Osteoarthritis and Cartilage

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Proteomic analysis of synovial fluid identifies periostin as a biomarker for anterior cruciate ligament injury

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SUMMARY

Objective: Emerging evidence suggests that injury to the anterior cruciate ligament (ACL) typically initiates biological changes that contribute to the development of osteoarthritis (OA). The molecular biomarkers or mediators of these biological events remain unknown. The goal of this exploratory study was to identify novel synovial fluid biomarkers associated with early biological changes following ACL injury distinct from findings in end-stage OA.

Methods: Synovial fluid was aspirated from patients with acute (≤30 days) and subacute (31–90 days) ACL tears and from patients with advanced OA and probed via tandem mass spectrometry for biomarkers to distinguish OA from ACL injury. Periostin (POSTN) was identified as a potential candidate. Further analyses of POSTN were performed in synovial fluid, OA cartilage, torn ACL remnants, and cultured cells and media by Western blot, PCR, immunostaining and ELISA.

Results: Synovial fluid analysis revealed that POSTN exhibited higher expression in subacute ACL injury than OA. POSTN expression was relatively low in cartilage/chondrocytes suggesting it is also produced by other intra-articular tissues. Conversely, high and time-dependent expression of POSTN in ACL tear remnants and isolated cells was consistent with the synovial fluid results.

Conclusions: Elevated POSTN may provide a synovial fluid biomarker of subacute ACL injury setting separate from OA. Increased expression of POSTN in ACL suggests that the injured ACL may play a pivotal role in POSTN production, which is sensitive to time from injury. Previous studies have shown potential catabolic effects of POSTN, raising the possibility that POSTN contributes to the initiation of joint degeneration and may offer a window of opportunity to intervene in the early stages of post-traumatic OA.

Introduction

The lifetime risk of developing osteoarthritis (OA) is one in two, and it is the third leading cause of years lived with a disability[1,2]. OA is a multifactorial disease and its pathophysiological mechanisms are complex and poorly understood, but it is now believed that the molecular changes to joint tissues begin much earlier than the clinical symptoms become apparent[3]. A prerequisite for developing disease modifying OA drugs is a reliable way for studying early pathogenic mechanisms[4]. While detection of early manifestations of primary OA is not currently possible due to complex nature of the disease, post-traumatic OA offers the opportunity to study early molecular changes following joint trauma as time and nature of initial trauma are generally known[5].

Anterior cruciate ligament (ACL) tears are a leading cause of joint trauma, and ACL-deficient knees have altered biomechanics that likely lead to cartilage damage[6] and eventually post-traumatic OA[7]. ACL reconstruction does not prevent OA progression[8,9], suggesting that the high risk of OA after ACL tear is likely due to factors

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other than joint instability[7,10,11]. Unfortunately, the biological underpinning of how ACL injury leads to OA remains an unanswered question. It is believed that paracrine factors released in the joint following joint injury − potentially from the ACL itself − could exert a biological effect on the joint which might contribute to the development of OA[12]. However, given the long lag between ACL injury and development of OA, the biological response of ACL tears appears to vary with time from injury[13,14]. For instance, we have reported that expression of periostin (POSTN) in human ACL tear remnants, which is elevated following ACL injury, changes with chronicity of tear[15].

POSTN is a secreted matricellular protein that has been shown to be expressed in a variety of connective tissues such as periodontal ligament[16,17], bone and periosteum[18], tendons[19] and skin[20], and is thought to influence collagen fibrillogenesis[17,21]. While reduced POSTN levels result in defective connective tissues[22], higher levels of POSTN have been implicated in human and rodent osteoarthritic cartilage[12,23]. In addition, higher levels of POSTN have been shown to correlate with severity of OA[24,25]. Certain studies have shown that POSTN is involved in cartilage matrix degradation via MMP-13[12,23] and MMP-9[26]. However, the levels of POSTN in knees after ACL injury has not been compared to levels in knees with OA.

The goal of this exploratory study was to identify novel synovial fluid biomarker candidates associated with early biological changes following ACL injury distinct from findings in end-stage OA. While it is thought that ACL tears initiate biological changes in the joint that contribute to the development of OA, the molecular mediators of these biological changes are not fully understood. We hypothesize that ACL tears result in a significant and predictable pattern of POSTN expression in the knee, which may be a critical mediator of biological changes in the knee after this injury. By cataloguing the proteins present in the synovial fluid of ACL tear and OA patients, we hope to characterize candidates for further study as potential targets for early therapeutic intervention.

Methods

Study design

First, we compared the synovial fluid proteomics data from 3 groups: (1) acute (≤30 days from injury) ACL tears; (2) subacute (31–90 days from injury) ACL tears; and (3) knee OA patients. POSTN was identified as a candidate marker. The findings were validated by multiplex bead assay and Western blot. Next, we measured and compared the expression of POSTN between acute and subacute ACL tears using synovial fluid (multiplex bead assay), ACL tear remnants (real-time PCR) and cells (real-time PCR, Western blot) and cell culture media (ELISA). Finally, we measured and compared the expression of POSTN between ACL tear remnants/cells and articular cartilage/chondrocytes using real-time PCR, Western blot, ELISA, and immunostaining.

Study patients

The study protocol was approved by the Institutional Review Board, and the surgical specimens were handled in accordance with the current ethical standards. All patients ($N = 124$) gave written and signed informed consent before inclusion in the study. Patients' characteristics are presented in Table I and inclusion and exclusion criteria are detailed in Supplementary Text.

Synovial fluid collection and processing

Synovial fluid was aseptically aspirated with an 18-gauge needle entering the joint from a superolateral approach with the knee in full extension. There was no gross evidence for iatrogenic contamination of the synovial fluid samples at the time of aspiration. Since all of the sample fluids were collected in the clinic from patients with effusions that warranted aspiration due to their volume, lavage was not necessary. The collecting syringe was capped and transferred to the lab within 30–60 min of collection. Synovial fluid was centrifuged at 3,000×g for 20 min at 4°C to remove cells and particulate material and clear supernatant was stored in cryovials at −80°C until assayed[27]. None of the synovial fluid samples were highly viscous to become a pellet after centrifugation.

Nano-LC-MS/MS analysis

Synovial fluid was depleted of 14 highly abundant proteins[28,29]. Fractionation, protein digestion, peptide preparation, and isobaric labeling were carried out using standard methods prior to *nano*-LC-MS/MS analysis. Data processing and analysis were performed using an R environment. The detail of the methods and analysis are given in Supplementary Text. Proteomic data have been deposited in the PRoteomics IDEntifications (PRIDE) PreoteomXchange and can be accessed by identifier PXD013043 (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp).

Multiplex assay

Synovial fluid was thawed, centrifuged at 15,000×g for 10 min at 4°C and diluted (1:8). Quantification of POSTN (and other proteins on the panel namely DKK-1, GDF-15, NSE, OPG, Osteonectin [SPARC], TRA5, TWEAK and YLKL-40) was performed using Milliplex MAP Luminex Assay Kit (Cat. #HCMBMAG-22K) by following the supplied protocol. The assay plate was read on Luminex® 200™ with xPONENT® software. Milliplex Analyst 5.1.0.0 was used as a curve fitting program. A 5-parameter logistic curve-fit and a linear polation logistic curve-fit were used to calculate the concentration in ng/mL from the standards.

Cartilage sampling and cell isolation

Cartilage was collected from knees of patients undergoing total knee arthroplasty. Chondrocytes were isolated via enzymatic digestion[12] as detailed in Supplementary Text.

ACL sampling and cell preparation

ACL remnants were removed from patients undergoing ACL reconstruction surgery. ACL progenitor cells that have been shown to express stem cell markers[30,31] were isolated as described before[32]. Moreover, we also isolated ACL fibroblasts as described previously[31] with slight modifications. Detailed procedures are described in Supplementary Text.

Collection of cell culture supernatant

Chondrocytes, ACL progenitor cells, and ACL fibroblasts were cultured in individual wells of 12-well plates at a density of 2×10^5 cells/well. After 4 days, the culture media was collected to measure the levels of POSTN.

RNA isolation and first strand cDNA synthesis

Total RNA was prepared from tissues and cells using TRIzol solution and RNeasy Mini kit (Qiagen) according to supplied protocol[12]. RNA quality and concentration were checked by measuring the ratio of optical densities at 260 and 280 nm using Nanodrop 2000. A total of 250 ng RNA was treated with DNase I (Invitrogen) to

Table I
Characteristics of study patients

Tissue	Condition	N	Age (years)	BMI (kg/m^2)	Sex	Time from injury (day) Mean [95% CI]	Material	Experiment	Data in
Synovial fluid	Acute (≤30 days)	3	35.3	22.8	3F, 0M	4.0 [1.74 to 6.26]	Synovial fluid	Proteomics, Western blot	Figs. 1(A)–(C), and 2(A), Tables II and V
Synovial fluid	Subacute (31–90 days)	3	47.0	24.1	1F, 2M	45.0 [17.6 to 72.4]	Synovial fluid	Proteomics, Western blot	Figs. 1(A), (B), (D), (F) and 2 (A), Tables III and V
Synovial fluid	OA	3	59.7	30.4	1F, 2M	–	Synovial fluid	Proteomics, Western blot	Fig. 1(A)–(D), and (F), Tables II and III
Synovial fluid	Acute (≤30 days)	9	32.9	25.8	6F, 3M	6.0 [3.5 to 8.5]	Synovial fluid	Multiplex ELISA	Figs. 1(E) and 2(B), Table IV
Synovial fluid	Subacute (31–90 days)	4	40.3	24.2	3F, 1M	43.7 [28.8 to 58.6]	Synovial fluid	Multiplex ELISA	Figs. 1(E) and 2(B), Table IV
Synovial fluid	OA	16	55.9	31.0	6F, 10M	–	Synovial fluid	Multiplex ELISA	Figs. 1(E) and 2(B), Table IV
ACL	Acute (≤30 days)	8	17.9	25.0	6F, 2M	21.8 [18.6 to 25.0]	ACL remnant	Real-time PCR	Figs. 2(C) and 3(D)
ACL	Subacute (31–90 days)	7	26.4	24.2	3F, 4M	53.1 [38.7 to 67.5]	ACL remnant	Real-time PCR	Figs. 2(C) and 3(D)
ACL	3–6 months	4	36.5	27.6	1F, 3M	206.5 [63.0 to 351.0]	ACL remnant	Real-time PCR	Fig. 2(C)
ACL	>6 months	3	32.0	29.6	0F, 3M	1550.3 [470.0 to 2630.0]	ACL remnant	Real-time PCR	Fig. 2(C)
ACL	Acute (≤30 days)	2	38.5	20.8	1F, 1M	21.0 [15.1 to 26.9]	Progenitors	Western blot	Fig. 2(D)
ACL	Subacute (31–90 days)	2	29.0	22.0	2F, 0M	70.5 [46.0 to 95.0]	Progenitors	Western blot	Fig. 2(D)
ACL	Acute (≤30 days)	3	17.7	24.2	2F, 1M	13.7 [8.35 to 19.0]	Progenitors	ELISA	Fig. 2(E)
ACL	Subacute (31–90 days)	3	47.0	24.1	3F, 0M	49.3 [38.5 to 60.1]	Progenitors	ELISA	Fig. 2(E)
ACL	ACL-T	6	29.8	26.8	2F, 4M	75.7 [25.9 to 126.0]	Progenitors	Real-time PCR	Fig. 3(A)
ACL	ACL-T	6	24.3	26.2	3F, 3M	29.3 [18.5 to 40.1]	Fibroblasts	Real-time PCR	Fig. 3(A)
Cartilage	OA	9	64.1	30.9	6F, 3M	–	Chondrocytes	Real-time PCR	Fig. 3(A)
ACL	ACL-T	2	27.0	27.8	1F, 1M	35.0 [-10.0 to 80.0]	Progenitors	Western blot	Fig. 3(B)
ACL	ACL-T	2	33.5	22.7	2F, 0M	34.0 [4.6 to 63.4]	Fibroblasts	Western blot	Fig. 3(B)
Cartilage	OA	2	63.5	33.9	1F, 1M	–	Chondrocytes	Western blot	Fig. 3(B)
ACL	ACL-T	6	32.3	24.1	5F, 1M	31.5 [14.9 to 48.1]	Progenitors	ELISA	Fig. 3(C)
ACL	ACL-T	3	31.0	26.4	2F, 1M	36.0 [16.1 to 55.9]	Fibroblasts	ELISA	Fig. 3(C)
Cartilage	OA	3	64.3	33.3	1F, 2M	–	Chondrocytes	ELISA	Fig. 3(C)
Cartilage	OA	8	60.9	36.9	4F, 4M	–	Cartilage	Real-time PCR	Fig. 3(D)
ACL	ACL-T	3	38.0	39.3	2F, 1M	34.7 [22.3 to 47.1]	Progenitors	Immunofluorescence	Fig. 3(E)
ACL	ACL-T	3	27.3	24.5	2F, 1M	41.3 [32.2 to 50.4]	Fibroblasts	IMmunofluorescence	Fig. 3(E)
Cartilage	OA	4	64.8	28.6	2F, 2M	–	Chondrocytes	Immunofluorescence	Fig. 3(E)

ACL = anterior cruciate ligament; ACL-T = ACL tear; BMI = body mass index; OA = osteoarthritis; M = male; F = female; CI = confidence interval.
PCR = polymerase chain reaction; ELISA = enzyme linked immunosorbent assay.

remove traces of genomic DNA. High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher-Scientific) and random primers were used to convert RNA to first strand of cDNA.

Real-time PCR

Real-time PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) as before[33]. The primer for POSTN included: *POSTN* (forward) 5′-TCTGCACTGCCAAGACTGAG-3′ and (reverse) 5′-TGGTCTTGCCATTCCTGGAC-3′. Peptidylprolyl isomerase A (*PPIA*) was used as housekeeping gene: (forward) 5′-CAACGCAGCGCTATTCTGAC and (reverse) 5′-CCAAGTTGTCC-CAAGCCTCA-3′. Detailed method is provided in Supplementary Text.

Enzyme-linked immunosorbent assay

The levels of POSTN were measured in the cell culture supernatant using a human POSTN ELISA Kit (Cat. # EHPOSTN, Thermo-Fisher-Scientific) as detailed in Supplementary Text.

Immunostaining and confocal microscopy

POSTN localization was assessed by immunofluorescence using chondrocytes, ACL progenitor cells, and ACL fibroblasts as detailed in Supplementary Text.

Western blot

Western blot was used for the detection of POSTN protein in synovial fluid samples as well as lysates prepared from cultured cells. A detailed description is provided in Supplementary Text.

Statistical analyses

All data were analyzed by non-parametric ANOVA (Kruskal-Wallis) test with Dunn's multiple testing or non-parametric Mann-Whitney U test. Results are presented as mean ± standard error of the mean unless indicated otherwise. Results with a $P < 0.05$ were considered statistically significant.

Results

Higher levels of POSTN in the synovial fluid of subacute ACL tears than OA patients

Tandem mass spectrometry on synovial fluid samples from acute and subacute ACL tear patients as well as OA patients quantified 491 proteins (Supplementary Table 1) from 5001 unique peptide sequences (Supplementary Table 2). To assess the levels of POSTN, we compared the ACL tear (acute and subacute) groups with the OA group. Interestingly, multidimensional scaling (MDS) analysis revealed a discrete separation of sample from three groups (acute and subacute tear groups without OA and knees with OA) based on differentially expressed proteins. Heat-maps further confirmed distinct segregation of samples [Fig. 1(A)]. We observed that 69 proteins were significantly differentially expressed in the synovial fluid from acute tears and OA comparison. Among these, 38 were higher in acute tears and 31 were lower in this group (Table II; Supplementary Table 3). While 20 proteins exclusively differed between acute tears and OA, 28 proteins overlapped with subacute tears and OA comparison, 19 proteins overlapped with acute and subacute tears comparison and 2 proteins were common to all three comparisons [Fig. 1(B)]. Our data demonstrated that POSTN was not significantly

differentially expressed in the synovial fluid from patients with acute tears compared to the OA group [Fig. 1(C)].

Comparison between subacute tears and OA revealed that 107 proteins were differentially expressed between the two groups. Among these, 49 were higher in subacute tears and 58 were lower in this group (Table III; Supplementary Table 4). While 62 proteins were exclusive to the comparison of subacute tears vs OA, 15 proteins overlapped with acute and subacute comparison, 28 proteins overlapped between acute tears and OA comparison [Fig. 1(B)]. The expression of POSTN was ~3.5 fold higher in subacute tears than in OA ($P < 0.0001$) [Fig. 1(D)].

Consistent with the proteomics results, POSTN was found to be significantly ($P = 0.001$) different between subacute (1096.02 ± 76.13 ng/mL) and OA (488.17 ± 55.70 ng/mL) groups based on multiplex kit analysis [Fig. 1(E)]. Interestingly, none of the other 7 proteins, except for osteonectin i.e., SPARC, were either significantly differentially expressed by proteomics or by multiplex assay between acute ACL tears and OA (Table IV). High concentration of POSTN in the subacute phase of injury was further confirmed by Western blot analysis using synovial fluid samples from acute, subacute and OA groups [Fig. 1(F)].

Temporal changes in synovial fluid POSTN in ACL injury

We examined the temporal changes in POSTN in synovial fluid samples from ACL tear patients. The MDS analysis showed a distinct separation of samples from two groups (acute and subacute) based on differentially expressed proteins. Unsupervised hierarchical clustering also showed segregation of samples [Fig. 1(A)]. In total, 52 proteins were significantly different between acute and subacute tears. Among these, 21 proteins were more highly expressed and 31 had less expression in subacute tears compared to acute tears (Table V; Supplementary Table 5). Sixteen proteins were exclusive to acute/subacute comparison while others also overlapped with subacute tears and OA comparison (15 proteins) and with acute tears and OA comparison (19 proteins) [Fig. 1(B)].

As shown in Fig. 2(A), the data demonstrated that POSTN was among the most highly significantly differentially expressed protein in the synovial fluid of patients in the subacute ACL tear group compared to the acute ACL tear group. The expression of POSTN was ~4 fold higher in subacute group than the acute group ($P < 0.001$). We also found that POSTN was strongly positively correlated with the time from injury based on proteomics data ($r = 0.94$, $P = 0.006$). As stated above, we validated the expression of POSTN protein using a multiplex kit that included POSTN and seven other proteins in a larger cohort of patients. Interestingly, only POSTN was found to be significantly ($P = 0.003$) different between acute (533.43 ± 107.21 ng/mL) and subacute (1096.02 ± 76.13 ng/mL) groups. Finally, we observed that POSTN was strongly positively correlated with the time from injury based on multiplex ($r = 0.78$, $P = 0.002$) data [Fig. 2(B)].

Temporal changes in POSTN in ACL tear remnants

Next, we assessed the changes in POSTN from ACL tear remnants over time from injury at the gene and protein level. Initial assessment of the natural history of gene expression from the time of ACL injury clearly suggests distinct levels of POSTN in the first 3 months after injury and more than 12 months from injury. Expression of POSTN was low for ≤30 days after ACL injury. Expression increased significantly 31−90 days from injury and remained at a similar high level of expression 3−6 months following injury. The expression started to decline 6 months after ACL injury [Fig. 2(C)]. Since ligament repair is unlikely to be a feasible option more than a year from injury, we
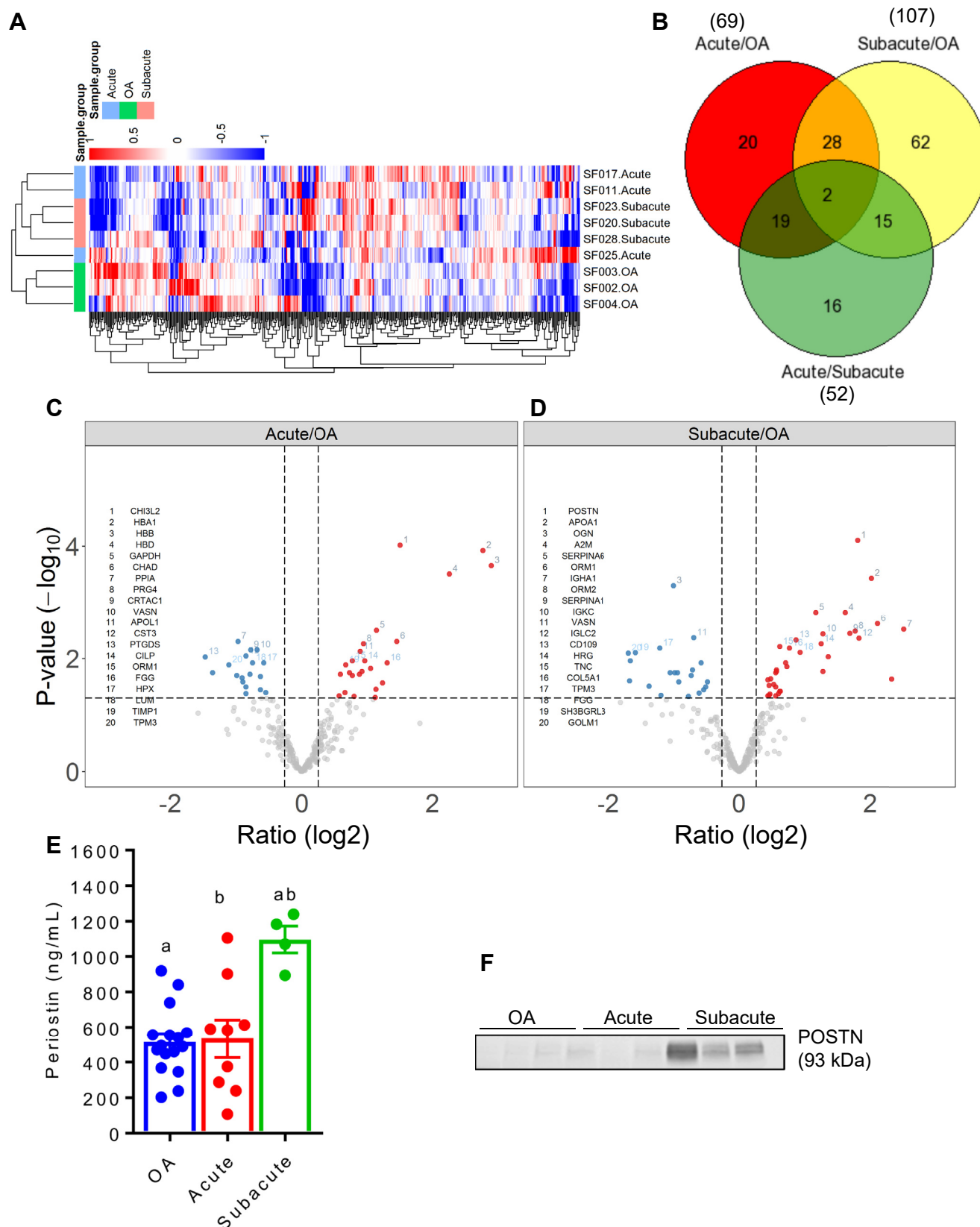
Fig. 1. A. Unsupervised clustering of the protein abundances relative to the reference standard generated from all samples analyzed using synovial fluid proteomic: acute ($N = 3$) and subacute ($N = 3$) tear groups and OA ($N = 3$) group. **B.** Venn diagram for the number of proteins that differed between any two groups and the number of proteins that were common to any two or all three comparisons. **C-D.** Volcano plots showing differentially expressed proteins between acute tear and OA groups (C) and between subacute tear and OA groups (D). The cutoffs for the horizontal dotted lines are at a P value of 0.05 and the cutoffs for the vertical dotted lines are at an absolute fold change of 1.2. Blue dots represent

Table II
Proteins differentially expressed in acute and OA groups[*]

Symbol	Name	Fold change	P value
Proteins elevated in acute group			
HBB	hemoglobin subunit beta	7.45	<0.001
HBA1	hemoglobin subunit alpha 1	6.83	<0.001
SIPA1L3	signal induced proliferation associated 1 like 3	6.35	<0.001
HBD	hemoglobin subunit delta	4.79	<0.001
SAA1	serum amyloid A1	4.70	0.013
THBS1	thrombospondin 1	3.41	0.001
CTSL	cathepsin L	3.22	0.006
CHI3L2	chitinase 3 like 2	2.84	<0.001
CHAD	chondroadherin	2.76	0.005
SELENBP1	selenium binding protein 1	2.49	0.013
Proteins repressed in acute group			
APOL3	apolipoprotein L3	−5.12	0.010
PEBP4	phosphatidylethanolamine binding protein 4	−2.98	0.002
PTGDS	prostaglandin D2 synthase	−2.77	0.009
PIGR	polymeric immunoglobulin receptor	−2.70	0.010
B2M	beta-2-microglobulin	−2.56	0.018
TPM3	tropomyosin 3	−2.16	0.013
SH3BGRL3	SH3 domain binding glutamate rich protein like 3	−1.98	0.020
PPIA	peptidylprolyl isomerase A	−1.97	0.005
IGFBP6	insulin like growth factor binding protein 6	−1.89	0.022
TBC1D1	TBC1 domain family member 1	−1.89	0.032

[*] Only top 20 differentially expressed proteins are shown here.

Table III
Proteins differentially expressed in subacute and OA groups[*]

Symbol	Name	Fold change	P value
Proteins elevated in subacute group			
IGHA1	immunoglobulin heavy constant alpha 1	5.75	0.003
NES	nestin	5.31	<0.001
ALB	albumin	5.06	0.023
ORM1	orosomucoid 1	4.32	0.002
APOA1	apolipoprotein A1	4.05	<0.001
IGLC2	immunoglobulin lambda constant 2	3.54	0.004
POSTN	periostin	3.51	<0.001
ORM2	orosomucoid 2	3.41	0.003
SERPINA1	serpin family A member 1	3.23	0.004
A2M	alpha-2-macroglobulin	3.08	0.002
Proteins repressed in subacute group			
APOL3	apolipoprotein L3	−8.54	0.013
NKPD1	NTPase, KAP family P-loop domain containing 1	−6.72	0.018
COL2A1	collagen type II alpha 1 chain	−4.43	0.006
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	−4.26	0.025
GSS	glutathione synthetase	−3.81	0.007
FKBP5	FK506 binding protein 5	−3.67	0.005
YWHAB	14-3-3 protein beta/alpha	−3.37	0.015
GOLM1	golgi membrane protein 1	−3.23	0.008
VIM	vimentin	−3.18	0.025
DEFA1	defensin alpha 1	−3.15	0.011

[*] Only top 20 differentially expressed proteins are shown here.

focused on two time intervals, acute (≤30 days after injury), and subacute (30–90 days after injury) for all subsequent studies. Follow up analysis demonstrated low levels of POSTN ≥30 days from injury (acute) and the highest levels of POSTN 31–90 days after injury (subacute). POSTN mRNA was significantly ($P = 0.017$) higher (1.8 fold) in the subacute group than the acute group [Fig. 2(C)]. This finding was further confirmed by Western blot analysis of proteins prepared from ACL progenitor cells from acute and subacute groups

detecting a 93 kDa protein band corresponding to POSTN protein in the subacute phase of injury [Fig. 2(D)]. Acute samples yielded only weak bands. To further explore if subacute ACL progenitor cells produce higher levels of POSTN protein, we measured the concentration of POSTN released in the culture media over 4 days using ELISA. As depicted in Fig. 2(E), subacute ACL progenitor cells generated significantly more POSTN ($P = 0.003$) compared to acute ACL progenitor cells (101.10 ± 3.77 ng/mL vs 64.55 ± 4.07 ng/mL).

proteins that are low in acute/OA comparison or subacute/OA comparison, while red dots show proteins that are expressed higher in these comparisons. **E.** Multiplex assays performed on synovial fluid from acute ($N = 9$) and subacute ($N = 4$) tear groups and OA ($N = 16$) group confirmed significantly higher concentration of POSTN protein in the subacute tear group compared to both acute tear and OA groups. Kruskal-Wallis with Dunn's multiple comparison test. Each bar graph is presented as mean ± standard error of the mean. Similar lowercase letters (a, b) indicate statistical significance across groups at $P < 0.05$. **F.** Western blot analysis of POSTN protein in the synovial fluid of selected patients further showed that the subacute tear group ($N = 3$) had strong bands corresponding to high POSTN protein compared to both acute tear group ($N = 3$) and OA groups ($N = 3$).

Table IV
Proteins differentially expressed in acute, subacute and OA groups as determined by multiplex assay

Symbol	Name	Group	N	Mean	S.E.M	95% Confidence Interval for Mean		P value		
						Lower Bound	Upper Bound	Acute vs Subacute	Acute vs OA	Subacute vs OA
GDF15	Growth Differentiation Factor 15	Acute	9	0.77	0.15	0.43	1.10	0.721	0.902	0.867
		Subacute	4	0.54	0.21	−0.11	1.10			
		OA	16	0.68	0.13	0.40	0.96			
DKK1	Dickkopf-related protein 1	Acute	9	0.36	0.05	0.24	0.48	0.800	0.695	0.409
		Subacute	4	0.27	0.08	0.03	0.51			
		OA	16	0.44	0.07	0.29	0.59			
NSE	Neuron-specific enolase	Acute	9	31.59	6.18	17.33	45.84	0.812	0.990	0.841
		Subacute	4	23.56	5.69	5.44	41.68			
		OA	16	30.37	6.14	17.28	43.45			
SPARK	Osteonectin	Acute	9	3448.58	528.82	2229.13	4668.04	0.055	**<0.001**	0.296
		Subacute	4	2000.10	211.37	1327.41	2672.71			
		OA	16	1159.78	138.84	863.84	1455.71			
POSTN	Periostin	Acute	9	533.43	107.21	286.21	780.66	**0.003**	0.902	**0.001**
		Subacute	4	1096.02	76.13	853.76	1338.28			
		OA	16	488.17	55.70	369.44	606.89			
TRAP5	tartrate-resistant acid phosphatase type 5	Acute	9	107.61	37.13	22.00	193.23	0.398	0.768	0.646
		Subacute	4	19.23	6.72	−2.14	40.60			
		OA	16	75.27	30.51	10.23	140.31			
OPG	Osteoprotegerin	Acute	9	31.87	9.66	9.59	54.15	0.262	0.646	0.550
		Subacute	4	6.02	2.59	−2.22	14.26			
		OA	16	21.83	7.04	6.82	36.84			
YKL49 (CHI3L1)	Chitinase 3 Like 1	Acute	9	4270.52	915.03	2160.46	6380.58	0.928	0.950	0.808
		Subacute	4	3599.47	1610.10	−1524.59	8723.53			
		OA	16	4655.36	779.16	2994.62	6316.11			
TWEAK	tumor necrosis factor-like weak inducer of apoptosis	Acute	9	0.88	0.17	0.49	1.27	0.285	0.123	0.988
		Subacute	4	0.51	0.17	−0.04	1.06			
		OA	16	0.54	0.08	0.37	0.72			

Values shown in boldface indicate statistical significance.
S.E.M = standard error of the mean; OA = osteoarthritis.

Comparative expression of POSTN in ACL tear remnants and articular cartilage

Finally, we compared expression of POSTN between ACL tear remnants and articular cartilage. We acknowledge that there is no direct physiological or pathophysiological link between the two tissues but comparing the levels of POSTN is informative as to the role of this molecule across the knee as a whole.

Real-time PCR and Western blot results respectively showed that ACL progenitor cells had significantly higher expression of POSTN mRNA [Fig. 3(A)] and protein [Fig. 3(B)] compared to chondrocytes. In addition, cell culture media collected 4 days after culture showed

Table V
Proteins differentially expressed in acute and subacute groups[*]

Symbol	Name	Fold change	P value
Proteins elevated in subacute group			
NES	nestin	7.01	<0.001
ALB	albumin	5.38	0.016
POSTN	periostin	4.08	<0.001
IGHA1	immunoglobulin heavy constant alpha 1	3.89	0.009
HP	haptoglobin	3.37	0.042
SERPINA1	serpin family A member 1	2.78	0.005
IGLC2	immunoglobulin lambda constant 2	2.66	0.011
ORM1	orosomucoid 1	2.51	0.023
MMP3	matrix metallopeptidase 3	2.07	0.004
IGKC	immunoglobulin kappa constant	1.98	0.012
Proteins repressed in subacute group			
SIPA1L3	signal induced proliferation associated 1 like 3	−6.02	0.008
HBB	hemoglobin subunit beta	−5.13	0.014
ALPL	alkaline phosphatase, liver/bone/kidney	−4.69	0.022
HBA1	hemoglobin subunit alpha 1	−4.22	0.010
HBD	hemoglobin subunit delta	−3.56	0.018
H3F3A	H3 histone family member 3A	−2.80	0.004
PZP	PZP, alpha-2-macroglobulin like	−2.72	0.019
PSMA3	proteasome subunit alpha 3	−2.49	0.013
YWHAB	14-3-3 protein beta/alpha	−2.46	0.044
CFHR2	complement factor H related 2	−2.30	0.027

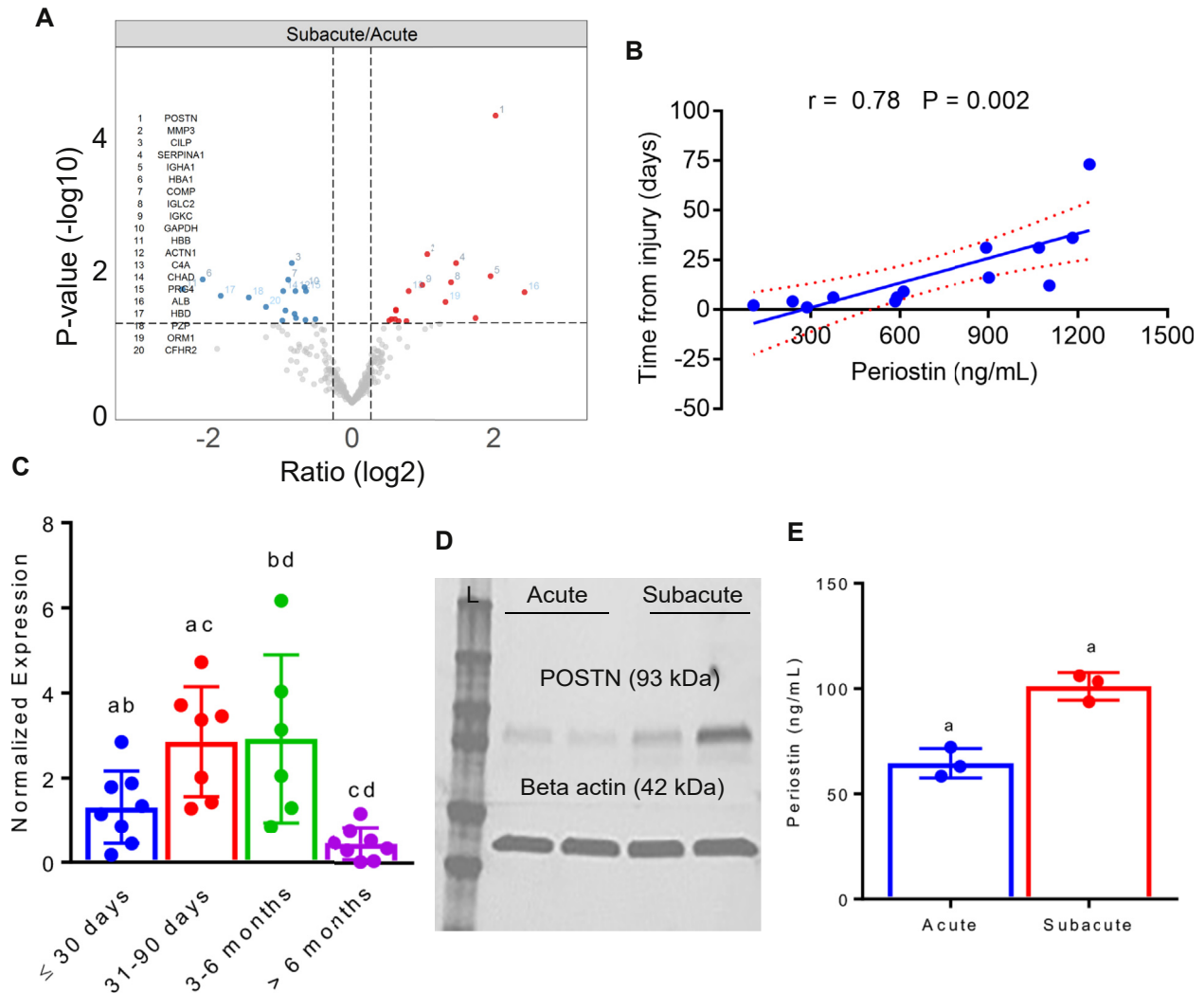[*] Only top 20 differentially expressed proteins are shown here.

Fig. 2. **A.** Volcano plot showing differentially expressed proteins between subacute and acute tear groups based on synovial fluid proteomic analysis. The cutoff for the horizontal dotted line is at a *P* value of 0.05 and the cutoff for the vertical dotted lines are at absolute fold change of 1.2. Blue dots represent proteins that are low in subacute/acute comparison, while red dots show proteins higher in this comparisons. **B.** Pearson's correlation between POSTN protein in the synovial fluid and time from ACL tear showed significantly positive correlation based on multiplex data. **C.** mRNA expression of POSTN in ACL tear remnants (ACL tissues) across four time from injury time points showed that subacute tears (31–90 days; $N = 7$) have significantly higher expression of POSTN than acute tears ($\leq$30 days; $N = 8$) as well as chronic tears (>6 months N = 8). ACL tears 3–6 months old ($N = 6$) showed higher levels of POSTN compared with acute and chronic tear groups. Kruskal-Wallis with Dunn's multiple comparison test. Each bar graph is presented as mean ± standard error of the mean. Similar lowercase letters (a, b, c, d) indicate statistical significance between groups at $P < 0.05$. **D.** Western blot analysis of POSTN protein for acute and subacute ACL tear groups using total protein extracted from ACL progenitor cells by RIPA buffer showed strong bands for the subacute tear group corresponding to high POSTN protein compared to acute tear group. **E.** Assessment of secretary POSTN protein from ACL progenitor cells revealed that after 4 days of culture there were significantly higher levels of POSTN in the cell culture media collected from subacute ACL tear remnants ($N = 3$) compared to those collected from acute ACL tear remnants ($N = 3$). Mann-Whitney U test. Each bar graph is presented as mean ± standard error of the mean. Similar lowercase letter (a) indicates statistical significance between groups at $P < 0.05$.

higher levels of POSTN protein in ACL cells (both progenitors and fibroblasts) than chondrocytes [Fig. 3(C)]. ACL tissues had higher expression of POSTN mRNA than cartilage [Fig. 3(D)]. Finally, we confirmed that ACL progenitor cells and fibroblasts also have significantly higher staining for POSTN protein than chondrocytes [(Fig. 3(E)].

Discussion

As expected, this study identified differential expression of a number of proteins in the synovial fluid of knees following ACL tear compared to knees with end-stage radiographic OA identifying biological differences between early changes following ACL tears and those with end-stage OA. POSTN stood out as a strong candidate biomarker because previous studies have demonstrated that

mRNA expression of POSTN in ACL remnants is very sensitive to time from injury[15], torn ACL remnants co-cultured with chondrocytes exert paracrine effects on chondrocytes function potentially via POSTN[12], and POSTN has been shown to be highly expressed in osteoarthritic cartilage[12,23,34]. However, there is no information on the source of POSTN in the knee and no comparison of POSTN levels in ACL injured knees with OA knees has been made.

This study demonstrated that the levels of POSTN were higher in the ACL injured knees especially in the subacute phase of injury (31–90 days) compared to knees with end-stage radiographic OA. This is the first comparison of POSTN levels between ACL deficient and OA knees. A number of other studies have reported that OA synovial fluid and serum had significantly higher concentrations of POSTN compared to that of rheumatoid arthritis (RA) patients and healthy controls, and that these higher levels are positively
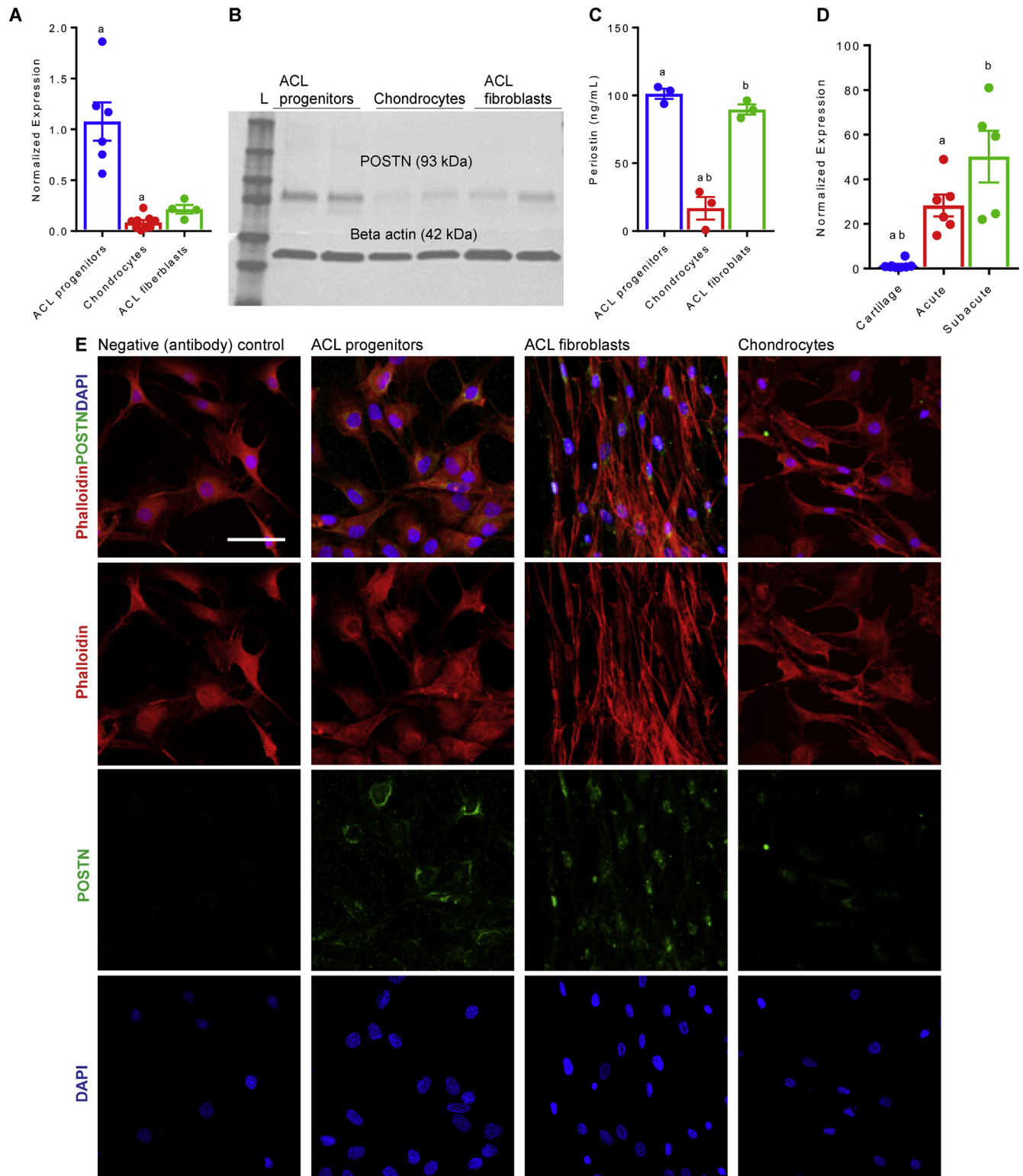
Fig. 3. A. mRNA expression of POSTN in ACL progenitor cells ($N = 6$), chondrocytes ($N = 9$) and ACL fibroblasts ($N = 4$) showed that ACL progenitor cells had significantly higher expression of POSTN mRNA than chondrocytes. Kruskal-Wallis with Dunn's multiple comparison test. Each bar graph is presented as mean ± standard error of the mean. Similar lowercase letter (a) indicates statistical significance between groups at $P < 0.05$. **B.** Western blot analysis of POSTN protein extracted from ACL progenitor cells, chondrocytes and ACL fibroblasts showed that ACL progenitor cells showed strong bands corresponding to more POSTN protein compared to chondrocytes. The intensity of POSTN bands was in between those of progenitor cells and chondrocytes for ACL fibroblasts. **C.** POSTN ELISA performed on cell culture media collected after 4 days of culture from ACL progenitor cells ($N = 3$), ACL fibroblasts ($N = 3$) and chondrocytes ($N = 3$) showed that chondrocytes had significantly lower concentration of POSTN protein than both ACL progenitor cells and ACL fibroblasts. Kruskal-Wallis with Dunn's multiple comparison test. Each bar graph is presented as mean ± standard error of the mean. Similar lowercase letters (a, b) indicate statistical significance between groups at $P < 0.05$. **D.** mRNA expression of POSTN was measured in ACL tear remnants and cartilage tissues demonstrating that both acute ($N = 6$) and subacute ($N = 5$) ACL tear remnants had significantly higher POSTN mRNA than cartilage ($N = 8$). Kruskal-Wallis with Dunn's multiple comparison test. Each bar graph is presented as mean ± standard error of the mean. Similar lowercase letters (a, b) indicate statistical significance across groups at $P < 0.05$. **E.** Immunofluorescent staining of ACL progenitor cells ($N = 3$), ACL fibroblasts ($N = 3$) and chondrocytes ($N = 3$) further showed that POSTN protein (green) was higher in ACL progenitor cells and fibroblasts than chondrocytes. Phalloidin (red) was used to stain F-actin while DAPI (blue) staining showed nucleus. Scale bar = 50 μm.

correlated with the severity and the risk of progression of knee OA[24,25,35]. One contrasting study reported higher levels of POSTN in the synovial fluid of RA patients than in OA patients[36]. Cartilage from human and mouse OA knees has been shown to exhibit higher expression of POSTN than normal cartilage[12,23]. This higher expression of POSTN was confined mainly to chondrocytes in the periphery of erosive lesions[34] and has been associated with higher expression of MMPs in chondrocytes[12,23] and synoviocytes[26]. There are some studies that report significant up-regulation of the POSTN gene in subchondral bone during OA[37–39]. However, none of these studies compared POSTN expression in OA patients with ACL tear patients. Given the fact that ACL tear increases the risk of developing OA[8,9] and that the biologic underpinnings of this elevated risk are unknown, our findings suggest that POSTN accumulation in the joint following ACL injury may contribute to the initiation of degenerative changes in the cartilage. If so, it may provide a therapeutic target for blocking the progress of OA at a very early stage following ACL injury, before cartilage degradation occurs.

Another interesting finding was that the expression of POSTN from subacute ACL tears was significantly higher than from acute ACL tears. Moreover, mRNA and protein level expression of POSTN was also significantly higher in ACL cells, tissues and culture media from the subacute injury group. These findings suggest that POSTN could serve as a marker of chronicity following ACL injury. These findings are consistent with our prior finding that ACL tear remnants express higher levels of POSTN mRNA, particularly within 3 months after ACL tear[15]. In the current study, differences in POSTN expression can be detected within 3 months of injury, which may identify a window of opportunity to intervene and delay or prevent post-traumatic OA following ACL tears. Our data showed relatively low levels of POSTN in the first month after injury, which increase within 3 months of injury, peak at 3–6 months following injury and then decline significantly. A similar expression pattern of POSTN mRNA has been shown in the medial compartment of the knee in a murine model of post-traumatic OA initiated by meniscus and ACL tears[23]. It is possible that this finding highlights a biological benefit from early surgical intervention following ACL injury, as stabilization within the first month of an ACL tear could reduce intra-articular accumulation of POSTN. If this accumulation contributes to the development of OA, early treatment of the tear could be chondroprotective.

The complete source of POSTN in the joint is not known although it is thought that many tissues in the joint express and produce POSTN such as bone[18], cartilage[12,23,34], ACL[12,15], meniscus[40] and synovium[36]. However, to date, no quantitative comparison of POSTN expression between various joint tissues has been reported. Comparing cartilage and ACL, we found elevated expression of POSTN in ACL tissues and cells suggesting that ACL tissue and cells are a more significant source of POSTN than cartilage and chondrocytes. Another study reported that POSTN was more highly up-regulated in ACL than in patellar tendon[41], consistent with our finding that the ACL is perhaps a major source of POSTN mRNA and protein in the knee.

This study has some limitations. First, while it is important to point out that not all patients with an ACL tear go on to develop OA, comparing the biologic response of the knee to ACL injury with the biology of OA is relevant because at least some subset of ACL tear patients go on to develop OA. This information is a first step towards understanding the initial effect of the ACL tear on the knee, as it is unknown exactly when and how knees diverge in their response to this common injury such that some develop OA and some do not. The study only included patients with isolated ACL tears without concomitant meniscus tears to minimize the confounding effect of these injuries. Second, we did not analyze the synovial fluid or tissues from healthy joints for practical and ethical

reasons. While the focus of the current study was on POSTN, the available data from other proteins offer the opportunity to study other candidate proteins in ACL injury and OA. Third, while we were not able to study the other proteins that showed significant differences between acute and subacute ACL tears or between OA and ACL tear groups, several other potential candidates emerged from proteomic analysis that deserve further investigation. For instance, nestin, which is an intermediate filament protein and a marker of neural progenitors[42], was elevated in the subacute group compared to acute ACL tears and OA patients, suggesting the subacute tear group may have an increased number of nestin-positive cells. The combination of elevated nestin and POSTN expression suggests a more substantial repair response. Another example is the expression of SPARC (osteonectin) protein. In this study SPARC was not significantly different between any groups in proteomic analysis, it was found to be significantly higher (~3-fold) in the acute group compared to the OA group by multiplex assay. SPARC is a secreted extracellular matrix protein that has been shown to be associated with ligament morphogenesis, modulation of tissue remodeling and extracellular matrix interaction[43]. SPARC expression also increased following ACL injury, suggesting that it may also play a role in the response of the knee to this trauma[44]. Another limitation is the lack of controls from non-injured knees. While this is an area that deserves further study, the significant differences between acute ACL tears, subacute ACL tears and OA represent novel information about the post-traumatic response of the knee to injury irrespective of if and how this differs from the uninjured controls. Lastly, the sample sizes are small. No *a priori* power calculation was undertaken, and so the study should be considered exploratory. However, the consistency of results across different methods of analysis in 124 discrete patients should give some confidence that they could be replicated. Other factors such as clearance rate across the synovial membrane could influence levels of POSTN over time and were not assessed in the current study. One area that deserves further evaluation is determining if there are sex based differences in the pattern of POSTN expression following ACL injury. While we did not find any evidence for a difference based on sex in our current analysis since that was not the focus of the investigation and more study is needed.

It remains unclear what POSTN tells us as a biomarker and what tissues truly contribute to POSTN in the knee. At this stage, we can only speculate about the role of POSTN based on the available literature. As outlined above, POSTN is a secreted matricellular protein that influences collagen fibrillogenesis and its reduced levels result in defective connective tissues. It appears that it is an important biomarker for synthesis of matrix components in fibrous tissues such as ACL. However, in articular cartilage, expression of POSTN is low in the normal state and is increased in OA. This suggests that higher levels in the cartilage are not desirable as it may lead to overexpression of MMP-13 that in turn results in cartilage degeneration and OA. Thus, POSTN likely plays a dual role in the knee, depending on which tissue and under what condition. Future mechanistic studies are necessary to expand our understanding of this complex, likely multifaceted, role. Based on our recent findings[32] and current observations, the injured ACL could be a major source of POSTN in the knee. However, screening of all knee joint tissues is necessary to make a final determination.

In conclusion, elevated expression of POSTN in knees with ACL tears compared to end-stage OA suggests that it is a distinct synovial fluid biomarker following this common injury. Further investigation is needed to determine the implications of this elevated POSTN expression for the knee and whether it has any predictive value for sequelae such as post-traumatic OA. The primary effect of ACL injury may be to induce the expression of POSTN from the injured ligament itself. In comparison with ACL tear remnants, cartilage

appears to have low levels of POSTN. The increased expression of POSTN in the injured ACL suggests the injured ACL plays a pivotal role in POSTN production, which is sensitive to time from injury. The implications of POSTN accumulation in the knee over time are unknown. Elevated levels of POSTN after ACL injury may serve as a marker of the repair/healing response, implying a benefit for the joint. Conversely, sustained overexpression of POSTN may contribute to the initiation of joint degeneration. In the latter case POSTN may be a predictor of and/or a possible target for early intervention to prevent post-traumatic OA.

Further investigation is needed to confirm these preliminary findings and better understand the cause and consequence of POSTN overproduction in knees after ACL injury, particularly to explore if it plays a critical role in the pathogenesis of OA.

Author contributions

All authors were involved in drafting and revision of the manuscript and all authors approved the final version to be published. Drs. Rai and Brophy had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Rai, Brophy, Guilak.

Acquisition of data: Brophy, Cai, Duan, Zhang, Townsend, Nunley, Rai.

Analysis and interpretation of data: Rai, Guilak, Cai, Zhang, Townsend, Nunley, Brophy.

Competing interests

The authors have no competing interest to declare.

Financial conflict of interest

All authors have nothing to disclose.

Supplementary data

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