

Notch Signaling in Osteoblast Progenitor Cells is Required for BMP-Induced Bone Formation

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DISCLOSURES: The authors do not have anything to disclose.

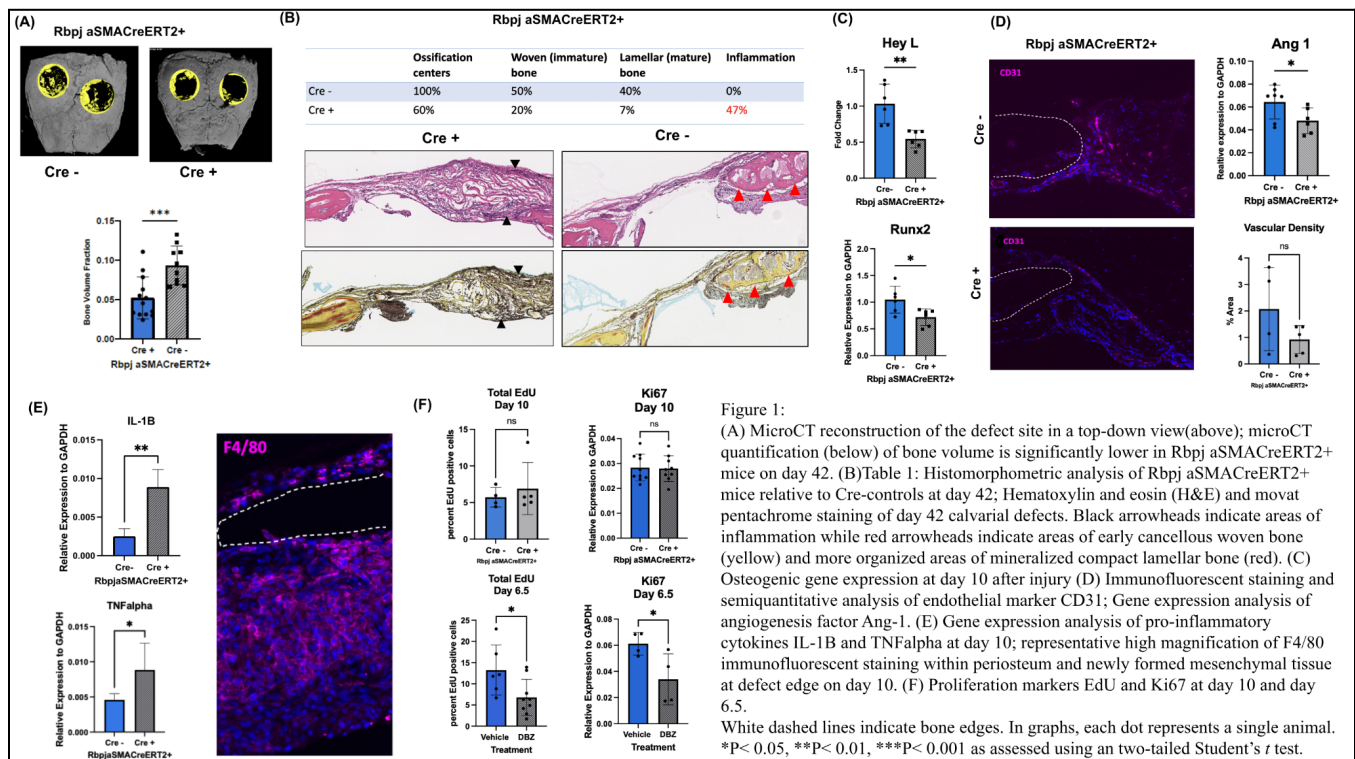
INTRODUCTION: Notch signaling is a cell-to-cell signaling mechanism by which a ligand (Jagged 1-2 or Delta like 1,3-4) binds to a receptor (Notch 1-4) on another cell to trigger an intracellular signaling cascade. We have previously shown that blocking Notch signaling in the osteoblast lineage during early BMP-induced osteogenesis, decreases osteoblast differentiation¹. Additionally, systemic treatment with the γ -secretase inhibitor (GSI), dibenzazepine (DBZ), which blocks Notch signaling, resulted in impaired BMP-induced bone formation in a calvarial critical size defect at day 42². Further, in a tibial fracture model we found that disruption of Notch signaling impairs bone healing while increasing inflammation in the fracture callus³. In this study, the Notch signaling pathway was inhibited post-injury in bone progenitor cells through conditional (tamoxifen regulated) Cre recombinase mediated recombination of *Rbpjk*, which codes for an essential Notch signaling transcription factor.

METHODS: With animal care and use committee approval, a tamoxifen-inducible alpha(α) Smooth Muscle Actin (α Sma) CreERT2 mouse model was used to disrupt the *Rbpjk* in progenitor cells responsible for calvarial bone regeneration. A calvarial defect model was utilized to study BMP-induced bone regeneration in these mice. Parietal bones were exposed by single midline sagittal incisions and the periosteum was scraped off to facilitate drilling. Bilateral 3mm critical-size defects were then created using a piezoelectric drill. A collagen sponge graft was loaded with a solution containing suboptimal (0.25 μ g) BMP2 and placed over the bone defect. Both cohorts were treated with 75mg/kg tamoxifen via intraperitoneal injection on 0, 2, and 4 days post-injury (dp). Whole calvariae were removed and fixed in 4% paraformaldehyde for 48 hours and processed for histology and immunohistochemistry on 6.5, 10, and 42 dp. Mice were injected with EdU 3 hours prior to harvest and EdU assays were performed using a Thermo Fisher Click-It EdU assay. The samples were microCT scanned and analyzed using either Parallax Microview or DragonFly software. Histological images were analyzed using NIS-Elements.

RESULTS: Ablation of *Rbpjk* in α SMA expressing cells during the early healing phase showed a ~50% decrease ($p < 0.001$) in bone volume fraction at 42 days post-injury (Fig 1A). Semi-quantitative histomorphometry results suggest an overall reduction in ossification centers, woven and lamellar bone, and overall repair score in α SMA CreERT2+ *Rbpjk* mice (Fig 1B). Surprisingly, areas of mixed inflammation were still seen in α SMA CreERT2+ *Rbpjk* mice at 42 days after injury signifying a prolonged inflammatory phase. To determine the potential mechanism driving this impaired bone healing, earlier time points were investigated using both immunofluorescence and gene expression analysis. There was significant downregulation of both Notch and BMP key signaling factors (*Hey L* and *Runx2* respectively) at 10 days post-injury (Fig 1C). Impaired angiogenesis was shown by a significant decrease in angiopoietin-1 (*Ang 1*) expression and CD31 immunostaining. Proliferation was evaluated with both EdU staining and *Ki67* gene expression at 10dp and there were no apparent (Fig 1F) differences between CreERT2+ and CreERT2- mice. Qualitative histological analysis at day 10 showed an increased mixed inflammatory cell populations (neutrophils & macrophages) in α SMA CreERT2+ *Rbpjk* mice relative to Cre-controls (40% vs 11% respectively). Similarly, gene analysis revealed a significant increase in pro-inflammatory cytokines *Tnfa* and *Il1b*, 10dp (Fig 1E). Our final experiment examined the impact of systemic Notch inhibition on BMP2-induced bone regeneration at an earlier time point of 6.5 dp. Inhibition of Notch signaling with DBZ significantly decreased the expression of the Notch target gene *Hey2* in the calvarial defects. A significant decrease in proliferation was seen in DBZ-treated mice through analysis of EdU-positive cells (Fig 1F).

DISCUSSION: These results definitively show that Notch signaling within α SMA+ osteoblast progenitors is required for BMP-induced bone formation. While there are cell-autonomous effects of decreased Notch signaling (decreased *Runx2*, decreased proliferation), there are also cell non-autonomous effects, including, increased and sustained inflammation and decreased vascularization. Cellular cross-talk amongst progenitors, osteoblast, inflammatory, and endothelial cells is a hallmark of bone healing, and future studies will probe how Notch signaling in α SMA+ regulates inflammation and angiogenesis.

SIGNIFICANCE: An increased understanding of the interaction between Notch signaling and BMP could lead to advances in the use of BMP as a treatment to repair bone as well as lead to new therapeutic targets which can be utilized to improve patient outcomes.



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