



# HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2020 March 01.

Published in final edited form as:

*Bone*. 2019 March ; 120: 327–335. doi:10.1016/j.bone.2018.11.014.

## SHP2 regulates intramembranous ossification by modifying the TGF $\beta$ and BMP2 signaling pathway

Lijun Wang, Jiahui Huang, Douglas C. Moore, Yueming Song, Michael G. Ehrlich, and Wentian Yang

Department of Orthopaedics, Brown University Alpert Medical School and Rhode Island Hospital, Providence, RI 02903

### Abstract

SHP2 is a ubiquitously expressed protein tyrosine phosphatase, which is involved in many signaling pathways to regulate the skeletal development. In endochondral ossification, SHP2 is known to modify the osteogenic fate of osteochondroprogenitors and to impair the osteoblastic transdifferentiation of hypertrophic chondrocytes. However, how SHP2 regulates osteoblast differentiation in intramembranous ossification remains incompletely understood. To address this question, we generated a mouse model to ablate SHP2 in the *Prrx1*-expressing mesenchymal progenitors by using “Cre-loxP”-mediated gene excision and examined the development of calvarial bone, in which the main process of bone formation is intramembranous ossification. Phenotypic characterization showed that SHP2 mutants have severe defects in calvarial bone formation. Cell lineage tracing and *in situ* hybridization data showed less osteoblast differentiation of mesenchymal cells and reduced osteogenic genes expression, respectively. Further mechanistic studies revealed enhanced TGF $\beta$  and suppressed BMP2 signaling in SHP2 ablated mesenchymal progenitors and their derivatives. Our study uncovered the critical role of SHP2 in osteoblast differentiation through intramembranous ossification and might provide a potential target to treat craniofacial skeleton disorders.

### Keywords

SHP2; intramembranous ossification; mesenchymal progenitors; osteoblast differentiation; TGF $\beta$  and BMP2 signaling

---

\*Corresponding Author: Wentian Yang, M.D., Ph.D. 1 Hoppin Street, Coro 402E Providence, RI 02903, 401-4445956 (Phone), wyang@lifespan.org.

Author Contributions

LW, JH, DCM and WY contributed to data collection; DCM conducted  $\mu$ CT data collection and analysis; YS and MGE contributed important intellectual content; LW, DCM and WY wrote the manuscript. All authors read and approve the final version of this manuscript.

**Disclosures:** All authors state that they have no conflicts of interest.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## 1. Introduction

The skull vault of mammals, providing essential protection of the brain and important sensory organs, consists of the frontal, parietal, inter-parietal and occipital bones, which all undergo intramembranous ossification[1, 2]. During development, the cell lineage analysis showed that the frontal bones are mainly contributed from the neural crest cells, while parietal and inter-parietal bones originate from the paraxial mesoderm[3–5]. These cells migrate into defined locations overlying the cerebral hemispheres, and subsequently differentiate into osteogenic mesenchyme, which is characterized by the expression of Runt-related transcription factor 2(RUNX2) and OSTERIX, the earliest molecular determinants of bone formation[6–9]. In contrast to the endochondral ossification that ensues from a cartilaginous anlagen, intramembranous ossification features the direct differentiation of mesenchymal progenitors into osteoblasts, responsible for the synthesis of collagen type 1(COL1 $\alpha$ 1)-rich bone extracellular matrix and its mineralization.

Protein tyrosine phosphatase non-receptor type 11(*PTPN11*), also known as SHP2, is a widely expressed SH2 domain-containing non-receptor protein tyrosine phosphatase. Accumulating evidences suggest a crucial role for SHP2 in regulation of skeletal development and homeostasis. SHP2 gain-of-function (GOF) mutations cause Noonan Syndrome (NS), which has calvarial abnormality including macrocephaly, oral malformation and hypertelorism[10–12]. Conversely, SHP2 loss-of-function(LOF) mutation cause benign cartilage tumor metachondromatosis both in human and in mice[13, 14]. Recently, we reported that SHP2 determines the cell fate of osteochondroprogenitors[15] and its specific deletion in collagen type 2(COL2a1) or collagen type 10(COL10a1) expressing chondrocytes impairs the osteoblast transdifferentiation of hypertrophic chondrocyte in endochondral ossification, which indicates SHP2 plays a crucial role in osteoblast differentiation[16]. SHP2 ablation in mesenchymal progenitors cause defects of calvarial bone formation[17], but the cellular and molecular mechanism how SHP2 regulates osteoblast differentiation and bone formation in calvarial development is not fully understood.

Calvarial development is a complex process consisting both endochondral ossification and intramembranous ossification. Here, we specifically delete SHP2 in mesenchymal progenitors through *Prrx1-Cre* to investigate the role of SHP2 in the development of parietal and inter-parietal bone, which undergo intramembranous ossification. During the early phases of the parietal and inter-parietal bone formation, mesenchymal progenitors first undergo proliferation and differentiation, leading to the formation of several bony anlagen margined by the osteogenic fronts and connected by the fibrous connective tissue sutures, which are rich in mesenchymal progenitors and fibroblasts. After acquiring the basic shape, each of the skull bony elements remains separated by sutures to ensures that calvarial expansion is coordinated with growth of the underlying brain. Synchronized with the osteoblast-mediated bone formation, calvarial bones also undergo dynamic modeling and remodeling through the coordinated resorptive activity of osteoclasts. These developmental processes at last establish the three-dimensional architecture of the skull.

Lines of genetic and molecular evidence suggest multiple signaling pathways and transcription factors as being important regulators of skull development, such as the signaling pathways evoked by BMP/TGF $\beta$ [18], FGFs[19], WNT[20] and hedgehog [21] et al. Among these signaling pathways, transforming growth factor beta (TGF $\beta$ ) and bone morphogenic protein2 (BMP2) are considered as one of the most important signaling pathways to regulate osteogenesis[18, 22], which are both the TGF $\beta$  superfamily members. TGF $\beta$  ligand binds to TGF $\beta$  receptor complex (TGF $\beta$ R I & TGF $\beta$ R II) and then phosphorylate SMAD2/3. Similarly, BMP2 ligand binds to BMP receptor complex (BMPRI & BMPRII) and then phosphorylate SMAD1/5/8 [23, 24], respectively. TGF $\beta$  signaling was demonstrated to promote proliferation and early differentiation of osteoprogenitor, however, it inhibits the maturation, mineralization and transit of osteoblast to osteocyte[18]. In addition, TGF $\beta$  treatment blocks osteoblast mineralization in vitro[25]. In contrast, BMP2 signaling has been proved to promote osteogenic differentiation of mesenchymal progenitors and bone formation [22, 24]. Furthermore, both TGF $\beta$  signaling and BMP2 signaling are reported to regulate osteoblast differentiation by modifying the activity of RUNX2 and OSTERIX[18, 26–28], the key osteogenic transcription factors regulating the expression of osteogenic gene programs[29].

In this study, we research on the role of SHP2 in intramembranous ossification of calvarial development at cellular and molecular level. And we demonstrate that SHP2 is critical for osteogenic differentiation of mesenchymal progenitors partially via modifying TGF $\beta$ /BMP2 signaling.

## 2. Materials and Methods

### 2.1 Animals

*Tg(Ptpn11<sup>fl/+</sup>)*[30], *Tg(Rosa26<sup>ZsG</sup>)*[31], *Tg(Prrx1-Cre)*[32] and *Tg(Sp7-mCherry)* (*Sp7<sup>mCherry</sup>*)[33] mice were described previously. PCR genotyping methods for the *Ptpn11* floxed allele, *Rosa26<sup>ZsG</sup>* and *Sp7<sup>mCherry</sup>* reporters, and Cre transgene are reported in the original publications and are available upon request. Mice bearing *Ptpn11* floxed alleles were bred to *Tg(Prrx1-Cre)* mice to generate offspring in which SHP2 was deleted in osteochondroprogenitors (OCPs) that express the paired related homeobox-1 protein PRRX1, and controls. The mice had the following genotypes and nomenclature: *Tg(Prrx1-Cre;Ptpn11<sup>fl/+</sup>)* (SHP2<sub>PRRX1</sub>CTR) and *Tg(Prrx1-Cre;Ptpn11<sup>fl/fl</sup>)* (SHP2<sub>PRRX1</sub>KO). SHP2<sub>PRRX1</sub>CTR and SHP2<sub>PRRX1</sub>KO mice were also bred with *Rosa26<sup>ZsG</sup>* and *Sp7<sup>mCherry</sup>* reporter mice for experiments designed to trace the fate of *Prrx1*-expressing cells *in vivo*. All of the mice were maintained on the C57BL/6J background.

Control and SHP2 mutant animals were sacrificed at the indicated time points and explanted skull tissue was processed for microcomputed tomographic imaging ( $\mu$ CT), histological sectioning, and biochemical and biological analyses. All animal work was reviewed and approved by the Rhode Island Hospital Institutional Animal Care and Use Committee and performed in accordance with PHS policy on the humane care and use of laboratory animals.

## 2.2 Osteoblast isolation and cultures

Primary osteoblastic cells were isolated from calvarias of the SHP2<sup>P<sub>TRX1</sub>CT</sup>R;Rosa26<sup>Z<sup>SG</sup></sup> and SHP2<sup>P<sub>TRX1</sub>KO</sup>;Rosa26<sup>Z<sup>SG</sup></sup> newborns. To do so, the parietal bones were collected under sterile condition and digested with 5 ml of Trypsin-EDTA (0.06%), and Collagenase II (285U/ml) for 4 hours. The digestion was halted by adding an equal volume of DMEM with 10% FBS, and the digestion solution was passed through a 40  $\mu$ m strainer to obtain single cells. Osteoblasts were collected by centrifugation and resuspended in fresh culture medium (DMEM with 10% FBS, 1% penicillin and streptomycin). Osteoblast cell preparations were cultured at 37° C under 5% CO<sub>2</sub>.

Primary osteoblasts were immortalized with retrovirus expressing pBabe(puro)/SV40 large T antigen prepared from 293T cells, as described previously [16], [30, 34]. Infected cells were cultured for 48 hours and then selected with puromycin for 7 days, after which the puromycin-resistant clones were pooled and expanded. Finally, the osteoblastic cells were enriched by FACS sorting based on the Rosa26<sup>Z<sup>SG</sup></sup> GFP fluorophore.

## 2.3 Reagents

Polyclonal antibodies against murine SHP2 (sc-280) and SMAD1 (sc-7965) were purchased from Santa Cruz Biotechnology, and monoclonal anti-Sp7/OSTERIX (ab22552) was purchased from Abcam. Monoclonal antibodies against murine phospho-SMAD2/3 (8828S) and total SMAD2/3 (3102S) were purchased from Cell Signaling Technology, p-SMAD1/5/8 (AB3848) was purchased from Millipore, and anti- $\beta$ -ACTIN (ab8226) was purchased from Abcam. Texas Red-X goat anti-rabbit IgG (T6391) was purchased from Invitrogen. 1% Alcian blue and 1% Alizarin Red staining solutions were purchased from Poly Scientific. Click-iT EdU Alexa Fluor 594 Imaging Kit (C10339) is purchased from Life Technology.

## 2.4 Histological, histochemical and immunostaining analysis

Skulls from P0.5 control and SHP2 mutants were fixed in 4% formaldehyde, soaked in 30% sucrose solution for 24 hours, and embedded in OCT. Frozen sections were stained with hematoxylin and eosin (H&E) and von Kossa (Millipore) to visualize general morphology and bone matrix mineralization, respectively. The fate of *P<sub>TRX1</sub>*-expressing mesenchymal cells *in vivo* was determined by direct visualization of the ZsGreen+ (green), mCherry+ (red), and ZsGreen/mCherry double positive (yellow) fluorophores on frozen sections of parietal bone from SHP2<sup>P<sub>TRX1</sub>CT</sup>R;Rosa26<sup>Z<sup>SG</sup></sup>;Sp7<sup>mCherry</sup> and SHP2<sup>P<sub>TRX1</sub>KO</sup>;Rosa26<sup>Z<sup>SG</sup></sup>;Sp7<sup>mCherry</sup> mice. DAPI was used for nucleus counterstaining. All fluorescent and phase contrast images were captured using a Nikon digital fluorescence microscope and Aperio slide scanner (Leica Biosystems).

For Immunofluorescent staining, frozen sections were blocked with 0.3% Triton X-100, and 10% goat serum in PBS for 2 hours at room temperature, after which they were incubated with primary antibody overnight (1:100 dilution). After washing, the primary antibodies were localized with Texas Red-X goat anti-rabbit IgG secondary antibody. Nuclei were counter-stained with DAPI.

## 2.5 *In situ* hybridization

mRNA expression was determined using RNAscope *in situ* hybridization, as described previously[16]. Probes against murine *Runx2*, *Sp7*, *Ctnnb1*, *Bmp2*, *Ibsp* and *Bglap* were synthesized and purchased through Advanced Cell Diagnostics (ACD). Visualization of the hybridized probes was achieved using the RNAscope® HD-Brown kit (Advanced Cell Diagnostics). Images of each section were acquired using the Aperio slide scanner.

Osteogenic gene expression was quantified on corresponding 200µm x 100µm areas in the same regions of the parietal bones from SHP2<sub>Prrx1</sub>CTR and SHP2<sub>Prrx1</sub>KO mice. The positively-stained area and the total bone tissue area were quantified using NIH ImageJ software.

## 2.6 Quantitative RT-PCR and micro-CT analyses

Total RNA was extracted from cultured osteoblasts using the RNeasy kit (Qiagen). cDNA was synthesized using 1 µg of total RNA with iScript™cDNA Synthesis Kit (Bio-Rad) and qRT-PCR was performed with RT<sup>2</sup>SYBR®Green kit on a Bio-Rad CFX machine. All samples were normalized to *Gapdh* and *Actin*, and gene expression was presented as fold increases or decreases compared with that of control. High resolution (20 µm isometric voxel size) 3D volume images were generated using a desktop µ-CT40 system (Scanco Medical AG, CH).

## 2.8 Western blot analysis

Cells were lysed in modified NP-40 lysis buffer (0.5% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris [pH 7.4]) supplemented with a protease inhibitor cocktail (1 mM PMSF, 10 mg/ml aprotinin, 0.5 mg/ml antipain, and 0.5 mg/ml pepstatin)[35]. Cell lysates (20–30 µg) were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with indicated primary antibodies for 2 hours or overnight at 4°C (primary antibody concentration is 1µg/ml); this was followed by HRP-conjugated secondary antibodies (Bio-Rad, 1:2000 dilution).

## 2.9 Statistical analysis

Differences between the SHP2 knockouts and controls were evaluated using Student's *t* tests, with *p* values <0.05 considered to be significant. Statistical analyses were performed by using Prism 5.0 (GraphPad, San Diego, CA) and Excel (Microsoft).

## 3. Results

### 3.1 SHP2 deletion in the *Prrx1*-expressing mesenchymal cells impairs calvarial bone ossification.

SHP2<sub>Prrx1</sub>KO mutants were born at the expected Mendelian ratios, but they were smaller than their control littermates[15]. Most of the SHP2 mutants died within 3 weeks after birth, likely due to difficulty with breathing and feeding, but a few of the mutant mice could survive until 6 weeks[15]. Compared with the SHP2<sub>Prrx1</sub>CTR mice, the skulls of SHP2<sub>Prrx1</sub>KO mice were incompletely formed. Alcian blue and Alizarin Red staining revealed marked defects in mineralization of the parietal, interparietal, and occipital bones

(Fig. 1A), as well as the tibiae, femur and humerus, which are of endochondral origin[15]. These findings were even more striking in the data from the  $\mu$ CT imaging, where the parietal and interparietal bones were totally absent in the models created from the thresholded  $\mu$ CT images of 6-week-old SHP2<sub>Prrx1</sub>KO mice (Fig. 1B, bottom), while the skulls of the SHP2<sub>Prrx1</sub>CTR mice appear entirely normal, with intact cranial vaults and well-defined sutures (Fig. 1B, top).

Histological analysis of stained frozen sections was performed to evaluate the microarchitecture of the skull elements. H&E and von Kossa staining of the sections from P0.5 SHP2<sub>Prrx1</sub>CTR mice revealed a sandwich-like morphology in which the fully mineralized parietal bone was covered by two layers of soft periosteal mesenchymal tissue (Fig. 1C left). In contrast, mineralization of the parietal bone was markedly reduced in P0.5 SHP2<sub>Prrx1</sub>KO mutants as the von Kossa staining image showed (Fig. 1C right). Instead, there was a dense layer of soft connective tissue filled with undifferentiated mesenchymal cells where the parietal bone should have been. Quantitative analysis revealed a ~70% reduction in the portion of the parietal “bone” tissue that was mineralized in the SHP2<sub>Prrx1</sub>KO mice compared to corresponding regions of SHP2<sub>Prrx1</sub>CTR mice ( $19.8 \pm 5.7\%$  vs  $63.3 \pm 0.6\%$ ,  $**p < 0.01$ ) (Fig. 1D). And finally, calcified osteogenic fronts appeared clearly in the SHP2<sub>Prrx1</sub>CTR mice but were absent in the SHP2<sub>Prrx1</sub>KO mice (Fig S1). Taken together, these data indicate that SHP2 expression in the *Prrx1*-expressing OCPs is essential for the mineralization of calvarial bones.

### 3.2 SHP2 is required for the osteogenic differentiation of the *Prrx1*+ mesenchymal progenitors.

Having established that lack of SHP2 in *Prrx1*-expressing OCPs inhibits intramembraneous ossification, we next sought to determine whether the cause might be lack of OCP differentiation. To start, we performed a dual fluorescent reporter-based cell lineage tracing study. SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> and SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup> mice were bred with *Sp7*<sup>mCherry</sup> reporter mice, with the idea that the resulting SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> and SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> mice would express ZsGreen in cells that expressed, or had expressed, *Prrx1*+ (i.e. osteochondral progenitors and their progeny), and mCherry in cells that expressed *Sp7* (i.e. osteoblast lineage cells). Importantly, if the *Prrx1*+ ZsGreen cells differentiated into *Sp7*-expressing osteoblastic cells, they would co-express the ZsGreen and mCherry reporters, which would appear yellow. As shown in Fig. 2A (left) & D(top), a large number of cells within the osteogenic fronts and the parietal bones fluoresced yellow in the SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> mice. The yellow cells at the osteogenic fronts were connected by soft tissue bridge rich in ZsGreen+ cells, which indicated they were *Prrx1*+ mesenchymal progenitors. In contrast, the number of yellow cells in the osteogenic fronts and in the parietal bone were significantly reduced in the SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> mutants ( $43.19 \pm 0.37\%$  vs.  $83.24 \pm 4.48\%$ ,  $***p < 0.001$ , Student's t-test) (Fig. 2A & D). Instead, a large quantity of green cells was observed in the SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> mutants. This suggests that the maturation of *Prrx1*+ green cells into osteoblasts was compromised in the absence of SHP2. The above observations were bolstered by immunostaining of the parietal bone frozen sections with the

Texas-red labeled anti-OSTERIX antibodies (SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> vs. SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup>: mean± SEM of percentage: 70.02±1.75 vs. 42.84±5.17, \*\**p*<0.01, Student's t-test) (Fig. 2B & E). Consistent with these observations, the width of sagittal suture significantly increased in the SHP2 mutants, compared to the controls (186.2±28.1µm vs. 293.9±24.9µm, \**p*<0.05, Student's t-test) (Fig. 2C). Taken together, these data suggest that lack of SHP2 in OCPs inhibits their osteogenic differentiation during intramembranous ossification.

### 3.3 SHP2 deletion in the *Prrx1*-expressing mesenchymal progenitors suppresses osteogenic gene expression.

Calvarial bones ossify through intramembranous ossification in which mesenchymal progenitors undergo progressive differentiation into osteoblasts, which express OSTERIX (*Sp7*), COL1a1, bone sialoprotein, and OSTEOCALCIN. RUNX2 has been considered the master gene of osteoblast differentiation[6, 8]. RUNX2 is considered essential for the osteogenic differentiation of the *Prrx1*+ mesenchymal progenitors but it is not required for the terminal differentiation of the *Colla1*+ osteoblasts[36]. Having demonstrated that SHP2 is indispensable for the differentiation of OCPs into functioning osteoblasts, we next explored whether the expression of *Runx2* and other osteogenic genes were affected in these mutants. RNAScope<sup>®</sup>-based in situ hybridization revealed significant reductions in *Runx2*, *Sp7*, *Ctnnb1*, *Bglap*, *Bmp2* and *Ibsp* transcripts in frozen sections of parietal bone from the SHP2<sub>Prrx1</sub>KO mutants, compared with the SHP2<sub>Prrx1</sub>CTR controls (Fig. 3.). These data are consistent with the von Kossa staining data showing an impaired mineralization of the parietal bones (Fig. 1C). Collectively, these data indicate that SHP2 regulates the osteogenic differentiation of OCPs in intramembranous ossification by modulating the expression of the osteogenic transcription factors, as well as downstream effecting osteoblastic genes.

### 3.4 SHP2 is required for osteogenic gene expression in the *Prrx1*+ progenitors and their derivatives

We used cell culture to gain additional insight into the molecular and cellular mechanisms through which SHP2 regulates intramembranous ossification. To do so we isolated ZsGreen + calvarial cells from the SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> and SHP2<sub>Prrx1</sub>KO;R26<sup>ZsG</sup> mice via FACS and immortalized them via the retroviral expression of SV40 large T antigen (Fig. 4A). Phase contrast images showed that immortalized calvarial cells from both control and SHP2 mutant mice display bipolar or multipolar and elongated shapes, and western blotting confirmed that the SHP2 protein level was significantly reduced in the osteoblastic cells from the SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup> mice, compared with SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> control animals (only about 30% of the controls) (Fig. 4B). qRT-PCR revealed significant reductions in the transcript abundance of osteogenic genes *Alp*, *Colla1*, *Ctnnb1*, *Sp7* and *Runx2* in the SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup> cells, compared to the SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> controls (Fig. 4C). ALP activity was also evaluated in the SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup> cells and SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> controls using a colorimetric assay. The number of positively-stained cells and total cells were counted in three fields of view and analyzed. The quantitative data showed that the ALP activity was markedly reduced in the SHP2 mutant cells (mean± SEM of percentage: 38.53± 1.80 vs. 4.00± 0.53, \*\*\**p*<0.001, Student's t-test)

(Fig. 4D). These data *in vitro* confirmed our *in vivo* findings that SHP2 is required for intramembranous ossification by promoting the osteogenic gene expression.

### 3.5 SHP2 regulates osteogenic gene expression by modifying the TGF $\beta$ and BMP2 signaling pathways.

TGF $\beta$  and BMP2 signaling has a fundamental role in regulation of embryonic skeletal development and maintaining postnatal bone homeostasis via the regulation of RUNX2 and osteogenic gene expression[18]. Because our data indicated that impaired osteogenic differentiation of SHP2-deficient OCPs involved reductions in *Runx2* and other osteogenic genes, we sought to investigate whether SHP2 might influence the TGF $\beta$ /BMP2 signaling axis. To do so, calvarial osteoblast cell lines from SHP2<sub>P<sub>TRX1</sub></sub>CTR and SHP2<sub>P<sub>TRX1</sub></sub>KO mice were starved overnight and then harvested 0, 15, 30, 60 and 120, minutes after stimulation with TGF $\beta$  (100ng/ml). Western blotting revealed that the SMAD2/3 phosphorylation was significantly increased in SHP2<sub>P<sub>TRX1</sub></sub>KO cells compared to the SHP2<sub>P<sub>TRX1</sub></sub>CTR control cells (Fig. 5A). Moreover, the phosphorylation level of SMAD2/3 returned to basal levels 120mins after stimulation in the SHP2<sub>P<sub>TRX1</sub></sub>CTR cells, but remained high in SHP2<sub>P<sub>TRX1</sub></sub>KO cells (Fig. 5A). We also starved SHP2<sub>P<sub>TRX1</sub></sub>CTR and SHP2<sub>P<sub>TRX1</sub></sub>KO cells overnight, stimulated them with BMP2 (100ng/ml) and then harvested them at 0, 5, 15, 30 and 60 minutes. The western blot data revealed that the phosphorylation level of SMAD1/5/8 was significantly decreased in SHP2<sub>P<sub>TRX1</sub></sub>KO cells, compared to SHP2<sub>P<sub>TRX1</sub></sub>CTR control cells under the same stimulation (Fig. 5B). These data indicate that SHP2 normally represses the phosphorylation of SMAD2/3 by TGF $\beta$ RI but promotes the phosphorylation of SMAD1/5/8 by BMPRI; these coupled but differential regulation of TGF $\beta$  and BMP2 signaling pathway to ensure the maturation and function of osteoblasts.

## 4. Discussion

Intramembranous ossification is regulated by a number of signaling pathways, in which protein tyrosine kinases are heavily involved [18, 19]. Increasing evidences suggest that protein tyrosine phosphatases play a crucial role in this process, too. SHP2 is a ubiquitously PTP. Early studies showed that SHP2 is a key modulator of the osteogenic differentiation of OCPs[15] and growth plate hypertrophic chondrocytes by modifying SOX9 abundance during endochondral ossification[16]. Here, we showed that SHP2 is indispensable for the osteogenic differentiation of OCPs in the calvarial bones. SHP2 deficient OCPs were halted at the primitive stages and failed to ossify the matrix of the forming calvarial vault (Fig. 1 & 2), rather than a defect in cell proliferation (Fig S3). Consistent with these morphological defects, transcript abundance of osteogenic transcription factors *Runx2* and *Osterix* and their downstream effectors (Fig. 3 & 4) were found significantly decreased in the SHP2 mutants. Importantly, SHP2 was uncovered to differentially regulate canonical BMP2 (promoting) and TGF $\beta$  (suppressing) signaling in OCPs and their derivatives *in vitro* (Fig. 5). Collectively, our study uncovered an important role for SHP2 in intramembranous ossification by modulating the osteogenic differentiation and maturation of OCPs, these actions, in part, are via the differential regulation of BMP and TGF $\beta$  signaling pathways.



BMP and TGF $\beta$  signaling pathways are well accepted as one of the most important signaling pathways to regulate the osteoblast differentiation and maturation. TGF $\beta$  signaling pathway promotes osteoblastic progenitor proliferation and early differentiation, but suppresses the osteoblastic maturation, mineralization and transit to osteocyte[18]. Upon TGF $\beta$  signaling pathway activation, SMAD2/3 is phosphorylated and then recruit class II deacetylases (HDACs 4 and 5) to repress the gene expression and activity of *Runx2* [37–40]. In contrast, activation of the BMP signaling pathway regulates osteoblast differentiation. Activated BMPR I phosphorylates SMAD1/5/8, which up-regulates the expression of *Runx2* and *Osterix* and drive osteoblastic differentiation [37–39]. Our immunoblotting data showed that SHP2 deficiency in osteoblast promoted TGF $\beta$ -evoked SMAD2/3 phosphorylation and suppressed BMP2-evoked SMAD1/5/8 activation (Fig. 5), indicating that SHP2 differentially regulates BMP and TGF $\beta$  signaling pathways and osteoblast differentiation and maturation. This could explain why there was a wider gap between the osteogenic fronts in the sagittal suture of SHP2 mutants and accumulation of primitive Prrx1+ osteochondroprogenitors. *Runx2* and *Osterix* are considered as the two master transcription factors during osteoblastogenesis[26–28]. The profound resemblance of the skull phenotype between SHP2 and RUNX2 mutants suggests that RUNX2 functions downstream SHP2 [36]. This notion was further supported by the reduction of RUNX2 responsive osteogenic gene expression (Fig. 4) in SHP2 mutants [18, 27, 41, 42].

TGF $\beta$  and BMP2 also signal via SMAD independent non-canonical pathways, in which, they activate TAK1 and TAK1-binding protein1(TAB1) to initiate the MKKs and ERK signaling cascade[22]. TGF $\beta$  and BMP2 evoked MKK activation could positively regulate *Runx2* expression and promote the differentiation of OCPs[43]. We therefore examined TGF $\beta$  and BMP2-evoked ERK activation in osteoblasts and found that they were comparable in the presence or absence of SHP2 (Fig. S2), suggesting that SHP2 regulation of BMP and TGF $\beta$  signaling primarily went through the canonical TGF $\beta$ /SMAD2/3 and BMP2/SMAD1/5/8 signaling pathways.

BMP and TGF $\beta$  signal through BMPR I/II and TGFR I/II respectively to phosphorylate SMAD1/5/8 and SMAD2/3 and activate their downstream transcription programs[44]. Altered SMAD phosphorylation in SHP2 deficient OCPs and their derivatives suggest that SHP2 acts either on a serine/threonine kinase upstream SMADs or a SMAD phosphatases. SHP2 functions a PTP, therefore the effect of SHP2 on BMP2- and TGF $\beta$ -evoked SMAD phosphorylation must be mediated via an indirect mechanism. Currently we are investigating whether TGF $\beta$  and BMP2 receptor kinase activity is modulated by phosphorylation of tyrosine residues[45].

In sum, SHP2 is found to be essential for the osteogenic differentiation and maturation of OCPs and intramembranous ossification, partially by differentially modifying the TGF $\beta$  and BMP signaling pathways. SHP2 and its signaling partner(s) may be potential to serve as pharmacological targets to treat craniofacial disorders, such as craniosynostosis and cleidocranial dysostosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This publication was made possible by NIH and the National Institute for General Medicine Sciences (NIGMS) Grant #8P20GM103468 and NIAMS RO1AR066746 (WY). This work was also in part supported by the Rhode Island Hospital Orthopaedic Foundation and Arthritis National Research Foundation (WY). LW is a pilot award recipient from NIGMS1P20 GM119943.

## References

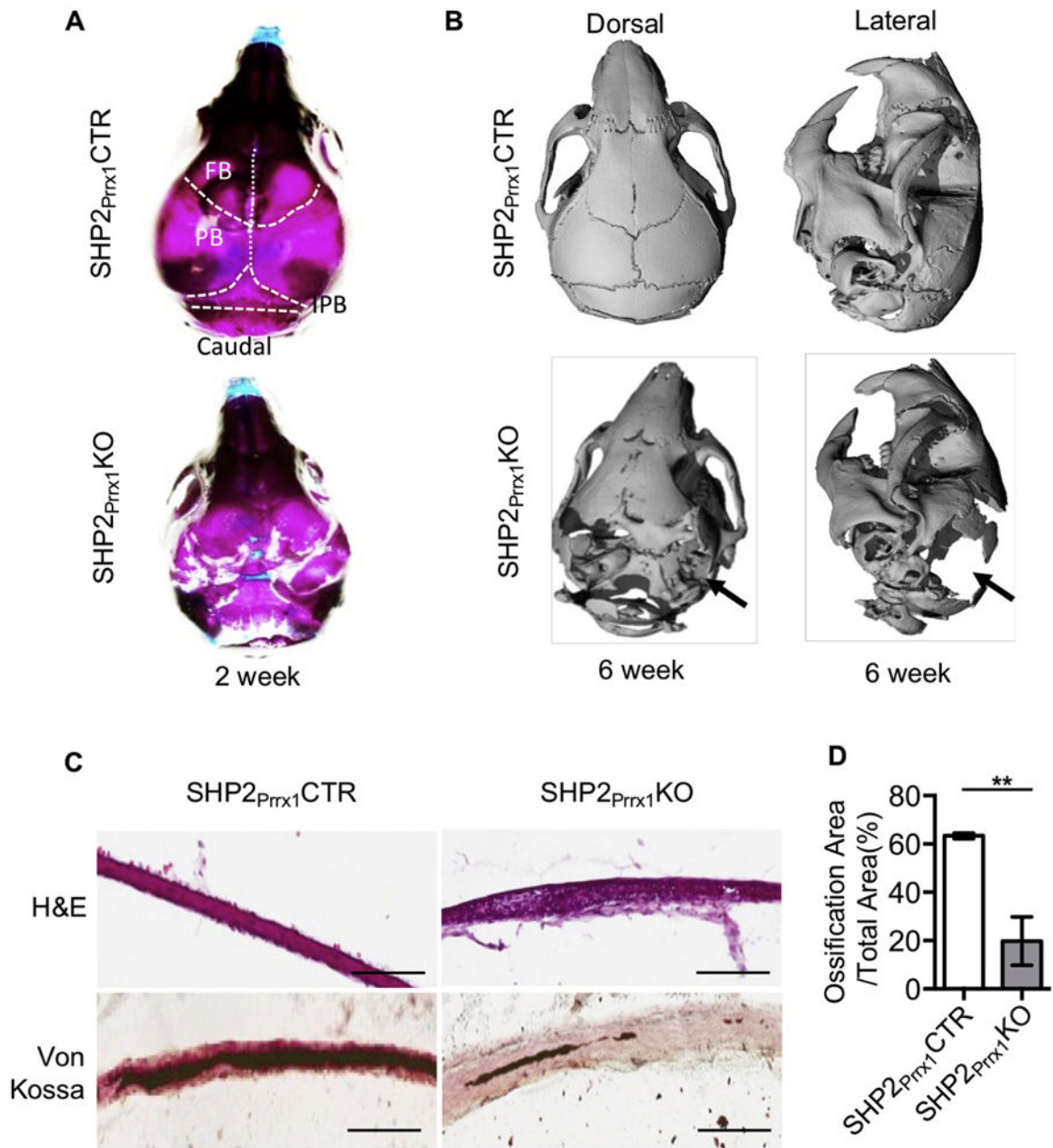
- [1]. Jin SW, Sim KB, Kim SD, Development and Growth of the Normal Cranial Vault : An Embryologic Review, *J Korean Neurosurg Soc* 59(3) (2016) 192–6. [PubMed: 27226848]
- [2]. Ishii M, Sun J, Ting MC, Maxson RE, The Development of the Calvarial Bones and Sutures and the Pathophysiology of Craniosynostosis, *Curr Top Dev Biol* 115 (2015) 131–56. [PubMed: 26589924]
- [3]. Jiang X, Iseki S, Maxson RE, Sucov HM, Morriss-Kay GM, Tissue origins and interactions in the mammalian skull vault, *Dev Biol* 241(1) (2002) 106–16. [PubMed: 11784098]
- [4]. Yoshida T, Vivatbutsi P, Morriss-Kay G, Saga Y, Iseki S, Cell lineage in mammalian craniofacial mesenchyme, *Mech Dev* 125(9–10) (2008) 797–808. [PubMed: 18617001]
- [5]. Deckelbaum RA, Holmes G, Zhao Z, Tong C, Basilico C, Loomis CA, Regulation of cranial morphogenesis and cell fate at the neural crest-mesoderm boundary by engrailed 1, *Development* 139(7) (2012) 1346–58. [PubMed: 22395741]
- [6]. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G, *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation, *Cell* 89(5) (1997) 747–54. [PubMed: 9182762]
- [7]. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrughe B, Nakashima K, Takayanagi H, NFAT and Osterix cooperatively regulate bone formation, *Nat Med* 11(8) (2005) 880–5. [PubMed: 16041384]
- [8]. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T, Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts, *Cell* 89(5) (1997) 755–64. [PubMed: 9182763]
- [9]. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B, The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation, *Cell* 108(1) (2002) 17–29. [PubMed: 11792318]
- [10]. Araki T, Mohi MG, Ismat FA, Bronson RT, Williams IR, Kutok JL, Yang W, Pao LI, Gilliland DG, Epstein JA, Neel BG, Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of *Ptpn11* mutation, *Nat Med* 10(8) (2004) 849–57. [PubMed: 15273746]
- [11]. Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H, van der Burgt I, Crosby AH, Ion A, Jeffery S, Kalidas K, Patton MA, Kucherlapati RS, Gelb BD, Mutations in *PTPN11*, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome, *Nat Genet* 29(4) (2001) 465–8. [PubMed: 11704759]
- [12]. Kosaki K, Suzuki T, Muroya K, Hasegawa T, Sato S, Matsuo N, Kosaki R, Nagai T, Hasegawa Y, Ogata T, *PTPN11* (protein-tyrosine phosphatase, nonreceptor-type 11) mutations in seven Japanese patients with Noonan syndrome, *J Clin Endocrinol Metab* 87(8) (2002) 3529–33. [PubMed: 12161469]
- [13]. Bowen ME, Boyden ED, Holm IA, Campos-Xavier B, Bonafe L, Superti-Furga A, Ikegawa S, Cormier-Daire V, Bovee JV, Pansuriya TC, de Sousa SB, Savarirayan R, Andreucci E, Vikkula M, Garavelli L, Pottinger C, Ogino T, Sakai A, Regazzoni BM, Wuyts W, Sangiorgi L, Pedrini E, Zhu M, Kozakewich HP, Kasser JR, Seidman JG, Kurek KC, Warman ML, Loss-of-function

- mutations in PTPN11 cause metachondromatosis, but not Ollier disease or Maffucci syndrome, *PLoS Genet* 7(4) (2011) e1002050. [PubMed: 21533187]
- [14]. Kim HK, Aruwajoye O, Sucato D, Richards BS, Feng GS, Chen D, King PD, Kamiya N, Induction of SHP2 deficiency in chondrocytes causes severe scoliosis and kyphosis in mice, *Spine (Phila Pa 1976)* 38(21) (2013) E1307–12. [PubMed: 23873233]
- [15]. Zuo C, Wang L, Kamalesh RM, Bowen ME, Moore DC, Dooner MS, Reginato AM, Wu Q, Schorl C, Song Y, Warman ML, Neel BG, Ehrlich MG, Yang W, SHP2 regulates skeletal cell fate by modifying SOX9 expression and transcriptional activity, *Bone Res* 6 (2018) 12. [PubMed: 29644115]
- [16]. Wang L, Huang J, Moore DC, Zuo C, Wu Q, Xie L, von der Mark K, Yuan X, Chen D, Warman ML, Ehrlich MG, Yang W, SHP2 Regulates the Osteogenic Fate of Growth Plate Hypertrophic Chondrocytes, *Sci Rep* 7(1) (2017) 12699. [PubMed: 28983104]
- [17]. Lapinski PE, Meyer MF, Feng GS, Kamiya N, King PD, Deletion of SHP-2 in mesenchymal stem cells causes growth retardation, limb and chest deformity, and calvarial defects in mice, *Dis Model Mech* 6(6) (2013) 1448–58. [PubMed: 24077964]
- [18]. Wu M, Chen G, Li YP, TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease, *Bone Res* 4 (2016) 16009. [PubMed: 27563484]
- [19]. Ornitz DM, Marie PJ, FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease, *Genes Dev* 16(12) (2002) 1446–65. [PubMed: 12080084]
- [20]. Regard JB, Zhong Z, Williams BO, Yang Y, Wnt signaling in bone development and disease: making stronger bone with Wnts, *Cold Spring Harb Perspect Biol* 4(12) (2012).
- [21]. Day TF, Yang Y, Wnt and hedgehog signaling pathways in bone development, *J Bone Joint Surg Am* 90 Suppl 1 (2008) 19–24. [PubMed: 18292352]
- [22]. Chen G, Deng C, Li YP, TGF-beta and BMP signaling in osteoblast differentiation and bone formation, *Int J Biol Sci* 8(2) (2012) 272–88. [PubMed: 22298955]
- [23]. Goumans MJ, Mummery C, Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice, *Int J Dev Biol* 44(3) (2000) 253–65. [PubMed: 10853822]
- [24]. Cao X, Chen D, The BMP signaling and in vivo bone formation, *Gene* 357(1) (2005) 1–8. [PubMed: 16125875]
- [25]. Matsunobu T, Torigoe K, Ishikawa M, de Vega S, Kulkarni AB, Iwamoto Y, Yamada Y, Critical roles of the TGF-beta type I receptor ALK5 in perichondrial formation and function, cartilage integrity, and osteoblast differentiation during growth plate development, *Dev Biol* 332(2) (2009) 325–38. [PubMed: 19501582]
- [26]. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC, Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12, *Mol Cell Biol* 20(23) (2000) 8783–92. [PubMed: 11073979]
- [27]. Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM, BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2, *Biochem Biophys Res Commun* 309(3) (2003) 689–94. [PubMed: 12963046]
- [28]. Kurata H, Guillot PV, Chan J, Fisk NM, Osterix induces osteogenic gene expression but not differentiation in primary human fetal mesenchymal stem cells, *Tissue Eng* 13(7) (2007) 1513–23. [PubMed: 17518720]
- [29]. Nakashima K, de Crombrughe B, Transcriptional mechanisms in osteoblast differentiation and bone formation, *Trends Genet* 19(8) (2003) 458–66. [PubMed: 12902164]
- [30]. Yang W, Wang J, Moore DC, Liang H, Dooner M, Wu Q, Terek R, Chen Q, Ehrlich MG, Quesenberry PJ, Neel BG, Ptpn11 deletion in a novel progenitor causes metachondromatosis by inducing hedgehog signalling, *Nature* 499(7459) (2013) 491–5. [PubMed: 23863940]
- [31]. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H, A robust and high-throughput Cre reporting and characterization system for the whole mouse brain, *Nat Neurosci* 13(1) (2010) 133–40. [PubMed: 20023653]

- [32]. Logan M, Martin JF, Nagy A, Lobe C, Olson EN, Tabin CJ, Expression of Cre Recombinase in the developing mouse limb bud driven by a Prx1 enhancer, *Genesis* 33(2) (2002) 77–80. [PubMed: 12112875]
- [33]. Strecker S, Fu Y, Liu Y, Maye P, Generation and characterization of Osterix-Cherry reporter mice, *Genesis* 51(4) (2013) 246–58. [PubMed: 23180553]
- [34]. Hahn WC, Dessain SK, Brooks MW, King JE, Elenbaas B, Sabatini DM, DeCaprio JA, Weinberg RA, Enumeration of the simian virus 40 early region elements necessary for human cell transformation, *Mol Cell Biol* 22(7) (2002) 2111–23. [PubMed: 11884599]
- [35]. Yang W, Klamann LD, Chen B, Araki T, Harada H, Thomas SM, George EL, Neel BG, An Shp2/SFK/Ras/Erk signaling pathway controls trophoblast stem cell survival, *Dev Cell* 10(3) (2006) 317–27. [PubMed: 16516835]
- [36]. Takarada T, Nakazato R, Tsuchikane A, Fujikawa K, Iezaki T, Yoneda Y, Hinoi E, Genetic analysis of Runx2 function during intramembranous ossification, *Development* 143(2) (2016) 211–8. [PubMed: 26657773]
- [37]. Li J, Tsuji K, Komori T, Miyazono K, Wrana JL, Ito Y, Nifuji A, Noda M, Smad2 overexpression enhances Smad4 gene expression and suppresses CBFA1 gene expression in osteoblastic osteosarcoma ROS17/2.8 cells and primary rat calvaria cells, *J Biol Chem* 273(47) (1998) 31009–15. [PubMed: 9812998]
- [38]. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R, TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation, *EMBO J* 20(9) (2001) 2254–72. [PubMed: 11331591]
- [39]. Hjelmeland AB, Schilling SH, Guo X, Quarles D, Wang XF, Loss of Smad3-mediated negative regulation of Runx2 activity leads to an alteration in cell fate determination, *Mol Cell Biol* 25(21) (2005) 9460–8. [PubMed: 16227596]
- [40]. Kang JS, Alliston T, Delston R, Derynck R, Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3, *EMBO J* 24(14) (2005) 2543–55. [PubMed: 15990875]
- [41]. Celil AB, Hollinger JO, Campbell PG, Osx transcriptional regulation is mediated by additional pathways to BMP2/Smad signaling, *J Cell Biochem* 95(3) (2005) 518–28. [PubMed: 15786511]
- [42]. Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM, Sung JH, Wozney JM, Kim HJ, Ryoo HM, BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression, *J Biol Chem* 278(36) (2003) 34387–94. [PubMed: 12815054]
- [43]. Lai CF, Cheng SL, Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells, *J Biol Chem* 277(18) (2002) 15514–22. [PubMed: 11854297]
- [44]. Wrighton KH, Lin X, Feng XH, Phospho-control of TGF-beta superfamily signaling, *Cell Res* 19(1) (2009) 8–20. [PubMed: 19114991]
- [45]. Heldin CH, Miyazono K, ten Dijke P, TGF-beta signalling from cell membrane to nucleus through SMAD proteins, *Nature* 390(6659) (1997) 465–71. [PubMed: 9393997]

### Highlights

- SHP2 is a key regulator of intramembranous ossification, its deficiency in the Prrx1+ mesenchymal progenitors causes severely developmental defects in the calvaria bones.
- SHP2 is required for osteogenic differentiation of mesenchymal progenitors but has minimal effect on their proliferation.
- SHP2 is required for BMP2-evoked SMAD1/5/8 activation but suppresses TGF $\beta$ -induced SMAD2/3 activation. Its deficiency in the Prrx1+ mesenchymal progenitors compromises the expression of osteogenic transcription factors *Runx2*, *Osterix* and other osteogenic genes.



**Figure 1. SHP2 ablation in the *Prrx1*+ mesenchymal cells impairs intramembranous ossification.**

**A.** Representative skull images of 2-week-old SHP2<sup>Prrx1</sup>CTR and SHP2<sup>Prrx1</sup>KO mice stained with Alcian blue and Alizarin Red. *n*=3. **B.**  $\mu$ -CT radiographs demonstrating the skull structure of 6-week-old SHP2<sup>Prrx1</sup>CTR and SHP2<sup>Prrx1</sup>KO mice. Note that parietal and interparietal bone ossification was defective (arrows) in the SHP2<sup>Prrx1</sup>KO mice, compared with age matched SHP2<sup>Prrx1</sup>CTR mice. *n*=3. **C.** Representative H&E (top) and von Kossa (bottom, counterstained by fast red) staining of murine parietal bone coronal frozen sections showing the appearance of a loose mesenchyme tissue and impaired mineralization of the parietal bone in the P0.5 SHP2<sup>Prrx1</sup>KO mice, compared with SHP2<sup>Prrx1</sup>CTR mice. Scale bar: 100 $\mu$ m. **D.** Bar graphs show the quantitative data of parietal bone matrix mineralization in

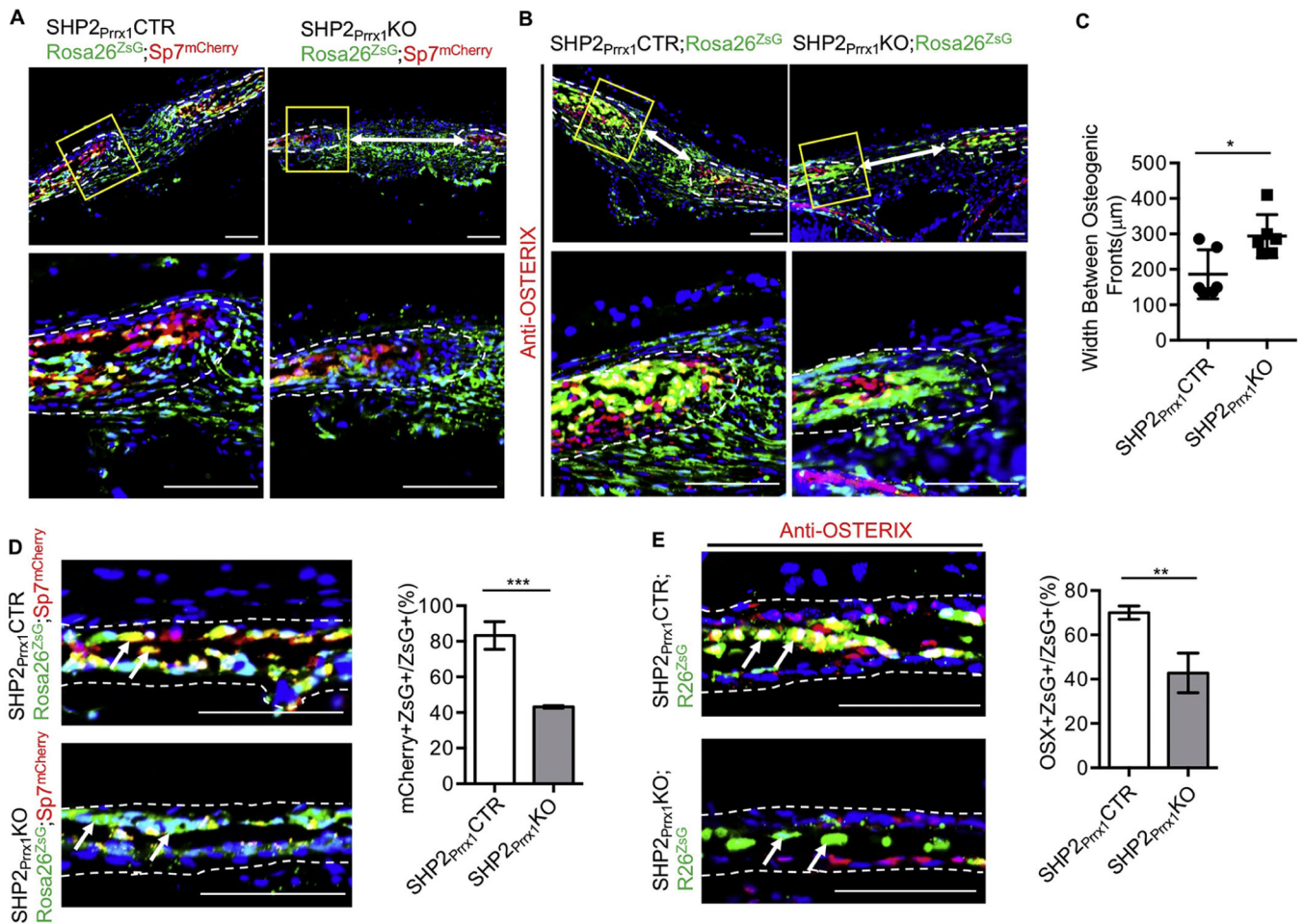
SHP2<sup>Prrx1</sup>CTR and SHP2<sup>Prrx1</sup>KO mice using NIH ImageJ software (n=3, \*\* $P < 0.01$ , Student's *t*-test).

Author Manuscript

Author Manuscript

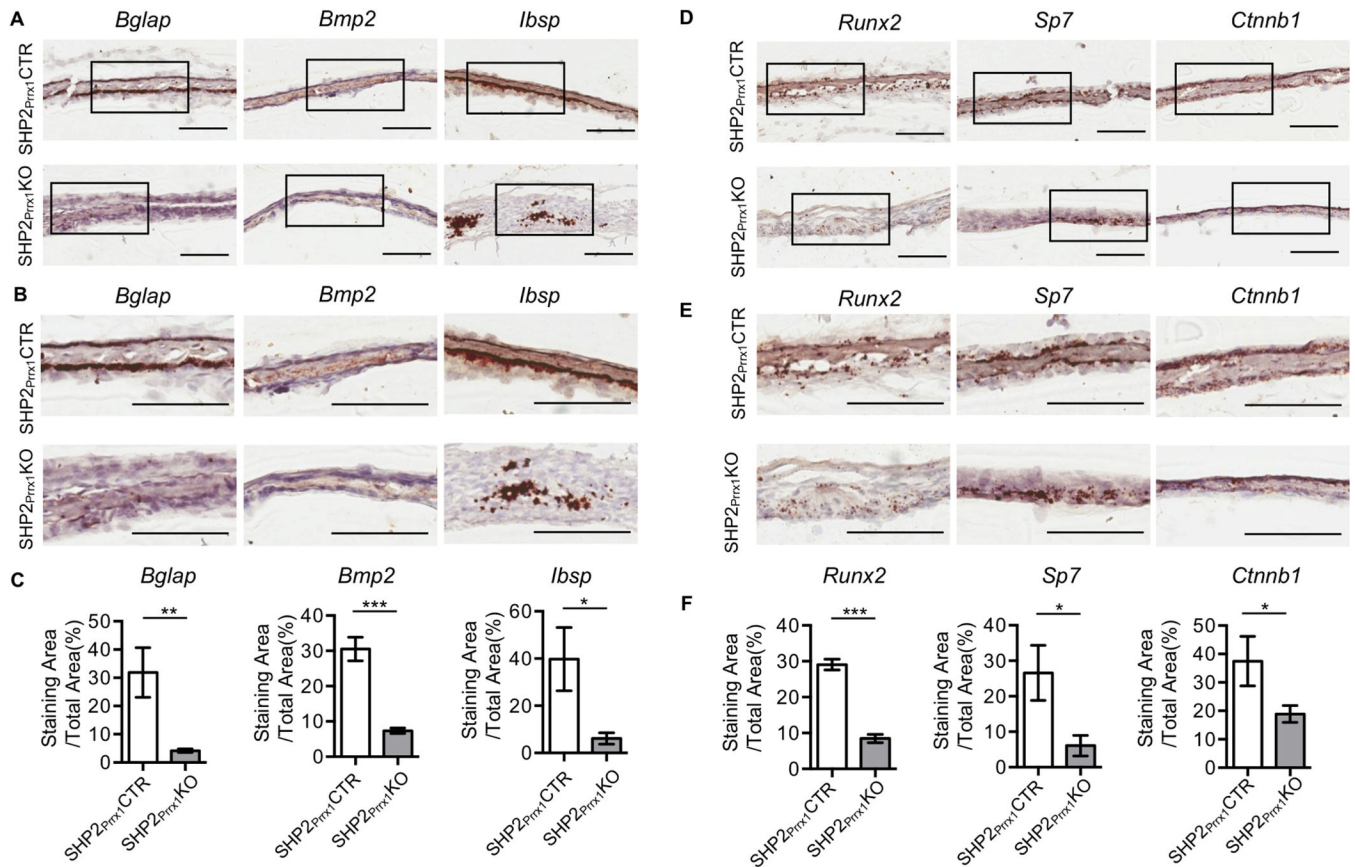
Author Manuscript

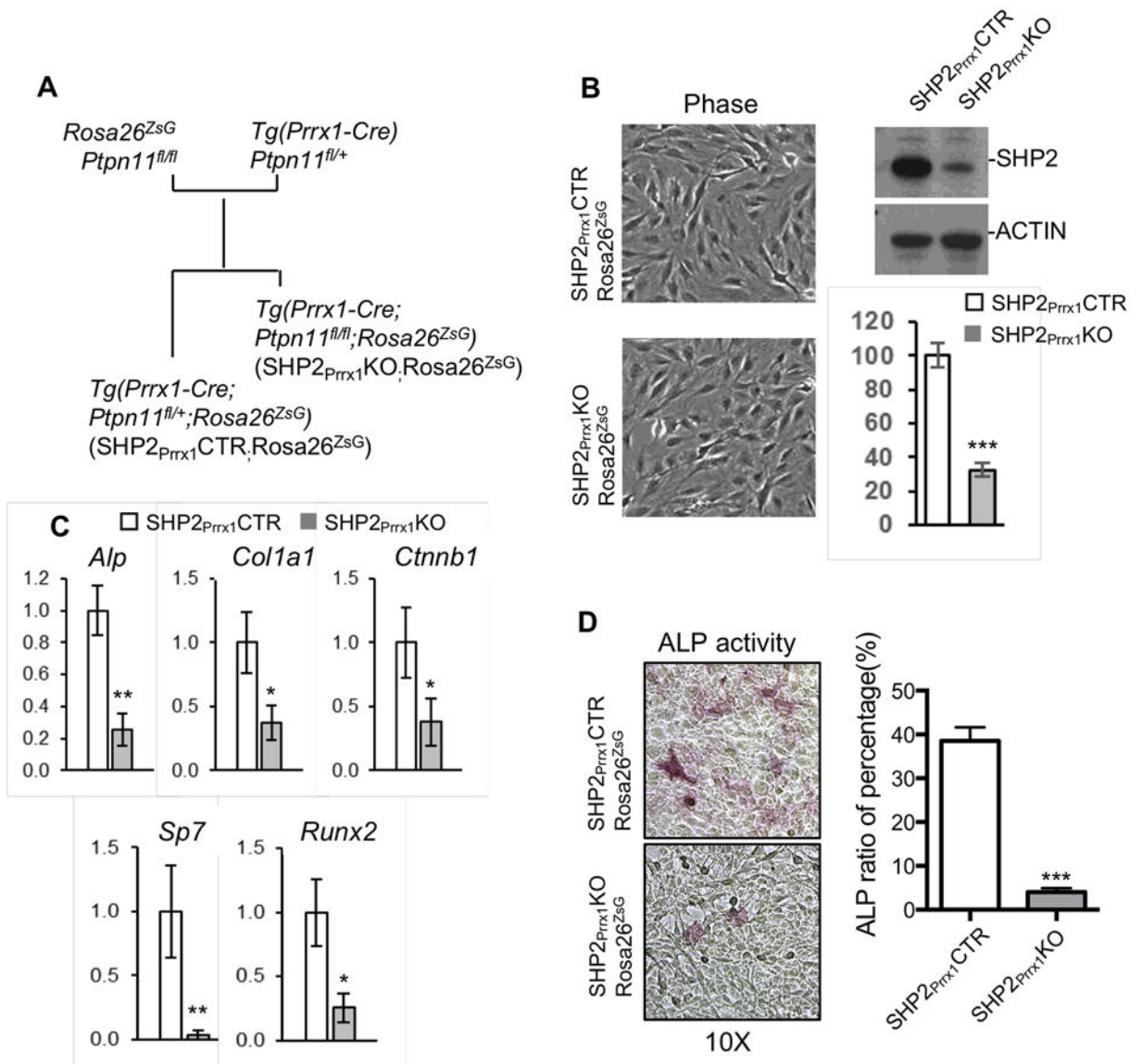
Author Manuscript



**Figure 2. SHP2 is required for the osteogenic differentiation of the *Prrx1*+ mesenchymal cells.** **A.** Fluorescent images of the sagittal sutures showing the number and location of *Prrx1*+ (green), *Osx*+ (red), and *Prrx1*/*Osx* double positive cells (yellow) at the osteogenic fronts and sutures. Note that the number of *Prrx1* and *Osx* double positive cells decreased and the width of the sagittal suture (white arrows increased significantly in SHP2<sup>Prrx1</sup>KO;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> mice, compared with the SHP2<sup>Prrx1</sup>CTR;Rosa26<sup>ZsG</sup>;Sp7<sup>mCherry</sup> controls. Images at the bottom are the enlarged views of the corresponding boxed areas in the top images. Scale bar: 100μm. **B.** Sagittal sutures of indicated mice immunostained with anti-OSTERIX antibody (red) showing the number and location of *Prrx1*+ (green), OSTERIX+ (red), and *Prrx1*/OSTERIX double positive cells (yellow) at the osteogenic fronts and sutures. Images at the bottom are the enlarged views of the corresponding boxed areas in the top images. Scale bar: 100μm. **C.** Bar graphs showing the quantitative data of sagittal suture width (images of A and B) in the P0.5 SHP2<sup>Prrx1</sup>CTR and the SHP2<sup>Prrx1</sup>KO mice (n=6, \**P*<0.05, Student's t-test). **D-E.** Fluorescent images of the parietal bone frozen sections showing the number and location of *Prrx1*+ (green), *Osx*+ (red), OSTERIX+ (red), and *Prrx1* and *Osx* (OSTERIX) double positive (yellow) cells in the indicated mice. Quantitative data are shown as bar graphs on the right (n=3, \*\*\**p*<0.001, Student's t-test) Scale bar: 100μm.

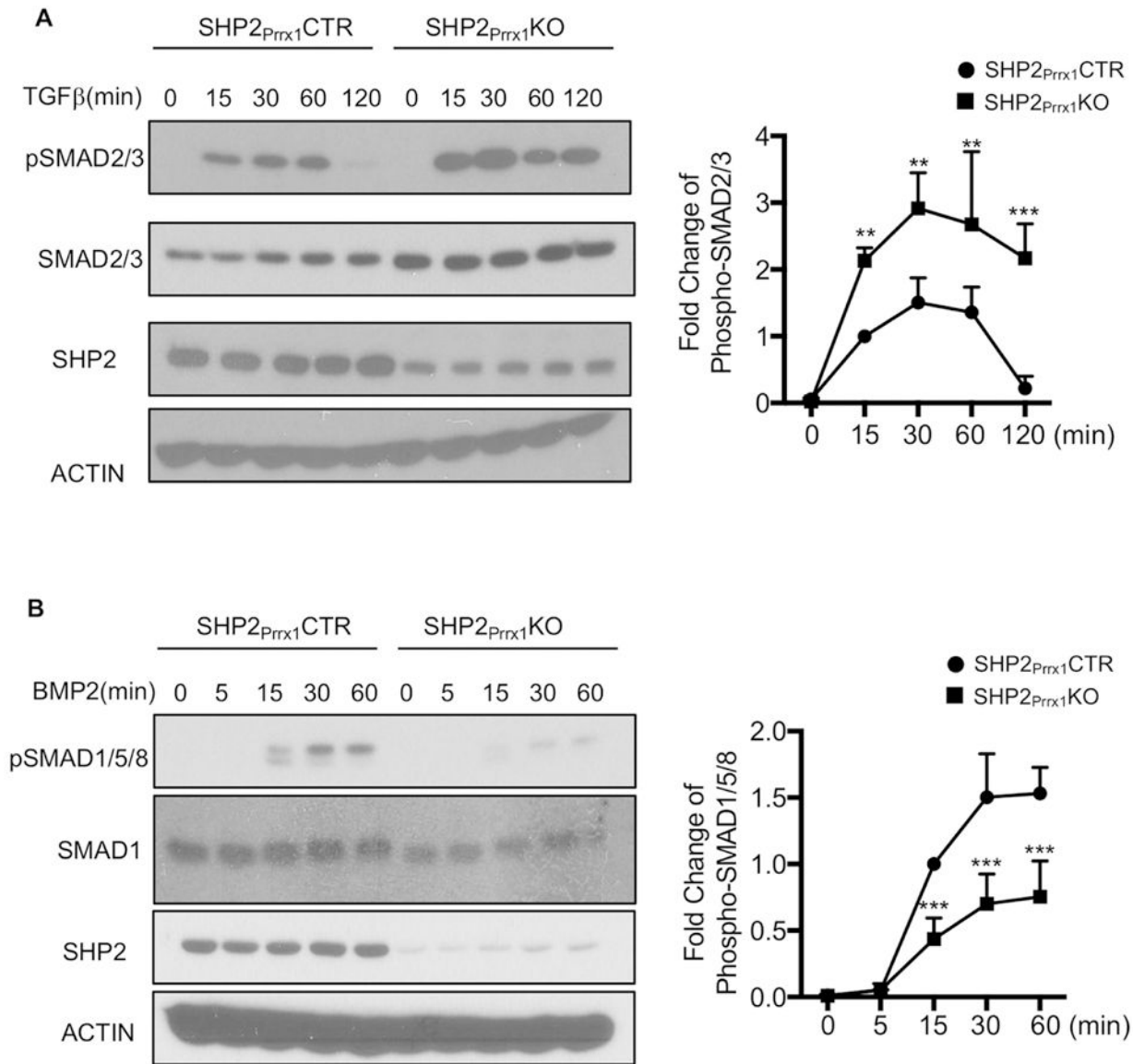






**Figure 4.** SHP2 deletion in the *Prrx1*-expressing cells compromises osteogenic genes expression *in vitro*.

**A.** Diagram denotes the strategy to generate SV40 large T antigen-immortalized osteoblasts from the SHP2<sub>Prrx1</sub>CTR;Rosa26<sup>ZsG</sup> and SHP2<sub>Prrx1</sub>KO;Rosa26<sup>ZsG</sup> mice. **B.** Phase contrast images (left) showing the morphology of immortalized osteoblasts. Western blot and bar graphs [44] demonstrating the level of SHP2 in the immortalized osteoblasts from indicated mice (n=3, \*\*\**P*<0.001, Student's t-test). **C.** Bar graphs showing the relative expression of indicated osteogenic genes in SHP2<sub>Prrx1</sub>CTR and SHP2<sub>Prrx1</sub>KO osteoblasts determined by qRT-PCR. (n=3, \**p*<0.05, \*\**p*<0.01, Student's t-test). **D.** Phase contrast images demonstrating ALP activity in the immortalized osteoblastic cells from indicated mice. Ratio of ALP positive cells vs total cells in the defined areas are presented as bar graphs on the right (n=3, \*\*\**p*<0.001, Student's t-test).



**Figure 5. SHP2 differentially regulates TGFβ and BMP signaling pathways.**

**A.** Immunoblotting data show that TGFβ-evoked SMAD2/3 phosphorylation was enhanced in the osteoblastic cells from SHP2<sub>Prrx1</sub>KO mice, compared to that from SHP2<sub>Prrx1</sub>CTR mice. Cells were starved overnight and stimulated with TGFβ (100ng/ml) for indicated time points. Phosphorylation of SMAD2/3 was quantified using NIH ImageJ software and normalized to the β-ACTIN controls, phosphorylation level of SMAD2/3 was corrected by the total level of SMAD2/3. (n=3, \*\**p*<0.01, \*\*\**p*<0.001, Student's t-test) **B.**

Immunoblotting data showing decreased SMAD1/5/8 phosphorylation in response to BMP2 in SHP2<sub>Prrx1</sub>KO osteoblastic cells, compared to SHP2<sub>Prrx1</sub>CTR osteoblastic cells. Cells were starved overnight and stimulated with BMP2 (100ng/ml) for indicated time points. Phosphorylation of SMAD1/5/8 was quantified as described in A (n=3, \*\**p*<0.01, \*\*\**p*<0.001, Student's t-test).