INTRODUCTION

More than 1,090,000 new cases of colon cancer (CC) are diagnosed annually, of which approximately 50% die due to the high rate of metastasis (Siegel et al., 2017; Bray et al., 2018). With changes in people’s lifestyle and diet, new cases of CC and young patients are increasing year by year (Zheng et al., 2017). Today, the mainstream of treatment for CC is segmental or total colectomy followed by anastomosis, possibly with the introduction of adjuvant chemotherapy if necessary (Benson et al., 2017; Sun et al., 2020). However, high recurrence/metastasis rates after surgery have not been effectively solved, which is a future direction for CC management.

Circular RNAs (circRNAs) take part in the occurrence, development, metastasis and invasion of CC, and confer diagnostic and prognostic values (Zhao et al., 2019; Han et al., 2021). Indeed, circRNAs are newly discovered in a variety of cells (Wang et al., 2022). Currently, the interaction between circRNAs and miRNAs has been a study focus (Lux & Bullinger, 2018). miRNAs primarily act on target mRNA 3’UTR and are key regulatory roles in CC (Wang et al., 2022; Siciliano et al., 2013; Hollis et al., 2015). Circ-POLA2, a cancer-associated circRNA, has been clarified to express abnormally in endometrial cancer (Fang et al., 2021), acute myeloid leukemia (Li et al., 2021), cervical squamous cell carcinoma (Cao et al., 2020) and lung cancer (Fan et al., 2020). But its relationship with CC has not been reported. Through multiple bioinformatics tools, miRNA (miR-138-5p) and mRNA (SEMA4C) of circ-POLA2 were predicted. miR-138-5p has been confirmed to have decreased expression in colorectal cancer (CRC) and have anti-tumor effects (Wei et al., 2021) and SEMA4C has been illustrated to promote CC progression (Hung et al., 2022). Based on this, circ-POLA2 is likely to competitively upregulate SEMA4C by sponging miR-138-5p, thereby participating CC. Here, circ-POLA2/miR-138-5p/SEMA4C axis-related function and mechanisms were explored, finding new insights into CC treatment.

METHODS

Clinical samples

Following Declaration of Helsinki, the study procedures proceeded with approval of the Ethics Committee of The First Affiliated Hospital of Baotou Medical College, Inner Mongolia University of Science and Technology. All patients has informed of the study and signed written informed consent. CC tumor tissues and adjacent tissues were operationally collected from 40 patients (19 males and 21 females) and immediately frozen in liquid nitrogen. None of patients received preoperative chemotherapy or radiotherapy. Patients with other intestinal diseases, primary cancers, or organ failure were excluded.

Cell lines and culture

SW480, SW620, HT29, and HCT116 were human CC cell lines purchased from ATCC, and NCM460 was normal human colonic mucosal epithelial cell line provided by INCELL Corporation LLC. In a Dulbecco’s modified Eagle’s medium made of 10% fetal bovine serum (Thermo Fisher Scientific, USA), 100 IU/mL penicillin (SigmaAldrich, USA), and 100 μg/mL streptomycin (SigmaAldrich), the cell lines were maintained.

Cell transfection

Short hairpin RNA (shRNA) stably against circ-POLA2 (sh-POLA2), circ-POLA2 overexpression (oe-POLA2), miR-138-5p mimic, miR-138-5p inhibitor, pcDNA-SEMA4C, and corresponding controls were obtained from Ribobio (Guangzhou, China). After Lipo...
fectamine 2000 (Invitrogen)-guided transient transfection, cells were amasses after 24 h.

**CCK-8**

On the 96-well plates, each well was covered with 5×10^4 SW480 cells and added with 10 μL of CCK-8 solution (Beyotime) after 72 h. The absorbance at 450 nm was recorded on a microplate reader (Tecan Sunrise, Mannedorf, Switzerland) after 4 h.

**Clone formation**

On the 6-well plates, each well was filled with 1×10^5 SW480 cells for 14 d until staining with 1% crystal violet (Corning). The stained colonies were counted using a microplate reader (Tecan Sunrise).

**Transwell**

SW480 cells (2×10^5) were seeded into upper chambers (Corning) containing serum-free medium. Matrigel (BD Biosciences, USA) was only coated for testing invasion. Fresh medium containing 10% FBS (Thermo Fisher Scientific) in the lower chamber aimed to attract cell movement. The transmembrane cells during 24 h were conditioned to crystal violet dyeing (0.1%) and microscopical counting.

**Flow cytometry**

On 1×10^6 SW480 cells, apoptosis was detected by the AnnexinV-fluorescein isothiocyanate/propidium iodide kit (BD Pharmingen, USA) and the apoptosis rate was measured by a BD FACS Aria (BD Biosciences).

**RT-qPCR**

All tissues and cells were processed for extracting total RNA using Trizol® reagent (Thermo Fisher Scientific). After determination of RNA quality and concentration by NanoDrop 2000 (Thermo Fisher Scientific), reverse transcription was done with SuperScript IV VILO kit (Invitrogen) and All-in-One First-Strand cDNA Synthesis kit (GeneCopeia, Guangzhou, China). With ABI SYBR Green Master Mix (Invitrogen), PCR was carried out to measure gene expression according to the calculation method 2-ΔΔCT. The primer sequences are shown in Table 1. The transmembrane cells during 24 h were conditioned to crystal violet dyeing (0.1%) and microscopical counting.

**Immunoblotting**

Sample lysates collected by RIPA lysis buffer (Pierce, USA) were measured by BCA assay kit (Beyotime, China). Proteins were separated by 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beyotime), loaded onto a polyvinylidene fluoride membrane (Millipore), after which 5% nonfat dry milk was added, and primary antibodies SEMA4C (1:10000; sc-136445; Santa Cruz Biotechnology) and GAPDH (1:1000; ab8245; Abcam), and horseradish peroxidase-conjugated secondary antibody (1:5000; sc-2054; Santa Cruz Biotechnology) were supplemented. Protein levels were detected by Bio-Rad Gel Imaging System using enhanced chemiluminescence (Thermo Fisher Scientific).

**Dual-luciferase reporter gene assay**

pmiR-GLO (Promega) was inserted with the amplified wild-type or mutant sequences (POLA2-WT, SEMA4C-WT, POLA2-MUT, SEMA4C-MUT) and co-transfected with miR-138-5p or miR-NC into SW480 cells with Lipofectamine 2000 (Thermo Fisher Scientific), and the luciferase activity was detected using a dual-luciferase reporter gene detection system (Genomeditech, Shanghai, China).

**RNA pull-down**

Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) was used. SW480 cells (1×10^5) were lysed in RIP lysis buffer (Thermo Fisher Scientific), followed by interaction with circ-POLA2 probe-coated C-1 magnetic beads. The final results were obtained by RT-qPCR.

**Statistical analysis**

SPSS 21.0 software and GraphPad Primer 6.0 were employed to perform statistical analysis. All data presented as mean ± standard deviation were analyzed by Student's t-test and one-way ANOVA. The Kaplan-Meier method was utilized to assess survival and analyzed by the Log rank test while Pearson analysis was to analyze the gene correlation. P<0.05 was considered statistically significant.

## RESULTS

### circ-POLA2 is associated with CC progression

High circ-POLA2 was expressed in CC patients’ tumor tissues (Fig. 1A), and circ-POLA2 expression was further increased in CC patients’ tumor tissues in TNM stage III and with lymph node metastasis (Fig. 1B, C). CC patients were divided into high or low circ-POLA2 expression groups defined by circ-POLA2 median value, and Kaplan-Meier analysis evaluated that patients with high circ-POLA2 expression had lower overall survival (Fig. 1D). These findings suggest that circ-POLA2 is up-regulated in tumor tissues of patients with CC and is associated with poor prognosis.

### Circ-POLA2 knockdown inhibits activities of CC cells

Circ-POLA2 in CC cells and normal colon epithelial cells was detected by RT-qPCR. The results showed that circ-POLA2 expression was increased in CC cell lines (SW480, SW620, HT29 and HCT116), and circ-

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**Table 1. Sequences used for PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences (5’-3’)</th>
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<tbody>
<tr>
<td>circ-POLA2</td>
<td>Forward; TGAGCTTGTAGTGAGTGGT</td>
</tr>
<tr>
<td></td>
<td>Reverse; GCAAGGGAGAATTGCGAGATGG</td>
</tr>
<tr>
<td>miR-138-5p</td>
<td>Forward; CGAGCTGGTGTTGTGTTGAAATC</td>
</tr>
<tr>
<td></td>
<td>Reverse; GCAGGGTGCCAGGTATTC</td>
</tr>
<tr>
<td>SEMA4C</td>
<td>Forward; ACCTTGTGCCGCGTAAGACAG</td>
</tr>
<tr>
<td></td>
<td>Reverse; CTGCAAGCTGTCAGTCAAGGAAGA</td>
</tr>
<tr>
<td>U6</td>
<td>Forward; CTGCTTTGCGCCGACCA</td>
</tr>
<tr>
<td></td>
<td>Reverse; AACGCTTCAAGAATTTCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward; CACCCACTCTCCCATCTTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse; CACCCCACCTGTGGCTGTAG</td>
</tr>
</tbody>
</table>

Note: circ-POLA2, circular RNA POLA2; miR-138-5p, microRNA-138-5p; SEMA4C, Semaphorin 4C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase
Circ-POLA2-mediated miR-138-5p/SEMA4C axis affects colon cancer

POLA2 expression was most significantly increased in SW480 cells (Fig. 2A). Therefore, SW480 cells were analyzed for subsequent experiments. After transfection of sh-POLA2 or oe-POLA2, circ-POLA2 in SW480 cells decreased or increased accordingly (Fig. 2B), indicating that the cells were successfully transfected. CCK-8 assay, colony formation assay, Transwell assay, and flow cytometry were then performed to explore the role of circ-POLA2 in CC cells. CCK-8 results showed that circ-POLA2 knockdown inhibited SW480 cell activity (Fig. 2C). Meanwhile, colony formation experiments determined that circ-POLA2 knockdown inhibited the proliferation of SW480 cells (Fig. 2D, E). Transwell analysis presented that circ-POLA2 knockdown inhibited SW480 cell migration and invasion (Fig. 2F, G). In addition, flow cytometry suggested that circ-POLA2 knockdown promoted apoptosis of SW480 cells (Fig. 2H, I). However, the effect of circ-POLA2 overexpression on CC cells was opposite to that of circ-POLA2 knockdown (Fig. 2C–I). In conclusion, circ-POLA2 plays a carcinogenic role by promoting proliferation, migration, and invasion of CC cells and inhibiting cell apoptosis.

Circ-POLA2 confers mediation of miR-138-5p

miR-138-5p was cross-screened on bioinformatics websites (CircBank, starBase, miRanda, and RNAhybrid) to be the potential miRNA of circ-POLA2 (Fig. 3A). Measurements of luciferase activity demonstrated that miR-138-5p inhibited the luciferase activity of POLA2-WT (Fig. 3B) and RNA pull-down test discovered that circ-POLA2 significantly increased miR-138-5p enrichment (Fig. 3C). Actually, miR-138-5p expression kept lowly in CC patients' tumor tissues (Fig. 3D), which was in an inverse correlation with circ-POLA2 expression (Fig. 3E). Likewise, miR-138-5p expression...
5p expression was downregulated in CC cells lines (SW480, SW620, HT29, and HCT116) (Fig. 3F). While miR-138-5p was up-regulated in SW480 cells intervened with sh-POLA2 and down-regulated in those transfected with oe-POLA2 (Fig. 3G). In conclusion, circ-POLA2 acts as a molecular sponge for miR-138-5p to inhibit miR-138-5p expression.

miR-138-5p prevents the aggressive activities of CC cells

After transfection with miR-138-5p mimic or inhibitor, miR-138-5p in SW480 cells increased or decreased accordingly (Fig. 4A). CCK-8 detection results showed that upregulating miR-138-5p inhibited the activity of SW480 cells (Fig. 4B). Colony formation assay found that upregulating miR-138-5p inhibited the proliferation
of SW480 cells (Fig. 4C, D). Transwell analysis showed that overexpressing miR-138-5p inhibited the migration and invasion of SW480 cells (Fig. 4E, F). Flow cytometry showed that upregulating miR-138-5p had opposite effects on CC cells compared with upregulation of miR-138-5p (Fig. 4G–H). These data indicate that miR-138-5p plays an antitumor function by inhibiting cell proliferation, migration, and invasion and promoting cell apoptosis.

**miR-138-5p has a modificatory action on SEMA4C expression**

On the bioinformatics website starBase, miR-138-5p had a targeted binding site with SEMA4C (Fig. 5A). Further detection results revealed that the luciferase activity of the SEMA4C-WT was inhibited by miR-138-5p transfection (Fig. 5B). High SEMA4C expression was measured in CC patients’ tumor tissues (Fig. 5C) in a negative correlation with miR-138-5p expression (Fig. 5D). The upregulation of SEMA4C was also seen in CC cell lines (SW480, SW620, HT29, and HCT116) (Fig. 5E) and cellular SEMA4C levels could be negatively modified by miR-138-5p (Fig. 5F). These results suggest that SEMA4C expression is negatively regulated by miR-138-5p.

**miR-138-5p/SEMA4C axis can intervene the influences of circ-POLA2 on CC**

miR-138-5p or SEMA4C expression was intervened in sh-POLA2-modified SW480 cells and the successful transfection was verified, as evidenced by miR-138-5p inhibitor-induced decline of miR-138-5p and SEMA4C-induced elevation of SEMA4C expression (Fig. 6A), CCK-8 assay showed that the sh-POLA2-mediated decrease in SW480 cell viability was reversed by anti-miR-138-5p or SEMA4C (Fig. 6B). Meanwhile, colony formation assay showed that anti-miR-138-5p or SEMA4C could attenuate sh-POLA2-induced proliferation inhibition in SW480 cells (Fig. 6C, D). Furthermore, anti-miR-138-5p or SEMA4C mitigated sh-Pola2-mediated decrease in SW480 cell migration and invasion (Fig. 6E, F). In addition, anti-miR-138-5p or SEMA4C mitigated an increase in sh-POLA2-mediated apoptosis in SW480 cells (Fig. 6G, H). In conclusion, circ-POLA2 can promote proliferation, migration, and invasion of CC cells and inhibit cell apoptosis by regulating miR-138-5p/SEMA4C axis.

**DISCUSSION**

Accumulating evidence suggests that circRNAs can act as tumor biomarkers and regulate CC progression (Chen and Shen 2020). Ju and others (Ju et al., 2019) analyzed 667 cases of stage II/III CC with R0 resection and found that circRNA could predict postoperative recurrence of stage II/III CC. Abnormal expression of circRNAs is indicative of patients’ prognosis in CC (Hsiao et al., 2017; Zhou et al., 2020; Zheng et al., 2019). In CC patients’ tumor, circ-POLA2 was upregulated, having an association with TNM stage III, lymph node metastasis, as well as low overall survival rate. Furthermore, our findings suggest that circ-POLA2 exerts oncogenic effects via miR-138-5p/SEMA4C axis.

The up-regulated expression and stability of circ-POLA2 make it a potential biomarker and diagnostic and therapeutic target for human cancer (Fang et al., 2021; Li et al., 2021; Cao et al., 2020; Fan et al., 2020). Furthermore, circ-POLA2 can sponge different miRNAs, including miR-31, miR-34a, miR-326 and miR-34a, to interfere tumor progression. Similarly, this study clarified a competitive binding of circ-POLA2 to miR-138-5p.

miR-138-5p is a cancer-related miRNA in gastric cancer (Zhang et al., 2020), prostate cancer (Huang et al., 2020),
Notably, it is known that miR-138-5p is decreased in CRC and that miR-138-5p serves to disrupt the aggressive activities of CRC cells (Wei et al., 2021). Here, miR-138-5p expression was reduced in CC and was anti-tumorigenic achieved by impeding cell malignancy.

SEMA4C, a member of the semaphorin family originally named M-SemaF, can mediate directional growth of axons and the development of myotubes (Yang et al., 2020). As miRNAs target, SEMA4C is associated with various tumors. For example, SEMA4C mediates chemoresistance in cervical cancer (Jing et al., 2019), induces tumor progression in pancreatic cancer (Fei et al., 2020), and is considered an epigenetic regulator involved in CC progression (Hung et al., 2022). Here, SEMA4C was upregulated in CC, and involved in circ-POLA2/miR-138-5p axis-regulated progression of CC. In detail, miR-138-5p downregulation or SEMA4C upregulation could attenuate the reduction of cell activities mediated by circ-POLA2 knockdown.

Although this study found that circ-POLA2/miR-138-5p/SEMA4C axis promotes CC progression in vitro, it has not been further validated in vivo. Circ-POLA2 expression in serum of CC patients should also be detected in the future to verify whether circ-POLA2 is of feasibility for blood biopsy of CC patients. Furthermore, circRNAs may encode proteins or peptides to participate in tumor progression (Qian et al., 2018; Peng et al., 2021; Wang et al., 2021; Wu et al., 2021). However, this research did not investigate the coding ability of circ-POLA2.

CONCLUSION

Circ-POLA2 has potent oncogenic activity in CC and an association with patients’ poor prognosis. Furthermore, circ-POLA2 upregulates SEMA4C expression by miR-138-5p, thereby promoting cancer development. This study extends the understanding of circRNA function in CC pathogenesis and proposes a novel circRNA as a therapeutic target for CC.

Declarations

Acknowledgments: Not applicable.

Funding Statements: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Data available: Data is available from the corresponding author on request.

REFERENCES


