Gene Expression in Torn Rotator Cuff Tendons Determined by RNA Sequencing

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Background: Although the cause of rotator cuff tearing is likely multifactorial and a genetic predisposition has been proposed, the biochemical basis remains unknown.

Purpose: To determine gene expression profiles in torn rotator cuff tendon tissue through use of RNA sequencing.

Study Design: Controlled laboratory study.

Methods: The supraspinatus tendon edge was biopsied in 24 patients undergoing arthroscopic rotator cuff repair for full-thickness supraspinatus rotator cuff tears. The supraspinatus tendon was also biopsied in 9 patients undergoing open reduction and internal fixation for a proximal humeral fracture (controls). Total RNA was extracted and sequenced. Differential gene expression was analyzed between the tear and control groups, and a secondary analysis was conducted between groups defined by an unbiased clustering.

Results: Tear and control transcriptomes demonstrated significant differential expression in more than 3000 genes. The identified differential genes were highlighted in pathways involved in inflammation in control patients and extracellular matrix generation in patients with tears. Secondary analysis using unsupervised and thus unbiased hierarchical clustering revealed 2 clusters (c2 and c3). Cluster c3 contained smaller ($P < .001$) and less retracted ($P = .018$) tears (ie, tears earlier in the progression of rotator cuff disease) with increased expression of hypoxia target genes. Cluster c2 contained larger, more retracted tears (ie, tears further in the progression of rotator cuff disease) with increased expression of endothelial cell markers and chronic inflammation target genes. Tears in c2 had significantly worse healing rates compared with tears in c3 (0% vs 89%; $P = .007$).

Conclusion: Smaller, less retracted tears had increased expression of hypoxia target genes and improved healing, whereas larger, more retracted tears were associated with endothelial cell markers and worse healing. Thus, hypoxia may be the inciting event for tear development, whereas with tear enlargement, a chronic, inflammatory, angiogenic process may predominate.

Clinical Relevance: Identification of differential gene expression in rotator cuff tears may be a reliable tool to predict repair healing in the future.

Keywords: rotator cuff; genetics; RNA sequencing; hypoxia; angiogenesis; inflammation; endothelial

The cause of rotator cuff disease is unknown but thought to include a combination of mechanical and biological factors. Possible causes of rotator cuff tearing include limited vascularity of the tendon, mechanical impingement on the undersurface of the acromion, and intrinsic degeneration. Sibling data and familiality data suggest a possible genetic origin of rotator cuff tearing.[6,14] Various genetic variants associated with rotator cuff tearing have been identified through use of candidate gene analysis of genomic DNA including tenascin-C and estrogen-related receptor β.[8,15] However, the differential expression of these genetic variants within the tear environment may provide even deeper insight into the pathogenesis of rotator cuff tearing.

Several genes have been identified to be upregulated in rotator cuff tendons in patients with tears, including tumor necrosis factor α (TNF-α) and interleukins 1α and 1β (IL-1α and IL-1β); matrix metalloproteinases (MMP)-1, -9, and -13; and apoptotic genes including caspase-3, -8, and -9.[1,9,10,12] Genome-wide survey of gene expression using microarray technique has identified certain gene expression profiles in torn rotator cuff tendons and identified upregulation of MMPs including MMP-3, -10, -12, -13, -15, -21, and -25 and interleukins including IL-3, -10, -13, and -15.[2]

Microarray techniques offer the ability to analyze the genome for differential gene expression based upon mRNA transcription.[7] Limitations include evaluation of a limited number of targets dependent on transcript-specific probes and the requirement for binding of mRNA to the targets to determine expression.[7] As a result, low signal transcripts or transcripts for which there is no probe may be missed.[11]

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RNA sequencing avoids these issues and offers superior accuracy in determining expression levels because total mRNA is sequenced and quantified. This allows detection of rare and low-abundance transcripts not measured by microarray techniques.[7,5] No prior studies have evaluated human genome-wide RNA sequencing of torn rotator cuff tissue.

The purpose of this study was to identify a genome-wide landscape of differential gene expression profiles in rotator cuff tendons, with tears compared with normal tendon tissue by using an RNA sequencing approach. We also conducted the gene set enrichment analysis with the differentially expressed gene to characterize possible mechanisms for tear development. We hypothesized that variations in gene expression would be identified in genes associated with the extracellular matrix, chemotaxis, and inflammatory and immune responses.

METHODS

This was a prospective comparative study. Rotator cuff tissue biopsies were conducted in 33 patients undergoing surgical treatment of their shoulder. Hospital investigational review board approval at the University of Utah was obtained before initiation of the study, and all patients signed informed consent. A total of 24 patients with full-thickness posterosuperior rotator cuff tears underwent arthroscopic rotator cuff repair (study group), and 9 patients underwent open reduction and internal fixation for a proximal humeral fracture (control group). Inclusion criteria included any patient willing to consent to the biopsy who was undergoing repair of a full-thickness posterosuperior rotator cuff tear or open reduction and internal fixation of a proximal humeral fracture. Exclusion criteria included any partial-thickness tear repairs, any revision rotator cuff repairs, and any patient with a proximal humeral fracture with a full-thickness rotator cuff tear as determined by surgical observation or with a history of prior shoulder surgical procedures.

All 24 patients with full-thickness posterosuperior rotator cuff tears were operated on by a single orthopaedic surgeon who was fellowship trained in shoulder and elbow surgery (R.Z.T.). All patients in the cohort had experienced symptoms for at least 3 months before repair. Each of the 9 patients with proximal humeral fractures underwent surgery by 1 of 3 orthopaedic surgeons with fellowship training in trauma surgery or by the surgeon who performed the rotator cuff repairs. Patients had surgery at an average of 5 days after the fracture. All surgeries were performed at the University of Utah, and the data were collected from February 2014 to May 2015.

Patient age, sagittal and coronal tear size, and fatty degeneration of the supraspinatus according to the Fuchs modification of the Goutallier classification were recorded for each patient with rotator cuff tear.[3,4] Sagittal tear size was recorded in an anteroposterior direction on the most lateral slice of the T2-weighted sagittal images including the entire greater tuberosity. Coronal tear size was measured from the lateral margin of the greater tuberosity to the lateral edge of the tendon on the coronal T2-weighted slice in the middle of the supraspinatus fossa. Goutallier classification was measured on the first sagittal T1-weighted image to include the entire scapular spine going from lateral to medial. Age and fracture type (Neer classification) were recorded for each patient with proximal humeral fracture.[11] Age was compared between groups (rotator cuff tear cases and fracture controls) by use of the Mann-Whitney U test. $P < .05$ was considered significant.

All patients from whom samples were collected at the time of rotator cuff repair had tissue removed arthroscopically. An arthroscopic tissue biter with a 3-mm width and depth was used to remove a piece of tissue at the edge of the torn tendon. The tissue was immediately placed into RNAlater (Qiagen) and stored until processing. The tissue collected at the time of open reduction and internal fixation was taken from the anterior edge of the supraspinatus at the rotator cuff interval as it was opened during the procedure. A scalpel was used to remove a 3×3–mm piece of supraspinatus tendon, which was placed in RNAlater and then stored until processing. All patients had 3- and 4-part fractures, and these involved the tuberosity in a location posterior to the biceps groove. The rotator cuff tendon at the rotator cuff interval was the location for tissue sampling, which in the setting of a 3- or 4-part fracture is preserved. Superficial inspection of the rotator cuff confirmed no evidence of rotator cuff injury.

Total RNA was extracted from the samples by use of the RNeasy Fibrous Tissue Mini Kit (Qiagen). Libraries were prepared through use of the Illumina TruSeq RNA kit, quality checked with the Agilent Bioanalyzer RNA 6000 chip (Agilent Technologies), captured by use of the Ribo-Zero method (Illumina), and sequenced by 50-cycle end-reads on an Illumina HiSeq 2000. Reference FASTA files were generated by combining the chromosome sequences from hg38 with all possible splice junction sequences, which

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Ethical approval for this study was obtained from the University of Utah School of Medicine (IRB No. 68284).

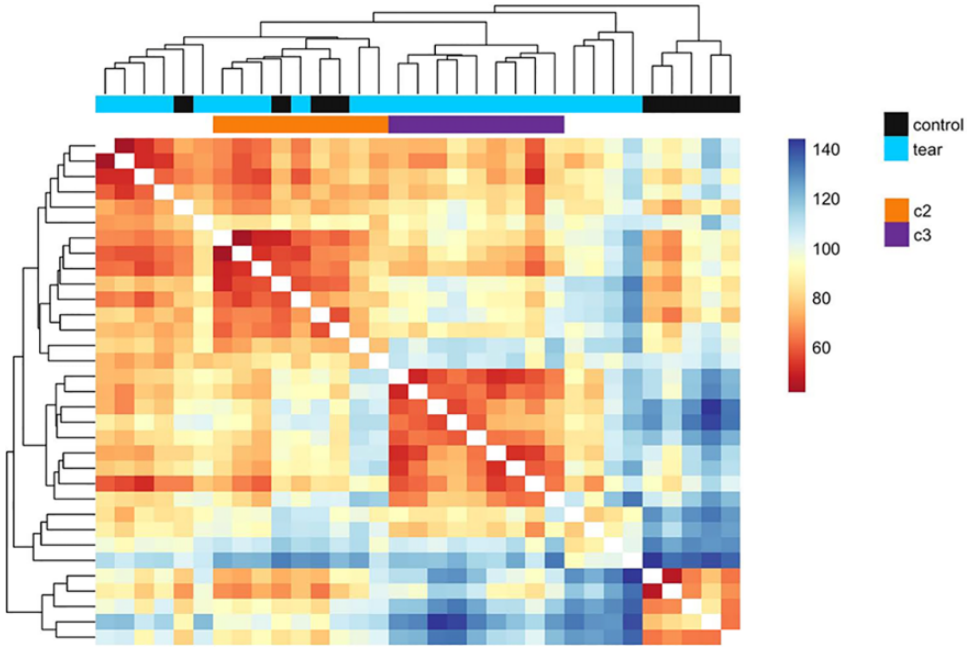

Figure 1. Heatmap of overall relatedness of samples. This heatmap indicates the pairwise relatedness between individual transcriptomes by a whole-genome raw read count analysis, depicted as a Pearson correlation coefficient on a colorimetric scale. The heatmap shows a very strong cluster of controls and another strong cluster of tears (c3), with a third cluster that has a few of both (c2).

were generated with USeq (v8.8.8) MakeTranscriptome using a radius of 46 and annotated with Ensembl transcripts from the UCSC browser. Reads were aligned with Novoalign (v2.08.01), allowing up to 50 alignments per read. The SamTranscriptomeParser in USeq selected the best alignment for each and converted the coordinates of reads aligning to splices back to genomic space. Differential gene expression was measured by use of the DefinedRegionDifferentialSeq in USeq. In brief, the number of reads aligned to each gene was calculated and normalized in DESeq2. Principal component analysis was used to measure distribution along 2 planes by sets of genes (components) among the 500 most differentially expressed genes. The R package "pheatmap" (v1.0.2) was used to generate heatmaps with color-based scales for either expression levels or pairwise comparisons. Log2 of the FPKM (fragments per kilobase per million reads) values were centered and scaled by gene. We performed a functional analysis using Ingenuity Pathway Analysis (Qiagen). Z scores are interpreted as the number of standard deviations away from the mean expression in the reference sample.

Postoperatively, patients were contacted to return for magnetic resonance imaging (MRI) of their shoulder to evaluate repair integrity at a minimum of 12 months from surgery. Anatomic assessment of tendon healing was performed by a shoulder surgeon who did not perform the surgical procedures (P.N.C.) using the grading scale described by Sugaya et al.[13] Grades 1 through 3 were considered healed, whereas grades 4 and 5 were considered unhealed.

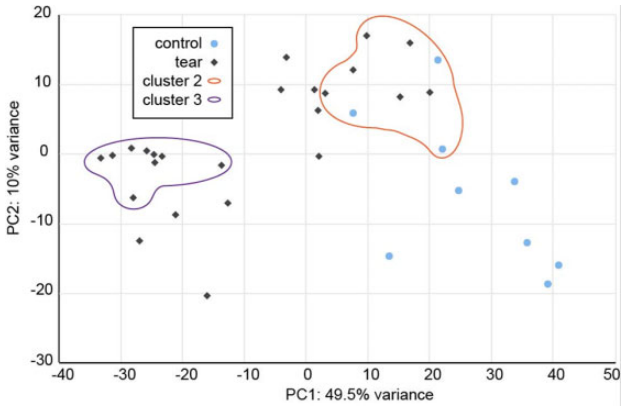

Figure 2. Principal component (PC) analysis in 2 dimensions, considering the 500 most differentially expressed genes. This demonstrates that torn rotator cuffs and control rotator cuffs from fractures separate from each other, almost entirely in a single principal component (or group of discriminating genes), but further with the second. The circled clusters of samples (c2 and c3) indicate groups of specimens identified by whole genome-wide clustering in Figure 1.

RESULTS

The average age of the patients with rotator cuff tear and the patients with proximal humeral fracture was 63.4 years (range, 49-77 years) and 62.2 years (range, 43-77 years), respectively ($P = .6$). The rotator cuff tear group had an

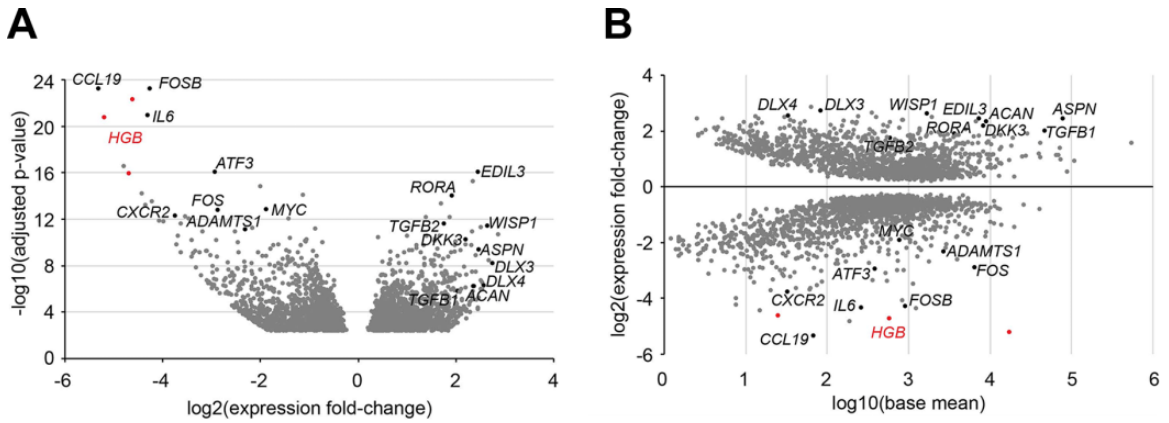

Figure 3. Differential expression of genes, tears versus controls. These 2 plots represent just over 3000 genes that were statistically significant in their differential expression between the 2 groups. (A) One dot per gene is positioned according to *P* value (higher is smaller, or a more significant *P* value) and fold-change, meaning those on the left of the volcano are more highly expressed in controls and those on the right are more highly expressed in tears. (B) The fold-change is plotted against the base mean, or roughly the overall expression level of the gene across all the samples.

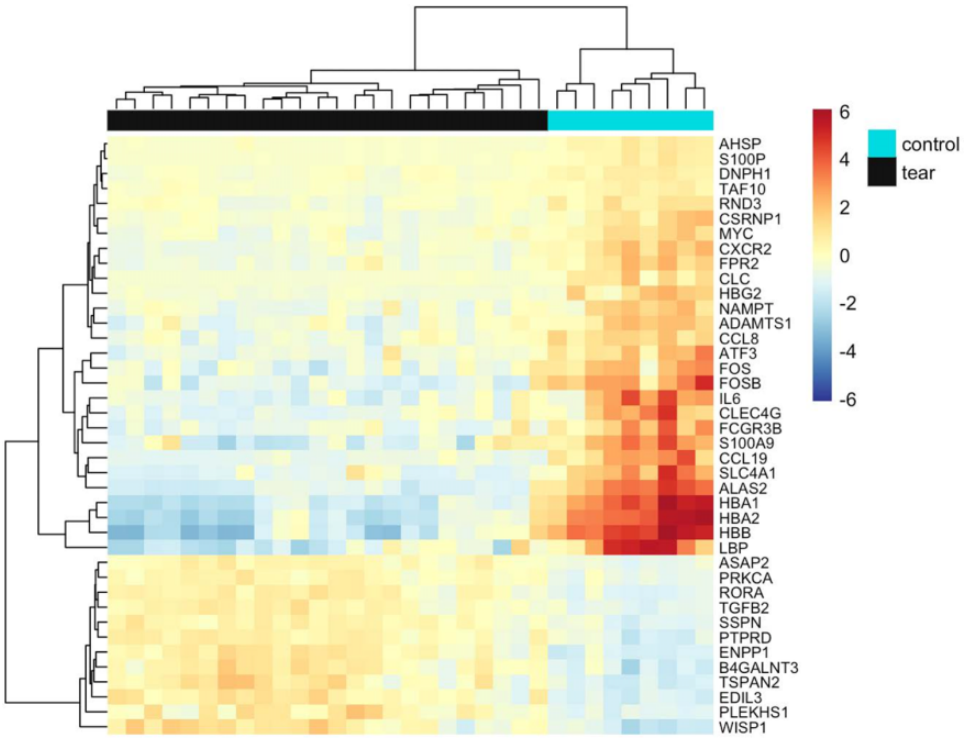

Figure 4. Heatmap of the expression levels of the 40 most significantly differentially expressed genes between tears and controls.

average sagittal tear size of 2.7 ± 1.1 cm and coronal tendon retraction of 2.4 ± 1.1 cm. Supraspinatus fatty degeneration was determined by Goutallier grading: There were 6 (25%) grade 0 patients, 12 (50%) grade 1 patients, 1 (4%) grade 2 patient, 2 (8%) grade 3 patients, and 3 (13%) grade 4 patients. The proximal humeral fracture group was comprised of one 2-part fracture, two 3-part fractures, and six 4-part fractures.

An unsupervised hierarchical clustering heatmap of all raw reads by pairwise correlation between individual transcriptomes demonstrated a strong correlation between 5 controls as a separate cluster and 3 distinct clusters of tears, into 2 of which the remaining controls also clustered (Figure 1). The 2 largest of these clusters (termed c2 and c3) were selected for additional secondary analysis.

The sequenced transcriptomes were also separated into 2-dimensional space by analysis of only the 500 most differentially expressed genes, sorted into 2 principal components (PCs). The first PC described almost half of the

TABLE 1
Ingenuity Pathway Analysis of Differential Expression in Tears Versus Controls[a]

Ingenuity Canonical Pathways	–log (*P* Value)	Ratio	*z* Score
Granulocyte adhesion and diapedesis	15	0.232	
Hepatic fibrosis/hepatic stellate cell activation	9.75	0.186	
Complement system	3.95	0.243	0.378
IL-6 signaling	3.35	0.134	3
Hematopoiesis from pluripotent stem cells	3.12	0.191	
HIF1α signaling	2.91	0.13	
Endothelin-1 signaling	2.59	0.107	2.683
RAR activation	2.18	0.1	
VEGF signaling	1.31	0.0971	1.265

[a]HIF, hypoxia-inducible factor; IL, interleukin; RAR, retinoic acid receptor; VEGF, vascular endothelial growth factor.

variation in the samples and the second PC another tenth. This unbiased analysis meaningfully separated the samples into tear and control (fracture) groups, with 3 controls intermingling with tears (Figure 2).

When we compared tear and control transcriptomes as groups, just over 3000 genes demonstrated significant differential expression (Figure 3). Although some of these differentially expressed genes were strongly expressed in at least 1 group, such as tumor growth factor β1 (TGF-β1) and aggrecan, others were different because of extremely low expression in 1 group and only very modest (although higher) expression in the other. These low-expression genes that were significantly lower in 1 group than the other included a number of transcription factors (which yield large impact even at very low levels of transcription in a tissue) as well as chemokines and their receptors (which represent infiltrating inflammatory cells in higher numbers in the fracture specimens than in torn rotator cuff tendons).

A few of the most highly overexpressed genes in the control samples included the components of hemoglobin (Figure 4).

Ingenuity pathway analysis of the differentially expressed genes highlighted pathways involved in inflammation and extracellular matrix generation, the former higher in the controls and the latter higher in the tears (Table 1).

A second analysis was undertaken of the groups defined by unsupervised hierarchical clustering according to pairwise comparisons. We identified 2 groups of tears from this analysis. One group (c2) included some control specimens within the cluster. The other group (c3) was more distinct and did not include any control samples. Strikingly, despite their smaller sample sizes, these 2 groups had more robust differential expression than was found between tears and controls as a whole (Figure 5). The c2 group of transcriptomes that included both controls and some tears expressed increased levels of markers of endothelial cells. These included genes such as *PECAM*, *SEPT4*, and *COL4A1*. This confirms the presence of increased vessels within these samples. The other group, c3, demonstrated a strongly

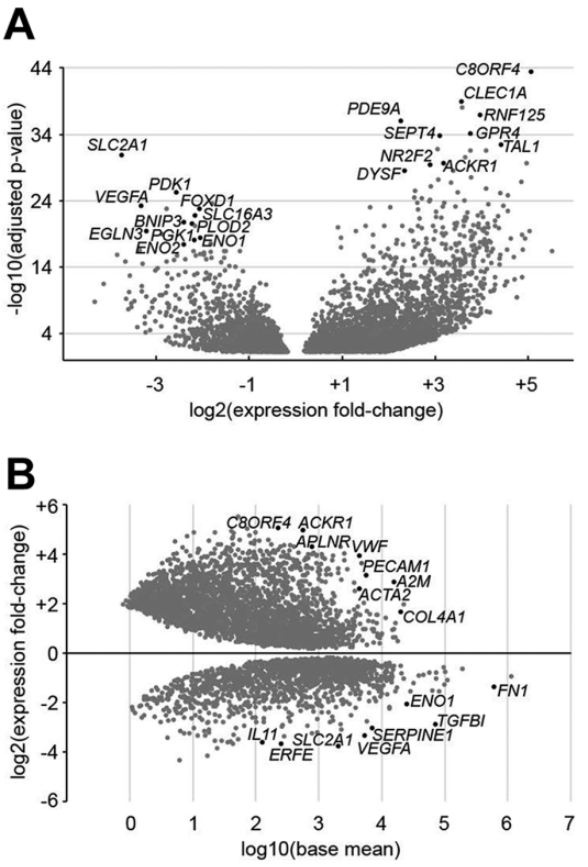

Figure 5. Differential expression of genes in c3 versus c2. These 2 plots represent more than 5000 genes that were statistically significant in their differential expression between the 2 groups. (A) *P* value and fold-changes, with the left of the volcano presenting genes more highly expressed in c2 (the cluster with tears and controls) and the right more highly expressed in c3 (the cluster with only tears). (B) Fold-change and base mean, or the overall expression level of each gene across all samples.

hypoxic gene signature, including many direct target genes of hypoxia-inducible factor 1α (HIF1α), such as *VEGFA*, *BNIP3*, and *ENO1* and *ENO2*. This finding suggests that these samples were producing high levels of chemoattractants of microvasculature but did not have as many vessels answering those signals because of the very low endothelial cell marker expression (Table 2 and Figure 6).

Comparing tear characteristics of these 2 groups, we found that c2, with the co-clustered control transcriptomes, included tears that were significantly larger, had significantly greater tendon retraction, and trended toward worse supraspinatus fatty degeneration than those in c3 that clustered as a separate group (Tables 3 and 4). Postoperative MRIs were obtained on 4 of 6 patients in cluster c2 and on 9 of 9 patients in cluster c3. Patients in cluster c3 (8/9 patients healed; 89%) had significantly greater healing rates compared with patients in cluster c2 (0/4 patients healed; 0%) ($P = .007$) (Table 5).

TABLE 2
Ingenuity Pathway Analysis of Differential Expression Between Clusters c2 and c3[a]

Ingenuity Canonical Pathways	–log (*P* Value)	Ratio	*z* Score
Hepatic fibrosis/hepatic stellate cell activation	13	0.182	
Nitric oxide signaling in the cardiovascular system	7.73	0.177	2.683
eNOS signaling	5.87	0.128	3.13
Hematopoiesis from pluripotent stem cells	5.77	0.234	
Endothelin 1 signaling	4.64	0.111	2.4
Inhibition of angiogenesis by TSP1	4.36	0.235	0.816
HIF1α signaling	4.17	0.127	
VEGF family ligand-receptor interactions	3.74	0.136	2.309
VEGF signaling	3.1	0.117	2.887

[a]eNOS, endothelial nitric oxide synthase; HIF, hypoxia-inducible factor; TPS, thrombospondin; VEGF, vascular endothelial growth factor.

DISCUSSION

In rotator cuff tendon tissue specimens obtained from 2 different scenarios—acute proximal humeral fractures undergoing operative fixation and rotator cuff tears undergoing arthroscopic repair—gene expression differed significantly. The expression profile in rotator cuff tears demonstrated an overabundance of extracellular matrix production and reduction in the drivers of cell division, suggesting a biology that may contribute to failure of healing after rotator cuff injury. Further, a secondary cluster analysis identified a subgroup of smaller tears with less tendon retraction with overexpression of genes within the hypoxia pathway, suggesting that hypoxia may play an important role in the early stages of rotator cuff disease. Interestingly, this group also had a very high propensity for healing, suggesting that repair in the early stages of disease may have a higher chance for repair success.

The sequenced transcriptomes from tears versus controls analyzed as groups suggested the relatively increased presence of hematologic cells and inflammation in the controls. The inflammation was not entirely surprising, given the fracture setting of the controls. Some of the most relatively overabundant transcripts in the controls were from the genes encoding hemoglobin. These are some of the only transcripts retained in erythrocytes that have otherwise become enucleated, thus demonstrating an increased presence of red blood cells in the control specimens as a whole. This information has 2 alternative interpretations. It may simply follow the fact that because the fracture specimens were obtained in an open operative field, instead of an arthroscopic operative field, they retained more field blood on the control specimens. It may alternatively suggest that the control samples have a denser network of vessels, therefore including more intravascular blood cells.

The tear group had increased expression of extracellular matrix genes. This may give some insight into why tears fail to heal in the setting of rotator cuff injury. The failing reparative cellular response appears to focus on high levels of matrix production with limited expression of the genes

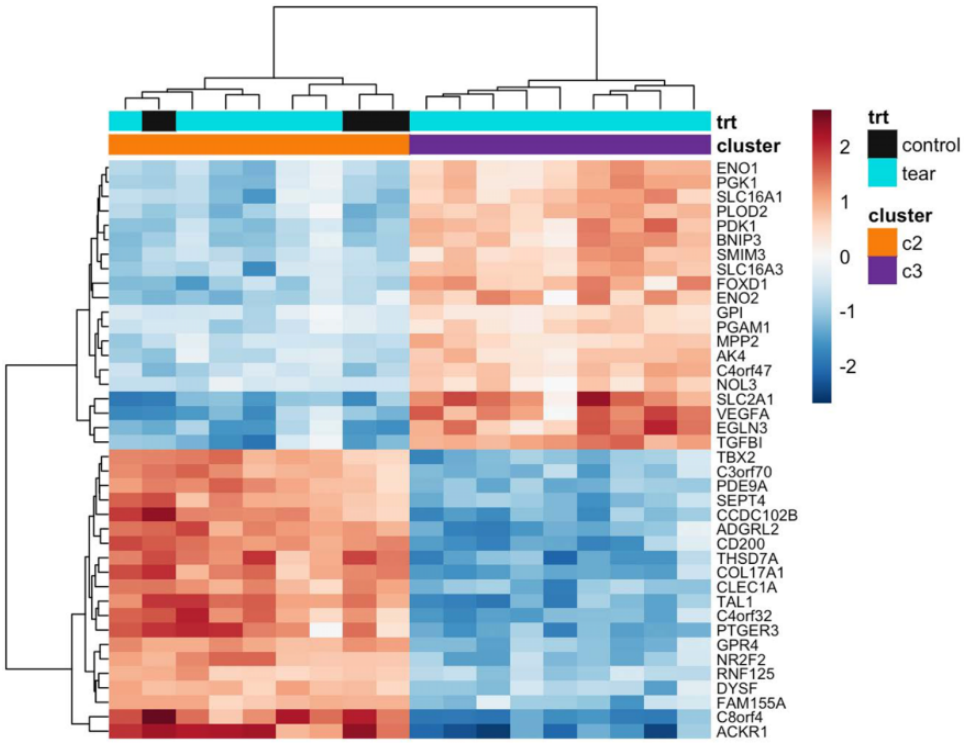

Figure 6. Heatmap of the expression levels of the 40 most significantly differentially expressed genes between c2 and c3. Controls were patients with proximal humeral fracture, and tears were patients with rotator cuff tear. trt, treatment.

TABLE 3
Tear and Patient Characteristics Between Clusters c2 and c3[a]

	Cluster c2	Cluster c3	*P* Value
Age, y	64	63	.819
Anteroposterior tear size, cm	4	2.2	.001
Tendon retraction, cm	3.4	2.1	.018

[a]Age, tear size, and tendon retraction compared using Student *t* test.

TABLE 4
Muscle Quality Between Clusters c2 and c3[a]

Cluster	Goutallier 0	Goutallier 1	Goutallier 2	Goutallier 3	Goutallier 4
c2	1	1	1	1	2
c3	4	5	0	0	0

[a]Pearson chi-square = 8.194. *P* value = .085.

TABLE 5
Tendon Healing Between Clusters c2 and c3[a]

Cluster	Healed	Unhealed
c2	0	4
c3	8	1

[a]Fisher exact test, 2-tailed *P* value = .007.

that drive cell division. Thus, it may be prudent to focus future biological augmentations for rotator cuff repairs on upregulating cell division instead of only matrix production by a stagnant population of cells.

We identified 2 distinct clusters (c2 and c3) within the tear patients, 1 of which (c2) also included 2 control patients. The c3 cluster, with only tears—but smaller with less tendon retraction—demonstrated increased expression in many hypoxia target genes. This finding suggests that hypoxia, so prominent in clinically "early" tears, may play an important role in the initiating cause of rotator cuff disease. The c2 cluster included both controls and tears. However, these tears were larger and more retracted, indicating further progression along the natural history of rotator cuff disease. This mixed c2 cluster demonstrated more endothelial cell marker gene expression. This suggests that as tears worsen, secondary inflammation develops and promotes ingrowth of granulation tissue, leading to increased vascularity and overall transcriptomes that more resemble the inflamed rotator cuffs of the acute fracture setting. Thus, the reduced endothelial cell markers and increased hypoxia gene expression in the transcriptomes from the cluster of smaller tears that are likely to occur earlier in the natural history of rotator cuff disease suggest that hypoxia and poor vascularity likely play a role in the cause of disease. As the disease progresses, there is greater evidence of inflammation and secondary angiogenesis, probably in granulation type tissue. This neo-angiogenesis unfortunately is not adequate to stimulate tendon healing, which is consistent with the common histologic appearance of angiofibroblastic hyperplasia seen in chronic tendinosis.

With almost complete healing in the c3 cluster and a complete lack of healing in the c2 cluster, tissue level of gene expression not only may explain the pathogenesis of tearing but also may be useful in predicting the success of repair. Once the genetic profile has shifted to an endothelial-predominant pattern, biological healing may be compromised. Reversal of this pattern of expression through biological inhibitors may be a strategy to improve healing rates in the tears in this cluster, although it has to be recognized that the findings can only be interpreted as associative and not necessarily causative.

Our dataset provides a wealth of differentially expressed genes, each of which provides avenues for future research. For instance, these data could be useful to future researchers for the creation of cellular and animal models to study degenerative rotator cuff tear. These data also may provide diagnostic targets to aid in the identification of transcription profiles associated with rotator cuff tear progression or nonhealing. The data provide vital information for future analyses, as they allow researchers to compare a specific subset of genes. Finally, these data provide the initial steps in identifying potential biological targets for future targeted therapies to improve healing in the setting of rotator cuff repair.

Limited data are available regarding differential gene expression using a genome-wide analysis of rotator cuff tears that can be compared with our results.[2] Chaudhury et al[2] used microarray analysis to evaluate gene expression between varying rotator cuff tear sizes and controls and reported some similar results to the current data. Extracellular matrix gene expression was elevated in tears compared with controls in both our data and those of Chaudhury et al. Similarly, Chaudhury et al reported that oxygen-related and inflammatory response groups played a larger role in their comparison of small and large tears. This was similar to our results showing that smaller tears had increased expression of hypoxic gene targets, whereas larger tears had increased expression of inflammatory markers.

Limitations of the current study include a relatively small sample size of tears and controls, potentially missing key genetic pathways. A second limitation is that some of the changes we evaluated may result from age. However, there were no significant differences in age among all groups we analyzed (tears vs controls and c2 vs c3). A third limitation is that a more in-depth analysis of signaling pathways associated with inflammation could be performed along with identification of potential genetic variants if whole exome DNA were available. We are currently recruiting patients back to attempt to obtain DNA for combined analysis with RNA seq data. Fourth, a variety of possible medical comorbidities and treatments including diabetes, thyroid dysfunction, smoking status, use of nonsteroidal anti-inflammatory drugs, and cortisone injections may potentially influence transcriptome analyses. Because these were not consistently reported, we elected to not

include these factors in our analyses; rather, we focused on factors that we could reliably measure (ie, imaging). Also, the small sample size limited our ability to evaluate multiple confounding factors. Fifth, expression data do not necessarily correlate with translation data; therefore, the relative significance of any study reporting transcription alone should be interpreted with some caution. Sixth, bulk cell analysis was performed as opposed to single-cell RNA sequencing; therefore, it is not possible to determine the cell type responsible for the expression, although the tissue was obtained from tendon only. Seventh, our controls were retrieved from a fracture population instead of age-matched controls without any disease or injury. Given the potential morbidity of taking biopsies of normal tendons, obtaining biopsies in the setting of a fracture was believed to represent the best compromise to obtain tissue of the anatomically matched portion of the tendon in a similarly aged cohort. The fracture population required the samples to be taken open instead of arthroscopically, which introduces another variable into the comparison between cases and controls.

CONCLUSION

Smaller, less retracted tears had increased expression of hypoxia target genes and improved healing, whereas larger, more retracted tears were associated with endothelial cell markers and worse healing. Thus, hypoxia may be the inciting event for tear development, whereas with tear enlargement, a chronic, inflammatory, angiogenic process may predominate.

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