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Lactoferrin activates BMP7 gene expression through the mitogen-activated protein kinase ERK pathway in articular cartilage

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease leading to destruction of cartilage in joints. Neutrophil granulocytes are the predominant cell type in the synovial fluid of affected joints. Neutrophils release stimulants that alter the chondrocyte metabolism. Lactoferrin (LF) is a marker of neutrophil granulocyte activation. Local concentrations of LF in synovial fluid are much higher in the joints of RA patients. However, the effect of LF on articular cartilage of joints is not well understood. Effect of LF on gene expression in primary chondrocytes of articular cartilage was investigated in this study. We found that LF preferentially activated BMP7 expression rather than BMP2 or BMP4, and that LF activated BMP7 expression in a dose-dependent and time-dependent manner. Interestingly, a specific mitogen-activated protein kinase ERK inhibitor U0126, but not JNK kinase inhibitor SP600125, abrogated LF activation of BMP7 gene expression. LF-induced increase in BMP7 protein level was in parallel with the phosphorylation of ERK in primary chondrocytes. Taken together, we provide the first evidence to demonstrate that LF activates BMP7 expression through the mitogen-activated protein kinase ERK pathway in primary chondrocytes of articular cartilage. Since BMP7 is important for the maintenance of homeostasis in articular cartilage, we speculate that there is a protective function of LF at the site of joint inflammation.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease leading to destruction of the joints and surrounding tissues characterized by infiltration of the synovium with activated T cells and macrophages. On the other hand, neutrophil granulocytes are the predominant cell type in the synovial fluid of affected joints [1]. Neutrophil granulocyte activation in the synovial fluid of patients with RA may be due to cell exposure to different stimuli, including immune complexes, rheumatoid factors, cytokines, and microbial antigens [2]. It has been reported that lactoferrin (LF, also known as lactotransferrin) may represent a marker of neutrophil granulocyte activation [3]. In acute arthritis, neutrophils release stimulants that affect the metabolism of synoviocytes and chondrocytes [4]. Chondrocytes are the sole producers of cartilage extracellular matrix synthesis. LF is abundant in the granules of neutrophils. While serum level of LF in healthy subjects ranges from 2 to 7 μg/ml, local concentrations in synovial fluid are much higher in the joints of RA patients, up to 100 μg/ml [5–7]. In animals, systemic levels of LF can reach concentrations as high as 200 μg/ml during inflammation [8]. However, the effect of LF on articular cartilage of joints is not well understood.

LF was first reported almost 50 years ago as an iron-binding glycoprotein of the transferrin family [9]. LF is one of the transferrin proteins that transfer iron to the cells and control the level of free iron in the blood and external secretions. It is a cell-secreted protein that is abundant in milk and most biological fluids. Apart from its main biological function, namely binding and transport of iron ions, lactoferrin also has antibacterial, antiviral, antiparasitic, catalytic, anti-cancer, anti-inflammatory, anti-allergic and radioprotecting functions and properties [10]. It bridges innate and adaptive immune function in mammals. This wide range of activities is made possible by mechanisms of action involving not only the capacity of LF to bind iron but also interactions of LF with molecular and cellular components of both hosts and pathogens. The large potential applications of LF have led scientists to develop this nutraceutical protein for use in feed, food and pharmaceutical applications. Clinical trials demonstrated the efficiency of LF for use in treating infections and inflammatory diseases. For example,

Abbreviations: LF, lactoferrin; BMP, bone morphogenetic protein; RA, rheumatoid arthritis; HSP90, heat-shock protein 90; DMEM, Dulbecco's modified Eagle's medium; LRP, lipoprotein-receptor-related protein.

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LF was tested as an adjuvant treatment against H. pylori in patients with recurring infections. The patients supplemented with LF showed a greater recovery from infection [11]. While many diverse physiological functions have been proposed for LF, the precise mechanism of action of LF in many of these processes remains unclear.

We are interested in investigating the effect of LF on gene expression in chondrocytes of articular cartilage. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor superfamily of cytokines that regulate different critical cellular processes, including differentiation and proliferation. Originally discovered by their ability to induce the formation of bone and cartilage, BMPs now constitutes a group of pivotal morphogenetic signals throughout the body. BMP2, BMP4 and BMP7 are considered to be the most osteoinductive [12]. In this study, we provide evidence to show for the first time that LF selectively activates gene expression of BMP7 over BMP2 or BMP4 in primary chondrocytes of articular cartilage, and that LF regulates BMP7 expression through the mitogen-activated protein kinase ERK pathway.

2. Materials and methods

2.1. Cartilage isolation

The use of animal tissues was approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Animal protocol number is 2010-0183. Yorkshire immature pigs aged 5–6 weeks old (6–8 kg) were used as previously described [13]. The animals were euthanized and articular cartilage of knee joint was dissected from distal femoral condyle. Use of discarded human tissues was approved by Institutional Review Board of the University of Texas Southwestern Medical Center. Study ID number is STU 01211-114. Cartilages were taken when joints were removed during polydactyly surgery. Participants provided their written informed consent to participate in this study.

2.2. Primary chondrocyte isolation and cultures

Primary chondrocytes were isolated from cartilage as previously described [14]. Briefly, cartilage was cut into small pieces (2–3 mm^3), washed with Dulbecco's modified Eagle's medium (DMEM, GIBCO), and treated for 15 min with trypsin (10% v/v) in a 37 °C water bath. Cartilage pieces were then transferred to DMEM containing 5% fetal calf serum, penicillin–streptomycin–Fungizone, and 2 mg/ml clostridial collagenase type IV and digested overnight on a shaker until the tissue fragments were dissolved. The cells were washed three times with DMEM and cultured in DMEM plus 10% fetal calf serum until confluent. All experiments used chondrocytes in the primary culture or within three passages following a 1:3 subculture. Primary chondrocytes were cultured and maintained in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin at 95% air/5% CO_2 humidified incubator. Primary chondrocytes were treated with LF (Sigma–Aldrich) at the indicated amounts. Specific kinase inhibitors U0126 and SP600125 were purchased from Sigma–Aldrich.

2.3. RNA isolation and quantitative real-time reverse transcription-PCR

Total RNA from primary chondrocytes was isolated directly using an RNeasy Mini Kit according to the manufacturer's protocols (Qiagen). RNA was subjected to quantitative RT-PCR using the TaqMan One-Step RT-PCR Master Mix reagent (Applied Biosystems) as previously described [15]. RT-qPCR was performed on a Thermal Cycler (iCycler, Bio-Rad) for 40 cycles at 95 °C for 15 s and at 48–58 °C, depending on the melting temperatures of the primers, for 30 s. Relative transcript levels were measured by real-time PCR in a 50 μl reaction volume on 96-well plates using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Heat-shock protein 90 (HSP90) was used to normalize the expression levels of various genes studied using primers from Applied Biosystems.

2.4. Protein purification and Western blot

Protein was isolated by acetone precipitation from the RNeasy cell lysates according to the manufacturer's protocol (QIAGEN). The protein pellet was dissolved in 1% SDS buffer, warmed for 15 min at 55 °C, and centrifuged for 5 min at 14,000 rpm. Protein concentrations in the supernatant were determined using a BCA Protein Assay Kit (Pierce). Proteins were separated on 10% SDS–PAGE gels and transferred to a PVDF membrane followed by Western blot analysis as previously described [16]. Briefly, 3% milk in TBS containing 0.1% Tween-20 was used to block non-specific binding. The blot was subsequently incubated with an anti-BMP7 rabbit polyclonal antibody (1:200, Abcam), an anti-phospho-ERK rabbit polyclonal antibody (1:200, Cell Signaling) or an anti-HSP90 rabbit polyclonal antibody (1:200, Abcam) followed by a secondary antibody (peroxidase-conjugated anti-rabbit IgG 1:2000, Sigma). After each antibody incubation, blots were extensively washed in TBS containing 0.1% Tween-20. For detection, the ECL kit (Amersham Life Sciences) was used according to the directions of the manufacturer.

2.5. Statistical analysis

All experiments were repeated a minimum of 3 times. Data was reported as the mean ± standard deviation (SD). Comparisons were made between groups by Student's t test. A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. LF effect on BMP2, 4, 7 expressions in primary chondrocytes of articular cartilage

To explore the effect of LF on gene expression in primary chondrocytes, we performed quantitative real-time RT-PCR on RNA from primary chondrocytes of articular cartilage of pig knee joint. Among BMP2, 4, 7 expressions, we observed that BMP7 expression increased by 9.9-fold after 200 μg/ml of LF stimulation for 24 h while BMP2 and BMP4 did not change significantly as shown in Fig. 1A. HIF-1α, as a negative control, remained the same after LF stimulation in chondrocytes. To confirm this observation, primary human chondrocytes from articular cartilage were used to test the effect of LF. Likewise, BMP7 expression was upregulated by 6.4-fold in the presence of LF as shown in Fig. 1B. In contrast, BMP2 and BMP4 did not change significantly, and as expected, HIF-1α was unchanged after LF stimulation. These data indicate that LF selectively activates BMP7 expression over BMP2 or BMP4.

3.2. LF activates BMP7 expression in chondrocytes in a dose-dependent manner

To further investigated the effect of LF on BMP7 expression, pig primary chondrocytes were treated with different amounts of LF. Fig. 2A showed that 50 μg/ml of LF was able to activate BMP7 expression by 4-fold. Increasing amounts of LF led to higher levels

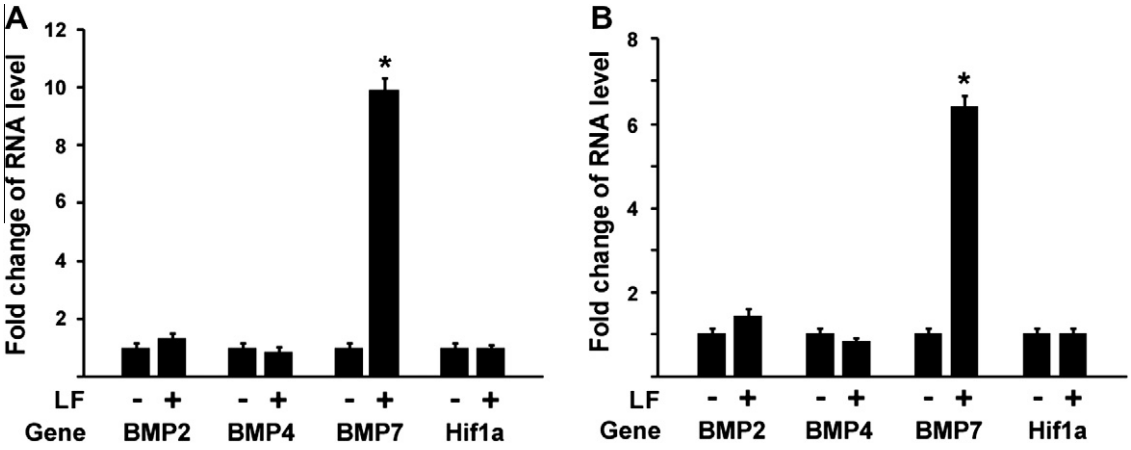

Fig. 1. LF effect on BMP2, 4, 7 expressions in primary chondrocytes of articular cartilage from pig (A) and human (B). The femur bones were obtained from Yorkshire immature pigs aged 5–6 weeks old. Pig primary chondrocytes were isolated from articular cartilage of knee joints. Human cartilages were taken when joints were removed during polydactyly surgery, and human primary chondrocytes were isolated after that. Primary chondrocytes were treated with 200 μg/ml of LF for 24 h. RNA was isolated and measured by quantitative real-time RT-PCR. Level of RNA from control group was normalized to a value of 1. *: A star indicates statistical significance compared to control group.

of BMP7 expression. Five hundred micrograms per milliliters of LF was able to activate BMP7 expression by 14.8-fold. This demonstrated that LF activated BMP7 expression in a dose-dependent manner. However, expression levels of BMP2 and BMP4 did not change significantly even at the presence of 500 μg/ml of LF as shown in Fig. 2B and C, respectively. Therefore, we focused on the investigation of LF effect on BMP7 expression in the following study.

3.3. LF activates BMP7 expression in chondrocytes in a time-dependent manner

Next, we examined effect of LF on BMP7 expression in different time points. Pig primary chondrocytes were treated with 200 μg/ml of LF. Fig. 3 showed that 4 h after LF treatment BMP7 expression increased by 2.7-fold. Longer time of LF treatment resulted in higher expression of BMP7 expression with the peak at 24 h after LF treatment, at which BMP7 expression was upregulated by 11.8-fold. At 48 h after LF treatment, BMP7 expression activation was down to 10.2-fold. This demonstrated that LF activated BMP7 expression in a time-dependent manner.

3.4. LF activates BMP7 through the mitogen-activated protein kinase ERK pathway

To explore molecular mechanisms of LF effect on BMP7 expression in chondrocytes, we used a loss-of-function approach to explore possible pathways involved. The following selective inhibitors were used: U0126 is a specific ERK inhibitor in mitogen-activated protein kinase pathway, and SP600125 is a specific inhibitor for JNK kinase pathway. Pig primary chondrocytes were treated with 200 μg/ml of LF. Different inhibitors were added in the culture medium as indicated. As shown in Fig. 4A, LF treatment led to BMP7 expression increase by 10.8-fold. In the presence of 2 μM U0126, BMP7 expression increase induced by LF was 5.4-fold. Addition of 50 μM U0126 abolished the increment of BMP7 expression induced by LF. We observed that LF-induced BMP7 activation was unchanged by treatment with SP600125 as shown in Fig. 4A. These data suggest that LF-induced BMP7 activation is mediated through the mitogen-activated protein kinase ERK pathway.

3.5. LF activates BMP7 expression in protein level

We then sought to examine LF regulation of BMP7 expression in protein level. Pig primary chondrocytes were treated with 200 μg/ml of LF for 24 h. Protein was isolated and analyzed by western blotting. As shown in upper panel of Fig. 4B, LF treatment led to an increase of BMP7 expression in protein level, and LF-induced BMP7 expression increase was inhibited by the specific ERK kinase inhibitor U0126. To address whether increased BMP7 protein levels by LF is in parallel with the phosphorylation of ERK in primary chondrocytes, we examined ERK phosphorylation after addition of LF using antibody against phosphorylated ERK. As shown in the middle panel of Fig. 4B, LF treatment resulted in the phosphorylation of ERK, and importantly, the ERK inhibitor U0126 markedly inhibited both the effects of LF on the levels of BMP7 protein and the phosphorylation of ERK. Either LF group or LF plus U0126 group did not affect the protein expression level of control HSP90 as indicated in the lower panel of Fig. 4B. These results support the hypothesis that the increase in BMP7 expression caused by LF is mediated through the ERK pathway.

4. Discussion

LF concentrations in synovial fluid become higher in the joints of RA patients. The effect of LF on gene expression in chondrocytes of articular cartilage has not been well elucidated. In this study, we report for the first time that LF activates BMP7 gene expression through the mitogen-activated protein kinase ERK pathway in primary chondrocytes.

We identified BMP7 as a LF selective regulating target gene. This is supported by our gene expression regulation studies which compared the effect of LF on BMP2, 4, and 7 expressions in primary chondrocytes. Primary chondrocytes cultures obtained from articular cartilage had markedly increased BMP7 gene expression after LF treatment while BMP2 and BMP4 did not have significant change after LF treatment. Importantly, the regulation of BMP7 gene transcription by LF was abolished when the specific ERK inhibitor U0126 in mitogen-activated protein kinase pathway was added in chondrocyte cultures, thus indicating that LF activates BMP7 gene expression through the mitogen-activated protein kinase ERK pathway. It will be interesting to address why BMP2 or BMP4 is not induced by LF in the future study. One possibility is that the promoters of BMP2 and BMP4 may be different

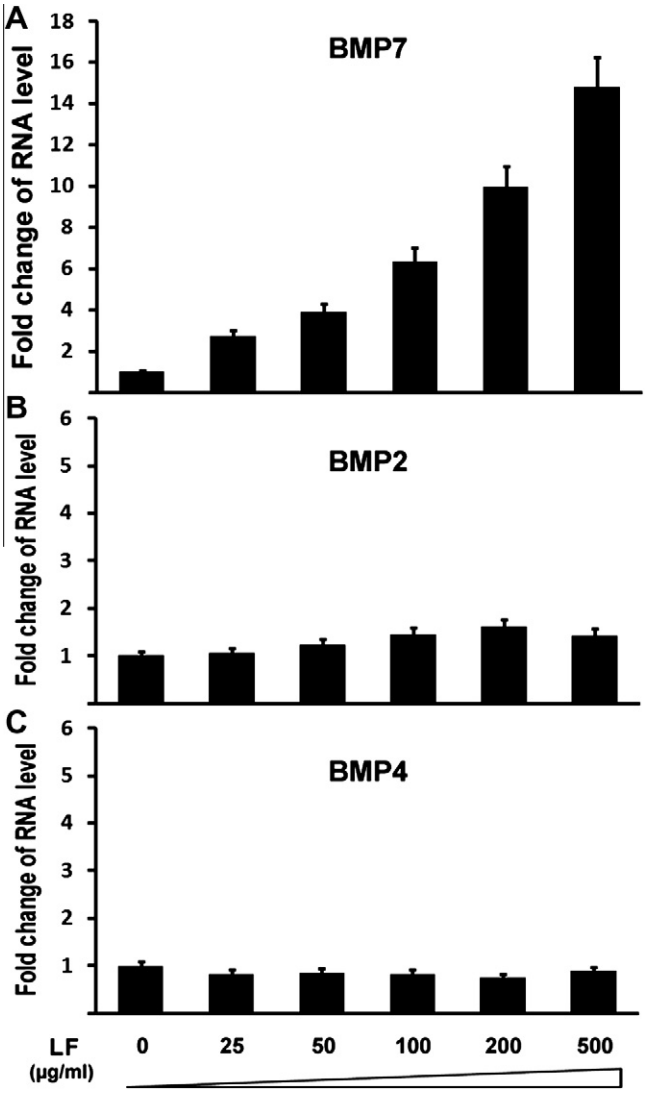

Fig. 2. Fold change in RNA levels of BMP7 (A), BMP2 (B) and BMP4 (C) in pig primary chondrocytes of articular cartilage. Pig primary chondrocytes were cultured and treated with different amounts of LF for 24 h. RNA was isolated and measured by quantitative real-time RT-PCR. Level of RNA from control group was normalized to a value of 1.

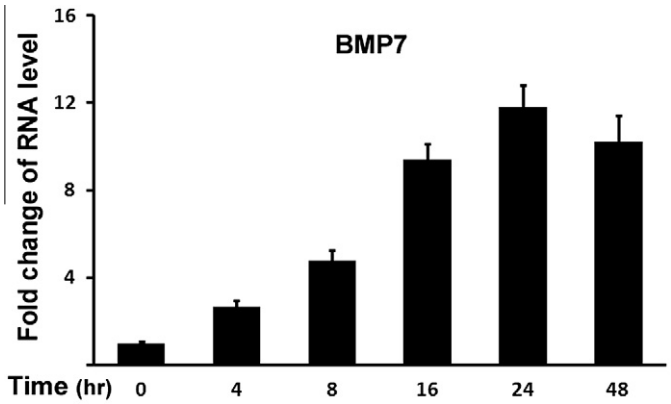

Fig. 3. LF activates BMP7 expression in a time-dependent manner. Pig primary chondrocytes were cultured and treated with 200 μg/ml of LF for different time points as indicated. RNA was isolated and measured by quantitative real-time RT-PCR. Level of RNA from control group was normalized to a value of 1.

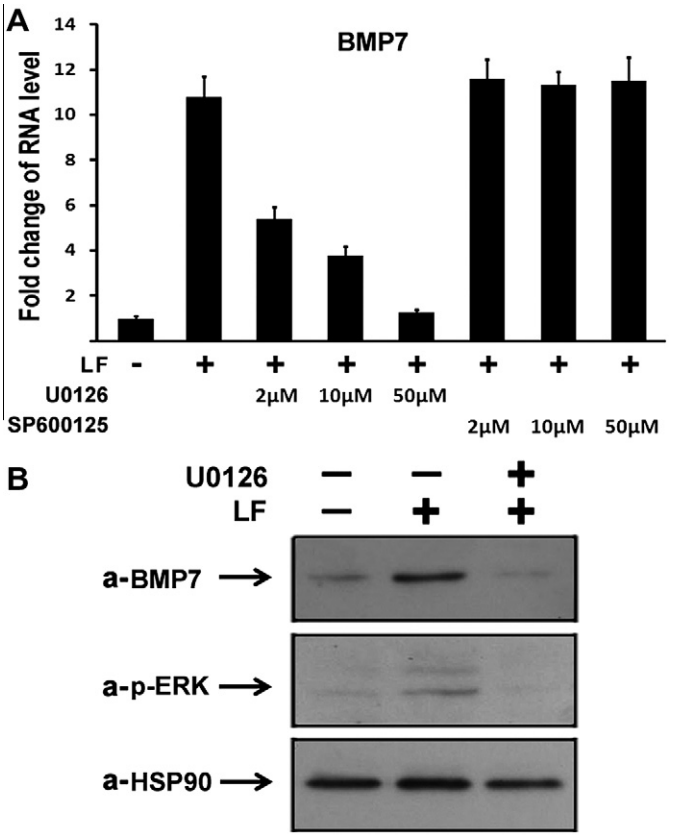

Fig. 4. LF activates BMP7 through the mitogen-activated protein kinase ERK pathway. (A) Fold change in RNA levels of BMP7. Pig primary chondrocytes were treated with 200 μg/ml of LF for 24 h. U0126 and SP600125 were added to the culture medium 2 h before LF treatment. RNA expression level of BMP7 was determined by quantitative real-time RT-PCR. Level of RNA from control group was normalized to a value of 1. (B) Inhibition by the ERK inhibitor U0126 of LF-induced increase in BMP7 protein and phosphorylation of ERK in primary chondrocytes. Confluent pig primary chondrocytes were cultured and incubated with or without 50 μM U0126 for 2 h before addition of 200 μg/ml LF for 24 h. Total cell lysates were prepared and analyzed by Western blot using antibodies that recognize BMP7, phosphor-ERK and HSP90.

from the promoter of BMP7, resulting in the difference of transcriptional regulation of gene expression of these different BMP family members.

LF has been reported for 50 years; however, its signaling pathway is not well understood. It has been reported that the actions of LF are mediated by signaling through the low-density lipoprotein-receptor-related protein (LRP) 1 [17], a multifunctional, multi-ligand receptor that belongs to a family of structurally related cell-surface receptors, the prototype of which is the LDL receptor. The same research group also found later that LF can function in an LRP1-independent pathway, suggesting that signaling through more than one LF receptor is responsible for the actions of LF [18]. A proliferative effect of LF was found in osteoblastic cells and also ovine chondrocytes [19].

BMP7 is important for the maintenance of homeostasis in articular cartilage. BMPs, particularly BMP7, show much promise as therapeutic agents for delaying the progression of arthritis [20]. The BMP7 isoform has been shown to promote cartilage matrix synthesis by chondrocytes, specifically collagen types II and VI, and proteoglycans. BMP7 also leads to an increase in Sox9 expression in articular cartilage [21]. Using animal models with chondral defects, it has been demonstrated that BMP7 alone or in combination with regenerative orthopedic procedures such as mosaicplasty and microfracture repair, is able to improve the histological

characteristics of newly formed cartilage matrix [20]. Under normal conditions, BMP7 appears in the superficial layer of articular cartilage concomitant with the expression of BMP receptors (BMPR-IA, IB, and II). There are several putative roles of BMP7 in inflammatory joint disease including preservation and repair of articular surfaces by enhancing the chondrocyte phenotype of dedifferentiated cells, increasing synthesis of tissue inhibitor of metalloproteinase, as well as leading to the expression of IGF-I and chondrocyte cytoskeletal proteins [20,22]. Levels of BMP7 have been found to be elevated in the synovial tissues derived from the joints of patients with RA [23]. However, the mechanisms of BMP7 upregulation in the joints of RA are not clear. In this study, we provide the evidence that demonstrates LF-induced activation of BMP7 expression in chondrocytes of articular cartilage. The animal studies need to be extended to include a systemic administration model to address possible LF-induced cartilage phenotype.

It has been demonstrated that LF is a marker of neutrophil granulocyte activation rather than a disease activity marker in RA [6]. The lack of correlation between LF expression of neutrophil granulocytes in synovial fluid and disease activity may indicate that cell activation markers at the site of inflammation are not always correlated with systemic disease activity. Our data indicated that LF activates BMP7 expression through the mitogen-activated protein kinase ERK pathway. Since BMP7 is important for the maintenance of homeostasis in articular cartilage, we speculate that there is a protective function of LF at the site of joint inflammation. Whether LF plays a functional or therapeutic role at the site of joint inflammation deserve further investigation.

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