

Noggin Promotes Cell Proliferation Through Up-regulating EGFR/HER2 in Pancreatic Cancer Cells

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Abstract

Background/Aim: Noggin is a secreted antagonist of bone morphogenetic proteins (BMPs) and plays a key role in regulating various developmental and homeostatic biological processes. BMPs have been linked to the development of several types of cancers. However, the impact of Noggin on cellular functions and its role in pancreatic cancer remain unclear. This study aimed to investigate the role of Noggin in pancreatic cancer and its underlying molecular mechanisms.

Materials and Methods: Noggin expression in both normal and cancerous pancreatic tissues was assessed using both quantitative and conventional PCR methods, alongside an analysis of publicly available gene expression array datasets. Correlations between Noggin expression and patient survival, TNM staging, tumor/stroma subtypes, and the expression of other cancer-related genes were examined. The influence of Noggin on cellular functions was evaluated in pancreatic cancer cell lines Mia PaCa-2 and PANC-1, which were genetically modified to overexpress Noggin.

Results: Noggin expression was found to be significantly higher in tumor tissues compared to normal pancreatic tissues. Elevated Noggin expression was associated with shorter overall survival in patients. Overexpression of Noggin led to increased proliferation of pancreatic cancer cells. Furthermore, elevated levels of EGFR and HER2 proteins were observed in the PANC-1 and Mia PaCa-2 cell lines, respectively. Treatment with EGFR and HER2 inhibitors reduced Noggin-induced proliferation in these cell lines.

Conclusion: Noggin is overexpressed in pancreatic cancer tissues and is linked to poor patient survival. Noggin promotes the proliferation of pancreatic cancer cells by up-regulating EGFR and HER2.

Keywords: Noggin, EGFR/HER2, pancreatic cancer, disease progression.



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Introduction

Pancreatic ductal adenocarcinoma (PDAC) has shown a 1% increase in incidence each year, with over 60,000 new cases reported in the United States in 2021 (1-3). The overall 5-year survival rate remains low, ranging from 2% to 9% depending on the region (4). Early detection is challenging due to non-specific symptoms such as jaundice, abdominal or back pain, and weight loss (5).

BMPs are key regulators of cell functions in pancreatic cancer. A recent study published in 2022 highlighted the importance of BMP signaling in epithelial cells in PDAC. The BMP antagonist GREM1 regulates epithelial cell populations in PDAC, and BMP2 has been shown to inhibit the epithelial-to-mesenchymal transition (EMT) process in PDAC by regulating GREM1 (6). Knockdown studies of GREM1 treated with BMP2 and the BMP receptor inhibitor LDN-193189 in organoid models showed an up-regulation of phosphorylated Smad1/5/8, which was diminished by LDN-193189 in the GREM1-deleted group. BMP2 also induced GREM1 expression in EPCAM+ pancreatic cancer cells (6). Additionally, reduced expression of Smad4 and BMPR1A has been associated with increased cell proliferation and invasiveness in PDAC (7). Conversely, BMP8B suppressed cell proliferation and induced apoptosis in PANC-1 cells, while BMP8B knockdown enhanced proliferation in BxPC-3 pancreatic cancer cells (8). BMP4 and BMP5 promote invasion and migration in PANC-1 cells through the canonical Smad signaling pathway (9). GREM1, increased by TGF- β , inhibits the BMP2-Smad1/5 pathway in pancreatic cancer (10). As a BMP antagonist, Noggin has been shown to improve acute pancreatitis and disrupt homeostasis (11). Despite recent studies on BMPs, BMP receptors, and BMP antagonists in PDAC, the precise roles and mechanisms remain poorly understood.

Noggin, a homodimeric glycoprotein encoded by the *NOG* gene on chromosome 17, has a molecular mass of 26 kDa (12, 13). It is a natural BMP antagonist and plays a crucial role in tissue differentiation, bone formation, and fetal development (14). While BMP activity has been implicated in various cancers (15-18), the regulatory role

of Noggin in cancer is not well-established. Previous research indicates that Noggin promotes bone metastasis in breast cancer (19) and increases proliferation in human skin cancer cells in xenograft mouse models (20). In prostate cancer, Noggin acts as a tumor suppressor by inducing osteoblast bone cell growth (21). In gastrointestinal malignancies, lower levels of Noggin, along with BMP-responsive gene *ID1*, are associated with better survival outcomes in colorectal cancer patients by inducing BMP9 expression. BMP9, in turn, can up-regulate tumor suppressor genes in colorectal cancer (22). Our team recently reported that Noggin promotes proliferation in gastric cancer cells through EGFR up-regulation (23). Given the shared embryological origin of the stomach and pancreas from the embryological foregut (24), further investigation into the role of Noggin in pancreatic cancer is provoked. The present study aimed to investigate the role played by Noggin in pancreatic cancer.

Materials and Methods

Cell lines. Pancreatic cancer cell lines PANC-1 and Mia PaCa-2 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium-F12 (DMEM/F-12) with 10% fetal calf serum (FCS) (Sigma-Aldrich, Dorset, UK).

Tissue samples. Human tissue samples of pancreatic tumors and adjacent background tissues were obtained from the Beijing Cancer Hospital shortly after surgery (and stored at -80°C until use). This cohort consisted of 136 tumor tissue samples, adjacent pancreatic tissue samples ($n=137$). This collection of pancreatic tissue samples was reviewed and approved by Beijing Cancer Hospital Research Ethic Committee (MTA01062008), and informed consent was obtained from the patients.

RNA sequencing and gene array expression data of pancreatic cancer tissues. RNA sequencing data was downloaded from the following publicly available pancreatic adenocarcinoma

dataset of The Cancer Genome Atlas (TCGA-PAAD). GSE15471 database was downloaded from Gene Expression Omnibus.

The data TCGA-PAAD was derived from pancreatic cancer samples in The Cancer Genome Atlas (TCGA) (<https://portal.gdc.cancer.gov>) database. This RNA sequencing dataset comprises 185 cases of adenomas, adenocarcinomas, cystic, mucinous, serous neoplasms, ductal and lobular neoplasms, epithelial neoplasms and NOS types. The cohort was employed as a dataset for analysis of correlation between *NOG* and *MYC*, *CCND1*. GSE15471, a gene expression database (25, 26) contains 36 pancreatic ductal adenocarcinoma tumors and paired normal pancreatic tissue samples derived from the Clinical Institute Fundeni (ICF) using whole genome chips (GPL570 Affymetrix U133 Plus 2.0).

Noggin overexpression cell models. Lentiviral *Noggin* overexpression and control vectors were obtained from Cyagen Biosciences (Santa Clara, CA, USA). To generate lentiviral particles, HEK 293T cells (ATCC) were used for packaging. Lentiviral particles containing *Noggin* overexpression or control vector were transduced into cells, followed by selection with hygromycin (250 µg/ml). After selection, the transduced cells were maintained in a medium with 100 µg/ml hygromycin and used for further experiments.

RNA extraction and reverse transcription (RT). Total RNA was extracted from cultured cells using TRI reagent (Sigma-Aldrich T9424, Merck KGaA, Darmstadt, Germany). For reverse transcription, 2 µg of total RNA per sample was used to synthesize cDNA for subsequent determination using conventional PCR and quantitative real-time PCR (qPCR).

Quantitative PCR and conventional PCR. Quantitative PCR (qPCR) was performed to measure gene expression levels using a SYBR Green master mix (Sigma-Aldrich). Each qPCR reaction mixture contained a total volume of 10 µl, consisting of 5 µl master mix, 0.3 µl forward primer, 0.3 µl reverse

primer, 0.4 µl PCR-grade water, and 4 µl of a 1:8 diluted cDNA solution. A second qPCR kit (uniprob master mix, Thermo Fisher Scientific, Cambridge, UK) was used for following reactions. Each 10 µl reaction comprises 5µl of master mix, 0.3 µl of forward primer (10 µM), 0.3 µl of reverse primer with Z sequence (1 µM), 0.3 µl of uniprobe (10 µM) and 4 µl of a 1:8 diluted solution of cDNA were added. Each sample was run in triplicate for all qPCR reactions, which were carried out using the StepOne Plus™ software (Applied Biosystems StepOne Plus™). Conventional PCR was used to confirm *EGFR* and *HER2* expression in pancreatic cancer cell lines. PCR products were visualized under ultraviolet light using SYBR safe dye (Sigma-Aldrich). The primers used are listed as follows: *NOG* F-TACAGATGTGGCTGTGGTCCG, R-TGCACTCGGAAATGATGGGG; *GAPDH* F-GGCTGCTTTTAACTCTGGTA, R-GACTGTGGTCATGAGTCCTT; *ACTIN* F-GGACCTGACTGACTACCTCA, ZR-ACTGAACCTGACCGTACAAGCTTCTCCTCCTTAATGTCAC; *MYC* F-TGCTCCATGAGGAGACAC, ZR-ACTGAACCTGACCGTACATGATCCAGACTCTGACCTTT; *CCND1* F-CGGTGTCTACTTCAAATGT, ZR-ACTGAACCTGACCGTACACAAAGCGGTCCAGGTAGTTC; *P21* F-GCGATGGAACCTTCGACTTTG, ZR-ACTGAACCTGACCGTACAGGGCTTCTCTTTGGAGAAGAT; *P27* F-GGAATAAGGAAGCGACCTG, ZR-ACTGAACCTGACCGTACACACCGTCTGAAACATTTTCTTC; *HER2* F-CGTTTGAGTCATGCCCAAT, ZR-ACTGAACCTGACCGTACACCCACGTCCGTAGAAAGGTA.

In vitro cell growth assay. Three 96-well plates were seeded with transduced cells. For each well, 2000 cells were seeded on day 0. Plates were incubated for 1, 3, and 5 days. On the respective days, the medium was removed, and cells were fixed using 4% formalin. After a minimum of 30 min, formalin was removed, and cells were stained with crystal violet for 10 min. Excess stain was washed off, and the remaining stain was dissolved in 200 µl of 10% acetic acid. Absorbance was measured at 595 nm using a spectrophotometer (BIO-TEK, Elx800, London, UK).

In vitro migration assay (wounding healing assay). Cells were seeded and cultured to reach 100% confluence. A fine tip was used to create a wound of approximately 200 µm

in the cell monolayer. Cell migration to close the wound was recorded using an EVOS FL Auto microscope and analyzed using ImageJ software (ImageJ).

Invasion assay. Invasion was performed using 24-well plates with 8 µm culture inserts (Greiner Bio-One® ThinCert™, Cat No. 662638). Matrigel was prepared at 10 mg/ml in pre-cooled sterile water, and 100 µl aliquots of Matrigel were added to each insert. Next, 30,000 cells were added to each insert and cultured for up to 72 h at 37°C. Cells that invaded into the Matrigel and migrated through the membrane were fixed with 4% formaldehyde for 15 min and stained with 0.5% crystal violet for 10 min. The non-invasive cells and Matrigel were removed using a cotton swab. Invasive cells were photographed and counted using ImageJ software.

Western blot. The proteins were extracted using RIPA buffer to lyse cells and the protein concentration of the cell lysates was determined using a DC protein assay kit (Bio Rad Laboratories, Inc., Watford, UK). Protein (30 µg) samples were separated on an SDS PAGE gel and then transferred onto PVDF membranes. The blotted membranes were incubated at 4°C overnight with primary antibodies, anti-Noggin (NBP1-47881, 1:1,000); anti-EGFR (SC-71034, 1:1,000); anti-HER2 (SC-33684, 1:1,000); anti-GAPDH (SC-32233, 1:5,000); anti-MYC (SC-70465, 1:100); anti-CCND1 (SC-8396, 1:100); anti-ERK1/2 (SC-514302, 1:1,000); anti-p-ERK (SC-7383, 1:1,000); anti-Akt1 (SC-5298, 1:1,000); anti-P27 (SC-1641, 1:1,000). Then with corresponding peroxidase conjugated secondary antibodies (1:1000; Sigma Aldrich; Merck KGaA Inc. cat. no. A5278; A6154 and A8919) for 1h at room temperature. Protein bands were visualized using enhanced chemiluminescence (EZ ECL) kit (Biological Industries, Beit Haemek Ltd., Beit HaEmek, Israel).

Statistical analysis. Comparisons between the two groups were performed using the Student's *t*-test for normally distributed data or the Mann-Whitney *U*-test for non-normally distributed data. One-way ANOVA was used for

Table I. *Noggin* expression in pancreatic cancer from the clinical cohort.

	N	Median (IQR)	<i>p</i> -Value
Normal	137	390 (27-5,421)	
Tumor	136	6,680 (885-45,165)	<i>p</i> =0.00
Histological classification			
Adeno carcinoma	119	383 (36-5,332)	<i>p</i> =0.8
Ductal carcinoma	5	30 (12-895,005)	<i>p</i> =1
Others carcinoma	10	238 (1-52,256)	
Lymph node metastasis			
Negative	51	352 (12-5,443)	
Positive	73	427 (48-6,992)	<i>p</i> =0.82
Distant metastasis			
M0	126	326 (21-5,039)	
M1	10	3,896 (981-23,194)	<i>p</i> =0.04
T stage			
T1-2	21	110 (2-994)	
T3-4	95	427 (30-5,443)	<i>p</i> =0.3
TNM stage			
TNM1-2	105	352 (24-5,144)	
TNM3-4	17	1,321 (117-8,469)	<i>p</i> =0.27
Embolism			
No	73	302 (28-5,398)	
Yes	41	416 (5-3,759)	<i>p</i> =0.78

IQR: Interquartile range.

comparisons across multiple groups. Kaplan-Meier analysis and log-rank tests were used to calculate patient survival. Correlations between *Noggin* and other genes were assessed using Pearson's correlation test for normally distributed data and Spearman's correlation test for non-normally distributed data.

Results

***Noggin* expression in pancreatic cancer and its implication in disease progression and prognosis.** Quantitative analysis of *Noggin* transcripts in the clinical cohort from Beijing Cancer Hospital revealed significantly elevated *Noggin* expression in pancreatic tumor tissues (n=136) compared to adjacent normal pancreatic tissues (n=137), with a *p*-value <0.001 (Table I). This result was supported by conventional PCR analyses, showing higher levels of *Noggin* in pancreatic tumor tissues (Figure 1A). Similarly, an independent dataset (GSE15471), comprising 36

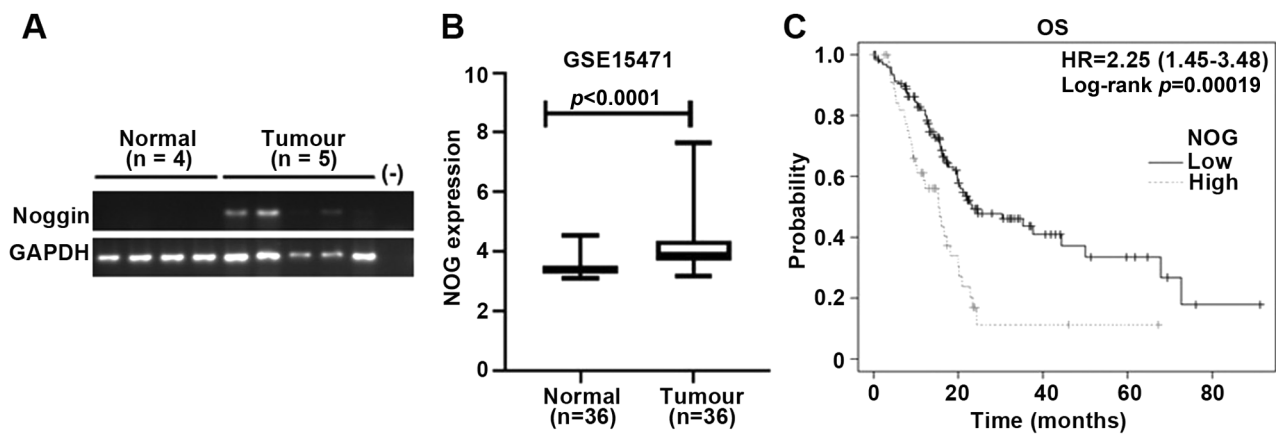


Figure 1. The expression of *Noggin* in pancreatic cancer. (A) Conventional PCR analysis shows higher *Noggin* expression in tumor tissues compared to normal tissues. (B) *Noggin* expression at the mRNA level in the GSE15471 cohort reveals significantly higher levels in tumor tissues ($n=36$) compared to paired normal tissues ($n=36$). (C) Kaplan-Meier survival analysis of high ($n=47$) and low ($n=130$) *Noggin* expression groups in the TCGA-PDAC cohort ($n=177$).

paired tumor and normal pancreatic tissue samples, demonstrated significantly increased *Noggin* expression in tumor tissues (Figure 1B).

Additionally, increased *Noggin* expression was observed in distant metastasis of pancreatic tumors (Table I). Survival analysis using the Kaplan-Meier method on the TCGA-PDAC database ($n=177$) revealed that patients with higher *Noggin* expression exhibited significantly shorter survival times compared to those with lower *Noggin* expression (Figure 1C).

Noggin promotes cell proliferation in pancreatic cancer cells. Overexpression of *Noggin* was established in both Mia PaCa-2 and PANC-1 cell lines using lentiviral vectors. This overexpression was confirmed at both mRNA and protein levels (Figure 2A-C). The overexpression of *Noggin* resulted in increased proliferation in both cell lines (Figure 2D, E). However, no significant changes in invasion and migration abilities were observed in either cell line (Figure 2F-I).

Noggin regulated *MYC*, *cyclin D1*, *P21* and *P27* in both Mia PaCa-2 and PANC-1 cell lines. *Noggin* overexpression resulted in increased levels of *MYC* and *cyclin D1* (*CCND1*) in both Mia PaCa-2 and PANC-1 cells, while levels of *P21* and *P27* were reduced (Figure 3A-D). In the TCGA

pancreatic cancer cohort, a positive correlation between *Noggin* and *CCND1* was observed, but no significant correlation was found between *Noggin* and *MYC* (Figure 3E, F). These findings were further verified at the protein level, where *CCND1* was up-regulated in Mia PaCa-2 cells overexpressing *Noggin* (Figure 3G).

Noggin promotes cell proliferation and mediates cell cycle by up-regulating *EGFR/HER2*. Given that *Noggin* promoted proliferation of gastric cancer cells through an up-regulation of *EGFR* (23), we explored the involvement of *EGFR* and *HER2* in pancreatic cancer cells. Moreover, the expression of *Noggin* was positively correlated with *EGFR* and *HER2* in the TCGA cohort ($n=177$, $p < 0.05$) (Figure 4A-B). Higher expression of *EGFR* and *HER2* was also observed in Mia PaCa-2 and PANC-1 cell lines at the mRNA level (Figure 4C). Further investigation of the activation of downstream signaling pathways, such as *ERK* and *AKT*, showed involvement of these pathways at the protein level (Figure 4D).

Treatment with *EGFR* inhibitor (gefitinib) and *HER2* inhibitor (CP724714) significantly decreased *Noggin*-induced proliferation in both cell lines, suggesting that *Noggin* promotes proliferation *via* *EGFR* and *HER2* pathways (Figure 4E-H).

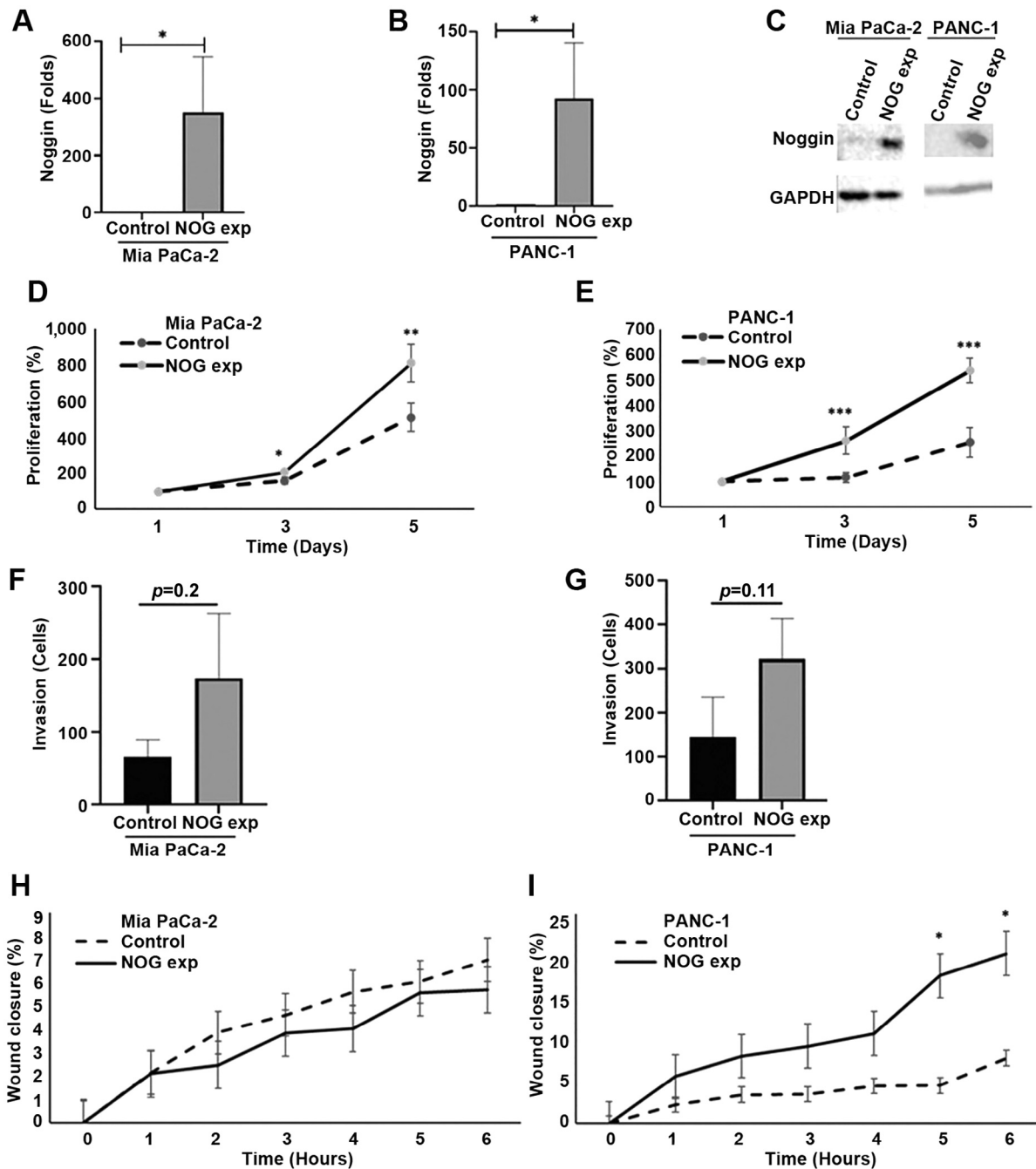


Figure 2. Overexpression of Noggin in pancreatic cancer cell lines. (A) Establishment of Noggin overexpression cell models in Mia PaCa-2 and (B) PANC-1 cells at the mRNA level. (C) Validation for both cell lines at the protein level using western blot. Effect of cellular functions of Noggin overexpression in pancreatic cancer cell lines. (D) Overexpressed Noggin in Mia PaCa-2 and (E) PANC-1 cell lines in vitro cell growth assays. Six repeats were performed for both cell lines on a 96-well plate. Three independent experiments were repeated. Growth percentage (%) = absorbance (Day3 or Day5) / absorbance (Day1) × 100. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars are standard deviation. (F-G) Transwell invasion assay was performed in both cell lines in triplicates. Cell numbers were counted using the Image J software. (H) Influence of Noggin overexpression on the migration in Mia PaCa-2 and (I) PANC-1 cells by wound healing assay in triplicates for each cell lines. Migration was examined by EVOS. Migration was captured automatically per hour for a duration of 6 h. The migration area was assayed by measuring the closing gap size. Error bars are standard deviation. All experiments were conducted three times for a validation of the findings.

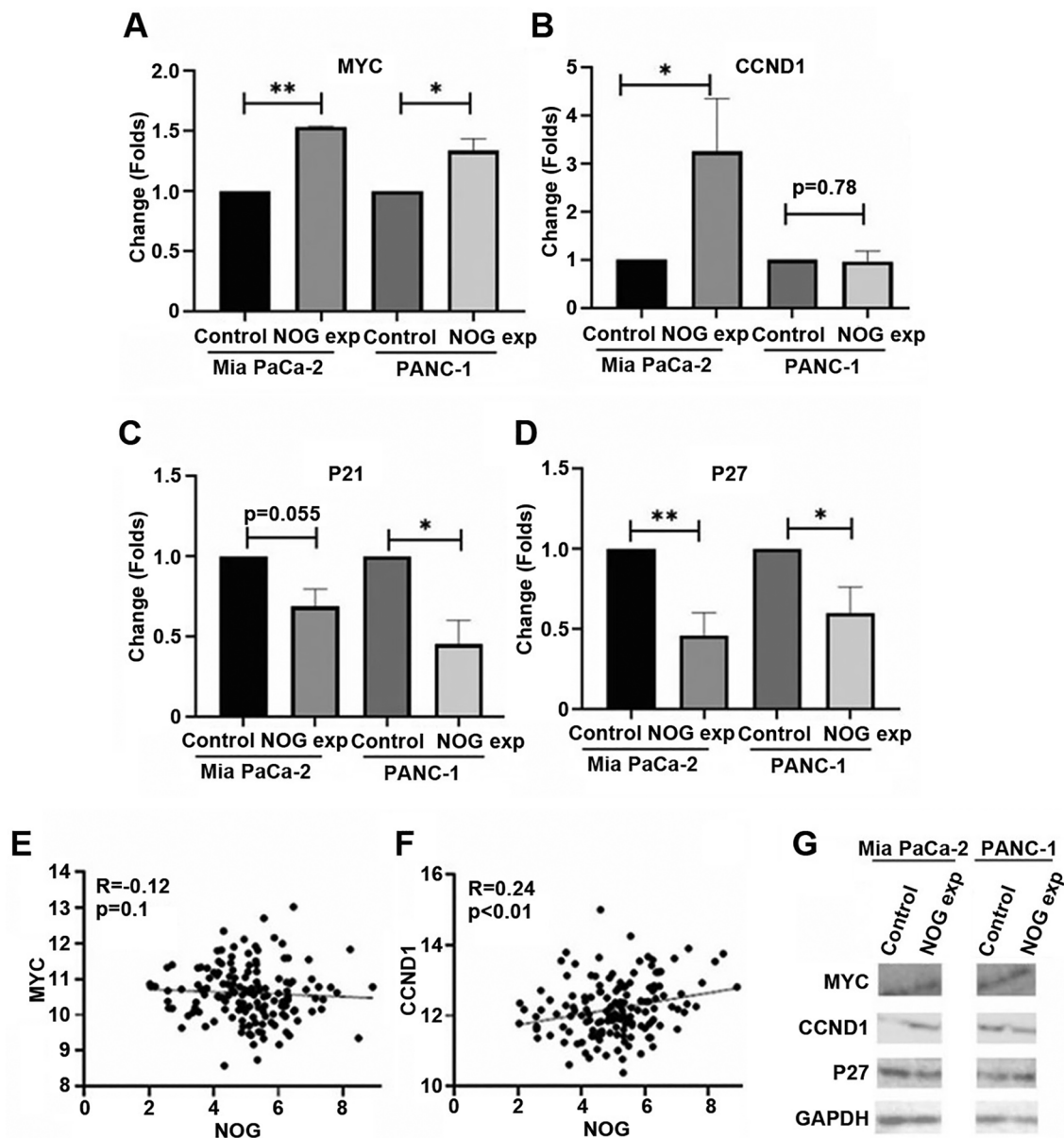


Figure 3. *Noggin* regulates the cell cycle in pancreatic cancer cell lines. Determination of cell cycle modulators: (A-D) MYC, CCND1, P21 and P27 expressions in Mia PaCa-2 and PANC-1 cells at mRNA level using QPCR in triplicates for each sample. (E-F) Scatter plots showed the correlation of *Noggin* with MYC and CCND1 in the TCGA cohort. (G) The expression of MYC, CCND1 and P27 were observed at the protein level using western blot. * $p < 0.05$, ** $p < 0.01$. Error bars are standard deviations.

Discussion

In this study, elevated *Noggin* expression was observed in human pancreatic tumor tissues compared to paired adjacent normal tissues. This finding is consistent with the

analysis of both the clinical cohort and the independent dataset (GSE15471). Furthermore, high levels of *Noggin* expression were significantly associated with poor overall survival in pancreatic cancer patients. Additional analyses revealed that *Noggin* expression was notably higher in

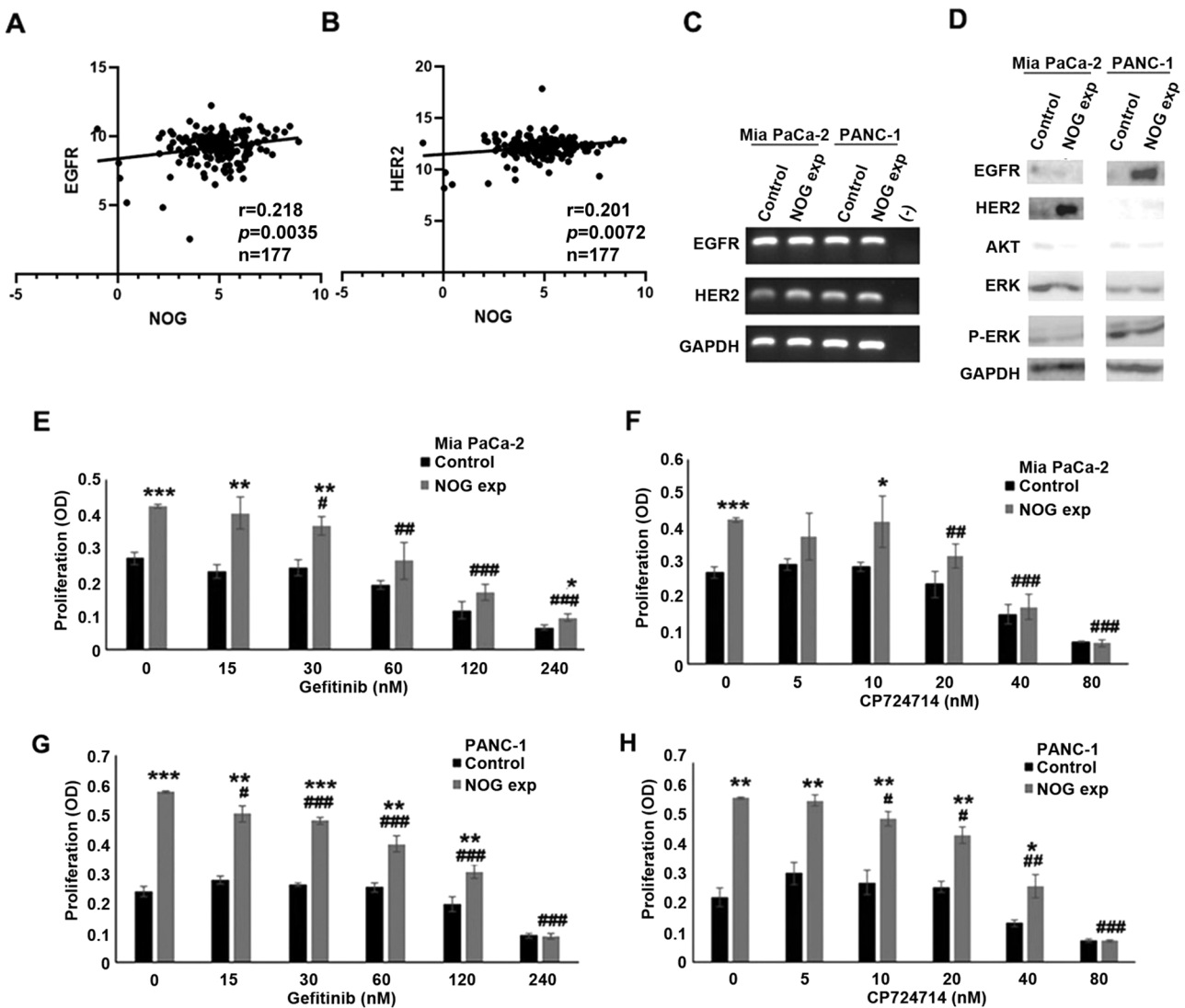


Figure 4. (A) Scatterplots exhibiting the expression of Noggin in correlation with EGFR and HER2 (B) in pancreatic cancer in the TCGA ($n=177$). (C) The expression of EGFR and HER2 was examined in both pancreatic cancer cell lines using normal PCR. (D) Protein expression and activation of EGFR, HER2, AKT and ERK were evaluated in both pancreatic cancer cell lines using western blot. Involvement of EGFR and HER2 in Noggin promoted cell proliferation was determined by using EGFR inhibitor (gefitinib) and HER2 inhibitor (CP724714) in (E-F) Mia PaCa-2 and (G-H) PANC-1 cells within 48 h with six repeats for each group of samples and treatment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

pancreatic tumors with distant metastasis, suggesting that Noggin may play a crucial role in the development and progression of pancreatic cancer.

Noggin has been implicated in various types of cancers. In non-small cell lung cancer (NSCLC) and prostate cancer, overexpression of Noggin led to reduced tumor growth in mouse models (27, 28). Dysregulation of Noggin has also

been shown to affect cell proliferation and migration in pituitary prolactinoma, prostate, and lung cancer cell lines (29-31). Similarly, Noggin has been shown to play an essential role in gastrointestinal cancers. Research from our lab previously demonstrated that Noggin promotes cell proliferation in gastric cancer cells by up-regulating EGFR expression (23). In the present study, Noggin was

found to promote cell proliferation in pancreatic cancer cell lines as well. This effect was confirmed through cell growth assays conducted using both Mia PaCa-2 and PANC-1 cell models with Noggin overexpression. Additionally, overexpression of Noggin was associated with up-regulated levels of MYC and cyclin D1 (CCND1) and down-regulated levels of cell cycle inhibitors P21 and P27 in both Mia PaCa-2 and PANC-1 cell lines.

Members of the ERBB receptor family, including EGFR (HER1) and HER2, are known to play a role in pancreatic ductal adenocarcinoma (PDAC). EGFR is overexpressed in more than 40% of pancreatic cancers (32-34), while HER2 expression has been detected in approximately 20% of PDAC cases through immunohistochemistry (IHC) analysis (34, 35). Additionally, inhibition of EGFR signaling has been shown to reduce cell growth in pancreatic tumor tissues (36). The expression of EGFR and HER2 was also evaluated in a cohort of 72 pancreatic tumor tissues using IHC, and it was found that a selective inhibitor of EGFR and HER2, lapatinib, might improve survival outcomes in EGFR-positive pancreatic ductal adenocarcinoma (37). Our study further supported these findings by demonstrating that Noggin-induced cell growth could be inhibited by treatment with EGFR inhibitor (gefitinib) and HER2 inhibitor (CP724714) in a concentration-dependent manner.

Several signaling pathways are involved in the malignancy and tumorigenesis of pancreatic cancer, such as the mitogen-activated protein kinase (MAPK), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and Notch pathways (38). In this study, increased expression of EGFR and HER2 was confirmed in both Mia PaCa-2 and PANC-1 cell lines. Interestingly, Noggin overexpression resulted in up-regulation of EGFR in PANC-1 cells, while an increased level of HER2 was observed only in Noggin-overexpressing Mia PaCa-2 cells. These results suggest that Noggin might promote cell proliferation through distinct mechanisms involving EGFR and HER2 pathways in different pancreatic cancer cell lines. Leucine-rich alpha-2-glycoprotein 1 (LRG-1), which is known to be critical in pancreatic cancer progression, has been shown to promote proliferation and

invasiveness of pancreatic cancer cells *in vitro* and *in vivo* via the EGFR/MAPK/P38 pathways (38). Although the activation of ERK and AKT was assessed in our study, no significant changes were observed among the different cell lines, indicating that Noggin may influence other downstream pathways in pancreatic cancer cells.

In summary, high Noggin expression is associated with poor prognosis in pancreatic cancer patients and promotes tumor cell proliferation through up-regulation of EGFR and HER2. These findings suggest that Noggin could be a potential prognostic marker and therapeutic target in pancreatic cancer. Further studies are warranted to explore the clinical implications of targeting Noggin in pancreatic cancer therapies.

Conflicts of Interest

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

Authors' Contributions

LY and WJ conceived the study. ML, CH sourced the clinical dataset, ML, LY and KC did the experiments, ML and AB did the statistical analysis. ML prepared the draft, which was revised by LY and WJ. All Authors have read and agreed to the published version of the manuscript. All Authors contributed to the article.

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Artificial Intelligence (AI) Disclosure

During the preparation of this manuscript, a large language model (ChatGPT, OpenAI) was used solely for language editing and stylistic improvements in select

paragraphs. No sections involving the generation, analysis, or interpretation of research data were produced by generative AI. All scientific content was created and verified by the authors. Furthermore, no figures or visual data were generated or modified using generative AI or machine learning-based image enhancement tools.

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