

# Involvement of the Osteoinductive Factors, Tmem119 and BMP-2, and the ER Stress Response PERK–eIF2 $\alpha$ –ATF4 Pathway in the Commitment of Myoblastic into Osteoblastic Cells

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**Abstract** The osteoinductive factors BMP-2 and Tmem119 that promote the differentiation of myoblasts into osteoblasts, each increase the levels of the other. However, the relative contributions of BMP-2 and Tmem119 to the osteogenic differentiation and the mechanisms involved are incompletely understood. In the present study, we examined the relationship among BMP-2, Tmem119, and the PERK–eIF2 $\alpha$ –ATF4 endoplasmic reticulum (ER) stress response pathway in the differentiation of C2C12 myoblasts into osteoblastic cells. Both BMP-2 and Tmem119 induced levels of the osteoblast markers Runx2, Osterix, Col1a1, ALP, and osteocalcin, as well as mineralization. BMP-2 activation of the ER stress sensor PERK stimulated phosphorylation of eIF2 $\alpha$  and led to increased biosynthesis of the osteoblast differentiation factor ATF4. When dephosphorylation of eIF2 $\alpha$  was blocked by the selective inhibitor salubrinal, the osteogenic effects of BMP-2 and Tmem119 were enhanced

further. Although BMP-2 stimulated both P-eIF2 $\alpha$  and ATF4 levels, Tmem119 had no effect on P-eIF2 $\alpha$  but stimulated ATF4 only. Reduction in endogenous Tmem119 levels by siRNA reduced both basal and BMP-2-stimulated levels of the ATF4 protein. In conclusion, BMP-2 stimulates differentiation of myoblasts into osteoblasts via the PERK–eIF2 $\alpha$ –ATF4 pathway but in addition stimulates Tmem119, which itself increases ATF4. Hence, BMP-2 stimulates ATF4 both dependently and independently of the PERK–eIF2 $\alpha$  ER stress response pathway.

**Keywords** Bone morphogenetic protein · Endoplasmic reticulum stress · Myoblast · Osteoblast · PERK–eIF2 $\alpha$ –ATF4 · Tmem119

Bone morphogenetic protein (BMP) 2 was originally identified as a molecule that induces bone regeneration and ectopic bone formation in vivo, and it enhances the differentiation of myoblasts into osteoblasts in vitro [1–3]. BMPs are members of the transforming growth factor (TGF)  $\beta$  superfamily of ligands that act through a complex of type I and type II transmembrane serine/threonine kinase receptors. After BMPs bind to the type II receptor, it phosphorylates the type I receptor, which phosphorylates regulated signaling molecules, Smads1/5/8 [4, 5]. Phosphorylated Smads1/5/8 form a complex with the common Smad, Smad4, and the complex translocates into the nucleus and regulates transcription of target genes such as those encoding Runx2, Osterix, and osteocalcin that are critical for bone formation [6, 7]. Use of C2C12 mouse myoblastic cells has provided many important insights into the differentiation of myoblasts on the one hand and osteoblasts on the other [8]. These cells express receptors responsive to members of the TGF- $\beta$  superfamily of ligands.

The authors report that they have no conflict of interest.

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Recently, transmembrane protein 119 (Tmem119) has been identified as an osteoinductive factor [9, 10]. Tmem119 came to attention as a novel skeletogenesis target gene in an analysis of the Runx2<sup>-/-</sup> mouse model [11], and Tmem119 induces the differentiation and mineralization of mouse osteoblastic MC3T3-E1 cells [9–11]. The rare autosomal-dominant disorder of skeletal malformations fibrodysplasia ossificans progressiva (FOP) is characterized by postnatal progressive heterotopic ossification in soft tissues [12–14]. We previously showed that Tmem119 is increased in C2C12 myoblasts overexpressing the constitutively activating BMP type 1 receptor (activin receptor-like kinase, ALK2) that carries the mutation (617G > A; R206H) responsible for the classic form of FOP [15]. We showed that Tmem119 promotes the differentiation of myoblasts into osteoblasts by inducing BMP-2 levels and interacting with the BMP signaling pathway [10, 15]. Tmem119 is a single-pass type 1a membrane protein and has a signal peptide of 18 amino acids, which directs the nascent polypeptide to the endoplasmic reticulum (ER), and amino acids 90–112 comprise the transmembrane helix. Previously, we observed perinuclear staining of Tmem119 consistent with its location in the ER membrane, with the carboxyl-terminal part being cytoplasmic [10]. However, the functions performed by Tmem119 as an ER protein are not known.

The ER is crucial for biosynthesis, folding, and modification of proteins [16]. The ER or unfolded stress response (unfolded protein response, UPR) is activated by accumulation of unfolded or misfolded proteins in the ER. The UPR aims to restore normal function by halting global mRNA translation while increasing production of factors that aid in protein folding. One of the key ER stress response systems is the PERK–eIF2 $\alpha$ –ATF4 pathway [17–19].

During osteoblast differentiation, high concentrations of secreted proteins such as osteocalcin are made, and the quality management of synthesized proteins in the ER is crucial at this time. The ER stress response mediated by PERK–eIF2 $\alpha$ –ATF4 is involved in BMP-2-induced osteoblast differentiation [20]. During the ER stress response, protein kinase RNA-like endoplasmic reticulum kinase (PERK) phosphorylates eukaryotic translation initiation factor 2 subunit alpha (eIF2 $\alpha$ ). P-eIF2 $\alpha$  mediates both a transient decrease in global mRNA translation and the up-regulation of translation of selected mRNAs one of which encodes the activating transcription factor (ATF) 4 that promotes osteoblast differentiation and bone formation [21]. Perk<sup>-/-</sup> mice have severe osteopenia similar to Atf4<sup>-/-</sup> mice [20, 21], and eIF2 $\alpha$  is required for the development of the skeletal system [22]. In wild-type primary calvarial osteoblasts, BMP-2 promotes ER stress that increases ATF4 protein. In contrast, in Perk<sup>-/-</sup> primary

calvarial osteoblasts levels of P-eIF2 $\alpha$  and ATF4 protein are reduced, as are target proteins such as osteocalcin. Perk<sup>-/-</sup> osteoblasts have decreased ALP activity and delayed mineralized nodule formation [20]. The abnormalities are corrected after introduction of ATF4 into the Perk<sup>-/-</sup> osteoblasts. Therefore ER stress occurs naturally in osteoblast differentiation and activates the PERK–eIF2 $\alpha$ –ATF4 pathway stimulating expression of genes essential for osteogenesis.

During the course of the ER stress response, P-eIF2 $\alpha$  is dephosphorylated by a complex containing the serine/threonine phosphatase PP1. The PP1 complex is inhibited by the small-molecule drug salubrinal, which selectively blocks dephosphorylation of P-eIF2 $\alpha$  [23]. Salubrinal has proved to be useful in studying the relationship between ER stress and the PERK–eIF2 $\alpha$ –ATF4 pathway in a variety of cellular processes [24, 25].

That BMP-2 affects osteogenesis via the ER stress response is known, and we have shown that there is an interaction between the ER osteoinductive factors Tmem119 and BMP-2. However, how BMP-2 and Tmem119 might together influence the ER stress response and osteogenesis is unclear. Therefore, in the present study, we have investigated the relationship between the osteoinductive factors, Tmem119 and BMP-2, and the ER stress response PERK–eIF2 $\alpha$ –ATF4 pathway in the differentiation of myoblastic into osteoblastic cells.

## Materials and Methods

### Materials

Human (h) recombinant BMP-2 was kindly provided by Astellas Pharmaceutical Co. Ltd. (Tokyo, Japan).  $\beta$ -Actin antibody was from Sigma-Aldrich Corp. (St. Louis, MO, USA). Antibodies against ALP, phospho-PERK, PERK, eIF2 $\alpha$ , and ATF4, Tmem119 (m) PERK, eIF2 $\alpha$ , and ATF4 siRNA s and control siRNA, as well as salubrinal, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-eIF2 $\alpha$  antibody was from Cell Signaling Technology. Col1a1 antibody was from Calbiochem and IngeneX Corp. (San Diego, CA, USA). All other chemicals used were of analytical grade.

### Cell Culture

Mouse myoblastic C2C12 cells (ATCC) were cultured in Dulbecco modified Eagle medium (Invitrogen) with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin. Mouse osteoblastic MC3T3-E1 cells were provided by Dr. H. Kodama (Ohu Dental College, Koriyama, Japan), and stromal ST2 cells were purchased from the Riken BioResource Center. The cells

were cultured in  $\alpha$ -MEM (containing 5.5 mmol/L glucose) with 10 % FBS and 1 % penicillin–streptomycin. The medium was changed twice a week.

#### Transient and Stable Transfection

Each vector was transfected into C2C12 cells with lipofectamine (Invitrogen), as previously described [15]. 6 h later, the cells were supplied with fresh DMEM containing 10 % FBS. 48 h later, the transiently transfected cells were used for experiments. To generate stably transfected C2C12 cells, after incubation in DMEM containing 10 % FBS for 48 h, the cells were passaged, and clones were selected in DMEM supplemented with G418 (0.3 mg/mL; Invitrogen) and 10 % FBS. Twenty-four clones were selected after 3 weeks of culture in G418. Several clones were selected after Western blot testing with Tmem119 antibody and semiquantitative RT-PCR. At least three independent clones for each stable transfection were characterized to rule out the possibility of clonal variation. Empty vector-transfected cell clones were used as control.

#### Protein Extraction and Western Blot Analysis

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1 % Triton X-100, and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20 % methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.1 % Tween-20, and 3 % dried milk powder. The membranes were immunoblotted with each primary antibody. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma-Aldrich Corp.) and an enhanced chemiluminescence detection system, LAS-4000 IR multi color (Fuji-film). The results depicted in each figure are representative of at least three independent cell preparations. Each experiment was repeated three times.

#### RNA Extraction and Real-Time PCR

Total RNA was prepared from cells using Trizol reagent (Invitrogen). cDNA was synthesized using a SuperScript-III cDNA synthesis kit (Invitrogen). Specific mRNA was quantified by real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems Inc.) with SYBR Premix Ex Taq™ II (Perfect Real Time) kits (TaKaRa) according to the manufacturer's standard protocol. The mRNA value for each gene was normalized to the mouse GAPDH mRNA levels in RNA samples. We performed SYBR Green PCR after recombinant DNase I

treatment to remove genomic DNA, and we additionally confirmed that there was no contamination by genomic DNA that could affect the results (data not shown). Primer sequences (forward and reverse) were as follows: GAPDH, 5'-GTGTACATGGTTCCAGTATGAGTCC-3' and 5'-AGTGAGTTGTCATATTTCTCGTGGT-3'; Tmem119, 5'-TGTTCTCCTCTGTCTCTGCT-3' and 5'-ATGATCCCTTCAGGAGGTT-3'; osteocalcin (OCN), 5'-CCTGAGTCTGACAAAGCCTTCA-3' and 5'-GCCGGAGTCTGTTCACTACCTT-3'; Runx2, 5'-AAATGCCTCCGCTGTTATGAA-3' and 5'-GCTCCGGCCCAAAATCT-3'; Osterix, 5'-AGCGACCACTTGAGCAAACAT-3' and 5'-GCGGCTGATTGGCTTCTTCT-3'.

#### Mineralization

Mineralization of C2C12 cells was determined in six-well plates using Alizarin Red staining. After confluent cells were grown in DMEM supplemented with 10 % FBS, 1 % penicillin–streptomycin, 5 mM  $\beta$ -glycerophosphate, 100  $\mu$ g/mL ascorbic acid, and BMP-2 (200 ng/mL) for 3 weeks, cells were fixed with ice-cold 70 % ethanol and stained with Alizarin Red (Sigma) to detect calcification. For quantitation, cells stained with Alizarin Red were destained with ethylpyridinium chloride (Wako Pure Chemical Industries Ltd.), the extracted stain transferred to a 96-well plate, and absorbance at 562 nm was measured using a microplate reader.

#### Small Interfering (si) RNA

Specific siRNAs and control siRNA were transfected as recommended by the supplier (Santa Cruz Biotechnology) into cells seeded at  $5 \times 10^5$  per well with Lipofectamine RNAi MAX (Invitrogen).

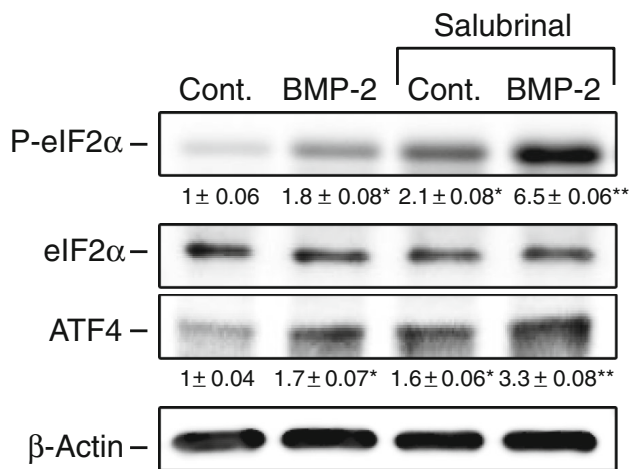
#### Statistical Analysis

All experiments were repeated at least three times. Data are expressed as mean  $\pm$  SEM. Statistical evaluation for difference between groups was carried out with one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test. A *p* value of  $<0.05$  was taken to indicate a significant difference.

## Results

#### Effects of BMP-2 on PERK–eIF2 $\alpha$ Pathway

BMP-2 stimulated the levels of the phosphorylated active form of eIF2 $\alpha$  (P-eIF2 $\alpha$ ), the translation initiation factor, which functions downstream of PERK, and increased the



**Fig. 1** BMP-2 up-regulates P-eIF2 $\alpha$  and ATF4 in myoblasts and the effect is enhanced by salubrin, a selective inhibitor of dephosphorylation of P-eIF2 $\alpha$ . Cells were pretreated or not with 25  $\mu$ M salubrin for 1 h and then with or without 200 ng/mL BMP-2 for 48 h. Cell extracts were made, Western blot analysis with P-eIF2 $\alpha$ , eIF2 $\alpha$ , ATF-4, and  $\beta$ -actin antibodies performed, and densitometric analysis made. \* $p < 0.01$ , relative to control; \*\* $p < 0.01$ , relative to salubrin-treated control

levels of the transcription factor, ATF4, in mouse myoblastic C2C12 cells. Addition of salubrin, an inhibitor of the phosphatases that dephosphorylate P-eIF2 $\alpha$ , enhanced both the basal and the BMP-2-stimulated levels of P-eIF2 $\alpha$  and ATF4 (Fig. 1). BMP-2 induced 1.8- and 1.7-fold increases in P-eIF2 $\alpha$  and ATF4 protein levels, respectively, and in the presence of salubrin, it induced 3.1- and 2.1-fold increases in P-eIF2 $\alpha$  and ATF4 protein levels, respectively (Fig. 1).

#### Effects of Salubrin on BMP-2-Induced Osteoblast Differentiation and Mineralization

We examined the effect of salubrin on the BMP-2-induced osteoblast differentiation in C2C12 cells. BMP-2 significantly increased the levels of Runx2, Osterix, and OCN mRNA, and this effect was significantly enhanced by salubrin (Fig. 2a). Moreover, BMP-2 increased the levels of the type 1 collagen (Col1a1) and ALP proteins, and salubrin enhanced the BMP-2-induced levels of these proteins further (Fig. 2b). BMP-2 induced 1.7- and 1.9-fold increases in Col1a1 and ALP protein levels, respectively, and in the presence of salubrin, it induced 3.6- and 3.4-fold increases in Col1a1 and ALP protein levels, respectively (Fig. 2b). Next, we examined the effects of salubrin on BMP-2-induced mineralization in C2C12 cells cultured in an osteogenic ascorbic acid and  $\beta$ -glycerophosphate medium. Although BMP-2 induced mineralization of myoblasts relative to control cells as indicated by Alizarin Red staining, the effect was more marked in the presence of salubrin (Fig. 2c). A quantitative analysis showed that salubrin

enhanced the BMP-2 effect 2-fold (Fig. 2d). Salubrin alone did not affect mineralization assessed by Alizarin Red staining (Fig. 2e, f) and cell number (data not shown) of C2C12 cells. However, salubrin alone induced the differentiation of mouse osteoblastic MC3T3-E1 cells and mouse stromal ST2 cells (Fig. 3a, b). Salubrin (25  $\mu$ M) increased the levels of Runx2, Osterix, and OCN mRNA as well as Col1a1 and ALP proteins in MC3T3-E1 cells (Fig. 3a). Moreover, BMP-2 and 25  $\mu$ M salubrin increased the levels of Runx2, Osterix, and OCN mRNA as well as Col1a1 and ALP proteins in ST2 cells (Fig. 3b).

#### Effects of Salubrin on Tmem119-Induced Osteoblast Differentiation and Mineralization

We examined whether salubrin would affect Tmem119-induced osteoblast differentiation using C2C12 cells overexpressing Tmem119. Transient Tmem119 overexpression significantly increased the levels of Runx2, Osterix, and OCN mRNA, and salubrin significantly further enhanced the levels of Runx2, Osterix, and OCN mRNA increased by Tmem119 (Fig. 4a). The transient Tmem119 overexpression increased the levels of Col1a1 and ALP proteins, and salubrin enhanced the levels of Col1a1 and ALP proteins increased by Tmem119 (Fig. 4b). The stable Tmem119 overexpression induced mineralization of C2C12 cells, relative to that in empty vector-transfected cells, and salubrin enhanced Tmem119-induced mineralization of myoblasts assessed by Alizarin Red staining in the presence of ascorbic acid and  $\beta$ -glycerophosphate (Fig. 4c). A quantitative analysis confirmed a greater than 2-fold effect of salubrin on the BMP-2 effect (Fig. 4d).

#### Effects of Tmem119 on PERK-eIF2 $\alpha$ -ATF4 Signaling in Myoblasts

We examined the effects of Tmem119 overexpression and salubrin on PERK-eIF2 $\alpha$ -ATF4 signaling in C2C12 cells. Transient Tmem119 overexpression enhanced the levels of ATF4 protein, although it did not affect the levels of P-PERK and P-eIF2 $\alpha$  (Fig. 5a). Tmem119 alone induced a 2.4-fold increase, and in the presence of salubrin, a 2.3-fold increase, in ATF4 protein levels. Hence, Tmem119 promoted similar fold increases in ATF4 levels irrespective of the phosphorylation status of eIF2 $\alpha$  influenced by salubrin.

#### Effects of a Reduction of Tmem119 by siRNA on BMP-2-induced PERK-eIF2 $\alpha$ -ATF4 Signaling in Myoblasts

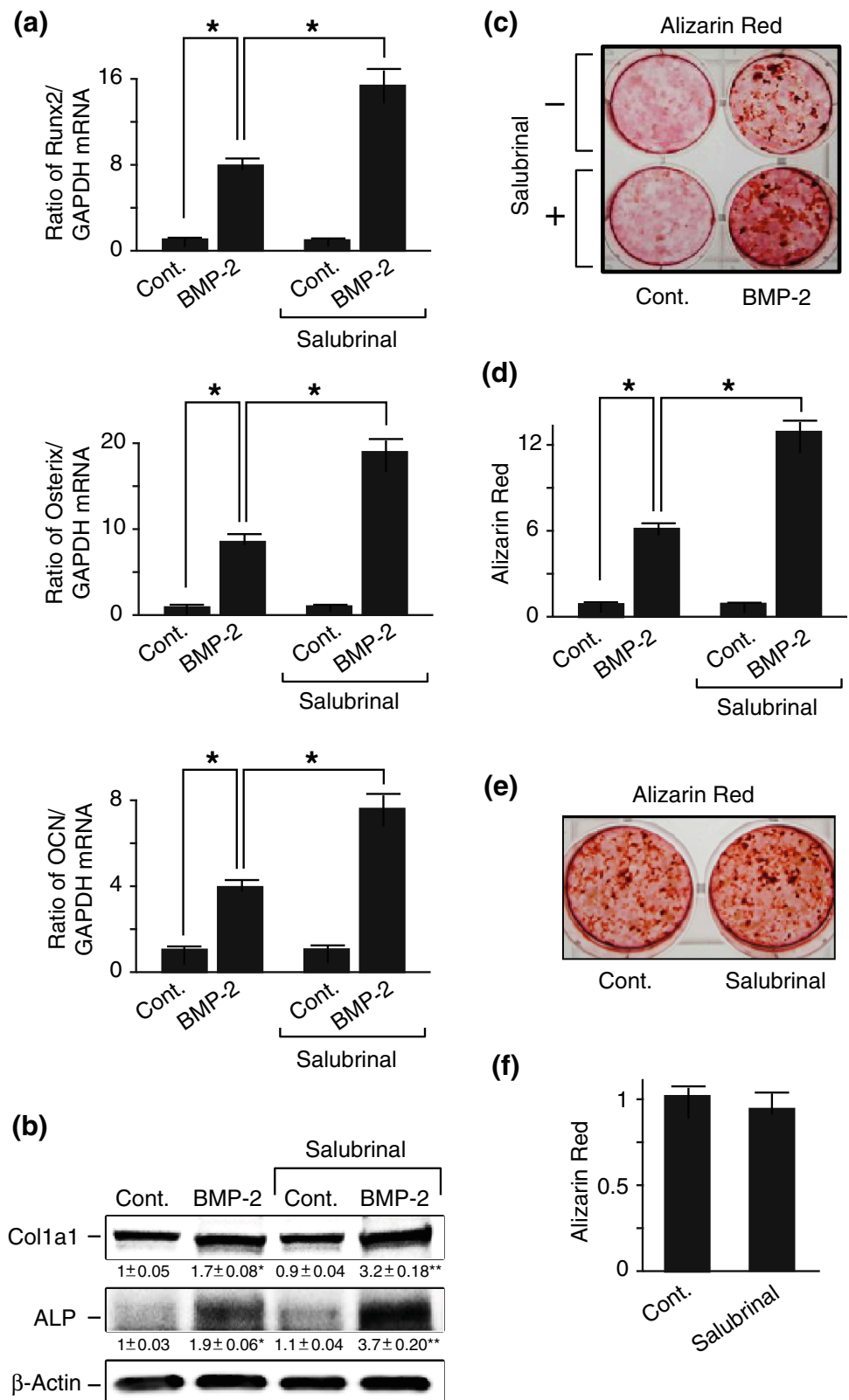
We examined the effects of a reduction in endogenous Tmem119 levels by siRNA on baseline and BMP-2-induced

**Fig. 2** Salubrinal enhances the BMP-2-induction of osteoblast phenotype and mineralization of myoblasts. **a** Total RNA was extracted from parental C2C12 cells pretreated or not with 25  $\mu$ M salubrinal for 1 h followed by treatment with or without 200 ng/mL BMP-2 for 48 h, and real-time PCR for Runx2, Osterix, OCN, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value.

\* $p < 0.01$ . **b** Total protein was extracted from parental C2C12 cells pretreated with or without 25  $\mu$ M salubrinal for 1 h followed by treatment with or without 200 ng/mL BMP-2 for 48 h. Cell extracts were made, Western blot analysis with Col1a1, ALP, and  $\beta$ -actin antibodies performed, and densitometric analysis made.

\* $p < 0.01$ , relative to control; \*\* $p < 0.01$ , relative to salubrinal-treated control. **c** Parental C2C12 cells treated with or without 200 ng/mL BMP-2 were cultured for 21 days in 5 mM  $\beta$ -glycerophosphate and 100  $\mu$ g/mL ascorbic acid in the presence (+) or absence (-) of 25  $\mu$ M salubrinal, and Alizarin Red staining was performed.

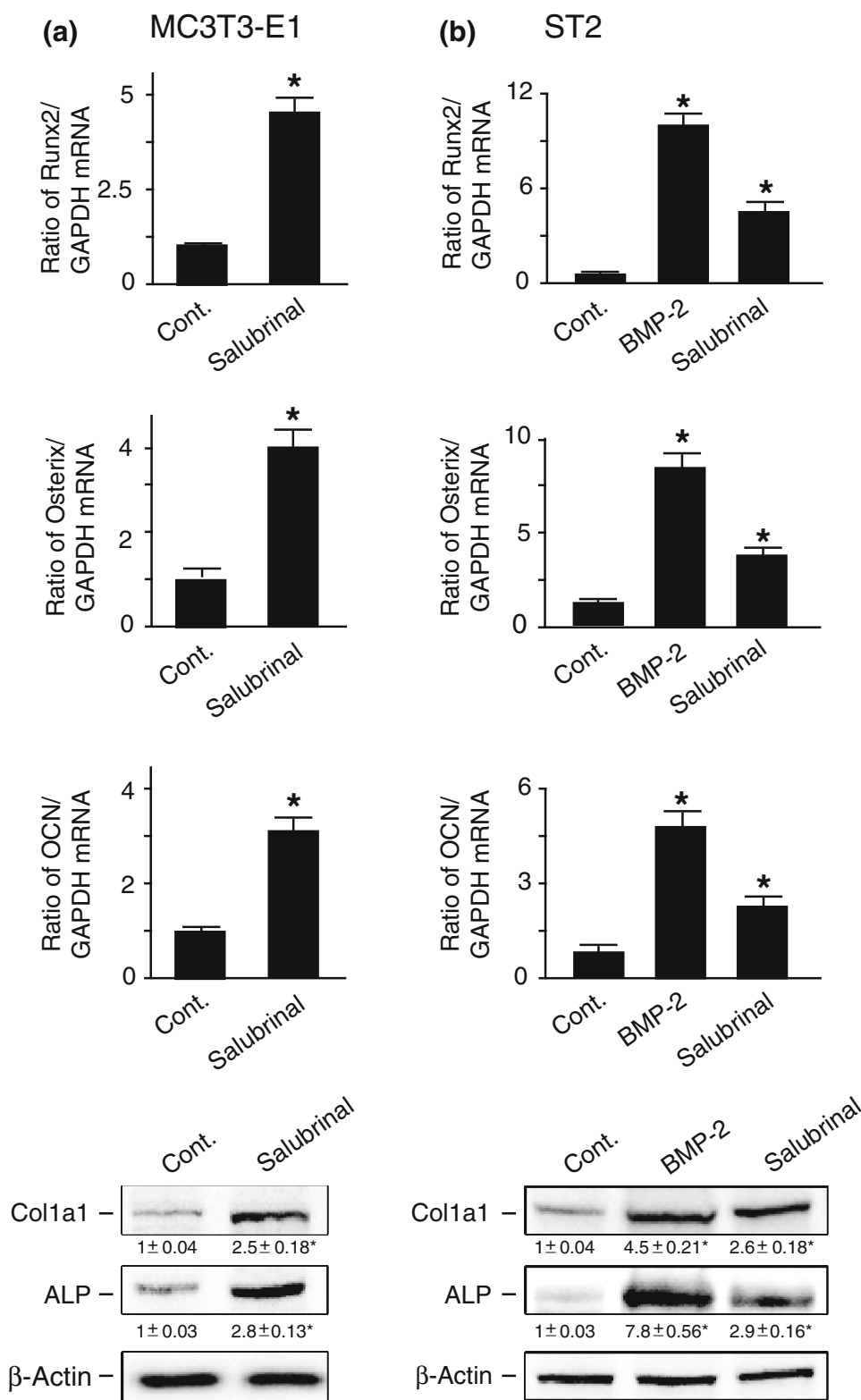
**d** Alizarin Red mineralization was quantitated as described in the “Materials and Methods” section. \* $p < 0.01$ . **e** C2C12 cells were cultured for 21 days in 5 mM  $\beta$ -glycerophosphate and 100  $\mu$ g/mL ascorbic acid in the presence (+) or absence (-) of 25  $\mu$ M salubrinal, and Alizarin Red staining was performed. **f** Alizarin Red mineralization was quantitated as described in the “Materials and Methods” section



PERK-eIF2 $\alpha$ -ATF4 signaling in C2C12 cells. In the absence of BMP-2, Tmem119 siRNA reduced the baseline ATF4 levels while those for P-eIF2 $\alpha$  and eIF2 $\alpha$  were no

different from control siRNA conditions (Fig. 5b). While BMP-2 enhanced the levels of ATF4 and P-eIF2 $\alpha$  proteins, a reduction in Tmem119 by siRNA led to decreased levels

**Fig. 3** Salubrinal alone induces differentiation of mouse osteoblastic MC3T3-E1 and stromal ST-2 cells. **a** Total RNA was extracted from MC3T3-E1 cells treated or not with 25  $\mu$ M salubrinal for 48 h, and real-time PCR for Runx2, Osterix, OCN, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value.  $*p < 0.01$ . Total protein was extracted from MC3T3-E1 cells treated or not with 25  $\mu$ M salubrinal for 48 h, Western blot analysis with Col1a1, ALP, and  $\beta$ -actin antibodies performed, and densitometric analysis made.  $*p < 0.01$ , relative to control. **b** Total RNA was extracted from ST2 cells treated or not with 200 ng/mL BMP-2 or 25  $\mu$ M salubrinal for 7 days, and real-time PCR for Runx2, Osterix, OCN, or GAPDH performed. Data are expressed relative to the GAPDH mRNA value.  $*p < 0.01$ . Total protein was extracted from ST2 cells treated or not with 200 ng/mL BMP-2 or 25  $\mu$ M salubrinal for 7 days, Western blot analysis with Col1a1, ALP, and  $\beta$ -actin antibodies performed, and densitometric analysis made.  $*p < 0.01$ , relative to control



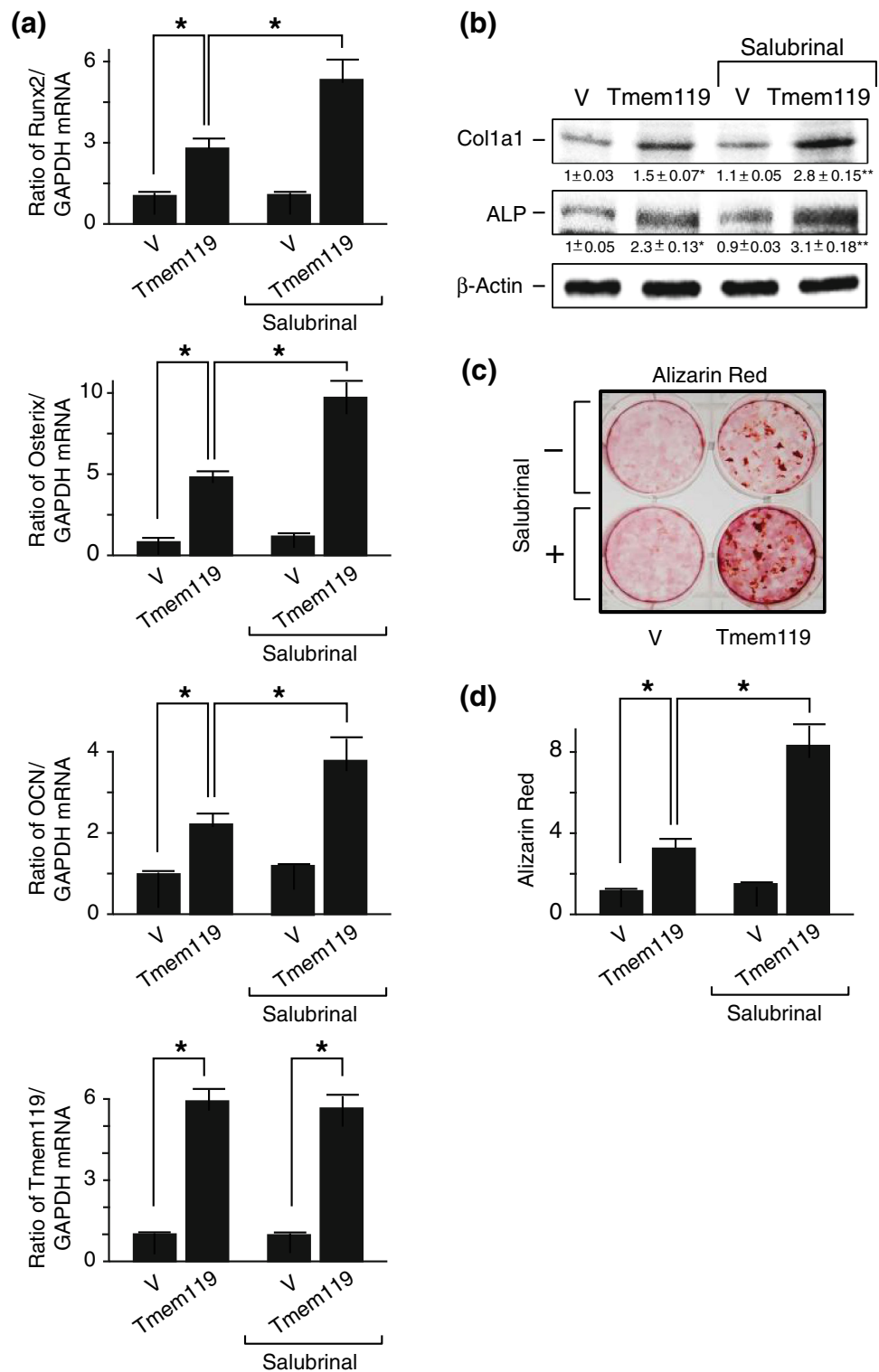
of ATF4 protein, to 40 %, and in the presence of BMP-2, to 34 %, of those of control siRNA transfected cells. By real-time PCR. The degree of efficiency of the knock-down of

Tmem119 was 80 % (Fig. 5c), leading to a significant reduction in the level of the BMP-2-induced Tmem119 mRNA in C2C12 cells.

**Fig. 4** Salubrinal enhances the Tmem119-induction of osteoblast phenotype and mineralization of myoblasts.

**a** Parental C2C12 cells transiently transfected with either empty vector (V) or Tmem119 expression vector (Tmem119) were treated with or without salubrinal for 48 h. Total RNA was extracted and real-time PCR for Runx2, Osterix, OCN, Tmem119, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value.

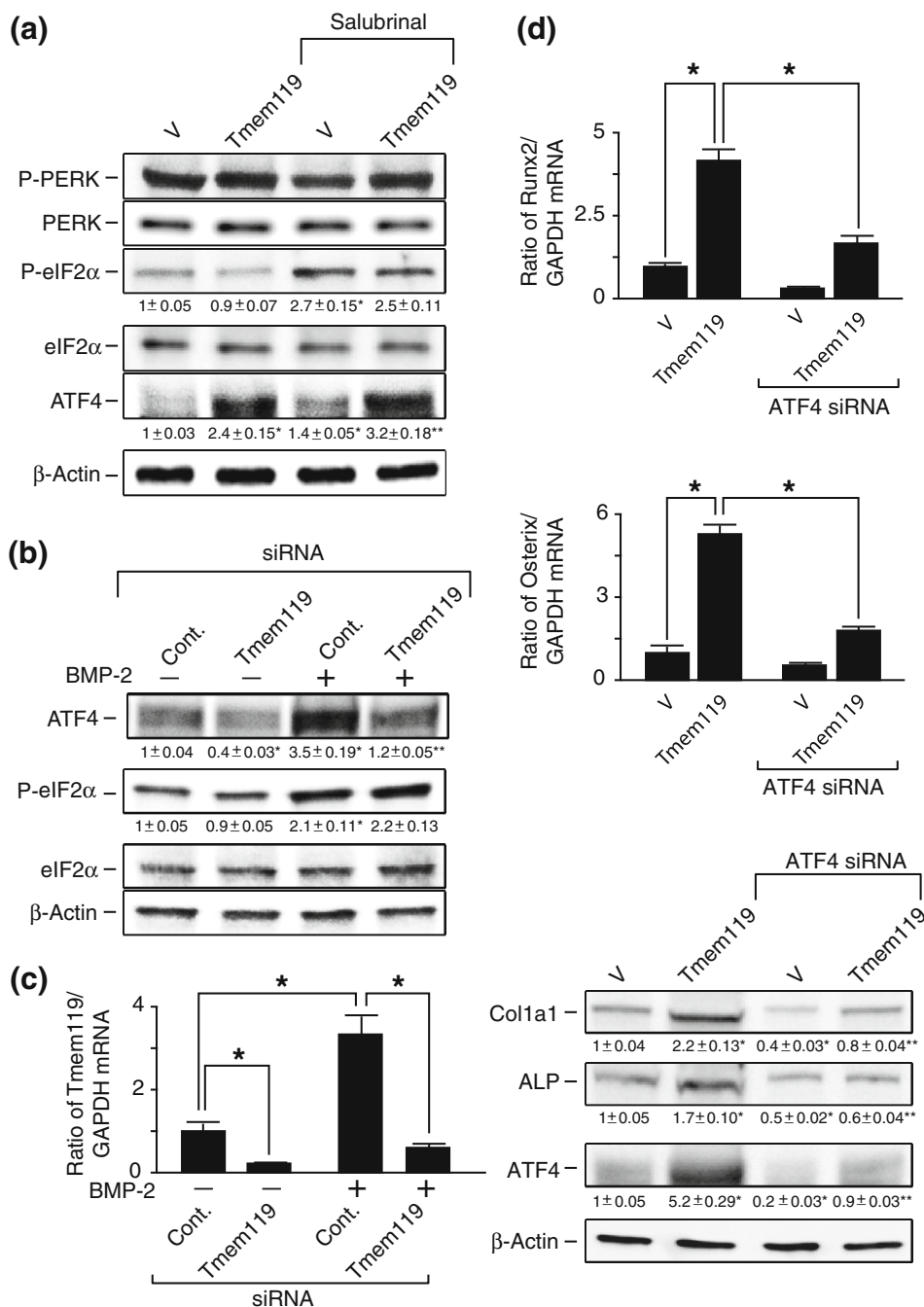
\* $p < 0.01$ . **b** Parental C2C12 cells transiently transfected with empty vector (V) or Tmem119 were treated with or without salubrinal for 48 h. Total protein was extracted, Western blot analysis with Col1a1, ALP, and  $\beta$ -actin antibodies performed, and densitometric analysis made. \* $p < 0.01$ , relative to control; \*\* $p < 0.01$ , relative to salubrinal-treated control. **c** C2C12 cells stably transfected with empty vector (V) or Tmem119 were cultured for 21 days in the presence of 5 mM  $\beta$ -glycerophosphate and 100  $\mu$ g/mL ascorbic acid. The cells were cultured in the presence (+) or absence (-) of 25  $\mu$ M salubrinal, and Alizarin Red staining was performed. Mineralization by the Alizarin Red method was quantitated as described in the “Materials and Methods” section. \* $p < 0.01$



We next examined the effects of a reduction in ATF4 levels by siRNA on Tmem119-induced osteoblast differentiation of myoblasts. A reduction in ATF4 level by siRNA, significantly inhibited the levels of Runx2 and Osterix mRNA in C2C12 cells (Fig. 5d). Moreover, a reduction in ATF4 level by siRNA suppressed the levels of Col1a1 and ALP proteins.

Effects of a Reduction in PERK, eIF2 $\alpha$ , or ATF4 on the BMP-2- or Tmem119-Induced Osteoblast Differentiation of Myoblasts

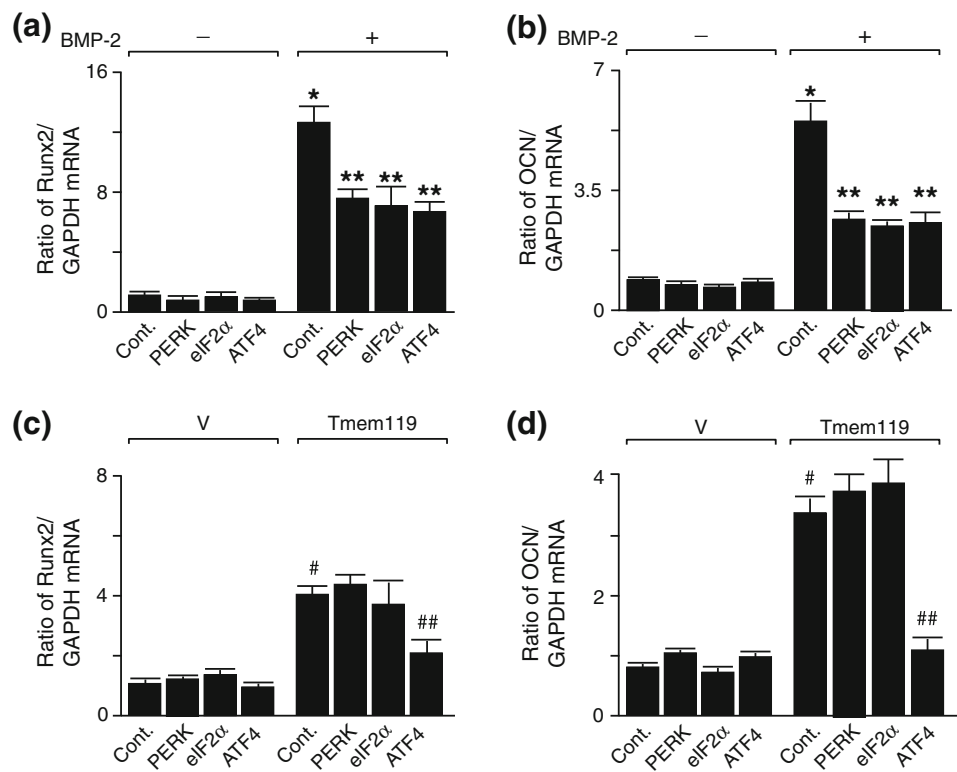
We examined the effects of reductions in PERK, eIF2 $\alpha$ , or ATF4 on the BMP-2- or Tmem119-induced osteoblast



**Fig. 5** Effects of Tmem119 on PERK–eIF2α–ATF4 signaling in myoblasts. **a** Parental C2C12 cells transiently transfected with empty vector (V) or Tmem119 expression vector (Tmem119) were treated without or with salubrinal for 48 h. Total protein was extracted, Western blot analysis with P-PERK, PERK, P-eIF2α, eIF2α, ATF-4, and β-actin antibodies performed, and densitometric analysis made. \**p* < 0.01, relative to V treated without salubrinal; \*\**p* < 0.01, relative to Tmem119 expression vector treated without salubrinal. **b** Control siRNA- or Tmem119 siRNA-transfected C2C12 cells were treated or not with 200 ng/mL BMP-2 for 48 h. Total protein was extracted, Western blot analysis with ATF4, P-eIF2α, eIF2α, and β-actin antibodies performed, and densitometric analysis made. \**p* < 0.01, relative to control siRNA-transfected C2C12 cells not treated with BMP-2; \*\**p* < 0.01, relative to control siRNA-transfected

C2C12 cells treated with BMP-2. **c** Control siRNA- or Tmem119 siRNA-transfected C2C12 cells were treated or not with 200 ng/mL BMP-2 for 48 h. Total RNA was extracted and real-time PCR for Tmem119 or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. \**p* < 0.01. **d** C2C12 cells stably transfected with empty vector (V) or Tmem119 expression vector (Tmem119) were transfected with control siRNA or ATF4 siRNA. Total RNA was extracted and real-time PCR for Runx2, Osterix, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. \**p* < 0.01. Total protein was extracted and Western blot analysis with Col1a1, ALP, ATF4, and β-actin antibodies performed, and densitometric analysis made. \**p* < 0.01, relative to V without ATF4 siRNA; \*\**p* < 0.01, relative to Tmem119 expression vector without ATF4





**Fig. 6** Effects of reduction in PERK, eIF2 $\alpha$ , or ATF4 on BMP-2 or Tmem119-induced osteoblast differentiation of myoblasts. **a, b** Control siRNA-, PERK siRNA-, eIF2 $\alpha$  siRNA-, or ATF4 siRNA-transfected parental C2C12 cells were treated (+) or not (-) with 200 ng/mL BMP-2 for 48 h. **c, d** Stably expressing empty vector (V) or Tmem119 expression vector (Tmem119) C2C12 cells were transfected with control siRNA-, PERK siRNA-, eIF2 $\alpha$  siRNA-, or

ATF4 siRNA. Total RNA was extracted and real-time PCR for Runx2, OCN, or GAPDH was performed. \* $p < 0.01$ , relative to control siRNA-transfected C2C12 cells. \*\* $p < 0.01$ , relative to control siRNA-transfected C2C12 cells with BMP-2. # $p < 0.01$ , relative to control siRNA-transfected empty vector (V) cells. ## $p < 0.01$ , relative to control siRNA-transfected Tmem119 expression vector (Tmem119) cells

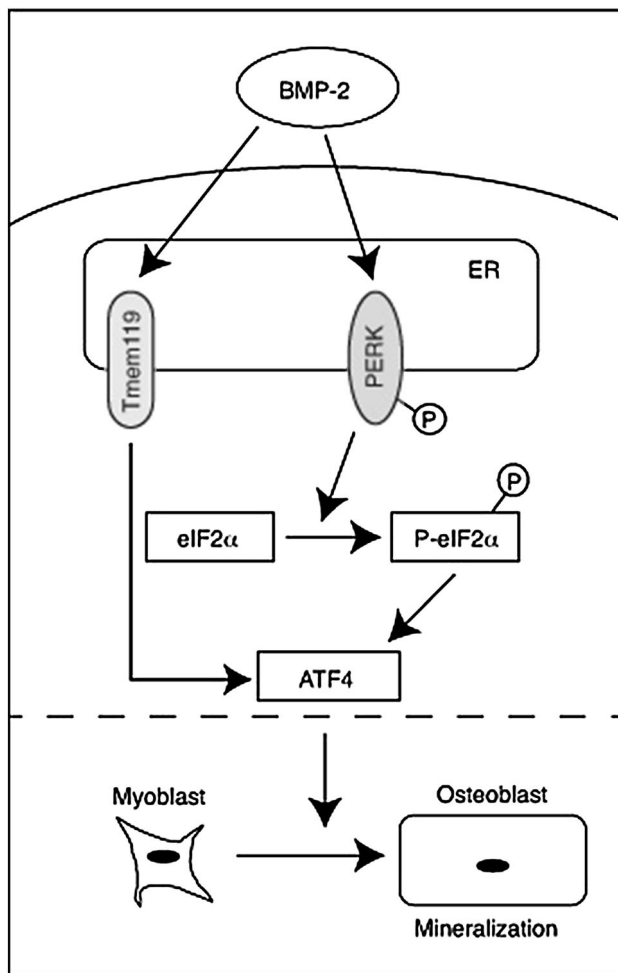
differentiation of myoblastic C2C12 cells. Reductions in PERK, eIF2 $\alpha$ , or ATF4 by siRNA significantly reduced the BMP-2-induced levels of Runx2 and OCN mRNA to 54 and 36 %, respectively, of those of the control siRNA treated parental C2C12 cells (Fig. 6a, b). In contrast, reductions in PERK or eIF2 $\alpha$  by siRNA had no effect on the increased levels of Runx2 and OCN mRNA in the C2C12 cells stably overexpressing Tmem119 relative to control siRNA treated cells (Fig. 6c, d). Only reduction in ATF4 by siRNA significantly reduced the increased levels of Runx2 and OCN mRNA to 36 and 12 %, respectively, relative to control siRNA, in the cells stably overexpressing Tmem119 (Fig. 6c, d).

## Discussion

In the present study, we have focused on the PERK–eIF2 $\alpha$ –ATF4 ER stress response pathway as potentially mediating osteoblast differentiation of myoblasts. We showed that in myoblastic cells, BMP-2 stimulates the phosphorylation of eIF2 $\alpha$ , which acts downstream of PERK, and increases the

osteoblast differentiating factor ATF4. Increasing the levels of basal and BMP-2-induced P-eIF2 $\alpha$  with the selective phosphatase inhibitor salubrinal, further increased the levels of ATF4. Salubrinal enhanced the BMP-2-induced osteoblast differentiation of myoblastic cells, as evidenced by increased expression of Runx2, Osterix, OCN, Col1a1, and ALP. In addition, the BMP-2-induced mineralization was increased further by the salubrinal treatment. Thus, salubrinal further enhances osteoblastic differentiation of myoblastic cells once they are directed to become osteoblasts.

Previously, we documented that BMP-2 stimulated the levels of the osteoinductive factor Tmem119, and that Tmem119 itself induced the differentiation of myoblasts into osteoblasts [15]. We hypothesized, therefore, that Tmem119 might carry out these actions through the PERK–eIF2 $\alpha$  pathway. Tmem119 induction of the differentiation of myoblasts into osteoblasts and their mineralization was enhanced by salubrinal. However, although salubrinal did enhance further the Tmem119-stimulated levels of ATF4, Tmem119 itself did not affect the levels of P-PERK and P-eIF2 $\alpha$ . These findings indicate that



**Fig. 7** Involvement of Tmem119 and BMP-2, and the ER stress response PERK-eIF2 $\alpha$ -ATF4 pathway in the commitment of myoblastic into osteoblastic cells. BMP-2 stimulates differentiation of myoblasts into osteoblasts via the PERK-eIF2 $\alpha$ -ATF4 pathway but in addition stimulates Tmem119 that itself increases ATF4. The mechanisms whereby Tmem119 directly and/or indirectly regulate ATF4 remain to be elucidated

Tmem119 acts on ATF4 independently of the PERK-eIF2 $\alpha$  pathway (Fig. 7). ATF4 plays a role in osteoblast differentiation along with Runx2 and Osterix, and it acts as a DNA-binding protein with a leucine zipper region. Tmem119 is a transmembrane protein with a signal peptide. The details of how Tmem119 influences ATF4 expression remain to be elucidated. Hence, Tmem119 does not seem to be a critical molecule acting downstream of the PERK-eIF2 $\alpha$  pathway in the BMP-2-induced differentiation of myoblasts into osteoblasts (Fig. 7). This conclusion is strengthened by the finding that while reduction of endogenous Tmem119 expression reduced the basal ATF4 levels, P-eIF2 $\alpha$  and eIF2 $\alpha$  levels were unchanged. Likewise, reduced endogenous Tmem119 levels led to an inability of BMP-2 to fully induce ATF4 levels while P-eIF2 $\alpha$  levels were increased as normal. Thus, Tmem119

seems to be necessary for BMP-2 to maximally induce ATF4 levels.

In conclusion, our study shows that the PERK-eIF2 $\alpha$ -ATF4 ER stress response pathway plays an important role in the differentiation of myoblasts into osteoblasts induced by BMP-2. Moreover, increases in ATF4 levels brought about by Tmem119 itself are implicated in the osteoblastic differentiation of myoblasts. Thus, BMP-2 and Tmem119 contribute separately and in concert to promote osteoblast differentiation (Fig. 7). Further studies that clarify the mechanism or mechanisms whereby they act may lead to development of novel treatments for FOP and other bone disorders.

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