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Upregulation of P63 inhibits chondrocyte autophagy thereby enhancing the malignant progression of osteoarthritis

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Loss of autophagy is suggested to play a key role in the progression of osteoarthritis (OA). P63 is a member of the P53 family, which is widely dysregulated in various tumors. However, the specific role of P63 in chondrocyte autophagy has never been fully understood. Here, the expression level of P63 in the articular cartilages of OA patients and chondrocytes treated with 3-MA was explored using western blot. Autophagy was determined using transmission electron microscopy and mRFP-GFP-LC3 assay. Fewer autophagic vesicles were identified in the articular cartilages of OA patients compared with that of normal control. Both the mRNA and protein levels of P63 was markedly increased in the articular cartilages of OA patients compared with that of normal control. MTT assay demonstrated that P63 overexpression markedly reduced chondrocyte viability at 24, 36 and 48 h, while inhibition of P63 inhibited cell viability at 24, 36 and 48 h, respectively. Furthermore, autophagic flux assay showed that transfection of ad-P63 markedly decreased the yellow dots in chondrocytes, while inhibition of P63 induced chondrycyte autophagy. In summary, we first demonstrated that upregulation of P63 in the cartilage tissues of OA patients inhibited chondrocyte autophagy thereby contributing to the malignant progression of OA.

1. Introduction

As a common bone-joint disease, the typical characteristics of osteoarthritis (OA) include the destruction of articular cartilage, synovial inflammation, impaired movement and ultimately disability (Takayama et al. 2014; Reyes et al. 2015). Recent study has demonstrated that autophagic response plays a key role in the disturbances of chondrocytes differentiation and proliferation. It is reported that autophagy declines with age and counteracts the aging process (Takayama et al. 2014; Lopez de Figueroa et al. 2015). Further study demonstrates that loss of autophagy progressive with aging induces accumulative damaged cells and inflammation, thereby causing OA (Lopez de Figueroa et al. 2015).

Increasing evidence has suggested that autophagy protects arthritic cartilage from progressive damage and loss of autophagy is demonstrated to contribute to OA (Martin et al. 2004). In the maturation progress of growth plate chondrocytes, autophagy is a transient stage which is modulated by adenosine 5'-monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signaling (Hosomi et al. 2015; Ryter and Choi 2015). Studies have demonstrated that autophagy allows the chondrocytes to mature before their elimination by apoptosis (Levine and Kroemer 2008). Even though, the specific mechanism regulating autophagy in OA progression has never been fully understood.

Here, we focused on a novel protein, P63, which is located on chromosome 3q27-29, including transactivation (TA) domain, DNA binding domain and oligomerization domain (Yao and Chen 2012). It is reported that P63 is involved in multiple physiological processes, such as oocyte death, autophagic cell death and epidermal morphogenesis (Bir et al. 2014; Choi et al. 2014; Leao et al. 2015; Vandormael-Pournin et al. 2015). In neural precursor cells, it is found that p63 induces cell apoptosis versus senescence in a p53-dependent manner, thereby modulating appropriate adult neurogenesis (Fatt et al. 2014). Among metastatic tumors, loss of p63 loss was shown to accelerate tumor genesis and metastatic spread (Urist et al. 2002; Koga et al. 2003). Moreover, the deficiency of p63 is also demonstrated to cause cellular senescence and induce aging phenotypes (Keyes et al. 2005). However, little is known about the association between p63 and OA progression. In the present study, we mainly explored the expression pattern of p63 in the cartilage tissues of OA patients compared with that of normal control. Then, we examined whether p63 induced autophagy in chondrocytes. Here, for the first time, the expression of p63 was demonstrated to be decreased with the reduction of autophagy in the progression of OA.

2. Investigations and results

2.1. P63 was overexpressed in the articular cartilages of OA patients

Firstly, we explored the autophagic response in the articular cartilages of OA patients and normal control. As shown in Fig. 1A, fewer autophagic vesicles were identified in the articular cartilages of OA patients compared with that of normal control. Then, we evaluated the expression of P63 in the articular cartilages of OA patients and normal control. Real time PCR analysis demonstrated that the mRNA levels of P63 was markedly increased in the articular cartilages of OA patients compared with that of normal control (Fig. 1B). Furthermore, the protein levels of P63 were significantly increased articular cartilages of OA patients compared to normal control (Fig. 1C). These data demonstrated that enhanced P63 expression may be correlated with reduction of autophagy in the articular cartilages of OA patients.

2.2. P63 upregulation correlated with suppressed cell autophagy in chondrocytes

To further explore whether abnormal expression of P63 is involved in autophagy, chondrocytes were treated with the autophagy inhibitor 3-MA (5 mM). As shown in Fig. 2A, pretreatment of 3-MA decreased the protein levels of LC3II, indicating suppressed autophagic responses. Then, adenovirus including mRFP-GFP-LC3 was used to determine the autophagic flux. After transfection, the

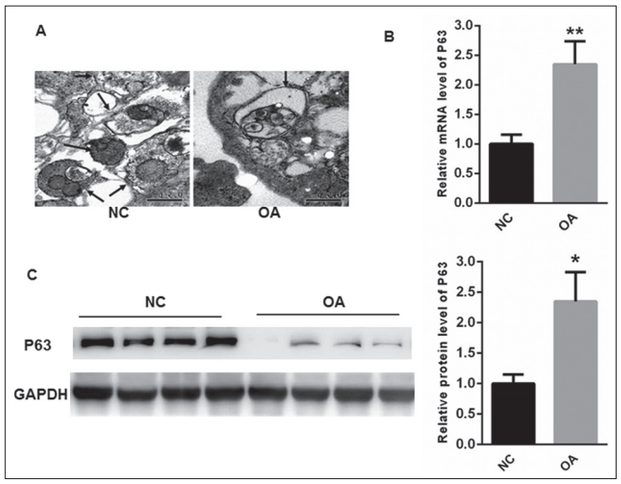

Fig. 1: P63 was overexpressed in the articular cartilages of OA patients. (A) Ultrastructural observations of autophagic vesicles. (B) Real time PCR analysis. (C) Western blot analysis. *p<0.05, ** p<0.01 vs. control.

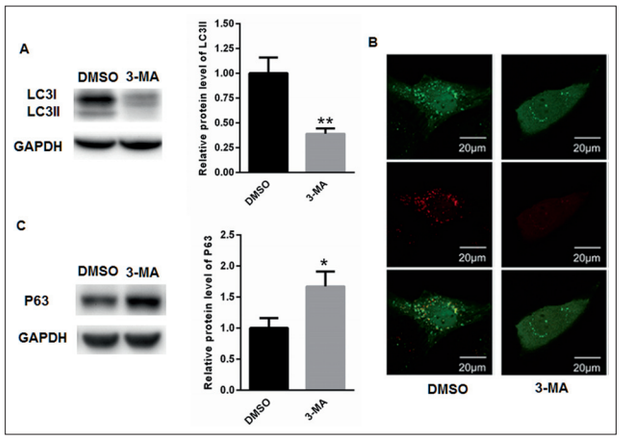

Fig. 2: P63 upregulation correlated with suppressed cell autophagy in chondrocytes. (A) Pretreatment of 3-MA decreased the protein levels of LC3II. (B) Analysis of autophagic flux following 3-MA treatment. (C) Western blot analysis showed that the protein level of P63 was markedly increased after 3-MA incubation. *p<0.05, ** p<0.01 vs. control.

autophagosomes in cells were shown as yellow dots. Following 3-MA treatment, the yellow autophagosomes were reduced, indicating the suppression role of 3-MA on autophagic flux in chondrocytes. More importantly, western blot analysis showed that the protein level of P63 was markedly increased after 3-MA incubation (Fig. 2C). These in vitro findings further support the correlation between P63 upregulation and suppressed cell autophagy in chondrocytes.

2.3. P63 suppressed chondrocyte viability

Then, we overexpressed P63 in chondrocytes and explored its effect on cell viability. As shown in Fig. 3A, transfection of ad-P63 significantly enhanced the protein level of P63 compared with that of ad-NC. Meanwhile, MTT assay demonstrated that P63 overexpression markedly reduced chondrocyte viability at 24, 36 and 48 h (Fig. 3B). In contrast, transfection of ad-shP63 markedly reduced P63 protein expression compared to ad-NC (Fig. 3C). Cell viability was also shown to be enhanced after inhibition of P63 in chondrocytes at 24, 36 and 48 h, respectively (Fig. 3D).

2.4. P63 upregulation induced chondrocyte autophagy

Then, we evaluated the autophagy responses in chondrocytes after transfection of ad-P63 or ad-NC. Western blot analysis demonstrated that overexpression of P63 significantly inhibited the protein level of LC3II, indicating a suppressed autophagy in chondrocytes (Fig. 4A). In comparison, after transfection with ad-shP63, the protein level of LC3II was significantly increased compared to ad-NC (Fig. 4B). These data indicated that overexpression of P63 in chondrocytes contributed to autophagy.

3. Discussion

As a cytoprotective pathway and an important anti-aging mechanism, loss of autophagy is suggested to play a key role in the progression of OA (Lotz and Carames 2011; Carames et al. 2012). Previous studies have shown that the protein levels of LC3-II were decreased in chondrocytes from OA cartilage compared to normal control, indicating suppressed autophagy responses (Srinivas et al. 2009; Carames et al. 2010). Therefore, in the current study, we first evaluated the autophagic responses in the cartilage tissues of OA patients. Here, we identified fewer autophagic vesicles in the articular cartilages of OA patients compared with that of normal control, indicating loss of autophagy in chondrocytes.

Autophagy is suggested to exert a protective effect on chondrocytes in OA (Takayama et al. 2014; Carames et al. 2015; Lopez de Figueroa et al. 2015). In the aging process, autophagy was reported to be decreased in senescent tissues (Wu et al. 2013). In normal status, autophagosomes form after the stimulation of certain stress

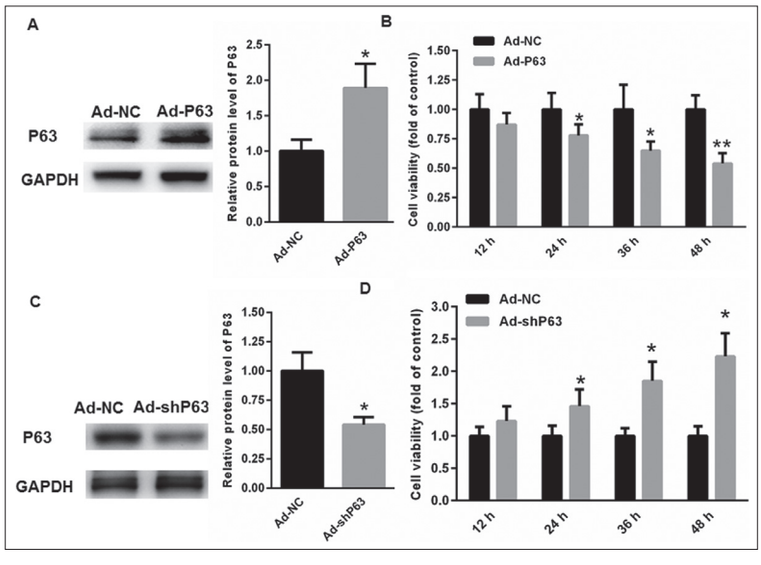

Fig. 3: P63 suppressed chondrocyte viability. (A) Transfection of ad-P63 significantly enhanced the protein level of P63 compared with that of ad-NC. (B) MTT assay demonstrated that P63 overexpression markedly reduced chondrocyte viability at 24, 36 and 48 h. (C) Transfection of ad-shP63 markedly reduced P63 protein expression than ad-NC. (D) Cell viability was also showed to be enhanced after inhibition of P63 in chondrocytes at 24, 36 and 48 h, respectively. *p<0.05, ** p<0.01, ***p<0.001 vs. control.

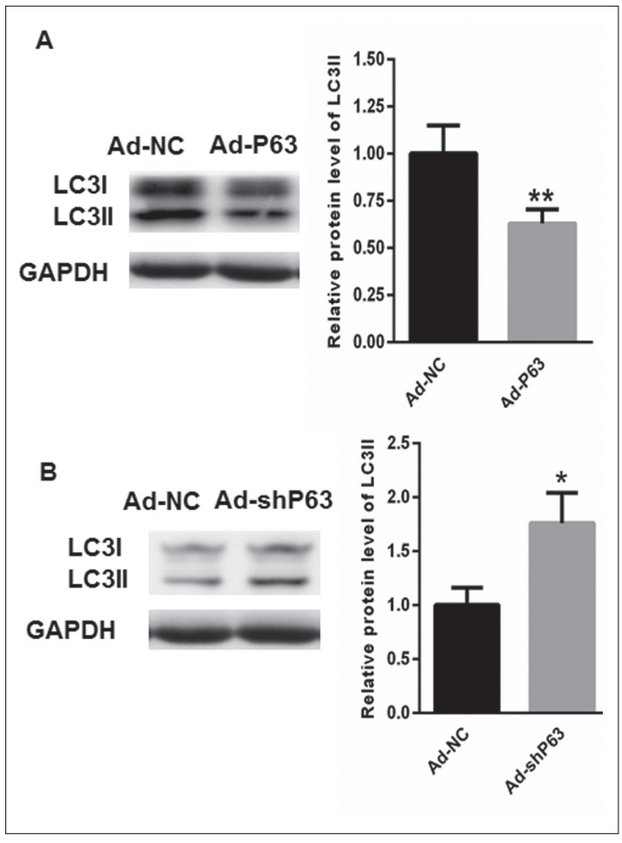

Fig. 4: P63 upregulation induced chondrocyte autophagy. (A) Western blot analysis demonstrated that overexpression of P63 significantly inhibited the protein level of LC3II. (B) After transfection with ad-shP63, the protein level of LC3II was significantly increased than ad-NC. *p<0.05, ** p<0.01, ***p<0.001 vs. control.

situations or bacterial invasion. Then, they could either fuse with endosomes or lysosomes, thereby inducing autolysosomes. In vitro studies have demonstrated that chondrocyte apoptosis could be significantly suppressed by autophagy (Sasaki et al. 2012). It is found that rapamycin treatment in vivo could induce chondrocyte autophagy and suppress chondrocyte damage in articular cartilage (Takayama et al. 2014; Lopez de Figueroa et al. 2015). Therefore, it is of great importance to elucidate the underlying mechanism which modulates chondrocyte autophagy in articular cartilage.

P63 is an important member of the P53 family, which is widely reported to be dysregulated in various tumors (Ratovitski 2013). However, the specific role of P63 in chondrocyte autophagy has never been fully understood. In the current study, we first explored the expression of P63 in the cartilage tissues of OA patients and normal control. Real time PCR and western blot analysis demonstrated that transcription and expression of P63 were significantly enhanced in the cartilage tissues of OA patients than that of normal control. In vitro study showed that overexpression of P63 suppressed cell autophagy in chondrocytes. Based on the above findings, we conclude that enhanced P63 expression correlates with reduction of autophagy in the cartilage tissues of OA patients. In the process of cell senescence, autophagy is proposed to prevent the aging process induced by heat shock protein (Ito et al. 2015). It has also been shown that 3-MA treatment activates senescence mainly through inhibit autophagy in chondrocytes. Therefore, we explored the effect of P63 on chondrocytes. Our data showed that enhanced P63 expression significantly suppressed chondrocyte viability, indicating a detrimental effect of P63 on chondrocytes. Moreover, autophagic flux assay showed that transfection of ad-P63 markedly decreased the yellow dots in chondrocytes, suggesting a suppressed autophagy response. These data indicated that P63 could inhibit chondrocyte autophagy thereby decreasing cell viability. It is suggested that P63 plays a decisive role in the post-transcriptional regulation of microRNAs, which then control cell proliferation, apotosis and autophagy (Melar-New and Laimins 2010, Ory and Ellisen 2011; Tucci et al. 2012). For instance, miR-30a was found to target BECN1 and miR-181a-5p was demonstrated to suppress ATG5 (Zhu et al. 2009; Fu et al. 2012). Thus, we propose that P63-induced chondrocyte autophagy may be mediated through microRNAs. Due to the limitation of the current study, further exploration is needed.

In conclusion, the current study first demonstrated that upregulation of P63 in the cartilage tissues of OA patients inhibited chondrocyte autophagy thereby contributing to the malignant progression of OA.

4. Experimental

4.1. Subject information and inclusion and exclusion criteria

Human articular cartilages were separated from six normal people who had suffered accidents (three females/three males, 57.0±8.1 years old), and six OA patients (three females/three males, 55.3±6.8 years old) undergoing total knee replacement surgery. At all of the subjects rheumatoid arthritis and genetic bone and cartilage diseases were excluded. This investigation was approved by the Human Ethics Committee of Xuzhou Central Hospital. Each donor signed an informed consent sheet.

4.2. Cartilage tissue collection and chondrocyte culture

The articular cartilages were collected from the bones and were rinsed in PBS supplemented with penicillin and streptomycin. To isolate chondrocytes, the cartilage tissues were cut into 5 mm^3 slices, and incubated with trypsin at room temperature for 30 min. Then, the trypsin was removed and washed by PBS for three times (5 min/time). And the tissue slices were treated for 16 h with type II collagenase. The cells were harvested and cultured at 37 °C in 5% CO_2 in DMEM/F-12 (1:1) supplemented with 10% (v/v) fetal calf serum (HyClone, Logan, Utah, USA), 100 units/mL penicillin and 100 mg/mL streptomycin.

4.3. Adenoviral vector construction

Recombinant adenoviruses expressing P63 (Ad-P63) or shRNA-P63 (Ad-shP63) or negative control (NC) adenovirus vector containing GFP (Ad-Con) were purchased from Shanghai Genechem Co., Ltd.

4.4. Western blot

Total proteins were isolated from tissues using a total protein extraction kit (Keygen, Nanjing, China). A total of 20 μg protein was separated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes and then blocked with 5% fat-free milk at room temperature for 2 h. The immune-blot was incubated with primary antibody detecting p63, LC3II, beclin (1:1000 dilution; CST) and GAPDH (1 : 1000 dilution; CST) was used as a control. The signals were detected using a Super ECL Plus Kit (Keygen) and determined by quantitative analysis using UVP software (UVP, LLC, Upland, CA, United States).

4.5. Electron Microscopy

Cell pellets were fixed in 2.3% glutaraldehyde, postfixed in 2% osmium tetroxide and 0.5% uranyl acetate, dehydrated and embedded in Spurs epoxy resin. Ultrathin sections (90 nm) were made and double-stained with uranyl acetate and lead citrate, and viewed with a Philips CM10 transmission electron microscope (Phillips Electronics, Amsterdam, the Netherlands).

4.6. Red fluorescent protein (RFP) -green fluorescent protein (GFP) -light chain 3 (LC3) assay

Before treatment, chondrocytes were transfected with monomeric (m)RFP-GFP-LC3 when the confluence was 50–70% using RFP-GFP-LC3 adenoviral vectors (HanBio Technology Co., Ltd., Shanghai, China) with the 100 multiplicity of infection (MOI). After transfection for 24 h at 37 °C, the transfected chondrocytes were infected with ad-P63 or ad-shP63 for another 48 h. Following treatment, autophagosomes and autolysomes in chondrocytes were observed under a confocal microscope (SP8; Leica Microsystems GmbH, Wetzlar, Germany).

4.7. Statistical analysis

Statistical analyses were performed using SPSS statistical software, version 16 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to analyze the differences between groups. Tukey's significance test was used to detect differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Conflicts of interest: None declared.

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