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Brief Report

Upregulation of lipocalin-2 (LCN2) in osteoarthritic cartilage is not necessary for cartilage destruction in mice

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SUMMARY

Objective: Lipocalin-2 (LCN2) is a recently characterized adipokine that is upregulated in chondrocytes treated with pro-inflammatory mediators and in the synovial fluid of osteoarthritis (OA) patients. Here, we explored the *in vivo* functions of LCN2 in OA cartilage destruction in mice.

Methods: The expression levels of LCN2 were determined at the mRNA and protein levels in primary cultured mouse chondrocytes and in human and mouse OA cartilage. Experimental OA was induced in wild-type (WT) or *Lcn2*-knockout (KO) mice by destabilization of the medial meniscus (DMM) or intra-articular (IA) injection of adenoviruses expressing hypoxia-inducible factor (HIF)-2α (Ad-*Epas1*), ZIP8 (Ad-*Zip8*), or LCN2 (Ad-*Lcn2*). The effect of LCN2 overexpression on the cartilage of WT mice was examined by IA injection of Ad-*Lcn2*.

Results: LCN2 mRNA levels in chondrocytes were markedly increased by the pro-inflammatory cytokines, interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), and by previously identified catabolic regulators of OA, such as HIF-2α and components of the zinc-ZIP8-MTF1 axis. LCN2 protein levels were also markedly increased in human OA cartilage and cartilage from various experimental mouse models of OA. However, overexpression of LCN2 in chondrocytes did not modulate the expression of cartilage matrix molecules or matrix-degrading enzymes. Furthermore, LCN2 overexpression in mouse cartilage via IA injection of Ad-*Lcn2* did not cause OA pathogenesis, and *Lcn2* KO mice showed no alteration in DMM-induced OA cartilage destruction.

Conclusions: Our observations collectively suggest that upregulation of LCN2 in OA cartilage is not sufficient or necessary for OA cartilage destruction in mice.

Introduction

During osteoarthritis (OA) pathogenesis, chondrocytes produce matrix-degrading enzymes, including matrix metalloproteinase (MMP) 3, MMP13, and ADAMTS5, and cease synthesizing cartilage matrix molecules, such as type II collagen and aggrecan[1]. Many soluble mediators have been reported to regulate these processes in chondrocytes. For instance, hypoxia-inducible factor (HIF)-2α, which is transcriptionally upregulated in OA chondrocytes in response to mechanical stress and pro-inflammatory cytokines, increases the expression of matrix-degrading enzymes[2,3]. Upregulation of the zinc importer, ZIP8, and its influx of zinc into chondrocytes also induces matrix-degrading enzymes and represses cartilage matrix synthesis[4,5]. Many studies have revealed that adipokines, which are mainly produced in white adipose tissue, are also produced in chondrocytes, where they act as soluble mediators of OA pathogenesis by regulating inflammation and cartilage destruction[6,7]. For example, visfatin causes OA cartilage destruction in mice by upregulating MMP3, MMP12, and MMP13 in chondrocytes[3].

Lipocalin-2 (LCN2; also known as neutrophil gelatinase-associated lipocalin, or NGAL) is a secreted glycoprotein that has been characterized as an adipokine. It binds to small substances, such as steroids and lipopolysaccharide (LPS), to mediate various physiological and pathophysiological processes, including metabolic homeostasis, apoptosis, and immune responses[6]. Although white adipose tissue is the main source of LCN2[6], the protein is also expressed in articular chondrocytes. Studies have shown that

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LCN2 expression in chondrocytes is increased by interleukin (IL)-1β, adipokines (leptin and adiponectin), LPS, and dexamethasone[6,8,9]. In addition, a complex comprising LCN2 and MMP9 is reportedly enriched in the synovial fluid of human OA knees, where it contributes to matrix degradation[10]. Thus, LCN2 is believed to play a significant functional role in OA cartilage[11] and may hold promise as a biomarker for cartilage degradation in arthritic disease[12].

Although LCN2 is believed to be associated with OA pathogenesis, however, no previous study has examined its effects on catabolic or anabolic genes during OA pathogenesis, and its *in vivo* functions in OA pathogenesis are unknown. Here, we examined the *in vivo* functions of LCN2 in mouse models of OA cartilage destruction. We report that although LCN2 is upregulated in OA cartilage, its upregulation is not sufficient or necessary for post-traumatic OA cartilage destruction in mice.

Materials and methods

Human OA cartilage and experimental OA in mice

Human OA cartilage was sourced from individuals undergoing arthroplasty, as described previously[2–5,13]. The Review Board of the Wonkwang University Hospital approved the use of these materials, and all individuals provided full written informed consent before the operative procedure. Male mice (C57BL/6) were used for experimental OA studies. *Lcn2*-knockout (KO) mice were obtained from Jackson Laboratory. All experiments were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee. Experimental OA was induced by destabilization of the medial meniscus (DMM) surgery or intra-articular (IA) injection of adenoviruses (once weekly for 3 weeks, 1×10^9 plaque forming units) expressing HIF-2α (Ad-*Epas1*), ZIP8 (Ad-*Zip8*), or LCN2 (Ad-*Lcn2*) (Vector Biolabs), as described previously[2–5,13]. At 8 weeks and 10 weeks after DMM surgery or 3 weeks and 8 weeks after the first IA injection, mice were sacrificed, and histological and biochemical analyses were performed. Cartilage destruction in mice was examined using alcian blue or safranin-O staining, and scored using the Osteoarthritis Research Society International (OARSI) grading system[2–5,13]. Cartilage sections were immunostained for HIF-2α (ab8365; Abcam), ZIP8 (sc-133415; Santa Cruz Biotechnology), and LCN2 (ab63929; Abcam).

Chondrocytes, adenoviral infection, and biochemical analysis

Chondrocytes were isolated from the femoral condyles and tibial plateaus of mice, and maintained as described previously[2–5,13]. On day 2 of culture, chondrocytes were treated as indicated or infected with adenoviruses at the indicated multiplicity of infection (MOI). Mouse LCN2 mRNAs were detected by reverse-transcription-polymerase chain reaction (RT-PCR) and measured by quantitative RT-PCR (qRT-PCR) using specific primers (sense 5′-AGACTTCCGGAGCGATCAGT-3′; antisense, 5′-ACTGGTTGTAGTCCGTGGTG-3′). The primer sequences for the other indicated transcripts were as described previously[2–5,13]. LCN2 protein levels were detected by Western blotting (ab63929; Abcam).

Statistical analysis

The *n* number indicated in each figure corresponds to the number of statistically independent observations or the number of mice used. Normal distribution of the data was confirmed using the Shapiro–Wilk test. For all qRT-PCR data, two-way analysis of variance (ANOVA) with *post-hoc* tests was used. Data quantified on an ordinal grading system, such as the OARSI grade, were analyzed using non-parametric Mann–Whitney *U* test. All data are presented as the mean and the 95% confidence intervals (CIs).

Results

LCN2 is upregulated in OA chondrocytes and cartilage

RT-PCR and qRT-PCR analyses revealed that mRNA levels of LCN2 were markedly increased in primary cultured mouse articular chondrocytes treated with IL-1β, tumor necrosis factor (TNF)-α, HIF-2α (encoded by *Epas1*)[2], and ZIP8[4] [Fig. 1(A) and (B)]. The protein levels of LCN2 were also markedly elevated in OA-affected regions of human cartilage, compared with undamaged areas of the same sample [Fig. 1(C)], and in OA cartilage from mice subjected to DMM surgery or IA injection of Ad-*Epas1* or Ad-*Zip8* [Fig. 1(D)]. These results suggest that LCN2 expression in chondrocytes might contribute to the pathogenesis of OA in mouse models of this disease.

Upregulation of LCN2 is not sufficient or necessary for OA cartilage destruction in mice

Based on the above results, we assessed whether LCN2 could regulate OA cartilage destruction. We first examined the ability of LCN2 to regulate the expression levels of matrix-degrading enzymes and cartilage matrix molecules in primary cultured mouse articular chondrocytes. However, the Ad-*Lcn2*-induced overexpression of LCN2 in chondrocytes did not alter the expression levels of catabolic matrix-degrading enzymes (e.g., MMP3, MMP13, and ADAMTS5) or anabolic cartilage matrix molecules (e.g., type II collagen or aggrecan) [Fig. 2(A)].

Next, we performed IA injection of Ad-*Lcn2* into mouse knee joints and examined the *in vivo* role of LCN2 in OA pathogenesis at 3 weeks or 8 weeks after IA injection. Consistent with our previous demonstration that this method could yield effective gene delivery[2–5,13], Ad-*Lcn2* injection caused LCN2 to be overexpressed in the cartilage, meniscus and synovium of injected joints [Fig. 2(B)]. However, cartilage destruction or synovial inflammation was not observed at both 3 weeks and 8 weeks after IA injection [Fig. 2(C) and (D)], indicating that LCN2 overexpression is not sufficient to initiate OA pathogenesis in this system.

Finally, we examined whether LCN2 expression is necessary for OA pathogenesis. However, we found that the siRNA-mediated knockdown of LCN2 in primary cultured mouse chondrocytes did not affect the IL-1β-induced upregulation of matrix-degrading enzymes or the concurrent downregulation of cartilage matrix molecules [Fig. 2(E)]. Similarly, knockdown of LCN2 did not affect HIF-2α- or ZIP8-modulated expression of catabolic and anabolic factors in chondrocytes [Fig. 2(E)]. Furthermore, WT and *Lcn2*-KO mice exhibited similar degrees of cartilage destruction and other manifestations of OA (e.g., osteophyte formation and subchondral bone sclerosis) at 8 weeks [Fig. 2(F) and (G)] or 10 weeks (data not shown) after DMM surgery. These results collectively suggest that LCN2 is not necessary for post-traumatic OA in mice.

Discussion

Several studies have suggested that LCN2 may be associated with OA pathogenesis. For example, pro-inflammatory mediators

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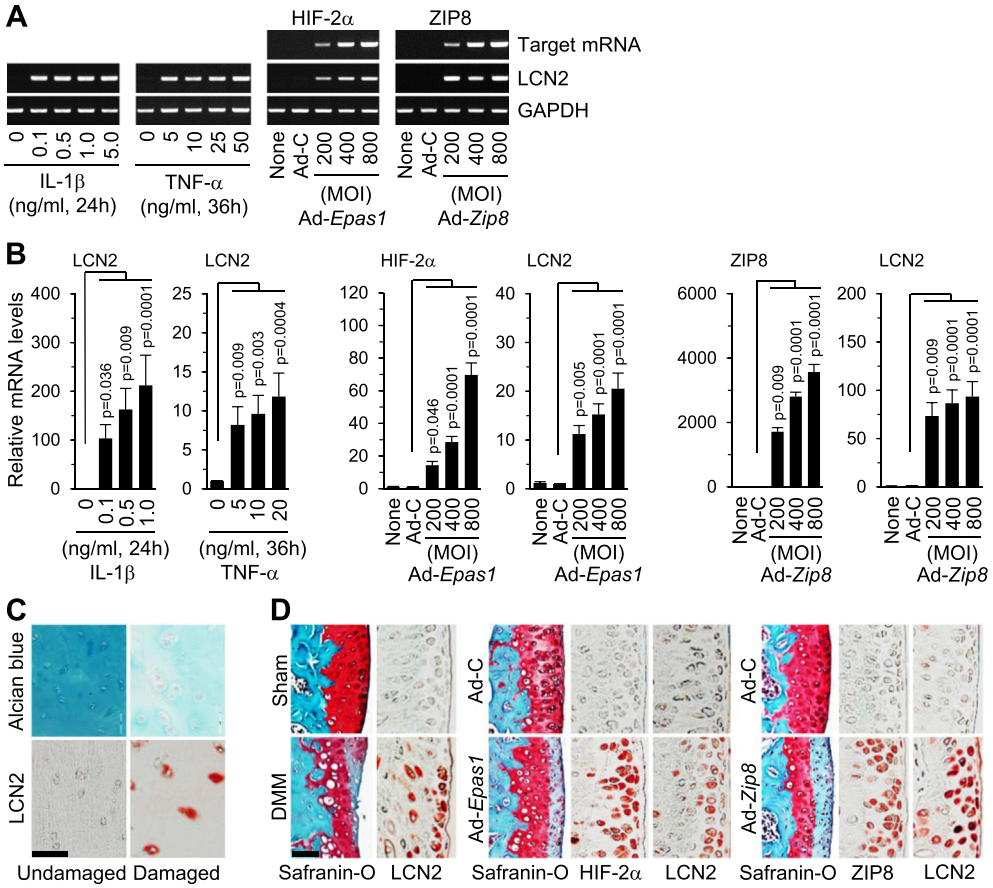

Fig. 1. LCN2 is upregulated in OA chondrocytes and cartilage. (A and B) LCN2 mRNA levels were detected by RT-PCR (A) and quantified by qRT-PCR (B) in chondrocytes stimulated with IL-1β ($n = 5$) and TNF-α ($n = 5$) or infected with the indicated MOIs of Ad-*Epas1* ($n = 7$) and Ad-*Zip8* ($n = 7$). (C) Representative images of alcian blue staining and immunostaining of LCN2 in damaged and undamaged regions of human OA cartilage ($n = 6$). (D) Representative images of safranin-O staining and immunostaining of LCN2 in cartilage sections from mice subjected to sham operation ($n = 4$), DMM surgery ($n = 10$), or IA injection with 1×10^9 plaque forming units of Ad-C ($n = 10$), Ad-*Epas1* ($n = 10$) or Ad-*Zip8* ($n = 10$). Values are the means ±95% CI. Scale bar: 50 μm.

(e.g., IL-1β, leptin, adiponectin, etc.) were shown to increase LCN2 expression in chondrocytes[6,8,9], and upregulation of LCN2 was found in synovial fluid from human OA patients[10]. However, the cause-and-effect relationship between LCN2 and OA pathogenesis was unknown, as was the function of LCN2 in OA pathogenesis *in vivo*. Here, we report that HIF-2α and ZIP8, two recently identified catabolic regulators of OA[2,4], also upregulate LCN2 in chondrocytes. Although LCN2 expression is increased in OA chondrocytes and cartilage, however, we found that LCN2 alone is neither sufficient nor necessary for post-traumatic OA cartilage destruction in mice.

We first employed IA injection of Ad-*Lcn2* to examine the *in vivo* function of LCN2. We previously used this approach to identify numerous catabolic regulators of OA, including HIF-2α, NAMPT, ZIP8, MTF1, metallothionein 2, and lysyl oxidase[2–5,13]. Here, we found that ectopic expression of LCN2 alone in the knee joint tissues of mice was not sufficient to cause cartilage destruction. This is consistent with our finding that overexpression of LCN2 in chondrocytes failed to alter the expression of matrix-degrading enzymes *in vitro*. Although LCN2 post-translationally regulates MMP9 activity[10], we found that it did not alter the chondrocytic expression levels of MMP3, MMP13 or ADAMTS5, all of which play crucial roles in cartilage destruction[1]. Moreover, we found that KO of *Lcn2* in mice did not alter OA in the DMM mouse model, indicating that the upregulation of LCN2 in OA cartilage is not a prerequisite for cartilage destruction. We examined cartilage destruction at 8 or 10 weeks after DMM surgery. Although it is possible that LCN2 may modify disease severity in later stages, we have previously found that KO of HIF-2α or ZIP8 effectively blocked cartilage destruction at 8 weeks after DMM surgery[2,4]. Consistent with our *in vivo* results, LCN2 knockdown in chondrocytes did not alter the IL-1β-, HIF-2α-, or ZIP8-induced upregulations of MMP3, MMP13, or ADAMTS5.

The expression of LCN2 is altered in several inflammatory diseases, and this factor has been proposed as a potential biomarker for various diseases, including acute kidney injury, lupus nephritis, and obesity-related metabolic diseases[6]. Although our results indicate that LCN2 is not a catabolic regulator of OA pathogenesis, its expression is upregulated in chondrocytes stimulated with various OA-causing catabolic regulators, and in the OA cartilage of human patients and various experimental mouse models of OA. This is consistent with the notion that LCN2 could be a possible biomarker for cartilage degradation in arthritic disease[12].

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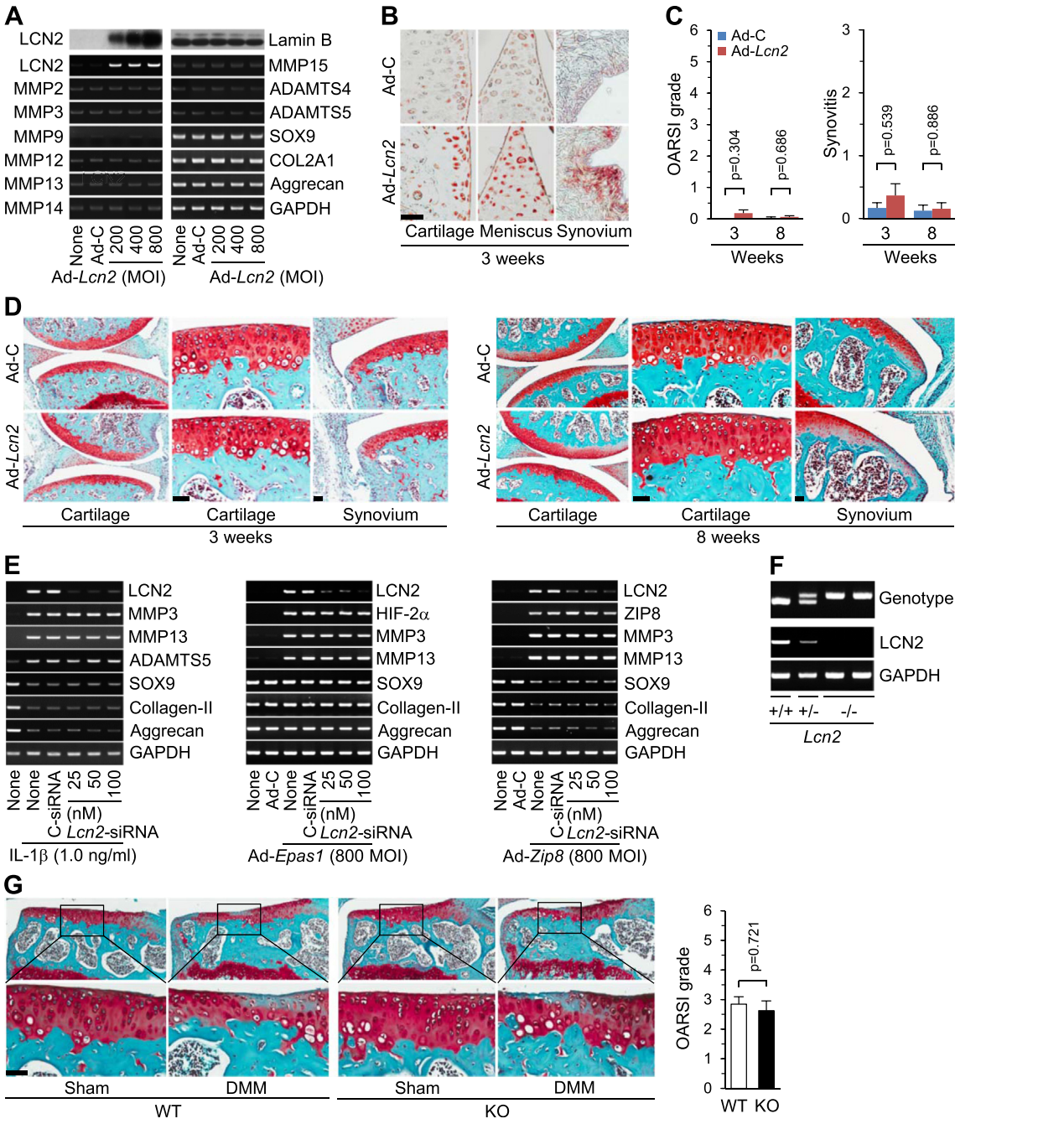

Fig. 2. Upregulation of LCN2 is not sufficient or necessary for OA cartilage destruction in mice. (A) Protein and mRNA levels of LCN2 in chondrocytes infected with Ad-C (800 MOI) or the indicated MOI of Ad-*Lcn2* ($n = 6$). (B–D) Mice were IA-injected with Ad-C or Ad-*Lcn2* (1×10^9 PFU, once per week for 3 weeks), and sacrificed 3 or 8 weeks after the first injection. Representative images of immunohistochemical staining for LCN2 in the joint tissues (B; $n = 10$). OARSI grade and synovial inflammation (C; $n = 10$). Cartilage destruction and synovitis detected by safranin-O/hematoxylin staining (D; $n = 10$). (E) RT-PCR analysis of the indicated mRNAs in chondrocytes transfected with or without the *Lcn2* siRNA and further treated with IL-1β (left; $n = 5$) or infected with 800 MOI of Ad-*Epas1* (middle; $n = 5$) or Ad-*Zip8* (right; $n = 5$). (F) Genotype and LCN2 mRNA levels in chondrocytes of WT and *Lcn2* KO mice. (G) Cartilage sections from sham- and DMM-operated WT ($n = 10$) and *Lcn2*-KO mice ($n = 10$) were stained with safranin-O, and cartilage destruction was quantified by OARSI grade. Values are the means ±95% CI. Scale bar: 50 μm.

Author contributions

WSC designed the study, acquired, analyzed and interpreted the data, and prepared and approved the manuscript. JSC acquired the funding, designed the study, interpreted the data, and prepared the manuscript. JSC (jschun@gist.ac.kr) takes responsibility for the integrity of this work.

Conflict of interest

The authors have no conflict of interest.

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