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The ZIC2-Claudin-18.2 Axis Stimulates Pancreatic Cancer Progression and Metastasis via Activation of the ERK1/2 Signaling Pathway

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Claudin-18.2 is considered a promising target in cancer treatment. However, studies on the role of Claudin-18.2 in pancreatic cancer are scarce, and a systematic exploration of its mechanisms of action in disease progression is missing. This research sought to reveal the detailed mechanisms by Claudin-18.2 impacts pancreatic cancer development and metastasis. Claudin-18.2 expression levels in pancreatic cancer were investigated through The Cancer Genome Atlas (TCGA) database, with subsequent validation by immunohistochemistry and quantitative reverse transcription polymerase chain reaction analysis. The potential regulatory transcription factors (TFs) for Claudin-18 were forecasted by the KnockTF database. Pearson's correlation was applied to ascertain the correlation between Claudin-18 and ZIC2, and the Molotool was utilized to analyze their potential binding sites. The TCGA database facilitated our analysis of ZIC2 expression levels within pancreatic cancer. Activation of the ERK signaling pathway was validated by western blot (WB). The colony formation assay evaluated cell proliferation, while the cell scratch and Transwell assays were used to determine cell migration and invasion abilities. WB was used to detect the expression of E-cadherin, N-cadherin and Vimentin associated with epithelial-mesenchymal transition. The ZIC2-Claudin-18.2 regulatory axis, via ERK1/2 signaling pathway activation, enhanced the malignant behaviors of pancreatic cancer. Claudin-18.2, when highly expressed in pancreatic cancer, facilitated tumor malignancy, primarily through activating the ERK1/2 signaling pathway. Additionally, ZIC2 was identified as an upstream regulatory molecule for Claudin-18.2. Our findings reveal that ZIC2, a TF, can upregulate Claudin-18.2, and initiate the ERK1/2 signaling pathway, eventually facilitating the malignant progression of pancreatic cancer.

Keywords: Claudin-18.2; ERK1/2; pancreatic cancer; ZIC2
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Introduction

Pancreatic cancer, one of the leading cancer types globally, is marked by a five-year survival rate of under 10% due to its silent early symptoms and high recurrence rate (Klein 2021). At present, the treatment paradigm for pancreatic cancer patients centered around total surgical resection complemented by systemic chemotherapy. However, most of them are diagnosed with unresectable or metastatic pancreatic cancer and suffer a high recurrence rate (Kolbeinsson et al. 2023), signaling an urgent need for new treatment strategies. Targeted therapies based on genetic testing can boost the quality and safety of surgeries applied to pancreatic cancer patients, but with few effective targets, their benefits are not universa (Wood et al. 2022). It is thus crucial to delve into the mechanisms of pancreatic cancer progression to identify and develop new treatment targets.

As members of the transmembrane protein family, Claudins are chiefly responsible for preserving cell polarity and have a strong link to the epithelial-mesenchymal transition (EMT) (Wang et al. 2023). Of the 27 identified family genes, the majority show reduced expression across various cancers (Kyuno et al. 2022). In contrast, Claudin-18 exhibits increased expression in many cancers, such as pancreatic (Wöll et al. 2014), gastric (Kayikcioglu et al. 2023), and

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esophageal cancer (Sahin et al. 2008). Claudin-18.2, a splice variant of Claudin-18, is a key element of tight junction proteins that are responsible for tissue permeability, paracellular transport, and signal transduction (Kubota et al. 2023). In pancreatic cancer research, several studies have indicated that Claudin-18.2 expression is upregulated at the mRNA and protein levels (Hong et al. 2020; Wang et al. 2022). Patients with high expression of Claudin-18.2 tend to have highly differentiated and metastatic tumors (Park et al. 2023; Lyu et al. 2024). Cell/antibody therapies against Claudin-18.2 targets have been initially validated in clinical trials (Qi et al. 2023; British Veterinary Association 2024). However, the current research on Claudin-18.2 is mainly focused on its potential as a clinical marker or an antibody target, with a relative lack of basic research. The concrete role of Claudin-18.2 in the development of pancreatic cancer has not been systematically explored, which greatly narrows the therapeutic benefits that targeting Claudin-18.2 could offer.

We have identified that Claudin-18.2, when upregulated in pancreatic cancer, accelerates its progression and metastasis, particularly through the activation of the ERK1/2 signaling pathway. In the early stage of this study, we identified ZIC2 as a potential transcription factor (TF) for Claudin-18.2, noting a positive correlation. We confirmed the detailed mechanism in which the ZIC2-Claudin-18.2 axis promotes pancreatic cancer development and metastasis via ERK1/2 pathway activation. This study provides new rationale for Claudin-18.2 as a therapeutic target in pancreatic cancer and offers fresh insights into increasing the therapeutic efficiency for cancer patients.

Materials and Methods

Bioinformatics analysis

In The Cancer Genome Atlas (TCGA) database, we compared the differential expression levels of Claudin-18.2 and ZIC2 in pancreatic cancer tissues vs. DM normal tissues. The potential TF ZIC2 for Claudin-18.2 was confirmed through the KnockTF database, and Pearson's correlation analysis was performed to assess the correlation between Claudin-18.2 and ZIC2 expression levels. The Molotool was used to examine the binding sites between Claudin-18.2 and ZIC2.

Cell selection and culture

Normal human pancreatic duct epithelial cells hTERT-HPNE and pancreatic cancer cell lines AsPC-1, HPAC, and MIA-Paca2 were procured. The hTERT-HPNE cells (SSRCC, China) were allowed to grow in high-glucose Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) added. The AsPC-1 pancreatic cancer cell line (SSRCC, China) was grown in Roswell Park Memorial Institute (RPMI) 1640 medium including 10% FBS and 1% P/S. The HPAC pancreatic cancer cell line (Jinyuan, China) was placed in DMEM medium with 10% FBS and 1% P/S. The MIA-Paca2 pancreatic cancer cell line (Procell, China) was cultured in DMEM medium supplemented with 10% FBS, 2.5% horse serum, and 1% P/S. The high-glucose DMEM, RPMI 1640 medium, P/S solution (double antibiotics), and horse serum were bought from Procell (China), and the FBS was purchased from Vivacell (Germany). All cultures were maintained under conditions of 37°C and 5% CO_2.

Cell transfection

Plasmids for the overexpression of Claudin-18.2 (oe-Claudin-18.2), ZIC2 (oe-ZIC2), along with their negative control (oe-NC), and small interfering RNAs (siRNAs) targeting Claudin-18.2 (si-Claudin-18.2) with a negative control (si-NC) were all synthesized by GenePharma (China). Pancreatic cancer cells in their logarithmic growth phase received the transfection of these nucleic acids according to the protocol provided with Lipofectamine 3000 (Thermo Fisher Scientific, USA). Further experimental procedures were carried out 48 h after transfection.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

From the cultured cells, total RNA was isolated employing the PureLink™ Pro 96 Total RNA Purification Kit (Thermo Fisher Scientific, USA). RNA concentration and purity were revealed by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Following this, the DNA was transformed into cDNA with the aid of the PrimeScript™ RT reagent Kit (TaKaRa, Japan). qRT-PCR analysis was conducted with the AceQ qPCR SYBR Green Master Mix (Vazyme, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA), employing the $2^{-\Delta\Delta Ct}$ method to evaluate the expression levels of the target genes. The specific primer sequences are detailed in Table 1.

Western blot (WB)

Total protein was extracted from cells with RIPA buffer (Beyotime, China), and protein levels detected using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, USA). Proteins were run on a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The blotting membrane was initially blocked with 5% BSA before being exposed to primary antibodies against E-cadherin, N-cadherin, Vimentin, ERK1/2, p-ERK1/2, and GAPDH (1:1,000 dilution, rabbit anti-human, Abcam, UK). This was followed by an incubation period with HRP-tagged secondary antibodies (goat anti-rabbit, Abcam, UK) for 1 h post-PBS washing. The protein bands were developed and photographed using a high-sensitivity ECL detection reagent (Beyotime, China) on the ChemiScope 6000 chemiluminescence imaging system (Clinx, China).

Table 1. qRT-PCR primers.

Primer	Forward (5′-3′)	Reverse (5′-3′)
Claudin-18.2	AGTTCAGGCTTCACCGAATG	GCATTTCAGGGCAAAGATGG
ZIC2	TCAAGATCCACAAAAGGACCC	CTTGCAGAGATAGGGCTTATCG
GAPDH	GGTGTGAACCATGAGAAGTATGA	CCTTCCACGATACCAAAGT

Colony formation assay for detecting cell proliferation

Drawing from earlier research (Wang et al. 2020), we performed a colony formation assay to measure the proliferative potential of the cells. Specifically, cells in the logarithmic growth phase were standardly digested, centrifuged, and the cell precipitate was gathered. These cells were then dispensed into a 12-well plate at a concentration of about 400 cells per well and cultured. The medium was refreshed, and the cells were cultured for 7-14 days before being stained with 0.5% crystal violet. The cell colonies were then photograghed and enumerated.

Wound-healing assay for measuring cell migration

Specifically, cells of various groups were plated into 6-well plates and grown to full confluence. At the 0-h mark, cells from each group were scraped with a 200 μL pipette tip. They were then rinsed with PBS to eliminate the separated cells and were co-cultured with complete growth medium. After 24 h, photographs of the cells that had migrated into the scraped region were taken with an ECLIPSE Ts2 inverted microscope (Nikon, Japan).

Transwell assays for measuring cell invasion

In line with previous work (Yan et al. 2019), we assessed the invasive potential of pancreatic cancer cells by the number of cells that invaded across the transwell chamber. To start with, pancreatic cancer cells that were serum-starved in a serum-free medium overnight (1 × 10^4) were inoculated into the upper compartment of a transwell coated with extracellular matrix gel, with 600 μL of complete culture medium in the lower chamber acting as a chemoattractant. After 48 h, the cells that had invaded to the lower compartment were fixed with 75% ethanol and stained using a 0.5% crystal violet solution. Finally, images of random fields were photographed with an ECLIPSE Ts2 inverted microscope (Nikon, Japan), and the invasive cell count was computed.

Immunohistochemistry (IHC) analysis

Ten pairs of pancreatic cancer tissues and para-cancerous tissues were all sourced from The First Affiliated Hospital of Yangtze University, with written informed consent given by all patients before the collection. The research adhered to the Helsinki Declaration and received approval from The First Affiliated Hospital of Yangtze University Ethics Committee (No. KY202394). Tumor tissue samples were fixed and embedded in paraffin, then sectioned into 4 μm thick slices. After dewaxing, the slices were rehydrated using a gradient ethanol series and processed with 3% H_2O_2 for 10 min at room temperature in the dark, followed by antigen retrieval. Once blocked, the sections were incubated with rabbit anti-Claudin-18.2 (Abcam, UK) at 4°C overnight in a moist environment. They were then incubated with peroxidase-conjugated goat anti-rabbit IgG (Abcam, UK) and stained with the DAB chromogenic substrate (Solarbio, China), followed by rinsing with distilled water, hematoxylin counterstaining for 20 s, dehydration through a graded series of ethanol, xylene clearing, and mounting with neutral resin. The slides were observed under an ECLIPSE Ts2 inverted microscope (Nikon, Japan).

Chromatin immunoprecipitation (ChIP)

Following the methodology of prior studies (Liu et al. 2023), a chromatin immunoprecipitation assay was executed utilizing the SimpleChIP® Plus Sonication Chromatin IP Kit (CST, USA). To begin, cells (about 5 × 10^6) were plated in a 10 cm petri dish and, once they achieved 90% confluence, were treated and collected. The cells were crosslinked with 1% formaldehyde for 10 min at 37°C. Crosslinking was halted with 1 M glycine, the cells were washed with PBS, and resuspended in SDS lysis buffer containing the protease inhibitor PMSF. Sonication was used to generate chromatin fragments of 2,000 bp. ChIP was performed using anti-ZIC2 (Thermo Fisher Scientific, USA) and anti-IgG (CST, USA) antibodies. The chromatin DNA was extracted and purified, and Claudin-18.2 expression levels were detected by qPCR with the primers for Claudin-18.2: TCCGGGATCATGTTCATTGTC (F) and ATCCAGAAGTTAGTCACCAGC (R).

Dual-luciferase reporter assay

The Dual-Luciferase Reporter Gene Assay System (Promega, USA) was employed to determine luciferase activity. The promoter fragments that were anticipated to interact with Claudin-18.2, as well as their mutated versions, were inserted into the MCS upstream of the luciferase gene in the pGL3 vector. These constructed plasmids were co-transfected with pRL-TK and oe-ZIC2 or oe-NC plasmids into the cells. Finally, the luciferase activity was determined using the Dual-Glo® Luciferase Reporter Gene Assay System (Promega, USA).

Statistical analysis

Data analysis was conducted via the GraphPad Prism 8.0 software. The results are expressed as "mean ± standard deviation (SD)", with T-tests employed for comparisons between groups and one-way ANOVA for multiple

comparisons. $P < 0.05$ was recognized as statistically significant.

Results

Upregulated claudin-18.2 expression in pancreatic cancer

Our analysis of the TCGA-Pancreatic Cancer database revealed that Claudin-18.2 expression is elevated in pancreatic cancer (Fig. 1A). Following this, we collected 10 pairs of pancreatic cancer tissues and adjacent normal tissues and used IHC to assess the expression levels of Claudin-18.2 protein. The results of the experiment showed that the staining of Claudin-18.2 in the tumor region was positive, and the staining of Claudin-18.2 in the non-tumor region was negative. Relatively speaking, the higher the stage of pancreatic cancer, the higher the expression level of Claudin-18.2 in tumor tissues of patients (Fig. 1B). Collectively, these results suggested that Claudin-18.2 was overexpressed in pancreatic cancer. Meanwhile, Claudin-18.2 expression profile may be associated with the development of pancreatic cancer.

Claudin-18.2 facilitates the growth and metastasis of pancreatic cancer

Proceeding with our experiments, we cultured normal human pancreatic duct epithelial cells hTERT-HPNE and pancreatic cancer cell lines AsPC-1, HPAC, and MIA-Paca2, and used qRT-PCR to detect Claudin-18.2 mRNA expression levels, confirming its upregulation in pancreatic cancer (Fig. 2A). To further investigate the biological function of Claudin-18.2 in pancreatic cancer, we knocked down Claudin-18.2 in the human pancreatic cancer AsPC-1 and HPAC cells, and the transfection efficiency was confirmed by qRT-PCR (Fig. 2B). A colony formation assay determined that Claudin-18.2 knockdown notably inhibited the proliferation of AsPC-1 and HPAC cells (Fig. 2C,D). Afterwards, we explored the role of Claudin-18.2 in the migration and invasion of pancreatic cancer cells. The wound-healing assay showed that Claudin-18.2 knockdown greatly fettered the wound healing ability of AsPC-1 and HPAC cells (Fig. 2E,F), illuminating the role of Claudin-18.2 in promoting pancreatic cancer cell migration. The Transwell assay for cell invasiveness confirmed the previous findings (Fig. 2G). Molecularly, the suppression of Claudin-18.2 impeded the advancement of the EMT, reflected in the upregulation of E-cadherin expression and the downregulation of N-cadherin and Vimentin expression (Fig. 2H), a critical process for the metastasis of cancer cells (Zhou et al. 2023). The results indicated that Claudin-18.2 aided in the malignant progression of pancreatic cancer cells.

Claudin-18.2 boosts pancreatic cancer growth and spread by activating ERK1/2

Given the connection between EMT activation and the altered expression of numerous genes, with the ERK1/2 members of the MAPK family seemingly have a pivotal role in the onset of EMT (Seton-Rogers 2016; Ji et al. 2021), we proceeded to explore whether the promotion of pancreatic cancer malignancy by Claudin-18.2 is related to the ERK1/2 signaling pathway. To begin, we used a cell line that overexpressed Claudin-18.2 and treated it with an ERK1/2 inhibitor (PD98059), resulting in the group designation: oe-NC + DMSO, oe-Claudin-18.2 + DMSO, and oe-Claudin-18.2 + PD98059. Using WB, we discovered that overexpression of Claudin-18.2 led to elevated phosphorylation levels of ERK1/2 proteins, and this effect can be reversed by treatment with PD98059 (Fig. 3A), implying that Claudin-18.2 may exert its effects through activation of the ERK1/2 signaling pathway. As a result, we next investigate if Claudin-18.2 modulates the ERK1/2 pathway to influence the aggressive progression of pancreatic cancer. Our findings showed that Claudin-18.2 overexpression enhanced the colony formation of AsPC-1 cells, and this enhancement was negated by PD98059 treatment (Fig. 3B). Additionally, treatment with the ERK1/2 inhibitor also counteracted the effect of Claudin-18.2 overexpression on promoting cell migration and invasion (Fig. 3C, D). Furthermore, we found that the overexpression of Claudin-18.2 in pancreatic cancer cells encouraged EMT, and this effect was reversed with the inclusion of PD98059 (Fig. 3E). These outcomes further supported that Claudin-18.2 contributed to the growth and metastasis of pancreatic cancer cells by activating the ERK1/2 signaling pathway.

Identification of ZIC2 as an upstream regulatory molecule of Claudin-18.2

To elucidate how Claudin-18.2 affects pancreatic cancer progression, we employed the KnockTF database to identify potential regulatory TFs for Claudin-18.2, identifying ZIC2 as a candidate (Fig. 4A). A subsequent Pearson analysis revealed a marked positive correlation between Claudin-18.2 and ZIC2 expression levels (Fig. 4B), and Molotool analysis confirmed the presence of a binding site for ZIC2 on the Claudin-18.2 promoter (Fig. 4C). Examination of the TCGA pancreatic cancer database demonstrated elevated ZIC2 expression in pancreatic cancer tissues (Fig. 4D). qRT-PCR analysis of ZIC2 mRNA levels in normal pancreatic ductal cells hTERT-HPNE and pancreatic cancer cell lines AsPC-1, HPAC, and MIA-Paca2 corroborated the upregulation of ZIC2 in pancreatic cancer (Fig. 4E). ChIP assay further validated the binding of ZIC2 to the Claudin-18.2 promoter (Fig. 4F). We also constructed luciferase reporter plasmids containing the wild-type and mutant forms of the Claudin-18.2 promoter and transfected them into the cells. Overexpression of ZIC2 was found to enhance the activity of the Claudin-18.2-WT promoter in a luciferase assay, while the mutant promoter was unaffected (Fig. 4G). Collectively, these findings indicated that ZIC2 transcriptionally activates Claudin-18.2 expression.

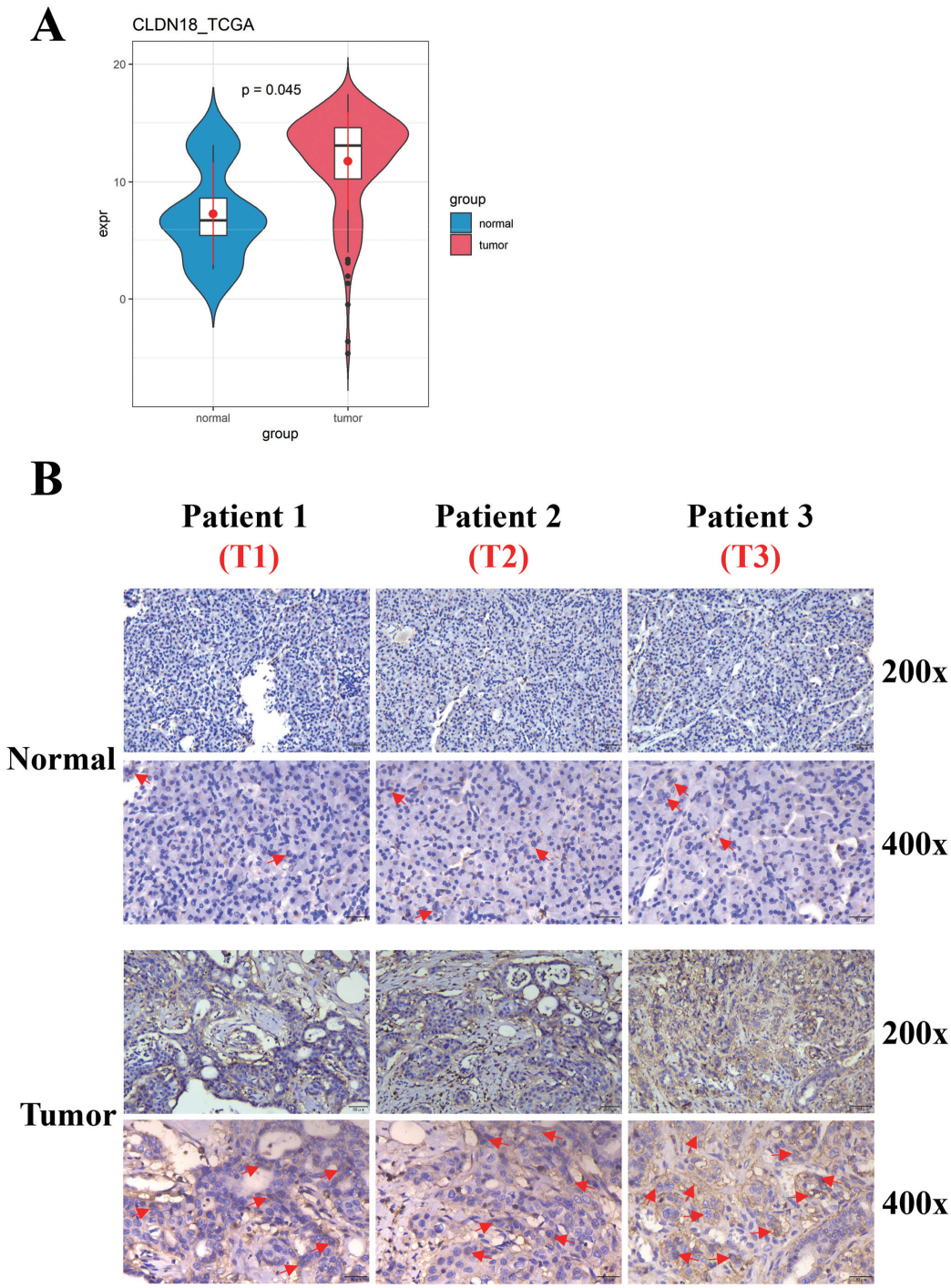

Fig. 1. Upregulated Claudin-18.2 expression in pancreatic cancer.
A: TCGA analysis of CLDN18 (Claudin-18.2) expression in pancreatic cancer; B: IHC detection of Claudin-18.2 protein levels in pancreatic cancer vs. adjacent normal tissues (sample images).

The ZIC2-Claudin-18.2 axis boosts pancreatic cancer growth and spread by activating ERK1/2

To probe into the function of ZIC2 in the proliferation of pancreatic cancer induced by Claudin-18.2, we overexpressed ZIC2 in pancreatic cancer cells where Claudin-18.2 was silenced, creating experimental groups of oe-NC + si-NC, oe-ZIC2 + si-NC, and oe-ZIC2 + si-Claudin-18.2, and confirmed transfection efficacy via qRT-PCR analysis (Fig. 5A). Overexpression of ZIC2 was observed to enhance the ERK1/2 signaling cascade, an effect mitigated by concurrent Claudin-18.2 silencing (Fig. 5B). Furthermore, ZIC2 overexpression markedly promoted the malignant behaviors of AsPC-1 cells, an effect that was dampened upon concurrent Claudin-18.2 knockdown (Fig. 5C-E). Expression analysis of EMT-associated protein showed that Claudin-18.2 silencing reduced ZIC2 overex-

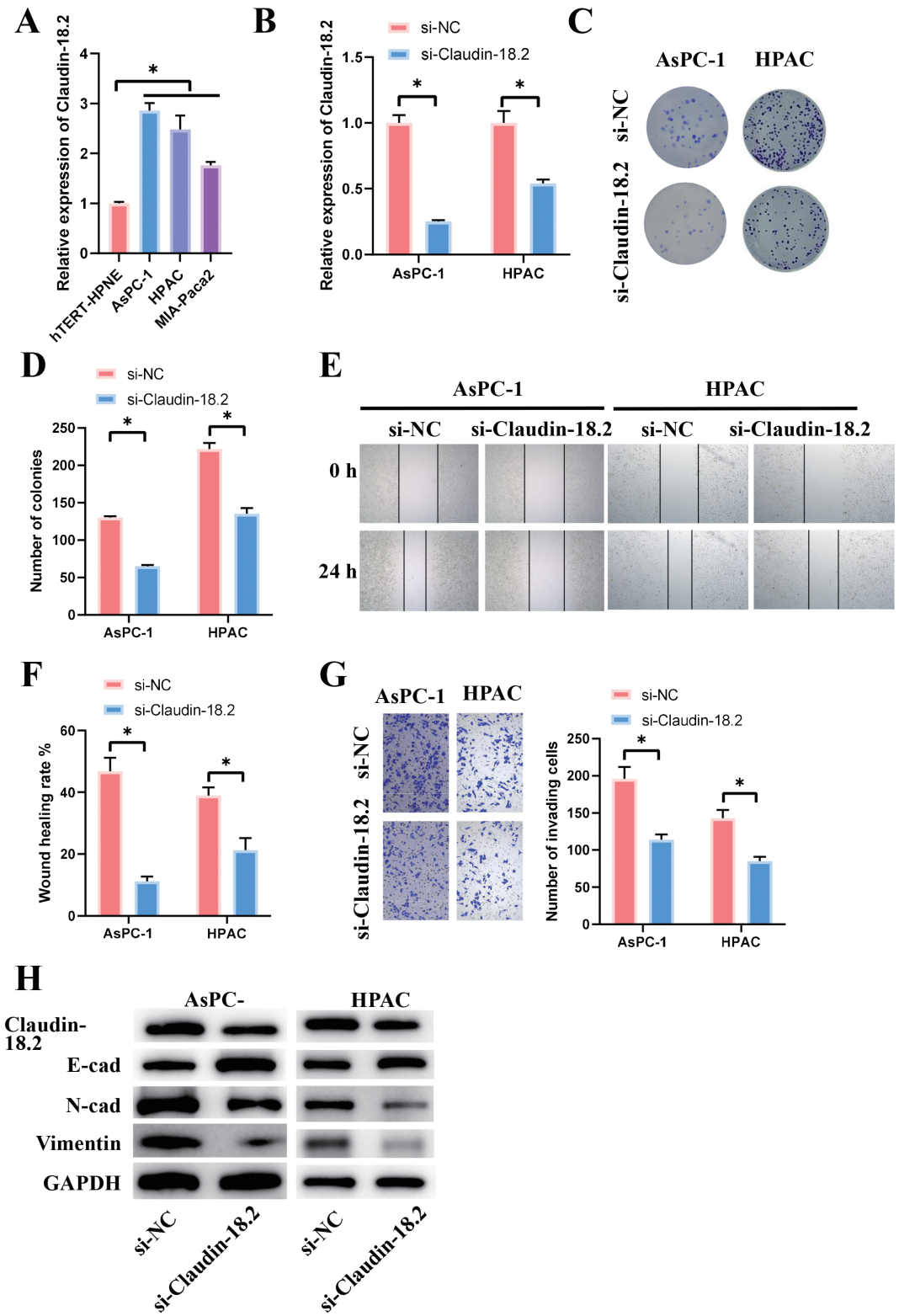

Fig. 2. Claudin-18.2 facilitates the growth and metastasis of pancreatic cancer.
A: qRT-PCR analysis of Claudin-18.2 mRNA levels in normal human pancreatic duct epithelial cells hTERT-HPNE and pancreatic cancer cell lines AsPC-1, HPAC, and MIA-Paca2; B: qRT-PCR assessed the efficiency of Claudin-18.2 knockdown; Groups included si-NC and si-Claudin-18.2; C,D: Colony formation assay evaluated cell proliferation across different groups; E,F: Wound-healing assay measured cell migration; G: Transwell assay tested cell invasiveness; H: WB analysis assessed the expression of EMT-associated proteins. *P < 0.05.

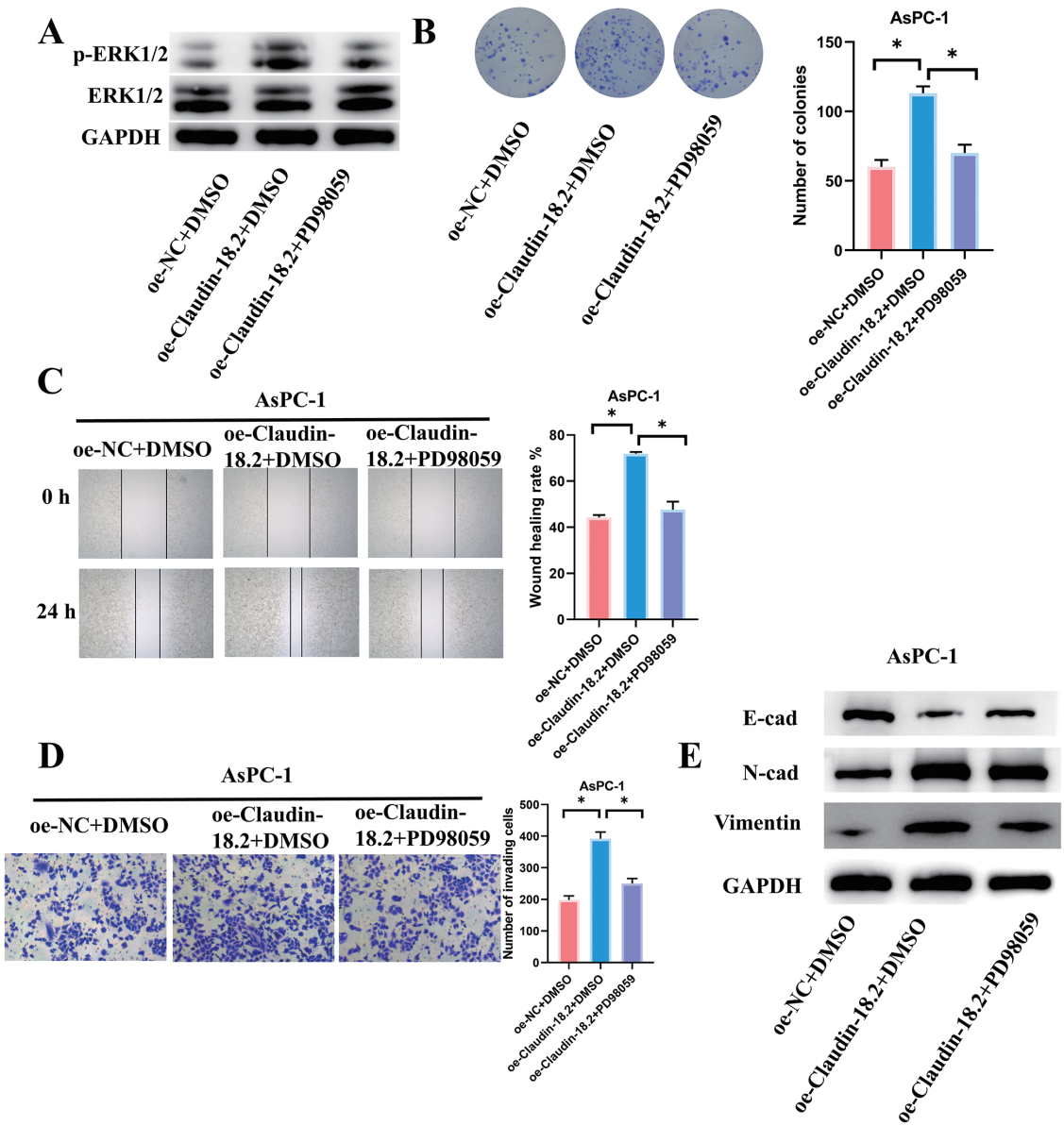

Fig. 3. Claudin-18.2 boosts pancreatic cancer growth and spread by activating ERK1/2.
Group setting: oe-NC+DMSO, oe-Claudin-18.2 + DMSO, and oe-Claudin-18.2 + PD98059 groups. A: WB detection of ERK1/2 protein phosphorylation levels; B: Cell proliferative capacity determined by colony formation assay; C: Cell migration ability tested via cell scratch assay; D: Cell invasive capacity assessed through transwell assay; E: WB detection of the expression of EMT-related proteins. *P < 0.05.

pression-induced activation of EMT. These discoveries point towards the ZIC2-Claudin-18.2 regulatory axis as a potential driver of pancreatic cancer malignancy through ERK1/2 pathway activation.

Discussion

The alarming increase in the incidence of pancreatic cancer, attributed to the escalation of risk factors such as obesity, diabetes, and smoking (Klein 2021), highlights an urgent demand for the development of effective treatment targets. On a positive note, Claudin-18.2 has been identified as a key factor in the progression of multiple cancer types. For instance, its presence has been linked to various clinical and molecular traits in gastric and gastroesophageal junction cancers (Kubota et al. 2023). Recent research indicates Claudin-18.2 is more prevalently and frequently expressed than the early pancreatic ductal tumor marker mucin 5AC (Wang et al. 2022), and its expression holds important prognostic value for pancreatic ductal adenocarcinoma patients undergoing surgery (Kayikcioglu and Yüceer 2023), underscoring its research significance in pancreatic cancer. Of interest, therapies targeting the Claudin-18.2 target have been initially validated in the clinic. For example, a monoclonal antibody (zolbetuximab) targeting Claudin-18.2 in combination with other therapies was able to prolong the overall survival of patients with

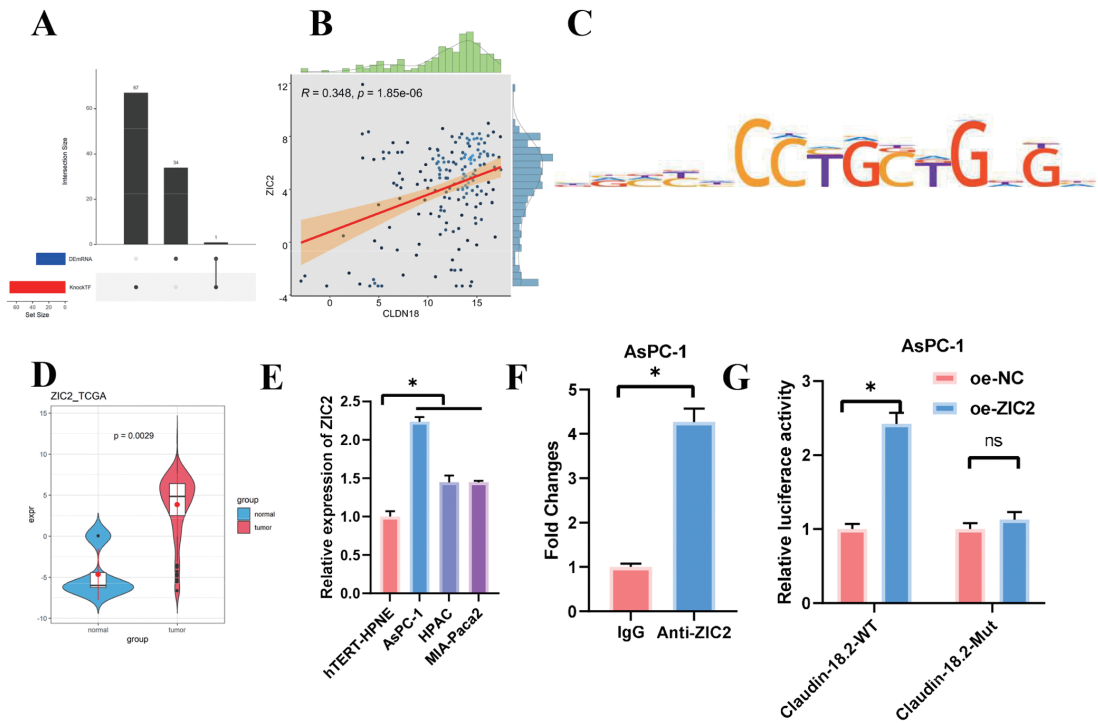

Fig. 4. ZIC2 is an upstream regulatory molecule of Claudin-18.2.
A: Prediction of potential TFs for Claudin-18.2 using the KnockTF database; B: Pearson correlation analysis of the expression of Claudin-18.2 and ZIC2; C: Analysis of binding sites between Claudin-18.2 and ZIC2 using the Molotool; D: Expression analysis of ZIC2 in the TCGA pancreatic cancer database; E: qRT-PCR validation of ZIC2 expression in human normal pancreatic ductal cells hTERT-HPNE and pancreatic cancer cell lines AsPC-1, HPAC, and MIA-Paca2; F: ChIP assay analysis of the binding between ZIC2 and Claudin-18.2; G: Dual-luciferase reporter assay to verify the transcriptional activation of Claudin-18.2 by ZIC2. *P < 0.05.

pancreatic cancer while improving their quality of life (Tureci et al. 2019; Zhou et al. 2024). Pancreatic cancer patients receiving Claudin-18.2 CAR-T therapy had significant reduction of metastatic foci and effective control of tumor progression (Qi et al. 2023).

However, despite the established concept of Claudin-18.2 as an antigenic target in cancer diagnosis, the investigation into its regulatory mechanisms in cancer progression is still in its nascent phase. Additionally, the ERK1/2 signaling pathway modulates the malignant behaviors of tumor cells (Guo et al. 2020). Claudin-1, a member of the same protein family, activates the ERK1/2 signaling pathway in liver cancer (Suh et al. 2013), and Claudin-2 regulates the progression of colorectal cancer by activating the ERK1/2 signaling pathway (Dhawan et al. 2011). Nonetheless, whether Claudin-18.2 can affect cancer progression through the ERK1/2 signaling pathway remains ambiguous. Thus, a thorough investigation into the regulatory influence of Claudin-18.2 on the ERK1/2 signaling pathway and its impact on pancreatic cancer progression is of great clinical significance. This study found that Claudin-18.2 could promote the development and metastasis of pancreatic cancer cells by activating ERK1/2 signaling pathway, suggesting its important role in the progression of pancreatic cancer.

More importantly, this study discovered through bioinformatics analysis that ZIC2 is a potential upstream regulator of Claudin-18.2 with a positive correlation between the two. However, the exact mechanisms by which ZIC2 acts in pancreatic cancer are still not clear. ZIC2 is known as a TF that exerts its effects by regulating the expression of downstream target genes (Hatayama and Aruga 2018). For example, ZIC2 directly binds to the promoter of p21-activated kinase 4 and modulates its activity, thus promoting tumor growth and metastasis in hepatocellular carcinoma (Lu et al. 2017). Interestingly, analyzed by Molotool tool, this study found that ZIC2 protein had a binding site with the promoter region of CLDN18 gene. This result implies that ZIC2 may act as a transcription factor to regulate the expression of Claudin-18. Subsequently, we verified the transcriptional activation of ZIC2 for Claudin-18 using CHIP and dual luciferase assays. The effect of this regulatory axis on pancreatic cancer development was further validated. In conclusion, we demonstrated that the ZIC2-Claudin-18.2 axis promotes the development and metastasis of pancreatic cancer via activating the ERK1/2 signaling pathway. These findings illuminated that targeting the ZIC2-Claudin-18.2 regulatory axis can improve pancreatic cancer patient survival.

To encapsulate, our research suggested that Claudin-18.2 was regulated by the transcriptional activator ZIC2, thereby activating pancreatic cancer development

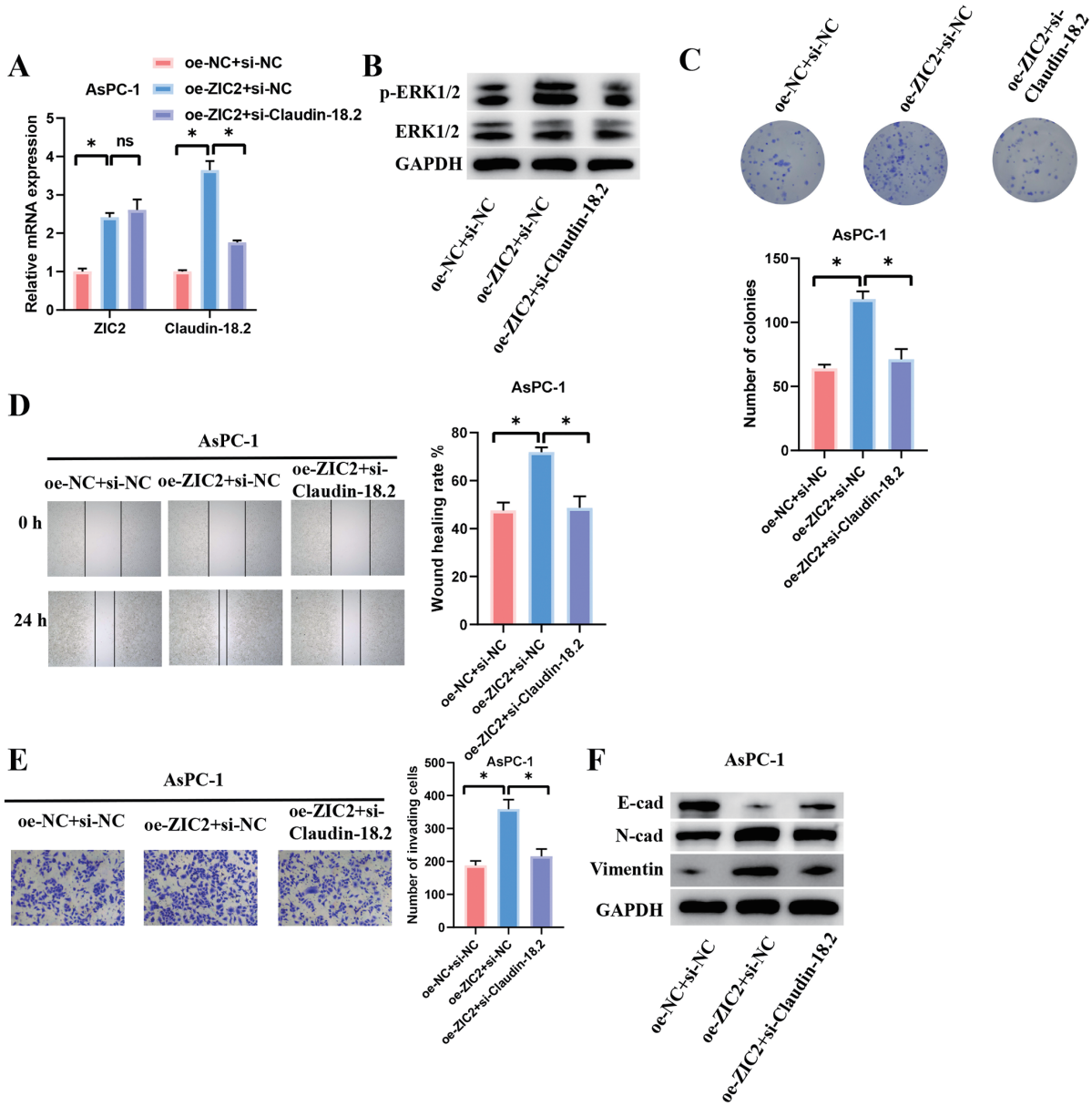

Fig. 5. The ZIC2-Claudin-18.2 axis boosts pancreatic cancer growth and spread by activating ERK1/2.
Group setting: oe-NC + si-NC, oe-ZIC2 + si-NC, oe-ZIC2 + si-Claudin-18.2; A: qRT-PCR detection of ZIC2 and Claudin-18.2 expression; B: WB detection of ERK1/2 protein phosphorylation level; C: Cell proliferation ability tested via colony formation assay; D: Cell migration ability assessed via scratch assay; E: Cell invasive capacity assessed by transwell assay; F: WB detection of the expression of EMT-related proteins. *P < 0.05.

and metastasis through the ERK1/2 signaling pathway. The notable overexpression of both ZIC2 and Claudin-18.2 in pancreatic cancer indicated their potential as viable targets for treatment. While our study has shed light on the impact of Claudin-18.2 on pancreatic cancer progression and its underlying mechanisms, it has only preliminarily outlined the specific mechanisms through which Claudin-18.2 acts, based on cellular experiments. Further animal experiments or more profound clinical studies are needed to validate these mechanisms, which is where our study falls short. In future research, we will delve deeper into the feasibility and efficiency of this mechanism in treating pancreatic cancer. Enhancements in screening technologies, alongside these efforts, are anticipated to mitigate the risks posed by many cancers, including pancreatic cancer. In summary, this study has uncovered the therapeutic potential of Claudin-18.2 in pancreatic cancer, offering new insights for the treatment of this disease.

Author Contributions

XPZ contributed to the study design. HCZ conducted the literature search. JX acquired the data. JX wrote the article. XPZ performed data analysis and drafted. JX revised the article. XPZ gave the final approval of the version to be submitted.

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Conflict of Interest

The authors declare no conflict of interest.

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