Differential Bone and Vessel Type Formation at Dura and Superior Periosteum During Cranial Bone Defect Repair

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INTRODUCTION: The neurocranium, also known as calvarium, consists of frontal, occipital and two parietal bones to form a protective cavity for brain. The outer surface of calvarial bone is covered by superior periosteum whereas the inner surface of calvarium is covered by dura. While the role of periosteum in repair and regeneration has been well described, the role of dura in cranial bone repair has only been superficially described. A considerable amount of data has suggested that dura could play a significant role in cranial bone repair and further contribute to regenerative activity of tissue engineered constructs. Using transgenic animal models, which allow for selective deletion of progenitor cell pools in bone tissue, here we examined bone defect healing at superior periosteum and dura in spontaneous repair as well as in the repair following intermittent administration of Teriparatide (rPTH). Our study showed that more new bone was formed at dura periosteal surface than superior periosteal surface during spontaneous bone healing. rPTH treatment for 5 weeks proportionally increased dura and periosteal bone formation, leading to thickened parietal bone at the repair site. Targeted deletion of PTH receptor PTHR1via SMA-CreER^{T2} and Col I (2.3)-CreER^{T2} showed selective reduction of bone formation at dura and periosteum site, indicating different progenitor cell pools in dura and superior periosteum. To further understand the differential role of dura and superior periosteum on bone healing, Multiphoton Laser Scanning Microscopy (MPLSM), which allows high resolution 3D analyses of cranial vasculature, was performed to examine blood vessel type distribution during repair. Our data showed differential vessel type distribution at superior and dura periosteum, underscoring the role of blood vessel type and function in bone progenitor cell survival, expansion and ultimate completion of cranial bone defect repair.

METHODS: Murine cranial defect repair model. Following aseptic technique, the calvarial bone was exposed and a 1mm circular full thickness defect was created using a Microdrill with a same-sized Busch inverted cone bur. Samples were harvested and processed for histologic, immunofluorescence and imaging analyses. Mouse strains used in the study: Col 1 (2.3) GFP mouse model which labels osteoblasts with GFP. PTH1R$^{f/f}$ mouse model which allows conditional deletion of PTH type I receptor. Tamoxifen inducible SMACreERT^2 and Col I (2.3) CreER mice crossed with Ai14 reporter mice. Tamoxifen (TM) was given via i.p. to all mice at a dose of 1mg/mice at 7 different time point, 2 days prior to surgery, and 2, 4, 6, 14, 21, 28 days after surgery. Intermittent Teriparatide (rPTH) treatment: Teriparatide rhPTH at a dose of 80μg/kg was administered daily for 4 weeks via Sub-Q injection. MPLSM: an Olympus FVMPE-RS system was used for high resolution imaging. The fluorescence of GFP, RFP, far-red RFP and Second Harmonic Generation (SHG) were collected with a 517/23-nm, a 605/25-nm, a 665/20nm, and a 390/20-nm bandpass filters, respectively. The analyses of the defect were performed using Imaris and Amira software.

RESULTS: Differential contribution of dura and superior periosteum during bone defect repair. Bone formation was examined at week 1, 3 and 5 in control and rPTH treated mice via MicroCT. rPTH treatment increased the thickness and volume of the cranial parietal bone at the repair site by 85% and further reduced the defect area by an average of 31 % (n=10, $p<0.05$). Histomorphometric analyses showed that the majority of bone was formed at the leading edge as well as along the inner surface of parietal bone (dura). Only ~10% of new bone was formed at the outer surface (superior periosteum). While rPTH markedly increased bone formation at all three sites, 3-fold more new bone was identified along the surface of dura than the surface of superior periosteum (n=8, $p<0.05$). Differential inhibition of bone formation at repair site following targeted deletion of PTH1R. To further delineate the response of bone forming cells to anabolic treatment of rPTH, we examined the rPTH enhanced repair following TM-mediated conditional deletion of PTH receptor type 1 (PTHR1) in mesenchymal progenitors via SMACreER^{T2} and osteoblasts via Col I (2.3)-CreER^{T2} mouse model. Using Ai14 reporter mice, we found that SMA-CreER^{T2} mouse model targeted superior periosteum, soft tissue, new bone formed along bone marrow cavity and at the leading edge of the cranial defect, with few cells targeting dura (Fig. 1A). Consequently, targeted deletion of PTH1R via SMA-CreER^{T2} significantly reduced new bone on the superior periosteal surface and at the leading edge of bone defect, but had little effects on bone formation along the dura surface following rPTH treatment (Fig. 1B-E). In comparison, targeted deletion of PTHR1 in osteoblasts via Col I (2.3)-CreER^{T2} led to reduction of new bone at all three sites in rPTH treated samples (Fig. 2, n=5-9 per group).
Differential blood vessel type distribution and function in dura and superior periosteum. Neovascularization at dura and superior periosteum was examined both prior to and following injury using high resolution MPLSM. Compared to superior periosteum which consisted of thin layer of poorly defined blood vessels, the vessels at dura was well-spaced and organized into a thick layer of network that consists of CD31$^+$EMCN$^-$ arterioles, and CD31$^+$EMCN$^+$ capillaries and CD31$^+$EMCN$^+$ venous vessels (Fig. 3A&B). Strong angiogenic response was identified at dura as early as day 3 post-injury. rPTH treatment markedly enhanced the volume of CD31$^+$EMCN$^+$ vessels coupling to Col I (2.3) GFP$^+$ osteoblasts and further enhanced the length of CD31$^+$EMCN$^-$ arterioles, leading to an orderly enhanced angiogenesis in bone. In contrast, angiogenic response at the superior periosteum was markedly delayed until day 10 with the majority of vessels coming from the dura. rPTH further enhanced small arterial capillaries as well as the distinct large diameter Type H vessels within marrow cavity at week 3 post surgery (Fig. 3 panel C&D). Quantitative analyses of vessel types from dura side showed significant induction of different vessel types at the repair site, with nearly 3-fold induction of type H and CD31$^+$EMCN$^-$ capillary vessels in bone tissue. rPTH treatment had no effect on vessels outside the bone tissue (Fig. 3E-L, n=4 $p<0.5$).

DISCUSSION: Cranial bone is distinct from long bone in its structure and composition. In our current study, we show a strong contribution of dura to bone defect repair, suggesting a bigger role of dura than superior periosteum in calvarial bone repair. Using transgenic mouse models that target different pools of bone forming cells, our data show that dura likely contains a diverse progenitor pool that is different from superior periosteum. These cells cannot be effectively labeled by SMACreER^{T2} but are more responsive to injury than progenitor cells at the superior periosteum. Consistently, vessel type distribution and vessel function are also markedly different in dura and superior periosteum, with dura consisting of well-organized vessel network that can quickly expand into functional vessel network to support progenitor cell expansion and bone formation. rPTH has a strong effect on the expansion and transformation of these vessels, leading to rapidly enhanced bone formation and remodeling during repair and regeneration. The poor healing response at the superior periosteum could be attributed to the poor organization and delayed neovascularization at the superior periosteum.

SIGNIFICANCE/CLINICAL RELEVANCE: Our current study demonstrates a differential response that depends on an organized angiogenesis coupling to osteoblasts at dura and superior periosteum during calvarial bone defect repair. The study offers new insights into the mechanisms of healing that could be used to direct repair and tissue engineering of bone tissue at the site of cranial bone defect.

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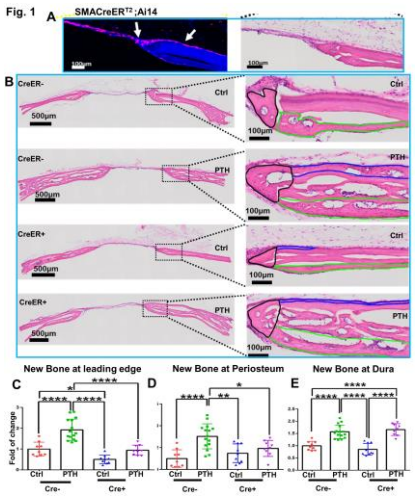

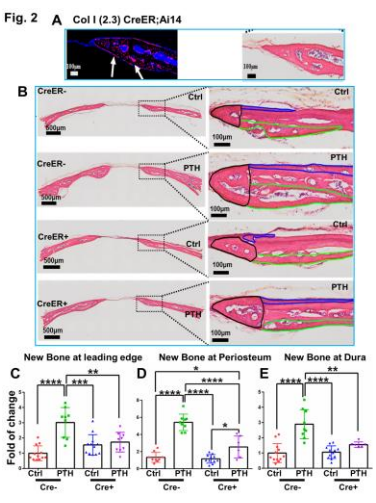

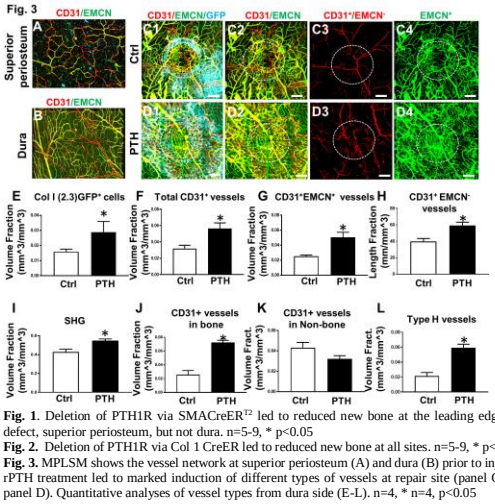

Fig. 1. Deletion of PTH1R via SMACreER^{T2} led to reduced new bone at the leading edge of defect, superior periosteum, but not dura. n=5-9, * $p<0.05$
Fig. 2. Deletion of PTH1R via Col 1 CreER led to reduced new bone at all sites. n=5-9, * $p<0.05$
Fig. 3. MPLSM shows the vessel network at superior periosteum (A) and dura (B) prior to injury. rPTH treatment led to marked induction of different types of vessels at repair site (panel C vs. panel D). Quantitative analyses of vessel types from dura side (E-L). n=4, * n=4, $p<0.05$