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

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Supplementary 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.