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ORIGINAL ARTICLE

Modulation of *PKM* alternative splicing by PTBP1 promotes gemcitabine resistance in pancreatic cancer cells

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and incurable disease. Poor prognosis is due to multiple reasons, including acquisition of resistance to gemcitabine, the first-line chemotherapeutic approach. Thus, there is a strong need for novel therapies, targeting more directly the molecular aberrations of this disease. We found that chronic exposure of PDAC cells to gemcitabine selected a subpopulation of cells that are drug-resistant (DR-PDAC cells). Importantly, alternative splicing (AS) of the pyruvate kinase gene (*PKM*) was differentially modulated in DR-PDAC cells, resulting in promotion of the cancer-related PKM2 isoform, whose high expression also correlated with shorter recurrence-free survival in PDAC patients. Switching *PKM* splicing by antisense oligonucleotides to favor the alternative PKM1 variant rescued sensitivity of DR-PDAC cells to gemcitabine and cisplatin, suggesting that PKM2 expression is required to withstand drug-induced genotoxic stress. Mechanistically, upregulation of the polypyrimidine-tract binding protein (PTBP1), a key modulator of *PKM* splicing, correlated with PKM2 expression in DR-PDAC cell lines. PTBP1 was recruited more efficiently to *PKM* pre-mRNA in DR- than in parental PDAC cells. Accordingly, knockdown of PTBP1 in DR-PDAC cells reduced its recruitment to the *PKM* pre-mRNA, promoted splicing of the PKM1 variant and abolished drug resistance. Thus, chronic exposure to gemcitabine leads to upregulation of PTBP1 and modulation of *PKM* AS in PDAC cells, conferring resistance to the drug. These findings point to PKM2 and PTBP1 as new potential therapeutic targets to improve response of PDAC to chemotherapy.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human cancers, being characterized by very low 5-year survival rate.[1] Lack of early symptoms and late diagnosis contribute to poor prognosis, with most patients presenting with metastasis. When surgical resection is unfeasible, chemotherapy with gemcitabine, administered either alone or in combination with other compounds, represents the clinical option for PDAC. Nevertheless, relapse always occurs with more aggressive features and insensitivity to chemotherapy, contributing to high lethality.[2,3] Thus, identification of new diagnostic markers and elucidation of the molecular pathways involved in acquisition of drug resistance represent clinical priorities for PDAC.[2,4]

Adaptation to variable stresses is a key feature of neoplastic cells. Recent evidence highlighted how cancer cells can flexibly modulate gene expression at the level of alternative splicing (AS) to withstand hostile conditions.[5–8] In this regard, changes in expression of some splicing factors have been directly linked to expression of oncogenic splice variants that confer various advantages to cancer cells.[9–14] Moreover, genotoxic stress was shown to modulate splicing regulation,[15] in some cases by affecting the localization or activity of specific splicing factors, such as SAM68(ref. 16) or EWS.[17] In the case of PDAC cells, it was previously shown that increased expression of the serine/arginine-rich protein kinase SRPK1, a prototypic splicing factor kinase, confers resistance to treatment with gemcitabine.[18] Notably, as SRPK1 modulates the activity of several serine/arginine protein splicing factors with implication in cancer,[19] including SRSF1,[20] it is likely that upregulation of this kinase contributes to the expression of oncogenic splice variants expressed in PDAC cells.[21]

Herein, we aimed at investigating the role of AS and splicing factors in the acquisition of a drug-resistant (DR) phenotype in PDAC cells. We observed that chronic treatment with gemcitabine promoted the formation of DR subpopulations highly resistant to drug-induced genotoxic stress. In order to understand the contribution of AS to the DR phenotype, we analyzed a group of cancer-related splice variants involved in oncogenic features.[5–8] We found that DR-PDAC cells exhibited a switch in *PKM* AS, a gene encoding two alternative splice variants, PKM1 and PKM2, through usage of mutually exclusive exons. PKM2 is typically expressed in cancer cells where it confers oncogenic features.[22–24] We show that splicing of PKM2 is favored in DR-PDAC cells with respect to the parental cells and promotes drug resistance, as interference with this splicing event in DR-PDAC cells restored sensitivity to gemcitabine and cisplatin. Mechanistically, we demonstrate that the polypyrimidine-tract binding protein PTBP1 is upregulated in DR-PDAC cells, and that its increased recruitment to the *PKM* pre-mRNA promotes PKM2 splicing. Knockdown of PTBP1 in DR-PDAC cells reduces its binding to *PKM* pre-mRNA, favors the expression of PKM1 and rescues drug sensitivity. Hence, our results indicate a positive role for PTBP1 and PKM2 in the acquisition of drug

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resistance, suggesting that this regulatory pathway represents a novel potential therapeutic target for PDAC.

RESULTS

Isolation of DR-PDAC cells

To isolate DR-PDAC cell sub-populations, we exposed to chronic treatment with gemcitabine (10 μM) the following two cell lines: Pt45P1, which displays higher sensitivity to the drug, and PANC-1, which is more resistant to treatment (Supplementary Figure S1A). As expected, gemcitabine caused massive cell death in both cell lines in the 7 days of treatment. However, 15 days after removal of the drug, few viable clones were visible in the plates of both cell lines. Clones were pooled, amplified and cultured by exposing them to a 24-h pulse of gemcitabine every other week, to maintain selection of the DR populations (Figures 1a and b).

To confirm that DR-PDAC cells were indeed more resistant to drug treatment than the parental cell line (PCL), we analyzed cell survival by colony formation assays. PCL- and DR-PDAC cells were cultured for 24 h with sub-optimal doses of gemcitabine and then allowed to grow in complete medium until they formed visible colonies (Figures 1c and d). Treatment with gemcitabine reduced the number of colonies in a dose-dependent manner in PCL cells, whereas DR cells were resistant to the lower dose of gemcitabine and less sensitive to the higher dose (Figures 1c and d). Analysis of cell death by Trypan blue cell count or by immunofluorescence analysis of the cleaved/activated form of caspase-3 confirmed that gemcitabine was more cytotoxic for PCL- than DR-PDAC cells (Supplementary Figures S1B and C). Collectively, these results indicate that the selected cell populations have acquired a DR phenotype.

PKM splicing is regulated in DR-PDAC cells

Recent evidence suggests a key role for misregulation of AS in the acquisition of oncogenic features and drug resistance by human cancer cells.[5–8] Thus, we tested whether PCL- and DR-PDAC cells display changes in splice variants of a subset of cancer-relevant genes. We selected a group of genes whose AS was reported to promote oncogenic features in cancer cells, such as the apoptotic genes *CASP9*,[25] *CASP2*,[26] *BCL-X*,[27] *BIM*[28] and *FAS*[29] (Figure 2a and Supplementary Figure 2A); genes involved in DNA repair and drug resistance, such as *USP5*[(ref. 30)] and *MKNK2*[(refs 31,32)] (Figure 2b and Supplementary Figure 2B); genes affecting basal metabolism, such as *PKM*[22] (Figure 2c); genes involved in cell migration and invasion, such as *RON*[10], *CD44*[(ref. 5)] and c-*MET*[33] (Figure 2d and Supplementary Figure 2C); or the cell cycle gene *CCND1*[(ref. 34)] (Figure 2e). Reverse transcription–PCR (RT–PCR) analysis showed that AS of most of these genes was either unchanged between

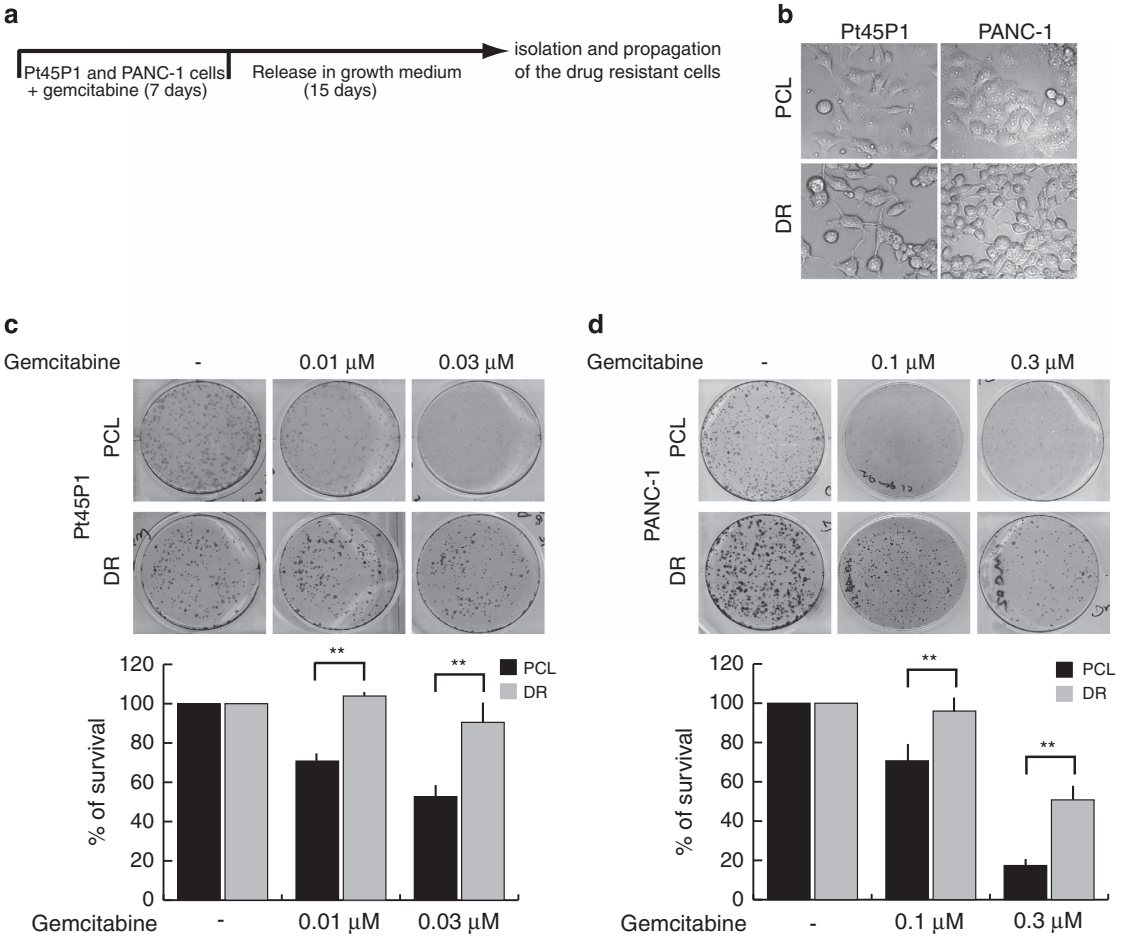

Figure 1. Chronic treatment with gemcitabine selects DR-PDAC cells. (**a**) Schematic representation of the protocol used to obtain DR-PDAC cells from parental PDAC cells (PCL). (**b**) Representative phase-contrast images of PCL- and DR-Pt45P1 (left panels) or PANC-1 (right panels) cells (×40 magnification). Representative images of the colony assay (upper panels) performed in PCL- and DR-Pt45P1 (**c**) or PANC-1 cells (**d**). (**c** and **d**) Bar graphs (bottom panels) show the percentage of survival with respect to untreated cells from three experiments (mean ± s.d.), as assessed by colony formation. Brackets indicate statistical comparison of the indicated samples. Statistical analyses were performed by the paired Student's *t*-test. $^{**}P \leqslant 0.01$.

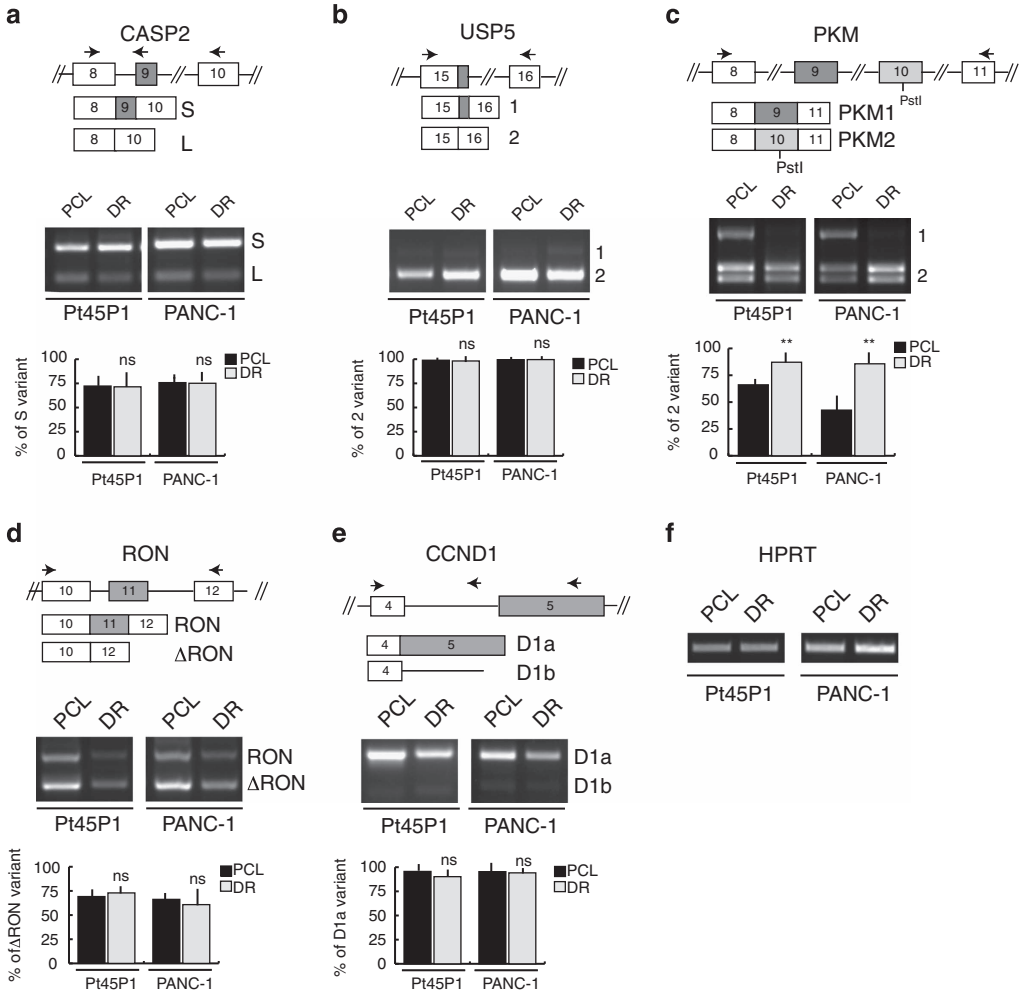

Figure 2. The PKM2 splice variant is promoted in DR-PDAC cells. (**a**–**e**) RT–PCR analysis in PCL- and DR-Pt45P1 or PANC-1 cells of splice variants encoded by the indicated cancer-related genes. Schematic representation of the cancer-related AS events analyzed is shown in the upper panels. Exons (boxes) and introns (lines) are indicated. Black arrows indicate primers used for the RT–PCR analysis (bottom panels). (**c**) RT–PCRs of *PKM* gene were followed by *Pst*I digestion in order to distinguish the amplicons. Bar graphs represent the percentage of the indicated AS variants, as assessed by densitometric analysis of the bands. Statistical analyses were performed by the paired Student's *t*-test comparing PCL- and DR-PDAC cell values (mean ± s.d., $n = 3$, $^{**}P < 0.01$; ns, not significant). (**f**) HPRT was used as loading control for RT–PCR analyses in **a**–**e**.

PCL- and DR-PDAC cells (*CASP2*, *CCND1*, *c-MET*, *USP5*, *MKNK2* and *RON*) or not modulated in the same direction in DR-PDAC cell lines (*CASP9*, *BCL-X*, *BIM*, *CD44* and *FAS*) (Figure 2 and Supplementary Figure 2). On the contrary, splicing of the PKM2 variant was favored with respect to PKM1 in both DR-PDAC cell lines (Figure 2c), suggesting that modulation of *PKM* AS correlated with acquisition of drug resistance in PDAC cells.

PKM2 protein is upregulated in DR-PDAC cells and correlates with relapse-free survival in PDAC patients

We focused on the regulation of *PKM* AS, because growing evidence supports a key role for this splicing event in tumorigenesis.[22,35] The PKM2 splice variant is prevalently expressed in cancer cells,[22,36] where it regulates processes spanning from cell metabolism[22,24] to transcription,[23] cell cycle[37] and cell death.[38,39] Differential expression of PKM1 and PKM2 in DR-PDAC cells was confirmed by RT–PCR analysis using primers positioned either in exon 9 (PKM1) or 10 (PKM2), to amplify each splice variant (Figure 3a). Furthermore, the switch in *PKM* splice variants was confirmed at the protein level, as DR-PDAC cells expressed higher levels of PKM2, whereas PKM1 was almost undetectable with respect to PCL-PDAC cells (Figure 3b). Notably, PANC-1 cells, which are more resistant to gemcitabine (Supplementary Figure S1A), also express higher levels of PKM2 and lower levels of PKM1 than the more sensitive Pt45P1 cells (Figure 3c). These observations indicate that the DR phenotype of PDAC cells correlates with increased expression of PKM2.

To assess the relevance of PKM2 *in vivo*, we investigated its expression levels by immunohistochemistry (IHC) in a cohort of 42 patients diagnosed with primary PDAC in the absence of metastases, who received radical surgery and subsequent gemcitabine-based adjuvant treatment. Our hypothesis was that patients expressing high levels of PKM2 could be more resistant to gemcitabine and display worse clinical outcome. The anti-PKM2 antibody was validated by immunofluorescence and western blot analyses of PANC-1 cells silenced for PKM2 and with mouse tissues expressing (embryonic) or not (adult) PKM2 (Supplementary Figures S3A–C). Titration analysis established 1:1600 as the optimal dilution for IHC (Supplementary Figure S3D). The neoplastic lesions of all 42 samples (100%)

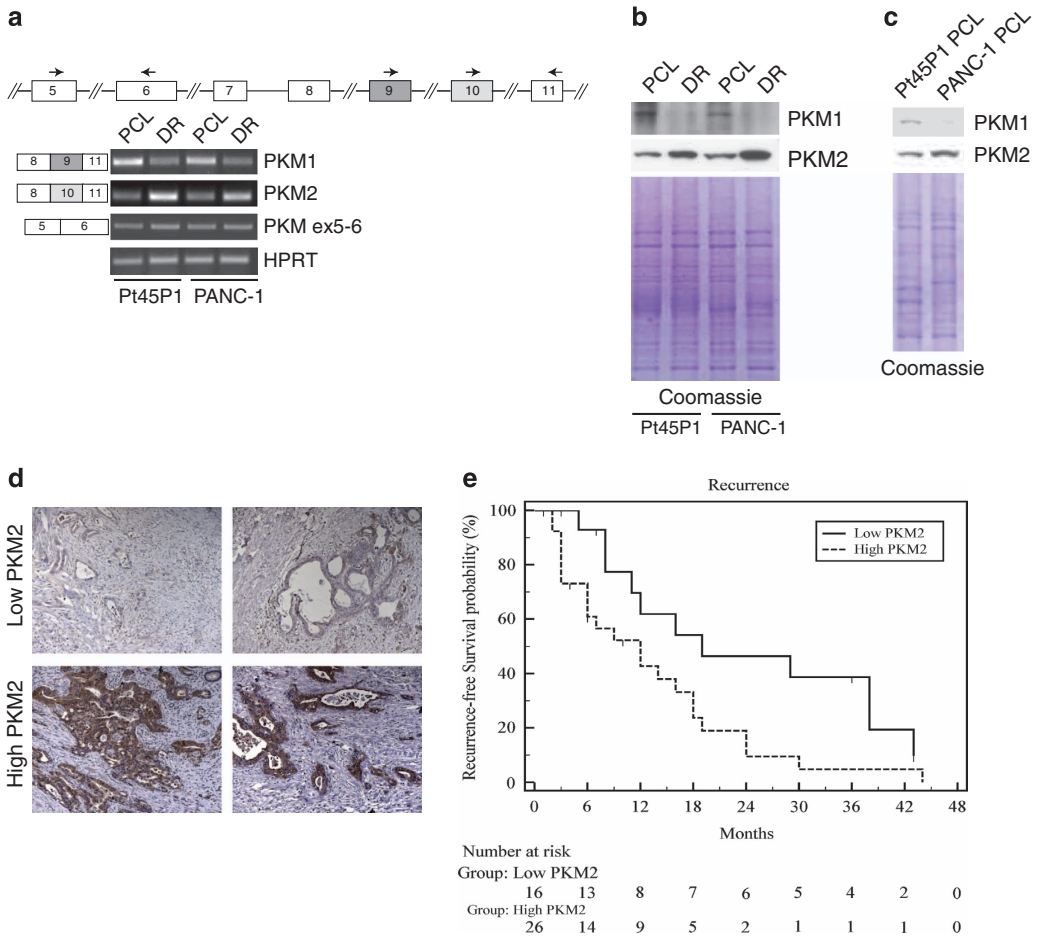

Figure 3. PKM2 protein expression in PDAC cells and PDAC tissues. RT–PCR (**a**) and western blot (**b**) analyses of PKM1 and PKM2 splicing variants in PCL- and DR-PDAC cells. Schematic representation of the *PKM* gene is shown in the upper panel; black arrows indicate the specific primers used to amplify the PKM1 and PKM2 in PCL- and DR-PDAC cells. HPRT and *PKM* exon 5–6 regions were used as loading control (**a**). Coomassie staining was used as loading control (**b**). (**c**) Western blot analysis of PKM1 and PKM2 protein in PCL-PDAC cells. Coomassie staining was used as loading control. (**d**) Representative images of PKM2 immunohistochemistry in PDAC tissues (×10 magnification). Upper panels show neoplastic glands with weak staining (low PKM2 group; score $\leqslant 3$); bottom panels show neoplastic glands with strong staining (high PKM2; group score >3). (**e**) Analysis of RFS of PDAC patients. Low PKM2 group comprised 16 patients (continuous line), whereas high PKM2 group comprised 26 patients (dotted line); $P=0.04$ at log-rank test.

showed cytoplasmic PKM2 staining (Figure 3d), whereas non-neoplastic pancreatic tissue occasionally displayed very weak PKM2 staining in normal ductal and acinar cells (Supplementary Figure S3D). A linear score of staining (range 0–5) was assigned to each sample (see Materials and Methods) and patients were subdivided in two groups: the 'low PKM2' group comprised 16 samples characterized by weak PKM2 staining (that is, $\leqslant 3$) (Figure 3d, upper panels), whereas the 'high PKM2' group comprised 26 samples displaying stronger PKM2 staining (that is, >3) (Figure 3d, lower panels). No differences regarding age, sex and pathological features (mean tumor size, grade, stage and resection margins) were found between the two groups (Supplementary Table S1). However, the recurrence free survival (RFS), defined as the time elapsing from surgery to disease recurrence, was significantly shorter in patients with 'high PKM2' (mean 11.6 months) as compared with the 'low PKM2' group (mean 19.8 months, $P=0.04$; Supplementary Table S1). Accordingly, RFS estimated by the Kaplan–Meier curve was significantly shorter in the first group (Figure 3e) and PKM2 was the only risk factor significantly associated with shorter RFS at a Cox proportional-hazards regression (hazard ratio: 1.12; 95% confidence interval: 1–4.4, $P=0.04$). These data suggest that tumors with higher PKM2 basal expression display more aggressive behavior and worse response to chemotherapy.

Modulation of *PKM* splicing impairs drug resistance of DR-PDAC cells

AS can be modulated in live cells by antisense short oligonucleotides (ASOs) directed against a specific regulatory region.[40] In the case of *PKM*, an ASO targeting exon 10 could efficiently induce splicing of PKM1 at the expense of PKM2.[38] We used this tool to modulate *PKM* splicing in PDAC cells and to evaluate the contribution of PKM2 to the DR phenotype. RT–PCR and western blot analyses indicated that AS of endogenous *PKM* could be efficiently modulated by transfection of the ASO in PDAC cells (Figures 4a and b). Analysis of cell death by immunofluorescence for the cleaved/activated form of caspase-3 (Figure 4c) showed that ASO-mediated switching of PKM AS in favor of PKM1 increased the sensitivity of DR-PDAC cells to gemcitabine without affecting the basal level of cell death (Figure 4c). Furthermore, overexpression of PKM2 in PCL-PDAC cells protected them from gemcitabine-induced cell death (Supplementary Figures S4A and B). These results indicate that PKM2 expression in DR-PDAC cells is required to maintain gemcitabine resistance.

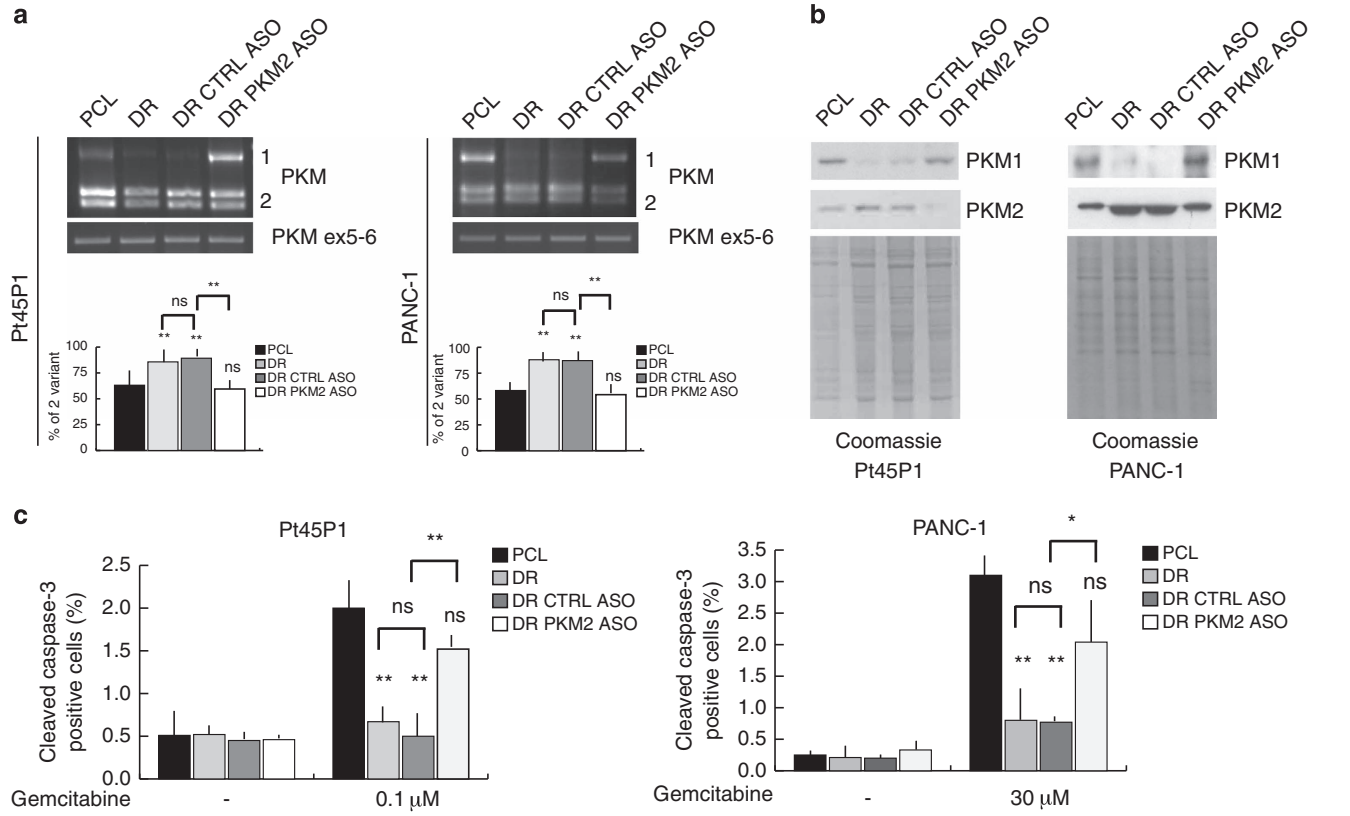

Figure 4. Modulation of *PKM* splicing enhances gemcitabine-induced cell death in DR-PDAC cells. RT–PCR (**a**) and western blot (**b**) analyses of *PKM* splicing variants performed in DR-Pt45P1 (left panels) and DR-PANC-1 (right panels) cells transduced with a control ASO (CTRL ASO) or with a specific ASO used to revert *PKM* splicing (PKM2 ASO) in favor of PKM1 (see Supplementary Table S1). (**a**) Bar graphs represent the percentage of PKM2 variant as assessed by densitometric analysis of the bands. Statistical analyses were performed by the paired Student's *t*-test comparing DR-PDAC cells values with those obtained in PCL-PDAC cells, whereas brackets indicate statistical comparison of the indicated samples (**$P \leqslant 0.01$; ns: not significant). (**b**) Coomassie staining was used as protein loading control. (**c**) Bar graphs show the percentage of cell death from three experiments (mean ± s.d.) as assessed by immunofluorescence analysis of the cleaved form of caspase-3 in PCL- and in DR-PDAC cells transduced with CTRL or PKM2 ASO and treated as indicated. Statistical analyses were performed by the paired Student's *t*-test, comparing DR-PDAC cell values with those obtained in PCL-PDAC cells treated with gemcitabine, whereas brackets indicate statistical comparison of the indicated samples (*$P \leqslant 0.05$, **$P \leqslant 0.01$; ns, not significant).

PTBP1 is upregulated in DR-PDAC cells

Three hnRNPs (hnRNPI/PTBP1, hnRNPA2/B1 and hnRNPA1) were shown to cooperate to suppress exon 9 inclusions in the *PKM* transcript, leading to exon 10 inclusion and expression of the PKM2 variant.[35] Notably, these splicing factors were upregulated in brain tumors and their expression strongly correlated with that of PKM2.[35] Thus, we investigated whether the expression of these hnRNPs was altered in DR-PDAC cells with respect to PCL cells. We found that only PTBP1 was markedly upregulated in both DR-Pt45P1 and DR-PANC-1 cells (Figures 5a and b). The highly homologous PTBP2 protein was not detected in PDAC cells (Figures 5a and b). By contrast, hnRNPA2/B1 levels were unchanged in PCL- and DR-PDAC cells, whereas hnRNPA1 was upregulated in DR-PANC-1 (Figure 5b) but slightly reduced in DR-Pt45P1 (Figure 5a). Furthermore, PTBP1 expression correlated with sensitivity of PDAC cells to gemcitabine, as it was higher in PANC-1 cells than in Pt45P1 cells (Supplementary Figure S5A). The correlation between PTBP1 expression and PKM2 splicing in both DR-PDAC cell lines was specific, as demonstrated by western blot analysis of other cancer-related serine/arginine proteins and hnRNPs in PCL- and DR-PDAC cells, which showed either marginal or inconstant alterations. For instance, upregulation of SRSF1 was detected in DR-Pt45P1 cells but not in DR-PANC-1 cells (Supplementary Figure S5B), possibly because PANC-1 cells are more resistant to drug treatment and express higher basal levels

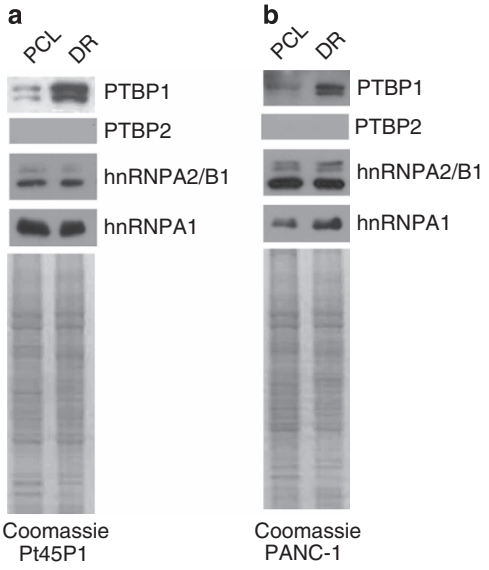

Figure 5. PTBP1 is upregulated in DR-PDAC cells. Western blot analysis of PTBP1, PTBP2, hnRNPA1 and hnRNPA2/B1 protein expression in PCL- and DR-Pt45P1 (**a**) or PANC-1 cells (**b**). Coomassie staining was used as loading control.

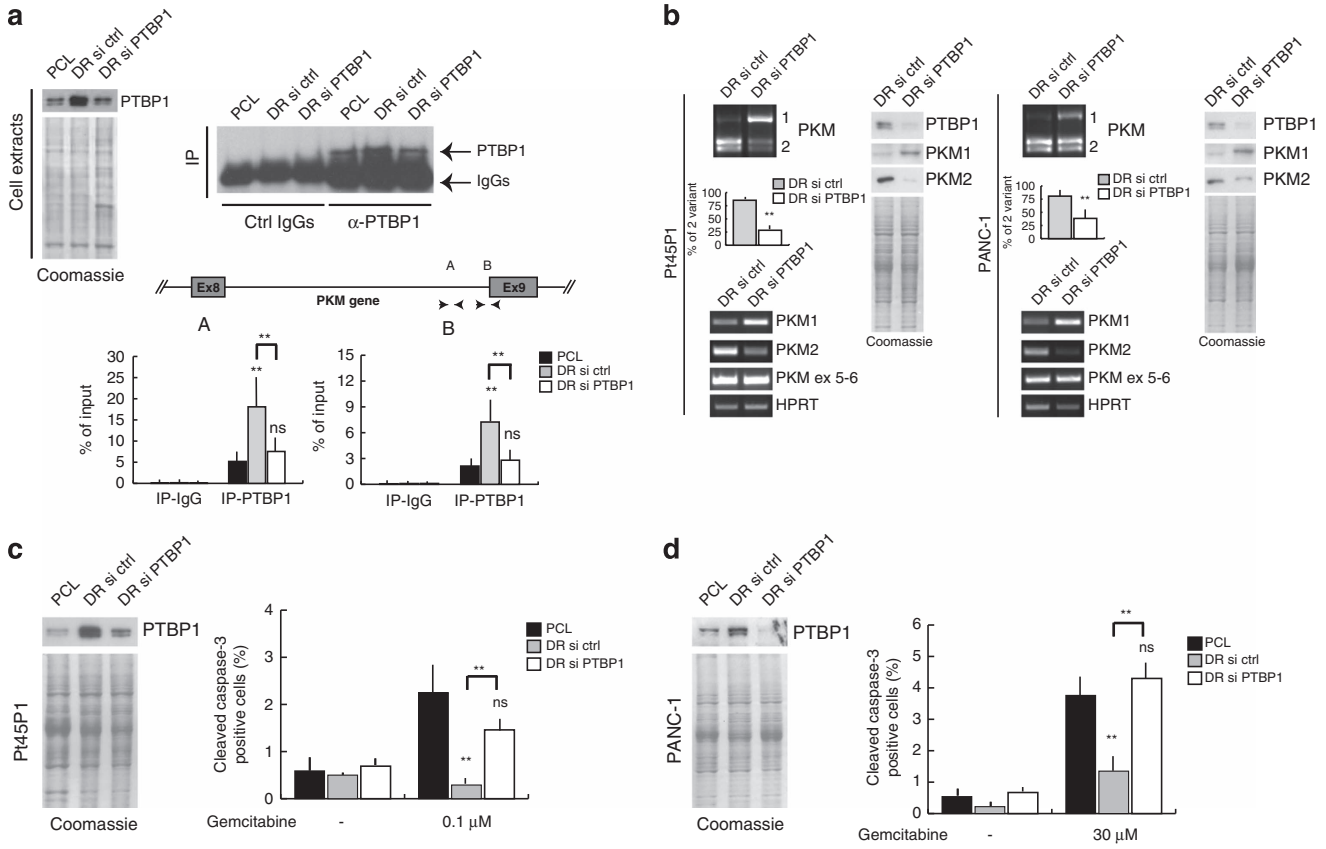

Figure 6. PTPB1 upregulation is required for PKM2 splicing and gemcitabine resistance in DR-PDAC cells. (**a**) CLIP of PTBP1 performed in PCL- or DR-Pt45P1 PDAC cells transfected with either a control (si-ctrl) or PTBP1 (si-PTBP1) small interfering RNAs (siRNAs), in the presence of RNaseI (1:1000). Associated *PKM* pre-mRNA was quantified by quantitative PCR; black arrows indicate primers used to amplify A and B regions (upper panels; see Supplementary Table S1). Data are represented as percentage of input (bottom panels; mean ± s.d.; $n = 3$). Statistical analyses were performed by the paired Student's *t*-test (**$P \leqslant 0.01$; ns, not significant). PTBP1 silencing in DR-Pt45P1 cells and IP efficiency were assessed by western blot analysis. (**b**) RT–PCR and western blot analyses to evaluate PKM1 and PKM2 expression in DR-PDAC cells transfected with either ctrl or PTBP1 siRNAs. Bar graphs represent the percentage of PKM2 variant, as assessed by densitometric analysis of the bands. Statistical analyses were performed by the paired Student's *t*-test comparing the values of DR-PDAC cells transfected with si-ctrl with those obtained in DR-PDAC cell transfected with si-PTBP1 siRNA (**$P \leqslant 0.01$; upper panels: mean ± s.d., $n = 3$, **$P \leqslant 0.01$). (**b**) PTBP1 silencing was assessed by western blot analysis. Coomassie staining was used as loading control. (**c** and **d**) Western blot analyses assessing PTBP1 expression levels in PCL- and DR-Pt45P1 or PANC-1 PDAC cells transfected with ctrl or PTBP1 siRNAs. Coomassie staining was used as loading control. (**c** and **d**) Bar graphs show the percentage of cell death from three experiments (mean ± s.d.) as assessed by immunofluorescence analysis of the cleaved form of caspase-3 in PCL-, DR-PDAC cells described in **c** and **d**, and treated as indicated for 72 h. Statistical analyses were performed by the paired Student's *t*-test comparing DR-PDAC cell values with those obtained in PCL-PDAC cells treated with gemcitabine, whereas brackets indicate statistical comparison of the indicated samples (**$P \leqslant 0.01$; ns, not significant).

of SRSF1. By contrast, SRSF6 was strongly upregulated in DR-PANC-1 but slightly reduced in DR-Pt45P1 (Supplementary Figure S5B). Thus, upregulation of PTBP1 appears to specifically correlate with the regulation of PKM2 splicing in DR-PDAC cells.

PTBP1 binds *in vivo PKM* intron 8 and its downregulation impairs PKM2 expression and sensitizes DR-PDAC cells to drug-induced cell death

To test whether PTBP1 was recruited to the *PKM* transcript more efficiently in DR-PDAC cells, we analyzed *in vivo* binding by UV-crosslinked and RNA immunoprecipitation (CLIP) assays in PCL- and DR-Pt45P1 cells, silenced or not for PTBP1 (Figure 6a). Binding of PTBP1 in intron 8 of *PKM* favors skipping of exon 9 in the mature transcript,[35] thereby generating the PKM2 isoform. Thus, we analyzed two regions in intron 8, named A and B (Figure 6a), which were identified as high PTBP1-bound sequences by CLIP sequencing analysis.[41] CLIP assays showed that PTBP1 was recruited more efficiently to *PKM* intron 8 in DR-Pt45P1 cells with respect to PCL-Pt45P1 cells (Figure 6a). Increased binding was specific and likely dependent on the higher expression of PTBP1 in DR cells, as it was suppressed by knockdown of the protein to levels comparable with those expressed in PCL cells (Figure 6a). Thus, upregulation of PTBP1 in DR-PDAC cells leads to increased recruitment of this splicing factor to *PKM* intron 8.

To evaluate the contribution of PTBP1 to the regulation of the PKM2 variant, we analyzed *PKM* AS in DR-PDAC cells knocked down for PTBP1. Knockdown of endogenous PTBP1 increased PKM1 splicing in DR-PDAC cells, resulting in a switch in PKM1 and PKM2 protein levels (Figure 6b). These results confirm that upregulation of PTBP1 promotes PKM2 splicing in DR-PDAC cells. To test whether PTBP1 affected additional splicing events in DR-PDAC cells, we analyzed a subset of genes whose AS is regulated by this splicing factor in other cell types.[41–43] RT–PCR analysis indicated that only the FGFR2 IIIc variant correlated with the higher expression of PTBP1 in both DR-PDAC cell lines (Supplementary Figure S6A). However, silencing of PTBP1 did not affect this splicing event (Supplementary Figure S6E), indicating that expression of FGFR2 IIIc correlates with but is not

dependent on high PTBP1 expression in DR-PDAC cells. By contrast, splicing of *EZH2*, *CTTN*, *RASSF8*, *MINK1*, *EIF4G2*, *FAM38A*, *CCDC138* and *TPM1* was either similar in PCL- and DR-PDAC cells or altered in one of the two DR cell lines (Supplementary Figures S6A–C). These findings indicate that splicing of *PKM* is specifically modulated by PTBP1 overexpression in DR-PDAC cells.

To investigate whether PTBP1 expression is required for the resistance of DR-PDAC to chemotherapeutic treatments, we analyzed gemcitabine-induced cell death in PTBP1-depleted DR-PDAC cells. Downregulation of PTBP1 expression significantly rescued the sensitivity of DR-PDAC cells to treatment with gemcitabine, reaching levels of cell death similar to those of PCL-PDAC cells (Figures 6c and d). We also tested resistance to cisplatin as prototype of a class of drugs currently used in clinical trials of combined chemotherapy for advanced PDAC.[44] Cell death analysis showed that DR-PDAC cells were more resistant to cisplatin treatment than PCL-PDAC cells (Supplementary Figure S7A). However, switching *PKM* splicing by either ASO transfection (Supplementary Figure S7B) or knockdown of PTBP1 (Supplementary Figure S6C) rescued sensitivity to cisplatin, suggesting that the PTBP1/PKM2 axis is involved in PDAC cell survival to multiple cytotoxic drugs.

Collectively, these results indicate that high PTBP1 expression levels are required for the maintenance of the DR phenotype of PDAC cells and suggest that this splicing factor mainly confers drug resistance to PDAC cells through the promotion of PKM2 splicing.

DISCUSSION

PDAC is a human cancer characterized by very poor prognosis. Chemotherapeutic approaches are largely ineffective and treatment with the elective agent gemcitabine slightly improves survival in patients with advanced disease, but does not represent a cure.[1] For this reason, understanding the biology of PDAC cells might shed light on novel therapeutic strategies for the management of advanced PDAC. In this work, we show that chronic gemcitabine treatment leads to isolation of DR-PDAC cells that display higher resistance not only to gemcitabine, but also to cisplatin, a prototype of cytotoxic drugs largely used in human cancer therapy, including PDAC.[44] These findings suggest that hostile stimuli promote the adaptive capabilities of PDAC cells, thus favoring the selection of DR populations.

In order to elucidate the molecular mechanisms involved in the acquisition of the DR phenotype by PDAC cells we focused on AS regulation, because this process is emerging as a key determinant of eukaryotic cell plasticity[45] that is often altered in human cancers.[5–8] Furthermore, genotoxic stresses such as those imposed by chemotherapeutic treatments can finely tune the expression of splice variants that protect cancer cells.[15] In this regard, our study identifies *PKM* splicing as a novel contributor to drug resistance acquired by PDAC cells during chronic chemotherapeutic treatment. We found that promotion of PKM2 is the AS event that correlates more closely with drug resistance among a subset of cancer-relevant splicing events analyzed. Importantly, PKM2 splicing and expression are functionally relevant for the resistance to chemotherapeutic treatment, as switching splicing toward PKM1 by ASO transfection restored sensitivity of DR-PDAC cells to both gemcitabine and cisplatin. These results point to PKM2 as a new potential prognostic marker and therapeutic target for PDAC. In support of this hypothesis, we also found that high PKM2 expression was the only risk factor significantly associated with shorter RFS in patients receiving radical surgery and adjuvant chemotherapy with gemcitabine. These results suggest that increased PKM2 expression might be responsible for lower response of residual cancer cells to chemotherapy. Although we did not find a significant correlation between PKM2 expression and overall survival, the observed trend suggests that patients expressing higher PKM2 levels also have a shorter survival rate (Supplementary Table S1). Studies with a larger cohort of patients will be required to further assess whether or not PKM2 can be used as marker for prediction of severity of the disease and response to treatments.

The role of PKM2 in cancer is not fully elucidated yet. Nevertheless, several observations pointed out that this splice variant is expressed at higher levels in cancer tissues versus their normal counterparts.[22,35] PKM2 protein was proposed as a potential molecular marker of PDAC, as immune reactivity toward this isoform was elevated in the blood from patients and positively correlated with metastatic disease.[46] We now show that PKM2 is barely detectable in areas of the pancreas with normal glands, whereas its expression is increased in neoplastic lesions. Moreover, our findings document that upregulation of PKM2 in DR-PDAC cells is required for survival in the presence of gemcitabine or cisplatin. Notably, depletion of PKM2 in several human cancer cell lines caused apoptosis even in the absence of chemotherapeutic treatments.[38,39] This effect was cancer specific, as depletion of PKM2 in non-cancerous cells did not affect their viability.[39] In the case of DR-PDAC cells, however, depletion of endogenous PKM2 *per se* does not trigger cell death, indicating that PDAC cells are somewhat less dependent on this enzyme for viability. Nevertheless, PKM2 was strictly necessary to withstand genotoxic stress in DR-PDAC cells. Importantly, PKM2 expression has been linked to response to chemotherapy also in lung cancer, as its depletion sensitized to apoptosis triggered by chemotherapeutic treatment in mouse xenograft models.[47] Our work also supports this scenario and suggests that modulation of *PKM* splicing by ASO treatment is a potential therapeutic tool to increase the efficacy of standard chemotherapy in advanced PDAC. This strategy might represent a promising approach, as ASOs are already in clinical trials for other splicing-caused diseases and improvement of their design and administration protocols might insure their use in cancer therapy in the near future.[40]

Aberrant expression of several splicing factors correlates with cancer onset, progression and/or response to therapeutic treatments.[48] Our findings indicate that the switch in *PKM* AS correlates with the upregulation of PTBP1 in DR-PDAC cells. A role for PTBP1 in *PKM* splicing was already shown in glioblastoma, where this splicing factor acted in concert with hnRNP A1 and A2/B1 to promote PKM2 splicing.[35] However, we found that neither of these hnRNPs was consistently modulated in DR-PDAC cells. Moreover, knockdown of PTBP1 to mimic the levels observed in PCL-PDAC cells was sufficient to raise PKM1 levels to those present in parental cells. Thus, PTBP1 is the main factor in the regulation of *PKM* AS during the acquisition of the DR phenotype by PDAC cells. Importantly, the effect of PTBP1 on PKM2 splicing in PDAC cells appears to be direct, as it correlates with the extent of PTBP1 recruitment to *PKM* intron 8. Furthermore, its effect on *PKM* AS can account for the acquired resistance to genotoxic drugs, as DR-PDAC cells knocked down for PTBP1 switched AS in favor of PKM1 and became sensitive to gemcitabine and cisplatin such as PCL-PDAC cells. Although the pro-survival effect of PTBP1 upregulation in PDAC cells might also involve other targets of this splicing factor, our observations suggest that splicing of PKM2 represents the main factor. Indeed, by monitoring a group of splice variants previously shown to be the target of PTBP1 in other cellular systems,[41,42,43] we did not observe striking and consistent changes correlating with the DR-PDAC phenotype. The only relevant change observed was promotion of the FGFR2 IIIc variant, which, however, was unaffected by knockdown of PTBP1 in DR-PDAC cells. Furthermore, selectively restoring the PCL pattern of *PKM* splicing by ASO transfection almost completely rescued the sensitivity of DR-PDAC cells to genotoxic stresses. Thus, our results suggest that *PKM* AS is particularly sensitive to changes in the expression levels of PTBP1 in PDAC cells, and that this splicing

event represent a key resource for these cells to acquire drug resistance.

In conclusion, our work characterizes a novel PTBP1/PKM2 pro-survival pathway triggered by chronic treatment of PDAC cells with gemcitabine. Interfering with this axis by repressing PKM2 splicing or PTPB1 expression can restore sensitivity of DR-PDAC cells to drug treatment. As development of therapeutic ASOs is already a clinical approach for other human diseases,[40] these findings might represent a promising strategy to improve therapeutic approaches for PDAC and to impact the resistance of cancer cells to current treatments.

MATERIALS AND METHODS

Cell culture, treatments and transfections

Pt45P1 and PANC-1 cells were obtained from the Centre for Molecular Oncology, Barts Cancer Institute (London, UK) in 2004 and authenticated in 2012. DR cells were obtained by treating PCL with 10 μM gemcitabine continuously for 7 days (medium replaced every 72 h) and then released in normal medium for 15 days. Resistant clones were pooled, amplified and cultured by performing a 24-h pulse of 10 μM gemcitabine every other week, to maintain selection. PCL and DR cells were maintained in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, gentamycin, penicillin and streptomycin. Gemcitabine (Eli Lilly & Company, Indianapolis, IN, USA) and cisplatin (Sigma-Aldrich, St Louis, MO, USA) were dissolved in water. For ASO transfection, cells were transduced by scraping delivery according to the manufacturer's instructions (Gene Tools, Philomath, OR, USA) with PKM2 or control ASO (10 μM for DR-PANC-1 cells and 15 μM for DR-Pt45P1 cells). For RNA interference, cells were transfected twice with 30 nM PTBP1 small interfering RNAs (On target plus human PTBP1 5725 siRNA, Dharmacon, Lafayette, CO, USA) using Lipofectamine RNAiMAX and Opti-MEM medium (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Colony formation assay and cell death analyses

Single-cell suspensions were plated in 6-well plates (500 cells/plate for Pt45P1, 750 cells/plate for PANC-1). After 1 day, cells were treated for 24 h with gemcitabine. Fresh medium was replaced every 48 h. After 10–12 days, cells were fixed in methanol for 10 min, stained overnight with 5% Giemsa (Sigma-Aldrich), washed in phosphate-buffered saline and dried. Pictures were taken using a digital camera and colonies were counted. For cell death analyses, cells were seeded at 70% confluence and treated as described for 72 h. Cells were then washed in phosphate-buffered saline and either trypsinized and incubated with 0.4% Trypan Blue Stain (Sigma-Aldrich) or processed for caspase-3 immunofluorescence using anti-cleaved caspase-3 antibody (1:500; Sigma-Aldrich) as previously described.[31,49] Positive cells were then counted using the Thoma's chamber (Trypan blue) or fluorescence microscopy (caspase-3). Five random fields were chosen for each treatment and at least 200 cells/field were counted.

PCR analyses

RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. After digestion with RNase-free DNase (Life Technologies), 1 μg of total RNA was retrotranscribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA), used as template for conventional PCR reactions (GoTaq, Promega). Products were analysed on agarose or acrylamide gels. RT–PCR images were collected with Biorad Universal Hood II using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). Real-time quantitative PCR analysis was performed using LightCycler 480 SYBR Green I Master and the LightCycler 480 System (Roche, Mannheim, Germany), according to the manufacturer's instructions. Primers used are listed in Supplementary Table S2.

Protein extracts and western blot analysis

Cells were resuspended in RIPA buffer: 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 1 mM dithiothreitol, 0.5 mM NaVO4 and protease inhibitor cocktail (Sigma-Aldrich). After 10 min on ice, extracts were centrifuged for 10 min at 12 000 *g* and supernatants were collected and used for western blotting as described.[49] Primary antibody incubation (1:1000) was carried out with the following antibodies: PKM1, hnRNPA1, hnRNPA2/B1 and hnRNPC1/C2 (Sigma-Aldrich); PKM2 (Cell Signaling Technology, Danvers, MA, USA); PTBP1, SRSF1, SRP20 and SRp40/p55/p75 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); hnRNPF/H (Abcam, Cambridge, UK). PTBP2 antibody was a generous gift of Professor Douglas L Black (UCLA, Los Angeles, CA, USA). Images of the western blotting were acquired as TIFF files.

IHC analysis

IHC was performed as previously described.[31] Briefly, formalin-fixed, paraffin-embedded tissue samples obtained from 42 primary non-metastatic PDAC patients receiving surgery with radical intent were investigated for PKM2 expression, on informed consent. All patients received gemcitabine-based adjuvant therapy after surgery. Clinical and histopathological data, time of tumor recurrence and survival for each patient were recorded. IHC was performed on 4-μm-thick sections. Antigen retrieval was carried out with EDTA at pH 8 (60 min at room temperature). Staining was carried out using anti PKM2 antibody (1:1600, Cell Signaling Technology), visualized by Envision-Flex (Dako, Glostrup, Denmark). Staining of PKM2 in neoplastic cells was scored based on distribution and intensity. Distribution was scored as 0 (0%), 1 (1%–50%) and 2 (51%–100%). Intensity was scored as 0 (no signal), 1 (mild), 2 (intermediate) and 3 (strong). Values were summed in a total score from 0 to 5. Samples were classified as 'low PKM2' expression (score $\leqslant 3$) and as 'high PKM2' expression (score >3). Statistical analysis was performed by MedCalc 9.6 (www.medcalc.be). Differences for continuous variables were evaluated by *t*-test and for categorical variables by Fisher's test. Analysis of RFS and of overall survival was performed by Kaplan–Meier method and analysed by log-rank test. Univariate and multivariate analyses for risk factors affecting survival were performed by Cox-proportional hazards regression model test; a *P*-value <0.05 was considered as statistically significant (Supplementary Table S1). Images were taken from a Zeiss axioskop 2 plus and elaborate with software Zeiss axiovision (Carl Zeiss Microscopy, Oberkochen, Germany).

CLIP assays

For CLIP assays, cell extractions were performed as previously described.[50,51] Half extract (1 mg) was treated with proteinase K for 30 min at 37 °C and RNA was purified (input). The remaining half (1 mg) was diluted to 1 ml with lysis buffer and immunoprecipitated by using anti-PTBP1 (Santa Cruz Biotechnology) antibody or IgGs (negative control), in the presence of protein-G magnetic dynabeads (Life Technologies). RNaseI 1:1000 (10 μl/ml; Life Technologies) were added. After immunoprecipitation and washes,[50,51] an aliquot (10%) of the sample was kept as control of immunoprecipitation, while the rest was treated with 50 μg of proteinase K and incubated for 1 h at 55 °C. RNA was then isolated.

Image acquisition and manipulation

Images in Figure 1b were taken from an inverted microscope (IX70; Olympus, Shinjuku, Tokyo, Japan) using an LCA ch 40 × /0.60 objective. Adobe Photoshop and Illustrator (Adobe System, San Jose, CA, USA) were used for composing the panels.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

SC, PB and IP performed the experiments and analyzed the data. EP and VF performed IHC experiments and score analysis. GC performed statistical analysis of patients; SC, GDF, GC and CS wrote the manuscript. SC and CS designed the study.

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