Autophagy regulates proliferation and biliary differentiation of hepatic oval cells via the MAPK/ERK signaling pathway

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Abstract. Hepatic oval cells (HOCs) are thought to possess self-renewal ability and a bipotential capacity for differentiation, which allows them to differentiate into hepatocytes and cholangiocytes. Autophagy serves an important role in self-renewal and differentiation of stem cells; however, how autophagy contributes to proliferation and differentiation of hepatic progenitor cells has yet to be elucidated. In the present study, autophagy was regulated by rapamycin (Rapa) and chloroquine (Chlo) administration. The results demonstrated that Chlo-treated HOCs exhibited decreased autophagic activity alongside a decreased tendency to proliferate, as determined by Cell Counting Kit-8. In addition, activation of autophagy by Rapa enhanced the biliary differentiation of HOCs. Furthermore, increased phosphorylated (p)-extracellular signal-regulated kinase (ERK)/p-p38 expression was observed following the induction of autophagy, thus indicating that the mitogen-activated protein kinase (MAPK)/ERK signaling pathway was activated by autophagy to exert effects on the stimulation of HOC proliferation and differentiation. In conclusion, the present study demonstrated that autophagy regulates proliferation and biliary differentiation of HOCs via the MAPK/ERK signaling pathway. These results suggest a role for autophagy in stimulating the proliferation and differentiation of HOCs.

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Abbreviations: CK19, cytokeratin 19; HOC, hepatic oval cell; Rapa, rapamycin; Chlo, chloroquine; SB, sodium butyrate; MAPK, mitogen-activated protein kinase

Key words: hepatic oval cells, autophagy, proliferation, differentiation, MAPK/extracellular signal-regulated kinase signaling

Introduction

Hepatic oval cells (HOCs) are thought to possess self-renewal ability and a bipotential capacity for differentiation, which allows them to differentiate into hepatocytes and cholangiocytes (1). The differentiation of HOCs has been reported to be regulated by various signaling pathways, including Notch, Wnt, transforming growth factor-β, bone morphogenetic protein, hepatocyte growth factor and fibroblast growth factor signaling pathways (2).

Macroautophagy, which is also referred to as autophagy, is a highly conserved and important metabolic process in eukaryotic cells. Autophagy has been characterized as an essential process associated with cellular homeostasis. Under stress or starvation conditions, unnecessary or dysfunctional cellular components are degraded and recycled through autophagy, thus promoting cell survival and growth (3). Autophagy, which is initiated by the microenvironment or cytokines, serves a role in various physiological and pathological processes, including cancer, metabolic disease, neurodegenerative disorders, cell growth, cell death, ontogeny, and cell differentiation and self-renewal (4). However, how autophagy contributes to proliferation and differentiation of HOCs remains to be elucidated.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases, which function as broad intracellular signal mediators of cell survival, proliferation, motility, apoptosis and differentiation (5). In addition, MAPKs are essential for stem cell self-renewal and commitment to lineage differentiation (6). Extracellular signal-regulated kinase (ERK) 1/2 is required for osteogenic mesenchymal stem cell differentiation and skeletal development (7). It has previously been reported that MAPK/ERK serves an important role in the proliferation of HOCs (8). Furthermore, induction of autophagy is associated with epidermal growth factor-induced MAPK phosphorylation. However, the effects of autophagy on the MAPK/ERK signaling pathways in HOC proliferation and biliary differentiation are currently not well understood. Therefore, to investigate these effects, as well as the underlying molecular mechanisms, the present study investigated the effects of autophagy on HOC proliferation, apoptosis and differentiation into cholangiocytes, and the role of the MAPK/ERK signaling pathway in these processes.

Materials and methods

Cell lines, culture and drug treatment. The WB-F344 HOC line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). This cell line possesses stem cell features and has been reported previously (9). WB-F344 cells were cultured in a 25 cm^3 flask with RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS) (both from (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37˚C. The culture medium was changed every second day and cells were passaged 1:3 using 0.05% trypsin plus 0.02% EDTA. To regulate autophagy in WB-F344 cells, chloroquine (Chlo; 100 mg) and rapamycin (Rapa; 1.0 mg/l) (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were administered to inhibit or induce autophagy, respectively (10). Briefly, WB-F344 cells were seeded in 6-well culture plates at $3x10^5$ cells/well and were cultured overnight. The following day, the cells were treated with or without Rapa (40 nM) or Chlo (50 μM) for 24 h at 37˚C. For the following experiments, Rapa and Chlo were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). Control cells were treated with an equal amount of DMSO used for drug treatment [not exceeding 0.1% (v/v)]. Morphology of WB-F344 cells was evaluated by a light microscope (Olympus CX21; Olympus Corporation, Tokyo, Japan) after Rapa or Chlo treatment.

Biliary differentiation of hepatic progenitor cells. WB-F344 cells had been used as a good model to study the mechanism of biliary differentiation (11). To induce biliary differentiation, WB-F344 cells which were seeded in 6-well culture plates at a density of $5x10^5$ cells/well were treated with 3.75 mM sodium butyrate (SB; Sigma-Aldrich; Merck KGaA) (11). The medium was changed every 2 days. The days of differentiation were numbered consecutively beginning at the first day of the SB treatment (0 day) to the last day (3 day).

Determination of cell viability. Cell viability was detected using the Cell Counting Kit (CCK)-8 assay. WB-F344 cells were plated in 96-well plates with three duplicate wells in each group. When the cells had grown to 70-80% confluency, the cells were treated with or without Rapa (30 and 700 nM) or Chlo (20-500 μM). The CCK-8 solution (10 μl) at a 1:10 dilution in FBS-free RPMI-1640 medium (100 μl) was added to each well, and the plates were incubated for 2 h at 37˚C. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The mean optical density (OD) of three wells in the indicated groups was used to calculate the inhibition rate of the cells as follows: Inhibition rate = $(OD_{control\ group} - OD_{treatment\ group})/(OD_{control\ group} - OD_{blank\ group})$ x 100%. The experiment was performed in quadruplicate.

Immunofluorescence staining. After treatment, $3x10^6$ cells in 6-well plates, were fixed with 4% paraformaldehyde and incubated with CK19 (cat. no. 3092, 1:200; Cell Signaling Technology, Leiden, the Netherlands) overnight at 4˚C. And then incubated with Alexa Fluor 488-labeled secondary antibodies (cat. no. A-11034, 1:200; Molecular Probes; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. DAPI (Sigma-Aldrich; Merck KGaA) was used to stain the nuclei. Five fields from each well were taken at magnification, x200. Fluorescence intensity was evaluated by using a confocal microscope (Leica TCS SP2; Leica Microsystems, GmbH, Wetzlar, Germany).

Flow cytometry to determine the effects of autophagy on apoptosis. To analyze cell cycle progression and to assess apoptosis, flow cytometric analysis was used to identify sub-G_1 cells/apoptotic cells and to measure the percentage of sub-G_1 cells after propidium iodide (PI) staining in hypotonic buffer as previously described (12). The cells were collected at 24 h after drug treatment and apoptotic cells were analyzed by flow cytometry using a fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection kit (cat. no. 556547; BD Biosciences, Franklin Lakes, NJ, USA). Briefly, following trypsinization and three washes in PBS, cells were resuspended in 500 μl 1X Annexin V binding buffer, 5 μl FITC-Annexin V and 5 μl PI, and were incubated in the dark for 15 min at 37˚C after gentle mixing. Following the addition of 200 μl 1X Annexin V binding buffer, apoptosis was analyzed by flow cytometry (BD FACSCanto II; BD Biosciences, San Jose, CA, USA) within 1 h. For cell cycle analysis after 24 h, after drug treatment cells were washed with cold PBS for three times and fixed in 70% ethyl alcohol at 4˚C overnight. Cells were then treated with 10 μg/ml RNase and were stained with 50 μg/ml PI for 30 min at room temperature in the dark. The cell cycle was then measured by BD FACSCanto II and the cell cycle distribution was analyzed by ModFit LT v3.3 software (BD Biosciences).

Western blot analysis. Western blot analysis was performed as described previously (12). Briefly, following cell lysis and protein extraction with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), protein concentration was measured using the bicinchoninic acid protein assay reagent. The samples (50 μg) were then denatured in sample buffer which containing SDS and the proteins were separated by 10-12% SDS-PAGE gels. The proteins were then electroblotted onto polyvinylidene difluoride membranes. After blocking with 5% skimmed milk for 1 h at room temperature, the blots were incubated with rabbit anti-microtubule-associated proteins 1A/1B light chain 3B (LC3B; cat. no. 4108, 1:1,000), rabbit anti-p62 (cat. no. 5114, 1:1,000), rabbit anti-cytokeratin 19 (CK19; cat. no. 13092, 1:1,000), and rabbit anti-cleaved PARP (cat. no. 9545, 1:1,000) (all from CST Biological Reagents Co., Ltd., Shanghai, China), rabbit anti-Bcl-2 (cat. no. ab59348, 1:1,000) or anti-GAPDH (CST Biological Reagents CO., Ltd., cat. no. 5174, 1:1,000) antibodies (1:2,000) overnight at 4˚C. Membranes were also incubated with anti-phosphorylated (p)-p38 MAPK (Thr180/Tyr182; cat. no. 9215, 1:1,000) and p-p44/42 MAPK (ERK1/2; cat. no. 41370, 1:1,000) (both from CST Biological Reagents Co., Ltd.) antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) at a 1:1,000 dilutions. Blots were stripped and re-probed with anti-p38 MAPK (cat. no. 9212l; Abcam, Cambridge, MA, USA) and anti-p44/42 MAPK (ERK1/2; cat. no. 4695, 1:1,000; CST Biological Reagents Co., Ltd.) antibodies at a 1:2,000 dilution. After three washes with

Tris-buffered saline containing 0.05% Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. GHPA002, 1:2,000; Xi'an Guanyu Bio-Tech Co., Ltd., Xi'an, China) for 1 h at room temperature. The membranes were washed a further three times for 5 min. Blots were visualized using the Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific, Inc.) followed by autoradiography. Grey value analysis was performed using Image Lab software (version 5.2.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). To ensure equal loading, membranes were probed with an anti-GAPDH antibody.

Statistical analysis. All experiments were repeated three times. Data are presented as the mean ± standard error and were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were statistically analyzed by one-way analysis of variance followed by Dunnett's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Pharmacological regulation of autophagic activity in WB-F344 cells. The present study detected the autophagic activity of WB-F344 cells following treatment with Chlo or Rapa for 24 h. Treatment with Rapa, which is known to induce autophagy, for 24 h promoted the ratio of LC3A/BII and LC3A/BI and the degradation of p62/sequestome 1, which is a selective substrate of autophagy. Conversely, treatment with the autophagy inhibitor Chlo significantly enhanced LC3A/BII and p62 expression (Fig. 1A and B). These results indicated that Rapa was able to enhance autophagy, whereas Chlo inhibited autophagy in WB-F344 cells.

Inhibition of autophagy decreases WB-F344 cell proliferation and induces WB-F344 cell apoptosis. Proliferation of WB-F344 cells was measured 24 h after Rapa or Chlo treatment. As presented in Fig. 2A and B, Chlo significantly decreased the proliferation of WB-F344 cells (P<0.05). The anti-proliferative effects of Chlo on WB-F344 cells were verified using the CCK-8 assay, which can be used to examine cell proliferation. When the cells were treated with 20-500 μmol/l Chlo for 24 h the proliferation of WB-F344 cells was inhibited in a time- and dose-dependent manner (Fig. 2B). However, Rapa, which induces autophagy, had no significant effect on viability (Fig. 2B). To determine whether cell cycle progression was affected by Rapa or Chlo treatment, a cell cycle analysis was conducted using flow cytometry. Rapa (0.4%±0.8) did not alter cell cycle progression, which is consistent with the findings of the CCK-8 assay. However, following treatment with 50 μM Chlo (2.8%±1.2), subG$_1$ cell cycle arrest was increased compare with the control group (0.5%±0.9, P<0.05; Fig. 2C). These findings indicated that Chlo induces apoptosis of WB-F344 cells. In order to confirm the apoptotic effects of Chlo, cellular apoptosis was determined by Annexin V-PI staining and flow cytometry. Flow cytometric analysis demonstrated that treatment with the autophagy inducer Rapa slightly reduced the apoptotic rate of WB-F344 cells (3.4%±0.6);

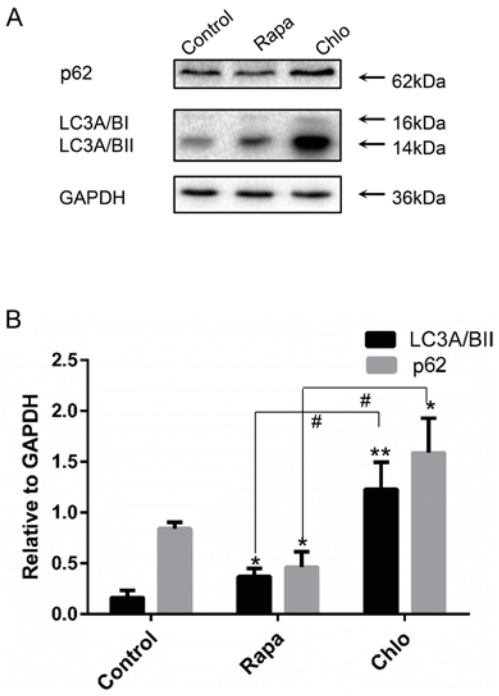

Figure 1. Effects of various drugs on autophagic activity of hepatic oval cells following 24 h incubation. (A) Cells were treated with various drugs for 24 h; p62 and LC3 were detected by western blotting. (B) Graphical presentation of the relative abundance of LC3-II and p62 following normalization with GAPDH. Results are presented as the mean ± standard error of the mean of four series of experiments. *P<0.05, **P<0.01 vs. the control group; #P<0.05 Rapa vs. Chlo group. Chlo, chloroquine; LC3, microtubule-associated protein 1A/1B-light chain 3; Rapa, rapamycin.

however, this was not significantly different compared with the control group. (5.4%±0.7; Fig. 2D). Following treatment with the autophagy inhibitor Chlo, the apoptotic rate of WB-F344 cells (16.6%±1.1) was significantly enhanced compared with the control group (P<0.05; 2D). In Fig. 2E and F, in the Rapa group, Rapa increased the expression of BCL-2 and decreased the expression of cleaved PARP (P<0.01). However, the results of chlo were the opposite of that of rapa (P<0.05). These results indicated that autophagy inhibits apoptosis, whereas inhibition of autophagy promotes apoptosis of WB-F344 cells. But in Fig. 2D. this was not significantly different compared with the control group. We believed that autophagy can reduce apoptosis, but autophagy can also promote cell death. Therefore, the apoptosis of Rapa group was not obvious compared with the control group.

Autophagy enhances the biliary differentiation of WB-F344 cells. Since autophagy can promote proliferation of WB-F344 cells, the present study aimed to determine whether autophagy exerts an effect on the differentiation of WB-F344 cells. Therefore, the effects of Rapa and Chlo were determined on the biliary differentiation of WB-F344 cells. WB-F344 cells were co-treated with sodium butyrate (SB) and Rapa or Chlo for 3 days. As presented in Fig. 3A, when WB-F344 cells were treated with SB, CK19 was slightly expressed in the cytoplasm; however, CK19 protein expression was significantly increased in the Rapa group compared with the control group (P<0.01). Furthermore, the results of the western blot analysis were consistent with the findings of immunofluorescence; upon

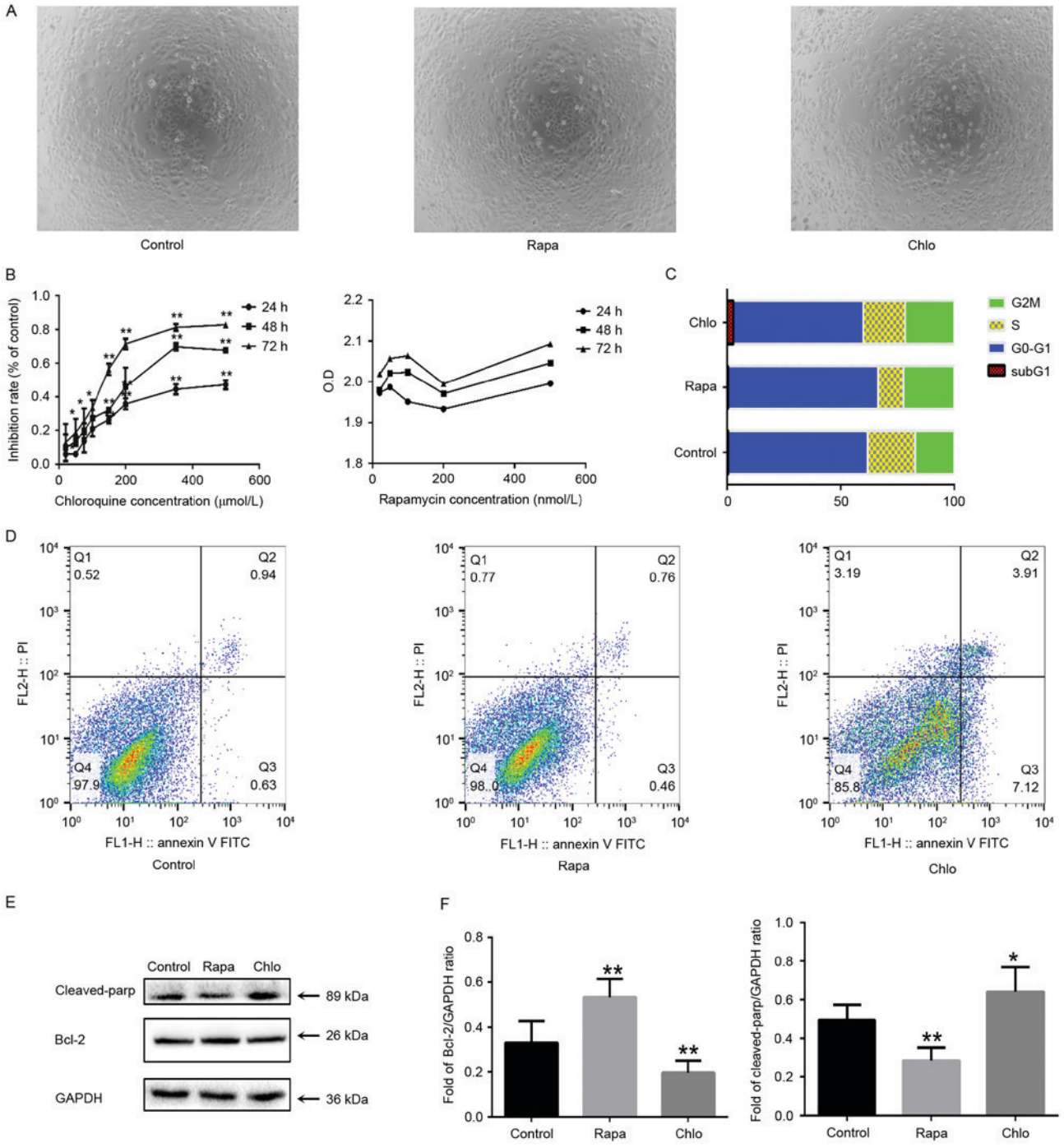

Figure 2. Chlo decreases the proliferation of WB-F344 cells and induces apoptosis. (A) Morphology of WB-F344 cells 24 h after Rapa or Chlo treatment (magnification, x40). (B) Chlo (20-500 μM) significantly decreased the proliferation of WB-F344 cells, as determined by Cell Counting Kit-8 assay. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group. (C) Cell cycle analysis indicated that 50 μM Chlo increased cell cycle arrest at SubG1, which may induce apoptosis. (D) WB-F344 cells were analyzed by Annexin V and PI staining followed by flow cytometry. (E and F) Western blot analysis of the expression levels of cleaved PARP and Bcl-2 in WB-F344 cells. *P<0.05, **P<0.01 vs. the control group. Bcl-2, B-cell lymphoma 2; Chlo, chloroquine; FITC, fluorescein isothiocyanate; OD, optical density; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; Rapa, rapamycin.

biliary differentiation, the expression of CK19 was increased in the Rapa-treated group compared with in the control group (P<0.01; Fig. 3B and C). These results indicated that activation of autophagy promotes the biliary differentiation of WB-F344 cells.

Effects of autophagy on the MAPK/ERK signaling pathway in WB-F344 cells. To determine the effects of MAPK/ERK signaling on autophagy-mediated enhancement of biliary differentiation of WB-F344 cells, western blotting was used to detect the expression levels of MAPK/ERK after modulation of autophagy. The results demonstrated that treatment with the autophagy inducer Rapa increased the phosphorylation of ERK1/2 and p38 (Fig. 4A) compared with in the control group. Semi-quantification of the blots indicated that the levels of p-ERK1/2 and p-p38 were increased ~2-fold

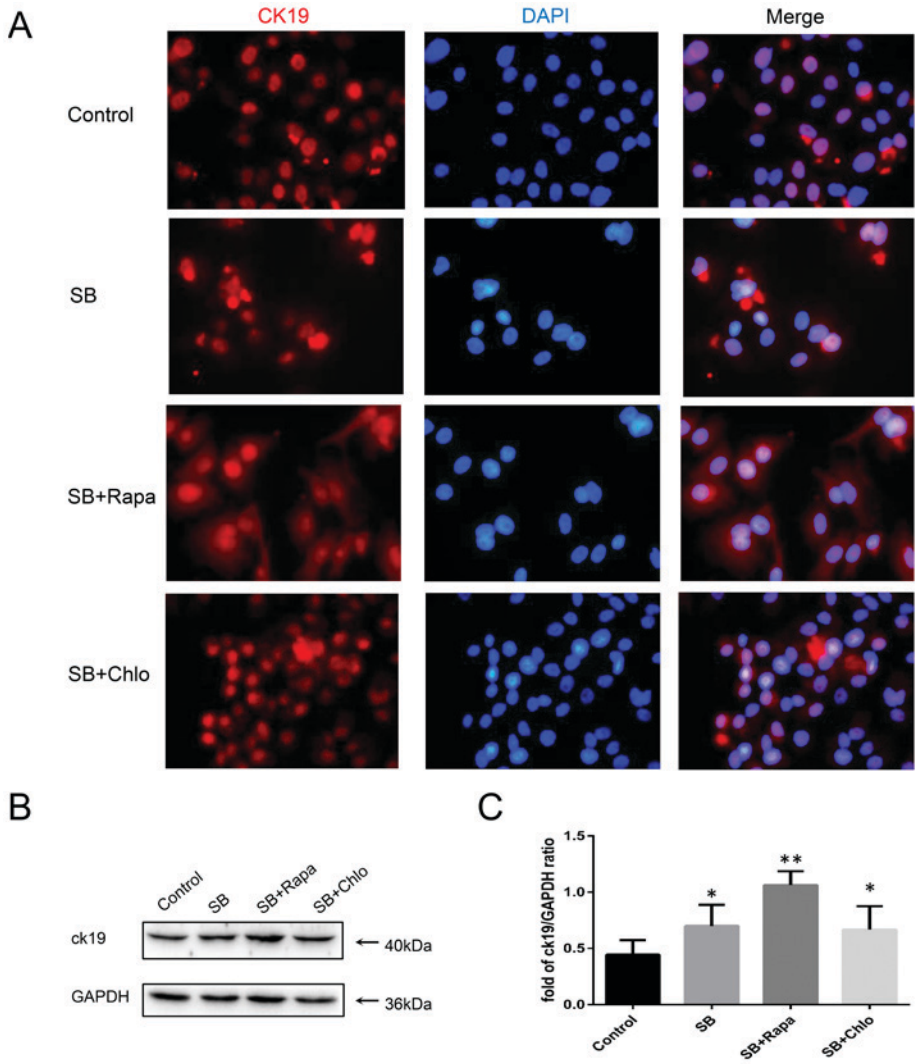

Figure 3. Effects of autophagy on differentiation of WB-F344 cells to bile duct epithelial cells *in vitro*. (A) Fluorescent immunocytochemistry of CK19 protein (red) and DAPI staining (blue) in WB-F344 cells (magnification, x200). (B) CK19 protein was detected by western blotting and was normalized to GAPDH. (C) Densitometric analysis of the protein bands (n=3/group). *P<0.05, **P<0.01 vs. the control group. Chlo, chloroquine; CK19, cytokeratin 19; Rapa, rapamycin; SB, sodium butyrate.

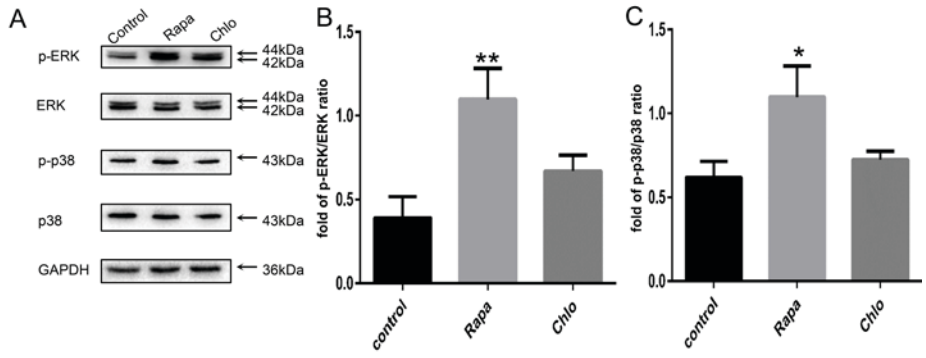

Figure 4. Effects of autophagy on the mitogen-activated protein kinase pathway in WB-F344 cells. Following incubation of WB-F344 cells with various drugs for 24 h, (A) protein expression levels of p-ERK1/2, ERK1/2, p-p38 and p38 were determined by western blotting. (B and C) Ratio of p-ERK/ERK and p-p38/p38 intensity. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 vs. the control group. Chlo, chloroquine; ERK, extracellular signal-regulated protein kinase; p, phosphorylated; Rapa, rapamycin.

following Rapa treatment compared with the control group (Fig. 4B and C). However, the expression levels of p-ERK1/2 and p-p38 were not significantly altered in the Chlo group. These results suggested that autophagy may promote differentiation of WB-F344 cells by upregulating the MAPK/ERK signaling pathway.

Discussion

Autophagy has been demonstrated to protect cells from stress conditions, including hypoxia, and to serve an important role in self-renewal, proliferation, differentiation and apoptosis of stem cells (13-16). In addition, autophagy can efficiently and rapidly downregulate enzymes, transcription factors, adhesion molecules and certain secreted proteins (17,18), which are important for stem cell proliferation and differentiation (19,20). Autophagy mediates the degradation of cellular components, including proteins and organelles, in lysosomes (21), and contributes to cellular homeostasis, quality control and maintenance of energetic balance, which are essential for cell proliferation (15). In epidermal stem cells, mesenchymal stem cells, hematopoietic stem cells and dermal stem cells, basal autophagy levels are higher (22-26). After the induction of differentiation, autophagic levels are decreased. Nevertheless, autophagy is increased during the differentiation of embryonic stem cells, neural stem cells and cardiac stem cells (27-32). It has also been reported that autophagy serves a crucial role in regulating the stemness of hepatic progenitor cells (HPCs) (33). High autophagic levels are detected in the majority of the stem cells when they differentiate into other cells, which is essential for the maintenance of cellular stemness and homeostasis. There are many studies of the role of pathways on autophagy (34,35). Therefore, the present study investigated the effects of autophagy on proliferation and differentiation of HOCs.

Stem cells undergo self-renewal and have the potential to differentiate into various cell populations. It has been established that a bipotential cell type, which has stem cell-like properties, exists in the adult liver and can differentiate into hepatic cells and cholangiocytes, these cells are referred to as HOCs or HPCs (36). HOCs are activated in reaction to various types of chronic liver disease (37).

It has been reported that autophagy regulates biliary differentiation of HPCs via the Notch1 signaling pathway (38). However, the role of autophagy in the proliferation, apoptosis and differentiation of WB-F344 cells remains to be elucidated. In the present study, Chlo was used to inhibit autophagy in WB-F344 cells, and was demonstrated to increase the $SubG_1$ population of cells and promote apoptosis, whereas treatment with Rapa exerted the opposite effects, thus suggesting that inhibition of autophagy inhibits WB-F344 cell proliferation. These results are consistent with previous studies, which reported that inhibition of autophagy suppresses proliferation and increases the population of apoptotic cells (39,40). Although autophagy and apoptosis are mutually inhibitory (41), in the present study, autophagy appeared to exert anti-apoptotic effects.

The present study also aimed to determine the effects of autophagy on HOC-cholangiocyte differentiation, the results confirmed that SB induced WB-F344 cell differentiation into a cholangiocyte phenotype and revealed that the expression of the biliary marker, CK19, was significantly increased in response to Rapa-induced autophagy. These findings suggested that autophagy may be involved in WB-F344 cell differentiation. Conversely, a previous study by Zeng *et al* (38) demonstrated that activation of autophagy may suppress the biliary differentiation of WB-F344 cells.

Further investigation suggested that Rapa-induced activation of autophagy may stimulate the MAPK/ERK signaling pathway. These results are consistent with those of previous studies, which indicated that activation of the MAPK/ERK pathway can be induced by Rapa (42,43), and may promote HOC proliferation (8). Therefore, the ERK signaling pathway may be considered an important regulator for the proliferation and differentiation of WB-F344 cells (8). These findings suggested that activation of MAPK/ERK signaling induced by autophagy is essential for cell differentiation.

HOCs are clinically significant, as they are potentially useful for cell and/or gene therapy for the treatment of metabolic liver diseases (37). However, the role of autophagy in WB-F344 cells involves alterations in signaling pathways, such as in Akt and Hedgehog, and the mechanisms are still highly heterogeneous (34,35). The present study, demonstrated that inhibition of autophagy can inhibit HOCs proliferation and autophagy promoting HOCs differentiation. In addition, MAPK/ERK pathway is likely to be involved. Future studies are required to explore how autophagy affects the MAPK/ERK pathway during liver regeneration, and to reveal the potential mechanisms underlying HOC proliferation and differentiation, thus providing subclinical strategies for HOC-mediated liver regeneration or the inhibition of HOC-mediated tumorigenesis.

Acknowledgements

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