Original papers

LncRNA *PCGEM1* mediates oxaliplatin resistance in hepatocellular carcinoma via *miR-129-5p/ETV1* axis in vitro

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Abstract

Background. Hepatocellular carcinoma (HCC) is one of the most severe malignant cancers that leads to high death rate worldwide. Recent research revealed that long non-coding RNAs (lncRNAs) exert a critical role regarding chemoresistance in numerous cancers, including HCC.

Objectives. Our research aimed to explore the function and molecular mechanism of lncRNA *PCGEM1* on oxaliplatin resistance of HCC in vitro.

Materials and methods. Expression of the lncRNA *PCGEM1*, together with *miR-129-5p*, and the mRNA level of *ETV1* and drug resistance-related genes including *LRPA*, *MDR1* and *MDR3* were determined using quantitative real-time polymerase chain reaction (qRT-PCR) in an oxaliplatin-resistant HCC cell line (Hep3B/OXA). Cell Counting Kit-8 (CCK-8) was employed to assess the viability and cell survival rate, and transwell assays were performed to measure the number of migrated or invaded cells. In addition, the relation among lncRNA *PCGEM1*, *miR-129-5p* and *ETV1* were determined using luciferase assay.

Results. Our data indicated that *PCGEM1* and *ETV1* expression were enhanced in Hep3B/OXA cells. Furthermore, knockdown of lncRNA *PCGEM1* significantly decreased the migration, invasion and mRNA expressions of *LRPA*, *MDR1* and *MDR3*, and the cell viability in Hep3B/OXA cells. The starBase online tool and luciferase assays verified that *miR-129-5p* targeted *PCGEM1* and ETV1, signifying that *PCGEM1* could enhance *ETV1* expression via suppressing *miR-129-5p*.

Conclusions. Our findings demonstrated that *PCGEM1* modulated oxaliplatin resistance by targeting the *miR-129-5p/ETV1* pathway in HCC in vitro, suggesting a potential strategy for the treatment of chemoresistant HCC.

Key words: hepatocellular carcinoma, *miR-129-5p*, oxaliplatin resistance, *PCGEM1*, *ETV1*

Background

Hepatocellular carcinoma (HCC) is a severe type of malignant tumor, leading to a large number of cancer-related deaths worldwide.[1,2] It is an invasive and fast-growing tumor that leads to higher recurrence and metastasis.[3] Despite advancements in the therapeutic techniques for HCC such as radiotherapy, chemotherapy and surgery, the prognosis is still unsatisfactory, with an overall five-year survival rate of 15–40%.[4,5] The underlying mechanism behind the pathogenesis of HCC is complicated and needs to be investigated for promising prognosis and early-stage detection.[6] Therefore, novel targets and diagnostic indicators of HCC treatment are urgently needed.

Along with the progression of microarray analysis and sequencing, numerous novel non-coding RNAs have been discovered. Non-coding RNAs are sequences of nucleotides transcribed from chromatin and lack protein-coding capacity.[7] Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs having a length of >200 nucleotides.[8] Previous research showed that lncRNAs exert critical functions in the pathogenesis of HCC.[9] For example, lncRNA UBE2CP3 was overexpressed in HCC and enhanced tumor metastasis through the modulation of EMT and induced migration and invasion.[10] Furthermore, other research revealed that overexpression of lncRNA UCA1 suppressed miR-216b expression and stimulated FGFR1/ERK pathways to enhance the pathogenesis of HCC.[11] Moreover, lncRNA AK021443 mediated the migration and proliferation of HCC cells via EMT regulation in vitro.[12] The lncRNA *PCGEM1* was identified to be highly expressed in prostate cancer, and acts to enhance its proliferation.[13,14] Also, *PCGEM1* was highly expressed in ovarian cancer tissues and enhanced the invasion, migration and proliferation, while decreasing the apoptosis rate.[15] Furthermore, *PCGEM1* induced metastasis and EMT via enhancing the SNAI1 expression in gastric cancer cells.[16] However, whether lncRNA *PCGEM1* exerts its function in the development and tumorigenesis of HCC remains unknown.

It has been previously reported that oxaliplatin-mediated chemotherapy prolonged survival and reduced mortality during treatment of HCC patients in an advanced stage of the disease.[17] Conversely, the resistance mechanism of oxaliplatin still needs to be elucidated. Numerous research has revealed that lncRNAs play a vital role in chemotherapy resistance in HCC. Several lncRNAs were highly expressed in chemoresistant HCC tissues and cells, and were shown to exert their function during oxaliplatin resistance and HCC pathogenesis.[18] For example, lncRNA ARSR increased doxorubicin resistance in HCC by regulating PTEN.[2] Conversely, lncRNA HULC increased chemosensitivity by inhibiting autophagy in HCC cells.[19]

Objectives

This study aimed to investigate the impact of lncRNA *PCGEM1* on oxaliplatin resistance in vitro in an HCC cell line. Our findings elucidated that lncRNA *PCGEM1* mediates oxaliplatin resistance in HCC via the *miR-129-5p/ETV1* axis, indicating that *PCGEM1* might be a therapeutic target for HCC treatment.

Materials and methods

Cell culture

The human liver cancer Hep3B cell line was acquired from American Type Cell Culture (ATCC, Manassas, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Biocompare, San Francisco, USA) containing 100 units/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, USA) and 10% fetal bovine serum (FBS; Biocompare) at 37°C in 5% CO_2.

Establishment of oxaliplatin-resistant Hep3B cells

Hep3B cells were exposed to increasing concentrations of oxaliplatin (2–25 μM) to establish the oxaliplatin-resistant Hep3B cells.[20] The IC_{50} of Hep3B cells was calculated after detection with Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China), and after that, the oxaliplatin (25 μM) group was selected as Hep3B/OXA. Hep3B and Hep3B/OXA cells were cultured in minimum essential medium (MEM) containing 10% FBS. A T25 culture flask was used to seed cells (2×10^6 cells/well) for 72 h per passage.

Cell transfection

Briefly, scrambled siRNA (si-control) and *PCGEM1* siRNAs (si-*PCGEM1*#1), (si-*PCGEM1*#2) and (si-*PCGEM1*#3), and pcDNA3.1 control (pcDNA/control) were all obtained from Invitrogen (Carlsbad, USA). The *miR-129-5p* mimics, mimic negative control (NC), *miR-129-5p* inhibitor, and inhibitor NC was bought from GeneCopoeia (Rockville, USA). *ETV1* mRNA 3'UTR and NC were obtained from Origene (Rockville, USA). Hep3B cells were cultured with 60–70% confluence at 37°C in 5% CO_2, and Lipofectamine 2000 (Invitrogen) was used to transfect these plasmids into the cells following the manufacturer's instructions (http://go.microsoft.com/fwlink/p/?LinkId=255141).

Mycoplasma detection

Hep3B cells and Hep3B/OXA cells were kept in an RPMI-1640 medium containing 10% FBS and antibiotic-antimycotic solution (Spectrum Chemical Manufacturing Corp.,

New Brunswick, USA) and cultured for 3 years with occasional freezing as previously described.[21] Blood agar plates and Mycoplasma IST2 kit (Biomereux Italia Spa, Florence, Italy) were used to perform titer of mycoplasmas as described previously.[21] Twelve well plates were used to add mycoplasmas (1×10^5 CFU/mL) into the mycoplasma-free Hep3B cells (2×10^3 cells/well). Infected cells were analyzed using MycoGuard™ Mycoplasma PCR detection kit (GeneCopoeia) for the detection of mycoplasmas using the polymerase chain reaction (PCR) method, as described previously.[21] Agarose gel electrophoresis was used to visualize amplified PCR products.

CCK-8 assay

Chemosensitivity and the rate of cell survival were examined using a CCK-8 assay. More specifically, 24-well plates were used to seed the cells at a density of 2×10^3 cells/well at 37°C. The CCK-8 solution (10 µL; Abcam, Cambridge, USA) was introduced into wells, and these plates were incubated for 1 h at 37°C. Proliferation was determined at 460 nm. The cell growth inhibition curve was detected by calculating the value of IC_{50} as (1-OD/$OD_{0\,\mu M}$) × 100%, as described previously.[22]

Transwell invasion and migration assay

Transwell chambers (Becton Dickinson, Franklin Lakes, USA) were used to perform invasion and migration assay on Hep3B/OXA cells. For performing the invasion assay, Hep3B/OXA cells were passaged in a serum-free media (200 µL) and kept in the higher compartment, followed by 600 µL of a medium comprising 10% FBS into the lower compartment. The cells were fixed with polyoxymethylene after incubation for 48 h. For the migration assay, 24-well plates were used to seed Hep3B/OXA cells, and 1×10^6 cells were re-suspended in a serum-free media (200 µL) and kept in the higher compartment. Media (600 µL) with 10% FBS were introduced into the lower compartment, and cells were incubated with 5% CO_2 for 48 h at 37°C. Finally, the invasion and migration of cells was visualized after staining with 20% Giemsa solution (Sigma–Aldrich, St. Louis, USA) and counted from 5 random chambers using an inverted microscope (Nikon Corp., Tokyo, Japan). All the experimentations were repeated in triplicate.

Luciferase assay

Online bioinformatics tools including miRcode (http://mircode.org/), starBase v. 2.0 (http://starbase.sysu.edu.cn/starbase2/index.php) and microRNA.org (https://bigd.big.ac.cn/databasecommons/database/id/1426) were used to predict the binding sites of *miR-129-5p* on *PCGEM1* as well as the *ETV1* binding sites on the *miR-129-5p* gene. Luciferase assay (BioAssay Systems, Hayward, USA) was

then performed to assess the luciferase activity according to the manufacturer's protocol (https://www.bioassaysys.com/Luciferase-Reporter-Assay-Kit-(1000-tests).html). A Mammalian Genomic DNA Miniprep Kit (Qiagen, Hilden, Germany) was used to generate the pGL3 plasmid (VWR, Radnor, USA), and once bound to their binding sites on the *miR-129-5p*, the RNA sequence of *PCGEM1* and 3'-UTR of *ETV1* were spliced into pGL3 to produce pGL3-*PCGEM1* (or *ETV1* mRNA) mutant or wild-type reporter vector. The 24-well plates were used to seed cells, and pGL3 mutant or wild type vector (100 ng) and *miR-129-5p* mimic or mimic NC, *miR-129-5p* inhibitor or inhibitor negative control (50 nmol/L) were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) into the cells. Renilla luciferase gene was taken as a standard. All the experiments were performed in triplicate.

qRT-PCR

Total RNA was extracted using Ribozol RNA extraction reagent (VWR) following the manufacturer's protocol (https://us.vwr.com/store/product/7437721/vwr-life-science-ribozoltm-rna-extraction-reagent). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) was used to examine the purity and RNA concentration at 260/280 nm. Reverse transcription of total RNA (2 µg) into cDNA was performed using the SuperScript First-Stand Synthesis System. ABI PRISM 7500 and SYBR Premix ExTaq II kit (Thermo Fisher Scientific) was used to perform quantitative real-time PCR (qRT-PCR). Primers sequences are given below in Table 1. All the experiments were performed 3 times. *GAPDH* acted as the internal control, and the expressions of genes were measured using the $2^{-\Delta\Delta Ct}$ method.[23]

Table 1. The primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Primers		Sequence
PCGEM1	forward	CTGTGTCTGCAACTTCCTCTAA
	reverse	TCCCAGTGCATCTCGTAGTA
miR-129-5p	forward	ACCCAGTGCGATTTGTCA
	reverse	ACTGTACTGGAAGATGGACC
ETV1	forward	TAGCCGTTCACTCCGCTATT
	reverse	CCTCGTTGATGTGACGTTCC
LRP1	forward	AATGGGCTAAGCCTGGACAT
	reverse	TGCCACTCCGATACTCAGTC
MDR1	forward	TCATTCGAGTAGCGGCTCTT
	reverse	TTCTTTGCTCCTCCATTGCG
MDR3	forward	TGCAGCCCACTTATTCATGC
	reverse	TTCTTCACCTCCAGGCTCAG
GAPDH	forward	GGTGTGAACCATGAGAAGTATGA
	reverse	GAGTCCTTCCACGATACCAAAG

Statistical analyses

Data are presented as the mean ± standard deviation (SD). IBM SPSS v. 19 (IBM Corp., Armonk, USA) and GraphPad Prism v. 8.0 (GraphPad Software, San Diego, USA) were used for statistical analyses of all data. The difference between the 2 groups was evaluated using Student's t-test while multiple-group comparisons were performed using analysis of variance (ANOVA) and Tukey's post hoc analysis. The general alpha value was set as 0.05. All the experimentations were performed 3 times.

Results

Effect of lncRNA *PCGEM1* and *ETV1* on oxaliplatin-resistant HCC cells

Hep3B cells were treated with oxaliplatin, and the *PCGEM1* expression was examined using qRT-PCR. Results indicated that *PCGEM1* expression was significantly enhanced in Hep3B/OXA cells compared to Hep3B cells without oxaliplatin treatment (Fig. 1A). The survival of Hep3B cells was assessed using a CCK-8 assay, and our data indicated that the percentage survival of Hep3B/OXA cells was greater than Hep3B control cells (Fig. 1B). Furthermore, *ETV1* mRNA expression was markedly enhanced in Hep3B/OXA cells compared to that of parental Hep3B cells (Fig. 1C). Next, oligonucleotides were
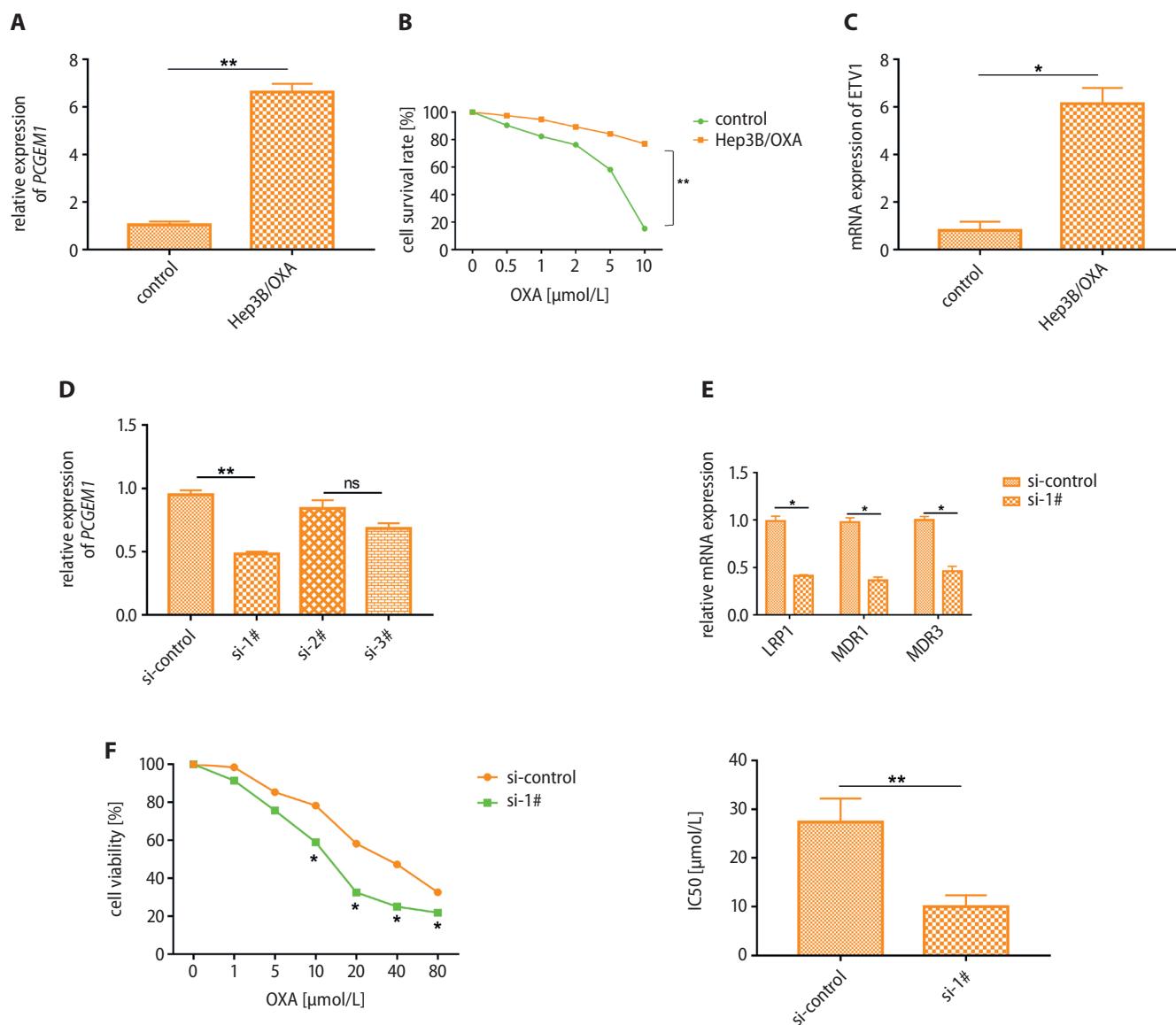

Fig. 1. Effect of lncRNA *PCGEM1* and *ETV1* in oxaliplatin-resistant hepatocellular carcinoma (HCC) cells. A. *PCGEM1* expression was determined in oxaliplatin-resistant Hep3B cells using quantitative real-time polymerase chain reaction (qRT-PCR); B. The cell survival rate was examined in oxaliplatin-resistant Hep3B cells using the Cell Counting Kit-8 (CCK-8) assay; C. The *ETV1* mRNA level in oxaliplatin-resistant Hep3B cells was examined using qRT-PCR; D. The *PCGEM1* expression was assessed after Hep3B cells were treated with interfering oligonucleotides (**p < 0.01, ns – not significant); E. qRT-PCR measured the mRNA level of *MDR1*, *MDR3* and *LRP1* (drug resistance-related genes); F. The IC$_{50}$ was measured in oxaliplatin-resistant Hep3B cells (*p < 0.03, **p < 0.002)
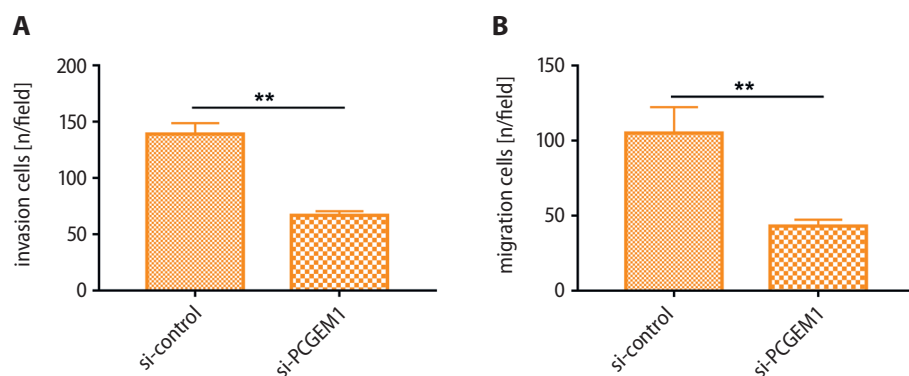
A

B

Fig. 2. Effect of *PCGEM1* knockdown on invasion and migration of oxaliplatin-resistant hepatocellular carcinoma (HCC) cells in vitro. A. Transwell assay detected the number of invasive cell in Hep3B/OXA cells transfected with *PCGEM1* siRNA compared to the cells transfected with empty vector (**p < 0.002); B. Transwell assay detected the number of migrative cell in Hep3B/OXA cells transfected with *PCGEM1* siRNA compared to the cells transfected with empty vector (**p < 0.002)
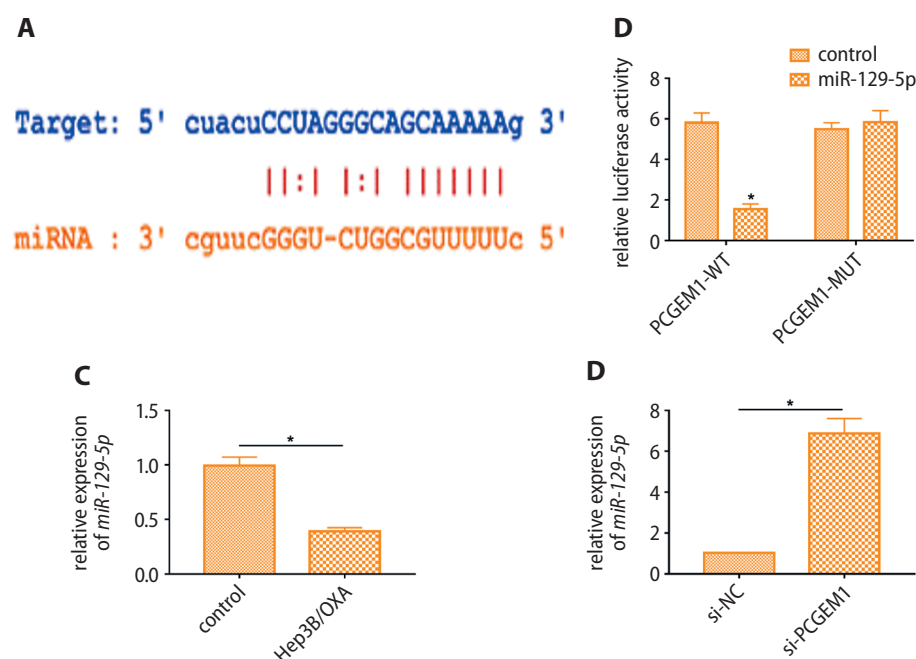
A

D

Fig. 3. *PCGEM1* directly targeted *miR-129-5p*. A and B. The binding sites between *PCGEM1* and *miR-129-5p* were predicted using online bioinformatic tools, including TargetScan website (http://www.targetscan.org) and starBase website (http://starbase.sysu.edu.cn/starbase2/index.php), and confirmed using a luciferase assay (*p < 0.03); C. The expression of *miR-129-5p* in Hep3B/OXA cells was examined using qRT-PCR (*p < 0.03); D. The *miR-129-5p* expression was determined in Hep3B/OXA cells transfected with empty vector or *PCGEM1* siRNA (*p < 0.03)

C

D

prepared and Hep3B/OXA cells were transfected with them to reduce the expression of *PCGEM1* (Fig. 1D). Moreover, our findings revealed that *PCGEM1* knockdown considerably decreased the mRNA expression of drug resistance-related genes, including low-density lipoprotein receptor-related protein 1 (*LRP1*), multidrug resistance gene 1 (*MDR1*) and multidrug resistance gene 3 (*MDR3*) compared to that of the control group (Fig. 1E). The CCK-8 assay was used to measure the cell growth inhibition curve by calculating the value of the inhibitory concentration (IC_{50}). Our findings elucidated that *PCGEM1* knockdown markedly suppressed the IC_{50} value in comparison to the control group (Fig. 1F). These data illustrated that *PCGEM1* knockdown markedly reduced the oxaliplatin resistance of HCC cells.

Effect of lncRNA *PCGEM1* on invasion and migration in the oxaliplatin-resistant HCC cells

Transwell assays indicated that *PCGEM1* knockdown considerably reduced the number of invasive cells in Hep3B/OXA cells compared to that of the empty vector group (Fig. 2A). Our findings also revealed that *PCGEM1* knockdown markedly reduced the number of migrating cells compared to the empty vector cells (Fig. 2B). These findings revealed that *PCGEM1* knockdown inhibited the invasion and migration in Hep3B/OXA cells.

MiR-129-5p was a target of *PCGEM1*

Online bioinformatics tools including miRcode (http://mircode.org/), starBase v. 2.0 (http://starbase.sysu.edu.cn/starbase2/index.php) and microRNA.org (https://bigd.big.ac.cn/databasecommons/database/id/1426), together with a luciferase assay, were used for the prediction and confirmation of binding between *PCGEM1* and *miR-129-5p*. Our data elucidated that the luciferase activity was significantly reduced in the *PCGEM1*-WT and *miR-129-5p* mimics group. At the same time, no significant change was observed in the rest of the groups (Fig. 3A,B). Moreover, *miR-129-5p* expression was reduced in Hep3B/OXA cells compared to that of parental Hep3B cells (Fig. 3C). Finally, *miR-129-5p* expression was significantly enhanced in Hep3B/OXA cells transfected with si-*PCGEM1* compared to that of the empty vector group (Fig. 3D).
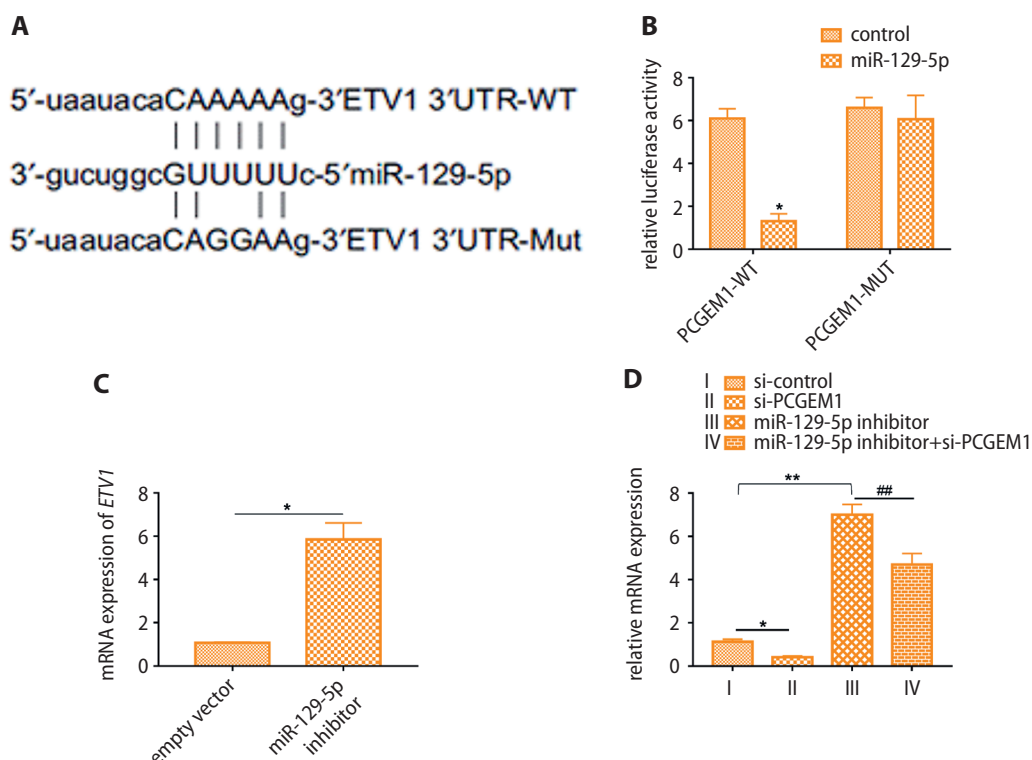
A

B

C

D

Fig. 4. LncRNA *PCGEM1* regulated *ETV1* expression by targeting *miR-129-5p*. A and B. The binding sites between *ETV1* and *miR-129-5p* were predicted using online bioinformatic tools including TargetScan website (http://www.targetscan.org) and starBase website (http://starbase.sysu.edu.cn/starbase2/index.php), and confirmed using a luciferase assay (*p < 0.03); C. The *ETV1* mRNA level was assessed in Hep3B/OXA cells transfected with *miR-129-5p* inhibitor using qRT-PCR (*p < 0.03, compared to si-control); D. The *ETV1* mRNA level was measured in Hep3B/OXA cells transfected with an empty vector, *miR-129-5p* inhibitor and *PCGEM1* siRNA (*p < 0.03, **p < 0.002 compared to si-control, ##p < 0.002 compared to *miR-129-5p* inhibitor)

LncRNA *PCGEM1* regulated *ETV1* expression via targeting *miR-129-5p*

The results from bioinformatic tools indicated that *miR-129-5p* shared binding sites with the 3′UTR of *ETV1* mRNA (Fig. 4A). Luciferase assay results revealed a decrease in luciferase activity after the co-transfection with *miR-129-5p* and *ETV1*-WT (100 ng), indicating molecular binding between *miR-129-5p* and *ETV1* (Fig. 4B). Furthermore, *ETV1* mRNA expression was increased in Hep3B/OXA cells transfected with *miR-129-5p* inhibitor (50 nmol/L) (Fig. 4C). Finally, the knockdown of *PCGEM1* reduced *ETV1* mRNA expression, while inhibition of *miR-129-5p* enhanced the mRNA level of *ETV1* in Hep3B/OXA cells (Fig. 4D).

Discussion

The HCC is a hepatic malignant neoplasm.[24] Despite great advancement in the therapeutic techniques for HCC, the overall outcomes remain unsatisfactory,[25] with a common obstacle being chemoresistance towards drugs such as oxaliplatin, doxorubicin and cisplatin, among others.[26] Our study explored the regulatory functions of *PCGEM1* in oxaliplatin resistance regarding *miR-129-5p*/*ETV1* interactions using oxaliplatin-resistant HCC cells (Hep3B/OXA) (Fig. 5).

The lncRNAs present a vital function in regulating tumorigenesis, as well as the chemoresistance of cancer cells.[27] Previous research has suggested that dysregulated lncRNA expression might mediate potential chemoresistance.[28] Our findings revealed that the *PCGEM1* expression was enhanced in Hep3B/OXA cells compared to that of its parental cell line. Furthermore, knockdown of lncRNA *PCGEM1* decreased the mRNA expression levels of *LRP1*, *MDR1* and *MDR3* in Hep3B/OXA cells, indicating that silencing of *PCGEM1* might reduce the oxaliplatin resistance. Moreover, *PCGEM1* knockdown significantly inhibited the proliferation, migration and invasiveness of Hep3B/OXA cells, which was in accordance with the hypothesis that downregulation of *PCGEM1* might lead to the decrease in chemoresistance in vitro. Similar to our findings, it has been reported that downregulation of another lncRNA, lncRNA *PVT1* was able to inhibit tumor progression and reduced chemoresistance to cisplatin in colorectal cancer patients.[29] In HCC, lncRNA *HULC* was significantly upregulated in HCC cells after treatment with drugs including cisplatin and oxaliplatin, and
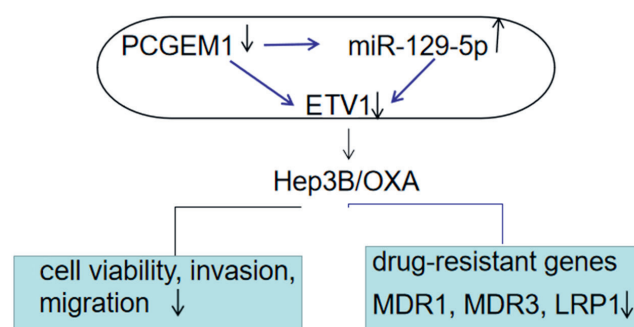

Fig. 5. The illustration of the hypothetical mechanism of *PCGEM1* in Hep3B/OXA cells

the additional knockdown of *HULC* enhanced chemoresistance of HCC cells through its interaction with *SIRT1*[19]. In this study, we found that the relative mRNA expression of *ETV1* was decreased by the knockdown of lncRNA *PCGEM1* in Hep3B/OXA cells. *ETV1* has been reported to function as an oncogene in various human malignant tumors.[30,31] A previous study indicated that *ETV1* enhanced the invasion, migration and proliferation of breast cancer cells.[32] Moreover, inhibitor of *ETV1* enhanced the cellular sensitivity to docetaxel in prostate cancer.[33] Similarly, ETV1 was reported to be modulated by Circ-ZNF609/miR-1224-3p in lung cancer cells.[34] We discovered that the expression of *ETV1* was positively regulated by *PCGEM1* in Hep3B/OXA cells, suggesting that knockdown of *PCGEM1* in HCC cells increased sensitivity to oxaliplatin, perhaps through the modulation of *ETV1*.

To predict the targeted sites between mRNA and lncRNA, bioinformatic prediction tools were used.[35] By using these online tools, we established that *miR-129-5p* targeted *PCGEM1*. The *miR-129-5p* overexpression has been shown previously to attenuate the proliferation of PC-3 cells,[36] while in prostate cancer, *miR-129-5p* was downregulated in tissues and cells in comparison with normal counterparts, and it specifically inhibits *ETV1*.[36] Furthermore, the upregulation of *miR-129-5p* was shown to curb the cell proliferation by suppressing *ETV1* in prostate cancer cells.[36] Finally, it was disclosed that *miR-129-5p* inhibited the development of colon cancer and osteosarcoma via regulating *HMGB1*.[37,38] In conclusion, our data revealed the impact of lncRNA *PCGEM1* in HCC oxaliplatin resistance in vitro. We have shown that *PCGEM1* and *miR-129-5p* expression levels were negatively correlated to each other, representing the enrichment and antagonistic function. *PCGEM1* downregulation led to an increase in *miR-129-5p* expression in Hep3B/OXA cells, while downregulation of *miR-129-5p* induced *ETV1* mRNA expression. In our work, *PCGEM1* downregulation reduced the oxaliplatin resistance in Hep3B/OXA cells via binding *miR-129-5p* and inhibiting *ETV1*.

Limitations

The limitation of this current study mainly exists in the absence of animal models. If respective animal models could be established to further validate the mechanism of the *PCGEM1/miR-129-5p/ETV1* pathway on HCC oxaliplatin resistance, the research would be more valuable and complete.

Conclusions

Our findings demonstrated the potential mechanism of the *PCGEM1/miR-129-5p/ETV1* pathway on HCC oxaliplatin resistance, suggesting the critical function of *PCGEM1* in HCC treatment and providing a new therapeutic target.

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