

# Epigenetic inactivation of *IRX4* is responsible for acceleration of cell growth in human pancreatic cancer

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## Abstract

Epigenetic gene silencing by aberrant DNA methylation is one of the important mechanisms leading to loss of key cellular pathways in tumorigenesis. Methyl-CpG-targeted transcriptional activation (MeTA) reactivates hypermethylation-mediated silenced genes in a different way from DNA-demethylating agents. Microarray coupled with MeTA (MeTA-array) identified seven commonly hypermethylation-mediated silenced genes in 12 pancreatic ductal adenocarcinoma (PDAC) cell lines. Among these, we focused on *IRX4* (Iroquois homeobox 4) because *IRX4* is located at chromosome 5p15.33 where PDAC susceptibility loci have been identified through genome-wide association study. *IRX4* was greatly downregulated in all of the analyzed 12 PDAC cell lines by promoter hypermethylation. In addition, the *IRX4* promoter region was found to be frequently and specifically hypermethylated in primary resected PDACs (18/28; 64%). Reexpression of *IRX4* inhibited colony formation and proliferation in two PDAC cell lines, PK-1 and PK-9. In contrast, knockdown of *IRX4* accelerated cell proliferation in an *IRX4*-expressing normal pancreatic ductal epithelial cell line, HPDE-1. Because *IRX4* is a sequence-specific transcription factor, downstream molecules of *IRX4* were pursued by microarray analyses utilizing tetracycline-mediated *IRX4* inducible PK-1 and PK-9 cells; *CRYAB*, *CD69*, and *IL32* were identified as *IRX4* downstream candidate genes. Forced expression of these genes suppressed colony formation abilities for both PK-1 and PK-9. These results suggest that DNA methylation-mediated silencing of *IRX4* contributes to pancreatic tumorigenesis through aberrant transcriptional regulation of several cancer-related genes.

## KEYWORDS

epigenetic gene silencing, *IRX4*, MeTA, methyl-CpG binding domain, pancreatic ductal adenocarcinoma

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## 1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive malignancy with an overall 5-year survival rate of less than 8% in Japan.<sup>1</sup> Although surgical resection is currently the only possible curative method for PDAC in the early stages, only a minority (10%-15%) of patients are amenable to such surgery at the time of diagnosis, because it is difficult to detect the early symptoms and the tumor cells in this disease are aggressive.<sup>2</sup> Identifying individuals at risk of developing PDAC and developing better diagnostic markers of pancreatic neoplasia will improve early diagnosis of precursor lesions and allow more patients to undergo curative surgical resection.<sup>3-5</sup> Previous studies have indicated that aberrant methylation of CpG islands contributes to PDAC development and progression.<sup>6,7</sup> In addition, aberrant DNA methylation increases during neoplastic development among the precursor lesions known as pancreatic intraepithelial neoplasm and intraductal papillary mucinous neoplasms.<sup>7</sup> Aberrantly hypermethylated genes in PDAC have been identified by comparing gene expression profiles of PDAC cells before and after treatment with DNA-demethylating agents<sup>6</sup> and by using promoter and CpG island arrays.<sup>8</sup> The detection of aberrantly methylated loci relative to normal tissues could improve the diagnosis of PDAC<sup>3</sup> and might also assist in identifying key regulatory genes and pathways as therapeutic targets.<sup>9</sup>

In order to identify critical hypermethylated and silenced genes in cancer cells, we previously developed a method called methyl-CpG targeted transcriptional activation (MeTA), which can globally reactivate hypermethylated genes in the genome.<sup>10-12</sup> In MeTA, a fusion gene encoding for the methyl-CpG binding domain (MBD) from MBD2 protein and the nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcriptional activation domain (AD) is transfected into cancer cells. The MBD specifically binds to the promoter regions of hypermethylated genes, and NF- $\kappa$ B (AD) recruits p300/CREB-binding protein and reactivates epigenetically silenced genes.<sup>13</sup> As one example, MeTA prompted the reactivation of methylated *MLH1* gene in human embryonic kidney cell line 293T (HEK293T) with the enhanced acetylation of histone H3 lysine 9/14 at the promoter region. Microarray coupled with MeTA (MeTA-array) provides not only information about hypermethylated genes but also about transcriptional repression in a single experiment.<sup>11</sup> In addition, because MeTA-array searches for hypermethylated genes by a completely different mechanism than does DNA-demethylating agent-based microarray, this method enables identification of hypermethylated genes that were difficult to find by the previous conventional methods.<sup>11,12</sup> In a previous study, we tested MeTA-array on three PDAC cell lines (AsPC-1, MIA PaCa-2, and PANC-1) as well as a normal pancreatic ductal epithelial cell line, HPDE-1, to identify commonly hypermethylated genes in PDAC.<sup>12</sup> Although this pilot study indicated the suitability of the MeTA method to identify hypermethylated and silenced genes, we needed to use many more PDAC cells in order to detect significant methylated genes in pancreatic ductal tumorigenesis. In the present study, we used 12 PDAC cell lines for MeTA and identified seven commonly hypermethylated and silenced genes. Among them, we

focused on the *IRX4* gene because *IRX4* has also been identified as a susceptibility gene in prostate cancer.<sup>14</sup>

A member of the Iroquois family of homeobox transcription factors, *IRX4* is expressed mainly in the developing ventricle of the heart and acts as an important mediator of ventricular differentiation during cardiac development.<sup>15</sup> Several *IRX* family members are known to be involved in tumorigenesis. For examples, *IRX1* expression is lost or reduced in gastric cancer,<sup>16</sup> *IRX3* is downregulated in androgen-insensitive prostate cancer cells,<sup>17</sup> and *IRX5* is suggested to be involved in the regulation of both the cell cycle and apoptosis in human prostate cancer cells.<sup>18</sup> Genome-wide association studies (GWAS) identified a number of prostate cancer susceptibility loci; *IRX4* is a possible causative gene of the prostate cancer susceptibility on chromosome 5p15.<sup>14</sup> In prostate cancer, variants in a putative enhancer region could alter *IRX4* expression. Knockdown of *IRX4* enhanced the growth of prostate cancer cells, whereas the forced expression of *IRX4* suppressed them. In PDAC, GWAS also identified a susceptibility locus on 5p15.33.<sup>19</sup> Although we found that *IRX4* is hypermethylated in PDAC in this study, the significance of *IRX4* hypermethylation in PDAC remains unclear. To examine the biological significance of the *IRX4* gene in pancreatic tumorigenesis, we constructed a tetracycline-mediated inducible gene expression system for *IRX4* by using two PDAC cells (PK-1 and PK-9) and asked whether the reactivation of *IRX4* suppresses cancer cell growth.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

Twenty-one human cell lines were used in this study: an immortal cell line derived from normal pancreatic ductal epithelium, HPDE-1, and 20 PDAC cell lines, AsPC-1, MIA PaCa-2, PANC-1, BxPC-3, PAN-09-JCK, PCI-6, PCI-24, PCI-35, PCI-43, PCI-55, PK-1, PK-8, PK-9, PK-12, PK-14, PK-36, PK-45P, PK-45H, PK-47, and PK-59. HPDE-1 was a kind gift from Dr M-S Tsao (University of Toronto, Canada)<sup>20</sup> and was grown in HuMedia-KG2 (Kurabo). AsPC-1, MIA PaCa-2, PANC-1, and BxPC-3 were purchased from ATCC. The remaining 16 cell lines have been described previously.<sup>21</sup> Most of the PDAC cell lines were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. MIA PaCa-2 and PANC-1 were grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS.

### 2.2 | Construction of plasmids

*Escherichia coli* strain DH5 $\alpha$ F<sup>+</sup> was used to propagate all of the plasmids. The plasmid to induce MeTA, pcDNA6-3xFLAG-NF- $\kappa$ B (AD)-MBD, was constructed previously.<sup>10</sup> pcDNA6/TR plasmid (Invitrogen) was used to establish cell lines that stably express the Tet repressor protein. The *IRX4*, *CRYAB*, *CD69*, and *IL32* cDNA clones

were PCR amplified by using the pooled human cDNA mix<sup>22</sup> or HPDE-1 cDNA as the templates and were cloned into the pcDNA6/myc-His vector for colony formation assay. Nucleotide sequences of the primers used for cDNA cloning are described in Table S1. To allow tetracycline-regulated expression of *IRX4*, the 70-bp fragment of 3xFLAG and the 1.6-kbp fragment of *IRX4* were cloned into the *Bam*HI and *Xho*I sites of pcDNA4/TO/myc-His (Invitrogen) vector to generate pcDNA4/TO/3xFLAG-*IRX4*.

### 2.3 | Transfection

Nine PDAC cell lines (PK-1, PK-8, PK-9, PK-45P, PK-45H, PK-59, PCI-6, PCI-35, and BxPC-3) were seeded in 6-well tissue culture dishes and transfected with an aliquot of 4  $\mu$ g of pcDNA6/myc-His vector or pcDNA6-3xFLAG-NF- $\kappa$ B (AD)-MBD using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's recommendations. The cells were harvested 48 hours after transfection for immunoblotting and microarray experiments. The other four cell lines (AsPC-1, MIA PaCa-2, PANC-1, and HPDE-1) had been previously analyzed.<sup>12</sup>

### 2.4 | Immunoblotting

Immunoblotting experiments were undertaken as described previously.<sup>23</sup> An Ab that specifically recognizes FLAG (F 1804; 1:1000, Sigma-Aldrich) or  $\beta$ -actin (A-5441; 1:3000, Sigma-Aldrich) was used.

### 2.5 | Microarray analyses

Microarray analyses were carried out according to methods described previously.<sup>12</sup> Nine PDAC cell lines (PK-1, PK-8, PK-9, PK-45P, PK-45H, PK-59, PCI-6, PCI-35, and BxPC-3) were transfected with the plasmid containing NF- $\kappa$ B (AD)-MBD or the vector alone, and PK-1-*IRX4*#28 and PK-9-*IRX4*#23 cells were treated with or without 1  $\mu$ g/mL tetracycline (Invitrogen) for 4 days. Total RNAs from these cells were purified and Cy-3 labeled cRNAs were prepared for hybridization to Human Gene Expression 4  $\times$  44K v2 Microarray Kit (Agilent Technologies). The cut-off value for twofold upregulation was used for selection of the genes. High-throughput microarray data are available in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession numbers GSE26215, GSE141246, and GSE141247.

### 2.6 | CpG island analyses

For CpG island (CGI) identification, we analyzed DNA sequences from 1 kb upstream of the transcription start site (TSS) to 1 kb downstream (CGI TSS  $\pm$  1000-bp). The CGI searcher (<http://cpgislands.usc.edu/>) was used for Takai and Jones' criteria: %GC  $\geq$  55, length  $\geq$  500 bp, and the observed CpG/expected CpG value  $\geq$  0.65.<sup>24</sup>

### 2.7 | Reverse transcription-PCR and Southern blotting

Total RNAs were extracted using RNeasy Mini Kit (Qiagen) and the single-stranded cDNA was synthesized as described previously.<sup>11</sup> Reverse transcription-PCR amplifications using intron-spanning primers and Southern hybridization were carried out as described previously.<sup>25</sup> Quantitative real-time RT-PCR analysis of *IRX4*, *CRYAB*, *CD69*, and *IL32* was carried out as described previously.<sup>26</sup> *B2M* was used as the internal control.<sup>27</sup> Nucleotide sequences of the primers used for RT-PCR and Southern hybridization are described in Table S1.

### 2.8 | Methylation-specific PCR

Genomic DNAs were extracted using the DNeasy Blood & Tissue Kit (Qiagen) and were bisulfite-treated using the EpiTect Bisulfite Kit (Qiagen). Methylation status of the *IRX4* gene was determined by methylation-specific PCR (MSP). The methylated and unmethylated band intensities were quantified by ImageJ software (NIH),<sup>28</sup> and the ratio between methylated and unmethylated was calculated for each sample; a twofold or higher increase in tumor as compared with corresponding normal sample was defined as tumor-specific methylation. The PCR products were separated in a 3% agarose gel. Primers used for MSP analyses are listed in Table S1.

### 2.9 | Tissue specimens

Primary PDACs and their corresponding normal tissue counterparts were obtained at surgery from a total of 28 patients with PDAC at Tohoku University Hospital (Sendai, Miyagi, Japan). These specimens were collected between January 2005 and September 2009, and their clinicopathologic characteristics are summarized in Table S2. The tissue specimens were snap-frozen in liquid nitrogen after resection and stored at  $-80^{\circ}\text{C}$ . Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of Tohoku University School of Medicine under the accession numbers of 2015-1-473 and 2015-1-474.

### 2.10 | Validation of *IRX4* methylation and expression statuses by The Cancer Genome Atlas and Gene Expression Omnibus databases

In order to confirm our results, we downloaded the methylation data (platform: Illumina HumanMethylation450 BeadChip) from The Cancer Genome Atlas (TCGA) and gene expression data (platform: Affymetrix Human Genome U133 Plus 2.0 Array) from Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) of the NCBI. TCGA database had registered 184 PDAC patients and 10 normal control specimens from PDAC patients. The SMART (Shiny Methylation Analysis Resource Tool) App<sup>29</sup> was used to analyze

DNA methylation data from TCGA. *IRX4* expression levels in 36 PDACs and matching normal pancreatic tissues from ICF pancreatic cancer patients (GSE15471) were also analyzed.

### 2.11 | Colony formation assay

PK-1 and PK-9 cells were seeded in 6-well tissue culture dishes 24 hours before transfection. Each aliquot of 4  $\mu\text{g}$  pcDNA6-derived DNA was transfected using Lipofectamine 2000 reagent (Invitrogen). After 24 hours of transfection,  $4 \times 10^4$  cells were replated onto 10-cm culture dishes and allowed to grow in culture medium containing 5  $\mu\text{g}/\text{mL}$  blasticidin (Invitrogen) for approximately 2 weeks. After colonies were observed, cells were washed in ice-cold PBS and fixed with ice-cold methanol for 10 minutes. Methanol was removed, and cells were stained with crystal violet solution (0.5% w/v) for 10 minutes at room temperature. Visible colonies were manually counted, and digital images of the colonies were obtained using a camera.

### 2.12 | Establishment of tetracycline-regulated *IRX4*-expressing cell lines

The PDAC cell lines PK-1 and PK-9 that allow tetracycline-regulated *IRX4* expression were constructed in two steps using the T-REx system (Invitrogen). First, cells were seeded in 6-well tissue culture dishes and transfected with 4  $\mu\text{g}$  pcDNA6/TR plasmid digested with *AhdI* using Lipofectamine 2000 reagent. Cells were selected with RPMI-1640 medium containing 5  $\mu\text{g}/\text{mL}$  blasticidin at 48 hours after transfection to obtain Tet repressor-expressing parental cell lines. Selection medium was changed every 3 days. PK-1\_TR4 and PK-9\_TR4 parental cells were selected because the immunoblotting experiment using the monoclonal TetR Ab (clone 9G9, 1:1000; Clontech Laboratories) showed strong bands corresponding to Tet repressor, and transient expression of pcDNA4/TO/myc-His/lacZ (Invitrogen) resulted in sevenfold or fourfold induction of  $\beta$ -galactosidase activities following addition of 1  $\mu\text{g}/\text{mL}$  tetracycline. Second, pcDNA4/TO/3xFLAG-*IRX4* plasmid was digested with *PvuI* and was transfected into PK-1\_TR4 or PK-9\_TR4 cells to yield PK-1\_IRX4#2 and #28 cells or PK-9\_IRX4#23 and #24 cells. PK1\_IRX4 and PK9\_IRX4 cells were maintained in RPMI-1640 medium containing 5  $\mu\text{g}/\text{mL}$  blasticidin and 250  $\mu\text{g}/\text{mL}$  zeocin (Invitrogen).

### 2.13 | Cell proliferation assay

For cell proliferation assay, cells were seeded in 24-well tissue culture dishes at a density of  $3 \times 10^3$  cells per well 1 day before the assay started. After the first assay, cells were treated with or without 1  $\mu\text{g}/\text{mL}$  tetracycline for 2, 4, or 6 days. Cell viability was determined by alamarBlue assay (Invitrogen). Every other following day, 0.5 mL medium containing 5% alamarBlue reagent was replaced

and absorbance was measured using 540/590 nm (ex/em) filter settings with a Fluoroskan Ascent Microplate Reader (Labsystems). Experiments were undertaken in quadruplicate and repeated three times.

### 2.14 | Cell cycle analyses

Cells were grown in the presence of 1  $\mu\text{g}/\text{mL}$  tetracycline for 2, 4, or 6 days. Tetracycline-containing medium was replaced every other day. Cells were harvested, washed in PBS, and fixed in ice-cold 70% ethanol for 2 hours at 4°C. Then  $1 \times 10^6$  cells were collected by centrifugation, suspended in 1 mL PBS containing 250  $\mu\text{g}/\text{mL}$  RNase A, and incubated for 60 minutes at 37°C. Cellular DNAs were stained with propidium iodide (final concentration, 50  $\mu\text{g}/\text{mL}$ ) for 30 minutes at 4°C. Cell cycle distributions of samples were determined by flow cytometric analysis (FACSCanto II; BD Biosciences).

### 2.15 | Knockdown experiments

Knockdown experiments were undertaken according to the methods described previously.<sup>30</sup> Double-stranded siRNA was purchased from Integrated DNA Technologies. Cells were seeded on 6-well tissue culture dishes and transfected with siRNA targeting against one of the following genes: *GL2* (a negative control targeting firefly luciferase),<sup>31</sup> *IRX4*, *CRYAB*, *CD69*, and *IL32*. Nucleotide sequences of the siRNAs used for knockdown experiments are shown in Table S1.

### 2.16 | Statistical analyses

A two-tailed Student's *t* test was used for statistical analyses of comparative data. Pearson's test was used for association between methylation status and clinicopathological parameters. The Kaplan-Meier method was used to analyze patient survival and significance was tested by the Wilcoxon test. *P* values of less than .05 were considered as significant. All analyses were carried out by using JMP (SAS Institute).

## 3 | RESULTS

### 3.1 | Methyl-CpG-targeted transcriptional activation array identifies seven commonly hypermethylated and silenced genes in PDAC cell lines

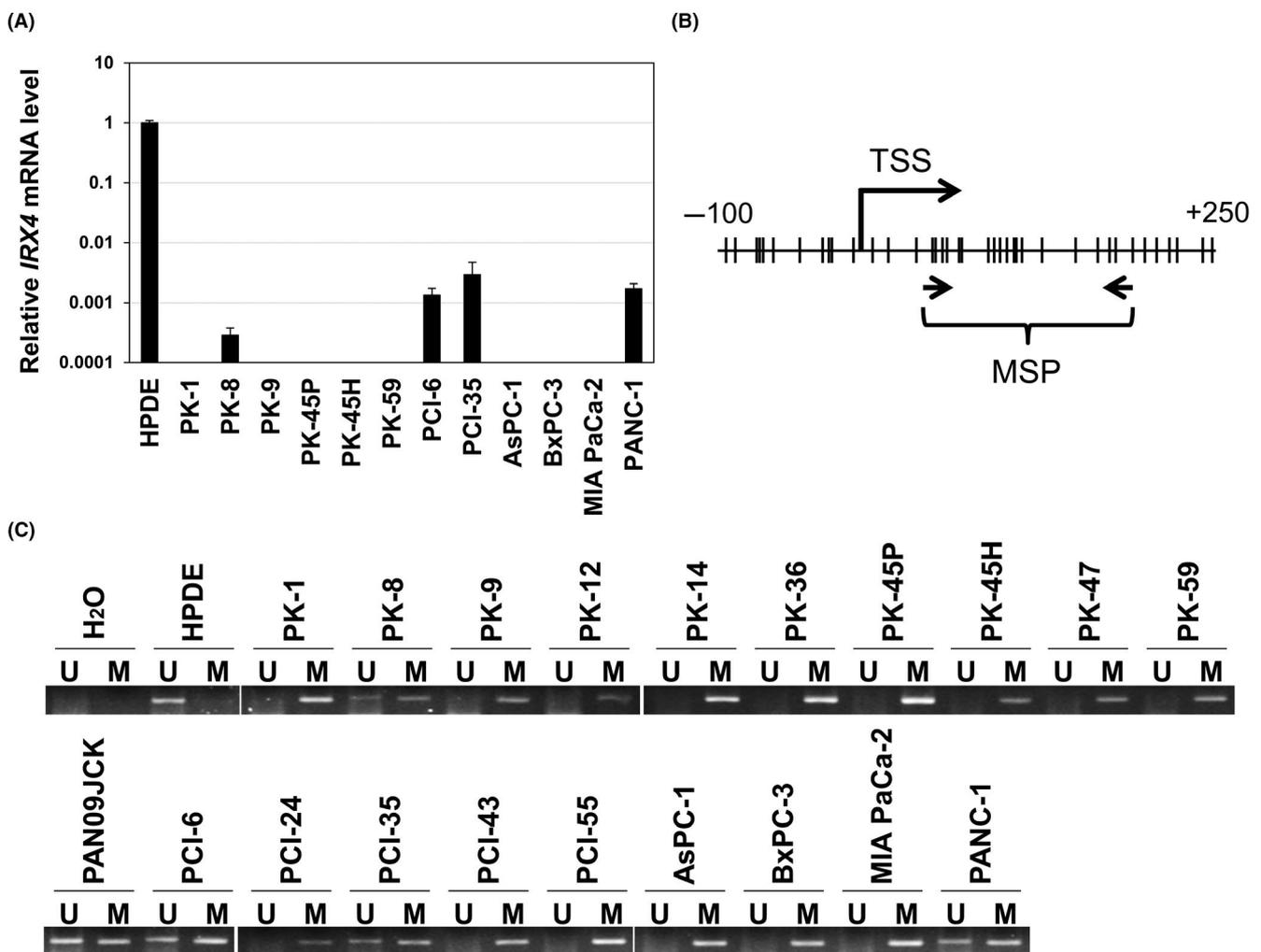
In the previous study, we applied MeTA-array to three PDAC cell lines, AsPC-1, MIA PaCa-2, and PANC-1, with normal pancreatic ductal epithelial cell line HPDE-1 as a control, and identified three hypermethylated genes, *CSMD2*, *SLC32A1*, and *TRH*, all specific for PDAC.<sup>12</sup> In this study, we tried to screen for more commonly

hypermethylated and silenced genes in PDAC by using nine more PDAC cell lines, BxPC-3, PCI-6, PCI-35, PK-1, PK-8, PK-9, PK-45P, PK-45H, and PK-59. We first transfected the empty vector or the MeTA-inducible plasmid containing NF- $\kappa$ B (AD)-MBD into the nine cell lines and confirmed the expression of the NF- $\kappa$ B (AD)-MBD fusion protein only in MeTA-inducible plasmid-transfected cell lines by immunoblot analysis (data not shown). Total RNA from each transfected cell line was also subjected to gene expression microarray to see whether the MeTA-array can detect hypermethylated and transcriptionally silenced genes in PDAC cells. A number of genes were upregulated twofold or more in these PDAC cell lines as well as in a nonneoplastic pancreatic ductal epithelial cell line HPDE-1. Among these, 31 genes were selected because they were commonly upregulated twofold or more by MeTA in six or more PDAC cell lines, but not in HPDE-1 (Table S3). These 31 genes contained CpG islands defined by Takai and Jones in the promoter regions with extremely high frequency (28/31, 90.3%); such CpG islands could be the

targets for aberrant DNA methylation in PDAC cells. Interestingly, 26 of these 31 genes have not been reported previously in the DNA-demethylating agent-mediated studies.<sup>6</sup> Seven of these 31 genes were further selected by RT-PCR followed by Southern hybridization (Figure S1): these seven genes were expressed moderately to strongly in HPDE-1 but were not expressed or weakly expressed in most PDAC cell lines. All seven genes reactivated by MeTA contained CGIs within  $\pm 1000$  bp of the TSS.

### 3.2 | *IRX4* is downregulated and hypermethylated in all 12 PDAC cell lines

Among the seven genes selected by MeTA-array, we selected the *IRX4* gene in PDAC for analysis. *IRX4* is a transcription factor with a homeobox domain that is known to play an important role in heart development. It has been mapped to one of the PDAC



**FIGURE 1** Reduced expression levels of *IRX4* through DNA methylation in pancreatic ductal adenocarcinoma (PDAC) cell lines. A, Expression levels of *IRX4* in 12 PDAC cell lines as well as in a nonneoplastic pancreatic ductal epithelial cell line HPDE-1 were analyzed by quantitative RT-PCR. B, CpG sites (solid bars) and methylation-specific PCR (MSP) primers (arrows) around the *IRX4* transcription start site (TSS) are indicated. C, MSP analyses of the *IRX4* gene in 20 PDAC cell lines and HPDE-1. *IRX4* was highly methylated in 100% (20/20) of PDAC cell lines but unmethylated in the nonneoplastic HPDE-1 cell. M, methylated-specific PCR products; U, unmethylated-specific PCR products

susceptibility loci by GWAS (5p15.33) and possibly plays a role in pancreatic tumorigenesis. Using semiquantitative (Figure S1) and quantitative RT-PCR (Figure ), we first measured the amounts of *IRX4* mRNA in 12 PDAC cell lines with HPDE-1 as the control and found that the expression level of *IRX4* was extremely low in all 12 lines in comparison with HPDE-1; PCI-35 cells, which showed the highest expression level of *IRX4* among the 12 PDAC cell lines, have only 1/400 expression level of HPDE-1. We designed a pair of MSP primers in the *IRX4* promoter region (Figure 1B) to assess whether *IRX4* is methylated specifically in PDAC cell lines, and tested 20 PDAC cell lines as well as HPDE-1 (Figure 1C). *IRX4* was unmethylated in HPDE-1, but aberrant methylation of *IRX4* was detected in all 20 PDAC cell lines (100%); it was completely methylated in 15 (75%) and partially methylated in five (25%) of the 20 cell lines. Interestingly, four of these PDAC cell lines with *IRX4* expression levels above the detection limits were all partially methylated. Although no *IRX4* expression of cell line PAN-09-JCK has yet been detected, it is likely that the expression level and promoter methylation level of *IRX4* are correlated in PDAC cell lines.

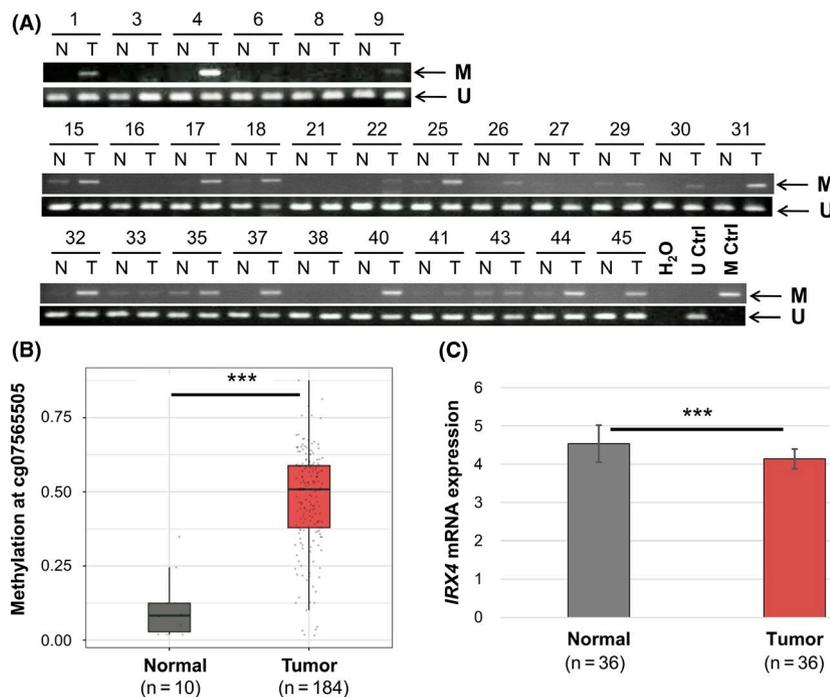
### 3.3 | *IRX4* aberrantly methylated in primary pancreatic carcinomas

To examine whether the aberrant methylation of *IRX4* was also present in primary PDACs, we analyzed the methylation statuses

of 28 paired cancerous and normal DNAs from patients with primary PDAC after resected specimens (Figure 2A). Although primary tumor specimens could contain a considerable quantity of contaminating normal cells because these samples were not microdissected, aberrant methylation of *IRX4* was clearly detected in a tumor-specific manner in 18 (64%) of 28 primary PDACs. Aberrant methylation of *IRX4* promoter region in primary PDACs was also observed in TCGA database (Figure 2B), and *IRX4* expression was suppressed in primary PDACs in GEO datasets (Figure 2C). We next compared the clinicopathologic features of PDAC patients showing *IRX4* gene methylation ( $n = 18$ ) and unmethylation ( $n = 10$ ) in terms of differentiation, staging, infiltration, and invasion (Table S2), but we found no significant relationships between methylation status and these characteristics. Furthermore, the cumulative survival rates of those two groups of PDAC patients did not show any significant differences based upon their methylation status, using the Kaplan-Meier method (Figure S2).

### 3.4 | Induced *IRX4* expression suppresses growths of PDAC cells

To examine whether *IRX4* can inhibit growth of PDAC cell lines, we first undertook colony formation assays using PK-1 and PK-9 cells. Compared with empty vector-transfected cells, *IRX4* expression vector-transfected cells formed significantly reduced numbers of colonies (Figure S3). To



**FIGURE 2** Aberrant DNA methylation and reduced expression of *IRX4* in primary pancreatic tumors. A, methylation-specific PCR analyses of *IRX4* in 28 pairs of primary pancreatic tumors (T) and normal pancreatic ductal epithelium (N). All primary tumors include amplification with the unmethylated-specific PCR product (U) primer set; results of the presence of normal contaminating cells. Note that *IRX4* was highly methylated in primary pancreatic ductal adenocarcinoma (PDAC) tissues. M, methylated-specific PCR product; M Ctrl, methylated DNA control; U Ctrl, unmethylated DNA control. B, DNA methylation statuses of *IRX4* promoter region in primary PDACs analyzed by The Cancer Genome Atlas cohort. Probe cg07565505 resides within 200 bp of the *IRX4* transcription start site. C, *IRX4* expression levels between normal and tumor tissues were analyzed by using Gene Expression Omnibus datasets

further examine the effects of reactivation of hypermethylated genes on cancer cell phenotypes, we constructed an inducible expression system of *IRX4* in PK-1 and PK-9 cells. The Tet repressor-expressing plasmid was transfected into PK-1 and PK-9 to generate stable cell lines PK-1\_TR4 and PK-9\_TR4. Then the plasmid containing *IRX4* with FLAG tag at the N-terminus was transfected into these two generated cell lines to yield stable cell lines PK-1\_IRX4#28 and PK-9\_IRX4#23. As indicated in Figure S4, immunoblot analyses using anti-FLAG Ab indicated that the FLAG-*IRX4* protein was produced in PK-1\_IRX4#28 and PK-9\_IRX4#23 cells in a tetracycline-dependent manner.

To examine the cell proliferation of PK-1 and PK-9 cells in response to *IRX4* induction, we used the alamarBlue assay that quantitatively measures chemical reduction of growth medium resulting from cell proliferation. Tet repressor-expressing cell lines PK-1\_TR4 and PK-9\_TR4 did not show any growth differences either with or without tetracycline (Figure S5). In contrast, the *IRX4* inducible cell lines PK-1\_IRX4#28 and PK-9\_IRX4#23 showed significant growth suppression 4–6 days after tetracycline addition (Figure 3A,B). We also used another set of cell lines (PK-1\_IRX4#2 and PK-9\_IRX4#24) and found that these results were reproducible (Figure S6). We next undertook FACS analysis of PK1\_IRX4#28 and PK9\_IRX4#23 cells to determine whether the growth suppression by *IRX4* induction affects the cell cycle of PDAC cells. Our results of flow cytometry analyses in PK1\_IRX4#28 cells are shown in Figure 3C; sub-G<sub>1</sub> fractions in PK1\_IRX4#28 cells increased on days 4 and 6 after tetracycline addition. The G<sub>1</sub> phase of cells decreased over time, whereas the G<sub>2</sub>/M phase of cells increased over time. In contrast, flow cytometry analyses in PK9\_IRX4#23 cells did not show any differences with or without tetracycline addition (data not shown). We additionally used siRNA-mediated knockdown of *IRX4* using normal pancreatic ductal epithelial cell line HPDE-1 to determine the influence on cell growth. Representative results of HPDE-1 are shown in Figure 3D,E. *IRX4* expression was significantly reduced by siRNA transfection (Figure 3D), and corresponding acceleration of cell proliferation was evident (Figure 3E).

### 3.5 | *IRX4* induction upregulates several genes with tumor suppressor function

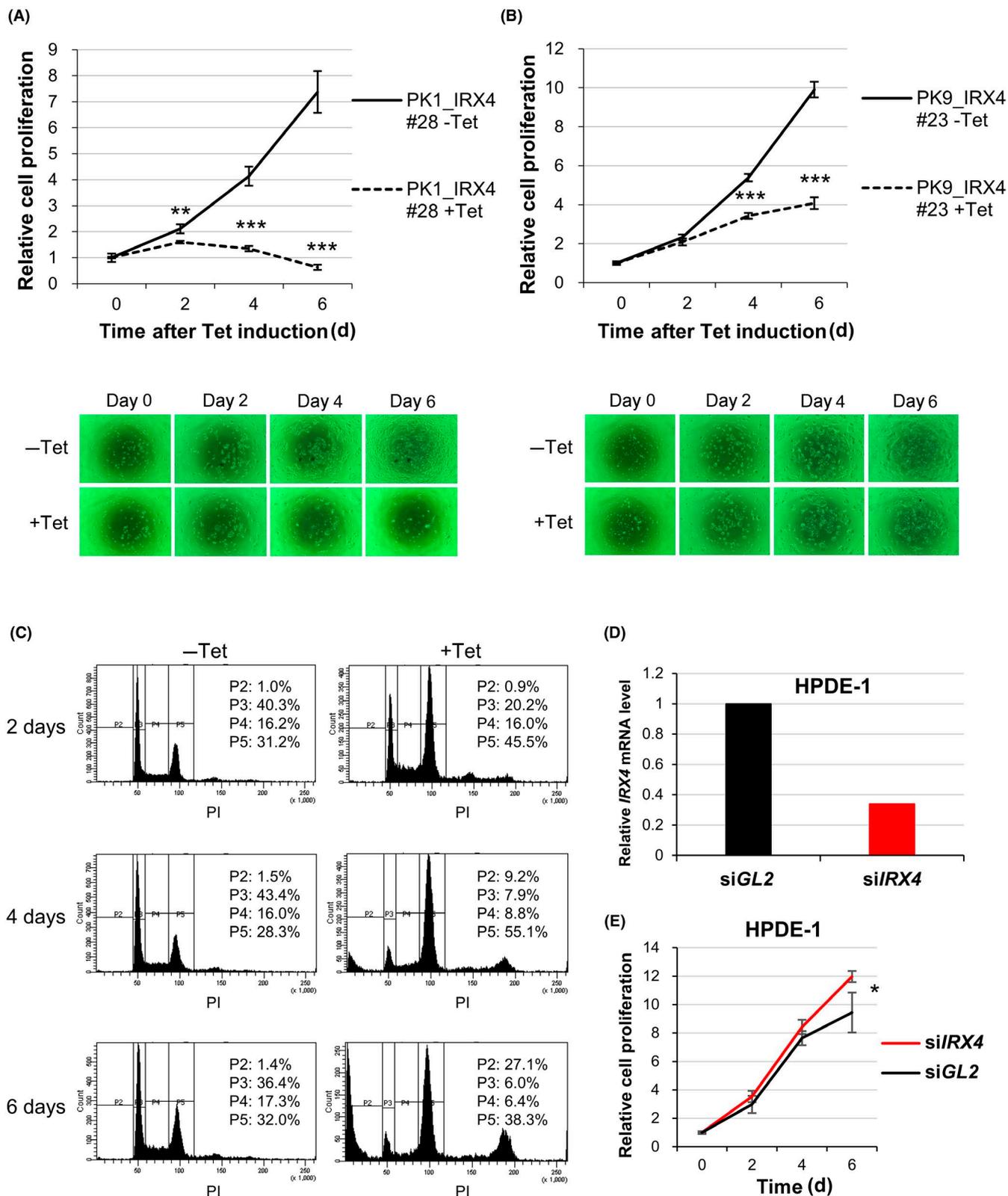
In order to search for downstream genes of *IRX4* in PDAC cells, we undertook gene expression microarray analyses using PK-1\_IRX4#28 and PK-9\_IRX4#23 cells with or without tetracycline. With tetracycline, a total of 99 genes were commonly upregulated twofold or more in these two cell lines. Among these candidate *IRX4* downstream genes, we selected six, *CRYAB* (crystallin alpha B), *CD69*, *IL32* (interleukin 32), *FST* (follistatin), *UCHL1* (ubiquitin C-terminal hydrolase L1), and *USP44* (ubiquitin specific peptidase 44), because these genes were known to have tumor suppressive function. As shown in Figure 4A, RT-PCR analyses of the *CRYAB*, *CD69*, and *IL32* genes were clearly upregulated with tetracycline induction of *IRX4* in both PK-1\_IRX4#28 and PK-9\_IRX4#23 cells. In order to confirm the tumor suppressive ability of these genes in PDAC cells, we undertook colony formation assays. The forced expression of each gene

reduced colony numbers compared with empty vector transfection (Figure 4B). The expression of *IL32* in both PK-1 and PK-9 cells suppressed colony formation more strongly than the expressions of *CRYAB* and *CD69*. To further confirm the ability of each candidate *IRX4* downstream gene, we assessed whether the knockdown of the gene can cancel the inhibition of cell growth in PK9\_IRX4#23 by *IRX4* induction. The *CRYAB*, *CD69*, and *IL32* genes successfully suppressed their expressions after knockdown (Figure 4C). Cell growths were partially recovered from the *IRX4* induction-mediated growth suppression by the knockdown of *CRYAB* and *CD69*, but *IL32* did not show such outcome (Figure 4D). In order to analyze downregulation of these candidate *IRX4* downstream genes in conjunction with suppression of *IRX4*, we surveyed GEO datasets on pancreatic cancer and found significant downregulation of *CRYAB* in cancerous tissue compared to normal tissue, but not in *CD69* or *IL32* (Figure S7).

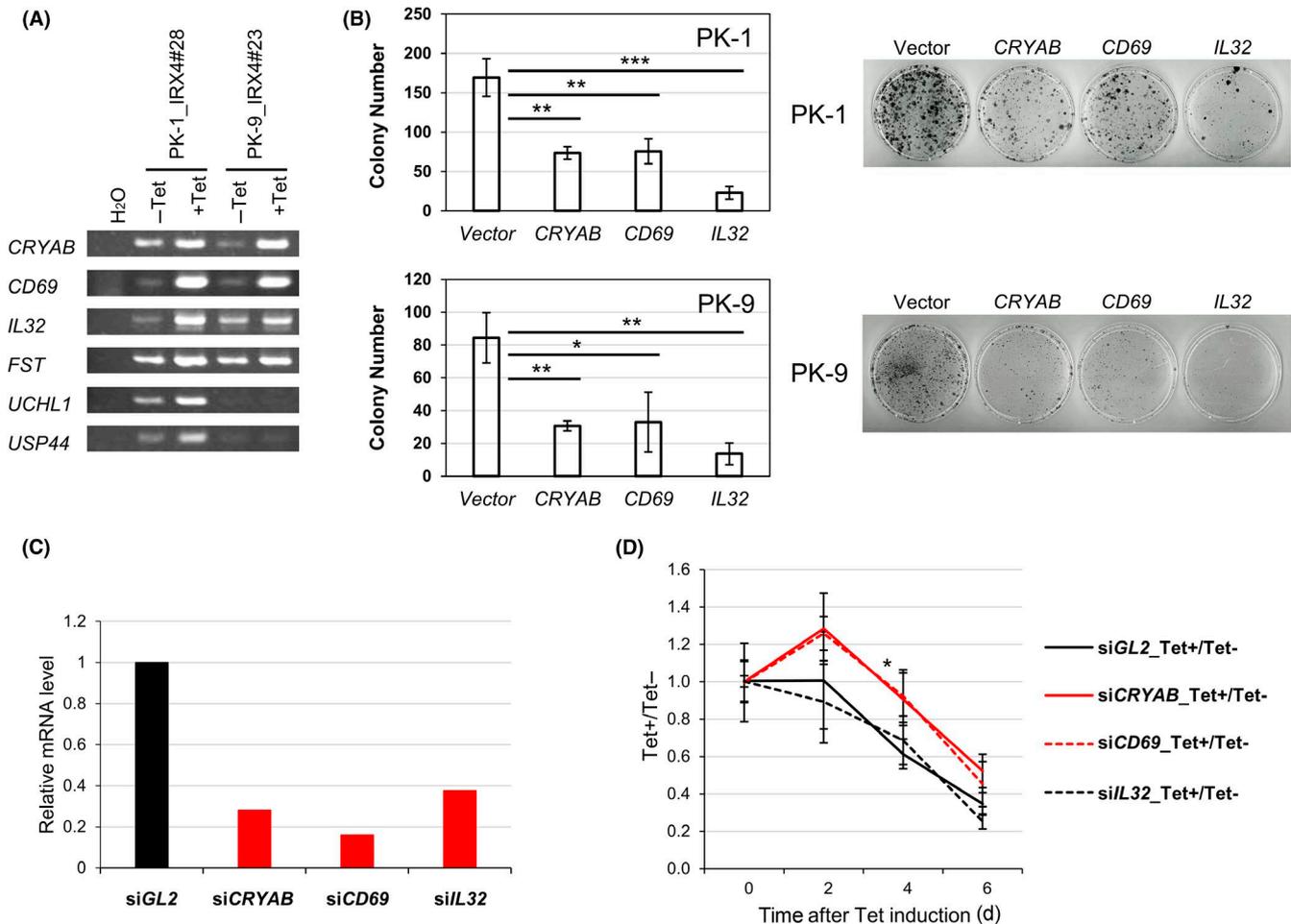
## 4 | DISCUSSION

The previous pilot study using three PDAC cell lines and a normal pancreatic ductal epithelial cell line indicated that MeTA-array could identify a number of hypermethylated genes specific for PDAC.<sup>12</sup> In addition, the overlapped number of genes selected by both MeTA and a conventional DNA-demethylating agent was not very high, probably because the properties of the two methods differed.<sup>11</sup> Methyl-CpG-targeted transcriptional activation upregulates hypermethylated genes by transcriptional activation, whereas DNA-demethylating agents upregulate hypermethylated genes by DNA demethylation. Therefore, a MeTA-array could lead to the discovery of significant hypermethylated genes in pancreatic tumorigenesis that were not detected using conventional methods. In this study, we examined 12 PDAC cell lines and a normal pancreatic ductal epithelial cell line HPDE-1 by MeTA-array and tried to detect more commonly hypermethylated genes among our series of PDAC cells. This MeTA-array analysis identified *TMEM204*, *NPTX2*, *NEFL*, *NEFM*, *NEFH*, *IRX4*, and *LHX6* as the most commonly methylated genes in PDAC; however, *TMEM204* was not methylated in a cancer-specific manner<sup>12</sup> and thus was eliminated from further analyses. The *NPTX2* gene has been reported to inhibit proliferation and invasion in PDAC,<sup>32</sup> and three neurofilament genes, *NEFL*, *NEFM*, and *NEFH*, are methylated in many kinds of cancers, including PDAC, and are associated with an aggressive phenotype of breast cancer.<sup>33</sup> The *LHX6* gene is also hypermethylated in cervical,<sup>34</sup> lung,<sup>35</sup> breast,<sup>36</sup> and pancreatic<sup>37</sup> cancers; its forced expression suppressed tumorigenic phenotypes such as cell proliferation, migration, and invasion in these cancer cells.

*IRX4* is reported as one of the prostate cancer susceptibility genes, but hypermethylation of *IRX4* has not been reported in prostate cancer.<sup>14</sup> Our strategy to identify the hypermethylated genes using the MeTA-array identified *IRX4* as being a highly probable candidate tumor suppressor gene in pancreatic tumorigenesis, and the cell proliferation assay, colony formation assay, and FACS analyses all demonstrated its tumor suppressor functions. Furthermore,



**FIGURE 3** A, B, Effects of *IRX4* induction on cell proliferation of pancreatic ductal adenocarcinoma cell lines, PK-1 and PK-9. Cell proliferation assay for PK-1\_ *IRX4* #28 (A) and PK-9\_ *IRX4* #23 (B) cells. Tetracycline (Tet) addition strongly inhibited cell proliferation of PK-1\_ *IRX4* #28 (A) and PK-9\_ *IRX4* #23 (B) cells. The panels below show the light microscopic appearance of PK-1\_ *IRX4* #28 (A) and PK-9\_ *IRX4* #23 (B) cells (40x magnification) at 0, 2, 4, and 6 days after treatment with or without tetracycline. C, Flow cytometry for PK-1\_ *IRX4* #28 cells. PK-1\_ *IRX4* #28 cells treated with tetracycline for 2, 4 and 6 days induced apoptotic events and G<sub>2</sub>/M arrest in a time-dependent manner. D, *IRX4* expression was analyzed by quantitative RT-PCR and confirmed suppressed expression by siRNA in HPDE-1 normal pancreatic ductal epithelial cells. E, Cell proliferation was accelerated by knockdown of *IRX4* in HPDE-1. \**P* < .05, \*\**P* < .01, \*\*\**P* < .001



**FIGURE 4** *IRX4* upregulates a subset of genes with tumor suppressive functions in pancreatic ductal adenocarcinoma (PDAC) cell lines. A, RT-PCR analyses of six genes showed that a subset of genes was upregulated after *IRX4* inducible expression in PK-1 and PK-9 cells. B, Colony formation assays of *CRYAB*, *CD69*, and *IL32* indicated that the expression of these genes significantly reduced the colony formation ability of PK-1 and PK-9 PDAC cell lines. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ . C, Expression levels of *CRYAB*, *CD69* and *IL32* in PK9\_IRX4#23 cells after siRNA-mediated knockdown were determined by quantitative RT-PCR analyses. D, Knockdown of *CRYAB* or *CD69*, but not *IL32*, partially recovers the inhibition of PK9\_IRX4#23 cell growth after *IRX4* induction. \* $P < .05$

three candidate downstream molecules have been identified with their growth suppressive functions. *IRX4* is a transcription factor with a homeobox domain and plays a crucial role in cardiac development. *IRX4* interacts with a heterodimer of vitamin D receptor (VDR) and retinoic X receptor (RXR) to regulate the expression of their target genes in cardiomyocytes.<sup>38</sup> However, VDR, but not RXR, can bind to *IRX4* in prostate cancer cells.<sup>14</sup> Furthermore, *IRX4* and VDR expressions can affect each other in prostate cancer cells; *IRX4* knockdown results in reduction of VDR at the mRNA level. Vitamin D receptor exerts antiproliferation and antidifferentiation effects in many types of cancers, including PDAC, but the molecular mechanisms of the tumor suppressive effects of the vitamin D and VDR pathways have not yet been elucidated. Recently, the expression of VDR in pancreatic normal and tumor tissues from a cohort of 61 patients with PDAC was analyzed to examine the potential correlation between VDR and pathological characteristics.<sup>39</sup> Interestingly, VDR expression was significantly lower or undetectable in 75.7% (28/37) of tissues with low differentiation ( $P = .004$ ). Kaplan-Meier analysis

has indicated that a low level of VDR expression in tumor tissues is associated with a poor prognosis ( $P = .037$ ). The expression levels of both *IRX4* and VDR in PDAC should be analyzed to identify their correlations. The understanding of their relationship could facilitate new approaches to preventing PDAC and improving survival.

In this study, we identified three genes, *CRYAB*, *CD69*, and *IL32*, that were upregulated with *IRX4* reexpression and have tumor suppressive functions. *CRYAB* has been reported to play a role as an oncogene or a tumor suppressor gene in different cancer types. *CRYAB* is downregulated in bladder cancer and inhibits migration and invasion through the PI3K/AKT and ERK pathways, whereas upregulation of *CRYAB* marginally affects bladder cancer cell proliferation and apoptosis.<sup>40</sup> Additionally, *CRYAB* suppresses nasopharyngeal carcinoma progression by associating with the cadherin/catenin adherens junctions and modulating  $\beta$ -catenin function.<sup>41</sup> Ketamine induces the apoptosis of lung adenocarcinoma cells by upregulating *CD69* expression.<sup>42</sup> The mechanism of *CD69*-mediated apoptosis remains unknown but could be explained by the association with an

N-terminal fragment of calreticulin at the cell surface.<sup>43</sup> In addition, microRNA-205 is silenced in prostate cancer; its reexpression induces apoptosis and cell cycle arrest through the specific activation of tumor suppressor genes *IL24* and *IL32*.<sup>44</sup> Interleukin 32 $\beta$  inhibits tumor growth by increasing cytotoxic lymphocyte numbers, and by inactivating the NF- $\kappa$ B and STAT3 pathways through changing of cytokine levels in tumor tissues.<sup>45</sup> Aberrant methylation of *IRX4* would downregulate the expression of *IRX4* and thereby downregulate downstream genes with tumor suppressive functions, such as *CRYAB*, *CD69*, and *IL32* in pancreatic tumorigenesis. Further analyses should clarify the precise roles of each gene in PDAC.

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## CONFLICT OF INTEREST

The authors have no conflict of interests.

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## REFERENCES

1. *Monitoring of Cancer Incidence in Japan – Survival 2006–2008 Report*. Tokyo, Japan: Center for Cancer Control and Information Services, National Cancer Center; 2016.
2. Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. *Gut*. 2007;56:1134-1152.
3. Hruban RH, Canto MI, Goggins M, et al. Update on familial pancreatic cancer. *Adv Surg*. 2010;44:293-311.
4. Matsubayashi H, Canto M, Sato N, et al. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. *Cancer Res*. 2006;66:1208-1217.
5. Canto MI, Goggins M, Hruban RH, et al. Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study. *Clin Gastroenterol Hepatol*. 2006;4:766-781.
6. Sato N, Fukushima N, Maitra A, et al. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res*. 2003;63:3735-3742.
7. Sato N, Ueki T, Fukushima N, et al. Aberrant methylation of CpG islands in intraductal papillary mucinous neoplasms of the pancreas. *Gastroenterology*. 2002;123:365-372.
8. Omura N, Li CP, Li A, et al. Genome-wide profiling of methylated promoters in pancreatic adenocarcinoma. *Cancer Biol Ther*. 2008;7:1146-1156.
9. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome—components and functional correlates. *Genes Dev*. 2006;20:3215-3231.
10. Fukushima S, Kondo E, Horii A. Methyl-CpG targeted transcriptional activation allows re-expression of tumor suppressor genes in human cancer cells. *Biochem Biophys Res Commun*. 2008;377:600-605.
11. Sato Y, Horii A, Fukushige S. Microarray coupled with methyl-CpG targeted transcriptional activation (MeTA-array) identifies hypermethylated genes containing the stringent criteria of CpG islands at high frequency. *Epigenetics*. 2011;6:752-759.
12. Shimizu H, Horii A, Sunamura M, et al. Identification of epigenetically silenced genes in human pancreatic cancer by a novel method “microarray coupled with methyl-CpG targeted transcriptional activation” (MeTA-array). *Biochem Biophys Res Commun*. 2011;411:162-167.
13. Fukushige S, Kondo E, Horii A. Methyl-CpG targeted recruitment of p300 reactivates tumor suppressor genes in human cancer cells. *Biochem Biophys Res Commun*. 2009;379:1021-1026.
14. Nguyen HH, Tanaka R, Akamatsu S, et al. *IRX4* at *5p15* suppresses prostate cancer growth through the interaction with vitamin D receptor, conferring prostate cancer susceptibility. *Hum Mol Genet*. 2012;21:2076-2085.
15. Bao ZZ, Bruneau BG, Seidman JG, et al. Regulation of chamber-specific gene expression in the developing heart by *lrx4*. *Science*. 1999;283:1161-1164.
16. Guo X, Liu W, Pan Y, et al. Homeobox gene *IRX1* is a tumor suppressor gene in gastric carcinoma. *Oncogene*. 2010;29:3908-3920.
17. Zhao H, Kim Y, Wang P, et al. Genome-wide characterization of gene expression variations and DNA copy number changes in prostate cancer cell lines. *Prostate*. 2005;63:187-197.
18. Myrthue A, Rademacher BL, Pittsenbarger J, et al. The Iroquois homeobox gene 5 is regulated by 1,25-dihydroxyvitamin D<sub>3</sub> in human prostate cancer and regulates apoptosis and the cell cycle in LNCaP prostate cancer cells. *Clin Cancer Res*. 2008;14:3562-3570.
19. Petersen GM, Amundadottir L, Fuchs CS, et al. A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet*. 2010;42:224-228.
20. Furukawa T, Duguid WP, Rosenberg L, et al. Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. *Am J Pathol*. 1996;148:1763-1770.
21. Gu Z, Inomata K, Ishizawa K, et al. The *FBXW7*  $\beta$ -form is suppressed in human glioma cells. *Biochem Biophys Res Commun*. 2007;354:992-998.
22. Mizuguchi Y, Saiki Y, Horii A, Fukushima S. Targeted TET oxidase activity through methyl-CpG-binding domain extensively suppresses cancer cell proliferation. *Cancer Med*. 2016;5:2522-2533.
23. Kondo E, Horii A, Fukushima S. The human PMS2L proteins do not interact with hMLH1, a major DNA mismatch repair protein. *J Biochem*. 1999;125:818-825.
24. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci USA*. 2002;99:3740-3745.
25. Mori Y, Shiwaku H, Fukushima S, et al. Alternative splicing of *hMSH2* in normal human tissues. *Hum Genet*. 1997;99:590-595.
26. Saiki Y, Yoshino Y, Fujimura H, et al. *DCK* is frequently inactivated in acquired gemcitabine-resistant human cancer cells. *Biochem Biophys Res Commun*. 2012;421:98-104.
27. Ogawa K, Shiga K, Saijo S, et al. A novel G106D alteration of the *SDHD* gene in a pedigree with familial paraganglioma. *Am J Med Genet A*. 2006;140:2441-2446.
28. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671-675.
29. Li Y, Ge D, Lu C. The SMART App: an interactive web application for comprehensive DNA methylation analysis and visualization. *Epigenetics Chromatin*. 2019;12:71.
30. Chen N, Sato D, Saiki Y, et al. *S100A4* is frequently overexpressed in lung cancer cells and promotes cell growth and cell motility. *Biochem Biophys Res Commun*. 2014;447:459-464.

31. Hata T, Furukawa T, Sunamura M, et al. RNA interference targeting aurora kinase A suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. *Cancer Res.* 2005;65:2899-2905.
32. Zhang L, Gao J, Li L, et al. The neuronal pentraxin II gene (*NPTX2*) inhibits proliferation and invasion of pancreatic cancer cells in vitro. *Mol Biol Rep.* 2011;38:4903-4911.
33. Calmon MF, Jeschke J, Zhang W, et al. Epigenetic silencing of neurofilament genes promotes an aggressive phenotype in breast cancer. *Epigenetics.* 2015;10:622-632.
34. Jung S, Jeong D, Kim J, et al. Epigenetic regulation of the potential tumor suppressor gene, *hLHX6.1* in human cervical cancer. *Int J Oncol.* 2011;38:859-869.
35. Liu WB, Jiang X, Han F, et al. *LHX6* acts as a novel potential tumour suppressor with epigenetic inactivation in lung cancer. *Cell Death Dis.* 2013;4:e882.
36. Hu Z, Xie L. *LHX6* inhibits breast cancer cell proliferation and invasion via repression of the Wnt/ $\beta$ -catenin signaling pathway. *Mol Med Rep.* 2015;12:4634-4639.
37. Abudurexiti Y, Gu Z, Chakma K, et al. Methylation-mediated silencing of the LIM homeobox 6 (*LHX6*) gene promotes cell proliferation in human pancreatic cancer. *Biochem Biophys Res Commun.* 2020;526:626-632.
38. Wang GF, Nikovits W Jr, Bao ZZ, et al. *Irx4* forms an inhibitory complex with the vitamin D and retinoic receptors to regulate cardiac chamber-specific slow MyHC3 expression. *J Biol Chem.* 2001;276:28835-28841.
39. Wang K, Dong M, Sheng W, et al. Expression of vitamin D receptor as a potential prognostic factor and therapeutic target in pancreatic cancer. *Histopathology.* 2015;67:386-397.
40. Ruan H, Li Y, Wang X, et al. *CRYAB* inhibits migration and invasion of bladder cancer cells through the PI3K/AKT and ERK pathways. *Jpn J Clin Oncol.* 2020;50:254-260.
41. Huang Z, Cheng Y, Chiu PM, et al. Tumor suppressor Alpha B-crystallin (*CRYAB*) associates with the cadherin/catenin adherens junction and impairs NPC progression-associated properties. *Oncogene.* 2012;31:3709-3720.
42. Zhou X, Zhang P, Luo W, et al. Ketamine induces apoptosis in lung adenocarcinoma cells by regulating the expression of *CD69*. *Cancer Med.* 2018;7:788-795.
43. Vance BA, Harley PH, Backlund PS, et al. Human *CD69* associates with an N-terminal fragment of calreticulin at the cell surface. *Arch Biochem Biophys.* 2005;438:11-20.
44. Majid S, Dar AA, Saini S, et al. MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. *Cancer.* 2010;116:5637-5649.
45. Yun H-M, Oh JH, Shim J-H, et al. Antitumor activity of IL-32 $\beta$  through the activation of lymphocytes, and the inactivation of NF- $\kappa$ B and STAT3 signals. *Cell Death Dis.* 2013;4:e640.

#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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