https://doi.org/10.1016/j.joca.2018.03.002

Knocking out or pharmaceutical inhibition of fatty acid binding protein 4 (FABP4) alleviates osteoarthritis induced by high-fat diet in mice

C.Zhang†‡K.Y.Chiu†B.P.M.Chan†T.Li†C.Wen§A.Xu||¶#C.H.Yan†††

†
Department of Orthopaedics & Traumatology, The University of Hong Kong, Hong Kong Special Administrative Region

‡
Department of Orthopedics, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China

§
Department of Biomedical Engineering, Faculty of Engineering, Hong Kong Polytechnic University, Hong Kong Special Administrative Region

||
State Key Laboratory of Pharmaceutical Biotechnology, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region

¶
Department of Medicine, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region

#
Department of Pharmacology and Pharmacy, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region

††
Shenzhen Key Laboratory for Innovative Technology in Orthopaedic Trauma, Department of Orthopaedics and Traumatology, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China

Summary
Objectives
Adipokines play roles in the pathogenesis of osteoarthritis (OA). Fatty acid binding protein 4 (FABP4) is a novel adipokine that is closely associated with obesity and metabolic diseases. The aim of this study was to discover the potential role of FABP4 in OA.

Methods
Seventy-two FABP4 knockout mice (KO) in C57BL/6N background and wild-type littermates (WT) (male, 6-week-old) were fed with a high-fat diet (HFD, 60% calorie) or standard diet (STD, 11.6% calorie) for 3 months, 6 months and 9 months (n = 6 each). In the parallel study, forty-eight 6-week-old male WT mice were fed with HFD or STD, and simultaneously treated with daily oral gavage of selective FABP4 inhibitor BMS309403 (15 mg/kg/d) or vehicle for 4 months and 6 months (n = 6 each). Serum FABP4 and cartilage oligomeric matrix protein (COMP) concentration was quantified. Histological assessment of knee OA and micro-CT analysis of subchondral bone were performed.

Results
HFD induced obesity in mice. After 3 months and 6 months of HFD, KO mice showed alleviated cartilage degradation and synovitis, with significantly lower COMP, modified Mankin OA score, and MMP-13/ADAMTS4 expression. After 6 months and 9 months of HFD, KO mice showed less osteophyte formation and subchondral bone sclerosis. Chronic treatment of BMS309403 for 4 months and 6 months significantly alleviated cartilage degradation, but had no effects on the subchondral bone. Knocking out or pharmaceutical inhibition of FABP4 did not have significant effects on lean mice fed with STD.

Conclusions
Knocking out or pharmaceutical inhibition of FABP4 alleviates OA induced by HFD in mice.

Keywords
OsteoarthritisFatty acid binding protein 4BMS309403ObesitySubchondral bone sclerosis

Introduction

Osteoarthritis (OA) is a debilitating joint disease that affects millions of individuals, and is estimated to be the fourth leading cause for disability by 2020 worldwide1. OA is characterized structurally by articular cartilage degradation and remodeling of the underlying subchondral bone, and is accompanied by joint inflammation, chronic pain and functional loss2. Current treatments are mainly symptom-modifying which focus on pain relieving and function improving. A better understanding of the cause of the disorder is critical towards developing prevention and treatment strategies.

OA is a multifactorial disease. Obesity is one of the modifiable risk factors for OA, and was conventionally believed to cause OA via the increased mechanical loading on weight-bearing joints3. However, recent studies have shown that obesity also contributes to joint degeneration by producing and releasing a plethora of factors termed adipokines4. Leptin, adiponectin, resistin and visfatin have been reported to induce expression of inflammatory cytokines in chondrocyte, including inducible nitric oxide synthase (iNOS) and prostaglandin E2 (PGE2)5, 6, 7, which lead to degradation of extracellular matrix (ECM).

Fatty acid binding protein 4 (FABP4) is an adipokine that is mainly expressed in adipocytes and macrophages8. The function of FABP4 is to act as a lipid chaperon to facilitate the transportation of lipids to specific compartments in the cell8. However, high level of FABP4 was found to be closely associated with obesity and metabolic diseases, including type 2 diabetes, atherosclerosis, or coronary heart disease9, 10, 11, 12, 13. In animals, the use of a FABP4 selective oral inhibitor (BMS309403) could significantly decrease the risk of atherosclerosis14.

Given all these facts, it poses possibility that FABP4, as an adipokine, may play roles in linking obesity to OA. However, to our knowledge, no study has examined the role of FABP4 in OA. The purpose of this study was to explore the potential role of FABP4 in OA, and to investigate if BMS309403 could be a potential drug therapy for OA. Briefly, we induced obesity in FABP4-deficient mice (KO) and their wild-type littermates (WT) with high-fat diet (HFD) for 3 months, 6 months, and 9 months. We assessed knee OA change including cartilage degradation, synovitis and subchondral bone remodeling. In the parallel study, we treated the WT mice with daily oral gavage of BMS309403 for 4 months and 6 months, to examine the potential effects of pharmaceutical inhibition of FABP4 on knee OA.

Materials and methods

Animals

All experimental protocols were approved by the local Ethics Committee (No: 3386-14). FABP4 knockout (KO) mice in C57BL/6N background were generated as previously described15. knockout mice (KO) and their littermates (WT) mice (All males, ages 6 weeks) were used in all the experiments. Mice were housed in a temperature-controlled facility (23 ± 1°C, 12-h light/dark cycle, 60–70% humidity) with ad libitum access to water and diet.

Two parallel studies were performed. In the first study, weight-matched KO and WT mice (n = 36 each, 18.0 g) were included. Animals were randomly allocated to two diet groups (n = 18 each): a HFD group (60% kcal from fat, 20% from carbohydrate, and 20% from protein. D12492, Research Diet, NJ, USA) and standard-diet group (STD, 11.6% kcal from fat, 52.9%

from carbohydrate, and 20% from protein. 5053, LabDiet, Brentwood, MO, USA). In each group, diet started from 6-week-old and sustained for 3 months, 6 months, and 9 months (n = 6 each).

In the parallel study, forty-eight weight-matched WT mice were enrolled, and randomly allocated to HFD or STD (n = 24 each, 18.0 g). Simultaneously, animals were treated with daily oral gavage of BMS309403 (15 mg/kg/d dissolved in PBS solution, Chemrenblock Technology, Jiangsu, China) or equivalent PBS solution (n = 12 each). In each group, treatment sustained for 4 months and 6 months (n = 6 each). The dosage, preparation, and administration of BMS309403 were referred to previous reports14, 16. Potential side effects of BMS309403 were strictly monitored during the whole study period.

Body composition measurement

At each time point, animals' body weight was measured, and body composition was determined in a nuclear magnetic resonance machine (Bruker, minispec, Germany). Body fat percentage was calculated as the fat mass divided by the body weight.

Quantification of serum FABP4 and cartilage oligomeric matrix protein (COMP)

Animals were anaesthetized and 1 ml of blood was collected by cardiac puncture. The blood was allowed to clot for 1 h at room temperature and centrifuged in 4°C for 10 min at 2,500 rpm. The supernatant (serum) were harvested and stored in aliquots at −80°C. Serum FABP4 and COMP was quantified in enzyme-linked immunosorbent assay (ELISA) kits (FABP4: RD291036200R, Biovendor, Czech Republic; COMP: AN-14-2004-86, IDS, UK).

Micro-CT analysis

Immediately after animals were euthanized, the bilateral knee joints were harvested and fixed in 10% neutral buffered formalin (NBF). The knees were scanned in a high resolution micro-CT system (1076, SkyScan, Kontich, Belgium) and 3D reconstructions were acquired as previously described17, 18. The data sets were then opened in DataViewer (version 1.4.4.0, SkyScan) and reoriented to analyze the sample in the coronal plane. After the selection of the volume of interest (VOIs) and regions of interest (ROIs), binarization of the images with a global thresholding of gray levels (75–255) was done. The calculation of morphological parameters was carried out in the CTAn software (version 1.13.2.1, SkyScan).

The subchondral bone of tibia plateau and femur condyles were manually selected for analysis. Measurements were done in four quadrants: medial femoral condyle (MFC), medial tibial plateau (MTP), lateral femoral condyle (LFC), and lateral tibial plateau (LTP). The ROI were achieved manually slice by slice. The bone mineral density (BMD, g/cm3), bone volume percentage (BV/TV), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, mm), and total porosity (%) were calculated. The BMD was calibrated by using the attenuation coefficient of two hydroxyapatite phantoms with defined mineral density of 0.25 and 0.75 g/cm3. The mean value of MFC, MTP, LFC, and LTP was determined, and the average value of the left and right knee was used for analysis.

Histological analysis

The specimen processing for histological analysis was performed as previously described17. Briefly, after fixation in 10% NBF for 24 h, the knee joints were rinsed thoroughly with tap water and immersed in 10% EDTA-Na (pH 7.4) for decalcification. Fresh solution was changed per 3 days for a total of 6 weeks, and confirmed by X-ray test. Tissues were embedded in paraffin and sectioned into 5 μm-thick slides. The slides were stained with Safranin O and Fast Green, and observed in a Nikon Eclipse 80i microscope (Nikon, Japan).

OA changes were assessed using the modified Mankin OA Scoring System for the following categories: changes in articular cartilage structure (score of 0–11), Safranin O staining (score of 0–8), tidemark duplication (score of 0–3) and hypertrophic chondrocytes (score of 0–2), for a maximum score of 2419, 20. The MFC, MTP, LFC, and LTP were assessed respectively, and the total OA score was averaged (Maximum 24). The area of osteophytes was measured by Image J (NIH, USA). For all the measurements, one in five sections with 60 μm of interval was assessed, and the mean value was calculated and averaged between left and right knee.

Immunohistochemistry staining (IHC)

IHC staining for MMP-13, ADAMTS4, CD68, IL6, and Caspase-3 were performed as previously described17. Briefly, the rehydrated sections underwent antigen retrieval in 20 μg/ml proteinase K (3115879001, Roche Life Science), and were blocked in peroxidase-blocking solution (S2023, Dako, Denmark) and 5% BSA (A5253, Sigma, MO, USA), followed by incubation with primary antibodies against MMP-13 (1:200, ab39012, abcam, Cambridge, MA), ADAMTS4 (1:100, ab185722, abcam, Cambridge, MA), CD68 (1:100, ab125212, abcam, Cambridge, MA), IL6 (1:50, ab7737, abcam, Cambridge, MA), Caspase-3 (1:100, 9662s, Cell Signaling Technology, Danvers, MA), and FABP4 (1: 500, AF1443, R&D systems, Minneapolis, MN) overnight at 4°C. The next day, slides were washed in PBS solution for three times and incubated with second antibody (K3467, Dako, Denmark) for 20 min. Color was developed using the Dako REAL EnVision Detection system (K3467, Dako, Denmark), and counterstained with hematoxylin. For FABP4, immunofluorescence staining was done, with donkey anti-goat second antibody (1:500, ab150129, abcam, Cambridge, MA) incubated for 1 h, then counter-stained with 4′,6-Diamidino-2-Phenylindole Dihydrochloride (DAPI). Digital images at 400 magnification were captured. The number of chondrocyte with positive MMP-13 and ADAMTS4 staining was measured in Image J. The percentage of positive cells, defined as the ratio of the number of positive cells to the total number of chondrocyte, was calculated as previously described21.

Synovitis scoring

Degree of synovitis was scored using a published synovitis scoring system22 that measured the enlargement of the synovial lining cell layer on a scale of 0–3 (0 = 1–2 cells, 1 = 2–4 cells, 2 = 4–9 cells and 3 = 10 or more cells) and cellular density in the synovial stroma on a scale of 0–3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity and 3 = greatly increased cellularity). Synovitis scores obtained from all four quadrants (medial tibia, medial femur, lateral tibia, and lateral femur) for both of the above parameters were averaged separately and then the sum of averages from both parameters was used for analysis (on a scale of 0–6).

Cell culture and treatment

The ATDC-5 murine chondrogenic cell line was cultured in 6-well plates in DMEM/Ham's F-12 medium (D2906, Sigma, MO, USA) supplemented with 10% fetal bovine serum (FBS). At 90% confluence, cell underwent 12-h serum starvation, and was then stimulated with serial concentrations of murine FABP-4 (0, 4.8, 24, 120, and 600 ng/ml, prepared as previously described23) for 24 h. Catabolic markers including MMP-3, ADAMTS4, and iNOS were analyzed by quantitative real-time PCR and Western Blot tests. The primer pairs used were (5′-3′): forward GTTGGAGAACATGGAGACTTTGT and reverse GGCTGAGTGGTAGAGTCCCA for MMP-3, forward CACTGACTTCCTGGACAATGGTTAT and reverse CACTGACTTCCTGGACAATGGTTAT for ADAMTS4, forward CTCACTGGGACAGCACAGAA and reverse TGGTCAAACTCTTGGGGTTC for iNOS. For western blotting, antibodies against MMP-3 (1:500, ab53915, abcam, Cambridge, MA), ADAMTS4 (1:500, ab185722, abcam, Cambridge, MA), iNOS (1:1000, ab178945, abcam, Cambridge, MA), and GAPDH (1:10,000, ab178945, abcam, Cambridge, MA) were used. The signals were detected using the MyECL Imager (Thermo Scientific).

Statistical analysis

Imaging analyses were evaluated in a blinded manner. Sections were randomly assigned identification numbers, and two experienced investigators respectively assessed the slides. Data was expressed as mean [95% confidence interval (CI)], and the error bars of all figures represent 95% CI. Data was analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons if two independent variables existed (diet & FABP4 KO or diet & FABP4 inhibitor), otherwise student's ttest was used. P value less than 0.05 was considered significant. Analyses were performed in SPSS 23.0 software (IBM, USA).

Results

1.

Knocking out or pharmaceutical inhibition of FABP4 significantly increased body weight and body fat percentage in animals fed with HFD

No animals died during the whole study period, nor were significant side effects of BMS309403 observed. Feeding HFD induced obesity in both the KO and WT mice, however, the KO mice showed significantly higher body weight than the WT mice after 3 months and 6 months of HFD. After 9 months, there was no significant difference [Fig. 1(A)]. In lean mice, body weight increased significantly after 6 months of STD ($P < 0.0001$), but remained unchanged after 9 months of STD ($P > 0.9999$). Such change was not significantly different between the KO and WT mice ($P = 0.0559$). Daily oral gavage of BMS309403 (15 mg/kg/d) for 4 months and 6 months also significantly increased animals' body weight under HFD [Fig. 1(B)]. Change in percentage of body fat was consistent to body weight. The KO mice showed significantly higher body fat percentage than the WT mice under HFD, at all time points observed [Fig. 1(C)]. Treatment of BMS309403 significantly increased animal's body fat percentage but only after 6 months of HFD [Fig. 1(D)].

Download : Download high-res image (468KB)Download : Download full-size image

Fig. 1. Knocking out or pharmaceutical inhibition of FABP4 significantly increased body weight and body fat percentage in mice fed with HFD. (A) Body weight of KO/WT mice fed

with HFD/STD for 3 months, 6 months, and 9 months. (B) Body weight of mice fed with HFD/STD and simultaneously treated with daily oral gavage of BMS309403 (15 mg/kg/d)/equivalent PBS for 4 months and 6 months. (C) Body fat percentage of KO/WT mice fed with HFD/STD for 3 months, 6 months, and 9 months. (D) Body fat percentage of mice fed with HFD/STD and treated with BMS309403/PBS for 4 months and 6 months. (E) Serum FABP4 concentration of KO/WT mice fed with HFD or STD for 3, 6 and 9 months. Serum FABP4 increased significantly with the duration of HFD in WT mice ($P < 0.0001$). (F) Serum FABP4 concentration of mice treated with HFD/STD and BMS309403/PBS for 4 months and 6 months. (G) Representative images of immunofluorescence staining of FABP4 in joint tissues of KO/WT mice after 6-month of HFD, and in mice treated with BMS309403/PBS. KO mice showed no positive staining whereas WT mice showed strong staining. The density was decreased in mice treated with BMS309403. (Bars are represented as mean ± 95%CI. There were six mice per group. Two-way ANOVA followed by multiple comparisons with Bonferroni's post hoc test was performed. Magnification = 400× and scale bar = 50 μm in G.

The serum FABP4 concentration was undetectable in all KO mice. In WT mice feeding with HFD, the FABP4 concentration significantly increased with the duration of diet [$P < 0.0001$, Fig. 1(E)]. Treatment of BMS309403 significantly decreased serum FABP4 concentration in mice feeding HFD, but had no effects on lean mice feeding STD [Fig. 1(F)]. Immunofluorescence staining of FABP4 in the cartilage of animals after 6-month of HFD showed no positive staining in KO mice whereas strong staining was observed in WT mice. The density was decreased in mice treated with BMS309403 [Fig. 1(G)].

2.

Knocking out or pharmaceutical inhibition of FABP4 significantly alleviated cartilage degradation at early stage of OA in animals fed with HFD

The coronal paraffin sections of knees were stained with Safranin O and Fast Green. Loss of Safranin O staining in cartilage indicated loss of glycosaminoglycan (GAG) content. After 3-month of HFD, staining loss of cartilage was observed in WT mice but not seen in KO mice [Fig. 2(A), (D)]. After 6-month of HFD, WT mice had serious staining deficiency while KO mice just started to undergo cartilage degradation [Fig. 2(B), (E)]. After 9-month of HFD, both groups experienced severe cartilage degradation with similar staining density observed [Fig. 3(C), (F)]. The KO mice had significantly lower serum COMP concentration than the WT mice after 3-month of HFD [Fig. 2(U)], had lower modified Mankin OA score after 3-month and 6-month of HFD [Fig. 2(V)], and lower osteophyte area after 6-month and 9-month of HFD [Fig. 2(W)]. In addition to cartilage degradation, significant subchondral bone sclerosis and osteophyte formation was noted in the WT mice after 6-month and 9-month of HFD [Fig. 2(B) and (C)]. However, animals with STD showed mild OA change, with no significant differences between KO and WT mice observed [Fig. 2(G)–(L)].

Download : Download high-res image (2MB)Download : Download full-size image

Fig. 2. Knocking out or pharmaceutical inhibition of FABP4 significantly alleviated cartilage degradation at early stage of OA in animals fed with HFD. The coronal sections of knees were stained with Safranin O and Fast Green. (A–F) Representative images of KO/WT mice

fed with HFD for 3 months, 6 months, and 9 months. After 3-month of HFD, staining loss of cartilage was observed in WT mice (arrow) but not seen in KO mice. After 6-month of HFD, WT mice had serious staining deficiency while KO mice had mild cartilage degradation. After 9-month of HFD, staining density was similar between WT and KO, but WT mice had significant osteophytes formation and subchondral bone sclerosis (arrow). (G–L) KO/WT mice fed with STD for 3 months, 6 months, and 9 months. No significant differences of OA changes were seen between KO and WT. (M–P) Representative images of mice fed with HFD and simultaneously treated with daily oral gavage of BMS309403 or PBS for 4 months and 6 months. Mice treated with BMS309403 had alleviated staining loss in the cartilage than the mice treated with PBS. (M–P) Representative images of mice fed with STD. There were no significant differences of OA changes between BMS309403 and PBS groups. (U) Serum COMP concentration of KO/WT mice fed with HFD or STD for 3 months, 6 months, and 9 months. (V) Modified Mankin OA Score of KO/WT mice fed with HFD or STD for 3 months, 6 months, and 9 months. (W) Osteophytes area of KO/WT mice fed with HFD or STD for 3 months, 6 months, and 9 months. (X) Serum COMP concentration of mice fed with HFD or STD and treated with BMS309403/PBS for 4 months and 6 months. (Y) Modified Mankin OA Score of mice fed with HFD or STD and treated with BMS309403/PBS for 4 months and 6 months. (Z) Osteophytes area of mice fed with HFD or STD and treated with BMS309403/PBS for 4 months and 6 months (Bars are represented as mean ± 95%CI. There were six mice per group. For modified mankin OA scoring in mice treated with BMS309403/PBS for 6 months, there were four mice per group. Two-way ANOVA followed by multiple comparisons with Bonferroni's post hoc test was performed. Magnification: 40×, Scale bar: 500 μm).

Download : Download high-res image (2MB)Download : Download full-size image
Fig. 3. Knocking out or pharmaceutical inhibition of FABP4 significantly alleviated cartilage degradation at early stage of OA in animals fed with HFD. Knee sections were performed with IHC staining for MMP-13, ADAMTS4, and Caspase-3. (A–C) KO mice and mice treated with BMS309403 had significantly lower expression of MMP-13, ADAMTS4, and Caspase-3 than WT mice at early stage of OA (arrow). (D) Determination of MMP-13 positive cells in KO/WT mice fed with HFD. KO mice had lower percentage of MMP-13 positive cells after 3 months of HFD. (E) Determination of MMP-13 positive cells in mice fed with HFD and treated with BMS309403/PBS. Mice treated with BMS309403 for 4 months had lower percentage of MMP-13 positive cells. (F) Determination of ADAMTS4 positive cells in KO/WT mice fed with HFD. KO mice had lower percentage of ADAMTS4 positive cells after 3 months of HFD. (G) Determination of ADAMTS4 positive cells in mice fed with HFD and treated with BMS309403/PBS (Bars are represented as mean ± 95%CI. There were six mice per group. Student's ttest was performed. Magnification: 400×, Scale bar: 50 μm).

Similar to the effects of knocking out FABP4, pharmaceutical inhibition of FABP4 significantly alleviated cartilage degradation in animals fed with HFD. Mice treated with 4-month and 6-month of BMS309403 showed less staining loss of cartilage compared to the animals treated with PBS [Fig. 2(M), (O), (N), (P)]. However, difference of serum COMP did not reach significance though higher concentrations were observed in the BMS309403 group [Fig. 2(X)]. The modified Mankin OA score was significantly lower in BMS309403 groups [Fig. 2(Y)]. However, no statistical difference was seen in osteophyte area [Fig. 2(Z)]. For animals

fed with STD, treatment of BMS309403 did not have significant effects on cartilage degradation [Fig. 2(Q)–(T)].

The IHC staining of MMP-13 showed after 3-month of HFD, the KO mice had significantly lower number of chondrocyte with positive MMP-13 staining than the WT mice [Fig. 3(A), (D)]. After 9-month of HFD, both groups underwent severe cartilage degeneration. Empty lacunas were widely seen, which indicated chondrocyte apoptosis and was confirmed by Caspase-3 staining [Fig. 3(C)]. Treatment of BMS309403 for 4 months also significantly decreased the number of chondrocytes with MMP-13 staining [Fig. 4(A), (E)]. The IHC staining for ADAMTS4 was consistent with that of MMP-13, in which the KO mice showed significantly lower number of positive cells after 3 months of HFD [Fig. 3(B), (F)].

Download : Download high-res image (1MB)Download : Download full-size image
Fig. 4. Knocking out or pharmaceutical inhibition of FABP4 significantly alleviated synovium hypertrophy and macrophage infiltration at early stage of OA in animals fed with HFD. Sections were performed with IHC staining for CD68 (macrophage marker) and IL6 (inflammatory marker). (A) Representative images of IHC staining for CD68 in KO/WT mice fed with HFD, and in mice treated with BMS309403/PBS. WT mice showed more severe synovium hypertrophy and macrophage infiltration than KO mice after 3 months and 6 months of HFD. For mice treated with BMS309403, synovium hypertrophy and macrophage infiltration were alleviated after 4 months of treatment. (B) Representative images of IHC staining for IL6. Changes were similar as seen in CD68. (C) Synovitis score of mice fed with HFD. (D) Synovitis score of mice fed with HFD and treated with BMS309403/PBS (Bars are represented as mean ± 95%CI. There were six mice per group. Student's ttest was performed. Magnification: 400×, Scale bar: 50 μm).

The IHC staining for CD68 and IL6 demonstrated that the KO mice had significantly decreased synovium hypertrophy and macrophage infiltration than the WT mice after 3 months and 6 months of HFD [Fig. 4(A) and (B)]. Treatment of BMS309403 for 4 months also showed similar effects. The synovitis score confirmed lowered scores in the KO mice after 3 months and 6 months of HFD, and in mice treated with BMS309403 for 4 months [Fig. 4(C) and (D)].

3.

Knocking out FABP4 significantly alleviated osteophyte formation and subchondral bone sclerosis at advanced stage of OA in animals fed with HFD

The coronal X-ray of knee demonstrated severe subchondral bone sclerosis in WT mice after 6 months and 9 months of HFD, especially in the medical compartment [Fig. 5(A)]. The 3D analysis of subchondral bone showed that after 3-month of HFD, there were no significant differences in BMD, BV/TV, trabecular thickness, trabecular number, trabecular separation, or porosity percentage between the KO and WT mice. However, after 6-month of HFD, KO mice had significantly lower BMD than WT mice. And after 9-month of HFD, KO mice showed significantly lower value in all the parameters above (Table I). For animals fed with STD, knocking out FABP4 did not have significant effects on the subchondral bone [Fig. 5(A)]. Daily oral gavage of BMS309403, either for 4 months or 6 months, did not seem to have

significant effects on the subchondral bone [Fig. 5(B), data of 3D analysis provided in the supplemental material].

4.

FABP4 induced the expression of catabolic markers in chondrocyte

Download : Download high-res image (689KB)Download : Download full-size image

Fig. 5. Knocking out FABP4 significantly alleviated osteophyte formation and subchondral bone sclerosis at advanced stage of OA in animals fed with HFD. Representative coronal X-rays of knees of KO/WT mice, and of WT mice treated with BMS309403/PBS were shown. (A) KO/WT mice after 3 months, 6 months and 9 months of HFD or STD. WT mice after 6-month and 9-month of HFD demonstrated significant subchondral bone sclerosis as well as osteophyte formation compared to the KO mice, especially in the medial compartment (arrow). (B) Mice treated with BMS309403/PBS for 4 months and 6 months. There were no significant differences in subchondral bone structure between these two groups.

Table I. 3D Micro-CT analysis of subchondral bone of knee in KO and WT mice after 3 months, 6 months, and 9 months of HFD (* = P < 0.05; ** = P < 0.01)

	Mean 3m	95% CI	P	Mean 6m	95% CI	P	Mean 9m	95% CI	P
BMD (g/cm3)	KO	0.93	0.89, 0.97	0.34	0.84	0.71, 0.97	0.01*	0.91	0.85, 0.98	0.0020**
WT	0.95	0.92, 0.97	1.00	0.92, 1.10	1.07	1.00, 1.14			
BV/TV	KO	43.89	35.30, 52.49	0.88	41.77	33.42, 50.11	0.43	34.20	24.70, 43.70	0.0012**
WT	44.69	32.44, 56.93	44.43	34.22, 54.63	53.67	50.27, 57.06			
Tb.Th (mm)	KO	0.10	0.09, 0.10	0.96	0.09	0.09, 0.10	0.15	0.09	0.08, 0.10	0.0026**
WT	0.10	0.08, 0.11	0.10	0.09, 0.11	0.11	0.11, 0.12			
Tb.N (1/mm)	KO	4.27	3.90, 4.20	0.85	4.41	3.57, 5.26	0.94	3.50	2.62, 4.37	0.0083**
WT	4.32	3.76, 4.88	4.39	3.50, 5.28	4.71	4.09, 5.32			
Tb.Sp (mm)	KO	0.20	0.18, 0.21	0.34	0.19	0.15, 0.23	0.53	0.23	0.19, 0.27	0.0109*
WT	0.19	0.17, 0.21	0.20	0.16, 0.23	0.18	0.16, 0.21			
Porosity (%)	KO	56.11	47.51, 64.70	0.88	58.23	49.89, 66.58	0.43	65.80	56.30, 75.30	0.0012**
WT	55.31	43.07, 67.56	55.57	45.37, 65.78	46.33	42.94, 49.73			

As shown by quantitative RT-PCR [Fig. 6(A)–(C)], incubation of FABP4 with murine chondrocyte was able to induce the expression of catabolic markers including MMP-3, ADAMTS4, and iNOS in chondrocyte (vs un-stimulated control). These results were confirmed in terms of protein expression, as shown by western blot analysis [Fig. 6(D)].

Download : Download high-res image (243KB)Download : Download full-size image

Fig. 6. FABP4 induced the expression of catabolic markers in chondrocyte. (A) Expression of iNOS mRNA as measured by qRT-PCR. Fold change relative to un-stimulated control (24 ng/ml *: P = 0.0156, 120 ng/ml *: P = 0.0194, 600 ng/ml **: P = 0.0095, 3,000 ng/ml *:P = 0.0117). (B) Expression of ADAMTS4 mRNA as measured by qRT-PCR. Fold change relative to un-stimulated control (24 ng/ml *: P = 0.01630, 120 ng/ml **: P = 0.0015, 600 ng/ml *: P = 0.0461, 3,000 ng/ml **:P = 0.0040). (C) Expression of MMP-3 mRNA as measured by qRT-PCR. Fold change relative to un-stimulated control (24 ng/ml *: P = 0.0186, 120 ng/ml **: P = 0.0053, 600 ng/ml **: P = 0.0004, 3,000 ng/ml *: P = 0.0044). (D) Expression of proteins as measured by Western blot (Bars are represented as mean ± 95%CI. three independent tests were performed. One-way ANOVA followed by multiple comparisons with Bonferroni's post hoc test was performed.).

Discussion

OA is the most common form of arthritis. However, the pathogenesis of OA is still poorly understood. Obesity is a modifiable risk factor for OA, and recent studies have suggested adipokines play important roles in linking obesity to OA24. This study aimed at investigating the role of a novel adipokine FABP4 in OA. We found that knocking out FABP4 in mice could significantly alleviate OA when feeding with HFD. KO mice, in comparison to WT mice, showed higher body weight and body fat percentage. Nevertheless, they demonstrated less severe cartilage degradation and synovitis in early-stage OA, which was represented by lower serum COMP level, modified Mankin OA scores, MMP-13/ADAMTS4 expression, and synovitis score. In addition, KO mice showed much attenuated subchondral bone sclerosis and osteophyte formation in advanced stage of OA. On the other hand, pharmaceutical inhibition of FABP4 with daily oral gavage of BMS309403 (15 mg/kg/d) could act similarly to knocking-out FABP4, in which animals showed less cartilage degradation than the mice treated with vehicle. We also found from the in vitro cell culture study that FABP4 directly induced chondrocyte degeneration. These data suggest that FABP4 plays important roles in linking obesity to OA. BMS309403, which is a potent and specific inhibitor of FABP4, might be used as a potential therapeutic strategy for OA.

FABP4 is a lipid chaperon that is responsible for the transportation and metabolism of free fatty acid in adipocyte8. However, increasing evidence have revealed positive correlation between serum FABP4 level with obesity and metabolic diseases in human9, 10, 11, 12, 13. Animal studies have shown that genetically knocking out FABP4 could protect mice from atherosclerosis, insulin resistance, diabetes and fatty liver diseases25, 26, 27, 28. FABP4 KO mice exhibited markedly reduced serum insulin, triglyceride, cholesterol, glucose levels, and lipid profiles, compared with WT controls23. However, the detailed mechanism of FABP4 in inducing metabolic diseases is not yet known. Pilot mechanistic studies demonstrated that FABP4 may be involved in the macrophage-mediated inflammatory responses29. FABP4-deficient macrophages exhibit suppressed inflammatory signaling, attenuated activation of the NF-kB pathway, and decreased ER stress. The biochemical processes are not understood but may be linked to the accumulation of intracellular unsaturated fatty acids, particularly palmitoleic acid30, 31.

Our data has shown that knocking out FABP4 or treatment of BMS309403 could significantly increase animals' body weight and body fat percentage, which is in accordance with previous reports8, 26, 27. One possible explanation is that the lipolytic capacity in adipocyte

is significantly decreased in FABP4 KO mice32. Bernlohr et al. have demonstrated knockout of FABP4 reduced the removal of fatty acids out of adipocytes thereby leading to greater storage of fat in adipose tissue and increased whole body fat mass and weight33. Despite higher body weight, KO mice had less cartilage degradation than WT mice, which added another evidence that OA is not simply a 'wear and tear' disease.

In addition, WT mice had higher serum FABP4 under HFD, and the level increased significantly with the duration of diet. Our previous study in human has also shown OA patients had significantly higher serum and synovial fluid FABP4 than non-OA patients34. Thus, though we were not able to harvest synovial fluid from the mice, it's likely that the WT mice also exhibited high synovial FABP4 which may contribute to cartilage degradation. Several studies have already shown adipokines, including leptin, adiponectin, resistin, and visfatin, could induce expression of inflammatory cytokines in chondrocyte, such as iNOS and prostaglandin E2 (PGE2)5, 6, 7, 35, which subsequently generated degradation of ECM. Our in vitro cell culture study demonstrated FABP4 could also directly induce the expression of catabolic markers in chondrocyte, which suggest that FABP4 may exert its function in a similar fashion. It's noted from the q-PCR tests and WB analysis that the expression levels were decreased under 600 ng/ml and 3000 ng/ml of FABP4, which suggests cytotoxic effects to chondrocyte under high concentration of FABP4. In addition to the direct effects of FABP4 on chondrocyte, a recently published study36 has shown that PPAR up-regulated FABP4 expression in chondrocytes and promoted changes in genes related to ECM homeostasis. Our immunofluorescence staining of FABP4 in joint tissues also showed positive staining of FABP4 in chondrocyte. These findings indicate FABP4 may also induce chondrocyte degeneration in an autocrine pathway. Further studies are required to examine the detailed mechanisms (signaling pathways, etc.) of FABP4 in generating chondrocyte degeneration, and if macrophage could also play a role in this process.

Subchondral bone remodeling is a characteristic feature of OA. Both cross-sectional and longitudinal epidemiological studies have consistently demonstrated positive association between high BMD and OA37. The strong association between adipokines and bone metabolism has been observed in many studies. Leptin was reported to directly induce bone marrow lesion as well as osteophytes formation38, or regulate bone formation via the sympathetic nervous system39. Adiponectin, visfatin, and several other adipokines have also been demonstrated to affect bone metabolism through different pathways4. We found from this study that the WT mice had active subchondral remodeling while knocking out FABP4 could attenuate this process. This suggests that bone marrow FABP4, which may be secreted from bone marrow adipose tissue (BMAT), was closely associated with subchondral bone remodeling. More studies are required to investigate the interactions between FABP4 and bone remodeling process.

BMS309403 is a potent and selective biphenyl azole inhibitor of FABP440. Oral gavage of BMS309403 has been shown to be an effective therapeutic strategy against atherosclerosis and diabetes in mouse models14, 41, 42. Our study showed that chronic treatment of BMS309403 could act similarly to knocking out FABP4 and alleviated the degradation of cartilage in fat mice. However, we did not observe significant changes in the subchondral bone. This may due to a lack of knowledge on the optimal administration of BMS309403. Different treatment protocols have been reported previously: 15 mg/kg/d for 6 weeks and 4

months, by Furuhashi and Ho14, 16; 40 mg/kg/d for 6 weeks, by Hu43, and 100 mg/kg (twice daily), by Suhre44. Besides oral gavage, whether BMS309403 can be administered by local injection remains interesting to investigate in future.

There are several limitations of this study that need to be acknowledged. First, we used a very high-fat diet to induce obesity in animals. This level of fat content is not common in human diets. Nevertheless, it is typical of some low-carbohydrate (e.g., “Atkins”) diets45. Second, we did not harvest specimens including synovial fluid and infrapatellar fat pad from the mice due to technical difficulties. If higher local level of FABP4 was found in mice with more advanced OA, it may reaffirm that FABP4 is directly involved in chondrocyte inflammation.

Conclusions

We concluded from this study that genetically knocking out FABP4 or pharmaceutical inhibition of FABP4 could significantly alleviate knee OA in mice fed with HFD. Drugs that function by inhibiting FABP4, including BMS309403, could be potential therapeutic strategy for OA.

Author contributions

The authors made specific contributions as follows: (1) Conception and design of study: C Zhang, KY Chiu, A Xu, CH Yan; (2) acquisition, analysis, and interpretation of the data: C Zhang, KY Chiu, BPM Chan, T Li, C Wen, A Xu, CH Yan; (3) drafting of the article: C Zhang; All authors have read and approved the final submitted manuscript.

Conflict of interest

The authors declare no conflict of interest.

Role of funding source

This study was supported by Shenzhen Science and Technology Funding (JCYJ20150331142757396), China, and Hong Kong Health and Medical Research Fund (14151651).

Acknowledgement

The authors would like to thank Mr Tony Liu for his assistance with the Micro-CT scanning and data interpretation.