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p53 regulates autophagic activity in senescent rat mesenchymal stromal cells

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ABSTRACT

The tumor suppressor protein p53 is an important player in the regulation of cell senescence, its functions are largely carried out by modulating its downstream genes. Emerging evidence has suggested that senescence and autophagy appear to be regulated by overlapping signaling pathways. Furthermore, autophagy markers have been observed in senescent cells. In this study, we sought to explore the effects of the expression pattern and function of p53 on the activity of autophagy and replicative senescence in bone marrow derived mesenchymal stromal cells (BMSCs). We found that more than 85% of BMSCs stained positive for SA-β-gal at passage 6 (senescent BMSCs) with increased expressions of senescence related genes ($p16^{ink4a}$ and $p21^{waf1}$). These results were accompanied by the up-regulation of p53, down-regulation of mammalian target of rapamycin (mTOR) and phosphorylation of Rb. Senescent BMSCs displayed an increased monodansylcadaverine (MDC) staining and autophagy related genes (LC3 and atg12) level compared with BMSCs at passage 2. Knockdown of p53 alleviated the senescent state and reduced autophagic activity during the progression of BMSC senescence, which was accompanied by significantly up-regulated levels of mTOR and phosphorylation of Rb. These results demonstrate that autophagy increases when BMSCs enter the replicative senescence state, and p53 contributes a crucial role in the up-regulation of autophagy in this state.

1. Introduction

As a rare heterogeneous subset of stromal cells, bone marrow derived mesenchymal stromal cells (BMSCs) possess the ability of self-renewal and differentiation. These cells can differentiate into multiple lineages, not only mesenchymal lineages, such as osteocytes, chondrocytes and adipocytes (Bianco et al., 2008; Galderisi and Giordano, 2014), but also cells representing other embryonic germ layers, including neural cells and endothelial cells. After in vivo administration, BMSCs home to or engraft to injured sites for tissue regeneration and repair. In addition, BMSCs possess a remarkable immunosuppressive activity, many scientists have attempted to use BMSCs to treat graft-versus-host disease (GvDH) and some autoimmune diseases (Salem and Thiemermann, 2010). However, BMSCs in primary tissues are extremely rare and thus require extensive in vitro expansion to obtain a sufficient number of cells for clinical use. However, BMSCs undergo replicative senescence after a limited number of cell divisions (Yoo et al., 2014). The senescent cells develop an enlarged and flat senescent morphology and ultimately stop dividing. Simultaneously, they lose their stem cell characteristics (Wagner et al., 2008). The senescence of BMSCs affects their clinical application. Therefore, the analysis of BMSC senescence and its implications are crucial for basic research as well as for therapeutic applications.

Cellular senescence is a complex process that involves massive changes in signaling pathways and effector mechanisms. As a major component of the cellular stress response, the cellular lysosomal degradation pathway of autophagy is reported to be an important component for establishing senescence (White and Lowe, 2009). Autophagy serves as a protective response under nutrient deprivation conditions and is also frequently activated upon the induction of senescence (Young et al., 2009). Markers of autophagy have been observed in senescent endothelial cells (Patschan et al., 2008), aging fibroblasts (Gerland et al., 2003), and senescent human dental pulp cells (Li et al., 2012). Senescence and autophagy share a number of common characteristics, including the protection of cells from external and internal forms of stress such as radiation, chemotherapy, and telomere shortening (Gewirtz, 2013). These similarities suggest that these processes could be regulated by overlapping signaling pathways.

The molecular mechanisms underlying senescence, especially those overlapping with autophagy, are still poorly understood. Interestingly, p53, a cell cycle regulating factor, is increased in senescent BMSCs and has been shown to be involved in functions controlling the cell-cycle, apoptosis and genomic stability (Armesilla-Diaz et al., 2009). Recently, high-throughput sequencing highlighted an extensive transcriptional

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network connection between p53 and autophagy (Kenzelmann and Attardi, 2013). Nonetheless, p53 controls autophagy in an ambiguous fashion. Several studies have shown that p53 contributes to the induction of autophagy in both transcription-dependent and -independent manners, whereas others have shown p53 can repress autophagy via poorly characterized mechanisms (Maiuri et al., 2010). The relationship between autophagy and p53 has not been well elucidated. However, it has been reported that the precise cellular response triggered by p53 is cell type dependent and relates to the intensity and type of activation signal (Levine and Abrams, 2008).

Autophagy and senescence both represent responses to stress that have cell protective functions, which indicates that p53 may play a role in autophagy and senescence. In this study, we analyzed whether p53 plays a pivotal role in the activity of autophagy along with BMSC replicative senescence.

2. Materials and methods

2.1. Isolation and expansion of BMSCs

The experimental protocols concerning animals were approved by the Institutional Animal Care Committee of Wuhan University. The rats were supplied by the Experimental Animal Center of Wuhan University (Wuhan, China). BMSCs were isolated and cultured from the femurs and tibias of Sprague-Dawley rats as previously described (Zheng et al., 2013). Briefly, muscle and extra-ostial tissue were trimmed and the ends of the bones were cut open. Bone marrow plugs were flushed out with 1 ml Hank's balanced salt solution, centrifuged at 250 *g* for 5 min, and resuspended with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transferred to culture flasks with DMEM containing 20% fetal bovine serum (FBS) and incubated at 37 °C with 5% CO_2. Non-adherent cells were removed by replacing the medium with DMEM containing 10% FBS after 3 days of culture. Cells were passaged with 0.25% trypsin plus 0.2% EDTA when they reached 80% confluence.

2.2. Senescence-associated β-galactosidase (SA-β-gal) staining

The SA-β-gal assay was performed using the Senescent Cell Staining Kit (C0602; Beyotime Biotech., Jiangsu, China) according to the manufacturer's instructions. The number of positive SA-β-gal cells (blue staining) were counted under a microscope (CKX41SF; Olympus, Japan). Experiments were performed in triplicate and at least 100 cells were scored in each treatment.

2.3. Labeling of autophagic vacuoles with monodansylcadaverine (MDC)

Autophagic vacuoles were labeled with MDC (600140; Cayman Chemical, Michigan, USA). Briefly, cells were incubated with 0.05 mM MDC in PBS at 37 °C for 10 min, washed 2 times with PBS, and immediately imaged with a CCD camera (DP80; Olympus, Japan). Images were analyzed using ImageJ software (http://rsb.info.nih.gov/ij).

2.4. Lentivirus vector-mediated RNA interference

Lentiviruses capable of generating p53 shRNA and a non-targeting control were purchased from GenePharma (Shanghai, China). Sequences that targeted rat p53 were (Sh1): 5′-GTC AGG GAC AGC CAA GTC TGT-3′ (Mukhopadhyay et al., 2009), (Sh2): 5′-GAG AAT ATT TCA CCC TTA A-3′ (Tsukamoto et al., 2006), and non-targeting control (NC): 5′-TTC TCC GAA CGT GTC ACG T-3′ (Zheng et al., 2013). The virus titer used for infection was 10^9 pfu/ml. Conditions for lentivirus infection were optimized to ensure target-specific efficiency, as previously described. Three days after the cells at passage 6 were seeded in the dish, the cells were transduced with lentiviral vectors (150 multiplicity of infection) in culture medium for 24 h. At 24 h the incubation medium was replaced with fresh culture medium. The cells were tested after an additional culture for 4 days.

2.5. Western blotting

BMSCs from each treatment group were collected with 0.25% trypsin containing 0.2% EDTA and were lysed for 30 min in RIPA buffer on ice. Total protein was extracted by centrifugation at 16,000 *g* for 5 min at 4 °C and quantified by BCA reagent (P0012S; Beyotime Biotech). The same amount of protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Membranes were incubated in blocking buffer (TBS-T containing 5% skim milk) for 1 h at room temperature and then incubated with specific primary antibodies (1:1000 dilutions) against microtubule-associated protein light chain 3 (LC3-I/II; 4108; Cell Signaling Technology, Inc., Danvers, MA, USA), Atg12 (4180; Cell Signaling Technology, Inc.), $p16^{ink4a}$ (BS6431, Bioworld Technology, Inc., Minneapolis, MN, USA), phospho(p)-p70S6K (BS4439; Bioworld Technology, Inc.), mTOR (AM831; Beyotime Biotech.), Phospho(p)-Rb (AR092; Beyotime Biotech.), Rb (BS1312; Bioworld Technology, Inc.) and $p21^{waf1}$ (BS1269; Bioworld Technology, Inc.) in blocking buffer overnight at 4 °C. After 3 washes with TBS-T, the membranes were incubated with horseradish peroxidase conjugated secondary antibody (1:3000, BS13278, Bioworld Technology, Inc.) in blocking buffer for 1 h at room temperature. The membranes were washed 3 times with TBS-T, and the bands were detected using an ECL kit (P0018; Beyotime Biotech). GAPDH was used as the internal control to normalize the loaded protein.

2.6. Autophagic flux assays

Bafilomycin A1 (BaF1, 88899-55-2; Cayman Chemical), which inhibits the fusion between autophagosomes and lysosomes, was utilized to inhibit autophagosome removal (Zhang et al., 2013). To measure autophagic activity, BMSCs were treated with 50 nmol BaF1 for 3 h, an equal volume of DMSO was used as a vehicle control. The LC3-II/LC3-I ratios were examined by Western blot and quantified with ImageJ software (http://rsb.info.nih.gov/ij/).

2.7. Reverse transcription-quantitative PCR analysis

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Approximately 1 μg of RNA was used for reverse transcription with the PrimeScript RT reagent kit (RR047; Takara Bio, Shiga, Japan). Quantitative PCR (qPCR) was performed using SYBR® Premix DimerEraser™ (RR091; Takara Bio, Shiga, Japan) on a Bio-Rad IQ2 Real-Time PCR system (Bio-Rad, Hercules, CA, USA). All of the assays were performed in triplicate. Relative gene expression was calculated by the $2^{-\Delta Ct}$ method, and the values were normalized to the levels of GAPDH. The sequences of the specific primers were the following: ATG12: 5′-AAA CGT GAG CCA AGG GGATT-3′(Forward), 5′-GGA AAC TTG GTG CTG CTT GG-3′(Reverse); p53: 5′-TCC TCC CCA ACA TCT TAT CC-3′-(Forward), 5′- GCA CAA ACA CGA ACC TCA AA-3′(Reverse); and GAPDH: 5′-GAT GGG TGT GAA CCA CGA GAAA-3′(Forward), 5′-ACG GAT ACA TTG GGG GTA GGA-3′(Reverse).

2.8. Statistical analysis

Each experiment was repeated at least three times. The Student's t-test was used to assess any statistical significance between treatment groups, and *p*-values less than 0.05 were considered to be statistically significant.