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Lysophosphatidic Acid Mediates Fibrosis In Injured Joints By Regulating Collagen Type I Biosynthesis

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Abstract

Objective—Articular cartilage is a highly specialized tissue which forms the surfaces in synovial joints. Full-thickness cartilage defects caused by trauma or microfracture surgery heal via the formation of fibrotic tissue characterized by a high content of collagen I (COL I) and subsequent poor mechanical properties. The goal of this study is to investigate the molecular mechanisms underlying fibrosis after joint injury.

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Author contributions

Conception and design: LW and DE; Analysis and interpretation of the data: LW, FP, SL, JB, BK and DE; Drafting of the article: LW and DE; Critical revision of the article for important intellectual content: AR, DM, JA, BH, GC and YL; Final approval of the article: DE; Provision of study materials or patients: FP, AR and BH; Statistical expertise: GC and YL; Obtaining of funding: DE; Administrative, technical, or logistic support: JA and DM; Collection and assembly of data: LW, FP, SL, JB and BK

Competing interest statement

The authors declare no financial support or any other benefits from commercial sources received for the work reported on in the manuscript. DE and FP have submitted a patent disclosure to the office of UCLA office of intellectual property

Design—Rat knee joint models are used to mimic cartilage defects after acute injury. Immunohistochemistry was performed to detect proteins related to fibrosis. Human fetal chondroyges and bone marrow stromal cells (BMSCs) were used to study the influence of lipid lysophosphatidic acid (LPA) on Collagen I (COL I) synthesis. Quantitative PCR, ELISA and immunohistochemistry were performed to evaluate the productions of COL I. Chemical inhibitors were used to block LPA signaling both *in vitro* and *in vivo*.

Results—After full-thickness cartilage injury in rat knee joints, stromal cells migrating to the injury expressed high levels of the LPA-producing enzyme autotaxin (ATX); intact articular cartilage in rat and humans expressed negligible levels of ATX despite expressing the LPA receptors LPAR1 and LPAR2. LPA-induced increases in COL I production by chondrocytes and BMSCs was mediated via the MAP kinase (MAPK) and PI3 Kinase (PI3K) signaling pathways. Inhibition of the ATX/LPA axis significantly reduced COL I-enriched fibrocartilage synthesis in full-thickness cartilage defects in rats in favor of the collagen II-enriched normal state.

Conclusion—Taken together, these results identify an attractive target for intervention in reducing the progression of post-traumatic osteoarthritis.

Keywords

autotaxin; lysophosphatidic acid; chondrocytes; cartilage injury; collagen type I; fibrosis

Introduction

Pathological and disorganized regeneration of tissue in a variety of organs after injury often results in deposition of excessive inert fibrotic tissue with inferior biomechanical properties. Full-thickness disruption of articular cartilage by trauma to synovial joints is one example in which highly specialized hyaline cartilage is replaced by biomechanically-inferior, disorganized fibrotic tissue enriched in collagen type I (COL I). Such traumatic cartilage defects often lead to degenerative joint disease or osteoarthritis[1]. One of the most popular treatments for small full-thickness cartilage defects is a bone marrow stimulation technique known as microfracture surgery. In the procedure, a surgical awl is used to create small holes in the subchondral plate, which allow bleeding into the defect from the subchondral bone marrow [2]. Thus far, this technique has demonstrated short-term clinical success in young and active patients but not in older, more sedentary adults [3]. In the process of healing, bone marrow mesenchymal stromal cells (BMSCs) migrate into the site of injury and form fibrocartilage, a scar-like tissue composed predominantly of COL I containing only modest amounts of glycosoaminoglycans (GAG) and collagen II (COL II) that are typically present in normal hyaline cartilage. Fibrocartilage demonstrates poor mechanical properties, and its integration to native cartilage is insufficient, often leading to degeneration of the repaired tissue [4] with subsequent deterioration and poorer clinical outcomes over the long term. The mechanisms by which BMSCs in the defect deposit disproportionate levels of disorganized COL I remain elusive. The identification of factors that drive the production of fibrocartilage at the site of repair represents is a major goal in the field of cartilage regeneration. One of the major questions remaining in this field is whether the disorganized reparative fibrocartilage generated by full thickness injury or microfracture is a result of i)

the limited intrinsic capacity of BMSCs to differentiate into COL II-producing chondrocytes or ii) the consequence of pathological “niche” in the site of cartilageinjury.

We have recently identified lysophophatidic acid (LPA) as a novel potential regulator of COL I biosynthesis that may play a mechanistic role in fibrocartilage formation [5]. LPA is a small lipid produced by an ectoenzyme autotaxin (ATX), also known as phospholipase D, concentrated on the outward face of the plasma membrane of articular chondrocytes and BMSCs[6]. The enzyme belongs to the family of nucleotide pyrophosphatases/ phosphodiesterases (NPPs) which convert lysophosphatidylcholine into LPA by cleavage of the choline group [7]. ATX knockout mice die at E9.5 due to severely impaired vasculature formation in yolk sac and embryo proper [8].

One of the best-documented effects of LPA under pathological conditions is excessive and disorganized COL I deposition in fibrotic tissue, leading to fibrosis of the lung, kidney, dermis and liver [9–12]. Six G-protein coupled LPA receptors have been identified so far; highly selective inhibitors for some of these receptors have been successfully developed and tested both *in vitro* and *in vivo* [13]. Pharmacological inhibitors of LPA signaling were found to prevent fibrotic transformation in several organs [14], suggesting the possibility that the same mechanism may play an important role during reparative neo-chondrogenesis. Based on these data, we hypothesized that the ATX/LPA axis is involved in fibrocartilage formation after full-thickness cartilage injury, and that the inhibition of LPA signaling during reparative neo-chondrogenesis may reduce COL I deposition and fibrosis in injured joints.

Here we show that the ATX/LPA axis regulates the synthesis of COL I in articular chondrocytes and BMSCs and that inhibition of this pathway reduces fibrocartilage formation following full-thickness microfracture of articular cartilage in rats. These data reveal a novel mechanism regulating cartilage healing and identify an attractive molecular target to improve widely-used clinical procedures for cartilage regeneration.

Methods

Animal model of cartilage defects in rat knee joints

Full thickness (involving the subchondral bone) cartilage defects were generated in the femoral intra-condylar region of a knee joint in rats using a previously described method [15]. Briefly, 11–12 week old Sprague-Dawley rats (Charles River, San Diego, CA) were anesthetized and medial para-patellar arthrotomy was carried out under a dissection microscope (Olympus, USA). One mm wide osteochondral defects were made with a Kirshner wire. At weeks 1, 2 and 4, rats were sacrificed and dissected for histological examination. For the ATX/LPA inhibition experiments, microspheres loaded with either BrP-LPA (“inhibitor” group, n=4) or its analog (“vehicle” group, n=4) were injected in the joints after closure of the arthrotomy but before skin closure. Local administration of BrP-LPA was combined with systemic intraperitoneal (IP) injection of the drug at a dose 5 mg/kg carried out every 3 days after surgery up to 21 days. Rats in “vehicle” group received IP injection of physiological saline at the same time points. Three weeks after surgery, rats of both groups were sacrificed for examination.

Cell culture and expansion

The use of human material has been approved by the UCLA Institutional Review Board. Fetal tissues were obtained from Novogenix Laboratories, LLC (Los Angeles, CA) following informed consent and anonymous donation. Articular chondrocytes were extracted from epiphyseal regions of 17-week tissues as previously described [16]. In short, cartilage was digested for 20–22 hr in collagenase type II (0.15% w/v) in DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). Chondrocytes were then cultured in chondrocyte expansion medium (DMEM/F12 supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin). Medium was refreshed every 3–4 days. Upon confluence, chondrocytes were sub-cultured. All reagents used for cell culture were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. Common chemicals were purchased from Sigma-Aldrich.

Fabrication of polymeric microspheres

PLGA (polylactic-co-glycolic acid) microspheres were fabricated according to previously published methods [17]. Briefly, 10% (w/v) PVA (polyvinyl alcohol), 10% (w/v) PVP (polyvinyl pyrrolidone) and 2% (w/v) 50:50 PLGA solutions were mixed. Microspheres were collected by centrifugation at 5000 RPM for 30 min. Microspheres were loaded with BrP LPA and re-suspended in DMEM and sterilized by passing through 0.22 μm filters. Each animal received 100 ng of BrP-LPA loaded onto PLGA microspheres reconstituted in 10 μL of PBS. This solution was injected into the knee as described above. Control animals were injected with microspheres prepared without BrP LPA. For drug release experiments, either BrP-LPA or the fluorescently tagged fatty acid C1-BODIPY 500/510 C12 (Invitrogen, Carlsbad, CA) was loaded onto the microspheres during fabrication. Drug release pilot experiments were performed first with C1-BODIPY 500/510 C12 to study the kinetics of drug release.

Knockdown of ATX by short hairpin RNA (shRNA) in fetal BMSCs

Four unique 29mer shRNA constructs against human ENPP2 (the gene encoding ATX) were cloned into a lentiviral vector also expressing GFP (Origen, Rockville, MD). Lentiviral supernatants containing shRNA constructs were packaged by triple transfection of HEK293T cells according to previous published protocols [18]. Fetal BMSCs were transduced by adding viral supernatants to the culture medium. Stably transduced cells were sorted by FACS based on expression of GFP (green fluorescent protein) on Day 7 after transduction and expanded for several passages.

Statistical analysis

All experiments were repeated at least three times. Statistical significance was determined either by paired Student's t test or one way ANOVA followed by Newman-Keuls or Dunnett tests. P values of < 0.05 were considered as statistically significant.

Descriptions for other methods are available in supplementary materials.

Results

The LPA-producing enzyme ATX is minimally expressed in normal human cartilage

To determine if the LPA signaling pathway may function during normal chondrogenesis, we first screened the expression of 6 LPA receptors in fetal chondrocytes (FCH) and bone morrow stromal cells (BMSC) by PCR. We found that LPA receptors 1 and 2 (LPAR 1 and 2) were the most abundant in FCH and BMSC (Fig. S1). Using immunohistochemistry, we further examined the expressions of the two receptors in articular chondrocytes and synovial tissue at various stages of human development and found that these two major receptors for LPA were abundantly present on articular chondrocytes and synovium (Fig. 1A and 1B). In contrast, ATX was minimally expressed by chondrocytes at all stages, although other cell types including vascular cells evidenced abundant expression (Fig. 1A and Fig. 1C). Our previous studies showed high levels of ATX expression by fetal and adult BMSCs [5]. To validate that these expression patterns were maintained in culture, we assessed the levels of ATX, LPAR 1 and LPAR 2 proteins on cultured primary articular chondrocytes and BMSCs isolated from human fetal joints (Fig. 1D). We found that human BMSCs expressed ATX as well as LPAR 1 and 2, while articular chondrocytes expressed the receptors and *minimally* expressed ATX (Fig. 1D). These data indicated that articular chondrocytes both *in vivo* and *in vitro* have little capacity to generate LPA, but do express the receptors required for responding.

The ATX protein is highly expressed in osteo-chondral defects in the rat knee

We hypothesized that the LPA pathway may influence fibrocartilage formation after cartilage injury. To assess this, the rat knee joint injury model was employed to study the levels of ATX expression after full-thickness cartilage injury. In this system, cells from the bone marrow migrate into the site of injury and form a scar that repairs the defect but has poor mechanical properties. Immunohistochemical staining confirmed little ATX expression by rat articular chondrocytes in uninjured joints (Fig. 2A); however, ATX was expressed at high levels by stromal cells filling the defect at Day 7 following injury (Fig. 2B). The expression of ATX at Day 14 and 28 after injury was significantly lower than at Day 7 (Fig. 2C and Fig. S2).

Significant COL I deposition at the site of cartilage injury was clearly present at Day 7 and further increased by Day 14 (Fig. 2B and 2C). Loose connective tissue filling the defect contained small blood vessels, as indicated by immunohistochemical staining for CD146, which is expressed by perivascular cells (Fig. S3). By Day 28, cartilage defects were filled with dense fibrotic tissue strongly positive for COL I (Fig. S2).

LPA stimulates COL I deposition by human chondrocytes and BMSCs

Next, we examined the effects of LPA on COL I deposition on cultured primary human chondrocytes and BMSCs. Chondrocyte pellets were cultured either in chondrogenic medium (control), in chondrogenic medium containing LPA (LPA) or chondrogenic medium containing both LPA and chemical inhibitor BrP-LPA (LPA+BrP-LPA). BrP-LPA (1-Bromo-3(S)-hydroxy-4-[(palmitoyloxy)butyl]phosphonate) is an α-halo-substituted phosphonate and a metabolically stable analog of LPA that has dual functions of antagonist

against LPA receptors and inhibitor for the lysophospholipase D activity of ATX [14]. Due to its specific inhibitory effects on ATX/LPA axis activity, it has been widely used to study to therapeutic potential of blocking ATX/LPA signaling pathway [19–21]. Results of histological examination indicated that both control and LPA-treated samples deposited cartilaginous matrix strongly stained with alcian blue (Fig. 3A). However, LPA-treated pellets produced high levels of COL I in addition to COL II, while control pellets primarily expressed COL II (Fig. 3A).

Inhibition of the ATX/LPA axis by BrP-LPA reduced the deposition of COL I by chondrocytes in pellets as documented by ELISA (Fig. 3B) and also decreased the size of the pellet (Fig. 3D). After normalization to DNA, LPA-treated pellets showed almost 3-fold higher levels of COL I deposition compared to control, while inhibition of the ATX/LPA axis abolished this increase. qPCR was performed next to examine the ratio of *COL II* and *COL I* gene expression in cultured pellets. After treatment with LPA, the *COL II*/*COL I* ratio in chondrocyte pellets decreased in a dose-dependent manner (Fig. 3C). Despite the increase in COL I mediated by LPA, no concomitant increase in matrix deposition of glycosaminoglycans (GAGs) was observed (Fig. S4). We next studied the effects of LPA on COL I deposition by BMSCs. Since BMSCs have considerable amount of endogenous ATX activity, the effects of LPA were studied in the presence of the ATX inhibitor S32826 (1 μM) added to all tested groups, which selectively inhibits the activity of ATX but not the LPA receptors [22]. LPA treatment resulted in almost 3-fold increase in COL I deposition by BMSCs and increase in pellet size (Fig. 3E and F), which was completely reversible by inhibiting the LPA receptors via BrP-LPA.

We then used shRNA to knock down the expression of ATX in fetal BMSCs. Four lentiviral shRNA-GFP constructs (named shRNA-A, -B, -C and –D) were delivered into cells via transduction; control cells were transduced with lentiviruses carrying scrambled shRNA and GFP sequences. To quantitate the amount of ATX activity present following transduction, the fluorogenic autotaxin substrate FS-3 was used. FS-3 is a doubly labeled analog of LPC where in the ground, uncleaved state the fluorophore is quenched through intramolecular energy transfer. Once ATX cleaves FS-3, the labeled analog of the ATX natural substrate lysophospatidylcholine (LPC), the fluorophore becomes liberated from the quencher, resulting in increased fluorescence[23]. This fluorogenic assay indicated that cells transduced with shRNA-B had the lowest enzymatic activity of ATX (Fig 4A). Analysis of the proliferation rate of cells after transduction showed that shRNA-B slightly reduced the proliferation of the cells (Fig. 4B). Immunofluorescent staining and qPCR were performed next to test the expression of ATX on GFP-positive cells sorted by FACS after transduction with shRNA-B vector. The expression of ATX is decreased by shRNA-B construct, as indicated by both immunoflorescent staining (Fig 4C) and qPCR (Fig 4D). We then made pellets of control and shRNA-B transduced cells. After 2 weeks culture in chondrogenic medium, ELISA results indicated that deposition of COL I in pellets of shRNA-B transduced cells was less than that of control cells (Fig. 4E). Together, these data indicate that the LPA signaling axis may be implicated in COL I deposition by BMSCs and chondrocytes at the site of cartilage injury.

LPA-induced COL I expression is mediated by MAP and PI3 kinase signaling pathways

To further characterize the downstream signaling pathways involved in LPA-induced COL I deposition, cultured human chondrocytes were treated with LPA (1 μM) in the presence of the selective chemical inhibitors of signaling pathways previously shown to regulate the intracellular activities of LPA [24–26]. To quantitatively detect COL I deposition in the presence or absence of the inhibitors, we used a recently developed method called Extracellular Matrix Domain Detection (EMDD), which permits the quantitative assessment of ECM production via flow cytometry [27]. COL I matrix domains were verified by immunofluorescent staining (Fig. S5) and quantified by flow cytometry. As expected, LPA treatment increased the percentage of chondrocytes depositing COL I-positive matrix domains (Fig. 5A and B).

Specific inhibitors for G-protein interaction with LPA G-protein coupled receptors (Pertussis Toxin), PI3 kinase (PI 828), MEK1/MEK2 (U0126) and p38 MAP kinase (SB230580) completely abolished the increase of COL I production induced by LPA, while the selective inhibitors for Phospholipase C (U73122) and Rho kinase (Y27623) did not block the effects of LPA. Next, we focused on PI3 kinase (PI3K) and p38 MAP kinase (p38 MAPK) pathways, as they demonstrated the most prominent inhibition of COL I deposition (Fig. 5A and B). qPCR confirmed that inhibition of PI3K, MEK1/MEK2 and p38 MAPK abolished the stimulatory effects of LPA on *COL I* gene expression (Fig. 5C). Using the same methods, we also tested the effects of the inhibitors alone on COL I deposition. Our data showed that blocking of PI3 kinase significantly reduced the COL I expression at both mRNA and protein level (Fig. S6). Next, we studied phosphorylation of PI3K and p38 MAPK in chondrocytes in response to LPA treatment at different time points by immunofluorescent staining using antibodies against phospho-PI3K and phospho-p38 MAPK. As shown in Fig. 5D, phosphorylation of both kinases peaked at 1 hour after LPA treatment and remained high for p38 MAPK for up to 24 hours, while phosphorylation levels of PI3K steadily decreased after 2 hours. These data independently validate the increase in COL I following LPA treatment of chondrocytes and identify the PI3K and MAPK signaling pathways as critical transducers of this effect.

Pharmacological inhibition of the ATX/LPA signaling reduces COL I synthesis and fibrosis in osteo-chondral defects

Finally, we studied the effects of ATX and LPA inhibition on the COL I deposition at the site of cartilage injury *in vivo*. BrP-LPA was administrated via local intra-articular application using PLGA microspheres loaded with BrP-LPA in combination with systemic delivery of the drug via IP injection. Our data demonstrated that PLGA microspheres loaded with a fluorescent analog of LPA provide prolonged release of the drug for up to 7 days in culture (Fig. S6). Control animals were injected with PLGA microspheres without the drug and also given IP injections of physiological saline. Three weeks post-surgery, COL I deposition in the defects was markedly inhibited by BrP-LPA (Fig. 6A). Notably, control defects were filled with fibrotic tissue rich in COL I fibers with a minimal cellular component, while BrP-LPA treated animals showed significantly higher numbers of cells expressing COL II but minimal levels of COL I in the area of the defect (Fig. 6A and B). These experiments revealed significant improvement of chondrogenesis though the

increased COL II deposition and tissue remodeling as well as reduction of the excessive COL I fibers in the site of injury in the presence of BrP-LPA.

Discussion

The data presented here define a direct mechanistic role of the ATX/LPA axis in the regulation of COL I biosynthesis both *in vitro* and *in vivo* in chondrocytes and BMSCs. Inhibitors of the MAPK and PI3K signaling pathways abolished LPA-induced COL I deposition by chondrocytes, indicating that both of these signaling pathways play a role in transmitting the intracellular effects of LPA on COL I biosynthesis. Our study also demonstrates that the ATX/LPA axis is activated after acute full-thickness cartilage injury resulting in deposition of fibrotic tissue at the site of injury. Inhibition of the ATX/LPA axis in injured rats significantly improved chondrogenesis at the site of injury, reducing COL I deposition and fibrotic tissue formation.

One of the major differences between fibrocartilage and hyaline cartilage is the composition and organization of collagen fibers. The reparative fibrocartilage that fills full-thickness cartilage defects contains very high levels of biologically inert and disorganized COL I, while normal hyaline cartilage is primarily comprised of COL II. In acute injury, COL I is secreted in large quantities as an important component in scar tissue to fill the gap in damaged tissue [28]. However, rapid generation of biomechanically inferior disorganized fibrotic tissue impairs the function of the organ and tissue over the long term. Fibrocartilage has been shown to have inferior biomechanical properties as compared to native hyaline cartilage and this may explain why the clinical results of microfracture surgery deteriorate with time [29].

MAPK and PI3K signaling pathways have been previously shown to regulate COL I biosynthesis in various cells types. In the current study, PI3K and p38 MAPK inhibitors significantly reduced COL I synthesis stimulated by LPA. Using a mouse model of injured skeletal muscle, Li et al. demonstrated that the PI3K/Akt signaling pathway plays an important role in scar tissue formation after acute contusion in mouse skeletal muscle by increasing COL I production. Blocking of PI3K by chemical inhibitors decreased scar tissue formation, which improved the function of injured skeletal muscle [30]. Our study demonstrates that the MAPK and PI3K pathways are implicated in the regulation of COL I biosynthesis by LPA in chondrocytes and may also be relevant targets to prevent fibrocartilage generation following injury or microfracture.

As one of the most popular bone marrow stimulation techniques [31], microfracture has been demonstrated to give good clinical results in 60–80% of patients [32, 33]. Evidence has shown that patients under 40 years old benefit the most from microfracture, possibly due to their intrinsically superior healing capacity as compared to older patients and the larger pool of available progenitor cells [34]. However, microfracture is not a curative treatment but rather a palliative procedure with clinical results deteriorating over time. Doubts remain over the durability of the repaired tissue produced, which is fibrocartilagenous tissue with its inferior biomechanical properties compared with hyaline tissue. Mithoefer et al. (2009) conducted a systematic review examining the clinical efficacy of microfracture in the knee, in which six

randomized control trials (RCT) were identified. All trials showed improved knee function during the first 24 months post-operation, but inconsistency in the long term subjective outcomes[35]. The current study describes a novel molecular mechanism regulating COL I deposition by chondrocytes and BMSCs in injured articular cartilage, which may potentially improve efficacy of microfracture. Reducing excessive COL I deposition and fibrocartilage formation via the selective blockade of ATX and/or LPA receptors or downstream targets of LPA signaling may significantly improve the quality of cartilage tissue formed after microfracture surgery. Previously published data suggest that paracrine interaction between chondrocytes and BMSCs may also enhance cartilage regeneration via trophic/supportive effect of BMSCs [36–38] and it is interesting to speculate that reduction of fibrosis may indirectly contribute to this paracrine crosstalk.

Recently published reports also demonstrate that ATX is highly upregulated in synovial fibroblasts from animal models of inflammatory arthritis [39]. Conditional knockout of ATX in synovial mesenchymal cells delays the process of rheumatic arthritis (RA) in animal models [40]. As a major driving factor for this arthritic model, tumor necrosis factor alpha (TNF-α) was shown to induce ATX expression at both the mRNA and protein level. It is believed that the synergistic effect of TNF-α on ATX expression is mediated NF-κB signaling pathway [41]. Not only TNF-α, but also other inflammatory cytokines, are reported to increase ATX expression [42,43]. Moreover, significant amounts of ATX were also detected in the serum, synovial fluid and synovial fibroblasts in human RA patients [44]. It is plausible to predict that LPA may contribute to the pathogenesis of joint stiffness in this group of patients as well. By selective inhibition of ATX/LAP signaling pathway, the risks of developing rheumatic arthritis due to exposure to inflammatory factors after injury may be reduced.

In conclusion, the current study demonstrates that LPA signaling plays a major role in the regulation of COL I biosynthesis by articular chondrocytes and BMSC both *in vivo* and *in vitro*. Our experimental data suggest that controlled inhibition of the ATX/LPA axis may significantly improve the quality of regenerated cartilage tissue via reduced COL I production and fibrosis in the site of injury and thus improve the existing clinical approaches for cartilage restoration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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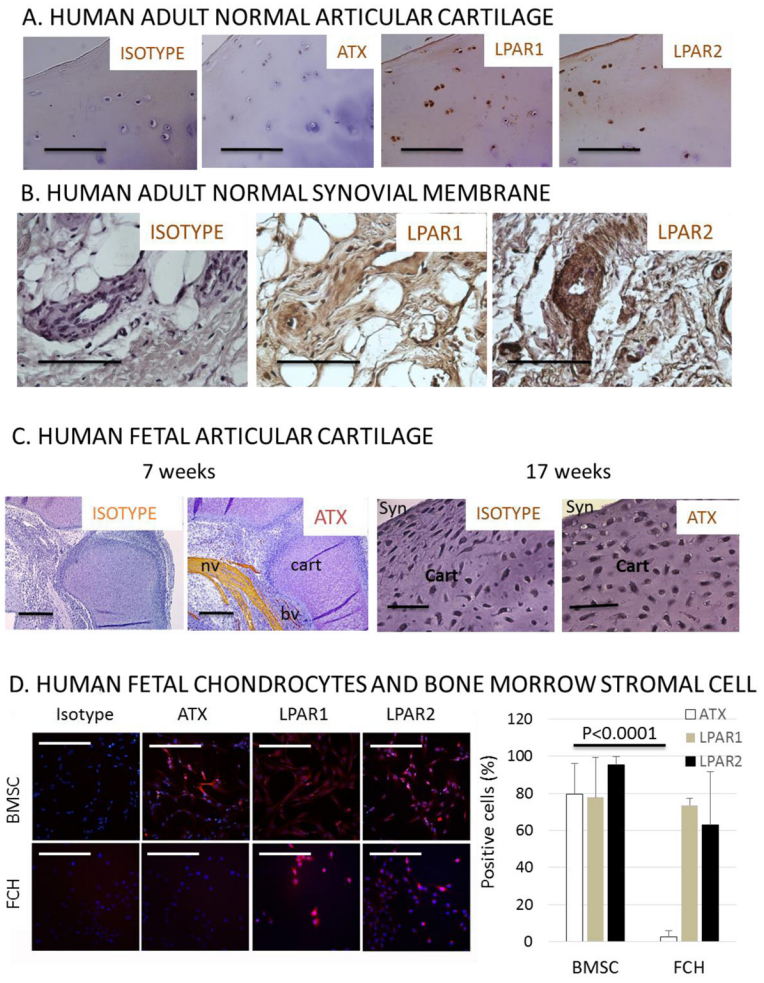

Figure 1. ATX expression is limited to bone marrow stromal cells in normal human joints

(A–B) Human adult articular chondrocytes express minimal levels of ATX, while the LPA receptors LPAR 1 and 2 are highly expressed by chondrocytes and synovial cells. (C–D) Fetal articular chondrocytes (FCH) do not express ATX but do express both LPAR1 and LPAR2; bone marrow stromal cells (BMSCs) express all three components of the ATX pathway. Positive signal is shown in RED, nuclei are counterstained with Dapi. nv=neural fiber, bv=blood vessel, Cart = cartilage, Syn = synovium; scale bars = 50 μm. Data presented as mean ± standard deviation (SD).

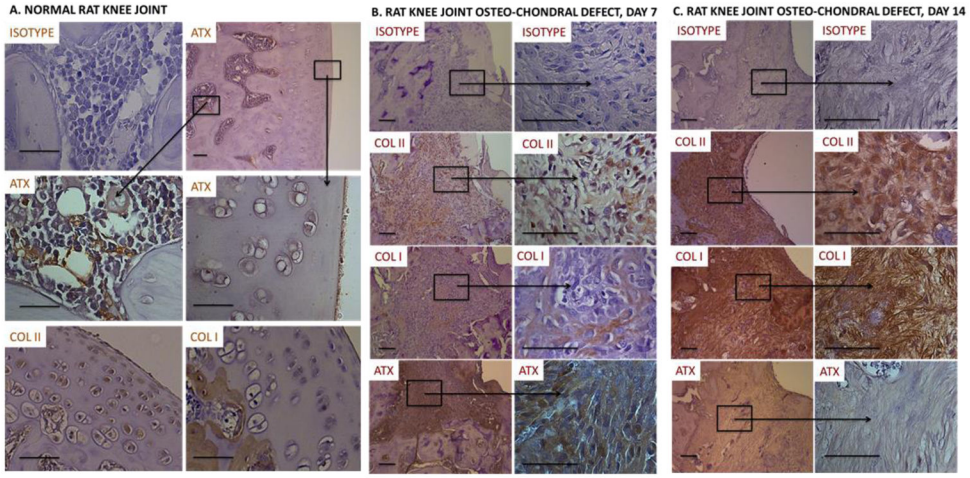

Figure 2. Fibrocartilage formation during the healing of full-thickness cartilage defects in rat knee joints

(A) Control rat knee joints only evidence ATX expression in bone marrow stromal cells, while articular chondrocytes express collagen II (COL II) and low levels of collagen I (COL I). (B) Seven days after the creation of full-thickness defects, ATX and COL I are abundantly expressed in the fibrotic tissue. By day 14 after injury (C), ATX expression had decreased in the injured area, while a rich fibrocartilagenous matrix highly positive for COL I was deposited. Arrows indicate higher magnification images of the boxed area. Scale bars = 50μm.

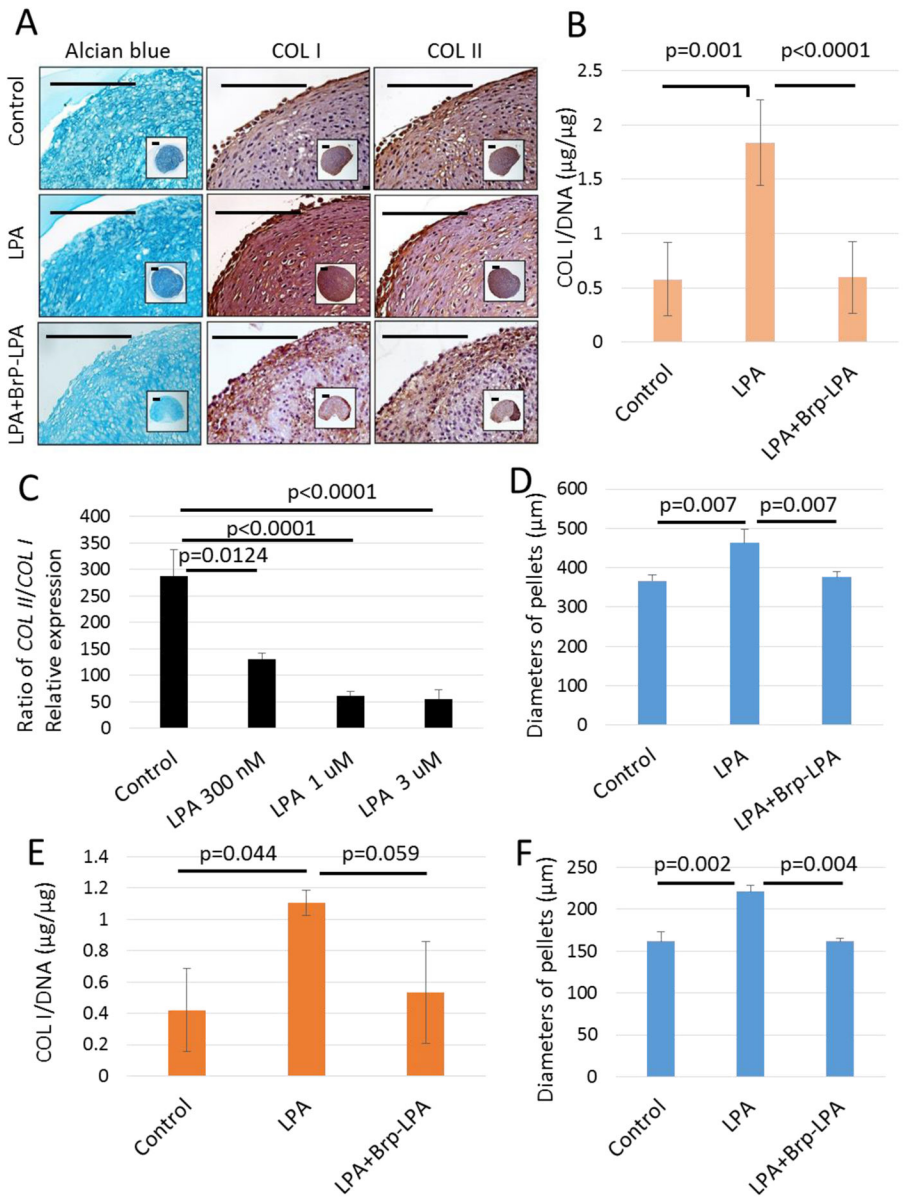

Figure 3. LPA treatment increases COL I expression by cultured human chondrocytes and BMSc

(A) Chondrocyte pellets cultured in the presence of LPA for 3 weeks deposit increased levels of COL I and evidence reduced levels of COL II; inclusion of the LPAR/ATX inhibitor BrP-LPA prevents these changes. Scale bar = 100 μm. (B) Quantitative analysis by ELISA demonstrated increased COL I in pellets cultured with LPA and reversal of this effect when BrP-LPA was included. The levels of COL I were normalized to total DNA of pellets (N = 4). (C) qPCR showed the ratio of COL II to COL I gene expression decreased after LPA treatment in a dose-dependent manner. (D) Pellets treated with LPA were larger than controls and this increase in size was dependent on LPA signaling (N = 4). (E) Quantitative analysis by ELISA demonstrated increased COL I in BMSCs pellet cultured with LPA and reversal of this effect when BrP-LPA was included. The levels of COL I were

normalized to total DNA (N = 4). (F) BMSCs treated with LPA were larger than controls and this increase in size was dependent on LPA signaling (N = 4). Data presented as mean ± standard deviation (SD).

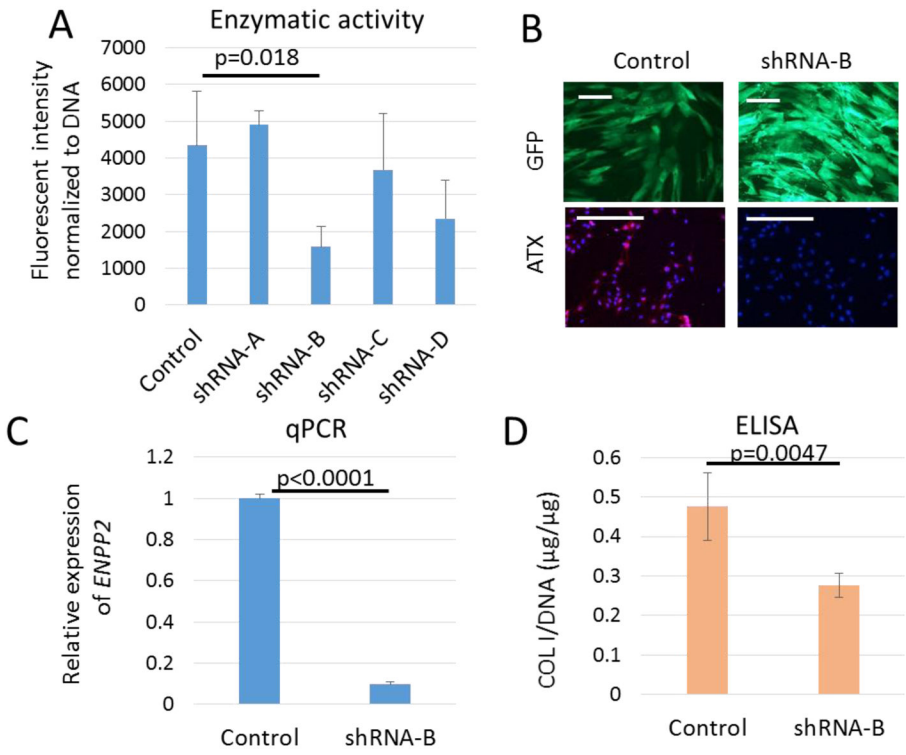

Figure 4. Knock down of ATX by shRNA reduces COL I deposition in pellets of fetal BMSCs

(A) Fluorogenic autotaxin substrate FS-3 was added to the medium of fetal BMSCs transdued by shRNA constructs. After 5 days, fluorescent intensity of medium was measured to reflect the activity of autotaxin. Total DNA was measured to for normalization (N = 4). (B)Fetal BMSCs were transduced by virus containing shRNA-B construct. GFP indicates the efficiency of transduction. Immunofluorescent staining against ATX shows knock down of ENPP2 gene in transduced cells. Scale bar = 50 μm. (C) qPCR showed the gene expression of ATX decreased after transduction of shRNA constructs (N=3). (D) Quantitative analysis by ELISA demonstrated decreased COL I in pellets made by shRNA-B transduced BMSCs. The levels of COL I were normalized to total DNA (N = 4). Data presented as mean ± standard deviation (SD).

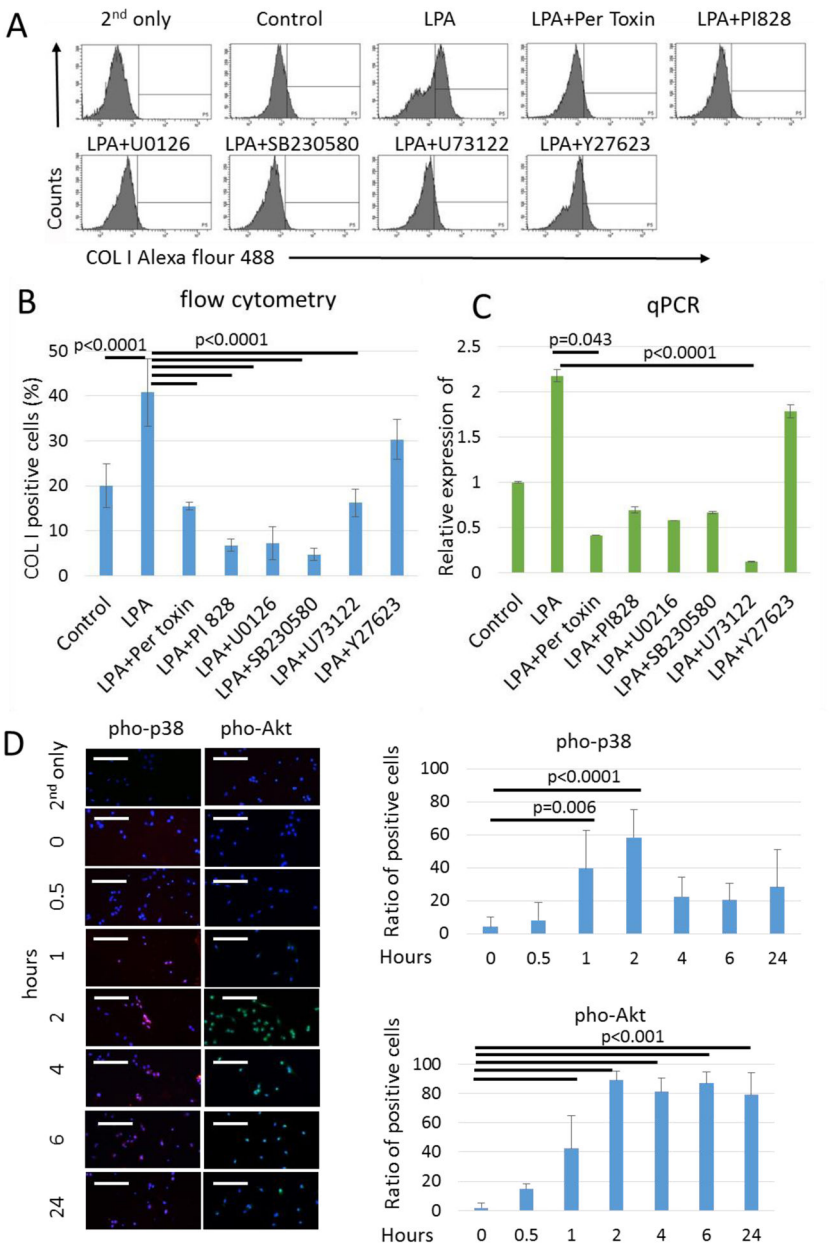

Figure 5. MAPK and PI3K signaling pathways mediate the effect of LPA on chondrocytes

(A) Analysis of COL I levels on chondrocytes using flow cytometry revealed that chemical inhibitors of the MAP (SB230580) and PI3 (PI 828) kinase pathways inhibited the increase of COL I protein following LPA treatment. Pertussis Toxin (Pert Toxin) inhibits interaction of G-proteins with G-protein coupled LPA receptors. Representative data of three replicates is shown. (B) Quantification of COL I+ cells as measured by flow cytometry (Mean ± S.D; N=3). (C) qPCR confirmed the function of the PI3K and MAPK pathways in transducing the effects of LPA on COL I gene expression (N=3). (D) LPA treatment induces phosphorylation of p38 MAPK (pho-p38) and PI3K (pho-Akt). LPA treatment of chondrocytes induced immediate phosphorylation of p38 MAPK (red) and PI3K (green);

activation of the PI3K pathway persisted longer after a single LPA treatment than MAPK. Nuclei are counterstained with Dapi (blue). Bar=25μm

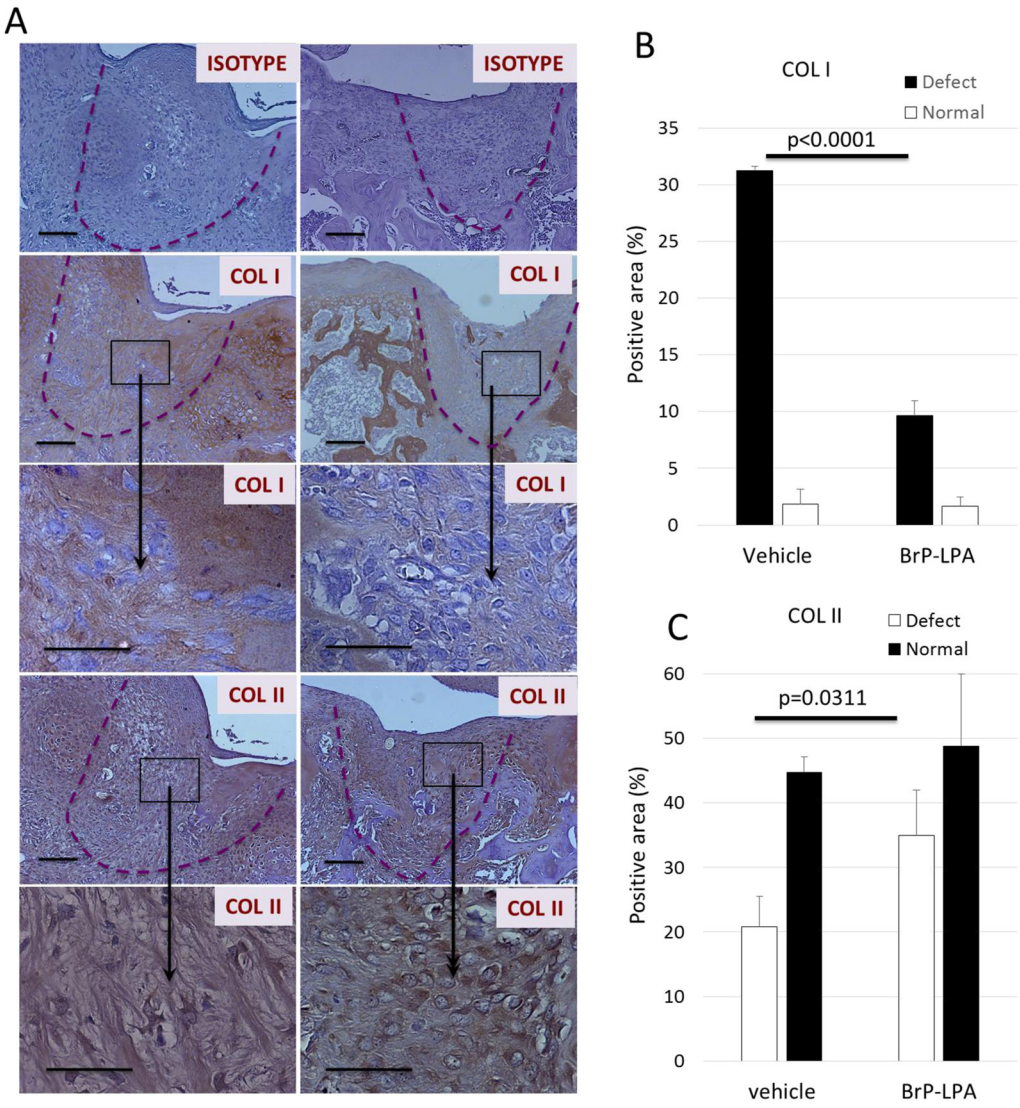

Figure 6. Pharmacological inhibition of the ATX/LPA axis reduces COL I deposition in the site of joint injury

(A) Three weeks following full-thickness oseto-chondral defects, rat knee joints treated with BrP-LPA showed less COL I accumulation and fiber formation while evidencing increased COL II deposition. Dashed line shows the initial site of injury. Arrows indicate enlarged images of the box area. Scale bar = 50 μm. (B) Quantitative analysis documented the concomitant decrease in COL I and increase in COL II following BrP-LPA treatment.