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Original Article

# Upregulation of FABP4 induced inflammation in the pathogenesis of chronic tendinopathy

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ABSTRACT

*Objectives:* Excessive inflammation contributes to the pathogenesis of tendinopathy. Fatty acid binding protein 4 (FABP4) is a pro-inflammatory adipokine mediating various metabolic and inflammatory diseases. This study aimed to examine the expression of FABP4 and its association with the expressions of inflammatory cytokines in tendinopathy. The effects of a single injection of FABP4 on tendon pathology and inflammation were examined. The effect of FABP4 on the expressions of inflammatory cytokines and the effect of IL-1β on the expression of FABP4 in tendon-derived stem/progenitor cells (TDSCs) were also investigated.

*Methods:* 1) Clinical patellar tendinopathy samples, healthy hamstring tendon samples, and healthy patellar tendon samples, 2) rotator cuff tendinopathy samples and healthy hamstring tendon samples; and 3) Achilles tendons of mice after saline or collagenase injection (CI) were stained for FABP4, IL-1β, IL-6, TNF-α and IL-10 by immunohistochemistry (IHC). For the rotator cuff tendinopathy samples, co-localization of FABP4 with IL-1β and TNF-α was done by immunofluorescent staining (IF). Mouse Achilles tendons injected with FABP4 or saline were collected for histology and IHC as well as microCT imaging post-injection. TDSCs were isolated from human and mouse tendons. The mRNA expressions of inflammatory cytokines in human and mouse TDSCs after the addition of FABP4 was quantified by qRT-PCR. The expression of FABP4 in TDSCs isolated from rotator cuff tendinopathy samples and healthy hamstring tendon samples was examined by IF. Mouse Achilles TDSCs were treated with IL-1β. The mRNA and protein expressions of FABP4 were examined by qRT-PCR and IF, respectively.

*Results:* There was significant upregulation of FABP4 in the patellar tendinopathy samples and rotator cuff tendinopathy samples compared to their corresponding controls. FABP4 was mainly expressed in the pathological areas including blood vessels, hypercellular and calcified regions. The expressions of IL-1β and TNF-α increased in human rotator cuff tendinopathy samples and co-localized with the expression of FABP4. Collagenase induced tendinopathic-like histopathological changes and ectopic calcification in the mouse Achilles tendinopathy model. The expressions of inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-10) and FABP4 increased in hypercellular region, round cells chondrocyte-like cells and calcified regions in the mouse Achilles tendons post-collagenase injection. A single injection of FABP4 in mouse Achilles tendons induced histopathological changes resembling tendinopathy, with increased cell rounding, loss of collagen fiber alignment, and additionally presence of chondrocyte-like cells and calcification post-injection. The expressions of IL1-β, IL-6, TNF-α and IL-10 increased in mouse Achilles tendons post-FABP4 injection. FABP4 increased the expressions of *IL10*, *IL6*, and *TNFa* in human TDSCs as well as the expressions of *Il1b*, *Il6*, and *Il10* in mouse TDSCs. Human tendinopathy TDSCs expressed higher level of FABP4 compared to healthy hamstring TDSCs. Besides, IL-1β increased the expression of FABP4 in mouse TDSCs.

*Conclusion:* In conclusion, an upregulation of FABP4 is involved in excessive inflammation and pathogenesis of tendinopathy. TDSCs is a potential source of FABP4 during tendon inflammation.

*Translation potential of this article:* FABP4 can be a potential treatment target of tendinopathy.

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## 1. Introduction

Tendinopathy is an unmet clinical challenge, causing significant pain and disability. The tendon tissue degenerates and undergoes spontaneous rupture. The accumulation of microinjuries causing failed tendon healing has been suggested to contribute to the disease pathogenesis [1].

Tendinopathy is previously considered as a degenerative disease with an absence of infiltration of inflammatory cells. However, recent studies have reported signs of inflammation in clinical samples of tendinopathy [2,3]. Tendon overuse initiates an inflammatory cascade and induces erroneous tendon-derived stem/progenitor cells (TDSC) differentiation, causing tissue metaplasia [4–8]. The TDSC pool for tenogenesis reduces, contributing to failed healing.

Fatty acid binding protein 4 (FABP4) is a pro-inflammatory adipokine expressed in various cell types and its circulating level is upregulated in various inflammatory and metabolic diseases such as obesity, insulin resistance and atherosclerosis [9]. Inhibition of FABP4 has been reported to improve these pathological conditions [10–12]. However, the roles of FABP4 in the pathogenesis of tendinopathy remain unclear. In a study screening for the molecular markers of Achilles tendinopathy, the mRNA expression of *FABP4* was significantly higher in the chronic rupture compared to the acute rupture tendons but the expression levels in both conditions were lower than the intact tendon group [13]. Confirmation of the findings at the protein level was not reported.

This study therefore aimed to examine the expression of FABP4 and its association with the expression of inflammatory cytokines in clinical samples and animal model of tendinopathy. The effects of a single injection of FABP4 on tendon pathology and inflammation were examined. The expressions of inflammatory cytokines in TDSCs after the addition of FABP4, the expression of FABP4 in rotator cuff tendinopathy TDSCs and the effect of IL-1β on the expression of FABP4 in TDSCs were also investigated. We hypothesized that there would be an upregulation of FABP4 in clinical samples and animal model of tendinopathy. Moreover, FABP4 would co-express with inflammatory cytokines in the tendinopathy samples. A single injection of FABP4 could induce tendon inflammation, and damages histologically and radiologically. FABP4 would induce inflammation in TDSCs and TDSCs is a potential cell source of FABP4 during tendon inflammation.

## 2. Materials and methods

The clinical study complied with the Declaration of Helsinki. The research protocol has been approved by the clinical research ethics committee (Ref no. 2018.109) of the authors' institution. Written informed consent was obtained from the subjects prior to interview and sample collection. The animal study was conducted in full compliance with the ARRIVE guidelines, local regulatory principles, and licensing regulations of the Government. The animal study was approved by the animal research ethics committee of the Animal Experimentation Ethics Committee of the authors' institution (Ref no. 22-287-GRF).

### 2.1. Collection of clinical samples of tendinopathy

Patients meeting inclusion and exclusion criteria were recruited from the affiliated hospital of the authors' institution after obtaining their consent. For the examination of expression of FABP4 in patellar tendinopathy, adult patients aged over 18 of both genders, with tendinopathy at the patellar tendons as verified by ultrasound (US) or magnetic resonance imaging (MRI), having tendon pain or weakness prior to surgery and unsatisfactory physiotherapy treatment were recruited (0F/6M; age 31.5 ± 8.7, 6 M). Guided by clinical findings and US or MRI scans, the pathological tendon tissue was excised. Patients in the control group shared similar subject inclusion and exclusion criteria except that they had no history or current clinical signs of tendon injury and tendon pain. Healthy tendons were obtained from the remnant of hamstring autograft (4F/2M; age 27.7 ± 9.7) and bone-patellar tendon-bone (BPTB) graft (2F/4M; age 23.2 ± 7.0) during anterior cruciate ligament reconstruction (ACLR). The expression of FABP4 in patellar tendinopathy, healthy hamstring tendon, and healthy patellar tendon samples was examined by immunohistochemistry (IHC) and image analysis. There were 6 samples in each group.

For the investigation of expression of FABP4 in rotator cuff tendinopathy, adult patients with tendinopathy at the rotator cuff tendons as verified by US or magnetic MRI, having tendon pain or weakness prior to surgery and unsatisfactory physiotherapy treatment were recruited (5M; age 58.2 ± 19.0; BMI: 25.0 ± 1.1 kg/m$^2$). Guided by clinical findings and US or MRI scans, the pathological tendon tissue was excised. Healthy control subjects with no history or current clinical signs of tendon injury and tendon pain were recruited (1F/4M; age 37.2 ± 9.5; BMI: 25.0 ± 3.2 kg/m$^2$). Healthy tendons were obtained from the remnant of hamstring autograft during ACLR. Both patients in the rotator cuff tendinopathy group and healthy tendon group were not taking any diabetic and lipid-lowering drugs. Fasting blood samples were additionally available for 2 patients each in the rotator cuff tendinopathy group and healthy tendon group. Except one tendinopathy patient with borderline high total cholesterol (5.6 mmol/l), the level of total cholesterol, high-density lipoprotein (HDL), total triglyceride, low-density lipoprotein (LDL), non-HD cholesterol and glucose were normal for these subjects. The expression of FABP4 in rotator cuff tendinopathy and healthy hamstring tendon samples was examined by IHC and image analysis. There were 5 samples in each group. The co-localization of FABP4 with IL-1β and TNF-α in the healthy tendon samples and rotator cuff tendinopathy samples was examined by immunofluorescent (IF) staining. There were 5 samples in each group.

### 2.2. Collagenase-induced (CI) Achilles tendon injury model of tendinopathy

Twenty-four male C57BL/6J mice (12-week-old, weight 29.7 ± 1.4 g) fed with standard chow (10 % kcal fat) were used in this part of the study. After anesthesia, 20 μl of 1 % bacterial collagenase I (0.1 mg) or saline was injected in the mid-substance of one Achilles tendon of each mouse. At week 2 and 8 after saline or collagenase injection, the injected Achilles tendons were harvested for hematoxylin & eosin (H&E) staining in histology and IHC of Sox9 (n = 5/group/time point). Ectopic calcification in tendon was examined by Von Kossa staining (n = 5/group) and microCT imaging (n = 6/group) at week 8 after collagenase or saline injection. The expressions of FABP4, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokine (IL-10) in mouse Achilles tendons were examined by IHC and image analysis.

### 2.3. Injection of FABP4 into the mouse Achilles tendon

Twenty-four male C57BL/6J mice (12-week-old, weight 30.1 ± 1.5 g) fed with standard chow (10 % kcal fat) were used in this part of the study. After anaesthesia, 20 μl of saline or FABP4 (0.5 μg) was injected into one Achilles tendon of each mouse. The tendon samples were collected at week 2 and week 8 for histology (n = 5/group/time point) and IHC of IL-1β, IL-6, TNF-α and IL-10 (n = 5/group/time point). Ectopic calcification in tendon was also examined at week 8 after injection by microCT imaging (n = 6/group). The dose of FABP4 was selected based on the literature [14] and our pilot study. Our pilot data showed that FABP4 at 0.5 μg was the lowest dose that induced loss of collagen birefringence, a marker of extracellular matrix degeneration, in mouse tendons at week 1 after injection (Supplementary Fig. 1). This dose was therefore selected for further analysis.

### 2.4. Histology and immunohistochemistry (IHC)

Histology was done according to the published protocol [15]. The human and mouse tendons were washed in PBS, fixed in buffered formalin and 100 % ethanol, dehydrated, and embedded in paraffin.

Five-micrometer-thick coronal sections in the middle of the mouse Achilles tendons and human tendon samples were cut and mounted on coated slides for haematoxylin and eosin (H&E) staining. Collagen fiber alignment, and hence extracellular matrix degeneration, was examined under polarized light. Tendon injury was evaluated using a modified validated histopathological scoring system with high intra- and inter-rater reliability [15]. The scoring system consists of 10 parameters (fiber arrangement, cellularity, cell alignment, cell rounding, vascularity, fiber structure, hyaline degeneration, inflammation, fat accumulation, ossification) and each parameter is scored 0–3. The total score is calculated as the sum of scores of these 10 parameters. A score of 0 indicates a healthy tendon and a score of 30 indicates a severely damaged or pathological tendon. The total score for each group at each time point was presented.

The animal samples were also stained with Von Kossa for confirmation of ectopic calcification and IHC staining of Sox9 for confirmation of the presence of chondrocytes. All the samples were examined under light microscopy (DM5500; Leica Microsystems GmbH, Wetzlar, Germany). Representative images were presented.

For IHC staining, 5-μm-thick sections were cut and mounted on coated slides. After deparaffination, the sections were rehydrated, decalcified, quenched of endogenous peroxidase activity, and treated with 10 mM citrate buffer at 65 °C for 20 min for antigen retrieval. After blocking with 1 % goat serum, the sections were incubated with specific antibodies against FABP4 (1:200) (Proteintech, IL, USA; catalog # 12802-1), IL-1β (1:200) (Origene, MD, USA; catalog # TA321162), IL-6 (1:100) (Abcam, Cambridge, UK; catalog # ab290735), TNF-α (1:200) (Affinity, OH, USA; catalog # AF7014-100), IL-10 (1:200) (Affinity, catalog # DF6894-100), or Sox9 (1:200) (Beyotime, Shanghai, China; catalog # AF2329) at 4 °C overnight. The primary antibodies were replaced with blocking solution in the controls. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:500) (Abcam, catalog # ab6721) was used and the immunopositivity was visualized using the 3, 3′-diaminobenzidine (DAB) substrate kit according to the manufacturer's protocol (Thermofisher, MA, USA; catalog # 34002). After counterstaining with haematoxylin, the sections were dehydrated with graded ethanol and xylene, mounted with p-xylene-bis-pyridinium bromide (DPX) permount (SigmaAldrich, St Louis, MO) and examined under light microscopy (DM5500; Leica Microsystems GmbH, Wetzlar, Germany). A positive signal was shown as brown color. All the incubation times and conditions were strictly controlled. The pathological and healthy tendons in each experiment were stained in the same batch. Representative images were presented. Image analysis was done using the Image Pro Plus 6.0 software (MediaCybernetics, Bethesda, MD, USA) according to the published protocol [16]. The immunopositive signal was measured and presented as integrated optical density of area of interest (IOD/mm$^2$).

### 2.5. microCT imaging

Ectopic calcification in tendons was examined using a cone-beam microCT system (Bruker Skyscan 1276 in vivo microCT system) [15]. The Achilles tendon was scanned with the calf muscle and the calcaneus bone as the landmarks. The images were then 3D-reconstructed after thresholding, using the built-in software. The Achilles tendon-only region was selected as the region of interest (ROI). The bone volume (BV) inside the Achilles tendon was measured.

### 2.6. Co-immunofluorescent (IF) staining of FABP4 with IL-1β in human tendon tissues

Co-immunofluorescent staining of FABP4 with IL-1β in healthy tendon samples and rotator cuff tendinopathy samples was done. The sections were stained with goat polyclonal antibodies against mouse/rat FABP4 (1:200) (R&D Systems, Inc., Minneapolis, MN, USA; catalog # AF1443) overnight at 4 °C, washed and stained with rabbit polyclonal antibodies against human/mouse/rat IL-1β (1:200) (Origene, catalog # TA321162) or rabbit polyclonal antibodies against human/mouse/rat TNF-α (1:200) (Affinity, OH, USA; catalog # AF7014-100) for 2 h at room temperature. After washing, the sections were stained with anti-rabbit Alexa Fluor® 488 (1:500) (Abcam; catalog # ab150077), and anti-goat Alexa Fluor® 555 (1:500) (Abcam, catalog #150130) for 60 min each. The sections were washed and counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen™, MA, USA) before examination with a fluorescent microscope (DM5500, Leica).

### 2.7. Isolation of TDSCs and treatment of human and mouse TDSCs with FABP4 or IL-1β

The procedures for the isolation of TDSCs are well-established.[5] Briefly, human rotator cuff tendinopathy tissue, healthy human hamstring tendon tissue or mouse Achilles tendons of male C57BL/6J mice (8-week-old) were minced, digested with type I collagenase to yield a single cell suspension, plated at an optimal low cell density for the isolation of stem cells and cultured to form colonies. Only TDSCs less than passage 5 were used for experiments. Human and mouse TDSCs were treated with FABP4 (120 ng/mL) under serum starvation for 24 h. Mouse TDSCs were also treated with IL-1β under serum starvation (2 % FBS for IF and 1 % FBS for qRT-PCR) for 48 h. IL-1β was used because it was overexpressed in clinical samples of tendinopathy and overuse-induced inflammation in tendon cells and animals [17–19]. The dose of FABP4 was selected based on the dose used in other cell types in the literature [20,21].

### 2.8. Immunofluorescent staining (IF) of FABP4 in human and mouse TDSCs

The cells were stained with primary antibodies against FABP4 (1:200) (Proteintech, catalog # 12802-1) and anti-rabbit Alexa Fluor® 555 (1:500) (ThermoFisher, catalog #A-31572) for 60 min each. The cells were washed and counterstained with DAPI (Invitrogen™, MA, USA) before examination with a fluorescent microscope (DM5500, Leica).

### 2.9. qRT-PCR of Fabp4 and inflammatory cytokines in TDSCs

Total RNA was extracted using the PuroLink™ RNA Mini kit (ThermoFisher, MA, USA). cDNA was synthesized from 700 ng of RNA using the PrimeScript RT Reagent kit with gDNA Eraser (Takara Bio, CA, USA). Afterwards, the target gene was amplified with 1 μl cDNA, the TB Green Premix Ex Taq II (Takara) and the following primer sequences mouse *Fabp4* (F) 5′ TCACCGCAGACGACAGGAAG (R) 5′ CACCACCAGCTTGTCACCA, mouse *Il1b* (F) 5′ TGTGTAATGAAAGACGGCAC (R) 5′ TCCACTTTGCTCTTGACTTC, mouse *Il6* (F) 5′ AGCCAGAGTCCTTCAGAGAG (R) 5′ CTTAGCCACTCCTTCTGTGAC, mouse *Il10* (F) 5′ AAGGCAGTGGAGCAGGTGAA (R) 5′ CCAGCAGACTCAATACACAC, human *IL1b* (F) 5′ GAAGCTGATGGCC-CTAAACAG (R) 5′ AGCATCTTCCTCAGCTTGTCC, human *IL6* (F) 5′ AGACAGCCACTCACCTCTTCA (R) 5′ CACCAGGCAAGTCTCCTCATT, and human *TNFa* (F) 5′ GTGCTTGTTCCTCAGCCTCTT (R) 5' ATGGGCTACAGGCTTGTCATC using the QS7-Pro 384 qPCR system (Applied Biosystems, ThermoFisher, MA, USA). The expressions of *Fabp4* and inflammatory cytokines were normalized to that of GAPDH/*Gapdh* gene. The relative gene expression was calculated using the $2^{-\Delta CT}$ formula.

### 2.10. Data analysis

Quantitative and semi-quantitative data were presented as mean ± SD and shown in boxplots. The comparison of two independent groups was done by Mann–Whitney U test. All the data analysis was done using the SPSS analysis software (SPSS Inc, 26.0). $p < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Expression of FABP4 in clinical samples of tendinopathy

The expression of FABP4 in healthy hamstring tendons and healthy patellar tendons was weak (Fig. 1A and C). There was no significant difference in the expression of FABP4 between healthy hamstring tendons and healthy patellar tendons ($p > 0.05$) (Fig. 1B). There was significant upregulation of FABP4 in both patellar tendinopathy samples ($p < 0.001$) and rotator cuff tendinopathy samples ($p < 0.01$) compared to their corresponding healthy controls (Fig. 1B and D). FABP4 was mainly expressed in the blood vessels, chondrocyte-like cells, hypercellular area and calcified region (Fig. 1A and C). The expressions of IL-1β and TNF-α increased in human rotator cuff tendinopathy samples and co-localized with the expression of FABP4 (Fig. 2).

### 3.2. Establishment of the mouse CI Achilles tendinopathy animal model

At week 2 after collagenase injection, there was an increase in cell rounding, cellularity, vascularity as well as infiltration of small, nucleated cells (Fig. 3Ai). There was loss of collagen fiber alignment, and hence extracellular matrix degeneration, as shown by polarized microscopy (Fig. 3Ai). At week 8, the infiltration of small, nucleated cells, cellularity and vascularity reduced. Chondrocyte-like cells characterized by large cytoplasmic lacunae and calcified regions were observed in CI tendons at week 8 (Fig. 3Ai). IHC showed expression of Sox9 in the round cells and chondrocyte-like cells in the CI tendons but not in the saline controls (Fig. 3Ai). At week 8, ectopic calcification, which stained positive for Von Kossa stain and surrounded by chondrocyte-like cells, was observed (Fig. 3Ai). This observation was further confirmed by microCT imaging, which showed that all samples (6/6) contained ectopic calcification in tendons after CI tendon injury (Fig. 3Bi). BV was significantly higher in the CI group compared to that in the saline group ($p < 0.05$) (Fig. 3Bii). The histopathological score was significantly higher after collagenase injection compared to saline injection at both week 2 and week 8 post-injection (both $p < 0.001$) (Fig. 3Aii).

### 3.3. Expression of inflammatory cytokines and FABP4 in the mouse CI animal model

Very weak to no expression of FABP4 and inflammatory cytokines were observed in the Achilles tendons at week 2 and week 8 after saline injection (Fig. 4A). The inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-10) in the mouse Achilles tendons increased, with concomitant upregulation in the expression of FABP4 at week 2 after CI tendon injury (Fig. 4A). The inflammatory cytokines and FABP4 were mainly expressed in the hypercellular region, chondrocyte-like cells, and small, nucleated cells (Fig. 4A). The expression levels of FABP4 and other inflammatory cytokines were similar at week 8 after CI tendon injury (Fig. 4A). Strong expression of FABP4 was observed mainly in the calcified region, in addition to the expression in the chondrocyte-like cells and hypercellular region at week 8 (Fig. 4A). There were significant higher expressions of FABP4 (week 2: $p < 0.01$; week 8: $p < 0.01$), IL-1β (week 2: $p < 0.01$; week 8: $p < 0.001$), TNF-α (week 2: $p < 0.05$; week 8: $p < 0.05$), IL-6 (week 2: $p < 0.05$; week 8: $p < 0.05$), and IL-10 (week 2: $p < 0.01$; week 8: $p < 0.001$) in the CI group compared to that in the saline group (Fig. 4B).

### 3.4. FABP4 injection on tendon histopathology and inflammation

A single injection of FABP4 induced cell rounding and loss of collagen fiber alignment in tendons at week 2 and week 8 (Fig. 5Ai). Calcified region and more chondrocyte-like cells were observed at week 8 after FABP4 injection (Fig. 5Ai). Fat accumulation was observed in one sample after FABP4 injection. The histopathological score was significantly higher in FABP4 group compared to the saline group at both week 2 and week 8 (both $p < 0.05$) (Fig. 5Aii). There was time-dependent increase in the histopathological score ($p < 0.01$) (Fig. 5Aii).

Ectopic calcification formed in tendon at week 8 after FABP4 injection (Fig. 5Bi) and was significantly higher in the FABP4 group compared to that in the saline group ($p < 0.05$) (Fig. 5Bii). Very weak to no expressions of IL-1β, TNF-α, IL-6, and IL-10 were observed in the saline groups at week 2 and week 8 post-injection (Fig. 5Ci). The expressions of these inflammatory cytokines increased in the mouse

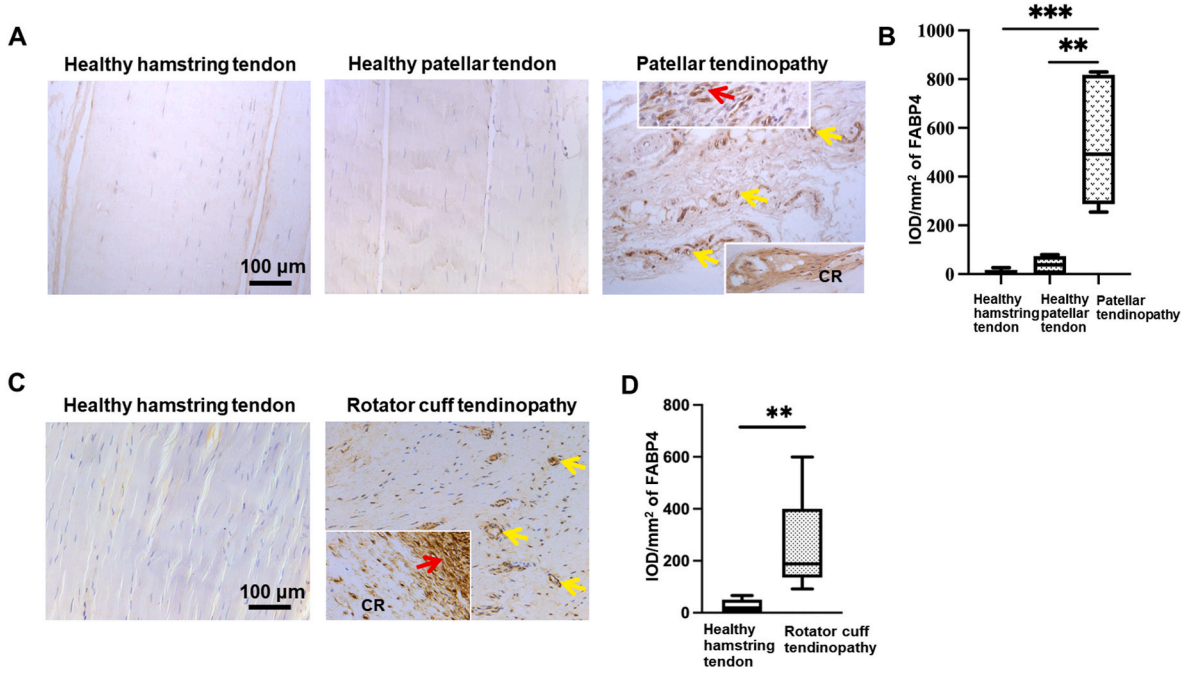


**Fig. 1.** Upregulation of FABP4 in patellar and rotator cuff tendinopathy. (A) Photomicrographs showing the immunohistochemical staining of FABP4 in (i) healthy hamstring tendon samples, (ii) healthy patellar tendon samples, and (ii) patellar tendinopathy samples. Scale bar = 100 μm, yellow arrow: blood vessel; red arrow: hypercellular area; CR: calcified region. (B) Boxplot showing IOD/mm$^2$ of FABP4 signal. n = 6/group; **$p < 0.01$; ***$p < 0.001$ (C) Photomicrographs showing the immunohistochemical staining of FABP4 in healthy human hamstring tendon samples and human rotator cuff tendinopathy samples. Scale bar = 100 μm; yellow arrow: blood vessel; red arrow: hypercellular area; CR: calcified region. (D) Boxplot showing IOD/mm$^2$ of FABP4 signal. n = 5/group; **$p < 0.01$.

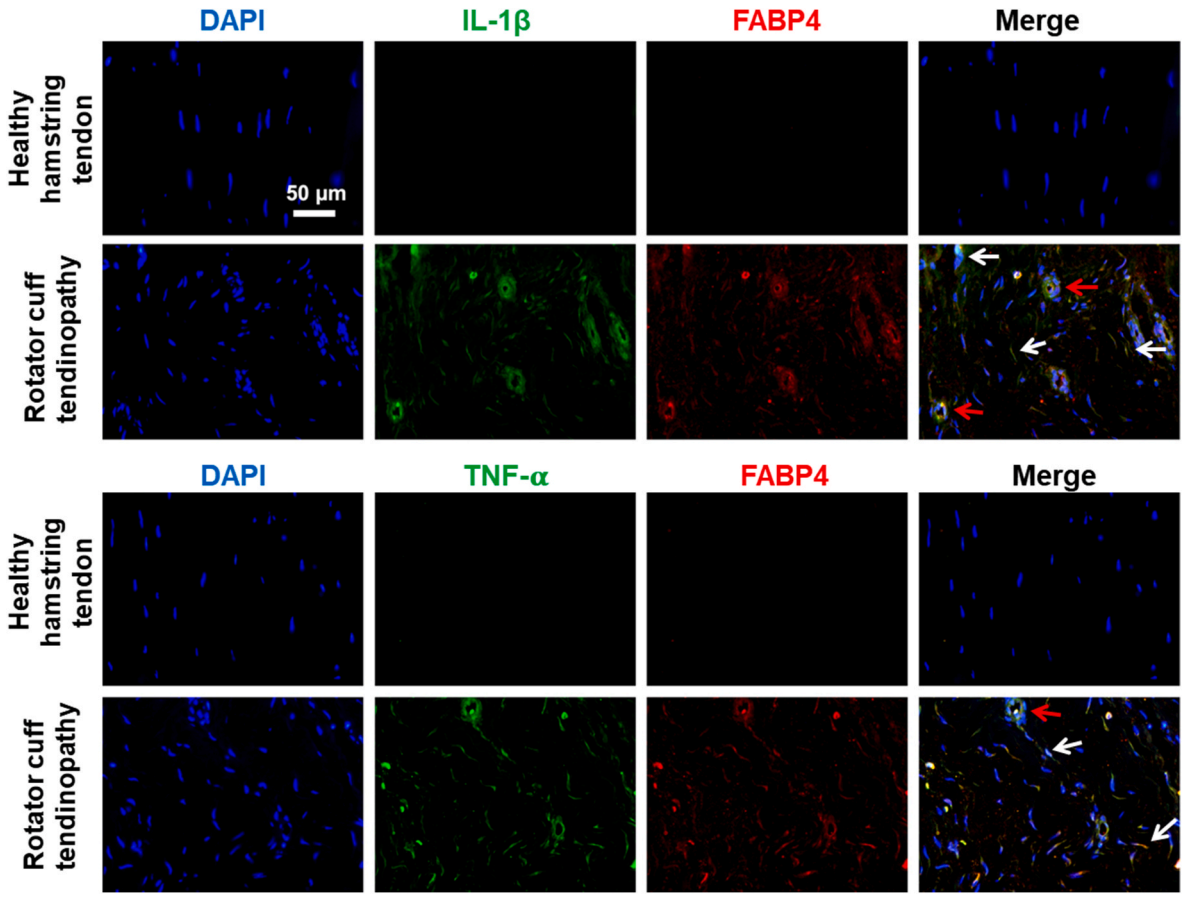


**Fig. 2.** Co-localization of FABP4 with inflammatory cytokines in rotator cuff tendinopathy. Photomicrographs showing co-localization of FABP4 with IL-1β or TNF-α in human rotator cuff tendinopathy samples. Scale bar = 50 μm; n = 5/group; white arrows: co-localized signal in tendon cells; red arrows: co-localized signal in blood vessels.

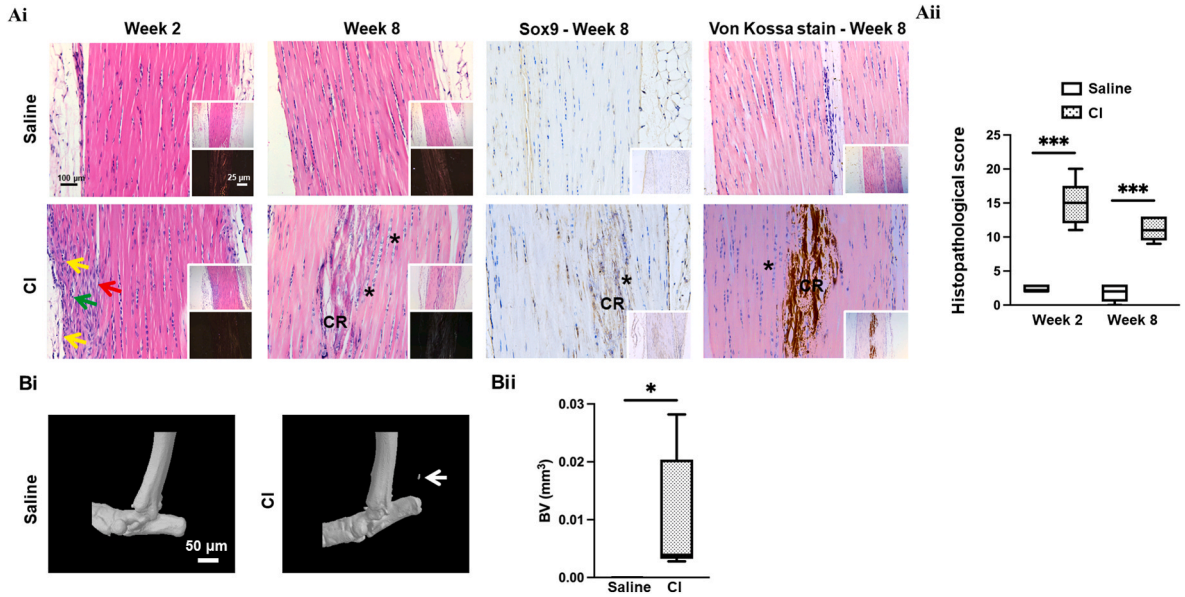


**Fig. 3.** Collagenase injection induced histopathological and radiological changes in mouse Achilles tendons resembling human tendinopathy. (Ai) Photomicrographs showing H&E staining and corresponding polarized images, IHC of Sox9 and Von Kossa staining of representative samples of mouse Achilles tendons at week 2 and 8 after saline or collagenase injection. (Aii) Histopathological scoring of H&E images. n = 5/group; Scale bar = 100 μm (insert 25 μm); yellow arrow: blood vessels; red arrow: hypercellular area; green arrow: small, nucleated cells; CR: calcified region; *: chondrocyte-like cells; black arrow: immunopositive cells; ***$p < 0.001$ (Bi) microCT images showing ectopic calcification inside Achilles tendons at week 8 after saline or collagenase injection. (Bii) Boxplot showing the bone volume (BV) inside tendon in different groups. n = 6/group; Scale bar = 50 μm; white arrow: ectopic calcification; *$p < 0.05$.

Achilles tendons at week 2 and remained high at week 8 post-FABP4 injection (Fig. 5Ci). Semi-quantitative image analysis showed significant higher expression of IL-1β (week 2: $p < 0.001$; week 8: $p < 0.05$), TNF-α (week 2: $p < 0.001$ week 8: $p < 0.05$), IL-6 (week 2: $p < 0.01$; week 8: $p < 0.01$) and IL-10 (week 2: $p < 0.001$; week 8: $p < 0.05$) in the FABP4 group compared to that in the saline group at both week 2 and week 8 post-injection (Fig. 5Cii).

### 3.5. Expressions of inflammatory cytokines in mouse TDSCs after FABP4 treatment

The addition of FABP4 significantly increased the expressions of *IL1b* ($p < 0.001$), *IL6* ($p < 0.01$), and *TNFa* ($p < 0.05$) in human TDSCs as well as the expressions of *Il1b*, *Il6*, and *Il10* in mouse TDSCs (all $p < 0.05$) (Fig. 6).

## 4. Expression of FABP4 in human tendinopathy TDSCs and IL-1β-treated mouse TDSCs

There was higher expression of FABP4 in human rotator cuff tendinopathy TDSCs compared to healthy hamstring tendon TDSCs (Fig. 7A). Treatment of mouse TDSCs with IL-1β increased the protein (Fig. 7B) and mRNA expressions (Fig. 7C) of FABP4. There was a significant higher expression of *Fabp4* in mouse TDSCs after treatment with IL-1β ($p = 0.014$) (Fig. 7C).

## 5. Discussion

Our data showed that FABP4 injection induced histopathological changes, increased the expression of inflammatory cytokines, and triggered ectopic calcification in tendon resembling tendinopathy. Moreover, FABP4 also increased the expressions of inflammatory cytokines in TDSCs. Both results showed that FABP4 is pro-inflammatory. The expression of FABP4 was weak in healthy tendons isolated from different anatomical locations. However, there was an upregulation of FABP4 in both patellar tendinopathy and rotator cuff tendinopathy as well as in the CI animal model of Achilles tendinopathy, supporting the universal involvement of FABP4 in the disease pathogenesis. IL-1β increased the protein and mRNA expressions of FABP4 in mouse TDSCs, suggesting that TDSCs might be a potential source of FABP4 during tendon inflammation. Similar to the results of immunohistochemical staining of FABP4 in rotator cuff tendinopathy tissue, there was higher expression of FABP4 in TDSCs isolated from rotator cuff tendinopathy tissue. FABP4 therefore can be a potential treatment target of tendinopathy.

In a study screening for the molecular pathological changes of Achilles tendons, the mRNA expression of *FABP4* in tendons that were

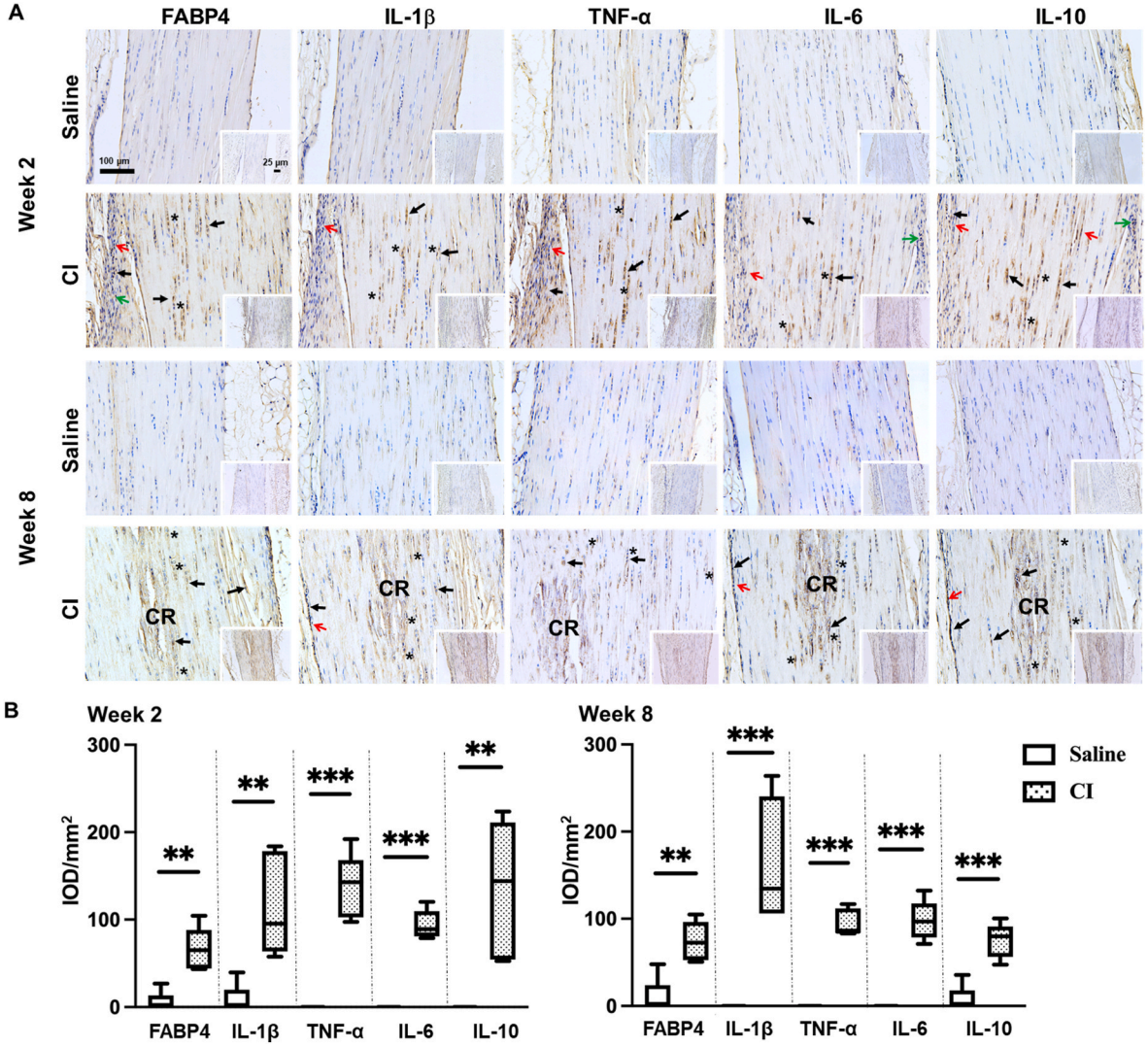


**Fig. 4.** Upregulation of FABP4 and inflammatory cytokines in mouse CI animal model.
Photomicrographs showing the immunohistochemical staining of FABP4, IL-1β, IL-6, TNF-α and IL-10 in representative samples of mouse Achilles tendons at (A) week 2 and week 8 after saline or collagenase injection. (B) Boxplots showing the IOD/mm$^2$ of target markers. n = 5/group; Scale bar = 100 μm (insert 25 μm); red arrow: hypercellular area; green arrow: small, nucleated cells; *: chondrocyte-like cells; CR: calcified region; black arrow: immunopositive cells; **$p < 0.01$; ***$p < 0.001$

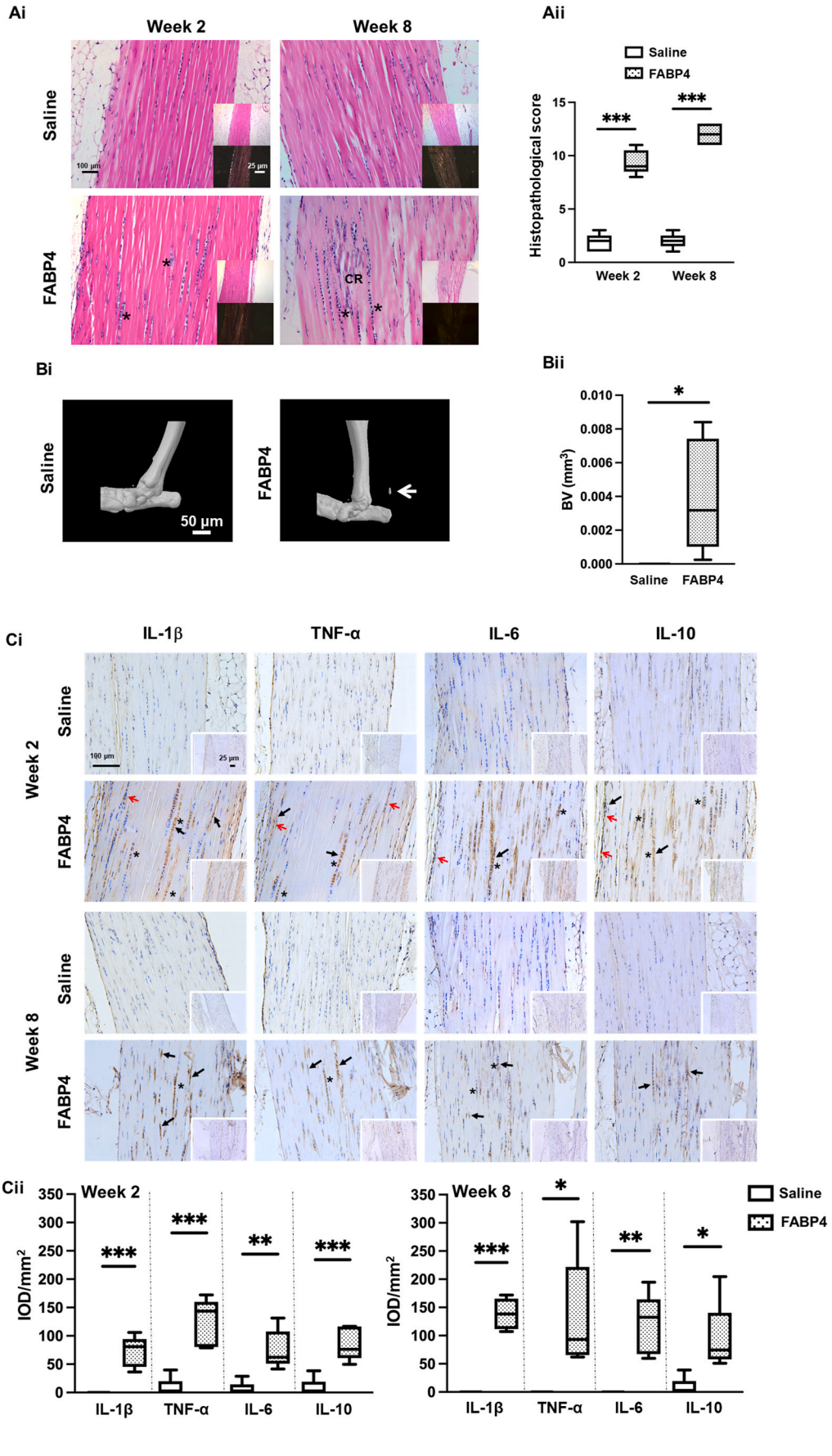


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**Fig. 5.** FABP4 injection induced damages, ectopic bone and inflammation in mouse Achilles tendons resembling human tendinopathy. (Ai) Photomicrographs showing H&E staining and corresponding polarized images of representative samples of mouse Achilles tendons at week 2 and week 8 after saline or FABP4 (0.5 μg) injection. (Aii) Histopathological scoring of H&E images. n = 5/group; Scale bar = 100 μm (insert 25 μm); yellow arrow: blood vessels; red arrow: hypercellular area; green arrow: small, nucleated cells; CR: calcified region; *: chondrocyte-like cells; ***p < 0.001. (Bi) microCT images showing ectopic calcification inside Achilles tendons at week 8 after saline or FABP4 (0.5 μg) injection. (Bii) Boxplot showing the bone volume (BV) inside tendon in different groups. n = 6/group; Scale bar = 50 μm; white arrow: ectopic bone; *p < 0.05. (Ci) Photomicrographs showing the immunohistochemical staining of IL-1β, IL-6, TNF-α and IL-10 in representative samples of mouse Achilles tendons at week 2 and week 8 after saline or FABP4 (0.5 μg) injection. (Cii) Boxplots showing the IOD/mm$^2$ of L-1β, IL-6, TNF-α and IL-10 at week 2 and week 8 after saline or FABP4 (0.5 μg) injection. n=5/group; Scale bar = 100 μm (insert 25 μm); red arrow: hypercellular area; *: chondrocyte-like cells; black arrow: immunopositive cells; *p < 0.05; **p < 0.01; ***p < 0.001.

intact, with tendinopathy, chronic ruptures or acute ruptures were compared. The mRNA expression of *FABP4* was significantly higher in the chronic rupture tendons compared to the acute rupture tendons [13]. However, the mRNA expression of *FABP4* was lower in all the injury groups compared to that in the intact group. Our study was different from the result of Klatte-Schulz et al.'s study [13]. The discrepancy might be due to the assessment of FABP4 at the mRNA and protein levels.

Obesity is a risk factor for the development of tendinopathy. The dysregulation of adipokines induces chronic, low-grade inflammation, which increases the risk of tendinopathy in obese patients [22]. However, the effects of adipokines on tendons are not limited to obese patients. Indeed, adipokines such as IL-1β, TNF-α and IL-6 are also overexpressed in tendinopathy samples of non-obese patients [17,23,24]. In this study, we observed an increased expression of FABP4 in the metabolically healthy rotator cuff tendinopathy samples and animal model of tendinopathy fed with standard chow, suggesting that FABP4 also contributed to the disease pathogenesis independent of obesity.

The expression of FABP4 co-localized with the expressions of IL-1β and TNF-α in the human rotator cuff tendinopathy samples. Similar results were observed in the CI animal model showing the concurrent expressions of FABP4, IL-1β, TNF-α, IL-6 and IL-10. FABP4 is a pro-inflammatory mediator of many inflammatory and metabolic diseases [9]. The circulating level of FABP4 is upregulated in diseases such as obesity, metabolic syndrome, insulin resistance, type 2 diabetes mellitus, ischemic stroke, atherosclerosis, peripheral arterial disease, and kidney diseases [9]. The adverse effects of FABP4 were supported by its upregulation both in vitro and in vivo models of these diseases [10–12]. Moreover, the administration of FABP4 triggered inflammation and damages in cell culture models [10]. We also reported the increased expressions of inflammatory cytokines in human and mouse TDSCs after the addition of FABP4 in this study. Therefore, the increased expression of FABP4 and its co-localization with inflammatory cytokines in this study were likely associated with its harmful pro-inflammatory effects in tendinopathy. Suppression of FABP4 therefore could be a novel strategy for treating tendinopathy. Indeed, pharmacological, or genetic ablation of FABP4 alleviated various inflammatory disorders such as high-fat diet-induced osteoarthritis [21], metabolic syndrome [25], kidney fibrosis [26] and cerebral ischaemia injury [27]. We will examine the effects of genetic and pharmacological inhibition of FABP4 on tendon healing in the CI animal model in future.

Although FABP4 is an adipokine, its production is not limited to adipocytes. FABP4 is also expressed in various cell types such as macrophages, capillary endothelial cells of the heart, skeletal muscles, and renal cells under physiological condition [9,28]. Our results showed that the expression of FABP4 increased in IL-1β-treated mouse TDSCs and in

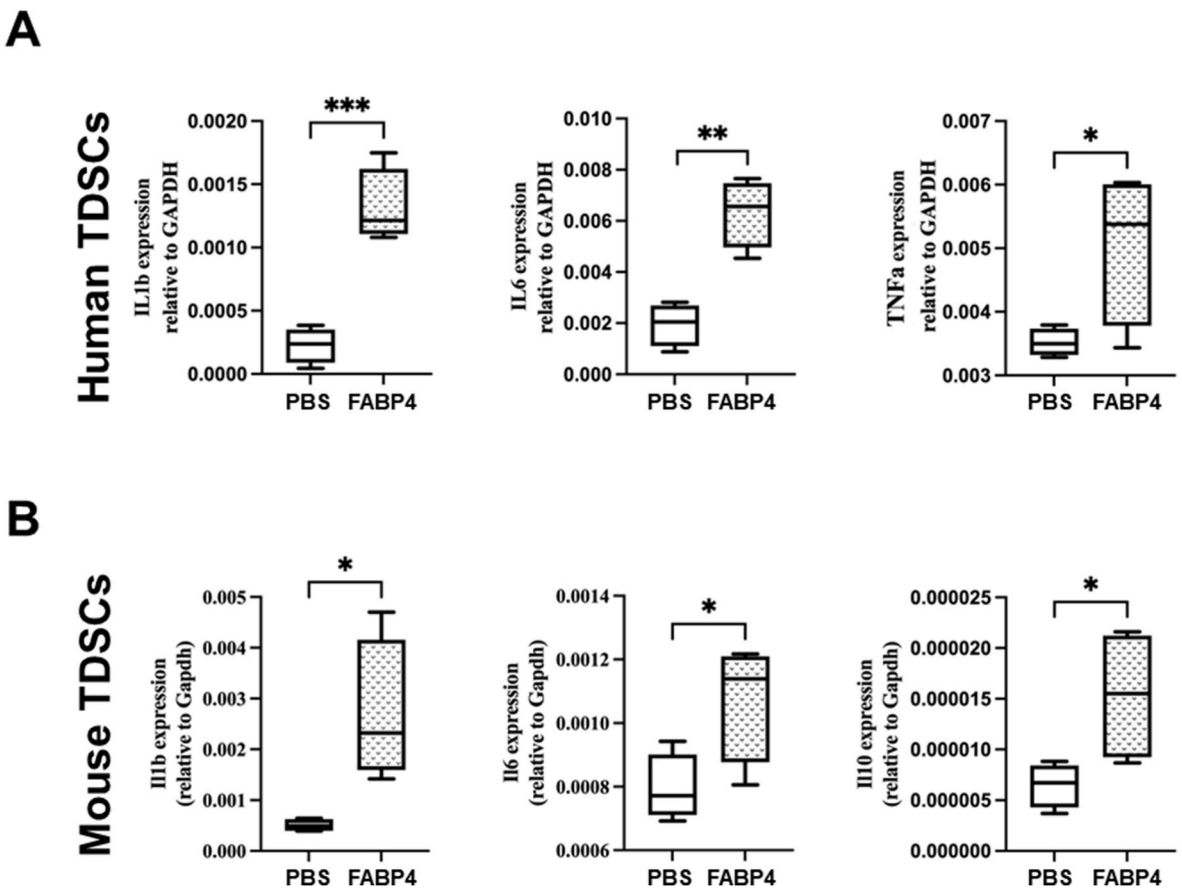


**Fig. 6.** FABP4 increased the mRNA expression of inflammatory cytokines. Boxplots showing (A) the expressions of *IL1b*, *IlL*, and *TNFa* in human TDSCs and; (B) the expressions of *Il1b*, *Il6*, and *Il10* in mouse TDSCs after the addition of FABP4 (120 ng/mL) for 24 h *p < 0.05; **p < 0.01; ***p < 0.001; n = 4/group.

human TDSCs isolated from the inflammatory and degenerative tendinopathy tissues, suggesting that TDSCs are a potential source of FABP4 under inflammation. Further study about the physiological and pathological roles of FABP4 on TDSCs will be our future research direction.

There is not enough evidence to show if FABP4 functions upstream or downstream of IL-1β in TDSCs. On one hand, IL-1β increased the expression of FABP4 in TDSCs. On the other hand, a single injection of FABP4 also increased the expression of IL-1β in the mouse Achilles tendon, and the addition of FABP4 increased the expressions of inflammatory cytokines in human and mouse TDSCs. They may share some common signaling mediators. Further research is needed to understand the cross-talks between FABP4 and IL-β1.

We observed an expression of FABP4 in the osteo-chondrogenic areas in the animal model and clinical samples of tendinopathy. Injection of FABP4 also induced fat accumulation (observed in one out of five samples) and ectopic calcification in all samples in the mouse Achilles tendon, supporting the potential role of FABP4 in the erroneous differentiation of resident TDSCs and tissue metaplasia. FABP4 is an adipogenic differentiation marker and inducer. A previous study has shown that ectopic expression of FABP4 gene induced adipogenesis of bovine muscle-derived stem cells [29]. There has been no study investigating the effects of FABP4 on the osteo-chondrogenic differentiation of stem/progenitor cells. The potential role of FABP4 in the erroneous differentiation of TDSCs requires further studies.

We also observed an expression of FABP4 in the vascular features in clinical samples of tendinopathy, suggesting that FABP4 might be produced by the capillary endothelial cells and regulate angiogenesis in tendinopathy. FABP4 has a pro-angiogenic role. It has been reported to stimulate proliferation, differentiation, and tube formation of trophoplastic cells in vitro [30] as well as increase the proliferation of hemangioma endothelial cells [31]. Treatment of endothelial cells with VEGF-A induced FABP4 expression [28].

We also reported an expression of FABP4 in the hypercellular area. FABP4 has been reported to increase the proliferation of pancreatic cancer cells [32]. Treatment of human coronary smooth muscle cells or human coronary artery endothelial cells (HCAECs) with FABP4 significantly increased the gene expression of inflammatory cytokines and proliferation- and adhesion-related molecules in cells, promoted the proliferation and migration of human coronary smooth muscle cells, and decreased the phosphorylation of nitric oxide synthase 3 in HCAECs [10]. Moreover, there was an upregulation of FABP4 in the cervical cancer tissues of patients compared to the normal cervical epithelia, and the proliferation, migration and invasion of cervical cancer cells were significantly inhibited after knocking down of FABP4 [33]. On the other hand, an increased expression of FABP4 in endometrial cell lines inhibited the proliferation, migration, and invasion in vitro and suppressed tumor growth in vivo [34]. The proliferative effects of FABP4 on cancer cells therefore varied with cancer types. The effect of FABP4 on the proliferation of tendon cells requires further research.

We selected TDSCs for the examination of the source of FABP4 because tenocytes and TDSCs are two major cell types in tendon. This was because tenocytes produce tendon matrix while the resident TDSCs maintain tendon homeostasis during growth and repair by cell proliferation and tenocyte differentiation [35]. Recent studies have shown that erroneous differentiation of TDSCs after overuse-induced inflammation is the key mediator in the pathogenesis of tendinopathy [36–38]. In our study, the expression of FABP4 was observed in various cell types including rounded cells, chondrocyte-like cells and calcified region in human tendinopathy and mouse Achilles tendon after collagenase injection (CI). The chondrocyte-like cells and calcified region were likely due to erroneous differentiation of TDSCs and the expression of FABP4 in these cell types suggested that it might play a role in the erroneous TDSC differentiation.

Our study is novel as the classical anti-inflammatory drugs such as

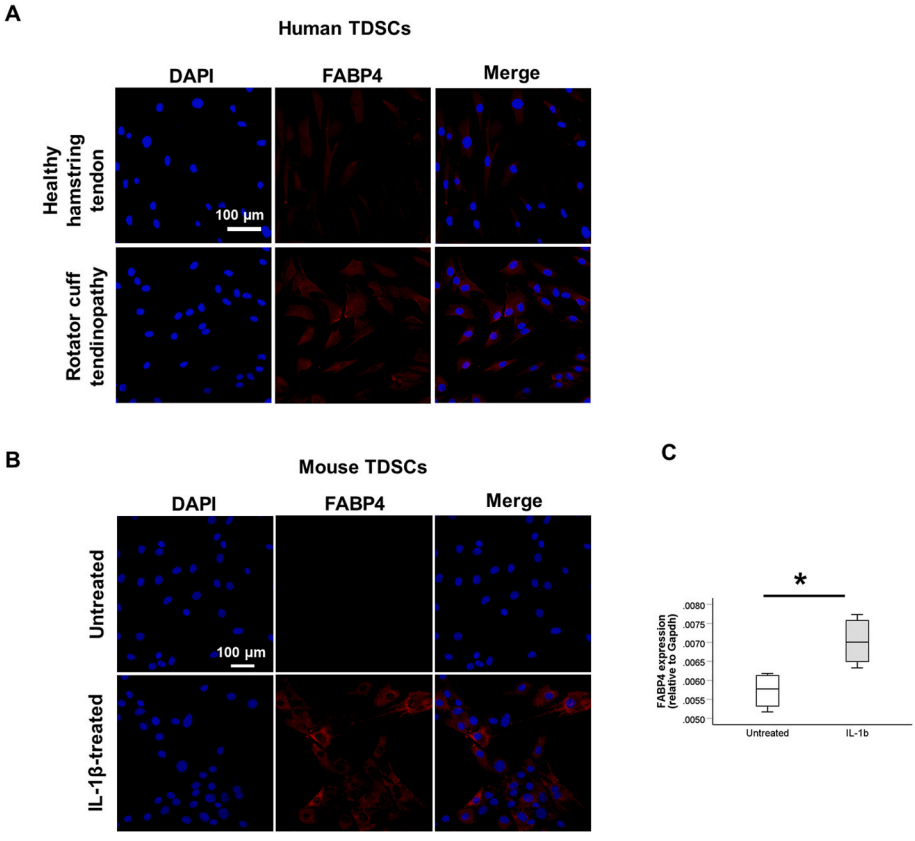


**Fig. 7.** Expression of FABP4 in human rotator cuff tendinopathy TDSCs and IL-1β-treated mouse TDSCs. (A) Photomicrographs showing protein expression of FABP4 in human rotator cuff tendinopathy TDSCs compared to healthy hamstring tendon TDSCs; Scale bar: 100 μm; n = 4. The cell nucleus was stained by DAPI. (B) Photomicrographs showing protein expression of FABP4 in mouse TDSCs treated with IL-1β (10 ng/mL) for 48 h; Scale bar: 100 μm; n = 6. The cell nucleus was stained by DAPI. (C) Boxplot showing mRNA expression of *Fabp4*. *p < 0.05; n = 4–5/group.

non-steroid anti-inflammatory drugs (NSAIDs) and steroids are not useful for treating tendinopathy. Although they are good immunosuppressive agents, they have detrimental effects on tendon cell survival, metabolism, senescence, and tendon healing [39–43]. Dexamethasone was reported to promote aberrant differentiation of TDSCs [44,45], and diclofenac and triamcinolone acetonide were shown to impair tenocyte differentiation of a fibroblast cell line [46]. A randomized controlled trial has shown that 1-week treatment with ibuprofen did not affect the gene expressions of collagens and TGF-β isoforms in human chronic tendinopathic tendons, tendon pain and function in patients [47]. Moreover, glucocorticoid use has been reported to increase the risk of tendon rupture [48]. The beneficial benefits of NSAIDs and steroids in pain relief, if any, were usually short-term [49]. Similarly, IL-1ra, an inhibitor of IL-1β, could not fully rescue the downregulation of mRNA expression of tenogenic markers and matrix metalloproteinases (MMPs) in equine tenocytes treated with IL-1β, and has no significant effects in reversing the adverse effects of IL-1β, TNF-α and IFN-γ in combination on the expression of tenogenic markers and MMPs [50]. IL-1ra injection in patients with chronic Achilles tendinopathy was reported to demonstrate little beneficial effects, with no significant reduction in pain or intratendinous blood flow, and the tendon thickness increased after treatment for 12 months [51]. Injection of IL-1ra to the carrageenan-induced rat patellar tendinopathy model improved histology but not mechanical properties of tendon [52]. Therefore, the existing anti-inflammatory drugs may not serve the therapeutic purpose. There is a need to find an intervention that both suppress tendon inflammation and promote tenogenic differentiation of TDSCs for treating tendinopathy. As the pro-inflammatory FABP4 affected stem cell differentiation in other tissues as stated above, inhibition of FABP4 may have the dual effects of suppressing inflammation and erroneous TDSC differentiation. Further research is required to confirm our hypothesis. In summary, this study is novel as we reported the role of a new pro-inflammatory adipokine, FABP4, in the pathogenesis of tendinopathy. Further research on the roles of FABP4 and its interaction with other inflammatory cytokines in tendon may identify a novel target for treating tendinopathy. However, this study is not without limitations. First, the case and control tendon samples used were isolated from different locations. It is unethical to get healthy rotator cuff tendons or Achilles tendons from human subjects. Hamstring tendon was used as the healthy controls for the rotator cuff tendinopathy group in this study because it is the common graft type during ACLR and can be obtained from the clinical waste. Our IHC results based on BPTB graft samples showed low expression of FABP4 in healthy patellar tendons, similar to the expression in the healthy hamstring tendons, supporting the upregulation of FABP4 in tendinopathy. Second, we haven't collected the metabolic information of patients with patellar tendinopathy and the corresponding hamstring and patellar tendon controls. It is possible that the patients have metabolic diseases that might affect the expression of FABP4. However, we observed very low expression of FABP4 in the hamstring control group and patellar tendon control group, and the patients in the three groups are relatively young that the chance of having metabolic disorders was relatively low compared to the aged patients. Third, the dose of FABP4 used in the mouse study was based on the concentration reported in the literature and our pilot study and might be different from the FABP4 concentration observed in the animal model and clinical samples of tendinopathy. Tendinopathy is a heterogeneous tendon disorder. The exact pathological and clinical presentation depends on a multitude of factors and hence the concentrations of FABP4 and inflammatory cytokines in the tissues might depend on the disease stages and the risk factors. In this study, we observed similar histopathological changes in the mouse Achilles tendons after injection of FABP4 compared to the tendon damages after collagenase injection in the mouse tendons and human tendinopathy, supporting the clinical relevance of the FABP4 concentration that we used in this study. Fourth, we didn't examine the pain-associated changes of gait pattern in the animals and mechanical properties of tendon after FABP4 injection. These experiments should be done in future. Finally, while we have reported TDSCs as one cell source of FABP4, we haven't examined other sources of FABP4 in tendons and the molecular mechanisms of FABP4 in the disease pathogenesis, which should be examined in future studies.

## 6. Conclusion

In conclusion, an upregulation of FABP4 is involved in excessive inflammation and pathogenesis of tendinopathy. TDSCs is a potential source of FABP4 during tendon inflammation.

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## Data availability statement

All data generated in this study are included in the manuscript.

## Institutional review Board Statement

The clinical study complies with the Declaration of Helsinki. The clinical research ethics committee of the authors' institution has approved the research protocol (Ref no. 2018.109).

The animal study was approved by the animal research ethics committee of the Animal Experimentation Ethics Committee (Ref no. 22-287-GRF) and the University Laboratory Safety Office of the Chinese University of Hong Kong.

## Informed consent Statement

Included.

## CRediT authorship contribution statement

**Zebin Ma:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing, Final approval. **Angel Yuk Wa Lee:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing, Final approval. **Cheuk Hin Kot:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing, Final approval. **Patrick Shu Hang Yung:** Investigation, Writing – review & editing, Final approval. **Ssu-chi Chen:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing, Final approval. **Pauline Po Yee Lui:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Final approval.

## Declaration of competing interest

We don't any potential conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2024.06.007.

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