Research Square

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Synovial transcriptomic signature associated with secondary arthrofibrosis of the shoulder

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Abstract

Background

Secondary arthrofibrosis (sAF) of the shoulder is a complication after trauma or surgery characterized by stiffness, pain, and restricted mobility; however, not all injuries lead to sAF. The underlying pathophysiology of sAF is not well understood. We sought to identify key molecular mechanisms associated with the development of shoulder sAF and to identify transcriptomic differences by conducting high throughput bulk RNA sequencing (RNA-Seq) of synovial tissue from patients with sAF and from patients showing no clinical signs of sAF following shoulder trauma or surgery.

Methods

We assembled a cohort of 33 patients after shoulder surgery or injuries, where n = 17 developed sAF, and n = 16 did not. Arthroscopic capsular release was performed on sAF patients, while non-sAF patients underwent different shoulder procedures. Synovia was collected during surgery and subjected to bulk RNA-Seq followed by statistical and bioinformatic analyses to identify differentially expressed genes (DEGs) and associated enriched pathways.

Results

Transcriptomic analyses revealed a total of 173 DEGs in sAF compared to the non-sAF group, with 119 upregulated and 54 downregulated. Pathway enrichment analysis showed upregulated DEGs were enriched for signal transduction, immune system, and endocrine system pathway categories, particularly involving Epithelial-to-Mesenchymal Transition regulation, nerve growth factor (NGF)-stimulated transcription, and insulin signaling.

Conclusions

We identified transcriptomic differences in synovia of patients who developed sAF of the shoulder compared to patients who did not. These transcriptomic differences were associated with unique biological functions and signaling pathways that may help to understand molecular mechanisms present in patients at risk of developing sAF.

Background

Secondary arthrofibrosis (sAF) is used to describe shoulder stiffness after surgery or trauma[1]. It is a common complication after surgeries or injuries of the glenohumeral joint[2]. The incidence of sAF after rotator cuff repair has been reported to be between 4.9% and 32.7%[3]. Stiff shoulder is clinically

described as global restricted range of motion (ROM), where both passive and active ROM are affected[4]. It is characterized by fibrosis and contracture of the glenohumeral joint capsule. Patients with sAF frequently suffer constant pain, severe physical limitations, and difficulty sleeping[2].

The exact pathophysiology associated with sAF is not well understood; however, studies have shown AF affected joints have dysregulated inflammatory processes and imbalances in extracellular matrix (ECM) degradation, remodeling and regeneration[5]. On a cellular level, arthrofibrosis (AF) is characterized by upregulated myofibroblast proliferation with reduced apoptosis, adhesions, and aggressive synthesis of ECM, especially type 1 collagen and proteoglycans[2]. Transcriptomic analyses of sAF have been limited, with available studies of primary and sAF suggesting a contribution of cellular transcriptomes to pathophysiology[6,7].

In this study, we aimed to identify putative molecular mechanisms associated with sAF of the shoulder. We established the Martin-Luther-University (MLU) shoulder cohort, which includes patients who had all undergone shoulder surgery or suffered shoulder injuries and either developed sAF or showed no clinical signs of the disease. Synovial tissue was obtained at the time of surgery. We performed bulk RNA-sequencing on the synovium of the glenohumeral joint capsule, identifying differentially expressed genes (DEGs) between sAF and non-sAF subjects and employed advanced computational assays to determine biological mechanisms and signaling pathways associated with the identified DEGs. To the best of our knowledge, this study is the first to evaluate transcriptomic differences and putative mechanisms associated with sAF in human shoulder synovium exclusively on sAF and non-sAF patients.

Methods

Subjects

Our cohort comprises 33 patients undergoing shoulder surgery after previous trauma or surgery. The study was approved by the Research Ethics Board for the Faculty of Medicine at the Martin-Luther-University, Halle (Saale), Germany [Nr. 2022-019]. Subjects provided informed written consent. Exclusion criteria included shoulder stiffness caused by glenohumeral osteoarthritis (including shoulder arthroplasty), shoulder arthrodesis, postoperative deep infection, primary arthrofibrosis, neurological disorder or complication. Demographic information collected included age and sex. Anthropometric measurements included body weight and height, from which body mass index (BMI, kg/m^2) was calculated. All subjects underwent shoulder surgery or had a shoulder injury at least 12 weeks prior to the start of the study. Subjects were tested on active and passive ROM, including anteflexion, abduction, external and internal rotation. Patients with passive external rotation of the shoulder ≤ 20 degrees and passive forward flexion ≤ 100 degrees were classified as having sAF [n = 17] and underwent arthroscopic capsular release (ACR) surgery after 12 weeks of prior unsuccessful physical therapy following the first shoulder trauma or surgery. Non-sAF subjects [n = 16] were identified as having no signs of sAF based on clinical examination, and were scheduled for a different shoulder surgery (e.g., stabilisation of the

shoulder or acromioclavicular joint). After the clinical diagnosis of sAF or non-sAF the diagnosis was confirmed during arthroscopy by the presence or absence of fibrotic tissue. Of note, non-sAF patients also had trauma or shoulder surgery, but did not develop sAF. Clinical examination and arthroscopic surgeries were performed by the same experienced orthopeadic and trauma surgeon between Feb 23, 2022 - Jan 23, 2024. All study participants are included in DART (German Speaking Arthroscopy Registry, German Clinical Trials Register). Patients' blood, synovial and bursa tissue, and synovial fluid were obtained intra-operatively, immediately stored at -80°C, and delivered to the Schroeder Arthritis Institute (University Health Network, Toronto, ON, Canada), where samples were stored at -80°C until use (Fig. 1A). For this study, only synovial tissue was evaluated. Differences in age and BMI between groups were assessed using the Wilcoxon test. Differences in sex distribution between groups were assessed using the chi-square test.

Total RNA Sequencing and Differential Gene Expression Profiling

RNA was extracted from synovial tissues using the Qiagen RNeasy Mini kit (Qiagen, Venlo, Netherlands) and quality of RNA was assessed using an RNA Nano chip on an Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA). Samples were fluorometrically quantified using the Qubit RNA BR assay (ThermoFisher) on the Denovix DS-11 spectrophotometer (Denovix, Wilmington, DE, USA). For each sample, 200ng RNA was used to prepare total RNA sequencing libraries using the TruSeq Stranded Total RNA with RiboZero per manufacturer's recommendations (Illumina, San Diego, CA, USA). Sequencing libraries were volumetrically pooled and sequenced on an Illumina NextSeq 550 sequencer for 150 paired end read-cycles at the Schroeder Arthritis Institute.

Demultiplexing of samples was performed using the bcl2fastq conversion tool (v2.20). During quality assessment, we maintained a minimum read length of 25 bp post-trimming of adapters using Cutadapt (v3.0) software, along with trimming of N's. Splice-aware alignment of reads was performed using HISAT2 software (v2.2.1 with parameters –rna-strandness RF –dta) against human reference genome (vGRCh38). StringTie (v2.1.4 with parameters -e -B -G referencetranscripts.gtf) was run to generate output as table format files. These files were processed using the script 'prepDE.py' from StringTie software to collect raw gene expression levels for downstream statistical analyses.

In total, 60,675 genes were measured across 33 samples in the original matrix of counts. Lowly expressed genes were filtered out based on genes expressed in < 2 patients or with a count per million ≤ 10, resulting in 10,029 genes remaining for further analysis. Differentially expressed genes (DEGs) between sAF and control groups were identified using DESeq2 package[8] v1.3 in R statistical software, which uses a negative binomial distribution[9]. Batches (runs) were corrected using ComBat_seq[10] and adjusted for as a covariate in the model. In addition, age, sex, and BMI were included in the model to control for potential confounding effects. Multiple comparisons were accounted for using the procedure proposed by Benjamini and Hochberg[11] to control the false discovery rate (FDR) and were visualized using volcano plots. Heatmaps were generated following the DEG analysis, where expression levels were

transformed via the variance stabilizing transformation in DESeq2 and standardized to have a mean of 0 and a variance of 1. Genes in heatmaps have an FDR-adjusted p-value less than 0.05 and abs($\log_2$ Fold-Change) greater than $\log_2(1.5)$.

Computational Analyses

Pathway enrichment analyses were performed using pathDIP 5 API in R v4.3.0. The literature-curated set was selected, and all pathway types except “Disease” and “Drugs and Vitamins” were considered, and KEGG was removed. Upregulated and downregulated genes were tested separately and combined, and all pathways with FDR: BH-method < 0.05 were considered. To highlight biological links among DEGs and EMT-pathways, we obtained EMT-related proteins from pathDIP version 5.2[12]. Four identified pathways (ACSN2 EMT Regulators, Reactome EMT during gastrulation, Reactome TGF-beta receptor signaling in EMT, and WikiPathways Hypoxia-mediated EMT and stemness resulted in 607 unique EMT-related proteins (**Supplementary Table 3A**). Up- and down-regulated DEGs and the EMT proteins were used to query Integrated Interaction Database (IID) v2025-05[13] to obtain physical protein-protein interactions among DEGs and EMT (**Supplementary Table 3B**). The resulting network was further annotated with Gene Ontology Biological Process (node color), with proteins related to Extra Cellular Matrix (ECM) from MatrixDB[14] and included 50 proteins annotated to matrix in Matrisome, Uniprot and Gene Ontology (**Supplementary Table 3C**). Final network was visualized in NAViGaTOR ver. 3.0.19[15,16]. Bubbleplots were generated in R using ggplot2 4.0.3.

Results

Cohort characteristics

The cohort included n = 33 individuals, with n = 17 subjects that developed sAF after shoulder surgery or trauma and n = 16 did not develop sAF (Table 1). In all sAF patients there was a presence of fibrotic tissue in the shoulder arthroscopy, while there was an absence of fibrotic tissue in the the non-sAF group. For representative intraoperative arthroscopic images please see **Supplementary Fig. 1**. Between sAF and non-sAF groups, no statistical differences were found for BMI or age ($p > 0.05$). Finally, no statistically significant difference in sex distribution between sAF groups and non-sAF was observed ($p > 0.05$).

Table 1
Baseline characteristics and ROM assessment of the shoulder in sAF and non-sAF groups

Age at surgery (years)	**Full Sample (n = 33)**	**sAF (n = 17)**	**Non-sAF (n = 16)**	**p-value**	**StatTest**
				0.53	**Wilcoxon Rank Sum**
Mean (sd)	52.5 (8.8)	53.2 (9.4)	51.7 (8.4)		
Median (Min,Max)	53 (35,66)	56 (35,66)	51.5 (35.0,62.0)		
Sex (m/f)				0.57	Chi Sq
f	13 (39)	8 (47)	5 (31)		
m	20 (61)	9 (53)	11 (69)		
BMI [kg/m2]				0.42	Wilcoxon Rank Sum
Mean (sd)	29.5 (6.1)	28.6 (5.6)	30.4 (6.6)		
Median (Min,Max)	27.7 (23.0,47.9)	27.0 (23.0,46.1)	27.9 (23.7,47.9)		
ROM [°]					
external rotation		0–20°	free ROM		
forward flexion		0-100°	free ROM		
Arthroscopic presence of fibrotic tissue [+/-]		+	-		

ROM, range of motion; sAF, secondary arthrofibrosis.

Transcriptomic differences in synovium of subjects with sAF vs. non-sAF

To determine transcriptomic differences of subjects exhibiting sAF vs. non-sAF, synovium was subjected to bulk RNA-seq (Fig. 1A). A total of 10,029 genes were analyzed after quality control and filtering, with 173 DEGs [q < 0.0' abs(log_2FC)>log2(1.5)] identified, of which 119 were upregulated and 54 were downregulated in sAF compared to non-sAF subjects (Fig. 1B**&C**). Top 10 upregulated genes sorted by log_2FC were ANKRD1, MYO18B, NRAP, LMOD2, MYL1, IGFN1, XIRP2, MYL2, IGHA1 and MYH7. Top 10 downregulated genes were AMTN, AC007952.4, CLEC12A, AC138811.2, SCARNA2, HHIP, CX3CR1, AC006064.5, SCARNA10 and LYVE1 (**Supplementary Table 1**). The presence of many muscle contraction genes, including MYO18B, MYL1 and MYL2, was identified in the top 10 upregulated genes.

Matrisome-related transcriptomic differences in sAF vs. non-sAF

Since excessive ECM is a key component associated with fibrosis and loss of ROM in patients with sAF, we investigated whether any identified DEGs overlapped with the matrix-related transcriptome (matrisome). Out of the 173 DEGs, 11 genes were linked to the matrisome, including COL6A6, CTSG, SPARC, CRELD1, COL14A1 and TGFB3 that were upregulated in the synovium of patients with sAF compared to non-sAF subjects, while EFEMP1, ADAM32, ADAMTS6, HHIP and CLEC12A were downregulated (**Supplementary Table 2**).

Pathway enrichment analyses

Pathway enrichment analysis was performed on up- and down-regulated DEGs to determine biological mechanisms putatively modified in patients with sAF. Among upregulated genes, pathway categories with at least five enriched pathways included signal transduction, immune system, endocrine system, muscular and bone system, and nervous system (Fig. 2). Of the top 15 enriched pathways ($q < 0.05$), Epithelial-to-Mesenchymal Transition (EMT) regulation, NGF-stimulated transcription/signaling by NTRK1, and insulin signaling were identified (Fig. 3B). Among downregulated DEGs, only three pathways were found enriched and were all related to Toll-like receptor signaling and MYD88 (Fig. 3A). An integrated protein interaction network among DEGs and EMT-related proteins was generated, highlighting the 11 up-regulated DEGs that overlap with EMT-annotated genes (ACTN2, MYL6B, MYL2, FOS, MYH10, ITGAX, TGFB3, MIB2, ATF3, MYH7, MYL1), with 4 of them corresponding to ECM-related DEGs (ACTN2, MYL6B, MYH10, TGFB3) (Fig. 3C**).**

Discussion

In this study, we created a unique cohort of individuals, which would help us understand whether there are any molecular transcriptomic differences in patients who develop sAF compared to those who have no clinical signs of sAF. Using this unique cohort, we performed gene expression profiling of synovial tissue from patients with sAF of the shoulder, identifying a distinct transcriptomic signature compared to non-sAF-patients that underwent shoulder surgeries but showed no signs of AF. Our study identified significant transcriptomic differences. This signature was linked to enriched pathways involving epithelial-to-mesenchymal transition, pain, hemostasis, and endocrine signaling. To our knowledge, this is the first study to use bulk RNA-seq to determine mechanisms exclusively in synovia of sAF patients compared to non-sAF surcical controls.

ANKRD1 was the most upregulated DEG found in the sAF group compared to non-sAF group. High expression of ANKRD1 has been reported in cells of the epidermis and dermis, including fibroblasts during the healing process[17]. Global deletion of ANKRD1 in mice delayed wound healing and decreased contraction due to a dysfunctional cell-matrix interaction in fibroblasts, supporting ANKRD1's role in fibroblast-associated wound healing[17]. ANKRD1 has also been identified as a mesenchymal-specific transcriptional coregulator in human dermal fibroblasts that promotes a myofibroblast cancer-associated fibroblast phenotype[18]. Our data also show upregulation of matrix-associated genes such as

TGFβ3, COL6A6, COL14A1, SPARC, CTSG, and NR4A1. NR4A1 is known to inhibit TGF-β signaling and fibrosis[19]. Thus, ANKRD1 and NR4A1 may contribute to sAF in opposing manners, suggesting that investigations to further define their roles in sAF are warranted.

Diabetes mellitus is a well-established risk factor for the development of arthrofibrosis, particularly in the shoulder joint[20]. While the underlying mechanisms remain incompletely understood, our data suggest alterations in the insulin signaling pathway and associated upregulated genes EGR1, FOS, PFKM, SLC2A4, SRF, VAMP2. Unfortunately, our cohort lacks subject diabetic status, but contributions of these DEGs to sAF may be of future interest.

We also observed broader transcriptomic differences, including genes involved in hemostasis. Although direct studies on hemostasis in arthrofibrosis are limited, general fibrosis research suggests a pro-fibrotic impact of coagulation pathways[21]. Many upregulated genes identified in our study involved in hemostasis overlap with the cell surface interactions at the vascular wall pathway (e.g. ATP1B1, IGHA1, IGKC, IGLC2, IGLC3, ITGAX, JCHAIN), a top five enriched pathway among upregulated genes identified. Thus, hemostatic mechanisms within the posttraumatic shoulder joint may contribute to fibrotic tissue remodeling and the development of arthrofibrosis.

Both Epithelial-to-Mesenchymal Transition (EMT) and nerve growth factor (NGF) signaling pathways were enriched in our upregulated DEG list. EMT is a common process among various fibrotic diseases where epithelial cells lose their characteristics and gain a more mesenchymal (fibroblast-like) phenotype. This process contributes to the formation of myofibroblasts, cells responsible for producing excess extracellular matrix (ECM) that contributes to fibrosis, and transforming growth factor–beta (TGF-β) is known to induce EMT[22]. However, TGF-β3, an upregulated ECM-annotated gene in our study, has been associated with reduced scarring and antifibrotic activity, in contrast to the profibrotic effects of TGF-β1[23]. Thus, upregulation of TGF-β3 in our sAF tissues could be a compensatory mechanism. NGF, known to promote pain, may also play a role in tissue repair/fibrosis. NGF has been shown to promote the transformation of fibroblasts into myofibroblasts[24]. Thus, DEGs and enriched pathways identified in our study are consistent with reported sAF-associated mechanisms of fibrosis and pain, with EMT and NGF signaling as potential target mechanisms for sAF therapy.

Although we found no differences in the demographic and anthropometric variables investigated between our groups, inherent heterogeneity of individual participants is likely to impact transcriptomic signatures, with larger cohort investigations needed to uncover smaller, yet potentially important signals. A clear separation between fibrotic tissue and the synovial membrane could also not be achieved during arthroscopy, and differential aquisition of muscle tissue during biopsy collection between groups cannot be ruled out, evidenced by identification of upregulated muscle contraction genes (TNNT1, MYL1, MYL2, and MYL6B) in the sAF vs non-sAF tissues; however, this may also be a pathological response to sAF. Finally, since most subjects were of European descent, transferring findings to other ethnic groups may be limited.

Conclusions

This study describes a unique cohort of patients with shoulder sAF and its transcriptomic profile, revealing key molecular mechanisms linked to the pathology. These DEGs and associated pathways may serve as putative therapeutic targets for patients with sAF. Nevertheless, further validation in larger patient cohorts and functional characterization of these genes are required to clarify their roles in disease pathogenesis.

Abbreviations

ACR	**arthroscopic capsular release**
AF	arthrofibrosis
BMI	body mass index
DART	German Speaking Arthroscopy Registry
DEGs	differentially expressed genes
ECM	extracellular matrix
EMT	Epithelial-Mesenchymal Transition
FDR	false discovery rate
HH	Humeral Head
IID	Integrated Interaction Database
LHB	Long Head of Biceps
MLU	Martin-Luther-University Halle-Wittenberg
NGF	nerve growth factor
RNA-Seq	RNA sequencing
ROM	range of motion
sAF	Secondary arthrofibrosis
SSC	Subscapularis Tendon
TGF-ß	transforming growth factor–beta

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Board for the Faculty of Medicine at the Martin-Luther-University, Halle (Saale), Germany [Nr. 2022-019].

Consent for publication

All participants provided written informed consent for publication.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the GEO data repository, [GEO accession ID: GSE344422].

Competing interests

M.K. is an officer and board member for the Osteoarthritis Research Society International (OARSI) and serves on the scientific advisory board for Chiron Inc and GlaxoSmithKline Inc.

The authors declare no other competing interests.

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Authors' contributions

FH contributed to study design, selection of sAF and non-sAF samples, acquisition of data, interpreting of analyses, bulk RNA-Seq experiments and writing of the manuscript and generation of figures. JSR contributed to the analysis and interpretation of all experiments and writing of the manuscript. KDS contributed to tissue preparations and the completion of bulk RNA-seq experiments and data analysis. PP contributed to the analysis and interpretation of bulk RNA-seq data. OEG contributed to the statisical analysis and interpretation of bulk RNA-seq data and the generation of associated figures. CP and IJ contributed to all GO biological process and pathway enrichment analysis, protein interaction network, and generation of associated figures. SV has contributed to the analysis pipeline used for bulk RNA-Seq data and generation of associated figures. AS and AR contributed to tissue preparations. JE and PK contributed to the editing and revision of the manuscript. KSD contributed to the acquisition of funding for this study. MA contributed to patient acquisation and performed shoulder ACR surgeries on all patients included in the study. MK and MA designed the original study design, contributed to the

conception and acquisition of funding for this study, directed the entire study and were involved in the interpretation of all experiments. MK and MA share equal senior authorship. All authors have contributed significantly to the critical revision of the manuscript and approved the final version of the manuscript.

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Figures

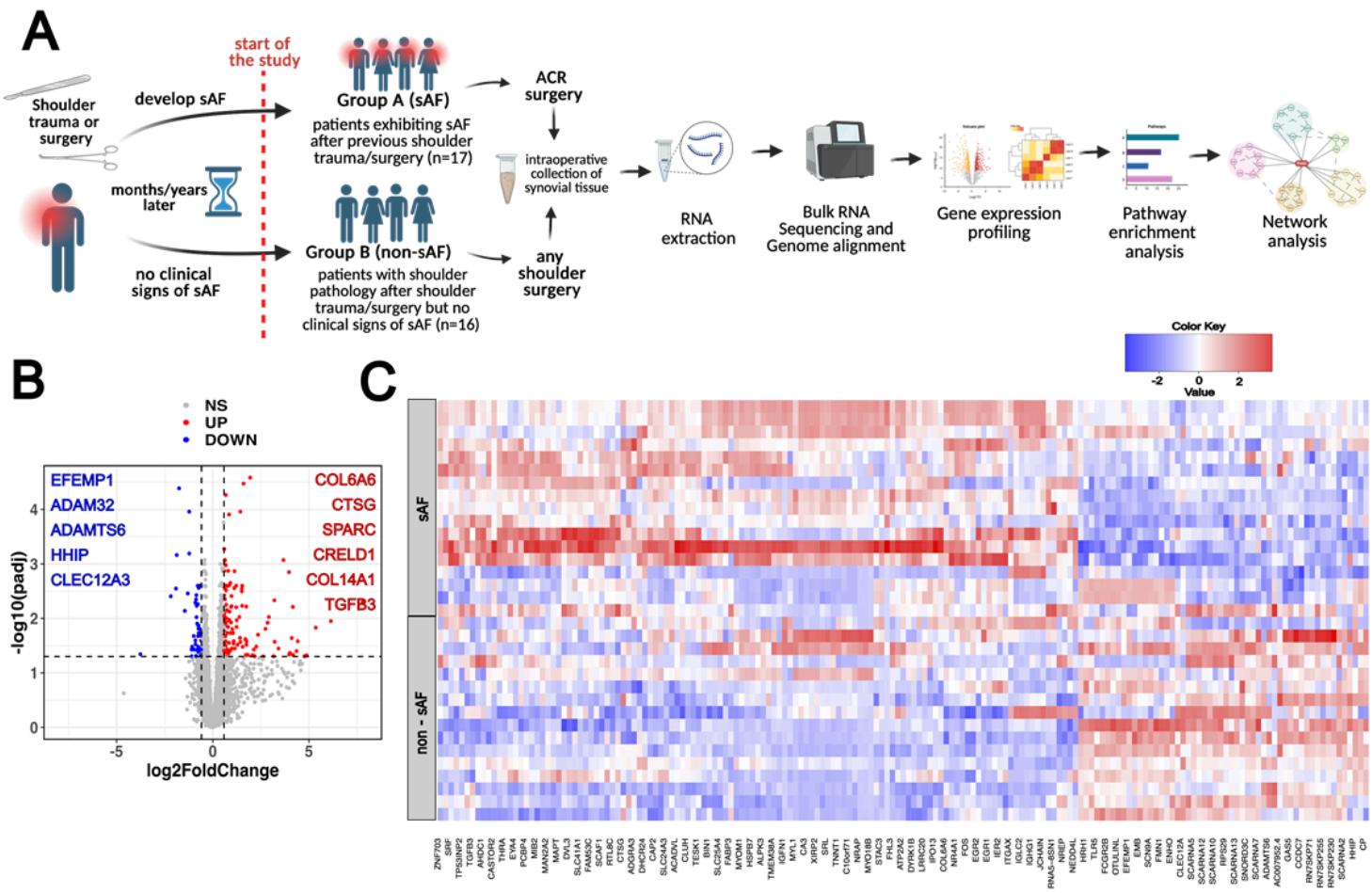


Figure 1

Schematic workflow and differentially expressed genes in synovia of sAF patients compared to non-sAF patients. (A) Schematic workflow to identify transcriptomic differences and enriched pathways. Group A (sAF) = patients exhibiting sAF after previous shoulder trauma/surgery; Group B (non-sAF) = patients who did not develop sAF after previous shoulder trauma/ surgery ; ACR = arthroscopic capsular release; any shoulder surgery = rotator cuff repair or repair of shoulder instability. **(B)**Joint distribution of the 173 differently expressed genes identified in sAF patients vs. non-sAF. 11 genes were identified that link to Matrisome (blue, downregulated, red upregulated). **(C)** Heatmap showing 119 upregulated and 54 downregulated DEGs in sAF patients compared to non-sAF.

Figure was created, in part, using Biorender.

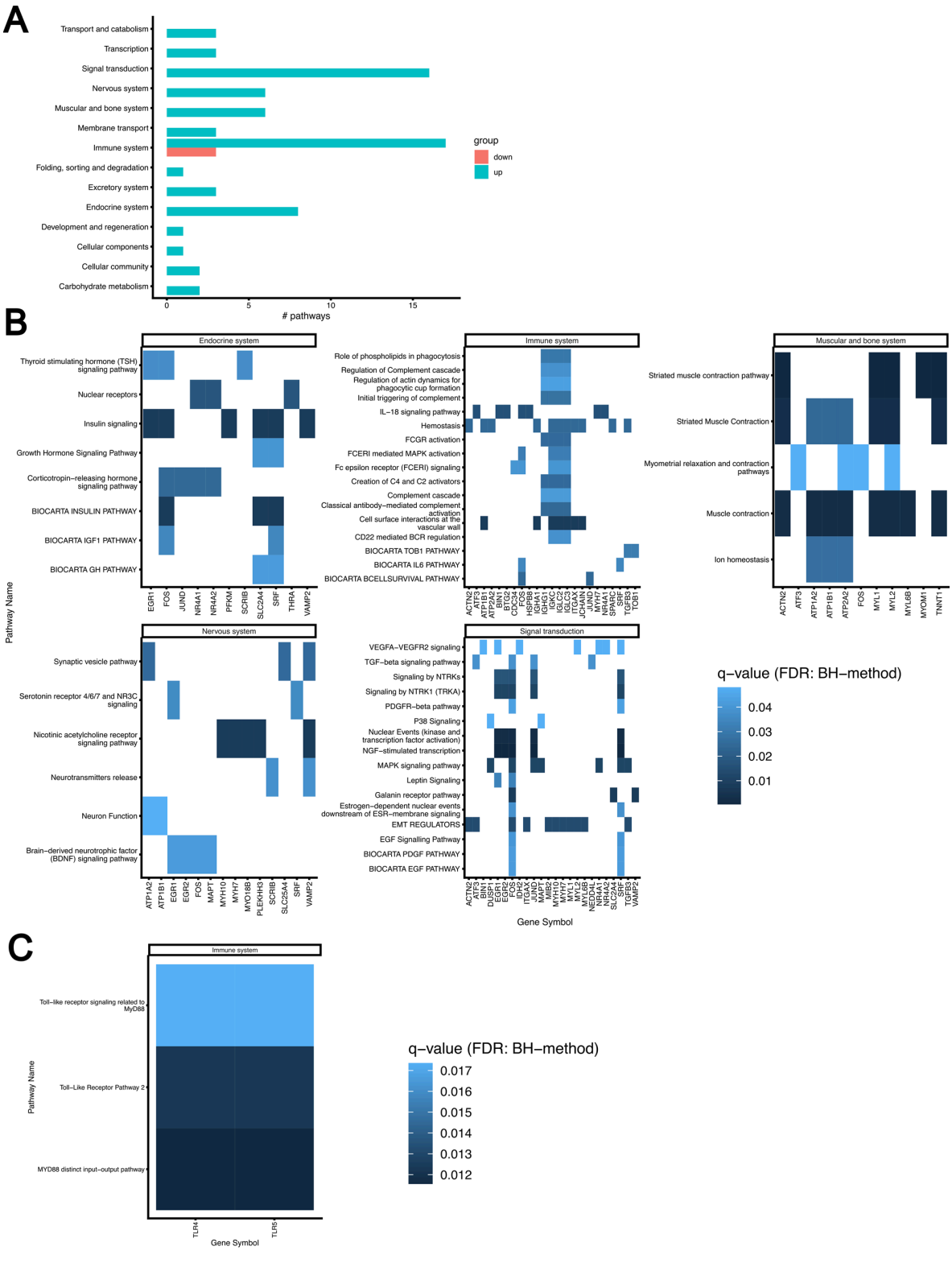


Figure 2

Enriched pathways of upregulated and downregulated genes of sAF patients compared to non-sAF patients. (A) Horizontal bar plot showing number of enriched pathways in each pathway category for down (orange) and up (turquoise) regulated genes. **(B)** Tile plots showing the pathways among the upregulated genes sorted by pathway category. **(C)**Tile plots showing the pathways among the downregulated genes sorted by pathway category.

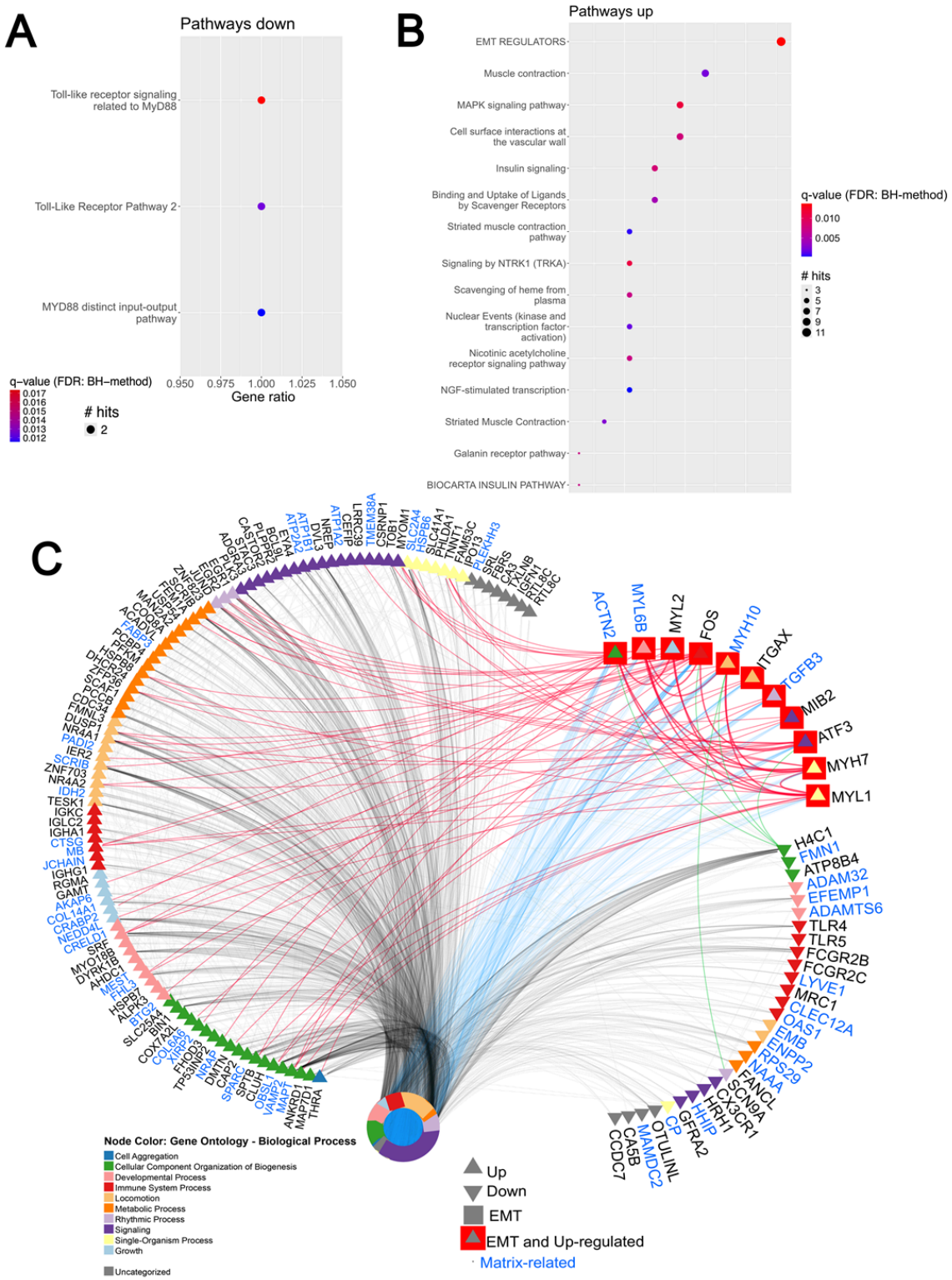


Figure 3

Enriched pathways associated with DEGs of sAF patients compared to non-sAF patients and Interactive Network. (A) Dot plots showing the top 3 pathways among the downregulated DEGs. **(B)** Dot plots showing the top 15 pathways among upregulated DEGs. **(C)** Integrated protein interaction network among DEGs and EMT-related proteins. Highlighted are 11 up-regulated DEGs that overlap with EMT proteins (upwards pointing triangles in red box), and ECM-releted DEGs (blue node text).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx
- SupplementaryFigure1.pdf