Different roles of Akt and mechanistic target of rapamycin in serum-dependent chondroprotection of human osteoarthritic chondrocytes

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Abstract. Despite various animal serums being used widely to culture chondrocytes, the regulatory mechanism of serum on chondrocyte activities has not been elucidated. In the present study, human osteoarthritis (OA) chondrocytes were used to perform *in vitro* investigations on the effect of different concentrations of bovine fetal serum on extracellular matrix synthesis, cell proliferation and autophagy using the Cell Counting Kit-8 analysis, a laser-scanning confocal microscope, and western blot analysis. The results demonstrated that 5% serum exerted a chondroprotective effect more than the other concentrations of serum, as it simultaneously promoted cell proliferation, autophagy, and ECM synthesis in human OA chondrocytes. Furthermore, the decreased mechanistic target of rapamycin (mTOR) and increased Akt were observed in 5% serum-treated OA chondrocytes. Either mTOR or Akt inhibitor influenced the effect of 5% serum on cell proliferation and autophagy in human OA chondrocytes, which was associated with LC-3B or B-cell lymphoma-2 (Bcl-2) signal molecules. Consistent with previous studies, the present study proposes that 5% serum promotes cell proliferation via the Akt/Bcl-2 axis and induces autophagy via the mTOR/LC-3B axis in human OA chondrocytes. Furthermore, the different roles of Akt and mTOR in the cell processes of human OA chondrocytes require consideration for preclinical and clinical therapy of OA.

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Introduction

Osteoarthritis (OA) is the most prevalent degenerative disease of the articulating joints, and deterioration of cartilage extracellular matrix (ECM) homeostasis is one of its pathological characteristics. As the sole cell type in cartilage, chondrocytes are responsible for regulating ECM anabolism and catabolism. Therefore, chondrocyte activities, including autophagy, apoptosis and proliferation, are crucial for preserving cartilage homeostasis. For example, autophagy, a lysosomal degradation process, is essential for survival, differentiation, development and homeostasis (1). It has been reported to control the secretion of type II collagen (Col 2), one of the primary components of cartilage ECM. In the absence of autophagy in growth-plate chondrocytes, Col 2 decreased due to the abnormal accumulation of collagen molecules in the endoplasmic reticulum (2). Increased autophagy is an adaptive response to protect chondrocytes from stresses (3). In addition, the blockade of chondrocyte apoptosis and the promotion of chondrocyte proliferation may be potential therapeutic strategies to treat OA. Berberine may ameliorate cartilage degeneration from OA by promoting cell survival and matrix production of chondrocytes in interleukin (IL)-1β-stimulated articular chondrocytes and in a rat OA model (4). Morroniside exerted chondroprotective effects on OA chondrocytes by promoting cell proliferation and ECM synthesis (5). Hyaluronic acid may suppress chondrocyte apoptosis in a dose-dependent manner in IL-1β-induced OA, which may be an underlying mechanism of the clinical action of intra-articular injection of hyaluronic acid in the treatment of OA (6).

The majority of cell signaling molecules are involved in regulating OA chondrocyte autophagy, proliferation and apoptosis, including protein kinase B (PKB/Akt) and mechanistic target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK). For example, mTOR targeting prevents physical harm to joints in diabetic mice by autophagy activation (7). Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from OA (8). Akt and ERK contribute to the chondroprotective effect of morroniside on OA chondrocytes by promoting chondrocyte proliferation and ECM synthesis (5,9,10). Therefore, present study predominantly focuses on elucidating the role and regulatory mechanism of

complicated cell signaling molecules involved in chondrocyte events.

Various animal serums are added to chondrocyte culture media as they contain numerous nutrient components and cytokines. Notably, in the mid-1990s, a novel therapeutic strategy, the intra-articular injection of autologous conditioned serum (ACS; Orthokine) was developed, and has recently been demonstrated to be safe and effective in a number of clinical studies, and is widely adopted in Europe (11,12). ACS contains enriched anti-inflammatory cytokines, including interleukin (IL)-1Ra, IL-10 and IL-13, and various growth factors, while maintaining low concentrations of pro-inflammatory cytokines, such as IL-1β and tumor necrosis factor (TNF)-α. However, the elevated concentrations of pro-inflammatory cytokines in ACS have no adverse effects on proteoglycan turnover in the cartilage explant cultures (12,13). As a result, understanding the regulatory mechanism of serum is needed to elucidate its effects on OA therapy.

The present study aims to evaluate the effect of different types of bovine fetal serum on human OA chondrocyte proliferation, autophagy and ECM metabolism, and to further investigate the regulatory mechanism associated with Akt and mTOR signaling molecules.

Materials and methods

Reagents and antibodies. Inhibitors [mTOR inhibitor Rapamycin (cat. no. 9904) and PI3K/Akt inhibitor LY294002 (cat. no. 9901)] and antibodies against mTOR (cat. no. 2983), phosphorylated (p)-mTOR (Ser2448; cat. on. 2971), Akt (cat. no. 4691), p-Akt (Ser473; cat. no. 4051), B-cell lymphoma-2 (Bcl-2, cat. no. 2870), p-S6 (Ser235/236; cat. no. 2211), Beclin 1 (cat. no. 4122), AMP-activated protein kinase (AMPK)α (cat. no. 5831), p-AMPKα (Thr172; cat. no. 2535) and LC-3B (cat. no. 3868) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-GAPDH antibody (cat. no. 0411) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies targeting Col 2 (cat. no. WH0001280M1) and aggrecan (cat. no. SAB4500662) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-proliferating cell nuclear antigen antibody (cat. no. BM0104) was purchased from Wuhan Boster Biological Technology., Ltd. (Wuhan, Hubei, China). Secondary antibodies (goat anti-mouse; cat. no. SA00001-1; and goat anti-rabbit; cat. no. SA00001-2) were purchased from Proteintech Group, Inc. (Chicago, IL, USA). Other commercially available reagents of the highest grade were obtained.

Human OA chondrocyte isolation and culture. Subsequent to receiving all patient consent and, in accordance with the ethical guidelines of Zhongshan Hospital of Xiamen University (Xiamen, China), human OA articular cartilage was obtained from advanced OA patients who were undergoing total knee replacement surgery in Zhongshan Hospital of Xiamen University (Xiamen, China). Under sterile conditions, the articular cartilage on the femoral condyle and tibial plateau was minced into small pieces and digested with 0.2% type II collagenase as described previously (14,15). The isolated chondrocytes were cultured in Dulbecco's modified Eagle's medium/F12 medium with 10% fetal bovine serum (FBS; v/v) plus 1% penicillin/streptomycin at 37˚C for 72 h in a water-saturated atmosphere of 5% CO_2. When chondrocytes were grown to 70-80% confluence, the cells were plated in 60-mm Petri dishes ($2x10^6$ cells/dish) or 96-well plates ($3x10^4$ cells/well), followed by the evaluation of chondrocytes using immunohistological techniques and treatment with or without different inhibitors. Ethical approval for the study was obtained from the Ethics Committee of the Medical School, Xiamen University (Xiamen, China).

Cell viability assay. Cells were plated in 96-well plates ($2x10^5$ cells/well) and starved with serum-free medium for 24 h. The medium was then replaced with fresh medium with different concentrations of serum in the presence or absence of inhibitors for a further 24 h. The Cell Counting Kit-8 (CCK8) assay (cat. no. C0037; Beyotime Institute of Biotechnology, Haimen, China) was then performed, as described previously (16). The end product was quantified spectrophotometrically at a wavelength of 450 nm. The optical density values correspond to the number of viable cells (17).

Western blot analysis. Cells were collected by centrifugation (825 x g, 7 min) at room temperature and lysed in Radioimmunoprecipitation assay lysis buffer containing 1% Triton X-100, 1% deoxycholate and 0.1% SDS (premixed solution; cat. no. P0013B, Byeotime Institute of Biotechnology). Lysates were then centrifuged at 19,071 x g or 30 min at 4˚C and the protein concentration was determined using a Pierce Bicinchoninic acid Protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein extracts were subjected to SDS-PAGE (8-12% according to the molecular weight of the protein) and transferred to polyvinylidene fluoride membranes (60 min, 100 V; EMD Millipore, Billerica, MA, USA) as described previously (18). The membranes were incubated with the indicated primary antibodies at 4˚C overnight and corresponding secondary antibodies at room temperature for 1-2 h in succession. The signal was monitored using a chemiluminescent detection system according to the manufacturer's protocol (cat. no. WBKLS0500; EMD Millipore).

Laser-scanning confocal microscope. Chondrocytes were fixed in 4% paraformaldehyde after harvesting. For staining of the endogenous LC-3B proteins, the cells were incubated with anti-LC-3B antibody (dilution, 1:200) followed by Cy3-conjugated secondary antibody (Merck KGaA) as described previously (18). The cells were stained with 50 mg/ml 4',6-diamidino-2-phenylindole (Sigma-Aldrich; Merck KGaA) to visualize the nuclei simultaneously at room temperature for 5 min (18). The stained cells were finally visualized under a confocal microscope (Leica TCS SP2 SE; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. Experimental data were formulated as the means ± standard error of the mean of three independent samples. Statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), using one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

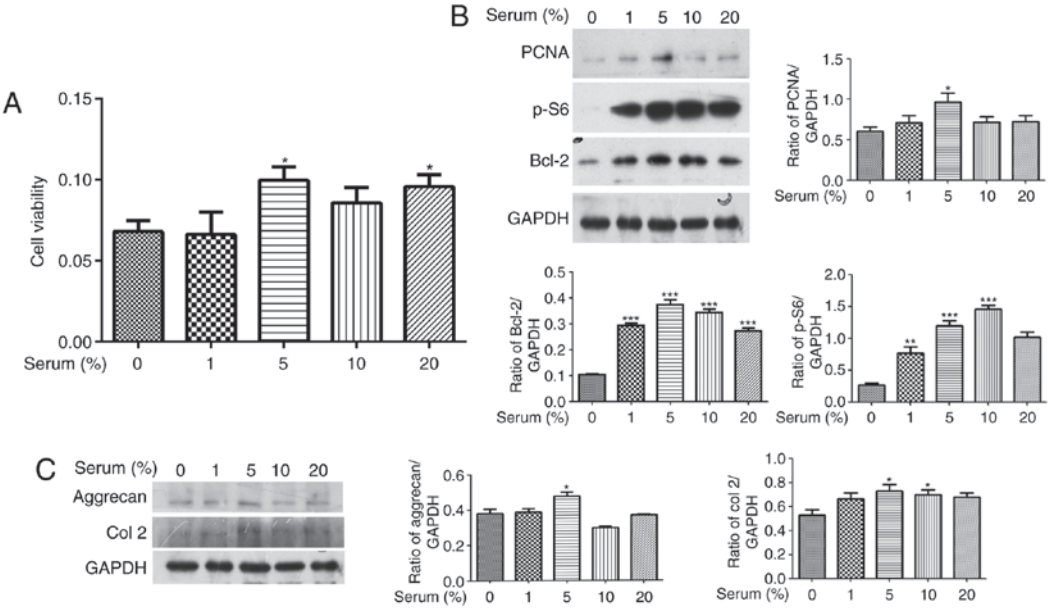

Figure 1. Effect of different concentrations of serum on cell proliferation in human OA chondrocytes. Cultured human OA chondrocytes were starved for 24 h and treated with different concentrations of serum (1, 5, 10 and 20%) for 24 h. (A) Cell viability was evaluated using a Cell Counting Kit 8 assay. (B) The expression levels of PCNA, p-S6, and Bcl-2 were detected by western blotting. The blots were normalized to an endogenous protein (GAPDH). (C) The expression levels of aggrecan and Col 2 were detected with western blotting. The blots were normalized to an endogenous protein (GAPDH). The data are representative of three independent experiments, each yielding similar results (*P<0.05, **P<0.01 and ***P<0.001 vs. serum-free group). OA, osteoarthritis; PCNA, proliferation cell nuclear antigen; p-, phosphorylated; Bcl-2, B-cell lymphoma 2; Col 2, type II collagen.

Results

Effect of different concentrations of serum on cell proliferation and ECM synthesis in human OA chondrocytes. To investigate the effect of serum on OA chondrocyte proliferation, cultured human OA chondrocytes were starved for 24 h and treated with different concentrations of serum (1, 5, 10 and 20%) for 24 h. The cell viability was measured using a CCK8 assay. Fig. 1A indicates that chondrocyte viability significantly increased in 5 and 20% serum-treated groups without any alteration observed in the other groups (P<0.05 vs. the serum-free group). Proliferation cell nuclear antigen (PCNA), Bcl-2 and S6 are predominant biomarkers of cell proliferation, and their expression levels were detected by western blotting. PCNA, Bcl-2 p-S6 expression levels increased in the 5% serum-treated group (Fig. 1B; P<0.05 vs. the serum-free group). 1, 10 or 20% serum led to increased Bcl-2 and p-S6 expression levels (Fig. 1B, P<0.05 vs. the serum-free group). In addition, the expression levels of the predominant components of the ECM, aggrecan and Col 2, were detected by western blotting analysis. The 5% serum simultaneously enhanced aggrecan and Col 2 expression levels in the human OA chondrocytes (Fig. 1C; P<0.05 vs. the serum-free group). The 10% serum only enhanced the expression level of Col 2 (Fig. 1C; P<0.05 vs. the serum-free group). Consequently, only 5% serum simultaneously significantly enhanced cell viability along with the increase in PCNA, Bcl-2 and p-S6 expression levels in human OA chondrocytes, and promoted ECM synthesis via the increase of aggrecan and Col 2 expression levels in human OA chondrocytes.

Effect of different concentrations of serum on autophagy in human OA chondrocytes. As autophagy is essential for survival, differentiation, development and homeostasis (1), the present study determined whether serum may regulate autophagy of OA chondrocytes. Cultured human OA chondrocytes were starved for 24 h and treated with different concentrations of serum (1, 5, 10 and 20%) for 4 h. These autophagy-associated markers, including LC-3B, Beclin 1 and AMPKα, were detected by western blotting. Fig. 2A demonstrated that LC-3B II, Beclin 1 and p-AMPKα expression levels increased in the 5% serum-treated group (P<0.05 vs. the serum-free group). The 1% serum led to increased LC-3B II and p-AMPKα expression levels. Additionally, the results of laser-scanning confocal microscopy demonstrated that the endogenous LC-3B expression level with anti-LC-3B antibody in 5% serum-treated OA chondrocytes was higher than the other concentration serum-treated groups, compared with the serum-free group (Fig. 2B). Overall, it was identified that only 5% serum could simultaneously enhance the expression levels of LC-3B, Beclin 1 and p-AMPKα in human OA chondrocytes.

Akt and mTOR participate in regulating cell proliferation and autophagy in 5% serum-treated human OA chondrocytes. Akt and mTOR have been implicated in OA pathogenesis; thus, the phosphorylation levels of mTOR and Akt were investigated in different concentrations of serum-treated human OA chondrocytes. Fig. 3A demonstrates that 5% serum caused a significant decrease in p-mTOR andincrease in p-Akt, while the other concentrationss did not alter the p-mTOR and p-Akt expression levels (P<0.05 vs. the serum-free group). To further investigate the role of mTOR and Akt on cellular proliferation and autophagy in 5% serum-treated human OA chondrocytes, the starved cells were treated with 50 nM rapamycin (mTOR inhibitor) or 10 mM LY294002 (phosphoinositide 3-kinase; PI3K/Akt inhibitor) for 2 h,

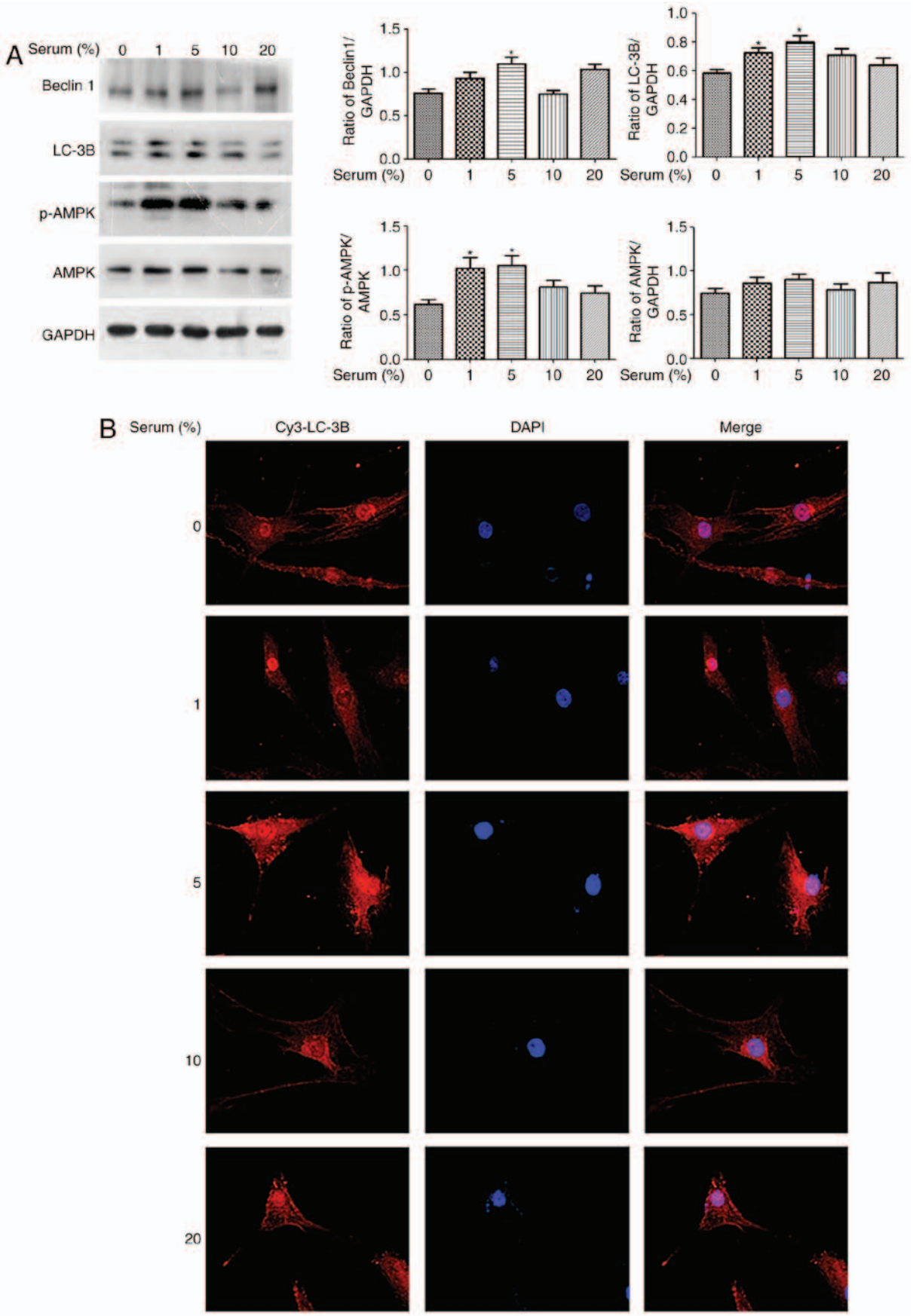

Figure 2. Effect of different concentrations of serum on autophagy in human OA chondrocytes. Cultured human OA chondrocytes were starved for 24 h and treated with different concentrations of serum (1, 5, 10 and 20%) for 4 h. (A) Expression levels of Beclin 1, LC-3B, p-AMPKα and AMPKα were detected by western blotting. The blots were normalized to an endogenous protein (GAPDH). The data are representative of three independent experiments, each yielding similar results (*P<0.05 vs. serum-free group). (B) Cells were fixed in 4% paraformaldehyde after harvesting. Cells were incubated with anti-LC3B antibody followed by Cy3-conjugated secondary antibodies. The endogenous LC-3B proteins (red) and nuclei (blue) were observed under a confocal microscope (Leica TCS SP2 SE). OA, osteoarthritis; AMPKα, AMP-activated protein kinase α; DAPI, 4',6-diamidino-2-phenylindole.

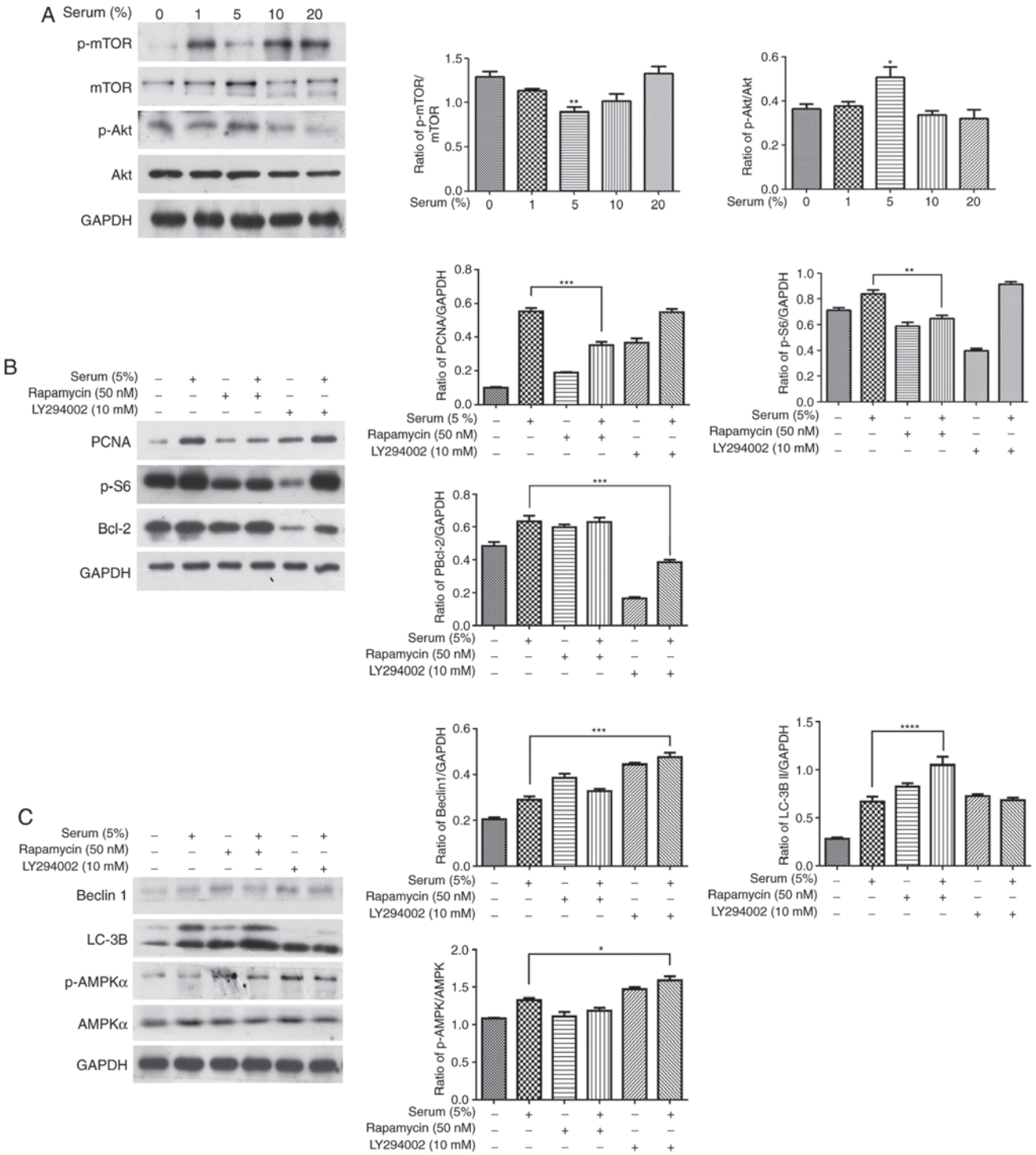

Figure 3. Effect of Akt and mTOR inhibitors on serum-associated cell proliferation and autophagy in human OA chondrocytes. (A) Cultured human OA chondrocytes were starved for 24 h and treated with different concentrations of serum (1, 5, 10 and 20%) for 24 h. The expression levels of Akt, p-Akt, mTOR and p-mTOR were detected with western blotting. (B and C) The starved chondrocytes were pretreated with 50 nM rapamycin (mTOR inhibitor) or 10 mM LY294002 (PI3K/Akt inhibitor) for 2 h, respectively, followed with 5% serum for 4 h. The expression levels of PCNA, p-S6, Bcl-2, Beclin 1, LC-3B, p-AMPKα and AMPKα were detected by western blotting. The blots were normalized to an endogenous protein (GAPDH). The data are representative of three independent experiments, each yielding similar results (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. serum-free group or untreated 5% serum-treated group). mTOR, mechanistic target of rapamycin; OA, osteoarthritis; p-, phosphorylated; PI3K, phosphoinositide 3-kinase; PCNA, proliferation cell nuclear antigen; Bcl-2, B-cell lymphoma 2.

respectively. This was followed by 5% serum treatment at 37˚C for 4 h in a water-saturated atmosphere of 5% CO_2. The results of Fig. 3B demonstrate that rapamycin reduced PCNA and p-S6 expression levels in the 5% serum-treated group (P<0.01 vs. the untreated 5% serum-treated group), while the Bcl-2 expression level did not significantly change. LY294002 significantly inhibited the level of Bcl-2 expression in the 5% serum-treated groups without any alterations to the PCNA and p-S6 expression levels (Fig. 3B; P<0.001 vs. the untreated 5% serum-treated group). Furthermore, Fig. 3C

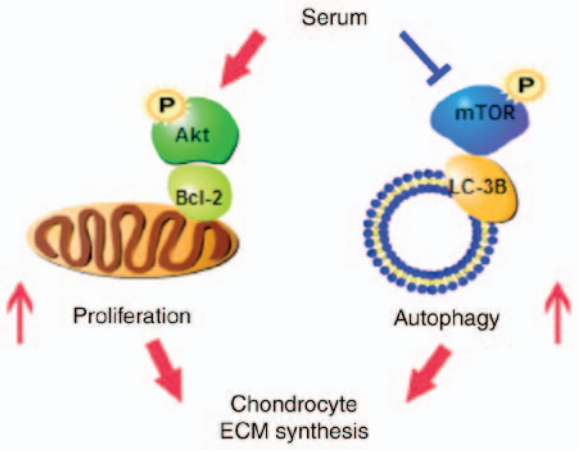

Figure 4. Proposed model of serum-dependent chondroprotection associated with Akt and mTOR. mTOR, mechanistic target of rapamycin; ECM, extracellular matrix; Bcl-2, B-cell lymphoma 2; p, phosphorylated.

demonstrated that rapamycin led to the increase in LC-3B II expression level in the 5% serum-treated group without any alteration to the p-AMPKα and Beclin 1 expression levels ($P<0.0001$ vs. the untreated 5% serum-treated group). Beclin 1 and p-AMPKα expression levels increased in the LY294002-treated 5% serum-treated group, while LC-3B II did not significantly change ($P<0.01$ vs. the untreated 5% serum-treated group). Therefore, the addition of either rapamycin or LY294002 modulated cell proliferation and autophagy in human OA chondrocytes, by targeting different signaling molecules.

Discussion

In the present study, the effect of different concentrations of serum on cell events was investigated in human OA chondrocytes. It is demonstrated that only 5% serum was able to simultaneously regulate cell proliferation, ECM synthesis and autophagy induction in human OA chondrocytes, compared with the other concentrations. Notably, decreased mTOR and increased Akt expression levels were observed in the 5% serum-treated group. Furthermore, either mTOR or Akt inhibition modulated cell proliferation and autophagy by targeting different signaling molecules in human OA chondrocytes. Combined with the findings of previous studies, it is proposed that 5% serum promotes cell proliferation via the Akt/Bcl-2 axis and induces autophagy via the mTOR/LC3B axis in human OA chondrocytes.

Although 10% serum is always used to culture chondrocytes, certain studies indicate that chondrocytes cultured with serum-free medium exhibit no proliferation difference when compared with those cultured with 10% FBS (19). Human nasal chondrocytes proliferate at comparable rates in different serum conditions, with no statistically significant differences observed between the lower percentage of autologous serum (2%) and 10% FBS (20). In the present study, treatment with 5% serum, not 10%, simultaneously resulted in increased cell proliferation, autophagy and ECM synthesis in human OA chondrocytes. Therefore, it is proposed that 5% serum may be more suitable for culturing chondrocytes than 10% serum, at least in human OA chondrocytes.

Under high nutrient conditions, mTOR suppresses cell autophagy by phosphorylating autophagy related 13 (Atg13) to prevent the interaction between Atg13 and unc-51 like autophagy activating kinase (ULK), or phosphorylating ULK Ser757 to disrupt the interaction between AMPK and ULK (21). Conversely, activated mTORC1, due to the inactivation of TSC complex subunit 2 with Akt, triggers the mTOR/S6 axis to regulate protein synthesis, promoting cell proliferation (22-24). Therefore, activated mTOR may perform different functions in cellular processes, including promoting cell proliferation and suppressing autophagy induction. In the present study, the cell viability, PCNA, Bcl-2, aggrecan and Col 2 expression levels increased in the 5% serum-treated group in Fig. 1, indicating that 5% serum may promote cell proliferation and ECM synthesis in human OA chondrocytes. However, 5% serum led to a significantly decreased p-mTOR expression level, implying that mTOR/S6-associated proliferation may be blocked and that decreased levels of mTOR expression, by 5% serum, did not inhibit cell proliferation. By contrast, the increase of LC-3B II, Beclin 1 and p-AMPKα expression levels in the 5% serum-treated group indicated that 5% serum may induce autophagy. Furthermore, the inhibitor of mTOR caused a significant increase in LC-3B II expression levels, demonstrating that decreased mTOR in 5% serum-treated chondrocytes may be involved in autophagy induction of OA chondrocytes. Thus, mTOR may be involved in autophagy induction in 5% serum-treated human OA chondrocytes, but not cell proliferation. The regulation of mTOR on chondrocyte autophagy is well known. Chondrocyte autophagy is stimulated by hypoxia-inducible factor-1-dependent AMPK activation and mTOR suppression (25). Inhibition of the PI3K/AKT/mTOR signaling pathway can promote autophagy of articular chondrocytes and attenuate inflammatory response in rats with OA (26). The intra-articular injection of rapamycin, an mTOR inhibitor, reduced mTOR expression levels, leading to a delay in articular cartilage degradation in an OA murine model (27). Therefore, the present study proposed that mTOR may predominantly be involved in regulating autophagy induction in 5% serum-treated chondrocytes.

The activation of Akt increases in normal chondrocytes more than in OA chondrocytes, and is required for basal and insulin-induced Col 2 expression in chondrocytes (28). Elevated production of tribbles pseudokinase 3, an inhibitor of Akt activation, has been reported to increase in OA chondrocytes (29). In addition, previous studies demonstrated that increased Akt expression levels by extracellular stimuli may protect articular cartilage from OA deterioration by promoting cell survival in human or rat OA chondrocytes (4,5). Thus, Akt is a positive element for chondroprotection. Consistent with these studies, 5% serum significantly enhanced p-Akt expression levels concomitantly with Bcl-2 depression caused by LY294002, exhibiting the promoting effect of Akt on serum-associated cell proliferation of human OA chondrocytes. Therefore, 5% serum is hypothesized to promote cell proliferation, to a lesser extent, by triggering the Akt/Bcl-2 signal axis. However, Hahn-Windgassen *et al* (30) demonstrated that Akt is a negative regulator of AMPK. Metformin, an AMPK activator, decreased the phosphorylation of Akt in

bladder cancer cells (31). Notably, Galasso *et al* (32) reported that in OA human cartilage, increased phosphatase and tensin homolog (Akt inhibitor), AMPK and autophagy reflected the chondrocyte responses observed during starvation and steroid depletion (32). The present data that LY294002 may upregulate p-AMPKα in 5% serum-treated human OA chondrocytes was consistent with the above-mentioned studies. Therefore, Akt may reduce autophagy induction by suppressing p-AMPKα. However, the induced autophagy of chondrocytes eventually increased in the 5% serum-treated group, implying that increased Akt may predominantly promote proliferation, rather than exert inhibitory effects on autophagy induction in 5% serum-treated human OA chondrocytes, which may be due to being interfered with by other signaling pathways, such as the mTOR/LC-3B axis.

The PI3K/Akt signaling pathway is an important activator of mTOR, although Akt-independent regulation of mTOR in response to mitogens, nutrient availability and conditions that deplete intracellular energy in cancer have been reported (33). Multiple signaling pathways are involved in regulating mTOR independently of Akt, such as LKB1/AMPK, AMPK/mTORC1, and calmodulin-dependent protein kinase kinase β/AMPK signaling pathways (33). Furthermore, mTOR inhibition may result in feedback activation of Akt in cancer (33-35), consistent with the present data of the opposite effect of 5% serum on Akt and mTOR in human OA chondrocytes (data not shown). Future studies are required to determine the interaction between Akt and mTOR on cell processes in 5% serum-treated human OA chondrocytes.

In conclusion, 5% serum may simultaneously promote cell proliferation and autophagy induction of OA chondrocytes with the increase of ECM synthesis by upregulating Akt and downregulating mTOR signaling molecules. Furthermore, decreased mTOR expression levels promoted autophagy induction in human OA chondrocytes via the mTOR/LC-3B axis, and increased Akt enhanced cell proliferation via the Akt/Bcl-2 axis (Fig. 4). Consequently, the effect of increased Akt and decreased mTOR expression levels on human OA chondrocytes should be considered simultaneously for the preclinical and clinical treatment of OA.

Acknowledgements

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