Neoplasma 2022; 69(5): 1101–1107

doi:10.4149/neo_2022_220324N333

SPARC induces M2 polarization of macrophages to promote proliferation, migration, and angiogenesis of cholangiocarcinoma cells

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Received March 24, 2022 / Accepted August 5, 2022

Cholangiocarcinoma (CCA) is a disease that includes a variety of epithelial neoplasms characterized by the differentiation of cholangiocytes. M2 polarization is imperative to the development of CCA cells. In this study, we investigated the influence of secreted protein acidic and rich in cysteine (SPARC) on M2 polarization and CCA cell growth. We found that the SPARC level was amplified in M2-polarized macrophages and TAMs. In addition, the downregulation of SPARC prevented the M2 polarization of macrophages. Silencing SPARC inhibited the M2 macrophage-mediated effects on the proliferation, migration, and angiogenesis of CCA cells. Additionally, SPARC knockdown blocked the M2 polarization of macrophages by inhibiting the PI3K/AKT signaling. Moreover, an activator of PI3K signaling repressed the effect of SPARC knockdown on the M2 macrophage-induced elevation of proliferation, migration, and angiogenesis in CCA cells. In conclusion, SPARC contributes to the M2 polarization of macrophages to promote proliferation, migration, and angiogenesis of CCA cells, which provides new insight into the treatment of CCA.

Key words: cholangiocarcinoma, SPARC, M2 polarization, PI3K/AKT signaling

Cholangiocarcinoma (CCA) is a malignant tumor of epithelial cells originating at different sites in the biliary tree [1]. In recent years, the incidence and mortality of CCA have significantly increased [2], which has significantly increased the interest of researchers in the pathogenesis of CCA [3]. In clinical work, surgical excision combined with drug therapy is the first treatment choice. However, CCA is generally less sensitive to drugs due to its complex vascular structure, rich lymphoid tissue, and the highly growth-promoting effect of nerve fibers [4]. The five-year survival rate for most CCA patients is very low [5]. Therefore, it is necessary for us to thoroughly study the pathogenesis of CCA and find suitable therapeutic targets as soon as possible, thus providing new possibilities for the treatment of CCA.

Tumor-associated macrophages (TAMs) are immune cells that infiltrate and activate around tumors and have strong tumor-promoting and immunosuppressive characteristics [6, 7]. Macrophages can change their polarization patterns in response to environmental signals. Previous studies have shown that macrophages can be polarized into two different conditions: a proinflammatory (M1) phenotype and an alternatively activated (M2) phenotype. Among them, M1 macrophages show proinflammatory and antitumor features. However, M2 macrophages are primarily stimulated by interleukin (IL)-4 or IL-13 and have a tumor-stimulating effect. The markers of M1 macrophages are iNOS and CD86, and the markers of M2 macrophages are Arg-1 and CD206 [8–10]. TAMs are involved in the development of various malignant tumors. For instance, Wang et al. demonstrated that TAMs induce cell growth in human hepatocellular carcinoma [11].

Secreted protein acidic and rich in cysteine (SPARC) encodes a cysteine-rich acidic matrix-associated protein [12]. SPARC is associated with a variety of cell biological behaviors, such as cell form, growth, and migration [13]. In addition, SPARC also plays a significant role in cancer development [14]. For instance, Lien et al. showed that the abundance of SPARC is elevated in breast cancer, and SPARC was linked with breast cancer cell epithelial-mesenchymal transition (EMT) [15]. Moreover, Gao et al. reported that the SPARC content is enhanced in liver cancer cells and that SPARC stimulates cell growth in liver cancer [12]. Furthermore, Ma et al. showed that SPARC increases cell 5-FU sensitivity in gastric cancer by controlling cell EMT and apoptosis [16]. Therefore, SPARC may serve as a therapeutic target for

pancreatic cancer [17]. However, the roles of SPARC in CCA are unclear and are the focus of this study. We also investigated the signaling pathway regulated by SPARC.

PI3K/AKT signaling is a key cell signaling pathway during the cell cycle. It has been linked to cellular stasis, growth, tumors, and longevity [18]. Xie et al. demonstrated that the PI3K/Akt signaling pathway is related to considerable erythropoiesis and glucose metabolism [18]. In addition, the PI3K/Akt pathway is essential for cell growth and apoptosis and plays a critical role in the expansion of cancers [19]. Here, we explore the role of PI3K/AKT signaling in CCA.

In this work, we investigate the influences of SPARC on the polarization of macrophages and demonstrate its effect on the proliferation, migration, and angiogenesis of CCA cells. We show that SPARC can facilitate the M2 polarization of macrophages. Furthermore, we demonstrate that knockdown of SPARC inhibits M2 macrophage-mediated effects on proliferation, migration, and angiogenesis of CCA cells, providing a promising target for CCA therapy.

Materials and methods

Cell culture and transfection. In this paper, a human peripheral blood monocyte cell line (THP-1), human cholangiocarcinoma cell lines (HCCC9810 and RBE), and human umbilical vein endothelial cells (HUVECs) were purchased from Procell (Wuhan, China) and cultured according to their guidelines. THP-1 cells were differentiated into M0 macrophages after treatment with PMA (100 ng/ml; Solarbio, Beijing, China) for 24 h. TAMs were generated when M0 macrophages were co-cultured with HCCC9810 cells for 48 h. To generate M2 polarization, M0 macrophages were exposed to IL-4 (20 ng/ml; Solarbio) and IL-13 (20 ng/ml; Solarbio) for 48 h.

Small interfering RNA (siRNA) binding SPARC (si-SPARC, 5'-AACAAGACCUUCGACUCUUCC-3'), control (si-NC, 5'-GCUCACAGCUCAAUCCUAAUC-3'), and 740 Y-P (an activator of PI3K signaling) were obtained from RiboBio (Guangzhou, China). When cells reached 70% confluence, Lipofectamine 3000 (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to carry out transfection according to the manufacturer's instructions. After incubation for 15 min at room temperature, the siRNA-lipid complex was added to M2-polarized macrophages. Then, gene expression was detected 48 h later. To activate the PI3K-Akt signaling pathway, M2-polarized macrophages were treated with 740 Y-P (30 μM; Selleck, Shanghai, China) for 24 h prior to transfection.

Table 1. Primers for PCR.

Name		Primers for PCR (5'-3')
iNOS	Forward	ACAGGAGGGGTTAAAGCTGC
	Reverse	GAGGCTCCGATCAATCCAGG
CD86	Forward	CCTTCCTGCTCTCTGCTAACTT
	Reverse	AAAGCCGCGTCTTGTCAGTT
Arg-1	Forward	ACTTAAAGAACAAGAGTGTGATGTG
	Reverse	CATGGCCAGAGATGCTTCCA
CD206	Forward	ACCTGCGACAGTAAACGAGG
	Reverse	TGTCTCCGCTTCATGCCATT
SPARC	Forward	TTCGGCATCAAGCAGAAGGAT
	Reverse	TGTCTCCAGGCAGAACAACA
GAPDH	Forward	TCCCATCACCATCTTCCAGG
	Reverse	GATGACCCTTTTGGCTCCC

Reverse transcription-quantitative PCR (RT-qPCR). Whole RNA was separated via TRIzol (Invitrogen). Next, the RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent Kit (Thermo Fisher Scientific, Cleveland, OH, USA). The abundance of genes was measured with RT-qPCR by PowerUp SYBR Green Mix (Applied Biosystems, Foster City, CA, USA). The primers are listed in Table 1. GAPDH served as an internal control. The relative content of the gene was standardized via the $2^{-\Delta\Delta CT}$ method.

Western blot. The cells were lysed with RIPA lysis buffer (Invitrogen), and then the supernatants comprising proteins were collected. The protein abundance was examined by a BCA reagent (Solarbio). Next, 50 μg of protein was electrophoresed in a 12% SDS-PAGE gel and transferred to a PVDF membrane (Invitrogen). After blocking, the membrane was incubated with primary antibodies at 4 °C. The antibodies were as follows: anti-SPARC (ab207743; Abcam, Cambridge, MA, USA), anti-p-PI3K (17366S; Cell Signaling Technology, Boston, MA, USA), anti-PI3K (ab86714; Abcam), anti-p-Akt (AF0016; Affinity Biosciences, Changzhou, China), anti-Akt (AF6261; Affinity Biosciences), and anti-GAPDH (AF7021; Affinity Biosciences). The membrane was then exposed to goat anti-rabbit IgG (S0001; Affinity Biosciences) for 1 h. The protein band was measured using an enhanced chemiluminescence kit (Invitrogen) and analyzed using ImageJ software.

Conditioned medium preparation. M2 macrophages were cultivated for 48 h, and the culture medium was collected as the M2 macrophage-conditioned medium (M2-CM). In addition, si-SPARC or si-NC was transfected into M2 macrophages, and the culture medium was collected 48 h later as si-SPARC/M2-CM or si-NC/M2-CM. The CM was stored at –80 °C until use. With or without 740 Y-P pretreatment, si-SPARC or si-NC was transfected into M2-polarized macrophages, and the expression of each gene was analyzed 48 h later.

Cell Counting Kit-8 (CCK8) assay. To evaluate cell viability, we seeded HCCC9810 and RBE cells (2.0×10^3/well) in 96-well plates. HCCC9810 and RBE cells were treated with different conditioned media for 48 h, and 10 μl of CCK-8 solution (Solarbio) was added to each well. After incubation for 2 h, the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

5-ethynyl-2'-deoxyuridine (EdU) assay. HCCC9810 and RBE cells were individually seeded in 24-well plates after

treatment with different conditioned media for 48 h. An EdU kit (Solarbio) was used to evaluate cell proliferation according to the manufacturer's instructions. Pictures were captured through a laser confocal microscope (Leica, Wetzlar, Germany). Nuclei were dyed with EdU (Solarbio) and DAPI (Solarbio) in order to measure cell proliferation.

Wound healing assay. HCCC9810 and RBE cells were exposed to different treatments for 24 h and then separately seeded in 6-well plates. The wound was made by applying the point of 10 µl pipette tips. Pictures of migrated cells were captured by phase-contrast microscopy (Leica) after 24 h.

Tube formation assay. To scrutinize cell angiogenesis ability, a tube formation assay was carried out. Matrigel matrix (Invitrogen) was equally decentralized in 24-well plates and incubated for 1 h. After treatment with different conditioned media for 48 h, HUVECs (1×10^5 cells/well) were plated on the matrix surface and cultivated for 24 h. Finally, the capillary-like structure was counted using an inverted microscope (Leica), and ImageJ software was used to analyze the branches.

Statistical assay. The data analyses were performed using SPSS 22.0. Quantitative statistics were scrutinized by Student's t-test or analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test. All figures are displayed as the means ± SD, and $p<0.05$ was categorized as significant.

Results

The expression of SPARC is elevated in M2-polarized macrophages and TAMs. To determine the association between SPARC content and CCA growth, the expression of SPARC in M0-polarized macrophages, M2-polarized macrophages, and TAMs was investigated. We observed that the contents of iNOS and CD86, markers of M1-polarized macrophages, were exceptionally reduced in the M2 polarization and TAM groups versus the M0 polarization group (Figure 1A). The levels of Arg-1 and CD206, markers of M2-polarized macrophages, were clearly augmented in the M2 polarization and TAM groups relative to the M0 polarization group (Figure 1A). These results demonstrated that we successfully induced M0-polarized macrophages, M2-polarized macrophages, and TAMs. In addition, the SPARC content was enhanced in M2-polarized macrophages and TAMs in contrast to the M0 polarization group (Figure 1B). Taken together, these results demonstrated that SPARC was highly expressed in M2-polarized macrophages and TAMs.

Knockdown of SPARC inhibits the M2 polarization of macrophages. To evaluate the influence of SPARC on macrophage polarization, si-SPARC or si-NC was transfected into M2-polarized macrophages, and gene expression was detected 48 h later. The abundance of SPARC was diminished by si-SPARC transfection (Figures 2A, 2B). Thus, we successfully silenced SPARC in M2-polarized macro-

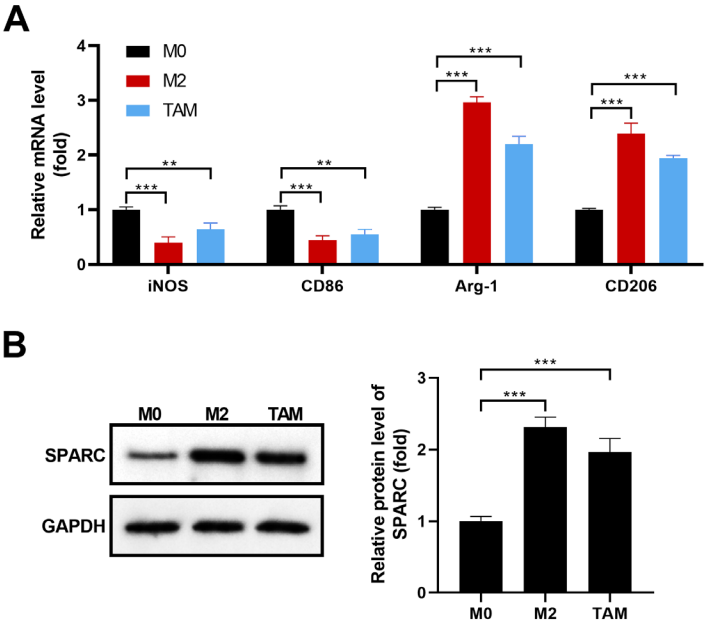

Figure 1. SPARC content is enhanced in M2 macrophages and TAMs. A) The iNOS, CD86, Arg-1, and CD206 expression levels were measured by RT-qPCR. B) SPARC expression was analyzed by western blot. **p<0.01, ***p<0.001

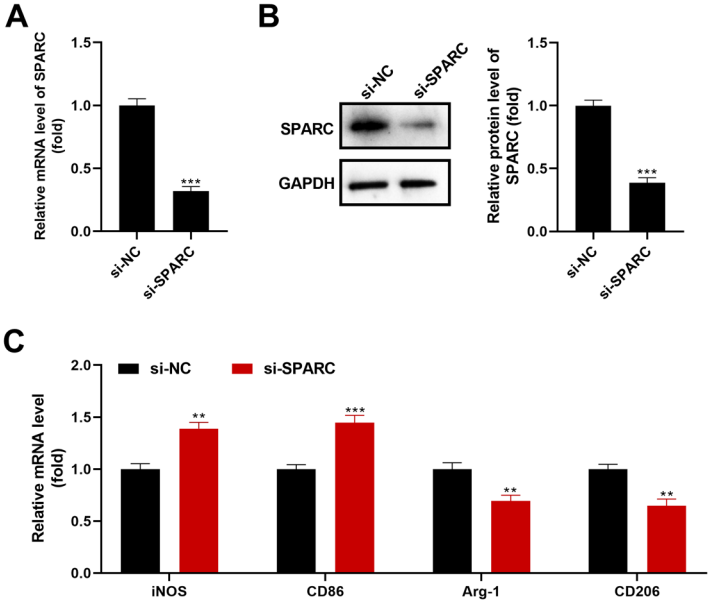

Figure 2. Silencing of SPARC inhibits the M2 polarization of macrophages. A, B) SPARC abundance was evaluated by RT-qPCR and western blotting. C) The iNOS, CD86, Arg-1, and CD206 levels were measured by RT-qPCR. **p<0.01, ***p<0.001

phages. After si-SPARC transfection, iNOS and CD86 levels were enhanced, but Arg-1 and CD206 levels were reduced in M2-polarized macrophages (Figure 2C). These results demonstrate that the downregulation of SPARC inhibited the M2 polarization of macrophages.

Knockdown of SPARC inhibits the M2 macrophage-mediated effects on the proliferation, migration, and angiogenesis of CCA cells. Next, we examined the effect of SPARC on CCA cells. In this part, HCCC9810, RBE, and HUVECs were treated with control, M2-CM, si-SPARC/M2-CM, and si-NC/M2-CM. The proliferation of CCA cells (HCCC9810 and RBE) was amplified by M2-CM treatment, but lessened by si-SPARC/M2-CM treatment (Figures 3A, 3B). In addition, the migration of CCA cells (Figure 3C) and angiogenesis function of HUVECs (Figure 3D) were both enhanced after M2-CM treatment but these effects were weakened by si-SPARC transfection. These results demonstrated that silencing SPARC lessened the M2 macrophage-induced influences on cell proliferation, migration, and angiogenesis.

Knockdown of SPARC reduces the M2 polarization of macrophages by inhibiting the PI3K/AKT signaling. Next, we investigated the signaling pathway involved in the SPARC-induced polarization of macrophages. After pretreatment with 740 Y-P, si-SPARC or si-NC was transfected into M2-polarized macrophages, and the content of each gene was detected 48 h later. The ratios of p-P13K/P13K and p-Akt/Akt were elevated in M2-polarized macrophages versus M0 and TAM groups, but these effects were abolished by si-SPARC transfection (Figure 4A). Moreover, 740 Y-P treatment weakened the inhibitory effect of si-SPARC transfection in M2-polarized macrophages (Figure 4A). In addition, 740 Y-P treatment also blocked the effects of SPARC knockdown on iNOS, CD86, Arg-1, and CD206 contents in M2-polarized macrophages (Figure 4B). Therefore, we suggest that silencing SPARC represses the M2 polarization of macrophages by inhibiting the PI3K/AKT signaling.

Activation of the PI3K/AKT signaling blocks the effect of SPARC silencing on the M2 macrophage-mediated promotion of proliferation, migration, and angiogenesis in CCA cells. To determine whether SPARC regulates M2 macrophage-mediated effects on CCA cells by modulating PI3K/AKT signaling, we conducted rescue experiments. HCCC9810, RBE, and HUVECs were individually treated with si-NC/M2-CM, si-SPARC/M2-CM, or 740 Y-P + si-SPARC/M2-CM. CCA cell proliferation (Figures 5A, 5B) and migration (Figure 5C) were decreased by si-SPARC/M2-CM treatment but improved by 740 Y-P co-treatment. Additionally, the angiogenic ability of HUVECs was inhibited after si-SPARC/M2-CM treatment, but this effect was inhibited by 740 Y-P (Figure 5D). Therefore, we confirmed that activation of the PI3K/AKT signaling repressed the effect of SPARC knockdown on M2 macrophage-induced facilitation of CCA cell development.

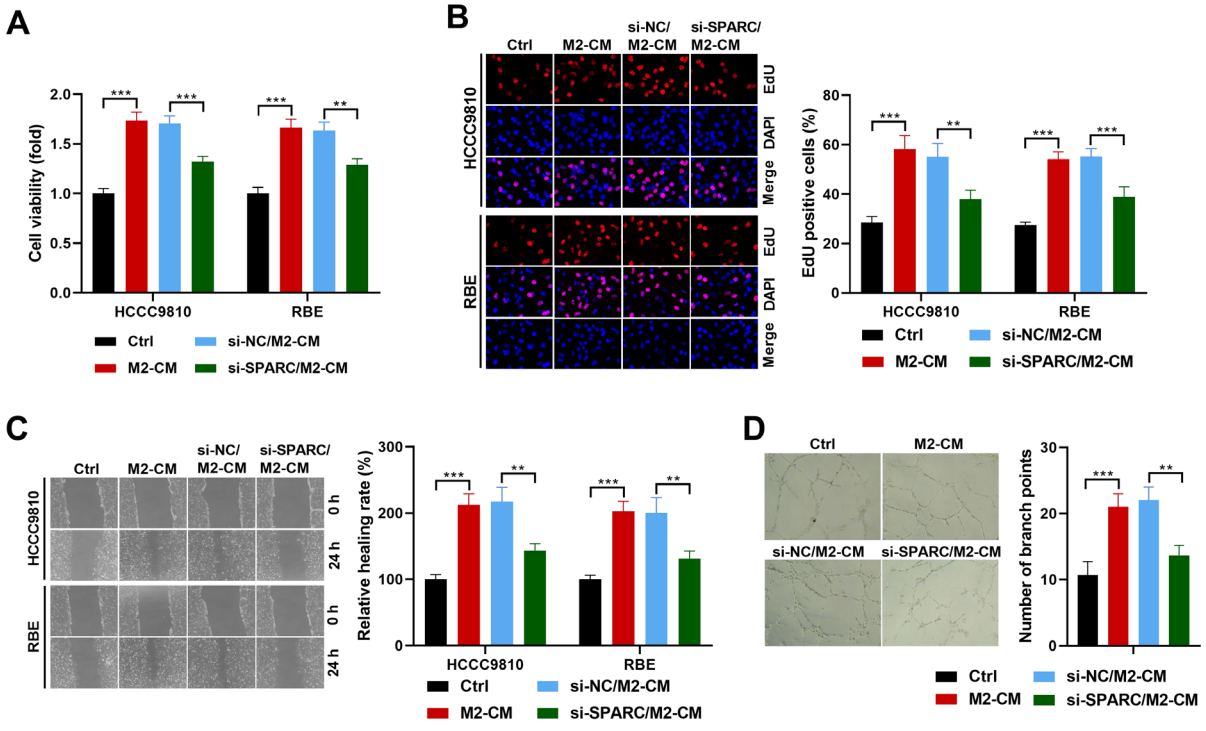

Figure 3. Silencing of SPARC inhibits the M2 macrophage-mediated effects on CCA cells. A) Cell viability was measured using a CCK-8 assay. B) Cell proliferation was examined using an EdU assay. C) Cell migration was analyzed using a wound healing assay. D) Cell angiogenesis was examined using a tube formation assay. *p<0.05, **p<0.01, ***p<0.001

Discussion

CCA is aggressive cancer. To make matters worse, most CCA patients are already at an advanced stage at diagnosis, missing the best time for treatment, and their treatment options are limited [20]. There are many risk factors for CCA, including hepatic steatosis, cirrhosis, gallstone disease, parasitic infection, inflammatory disease, smoking, alcohol consumption, obesity, and genetic factors [21]. Most CCA patients have a poor prognosis even when diagnosed at an early stage [22]. To reduce the global mortality rate of CCA, multifaceted efforts are needed. At present, the treatment focus of CCA is mainly on prevention, early diagnosis of high-risk populations, and molecular targeted therapy for confirmed diseases. At present, some molecular targeted drugs are being actively studied, such as small-molecule inhibitors of FGFRs, including derazantinib, TAS-120, Debio 1347, and INCB054828 [23]. In addition, ponatinib has also displayed encouraging efficacy in patients with CCA [24]. In this paper, we investigated the role of SPARC in CCA.

Previous studies have shown that SPARC plays an important role in a variety of cancers. Li et al. showed that SPARC is a hub gene in stomach adenocarcinoma (STAD), and the abundance of SPARC predicted a poor prognosis of STAD

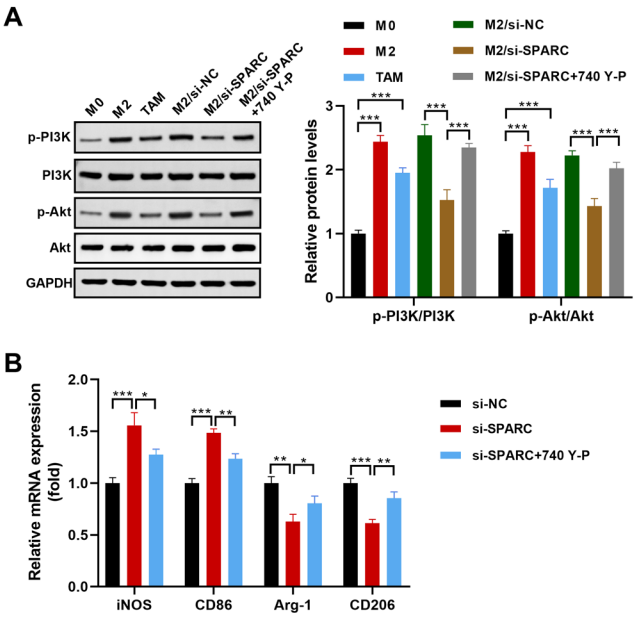

Figure 4. Silencing of SPARC reduces the M2 polarization of macrophages by inhibiting PI3K/AKT signaling. A) The activity of PI3K/AKT signaling was analyzed using western blot. B) The iNOS, CD86, Arg-1, and CD206 levels were analyzed by RT-qPCR. *p<0.05, **p<0.01, ***p<0.001

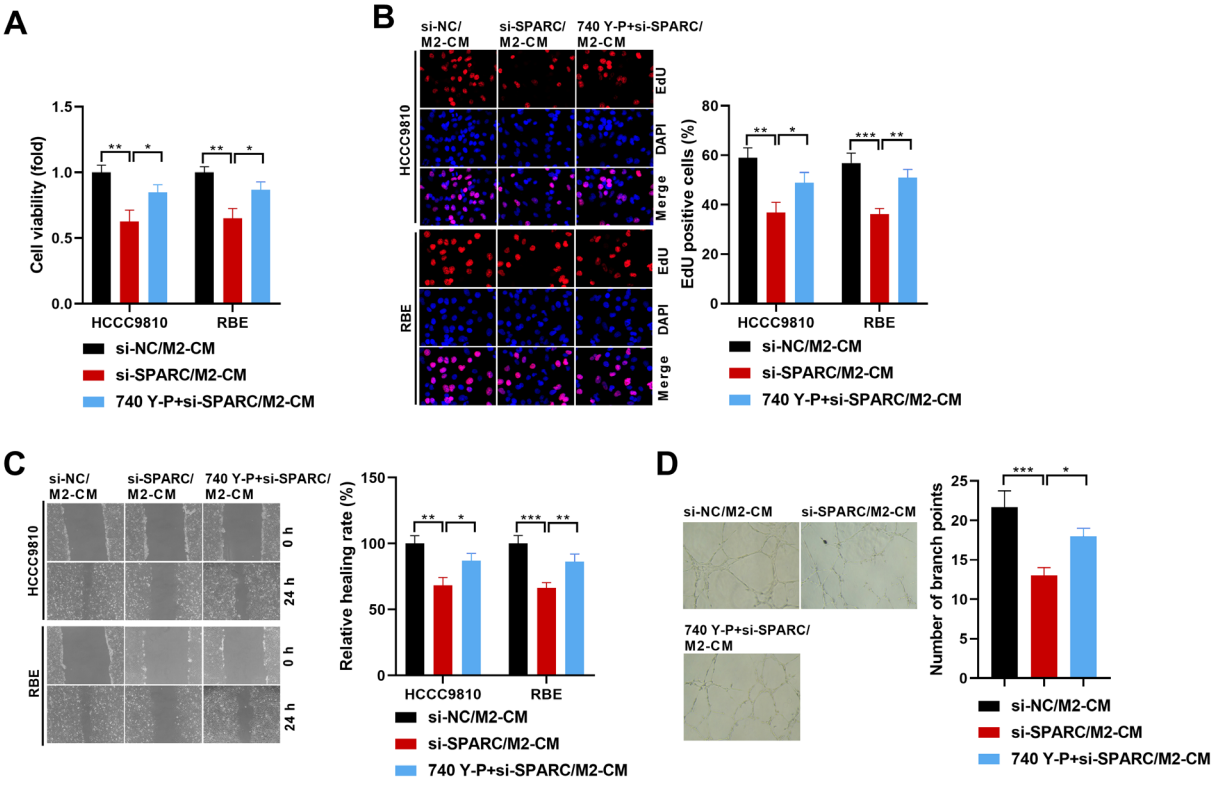

Figure 5. Inhibition of PI3K/AKT signaling blocks the influence of SPARC silencing on the M2 macrophage-mediated effects on CCA cells. A) Cell viability was analyzed using a CCK-8 assay. B) Cell proliferation was analyzed using an EdU assay. C) Cell migration was assessed using a wound healing assay. (D) Cell angiogenesis was examined using a tube formation assay. *p<0.05, **p<0.01, ***p<0.001

[25]. In addition, Carriere et al. demonstrated that SPARC treatment of HCT116 cells from colon adenocarcinoma enhanced the regulation of PTHrP and E-cadherin expression and cell migration [26]. In addition, Gao et al. showed that the expression of SPARC mRNA is elevated in liver hepatocellular carcinoma (LIHC) tumor tissues, and upregulated SPARC may facilitate LIHC cell growth. However, the serum SPARC content was shown to be lower in LIHC patients than in healthy controls [12]. Moreover, Chen et al. reported that the content of SPARC is enhanced in ovarian serous cystadenocarcinoma (OV) tissues and that silencing SPARC prominently repressed OV cell growth, invasion, and metastasis, but expedited cell apoptosis [27]. Furthermore, Menon et al. showed that SPARC is upregulated in gliomas (grades II–IV) and contributes to cell invasion *in vitro* [28]. In our study, downregulation of SPARC inhibited the M2 polarization of macrophages but did not completely reverse the phenotype of macrophages. In our future work, we will investigate in more depth the molecular regulatory mechanism by which SPARC regulates macrophage polarization.

According to Johannes et al., the abundance of SPARC is heightened in the distal CCA, which was linked with lymph node metastasis [29]. In addition, Deng et al. confirmed that the SPARC content is increased in CCA tissues and cells, and SPARC elevated CCA cell proliferation, metastasis, and EMT [30]. In addition, a lack of SPARC was linked with EMT and low differentiation conditions in biliary tract cancer [31]. However, Hu et al. showed that SPARC upregulation in M2-type macrophages can inhibit M2-mediated promotion of growth, migration, and antiapoptotic effects in gastric cancer (GC). SPARC is a key tumor suppressor in GC, which demonstrates the high specificity of SPARC in different cancers [32]. Here, we showed that the expression of SPARC was increased in M2-polarized macrophages and TAMs, which is similar to the results of Johannes et al. and Deng et al. [29, 30]. In addition, we first discovered that the knockdown of SPARC inhibited the M2 polarization of macrophages. Moreover, a lack of SPARC inhibited M2 macrophage-mediated effects on the proliferation, migration, and angiogenesis of CCA cells, which is similar to the results of Deng et al. [30]. Therefore, we continued to study the signaling pathways that SPARC may regulate in CCA.

PI3K-AKT signaling has a critical significance for the course of many cancers. Yue et al. showed that the SPARC content is enhanced in oral squamous cell carcinoma (OSCC) tissues and that SPARC can activate PI3K/AKT signaling to facilitate OSCC cell growth and metastasis [33]. Deng et al. also demonstrated that SPARC facilitated CCA cell proliferation, metastasis, and EMT by activating PI3K-AKT signaling [30]. In this paper, we showed that the downregulation of SPARC inhibited the M2 polarization of macrophages by hindering the PI3K/AKT signaling. Moreover, the activation of PI3K/AKT signaling curbed the influence of SPARC knockdown on M2 macrophage-mediated promotion of proliferation, migration, and angiogenesis in CCA cells, which is in agreement with the results of Deng et al. and Yue et al. [30, 33].

In summary, we demonstrated that the SPARC content was amplified in M2-polarized macrophages and TAMs. Moreover, SPARC induced the M2 polarization of macrophages. SPARC modulated M2 macrophage-mediated effects on the proliferation, migration, and angiogenesis of CCA cells via the PI3K/AKT signaling. Our research shows that SPARC might be a prospective therapeutic target for CCA.

Acknowledgments: This research was supported by the Yunnan Provincial High-Level Medical Technical Talents Training Project (H-2019038), Joint project of Yunnan Provincial Department of Science and Technology and Kunming Medical University on applied basic research (202101AY070001-233), Scientific Researching Fund of First People's Hospital of Yunnan Province (KHBS-2020-019), Yunnan Provincial High-level Medical Technical Talents Training Project (L-2019016), The National Natural Science Foundation of China (No. 81960514), The Young and Middle-aged Academic and Technical Leaders in Yunnan Province/Reserve Talent Training (Program No. 202005AC160017), Open Project of Gastroenterology Clinical Medicine Center (No.2021LCZXXF-XH02), The Project of Kunming Medical joint (No. 2019FE001-010).

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