Abstract. Sphingosine kinase 1 (SphK1) is a master kinase that catalyzes the synthesis of sphingosine 1 phosphate and participates in the regulation of cell proliferation and autophagy. The present study aimed to assess the effects of the activation of the SphK1/extracellular signal-regulated kinase (ERK)/phosphorylated (p-ERK) pathway in the regulation of autophagy in colon cancer (HT-29) cells. Inverted fluorescence microscopy was used to detect the expression of green fluorescent protein (GFP) in the SphK1-overexpressing HT-29 cells [SphK1(+)-HT-29] and the negative control HT-29 cells (NC-HT-29). Western blotting was used to detect the protein expression levels of SphK1, ERK1/2, p-ERK1/2, as well as those of the autophagy-associated markers LC3A, ATG5, and ULK1. Protein localization and expression of the LC3A antibody were detected by immunofluorescence. The results demonstrated that GFP was similarly expressed in SphK1(+)-HT-29 and NC-HT-29 cells. However, significantly increased SphK1 mRNA and protein expression levels were detected in SphK1(+)-HT-29 cells compared with in NC-HT-29 cells, which resulted in upregulated ERK/p-ERK. Furthermore, the protein expression levels of the three autophagy-associated markers increased. LC3A protein was localized in the cytoplasm of SphK1(+)-HT-29 cells, indicating autophagy. In summary, the findings of the present study suggested that activation of the SphK1/ERK/p-ERK pathway promotes autophagy in colon cancer HT-29 cells.

Introduction
Colon cancer is a type of malignant epithelial cell tumor, and presents a major health concern worldwide. The inhibition of cancer cell proliferation is an essential strategy in the treatment of colon cancer (1). However, the molecular mechanisms of colon cancer cell proliferation remain unresolved.

Autophagy is an evolutionarily conserved process in eukaryotes. During autophagy, a nascent double membrane-bound vesicle called an autophagosome encloses a portion of the cytoplasm and the outer membrane of autophagosomes then fuses with the vacuolar or lysosomal membrane to release the inner-membrane structures called autophagic bodies, into the vacuolar or lysosomal lumen for digestion (2). Autophagy serves an important role in the proliferation of colorectal cancer cells (3), and a number of studies have suggested that autophagy prevents metabolites from damaging cells and genomes (4,5). Conversely, other studies have suggested that autophagy contributes to the supply of nutrients and reused metabolites to tumor cells, therefore promoting their survival and proliferation (6,7). Although autophagy has been demonstrated to affect the proliferation of tumor cells, the regulatory mechanism underlying autophagy in colon cancer cells has not been fully investigated.

Sphingosine kinase-1 (SphK1), is an important enzyme that maintains the intracellular sphingolipid balance and has a role in the development of multiple malignancies, plays an important role in resistance to therapies, tumor growth, tumor neovascularization and metastatic spread (8,9). Recently, a study reported that SphK1 regulates LC3 expression and autophagy in neuroblastoma cells (10). Despite the involvement of SphK1 in autophagy, its specific role and associated regulatory mechanism in colon cancer cells remain unclear.

A number of studies have suggested that increased extracellular signal-regulated kinase (ERK) phosphorylation levels induce autophagy in cells (12,13), and that SphK1 promotes the proliferation of colon cancer cells through activation of the ERK/phosphorylated (p-)ERK cascade (14). In the present study, the hypothesis that the activation of the SphK1/ERK/p-ERK pathway promotes autophagy in HT-29...
cells was examined. In order to investigate this, the protein expression levels of SphK1, ERK1/2 and p-ERK1/2, and those of the autophagy-associated markers LC3A, ATG5, and ULK1, were analyzed following the upregulation of SphK1 in HT-29 cells. Additionally, the protein localization and expression patterns of intracellular LC3A, a key marker of autophagy, were assessed.

**Materials and methods**

**Cell lines and culture.** The human colorectal cancer cell line HT-29, Caco-2, RKO and HCT116 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Excell Bio, Inc., Shanghai, China) at 37°C with 5% CO₂.

**Cell transfection.** The Lentiviral vector PLenti-SPHK1-IRES-EGFP and the blank vector (NC; R&S Biotechnology Co., Ltd., Shanghai, China) were used for infection of cancer cells, the cells inoculated with lentivirus at a multiplicity of infection (MOI) of 20 for 48 h, and the percentage of infected cells was approximately 90% at this MOI. Blasticidins (2 µg/ml) (Merck KGaA, Darmstadt, Germany) was added for 2 weeks. The SphK1-overexpressing HT-29 cells [SphK1(+)-HT-29] and the corresponding negative control HT-29 cells (NC-HT-29) were detected by fluorescence-activated cell sorting. The stabilized transfected SphK1(+)-HT-29 and NC-HT-29 cells were stored in liquid nitrogen (Jinfeng liquid Nitrogen Container Co., Ltd., Chengdu, China) and were used within 3 months between transfection and subsequent experimentation. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS at 37°C with 5% CO₂.

**Inverted fluorescence microscopy analysis.** SphK1(+)-HT-29 and NC-HT-29 cells were seeded onto a 6-well plate and cells covered ~95% of each well. The cells were observed under an inverted fluorescence microscope (TS100-F; Nikon Corporation, Tokyo, Japan) at ×100 magnification. The NIS-Elements software (version 4.0; Nikon Corporation, Tokyo, Japan) was used for cell imaging, according to the manufacturer's protocol. The transfection efficiency of cells was calculated as follows: The number of cells in 3 randomly selected fields that expressed green fluorescent protein (GFP)/the total number of cells.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** RNA isolation was performed using the Total RNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. cDNA synthesis was performed using the Reverse Transcription Kit (Takara Bio, Inc., Otsu, Japan). A fluorescence-based qPCR method was performed using 2 µl cDNA, 10 µl SYBR Green (Takara Bio, Inc.), 0.6 µl PCR forward primer (10 µM), 0.6 µl PCR reverse primer (10 µM) and 6.8 µl dH₂O, in a 20 µl PCR reaction volume. The RT-qPCR reaction was run on a StepOne Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling parameters were as follows: Denaturing at 95°C for 30 sec, 40 cycles at denaturing at 95°C for 5 sec, primer annealing at 60°C for 34 sec, and extension temperature at 95°C for 15 sec; final extension at 60°C for 1 min and final denaturing at 95°C for 15 sec. Gene expression levels were determined via the 2⁻ΔΔCq method (15), using GAPDH as a reference gene, with the GAPDH gene expression level in NC-HT-29 cells set to 1. The primers of GAPDH and SphK1 were obtained from Takara Bio, Inc. (Otsu Japan). GAPDH, forward: 5'-GCACCGCAAGGCTGAGAAC-3', and reverse: 5'-TGGTGAAGCCGCATGGA-3'; SphK1, forward: 5'-GGCTTTATTGCCTGATGTTGAA-3', and reverse: 5'-AGGAGAAGGTGCCAACGAGTGA-3'.

**Western blotting analysis.** Total proteins were extracted using radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were measured by bicinchoninic acid assay (Solarbio Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. A total of 30 µg of protein from each sample was separated via 12% SDS-PAGE (Beyotime Institute of Biotechnology, Haimen, China) for 1 h at 100 V, and then transferred onto nitrocellulose membranes. Samples were blocked with 5% nonfat-milk in Tris-buffered saline with Tween-20 (Solarbio Biotech Co., Ltd., Beijing, China) for 1 h at room temperature. The membranes were incubated overnight at 4°C with antibodies diluted in WB Antibody Diluent (Beyotime Institute of Biotechnology, Haimen, China). Subsequently, the membranes and secondary antibodies were incubated for 1 h at room temperature. Bands were quantified by Odyssey infrared imaging (LICOR Biosciences, Lincoln, NE, USA) and GAPDH acted as an internal reference. Rabbit polyclonal anti-SphK1 (dilution 1:1,000; catalog no. A0139), mouse monoclonal anti-ERK1/2 (dilution 1:1,500; catalog no. A10613), rabbit polyclonal anti-p-ERK1/2 (dilution 1:1,500; catalog no. AP0472), mouse monoclonal anti-ATG5 (dilution 1:1,000; catalog no. A2468) and rabbit polyclonal anti-ULK1 (dilution 1:2,000; catalog no. A8529) were purchased from aBclonal, Inc. (Woburn, MA, USA). Rabbit monoclonal anti-LC3A (dilution 1:1,000, 4599) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit polyclonal anti-GAPDH (dilution 1:2,000; catalog no. 10494-1-AP) was purchased from ProteinTech Group (Rosemont, IL, USA). The secondary antibodies horseradish-peroxidase (HRP)-conjugated Goat Anti-Rabbit IgG (dilution 1:10,000; catalog no. AS014) and HRP-conjugated Goat Anti-Mouse IgG (dilution 1:10,000; catalog no. AS003) were purchased from aBclonal Inc.

**Immunofluorescence.** The Cell slide (Solarbio Biotech Co., Ltd., Beijing, China) was placed in 24-well plates and then cells were seeded at a density of 1x10⁵. Routinely cultured overnight at 37°C with 5% CO₂, the cells were washed with PBS, fixed in a 4% paraformaldehyde solution (Solarbio Biotech Co., Ltd., Beijing, China) for 20 min, permeabilized with 0.5% Triton X-100 for 10 min, sealed with 10% FBS diluted with 10% PBS (Excell Bio, Inc., Shanghai, China) for 20 min, and then incubated overnight at 4°C with rabbit polyclonal anti-LC3A (dilution 1:500; catalog no. 4599, CST, USA). Subsequently, the cells were incubated with Anti-Rabbit IgG Fab2 Alexa Flour®594 (dilution 1:500; catalog no. 8889S;
Cell Signaling Technology, Inc.) for 1 h at 37˚C. Cells were stained with DAPI (Beyotime Institute of Biotechnology) for 1 min, and then covered with anti-fluorescent quenching fluid (Beyotime Institute of Biotechnology). An Olympus BX53 (Olympus Corporation, Tokyo, Japan) polarizing microscope was used to observe the cells under x600 magnification and obtain images for further analysis.

Statistical analysis. Each immunofluorescence assay was performed a minimum of three times. Statistical analysis was based on the unpaired Student's t test or the one-way analysis of variance test using SPSS v.16.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

SphK1 expression is upregulated in transfected HT-29 cells. In a previous study, the relative mRNA expression of SphK1, when compared with that of the reference gene GAPDH, was 0.96±0.02 in Caco2 (colon adenocarcinoma) cells, 0.61±0.07 in HT-29 cells, 0.92±0.05 in RKO (colon carcinoma) cells and 0.97±0.02 in HCT116 cells (16). Therefore, the lowest expression of SphK1 occurred in the HT-29 cell line. To avoid cell autophagy caused by chemical stress, a pLenti-SPHK1-IRES-EGFP vector and a blank vector (NC) were used to transform HT-29 cells in order to obtain an increase in SphK1 expression. The SphK1(+)‑HT-29 and NC‑HT-29 cells expressed GFP (Fig. 1A) with a transfection efficiency of 92% in NC‑HT-29 cells and 95% in SphK1(+)‑HT-29 cells. RT-qPCR results, using GAPDH as a reference gene and the SphK1 expression level of NC-HT-29 cells set to 1, demonstrated that the relative expression of SphK1 in SphK1(+)‑HT-29 cells was significantly increased (Fig. 1B). These results also indicated that SphK1(+)‑HT-29 and NC-HT-29 cells were suitable for the subsequent experiments.

The SphK1/ERK/p-ERK pathway is activated in HT-29 cells. The protein expression levels of SphK1, total ERK1/2 and p-ERK, as detected by western blotting, are illustrated in Fig. 2. SphK1 and p-ERK protein expression was increased in SphK1(+)-HT-29 cells, compared with in NC-HT-29 cells, while there were no significant differences in total ERK1/2 expression. These results suggest that SphK1 activates ERK by phosphorylation.

Autophagy in HT-29 cells is induced by activation of the SphK1/ERK/p-ERK pathway. LC3A protein is a recognized marker for autophagy (17-19), which, upon staining, presents a spotted aggregation pattern under fluorescence microscopy (20,21). As shown in Fig. 3, the protein expression of LC3A appeared as a spotted aggregation in the cytoplasm of SphK1(+)-HT-29 cells, but not in NC-HT-29 cells, suggesting that SphK1 promotes autophagy in HT-29 cells. ATG5 and ULK1 proteins are other important autophagy-associated markers (21-23). In the present study, the protein expression of LC3A, ATG5 and ULK1 was increased in SphK1(+)-HT-29 cells when compared with in NC-HT-29 cells (Fig. 4). Previous studies have reported that increased expression of p-ERK decreases the levels of mTOR/p-mTOR, which then results in increased expression of ULK1 (24,25). When analyzed together, these results suggested that autophagy in HT-29 cells is induced via activation of the SphK1/ERK/p-ERK pathway.

Discussion

Increasing evidence suggests that the sphingosine kinase-1 (SphK1) serves an important role in the development of cancer, including cell proliferation, apoptosis, metastasis, angiogenesis and chemotherapeutic resistance (9). It has also been reported that SphK1 promotes the proliferation and metastasis of colon cancer (14). SphK1 is involved in the regulation of sphingolipid metabolism via the production of sphingosine-1 (SIP) (26,27). SphK1/SIP regulates cell proliferation through multiple pathways including the ERK, P38 mitogen-activated
protein kinase (MAPK) and Akt pathways (28). In the present study, SphK1 expression upregulated ERK phosphorylation, as previously hypothesized. This is consistent with the results of a previous study, which reported that the activation ERK is elevated by the upregulation of SphK1 and attenuated by the suppression of SphK1, while the blocking of the ERK pathway suppressed the effects that are mediated by the overexpression of SphK1 (14). These results suggest that SphK1 modulates the ERK/p-ERK pathway.

ERK is an important component of the MAPK system, which is one of the most important signaling cascades, and has been identified as frequently dysregulated in tumors (29). Additionally, an increase in the expression of ERK and p-ERK led to a decrease in the levels of mTOR and p-mTOR (24). mTOR is an inhibitor of ULK1 (25), which induces the initiation of autophagy (23). Similarly, in the present study, increased levels of p-ERK were observed to promote an increase in ULK1 protein expression. Furthermore, it has been reported that ERK and its upstream kinase MEK are localized in the extra-luminal face of the autophagosomes, and that ERK interacts with autophagy proteins via its substrate-binding domains (20). Overall,
these results suggest that increasing ERK phosphorylation induces cell autophagy (12,13).

Autophagy is a degradation system that supplies cytoplasmic components into the lysosome or vacuole, where the degradation of lipid droplets is known to occur (30). Autophagy eliminates incorrectly translated proteins, metabolic waste and toxic oxygen free-radicals in cancer cells in order to achieve self-renewal and to promote the growth and development of cells (31). Cell autophagy begins with the formation of a bilayer structure termed an autophagosome. Subsequently, two ubiquitination systems are activated, including the ATG8/LC3 phosphatidylethanolamine conjugate system and the ATG12-ATG5 conjugate system (22). LC3 and ATG5 proteins are, consequently, regarded as the principal autophagy-associated proteins. Ubiquitination systems are widely known to be involved in various physiological processes, including cell proliferation, apoptosis and autophagy (32). In the present study, the LC3 protein formed a spotted aggregation pattern in SphK1(+)-HT-29 cells under fluorescent microscopy, which suggested autophagy; this was in accordance with the results of previous studies (20,21). Furthermore, the results of the present study demonstrated that the protein expression of LC3A, ATG5 and ULK1 were increased in the SphK1-upregulated HT-29 cells. These results suggested that SphK1 regulates the expression of LC3 and promotes the autophagy process in colon cancer cells, which is consistent with SphK1 induced autophagy in neuroblastoma and breast cancer cells (13,14).

In summary, the present study demonstrated that activation of the SphK1/ERK/p-ERK pathway promotes autophagy in colon cancer HT-29 cells. An increase in SphK1 lead to an upregulation of ERK/p-ERK by increasing ERK phosphorylation, which in turn resulted in an increase in the expression level of the autophagy-associated markers LC3, ATG5 and ULK1 in SphK1(+)-HT-29 cells. These findings provide a rationale for the development of SphK1 inhibitors, or other cell autophagy inhibitors, as part of a therapeutic strategy for patients with colorectal cancer or other epithelial tumor types. Furthermore, in order to further investigate the role of autophagy in colorectal cancer cells, gene regulation of ERK expression or change cells culture conditions is need in future studies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

CYX and WLZ conceived and designed the study and conducted the experiments. CYX assisted with drafting the manuscript. WHW and MBQ performed the statistical analysis. JAH and SQL interpreted the statistical analysis, reviewed and made final approval of the version to be published. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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