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BMP signaling is required for adult skeletal homeostasis and mediates bone anabolic action of parathyroid hormone☆,☆☆

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ABSTRACT

Bmp2 and *Bmp4* genes were ablated in adult mice (*KO*) using a conditional gene knockout technology. Bones were evaluated by microcomputed tomography (μCT), bone strength tester, histomorphometry and serum biochemical markers of bone turnover. Drill-hole was made at femur metaphysis and bone regeneration in the hole site was measured by calcein binding and μCT. Mice were either sham operated (ovary intact) or ovariectomized (OVX), and treated with human parathyroid hormone (PTH), 17β-estradiol (E2) or vehicle. *KO* mice displayed trabecular bone loss, diminished osteoid formation and reduced biomechanical strength compared with control (expressing *Bmp2* and *Bmp4*). Both osteoblast and osteoclast functions were impaired in *KO* mice. Bone histomorphomtery and serum parameters established a low turnover bone loss in *KO* mice. Bone regeneration at the drill-hole site in *KO* mice was lower than control. However, deletion of *Bmp2* gene alone had no effect on skeleton, an outcome similar to that reported previously for deletion of *Bmp4* gene. Both PTH and E2 resulted in skeletal preservation in control-OVX, whereas in *KO*-OVX, E2 but not PTH was effective which suggested that the skeletal action of PTH required Bmp ligands but E2 did not. To determine cellular effects of Bmp2 and Bmp4, we used bone marrow stromal cells in which PTH but not E2 stimulated both Bmp2 and Bmp4 synthesis leading to increased Smad1/5 phosphorylation. Taken together, we conclude that Bmp2 and Bmp4 are essential for maintaining adult skeletal homeostasis and mediating the anabolic action of PTH.

1. Introduction

Osteoporosis is characterized by low bone mass, consequent occurrence of low impact fractures and compromised fracture healing. Therapy against osteoporosis predominantly involves anti-catabolic drugs which suppress osteoclast-mediated bone resorption [1]. Clinical outcome with anti-catabolic therapies alone is unsatisfactory as they fail to regenerate the bone that has already been lost [2]. Stimulating bone formation requires anabolic therapy and intermittent PTH administration is only such therapy in clinical use [3]. However, the exact mechanism of intermittent PTH-mediated bone formation is still unclear as the hormone stimulates bone resorption for the maintenance of systemic calcium homeostasis [4].

Allelic series of limb specific (using *Prx1*::*Cre*) embryonic knockout/ablation of *Bmp2*, *Bmp4* and *Bmp7* as well as combinations of these mutations revealed that BMP signaling is essential for embryonic bone formation [5]. In this study, it was observed that depletion of any of these ligands individually or *Bmp2* and *Bmp7* or *Bmp4* and *Bmp7* in combination did not compromise embryonic bone development. However, depletion of *Bmp2* and *Bmp4* together severely impaired embryonic bone development. These data when analyzed in the light of reported expression patterns of these molecules in developing skeletal anlagen suggest

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that Bmp ligands are functionally redundant and depletion of both Bmp2 and Bmp4 are necessary to lower the level of BMP signaling below the critical threshold necessary for supporting embryonic bone development. Interestingly, although deletion of *Bmp2* gene (*Bmp2*$^{c/c}$; *Prx1*::*Cre*) alone in limb mesenchymal cells largely spared embryonic bone development, these mice when attained their adult life suffered from low bone mass, spontaneous fractures and lack of fracture healing ability [6]. What remains unclear is whether the defects observed in adult animals are a result of subtle defect(s) in embryonic bone development or defects in adult bone homeostasis.

In adult skeleton, the role of BMPs has been studied with respect to fracture healing of non-unions. Healing of non-unions in presence of BMPs undergo endochondral bone formation and recapitulates developmental events. Among the various BMPs, BMP-2 or BMP-7 is clinically used for the treatment of long bone non-unions [7]. In a recent clinical study comparing for the first time the efficacy of BMP-2 and BMP-7 in tibial non-unions observed that the former group healed and bore weight sooner than the latter group [8], thus suggesting that BMP-2 had greater osteogenic impact. BMP signaling is regulated by various endogenous antagonists including noggin, gremlin and chordin. BMP-2, noggin and chordin are co-expressed in human fractures suggesting their coordinated roles in the process of bone healing [9]. Taken together, the literature pertaining to bone healing in adults by BMPs does not accurately address bone remodelling, which is the predominant event in adult skeleton.

To investigate the role of BMP signaling exclusively in adult bones it would be necessary to inactivate BMP signaling pathway once the animal has attained skeletal maturity. Till date the only such experiment has been conducted by Yuji Mishina's group [10]. In this study BMP receptor 1a (Bmpr1a) was knocked out in 8 week old mice using *Col1*::*CreER(T)* line. In these mutants the bone mass actually increased. However, what is not clear is whether BMP signaling was effectively depleted in these mutants considering the reported functional redundancy between *Bmpr1a* and *Bmpr1b* [11]. This study also did not allow for investigation of the possible role(s) of BMP signaling in adult bone precursor cells that are likely to be Col1a1 negative. In fact the identity of bone progenitor cells have not been conclusively determined. Many cell populations including Nestin expressing bone marrow cells are proposed to be adult bone progenitors [12]. Therefore, to assess whether BMP signaling has any role exclusively in adult bone, this signaling pathway must be inactivated in a systemic manner in mice that have already attained skeletal maturity which has never been done before.

According to existing literature Bmp2 and Bmp4 are the most potent osteogenic ligands [5]. Unlike *Bmp2*, limb-specific embryonic deletion of *Bmp4* (*Bmp4*$^{c/c}$; *Prx1*::*Cre*) alone does not cause defects in embryonic or adult skeleton of mice [13]. Thus if BMP signaling is at all important for maintenance of adult bone homeostasis it must be accomplished through expression of Bmp2 alone or Bmp2 and Bmp4 in combination. In this context it is relevant to note that bone marrow stromal cells (possible adult osteoprogenitor cell niche) express both Bmp2 and Bmp4 [14,15]. Bmp2 is known to be produced by mouse bone marrow macrophages, osteoclasts and bone marrow hematopoietic stem cells (HSC) [16]. Further, Bmp2 produced by HSCs induce osteogenic differentiation of mesenchymal stem cells (MSC) [17]. Therefore, knocking out *Bmp2* and *Bmp4* only in osteoblasts (using an osteoblast-specific Cre such as Coll-Cre) may not be effective. In fact, as mentioned above, conditional knockout of Bmpr1a in Coll expressing adult bone cells resulted in increased bone mass [10]. Thus in this study we used a tamoxifen inducible ubiquitously expressed Cre recombinase, *ROSA26CreER(T)* [18] to delete both *Bmp2* and *Bmp4* alleles in adult mice. Using similar approach, we also deleted *Bmp2* allele in adult mice. In these mice we: (1) characterized the bone phenotype and bone cells and (2) evaluated the skeletal response of PTH and E2 after ovariectomy in order to determine whether these two hormones mediate their skeletal response via Bmp2 and Bmp4. Using ex vivo cultures of bone marrow stromal cells and its immortalized form [19], we sought to determine whether the effects of E2 and PTH are mediated by osteoblast produced Bmp2 and Bmp4.

2. Materials and methods

2.1. Reagents and kits

Cell culture media and all fine reagents were from Sigma Aldrich (St. Louis, MO); FBS was from Invitrogen (Carlsbad, CA); human PTH (1–34) from Calbiochem (USA); ELISA kits for mouse C-terminal telopeptidase type-I collagen (CTX), procollagen type 1 propetides (P1NP) and tartrate-resistant acid phosphatase subunit 5 (TRAPc5) were from MyBioSource (San Diego, CA) and that of Bmp2, Bmp4 and IGF-1 were from Abcam (Cambridge, MA). Serum 1,25-dihydroxyvitamin D kit from Immunodiagnostic Systems (Scottsdale, AZ, USA). Recombinant mouse macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappaB ligand (RANKL) were from Sigma Aldrich (St. Louis, MO); recombinant human Bmp-2 and Bmp-4 were purchased from Abcam (Cambridge, MA). Primary antibodies against Bmp-2 and Bmp-4 were from Abcam (Cambridge, MA), antibody for p-Smad 1/5 and all secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Smad 1/5 and β-actin antibodies were from Santa Cruz Biotechnology (Dallas, TX).

2.2. Generation of Bmp2 and Bmp4 conditional null allele

R26CreER/R26CreER and *Bmp2*$^{C/C}$ mouse strains have been reported before [5,20]. *Bmp2*$^{C/C}$; *R26CreER/+* has been generated in this study by crossing *R26CreER/R26CreER* and *Bmp2*$^{C/C}$. *Bmp2*$^{C/C}$; *Bmp4*$^{C/C}$; *R26CreER/+* (*Bmp2/4 DCKO*) as described previously [21]. All animal care and experimental procedures were approved by Institutional Animal Ethical Committee guidelines (approval # IAEC/2012/65). Mice were kept in individually ventilated cage (one mouse/cage) systems at low ammonia and CO_2 concentrations to support a low relative humidity, and to reduce spread of infective agents and allergenic contaminants. Temperature was controlled (22–24 °C) with room having fresh air supply with 100% exhaust air to the outside. Room had diffuse lighting in the range of 200–300 lx and equipped for automatic maintenance of a diurnal 12 h light cycle. The animals were fed γ irradiated ad libitum maintenance diet (Provimi Animal Nutrition, India) and had free access to RO water.

2.3. Experimental design for in vivo studies and related methods

All studies were done on female mice (10–12 weeks old) weighing 25 ± 3 g. The animals were fed γ irradiated ad libitum maintenance diet (Provimi Animal Nutrition, India) and had free access to RO water.

To knock out *Bmp2* gene, *Bmp2*$^{C/C}$; *R26CreER/+* mice were injected with tamoxifen in corn oil (vehicle) (2.5 mg/20 g of mice, i.p. with corn oil) for five consecutive days (*Bmp2 KO*). Littermate *Bmp2*$^{C/C}$; *R26CreER/+* mice that were injected with corn oil alone to serve as control. Similarly *Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$ mice were injected with tamoxifen to obtain knock-out *Bmp2/4* DC*KO* mice, to be called *KO* mice henceforth. *Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$ mice were treated with tamoxifen one day after ovariectomy (OVX) to obtain *KO*-OVX mice as described below. Littermate *Bmp2*$^{c/c}$/*Bmp4*$^{c/c}$ mice were injected only with corn oil (vehicle) to serve as control. The parental Cre-mouse strain containing *R26CreER(T)* transgene was similarly treated with tamoxifen (referred to as P1 in the results). All surgical procedures were performed under anaesthesia with xylazine (15 mg/kg, s.c.) and ketamine (100 mg/kg, i.p.) [22].

Further, control and *KO* mice were sham operated or OVX and were weight-randomized into different groups (n = 6/group); control-sham, control-OVX, control-OVX + E2, control-OVX + PTH, *KO*-sham, *KO*-OVX, *KO*-OVX + E2 and *KO*-OVX + PTH. The dosing and regimen of PTH (40 μg/kg; 5 days a week, s.c.) and E2 (10 μg/kg/d, s.c.) were based on previous reports [23,24]. After 6 weeks of various treatments,

all groups were sacrificed, and femurs, 5th lumbar vertebra (L5) and serum samples were collected for further analysis. Euthanasia and disposal of carcasses were in compliance with the IAEC guidelines.

2.4. Bone histomorhometry

For dynamic histomorphometry, each animal received calcein (5 mg/kg, i.p.) on the fifteenth and second days before sacrifice. Mineralizing surface per bone surface (MS/BS), mineral appositional rate (MAR) and bone formation rate/bone surface (BFR/BS) at distal femur (sections of undecalcified bone, 50 μM) were determined according to our previously published protocol in accordance to the American Society for Bone and Mineral Research [25].

To measure bone volume and unmineralized osteoid, 5 μm undecalcified sections were prepared from distal femurs embedded in polymerized methyl methacrylate and stained with Goldner's trichrome as described before [26]. Trabecular bone volume (BV/TV), osteoid volume per bone volume (OV/BV) and oteoid width (O.Wi) were measured using Bioquant Osteo Software (Bioquant Image Analysis, Nashville, TN, USA).

To investigate effects on osteoclast, longitudinal sections of distal femur (5 μm, decalcified) were stained for tartrate-resistant acid phosphatase (TRAP). OC surface/bone surface (%) and number of osteoclasts (OC)/bone perimeter (N.Oc/B.Pm) were analyzed using Bioquant Osteomeasure software version 12.5.6 (Nashville, TN), following a previously described method [27].

2.5. Drill-hole injury

For bone regeneration study, a drill-hole of 0.5 mm diameter was created in anterior femur metaphysis of mice under anaesthesia [28]. After 2 weeks, all mice were sacrificed and their femurs collected for the measurement of bone volume (BV/TV) in the drill hole site using 3D μCT analysis (SkyScan 1076, Aartselaar, Belgium) as described below. Callus regeneration at the fracture site was measured with confocal microscopy (LSM 510 Meta, Carl Zeiss, Inc., Germany) on bone sections (50 μm) made through drill-hole. All animals were given calcein (5 mg/kg, i.p.) 24 h before sacrifice and the intensity of calcein binding was calculated using Carl Zeiss AM 4.2 image-analysis software.

2.6. μCT

μCT scans (Skyscan 1076, Aartselaar, Belgium) of live animals and excised bones were performed to assess trabecular and cortical bones as described before [27,29]. Trabecular BMD of femora was determined from the volume of interest made for the trabecular region, using μCT scans. For calibration, the hydroxyapatite phantom rods of 2 mm diameter with known BMD (0.25 g/cm^3 and 0.75 g/cm^3) were employed [30]. Coronal images of femur distal region were used for node-strut analysis. Node was characterized as the intersection point of three or more trabeculae; terminus area as the bone area within the range of mean length of individual trabecular thickness from the free end of a trabecula; and strut area as the residual bone area [31]. Number of nodes (N.Nd), node to node strut length (Nd.Nd; 1/mm^2 of tissue area) and terminus-to-terminus strut length as a percentage of total strut length (Tm.Tm/TSL) were calculated with Skyscan data viewer software [32].

2.7. Bone strength

L5 was subjected to compression test with bone strength tester model TK252C (Muromachi Kikai Co. Ltd., Tokyo, Japan) to determine various strength parameters including energy to failure and stiffness following a protocol described before [27].

2.8. Bone turnover markers

Commercially available ELISA kits were used to measure serum levels of collagen type 1 cross-linked C-telopeptide (CTX), TRAPc5, N-terminal type 1 procollagen (P1NP), 1,25 $(OH)_2$-vitamin D_3 and insulin-like growth factor 1 (IGF-1), and Bmp2 and Bmp4 levels in the conditioned medium. Serum calcium was determined by *o*-cresolphthalein-complex one kit and (Sigma-Aldrich, St. Louis, MO) serum phosphorus by ammonium molybdate method (Sigma-Aldrich).

2.9. In vitro methods

2.9.1. Osteoclast differentiation

Non-adherent bone marrow cells were differentiated to osteoclasts using M-CSF and RANKL following a previously described protocol [27]. At the end of treatments, cultured bone marrow derived cells were subjected to qPCR analysis and were fixed and incubated in acetate buffer containing naphthol AS-BI phosphate, fast red violet LB salt and sodium tartrate. TRAP-positive mononuclear cells and TRAP-positive multinucleated cells (more than three nuclei) were scored under light microscope as described previously [27,33].

2.9.2. Nodule formation

For ex vivo mineralized nodule formation, stromal cells from bone marrow (BMSC) were harvested from long bones, seeded and cultured in osteoblast differentiation media (α-MEM containing 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and 100 nM dexamethasone) with 10% FBS for 21 days and the medium was changed at every 48 h. After 21 days, plates were fixed in 4% PFA and the mineralized nodules were stained by alizarin red-S. Following photography of the nodules, alizarin dye was dissolved in 10% CPC (cetyl pyridium chloride) and read at 595 nm [30].

2.9.3. Generation of immortalized TVA-BMSCs

BMSC of *Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$; *R26CreER*/*R26CreER* mouse were immortalized using a retrovirus expressing SV40 large T-antigen. These cells are also engineered to express an avian receptor called TVA receptor and named as TVA-BMSC. These cells can be differentiated into chondrogenic, osteogenic, and adipogenic lineages when cultured under appropriate conditions [19]. *Bmp2* and *Bmp4* genes in TVA-BMSC were deleted by the treatment of 1 μM 4-OH-tamoxifen (4-OHT) for 24 h.

2.9.4. qPCR analysis

qPCR was performed using SYBR green chemistry as previously described [34]. Total RNA was isolated using TRIzol (Gibco BRL, Gaithersburg, MD, USA). cDNA was synthesized from aliquots of 2 μg total RNA/sample, with the Revertaid cDNA Synthesis Kit (Fermentas, Aust in, TX, USA). The design of primers was based on published cDNA

Table 1
Primer sequences of various genes used for qPCR.

Gene name	Primer sequence	Accession number
Bmp2	F - CGGACTGCGGTCTCCTAA R - GGGGAAGCAGCAACACTAGA	NM_007553.2
Bmp4	F - GAGGAGTTTCCATCACGAAGA R - GCTCTGCCGAGGAGATCA	NM_007554.2
Sost (*sclerostin*)	F - TCCTGAGAACAACCAGACCA R - GCAGCTGTACTCGGACACATC	NM_024449.5
Rank	F - GTGCTGCTCGTTCCACTG R - AGATGCTCATAATGC CTCTCCT	NM_009399.3
Rankl	F - TGAAGACACACTACCTGACTCCTG R - CCCACAATGTGTTGCAGTTC	NM_011613.3
Opg	F - GTTTCCCGAGGACCACAAT R - CCATTCAATGATGTCCAGGAG	NM_008764.3
Gapdh	F - AGCTTGTCATCAACGGGAAG R - TTTGATGTTAGTGGGGTCTCG	DQ403054.1

sequences using the Universal Probe Library (Roche Applied Science, Indianapolis, IN, USA). Primer pairs used in PCR reactions are described in Table 1.

2.9.5. Western blotting

Western blotting was performed following our previously described protocol [34]. Briefly, TVA-BMSCs at 60–70% confluence were exposed to various treatments for 48 h. Cells were lysed using Cellytic M Cell lysis buffer (Sigma-Aldrich, St. Louis, MO) supplemented with 1 × protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and total protein was quantified by BCA assay. Cell lysates were resolved on an SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated with different primary antibodies against Bmp2 (ab6285), Bmp4 (ab39973), Smad1/5 (sc-6031-R) and p-Smad1/5 (9516S) (all at 1:2000 dilution) and β-actin (A3854) (1:5000 dilution). Relative density of the respective blots was determined with Image J software (NIH, Bethesda).

2.10. Data analysis and statistics

Results are expressed as mean ± SEM. All data were analyzed using GraphPad Prism 5.0 (GraphPad, San Diego, CA). One-way ANOVA followed by Tukey multiple comparison test was used to analyse the data involving more than two-groups. In vitro or in vivo data with only two experimental groups were analyzed using two-tailed unpaired Student *t*-test.

3. Results

3.1. Bmp2 and Bmp4 are necessary for remodelling of adult skeleton

Limb mesenchyme specific inactivation of *Bmp2* and *Bmp4* (*Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$; *Prx1*::*Cre*) blocks embryonic bone formation [5]. On the other hand, limb mesenchyme specific inactivation of *Bmp2* alone (*Bmp2*$^{c/c}$; *Prx1*::*Cre*) while spares embryonic bone formation compromises bone health in the adult [5,6]. Based on these two reports we speculated that these BMP ligands may be important for maintenance of homeostasis of adult bone. Since limb specific embryonic deletion of *Bmp4* did not produce any osteopenic phenotype in adult mice [13], we did not investigate if adult specific inactivation of Bmp4 alone would have an effect on adult bone health. To test if *Bmp2*, by itself, was necessary for the maintenance of adult bone, we treated skeletally mature (10–12 weeks old) female *Bmp2*$^{C/C}$; *ROSA26CreER(T)* mice with tamoxifen and analyzed bones after six weeks. Trabecular bone parameters in L5 and distal femur assessed by μCT were not different between *Bmp2 KO* and control mice (Fig. 1A). Cortical parameters at femur mid-diaphysis were also not different between these two groups (Fig. 1B).

During embryonic development, inactivation of *Bmp2* and *Bmp4* alleles together revealed the essential role of BMP signaling in bone morphogenesis [5,35]. Likewise, it is possible that to decipher the role of BMP signaling in adult bone homeostasis, deletion of both these genes was necessary. For this purpose, skeletally mature female mice were taken and recombination at *Bmp2* and *Bmp4* loci were induced by tamoxifen injection to generate *KO* mice and comparisons were made with corn oil injected control mice (having intact expression of *Bmp2* and *Bmp4* genes) [36]. Mice were scanned before and after tamoxifen treatment by μCT to assess bone parameters. After tamoxifen treatment, skeletal response to *Bmp2/4* deletion was monitored every 3 weeks and compared with the pre-tamoxifen treatment values (baseline; BL). Six-week post-tamoxifen, a significant bone loss was observed at distal femur compared with BL (Fig. 2).

To ensure that the skeletal effect observed in *KO* mice was not an effect of tamoxifen or Cre recombinase, the parental Cre-mouse strain (*ROSA26CreER(T)*) was similarly treated with tamoxifen (henceforth referred to as P1) and trabecular bone parameters determined by 3D-μCT of isolated bones of P1 were comparable to age-matched female control mice (Fig. 3).

Six weeks post-tamoxifen treatment, when bone loss was observed in *KO* mice, there was no difference in body and uterine weights, femur length and snout-to-tail length between the P1, control and *KO* mice (Table 2). Serum total calcium, phosphate and 1,25-$(OH)_2$ vitamin D_3 levels were not different between control and *KO* suggesting that systemic calcium homeostasis was unaffected in *KO* mice (Table 3). IGF-1, which is a regulator of osteoblast function [37] and required for bone development and maintenance [38], and a mediator of PTH action in bone [39] showed comparable serum levels between control and *KO* mice (Table 3).

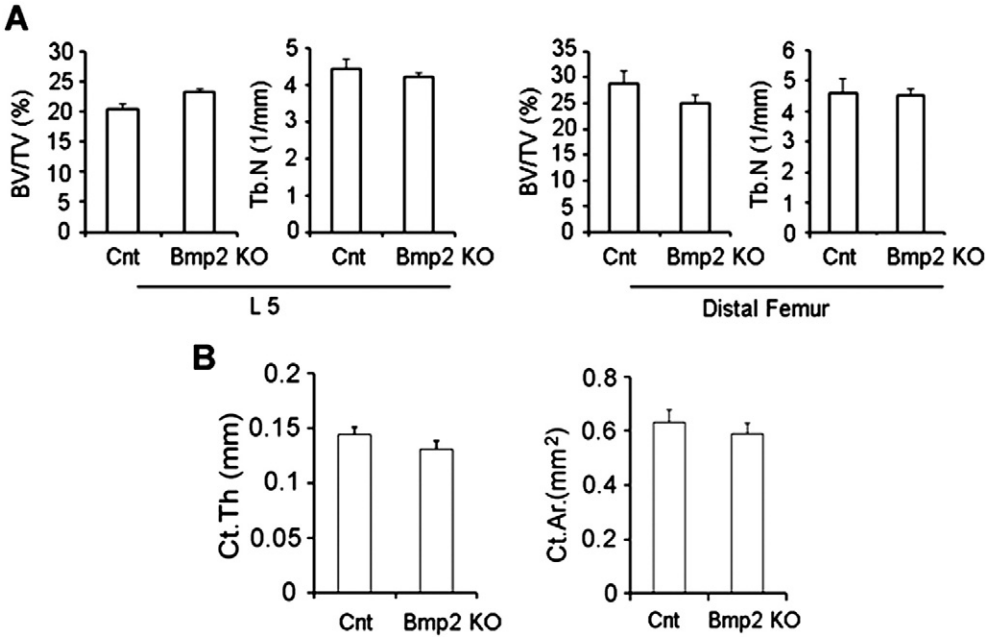

Fig. 1. *Bmp2* deletion does not alter trabecular and cortical bones in adult mice. *Bmp2*$^{C/C}$; *R26CreER(T)* female mice (10–12 weeks) were injected with tamoxifen (*Bmp2 KO*) and after 6 weeks femur trabecular (A) and cortical (B) parameters were compared with age-matched control (Cnt) mice. Tamoxifene in corn oil was dosed 2.5 mg/20 g of mice, i.p. for 5 consecutive days; Cnt goup was given corn oil treatment. Data are mean ± SEM.

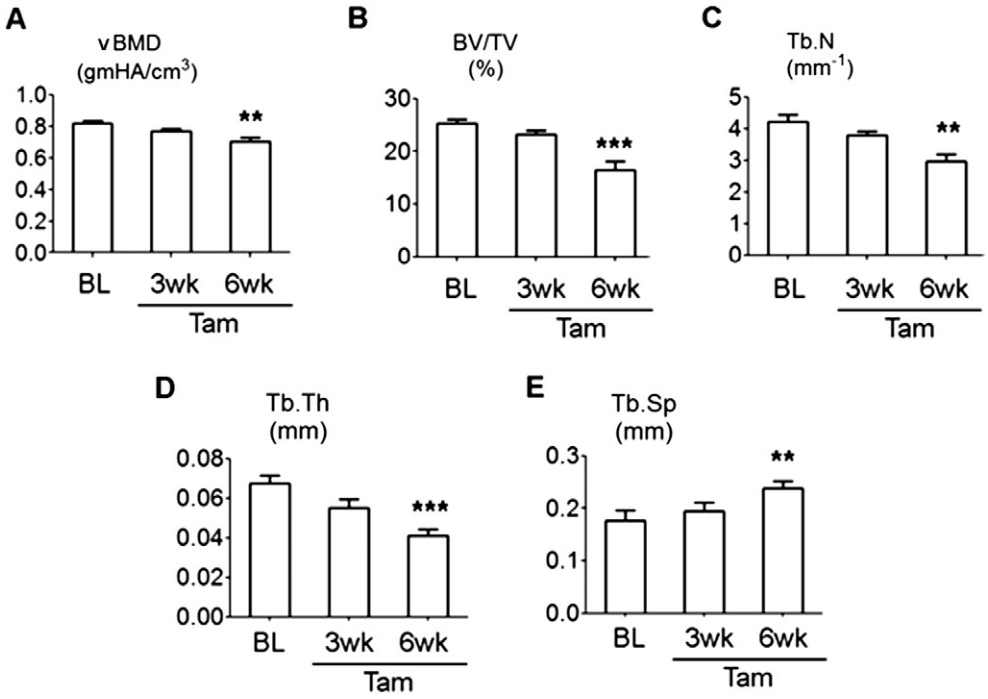

Fig. 2. Bone loss in skeletally mature *Bmp2/4 DCKO* female mice in which both *Bmp2* and *Bmp4* alleles are deleted by tamoxifen (Tam) administration. (A–E) μCT analysis of mice (10–12 weeks) showing trabecular bone parameters of distal femur at day 0 (baseline, BL), 3wk and 6wk post-Tam treatment (to delete *Bmp2* and *Bmp4* genes); n = 6 mice per time point, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus BL.

At the cellular level, impact of *Bmp2/4* ablation on osteoblast differentiation was assessed using bone marrow stromal cells (BMSC). Immunoblotting revealed absence of Bmp2 and Bmp4 proteins in *KO* BMSC, while both control and P1 expressed them (Fig. 4A). BMSC from P1 and control mice showed a strong basal anti-p-Smad1/5 immunoreactivity but it was undetectable in cells derived from *KO* mice (Fig. 4B). That the stromal cells had intact BMP signaling apparatus despite *Bmp2/4* deletion has been demonstrated by two ways. In the first set of experiments, TVA-BMSC line made from BMSC of *KO* mice was used, which when treated with 4-OHT would result in the depletion of *Bmp2/4* gene products in vitro. We confirmed that 4-OHT treatment to TVA-BMSCs completely depleted Bmp2 and Bmp4 (assessed by immunoblotting) as the expression was lost after 48 h and did not recover up to day 120 h (5 days) (Fig. 4C), thus suggesting that loss of Bmp2/4 expression was irreversible. TVA-BMSC line with intact Bmp2/4 (without 4-OHT treatment) had a strong basal p-Smad1/5 expression while the same line after 4-OHT treatment had a very low p-smad1/5 level (Fig. 4D). However, exogenous Bmp2 or Bmp4 robustly induced p-

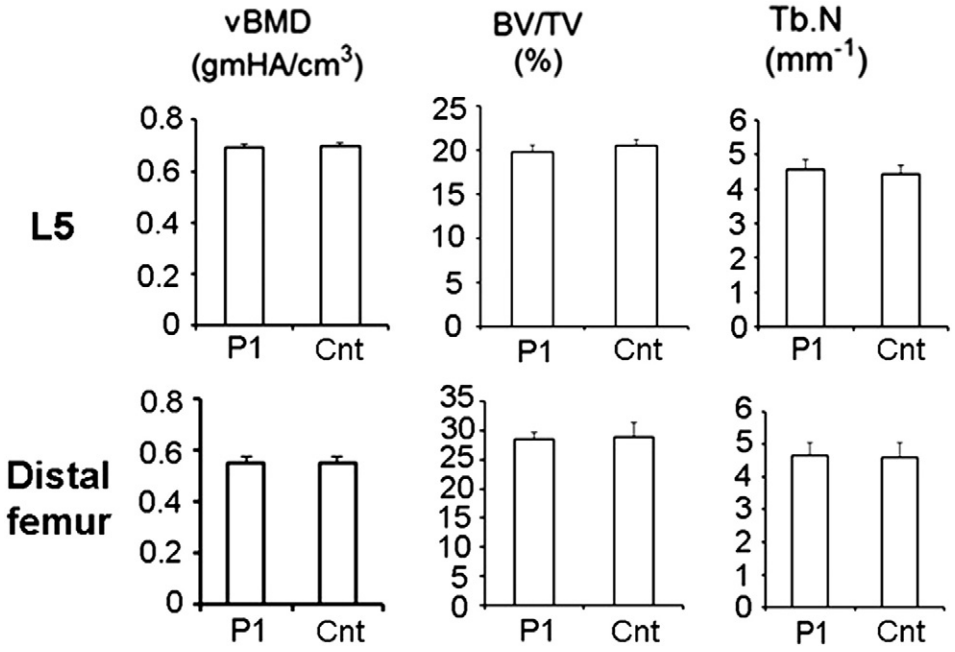

Fig. 3. Tamoxifen (Tam) treatment has no effect on trabecular bones. Parental Cre-recombinase strain of female mice (*ROSA26CreER(T)*) (10–12 weeks) were injected with Tam (P1) and after 6 weeks they were killed, and skeletal parameters were compared with age-matched female control (Cnt) mice.

Table 2
Body weight, uterine weight and morphological topographies.

Parameters	P1	*Cnt*	*KO*
Body weight (gm)	23.3 ± 0.46	23.2 ± 0.71	21.2 ± 0.82
Uterine weight (mg)	119.8 ± 7.9	119.3 ± 8.9	122 ± 6.0
Snout-to-tail length (cm)	17.3 ± 0.13	17.4 ± 0.10	17.4 ± 0.15
Femur length (mm)	1.8 ± 0.03	1.8 ± 0.04	1.8 ± 0.02

Female mice (10–12 weeks) were used for the study. Data presented as mean ± SEM; n = 6.

Smad1/5 in TVA-BMSC line lacking Bmp2/4 expression (Fig. 4D). Cultures of mouse primary calvarial osteoblasts when treated with 4-OHT showed no effect on osteoblast function (growth and differentiation) and Smad1/5 phosphorylation (Fig. 4E), which suggested that 4-OHT had no effect on osteoblast function. In the second experiment, we found that BMSC from *KO* mice displayed a significant decrease in the formation of mineralized nodules compared with control, however, exogenous Bmp2 treatment stimulated nodule formation by these cells (Fig. 4F). Together, our data demonstrate that Bmp2/4-depleted osteoblastic cells possess intact BMP signaling apparatus.

As osteogenic differentiation was impaired in the *KO* BMSC, we assessed if osteoinduction at the site of bone wound could be compromised in these mice. To this effect, we made drill-hole at femur metaphysis to study bone wound healing and observed that calcein binding intensity at the callus in the *KO* mice was only 30% of the control or P1 (Fig. 5A). In addition, at the drill-hole site, a reduced percentage of bone volume/tissue volume (BV/TV %) was observed in *KO* compared with control or P1 (Fig. 5B).

We next assessed the impact of *Bmp2/4* depletion on trabecular bones of skeletally mature mice. At distal femur, bone volume fraction was significantly reduced in *KO* compared to control mice (Fig. 6A). Because, osteoblasts first produce mineralized osteoids in the primary spongiosa (PS) which is later remodelled to secondary spongiosa (SS) and we observed impaired osteoblast function in *KO* mice, we thus studied the spongiosa regions of femur metaphysis by node-strut analysis to assess structural connectivity of bony elements. A dramatic loss in connectivity at PS was observed in the *KO* as the number of nodes (N.Nd) and node-to-node strut length (Nd.Nd) per unit area were reduced and terminus-to-terminus strut length (Tm.Tm/TSL) was increased compared to control (Fig. 6B). As osteoblasts and osteoprogenitor cells reside in PS in abundance and contribute to the formation of woven bone, reduced bony elements in PS of *KO* mice was consistent with impaired osteoblast function. The severity of connectivity impairment at SS was relatively less in *KO* mice, as the decrease in Nd.Nd and increase in Tm.Tm/TSL were modest compared to control (Fig. 6B), and this could be due to fewer osteoblasts and osteoprogenitor cells that normally reside in SS compared with PS.

Histomorphomtery analysis of distal femur following Goldner's trichrome (GT) staining showed reduced BV/TV, confirming the μCT data showing trabecular osteopenia in *KO* mice (Fig. 6C). In addition, OV/BV (osteoid volume per bone volume, percent of a given volume of bone tissue that consists of unmineralized bone) and O.wi (osteoid width) were also reduced in *KO*, suggesting impaired osteoblast function (Fig. 6C). Further, assessment of mineral apposition and bone formation by dynamic histomorphometry affirmed decreased osteoblast function in *KO* mice. Mineralizing surface/bone surface (MS/BS) reflects active mineralization on a given bone surface, mineral apposition rate (MAR) measures linear rate of bone deposition and bone formation rate/bone surface (BFR/BS) reflects the amount of new bone formed at a given time per unit bone surface. All three parameters were decreased in *KO* mice compared with control (Fig. 6D). Cortical parameters including vBMD, cortical thickness (Ct.Th) and cortical area (Ct.Ar), however, were not different between the control and *KO* (Fig. 6E).

Adult bone homeostasis is characterized by remodelling process which is initiated by osteoclasts. BMP signaling has been implicated in osteoclast differentiation [10,40]. Upon induction of osteoclastic differentiation of bone marrow cells, *KO* mice showed significantly reduced TRAP-positive cells and RANK mRNA expression compared with control (Fig. 6F). Consistent with these ex vivo data, histomorphometric analysis of distal femur demonstrated that the proportion of total TRAP-positive cells per bone area, indicating osteoclast number (Oc.N/B.Pm), was less in *KO* compared with control (Fig. 6G). The ratio of osteoclast surface to bone surface (Oc.S/Bs), a surrogate of osteoclastic activity was diminished in *KO* compared to control (Fig. 6G). RANKL and OPG mRNA levels were determined in femur trabecular region and their ratio was not different between control and *KO* mice (Fig. 6H), suggesting that reduced osteoclast differentiation/function in *KO* was independent of osteoblastic influence.

In addition to femur (a part of appendicular skeleton), we also studied the 5th lumbar vertebra (L5) which is a part of axial skeleton. μCT analysis showed decreased vBMD, BV/TV% and Tb.N in *KO* compared with control (Table 4). Histomorphometry of L5 following GT stain corroborates the μCT data showing reduced BV/TV% in *KO* compared with control. Both OV/BV % and O.Wi were decreased in *KO* (Table 4). Compression test of L5 showed a significant decrease in energy-to-failure and stiffness in *KO* mice compared with control mice (Table 4).

3.2. Bmp2/4 are required for skeletal action of PTH but not E2

We next studied the effect of depletion of endogenous Bmp2/4 on the actions of PTH and E2, the hormones involved in regulating adult skeletal homeostasis. In vitro, E2 has been shown to promote osteogenic differentiation of preosteoblasts and MSCs by enhancing transcription of *Bmp2* [41]. PTH has been shown to upregulate *Bmp2* expression via activation of the transcription factor cAMP response element binding protein (CREB) in cultured osteoblastic cells [42,43]. However, the effects of PTH and E2 on Bmp2 and Bmp4 have never been investigated in vivo.

Compared to ovary intact control (control-sham) mice, BV/TV % was reduced in control-OVX (74.5%, $P < 0.001$), *KO*-sham (46.9%, $P < 0.001$) and *KO*-OVX mice (86.8%, $P < 0.001$) (Fig. 7A). Reduction in BV/TV was contributed by decreases in Tb.N and trabecular thickness (Tb.Th, mean distance across individual trabeculae) in control-OVX, *KO*-sham and *KO*-OVX compared with control-sham, and all three parameters were lowest in *KO*-OVX (Fig. 7A–C). Trabecular spacing (Tb.sp, mean distance between trabeculae) was consequently increased in these three groups compared with control-sham. *KO*-OVX mice had the highest Tb.sp (Fig. 7D). From these data it appeared that *KO*-OVX displayed bone loss that was more than control-OVX and *KO*-sham. None of the parameters were different between control-sham and control-OVX + PTH suggesting complete skeletal preservation, following OVX, by PTH. Except Tb.Th, which was reduced in the control-OVX + E2 group compared with control-sham, other parameters were comparable between the two groups. None of the parameters were different between *KO*-sham and *KO*-OVX + E2, suggesting complete bone preservation by E2 in mice lacking *Bmp2/4*. By contrast, PTH failed to improve any of these parameters over *KO*-OVX (compare, *KO*-OVX + PTH vs. *KO*-OVX). From the data, it appeared that the skeletal action of PTH was completely dependent on Bmp2,4 whereas E2 effect was independent of these two proteins.

Serum TRAPc5b (an isoform of TRAP that is characteristic of osteoclasts) and CTX represent osteoclast number [44] and activity [45]

Table 3
Serum biochemistry.

Parameters	*Cnt*	*KO*
Serum calcium (mg/dl)	8.1 ± 0.06	8.05 ± 0.07
Serum phosphate (mg/dl)	5.4 ± 0.20	5.5 ± 0.25
1,25-dihydroxyvitamin D_3 (pg/ml)	168.5 ± 8.7	175.8 ± 15.1
IGF-1 (pg/ml)	539.9 ± 31.5	559.9 ± 45.9

Female mice (10–12 weeks) were used for the study. Data presented as mean ± SEM; n = 6.

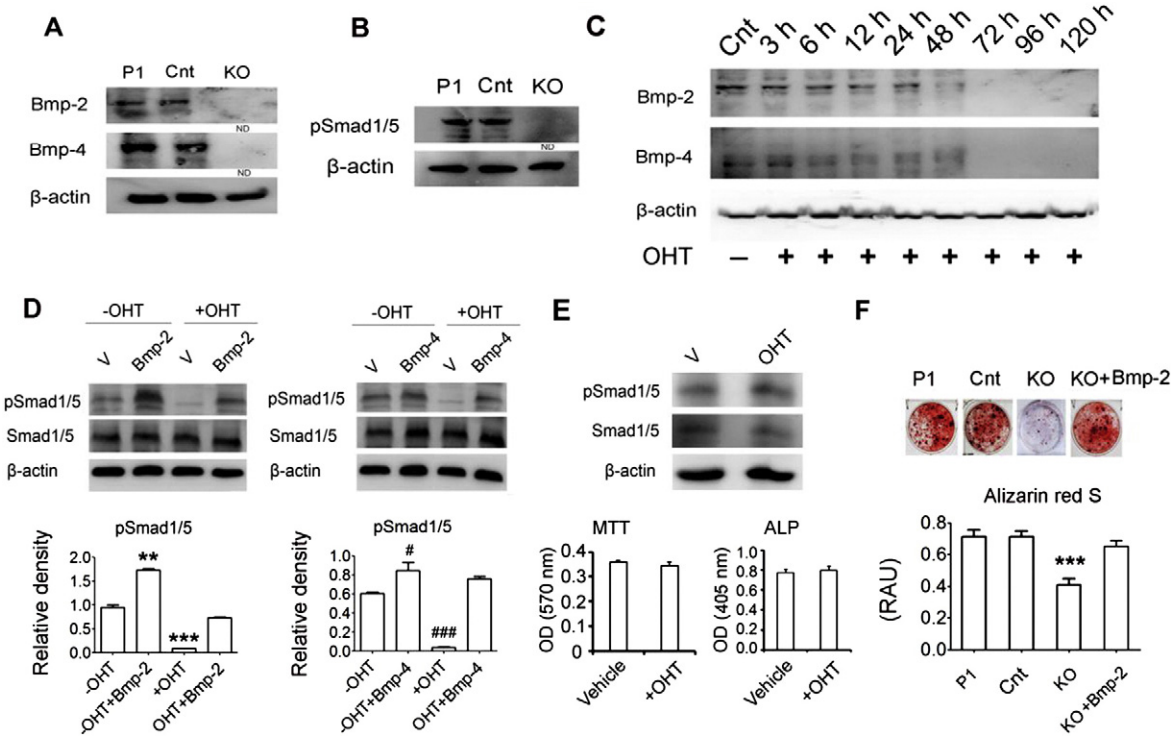

Fig. 4. *Bmp2/4* deletion resulted in impaired osteogenic differentiation. (A) Representative Western blots showing expression of BMP-2, BMP-4 and (B) p-Smad1/5 in the lysates of BMSC (stromal cells harvested from bone marrow of mice of various genotypes). None of the three proteins were detected in BMSC from *KO* mice. Control (Cnt): *Bmp2/4 DCKO* (10–12 weeks) injected with corn oil (V); *KO*: *Bmp2,4* DC*KO* injected with Tam and P1: *Cre*-mouse (*ROSA26CreER(T)*) (10–12 weeks) injected with Tam (similar treatment as in *KO*). (C) Representative western blots showing loss of BMP-2 and BMP-4 expression after 48 h treatment of 4-OHT to TVA-BMSC. (D) Increase in p-Smad1/5 levels in TVA-BMSC in response to BMP-2 and BMP-4. TVA-BMSC line was made from BMSC of control mice in which 4-OHT treatment resulted in the loss of *Bmp2* and *Bmp4* expression (depleted cells) whereas 4-OHT untreated cells (-OHT) expressed *Bmp2* and *Bmp4* (intact cells). Bmp2 and Bmp4 increased p-Smad1/5 in both depleted and intact cells. Data are mean ± SEM of 3 independent experiments. $^{**}P < 0.01$, BMP-2 treated vs. untreated intact cells; $^{***}P < 0.001$, Bmp2 depleted (OHT treated) vs. depleted cells treated with BMP-2; $\#P < 0.05$, Bmp4 treated vs. untreated intact cells; $\#\#\#P < 0.001$, Bmp4 depleted (OHT treated) vs. depleted cells treated with BMP-4. (E) Osteoblast functions were unaffected with 4-OHT; primary mouse calvarial osteoblasts were treated with 4-OHT for 48 h and thereafter Smad phosphorylation (upper panel), osteoblast viability (MTT) and differentiation (ALP assay) were measured. Data are mean ± SEM of 3 independent experiments. (F) Assessment of osteogenic differentiation of BMSC by alizarin red S staining (upper panel; representative photomicrograph and lower panel; quantification of the extracted dye), n = 3 mice/group; $^{***}P < 0.001$.

respectively, and both were decreased in *KO* compared with control (Fig. 8A, B) which was consistent with our findings from histomorphometry showing reduced osteoclast number and surface (Fig. 6G). Together, these data attested to osteoclastogenesis and resorption being dependent on Bmp2/4. As expected, both TRAPc5b and CTX were increased in the control-OVX compared with control-sham. Further, E2 but not PTH suppressed both resorption markers in control-OVX. The OVX-induced rise in these markers in *KO* mice showed a response similar to that observed with control-OVX treated with E2 or PTH (Fig. 8A, B).

Serum P1NP, the bone formation marker was decreased in both *KO*-sham and control-OVX mice compared with control-sham. In control-OVX, PTH treatment resulted in twice as much increase in P1NP as compared to control-sham (Fig. 8C). P1NP level was comparable between control-sham and control-OVX + E2. PTH treatment however did not increase P1NP in *KO*-OVX mice (Fig. 8C). These data suggested that PTH-induced stimulation of serum P1NP required Bmp2/4.

Ex vivo nodule formation by BMSC was reduced in *KO*-sham, control-OVX and *KO*-OVX compared with the control-sham (Fig. 8D).

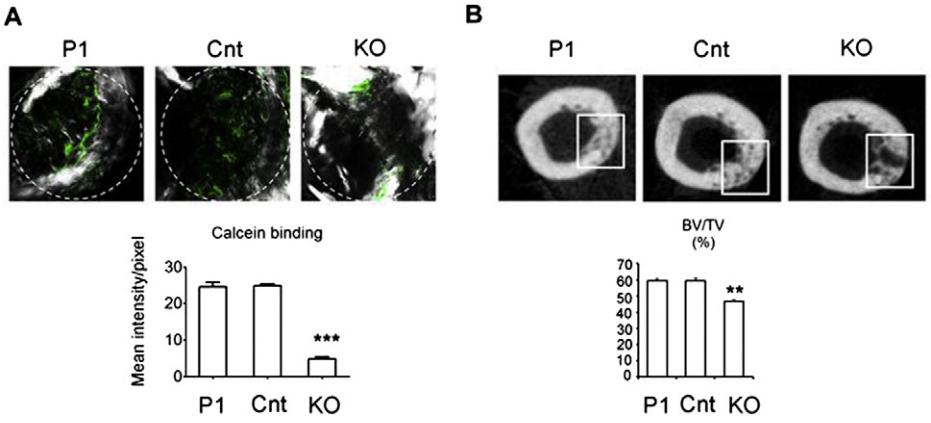

Fig. 5. Bmp2,4 deletion reduced bone regeneration at the site of osseous wound. All mice were 10–12 weeks old and control (Cnt) and *KO* were obtained as described in Fig. 4. (A) Upper panel showing representative confocal images of calcein label at the site of wound in various groups (region of interest within white dotted circle) and lower panel showing quantification of calcein binding intensity. (B) Representative μCT images of the callus (upper panel, boxed area) in various groups and quantification of bone volume (BV/TV%) (bottom panel). For A and B, data are mean ± SEM; n = 6; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control or P1.

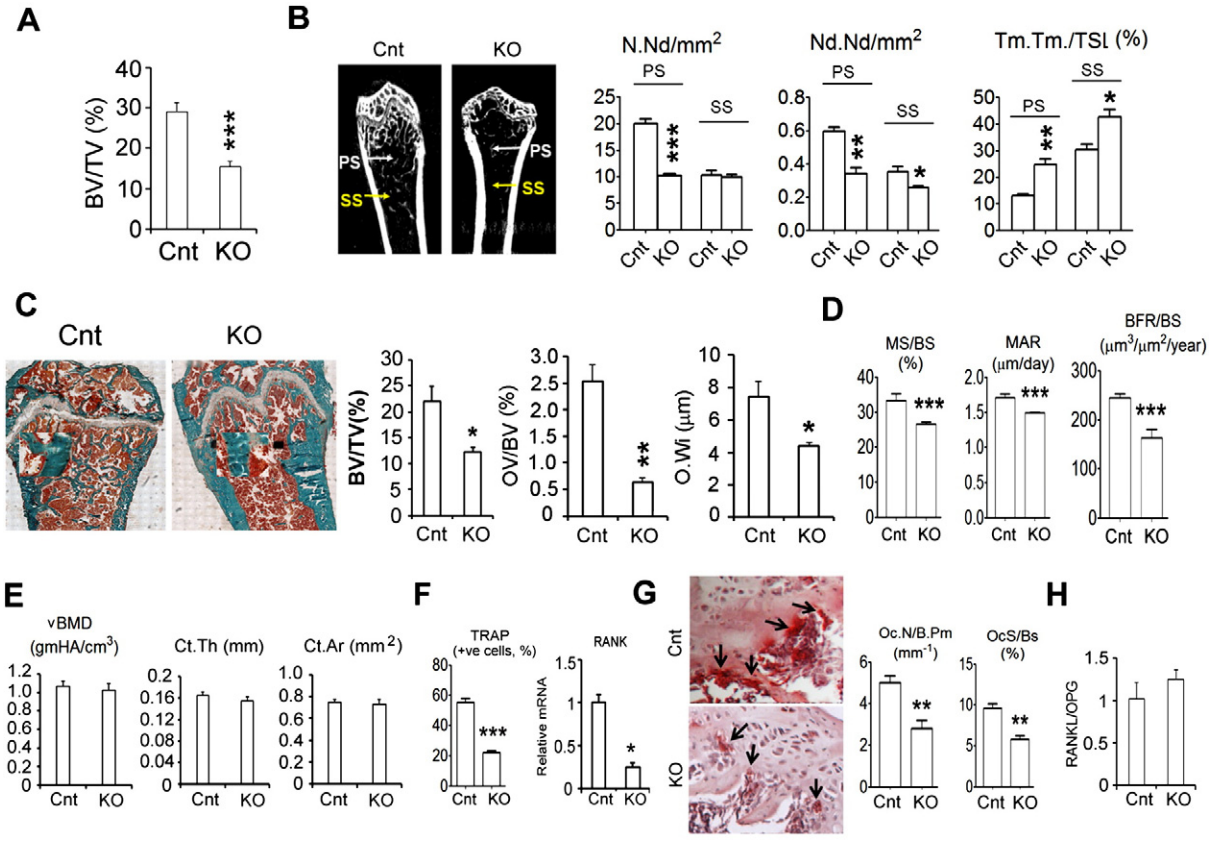

Fig. 6. Deletion of *Bmp2,4* genes caused low turnover bone loss in skeletally mature female mice. All mice were 10–12 weeks old and control (Cnt) and *KO* were obtained as described in Fig. 4. (A) Bone volume fraction (BV/TV %) of excised distal femur showing significant decrease in *KO* compared with control. (B) Representative 3D-µCT coronal images of distal femur metaphysis of mice (left panels) and quantification of trabecular strut as number of nodes per unit area (N.Nd), node-to-node strut length per unit area (Nd.Nd) and terminus-to-terminus strut length (Tm.Tm/TSL) are shown. In *KO* mice, trabecular connectivity was more impaired at PS than SS. A and B, data are as mean ± SEM; n = 6 mice/group; *P < 0.05, **P < 0.01 and ***P < 0.001. (C) GT staining of distal femur (left panels, representative photomicrograph at 40×) and histomorphometric quantification using Bioquant software (right panels). BV/TV %, OV/BV % and O.Wi µm were decreased in *KO* mice compared with control. Data are mean ± SEM, n = 3 mice/group. *P < 0.05 and **P < 0.01. (D). Double fluorochrome labelling-based dynamic measures of osteoblast function using Bioquant Osteo-measure software at distal femur showing decrease in MS/BS, MAR and BFR/BS in *KO* compared with control mice. Data are mean ± SEM (n = 6 bones/group; 5 fields/bone); ***P < 0.001. (E) µCT analysis show comparable cortical parameters between the control and *KO*. (F) Percentage of multinucleated (>3 nuclei) TRAP-positive osteoclasts differentiated from bone marrow cells and RANK mRNA expression were less in *KO* compared with control. n = 6 mice/genotype; ***P < 0.001for TRAP-positive cells and 3 independent experiments in triplicate for mRNA expression; *P < 0.05 versus control. (G) TRAP staining of distal tibia (left panels, representative photomicrograph where osteoclasts are indicated by arrows) and quantification using Bioquant software (right panels). Oc.N/B.Pm and Oc.S/BS were decreased in *KO* compared with control mice. Data are mean ± SEM (n = 6 bones/group; 4 fields/bone); **P < 0.01. (H) qPCR analysis show comparable RANKL/OPG mRNA ratio in femur metaphysis of control and *KO*.

Nodule formation in control-OVX + PTH group was comparable to control-sham group whereas the same in control-OVX + E2 was less than control-sham. The observed stimulatory effect of nodule formation by PTH on control-OVX BMSC was lacking in *KO*-OVX group, further demonstrating that osteogenic effect of PTH required Bmp2/4 (Fig. 8D).

PTH administration has previously been shown to have osteogenic effect in gonad intact mice [24,46]. We observed lack of osteogenic response of PTH in ovary intact skeletally mature *KO* mice as trabecular bone volume (BV/TV %) at distal femur and periosteal cortical thickness at femur diaphysis were significantly increased by PTH in control but not in *KO* mice (Fig. 8E, F), which further confirmed that the bone anabolic action of PTH in vivo required Bmp2/4.

Table 4
L5 parameters of female mice (10–12 weeks).

	Cnt	*KO*
µCT parameters		
vBMD (gHA/cm^3)	0.7 ± 0.01	0.6 ± 0.02*
BV/TV (%)	21.1 ± 0.9	15.4 ± 1.1**
Tb.N (1/mm)	4.5 ± 0.3	3.4 ± 0.1**
Tb.Th (mm)	0.05 ± 0.003	0.04 ± 0.003*
Histomorphometry parameters		
BV/TV (%)	0.2 ± 0.002	0.1 ± 0.02*
OV/BV	0.03 ± 0.003	0.007 ± 0.002**
O.Wi (µm)	9.0 ± 0.6	6.8 ± 0.1*
Compression parameters		
Energy (mJ)	5.4 ± 0.3	3.8 ± 0.3**
Stiffness (N/mm)	154.2 ± 8.6	121.4 ± 3.9**

Data presented as mean ± SEM; n = 6 for µCT and n = 3 for other parameters.
** P < 0.01 compared to control (Cnt).
* P < 0.05 compared to control (Cnt).

3.3. PTH stimulates Bmp2/4 synthesis and elicits BMP signaling in osteoblasts

As our data showed that the osteogenic action of PTH was dependent on the presence of Bmp2 and Bmp4, we surmised that PTH could trigger BMP signaling in osteoblasts by stimulating Bmp2/4 production. Thus, we treated TVA-BMSCs with PTH or E2 and measured *Bmp2* and *Bmp4* transcript levels. As shown in Fig. 9A, mRNA levels of *Bmp2* and *Bmp4* were increased by PTH but not E2. We next treated TVA-BMSC line with PTH or E2 and measured Bmp2 and Bmp4 proteins and immunoblot data showed that both were increased by PTH and not E2 (Fig. 9B). In addition, abundance of BMP2 and BMP4 were increased in the conditioned medium by PTH and not E2 (Fig. 9C), suggesting PTH

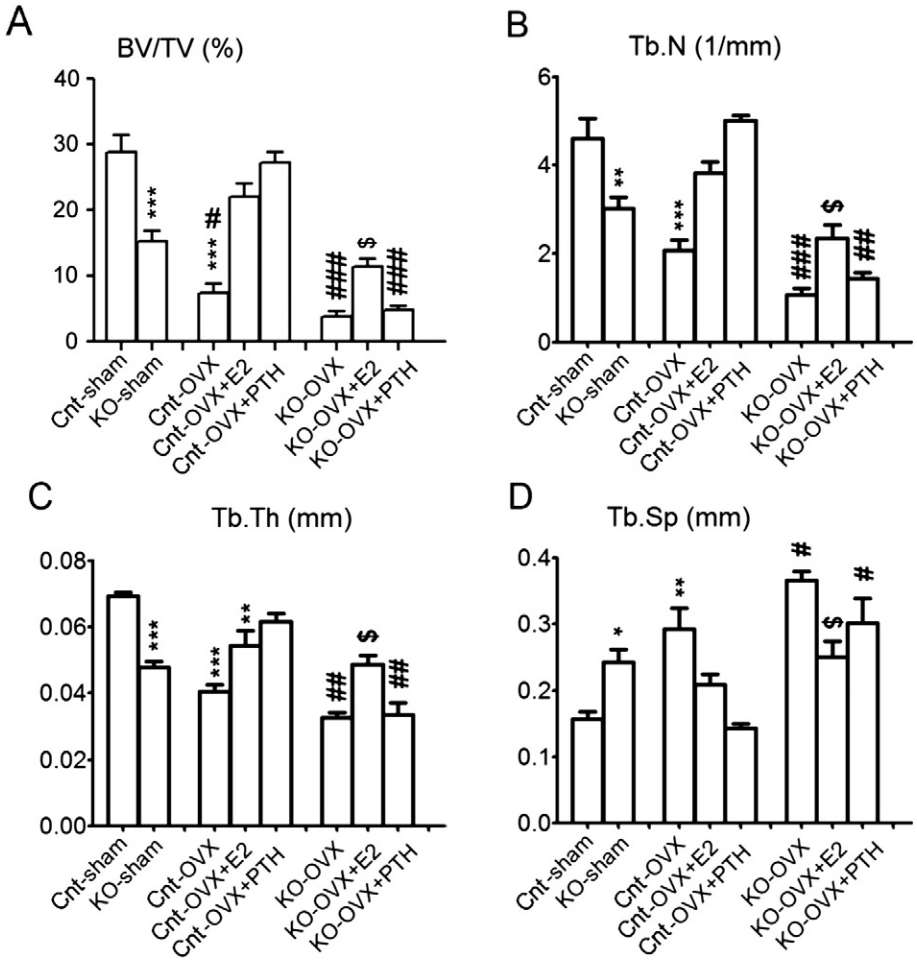

Fig. 7. *Bmp2,4* are required for trabecular effects of PTH but not E2 in OVX mice. All mice were 10–12 weeks old and control (Cnt) and *KO* were obtained as described in Fig. 4. 3-D μCT assessment of distal femur in various groups. (A) BV/TV, (B) Tb·N and (C) Tb·Th showing complete preservation by PTH in control-OVX but not in *KO*-OVX, whereas E2 was effective in both groups. (D) OVX-induced increase in Tb·Sp was maintained in control-OVX but not *KO*-OVX mice when treated with PTH whereas E2 maintained this parameter in both groups. Data are mean ± SEM, n = 6 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control-sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. *KO*-sham; $P < 0.05 vs. *KO*-OVX.

treated TVA-BMSCs secreted more BMP2/4. Next, we assessed the levels of p-Smad1/5, as a measure of canonical BMP signaling in TVA-BMSC line treated with PTH or E2. PTH but not E2 treatment enhanced p-Smad1/5 in TVA-BMSCs (Fig. 9D). The stimulation of canonical BMP signaling by PTH was blocked by noggin (Fig. 9E), a secreted inhibitor of BMP signaling [47]. Taken together, our data demonstrate that PTH stimulates transcription of *Bmp2/4* which in turn stimulates canonical BMP signaling in osteoblasts.

4. Discussion

BMP signaling has critical roles in embryonic development of cartilage [48] and bone [49]. Importance of BMP signaling in bone formation is further supported by the observations that overexpressing BMP antagonists such as noggin [50] or gremlin [51] and deleting gremlin [52] at the embryonic stage in mice resulted in loss and gain of bone formation, respectively. However, what has not been investigated thus far is whether this signaling pathway continues to play an important role in adult skeleton. Mice with limb specific embryonic deletion of *Bmp2* are born with apparently normal skeleton but suffer loss of BMD and spontaneous limb bone fracture in the adult life [6]. In addition, it appears that deletion of *Bmp4* alone at the embryonic stage does not alter adult skeleton [13]. We observed that deletion of *Bmp2* allele in adult mice caused no skeletal phenotype. However, when both *Bmp2* and *Bmp4* alleles were deleted in skeletally mature mice, we observed significant osteopenia suggesting that BMP signaling has a critical role in adult bone homeostasis. $Bmp2^{c/c}$; *Prx1-Cre* mice were compromised in fracture healing [6] while in our case some bone regenerative response following bone injury was observed in *KO* mice, albeit significantly lesser than control mice. This difference in bone injury healing response between these two strains may be attributed to certain subtle defects in $Bmp2^{c/c}$; *Prx1-Cre* mice caused by lack of optimal level of BMP signaling during embryonic development. Our study, taken together with available literature, further reveals that just like in the embryos, absence of either of *Bmp2* or *Bmp4* gene alone does not affect adult skeleton [13, 53] but when deleted together, results in the development of osteopenia reflected by compromised trabecular integrity and reduced vertebral compression strength.

Static histomorphometric analyses (GT staining and μCT) showed decreased osteoid volume, osteoid width and trabecular width in *KO* compared with control, and from dynamic histomorphometry MS/BS, MAR and BFR/BS were decreased in *KO* compared with control, suggesting osteoblast function was impaired in *KO* mice. MS/BS is often considered a determinant of osteoblast activity such that an intervention that would affect osteoblast proliferation and/or differentiation is expected to change this parameter. In our case, MS/BS was reduced in *KO* which suggested reduction in both osteoblast number and/or differentiation. We also observed that osteoblast differentiation of BMSC was decreased in *KO*. Further, because MAR represents osteoblast vigour, a decreased MAR in *KO* therefore, indicated impaired osteoblast function, which appeared to have contributed in the development of osteopenia in *KO* mice. As to the development of osteopenia, a diminished osteoblast

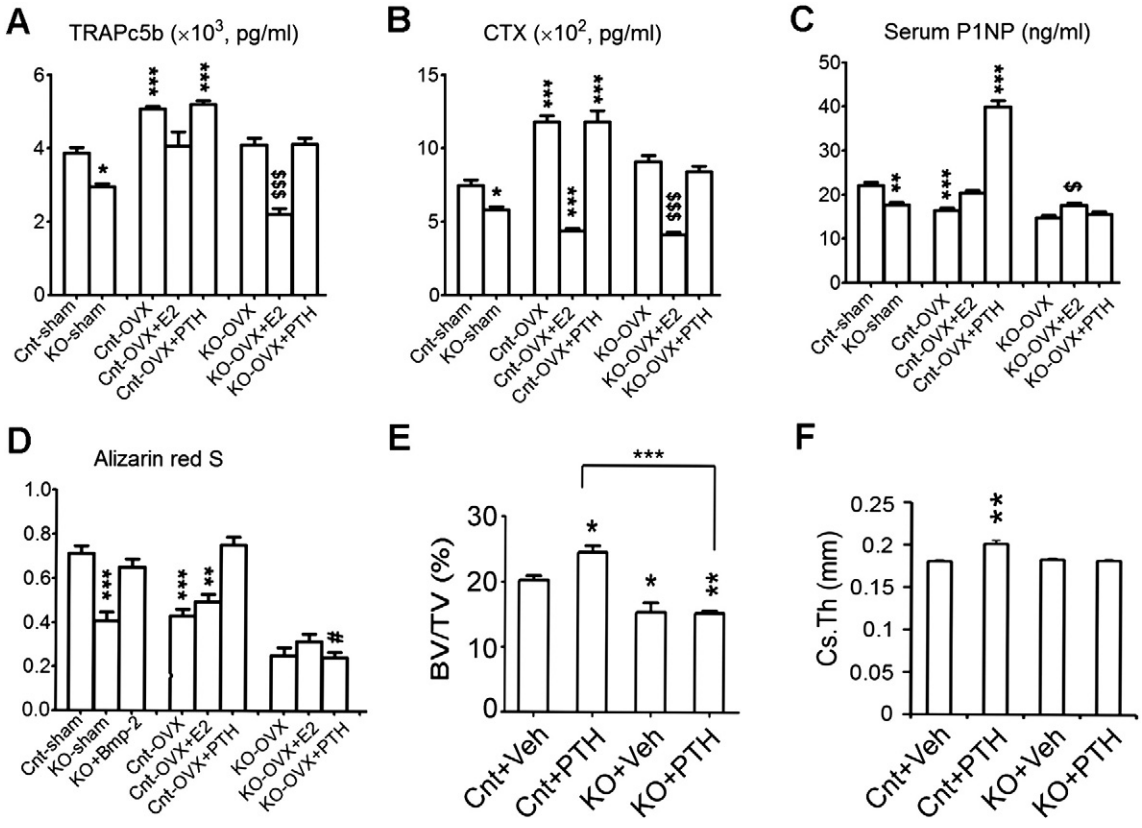

Fig. 8. *Bmp2/4* are required for osteogenic response of PTH. Serum levels of (A) TRAPc5b, (B) CTX-1 and (C) P1NP in various groups are shown. E2 strongly suppressed OVX-induced increase in both TRAPc5b and CTX-1 but PTH did not. P1NP was robustly increased by PTH in control-OVX but not in *KO*-OVX. (D) Ex vivo nodule formation from BMSC cultures of indicated groups of mice by alizarin red S staining. BMSC from control-OVX treated with PTH displayed robust increase in nodule formation but that from *KO*-OVX + PTH mice failed; n = 6 mice/genotype. (E) μCT measurements of distal femoral BV/TV % and (F) femur diaphyseal cortical thickness (Cs.Th) showed osteogenic response of PTH in skeletally mature ovary intact mice (Cnt) but not in *KO* mice. Data are represented as mean ± SEM, n = 6 mice/group, $^{*}P < 0.05$, $^{**}P < 0.01$and $^{***}P < 0.001$ versus Cnt-sham; $^{\#}P < 0.05$ versus *KO*-sham; $^{\$}P < 0.05$ and $^{\$\$\$}P < 0.001$ versus *KO*-OVX.

number might have contributed as well in *KO* mice which however, have not been measured.

Osteoclasts express BMP receptors [16,54–56], and several reports demonstrate that BMPs directly regulate the formation and activity of osteoclasts [16,54,57]. Moreover, noggin, a BMP antagonist has been shown to inhibit murine osteoclast formation [58]. Our data show that in *KO* mice not only were the osteoblast functions impaired but also the formation and function of osteoclasts were impacted negatively, thus provided evidence for the first time that Bmp2/4 were required for osteoclast function in vivo. In addition, given that RANKL-to-OPG ratio in bones of *KO* and control mice were similar it therefore, appeared that Bmp2/4 deletion affected osteoclast function without compromising osteoblast support system. Overall impact of the impairments in osteoclast and osteoblast functions in *KO* mice was bone loss, specifically the trabecular bones. Parameters of Ca^{2+} homeostasis, uterine weight (a surrogate of E2 function) and serum IGF-1 were not different between control and *KO*, which suggested that the osteopenia due to *Bmp2/4* deletion was not a secondary outcome of the alterations in these important physiological regulators of bone.

We next investigated the effect of depletion of endogenous Bmp2/4 in the pathogenesis and treatment of postmenopausal bone loss. Our data showed that E2 deficiency was a stronger stimulus for trabecular osteopenia than *Bmp2/4* deletion as all the skeletal parameters in control-OVX were worse than the ovary intact *KO* mice. In addition, trabecular deterioration was greater in *KO*-OVX compared with *KO*-sham, likely produced by an additive effect of two negative stimuli (E2 deficiency and *Bmp2/4* deletion) and suggested that the mechanisms for osteopenia by these two stimuli were different. Moreover, although osteoclast function was suppressed in *KO* mice, OVX of *KO* mice increased serum surrogates of osteoclast number and activity (TRAPc5 and CTX), reiterating the presence of different mechanisms by which E2 and Bmp2/4 regulate skeletal homeostasis in adults.

Indeed, trabecular bones were preserved and resorption markers were suppressed in *KO*-OVX mice treated with E2 compared with vehicle treated *KO*-OVX, suggesting a significant skeletal preservation being achieved by E2 through its recognized anti-catabolic action. Primarily based on in vitro analyses, E2 and estrogenic compounds (phytoestrogens) have been widely shown to increase Bmp2 and/or Bmp4 production in MSC and osteoblastic cells [59–61]. However, our finding that the effect of E2 in adult skeleton is independent of Bmp2/4 raises uncertainty regarding the in vivo significance of the reported in vitro stimulatory effect of E2 on Bmp2/4. By contrast, PTH maintained trabecular bones in control-OVX to the level of control-sham but failed to preserve bones in *KO*-OVX, which suggested that the bone anabolic action of PTH in vivo was dependent on the presence of Bmp2/4. In vitro, PTH has been shown to augment *Bmp2* expression by CREB transactivation in osteoblasts [42] which could explain the observed lack of bone anabolic effect of PTH in *KO* mice. In osteoblasts (TVA-BMSC), we observed that PTH but not E2 stimulated the synthesis and consequently the secretion of both Bmp2 and Bmp4. Also, our data demonstrates that PTH stimulates canonical BMP signaling in osteoblasts. Thus our in vivo and in vitro data taken together with existing literature [42,62] strongly suggest that the bone anabolic function of PTH is mediated by its ability to stimulate *Bmp2* and *Bmp4* transcription in osteoblasts/osteprogenitor cells. This, if holds true in humans as well, it will necessitate assessment of Bmp2/4 status in osteoporotic patients before prescribing PTH therapy as there is no predictor of teriparatide (PTH) treatment failures in patients with low bone mass [63].

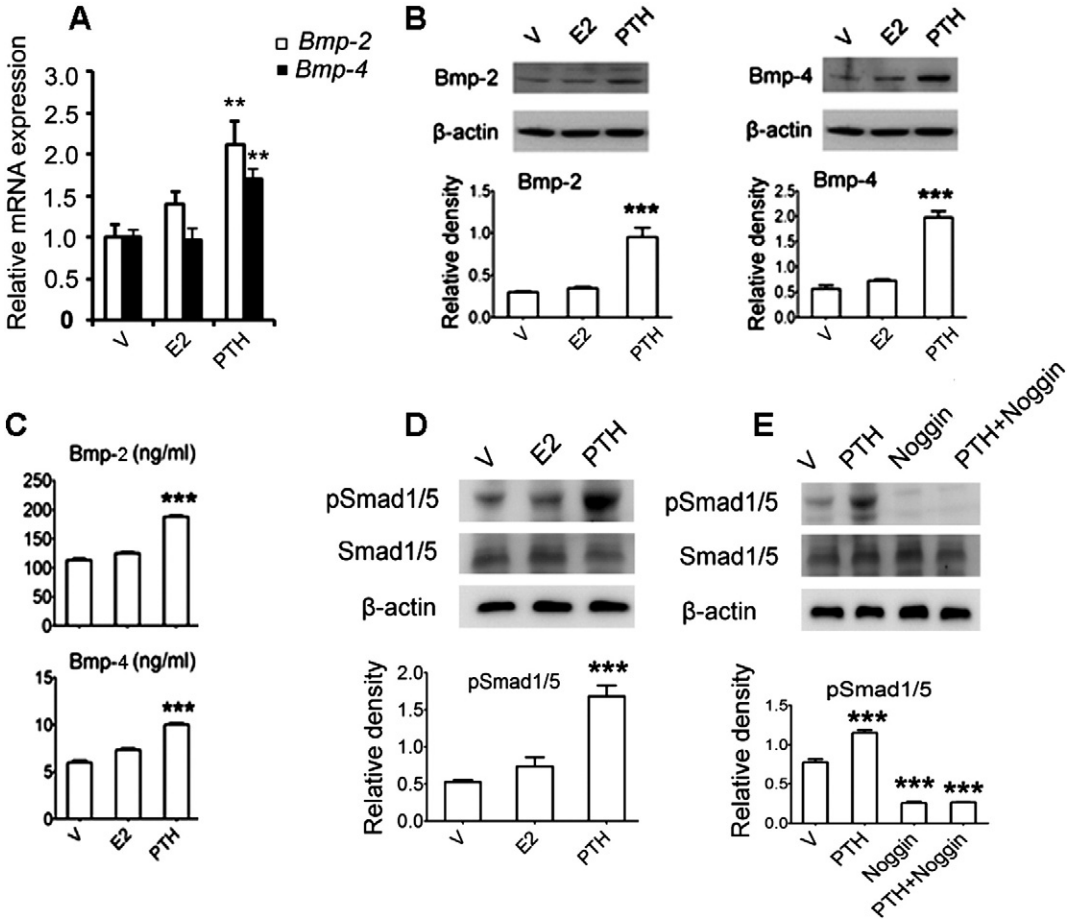

Fig. 9. Activation of BMP signaling in osteoblasts by PTH but not E2 via increased production of Bmp2/4. (A) qPCR analysis show increased mRNA levels of *Bmp2* and *Bmp4* in TVA-BMSCs by PTH but not E2 treatment. (B) Immunoblot data showing increased BMP-2 and BMP-4 in the lysates of TVA-BMSC line treated with PTH but not E2. (C) Determination of Bmp2 and Bmp4 by ELISA from the conditioned media of TVA-BMSC line after 48 h of indicated treatments. (D) Immunoblot data showing increased p-Smad1/5 by PTH but not E2. Lysates were made from TVA-BMSCs following various treatments for 48 h. (E) Immunoblot data showing abrogation PTH-induced p-Smad1/5 in TVA-BMSCs by noggin (inhibitor of Bmp-2 and Bmp-4). Lysates made from TVA-BMSCs after indicated treatments for 48 h. β-Actin was used for normalization in relative density plots. Data are mean ± SEM of 3 independent experiments done in triplicate; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus V, vehicle (control).

We have a few caveats in the study. Firstly, we have not tested whether the deletion of BMP receptor 1, which transduces intracellular Bmp2,4 signaling had a similar skeletal effect as deletion of its ligands in adult mice and involved in mediating the osteoanabolic action of PTH. Second, because live animal scanning showed appearance of a significant trabecular but not cortical bone loss at 6 wk post-tamoxifen treatment, we killed them at that time point. Whether the cortical bones are impacted by *Bmp2/4* deletion require prolonging the skeletal assessment of mice beyond 6 wk post-tamoxifen treatment. Third, in the bone regeneration study following drill-hole injury, we have not assessed whether growth factor supply from hematoma and chondrogenic activity that precede formation of mineralized bone were perturbed. Fourth, the assessment of gonadal function in *KO* mice was based only on uterine weight, and not on gonadal hormone levels or more appropriately, their ability to reproduce. Fifth, we have not studied whether PTH could activate non-canonical BMP signaling such as MAP kinases in osteoblasts. Finally, we have not studied whether IGF-1 and TGF-β signaling pathways, the reported mediators of the osteogenic action of PTH [37,39,64,65], were disturbed in the osteoblasts of *KO* mice.

In conclusion, this study strongly supports the key role of BMP2/4 in adult skeletal homeostasis and demonstrates that the osteoanabolic effect of PTH requires BMP2/4 while the skeletal action of E2 is independent of these two BMPs.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

Study design: NC, AB and SS. Study conduct: MPK, PSY, PP, KK, AKS, MCT, GKN, DT, AN. Data analysis: MPK, KK and AKS. Histomorphometry: SPC and MM. Data interpretation: NC, AB, SS and AKT. Drafting manuscript: NC, AB, SS, MPK and KK. Approving final version of manuscript: NC and AB. MPK take responsibility for the integrity of data.

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