The IRE1 α –XBP1 pathway is essential for osteoblast differentiation through promoting transcription of *Osterix*

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During skeletal development, osteoblasts produce large amounts of extracellular matrix proteins and must therefore increase their secretory machinery to handle the deposition. The accumulation of unfolded protein in the endoplasmic reticulum induces an adoptive mechanism called the unfolded protein response (UPR). We show that one of the most crucial UPR mediators, inositol-requiring protein 1 α (IRE1 α), and its target transcription factor X-box binding protein 1 (XBP1), are essential for bone morphogenic protein 2-induced osteoblast differentiation. Furthermore, we identify *Osterix* (*Osx*, a transcription factor that is indispensable for bone formation) as a target gene of XBP1. The promoter region of the *Osx* gene encodes two potential binding motifs for XBP1, and we show that XBP1 binds to these regions. Thus, the IRE1 α –XBP1 pathway is involved in osteoblast differentiation through promoting *Osx* transcription.

Keywords: IRE1 α ; osteoblast differentiation; *Osterix*; unfolded protein response; XBP1

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INTRODUCTION

To manage the burden of protein synthesis, cells augment the folding and secretory capacity of the endoplasmic reticulum (ER) in response to the accumulation of misfolded proteins. This system, known as the unfolded protein response (UPR), is a highly conserved cellular mechanism in eukaryotic cells. In mammalian cells, there are three classes of ER stress transducers involved in the UPR including inositol-requiring protein 1 α (IRE1 α), activating transcription factor 6 (ATF6) and pancreatic ER kinase (PERK), and their functions in the UPR have been a subject of intensive study in the past decade (Rutkowski & Kaufman, 2004; Xu *et al*, 2005; Ron & Walter, 2007; Hetz & Glimcher, 2009).

The activation of the UPR is especially important in cells specialized to secrete proteins including plasma cells, endocrine cells and osteoblasts. Past studies have shown that PERK (Zhang *et al*, 2002) and OASIS (old astrocyte specifically induced substance), an ATF6 homologue (Murakami *et al*, 2009), are involved in skeletal development. However, a potential role for IRE1 α in osteoblast differentiation remains uninvestigated. IRE1 α is a type-1 transmembrane protein harbouring both a kinase domain and an endoribonuclease domain. The former is involved in the recruitment of tumour necrosis factor receptor-associated factor 2 (TRAF2)—which in turn activates JUN amino-terminal kinase (JNK)—and the latter is involved in the processing of X-box binding protein 1 (XBP1) mRNA. The consequences of JNK activation through IRE1 α signalling are not fully understood; however, it is probably involved in the initiation of autophagy (Ogata *et al*, 2006) and in the promotion of apoptosis (Yoneda *et al*, 2001). XBP1 derived from spliced *Xbp1* mRNA (XBP1s) translocates to the nucleus and binds to UPR elements to induce the transcription of a variety of UPR genes that mainly function to alleviate ER stress (Yoshida *et al*, 2001; Acosta-Alvear *et al*, 2007). Mice lacking IRE1 α or XBP1 were embryonically lethal and died

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Fig 1 | *Xbp1* and *BiP* transcripts are upregulated during BMP2-induced osteoblast differentiation. Wild-type MEFs were incubated in the presence of BMP2 (200 ng/ml) for the designated time period. The expression levels of the transcripts for (A) osteoblast differentiation markers and (B) UPR-related genes were analysed by quantitative RT-PCR and western blot analysis (IRE1 α). The expression level of each gene at baseline was set to one. (C) Western blot analysis (left panel) and immunostaining (right panel) of XBP1 in BMP2-treated or untreated (–) MEFs. Scale bar, 20 μ m. (D) Fluorescent activity in BMP2 (500 ng/ml)-treated calvariae collected from control and ERAI-transgenic (ERAI-tg) newborn mice. Upper panel, light image; lower panel, fluorescence image. Scale bar, 3 mm. BMP2, bone morphogenic protein 2; ERAI, endoplasmic reticulum stress-activated indicator; IRE1 α , inositol-requiring protein 1 α ; MEF, mouse embryonic fibroblast; RT, reverse transcription; UPR, unfolded protein response; XBP1, X-box binding protein 1.

at around 9.5–11.5 or 11.5–14.5 days post-coitum (dpc), respectively, supporting the idea that these genes are important *in vivo* (Reimold et al, 2000; Urano et al, 2000).

We investigated the potential role of IRE1 α in osteoblast differentiation and found that the IRE1 α –XBP1 pathway is essential for bone morphogenic protein 2 (BMP2)-induced osteoblast differentiation. Moreover, we identified *Osterix* (*Osx*)—one of the essential transcription factors for osteoblast differentiation (Nakashima et al, 2002)—as a target gene for XBP1s. In addition, we found that thapsigargin, a potent inducer of ER stress, can promote *Osx* transcription in BMP2-treated fibroblasts and in an osteoblast cell line—MC3T3-E1—in an IRE1 α –XBP1-dependent manner. Taken together, these observations show a previously unknown relationship between the IRE1 α –XBP1 pathway and *Osx*, and might provide new insights into the mechanisms underlying UPR and skeletal development.

RESULTS AND DISCUSSION

To examine the possible involvement of IRE1 α signalling in osteoblast-differentiation, we performed *in vitro* osteoblast-

differentiation experiments using mouse embryonic fibroblasts (MEFs) and recombinant BMP2, which is known to stimulate osteogenic differentiation processes in undifferentiated fibroblasts *in vitro*. The expression levels of two transcription factors that are essential for osteoblast differentiation, *Runx2* and *Osx* (Komori et al, 1997; Nakashima et al, 2002), and the bone extracellular matrix proteins COL1A1 and osteocalcin (OCN; markers for early and late stages of osteoblast differentiation, respectively), were evaluated to monitor the degree of differentiation. The expression levels of these osteoblastic markers in the MEFs—which were almost undetectable when cultured in maintenance medium—were significantly increased by the addition of BMP2 (Fig 1A). By contrast, the expression level of IRE1 α showed no change during the course of the experiment (Fig 1B). However, we found that mRNA expression levels of total *Xbp1* (including both unspliced (*Xbp1u*) and spliced *Xbp1* (*Xbp1s*) mRNA), *Xbp1s* and *BiP*/GRP78 chaperone were significantly upregulated, indicating that the UPR was induced by BMP2 in MEFs, probably to deal with the increase in protein synthesis that is known to occur in these conditions. There was also an increase in the expression

Fig 2 | The IRE1-XBP1, but not the IRE1-TRAF2, pathway is required for BMP2-induced osteoblast differentiation. (A) Wild-type and *Ire1α*^{-/-} MEFs were incubated in the presence or absence of BMP2 (200 ng/ml) for 48 h. ALP activity was evaluated by cell staining with BCIP/NBT or by colorimetry (right panel) as described in the supplementary methods online. Cell staining with crystal violet (CV) showed a similar amount of cells in each well. (B) mRNA expressions of total *Xbp1* and *Xbp1s* in BMP2-treated wild-type and *Ire1α*^{-/-} MEFs. (C) Quantitative analysis of the transcripts of osteoblast markers in BMP2-treated wild-type and *Ire1α*^{-/-} MEFs. The expression level of each gene in wild-type MEFs at baseline was set to one. (D) Wild-type MEFs treated with control (Ctrl) *Ire1α*- or *Xbp1*-siRNA were incubated with or without BMP2 for 48 h. ALP activity was evaluated by cell staining (left panel) and colorimetry (right panel). (E) Quantitative analysis of *Xbp1s* in siRNA-introduced MEFs incubated with or without BMP2. (F) Quantitative analysis of the transcripts of osteoblast markers in *Xbp1*-siRNA-treated wild-type MEFs incubated in the presence of BMP2 for 48 h. (G) No difference in ALP activity between control (Ctrl) and *Traf2*-siRNA-treated wild-type MEFs incubated with BMP2 for 48 h (left panel) was observed. Quantitative PCR analysis showed a significant decrease in *Traf2* transcript levels in *Traf2*-siRNA-treated MEFs (right panel). (H) Quantitative PCR analysis of osteoblast markers showed no difference between control (Ctrl) and *Traf2*-siRNA-treated wild-type MEFs incubated with BMP2. The expression level of each gene in the control siRNA-transfected MEFs was set to one. **P* < 0.05. ALP, alkaline phosphatase; BMP2, bone morphogenetic protein 2; Ctrl, control; IRE1 α , inositol-requiring protein 1 α ; MEF, mouse embryonic fibroblast; NS, not significant; Ocn, Osteocalcin; Osx, Osterix; RT, reverse transcription; siRNA, small-interfering RNA; TRAF, tumour necrosis factor receptor-associated factor 2; UPR, unfolded protein response; XBP1, X-box binding protein 1.

levels of other UPR-related genes, including *Gadd34*, *Chop*/*Gadd153*, *Ero1a* and *Wfs1* (data not shown). In addition, we found that BMP2 treatment can upregulate the expression of genes in the other branches of ER stress inducers (PERK-ATF4 and ATF6; supplementary Fig S1 online). The increase in the level of XBP1

protein was also confirmed by western blot (Fig 1C, left panel), and immunocytochemistry of XBP1 in BMP2-treated MEFs showed an accumulation of XBP1 protein in the nucleus (Fig 1C, right panel). The processing of *Xbp1* in differentiating osteoblasts was also evaluated by using ER stress-activated indicator (ERAI)-transgenic

Fig 3 | XBP1 promotes *Osterix* transcription. (A) Reporter assays using the *Runx2*, *Osx*, *Col1a1* and *Ocn* promoter constructs. Mouse embryonic fibroblast cell line C3H10T1/2 was co-transfected with the indicated reporter constructs and an empty vector (–) or with an expression vector bearing either XBP1s or XBP1u complementary DNA. **P* < 0.05. (B) Comparison of XBP1-binding sequence and UPR element-like sequence in the promoter region of the *Osx* genome between species. (C) Schematic representations of the *Osx* reporter constructs and the mutants (left panel). Reporter assays using the indicated reporter construct and an expression vector bearing XBP1s complementary DNA (right panel). Luciferase activity of the empty reporter construct (–)-transfected cells was set to one. (D) Schematic representation of the *Osx* promoter region and the expected PCR products (upper panel). Chromatin immunoprecipitation assays using a control rabbit IgG, XBP1 antibody or RUNX2 antibody (RX2, used as a negative control; lower panels). A set of primers that amplifies the region outside the putative binding sites was used as a negative control for the experiment. IgG, immunoglobulin G; IP, immunoprecipitation; mEF, mouse embryonic fibroblast; NS, not significant; Ocn, Osteocalcin; Osx, Osterix; POB, primary osteoblasts; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1s, spliced X-box binding protein 1; XBP1u, unspliced X-box binding protein 1.

mice (Iwawaki et al, 2004), in which fluorescent protein is induced on activation of IRE1 α . As shown in Fig 1D, there was a significant increase in fluorescence activity in the BMP2-treated calvarial bone collected from newborn ERAI-transgenic mice, especially in the junction of the sagittal and coronal sutures, where bone formation is achieved through direct differentiation of mesenchymal cells to osteoblasts. This observation illustrates that *Xbp1* transcripts are processed in the activated osteoblasts.

Next, we used MEFs lacking IRE1 α to investigate how the absence of IRE1 α in MEFs would affect their osteoblast-differentiation capacity and the production of extracellular matrix proteins. As shown in Fig 2A, the addition of BMP2 significantly increased alkaline phosphatase (ALP) activity in wild-type MEFs, compared with minimal or no increase in *Ire1 α* ^{–/–} MEFs. These results indicate that BMP2-induced osteoblast differentiation is hampered by the absence of IRE1 α . The absence of *Xbp1*s mRNA expression in *Ire1 α* ^{–/–} MEFs was confirmed by reverse transcription (RT)–PCR (Fig 2B). A time course expression analysis of the aforementioned osteoblast markers by quantitative RT–PCR in

BMP2-treated MEFs showed reduced expression in *Ire1 α* ^{–/–} MEFs, compared with control MEFs (Fig 2C). The expression levels of other osteoblast markers—including bone sialoprotein, osteopontin and osteonectin—were also significantly reduced in *Ire1 α* ^{–/–} MEFs, compared with control MEFs (supplementary Fig S2 online). As it is also conceivable that the hampered osteoblast differentiation in *Ire1 α* ^{–/–} MEFs was derived from an increase in apoptosis, we evaluated the apoptosis in BMP2-treated *Ire1 α* ^{–/–} and wild-type MEFs. As expected, there was an increase in the apoptosis rate in *Ire1 α* ^{–/–} MEFs compared with wild-type MEFs; however, the overall ratio of the apoptotic cell rate was low (1.7% and 3.5% in wild-type and *Ire1 α* ^{–/–} MEFs, respectively), suggesting that the increased apoptosis in the absence of IRE1 α was not the primary cause for the defect in differentiation (supplementary Fig S3 online). Notably, even in the absence of IRE1 α , there was a transient increase (peaking at around 48 h) in the expression levels of *Runx2* and *Col1a1* after BMP2 treatment, whereas the expression levels of *Osx* and *Ocn* (late-stage markers of osteoblast differentiation) remained almost unchanged. Taken

Fig 4 | *Osterix* transcription is induced by thapsigargin in BMP2-treated mouse embryonic fibroblasts and MC3T3-E1 cells. (A) Non-pretreated or BMP2-pretreated (BMP(+)) wild-type and *Ire1α*^{-/-} MEFs were incubated with or without thapsigargin for 3 or 6 h. The expression level of the *Osx* gene was evaluated by quantitative PCR. (B) Quantitative analysis of *Osx* transcripts in MC3T3-E1 cells incubated with or without thapsigargin (Tg) for 3 h. (C) Quantitative analysis of *Osx* transcripts in MC3T3-E1 cells treated with control or *Xbp1*-siRNA in the presence or absence of thapsigargin. (D) RT-PCR analysis of *Xbp1s*, *Osx* and *Runx2* transcripts in the control vector (-) or XBP1s-expression vector-introduced MC3T3-E1 cells (left panel). The expression level of *Osx* was also evaluated by quantitative RT-PCR (right panel). (E) Wild-type and *Ire1α*^{-/-} MEFs transfected with a control (-) or OSX expression vector (OSX) were incubated in the presence or absence of BMP2 (200 ng/ml) for 5 days. ALP activity was evaluated by cell staining with BCIP/NBT and by colorimetry (right panel). **P* < 0.05. (F) A schematic model showing the role of IRE1 α -XBP1-mediated UPR in osteoblast differentiation. ALP, alkaline phosphatase; BMP2, bone morphogenic protein 2; CV, crystal violet; ER, endoplasmic reticulum; IRE1 α , inositol-requiring protein 1 α ; Ocn, Osteocalcin; *Osx*, Osterix; RT, reverse transcription; UPR, unfolded protein response; siRNA, small interfering RNA; XBP1, X-box binding protein 1.

together, these observations indicate that the maturation of osteoblasts is inhibited in the absence of IRE1 α , whereas the initial stage of differentiation—in which undifferentiated mesenchymal cells commit to the osteoblast lineage—is not affected.

It has been shown previously that IRE1 α not only processes *Xbp1* mRNA, but can also activate stress-induced JNK through recruitment of TRAF2 (Urano et al, 2000). Next, we asked which of these pathways is more involved in BMP2-induced osteoblast differentiation. We used small interfering RNA (siRNA)-mediated gene silencing to suppress the expression of XBP1 and TRAF2 in wild-type MEFs and subjected these cells to *in vitro* osteoblast-differentiation experiments. In *Ire1α*- and *Xbp1*-siRNA-transfected MEFs, the increase in ALP activity after BMP2 treatment was reduced to a level comparable to that observed in *Ire1α*^{-/-} MEFs (Fig 2D). The reduction in the amount of *Xbp1s* mRNA was confirmed by quantitative RT-PCR (Fig 2E). In addition, we found that the mRNA expression levels of *Col1a1* and *Osx* were significantly reduced, whereas that of *Runx2* remained unaltered (Fig 2F). Conversely, introduction of *Traf2*-siRNA into MEFs had little effect on their potential to induce ALP after BMP2 treatment (Fig 2G, left panel). The suppression of the transcript level of *Traf2*

in siRNA-transfected MEFs was confirmed by quantitative RT-PCR (Fig 2G, right panel). Consistently, there was no change in mRNA expression levels in any of the osteoblast markers in *Traf2*-siRNA-treated cells (Fig 2H). Taken together, these observations suggest that the IRE1 α -XBP1, but not the IRE1 α -TRAF2, pathway is essential for BMP2-induced osteoblast differentiation.

To gain insight into the regulatory mechanism underlying IRE1 α -XBP1 and osteoblast differentiation, we performed a reporter assay using reporter constructs carrying the promoter of *Runx2*, *Osx*, *Col1a1* and *Ocn*. Unexpectedly, we found that reporter activity was highly induced in cells transfected with an *Osx*-reporter vector and XBP1s expression vector, and to a lesser extent in the cells transfected with a *Col1a1*-reporter vector (Fig 3A). By contrast, there was no change in reporter activity in the cells transfected with a *Runx2*- or *Ocn*-reporter vector. Introduction of the unspliced version of XBP1—which could potentially interfere with the activity of XBP1s (Yoshida et al, 2006)—had no effect on reporter activity in the cells transfected with any of the four reporter constructs. In addition, co-transfection of ATF4 (which functions downstream of PERK) or ATF6 expression vector with the *Osx*-reporter construct did not show any change

in reporter activity (data not shown). Interestingly, comparative genomic sequence analysis revealed that the promoter region of *Osx* encodes a UPR element (TGACGTGG/A)-like sequence (TGAGCTGG; Yoshida *et al*, 2001) and an XBP1-binding sequence (ACGT; Acosta-Alvear *et al*, 2007), which are highly conserved among species (Fig 3B). On the basis of these sequence data, we generated several mutant reporter constructs and performed a luciferase assay. As shown in Fig 3C, the shorter reporter constructs—which lack one (*Osx* –786) or both (*Osx* –355) of the aforementioned putative binding motifs—showed a decrease in luciferase activity, compared with the full-length construct (*Osx* –1269). Furthermore, the mutant constructs—which lack either one (*Osx* 1269 Δ 504–491 and *Osx* –1269 Δ –830–821) or both (*Osx* –1269 Δ –830–821, 504–491) binding motifs—also showed a significant decrease in the XBP1s-driven luciferase activity. Chromatin immunoprecipitation assays confirmed that XBP1 binds to the promoter region of *Osx* containing either one of the putative binding motifs in MEFs, MC3T3-E1 cells and primary osteoblasts from wild-type newborn mice (Fig 3D).

The observations that XBP1s promotes *Osx* transcription in the reporter assay led us to ask whether ER stress alone is capable of inducing *Osx* transcription. We repeated the reporter assays using the aforementioned *Osx* reporter constructs and found that ER stress induced by thapsigargin is able to promote *Osx* reporter activity (supplementary Fig S4 online). Furthermore, this activity was abolished in *Ire1 α* ^{–/–} MEFs, in which no *Xbp1*s mRNA is expressed (Fig 2B), indicating that ER stress-induced *Osx* reporter activity is dependent on IRE1 α , presumably involving IRE1 α -mediated induction of XBP1s. Next, we incubated wild-type and *Ire1 α* ^{–/–} MEFs with thapsigargin for 3 or 6 h and then examined the transcript level of *Osx* by quantitative RT–PCR. As shown in Fig 4A, there was no change in *Osx* transcripts in either wild-type or *Ire1 α* ^{–/–} MEFs; however, when the cells were preincubated with BMP2 for 16 h, the addition of thapsigargin significantly induced the *Osx* transcripts in wild-type, but not in *Ire1 α* ^{–/–}, MEFs. Furthermore, we found that treatment with thapsigargin alone was sufficient to promote *Osx* transcripts in a preosteoblastic cell-line, MC3T3-E1 (Fig 4B). The thapsigargin-induced *Osx* transcripts were abolished by gene silencing of *Xbp1* (Fig 4C), indicating that upregulation of *Osx* transcripts by thapsigargin is XBP1s-dependent. Consistently, overexpression of XBP1s was also capable of inducing *Osx* transcripts, but not those of *Runx2*, in MC3T3-E1 cells (Fig 4D). These observations suggest that after a cell differentiates into a preosteoblast, *Osx* becomes inducible by XBP1-dependent UPR signalling.

Finally, to consolidate our observations, we examined whether the introduction of *Osx* to *Ire1 α* ^{–/–} MEFs could rescue the defect in BMP2-induced osteoblastic differentiation. As shown in Fig 4E, we found that enhanced expression of *Osx* can partly restore ALP activity in the BMP-treated *Ire1 α* ^{–/–} MEFs, further supporting a functional link between the IRE1 α –XBP1 pathway and *Osx*.

In summary, this study indicates that UPR has two functions during osteoblast differentiation: (i) to expand the capacity of the ER to cope with the increase in the production of extracellular matrix proteins, and to avoid the cells producing matrix proteins from apoptosis induced by ER overload (Hino *et al*, 2010); (ii) to stimulate *Osx* expression through the IRE1 α –XBP1 pathway to promote the maturation of preosteoblasts into osteoblasts (Fig 4F). In comparison to *Runx2*, the functions and the molecular

regulation of *Osx* are poorly understood, although both genes are equally indispensable for skeletogenesis (Komori, 2006; Karsenty, 2008). Therefore, the unexpected link between the IRE1 α –XBP1 pathway and *Osx* identified here might shed new light on the understanding of the UPR and osteoblast differentiation.

METHODS

Cells and reagents. The generation of *Ire1 α* ^{–/–} and wild-type MEFs was described previously (Urano *et al*, 2000). Primary osteoblasts were collected from the calvaria from 18.5 dpc wild-type embryos. Recombinant human BMP2 was purchased from Wako (Osaka, Japan). IRE1 α antibody (14C10), XBP1 antibody (M-186), RUNX2 antibody (M-70) and actin antisera were from Cell Signaling, Santa Cruz (M-186 and M-70) and Sigma-Aldrich, respectively. *Ire1 α* , *Xbp1*, *Traf2* and non-targeting control siRNA were purchased from Sigma Genosys.

Luciferase assay. Luciferase assay was performed using a dual luciferase assay system (Promega). pRL-SV40 (Promega) was used as a transfection efficiency control. The assays were performed 16 h after the transfection of reporter constructs and expression vectors. Transcriptional activity was expressed as a ratio of firefly:Renilla luciferase activity.

Chromatin immunoprecipitation assay. MC3T3-E1 cells, MEFs and primary osteoblasts were cultured in BMP2-supplemented medium for 72 h. The cells were fixed by 1% paraformaldehyde/phosphate-buffered saline for 10 min at 23 °C. Chromatin shearing and immunoprecipitation were performed using ChIP-IT Express Chromatin Immunoprecipitation Kits (Active Motif), as instructed by the manufacturer. The isolated DNA fragments were used as templates for PCR amplification (see Fig 3D for a schema showing the target region for PCR and supplementary Table S1 online for the PCR primers used in this study).

Statistical analysis. A student's *t*-test for two samples assuming equal variances was used to calculate *P*-values. *P*-values <0.05 were considered statistically significant. All experiments, including the reporter assay, quantitative RT–PCR and chromatin immunoprecipitation assay, were repeated at least three times. Error bars indicate standard deviation.

Detailed description of the constructs and the protocols for immunostaining, evaluation for ALP activity, *ex vivo* culture of calvariae and quantitative RT–PCR are provided in the supplementary information online.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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