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ORIGINAL ARTICLE

Basic Study

Loss of monopolar spindle-binding protein 3B expression promotes colorectal cancer invasiveness by activation of target of rapamycin kinase/autophagy signaling

Juan Sun, Jin-Xiu Zhang, Meng-Shi Li, Meng-Bin Qin, Ruo-Xi Cheng, Qing-Ru Wu, Qiu-Ling Chen, Dan Yang, Cun Liao, Shi-Quan Liu, Jie-An Huang

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Abstract

BACKGROUND

Monopolar spindle-binding protein 3B (MOB3B) functions as a signal transducer and altered MOB3B expression is associated with the development of human cancers.

AIM

To investigate the role of MOB3B in colorectal cancer (CRC).

METHODS

This study collected 102 CRC tissue samples for immunohistochemical detection of MOB3B expression for association with CRC prognosis. After overexpression and knockdown of MOB3B expression were induced in CRC cell lines, changes in cell viability, migration, invasion, and gene expression were assayed. Tumor cell autophagy was detected using transmission electron microscopy, while nude mouse xenograft experiments were performed to confirm the *in-vitro* results.

RESULTS

MOB3B expression was reduced in CRC *vs* normal tissues and loss of MOB3B expression was associated with poor CRC prognosis. Overexpression of MOB3B

protein *in vitro* attenuated the cell viability as well as the migration and invasion capacities of CRC cells, whereas knockdown of MOB3B expression had the opposite effects in CRC cells. At the molecular level, microtubule-associated protein light chain 3 II/I expression was elevated, whereas the expression of matrix metalloproteinase (MMP)2, MMP9, sequestosome 1, and phosphorylated mechanistic target of rapamycin kinase (mTOR) was downregulated in MOB3B-overexpressing RKO cells. In contrast, the opposite results were observed in tumor cells with MOB3B knockdown. The nude mouse data confirmed these *in-vitro* findings, *i.e.*, MOB3B expression suppressed CRC cell xenograft growth, whereas knockdown of MOB3B expression promoted the growth of CRC cell xenografts.

CONCLUSION

Loss of MOB3B expression promotes CRC development and malignant behaviors, suggesting a potential tumor suppressive role of MOB3B in CRC by inhibition of mTOR/autophagy signaling.

Key Words: Colorectal cancer; Monopolar spindle-binding protein 3B; Mechanistic target of rapamycin kinase; Autophagy; Prognosis

Core Tip: Monopolar spindle-binding protein 3B (MOB3B) functions as a signal transducer and altered MOB3B expression is associated with the development of human cancers. In this study, we investigated the effects of MOB3B in colorectal cancer (CRC). We found that loss of MOB3B expression promoted CRC development and malignant behaviors, suggesting a potential tumor suppressive role of MOB3B in CRC by inhibition of mechanistic target of rapamycin kinase/autophagy signaling.

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INTRODUCTION

To date, colorectal cancer (CRC) still accounts for approximately 10% of all annually diagnosed human cancers and cancer-related deaths globally[1]. CRC is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths, with an estimated 1.9 million new cases and 935000 deaths in 2020 worldwide[1]. Molecularly, CRC is a heterogeneous disease, and the majority of CRC cases are "sporadic" cancers developed following the conventional adenoma-to-carcinoma sequence[2]. CRC research and scientific breakthroughs have significantly reduced CRC mortality due to improvement of early cancer diagnosis, prevention, and advanced treatment strategies[3,4]; however, a major cause of CRC deaths is cancer metastasis[5]. Thus, further research on the identification of potentially novel therapeutic targets could help medical oncologists to control CRC metastasis and reduce CRC mortality.

Accumulated evidence has revealed that aberrant autophagy is closely related to cancer metastasis[6,7]. Autophagy *per se* is an evolutionarily conserved proteolytic process that regulates the lysosomal degradation and recycling of any damaged cellular components to save energy and maintain cellular homeostasis[8,9]. At the cellular level, autophagy includes three main types, *i.e.*, macroautophagy, microautophagy, and chaperone-mediated autophagy[10]. However, dysregulated autophagy can result in the development of various human diseases, such as neurodegenerative disorders [11] and cancer[12]. In human cancer, autophagy is a double-edged sword as it can both promote and inhibit tumorigenesis; for example, an increase in cell autophagy might inhibit cancer development due to its ability to remove the damaged organelles in transformed cells, thereby protecting cells from oxidative stress and preventing malignant cell transformation[13]. In contrast, an altered autophagy level might support tumor progression by enhancing tumor cell survival and inhibiting tumor cell apoptosis, thus promoting chemoresistance and epithelial-mesenchymal transition-induced cancer metastasis[14,15]. Furthermore, mammalian target of rapamycin (mTOR), a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases, is considered one of the key regulators of autophagy as the two distinct complexes mTORC1 and mTORC2 form the catalytic core that regulates various cellular processes, including gene transcription, protein synthesis, and autophagy, which change the rates of cell growth, motility, survival, and apoptosis[16-19]. Previous studies have shown that targeting mTOR signaling can be effectively used to control and treat primary and metastatic CRCs[20,21]. In addition, the monopolar spindle-binding protein (MOB) family of proteins regulate cell signal transducers *via* important intracellular pathways[22] to regulate the cell cycle[23], DNA damage repair[23,24], apoptosis, and autophagy[25]. MOB3B is a member of the MOB family[26], and knockdown of MOB3B expression has been demonstrated to reduce the viability of breast cancer cells[27]. Moreover, MOB3B expression

and activation in cancer cells have conferred resistance to vemurafenib[28] and to epidermal growth factor receptor tyrosine kinase inhibitors[29]. Furthermore, MOB3B has been suggested as a candidate methylation marker for prostate cancer[30].

In this study, we assessed the role of MOB3B and underlying molecular events in CRC development and progression by first detecting MOB3B expression in CRC tissue specimens for association with CRC prognosis. Subsequently, we assessed the changes in CRC cell viability, migration, invasion, and gene expression in CRC cells with MOB3B overexpression and knockdown as well as in CRC cell xenografts grown in nude mice. In particular, the role of MOB3B in regulating mTOR/autophagy signaling in CRC cells was determined. The results of this study are expected to provide novel and useful information regarding the loss of MOB3B expression in CRC development and progression.

MATERIALS AND METHODS

Patients and samples

In this study, we collected 102 CRC tissue specimens and 60 distant normal colonic mucosal specimens (more than 10 cm away from the CRC lesions) from CRC patients at the First Affiliated Hospital, Guangxi Medical University (Nanning, China) between April 2013 and July 2017. These patients were diagnosed with CRC, and their tumor stages were classified according to the 8th edition of the American Joint Committee on Cancer staging manual[31]. In addition, these patients had not received radiotherapy, chemotherapy, or biological immunotherapy before surgical resection of their tumor lesions. Their clinicopathological and follow-up data were retrieved from their medical records. This study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (Approval No. 2022-E335-01) and was conducted in accordance with the guidelines of the Declaration of Helsinki. All patients signed a written informed consent form before enrollment into this study.

Antibodies and major reagents

Polyclonal rabbit anti-GAPDH (Cat. #AC001) and anti-phospho-mTOR-S2448 (Cat. #AP0115) antibodies were purchased from Abclonal Technology (Wuhan, China), while monoclonal rabbit anti-light chain (LC)3B(D11) (Cat. #3868) and mouse anti-sequestosome 1 [SQSTM1/p62(D5L7G)] (Cat. #88588) antibodies were obtained from Cell Signaling Technology (Boston, MA, United States). Polyclonal rabbit anti-matrix metalloproteinase (MMP)2 (Cat. #10373-2-AP) and anti-MMP9 (Cat. #10375-2-AP) antibodies were from Proteintech (Wuhan, China).

Furthermore, Dulbecco's modified Eagle's medium (DMEM), puromycin, and protein phosphatase inhibitor were purchased from Meilunbio (Dalian, China); fetal bovine serum (FBS) was from VivaCell (Shanghai, China); and serum-free freeze drying solution, cell counting kit-8 (CCK8), and 0.25% EDTA-trypsin were obtained from NCM-Biotech (Shanghai, China). Phosphate-buffered saline (PBS) was purchased from Servicebio (Wuhan, China), and radioimmunoprecipitation assay (RIPA) buffer, phenylmethane sulfonyl fluoride (PMSF), Tris-based saline, glycine, sodium dodecyl-sulfate (SDS), and 0.1% crystal violet/ammonium oxalate solution were all from Solarbio Biotech (Beijing, China). Antibody dilution buffer, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer, and enhanced bicinchoninic acid (BCA) protein assay kit were purchased from Beyotime (Shanghai, China). Polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, MA, United States), and the Splink Detection kits (Cat. #SP-9000) were purchased from OriGene Technologies (Beijing, China). MHY1485 (Cat. #HY-B0795) and rapamycin (Cat. #HY-10219) were obtained from MedChemExpress (Shanghai, China).

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue blocks were retrieved from our Pathology Department and prepared into 4-μm-thick sections. For immunohistochemistry, these tissue sections were first deparaffinized, rehydrated, and then subjected to antigen retrieval by boiling in a pressure cooker with EDTA-antigen retrieval solution (pH = 8.0) for 10 min. After that, the sections were blocked in 3% hydrogen peroxide for 30 min at room temperature to remove any potential endogenous peroxidase activity and then in normal goat serum for 30 min to block any nonspecific binding. Next, the sections were incubated with a polyclonal anti-MOB3B antibody at a dilution of 1:400 at 4 °C overnight. On the following day, these sections were washed with PBS three times and incubated with biotin-labeled goat anti-rabbit immunoglobulin G polymer, horseradish peroxidase-labeled streptomycin-working solution, and 3,3'-diaminobenzidine chromogenic solution, in succession. Subsequently, the sections were counterstained with hematoxylin, dehydrated with a gradient alcohol series, and sealed with neutral resin and a coverslip. These immunostained sections were then reviewed and scored under a light microscope (Olympus, Tokyo, Japan). The immunoreactivity scoring of the data was carried out semi-quantitatively according to a method reported previously[32] using the following formula: Score = staining intensity × percentage of positive cells.

Cell culture and treatment

The human RKO cell line and DLD1 cell line were originally obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator containing 50 mL/L CO_2 at 37 °C.

Establishment of CRC cell sublines with stable MOB3B overexpression or knockdown

Lentivirus carrying MOB3B siRNA (LV-MOB3B-RNAi) or the negative control (CON077) as well as lentivirus carrying

MOB3B cDNA (LV-MOB3B) or vector-only (as a negative control; CON238) were purchased from Genechem (Shanghai, China). For cell infection, RKO and DLD1 cells were grown and infected with these lentiviruses at a multiplicity of infection of 10, according to the manufacturer's instructions, and then selected in DMEM containing puromycin for 7 d. The individual cell colonies were picked, expanded in new cell culture plates, and reselected in DMEM containing puromycin. The colonies were named "RKO-OE" and "RKO-NC+" for MOB3B-overexpressing RKO cells and negative control RKO cells, respectively, or "RKO-KD" and "RKO-NC-" for RKO cells with MOB3B knockdown and negative control RKO cells, respectively. Similarly, the colonies were named "DLD1-OE" and "DLD1-NC+" for MOB3B-overexpressing DLD1 cells and negative control DLD1 cells, respectively, or "DLD1-KD" and "DLD1-NC-" for DLD1 cells with MOB3B knockdown and negative control DLD1 cells, respectively.

Quantitative reverse transcription-polymerase chain reaction

Total cellular RNA was isolated from RKO and DLD1 cells using an Eastep™ Super Total RNA Extraction Kit (Promega, Madison, WI, United States) and reversely transcribed into cDNA using a Reverse Transcription Kit (Promega). The resultant cDNA samples were subjected to qPCR amplification using the Power SYBR Green Master Mix (Promega) on a StepOnePlus™ Real-Time PCR System. The qPCR conditions were set to an initial denaturation step at 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer sequences of the genes were synthesized by Sangon (Shanghai, China; see Table 1). The relative level of each mRNA was quantitated using the $2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$ method and compared to that of the housekeeping gene *GAPDH*.

Western blot analysis

Total cellular protein was isolated from cells using RIPA buffer containing 1% phosphatase inhibitor and 1% PMSF and then quantified using a BCA protein assay kit. Equal amounts (30-40 μg/Lane) of these protein samples were separated on 10% or 15% SDS-PAGE gels and transferred onto 0.22-μm PVDF membranes. Next, these membranes were subjected to Western blot analysis, and the protein levels were quantified based on the band density of GAPDH protein, according to our previous study[33]. The antibodies against the following proteins were used at different dilutions, *i.e.*, GAPDH (1:10000), p-mTOR-S2448 (1:1000), LC3B(D11) (1:1000), SQSTM1/p62(D5L7G) (1:1000), MMP2 (1:800), and MMP9 (1:800).

Cell proliferation assay

A CCK8 assay was performed to assess the changes in cell viability and proliferation rates. Stable cell sublines were inoculated into 96-well cell culture plates at a density of 8000 cells per well, grown for up to 72 h, and subjected to incubation with the reagents in the CCK8 kit, according to the manufacturer's instructions. All experiments were performed in triplicate and independently repeated three times.

Transwell and wound healing assays

Transwell assays were performed to assess the change in cell migration/invasion capacity using 24-well inserts with 8.0-μm polycarbonate filter chambers (Corning Costar, Corning, NY, United States). In brief, serum-starved cells (6×10^4 cells in 100 μL/well) were added into the upper chamber, while 600 μL of DMEM containing 20% FBS was added into the lower chamber, and the cells were incubated for 48 h at 37 °C in a humidified incubator containing 50 mL/L CO_2. At the end of the experiment, the non-invading or migrating cells on surface of the upper chamber were gently wiped away with a cotton swab, and the cells that migrated or invaded into the lower chamber were fixed in 0.4% paraformaldehyde for 30 min at room temperature and then stained with 0.1% crystal violet solution for 30 min. After washing in tap water and air-drying, the cells were photographed in five randomly selected fields at a magnification of 200 × under an inverted microscope (Life Technologies, United States). The data were quantified using ImageJ software. For the Transwell cell invasion assay, the chamber membranes were precoated with Matrigel (Corning Costar).

For cell wound healing assay, the cells were inoculated into a 6-well plate and three horizontal lines were drawn 1 cm apart on the bottom of the plate using a sterile ruler. The cells were then grown to approximately 95% confluency and wounded by scratching with a sterile 10-μL pipette tip along the sterile ruler perpendicular to the bottom horizontal line. The cells were washed with PBS three times and further cultured in serum-free DMEM for 48 h. Four fixed microscopic fields were randomly selected to be photographed at 0 h and 48 h. The wound-healing area was measured with ImageJ software. All experiments were performed in triplicate and independently repeated three times.

Transmission electron microscopy

RKO, RKO-NC-, RKO-KD, RKO-NC+, and RKO-OE cells were grown overnight, washed with ice-old PBS, fixed with 2.5% glutaraldehyde, treated with 1% osmium tetroxide at 4 °C for 4 h, dehydrated in a graded series of acetone (30% to 100%), embedded in EMbed812, and baked at 60 °C for 24 h. Ultra-thin cell sections were prepared using an ultramicrotome, stained with uranium acetate for 10-15 min and then lead citrate for 2 min, and reviewed by transmission electron microscopy (TEM), with the images being captured.

Nude mouse CRC cell xenograft assay

This animal study was approved by the Institutional Animal Care and Use Committee of Guangxi Medical University (Protocol No. 202108006) and performed in accordance with the Guidelines of the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research. In particular, 20 male BALB/c nude mice, aged 5-6 wk, were purchased from the Guangxi Medical University Laboratory Animal Center (Nanning, China) and randomly assigned into four groups (n = 5 per group); they were subcutaneously injected with RKO-NC-, RKO-KD, RKO-NC+, and RKO-OE cells at a density of 5×10^6 in 200 μL, respectively. The mice were housed in a specific pathogen-free animal room at a

Table 1 Primer sequences for polymerase chain reaction

Gene	DNA sequence
MOB3B	5′-CGGTGGCAGGATGATCTCAAGTATAAG-3′
	5′-CGTTGTTGATCTGAACCTCAATCCAATC-3′
GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′
	5′-TGGTGAAGACGCCAGTGGA-3′

MOB3B: Monopolar spindle-binding protein 3B.

temperature of 22-25 °C with *ad libitum* access to water and food. The xenograft formation and growth were assessed daily for 20 d, and the xenograft size was measured for the length (L) and width (W) every 2 d so that the tumor volume could be calculated according to the following formula: Tumor volume = (L × W^2)/2. At the end of the 20-d experiment, the mice were sacrificed, and the tumor cell xenografts were resected and weighed.

Statistical analysis

All data are summarized as the mean ± SD and were statistically analyzed using the SPSS 26.0 software package (SPSS, Chicago, IL, United States) or GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, United States). The associations of MOB3B expression with different clinicopathological data from CRC patients were analyzed using the χ^2 test, while the association of MOB3B expression with the overall survival of patients was analyzed using Kaplan-Meier curves and the log-rank test. The unpaired Student's *t*-test and analysis of variance (ANOVA) test were performed to determine the statistical significance between two groups and among more than two groups, respectively. The data from the ANOVA test were further analyzed using the *post hoc* Tukey's honestly significant difference test. A P value < 0.05 was considered statistically significant.

RESULTS

Downregulated MOB3B expression is associated with a poor CRC prognosis

In this study, we performed immunohistochemistry to assess MOB3B protein expression in 102 cases of CRC tissues and 60 cases of distant normal tissues and confirmed lower MOB3B expression in the CRC tissues than in the normal tissues (P = 0.002; Figure 1A and Table 2). The loss of MOB3B protein expression was associated with CRC lymph node metastasis, distant metastasis, and advanced tumor-node-metastasis stage (P < 0.001; Table 3); however, it was not associated with age, sex, or tumor size (P > 0.05; Table 3). Furthermore, the loss of MOB3B protein expression in CRC tissues was associated with a short patient survival (P = 0.0005; Figure 1B).

Effects of MOB3B expression on CRC cell proliferation, migration, and invasion in vitro

Next, we assessed the role of MOB3B in regulating CRC cell proliferation, migration, and invasion *in vitro* by overexpressing and knocking down MOB3B in CRC cells (Figure 2). Our data showed that MOB3B overexpression significantly alleviated cell viability (Figure 3A and B), whereas knockdown of MOB3B enhanced tumor cell proliferation (Figure 3C and D). Our tumor cell Transwell assay data also revealed that MOB3B overexpression significantly reduced CRC cell migration and invasion (Figure 3E and G) and that knockdown of MOB3B expression had the opposite effects on CRC cells (Figure 3F and H).

MOB3B suppresses CRC cell xenograft growth in nude mice

To confirm our *ex-vivo* and *in-vitro* data, we performed a nude mouse CRC cell xenograft assay and found that the tumor cell xenograft size and weight were significantly reduced in the RKO-OE xenograft mice compared to the RKO-NC+ xenograft mice (Figure 4A-C). In contrast, the tumor size and weight were significantly greater in the RKO-KD xenograft mice than in the RKO-NC- xenograft mice (Figure 4D-F).

MOB3B induces mTOR-dependent autophagy in vitro

To explore the underlying molecular events of the antitumor activity of MOB3B, we first assessed the levels of autophagy-related proteins in CRC cells with MOB3B overexpression or knockdown and found that LC3B II/I expression was significantly induced, whereas the expression of the autophagic substrate SQSTM1/p62 was decreased in the MOB3B-overexpressing cells *vs* those of control cells (Figure 5A and B). In contrast, the opposite effects occurred in cells with MOB3B knockdown (Figure 5C and D). Moreover, we found that MOB3B expression was able to suppress the p-mTOR level (Figure 5A and B), whereas knockdown of MOB3B expression enhanced the p-mTOR level in CRC cells (Figure 5C and D). Interestingly, the expression of MMP2 and MMP9 decreased in the RKO-OE group compared with the RKO-NC+ group (Figure 5A and B), while the expression of MMP2 and MMP9 increased in the RKO-KD group compared with the RKO-NC- group (Figure 5C and D). Indeed, our TEM data showed that MOB3B overexpression

Table 2 Downregulation of monopolar spindle-binding protein 3B expression in colorectal cancer *vs* normal tissues, *n* (%)

MOB3B protein	CRC, *n* = 102	Normal tissues, *n* = 60	*P* value
Negative (-)	53 (52.0)	16 (26.7)	0.002[a]
Positive (+)	49 (48.0)	44 (73.3)	

[a]$P < 0.05$, χ^2 test.
MOB3B: Monopolar spindle-binding protein 3B; CRC: Colorectal cancer.

Table 3 Association of monopolar spindle-binding protein 3B expression with clinicopathological characteristics of colorectal cancer patients

Clinicopathological characteristic	MOB3B		*P* value
	+	-	
Sex			0.12
Male	28	38	
Female	21	15	
Age (years)			0.53
≥ 60	21	26	
< 60	28	27	
Tumor size (cm)			0.35
≥ 5.0	27	34	
< 5.0	22	19	
Lymph node metastasis			< 0.001[a]
N0	41	22	
N1 + N2	8	31	
Distant metastasis			0.001[a]
M0	48	40	
M1	1	13	
Tumor-node-metastasis stage			< 0.001[a]
I + II	41	18	
III + IV	8	35	

[a]$P < 0.05$, χ^2 test.
MOB3B: Monopolar spindle-binding protein 3B.

increased CRC cell autophagic flux (Figure 6A and B), whereas knockdown of MOB3B decreased CRC cell autophagic flux (Figure 6C and D).

MOB3B inhibits CRC cell malignant behaviors by regulating mTOR/autophagy signaling

To verify our above experimental findings, we treated the cells with an mTOR agonist (MHY1485) or inhibitor (rapamycin) to find out whether MOB3B affects CRC malignant behaviors through mTOR-dependent autophagy. After treating RKO-OE cells with the mTOR agonist, LC3B II/I expression was downregulated, whereas the expression of p-mTOR, SQSTM1/p62, and MMP9 was either restored or upregulated (Figure 7A and B). In addition, our TEM data confirmed suppression of autophagic flux (Figure 6A and B). Moreover, after RKO-KD cells were treated with the mTOR inhibitor, their migratory and invasive abilities were significantly inhibited compared to those of control cells (Figure 7C-F), and LC3B II/I expression was upregulated or restored; however, the expression of p-mTOR, QSTM1/p62, and MMP9 was restored or downregulated (Figure 7G and H). Our TEM data revealed a significant increase in the autophagic flux in CRC cells (Figure 6C and D).

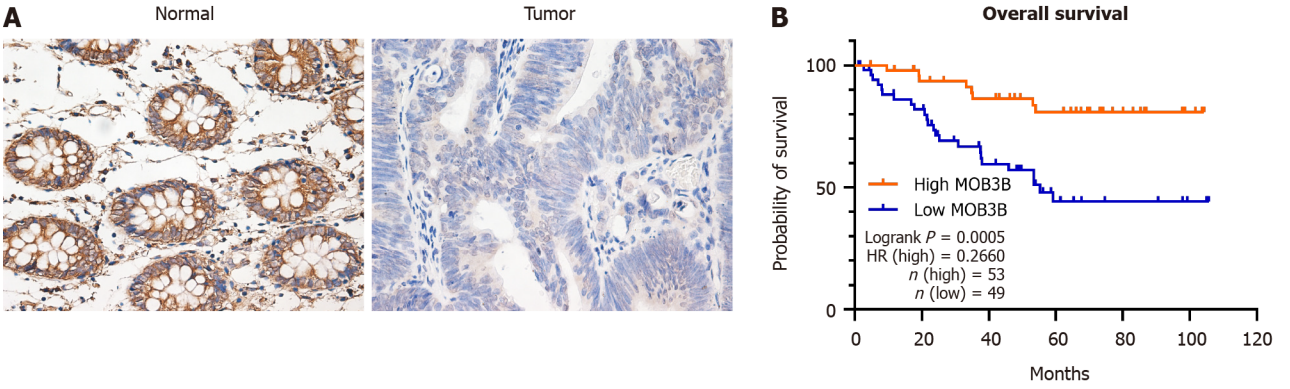


Figure 1 Monopolar spindle-binding protein 3B downregulation is associated with a poor colorectal cancer prognosis. A: Immunohistochemistry images. Monopolar spindle-binding protein 3B (MOB3B) protein was detected in colorectal cancer and distant normal tissues (magnification, 400 ×); B: Kaplan-Meier curves stratified by MOB3B expression and the log-rank test.

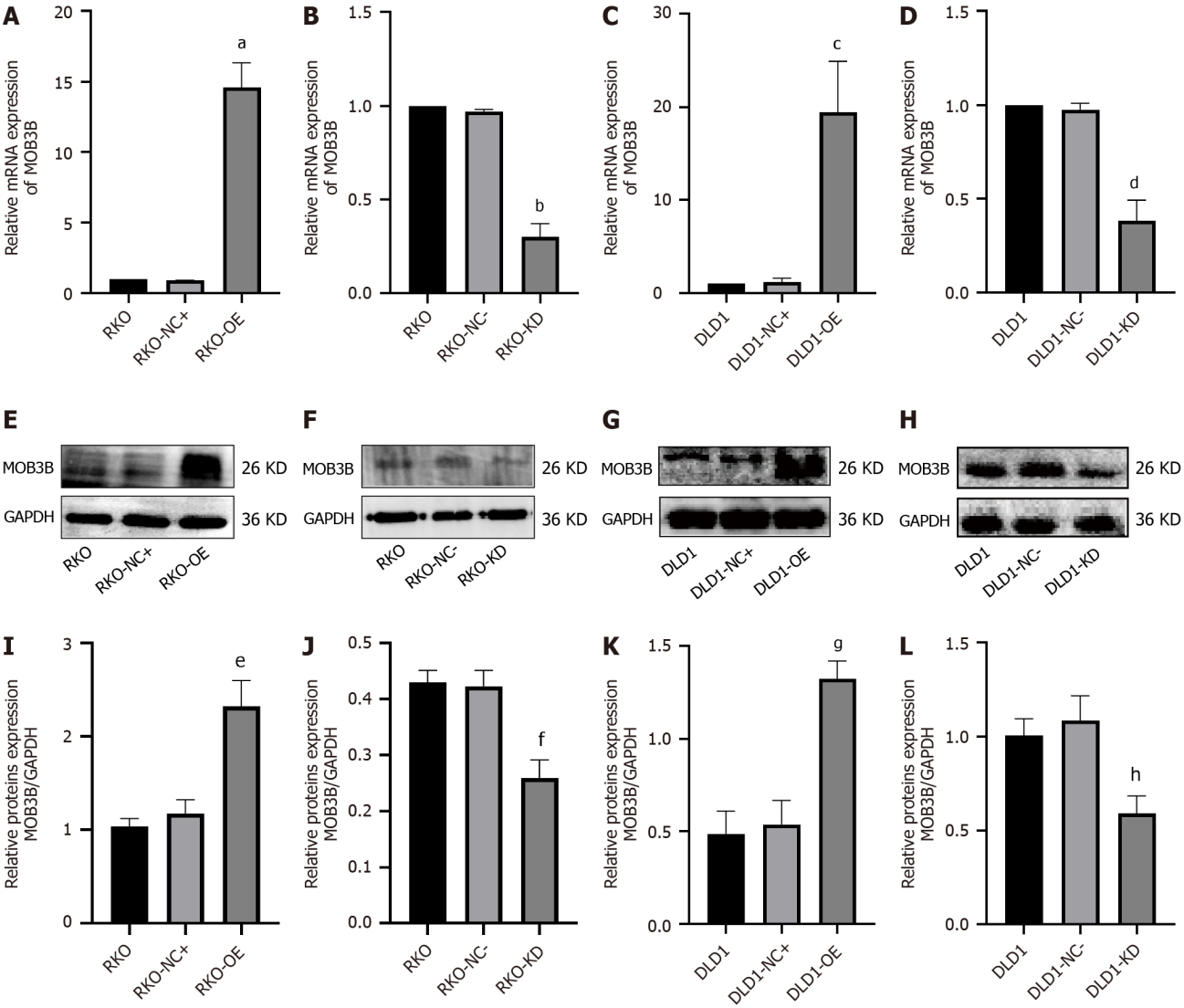
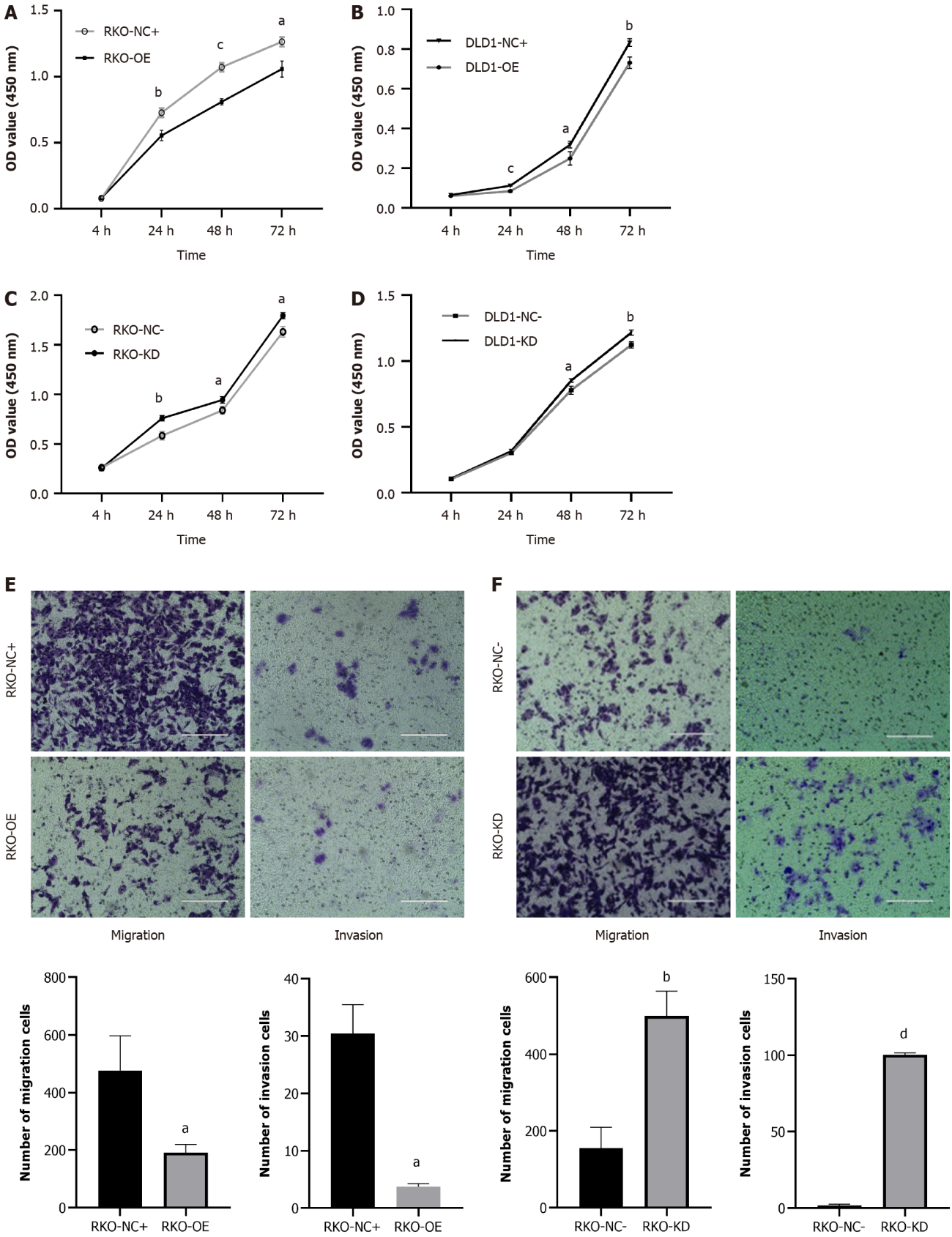


Figure 2 Establishment of RKO and DLD1 cell subline with stable monopolar spindle-binding protein 3B overexpression or knockdown. A-D: Quantitative reverse transcription-polymerase chain reaction; E-H: Western blot; I: Quantified data of E; J: Quantified data of F; K: Quantified data of G; L: Quantified data of H. The data are summarized as the mean ± SD of three independent experiments. [a]$P < 0.0001$ *vs* RKO-NC+, [b]$P < 0.0001$ *vs* RKO-NC-, [c]$P < 0.001$ *vs* DLD1-NC+, [d]$P < 0.0001$ *vs* DLD1-NC-, [e]$P < 0.001$ *vs* RKO-NC+, [f]$P < 0.001$ *vs* RKO-NC-, [g]$P < 0.001$ *vs* DLD1-NC+, [h]$P < 0.01$ *vs* DLD1-NC-.


A
RKO-NC+
RKO-OE
OD value (450 nm)
Time
4 h
24 h
48 h
72 h
B
DLD1-NC+
DLD1-OE
C
RKO-NC-
RKO-KD
D
DLD1-NC-
DLD1-KD
E
RKO-NC+
RKO-OE
Migration
Invasion
F
RKO-NC-
RKO-KD
Number of migration cells
Number of invasion cells

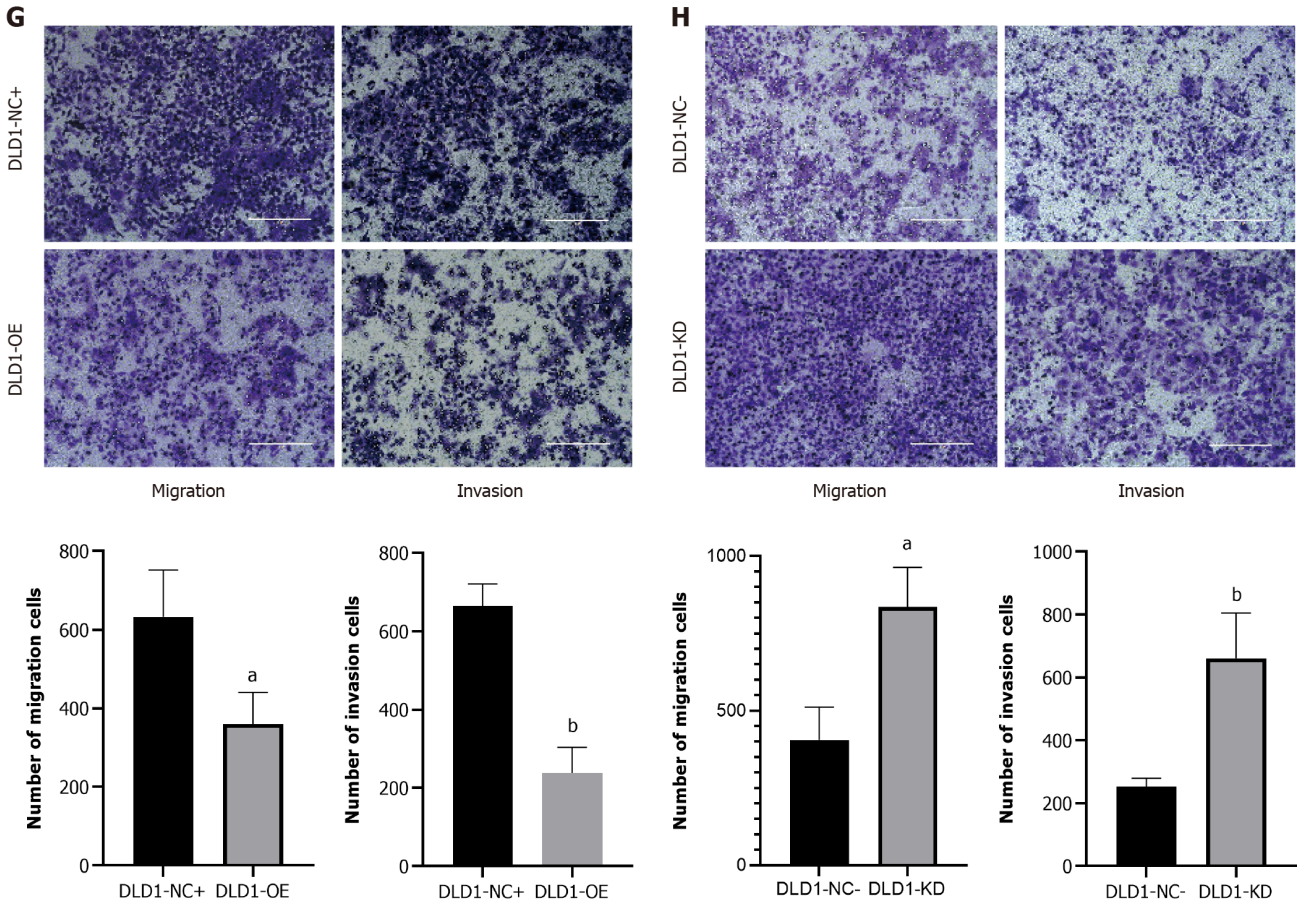


Figure 3 Effect of monopolar spindle-binding protein 3B on proliferation, migration, and invasion of RKO and DLD1 cells. A-D: Cell counting kit-8 assay; E-H: Transwell assay. The data are presented as the mean ± SD of three independent experiments. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001, [d]*P* < 0.0001.

DISCUSSION

In the current study, we demonstrated that loss of MOB3B expression in CRC tissue specimens was associated with a poor prognosis of CRC patients. Our *in-vitro* and *in-vivo* data revealed that overexpression of MOB3B attenuated CRC cell proliferation, migration, and invasion, whereas knockdown of MOB3B expression caused the opposite effects in CRC cells. At the molecular level, MOB3B regulated mTOR/autophagy signaling, *i.e.*, MOB3B overexpression induced LC3 II/I expression but downregulated the expression of MMP2, MMP9, SQSTM1/p62, and p-mTOR, whereas knockdown of MOB3B expression had the opposite effects. Taken together, these data suggest that MOB3B is a tumor suppressor in CRC or at least possesses anti-CRC activity through regulation of mTOR/autophagy signaling and that loss of MOB3B expression contributes to CRC development and progression.

In 2005, Lai *et al*[34] identified a Mob superfamily protein, Mats, in Drosophila and revealed that loss of Mats expression induced cell proliferation, apoptosis suppression, and tissue overgrowth. At the molecular level, Mats protein interacts with Wts, another tumor suppressor. The family of MOB proteins plays an important role in the suppression of tumorigenesis, and MOB1 has been shown to assist the nuclear Dbf2-related kinases 1/2 in coordinating autophagic and apoptotic events in human cells and flies[25]. MOB3 shares a high sequence similarity with MOB1[26], and Rual *et al*[35] have revealed that MOB3B is able to interact with the 5'-nucleotidase, cytosolic 2 (NT5C2) protein; the latter has been determined to be a prognostic marker in lung cancer[36] and hematologic malignancies[37,38], suggesting that the link between MOB3B and NT5C2 proteins might be associated with the development of lung and hematologic cancers. Knockdown of MOB3A, MOB3B, or MOB3C expression has been demonstrated to individually reduce the viability of breast cancer cells[27], while the *MOB3B* promoter is hypermethylated in prostate cancer samples[30], leading to a reduction of *MOB3B* mRNA levels in prostate cancer[39]. Indeed, our current study also showed that MOB3B expression was significantly downregulated in CRC tissue samples and that CRC cells with MOB3B overexpression had significantly reduced tumor cell proliferation, migration, and invasion abilities, further supporting a tumor-suppressing role of MOB3B in human cancers, including CRC. A recent review has summarized and discussed the MOB-regulated signaling in cells and its regulation of tissue growth, morphogenesis, and cell polarity of unicellular to multicellular organisms[40]. It also has been demonstrated that MOB protein expression is associated with an even broader disease spectrum as it regulates post-translational modifications, protein-protein interactions, and cellular processes that are possibly linked to cancer and other diseases[26]. Another recent review has linked MOB expression to the regulation of Hippo signaling[41].

Cancer metastasis is an important cause of death for CRC patients[42]. MMPs are able to degrade the extracellular matrix and basement membrane to facilitate tumor cell migration, invasion, and metastasis[43]. Thus, the overexpression

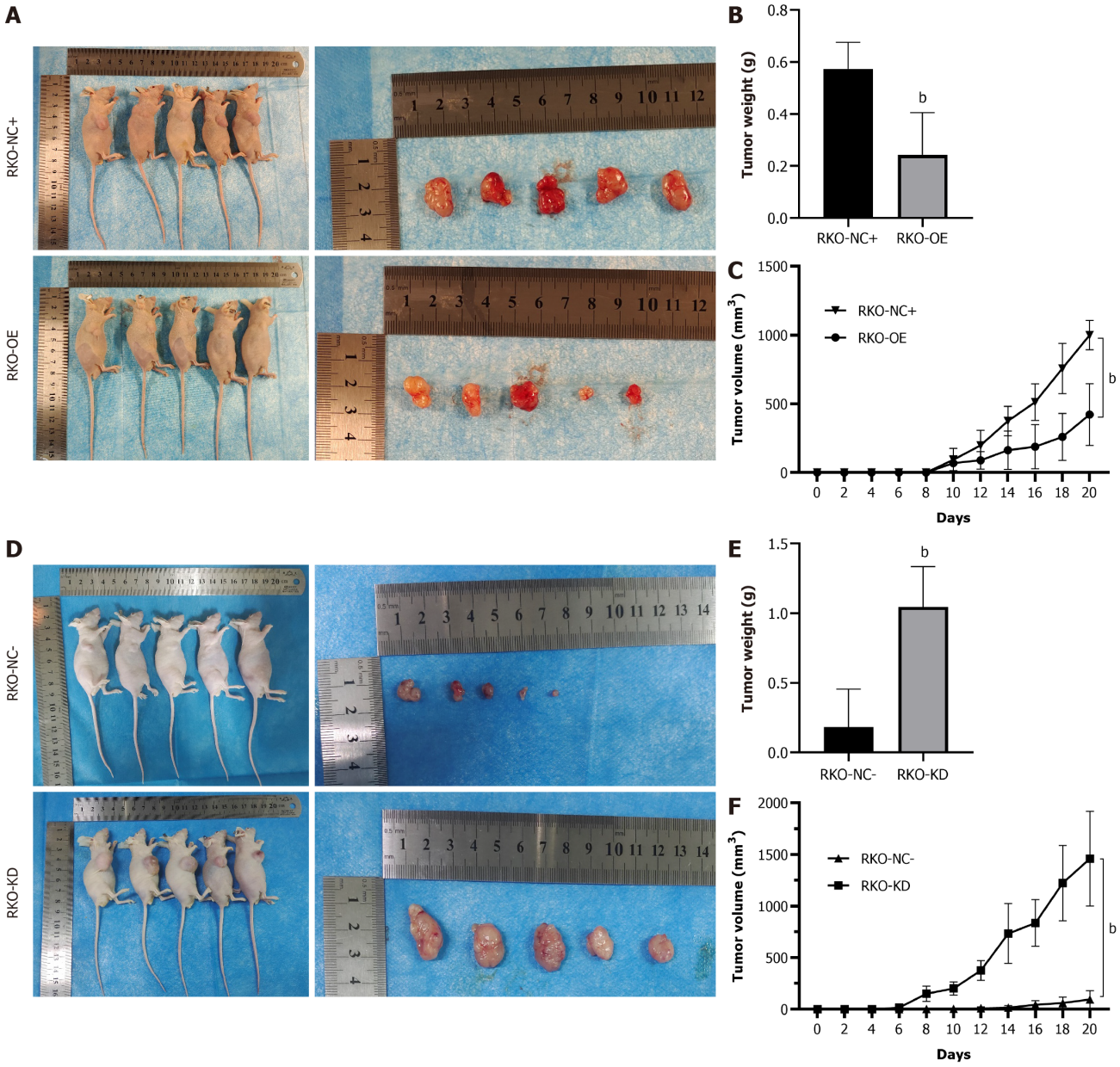


Figure 4 Antitumor activity of monopolar spindle-binding protein 3B in nude mice. A: Tumor cell xenograft assay using RKO-OE and RKO-NC+ cells; B: Weight of xenografts from RKO-OE *vs* RKO-NC+ cells; C: Tumor cell xenograft growth curves of RKO-OE and RKO-NC+ cells; D: Tumor cell xenograft assay using RKO-KD and RKO-NC- cells; E: Weight of xenografts from RKO-KD *vs* RKO-NC- cells; F: Tumor cell xenograft growth curves of RKO-KD *vs* RKO-NC- cells. $n = 5$, $^{b}P < 0.01$.

of MMP2 and MMP9, important members of the MMP family of enzymes, is associated with tumor progression and metastasis[44]. Our current study demonstrated that MOB3B overexpression was able to suppress MMP2 and MMP9 expression as well as reduce CRC cell proliferation, migration, and invasion, whereas knockdown of MOB3B upregulated MMP2 and MMP9 expression and CRC cell proliferation, migration, and invasion. However, further investigation is needed to determine how MOB3B regulates MMP2 and MMP9 expression in CRC cells.

Altered autophagy occurs in various human cancers, including CRC[45]. LC3 is the central protein that regulates autophagy. During autophagy, cytoplasmic LC3 (namely, LC3-I), through a series of ubiquitin-like reactions, is conjugated to its lipid phosphatidylethanolamine head to form LC3-II, which is involved in expansion and fusion events of the autophagosome membrane[46]. However, the exact function of LC3 in autophagy remains controversial. In any case, the LC3-II/I ratio can be used to estimate the level of autophagy in cells[47,48]. Furthermore, the SQSTM1/p62 protein, a known autophagy factor, is involved in autophagosome formation and is degraded in the middle and late stages of autophagy, so there is an inverse association between the overall p62 levels and autophagic activity in cells[48]. In addition, mTOR, a serine/threonine kinase, negatively regulates autophagy[17], *i.e.*, inhibited mTOR function or expression leads to conversion of LC3-I to LC3-II and autophagy[49]. Rapamycin is able to inhibit mTOR activity to promote cell autophagy, whereas MHY1485 is a potent cell-permeable mTOR agonist that targets the mTOR's ATP structural domain to inhibit cell autophagy through suppression of autophagosome and lysosome fusion. Previous studies have demonstrated that MMP expression and activation are dependent on mTORC1-mediated translation[50] and

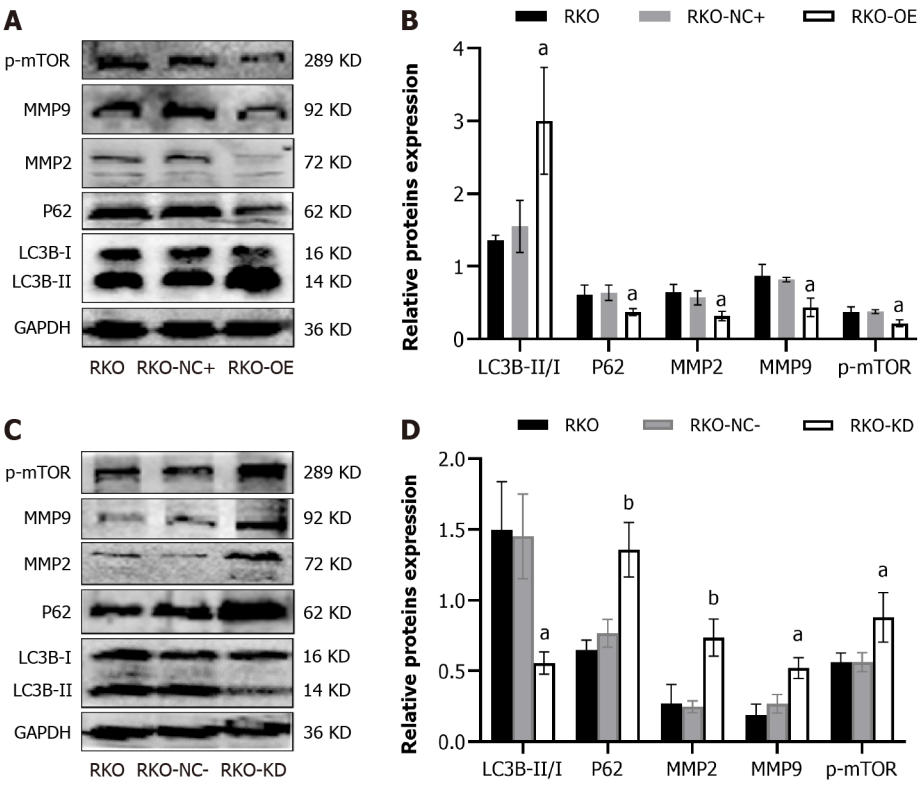


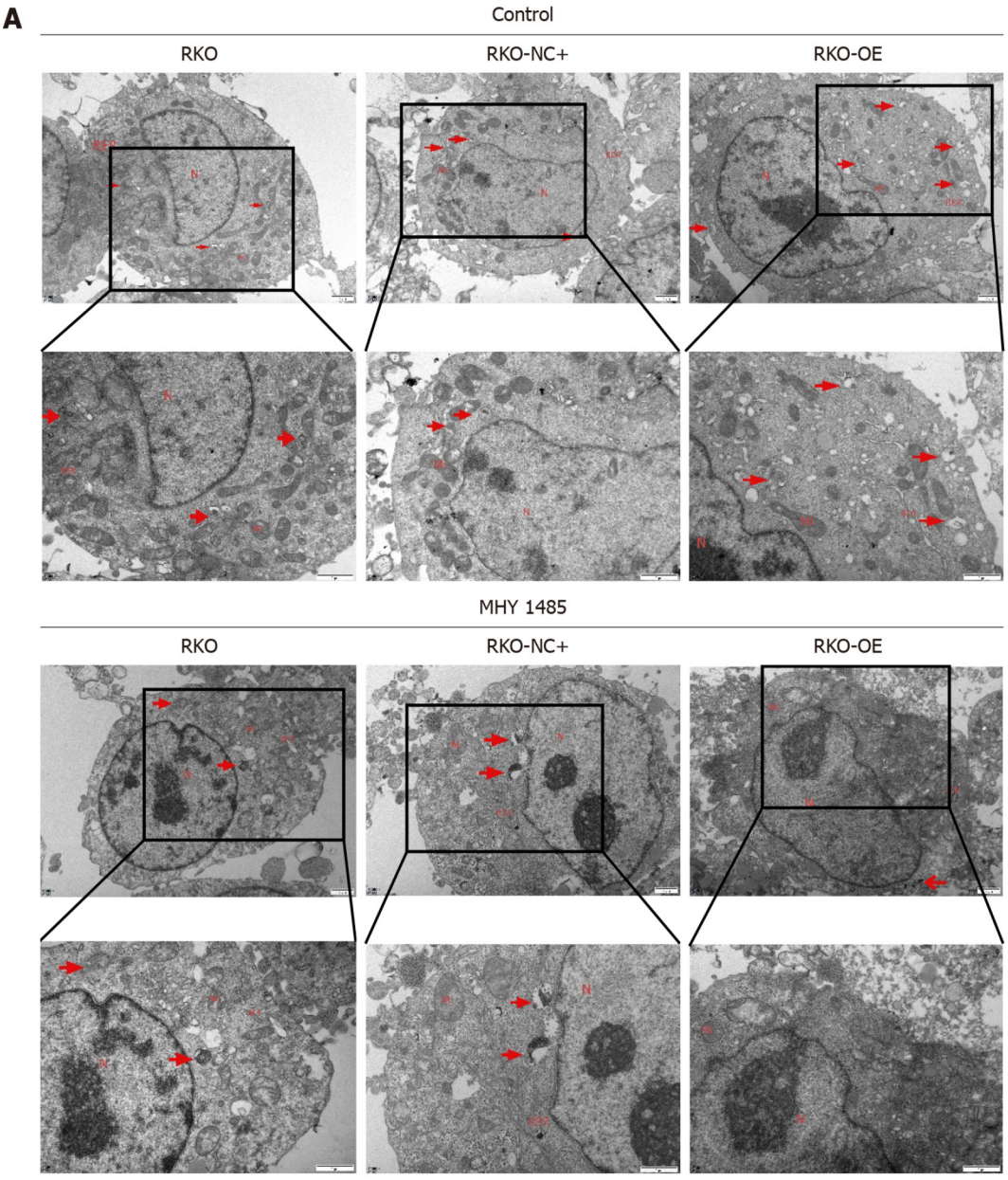
Figure 5 Effects of monopolar spindle-binding protein 3B on expression of matrix metalloproteinase 2, matrix metalloproteinase 9, phosphorylated mechanistic target of rapamycin kinase, and autophagy related proteins in RKO cells. A: Representative Western blot images. Stable monopolar spindle-binding protein 3B (MOB3B)-overexpressing RKO cells were grown and subjected to Western blot analysis; B: Quantified data of A; C: Representative Western blot images. RKO cells with stable MOB3B knockdown were grown and subjected to Western blot analysis; D: Quantified data of C. The data are summarized as the mean ± SD of three replicates. $^{a}P < 0.05$, $^{b}P < 0.01$. mTOR: Mechanistic target of rapamycin; MMP: Matrix metalloproteinase.

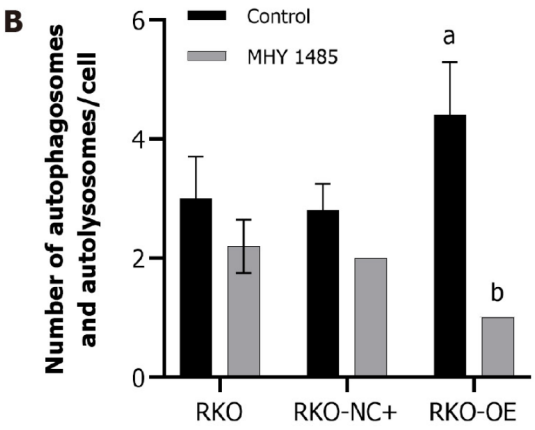
that the mTOR-p70S6K pathway induces MMP expression[51,52]. Moreover, it has been documented that autophagy is able to inhibit cell migration and invasion through MMPs and integrins[6]. In our study, we found that MOB3B overexpression decreased the p-mTOR, MMP2, MMP9 and SQSTM1/p62 expression but induced the conversion of LC3-I to LC3-II. Meanwhile, our TEM results revealed that the autophagy flux was increased. In parallel, CRC cell proliferation, migration, and invasion were reduced, whereas knockdown of MOB3B had the opposite effects in tumor cells. In this context, MOB3B-overexpressing CRC cells treated with MHY1485 activated mTOR activity but inhibited autophagy, whereas treatment of the MOB3B-knocked-down CRC cells with rapamycin inhibited mTOR activity but activated autophagy. These results suggest that MOB3B could be involved in regulating mTOR/autophagy signaling to alter CRC cell malignant biological behaviors.

Nevertheless, our current study does have some limitations; for example, our mechanistic data are mainly based on *in-vitro* experiments. Future investigation will focus on how this signaling pathway facilitates the anticancer activity of MOB3B. Moreover, our current *ex-vivo* data need to be confirmed by studies with a larger sample size from multiple institutions before MOB3B can be used as a prognostic marker for CRC patients.

CONCLUSION

In conclusion, our current study demonstrated the antitumor activity of MOB3B protein through inactivation of mTOR/autophagy signaling. The main findings are as follows: (1) Loss of MOB3B expression was observed in CRC tissues; (2) Loss of MOB3B expression was associated with a poor CRC prognosis; (3) MOB3B overexpression suppressed CRC malignant behaviors *in vitro* and *in vivo*, whereas knockdown of MOB3B showed the opposite effects in CRC cells; and (4) MOB3B regulated the expression of autophagy- and cell mobility-related proteins, like the LC3-II/LC3-I ratio and MMP2 and MMP9, in CRC cells. Our data may provide a theoretical basis for the use of MOB3B as a novel prognostic marker or therapeutic target for CRC.


A
Control
RKO
RKO-NC+
RKO-OE
MHY 1485
RKO
RKO-NC+
RKO-OE


B
Number of autophagosomes and autolysosomes/cell
Control
MHY 1485
6
4
2
0
a
b
RKO
RKO-NC+
RKO-OE

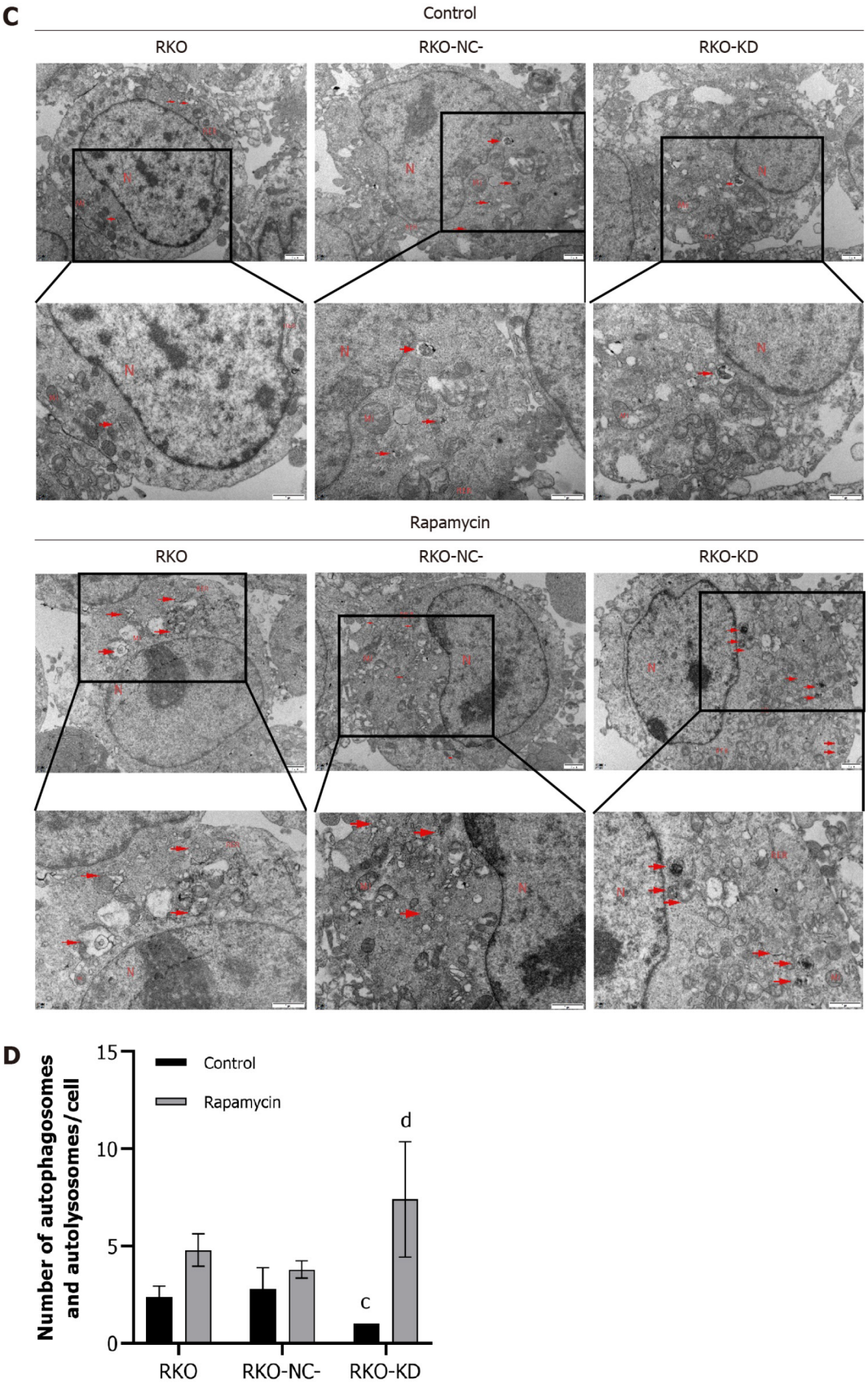
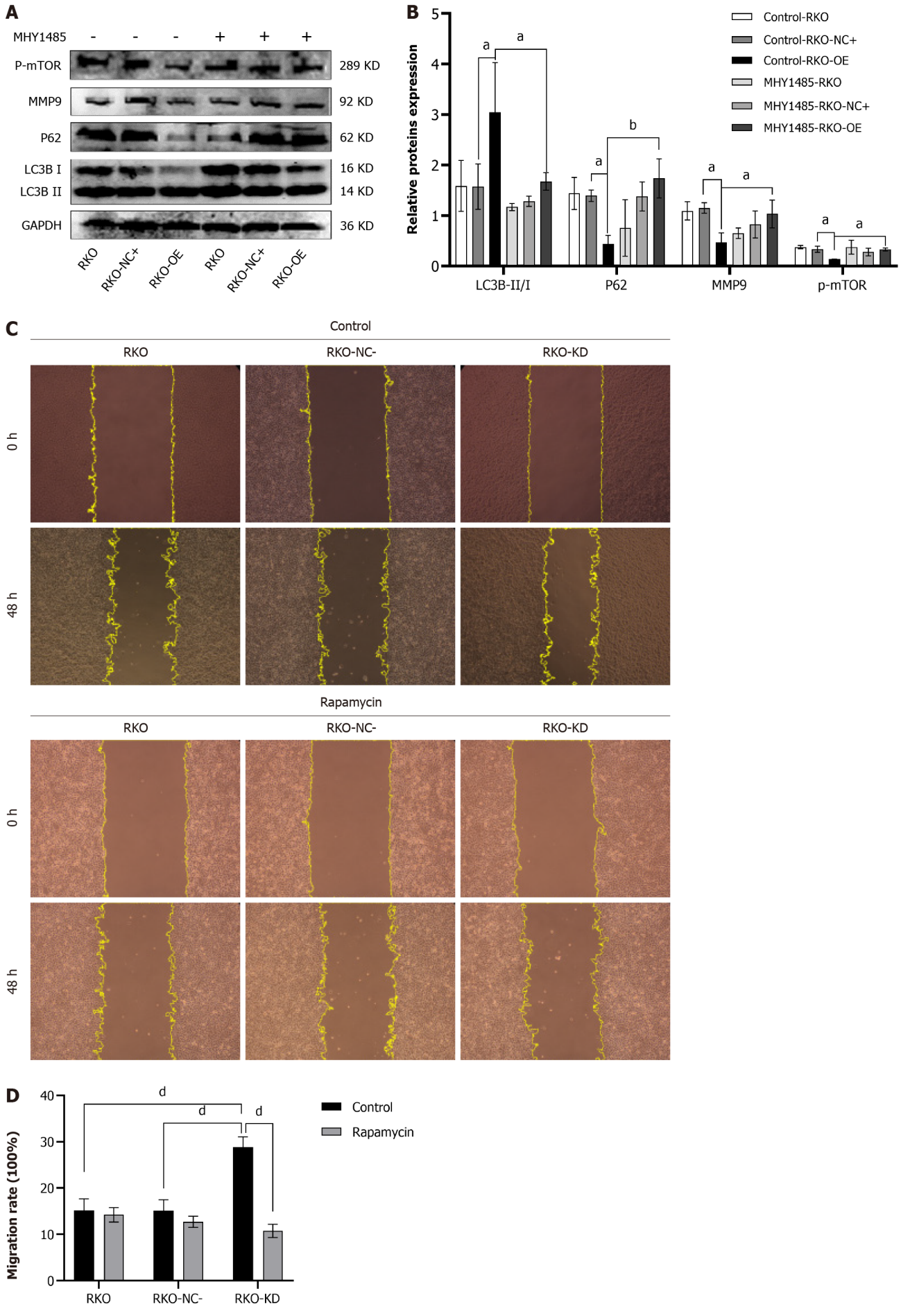


Figure 6 Effect of monopolar spindle-binding protein 3B on number of autophagosomes/autophagy lysosomes in RKO cells. A: Transmission electron microscopy (TEM) images. Stable monopolar spindle-binding protein 3B (MOB3B)-overexpressing RKO cells were grown and treated with MHY 1485 (50 μM), a mechanistic target of rapamycin kinase (mTOR) agonist, for 48 h, and then subjected to TEM analysis of cell morphology; B: Quantified data of A; C: TEM images. RKO cells with stable MOB3B knockdown were grown and treated with rapamycin (100 nM), an mTOR inhibitor, for 48 h, and then subjected to TEM analysis of cell morphology; D: Quantified data of C. The arrows show autophagosomes/autophagy lysosomes. The data are shown as the mean ± SD of five replicates. $^{a}P < 0.05$ *vs* RKO-NC+, $^{b}P < 0.05$ *vs* RKO-OE, $^{c}P < 0.05$ *vs* RKO-NC-, $^{d}P < 0.05$ *vs* RKO-KD.


A
MHY1485 - - - + + +
P-mTOR
MMP9
P62
LC3B I
LC3B II
GAPDH
289 KD
92 KD
62 KD
16 KD
14 KD
36 KD
RKO
RKO-NC+
RKO-OE
RKO
RKO-NC+
RKO-OE
B
Relative proteins expression
Control-RKO
Control-RKO-NC+
Control-RKO-OE
MHY1485-RKO
MHY1485-RKO-NC+
MHY1485-RKO-OE
LC3B-II/I
P62
MMP9
p-mTOR
C
Control
RKO
RKO-NC-
RKO-KD
0 h
48 h
Rapamycin
RKO
RKO-NC-
RKO-KD
0 h
48 h
D
Migration rate (100%)
Control
Rapamycin
RKO
RKO-NC-
RKO-KD

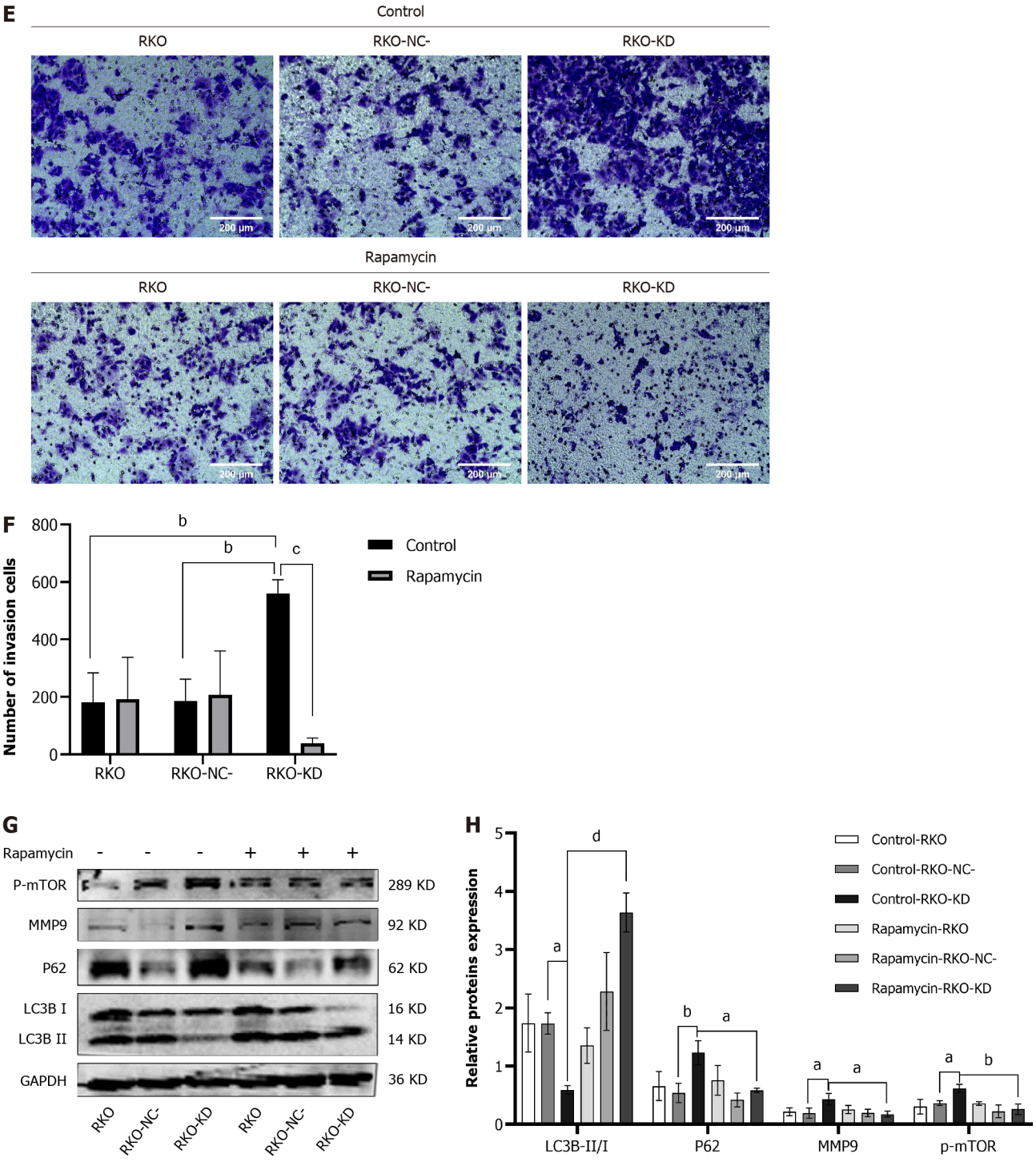


Figure 7 Monopolar spindle-binding protein 3B inhibition of colorectal cancer cell malignant behaviors through inactivation of mechanistic target of rapamycin kinase/autophagy signaling. A: Western blot images. Stable monopolar spindle-binding protein 3B (MOB3B)-overexpressing RKO cells were grown, treated with MHY 1485 (50 μM) for 48 h, and then subjected to Western blot analysis; B: Quantified data of A; C-H: RKO cells with with stable MOB3B knockdown were grown, treated with rapamycin (100 nM) for 48 h, and then subjected to wound healing assay (C and D), Transwell assay (E and F), and Western blot analysis (G and H). The data are presented as the mean ± SD of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. mTOR: Mechanistic target of rapamycin; MMP: Matrix metalloproteinase; LC: Light chain.

FOOTNOTES

Author contributions: Sun J and Zhang JX contributed equally to this study; Sun J, Zhang JX, and Huang JA developed the original hypothesis, supervised the experimental design, and wrote and revised the manuscript; Sun J, Zhang JX, Li MS, and Qin MB performed *in vitro* and *in vivo* experiments; Qin MB, Cheng RX, Wu QR, and Liao C participated in the clinical specimens collection; Sun J, Zhang JX, Chen QL, Yang D, and Liu SQ analyzed the data and performed statistical analysis; and all authors read and approved the final manuscript.

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