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Original Paper

The Protective Role of Autophagy in *Heterocephalus glaber* Hepatic Stellate Cells Exposed to H_2O_2 or Nutritional Stress

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Key Words
Heterocephalus glaber • Hepatic Stellate Cells • Autophagy • H_2O_2 • Nutritional Stress

Abstract
Background/Aims: Naked mole rats (NMRs) survive and thrive in dark, dank environments with low levels of oxygen and poor quality nutrition. Their long lifespan is attributed to sustained good health and pronounced resistance to cancer. Physiological and biochemical processes, such as autophagy, may contribute to the successful aging of this exceptionally long-lived species. We demonstrated that NMRs have higher levels of autophagy than short-lived C57BL/6 mice, and this may play an important role in the maintenance of cellular protein quality and the defense of cells against intracellular and extracellular aggressors in NMRs. The present study assesses autophagy as a means for cells to flexibly respond to environmental changes (H_2O_2 treatment and a shortage of nutrients). ***Methods:*** Primary NMR HSCs were isolated from liver and treated with serum-free medium. Cells in the experimental group were incubated with different concentrations of hydrogen peroxide (H_2O_2) in the presence and / or absence of 3-MA (5 mM).The LC3-II/LC3-I ratio was determined by western blot analysis. Western blotting was performed to analyze the expression level of Beclin 1 protein. Apoptosis and cell-cycle progression were analyzed by flow cytometry. ***Results:*** Our data reveal that both poor quality nutrition and H_2O_2 treatment induces apoptosis and autophagy in NMR hepatic stellate cells(HSCs). ***Conclusion:*** NMR cells have the capacity to induce cell death through apoptosis and downregulate the energy consuming processes through inhibition of proliferation when they become superfluous or irreversibly damaged.

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Introduction

Naked mole rats (NMRs; *Heterocephalus glaber*) are the longest-living rodents known, with a maximum lifespan of 30 years, which is five times longer than expected on the basis of body size [1]. NMRs can survive and thrive, maintaining normal function and reproduction, in dark, dank environments in the arid and semiarid regions of northeast Africa. NMRs have high levels of oxidative stress and extreme resistance to oxygen deprivation and hypoxia [1, 2]. Physiological and biochemical processes in this species have evolved to significantly extend both their good health and lifespan. In other words, NMRs must continuously adapt to environmental stress such as oxidative stress and hypoxia. An understanding of the cellular and molecular mechanisms underlying the health of NMRs may provide insights into human disease.

It has been demonstrated that autophagy plays a critical role in the maintenance of cellular homeostasis and adaption to environmental stress, such as oxidative stress, starvation, hypoxia and infection [3]. Autophagy is thought to promote survival by recycling amino acids and fatty acids for energy utilization in stressful conditions, such as hypoxia, starvation and oxidative stress, and is induced as an adaptive response to drive the catabolism of proteins, lipids and carbohydrates for de novo biosynthesis of biomacromolecules [4, 5]. We demonstrated that NMRs have higher levels of autophagy than the short-lived C57BL/6 mice [6]. The purpose of this study was to determine whether H_2O_2 treatment and serum starvation can induce programmed cell death in NMR hepatic stellate cells (HSCs) to determine the relationship between autophagy and apoptosis during this process. We observed that serum starvation and H_2O_2 induced autophagy as evidenced by the increase in conversion of light chain 3 (LC3)-I to the membrane form LC3-II.

Materials and Methods

Cell culture and culture conditions

Primary NMR HSCs were isolated from liver using methods similar to those previously reported [7]. All primary HSCs were maintained at 37°C and in Dulbecco's modified Eagle's low-glucose medium (DMEM; GIBCO, Life Technologies Ltd, UK) and supplemented with 10% fetal bovine serum (FBS; GIBCO, Life Technologies Ltd, UK), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator under 92% N_2, 5% CO_2, and 3% O_2. 3-Methyladenine (3-MA) was purchased from Sigma Aldrich. All cell lines were used at early passage (<9–15 population doublings).

Cell culture treatments

When the HSCs reached 70-80% confluence, they were randomly divided into different groups. To investigate the effects of serum starvation, starvation was induced by incubating cells in medium without glucose (DMEM-no glucose, GIBCO, Life Technologies Ltd, UK). When 3-MA was used in combination with starvation, cells were treated with 5mM 3-MA during subsequent incubations. Cells in the experimental group were incubated with different concentrations of hydrogen peroxide (H_2O_2) in the presence and / or absence of 3-MA (5 mM). 3-MA was added to the culture medium before H_2O_2 exposure. Cells were harvested at 12, 24 and 48-h intervals, respectively.

Cell cycle analysis and apoptosis assay

For cell cycle analysis, the collected cells were fixed in 90% ethanol and stored overnight at -20°C. Fixed cells were centrifuged to remove ethanol, and the pellets were resuspended in propidium iodide and RNase A in PBS for 20 min at 37°C. The DNA content of nuclei was analyzed by FACS. Cell proliferation was assessed by the percentage of S + G2 phase cells in the cell cycle. The annexin V-FITC assay was used to measure apoptotic cells by flow cytometry (FCM) according to the manufacturer's instructions (Nanjing Keygen Biotech, KGA108). Briefly, the cells were collected by trypsinization, washed with ice-cold PBS twice and resuspended in 300 μL of 1× binding buffer containing 5 μL of annexin V and 5 μL of PI for 30 min at

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room temperature in the dark. All samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The results were expressed as the percentage of early apoptotic cells (PI-negative and annexin V-positive cells). Apoptosis was detected on the basis of the nuclear morphology observed in cells stained with diaminopimelic acid (DAPI), and the cells with condensed and fragmented nuclei were identified as apoptotic.

Staining dye protocols for detecting autophagy

Autophagic vacuoles were detected by monodansylcadaverine (MDC) staining as described previously [8, 9]. Briefly, NMR HSCs were cultured in a 96-well plate. At 12 h after treatment, the cells were incubated with 0.05 mM MDC in PBS at 37˚C for 10 min. After incubation, the cells were rinsed three times with PBS and analyzed immediately using a fluorescent microscope.

Western blot analysis

At the end of the designated treatments, NMR HSCs were lysed in RIPA lysis buffer with 1 mM PMSF. Equal amounts of protein were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A wet transfer method was used to electrophoretically transfer proteins from the native gel to nitrocellulose membranes, which were then probed with anti-Beclin 1 (1:1000, Abcam, Cambridge, UK) and anti-LC3 (1:1000, Cell Signaling Technology, Shanghai, China) antibodies, and developed with the BeyoECL Plus substrate system (Beyotime, Shanghai, China). The blots were stripped and re-probed with β-actin antibody (1:2000, Wuhan Boster Biotech, Wuhan, China) to confirm equal protein loading. Bands were visualized using the Kodak Gel Logic 4000 R Imaging System (Carestream, USA).

Small interfering RNA transfection

Small interfering RNA (siRNA) was synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, China). HSCs were seeded in six-well plates at a density of 2×10^5 cells/well in antibiotic-free normal growth medium supplemented with FBS. The cells were incubated at 37˚C in a humidified incubator under 92% N_2, 5% CO_2, and 3% O_2 until 60–80% confluent. HSCs were transfected with 50 nmol/L of siRNA using lipofectamine 2000 according to manufacturer's protocol. Lipofectamine-mediated transfections were performed at a siRNA concentration of 50 nM following the manufacturer's recommendations. The cells were incubated for 6 h. The transfection mixture was then removed and replaced with normal growth medium. After 24 h incubation, the cells were ready for treatment. The mRNA levels were detected by reverse transcription-polymerase chain reaction (RT-PCR) following siRNA knockdown, 24 h post-transfection. Western blotting was performed to detect protein levels of Beclin-1 and LC3, 48 h post-transfection. The sequences of double strand siRNA targeting Beclin 1 were 5'-CUCAAGUUCAUGCUGACCAdTdT-3' and 5'-UGGUCAGCAUGAACUUGAGdTdT-3'.

RT-PCR to assess gene expression

Beclin 1 and GAPDH mRNA levels were quantified by RT-PCR. Following experimental procedures, total RNA was extracted by TRIzol extraction (Life Technologies, Inc.) according to the manufacturer's instructions. Quantification and purity of total RNA was assessed by A260/A280 absorption. cDNA was generated from total RNA (2 μg) using the SuperScript® III First-Strand synthesis kit purchased from Tiangen (Beijing, China). Transcript expression levels were quantified by the StepOne Plus Real-Time PCR Detection System (Applied Biosystems, Warrington, UK) using SYBR® Green Master Mix (Tiangen, Beijing, China). Beclin 1 transcript levels were then adjusted relative to GAPDH expression. The PCR primers were manufactured by Shenggong Inc. (Shanghai, China) and the sequences were as follows: for NMR Beclin 1: 5'-GTTCAAAGAGGAGGTGGAGAAG-3' and 5'-GAGGAAACCCAGGCAAGAC-3'; for NMR GAPDH: 5'-CGCCTGCTTCACCACCTT-3' and 5'-CCTGCCGCCTGGAGAAA-3'.

To allow comparisons of mRNA expression levels, the real-time PCR data were analyzed with the $\Delta\Delta$Ct method and normalized to the amount of GAPDH cDNA as the endogenous control.

Statistical analysis

Results shown are the mean and standard deviation (SD). The statistical significance of the differences between the means was assessed by the least significant difference (LSD) t test. P values of <0.05 were considered statistically significant.

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Results

Autophagy is involved in nutritional stress response

To confirm that serum-free medium induced autophagy, NMR HSCs were treated with serum-free medium for various lengths of time and the LC3-II/LC3-I ratio was determined by western blot analysis. The data showed that there was a higher LC3-II/ LC3-I ratio at each time point examined during starvation, as compared with their respective controls (Fig. 1A and 1B). Cells treated with starvation in the presence or absence of 3-MA had significantly higher levels of apoptosis than the control group (Fig.1E and 1F). MDC is an autofluorescent compound that has been proposed for use as a tracer in autophagic vacuoles. We studied the incorporation of MDC into HSCs using fluorescence microscopy. As shown in Figure 1G, increased numbers of autophagic vacuoles shown by MDC staining were seen in the starvation group compared with the controls at 12 h. Furthermore, cell DNA content was measured by FCM to determine the cell cycle distribution. Compared to full culture medium, NMR HSCs treated with serum-free medium exhibited a significant proportion of cells reaching the S and G2 stages ($P < 0.05$) (Fig. 1C and 1D). This confirmed that cell growth was arrested by starvation in serum-free medium. DAPI staining also showed more condensed and shrunken nuclei in cells in the starvation group (Fig. 1H). Both the results from DAPI staining and Annexin V-FITC assay showed that there were more apoptotic cells during starvation.

Oxidative stress following exposure to H_2O_2 induces apoptosis and autophagy in NMR HSCs

Accumulation of reactive oxygen species (ROS), such as H_2O_2, is an oxidative stress response induced by various defense mechanisms or programmed cell death (PCD). Our research shows that H_2O_2 induced both autophagy and apoptosis in NMR HSCs. As shown in Figures 2A and 2B, NMR HSCs treated with different concentrations of H_2O_2 for 12 h and 24 h, exhibited dose-dependent cell death. Exposure of cells to H_2O_2 increased the extent of cell apoptosis in a time-dependent manner. Our results showed that administration of H_2O_2 significantly increased LC3 II/LC3 I ratio compared to control at each time point, indicating that H_2O_2 induced autophagy (Fig. 2C and 2D; $p < 0.05$). An increased number of autophagic vacuoles shown by MDC staining were seen in cells exposed to H_2O_2 compared with the controls at 12 h (Fig. 2E). DAPI staining also showed more condensed and shrunken nuclei in cells in the starvation group (Fig. 2F)

3-MA has a dual role in modulation of autophagy

The LC3-II/LC3-I ratio in cells treated with 3-MA under starvation was significantly elevated compared with the controls or the starvation condition ($P < 0.05$) (Fig. 1A and 1B). In Figures 2C and 2D, we found that the LC3-II/LC3-I ratio was increased at 12 h or 24 h following exposure to 3-MA compared with the other groups ($P < 0.05$). More autophagic vesicles were formed in the cells treated with 3-MA than in cells treated with H_2O_2 (Fig. 2E). However, a lower level of the LC3-II/LC3-I ratio was detected in the H_2O_2+3-MA treatment than with H_2O_2 or 3-MA alone ($P < 0.05$). These results demonstrate that prolonged treatment with 3-MA in full medium leads to an increase in the LC3-II/LC3-I ratio compared with the control group, and was still effective in suppressing LC3-II in the same cell type induced by H_2O_2. 3-MA has different roles in the modulation of autophagy under different conditions.

siRNA inhibition of Beclin 1 expression

To determine the effect of Beclin 1 inhibition in siRNA transduced NMR HSCs, real-time PCR was also used to detect Beclin 1 mRNA expression, and western blotting was performed to analyze the expression level of Beclin 1 protein. Compared to the negative control (NC)-siRNA group, less Beclin 1 mRNA were detected in the Beclin 1-siRNA transfected NMR HSCs after transfection for 24 h (Fig. 3A). From the three Beclin 1-siRNA sequences provided, Beclin 1-siRNA$_3$ was selected for subsequent experiments (Fig. 3B). Less Beclin 1 protein was detected in the Beclin 1-siRNA$_3$ after transfection for 48 h (Fig. 3C) and the amount

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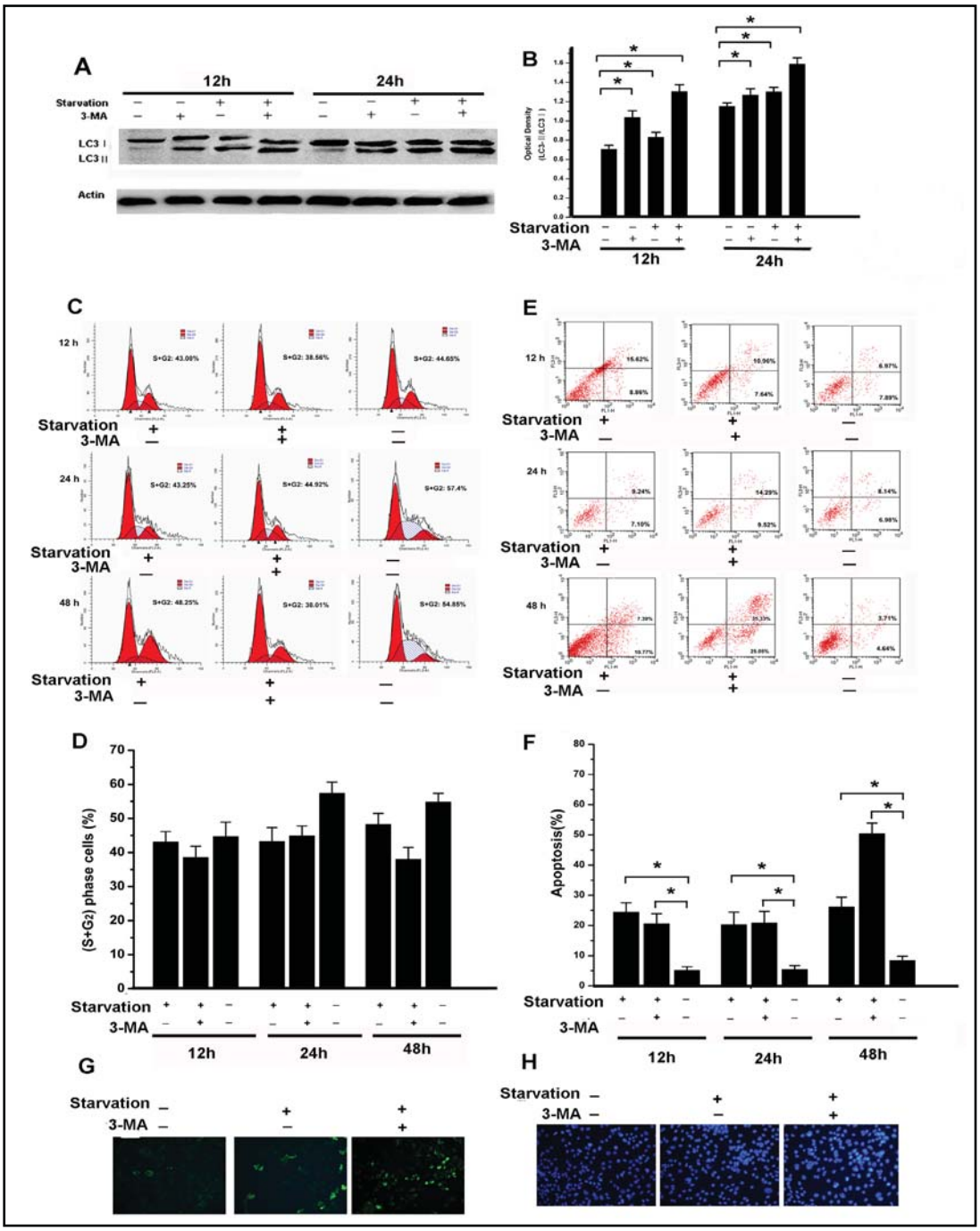

Fig. 1. Autophagy is involved in the tolerance of hepatic stellate cells to nutrient deprivation. (A) LC-3 detected by western blot in the control group and experimental group. β-actin was used as the loading control. (B) Quantitative analysis of the levels of the LC3-II/LC3-I ratio in each group. Bars represent mean ±SD of at least three experiments. *P< 0.05. (C) Flow cytometry (FCM) analysis of the cell cycle distribution of cells in the three groups. The fraction of cells in the S + G2 phases of the cell cycle was determined in each individual lobe as an indicator of the proliferation rate. (D) The quantification of FCM results of HSCs in the different groups. *P< 0.05. (E) Apoptosis was analyzed by annexin V and PI staining and FCM. (F) Graph indicates the percentage of annexin V-positive/PI-negative cells and annexin V-positive/PI-positive cells. *P< 0.05. (G) Labeling of autophagic vacuoles with MDC (all panels, 200× magnification). (H) Detection of apoptosis by DAPI staining. Fluorescence microscopy was performed to observe cell morphology following DAPI staining (all panels, 200× magnification).

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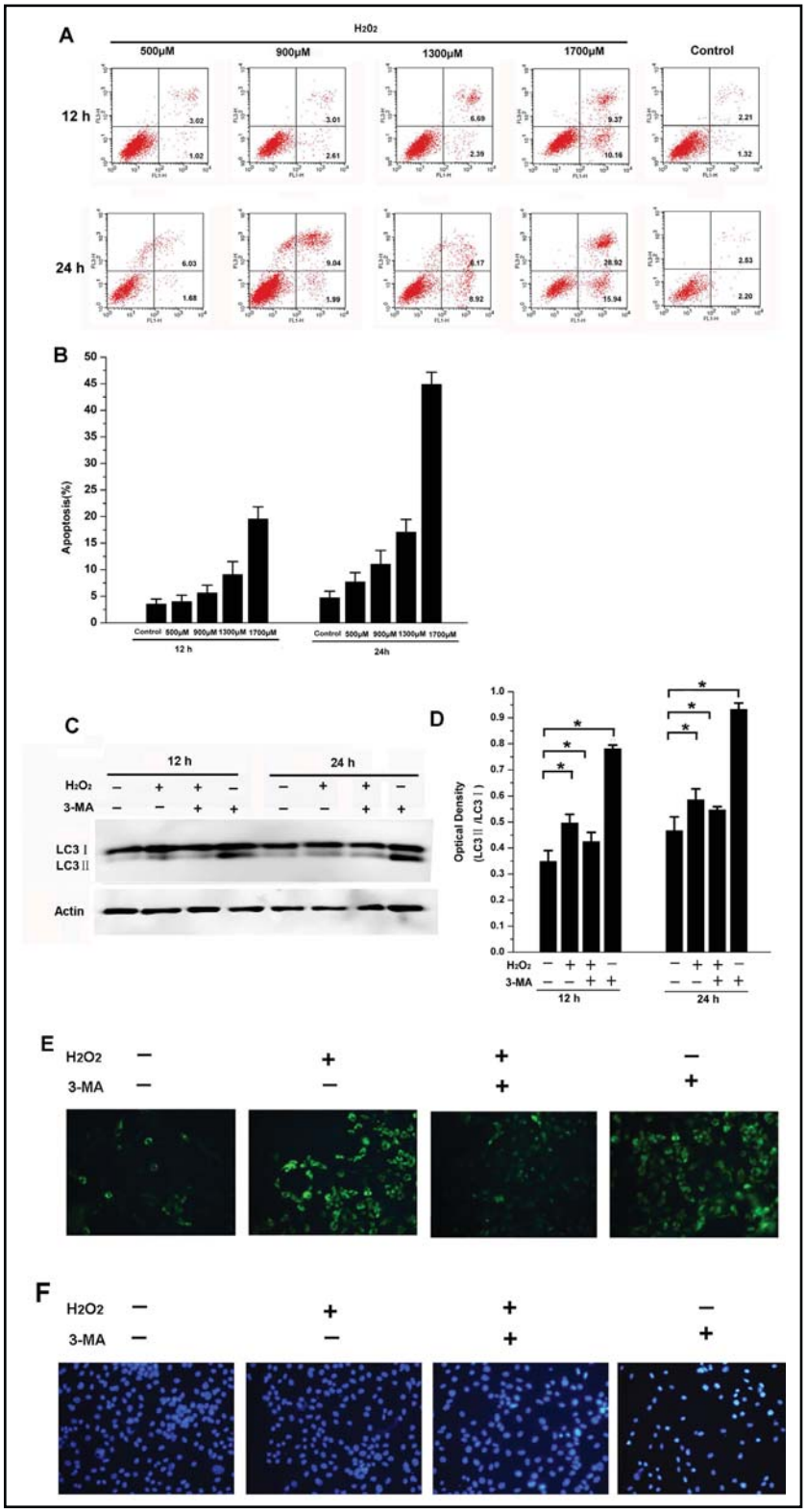

Fig. 2. Effect of H_2O_2 on autophagy in hepatic stellate cells. (A) The effect of various concentrations of H_2O_2 on cell apoptosis.(B) Graph indicates the percentage of annexin V-positive/PI-negative cells and annexin V-positive/PI-positive cells. *P< 0.05. (C) One representative western blot is shown. (D) Quantitative detection of the LC3II/I ratio in HSCs. (E) MDC staining to detect the accumulation of autophagic vacuoles (all panels, 200× magnification). (F) Detection of apoptosis by DAPI staining. Fluorescence microscopy was performed to observe cell morphology following DAPI staining (all panels, 200× magnification).

of LC3-II was increased in Beclin 1-siRNA$_3$ transduced NMR HSCs compared with the NC-siRNA, and the LC3-II/LC3-I ratio increased accordingly after transfection for 48 h (Fig. 3D).

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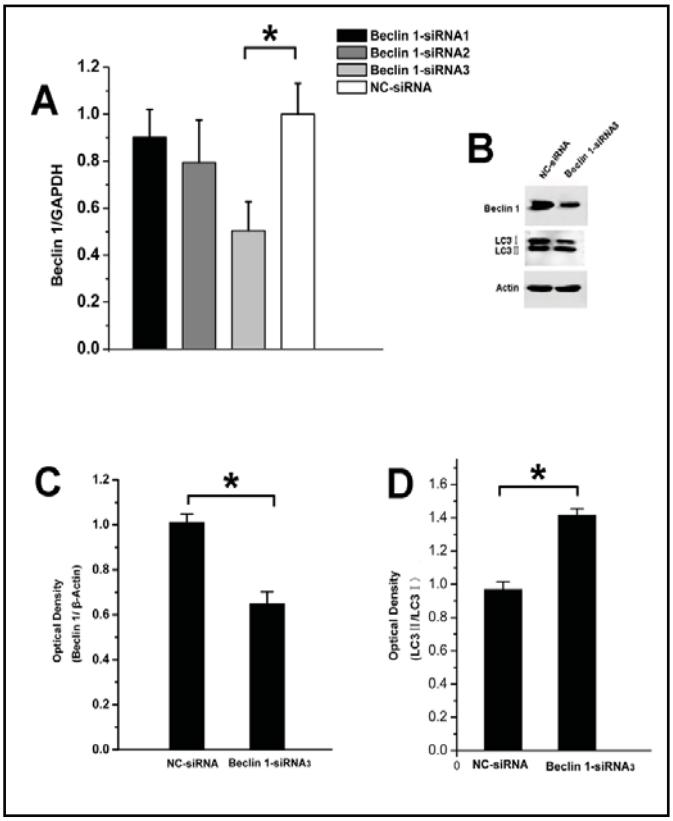

Fig. 3. Detection of Beclin 1 expression in Beclin 1-siRNA transfected HSCs. (A) Beclin 1 mRNA expression analysis by real-time PCR in HSCs transfected with Beclin 1-siRNA and NC-siRNA for 24 h. GAPDH was used to ensure equal loading. Beclin-siRNA$_3$ was found to be the preferred sequence.* $P < 0.05$ vs. NC-siRNA group. (B) LC3 and Beclin 1 protein expression analysis by western blotting for 48 h. β-actin was used to ensure equal loading. (C) Beclin 1 expression in HSCs transfected with Beclin 1-siRNA and NC-siRNA for 48 h. *P< 0.05 vs. NC-siRNA group. (D) Quantitative detection of the LC3II/I ratio in HSCs transfected with Beclin 1-siRNA and NC-siRNA for 48 h.

Discussion

Autophagy can be classified into two modes: basal and induced [10]. In normal cells, the basal levels of autophagy maintain homeostasis by eliminating excessive or unnecessary proteins and damaged or aged organelles. On the other hand, autophagy is observed under some pathological conditions, including some aggressive malignant tumors [9, 10] in which cancer cells are likely to encounter a shortage of nutrients. Both basal and induced levels of autophagy are important in human health and disease. NMRs are the longest-living rodents known, with a maximum lifespan of 30 years, which is five times longer than expected on the basis of body size [1]. NMRs maintain normal activity and body composition for at least 80% of their lives, and they have no obvious age-related increases in morbidity or mortality rate, even though they survive and thrive in dark, dank environments with low levels of oxygen and poor quality nutrition. Their long lifespan is attributed to sustained good health and pronounced resistance to cancer. Physiological and biochemical processes, such as autophagy, may contribute to the successful aging of this exceptionally long-lived species. We demonstrated that NMRs have higher levels of autophagy than short-lived C57BL/6 mice, this may play an important role in the maintenance of cellular protein quality and the defense of cells against intracellular and extracellular aggressors in NMRs. The present study assessed autophagy as a means for cells to flexibly respond to environmental changes, such as reactive oxygen species. The hepatic stellate cells comprise approximately one-third of the nonparenchymal cell population and 15% of the total number of resident cells in normal liver. This cell type is a remarkably versatile mesenchymal cell which is vital to hepatocellular function and the liver's response to injury [11]. We tried to elucidate the possible role of autophagy against H_2O_2 treatment and shortage in nutrient supply using naked mole rat HSCs.

Mammalian cells respond to nutrient deprivation by downregulating the energy consuming processes, such as proliferation and protein synthesis, and in turn, by stimulating catabolic processes such as autophagy [12-15]. The formation of autophagosomes requires LC3 modification, which serves as a widely used marker for autophagosomes [16-18]. Con-

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sequently, immunoblotting of LC3 usually gives two bands: LC3-I (apparent MW is 18 kD) and LC3-II (apparent MW is 16 kD) [19]. LC3-II is the only protein marker that is reliably associated with completed autophagosomes, and is also localized in phagophores. As the amount of LC3-II or the ratio of LC3-II versus LC3-I correlates with the number of autophagosomes, the immunoblot analysis of LC3 is a good and simple method to predict autophagic activity of mammalian cells [19-21]. In this study we proved that NMR HSCs respond to starvation through a mechanism that involves autophagy, which modulates cell cycle arrest and apoptosis. Starvation induced an increase in autophagic vacuole-specific protein LC-3 expression, and a higher LC3-II/LC3-I ratio was detected after 12h and 24h of starvation, as compared with their respective controls, indicating starvation induced autophagy. Rodriguez et al. [2] also showed that macroautophagy is substantially higher in NMR fibroblasts maintained under serum starvation than in fibroblasts from shorter-lived mice. Similarly, when autophagy was assessed by monitoring markers of vacuole development (i.e., the conversion of LC3-I to LC3-II), the LC3-II/LC3-I ratio induced by serum deprivation was approximately two-fold higher in NMR cells [22]. Apoptosis and autophagy are genetically-regulated, evolutionarily-conserved processes that regulate cell fate [23]. There are many connections between autophagy and apoptosis. The same regulators can sometimes control both apoptosis and autophagy [24]. Inhibition of either pathway by both genetic and chemical means can also result in the activation of the other; that is, blocking apoptosis in cells that normally would die can trigger autophagy and survival, whereas blocking autophagy induction in cells that normally would survive rapidly induces apoptosis [25]. For example, some experiments have shown that increased autophagy in nutrient-deprived or growth factor-withdrawn cells allows cell survival by inhibiting apoptosis [26, 27]. Autophagy also protects cells from various other apoptotic stimuli. Autophagic processes can also induce apoptotic cell death [27]. We detected apoptosis after starvation when assessing the relationships between autophagy and apoptosis. HSCs starved for 12h, 24h and 48h showed an increased level of apoptosis compared with the control group. Accumulation of ROS such as H_2O_2, is an oxidative stress response induced by various defense mechanisms or programmed cell death (PCD). Our study showed that H_2O_2 also induces apoptosis and autophagy in NMR HSCs. We observed that NMR HSCs treated with H_2O_2 led to a time-dependent conversion of LC3 protein from LC3-I to LC3-II and an increase in autophagy flux. Labinskyy et al. [28] confirmed that arteries from NMRs showed less apoptotic cell death than C57 mouse vessels at the same concentrations of H_2O_2, indicating that NMR vessels are resistant to the proapoptotic effects of H_2O_2. NMRs are extremely tolerant to oxidative and other types of damage as they may possess very effective repair mechanisms. Autophagy might degrade cellular components in NMR cells eventually leading to activation of the apoptosis machinery. These data demonstrate that HSCs utilize autophagy to prevent apoptosis and maintain their functionality. Some experiments have proved that autophagy can retard cell cycles at the G0/G1phase [29, 30]. We observed a marked decrease in the proportion of the S and G2 population after starvation, indicating that starvation autophagy effectively inhibitsthe proliferation of HSCs. This indicated that NMR cells respond to nutrient deprivation by downregulating the energy consuming processes, such as proliferation [15]. Autophagy may have anti-proliferative roles in HSCs. Triggering autophagy in response to nutrient scarcity may represent an adaptive response by generating recycled metabolic substrates to maintainenergy homeostasis [31, 32]. These data demonstrate that NMR cells have the capacity to induce cell death through apoptosis and downregulate the energy consuming processes through inhibition of proliferation when they become superfluous or irreversibly damaged.

Recently, a large number of studies on the interconnection between Beclin 1, autophagy and tumorigenesis have been analyzed. Numerous observations have demonstrated that Beclin 1 is implicated in tumorigenesis, and plays a role in autophagy and cellular proliferation [33]. Many observations have confirmed that the expression of Beclin 1 coincidentally regulates the autophagy process [33-36]. Heterozygous disruption of Beclin 1 in mice results in increased cellular proliferation and reduced autophagy [37]. Beclin 1 overexpression consistently increases autophagosome numbers [33, 37]. In our previous stud-

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ies, Beclin 1 expression in NMR tissues was lower than that in tissues from C57BL/6 mice [6]. In order to determine whether downregulation of Beclin 1 plays a role in the regulation of autophagy, we initially determined a change in the expression level of the microtubule-associated protein LC3-II in the Beclin 1-siRNA transduced NMR HSCs using immunoblot analysis. As shown in Figure 3B and 3D, the amount of LC3-I was decreased accordingly, and the LC3-II/ LC3-I ratio was increased in Beclin 1-siRNA transduced NMR HSCs as compared to NC-siRNA, which meant that Beclin 1 inhibition may promote autophagy in NMR HSCs. Li et al. [33] confirmed that downregulation of Beclin 1 increases LC3-II protein expression and the formation of autophagosomes in Miapaca2 cells. These results demonstrated that Beclin 1 plays a role in the regulation of autophagosome formation in HSCs. Beclin 1 is involved in the formation of pre-autophagosomal structures, and following downregulation of Beclin 1 is unable to contribute directly to the inhibition of LC3-II protein expression. Therefore, the amount of Beclin 1 did not correlate well with the number of autophagosomes.

An interesting finding in our study is that the LC3-II/LC3-I ratio in cells treated with 3-MA under starvation was significantly elevated compared with the controls or starvation condition. The LC3-II/LC3-I ratio was increased at 12 h or 24 h following treatment with 3-MA compared with the controls and H_2O_2 treatment. However, a lower level of the LC3-II/ LC3-I ratio was detected following H_2O_2+3-MA treatment compared with H_2O_2 or 3-MA alone. These results demonstrate that prolonged treatment with 3-MA in full medium leads to an increase in the LC3-II/LC3-I ratio compared with the control group, and was still effective in suppressing LC3-II conversion in the same cell type induced by H_2O_2. These data indicate that 3-MA promotes autophagy when the treatment period is prolonged in NMR HSCs, and is still capable of suppressing starvation-induced autophagy. A similar result was obtained by Wu et al. [38]. These authors showed that prolonged treatment with 3-MA (up to 9 h) in full medium induced autophagy in mouse embryonic fibroblasts (MEFs), L929 mouse fibrosarcoma cells and human renal epithelial 293T cells [38]. When HSCs were treated with 3-MA, the apoptosis ratio was significantly enhanced in a time-dependent manner as compared with the control group [6]. This maybe the result of an accumulation of toxins caused by 3-MA. These results demonstrate that 3-MA has different roles in the modulation of autophagy under different conditions, and has a dual role in the modulation of autophagy: promoting autophagy or suppressing autophagy. The probable mechanism of this process interests us and will form the basis of further studies.

In conclusion, our data reveal that NMR cells have the capacity to induce cell death through apoptosis and downregulate the energy consuming processes through inhibition of proliferation in response to poor quality nutrition or H_2O_2 treatment. Beclin 1 downregulation by siRNA is unable to contribute directly to the inhibition of LC3-II protein expression, though it plays a role in the regulation of autophagosome formation.

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