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Transcription factor HBP1: A regulator of senescence and apoptosis of preadipocytes

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ABSTRACT

Background: /aim: HMG-box protein 1 (HBP1) plays an important role in the senescence and apoptosis of mammalian cells, but its role in chicken cells remains unclear. The aim of this study was to investigate the effects of HBP1 on senescence and apoptosis of chicken preadipocytes. **Methods:** The immortalized chicken preadipocyte cell line (ICP2) was used as a cell model. Chicken HBP1 knockout and overexpressing preadipocyte cell lines were established using CRISPR/Cas9 gene editing technology and lentiviral infection. Western blotting was used to detect the protein expression of HBP1 and senescence markers p16 and p53. Cell senescence was measured by Sa-β-Gal staining and apoptosis was detected by flow cytometry. **Results:** HBP1 was highly expressed in senescent ICP2 cells compared with young ICP2 cells. After the deletion of HBP1, the degree of senescence, the apoptosis rate and the protein expression levels of p16 and p53 were significantly reduced. After the overexpression of HBP1, the degree of senescence, the apoptosis rate and the protein expression levels of p16 and p53 were significantly increased. **Conclusion:** HBP1 promotes the senescence and apoptosis of chicken preadipocytes.

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1. Introduction

The rapid growth of broilers is accompanied by an increase in abdominal fat deposition, leading to many adverse consequences, such as reduced feed efficiency [1] and reproductive performance [2]. Therefore, one of the main goals of poultry breeding is to control the excessive deposition of abdominal fat in broilers. The increase in adipose tissue results from an increase in the number and size of adipocytes [3]. The size of adipocytes is mainly regulated by the differentiation of preadipocytes, and the number of preadipocytes is mainly regulated by the proliferation, senescence and apoptosis of preadipocytes. Research on preadipocyte proliferation, senescence and apoptosis has mainly focused on humans and rodents [4–8], and not on birds. Our previous study found that HMG-box protein 1 (HBP1) may be a key protein affecting the growth and development of chicken adipose tissue (data not published). However, the role of HBP1 in regulating the development of chicken adipocytes remains unclear.

HBP1 is a ubiquitous transcription factor, belonging to the high mobility (HMG) family of DNA binding proteins [9]. Studies in mammals have shown that HBP1 plays an important role in cell cycle transformation, differentiation, senescence and apoptosis. In a study of rat cardiomyocytes, HBP1 inhibited the G1-S phase of the cell cycle by regulating key genes involved in cell proliferation [10]. HBP1 also plays an important role in the cell cycle by inhibiting the Wnt/β-catenin pathway in various types of cells such as NIH3T3 and 293T cells [11]. HBP1 was increased during the differentiation of mouse C2C12 muscle satellite cells and Ob1771 preadipocytes [12]. HBP1 also promotes the differentiation of leukemia bone marrow cell lines, megakaryocytes and red blood cells [13,14]. Studies in 3T3-L1 and MEF cells found that HBP1 can induce terminal differentiation of preadipocytes by regulating C/EBPα expression [15]. A study in WI-38 human lung fibroblasts found that HBP1 is required for cell cycle arrest in Ras-induced senescence and triggers cell senescence [16]. In 2BS (human diploid fibroblasts), HeLa (cervical cancer) and in U2OS (osteosarcoma) cells, HBP1 and Pim-1 can form a positive feedback loop, which regulates cell senescence and apoptosis induced by hydrogen peroxide. Further studies revealed that the Pim-1-HBP1 positive feedback loop exerts its effects by regulating the expression of senescence

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marker p16 and apoptosis marker Bax [17]. Studies on 293T cells showed that HBP1 can also participate in apoptosis as a substrate of p38 MAPK [18]. In addition, HBP1 regulates chromatin remodeling by inhibiting the expression of DNMT1, resulting in overall DNA hypomethylation in some cell lines [19].

To date, no studies have been reported on the relationship between HBP1 and senescence and apoptosis of preadipocytes. Therefore, the aim of this study was to investigate the effects of the chicken HBP1 on the senescence and apoptosis of preadipocytes.

2. Materials and methods

2.1. Cell culture

The immortalized chicken preadipocyte cell line (ICP2) was preserved in our laboratory [20]. The cells were cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and were maintained at 37 °C in a humidified, 5% CO_2 atmosphere.

2.2. Establishment of HBP1 knockout preadipocyte cell line

The chicken preadipocyte cell line with HBP1 knockout was constructed using Cas9 protein/gRNA ribonucleoprotein complex (Cas9 RNP). Three gRNAs were designed target to the Exon 2 of chicken HBP1 gene by using the CRISPR RGEN tools (http://www.rgenome.net/about/). The oligonucleotide sequences of the gRNAs were shown in Table 1. The primers designed against the target sites were shown below. Target 1 and target 2: were HBP1-Exon2-1/2F:5′-TGTGGGAAGTGAAGACGA-3′, HBP1-Exon2-1/2R:5′-GAGGACTTGTGGCGATG-3'; target 3: HBP1-Exon2-3F:5′-CAAACAGCGATTCAGAAAG-3′, HBP1-Exon2-3R:5′-AAGCACAAACGAGACCCT-3'. The GeneArt™ Precision gRNA Synthesis Kit (A29377; Invitrogen) was used for *in vitro* transcription and the purification of the gRNAs, according to the manufacturer's instructions. ICP2 cells were seeded into a 12-well cell culture plate. When cells reached 70% confluency, the Cas9 protein (TrueCut™ Cas9 Protein v2, A36497; Invitrogen) was premixed with the gRNA (Cas9: gRNA = 1:1.5) using a transfection reagent (Lipofectamine™ CRISPRMAX™ Transfection Reagent, CMAX00001; Invitrogen) and added to the cell culture medium. After 48 h, genomic DNA was extracted from the cells. Then the genome sequences containing the target sites were amplified by PCR. The GeneArt® Genomic Cleavage Detection Kit (A24372; Invitrogen) was used to identify the cleavage efficiency of each gRNA. The gRNA with the highest cleavage efficiency was chosen for monoclonal screening. Monoclonal cell line was obtained by limiting dilution. The types of HBP1 gene editing in the monoclonal cell line were detected by TA cloning and sequencing. The knockout effect of HBP1 was envaluated by Western blotting.

2.3. Establishment of a preadipocyte cell line stably over-expressing HBP1

A lentivirus over-expressing HBP1(HBLV-GFP-HA-HBP1) and a control lentivirus (HBLV- GFP-HA) were constructed by Hanbio (Shanghai, China). When ICP2 cells achieved 60% confluency, they were infected with HBLV-GFP-HA-HBP1 or HBLV-GFP-HA at different multiplicity of infection (MOI). After 72 h, the infection efficiency was evaluated based on GFP fluorescence intensity and the optimum MOI was confirmed. The positive cells were selected based on the presence of the GFP gene in these constructs. Briefly, 72 h after infection with the optimum MOI, GFP-positive cells were sorted into one well of a six-well plate by flow cytometry. Overexpression of HBP1 was confirmed by Western blotting.

Table 1
Oligonucleotide sequences used in the construction of HBP1-Exon2-sgRNA.

gRNA	Oligonucleotide sequence (5′-3′)
HBP1-Exon2-gRNA1	F: AATCGCTGTTTGAGGTATGT R: ACATACCTCAAACAGCGATT
HBP1-Exon2-gRNA2	F: TTTCACATCAGGAACGTTCA R: TGAACGTTCCTGATGTGAAA
HBP1-Exon2-gRNA3	F: AGTGCTCATTCTTGTGAGAA R: TTCTCACAAGAATGAGCACT

2.4. Western blotting

Total protein was extracted from preadipocytes after lysis using radio immunoprecipitation assay buffer. Cellular extracts were supplemented with protease inhibitor cocktail and protein levels weremeasured and equal amounts were loaded onto SDS-PAGE gels. After transfer to nitrocellulose membranes, blots were probed overnight at 4 °C with the appropriate primary antibody (*anti*-HBP1, 1:1000, Abcam; *anti*-p16, 1:1000, USCN; *anti*-p53, 1:1000, USCN; *anti*-β-actin, 1:1000, TransGen Biotech) followed by a HRP-conjugated secondary antibody (1:5000, TransGen Biotech). Specific protein bands were visualized using the BeyoECL Plus kit (Beyotime) and a chemiluminescence system (Sagecreation, Beijing, China).

2.5. Sa-β-Gal staining

Cell senescence was detected using the Senescence β-Galactosidase Staining Kit (Beyotime, C0602). Three biological replicates were set for each treatment group and three visual fields under the microscope were randomly selected for each biological replicate. Cytoplasmic with blue staining were senescent cells. The senescent cells and normal cells were counted by Image J software.

2.6. Flow cytometry

Apoptosis was analyzed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, C10625). Each treatment group was assessed in triplicate.

2.7. Statistical analysis

All experiments were repeated three times. Experimental data were analyzed using the analysis of variance (ANOVA) module of the SPSS16.0 statistical software. The data was expressed as means ± standard deviations. $^{*}P < 0.05$ represented a significant difference and $^{**}P < 0.01$ represented a highly significant difference.

3. Results

3.1. Establishment of HBP1 knockout preadipocyte cell line

The gRNA obtaind by *In vitro* transcription (Fig. 1A) was mixed with Cas9 protein to form Cas9 RNP, and were then transfected into ICP2 cells. The result of genome cleavage activity analysis showed that gRNA1 has the highest cleavage efficiency (Fig. 1B). Therefore, gRNA1-transfected cells were selected for monoclonal screening. Two monoclonal cell lines were obtained, one of them was wild-type and the other one was homozygous with a 219-bp deletion of exon 2 of chicken HBP1 gene and was named as $HBP1^{-/-}$ (Fig. 1C). Western blotting confirmed that the lack of HBP1 protein expression in $HBP1^{-/-}$ cells (Fig. 1D).

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Fig. 1. Establishment of HBP1 knockout preadipocyte cell line. (A) gRNAs were synthesized by using the GeneArt Precision gRNA Synthesis Kit. (B)The cleavage efficiencies of gRNAs were detected by using the GeneArt® Genomic Cleavage Detection Kit 48 h after transfection of Cas9 RNP. (C) The types of gene editing in monoclonal cell lines were detectd by TA cloning and sequencing. (D) The expression level of HBP1 was detected by Western blotting.

3.2. Establishment of HBP1 over-expression preadipocyte cell line

The optimum MOI of ICP2 cells infected with the lentivirus was chosen at 100, since the GFP fluorescence intensity was the strongest both in the control group and the HBP1 overexpression group at this degree of MOI (Fig. 2A). 72 h after infections with the optimum MOI, GFP positive cells were enriched by flow cytometric. The result of western blotting showed that the expression level of HBP1 was significantly increased in the overexpression group compared with that of the control group (Fig. 2B).

3.3. Chicken HBP1 is highly expressed in senescent preadipocytes

The literature indicates that the degree of cell senescence increases with continuous cell division [21]. So ICP2 cells were designated the young group at 0 h of culture, and cells cultured for 48 h were designated the senescence group. The result of Sa-β-Gal staining showed that the senescence degree of senescence group was significantly higher than that of the young group (Fig. 3A). Western blot analysis revealed that HBP1 expression was significantly increased in the senescent group compared with that in the young group (Fig. 3B).

Fig. 2. Establishment of HBP1 over-expressing preadipocyte cell line. (A) GFP positive cells were observed by using fluorescence microscopy at different degree of MOI. Scale bar: 200 μm. (B) The expression level of HBP1 expression was detected by western blotting.

3.4. HBP1 promotes the senescence and apoptosis of preadipocytes

Sa-β-Gal staining revealed that the degree of cell senescence decreased remarkably after HBP1 knockout while increased significantly after HBP1 overexpression (Fig. 4A). Western blotting revealed that the expression levels of p16 and p53 decreased after HBP1 knockout while increased after overexpression of HBP1 (Fig. 4B). The result of flow cytometry showed that the percent of apoptotic cells decreased significantly after HBP1 knockout while significantly increased after HBP1 overexpression (Fig. 4C).

4. Discussion

To our knowledge, there are few studies on gene editing using Cas9 RNP, and most of them focus on mammals. There are many advantages to using the RNP as a CRISPR/Cas9 delivery system compared with Cas9 plasmid, Cas9 mRNA and Cas9 lentiviral. First, when the RNP is paired with a DNA templete, a "total package" is formed without the need for a cell environment to synthesize Cas9 protein and sgRNA, which enables faster and more efficient gene editing [22]. Second, when genome editing is completed by Cas9 RNP, the unnecessary Cas9 protein can be quickly removed by the cells, thus greatly reducing non-target cleavage [22]. Third, Cas9 RNP avoid the damage of cells caused by random integration of plasmid sequece into the genome of the host cells [22]. Cas9 nuclease recognizes the adjacent motifs (PAM) of the prototype spacer region adjacent to gRNA and produces double-strand breaks, which are rapidly repaired by non-homologous end-junction (NHEJ) or homologous recombination. NHEJ-mediated double-stranded DNA repair usually produces point mutations, insertions or deletions of short fragments, but occasionally produces insertions or deletions of long fragments [23–25]. In this study, for the first time, we first used the Cas9 RNP as a CRISPR/Cas9 delivery system for gene editing in poultry cells, and obtained a monoclonal preadipocyte cell line with HBP1 knockout (Fig. 1C–D). We believe

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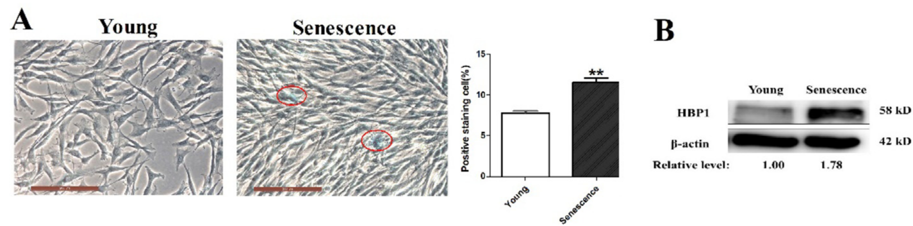

Fig. 3. Expression of HBP1 in young and senescent preadipocytes. (A) Sa-β-Gal staining of ICP2 cells in the young group and the senescence group. Scale bar: 200 μm. The red circle indicates the senescent cells. (B) Western blotting was used to detect the expression level of HBP1.

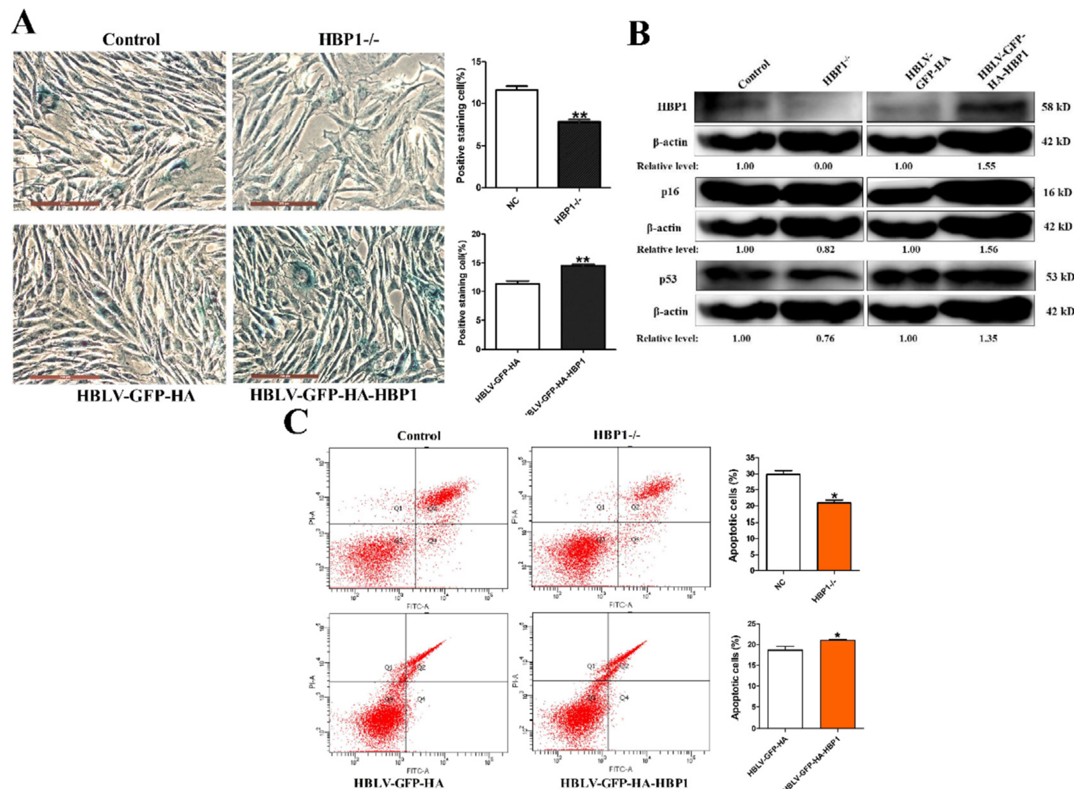

Fig. 4. Effects of HBP1 on the senescence and apoptosis of preadipocytes. (A) Cell senescence was determined by Sa-β-Gal staining. Scale bar: 200 μm. (B) Western blot analysis of p16 and p53 in the preadipocytes. (C) Cell apoptosis was determined by Annexin V-FITC/PI staining followed by flow cytometry.

that Cas9 RNP delivery system will play an important role in genome editing of agricultural animals in the near future.

Cell senescence and apoptosis are conceptually similar, both being self-protective mechanisms of cells. The most commonly studied pathways of cell senescence were p53-p21 and p16-Rb. The p53-p21 pathway mainly participates in the process of cell senescence through the following two mechanisms. First, p53 plays an important role in maintaining cell senescence induced by telomere shortening [26]. Second, when cells are damaged, p53 regulates the cell cycle and promotes cell senescence [27]. The involvement of p16 in cell senescence is mainly related to the Rb signaling pathway. Indeed, p16 regulates cell senescence by promoting the binding of Rb protein to E2Fs family transcription factors [28]. Studies have shown that p53 not only regulates cell senescence, but also plays an important role in regulating cell apoptosis [29].

Mammalian studies have shown that HBP1 can promote the senescence of WI-38 pulmonary fibroblasts and the apoptosis of K562 myeloblasts [30], but its role in regulating the senescence and apoptosis of preadipocytes is unclear. In this study, we found that HBP1 was highly expressed in senescent preadipocytes (Fig. 3A and B), which suggested that HBP1 might play a role in senescence of chicken preadipocytes. In HBP1 knockout and overexpressing preadipocyte lines, we found that HBP1 promotes the senescence of chicken preadipocytes and upregulates the expression of senescence markers p16 and p53 [26] (Fig. 4A and B). In view of this, we speculate that HBP1 may affect the senescence of chicken preadipocytes by regulating the expression of p16 and p53. As is known, cell senescence is often accompanied by apoptosis, and p53 is not only a marker gene of cell senescence, but also a critical molecular of the apoptotic pathway [29]. Therefore, we supposed that HBP1 may play a role in the apoptosis of chicken preadipocytes. The result of flow cytometry confirmed our hypothesis that HBP1 promotes the apoptosis of preadipocytes in chickens (Fig. 4C).

In summary, we first reported that HBP1 positively regulated senescence and apoptosis of preadipocytes in birds. However, the underlying mechanisms need to be further studied.

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Conflicts of interest

The authors declare no conflict of interest.

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