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Osteoarthritis and Cartilage

Selective interference of mTORC1/RAPTOR protects against human disc cellular apoptosis, senescence, and extracellular matrix catabolism with Akt and autophagy induction

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S U M M A R Y

Objective: The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that integrates nutrients to execute cell growth and protein synthesis. We hypothesized that mTOR is essential for the intervertebral disc, the largest avascular, low-nutrient organ. Our objective was to elucidate roles of mTOR signaling in human disc cells.
Design: The mTOR exists in two complexes: mTORC1 containing the regulatory-associated protein of mTOR (RAPTOR) and mTORC2 containing the rapamycin-insensitive companion of mTOR (RICTOR). To analyze their functions in human disc nucleus pulposus cells, RNA interference (RNAi) of mTOR targeting mTORC1 and mTORC2, RAPTOR targeting mTORC1, or RICTOR targeting mTORC2 or rapamycin, a pharmacological mTORC1 inhibitor, was applied. First, mTOR signaling including Akt, p70/ribosomal S6 kinase (p70/S6K), and autophagy were assessed. Then, apoptosis, senescence, and matrix metabolism were evaluated under pro-inflammatory interleukin-1 beta (IL-1β) stimulation.
Results: Western blotting showed significant decreases in specific proteins by each RNAi (all $P < 0.0001$). In mTOR signaling, RNAi of mTOR and RICTOR decreased p70/S6K and Akt phosphorylation, whereas RAPTOR RNAi decreased p70/S6K but increased Akt phosphorylation. All RNAi treatments increased light chain 3 (LC3)-II and decreased p62/sequestosome 1 (p62/SQSTM1), indicating enhanced autophagy. In apoptosis, IL-1β-induced terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells and poly (ADP-ribose) polymerase (PARP) and caspase-9 cleavage decreased by RAPTOR RNAi. In senescence, IL-1β-induced senescence-associated beta-galactosidase (SA-β-gal)-positive cells and p16/INK4A expression also decreased by RAPTOR RNAi. In matrix metabolism, RAPTOR RNAi reduced IL-1β-induced catabolic matrix metalloproteinase (MMP) release and activation and up-regulated anabolic gene expression. These findings were all consistent with rapamycin administration. Additional disc-tissue analysis detected expression and phosphorylation of mTOR-signaling molecules in varying ages.
Conclusion: Selective interference of mTORC1/RAPTOR protects against inflammation-induced apoptosis, senescence, and matrix catabolism possibly through Akt and autophagy induction in human disc cells.
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Introduction

Up to 85 % of people experience low back pain during their lives[1]. Low back pain causes disability that increases medical expenses and affects the workforce[1]. Health care costs related to low back pain are approximately $100 billion/year in the US[2]. Although the mechanism of low back pain is multifactorial, intervertebral disc degeneration is one of the independent causes[3].

The intervertebral disc consists of the nucleus pulposus (NP) encapsulated by the annulus fibrosus (AF) and endplates. The disc is anatomically the largest avascular organ in the body[4]; therefore, nutrient supply depends on diffusion through the endplates. Endplate calcification and subchondral bone sclerosis with aging limit nutrient supply[5]. Such additional nutrient deprivation is a suspected contributor to disc degeneration[5].

Disc degeneration is biochemically characterized by extracellular matrix degradation[4,6]. Matrix metabolism is regulated by the balance between catabolic enzymes, matrix metalloproteinases (MMPs) and a disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs), and their anti-catabolic inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[7]. Increased MMPs and ADAMTSs relative to TIMPs are often observed in human clinical[8–10] and rodent experimental disc degeneration[11–13], resulting in degraded matrix components including proteoglycans, principally aggrecan, and collagens, predominantly type 2 in the NP[6].

Cell decrease is another major characteristic of disc degeneration, which primarily results from programmed cell death, apoptosis[14]. A high incidence of apoptosis is observed in human[15] and rodent disc aging and degeneration[13,16]. The incidence of irreversible cell growth arrest by aging, senescence[17], also increases with human disc degeneration[18]. Furthermore, the involvement of autophagy, the process by which cells break down and recycle damaged components[19], has gained increasing attention in the disc[20]. Autophagy is an important cell survival mechanism to sustain metabolism and prevent the accumulation of damaged toxic proteins and organelles under stress, primarily nutrient deprivation[19]. In molecular signaling, autophagy is under the tight negative regulation of the mammalian target of rapamycin (mTOR)[21]. Therefore, we hypothesized that resident disc cells would utilize autophagy and mTOR signaling to cope with the harsh, low-nutrient environment.

The mTOR is a serine/threonine kinase that integrates nutrients to execute cell growth and division[21]. The mTOR exists in two complexes: mTOR complex 1 (mTORC1) containing the regulatory-associated protein of mTOR (RAPTOR) and mTOR complex 2 (mTORC2) containing the rapamycin-insensitive companion of mTOR (RICTOR)[21]. Down-stream mTORC1 effectors including p70/ribosomal S6 kinase (p70/S6K) regulate cell proliferation, messenger RNA (mRNA) translation, and protein synthesis[21]. Then, mTORC1 signaling is regulated by up-stream Akt, an essential pro-survival mediator by suppressing apoptosis[22]. Roles of mTORC2 still remain unclear; however, mTORC2 is known to regulate Akt (Fig. 1)[22].

Recent studies have reported that rapamycin, an mTORC1 inhibitor, extends mammalian lifespan[23] and has protective roles in human chondrocytes[24]. However, inhibitory effects of mTOR signaling on human disc cells are poorly understood. More importantly, little evidence exists regarding which subunit(s) of mTOR-signaling components exerts these beneficial effects on disc or cartilage cells. Thus, an *in vitro* study of human disc cells treated by (1) RNA interference (RNAi) and (2) rapamycin was designed to analyze cascade-specific roles of mTOR signaling. The *in vivo* involvement of mTOR signaling was also explored in human disc tissues.

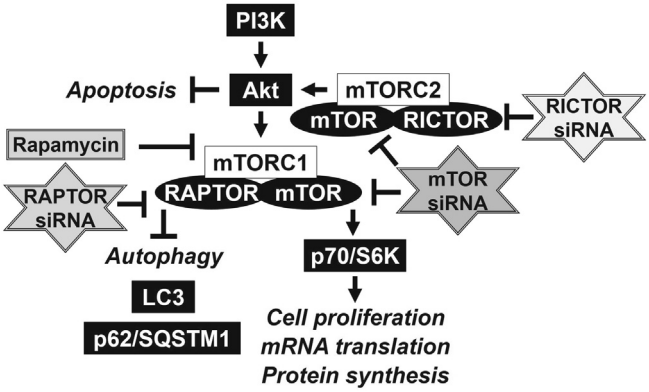

Fig. 1. Schematic illustration of the mTOR signaling pathway. The mTOR is a serine/threonine kinase that integrates nutrients to execute cell growth and division. The mTOR exists in two complexes of mTORC1 containing RAPTOR and mTORC2 containing RICTOR. Down-stream effectors of mTORC1 including p70/S6K regulate cell proliferation, mRNA translation, and protein synthesis. Autophagy, as assessed by LC3 and p62/SQSTM1, is under the tight negative regulation of mTORC1. The mTORC1 is regulated by up-stream class I PI3K followed by Akt, an essential pro-survival mediator by suppressing apoptosis. To analyze cascade-dependent roles of mTOR signaling, RNAi using the siRNA against mTOR targeting mTORC1 and mTORC2, RAPTOR targeting mTORC1, or RICTOR targeting mTORC2 or rapamycin, an pharmacological mTORC1 inhibitor, was applied.

Materials and methods

Ethics statement

All experimental procedures were performed under the approval and guidance of the Institutional Review Board (160004) at Kobe University Graduate School of Medicine. Written informed consent was obtained from each patient in accordance with the principles of the Declaration of Helsinki and the laws and regulations of Japan.

Antibodies and reagents

The antibodies and reagents used are listed in Supplemental Table 1.

Cells

Two types of cells were applied. To conduct comprehensive examinations for multi-cascade analysis of mTOR signaling by RNAi, a human disc NP cell line was used. This was developed using a recombinant SV40 adenovirus vector by Dr Daisuke Sakai (Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Isehara, Japan) and characterized previously[25,26]. Monolayer cells were grown to ~80% confluence in 1% penicillin/streptomycin-supplemented Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C under 2% O_2 to simulate the physiologically hypoxic disc environment[5], and then underwent subculture. Cells at passage ≤8 were characterized and used for experiments following 72-h pre-culture.

To assess clinical relevance of cell-line findings, human disc NP cells obtained from patients who underwent lumbar interbody fusion surgery for degenerative disease were used (n = 40: age, 60.8 ± 4.7 [25–82] years; 20 males and 20 females; Pfirrmann degeneration grade[27], 3.5 ± 0.2 [3–4]). Immediately after surgery, human disc NP tissues were carefully collected from discarded surgical waste and digested in 1% penicillin/streptomycin-supplemented DMEM with 10% FBS and 0.114% collagenase type 2 for 1 h at 37°C. Isolated cells were grown to ~80% confluence as a

monolayer in 1% penicillin/streptomycin-supplemented DMEM with 10% FBS at 37°C under 2% O_2 for 240 h. Only first-passage cells were used for experiments following 72-h pre-culture.

Subsequently, 1.5×10^5/well (6-well plate) for protein extraction and RNA isolation, 1.2×10^4/well (8-well chamber) for staining, and 5.0×10^3/well (96-well plate) for cell proliferation and viability were prepared. As treatment, RNAi was applied. Cell proliferation and viability were assessed after up to 48-h treatment. Protein analysis was conducted after 36-h treatment. To simulate a clinically relevant disease condition[28], cells were treated for additional 24 h by interleukin-1 beta (IL-1β) at 10 ng/ml[24] with media change to serum-free DMEM to analyze released proteins. The IL-1β is a pro-inflammatory cytokine closely linked to the pathogenesis of disc degeneration[28], showing increased production with its severity[29]. After treatment, cells and supernatants were collected for protein extraction and mRNA isolation. Cells were also applied to staining assays.

As an alternative treatment, a common mTORC1 inhibitor of rapamycin was applied for 24–48 h, combined with IL-1β depending on the assays. Then, similar assessments were performed.

Tissues

Portions of human disc NP tissues surgically obtained from the lumbar spine were carefully dissected and directly used for protein extraction ($n = 8$: age, 58.6 ± 12.4 [27–80] years; five males and three females; Pfirrmann degeneration grade, 3.1 ± 0.6 [2–4]).

RNAi

To knock-down specific signals in the mTOR pathway (Fig. 1), RNAi using the small interfering RNA (siRNA) against mTOR targeting mTORC1 and mTORC2, RAPTOR targeting mTORC1, or RICTOR targeting mTORC2 was applied. Non-targeting siRNAs were used as a negative control. To exclude off-target effects of RNAi, consistent findings were confirmed using at least two siRNAs with different sequences in all experiments. The siRNA sequences are listed in Supplemental Table 2.

To deliver siRNAs effectively, the reverse transfection technique was used. Cells suspended in DMEM with 10% FBS were added to Opti-minimal essential medium I with the siRNA and Lipofectamine RNAiMAX, cultured for 36–48 h. Applied amounts of siRNAs were 60 (6-well plate), 4.8 (8-well chamber), and 2 (96-well plate) pmol/well.

Cell proliferation assay

The number of adherent cells was counted in six random low-power fields (×100) under the BZ-X700 microscope repeatedly at 12, 24, and 48 h after treatment.

Cell viability and mitochondrial activity assays

Cell viability and metabolism were assessed by the cell counting kit-8 (CCK-8) based on detecting total mitochondrial activity, the absorbance of which (450 nm) was measured using the Model 680 microplate reader at 48 h after treatment. Then, mitochondrial activity per cell was calculated as CCK-8 value normalized to cell number.

Protein extraction

Cells were scraped off on ice in the 3-(*N*-morpholino)propanesulfonic acid buffer containing protease and phosphatase inhibitors. Soluble proteins were collected after centrifugation at 20,000 *g* for 15 min at 4°C. Non-serum-containing culture media were also collected, centrifuged at 1000 *g* for 10 min at 4°C to remove cellular debris, and concentrated using Amicon Ultra spin columns.

Tissues were homogenized using the MS-100R bead-beating disrupter for 30 s twice at 4°C in the T-PER tissue protein extraction reagent with protease and phosphatase inhibitors. Soluble proteins were collected after 20,000-*g* cold centrifugation for 15 min.

Protein concentration was determined by the bicinchoninic acid assay. Samples were stored at −80°C.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting

Equal 30-μg amounts of protein were mixed with the electrophoresis sample buffer and boiled for 5 min before loading onto a 7.5–15.0% polyacrylamide gel. Separated proteins in the Tris–glycine–SDS buffer system were transblotted electrically and probed with primary antibodies for 12 h in 4°C (1:200–1:1000 dilution) followed by secondary antibodies (1:2000 dilution). Signals were visualized by enhanced chemiluminescence. Images were obtained using the Chemilumino analyzer LAS-3000 mini. Relative band intensity was quantified using the ImageJ software (http://rsweb.nih.gov/ij/).

Western blotting was designed to analyze intracellular expression of disc NP-associated notochordal markers, brachyury and CD24[30] (both 1:1000 dilutions), mTOR signaling-related mTOR, RAPTOR, RICTOR, Akt, phosphorylated Akt, p70/S6K, and phosphorylated p70/S6K[21] (all 1:1000 dilutions), autophagy-related light chain 3 (LC3) and p62/sequestosome 1 (p62/SQSTM1)[31] (both 1:1000 dilutions), apoptosis-related cleaved poly (ADP-ribose) polymerase (PARP)[32] and cleaved caspase-9[33] (both 1:1000 dilutions), senescence-related p16/INK4A[34] (1:1000 dilution) in total cell or tissue protein extracts. Western blotting was also designed to analyze released protein expression of catabolic MMP-3, MMP-13, MMP-2, and MMP-9 (all 1:1000 dilutions) and anti-catabolic TIMP-1 (1:200 dilution) and TIMP-2 (1:1000 dilution) in supernatant protein extracts.

Zymography

Equal 30-μg amounts of protein were mixed with the sample buffer and loaded onto a 10% gel containing gelatin. After electrophoresis, the gel was equilibrated with the zymogram renaturing buffer for 30 min and incubated with the zymogram developing buffer for 12 h at 37°C. Signals were visualized by staining the gel with SimplyBlue SafeStain. Image acquisition and band quantification were performed using the LAS-3000 mini and the ImageJ, respectively.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptotic cells were identified using a fluorescein-labeled TUNEL assay kit[35]. Cells were fixed with 4% paraformaldehyde for 10 min. The 4′,6-diamidino-2-phenylindole (DAPI) was used for counterstaining. Images were photographed using the BZ-X700 microscope. The percentage of TUNEL-positive cells was calculated as relative to the number of DAPI-positive total cells, which were both counted in six random low-power fields (×100) using the ImageJ.

Senescence-associated beta-galactosidase (SA-β-gal) staining

Cytochemical staining was performed to detect senescence using a SA-β-gal staining kit at pH 6[36]. The percentage of SA-β-gal-positive cells was similarly calculated in six random low-power fields (×100).

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was extracted using the RNeasy mini kit, and 0.1 μg of total RNA was reverse-transcribed with random primers. Relative mRNA expression levels of catabolic *MMP-3*, *MMP-13*, *ADAMTS-4*, and *ADAMTS-5*, anti-catabolic *TIMP-1* and *TIMP-3*, and anabolic *ACAN* encoding aggrecan and *COL2A1* encoding collagen type II alpha 1 chain relative to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were assessed by real-time RT-PCR using SYBR Green fluorescent dye. Good feasibility of *GAPDH* as an endogenous control for disc cells was established previously[37]. Measurements were performed in duplicate using the Applied Biosystems 7500 real-time PCR system. The primer sequences were obtained from prior reports[38–40] or purchased from Takara Bio, as listed in Supplemental Table 2. Melting curve analysis was performed using the Dissociation Curves software to ensure the amplification of only a single product. Relative mRNA expression was analyzed using the $2^{-\Delta\Delta Ct}$ method[41]. The value of the non-targeting siRNA-treated control sample was set as 1.

Statistical analysis

Data are expressed as the mean ± 95% confidence interval (CI) of six independent samples in duplicate or triplicate, consisting of experiments using two siRNAs with different sequences in RNAi, unless indicated otherwise ($n = 6$). Multi-way analysis of variance (ANOVA) with the Tukey–Kramer post-hoc test or regression analysis was used. Mixed-design ANOVA or paired *t* test was used for the comparison between within-variable groups. The *P*-values <0.05 were regarded as statistically significant using IBM SPSS Statistics 23.0 (IBM, Armonk, NY, USA).

Results

Selective interference of mTOR signaling induces consistent autophagy and differential Akt activation in human disc NP cell-line cells

First, to validate the human disc NP cell-line cells, we assessed expression of disc NP-associated notochordal markers. Although there have been no definitive disc NP phenotypic markers[42], the disc NP development from the notochord results in a relatively high specificity of brachyury and CD24[30]. Consequently, this cell line expressed brachyury and CD24 consistently through passage 2–8, suggesting the maintained phenotype [Fig. 2(A)].

Next, we assessed whether RNAi effectively knocked-down target proteins. Western blotting showed specific decreases in expression of target proteins by the corresponding siRNAs—control, 100%; mTOR, 43.0 ± 3.3%; RAPTOR, 35.8 ± 4.2%; RICTOR, 47.4 ± 5.8% (all $P < 0.0001$) [Fig. 2(B)].

Then, we assessed how RNAi modulated mTOR signaling. Western blotting showed that mTOR and RICTOR siRNAs decreased p70/S6K and Akt phosphorylation, whereas RAPTOR siRNA decreased p70/S6K but increased Akt phosphorylation [Fig. 2(C)].

Furthermore, we assessed how RNAi modified autophagy. Western blotting showed that all siRNA treatments increased LC3-II and decreased p62/SQSTM1. The phosphatidylethanolamine-conjugated form of LC3, LC3-II (unlike its cytosolic form, LC3-I), is the only protein marker reliably associated with completed autophagosomes[31]. The p62/SQSTM1 and p62/SQSTM1-bound poly-ubiquitinated proteins become incorporated into completed autophagosomes and degraded in autolysosomes, demonstrating a negative correlation with autophagy[31]. Thus, the present findings indicate enhanced autophagy [Fig. 2(D)].

Selective interference of mTOR signaling induces differential effects on proliferation, apoptosis, senescence, and matrix metabolism in human disc NP cell-line cells

To understand modified cellular physiology by RNAi, we counted cell numbers. After 24-h transfection, the number of cells decreased by siRNAs against mTOR ($P = 0.03$) and RICTOR ($P = 0.0004$) but not RAPTOR. After 48-h transfection, this trend toward decrease by mTOR and RICTOR siRNAs was more remarkable (both $P < 0.0001$), indicating reduced cell proliferation by mTOR inhibition except RAPTOR interference [Fig. 3(A)]. Similar findings were observed in CCK-8 mitochondrial activity-based cell viability after 48-h mTOR ($P = 0.006$) and RICTOR ($P = 0.004$) siRNA transfection [Fig. 3(B)]. However, mitochondrial activity per cell, calculated as CCK-8 normalized to cell number, demonstrated no significant differences between all siRNA treatments, indicating maintained mitochondrial activity by mTOR inhibition [Fig. 3(B)].

Then, we assessed modified cell death, aging, and matrix metabolism by RNAi. First, effective modulation of mTOR signaling and autophagy by IL-1β in human disc NP cell-line cells was confirmed [Supplemental Fig. 1(A)]. Then, the percentage of apoptotic TUNEL-positive cells increased by IL-1β ($P < 0.0001$). This change was suppressed by RAPTOR siRNA only ($P < 0.0001$) [Fig. 3(C)]. Western blotting also showed IL-1β-induced increases in PARP and caspase-9 cleavage, indicating mitochondrial apoptosis, decreased by RAPTOR siRNA only [Fig. 3(D)]. Meanwhile, Western blotting showed that IL-1β-induced increases in senescent p16/INK4A decreased by all mTOR, RAPTOR, and RICTOR siRNAs [Fig. 3(D)]. In culture supernatants, IL-1β induced a shift in matrix metabolism toward catabolism, showing drastically increased catabolic MMP-3 and MMP-13 relative to anti-catabolic TIMP-1 and TIMP-2. This MMP-3 and MMP-13 release was markedly reduced by all siRNAs; however, TIMP-1 and TIMP-2 were less affected [Fig. 3(E)]. Gelatin zymography further showed that IL-1β-induced increases in the MMP-2 and MMP-9 active-forms were most effectively suppressed by RAPTOR siRNA [Fig. 3(E)].

Selective interference of mTORC1/RAPTOR protects against apoptosis and senescence in human disc NP cells

Based on the human disc NP cell-line findings, RNAi of RAPTOR targeting mTORC1 was considered as a candidate to treat disc disease. Therefore, to confirm its clinical efficacy, we assessed RNAi effects of RAPTOR on human disc NP cells. First, we validated human disc NP cells obtained surgically, revealing relatively preserved expression of notochord-phenotypic brachyury and CD24 [Fig. 4(A)]. Next, we confirmed that human disc NP cells expressed mTOR, RAPTOR, and RICTOR [Fig. 4(A)]. Then, we examined the knock-down efficacy by RAPTOR RNAi, demonstrating reduced RAPTOR protein expression by two different siRNA sequences—control, 100%; RAPTOR no. 1, 33.7 ± 5.5% ($P < 0.0001$); RAPTOR no. 2, 36.3 ± 7.9% ($P < 0.0001$) [Fig. 4(B)]. Furthermore, we assessed modulated mTOR signaling and autophagy by RAPTOR RNAi,

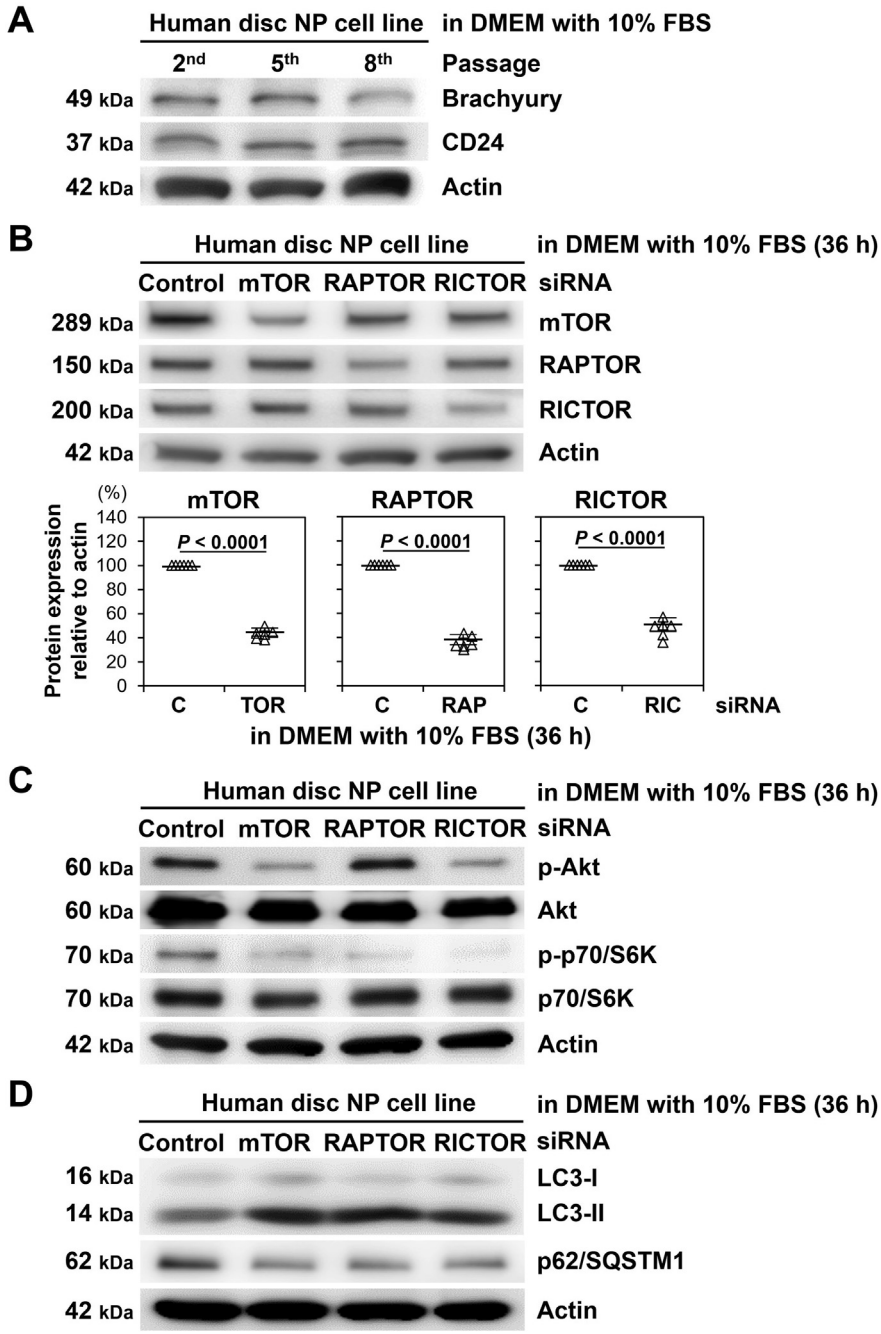

Fig. 2. Selective interference of mTOR signaling induces consistent autophagy and differential Akt activation in human disc NP cell-line cells. (A) Western blotting for disc NP-phenotypic brachyury and CD24 in total protein extracts from human disc NP cell-line cells at passage ≤8 after 72-h culture in DMEM with 10% FBS. Actin was used as a loading control. (B) Western blotting for mTOR signaling-related mTOR, RAPTOR, and RICTOR and actin in total protein extracts from human disc NP cell-line cells after 36-h transfection of mTOR (TOR), RAPTOR (RAP), RICTOR (RIC), or control (C) siRNA in DMEM with 10% FBS. Changes in protein expression of mTOR, RAPTOR and RICTOR relative to actin are shown. Data are the mean ± 95% CI ($n = 6$). The paired t test was used. (C) Western blotting for mTOR signaling-related Akt, phosphorylated Akt (p-Akt), p70/S6K, phosphorylated p70/S6K (p-p70/S6K), and actin in total protein extracts from human disc NP cell-line cells. (D) Western blotting for autophagy-related LC3 and p62/SQSTM1 and actin in total protein extracts from human disc NP cell-line cells. All immunoblots shown are representative of experiments with similar results ($n = 6$).

exhibiting decreased p70/S6K phosphorylation and increased Akt phosphorylation [Fig. 4(B)]. Autophagic increases in LC3-II and decreases in p62/SQSTM1 were also observed [Fig. 4(B)]. These findings were all consistent with human disc NP cell-line cells.

Then, we assessed modified apoptosis and senescence by RAPTOR RNAi. The relationship between IL-1β stimulation and mTOR signaling in human disc NP cells was similar to the cell-line findings [Supplemental Fig. 1(B)]. Western blotting showed that IL-1β-induced mitochondrial apoptosis was suppressed by RAPTOR siRNA [Fig. 4(C)]. Next, IL-1β-induced senescence was suppressed by RAPTOR siRNA [Fig. 4(C)]. Additionally, SA-β-gal staining demonstrated that the positive cell percentage increased by IL-1β ($P < 0.0001$), which was suppressed by RAPTOR siRNA ($P < 0.0001$) [Fig. 4(D)].

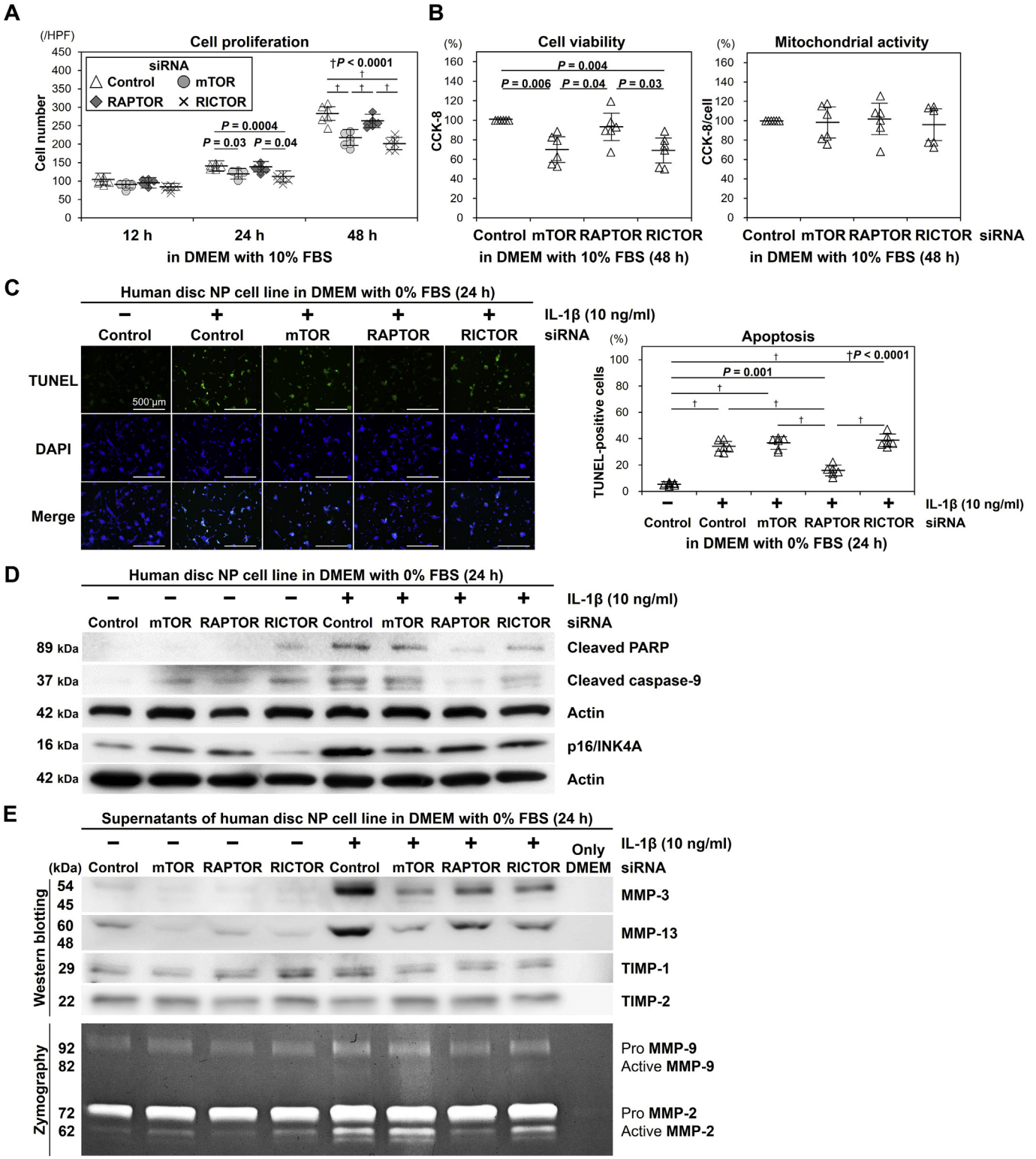

Fig. 3. Selective interference of mTOR signaling induces differential effects on proliferation, apoptosis, senescence, and matrix metabolism in human disc NP cell-line cells. (A) Changes in the number of human disc NP cell-line cells at 12, 24, and 48 h after transfection of mTOR, RAPTOR, RICTOR, or control siRNA in DMEM with 10% FBS. Data are the mean ± 95% CI ($n = 6$). Two-way mixed-design ANOVA with the Tukey–Kramer post-hoc test was used. (B) Changes in the percentage of total viability and individual mitochondrial activity of human disc NP cell-line cells using CCK-8 after 48-h transfection of mTOR, RAPTOR, RICTOR, or control siRNA in DMEM with 10% FBS. Data are the mean ± 95% CI ($n = 6$). One-way repeated-measures ANOVA with the Tukey–Kramer post-hoc test was used. (C) Immunofluorescence for apoptotic TUNEL (green), nuclear DAPI (blue), and merged signals in human disc NP cell-line cells after 36-h transfection of mTOR, RAPTOR, RICTOR, or control siRNA in DMEM with 10% FBS followed by 24-h culture in serum-free DMEM with 10-ng/ml IL-1β. Changes in the percentage of TUNEL-positive cells in DAPI-positive cells are shown. The number of cells was counted in six random low-power fields (×100). Data are the mean ± 95% CI ($n = 6$). One-way ANOVA with the Tukey–Kramer post-hoc test was used. (D) Western blotting for apoptotic cleaved PARP and cleaved caspase-9 and senescent p16/INK4A in total protein extracts from human disc NP cell-line cells. Actin was used as a loading control. (E) Western blotting for catabolic MMP-3 and MMP-13 and anti-catabolic TIMP-1 and TIMP-2 in supernatant protein extracts from human disc NP cell-line cells. Gelatin zymography for MMPs in supernatant protein extracts from human disc NP cell-line cells. All immunoblots and zymographs shown are representative of experiments with similar results ($n = 6$).

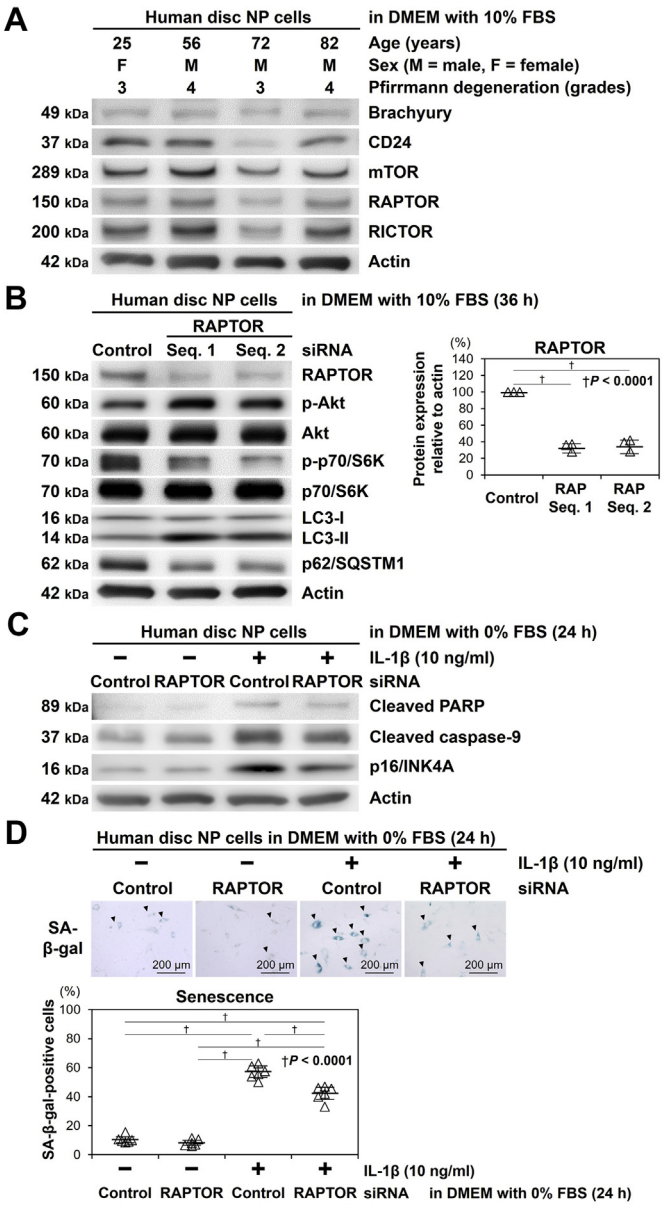

Fig. 4. Selective interference of mTORC1/RAPTOR protects against apoptosis and senescence in human disc NP cells. (A) Western blotting for disc NP-phenotypic brachyury and CD24 and mTOR signaling-related mTOR, RAPTOR, and RICTOR in total protein extracts from human disc NP cells of patients who underwent lumbar spine surgery for degenerative disease after 240-h culture in DMEM with 10% FBS. Actin was used as a loading control. Immunoblots show samples randomly selected ($n = 4$). (B) Western blotting for mTOR signaling-related RAPTOR, Akt, phosphorylated Akt (p-Akt), p70/S6K, and phosphorylated p70/S6K (p-p70/S6K), autophagy-related LC3 and p62/SQSTM1, and actin in total protein extracts from human disc NP cells after 36-h transfection of RAPTOR siRNA with two different sequences or control siRNA in DMEM with 10% FBS. Changes in protein expression of RAPTOR relative to actin are shown. Data are the mean ± 95% CI ($n = 3$). One-way repeated-measures ANOVA with the Tukey–Kramer post-hoc test was used. (C) Western blotting for apoptotic cleaved PARP and cleaved caspase-9, senescent p16/INK4A, and actin in total protein extracts from human disc NP cells after 36-h transfection of RAPTOR or control siRNA in DMEM with 10% FBS followed by 24-h culture in serum-free DMEM with 10-ng/ml IL-1β. (D) SA-β-gal staining in human disc NP cells. Arrows indicate SA-β-gal-positive cells. Changes in the percentage of SA-β-gal-positive cells in total cells are shown. The number of cells was counted in six random low-power fields (×100). Data are the mean ± 95% CI ($n = 6$). One-way ANOVA with the Tukey–Kramer post-hoc test was used. In (B) and (C), immunoblots shown are representative of experiments with similar results ($n = 6$).

Selective interference of mTORC1/RAPTOR protects against matrix catabolism in human disc NP cells

We further assessed modified matrix metabolism by RAPTOR RNAi in human disc NP cells. Western blotting in culture supernatants showed that IL-1β-induced release of the MMP-3, MMP-13, MMP-2, and MMP-9 pro-forms and MMP-3, MMP-13, and MMP-2 active-forms was all suppressed by RAPTOR siRNA, while TIMP-1 and TIMP-2 were less sensitive [Fig. 5(A)]. Gelatin zymography also showed that IL-1β-induced increases in the MMP-2 and MMP-9 active-forms were suppressed by RAPTOR siRNA [Fig. 5(A)]. These findings were common between human disc NP cells and cell-line cells.

Real-time RT-PCR demonstrated that IL-1β-induced mRNA up-regulation of catabolic *MMP-3* ($P < 0.0001$), *MMP-13* ($P = 0.04$), and *ADAMTS-4* ($P < 0.0001$) was suppressed by RAPTOR siRNA [Fig. 5(B)]. Meanwhile, catabolic *ADAMTS-5* and anti-catabolic *TIMP-1* and *TIMP-3* mRNA expression was less affected by IL-1β and RAPTOR siRNA [Fig. 5(B)]. Then, IL-1β-induced down-regulation of anabolic *ACAN* and *COL2A1* was not significantly rescued by RAPTOR siRNA; however, RAPTOR siRNA treatment resulted in a trend toward up-regulation of these anabolic genes with statistical significance for *COL2A1* ($P = 0.02$) [Fig. 5(B)].

Pharmacological inhibition of mTORC1 by rapamycin protects against apoptosis, senescence, and matrix catabolism in human disc NP cells

Rapamycin was used as a positive control of mTORC1 inhibition. First, CCK-8 assay demonstrated significant decreases in cell viability by rapamycin ≥1 μM ($P = 0.004$). Thus, 100 nM was selected as an effective but non-toxic concentration [Fig. 6(A)]. Then, Western blotting showed that rapamycin decreased p70/S6K and increased Akt phosphorylation in mTOR signaling and increased LC3-II and decreased p62/SQSTM1 in autophagy, which are similar findings to RAPTOR siRNA indicating mTORC1 suppression [Fig. 6(B)]. Furthermore, rapamycin decreased mitochondrial apoptosis markers and senescence markers, also similar to RAPTOR siRNA [Fig. 6(C)]. Finally, rapamycin decreased expression and activity of catabolic MMPs whereas anti-catabolic TIMPs were relatively unchanged, consistent with mTORC1/RAPTOR interference [Fig. 6(D)].

mTOR signaling is detectable in human disc NP tissues

Additionally, we examined mTOR-signaling involvement in human disc NP surgical specimens. Western blotting showed maintained expression of disc NP-phenotypic brachyury and CD24. Expression of mTOR, RAPTOR, RICTOR, Akt, and p70/S6K and phosphorylation of Akt and p70/S6K were also detected in all patient samples with 20–80 in ages and 2–4 in degeneration grades (Fig. 7). Regression analysis demonstrated age-dependent decreases in Akt expression ($P = 0.049$) and phosphorylation ($P = 0.046$) (Fig. 7).

Discussion

This is the first mechanistic study to demonstrate cascade-dependent, differential roles of mTOR signaling in human intervertebral disc cells. The RNAi technique enabled specific suppression of respective pathway constituents. In mTOR signaling, while mTOR, RAPTOR, and RICTOR siRNA treatments all diminished mTOR-downstream p70/S6K phosphorylation and activated

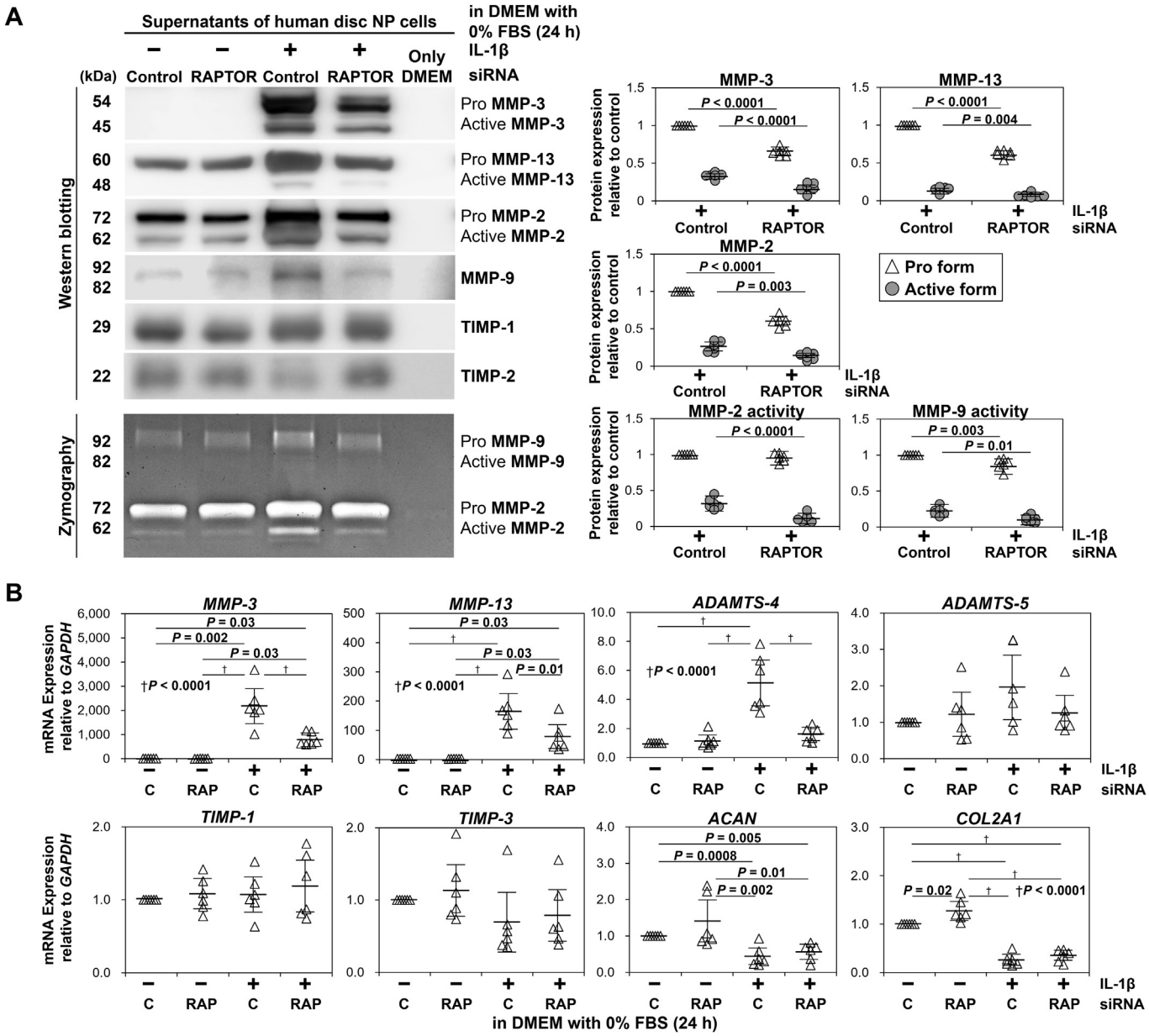

Fig. 5. Selective interference of mTORC1/RAPTOR protects against matrix catabolism in human disc NP cells. (A) Western blotting for catabolic MMP-3, MMP-13, MMP-2, and MMP-9 and anti-catabolic TIMP-1 and TIMP-2 in supernatant protein extracts from human disc NP cells after 36-h transfection of RAPTOR or control siRNA in DMEM with 10% FBS followed by 24-h culture in serum-free DMEM with 10-ng/ml IL-1β. Gelatin zymography for MMPs in supernatant protein extracts from human disc NP cells. Changes in protein expression of the pro-form and active-form of MMP-3, MMP-13, and MMP-2 and activity of the pro-form and active-form of MMP-2 and MMP-9 relative to the control siRNA-transfected pro-form under IL-1β treatment are shown. Data are the mean ± 95% CI. One-way repeated-measures ANOVA with the Tukey–Kramer post-hoc test was used. Immunoblots and zymographs shown are representative of experiments with similar results ($n = 6$). (B) Real-time RT-PCR for catabolic *MMP-3*, *MMP-13*, *ADAMTS-4*, and *ADAMTS-5*, anti-catabolic *TIMP-1* and *TIMP-3*, and anabolic *aggrecan* (*ACAN*) and *collagen type 2-α1* (*COL2A1*) in total RNA extracts from human disc NP cells. *GAPDH* was used as an endogenous control. Data are the mean ± 95% CI expressed as the fold change relative to the condition of control siRNA without IL-1β ($n = 6$). One-way repeated-measures ANOVA with the Tukey–Kramer post-hoc test was used.

autophagy, only RAPTOR siRNA enhanced mTOR-upstream Akt phosphorylation. Cell proliferation was reduced by mTOR and RICTOR siRNAs but not RAPTOR siRNA. Under inflammation, cellular senescence and extracellular matrix catabolism were suppressed by all siRNAs; however, cellular apoptosis was blocked by RAPTOR siRNA only. These protective effects of RAPTOR RNAi against cell death, aging, and matrix catabolism were consistent with pharmacological mTORC1 inhibition by rapamycin, suggesting mTORC1/RAPTOR interference as a potent molecular treatment strategy for disc disease.

The observed consistent RNAi findings of mTOR, RAPTOR, and RICTOR were pro-autophagic, anti-senescent, and anti-catabolic (Fig. 8). Autophagy is negatively regulated by mTORC1[21], which can explain our pro-autophagic findings directly. Cell cycle and proliferation are controlled positively by translation-stimulating p70/S6K, receiving positive regulation of mTORC1, and negatively by translation-repressing eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), receiving negative regulation of mTORC1[21]. The 4E-BP1 is required for dietary restriction-induced lifespan extension[43]. Deletion of p70/S6K also extends lifespan[44].

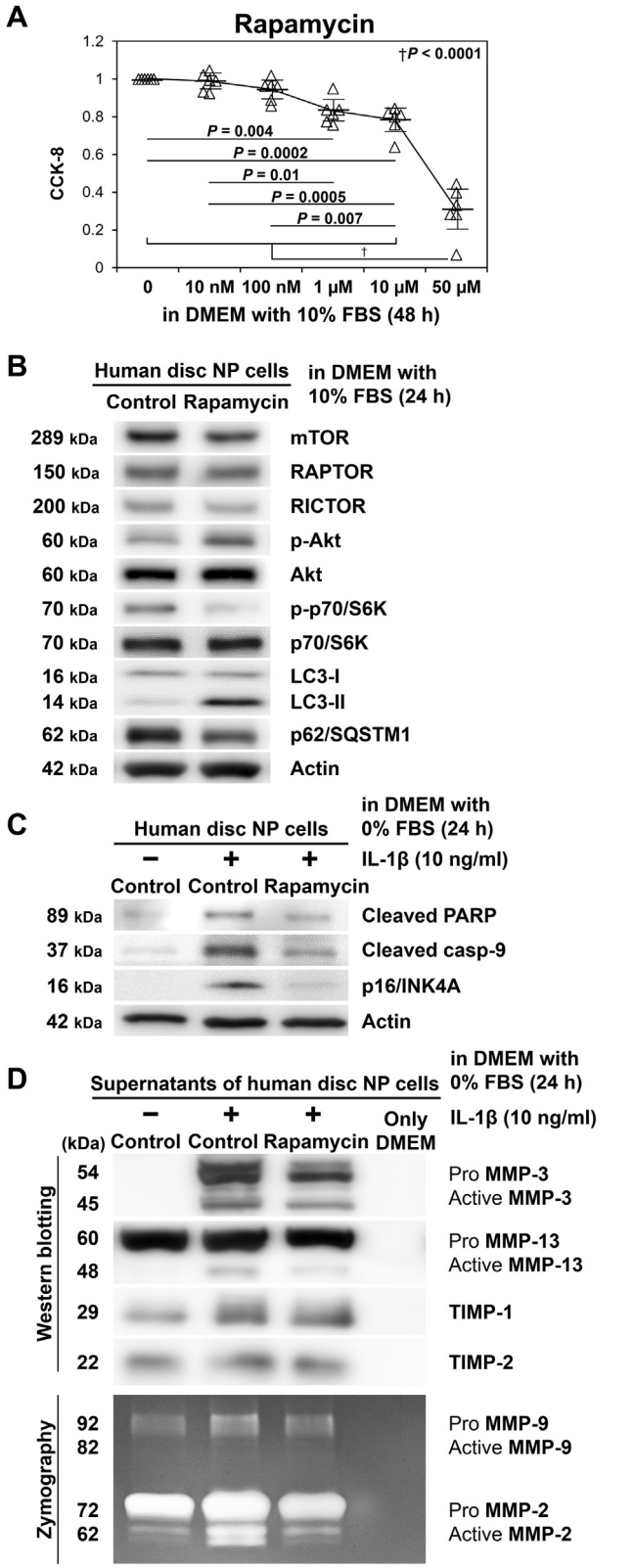

Fig. 6. Pharmacological inhibition of mTORC1 by rapamycin protects against apoptosis, senescence, and matrix catabolism in human disc NP cells. (A) Changes in the percentage of viability of human disc NP cells form surgical specimens using CCK-8 after 48-h treatment of 0–50 μM rapamycin in DMEM with 10% FBS. Data are the mean ± 95% CI ($n = 6$). One-way repeated-measures ANOVA with the Tukey–Kramer post-hoc test was used. (B) Western blotting for mTOR signaling-related mTOR, RAPTOR, RICTOR, Akt, phosphorylated Akt (p-Akt), p70/S6K, and phosphorylated p70/S6K (p-p70/S6K) and autophagy-related LC3 and p62/SQSTM1 in total protein extracts from human disc NP cells after 24-h treatment of 100-nM rapamycin in DMEM with 10% FBS. Actin was used as a loading control. (C) Western blotting for apoptotic cleaved PARP and cleaved caspase-9, senescent p16/INK4A, and actin in total protein extracts from human disc NP cells after 24-h treatment of 100-nM rapamycin in serum-free DMEM with 10-ng/ml IL-1β. (D) Western blotting for catabolic MMP-3 and MMP-13 and anti-catabolic TIMP-1 and TIMP-2 in supernatant protein extracts from human disc NP cells. Gelatin zymography for MMPs in supernatant protein extracts from human disc NP cells. All immunoblots and zymographs shown are representative of experiments with similar results ($n = 6$).

Mice with hypomorphic mTOR (approximately 25% of wild-type mice) have shown increased lifespan and reduced aging tissue biomarkers including p16/INK4A[45]. Therefore, our anti-senescent findings can be explained by mTORC1-mediated proliferation. Meanwhile, few reports have described the regulation and mechanism of matrix metabolism by mTOR. However, our anti-catabolic findings suggest that expression and activation of MMPs, possibly rather than ADAMTSs and TIMPs, depend on mTORC1-mediated translation. Further investigations need to be conducted; however, mTORC1-mediated proliferation and translation provide a plausible interpretation with reduced disc cell aging and matrix catabolism by mTOR/RAPTOR/RICTOR interference.

The differential anti-apoptotic RNAi findings of RAPTOR, resulting in mTORC1 inhibition, from mTOR and RICTOR, resulting in mTORC1 and mTORC2 inhibition, can be explained by contrasting phosphorylation levels of Akt (Fig. 8). There is a negative feedback loop from down-stream p70/S6K to up-stream class I phosphoinositide 3-kinase (PI3K)[46]. The PI3K is responsible for various cellular functions related to the competence of class I PI3K to activate Akt. Therefore, suppressed mTORC1 and then p70/S6K by RAPTOR RNAi lead to the loss of this feedback for class I PI3K, resulting in Akt activation. The Akt enhances cell survival directly by blocking pro-apoptotic proteins including the Bcl-2-associated death promoter protein and through effects on transcription factors such as forkhead box O and p53[22]. In addition, Akt increases cell proliferation through not only mTORC1 induction (p70/S6K induction and 4E-BP1 inhibition) but also other pathways including inhibition of negative cell-cycle regulators p27/KIP1 and p21/CIP1[22]. This may explain why RAPTOR RNAi relatively maintained cell proliferation despite reduced p70/S6K phosphorylation. Thus, Akt-mediated survival and proliferation strongly support mTORC1/RAPTOR interference as a potential therapeutic application to disc disease.

However, it is controversial whether apoptosis is under the direct regulation of Akt. In this study, mTOR and RICTOR siRNAs did not increase apoptotic markers despite decreased Akt phosphorylation through mTORC2 suppression. There are many points of cross-talk and redundancy in the AKT-signaling network[47]. The mTORC1 and/or mTORC2 inhibition-mediated autophagy induction might prevent excessive disc cell apoptosis as shown previously[48]. Further mechanistic analyses are required to identify the optimal intervention in mTOR signaling of disc cells for treatment.

Articular cartilage-specific deletion of mTOR in mice has shown to be protective against destabilized medial meniscus-induced osteoarthritis, resulting in enhanced autophagy, decreased apoptosis, and increased catabolic MMP-13 expression[49]. Rapamycin, inhibiting mTORC1, has been reported to activate autophagy, suppress apoptosis, and reduce osteoarthritis-related gene expression changes in human chondrocytes[24]. The majority of our results are consistent with these data, providing more mechanistic interpretations and speculations. Although these prior studies place importance on mTORC1 suppression-induced autophagy activation for chondrocyte protection, the current study highlights Akt activation by mTORC1/RAPTOR interference. Future studies will

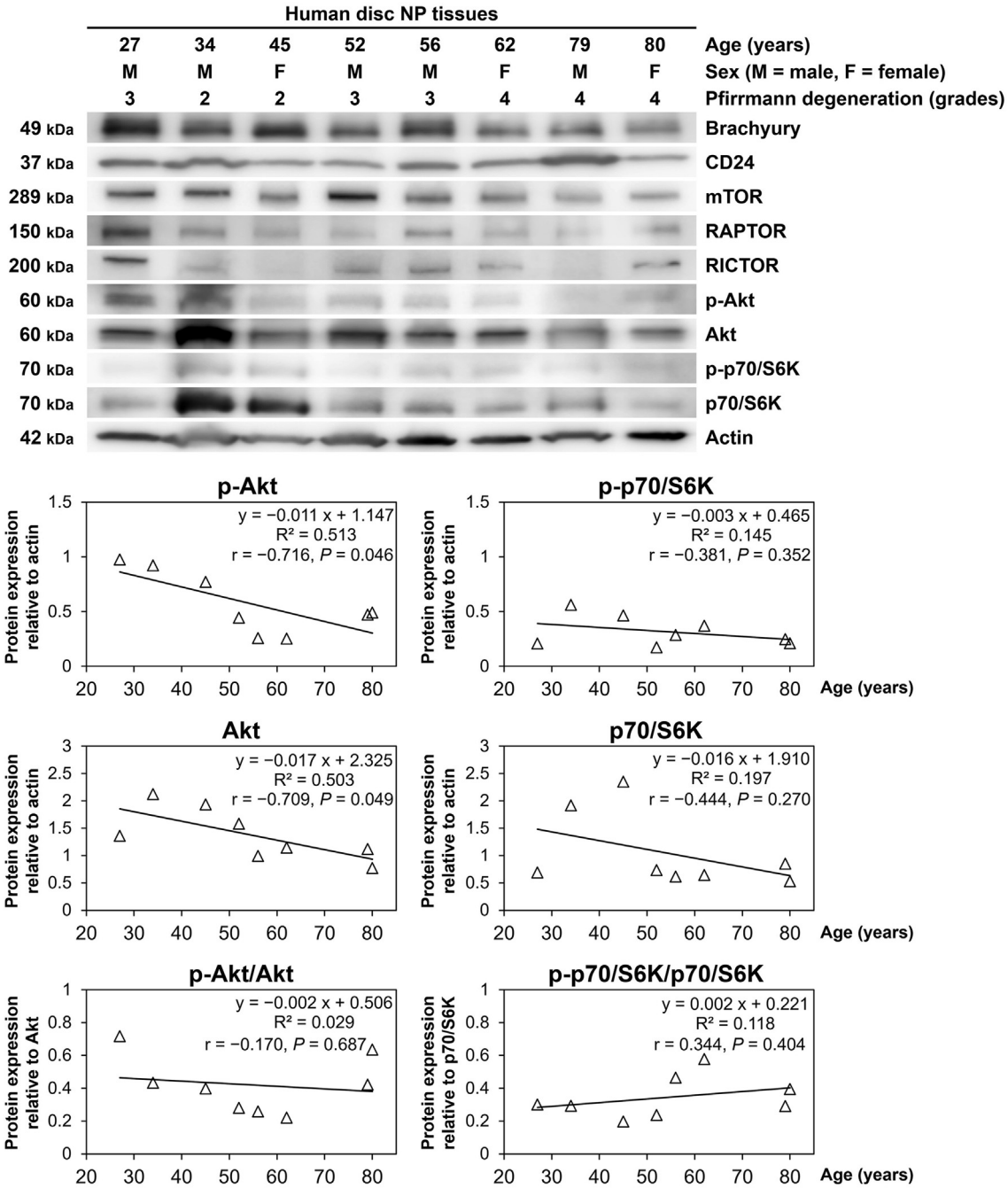

Fig. 7. mTOR signaling is detectable in human disc NP tissues. Western blotting for disc NP-phenotypic brachyury and CD24 and mTOR signaling-related mTOR, RAPTOR, RICTOR, Akt, phosphorylated Akt (p-Akt), p70/S6K, and phosphorylated p70/S6K (p-p70/S6K) in total protein extracts from human disc NP tissues of patients who underwent lumbar spine surgery for degenerative disease. Actin was used as a loading control. Immunoblots show samples randomly selected ($n = 8$). Changes in protein expression, phosphorylation, and phosphorylation/expression ratio of Akt and p70/S6K relative to actin are shown. Regression analysis was used.

be designed to determine whether Akt or autophagy is essential to protect cells against apoptosis, senescence, and matrix catabolism. Then, as shown in this study, the findings in prior studies can be explained by mTORC1 suppression. However, mTOR is the central signal integrator for nutrients and energy, playing critical roles in cell growth and division[21]. The present study indicates that complete inhibition of mTOR signaling may be harmful because of reduced cell proliferation and viability. In fact, homogenous deletion of mTOR results in embryonic lethality[50]. Therefore, incomplete inhibition of mTORC1 only, facilitating maintained reduced cell proliferation and viability with apoptosis suppression, is recommended.

Our disc-tissue Western blotting successfully detected mTOR-signaling molecules showing age-dependent decreases in Akt expression and phosphorylation. Despite the increased attention of mTOR signaling[21], its actual involvement in disc degeneration is largely unknown. The observed, reduced expression and phosphorylation of mTOR-signaling molecules with aging is potentially supportive to disclose the involvement in disc health. Future examinations with larger sample sizes are required.

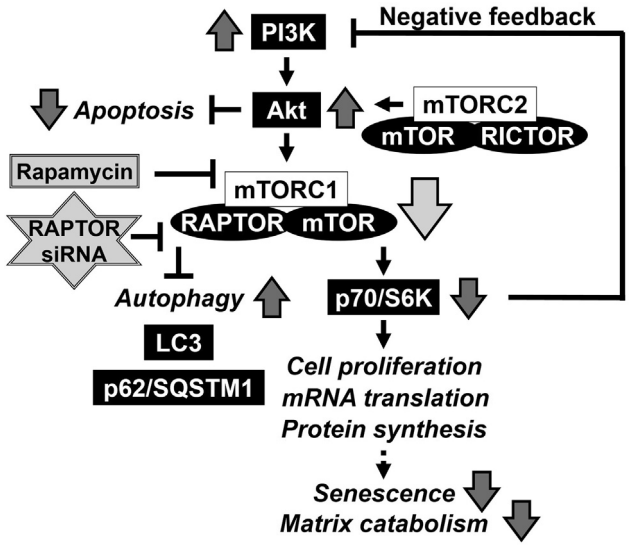

Fig. 8. Schematic illustration of effects of mTORC1/RAPTOR interference on human disc NP cells. The mTOR is a serine/threonine kinase that integrates nutrients to execute cell growth and division. The mTOR exists in two complexes of mTORC1 containing RAPTOR and mTORC2 containing RICTOR. Down-stream effectors of mTORC1 including p70/S6K regulate cell proliferation, mRNA translation, and protein synthesis. Autophagy, as assessed by LC3 and p62/SQSTM1, is under the tight negative regulation of mTORC1. The mTORC1 is regulated by up-stream Akt, an essential pro-survival mediator by suppressing apoptosis. In human disc NP cells, selective suppression of mTORC1 by RAPTOR siRNA or rapamycin decreased p70/S6K phosphorylation, increased Akt phosphorylation through the negative feedback loop for class I PI3K, and enhanced autophagy, which further inhibited senescence and matrix catabolism possibly through disruption of p70/S6K and other mTORC1 effectors and also apoptosis possibly through Akt activation.

A limitation of this study is the lack of *in vivo* RNAi or rapamycin data. Further studies using animal models of disc degeneration[11–13,16,37] may reveal the effectiveness of mTORC1/RAPTOR-suppressing therapies. Another limitation is the monolayer cell culture. Three-dimensional culture systems are favorable to simulate the physiological environment of the disc NP, although it is actually difficult to analyze detailed molecular signaling using alginate gel beads. An additional limitation is no consideration of the variety among subjects with different ages, genders, and degeneration grades, although our findings of *in vitro* experiments were consistent regardless these parameters. These are subjects to be studied in the future.

In conclusion, selective interference of mTORC1/RAPTOR protects against inflammation-induced apoptosis, senescence, and matrix catabolism in human disc cells. These beneficial effects depend on activation of Akt as well as autophagy, providing insights into biological therapy.

Authors' contributions

All authors have made substantial contributions to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted. The specific contributions of the authors are as follows:

(1) Conception and design of the study: MI, TY.
(2) Analysis and interpretation of the data: MI, TY, KK, KM, TT, YTerashima, YK, YTakeoka, SM, RK, KN.
(3) Drafting of the article: MI, TY.
(4) Critical revision of the article for important intellectual content: KK, KM, TT, YTerashima, YK, YTakeoka, SM, RK, KN.
(5) Final approval of the article: MI, TY, KK, KM, TT, YTerashima, YK, YTakeoka, SM, RK, KN.
(6) Statistical expertise: MI, TY.
(7) Collection and assembly of data: MI, TY.

Conflict of interest

The authors have no competing interests to declare.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2017.08.019.

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