DMP1 Lineage Connexin43 Deficiency Attenuates Decreases in Skeletal Muscle Strength and Fracture Callus Mineralization During Hindlimb Unloading

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INTRODUCTION: Preclinical studies have shown that musculoskeletal unloading by hindlimb suspension (HLU) decreases hindlimb bone and skeletal muscle mass and alters fracture healing. Our lab and others have shown that deletion of connexin 43 (Cx43), the predominate gap junction protein in bone, in early osteoblasts/osteocytes via (Col1A1 or Osteocalcin) decreases postnatal skeletal muscle mass and impairs fracture healing.[1,2] While deletion of Cx43 using Osteocalcin Cre protects against HLU associated bone loss, the role that mature DMP1 lineage osteoblast/osteocyte Cx43 deficiency plays in muscle atrophy and fracture healing during HLU remains unclear. We hypothesized that mature osteoblast and osteocyte Cx43 deficiency (Dmp1-Cre) would decrease bone healing and hindlimb musculoskeletal tissue mass and strength during normal ambulatory conditions, but is protective during hindlimb unloading.

METHODS: All animal procedures were approved by the VCU IACUC. Cx43 cKO animals consisted of female C57BL/6J mice harboring the noninducible 10 kb *Dmp1-Cre* transgene, *Gja1*$^{fl/fl}$ (Cx43 floxed) and *Ai14* (TdTomato) alleles. Littermate Cre-negative mice (WT) served as controls. All mice underwent HLU or normal weight-bearing activity (controls) for 3 weeks following skeletal maturity (26 weeks) to simulate extended disuse. Mice then had their right femur fractured by open surgical dissection (stabilized with 24-gauge pin), and were allowed to heal for 14 days (DPF14). At DPF14, *Gja1* conditional gene deletion and Cx43 protein expression was assessed in uninjured muscle and bone by qPCR and Immunohistochemistry (IHC). Hindlimb skeletal muscle strength was assessed *in vivo* using the anterior crural muscles to elicit maximum twitch (40ms frequency) and tetanic torque (150ms frequency). Fractured femoral bone formation were analyzed by micro-CT (Bruker Skyscan 1276), bone turnover by ELISA (CTX and BGLAP) and osteoclast indices in the callus by TRAP$^+$ staining, respectively. Data were analyzed by 2-WAY repeated measures using ANOVA with Tukey's multiple comparisons test (*p< 0.05).

RESULTS: At DPF14, qPCR and IHC demonstrated significant knockdown of *Gja1* expression (**Fig 1A**) and Cx43 protein (**Fig 1B**) in uninjured cortical bone (especially osteocytes and periosteal cells), but not skeletal muscle cells, confirming bone-specific deletion. HLU mice demonstrated significantly reduced normalized gastrocnemius mass and soleus independent of Cx43 deficiency (**Fig 1C**). Surprisingly, Cx43 deficiency increased *in vivo* twitch force compared to control mice and significantly attenuated tetanic torque with HLU compared to WT (**Fig IC**). Fractured Cx43 deficient femurs showed significantly reduced callus mineralization compared to WT, with trending decreases in systemic bone turnover as well (**Fig 2A**). Cx43 cKO fractured femurs showed significant attenuation of osteoclasts in woven bone regions of the callus, especially during HLU (Fig **2B**).

DISCUSSION: Our results suggest that loss of Cx43 in mature osteoblasts and osteocytes using DMP1 Cre decreases fracture callus mineralization *in vivo* while attenuating trends due to HLU, thereby confirming our hypothesis. These data are similar to findings seen using Osteocalcin-Cre and suggest that Cx43 in later stage osteoblast lineage cells (mainly osteocytes and woven bone lining cell) is a prerequisite for successful callus mineralization and remodeling.[2] However, contrary to our other hypothesis, Dmp1-Cre Cx43 deficiency didn't significantly decrease *in vivo* muscle strength or muscle mass under control conditions similar to reports by others using Col1a1 Cre[1]; in fact it protected against HLU induced deficits in muscle contraction. Although prior reports suggest that Cx43 deficiency in earlier osteoblast lineage cells (Col1a1)[1] and late osteoblast lineage (DMP1)[3] impair muscle development and function with aging, our results were different. These findings are novel, exciting, and suggest altered mechanosensation of skeletal muscle and callus tissues with disuse compared to aging that warrants further mechanistic study.

REFERENCES: 1. Shen et al. J Bone Miner Res. 2015 **2.** Loiselle et al. J Orthop Res. 2013 **3.** Li et al. Int J Mol Sci. 2022

SIGNIFICANCE/CLINICAL RELEVANCE: These findings suggest that DMP1 lineage Cx43 deficiency may be a means to selectively increase muscle strength while decreasing fracture callus sensitivity to mechanical unloading.

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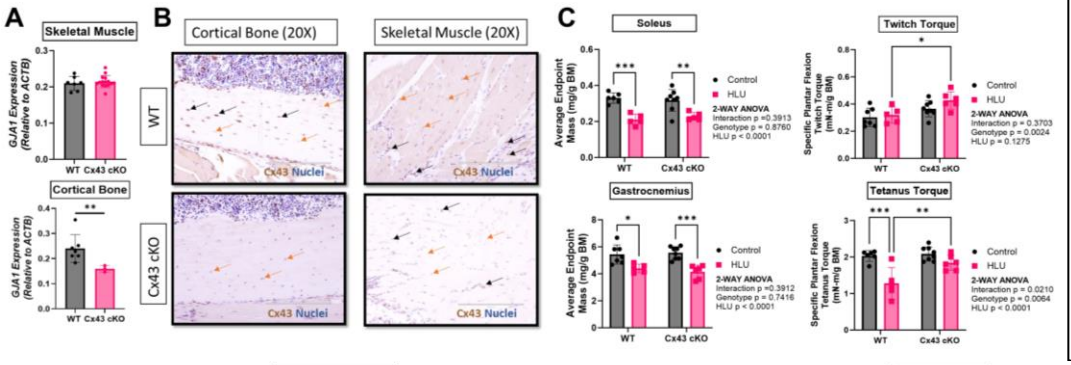

Figure 1. DMP1 mediated Cre activity deletes CX43 in cortical bone but not skeletal muscle via **Panel A:** whole tissue qPCR (normalized to ACTB) and **Panel B:** cell phenotyping by immunohistochemistry. Black arrow = Cx43$^+$ cell; Orange arrow = Cx43$^-$ cell. ** p <0.005 by student t-test. **Panel C:** DMP1 mediated Cx43 knockdown leads doesn't protect against HLU mediated muscle mass loss but does increase torque generation from *in vivo* muscle stimulation using Aurora 1300. *** p <0.0005; ** p <0.005; * p <0.05 by 2-WAY ANOVA with Tukey.

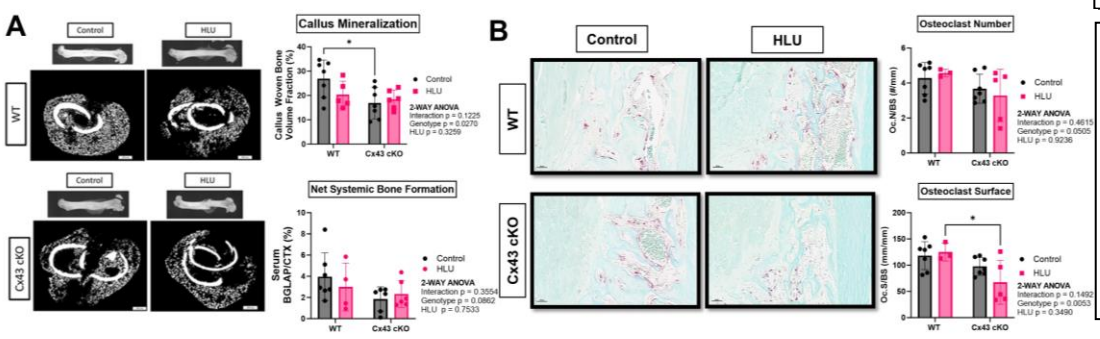

Figure 2. Panel A: DMP1 mediated Cx43 knockdown leads to reduced fracture femur callus mineralization via microCT scanning and trends toward reduced systemic bone turnover via ELISA. **Panel B:** Histology supports attenuated bone remodeling in Cx43 cKO mice by reduced osteoclastogenesis on TRAP$^+$ stained sections in woven bone regions of the callus. * p <0.05 by 2-WAY ANOVA with Tukey Post-hoc.