# Inhibition of CGRP-CLR Pathway on Bone Fracture Healing

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**Disclosures:** No conflict of interest

INTRODUCTION: Calcitonin gene-related peptide (CGRP), a neuropeptide, plays various neuroendocrine roles such as vasodilation and pain modulation. Studies have indicated effects of CGRP on enhancing bone formation, inhibiting bone resorption, and promoting vascular growth. CLR (gene *Calcrl*), a member of the G-protein coupled receptor (GPCR) family, dimerizes with the receptor activity modifying protein 1 (*Ramp1*) creating CGRP receptor. We previously demonstrated increased cells differentiation in response to CGRP stimulation, negative effect of CGRP inhibitor on bone fracture healing, expression of *Calcrl* in periosteal and endothelial cells (ECs), and delayed bone fracture healing process when CLR is deleted in mesenchymal lineage (αSMA-CreER/CLR$^{fl/fl}$) mice [1]. The formation of fracture callus is highly dependent on ECs through angiogenesis establishing sufficient tissue supply and oxygenation. In this study, our aim is to investigate the effects and signaling of targeted CLR deletion in ECs on bone fracture healing.

METHODS: Cdh5-CreER mice were bred with CLR$^{fl/fl}$ mice to selectively target deletion in ECs. Transversal femoral fractures were made in 8-10 weeks old mice. To induce CLR deletion, both Cre$^{-}$ and Cre$^{+}$ mice were treated with 75 mg/kg tamoxifen on -2/0/2 days post fracture (DPF). DNA recombination was confirmed in callus tissue on 7DPF in both male and female mice but significant deletion in Cre$^{+}$ mouse was only found in male. To achieve the goals, real-time qPCR, immunohistology, 10X single cell RNA sequencing (scRNA-seq), bulk RNA sequencing, microcomputed tomography, and mechanical testing were all conducted in male mice at different fracture timepoints. 10X scRNA-seq was performed using CD45$^{-}$/Ter119$^{-}$ cells and data were analyzed using Seurat package in RStudio. Bulk RNA-seq was conducted on sorted endothelial cells (CD45$^{-}$/Ter119$^{-}$/CD31$^{+}$) and mesenchymal (CD45$^{-}$/Ter119$^{-}$/CD31$^{-}$) populations isolated from periosteum callus at 4DPF from Cdh5Cre/CLR$^{fl/fl}$ mice (Cre$^{-}$ & Cre$^{+}$), and raw data were processed on Xanadu high-performance computing cluster and analyzed in RStudio using DESeq2 package. Data visualization and statistical plots were generated using the ggplot2 package in R. 3-4 mice samples were pooled for populations sorting and cells collection. To perform statistical analysis, GraphPad Prism 10 software was used. An unpaired, 2-tailed Student's t test was performed to determine differences between 2 groups, with *p*-value less than 0.05 set as the threshold for statistical significance between the tested groups. Data are presented as mean value ± standard error of the mean (SEM). The number of bone samples included in each experiment is listed in result section. All animal procedures were approved by IACUC.

RESULTS: The reduced gene expression of *Calcrl* and *Ramp1* in 7DPF callus tissue of Cre$^{+}$ mouse was confirmed (n=6-9, $p<0.05$) (Figure 1A). The significantly decreased cartilage area was found in Cre$^{+}$ mouse at 7DPF by Safranin O staining (n=11-12, $p<0.05$) with no difference in bone strength and stiffness, callus bone mass, and bone total volume at 21DPF (n=6-8). Unsupervised clustering of 10X scRNA-seq data determined clusters of undifferentiated mesenchymal (MSCs), chondroblasts, chondrocyte, osteoblasts, endothelial cells, satellite cells, smooth muscle cells, tenocytes clusters, macrophages, and erythroid cells (Figure 1B). Deletion of CLR within ECs led to significantly decreased expression of multiple chondrogenesis related genes such as *Col11a1*, *Col11a2*, *Col2a1*, *Ibsp*, *Acan*, *Comp* in MSCs, osteoblast and chondrocyte clusters (Figure 1B). Gene Set Enrichment Analysis (GSEA) detected multiple down-regulated biological pathways from Gene Ontology (GO) including mesenchymal cell differentiation, ossification, cartilage development, chondrocyte differentiation, skeletal system morphogenesis (Figure 1C). KEGG pathway analysis showed down-regulation of ECM-receptor interaction and PI3K-Akt signaling in those clusters from Cre$^{+}$ mouse, suggesting suppressed cell proliferation activity. Histological analysis of Ki67 staining was performed at 4DPF, revealing lower number of Ki67$^{+}$ cells ($p=0.06$) in Cre$^{+}$ calluses. Furthermore, KEGG pathway analysis of bulk RNA-seq data revealed enrichment of TNF and chemokine signaling pathway within CD31$^{+}$cells from Cre$^{+}$ mice at 4DPF (n=3-4). Consistently, a significant upregulation *Ccl2* expression was observed in 7DPF callus tissue from Cre$^{+}$ mice, indicating sustained inflammation activation.

DISCUSSION: Our results demonstrate that endothelial-specific deletion of CLR suppresses cell proliferation, mesenchymal differentiation and chondrogenesis, while promoting prolonged inflammation during fracture healing. Impaired CGRP-CLR pathway in ECs impairs the activity and function of mesenchymal progenitor cells, which potentially results in impaired bone fracture healing process early in a fracture healing process. Disruption of the CGRP–CLR signaling pathway in endothelial cells impairs mesenchymal progenitor cell activity and function, thereby leading to impaired bone fracture healing.

SIGNIFICANCE/CLINICAL RELEVANCE: These findings highlight a previously unrecognized regulatory role of CGRP–CLR signaling in endothelial cells to coordinate bone regeneration during fracture repair. Understanding this pathway may identify new therapeutic targets to enhance bone healing and improve outcomes in patients with impaired fracture repair or inflammatory bone disorders.

REFERENCES:

1. Wee, N.K.Y., et al., *Inhibition of CGRP signaling impairs fracture healing in mice.* J Orthop Res, 2023. **41**(6): p. 1228-1239.

IMAGES AND TABLES:

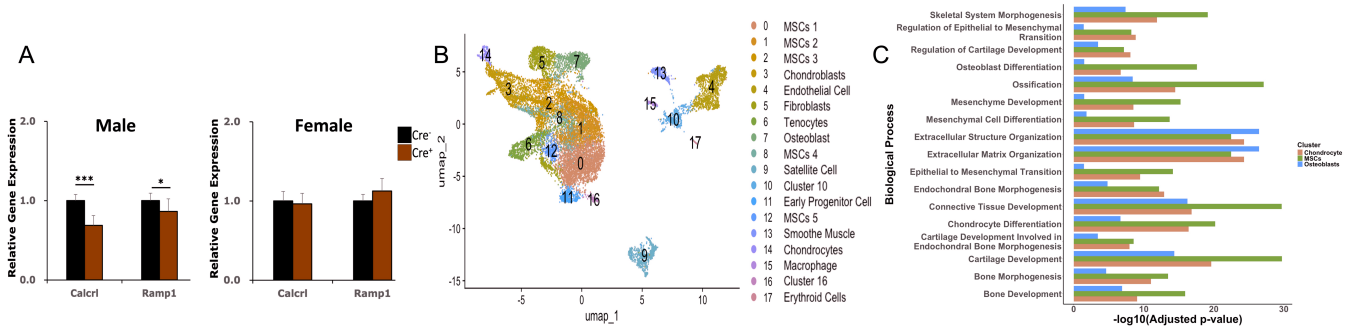


**Figure 1**. **A**) Confirmation of CLR deletion. **B**) Unsupervised clustering of 10X scRNA-seq data determined undifferentiated mesenchymal (MSCs), chondrocyte, osteoblasts, endothelial cells, satellite cells, smooth muscle cells, etc. **C**) GO Analysis detect multiple significantly down-regulated biological pathways, including mesenchymal cell differentiation, ossification, cartilage development, chondrocyte differentiation, skeletal system morphogenesis in those MSCs, Osteoblasts, undifferentiated and mature Chondrocyte clusters from Cre$^{+}$ mice.