Notch signaling induced by endothelial cells regulates fracture healing

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INTRODUCTION. Bone has great regenerative capacity that depends on a pool of skeletal stem/progenitor cells (SSPC) and interactions between mesenchymal, endothelial, and hematopoietic cells. Upon bone injury there is an influx of hematopoietic cells, followed by dramatic expansion of SSPC and endothelial cells and SSPCs commitment to chondrogenic and osteogenic lineages. It has been shown that Notch signaling has a major role in maintaining progenitor pool and differentiation into mature lineages. Forced overexpression of Notch 1 intracellular domain in SSPC improves fracture healing, while inhibition of Notch1 signaling by systemic neutralizing antibody or targeted deletion in SSPC of the downstream transcriptional effector of the NOTCH signaling pathway, Rbpjk, impairs healing. However, molecular pathways and cell-to-cell signaling mechanisms controlling progenitor cell activation are still unclear. It is critical to understand the early events regulating fracture healing. Particularly, specificity of ligand and receptor activity in the heterogenous callus have been poorly described. We aimed to 1) determine the molecular changes and mechanisms involved in improved healing process with inducible NICD1 overexpression in αSMA osteoprogenitors, and 2) evaluate the fracture healing process with targeted deletion of Notch ligand *Dll4* in endothelial cells.

METHODS. We have completed single cell RNA sequencing (scRNA-seq) from intact and fractured periosteal cells (3 days after the fracture) in αSMACre+/NICD1 and Cre-/NICD1 mice. Live, mesenchymal (CD45-) and hematopoietic (CD45+) cells were sorted and single cell RNA-seq performed (10x Genomics). Rstudio and Seurat package were used for data analysis. Principal component (PC) analysis was performed, and clusters were identified using 40 PCs at the resolution of 0.25 for CD45- cells and 0.4 for CD45+ cells.

To evaluate the effects of Notch ligand Dll4 in fracture healing, we used Cdh5CreERT2 to induce targeted deletion of Dll4 (Dll4Δ) in endothelial cells (EC). Fracture healing was assessed in 8–10-week-old male mice. Cre activity was induced by intraperitoneal injection of tamoxifen (75 mg/kg) on 0-, 2- and 4-days post fracture (DPF) and healing evaluated by real time qPCR, histology and μCT at different time points. All procedures were approved by the UConn Health Institutional Animal Care and Use Committee.

RESULTS. scRNA-seq analysis of periosteal cells from intact and fractured periosteum identified endothelial, satellite and muscle cell clusters and trajectories from SSPCs into chondrocytes and osteoblasts. A quiescent cluster termed MSC1 with characteristics of stem cells (inhibition of cell cycle genes and mitosis) initiates proliferation after fracture and transitions into highly proliferating MSCs (cluster 2, 3 and 4). MSCs from injured periosteum of NICD1 overexpressing animals (Cre+) had significantly increased expression of *Alpl* and *Ibsp* compared to injured Cre- callus. Overexpression of NICD1 lead to increases in interferon signaling genes (*Ifit1, Ifit3, Iigp1, Ifi203, Ifi27l2a*) in mesenchymal and endothelial clusters. We show increased *Isg15* and *Ifit1* mRNA in isolated periosteal cells upon Jag1 treatment, confirming the scRNA-seq data. In hematopoietic population NICD1 overexpression in the αSMA expressing cells resulted in decreased proinflammatory signals in CD45+ cells (*Tnf* and *Il6*) after bone injury confirming cellular interactions between mesenchymal and hematopoietic cells partially regulated by Notch signaling. We confirmed *Dll4* and *Jag2* expression in EC while *Jag1* was also expressed in mesenchymal cells. We did not detect significant expression of *Dll1* nor *3* in periosteum. *Notch1* has strong expression in EC, and in committed mesenchymal progenitors and preosteoblasts, satellite and muscle cells while low level of *Notch2* is present in all CD45- cells within the callus (not shown).

Based on gene expression analysis, we decided to use conditional knockout of Dll4 (Dll4Δ) in endothelial cells (EC), using Cdh5CreERT2. *Dll4* deletion in EC led to decreased expression of *Hey1*, *Hes1* and *OCN* compared to Cre- mice 7 days post fracture (dpf). Evaluating proliferation by injecting EdU on day 2 and 3 post fracture, we determined that deletion of *Dll4* in endothelial cells led to decreased number of proliferating cells within the periosteal callus (4 dpf) ($p<0.05$). Cre+ mice have smaller callus at 4 dpf, as well as 7 dpf with significantly less cartilage. However, at 7 dpf, proportion of EdU+ cells within the smaller Cre+ callus was higher than in Cre- mice, displaying phenotype of delayed healing. Further micro-CT analysis at 14 dpf showed that Cre+ animals have decreased callus volume and bone mass within the callus. Histology at that time point confirmed decreased callus area with less mineral tissue ($p<0.001$). No significant difference in bone volume at 3 weeks post fracture was determined by micro-CT analysis, as *Jag1* ligand could have potential to compensate for *Dll4*. Mechanical testing is needed to confirm impaired healing process with deletion of Dll4.

DISCUSSION. Induced Notch1 activation in SSPCs led to increased expression of osteogenic genes within CD45- and decreased proinflammatory genes within CD45+ populations. We confirmed the importance of Notch signaling in osteoprogenitors which leads to distinct transcriptional changes affecting not only osteoprogenitors and their lineage, but also hematopoietic cells during the healing process. We showed a negative effect on the early fracture healing process when *Dll4* is deleted in EC. This data indicates that early Notch signaling through endothelial *Dll4* expression is important during the initial fracture healing phase and potentially during osteoprogenitor differentiation.

SIGNIFICANCE/CLINICAL RELEVANCE. Data presented indicate that activating the Notch signaling pathway early in fracture healing could improve fracture healing through regulation of periosteal cell proliferation, modulation of proinflammatory signals within hematopoietic cells and induction of commitment of SSPCs into osteoprogenitors expressing *Ibsp* and *Alpl*.

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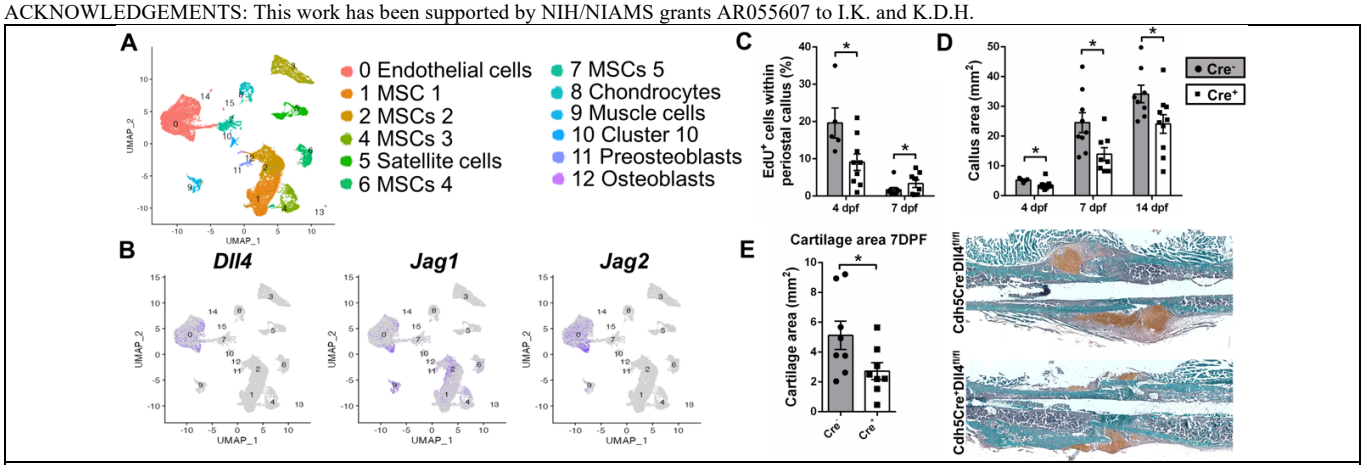

Figure 1. A. UMAP projection of clusters in periosteum. **B.** UMAP of Notch ligands present in non-hematopoietic cells within periosteum. Dll4 from endothelial cells affects fracture healing. **C.** Cell proliferation within periosteal callus in Cdh5CreERT2 Cre+ and Cre- littermate control at 4- and 7-days post fracture (dpf). **D.** Callus area at 4, 7 and 14 dpf in Cre- and Cre+ mice. **E.** Histological evaluation of cartilage with representative Safranin O staining in Cre- and Cre+ samples one week post fracture.