Spatial transcriptomics reveal distinct gene expression patterns and treatment targets during fracture healing in (non)-union models in mice

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ABSTRACT INTRODUCTION: Non-union bone fractures remain a challenging clinical problem. Combining novel omics technologies with mechanically-controlled preclinical animal models have the potential to capture, understand and target underlying biological changes during fracture healing. The objective of this study was to apply spatial transcriptomics (ST) to characterize spatiotemporal gene expression patterns in non-union and union fractures in mice while monitoring healing progression by longitudinal *in vivo* stiffness measurements and micro-computed tomography (micro-CT).

METHODS: Female 20-week-old C57BL/6J mice (body weight: 25±2g; pair-housed, enrichment: nesting material, wooden sticks, tunnel; 3Rs: tunnel handling as refinement) received a femur osteotomy (0.7mm) or a segmental defect (2.4mm) stabilized with an external fixator (n=14/group; license 22/2022, Grisons CH) using established protocols for Sevoflurane anesthesia and analgesia (Tramadol, 30mg/l drinking water, [1]). To monitor healing progression, *in vivo* micro-CT [2] and stiffness measurements [3] were performed on days 0, 3, 7, 10, 14, 21, 28, 49 (statistics with GraphPad Prism10). No adverse events and no dropouts occurred throughout the study. Mice were euthanized (cervical dislocation under deep sevoflurane anesthesia) at postoperative days 3, 7, 14, 21 (all n=3/group) and 49 (n=2/group). ST was performed from formalin-fixed paraffin-embedded tissue samples (day 3, 7, 14, 21, n=2 per timepoint; Visium CytAssist FFPE; 10x Genomics) using a recently established sample pre-treatment protocol [4]. Sequencing data was processed in SpaceRanger with downstream statistical analyses and visualization in R using Seurat and Bioconductor packages.

RESULTS SECTION: In the osteotomy group, *in vivo* stiffness in the defect region continuously increased indicating bony bridging by day 21 (0.03N/µm±0.004, confirmed by micro-CT), whereas it remained at low levels in segmental defects (day 21, $p<0.01$, unpaired t-test+Welch-correction). Histology/ST substantiated this finding with distinct healing-associated gene expression clusters in both groups (Fig. 1A, t-SNE projection for day 14). Differential gene expression analysis in the defect region of non-unions vs. unions revealed >600 differentially expressed genes (DEGs) during the post-operative monitoring period reaching from the inflammation phase (day 3) via the reparative phase (days 7+14) to the remodeling phase (day 21; see Venn diagram in Fig. 1A). When looking at the temporal pattern, 82% of the DEGs were specific to one of the four assessed post-operative days, whereas 18% of DEGs were differentially expressed at multiple timepoints, indicating the importance of temporal aspects, when selecting treatment targets and settings for specific healing phases. In unions, characteristic spatiotemporal expression patterns of genes involved in endochondral ossification, mineralization and remodeling were seen (e.g. *Vegfa*, *Bglap*, *Mmp9*, *Dmp1*, ***Col1a1/2***, ***Acp5***, *Ctsk;* for genes in bold see Fig. 1B). Non-unions were histologically characterized by prolonged persistence of hematoma tissue (until day 7) associated with significantly lower expression of genes involved in endochondral ossification in the defect region (e.g. *Acp5:* day 7-21, *Col1a2:* day 3-21*;* Fig. 1B). Additionally, high marrow adiposity adjacent to the defect site (cluster 9, Fig. 1A) largely displaced the marrow clusters seen in unions (clusters 4+8) by day 14. Interestingly, ST captured early and distinct molecular deviations in non-unions comprising genes involved in amino-acid metabolism, cellular organization and pathway regulation during bone development and repair (e.g. *Arg1*: day 3, *Dym:* days 3+7, ***Postn****:* days 7+14, for genes in bold see Fig. 1B). Further, ST showed distinct expression patterns of mechanically-regulated markers (e.g. *Esr1/2*, *Piezo1/2*) giving indications for potential treatment targets, initiation timepoints and treatment duration for impaired healing conditions.

DISCUSSION: ST combined with longitudinal *in vivo* stiffness measurements comprehensively captured (mechano)-molecular features during bone healing. Via distinct spatiotemporal gene expression patterns in non-union and union fractures, ST enables identifying and understanding molecular deviations from normal healing. With increasing spatial resolution of ST platforms and rapid development in downstream analyses (e.g. RNA velocity), integration of single cell analyses will provide options to capture, understand and model distinct treatment targets to improve impaired healing conditions.

SIGNIFICANCE: ST comprehensively characterizes spatiotemporal gene expression patterns in non-union and union fractures relevant for individualized treatments to improve impaired bone healing.

REFERENCES: [1] Wehrle *et al.* Scientific Reports 2021, 11: 23037; [2] Wehrle *et al.* Scientific Reports 2019, 9:17445; [3] Barcik *et al.* ESB 2024; [4] Wehrle *et al.* STAR Protocols 2024, 5: 102986.

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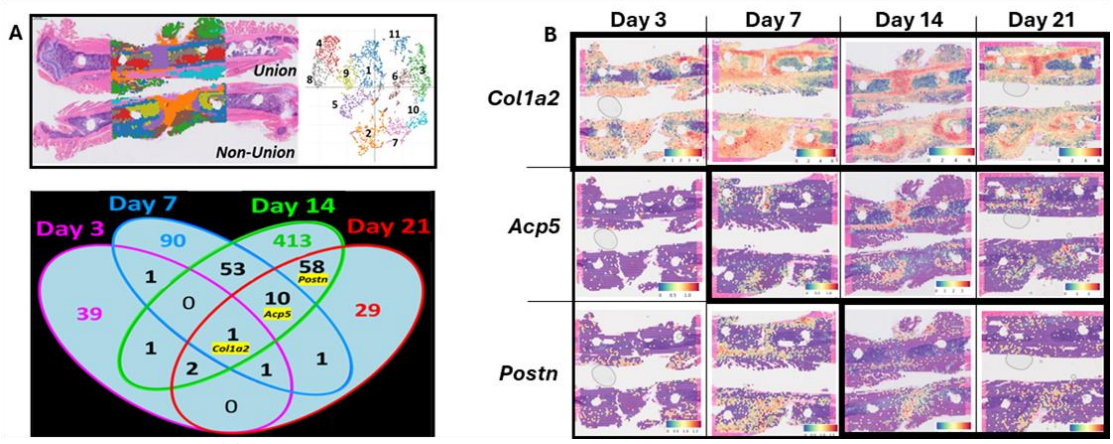

Fig. 1 A: Spatial transcriptomics of fracture healing in mouse (non)-union models. Top: H&E-stained sections and clustering, t-SNE projection (day 14). Bottom: Venn diagram depicting the number of unique and shared DEGs between non-union and union samples during bone healing on days 3, 7, 14 and 21; spatiotemporal patterns of DEGs highlighted in yellow are depicted in **B:** Spatial distribution of molecular markers during bone healing in (non)-union models in mice (days 3, 7, 14 and 21, n=2 sections/day, per capture area: union – top, non-union – bottom; gene expression: low (blue) → high (red)).