation via a) acting as a co-factor of Runx2 and enhancing Runx2-incited hypertrophic chondrocyte differentiation, and b) affecting IHH/PTHrP signaling.

Keywords

ATF6a, chondrocyte hypertrophy, UPR, transcription factor, regulation
Introduction

In eukaryotic cells, ER stress is known to initiate a kind of signaling pathways named as the unfolded protein response (UPR) (Hetz C, 2012; Zhang K and Kaufman RJ, 2004; Ron D and Walter P, 2007). Three signaling pathways in UPR, i.e. the inositol-requiring enzyme-1 (IRE1), PKR-like ER resistant kinase (PERK) and activation transcription factor 6 (ATF6), act in concert to limit new protein synthesis and to increase the levels of chaperones. These three different pathways are triggered by ER stress to reduce misfolded protein levels (Kaufman RJ 2002; Kim R et al., 2006; Kaufman RJ.1999). In addition to induction of autophosphorylation and activation of IRE1 and PERK, ER stress also causes ATF6 transit through the Golgi complex, where it is activated through protelytic cleavage by the enzymes S1P and S2P. The cleaved N-terminal cytoplasmic domain of ATF6 (ATF6α) is released from Golgi complex, followed by translocation to the nucleus (Li M et al., 2000; Nakanishi Keiko et al., 2005).

Chondrogenesis is an essential process for cartilage development and endochondral bone growth (Lui JC et al., 2010; Lefebvre V and Smits P, 2005). Bone morphogenetic proteins, in particular BMP2, are believed to play important roles in mediating chondrocyte differentiation, proliferation and function (Chen D et al., 2004; Yu L et al., 2012). BMP2 is known to activate UPR-signaling molecules, including PERK, CHOP (C/EBP homologous protein), and IRE1α. In addition, the unfolded protein response in ER stress was reported to mediate BMP2-signaling in osteoblasts (Lai CF and Cheng SL, 2002; Murakami et al., 2009).

Previously we also reported that XBP1S is a critical inducer in the BMP2 signal pathway and involved in BMP2-triggered chondrogenesis and bone formation. XBP1S stimulates chondrocyte hypertrophy, maturation and bone growth through GEP growth factor (Guo et al., 2014). Furthermore, BMP2 was known to activate ER stress sensors, including ATF6 and OASIS. BMP2 can stimulate osteoblast differentiation and extracellular matrix mineralization via Runx2-induced ATF6 expression, which enhances osteocalcin transcription and expression (Jang et al., 2012). In this study, we examined the expression of ATF6 in the course of chondrogenesis, the regulation of chondrocyte differentiation, especially hypertrophy, by ATF6 and its cleaved ATF6α, as well as the molecular mechanisms involved.

Materials and methods

Construction of Reporter gene vectors and Adenoviruses

To construct wild type and two mutants of the pGL3-ATF6-luc reporter plasmid, the target sequences were amplified using PCR with the following primers:

- 5’-GGTACCGCTGCAGTGAGCTGAGATGGCT-3’ and 5’-CTGGAGATCACCCGGTACTTCCCCAGTG-3’ for wild type ATF6-luc (-2103~+321);
- 5’-GGTACCGTTCTGAGATAGCCACGCTGTGG-3’ and 5’-CTGGAGATCACCCGGTACTTCCCCAGTG-3’ for wild type ATF6-luc (-384~+321);
- 5’-TATTTTTACCACTGGCAGTTGATTG-3’ and 5’-CTGGAGATCACCCGGTACTTCCCCAGTG-3’ for wild type ATF6-luc (-384~+321);
- 5’-CTTCCCCCGCCTagtagTAAACACGGGGAC-3’ and 5’-GTGGGCGGAAGTAGGGAGGAAGA-3’ for mut1;
- 5’-TATTTTTAATATATATACCCGCGGAGGGGAC-3’ and 5’-GTGGGCGGAAGTAGGGAGGAAGA-3’ for mut2.
A-3’ for mut2 (the mutated nucleotides in the primers are underlined). PCR products were inserted into the pGL3 vector.

Adenovirus ATF6a (Ad-ATF6a), Adenovirus ATF6a siRNA (siATF6a), Adenovirus ATF6 (Ad-ATF6), and adenovirus encoding Runx2, Sox6 were constructed, respectively, with methods described previously (Guo et al., 2014). All constructs were verified by nucleic acid sequencing; subsequent analysis was performed using BLAST software (National Institutes of Health).

**Isolation and Culture of Mouse Bone Marrow Stromal Cells (BMSCs)**

Mouse bone marrow was isolated by flushing the femurs and tibiae of 8-week-old female BALB/c mice with 0.6 ml of improved minimal essential medium (Sigma-Aldrich), supplemented with 20% fetal bovine serum, 100 units/ml penicillin,100 µg/ml streptomycin (Invitrogen), and 2 mM glutamine (Invitrogen), and then it was filtered through a cell strainer (Falcon, BD Biosciences). Cells were centrifuged, resuspended, and plated out at a density of ~2×10^6 cells/cm^2. Then cells were incubated at 37°C in 5% CO2. After 72 h, nonadherent cells and debris were removed, and the adherent cells were cultured continuously. Nondetached cells were discarded, and the remaining cells were regarded as passage 1 of the BMSC culture.

**Immunohistochemistry**

For histological examination, the sections of postcoital day 12.5, 14.5, 15.5, 17.5 embryos and newborn mice, day 10 mice were immunostained for ATF6 after serum blocking for 30 min at 37°C. For detection, biotinylated secondary antibody and horseradish peroxidase (HRP)-streptavidin complex (Santa Cruz Biotechnology, CA) were used. A total of 0.5mg/ml 3,3'-diaminobenzidine (DAB) in 50mM Tris-Cl substrate was used for visualization, then sections were counterstained with Mayer’s hematoxylin.

**RNA Preparation and Quantitative Real-time PCR**

To examine the effects of Runx2 and Sox6 on the expression of the ATF6 gene, total RNA was extracted from C3H10T1/2 cells infected with Ad-Runx2, Ad-Sox6 or control Ad-GFP using RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA per sample was reverse transcribed into cDNA. The following sequence-specific primers were synthesized: sense (3’-AACGAGAACGAGCTGAGTG-5’) and antisense (3’-AAAGGAGGCCAGATGACAGAT-5’) for collagen II; sense (3’-TACCACGT GCATGTAAGGG-5’) and antisense (3’-GGAGC CACTAGGAAATCCTGAG-5’) for collagen X; sense (3’-GAGTCCCCAAGAGCCACCCA-5’) and antisense (3’-TGTTG GCTGATAGGGTGCCGC-5’) for IHH; sense (3’-ATG GTGCAGGAGGCTGGATC-5’) and antisense (3’-GCACGGAGTAGCTGAGCAGG AA-5’) for PTHrP. The following pair of oligonucleotides was used as internal controls: 5’-ACCACAGTCCA TGCCATCAC-3’ and 5’-TCCACCACCTGTGTTG CTGTA-3’ for GAPDH. Real-time PCR was performed with the SYBR Green PCR kit in a 96-well optical reaction plate formatted in the ABI 7400 system according to the Manufacturer’s protocol. The transcript of GAPDH mRNA was employed as an internal control for RNA quality. PCR
cycling conditions were as follows: initial incubation step of 2 min at 50°C, reverse transcription of 60 min at 60°C and 94°C for 2 min, followed by 40 cycles of 15 s at 95°C for denaturation and 2 min at 62°C for annealing and extension. For each gene, three independent PCRs from the same reverse transcription sample were examined.

**Western Blotting**

Total cell lysates of C3H10T1/2 treated with BMP2 (300ng/ml) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and examined by Western blotting with either mouse monoclonal anti-ATF6 antibody (diluted 1:500; BioLegend) or rabbit polyclonal anti-collagen X (diluted 1:500; Santa Cruz Biotechnology, Inc.) for 1 h, then followed by anti-mouse immunoglobulin G (IgG)-conjugated horseradish peroxidase, anti-rabbit IgG-conjugated horseradish peroxidase at a 1:1,000 dilution. After washing, the signals were detected using the enhanced chemiluminescence system (Amersham Biosciences).

**Electrophoretic Mobility Shift Assays**

Nuclear proteins from C3H10T1/2 cells infected with Ad-Runx2 or Ad-Sox6 were extracted as described previously, according to the manufacturer’s protocol. After 48 h, the C3H10T1/2 cells were scraped by cold PBS from the plate to 10ml tubes, and centrifuged at 4000 rpm for 10 min. The cell pellet was resuspended in 400µl of cold buffer and 19 protease inhibitors (Roche Applied Science) and placed on ice for 15 min. Then the cell pellet were added 25 µl of 10% Nonidet P-40, vortexed vigorously for 10 s, centrifuged for 1 min at 14,000 rpm, and kept the pellet. The nuclear pellet were resuspended in a 50-ml ice-cold buffer (same as above) and rocked the tube for 30 min at 4°C. It was then centrifuged for 15 min at 14,000 rpm at 4°C. Oligonucleotides containing the first Runx2-binding site from -240 to -233, and the first Sox6-binding site from -294 to -285 within the 5’-flanking region of the ATF6 promoter were synthesized. The probes were labeled with digoxigenin (DIG)-11-ddUTP, and electrophoretic mobility shift assays (EMSAs) were performed using a DIG gel shift kit (Roche Applied Science). Competition experiments were performed by preincubating nuclear extract with excess unlabeled probes before adding labeled oligonucleotides. In supershift assays, 5 µg of anti-Runx2 or anti-Sox6 antibody (Santa Cruz Biotechnology, Inc.) were incubated with the reaction mixture for 15 min before the addition of the digoxigenin-labeled probe. Reaction mixtures were incubated for 20 min at room temperature. Samples were subjected to electrophoresis on a native 5% polyacrylamide gel run in 0.5×TBE for 2.5 h at 100 V. The signal was detected using a chemiluminescence detection system (Roche Applied Science).

**Chromatin Immunoprecipitation (ChIP) Assay**

C3H10T1/2 cells infected with Ad-Runx2 or Ad-Sox6 or treated with BMP2, were fixed by 1% formaldehyde for 10 min before cell lysis. Then cells were lysed, the chromatin was subsequently sonicated and protein-DNA complexes were immunocoprecipitated using IgG (control) as well as anti-Runx2 antibody. The DNA recovered from the immunoprecipitation was then amplified by PCR using primers that span the Runx2-binding site and the Sox6-binding site of the ATF6 gene promoter. The sequences
of primers are following: sense 5’-GTTTAGTCGAATTGATGTCTGCG-3’, antisense 5’-CAGAGACTAAGCAATTGGTA-3’ (for first Runx2-binding site); sense 5’-CGCTG TGGCATTAAGAAGGA-3’, antisense 5’-GGGAAGACACGCAGACATCA-3’ (for first Sox6-binding site). The input (1% of the supernatant) was used in PCR as a positive control. PCR was performed under the following conditions: 94°C for 5 min, 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s.

**Luciferase Reporter Gene Assays**

C3H10T1/2 and ATDC5 cells were plated at a density of 3×10⁵ cells/well in 6-well tissue culture plates and were transfected with ATF6-specific reporter plasmids (pGL3-ATF6-luc, pGL3-ATF6(mut1)-luc, or pGL3-ATF6-(mut2)-luc), pcDNA3.1(-)-Runx2, pcDNA3.1(-)-Sox6 and pCMV-gal (an internal control for transfection efficiency). 48 h 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 analyzed as described above.

**Fetal mouse bone explants culture**

Fetal mouse metatarsals were extracted from 15-day-old pregnant fetal FVB/N mice and cultured in DMEM (Gibco) including 1% heat-inactivated FCS and 100 U/ml penicillin/streptomycin per milliliter with or without of various stimuli for 5 days, as shown in Figure 5. For safranin O-fast green staining and alizarin red/alcian blue staining, the methods were explained formerly (Feng et al., 2010; Guo et al., 2014).

**Assay for chondrogenesis of human mesenchymal stem cells (hMSCs)**

Chondrogenic differentiation was induced by placing 2.5×10⁵ hMSCs into the defined chondrogenic medium and subjecting the cells to gentle centrifugation (800 g for 5 min) in a 15-ml conical polypropylene tube, after which the cap was loosened, and the tube was placed in the incubator, where the cells adhered to one another and consolidated into a cell pellet within 24 h. Three to 4 d later, the hMSCs had formed a 1-mm ball in the bottom of the tube. The chondrogenic medium was made with fresh BMP2 or Ad-ATF6 or Ad-ATF6a every 3–4 d, and the medium was changed by careful aspiration, because the cell pellets were free floating. Three weeks later, for histological analysis, the pellets were fixed in 4% formaldehyde, paraffin-embedded, sectioned, and analyzed by immunostaining for collagen II or collagen X expression. Sections can also be stained with 0.1% Safranin O for detection of proteoglycans (Feng et al., 2010).

**Coimmunoprecipitation (CoIP) Assay**

Micromass culture of C3H10T1/2 cells or BMSCs were treated with BMP2 (300ng/ml). Cells were harvested after incubation in DMEM, 10% FCS for 5 days, and then they were lysed in lysis buffer (1% NP-40, 50 mmol/l Tris-HCl, pH7.2, 0.15 mol/l NaCl, 0.01 mol/l sodium phosphate, pH 7.2, 1% Trasylol, and protein inhibitor cocktails, Sigma-Aldrich). Approximately 500μg of protein samples were incubated with anti-Runx2 (25μg/ml; Santa Cruz Biotechnology, Inc.) or control rabbit IgG (25μg/ml) antibodies for 1 h, followed by
incubation with 30μl of protein A-agarose (PerkinElmer Life Sciences) at 4°C overnight. The immunoprecipitated complex was detected using Western blotting with anti-ATF6a antibody.

**Statistical Test**

Results were expressed as mean ±S.D. from at least three independent experiments. One-way ANOVA was performed using R software to determine the significant differences ($F > 3.35$, $\alpha_\alpha < 0.05$) of the activity among different doses. In addition, Tukey’s test was also used in conjunction with an analysis of variance to find significant differences ($P < 0.05; P < 0.01$) of the levels of genes of interest.

**Results**

**ATF6 expression in chondrocytes during both embryonic and postnatal development stages**

We first examined ATF6 expression during cartilage development using immunohistochemistry at various time points, such as embryonic day (E) E12.5, E14.5, E15.5, E17.5, newborn and day 10. As showed in Figure 1A, ATF6 is undetectable at E12.5, and becomes to be detectable in the center and surrounding of the condensation part at E14.5. It is highly expressed in prehypertrophic chondrocytes at E15.5 and E17.5. ATF6 is clearly expressed all over the entire growth plate in newborn and day 10 mice. These expression profiling of ATF6 in growth plates during development suggest that ATF6 may be greatly related to the whole chondrogenic process.

**Expression profiles of ATF6a and ATF6 in chondrocyte differentiation in vitro**

We then detected the expression profiles of ATF6a and ATF6 during chondrocyte differentiation using the C3H10T1/2 cell line, a pluripotent murine stem cell line widely used for in vitro chondrogenic studies (Zhang Y et al., 2008; Johnson et al., 2008; Meirelles Lda et al., 2003). Firstly, BMP2 (300 ng/ml) induced micromass cultures of C3H10T1/2 cells, then these cells were collected at different time points followed by realtime PCR for measurements of ATF6a, BBF2H7 (box B-binding factor-2 human homolog on chromosome 7) and collagen X (Figure 1B). As showed in Figure 1B, the ATF6a mRNA level was comparably low until day 5, then it remained at high levels in the differential stage. BBF2H7, the endoplasmic reticulum (ER) stress transducer, was expressed relatively low at day 1, and markedly increased at day 3 with a subsequent reduction. It was reported that BBF2H7 is preferentially expressed in chondrocytes of developing cartilage, and was the target of Sox9 and Col II. BBF2H7 was induced in resting chondrocytes and proliferating chondrocytes (Kenta et al. 2014; Saito et al. 2009). Conversely, the Type X collagen (Col X), a hypertrophic chondrocyte marker, was obviously up-regulated at day7 to day 9. Interestingly, the expression of ATF6a increased significantly at day5 while BBF2H7 was clearly reduced, the peak level of ATF6a came two days earlier than that of collagen X; the results indicate that ATF6a may regulate
collagen X expression during BMP2-induced chondrocyte differentiation. Additionally, similar expression profiling was also observed in the course of chondrogenesis of primary BMSCs (Figure 1C).

Then we detected the expression of ATF6a and ATF6 at protein level during chondrocyte differentiation. As revealed in Figure 1D, the expression of ATF6 was reduced while the ATF6a (cleaved ATF6) was increasing with the elongation of BMP2 treatment, ATF6a protein was not examined until day 5 in BMP2-stimulated chondrocyte differentiation of C3H10T1/2 cells. It was suggested that BMP2 induced ATF6 cleavage and produced ATF6a during chondrogenesis. Furthermore, ATF6a expression was two days earlier than that of collagen X, which was also immunopositive at day 7. It was demonstrated that the expression of ATF6a is specific for the hypertrophic and prehypertrophic chondrocyte. Similar results were also observed in the chondrogenesis of BMSCs (Figure 1E).

**Runx2 and Sox6 bind to the ATF6 gene promoter in vitro and in vivo**

We then manage to clarify the molecular mechanism modulating hypertrophic chondrocyte expression of ATF6. The sequence analysis showed that there exist three Runx2-binding elements (RBE) and three Sox6-binding elements (SBE) in the promoter of ATF6 gene according to the report (Cantu’C et al., 2011; Roca H et al., 2005). In addition, both Runx2 and Sox6 are reported to regulate the hypertrophic chondrocyte formation as the essential transcriptional factor (Cantu’C et al., 2011; Yoshida CA. et al., 2002). These findings pushed us to detect whether Runx2 and/or Sox6 connects with the promoter of ATF6 gene. We firstly determined whether Runx2 and Sox6 combined with ATF6 promoter using electrophoretic mobility shift assay (EMSA) (Figure 2A).

Two digoxigenin-labeled probes targeting the first Runx2-binding site (Figure 2A), and the Sox6-binding site (Figure 2B) incubated with the nuclear proteins from C3H10T1/2 cells infected with Ad-Runx2 or Ad-Sox6, a process resulting in a particular Runx2/DNA complex (Figure 2A, lane 3 and 4) or Sox6/DNA complex (Figure 2B, lane 3 and 4). Following the addition of anti-Runx2 antibodies, the antibody-Runx2-RBE band was found to be supershifted (Figure 2A, lane 4). And the antibody-Sox6-SBE band was also found to be supershifted (Figure 2B, lane 4). Besides, the binding of corresponding probes to Runx2 (Figure 2A, lane 2) or Sox6 (Figure 2B, lane 2) in vitro were totally eliminated by excess unlabeled probes. It was demonstrated that the binding of Runx2 or Sox6 to their corresponding binding motifs are sequence-specific.

Then to examine whether Runx2 and Sox6 also combined to ATF6 promoter in vivo, ChIP assays were performed in C3H10T1/2 cells infected with Ad-Runx2 or Ad-Sox6. Firstly, cell lysates were immunoprecipitated with either control IgG (negative control), anti-Runx2 or anti-Sox6 antibodies after crosslinking with formaldehyde, then the DNA purified from this immunoprecipitation was analyzed by PCR with PCR primers that spanned the first Runx2-binding site (Figure 2C), and Sox6-binding sites (Figure 2D) respectively in the ATF6 promoter. It was showed that the clear PCR product was observed in the DNA isolated from immunoprecipitated complexes with anti-Runx2
(Figure 2C, lane3) or anti-Sox6 (Figure 2D, lane3) antibodies but not with control IgG (Figure 2C, lane4; Figure 2D, lane4). And it was demonstrated that both Runx2 and Sox6 combine to their corresponding elements in the ATF6 promoter in the infected living cells. To further testify their binding under physiological condition, we also collected the cell lysate of micromass culture of C3H10T1/2 cells treated with BMP2. As revealed in Figure 2E and 2F, it was showed that both Runx2 and Sox6 can particularly bind to the promoter of ATF6 during the course of chondrocyte differentiation.

Runx2 activates, whereas Sox6 inhibits, the transactivation of ATF6 gene

To examine whether Runx2 regulates transcription of the ATF6 promoter, we used two reporter gene constructs, ATF6-3R3SBE-luc (pGL3-ATF6(-2103)-luc) and ATF6-1R1SBE-luc (pGL3-ATF6(-384)-luc), in which segments with RBEs (Runx2-binding elements) and SBEs (Sox6-binding elements) sequences were placed upstream of a gene encoding luciferase in the pGL3 vector respectively (Figure 3A, B). Co-transfection of C3H10T1/2 cells and ATDC5 cells with these two reporter plasmids and a Runx2 expression plasmid strongly increased reporter gene expression in a dose-dependent manner (Figure 3C, E). In the case of Sox6 transcription factor, Sox6 inhibited both pATF6-specific reporter gene constructs, and this inhibition was also dose-dependent (Figure 3D, F).

To dissect the importance of the Runx2-binding site and the Sox6-binding site in the 5′-flanking region of ATF6 gene, several point mutation reporter constructs of pGL3-ATF6-1R1SBE-luc were produced and their transcriptional activity by Runx2 or Sox6 tested (Figure 3G). The mutations of the six AGTGTG with TCCCCA resulted in an obvious reduction in reporter gene activities (Figure 3H). Whereas the alteration of five nucleotides (CCAGC to TTGAT) clearly reduced the inhibitory effect of the reporter gene by Sox6 (Figure 3I). These data confirmed that both the Runx2-binding element and the Sox6-binding site are responsible for driving ATF6 expression mediated by Runx2 and Sox6.

Runx2 induces, whereas Sox6 depresses, the endogenous ATF6 gene expression

We then detect whether it is right for the expression of endogenic ATF6 gene. As shown in Figure 4A, after Ad-Runx2 infection for 48 h, the ATF6 gene mRNA level was remarkably increased approximately three- to four fold increase. However, infection of Ad-Sox6 resulted in a nearly 50% reduction in the ATF6 mRNA level (Figure 4B). These data were also testified at protein level, the expression of ATF6 and the cleaved ATF6 (ATF6a) were prominently enhanced after Ad-Runx2 infection, and remarkably reduced after Ad-Sox6 infection (Figure 4C, D). It was clearly suggested that Runx2 and Sox6 can modulate the endogenic ATF6 gene expression and splicing.

Overexpressing ATF6a promotes chondrocyte differentiation and cartilage bone growth

Next, we determined whether ATF6a and ATF6 can influence chondrogenesis. We first explore on the effect of ATF6a, ATF6 and BMP2 in chondrocyte differentiation in high-density micromass cultures of C3H10T1/2 and BMSCs, which possess the
multipotent differentiation into various lineages of tissue cells (Liu CJ et al., 2004; Atkinson et al., 1997). In brief, high-density culture of C3H10T1/2 or BMSC cells were delt with BMP2 (positive control, 300 ng/ml), Ad-ATF6a, Ad-ATF6 and control GFP (Ad-GFP) for 3 or 7 days, respectively, then, RNA was brought out for quantitative RT-PCR. As shown in Figure 5A and B, chondrogenesis was monitored by analysing the expression of marker genes specific for chondrocyte maturation, including collagen II, Aggrecan, MMP13 and collagen X (Canalis E et al., 2003; Welch RD et al., 1998; Colnot, 2005). As for BMP2, ATF6a and ATF6 remarkably promoted the expression of collagen II, Aggrecan, MMP13 and collagen X. It was suggested that both ATF6a and ATF6 are the favorable mediator for hypertrophic chondrocyte differentiation.

Using a long-term culture system (3 wk) of high density hMSC pellets, we demonstrated that ATF6a and ATF6, like BMP2, induced chondrogenesis as reflected by positive stains with Safranin O (pink color, left panel, Figure 5C) and immunostains for collagen II and collagen X (right panels, Figure 5C). Then the 15-day-old mouse fetus metatarsal bones were cultured and detected the effect of ATF6a and ATF6 on endochondral bone growth. These explants model included undifferentiated cartilage. During a 5-day explantation culture of Ad-ATF6a and Ad-ATF6 stimulation, these explants went through all phases of endochondral bone growth and formation. As revealed in Figure 5D, both ATF6a and ATF6 remarkably energized chondrocyte hypertrophy, mineral conversion and bone length.

**Knockdown of ATF6a suppresses chondrocyte hypertrophy**

Based on our observation that ATF6a augments chondrogenesis, we next sought to examine whether suppression of its expression via a small-interference RNA approach would also affect hypertrophic chondrocyte differentiation. The expression of siATF6a, which encodes ATF6a-siRNA in adenoviruses, efficiently inhibited endogenous ATF6a expression in C3H10T1/2 cells (77%) and BMSC cells (73%). High-cell-density cultures of C3H10T1/2 cells or BMSC cells were stimulated with BMP2 for various time points, then infected with either control adenovirus (siRFP, MOI 50) or siATF6a adenovirus (MOI 50). The result showed that knockdown of ATF6a largely blocked the collagen II (Figure 6, B and G), Aggrecan (Figure 6, C and H), collagen X (Figure 6, D and I) and MMP13 (Figure 6, E and J) expression in the course of chondrogenesis. The outcome obviously demonstrated that endogenous ATF6a is necessary for chondrocyte hypertrophy and chondrogenesis.

**ATF6a combines with Runx2 in chondrocyte differentiation**

We then examined whether ATF6a combines with Runx2 in chondrogenesis via CoIP assay. Micromass culture of C3H10T1/2 cells were induced with BMP2 for 5 days, then the treated cell extracts were incubated with control IgG or anti-Runx2 antibodies, and the complexes were detected with anti-ATF6a antibody. A specific ATF6a band was immunoprecipitated by anti-Runx2 from the cell lysated (Figure 7A, lane 2) but not by control antibodies. Note that ATF6a specifically interacts with Runx2 in BMP2-triggered chondrogenesis. Control IgG and cell lysates were negative and positive control.
Next to detect whether ATF6a also combines with Runx2 in BMP2-induced BMSCs, micromass culture of BMSCs cells were stimulated with BMP2 for 5 days, then the treated cell extracts were incubated with the IgG control (Figure 7B, lane 3) or the anti-Runx2 antibody (Figure 7B, lane 2), and the complexes were detected with the antibody of anti-ATF6a. It was indicated that ATF6a binds to Runx2 in BMP2-induced chondrogenesis in BMSCs cells.

Then we detected whether ATF6 combine with Runx2 without BMP2. The cell extracts from C3H10T1/2 were incubated with the IgG control (Figure 7C, lane 3) or the antibody of anti-Runx2 (Figure 7C, lane 2), the complexes were examined with the antibody of anti-ATF6. The result displayed that the anti-Runx2 antibody can not bring down ATF6 protein (Figure 7C), indicating that ATF6 can not interact with Runx2 without BMP2. Taken together, ATF6a and Runx2 can constitute a new protein complex during BMP2-triggered chondrocyte hypertrophy and chondrogenesis. Without BMP2 stimulation, this complex can not be formed.

**ATF6a enhances Runx2-dependent hypertrophic chondrocyte differentiation**

It is reported that Runx2 is necessary for chondrocyte hypertrophy via collagen X, IHH and MMP13 activation (Zheng Q et al., 2003). After high density culture of C3H10T1/2 cells were infected with adenovirus encoded with ATF6a (Ad-ATF6a), ATF6 (Ad-ATF6), Runx2 (Ad-Runx2), or a combination, then RNA was extracted for real-time PCR. As shown in Figure 7, D, E and F, the expressions of collagen X, MMP13 and IHH were remarkably increased in cells infected with Ad-ATF6a+Ad-Runx2 , Ad-ATF6+Ad-Runx2 compared with those in Runx2-infected cells. It was suggested that both ATF6a and ATF6 significantly augmented the expression of collagen X, MMP13 and IHH induced by Runx2, and the former (ATF6a) enhanced that more obviously. The results illustrated that ATF6a is a cofactor of Runx2 in regulating chondrocyte hypertrophy and chondrogenesis. The promoting effect of ATF6 might be achieved by increasing the splicing of ATF6 and the production of ATF6a in chondrocyte differentiation.

**ATF6a regulates IHH/PTHrP signaling in chondrocyte hypertrophy**

It is well known that Indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHrP) cooperatively controls the process of hypertrophic chondrocyte differentiation. We then determined whether ATF6a influences this signaling pathway in hypertrophic chondrocyte differentiation. Firstly, the high density micromass of BMSC cells (Figure 8A,C) or C3H10T1/2 (Figure 8B, D) infected with Ad-ATF6a or control (CTR) were cultured in the stimulation of BMP2 for different time points respectively, then the real-time PCR was performed. The result indicated that ATF6a increased the IHH expression (Figure 8A, B), whereas it obviously inhibited the PTHrP mRNA level (Figure 8C, D) compared with the control. On the contrary, lowering expression of ATF6a via siRNA completely suppressed IHH mRNA level and augmented PTHrP expression in hypertrophic chondrocyte differentiation of both BMSC (Figure 8E, G) and C3H10T1/2 cells (Figure 8F, H). Above all, these results demonstrated that ATF6a can influence
chondrogenesis through IHH/PTHrP, an important signaling pathway in hypertrophic chondrocyte differentiation.

**Discussion**

ER is a cellular organelle responsible for the folding and posttranslational modifications of proteins, and its homeostasis is disturbed when ER stress occurs. ER stress triggers a conserved response, referred to as UPR, which mitigates ER stress (Bernales S et al., 2006; Urano F et al., 2000). Accumulated evidences have shown that factors influencing cell fate and/or differentiation are activated in ER stress (Liu et al., 2000; Korennykh AV et al., 2009; Lin JH et al., 2007), but whether and how such factors affect chondrocyte differentiation remains largely unknown. Therefore, in this study we examined the role of ATF6 and ATF6a, one branch of the UPR, in hypertrophic chondrocyte differentiation and the molecular mechanism.

ATF6 is a multifunctional transcription factor, implicated in the regulation of cell cycle and cell differentiation of many types of cells such as osteoblasts, and skeletal muscle myotubes (Jang et al., 2012; Kim et al., 2014; Vekich et al., 2012; Glembotski, 2014; Howarth et al., 2014; Wu J et al., 2011). ATF6a is the cleaved N-terminal cytoplasmic domain of ATF6, produced by proteases cleavage of ATF6 in ER stress (Li M et al., 2000; Nakanishi Keiko et al., 2005). Herein we found that ATF6 demonstrated clear expression in the entire growth plate chondrocyte in vivo (Figure 1A) and was differentially induced during BMP2-triggered chondrogenesis in vitro. (Figure1, B, C). Interestingly, the expression of ATF6 was reduced while the ATF6a (cleaved ATF6) was increasing with the elongation of BMP2 treatment, ATF6a protein was not detected until day 5 in BMP2-induced chondrocyte differentiation. The different expression profiles of ATF6 and ATF6a during chondrocyte differentiation demonstrate that posttranscription regulations might also be involved in the regulation of ATF6 and ATF6a expression during chondrogenesis.

ATF6 is an ER membrane-bound bZIP transcription factor. Under normal conditions, it is bound to the ER membrane through an interaction with chaperone BiP/GRP78 (Murakami et al., 2009; Li et al., 2000; Nakanishi et al., 2005). Upon the accumulation of unfolded or misfolded proteins in ER stress, ATF6 is released from BiP and transits to the Golgi, where it is cleaved by proteolysis, and nuclear translocation of its N-terminal fragment (Jang et al., 2012; Roca et al., 2005). In this study, we also found that ATF6 was differentially induced and cleaved in BMP2-mediated chondrocyte differentiation. Runx2 transcription factor appears to activate, whereas Sox6 inhibits, the expression of ATF6 gene in the BMP-2 stimulated chondrogenesis (Figures 3–5). There exist three Runx2-binding consensus sequence, RCCRC(A/T), and three Sox6-binding motif, (A/T)(A/T)CAA(A/T)G (Cantu’ C et al., 2011; Roca et al., 2005), in the ATF6 gene promoter. (Figures 2). Our assays, including both electrophoretic mobility shift assays and chromatin immunoprecipitation, indicated that Runx2 and Sox6 bind to their binding sites
in the regulatory region of ATF6 gene and mediate the delicate expression of ATF6 gene during BMP-2 trigged chondrogenesis (Figures 2-4).

Our in vitro chondrogenesis indicated that ATF6a and ATF6 are positive regulators of chondrocyte differentiation, since their overexpression enhances, while their suppression inhibits, BMP2-induced expressions of marker genes for chondrogenesis, including Aggrecan, collagen II, collagen X and MMP13. The concept that ATF6a and ATF6 are the stimulator of chondrocyte differentiation was further supported by ex vivo and in vivo studies demonstrating that ATF6a and ATF6 stimulate mineralization and endochondral bone growth (Figures 5, 6). Interestingly, ATF6a was found to associate with Runx2 and to act as a co-factor of Runx2 in the course of chondrogenesis. This finding is in accordance with numerous previous publications that a number of Runx2-binding proteins could modify its transcriptional function (Yoshida et al., 2004; Drissi, et al., 2003) (Cui CB et al., 2003; Gutierrez et al., 2002; Schroeder et al., 2004; Yoshida et al., 2002). Here our data showed ATF6a associates with Runx2 and enhances Runx2-induced chondrocyte hypertrophy. In addition, this interaction is BMP-2 dependent, since this complex is undetectable without BMP2 stimulation (Figure 7).

It is reported that IHH/PTHrP signaling regulates the process of hypertrophic chondrocyte differentiation through a negative feedback loop in growth plate endochondral ossification. PTHrP suppresses chondrocyte hypertrophy and negatively regulated endochondral bone development (Huang et al., 2001; Karp et al., 2000; Kronenberg, 2006). IHH is expressed at the prehypertrophic-hypertrophic chondrocytes and stimulates PTHrP production (Hu et al., 2007; Vortkamp et al., 1996). In PTHrP null embryonic mice, chondrocytes block the premature proliferation, and accelerate differentiation (MacLean et al., 2004). In the mice lacking either PTHrP or the parathyroid hormone (PTH)-PTHrP receptor, the proliferating zones of growth plate of metatarsal bones were decreased, and the hypertrophic chondrocytes differentiation were premature (Amizuka et al., 1994; Lanske et al., 1996; Lee et al., 1996; Weir et al., 1996). In contrast, chondrocyte-specific PTHrP transgenic mice displayed delayed hypertrophied chondrocyte differentiation (Vortkamp et al., 1996). It was known that the primary binding sites for ATF6a include the ERSE, ERSEII and the UPR element on basis of its combining feature. Interestingly, PTHrP gene promoter contains one UPR element sequence (TGACGT (T/G) and five ERSEII sequences (CCAC (G/A). In addition, the IHH promoter also contains two UPR element sequences and four ERSEII sequences (Yamamoto et al., 2004; Yoshida et al., 2000). These results suggest that ATF6a, as a transcription factor, may modulate IHH and PTHrP transcription and expression through combining with the specific cis-elements in their promoter regions. Our studies have demonstrated that overexpressing ATF6a enhances the expression of IHH, whereas it inhibits PTHrP; However, knockdown of ATF6a reduces the expression of IHH and increases PTHrP (Figure 8A-H). Whether regulation of PTHrP and IHH by ATF6a requires the direct binding of ATF6a to their promoters needs to be further delineated.
Based on the findings in this study, together with the literature (Ron D and Walter P, 2007; Murakami et al., 2009; Han et al., 2014; Cantu C et al., 2011; Roca et al., 2005; Kronenberg, 2006; Yamamoto et al., 2004), as well as our previous report, we propose a model for illustrating the role and regulation of ATF6a in the course of hypertrophic chondrocyte differentiation (Figure 8I). In brief, Runx2 activates the expression of ATF6 and enhances the ATF6 splicing. ATF6a, derived from ATF6, combines with Runx2, and function as the cofactors of Runx2 in mediating the chondrocyte hypertrophy. ATF6a and Runx2 form a positive feedback control loop. However, Sox6 prohibits the ATF6 expression and cleavage. Furthermore, ATF6a also regulates the expressions of IHH and PTHrP, modulating the process of chondrocyte hypertrophy and chondrogenesis as an opposing feedback loop. In summary, results in this study testify firstly that ATF6a is a previously unrecognized modulator of chondrocyte differentiation, and it exerts its functions in mediating chondrogenesis through, at least in part, acting as the co-activator of Runx2 and affecting IHH/PTHrP signaling.
Acknowledgments
This work was aided by the National Science Foundation of China (No. 81371928, No. 81171697); and New Century Excellent Talent Support Project of Education Ministry of China (NCET-12-1090).

Footnotes
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The abbreviations used are: BMP2, Bone morphogenetic protein 2; ATF6, activating transcription factor 6; ERS, ER stress; UPR, unfolded Protein Response; PERK, PKR-like ER resistant kinase; IRE1α, inositol requiring enzyme 1α; XBP1S, X-box binding protein1 spliced; IHH (Indian hedgehog) and PTHrP (parathyroid hormone-related peptide).

Conflicts of interest
All authors state that they have no conflicts of interest.
References


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Figure 1  Expression of ATF6a and ATF6 in the growth plate chondrocytes in vivo and in chondrocyte differentiation in vitro. (A), Temporal and spatial expression of ATF6 during chondrogenesis in vivo, assayed by immunohistochemistry. The sections of long bone from various embryonic and postnatal developmental stages of postcoital day 12.5 mouse embryo (E12.5; a), postcoital day 14.5 mouse embryo (E14.5; b), and then at the differentiation stage (E15.5, c) and continues during entire chondrogenic developmental stages in both proliferating and hypertrophic zones (from E17.5 to 10 days old, E17.5, d; Newborn, e; Day10, f). Microphotographs are shown of sections stained with anti-ATF6 antibody (brown) and counterstained with hematoxylin (blue). Immunostaining reveals positive nuclear staining in the entire chondrogenic developmental stages in both proliferating and hypertrophic zones, and the scale bars represent 100 µm. (B and C), Expression of ATF6a, BBF2H7 and type X collagen were examined in the course of chondrogenesis of a micromass cultures of C3H10T1/2 cells (B) and BMSCs (C) in the presence of 300 ng/ml recombinant BMP2 for various time points, as indicated, and the mRNA levels of ATF6a, BBF2H7 and type X collagen and GAPDH (serving as an internal control) were detected by real-time PCR. (D and E), Differential expression of ATF6a, ATF6 and type X collagen during chondrogenesis in C3H10T1/2 cells (D) and BMSCs (E) with 300 ng/ml BMP2 for the times indicated, and the levels of ATF6a, ATF6 (a and d), collagen X (b and e), and tubulin(c and f; serving as an internal control) were detected by immunoblotting.
Figure 2 Runx2 and Sox6 bind to the promoter of ATF6 gene. (A). Runx2 binds to the ATF6 promoter in vitro (EMSA). Ten micrograms of nuclear extracts (NE) prepared from C3H10T1/2 cells infected with Ad-Runx2 were incubated with digoxigenin-labeled Runx2-binding site in reaction buffer (20μl). For competition experiments, a 100-fold excess of wild type oligodeoxynucleotide was added. For supershift assays, anti-Runx2 (0.5μg) was included. After 15 min of incubation, the digoxigenin-labeled probe was added, and the reaction mixture was incubated for an additional 15 min and analyzed by gel electrophoresis. The positions of the supershifted complex (supershift), the DNA/protein complex (shift), and the free DNA probe (probe) are indicated. Arrows indicate free DNA probe (bottom) and DNA-protein complex (top). (B). Same procedure was followed in addition that Dig-labeled Sox6-binding site was employed as probe. Arrows indicate free probe (bottom) and DNA/protein complex (up). (C). Runx2 binds to the ATF6 promoter in vivo (ChIP). C3H10T1/2 cells infected with Ad-Runx2 were cross-linked by formaldehyde treatment and lysed. Cell lysates were subjected to immunoprecipitation with control IgG (lane 4) or anti-Runx2 antibodies (lane 3). Input DNA (lane 2; serving as positive control) and DNA recovered from the immunoprecipitation were amplified by PCR with the primers spanning the Runx2-binding site in the ATF6 promoter. (D). Same procedure was followed in addition that primers spanning Sox6-binding site were employed. (E and F), Runx2 and Sox6 bind to the ATF6 promoter in chondrogenesis. Micromass cultures of C3H10T1/2 cells were treated with 300 ng/ml BMP2 for 5 days, and cultures were processed and analyzed as in C.
Figure 3 Runx2 activates whereas Sox6 represses the transactivation of ATF6-specific reporter genes. (A and B) Schematic representation of three ATF6-specific reporter gene constructs. The indicated segments from the 5'-flanking region of the ATF6 gene were linked to an SV40 promoter (‘SV’) and a DNA segment encoding luciferase (‘Luc’). Black and open ovals indicate RBEs (Runx2-binding elements) and SBEs (Sox6-binding elements); numbers indicate distances in nucleotides from the first nucleotide of intron 1. (C) Runx2 activates the longer ATF6-specific reporter construct pATF6-luc in both C3H10T1/2 cells and ATDC5 cells. The reporter gene and the pCMV-gal internal control plasmid were transfected into cells together with the pcDNA3.1 (-)-Runx2 expression plasmid. At 48 h after transfection, the cultures were harvested and the luciferase and β-galactosidase activities were determined; the data shown are the mean levels of luciferase activity from three independent experiments, analyzed in triplicate and normalized by β-gal activity. *P<0.05. (D) Sox6 inhibits the activity of ATF6-specific reporter construct pATF6-luc in both C3H10T1/2 and ATDC5. The same procedure as
described in (C) was followed; (E) Runx2 activates the shorter ATF6-specific reporter construct pATF6-1SBE-1RBE-luc in C3H10T1/2. The same procedure as described in (C) was followed; (F) Sox6 inhibits the shorter ATF6-specific reporter construct pATF6-1SBE-1RBE-luc in C3H10T1/2. The same procedure as described in (C) was followed; (G) Diagrams show the alterations in the first of the Sox6-binding sites and the first of the Runx2-binding sites in the ATF6-1SBE-1RBE-luc reporter gene. Mutant nucleotides are indicated by arrows. (H) Runx2-dependent transactivation of pATF6 gene was dramatically reduced when Runx2-binding site was mutated (Mut1). The wild-type or mutant reporter gene specified and the pCMV-gal internal control plasmid were transfected into C3H10T1/2 together with pcDNA3.1 (-) vector (control) or pcDNA3.1 (-)-Runx2 expression plasmid, and the same procedure as described in (C) was followed; (I) Sox6-dependent inhibition of pATF6 gene was clearly disappeared when Sox6-binding site was mutated (Mut2).
Figure 4 Runx2 induces whereas Sox6 inhibits the expression of endogenous ATF6 gene. (A) Runx2 increases the level of ATF6 mRNA. C3H10T1/2 cells infected with Ad-Runx2 or control Ad-GFP were cultured for 48 h and endogenous ATF6 gene expression was determined by real-time PCR. Expression of ATF6 was normalized against the 18S rRNA endogenous control. The normalized values were then calibrated against the control value. The units are arbitrary, and the left bar indicates a relative level of ATF6 mRNA of 1; *P <0.05. (B) Sox6 reduces the level of ATF6 mRNA. C3H10T1/2 cells infected with Ad-Sox6 or control Ad-GFP were processed and analyzed as described in (A). (C) Runx2 increases the protein level of ATF6 and ATF6a. The same cultures were used to detect the protein level of ATF6 by western blotting. Tubulin protein serves as an internal control; (D) Sox6 reduces the protein level of ATF6 and ATF6a. C3H10T1/2 cells infected with Ad-Sox6 or control Ad-GFP were processed and analyzed as described in (C).
Figure 5  ATF6a and ATF6 promote hypertrophic chondrocyte differentiation, mineralization and endochondral bone growth. (A). Comparisons of ATF6a, ATF6 and BMP2 in stimulations of chondrogenesis of murine C3H10T1/2. C3H10T1/2 aggregate cultures were infected with either adenovirus encoding either GFP (Ad-GFP; serving as a control) or ATF6a (Ad-ATF6a) or ATF6 (Ad-ATF6) or presence of either 300 ng/ml BMP2, as indicated, for 3 or 7 days, followed by quantitative measurements of aggrecan, collagen II (Col II), collagen X (Col X) and MMP13 using real-time PCR. Units are arbitrary; normalized values were calibrated against controls, here given the value of 1. (B) Comparisons of ATF6a, ATF6 and BMP2 in stimulations of chondrogenesis of murine BMSCs, and the same procedure as described in (A) was followed; (C) Comparable potency of ATF6a, ATF6 and BMP2 in stimulation of chondrogenesis and activation of collagen II and collagen X expressions. hMSC aggregate cultures were incubated in the absence (CTR) or presence of 300 ng/ml BMP2 or Ad-ATF6 or Ad-ATF6a for 3 wk followed by Safranin O staining (left, red) or immunostains of collagen II (middle, green) or collagen X (right, green). (D)ATF6 (Ad-ATF6) and ATF6a (Ad-ATF6a) stimulates chondrocyte hypertrophy, mineralization and endochondral bone growth. (a and b) Safranin O/Fast Green staining of metatarsal bones. Metatarsals were explanted from 15-day-old mouse embryos and cultured in the presence of control (CTR), Ad-ATF6 (MOI 50) or Ad-ATF6a (MOI 50). After 5 days of culture, the explants were fixed and stained with Safranin O/Fast Green, shown in low-power (a) and high-power (b) microphotographs. (c) Length of proliferative zone and hypertrophic zone in metatarsal
bones. *P < 0.05 versus control; (d) Alizarin red/Alcian blue staining of metatarsals. The explants were fixed and processed for staining, and a representative photograph of an explanted metatarsal after 5 days of culture is presented. (e) Percentage changes in total (T) and mineralization (M) length of metatarsal bones. Percentage changes in bone length were calculated as (length at d5-length at d0)/length at d0. *P < 0.05 versus control; scale bar = 100µm.
Figure 6 Knockdown of ATF6a via the siRNA method largely abolishes hypertrophic chondrocyte differentiation. (A and F) Ad-ATF6a siRNA effectively prohibited expression of endogenous ATF6a in both C3H10T1/2(A) and BMSC cells (F). Cells were infected with Ad-ATF6a siRNA or control adenovirus (CTR), and mRNA was detected by real-time PCR. Expression of ATF6a was normalized against the GAPDH endogenous control. The normalized values were then calibrated against the control value, here set as 1. *, p<0.05. (B and G) Reduction of ATF6a obviously blocked BMP2-induced collagen II expression in C3H10T1/2 (B) and BMSC (G) cells. The mRNA level of collagen II was detected by real-time PCR, mRNA was isolated from micromass cultures of C3H10T1/2 (B) or BMSC (G) cells infected with siATF6a or control adenovirus in the presence of 300ng/ml BMP2 at various time points, as indicated. *, p<0.05. (C and H) Decrease of ATF6a clearly blocked BMP2-induced aggrecan expression in C3H10T1/2 (C) and BMSC (H) cells. Using the methods B and G, we detected the mRNA level of aggrecan by real-time PCR. *, p<0.05. (D and I) Reduction of ATF6a largely prohibited BMP2-induced collagen X expression in C3H10T1/2 (D) and BMSC (I) cells. We detected the mRNA level of collagen X with the same methods B and G. *, p<0.05. (E and J) Reduction of ATF6a remarkably blocked BMP2-induced MMP13 expression in C3H10T1/2 (E) and BMSC (J) cells. We detected the mRNA level of MMP13 with the same methods B and G. *, p<0.05. Error bars, S.D.
Figure 7  ATF6a combines with Runx2 in chondrocyte differentiation and ATF6a augments Runx2-induced hypertrophied chondrocyte. (A) ATF6a conjoins Runx2 in BMP2-induced C3H10T1/2 cells for 5 days. Cell lysates were collected from micromass culture of BMP2-induced C3H10T1/2 cells, and then were incubated with either control IgG (lane 3) or Runx2 antibodies (lane 2), followed by protein A-agarose. The immunoprecipitated (IP) protein complex and cell extracts (lane 1; serving as a positive control) were detected by Western blotting with anti-ATF6a antibody. (B) ATF6a associates with Runx2 in BMP2-mediated BMSC cells for 5 days, with the identical method in A. (C) ATF6 can not bind to Runx2 in vivo without BMP2. C3H10T1/2 cells lysates were incubated with either control IgG (lane 3) or Runx2 antibodies (lane 2), followed by protein A-agarose. The immunoprecipitated (IP) protein complex and cell extracts (lane 1; serving as a positive control) were examined by Western blotting with anti-ATF6 antibody. (D, E, F) ATF6a increased Runx2-induced collagen X (D), MMP13 (E), IHH (F) expression in C3H10T1/2 cells. The mRNA levels of collagen X, MMP13 and IHH were detected by real-time PCR, mRNA was isolated from C3H10T1/2 cells infected with Ad-GFP, Ad-Runx2, Ad-ATF6, Ad-ATF6+Ad-Runx2, Ad-ATF6a and Ad-ATF6a+Ad-Runx2, respectively, as indicated. *, p<0.05. Error bars, S.D.
Figure 8 ATF6a influences the expression of IHH and PTHrP. (A and B) Ad-ATF6a enhances the expression of IHH in chondrocyte differentiation of BMSCs (A) and C3H10T1/2 (B) cells. Micromass cultures of BMSCs (A) and C3H10T1/2 (B) cells infected with either Ad-GFP (CTR) or Ad-ATF6a were incubated with 300 ng/ml BMP2 for various times, as indicated, and the level of IHH was measured by real-time PCR. *, p <0.05. (C and D) Overexpression of Ad-ATF6a leads to remarkable reduction of PTHrP expression in chondrocyte differentiation of BMSCs (C) and C3H10T1/2 (D) cells. The same cultures as described in A and B were used to examine the expression of PTHrP using real-time PCR. *, p <0.05. (E and F) Knockdown of ATF6a via the siRNA approach completely prohibited IHH induction in chondrogenesis of BMSCs (E) and C3H10T1/2(F) cells. Micromass cultures of BMSCs(E) and C3H10T1/2(F) cells infected with either control RFP adenovirus (CTR) or siATF6a adenovirus were incubated with 300 ng/ml BMP2 for various times, as indicated, and the level of IHH was determined by real-time PCR. *, p <0.05. (G and H) Knockdown of ATF6a via the siRNA approach enhanced PTHrP expression in chondrocyte differentiation of BMSCs (G) and C3H10T1/2 (H) cells. The same cultures as described in A and B were used to examine the expression of PTHrP using real-time PCR. *, p <0.05. Error bars, S.D. (I) A proposed model for explaining the role and regulation of ATF6a in chondrogenesis. ATF6a, connecting with Runx2, acts as a necessary cofactor of Runx2 in stimulating hypertrophic

Sox6 → ATF6a ← Runx2 → Chondrocyte hypertrophy

Ihh → PTHrP
chondrocyte differentiation. ATF6a and Runx2 form a positive feedback regulation loop, whereas Sox6, suppresses the expression of ATF6a gene. The symbol ‘$\Rightarrow$’ and ‘$\neg$’ indicate ‘stimulation’ and ‘inhibition’ respectively.