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Bmi-1 is related to proliferation, survival and poor prognosis in pancreatic cancer

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B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) is a member of the polycomb group of transcriptional repressors. Until now, its expression and functional significance in pancreatic carcinogenesis was unknown. In the present study, we demonstrated that expression of BMI1 was markedly up-regulated in pancreatic cancer cell lines and surgically resected cancer specimens. In addition, BMI1 expression levels correlated positively with the presence of lymph node metastases and negatively with patient survival rates, suggesting a role for BMI1 in the progression of pancreatic cancer. Furthermore, stable down-regulation of BMI1 suppressed cell growth, delayed the G1/S transition, and enhanced the susceptibility of different pancreatic cell lines to apoptosis following expression of a lentiviral-mediated shRNA targeted for BMI1. Expression of the short-hairpin RNA also correlated with the up-regulation of p21 and Bax and the down-regulation of cyclin D1, cyclin-dependent kinase (CDK)-2 and -4, Bcl-2, and phospho-Akt. Finally, growth suppression following BMI1 depletion was confirmed in a nude mouse model. In conclusion, our findings indicate that BMI1 plays an important role in the late progression of pancreatic cancer and may represent a novel therapeutic target for the treatment of pancreatic cancer. (*Cancer Sci* 2010; 101: 1754–1760)

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal diseases worldwide.[1–3] Despite recent progress in its diagnosis and treatment, patient prognosis still remains unsatisfactory and unpredictable due to the invasive phenotype, early metastasis, and profound resistance to existing chemo-radiation therapies associated with PDAC.[4] Consequently, most patients who undergo surgery do not survive more than a year, resulting in a cumulative 5-year survival rate of 1–4%.[5] Thus, identification of key molecules or pathways specifically expressed in PDAC that are essential for the growth and survival of cancer cells may provide novel therapeutic targets and ultimately lead to improved survival.

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) is a member of the polycomb group of transcriptional repressors and was originally identified as an oncogene associated with c-*myc* in the development of murine lymphoma.[6,7] Subsequent studies have identified a role for BMI1 in embryonic development and have demonstrated that BMI1 is essential for the maintenance and self-renewal of both hematopoietic and neural stem cells.[8–10] Additional work has revealed that BMI1 potentially regulates a diverse number of cellular processes including cell cycle progression, apoptosis, and senescence, as well as immortalization through repression of the inhibitor of cyclin-dependent kinase 4a(INK4a)/alternative reading frame (ARF) locus encoding $p16^{INK4a}$/$p19^{ARF}$ and/or induction of telomerase.[11–13] More recently, BMI1 has been associated with tumor development and progression.[14] For example, BMI1 alone or in coordination with other molecules has been shown to induce malignant transformation and tumor initiation in several types of non-tumorigenic immortalized cell lines via $p16^{Ink4a}$-dependent/independent mechanisms.[15–17] Up-regulation of BMI1 can also promote cell proliferation and prevent apoptosis triggered by extraneous injury,[12,18] whereas BMI1 depletion reduces cell viability and promotes cancer-specific cell death.[19] Moreover, BMI1 can repress p53-dependent apoptosis signaling and the production of reactive oxygen species to confer radioresistance in nasopharyngeal carcinoma.[20] Because elevated expression of BMI1 is associated with cancer cell proliferation, vascular infiltration, and lymph node metastases, BMI1 is a factor associated with an adverse clinical prognosis.[21,22] Together, these data strongly suggest that BMI1 may be specifically activated in tumor cells to affect cellular growth and/or survival. Indeed, aberrant expression of BMI1 has been reported in a variety of human tumors[23–27] including PDAC.[28,29] However, the clinical significance and functional role of BMI1 *in vivo* remain unclear in PDAC carcinogenesis. Therefore, the prognostic value of changes in BMI1 expression in PDAC, and the use of BMI1 as a potential target for cancer therapy, warrants further research.

In the current study, we investigated the clinical significance of BMI1 expression in PDAC patients and the correlation between BMI1 expression and clinicopathological characteristics by immunohistochemistry. The effects of BMI1 depletion on the growth and survival of PDAC cells both *in vitro* and *in vivo* were also investigated, with the goal of clarifying potential molecular mechanisms of BMI1.

Materials and Methods

Tissue specimens and immunohistochemical analysis. Seventy-two PDAC tissues and five normal pancreatic tissues were obtained from the Department of Hepatobiliary Surgery, Xijing Hospital of the Fourth Military Medical University (Xi'an, China), between January 2001 and September 2003. Patient consent was obtained for the collection of specimens, and all study protocols were approved by the Ethics Committee for Clinical Research of the Fourth Military Medical University.

Immunohistochemistry protocols were performed as previously described.[30] In brief, slides were incubated with an anti-BMI1 monoclonal antibody (1:50; clone F6; Upstate, Lake Placid, NY, USA) followed by incubation with a horseradish peroxidase-conjugated antimouse secondary antibody (1:200; Dako, Glostrup, Denmark). Antibody binding was visualized using 3,3′-diaminobenzidine and counterstained with hematoxylin. Negative control sections were incubated with PBS instead of the primary antibody. Immunostained results were independently evaluated by two pathologists who were blinded with respect to the clinical and histopathologic features. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1)

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nuclear accumulation was quantified as a percentage of the total number of nuclei detected in at least 4–5 random high power fields (×400) in each section. Cases with >10% of cells staining for BMI1 were scored as positive samples.[21]

Cell culture. Five human pancreatic cancer cell lines, PANC-1, BxPC-3, ASPC-1, CFPAC-1, and SW1990, and a normal pancreas cell line, HPDE6c7, were maintained at 37°C and 5% CO_2 in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin (100 U mL/L), and streptomycin (0.1 mg mL/L).

Lentiviral vector construction and transduction. Potential target sequences for RNA interference were chosen using the BLOCK-iT RNAi Designer. Specific sequences for targeting BMI1 (accession no. NM_005180.5) included the following: si1 (5′-GGAGGAGGTGAATGATAAA-3′) and si2 (5′-AGAATTGGTTTCTTGGAAA-3′). An invalid RNAi sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as a negative control. Short-hairpin RNAs (shRNAs) were synthesized and cloned into the pENTR/U6 entry plasmid, and shRNAs were then recombined into the pLenti6/BLOCK-iT expression vector. Inserted sequences were checked with restriction digests and sequencing. Lentivirus production, amplification, and titer were performed using BLOCK-iT Lentiviral RNAi Expression System protocols (catalog no. K4944-00; Invitrogen). CFPAC-1 and PANC-1 cells were transduced with recombinant lentiviruses harboring BMI1 shRNA or control lentiviral vectors, and pooled stable clones were selected using blasticidin (Sigma, St. Louis, MO, USA). Clones expressing BMI1si1 and BMI1si2 as well as control clones were obtained for both the PANC-1 and CFPAC cell lines.

Cell growth assays. Cell growth was assessed using MTT [3-(4,5-dimethylthiahiazol-2-y1)-2,5-diphenytetrazolium bromide; Sigma] assays. Briefly, 2000 cells/well were seeded into 96-well plates, and cell viability was assayed on Days 1–4 following seeding. Absorption values were determined using an enzyme-linked immunosorbent assay reader (Dasit, Milan, Italy) at 490 nm.

Colony formation assay and *ex vivo* tumor inhibition. Approximately 3×10^2 cells from each stably transfected cell line were plated in six-well dishes. After 2 weeks, cells were fixed with 20% methanol and stained with 1% crystal violet. Colonies consisting of more than 50 cells were counted per well, and each experiment was performed in triplicate. For *in vivo* tumorigenicity assays, stably transfected cells (3×10^6 cells/mouse) were prepared in a 0.1 mL volume of PBS and were subcutaneously injected into 5-week-old male BALB/c nude mice (four mice per group; BiKai, Shanghai, China). Four weeks after the initial implantation, mice were sacrificed, and the tumors were recovered. Tumor size was monitored using micrometer calipers, and tumor volume was calculated as follows: Volume = length × $(\text{width})^2/2$. Animal experiments were performed in full accordance with the Medicine Institutional Guidelines of the Fourth Military Medical University.

Analysis of cell cycle and apoptosis. Both cell cycle distribution and spontaneous apoptosis events were detected using a FACScaliber II sorter and Cell Quest FACS system (BD Biosciences, San Jose, CA, USA). To analyze cell-cycle distribution, cells were synchronized using serum starvation for 24 h and stimulated with complete medium for 24 h before being harvested. Cells were fixed with 70% ethanol at 4°C overnight, washed twice with PBS, and resuspended in Staining Solution (50 μg/mL propidium iodide, 1 mg/mL RNase A, 0.1% Triton X-100 in PBS) for 30 min at 37°C in the dark. To detect the extent of apoptosis under stress conditions, cells were grown in serum-free medium for 72 h and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection kit (Jingmei, Shanghai, China) according to the manufacturer's protocol.

Western blot analysis. Cells were solubilized in RIPA lysis buffer (50 mM Tris–HCl [pH 7.4], 1% Triton X-100, 5 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4) and centrifuged at 20 000 *g* for 30 min at 4°C to remove debris. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL, USA). Protein extracts were separated using SDS–PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween-20 for 1 h, then incubated overnight at 4°C with primary antibodies raised against the following proteins: BMI1 (Upstate); cyclin D1, cyclin E, Cdk2, Cdk4, Cdk6, p21, p27, Akt, Ser^{473}phospho-Akt, Bcl-2, and Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and GAPDH (Kangchen, Shanghai, China). Immune complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Amersham Life Science, Piscataway, NJ, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control.

Statistical analysis. Correlations between categorical variables were analyzed using Pearson's chi-square tests, and two-tailed *t*-tests were used for continuous variables. Survival curves were plotted using the Kaplan–Meier method and were compared using the log-rank test. All statistical analyses were performed using the SPSS software package (SPSS, Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

Results

Increased BMI1 expression in PDAC. The expression of BMI1 in PDAC and normal pancreas tissues was analyzed by immunohistochemistry. Consistent with previous reports,[28] BMI1 was mainly expressed in neoplastic epithelial cell nuclei and occasionally in stromal cells neighboring the neoplastic cells (Fig. 1b,c). In contrast, no staining or only weak staining was seen in normal duct or acinar cells. Infiltrating lymphocytes known to express BMI1 served as internal positive controls (Fig. 1a).[21] For the 72 PDAC patients, increased nuclear accumulation of BMI1 was found in 35 (48.61%) specimens. Furthermore, BMI1 expression was compared between five pancreatic cancer cell lines (PANC-1, BxPC-3, ASPC-1, CFPAC-1, and SW1990) and an immortal pancreatic epithelial cell line (HPDE6c7). The levels of BMI1 expression in the PDAC cell lines were higher than those for the HPDE-6 cell line (Fig. 1f).

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) overexpression is associated with the progression and adverse prognosis of PDAC. To investigate the clinical role of BMI1 during pancreatic carcinogenesis, BMI1 expression was compared with clinical pathological features of patients with PDAC. As shown in Table 1, the nuclear accumulation of BMI1 in PDAC was significantly associated with lymph node metastases ($P < 0.05$), indicating a correlation between BMI1 expression and PDAC invasion and metastasis. However, there was no difference between the expression of BMI1 and other clinical features such as patient age, sex, pathologic stage, and histology grade ($P > 0.05$ for all comparisons). Further, Kaplan–Meier survival analysis demonstrated that patients harboring BMI1 overexpression had a significantly shorter overall survival than patients with lower levels of BMI1 expression ($P = 0.007$, log-rank test; Fig. 1e). These observations indicate that increased BMI1 is associated with PDAC clinical progression.

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) knockdown inhibited the growth of PDAC cells both *in vitro* and *in vivo*. To determine whether BMI1 effects tumor cell growth, two BMI1-specific shRNAs were constructed and inserted in lentiviral vectors. PANC-1 and CFPAN-1 cells, two cell lines with high levels of BMI1 expression, were infected

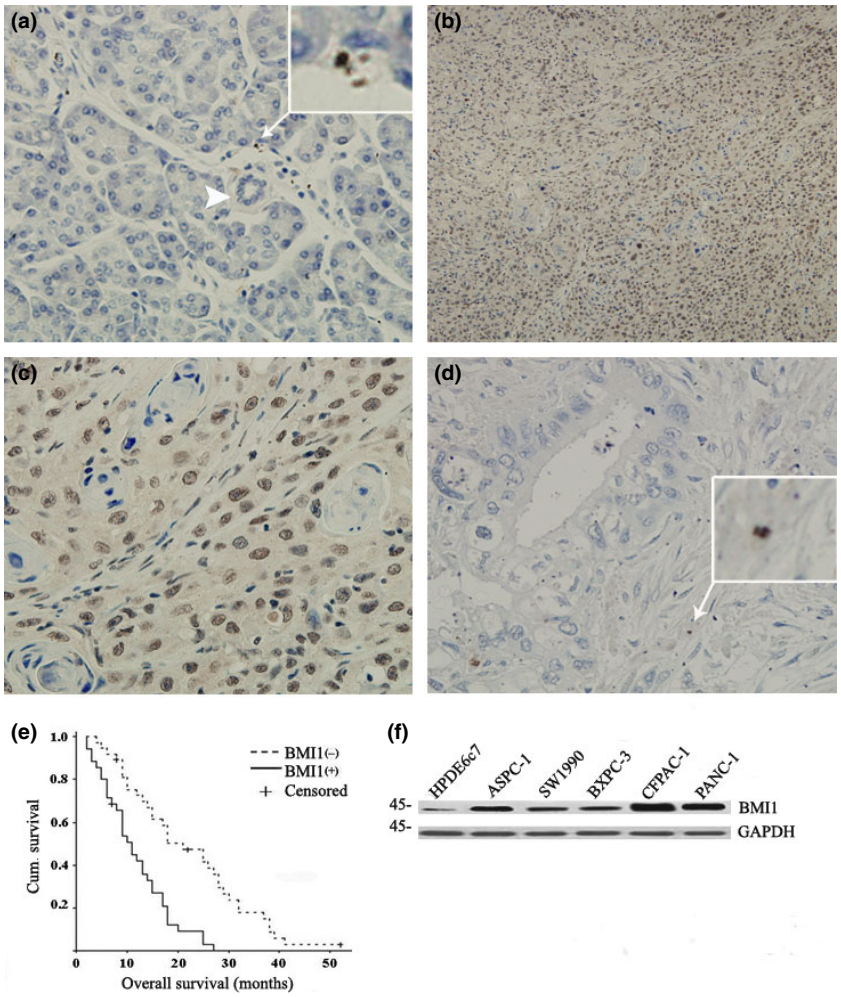

Fig. 1. Expression of B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) in human pancreas specimens. (a–d) Immunohistochemical analysis of BMI1 expression in normal adult pancreas (a) and pancreatic ductal adenocarcinoma (PDAC) tissues (b–d). B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) staining is mainly localized in the nuclei of cancerous cells and scattered infiltrating lymphocytes (arrows). (a) Weak or absent BMI1 in a normal duct (arrowhead) and acinar cells. (b,c) Strong BMI1 staining in PDAC. (d) B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1)-negative staining in PDAC. Insets show high magnification of structures indicated by arrows. Magnification: (a,c,d) ×400; (b) ×100. (e) Kaplan–Meier overall survival curves for 72 patients with PDAC. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) overexpression was associated with a significantly shorter overall survival ($P < 0.01$, log-rank test). (f) Western blot analysis of BMI1 expression in five pancreatic cancer cell lines and a normal human pancreatic epithelial cell line, HPDE6c7. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Size markers (in kDa) are shown on the left side of each panel.

Table 1. Association between BMI1 expression and clinicopathological variables of patients with PDAC

Variables	BMI1 expression		*P*-value
	Positive	Negative	
Age, years			
>60	24	22	0.469
≤60	11	15	
Gender			
Male	19	23	0.633
Female	16	14	
Histological grade			
Well or moderated	26	23	0.318
Poorly	9	14	
TNM stage			
I–II	30	26	0.158
III–IV	5	11	
Lymph node metastasis			
Positive	23	15	0.037
Negative	12	22	

BMI1, B-cell-specific Moloney murine leukemia virus insertion site 1; PDAC, pancreatic ductal adenocarcinoma.

with the lentiviral constructs and selected with blasticidin for 10 days. Expression of BMI1 was subsequently determined by western blot analysis. As shown in Figure 2(a), the shRNA BMI1si2 more effectively down-regulated the expression of BMI1 in both PANC-1 and CFPAC-1 cells than did BMI1si1. Furthermore, the control vectors had no effect on the expression of BMI1 in either cell line. As a result, PANC-1-BMI1si2 cells and CFPAC-1-BMI1si2 cells were further characterized.

We first examined the effect of BMI1 depletion on cell viability using MTT assays. As shown in Figure 2(b), BMI1 depletion significantly suppressed the growth of PANC-1 and CFPAC-1 cells compared with control cells ($P < 0.05$). In contrast, BMI1 depletion did not affect the viability of HPDE6c7 cells (data not shown). Colony formation analysis showed that BMI1-depleted tumor cells had a greatly reduced capacity to form colonies compared with the control cells ($P < 0.05$; Fig. 2c). To confirm these findings *in vivo*, xenograft tumor growth assays were performed in nude mice. Compared with the control cells, injection of PANC-1 (CFPAC-1) BMI1si2 cells led to dramatically decreased tumor volume ($P < 0.05$; Fig. 2d). Taken together, these data show that decreased expression of BMI1 in PDAC cells effectively suppressed the growth of PDAC cells both *in vitro* and *in vivo*.

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) knockdown arrested PDAC cells in the G0-G1 phase. Our flow cytometry analysis showed that the cell-cycle distribution of PANC-1 and CFPAC-1 cells was significantly affected following BMI1 depletion. As shown in Figure 3, there was a higher proportion of G0-G1 phase cells (56.92%) in PANC-1-BMI1si2 cells than in the control (42.03%). A compensatory decrease in the S (35.73%) and G2/M phase (7.35%) proportions was also detected as compared with the control in S (41.34%)

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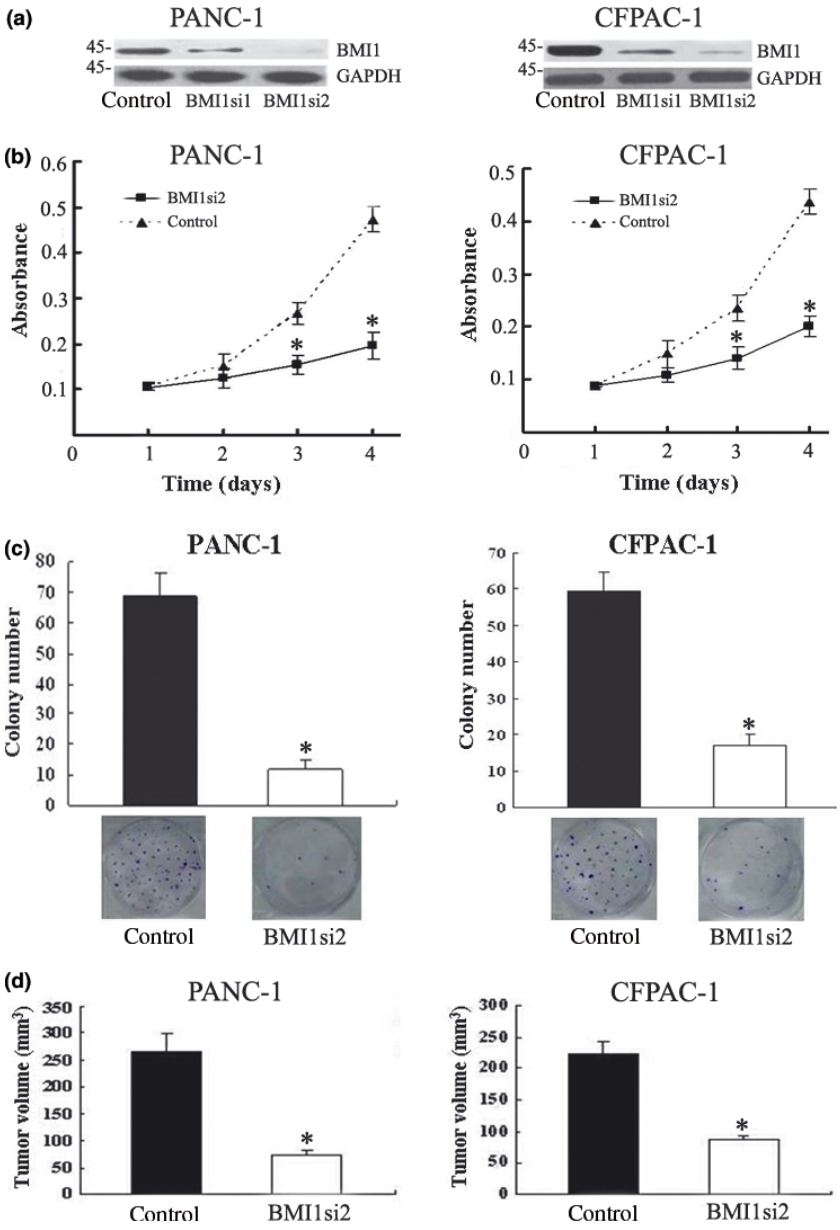

Fig. 2. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) depletion caused inhibition of growth and tumorigenesis of pancreatic ductal adenocarcinoma (PDAC) cells. (a) Western blot analysis of PANC-1 and CFPAC-1 cells infected with BMI1si1 and BMI1si2 and control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Size markers (in kDa) are present on the left side of each panel. (b) MTT [3-(4,5-dimethylthiahiazol-2-y1)-2,5-diphenytetrazolium bromide] assays of PANC-1 and CFPAC-1 cells infected with BMI1si2 or control shRNA. Values are presented at the indicated time points as the mean absorbance with a SD of four wells. (c) Colony formation assays of PANC-1 and CFPAC-1 cells infected with BMI1si2 or control. The count number of the colonies is shown in the diagram. (d) Effect of BMI1si2 on tumorigenicity in nude mice of PANC-1 and CFPAC-1 cells. Average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. *$P < 0.05$ compared with control.

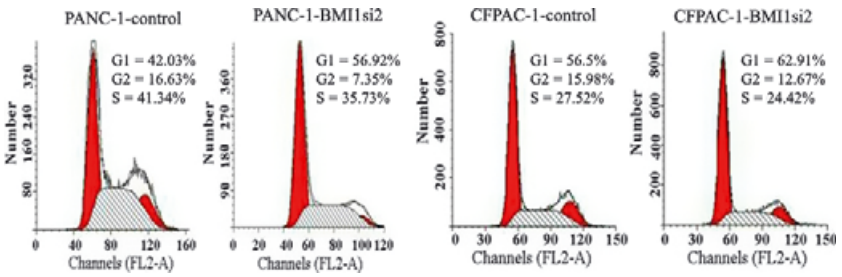

Fig. 3. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) depletion arrested the accumulation of pancreatic ductal adenocarcinoma (PDAC) cells in the G0-G1 phase. Following synchronization, the cell cycle distribution of PANC-1-control, PANC-1-BMI1si2, CFPAC-1-control, and CFPAC-1-BMI1si2 cells was determined by flow cytometry.

and G2/M phases (16.63%). Similar results were obtained in CFPAC-1 cells (Fig. 3). These results suggest that BMI1 silencing inhibited the entry of cells into the S phase and therefore suppressed cell growth. Furthermore, western blotting was performed to explore the cell cycle regulatory role of BMI1. Following BMI1 silencing, the expression of cyclin D1, cyclin-dependent kinase (CDK)-2, and CDK4 were decreased, while the level of p21 was increased (Fig. 4). Additionally, the levels of cyclin A, cyclin E, and p27 were also detected but were not significantly altered (data not shown).

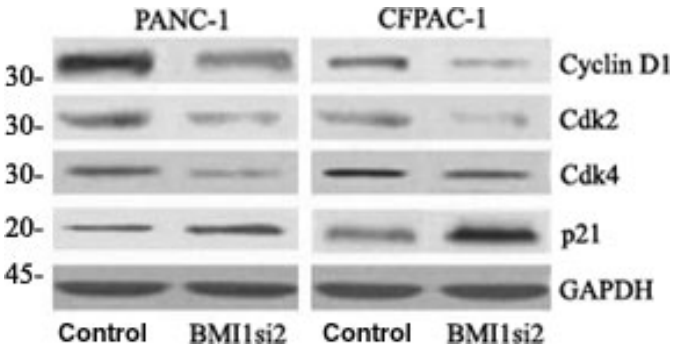

Fig. 4. Changes in cell cycle-related protein expression with B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) silencing. Western blot analysis was used to detect the expression of several cell cycle-related regulatory factors in PANC-1 and CFPAC-1 cells infected with BMI1si2 *vs* control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Size markers (in kDa) are present on the left side of each panel. CDK, cyclin-dependent kinase.

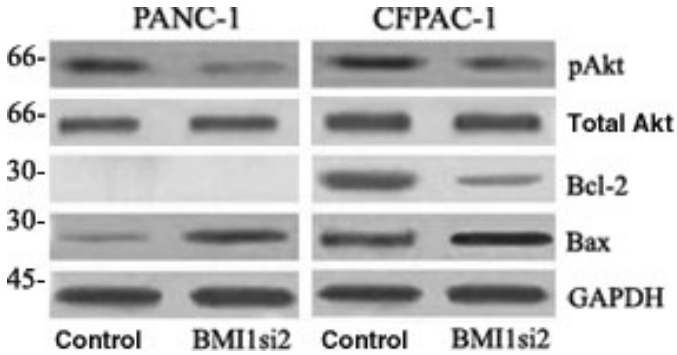

Fig. 6. Changes in survival-related protein expression with B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) silencing. Western blot analysis was used to detect the expression of several survival-related regulatory factors in PANC-1 and CFPAC-1 cells infected with BMI1si2 *vs* control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Size markers (in kDa) are present on the left side of each panel.

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) knockdown increased apoptosis of PDAC cells under serum depletion. To further explore the functional mechanisms by which BMI1 promotes PDAC tumor growth, BMI1si2-infected PANC-1 and CFPAC-1 cells were maintained in serum-free media for 72 h. A dramatic increase in apoptosis was detected in both cell lines compared to controls (Fig. 5). The percentage of apoptotic cells (Annexin-V+/PI− and Annexin-V+/PI+) in PANC-1-BMI1si2 (CFPAC-1-BMIsi2) cells was 28.38% (30.24%), while the percentage of apoptosis was 10.2% (12.84%) in controls. These results suggest that BMI1 protects PDAC cells from stress-induced death. Furthermore, the expression of several cell survival-related proteins was evaluated by western blot (Fig. 6). Following BMI1 silencing, the expression of phospho-Akt and Bcl-2 were decreased, while the level of Bax was increased. Additionally, there was no significant change in Bcl-2 protein levels between PANC-1-BMI1si2 cells and control cells. This lack of difference may be due to the absence or low levels of expression of Bcl-2 in PANC-1 cells[31–33] that could have impeded our examination of the effects of BMI1 silencing.

Discussion

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1), a member of the polycomb group of transcriptional repressors, may play an important role in carcinogenesis, including in PDAC. The possible clinical significance and functional role of BMI1 were poorly known in the late progression of pancreatic cancer, although prior studies have shown that BMI1 is overexpressed in PDAC compared with normal pancreas tissues.[28,29] In the current study, we provided additional clinicopathological evidence and a mechanistic basis for the role of BMI1 in human PDAC progression. We found that BMI1 was overexpressed in both PDAC tissues and cell lines compared with normal pancreas tissues and a normal pancreatic epithelial cell line. A prior study has also shown evidence of BMI1 expression in normal pancreas duct or islet cells.[29] Although even in our study normal pancreatic tissues contained weak or absent BMI1 expression, these results suggest that the cellular population of the normal pancreas is probably heterogeneous and may reflect different genetic expression patterns due to distinct stages of cell differentiation. Another possible explanation for the discrepancies could be in part due to the different protocol used for immunohistochemistry. Further investigation would be needed to clarify this point. Of note, the previous study reported that increased BMI1 was also found in pancreatitis and pancreatic intraepithelial neoplasia, implying that BMI1 is involved in the early progression of PDAC.[29] Here, we further demonstrated that aberrant nuclear overexpression of BMI1 was positively correlated with lymph node metastases but negatively correlated with patient survival, underscoring the role of BMI1 in the late progression of PDAC. Indeed, this association between BMI1 up-regulation and lymph node metastasis and an adverse prognosis is not unprecedented, as similar findings have been reported in other tumor types.[25,34,35] However, there is no statistically significant correlation between Bmi-1 overexpression with the grade or stage of PDAC. The reason may be due to the small number of cases examined in our study, and therefore additional specimens would need to be analyzed to confirm our

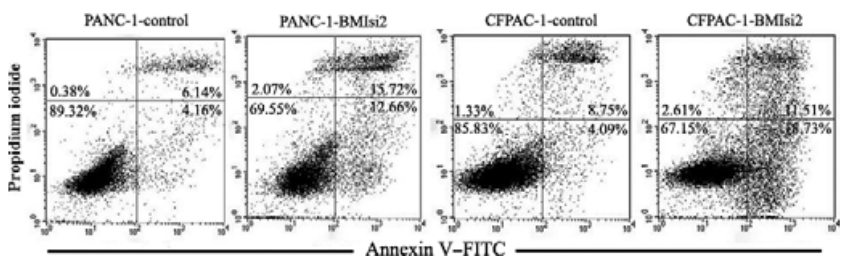

Fig. 5. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) depletion enhanced apoptosis of pancreatic ductal adenocarcinoma (PDAC) cells under stress conditions. Following 72 h of culturing in serum-free medium, apoptotic cells of PANC-1-control, PANC-1-BMI1si2, CFPAC-1-control, and CFPAC-1-BMI1si2 were measured by flow cytometry. The cell populations of Annexin-V+/PI− and Annexin-V+/PI+ were used to assess apoptotic events.

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results. Nevertheless, our clinical evidence clearly suggests that Bmi-1 may be an important late mediator of PDAC progression.

To further confirm the functional significance of increased BMI1 expression in the progression of PDAC, we stably down-regulated BMI1 expression in two PDAC cell lines using lentiviral-mediated expression of a BMI1-targeted shRNA. Consequently, BMI1 depletion significantly inhibited the proliferation and growth of PDAC cells *in vitro* and tumorigenicity in animal models. Because BMI1 depletion significantly reduced cell growth, we wondered whether cell growth inhibition was due to cell cycle arrest in any specific phase of the cell cycle. Indeed, we found that BMI1 depletion resulted in a dramatic accumulation of cells in the G0/G1 phase and a marked reduction in S and G2/M phase cells. We also tested the expression of several known G0/G1 cell cycle-related molecules in the BMI1 stable depletion cells and the control. As a result, significant decreases in the levels of cyclin D1, Cdk2, and Cdk4, all of which are frequently deregulated in PDAC, were detected in BMI1 siRNA-infected cells.[36] Marked up-regulation of p21 was also observed. p21 is a member of the Cip/Kip families of CDK inhibitors that are associated with mediating negative effects on cell cycle progression through the binding of various cyclin–CDK complexes and blocking of their activities.[37] In this study, up-regulation of the CDK inhibitor p21CIP and down-regulation of cyclin D1, Cdk2, and Cdk4 were consistent with the inhibition of cell growth and altered cell cycle distribution exhibited by BMI1-depleted cells. Therefore, these results suggest that BMI1-mediated promotion of tumor progression is associated with increased PDAC cell proliferation via regulation of the expression of some cyclins (cyclin D1, Cdk2, and Cdk4) and a CDK inhibitor (p21CIP).

The inner microenvironment of solid tumors is characterized by regions of fluctuating hypoxia, low pH, and nutrient deprivation. Cancer cell adaptation to a variety of stresses in a solid tumor is critical to the tumor progression. Our studies demonstrated that BMI1 is an important survival factor for PDAC cells under stress conditions. We found that BMI1 depletion led to a significant decrease in the survival of pancreatic cancer cells under serum-starved conditions. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) depletion also resulted in up-regulation of Bax as well as down-regulation of Bcl-2 and phospho-Akt. Although the speculative role of the PI3K/Akt pathway in BMI1-mediated pancreatic cancer survival remains to be investigated, several previous studies have suggested a cross-talk between BMI1 and PI3K/Akt signals in other types of tumors.[38,39] The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a key regulator of various cellular processes such as survival, proliferation, and tumorigenesis, and therefore disruption of this pathway can at least in part account for the consequence of BMI1 knockdown. Interestingly, we also detected decreased expression of Bcl-2, a downstream target of the nuclear factor-kappa B (NF-κB) pathway, in BMI1-depleted cells. A recent report showed that BMI1 was involved in NF-κB signals,[40] implying that BMI1 may serve as a mediator in signal pathways to confer its survival functions. Considering the importance of Bcl-2 and/or its family members in pancreatic cancer,[33,41] BMI1-mediated Bcl-2 expression may serve as a novel pathway to contribute to pancreatic cancer progression. Moreover, a recent study showed that BMI1 silencing significantly sensitized nasopharyngeal cancer cells to chemotherapeutic drugs *in vitro* at least in part through the regulation of Bcl-2 family proteins.[38] Collectively, our results suggest that the underlying mechanism of BMI1-mediated promotion of tumor progression may be to associate with the PI3K/Akt pathway and Bcl-2 family proteins to enhance cell survival under stresses.

As described earlier, BMI1 functions are mainly attributed to its repressive action on the INK4a/ARF locus that contributes to cell cycle regulation and apoptosis through modulation of the retinoblastoma and p53 pathways.[11,14,19,20] However, deletion or mutation of the INK4a/ARF locus and p53 is one of the most common alterations in pancreatic cancers, including PANC-1 cells, which were used in this study.[42,43] Consistent with this, silenced INK4a and mutant p53 were also found in the CFPAC-1 cells.[42,44] All these data presented here imply that intact an INK4a/ARF locus is not required for BMI1-mediated growth and survival in both PDAC cell lines. Therefore, BMI-1 functioned to maintain the growth and survival of PDAC via an INK4a/ARF-independent manner in our study.

To the best of our knowledge, this study is the first detailed survey of BMI1 expression and its clinical significance in PDAC. B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) was found to be overexpressed in PDAC cell lines and was associated with an unfavorable prognosis for patients with PDAC. When BMI1 was down-regulated, cell growth was suppressed as a result of cell cycle arrest, and susceptibility to apoptosis was enhanced. In combination, these results indicate that BMI1 plays an important role in PDAC progression and may represent a potential therapeutic target in the treatment of PDAC.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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