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Epigenetic silencing *ZSCAN23* promotes pancreatic cancer growth by activating Wnt signaling

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the most malignant tumor. Zinc finger and SCAN domain-containing protein 23 (*ZSCAN23*) is a new member of the SCAN domain family. The expression regulation and biological function remain to be elucidated. In this study, we explored the epigenetic regulation and the function of *ZSCAN23* in PDAC. *ZSCAN23* was methylated in 60.21% (171/284) of PDAC and its expression was regulated by promoter region methylation. The expression of *ZSCAN23* inhibited cell proliferation, colony formation, migration, invasion, and induced apoptosis and G1/S phase arrest. *ZSCAN23* suppressed Panc10.05 cell xenograft growth in mice. Mechanistically, *ZSCAN23* inhibited Wnt signaling by interacting with myosin heavy chain 9 (MYH9) in pancreatic cancer cells. *ZSCAN23* is frequently methylated in PDAC and may serve as a detective marker. *ZSCAN23* suppresses PDAC cell growth both *in vitro* and *in vivo*.

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ZSCAN23; DNA methylation; pancreatic ductal adenocarcinoma; Wnt signaling pathway; tumor suppressor

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is currently the third leading cause of cancer-related deaths in the United States and is predicted to be the second leading cause of cancer-related deaths by 2030. Only 15–20% patients are eligible for surgical resection, with approximately 75% of these cases experiencing recurrence within 2 years. Regrettably, systemic therapies remain largely ineffective.[1–3] Although oncogenic *KRAS* mutations are found in more than 90% of PDAC cases, the development of inhibitors remains a challenge. Familial pancreatic cancer accounts for 5–10% of cases with mutations in DNA damage repair genes, including breast cancer type 1 (*BRCA1*), *BRCA2*, partner and localizer of *BRCA2* (*PALB2*), serine/threonine kinase 11 (*STK11*), and MutL homolog 1 (*MLH1*).[4–6] However, the utilization of precision-based therapy is limited to only 7% of patients.[7,8] Therefore, the identification of novel biomarkers remains a critical topic in PDAC diagnosis and therapy.

Epigenetic regulation is involved in carcinogenesis, tumor development, and metastasis.[9,10] Epigenetic heterogeneity increases the complexity of cancer diagnosis and therapy.[11,12] Epigenetic regulators consist of three distinct categories: writers, readers and erasers. Writer is responsible for the addition of chemical groups to DNA or histone (such as DNA methyltransferases, DNMTs). Eraser, such as histone deacetylases (HDACs), is involved in the removal of these modifications. Reader, such as methyl-binding domain protein, is to recognize specific epigenetic marks and acts as an effector.[13,14] Epidrugs are small molecular inhibitors to target the epigenome or epigenetic regulatory enzymes. The role of epidrugs has been extensively investigated in various cancers. However, their application remains restricted to hematological malignancies and is infrequently implemented in clinical practice.[10,13] Oncologists have been pursuing the precise targeting of cancer cells without damaging normal cells. Many new anticancer drugs have been registered for clinical trials by combination of epidrugs with other therapeutics in solid tumors, yet a significant portion of them have ultimately been excluded (such as CRUKD/07/065, NCT00481078, NCT01100944).[15–17]

This limitation is mainly attributed to the complexity of diverse cellular compositions and imprecise targeting. "Synthetic lethality" based on epigenetic loss-of-function is a novel strategy for cancer therapy.[15,18,19] Therefore, understanding the abnormal epigenetic changes involved in the signaling pathways and regulatory networks becomes imperative.

Zinc finger and SCAN domain-containing protein 23 (*ZSCAN23*) is a member of the SCAN domain zinc-finger family, which is located on chromatin 6p22.1, a tumor-susceptible locus.[20] The ZSCAN23 protein contains two domains: a zinc- finger domain at the C-terminus and a SCAN domain at the N-terminus.[21] The zinc-finger domain

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binds to specific DNA segments and interacts with other cellular factors to regulate the transcription of target genes.[22] The SCAN domain, a highly conserved vertebrate-specific protein domain, is involved in protein-protein interactions via self-binding and mediates oligomerization, leading to formation of homodimers or heterodimers that regulate various transcriptional activities.[23–26] Other members of the *ZSCAN* family are involved in cancer development, and their functions differ in various cancers.[27–29] The role of *ZSCAN23* in cancer remains unclear. Therefore, the aim of this study is to elucidate the role of *ZSCAN23* in PDAC development.

Results

ZSCAN23 was frequently methylated in PDAC and its expression was regulated by promoter region methylation

The TCGA database was used to analyze the association between *ZSCAN23* mRNA expression and CpG site methylation using the extracted data of 194 PDAC cases (http://xena.ucsc.edu/). The mRNA levels were inversely associated with methylation at CpG sites around the transcription start site (cg21486944, cg06818710, cg00651523, and cg07836142; all $p < .0001$; Figure 1a,b), suggesting that *ZSCAN23* expression is regulated by promoter region methylation.

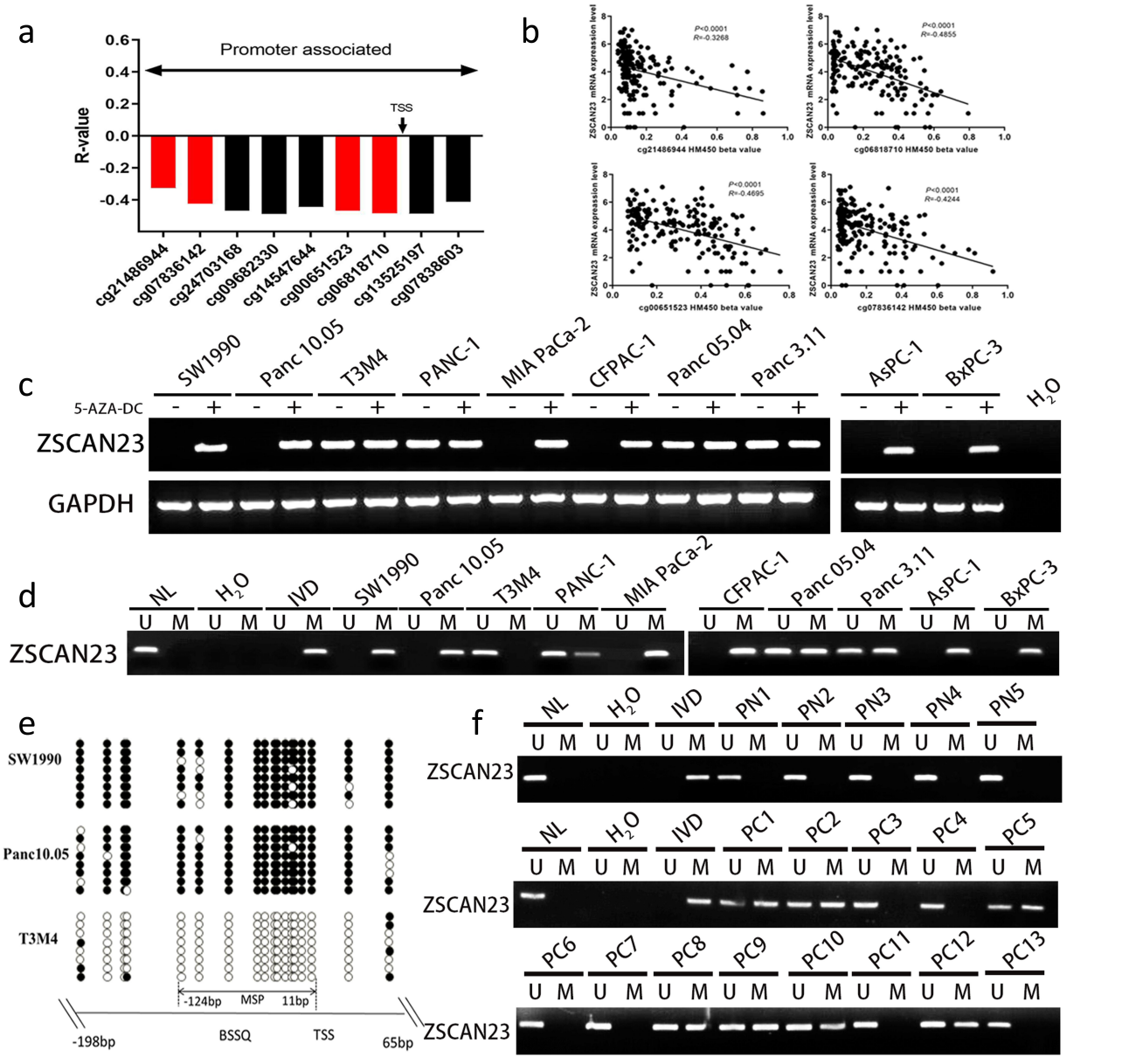

Figure 1. Expression and methylation status of *ZSCAN23* in PDAC. a, association of *ZSCAN23* expression and methylation status of CpG sites around the TSS site. b, scatter plots show that *ZSCAN23* expression is negatively associated with representative CpG site methylation status (cg21486944, cg06818710, cg00651523, and cg07836142). c, detection of *ZSCAN23* expression in PDAC cells via semi-quantitative RT-PCR. 5-AZA-DC: 5-aza-2′-deoxycytidine; *GAPDH*: internal control of RT-PCR; H_2O: double distilled water; (-): absence of 5-AZA-DC; (+): presence of 5-AZA-DC. d, MSP results of *ZSCAN23* in PDAC cells. U: unmethylated alleles; M: methylated alleles; IVD: in vitro methylated DNA, serves as methylation control; NL: normal lymphocytes DNA, serves as unmethylation control. e, Bisulfite sequencing results of *ZSCAN23* in SW1990, Panc10.05, and T3M4 cells. The size of unmethylated MSP products was 136 bp. Bisulfite sequencing was performed in a 264 bp region around the *ZSCAN23* transcription start site (−198 bp to 65 bp). Filled circles: methylated CpG sites; open circles: unmethylated CpG sites; TSS: transcription start site. f, representative MSP results of *ZSCAN23* in primary PDAC samples. PN: adjacent normal tissue of pancreas solid pseudopapillary neoplasm; PC: primary PDAC samples.

ZSCAN23 was found to be highly expressed in T3M4, PANC-1, Panc 05.04, and Panc3.11 cells and no expression was detected in SW1990, Panc10.05, MIAPaCa-2, CFPAC-1, AsPC-1, and BxPC-3 cells, determined by the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) detection (Figure 1c). *ZSCAN23* was unmethylated in T3M4 cells, completely methylated in SW1990, Panc 10.05, MIAPaCa-2, CFPAC-1, AsPC-1, and BxPC-3 cells, and partially methylated in PANC-1, Panc 05.04, and Panc3.11 cells (Figure 1d). The loss of *ZSCAN23* expression correlated with complete methylation. *ZSCAN23* expression was restored after 5-AZA-2′-deoxycytidine (5-AZA-DC) treatment in SW1990, Panc10.05, MIAPaCa-2, CFPAC-1, AsPC-1, and BxPC-3 cells, whereas no changes were observed in T3M4, PANC-1, Panc 05.04, and Panc3.11 cells (Figure 1c), demonstrating the epigenetic regulation of *ZSCAN23* expression. Validation of the methylation-specific PCR (MSP) primer efficiency and methylation density was performed using bisulfite sequencing in SW1990, Panc 10.05, and T3M4 cells (Figure 1e).

No methylation was found in the 17 cases of adjacent tissue samples of serous cystadenoma (benign cyst in pancreas), excluding tissue/cell type-specific methylation. *ZSCAN23* was methylated in 60.21% (171/284) of the PDAC samples (Figure 1f). No association was found between *ZSCAN23* methylation and clinical factors, including sex, age, tumor size, differentiation, TNM stage, tumor location, smoking or alcohol consumption (all $p > .05$, Table 1).

ZSCAN23 *expression inhibited PDAC cell proliferation*

MTT assays were used for evaluating the effect of *ZSCAN23* on cell proliferation. The optical density (OD) values before and after restoration of *ZSCAN23* expression were 1.245 ± 0.052 vs 1.034 ± 0.050 in SW1990 cells ($p < .001$) and 0.799 ± 0.014 vs 0.719 ± 0.024 in Panc10.05 cells ($p < .0001$) (Figure 2a). The OD value was reduced in *ZSCAN23*-re-expressed SW1990 (16.9%) and Panc10.05 cells (10.0%), indicating an inhibitory role of *ZSCAN23* expression in cell proliferation.

In colony formation analysis, the colony number before and after restoration of *ZSCAN23* expression was 384.000 ± 22.627 vs 199.500 ± 14.849 in SW1990 cells ($p < .05$) and 261.333 ± 14.012 vs 99.333 ± 17.388 in Panc10.05 cells ($p < .001$) (Figure 2b). The number of colonies reduced after restoring *ZSCAN23* expression in SW1990 (48.0%) and Panc10.05 cells (62.0%). These data indicated that *ZSCAN23* expression inhibited PDAC cell proliferation.

ZSCAN23 *expression inhibited PDAC cell migration and invasion*

Cell migration and invasion were evaluated using a Transwell assay. The number of migrating cells with and without *ZSCAN23* expression was 261.667 ± 18.930 vs 63.667 ± 4.933 cells (SW1990 cells; $p < .0001$) and 236.000 ± 7.350 vs 74.000 ± 6.928 cells (Panc10.05 cells; $p < .0001$). Cell migration was decreased in SW1990 (75.7%) and Panc10.05 cells (68.6%) after restoring *ZSCAN23* expression (Figure 2c). The number of invading cells with and without *ZSCAN23* expression was 234.333 ± 19.140 vs 49.000 ± 6.245 cells (SW1990 cells; $p < .0001$) and 190.333 ± 8.327 vs 44.333 ± 8.859 cells (Panc10.05 cells; $p < .0001$). The number of invading cells was reduced in SW1990 (79.1%) and Panc10.05 cells (76.6%) after restoring *ZSCAN23* expression (Figure 2c), demonstrating that *ZSCAN23* expression inhibited cell invasion.

Table 1. Clinical factors and *ZSCAN23* methylation status in PDAC.

		Methylation status		
Clinical parameter	NO. 284	Unmethylated $n = 113$ (39.79%)	Methylated $n = 171$ (60.21%)	*P* value
Sex				
Male	184	67	117	0.115
Female	100	46	54	
Age				
≤50	44	18	26	0.869
>50	240	95	145	
Differentiation				
Well/Moderately	137	57	80	0.546
Poorly	147	56	91	
TNM stage				
I/II	255	104	151	0.309
III/IV	29	9	20	
Lymph node metastasis				
Negative	189	79	110	0.329
Positive	95	34	61	
Tumor size				
≤4 cm	219	88	131	0.803
>4 cm	65	25	40	
Tumor location				
Head	172	62	110	0.110
Body	112	51	61	
Smoking				
Yes	113	43	70	0.627
No	171	70	101	
Alcohol consumption				
Yes	133	53	80	0.984
No	151	60	91	

p values are obtained from χ^2 test, significant difference, $p < .05$.

To further validate the inhibitory role of *ZSCAN23*, the migration and invasion related proteins, matrix metallopeptidase-2 (MMP2), MMP7 and MMP9 were detected using western blotting. The levels of MMP2, MMP7 and MMP9 were reduced after restoration of *ZSCAN23* expression (Figure 2d).

ZSCAN23 *expression induced G1/S arrest in PDAC cells*

The cell cycle phase distributions of G0/G1, S, and G2/M in *ZSCAN23* unexpressed and overexpressed SW1990 cells were 53.0 ± 0.0% vs 59.7 ± 1.2% ($p < .001$), 30.2 ± 0.6% vs 24.8 ± 0.8% ($p < .001$), and 16.8 ± 0.7% vs 15.5 ± 0.9%, respectively. In *ZSCAN23* unexpressed and re-expressed Panc10.05 cells, the distributions of G0/G1, S, and G2/M cell phases were 29.0 ± 0.1% vs 39.6 ± 2.2% ($p < .01$), 48.2 ± 2.5% vs 40.8 ± 1.2% ($p < .01$), and 22.8 ± 1.8% vs 19.6 ± 1.7%, respectively (Figure 3a). The proportion of G0/G1 phase cells increased in SW1990 (6.7%) and Panc10.05 cells (10.6%), and that of S phase cells decreased in SW1990 (5.4%) and in Panc10.05 cells (7.4%) upon restoration of *ZSCAN23* expression. These results indicated that *ZSCAN23* induced G1/S arrest. Cyclin D1, cyclin E1, cyclin A2, and cyclin-dependent kinase 2 (CDK2) are G1/S checkpoint regulators and their expression was detected by western blotting. The expression of cyclin D1, cyclin E1, cyclin A2, and CDK2 was reduced following re-expression of *ZSCAN23* in SW1990 and Panc10.05 cells (Figure 3b), validating that *ZSCAN23* expression induced G1/S arrest in PDAC cells.

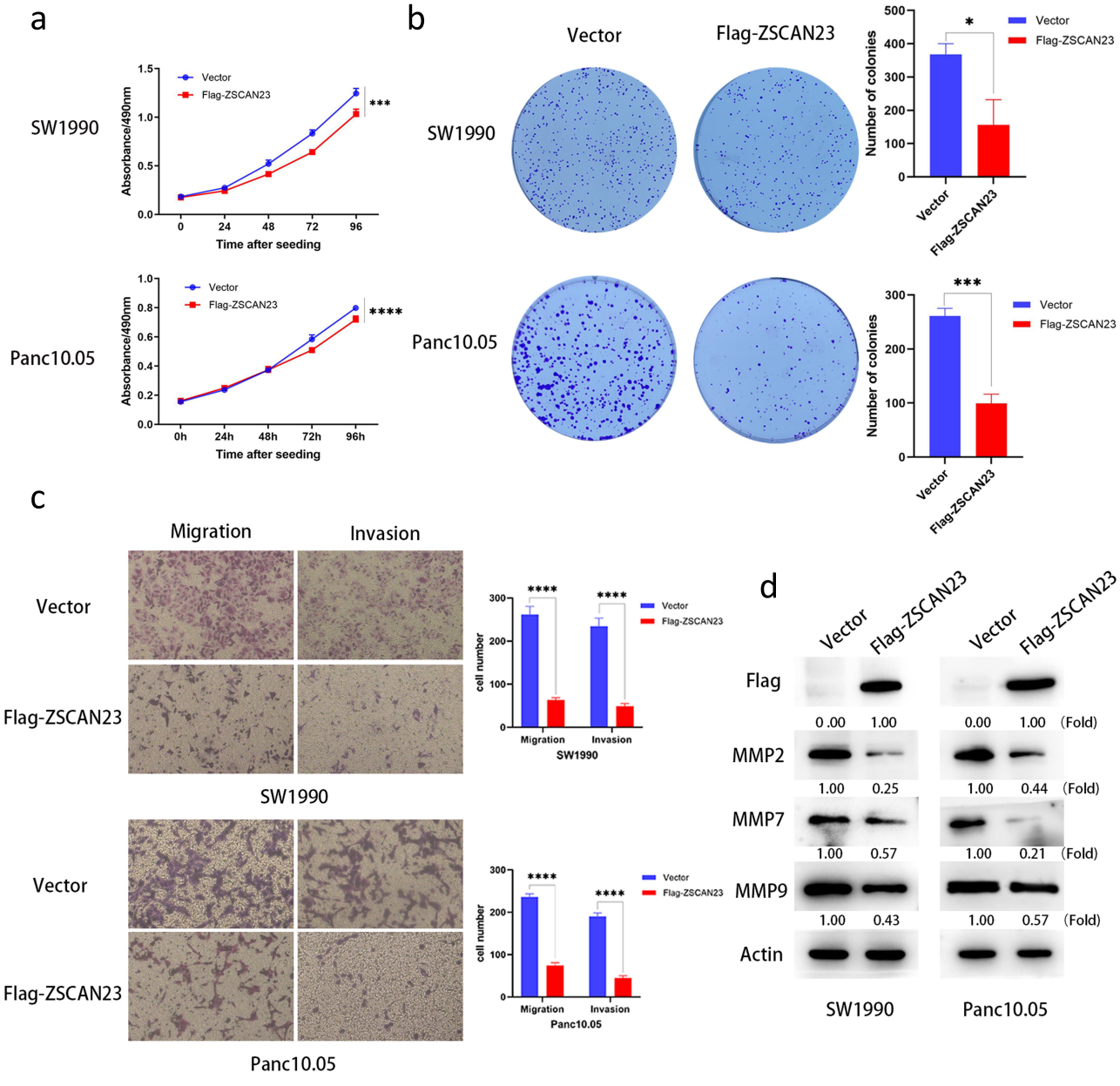

Figure 2. Effect of *ZSCAN23* expression on cell proliferation, invasion, and migration. a, growth curves represent the effects of *ZSCAN23* expression on PDAC cells. b, the average number of cell clones is presented in the bar diagram. c, cell migration and invasion experiments on SW1990 and Panc10.05 cells before and after re-expression of *ZSCAN23*. The average number of migrating cells is presented in the bar diagram. Each experiment was repeated in triplicate. *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$. D, the expression levels of flag, MMP2, MMP7, and MMP9 were detected via western blotting.

ZSCAN23 expression induced PDAC cell apoptosis

Flow cytometry analysis revealed that the proportions of apoptotic cells among *ZSCAN23* silenced and overexpressed cells were 12.5 ± 1.2% vs 25.2 ± 1.0% (SW1990 cells; $p < .001$) and 8.6 ± 1.2% vs 17.8 ± 1.9% (Panc10.05 cells; $p < .05$) (Figure c). These results indicated that *ZSCAN23* expression induces apoptosis. Subsequently, the levels of apoptosis-related proteins were analyzed. Increased levels of BAX and Cleaved caspase-3 along with reduced levels of Bcl-2 were detected after re-expression of *ZSCAN23* in SW1990 and Panc10.05 cells (Figure 3d), further demonstrating that *ZSCAN23* induced apoptosis.

ZSCAN23 expression suppressed Panc10.05 cell xenograft growth

A mouse xenograft model was used to evaluate the role of *ZSCAN23* in PDAC development. Before and after re-expression of *ZSCAN23*, the tumor volume was 570.874 ± 118.293 mm^3 and 340.482 ± 32.191 mm^3 in Panc10.05 cell xenografts ($p < .01$; Figure 4a), respectively. The tumor volume decreased after restoring *ZSCAN23* expression (Figure 4b). The tumor weight in *ZSCAN23* unexpressed and re-expressed xenografts was 0.396 ± 0.160 g and 0.146 ± 0.050 g, respectively ($p < .05$). Tumor weight

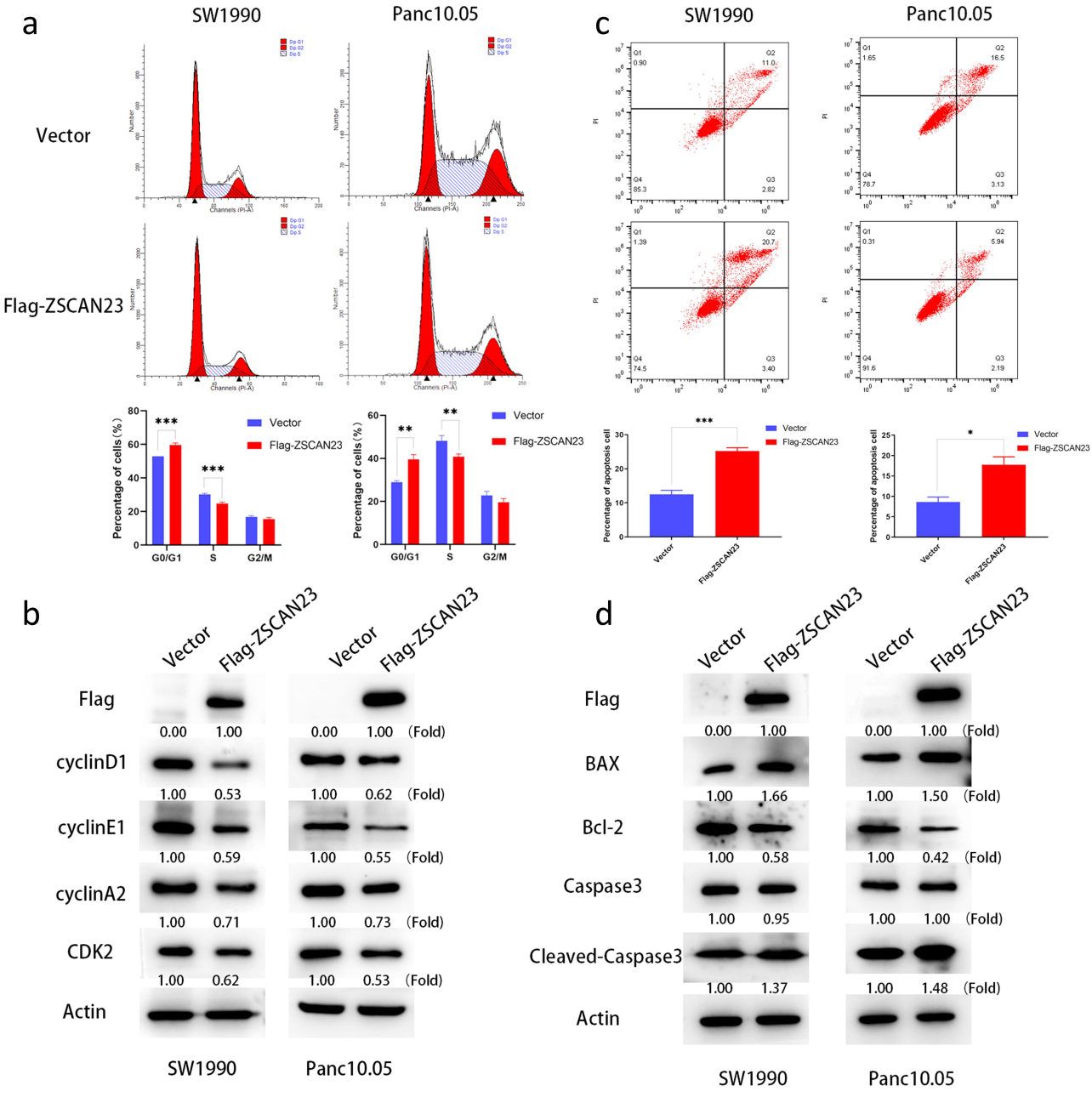

Figure 3. Effect of *ZSCAN23* expression on cell cycle and apoptosis. a, the effect of *ZSCAN23* expression on cell cycle phase in PDAC cells. The bar diagram presents the proportions of cells at different cell cycle phases. b, Western blots present the levels of flag, cyclin D1, cyclin E1, cyclin A2, and CDK2. c, effect of *ZSCAN23* expression on apoptosis in PDAC cells. Each experiment was repeated in triplicate. *$p < .05$, **$p < .01$, and ***$p < .001$. d, Western blotting shows the levels of flag, BAX, Bcl-2, Caspase 3 and Cleaved-caspase 3.

was reduced after *ZSCAN23* re-expression (Figure 4c), suggesting that *ZSCAN23* expression suppressed PDAC growth *in vivo*.

ZSCAN23 expression inhibited Wnt signaling by interacting with MYH9

Co-immunoprecipitation assays were performed to further evaluate the mechanism of action of *ZSCAN23* in PDAC. An extra band, which was pulled down using a Flag antibody, was excised and analyzed using mass spectrometry (Figure 4d). The protein with the highest score was myosin heavy chain 9 (MYH9), and its interaction with ZSCAN23 was validated using co-IP and reciprocal co-IP assay (Figure 4e). MYH9 has been recently reported to promote the progression of PDAC and other cancers via Wnt signaling.[30–32] By analyzing the potential molecules in the mass spectrometry results, Wnt signaling proteins were found to represent an important part of the complex (Supplementary Table S1 and Supplementary Figure S1). Subsequently, we analyzed the role of *ZSCAN23* in Wnt signaling. The expression levels of β-catenin, c-myc, and cyclin D1 were reduced, while *p*-β-catenin expression level was increased after re-expression of *ZSCAN23* in SW1990 and Panc10.05 cells (Figure 4f). The effect of *ZSCAN23* on Wnt signaling via MYH9 was validated by knocking down *MYH9* in these cells (Figure 4g,h). These results demonstrate that *ZSCAN23* inhibited Wnt signaling by interacting with MYH9 in PDAC cells.

Discussion

Epigenetic based "synthetic lethality" represents a novel strategy for cancer therapy. Applying this theory to cell fate or DNA damage repair genes with abnormal epigenetic changes may provide additional opportunities for targeted therapy without damaging normal cells.[18,19] Therefore, the discovery of novel epigenetic markers involved in cell fate determination or DNA damage repair is becoming increasingly important. The SCAN domain proteins play multifaceted roles in embryonic development, DNA damage repair, and cancers.[33–35] However, most of these members have not been studied extensively. *ZKSCAN3* is involved in Wnt/β-catenin signaling and causes chromosomal instability.[36] *ZSCAN18* is a tumor suppressor in breast cancer and has been found to inhibit Wnt/β-catenin and glycolysis signaling pathways.[34] *ZSCAN23* is a new member of this family and has recently been reported to contain a C2H2 zinc-finger structure.[21] However, its regulation of expression and role in cancer remain unclear. In this study, we found that *ZSCAN23* was frequently methylated in PDAC and its expression was regulated by promoter region methylation, suggesting that *ZSCAN23* methylation is a potential PDAC diagnostic marker and epigenetic regulation involves in PDAC development. Then, we studied the function of *ZSCAN23* in PDAC. It was observed that *ZSCAN23* exerted inhibitory effects on cell proliferation and colony formation in PDAC cells. Furthermore, the use of a transwell assay demonstrated that *ZSCAN23* suppressed cell migration and invasion, which was further confirmed at the molecular level through the assessment of MMP2, MMP7 and MMP9 expression in PDAC cells. G1/S arrest and apoptosis were induced by *ZSCAN23*. The G1/S checkpoint regulatory proteins, cyclin D1, cyclin E1, cyclin A2 and CDK2, were reduced. Pro-apoptotic proteins, BAX and cleaved caspase-3, were increased. While Bcl-2, an anti-apoptotic protein, was decreased by restoration of *ZSCAN23* expression. *ZSCAN23* suppressed Panc10.05 cell xenografts growth in mice. To further understand the mechanism of *ZSCAN23* in PDAC, immunoprecipitation and mass spectrometry assay were applied. The interaction of ZSCAN23 and MYH9 was discovered, and further validation was performed using reciprocal co-IP assay and western blotting. MYH9 was reported to be involved in Wnt, PI3K-AKT and NOTCH signaling pathways in cancers.[31,37–40] The analysis of the binding proteins in the complex of ZSCAN23 antibody revealed the presence of significant components related to Wnt signaling. Wnt signaling is a well-known signaling pathway for development and disease.[41] It is divided into canonical and non-canonical pathways. The canonical pathway is involved in cell fate, proliferation, differentiation and migration. While the non-canonical pathway regulates cell polarity and migration.[42] Recent findings have indicated that inhibiting Wnt signaling can induce a *BRCA*-like state in cancer.[43,44] The dysregulation of Wnt signaling varies across different types of cancer, with hyperactivation of the canonical Wnt pathway observed in colorectal, pancreatic, ovarian and gastric cancers.[44–46] Then, we studied the role of *ZSCAN23* on Wnt signaling. Our findings revealed that *ZSCAN23* acted as an inhibitor of Wnt signaling by interacting with MYH9, and epigenetic silencing of *ZSCAN23* activated Wnt signaling in PDAC cells. The impact of *ZSCAN23* on Wnt signaling through MYH9 was validated by small interfering RNAs (siRNAs) knockdown technique. The complexity of epigenetics in cancer requires a more extensive study. Epigenetic-based synthetic lethality is becoming a new therapeutic strategy in human cancer.[18,47–49] Our finding paves the way for further investigation of synthetic lethality in PDAC. Better understanding the role of *ZSCAN23*, especially in the regulatory network, may improve the therapeutic strategies in PDAC in the future.

In conclusion, *ZSCAN23* is frequently methylated in PDAC, and its expression is regulated by promoter region methylation. *ZSCAN23* serves as a novel tumor suppressor that inhibits Wnt signaling in PDAC.

Materials and methods

Human tissue samples and cell lines

A total of 284 cases of primary PDAC and 17 cases of adjacent tissue of serous cystadenoma were obtained from surgically resected tissue samples at the Chinese PLA General Hospital without chemo-radiotherapy before surgery. Tumors were classified according to the standard TNM staging system (AJCC on Cancer 8th Edition Cancer Staging Manual). All human tissue samples were collected according to the

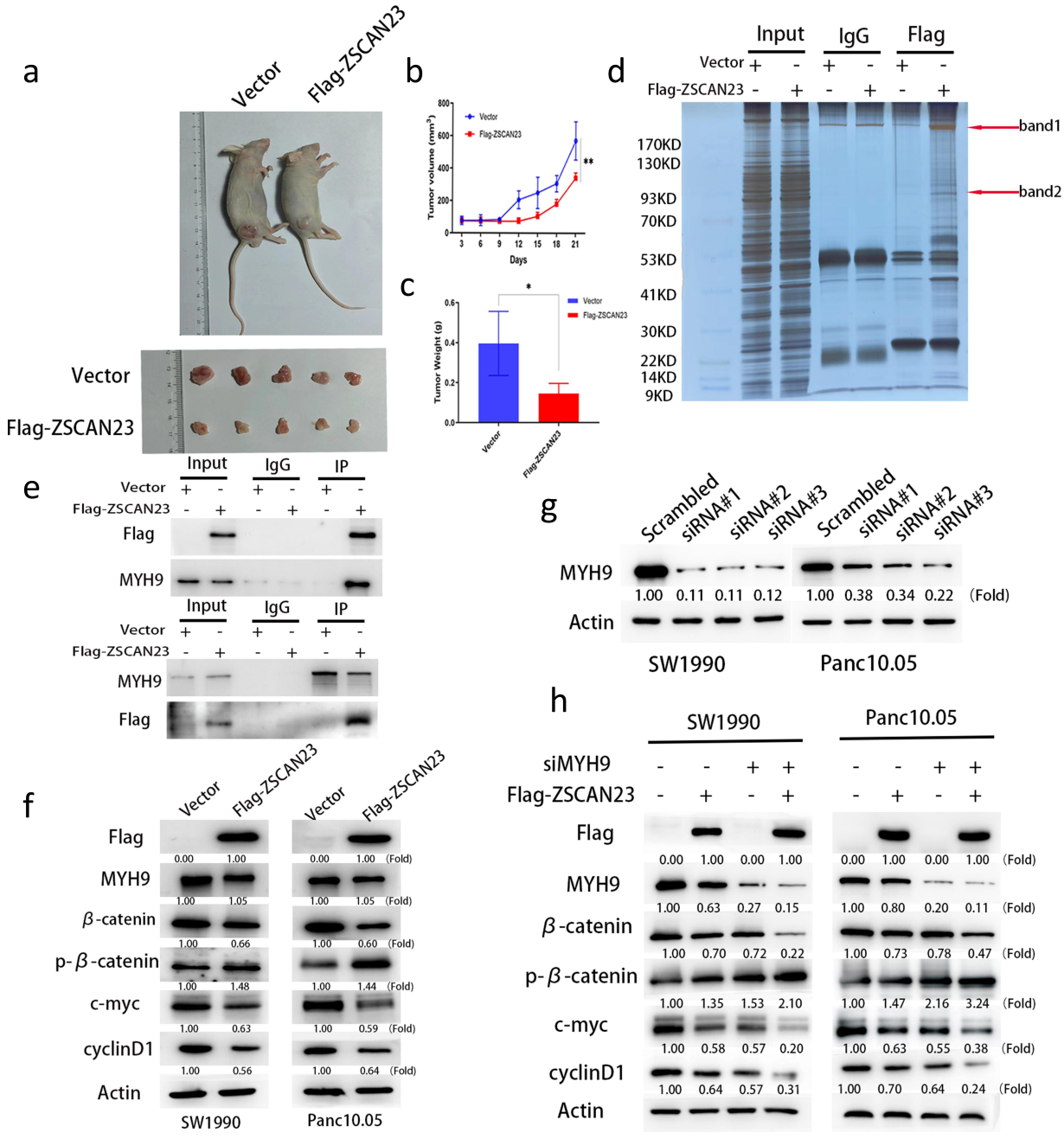

Figure 4. *ZSCAN23* suppresses human PDAC cell xenograft growth in mice and ZSCAN23 suppresses the wnt signaling pathway through MYH9. a, representative tumors from *ZSCAN23* unexpressed and re-expressed Panc10.05 cell xenografts. b, tumor weights of nude mice at the 21st day after inoculation of *ZSCAN23* unexpressed and re-expressed Panc10.05 cells. Bars present the mean values for five mice. $^{**}p < .01$. c, tumor growth curves of *ZSCAN23* unexpressed and *ZSCAN23* re-expressed Panc10.05 cells. $^{*}p < .05$. d, Co-IP assay and silver staining. e, Co-IP assay shows the binding between ZSCAN23 and MYH9 in SW1990 cells. IgG: negative control. f, Western blotting shows the levels of flag, MYH9, β-catenin, *p*-β-catenin, c-myc and cyclinD1 in PDAC cells. g, Western blotting shows the effects of *MYH9* knockdown by different siRNAs. Scrambled: siRNA negative control; siRNA#1 and siRNA#2: siRNA for *MYH9*. h, Western blotting shows the levels of flag, MYH9, β-catenin, *p*-β-catenin, c-myc and cyclinD1 before and after knockdown of *MYH9* in PDAC cells.

guidelines approved by the Institutional Review Board of the Chinese PLA General Hospital.

Ten PDAC cell lines were established from primary pancreatic cancer cells and cultured in RPMI-1640 medium (Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (GeminiBio, West Sacramento, CA, USA).

5-AZA-2'-deoxycytidine treatment, RNA preparation, and semiquantitative RT-PCR

5-AZA-DC (Sigma Aldrich, St. Louis, MO, USA) treatment for cell lines, total RNA preparation, and first-strand cDNA synthesis were performed as described previously.[50] The semiquantitative RT-PCR primer sequences used for *ZSCAN23*

were 5′-CATCTCCTCTCACGGATGCC-3′ (F) and 5′-TCTGAAGGGTCAAGGTTATGGC-3′ (R). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primers were used as described previously.[48]

Sodium bisulfite treatment and methylation detection

DNA extraction, sodium bisulfite, MSP, and bisulfite sequencing were performed as previously described.[51] The following primers were used: 5′-AAGATTATAGA CGGGATTTAGAGTGAC-3′ (methylation forward) and 5′-ACTACAAAAACCGAAAATACGTCACCG-3′ (methylation reverse); 5′-TTAAGATTATAGATGGGATTTAGAG TGAT-3′ (unmethylation forward) and 5′-TAAACT ACAAAAACCAAAAATACATCACCA-3′ (unmethylation reverse). The following bisulfite sequencing primers were used: 5′-AATAYGTGTTAAAATATTATGTTGG-3′ (forward) and 5′-CTAACRCAAATCACATCTACTC-3′ (reverse).

Construction of ZSCAN23 expression plasmid and screening of ZSCAN23 stably expressing cells

The full-length CDS sequence (NM_001012455.2) and 3 × Flag sequences of *ZSCAN23* were synthesized by Azenta Life Sciences (Suzhou, China), and cells that stably expressed *ZSCAN23* were generated by constructing pCDH-CMV-MCS-puro vectorand screening cells via puromycin-based selection for 3 d.[52] The puromycin concentration used was 6.0 μg/ml for SW1990 cells and 2.0 μg/ml for Panc10.05 cells; monoclonal cells were selected via dilution in 96-well plates.

Cell proliferation analysis

A total of 1×10^3 SW1990 and 2×10^3 Panc10.05 cells (*ZSCAN23*-silenced and overexpressed) were seeded in 96-well plates and grown for 4 d. The OD values were measured using a microplate reader. Six-well plates were used for colony formation analysis, as previously described.[53]

Transwell, cell cycle, and apoptosis analysis

For cell migration analysis, 4×10^4 SW1990 and 2×10^4 Panc10.05 cells (*ZSCAN23*-silenced and overexpressed) were used for Transwell analysis. Each experiment was repeated three times. After coating the upper chamber with matrigel, the same cell numbers and procedures were used for studying cell invasion. Cell cycle and apoptosis were analyzed as described previously.[50]

Western blotting

Cell lysis was prepared as previously described.[54] Antibodies were used as follows: MMP2 (Huaxingbio, China), caspase 3/cleaved-caspase 3, MMP9, c-myc, MYH9, β-catenin, actin (Proteintech, Rosemont, IL, USA), Flag Tag Rabbit mAb, MMP7, and *p*-β-catenin (ZENBIO, China).

PDAC cell xenograft mice

Four-week-old BABL/c nude mice were used to establish *ZSCAN23*-silenced and overexpressing Panc10.05 cells (4.5×10^6 cells) in a xenograft tumor model by following previously described procedures, according to the Animal Ethics Committee of the Chinese PLA General Hospital.[55]

Co-immunoprecipitation and mass spectrometry

ZSCAN23 silenced and re-expressing SW1990 cells were lysed using cold IP buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl, 0.1% DTT, 1% PIC, 1% PI, and 1% PMSF). Specific antibodies or IgG (Beyotime Biotech, China) were added to the cell lysates and incubated overnight. Protein A/G beads (Yeasen Biotechnology, Shanghai, China) were incubated with the antibody – protein complex for at least 4 h. The bead-antibody-protein complex was washed with IP buffer, and then the protein loading buffer was added to the complex. The IP products were analyzed via silver staining (Thermo Fisher Scientific, Waltham, MA, USA) and mass spectrometry, and then validated by western blotting.

RNA interference assay

SiRNAs used to target *MYH9* and scrambled control duplex sequences were as follows: siRNA#1: 5′-GCAAGCUGCCGAUAAGUAUTT-3′ (sense, S) and 5′-AUACUUAUCGGCAGCUUGCTG-3′ (antisense, AS); siRNA#2: 5′-CCAGAAGGCGCAGAC UAAATT-3′ (S) and 5′-UUUAGUCUGCGCCUUCU GGAC-3′ (AS); siRNA#3: 5′-CCAGUCCUCUGACAA GUUUTT-3′ (S) and 5′-AAACUUGUCAGAGGAC UGGTG-3′ (AS); siRNA scrambled control duplex: 5′-UUCUCCGAACGUGUCACGUTT-3′ (S) and 5′-ACGUGACACGUUCGGAGAATT-3′ (AS).

Statistical analysis

SPSS software (version 22.0; IBM, Armonk, NY, USA) was used for the statistical analysis. The association between *ZSCAN23* methylation and clinicopathological factors was analyzed using the chi-squared test. Quantitative data were evaluated using the Student's t-test. Values of $p < .05$ were considered significant.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the Chinese PLA General Hospital.

Availability of data and materials

The data in the present study are available from the corresponding author.

Author contributions

QD and AG performed experiments. QD and MZ helped analyze the data. QD and MG wrote the manuscript. MG designed the study and edited the manuscript. TH and MG interpreted the data. All the authors have read and approved the final version of the manuscript.

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