The Mohawk homeobox gene represents a marker and osteo-inhibitory factor in cranial suture stem cells

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INTRODUCTION: The regeneration of craniofacial bones of the mammalian skeleton necessitates the action of both intrinsic and extrinsic inductive factors from multiple cell types, which function in a hierarchical and temporal fashion to control the differentiation of osteogenic progenitors. Single-cell transcriptomics of developing mouse cranial suture recently identified a suture mesenchymal progenitor population with previously unappreciated tendon- or ligament-associated gene expression profile. Among teno-ligamentous genes is the transcription factor Mohawk (*Mkx*), an IRX-family homeobox protein involved in vertebrate developmental patterning and critical for tenogenesis. *Mkx* regulates cellular processes such as cell adhesion and migration. *Mkx* has also been implicated in homeostatic maintenance of the teno-ligamentous microenvironments, such as the periodontal ligament and Achilles tendon. However the regulatory role of *Mkx* in other tissues has not been explored. In this study, we used transgenic mouse models and single cell transcriptomics to investigate the role of *Mkx*$^{+}$ cranial suture cells.

METHODS: All experiments were conducted under IACUC approval within the Johns Hopkins University. We engineered *Mkx* reporter mice *Mkx*^{CG}; R26R^{tdT} to examine *Mkx*$^{+}$ cell distribution in the cranial vault. Then, single-cell RNA sequencing (ScRNA-Seq) was performed on cells from calvarial bones of uninjured, 7 d, and 28 d post-injury in *Mkx* reporter mice. The transcriptional characteristics of calvarial cells and *Mkx*$^{+}$ cells were analyzed during defect healing. Next, a cell ablation strategy was used to assess the requirement of *Mkx*-expressing cells for bone repair by crossbreeding *Mkx*^{tdT} mice with previously validated iDTR mice (*Mkx*$^{tdT/iDTR}$). *Mkx*$^{+}$ cell ablation was achieved by local diphtheria toxin (DTX) administration. *Mkx* gene knockout (KO) were performed in *Mkx*$^{fl/fl}$ animals by adenovirus-encoding Cre recombinase (Ad-Cre). Calvarial bone healing was assessed following either *Mkx*$^{+}$ cell ablation in *Mkx*^{tdT} and *Mkx*$^{tdT/iDTR}$ mice or local *Mkx* gene deletion in *Mkx*$^{fl/fl}$ animals over a 28 d period, and bone healing was assessed by high resolution micro computed tomography (microCT), as well as histology and immunohistochemistry for Osteocalcin (OCN) and CD31. Unpaired two-tailed Student t-test was used for a two-group comparison.

RESULTS: We demonstrated that *Mkx* reporter identifies an adult cranial suture resident cell population that gives rise to calvarial osteoblasts and osteocytes during homeostatic conditions (**Fig.1**). ScRNA-Seq data reveal that *Mkx*$^{+}$ suture cells display a stem-like phenotype with expression of teno-ligamentous genes. Bone injury with *Mkx*$^{+}$ cell ablation showed significantly impaired bone healing among *Mkx*$^{tdT/iDTR}$ mice. Micro-CT reconstructions and cross-sectional images demonstrated impaired re-ossification (**Fig.2A**), including bone volume (BV, 40.2% reduction, **Fig.2B**), fractional BV (BV/tissue volume (TV), 39.9% reduction, **Fig.2C**), mean diameter of the bone defect area (36.3% increase, **Fig.2D**), and bone fractional area (BFA, 36.4% decrease, **Fig.2E**). The reduction in bone healing in *Mkx*$^{tdT/iDTR}$ animals was further confirmed by OCN immunostaining (**Fig.2F, G**), and associated with reduced angiogenesis at the healing edge, shown as decreased CD31+ blood vessels (54.3% reduction). Remarkably, *Mkx* gene played a critical role as an osteo-inhibitory factor in cranial suture cells, as *Mkx* knockdown or knockout resulted in increased osteogenic differentiation *in vitro* in cranial suture cells (**Fig. 3A, B**). Furthermore, localized deletion of *Mkx in vivo* resulted in robustly increased calvarial defect repair (**Fig.3C-G**), including BV (93.7% increase), BV/TV (92.7% increase), mean diameter of the bone defect area (25.6% reduction), and BFA (50.5% increase).

DISCUSSION: In summary, we have identified *Mkx*$^{+}$ cells within the suture mesenchyme with a stem/osteoprogenitor phenotype and a teno-ligamentous gene profile that participates in calvarial bone turnover and bone repair. Depletion of this *Mkx*$^{+}$ progenitor cell population hampered the repair of calvarial defects, yet *Mkx* itself also functions as an osteogenic inhibitor. When *Mkx* was deleted locally, strikingly enhanced cranial bone repair was observed.

SIGNIFICANCE/CLINICAL RELEVANCE: Further characterization of *Mkx*$^{+}$ cells may lead to new insights into the regulatory role of *Mkx* in cranial vault patterning and regeneration, as well as new mechanisms to speed skeletal repair or address craniofacial deformities.

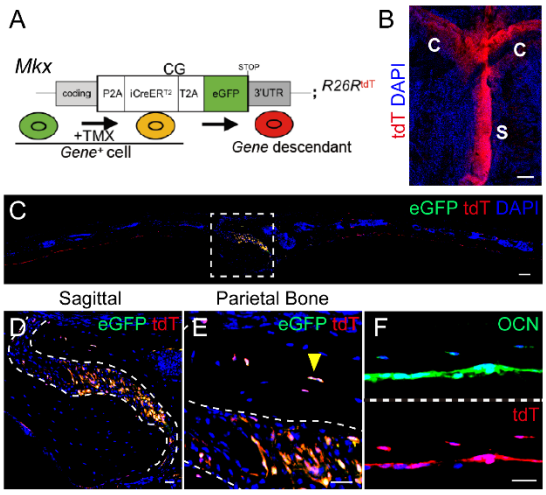

Fig 1. ***Mkx*$^{+}$ *cells populate the cranial suture mesenchyme and contribute to cranial bone turnover.*** **(A)** Diagrams of the *Mkx*^{CG} driver. *Mkx*-expressing cells are labeled with GFP. Following tamoxifen (TM) administration, *Mkx*-expressing cells will be permanently labeled with TdTomato (tdT). **(B)** Top down whole-mount image of calvarial bones 2 wks after TM injection. C: coronal and S: sagittal suture. GFP not shown. **(C-F)** Representative images of *Mkx* reporter activity within **(C)** Tile scan of parietal bone, **(D)** sagittal suture and **(E)** suture adjacent osteocytes at 6 mo after TM administration. Yellow arrowheads indicate reporter positive osteocyte. **(F)** OCN immunostaining and *Mkx* reporter activity at 6 months after TM administration. GFP reporter activity not shown.

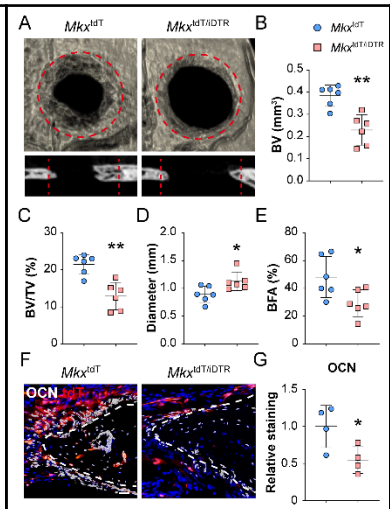

Fig 2. ***Mkx+ cell ablation leads to compromised injury repair.*** **(A)** µCT reconstructions of the defect site. (**B-E**) µCT quantification of bone healing among *Mkx*^{tdT} and *Mkx*$^{tdT/iDTR}$ mice, including (**B**) bone volume (BV), (**C**) bone volume/tissue volume (BV/TV), (**D**) residual defect diameter, and (**E**) bone fractional area (BFA). (**F**) Immunostaining of OCN at the defect edge and (**H**) quantification. White dashed lines indicate healing bone edges. Scale bar=100 µm. *$p < 0.05$; **$p < 0.01$.

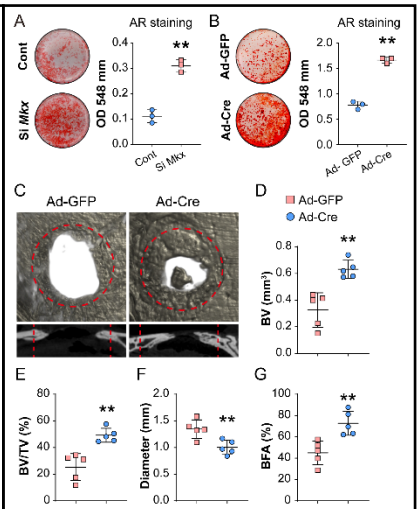

Fig 3. ***Mkx knockout enhances calvarial bone repair.*** **(A, b)** Representative Alizarin red staining at d 10 of osteogenic differentiation (left) and quantification (right) within cranial suture cell post (**A**) siRNA mediated *Mkx* KD, and (**B**) Ad-Cre mediated *Mkx* KO. (**C**) µCT reconstructions of the defect site. (**D-G**) Quantification of bone healing among control and Ad-Cre treated mice, including (**D**) BV, (**E**) BV/TV, (**F**) residual defect diameter, and (**G**) BFA. **$p < 0.01$.