Occurrence of cellular senescence in chronic human shoulder tendinopathies and its attenuation ex vivo by inhibition of Enhancer of Zeste 2

From University Hospital Basel and University of Basel, Basel, Switzerland

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Correspondence should be sent to Karoliina Pelttari
karoliina.pelttari@usb.ch

D. Bühler,[1,2] M. Hilpert,[1] A. Barbero,[1] A. M. Müller,[2,3] S. A. Müller,[4,5] I. Martin,[1,6] K. Pelttari[1]

[1]Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland
[2]Department of Orthopaedics and Traumatology, Basel, Switzerland
[3]Department of Surgery, University Hospital Basel, Basel, Switzerland
[4]Department of Orthopedic Surgery and Traumatology, Kantonsspital Baselland, Bruderholz, Switzerland
[5]Faculty of Medicine, University of Basel, Basel, Switzerland
[6]Department of Biomedical Engineering, University of Basel, Allschwil, Switzerland

Aims

Our aim was to investigate occurrence of senescent cells directly in tendon tissue biopsies from patients with chronic shoulder tendinopathies, and to correlate senescence with Enhancer of zeste 2 (EZH2) expression, the functional subunit of the epigenetic master regulator polycomb repressive complex.

Methods

Human proximal long head of biceps tendons from patients with different chronic shoulder pathologies (n = 22), and controls from patients with humerus fracture (n = 6) and pathology (n = 4), were histologically scored for degeneration and analyzed for gene and protein expression of tendon specific factors, senescence markers, and EZH2. Tissues were further exposed to senotherapeutic compounds and the USA Food and Drugs Administration-approved selective EZH2 inhibitor EPZ-6438 and their senescence-associated secretory phenotype (SASP) assessed.

Results

Expression of senescence markers (*CDKN2A*/p16, *CDKN2D*/p19) and *EZH2* was significantly higher in tendinopathies compared to fracture or healthy tissue controls and positively correlated with the degree of tissue degeneration. Immunofluorescent stainings demonstrated colocalization of p16 and p19 with EZH2 in tenocytes. Treatment of tendon biopsies with EPZ-6438 reduced secretion of a panel of SASP factors, including interleukin-6 (IL6), IL8, matrix metalloproteinase-3 (MMP3) or GRO1, similarly to the senotherapeutic compound AG490.

Conclusion

We demonstrate that senescence traits accumulate in pathological tendon tissues and positively correlate with tissue degeneration. Increased expression of *CDKN2A*/p16 and *CDKN2D*/p19 coincides with EZH2 expression, while its inhibition decreased the secretion of SASP factors, indicating a possible regulatory role of EZH2 in tenocyte senescence in tendinopathies. Reduction of cellular senescence, e.g. with EPZ-6438, opens ways to new potential therapeutic approaches for enhancing regeneration in chronic tendinopathies.

Article focus

- Assessment of cellular senescence in tendon biopsies from patients with shoulder tendinopathies.
- Correlation of epigenetic regulator Enhancer of Zeste 2 (EZH2) with senescence markers in tendinopathies.

Bone & Joint Research

Key messages

- Senescence traits accumulate in pathological tendon tissues and positively correlate with tissue degeneration stage.
- EZH2 colocalizes within cells expressing senescent markers.
- Inhibition of EZH2 reduces the secretion of factors characteristic for senescent cells (SASP) similarly to common senolytics, indicating the regulatory role of EZH2 on cellular senescence.

Strengths and limitations

- Human tissue biopsies from patients with different shoulder tendinopathies were compared to healthy tissue of the same anatomical location: the long head of biceps tendon.
- The occurrence of cellular senescence was identified in tendon tissues in situ from patients with different types of tendinopathies.
- Linking epigenetic regulator EZH2 to cellular senescence in tendinopathies identifies epigenetic regulators as a potential new therapeutic target.
- Demonstrating SASP reduction by the USA Food and Drugs Administration-approved selective EZH2 inhibitor EPZ-6438 could improve treatment strategies for patients.
- There was a relatively low number of replicates per disease group.
- There was a predetermined selection bias, since only patients with insufficient endogenous healing capacity who underwent surgery were assessed in this study.
- Age bias due to the epidemiological prevalence of different shoulder pathologies.

Introduction

Due to ageing or accidents, tendinopathies are a frequent challenge in orthopaedics with a considerably high socioeconomic burden.[1] Tendon healing is slow and often results in mechanically inferior scar tissue, which is believed to account for high postoperative retear rates,[2,3] eventually leading to impaired mobility and reduced quality of life for the patient.[4-6] Common chronic shoulder tendinopathies causing pain and disabilities include rotator cuff tears (RCT) or lesions at the intra-articular origin of the long head of biceps tendon, so-called superior labrum anterior to posterior (SLAP) lesions. In both cases, initial conservative treatment aims to improve mobility and reduce persistent pain due to ongoing joint inflammation. However, chronic impairment can lead to osteoarthritis (OA) in the glenohumeral joint, which may result in the need of joint arthroplasty surgery.[7] So far, optimization of surgical refixation techniques and rehabilitation programmes are the common targets to improve clinical outcomes for patients.[8,9] Meanwhile, underlying mechanisms of tendon regeneration remain poorly investigated, yet their better understanding is essential to identify and develop new therapeutic strategies for tendon repair and treatment of tendinopathies.[10,11]

The strong correlation of tendon retear rates with patient age suggests that processes associated with tissue ageing, including changes in extracellular matrix composition and declined structural and functional properties,[12,13] are involved in impaired tendon healing.[14] However, underlying molecular mechanisms of ageing have hardly been studied in human tendon.[15] Cellular senescence is defined as a state of irreversible cell cycle arrest that is further characterized by their secretion of a panel of pro-inflammatory and catabolic factors, defined as the senescence-associated secretory phenotype (SASP).[16] In age-related pathologies, the importance of cellular senescence is widely acknowledged and suggested to play an important role in degenerative joint diseases, such as OA.[17] Accelerated senescence may also occur independently of ageing, e.g. following cartilage injury.[18] However, its role in acute or chronic tendinopathies has so far been poorly investigated. Induction of senescence in mouse models resulted in tendon atrophy, loss of orientation, and collagen fibre degeneration.[19] In ageing-tendon rat models, the senotherapeutic compound AG490 (a JAK2/STAT3 inhibitor) restored age-related dysfunction and improved regeneration.[20] Therefore, increased cellular senescence could possibly account for the insufficient tendon repair in chronic tendinopathies.[21] Senescence was described to increasingly accumulate in tendon progenitor cells with age,[15] and was further described also in different types of human chronic tendinopathies.[22,23] These studies showed increased senescence in isolated and culture-expanded human tenocytes from degenerative supraspinatus tendon compared to healthy hamstring tendon, and demonstrated the influence of senescence on tenogenic differentiation capacity in vitro. Occurrence of cellular senescence in situ was recently shown in tendon tissues of patients with rotator cuff tears.[24,25]

The polycomb repressive complex (PRC) is known to epigenetically control gene expression through chromatin remodelling during important processes in development and differentiation.[26] Its enzymatically active subunit, EZH2, functions as a histone methyltransferase to repress transcription of a variety of genes.[27] While downregulation of EZH2 was demonstrated to trigger senescence in human fibroblastic cell lines,[28] more recent publications demonstrate a positive correlation of EZH2 expression and senescence in injured or cancerous tissues.[29,30] Increased levels of EZH2 were also identified in patients with OA, a degenerative disease during which senescent chondrocytes also accumulate. Amelioration of OA development has been achieved in mouse models following clearance of senescent cells[18] or upon inhibition of EZH2.[31,32] EZH2's possible role in cellular senescence in tendinopathies has not been addressed yet.

The aim of this study was to assess the occurrence of cellular senescence in native tendon tissues from patients with chronic shoulder tendinopathies and its possible correlation with EZH2 expression. We further evaluated the capacity of a selected senotherapeutic compound as well as EPZ-6438, a USA Food and Drug Administration (FDA) approved inhibitor of EZH2, to modulate senescence and reduce the secretion of SASP factors in human tendon tissue. These insights represent the basis of better understanding the processes associated with tendon degeneration, and could support identification of new therapeutic targets to improve tendon healing and regeneration.

Methods

Collection of material

All individuals/their relatives (for tissues collected at the Pathology of the University of Basel) gave informed consent

to participate in this study, which was approved by the local ethical authority and performed in accordance with the Declaration of Helsinki.[33]

Proximal long head of biceps tendon biopsies were collected from patients with proximal humerus fracture (n = 6), superior labrum anterior to posterior tear (n = 8), rotator cuff tear (n = 10), or OA (n = 4). Healthy tendons without macroscopic signs of degeneration were collected post-mortem from cadavers as a control group ('healthy'; n = 4). Details on patients' demographics are summarized in Supplementary Table i. Patient inclusion and exclusion criteria are also presented in the Supplementary Material. Samples of 4 mm length from intra-articular, bicipital groove, and extra-articular regions (the latter only when biopsy length exceeded > 4 cm) (Figures 1a and 1b) were horizontally cut in half; one half was formalin-fixed and paraffin-embedded for immunohistochemistry/histology, and the other half was snap-frozen for gene expression analyses.

Ex vivo tissue culture with senotherapeutics and EZH2 inhibitor

Tendon tissues (from n = 2 rotator cuff tear (RCT), n = 1 SLAP, n = 1 OA) were chopped and the resulting pieces (of approx. 5 × 5 × 5 mm) cut in half, with one half left untreated as the control and the other half treated as described below (see also schematic experimental setup in Supplementary Figure aa). Each piece was further cut to increase its surface area as much as possible. All tissue pieces were placed in agarose-coated 24-well plates for two to three days with 1 ml complete medium.[31] Control tissues were kept in complete medium while treated tissue pieces were exposed to ABT263 (12.5 µM; ab218114), AG490 (1 mM; ab120950), and (in combination) dasatinib (2.5 µM, ab142050) and quercetin (500 nM; ab120247), as well as to the EZH2 inhibitor EPZ6438 (100 µM; AG-CR1-3743, AdipoGen Life Sciences, USA) for six days with a media change on day 3. All factors were reconstituted in dimethylsulfoxide (DMSO), and the same volume of DMSO (10 µl/ml medium) was applied to control samples. Samples were analyzed for gene expression (Supplementary Figure a) and the expression levels directly compared to those of the corresponding control. Tendon pieces from five additional donors (n = 4 RCT, n = 1 SLAP) were cultured in complete medium (untreated control) and in the presence of 1 ng/ml IL1β (interleukin 1 β) alone (control + IL1) or in combination with AG490 or EPZ-6438. Collected supernatants were frozen at -80°C for quantification of SASP.

Quantification senescence-associated secretory phenotype proteins

Luminex Magnetic Assay (R&D Systems, USA) was used to quantify the following selected SASP factors in the supernatant (mean of technical duplicates each for n = 5 donors): A Disintegrin and Metalloproteinase with Thrombospondin motifs 13 (ADAMTS13), matrix metalloproteinase 3 (MMP3), MMP13, interleukin 6 (IL6), tumour necrosis factor α (TNFα), monocyte chemoattractant protein 1/C-C chemokine ligand 2 (MCP1/CCL2), MCP3/CCL7, MCP3/CCL8, growth-related oncogene 1/chemokine C-X-C motif ligand 1 (GRO1/CXCL1), GRO2/CXCL2, and macrophage inflammatory protein 1 (MIP1α/CCL3). IL8 was quantified with human IL8 enzyme-linked immunosorbent assay (ELISA) Kit (BD OptEIA; BD BioSciences, USA).

Histology/immunohistochemistry

H&E stainings were acquired by brightfield and polarized light microscopy for blinded grading by two independent observers (DB, MH) (inter-rater reliability: Spearman's $r = 0.96$, $p < 0.001$) according to a Modified Bonar Score (Supplementary Table ii), adapted from previously described protocols.[34,35]

Immunofluorescence stainings were performed with primary antibodies for CDKN2A/p16 (ab54210, Abcam, UK), CDKN2D/p19 (P4354, Sigma-Aldrich, USA), EZH2 (ab191080, Abcam) (1:250), or γH2AX (80,312 S, Cell Signaling Technology) (1:100) using a previously described protocol for immunofluorescent stainings.[36] As a secondary antibody, goat anti-mouse IgG Alexa 546 (A11030, Invitrogen) was used for p16/p19/γH2AX and goat anti-rabbit IgG Alexa 647 (A21245; Invitrogen, USA) for EZH2 (1:200). Controls without primary antibody, as well as with matched IgG controls, were performed to exclude non-specific staining. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Tissue autofluorescence was reduced using TrueVIEW Quenching Kit (SP-8400-15, Vector Laboratories, USA).

Cells expressing p19 and/or EZH2 were quantified using QuPath, with QuPath's *Cell detection* command to identify cells across all cores based on nuclear staining. Two-way classifier was interactively trained to distinguish cells as either positive or negative for p19 and EZH2 (n = 4 to 7 sections/group). Intensity thresholds were set based upon mean nuclear intensity.

Gene expression

Tissue pieces (1 × 1 × 1 mm) were homogenized in 1 ml TRIzol with two ceramic beads (Lysing Matrix M, MP Biomedicals) using MP FastPrep 5 G system (MP Biomedicals, USA) for 7 × 20 sec at 4 m/s, with breaks of ten minutes in a thermomixer (RT, 800 rpm) after rounds three and six. Chloroform was added to subsequent supernatant and after phase separation, up to 1 µg total RNA per samples was extracted with RNeasy Fibrous Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions, and NanoDrop One (Thermo Fisher Scientific, USA) used to monitor its quality and quantity. Messenger RNA (mRNA) was transcribed to complementary DNA (cDNA), and the expression of target genes (Supplementary Table iii) analyzed by quantitative polymerase chain reaction (PCR) (Applied Biosystems 7500 Fast Real-Time PCR, Thermo Fisher Scientific) and displayed as δ Ct ($\Delta CT = CT^{\text{gene of interest}} - CT^{\text{reference}}$ for normalization (mean of technical triplicates) and subsequent $\log_2$ transformation). Out of multiple tested reference genes, only *GAPDH* provided consistent and high expression levels under our applied conditions,[37] and was selected as a single reference gene also due to constrained sample material. All procedures followed standardized protocols of our laboratory to ensure quality standards.

Statistical analysis

Association of patient age with degeneration stage by Modified Bonar Score was analyzed using linear mixed models, with age and Modified Bonar Score as fixed effects and patient

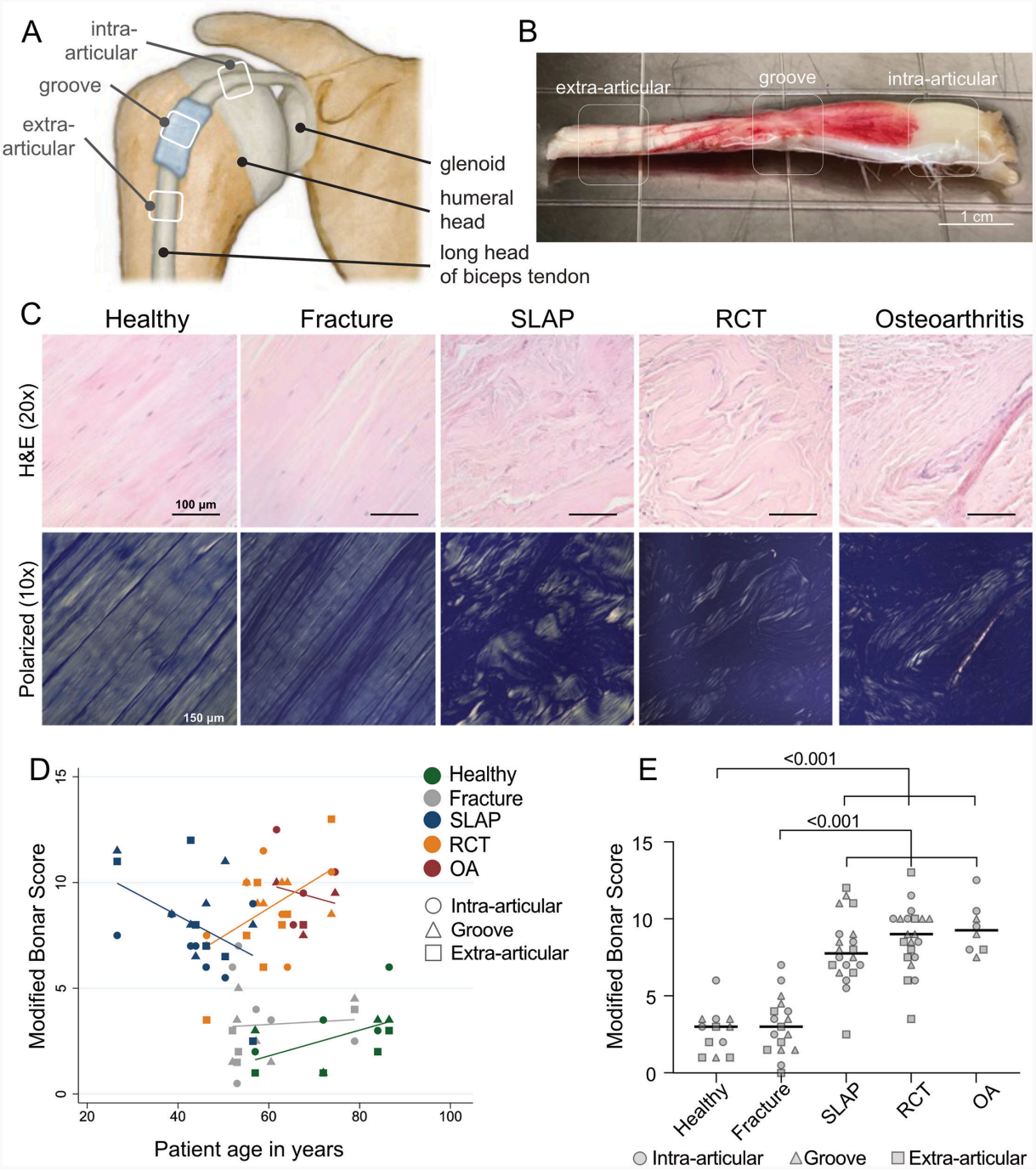

Fig. 1

a) Anatomical illustration of the proximal long head of biceps tendon originating from inside the shoulder joint. The three regions of interest (intra-articular, bicipital groove, extra-articular) are marked. b) Representative image of a tendon biopsy from a patient with rotator cuff tear (RCT). The increased amount of capillaries indicates ongoing inflammatory processes. The three regions of interest are indicated. Scale bar = 1 cm. c) Representative haematoxylin and eosin (H&E) stainings and polarized light microscopy images of all five patient groups. Scale bars = 100 µm for H&E images and 150 µm for polarized light microscopy images. d) Scatterplot depicting distribution of tendon degeneration (by Modified Bonar Score adapted from Fearon et al[34] and Streit et al,[35] see also Supplementary Table ii) with aetiology (patient group) and age. Patient groups are colour-coded, and different locations of origin are displayed by geometrical shapes (see key). Note that for each patient the assessed regions are aligned vertically due to patient age. e) Comparison of Modified Bonar scores from different shoulder tendinopathies irrespective of age. Lines represent mean scores; statistical significance was defined using linear mixed models as decribed in the Methods section. P-values are indicated if $p \leq 0.05$. OA, osteoarthritis; SLAP, superior labrum anterior to posterior tear.

identifier as random effect in the model, allowing us to analyze all assessments while accounting for non-independence in the data, due to the fact that two or three assessments relate to the same patient. To address the question of whether location of the tendon segment has an impact on the Modified Bonar Score, we included location as a covariate into a linear mixed model and adjusted for age and aetiology which, given the small sample size, we simplified to degenerative yes/no, grouping fracture patients and healthy subjects as "no", and SLAP, RCT, and OA as "yes". Analyses were conducted using Stata 16.0 (StataCorp, USA). p-values were defined by Student's *t*-test on the estimated coefficient divided by its

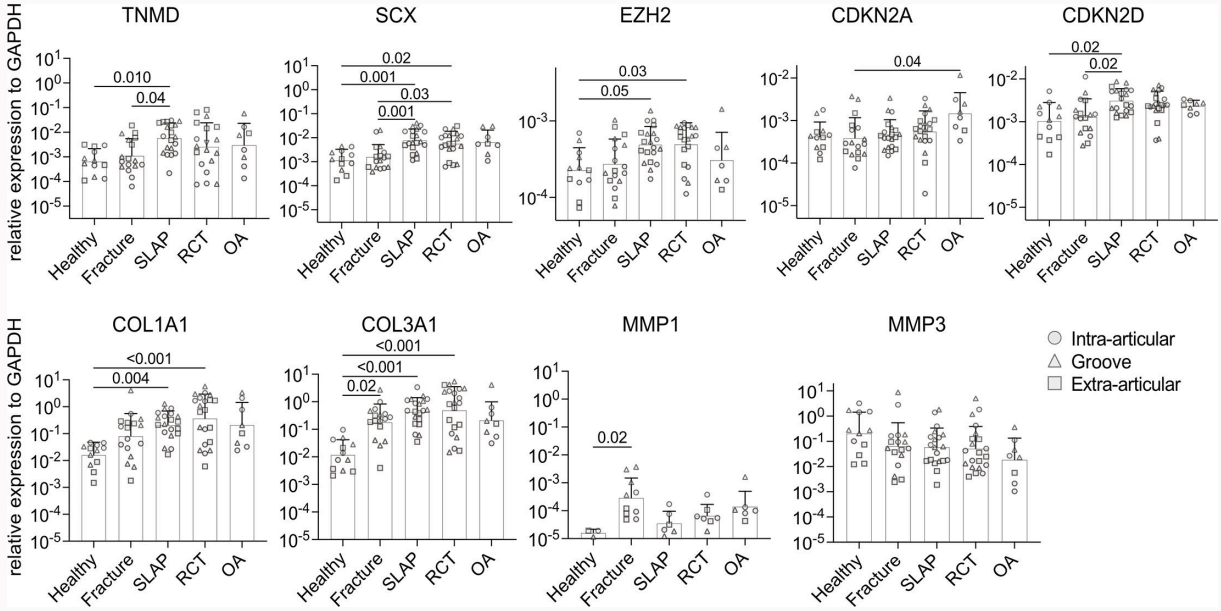

Fig. 2
Gene expression analysis of native tendon biopsies in the different regions (indicated by the symbols; 2 to 3 regions/donor, n = 4 to 7 donors/group) for tendon-specific anabolic (tenomodulin (*TNMD*), *SCX*, collagen type I alpha 1 chain (*COL1A1*), and *COL3A1*) and catabolic genes (matrix metalloproteinase (*MMP*)1 and *MMP3*), Enhancer of Zeste 2 (*EZH2*), as well as senescent markers *CDKN2A* and *CDNK2D*. Expression levels are relative to reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Kruskal-Wallis testing was performed for overall comparison with post-hoc Dunn's multiple comparison test. p-values are indicated if $p \leq 0.05$. The bar graphs display geometrical means (geometrical SDs). OA, osteoarthritis; RCT, rotator cuff tear; SLAP, superior labrum anterior to posterior tear.

standard error as part of the mixed model statistical analysis. All other analyses were performed with GraphPad Prism: following Shapiro-Wilk test for normal distribution, parametric data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's (when comparing means to the control mean) or by Tukey's (when comparing all means with each other) multiple comparison test. Non-parametric data were tested by Kruskal-Wallis with post-hoc Dunn's test for multiple comparisons. Spearman's rank correlation was applied for correlation analysis and non-parametric Mann-Whitney U test for fold change (FC) analyses upon treatment. p-values ≤ 0.05 were considered significant and reported as actual values, unless $p < 0.001$. Data are presented as mean values and SDs (linear scaled graphs) or geometrical means and geometrical SD (logarithmic scaled graphs).

Results

Tendon degeneration

Representative H&E stainings and polarized light microscopy images showed that tendon tissues from healthy and fracture groups displayed no signs of tendon degeneration, and a well-organized collagen fibre alignment, visualized by tissue birefringence. Chronic shoulder tendinopathies (SLAP, RCT, and OA) instead showed clear signs of degeneration and disruption in collagen fibre alignment (Figure 1c). Modified Bonar Scores increased by a mean 0.02 points per year of patient age, showing no strong association of tissue degeneration and age ($p = 0.509$, mixed model statistical analysis). Specifically, Modified Bonar Scores of fracture patients and healthy donors were similar (mean of modelled values (SD): 3.28 (SD 0.163) and 2.71 (SD 0.196)), while scores of SLAP (8.00 (SD 0.146)), RCT (8.76 (SD 0.129)), and OA (9.44 (SD 0.078)) patients were approximately five points higher (all $p < 0.001$ compared to fracture as well as compared to healthy) (Figures 1d and 1e, Supplementary Table iv). Modified Bonar Scores did not differ between the different locations, namely intra-articular, bicipital groove, and extra-articular, when adjusted for age and stage of degeneration (Supplementary Table v).

Expression of EZH2 and senescence markers in tendinopathies

In pathological tendon tissues, higher gene expression levels were demonstrated for tendon markers tenomodulin (*TNMD*) (SLAP vs Healthy: $p = 0.010$; SLAP vs Fracture: $p = 0.040$) and *SCX* (SLAP and RCT vs Healthy: $p = 0.001$ and $p = 0.020$, respectively; SLAP and RCT vs Fracture: $p = 0.001$ and $p = 0.030$, respectively), and of the extracellular matrix components *COL1A1* and *COL3A1* (*Col1A1*: SLAP and RCT vs Healthy: $p = 0.004$ and $p < 0.001$, respectively; *Col3A1*: SLAP and RCT vs healthy: $p < 0.001$, Kruskal-Wallis with post-hoc Dunn's multiple comparison test) (Figure 2). Increased *COL3A1* expression was also observed in the fracture group (Fracture vs Healthy: $p = 0.020$), indicating ongoing remodelling processes in tendons of acutely injured joints. Expression of *MMP1* remained undetectable in most of the samples, and if expressed, levels remained generally low. Significantly higher *MMP1* expression was only observed in fracture samples. Compared to healthy samples, chronic tendinopathies displayed significantly increased levels of *EZH2* (SLAP and RCT vs Healthy: $p = 0.050$ and $p = 0.030$, respectively), as well as increased levels of well-established cellular senescence markers *CDKN2A*, encoding for p16 (OA vs Fracture: $p = 0.040$), and *CDKN2D*, encoding for p19 (SLAP vs Healthy and Fracture: both $p = 0.020$). Overall, gene expression analyses revealed an increase in cellular senescence markers in tendon tissue from

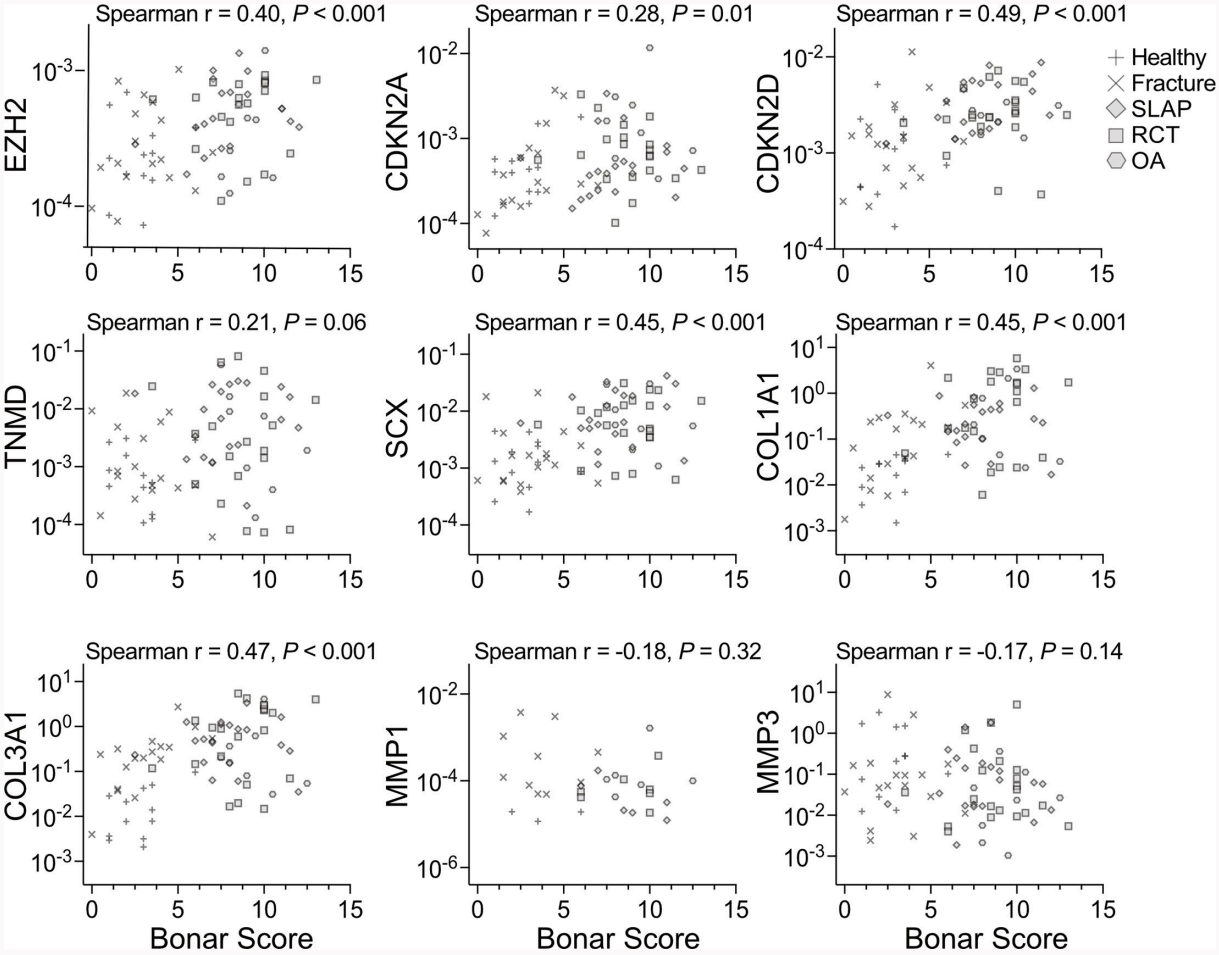

Fig. 3
Correlation of gene expression in native tendon biopsies to Modified Bonar score for all patient groups. Spearman's test with corresponding p-value is indicated in each graph. *COL1A1*, collagen type I alpha 1 chain; *EZH2*, Enhancer of Zeste 2; *MMP1*, matrix metalloproteinase 1; OA, osteoarthritis; RCT, rotator cuff tear; SLAP, superior labrum anterior to posterior tear; *TNMD*, tenomodulin.

patients with tendinopathies, together with an upregulation of *EZH2*.

Correlation of EZH2 and senescence factors with tendon degeneration and patient age

Considering the inter-donor heterogeneity of degeneration stages and age within each group, correlation analyses showed a significant positive correlation between the degree of tendon degeneration with *EZH2* ($p < 0.001$), as well as with senescence markers *CDKN2A* ($p = 0.010$) and *CDKN2D* ($p < 0.001$). Expression levels of *SCX, COL1A1*, and *COL3A1* correlated with the extent of degeneration (all $p < 0.001$), while no correlations of Modified Bonar Scores were observed with *TNMD, MMP1*, or *MMP3* (Figure 3).

EZH2 ($p = 0.010$) and *CDKN2D* ($p = 0.005$) expression further showed a correlation with patient age, unlike the other senescent marker *CDKN2A* (Figure 4a).

Coexpression analysis of EZH2 and p16/p19/γH2AX

Correlation analyses revealed a connection between *EZH2* and senescence markers *CDKN2A* and *CDKN2D* gene expression (Figure 4b). Further association of EZH2 with cellular senescence at protein level was addressed immunohistochemically in RCT tendon tissue for EZH2, p16, p19, and an additional senescence marker (γH2AX), detecting DNA breaks typically accumulating in senescent cells (Figure 5).

Co-stainings for EZH2/p16, EZH2/p19, and EZH2/γH2AX showed a clear dissection into double-positive senescent tenocytes and double-negative non-senescent tenocytes. The majority of senescent cells, irrespective of the senescent marker used, also expressed EZH2. To provide proof of principle of EZH2 co-localization with markers of senescence in the different disease groups, co-staining of EZH2/p19 was further performed for two randomly selected donors from each group for quantification (Figure 6, Supplementary Table vi). Our observations corroborate cellular co-localization of senescent markers with EZH2, indicating a direct association of EZH2 with tenocyte senescence and further suggesting a common regulatory pathway.

Senotherapeutic treatment of native tendon tissue

To further evaluate the role of EZH2 in cellular senescence, the effect of its selective inhibitor EPZ-6438 was assessed on native tendon biopsy cultures, and compared to treatments with commonly used senolytics Dasatinib and Quercetin (D + Q; clinically used senolytic combination of tyrosine kinase inhibitor and an antioxidant, respectively), ABT-263 (Navitoclax; Bcl-2 inhibitor), and AG490 (JAK2/STAT3 inhibitor)

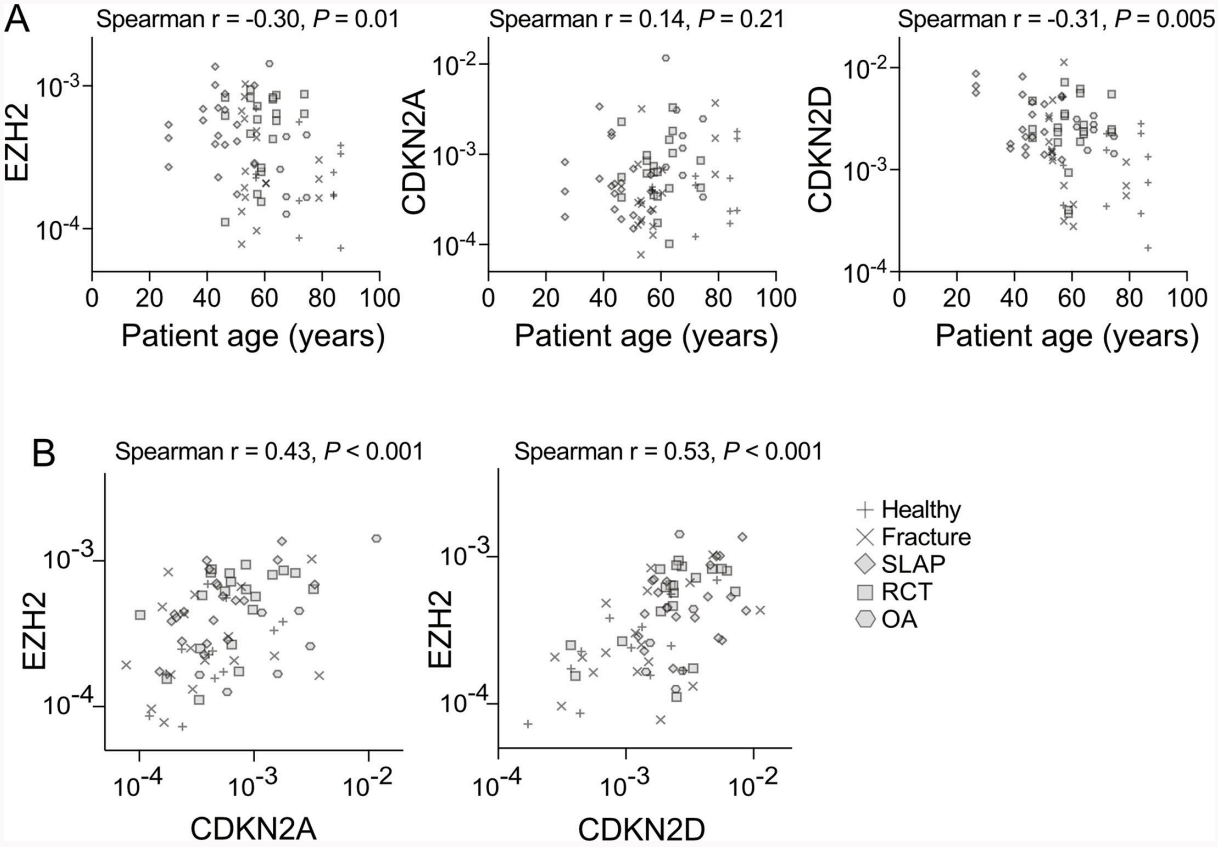

Fig. 4
a) Gene expression in native tendon biopsies in correlation to patient age and b) correlation of Enhancer of Zeste 2 (*EZH2*) to *CDKN2A* and *CDKN2D* gene expression. Spearman's test with corresponding p-value is indicated in each graph. OA, osteoarthritis; RCT, rotator cuff tear; SLAP, superior labrum anterior to posterior tear.

(Supplementary Figure a). The senolytic AG490 reduced mean expression of *CDKN2A* (FC to control: 0.46 (SD 0.12), p = 0.029) and correlated with downregulation of the antiapoptotic factors *BCL2* (FC: 0.16 (SD 0.06), p = 0.029) and *BCL-xL/BCL2L1* (FC: 0.21 (SD 0.07), p = 0.029). EPZ-6438 also reduced expression of *CDKN2A* (FC: 0.23 (SD 0.14), p = 0.029), *CDKN2D* (FC: 0.43 (SD 0.31), p = 0.049), and the anti-apoptotic factor *BCL2* (FC: 0.20 (SD 0.12), p = 0.029; p-values were defined for all aforementioned gene expression data by Mann-Whitney U tests). No significant effect of EPZ-6438 was observed on expression of *TNMD* and *SCX*. Other senolytic compounds (Dasatinib/Quercetin, ABT-263) did not show significant senolytic effects at gene expression level in our tendon tissue culture system. A possible modulatory effect of EZH2 inhibition on the secretion of SASP components was then investigated in a similar tissue culture, where secretion of the pro-inflammatory and catabolic factors was stimulated in the presence of IL1β (Figure 7, Supplementary Table vii). Out of the 12 selected factors representing the SASP, the senolytic AG490 significantly reduced secretion of all of them – except MMP13 – including MMP3, IL6, IL8, TNFα, and MCP1. Interestingly, inhibition of EZH2 also reduced six of the assessed factors, namely MMP3 (FC: 1.3 (SD 0.1), p = 0.008), IL6 (FC: 6.7 (SD 8.4), p = 0.005), IL8 (FC: 3.8 (SD 4.1), p = 0.046), TNFα (FC: 1.4 (SD 0.1), p = 0.036), GRO1 (FC: 141.5 (SD 160.5), p < 0.001; p-values were defined for all aforementioned factors in this paragraph by one-way ANOVA with Dunnett's multiple comparison test), and MCP2 (FC: 5.5 (SD 5.3), p = 0.049, defined by Kruskal-Wallis followed by Dunn's post-hoc test for multiple comparisons).

Taken together, these data show that cellular senescence in chronic tendinopathies can be reduced not only by the senolytic compound AG490, but also by the EZH2 inhibitor EPZ-6438, suggesting that EZH2 is actively involved in regulation of cellular senescence in tenocytes.

Discussion

In this study, we demonstrated that cellular senescence occurs in tendon tissues of patients with chronic tendinopathies, and positively correlates with EZH2, while its inhibition reduces senescent traits to a similar extent as common senotherapeutics. Since the majority of the analyzed cells were either co-expressing EZH2 and p19 or double-negative, and EZH2 inhibition further reduced secretion of SASP factors, we hypothesize a possible common regulatory pathway where EZH2 could directly regulate cellular senescence in tendons.

Cellular senescence in degenerative joint diseases is known to impact tissue regeneration processes,[17,18] but was only recently addressed in the tendon, where an increase of senescent cells was observed in expanded cell culture systems.[22,23] Complementing these in vitro findings, and confirming recent observations in tendon tissues of RCT patients,[24] we here demonstrated the occurrence of senescent traits in native tissues from patients with shoulder tendinopathies to correlate with the degree of tendon degeneration. Interestingly, tendon degeneration was equally present in all

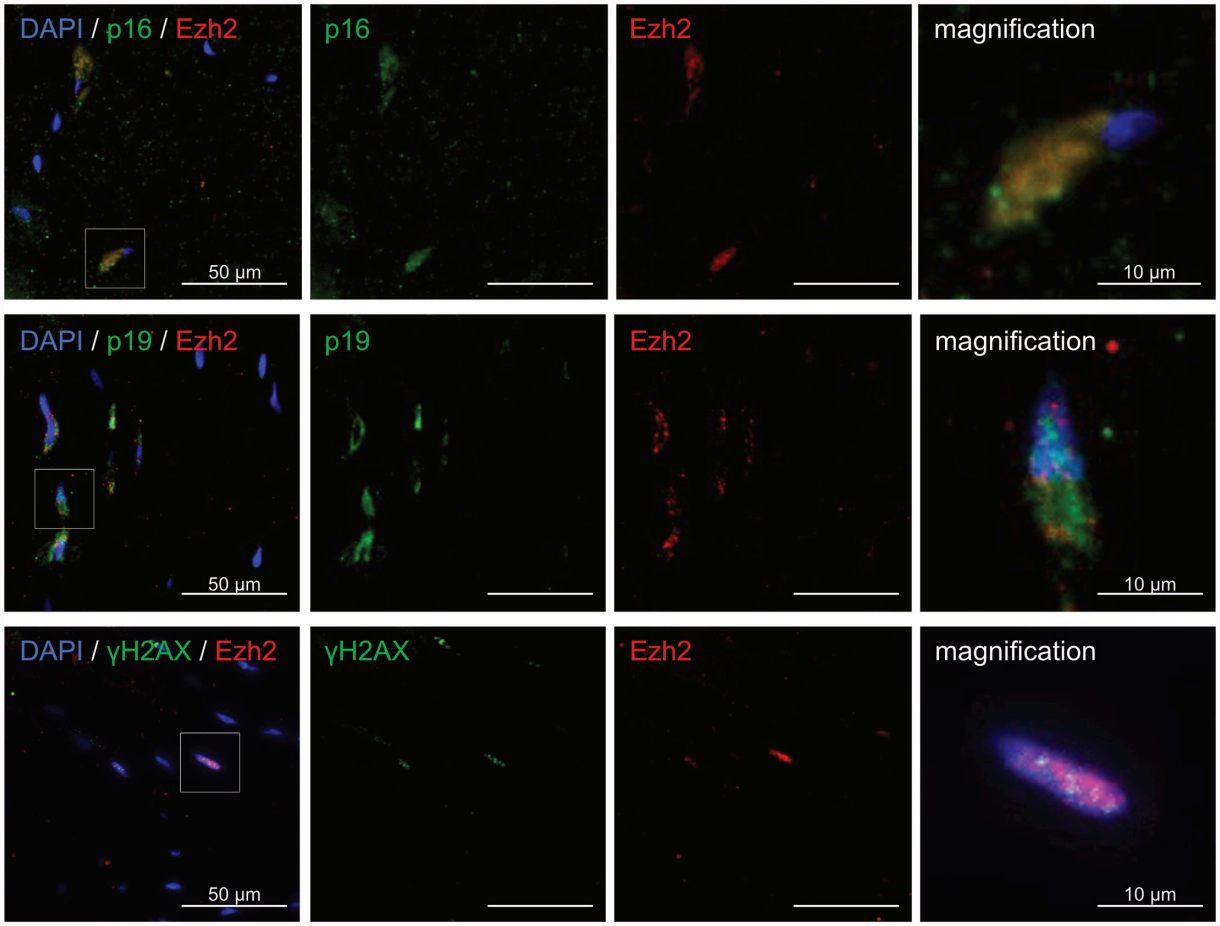

Fig. 5
Representative immunofluorescent co-stainings of Enhancer of Zeste 2 (EZH2) with p16 (upper row), p19 (middle row), and γH2AX (lower row) for a rotator cuff tear (RCT) biopsy. Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI). Images at the right show a magnification of the area with a selected tenocyte, indicated by a white box on the left. Scale bars equal 50 µm unless otherwise indicated.

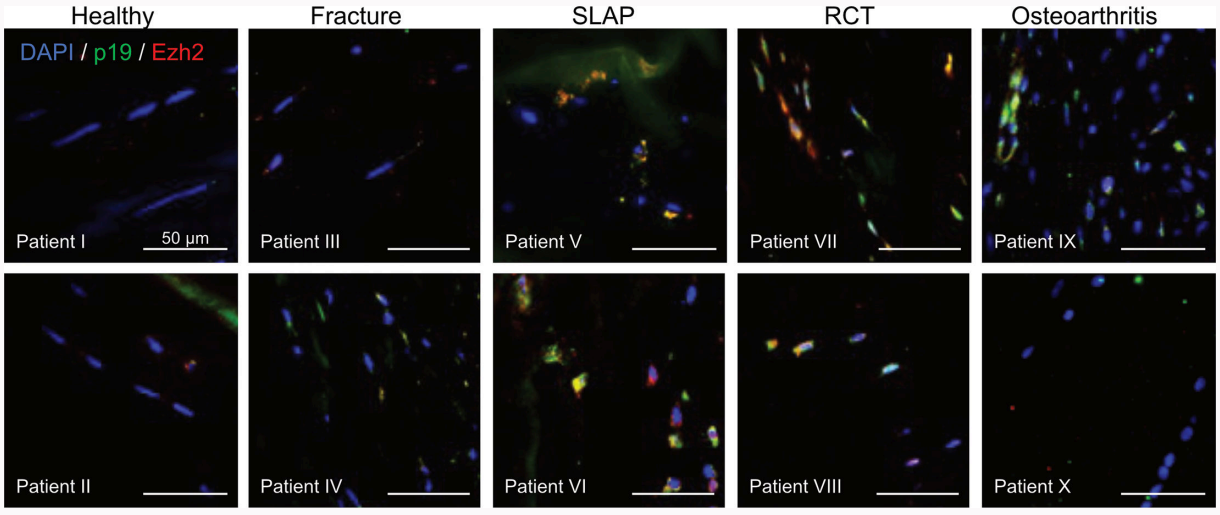

Fig. 6
Immunofluorescent co-stainings of Enhancer of Zeste 2 (EZH2) and p19. For each group, stainings of two individual patient samples are shown; images are representative for that patient sample. Scale bars equal 50 µm. DAPI, 4',6-diamidino-2-phenylindole; RCT, rotator cuff tear; SLAP, superior labrum anterior to posterior tear.

assessed patient groups and regions, irrespective of whether the biceps tendon was affected directly (SLAP lesion) or only indirectly (RCT or OA), or whether the biopsy originated from intra- or extra-articular sites. This further supports a recent postulation that tissue degeneration disseminates along

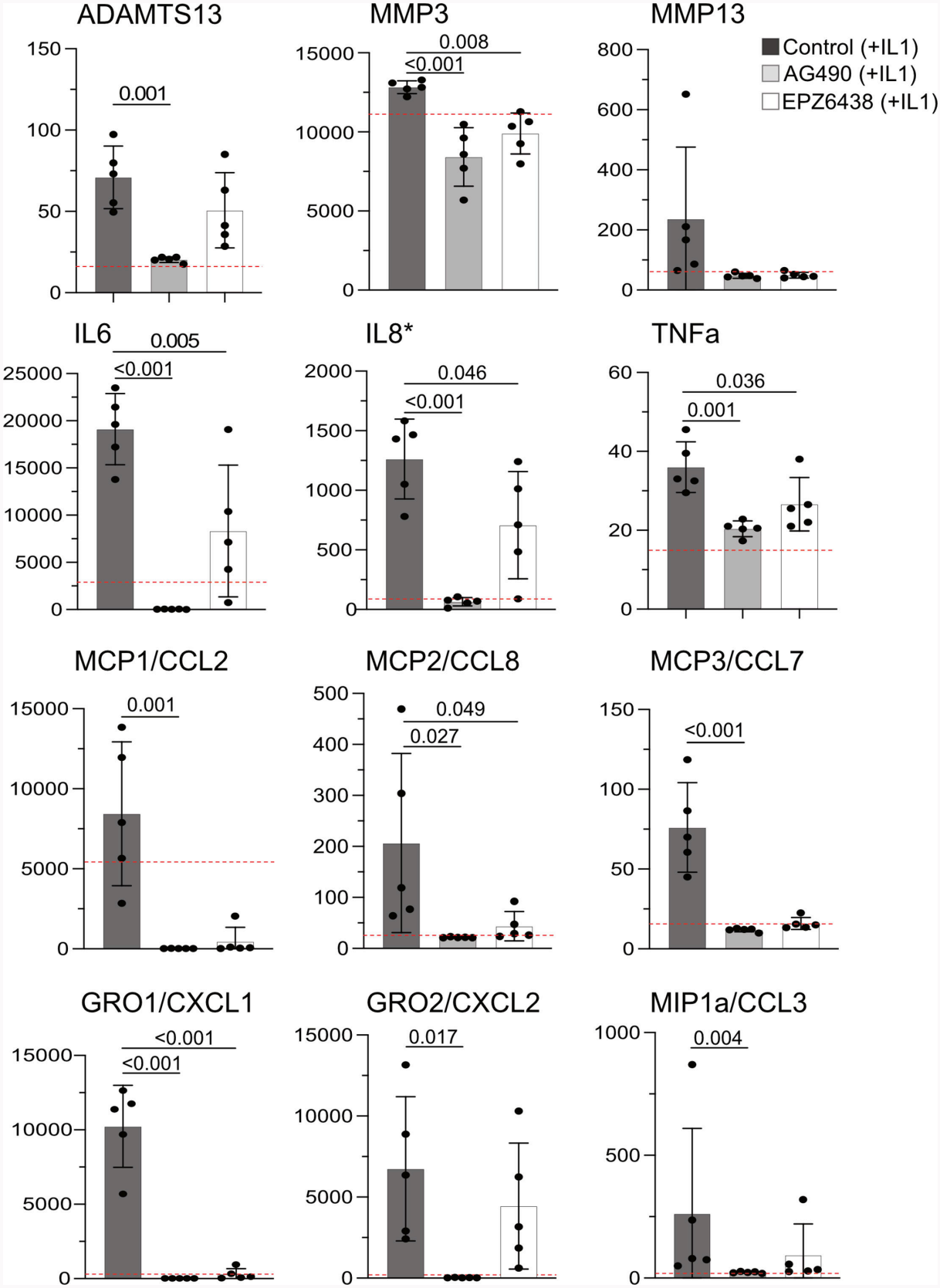

Fig. 7

Quantification of proteins of the senescence-associated secretory phenotype (SASP) (in pg/ml, except for IL8,* in ng/ml) after incubation in standard culture medium with interleukin (IL)1β alone (control + IL1) or in combination with AG490 or EPZ-6438. Dashed red lines indicate levels of expression in control medium in the absence of IL1β. One-way analysis of variance with Dunnett's multiple comparison test was performed, except for MCP1/CCL2, MCP3/CCL7, and MIP1a/CCL3, which were not normally distributed according to Shapiro-Wilk test, and thus analyzed with non-parametric Kruskal-Wallis followed by Dunn's post-hoc test for multiple comparisons. p-values are indicated if $p \leq 0.05$. Values are presented as mean (SD). MMP3, matrix metalloproteinase 3; TNFa, tumour necrosis factor alpha.

the biceps tendon in chronic joint diseases, also affecting extra-articular tendon regions.[38,39]

It is a commonly accepted idea that signs of tendon degeneration accumulate with age, even in asymptomatic patients.[21,40] However, we observed low Bonar scores in our elderly patient population of the control groups ('fracture' and 'healthy' from cadavers), compared to high Bonar scores even in relatively young patients, but with increased symptom duration (SLAP). Since we only characterized tendon injuries from patients with insufficient endogenous healing capacities who developed chronic tendinopathy, no conclusion can be drawn about the mechanisms of senescence in asymptomatic patients with completely healed tendon injuries, or possible age-related differences in these patients.

Excessive expression of EZH2 has been shown to promote pathogenesis of rheumatoid diseases,[41] and to increase in degenerated OA cartilage, while its inhibition showed chondroprotective results in an OA mouse model.[31,32,42] Interestingly, we did not observe accumulation of either senescent or EZH2 expressing cells in OA tendon tissues, which could be – in addition to the small sample size – attributed to high aetiological heterogeneity regarding risk factors, and the long-lasting persistence of symptoms in OA.[43]

In line with a previously described work using the JAK2/STAT3 inhibitor AG490 in a tendon ageing mouse model,[20] we confirmed its similar senolytic effect on human tendon tissues, shown by reduced CDKN2A/p16 expression and induced expression of apoptotic factors as well as reducing components of SASP, a hallmark of cellular senescence. A comparable senolytic effect, reflected by down-regulation of CDKN2D/p19 and CDKN2A/p16, as well as the reduction of secreted pro-inflammatory cytokines and catabolic SASP components, was achieved with the selective EZH2 inhibitor EPZ-6438 – a methyltransferase inhibitor which was approved as Tazemetostat in 2020 by the FDA to treat relapsed epithelioid sarcoma. Targeting cellular senescence through epigenetic regulation, i.e. via EZH2 modulation, could affect a broader range of senescence mediators, compared to conventional senolytics that more specifically target distinct pathways, such as the JAK/STAT or apoptotic pathways.

Overall, our findings hint at a direct regulatory mechanism of EZH2 and cellular senescence in tendinopathies, and warrant further investigation in other musculoskeletal tissues under degenerative conditions. This hypothesis is corroborated by our observation that EZH2 inhibition was able to reduce the secretion of several SASP compounds known to play a crucial role in cellular senescence in general, and in tendon tissues in particular,[24,44] although not to the same extent as with the established senolytic AG490. This could be due to a different mechanism of action, which may go beyond the highly specific targeting of the defined JAK2/STAT3 pathway by AG490, and remains to be elucidated for EPZ-6438.

The observed increase of anabolic factors (TNMD, SCX) in the presence of senotherapeutics or EPZ-6438 could be interpreted as a sign of increased regenerative activity of the non-senescent tenocytes, a phenomenon well known in early stages of other degenerative diseases such as OA.[45] Analyses at the single-cell level would be necessary to highlight the processes activated by these treatments in defined cell populations, which may occur in parallel with the induction of apoptosis in senescent cells.[46] Further investigations are also required to demonstrate a functional role of EZH2 in tissue regeneration, e.g. by assessment of altered histone methylase activity, and of the effect of removal/reduction of senescent cells within the tendon tissue. Our observation that EZH2 inhibition can affect the SASP, and thus prevent senescent cells contributing to the chronic inflammation status in an injured or diseased joint, could positively affect tendon healing and/or regeneration. Whether attenuation of cellular senescence in response to selected compounds can structurally and functionally enhance tendon regeneration should thus be addressed in future studies, e.g. using in vitro bioreactor systems to reproducibly engineer tendon-like tissues.

The main limitations of our study are the relatively low number of patients per disease group and the methodologically predetermined selection bias, since we only operate on patients with insufficient endogenous healing processes. Furthermore, the entire age spectrum per group could not be covered; certain diseases like SLAP occur more frequently in younger individuals, while others, such as OA, are more common in elderly individuals – our biopsy collection thus reflects the epidemiological prevalence of the different shoulder pathologies. All our assessments were further performed using native tendons, reflecting the in situ state within the tissue. While senescence may occur in response to applied culture conditions in in vitro cell culture systems,[47] such experiments with isolated and expanded tenocytes are required in future studies to unravel underlying mechanisms and cellular responses, i.e. to senolytic treatments and EZH2 inhibition. While our study lays the foundation, in-depth characterization of the underlaying mechanisms demands further investigation in future research.

Supplementary material

Patient/donor inclusion and exclusion criteria, a supplementary figure, and tables of patient demographic data, Modified Bonar score, Taqman assays used for gene expression analyses, association of age and tissue degeneration, association of location and tissue degeneration, and quantification of p19 and/or EZH2 expressing cells.

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Author information

D. Bühler, MD, Orthopaedic Surgeon,
Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland;
Department of Orthopaedics and Traumatology, Basel, Switzerland.

M. Hilpert, PhD, Scientist, Head of Flow Cytometry Core Facility
A. Barbero, PhD, Professor, Group Leader Cartilage Engineering
K. Pelttari, PhD, Lecturer, Senior Scientist
Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland.

A. M. Müller, MD, Professor, Orthopedic Surgeon, Head of Orthopedic Surgery and Traumatology, Department of Orthopaedics and Traumatology, Basel, Switzerland; Department of Surgery, University Hospital Basel, Basel, Switzerland.

S. A. Müller, MD, Orthopedic Surgeon, Lecturer, Department of Orthopedic Surgery and Traumatology, Kantonsspital Baselland, Bruderholz, Switzerland; Faculty of Medicine, University of Basel, Basel, Switzerland.

I. Martin, PhD, Professor, Group Leader Tissue Engineering, Head of Department of Biomedicine, Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland; Department of Biomedical Engineering, University of Basel, Allschwil, Switzerland.

Author contributions

D. Bühler: Data curation, Formal analysis, Funding acquisition, Visualization, Writing – original draft.
M. Hilpert: Data curation, Formal analysis, Visualization, Writing – review & editing.
A. Barbero: Writing – review & editing.
A. M. Müller: Resources, Writing – review & editing.
S. A. Müller: Resources, Writing – review & editing.
I. Martin: Conceptualization, Supervision, Writing – review & editing.
K. Pelttari: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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ICMJE COI statement

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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Ethical review statement

The present study was reviewed and approved by the the local ethical authority (EKNZ Ethikkommission Nordwest- und Zentralschweiz 2020-02934). All individuals/their relatives gave written informed consent to participate in this study.

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