Role of Sfrp4, a Wnt Antagonist, in Bone Repair and Regeneration

Citation

Ahn, Chiho. 2021. Role of Sfrp4, a Wnt Antagonist, in Bone Repair and Regeneration. Doctoral dissertation, Harvard University School of Dental Medicine.

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Role of Sfrp4, a Wnt Antagonist, in Bone Repair and Regeneration

A Thesis Presented by

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to

The Faculty of Medicine
In partial fulfillment of the requirements
for the degree of
Doctor of Medical Sciences

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Boston, Massachusetts

April 2021

Dedication

This thesis is devoted to my family for their constant love and support.

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Abstract

Sfrp4 (<u>S</u>ecreted <u>F</u>rizzled <u>R</u>eceptor <u>P</u>rotein 4) serves as a decoy receptor for Wnts and differently from *sclerostin* (*Sost*) that blocks canonical (c)Wnt signaling, it suppresses cWnt and non-cWnt cascades. Loss of function mutations in *Sfrp4* cause Pyle disease, a skeletal disease-causing cortical thinning and fractures. Using *Sfrp4-/-* mice, we demonstrated cortical thinning is by decreased periosteal bone formation and increased endosteal remodeling through non-cWnt/Ror2/Jnk cascade activation. Given that periosteum contains stem cells which forms new bone to injury, we investigated role of activation of cWnt and non-cWnt signaling (*Sfrp4-/-* mice) in bone regeneration. We created calvarial critical defects (which don't heal spontaneously) or subcritical defects (which heal spontaneously). We used *Sost-/-* mice for cWnt signaling activation. We confirmed cWnt activation in *Sost-/-* and *Sfrp4-/-* calvariae, and non-cWnt/Jnk activation only in *Sfrp4-/-* calvariae. MicroCT analyses indicate while cWnt activation (*Sost* deletion) favors bone regeneration (BV/TV(%)) within initial critical defect ($p<0.0001$) 6-wk after surgery, *Sfrp4* deletion did not. In subcritical defects, cWnt activation (*Sost-/-* mice) led to accelerated bone regeneration ($p<0.01$), while *Sfrp4-/-* mice did not. *Sfrp4* deletion leads to significant decrease in percentage and function of Cathepsin K (*Ctsk+*) labelled calvarial periosteal stem cells (PSCs). We investigated effect of *Sfrp4* deletion in response to injury by using *CtskCre;mTmG;wt* and *CtskCre;mTmG;Sfrp4-/-* mice. Preliminary confocal analyses indicate *Sfrp4* deletion impairs response of *Ctsk+* PSCs. We explored whether activation of *Ror2* cascade in *Sfrp4* null mice might improve bone formation in subcritical defects. We generated mice lacking *Ror2* receptor in *Ctsk+* cells in *Sfrp4* null background. Preliminary findings suggest *Ror2* signaling does not impact response to injury found in *Sfrp4-/-* mice. Our findings demonstrate Wnt signaling fine-tuning is critical for bone responses and activation of non-cWnt signaling in *Sfrp4-/-* mice might be responsible for improper function of stem cells within sutures and periosteum.

Introduction

The periosteum is required for appositional bone growth during skeletal development and plays a critical role in bone repair and bone regeneration.[1, 2] The periosteum contains a niche of stem cells which are involved in the response to injury.[1, 2] Compelling evidence shows that sutures host stem cells which serve as growth centers for bone formation during bone development but are also activated upon injury to support new bone formation and homeostasis between cortical bone and trabecular bone is critical to achieve proper bone stability.[3-7] It also has been demonstrated that healing rate of the calvarial bone is fast as the injury site is closer to the suture confirming that the suture is a major location of mesenchymal stem cells involved in bone regeneration.[8] Within the periosteum and the sutures, Wnt responding cells are induced by local Wnt stimuli and are responsible for bone repair as indicated by study showing that ablation of these cells leads to disruption in the rate of repair after injury.[9, 10] Wnt signaling is one of the most important developmental signaling pathways that controls cell fate decision and tissue homeostasis.[9, 10] Not surprising, the last decade has provided abundant data implicating the Wnt pathway also in bone development, in the regulation of bone mass and in bone regeneration.[9, 10] Wnts are secreted cysteine-rich glycoproteins loosely classified as either "canonical" or "non-canonical" depending on whether they activate β-catenin-dependent or -independent signaling events, respectively (Fig. 1).
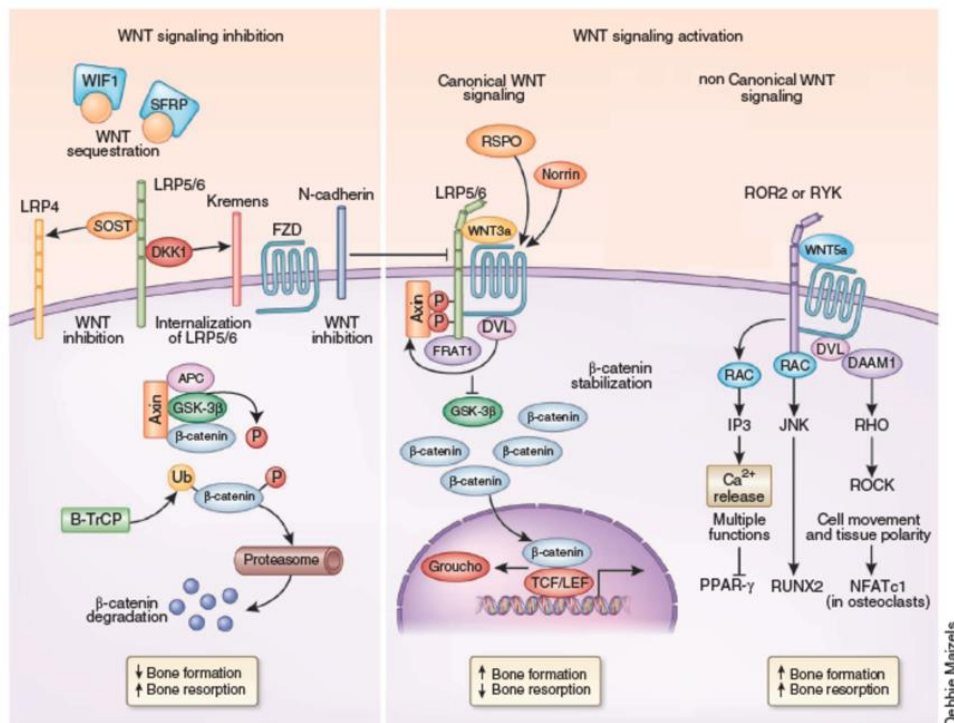

Figure 1. Wnt signaling pathways adapted from Baron and Kneissel.[9]

Canonical signaling is initiated by Wnt ligands that bind to dual receptor complex of frizzled (FZD) and one of Lrp5 or Lrp6.[9] This inactivates the β catenin destruction complex that allow β-catenin to translocate into the nucleus and associates with transcription factors to promote target gene transcription.[9] In the non-canonical Wnt signaling, Wnt ligands engage FZD receptors alone or together with co-receptors such as the receptor tyrosine kinase-like orphans *Ror2* or *Ryk* and trigger cascades such as the *Wnt*-Ca^{+2} and Wnt-Jnk which in turn lead to activation of specific target gene expression.[9] Given that Wnt signaling can be the targeted for therapeutic intervention to improve bone mass and bone regeneration,[9] it is important to explore the various components within the Wnt pathway and their function. Wnt ligands function with an entourage of receptors, co-receptors, agonists and antagonists that either enable or prevent Wnt signaling activation.[9]

Among the Wnt antagonists is the family of S̲ecreted F̲rizzled R̲elated P̲roteins (*Sfrp*1 to 5), which bind directly to Wnts and block their receptor complexes.[9, 11] Therefore, differently from the other two well-known Wnt antagonists, *sclerostin (Sost)* and *Dkk1*, that bind to Lrp5/Lrp6 and block canonical signaling, *Sfrps* suppress both canonical and non-canonical Wnt cascades (Fig. 2). Consequently, they may have more pleiotropic effects and broader influence on tissue development and homeostasis.
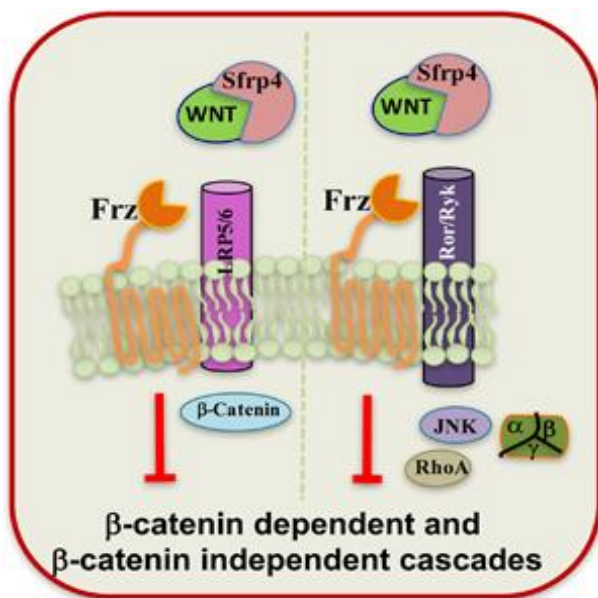

Figure 2. *Sfrps* are secreted glycoproteins that function as Wnt decoy receptors and can therefore block canonical and non-canonical Wnt-cascades.

Among the *Sfrps*, *Sfrp4* has been found to be associated with bone mineral density (BMD) in GWAS studies.[12] We have recently reported that in human loss of function mutations in *Sfrp4* cause Pyle disease.[7] Pyle disease is characterized by limb deformity with long bones with wide and expanded trabecular metaphyses, thin cortical bone and bone fragility (Fig. 3*A-E*). Importantly for these studies, in mice, *Sfrp4* genetic inactivation causes skeletal deformities closely mimicking those seen in humans: increased trabecular bone formation and decreased cortical thickness, due to impaired periosteal and endosteal bone formation and increased endosteal resorption (Fig. 3*F-G*).[7] We have shown that in the *Sfrp4*-null mouse Pyle disease model, activation of the non-canonical Wnt/Jnk signaling cascade, together with increased BMP signaling and sclerostin levels, leads to decreased

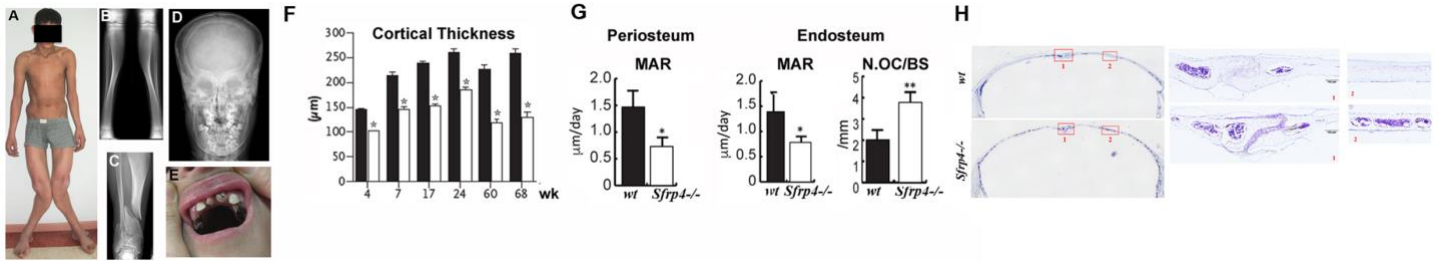periosteal bone formation and deregulation of endosteal bone remodeling.[7]

Figure 3. Clinical features of Pyle disease (*Sfrp4*-loss of function) and cortical bone features of *Sfrp4-/-* mice.[7] (*A*) Patient 1. (*B*) Radiograph of the lower limbs in Patient 1 showing expanded metaphyses of the proximal and distal tibiae with extremely thin cortexes, (*C*) fracture in Patient 3 and (*D*) skull of Patient 2 showing diploe expansion. (*E*) Dental problems in Patient 2. (*F*) microCT analysis of cortical thickness in femur midshaft of *wt* (black bars) and *Sfrp4-/-* (open bars) mice (n=5). (*G*) Cortical bone histomorphometry (10wk, n=5). *=P<0.05, **=P<0.01 vs wt.* (*H*) Representative images of calvarial H&E sections (1=sagittal suture, 2=parietal bone).

Individuals affected by Pyle disease and *Sfrp4-/-* mice present with decreased calvarium thickness and increased diploe (porosity), supporting a role for *Sfrp4* also in calvarium growth and craniofacial biology (Fig. 3*D,H*).[7] Furthermore, smaller head circumference, prominence of the frontal bones, and delayed eruption of permanent teeth are also found in patients with Pyle disease.[7] Importantly and supporting findings in humans linking *Sfrp4* with craniosynostosis,[13] we found that *Sfrp4-/-* mice can present with a variable degree of suture fusion (Fig. 3*H*).[7] It is thought that sutures host stem cells and serve as growth centers for bone formation and homeostasis between cortical bone and trabecular bone are important to achieve proper bone thickness .[3-7] Tight signals between the suture mesenchyme, periosteum, osteogenic front and dura are pivotal for proper skull formation and maintenance as well as for bone regeneration.[3-6] If a key role for canonical Wnt signaling in the suture mesenchyme and periosteum has been clearly demonstrated as well as the presence of *Axin2+* cells,[5] our understanding of the role of non-canonical Wnt in the suture and periosteum in bone regeneration is limited. We have reported that *Sfrp4*-null calvarial osteoblasts (cOBs) display activation of both canonical Wnt/⬜-catenin and non-canonical Wnt/Jnk cascades[7] and confirming *in vivo* phenotype of thinner calvarial bone, we found that their osteoblasts differentiation *in vitro* is impaired (Fig. 4*A-C*).[7]
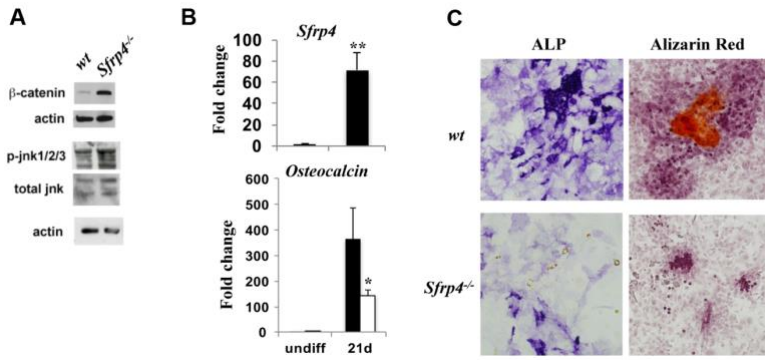
Figure 4. Functional results of *Sfrp4* deficiency in cOBs.[7] (*A*) Western analysis of on canonical and non-canonical Wnt signaling cascades in cOBs. (*B*) RNA expression of *Sfrp4* and osteocalcin expression in calvarial osteoblasts. (Black bars =wt cOBs and open bar=Sfrp4-/- cOBs). Data are the mean +SEM. (n=3) *=P<0.05, **=P<0.01 *vs wt by* Student T-test. (*C*) Representative images of ALP and Alizarin Red staining.

Importantly in the context of the studies performed here, *Sfrp4* is expressed in the periosteum (Fig. 5*A*).[14] In collaboration with Dr. Greenblatt, who, using a combination of lineage tracing of cells expressing CathepsinK (*Ctsk*) and their progeny has identified three distinct periosteal mesenchymal subpopulations 1) periosteal stem cells (PSCs) 2) periosteal progenitors 1 (PP1) and 3) periosteal progenitors 2 (PP2),[14] we found that *Sfrp4* is expressed in these cell populations and prevalently in PP1 and PP2 cells, which are also *Sca1+*. *Ctsk+* periosteal stem cells are involved in intramembranous ossification and participate in bone healing.[14] *Ctsk+* PSC, PPI and PP2 are present in the periosteum of the long bones, in the calvaria and in the sagittal suture mesenchyme and their number decreases with age.[14] Importantly, *Wnt5a*, a typical non-canonical ligand, is expressed mainly in the PSC pool, while *Wnt1* and *Wnt3a*, typical canonical Wnt signaling ligands, are not expressed in these periosteal populations (Fig. 5*B*).[14] In addition, *Ror2* is one of the coreceptors for *Wnt5a* that is expressed at similar levels in all the three populations (Fig. 5*B*).[14] Therefore, it is possible that a subset of periosteal cells secretes locally *Sfrp4*, which in turn contributes to intramembranous ossification likely regulating, via a *Sfrp4/Wnt5a* axis, the expansion, differentiation and function of progenitor cells.
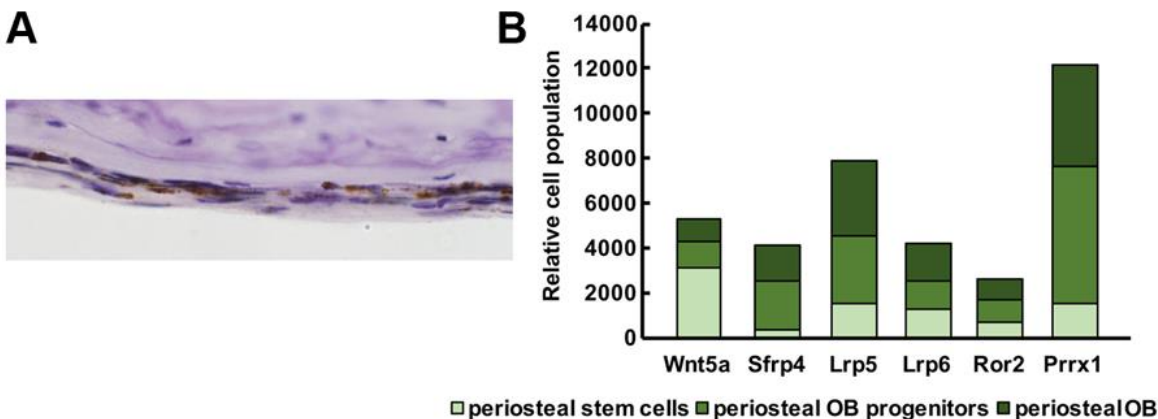

Figure 5. Sfrp4 in the periosteum.[14] (*A*) Periosteal *Sfrp4* mRNA (RNAscope) in 10-wk old tibia (n=3). (*B*) Relative gene expression in separate periosteal cell populations isolated from P10 long bone periosteum (bulk RNA-seq) (n=4).

Importantly, studies in the lab have shown that *Sfrp4* deficiency leads to a significant decrease in the percentage of PSCs and increase in the percentage of PP1 and PP2 cells in the calvarium (including periosteum and sutures) (Fig. 6) and in the long bones (data not shown). Further studies performed using the periosteum of long bones demonstrated that *Sfrp4* deletion decreases the pool of *bona fide* stem cells while favoring their transition to non-stem progenitors with impaired differentiation in mature and functional osteoblasts. (Chen et al. ASBMR_2020).
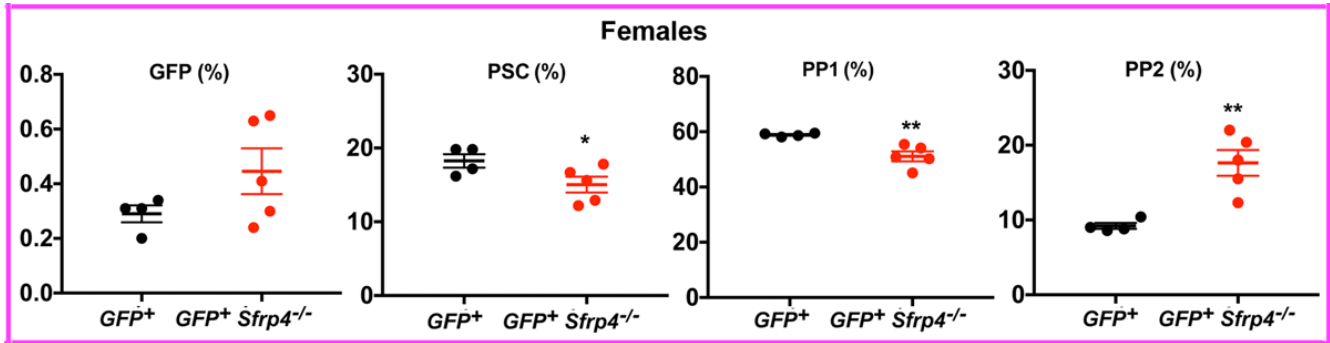

Figure 6. *Sfrp4* and calvarium (periosteum+suture) % of *Ctsk⁺* subpopulations. (n=5. each point=1 mouse). Data: mean±SEM, *p<0.05, **p<0.01 by Student T-test. (Courtesy of Ruiying Chen).

However, how *Sfrp4* modulates the functions of cells within the periosteum and the suture mesenchyme responsible for bone regeneration is not known. Thus, we asked whether 1) *Sfrp4* mediated signaling is required for proper bone regeneration in response to injury and 2) *Sfrp4*-mediated signaling ensure proper bone regeneration in response to skeletal injury by affecting stem cells and osteoblast function. To this end, we chose the calvarial injury repair model over long bone fractures as 1) it allows to study bone healing without the need to stabilize the defect, a challenge when long bone critical size fracture models are used, 2) minimal loading baring in comparison to long bones, and 3) the repair site recapitulates intramembranous bone formation (consistent with the known developmental origin of calvaria). Thus, we created critical (absence of spontaneous regeneration) size defects and subcritical (spontaneous regeneration)[15,16] on the surface of mouse calvarium and investigated bone healing and regeneration.

Materials and Methods

Mice

For all studies *Sfrp4+/-* mice were interbred to obtain *Sfrp4+/+ (wt), Sfrp4+/-* and *Sfrp4-/-* mice. Similarly, *Sost+/-* mice were interbred to obtain *Sost+/+(wt), Sost+/-* and *Sost-/-* mice. Cathepsin Cre (*CtskCre*) mice were kindly provided by Dr. Kato (Japan). *CtskCre* and their progeny with *mTmG* reporter (*CtskCre;mTmG)* and *Sfrp4-/-* mice were also used to generate *CtskCre;mTmG;wt* and *CtskCre;mTmG;Sfrp4-/-* mice. *CtskCre;mTmG* were crossed with *Ror2fl/fl* and *Sfrp4+/-* mice to assess the role of the *Ror2* cascade in the *Sfrp4-/-* mice as previously done.[17] *CtskCre;mTmG;Ror2fl/fl;wt (Ror2del)*, *mTmG;Ror2fl/fl;wt(Control)*, *CtskCre;mTmG;Ror2fl/fl;Sfrp4-/- (Ror2del;Sfrp4del), and mTmG;Ror2fl/fl;Sfrp4-/-(Sfrp4del),* mice were then used. All mice are on the C57BL/6J background in order to enhance reproducibility. Animal studies were approved by the Harvard University Institutional Animal Care and Use Committee.

Total RNA extraction and analysis of mRNA levels

Total RNA from calvarial bone of *Sfrp4-/-* and *Sost-/-* and mice and their *wt* littermates were extracted using Trizol reagent (Invitrogen) followed by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Quantitative real time PCR was performed using using StepOnePlus Real-Time PCR system (Applied Biosystems). mRNA levels encoding each gene of interest were normalized for actin mRNA in the same sample and the relative expression of the genes of interest were determined using the formula of Livak and Schmittgen.[18]

Critical and subcritical calvarial defects

Critical and subcritical calvarial defects were performed in 8 weeks old male and female mice. Mice were euthanized, and samples were analyzed 6 weeks after surgery (Fig. 7*A*). Briefly, once the mice were under anesthesia, hairs were removed starting from the region between the eyes to the posterior end of the skull using an electric shaver (Fig. 7*B-a*). Using 15 sterile blade, a deep longitudinal skin incision was made on the scalp of the mouse starting from behind the eyes down to midsagittal area of the skull and the skin was then pulled laterally to expose the parietal bones (Fig. 7*B-b*). Using a dental low-speed dental drill (~1500rpm), the left parietal bone was scored on the central region of the parietal bone (2mm away from sagittal suture) with a critical defect injury of 2.3mm with a dental trephine bur or subcritical defect injury

of 0.8mm with a round bur (Fig. 7*B-c.*). Upon visualizing the thinning of the calvarium, a blunt forcep was used around defect and scoop out the trephined bone (Fig. 7*B-d*). The surgical site was washed with sterile saline and the periosteum and skin tissues were closed with simple interrupted suture patterns using 5-0 Vicryl suture (Fig. 7*B-e*).
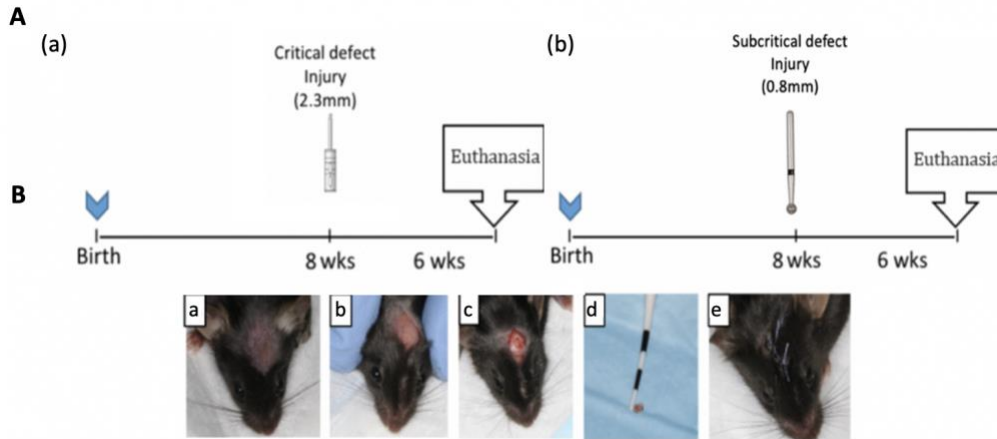

Figure 7. Methods for the surgical procedure. (*A*) Timeline of the subcritical and critical defects. (*B*) Images showing the main steps of the surgical procedure.

Evaluation of bone regeneration by microCT (µCT)

To explore the effect of *Sfrp4* deletion on new bone formation within the defect site, we performed µCT 6 weeks post-surgery. For µCT scanning, a high-resolution desktop micro-tomographic imaging system (µCT50, Scanco Medical AG, Brüttisellen, Switzerland) was used. Scans were acquired using a 10 μm^3 isotropic voxel size, 70 kVP, 114 µA, 200 ms integration time. Images were subjected to Gaussian filtration and segmented using a fixed threshold of 700 mgHA/cm^3. Image acquisition and analysis protocols adhered to the JBMR guidelines.[19] New bone formed within the defect will be quantified and expressed as bone volume over total volume (BV/TV(%)).

Evaluation of Cathepsin K (*Ctsk*+) labelled calvarial periosteal stem cells (PSCs)

Lineage tracing was performed to detect *Ctsk*+ calvarial periosteal stem cells (PSCs) in response to subcritical defect injury by using *CtskCre;mTmG;wt* and *CtskCre;mTmG;Sfrp4-/-* mice . Mice were sacrificed 2, 7 and 14 days after surgery. *CtskCre;mTmG;wt and CtskCre;mTmG;Sfrp4-/-* mice without surgery were used to assess the *Ctsk*+ PSC at basal level. Calvariae were decalcified and processed for frozen embedding and sectioning. Confocal imaging was performed with a Leica SPE with a high-resolution detector confocal microscope at the Neurobiology Imaging Facility at Harvard Medical School.

Statistical Analysis

Data were expressed as boxplot with median, interquartile range, maximum and minimum values including all data points plotted. Statistical analysis was conducted using unpaired two-tailed Student's t test. GraphPad Prism 9 was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

***Sfrp4* and *Sost* deletion similarly activate canonical Wnt signaling while *Sfrp4* deletion activates also non-c Wnt/Jun cascade in calvariae**

We assessed the level of activation of canonical Wnt and non-cononical cascades in freshly calvarial bones isolated from both *Sost$^{-/-}$* and *Sfrp4$^{-/-}$* mice. To this end, the expression of canonical Wnt signaling downstream target genes such as *Dkk1* (dickkopof-1) and *Tcf1* (transcription factor T cell factor 1), and that of the non-canonical downstream target gene, *c-Jun* (transcription factor c-Jun) was evaluated by Real Time Quantitative PCR (RT-qPCR).
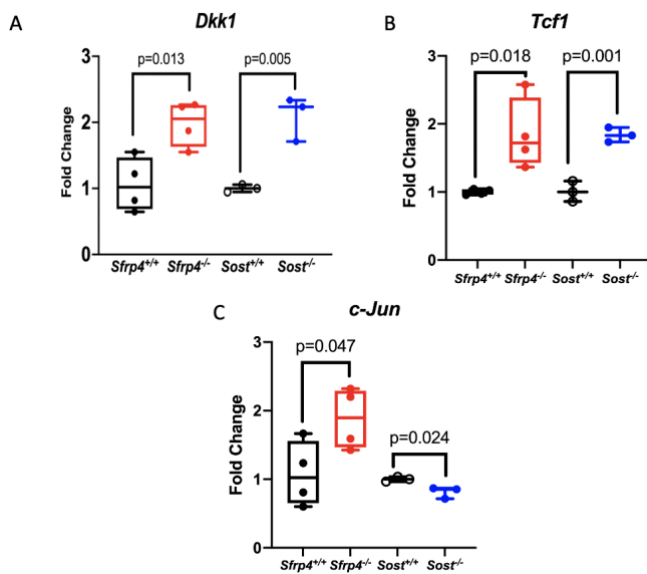
While the expressions of the canonical Wnt target genes, *Dkk1* and *Tcf1*, were significantly upregulated in both *Sfrp4$^{-/-}$* and *Sost$^{-/-}$* calvaria, the expression of *c-Jun*, known to be a downstream target gene of the Wnt/Ror2 non-canonical Wnt cascade, was significantly upregulated only in *Sfrp4$^{-/-}$* calvaria (Fig. 8*A-C*).

Figure 8. Wnt signaling cascade activation. RT-qPCR analysis of (*A*) *Dkk1*, (*B*) *Tcf1*, and (*C*) *c-Jun* mRNA levels. Data are expressed as fold changes compared to *wt* littermates and normalized to Actin. Statical analysis included unpaired Student T-test, p value comparing groups using boxplot with median, interquartile range, max and min values and all data points included (n=3-4).

These data confirmed that *Sfrp4* deletion activates both canonical and non-canonical pathway, while *Sost* deletion only activates canonical Wnt pathway. In addition, these results indicate that in calvarial bones the expression of two canonical Wnt signaling target genes, *Dkk1* and *Tcf1*, is similarly regulated by *Sfrp4* and *Sost* deletion.

Differently from *Sost* deletion, *Sfrp4* deletion does not allow bone regeneration of critical size defects

To explore the effect of *Sfrp4* deletion on healing of critical bone defects, we created a 2.3mm diameter calvarial critical defect in the left central region of the parietal bone as detailed above (Fig. 7*B*). In accordance with investigations on calvarial healing in mice, there is no spontaneous healing of circular

defects sized 1.8mm and 2mm after 6 and 12 weeks.[15] Therefore, a 2.3mm diameter was chosen as a choice of critical defect size. *Wt* and *Sfrp4*^{-/-} mice were used in these studies and calvarial critical defects and analysis were performed as detailed above. We used *Sost*^{-/-} mice in which critical-sized defects heal,[15] as a control for our methods, and as a model for canonical Wnt signaling activation. Confirming previous findings,[15] activation of canonical Wnt signaling (*Sost* deletion) favors bone regeneration within the initial critical defect (Fig. 9*A,C*). Contrary to our expectations, deletion of *Sfrp4* showed no regeneration in which BV/TV (%) which was similar to that of *wt* mice (Fig. 9*B,C*).
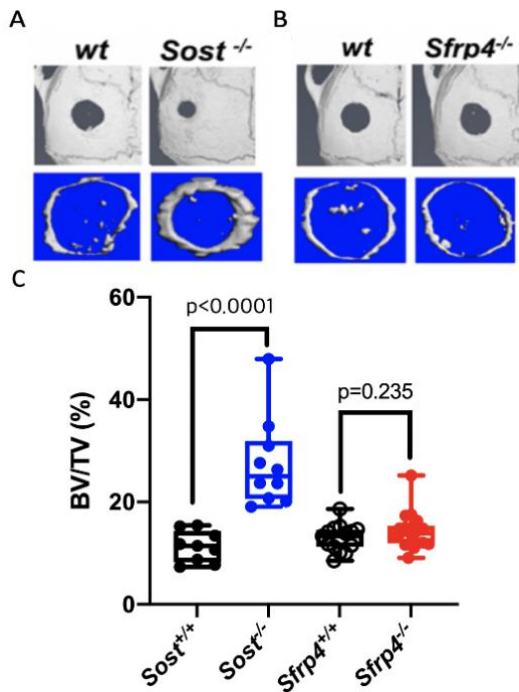

Figure 9. Bone regeneration of calvaria critical defects. Representative images of microCT of (*A*) *Sost*^{-/-} and (*B*) *Sfrp4*^{-/-} and their respective *wt* littermates 6 weeks after surgery. (*C*) Quantification of new bone formation BV/TV (%) within the critical defects. Statical analysis included unpaired Student T-test, p value comparing groups using boxplot with median, interquartile range, max and min values and all data points included (n=9-18).

These results indicate that while activation of canonical Wnt signaling (*Sost*^{-/-}) allows for bone regeneration in the critical defect, deletion of *Sfrp4* and therefore activation of both cWnt and non-cWnt cascade does not.

Differently from *Sost* deletion, *Sfrp4* deletion does not accelerate bone regeneration of subcritical size defects

To explore the effect of *Sfrp4* on healing of subcritical bone defects, we created a 0.8mm diameter calvarial subcritical defect in the left central region of the parietal bone, which has been shown to heal spontaneously.[16] Our studies demonstrated that in subcritical defects, while activation of canonical Wnt signaling (*Sost*^{-/-} mice) led to accelerated bone regeneration, as indicated by increased BV/TV (%) 6 weeks after surgery (Fig. 10*A,C*), *Sfrp4*^{-/-} mice showed only a response similar to *wt* mice (Fig. 10*B,C*).
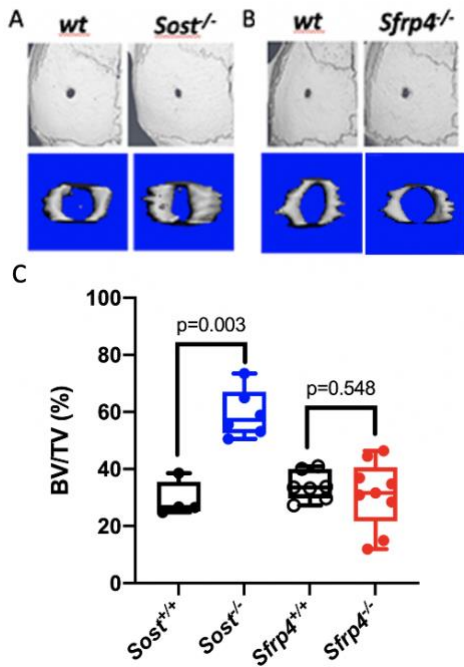
Figure 10. Bone regeneration of calvaria subcritical defects. Representative images of microCT of (*A*)*Sost⁻/⁻*, and (*B*) *Sfrp4⁻/⁻* and their respective wt littermates 6 weeks after surgery (n=4-9). (*C*) Quantification of new bone formation (BV/TV(%)) within the defect 6 weeks after surgery in *Sost⁻/⁻* and *Sfrp4⁻/⁻* and their respective *wt* male and female littermates. Statical analysis included unpaired Student T-test, p value comparing groups using boxplot with median, interquartile range, max and min values and all data points included (n=4-9).

We also investigated potential changes in bone formation in the *Sfrp4* null mice overtime. As shown in Figure 11, no differences in BV/TV (%) were observed 2 and 4 weeks after surgery.
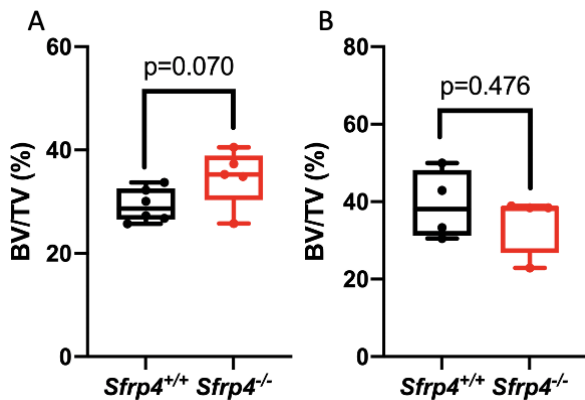

Figure 11. Quantification of new bone formation (BV/TV(%)) within the defect (*A*) 2 weeks (*B*) 4 weeks after surgery in *Sfrp4⁻/⁻* and their respective *wt* male and female littermates. Statical analysis included unpaired Student T-test, p value comparing groups using boxplot with median, interquartile range, max and min values and all data points included (n=4-9).

These results demonstrate that while new bone formation (BV/TV (%)) within the subcritical defect was significantly accelerated in *Sost⁻/⁻* mice, deletion of *Sfrp4* did not lead to acceleration of bone regeneration.

Sfrp4 deletion impairs the response of *Ctsk⁺* periosteal stem cells

The periosteum and the suture contain stem cells which contribute to bone repair and regeneration after injury.[8] Given that we have shown that *Sfrp4* regulates *Ctsk⁺* periosteal stem cell stemness, favoring the accumulation of periosteal progenitors with impaired capability to differentiate into mature mineralizing osteoblasts (Fig. 6 and data not shown), we investigated the response of the *Ctsk⁺* labelled calvarial periosteal stem cells in the absence of *Sfrp4* in response to the injury of subcritical defect. For these studies we used the *mTmG* reporter mouse in which all cells are labelled by dtTomato while the green fluorescent protein (GFP) is expressed only upon Cre recombination (Fig. 12).[21] When these mice are crossed with *Ctsk-Cre* mice, only the cells expressing *Ctsk* (*Ctsk⁺*) are labelled by GFP. This dual labelling allowed us the visualization of *Ctsk⁺* periosteal stem cells (GFP⁺) while *Ctsk* negative cells remain tdTomato⁺.
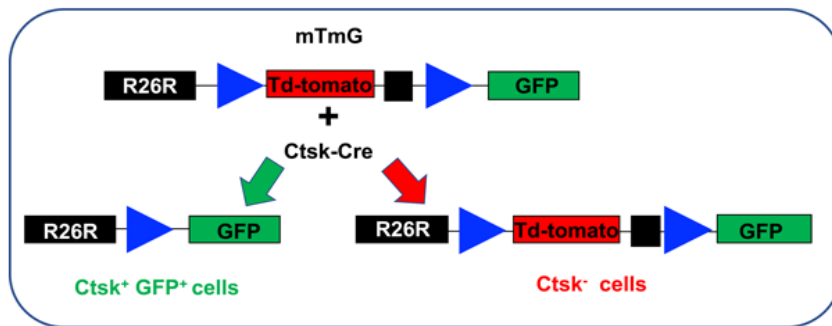

Figure 12. The generation of mouse strain bearing *Ctsk⁺*-GFP cells and *Ctsk⁺* cells using *Cre* activable dual fluorescent reporter gene in the *R26* locus.

Preliminary confocal analyses show a small number of *Ctsk⁺* calvarial periosteal stem cells (GFP⁺) in the sagittal suture mesenchyme at steady state in 8 weeks old mice in both the *CtskCre;mTmG;wt* and *CtskCre;mTmG;Sfrp4⁻ᐟ⁻* mice (Fig. 13*A,B*). Two days after surgery we observed a marked increase in *Ctsk⁺* cells in the sagittal suture mesenchyme of *CtskCre;mTmG;wt* mice(Fig. 13*C*). In contrast, this response was markedly lower in the absence of *Sfrp4* (Fig. 13 *C,D*). A similar response was also seen at 7 and 14 days after surgery (Fig. 13 *E-H*).

Altogether these studies indicate that deletion of *Sfrp4* impairs the response of *Ctsk⁺* cells to injury. The findings that bone repair is impaired in *Sfrp4* null mice in comparison to *Sost* null mice might be a consequence of lack of response of *Ctsk⁺* cells lacking *Sfrp4*.
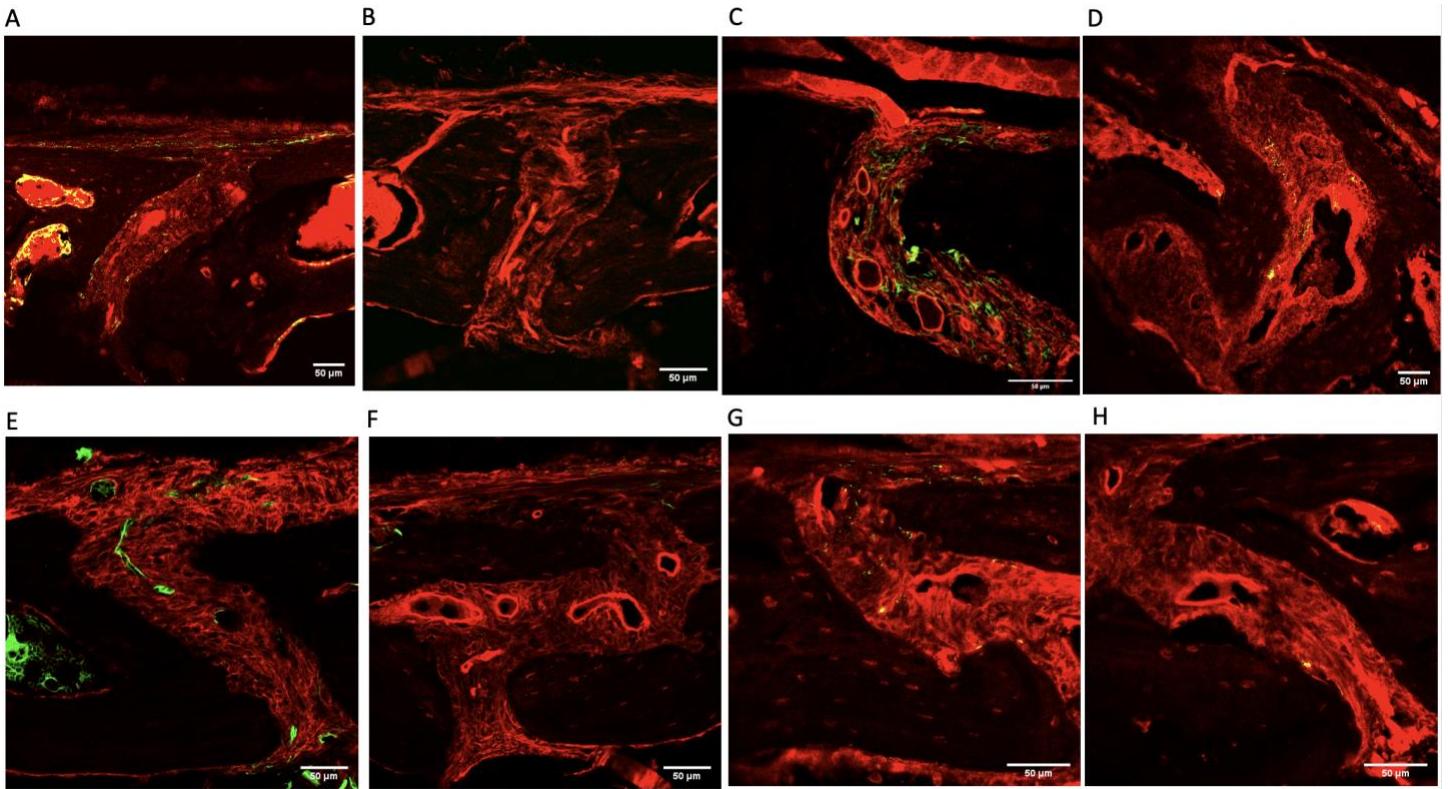
Figure 13. *Ctsk⁺* PSCs in the sagittal suture. *A-B*) Representative confocal images of sagittal suture mesenchyme at steady state in *CtskCre;mTmG;wt* (*A*) and *CtskCre;mTmG;Sfrp4⁻/⁻* mice (*B*). *C-H*) *Ctsk⁺* PSCs (labelled in green) in *CtskCre;mTmG;wt* mice (*C,E* and *G*) and *CtskCre;mTmG;Sfrp4⁻/⁻* mice (*D,F* and *H*) 2, 7 and 14 days after subcritical defect respectively (n=2).

Deletion of the non-cWnt/Ror2 signaling does not ameliorate the bone healing and regeneration responses of *Sfrp4⁻/⁻* mice

We have shown that Ror2/Jnk cascade is involved in the *Sfrp4*-dependent regulation of cortical bone. [7, 20]. We, therefore, asked the physiological relevance of non-canonical Wnt signaling activation in the absence of *Sfrp4* in bone regeneration. To block the Wnt/Ror2 cascade in *Ctsk⁺* stem cells in the suture and calvarial periosteum of the *Sfrp4* null mice, we generated *CtskCre;mTmG;Ror2ᶠˡ/ᶠˡ;wt (Ror2del)*, and *CtskCre;mTmG;Ror2ᶠˡ/ᶠˡ;Sfrp4⁻/⁻ (Ror2del;Sfrp4del)* and performed critical defect as shown in Figure 7*B*. *mTmG;Ror2ᶠˡ/ᶠˡ;wt(Control), mTmG;Ror2ᶠˡ/ᶠˡ;Sfrp4⁻/⁻(Sfrp4del)*, mice did also go under surgery (Fig. 7*B*). Our preliminary data show lack of bone regeneration in all groups (Fig. 14*A,B*). More samples will be needed to make any significant conclusions. These studies are currently underway.
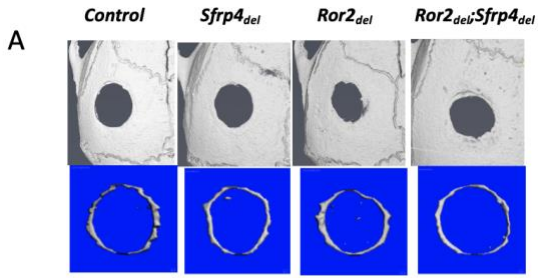
Figure 14. Bone regeneration of calvaria critical defects. Representative images of mCT to assess the role of non-canonical Wnt/Ror2 signaling activation in the *Sfrp4*$^{-/-}$ mice response to bone injury. (*A*) Representative images of mCT of *Control, Sfrp4*$_{del}$, *Ror2*$_{del}$, *Ror2*$_{del}$;*Sfrp4*$_{del}$ of male and female mice (n=1-20). (*B*) Quantification of new bone formation BV/TV (%) within the critical defects. Statical analysis included unpaired Student T-test, p value comparing groups using boxplot with median, interquartile range, max and min values and all data points included (n=1-20).
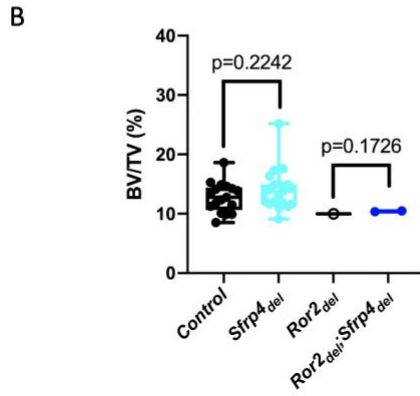
Discussion

Our studies have established that loss of function mutations in the Wnt inhibitor *SFRP4* are the cause of Pyle disease, a rare skeletal disease characterized by limb deformity and fragility fractures (OMIM-265900).[7] *Sfrp4* deletion in mice causes skeletal deformities closely mimicking those seen in individuals with Pyle disease.[7] Using these mice, we demonstrated that activation of distinct Wnt signaling pathways is bone surface-specific and that cortical thinning is due to decreased periosteal bone formation as well as uncoupled remodeling on the endosteal surface, mainly due to the activation of the non-canonical Wnt/Jnk cascade.[7]

The periosteum, a thin layer surrounding the cortical bone, contains a niche of stem cells and progenitors which contributes to cortical expansion during growth, to cortical homeostasis in the adult skeleton, to the response to anabolic drugs and to injury.[22-24] In spite of its clinical significance, our basic understanding of periosteal cellular characteristics, local or paracrine regulatory factors remain elusive.

Here we focused to explore the effect of *Sfrp4* deletion in the periosteal response to injury and subsequent bone regeneration. To this end we used critical calvarial defects as a tool to investigate the mechanism by which *Sfrp4* is involved in bone regeneration. Bone regeneration following bone injuries is a highly coordinated process involving an ensemble of molecules and signaling pathways originating from the suture mesenchyme and periosteum that interact with each other to ultimately regenerate bone. There is evidence that activation of canonical Wnt signaling favors new bone formation at critical defect sites.[15] However, whether non-canonical Wnt signaling is involved in this process remains elusive. Importantly, in what could be a paradigm shift, our data suggest that *Sfrp4* deficiency (which leads to activation of both canonical and non-canonical Wnt cascades) is not sufficient to induce bone regeneration of critical-size defects as seen in the absence of *Sost*. Given that a similar activation of canonical Wnt signaling is observed in *Sfrp4* and *Sost* null mice, we found that non-canonical Wnt signaling needs to be blocked by *Sfrp4* within the sutures and/or periosteum to achieve efficient response after injury. In this study we have investigated the role of *Sfrp4* deletion in bone repair using both a critical and subcritical calvarial defect as a model of bone repair.

Our studies demonstrated that: 1) deletion of *Sfrp4* and *Sost* similarly activates canonical Wnt signaling while *Sfrp4* deletion also activates non-canonical Wnt/Jun cascade in calvariae, 2) activation of canonical Wnt signaling (*Sost* deletion) favors bone regeneration within the initial critical defect while deletion of *Sfrp4* does not and 3) activation of canonical Wnt signaling (*Sost* deletion) led to accelerated bone regeneration while *Sfrp4* deletion showed a response similar to *wt* mice in the subcritical defects.

Importantly we showed that deletion of *Sfrp4* and *Sost* similarly activates canonical Wnt signaling

while *Sfrp4* deletion also activates non-canonical pathway (Fig. 8*A-C*). Our findings show that both *Dkk1*, a Wnt target genes and an inhibitor of the Wnt canonical pathway, and *Tcf1*, a transcription factor of canonical Wnt signaling pathway, are similarly expressed when *Sfrp4* and *Sost* are deleted, while *c-Jun*, a downstream target gene of non-canonical Wnt signaling pathway is highly expressed only when *Sfrp4* is deleted (Fig. 8*A-C*).

We found that *Sfrp4* is expressed in a newly identified population of periosteal *Ctsk*^{+} cells and confocal analyses showed, as expected given that we analyzed 8 weeks old mice, a small number of *Ctsk*^{+} cells at steady state in the suture mesenchyme and in the periosteum (Fig. 13*A,B*). Importantly, our investigations suggest that 1) *Ctsk*^{+} cells respond to the subcritical defect (shown by increased in GFP^{+} cells in the suture) and 2) that lack of *Sfrp4* in these cells impairs their response to injury. Given that differently from *Sost* deletion, *Sfrp4* deficiency does not lead to increased bone formation, it is possible that in the absence of *Sfrp4*, these *Ctsk*[+] periosteal stem cells cannot contribute to bone regeneration like they do when *Sfrp4* is normally expressed (i.e. *Sost* null mice). Further studies will be required to confirm this hypothesis.

We have shown that the Ror2/Jnk cascade is involved in the *Sfrp4*-dependent regulation of cortical bone.[7,17] Thus, we explored the physiological relevance of non-canonical Wnt signaling activation in the absence of *Sfrp4* in our model of bone regeneration. To this end, we used *CtskCre;mTmG;Ror2*^{fl/fl}*;wt* and *CtskCre;mTmG;Ror2*^{fl/fl}*;Sfrp4*^{-/-}, to block the *Ror2* cascade in periosteal and suture *Ctsk*^{+} cells and assess *in vivo* whether new bone formation occurs within the defect of *Sfrp4*^{-/-} mice. Our hypothesis was that if deletion of *Ror2* favors bone regeneration in the *Sfrp4*^{-/-} mice we might conclude that *Sfrp4* suppresses non-canonical Wnt/Ror2 cascade in the suture, an event that is required for proper new bone formation following bone injury. On the other hand, if deletion of the *Ror2* cascade in these cells does not rescue the lack of bone formation seen in the absence of *Sfrp4*, we might conclude that non-canonical Wnt/Ror2 signaling is not involved in the effect that *Sfrp4* deficiency and that other signaling cascades regulated by *Sfrp4* are involved in this process. Our very preliminary data support our second hypothesis (Fig. 14*A,B*). However, more studies need to be done to make any significant conclusion.

In summary, this study demonstrated that fine-tuning of Wnt signaling is critical to achieve proper bone responses. Given that activation of canonical Wnt signaling in the calvaria is similar in both *Sost* and *Sfrp4* null mice, activation of non-canonical Wnt signaling may be responsible for improper function of stem cells within suture and progenitors in the periosteum. Further investigations are focused to find whether *Sfrp4* regulates the function of stem cells in the sutures and in the periosteum in response to bone injury.

Acknowledgments

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