The IRE1α-XBP1 Pathway Positively Regulates Parathyroid Hormone (PTH)/PTH-related Peptide Receptor Expression and Is Involved in PTH-induced Osteoclastogenesis*

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Background: XBP1 is a transcription factor that is activated by one of the ER stress sensors, IRE1α.

Results: XBP1 promotes the expression of PTH/PTH-related peptide receptor in osteoblasts and renders these cells responsive to PTH.

Conclusion: The IRE1α-XBP1 pathway indirectly regulates PTH-induced osteoclastogenesis.

Significance: This study may be the first to reveal the potential link between ER stress sensors and the regulation of osteoclastogenesis.

Osteoblasts produce a large amount of extracellular matrix proteins during skeletal development. The high level of protein synthesis leads to an accumulation of unfolded proteins in the endoplasmic reticulum (ER), a condition referred to as “ER stress” (1–3). To alleviate the overload in protein synthesis and the accumulation of unfolded proteins, cells under ER stress conditions temporarily suppress protein synthesis, remove the unfolded proteins, and reinforce the capacity of the ER to perform its functions. This homeostatic regulation of protein synthesis is called the unfolded protein response and is highly conserved across species. In mammalian cells, there are three major classes of stress sensors located in the ER that are involved in the transmission of ER stress signals to the nucleus: inositol-requiring protein-1α (IRE1α), activating transcription factor 6 (ATF6), and pancreatic ER kinase (PERK).

In addition to their roles in the maintenance of ER capacity and the quality of protein synthesis, recent studies have revealed that the unfolded protein response exhibits biological functions that are not directly related to ER stress (4), including the regulation of innate immunity, energy metabolism, and cell differentiation (5–10). In osteoblasts, it has been shown that pancreatic ER kinase and old astrocyte specifically induced substance (OASIS), a homologue of ATF6, are involved in osteoblast differentiation by inducing the transcription of Osteocalcin and type 1 collagen, respectively (11–13). Furthermore, we previously showed that IRE1α and its downstream transcription factor X-box-binding protein-1 (XBPI) are essential for BMP-induced osteoblast differentiation through promoting the transcription of Osterix (Oxx) (5), a transcription factor that is indispensable for osteoblast differentiation (14). IRE1α is a type-1 transmembrane protein that contains both a kinase domain and an endoribonuclease domain in the cytoplasmic tail (15). Upon activation, IRE1α activates JUN N-terminal kinase and processes the transcripts of Xbp1 by removing 26-bp-long nucleotides. The spliced transcripts of Xbp1 are translated to produce the full-length XBP1 protein, which exhibits potent transcriptional activity. The IRE1α-XBP1 pathway is the most highly conserved ER stress sensor in eukaryotes, and mice lacking IRE1α or XBP1 were shown to be embryonic lethal at an early developmental stage (16, 17).

The aim of the present study was to identify novel target genes of the IRE1α-XBP1 pathway in osteoblasts and investigate their potential roles in osteoblast differentiation and function. In a preliminary cDNA microarray experiment using transcripts from BMP2-treated wild-type and IRE1α−/− mouse
embryonic fibroblasts (mEFs), we unexpectedly found that the transcripts for PTH/PTH-related peptide (PTHRP) receptor (parathyroid hormone 1 receptor, PTHR1) were expressed at significantly lower levels in Ire1α−/− mEFs than in wild-type mEFs. PTHR1 is a member of the G-protein-coupled receptor family proteins and functions as a common receptor for both PTH and PTHrP (18). PTH is a crucial hormone for the regulation of calcium metabolism and is primarily produced in the parathyroid gland. PTHrP has diverse functions, including endochondral ossification, mammary gland development, and tooth eruption, and is produced by various cell types and organs (19, 20). In osteoblasts, both PTHR1 and PTHrP promote the expression of RANKL, a membrane-bound ligand that is essential for osteoclastogenesis, through PTHR1 signaling and thereby stimulate osteoclast formation and bone resorption (20). Of note, PTHrP is often highly secreted from cancer cells, and this overproduction of PTHrP is casually related to the pathogenesis of skeletal metastasis and hypercalcemia in malignancies (21, 22).

In this study, we show that the Ire1α-XBP1 pathway positively regulates the transcription of PTHR1 in osteoblasts and the osteoblast-like cell line MC3T3-E1 and that the suppression of Xbp1 transcripts abolishes PTH-induced Rankl transcripts and osteoclast formation in an in vitro model of osteoclastogenesis. The present study thus reveals the previously undescribed involvement of an ER stress sensor in the regulation of PTH-induced osteoclastogenesis.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cells**—Recombinant human BMP2 and PTH were purchased from Wako (Osaka, Japan). The anti-XBP1 antibody (M-186) and anti-β-actin antiserum were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, respectively. Ire1α, Xbp1, Traf2, and nontargeting control siRNAs were purchased from Sigma-Genosys. The generation of Ire1α−/− and wild-type mEFs was previously described (16). Primary osteoblasts (POBs) were collected from the calvaria of 18.5 days postcoitum wild-type embryos. STF-083010 was provided by the Ruga Corp. (Palo Alto, CA) (23).

Quantitative RT-PCR—cDNA was generated using RNAiso (Takara Bio, Shiga, Japan) and ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. PCR amplification and quantification were performed using SYBR premix Ex Taq II (Takara Bio) and the 7300 real-time PCR system (Applied Biosystems). Relative mRNA expression levels were calculated by normalizing to β-actin transcript expression levels. The nucleotide sequences of the oligonucleotides used in the current study will be provided upon request.

**Constructs**—The expression vector for XBP1s was generated as described previously (5). For retroviral gene transfer, the cDNA of XBP1s was cloned into the pMXs-IG retroviral vector, which harbors an internal ribosome entry site and a green fluorescent protein sequence downstream of the multiple cloning site to facilitate the detection of virus-transfected cells (24). The Pth1r P2 promoter (P2-1812; see Fig. 1, E and F) was cloned through PCR into the pGL3 luciferase reporter vector (Promega) using C57BL/6 genomic DNA as a template. Deletion mutants of the Pth1r promoter were generated by a PCR-based method using a KOD-Plus-mutagenesis kit (Toyobo) according to the manufacturer’s instructions.

**Luciferase Assay**—The luciferase assay was performed with a Dual-Luciferase assay system (Promega), and pRL-SV40 (Promega) was used as a transfection efficiency control. C3H10T1/2 cells were transfected with the reporter constructs and the XBP1s expression vector using FuGENE HD (Roche Applied Science) and incubated overnight. Luciferase activity was measured using GloMax-20/20 (Promega). The transcriptional activity was expressed as the ratio of firefly:Renilla luciferase activity.

**Chromatin Immunoprecipitation Assay**—MC3T3-E1 cells and POBs were cultured in the presence of BMP2 (200 ng/ml) for 72 h. After incubation, the cells were fixed in 1% paraformaldehyde/PBS for 10 min at room temperature. Chromatin shearing and immunoprecipitation were performed using ChIP-IT express chromatin immunoprecipitation kits (Active Motif) according to the manufacturer’s instructions. The immunoprecipitated DNA fragments were used as templates for PCR amplification.

**In Vitro Osteoclastogenesis Assay**—Bone marrow cells harvested from the femurs of 8-week-old wild-type mice were grown in Minimum Essential Medium α medium with 10% FCS, antibiotics, and 30 ng/ml recombinant murine CSF-1 (Wako) for 3 days on Petri dishes. The adherent cells were used as bone marrow macrophages (BMMs). POBs treated with siRNA against Ire1α, Xbp1, or control siRNA were incubated overnight. BMMs were subsequently added to the siRNA-pretreated POBs and further incubated in the presence of PTH for 4 days. After incubation, the cells were fixed with 10% formaldehyde/PBS for 5 min, dehydrated with 1:1 ethanol/acetone solution for 1 min, and air-dried for 30 min. The cells were stained for tartrate acid-resistant acid phosphatase (TRAP), and the number of osteoclasts (defined as TRAP-positive multinucleated cells with more than three nuclei) was counted under a microscope.

**Determination of Cellular cAMP Levels**—POBs were treated with siRNA against Xbp1 or control siRNA and cultured overnight. On the following day, the siRNA-treated cells were incubated in serum-free medium for 5 h and subsequently incubated with PTH-supplemented serum-free medium for 20 min. The cellular cAMP levels were determined using the bioluminescent CAMP-Glo assay kit (Promega) according to the manufacturer’s instructions.

**Retroviral Gene Transfer**—The pMXs-IG retroviral vector harboring Xbp1s cDNA or an empty vector was introduced into PLAT-E retrovirus packaging cells (24) using FuGENE HD (Roche Applied Science). The viral supernatants were collected on day 3 and added to wild-type mEFs. A transfection rate of ~70% was achieved in the present study.

**Statistical Analysis**—Student’s t test for two samples assuming equal variances was used to calculate the p values. p values less than 0.05 were considered to be statistically significant. All of the experiments were repeated at least three independent times with similar results. Error bars indicate the S.D.
scripts were highly induced in wild-type mEFs after the addition of BMP2-driven osteoblast differentiation model, related genes, including Osx, were activated upon BMP2 stimulation (Fig. 1A, left panel). Wild-type and Ire1α−/− mEFs were incubated with BMP2 (200 ng/ml) for the designated time period (right panel). The expression levels of Pth1r transcripts were analyzed by quantitative RT-PCR. B, MC3T3-E1 cells were treated with control siRNAs (Ctrl) or siRNAs against Ire1α or Xbp1 and incubated with BMP2 (200 ng/ml) for 48 h. The expression levels of Pth1r (left panel) and Xbp1s (middle panel) transcripts were analyzed by quantitative RT-PCR. The decrease in Xbp1s protein in Ire1α or Xbp1 siRNA-treated cells was confirmed by Western blot analysis (right panel). C, left and middle panels, quantitative analysis of the expression levels of Pth1r (left panel) and Xbp1s (middle panel) in siRNA-treated POBs. Right panel, Western blot analysis of Xbp1s protein in siRNA-treated POBs. D, quantitative analysis of the expression levels of Pth1r and Osx in wild-type and Ire1α−/− mEFs transfected with empty (−) or Osx expression vector (Osx). The cells were incubated with BMP2 (B2, 200 ng/ml) or vehicle (v) for 48 h. E, a schematic of the P2 promoter of Pth1r. Two putative binding sites for XBP1 and the expected PCR products for the chromatin immunoprecipitation assays performed in the present study are shown. F, reporter assays using the indicated reporter construct and an expression vector bearing Xbp1s cDNA (XBP1s) or a control empty vector (−). G, chromatin immunoprecipitation assays using the nuclear extracts from POBs and MC3T3-E1 cells. The extracts were immunoprecipitated (IP) with a control rabbit IgG antibody (IgG) or the anti-XBP1 antibody (aXBP1). H, the mEFs transfected with an empty vector (−) or the pMXs-IG vector harboring Xbp1s cDNA (XBP1s) were cultured with vehicle or BMP2 (200 ng/ml) for 48 h. The expression levels of Pth1r were analyzed by quantitative RT-PCR. *, p < 0.005. n.s., not significant.

**RESULTS AND DISCUSSION**

**Pth1r Expression Is Suppressed in Ire1α−/− mEFs**—To analyze how the lack of Ire1α would affect the gene expression pattern in BMP2-treated mEFs, we previously performed a gene chip experiment using the transcripts from wild-type and Ire1α−/− mEFs. Pth1r was among several bone metabolism-related genes, including Osx (5), that showed a significantly lower expression in Ire1α−/− mEFs than in wild-type mEFs. Because PTH/PTHrP-PTH1R signaling is a critical regulator for both calcium metabolism and osteoclastogenesis, we further explored the potential link between Ire1α and the transcriptional regulation of PTH1R.

Consistent with our previous study (5), we found that treatment with BMP2 significantly increased the expression of both unspliced and spliced Xbp1 (Xbp1s) transcripts in wild-type mEFs, confirming that the Ire1α-XBP1 pathway becomes activated upon BMP2 stimulation (Fig. 1A, left panel). In this BMP2-driven osteoblast differentiation model, Pth1r transcripts were highly induced in wild-type mEFs after the addition of BMP2 (Fig. 1A, right panel). In contrast, this increase in Pth1r transcripts was significantly suppressed in mEFs lacking Ire1α. To further validate the observation that the abrogation of Ire1α leads to the suppression of Pth1r transcripts, we performed gene silencing experiments using siRNAs against Ire1α and Xbp1. As shown in Fig. 1B, we found that the gene silencing of Ire1α and Xbp1 in MC3T3-E1 cells led to a decrease in Pth1r transcription. The gene silencing of Traf2, a target substrate of Ire1α kinase (15, 16), did not show any effect on Pth1r expression (data not shown). The decrease in Xbp1s transcripts and protein in the cells treated with siRNAs against Ire1α and Xbp1 was confirmed by quantitative RT-PCR and Western blot analysis, respectively (Fig. 1B). The decreased expression of Pth1r transcripts was also observed in Xbp1 siRNA-treated POBs (Fig. 1C).

Because mEFs lacking Ire1α are defective in inducing Osx transcripts when osteoblastic differentiation is driven by BMP2 (Fig. 1D, right panel) (5), we examined whether the forced expression of Osx would rescue the defective Pth1r expression
in Ire1α−/− mEFs. However, the reintroduction of Osx showed little effect on the expression levels of Pth1r in BMP2-treated Ire1α−/− mEFs (Fig. 1D, left panel). A high expression of Osx transcripts in Osx expression vector-transfected Ire1α−/− mEFs was confirmed by quantitative RT-PCR (Fig. 1D, right panel). These observations indicate that the decrease in Pth1r transcripts in Ire1α−/− mEFs was not secondarily derived from defective Osx expression in these cells.

**XBP1s Binds to the P2 Promoter of Pth1r and Induces Transcriptional Activity**—The observation that Pth1r expression is decreased in Ire1α−/− mEFs and suppressed by the gene silencing of Ire1α and Xbp1 in wild-type cells implied that the transcription of Pth1r is, at least in part, regulated by the Ire1α-XBP1 pathway. It has been shown that the promoter sequence of Pth1r consists of two major components, the P1 and P2 promoters (25). The expression of Pth1r in the kidney is regulated by both the P1 and the P2 promoters, whereas in osteoblasts, expression is primarily dependent on the P2 promoter (Fig. 1E).

We therefore performed luciferase promoter assays using a luciferase reporter plasmid harboring the P2 promoter sequence (Fig. 1F, P2-1812). As shown in Fig. 2B, we observed a sharp increase in luciferase activity when the XBP1s expression vector was co-transfected with the reporter plasmid, suggesting that XBP1s directly up-regulates the transcription of Pth1r. Nucleotide sequence analysis revealed two potential binding sites for XBP1s (26) in the P2 promoter region that were located 4 bp apart from one another: 245–242 and 237–232 bp upstream from the start codon, respectively (Fig. 1E). The abrogation of either or both of these putative binding sites (reporter construct: P2Δ-237–232, P2Δ-245–242, and P2Δ-245–232) resulted in a significant decrease in Pth1r transcriptional activity (Fig. 1F), indicating that XBP1s exerts its transcriptional activity via directly binding to these sites. Furthermore, we performed chromatin immunoprecipitation experiments using POBs and MC3T3-E1 cells and found that XBP1s indeed binds to the promoter of Pth1r containing these two putative binding sites (Fig. 1G). In line with these observations, the introduction of the XBP1s expression vector alone was sufficient to promote Pth1r transcription in untreated or BMP2-treated MC3T3-E1 cells (Fig. 1F).

**Gene Silencing of Xbp1 Suppresses PTH-induced Rankl Expression and Osteoclastogenesis**—Because the Ire1α-XBP1 pathway positively regulates Pth1r expression, we next sought to elucidate more biologically relevant functions of this regulatory mechanism. To this end, we first examined whether the expression levels of Pth1r in siRNA-treated cells correlated with the degree of PTH1R signaling. We measured the cellular levels of cAMP, a second messenger of PTH1R, and found that POBs treated with Xbp1 siRNA produced significantly less cAMP upon PTH stimulation as compared with the control siRNA-treated POBs (Fig. 2A). This observation indicates that the suppression of Xbp1 transcripts down-regulates PTH1R expression and consequently diminishes PTH-PTH1R signaling.

Both PTH and PTHrP induce RANKL expression in stromal cells and osteoblasts and support osteoclast formation and bone resorption. Because inhibition of the Ire1α-XBP1 pathway suppresses PTH1R signaling, we hypothesized that osteoblasts treated with Xbp1 siRNA would become less responsive to PTH and have lower Rankl expression upon PTH stimulation. As shown in Fig. 2B (left panel), the incubation of POBs with PTH significantly increased Rankl expression levels; how-
ever, this increase was offset by the gene silencing of Xbp1. The suppression of PTH-induced Rankl expression was also observed in cells treated with an IRE1α endonuclease inhibitor, STF-083010 (Fig. 2B, right panel) (23). To determine whether the inhibition of the IRE1α-XBP1 pathway in osteoblasts would render the cells less potent in supporting PTH-induced osteoclastogenesis, we performed an in vitro osteoclast-forming assay using BMMs. TRAP-positive osteoclasts were induced when BMMs and control siRNA-pretreated POBs or untreated POBs (−) were co-cultured in the presence of PTH; however, the formation of osteoclasts was almost completely abrogated when the POBs were pretreated with Ire1α or Xbp1 siRNA (Fig. 2, C and D). Similar results were observed using the osteoclast progenitor-like cell line, RAW264.7 (data not shown).

Taken together, the present study demonstrates that XBP1s positively regulates the transcription of Pth1r by directly binding to the P2 promoter of Pth1r and suggests that the IRE1α-XBP1 pathway in osteoblasts is involved in the regulation of PTH-induced osteoclastogenesis through promoting the transcription of Pth1r. This study thus reveals an unexpected link between the IRE1α-XBP1 pathway and PTH-induced osteoclastogenesis (Fig. 2E). These findings may also have an important clinical implication because PTH1R signaling is deemed critical for the establishment of the skeletal metastasis of cancer cells through stimulating osteoclast formation and bone resorption by promoting RANKL expression in osteoblasts and stromal cells (18, 21).

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