BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-18-058

Title: 29-kDa FN-f inhibited autophagy through modulating localization of HMGB1 in human articular chondrocytes

Article Type: Article

Keywords: Fibronectin fragment; Fibronectin fragment; autophagy; chondrocyte; osteoarthritis

Corresponding Author: Hyun Ah Kim

Authors: Hyun Sook Hwang[1,2], Min Ha Choi[1,2], Hyun Ah Kim[1,2,*]

Institution: [1]Department of Internal Medicine, Hallym University Sacred Heart Hospital,
[2]Institute for Skeletal Aging, Hallym University,

29-kDa FN-f inhibited autophagy through modulating localization of HMGB1 in human articular chondrocytes

Hyun Sook Hwang[a,b], Min Ha Choi[a,b], Hyun Ah Kim[a,b] *

[a]Division of rheumatology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, Kyunggi, 14068, Korea; [b]Institute for Skeletal Aging, Hallym University, Chunchon 24251, Korea

Running title: autophagy signaling regulated by 29-kDa FN-f

Keywords: Fibronectin fragment, HMGB1, autophagy, chondrocyte, osteoarthritis

***Corresponding author:**

Hyun Ah Kim

Division of rheumatology, Department of internal medicine, Hallym University Sacred Heart Hospital, 896, Pyungchon, Anyang, Kyunggi, 14068, Korea

Phone: 82-31-380-1826; Fax: 82-31-381-8812; E-mail: kimha@hallym.ac.kr

ABSTRACT

Fibronectin fragments found in synovial fluid of patients with osteoarthritis (OA) induce the catabolic responses in cartilage. Nuclear high mobility group protein Box 1 (HMGB1), a damage-associated molecular pattern, is responsible for the regulation of signaling pathways related with cell death and survival in response to various stimuli. In this study, we investigated whether 29-kDa amino-terminal fibronectin fragment (29-kDa FN-f)-induced change in HMGB1 expression influences the pathogenesis of OA through HMGB1-modulated autophagy signaling pathway. Human articular chondrocytes were enzymatically isolated from articular cartilage. The level of mRNA was measured by quantitative real-time PCR. The expression of proteins was examined by western blot analysis, immnunofluorescence assay, and enzyme-linked immunosorbent assay. Interaction of proteins was evaluated by immunoprecipitation. The HMGB1 level was significantly reduced in human OA cartilage compared to normal cartilage. Although 29-kDa FN-f significantly reduced the HMGB1 expression at the mRNA and protein levels 6 h after treatment, the cytoplasmic level of HMGB1 was increased in 29-kDa FN-f-treated chondrocytes. 29-kDa FN-f significantly inhibited the interaction of HMGB1 with Beclin-1 but increased the interaction of Bcl-2 with Beclin-1, together with decreased levels of Beclin-1 and phosphorylated Bcl-2. In addition, the level of microtubule associated protein 1 light chain 3-II, an autophagy marker, was down-regulated in 29-kDa FN-f-treated chondrocytes, whereas the effect was antagonized by mTOR knockdown. Furthermore, prolonged treatment with 29-kDa FN-f significantly increased the release of HMGB1 into the culture medium. These results demonstrated that 29-kDa FN-f inhibits chondrocyte autophagy through modulation of HMGB1 signaling pathway.

INTRODUCTION

Osteoarthritis (OA), the most common type of degenerative joint disease, causes joint pain and disability (1). Several risk factors, including aging, gender, genetic predisposition, and obesity, are involved in the pathogenesis of OA. Degeneration of cartilage extracellular matrix (ECM), the major component of which are proteoglycans and collagens, is a key pathologic finding in OA. A variety of cytokines, such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α), lead to imbalance of cartilage homeostasis, subsequently degrading ECM by increasing inflammatory mediators, such as nitric oxide (NO) and prostaglandin E_2, and catabolic enzymes, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (2).

Autophagy is an important process for the maintenance of cellular homeostasis in response to various types of stress. Several studies demonstrated that the defect of autophagy process leads to diverse diseases, including neurodegenerative diseases and OA. There is accumulating evidence that disturbance of autophagy is related with chondrocyte death and survival. Because chondrocytes, the single type of cells embedded in articular cartilage, are responsible for ECM homeostasis such as synthesis and turnover, regulation of chondrocyte survival is crucial in the pathogenesis of OA (3). Autophagic markers in articular cartilage of normal human, sham and young mice were higher than those in OA human, surgically-induced-OA and aging mice, respectively, indicating that autophagy may serve as a protective mechanism (4, 5). Autophagy was suppressed in the superficial zone of articular cartilage in response to mechanical injury (6).

Fibronectin (FN) is an ECM glycoprotein present in cartilage and synovial fluid (7). Fibronectin fragments (FN-fs) of 29-, 45-, 120-, and 200-kDa derived from FN are found in OA cartilage and synovial fluid and they stimulate the production of various inflammatory cytokines, such as TNF-α and IL-1β (8, 9). In particular, 29-kDa fibronectin fragment (29-

kDa FN-f), the most potent molecule, is associated with proteoglycan breakdown by upregulating NO, catabolic cytokines, and MMP family proteins in articular chondrocytes (10, 11). Previously, we reported that 29-kDa FN-f stimulates expression of catabolic factors through toll-like receptor 2 (TLR-2)-dependent signaling pathway (12). However, it is unknown whether 29-kDa FN-f regulates autophagy pathway in chondrocytes.

High mobility group box 1 (HMGB1), a non-histone DNA-binding protein in the nuclei, regulates transcription and DNA repair in nucleus (13). Previous studies demonstrated that HMGB1 is a potent regulator of autophagy and translocation of nuclear HMGB1 into cytoplasm by cellular stress induces autophagy (13). Cytoplasmic HMGB1 by binding to Beclin1 leads to autophagy, while a complex of Beclin-1 and Bcl-2 suppresses it (14). In addition, HMGB1 is released into extracellular spaces in response to a variety of stimuli and works as a damage-associated molecular pattern (DAMP). For example, HMGB1 induces cellular senescence after release in a p53-dependent manner (15, 16). Previous report showed that HMGB1 enhances pro-inflammatory responses in chondrocytes in the presence of 29-kDa FN-f compared to treatment with HMGB1 alone (17).

In this study, we investigated whether 29-kDa FN-f regulates HMGB1 expression, its extracellular release and HMGB1-mediated autophagy in human articular chondrocytes.

RESULTS

29-kDa FN-f altered HMGB1 expression in human articular chondrocytes

To reveal the effect of HMGB1 on the pathogenesis of OA, the level of HMGB1 in the normal and OA cartilages was examined. HMGB1 expression in OA cartilage was significantly reduced compared to that of normal cartilage (Fig. 1A). We next examined the effect of 29-kDa FN-f on

HMGB1 expression in primary human chondrocytes. HMGB1 expression was maximally induced at 3 hours in control culture while 29-kDa FN-f suppressed it at 3, 6 and 24 hours (Fig. 1B). On the other hand, the level of HMGB1 protein increased at the early phase (1 and 3 h incubation) and decreased at the late phase (6 and 24 h incubation) in 29-kDa FN-f-treated chondrocytes (Fig. 1C).

29-kDa FN-f induced cytoplasmic translocation of HMGB1 and subsequently released HMGB1 into extracellular space

We examined whether 29-kDa FN-f induces the extracellular secretion of HMGB1. Western blot analysis showed that the level of HMGB1 in the cytoplasmic fractions was increased while that of nuclear fraction decreased at 24 h after stimulation with 29-kDa FN-f (Fig. 2A). Fluorescence microscopy data revealed the downregulation of nuclear HMGB1 in 29-kDa FN-f-stimulated cells (Fig. 2B). Enzyme-linked immunosorbent assay data showed that HMGB1 was released into the culture media throughout the 48 h culture period and 29-kDa FN-f led to its increase (Fig. 2C). The results demonstrate that 29-kDa FN-f stimulates the translocation of nuclear HMGB1 into the cytoplasm and subsequent release of HMGB1 into the extracellular space.

29-kDa FN-f inhibited autophagy through mammalian target or rapamycin (mTOR)/HMGB1-dependent signaling pathway

We investigated whether 29-kDa FN-f inhibits autophagy signaling pathway. We measured the level of microtubule associated protein 1 light chain 3-II (LC3-II), an autophagy marker, using immmunoblot and fluorescence microscopy analysis. The level of LC3-II significantly decreased by 24 h treatment with 29-kDa FN-f (Fig. 3A and B), indicating that 29-kDa FN-f

significantly suppresses autophagy. Western blot analysis demonstrated that 29-kDa FN-f elevated phosphorylation of mTOR, an inhibitor of autophagy (Fig. 3C). In addition, the level of phospho-eIF4E-binding protein 1 (4E-BP1), the substrate of mTOR, was increased in the presence of 29-kDa FN-f (Fig. 3C).

HMGB1 leads to autophagy when it forms a complex with Beclin-1. On the other hand, the association of Beclin-1 and Bcl-2 inhibits autophagy (14, 18). We examined whether 29-kDa FN-f affects the formation of Beclin-1/Bcl-2 and Beclin-1/HMGB1 complex. After 24 h exposure to 29-kDa FN-f, cell lysates were immunoprecipitated with antibodies against Beclin-1 or HMGB1. Immunoprecipitation (IP) data showed that 29-kDa FN-f elevated the formation of Bcl-2/Beclin-1 complex, which inhibits autophagy pathway, and suppressed the formation of HMGB1/Beclin-1 complex, which induces HMGB1-dependent autophagy pathway (Fig. 3D). Therefore, these results demonstrated that 29-kDa FN-f not only activated mTOR signaling pathway, but also induced the interaction of Bcl-2/Beclin-1 rather than the association of HMGB1/Beclin-1 complex, subsequently inhibiting mTOR/HMGB1-dependent autophagy pathway.

mTOR knockdown antagonized 29-kDa FN-f-inhibited autophagy signaling pathway

To determine whether 29-kDa FN-f regulates autophagy and catabolic responses through mTOR signaling pathway, chondrocytes were transfected using si-mTOR or control siRNA, followed by stimulation with 29-kDa FN-f (300 nM) for 24 h. Western blot analysis revealed that mTOR silencing led to the decrease in phosphorylation of 4E-BP1 and the subsequent increase in the level of LC3-II, an autophagy marker, compared to si-control-transfected chondrocytes in control culture (Fig. 4A). While the phosphorylation of 4E-BP1 increased and the expression of LC3-II and HMGB1 decreased in 29-kDa FN-f-treated chondrocytes,

mTOR knockdown attenuated the phosphorylation of 4E-BP1 and reversed the level of LC3-II and HMGB1 (Fig. 4A). In addition, 29-kDa FN-f strongly upregulated MMP-1 and MMP-3, whereas mTOR knockdown significantly suppressed both MMP-1 and -3. These results indicate that 29-kDa FN-f negatively modulated autophagy as well as catabolic response via mTOR signaling pathway.

DISCUSSION

We previously examined that 29-kDa FN-f potently induced the expression of catabolic factors, including MMP-1, -3, and -13 (12). In this study, we investigated whether 29-kDa FN-f regulates HMGB1 expression and cytoplasmic translocation. The modulation of HMGB1-dependent autophagy signaling in human articular chondrocytes was also investigated. Our results show that 29-kDa FN-f elevates extracellular release of HMGB1 and inhibits autophagy signaling via activation of mTOR signaling pathway as well as suppression of HMGB1/Beclin-1 complex (Fig. 4B).

The role of DAMPs such as HMGB1, hyaluronan fragments, and FN-fs in the induction of vicious cycle of OA cartilage destruction has been postulated, because they are produced in the process of matrix degradation and in turn increase expression of inflammatory cytokines and MMPs (19). Increased levels of FN and FN-fs of 30-200 kDa are found in cartilage and synovial fluid from patients with OA and RA (9, 20, 21). In line with its role as a DAMP, 29-kDa FN-f enhanced the expression of MMPs via TLR-2 signaling pathway (12). Of the HMGB protein family members, including HMGB1, 2, 3, and 4, HMGB1 is ubiquitously expressed and up-regulates the expressions of MMP-1, -3, and -9 by interacting with multiple TLRs (22-25). In addition, HMGB1 functions as a nuclear DNA chaperone to support DNA replication within

nucleus (24, 26) and as a protein chaperon to prevent protein aggregation (27). HMGB1 can be translocated from the nucleus to the cytosol and then be extracellularly released during cell death, including necrosis, apoptosis, and pyroptosis (28). Several studies suggested that synovial fluid level of HMGB1 is correlated with OA severity. Research using bovine cartilage and synovial fluid showed that OARSI score of joint correlated with the extent of HMGB1 labeling in the ECM, chondrocyte nuclei and cytoplasm, indicating that the switch of HMGB1 from DNA-binding nuclear protein to an extracellular alarmin/cytokine correlates with the destruction of cartilage (29). In line with the suggested role of HMGB1 as a DAMP, HMGB1 neutralization by injection with anti HMGB1 antibody protected lipopolysaccharide-induced lethality in mice (30). In our data, early treatment with 29-kDa FN-f induced the increased cellular level of HMGB1 and then longer treatment significantly decreased cellular level of HMGB1. It was due to sharp increase in translocation of nuclear HMGB1 into the cytoplasm as well as extracellular release of HMGB1. Our result show that 29-kDa FN-f may function as an amplifier of damage response in OA by upregulating HMGB1 extracellular release.

29-kDa FN-f was also found to inhibit chondrocyte autophagy by inducing the formation of Bcl-2:Beclin-1 than HMGB1:Beclin-1 complex. Because chondrocytes maintain fine balance of synthesis and turnover of ECM, regulation of cell metabolism and survival by autophagy is considered an important mechanism for maintaining cartilage integrity (31). Decreased autophagy and cellularity with increased apoptosis were found in aged mouse knee joints (4). Human OA cartilage and animal OA models revealed highly expressed mTOR, increased apoptosis, and reduced expression of autophagic genes (32). Furthermore, mechanical injury also suppresses autophagy, with decrease of Beclin-1 and LC3 expression, in the superficial zone of cartilage with induction of cell death and loss of soluble glycosaminoglycan (6). In

contrast, another study showed that autophagy in the OA pathogenesis may function differently in young and OA cartilage, leading to chondrocyte protection or death depending on age (33).

Auophagy and apoptosis are modulated by both Bcl-2 and Beclin-1 expression levels and localization of Bcl-2:Beclin-1 complex. In particular, Bcl-2 inhibits assembly of autophagogomal structure by binding to Beclin-1, subsequently suppressing autophagy, while HMGB1 disrupts interaction of Bcl-2:Beclin-1 by competing with Bcl-2 for interaction with Beclin-1 and driving Beclin-1 to autophagosomes, leading to autophagy pathway (14, 34). Several studies showed that mTOR signaling pathway was considered a key regulator of autophagy in OA cartilage. Increase in the expression of mTOR in peripheral blood mononuclear cells of patients were related to disease activity represented by synovitis and significant upregulation of mTOR levels were found in the lesional area of the OA cartilage compared to the non-lesional area (35, 36). Beclin-1 overexpression mitigated phsophoinositol-3 kinase (PI3K) /protein kinase B (AKT)/mTOR signaling pathway, which led to increased cell viability, and inhibition of apoptosis and MMP expression (37). Furthermore, inhibition of mTOR by treatment with rapamycin led to significant suppression of cartilage degeneration in surgically induced OA mouse model and chondroprotection in human OA chondrocytes, (38, 39). In our experiments, 29-kDa FN-f down-regulated LC3-II level through activation of mTOR signaling pathway.

In summary, our results demonstrate that 29-kDa FN-f regulated the expression of HMGB1, the cytoplasmic translocation of HMGB1, and subsequent extracellular secretion of HMGB1. In addition, 29-kDa FN-f inhibits autophagy pathway through modulating mTOR/HMGB1-dependent pathway, thereby conferring the detrimental effect on articular chondrocytes. Modulation of autophagy pathway inhibited by 29-kDa FN-f may be a useful strategy in the treatment of OA cartilage degradation.

MATERIALS AND METHODS

Materials, quantitative real-time reverse transcription polymerase chain reaction analysis, immunofluorescence microscopy, and IP assay are described in the Supplementary Materials.

Primary chondrocytes isolation and culture

OA cartilage samples were obtained from the knee joints of OA patients (n= 20, 74.1 ± 5.4 years of age) at the time of total knee replacement surgery. Patient diagnoses were determined using the criteria set forth by the American College of Rheumatology. Normal cartilage samples were obtained from the femoral head of patients (n= 9, 73.7 ± 10.1 years of age) with femoral neck fractures and no known history of OA or RA. The collection and use of human tissue samples was reviewed and approved by the Institutional Review Board of Hallym University Sacred Heart Hospital, Anyang, Korea (approval number 2013-I022). All patients provided written informed consent for the use of the discarded cartilage samples.

Human primary chondrocytes were isolated from the articular cartilage and cultured as previously described (12).

Western blot analysis

Cell lysates were prepared with radioimmunoprecipitation assay (RIPA) lysis buffer and western blot analysis was performed as described previously (40).

ELISA

The level of HMGB1 released from chondrocytes into culture medium was measured using Human HMGB1 ELISA kits (Elabscience Biotechnology, Houston, TX, USA). Briefly, 100 μl of culture medium was added to each well of 96-well plate and 100 μl of biotinylated detection antibody were also added for 30 min at 37°C. After washing with wash buffer, 100 μl of HRP conjugate was added to each well for 30 min at 37°C followed by incubation with substrate reagent for 15 min at 37°C. The absorbance was measured at 450 nm using ELISA reader.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Mann-Whitney U test or two-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

ACKNOWLEDGEMENTS

This study was supported by a grant (HI16C0287**)** from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea, the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education (2017R1A2B200188), and in part by Hallym University Research Fund

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Goldring MB and Goldring SR (2007) Osteoarthritis. J Cell Physiol 213, 626-634
2. Kim HA, Cho ML, Choi HY et al (2006) The catabolic pathway mediated by Toll-like receptors in human osteoarthritic chondrocytes. Arthritis Rheum 54, 2152-2163
3. Loeser RF (2009) Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. Osteoarthritis Cartilage 17, 971-979
4. Carames B, Olmer M, Kiosses WB and Lotz MK (2015) The relationship of autophagy defects to cartilage damage during joint aging in a mouse model. Arthritis Rheumatol 67, 1568-1576
5. Carames B, Taniguchi N, Otsuki S, Blanco FJ and Lotz M (2010) Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. Arthritis Rheum 62, 791-801
6. Carames B, Taniguchi N, Seino D, Blanco FJ, D'Lima D and Lotz M (2012) Mechanical injury suppresses autophagy regulators and pharmacologic activation of autophagy results in chondroprotection. Arthritis Rheum 64, 1182-1192
7. Wang CC, Lee CH, Peng YJ, Salter DM and Lee HS (2015) Platelet-Rich Plasma Attenuates 30-kDa Fibronectin Fragment-Induced Chemokine and Matrix Metalloproteinase Expression by Meniscocytes and Articular Chondrocytes. Am J Sports Med 43, 2481-2489
8. Xie DL, Hui F, Meyers R and Homandberg GA (1994) Cartilage chondrolysis by fibronectin fragments is associated with release of several proteinases: stromelysin plays a major role in chondrolysis. Arch Biochem Biophys 311, 205-212
9. Xie DL, Meyers R and Homandberg GA (1992) Fibronectin fragments in osteoarthritic synovial fluid. J Rheumatol 19, 1448-1452

10. Homandberg GA and Hui F (1996) Association of proteoglycan degradation with catabolic cytokine and stromelysin release from cartilage cultured with fibronectin fragments. Arch Biochem Biophys 334, 325-331

11. Pichika R and Homandberg GA (2004) Fibronectin fragments elevate nitric oxide (NO) and inducible NO synthetase (iNOS) levels in bovine cartilage and iNOS inhibitors block fibronectin fragment mediated damage and promote repair. Inflamm Res 53, 405-412

12. Hwang HS, Park SJ, Cheon EJ, Lee MH and Kim HA (2015) Fibronectin fragment-induced expression of matrix metalloproteinases is mediated by MyD88-dependent TLR-2 signaling pathway in human chondrocytes. Arthritis Res Ther 17, 320

13. Lee SA, Kwak MS, Kim S and Shin JS (2014) The role of high mobility group box 1 in innate immunity. Yonsei Med J 55, 1165-1176

14. Marquez RT and Xu L (2012) Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. Am J Cancer Res 2, 214-221

15. Davalos AR, Kawahara M, Malhotra GK et al (2013) p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes. J Cell Biol 201, 613-629

16. Jeon OH, Kim C, Laberge RM et al (2017) Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. Nat Med 23, 775-781

17. Ding L, Buckwalter JA and Martin JA (2017) DAMPs Synergize with Cytokines or Fibronectin Fragment on Inducing Chondrolysis but Lose Effect When Acting Alone. Mediators Inflamm 2017, 2642549

18. Yu C, Yu X, Zhu HW et al (2016) Expression pattern of HMGB1 and its association

with autophagy in acute necrotizing pancreatitis. Mol Med Rep 14, 5507-5513

19. Rosenberg JH, Rai V, Dilisio MF, Sekundiak TD and Agrawal DK (2017) Increased expression of damage-associated molecular patterns (DAMPs) in osteoarthritis of human knee joint compared to hip joint. Mol Cell Biochem 436, 59-69
20. Yasuda T (2006) Cartilage destruction by matrix degradation products. Mod Rheumatol 16, 197-205
21. Homandberg GA, Wen C and Hui F (1998) Cartilage damaging activities of fibronectin fragments derived from cartilage and synovial fluid. Osteoarthritis Cartilage 6, 231-244
22. Fang F and Jiang D (2016) IL-1beta/HMGB1 signalling promotes the inflammatory cytokines release via TLR signalling in human intervertebral disc cells. Biosci Rep 36,
23. Park JS, Gamboni-Robertson F, He Q et al (2006) High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol 290, C917-924
24. Stros M (2010) HMGB proteins: interactions with DNA and chromatin. Biochim Biophys Acta 1799, 101-113
25. Taniguchi N, Kawakami Y, Maruyama I and Lotz M (2018) HMGB proteins and arthritis. Hum Cell 31, 1-9
26. Javaherian K, Liu JF and Wang JC (1978) Nonhistone proteins HMG1 and HMG2 change the DNA helical structure. Science 199, 1345-1346
27. Min HJ, Ko EA, Wu J et al (2013) Chaperone-like activity of high-mobility group box 1 protein and its role in reducing the formation of polyglutamine aggregates. J Immunol 190, 1797-1806
28. Magna M and Pisetsky DS (2015) The Role of Cell Death in the Pathogenesis of SLE: Is Pyroptosis the Missing Link? Scand J Immunol 82, 218-224

29. Heinola T, de Grauw JC, Virkki L et al (2013) Bovine chronic osteoarthritis causes minimal change in synovial fluid. J Comp Pathol 148, 335-344

30. Wang H, Bloom O, Zhang M et al (1999) HMG-1 as a late mediator of endotoxin lethality in mice. Science 285, 248-251

31. Almonte-Becerril M, Navarro-Garcia F, Gonzalez-Robles A, Vega-Lopez MA, Lavalle C and Kouri JB (2010) Cell death of chondrocytes is a combination between apoptosis and autophagy during the pathogenesis of Osteoarthritis within an experimental model. Apoptosis 15, 631-638

32. Zhang Y, Vasheghani F, Li YH et al (2015) Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. Ann Rheum Dis 74, 1432-1440

33. Chang J, Wang W, Zhang H, Hu Y, Wang M and Yin Z (2013) The dual role of autophagy in chondrocyte responses in the pathogenesis of articular cartilage degeneration in osteoarthritis. Int J Mol Med 32, 1311-1318

34. Tang D, Kang R, Livesey KM et al (2010) Endogenous HMGB1 regulates autophagy. J Cell Biol 190, 881-892

35. Katsara O, Attur M, Ruoff R, Abramson SB and Kolupaeva V (2017) Increased Activity of the Chondrocyte Translational Apparatus Accompanies Osteoarthritic Changes in Human and Rodent Knee Cartilage. Arthritis Rheumatol 69, 586-597

36. Tchetina EV, Poole AR, Zaitseva EM et al (2013) Differences in Mammalian target of rapamycin gene expression in the peripheral blood and articular cartilages of osteoarthritic patients and disease activity. Arthritis 2013, 461486

37. Song B, Song H, Wang W et al (2017) Beclin 1 overexpression inhibits chondrocyte apoptosis and downregulates extracellular matrix metabolism in osteoarthritis. Mol

Med Rep 16, 3958-3964

38. Carames B, Hasegawa A, Taniguchi N, Miyaki S, Blanco FJ and Lotz M (2012) Autophagy activation by rapamycin reduces severity of experimental osteoarthritis. Ann Rheum Dis 71, 575-581

39. Zhang T, Liu J, Zheng X, Zhang B and Xia C (2018) Different roles of Akt and mechanistic target of rapamycin in serumdependent chondroprotection of human osteoarthritic chondrocytes. Int J Mol Med 41, 977-984

40. Hwang HS, Park IY, Kim DW, Choi SY, Jung YO and Kim HA (2015) PEP-1-FK506BP12 inhibits matrix metalloproteinase expression in human articular chondrocytes and in a mouse carrageenan-induced arthritis model. BMB Rep 48, 407-412

FIGURE LEGENDS

Figure 1. High mobility group box 1 **(**HMGB1) expression was modulated by 29-kDa FN-f. (A) Relative expression of HMGB1 in human normal and osteoarthritis (OA) cartilage. Relative expression of HMGB1 in normal and OA cartilage was measured using SYBR Green-based real-time polymerase chain reaction (PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. *P < 0.05 vs. normal cartilage. Data are presented as the mean ± standard deviation (SD) of data from duplicate experiments using cartilage from different donors (normal cartilage, n = 9 and OA cartilage, n= 20). (B), (C) mRNA and protein expression of HMGB1 altered by 29-kDa FN-f. Chondrocytes were stimulated with 29-kDa FN-f (300 nM) for 1, 3, 6, and 24 hours. mRNA and protein levels of HMGB1 were measured using (B) qRT-PCR and (C) western blot analysis, respectively. *P < 0.05 and ***P < 0.005 vs. untreated cells at each time point. Data are presented as the mean ± SD of data from duplicate data using chondrocytes from three different donors.

Figure 2. Translocation of nuclear HMGB1 was increased by 29-kDa FN-f. (A), (B) 29-kDa FN-f induced the cytoplasmic localization of HMGB1. Cytoplasmic and nuclear fractions were isolated from chondrocytes treated with 29-kDa FN-f for 6 and 24 h. TATA-binding protein (TBP) and β-actin were used as loading controls for nuclear and cytoplasmic fraction, respectively. (B) Localization of HMGB1 was evaluated in primary chondrocytes in the presence or absence of 29-kDa FN-f using fluorescence microscopy. Nuclei were stained with DAPI. Scale bars = 20 μm. (C) Release of HMGB1 into the extracellular milieu was increased by prolonged treatment with 29-kDa FN-f. Culture medium was collected from primary chondrocytes treated with 29-kDa FN-f. The levels of HMGB1 released into the medium were measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as the mean ±

SD of duplicate data from more than three independent experiments. ns, not significant, *P < 0.05, ***P < 0.005, and ****P < 0.001 vs. untreated cells.

Figure 3. Autophagy was inhibited by 29-kDa FN-f. (A), (B) LC3-II level was decreased by 29-kDa FN-f. LC3-II level was measured by (A) immunofluorescence microscopy and (B) western blot analysis. Nuclei were stained with DAPI. (C) Activation of mTOR and 4E-BP1 by 29-kDa FN-f. Phosphorylation of mTOR and 4E-BP1 in 29-kDa FN-f-treated chondrocytes was measured by western blot analysis. (D) The formation of HMGB1/Beclin-1 complex was diminished by 29-kDa FN-f. Interaction with HMGB1 and Beclin-1 in untreated and 29-kDa FN-f-treated chondrocytes was determined using immunoprecipitation (IP) assay. After IP of cell lysates with an antibody against HMGB1, Beclin-1 or IgG, protein samples were subjected to western blot assay. IP with IgG served as a negative control.

Figure 4. mTOR silencing restored 29-kDa FN-f-suppressed LC3-II level and HMGB1 in primary chondrocytes. (A) Chondrocytes were transfected with control siRNA (scRNA) and small interfering mTOR RNA (si-mTOR). Forty-eight hours later chondrocytes were stimulated with 29-kDa FN-f for 24 h. The protein levels were measured using western blot analysis. β-actin served as loading controls. (B) Schematic diagram of mechanism leading to 29-kDa FN-f – suppressed HMGB1-dependent autophagy pathway in primary chondrocytes.

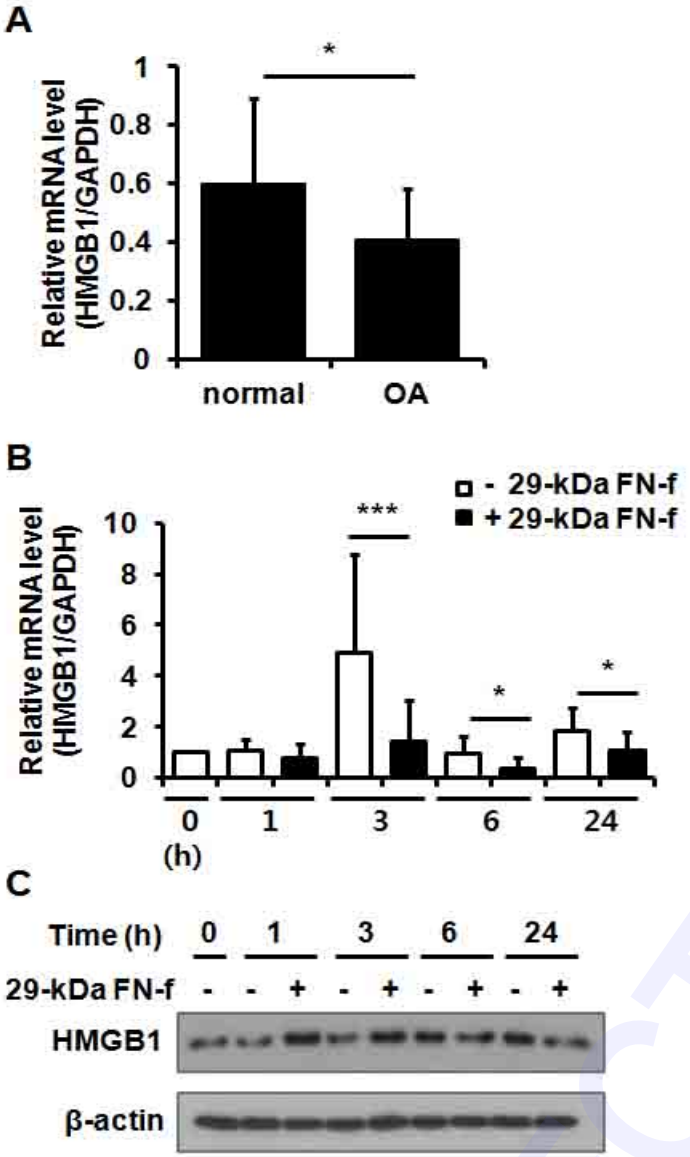

Fig. 1

Fig. 1.

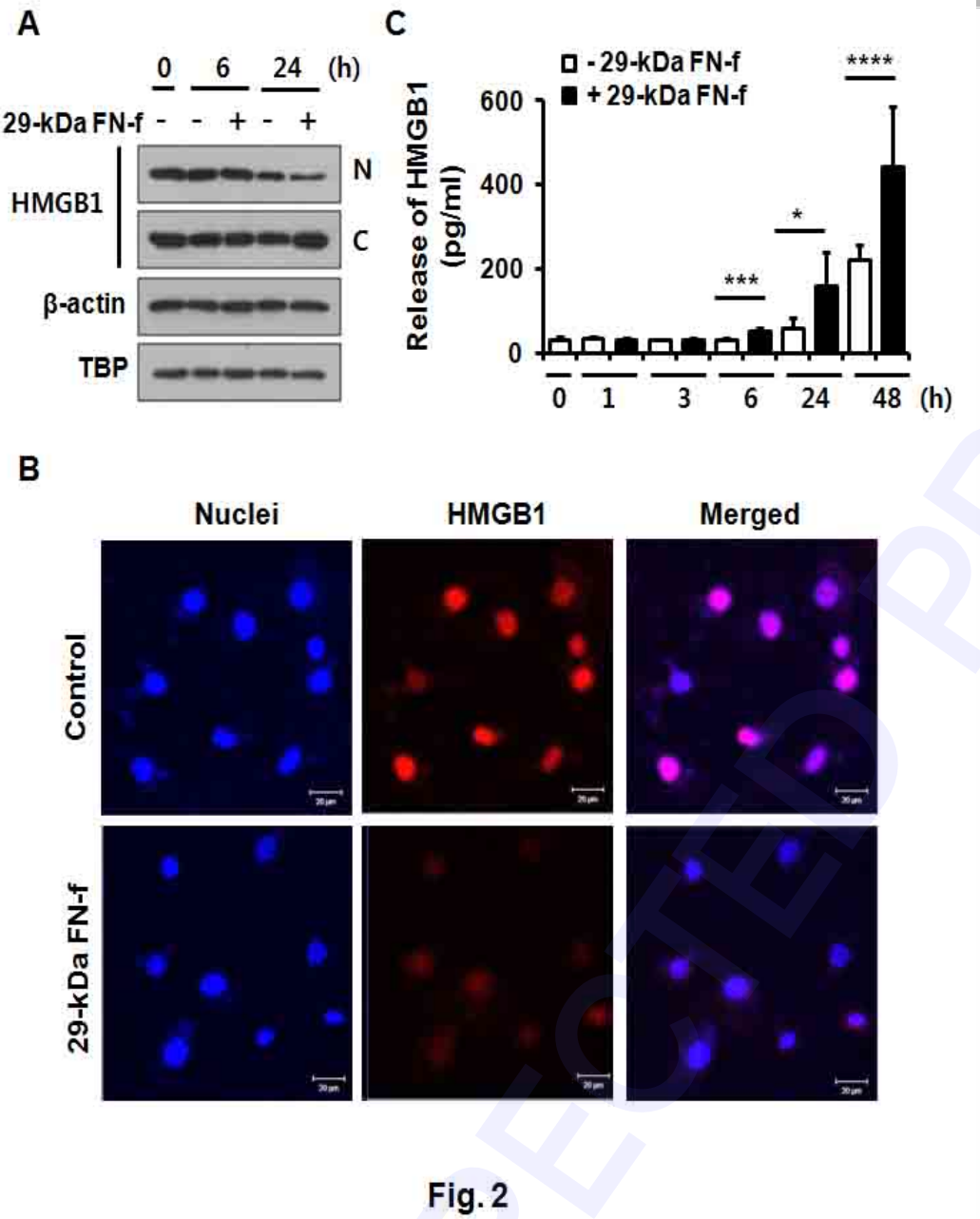

Fig. 2

Fig. 2.

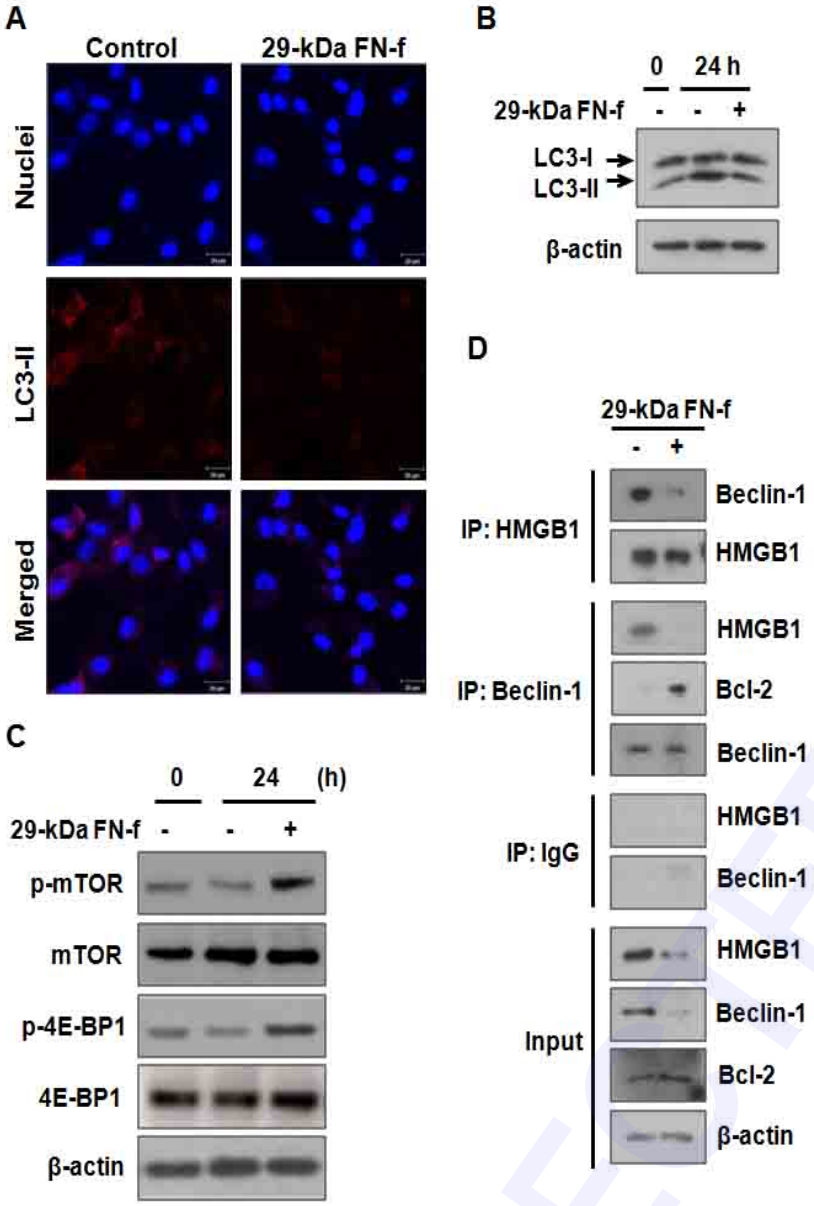

Fig. 3

Fig. 3.

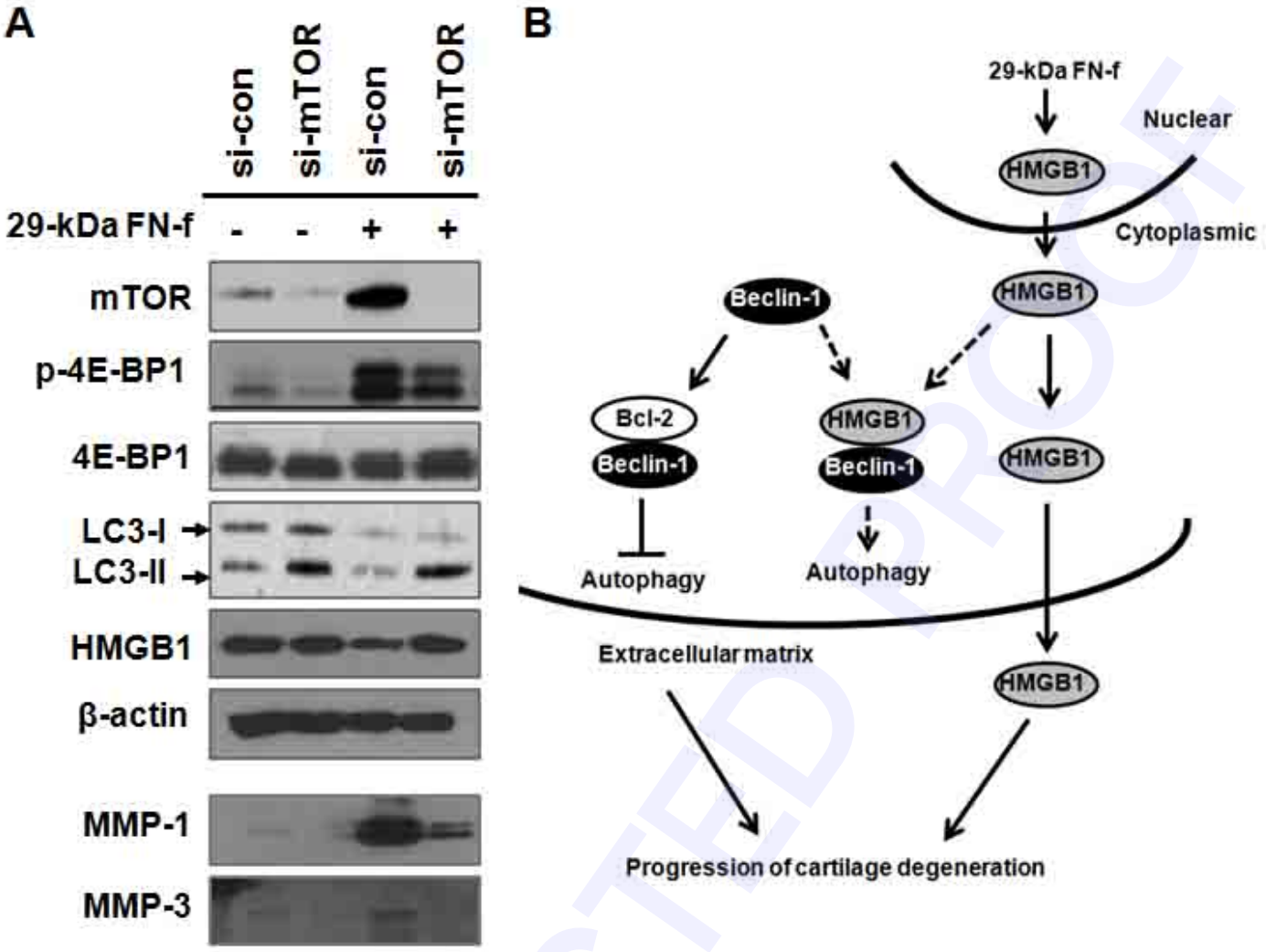

Fig. 4

Fig. 4.

Materials

Antibodies against Beclin-1, Bcl-2, p-mTOR, TOR, p-4EBP1, 4EBP1, and LC3 were purchased from Cell Signaling Technology (Danvers, MA, USA) and antibodies against HMGB1 and TATA binding protein were obtained from Abcam (Cambridge, UK). An antibody against β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Antibodies to MMP-1 and MMP-3 were obtained from R&D Systems (Minneapolis, MN, USA). Small interfering RNAs (mTOR and control) were purchased from Bioneer (Daejeon, Korea). Primers of HMGB1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Cosmogenetech (Seoul, Korea).

Quantitative real-time reverse transcription polymerase chain reaction (qrt-PCR) analysis

Total RNA from cartilage tissues was prepared by grinding cartilage samples to a fine powder in liquid nitrogen and deproteinizing using TRIzol reagent. In case of chondrocytes total RNA was extracted from chondrocytes using TRIzol reagent. cDNA was synthesized from 2 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR analysis was performed with a QuantiFast SYBR Green PCR Kit containing cDNA, primers, and SYBR Green PCR master mix (Qiagen, Hilden, Germany) and data was acquired under condition of two-step cycling, such as denaturation (95°C, 10 s) and combined annealing/extension (58°C, 30 s), for 40 cycles using a StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, USA). GAPDH was used as an internal control. Primer sequences were as follows: HMGB1 forward 5'-GAT-CCC-AAT-GCA-CCC-AAG-AG-3', HMGB1 reverse 5'-GGG-CGA-TAC-TCA-GAG-CAG-AAG-A-3'; GAPDH forward 5′-ATG-GAA-ATC-CCA-TCA-CCA-TCT-T-3′, GAPDH reverse 5′-CGC-CCC-

ACT-TGA-TTT-TGG-3′.

Immunofluorescence microscopy analysis

Chondrocytes were seeded on 8 chamber slides and exposed to 29-kDa FN-f for 24 h. The cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.2% Triton X-100 for 10 min. Chondrocytes were incubated overnight with primary antibodies against HMGB1 (1:600 dilution) and LC3 I/II (1:200 dilution), respectively. Dylight 594-conjugated goat anti-rabbit IgG secondary antibody (1:400 dilution, Bethyl Laboratories, Montgomery, TX, USA) was added to each well of slides for 1 h and nuclei were stained with 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) (Roche, Mannheim Germany) for 5 min. Fluorescence signals were analyzed by confocal microscopy using LSM 700 microscope (Zeiss, Germany).

Immunorpecipitation assay

Chondrocytes were washed with ice-cold PBS and lysed with RIPA buffer containing protease inhibitors. After centrifugation of cell lysates at 13,000 g for 10 min at 4°C, the supernatant was collected. Equal amounts of samples were pre-cleared with protein A agarose (Sigma-Aldrich) bead slurry for 4 h at 4°C on a rotator. The pre-cleared samples (1 μg) were incubated with specific antibody against HMGB1, Beclin-1 or IgG in the presence of protein A agarose beads at 4°C overnight with gentle rotation and subsequently proteins were eluted by boiling in 2x SDS sample buffer. A portion of the lysates was used as an input control. Eluted protein samples were subjected to SDS-PAGE and western blot analysis.