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RESEARCH ARTICLE

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Rapamycin mitigates inflammation-mediated disc matrix homeostatic imbalance by inhibiting mTORC1 and inducing autophagy through Akt activation

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Abstract

Background: Low back pain is a global health problem that originated mainly from intervertebral disc degeneration (IDD). Autophagy, negatively regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway, prevents metabolic and degenerative diseases by removing and recycling damaged cellular components. Despite growing evidence that autophagy occurs in the intervertebral disc, the regulation of disc cellular autophagy is still poorly understood.

Methods: Annulus fibrosus (rAF) cell cultures derived from healthy female rabbit discs were used to test the effect of autophagy inhibition or activation on disc cell fate and matrix homeostasis. Specifically, different chemical inhibitors including rapamycin, 3-methyladenine, MK-2206, and PP242 were used to modulate activities of different proteins in the PI3K/Akt/mTOR signaling pathway to assess IL-1β-induced cellular senescence, apoptosis, and matrix homeostasis in rAF cells grown under nutrient-poor culture condition.

Results: Rapamycin, an inhibitor of mTOR complex 1 (mTORC1), reduced the phosphorylation of mTOR and its effector p70/S6K in rAF cell cultures. Rapamycin also induced autophagic flux as measured by increased expression of key autophagy markers, including LC3 puncta number, LC3-II expression, and cytoplasmic HMGB1 intensity and decreased p62/SQSTM1 expression. As expected, IL-1β stimulation promoted rAF cellular senescence, apoptosis, and matrix homeostatic imbalance with enhanced aggrecanolysis and MMP-3 and MMP-13 expression. Rapamycin treatment effectively mitigated IL-1β-mediated inflammatory stress changes, but these alleviating effects of rapamycin were abrogated by chemical inhibition of Akt and mTOR complex 2 (mTORC2).

Conclusions: These findings suggest that rapamycin blunts adverse effects of inflammation on disc cells by inhibiting mTORC1 to induce autophagy through the PI3K/Akt/mTOR pathway that is dependent on Akt and mTORC2 activities. Hence, our

findings identify autophagy, rapamycin, and PI3K/Akt/mTOR signaling as potential therapeutic targets for IDD treatment.

KEYWORDS
autophagy, disc degeneration, inflammation, intervertebral disc, PI3K/Akt/mTOR signaling pathway, rapamycin, spine

1 | INTRODUCTION

Back pain is a global health problem with 15%–45% morbidity[1] and socioeconomic burden of over $100 billion annually in the United States.[2] The cause of back pain is multifactorial.[3] However, a recent UK twin study has shown that intervertebral disc degeneration (IDD) is a main risk factor for back pain.[4] Furthermore, IDD is associated with neurological impairments including radiculopathy, myelopathy, and paralysis[3] and impaired daily activities of the elderly.[5]

Biochemically, IDD is characterized by the loss of extracellular proteoglycan matrix due to increased catabolism from persistent matrix homeostatic imbalance.[6] Matrix degradation is a consequence of increased activities of catabolic enzymes, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), and/or decreased activities of tissue inhibitors of metalloproteinases (TIMPs).[7–9] Disc matrix imbalance can be induced by inflammation from cytokines such as tumor necrosis factor-α, interleukin (IL)-1α, IL-1β, IL-6, and IL-17.[10] In addition, IDD is associated with increased apoptosis and cellular senescence, characterized by irreversible cell growth arrest due to the accumulation of DNA damage.[11–15] Apoptosis acts as a quality control mechanism for the maintenance of tissue homeostasis by eliminating defective cells.[16] Senescence serves as a protective mechanism against cell proliferation to prevent the propagation of damaged DNA.[17] However, cellular senescence acquires the senescence-associated secretory phenotype (SASP) that produces an abundance of matrix imbalance-inducing pro-inflammatory cytokines and MMPs.[17] Although the pathogenesis of IDD remains unclear, growing evidence suggest that degenerative disc cells acquire the phenotype, exhibiting all the key features of matrix homeostatic imbalance.

Autophagy, the intracellular process by which cells recycle their own damaged components, is an important cell survival mechanism that is activated under stress.[18] Autophagy is negatively regulated primarily by the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway.[19] There is growing evidence for the involvement of PI3K/Akt/mTOR signaling in various diseases,[19] but roles of this pathway in musculoskeletal disorders are not well understood.[20] The harsh disc microenvironment of hypoxia, mechanical overload, and low nutritional stress,[21] raises the possibility that resident disc cells utilize autophagy to cope with these stressful conditions.[22–24] The autophagy machinery contains proteins encoded by autophagy-related (Atg) genes, which are negatively regulated by mTOR under typical physiological conditions.[25] The mTOR is a serine/threonine kinase whose catalytic subunit consists of mTOR complexes 1 (mTORC1) and 2 (mTORC2), providing a key role in cell growth and homeostasis.[19] The mTORC1 negatively regulates autophagy.[19] The p70/ribosomal S6 kinase (p70/S6K) is a primary downstream effector of mTORC1, directly regulating protein synthesis.[19] Upstream regulation of mTORC1 is primarily mediated by the class I PI3K and Akt,[19] with Akt being an essential pro-survival regulator.[26]

Under stress, mTORC1 is suppressed and Atg proteins are activated for the formation and maturation of the autophagosome, an important step in autophagy.[18] The microtubule-associated protein 1 light chain 3 (LC3; Atg8 homolog) is a ubiquitin-like protein. Unlike its LC3-I cytosolic form, LC3-II is a phosphatidylethanolamine-conjugated form of LC3 that serves as a key protein marker of autophagy because LC3-II reliably associates with completed autophagosomes.[27] In addition, cytoplasmic release of high mobility group box 1 (HMGB1), a DNA-binding nuclear protein that plays a role in transcriptional regulation, has been established as a regulator and sensor of autophagy.[28] In response to stress, HMGB1 translocates from the nucleus to the cytoplasm, resulting in the initiation of the autophagosome formation.[28] The autophagosome fuses with the lysosome, upon which the enclosed cargo, autolysosome, is degraded and released to be reutilized.[18] The p62/sequestosome 1 (p62/SQSTM1) is a ubiquitin-binding protein that serves as a link between LC3 and ubiquitinated substrates.[29] Selectively, p62/SQSTM1 and p62/SQSTM1-bound polyubiquitinated proteins become incorporated into completed autophagosomes and then degraded in autolysosomes.[29] Decreasing p62/SQSTM level thus serves as an important marker of active autophagy.[29] Monitoring of this dynamic process, autophagic flux, is essential to understand the involvement and roles of autophagy.[30]

Rapamycin, a key inducer of autophagy by inhibiting mTORC1, protects against aging-associated diseases.[19] Rapamycin is a clinically approved immunosuppressant agent, that is widely reported to increase the lifespan in various clinical settings[31] and known for cancer prevention in animal models and humans.[32] Recent studies have shown its protective effects on human articular chondrocytes[33] and disc cells.[15,20,34,35] However, the mechanisms by which rapamycin affects these cells remain poorly understood. Moreover, studies using human disc surgical specimens generate variable findings that are difficult to interpret due to the heterogeneous cell populations from different age, sex, and degeneration severity of tissues. Therefore, we here performed an in vitro study using a healthy female rabbit disc annulus fibrosus (rAF) cell culture model to elucidate the effects of inhibiting or inducing autophagy on disc cell fate and matrix homeostasis. Specifically, activities of different proteins in the PI3K/Akt/mTOR signaling pathway were systematically modulated

by using different chemical inhibitors including rapamycin, 3-methyladenine (3-MA), MK-2206, and PP242. Autophagy induction by rapamycin-mediated mTORC1 inhibition played a pivotal role in mitigating the detrimental inflammatory effects on rAF cells, but this rapamycin therapeutic action required Akt activation to be non-inhibited. Our results provide important insights into potential biological disc therapeutic targets.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experiments were performed under the approval and guidance of the University of Pittsburgh Institutional Animal Care and Use Committee (1001336A-2 and 1001336B-2). This study was conducted in accordance with the principles of the Declaration of Helsinki and with the laws and regulations of the United States.

2.2 | Reagents

The antibodies against the following protein antigens were obtained for this study: mTOR (#2983: rabbit-monoclonal), mTOR phosphorylated at serine 2448 (#5536: rabbit-monoclonal), mTOR phosphorylated at serine 2481 (#2974: rabbit-polyclonal), p70/S6K (#2708: rabbit-monoclonal), p70/S6K phosphorylated at threonine 389 (#9234: rabbit-monoclonal), Akt (#4691: rabbit-monoclonal), Akt phosphorylated at serine 473 (#4060: rabbit-monoclonal), cleaved caspase-3 (#9664: rabbit-monoclonal), and poly (ADP-ribose) polymerase (PARP) (#9532: rabbit-monoclonal) from Cell Signaling Technology (Danvers, MA); p62/SQSTM1 (ab56416: mouse-monoclonal), MMP-3 (ab73955: rabbit-polyclonal), MMP-13 (ab39012: rabbit-polyclonal), and TIMP-1 (ab38980: rabbit-polyclonal) from Abcam (Cambridge, UK); LC3 (L8918: rabbit-polyclonal), HMGB1 (H9537: mouse-monoclonal), and β-actin (A2228: mouse-monoclonal) from Sigma-Aldrich (St. Louis, MO); LC3 (sc-271 625: mouse-monoclonal) and p16/INK4A (sc-1661: mouse-monoclonal) from Santa Cruz Biotechnology (Santa Cruz, CA). Then, the antibody for aggrecan G1-domain (rabbit-polyclonal) was a kind gift from Dr. Peter J. Roughley (Genetics Unit, Shriners Hospital for Children, Montreal, Quebec, Canada).[36]

The fluorescein-labeled terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (#11684795910), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (#203782) for the senescence-associated β-galactosidase (SA-β-gal) activity detection, Cell Counting Kit-8 (CCK-8; CK04), and PicoGreen double-stranded DNA quantification assay kit (P11496) were purchased from Roche Diagnostics (Mannheim, Germany), EMD Millipore (Billerica, MA), Dojindo Molecular Technologies (Kumamoto, Japan), and Life Technologies (Carlsbad, CA), respectively. Immunofluorescent reagents including donkey-derived Alexa Fluor 488 (A21206: anti-rabbit) and 555 (A31570: anti-mouse) dyes and Hoechst 33342 (H1399) were purchased from Life Technologies. Western blotting reagents including the bicinchoninic acid (BCA) protein assay kit (#23227) were purchased from Thermo Fisher Scientific (Rockford, IL). The RNA extraction kit (#73404) was purchased from Qiagen (Hilden, Germany). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) SYBR Green dye (#170-8893) and custom primers were purchased from Bio-Rad Laboratories (Hercules, CA) and Life Technologies, respectively. ^{35}S-sulfate (ARS0105) and ^{3}H-proline (ART0475) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Pronase (#53702) and collagenase-P (#12593123) were purchased from EMD Millipore and Roche Diagnostics, respectively. Dimethyl sulfoxide (DMSO) (D8418), trypan blue solution (T8154), paraformaldehyde (P6148), chondroitinase ABC (C2905), endo-β-galactosidase (G6920), papain (P3375), and chondroitin-6-sulfate (C8529) were obtained from Sigma-Aldrich.

Rapamycin (#553210) and recombinant human interleukin (IL)-1β (#407615) were purchased from EMD Millipore. Then, 3-MA (M9281), chloroquine (C6628), and PP242 (P0037) were purchased from Sigma-Aldrich. In addition, MK-2206 (S1078) was purchased from Selleck Chemicals (Houston, TX).

Ham's F-12 nutrient mixture medium (F-12) containing 1 mM L-glutamine (#11765) and Dulbecco's modified Eagle's medium (DMEM) containing 25 mM D-glucose and 4 mM L-glutamine (#11965) were obtained from Life Technologies. Fetal bovine serum (FBS) (S12450) was purchased from Atlanta Biologicals (Lawrenceville, GA). Penicillin and streptomycin (#15140) were obtained from Life Technologies and added to all media at 1%.

2.3 | Cells

Discs from lumbar spines of 6-month-old female New Zealand White rabbits (~2.5 kg) were harvested immediately postmortem. Annulus fibrosus tissues were dissected and digested at 37°C in F-12 with 5% FBS and 0.2% Pronase for 1 h and then 0.02% collagenase-P for 12 h. Isolated rAF cells were cultured in F-12 with 10% FBS in a 5% CO_2 incubator, and 80% confluent cells were trypsinized and prepared for experiments.

First passage, monolayer cultures of rAF cells were used for evaluation. For cell proliferation assay, 5×10^4 cells/well were directly treated for up to 336 h. For other assays, cells were precultured in DMEM with 1% FBS for 24 h followed by 10% FBS for 72 h (~80% confluence), and then treated for up to 48 h. All experiments were performed in a 5% O_2 (5% CO_2) incubator to simulate relatively hypoxic physiological conditions of rAF cells (4%–7% oxygen).[37]

2.4 | Treatments

Cultures of rAF cells were pharmacologically treated with rapamycin to inhibit mTORC1 to induce autophagy,[30] 3-MA to inhibit the class III PI3K to block autophagy,[30] MK-2206 to inhibit Akt,[38] and PP242 to inhibit both mTORC1 and mTORC2.[39] The DMSO was used as the vehicle of rapamycin, MK-2206, and PP242 because of their poor

water solubility. A lysosomotropic compound, chloroquine, was also used at 15 μM for autophagic LC3 turnover assessment.[30] Further, a pro-inflammatory cytokine, IL-1β, was added at 10 ng/mL to simulate inflammatory conditions often observed in IDD.[10] Vehicle control was used for all treatment agents.

2.5 | Cell proliferation assay

In a 6-well plate, 5×10^4 cells/well were cultured in 10% FBS-supplemented DMEM with (i) 100 nM or 1 μM rapamycin, (ii) 2.5 or 5 mM 3-MA, or (iii) vehicle control for 0, 48, 96, 144, 192, 240, 288, or 336 h with media change every 48 h. Cells were trypsinized and the number was counted using the trypan blue exclusion method.

2.6 | Dehydrogenase activity, DNA amount, and cell metabolic activity assays

Cells were cultured in 1%-supplemented DMEM with (i) 0–50 μM rapamycin, (ii) 0–50 mM 3-MA, (iii) 0–50 μM MK-2206, (iv) 0–50 μM PP242, or (v) vehicle control for 48 h. Total dehydrogenase activity was assessed by CCK-8. Total DNA amount was assessed by PicoGreen. The CCK-8 absorbance (450 nm) and PicoGreen fluorescence (fluorescein) were measured using the VICTOR X3 multilabel plate reader (PerkinElmer, Waltham, MA). Cell metabolic activity (per cell) was calculated as dehydrogenase activity normalized to DNA amount.

2.7 | Imaging cytometry

Cells were cultured in 1% FBS-supplemented DMEM with (i) 100 nM rapamycin, (ii) 2.5 mM 3-MA, or (iii) vehicle control for 0, 3, 6, 12, 24, or 48 h. Cells were fixed in 4% paraformaldehyde and stained with primary antibodies for LC3 and HMGB1 followed by staining with Alexa Fluor secondary antibodies. Hoechst 33342 was used for counterstaining. To assess autophagic flux, the number of LC3 puncta and nuclear and cytoplasmic intensity of HMGB1 per cell were measured by the Thermo Scientific Cellomics ArrayScan VTI automated fluorescent microscopic imaging cytometry system (Thermo Fisher Scientific) as described previously.[28,40]

2.8 | Western blotting

Cells were cultured in 1% FBS-supplemented DMEM with (i) 100 nM rapamycin, (ii) 2.5 mM 3-MA, (iii) 100 nM rapamycin combined with 5 μM MK-2206, (iv) 100 nM PP242, or (v) vehicle control for 0, 12, 24, or 48 h for autophagy, apoptosis, and senescence analysis, or in 0%-supplemented DMEM with the same treatments for 48 h for matrix degradation analysis. Protein extracts from total cell lysates were resolved on a 4%–20% gel for autophagy, senescence, and apoptosis markers. Serum-free conditioned media containing protein released from cells were concentrated using spin columns (EMD Millipore). Protein extracts from supernatants were resolved on a 4%–20% gel for catabolic and anti-catabolic molecules. Both protein extracts from cells and supernatants were resolved on an 8% gel for aggrecan analysis following deglycosylation by chondroitinase ABC and endo-β-galactosidase. Protein concentration was determined by BCA protein assay. Separated proteins were transferred to a polyvinylidene difluoride membrane. After blocking, the membrane was incubated with primary antibodies for LC3, HMGB1, p62/SQSTM1, mTOR, phosphorylated mTOR, p70/S6K, phosphorylated p70/S6K, Akt, phosphorylated Akt, PARP, p16/INK4A, MMP-3, MMP-13, TIMP-1, aggrecan G1-domain, and actin, followed by incubation with peroxidase-conjugated secondary antibodies. Signals were visualized by enhanced chemiluminescence and images were taken using the ChemiDoc MP system (Bio-Rad Laboratories).

2.9 | Immunofluorescence

Cells were cultured in 1% FBS-supplemented DMEM with (i) 100 nM rapamycin, (ii) 2.5 mM 3-MA, or (iii) vehicle control for 48 h. After fixation, apoptotic cells were determined by immunofluorescent TUNEL assay[41] or staining for an apoptosis marker, cleaved caspase-3.[42] Senescent cells were identified by immunofluorescence for a senescence marker, p16/INK4A.[43] Hoechst 33342 was used for counterstaining. Images were obtained by the Eclipse E800 microscope (Nikon, Tokyo, Japan) and Northern Eclipse software (Empix Imaging, Mississauga, Canada). The number of TUNEL-positive cells and immunopositive cells for cleaved caspase-3 and p16/INK4A was divided by the total cell number, counted in 10 random low-power fields (×100) using the ImageJ (https://imagej.nih.gov/ij/).

2.10 | SA-β-gal assay

Cells were cultured as in immunofluorescent experiments. Senescent cells were identified by SA-β-gal assay using 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside at pH 6.[44] The percentage of SA-β-gal-positive cells was calculated as described above.

2.11 | Quantitative real-time reverse transcription-polymerase chain reaction

Cells were cultured in 0%–10% FBS-supplemented DMEM with (i) 100 nM rapamycin, (ii) 2.5 mM 3-MA, or vehicle control for 48 h. Total RNA was isolated, and messenger RNA (mRNA) expression levels of catabolic MMP-3, MMP-13, ADAMTS-4, ADAMTS-5, anti-catabolic TIMP-1, TIMP-3, anabolic aggrecan-1, collagen type 1-α1, and collagen type 2-α1 relative to 18S ribosomal RNA (rRNA) were assessed by SYBR Green-based qRT-PCR using the iQ5 real-time PCR detection system (Bio-Rad Laboratories).[45] A good feasibility of 18S rRNA as an endogenous control in rabbit disc cells has been

reported.[46] The primer sequences were designed using the Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA) based on the sequences in the database (GenBank; http://www.ncbi.nlm.nih.gov/genbank/) and the specificity was confirmed using the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Table S1). Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method[47] for drug-treated conditions relative to the vehicle control condition.

2.12 | Proteoglycan, collagen, and total protein synthesis assays

Cells were cultured in 1% FBS-supplemented DMEM with (i) 100 nM rapamycin, (ii) 2.5 mM 3-MA, (iii) 5 μM MK-2206, (iv) 100 nM rapamycin with 5 μM MK-2206, (v) 100 nM PP242, or (vi) vehicle control for 48 h, followed by 8-h labeling incubation with 10 μCi/mL ^{35}S-sulfate for newly synthesized proteoglycan detection or by 24-h labeling incubation with 10 μCi/mL ^{3}H-proline for newly synthesized collagen and total protein detection. After extraction, radioactivity in synthesized products was measured using the Tri-Carb 2100TR liquid scintillation counter (PerkinElmer) and normalized to DNA amount as described previously.[48]

2.13 | Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of four independent samples in duplicate or triplicate unless otherwise indicated. Multi-way analysis of variance (ANOVA) with the Tukey–Kramer post hoc test was used to assess changes for the effects of treatment, time, and serum concentration. Mixed-design ANOVA was applied to dehydrogenase activity, DNA amount, and cell metabolic activity assays and qRT-PCR analysis because of within-subject variables. Statistical significance was assessed with $p < 0.05$ and $p < 0.01$ using SPSS Statistics 17 (SPSS, Chicago, IL).

3 | RESULTS

3.1 | Rapamycin and 3-MA both induce autophagic flux in rAF cells via differing mechanisms

Rabbit disc AF cell cultures, grown in DMEM with 1% FBS under 5% O_2 to mimic physiologic harsh conditions of low nutrition and hypoxia,[21] were treated with 100 nM rapamycin or 2.5 mM 3-MA for different durations to study autophagic flux. We chose rapamycin to induce autophagy through its inhibition of mTORC1, and 3-MA with the intention to block autophagy by its reported inhibition of the autophagosome formation via the inhibition of the class III PI3K.[49] These drug concentrations were chosen in this study as they are subtoxic but has an impact on rAF cell metabolism (Figure S1A–C).

To monitor autophagic flux, we initially performed time-course LC3 puncta counting and nuclear and cytoplasmic HMGB1 intensity measurement in rAF cell cultures using imaging cytometry (Figure 1A).[40] Increased LC3 punctae indicate increased completed autophagosomes.[30] Increased cytoplasmic HMGB1 indicates the activation of beclin1, Atg6 homolog, which is essential to initiate the autophagosome formation.[28,30] Cytoplasmic HMGB1 binds beclin1, resulting in the dissociation of beclin1 and B-cell lymphoma 2 and subsequent induction of autophagy.[28] We also previously established total HMGB1 expression as an autophagy marker in rAF cells.[50]

Nutrients supplied by 1% FBS-supplemented DMEM under 5% O_2 have been shown to be sufficient to maintain the balance between rAF cell proliferation and death in our previous study.[50] We evaluated cell number by counting Hoechst-positive nuclei, which demonstrated that the number of cells remained relatively unchanged though the time course in every group (Figure 1B,C). The number of LC3 puncta per cell, which showed modest increases at 12 h and later time points in the vehicle control (Figure 1B,C), suggesting a dynamic involvement of autophagy in our cell culture conditions. More pronounced increases in LC3 puncta number by rapamycin treatment were observed at 6 h and later (Figure 1B,C). Although 3-MA did not increase LC3 punctae at 6–12 h, unexpectedly 3-MA subsequently showed marked increases in LC3 puncta at 24–48 h, suggesting the induction of autophagy (Figure 1B,C). Nuclear HMGB1 intensity increased transiently at 12 h in every group, although this change was more remarkable in rapamycin and 3-MA treatments (Figure 1B,C). While cytoplasmic HMGB1 intensity showed modest increases at 12 h and later in the control group, rapamycin and 3-MA treatment both induced more progressive, time-dependent increases in cytoplasmic HMGB1 level with statistical significance at 48 h when compared to the control (Figure 1B,C). Thus, rapamycin and surprisingly 3-MA activate the early (HMGB1 translocation) and middle (LC3 puncta formation) phases of autophagic flux in disc cells.

To further evaluate autophagy induction, we performed Western blotting to measure autophagy biomarkers of LC3, HMGB1, and p62/SQSTM1. Western blotting demonstrated a time-dependent increase in expression of a key autophagy marker LC3-II with a peak at 12–24 h by rapamycin (Figure 1D). For reasons unknown, very low LC3-I was immunodetected in rAF cells, which is consistent with our previous report.[50] Further, expression of HMGB1 showed a similar pattern to LC3-II expression (Figure 1D). Then, p62/SQSTM1, an autophagy-selective substrate that gets degraded during the late phase of autophagic flux (autophagosome degradation),[30] also decreased over time starting at 12 h in cells after being treated by rapamycin compared to the vehicle control (Figure 1D). Similarly, 3-MA treatment increased LC3-II and HMGB1 as well as decreased p62/SQSTM1 expression, but at a later time window of 24–48 h (Figure 1D).

To additionally confirm the induction of autophagy in rAF cells by rapamycin and 3-MA, we performed an LC3 turnover assay using a lysosomotropic compound, chloroquine, that blocks lysosomal acidification and induces the accumulation of autophagosomes containing LC3-II.[30] Chloroquine treatment increased LC3-II level in rapamycin-treated cells and 3-MA-treated cells at 48 h posttreatment (Figure 1E), unequivocally indicating increased autophagic flux

through the induction of the autophagosome formation, maturation, and degradation by rapamycin and 3-MA.

In the PI3K/Akt/mTOR signaling pathway, mTOR is phosphorylated at serine 2448 by PI3K/Akt signaling[51] and is also auto-phosphorylated at serine 2481.[52] The p70/S6K is a direct downstream protein target of mTORC1 which phosphorylates p70/S6K primarily at threonine 389[53]; thus, S6K phosphorylation directly indicates mTORC1 activation. The Akt is activated by the class I PI3K and mTORC2, resulting in Akt phosphorylation at serine 473.[26] Although 3-MA targets both of the classes I and III PI3Ks indiscriminately,[49,54] it has been proposed to suppress autophagy by inhibiting the class III PI3K activity,[55] which is essential for the initiation of autophagy via the recruitment of other Atg proteins at the isolation membrane or phagophore.[56] In disc cells, our results suggest that 3-MA preferentially blocks the class I PI3K over the class III PI3K to induce rather than inhibit autophagy. Indeed, Western blotting results showed the inhibition of mTOR and its substrate p70/S6K phosphorylation by rapamycin and 3-MA (Figure 1F). These findings

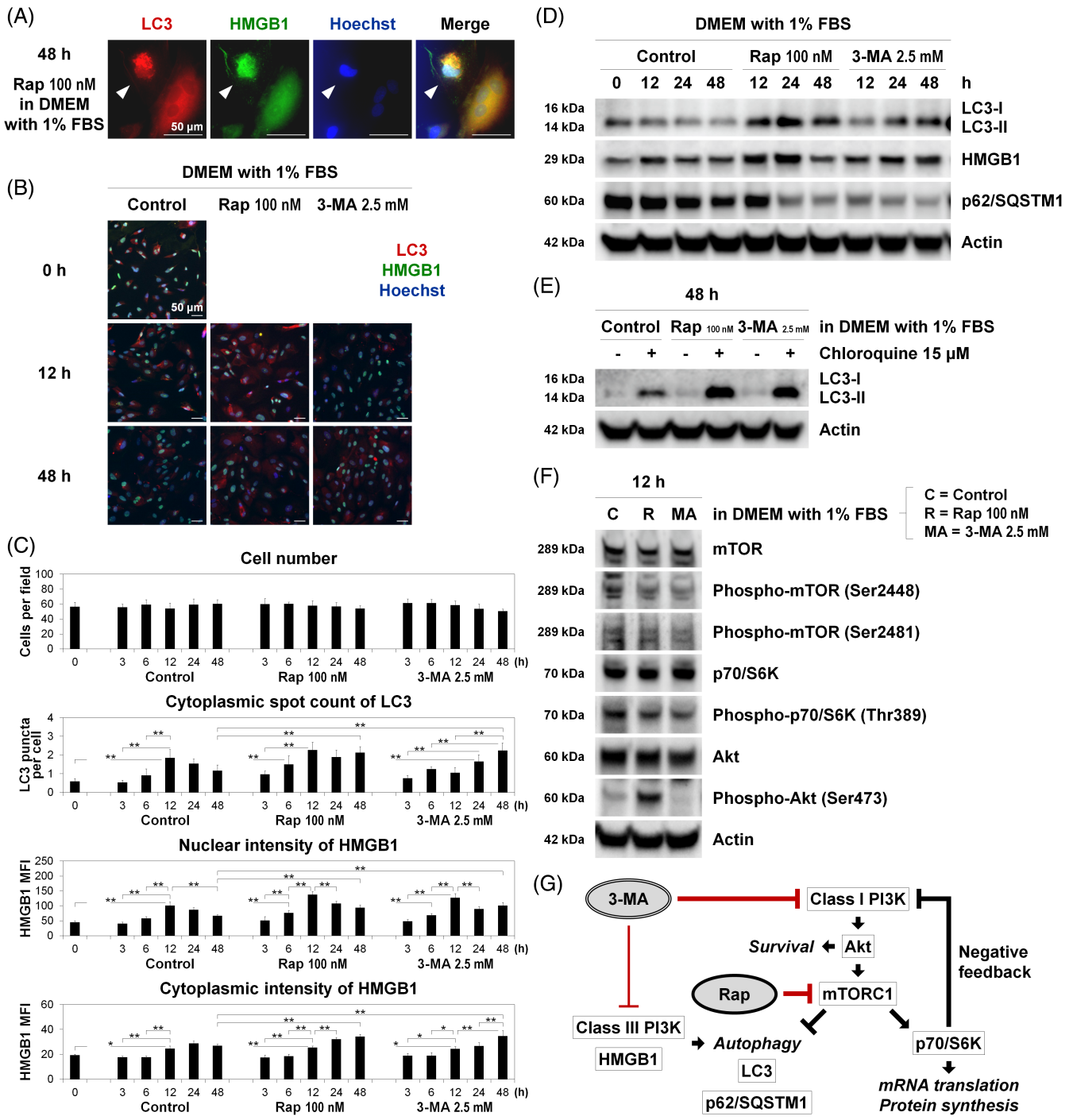

FIGURE 1 Legend on next page.

suggest that 3-MA predominantly blocks the class I PI3K, consistent with the finding that rapamycin blocks mTORC1 with the p70/S6K-mediated negative feedback effect on phosphorylated Akt,[57] while 3-MA treatment completely abolished Akt phosphorylation, depending on the class I PI3K activity (Figure 1G).

3.2 | Rapamycin decreases while 3-MA increases apoptosis and senescence in rAF cells under nutritional and inflammatory stress

Rabbit disc AF cells, grown under 1% FBS-supplemented DMEM analogous to the culture conditions used above, were treated with rapamycin or 3-MA to assess the effects of modulating autophagy on the phenotype. Rapamycin decreased while 3-MA increased the percentage of apoptotic cells as shown by TUNEL assay and immunofluorescence for nuclear cleaved caspase-3 (Figure 2A,B). The TUNEL reactivity was localized in the nucleus, indicating nuclear DNA fragmentation.[41] Caspase-3, which is cleaved and activated by upstream caspases in the cytoplasm[16] and translocated into the nucleus for the cleavage of its nuclear substrates,[42] showed moderately increased signal translocated from the cytoplasm to the nucleus after 1% FBS-supplemented DMEM exposure. This effect was further enhanced by 3-MA; however, this translocation was consistently suppressed by rapamycin. Together, the observed results indicate that rapamycin decreases but 3-MA increases disc cell apoptosis.

The percentage of SA-β-gal-positive cells and p16/INK4A-immunopositive cells decreased by rapamycin but increased by 3-MA treatment (Figure 2A,B). Most SA-β-gal-positive cells were also morphologically flat and enlarged, consistent with the cellular senescent phenotype.[44] Then, SA-β-gal reactivity was localized in the cytoplasm while p16/INK4A signal was localized in the nucleus. These findings are consistent with reported evidence in other cell types,[43,44,58] suggesting disc cellular senescence decreased by rapamycin but increased by 3-MA.

To further investigate the effects of modulating autophagy on rAF cells under clinical conditions of limited nutrition and inflammation, rapamycin or 3-MA treatment was applied in 1% FBS-supplemented DMEM under the presence of IL-1β. Western blotting demonstrated stress-response increases in an apoptotic marker of cleaved PARP and senescence marker of p16/INK4A (Figure 2C). The PARP is a nuclear DNA-binding zinc finger protein involved in DNA repair, cleavage of which facilitates cellular disassembly and serves as a marker of apoptosis induction.[59] The p16/INK4A acts as a tumor suppressor by inhibiting cell cycle progression, and its expression level serves as a key biomarker of cellular senescence.[43] Then, stress-induced apoptosis and senescence were clearly suppressed by rapamycin but further amplified by 3-MA (Figure 2C). Furthermore, anti-apoptotic and anti-senescent effects of rapamycin were dose-dependent, as 1 μM rapamycin produced greater effects than 100 nM rapamycin (Figure 2D). Thus, rapamycin and 3-MA have contrasting effects on disc cell fate under low-nutrient and inflammatory conditions, with rapamycin being anti-apoptotic and anti-senescent while 3-MA is pro-apoptotic and pro-senescent. The upregulation of autophagy is suggested to be beneficial in suppressing disc cell apoptosis and senescence.

3.3 | Rapamycin decelerates while 3-MA accelerates matrix catabolism in rAF cells under nutritional and inflammatory stress

To understand the effects of autophagy modulation by rapamycin and 3-MA on disc extracellular matrix homeostasis, we assessed catabolic,

FIGURE 1 Rapamycin and 3-methyladenine (3-MA) both induce autophagic flux in rabbit disc annulus fibrosus (rAF) cells with differing mechanisms. (A) Imaging cytometry for LC3 (red) and HMGB1 (green) in rAF cells after 48-h culture in 1% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DMEM) with 100 nM rapamycin (shown as Rap in the figure). Hoechst (blue) was used for counterstaining. Autophagic cells (white arrow) showed the formation of LC3 puncta and cytoplasmic release of HMGB1. (B) Time-course imaging cytometry for LC3 (red), HMGB1 (green), and Hoechst (blue) in rAF cells after 0–48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control. (C) Changes in cell number per field, cytoplasmic spot count of LC3 per cell, and mean fluorescent intensity (MFI) of HMGB1 in the nucleus and cytoplasm per cell. Imaging cytometric data were automatically collected from up to 36 fields and analyzed according to an established algorithm. Data are shown as the mean ± SD ($n = 4$). Two-way ANOVA with the Tukey–Kramer post hoc test was used. $^{*}p < 0.05$; $^{**}p < 0.01$. (D) Time-course Western blotting for LC3, HMGB1, and p62/SQSTM1 in total protein extracts of rAF cells after 0–48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control. Actin was used as a loading control. (E) Western blotting for LC3 and Actin to assess LC3 turnover using 15 μM chloroquine in total protein extracts of rAF cells after 48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control. (F) Western blotting for mTOR, mTOR phosphorylated at serine 2448 (phospho-mTOR [Ser2448]), mTOR phosphorylated at serine 2481 (phospho-mTOR [Ser2481]), p70/S6K, p70/S6K phosphorylated at threonine 389 (phospho-p70/S6K [Thr389]), Akt, Akt phosphorylated at serine 473 (phospho-Akt [Ser473]), and Actin in total protein extracts of rAF cells after 12-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control. (G) Schematic illustration summarizing the effects of rapamycin and 3-MA on the PI3K/Akt/mTOR signaling pathway in rAF cells. Autophagy induction by rapamycin results from its inhibitory effect on mTOR complex 1 (mTORC1). The primary effect of 3-MA is to suppress the classes I and III PI3Ks, leading to autophagy inhibition only when the class III PI3K is successfully blocked. The PI3K/Akt/mTOR pathway regulates cell survival via Akt and mRNA translation and protein synthesis via p70/S6K. There is the p70/S6K-mediated negative feedback loop for the class I PI3K. Further, PI3K/Akt/mTOR signaling negatively regulates autophagy. In response to stress, HMGB1 translocates from the nucleus to the cytoplasm, resulting in the initiation of the autophagosome formation. The conjugation of phosphatidylethanolamine with LC3 drives the autophagosome maturation. An autophagy substrate, p62/SQSTM1, negatively correlates with the autophagosome degradation. In (A), (B), (D), (E), and (F), all images and immunoblots shown are representative of four experiments with similar results.

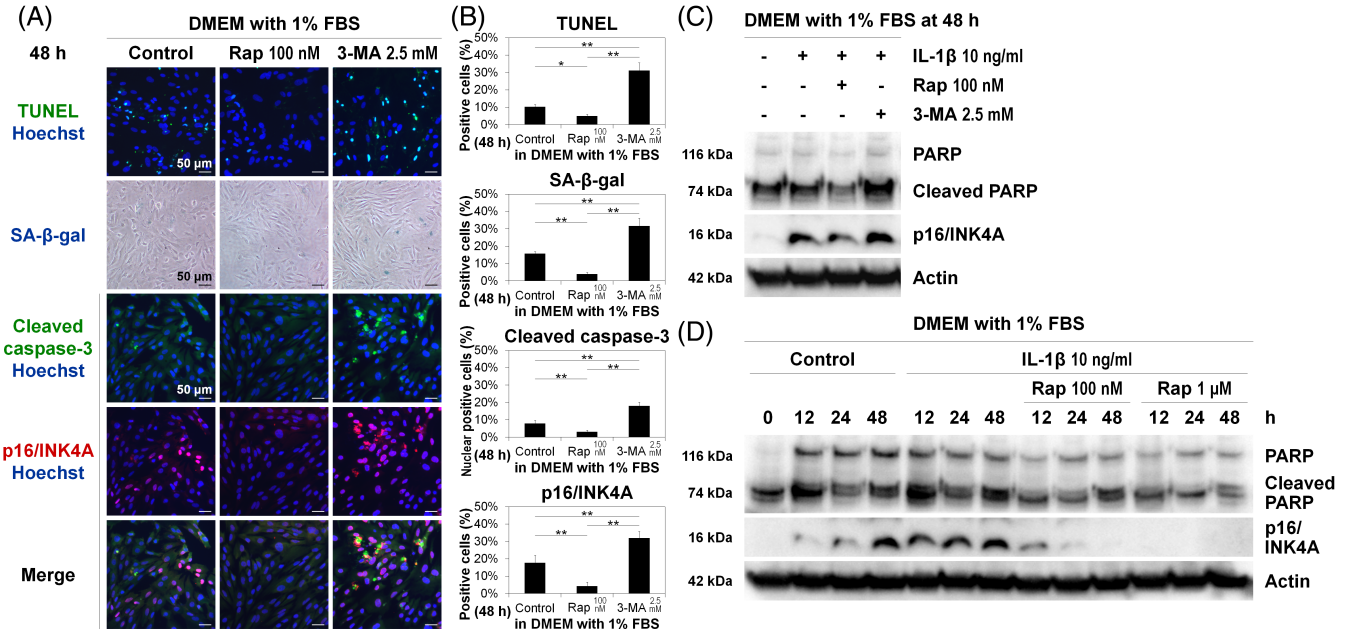

FIGURE 2 Rapamycin decreases while 3-methyladenine (3-MA) increases apoptosis and senescence in rabbit disc annulus fibrosus (rAF) cells under nutritional and inflammatory stress. (A) Immunofluorescence for TUNEL (green), colorimetric staining for SA-β-gal (blue), and immunofluorescence for cleaved caspase-3 (green) and p16/INK4A (red) in rAF cells after 48-h culture in 1% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DMEM) with 100 nM rapamycin (shown as Rap in the figure), 2.5 mM 3-MA, or vehicle control. Hoechst (blue) was used for immunofluorescent counterstaining. (B) Changes in the percentage of positive cells for TUNEL, SA-β-gal, nuclear cleaved caspase-3, and p16/INK4A. The number of total and positive cells was counted in 10 random low-power fields (×100). Data are shown as the mean ± SD ($n = 4$). One-way ANOVA with the Tukey–Kramer post hoc test was used. *$p < 0.05$; **$p < 0.01$. (C) Western blotting for cleaved PARP and p16/INK4A in total protein extracts of rAF cells after 48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control under the presence of 10 ng/mL IL-1β. Actin was used as a loading control. (D) Time-course Western blotting for cleaved PARP, p16/INK4A, and Actin in total protein extracts of rAF cells after 0–48-h culture in 1% FBS-supplemented DMEM with 100 nM or 1 μM rapamycin or vehicle control under the presence of 10 ng/mL IL-1β. In (A), (C), and (D), all images and immunoblots shown are representative of four experiments with similar results.

anti-catabolic, and anabolic gene expression by qRT-PCR. We performed this analysis on rAF cells cultured in DMEM with varying 0%–10% FBS because PI3K/Akt/mTOR signaling and autophagy have the close relationship with the abundance of nutrients and growth factors.[19] Rapamycin downregulated catabolic MMP-3 and MMP-13 mRNA expression under all serum conditions whereas 3-MA upregulated expression of these genes under more stressful, nutrient-limited 0% and 1% FBS conditions (Figures 3A and S2). While modest mRNA up-regulation of anti-anabolic TIMP-1 by rapamycin was observed regardless of serum concentrations, TIMP-1 expression was unchanged or downregulated by 3-MA (Figure S2). Catabolic ADAMTS-4 mRNA expression was downregulated, but ADAMTS-5 and anti-catabolic TIMP-3 expression was unresponsive to rapamycin and 3-MA treatment in 0%–1% FBS-supplemented DMEM (Figures 3A and S2). Overall, rapamycin but not 3-MA has the potential to suppress matrix catabolism induced by MMP-3 and MMP-13 which are major catabolic enzymes driving disc degeneration.[6] Both rapamycin and 3-MA generally suppressed matrix anabolism of rAF cells cultured under limited nutrients of 0%–1% FBS, including downregulated mRNA expression of anabolic aggrecan-1 and collagen types 1-α1 and 2-α1 genes (Figures 3A and S2). Then, new matrix protein synthesis, including collagen and proteoglycan, by radioisotopic assays also decreased in rAF cells treated with rapamycin and 3-MA under the conditions (Figure 3B). Hence, under physiologic conditions of nutrient deprivation, autophagy upregulation is protective against a shift toward matrix catabolism induced by nutritional stress, but it also limits matrix anabolism.

To determine the effects of autophagy modulation on disc cell matrix homeostasis under inflammatory stress often found in IDD, rAF cells treated with IL-1β were exposed to rapamycin or 3-MA. Western blotting of proteins from nonserum culture conditioned media of rAF cells showed that IL-1β drastically increased pro-MMP-3 and MMP-13 expression (Figure 3C). Rapamycin reduced IL-1β-induced MMP increases while 3-MA further promoted increases in pro and active MMPs (Figure 3C). Although IL-1β increased TIMP-1 expression slightly (Figure 3C), it was not affected by rapamycin but decreased by 3-MA treatment (Figure 3C). Finally, Western blotting using the aggrecan G1-domain antibody[36] showed that IL-1β increased MMP-cleaved aggrecan fragments (~63 kDa)[60] released in rAF cell culture conditioned media (Figure 3D), which decreased by rapamycin but further increased by 3-MA treatment (Figure 3D). The disc specificity of aggrecan fragments detected by the G1-domain antibody was confirmed by comparing with aggrecan neoepitopes using the anti-NITEGE (aggrecanase cleavage) and anti-VDIPEN (MMP cleavage) antibodies.[61] These findings suggest that autophagy upregulation also dampers matrix catabolism induced by inflammatory stress in disc cells.

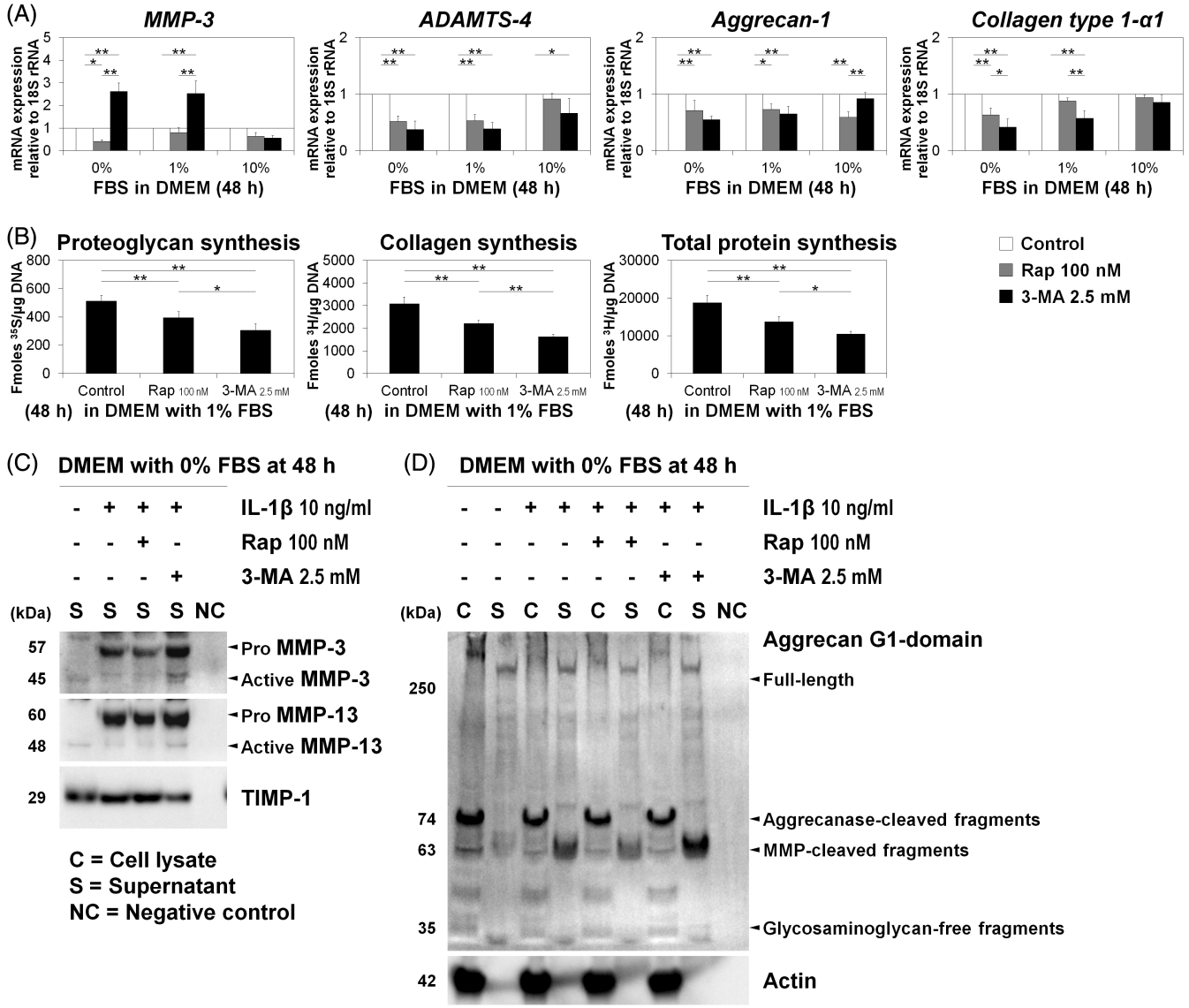

FIGURE 3 Rapamycin decelerates while 3-methyladenine (3-MA) accelerates matrix catabolism in rabbit disc annulus fibrosus (rAF) cells under nutritional and inflammatory stress. (A) The mRNA expression of MMP-3, ADAMTS-4, aggrecan-1, and collagen type 1-α1 relative to 18S rRNA in total RNA extracts of rAF cells after 48-h culture in 0%–10% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DMEM) with 100 nM rapamycin (shown as Rap in the figure), 2.5 mM 3-MA, or vehicle control. Data are the mean ± SD shown as the fold change relative to those in the vehicle control condition ($n = 4$). Mixed-design ANOVA with the Tukey–Kramer post hoc test was used. $^{*}p < 0.05$; $^{**}p < 0.01$. (B) ^{35}S-incorporated proteoglycan and ^{3}H-incorporated collagen and total protein synthesis normalized to PicoGreen DNA amount in rAF cell lysates and conditioned media after 48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control. Data are shown as the mean ± SD ($n = 4$). One-way ANOVA with the Tukey–Kramer post hoc test was used. $^{*}p < 0.05$; $^{**}p < 0.01$. (C) Western blotting for MMP-3, MMP-13, and TIMP-1 in supernatant protein extracts of rAF cells after 48-h culture in 0% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control under the presence of 10 ng/mL IL-1β. (D) Western blotting using the aggrecan G1-domain antibody in rAF cell and supernatant protein extracts after 48-h culture in 0% FBS-supplemented DMEM with 100 nM rapamycin, 2.5 mM 3-MA, or vehicle control under the presence of 10 ng/mL IL-1β. Full-length aggrecan (~250 kDa) and aggrecanase-cleaved (~74 kDa), MMP-cleaved (~63 kDa), and glycosaminoglycan-free (~35 kDa) aggrecan fragments were identified. Actin was used as a loading control. In (C) and (D), immunoblots shown are representative of four experiments with similar results.

3.4 | Inhibition of Akt or mTORC2 abolishes mTORC1 suppression-mediated protective effects of rapamycin against apoptosis and matrix catabolism in rAF cells under nutritional and inflammatory stress

To understand autophagy regulation by the PI3K/Akt/mTOR signaling pathway, rAF cells cultured in limited nutrients of 1% FBS were treated with rapamycin, rapamycin together with MK-2206, or PP242, effective but sub-toxic drug concentrations of which were selected (Figure S1). Rapamycin primarily inhibits mTORC1 while PP242 inhibits both mTORC1 and mTORC2 resulting in the complete inhibition of PI3K/Akt/mTOR signaling (Figure 4A).[39] Then, MK-2206 is a selective Akt inhibitor (Figure 4A).[38] As expected, rapamycin decreased phosphorylation of mTOR and p70/S6K but increased Akt

phosphorylation while rapamycin and MK-2206 combination or PP242 decreased phosphorylation of all these proteins in rAF cells (Figure 4B). The inhibition of mTORC1 by rapamycin prevents p70/S6K phosphorylation responsible for the p70/S6K-mediated negative feedback inhibition of the class I PI3K, leading to Akt activation.[57] Thus, our pharmacological modulation using these drugs on the PI3K/Akt/mTOR signaling pathway works effectively. Western blotting demonstrated increased LC3-II by rapamycin and more pronouncedly by rapamycin and MK-2206 or PP242 (Figure 4C). Moreover, p62/SQSTM1 level decreased by rapamycin and more pronounced by rapamycin and MK-2206 or PP242 (Figure 4C). Therefore, autophagy is substantially enhanced in disc cells treated with these drugs which suppresses PI3K/Akt/mTOR signaling.

Further, IL-1β elevated expression of apoptotic cleaved PARP and senescent p16/INK4A in rAF cells. These IL-1β-induced changes were moderately suppressed by rapamycin. Rapamycin and MK-2206 or

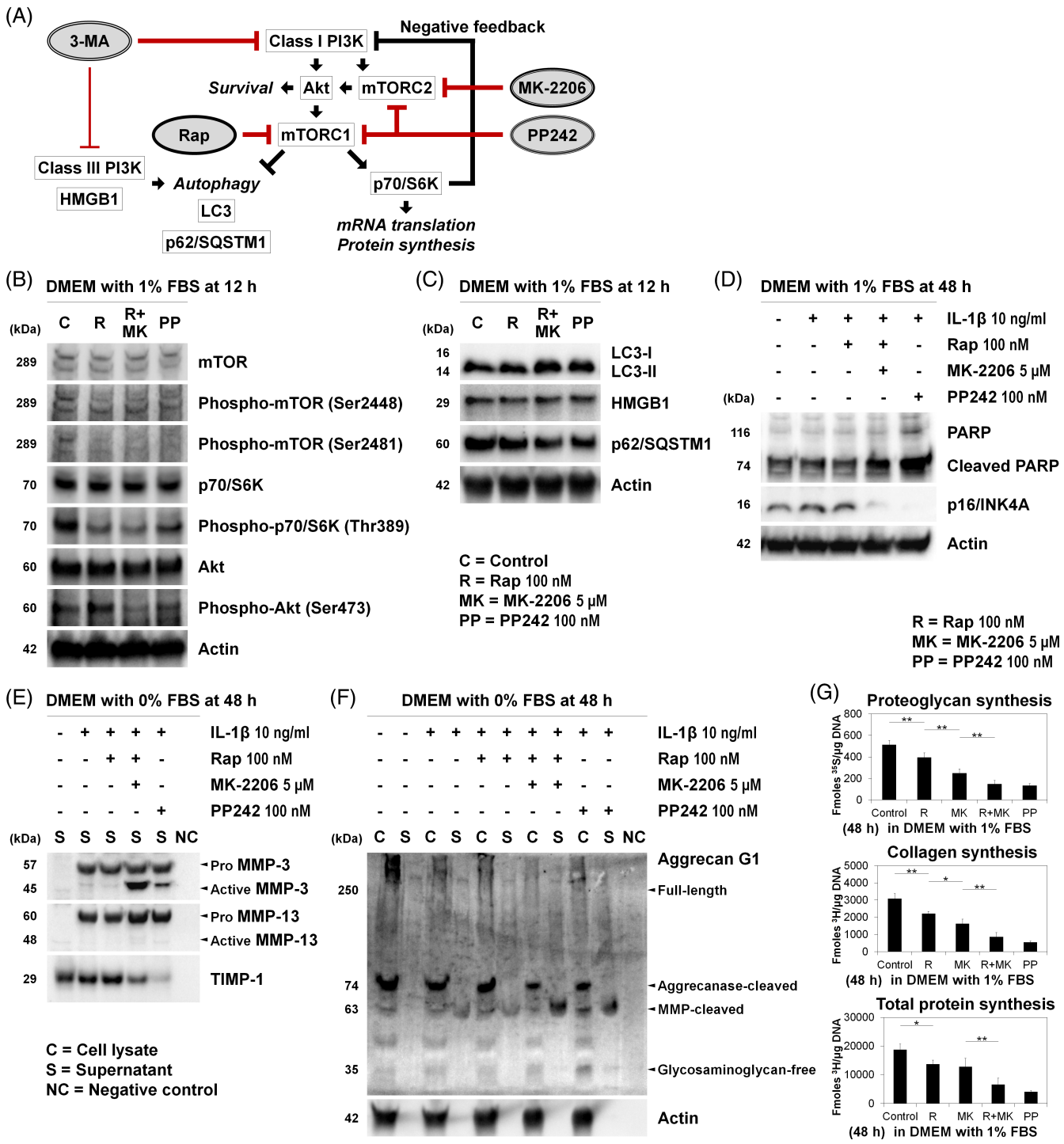

FIGURE 4 Legend on next page.

PP242 treatment markedly suppressed p16/INK4A while drastically increased cleaved PARP level (Figure 4D). These results suggest that the inhibition of Akt and mTORC2 suppresses senescence but induces apoptosis in disc cells under inflammation. Thus, PI3K/Akt/mTOR signaling differentially regulates disc cell fate, depending on the activity of specific protein regulators. Consequently, mTORC1 inhibition is anti-apoptotic and anti-senescent while the inhibition of Akt or mTORC2 is pro-apoptotic but anti-senescent.

Finally, IL-1β drastically increased catabolic MMP-3 and MMP-13 levels in conditioned media of rAF cells cultured grown in 0% FBS (Figure 4E). Rapamycin treatment modestly reduced IL-1β-induced MMP expression but rapamycin and MK-2206 or PP242 further enhanced MMP expression (Figure 4E). Compared to rapamycin treatment alone, rapamycin and MK-2206 or PP242 also substantially suppressed expression of anti-catabolic TIMP-1 (Figure 4E). These findings suggest that rapamycin requires Akt and mTORC2 activities to blunt matrix catabolism induced by inflammation in disc cells. This is supported by the observation that rapamycin reduced IL-1β-induced production of MMP-generated aggrecan fragments (~63 kDa) in conditioned culture media of rAF cells but treatment with rapamycin and MK-2206 or PP242 further increased the production of this aggrecan fragmentation (Figure 4F). Radioisotope incorporation assays also revealed that rapamycin, markedly rapamycin and MK-2206, and especially PP242 all reduced newly synthesized proteoglycans, collagens, and total proteins (Figure 4G). These results are consistent with the fact that protein synthesis depends on phosphorylation of p70/S6K, which is successfully inhibited by these drug treatments.

4 | DISCUSSION

Autophagy is primarily regulated by the PI3K/Akt/mTOR pathway, a central signal integrator for nutrients and energy.[18,25] The pro-survival role of autophagy has elegantly been shown in Atg5-deficient mice which lack autophagy machinery.[62] In this study, we systematically assessed specific protein targets within the PI3K/Akt/mTOR signaling pathway that regulates autophagy, cell fate, and matrix homeostasis in healthy rAF cells cultured in conditions that mimic normal physiology of limited nutrition as well as degenerative condition of inflammatory stress. Rapamycin suppressed IL-1β-induced increases in rAF cell apoptosis, senescence, and extracellular matrix degradation by MMPs while 3-MA further elevated these inflammatory changes, suggesting autophagy as an important therapeutic target and rapamycin as a promising drug to treat IDD. Our findings suggest that the inhibition of mTORC1 by rapamycin upregulates autophagic flux which counteracts the detrimental effects of inflammation. Importantly, we also uncovered protective effects of rapamycin depend on the intact function of Akt and mTORC2 as their inhibition abrogated rapamycin's therapeutic action in rAF cells. Hence, the regulation of disc cell autophagy and matrix homeostasis by the PI3K/Akt/mTOR pathway is complex and require crosstalk between multiple partners.

FIGURE 4 Inhibition of Akt or mTOR complex 2 (mTORC2) abolishes mTOR complex 1 (mTORC1) suppression-mediated protective effects of rapamycin against apoptosis and matrix catabolism in rabbit disc annulus fibrosus (rAF) cells under nutritional and inflammatory stress. (A) Schematic illustration summarizing the effects of PI3K/Akt/mTOR signaling modulators on rAF cells. The mTOR is a serine/threonine kinase whose catalytic subunit consists of mTORC1 and mTORC2. The mTORC1 is regulated by Akt. The Akt is further subject to mTORC2 control. The PI3K/Akt/mTOR signaling pathway regulates cell survival via Akt and mRNA translation and protein synthesis via mTORC1 and p70/S6K. There is a negative feedback loop for the class I PI3K mediated by p70/S6K. The mTORC1 also negatively regulates autophagy. In response to stress, HMGB1 translocates from the nucleus to the cytoplasm, resulting in initiating the autophagosome formation. The conjugation of phosphatidylethanolamine with LC3 drives the autophagosome maturation. An autophagy substrate, p62/SQSTM1, negatively correlates with the autophagosome degradation. Rapamycin (shown as Rap in the figure) blocks only mTORC1 but not mTORC2. Then, 3-methyladenine (3-MA) is a PI3K inhibitor against the classes I predominantly and III at the optimal concentration. While MK-2206 is an allosteric inhibitor of Akt, PP242 blocks the active site of mTOR in both mTORC1 and mTORC2. (B) Western blotting for mTOR, mTOR phosphorylated at serine 2448 (phospho-mTOR [Ser2448]), mTOR phosphorylated at serine 2481 (phospho-mTOR [Ser2481]), p70/S6K, p70/S6K phosphorylated at threonine 389 (phospho-p70/S6K [Thr389]), Akt, and Akt phosphorylated at serine 473 (phospho-Akt [Ser473]) in total protein extracts of rAF cells after 12-h culture in 1% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DMEM) with 100 nM rapamycin, 100 nM rapamycin and 5 μM MK-2206, 100 nM PP242, or vehicle control. Actin was used as a loading control. (C) Western blotting for LC3, HMGB1, p62/SQSTM1, and Actin in total protein extracts of rAF cells after 12-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 100 nM rapamycin and 5 μM MK-2206, 100 nM PP242, or vehicle control. (D) Western blotting for cleaved PARP and p16/INK4A in total protein extracts of rAF cells after 48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 100 nM rapamycin and 5 μM MK-2206, 100 nM PP242, or vehicle control under the presence of 10 ng/mL IL-1β. (E) Western blotting for MMP-3, MMP-13, and TIMP-1 in supernatant protein extracts of rAF cells after 48-h culture in 0% FBS-supplemented DMEM with 100 nM rapamycin, 100 nM rapamycin and 5 μM MK-2206, 100 nM PP242, or vehicle control under the presence of 10 ng/mL IL-1β. (F) Western blotting using the aggrecan G1-domain antibody in rAF cell and supernatant protein extracts after 48-h culture in 0% FBS-supplemented DMEM with 100 nM rapamycin, 100 nM rapamycin and 5 μM MK-2206, 100 nM PP242, or vehicle control under the presence of 10 ng/mL IL-1β. Full-length aggrecan (~250 kDa) and aggrecanase-cleaved (~74 kDa), MMP-cleaved (~63 kDa), and glycosaminoglycan-free (~35 kDa) aggrecan fragments were identified. Actin was used as a loading control. (G) ^{35}S-incorporated proteoglycan and ^{3}H-incorporated collagen and total protein synthesis normalized to PicoGreen DNA amount in rAF cell lysates and conditioned media after 48-h culture in 1% FBS-supplemented DMEM with 100 nM rapamycin, 5 μM MK-2206, 100 nM rapamycin and 5 μM MK-2206, 100 nM PP242, or vehicle control. Data are shown as the mean ± SD ($n = 4$). One-way ANOVA with the Tukey–Kramer post hoc test was used. $^{*}p < 0.05$; $^{**}p < 0.01$. In (B), (C), (D), (E), and (F), all immunoblots shown are representative of four experiments with similar results.

The PI3K/Akt/mTOR pathway is a key signal integrator regulating cell growth and division.[19] Therefore, the observed changes in rAF cell proliferation and metabolic activity by rapamycin and 3-MA are potentially related to PI3K/Akt/mTOR modulation. Rapamycin treatment decreased dehydrogenase activity without changing DNA quantity, indicating effective mTORC1 suppression by rapamycin. On the other hand, 3-MA reduced DNA amount without affecting dehydrogenase activity, suggesting a pro-apoptotic condition with effective PI3K/Akt suppression. Moreover, the observed anti-apoptosis by rapamycin and pro-apoptosis by rapamycin and MK-2206 and PP242 through the inhibition of Akt and mTORC2 can be explained by Akt phosphorylation levels. However, the observed senescence finding is less clear. Suppressed PI3K/Akt signaling leads ultimately to senescence.[63] Therefore, it is suggested that decreased senescence by rapamycin and MK-2206 and PP242 results from excessive apoptosis induction that overtakes the formation of cellular senescence. Further mechanistic investigation is required; however, anti-apoptotic and anti-senescent effects of rapamycin are influenced by the induction of Akt in conjunction with enhanced autophagy, rather than by autophagy alone.

Autophagy is negatively regulated by PI3K/Akt/mTOR signaling (Figures 1G and 4A).[19] The PI3K has several classes, including the class I which is an upstream regulator of Akt and mTOR[19] and the class III which is required for autophagy activation.[18] Thus, the classes I and III PI3Ks have contrasting roles in autophagy machinery. Rapamycin induces autophagy through its inhibitory effect on the mTORC1.[19,30,64] Although 3-MA inhibits autophagy largely via blocking the class III PI3K, it is also known to suppresses the class I PI3K.[30] Interestingly, our findings demonstrated that in rAF cells, autophagy was induced rather than inhibited by 3-MA at longer treatment time points of 24–28 h, most likely through the preferential inhibition of the class I PI3K over the class III PI3K by 3-MA. A report has shown that 5 mM 3-MA increases autophagic flux in mouse embryonic fibroblasts and cancer cells in 10% FBS-supplemented DMEM through 9 h while autophagy inhibition by 3-MA is consistent in serum-starved Earle's balanced salt solution.[65] Further, these unexpected findings are consistent with the explanation that 3-MA induces a persistent block of the class I PI3K and transient block of the class III PI3K.[65] In this study, rAF cells were treated by 3-MA at 2.5 mM in 1% FBS-supplemented DMEM through 48 h which was optimized for the maintenance. Autophagy induction under nutrient-limited condition, which has been reported to promote the class I PI3K activity, might be due to the inhibition of the class I PI3K inhibition by a prolonged treatment of disc cells with 3-MA.[30]

Differential regulation of Akt phosphorylation likely explains the opposite effects of rapamycin and 3-MA on disc cell apoptosis and senescence, despite autophagy induction by both drugs. The Akt plays a critical role in promoting cell survival and inhibiting apoptosis.[26] Our results also demonstrated that suppressed PI3K/Akt signaling by MK-2206-mediated Akt inhibition resulted in apoptotic cell death. This further supports that anti-apoptotic and anti-senescent effects of rapamycin potentially depend on Akt activation as well as autophagy

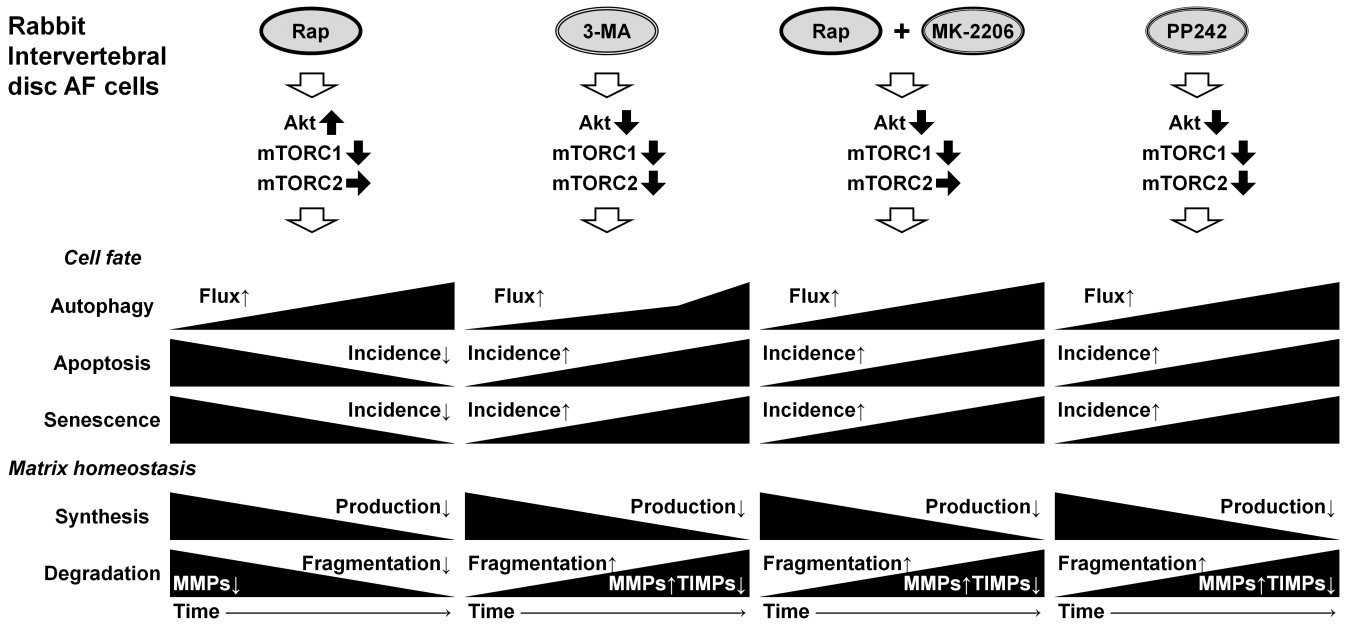

FIGURE 5 Schematic illustration summarizing the comparison of effects between PI3K/Akt/mTOR signaling modulators on rabbit disc annulus fibrosus (rAF) cell fate and matrix homeostasis. Unlike 3-methyladenine (3-MA) inhibiting the classes I and III PI3Ks, MK-2206 inhibiting Akt, and PP242 inhibiting mTOR complexes 1 (mTORC1) and 2 (mTORC2), rapamycin (shown as Rap in the figure) inhibiting mTORC1 is suggested to be a promising drug to treat disc disease by suppressing the incidence of cellular apoptosis and senescence as well as the degradation/fragmentation, relative to the synthesis/production, of extracellular proteoglycan matrix through decreased mTORC1 and p70/S6K signaling but increased autophagic flux and Akt signaling. This study further suggests that rAF cell-protective effects of rapamycin depend on Akt activation rather than autophagy induction.

induction. In addition, rapamycin and 3-MA have contrasting effects on disc extracellular matrix homeostasis. Generally, mRNA downregulation of many tested genes could be explained by suppressed p70/S6K phosphorylation, which are mediated through rapamycin-induced mTORC1 inhibition and 3-MA-induced class I PI3K, Akt, and then mTOR inhibition.[19] Consistently reduced mRNA expression and protein synthesis of anabolic matrix components suggest that extracellular matrix production is p70/S6K-dependent. While no obvious trends in the ADAMTSs/TIMP-3 balance were observed in the current study, we found a clear trend that rapamycin blunts while 3-MA promotes MMPs/TIMP-1 imbalance. This is evidenced by decreased MMP expression and aggrecanolysis by rapamycin and the opposite effects by 3-MA. It is possible that these effects are the result of suppression of disc cellular senescence by rapamycin. Senescent cells with SASP produce and secrete an abundant amount of pro-inflammatory cytokines and proteases, including MMPs.[66] Apoptotic cells can also release proteases during late phases of apoptosis (secondary necrosis).[50] Thus, rapamycin, but not 3-MA, is protective against disc cell matrix catabolism possibly through Akt-associated anti-apoptotic and anti-senescent effects. This is consistent with decreased MMP expression and aggrecanolysis following rapamycin but increased MMP expression and aggrecanolysis following the combination treatment of disc cells with rapamycin and MK-2206 and PP242, which blocks Akt. Moreover, reduced synthesis of anabolic matrix components by rapamycin and more consistently by rapamycin and MK-2206 and PP242 suggests that extracellular matrix production is p70/S6K-dependent.

Rapamycin is a clinically approved drug used specifically in renal transplant patients.[32] Also, its significant role in the prevention of age-related disease is well documented.[31] Therefore, rapamycin could be an important drug candidate to slow down aging-associated IDD. Although using rAF cells as a model for disc research has significant values in the field, using pathological human disc cells as well as appropriate animal models including aging mice would provide more solid evidence for rapamycin to be used to treat IDD.

In conclusion, this study presents the importance of the PI3K/Akt/mTOR signaling pathway in regulating rAF cell fate, survival, and matrix homeostasis under nutritional and inflammatory stress (Figure 5). Disc cells generally live under extremely stressful conditions consisting of low nutrition, pH, and oxygen concentration.[21] Therefore, a major cellular stress–response mechanism of autophagy is speculated to play a pivotal role in maintaining disc health. Autophagy-inducing rapamycin protects against disc cellular apoptosis, senescence, and extracellular matrix degradation by inhibiting mTORC1, but these protective effects intimately depend on Akt activity. Our findings identify autophagy, rapamycin, and PI3K/Akt/mTOR signaling as potential therapeutic targets for treating IDD.

AUTHOR CONTRIBUTIONS

Takashi Yurube conceived of the study, carried out all the experiments, participated in the design and coordination of the study and the interpretation of the data, performed the statistical analysis, and drafted the manuscript. William J. Buchser carried out the experiments in imaging cytometry and participated in the interpretation of the data. Zhongying Zhang participated in the interpretation of the data. Prashanta Silwal participated in the interpretation of the data and helped to draft the manuscript. Michael T. Lotze participated in the design and coordination of the study and the interpretation of the data. James D. Kang secured funding and participated in the interpretation of the data. Nam V. Vo and Gwendolyn A. Sowa supervised the conduct of the study, participated in the design and coordination of the study and the interpretation of the data, and helped to draft the manuscript. All authors proofread the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

Takashi Yurube, William J. Buchser, Zhongying Zhang, Prashanta Silwal, Michael T. Lotze, and Nam V. Vo declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. James D. Kang has received research grants from Stryker and Synthes. Gwendolyn A. Sowa has received a manuscript royalty from UpToDate and a speaking honorarium from Cytonics.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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