# Thrombospondin signaling links interfragmentary strain to fibroblast activation in nonunion

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**INTRODUCTION**: Musculoskeletal injuries are a leading source of disability worldwide, and 5-15% of the 7 million annual fractures progress to delayed healing or nonunion. Hypertrophic nonunion is the most common clinical subtype, driven by excessive interfragmentary motion and tissue strain. While early inflammation is essential to initiate repair, heightened mechanical instability can skew immune cell phenotypes, fostering fibrosis and impairing functional regeneration. Yet the cellular pathways and mechanisms that derail the healing cascade remain incompletely defined. Clarifying how strain modulates inflammatory responses within the fracture milieu could guide the design of immunomodulatory strategies to prevent nonunion. Here, we establish a murine model of delayed healing that recapitulates the biomechanics underlying hypertrophic nonunion. By integrating spatial transcriptomics of callus tissue with a 3D *in vitro* co-culture model, we delineate strain-associated immune dysregulation and identify profibrotic programs that emerge during delayed healing, consistent with a collagen-rich callus and impaired repair.

**METHODS**: C57BL/6 mice received transverse femoral fractures and intramedullary stabilization with low- or high-stiffness NiTi nails to impose low or high interfragmentary strain; calluses were collected at predefined times, including week 3 for spatial analyses. Fibrillar collagen was assessed by picrosirius red under polarized light. Callus cells were profiled by 10x Genomics Visium spatial transcriptomics, processed with Cell Ranger and analyzed in Seurat for niche annotation, differential expression, and Gene Ontology enrichment; cell–cell signaling was inferred with CellChat. To isolate strain effects, macrophage-fibroblast co-cultures were embedded in fibrin gels and subjected to 0, 5, or 15% uniaxial strain in silicone wells, with digital image correlation confirming strain transfer. TSP-1 signaling was perturbed with the LSKL peptide, and Thbs1 and Col3a1 expression were quantified by RT-qPCR.

**RESULTS SECTION**: Spatial transcriptomics of week 3 fracture callus showed that interfragmentary strain reshapes cellular niches (**Fig. 1A**). Unsupervised clustering identified fibroblast-dense regions comprising 5.79% of high-strain calluses, which was about 14-fold higher than low-strain controls at 0.39%. These regions colocalized with picrosirius red positive fibrillar collagen, consistent with fibrotic matrix. High-strain calluses also showed a 1.7-fold expansion of osteoclast-rich areas (11.58% vs. 6.96%) positioned adjacent to fibroblast clusters, suggesting coupled remodeling of fibrotic and mineralized matrices. Although the gross spatial organization of niches was similar across strain conditions, transcriptional programs diverged. Fibrosis-associated genes were enriched in high-strain calluses, with osteoclast Mmp9 upregulation ($p < 0.0001$) and increases in Spp1, Pdgfrb, and Lgals3 within macrophage zones ($p < 0.0137$). Gene ontology analysis highlighted PDGF signaling and collagen fibril organization driven largely by macrophage transcripts, while osteoclasts and osteoblasts were enriched for extracellular matrix disassembly and ossification pathways, respectively. These findings implicate mechanical strain as a driver of maladaptive immune–stromal remodeling that sustains fibrosis and impedes repair. Inference of cell to cell communication demonstrated globally elevated signaling in high-strain callus, with fibroblasts, macrophages, and osteoclasts as major hubs (**Fig. 1B(i)**). Sixteen mechano-responsive pathways were preferentially engaged, including TGFβ, noncanonical WNT, PDGF, SPP1, FN1, TENASCIN, THBS, and COLLAGEN networks. A THBS or COMP to SDC4 axis and TENASCIN signaling were selectively activated in high-strain tissue, with macrophage and osteoclast ligands Thbs1 or Thbs2, Tnc, and Comp targeting fibroblast Sdc4 (**Fig. 1B(ii)**). These interactions were absent in low-strain calluses. To isolate these interactions, we developed a 3D in vitro model of uniaxial strain (**Fig. 1C(i)**). Digital image correlation confirmed that strain applied to the silicone well was transmitted to the fibrin hydrogel (**Fig. 1C(ii)**). In macrophage and fibroblast co-culture, increasing strain upregulated Thbs1 and Col3a1. Pharmacologic inhibition of TSP-1 with the LSKL peptide reversed this response, significantly reducing Col3a1 and dampening Thbs1 expression (**Fig. 1C(iii)**). In contrast, macrophage or fibroblast monocultures showed minimal transcriptional responses to strain.

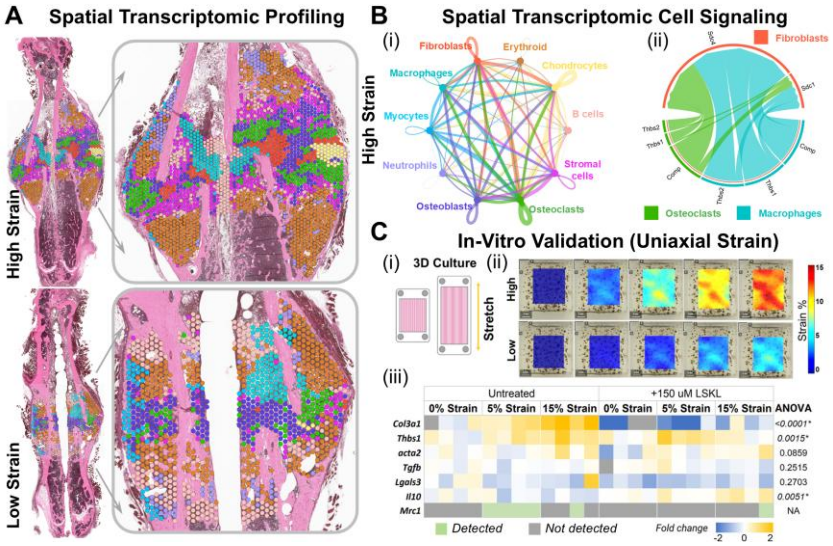


***Fig. 1. Transcriptomics of the fracture callus***. *(A) Spatial analysis of high (top) and low strain (bottom) callus annotated cell populations based on known gene markers. (Bi) CellChat interaction networks in high-strain callus. Line thickness denotes interaction strength. (Bii) Chord diagram highlighting major signaling interactions in high-strain calluses. (Ci) Schematic of the 3D fibrin hydrogel co-culture subjected to uniaxial stretch. (Cii) Digital image correlation strain maps for low and high stretch conditions shown over time. (Ciii) Heatmap of transcript levels in co-cultures exposed to 0%, 5%, or 15% strain, either untreated or treated with the TSP-1 inhibitor LSKL.*

**DISCUSSION**: Mechanical strain reprograms the fracture callus toward a fibrotic state by expanding fibroblast niches, elevating osteoclast activity, and enriching profibrotic transcripts within macrophage and osteoclast regions. Spatial colocalization of fibroblast clusters with fibrillar collagen supports a structural shift toward stiff extracellular matrix, while gene ontology highlights PDGF signaling and collagen organization as central processes. Cell–cell communication analysis points to a strain responsive network in which macrophages and osteoclasts deliver THBS, COMP, and TENASCIN cues to fibroblast SDC4 receptors, providing a mechanistic route for sustained fibroblast activation and matrix remodeling. The in vitro uniaxial strain model shows that strain is sufficient to induce Thbs1 and Col3a1 in macrophage–fibroblast co-culture and that LSKL inhibition attenuates both, indicating that TSP-1 activity is required for this response. Minimal responses in monocultures suggest that strain driven fibrosis depends on reciprocal immune–stromal signaling rather than cell intrinsic programs alone. Together, these findings position THBS–SDC4 signaling as a tractable target for interrupting maladaptive remodeling, and they motivate combined strategies that modulate mechanical environment and block mechanosensitive ligand–receptor pathways. Translationally, the integrated spatial and functional data indicate a concise set of pathways and cell interactions that can be monitored as early indicators of nonunion risk and trialed for therapeutic intervention. Future work should extend these observations across additional time points and outcomes to define when strain modulation or targeted inhibition most effectively redirects healing toward regeneration.

**SIGNIFICANCE/CLINICAL RELEVANCE**: Our findings elucidate the mechanisms underlying the strain-induced immune dysregulation observed in recalcitrant fractures. This work underscores the potential of our research to make a significant impact on clinical practice and patient outcomes.