PGM5-AS1 Inhibits the Malignant Phenotype of Colon Cancer Cells by Regulating PAEP and NME1

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Research

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Abstract

Background: An increasing number of long non-coding RNAs (lncRNAs) is recognized to be associated with drug resistance in CRC.

Methods: For identifying differentially expressed target genes regarding PGM5-AS1, RNA transcriptome sequencing was performed. The mechanism by which PGM5-AS1 regulates its target genes was explored by performing experiments such as fluorescent in situ hybridization assay, dual luciferase reporter gene assay and RNA immunoprecipitation.

Results: The lncRNA PGM5-AS1 was identified by analyzing data from the original microarray data set of colon cancer (GSE75970). PGM5-AS1 additionally suppressed acquired oxaliplatin resistance in CRC cells. Malignant phenotype of PGM5-AS1 was inhibited by recruiting SRSF3 to activate alternative splicing and being a sponge specific to hsa-miR-423-5p.

Conclusions: Downregulation of PGM5-AS1 in oxaliplatin-resistant colon cancer tissues and cell lines is induced by transcriptional inhibition of GFI1B. PGM5-AS1 recruited SRSF3 to activate alternative splicing to downregulate the expression of PAEP. In addition, PGM5-AS1 could competitively bind with hsa-miR-423-5p to upregulate the expression of NME1. PGM5-AS1 inhibits the proliferation, invasion, migration and acquired oxaliplatin resistance of colon cancer cells through these two pathways.

Background

Colorectal cancer (CRC) ranks 3rd in common cancer types related to men and women, and also the major cause of death from cancer in the U.S. Furthermore, morbidity and mortality associated with CRC are gradually increasing among the young and middle-aged population[1]. Chemotherapeutic regimens based on oxaliplatin, such as the administration of fluorouracil, calcium folinate, and oxaliplatin (FOLFOX), or capecitabine plus oxaliplatin (CAPOX), are used to treat patients suffering stage III colon cancer postoperatively[2-4]. However, in almost all cases of metastatic CRC, resistance to oxaliplatin eventually develops[5]. As a commonly used third-generation platinum analog, oxaliplatin induces cell cycle arrest as well as cell death in proliferative cells[6, 7]. Elucidation of the mechanism by which drug resistance to oxaliplatin develops may enable to confirm novel therapeutic targets as well as colorectal tumor sensitivity to oxaliplatin.

Long non-coding RNA (lncRNAs) are long RNA transcripts (> 200 nucleotides) that do not possess protein-coding capabilities. As high-throughput sequencing (HTS) technology has made some advances, an increasing number of lncRNAs associated with cancer are identified as well as verified[8, 9], including those associated with drug resistance in CRC. For instance, linc00152 promotes tumorigenesis in vitro and in vivo, as well as resistance of tumor cells to oxaliplatin-induced apoptosis[10]. SNHG5 knockdown leads to CRC cell cycle arrest as well as apoptosis in vitro, and suppresses growth of tumor in vivo. In contrast, the overexpression of SNHG5 inhibits oxaliplatin-induced apoptosis[11]. The lncRNA CCAL, which is expressed by carcinoma-associated fibroblasts (CAF) of colorectal tumor stroma, increases the

resistance of tumor cells to chemicals[12]. Because lncRNAs regulates a subset of genes and can themselves be regulated by transcription factors, cancer cell death can be induced more effectively by targeting lncRNAs than by targeting an individual gene[13]. Therefore, investigation of the role of lncRNAs in oxaliplatin resistance, and the specific regulatory mechanisms, have received widespread attention worldwide.

In the current study, we conducted a detailed analysis of RNA expression profile data for examining the function possessed by lncRNA PGM5-AS1 in colon cancer and investigating its expression levels in both oxaliplatin-resistant colon cancer tissues and CRC cell lines. We collected data on the clinicopathological characteristics of patients with colon cancer and revealed that the expression of PGM5-AS1 is regulated by a transcriptional repressor. The study results help to better understand how PGM5-AS1 regulates the proliferation, invasion, migration, and acquired oxaliplatin resistance of colon cancer cells, as well as reveal molecular biological mechanisms that may underlie such effect. Our research advances knowledge of the mechanisms underlying resistance to oxaliplatin in CRC, and may be valuable for the development of more effective therapeutic strategies against this disease.

Methods

Analysis of the RNA expression profile in colon cancer

We downloaded the microarray data together with relevant clinical information from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/gds/) database. The CRC gene expression dataset (GSE75970) was obtained from GEO and analyzed to compare the strength of the RNA standardized probe levels of four human CRC tissues and four corresponding adjacent tissues. Next, differentially expressed lncRNAs ($P < 0.05$, the absolute value of logFC> 2) were identified. Based on the results, HELM software processed the normalized signal data in original state, and a heat map was generated.

Patient samples

All 62 CRC patients in this study received oxaliplatin. The clinical pathological information for the patients is shown in Table 1. We collected the cancer tissues together with adjacent normal tissues from January 2008 to January 2018, which were verified by a professional pathologist, and stored in the liquid nitrogen at -80℃. This research plan had obtained the approval of the Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu), and all experiments were conducted following the Declaration of Helsinki. We had obtained the written informed consent from all participants. We conducted long-term follow-up of these patients and determined the overall survival time from the surgery time to the death of patients or last follow-up. In addition, progression-free survival (PFS) from surgery time to the occurrence of the first relapse or the last follow-up was determined.

Cell culture

Human colon normal epithelial cell lines (HCoEpiC), colon cancer cell lines (DLD1, SW480, LOVO), as well as corresponding drug-resistant cell lines (DLD1-OR, SW480-OR, LOVO-OR) were provided by the Chinese Academy of Sciences Cell Bank (Shanghai, China). We cultured the cells in HyClone Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (10%, FBS) (Sigma-Aldrich, St. Louis, Missouri, USA), 100 U/ml penicillin, and 100 μg/ml chain A complete culture medium composed of amycin (Life Technologies, Grand Island, NY) in a cell incubator containing 5% CO_2 at 37℃.

Extraction of RNA and real-time quantitative PCR analysis

TRIzol reagent (Invitrogen, California, USA) assisted in extracting the total RNA from tissues and cells. A PrimeScript RT kit (Takala, Nanjing, China) was applied to the synthesis of synthetic cDNA. Real-time quantitative PCR (qRT-PCR) used an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA) together with SYBR Green Premix Ex Taq reagent (Takala, Nanjing, China) to determine relative RNA expression levels. In addition, total RNA levels were standardized via glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Supplementary Table S2 lists the primer sequence. In particular, in order to distinguish different transcript variants of PAEP, we designed specific primers to detect PAEP pre-mRNA (PAEP-p), PAEP transcript 1 (PAEP-1), transcript 3 (PAEP-3), and PAEP 3 transcripts (PAEP-all). Because it was not possible to design primers that specifically detect transcript 2, this study only discusses the transcription levels of transcripts 1 and 3. The $2^{-\Delta\Delta CT}$ method assisted in determined the relative quantitative value possessed by each gene.

Construction of a cell line stably expressing PGM5-AS1

In order to stably overexpress PGM5-AS1 in human CRC cells, we constructed a lentivirus (Hanheng, Shanghai, China) vector to obtain a stable overexpression cell line. The transfection step was performed strictly in accordance with the supplier’s instructions, and puromycin was used for screening.

Plasmid construction and cell transfection

In this study, we used Lipofectamine 3000 (Invitrogen) and p3000 for transfection of siRNA and plasmids into CRC cells. The small interfering RNA (siRNA) sequences designed for PGM5-AS1, GFI1B, SRSF3, PAEP, and NME1 are listed in Supplementary Table S2. To ensure the effectiveness of knockdown, different siRNAs (three types) and si-NCs were designed for different sites of PGM5-AS1. The full-length complementary cDNAs were synthesized for GFI1B, SRSF3, PAEP, and NME1 in human body, which were then cloned into pcDNA3.1, an expression vector (Invitrogen). In addition, RiboBio (Guangzhou, China) helped to design and synthesize the miR-423-5p mimetics, miR-423-5p inhibitors, as well as related controls.

Cell proliferation analysis

CCK-8 assay (Dojindo, Kumamoto, Japan) assisted in examining cell proliferation ability, in line with the instructions of manufacturer. After counting cells undergoing different treatments, we seeded 5000 cells

on 96-well culture plates. We added CCK-8 reagent to culture plate at certain time point. Cells received 2 h of incubation in a 37℃ incubator. An Infinite M200 spectrophotometer (Tecan, Switzerland) measured the absorbance at 450 nm.

Colony formation experiment

Cells subjected to various treatments were evenly distributed in each well of a six-well plate, which was then added with an appropriate amount of culture medium, and placed in an incubator for two weeks. During this period, we changed the medium each 2-3 days. After removing the culture plate from the incubator, cells were fixed for twenty minutes by using methanol and stained for fifteen minutes by using phosphate buffer (PBS) that contained 0.1% crystal violet (Sigma), and the results of the study were photographed.

5-ethynyl-2'-deoxyuridine (EdU) assay

For the EDU assay, cells used in the experiment were pre-plated in a 24-well plate on the basis of instructions of manufacturer. Next, the EDU solution was configured, 10 μM EdU was added to each well, and the culture plate received 2 h of incubation. After 2 hours, cells were fixed with 4% formaldehyde for 30 minutes. Then, we used special reagents for EDU staining; this step was carried out for 30 minutes. DAPI was used to stain cells at last. After 10 minutes, the culture plates were observed with a fluorescence microscope (Olympus), and Image-Pro Plus 6.0 software (Media Cybernetics) helped to calculate the ratio of the EdU-positive cells to the total DAPI-positive cells (i.e. blue cells).

Xenograft mouse model

Five male BALB/C nude mice (four weeks old), provided by the Animal Center of Nanjing University (Nanjing, China), were used in this study. Mice were kept in a laminar flow cabinet at 28℃ and under 50% humidity, in a pathogen-free environment. We used DLD1 cells with stable overexpression of PGM5-AS1 and a control group to perform cell proliferation assays in nude mice. We extracted cells in the two groups and resuspended them at a density of 3×10^6 cells/ml. Every nude mouse received subcutaneous vaccination with 3×10^6 cells on both sides of its armpits. Observations were performed every 5 days to observe tumor formation and growth in nude mice axilla, with the tumor volume being recorded. The tumor volumes = length × width ^{2} × 0.5. After the 30th day, the asphyxiated nude mice were dissected, sampled, and analyzed. The entire experimental process strictly obeyed the National Institutes of Health (NIH) guidelines specific to the use of laboratory animals.

Immunohistochemical analysis

We embedded tumor tissue samples in paraffin, and sliced them later. Hematoxylin eosin (HE) helped to stain the sliced samples. Regarding to the immunohistochemical study, samples were incubated by using human anti-Ki67 antibody at 4℃ overnight. The next day, after being washed, samples were incubated in a water bath at 37℃; next, a second antibody was added dropwise, and the samples were then washed

with PBS. Subsequently, samples were treated with hematoxylin and DAB solution, washed, sliced, dehydrated, treated by using a transparent medium, and at last fixed on a glass slide. A microscope was used to visualize the slide and visualized. With ≥50% of cancer cells being stained, we consider the result positive.

Flow cytometry

In this study, flow cytometry assisted in analyzing the cell cycle stage as well as identifying apoptotic cells. First, we used trypsin to digest the cells. Based on the protocol of manufacturer, cells were stained by virtue of FITC-Annexin V Apoptosis Detection Kit (BD Biosciences) using FITC-Annexin V together with propidium iodide; then, flow cytometry (FACScan; BD Biosciences, Franklin Lakes, New Jersey, USA) was carried out. The results allowed CRC cells in different cell cycle stages to be distinguished. In addition, we compared the proportion of early apoptotic cells among all cells between the two groups. In line with the protocol of manufacturer, when performing cell cycle experiments, we used aCycleTEST PLUS DNA kit (BD Biosciences) for the calculation and comparison of cells in G0–G1, S, and G2–M phase in the two group via FACScan analysis.

Cell invasion and migration experiment

For detecting the invasion and migration of cells, 1×10^4 CRC cells subjected to various treatments were evenly spread the upper chamber (pore size 8 μm; Millipore, Billerica, MA, USA), together with being cultured in medium free of serum. The lower chamber was added with medium that contained 10% FBS. 24 hours later, a cotton swab was used to scrap off the CRC cells remaining on the upper membrane, which were fixed (15 min) and stained (20 min) by using 4% methanol and 0.1% crystal violet, respectively. Next, an inverted microscope (Olympus, Tokyo, Japan) was used to observe these cells.

RNA Sequencing

We extracted RNA from si-PGM5-AS1-transfected SW480 cells and the control to be used for RNA sequencing. FPKM values were log10-transformed and processed using HELM software to generate heat maps.

Western blot analysis

Cells subjected to various treatments were collected in order to prepare protein samples; then, buffer was added and the protein samples were sampled. Subsequently, based on the protocol of manufacturer, protein samples underwent electrophoretic separation with 10% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. At room temperature, the membrane was blocked for one hour in 5% skimmed milk, followed by incubation by using primary antibody at 4°C overnight. In the next day, after being washed, membrane received one hour of incubation by using secondary antibody at room temperature on a shaker. Subsequently, the PVDF film was

developed using ECL chemiluminescence reagents. The intensity of the band was observed and determined by the optical density method (Quantity One software; Bio-Rad, Hercules, CA, USA).

Fluorescence in situ hybridization assay

We used 4% formaldehyde to fix SW480 cells for 15 minutes, and used PBS to wash the fixed cells. Pepsin (1% in 10 mmol/l HCl) was employed to treat these cells, followed by continuous dehydration with ethanol. The FISH probe (40 nmol/l; U6, 18s, PGM5-AS1) and the continuously dehydrated cells were then mixed in hybridization buffer, followed by 2 min of incubation at 80 ° C, and subsequently at 55 ° C. After 2 hours, cells were first washed and then dehydrated. At last, DAPI and Prolong Gold Antifade Reagent were used for observation and detection. The RNA FISH probe came from Ribobio. The experiment took IgG as a negative control.

Separation of cytoplasmic and nuclear RNA

Based on the instructions of manufacturer, the PARIS kit (Life Technologies) assisted in isolating the nucleus and cytoplasm from the CRC cells. The extract was used for reverse transcription as well as RT-PCR (SYBR Pre-mix Ex Taq; TaKaRa) RNA. Supplementary Table S2 lists the sequence of primers.

RNA immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP)

We prepared CRC cell lysates with the help of the Magna RIP RNA-binding protein immunoprecipitation kit as well as incubated them with RIPA buffer that contained magnetic beads and antibodies. Normal mouse IgG was used as the negative control. RNA fractions precipitated with RIPA buffer were analyzed by qPCR. For ChIP analysis, we crosslinked CRC cells by using 1% formaldehyde for ten minutes, followed by quenching them by using glycine. Sonication on cell lysates produced the chromatin fragments, followed by the immunoprecipitate by using antibodies overnight at 4°C. After they were rinsed and eluted, the EZ ChIP kit (Millipore) was employed to remove the cross-linked chromatin in accordance with instructions of manufacturer. IgG antibodies were used as negative controls for qRT-PCR. Supplementary Table S2 lists the sequence of ChIP primers.

Luciferase reporter gene detection

In order to detect the gene binding to the PGM5-AS1 promoter region, a reporter gene plasmid that inserts the PGM5-AS1 promoter full length/PGM5-AS1 promoter binding sequence deletion in front of the luciferase expression sequence was constructed. Via luciferase reporter gene experiments, SW480 cells under the transfection of pcDNA-GFI1B/empty vector or si-GFI1B/si-NC were detected. In the luciferase reporter gene analysis of miRNA target gene, we synthesized PGM5-AS1 and NME1 wild-type sequences containing potential miR-423-5p binding sites or mutations at each binding site and co-transfected them into 293T cells. A dual-luciferase reporter gene detection system (Promega) helped to measure the luciferase activity.

Statistical analysis

We used GraphPad software version 7.0 (San Diego, California, USA) to analyze experimental data. The chi-square test, Student's t-test, or one-way analysis of variance (ANOVA) were adopted for estimating the significance of differences according to specific data types. The log-rank test assisted in calculating the overall survival (OS) of different types of patients. Pearson correlation coefficient was used to determine correlations between variables. $P < 0.05$ denoted statistical significance.

Results

Identification of PGM5-AS1 by analysis of RNA expression profile data of CRC

To identify lncRNAs that play a regulatory role in CRC, the original microarray data set (GSE75970) from the GEO database, which contains data for 4 pairs of colorectal cancer tissues and paraneoplastic tissues, was downloaded. We focused on ncRNAs (including pseudogene-derived ncRNAs) with differential expression (logFC>2 or logFC<-2; $P<0.05$ indicating significant difference) between tumor tissues and paraneoplastic tissues. Figure 1A shows a heat map generated by processing the signal intensities (normalized signal intensities) of these ncRNAs using HELM software. MNX1-AS1 (NR_038835), the lncRNA with the most significant upregulation of expression, is reported to inhibit carcinogenesis in glioblastoma by suppressing miR-4443[14]. MNX1-AS1 partly regulates BTG2 and BCL2, thereby facilitating gastric cancer progression[15]. The up-regulated lncRNA UCA1 (NR_015379) promotes immune escape in gastric cancer by binding to anti-tumor miRNA[16]. In lung cancer, UCA1 promotes tumorigenesis by competitively binding to miR-193a-3p[17]. In colon cancer, UCA1 enhances tumor cell proliferation by modulating the miR-28-5p/HOXB3 axis[18]. HAND2-AS1 (NR_003679) promotes liver cancer stem cell autologous renewal via BMP signaling pathway activation[19]. HAND2-AS1 targets miR-1275 and up-regulates KLF14 to exert a tumor-suppressive effect in CRC[20] and it also modulates the miR-20a/PDCD4 axis for inhibiting the 5-fluorouracil resistance in CRC[21]. Numerous other lncRNAs in the GSE75970 dataset have not been characterized in terms of their roles in colon cancer. For example, PGM5-AS1 (NCBI, Gene, NR_015423) has been reported to regulate the miR-466/PTEN axis to exert tumor-suppressive effects in esophageal squamous cancer[22]; furthermore, abnormally low expression of PGM5-AS1 is related to the poor prognosis of renal clear cell carcinoma[23]. Zhu et al.[24]downloaded the original microarray data set (GSE64857) containing analysis data of gene expression of patients suffering recurrent and non-recurrent CRC, and performed bioinformatics analysis to show that PGM5-AS1 was highly co-expressed with differentially expressed mRNAs. However, no further studies of the role and underlying molecular mechanism of PGM5-AS1 in colon cancer has been carried out. Therefore, we comprehensively investigated the function possessed by PGM5-AS1 in this context. PGM5-AS1 (PGM5 antisense RNA 1), which is also known as FAM233A, is located at 9q21.11, variant 3 (NR_015423, hereinafter referred to as PGM5-AS1) employs another splice site on the 5' exon. Relative to variant 1, variant 3 has a shorter transcript, whose length is 586 bp. We input the primary sequence of PGM5-AS1 into the ViennaRNA Web Services server (http://rna.tbi.univie.ac.at/) for calculating as well as predicting its RNA secondary structure (Figure 1B).

PGM5-AS1 is lowly expressed in oxaliplatin-resistant CRC tissues and cell lines

Efforts are necessary to overcome lncRNA-mediated oxaliplatin resistance to ensure the effectiveness of therapeutic interventions in colon cancer[11, 25]. linc00152 is resistant to oxaliplatin in colon cancer being a competitive endogenous RNA[10]. To determine whether PGM5-AS1 plays a role in colon cancer-acquired oxaliplatin resistance, qRT-PCR was conducted in human colon normal epithelial cell lines (HCoEpiC), CRC cell lines (LOVO, SW480, DLD1), as well as 3 colon cancer cell lines resistant to oxaliplatin for examining PGM5-AS1 expression after oxaliplatin treatment. Test results showed that PGM5-AS1 was significantly down-regulated in CRC cell lines compared with HCoEpiC cell line (Figure 1C). Furthermore, relative to respective parental cell lines, PGM5-AS1 exhibited an obvious down-regulation in established oxaliplatin-resistant cell sublines (Figure 1D). Next, we tested the levels of PGM5-AS1 in 62 paracancerous normal tissues, 42 primary colon cancer tissues, together with 20 recurrent colon cancer tissues (not sensitive to oxaliplatin treatment) by qRT-PCR. Compared with that in normal tissues, PGM5-AS1 was under-expressed in colon cancer tissues. In addition, in recurrent colon cancer tissues, PGM5-AS1 expression was significantly reduced compared with that in primary colon cancer tissues (Figure 1E and S1A).

Colon cancer tissues was divided into two groups: group with a high PGM5-AS1 expression and group with a low PGM5-AS1 expression. Grouping was performed considering the median PGM5-AS1 expression (Figure 1F). Then, a chi-square test was performed for comparing the difference between them regarding the clinicopathological characteristics. Results were as follows: the downregulation of PGM5-AS1 expression in CRC patients is related to colon depth of tumor ($P = 0.03951$), advanced TNM stage ($P = 0.01105$), regional lymph node invasion ($P = 0.00484$), and distant metastasis ($P = 0.02115$) (Table 1). Subsequently, Kaplan-Meier analysis assisted in confirming the impact of PGM5-AS1 expression on the prognosis of patients. Based on results, higher PGM5-AS1 expression in colon cancer patients leads to the higher OS (Figure 1G). Besides, patients whose PGM5-AS1 expression was high showed better OS after oxaliplatin treatment, while patients whose PGM5-AS1 expression was low responded poorly to oxaliplatin treatment (Figure 1G). In summary, lncRNA PGM5-AS1 is down-regulated in oxaliplatin-resistant colon cancer cell lines and tissues, suggesting the association of PGM5-AS1 with acquired oxaliplatin resistance in this cancer.

Table 1

Characteristics	Expression of PGM5-AS1		P value*
	low(n=31)	high(n=31)	Chi-squared test
Sex			0.12731
Male	13	19	
Female	18	12	
Age			0.07297
≤60	10	17	
>60	21	14	
Histological grade			0.44514
low or undiffer	16	13	
middle or high	15	18	
TNM stage			0.01105*
I and II	11	21	
III and IV	20	10	
Depth of tumor			0.03951*
T1 and T2	14	22	
T3 and T4	17	9	
Regional lymph node invasion			0.00484**
Negtive	12	23	
Positive	19	8	
Distant metastasis			0.02115*
Negtive	13	22	
Positive	18	9	

GFI1B suppresses transcription of PGM5-AS1

For elucidating the potential mechanisms of the abnormally low PGM5-AS1 expression in colon cancer, we used the JASPAR database (http://jaspardev.genereg.net/) and calculated and screened transcription factors or transcriptional inhibitors that may bind to the promoter region of PGM5-AS1 on this basis. The results showed that a putative binding site of growth factor independent 1B transcriptional repressor (GFI1B) was present in the promoter region of PGM5-AS1 (Supplementary Table S1). GFI1B is an inhibitory transcription factor that regulates the quiescence of hematopoietic stem cells and

differentiation of red blood cells and platelets[26]. Plasmid (pcDNA-GFI1B) was used to overexpress GFI1B, and small interfering RNA (si-GFI1B) assisted in knocking down GFI1B (Figure S1B). We then examined whether GFI1B induces reduced expression of PGM5-AS1. qRT-PCR results showed that lower levels of PGM5-AS1 were induced in CRC cells transfected with the GFI1B vector (Figure 1H). Knockdown of GFI1B with siRNA resulted in upregulation of PGM5-AS1 expression (Figure 1H). In order to verify the expression of GFI1B in tumor tissues, GFI1B expression in 62 pairs of cancer tissues as well as the adjacent normal tissues was checked, finding elevated GFI1B expression in CRC tissues (Figure 1I). Further, PGM5-AS1 expression showed a negative relation to GFI1B expression in CRC tissues (Figure 1J). ChIP assay was used to verify whether GFI1B could suppress the transcription of PGM5-AS1. We found that GFI1B can bind to the promoter region of PGM5-AS1 (Figure 1K). We then inserted the full-length PGM5-AS1 promoter/PGM5-AS1 promoter binding sites-deleted sequence in front of the luciferase expression sequence to construct a reporter gene plasmid. The luciferase reporter assay helped identify SW480 cells under the transfection of pcDNA-GFI1B/empty vector. Compared with that for the empty vector, the activity of the full-length PGM5-AS1 promoter of the pcDNA-GFI1B group decreased. After transfection with pcDNA-GFI1B, compared with that in group with the complete PGM5-AS1 promoter, enhanced promoter activity was observed in the group with deletion of the PGM5-AS1 promoter binding sequence (Figure 1L). In conclusion, highly expressed GFI1B in CRC can bind to the cis-acting element of the PGM5-AS1 promoter region, thereby inhibiting the transcription of the latter, which partially explains the abnormally low PGM5-AS1 expression in colon cancer.

PGM5-AS1 inhibits proliferation, invasion, and migration of colon cancer cells

Aiming at studying the biological function possessed by PGM5-AS1 in colon cancer, PGM5-AS1 was ectopically expressed using a lentiviral infection system (Figure S1C) to establish a colon cancer cell line DLD1 that stably overexpressed PGM5-AS1 (Figure S1C). To ensure the effectiveness of knockdown, we designed three siRNAs targeting different sites (si-PGM5-AS1 1 #, si-PGM5-AS1 2 #, and si-PGM5-AS1 3 #) to silence PGM5-AS1 expression in SW480 cells (Figure S1D). si-PGM5-AS1 1 # with the highest silencing efficiency was used in subsequent studies. As indicated by the CCK-8 assay, PGM5-AS1 overexpression reduced colon cancer cells' proliferation ability (Figure 2A). In contrast, the proliferation ability regarding SW480 cells under transfection of si-PGM5-AS1 presented an obvious improvement (Figure 2A). The same results were obtained in the clone formation experiment and the EdU assay (Figure 2B-2C). For confirming whether PGM5-AS1 upregulation inhibited colon cancer cell growth in vivo, DLD1 cells stably overexpressed PGM5-AS1 were subcutaneously implanted in nude mice (Figure 2D). Tumors in nude mice inoculated with DLD1/PGM5-AS1 cells grew more slowly than those in nude mice inoculated with DLD1/mock cells (Figure 2E). After 30 days, the DLD1 / PGM5-AS1 cells group harbored a lower tumor weight relative to the DLD1/mock cells group (Figure 2F). Immunohistochemical analysis showed that, compared with that in the DLD1/mock cells group, the Ki-67 level was lower in the tumors of the DLD1/PGM5-AS1 group (Figure 2G). Overall, our results indicate that PGM5-AS1 suppresses colon cancer cell growth in vitro as well as in vivo.

In addition, to analyze how PGM5-AS1 affects the cell cycle, we used flow cytometry for further experiments. PGM5-AS1 significantly reduced S-phase cell proportion in DLD1 cells, and increased the ratio of G0/G1 cells. Consistent with this result, si-PGM5-AS1 significantly reduced the proportion of SW480 cells in the G0/G1 phase (Figure 2H). Further, we also used flow cytometry to detect the effect of PGM5-AS1 on apoptosis. Overexpression of PGM5-AS1 led to a higher percentage of apoptosis, while si-PGM5-AS1 significantly reduced apoptosis rates in SW480 cells (Figure 2I).

In addition, we further investigated whether PGM5-AS1 affects colon cancer cell migration and invasion. Transwell assay results showed that PGM5-AS1 overexpression significantly reduced colon cancer cell invasion (Figure 3A). We studied how PGM5-AS1 affects cell migration through the Transwell analysis method together with wound healing test to show that PGM5-AS1-overexpressing cells exhibited reduced migration ability (Figure 3B-3C), while si-PGM5-AS1 significantly enhanced the migration and invasion ability of cancer cells (Figure 3A-3C). Therefore, PGM5-AS1 inhibits migration and invasion regarding colon cancer cell in vitro.

PGM5-AS1 suppresses acquired oxaliplatin resistance in colon cancer cells

With the purpose of elucidating the function possessed by PGM5-AS1 in the oxaliplatin resistance in colon cancer cells, we overexpressed or knocked down PGM5-AS1 in oxaliplatin-resistant colon cancer cells as well as used oxaliplatin to treat them (Figure 3D). Based on CCK8 analysis, PGM5-AS1 overexpression showed a strong inhibiting effect on DLD1-OR cells' proliferation ability (Figure 3E). The results of clone formation experiments showed that PGM5-AS1 overexpression remarkably suppressed the survival rate regarding these cells (Figure 3F). The same result was obtained in the EdU assay (Figure 3G). Transwell analysis showed that PGM5-AS1 overexpression suppressed DLD1-OR cells in terms of invasion and migration compared with the control group (Figure 3H). We additionally studied the effect of PGM5-AS1 knockdown on oxaliplatin resistance in SW480 parental cells. We treated SW480 cells with different concentrations of oxaliplatin, and found that PGM5-AS1 knockdown enhanced the proliferation ability of these cells. However, overexpression of PGM5-AS1 reduced the proliferation ability of DLD1 cells (Figure 3I). The above results indicate that PGM5-AS1 can enable colon cancer cells to be more sensitive to oxaliplatin in vitro.

PGM5-AS1 inhibits the malignant phenotype of CRC by down-regulating PAEP and up-regulating NME1 expression

In order to determine the mechanism involved in the inhibition of CRC by PGM5-AS1 and to explore the changes in gene expression mediated by PGM5-AS1, we used RNA transcriptome sequencing to assess the overall effect of PGM5-AS1 knockout and found that 315 up-regulated genes ($\log_2$FoldChange$\geq$1, P$\leq$0.05), and 293 down-regulated genes ($\log_2$FoldChange$\leq$-1, P.$\leq$0.05), in SW480 cells (Figure 4A). In regard to the RNA-seq data for PGM5-AS1 knockdown, gene ontology (GO) analysis revealed that common biological activities involved include pathways related to cell growth/death, cell motility, transport and catabolism, and cellular community-eukaryotes (Figure 4B). In terms of the magnitude of

up-regulation and down-regulation, the FPKM values of the 50 mRNAs with the largest changes are shown in Figure 4C after log_{10}-conversion. The downregulated NME1 with the fourth most significant difference was also known as NM23-H1[27]. NME1 expression might make cancer cells more sensitive to cisplatin[28]. Overexpression of PAEP by up-regulated genes promotes melanoma tumor progression, invasion, and metastasis[29]. Colon cancer patients harbored a higher serum expression of PAEP relative to healthy controls[30]. The human *PAEP* gene (NCBI, Gene ID: 5047) generates three different transcript variants through alternative splicing events, among which variant 1 and 2 encode the same protein (isoform 1, 20 kDa). Variant 3 encodes isoform 2 (17 kDa) which is shorter than isoform 1. Because the probes used for microarray analysis cannot distinguish different transcript variants of PAEP, we designed specific primers to detect PAEP pre-mRNA (PAEP-p), PAEP transcript 1 (PAEP-1), transcript 3 (PAEP-3), and all three transcripts (PAEP-all). In particular, because specific primers for detecting transcript 2 could not be designed, we only discussed the levels of transcripts 1 and 3 in this study. After knocking down PGM5-AS1 in SW480 cells, qRT-PCR was employed for testing the PAEP and NME1 expression. As found, with NME1 expression being down-regulated, the expression of PAEP-3 expression increased significantly, while that of PAEP-1 did not show much difference (Figure 4D). After overexpression of PGM5-AS1 in DLD1 cells, a decrease in PAEP-3, little change in the expression of PAEP-1, and an increase in NME1 expression was detected by qRT-PCR (Figure 4E). In addition, western blotting analysis confirmed that after PGM5-AS1 knockdown, PAEP isoforms 1 and 2 were up-regulated in SW480 cells, and NME1 was down-regulated, at the protein level. In contrast, after overexpressing PGM5-AS1 in DLD1 cells, the opposite result was obtained by western blotting (Figure 4F). We detected the expression of PAEP and NME1 in CRC tissues and analyzed the correlation between PGM5-AS1 and PAEP/NME1. As shown in Fig. S2A and S2B, the expression level of PGM5-AS1 was negatively correlated with the level of PAEP (P=0.0137, Pearson r= -0.3114) and positively correlated with the level of NME1 (P=0.0021, Pearson r= 0.3829) in CRC tissues.

To investigate whether PGM5-AS1 down-regulated PAEP or up-regulated NME1 to inhibit CRC cell proliferation, invasion and migration, and chemical resistance, PAEP vector or si-NME1 was co-transfected into DLD1 cells stably overexpressing PGM5-AS1. As demonstrated by CCK8 assay (Figure 4G), clone formation experiments (Figure 5A), and Transwell assay (Figure 5B), co-transfection to some extent improved the reduced proliferation and invasion, and resistance to oxaliplatin induced by PGM5-AS1. si-PGM5-AS1 and si-PAEP or NME1 vector were used to co-transfect SW480 cells; results showed that after co-transfection, si-PGM5-AS1-induced proliferation, invasion, and oxaliplatin resistance were rescued (Figures 4H, 5C, and 5D). In summary, PGM5-AS1 inhibits the malignant phenotype of CRC by down-regulating PAEP and up-regulating NME1.

PGM5-AS1 downregulates PAEP expression by recruiting SRSF3 to activate alternative splicing

Many lncRNAs exhibit clear subcellular localization[31], and their function is related to their unique subcellular localization patterns[32]. Therefore, we conducted a FISH experiment (Figure 5E) and a nuclear-plasma separation experiment (Figure 5F) and found that the nucleus of colon cancer cells mainly saw the distribution of PGM5-AS1. Functions of nuclear lncRNA mainly involve chromatin

interaction, transcriptional regulation, and RNA processing[33], most of which require interaction with one or more RNA-binding proteins (RBPs)[34]. Therefore, we conducted bioinformatics analysis (http://starbase.sysu.edu.cn/index.php) for predicting RBPs that may interact with PGM5-AS1, and found that CLIP-seq data supported its interaction with SRSF3 and DKC1 (Supplementary Table S3). SRSF3, the smallest of the SR protein family, is essential for the formation of pre-mRNA and the alternative splicing of many transcripts, including its own[35-37]. At present, research on SR protein mainly focuses on its role in the nucleus, e.g. SR protein can affect transcription, co-transcription of mRNA processing, genome stability control, etc.[38, 39]. Detection of si-NC- or si-SRSF3-transfected SW480 cells using qRT-PCR, found the increase in PAEP transcript 3 expression in response to knockdown and overexpression of SRSF3. SRSF3 induced the down-regulation of PAEP transcript 3 in DLD1 cells (Figure 5G). In contrast, PAEP transcription 1 levels were not significantly different from those of control cells (Figure 5G). Western blotting data showed that the levels of both PAEP isoforms decreased in response to SRSF3 overexpression, and were up-regulated in response to SRSF3 knockdown (Figure 6A). We detected the expression of SRSF3 in CRC tissues and analyzed the correlation between SRSF3 and PAEP. As shown in Fig. S2C, the expression level of PAEP was negatively correlated with the level of SRSF3 in CRC tissues (P=0.0187, Pearson r= -0.2978).

Given that SRSF3 or PGM5-AS1 can alter the levels of PAEP transcript 3 and PAEP isoforms in cells, we asked whether PGM5-AS1 affects the alternative splicing of PAEP by interacting with SRSF3. We performed RIP analysis to confirm that PGM5-AS1 can interact with SRSF3 (Figure 6B). Therefore, we hypothesized that PGM5-AS1 could down-regulate PAEP expression by recruiting SRSF3 to PAEP pre-mRNA and activating alternative splicing. To verify this hypothesis, we conducted RIP experiments by overexpressing PGM5-AS1, and demonstrated that PGM5-AS1 increases SRSF3 enrichment on pre-PAEP mRNA (Figure 6C). In summary, PGM5-AS1 recruits SRSF3 to activate alternative splicing, thus down-regulating the expression of PAEP and suppressing the malignant phenotype of CRC.

Hsa-miR-423-5p up-regulates NME1 expression using PGM5-AS1 as a sponge

As shown in Figure 5E and 5F, PGM5-AS1 is not only distributed in the nucleus of CRC cells, but also in the cytoplasm; however, the regulatory role of PGM5-AS1 in the cytoplasm has not been elucidated so far. Based on many researches, some lncRNAs, inclusive of terminal tissue-induced differentiation ncRNA (TINCR)[40] and competition for endogenous RNA (ceRNA)[41], possess established cytoplasmic functions[31]. The ceRNA hypothesis, which has been widely used to predict lncRNA functions, assumes that ceRNAs are specific RNAs that can weaken the activity of microRNA (miRNA) through chelation, allowing the target gene of the miRNA to be highly expressed[42]. The online software programs LncBase v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex), ENCORI (http: //starbase.sysu. edu.cn/) together with lncRNASNP2 (http://bioinfo.life.hust.edu.cn/lncRNASNP#!/) were adopted, showing that miR-423-5p contains complementary sites to PGM5-AS1-miRNA-NME1 (Figure 6D, listed in Supplementary Table S4). miR-423-5p can promote malignant phenotype and temozolomide resistance in glioblastoma[43], play a role in the occurrence of brain metastases in lung adenocarcinoma, and inhibit the expression of MTSS1[44]. We

performed a 3'-untranslated region (3'-UTR) dual-luciferase reporter gene assay to determine whether direct binding of PGM5-AS1 to hsa-miR-423-5p occurred. We constructed a 3'-UTR luciferase reporter plasmid with wild-type as well as mutant PGM5-AS1 fragments (Figure 6E). Then, we co-transfected hsa-miR-423-5p mimics as well as PGM5-AS1 wild-type or mutant reporter genes into 293T cells and found that, when compared with that in the control and mutant groups, the activity of wild-type luciferase reporter molecules was significantly reduced (Figure 6F). Therefore, we confirmed the direct binding relationship between PGM5-AS1 and hsa-miR-423-5p by 3'-UTR luciferase reporter gene analysis. In addition, PGM5-AS1 overexpression in DLD1 cells led to obviously decreased hsa-miR-423-5p expression, whereas PGM5-AS1 silencing caused an obvious increase in the hsa-miR-423-5p expression (Figure 6G). We detected the expression of miR-423-5p in CRC tissues and analyzed the correlation between miR-423-5p and PGM5-AS1. As shown in Fig. S2D, the expression level of miR-423-5p was negatively correlated with the level of PGM5-AS1 in CRC tissues (P=0.0097, Pearson r= -0.3259). Taken all together, PGM5-AS1 directly interacts with hsa-miR-423-5p and acts as a sponge for hsa-miR-423-5p. Hsa-miR-423-5p expression mediated by mimics is used for exogenously regulating the hsa-miR-423-5p expression in CRC cell lines, and hsa-miR-423-5p inhibitors are used to inhibit the hsa-miR-423-5p expression in these cells (Figure S2E). Based on Figure 6H, hsa-miR-423-5p inhibitors and hsa-miR-423-5p mimics upregulated and downregulated NME1 protein level, respectively. We analyzed the correlation between miR-423-5p and NME1. As shown in Fig. S2F, the expression level of NME1 was negatively correlated with the level of miR-423-5p in CRC tissues (P=0.0159, Pearson r= -0.3051). In the subsequent luciferase reporter gene assay, we mutated the binding site in 3'-UTR of NME1 (Figure 6E). As demonstrated by results, the hsa-miR-423-5p mimic lowered the activity exhibited by luciferase reporter vector with NME1 3'-UTR-WT sequence. However, the mutant NME1 3'-UTR luciferase reporter vector did not produce a corresponding response (Figure 6I). Furthermore, the increase in NME1 expression induced by PGM5-AS1 overexpression was reversed by the hsa-miR-423-5p mimic (Figure 6J). Accordingly, we reasonably doubt that PGM5-AS1 may regulate NME1 expression through absorbing hsa-miR-423-5p in CRC. To test this hypothesis, luciferase reporter gene detection assisted in further studying how PGM5-AS1, hsa-miR-423-5p, and NME1 interacted with each other. The activity of the 3'-UTR luciferase reporter gene increased significantly after PGM5-AS1 overexpression; however, PGM5-AS1 and hsa-miR-423-5p co-transfection counteracted this effect of the PGM5-AS1 on the WT NME1 sequence. Mutation underwent by the hsa-miR-423-5p binding site also abrogated this effect (Figure 6K). These results indicate that hsa-miR-423-5p up-regulates NME1 expression taking PGM5-AS1 as the sponge, thus suppressing the malignant phenotype of CRC.

Discussion

Standard clinical treatments for colon cancer currently include surgery, radiotherapy, and chemotherapy. Although postoperative chemotherapy improves the duration of survival of colon cancer patients to a certain extent, acquired oxaliplatin resistance in colon cancer reduces therapeutic effectiveness. Clarification of the molecular mechanisms involved should enable the development of new strategies for blocking chemotherapeutic resistance in CRC. Recently, the role of lncRNA in various cancer-related processes, including in chemical resistance, has been extensively studied[45, 46]. In the study, oxaliplatin-

resistant cells and tissues harbor an abnormally low PGM5-AS1 expression. Studies have confirmed the inhibiting effect of PGM5-AS1 on colon cancer cell growth in vitro and in vivo. This effect may induce cell cycle arrest as well as apoptosis due to PGM5-AS1. PGM5-AS1 can also inhibit colon cancer cells with regard to migration and invasion and acquired oxaliplatin resistance in CRC. Upregulation of PGM5-AS1 can make oxaliplatin-resistant cells more sensitive to oxaliplatin, suggesting the involvement of this lncRNA in the induction of oxaliplatin resistance and its potential utility as a therapeutic target.

According to previous studies, abnormally high expression of lncRNA may be induced by transcription activators, stress, DNA methylation, and exosomes[47-51]. For example, the tumor suppressor gene p53 can transcriptionally activate lncRNA Pvt1b[47]. E2F1-mediated overexpression of the lncRNA PLACT1 continuously activates the NF-kappaB signaling pathway through a positive feedback loop to regulate the process of pancreatic ductal adenocarcinoma (PDAC)[48]. Stress-induced lncRNA LASTR is up-regulated in hypoxic breast cancer[49]. Upregulation of lncRNA SNHG12, mediated by DNA methylation, thereby activating and increasing temozolomide resistance in glioblastoma[50]. Exosome-mediated lncRNA AFAP1-AS1 activates ERBB2 translation[51]. However, few studies have explored the mechanism underlying the abnormally low expression of lncRNA. Here, we found that GFI1B, a transcription repressor, can bind to the cis-acting element of the PGM5-AS1 promoter region to suppress the transcription of PGM5-AS1. Previous research on GFI1B mainly focused on its role in the circulatory system, e.g. inhibition of the expression of Gata3, whose upregulation promotes the survival of lymphoma and T lymphocyte progenitor cells[52]. Blood progenitor cells in embryos lacking GFI1 and GFI1B can maintain endothelial gene expression[53]. GFI1B controls the cellular structure and functional integrity exhibited by hematopoietic stem cells and megakaryocytes[54]. Here, we hypothesized that GFI1B suppresses PGM5-AS1 transcription in colon cancer cells, and performed ChIP experiments and luciferase reporter gene experiments to verify this. GFI1B may transcriptionally regulate other genes in colon cancer and other cancers, and have diagnostic utility or serve as a therapeutic drug target for certain tumors. Further research is warranted to explore these possibilities.

It is widely accepted that lncRNAs have regulatory effects on the transcription and post-transcription of gene expression, in various cellular environments and biological processes[32]. The molecular function of lncRNAs mainly depend on their subcellular localization[55]. Therefore, in order to elucidate how PGM5-AS1 affects its downstream target genes, the localization of PGM5-AS1 in CRC cells was examined. We found that PGM5-AS1 is distributed in the nucleus and cytoplasm, with higher levels found in the nucleus. lncRNAs are capable of interacting with RNA, DNA and proteins to maintain the integrity of nuclear structure[32]. Our findings show that PGM5-AS1 recruits SRSF3 in colon cancer to activate the alternative splicing of PAEP pre-mRNA, thereby down-regulating the levels of PAEP transcript 3, PAEP isoform 1, and isoform 2 to inhibit CRC malignant phenotype. SRSF3 has been shown to prevent fibrosis by regulating splicing, mitogenic splicing, and epithelial-mesenchymal transition (EMT) to prevent the occurrence of liver cancer[56]. The upregulation of CCDC50S, under the mediation of HBx/SRSF3/14-3-3beta complex, could enhance the carcinogenicity presented by HCC[57]. YTHDC1 recruits SRSF3, hence facilitating exon inclusion in target mRNA[58]. Here, we report for the first time that lncRNA PGM5-AS1 mediates the

assembly of SRSF3 and PAEP pre-mRNA and affects the alternative splicing of the latter. Whether PGM5-AS1 can interact with other proteins, RNA, and DNA in the nucleus remains to be further explored.

In addition, the ceRNA hypothesis describes a new mechanism of RNA interaction. This hypothesis has received extensive attention because it unifies the functions of long noncoding RNA, pseudogene transcripts and circular RNA, and an alternative function of messenger RNA[42]. We found that PGM5-AS1, miR-423-5p, and NME1 contained related complementary sites, and showed that PGM5-AS1 competitively adsorbed miR-423-5p as well as partially relieved its binding from NME1 mRNA. Transcription levels of NME1 mRNA are downregulated in cells with high levels of metabolism[59]. NME1 is transcribed together with NME2 to produce a natural transcript. The protein encoded by this transcript is a fusion protein that contains sequences that share identity with each individual gene product[60, 61]. Whether PGM5-AS1 can interact with other miRNA to regulate NME1 expression in CRC remains to be further explored.

Conclusion

In summary, the findings of this study show that lncRNA PGM5-AS1 is under-expressed in oxaliplatin-resistant colon cancer tissues and cells, and that the transcription inhibitor GFI1B induces this downregulation in expression. PGM5-AS1 has the function of suppressing colon cancer cells with regard to their proliferation, invasion, migration, as well as acquired oxaliplatin resistance. PGM5-AS1 inhibits the malignant phenotype of CRC by recruiting SRSF3 to activate alternative splicing, thereby down-regulating the expression of PAEP and being a sponge of hsa-miR-423-5p in the up-regulation of NME1 expression (Figure 7). In addition, we determined that PGM5-AS1 expression is associated with better clinical overall survival and drug sensitivity. In summary, PGM5-AS1 is likely to be a biomarker of CRC and may help address the problem of oxaliplatin resistance in this cancer.

Abbreviations

CRC: colorectal cancer; lncRNAs: long non-coding RNAs; FOLFOX: fluorouracil, calcium folinate, and oxaliplatin; CAPOX: capecitabine plus oxaliplatin; HTS: high-throughput sequencing; CAF: carcinoma-associated fibroblasts; GEO: Gene Expression Omnibus; PFS: progression-free survival; DMEM: Dulbecco's Modified Eagle Medium; FBS: fetal bovine serum; qRT-PCR: Real-time quantitative PCR; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; siRNA: small interfering RNA; PBS: phosphate buffer; EdU: 5-ethynyl-2'-deoxyuridine; NIH: National Institutes of Health; SDS-PAGE: polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; RIP: RNA immunoprecipitation; ChIP: chromatin immunoprecipitation; OS: overall survival; PGM5-AS1 (PGM5 antisense RNA 1; GFI1B: growth factor independent 1B transcriptional repressor; GO: gene ontology; RBPs: RNA-binding proteins; TINCR: tissue-induced differentiation ncRNA; ceRNA: competition for endogenous RNA; miRNA: microRNA; 3'-UTR: 3'-untranslated region; PDAC: pancreatic ductal adenocarcinoma; EMT: epithelial-mesenchymal transition

Declarations

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Not applicable.

Authors' contributions

BQH designed research ideas and completed most of the experiments. CL wrote the manuscript and JW reviewed the manuscript. YTX, HJ, XFL designed and conducted animal experiments, LYX, JWW, YCY helped to collect and analyze data, YHG and KMW mainly provide administrative, technical and material support. The final manuscript has been read and approved by all authors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Nanjing Medical University and written informed consent was obtained from all patients. All international, national, and/or institutional guidelines for animal care and use were strictly followed during the course of the experiment. All procedures conducted in studies involving human participants were in accordance with the ethical standards of institutional and/or national research committees, as well as the 1964 Helsinki Declaration and its subsequent revisions or similar ethical standards.

Consent for publication

We have obtained consents to publish this paper from all the participants.

Competing interests

The authors declare that they have no competing interest.

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Figures

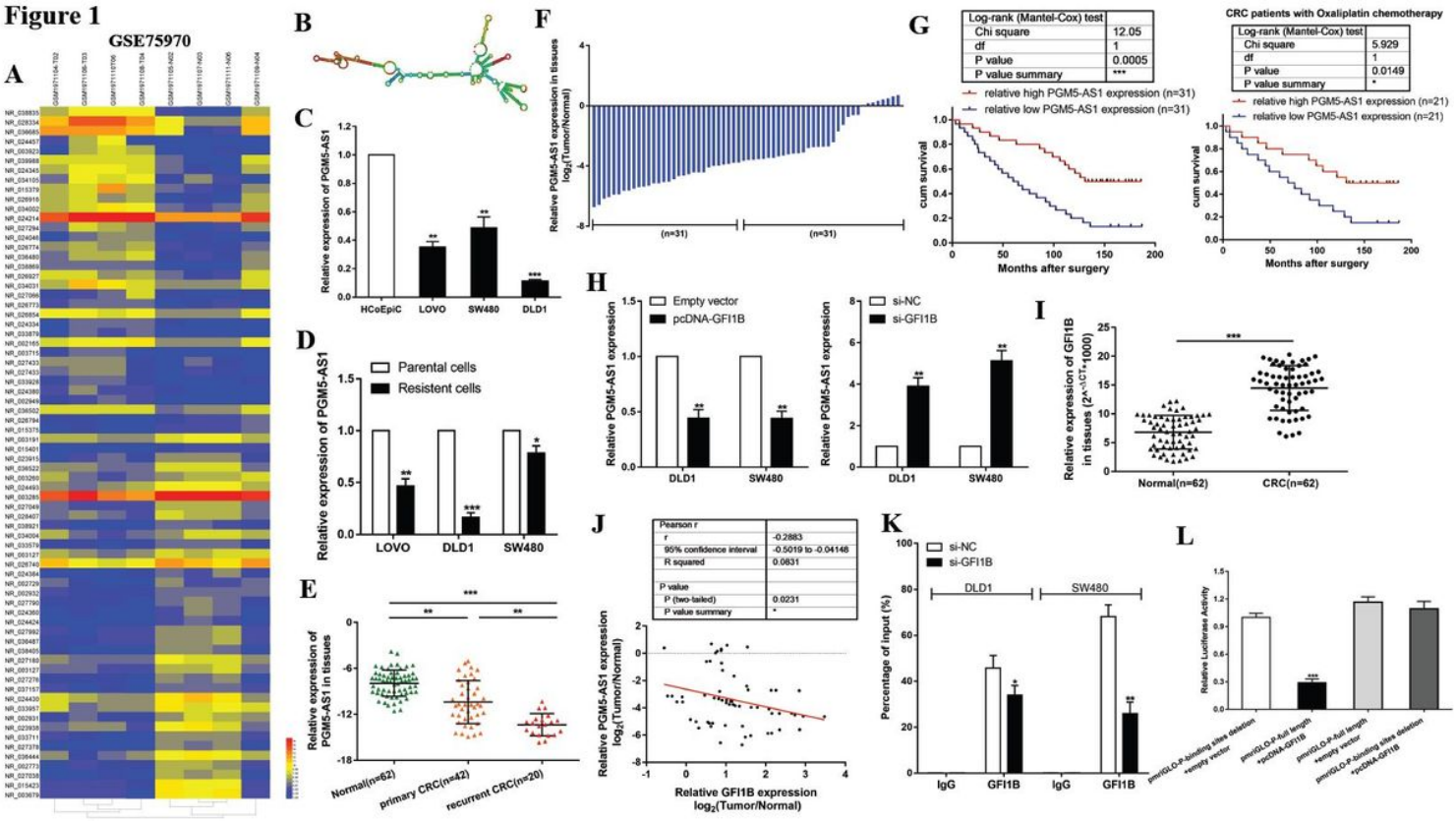

Figure 1

GFI1B suppresses transcription of PGM5-AS1 in CRC. A. Heat map generated by processing the signal intensities of ncRNAs with differential expression in the GSE75970 dataset from the GEO database. B. The ViennaRNA Web Services server was used to predict the RNA secondary structure of PGM5-AS1. C. The level of expression of PGM5-AS1 was examined in the CRC cell lines and HCoEpiC cell line. D. Expression level of PGM5-AS1 was examined in the established oxaliplatin-resistant cell sublines and the respective parental cell lines. E. Expression level of PGM5-AS1 was examined in primary colon cancer tissues, tissues from patients with recurrent colon cancer, and normal tissues. F. The samples were divided into two groups according to the median expression level of PGM5-AS1 in colon cancer tissues.

G. Kaplan-Meier overall survival (OS) curves of patients with colon cancer according to PGM5-AS1 expression level. H. The expression level of PGM5-AS1 was examined in CRC cell lines transfected with pcDNA-GFI1B or si-GFI1B. I. The expression level of GFI1B was examined in CRC tissues and adjacent normal tissues. J. The expression level of PGM5-AS1 in CRC tissues was negatively correlated with the level of GFI1B. K. The ChIP assay results showed that GFI1B could bind to the promoter region of PGM5-AS1. L. Luciferase reporter gene experiment was carried out to identify SW480 cells transfected with pcDNA-GFI1B/empty vector. *P < 0.05, **P < 0.01, ***P < 0.001.

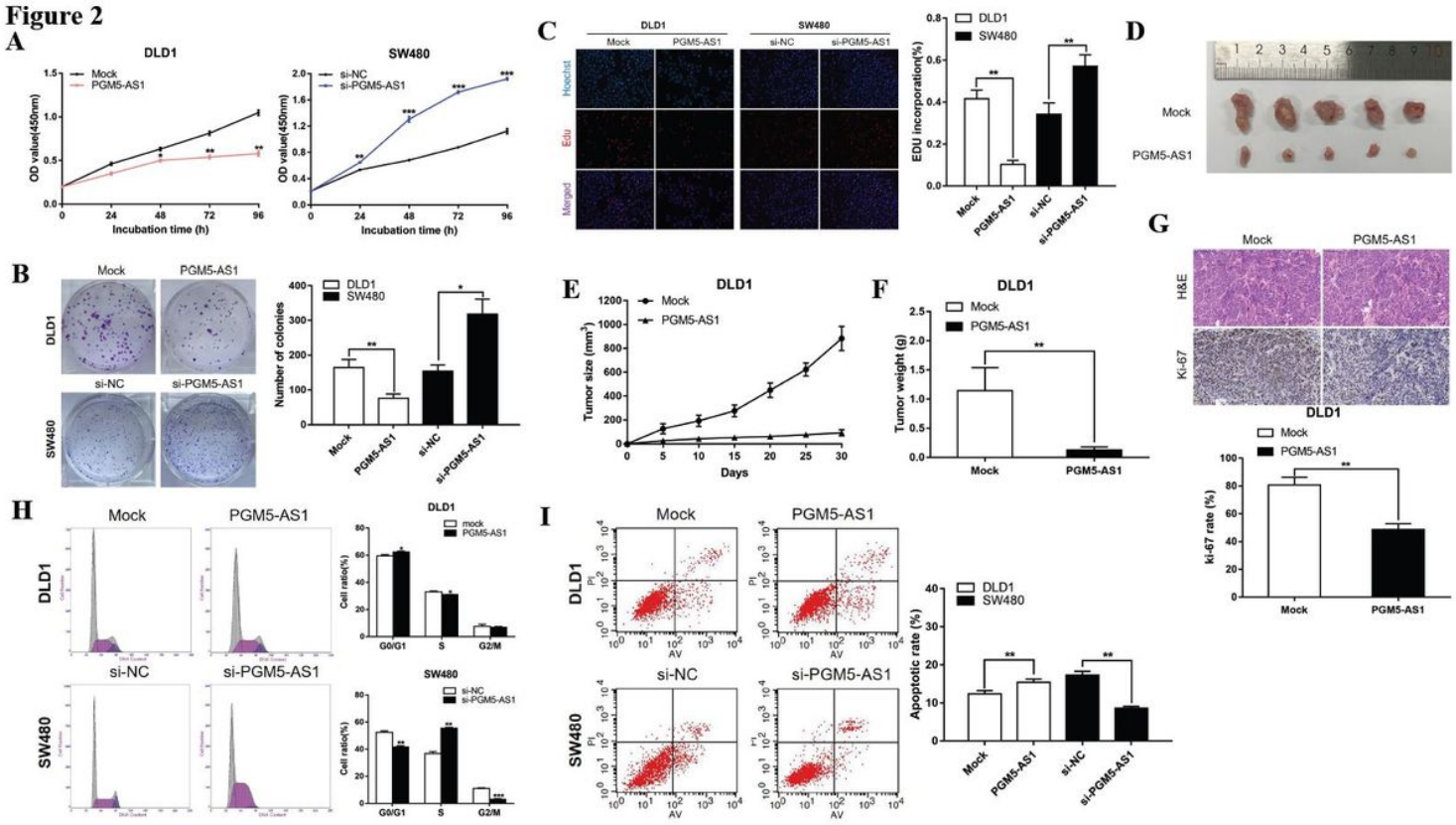

Figure 2

PGM5-AS1 affects colon cancer cell proliferation, cell cycle, and apoptosis. A-C. CCK-8 analysis, clone formation experiments, and the EDU assay were used to detect the proliferation of colon cancer cells. D. A subcutaneous xenotransplanted tumor model was used to investigate the growth of colon cancer cells in vivo. E. Tumor volumes were measured and recorded every 5 days after injection. F. Tumors were removed, weighed, and recorded on day 30 after injection. G. The Ki-67 level of the tumors was investigated by immunohistochemical analysis. H. The effect of PGM5-AS1 on the cell cycle was analyzed using flow cytometry. I. The effect of PGM5-AS1 on apoptosis was analyzed by flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001.

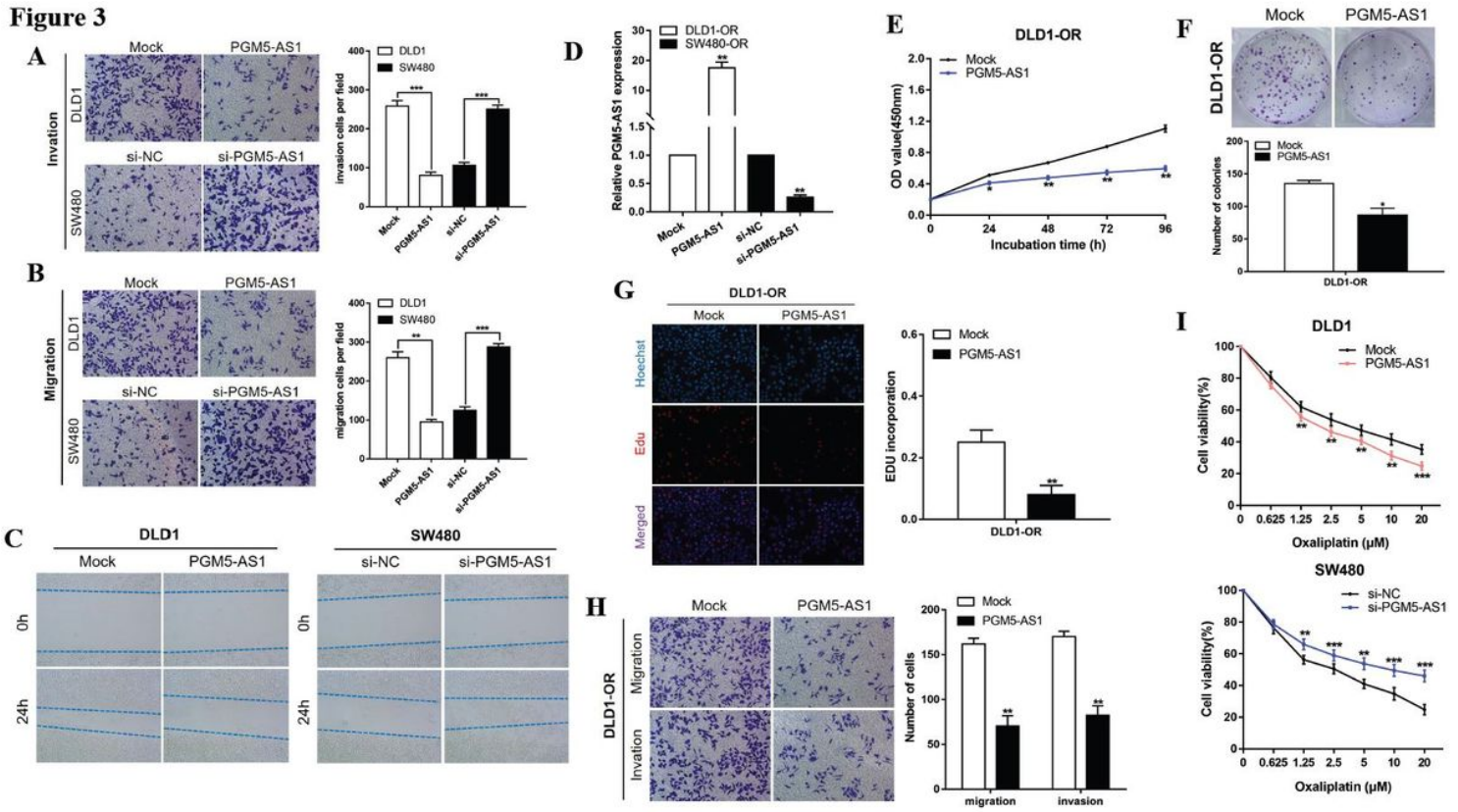

Figure 3

PGM5-AS1 inhibits invasion, migration, and acquired oxaliplatin resistance in CRC cells. A. The effect of PGM5-AS1 on cell invasion was detected by Transwell assay with a Matrigel-coated upper compartment. B. The effect of PGM5-AS1 on cell migration was examined by Transwell assay without a Matrigel-coated upper compartment. C. The effect of PGM5-AS1 on cell migration was examined using a wound healing assay. D. PGM5-AS1 was overexpressed or knocked down in oxaliplatin-resistant colon cancer cells. E-G. CCK-8 analysis, clone formation experiments, and the EDU assay were used to detect the proliferation of DLD1-OR cells. H. The Transwell experiment was carried out to detect the invasion and migration of DLD1-OR cells. I. The proliferation ability of DLD1 and SW480 cell lines treated with different concentrations of oxaliplatin. *P < 0.05, **P < 0.01, ***P < 0.001.

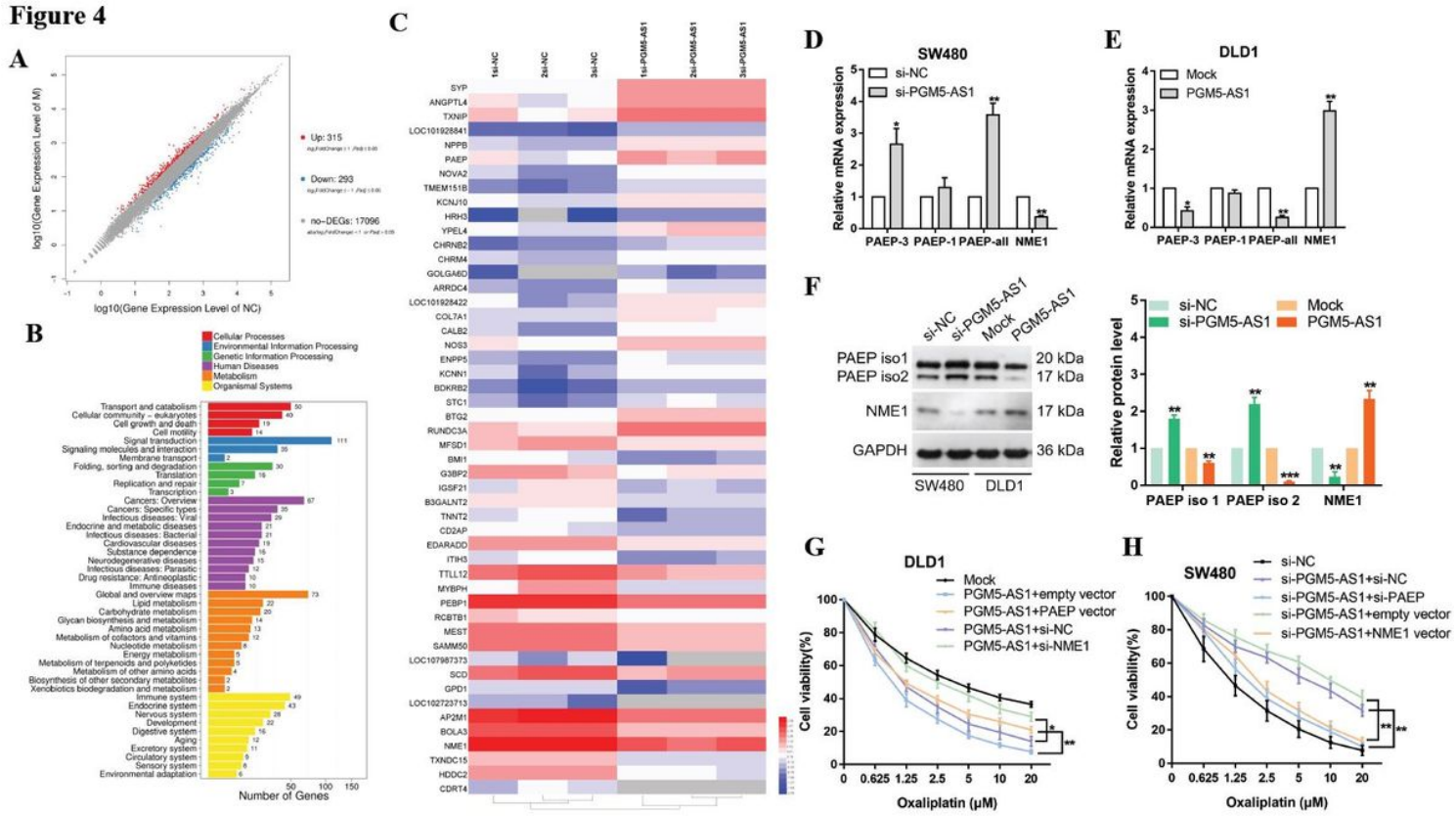

Figure 4

PGM5-AS1 inhibits acquired oxaliplatin resistance of CRC by down-regulating PAEP and up-regulating NME1 expression. A. RNA transcriptome sequencing results showed that 315 genes were significantly up-regulated and 293 genes were significantly down-regulated in SW480 cells transfected with si-PGM5-AS1. B. Gene ontology (GO) analysis revealed the biological processes involved in the RNA-seq results. C. Heat map generated with the FPKM values of the 50 mRNAs with the largest changes were subject to log10 conversion. D-E. The expression levels of PAEP and NME1 were detected by qRT-PCR in SW480 and DLD1 cells. F. Protein levels of PAEP and NME1 were detected by western blotting in SW480 and DLD1 cells. G. CCK8 assay data showed that co-transfection partially rescued PGM5-AS1-induced reduction in proliferation. H. CCK8 assay data showed that co-transfection partially rescued the increase in proliferation induced by si-PGM5-AS1. *P < 0.05, **P < 0.01, ***P < 0.001.

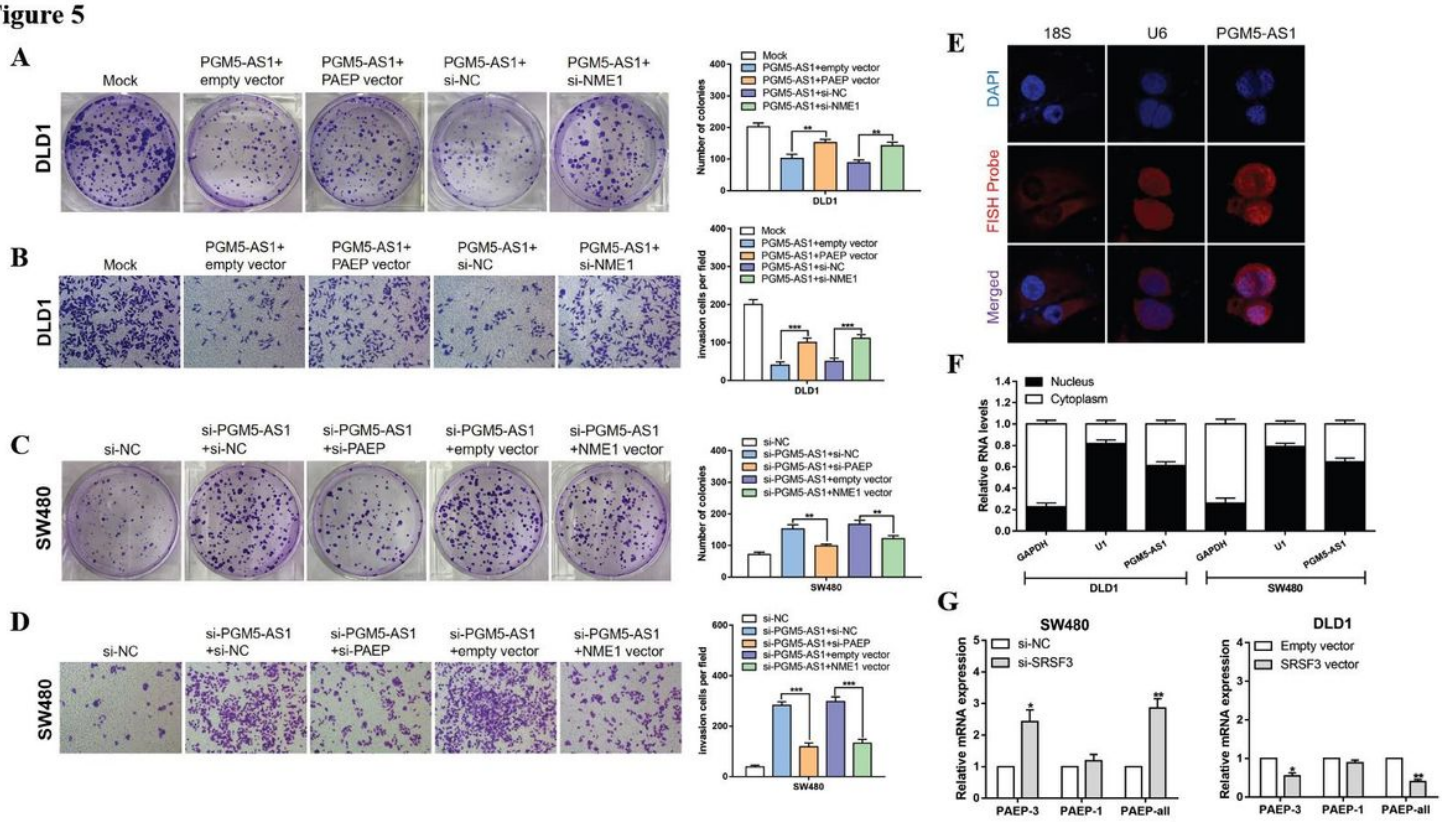

Figure 5

PGM5-AS1 suppresses the malignant phenotype of CRC by down-regulating PAEP and up-regulating NME1 expression. A-B. Clone formation experiments and Transwell assay showed that co-transfection partially rescued the PGM5-AS1-induced reduction in proliferation and invasion. C-D. Clone formation experiments and Transwell assay showed that co-transfection partially rescued the increase in proliferation and invasion induced by si-PGM5-AS1. E-F. FISH and nuclear-plasma separation experiments were performed to examine the subcellular localization of PGM5-AS1. G. The expression levels of different transcripts of PAEP were examined in CRC cells treated with SRSF3 vector or si-SRSF3. *P < 0.05, **P < 0.01, ***P < 0.001.

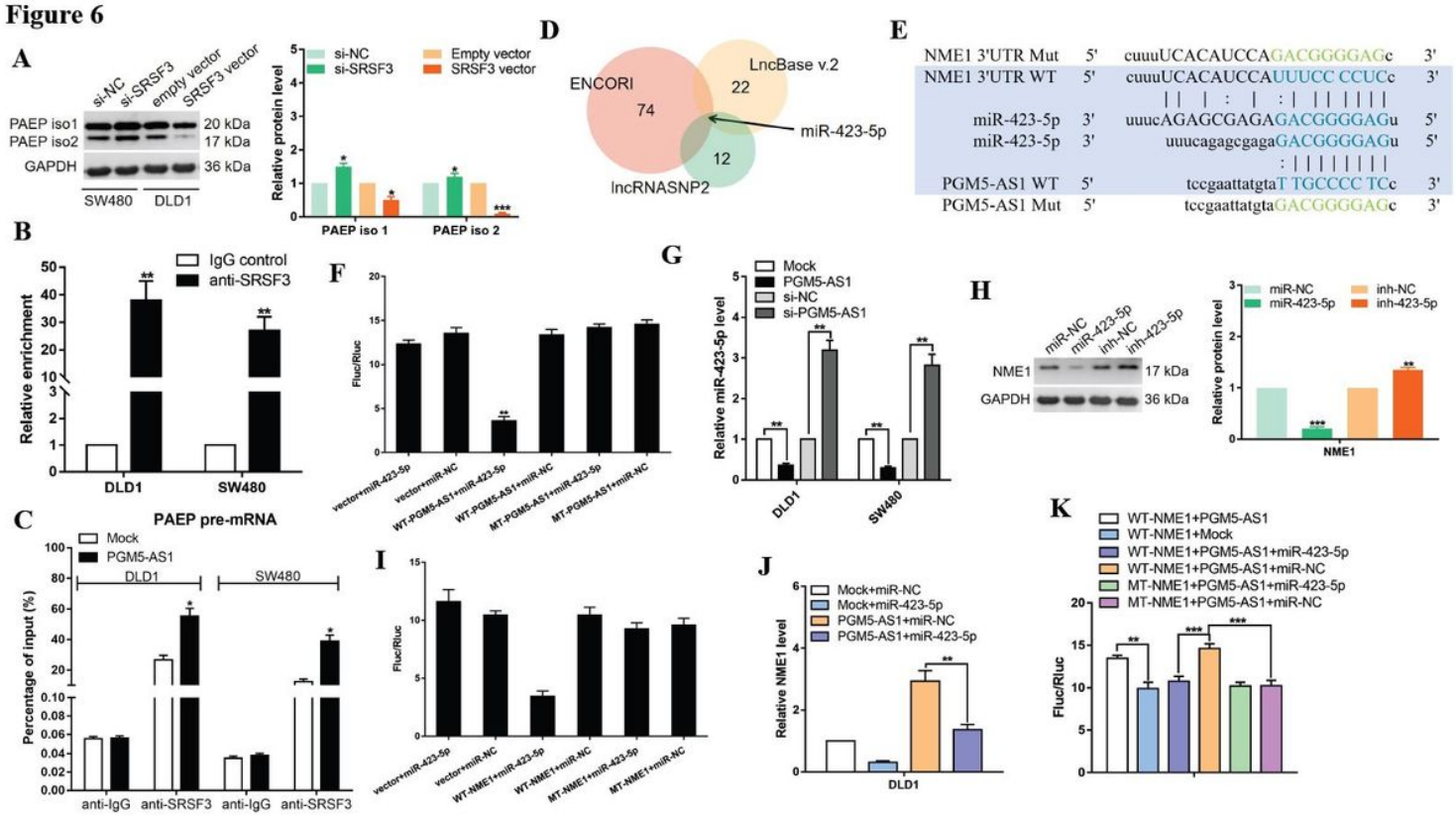

Figure 6

PGM5-AS1 recruits SRSF3 and acts as a sponge for miR-423-5p. A. The protein levels of different isoforms of PAEP were detected in CRC cells transfected with SRSF3 vector or si-SRSF3. B. RIP analysis was performed to confirm that PGM5-AS1 can interact with SRSF3. C. RIP analysis was conducted to confirm that PGM5-AS1 increased the level of SRSF3 enrichment to PAEP pre-mRNA. *P < 0.05, **P < 0.01, ***P < 0.001. E. miR-423-5p contains complementary sites to PGM5-AS1-miRNA-NME1. F. We co-transfected 293T cells with miR-423-5p mimics and wild-type PGM5-AS1 or mutant PGM5-AS1 reporter genes to carry out luciferase reporter gene assay. G. Overexpression or knockdown of PGM5-AS1 in CRC cells resulted in changes in miR-423-5p levels. H. Treatment with miR-423-5p inhibitors or mimics resulted in changes in the protein level of NME1. I. We co-transfected 293T cells with miR-423-5p mimics and wild-type NME1 or mutant NME1 reporter genes to perform a luciferase reporter gene assay. J. The increase in NME1 induced by PGM5-AS1 overexpression was reversed by the miR-423-5p mimic. K. We co-transfected PGM5-AS1-overexpressed cells with miR-423-5p mimics and wild-type NME1 or mutant NME1 reporter genes to carry out a luciferase reporter gene assay. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

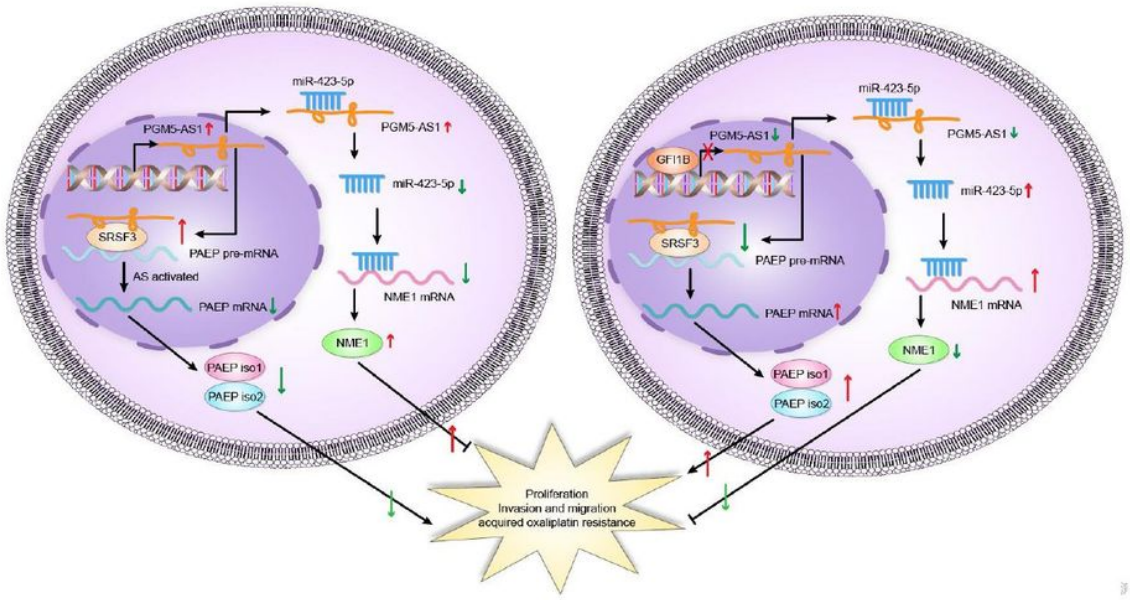

Figure 7

Reduction in the expression of PGM5-AS1 in CRC is induced by transcriptional inhibition of GFI1B. PGM5-AS1 suppresses the proliferation, invasion, migration, and acquired oxaliplatin resistance of CRC cells by recruiting SRSF3 to activate alternative splicing, down-regulating the level of PAEP and acting as a sponge for miR-423-5p to up-regulate the expression of NME1. *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

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