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Basic Study

CALD1 facilitates gastric cancer epithelial-mesenchymal transition progression by modulating the PI3K-Akt pathway

Ma WQ *et al*. CALD1 facilitates GC EMT progression

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Abstract

BACKGROUND

CALD1 was discovered to be abnormally expressed in a variety of malignant tumors, including prostate, bladder, ovarian and gastric cancers (GCs), and is associated with tumor progression and immune infiltration; however, the roles and mechanisms played by CALD1 in epithelial-mesenchymal transition (EMT) of GCs are unknown.

AIM

To investigate the role and mechanism of the CALD1 in GC progress, nvasion and migration.

METHODS

In this study, the relationship between CALD1 and GC, as well as the possible network regulatory mechanisms of CALD1 were investigated and validated by bioinformatics. CALD1-siRNA was tested and synthesized, and GC cell lines were transfected; cell activity was measured using the CCK-8 method; cell migration and invasive ability was measured using the Wound assay and Transwell assay; and the expression of relevant genes and protein levels of each group of cells were measured using qRT-PCR and Western blot. The experiment of subcutaneous tumor transplantation in mice was validated.

RESULTS

Bioinformatics results showed that CALD1 was highly expressed in GC tissues, and CALD1 was significantly higher in EMT-type GC tissues than in other types of GC tissues; the prognosis of patients with high expression of CALD1 was worse than that of patients with low expression, and prognostic models were constructed and evaluated. The validation results were consistent with the results of the raw letter. Among the cell lines, the expression level of CALD1 in GC cell lines was all higher than those in gastric epithelial cell line GES-1, with the strongest expression was found in AGS and MKN45.

Cell activity was significantly reduced after CALD1-siRNA transfection of AGS and MKN45. The cell's ability to migrate and invade was reduced after CALD1-siRNA transfection of AGS and MKN45, and the corresponding mRNA and protein expression were altered. According to bioinformatics findings in GC samples, the *CALD1* gene was significantly associated with the expression of members of the PI3K-AKT-mTOR signalling pathway as well as the EMT signalling pathway, and was closely related to the PI3K-Akt signalling pathway. Experimental validation revealed that upregulation of CALD1 increased the expression of PI3K, p-AKT and p-mTOR, members of the PI3K-Akt pathway, was enhanced after up-regulation of CALD1, while decreasing the expression of PTEN; overexpression of CALD1+ inhibitor decreased the expression of PI3K, p-AKT and p-mTOR (still higher than that in the normal group), but increased the expression of PTEN (still lower than that in the normal group). CCK-8 results revealed that the effect of CALD1 on tumor cell activity was decreased by the addition of the inhibitor. Scratch and Transwell experiments showed that the effect of CALD1 on tumor cell migration and invasion was weakened by the addition of the inhibitor. mRNA and protein levels of EMT-related genes in AGS and MKN45 were greatly altered by the addition of overexpression of CALD1, whereas the effect of overexpression of CALD1 was significantly weakened by the addition of the inhibitor. Animal experiments showed that tumour growth was slow after inhibition of CALD1, and some PI3K-Akt and EMT pathway protein expression was altered.

CONCLUSION

Increased expression of CALD1 is a key factor in the progression and invasion and metastasis of GC, and it may be achieved by regulating the *PI3K-Akt* gene to promote EMT.

Key Words: Gastric tumor; CALD1; Epithelial-mesenchymal transition; Gene disruption; Invasion; Migration; Bioinformatics

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Core Tip: In this study, the relationship between CALD1 and gastric cancer (GC) and the possible network regulatory mechanisms of CALD1 were explored and validated by bioinformatics. We conducted functional analysis and verification through tissue and cell experiments, delving into its pathways and mechanisms, it was showed that CALD1 may participate in the proliferation, invasion, and migration and epithelial-mesenchymal transition (EMT)-related gene and protein expression of GC cells. Our study propounds that CALD1, through PI3K-Akt signaling pathway activation, may regulate the EMT process in GC cells, thereby presenting a potentially novel target for GC treatment.

INTRODUCTION

Gastric cancer (GC) is a major malignancy of the digestive tract that ranks fifth in incidence and fourth in mortality, thereby imposing a significant societal burden[1,2]. The rise in health consciousness, as well as advances in diagnostic technologies such as endoscopy and computed tomography imaging have improved the early detection and treatment of GC. Nonetheless, the survival rate for patients diagnosed at advanced stages is less than 40%[3], indicating a bleak prognosis. The aggressive nature of GC, which is characterized by rapid progression, increased metastatic potential, and recurrent manifestations, is associated with the accelerated cell growth, robust invasiveness, and antiapoptotic properties of GC cells[3-6]. It is a critical to identify precise and sensitive molecular targets, investigate their regulatory roles in GC invasion and metastasis, and thus lay the groundwork for comprehensive, personalized treatment strategies and prognostic assessments.

CALD1 encodes the Caldesmon protein, which is divided into high and low molecular weight variants and functions as a cytoskeletal-associated protein. It is

involved in cellular adhesion, cytoskeletal organization, and angiogenesis, as well as cell proliferation, apoptosis, motility, and adhesion. As a result, it may influence tumor proliferation, invasion, and metastasis[7-9]. CALD1 expression is abnormal in various cancers, including bladder, breast, colorectal, and GCs, show abnormal CALD1 expression, and it correlates with tumor staging and prognosis[8-13]. In GC research, CALD1 expression has been linked with immune infiltration and prognosis, though its specific mechanisms remain unknown[8].

Tumor cells can acquire invasive mesenchymal characteristics via the epithelial-mesenchymal transition (EMT) process, resulting in increased motility and invasiveness, decreased adhesion, and altered cell polarity, all of which can lead to metastasis from the primary tumor site[14]. EMT is triggered by key transcription factors such as SNAIL and Zinc finger E-box binding, and involves changes in gene expression mediated by the TGF-β family signaling, PI3K-Akt, and ERK-MAPK pathways, among others[15,16]. However, the exact role and mechanism of CALD1 in GC-related EMT are unknown. Therefore, this study aims to investigate the relationship between CALD1 and GC using the TCGA and GEO databases. We assessed CALD1 mRNA and protein expression in GC tissues, explored the relationship between CALD1 expression and clinical pathological features, and investigated the role and mechanism of CALD1 in GC tissues and cell lines through a combination of experimental and bioinformatics approaches.

MATERIALS AND METHODS

Clinical tissue collection

This study registered 80 GC patients undergoing radical surgery from January to December 2022 at the Department of Surgery, Fourth Hospital of Hebei Medical University. Postoperative confirmation revealed that all patients had gastric adenocarcinoma, with no preoperative treatments or having concurrent secondary tumors. Uniformly sized tumors and adjacent non-tumor tissues were excised, placed in cryopreservation tubes, and stored at -80 °C in liquid nitrogen. Additionally, tissues

from a previous cohort of 60 patients were included in immunohistochemical staining and follow-up studies. The Medical Ethics Committee of The Fourth Hospital of Hebei Medical University granted ethical approval, and all patients provided informed consent.

Cell line culture and passaging

Human GC cell lines HGC27, NCI-N87, AGS, MKN45, and the gastric epithelial cell line GES-1, after being obtained from the National Cell Resource Center of the National Biomedical Resource, were cultured in our laboratory. These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified 37 °C incubator with 5% CO_2. Cell growth and sterility were monitored daily, with medium changes every 1-2 d. For cell detachment during passaging or subsequent experiments, 0.25% trypsin was used.

Cell transfection

Single-cell suspensions of 1 × 10^6 cells/mL were prepared, seeded into 6-well plates, and cultured to complete fusion. siRNA and overexpression plasmids were introduced using Lipofectamine 2000 according to the manufacturer's instructions. CALD1-shRNA was obtained from a gene pharmaceutical company in Shanghai, China, and was used for CALD1 knockdown. For overexpression, lentiviral particles were produced by co-transfecting plasmids psPAX2, pMD2G, and pcDNA3.1/CALD1 into 293T cells and harvested 48 h post-transfection. The PI3K-Akt inhibitor from Sigma was used according to the manufacturer's instructions.

Immunohistochemical staining

Paraffin-embedded GC tumor and adjacent tissue specimens were sectioned at 4 μm, deparaffinized, and had antigen retrieval. The staining was done according to the kit instructions, and the results were evaluated by board-certified pathologists evaluating

the outcomes. The scoring system considered the percentage of positive cells and staining intensity to caculate the cumulative score.

Western blot analysis for target protein expression

Cells were harvested and lysed for protein extraction, and the BCA method was used to quantify them. Proteins were denatured, before being separated using SDS-PAGE and transferred to PVDF membranes. Membranes were blocked and then incubated with primary and secondary antibodies before being imaged with the Odyssey dual-color infrared fluorescence scanning system. Relative protein expression was assessed by comparing grayscale values, with β-actin serving as an internal reference. This procedure was repeated three times.

RNA extraction and RT-qPCR analysis

Total RNA was extracted using Trizol, quantified, and reverse-transcribed into cDNA. qPCR amplification was performed according to the manufacturer's instructions, and mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell viability assessment via CCK-8 assay

Cells were seeded in 96-well plates and incubated for 48 h. CCK-8 reagent was added at different time points, and incubated, and the optical density (OD) at 450 nm was measured using a microplate reader.

Cell scratch assay

After seeding single-cell suspensions in 24-well plates and reaching confluence, cells were transfected. Once the celss were confluent, a scratch was made, followed by washing, serum-free medium addition, and microscopic examination at 0 and 48 h post-scratch.

Transwell chamber invasion assay

Single-cell suspensions were seeded into Transwell chambers in a 24-well plate. After 24 h, cells on the inner surface were removed, fixed, stained, and examined under an inverted phase-contrast microscope.

GC cell xenograft model establishment

Single-cell suspensions of lentivirus-infected cells were subcutaneously injected into BALB/c nude mice. Tumor growth was tracked, and tumors were dissected for analysis at the end of the experiment.

Bioinformatics analysis

Various bioinformatics analyses were performed, including Gene Set Enrichment Analysis, Protein-Protein Interaction (PPI) network analysis, differential gene expression enrichment analysis, Pearson correlation analysis, and Cox proportional hazards models. The goal of these studies was to determine the effect of CALD1 expression on survival and to create a predictive nomogram.

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Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics software version 25.0. The *t*-test, one-way ANOVA, and Kaplan-Meier method were used for certain data comparisons and survival curve generation. The data is presented as the mean ± SD.

RESULTS

Analysis of CALD1 expression in GC revealed its higher levels in GC cases, particularly in the EMT subtype, according to the Asian Cancer Research Group (ACRG) classification (Figure 1A). Patients with higher levels of CALD1 expression had a worse prognosis than those with lower levels (Figure 1B). A prognostic model, that included age, gender, and CALD1 expression, was developed and validated for accuracy using a calibration curve (Figure 1C). Immunohistochemical analysis revealed increased CALD1 protein expression in GC tissues compared to adjacent non-cancerous tissues in

a subset of 60 cases (Figure 1D). Both CALD1 mRNA and protein levels were significantly higher in the EMT subtype of GC, as demonstrated by qPCR and Western blot in 20 cases (Figure 1E), and high expression was associated with a poor prognosis in another subset of 60 cases (Figure 1F).

Inhibition of CALD1 reduced proliferation, invasion, and migration in GC cells. Among the various cell lines tested, AGS and MKN45 had significantly higher CALD1 expression and were chosen for further investigation (Figure 2A). CALD1-siRNA had a significant inhibitory effect in these cell lines (Figure 2B). Post-transfection, there was a significant decrease in cell activity, migration, and invasion was observed (Figure 2C). CALD1 inhibition also increased E-cadherin and Claudin-1 expression while decreasing N-cadherin, Vimentin, and Dickkop-1 (DKK-1) mRNA and protein levels, as confirmed by RT-qPCR and Western blot (Figure 2D).

Bioinformatics analysis revealed significant associations of CALD1 with the PI3K-Akt-mTOR and EMT signaling pathways in GC samples (Figure 3A). A PPI network revealed multiple proteins that interact with CALD1 (Figure 3B). GO and KEGG enrichment analyses revealed that CALD1 was involved in a network of various biological processes, that included Protein Kinase B signaling and protein tyrosine kinase activity (Figure 3C). Single-cell data showed high CALD1 expression in fibroblasts, as evidenced by bulk data analysis, which revelaed a positive correlation between CALD1 and fibroblast surface molecule expression (Figure 3D).

Additional studies confirmed CALD1's role in enhancing GC cell proliferation, invasion, and migration *via* the PI3K-Akt pathway. Western blot analysis revealed that CALD1 overexpression increased PI3K, p-AKT, and p-mTOR expression, while decreasing PTEN expression. However, integrating CALD1 overexpression with PI3K-Akt pathway inhibition moderated these effects (Figure 4A). The CCK-8 assay results showed that the effect of CALD1 on tumor cell activity was reduced after the addition of a PI3K-Akt inhibitor addition (Figure 4B). Scratch and Transwell assays revelaed that CALD1 had a reduced impact of CALD1 on cell migration and invasion after inhibitor treatment (Figure 4C). In AGS and MKN45 cells, CALD1 overexpression altered the

expression of EMT-related genes and proteins, an effect that was mitigated by the PI3K-Akt inhibitor (Figure 4D).

In an animal model, CALD1 inhibition led to reduced tumor growth in nude mice. Tumors with CALD1-shRNA-transfected cells were significantly lighter and smaller than those in the control group ($P < 0.05$), as shown by Western blot analysis, which also revealed changes in EMT-related protein expression (Figure 5A and B), thus demonstrating the suppressive effect of CALD1 inhibition on GC tumor growth.

DISCUSSION

GC is a complex, multifaceted disease that progresses through multiple steps and stages, with tumor invasion and metastasis playing important roles in the high mortality rates observed in advanced stages of GC[3-6]. It is critical to comprehend the underlying mechanisms of GC development is critical. Identifying molecules integral to its initiation and progression, as well as discovering specific and sensitive molecular targets, is critical to improve diagnosis, comprehensive treatment, and prognostic evaluation. This is especially important for patients with unresectable GC who are not candidates for surgical intervention.

The ACRG divides GC into distinct molecular subtypes: MSS/EMT, MSS/TP53+, MSI/TP53+, and MSS/TP53-. Each subtype is associated with varying prognostic outcomes, with the MSS/EMT subtype being linked to the poorest prognosis[17].

Our study employed bioinformatics analysis to establish that CALD1 is significantly overexpressed in GC compared to normal tissues. Further investigation, consistent with the ACRG molecular classification, revealed a significantly higher expression of CALD1 in the EMT subtype of GC than in other subtypes. Futhermore, increased CALD1 expression was linked to a worse prognosis, implying a role for CALD1 in the progression of EMT subtype GC. This suggests CALD1's role in the development of aggressive EMT subtype GC. To substantiate these observations, we procured GC and adjacent normal tissue samples for bioinformatics analysis, which corroborated our

initial findings. The correlation of high CALD1 expression with poorer patient outcomes implicates its significant role in GC progression.

Further validation using qPCR experiments showed increased CALD1 expression in GC cell lines compared to gastric epithelial cell lines. Functional assays, including CCK-8, scratch, and Transwell assays, revealed that siRNA-mediated CALD1 knockdown significantly reduced cell viability, migration, and invasion. This demonstrates a strong link between CALD1 and GC cell activity, invasion, and metastasis, emphasizing its critical role in the initiation and progression of GC.

It is well-established that the progression of GC is closely associated with the EMT process in GC cells. EMT, which is triggered by various genes and pathways, allows GC cells to switch from an epithelial to a more aggressive mesenchymal phenotype, thereby facilitating tumor progression[18]. This process involves the downregulation of epithelial cell markers and the upregulation of mesenchymal cell markers, both of which are important factors promoting tumor metastasis[19,20].

In our investigation, CALD1 expression inhibition in AGS and MKN45 cells, specifically, we observed an upregulation of epithelial markers E-cadherin and Claudin-1 and a downregulation of mesenchymal markers N-cadherin, Vimentin, and DKK-1. This resulted in increased expression of epithelial cell markers, decreased mesenchymal cell marker expression, and decreased tumor invasion, migration, and EMT-related mRNA and protein levels. Animal experiments confirmed these findings, demonstrating delayed tumor formation, decreased tumor volume and weight, and corresponding changes in cell marker expressions in response to CALD1 inhibition, implying that CALD1 inhibition could impede GC growth and that CALD1 may contribute to GC invasion and metastasis by modulating EMT-related genes and proteins. These results also underscore the pivotal role of CALD1 in highlighting its potential as a therapeutic target to promote GC progression and metastasis.

CALD1, which is found at 7q33, encodes the Caldesmon protein, which exists in two isoforms with different molecular weights and cellular origins. Caldesmon has diverse roles in cellular processes such as migration, invasion, and proliferation by regulating

actin cytoskeleton remodeling[8,9,21,22]. Previous research has linked CALD1 to tumor angiogenesis[13], and its aberrant expression has been observed in various solid tumors[8,9,12,13,23-25], notably in extensive studies on bladder cancer[9,12]. However, the precise mechanisms and pathways of CALD1 function remain unclear. In this study, we undertook functional analyses and validations using tissue and cell experiments to explore its pathways and mechanisms.

Our findings, using bioinformatics analysis, the PPI network of CALD1 was established and GO and KEGG data analysis was performed, revealed CALD1 enrichment in the PI3K-Akt signaling pathway, which is known for its significant influence on GC development. Previous researche has shown that the PI3K-Akt signaling pathway is significantly implicated in the onset and progression of GC. This pathway contributes to the advancement of GC by inhibiting apoptosis, metastasis, EMT, and angiogenesis[26-31]. To verify CALD1's involvement in this pathway, we chose HGC27 cells, which exhibit low CALD1 expression, for overexpression experiments. These experiments were conducted both independently and in combination with PI3K-Akt pathway inhibition. Our results indicated that CALD1 upregulation enhanced expression of pathway members, PI3K, p-AKT and p-mTOR were positively correlated, while PTEN expression was negatively correlated, promoting cell viability, migration, and invasion, which were mitigated post pathway inhibitor addition, but there are still differences with the control group. The congruence between bioinformatics analysis and cell experiment results suggests that CALD1 may regulate the EMT process in GC cells through the activation of the PI3K-Akt signaling pathway. This activation potentially enhances the activity and invasive capabilities of these cells. Consequently, CALD1 emerges as a potential novel target for GC therapy, offering promising avenues for the development of new treatment strategies aimed at targeting this pathway to curb the aggressiveness of GC. Our bioinformatics analysis also underscored a significant CALD1 expression in fibroblasts, the bulk dataset analysis showed that CALD1 was significantly positively correlated with the surface molecular expression of fibroblasts, which, alongside literature, suggests a potential interaction between CALD1

and fibroblasts in GC[32,33], necessitating further thorough investigation. The specific mechanism of action between CALD1 and fibroblasts in GC can be further studied as a follow-up research direction.

However, our study has limitations, while the study suggests that CALD1 is involved in EMT and the PI3K-Akt pathway, the exact molecular mechanisms remain unknown, and its role in the tumor microenvironment requires further exploration. Further research need more patients to be included in the verify the conclusions obtained by this study.

CONCLUSION

In summary, our findings show that CALD1 is upregulated in GC tissues and cell lines, and that high CALD1 expression is associated with a poor prognosis in GC patients. Alterations in CALD1 expression resulted in changes in cell activity, invasion, migration, and the expression of EMT-related genes and proteins. The PI3K-Akt signaling pathway was discoverd to be a key mediator of CALD1's effects, with results from bioinformatics analyses and cell experiments showing alignment. Our study suggests that CALD1 may regulate EMT in GC cells by activating the PI3K-Akt signaling pathway, increasing their invasive properties and thereby presenting a potential novel target for GC therapy.

ARTICLE HIGHLIGHTS

Research background

This study has verified that through bioinformatics analysis, as well as *in vivo* and *in vitro* experiments, this study has confirmed that CALD1 promotes the epithelial-mesenchymal transition (EMT) in gastric cancer (GC) by affecting the PI3K-Akt signaling pathways. These findings underscore its significance in tumor progression and potential as a target for future therapeutic approaches. However, the investigation into the regulatory function of these pathways in this research was not comprehensive, leaving the full mechanism of action somewhat unclear. To validate the study's

conclusions more robustly, further research involving a larger patient cohort is necessary.

Research motivation

By influencing the PI3K-Akt pathway, CALD1 plays a pivotal role in advancing the EMT in GC.

Research objectives

The bioinformatics analysis revealed that CALD1 expression is significantly elevated in GC tissues, particularly in those exhibiting the EMT type. Additionally, the *CALD1* gene was found to be associated with the PI3K-Akt signaling pathway and other EMT components. Compared to gastric epithelial cell lines, GC cell lines show higher levels of CALD1 expression. Suppressing CALD1 and the PI3K-Akt pathway results in reduced viability, invasion, and migration of GC cells. These experimental findings elucidate the role of CALD1 and the PI3K-Akt pathway in GC, laying a foundation for further molecular mechanism studies of this disease.

Research methods

This study investigated the relationship between CALD1 and GC and the possible network regulatory mechanisms of CALD1 were explored and validated by bioinformatics. CALD1-siRNA was assayed and synthesised, and GC cell lines were transfected; cell activity was detected by CCK-8 method; cell migration and invasive ability was detected by Wound assay and Transwell assay; and the expression of relevant genes and protein levels of each group of cells were detected by qRT-PCR and Western blot. The experiment of subcutaneous tumor transplantation in mice was verified.

Research results

This study aims to investigate the role and mechanism of the CALD1 in GC progress, nvasion and migration.

Research conclusions

The motivation behind this research is to explore and better comprehend how CALD1 functions and interacts within the context of GC.

Research perspectives

CALD1 is known for its abnormal expression in various malignant tumors, this expression is linked to tumor growth and immune system infiltration. However, the specific functions and underlying mechanisms of CALD1 in the EMT process in GCs remain unclear.

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Footnotes

Institutional review board statement: The study design was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure Legends

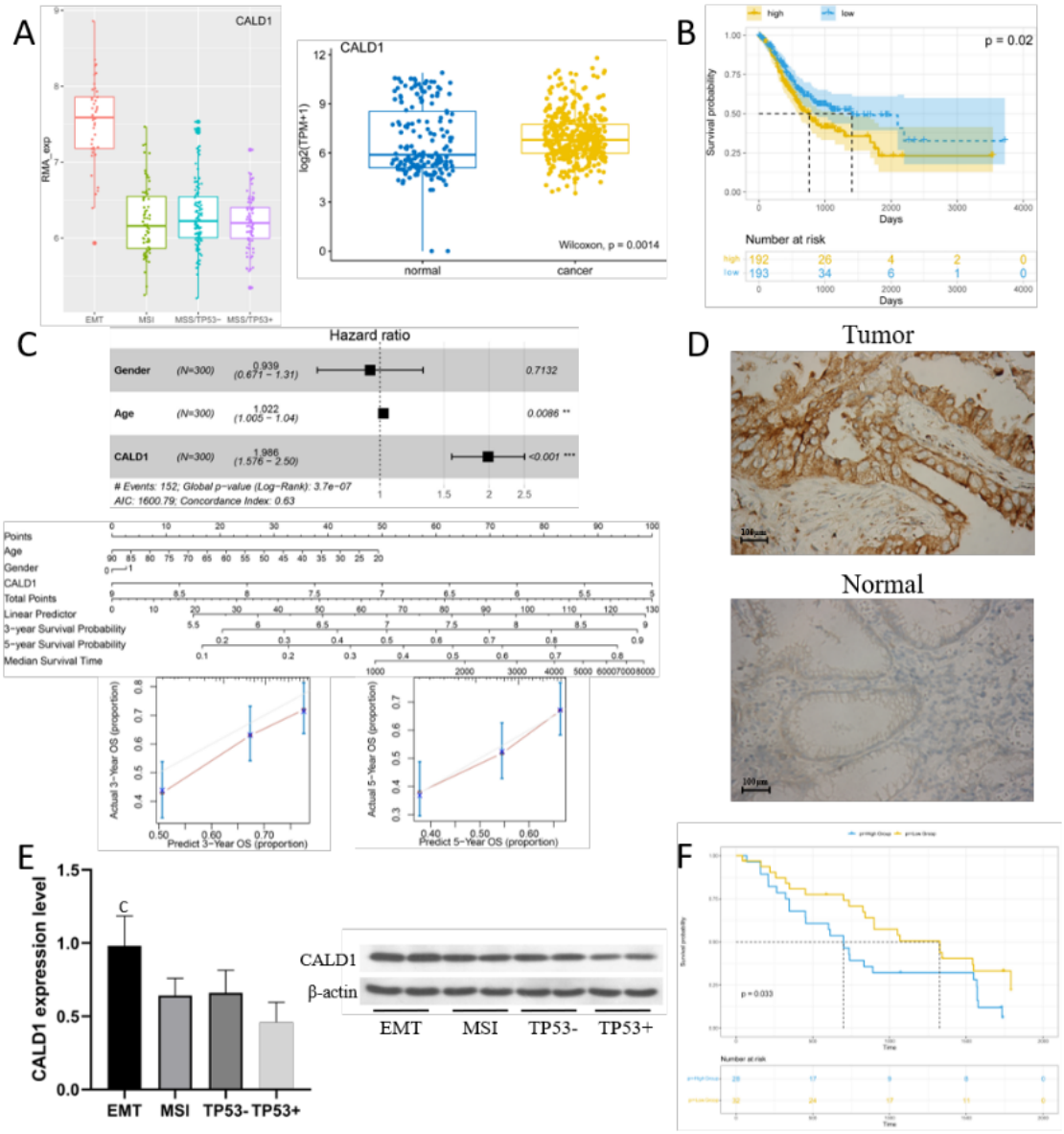


Figure 1 CALD1 Overexpression in gastric cancer: correlation with epithelial-mesenchymal transition type, prognostic implications, and enhanced protein expression in tissues. A: Bioinformatics results showed that CALD1 was highly expressed in gastric cancer (GC), and CALD1 in GC tissues of epithelial-mesenchymal transition (EMT) type was significantly higher than that in GC tissues of other types; B

and C: Patients with high expression of CALD1 had poorer prognosis than those with low expression, and prognostic models were constructed and evaluated; D: Validation showed that the protein (expression of CALD1 in GC tissues was enhanced compared with that in paracancerous tissues (60 cases); E: EMT type CALD1 mRNA (qPCR) and protein (Western blot) in GC tissues were significantly higher than those of other types (20 cases); F: Patients with high CALD1 expression had a poorer prognosis than those with low expression (60 cases). $^{c}P < 0.001$.

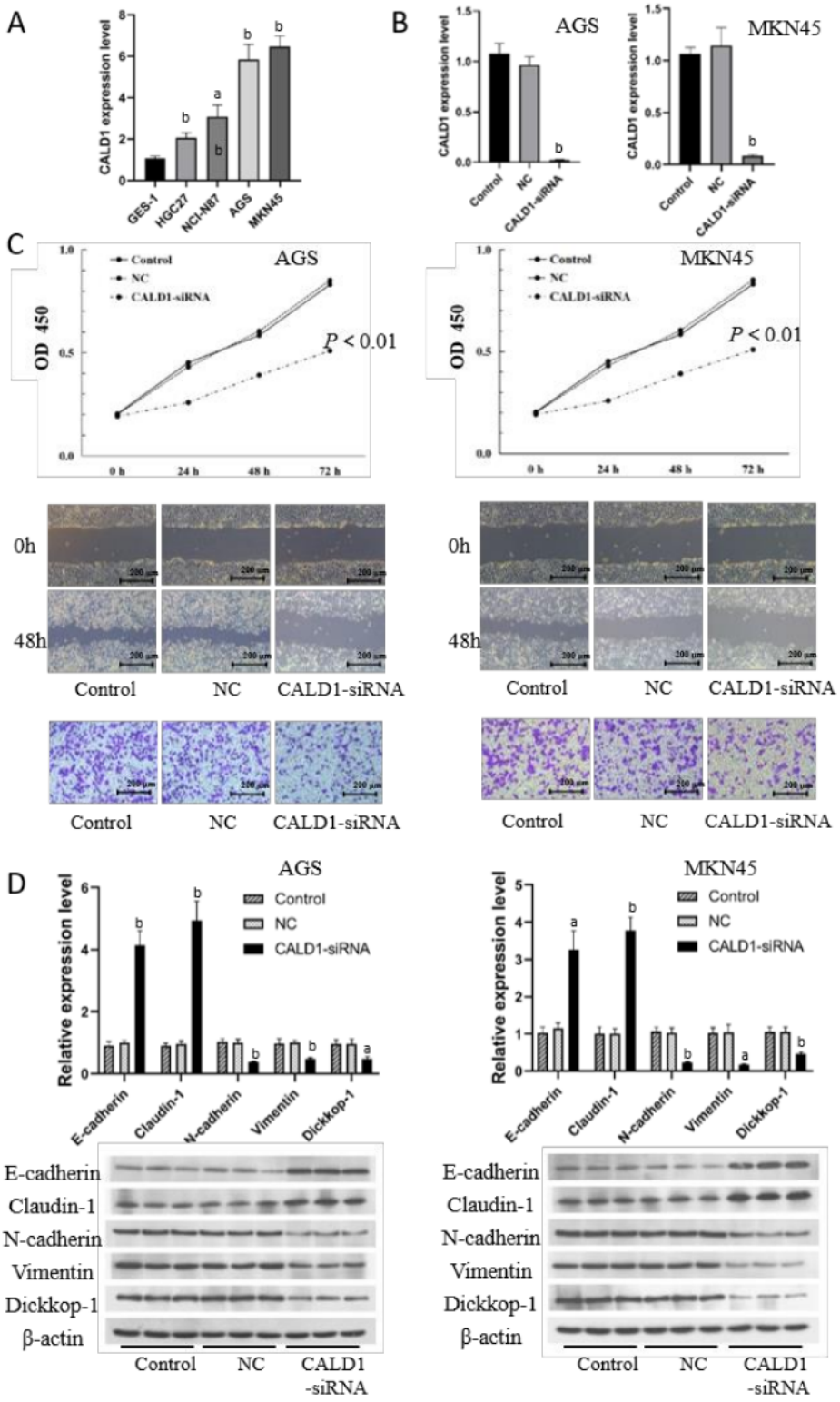
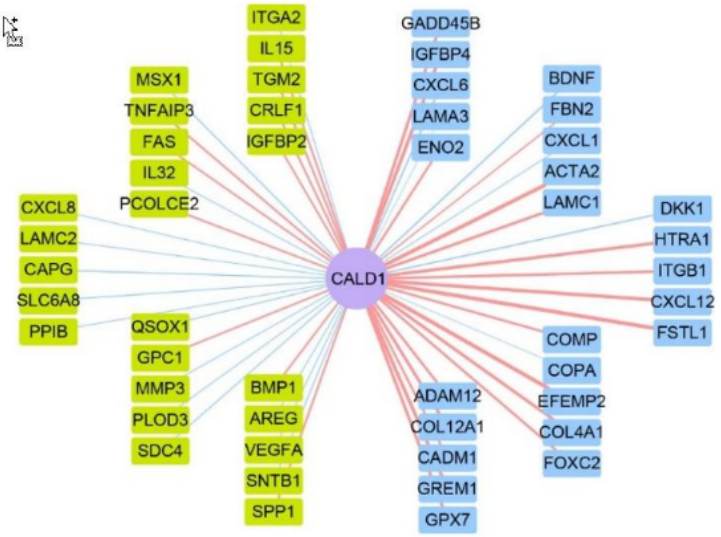

A
CALD1 expression level
GES-1
HGC27
NCI-N87
AGS
MKN45
B
AGS
MKN45
CALD1 expression level
Control
NC
CALD1-siRNA
C
AGS
MKN45
Control
NC
CALD1-siRNA
OD 450
P < 0.01
0 h
24 h
48 h
72 h
0h
48h
Control
NC
CALD1-siRNA
D
AGS
MKN45
Relative expression level
E-cadherin
Claudin-1
N-cadherin
Vimentin
Dickkop-1
β-actin
Control
NC
CALD1
-siRNA

Figure 2 Inhibition of CALD1 in gastric cancer cell lines reduces cell activity, migration, invasion, and alters epithelial-mesenchymal transition marker expression. A: Among cell lines, CALD1 expression levels were higher in gastric cancer cell lines HGC27, NCI-N87, AGS, and MKN45 than in gastric epithelial cell line GES-1, and the strongest expression was found in AGS, and MKN45; B: The inhibitory effect of CALD1-siRNA was verified; C: After transfection of AGS, and MKN45 with CALD1-siRNA, cells' activity was significantly reduced, and the migration and invasion ability was decreased; D: The expression of E-cadherin and Claudin-1 was increased in AGS and MKN45 after inhibiting the expression of CALD1; the expression of N-cadherin, Vimentin, and Dickkop-1 mRNA and protein was decreased. $^{a}P < 0.05$, $^{b}P < 0.01$.

A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TGM2
CRLF1
IGFBP2
GADD45B
IGFBP4
CXCL6
LAMA3
ENO2
MSX1
TNFAIP3
FAS
IL32
PCOLCE2
BDNF
FBN2
CXCL1
ACTA2
LAMC1
CXCL8
LAMC2
CAPG
SLC6A8
PPIB
CALD1
DKK1
HTRA1
ITGB1
CXCL12
FSTL1
QSOX1
GPC1
MMP3
PLOD3
SDC4
BMP1
AREG
VEGFA
SNTB1
SPP1
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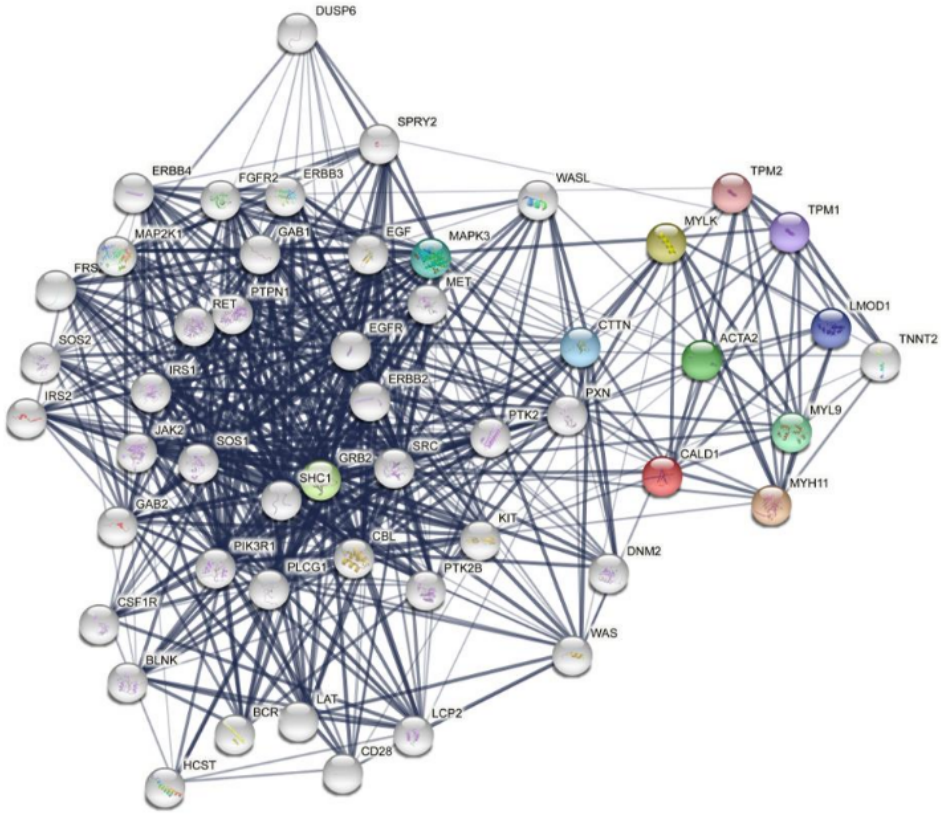
COPA
EFEMP2
COL4A1
FOXC2
ADAM12
COL12A1
CADM1
GREM1
GPX7

B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SPRY2
ERBB4
FGFR2
ERBB3
WASL
TPM2
TPM1
MYLK
MAP2K1
GAB1
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MAPK3
FRS2
MET
PTPN1
RET
LMOD1
EGFR
CTTN
ACTA2
TNNT2
SOS2
IRS1
IRS2
ERBB2
PXN
MYL9
JAK2
SOS1
PTK2
SRC
GRB2
CALD1
SHC1
MYH11
GAB2
KIT
CBL
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PLCG1
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CSF1R
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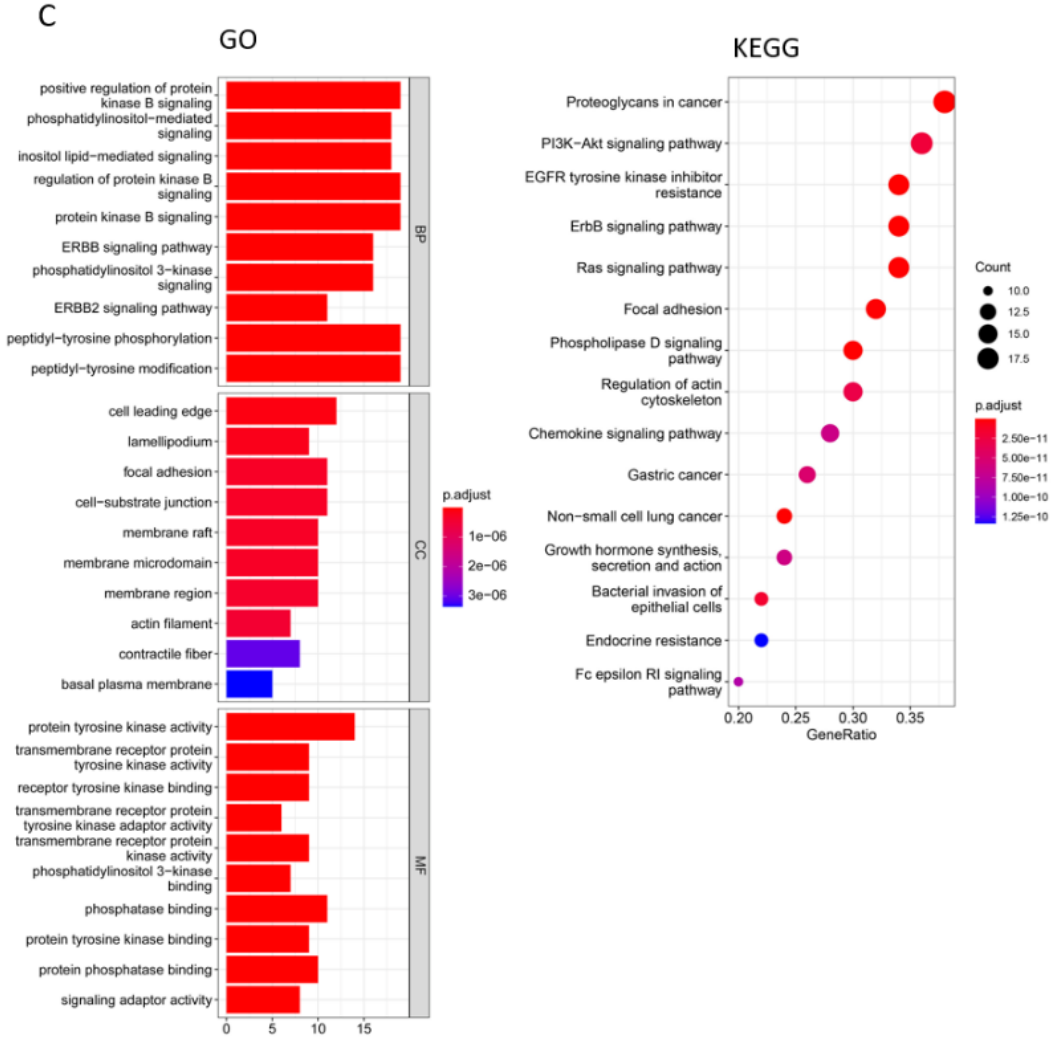
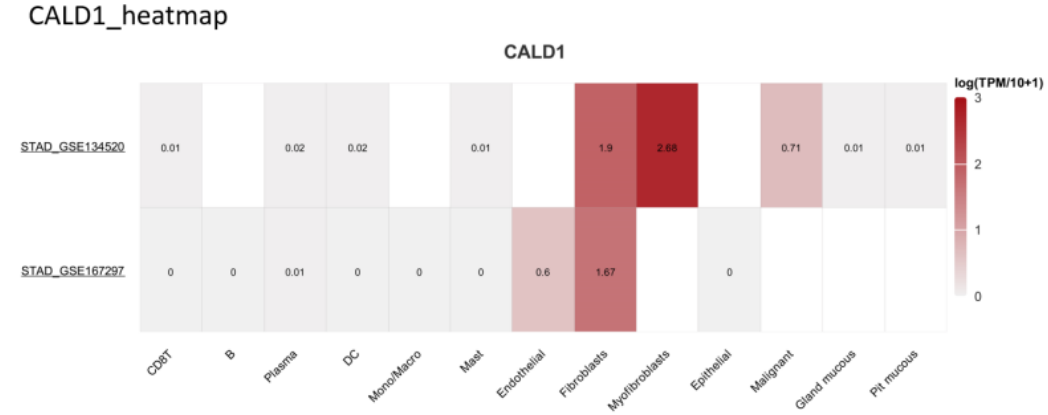
BLNK
LAT
BCR
LCP2
CD28
HCST

C


GO
positive regulation of protein kinase B signaling
phosphatidylinositol−mediated signaling
inositol lipid−mediated signaling
regulation of protein kinase B signaling
protein kinase B signaling
ERBB signaling pathway
phosphatidylinositol 3−kinase signaling
ERBB2 signaling pathway
peptidyl−tyrosine phosphorylation
peptidyl−tyrosine modification
BP
cell leading edge
lamellipodium
focal adhesion
cell−substrate junction
membrane raft
membrane microdomain
membrane region
actin filament
contractile fiber
basal plasma membrane
CC
protein tyrosine kinase activity
transmembrane receptor protein tyrosine kinase activity
receptor tyrosine kinase binding
transmembrane receptor protein tyrosine kinase adaptor activity
transmembrane receptor protein kinase activity
phosphatidylinositol 3−kinase binding
phosphatase binding
protein tyrosine kinase binding
protein phosphatase binding
signaling adaptor activity
MF
0 5 10 15
p.adjust
1e−06
2e−06
3e−06
KEGG
Proteoglycans in cancer
PI3K−Akt signaling pathway
EGFR tyrosine kinase inhibitor resistance
ErbB signaling pathway
Ras signaling pathway
Focal adhesion
Phospholipase D signaling pathway
Regulation of actin cytoskeleton
Chemokine signaling pathway
Gastric cancer
Non−small cell lung cancer
Growth hormone synthesis, secretion and action
Bacterial invasion of epithelial cells
Endocrine resistance
Fc epsilon RI signaling pathway
0.20 0.25 0.30 0.35
GeneRatio
Count
10.0
12.5
15.0
17.5
p.adjust
2.50e−11
5.00e−11
7.50e−11
1.00e−10
1.25e−10

D


CALD1_heatmap
CALD1
log(TPM/10+1)
3
2
1
0
STAD_GSE134520
0.01 0.02 0.02 0.01 1.9 2.68 0.71 0.01 0.01
STAD_GSE167297
0 0 0.01 0 0 0 0.6 1.67 0 0
CD8T
B
Plasma
DC
Mono/Macro
Mast
Endothelial
Fibroblasts
Myofibroblasts
Epithelial
Malignant
Gland mucous
Pit mucous

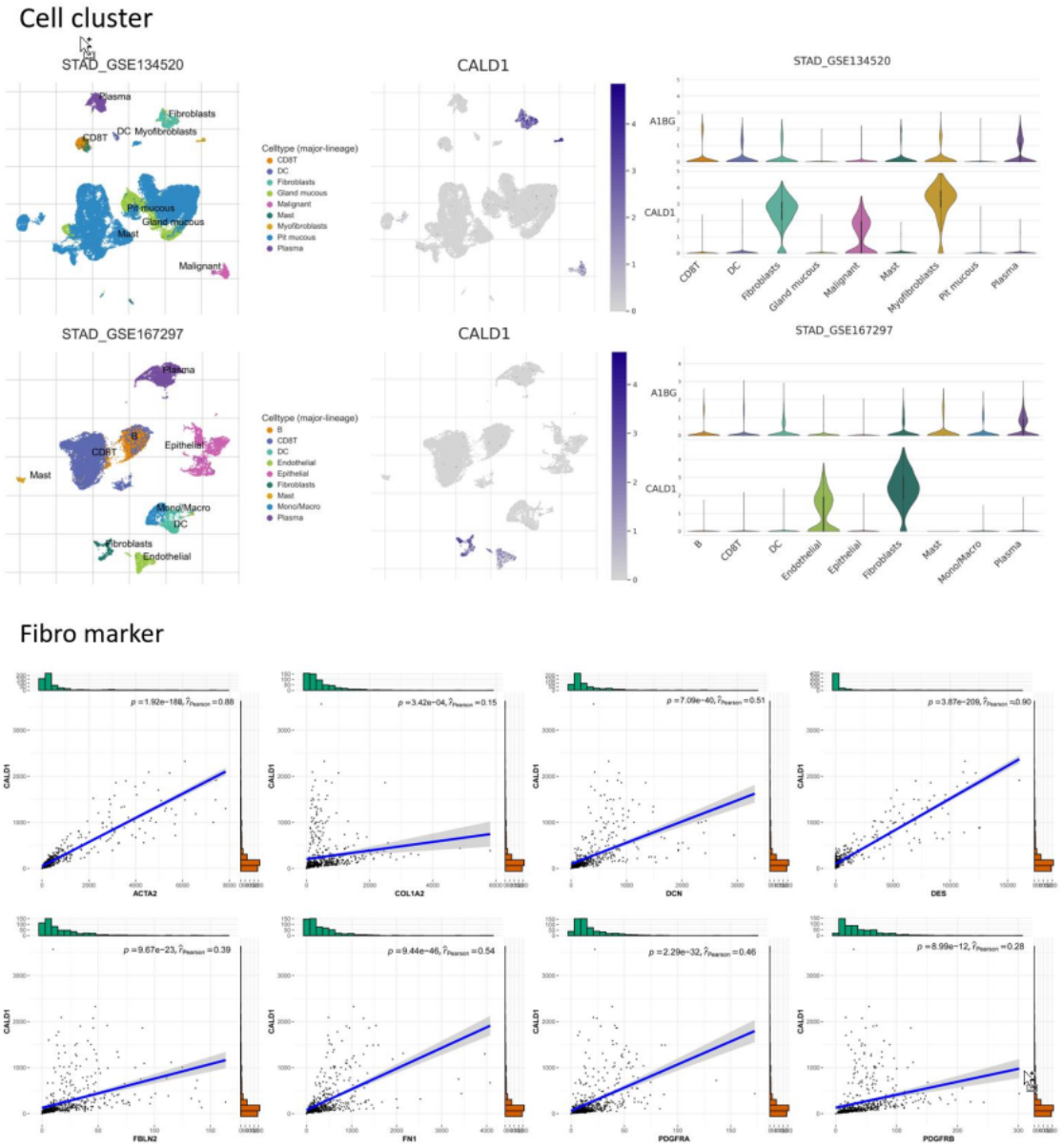
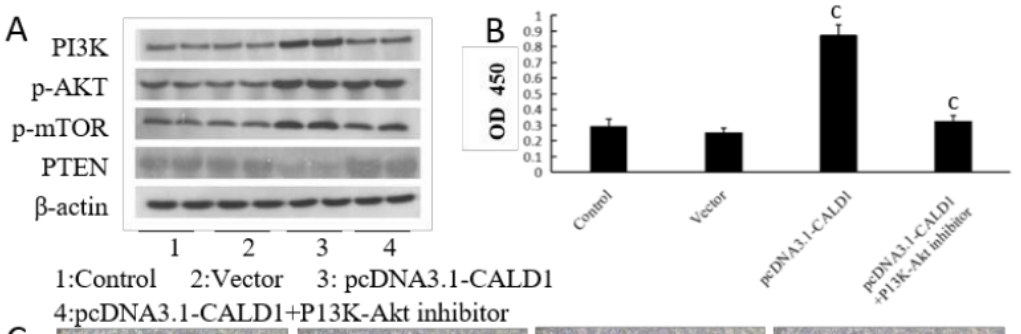
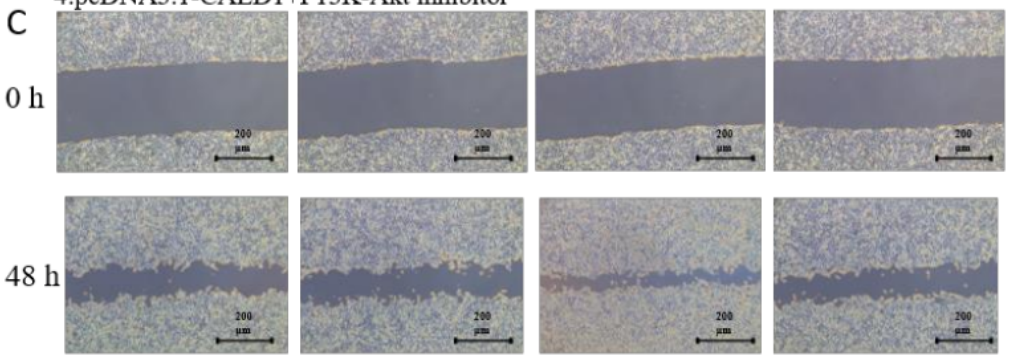


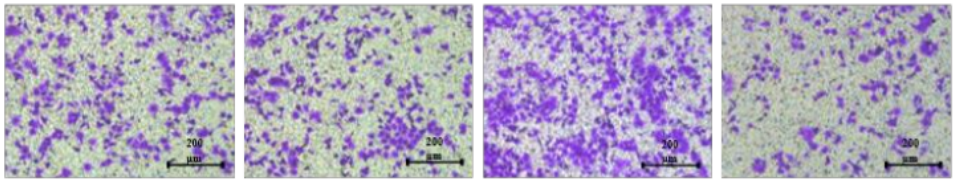
Figure 3 *CALD1* gene association with PI3K-Akt-mTOR and epithelial-mesenchymal transition pathways in gastric cancer: insights from bioinformatics and protein interaction studies. A: Bioinformatics results showed that the CALD1 gene was significantly associated with the expression of members in the PI3K-Akt-mTOR signalling pathway as well as the epithelial-mesenchymal transition signalling pathway in gastric cancer; B: The PPI network of CALD1 showed that it interacted with a variety

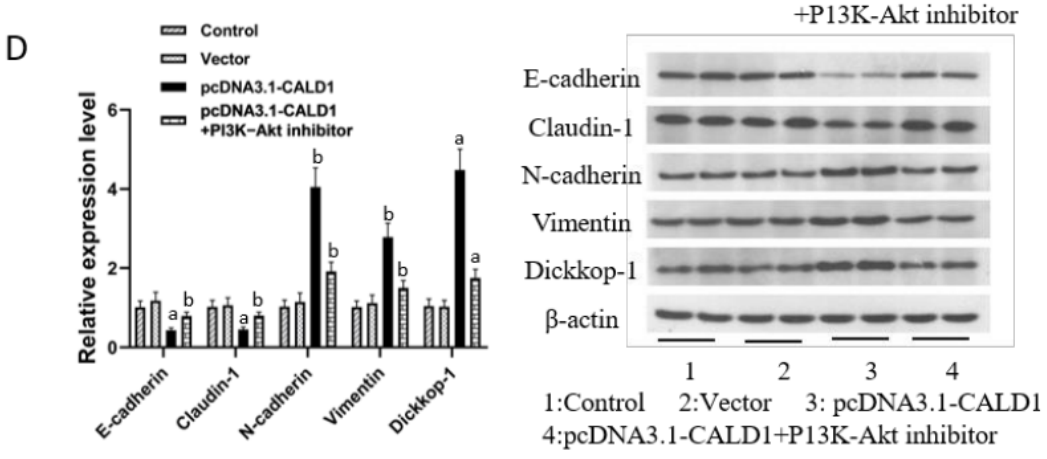
of proteins; C: GO and KEGG enrichment analyses showed that CALD1 was closely associated with the PI3K-Akt signaling pathway was closely related; D: In-depth analysis revealed that CALD1 was highly expressed in fibroblasts and was significantly positively correlated with the expression of fibroblast surface molecules.



1:Control 2:Vector 3: pcDNA3.1-CALD1
4:pcDNA3.1-CALD1+P13K-Akt inhibitor







1:Control 2:Vector 3: pcDNA3.1-CALD1
4:pcDNA3.1-CALD1+P13K-Akt inhibitor

Figure 4 Effects of CALD1 modulation and PI3K-Akt inhibition on tumor cell activity, migration, and EMT-related gene expression. A: Up-regulation of CALD1 enhanced the expression of PI3K, p-AKT, and p-mTOR members of the PI3K-Akt pathway, whereas PTEN expression was weakened; the addition of the inhibitor attenuated the expression of PI3K, p-AKT, and p-mTOR, while the expression of PTEN was enhanced; B: CCK-8 results showed that the addition of inhibitor CCK-8 results showed that the effect of CALD1 on tumour cell activity was weakened after adding the inhibitor (blank group-moderate activity, negative group-moderate activity, CALD1 overexpression group-high activity, CALD1 overexpression + inhibitor group-moderate activity or slightly high activity); C: Scratch and Transwell results showed that the effect of CALD1 on tumour cell migration and invasion was weakened after adding the inhibitor (blank group-moderate, negative group-moderate, CALD1 overexpression group-strong, CALD1 overexpression + inhibitor group-moderate or slightly strong); D: Correlative treatment of CALD1 and PI3K-Akt resulted in corresponding changes in EMT-related genes and proteins in AGS, MKN45. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

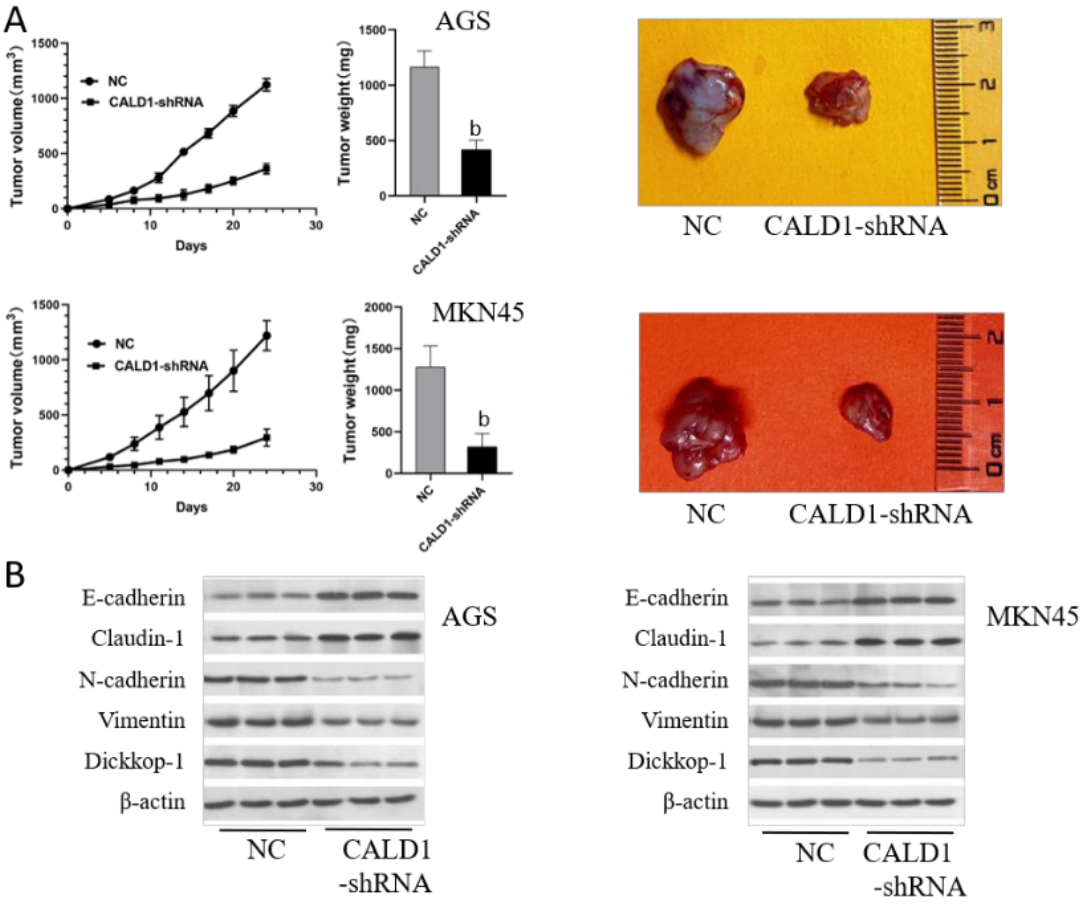


Figure 5 Impact of CALD1-shRNA on tumor growth and metastasis-related protein expression: results from animal experiments. A: Animal experiments the mean weight of transplanted tumours in the CALD1-shRNA-transfected group was significantly lower than that in the empty vector-transfected group ($P < 0.05$), with a delayed growth curve and smaller final subcutaneous tumours; B: Compared with the empty vector-transfected group, the expression of N-cadherin, Dickkop-1, and Vimentin was reduced, whereas that of Claudin-1 and E-cadherin expression increased. $^{b}P < 0.01$.

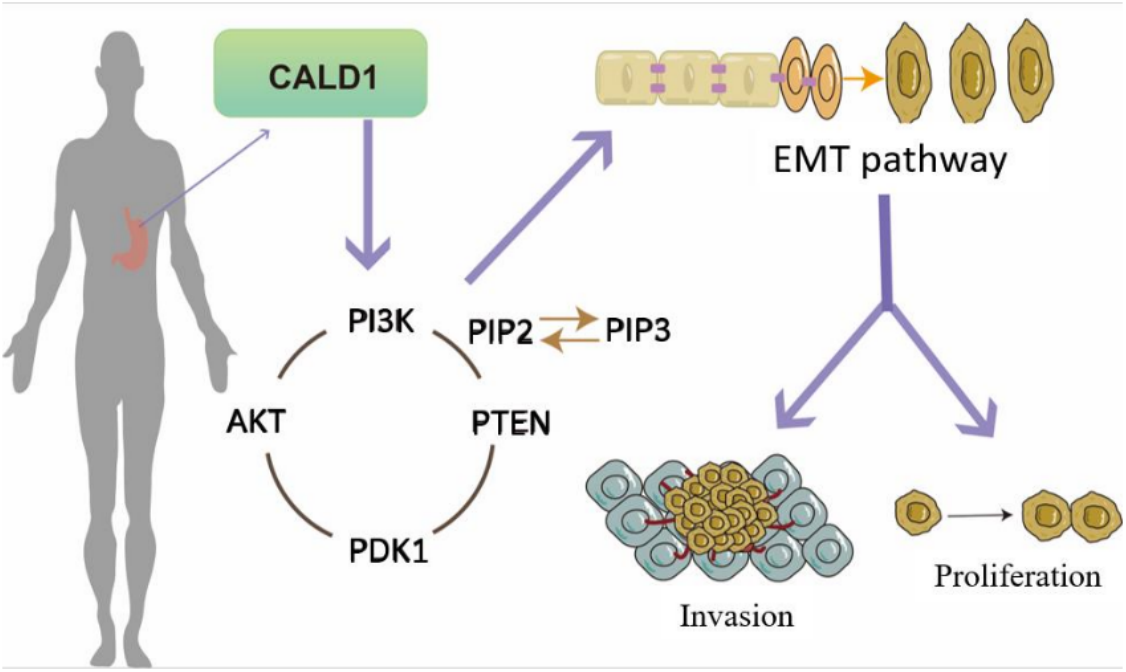


Figure 6 Pathway diagram. Simple pathway diagrams were made of the studies.

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