Cdk8 Underpins Delayed Healing During Ischemic Fracture Repair

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INTRODUCTION: Non-union fractures occur in ~10% of long bone injuries, however this number is increased up to 46% under ischemic conditions[1]. We previously performed single cell RNA sequencing (scRNA-seq) of the intact and ischemic fracture callus and identified Cyclin Dependent Kinase 8 (Cdk8) as a gene of interest due to its overexpression under ischemic conditions[2]. Cdk8 is a cyclin-dependent protein that functions as part of the Mediator Complex to regulate cell metabolism. Cdk8 also regulates signaling processes upstream of TGF**β** and BMP, and recent studies have demonstrated a regulatory role for Cdk8 in bone homeostasis[3-6]. Despite these observations, the role of Cdk8 in the context of chondrogenesis and fracture callus formation are not clear. We hypothesize that inhibiting Cdk8 will improve chondrogenesis, endochondral ossification, and impaired fracture repair.

METHODS: With animal care and use approval, C57 Bl/6 WT mice were subjected to an ischemic injury prior to a left transverse tibia fracture. The femoral artery was isolated; a 1 mm section was resected, limiting perfusion to the distal limb. Fractures were generated using a three-point-bend apparatus. Mice with intact vasculature were fractured as controls. scRNA-seq was performed, and Seurat was utilized to identify cellular subsets within the callus. Chondrogenic populations were identified by expression of chondrogenic markers, namely Sox9 and Aggrecan. Intact and ischemic fractures also underwent formalin fixation paraffin embedding and sectioning. Immunofluorescent quantification of Sox9 was performed on 10X stitches of whole callus regions using Cell Profiler. Cdk8 expression was assessed in the stromal population through scRNA-seq read counts. Human mesenchymal progenitor cells (hMSCs) underwent pelleted 3D chondrogenic differentiation in the presence of Cdk8 inhibitor, Senexin B. Chondrogenic gene expression was assessed via gene analysis of Aggrecan, Collagen II, and Collagen X. Content of sulfated glycosaminoglycans (sGAGs) was quantified through DMMB analysis. To assess the effect of Cdk8 inhibition *in vivo*, mice underwent ischemic fractures as described above and underwent systemic (IP) treatment with 36 mg/kg Senexin B starting at day -1 to day 8 post fracture. Mice were harvested at 10 days post fracture. µCT analysis and histomorphometry was performed to phenotype the callus.

RESULTS: Assessment of the stromal population through scRNAseq reveals decreased chondrogenic cells under ischemic conditions **(Fig 1A)**. This is supported by a trend of decreased Sox9 positive nuclei with ischemia **(Fig 1B)**. *Cdk8* expression was found to be upregulated under ischemic conditions **(Fig 1C)**. Alcian blue staining of hMSCs reveals robust chondrogenesis under control differentiation and treatment with the Cdk8 inhibitor, Senexin B by day 21 **(Fig D).** At 14 days, Senexin B increased the expression of chondrogenic markers Aggrecan, and Collagen II and improved sGAG content **(Fig 1E-F)**. This persists to day 21 **(Fig 1F**), suggesting that Cdk8 inhibition via Senexin B promotes chondrogenesis and matrix deposition. *In vivo* treatment with Senexin B increased cartilage content in the fracture callus as demonstrated through increased Safranin-O staining (**Fig 1G**). Additionally, there is less mineralization at the edges of the Senexin B-treated callus compared to Vehicle. µCT analysis further corroborates this, with distinctly less peri-cortical mineral present in Senexin B-treated calluses, and a decrease in total bone volume was observed **(Fig 1H)**.

DISCUSSION: Our findings highlight decreased chondrogenesis under ischemic conditions with associated increased expression of Cdk8. Inhibition of Cdk8 appears to increase chondrogenesis of hMSCs *in vitro* and in the murine fracture callus *in vivo*. While previous studies have demonstrated that Cdk8 inhibition promotes osteogenesis, our data suggest that in the context of bone healing, Cdk8 inhibition may sustain chondrogenesis, and either limit endochondral and/or intramembranous bone formation[3]. It is also possible that Cdk8 is not deleterious to fracture healing and that its overexpression with ischemia functions as a protective response under impaired conditions and that herein, our Cdk8 inhibition further delays the healing response and increases risk of nonunion. Further work will involve interrogation of Cdk8 inhibition and overexpression temporally in fracture healing as well as explore Cdk8 expression in other impaired healing scenarios, such as aging. As well, we will pursue local therapeutic regulation of Cdk8 to the fracture site, as one shortcoming of Senexin B treatment in this study was the systemic administration.

SIGNIFICANCE/CLINICAL RELEVANCE: Nonunion fractures result in prolonged healing for the patient, with enormous societal and individual burden. While we observed Cdk8 upregulation within our ischemic fracture group, Cdk8 upregulation has also been identified in aged MSCs, suggesting that this phenotype of increased Cdk8 may be prevalent across impaired healing conditions[5]. Therapeutic targeting of Cdk8 may represent a method to improve callus formation during impaired healing conditions.

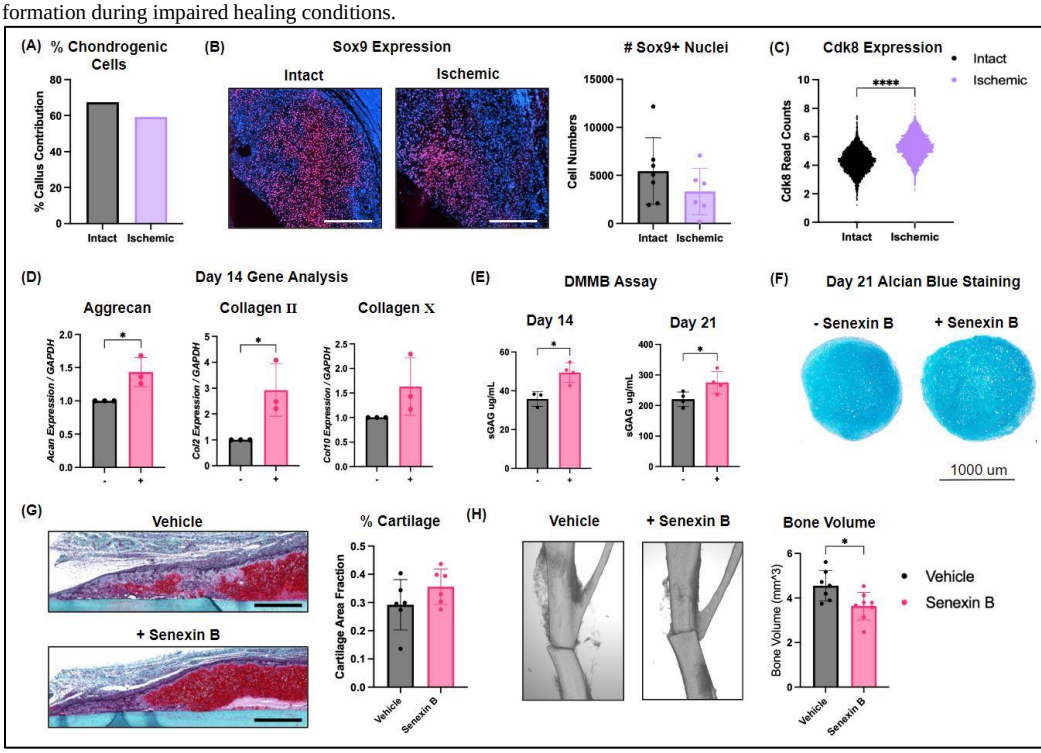

Figure 1: Ischemia upregulates Cdk8 expression within the early fracture callus resulting in decreased chondrogenesis (A) Percent chondrogenic cells within the intact and ischemic fracture callus at 7 days post fracture. **(B)** Number of Sox9+ Nuclei in the intact and ischemic fracture callus quantified through histomorphology. Scale bar = 500 µm **(C)** Cdk8 read counts in the stromal population of the intact and ischemic fracture callus at 7 days post fracture. **(D)** Gene expression of chondrogenic markers - Aggrecan, Collagen II, and Collagen X in hMSCs treated with chondrogenic media in the presence of Senexin B. **(E)** Quantification of sulfated glycosaminoglycans in the hMSC chondrogenic pellets treated with Senexin B. **(F)** Representative alcian blue staining of chondrogenic pellets. **(G)** 10X stitch and cartilage quantification of Safranin-O staining of ischemic fracture calluses treated with Senexin B 10 days post-fracture. Scale bar = 500 µm. **(H)** µCT analysis of mineralization at 10 days post fracture of ischemic fractures treated with Senexin B and bone volume quantification. $*P<0.05$, $**P<0.01$, $***P<0.001$, two-sided t test.

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