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Autophagy in Hypoxia Protects Cancer Cells Against Apoptosis Induced by Nutrient Deprivation Through a Beclin1-Dependent Way in Hepatocellular Carcinoma

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ABSTRACT

Oxygen deficiency and nutrient deprivation widely exists in solid tumors because of the poor blood supply. However, cancer cells can survive this adverse condition and proliferate continuously to develop. To figure out the way to survive, we investigated the role of autophagy in the microenvironment in hepatocellular carcinoma. In order to simulate the tumor microenvironment more veritably, cells were cultured in oxygen-nutrient-deprived condition following a hypoxia preconditioning. As a result, cell death under hypoxia plus nutrient deprivation was much less than that under nutrient deprivation only. And the decreased cell death mainly attributed to the decreased apoptosis. GFP-LC3 and electron microscopy analysis showed that autophagy was significantly activated in the period of hypoxia preconditioning. However, autophagic inhibitor—3-MA significantly abrogated the apoptosis reduction in hypoxia, which implied the involvement of autophagy in protection of hepatocellular carcinoma cells against apoptosis induced by starvation. Furthermore, Beclin 1 was proved to play an important role in this process. siRNA targeting Beclin 1 was transfected into hepatocellular carcinoma cells. And both data from western blot detecting the expression of LC3-II and transmission microscopy observing the accumulation of autophagosomes showed that autophagy was inhibited obviously as a result of Beclin 1 knockdown. Besides, the decreased apoptosis of starved cells under hypoxia was reversed. Taken together, these results suggest that autophagy activated by hypoxia mediates the tolerance of hepatocellular carcinoma cells to nutrient deprivation, and this tolerance is dependent on the activity of Beclin 1. J. Cell. Biochem. 112: 3406–3420, 2011.

KEY WORDS: AUTOPHAGY; HYPOXIA; APOPTOSIS; HEPATOCELLULAR CARCINOMA; NUTRIENT DEPRIVATION

Oxygen deficiency and nutrient deprivation are typical characteristics of solid tumor microenvironment [Suzuki et al., 2005; Chen et al., 2008], which run through the development of cancer. In the early phase, cancer cells are exposed to hypoxic and nutrient-deprived environment generated by insufficient blood supply [Drogat et al., 2007]. Angiogenesis is one of the ways to increase oxygen and nutrient supply in blood vessels [Sato et al., 2007]. However, even after new blood vessels have rebuilt, both oxygen and nutrient supply are still insufficient in locally advanced cancers [Harris, 2002; Jain, 2003], because tumor microvasculature is structurally and functionally abnormal, which leads to insufficient supply of oxygen and nutrient needed by the aggressively proliferating cancer cells [Greijer and van der Wall, 2004]. Developing further in the metastatic foci, some tumors are clinically hypovascular, such as the liver metastases [Warshaw and Fernandez-del Castillo, 1992]. Thus oxygen deficiency and nutrient

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deprivation exists throughout the whole process of cancer, from oncogenesis, development to metastasis.

However, oxygen deficiency and nutrient deprivation do not happen at the same time. Blood flow delivers oxygen and nutrient to the metabolized cancer cells by diffusion from the blood vessels across the basement membrane into layers of cancer cells [Gatenby and Gillies, 2004]. Oxygen is delivered through simple diffusion; nutrients, such as glucose and amino acids [Kallinowskil et al., 1985; Roslin et al., 2003], are taken up by specific transporters [Gatenby and Gillies, 2004], which are faster than the simple diffusion. So pO_2 declines more rapidly than nutrient level with distance from blood vessels. On the other hand, nutrients could be taken up through alternative ways, while oxygen could not. Then, different from nutrient deprivation, low oxygen concentration may be the earlier substrate limitation that cancer cells have to confront [Gatenby and Gillies, 2008].

Once cancer cells sense the low oxygen, they may activate some genes [Vaupel and Hockel, 2000], whose expression will develop a range of metabolic adaptations for surviving hypoxia [Greijer and van der Wall, 2004; Fujii et al., 2008]. Therefore, the cells viability is enhanced and, subsequently, the cells become tolerant to the nutrient deprivation which occurs later [Hashimoto et al., 2002]. For example, under starvation, human hepatoma cell lines exhibited acute cell death [Kato et al., 2002], but hypoxia could allow the cells to survive the starvation [Esumi et al., 2002]. Cells and tissues in hypoxia react by vasodilatation, angiogenesis, erythropoiesis, and glycolysis [Papandreou et al., 2008]. However, these reactions cannot satisfactorily explain how cancer tissues survive under hypoxic condition that is always associated with an insufficient supply of nutrients [Sato et al., 2007]. Then cancer cells may activate other metabolic processes to obtain energy, such as autophagy, for their survival in the hypoxic and nutrient-deprived microenvironment.

Autophagy is a catabolic process that enables cells to recycle amino acids and other intracellular nutrients, and to obtain energy from recycled materials, which is evolutionarily conserved in eukaryotes ranging from yeast to mammal [Tang et al., 2009]. The autophagy process occurs in three steps: (1) autophagosome formation, that autophagic vacuoles envelope and sequester cytoplasmic components; (2) lysosomal fusion with the autophagosome; and (3) lysosomal degradation that process regenerates metabolic precursors, such as amino acids and fatty acids, which can be reutilized for de novo synthesis of macromolecules and energy generation [Shintani and Klionsky, 2004]. So far, autophagy has been implicated in various biological processes, including development, differentiation, immunity, aging, tumorigenesis, chemoresistance, adaptation to changing environmental conditions, and lifespan extension [Carew et al., 2010].

Beclin 1 is the first identified mammalian autophagy gene product whose ortholog in yeast is Atg6/Vps30 [Yue et al., 2003], who plays an important role during the formation of autophagosome [Kihara et al., 2001]. Beclin 1 can complement the defect in autophagy in yeast strains and stimulate autophagy when it is overexpressed in mammalian cells [Pattingre et al., 2005]. It is reported that Beclin 1 possesses a so-called BH3 domain that mediates its interaction with Bcl-2 family members, such as Bcl-2, Bcl-XL, and Mcl-1 [Liang et al., 1998; Levine and Yuan, 2005; Maiuri et al., 2007b]. And these interactions are related with the central role of Beclin 1 in coordinating the cytoprotective function of autophagy and in opposing the cellular death process of apoptosis [Cao and Klionsky, 2007].

Although autophagy has been studied extensively, the role of autophagy in hypoxia followed by nutrient deprivation has not been understood clearly. In the present study, we hypothesized that cancer cells within the tumor tissue may firstly sense the primary substrate limitation—hypoxia, hypoxia then triggers the protective mechanism autophagy, autophagy enhances cancer cells viability so that these cells become tolerant to the following starvation. We investigated the relationship between hypoxia with hypoxia preconditioning and the following starvation, and the role of autophagy within this relationship in hepatocellular carcinoma cells. And our results demonstrate that the hypoxia with hypoxia preconditioning help the hepatocellular carcinoma cells escape from nutrient deprivation-induced apoptosis, which is mediated by autophagy in a Beclin 1-dependent way. This explains the phenomenon well that hepatocellular carcinoma cells could survive and proliferate continually in the ischemic microenvironment, even with extreme nutrient limitation.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human hepatocellular carcinoma cell lines SMMC-7721, HepG2, Huh-7, and Hep3B were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) (GIBCO, Invitrogen) and supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen), 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator under 95% air and 5% CO_2 at 37°C. For hypoxic condition, cells were cultured in a CO_2 incubator (SANYO Co., Ltd.) maintained at 94% N_2, 5% CO_2, and 1% O_2 for 24 h, or treated with hypoxia mimetic: 100 μM $CoCl_2$ (Sigma–Aldrich) for 24 h. Nutrient deprivation, that is, starvation was induced through incubating cells in DMEM without glucose (DMEM-no glucose, GIBCO, Invitrogen) and serum. And the cells under starvation were incubated in this medium for 16 h. For hypoxia plus starvation treatment, cells were firstly cultured under hypoxia but non-starvation for 8 h, and then in oxygen-nutrient-deprived condition for 16 h. Both DMSO and 3-MA were purchased from Sigma–Aldrich, and 3-MA was used at 5 mM.

CELL COUNTING KIT-8

The measurement of viable cell mass was performed with Cell Counting Kit-8 (Dojindo, Japan), and living cells was counted with WST-8. Cells (5×10^3 cells/well) were firstly seeded in 96-well flat-bottomed plates for overnight, then exposed to 1% O_2 or hypoxia mimetic $CoCl_2$ (100 μM) in complete medium for 8 h. Later, we changed the medium with glucose-free and serum-free DMEM and continued to culture these cells for 16 h in hypoxia. As soon as the treatment was completed, 10 μl solution from Cell Counting Kit-8 was added to each well. These plates were continuously incubated for 2 h in a humidified CO_2 incubator at 37°C. Finally, the absorbance of sample taken from each well was measured on a microplate reader (Synergy HT, Bio-Tek) at 450 nm, on the basis of

which the percentage of surviving cells of each treated group to the untreated one was plotted.

CELL DEATH ANALYSIS

The percentage of apoptotic or necrotic cells was assessed by Apoptosis and Necrosis Assay Kit (Beyotime, China). After incubation, cells were stained with Hoechst 33342 and propidium iodide (PI) and then examined by fluorescence microscopy. At least 1,000 cells were counted and distinguished as viable, apoptotic and necrotic cells.

CELL APOPTOSIS ASSAY

For cell apoptosis analysis, approximate 1×10^6 cells were collected. Annexin V-fluorescein isothiocyannate (FITC) assay was used to measure apoptotic cells by flow cytometry according to the manufacturer's instructions (Nanjing Keygen Biotech, China). Briefly, cells were collected by trypsinization, washed with ice-cold phosphate-buffered saline (PBS) twice and resuspended in 300 μl 1× binding buffer containing 5 μl Annexin V and 5 μl PI for 30 min at room temperature in the dark. After incubation, at least 10,000 cells were measured on a BD FACSAria flow cytometer (Becton Dickinson). Results were expressed as the percentage of apoptotic cells at early stage (PI negative and Annexin V positive). Meanwhile apoptosis was detected by staining cells with 4′, 6′-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) based on the nuclear morphology. Identify those cells with condensed and fragmented nuclei as apoptotic.

TRANSIENT TRANSFECTION AND IDENTIFICATION OF AUTOPHAGY

GFP-tagged microtubule-associated protein 1 light chain 3 (LC3) expression vector has recently been utilized to demonstrate the occurrence of autophagy. SMMC-7721 and HepG2 cells were seeded (1×10^4 cells/well) in 96-well plates for overnight, then GFP-LC3 expressing plasmids were transiently transfected into the cells using Fugene HD transfection reagent (Roche) according to the manufacturer's instructions. Continuing to be cultured for 24 h to ensure the expression of GFP-LC3, the cells were subjected to the indicated treatment. At the end of the treatment, autophagy was measured by analyzing the percentage of cells with GFP-LC3-positive dots under a fluorescence microscope (Olympus IX71). A minimum of 200 cells per sample was counted in triplicate for each experiment.

ELECTRON MICROSCOPY

The cells were fixed in 2.5% glutaraldehyde acid in 0.1 M PBS buffer (pH 7.4) for 2 h or more, incubated in 1% osmium tetroxide in 0.1 M PBS buffer (pH 7.4) for 2–3 h, dehydrated in solutions of ethanol and acetone, then embedded in Araldite and finally solidified. Fifty to sixty nanometer sections were cut on a LKB-I ultramicrotome and picked up on copper grids, post-stained with uranyl acetate and lead citrate, and observed in a Philips CM-120 transmission electron microscopy (TEM).

siRNA

The Stealth™ RNAi negative control duplex and Stealth RNAi™ small interfering RNA (siRNA) duplex oligoribonucleotides targeting human Beclin 1 were obtained from Invitrogen. The siRNA was transfected into SMMC-7721 or HepG2 cells using siRNA transfection reagent (Santa Cruz) according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates at a density of 2×10^5 cells/well in antibiotic-free normal growth medium supplemented with FBS. Incubate the cells at 37°C in a CO_2 incubator until the cells were 60–80% confluent. After washing cells once with siRNA transfection medium, 1 μg/well of either siRNA targeted Beclin 1 or siRNA negative control duplex mixed with siRNA transfection reagent and siRNA transfection medium was added to the cells. Incubate the cells for 6 h. Then remove the transfection mixture and replace with normal growth medium. After 24 h incubation, cells were ready for the treatment.

WESTERN BLOT ANALYSIS

At the end of the treatments, cells were lysed in RIPA lysis buffer (Beyotime) with 1 mM PMSF. Equal amount of protein was separated by SDS–PAGE and transferred to NC membrane. After blocking with 5% non-fat milk, the protein on membrane was probed by anti-Beclin 1 (Novus Biologicals, Inc) and anti-LC3 (Novus Biologicals, Inc), then developed with the BeyoECL Plus substrate system (Beyotime). Blots were stripped and re-probed with β-actin antibody (Santa Cruz) to confirm equal protein loaded.

STATISTICAL ANALYSIS

All of experiments were repeated at least three times. The data were expressed as means ± SD. Statistical analysis was performed by using Student's *t*-test (two-tailed). The criterion for statistical significance was taken as $P < 0.05$.

RESULTS

HYPOXIA CONFERS THE TOLERANCE OF HEPATOCELLULAR CARCINOMA CELLS TO NUTRIENT DEPRIVATION

We previously reported that hepatocellular carcinoma cells in hypoxia showed less chemosensitivity than those in normoxia [Song et al., 2009]. Hypoxia contributes aggressively to oncogenesis and tumor development. Low oxygen tension and nutrient deprivation typically characterize ischemic microenvironment in solid tumors [Chen et al., 2008] and also occur at different times. Low oxygen tension occurs earlier than nutrient deprivation [Gatenby and Gillies, 2008]. So there may be some interaction between these two environmental factors. To test this hypothesis, hepatocellular carcinoma cells SMMC-7721 were cultured under hypoxia for 8 h at first, and then the cells were subjected to hypoxic and nutrient-deprived condition for 16 h. The nutrient-deprived condition was attained by culturing cells in medium without glucose and serum, components of ischemia in vivo [Bialik et al., 1999]. Remarkably, at the end of the treatment, lots of cells under glucose/serum starvation in normoxia revealed detachment, shrinkage, and cell rounding, but much less cells with such morphological characters were shown within the cells under starvation plus hypoxia (Fig. 1A). And what is worth mentioning is, the cells incubated in hypoxic and nutrient-deprived condition without hypoxia preconditioning grew bad (data not shown), a little worse than that under nutrient deprivation, although the ones

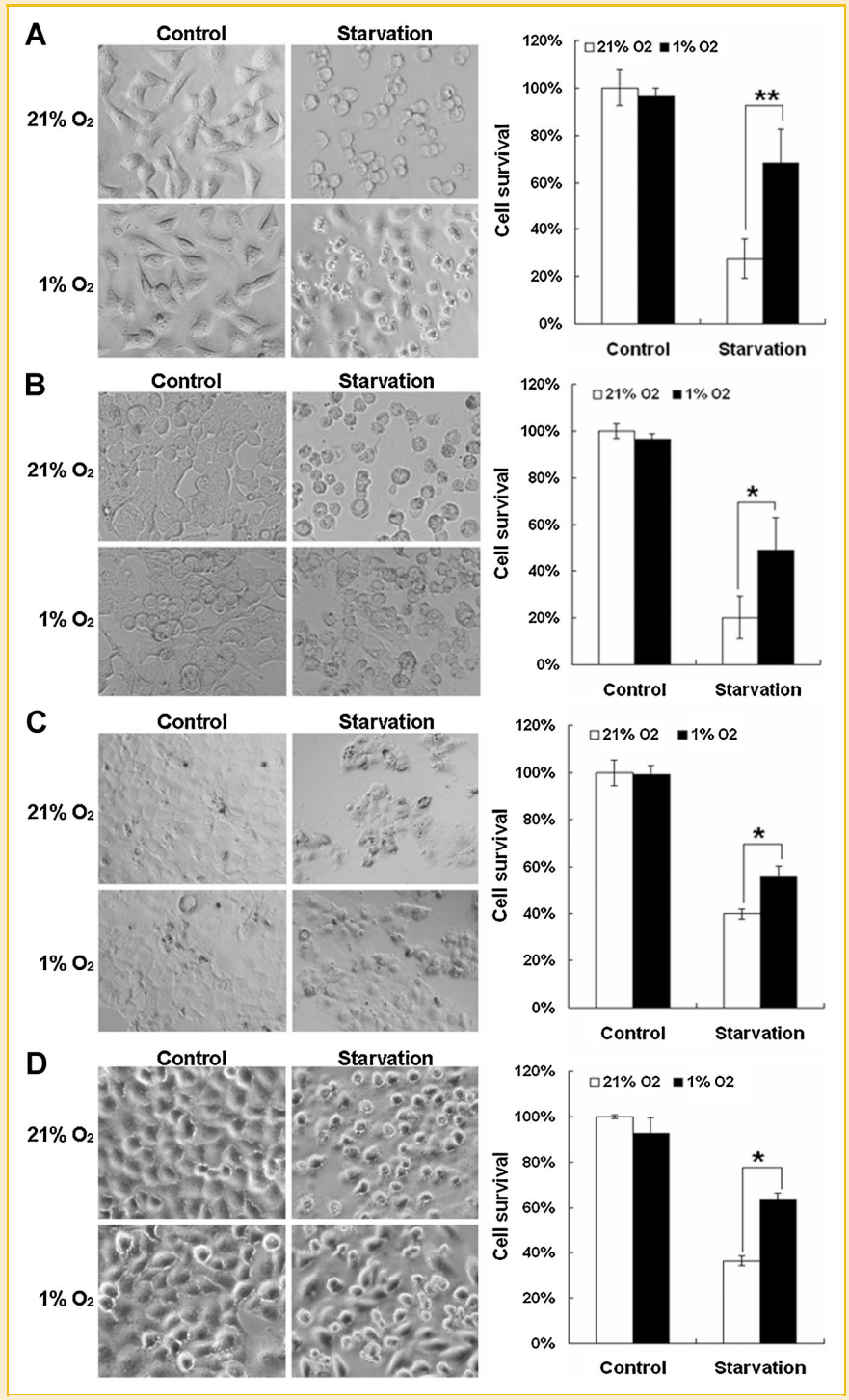

Fig. 1. Cell death induced by nutrient deprivation decreased in hypoxia. The hepatocellular carcinoma cell lines SMMC-7721 (A), HepG2 (B), Huh-7 (C), and Hep3B (D) were cultured under normoxia (21% O_2, control), hypoxia (1% O_2), starvation or hypoxia plus starvation. At the end of treatment, their morphology was captured by light microscope, and cell death was measured by cell counting kit-8. The cells viability is shown as means ± SD of six independent determinations. Statistically significant differences are marked $^{*}P < 0.05$ or $^{**}P < 0.01$.

in hypoxic and nutrient-deprived condition with hypoxia preconditioning grew better. It is possible that hypoxia, especially hypoxia with hypoxia pre-treatment protects SMMC-7721 cells from cell death induced by starvation. Moreover, cells viability analysis by cell counting kit-8 showed that 68.37% SMMC-7721 cells under hypoxia survived the starvation, while just 27.55% survived the starvation under normoxia (Fig. 1A). In order to investigate further, we extended this experiment in other hepatocellular carcinoma cell lines, such as HepG2, Huh-7, and Hep3B. Similarly, the viability of starved cell lines HepG2, Huh-7,

and Hep3B under hypoxia was 48.79%, 55.71%, and 63.30%, respectively, while only 20.14%, 39.75%, and 36.26% under normoxia (Fig. 1B–D). All the data above suggest that hepatocellular carcinoma cells in hypoxia become resistant to the nutrient insufficiency; hypoxia may make cells adaptable to the following nutrient deprivation that more cells under hypoxia plus starvation survived.

THE TOLERANCE OF HEPATOCELLULAR CARCINOMA CELLS TO NUTRIENT DEPRIVATION ATTRIBUTES TO THEIR DECREASED APOPTOSIS IN HYPOXIA

As more cells under hypoxia plus starvation survived than cells under starvation only, we are very interested in finding out the reason. Tumor microenvironment, in fact, is a ''poor soil'' because of limited blood supply. Because of this extreme nutrient limitation, cancer cells often die through necrosis or apoptosis. Then the reduced cell death may attribute to decreased necrosis, or decreased apoptosis, or both of them. To clarify this question, the numbers of cells undergoing the apoptotic or necrotic process under different stress conditions were determined by Hoechst 33342/PI staining procedure. PI positive cells are regarded as necrotic ones, and cells, who show PI negative, Hoechst 33342 strong positive and condensed and fragmented nuclei, are considered as apoptotic ones. Compared with nutrient-deprived cells in normoxia, there were similar amount of cells in oxygen-nutrient-deprived condition underwent necrosis, however, there were significantly less cells underwent apoptosis, and this phenomenon appeared in all the four hepatocellular carcinoma cell lines, which suggests that this may widely exist in solid tumors, at least in hepatocellular carcinoma (Fig. 2A,F). In order to show the contrast more clearly, the necrotic or apoptotic cells were quantified, respectively, and data are presented as percentage of necrotic or apoptotic cells (Fig. 2B–E). For example, 46.67% SMMC-7721 cells under starvation were apoptotic, while only 18.18% apoptosis in oxygen-nutrient-deprived condition, but the percentage of necrotic cells under these two conditions was similar to each other, 21.54% and 20.76% respectively (Fig. 2B). So we can conclude that the decreased cell death is mainly due to the reduced apoptosis, but not necrosis. To confirm this result, apoptosis was examined with two additional independent methods, Annexin V-FITC assay and DAPI staining. Results of Annexin V-FITC assay with flow cytometry showed that in all four hepatocelluar carcinoma cell lines, the percentage of Annexin V positive and PI negative cells—apoptotic cells at early stage—under hypoxia plus starvation was less than that under starvation in normoxia (Fig. 3A,B). Apoptosis under hypoxia plus starvation decreased nearly 30% in SMMC-7721 and HepG2 cells (Fig. 3A,B). In addition, results of DAPI staining showed that more cells under starvation than that under hypoxia plus starvation exhibited nuclear shrinkage, fragmentation, and chromatin condensation (Fig. 3C–F). In conclusion, hypoxia, especially the hypoxia with hypoxia preconditioning decreased the hepatocellular carcinoma cells apoptotic potential that they became more adaptable to the following starvation, so more cells under hypoxia plus starvation than that under starvation in normoxia escaped from cell death.

INVOLVEMENT OF AUTOPHAGY IN HYPOXIA-INDUCED TOLERANCE TO NUTRIENT DEPRIVATION

In order to explain the decreased apoptotic potential which makes hepatocellular carcinoma cells tolerant to nutrient deprivation, there may be some mechanism that enhances the cells viability in hypoxia. Stimulated by low oxygen concentration, cancer cells activate various mechanisms [Hippert et al., 2006] to promote cell survival as well as suppress apoptosis [Wu et al., 2007]. Autophagy was reported to be an protective mechanism that enhances cells viability in adverse conditions [Abedin et al., 2007], including hypoxia [Pursiheimo et al., 2008; Zhang et al., 2008]. Then whether autophagy is involved in the adaptation of hepatocellular carcinoma cells to nutrient deprivation in hypoxia? To detect this hypothesis, the autophagic activity of cells in hypoxia was analyzed with an expression vector encoding GFP-LC3, which is concentrated in autophagic vacuoles, and cells undergoing autophagy result in punctate green fluorescence. Transiently transfected with GFP-LC3 plasmids, SMMC-7721, and HepG2 cells were incubated, 24 h later, the cells were exposed to normoxia or hypoxia. Observed under a fluorescence microscope, cells under hypoxia exhibited significant high percentage of punctate GFP, while cells under normoxia showed primarily diffused (Fig. 4A). This result was further confirmed by data from TEM. The cells revealed a marked accumulation of autophagic vacuoles where cytoplasmic material and/or membrane vesicles were encapsulated after incubation under hypoxia (Fig. 4B–D). Moreover, examination of LC3-I to LC3-II protein processing, a typical hallmark of autophagy, showed that the level of endogenous LC3-II was markedly increased in cells incubated at 1% O_2 (Fig. 6A, right panel). So it is undoubted that autophagy was activated in response to hypoxia, and it may be the protective mechanism of hepatocellular carcinoma cells against the following nutrient deprivation.

To further analyze whether autophagy is really involved, the effect of autophagy inhibition on cells viability was tested. 3-MA, known pharmacological autophagy inhibitor [Seglen and Gordon, 1982], was used to inhibit autophagy. Although incubated under hypoxia, the hepatocellular carcinoma cells did not show any autophagic activity after the treatment of 3-MA (Figs. 4E, 6A, right panel and 6D, c). What deserved our notice, when autophagy was inhibited, the amount of apoptotic cells under hypoxia plus starvation increased, up to more than two times of that without the treatment of 3-MA (Fig. 4F,G). Undoubtedly, autophagy is involved and it protects cells from apoptosis. However, as we all know, not only hypoxia, but also starvation can induce autophagy. And as we have mentioned earlier, only the hypoxia with hypoxia preconditioning plays the protective role but not the hypoxia without hypoxia preconditioning. Then what is the difference between the autophagy induced during the glucose–serum-deprivation ± hypoxia preconditioning. To make it clear, an LC3 flux assay [Klionsky et al., 2008] at different time points following glucose–serum-deprivation was performed. Autophagy was significantly induced by hypoxia pretreatment in the time point of 0 or 4 h after glucose and serum removal, but when the time was extended to 8 or 16 h, the expression of LC3-II between the groups ± hypoxia pretreatment showed little

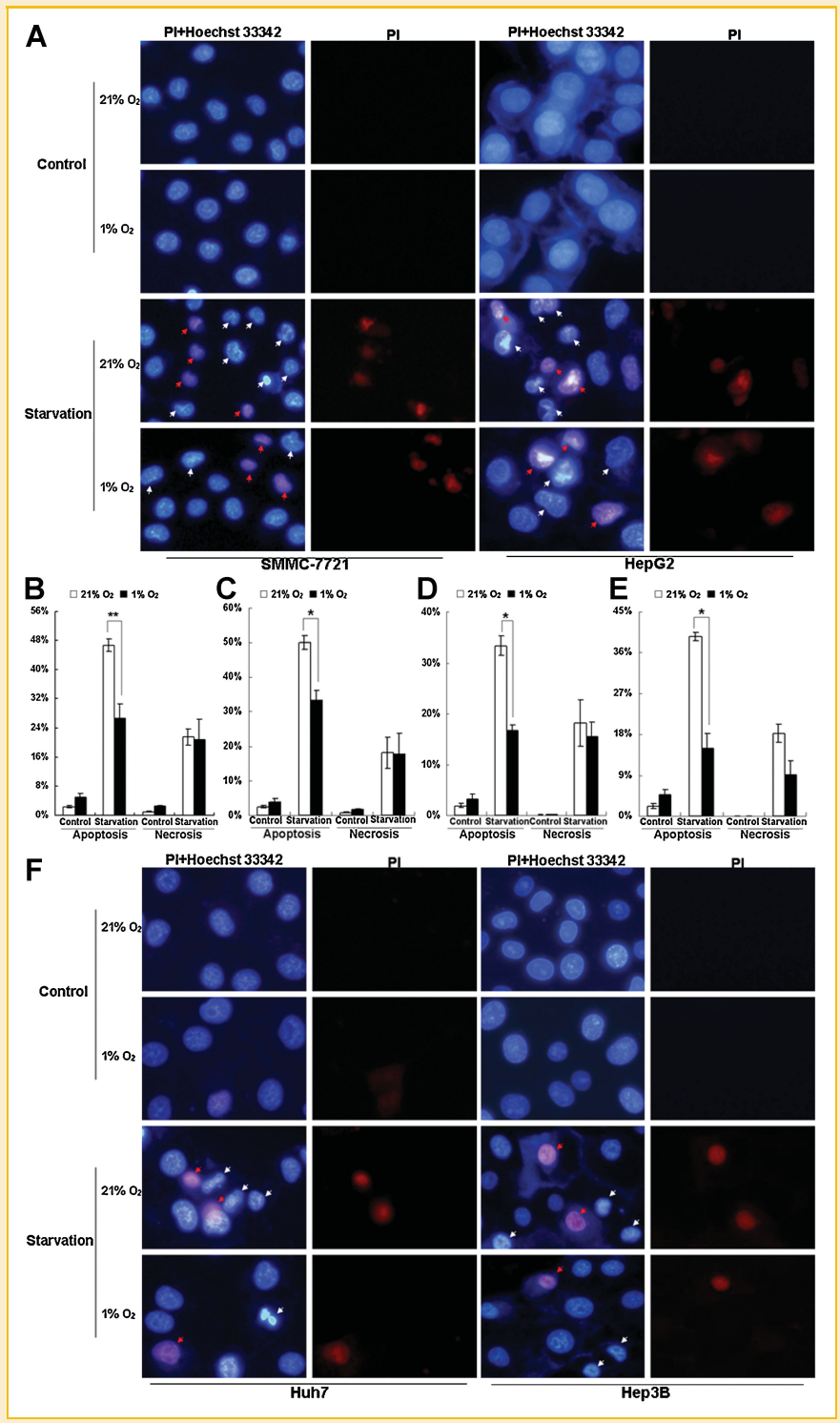

Fig. 2. The number of apoptotic or necrotic cells was analyzed, respectively, after starvation under hypoxia. SMMC-7721 (A,B), HepG2 (A,C), Huh-7 (F,D), and Hep3B (F,E) cells were grown in control conditions (21% O_2, non-starvation) or subjected either to hypoxia (1% O_2), starvation or to hypoxia plus starvation, then the number of apoptotic or necrotic cells was assessed by Hoechst 33342/PI staining. The number of apoptotic or necrotic cells is reported as the percentage of total cells at the end of the experimentation under each condition. Values are the mean value ± SD of three independent determinations. $^{*}P < 0.05$ or $^{**}P < 0.01$.

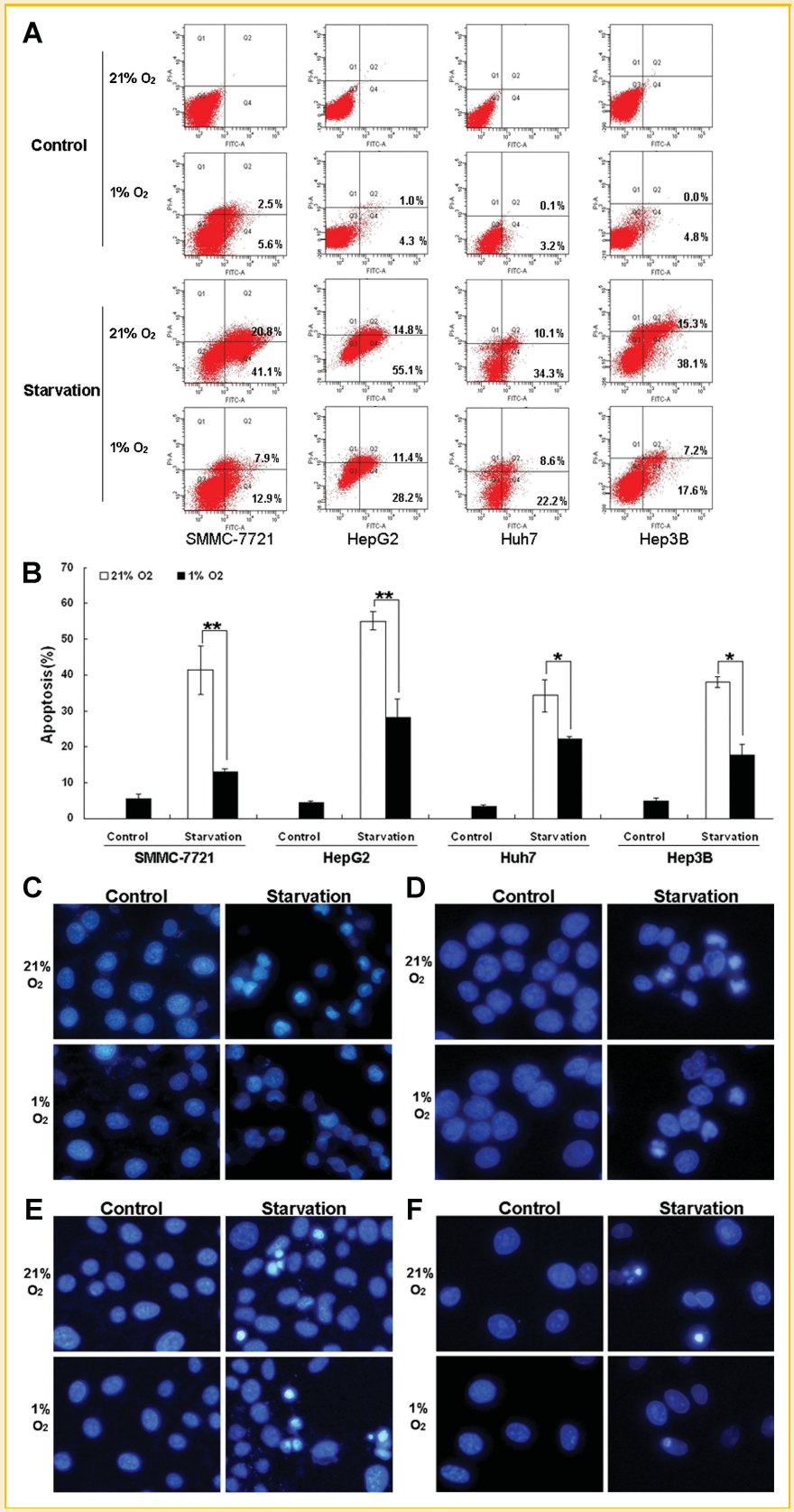

Fig. 3. Apoptosis of hepatoma cells induced by starvation decreased in hypoxia. A: The hepatoma cell lines were treated just as in Figure 2. Then the apoptosis was analyzed by Annexin-V and PI staining with flow cytometry. B: Apoptotic cells at early stage that are Annexin V+/PI− were quantified. Mean data (±SD) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$. C–F: SMMC-7721 (C), HepG2 (D), Huh-7 (E), and Hep3B (F) cells were treated just like that in Figure 2. The cells apoptosis was detected by DAPI staining. At the end of treatment, cell morphology was observed under a fluorescent microscope.

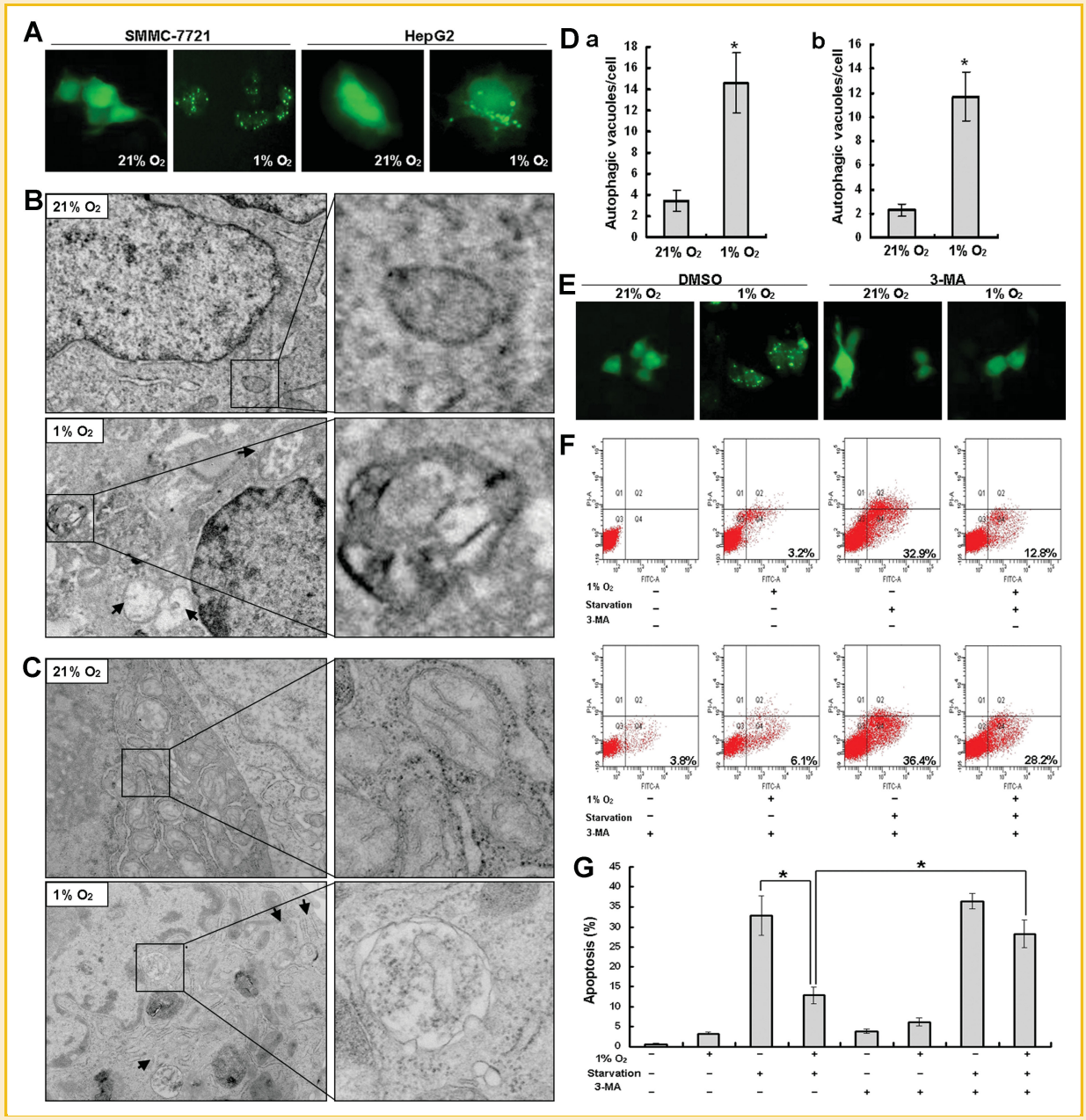

Fig. 4. Autophagy is involved in the tolerance of hepatoma cells to nutrient deprivation. A: SMMC-7721 and HepG2 cells were transfected with GFP-tagged LC3; 24 h after transfection, cells were incubated at 20% or 1% O_2 for 8 h. Images were taken under a fluorescence microscopy. B: Electron micrographs showing the ultrastructure of SMMC-7721 cells incubated at 20% or 1% O_2 for 8 h. Black arrows indicate the autophagic vacuoles in the cytoplasm. Magnification, 10,000×. C: Electron micrographs showing the ultrastructure of HepG2 cells incubated at 20% or 1% O_2 for 8 h. Black arrows indicate the autophagic vacuoles in the cytoplasm. Magnification, 10,000×. D: The number of autophagic vacuoles per cell was analyzed in SMMC-7721(a) and HepG2(b) cells that were incubated under normoxia or hypoxia for 8 h. At least 30 cells were analyzed per group. $^{*}P < 0.05$. E: SMMC-7721 cells transiently expressing GFP-LC3 were cultured under normoxia or hypoxia in the presence or absence of 3-MA for 8 h. Representative fluorescent images were shown. F,G: SMMC-7721 cells were firstly incubated under normoxia or hypoxia in the presence or absence of 3-MA for 8 h, then the cells were continued in normoxia or hypoxia but in nutrient deprived medium or not in the presence or absence of 3-MA for 16 h. Annexin V-FITC staining following FACS analysis was used to examine the cells apoptosis. Data are shown as means (±SD) of at least three independent determinations. $^{*}P < 0.05$.

difference (Fig. 6C). Combined with the results that cells under starvation plus hypoxia grew better than cells under starvation in normoxia, and the result only hypoxia with hypoxia preconditioning protected the cells from cell death, we may draw the conclusion that autophagy activated by hypoxia with hypoxia preconditioning dominates in the protection. All of these results provide evidences for the involvement of autophagy which is activated by hypoxia. It promotes the survival of hepatocellular carcinoma cells and helps them obviate apoptosis induced by starvation. Autophagy mediates the protective role of hypoxia in the tolerance of hepatocellular carcinoma cells to nutrient deprivation.

In addition, another hypoxia model in which hypoxia was achieved by hypoxia mimetic, 100 μM $CoCl_2$, was used to investigate the role of hypoxia and hypoxia-induced autophagy in the adaptation of hepatocellular carcinoma cells to nutrient deprivation. After the treatment by $CoCl_2$, cell viability was analyzed by CCK-8, and cell morphology was observed under light microscope. Just like the results at 1% O_2, there was less cell death in the glucose–serum-deprived medium with $CoCl_2$ (Fig. 5A,B). Additionally, both results from DAPI staining and Annexin V-FITC assay showed that there were less apoptotic cells under starvation with the treatment of $CoCl_2$ (Fig. 5C,D). Further, the involvement of autophagy in this context was investigated by GFP-LC3 plasmid. As shown in Figure 5E,F, there were a lot of GFP puncta in cells exposed to $CoCl_2$, which indicated the occurrence of autophagy. This is consistent with the report of Chen et al. [2008] whose data showed that DFO, another hypoxia-mimetic agent, could

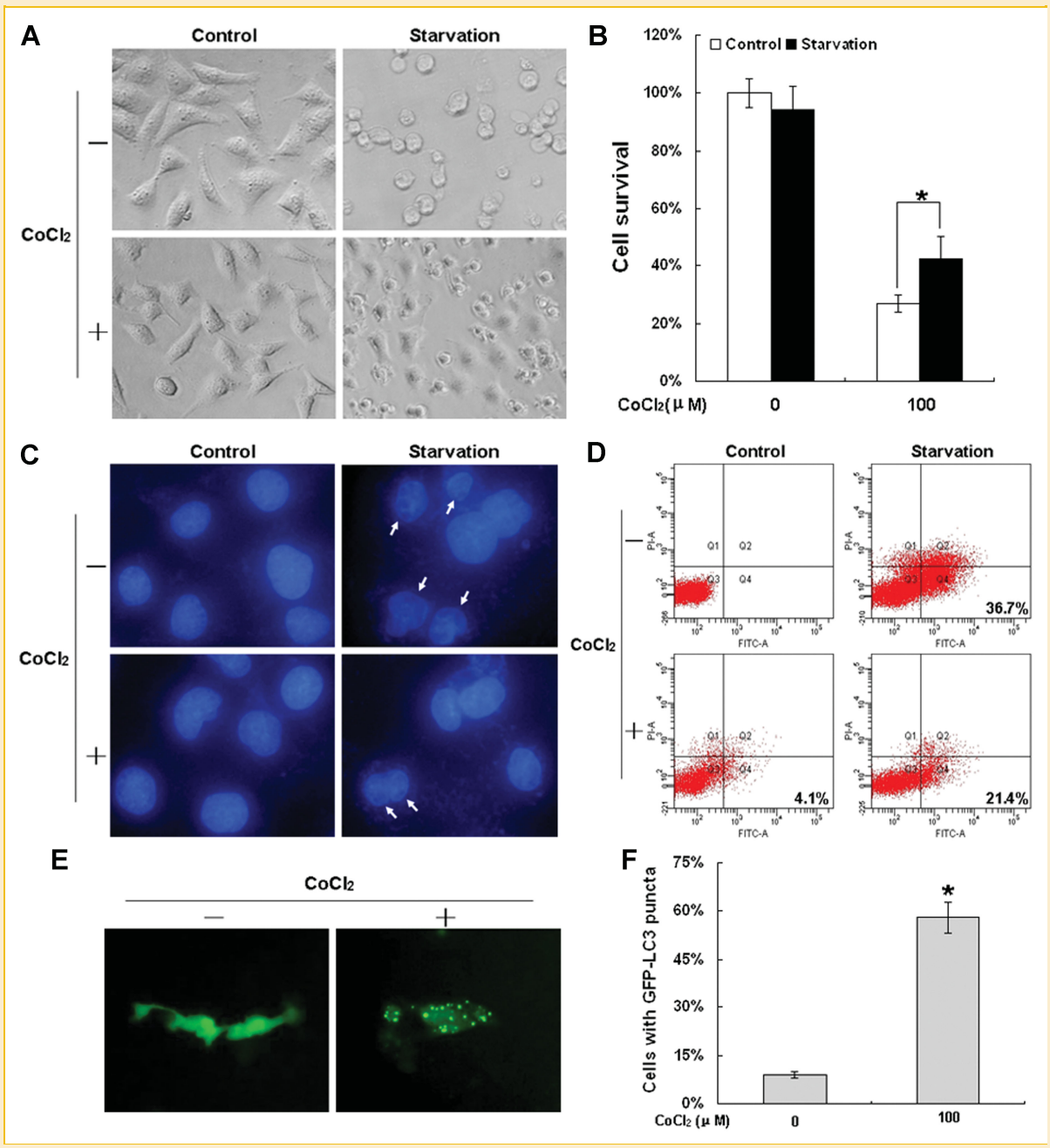

Fig. 5. Autophagy also confers tolerance of hepatoma cells to starvation when hypoxia is induced by hypoxic mimetics. A: SMMC-7721 cells were cultured first with or without $CoCl_2$ for 8 h, then they were starved or not and continued in the presence or absence of $CoCl_2$ for 16 h. Their morphology was captured by light microscope. B: The cell death was measured by cell counting kit-8. Cells apoptosis was determined by DAPI staining (C) and Annexin V-FITC assay (D), respectively. The arrows indicated the condensed and fragmented nuclei. E: SMMC-7721 cells transiently expressing GFP-LC3 were cultured in the presence or absence of $CoCl_2$ for 8 h. Then the puncta was observed under a fluorescent microscope. F: The percentage of cells with punctuate GFP-LC3 was measured. Data of at least three replicates are shown as means (±SD). $^*P < 0.05$.

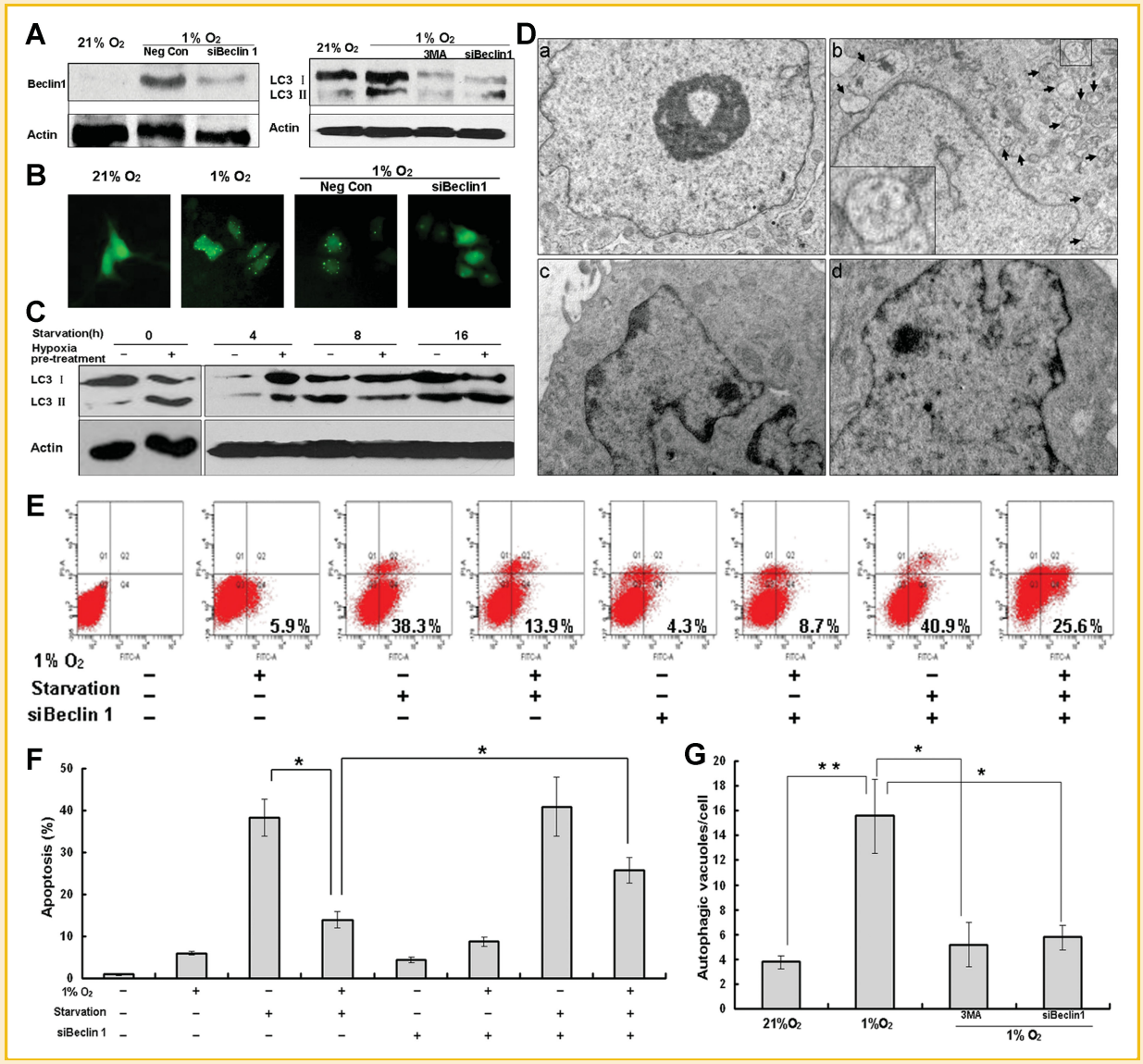

Fig. 6. Hypoxia-induced autophagy mediates the tolerance to starvation in a Beclin1-dependent way in SMMC-7721 cells. A: SMMC-7721 cells were cultured at 20% or 1% O_2 for 8 h after transfection of siRNA negative control (Neg Con) or siBeclin 1 or treatment with 3-MA or not. Whole cell lysates were subjected to western blot to detect the expression of Beclin 1 and the conversion from LC3-I to LC3-II. B: SMMC-7721 cells transiently expressing GFP-LC3 were transfected with siRNA negative control or siRNA against Beclin 1, cultured at 20% or 1% O_2 for 8 h, and analyzed by fluorescence microscopy. C: SMMC-7721 cells were cultured in nutrient deprived medium for indicated times with or without hypoxia pre-treatment (1% O_2, 8 h), and then subjected to western blot using anti-LC3 antibody. D: SMMC-7721 cells were incubated with 3-MA (c) or siBeclin 1 (d) in hypoxia for 8 h, or directly incubated in normoxia (a) or hypoxia (b) for 8 h. Electron micrographs showing the cells ultrastructure were made. Black arrows indicate the autophagic vacuoles in the cytoplasm. Magnification, 10,000×. E,F: SMMC-7721 cells were first incubated with transfected siBeclin 1 or negative control under normoxia or hypoxia for 8 h, then the cells were continued in normoxia or hypoxia but in nutrient deprived medium or not with or without siBeclin 1 or negative control or not for 16 h. Annexin V-FITC staining following FACS analysis was used to examine the cells apoptosis. Data of at least three replicates are shown as means (±SD). $^{*}P < 0.05$. G: The quantification of autophagic vacuoles per cell of SMMC-7721 cells in (D). At least 30 cells were analyzed per group. $^{*}P < 0.05$; $^{**}P < 0.01$.

induce autophagic process. Therefore, it is demonstrated primarily that autophagy is associated with the starvation resistance of hepatocellular carcinoma cells under hypoxia either achieved by 1% O_2 or by hypoxia mimetic.

AUTOPHAGY PROTECTS CELLS AGAINST NUTRIENT DEPRIVATION IN A BECLIN1-DEPENDENT WAY

The first identified mammalian autophagy gene, Beclin 1, plays a central role in coordinating the cytoprotective function of autophagy and in opposing the cell death process of apoptosis [Cao and Klionsky, 2007]. Also, Beclin 1 was demonstrated to be an essential gene in hypoxia-induced autophagy in hepatocellular carcinoma cells in our previous study [Song et al., 2009]. Accordingly, it is possible that Beclin 1 is pivotal to the autophagy in this study too. So the effect of Beclin 1 knockdown was examined. Beclin 1 siRNA was transfected into hepatocellular carcinoma cells, and result from western blot showed that Beclin 1 was up-regulated obviously in hypoxia and the Beclin 1 siRNA inhibited its expression

effectively, while the siRNA negative control not (Fig. 6A, left panel). Meanwhile, the Beclin 1 silence inhibited autophagy significantly. As a result of Beclin 1 knockdown, the expression of LC3-II decreased a lot (Fig. 6A, right panel). Further independent assays, analysis of punctuate GFP and ultrastructure observation by TEM also showed that autophagy in hepatocellular carcinoma cells: SMMC-7721 (Fig. 6B,D-d) and HepG2 (Fig. 7A,B,E) were significantly inhibited by Beclin 1 siRNA. Subsequently, the effect of Beclin 1 knockdown on survival of hepatocellular carcinoma cells was analyzed. The apoptosis of SMMC-7721 cells under hypoxia plus starvation elevated significantly as a result of Beclin 1 silence (Fig. 6E,F). In order to identify the importance of Beclin 1 in hypoxia-induced autophagy in hepatocellular carcinoma cells, experiment in another hepatoma cell line HepG2 was carried out. And similar results were obtained (Fig. 7C,D). That is to say inhibition of autophagy by siBeclin 1 restores hepatoma cells sensitivity to nutrient deprivation. These results suggest that autophagy activated by hypoxia confers the tolerance of hepatocellular carcinoma cells to the following starvation in a Beclin 1 dependent way.

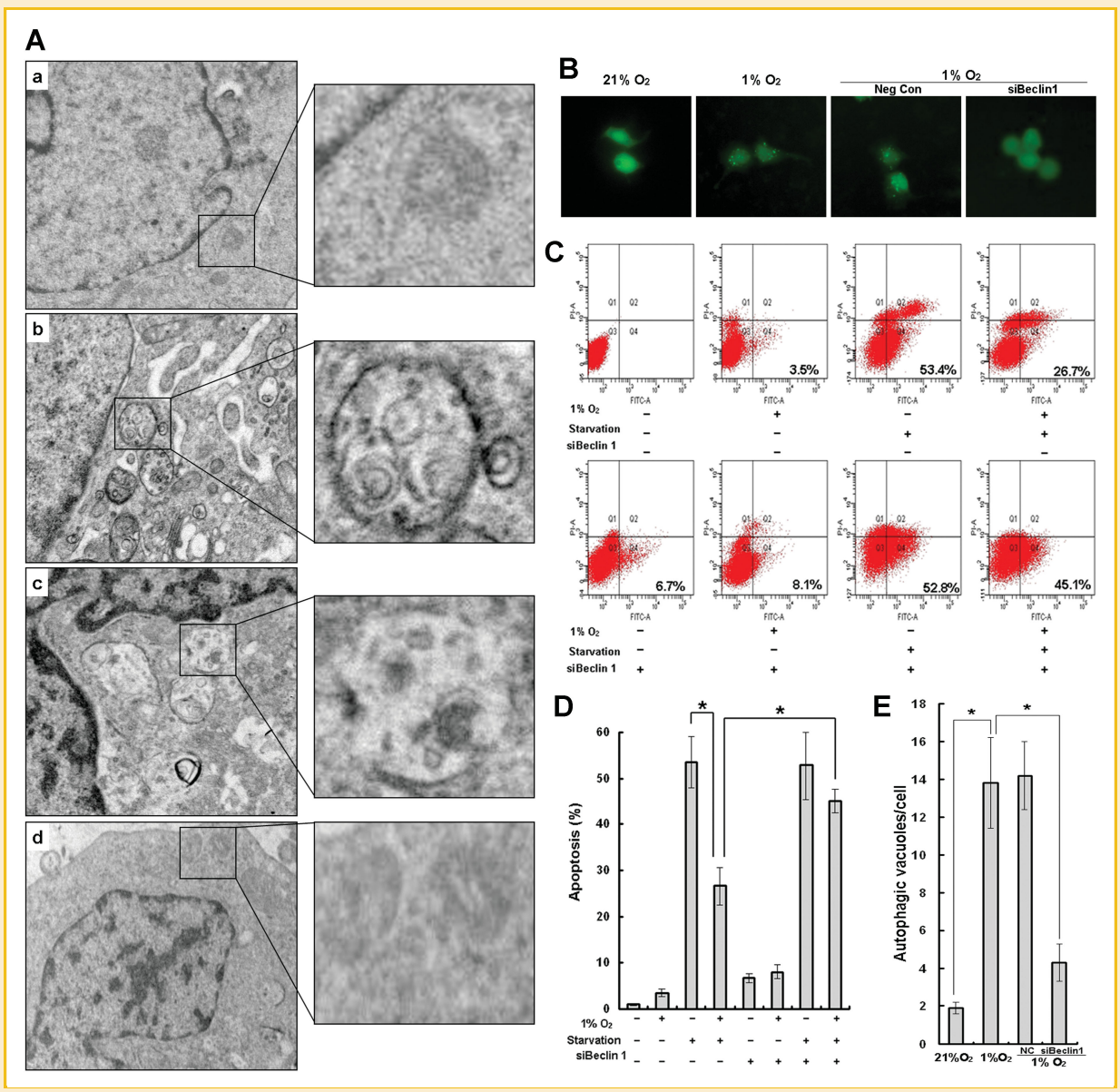

Fig. 7. Autophagy induced by hypoxia mediates the starvation resistance in a Beclin1-dependent way in HepG2 cells. A: HepG2 cells were incubated with siRNA negative control (c) or siBeclin 1 (d) for 8 h, or directly incubated in normoxia (a) or hypoxia (b) for 8 h. Representative electron micrograph images showing autophagic vacuoles were made. Black arrows indicate the autophagic vacuoles. Magnification, 10,000×. B: HepG2 cells transiently expressing GFP-LC3 were transfected with siRNA negative control or siRNA against Beclin 1, cultured at 20% or 1% O_2 for 8 h, and analyzed by fluorescence microscopy. C,D: HepG2 cells were first incubated with transfected siBeclin 1 or negative control under normoxia or hypoxia for 8 h, then the cells were continued in normoxia or hypoxia but in nutrient deprived medium or not with or without siBeclin 1 or negative control or not for 16 h. Annexin V-FITC staining following FACS analysis was used to examine the cells apoptosis. Data of at least three replicates are shown as means (±SD). $^{*}P < 0.05$. E: The quantification of autophagic vacuoles per cell of HepG2 cells in (D). At least 30 cells were analyzed per group. $^{*}P < 0.05$.

DISCUSSION

Tumor microenvironment is characterized by decreased available oxygen and loss of nutrient supply [Helmlinger et al., 1997]. These characteristics regulate cell behavior and induce stress responses, such as angiogenesis, shift to glycolytic metabolism, expression of genes modulating apoptotic process, and so on, to promote cell survival [Cosse and Michiels, 2008]. In this study, it has been elucidated that autophagy activated by hypoxia which appeared early in tumor could enhance cancer cells tolerance to the following nutrient deprivation in a Beclin 1-dependent way.

Ischemia often occurs in solid tumors because of inadequate blood flow, tumor expansion, and increased diffusion distance. Low oxygen tension and nutrient deprivation resulted from ischemia participate in carcinogenesis and contribute to aggravation of the tumor phenotype [Brown and Giaccia, 1998]. Hepatocellular carcinoma cells in hypoxia showed apparent tolerance to nutrient deprivation (Fig. 1), which is consistent with reports that exposure to hypoxia caused cellular tolerance against glucose starvation [Esumi et al., 2002; Suzuki et al., 2005]. Nonetheless, it should be pointed out that hypoxia preconditioning is necessary in our study. The hepatocellular carcinoma cells cultured in hypoxic and nutrient-deprived condition without hypoxia preconditioning did not show the tolerance but showed a little more cell death than that under starvation only (data not shown). On the other hand, the nutrient and oxygen deprived condition with hypoxic preconditioning simulates the microenvironment in solid tumors more veritably.

Cells adapt to an insufficient supply of nutrients mainly in two ways—increasing supply or tolerating insufficiency [Izuishi et al., 2000]. Compared with neovascularization by which supply is increased, less attention has been paid to the tolerance to poor nutrition, although tolerance as well as angiogenesis is important for tumor progression under ischemic condition [Izuishi et al., 2000]. It has been found that cancer cells are resistant to nutrient deficiency in various tumors, including pancreatic cancer, colorectal cancer, gastric cancer, and liver cancer [Fujii et al., 2008]. This starvation-resistant phenotype is named as austerity which may contribute to cancer cell survival in a nutrient-deficient microenvironment [Izuishi et al., 2000; Esumi et al., 2002]. But how do cancer cells tolerate the nutrient deprivation and keep survival?

As both oxygen and nutrient are deficient in ischemic tumor microenvironment, oxidative respiration, and anaerobic glycolysis, by which cancer cells often attain energy for survival in hypoxia, are inhibited to a great extent that there must be an alternative metabolic pathway providing energy for cancer cells [Izuishi et al., 2000; Esumi et al., 2002]. Autophagy providing nutrients by degrading existing cellular components is referred to as an adaptive response to various cell stresses, including hypoxia and starvation. Our published and current data demonstrate the substantial role of autophagy in hypoxia not only confers the chemoresistance but also mediates the tolerance against nutrient deprivation in hepatocellular carcinoma cells. Meanwhile autophagy has been mentioned to be an indispensable physiological reaction for sustaining cell viability during starvation [Kanamori et al., 2009]. The $Atg5^{-/-}$ mice that display impaired autophagy were more susceptible to cardiac dysfunction following starvation [Kuma et al., 2004]. And suppression of autophagy was shown to promote apoptosis and caspase-3 activation in starved HeLa cells [Boya et al., 2005; Ryter and Choi, 2010]. As to the present study, autophagy activated by hypoxia with hypoxia preconditioning promotes the cell survival and mediates the tolerance to nutrient deprivation in hepatocellular carcinoma cells. Since not only hypoxia, but also starvation can induce autophagy, it is necessary to clarify the importance of autophagy activated by hypoxia with hypoxia preconditioning in this study. First of all, cells under starvation plus hypoxia grew better than cells under starvation in normoxia (Fig. 1). Second, hepatocellular carcinoma cells cultured in hypoxic and nutrient-deprived condition without hypoxia preconditioning did not show the tolerance but showed a little more cell death than that under starvation only (data not shown). Third, there was not significantly more apoptosis in cells under starvation when autophagy was inhibited (Figs. 4F,G, 6E,F, and 7C,D). Forth, the LC3 flux assay at different time points following starvation showed that, during the starvation ± hypoxia preconditioning, autophagy was significantly induced by hypoxia pretreatment in the time point of 0 and 4 h, but there was little difference between the groups ± hypoxia pre-treatment at the time point of 8 and 16 h (Fig. 6C). For explanation, firstly stressed by hypoxia for a period, the activated autophagy could enhance the viability of hepatocellular carcinoma cells and make them powerful enough to response to the following extreme starvation in hypoxia. For the autophagy flux, it may be that, before starvation or at the early age of starvation, autophagy was mainly activated by hypoxia and played a typical protective role. As the time for starvation went on, autophagy was induced by two stimuli: hypoxia and starvation, at this time, autophagy was over-induced. It was also at this time, there was no difference in autophagic activity between cells with or without hypoxia pre-treatment because the glucose–serum-deprivation for a long time was such strong stimuli of autophagy that the role of hypoxia pre-treatment was weakened greatly. The over-stimulated autophagy could not protect cells from cell death because autophagic cell death would occur when autophagy was activated excessively [Puissant et al., 2010]. So autophagy activated by hypoxia with hypoxia preconditioning protected hepatocellular carcinoma cells from apoptosis induced by starvation. Meanwhile, it has been demonstrated that autophagy can keep tumor cells alive under hypoxia-associated nutrient deprivation by providing energy and macromolecular precursors required for adaptation [Moretti et al., 2007], which corroborates our results.

However, the relevance of autophagy with cell survival and cell death is still controversial. Different from the pro-survival role of autophagy mentioned above, autophagy was reported to may represent a form of non-apoptotic cell death on death stimuli, when apoptosis is inhibited [Shimizu et al., 2004]. Recently, autophagy has grabbed much attention by autophagic death [Baehrecke, 2005] which eliminates damaged and/or harmful cells, such as cancer cells damaged by anti-cancer reagents, or cells infected with pathogenic microorganisms [Kondo et al., 2005]. It is speculated that the role of autophagy may vary depending on cell type, microenvironment, and extent of induced autophagy. For instance, autophagy induced by TGF-β1 could protect glomerular mesangial cells from apoptosis during serum deprivation, while excessive autophagic activity leads

to autophagic cell death, and thereby contributes to the pathology of diseases [Kim et al., 2002].

Further, we demonstrate that the hypoxia-induced autophagy protects hepatocellular carcinoma cells against nutrient deprivation in a Beclin1-dependent way. Beclin 1, a mammalian homologue of yeast Atg6, is a major regulator of autophagy [Kuma et al., 2004]. The overexpression of Beclin 1 increases cell survival through inhibition of apoptosis [Liang et al., 1998; Shimizu et al., 2004], and its silence results in cells increased sensitivity to the stress [Mizushima et al., 2004], which is consistent with our results. On the contrary, what has been proved in breast, ovarian, and prostate cancers is that Beclin 1 is a haploinsufficient tumor suppressor gene, whose hemiallelic loss leads to tumorigenesis [Yue et al., 2003]. In order to clarify this contradiction, we could refer to the achievements in genome study. The Beclin 1 gene was mapped to the human chromosome 17q21, which is monoallelically deleted in 40–75% of human breast, ovarian, and prostate cancers, and Beclin 1 is commonly expressed at reduced levels in those tumors [Aita et al., 1999], while interestingly the17q21 locus in liver cancer is subjected to allelic gain rather than loss [Chen et al., 2000]. And results from complementary DNA (cDNA) microarray screen and northern blot analysis demonstrate that the Beclin 1 gene was up-regulated in hepatocellular carcinoma tissues [Song et al., 2004]. According to these reports, we could determine that the role of Beclin 1 as an essential autophagy gene dominates in hepatocellular carcinoma, although it has multiple function and its role of haploinsufficient tumor suppressor gene dominates in some tumors.

However, further study is needed on Beclin 1 working in this hypoxia-induced autophagy against nutrient deprivation model. HIF-1, composed of HIF-1α and HIF-1β(ARNT) subunits [Salceda and Caro, 1997], is the major transcription factor specifically activated during hypoxia. Bohensky et al. [2007] demonstrated that HIF-1 activity profoundly influenced the expression of Beclin 1 as well as the interaction between Beclin 1 and members of the Bcl-2 family. On one hand, HIF-1 serves to regulate autophagy directly that HIF-1 silence caused a decrease in Beclin 1 expression. On the other hand, HIF-1 modulates autophagy indirectly through its target gene BNIP3 and BNIP3L who are BH3-only proteins belonging to Bcl-2 family [Zhang et al., 2008]. Knockdown of BNIP3 and BNIP3L by siRNA inhibited the hypoxia-induced autophagy, whereas overexpression of BNIP3 promoted autophagy under normoxia [Bellot et al., 2009]. And recent studies indicate that BNIP3 induces autophagy by disrupting the interaction of Beclin 1 with Bcl-2 and Bcl-XL [Maiuri et al., 2007a]. Contrast to this, Chen et al. [2008] demonstrated that autophagy induced by hypoxia was in a BNIP-independent way, as autophagy induction occurs prior to the maximal induction of BNIP3 in response to hypoxic stress. It is possible that the disparity may stem from differences in the experimental conditions and/or cell types analyzed.

Meanwhile, there are investigations demonstrating that AMPK is induced by hypoxia and is implicated in hypoxia-induced autophagy [Papandreou et al., 2008]. AMPK, in response to hypoxia-induced energy depletion, down-regulates mTOR through the activation of TSC complex [Liu et al., 2006; Cosse and Michiels, 2008], which just regulates autophagy positively through mTOR-dependent way. Moreover, recent studies specify a novel role for PKC-dependent signaling in hypoxic regulation of autophagy [Chen et al., 2008]. So someone concluded that PKC/JNK1-dependent but BNIP3- and AMPK-independent pathway is required for autophagy in the early response to hypoxic stress, whereas a BNIP3-dependent pathway is required for autophagy in response to prolonged hypoxia [Zhang et al., 2008].

Anyway, manipulation of autophagy or its capital regulatory proteins may represent a novel therapeutic strategy in the treatment of hepatocellular carcinoma. Anti-angiogenesis therapy has been widely used in cancer therapy as it could induce cell death by oxygen and nutrient deprivation, though the therapeutic effect is not satisfied currently because some cancer cells may survive through autophagy and finish the malignant transformation which is responsible for tumor recurrence and metastasis. But if we combined anti-angiogenesis therapy with autophagy inhibition, the cancer cells could be killed thoroughly, which may improve the therapeutic effect apparently. Therefore, our results may help to suggest a new therapeutic strategy designed to prevent adaptation of cancer cells in stressed condition, and inhibition of autophagy may be a promising novel strategy with broad applications in cancer therapy.

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