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Downregulation of COPB2 by RNAi inhibits growth of human cholangiocellular carcinoma cells

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Abstract. – OBJECTIVE: The present study aimed to explore the contribution of COPB2 (coatomer subunit beta) towards the tumorigenesis of cholangiocellular carcinomas and to elucidate the underlying mechanism(s).

MATERIALS AND METHODS: Expression of COPB2 mRNA by RBE and QBC939 cholangiocellular carcinoma cell lines was determined by qRT-PCR. We, then, silenced COPB2 expression in RBE cells by infection with a COPB2-siRNA lentivirus and measured the proliferation, cell-cycle distribution, and apoptosis of transduced cells.

RESULTS: COPB2 was highly expressed in RBE and QBC939 cholangiocellular carcinoma cell lines. Infection with COPB2-siRNA lentivirus in RBE cells significantly decreased COPB2 expression. More so, silencing of COPB2 by COPB2-siRNA significantly suppressed the proliferation and promoted the apoptosis of RBE cells by arresting transduced cells in the G1 phase.

CONCLUSIONS: Our results demonstrate that the COPB2 gene is highly expressed in cholangiocellular carcinoma cell lines, wherein knockdown inhibited the proliferation and promoted the arrest of cell-cycle progression and the apoptosis of cholangiocellular carcinomas. COPB2 may constitute an attractive target for therapeutic strategies against cholangiocellular cancers.

Key Words:

Apoptosis, Cell cycle, Cholangiocellular carcinoma, COPB2.

Introduction

Cholangiocellular carcinoma, also known as cholangiocarcinoma, is a malignant epithelial tumor arising from the diverse anatomical zones within the biliary tract[1]. Cholangiocarcinoma is one of two primary subtypes and the second most common primary liver cancer, accounting for 15% of the overall liver-cancer burden worldwide[2-4]. Although relatively uncommon, the incidence of cholangiocarcinoma has increased in recent decades wherein it has exceeded hepatocellular carcinoma as the main cause of death of all primary hepatobiliary tumors[5]. Currently, its molecular features are still poorly understood.

Surgical resection is thought to be the only curative treatment protocol[6,7], but only 5% of these patients survive 5 years[8]. These dismal outcomes largely arise from the high rates of tumor recurrence[9]. Accordingly, there is an urgent demand for targeted therapies able to ameliorate patients prognosis and reduce the likelihood of tumor recurrence.

The coatomer protein complex 1 (COP I) is a complex of seven cytosolic protein subunits (α, β, β', γ, δ, ε, ζ)[10,11]. It binds to dilysine motifs and reversibly associates with Golgi non-clathrin-coated vesicles. Its β-coat protein complex (β-COP) interacts with the ADP ribosylation factor (ARF) when mediated by the Golgi-attached nucleotide exchange protein. Coatomer and ARF exist separately in the cytosol but co-assemble to form coats[12]. The product of such reaction is the coating of COPI-coated vesicles[13], which act to regulate the transport of proteins across the Golgi stack[14,15] and between the Golgi and the endoplasmic reticulum[16]. Then, the vesicles will go up to the trans Golgi network. Some COP subunits have been demonstrated to play important roles in many important biological and physiological functions. The alpha COPI subunit (α-COP), for example, is required for forming the concentric whorls of the rough endoplasmic reticulum[17], and its ring-like domain within the C-terminal may aid the oligomerization of coats[18]. Meanwhile, the γ subunit of COPI (γ-COP) plays an important role in epithelial morphogenesis in *Drosophila melanogaster*[19].

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Gene silencing of the coatomer protein complex subunit ζ2 (ζ2-COP) induces the apoptosis of both proliferating and non-dividing tumor cells[20], thereby attracting much attention.

The COPB2 (Coatomer Subunit Beta) encoded by the coatomer protein complex β' (β'-) gene, p102, comprises a brefeldin A-sensitive component of the Golgi membrane that is involved in mediating transmembrane transport in the essential exocytic pathway[21]. Notably, COPB2 (p102) has been reported to be overexpressed in human sarcomas and neoplastic tissues, thereby potentially implicating COPB2 in tumorigenesis[22]. Accordingly, the present work aimed to determine the expression of COPB2 in human RBE and QBC939 cholangiocellular carcinoma cell lines. We also sought to study the impact of COPB2 knockdown with a specific COPB2-siRNA on the proliferation, apoptosis and cell-cycle distribution of transduced RBE cells.

Materials and Methods

Cell Cultures

RBE and QBC939 cells purchased from Shanghai Cell Bank, (Shanghai, China) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Construction of COPB2-siRNA Lentivirus

COPB2-specific siRNA and non-silencing control sequences were cloned into pGCL-GFP-Lentivirus vectors, generating pGCSIL-GFP-shCOPB2 and pGCSIL-GFP-NC lentiviral plasmids, respectively. The COPB2 target sequence was 5'- AGA TTA GAG TGT TCA ATT A-3'. The negative control (NC), non-silencing sequence was 5'- AAT TCT CCG AAC GTG TCA CGT -3'.

COPB2 mRNA Expression

Total RNA was isolated from RBE and QBC939 cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) used in accordance with the manufacturer's instructions. RNA was reverse transcribed to cDNA using M-MuLV One-Step RT-PCR Kit (Sangon Biotechnology Co. LTD, Shanghai, China). This cDNA was subsequently assayed by PCR using the following primer sequences: COPB2 (forward 5'-GTG GGG ACA AGC CAT ACC TC-3' and reverse 5'-GTG CTC TCA AGC CGG TAG G-3'); GAPDH (forward 5'-TGA CTT CAA CAG CGA CAC CCA-3' and reverse 5'-CAC CCT GTT GCT GTA GCC AAA-3'). COPB2 mRNA expression was determined in accordance with the $2^{-\Delta\Delta CT}$ method.

Infection of RBE Cells with COPB2-siRNA Lentivirus

Human RBE cholangiocellular carcinoma cells were seeded into 6-well plates and incubated with either a COPB2-siRNA or control-payload lentiviruses. Seventy-two hours post-infection, infected cells were observed under a fluorescent imaging microscope (Olympus, Tokyo, Japan) by counting green cells based on green fluorescent protein (GFP) intensity. The efficiency of this infection was determined by RT-PCR and Western blotting.

Western Blotting

RBE cells were homogenized in ice-cold Mammalian Cell Total Protein Lysis Buffer (Sangon Biotechnology Co. Ltd, Shanghai, China) for 5 min. Total protein was isolated by centrifugation of lysates at 12,000 g for 20 min at 4°C. Protein extracts were resolved by 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto PVDF (polyvinylidene difluoride) membranes. Membranes were incubated overnight at 4°C with rabbit anti-COPB2 polyclonal (1:2000 dilution; ab131885, Abcam, Cambridge, MA, USA) and anti-GAPDH monoclonal antibodies (1:5000 dilution; sc-32233, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) dissolved in blocking buffer with 5% non-fat milk. Membranes were then incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:2000 dilution; sc-2005, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 1 h. Blotting was detected developed with a DAB substrate (Bio Basic Inc., Markham, ON, Canada).

Cell Growth

Ten-days post-infection with either pGCSIL-GFP-shCOPB2 or pGCSIL-GFP-NC lentiviral plasmids, RBE cells were cultured in 96-well plates for 5 days at 37°C and 5% CO_2. Cell growth was determined by measuring the intensity and distribution of fluorescence emitted by each individual cell by fluorescence microscopy. Images were obtained from the Microsoft SQL database.

Methyl-Thiazol-Tetrazolium (MTT) Cell Proliferation Assay

Infected RBE cells (2×10^3 cells) were seeded into 96-well plate suspended 100 μL medium per well, and cultured at 37°C. The proliferation of cells was detected at days 1, 2, 3, 4 and 5. Briefly, 20 μL MTT (5 mg/mL) were added to each well and incubated for 4 h at 37°C. Then, the cell media in each well was removed from each well, to which 150 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were added for dissolution of the crystals. The absorbance was measured at 570 nm.

Cell-Cycle Distribution

Infected RBE cells were seeded into 6-well plates and cultured at 37°C for 5 days. Briefly, cells were centrifuged at 1000 g for 5 min, rinsed twice with ice-cold phosphate-buffered saline (PBS), fixed with cold 70% ethanol, and stained with propidium iodide (PI) in the presence of RNase (100 μg/mL) at 4°C for 30 min. The cell-cycle distribution was then determined by flow cytometric analysis using Guava InCyte Software (Guava easyCyte HT, Merck, Shanghai, China).

Annexin V-APC Apoptosis Assay

To study the impact COPB2 knockdown has on cell apoptosis, the proportion of RBE cells infected with either a COPB2-siRNA lentivirus or control lentivirus undergoing apoptosis was determined with the Annexin V Apoptosis Detection Kit APC (eBioscience, San Diego, CA, USA). Briefly, cells were stained with Annexin V-APC reagent for 15 min at 25°C, which was subsequently detected by flow cytometry.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Comparisons between variables were undertaken using one-way analysis of variance (ANOVA) with the method of Least-Significant Difference and the Student's *t*-test. All analyses were undertaken within SPSS, 16.0 software (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

Results

Measurement of COPB2 mRNA in Cholangiocellular Carcinoma Cell Lines

qRT-PCR was used to determine the expression of COPB2 mRNA in the RBE and QBC939 cholangiocellular carcinoma cell lines. COPB2 mRNA was expressed in these two lines of cholangiocellular carcinoma, and was about two-fold greater in RBE over QBC939 cells, though this difference was not significant (Figure 1A).

Infection Efficiency of COPB2-siRNA

RBE cells were infected with either shCOPB2 or shCtrl lentivirus. First and foremost, the infection efficiency (> 80%) was high, as indicated by measuring GFP expression via fluorescence microscopy (Figure 1B). Expression of COPB2 at the mRNA (Figure 1C) and protein levels (Figure 1D) was significantly lower in cells infected with COPB2-siRNA lentivirus over those infected with a control vector ($p < 0.01$).

COPB2-siRNA Inhibited the Proliferation of RBE Cells

The proliferation of RBE cells post-knockdown of COPB2 was determined to define the contribution of COPB2 towards tumorigenesis. Downregulation of COPB2 notably suppressed the expansion of these cells (Figure 2A). By days 4 and 5, this suppression of proliferation was statistically significant over control infections ($p < 0.01$; Figure 2B). These data suggest that the knockdown of COPB2 inhibits the proliferation of RBE cholangiocellular carcinoma cells.

Downregulation of COPB2 Arrests the Cell Cycle in the G1 Phase

The cell-cycle distribution of cells infected with either shCOPB2 or shCtrl lentivirus was explored in an attempt to explain its suppression of proliferation. The number of COPB2-knockdown cells within the G1 phase was significantly greater than unaffected cells, whilst the number in the S phase was markedly lower ($p < 0.01$; Figure 3).

Knockdown of COPB2 Seemingly Promotes Apoptosis

Within our RBE cells transduced to have COPB2 knocked down, we determined the proportion of cells undergoing apoptosis and made comparisons to controls cells with COPB2 intact. Infection with a shCOPB2 lentivirus resulted in a significantly greater proportion of cells undergoing apoptosis in comparison to control cells ($p < 0.01$; Figure 4). COPB2 knockdown seemingly promotes human RBE cholangiocellular carcinoma cells to undergo apoptosis.

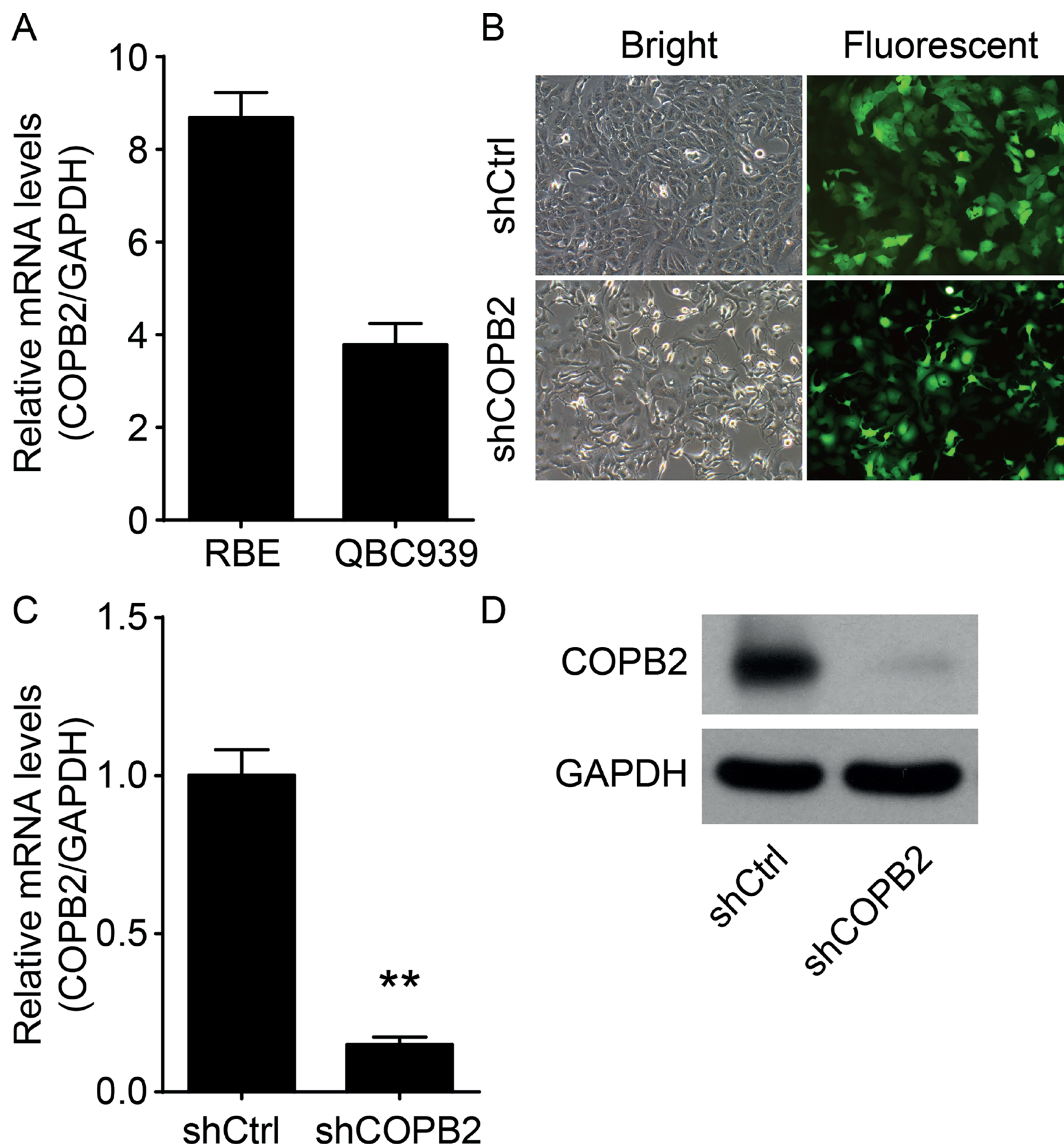

Figure 1. Expression of COPB2 in cholangiocellular carcinoma cell lines. ***A,*** High expression of COPB2 mRNA in RBE and QBC939 cells. ***B,*** Representative bright and fluorescent micrographs (×100) of RBE cells at 3-days post-infection. ***C,*** COPB2 mRNA expression was significantly suppressed 5-days post-infection with a COPB2-siRNA-knockdown payload (** $p < 0.01$). ***D,*** Western blots illustrating COPB2 protein levels post infection with shCtrl or shCOPB2 plasmids. GAPDH was used as the internal control.

Discussion

Cancer cells generally carry several genes of abnormal function, but their proliferation and survival can be highly dependent on the function of a single oncogene. Accordingly, the exploration for tumor-specific targets provides a rationale for molecular therapies against tumors (23), including cholangiocarcinoma. However, the molecular and/or clinical features of cholangiocarcinoma remain unclear. Thus, there is an urgent need to develop novel treatment strategies for cholangiocarcinoma.

COPB2 as a coatomer complex protein subunit, mediates the continuous constitutive protein transport *in vivo*.

It has been reported that COPB2 has homology to the β-subunits of the trimeric G protein, wherein it is associated with the reversible binding of the coatomer protein complex to ARF in

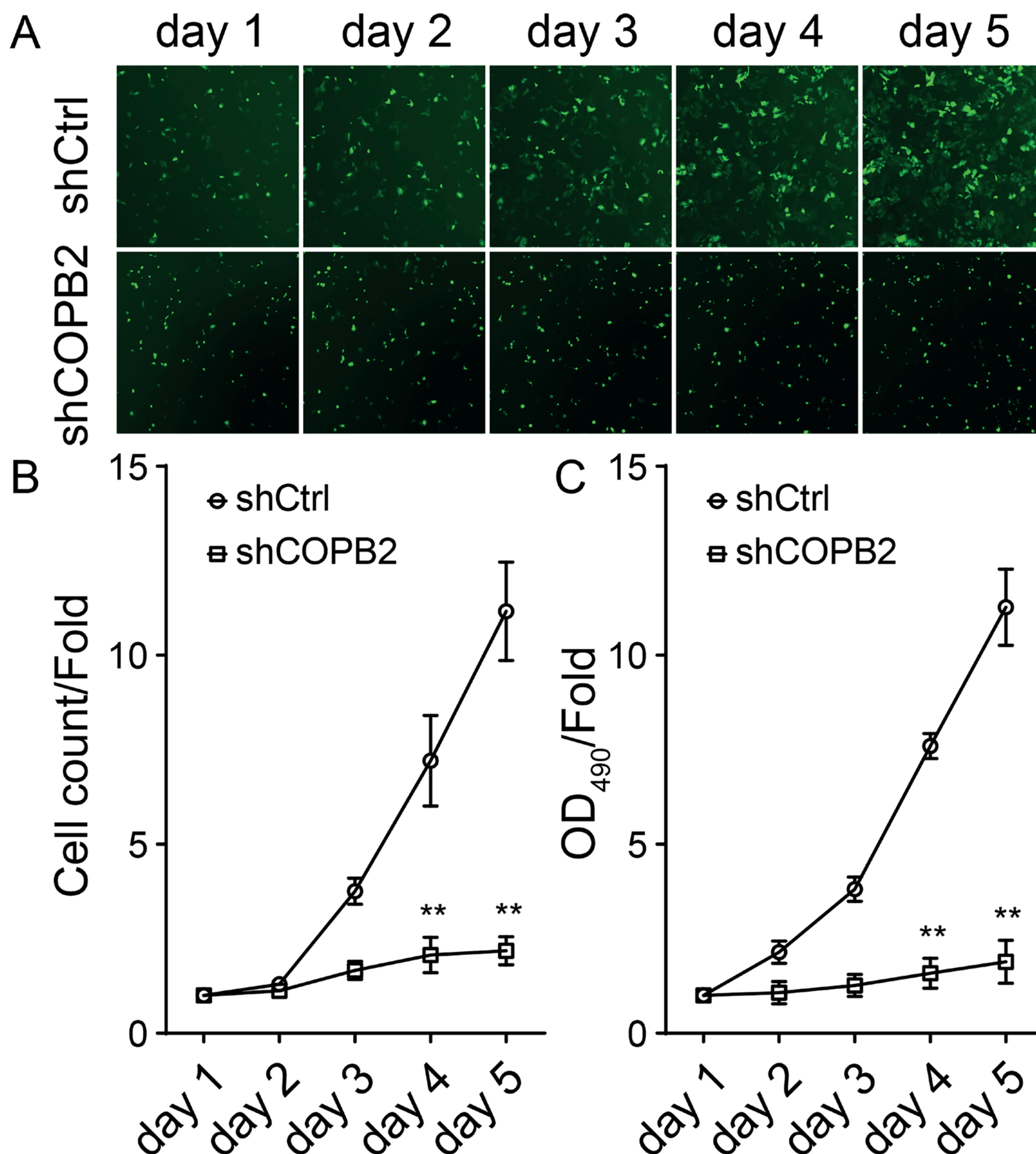

Figure 2. COPB2 knockdown suppresses the proliferation of cholangiocellular carcinoma cells. ***A,*** GFP labeled fluorescent micrographs of cell growth. ***B,*** RBE cell count at days 1-5 post-infection (shCtrl vs. shCOPB2, $p < 0.01$). ***C,*** Cell growth rates over days 2-5 (shCtrl *vs.* shCOPB2, $p < 0.01$).

normal cells[24]. In mammals, the coatomer can only be recruited by membranes associated to ARFs, which are small GTP-binding proteins. Furthermore, overexpression of SND p102 in hepatocytes accelerates phospholipid secretion into lipoprotein[25]. Brown et al[22] observed that COPB2 is overexpressed in neoplastic tissues, but is under-expressed in melanoma and carcinomas of the lung and colorectum. Many studies have highlighted that COPB2 functions to transport dilysine-tagged protein between the Golgi and the endoplasmic reticulum[26,27]. However, its effect in cholangiocarcinoma has yet to be reported. Cell proliferation was significantly inhibited in COPB2-knockdown RBE and QBC939 cells.

In the present work, we determined the expression of COPB2 in the human RBE and QBC939 cholangiocarcinoma cell lines. We also sought to study the effect of COPB2 knockdown by a COPB2-siRNA lentivirus on the proliferation, cell-cycle distribution, and apoptosis of RBE cells. Our data showed that COPB2 was highly expressed at the mRNA level in two cholangiocarcinoma cell lines. Furthermore, we also

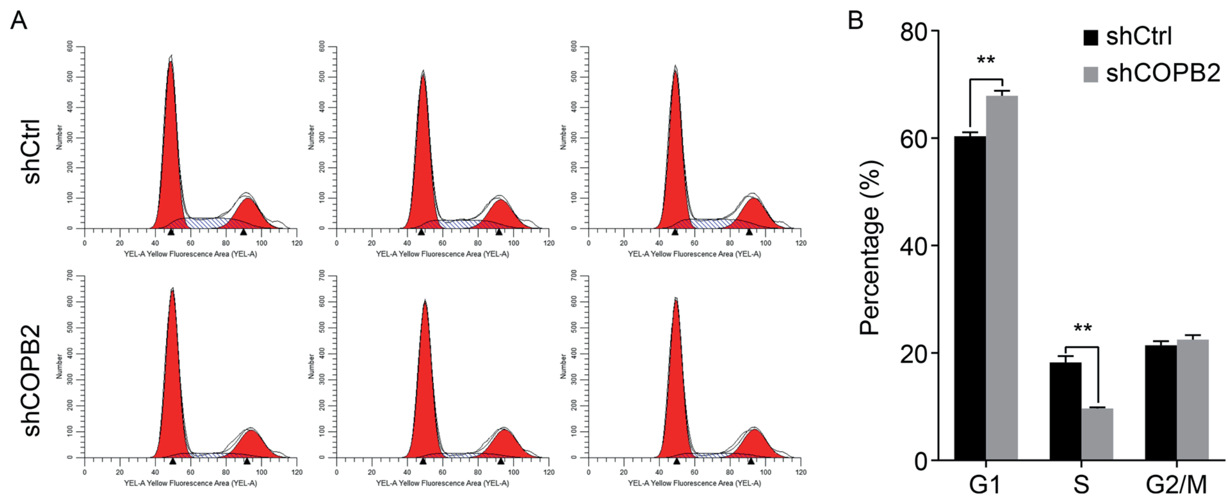

Figure 3. Knockdown of COPB2 arrested cells in the G1 phase. ***A,*** Cell-cycle analysis of RBE cells was determined by flow cytometry. ***B,*** Cell-cycle distribution of infected cells. The proportion of cells in the G1 phase was significantly greater in cells infected with COPB2-siRNA (**$p < 0.01$).

demonstrated that silencing the expression of COPB2 suppressed proliferation, arrested cells in the G1 phase, and increased apoptosis.

Our results suggest that COPB2 may be connected with cell-cycle checkpoints in RBE cholangiocarcinomas. Typically, non-neoplastic cells maintain genomic fidelity and integrity by requiring cells to pass a series of cell-cycle checkpoints before they are able to proliferate. Upon recognition of DNA damage, the checkpoint guarding exit from the G1 phase is activated, thereby preventing a cells progression and limiting the impact of genome damage[28,29]. This transient arrest requires a cell to repair the damage before re-entry to the cell cycle, but in instances of irreparable damage, the cell will activate apoptosis[30].

Human cancers are driven by pathological genetic alterations, and proteins encoded by many of the genes mediate the progression through the G1 phase of the cell cycle[31,32]. Both protein synthesis and cell growth occurred during the G1 phase. Both COPB2 and trans-Golgi network clathrin are recruited by the GTPase protein ARF1.

Our data showed that treatment with a COPB2-siRNA increased both the proportion of RBE cells in the G1 phase and the proportion undergoing apoptosis. Taken together, apoptosis is

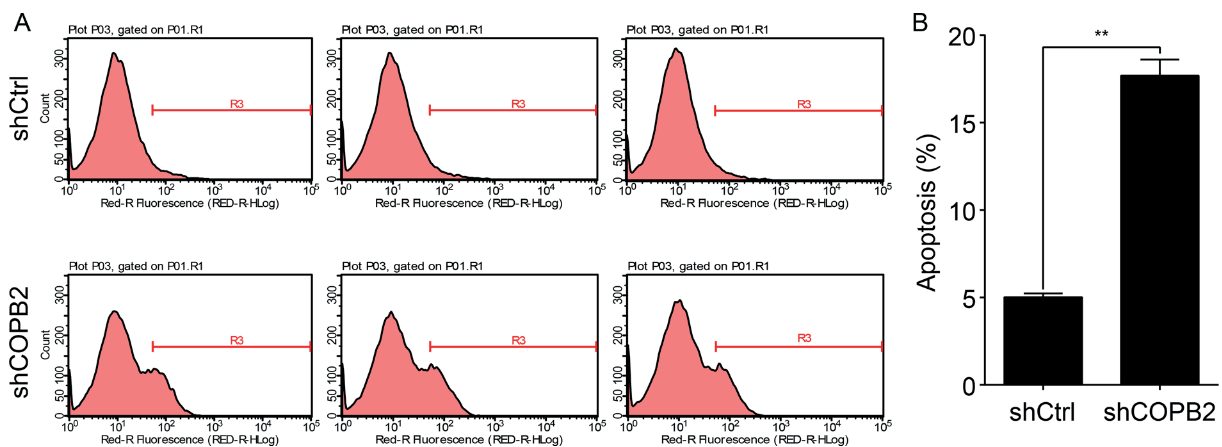

Figure 4. Knockdown of COPB2 induced cells to undergo apoptosis. ***A,*** Measurements of the fluorescent Annexin V stain indicative of apoptotic cells at early stage. ***B,*** Proportion of cells undergoing apoptosis was markedly greater in cells infected with COPB2-siRNA compared to those infected with a control plasmid (**$p < 0.01$).

seemingly induced by the knockdown of COPB2, possibly by arresting cells in the G1 cell-cycle phase. COPB2 has a tryptophan-aspartic acid (WD)-repeat motif and belongs to a conserved WD protein family in all eukaryotes and is implicated in a variety of functions, including regulation of cell cycle control and apoptosis[33,34]. Accordingly, the COPB2-knockdown designates to the oligopeptides, which bound to cholangiocarcinoma cells and the target molecules, interacts with the clathrin heavy chain.

Therefore, COPB2 appears to be an attractive target for molecular therapies against cholangiocarcinomas. However, to our knowledge, there is no information about the relationship between cell cycle arrest and cell cycle-related molecules by down-regulation of COPB2. Thus, further investigation of COPB2 knockdown may lead to the elucidation of the potential molecular mechanisms of apoptosis.

Conclusions

We have highlighted the potential roles that COPB2 has in the tumorigenesis of human cholangiocellular carcinomas. The knockdown of COPB2 suppressed the proliferation and enhanced the apoptosis of cholangiocarcinomas, whilst promoted the arrest of cell-cycle progression. Altogether, COPB2 may have a potential basis for targeted therapies for the treatment of cholangiocellular cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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