

Clonorchis sinensis-derived extracellular vesicle (CsEV) miRNA Csi-miR-190-3p exerts dual roles in cholangiocarcinoma progression via cell cycle modulation and CXCL5 signaling suppression

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Abstract

Clonorchis sinensis, a Class 1 carcinogen classified by International Agency for Research on Cancer (IARC), is strongly linked to cholangiocarcinoma (CCA) development. Extracellular vesicles of *C. sinensis* (CsEVs) facilitate parasite-host interactions by transferring microRNAs (miRNAs). This study investigated the role of *Csi*-miR-190-3p, a CsEV-enriched miRNA, in regulating CCA progression. We transfected *Csi*-miR-190-3p mimics or inhibitors into RBE cells, and evaluated cellular functions (proliferation, migration, invasion) using CCK-8, EdU-488, colony formation, flow cytometry, wound healing and Transwell assays. A BALB/c nude mouse lung metastasis model was established to evaluate *in vivo* effects. Target genes were predicted using bioinformatics tools and validated by dual-luciferase reporter gene assays. RT-qPCR was employed to assess gene expression and signaling pathways. Results showed that *Csi*-miR-190-3p significantly promoted CCA cell proliferation via cell cycle acceleration, while potently inhibiting cell migration and invasion *in vitro* and *in vivo*. *Csi*-miR-190-3p directly bound to the 3'UTR of CXCL5 and downregulated its expression, thereby inhibiting metastasis. These findings uncover a novel dual regulatory mechanism of CsEV-derived *Csi*-miR-190-3p in CCA progression, and identify *Csi*-miR-190-3p/CXCL5 axis as a potential therapeutic target for CCA.

1. Introduction

Clonorchis sinensis, as a foodborne parasite, has its adults living in the host's biliary ducts and causes clonorchiasis ^[1]. Humans are frequently infected by consuming raw or undercooked freshwater fish or shrimp that harbor metacercariae of *C. sinensis* ^[2]. Currently, there are approximately 15 million cases of *C. sinensis* infection worldwide, primarily distributed in East Asian regions such as China, South Korea, and northern Vietnam, as well as the Russian Far East ^[3]. Cholangiocarcinoma (CCA), a lethal malignancy originating from biliary epithelia, is epidemiologically linked to chronic infection with *C. sinensis* ^[4]. The epidemiological landscape of CCA is varied across different regions; however, in Southeast Asia and China, *C. sinensis* emerges as a significant etiological factor ^[5]. The International Agency for Research on Cancer (IARC) has classified *C. sinensis* as a Class 1 carcinogen.

As an important pathway of pathogen-host interaction, the role of extracellular vesicles of *C. sinensis* (CsEVs) in the pathogenicity of *C. sinensis* has received widespread attention ^[6, 7]. CsEVs encapsulate bioactive molecules, including proteins, lipids, and nucleic acids, which are delivered to host cells to regulate pathological processes such as inflammation, fibrosis, and malignant transformation ^[8]. Among these cargo components, microRNAs (miRNAs) have emerged as key post-transcriptional regulators of host gene expression. Yan et al. ^[9] demonstrated that CsEVs promote NF- κ B pathway activation and inflammatory macrophage polarization through the delivery of *Csi*-let-7a-5p, which simultaneously targets *Clec7a* and *SOCS1* in host macrophages. Zhang et al. ^[10] have identified 37 known and 3 novel miRNAs in CsEVs from 24/48h-cultured *C. sinensis* adults. Target prediction implicated these miRNAs in oncogenic pathways, suggesting CsEV-mediated miRNA transfer modulates host immunity and contributes to pathogenesis. Furthermore, CsEV-carried *Csi*-miR-96-5p can promote CCA proliferation

and migration by suppressing PTEN-mediated ferroptosis^[11]. This suggests that the miRNAs encapsulated within CsEVs play a significant role in their pathogenicity. However, the specific CsEV-derived miRNAs that drive CCA malignant progression, as well as their underlying molecular mechanisms regulating CCA cell proliferation, migration and invasion, remain largely unknown. To address this knowledge gap in our preliminary research, transcriptomic high-throughput sequencing analysis of CsEVs was performed^[12]. Among the top 23 ranked miRNAs, three candidates were selected for preliminary experiments. The results demonstrated that *Csi*-miR-190-3p significantly promoted the proliferation of CCA cells and was notably present with high expression levels in CsEVs. Based on these findings, this miRNA was consequently chosen as the research subject for further investigation. *Csi*-miR-190-3p has a highly similar sequence to hsa-miR-190 (Gene ID: 406965), which has been shown to play various roles in different cancers. For instance, Jin et al.^[13] found that Long Non-Coding RNA PART1 exerts tumour suppressive functions in glioma via sponging miR-190a-3p and inactivation of the PTEN/AKT pathway; Wang et al.^[14] found that the CBX4/miR-190 regulatory loop inhibits lung cancer metastasis; Huang et al.^[15] found that miR-190 may promote the malignant progression of human urothelial cells by downregulating CDKN1B. From the perspective of molecular evolution and functional conservation, sequence similarity often implies potential similarities in functional mechanisms. Based on these observations, we hypothesized that CsEV-enriched *Csi*-miR-190-3p modulates *C. sinensis*-associated CCA progression, and herein we investigated its functional roles and molecular mechanisms in CCA cell proliferation and metastasis.

Here, we investigated the functional roles and molecular mechanisms of *Csi*-miR-190-3p in CCA progression. Using *in vitro* and *in vivo* models, we demonstrate that *Csi*-miR-190-3p exhibits dual regulatory effects: promoting proliferation via cell cycle acceleration, while inhibiting migration and invasion by targeting CXCL5. These findings not only advance our understanding of *C. sinensis*-driven cholangiocarcinogenesis but also highlight *Csi*-miR-190-3p as a potential therapeutic target for CCA management.

2. Materials and Methods

Cell Culture

The human CCA cell line (RBE) was sourced from Procell Corporation (China) with the catalog number CL-0191, while the human embryonic kidney cell line 293 (HEK-293T) was derived from the same company with the product number CL-0005. Both cell lines used were free from contamination. The Research Resource Identifier (RRID) of RBE is CVCL_4896, and the RRID of HEK-293T is CVCL_0063. The RBE cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, USA), while the HEK-293T cells were cultured in DMEM medium (Gibco, Carlsbad, USA). Both media contained 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (P/S, HyClone, USA), and all cells were cultured in a 37 °C incubator with 5% CO₂.

Cell transfection

The cells underwent trypsin digestion and were subsequently seeded into 6-well dishes at a density of 1×10^6 cells per well. They were then incubated in an incubator at 5% CO₂ until 80% confluence was achieved. The *Csi*-miR-190-3p mimics, inhibitors, and Scramble-miRNA mimics were synthesized by RiboBio (Guangzhou, China) (Table S1), while the pcDNA3.1-CXCL5 plasmid was synthesized by HanYi (Guangzhou, China). Lipofectamine 3000 (Invitrogen, California, USA) was utilized for transfection, and the transfection efficiency was assessed 24 h post-transfection. The miRNA mimic was transfected at a concentration of 20 nM, while plasmid DNA was delivered at 20 ng per well.

RT-qPCR

Total RNA was extracted from the samples using the EZ-press RNA Purification Kit (EZBioscience, USA) according to the manufacturer's instructions and RNA was quantified by an Epoch microplate spectrophotometer (BioTek, USA). The miRNA was reverse transcribed using riboSCRIPT™ Reverse Transcription Kit (Reebok, China) and the mRNA was reverse transcribed using 5× *Evo M-MLV* RT Master Mix (AGBio, China) according to the manufacturer's instructions. Quantitative analyses were performed on a CFX96 real-time PCR system (Bio-Rad) using a SYBR Green Premix Pro Taq HS qPCR kit (AGBio, China), with the *Csi*-miR-190-3p endogenous gene being U6 and the CXCL5 endogenous gene being GAPDH. $2^{-\Delta\Delta C_t}$ formula was used to calculate the relative miRNA or mRNA expression, and all experiments were performed in triplicate. The specific primers used for miRNA in this study were synthesised by RiboBio (Guangzhou, China), and the specific primers used for mRNA were synthesised by Sangon Biotech (Shanghai, China) (Table S2). The 20 µL RT-qPCR reaction system contained 10 µL SYBR Green Premix, 0.4 µL each primer (10 µM), 2 µL cDNA template and 7.2 µL nuclease-free water. The cycling conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

miRNA target prediction

miRNA target gene prediction and analysis were performed using TargetScan (<http://www.targetscan.org/>), miRTarBase (<https://mirtarbase.cuhk.edu.cn/>), and miRDB (<https://mirdb.org/>).

The interaction site between *Csi*-miR-190-3p and the target gene's 3' UTR was predicted using the TargetScan database. The optimal binding region was subsequently validated through the RNAhybrid online tool (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid>).

Dual-luciferase reporter gene assay

The CXCL5 wild-type plasmid was constructed based on the binding sites predicted by the TargetScan database, and random mutation was introduced at this binding site to generate the CXCL5 mutant plasmid. The pmirGLO CXCL5 3' UTR-WT and pmirGLO CXCL5 3' UTR-MUT constructs were built by Genecreate Company (Wuhan, China). The luciferase gene was cloned in the downstream position of the wild-type or mutant region of the CXCL5, and the cells were co-transfected with the *Csi*-miR-190-3p mimics or Scramble mimics. 48 h post-transfection, the cells were lysed and the firefly luciferase activity

was measured using a dual-luciferase assay kit (Servicebio, China) in accordance with the manufacturer's instructions. Renilla luciferase was used as the internal reference.

CCK-8 assays

Cell Counting Kit 8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was used to detect cell proliferation. Briefly, RBE cells were seeded into 96-well plates at approximately 2×10^3 cells/well and incubated overnight. After 0 h, 24 h, 48 h and 72 h of culture, the medium was removed and 100 μ L of serum-free RPMI-1640 medium supplemented with 10 μ L CCK-8 reagent was replaced in each well, and then the cells were incubated in the dark at 37°C for 1 to 4 h. The absorbance value at 450 nm wavelength was detected by a microplate reader (Molecular Devices, CA).

EdU-488 assays

EdU-488 incorporation assay kit (C0071S; Beyotime, China) was employed to estimate cell proliferation. Briefly, RBE cells with or without *Csi-miR-190-3p* treatment were incubated with EdU medium diluent for 2 h according to the product manual. After being fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100, cells were labeled with EdU-488 through Click reaction, and the cell nuclei were stained with Hoechst 33342. The stained cells were observed under a fluorescence microscope (Leica DMI8, Wetzlar, Germany) and EdU positive cells were analyzed by ImageJ software (National Institutes of Health, USA).

Colony formation assays

Colony formation assays were used to test the effects of *Csi-miR-190-3p* on the proliferation ability of RBE cells. In brief, RBE cells with or without *Csi-miR-190-3p* treatment were seeded into 6-well plates at about 1000 cells/well and cultured for 2 weeks with medium replacement every 3 days. After being washed twice with PBS, cell colonies were fixed with 4% paraformaldehyde and then stained with 1% crystal violet stain solution (Solarbio, Beijing, China) for 30 min. Unbound dye was washed off. The cell colonies were counted via ImageJ software (NIH).

Cell cycle analysis

Cell cycle staining kit [MultiSciences (Lianke) Biotech, China] was used to analyze cell cycle distribution. Firstly, 1×10^6 cells with or without *Csi-miR-190-3p* treatment were collected and washed once with PBS. Then, Propidium iodide (PI) with RNase A and permeabilization solution were added and incubated at room temperature in darkness for 30 min to stain the cells. The samples were detected using flow cytometry (CytoFLEX S, Beckman Coulter, USA) and the data was analyzed with the ModFit LT 4.1 program (Verity Software House, Topsham, USA).

Wound healing assays

Wound healing assays were used to analyze the effects of *Csi-miR-190-3p* on the migration of RBE cells. Briefly, RBE cells were seeded into 6-well plates at about 5×10^5 cells/well and incubated to 80% confluence, followed by treatment with or without *Csi-miR-190-3p*. After that, scratches were created vertically with a 200- μ L pipette tip and suspension cells were washed off with PBS. The cells were cultured in serum-free RPMI-1640 medium and photographed under a microscope (Leica DMI4000B, Wetzlar, Germany) at 0 h, 24 h and 48 h after scratching. Finally, the scratch area was measured with ImageJ software (NIH).

Transwell assays

The transwell chambers (Corning, NY, USA) were used to evaluate the migration and invasion ability of RBE cells. For the transwell migration assays, 1×10^5 treated cells were seeded into the upper chamber in serum-free RPMI-1640 medium, while the lower chamber was inserted into 24-well plates supplemented with 600 μ L of medium containing 10% FBS. After incubation for 24 h, the migrated cells were fixed with 4% paraformaldehyde for 15 min, and 1% crystal violet solution (Solarbio, Beijing, China) was used to stain. The cells on the upper membrane surface were wiped off, and then the lower membrane surface was randomly imaged with a microscope (Leica DMI4000B, Wetzlar, Germany). The migrated cells were calculated with ImageJ software (NIH). The procedure for the transwell invasion assays was the same as for the migration assays, but the upper chamber was precoated with Matrigel (Corning, NY, USA).

In vivo metastasis assays

Twenty athymic male BALB/c nude mice (5-week-old) were purchased from the Laboratory Animal Center of Sun Yat-sen University. The following experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Sun Yat-sen University (No. SYSU-IACUC-2024-002026). All mice were housed under specific pathogen-free (SPF) conditions with a 12 h light/dark cycle, free access to standard chow and sterile water, and acclimatized for 1 week before experiments. For the lung metastasis model, approximately 2×10^6 control, CsEVs with Scramble-miRNA mimics or CsEVs with *Csi-mir-190-3p* inhibitors treated-RBE cells were resuspended in 200 μ L PBS and injected into the nude mice through the tail vein ($n = 6$ /per group). After about 4 weeks of inoculation, the mice were sacrificed by cervical dislocation after anesthetization via intraperitoneal injection with 1% sodium pentobarbital, and their lungs were separated for the subsequent histopathologic analyses with hematoxylin-eosin staining.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 8.0 (GraphPad, San Diego, USA). In the case of comparison of two groups, the differences were evaluated using a two-tailed Student's t-test, and One-way ANOVA analysis was used for comparison of differences among more than two groups. All results were from three independent experiments and the data were expressed as Mean $\pm$ SD. The differences were statistically significant under the following circumstances: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

3. Results

3.1 *Csi-miR-190-3p* promotes the proliferation of CCA cells *in vitro*

RT-qPCR analysis showed that *Csi-miR-190-3p* expression was significantly upregulated in RBE cells after transfection with its mimics ($p < 0.0001$) (Fig. 1A). To evaluate its functional impact on cellular growth, a series of proliferation assays were performed. The CCK-8 assay demonstrated enhanced viability in mimic-treated cells compared to the scramble control group at 24 h, 48 h, and 72 h post-transfection ($p < 0.001$, $p < 0.0001$) (Fig. 1B). Consistent with these findings, the EdU-488 assay showed a markedly higher proportion of EdU-positive cells in the mimic group ($p < 0.0001$) (Fig. 1C), while colony formation assays revealed a significant increase in clonogenic capacity ($p < 0.001$) (Fig. 1D). Collectively, these data indicate that *Csi-miR-190-3p* significantly promotes RBE cell proliferation.

Further mechanistic insights were obtained through cell cycle analysis. Flow cytometry detected increased proportions of cells in S-phase and G2/M-phase, accompanied by an elevated proliferation index in the mimic group ($p < 0.01$) (Fig. 1E). These observations suggest that *Csi-miR-190-3p* may enhance cell cycle progression and proliferation by accelerating cell cycle progression.

3.2 *Csi-miR-190-3p* inhibits CCA cells migration and invasion *in vitro*

We subsequently evaluated the effects of *Csi-miR-190-3p* on the migration and invasion of CCA cells *in vitro*. We employed matrigel-free transwell assays to assess cell migration and matrigel-coated transwell assays to evaluate cell invasion. The results indicated a significant reduction in the number of migrated/invaded cells within 24 h post-treatment with *Csi-miR-190-3p* ($p < 0.0001$) (Fig. 2A). Consistent with these findings, the wound healing assay also demonstrated that *Csi-miR-190-3p* significantly reduced the migration rate of RBE cells at 24 h compared to the control group ($p < 0.001$) (Fig. 2B). These experiments demonstrate that *Csi-miR-190-3p* inhibits the migration and invasion capabilities of CCA cells *in vitro*.

3.3 *Csi-miR-190-3p* inhibition attenuates the pro-proliferative effects of CsEVs in CCA

To investigate the regulatory role of *Csi-miR-190-3p* in CsEVs-mediated CCA proliferation, functional assays were performed under three conditions: 10 $\mu\text{g/ml}$ CsEVs with *Csi-miR-190-3p* inhibitors, 10 $\mu\text{g/ml}$ CsEVs with Scramble-miRNA mimics and phosphate-buffered saline (PBS)-treated control. CCK-8 assays revealed that *Csi-miR-190-3p* inhibition significantly attenuated CsEV-induced increase in RBE cell viability over 72 h compared to the scramble control ($p < 0.0001$) (Fig. 3A). EdU-488 staining demonstrated a marked reduction in proliferative cells upon *Csi-miR-190-3p* suppression, abrogating the

pro-proliferative effects of CsEVs($p < 0.001$) (Fig. 3B). Consistent with these findings, colony formation assays showed that CsEVs-induced clonogenicity was substantially diminished by *Csi*-miR-190-3p inhibition ($p < 0.001$) (Fig. 3C).

3.4 *Csi*-miR-190-3p inhibition enhances the pro-migratory and invasive effects of CsEVs in CCA

We investigated the regulatory role of *Csi*-miR-190-3p in CsEV-mediated migration and invasion of CCA cells through *in vitro* functional assays and an *in vivo* lung metastasis model. The wound healing assay revealed that CsEV treatment substantially enhanced the migratory capacity of CCA cells, while co-treatment with the *Csi*-miR-190-3p inhibitors markedly enhanced this pro-metastatic phenotype with progressively amplified disparities over time ($p < 0.01, p < 0.001$) (Fig. 4A). The Transwell assay corroborated this trend, demonstrating that CsEVs plus *Csi*-miR-190-3p inhibitor group exhibited significantly higher migratory and invasive activities compared to other experimental groups ($p < 0.01, p < 0.001$) (Fig. 4B). The *in vivo* lung metastasis model further confirmed that the CsEVs combined with *Csi*-miR-190-3p inhibitors group developed markedly more lung metastatic nodules compared to the CsEVs plus Scramble control group (Fig. 4C). Collectively, these findings demonstrate that suppression of *Csi*-miR-190-3p expression significantly enhances the pro-metastatic effects of CsEVs both *in vitro* and *in vivo*, suggesting that this miRNA may serve as a key negative regulator in CCA migration and invasion.

3.5 *Csi*-miR-190-3p targets and downregulates the expression of CXCL5

To identify the target gene of *Csi*-miR-190-3p regulating CCA cell behavior, we predicted potential targets using TargetScan, miRTarBase, and miRDB databases, identifying 420 overlapping candidate target genes(Fig. 5A; Table S4). The top three predicted targets (DENR, SLC35E3, and CXCL5) were screened by RT-qPCR, revealing significant suppression of CXCL5 mRNA expression ($p < 0.0001$) (Fig. 5B). Bioinformatic predictions using TargetScan and RNAhybrid revealed a specific binding site between *Csi*-miR-190-3p and the CXCL5 3'UTR(Fig. 5C).Dual-luciferase reporter gene assays confirmed a direct binding interaction between *Csi*-miR-190-3p and CXCL5 3' UTR, where CXCL5-WT co-transfection with *Csi*-miR-190-3p mimics resulted in markedly reduced luciferase activity markedly reduced luciferase activity compared to scramble controls, while mutant constructs exhibited no significant difference ($p < 0.001$) (Fig. 5D). RT-qPCR further demonstrated that *Csi*-miR-190-3p overexpression significantly downregulated CXCL5 mRNA levels ($p < 0.001$) in RBE cells (Fig. 5E). These results collectively establish CXCL5 as a direct functional target of *Csi*-miR-190-3p in CCA through 3' UTR-mediated regulation.

3.6 Overexpression of CXCL5 attenuates the proliferation-promoting effect of *Csi*-miR-190-3p

To investigate the changes in cellular proliferation capacity, RBE cells were co-transfected with pcDNA3.1-CXCL5 (CXCL5 overexpression plasmid) and *Csi-miR-190-3p* mimics. RT-qPCR analysis confirmed a significant increase in CXCL5 expression following transfection with the pcDNA3.1-CXCL5 plasmid ($p < 0.0001$) (Fig. 6A). The CCK-8 assays revealed that cell viability in the CXCL5 + mimic group was lower than in the mimic group at 24 h, 48 h, and 72 h post-transfection ($p < 0.05$) (Fig. 6B). The EdU-488 assay staining results further demonstrated a reduced proportion of EdU-positive cells in the CXCL5 + mimic group compared to the mimic group ($p < 0.05$) (Fig. 6C). Consistent with these findings, colony formation assays showed a decrease in the number of cell clones in the CXCL5 + mimic group relative to the mimic group ($p < 0.01$) (Fig. 6D). Collectively, these experimental results indicate that overexpression of CXCL5 effectively inhibits the proliferation-promoting effect of *Csi-miR-190-3p*.

The CXCL5 expression after transfection of pcDNA3.1-CXCL5 plasmid in RBE cells. **(B)** The CCK-8 assay was employed to detect cell proliferation. RBE cells were detected at 0, 24, 48, and 72 h post-treatment. **(C)** The colony formation assay were conducted to determine the proliferation capacity of RBE cells. **(D)** EdU-488 assays were used to detect the proliferative ability of CCA cells. Representative images are shown, and the summary results are from three independent experiments.

3.7 Overexpression of CXCL5 rescued the inhibitory effect of *Csi-miR-190-3p* on the migration and invasion

Functional assays revealed that *Csi-miR-190-3p* significantly inhibited the migratory and invasive capacities of CCA cells, while co-expression of CXCL5 effectively rescued these phenotypes. The wound healing assay indicated that compared with the mimic group, the CXCL5 + mimic group significantly increased the migration rate of RBE cells at 24 and 48 h ($p < 0.001$) (Fig. 7A). Consistent with these findings, we used transwell assays without matrigel to assess cell migration and transwell assays with matrigel-coated membranes to evaluate cell invasion. The results showed that the number of cells passing through the chambers in the CXCL5 + mimic group was significantly higher than that in the mimic group ($p < 0.001, p < 0.0001$) (Fig. 7B). Given that *Csi-miR-190-3p* targets CXCL5 and the CXCL5-CXCR2 axis is known to activate ERK/Elk-1 signaling to promote metastasis^[16], we speculate that it may regulate migration and invasion through the CXCL5/ERK axis. These findings suggest that *Csi-miR-190-3p* may regulate the migration and invasion of CCA by targeting CXCL5, potentially involving the ERK signaling pathway.

4. Discussion

Cancer progression is a complex process involving multiple genes and signaling pathways. The involvement of miRNAs in cancer development and progression has been systematically documented, with resources such as the Cancer miRNA Census providing a curated list of cancer-related miRNA genes^[17]. Recent studies have highlighted the importance of miRNAs in CCA, a devastating malignancy with limited therapeutic options^[18]. *C. sinensis* is one of the causative factors of CCA, promoting

carcinogenesis through multidimensional mechanisms including mechanical injury, metabolic byproduct stimulation, and immune microenvironment remodeling ^[19]. CsEVs have emerged as pivotal mediators in parasite-host interactions, with their parasite-derived miRNAs being increasingly implicated in CCA pathogenesis ^[8, 10, 20].

Notably, specific miRNAs such as *Csi-miR-96-5p* and *Csi-let-7a-5p* have been experimentally validated to contribute to *C. sinensis* -driven oncogenesis by modulating host signaling cascades and inflammatory responses ^[9, 11]. Our study focused on the role of *Csi-miR-190-3p* from CsEVs in the progression of CCA and its interaction with CXCL5. Notably, CXCL5 has been demonstrated to dually promote both proliferation and metastasis in lung adenocarcinoma through NAT10-mediated ac4C RNA acetylation that stabilizes its mRNA ^[21]. By conducting a series of functional experiments, we have confirmed that *Csi-miR-190-3p* can promote the proliferation of CCA cells and target CXCL5 to inhibit the migration and invasion of CCA. This finding represents a novel insight into the molecular mechanisms underlying CCA progression and suggests potential therapeutic targets (Fig. 8).

In vitro functional assays, including CCK-8, EdU-488, and colony formation, confirmed that *Csi-miR-190-3p* overexpression significantly enhances CCA cell proliferation. Flow cytometric analysis further revealed accelerated cell cycle progression via increased S-phase and G2/M-phase populations. However, Transwell assays, wound healing assays, and nude mouse lung metastasis models consistently demonstrated its capacity to suppress CCA cell migration and invasion in both *in vivo* and *in vitro*. These findings suggest a unique regulatory equilibrium within the tumor microenvironment: while promoting localized tumor growth through cell cycle acceleration, *Csi-miR-190-3p* simultaneously impedes metastatic dissemination. This phenomenon may reflect an evolutionary adaptation to balance tumor expansion with delayed systemic spread, potentially aligning with immune evasion strategies during tumor immunoediting to avoid premature immune clearance ^[22, 23]. Beyond its direct effects on tumor cells, the CXCL5 signaling axis also plays a critical role in modulating the tumor immune microenvironment. For instance, targeting the STC1/p65/CXCL5 axis has been shown to inhibit malignant pleural effusion by reprogramming tumor-associated macrophages ^[24]. This raises the intriguing possibility that, in addition to directly targeting CXCL5 in CCA cells, *Csi-miR-190-3p* might also influence the behavior of stromal or immune cells within the tumor microenvironment, a hypothesis that merits further investigation.

This study confirmed CXCL5 as a direct target of *Csi-miR-190-3p* through dual-luciferase reporter gene assays and RT-qPCR. As a member of the chemokine family, CXCL5 plays critical roles in cancer progression, metastasis, and immune regulation ^[23, 24]. Mechanistically, CXCL5 promotes tumor angiogenesis by activating the AKT/NF- κ B pathway, enhances cancer cell migration and invasion ^[25, 26], and facilitates immune evasion by recruiting myeloid-derived suppressor cells (MDSCs) and neutrophils to suppress CD8⁺ T-cell function ^[27]. Functional rescue experiments demonstrated that CXCL5 co-expression marginally attenuated the pro-proliferative effect of *Csi-miR-190-3p* while significantly

reversing its inhibitory effects on migration and invasion. These findings indicate that *Csi-miR-190-3p* suppresses CCA cell migration and invasion by targeting CXCL5.

Existing studies indicate that the ERK signaling pathway is a potential downstream target of CXCL5 and is closely associated with tumor cell migration and invasion [28, 29]. For instance, the CXCL5-CXCR2 axis has been shown to promote epithelial-mesenchymal transition (EMT) via ERK/Elk-1 signaling, thereby driving gastric inflammation-associated carcinogenesis [16]. Furthermore, *C. sinensis* itself has been shown to activate ERK signaling through its secreted protein granulin, which promotes malignant transformation of hepatocytes via the EGFR-mediated RAS/MAPK/ERK pathway [30]. Therefore, we hypothesize that *Csi-miR-190-3p* suppresses CCA cell migration and invasion by targeting CXCL5 to downregulate ERK signaling; this hypothesis aligns with the well-characterized role of CXCL5 in activating ERK signaling. The ERK pathway, a core component of the Mitogen-Activated Protein Kinase (MAPK) cascade, is frequently hyperactivated in tumors, significantly enhancing metastatic potential [31]. ERK promotes migration and invasion through two primary mechanisms: (1) phosphorylation of transcription factors to induce epithelial-mesenchymal transition (EMT), thereby disrupting E-cadherin-mediated cell adhesion and enhancing motility; and (2) upregulation of matrix metalloproteinases to degrade extracellular matrix, facilitating tissue barrier penetration [32, 33]. Conversely, ERK pathway inhibition robustly suppresses these pro-metastatic processes [34]. The discovery that *Csi-miR-190-3p* indirectly modulates ERK signaling via CXCL5 inhibition provides novel therapeutic insights. Given that ERK signaling plays a pivotal role in tumor metastasis, therapeutic strategies targeting this pathway have been systematically evaluated in various cancers [35]. For instance, combinatorial use of ERK inhibitors and *Csi-miR-190-3p* mimics could synergistically enhance anti-metastatic efficacy while preserving physiological ERK functions in normal cells. However, direct evidence of ERK pathway modulation in this context, such as detection of ERK phosphorylation, remains to be experimentally validated.

The dual regulatory role of *Csi-miR-190-3p* in CCA progression may be related to the synergistic effects of multiple components in *CsEVs*, where *Csi-miR-190-3p* potentially counteracts the pro-invasive effects of other parasite-derived molecules by targeting metastasis-promoting factors. Additionally, besides CXCL5, *Csi-miR-190-3p* may regulate CCA cell proliferation by interacting with multiple target genes. Such functional separation is not uncommon in tumor-associated miRNAs. For example, Xu *et al.* [35] demonstrated that overexpression of miR-490-5p in RBE cells significantly inhibits CCA cell migration and invasion, while showing no apparent effects on proliferation, clonogenicity, or apoptosis. This indicates that although tumor proliferation and metastasis are closely linked, they can form functionally distinct regulatory networks. Notably, this study has several limitations: first, the pro-proliferative effect of *Csi-miR-190-3p* was only validated *in vitro*, and *in vivo* validation using CCA xenograft models is required to confirm its role in tumor growth; second, we only identified CXCL5 as a direct target of *Csi-miR-190-3p*, and additional targets regulating cell cycle progression remain to be screened; third, no clinical correlation between *Csi-miR-190-3p* and CXCL5 expression in human CCA tissues was investigated, which limits the translational potential of our findings. and its target genes and underlying signaling pathways require further exploration. Additionally, we lack sufficient *in vivo* validation to substantiate its

proliferation-promoting effects. Future studies should employ xenograft mouse models to investigate the role of *Csi*-miR-190-3p in malignant proliferation and tumor microenvironment remodeling in CCA. And the absence of clinical correlation analysis between *Csi*-miR-190-3p and CXCL5 expression in human CCA tissues is another limitation. Subsequent cohort studies are required to validate their biomarker potential and pathological relevance.

5. Conclusion

This study elucidates the dual regulatory role of *C. sinensis*-derived *Csi*-miR-190-3p in cholangiocarcinoma progression: it drives cell cycle progression to positively regulate tumor cell proliferation, while negatively modulating tumor cell migration and invasion through the targeted suppression of CXCL5. This finding furnishes novel mechanistic insights into how *C. sinensis*-derived miRNAs orchestrate oncogenic transformation in host cells, and lays a pivotal molecular foundation for deciphering the pathogenic mechanisms underlying parasite-associated tumorigenesis.

Declarations

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Statement: All experimental procedures were approved by the Research Ethics Board of Zhongshan School of Medicine. The animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University (SYSU-IACUC-2024-002026). All animal studies have followed the ARRIVE guidelines.

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Author contributions:

Anyuan Xu: Conceptualization, methodology, investigation, formal analysis, writing-original-draft, writing-review & editing. **Aoxun Wu:** Methodology, investigation, formal-analysis, writing-review & editing. **Xiaowen Pan:** Investigation, formal analysis, writing-review & editing. **Linya Huang:** Writing-review & editing. **Shu Fang:** Writing-review & editing. **Chunyan Xu:** Writing-review & editing. **Chenlin Huang:** Writing-review & editing. **Meiyu Li:** Writing-review & editing. **Zifeng Zhong:** Writing-review & editing. **Yinjuan Wu:** Conceptualization, investigation, formal analysis, validation, writing-review & editing, supervision, funding acquisition. **Xuerong Li:** Conceptualization, validation, writing-review & editing, supervision, funding acquisition. All authors read and approved the final manuscript.

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

Consent to Participate declaration:Not applicable. This study involved parasite culture, in vitro cell experiments and animal experiments. No human participants or human clinical samples were used, so informed consent from human subjects is not required.

Consent to Publish declaration:All co-authors have read and approved the final manuscript, and consented to its submission and publication in this journal. No personal identifiable human data is involved in this study.

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Figures

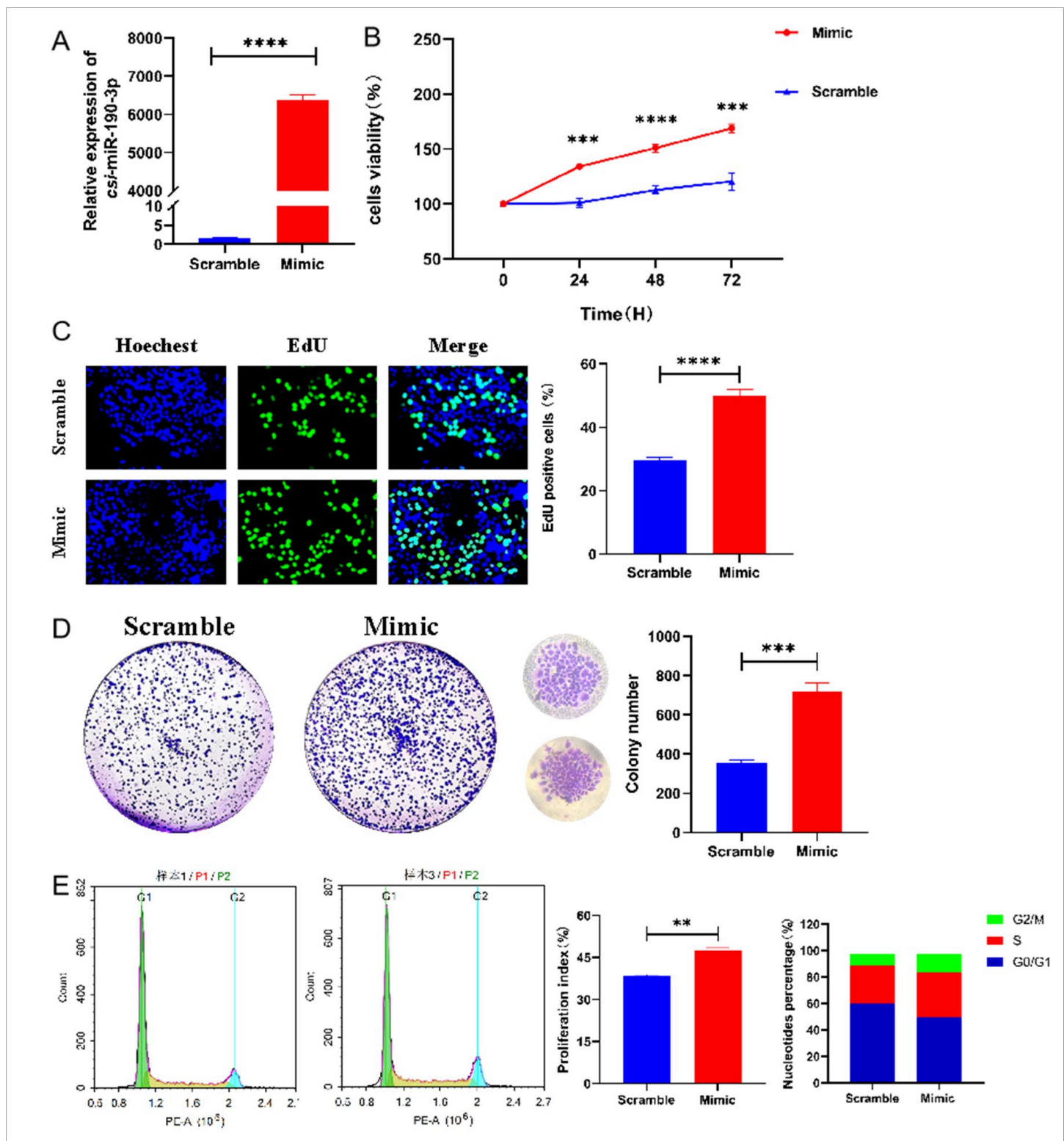


Figure 1

Csi-miR-190-3p induces the malignant proliferation of CCA cells *in vitro*. (A) The *Csi-miR-190-3p* expression profiles after transfection of *Csi-miR-190-3p* mimics in RBE cells. (B) The CCK-8 assay was employed to detect cell proliferation. RBE cells were treated with or without *Csi-miR-190-3p* mimics and were detected at 0, 24, 48, and 72 h post-treatment. (C) The EdU-488 assay and (D) colony formation assay were conducted to determine the proliferation capacity of RBE cells after treatment with or without

Csi-miR-190-3p mimics for 24 h, respectively. Representative images are shown, and the summary results are from three independent experiments. (E) Cell cycle distribution was analyzed after treatment with or without Csi-miR-190-3p mimics for 24 h using flow cytometry. The percentage of cells in each cell cycle phase and the proliferation index (PI) were calculated and presented. $PI = (S+G2/M)/(G0/G1+S+G2/M)$.

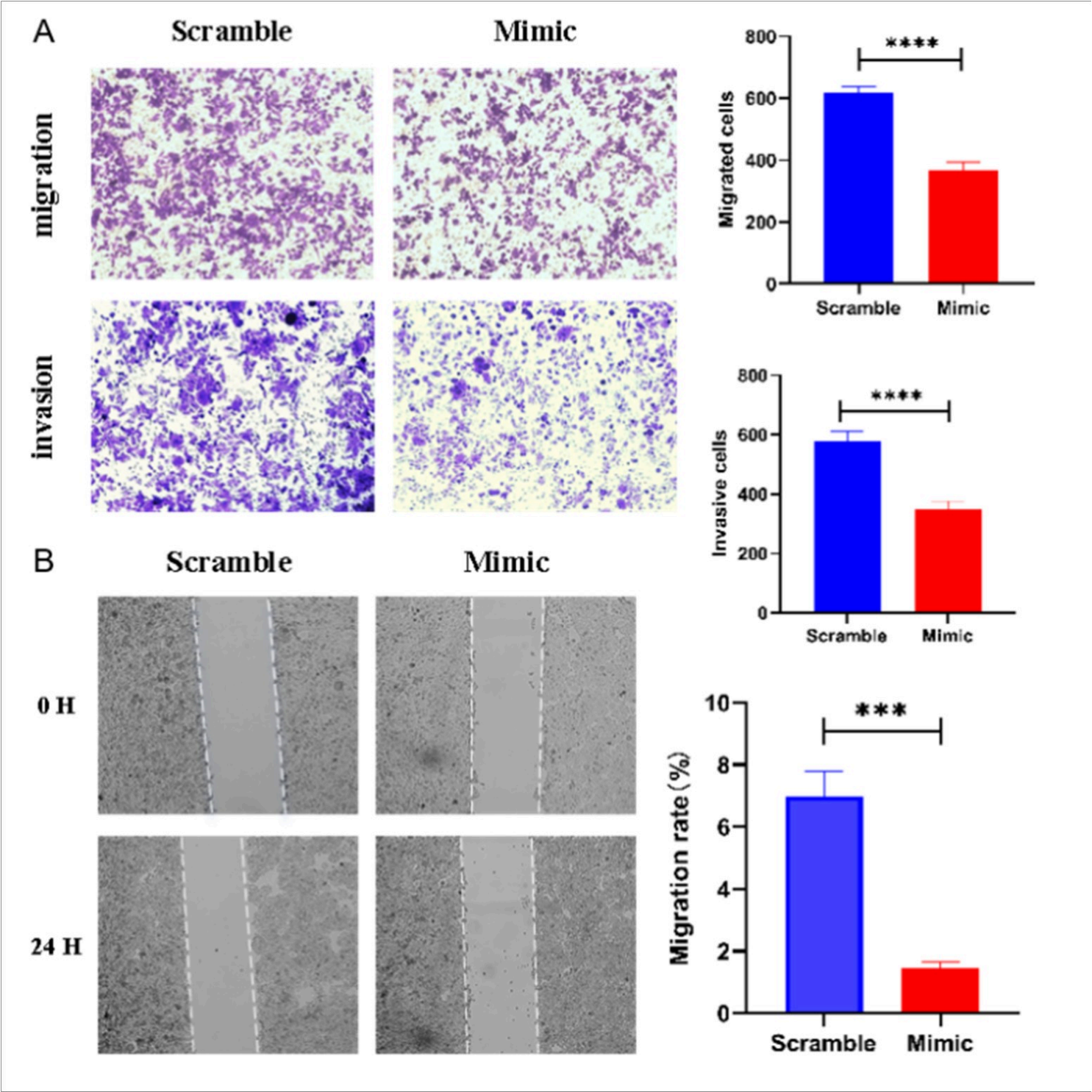


Figure 2

Csi-miR-190-3p inhibits the migration and invasion of CCA cells in vitro.(A)Transwell assay was conducted to determine the migration and invasion abilities of RBE cells after treatment with or without Csi-miR-190-3p mimics for 24 h. Representative images of matrigel-free (for migration) and matrigel-coated (for invasion) transwell assay were shown. Each group underwent three independent experiments. (B) Wound healing assay for RBE cells showed motility changes after treatment with or without Csi-miR-190-3p mimics for 24 h. Images were captured at 0 h and 24 h after scratching, with representative pictures displayed.

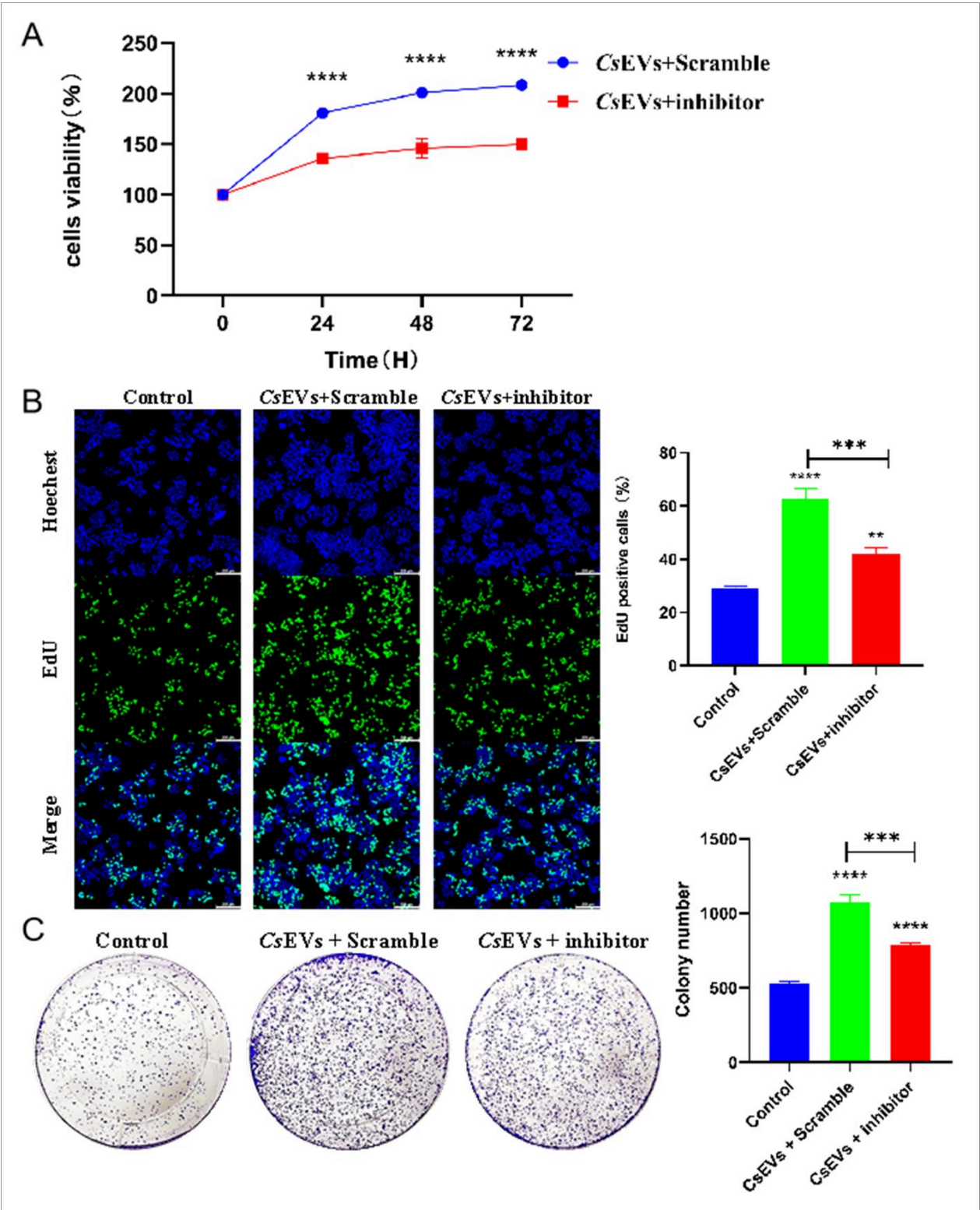


Figure 3

Inhibition of *Csi*-miR-190-3p expression in CsEVs diminishes their ability to promote cell proliferation. (A) CCK-8 assays were used to detect the proliferative ability of CCA cells. (B) EdU-488 assays were used to detect the proliferative ability of CCA cells. (C) Colony formation assays were used to detect the proliferative ability of CCA cells. The Control group served as the negative control.

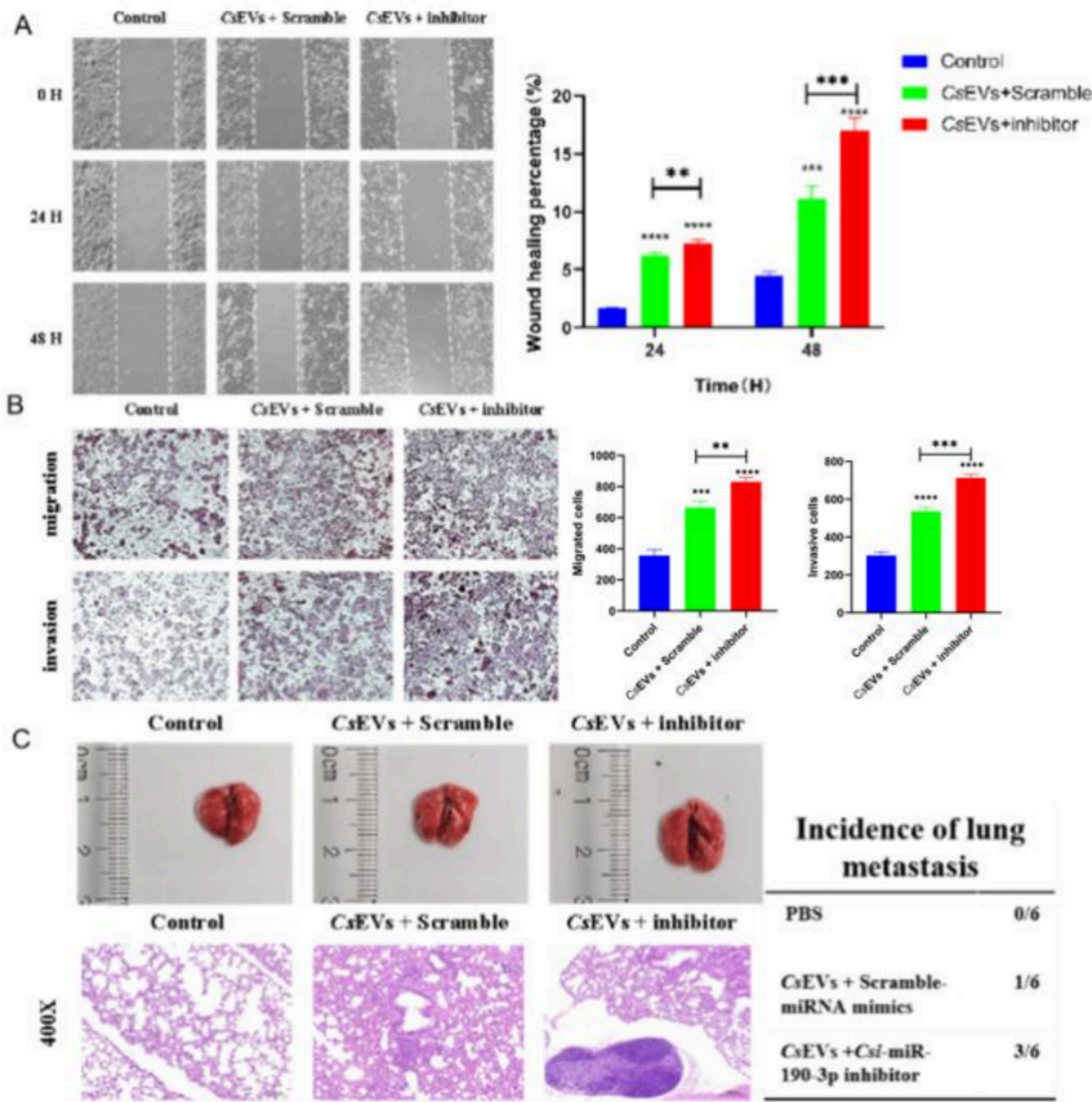


Figure 4

Prediction and verification of CXCL5 as a target gene of Csi-miR-190-3p. (A) The intersection of target genes predicted by TargetScan, miRTarBase, and miRDB yielded 420 common candidates. (B) RT-qPCR were performed to identify potential target genes of Csi-miR-190-3p. (C) Prediction of Csi-miR-190-3p and the potential binding sites on the 3' UTR of CXCL5. (D) Detection of relative luciferase activity in cells transfected with Csi-miR-190-3p mimics or scramble mimics via dual-luciferase reporter gene assay. (E) RT-qPCR showing the impact of Csi-miR-190-3p on CXCL5 mRNA expression levels in CCA cells.

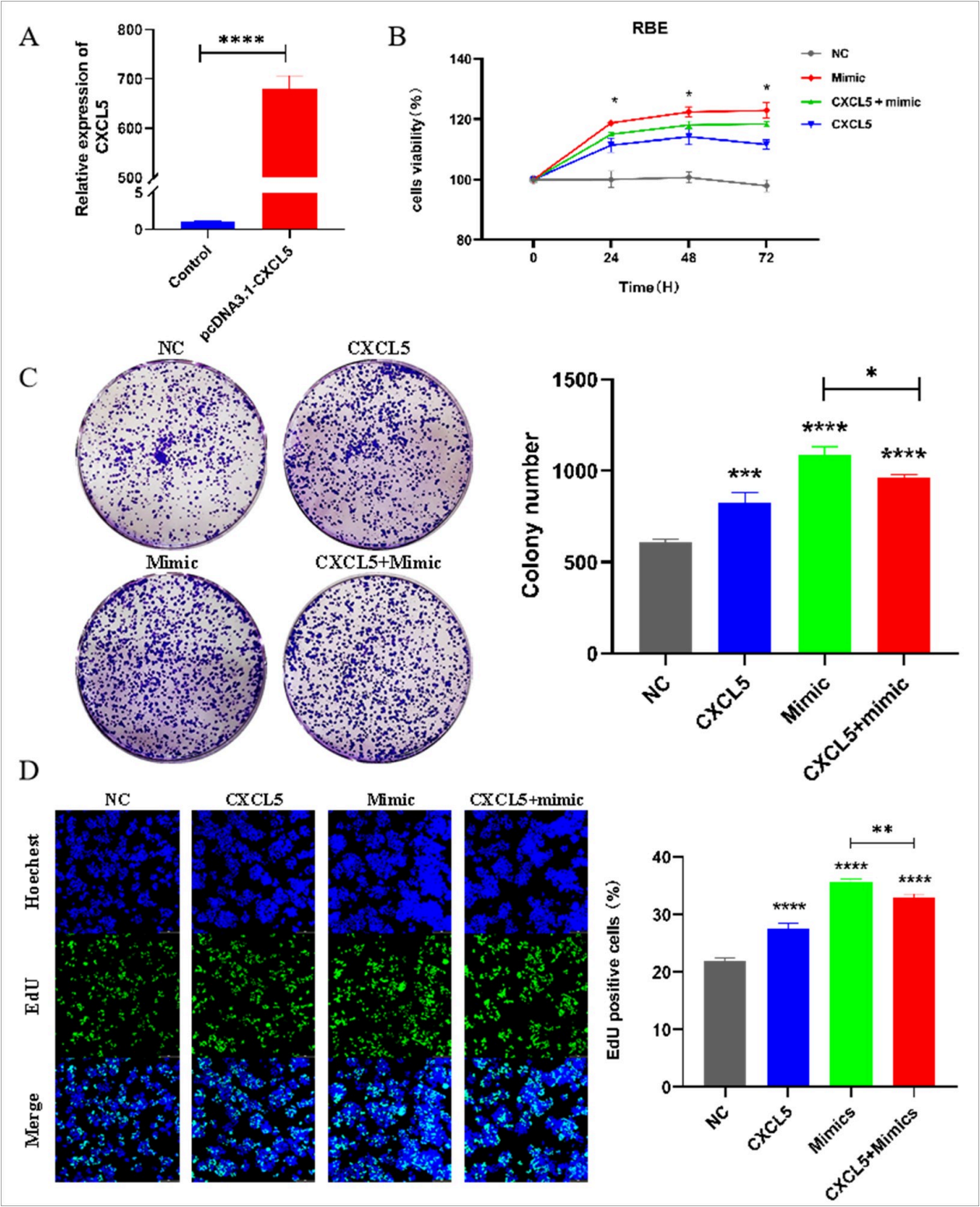


Figure 6

Co-transfect functional rescue experiment of Csi-miR-190-3p and CXCL5.

(A) The CXCL5 expression after transfection of pcDNA3.1-CXCL5 plasmid in RBE cells. (B) The CCK-8 assay was employed to detect cell proliferation. RBE cells were detected at 0, 24, 48, and 72 h post-treatment. (C) The colony formation assay were conducted to determine the proliferation capacity of RBE cells. (D) EdU-488 assays were used to detect the proliferative ability of CCA cells. Representative images are shown, and the summary results are from three independent experiments.

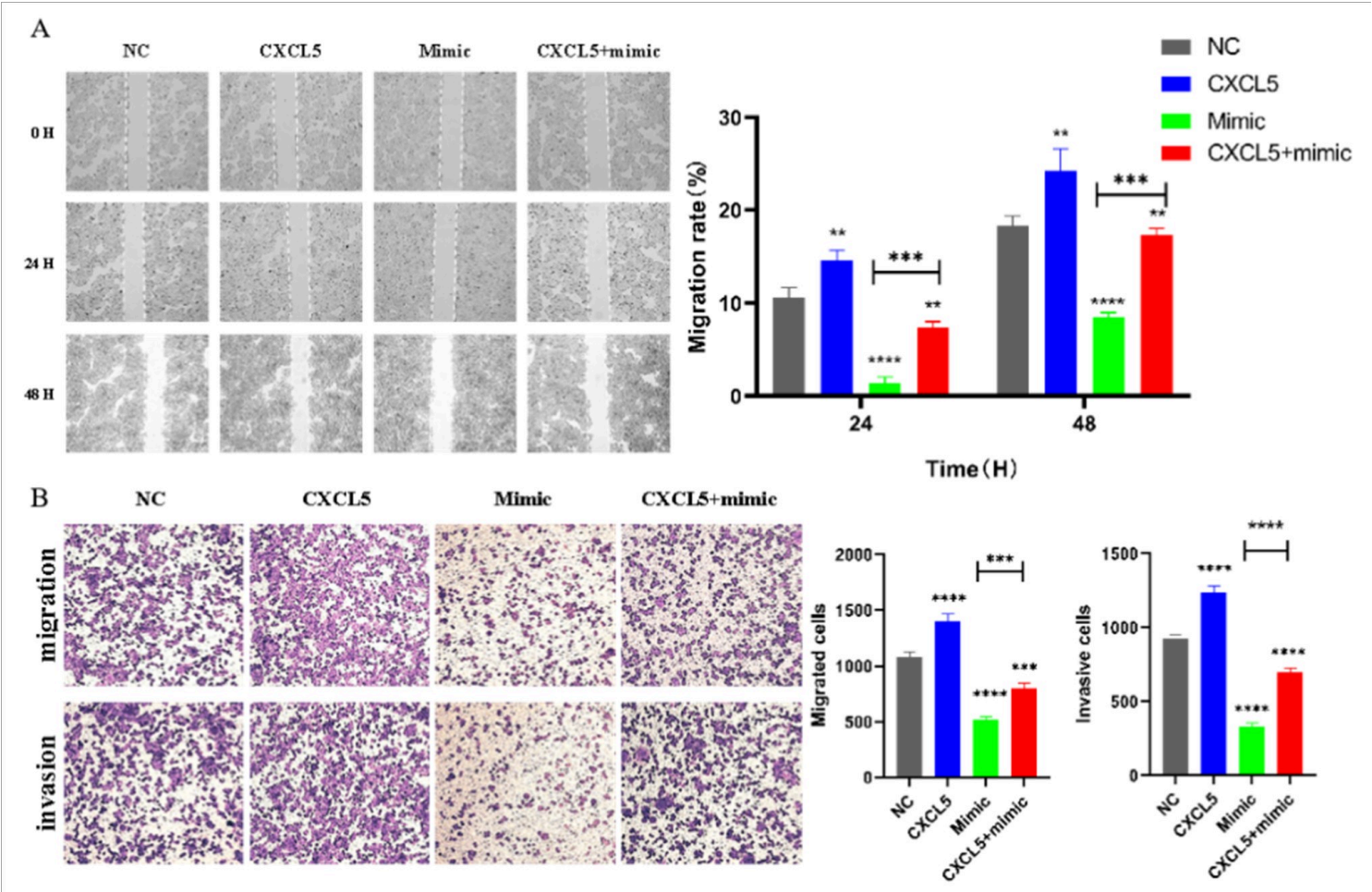


Figure 7

CXCL5 overexpression rescues the inhibitory effects of Csi-miR-190-3p on migration and invasion in CCA cells. (A) Wound healing assay for RBE cells showed motility changes. Images were captured at 0 h, 24 h and 48 h after scratching, with representative pictures displayed. (B) Transwell assay was conducted to determine the migration and invasion abilities of RBE cells after treatment. Representative images of matrigel-free (for migration) and matrigel-coated (for invasion) transwell assay were shown. Each group underwent three independent experiments.

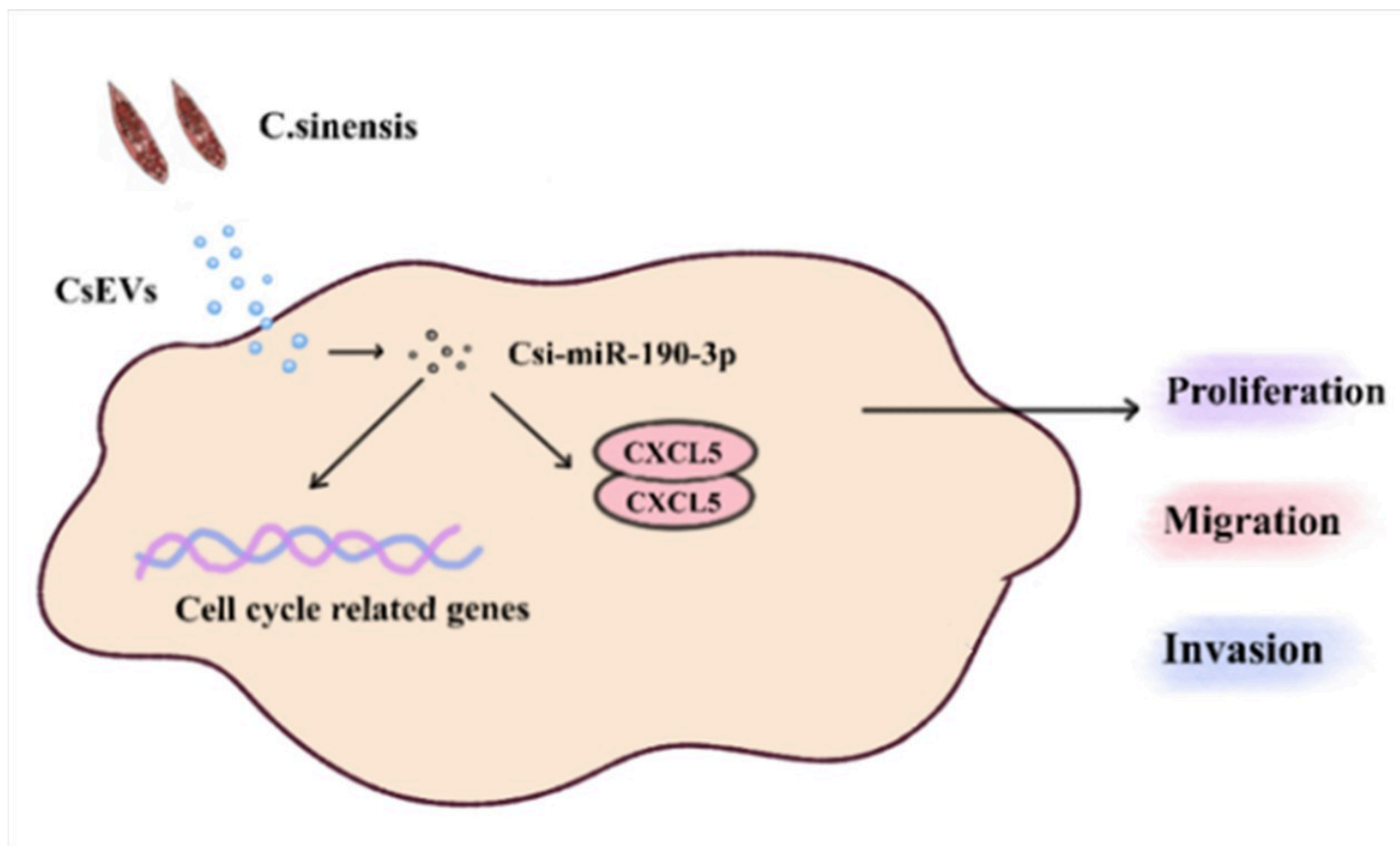


Figure 8

Schematic model of *Csi*-miR-190-3p-mediated dual regulation of CCA proliferation, migration and invasion

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