Regulation of Pancreatic Tumor Cell Proliferation and Chemoresistance by the Histone Methyltransferase Enhancer of Zeste Homologue 2

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Abstract

Purpose: Enhancer of zeste homologue 2 (EZH2), a histone methyltransferase, plays a key role in transcriptional repression through chromatin remodeling. Our objectives were to determine the expression pattern of EZH2 and to assess the anticancer effect of EZH2 depletion in pancreatic cancer cells.

Experimental Design: Immunohistochemistry and cytosolic/nuclear fractionation were done to determine the expression pattern of EZH2 in normal pancreas and human pancreatic tumors. We used RNA interference, Western blotting, reverse transcription-PCR, and chromatin immunoprecipitation to study the effect of EZH2 depletion on pancreatic cancer cell proliferation and survival.

Results: We detected nuclear overexpression of EZH2 in pancreatic cancer cell lines and in 71 of 104 (68%) cases of human pancreatic adenocarcinomas. EZH2 nuclear accumulation was more frequent in poorly differentiated pancreatic adenocarcinomas (31 of 34 cases; $P < 0.001$). We found that genetic depletion of EZH2 results in reexpression of $p27^{Kip1}$ and decreased pancreatic cancer cell proliferation. Moreover, we showed that EZH2 depletion sensitized pancreatic cancer cells to doxorubicin and gemcitabine, which leads to a significant induction of apoptosis, suggesting that the combination of EZH2 inhibitors and standard chemotherapy could be a superior potential treatment for pancreatic cancer.

Conclusions: Our results show nuclear accumulation of EZH2 as a hallmark of poorly differentiated pancreatic adenocarcinoma; identify the tumor suppressor $p27^{Kip1}$ as a new target gene of EZH2; show that EZH2 nuclear overexpression contributes to pancreatic cancer cell proliferation; and suggest EZH2 as a potential therapeutic target for the treatment of pancreatic cancer.

Pancreatic cancer, the fourth leading cause of cancer deaths in the United States, kills more than 30,000 Americans every year. Not only is there no cure but there are also no effective treatments for this disease. The 5-year survival rate for people with pancreatic cancer is 3% (1).

Deciphering the cancer epigenetic code promises to dramatically change our understanding of pancreatic cancer, leading to the discovery of new oncomarkers and targets to develop superior diagnostic and treatment strategies. Recent evidence suggests that epigenetic silencing of tumor suppressor genes plays a significant role in tumor development (2). Epigenetic control of gene expression occurs in two main ways: either the DNA itself is chemically altered (usually methylation of cytosines by DNA methyltransferases) or the histones, proteins that package DNA into chromatin (the main component of chromosomes), are modified (2). Posttranslational modification of histones determines whether the chromatin is tightly packed, leading to gene repression, or relaxed, leading to gene expression (2). The Polycomb group (PcG) proteins repress gene expression through the formation of multiple, unique complexes, which ultimately lead to the methylation of both histones and DNA (2–4). Specifically, PcG complexes containing the histone methyltransferase enhancer of zeste homologue 2 (EZH2) silence chromatin via methylation of histone H3-lysine 27 (H3-K27; ref. 3). Thus, EZH2 is thought to have the potential to silence genes that could be involved in tumorigenesis. Indeed, *EZH2* gene amplification was first reported in hematologic malignancies (5, 6), and EZH2-catalyzed methylation of H3-K27 is frequently associated with PcG-mediated silencing of tumor suppressor genes, such as *hMLH1*, *ARHI*, and *RASSF1A* in ovarian cancer (7) and *E-cadherin* in gastric cancer (8).

Whereas recent studies suggest EZH2 overexpression as an important factor of prostate (9) and breast (10) carcinoma progression, the expression pattern of EZH2 in human pancreatic cancer and the role of EZH2 in the proliferation, survival, and chemoresistance of pancreatic cancer cells remain

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Translational Relevance

Our study provides novel insight into the mechanism by which the histone methyltransferase enhancer of zeste homologue 2 (EZH2) contributes to both pancreatic tumor cell proliferation and chemoresistance. We have found EZH2 nuclear accumulation in most pancreatic adenocarcinomas, whereas EZH2 expression is undetectable in normal pancreatic tissue, thus identifying EZH2 as a potential diagnostic oncomarker of pancreatic cancer. Immunohistochemical detection of EZH2 nuclear accumulation in biopsy specimens obtained by pancreatic needle or brush biopsy might be a useful method for pathologic diagnosis of pancreatic cancer. We identify EZH2 as a positive regulator of pancreatic cancer proliferation. Moreover, we show for the first time that RNA interference–mediated gene silencing of EZH2 sensitizes pancreatic cancer cells to doxorubicin and gemcitabine resulting in a significant induction of apoptosis, thus identifying EZH2 as a protein that contributes to pancreatic cancer chemoresistance. Taken together, our findings suggest that the combination of EZH2 inhibitors and standard chemotherapy could be a superior treatment for human pancreatic cancer.

unknown. Here, we show aberrant EZH2 nuclear overexpression in pancreatic cancer cell lines and most pancreatic adenocarcinomas. We show that depletion of EZH2 results in reexpression of the $p27^{Kip1}$ tumor suppressor and decreased pancreatic cancer cell proliferation. Furthermore, for the first time, we show that EZH2 plays a role in pancreatic cancer chemoresistance, suggesting that combination of EZH2 inhibitors with standard chemotherapy could be a superior potential therapy for pancreatic cancer.

Materials and Methods

Reagents, plasmids, and cells. All chemicals were obtained from Sigma. An EZH2-specific targeting short hairpin RNA vector was generated as previously described (11) using the target sequence 5′-GACTCTGAATGCAGTTGCT-3′. All cell lines were obtained from the American Type Culture Collection.

Immunohistochemistry. The Institutional Review Board at the Mayo Clinic approved all studies carried out on human specimens. EZH2 antibody was obtained from BD Biosciences PharMingen. EZH2 immunostaining was done on 104 resected primary pancreatic adenocarcinoma specimens. Two pathologists (A.V.O. and D.D.B.) independently reviewed all cases and classified the tumors as well differentiated (n = 20), moderately differentiated (n = 50), or poorly differentiated (n = 34). For each case, the most representative section reflecting the major features of the primary pancreatic tumor (i.e., histologic type) was selected for immunohistochemical examination to determine the expression of EZH2. Immunohistochemical staining was done as described (12). EZH2 nuclear accumulation was defined as positive staining of more than 10% of cancer cell nuclei throughout the tumor regardless of cytoplasmic staining.

Immunoblot analysis and antibodies. For immunoblots, cells were lysed as described previously (13). Nuclear/cytosolic fractionation was done by the Dignam method (14). Protein sample concentration was quantified and equal amount (50 μg of whole, nuclear, or cytosolic protein extract) of protein was loaded in each well of SDS-polyacrylamide gel. Cell or tissue extracts were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane (PVDF), and probed as indicated. Antibodies for immunoblot analysis were obtained from the following suppliers: EZH2, poly(ADP-ribose) polymerase, and $p27^{Kip1}$ from BD Biosciences PharMingen; IκBα from Santa Cruz Biotechnology; trimethyl-K27 histone H3 and histone H3 from Upstate; Cu/Zn superoxide dismutase from Stressgen; and β-actin from Novus. Bound antibodies were detected as previously described.

Reverse transcription-PCR. Expression of mRNA of $p27^{Kip1}$ and MYT1 was determined by reverse transcription-PCR. cDNA was generated from 2 μg of total RNA by reverse transcription using a Reverse Transcription System Kit (Promega). To quantify the expression in each cDNA sample, the target was amplified by PCR in parallel with an internal control, glyceraldehyde-3-phosphate dehydrogenase. Increased expression of MYT1, EZH2 target gene, was used as a positive control of EZH2 depletion. The following primers were used: $p27^{Kip1}$ upstream, 5′-AGGATGTC AGCGGGAGCCGG-3′, and downstream, 5′-CTTCTTGGGCGTCTGCTCCA-3′; MYT1 upstream, 5′-ATCCCAGTCC-CAGCCTACTT-3′ and downstream, 5′-GTCTCCCTCCTG GACCTCAC-3′. Primer pairs that detect glyceraldehyde-3-phosphate dehydrogenase were used as previously described (13). Amplification was done by using a thermal cycling program with varying numbers of cycles for $p27^{Kip1}$ (22 cycles) and MYT1 (22 cycles) in which each cycle consisted of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min. Amplification for glyceraldehyde-3-phosphate dehydrogenase (22 cycles) was done following a cycling program, in which each cycle consisted of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min. All PCR products were subjected to electrophoresis through a native 8% polyacrylamide gel and were visualized by staining with ethidium bromide.

Chromatin immunoprecipitation assay. Panc04.03 cells were cross-linked with formaldehyde for 15 min at 25°C, harvested in SDS lysis buffer (Upstate Biotechnology), and sheared to fragment DNA (500-1,000 bp). Samples were then immunoprecipitated with an agarose-conjugated EZH2, trimethyl-histone H3 (Lys^{27}) antibody, acetyl-histone H3 (Lys^{14}) antibody, or rabbit control IgG at 4°C overnight. Antibodies for chromatin immunoprecipitation analysis were obtained from the following suppliers: trimethyl-H3-K27 and acetyl-H4-K20 from Upstate Biotechnology, and EZH2 from BD PharMingen. Following immunoprecipitation, samples were washed and eluted using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) according to the manufacturer's instructions. Cross-links were removed at 65°C for 6 h, and immunoprecipitated DNA was purified by phenol/chloroform extraction and ethanol precipitation. In the immunoprecipitated samples, 212 bp of the $p27^{Kip1}$ promoter and 154 bp of the $p27^{Kip1}$ exon 1 were detected by PCR. PCR products were separated on a native 10% polyacrylamide gel and visualized by staining with ethidium bromide.

Statistical analysis. Data were analyzed using the Prism software package (GraphPad, Inc.). Associations between EZH2 nuclear accumulation and degree of tumor differentiation were analyzed by Fisher's exact test for 2 × 2 contingency tables or by χ^2 test for larger tables. Two-sided tests were used. The χ^2 critical value for 0.05 probability level is 3.841; $\chi^2 > 3.841$ and $P < 0.05$ were considered to indicate statistical significance.

Results

EZH2 accumulates in the nuclei of pancreatic cancer cells. Us-Using immunohistochemical staining for EZH2, we found weak cytoplasmic EZH2 expression in normal human pancreatic ductal and acinar cells (Fig. 1A). Nuclear accumulation of EZH2 was found in well-, moderately, and poorly differentiated adenocarcinomas in 11 of 20 (55%), 29 of 50 (58%), and 31 of 34 (91%) cases, respectively (Fig. 1B-F). EZH2 nuclear accumulation was significantly associated with dedifferentiation of tumors (χ^2 test for independence, 12.296; P = 0.0021). It was significantly more frequent in poorly differentiated

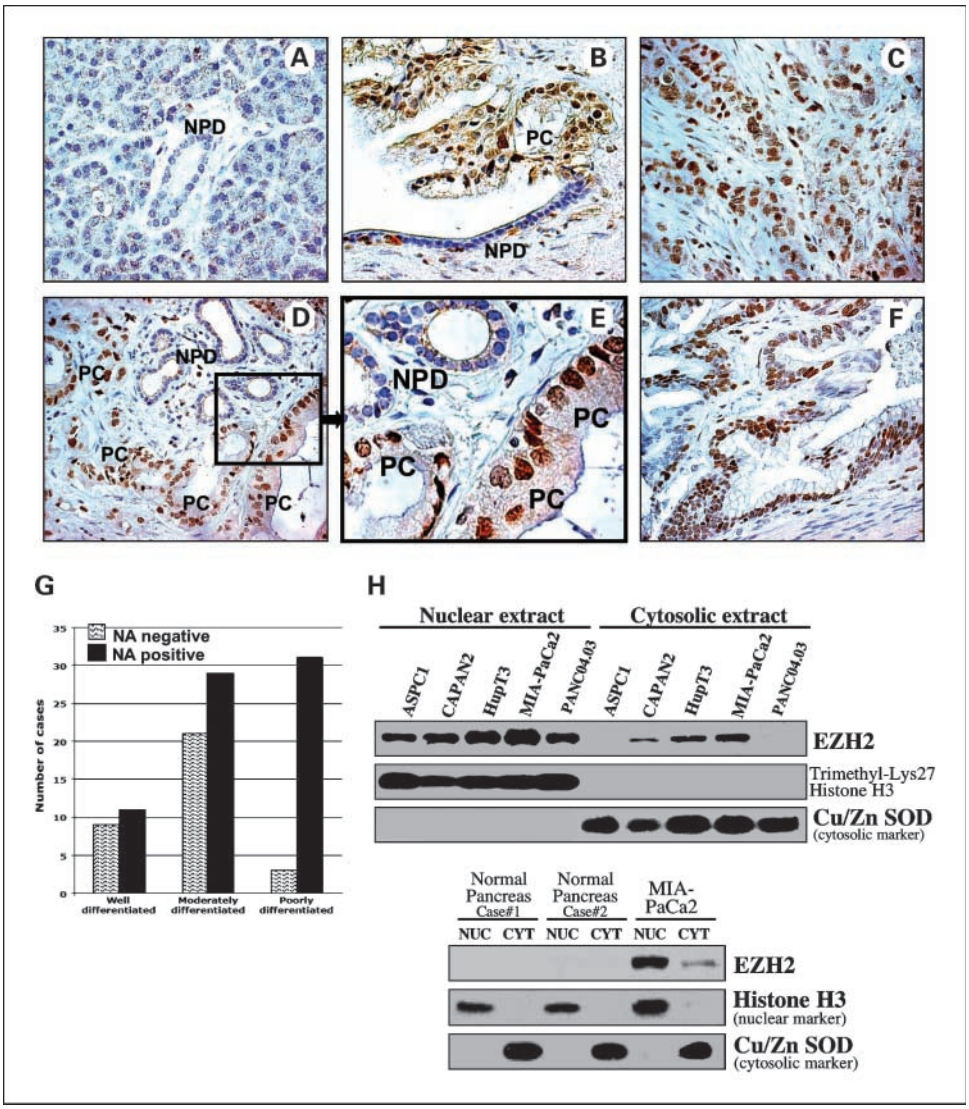

Fig. 1. EZH2 is overexpressed and accumulated in the nuclei of pancreatic cancer cells. *A* to *F*, immunohistochemical analysis of EZH2 expression and localization in normal human pancreas (*A*) and pancreatic adenocarcinoma specimens (*B* – *F*). *A*, normal pancreatic duct (*NPD*) is indicated. *B*, malignant pancreatic duct shows nuclear accumulation of EZH2, whereas adjacent normal pancreatic ductal cells show no nuclear EZH2 staining. PC, pancreatic cancer cells. *C*, EZH2 nuclear accumulation in a poorly differentiated pancreatic adenocarcinoma. *D*, nuclear accumulation of EZH2 found in cancer cells of moderately differentiated pancreatic adenocarcinoma but not in adjacent normal pancreatic ductal cells. *E*, higher magnification of the delineated inset in *D*. *F*, nuclear accumulation of EZH2 in cancer cells of well-differentiated pancreatic adenocarcinoma. *G*, distribution of EZH2 staining patterns in pancreatic carcinomas. NA, nuclear accumulation. *H*, equivalent amounts (50 μg) of nuclear and cytosolic proteins isolated from the indicated pancreatic cancer cell lines and normal human pancreas tissue were separated by SDS-PAGE and immunoblotted.

adenocarcinomas [Fig. 1G; $P = 0.0003$; odds ratio, 7.750 (95% confidence interval, 2.162-27.775); relative risk, 1.614 (95% confidence interval, 1.280-2.034)]. Our results suggest that nuclear accumulation of EZH2 is associated with the loss of pancreatic cancer differentiation.

Using nuclear/cytosolic fractionation, we found nuclear expression of EZH2 in pancreatic cancer cell lines ASPC1, CAPAN2, HupT3, MIA-PaCa2, and Panc04.03 (Fig. 1H, *top*). In contrast to these findings, EZH2 expression was not detected in normal human pancreatic cells (Fig. 1H, *bottom*). Thus, nuclear overexpression of EZH2 seems to be a feature of pancreatic cancer cells.

Suppression of EZH2 inhibits pancreatic cancer cell proliferation. Our data identify overexpression of EZH2 in pancreatic cancer cells, but its role in regulating pancreatic cancer cell proliferation and survival is unknown. To address this question, we depleted EZH2 using short hairpin RNA interference (Fig. 2A) and assayed the ability of cells to proliferate using a well-established colorimetric technique [3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay] and trypan blue staining. We observe that transfection of an EZH2-targeting construct (shEZH2) into MIA-PaCa2 and Panc04.03 pancreatic cancer cells resulted in decreased proliferation in these pancreatic tumor cell lines as compared with vector control (shVector)–transfected cells (Fig. 2B and C). We did not observe an increased apoptosis in EZH2-depleted cancer cells (data not shown). These results suggest that EZH2 participates in the regulation of signaling cascades that positively influence pancreatic cancer cell proliferation.

Depletion of EZH2 leads to reexpression of the $p27^{Kip1}$ tumor suppressor gene in pancreatic cancer cells. Using reverse transcription-PCR and immunoblotting, we screened for tumor suppressor genes, which might be reexpressed on depletion of EZH2 in pancreatic cancer cells. Because EZH2 suppression leads to decreased pancreatic cancer cell proliferation, we analyzed the expression levels of the cyclin-dependent kinase inhibitors p15, p16, p21, p27, and p57 in pancreatic cancer cells, as well as the known EZH2 target gene MYT1 as a control for EZH2 suppression. Suppression of EZH2 in MIA-PaCa2 and Panc04.03 resulted in an increase in MYT1 gene expression consistent with the known role of EZH2 in the repression of

MYT1 gene transcription. However, although EZH2 depletion resulted in the reexpression of multiple cyclin-dependent kinase inhibitor genes in several pancreatic cancer cell lines tested (data not shown), only $p27^{Kip1}$ was uniformly and significantly reexpressed at both the transcriptional and protein levels after depletion of EZH2 in all of the pancreatic cancer cell lines examined (Fig. 3A and B, and data not shown). Whereas it has been suggested that loss of $p27^{Kip1}$ protein in human cancer is not related to its gene alterations but mainly resulting from increased degradation of the $p27^{Kip1}$ protein (15, 16), we found that treatment of pancreatic cancer cells with the proteasome inhibitor MG132 did not increase the level of $p27^{Kip1}$ protein expression (Fig. 3C). Our results identify the tumor suppressor $p27^{Kip1}$ as a new target gene of EZH2 in human pancreatic cancer.

EZH2 depletion affects repressive chromatin status, leading to reexpression of $p27^{Kip1}$ in pancreatic cancer cells. EZH2-mediated recruitment of DNA methyltransferases to target gene promoters is one mechanism by which EZH2 can repress gene transcription (4). To investigate whether $p27^{Kip1}$ may be inactivated by DNA hypermethylation through EZH2-mediated recruitment of DNA methyltransferases to the *$p27^{Kip1}$* gene in pancreatic cancer cells, we examined the CpG islands located in both the promoter and exon 1 of the *$p27^{Kip1}$* gene for hypermethylation by methylation-specific PCR. We did not detect hypermethylation at the $p27^{Kip1}$ promoter or exon 1 in Panc04.03 cancer cells (data not shown), suggesting that reexpression of $p27^{Kip1}$ in EZH2-depleted pancreatic cancer cells is not dependent on the DNA methylation status of the $p27^{Kip1}$ promoter or exon 1.

Opening of chromatin to allow transcription factors to gain access to gene promoters and regulate gene transcription is one of the major functions of histone modifications (i.e., phosphorylation, acetylation, and methylation; ref. 2). PcG complexes containing the histone methyltransferase EZH2 silence chromatin via methylation of histone H3-K27 (3). Methylation H3-K27 is a known feature associated with repressive chromatin status and epigenetic gene silencing (3). To evaluate the possibility that suppression of EZH2 alters its ability to silence the *$p27^{Kip1}$* gene through transcriptional repression, we used chromatin immunoprecipitation to assess EZH2 binding and H3-K27 methylation at the $p27^{Kip1}$ promoter and exon 1 in Panc04.03 cancer cells transfected with the shEZH2 or control vector. We found that EZH2 is bound to $p27^{Kip1}$ at exon 1, but not within the $p27^{Kip1}$ promoter region (Fig. 3D). Consistently, EZH2-depleted cancer cells showed a significant decrease in methylation of H3-K27 bound to exon 1 of $p27^{Kip1}$, but not to its promoter (Fig. 3E, *top*). It has also been shown that PcG (EED/EZH2) mediated the repression of gene activity by interacting with histone deacetylases (17). Acetylation of H3-K14 is associated with open, actively transcribed genomic regions (18). We found that EZH2-depleted cancer cells show increased acetylation of H3-K14, a marker of active transcription, at exon 1 of $p27^{Kip1}$ (Fig. 3E, *bottom*). These results suggest that EZH2 regulates $p27^{Kip1}$ expression through the maintenance of inactive chromatin at the *$p27^{Kip1}$* gene.

Nuclear overexpression of EZH2 is significantly associated with loss of p27 expression in human pancreatic adenocarcinomas. To test the hypothesis whether EZH2 nuclear overexpression leads to loss of $p27^{Kip1}$ expression in human pancreatic tumors *in vivo*, we have done immunohistochemical analysis of EZH2 and $p27^{Kip1}$ expression in a series of human pancreatic adenocarcinomas. Using a pair of serial sections, we detected nuclear accumulation of EZH2 and loss of $p27^{Kip1}$ expression in pancreatic cancer cells, whereas nuclear accumulation of $p27^{Kip1}$, but not EZH2, was observed in benign pancreatic lesions (Fig. 3F and G). We found aberrant EZH2 nuclear accumulation and loss of $p27^{Kip1}$ expression in 61% and 67% of pancreatic carcinomas, respectively (Fig. 3F and G). Loss of $p27^{Kip1}$ expression was more frequently found in tumors with nuclear accumulation of EZH2 ($P < 0.001$; Fig. 3F and G) than in tumors without nuclear accumulation of EZH2. Moreover, simultaneous EZH2 nuclear accumulation and loss of $p27^{Kip1}$ expression are associated with the loss of pancreatic cancer differentiation ($P < 0.001$). Consistent with our *in vitro* data, our *in vivo* results show that aberrant nuclear overexpression of EZH2 is significantly associated with loss of $p27^{Kip1}$ expression in human pancreatic cancer cells.

EZH2 depletion affects pancreatic cancer chemoresistance. Pancreatic cancer is less chemosensitive than other common solid malignancies, with partial response to chemotherapy of <10% (19). The role of chemotherapy pancreatic cancer treatment is limited because pancreatic cancer rapidly develops a chemoresistance leading to fatal patient outcome (19, 20). With any chemotherapy regimen, average survival rates are between 3 and 6 months (20). Thus, a better understanding of tumor chemoresistance enables the discovery of novel targets to

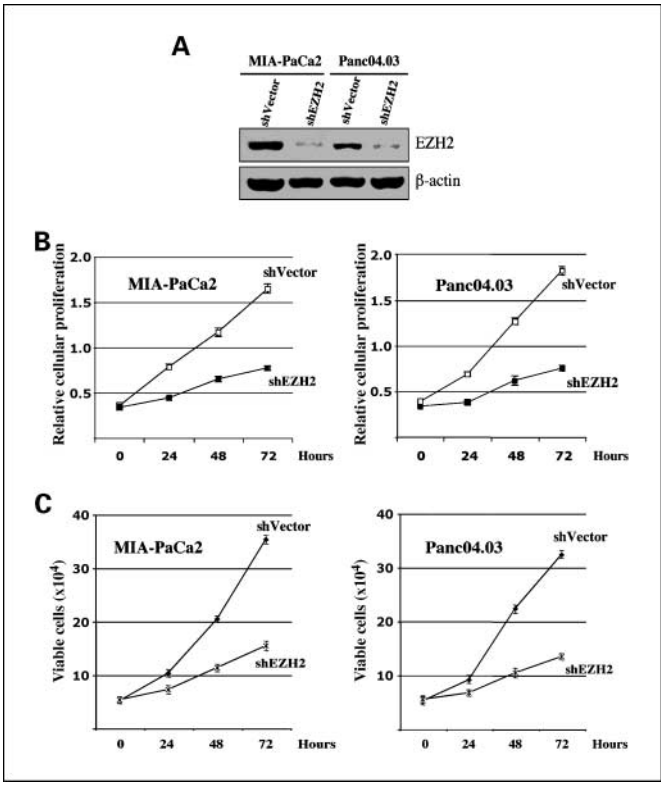

Fig. 2. Suppression of EZH2 inhibits pancreatic cancer cell proliferation. MIA-PaCa2 and Panc04.03 pancreatic cancer cells were transfected with a control vector (*shVector*) or the shEZH2 silencing vector (*shEZH2*). *A,* at 48 h posttransfection, the cell pellet was collected and protein was obtained. Cell lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with antibodies to indicated proteins. *B,* relative cell viability was measured by colorimetric 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay at the indicated times as described (13). *C,* numbers of viable cells were counted by the trypan blue exclusion assay as described (30).

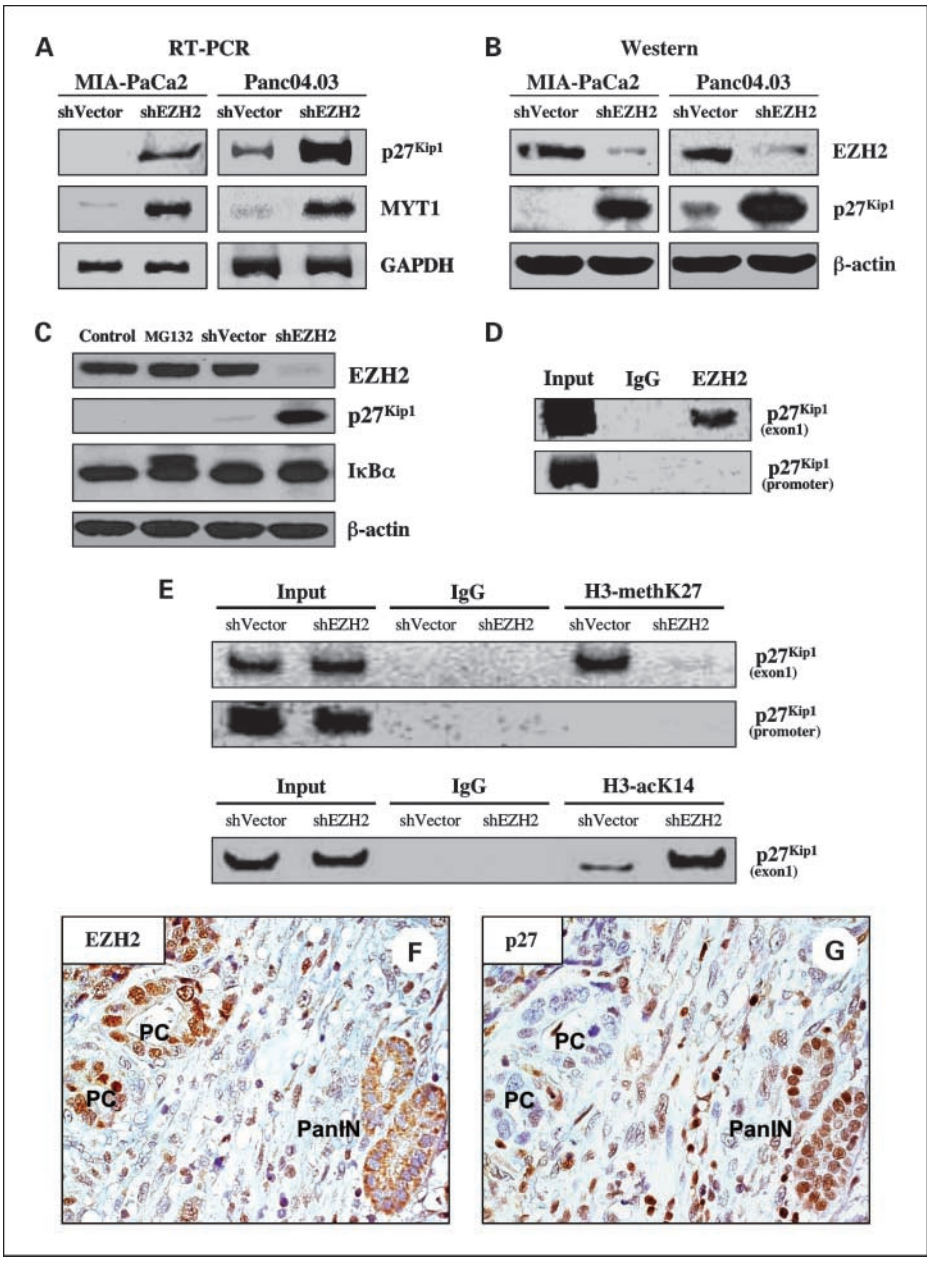

Fig. 3. Depletion of EZH2 leads to reexpression of $p27^{Kip1}$ in pancreatic cancer cells. MIA-PaCa2 and Panc04.03 pancreatic cancer cells were transfected with a control vector or the shEZH2 silencing vector. *A,* at 48 h posttransfection, the cell pellet was collected; mRNA was obtained; and reverse transcription-PCR was done, as described in Materials and Methods. Increased expression of MYT1, a known EZH2 target gene, was used as a positive control. *B,* at 48 h posttransfection, the cell pellet was collected and protein was obtained. Cell lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with antibodies to indicated proteins. *C,* at 48 h posttransfection, Panc04.03 cell pellet was collected. Panc04.03 cancer cells were treated with DMSO (control) or MG132 (10 μmol/L) for 6 h. Cell lysates were prepared, separated by SDS-PAGE (50 μg/well), transferred onto PVDF membrane, and immunoblotted as indicated. An increase in IκBα expression was used as a positive marker of MG132 treatment. *D,* binding of EZH2 to the promoter or exon 1 of *$p27^{Kip1}$* gene was assayed in Panc04.03 cancer cells by chromatin immunoprecipitation. *E,* Panc04.03 cancer cells were transfected with a control vector or the shEZH2 silencing vector. At 48 h posttransfection, genomic chromatin fragments were immunoprecipitated with trimethyl-H3-K27 and acetyl-H3-K14 at the $p27^{Kip1}$ promoter or exon 1. PCR analysis on input chromatin (*first two lanes*) confirmed that equal chromatin amounts were used for chromatin immunoprecipitation. *F* and *G,* immunohistochemical staining of a pair of serial sections from the same pancreatic tumor shows nuclear localization of EZH2 (*F*) and loss of $p27^{Kip1}$ expression (*G*) in the same cancer cells, whereas absence of EZH2 nuclear staining but $p27^{Kip1}$ nuclear accumulation has been observed in benign pancreatic lesions (*G* and *F*). PanIN, pancreatic intraepithelial neoplasia.

develop a superior pancreatic cancer therapy. Gemcitabine, a deoxycytidine nucleoside analogue, is currently the standard chemotherapeutic agent used in the treatment of metastatic pancreatic cancer (20). Doxorubicin, an anthracycline antibiotic, has been used as a chemotherapeutic agent in a variety of carcinomas including pancreatic cancer. Interestingly, we found that treatment of Panc04.03 and SU86.86 cells with either doxorubicin or gemcitabine induces the nuclear accumulation of EZH2 in pancreatic cancer cells (Fig. 4A). Moreover, although treatment of pancreatic cancer cells with doxorubicin (1 μmol/L) or gemcitabine (100 nmol/L) did not induce apoptosis in control transfected cells, EZH2 depletion sensitized cancer cells to doxorubicin and gemcitabine, leading to a significant increase in apoptosis of pancreatic cancer cells (Fig. 4B and C). Our results suggest that nuclear overexpression of EZH2 might participate in pancreatic cancer chemoresistance and that the combination of EZH2 inhibitors with gemcitabine or doxorubicin could be superior to conventional therapy for pancreatic cancer.

Discussion

Epigenetic silencing of tumor suppressor genes can prevent programmed cell death and lead to uncontrolled proliferation in human tumors (2, 18). Recent studies implicate EZH2, a member of the PcG regulators of gene activity, in the pathogenesis of human cancer, including prostate (9), breast (10), gastric (21), and bladder (22) carcinomas, whereas the role of EZH2 in pancreatic cancer remains unknown.

In the present study, we show aberrant nuclear overexpression of EZH2 in pancreatic cancer cell lines and in 71 of 104 (68%) cases of human pancreatic adenocarcinomas, thus

identifying EZH2 as a potential oncomarker in pancreatic cancer. We find that EZH2 nuclear accumulation is strongly associated with poorly differentiated pancreatic adenocarcinoma, suggesting EZH2 as one of the molecular determinants of pancreatic tumor dedifferentiation. Our findings are supported by other studies showing overexpression of EZH2 in advanced prostate (9) and breast (10) cancers, whereas nuclear accumulation of EZH2 was more frequently found in poorly differentiated breast carcinomas (23).

We identify EZH2 as a positive regulator of pancreatic cancer cell proliferation. We find that EZH2 depletion inhibits the proliferation, but not the survival, of pancreatic cancer cells. Our data are in agreement with another study showing that EZH2 knockdown in prostate cancer cells results in decreased proliferation with no effect on cancer cell survival (9). A previous study has also shown that EZH2 contributes to the proliferation of breast cancer cells (23). Our findings of EZH2 nuclear accumulation in most cases (31 of 34) of poorly differentiated pancreatic adenocarcinoma, a highly proliferating tumor fraction, support the hypothesis that EZH2 could play an important role in pancreatic cancer proliferation.

However, it is still unclear how EZH2 controls cancer cell proliferation. To address this question, we performed screening of numerous tumor suppressor genes, which might be reexpressed on depletion of EZH2 in pancreatic cancer cells. We identified the $p27^{Kip1}$ tumor suppressor as a novel EZH2-regulated gene. We are unaware of any previous report that has assessed the role of EZH2 in the regulation of $p27^{Kip1}$ expression. The cyclin-dependent kinase inhibitor $p27^{Kip1}$ is a tumor suppressor that regulates cell cycle by binding to cyclin-dependent kinases (24). Reduced $p27^{Kip1}$ expression is correlated with poor disease outcome in cases of lung, colon, prostate, ovary, and breast carcinomas (25). A decrease or loss of $p27^{Kip1}$ expression is frequently observed in human pancreatic cancer (26). Although $p27^{Kip1}$ expression seems to be an important prognostic factor in numerous human malignancies, it has been suggested that loss of $p27^{Kip1}$ protein is not related to its gene alterations but mainly resulting from increased degradation of the $p27^{Kip1}$ protein (15, 16). However, we found that treatment of pancreatic cancer cells with the proteasome inhibitor MG132 did not increase the level of $p27^{Kip1}$ protein expression, whereas $p27^{Kip1}$ expression was significantly up-regulated in EZH2-depleted pancreatic cancer cells at both the transcriptional and protein levels. Our findings suggest that EZH2 nuclear overexpression contributes to epigenetic silencing of *$p27^{Kip1}$* gene that leads to down-regulation of $p27^{Kip1}$ mRNA and protein levels in pancreatic cancer cells. To confirm our *in vitro* results showing reexpression of $p27^{Kip1}$ in EZH2-depleted cancer cells, we show *in vivo* that EZH2 nuclear accumulation is significantly associated with loss of $p27^{Kip1}$ expression in human pancreatic adenocarcinomas. These results support a speculation that EZH2 might

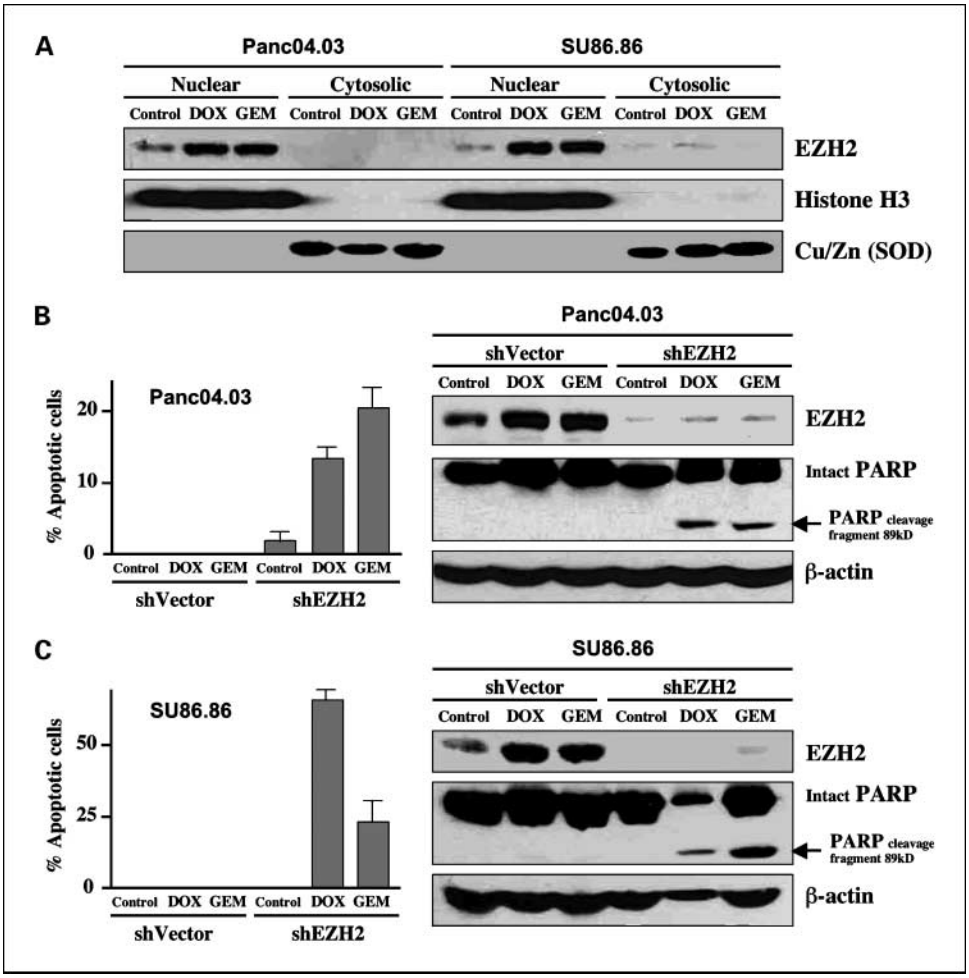

Fig. 4. EZH2 depletion affects pancreatic cancer chemoresistance. *A,* Panc04.03 and SU86.86 pancreatic cancer cells were treated with DW (control), 1 μmol/L doxorubicin (*DOX*), or 100 nmol/L gemcitabine (*GEM*) for 24 h. Nuclear and cytosolic fractions were prepared, separated by SDS-PAGE (50 μg/well), transferred onto PVDF membrane, and probed with the indicated antibodies. *B* and *C,* Panc04.03 (*B*) and SU86.86 (*C*) pancreatic cancer cells were treated with DW (control), doxorubicin (1 μmol/L), or gemcitabine (100 nmol/L) for 48 h. Whole cell lysates were prepared, separated by SDS-PAGE (50 μg/well), transferred onto PVDF membrane, and immunoblotted as indicated. The percentage of apoptotic cells was determined by Hoechst staining as previously described (31). Columns, mean; bars, SD.

contribute to pancreatic cancer cell proliferation by epigenetic silencing of tumor suppressor genes, in part through suppression of $p27^{Kip1}$. Whether EZH2 could suppress $p27^{Kip1}$ expression in a similar manner in other human malignancies remains to be determined.

Pancreatic cancer is one of the most drug-resistant tumors (19, 20). Our results identify EZH2 as an important factor of pancreatic cancer cell chemoresistance. We found that EZH2 depletion sensitized cancer cells to doxorubicin and gemcitabine, leading to a significant decrease in survival of pancreatic cancer cells. Our findings are supported by another study showing that loss of EZH2-mediated methylation of H3-K27 resensitizes ovarian cancer cells to cisplatin (7). It has also been shown that trimethylation of H3-K27 is an important prognostic indicator for clinical outcome in patients with pancreatic cancer (27). Increased sensitivity of EZH2-depleted pancreatic cancer cells to doxorubicin and gemcitabine seems to be mediated, in part, by changes in gene expression. We found that EZH2 downregulates the expression of $p27^{Kip1}$ tumor suppressor in pancreatic cancer cells. Although the speculative role of 27^{Kip1} in pancreatic cancer chemoresistance remains to be investigated, reduced $p27^{Kip1}$ correlates with poor survival after platinum-based chemotherapy for non–small-cell lung and ovarian carcinomas (28, 29). Because EZH2-mediated H3-K27 methylation is a mark of heterochromatin, it is also possible that loss of chromatin compaction could allow increased DNA damage at lower doxorubicin and gemcitabine doses, leading to a decreased survival in EZH2-depleted pancreatic cancer cells.

In summary, our study provides novel information on the mechanisms underlying the growth and chemoresistance of pancreatic cancer, and urges a demand for the development of pharmacologic inhibitor of EZH2 as a potential anticancer agent for the chemotherapy of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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