Spatial Transcriptomic Comparison Shows Spatially Distinctive Gene Expression in the Murine Fracture Callus in Normal and Breast Cancer-Laden Femurs

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Introduction: Advanced stage breast cancers often metastasize to the bone, predisposing patients to a high risk of pathological fracture due to osteolytic destruction. The presence of breast cancer cells in the bone may lead to impaired bone healing following pathological fracture. Pathological fractures do not adhere to the same fundamental fracture healing principles as seen in fractures of healthy patients. Limited reports using microarray and single-cell RNA sequencing (scRNA-Seq) have suggested that the presence of breast cancer cells in bone produces a unique transcriptomic signature and activates various molecular pathways to induce an altered skeletal microenvironment. However, these studies are inherently limited in their ability to assess transcriptomic expression with respect to tissue architecture. Spatial transcriptomics represents an emerging novel platform to provide direct analysis of localized transcriptomic expression whilst preserving spatial context. This study provides the first characterization of spatially relevant gene expression of a metastatic breast cancer pathological fracture callus to better understand the altered molecular signature and fracture microenvironment of osteolytic metastases.

Methods: *Tissue Sample:* Animal experiments were approved by the Institutional Animal Care and Use Committee of Yale University. Nude mice (age 10-12 weeks) were acquired from Charles River Laboratories. Femoral osteotomy was performed with ketamine (10 mg/mL) and xylazine (1 mg/mL) for anesthesia and a 0.22 mm diameter Gigli saw was used to create a transverse midshaft femoral osteotomy. Pathological fracture healing model was given an intramedullary injection of 0.5 x 10^6 cells of MDA-MB-231. Mice were sacrificed at 2-weeks post-operative and harvested tissue was fixed in 4% paraformaldehyde and PBS. Decalcification was performed with 10% EDTA (pH 7.2-7.4) for 2 weeks before embedding in paraffin. RNA curls off the paraffin block were sent for quality verification. Slides were then sent off to 10x Genomics for use with Visium CytAssist spatial transcriptomic protocol. *Data Analysis:* Raw data was processed and filtered by a bioinformatician using SpaceRanger v2.0. After, the filtered feature matrix was imported into Partek Flow. Normalization was performed by *SCTransform* and batch effect correction was applied using a *Seurat* package. Three regions of interest within the fracture callus (hard callus, soft callus, and interzone) were drawn based upon histology. Filtered differential expression gene lists were generated by ANOVA (false discovery rate ≤ 0.01, p-value < 0.05, log fold change at least -1 to 1). Gene lists were then exported for molecular function analysis based upon K-means clustering (StringDB v12.0). Gene expression data was exported for biological pathway analysis (QIAGEN Ingenuity Pathway Analysis).

Results: 19,465 genes were included in the analysis. A total of 444 gene were found to be differentially expressed between the normal control and the MDA231 fracture callus (FDR≤0.01). Amongst the regions of interest, the hard callus, soft callus, and interzone contained 111, 58, and 275 differentially expressed genes respectively. Within the hard callus, 6 of 111 genes were upregulated in the MDA231 callus (Hba-a2, Hbb-bt, Sem1, Slpi, Sod3, and Saa3), functionally involved in hemoglobin regulation, BRCA2 association, epithelial protection, redox homeostasis, and inflammation. In the soft callus, 2 of 58 genes were upregulated (Myh4, Mylpf). In the interzone, 19 of 275 genes were upregulated (Mylpf, Acta1, Hbb-bs, Tnnc2, Hba-a2, Myh4, Car3, Hbb-bt, Ckm, Tnnt3, Pvalb, Myl1, Eno3, Atp2a1, Ttn, Actn3, Pdlim3, Ank1, Sln). Clustering showed functionally distinct regions of gene expression. Pathway analysis of the MDA231 hard callus genes showed metabolic downregulation (glycolysis, sirtuin signaling, gluconeogenesis, IGF-1) and downregulated immunoinflammatory pathways (interleukins (4, 10, 12, 17A), microautophagy, oxidative stress response). The soft callus and interzone both primarily showed downregulated expression of varying signaling pathways such as wound healing, CLEAR, protein kinase A, immune pathways.

Discussion: Isolation of fracture callus gene expression with intact architectural context was achieved through a novel spatial transcriptomics platform. Distinct transcriptomic and functional gene signatures were identified based upon the hard callus, soft callus, and interzone, emphasizing the value of spatial context in characterizing differential gene expression. The pathological fracture hard callus, calcified immature bone, showed downregulated metabolism and immunosuppression pathways contributing towards impaired fracture healing. Normal physiology maintains a precise balance of inflammation and upregulated metabolism to facilitate fracture repair. As part of endochondral ossification, the soft callus governs osteo-cellular differentiation. In this case, the MDA231 callus showed disrupted cellular signaling that may prevent proper differentiation for cellular repair. The fibrous interzone proved to be the most distinct region with the highest number of differentially expressed genes between the control and MDA231 callus. From pathway analysis, the interzone showed the highest number of differentially expressed genes and may be directly involved in mediating osteo-cellular as a bridging zone. Additional studies are required to unpack mechanistic understandings of differential gene expression.

Significance: Pathological fractures of metastatic cancer disrupts the normal fracture callus microenvironment and activates aberrant pathways that may contribute towards impaired healing. Spatial transcriptomics is a novel platform that can identify architecturally relevant gene expression to guide biomarker discovery and rescue impaired fracture healing.

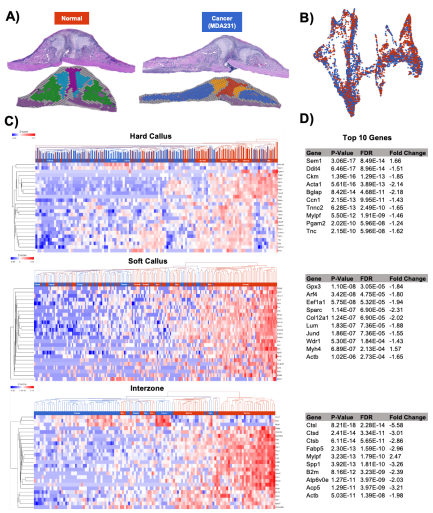

Figure 1. A) Hematoxylin and eosin-stained histological slide of two-week normal fracture healing mouse and two-week GFP MDA231 nude mouse. Regions of interest were selected based upon histology including the following: hard callus, soft callus, and fibrous interzone. B) UMAP 3D plot of all gene expression after SCTransform (Seurat) and batch effect correction applied. Red is expression of normal fracture healing. Blue is expression of MDA231 pathological fracture healing. C) Heatmap of top 25 significant differential expression genes in each region of interest comparing cancer to normal. D) Top 10 genes that are significantly different across each region of interest with p-value, false discovery rate, and fold change comparing cancer to normal.

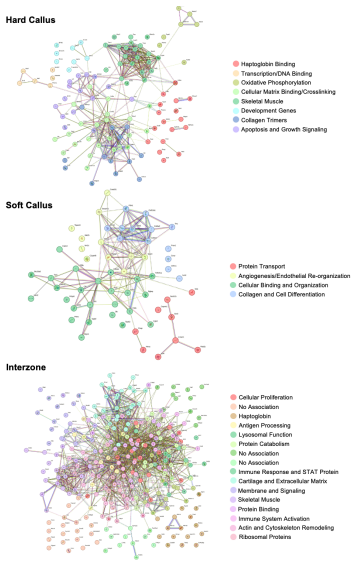

Figure 2. K-Means Clustering (StringDB v12.0) of differentially expressed genes within the hard callus, soft callus, and interzone regions of interest with gene list generated from cutoff of False Discovery Rate set at 0.01, fold change at least -1 to 1, p-value <0.05 (hard callus=111, soft callus=58, interzone=275). Protein-Protein Interaction Enrichment p-value <0.0001 for network analysis.

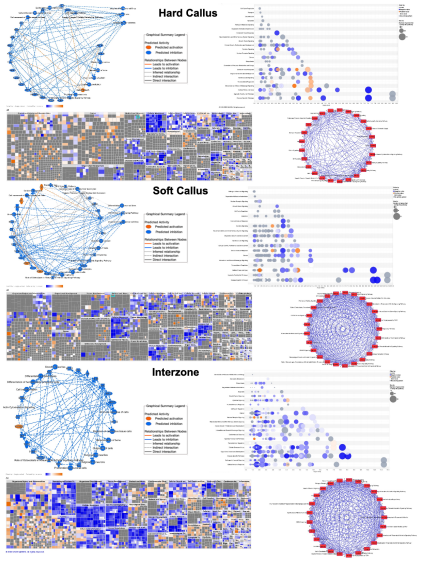

Figure 3. Pathway analysis (QIAGEN Ingenuity Pathway Analysis v01-22-01) results of differentially expressed genes within the hard callus, soft callus, and interzone. Four panels show graphical summary of major gene regulators and associated pathways, bubble plot of significant pathways (p-value <0.05), heat map of activated and inhibited pathways and their cellular functions (colored by -log(p-value), and shared network map of major canonical pathways and the common genes between each pathway.