Mechanical Loading Disrupts Focal Adhesion Kinase Activation in Mandibular Fibrochondrocytes During Murine Temporomandibular Joint Osteoarthritis

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Purpose: Mechanical overloading is a key initiating condition for temporomandibular joint (TMJ) osteoarthritis (OA). The integrin-focal adhesion kinase (FAK) signaling axis is implicated in the mechanobiological response of cells through phosphorylation at Tyr397 (pFAK) but poorly defined in TMJ health and disease. We hypothesize that mechanical overloading disrupts TMJ homeostasis through dysregulation of FAK signaling.

Materials and Methods: To assess if FAK and pFAK are viable clinical targets for TMJ OA, peri-articular tissues were collected from patients with TMJ OA receiving a total TMJ replacement. To compare clinical samples with preclinical in vivo studies of TMJ OA, the joints of c57/bl6 mice were surgically destabilized and treated with and without inhibitor of pFAK (iFAK). FAK signaling and TMJ OA progression was evaluated and compared using RT-PCR, western blot, immunohistochemistry, and histomorphometry. To evaluate mechanical overloading in vitro, primary murine mandibular fibrochondrocytes were seeded in a 4% agarose-collagen scaffold and loaded in a compression bioreactor with and without iFAK.

Results: FAK/pFAK was mostly absent from the articular cartilage layer in the clinical sample and suppressed on the central condyle and elevated on the lateral condyle in murine TMJ OA. In vitro, compressive loading lowered FAK/pFAK levels and elevated the expression of TGFβ, NG2, and MMP-13. iFAK treatment suppressed MMP13 and Col6 and elevated TGFβ, NG2, and ACAN in a load independent manner. In vivo, iFAK treatment moderately attenuated OA progression and increased collagen maturation.

Conclusion: These data illustrate that FAK/pFAK is implicated in the signaled dysfunction of excessive mechanical loading during TMJ OA and that iFAK treatment can moderately attenuate the progression of cartilage degeneration in the mandibular condyle.

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Arthopathies affect more than 10% of the population, and are the most common source of disability in the USA associated with an annual total healthcare burden of $81 billion. Osteoarthritis is the most common arthopathy, and has a substantial impact on orofacial healthcare expenditures and disability in the USA. Temporomandibular disorders, broadly defined, affect between 3 to 7% of the population with 11% of these patients presenting with arthritis and/or arthralgia. There are currently no disease-modifying therapeutic interventions available, although some have shown promise (e.g., platelet-rich plasma). Current therapies are aimed at palliation and prevention of disease progression and not disease reversal. Despite these treatments, degenerative joint disease and temporomandibular joint (TMJ) dysfunction may culminate in the need for total TMJ replacement. Contemporary molecular targets for clinical preventative or interventional approaches are needed that consider the etiopathophysiology of TMJ osteoarthritis (OA) strongly in their approach to care.

To date, although systemic illness, aging processes, hormonal factors, and behavioral factors are all associated with TMJ OA, growing evidence suggests that mechanical overload of the articular fibrocartilage is a key initiating factor for a series of degenerative changes in the TMJ that culminate in condylar resorption, deformation, and functional limitations. These factors, as well as the resultant bony changes, lead to a secondary inflammatory response culminating in pain and joint dysfunction. Degeneration occurs when functional overloading of the joint exceeds the normal adaptive capacity of the tissues, resulting in an imbalance away from homeostasis, and towards dysfunctional remodeling of the extracellular matrix.

This hypothesis is consistent with reports of remodeling of the superficial layer of articular cartilage present in OA of both the limbs as well as in clinical TMJ dysfunction. In a preclinical surgical destabilization murine model of TMJ OA, the superficial layer of the mandibular articular cartilage changes in organization, orientation, and composition due to the formation of a neo-matrix that is rich in collagen types I, III, and VI. The molecular mechanisms that regulate the mechanical induction of a neo-matrix in the TMJ following injury has yet to be fully elucidated.

During dermal wound healing, mechanical loading induces fibrogenic remodeling of the extracellular matrix through the focal adhesion kinase (FAK) signaling axis. FAK is an important mechano-sensing and mechano-transductive molecule serving as a signaling hub between extracellular integrin’s and downstream pathways including p38, ERK, and MAPK. Modeling studies implicate FAK directly in mechanotransduction through PIP2 mediated enzymatic reactions requiring autophosphorylation at Tyr397 for pathway activation. Further, FAK is implicated in the activation of yes-associated protein (YAP), a key transcription factor that transduces mechanical loading to the nucleus. This integrin-FAK signaling axis regulates diverse mechanically mediated behaviors including cellular attachment, survival, proliferation, and motility.

Growing evidence supports the role of FAK as a critical mediator of chondrocyte health and disease. Small molecule inhibition of FAK is chondroprotective in a post-traumatic impact model of knee OA, and facilitates the crosstalk between TGF-β signaling and type II collagen. In the TMJ, mechanical activation of FAK by phosphorylation at Tyr397 (pFAK) enhances cellular viability and upregulates pro-inflammatory cytokines including COX-2, IL1β, and TNF-α. Further, inhibition of pFAK at Tyr397 is chondroprotective in a static loading mandibular condylar cartilage, ex vivo organ culture loading model, suppressing IL1β, MMP-13, and apoptosis in hypertrophic cells. A similar chondroprotective effect was demonstrated using in vitro mechanical loading of mandibular fibrochondrocytes treated with and without an antagonist of integrin. However, the spatiotemporal distribution and role of FAK/pFAK in potentiating fibrogenic signaling has not yet been characterized in preclinical models of TMJ OA or clinical TMJ OA samples.

The purpose of this study is to address these key gaps in knowledge regarding the role of integrin-FAK signaling in regulating the mechanical induction of matrix remodeling during TMJ OA by: 1) characterizing the spatiotemporal expression of FAK phosphorylation at Tyr397 in an in vivo preclinical murine model of TMJ OA, 2) comparing these preclinical data with clinical samples obtained from patients with end-stage TMJ OA receiving total TMJ replacement, 3) determining the effect of mechanical loading and chemical inhibition of FAK at Tyr397 on cell-matrix signaling, and 4) determining if intra-articular delivery of a small molecule inhibitor of FAK phosphorylation at Tyr397 attenuates the progression of TMJ OA.

MATERIALS AND METHODS

CLINICAL HUMAN SAMPLES FOR COMPARISON WITH PRECLINICAL DATA

Peri-articular tissue samples were collected intraoperatively from the clinical practice of the UIC Department of Oral and Maxillofacial Surgery during total TMJ replacement surgery. Inclusion criteria included patients between the age of 18 to 65 years receiving total TMJ replacement, and radiographic evidence of severe joint degeneration in 1 or both TMJs. Exclusion criteria included patients that...
had previously received surgical treatment of the TMJ, subjects from vulnerable populations including pregnant women, children under the age of 18, prisoners, institutionalized individuals, mentally handicapped, subjects with a diagnosis of juvenile idiopathic arthritis, and subjects with a history of systemic inflammatory, and/or rheumatoid, conditions, including rheumatoid arthritis. All tissue were collected in the operating suite, placed in ice-cold 1x PBS, and then prepared for histomorphometry and immunohistochemistry. All tissue collection was approved by the institutional review board of the University of Illinois at Chicago (IRB Protocol No. 2017-0033).

**PRECLINICAL SURGICAL INSTABILITY MODEL OF TMJ OA AND FAK INHIBITION**

TMJ OA was induced by unilateral partial discectomy according to the methods in Xu et al.27 and our previous publications.12,28,29 In short, skeletally mature 16-week-old male and female c57 BL/6 mice were anesthetized with ketamine (100 mg/kg, Henry Schein, Dublin, Ohio) and xylazine (5 mg/kg, Akorn, Lake Forest, IL). The skin over the TMJ was shaved and cleaned with 70% ethanol and betadine. A 3 to 5 mm incision was made over the TMJ. The lateral capsule was exposed, the articular disc excised, and the joint was irrigated with sterile 1x PBS. An injection of saline (no-treatment), vehicle control (DMSO, 1 μL, Sigma, St. Louis, MO), or inhibitor of FAK phosphorylation at Tyr397 (PF-573228, 50 μM, 1 μL, Tocris, Minneapolis, MN) was administered through intra-articular injection. The skin incision closed with 5−0 nylon suture (Ethicon, Bridgewater, NJ). Sham control surgeries were identical except the disc remained intact. One hundred forty mice were included in the study, with 60 undergoing unilateral partial discectomy, 60 undergoing sham surgery, and 20 serving as nonsurgical controls. The mice received re-dosing of saline, vehicle control, or FAK Tyr397 inhibitor every 14 days through intra-articular injection. Experimental endpoints included 4- and 16- weeks postoperative. All in vivo data were compared with age matched non-surgical controls. Experiments using vertebrate animals were approved by the University of Illinois at Chicago Animal Care Committee and performed in accordance with the relevant guidelines and regulations (UIC ACC #20-068).

**HISTOMORPHOMETRY AND GRADING CARTILAGE DEGENERATION**

To characterize changes in the collagen of the mandibular condylar cartilage during the progression of TMJ OA, Herovici collagen staining was used to identify new and mature collagens. Tissue samples were fixed in 4% PFA overnight, decalcified with 4.5% EDTA for 28 days, paraffin embedded, and sectioned at 8 μm. For histomorphometric analysis, sections from the middle of the condyle were selected, deparaffinized, and stained with either safranin-o/fast green or with a Herovici’s polychrome collagen stain (NC1049318, StatLab, McKinney, TX). Cartilage degeneration was staged for each experimental group using a Modified Mankin score according to published methods27-29 and listed in Table 1. For characterizing collagen maturity, Herovici stained slides were evaluated for mature collagen staining red or young/new collagen in blue and quantified using color deconvolution. Four biological replicates for each experimental group were used for the Modified Mankin scoring analysis and statistically compared using a 1-way ANOVA with Bonferroni corrections.

**PRIMARY MANDIBULAR FIBROCHONDROCYTE ISOLATION**

The isolation of primary mandibular fibrochondrocytes followed published methods28 and followed the established protocols for isolating primary chondrocytes.30,31 In short, mandibular condyles were collected from 10 to 14 day old mice in CO2 independent collection medium (18045 to 025, Gibco, Gaithersburg, MD), washed in 1x sterile PBS supplemented with 25 mg/mL Plasmocin (ant-mmp, Invivo-Gen, San Diego, CA), 50 U/mL penicillin, and 0.05 mg/mL streptomycin (P0781, Sigma, St. Louis, MO) for 5 minutes, transferred to a 3 mg/mL type II collagenase (S004174, Worthington Biochemical, Lakewood, NJ) in DMEM (11966 to 025, Gibco, Gaithersburg, MD) digestion medium for 1 hour, and

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<th>Table 1. MODIFIED MANKIN SCORING FOR GRADING CARTILAGE DEGENERATION IN MURINE MODEL OF POST-TRAUMATIC TMJ OA</th>
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then transferred to a 1.5 mg/mL collagenase digestion medium overnight in 5% CO2 at 37˚C. After overnight digestion, cells were dispersed by agitation, filtered through a 40 μm cell strainer, and centrifuged at 10,000 g for 15 minutes at room temperature. The pellet was re-suspended and washed in supplemented sterile 1x PSB, centrifuged again, re-suspended in 10 mL of FBS supplemented advanced DMEM (12492-013, Gibco, Gaithersburg, MD), and plated for expansion. All experiments were carried within the first 6 passages of the cells.

GENERATING CELL-AGAROSE-COLLAGEN SCAFFOLDS AND MECHANICAL LOADING

To determine if mechanical loading changes the levels of FAK and pFAK at Tyr 397 in mandibular fibrochondrocytes, primary mandibular condylar fibrochondrocytes seeded in agarose-collagen scaffolds were loaded in a compression bioreactor. Confluent cells in a 150 mm dish were trypsinized, centrifuged at 10,000 g for 10 minutes at room temperature, and resuspended in 1 mL of supplemented advanced DMEM (12492-013, Gibco, Gaithersburg, MD). A 5% low-gelling temperature agarose was prepared separately in 5 mL 1x PBS. Once cooled to 45˚C, the agarose solution was combined with 0.25 mg/mL rat tail collagen (A1048301, Thermo Fisher, Waltham, MA) and the cell suspension to create a 4% agarose and/or collagen solution with a cell density of 4×105 /mm3. The cell-agarose-collagen solution was then poured into a 1 mm thick glass spacer plate (1653511, Bio-Rad, Des Plaines, IL). A glass cover plate was placed over the space plates to cast a scaffold of uniform 1 mm thickness. The cell-agarose-collagen scaffold was then left at room temperature for 30 minutes for cross-linking. Once set, the cover plate was removed, and a punch was used to generate plugs measuring 17 mm in radius. The cell-agarose-collagen scaffold plugs were immediately placed in supplemented advanced DMEM (12492 to 013, Gibco, Gaithersburg, MD) and cultured for 72 hours at 37˚C and 5% CO2. Cell-agarose-collagen scaffolds were loaded in a compression bioreactor housed inside of a cell incubator at 37˚C and 5% CO2. All samples were loaded in constrained, uniaxial compression at 2 N for 2 hours with and without vehicle control (DMSO, 1 μL, Sigma, St. Louis, MO) or inhibitor of FAK phosphorylation at Tyr397 (PF-573228, 50 μM, 1 μL, Tocris, Minneapolis, MN).

IMMUNOHISTOCHEMISTRY FOR ANALYZING SPATIOTEMPORAL EXPRESSION OF FAK PHOSPHORYLATION

To characterize temporospatial distribution of FAK and pFAK at Tyr-397, immunolabeled paraffin sections were imaged using fluorescent microscopy at 4-weeks. Paraffin sections from the center of the condyle were deparaffinized, permeabilized with methanol and 0.5% Triton (v/v), blocked in 5% donkey serum (D9663, Sigma, St. Louis, MO) for 2 hours and incubated with primary antibodies against FAK (1:100, sc-1688, SCBT, Santa Cruz, CA) and pFAK at Tyr397 (1:200, 44-624G, Thermo Fisher, Waltham, MA). All secondary labeling was with Alexa Fluor donkey anti-mouse 488 and donkey anti-rabbit 568 (1:500, Invitrogen, Invitrogen, Carlsbad, CA). Nuclei were labelled with DAPI (D9542-1MG, 1 μg/μL, Sigma, St. Louis, MO). Sections were imaged using an inverted fluorescent microscope using a 10x objective (DMI6000B, Leica, Buffalo Grove, IL). Laser intensity, gain, and magnification were standardized for all acquisitions. Brightness and contrast settings were standardized for all images during post-processing. All data were compared to a no primary antibody control and isotype control. Autofluorescence was detectable in the subchondral vasculature but negligible in the mandibular condylar cartilage for the brightness and contrast settings used during data acquisition.

RT-PCR

For quantifying gene expression changes from in vitro and homogenized tissues, samples were placed in 2 mL of lysis reagent (QIAzol, Qiagen, Germantown, MD) for 2 hours at room temperature and vortexed every 20 minutes. To extract and purify mRNA from agarose samples, lysate was processed using the RNeasy Plant Mini Kit (74903, Qiagen, Germantown, MD). The plant kit was recommended for high integrity extraction from cell-agarose constructs due to the high levels of polysaccharides. RNA was generated from 1 μg total RNA using a High Capacity Reverse Transcription Kit (4368814, Applied Biosystems, Waltham, MA). For cells and tissues, lysate was processed using the RNeasy Mini Kit (74104, Qiagen, Germantown, MD). Target genes were amplified using SYBR Select Master Mix (4385610, Applied Biosystems, Waltham, MA) in a Bio-Rad iQ5 (Bio-Rad, Des Plaines, IL). The specific primer sequences are listed in Table 2. Fold change in gene expression was calculated by comparative threshold cycle method. Four biological replicates were used for each experimental group, with the biological sample size calculated using sample size and power analysis. Statistical significance was calculated by 1-way ANOVA with post hoc Bonferroni corrections. (SPSS, Chicago IL). Negative controls substituting saline for cDNA were carried out for each primer for standard quality control.

WESTERN BLOT ANALYSIS

For in vitro protein isolation of cultured cells, plates were washed in ice-cold, 1x PBS, lysed using
an extraction reagent (M-PER, 78501, Thermo Fisher, Waltham, MA) with protease (cOmplete, 4693116001, Sigma, St. Louis, MO) and phosphatase (PhosSTOP, 4906845001, Sigma, St. Louis, MO) inhibitors. For in vitro protein isolation of the cell-agarose scaffolds, samples were rinsed in 1x PBS for 20 minutes, placed in Laemmli Buffer, boiled for 5 minutes, cooled on ice, and spun down for 2 hours using a mini-spin column (Pierce Spin Cups, 69700, Thermo Fisher, Waltham, MA). For in vivo analyses, full TMJ tissue was excised, washed in ice cold sterile 1x PBS, and homogenized in ice cold NP40 lysis buffer (FNN0021, Invitrogen, Carlsbad, CA) supplemented with Halt Protease Inhibitor Cocktail (78442, Thermo Fisher, Waltham, MA), and lysed for 3 hours. For all samples, lysate insolubles were removed by centrifugation at 14000 g for 15 minutes at 4˚C. The supernatant was incubated with Chondroitinase ABC (100330-1, AMSBio, Cambridge, MA) added at 0.05 units/mL for 3 hours at 37˚C. Lysates were adjusted to a 1x Protein Sample Loading Buffer (928 to 40004, Licor, Lincoln, NE), heated at 100˚C 5 minutes, run on a 4 to 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and analyzed by western blot with antibodies against FAK (1:500, AHO0502, Thermo Fisher, Waltham, MA), pFAK at Tyr397 (1:500, 44 to 624G, Thermo Fisher, Waltham, MA) and a β-actin mouse monoclonal control (1:1000, 926 to 42210, Licor, Lincoln, NE). Blots were imaged using a Licor Fluorescence Quantitative western blot. Fluorescence values were normalized to β-actin and standardized to unloaded, untreated control samples. Four biological replicates were used for all western blots. Statistical significance was assessed by 1-way ANOVA with post hoc Bonferroni test. (SPSS, Chicago IL).

RESULTS

FAK, PFAK, AND THE CARTILAGE EXTRACELLULAR MATRIX IN A CLINICAL SAMPLE OF TMJ OA

Immunohistochemistry of clinical human TMJ OA tissue illustrates that there was low cellularity in the samples due to the advanced stage of the disease (Fig 1A). Cells in the deep layer of the mandibular condylar cartilage have some FAK and pFAK positive cells but this staining is mostly absent from superficial layer cells in the articular and proliferative layers. Safranin-o/fast green staining illustrates high levels of fibrillation in the superficial layer, diminished extracellular proteoglycans, and elevated pericellular proteoglycans. These are all signs of advanced TMJ OA (Table 1, Fig 1D). Herovici’s polychrome staining with deconvolution analysis illustrated that most the collagen present is mature (Fig. 1E-F).

EARLY STAGE PRECLINICAL TMJ OA IS CHARACTERIZED BY TEMPOROSPATIALLY SUPPRESSED FAK AND PFAK

TMJ OA was induced in a murine animal model using unilateral partial discectomy (Fig. 2A-D). Immunostaining of the preclinical murine model of TMJ OA illustrates that the temporospatial distribution of FAK and pFAK at Tyr397 was heterogeneously distributed. FAK phosphorylation was suppressed on the center of the condyle and elevated on the lateral and medial condyle 4-weeks after discectomy when compared to the nonsurgical and sham control samples (Fig. 2E-R). Quantification of changes in FAK and pFAK in total joint samples using western blot illustrated that total FAK and pFAK were significantly elevated in the sham joints compared to the non-surgical controls (P < .001; n = 3) and TMJ OA (P < .01; n = 3) joints (Fig. 2S-T). There was no significant difference in the ratio of pFAK/FAK (Fig 2U).

EARLY STAGE PRECLINICAL TMJ OA IS ASSOCIATED WITH CHANGES IN THE CARTILAGE EXTRACELLULAR MATRIX AND CELL-MATRIX SIGNALING AXIS

Quantification of the Herovici’s polychrome stained tissues with color deconvolution illustrated significantly more mature collagen 4-weeks after discectomy when compared to non-surgical and sham controls. The 2-week post-discectomy tissue illustrated the lateral and medial condyle are the first to undergo changes in the composition of collagens. By 4-weeks, the entire superficial layer was
remodeled (Fig 3). RT-qPCR on full joints showed significant increases in transforming growth factors-β (TGFβ; \( P < 0.05, n = 4 \)), Neuron/Glial antigen-2 (NG2; \( P < 0.05, n = 4 \)), and platelet derived growth factor receptor (PDGFrβ; \( P < 0.05, n = 4 \)). We also found non-significant increases in matrix metalloproteinase-13 (MMP13), aggrecan (ACAN), and type VI collagen (Col6a1) (Fig 4).

CONstrained STATIC COMPRESSIVE LOADING SUPPRESSES FAK AND pFAK AND CHANGES THE CELL-MATRIX SIGNALING AXIS

In vitro loading significantly suppressed the level of both total FAK and pFAK at Tyr397 (\( P < 0.05; n = 4 \)) (Fig 5A). Loading the samples increased expression of TGFβ (\( P < 0.05; n = 4 \)), NG2 (\( P < 0.05; n = 4 \)), and MMP13 (\( P < 0.05; n = 4 \)) when compared without Bonferroni corrections (Fig 5D-I). Note that these genes were upregulated in a similar manner in the in vivo data. To evaluate the load dependent and independent effects of pFAK inhibition on the cell-matrix signaling axis, we quantified gene expression using RT-qPCR from loaded and unloaded cell-agarose-collagen scaffolds treated with and without the pFAK inhibitor. These data illustrate load independent increases in TGFβ, NG2, and ACAN that were lowered after loading (\( P < 0.05; n = 4 \)) and load independent suppression of MMP13 (\( P < 0.05; n = 4 \)) and...
FIGURE 2. TMJ OA is associated with a temporospatially heterogeneous distribution of FAK and FAK phosphorylation at Tyr397.

(A) Micro-surgical approach of the TMJ for unilateral partial discectomy illustrating the position of the facial nerve and superficial temporal artery/vein.

(B) The masseter muscle is reflected to expose the lateral capsule and the posterior/lateral capsule is entered. (C) The posterior band of the articular disc is pulled laterally and the discal attachments are divided.

(D) The joint cavity is irrigated with saline and the skin is sutured. A pseudocapsule is seen 5 days after surgery. Diagrams (A-D) courtesy of Rachel Poli.

(E) FAK immunostaining in preclinical murine non-surgical control tissue
(F) pFAK at Tyr 397 immunostaining in preclinical murine non-surgical control (NSC) tissue
(G) Zoomed in pFAK at Tyr 397 immunostaining in preclinical murine NSC tissue on the center condyle
(H) Zoomed in pFAK at Tyr 397 immunostaining in preclinical murine NSC tissue on the medial condyle
(I) FAK immunostaining in preclinical murine 4-week sham control (Sham 4w) tissue
(J) pFAK at Tyr 397 immunostaining in preclinical murine Sham 4w tissue
(K) Zoomed in pFAK at Tyr 397 immunostaining in preclinical murine Sham 4w tissue on the center condyle
(L) Zoomed in pFAK at Tyr 397 immunostaining in preclinical murine Sham 4w tissue on the medial condyle

(M) FAK immunostaining in preclinical murine 4-week TMJ OA (TMJ OA 4w) tissue
(N) PFAK AT TYR 397 IMMUNOSTAINING IN PRECLINICAL MURINE TMJ OA 4W TISSUE
(O) Zoomed in pFAK at Tyr 397 immunostaining in preclinical murine TMJ OA 4w tissue on the center condyle
(P) Zoomed in pFAK at Tyr 397 immunostaining in preclinical murine TMJ OA 4w tissue on the medial condyle
(Q) Heat map of the pFAK immunofluorescence images in preclinical murine NSC tissue
(R) Heat map of the pFAK immunofluorescence images in TMJ OA 4w tissue illustrating a concentration of high intensity staining on the lateral and medial margins of the condyle and synovium and low staining on the center of the condyle.

(S) Western blot analysis illustrating changes in protein levels in FAK from fluorescence values and statistically compared using a 1-way ANOVA with Bonferroni correction in NSC, Sham 4w, and TMJ OA 4w (n = 4; * P < 0.05).
(T) Western blot analysis illustrating changes in protein levels in pFAK at Tyr 397 in NSC, Sham 4w, and TMJ OA 4w (n = 4; * P < 0.05).
(U) Western blot analysis illustrating changes in protein levels in FAK/pFAK ratio in NSC, Sham 4w, and TMJ OA 4w (n = 4; * P < 0.05).

Col6a1 ($P < 0.05$; $n = 4$). No significant changes in PDGFrβ were identified (Fig 5D-I).

INTRA-ARTICULAR INJECTION OF AN INHIBITOR OF FAK PHOSPHORYLATION AT TYR397 ATTENUATED CARTILAGE DEGENERATION

Cartilage degeneration scoring showed that the 4-week vehicle control had significantly more degeneration than the non-surgical control, sham controls, and the iFAK treated group after Bonferroni corrections ($P < 0.05$; $n = 4$). However, cartilage degeneration in the 4-week discectomy iFAK treated group was not significantly lower than the 4-week discectomy no-treatment group (Fig 6A-E). At 16-weeks post-discectomy, cartilage degeneration in the iFAK treated group was significantly lower than the 16-week discectomy no treatment group ($P < 0.05$; $n = 4$), while the vehicle control treated group was not (Fig 6F-J). However, both of the iFAK and vehicle control treatment groups were lower and the difference between the 2 was not significant (Fig 6K-L). The 4-week tissue was further evaluated using the Herovici’s polychrome staining with color deconvolution analysis because the tissue was intact and cellular. Treatment with the pFAK inhibitor resulted in a significant increase in the amount of mature collagen, shown in other studies to correlate with type I collagen synthesis (Fig 6M-Q; $P < 0.05$; $n = 3$).

DISCUSSION

The results of this study illustrate that FAK signaling in the TMJ is responsive to mechanical loading and can regulate key genes implicated in the matrix synthesis associated with mechanical injury of the joint. The many findings of this study, including the shift in the composition of the ECM, in vitro and in vivo expression of growth factors, and changes in spatio-temporal distribution and levels of FAK, all support the key role of FAK signaling in mechanical loading to the TMJ. The change in the composition of the extracellular matrix of the mandibular condylar cartilage after injury toward a more type I collagen-rich, fibrogenic phenotype is supported by the gene expression data. Here we illustrate that 4-weeks after the induction of TMJ OA through surgical destabilization, there is a significant increase in the expression of TGFβ in the TMJ (Fig 4). TGFβ signaling is a key growth factor that regulates the synthesis of type I collagens,34,35 is a key regulator of the fibrotic injury response of the cells,36,37 is implicated in the mechanobiological response of chondrocytes,38 and is a critical mediator
of TMJ tissue homeostasis and OA etiopathophysiology. TGFβ engagement with receptors activates SMAD signaling and involves crosstalk between multiple canonical and non-canonical pathways. In chondrocytes, FAK-SMAD2/3 signaling mediates crosstalk between TGFβ and type II collagen in control of GAG synthesis. There is support for TGFβ being linked mechanistically with mechanical loading through FAK signaling. In dermal tissues, excessive TGFβ is implicated in the tissue injury response and fibrosis via non-canonical FAK pathways, mediating myofibroblast differentiation, and providing a potential therapeutic target for attenuating the fibroproliferative phenotype. Mechanical loading also significantly increased the expression of neuron-glial antigen 2 (NG2/CSPG4), a transmembrane proteoglycan that binds with Col6, is internalized during the early stages of TMJ OA, and can activate the mTORC1 signaling pathway. NG2 is mechanistically linked with FAK signaling through the protein kinase C pathway. The lack of change in PDGFrβ was likely due to the lack of vasculature in this type of cell population. Taken together, these data indicate that there are multiple convergent lines of evidence linking FAK with mechanically induced matrix remodeling.

To mechanistically evaluate the effect of mechanical loading on FAK/pFAK levels in mandibular fibrochondrocytes, we applied a controlled compressive load on cell-agarose-collagen scaffolds in a bioreactor. There have been numerous studies on the role of mechanical loading on FAK autophosphorylation at Tyr397 (pFAK), with contradictory findings. In sum, these studies illustrate the cells that are loaded in monolayer culture system typically yield an increase in pFAK at Tyr397 including periodontal fibroblasts, SW620 colonocytes on a COL1 matrix, A549 cells, Caco-2 cells, bovine pulmonary artery endothelial cells and ATDC5 cells. However, there are temporal dynamics associated with FAK and pFAK at Tyr397 levels under mechanical stimulation. Mechanical stretching of airway epithelial cells with a cyclic strain of 20% and a frequency of 30 Hz resulted in a transient increase in FAK phosphorylation and then subsequent decrease. It has been hypothesized that the short-term stimulation of FAK activation is related to mobilization of the focal adhesion complex immediately after mechanical loading followed by disassociation of the complex with continued mechanical disruption. Conversely, cells that are loaded in a 3-dimensional scaffold yield a decrease in pFAK at Tyr397 including mesenchymal stem cells and chondrocytes. Interestingly, the presence of collagen in a 3-dimensional agarose scaffold affects pFAK at Tyr397 when compared to agarose alone, resulting in a pFAK/FAK ratio

**FIGURE 4.** Early stage TMJ OA is associated with upregulation of key growth factors associated with synthesis and degradation of the extracellular matrix. RT-qPCR quantification of key genes implicated in early stage TMJ OA quantified from joints in non-surgical control (NSC), 4-week sham (4s), and 4-week post-surgical discectomy (4e). Data standardized to the NSC sample using the ∆∆Cq method and statistically compared using a 1-way ANOVA with Bonferroni corrections (n = 4; * P < 0.05).

that is initially high but decreases in total FAK and pFAK at Tyr397 following loading. It was hypothesized that this was a result of enhanced cell adhesion to the scaffold matrix due to the presence of collagen, supporting previous reports that 3-dimensional cell adhesions interact and respond to matrix engagement in different ways than monolayer cells. Thus, the incorporation of a complex 3-dimensional microfabric of collagens, matching the higher-order structure of the extracellular matrix of the joint, may be critical for eliciting and interpreting the mechanobiological response of the mandibular condylar cartilage using in vitro assays.

Our study findings agree with other reports from 3-dimensional cell culture systems loaded in a compression bioreactor, illustrating that static compression lowers total FAK and pFAK Tyr397 when compared to unloaded control samples. Loading induced increases in gene expression were identified in TGFβ, NG2, and MMP13 (Fig 5), matching the trends identified from the in vivo preclinical animal model data (Fig 4). Small molecular inhibition of pFAK at Tyr397 increased the expression of TGFβ, NG2, and ACAN and suppressed the expression of MMP13 and Col6a1 independent of loading. Changes in the organization, composition, and orientation of Col6a1 are notable in early stage TMJ OA, since this microfibrilar collagen has been shown to be important for facilitating cell migration in response to injury and as a modulator of the mechanical microenvironment of the cell. Neuron/glial antigen 2 (NG2) is a transmembrane proteoglycan that binds with type VI collagen, can regulate cell viability and matrix mediated cell death, and co-regulates the FAK

FIGURE 5. Constrained axial compression suppresses FAK and FAK phosphorylation at Tyr397 and promotes the cell-matrix signaling axis in a FAK dependent manner.

(A) Western blot analysis from cell-agarose-collagen scaffolds comparing FAK and pFAK at Tyr397 protein levels in an unloaded and loaded sample illustrating that static compression suppresses FAK and pFAK at Tyr397.

(B) Western blot analysis from primary mandibular fibrochondrocytes treated with an inhibitor of pFAK at Tyr397 [iFAK] added at various concentration, with fluorescence values normalized to a β-actin control and reported relative to a vehicle control.

(C) Western blot analysis from primary mandibular fibrochondrocytes treatment with the optimized concentration of iFAK for various durations, with fluorescence normalized to a β-actin control reported relative to a vehicle control.

(D) RT-qPCR of loaded and unload samples with and without iFAK treatment illustrating changes in gene expression of key genes in the cell-matrix signaling axis. Data standardized to the NSC sample using the ΔΔCq method and statistically compared using a 1-way ANOVA with Bonferroni corrections (n = 4; * P < 0.05).

FIGURE 6. Intra-articular injection of FAK-Tyr397 inhibitor attenuates cartilage degeneration on the mandibular condyle.

[A] Safranin-o/fast green staining of mouse TMJs in nonsurgical control (NSC), 4-week sham controls with vehicle control treatment (4s-vc), 4-week sham control with FAK-Tyr397 treatment (4s-iFAK), 4-week disectomy with vehicle control treatment (4e-vc), 4-week disectomy with FAK-Tyr397 treatment (4e-iFAK), 16-week sham controls with vehicle control treatment (16s-vc), 16-week sham control with FAK-Tyr397 treatment (16s-iFAK), 16-week disectomy with vehicle control treatment (16e-vc), 16-week disectomy with FAK-Tyr397 treatment (16e-iFAK), 16-week disectomy with no treatment (16e-nt).

[B] Box plot illustrating changes in cartilage degeneration as measured by a Modified Mankin score in each group (n = 4).

[C] Results from a 1-way ANOVA with post-hoc Bonferroni corrections. Significant changes are denoted by the reported P-value in the table, with significant increases labeled in red and significant decreases labeled in blue.

[D] Herovici's polychrome staining of a 4-week TMJ OA condyle compared to an iFAK treated TMJ OA condyle.

[E] Quantification of a color deconvolution analysis of the Herovici's polychrome stained samples statistically compared using a 1-way ANOVA with Bonferroni corrections (n = 4; * P < 0.05) illustrating that the purple and/or red mature collagen frequency range is significantly increased in the iFAK treated condyle when compared to the vehicle control.

signaling axis. TGF-β1 is a critical regulator of the fibrotic response of cells both in vivo and in vitro, and is elevated in TMJ-OA condyles. Further, TGF-β1 knockout mice have attenuated OA progression. This gene expression data is consistent with work illustrating that mesenchymal stem cells seeded in a PLCL/chitosan scaffold and loaded in cyclic compression are associated with the suppression of pFAK at Tyr397 and activation of the TGF/Activin/Nodal pathway. Thus, the mechanical induction of fibrogenic signaling through TGFβ may be associated with mechanobiological pathways suppressing the FAK signaling axis.

In vivo, we have shown that the progression of cartilage degeneration following surgical destabilization of the condyle is exacerbated by mechanical loading on the joint. Thus, it would be predicted that the preclinical mouse model would have lower levels of FAK and pFAK at Tyr397. This is in contrast to reports that mechanical and inflammatory injury of cartilage in the mandible, cartilage of the intervertebral disc, and skin are associated with an increase in the level of pFAK at Tyr397. Western blot analysis of the whole joint after TMJ OA illustrates the largest increase in FAK and pFAK at Tyr397 in sham control tissues and no significant difference between the TMJ OA and non-surgical control samples. While our data may seem to be in conflict with these other injury model studies, we believe that the temporospatial patterns must be analyzed to further elucidate the role of FAK, and our results in the immunohistochemical staining support convergent interpretations.

Immunostaining illustrated that FAK/pFAK is suppressed on the center condyle and elevated in the lateral and medial condyle and the adjacent enthesis during TMJ OA. This mirrors the patterns of collagen compositional change found in our histomorphometric analysis. This, along with the heterogeneous distribution of FAK/pFAK, likely reflect spatial differences in the loading on the articular cartilage, as well as functional adaptation to a new joint space after discectomy. Removal of the disc will result in greater loading on parts of the condyle during function, as well as adaptive changes to the masticatory musculature, articular and peri-articular soft tissues. The concentration of FAK in the lateral and medial condyle could reflect the mobilization of migratory progenitor and/or reparative, fibroproliferative cells activated in the injury response of the tissue, or these cells may simply be experiencing lower mechanical stress since destabilization of the condyle causes rotation of the lateral pole outside of the glenoid fossa. Alternatively, increased tensile forces exerted on the enthesis (due to adaptation to a new joint space post-discectomy), could possibly account for localized elevation of FAK laterally. In the sham control tissue, inflammation would promote FAK and pFAK without mechanically induced suppression, causing higher FAK/pFAK levels when total protein is measured from the whole joint, without any regional increases within the joint.

Other studies have proposed that FAK inhibition as a therapeutic strategy for fibrogenic disorders and for TMJ OA. To evaluate the role of FAK in the in vivo progression of TMJ OA, we treated surgically destabilized mice with a small molecule inhibitor of pFAK at Tyr397 through intra-articular injection. Quantification of cartilage degeneration illustrates a modest but significant improvement after 16-weeks over the vehicle control. Interestingly, there was a significant improvement of both the vehicle control and iFAK treated joints over a nontreated joint. We hypothesize that this is the result of flushing inflammatory cytokines and proteases from the joint space, having the same therapeutic benefit as arthrocentesis. The similarities between iFAK and vehicle control may also illustrate the temporality of FAK upon mechanical stimulation. That is, as shown in in vivo studies using 3-dimensional cell culture scaffolds, episodic and nonrepetitive compressive forces may only induce transient elevation of FAK and pFAK with a subsequent decrease. This also aligns with the fact that by the time the spatiotemporal distribution of FAK was analyzed (4 weeks), cartilage maturation was complete, indicating maturation and adaptation of the cartilaginous surfaces. With these explanations, the same pattern of suppression can be expected from iFAK treatment and flushing (with vehicle control) if FAK is suppressed after a brief elevation from early trauma. At 4-weeks, there was no significant difference between the vehicle control and iFAK treated joints; however, degeneration in the vehicle control was significantly higher than the nonsurgical control while the iFAK treated joint was not. We failed to recover any significant differences in gene expression between the treated and untreated samples. This may be because tissues were collected after 2-weeks following in the administration of the inhibitor, well after the half-life of the drug.

These data support the hypothesis that the iFAK treatment can attenuate the progression of cartilage degeneration, possibly because it amplifies these intrinsic protective mechanisms of the fibrocartilage, promoting the synthesis of a low compliance, high stiffness neo-matrix. Further, iFAK treatment suppresses MMP13 in a loading independent manner and attenuates matrix proteolysis. This hypothesis is consistent with data from a rat
mandibular condyle explant model loaded under static compression with and without FAK inhibitor PF0455487, illustrating FAK dependent suppression of inflammatory biomarkers, MMP13 mediated proteolysis, and TUNEL staining in the chondroblastic and hypertrophic cell layer.\textsuperscript{25} The inhibition of pFAK at Tyr597 is also associated with an increase in TGFβ. The role of TGFβ in regulating tissue homeostasis in the TMJ has yet to be fully resolved. While the expression of TGFβ is associated with synthesis of type I collagen and fibrogenic pathways, the overexpression in the subchondral bone of the mandible increases in the number of MMP-13 positive cells and promotes cartilage degeneration.\textsuperscript{40} Taken together, the therapeutic benefits of inhibiting pFAK at Tyr597 in the post-traumatic environment following discectomy may lie in promoting the chondroprotective fibrogenic neo-matrix in the superficial layer of the articular cartilage while suppressing catabolic proteolysis associated with mechanical overloading. The exact mechanism and role of FAK in TMJ OA remains unclear, however, results of our study indicate a complex interaction with multiple molecular pathways, with temporal dynamics.

We chose to evaluate pFAK inhibition using a small molecule drug because this is a delivery mechanism that is scalable for introduction into the TMJ joint space via minimally-invasive TMJ procedures (arthrocentesis and arthroscopy) that are typically performed before the disease has progressed to end stage TMJ OA. Here we have demonstrated some insight into a potential mechanism of action and modest efficacy of the inhibitor for attenuation of TMJ OA progression. These results are encouraging but additional experiments are needed to evaluate the downstream effects of inhibiting the FAK regulatory network in both acute and chronic TMJ disorders, characterizing the half-life of the inhibitor in vivo, evaluating alternative delivery approaches, evaluating similar related small molecule inhibitors, and determining the long-term costs and benefits of promoting remodeling of the mandibular cartilage.

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