CUX1: a modulator of tumour aggressiveness in pancreatic neuroendocrine neoplasms

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Abstract

Pancreatic neuroendocrine neoplasms (PNEs) constitute a rare tumour entity, and prognosis and treatment options depend on tumour-mediating hallmarks such as angiogenesis, proliferation rate and resistance to apoptosis. The molecular pathways that determine the malignant phenotype are still insufficiently understood and this has limited the use of effective combination therapies in the past. In this study, we aimed to characterise the effect of the oncogenic transcription factor Cut homeobox 1 (*CUX1*) on proliferation, resistance to apoptosis and angiogenesis in murine and human PNEs. The expression and function of *CUX1* were analysed using knockdown and overexpression strategies in Ins-1 and Bon-1 cells, xenograft models and a genetically engineered mouse model of insulinoma (RIP1Tag2). Regulation of angiogenesis was assessed using RNA profiling and functional tube-formation assays in HMEC-1 cells. Finally, *CUX1* expression was assessed in a tissue microarray of 59 human insulinomas and correlated with clinicopathological data. *CUX1* expression was upregulated during tumour progression in a time- and stage-dependent manner in the RIP1Tag2 model, and associated with pro-invasive and metastatic features of human insulinomas. Endogenous and recombinant *CUX1* expression increased tumour cell proliferation, tumour growth, resistance to apoptosis, and angiogenesis *in vitro* and *in vivo*. Mechanistically, the pro-angiogenic effect of *CUX1* was mediated via upregulation of effectors such as HIF1 α and MMP9. *CUX1* mediates an invasive pro-angiogenic phenotype and is associated with malignant behaviour in human insulinomas.

Key Words

- CUX1
- pancreatic neuroendocrine neoplasms
- angiogenesis
- RIP1Tag2
- insulinomas

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Introduction

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) represent a heterogeneous group of solid cancers, on the basis of on embryogenic, morphological and biochemical findings (Modlin *et al.* 2008, Rindi & Wiedenmann 2012). The incidence and prevalence of

neuroendocrine neoplasms (NENs) are constantly increasing, with an estimated number of 5.76/100 000 and 35/100 000 respectively (Yao *et al.* 2008, Lawrence *et al.* 2011). The subgroup of pancreatic NENs (PNEs) is one of the top three entities in NEN, with a reported prevalence

of between 6 and 25% (Pape *et al.* 2008, Lawrence *et al.* 2011). The prognosis, time to progression and response to treatment of PNENs largely depend on tumour-mediating hallmarks such as proliferation rate and resistance to drug-induced apoptosis. Metastasised PNENs show a poor 5-year survival rate of 37.6% with a median overall survival (OS) of 18.9 months (Panzuto *et al.* 2011, 2012, Falconi *et al.* 2012, Larghi *et al.* 2012).

The most frequent genetic alterations in PNENs are inactivating mutations of the *MEN1* and *DAXX/ATRX* genes, dysregulation of the PI3K/AKT/mTOR signalling pathway and overactivation of growth factors and their receptors, such as VEGF PDGF, IGF and c-KIT (Grothey & Galanis 2009, Missiaglia *et al.* 2010, Jiao *et al.* 2011). Increased tumour angiogenesis is a hallmark histological feature of PNENs and is generally correlated with aggressive tumour biology and disease progression (Grothey & Galanis 2009). Preclinical data for an established genetically engineered mouse model of insulinomas (RIP1Tag2) revealed promising activity of the multi-tyrosine-kinase inhibitor sunitinib, which has been approved by the FDA for the treatment of progressive, unresectable, locally advanced or metastatic PNENs (Olson *et al.* 2011, Raymond *et al.* 2011). However, although sunitinib significantly prolonged the progression-free survival (PFS) in metastasised PNENs, it failed to improve the OS, and surrogate markers that predict the response to treatment are currently not available (Raymond *et al.* 2011). Therefore, further molecular characterisation and functional validation of novel targets are urgently needed and hold promise of classifying PNENs into several prognostic and therapeutic subgroups that can be employed for combined and personalised treatment strategies in the future (Capurso *et al.* 2009, 2012).

The transcription factor Cut homeobox 1 (*CUX1*), also called CCAAT-displacement protein (*CDP*) or Cut-like1 (*CUTL1*), is located on chromosomal band 7q22.1 and is expressed in multiple isoforms. Hence, *CUX1* is involved in repressive and activating transcriptional processes depending on the promoter regulation. Results described in recent reports have indicated an important role for *CUX1* in tumorigenesis and tumour progression (Hulea & Nepveu 2012). We have shown that *CUX1* directly mediates tumour cell proliferation, migration and invasiveness and also acts via the target genes *GRIA3* and *WNT5A* in the development of pancreatic ductal adenocarcinomas (PDACs; Aleksic *et al.* 2007, Ripka *et al.* 2007, 2010a, b, Griesmann *et al.* 2013). Moreover, *CUX1* expression is strongly associated with a less differentiated phenotype and decreased survival in patients with breast

cancer (Michl *et al.* 2005). So far, the expression and function of *CUX1* in PNENs have not, to our knowledge, been investigated. In this study, we aimed to elucidate the effects of *CUX1* on tumour growth and progression in neuroendocrine tumour cell lines as well as in a xenografts and the RIP1Tag2 model *in vitro* and *in vivo*. In addition, we investigated potential mechanisms by which *CUX1* mediates angiogenesis, and assessed *CUX1* expression in a series of human insulinomas.

Materials and methods

Material and cell lines

All media contained 10% fetal bovine serum and 40 µg/ml gentamicin. The cell lines were cultured in the indicated media: human Bon-1 carcinoid cells (a kind gift of R Göke, University of Marburg, Germany) (Parekh *et al.* 1994, passage 10–30) were cultured in DMEM/HAM's F12 medium; rat Ins-1 insulinoma cells (donated by C B Wollheim, University of Geneva, Switzerland) (Asfari *et al.* 1992, Lankat-Buttgereit *et al.* 2004, passage 80) were cultured in RPMI1640 medium supplemented with additional 1 mM sodium pyruvate, 10 mM HEPES and 0.05 mM 2-β-mercaptoethanol; HMEC-1 human immortalised microvascular endothelial cells (courtesy of Dr R Köhler, Department of Internal Medicine – Nephrology, Philipps-University, Marburg, Germany (Ades *et al.* 1992)) were cultured in DMEM high-glucose with L-glutamine and 1 mM sodium pyruvate. All cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C. The cell number was analysed by counting the cells in a Neubauer chamber. Thereafter, in our laboratory, the cells were used at passages 8–11. The conditioned media were obtained from stably *CUX1*-expressing Bon-1 cells cultured for 3 days with approximately 70% confluency. The amphotropic packaging cell line LinX was maintained in DMEM, 10% fetal bovine serum, 250 µg/ml gentamicin and 100 µg/ml hygromycin B (Roth, Karlsruhe, Germany). 5-Fluorouracil (5-FU) was purchased from Sigma-Aldrich and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) was obtained from Lilly (Bad Homburg, Germany). Synchronization of cells was not performed before functional assays.

Plasmids, siRNA and retroviral infection

CUX1 was transiently suppressed by using two different oligonucleotides (*CUX1_1* and *CUX1_2*) as described previously (Michl *et al.* 2005). 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, TX, USA). Both silencing sequences resulted in a knockdown efficiency of over 70% (Ripka *et al.* 2010a). Stable Bon-1 and Ins-1 cells were generated using retroviral systems. For retroviral expression, *CUX1* was amplified and cloned into the pENTR-vector using the pENTR/D-TOPO Cloning Kit (Invitrogen) and recombined into a Gateway competent pQCXIP (a gift from T Stiewe). To produce retroviruses, we transfected LinX packaging cells with 5 µg of retroviral vectors; 48 and 72 h after transfection, the retrovirus-containing supernatant was harvested, filtered and supplemented with 8 µg/ml polybrene (Sigma). The target cells were transduced by spin infection at 252 g, at 37 °C for 1 h and selected with puromycin and G418.

RNA isolation, cDNA synthesis and real-time PCR

Murine RIP1Tag2 RNA isolation was done as previously described (Fendrich *et al.* 2011). The 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 carried out using an Applied Biosystems 7500 Fast Real-time PCR machine using the SYBR Green PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions. Sequence-specific primer pairs were designed using the Primer Express Software (Applied Biosystems). RPLP0 was used as an internal standard (XS-13: forward, 5'-GTC GGA GGA GTC GGA CGA G-3' and reverse, 5'-GCC TTT ATT TCC TTG TTT TGC AAA-3'). The primer sequences for *CUX1* are forward 5'-GAC GTG TTG CGC ACT TAA CG-3' and reverse 5'-ACG ACA TAG ATT GGG CTT AAT GCT-3'. Human angiogenesis-pathway-focused gene expression profiling comprising 84 genes was performed using the RT² Profiler PCR Array System (Qiagen) according to the manufacturer's instructions. A list of the genes analysed in this profiler is available online (<http://sabiosciences.com/Manual/1062756>).

Immunoblotting

The cells were lysed in RIPA buffer supplemented with complete protease inhibitor cocktail (Roche). Nuclear and cytoplasmic fractions were obtained using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, St Leon-Rot, Germany). Immunoblottings were carried out as described previously (Ripka *et al.* 2010a).

Viability, proliferation and apoptosis assays

Bromodeoxyuridine (BrdU) incorporation was measured by using the colorimetric BrdU Cell Proliferation ELISA (Roche Diagnostics) according to the manufacturer's instructions. Histone-associated DNA fragments were quantified using Cell Death Detection ELISA PLUS (Roche) according to the manufacturer's instructions. Cell viability was determined by using the MTT Cell Proliferation Kit (Roche Diagnostics) according to the manufacturer's instructions.

Cell migration experiments

The cell migration experiments were carried out as described previously (Michl *et al.* 2005). Briefly, 40 000 cells in 500 µl/insert on a 24-well plate were used and incubated for 4 h. The inserts were then rinsed with PBS, dried at room temperature and fixed with methanol. Migration was measured by counting ten fields of view under a microscope covering about 70–80% of the insert area of the fixed and stained cells (0.2% crystal violet) migrating towards the conditioned media and normalised to the number of cells that migrated towards mock conditioned media. The number of migrated cells was also validated using the via TimeLapseAnalyzer program (Huth *et al.* 2010).

Tube formation assays

BD Matrigel Basement Membrane Matrix Growth Factor Reduced (BD Bioscience, Heidelberg, Baden-Württemberg, Germany) was thawed and plated according to the supplier's protocol. Briefly, 20 000 cells of the human microvascular endothelial cell line HMEC-1 suspended in the appropriate conditioned media of *CUX1* overexpressing Bon-1 cells were plated onto the Matrigel. After incubation for 24 h, at 37 °C, the wells were photographed at a fourfold magnification using a Canon 450 EOS camera connected to the microscope (Olympus IMT2). The length and junctions of the generated tubes were analysed using the TimeLapseAnalyzer program (Huth *et al.* 2010). Each experiment was carried out at least in triplicate with independently obtained conditioned media.

Immunohistochemistry, construction of tissue arrays and evaluation

In brief, paraffin sections were stained after antigen retrieval (microwave in antigen-unmasking solution, Vector Laboratories, Burlingame, CA, USA) with rabbit polyclonal anti-CUX1 (1:200), as described previously

(Michl *et al.* 2005). In addition, CD31 (1:20; Dianova; Hamburg, Germany) and Ki-67 (1:200; Clone SP6; Thermo Fischer Scientific, Fremont, CA, USA) antibodies were used. The binding of antibody was visualised using a biotinylated secondary antibody, avidin-conjugated peroxidase (ABC method; Vector Laboratories), 3,30-diaminobenzidine tetrachloride (DAB) as a substrate and H&E as a counterstain. The insulinoma tissue samples (FFPE) were obtained from the neuroendocrine tumour archives of the Departments of Pathology in Zürich (Switzerland), Düsseldorf (Germany) and Kiel (Germany) from 1975 to 2006 according to the guidelines of the local ethics committee. The 1.5-mm tissue cores were taken from representative areas and inserted into four paraffin blocks. The four MTAs contained a total of 286 cores and additional human tonsil orientation/control cores. The construction was performed using MTA1 tissue arrayer equipment (Beecher Instruments, Sun Prairie, WI, USA). The MTAs were routinely processed for paraffin sectioning. The paraffin sections were deparaffinised and rehydrated and immunohistochemically stained according to routine methods. The analysis was performed by a pathologist with expertise in endocrine and pancreatic tumours (B S) in a blinded fashion regarding tumour parameters. The intensity of the reactions was scored as mild, moderate or strong (score 1, 2 or 3 respectively). The proportion of the positive cells in ducts and tumour areas was estimated as

percentage and divided into scores (<10%, 1; 10–50%, 2; 51–80%, 3 and >80%, 4). The final score was determined as a product of the intensity of the staining and the proportion of positive cells (minimum 0 and maximum 12; Remmele *et al.* 1986). The scores for cores derived from the same tissue were averaged.

Animals and xenografts

Athymic female nude mice (nu/nu mice) were purchased from Charles River (Sulzfeld, Germany). The mice were housed in a climate-controlled SPF facility. All animal experiments were approved by the local government authorities and were carried out according to the guidelines of the animal welfare committee. The nude mice received subcutaneous injections of 1×10^6 CUX1-overexpressing or WT Bon-1 cells (10^6 cells of each clone) dispersed in 0.1 ml of normal saline at each flank. Six mice per group received injections of cells stably transfected with either CUX1 or empty vector. The size of the tumours was measured during the course of observation (twice a week) and after the animals had been killed.

Statistical analyses

All data are presented as mean $\pm$ S.E.M. Two-tailed paired Student's *t*-test was used for statistical evaluation of

Figure 1

CUX1-expressing human and murine insulinoma tissues. (A) Representative sections of human invasive insulinomas immunohistochemically stained for CUX1 demonstrating a prominent expression of the target (upper row ten $\times$ and bottom row 40 $\times$ magnifications; $n=10$). Arrows indicate the invasive front and highly proliferative areas (B). Increasing CUX1 expression in correlation with the islet tumour stage ranging from normal islets, hyperplastic islets to invasive insulinomas, depicted at two different

magnifications (upper row 10 $\times$ and bottom row 20 $\times$; $n=10$).

(C) CUX1 mRNA levels as measured by RT-PCR of five 10-week-old RIP1Tag2 mice (islet hyperplasia stage) compared with five 13-week-old mice (invasive insulinoma stage). Data were normalised to RPLP0 and are representative of at least five independent experiments and presented as relative expression levels $\pm$ s.d. (mean: 1.0 vs 1.74; $n=5$, s.d.=0.2; $P=0.011$). * $P<0.05$; IHC, immunohistochemistry.

the data. The one-way and non-parametric ANOVA test was used to calculate the *P* value for more than two groups. A *P* value <0.05 was considered statistically significant.

Results

CUX1 is expressed in human insulinomas and is upregulated during tumour progression in the RIP1Tag2 model

Immunohistochemistry revealed that *CUX1* is strongly expressed in a series of human insulinomas and shows a particular intense immunoreactivity at the invasive front and in highly proliferative areas (Fig. 1A). To assess *CUX1* expression during tumour progression, we employed an established genetically engineered mouse model of insulinoma (RIP1Tag2 model). The RIP1Tag2 model is a tumour progression model, which has been generated to develop invasive neuroendocrine pancreatic islet neoplasms via hyperplastic and angiogenic precursor stages that closely recapitulate the development of human disease (Hanahan 1985). In this murine model, *CUX1* mRNA and *CUX1* protein were increasingly expressed during tumour progression, with the highest levels in invasive insulinomas in comparison with islet hyperplasia and native islets (Fig. 1B and C: statistical analysis of relative *CUX1* mRNA expression: control vs *CUX1*, mean 1.0 vs 1.7, *P*=0.011). These results indicate that *CUX1* is highly expressed in murine and human PNENs and that *CUX1* may play a role in tumour progression.

CUX1 modulates proliferation, basal and TRAIL-induced apoptosis in neuroendocrine tumour cell lines

In order to study the functional role of *CUX1* in neuroendocrine tumour cells, *CUX1* was genetically ablated in Ins-1 and Bon-1 cells by transient transfection with two different siRNAs. Knockdown of *CUX1* resulted in a significant reduction in cell viability as well as proliferation rate in both cell lines (Fig. 2A, B and C, Supplementary Figure 1A, B and C, see section on supplementary data given at the end of this article: statistical analysis for siCUX1 versus siC in Ins-1 for MTT, mean: 65% versus 100%, *P*=0.013 and BrdU: mean: 81% versus 100%, *P*=0.023 and Bon-1 for MTT: mean: 74% versus 100%, *P*=0.027 and BrdU: 68% versus 100%, *P*=0.012). Next, we overexpressed *CUX1* by stable transfection of Ins-1 and Bon-1 cells using two different clones with an appropriate empty vector control for each cell line (Fig. 2D and Supplementary Figure 1D).

MTT *in vitro* assays (Fig. 2E and Supplementary Figure 1E: statistical analysis for Mock versus *CUX1*-overexpressing after 72 h in Ins-1: mean: 461% versus 657%, *P*=0.002 and Bon-1: mean: 609% versus 741%, *P*=0.023) and BrdU experiments (Fig. 2F and Supplementary Figure 1F: Mock versus *CUX1* in Ins-1: mean: 100% versus 123%, *P*=0.0003 and Bon-1: mean: 100% versus 123%, s.d. 19; *P*=0.009) unequivocally showed robust and statistically significant increase in proliferation in both cell lines compared with the relevant mock control group.

Next, we investigated the effect of *CUX1* on basal and TRAIL-induced apoptosis in Ins-1 and Bon-1 cells using the same experimental setting. Knockdown of *CUX1* induced basal apoptosis in both cell lines, as determined by cell death detection assay (Fig 3A and Supplementary

Figure 2

CUX1 affects proliferation in insulinoma cells. (A) Ins-1 cells were transiently transfected with *CUX1* siRNA (siCUX1) or control siRNA (siC). Protein isolation and western blotting analysis were carried out demonstrating the *CUX1* knockdown. (B) Relative cell viability was measured via MTT after 24 h (*n*=4; mean: 65% versus 100%; s.d. 7.5; *P*=0.013). (C) Proliferation after suppression of *CUX1* in Ins-1 cells after 24 h was assessed by BrdU assay (*n*=4; mean: 81% versus 100%; s.d. 14.9, *P*=0.023). (D) Confirmation of stable overexpression of *CUX1* in Ins-1 cells by western blot analysis. (E) The relative cell viability of *CUX1* overexpressing (CUX1) and empty vector control-transfected cells (Mock) was detected after 24 h (*n*=6; mean: 100% versus 123%, s.d. 4) and 72 h (*n*=6; mean: 461% versus 657%, s.d. 13, *P*=0.002) via MTT assay. (F) Similarly, the relative proliferation of *CUX1*-overexpressing cells in comparison with mock-transfected cells was quantified by BrdU assay (*n*=5; mean: 100% versus 123%, s.d. 16, *P*=0.0003). Averages of the two stable clones each were evaluated and presented. Data are representative of at least three independent experiments and are shown as mean ± s.d. **P*<0.05 as compared with control cells.

Figure 3

CUX1 overcomes basal and drug-induced apoptosis in insulinoma cells. (A) Knockdown of *CUX1* in Ins-1 cells via siRNA (siCUX1) in comparison with empty vector (siC) ($n=3$; mean: 166 vs 100, s.d. 10, $P=0.001$). Apoptosis was determined by using a specific cell death detection ELISA quantifying the histone-bound DNA fragmentation. (B) Apoptosis after knockdown of *CUX1* by siRNA and apoptosis induced by TRAIL (100 ng) after 24 h, as assessed by a caspase-3/7 activation assay ($n=4$; mean: 100% versus 200% and 124% versus 227%; s.d. 26, 35, 24; siC/siCUX1, $P=0.019$; siC+TRAIL/siCUX1+TRAIL, $P=0.0043$). (C) Apoptosis in Ins-1 cells stably overexpressing *CUX1* and mock-transfected control cells +/- TRAIL and +/- 5-FU (0.2 mM) for 24 h as assessed by quantification of histone-bound DNA fragmentation ($n=3$; mean: 100 vs 78; 147 vs 101; 156 vs 62; s.d. 1 vs 3.7; 4.6 vs 0.6; 5.7 vs 2.3; Mock/CUX1, $P=0.04$; Mock/CUX1+5-FU, $P=0.011$; Mock/CUX1+TRAIL, $P=0.0023$). Data are representative of at least three independent experiments and are shown as mean $\pm$ s.d. * $P<0.05$ as compared with control cells.

Figure 2A: statistical analysis siCUX1 versus siC in Ins-1: mean: 166% versus 100%, $P=0.001$ and Bon-1: mean: 133% versus 100%; $P=0.021$). Interestingly, Ins-1 cells were more sensitive than Bon-1 cells to the suppression of *CUX1*, resulting in a significant increase in apoptosis without any apoptotic stimulus. In addition, knockdown of *CUX1* significantly enhanced TRAIL-induced apoptosis, whereas treatment with TRAIL alone had only a minor effect on apoptosis induction, as measured by effector caspase-3/7 activity (Fig. 3B and Supplementary Figure 2B: statistical analysis for Ins-1: mean: 100% versus 200% and 124% versus 227%, siC/siCUX1, $P=0.019$; siC+TRAIL/

siCUX1+TRAIL, $P=0.0043$ and Bon-1: mean: 100% versus 298% and 110% versus 413%, siC/siC+TRAIL, $P=0.001$; siCUX1/siCUX1+TRAIL, $P=0.0031$). TRAIL-induced apoptosis was more pronounced in Bon-1 cells, probably due to the fact that these cells were highly proliferative and less differentiated. To confirm the anti-apoptotic effects of *CUX1*, we additionally carried out western blotting for cleaved caspase-3: knockdown of *CUX1* increased cleaved caspase-3 levels (Supplementary Figure 3A).

To exclude the possibility that only siRNA-mediated suppression of endogenous *CUX1* affects apoptosis due to off-target effects, we assessed whether overexpression of *CUX1* is able to protect cells from TRAIL- and 5-FU-induced apoptosis. Upon *CUX1* overexpression, basal apoptosis rates were strongly reduced, as determined by cell death detection assays (Fig. 3C and Supplementary Figure 2C, see section on supplementary data given at the end of this article: statistical analysis for Ins-1: Mock/CUX1, Mock/CUX1+5-FU and Mock/CUX1+TRAIL: mean: 100% versus 78%, 147% versus 101%, and 156% versus 62%; $P=0.04$, $P=0.011$, and $P=0.0023$, respectively and Bon-1: Mock/CUX1, Mock/CUX1+5-FU and Mock/CUX1+TRAIL: mean: 100% versus 43%, 30% versus 32%, and 167% versus 129%; $P=0.003$, $P=0.8$, and $P=0.024$, respectively). Furthermore, *CUX1* expression protected Ins-1 and Bon-1 cells from TRAIL-induced apoptosis (Fig. 3C and Supplementary Figures 2C and 3B). Interestingly, while *CUX1* affected cell viability and protected cells from TRAIL-induced apoptosis in both cell lines, it affected 5-FU-induced apoptosis only in Ins-1 cells (Fig. 3C and Supplementary Figure 2C). Taken together, these results indicate that *CUX1* mediates pro-proliferative and anti-apoptotic effects in neuroendocrine tumour cell lines.

CUX1 increases tube formation in HMEC-1 cells via tumour cell-derived secreted pro-angiogenic mediators

One of the most important features of NENs is the high vascularisation that is established and maintained by a complex molecular and cellular crosstalk between neoplastic cells and endothelial cells. To assess the potential angiogenic effects of *CUX1*, we used an established tube formation assay. The conditioned medium from *CUX1*-overexpressing neuroendocrine tumour cells was used to stimulate human immortalised microvascular endothelial cells (HMEC-1) in order to assess the ability of endothelial cells to generate vessels. After 24 h stimulation with Ins-1 and Bon-1 cell supernatants, HMEC-1 cells displayed

Figure 4

CUX1 augments angiogenesis in tube formation assays. (A) Representative pictures of HMEC-1 cells incubated with mock or CUX1-conditioned medium from Bon-1 cells. Analysis of tube formation assay by determining length (red lines) and junctions (yellow dots) of tubes evaluated using TimeLapseAnalyzer program (B and C). Calculation of length (mean: 100% versus 116%; s.d. 14.6; $P=0.044$) and junctions (mean: 100% versus 128%; s.d. 18.9; $P=0.022$) from pictures were done from at least nine independent experiments. Photographs were taken at 4 $\times$ magnification. Tube formation was performed with conditioned medium from Ins-1 cells stably transfected with CUX1 compared with mock-transfected cells. * $P<0.05$ as compared with control cells.

significant induction of tube formation in a CUX1-dependent manner (Fig. 4A, B and C, [Supplementary Figure 4A and B](#), see section on [supplementary data](#) given at the end of this article: statistical analysis of Ins-1 Mock CM and CUX1 CM: length: mean: 100% versus 116%; $P=0.044$ and junctions: mean: 100% versus 128%; $P=0.022$ and Bon-1: length: mean: 100% versus 115%; $P=0.012$ and junctions: mean: 100% versus 132%; $P=0.006$). Analysis of the assays, using the TimeLapseAnalyzer program, from at least five independent experiments demonstrated a remarkable increase in tube length and junctions (Fig. 4A, B and C and [Supplementary Figure 4A and B](#)). In contrast

to tube length and formation of junctions, HMEC-1 migration was not altered significantly ([Supplementary Figure 5](#): statistical analysis of Ins-1-CM: mean: 100% versus 118%; $P=0.08$ and Bon-1-CM: mean 100% versus 120%; $P=0.06$).

To elucidate the transcriptional alterations underlying this CUX1-dependent pro-angiogenic phenotype, we used a human angiogenesis-pathway-focused gene expression profiler comprising 84 genes to examine potential CUX1-regulated targets at the mRNA level in CUX1-overexpressing Bon-1 cells ([Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). Among others, the hypoxia-induced transcription factor

Figure 5

CUX1 modulates the expression of angiogenic markers in neuroendocrine cells. (A) QRT-PCR analysis for *HIF1α* and *MMP9* mRNA in CUX1-over-expressing and mock transfected Bon-1 cells. *HIF1α*: $n=3$; mean: 0.09 vs 0.26; s.d. 0.009 vs 0.11; $P=0.1$ and *MMP9*: $n=3$; mean: 1.0 vs 1.9; s.d. 0.23 vs 1.2 $P=0.05$. (B) Confirmation of the CUX1-dependent regulation of *HIF1α* and *MMP9* at the protein level by western blotting analysis. Actin served as a control for equal protein loading.

HIF1 α as well as the matrix metalloproteases *MMP9* were consistently induced by CUX1. Both genes could be confirmed to be modulated by CUX1 at the mRNA and protein level (Fig. 5A and B: statistical analysis for mRNA expression relative to GAPDH in Bon-1 Mock and CUX1-overexpressing cells: *HIF1 α* : mean: 0.09 vs 0.26; $P=0.1$ and *MMP9*: 1.0 vs 1.9; $P=0.05$). These results indicate that CUX1 expression stimulates angiogenesis in endothelial cells *in vitro*, with a number of differentially regulated pro-angiogenic effector molecules indicating an important mechanistic crosstalk between the secretome of neuroendocrine tumour cells and endothelial cells during angiogenesis (Supplementary Figures 6, 7 and 8).

CUX1 expression correlates with tumour-mediating hallmarks in murine and human insulinomas

To validate our *in vitro* findings on the role of CUX1 as a regulator of proliferation and angiogenesis, xenograft

experiments were carried out on nude mice using CUX1-transfected Bon-1 cells. Expression of CUX1 was confirmed by western blotting analysis in $n=4$ mice each at endpoint (Fig. 6B). CUX1 tumours showed a marked trend towards increased tumour volumes 7 weeks after implantation which, however, did not reach statistical significance due to the limited number of animals (Fig. 6A: statistical analysis: mean: 134 mm versus 226 mm; $P=0.26$).

In line with our *in vitro* data, CUX1 expression *in vivo* caused a significant increase in tumour cell proliferation (Mock versus CUX1: mean: 14.6% versus 37.5%; $P=0.0045$), as assessed by Ki-67 immunohistochemistry, but failed to increase mean vessel density (Mock versus CUX1: mean: 26.4 versus 38.6 positive cells per visual field; $P=0.074$), as determined by CD31 immunohistochemistry (Fig. 6C and E). Despite the fact that grafting of CUX1-expressing Bon-1 cells resulted in larger and more proliferative xenograft tumours, systematic histological analysis of H&E sections showed a significant reduction in the necrotic areas of CUX1-expressing xenografts (Fig. 6D: statistical analysis Mock versus CUX1: mean: 49.7% versus 24.8%; $P=0.027$).

As CUX1 is involved in the tumour progression *in vitro* and *in vivo*, we sought to elucidate the clinical relevance of CUX1 in tumour progression and metastasis by studying CUX1 expression in human insulinoma samples. To this end, we used MTAs containing 59 different human insulinomas tissues for immunohistochemical analysis. In the entire cohort, samples from 39 female and 20 male patients were included (Supplementary Table 2, see section on supplementary data given at the end of this article): 52 primary tumours, three lymph nodes and four liver metastases were immunohistochemically investigated. Overall, 45 tumours exhibited a benign behaviour in comparison with 14 malignant tumours. CUX1 showed a distinct nuclear expression in islets, as well as benign and malignant insulinomas. Out of 59 insulinomas, 58 were CUX1 positive. Importantly, malignant tumours revealed significantly increased immunoreactivity for CUX1 compared with those with benign behaviour (immunoreactivity score (IRS) 8.8 versus 5.8, $P=0.002$; Table 1, Fig. 7A and B). Metastases scored higher for CUX1 positivity than primary tumours ($P=0.018$), independently of their localisation (lymph node or liver). In addition, a significant correlation between CUX1 expression and G1/G2 grading was observed ($P=0.038$), indicating a connection between CUX1 and proliferation, because tumour grading is based on the Ki-67 proliferation index. Among the 14 patients with malignant insulinoma,

Figure 6

CUX1 influences tumour growth via acceleration of proliferation and neovascularisation *in vivo*. (A) Nude mice received subcutaneous injections of 1×10^6 CUX1-overexpressing or mock-transfected Bon-1 cells (10^6 cells of each clone). Six mice per group received injections. Tumours were measured *ex vivo* after the animals had been killed (mean: 134 mm versus 226 mm; s.d. 42 vs 65; $P=0.26$). (B) Representative expression of CUX1 in protein lysates of CUX1 overexpressing or mock-transfected Bon-1 xenografts, for which material for protein lysates was available. (C, D and E) Immunohistochemistry of Ki-67 and CD31 as well as H&E evaluation in tumour tissues +/- CUX1 overexpression. (C) The proliferation index of the xenograft tumours was estimated using the antibody against Ki-67 ($n=8$; mean: 14.6% versus 37.5%; s.d. 3.1 versus 4.8; $P=0.0045$). (D) Necrosis areas were quantified and evaluated comparing CUX1- and mock-transfected xenografts ($n=8$; mean: 49.7% versus 24.8%; s.d. 12.2 versus 3.1; $P=0.027$). (E) Proportion of endothelial cells per visual field in sections of CUX- or mock-tumours as measured using the CD31 antibody which detects endothelial cells ($n=8$; mean: 26.4 versus 38.6 CD31-positive cells per visual field; s.d. = 2.4 vs 4.6; $P=0.074$). Data are shown as mean $\pm$ s.d. * $P<0.05$ as compared with mock plasmid.

Table 1]CUX1 expression is increased in malignant insulinomas. CUX1 expression was evaluated using the Remmele and Stegner immunoreactive score as described in 'Materials and methods'

WHO 2010	CUX1 score (0–12)		ANOVA
	Mean	S.E.M.	
Grade 1 (n=43)	5.93	3.42	P=0.038
Grade 2 (n=12)	8.07	2.76	
Site			
Primary benign (n=45)	5.84	3.1	P=0.018
Primary malignant (n=7)	8.42	3.91	
Node metastasis (n=3)	10	1.73	
Liver metastasis (n=4)	9.25	3.77	
Behaviour			P=0.002
Benign (n=45)	5.84	3.1	
Malignant (n=14)	9.0	3.35	

Data are shown as mean $\pm$ S.E.M.

five disease-related deaths occurred, none in the benign group (Supplementary Table 2). The OS of 36.0 months in patients with malignant insulinomas demonstrated the poor prognosis of this tumour entity.

In summary, our *in vivo* data indicate that CUX1 mediates tumour progression and angiogenesis in murine neuroendocrine tumours and is associated with malignant behaviour in human insulinomas.

Discussion

PNENs represent the second most common malignant disease of the pancreas with the majority of patients presenting at a metastasised and unresectable stage. The mTOR inhibitor everolimus and the multi-target anti-angiogenic agent sunitinib have recently been introduced for the treatment of progressive, unresectable, locally advanced or metastatic PNENs, but still fail to prolong OS (Raymond *et al.* 2011, Yao *et al.* 2011). Therefore, novel therapeutic approaches and predictive biomarkers are needed to classify patients into subgroups to better predict treatment success and to guide treatment by molecular rationales. Furthermore, molecular and biochemical characterisation of novel targets in PNENs may aid in devising new treatment approaches and treatment combinations that can be deployed upon primary and secondary chemoresistance.

CUX1 has been identified as a transcriptional activator as well as a transcriptional repressor, and its activity has been associated with tumour progression and shortened survival in several tumour entities (Michl *et al.* 2005, Ripka *et al.* 2010a).

Based on several studies on PDAC, CUX1 was found to co-opt Src and other downstream signalling molecules such as WNT5A to induce an invasive phenotype in pancreatic cancer (Aleksic *et al.* 2007, Ripka *et al.* 2007, 2010b, Griesmann *et al.* 2013). Moreover, CUX1 has been described as transcriptional target of TGFB, mediating cell motility via influencing migration and invasion in diverse *in vitro* and *in vivo* systems (Michl *et al.* 2005). Previous results indicated that CUX1 is also a major mediator of PI3K/AKT-induced tumour cell survival (Ripka *et al.* 2010a).

Although PDAC and PNENs are distinctly different in terms of molecular evolution, clinical behaviour, prognosis and treatment options, there seems to be a considerable molecular overlap in terms of dysregulated pathways in PDAC and PNENs. For instance, the PI3K/AKT/mTOR pathway, the TGFB pathway and Src family kinase activity have been implicated in the pathogenesis of PNENs (Di Florio *et al.* 2007, 2011, Ghayouri *et al.* 2010, Missiaglia *et al.* 2010, Capdevila &

Figure 7

CUX1 expression is increased in malignant insulinomas. (A) Comparison of representative CUX1 IHC in benign and malignant insulinomas at 10 $\times$ magnification. (B) CUX1 score assessed in human benign and malignant insulinomas (mean: 5.8 versus 8.8; S.E.M. 0.47 versus 0.98; $P=0.002$). Data are shown as mean $\pm$ S.E.M. (B). * $P<0.05$ as compared with benign insulinomas.

Tabernero 2011). Therefore, we aimed to investigate the expression and molecular function of the oncogenic transcription factor *CUX1* in neuroendocrine tumours.

We showed that *CUX1* is a mediator of tumour progression in two neuroendocrine tumour cell lines and xenograft tumours by promoting proliferation, tumour growth and resistance to apoptosis. Importantly, 5-FU-containing regimens represent a current standard of care treatment in combination with streptozocin (Moertel *et al.* 1992, Falconi *et al.* 2012), and in fact *CUX1* protected cells against 5-FU-induced apoptosis in the insulinoma cell line thus indicates the potential therapeutic relevance of our findings.

Interestingly, in xenograft experiments, *CUX1* overexpression reduced necrotic areas which might be attributable to *CUX1*-induced improvements in perfusion and angiogenesis in this experimental setting.

Furthermore, we identify *CUX1* as novel modulator of tumour angiogenesis *in vitro* and *in vivo* by paracrine stimulation of endothelial cells via a *CUX1*-dependent, pro-angiogenic secretome in neuroendocrine tumour cells. Surprisingly, the classic mediators of angiogenesis, VEGF, FGF and PDGF, revealed no differences in response to *CUX1* expression. However, we found that MMP9 and HIF1 α were both upregulated in *CUX1*-overexpressing cells, and both proteins have been implicated in tumour progression and angiogenesis (Couvelard *et al.* 2008). For instance, MMP9 has been identified as critical modulator of the angiogenic switch in the RIP1Tag2 model (Bergers *et al.* 2000). Interestingly, MMP9 does not only promote angiogenesis but also interacts with VEGF, facilitating its expression and release (Bergers *et al.* 2000).

Finally, to evaluate the clinical significance of *CUX1* in human PNENs, we carried out *CUX1* immunohistochemistry on a large cohort of human insulinomas. Our results indicate that *CUX1* is strongly expressed in the majority of insulinoma tissues. Moreover, malignant invasive insulinomas expressed more *CUX1* compared with those with benign behaviour, and metastatic tumours were slightly more positive for *CUX1* than primary tumours. In analogy to our findings, *CUX1* has been reported to be overexpressed in breast and pancreatic PDAC, and inversely correlated with the patients' outcome in breast cancer (Michl *et al.* 2005, Ripka *et al.* 2010a). Data on the clinical follow-up of our patients confirmed that surgical cure is feasible in most cases of patients with benign insulinomas. However, malignant insulinomas presented with an aggressive behaviour associated a shortened OS of approximately 35 months. Based on our data on insulinomas, we are currently planning to investigate and

correlate *CUX1* expression with clinicopathological features and outcome in patients with human non-functional pancreatic neoplasms.

In summary, our results indicate for the first time, to our knowledge, an involvement of the transcription factor *CUX1* in NENs. *CUX1* promotes carcinogenesis via regulation of growth, apoptosis and angiogenesis by multiple mechanisms. Mechanistically, we found that *CUX1* promotes tumour angiogenesis via paracrine stimulation of endothelial cells. Tumour angiogenesis represents a hallmark feature of PNENs, and anti-angiogenic drugs are currently the most promising candidates for treatment of PNENs. Therefore, *CUX1*-dependent pathways may constitute attractive targets for future therapeutic strategies for PNENs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0152>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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