CUX1: target of Akt signalling and mediator of resistance to apoptosis in pancreatic cancer

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Revised 10 November 2009
Accepted 23 December 2009
Published Online First
4 May 2010

ABSTRACT

Background and aims The transcription factor CUX1 is known as a regulator of cell differentiation and cell cycle progression. Previously, CUX1 was identified as a modulator of invasiveness in various cancers. Based on expression profiles suggesting a role for CUX1 in mediating chemoresistance, the aim of this study was to characterise the effect of CUX1 on apoptosis as well as its regulation by signalling pathways modulating drug resistance in pancreatic cancer.

Methods The effect of CUX1 on TRAIL- (tumour necrosis factor-related apoptosis-inducing ligand) and drug-induced apoptosis was analysed using overexpression and knock-down strategies. Regulation of CUX1 by phosphatidylinositol-3-kinase (PI3K)/Akt signalling was examined at the mRNA and protein level. The effect of CUX1 knock-down by nanoparticle-complexed small interfering RNA (siRNA) in vivo was analysed in a murine xenograft model. Furthermore, *CUX1* RNA and protein expression was evaluated in human pancreatic cancer and adjacent normal tissues.

Results Knock-down of CUX1 resulted in significantly enhanced TRAIL- and drug-induced apoptosis, associated with increased PARP (poly ADP-ribose polymerase) cleavage and caspase activity. Vice versa, overexpression of CUX1 inhibited apoptosis. CUX1 expression was induced by activation of Akt/protein kinase B signalling, and decreased by PI3K inhibitors. The antiapoptotic effect of CUX1 was associated with upregulation of BCL2 and downregulation of tumour necrosis factor α. CUX1 was significantly overexpressed in pancreatic cancers, as analysed by in situ hybridisation and immunohistochemistry. In vivo, silencing of CUX1 by intratumourally administered polyethylenimine-complexed siRNA led to reduced tumour growth and increased apoptosis in pancreatic cancer xenografts.

Conclusion CUX1 was identified as an important mediator of tumour cell survival in pancreatic cancer in vitro and in vivo.

INTRODUCTION

Pancreatic cancer is a devastating disease with a dismal prognosis, representing the fifth leading cause of cancer-related deaths in the Western world. Histologically, ductal adenocarcinomas of the pancreas (PDAC) account for >95% of all exocrine pancreatic cancers. PDAC is almost uniformly fatal, with a 5-year survival rate of <5%.[1] For advanced stages of the disease, a large number of single and combined radiotherapeutic or chemotherapeutic regimens have been tested during the past decades, and at most achieve palliation and/or modest, if any, prolongation of survival.[2] This is largely due to the high level of chemoresistance of this tumour entity. In this appalling situation new therapeutic targets to overcome chemoresistance are urgently needed.[2]

The transcription factor CUX1, also known as CUTL1 or CDP (CCAAT displacement protein), belongs to a family of homoeobox transcription factors involved in the regulation of cell proliferation, embryonic development and cell differentiation.[3 4] CUX1 is present in all metazoans and is expressed as multiple isoforms. It contains up to four evolutionarily conserved DNA-binding domains, three of which are known as Cut repeats and one as a Cut homeodomain (HD).[5]

Transcriptional activation of CUX1 leads to increased cellular proliferation and cell cycle progression in various cell systems.[6–8] In vivo, knockout studies revealed a phenotype characterised by reduced growth, retarded differentiation of the lung epithelia, hair follicle defects, reduced male fertility and deficient T and B cell function.[9–11] In contrast, mice transgenic for Cux1 showed organomegaly and multiorgan hyperplasia.[12]

Recent reports suggest an important role for CUX1 in tumourigenesis and tumour progression.[4] Goulet *et al* described a tissue-specific CUX1 isoform that appears to be strongly expressed in some breast tumours and, when overexpressed, inhibited tubule formation of breast cancer cells in vitro, indicating that CUX1 might enhance dedifferentiation of tumours.[13] We could show that CUX1 stimulates tumour cell migration and invasiveness in vitro and in vivo, and acts as an important effector of transforming growth factor β signalling.[14 15] The important role of CUX1 in promoting cell motility and invasiveness is underlined by the fact that CUX1 expression is strongly associated with a less differentiated phenotype and decreased survival in invasive breast cancer.[14] Given the role of CUX1 as a transcriptional regulator, we could identify several downstream targets of CUX1, such as the Wnt family member WNT5A, which mediate the proinvasive effects of CUX1 in various tumour cells.[16 17]

Interestingly, analysis of gene expression profiles available in the literature revealed the CUX1 is increased in carcinomas resistant to chemotherapeutic drugs such as oxaliplatin-resistant ovarian cancers as compared with drug-sensitive tumours.[18] Furthermore, CUX1 was found to be decreased after chemotherapy-induced apoptosis.[19] Based on these data, we were interested to discover whether CUX1 interferes with tumour cell survival and resistance to apoptosis, which could help to explain the striking association of CUX1 expression with

enhanced tumour progression and poor prognosis we had observed before in breast cancer.[14] In this study, we could show a significant impact of CUX1 on tumour cell survival in pancreatic cancer cells in vitro and in vivo, and identified CUX1 as a transcriptional target of phosphatidylinositol-3-kinase (PI3K)/Akt signalling.

MATERIALS AND METHODS

Materials and cell lines

Recombinant human TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) and insulin-like growth facor 1 (IGF1) were obtained from R&D Systems (Minneapolis, Minnesota, USA). The PI3K inhibitor LY294002 was purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). PANC1 and HEK293 cell lines were obtained from the ATCC (Rockville, Maryland, USA). ImimPC1 cells were kindly provided by FX Real (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). Cells were maintained in Dulbecco`s modified minimal essential medium (Gibco, Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal calf serum (Gibco), 100 µg/ml streptomycin (Sigma-Aldrich, St Louis, Missouri, USA) and 100 U/ml penicillin (Sigma). All cell lines were grown at 37°C in 5% CO_2. 5-Fluorouracil (5-FU) was purchased from Sigma and gemcitabine was obtained from Lilly (Bad Homburg, Germany).

Plasmids and small interfering RNA (siRNA) oligonucleotides

Myc-tagged full-length human CUX1 in pMX vectors and C-terminal CUX1 in pXJ vectors were a kind gift of A Nepveu (McGill University, Canada). The AKT2/pcDNA4 expression plasmid was a kind gift of A Schulze (Cancer Research UK, London Research Institute, UK). Plasmids were transfected into PANC1 and HEK293 cells using Transfast transfection reagent (Promega, Madison, Wisconsin, USA).

CUX1 was transiently suppressed by using two different oligonucleotides (hCUX1_1 and hCUX1_2) as described previously.[14] All assays were confirmed using two different siRNA sequences to minimise the risk of possible off-target effects. siRNA oligonucleotides were purchased from Ambion (Austin, Texas, USA). Both silencing sequences resulted in a knock-down efficiency >70% (supplementary figure 1). PANC1 cells were transfected using Transmessenger transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To optimise transfection efficacy, cells were transfected with siRNA twice, with an interval of 24 h. ImimPC1 cells were transfected using XtremeGene (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. As non-silencing controls, silencer-negative control siRNAs from Ambion were used.

Quantitative real-time PCR (RT-PCR)

RNA was extracted using the RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesised using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed with an Applied Biosystems 7500 Fast Real time PCR using the SYBR Green PCR Master Mix kit (Applied Biosystems, Wellesley, Massachusetts, USA) according to the manufacturer's instructions. Sequence-specific primer pairs were designed using the PrimerExpress software (Applied Biosystems). The ribosomal protein RPLP0 (NM_001002) was used as internal standard. Primer sequences for CUX1 and RPLP0 are shown in supplementary table 1.

Apoptosis pathway-focused gene expression profiling comprising 84 genes was performed using the RT^2 Profiler PCR Array System (SuperArray Bioscience, Frederick, Maryland, USA) according to the manufacturer's instructions. A list of the genes analysed in this profiler is available online (<http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-012A.html>).

Immunoblotting

Cells were incubated in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM $Na_4P_2O_7$, 10% glycerol, 1% Triton X-100) supplemented with a cocktail of protease inhibitors (Complete, Roche Applied Science). Proteins were separated by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, USA). Immunoblots were probed with primary antibodies against CUX1 (custom-made rabbit polyclonal antibody as previously described[14]) caspase-3, cleaved caspase-3, poly ADP-ribose polymerase (PARP), bcl-2 (Cell Signalling Technology), pAKT (Cell Signalling Technology) and β-actin (Sigma-Aldrich) and subsequently incubated with peroxidase-conjugated secondary antibodies (Amersham Biosciences, Freiburg, Germany). Blots were detected by ECL chemiluminescence (Amersham).

Apoptosis assays

Apoptosis was assessed in three ways: (1) by fluorescence-activated cell sorting (FACS) analysis to determine DNA fragmentation of propidium iodide-stained nuclei[20]; (2) using the Cell Death Detection Elisa^{PLUS} for quantification of histone-associated DNA fragments (Roche Diagnostics); and (3) by the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Using the DeadEnd TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labelling) assay, the labelled DNA was visualised directly by fluorescence microscopy.

Caspase activity

Caspase-3 and caspase-7 activities were measured using the luminescent assay Caspase-Glo3/7 (Promega), according to the manufacturer's instructions. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. Luminescence is proportional to the amount of caspase activity present.

Tumour necrosis factor α (TNFα) ELISA

TNFα secretion by PANC1 cells over 24 h with or without knock-down of CUX1 was measured in the cell culture supernatant using a TNFα ELISA (BioSource Europe, Nivelles, Belgium) according to the manufacturer's instructions.

In vivo experiments

All animal experiments were approved by the local government authorities and were performed according to the guidelines of the animal welfare committee. Twenty tumours were induced in 10 female NMRI nu/nu mice (left and right flank each) by subcutaneous injection of 10^6 CAPAN1 cells/0.1 ml of phosphate-buffered saline (PBS) each. After the tumours had reached a volume of ~60 mm^3, mice were randomised into two groups and 10 µg polyethylenimine (PEI)-complexed siRNA against *CUX1* or PEI-complexed non-silencing control siRNA, dissolved in 50 µl of PBS, were injected intratumourally (three times a week over 3 weeks, 10 tumours per group). PEI–siRNA complexes were based on PEI F25-LMW[21] and were generated as described previously.[22] Tumours were measured during the course of treatment, and, after the animals had been sacrificed,

tumours were paraffin embedded and immunohistologically evaluated for necrotic areas (H&E staining), apoptosis (M30 CytoDEATH antibody, Roche) and CUX1 expression (mouse monoclonal anti-CUX1, Abcam, Cambridge, UK).

In situ hybridisation (ISH)

ISH for hCUX1 mRNA was performed as described previously.[14 23] The human CUX1 probe (nucleotides 330–1040) was PCR amplified and cloned into the *Kpn*I and *Sph*I sites of the pGEM3Z vector (Promega). Multiple tissue arrays (MTAs) comprising 49 tumour samples and paired normal control tissues were prepared at the Department of Surgery, University of Liverpool, UK, according to the guidelines of the local ethics committee. Expression within the tumour epithelium was scored by two investigators blinded to the H&E sections as negative or positive, as described previously.[14] As a positive control, ACTB (β-actin) mRNA was detected on a near serial section for each tissue.

Immunohistochemistry

For immunohistochemical analysis, an independent set of eight human pancreatic adenocarcinoma tissues and normal pancreatic tissues were provided by the Institute of Pathology of the University of Marburg according to the guidelines of the local ethics committee. Immunohistochemical analysis was performed as previously described.[24] In brief, paraffin sections were stained after antigen retrieval (microwave in antigen-unmasking solution, Vector Laboratories, Burlingame, California, USA) with rabbit polyclonal anti-CUX1 (1:200), as described previously.[14] Antibody binding was visualised using a biotinylated secondary antibody, avidin-conjugated peroxidase (ABC method; Vector Laboratories), 3,3′-diaminobenzidine tetrachloride (DAB) as a substrate, and H&E as counterstain.

Statistical analysis

For the in vitro experiments, statistical analyses were performed using the double-sided unpaired Student t test after Bonferroni correction for multiple testing, where appropriate. Differences in tumour growth in the mouse xenografts were analysed using paired t test/Wilcoxon matched pairs test.

RESULTS

Knock-down of CUX1 enhances TRAIL- and drug-induced apoptosis in pancreatic cancer cell lines

We first evaluated the effect of CUX1 on basal and TRAIL-induced apoptosis in two different pancreatic cancer cell lines, PANC1 and ImimPC1. Knock-down of CUX1 by specific siRNAs significantly induced basal apoptosis rates in both cell lines, as determined by FACS analysis of the sub-G_1 population (figure 1A, B). ImimPC1 cells were more sensitive than PANC1 cells to knock-down of CUX1, resulting in a significant increase in apoptosis without any apoptotic stimulus. Further, knock-down of CUX1 significantly enhanced TRAIL-induced apoptosis, whereas treatment with TRAIL alone had only a minor effect on apoptosis induction (figure 1A,B). The use of two independent CUX1-specific siRNA oligonucleotides, which had been validated previously,[14] revealed comparable results (data not shown). The effects of CUX1 siRNA on apoptosis were confirmed by quantification of histone-associated DNA fragments using a specific ELISA (supplementary figure 2) and by TUNEL staining, which both revealed very similar results (supplementary figure 3). All assays verified that knock-down of CUX1 by specific siRNAs significantly enhanced basal and TRAIL-induced apoptosis.

TRAIL is known to induce the death receptor pathway of apoptosis via binding to its cognate surface receptors (extrinsic

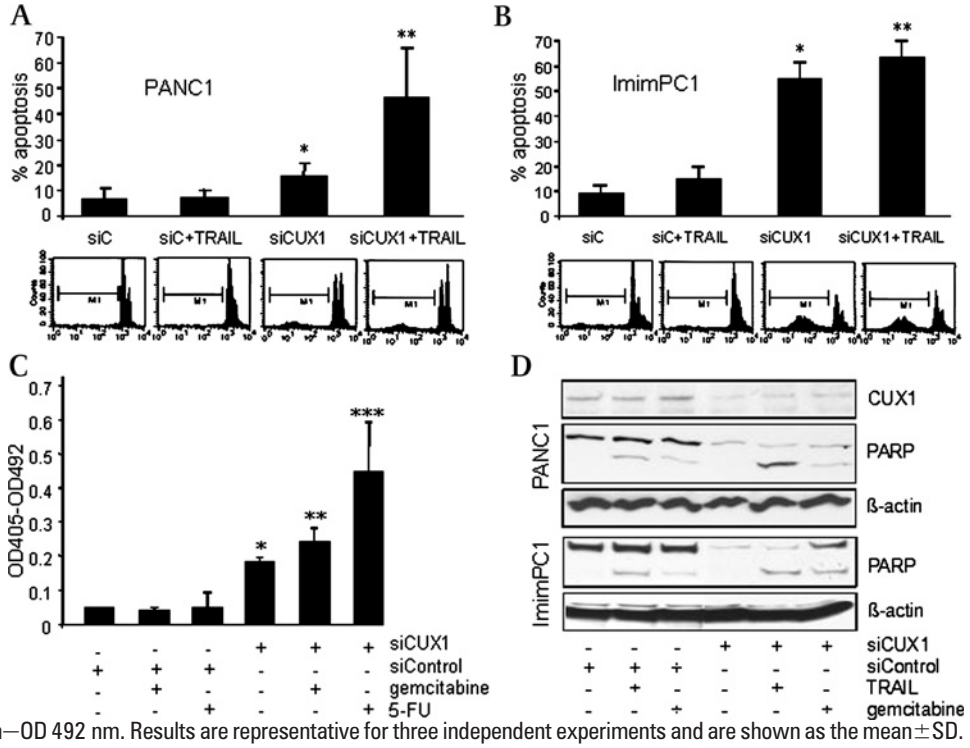

Figure 1 (A and B) Knock-down of *CUX1* increases TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-induced apoptosis. Fluorescence-activated cell sorting (FACS) analysis of PANC1 (A) and ImimPC1 (B) cells, transiently transfected with *CUX1* small interfering RNA (siRNA) (siCUX1) or non-silencing control siRNA (siC), and subsequently treated with 100 ng/ml (PANC1) or 75 ng/ml (ImimPC1) TRAIL for 20 h. DNA fragmentation was quantified by FACS analysis of propidium iodide-stained DNA in sub-G_1 phase (M1). Results are representative for four independent experiments and are shown as the mean±SD. *p<0.05 compared with siC cells; **p<0.05 compared with siC+TRAIL cells. (C) Knock-down of *CUX1* enhances drug-induced apoptosis, as detected by ELISA for histone-associated DNA fragments. PANC1 cells transiently transfected with siCUX1 or siC were treated with gemcitabine (80 μg/ml) or 5-fluorouracil (5-FU; 50 μg/ml) for 20 h. Quantification of histone-associated DNA fragmentation using a specific ELISA was measured at OD 405 nm−OD 492 nm. Results are representative for three independent experiments and are shown as the mean±SD. *p<0.05 compared with siC control cells, **p<0.05 compared with siC+gemcitabine; ***p<0.05 compared with siC+5-FU cells. (D) Knock-down of *CUX1* leads to increased poly ADP-ribose polymerase (PARP) cleavage. After transfection of *CUX1* (siCUX) or control (siC) siRNA, PANC1 and ImimPC1 cells were treated with TRAIL or gemcitabine for 20 h. PARP protein was analysed by immunoblotting with anti-PARP, with cleaved PARP detected as smaller band below the uncleaved form. CUX1 levels were assessed by immunoblotting to confirm the knock-down (similar results in ImimPC1 cells are not shown). The blot was reprobed with the β-actin antibody to assess equality of loading.

pathway). By comparison, chemotherapeutic drugs characteristically trigger apoptosis via induction of DNA damage and activation of the intrinsic apoptosis pathway. To test the effect of CUX1 in drug-induced apoptosis, we treated PANC1 cells±CUX1 siRNA with gemcitabine or 5-FU, two drugs commonly used in pancreatic cancer patients. Similar to TRAIL-induced apoptosis, knock-down of CUX1 sensitised both cell lines to apoptosis induced by gemcitabine or 5-FU (figure 1C).

CUX1 siRNA-induced apoptosis is associated with enhanced PARP cleavage and activation of effector caspases

To delineate further the downstream events in CUX1-modulated apoptosis, we analysed characteristic features of apoptosis such as cleavage of PARP and activation of caspases.

Downregulation of CUX1 led to enhanced PARP cleavage, after treatment with both TRAIL and various drugs including gemcitabine (figure 1D). Similar effects of CUX1 could be observed on activation of effector caspase-3 and caspase-7. Knock-down of CUX1 led to decreased levels of uncleaved caspase-3 accompanied by an increase of cleaved caspase-3 in both cell lines after treatment with TRAIL (figure 2A). In analogy to differences between PANC1 and ImimPC1 cells seen in FACS and TUNEL assays, CUX1 knock-down alone was sufficient to induce a marked cleavage of caspase-3 in ImimPC1 cells. In contrast, PANC1 cells further required induction of apoptosis by TRAIL (figure 2A), indicating that ImimPC1 cells are more susceptible to undergo spontaneous apoptosis after CUX1 withdrawal. In addition to detecting differences in caspase-3 cleavage by immunoblotting, we analysed activation of caspase-3 and caspase-7 using a specific assay which measures cleavage of a luminogenic caspase-3/7 substrate. In this assay it could be confirmed that caspase-3/7 activity was stimulated by knock-down of CUX1 in both PANC1 (figure 2B) and ImimPC1 cells (figure 2C). siRNA for CUX1 significantly enhanced TRAIL-induced activation of caspase-3/7 in both cell lines (figure 2B, C).

These results indicate that downregulation of CUX1 by siRNA effectively enhances the sensitivity of pancreatic cancer cell lines to apoptosis induced by different stimuli that trigger apoptosis via the extrinsic (TRAIL) or intrinsic (gemcitabine and 5-FU) pathway of apoptosis.

Expression of CUX1 protects from TRAIL-induced apoptosis

To exclude the possibility that only knock-down of endogenous CUX1 affects apoptosis, for example by withdrawing a transcription factor required for basal cellular maintenance, we examined whether overexpression of CUX1 protects from TRAIL-induced apoptosis. We first chose HEK293 cells, known to be transfectable with high efficiency, to verify that transient CUX1 overexpression was able to rescue HEK293 cells from TRAIL-induced apoptosis (figrue 3A). To confirm the CUX1-induced rescue from apoptosis also in pancreatic carcinoma cells, we analysed PARP cleavage after transient overexpression of C-terminal CUX1 in PANC1 cells. After CUX1 overexpression, TRAIL-induced PARP cleavage was markedly reduced. Furthermore, we used PANC1 clones stably overexpressing CUX1: in CUX1-overexpressing clones, TRAIL-induced apoptosis was significantly reduced compared with mock-transfected clones, as determined by DNA fragmentation assays (figure 3C). This indicates that CUX1 is able to rescue cells from TRAIL-induced apoptosis.

Apoptosis pathway-focused gene expression profiling identifies CUX1-regulated genes

To identify CUX1 target genes involved in apoptosis, we used a commercial pathway profiler based on quantitative RT-PCR comprising 84 genes involved in the regulation of apoptosis. A description of all 84 genes analysed in this profiler is available online (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-012A.html). ImimPC1 cells transfected with CUX1 siRNA or non-silencing control siRNA were used to obtain RNA. The assay was performed three times for each condition and statistical analysis was performed with the software accompanying the PCR profiler. Genes that were significantly regulated by CUX1 are shown in figure 4A. Interestingly, all genes repressed by CUX1 are involved in the TNF signalling pathway, including TNFα itself, TNFSF7, a member of the TNF superfamily, and TNFRSF9, a member of the TNF receptor superfamily, as well as BIRC3, which has been shown to be induced by TNFα.[25] In addition to genes downregulated by CUX1, we identified the antiapoptotic mitochondrial membrane protein BCL2 as significantly upregulated by CUX1.

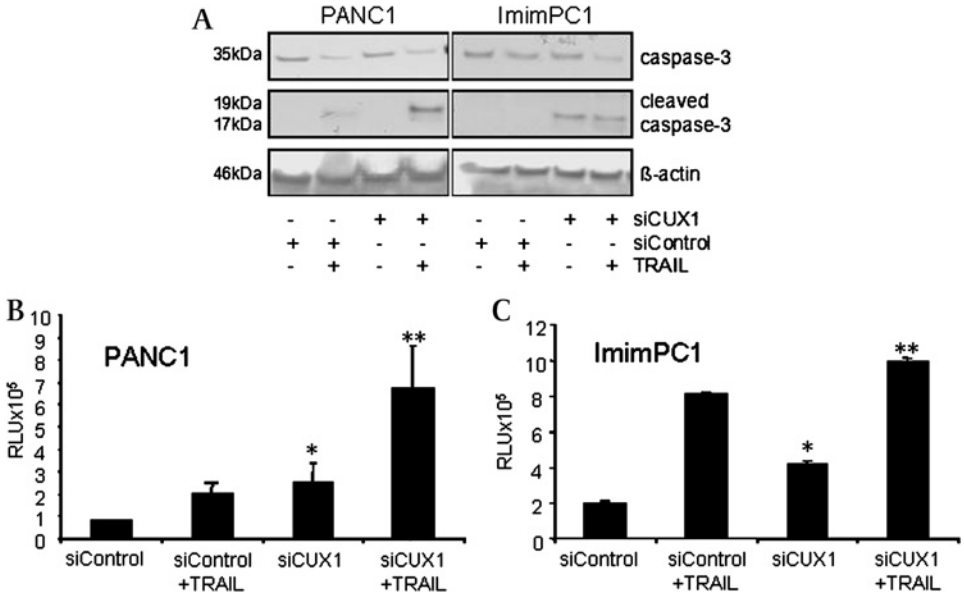

Figure 2 Knock-down of *CUX1* increases activity of caspase-3 and caspase-7. (A) After knock-down of *CUX1* with small interfering RNA (siRNA), PANC1 and ImimPC1 cells were treated with TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) for 20 h. Caspase-3 levels were detected by immunoblotting using antibodies against uncleaved and cleaved caspase-3. The blot was reprobed with the β-actin antibody to control for equal loading. (B and C) Caspase-3/7 activity was analysed with the Caspase-Glo3/7 assay using a specific substrate in PANC1 (B) and ImimPC1 (C) cells. Results are representative for three independent experiments and are shown as the mean±SD. *$p<0.05$ compared with control cells; **$p<0.05$ compared to non-silencing control siRNA (siC) cells+TRAIL.

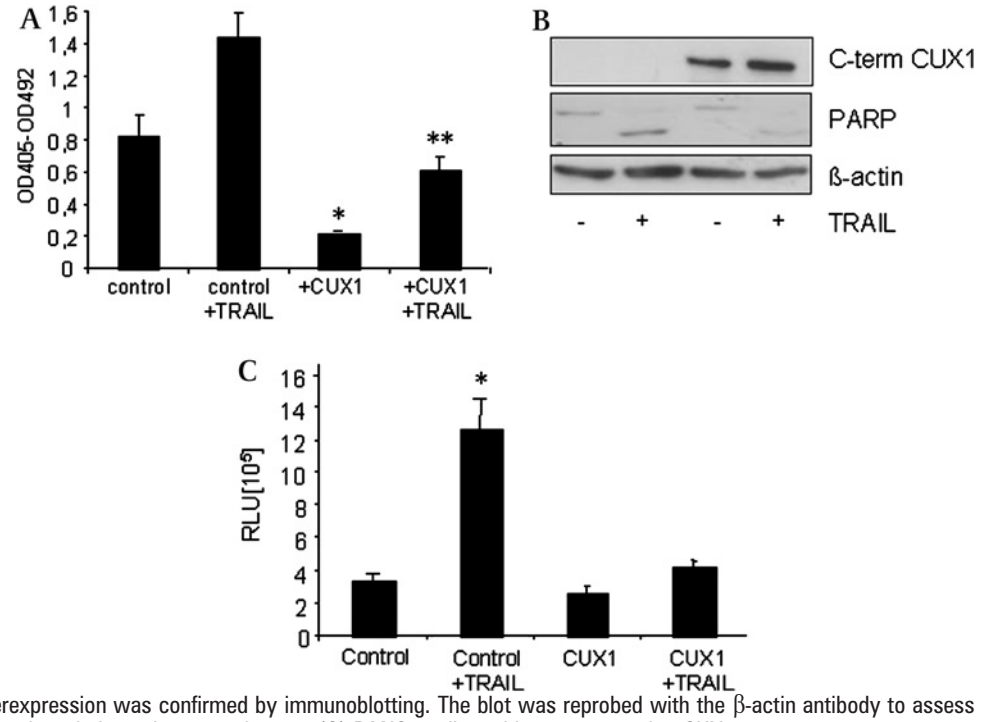

Figure 3 CUX1 expression rescues cells from TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-induced apoptosis. (A) HEK293 cells were transiently transfected with a full-length CUX1 expression plasmid or an empty vector. Apoptosis was induced with TRAIL (50 ng/ml) for 20 h. DNA fragmentation was measured by using Cell Death Detection Elisa^{PLUS}. Results are representative for three independent experiments and are shown as the mean±SD. *$p<0.05$ compared with mock-transfected cells; **$p<0.05$ compared with mock-transfected cells+TRAIL. (B) Overexpression of CUX1 leads to decreased poly ADP-ribose polymerase (PARP) cleavage. PANC1 cells were transiently transfected with the transcriptionally active C-terminal CUX1 expression plasmid or an empty vector and treated with TRAIL for 20 h. PARP protein was analysed by immunoblotting with anti-PARP, with cleaved PARP detected as a smaller band below the uncleaved form. CUX1 overexpression was confirmed by immunoblotting. The blot was reprobed with the β-actin antibody to assess equality of loading and is representative for three independent experiments. (C) PANC1 cells stably overexpressing CUX1 or an empty vector were treated with TRAIL (50 ng/ml) for 20 h. DNA fragmentation was measured by using Cell Death Detection Elisa^{PLUS}. Results are representative for three independent experiments with two different clones each and are shown as mean± SD. *$p<0.05$ compared with control cells.

To confirm the regulation of BCL2 by CUX1 at the protein level, we performed immunoblots with or without CUX1 siRNA. Knock-down of CUX1 led to a marked decrease in BCL2 protein levels in both PANC1 and ImimPC1 cells (immunoblot, figure 4B; semiquantitative densitometry, supplementary figure 4), corroborating the profiler result at the protein level and suggesting that CUX1 protects cells from apoptosis by modulating BCL2 levels as a downstream effector.

The effect of CUX1 on the levels of secreted TNFα could be verified by a specific ELISA. Knock-down of CUX1 significantly increased TNFα levels in the supernatant of PANC1 cells collected over 24 h. This indicates that CUX1 is able to suppress TNFα expression (figure 4C).

These results suggest that CUX1 initiates a transcriptional programme which enhances expression of prosurvival factors and decreases expression of proapoptotic genes, thereby shifting the balance of proapoptotic and antiapoptotic genes towards cell death.

CUX1 is regulated by the anti-apoptotic IGF/PI3K/Akt signalling cascade

After demonstrating the protective effects of CUX1 on apoptosis, we speculated that CUX1 expression is modulated by upstream signalling pathways regulating cell survival and resistance to apoptosis. One of the cardinal signalling cascades in this context is the Akt/protein kinase B (PKB)-dependent prosurvival pathway. Various ligands such as IGF1 activate receptor tyrosine kinases such as IGF1R, which leads to activation of PI3K. PI3K in turn activates Akt/PKB, which induces effector cascades resulting in enhanced survival and resistance to apoptosis in multiple cancers.[26]

Incubation of ImimPC1 cells with IGF1 resulted in a significant upregulation of the CUX1 mRNA level which could be inhibited by co-incubation with the PI3K inhibitor LY294002 (figure 5A). These effects could be confirmed at the protein level by immunoblotting: IGF1 markedly increased CUX1 protein levels in both PANC1 and ImimPC1 cells, which was accompanied by an increase in phosphorylated Akt/PKB (figure 5B; semiquantitative densitometry, supplementary figure 5). PI3K inhibition reduced CUX1 protein levels associated with decreased phosphorylation of Akt/PKB. Co-incubation with both IGF1 and LY294002 partly reversed the inhibition induced by LY294002 alone (figure 5B).

To confirm involvement of Akt/PKB downstream of PI3K, we transiently overexpressed AKT2 in PANC1 cells. Overexpression of AKT2 increased CUX1 protein levels, confirming direct involvement of Akt/PKB in the upstream regulation of CUX1 (figure 5C; semiquantitative densitometry, supplementary figure 6). To examine the relevance of CUX1 as a downstream effector in PI3K/Akt-induced resistance to apoptosis, we modulated CUX1 expression either directly by siRNA or by inhibiting PI3K upstream of CUX1 with LY294002. CUX1 levels inversely correlated with apoptosis in PANC1 cells, measured by histone-bound DNA fragmentation (figure 5D). Interestingly, the siRNA-induced decrease in CUX1 was further reduced by PI3K inhibition, resulting in a further increase in apoptosis. Similarly reduced CUX1 levels, regardless of whether they resulted from direct CUX1 inhibition or from PI3K inhibition, led to comparable apoptosis rates (figure 5D). In addition, we used PANC1 cells stably overexpressing the transcriptionally active C-terminal CUX1 compared with mock-transfected control cells. PI3K/Akt inhibition by LY294002 resulted in significantly increased apoptosis in mock-transfected cells. However, this effect was markedly diminished in CUX1-overexpressing cells (figure 5E). These experiments suggest that CUX1 plays a major role as a downstream effector of PI3K/Akt-induced protection from apoptosis.

CUX1 is highly expressed in pancreatic cancer tissues

To examine the clinical relevance of CUX1 expression in human tissues, we performed ISH experiments using a *CUX1*-specific

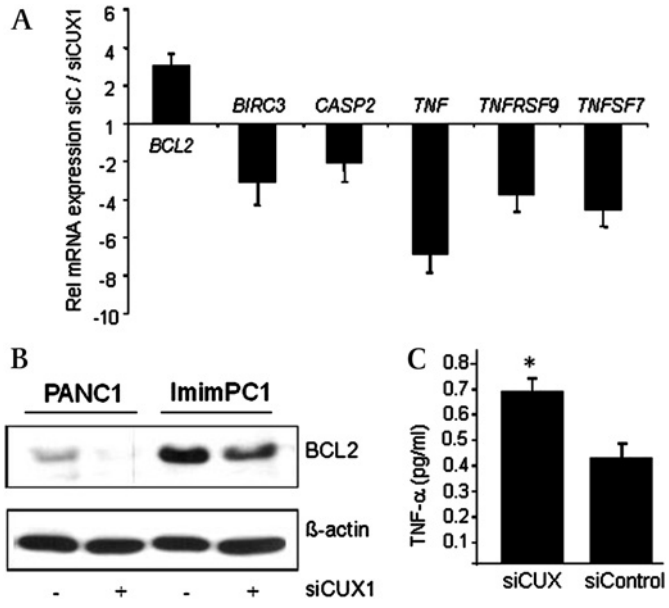

Figure 4 CUX1 modulates transcription of apoptosis-regulating genes. (A) ImimPC1 cells were transfected with *CUX1* small interfering RNA (siCUX1) RNA or control small interfering RNA (siC) and expression profiling of 84 apoptosis pathway-focused genes was performed using the RT^2 Profiler PCR Array System. Results are shown as the expression ratios between siC/siCUX1-treated cells and are representative for three independent experiments. (B) Regulation of BCL2 protein analysed by immunoblotting 48 h after knock-down of *CUX1* by siRNA in PANC1 and ImimPC1 cells. The blot was reprobed with the β-actin antibody to assess equality of loading. The blot is representative for three independent experiments. (C) Tumour necrosis factor α (TNFα) protein levels in the supernatant of PANC1 cells, collected over 24 h, was detected by specific ELISA after transfection with *CUX1* siRNA or siC. Data are representative for three independent experiments and expressed as the mean ± SEM. *$p<0.05$ compared with control siRNA-transfected cells.

riboprobe in multiple tissue arrays containing 49 matched pairs of pancreatic cancer and adjacent normal pancreatic tissues. *CUX1* mRNA expression within the tumour epithelium was scored as negative or positive by two investigators (RP and PM) blinded to the H&E sections, as described previously.[14] Figure 6A depicts *CUX1* ISH (left panel) and corresponding H&E staining (right panel) of three representative positive tumour tissues. A total of 31/49 (63%) of the tumour specimens were scored positive for *CUX1* mRNA as compared with 15/49 (30%) of benign pancreatic tissues (figure 6B). This indicates that *CUX1* is highly overexpressed in pancreatic cancer tissues. To confirm CUX1 expression at the protein level, we performed immunohistochemistry with a CUX1-specific antibody in an independent series of eight pancreatic cancer and normal pancreatic tissues. In this series, we could confirm high expression levels of CUX1 protein in 5/8 cancers compared with normal pancreas (figure 6C). In tumour tissues, we saw a predominant staining of the epithelial tumour cells. However, some cells within the adjacent stroma also appeared to be positive. In normal pancreas tissues, we detected only a faint staining of normal ducts and, to a lesser extent, in acinar cells (figure 6C).

Therapeutic targeting of CUX1 by PEI-complexed siRNA reduces tumour growth and increases apoptosis in vivo

Based on the high expression levels of CUX1 in pancreatic cancers and given the striking in vitro effects of CUX1 knock-down, we aimed to investigate the therapeutic efficacy of the specific knock-down of CUX1 expression in vivo. For the protection and delivery of siRNA oligonucleotides in vivo, we used PEI to form nanoscale complexes, which contained either siRNA oligonucleotides directed against CUX1 (specific treatment group) or non-silencing oligonucleotides (control group). For our in vivo experiments, we used the CUX1-positive cell lines CAPAN1 known to form subcutaneous xenograft tumours reproducibly in nu/nu mice. CAPAN1 cells treated with siRNA against CUX1 showed similar in vitro effects on apoptosis to those described above for PANC1 and ImimPC1 cells (supplementary figure 7). After establishment of subcutaneous tumours, CUX-specific PEI—siRNA complexes or non-silencing control PEI—siRNA complexes were injected intratumourally three times per week over 3 weeks. Tumour size was measured regularly at the time points indicated in figure 7A. After sacrificing the mice, tumours were stained for H&E and CUX1 expression and assessed for apoptosis using the M30 antibody which detects cleaved cytokeratin-17.

Treatment with CUX1-specific PEI—siRNA complexes resulted in a significantly decreased tumour volume, as depicted in figure 7A. This was accompanied by a marked reduction in CUX1 expression compared with the control group (tumours treated with non-specific PEI-complexed siRNAs), as detected by immunohistochemistry (figure 7B) and quantitative RT-PCR (figure 7C). Furthermore, we observed a marked increase in apoptotic cells in CUX1 PEI—siRNA-treated tumours compared with the control tumours, as detected by staining with the M30 antibody (figure 7D). These results confirm our in vitro data and demonstrate that reducing CUX1 expression impairs tumour growth and enhances apoptosis in vivo. As a proof of concept, we could show that therapeutic targeting of CUX1 by nanoparticle-complexed siRNA in vivo reduces tumour growth and is associated with increased apoptosis.

DISCUSSION

In this study, we could demonstrate that CUX1 plays an important role in protecting pancreatic cancer cells from apoptosis induced by various stimuli such as TRAIL and chemotherapeutic drugs, engaging both the extrinsic and the intrinsic apoptosis pathways. The prosurvival effect of CUX1 was associated with upregulation of antiapoptotic proteins such as BCL2 and downregulation of proapoptotic proteins such as TNFα, thereby favouring an antiapoptotic state. Furthermore, we identified CUX1 as a downstream effector of PI3K/Akt signalling, indicating an important role for CUX1 in PI3K/Akt-mediated resistance to apoptosis. In vivo, targeting CUX1 by siRNA led to increased apoptosis and reduced tumour growth in murine xenograft models. The important role of CUX1 in pancreatic cancer progression is evidenced by its high expression levels in pancreatic cancer samples as compared with matched normal pancreatic tissue.

Our data are in accordance with reports in the literature suggesting that CUX1 might play a role in modulating thymocyte cell survival. By constructing a hypomorphic allele of CUX1, Sinclair *et al* generated mutant mice lacking the CUX1 HD. Homozygous mice had dramatically reduced thymic cellularity due to enhanced apoptosis, with a preferential loss of CD4(+)CD8(+) thymocytes. In contrast to the lymphoid demise, however, these mice demonstrated myeloid hyperplasia.[11]

Alcalay *et al* investigated the effect of a truncated version of CUX1 lacking Cut repeat-1 (Cux1 CR1) on the progression of polycystic kidney disease (PKD) by crossing the mutant Cux1 CR1 in a PKD mouse model (*Cys1^{cpk}* mice).[27] Interestingly, Cux1

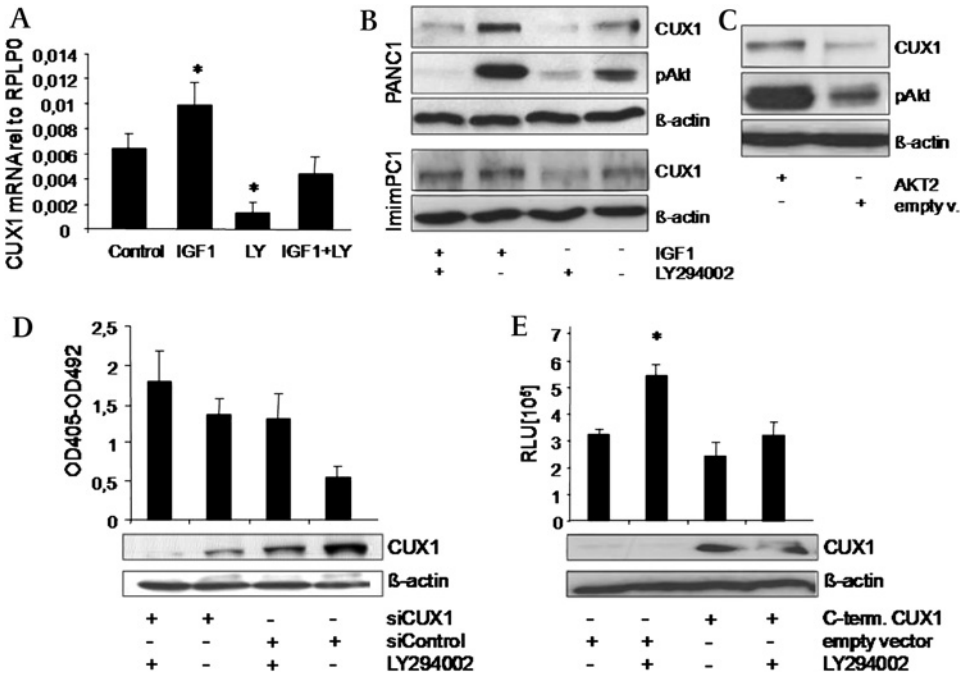

Figure 5 *CUX1* mRNA expression is regulated by insulin-like growth factor 1 (IGF1) and phosphatidylinositol-3-kinase (PI3K). (A) ImimPC1 cells were treated with IGF1 (10 ng/ml) and/or the PI3K inhibitor LY294002 (50 μM) for 24 h. *CUX1* mRNA was quantified by quantitative real-time PCR (RT-PCR). *CUX1* mRNA levels were normalised to *RPLP0* expression as the housekeeping gene. *$p<0.05$ compared with untreated control cells. Results are expressed as the mean±SD and are representative for three independent experiments. (B) Phospho-Akt and CUX1 levels were detected by immunoblotting following treatment with IGF1 and LY294002 for 15 min (pAkt) or 20 h (CUX1). The blots were reprobed with the β-actin antibody to control for equal loading. The blot is representative for three independent experiments. (C) PANC1 cells were transiently transfected with Akt2-pcDNA4 expression plasmid or empty control plasmid. CUX1 and phospho-Akt levels were detected by immunoblotting after 24 h in 5% serum. β-Actin was used as a marker for equal loading. (D) Apoptosis correlates with CUX1 levels modulated either by PI3K inhibition or by CUX1 small interfering RNA (siRNA) knock-down. PANC1 cells transiently transfected with hCUX1 siRNA (siCUX1) or control siRNA (siControl) were incubated with the PI3K inhibitor LY294002 (50 μM) for 24 h. DNA fragmentation was quantified using Cell Death Detection Elisa^{PLUS}. CUX1 and β-actin expression was analysed by immunoblotting. Results are representative for three independent experiments. (E) CUX1 rescues cells from apoptosis induced by PI3K inhibition. PANC1 cells stably overexpressing C-terminal CUX1 were incubated with LY294002 (50 μM) for 24 h. Apoptosis was quantified by measuring caspase-3 and caspase-7 activities using the luminescent assay Caspase-Glo3/7. Results are representative for three independent experiments and are shown as the mean±SD. *$p<0.05$ compared with untreated empty vector-transfected control cells.

CR1, which lacked CR1 but still contained CR2, CR3 and the HD, accelerated progression of PKD which was associated with strong proliferation but also with enhanced apoptosis in cystic epithelial cells.[27] Most probably, the increased apoptosis seen in this mouse model is a secondary effect due to higher cell turnover. Our data using CUX1 expression plasmids suggest that the C-terminal fragment of CUX1 encompassing CR2, CR3 and the HD is clearly protecting from apoptosis.

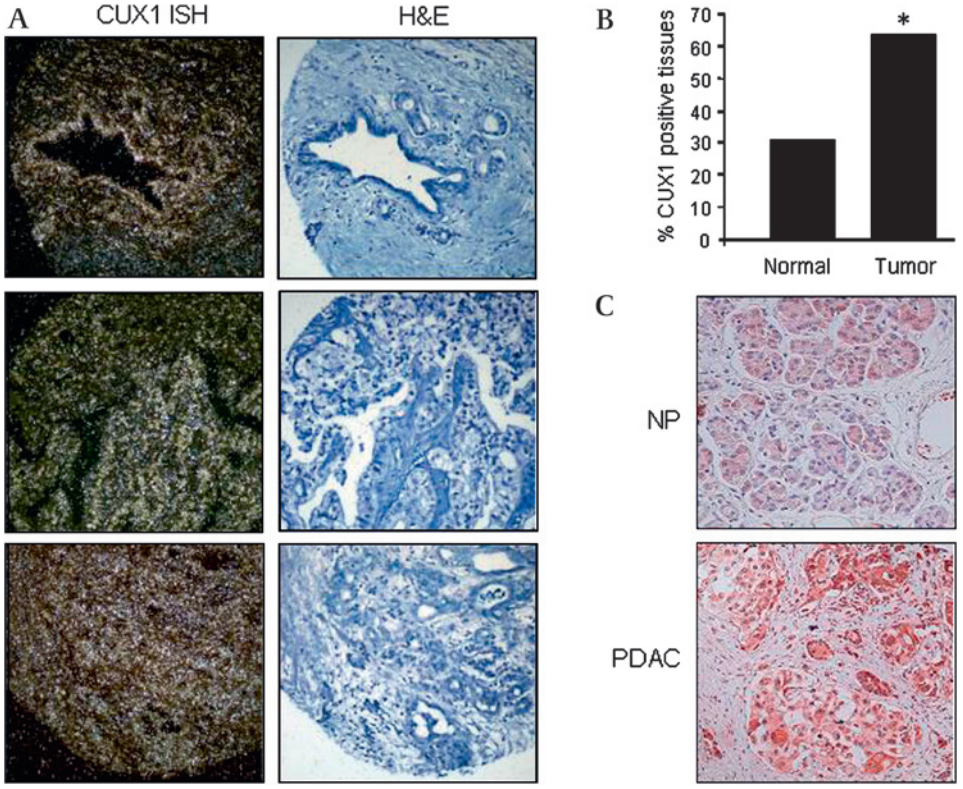

Figure 6 *CUX1* is highly expressed in pancreatic cancer tissues. (A) A total of 49 pancreatic tumour tissues and 49 paired adjacent normal pancreatic tissues were probed with a 35S-labelled CUX riboprobe by in situ hybridisation (ISH). Three representative sections of pancreatic cancer tissues are depicted: *CUX1* expression visualised by dark-field microscopy (left panel) and corresponding H&E sections (right panel). (B) Comparison of *CUX1* expression in pancreatic cancer tissue versus adjacent normal tissue, detected by ISH and scored as negative or positive, shown as a percentage of positive tissues. (C) Representative immunohistochemical stainings of one normal pancreatic tissue and one pancreatic cancer tissue using the rabbit polyclonal anti-CUX1 antibody. NP, normal pancreas; PDAC, ductal adenocarcinomas of the pancreas.

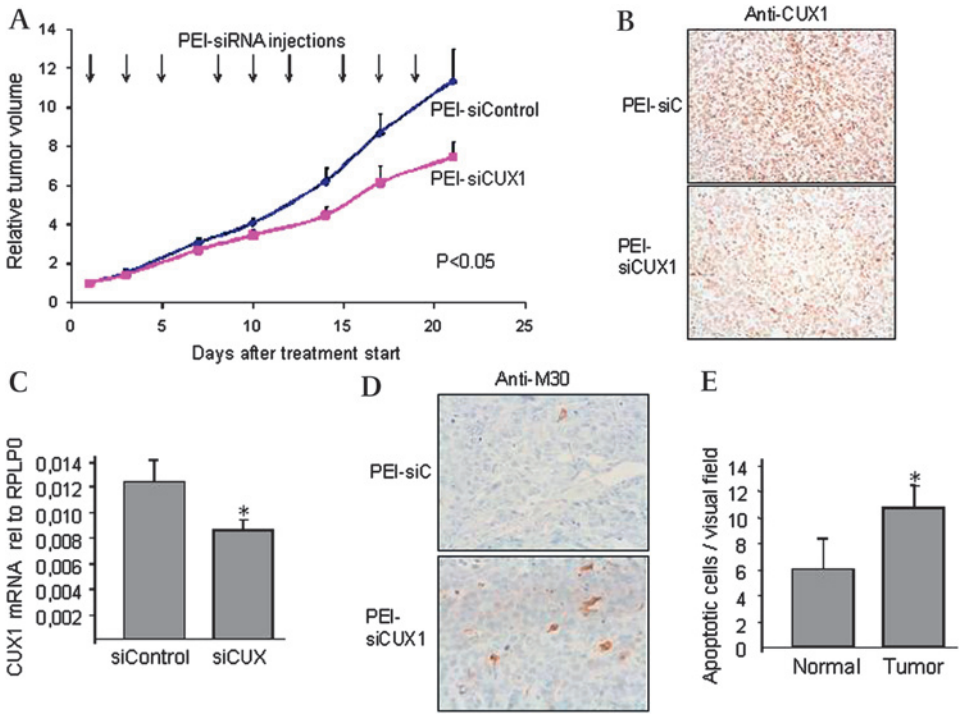

Figure 7 Intratumoural application of polyethylenimine (PEI)-complexed small interfering RNA (siRNA) reduces tumour growth associated with increased apoptosis. (A) Subcutaneous CAPAN1 xenografts were injected intratumourally with PEI-complexed siRNA against *CUX1* or non-silencing control siRNA (three times a week over 21 days, 10 tumours per group). Tumour volume was determined regularly until day 21 after treatment initiation. Tumour volume was normalised to the tumour volume on day 1 and is shown as mean±SEM. $p<0.05$ indicates a significant difference between both groups as determined by paired t test/Wilcoxon matched pairs test. (B) Representative sections of xenograft tumours treated with PEI—siRNA against hCUX1 or non-silencing control stained with anti-CUX1 mouse monoclonal antibody. Original magnification ×200. (C) Quantitative real-time PCR (RT-PCR) of *CUX1* mRNA levels in *CUX1* PEI—siRNA-treated tumours versus control PEI—siRNA-treated tumours, normalised to *RPLP0* expression as the housekeeping gene. *$p<0.05$ compared with control PEI—siRNA-treated cells. Results are expressed as the mean±SD. (D+E) Proportion of apoptotic cells per visual field in sections of tumours treated with PEI—siRNA against CUX or control PEI—siRNA, as measured using the M30 antibody which detects cleaved cytokeratin 17. Two representative pictures (original magnification x200) are shown (D). Five visual fields per tumour and 10 tumours per group were quantified and shown as mean±SD (E).

Recently, Truscott *et al* described proteolytic cleavage of CUX1 by caspases at its C-terminus. This cleavage was observed in the absence of apoptotic stimuli and was associated with increased DNA binding activity, activation of proproliferative target genes of CUX1 and accelerated entry into S-phase.[28] In that study, which was performed in fibroblasts and T cells, caspase activity led to enhanced proliferation even in the absence of apoptosis. The significance of these findings in the presence of apoptotic stimuli remains to be elucidated. It may be speculated that, depending on the cellular context, initial apoptotic stimuli resulting in activation of distinct caspases might also lead to stimulation of CUX1 activity as a prosurvival feedback mechanism.

Our results indicate that CUX1 is a downstream target of PI3K/Akt signalling, one of the cardinal prosurvival pathways mediating resistance to apoptosis in most malignancies including gastrointestinal carcinomas.[26] We could show that activation of PI3K/Akt signalling by ligands such as IGF1 led to transcriptional upregulation of CUX1 at the mRNA and protein level which could be blocked by inhibition of PI3K. Similar effects were seen after transfection of constitutively active Akt2, which has been shown to play an important role in pancreatic cancer progression.[29] Modulating CUX1 levels either directly by siRNA or by inhibition of PI3K activity led to comparable effects on CUX1 levels as well as on apoptosis. This suggests that CUX1 is a major mediator of PI3K/Akt-induced tumour cell survival. We have not yet addressed which effector pathway downstream of Akt is utilised for the transcriptional regulation of CUX1. Several phosphorylation targets of Akt have been implicated in the regulation of apoptosis, such as FOXO1,[30] IKK[31] and CREB,[32] which are worthwhile candidates to investigate for interactions with the CUX1 promoter.

To analyse downstream effectors of CUX1 mediating its prosurvival actions, we employed a quantitative RT-PCR-based screening approach for key targets known to be involved in the modulation of apoptosis. We found that CUX1 significantly upregulates the antiapoptotic protein BCL2 which is localised to the mitochondrial membrane and inhibits membrane permeabilisation by interacting with other, partly proapoptotic members of the same protein family.[33] High levels of BCL2 have been associated with a more aggressive malignant phenotype and/or drug resistance to various categories of chemotherapeutic agents in haematological malignancies and solid tumours.[33] Transcriptional upregulation of BCL2 may well explain the drug-resistent phenotype induced by CUX1 observed in our cell systems. In addition, our quantitative RT-PCR screen also revealed significant downregulation of several genes of the TNF superfamily including TNFα. It is well known that TNFα plays a dual role in modulating cancer progression. Depending on the cellular context, it may be able to promote cell growth and invasion by activating nuclear factor-κB (NF-κB) signalling.[34 35] On the other hand, if NF-κB activation is not successful, TNFα has been shown to be a potent inducer of cell death by inducing activation of caspase-8 and sustained JNK (Jun N-terminal kinase) activation.[36] The apoptosis-inducing effect of TNFα has been confirmed in various cancers including pancreatic cancer.[37 38] Further studies are underway to decipher the effects of CUX1-induced TNFα downregulation in pancreatic cancer.

To evaluate the clinical significance of CUX1 in human pancreatic cancer specimens, we performed ISHs using a *CUX1*-specific probe on a large cohort of pancreatic cancer tissues and paired adjacent normal pancreatic tissue analysed as MTAs. Our data indicate that CUX1 is overexpressed in the majority of pancreatic cancer tissues compared with normal pancreas tissues. Previously, we showed strong expression of CUX1 in a large series of breast cancer tissues.[14] In this series, CUX1 expression was inversely correlated with patient survival, indicating a significant impact of CUX1 expression on tumour

progression.[14] Since most patients with breast cancer underwent multiple chemotherapies during the course of their disease, it may be inferred that inverse correlation of CUX1 with survival is also related to its possible impact on drug resistance. In our current MTA collection of pancreatic cancer specimens, data on the clinical follow-up were also available. However, we could not observe a significant correlation with survival (data not shown). Most probably this is due to the fact that overall survival is considerably shorter in pancreatic cancer compared with breast cancer, precluding the detection of a statistically significant difference in survival. At the protein level, we previously had observed strong CUX1 expression in a small series of pancreatic cancer tissues.[14] In our current series, we could confirm the high expression levels of CUX1 protein in pancreatic tumours compared with normal pancreatic tissues. Taken together, these expression data suggest a significant impact of CUX1 in the progression of various carcinomas including pancreatic cancer.

Based on the high expression of CUX1 in pancreatic cancer tissues, we sought to determine whether therapeutic targeting of CUX1 impacts on tumour growth and apoptosis in vivo. By using a xenograft model, we could show that therapeutic application of *CUX1* siRNA in subcutaneous tumours reduced tumour growth and increased the rate of apoptosis in the tumours. This indicates that CUX1 plays an important role in promoting tumour cell survival in vivo. Interestingly, therapeutic targeting of CUX1 expression also significantly enhances apoptosis in vivo, resulting in decreased tumour growth.

In summary, our data indicate that CUX1 represents an important survival factor downstream of PI3K/Akt, which orchestrates a transcriptional programme mediating resistance to apoptosis in pancreatic cancer. If therapeutic targeting of transcription factors becomes technically more feasible, for example by optimising strategies for systemic delivery of siRNAs, CUX1 would be a promising candidate. Moreover, screening experiments are currently underway in our group to identify further downstream effectors of CUX1, mediating its effects on tumour cell survival. These effectors can be explored as druggable targets to overcome the highly drug-resistant phenotype of pancreatic cancer, which determines the current appalling outcome of patients suffering from this tumour.

Acknowledgements We are grateful to Dorothee Bonorden and Susanne Braun for excellent technical assistance, and to Sabrina Höbel for preparing the PEI—siRNA complexes.

Funding This work was supported in part by grants of the Deutsche Forschungsgemeinschaft (DFG) (to PM), the Behring-Roentgen Foundation (to PM), the Deutsche Krebshilfe (to AA) and the European Commisson FP6 grant LSHB-CT-2006-018771 (Integrated Project 'MolDiag-Paca', to TG). This publication reflects only the authors' views. The European Community is not liable for any use that may be made of the information herein.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Michl P,** Pauls S, Gress TM. Evidence-based diagnosis and staging of pancreatic cancer. *Best Pract Res Clin Gastroenterol* 2006;**20**:227—51.
2. **Gress TM.** The EPC-RC. In: Gress TM, Neoptolemos J, Lemoine NR, *et al*, eds. *Exocrine pancreas cancer. The European Pancreatic Cancer-Research Cooperative (EPC-RC)*. Hannover: Felsenstein, 2005:42—59.
3. **Nepveu A.** Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. *Gene* 2001;**270**:1—15.
4. **Sansregret L,** Nepveu A. The multiple roles of CUX1: insights from mouse models and cell-based assays. *Gene* 2008;**412**:84—94.
5. **Harada R,** Dufort D, is-Larose C, *et al*. Conserved cut repeats in the human cut homeodomain protein function as DNA binding domains. *J Biol Chem* 1994;**269**:2062—7.
6. **van Wijnen AJ,** Cooper C, Odgren P, *et al*. Cell cycle-dependent modifications in activities of pRb-related tumor suppressors and proliferation-specific CDP/cut homeodomain factors in murine hematopoietic progenitor cells. *J Cell Biochem* 1997;**66**:512—23.
7. **Coqueret O,** Berube G, Nepveu A. The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates p21WAF1/CIP1/SDI1 in S phase. *EMBO J* 1998;**17**:4680—94.
8. **Truscott M,** Raynal L, Premdas P, *et al*. CDP/Cux stimulates transcription from the DNA polymerase alpha gene promoter. *Mol Cell Biol* 2003;**23**:3013—28.
9. **Ellis T,** Gambardella L, Horcher M, *et al*. The transcriptional repressor CDP (Cutl1) is essential for epithelial cell differentiation of the lung and the hair follicle. *Genes Dev* 2001;**15**:2307—19.
10. **Luong MX,** van der Meijden CM, Xing D, *et al*. Genetic ablation of the CDP/Cux protein C terminus results in hair cycle defects and reduced male fertility. *Mol Cell Biol* 2002;**22**:1424—37.
11. **Sinclair AM,** Lee JA, Goldstein A, *et al*. Lymphoid apoptosis and myeloid hyperplasia in CCAAT displacement protein mutant mice. *Blood* 2001;**98**:3658—67.
12. **Vanden Heuvel GB,** Brantley JG, Alcalay NI, *et al*. Hepatomegaly in transgenic mice expressing the homeobox gene Cux-1. *Mol Carcinog* 2005;**43**:18—30.
13. **Goulet B,** Watson P, Poirier M, *et al*. Characterization of a tissue-specific CDP/Cux isoform, p75, activated in breast tumor cells. *Cancer Res* 2002;**62**:6625—33.
14. **Michl P,** Ramjaun AR, Pardo OE, *et al*. CUTL1 is a target of TGF(beta) signaling that enhances cancer cell motility and invasiveness. *Cancer Cell* 2005;**7**:521—32.
15. **Michl P,** Knobel B, Downward J. CUTL1 is phosphorylated by protein kinase A, modulating its effects on cell proliferation and motility. *J Biol Chem* 2006;**281**:15138—44.
16. **Aleksic T,** Bechtel M, Krndija D, *et al*. CUTL1 promotes tumor cell migration by decreasing proteasome-mediated Src degradation. *Oncogene* 2007;**26**:5939—49.
17. **Ripka S,** Konig A, Buchholz M, *et al*. WNT5A—target of CUTL1 and potent modulator of tumor cell migration and invasion in pancreatic cancer. *Carcinogenesis* 2007;**28**:1178—87.
18. **Peters GJ,** Van Moorsel CJ, Lakerveld B, *et al*. Effects of gemcitabine on cis-platinum—DNA adduct formation and repair in a panel of gemcitabine and cisplatin-sensitive or -resistant human ovarian cancer cell lines. *Int J Oncol* 2006;**28**:237—44.
19. **Carson JP,** Zhang N, Frampton GM, *et al*. Pharmacogenomic identification of targets for adjuvant therapy with the topoisomerase poison camptothecin. *Cancer Res* 2004;**64**:2096—104.
20. **Nicoletti I,** Migliorati G, Pagliacci MC, *et al*. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991;**139**:271—9.
21. **Werth S,** Urban-Klein B, Dai L, *et al*. A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes. *J Control Release* 2006;**112**:257—70.
22. **Hobel S,** Prinz R, Malek A, *et al*. Polyethylenimine PEI F25-LMW allows the long-term storage of frozen complexes as fully active reagents in siRNA-mediated gene targeting and DNA delivery. *Eur J Pharm Biopharm* 2008;**70**:29—41.
23. **Poulsom R,** Longcroft JM, Jeffery RE, *et al*. A robust method for isotopic riboprobe in situ hybridisation to localise mRNAs in routine pathology specimens. *Eur J Histochem* 1998;**42**:121—32.
24. **Wagner M,** Kunsch S, Duerschmied D, *et al*. Transgenic overexpression of the oncofetal RNA binding protein KOC leads to remodeling of the exocrine pancreas. *Gastroenterology* 2003;**124**:1901—14.
25. **Horrevoets AJ,** Fontijn RD, van Zonneveld AJ, *et al*. Vascular endothelial genes that are responsive to tumor necrosis factor-alpha in vitro are expressed in atherosclerotic lesions, including inhibitor of apoptosis protein-1, stannin, and two novel genes. *Blood* 1999;**93**:3418—31.
26. **Michl P,** Downward J. Mechanisms of disease: PI3K/AKT signaling in gastrointestinal cancers. *Z Gastroenterol* 2005;**43**:1133—9.
27. **Alcalay NI,** Sharma M, Vassmer D, *et al*. Acceleration of polycystic kidney disease progression in cpk mice carrying a deletion in the homeodomain protein Cux1. *Am J Physiol Renal Physiol* 2008;**295**:F1725—34.
28. **Truscott M,** Denault JB, Goulet B, *et al*. Carboxyl-terminal proteolytic processing of CUX1 by a caspase enables transcriptional activation in proliferating cells. *J Biol Chem* 2007;**282**:30216—26.
29. **Yamamoto S,** Tomita Y, Hoshida Y, *et al*. Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2004;**10**:2846—50.
30. **Yang Y,** Zhao Y, Liao W, *et al*. Acetylation of FoxO1 activates Bim expression to induce apoptosis in response to histone deacetylase inhibitor depsipeptide treatment. *Neoplasia* 2009;**11**:313—24.
31. **Sethi G,** Ahn KS, Sung B, *et al*. SH-5, an AKT inhibitor potentiates apoptosis and inhibits invasion through the suppression of anti-apoptotic, proliferative and metastatic gene products regulated by IkappaBalpha kinase activation. *Biochem Pharmacol* 2008;**76**:1404—16.
32. **Sakamoto KM,** Frank DA. CREB in the pathophysiology of cancer: implications for targeting transcription factors for cancer therapy. *Clin Cancer Res* 2009;**15**:2583—7.
33. **Kang MH,** Reynolds CP. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res* 2009;**15**:1126—32.

34. **Arlt A,** Gehrz A, Müerköster S, *et al*. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. *Oncogene* 2003;**22**:3243—51.
35. **Liptay S,** Weber CK, Ludwig L, *et al*. Mitogenic and antiapoptotic role of constitutive NF-kappaB/Rel activity in pancreatic cancer. *Int J Cancer* 2003;**105**:735—46.
36. **Balkwill F.** Tumour necrosis factor and cancer. *Nat Rev Cancer* 2009;**9**:361—71.
37. **Lyu MA,** Kurzrock R, Rosenblum MG. The immunocytokine scFv23/TNF targeting HER-2/neu induces synergistic cytotoxic effects with 5-fluorouracil in TNF-resistant pancreatic cancer cell lines. *Biochem Pharmacol* 2008;**75**:836—46.
38. **Takahashi H,** Funahashi H, Sawai H, *et al*. Synthetic serine protease inhibitor, gabexate mesilate, prevents nuclear factor-kappaB activation and increases TNF-alpha-mediated apoptosis in human pancreatic cancer cells. *Dig Dis Sci* 2007;**52**:2646—52.

Editor's quiz: GI snapshot

ANSWER

From the question on page 1093

The patient was diagnosed with an iatrogenic, tracheo-oesophageal fistula (TEF) secondary to pressure on the trachea from the tracheostomy tube. A TEF is a rare long-term complication of either endotracheal intubation or tracheostomy that occurs as a result of either tracheal posterior wall perforation during a procedure or posterior wall erosion caused by excessive cuff pressures or tube abrasion.[1] Usually the treatment is endoscopic, including stent placement, suturing devices and fibrin glue.[2—5] The endoscopic management generally results in a rapid TEF closure, provides early oral nutrition and may avoid the potential morbidity of surgery.[1] In this case the huge dimension of the TEF necessitated a surgical approach. Before surgery, the patient underwent percutaneous endoscopic gastrostomy (PEG) for feeding with the aim of decompressing the stomach, reducing gastro-oesophageal reflux and hence the risk of aspiration pneumonia. He had started parenteral nutrition and antibiotic treatment (piperacillin, tazobactam and levofloxacin). Twenty days after admission, when the patient's general condition had improved, he underwent surgery. Three surgical accesses (cervical, thoracic and abdominal) were performed. The oesophagus was closed proximally and distally to the TEF and was used as a new 'membranous' wall of the trachea. Gastrointestinal continuity was achieved by cervical oesophagogastric anastomosis. Despite apparent good postoperative recovery, he unfortunately died on the fourth day from a myocardial arrhythmia.

Gut 2010;**59**:1110. doi:10.1136/gut.2009.178129a

REFERENCES

1. **Reed MF,** Mathisen DJ. Tracheoesophageal fistula. *Chest Surg Clin N Am* 2003;**13**:271—89.
2. **Richter GT,** Ryckman F, Brown RL, *et al*. Endoscopic management of recurrent tracheoesophageal fistula. *J Pediatr Surg* 2008;**43**:238—45.
3. **Moses FM,** Wong RK. Stents for esophageal disease. *Curr Treat Options Gastroenterol* 2002;**5**:63—71.
4. **Rocca R,** Fracchia M, Daperno M, *et al*. Treatment of tracheoesophageal fistula in a child by using an endoscopic suturing device. *Gastrointest Endosc* 2007;**65**:932—5.
5. **Scappaticci E,** Ardissone F, Baldi S, *et al*. Closure of an iatrogenic tracheo-esophageal fistula with bronchoscopic gluing in a mechanically ventilated adult patient. *Ann Thorac Surg* 2004;**77**:328—9.