Laboratory Investigation (2011) 91, 1472–1479

MicroRNA-148a is down-regulated in human pancreatic ductal adenocarcinomas and regulates cell survival by targeting *CDC25B*

Sven-T Liffers*[,1], Johanna B Munding*[,1], Markus Vogt[1], Jan D Kuhlmann[2], Berlinda Verdoodt[1], Sandeep Nambiar[1], Abdelouahid Maghnouj[2], Alireza Mirmohammadsadegh[1], Stephan A Hahn[2] and Andrea Tannapfel[1]

MicroRNAs (miRNAs: short non-coding RNAs) are emerging as a class of potential novel tumor markers, as their dysregulation is being increasingly reported in various types of cancers. In the present study, we investigated the transcription status of *miRNA-148a* (*miR-148a*) in human pancreatic ductal adenocarcinoma (PDAC) and its role in the regulation of the dual specificity protein phosphatase *CDC25B*. We observed that *miR-148a* exhibited a significant 4-fold down-regulation in PDAC as opposed to normal pancreatic ductal cells. In addition, we observed that stable lentiviral-mediated overexpression of *miR-148a* in the pancreatic cancer cell line IMIM-PC2, inhibited tumor cell growth and colony formation. Furthermore, *CDC25B* was identified as a potential target of *miR-148a* by *in silico* analysis using PicTar, Targetscan and miRanda in conjunction with gene ontology analysis. The proposed interaction between *miR-148a* and the 3′ untranslated region (*UTR*) of *CDC25B* was verified by *in-vitro* luciferase assays. We demonstrate that the activity of a luciferase reporter containing the 3′*UTR* of *CDC25B* was repressed in the presence of *miR-148a* mimics, confirming that *miR-148a* targets the 3′*UTR* of *CDC25B*. Finally, *CDC25B* was down-regulated at the protein level in *miR-148a* overexpressing IMIM-PC2-cells, and in transiently transfected pancreatic cell lines (as detected by Western blot analysis), as well as in patient tumor samples (as detected by immunohistochemistry). In summary, we identified *CDC25B* as a novel *miR-148a* target which may confer a proliferative advantage in PDAC.

Laboratory Investigation (2011) **91,** 1472–1479; doi:10.1038/labinvest.2011.99; published online 27 June 2011

KEYWORDS: CDC25B; miR-148a; pancreatic ductal adenocarcinoma (PDAC)

MicroRNAs are approximately 18–23 nucleotides long small non-coding RNA molecules, which post-transcriptionally regulate the expression of more than 30% of protein coding genes by translational repression.[1] MicroRNAs regulate the expression of several putative target genes by binding to a complementary sequence predominantly in their 3′UTR. This post-transcriptional regulation within the context of tumor development has been reported for the regulation of genes which have an impact on cell differentiation, apoptosis and neoplastic transformation.[2] This led to the categorization of cancer-specific miRNAs known as oncomiRs (ie microRNAs with oncogenic or tumor-suppressive activity).[3] The central role of miRNAs in cell homeostasis and tissue-specific expression profiles envisage their utility as makers of therapeutic success during cancer treatment, in addition to their role as discriminators of cancer from non-cancerous tissue. For instance, previous studies have reported that the expression ratio of miR-196 and miR-217 can-not only distinguish normal from cancer but also chronic pancreatitis from each other. Furthermore, miR-196-expression also harbors a prognostic relevance in pancreatic carcinomas.[4]

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the Western world owing to late diagnosis, fast tumor progression and low response rate to recent chemotherapeutic strategies. Hence, further investigation of pancreatic cancer biology is warranted to gain plausible insights into the patho-physiologic mechanisms underlying PDAC-development. An in-depth investigation of the functional role of deregulated microRNAs in PDAC is essential for a deeper understanding of this complex disease. *Mir-148a* has

[1]Institute of Pathology, Ruhr-University Bochum, Bochum, Germany and [2]Department of Molecular GI-Oncology, Ruhr-University Bochum, Bochum, Germany
Correspondence: Professor A Tannapfel, Institute of Pathology, Ruhr-University Bochum, Buerkle-de-la-Camp-Platz 1, 44789 Bochum, Germany.
E-mail: andrea.tannapfel@rub.de and SA Hahn, Department of Molecular GI-Oncology, Ruhr-University Bochum, Universitätsstraße 150, 44780 Bochum, Germany.
E-mail: stephan.hahn@rub.de
*These authors equally contributing to this work.

Received 22 December 2010; revised 11 April 2011; accepted 2 May 2011

been earlier described to be down-regulated in several types of solid cancers including gastrointestinal,[5] colon, breast, lung, head and neck cancer, as well as melanoma[6] and hepatoblastoma.[7] The generally observed reduction in *miR-148a* abundance in several cancers lead to the assumption that *miR-148a* confers a tumor suppressive role and is important for tumorigenesis. To date there are only limited reports of direct *miR-148a* targets namely: *TGIF2,*[6] *DNMT1*,[8] *DNMT3b*[9] and *MSK1*.[10]

CDC25-proteins are highly conserved dual specificity phosphatases that are vital for appropriate cell cycle progression. In mammals, the CDC25-family consists of three different isoforms (*CDC25A*, *CDC25B* and *CDC25C*) that control the specific activation of distinct CDK/cyclin complexes at different time points during the cell cycle by removing inhibitory phosphate residues from target cyclin-dependent kinases (Cdk).[11–13] Among these, the *CDC25B* isoform activates the CDK1/cyclinB complex at the G2/M checkpoint, thereby initiating mitosis.[14,15] The tight regulation of *CDC25B* is pivotal to temporal orchestration of cell cycle progression and hence its over-expression correlates not only with DNA-damage checkpoint response abrogation but also with increased genomic instability.[12] Not surprisingly, the *CDC25B*-induced elevated genomic instability correlates with its oncogenic effects,[11,16,17] although *CDC25B* over-expression alone does not lead to malignant tumor formation. *CDC25B*, in combination with other oncogenes (eg, *HRAS* mutation or the loss of *RB1* in mouse fibroblasts) does serve as an important factor for transformation.[18]

We investigated if *miR-148a* directly targeted the 3′*UTR* of *CDC25B* and if overexpression of *miR-148a* impacted the expression of *CDC25B* on the protein level. We also focused on the impact of *miR-148a* re-expression in IMIM-PC2 cells and its potential role in proliferation and anchorage-independent growth.

MATERIALS AND METHODS

Cell Culture

The human embryonic kidney (HEK) 293T cell line, the human pancreatic cancer cell line IMIM-PC2 and the human osteosarcoma cell line U2-OS were cultured in DMEM-media supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco, Karlsruhe, Germany). Cells were maintained in a humified incubator with 5% CO_2 at 37 °C.

Microdissection and RNA Isolation

Fresh frozen and microdissected normal ductal cells ($n = 10$), and pancreatic adenocarcinoma cells ($n = 9$) were included in our study, respectively. The sample collection was performed according to a protocol approved by the ethics committee of the Ruhr-University Bochum (permission no. 3534–09 and 2392–04). All samples were reviewed by a pathologist (JBM).

Surgical pancreatic resections were immediately placed on ice, and subsequently snap-frozen and stored at −80 °C. For the identification of normal ductal cells, 5 μm frozen sections prepared from tissue blocks of pancreatic parenchyma, in particular from the resection margins, were subsequently placed in RNase free 95% ethanol (Merck, Darmstadt, Germany), stained with H&E and diagnosed by a pathologist (JBM). For normal ductal cell isolation, medium-sized interlobular ducts were selected by preference in order to avoid contamination by acinar tissue. Tissue blocks containing the cells of interest (ie normal ductal cells and pancreatic adenocarcinoma cells, respectively) were serially sectioned (10 μm sections). The slides were stained with methyl green and immediately stored at −20 °C. Areas with cells of interest were manually microdissected under a microscope (BH2, Olympus, Wetzlar, Germany) using a sterile injection needle (size 0.65 × 25 mm). Microdissected cells were placed in 50 μl extraction buffer from the RNAqueous-Micro kit (Ambion/Applied Biosystems, Darmstadt, Germany) and kept on ice. For each sample microdissected cells were collected from 10 to 20 serial sections (each 10 μm). RNA isolation from microdissected cells was performed using the RNAqueous-Micro kit according to the manufacturer's instructions. Total RNA concentration and purity of total RNA samples were measured with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific).

Quantitative RT-PCR

Mature miRNA expression of micro-dissected PDAC-samples, normal ducts and IMIM-PC2-cells were analyzed by TaqMan miRNA-assays (Applied Biosystems). For cDNA-synthesis 50 ng of total RNA were reverse transcribed using the specific looped primer. All analyzed samples were run in triplicates using a 7500 Real-Time PCR System (Applied Biosystems) and were normalized as indicated. Relative fold changes were calculated based on the $2^{\Delta\Delta Ct}$ method. For absolute quantification C_T values were converted into copy numbers using a standard curve. The standard curve was generated by spiking a serial dilution of synthetic miR-148a RNA oligonucleotides ranging from 10^4 up to 10^8 molecules per reaction into 50 ng *E. coli* tRNA (Sigma-Aldrich, Taufkirchen, Germany) and analyzed by TaqMan miRNA-assays (Applied Biosystems). The copy number per cell was calculated according to Cheng *et al.*[19]

5-aza-2′Deoxycytidine (5-aza-dC) Treatment

IMIM-PC2-cells were treated with the DNA-methylase-inhibitor 5-aza-dC for 5 days. 4×10^5 cells were seeded on 10 cm plates. The following day, cells were treated with 2.5 μM 5-aza-dC and the medium was renewed daily. After 5 days, cells were washed with PBS and subsequently lysed with 3 ml of lysis-buffer (4 M Guanidiniumthiocyanate, 25 mM Sodium Citrate, 0.5% N-Lauroyl-Sarcosin, 0.72% β-mercaptoethanol). Total RNA was then purified by acidic chlorophorm-phenol extraction.

Stable Over-Expression of sh*miR-148a* in IMIM-PC2 cells

MiR-148a over-expression was performed by cloning the hairpin sense: 5′-ccggacaaagttctgtagtgcactgactcgagtcagtgcacta cagaactttgtttttttg-3′ and antisense: 5′-aattcaaaaaacaaagttctgtagt gcactgactcgagtcagtgcactacagaactttgt-3′ into the AgeI/EcoRI site of the *pLKO.1* puro-vector. Lentiviruses were produced by transfecting packaging cells (HEK293T) with a 3-plasmid system. DNA for transfections was prepared by mixing 12 μg *pCMVΔRR8.2*, 1 μg *pHIT G* and 12 μg *pLKO.1* plasmid DNA with 62 μl of 2 M $CaCl_2$ in a final volume of 500 μl. Subsequently 500 μl of 2 × HBS phosphate buffer was dropwise added to the mixture and incubated for 10 min at RT. The 1 ml transfection mixture was then added to 50% confluent HEK293T-cells (seeded the day before) into a 6 well plate. Cells were incubated for 16 h (37 °C and 5% CO_2), and the medium was changed to remove remaining transfection reagent. Lentiviral-supernatants were collected 36 h post-transfection and for each infection 3 ml supernatant containing 4 μg/ml polybrene was immediately used to infect target cells seeded the day before in 6 well plates to reach 70% confluency on the day of infection. Cells were incubated for 24 h, and then the medium was changed to remove virus particles. To control infection rate, a parallel infection under identical conditions and targeting the same cell line was prepared using a lentiviral-GFP expression control vector (*pRRLU6-CPPT-pSK-GFP*). Six days after infection 2 μg/ml puromycin was added to the cell culture media.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

IMIM-PC2-cells stably over-expressing sh*miR-148a* and mock-transduced cells were seeded at 5×10^3 cells per well in 12-well plates, respectively. Cell growth activity was monitored after 48, 96 and 120 h by adding MTT reagent (Sigma-Aldrich) (5 mg/ml in PBS) to a final concentration of 0.8 mg/ml for 4 h at 37 °C. After incubation cells were lysed by adding 500 μl Triplex solution (10% (w/v) SDS; 5% (v/v) isobutanol; 12 mM HCl) and incubated overnight at 37 °C. Absorbance was measured at 562 nm with background substraction at 630 nm. All measurements were done in triplicates and normalized to the corresponding t_0 value.

Anchorage Independent Growth Assay

For anchorage independent growth 2×10^5 IMIM-PC2-cells were seeded per well on 0.7%. agarose (Invitrogen) in a 0.3% agarose-solution in a 6 well plate. Cells were fed every third day and cultivated over 4 weeks. Cells were stained with Iodonitrotetrazolium-chloride solution overnight. The stained cells were scanned on an Epson scanner.

Luciferase Reporter Assay

Luciferase constructs were made by ligating 200 bp 3′ of the 3′*UTR* of *CDC25B* as well as the mutated version after the luc orf in the *pGL3*. Cells were triple-transfected using Attractene (Qiagen, Hilden, Germany) with 41.5 ng of *pGL3* containing either the wild type or mutant *CDC25B* sequence, in combination with 6.5 nM *miR-148a* mimics, 20 ng *pRL-TK* (Promega, Mannheim, Germany) served as transfection control. As a negative control 6.5 nM AllStars siRNA oligos (Qiagen) was used. Firefly and Renilla activity was measured 32 h post transfection. The luciferase-signal was measured for 10 s (Tecan M2000, Crailsheim, Germany). The Renilla-signal was used for normalization. Mean values and s.e.m. were calculated in triplicates.

Transient Transfection of miR-148a Mimics

For transient knock-down experiments 1×10^5 pancreatic carcinoma cells (AsPC1, Capan-1, MIA PaCa-2, Colo357 and PaTu 8092) were seeded in 6 well plates. Cells were transfected in solution with 10 nM *miR-148a* mimics (Qiagen) using Attractene (Qiagen) according to the manufacturer's recommendations; as control served 10 nM AllStars (Qiagen). After 24 h the media was replaced. Cells were harvested in RIPA-buffer, 96 h post transfection and subjected to Western blot analysis using CDC25B antibody.

Western Blot Analysis

Sh*miR-148a* stably over-expressing and mock transducted IMIM-PC2-cells were grown to 80% subconfluency and lysed in RIPA buffer (50 mM Tris-HCl; pH 7.6, 150 mM NaCl, 1% NP40 (Sigma-Aldrich), 0.1% SDS, 0.5% Sodium deoxycholate, 1 mM EDTA, and 1 mM EGTA) with protease inhibitors (Thermo Scientific, Karlsruhe, Germany), and sonicated (Bioruptor, Liége, Belgium) for 15 min with 30 pulses in iced water to prepare whole cell lysates. 30 μg whole cell lysates were subjected to 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Schwalbach, Germany) with 2 mA/cm^2 for 1 h. After protein transfer membranes were blocked in PBS-T, containing 5% (w/v) skimmed milk, for 1 h and incubated with anti-*CDC25B* antibody (Cell Signalling, Frankfurt am Main, Germany) overnight (1:1000 in PBS-T). As loading control anti-α-tubulin antibody (Sigma-Aldrich) was used at 1:2500 dilution in PBS-T for 1 h at room temperature. Membranes were incubated for detection with secondary antibodies raised against rabbit labeled with CyDye800 (Li-cor, Bad Homburg, Germany) and mouse labeled with CyDye700 (Li-cor) for 1 h at room temperature. Signals were detected by Odyssey Scanner (Li-cor).

Immunohistochemistry

Two μm thick formalin fixed paraffin embedded (FFPE) sections were deparaffinized and rehydrated. Sections were subsequently cooked in a microwave oven for 10 min in target retrieval solution; pH 6.0 (DAKO, Hamburg, Germany). Pretreated sections were then blocked with 2% BSA in TBS-T for 30 min at room temperature before overnight incubation at 4 °C with the *CDC25B* antibody (1:200; Cellt Signalling, Frankfurt am Main, Germany). After washing with TBS-T the secondary antibody was incubated for 20 min

at room temperature followed by coupling the alkaline phosphatase by the DAKO Real™ Detection System, Alkaline Phosphatase/Red, rabbit/mouse (DAKO) according to the manufacturer's manual. *CDC25B* was detected by chromogen red staining for 4 min. Sections were finally counter stained with Hematoxylin (DAKO).

Statistical Analysis

Data are expressed as mean ± s.d. unless otherwise noted. MicroRNA expression of patient samples was statistical analyzed by Mann-Whitney *U*-Test. For tissue culture based assays the differences between groups were statistically analyzed by a two tailed unpaired Student's *t*-test. The level of statistical significance was set at $P<0.05$.

RESULTS

Quantitative Analysis of *miR148a* Expression in Ductal Pancreatic Adenocarcinoma

Using miRNA-array analysis we identified *miR-148a* to be frequently down-regulated in PDAC.[20] To confirm and validate the miRNA-array data on *miR-148a*, we first compared the transcription level of *miR-148a* in micro-dissected samples of 9 patients with PDAC to 10 samples of normal pancreatic epithelia. The mean transcription level of *miR-148a* was significantly lower in PDACs compared with non tumor samples by a factor of four ($P=0.008$) (Figure 1a). To further investigate the potential mechanism underlying the *miR-148a* down-regulation in PDAC,[6,21] we investigated its expression in the pancreatic cancer tissue cell line IMIM-PC2 post 5-aza-treatment a demethylating reagent. After five days of 5-aza-dC treatment, IMIM-PC2-cells demonstrated a significant 8-fold increase in *miR-148a*-expression ($P<0.05$) (Figure 1b). This confirms that *miR-148a* is silenced in IMIM-PC2 cells through promoter-methylation enabling the use of this cell line as a model system for miR148a overexpression experiments.

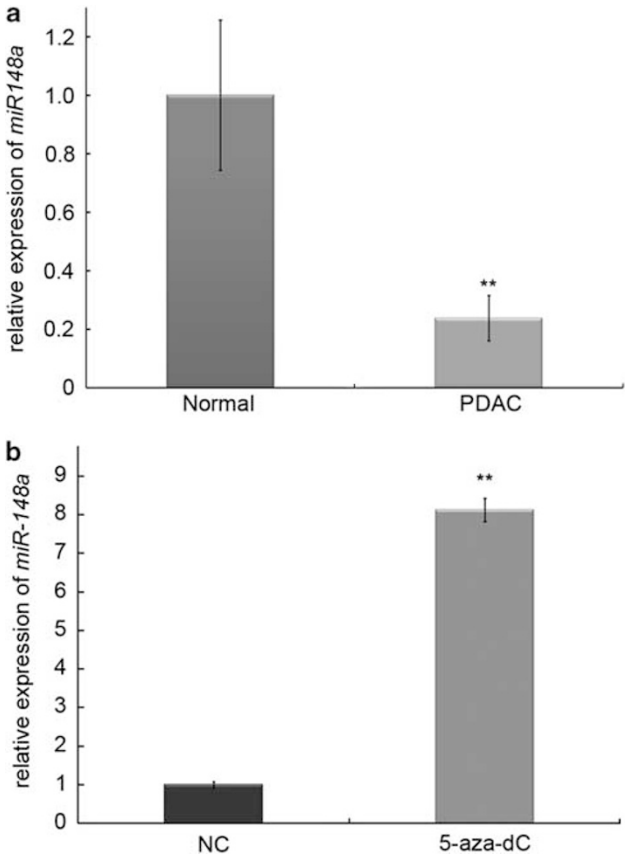

Figure 1 The *miR-148a*-expression is significantly down regulated in PDAC. (**a**) *MiR-148a* relative expression levels were determined using TaqMan real time qRT-PCR in normal ductal pancreas epithelia ($n=10$) and PDAC ($n=9$). (**$P<$ 0.01 by Mann-Whitney U-Test) (**b**) Induction of *miR-148a* expression in IMIM-PC2-cells post 5-aza-dC-treatment for 5 days. Relative expression levels were determined using *RNU44a* as endogenous internal control. The histogram represents mean of three replicates. (**$P<0.01$ by unpaired two-tailed Student's *t*-test).

MiR-148a Re-expression Suppressed Cell Growth in IMIM-PC2 Cells

As *miR-148a*-expression was silenced by promoter methylation in IMIM-PC2-cells, we analyzed the functional impact of *miR-148a* in the pancreatic cancer cell line IMIM-PC2 as a relevant *in vitro* model for PDAC. We generated stably *miR-148a* over-expressing IMIM-PC2-cells by lentiviral infection. The validity of *miR-148a*-expression was confirmed by quantitative RT-PCR: we observed a 160-fold increased *miR-148a*-expression in comparison to the respective mock transduced cells (Figure 2a). This relative fold change correlates with a re-expression of 1.27×10^5 *miR-148a* molecules/cell in IMIM-PC2 cells compared to 2.83×10^5 *miR-148a* molecules/cell in normal pancreatic ductal epithelia. Having shown that our *miR-148a* level did not exceed the physiological range in normal cells, we analyzed the impact of *miR-148a* re-expression on IMIM-PC2 cells using MTT and colony-forming assay in this system (Figure 2b and c). After 96 h the MTT assay revealed a significant change in the cell growth ($P=0.003$). At the end of the assay we observed a reduction in cell growth of 22.83% in the *miR-148a* over-expressing cells compared to the mock transducted ones ($P=0.047$). This leads to the assumption that *miR-148a* overexpression significantly reduces the tumor cell viability (Figure 2b). The colony forming assay showed a reduction of foci for the *miR-148a* overexpressing IMIM-PC2 cells in comparison to the mock transducted cells after 4 weeks (Figure 2c upper panel). Microscopical examination of the cells revealed that in addition to the increased number of foci the mock transducted cells also form bigger colonies (Figure 2c lower panel). Together, these findings led us to propose that *miR-148a* might be an important negative regulator in PDAC in terms of growth and proliferation.

CDC25B is a Candidate Target of *miR-148a*

To elucidate the molecular mechanism underlying *miR-148a* mediated regulation of proliferation we used *in silico* analysis based on the computer-aided algorithms: PicTar,[22]

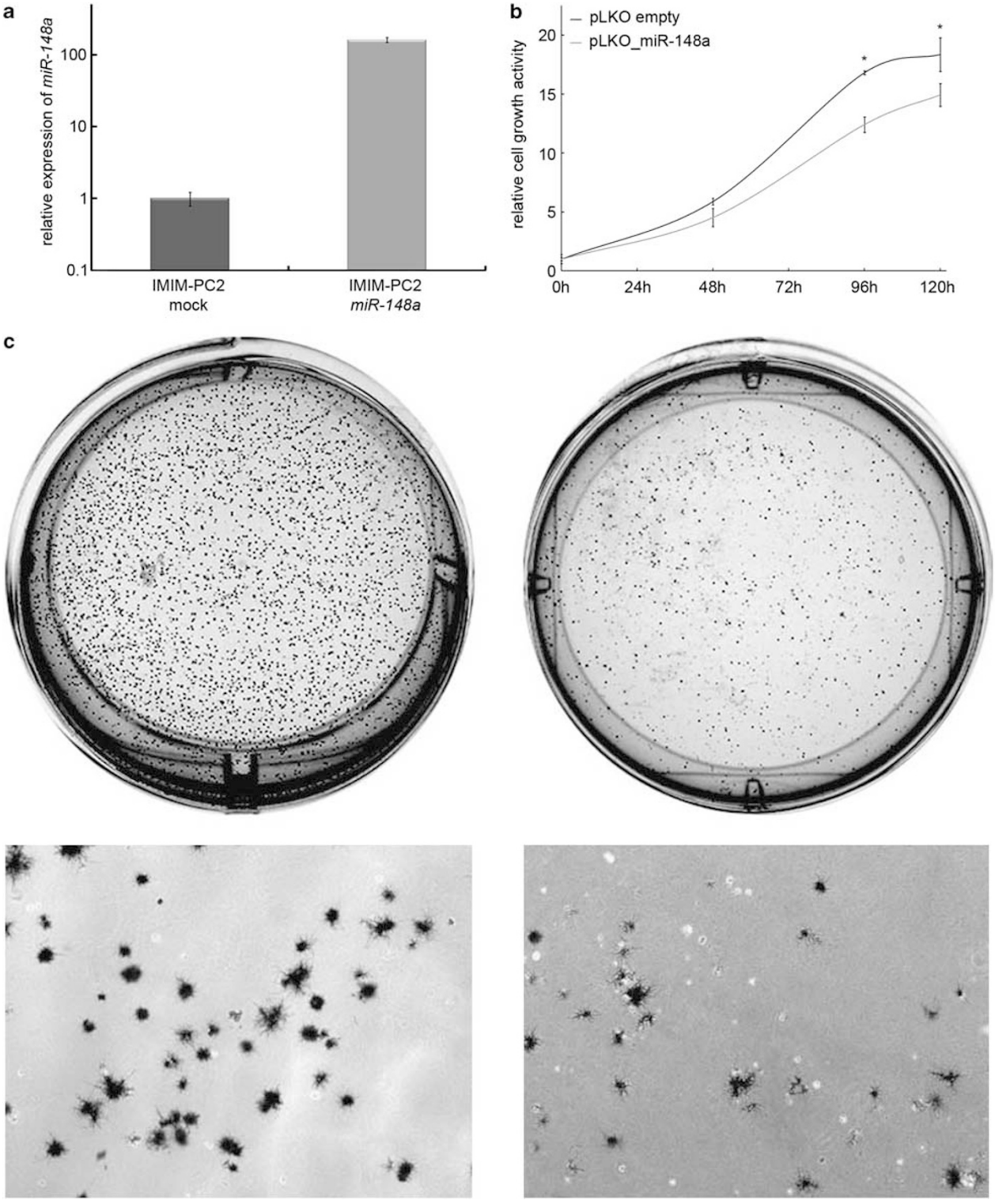

Figure 2 *MiR-148a* reduced cell growth and colony formation. (**a**) Average *miR-148a* relative expression levels were determined using TaqMan real time qRT-PCR in IMIMPC2-cells stably over-expressing *miR-148a*. (**b**) Growth kinetics of mock-IMIM-PC2 and *miR-148a* overexpressing-IMIM-PC2 over 5 days using MTT assay (*$P<0.05$ by unpaired two-tailed Student's *t*-test). (**c**) Anchorage independent growth of mock-IMIM-PC2 and *miR-148a* overexpressing-IMIM-PC2 over 5 days using colony forming assay. All experiments were performed in triplicates and mean values are shown with s.d. of triplicates.

Targetscan[23–25] and miRanda[26] in conjunction with Gene Ontology-analysis for predicted target genes. To attribute a gene as a potential target of *miR-148a*, it had to be predicted independently by at least two of the three applied algorithms and simultaneously be an established positive regulator of cell proliferation. The most promising candidate fulfilling the above criteria was *CDC25B*, which also contained a putative *miR-148a* binding site at the end of its 3′*UTR* (Figure 3a). To prove the functionality of this *miR-148a* binding-site, we chose to perform a luciferase based reporter assay. Therefore we cloned the 200 bp 3′ of the *CDC25B* 3′*UTR* containing the predicted seed sequence downstream of the luciferase (luc) reporter-gene. 32 h post-transfection the luciferase-activity was monitored in the presence of *miR-148a* mimics or AllStar oligos as a negative control in the human osteosarcoma cell line U2-OS, respectively. The luciferase-reporter activity decreased approximately 38% in U2-OS cells containing the *CDC25B* wild-type 3′*UTR* fragment (Figure 3b). In contrast; we observed no significant change in the luciferase-activity in cells expressing the mutated 3′*UTR* (Figure 3b). These data confirm the direct interaction between *miR-148a* and the 3′*UTR* of *CDC25B* mRNA. Next, we determined the impact of *miR-148a* on *CDC25B* protein expression by Western blot analysis in *miR-148a* stable overexpressing IMIM-PC2 cells. Concordant with our earlier results, we observed that the over-expression of *miR-148a* suppressed the endogenous *CDC25B* protein level in IMIM-PC2 cells below the detection limit (Figure 4a). Furthermore, we opted to analyze the impact of *miR-148a* on the *CDC25B* expression in a panel of different pancreatic carcinoma cell lines. Therefore, we analyzed the *CDC25B* level in the following pancreatic carcinoma cell lines: AsPC1, Capan-1, MIA PaCa-2, Colo-357 and PaTu 8092 in the presence and absence of *miR-148a* mimics. After 96 h a distinct reduction in the *CDC25B* level was shown in three out of five analyzed cell lines (Figure 4a), whereas Capan-1 cells showed only a slight reduction. The cell line AsPC-1 showed no alteration in the CDC25B expression. In addition to the *CDC25B* repression in our tissue culture experiments we determined the *CDC25B* expression in formalin fixed paraffin embedded PDAC samples with different *miR-148a* expression levels. This analysis showed a predominant nuclear CDC25B staining in samples of PDAC with a reduced *miR-148a* level (Figure 4c), compared to normal ductal cells and to PDAC with high *miR-148a* expression (Figure 4b and d). Collectively, we were able to show that the microRNA *miR-148a* down-regulated the expression of *CDC25B* in different pancreatic cell lines (ie IMIM-PC2, Capan-1, MIA PaCa-2, Colo347 and PaTu 8092), as well as in patient samples of PDAC.

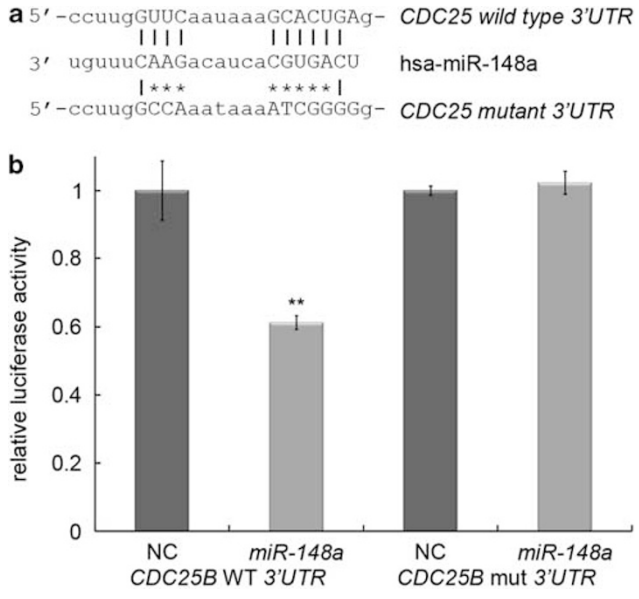

Figure 3 Suppression of *CDC25B* by *miR-148a*. (**a**) Sequence complementarity between *hsa-miR-148a* and the 1135–1141 bp of the *CDC25B 3′UTR*. * indicates mutated base pairs. (**b**) Interaction between *miR-148a* and 3′*UTR* of *CDC25B*. U2-OS cells were transfected with wild-type (WT) and mutant (Mut) 3′*UTR* of *CDC25B* containing luciferase reporter vector together, *miR-148a* and control vector respectively as indicated. (**$P<0.01$ by unpaired two-tailed Student's *t*-test)

DISCUSSION

Mir-148a has been reported as a microRNA with tumor-suppressor activity in several types of cancers in which it is down-regulated through epigenetic silencing by promoter hypermethylation.[6,21,27] In colon, lung, breast, and head and neck carcinoma and melanoma, *miR-148a*-promoter hypermethylation has been correlated with a higher metastatic potential.[6] Hanoun *et al* have reported that hypermethylation of the promoter region encoding *miR-148a* is responsible for its repression not only in PDAC samples but also in preneoplastic-pancreatic-intraepithelial-neoplasia (PanIN) and can serve as an ancillary marker for the differential diagnosis of PDAC and chronic pancreatitis.[21] Consistent with this observation, we report that *miR-148a* is significantly down-regulated in micro-dissected PDAC samples. Therefore the expression of *miR-148a* in TGFβ non-responsive IMIM-PC2 pancreatic cancer cells is probably regulated by DNA-methylation. In the present study, we first established an *in vitro* model based on lentiviral-mediated stable *miR-148a* overexpression in the pancreatic carcinoma cell line IMIM-PC2 (in which cellular *miR-148a* is silenced by promoter-methylation). We subsequently exploited this *in vitro* model to validate the tumor-suppressor role of *miR-148a* in PDAC by demonstrating that *miR-148a* over-expression had an inhibitory influence on the growth potential of pancreatic cancer cells.

Recently Fujita *et al* have demonstrated that *miR-148a,* which is part of a reported DNA methylation-signature for human cancer metastasis, attenuates paclitaxel-resistance of hormone-refractory, drug-resistant PC3PR cells in part by regulating *MSK1* expression.[6,10] However, as miRNAs are expected to have multiple targets, we sought to further

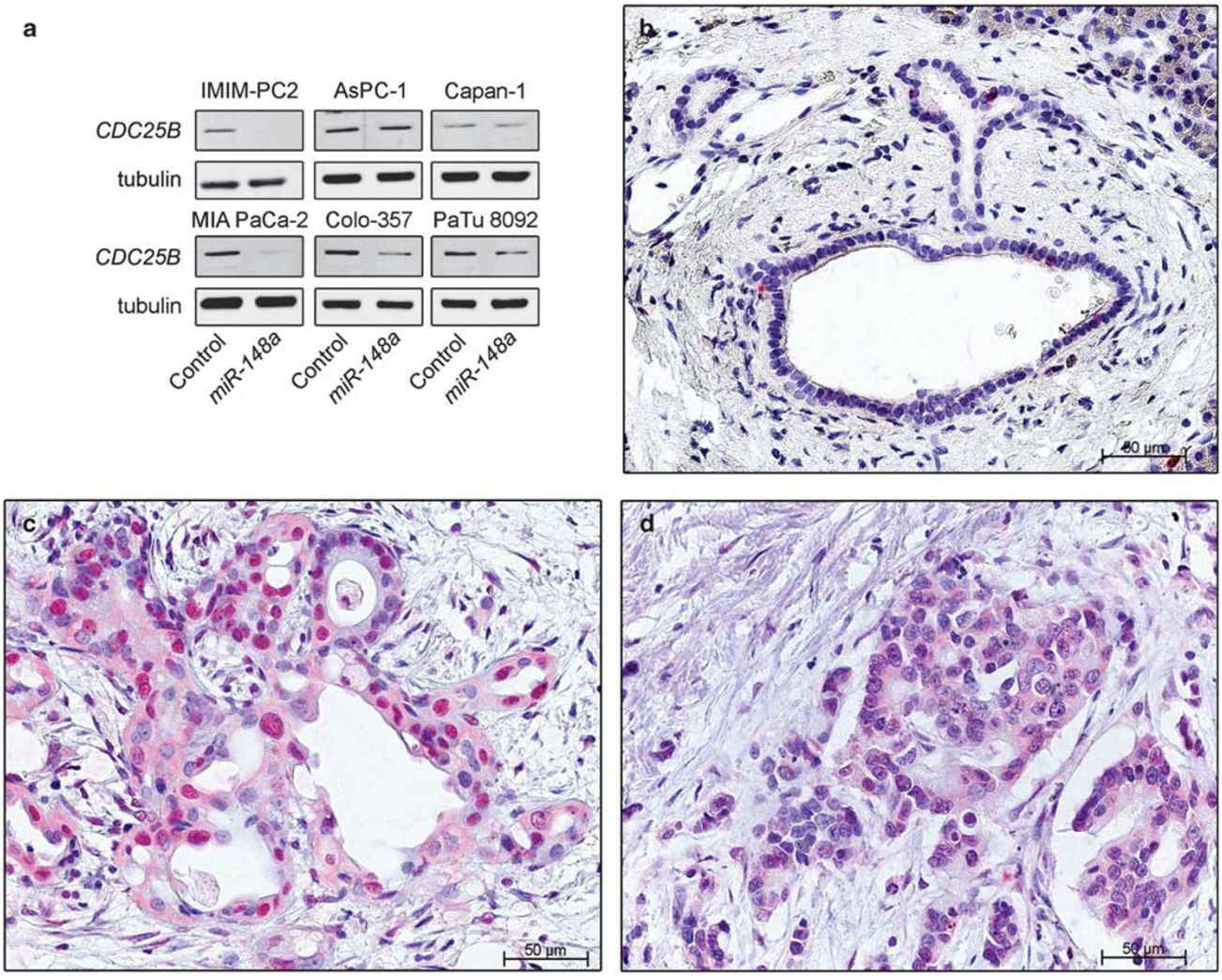

Figure 4 Alteration of the *CDC25B* expression by *miR-148a*. (**a**) Western blot analysis demonstrating reduction in CDC25B protein in IMIM-PC2 cells stably overexpressing *shmiR-148a.* For transient CDC25B knock-down experiments, the pancreatic carcinoma cell lines AsPC-1, Capan-1, MIA PaCa-2, Colo-357 and PaTu 8092 were transfected with *miR-148a* mimics or control (10 nM). After 96 h the cells were harvested and the CDC25B levels were determined by Western blot analysis. Anti-α-tubulin served as loading control. CDC25B immunohistochemical staining of normal ductual cells (**b**) compared to PDAC section with down-regulated miR148a (**c**) and PDAC with high *miR148a* transcription-level (**d**). The most intense CDC25B signal intensity was shown for section (**c**), further supporting the link between miR148a transcription and down-regulation of CDC25B expression.

investigate and expand the putative targets of *miR-148a* in PDAC. *In silico* analysis revealed five putative targets, which have positive effects on cell cycle progression and contain a seed sequence for *miR-148a* binding in their 3′*UTR*. Of these candidates, *CDC25B* emerged as the most promising novel candidate.

CDC25B is frequently dysregulated in a broad variety of cancers,[12,28] and its overexpression has been reported in PDAC.[29,30] Further *CDC25B* has been reported to have prognostic relevance in breast,[31] prostate,[32] colorectal[33,34] and gastric cancer,[35] in which the overexpression of *CDC25B* seemed to correlate with a poor prognosis. However, the mechanism which leads to the dysregulation of the *CDC25B* within the specific context of tumor proliferation and progression has largely remained elusive. The poor correlation between *CDC25B* mRNA and its corresponding protein abundance has often led to the speculation that the deregulation of *CDC25B* may occur at any stage between its transcription and translation.[12] Our results for the first time indicate a down-regulation of *CDC25B* by *miR-148a* at the post-transcriptional level by translational repression. This proposed interaction is evidenced from our reporter assay based studies where *miR-148a* has been directly shown to influence the expression of the luc reporter by binding to the predicted target sequence in the *CDC25B*-3′*UTR*. Interestingly, increased *CDC25B* level can lead to a premature exit of the G2/M checkpoint,[16] resulting in the loss of DNA damage repair mechanisms or the initiation of PCD and hence *miR-148a-CDC25B*-association is consistent with a model of pancreatic cancer progression in which *miR-148a*

is lost in PanIN pre-neoplastic lesions. Accordingly, in PDAC it has been shown that there was no correlation between the percentage of *CDC25B* positive cells and tumor stage/grade implicating dysregulation of *CDC25B* as an early event in the current multi-step PDAC progression model.[30] Based on these collective observations, we propose a model in which the control of the *CDC25B* expression is epigenetically pre-regulated in PanINs *via miR-148a* promoter-methylation and continues to confer proliferative advantage in PDAC *via CDC25B*. Further this early repression of *miR-148a* and the resultant increase in *CDC25B* expression may additionally confer an increased propensity for genomic instability at an early stage in the PDAC progression model, thus facilitating a higher risk to accumulate genetic defects and subsequent enhanced tumor progression.

In summary, using an *in vitro* model based on lentiviral-mediated stable *miR-148a* over-expression in the pancreatic carcinoma cell line IMIM-PC2, we validate the tumor-suppressor role of *miR-148a* in PDAC. Further, our results for the first time demonstrate that *CDC25B* is a direct target of *miR-148a*. Our data link the postulated promoter-methylation of *miR-148a* at early stages of pancreatic-tumorigenesis (PanIN)[21] to an increased *CDC25B* expression in PanINs[30] which leads to a PDAC model in which the transcriptional loss of *miR-148a* and the resultant increase in *CDC25B* expression facilitates increased genomic instability at an early stage of tumor development.

ACKNOWLEDGEMENTS

We thank Dr Sheila Stewart, Department of Cell Biology & Physiology, Washington University School of Medicine for providing *pLKO.1-puro* and *pRRLU6-CPPT-pSK-GFP* vectors. A T and SA H were supported by Bundesministerium für Bildung und Forschung (01GS08118) and the Deutsche Krebshilfe (70-2988). This work was funded by the Protein Research Unit Ruhr within Europe, PURE.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Carthew RW. Gene regulation by microRNAs. Curr Opin Genet Dev 2006;16:203–208.
2. Drakaki A, Iliopoulos D. MicroRNA gene networks in oncogenesis. Curr Genomics 2009;10:35–41.
3. Hammond SM. RNAi, microRNAs, and human disease. Cancer Chemother Pharmacol 2006;58(Suppl 1):s63–s68.
4. Szafranska AE, Doleshal M, Edmunds HS, *et al.* Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. Clin Chem 2008;54:1716–1724.
5. Chen Y, Song Y, Wang Z, *et al.* Altered expression of MiR-148a and MiR-152 in gastrointestinal cancers and its clinical significance. J Gastrointest Surg 2010;14:1170–1179.
6. Lujambio A, Calin GA, Villanueva A, *et al.* A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 2008;105:13556–13561.
7. Magrelli A, Azzalin G, Salvatore M, *et al.* Altered microRNA expression patterns in hepatoblastoma patients. Transl Oncol 2009; 2:157–163.
8. Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 2010;51:881–890.
9. Duursma AM, Kedde M, Schrier M, *et al.* miR-148 targets human DNMT3b protein coding region. Rna 2008;14:872–877.
10. Fujita Y, Kojima K, Ohhashi R, *et al.* MiR-148a attenuates paclitaxel-resistance of hormone-refractory, drug-resistant prostate cancer PC3 cells by regulating MSK1 expression. J Biol Chem 2010;285:19076–19084.
11. Boutros R, Dozier C, Ducommun B. The when and wheres of CDC25 phosphatases. Curr Opin Cell Biol 2006;18:185–191.
12. Boutros R, Lobjois V, Ducommun B. CDC25 phosphatases in cancer cells: key players? Good targets? Nat Rev Cancer 2007;7:495–507.
13. Strausfeld U, Labbe JC, Fesquet D, *et al.* Dephosphorylation and activation of a p34cdc2/cyclin B complex *in vitro* by human CDC25 protein. Nature 1991;351:242–245.
14. Gabrielli BG, De Souza CP, Tonks ID, *et al.* Cytoplasmic accumulation of cdc25B phosphatase in mitosis triggers centrosomal microtubule nucleation in HeLa cells. J Cell Sci 1996;109(part 5):1081–1093.
15. Lindqvist A, Kallstrom H, Lundgren A, *et al.* Cdc25B cooperates with Cdc25A to induce mitosis but has a unique role in activating cyclin B1-Cdk1 at the centrosome. J Cell Biol 2005;171:35–45.
16. Bugler B, Quaranta M, Aressy B, *et al.* Genotoxic-activated G2-M checkpoint exit is dependent on CDC25B phosphatase expression. Mol Cancer Ther 2006;5:1446–1451.
17. Karlsson C, Katich S, Hagting A, *et al.* Cdc25B and Cdc25C differ markedly in their properties as initiators of mitosis. J Cell Biol 1999;146:573–584.
18. Galaktionov K, Lee AK, Eckstein J, *et al.* CDC25 phosphatases as potential human oncogenes. Science 1995;269:1575–1577.
19. Chen C, Ridzon DA, Broomer AJ, *et al.* Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
20. Szafranska AE, Davison TS, John J, *et al.* MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. Oncogene 2007;26:4442–4452.
21. Hanoun N, Delpu Y, Suriawinata AA, *et al.* The silencing of MicroRNA 148a production by DNA hypermethylation is an early event in pancreatic carcinogenesis. Clin Chem 2010;56:1107–1118.
22. Krek A, Grun D, Poy MN, *et al.* Combinatorial microRNA target predictions. Nat Genet 2005;37:495–500.
23. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15–20.
24. Grimson A, Farh KK, Johnston WK, *et al.* MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007;27:91–105.
25. Friedman RC, Farh KK, Burge CB, *et al.* Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19:92–105.
26. Enright AJ, John B, Gaul U, *et al.* MicroRNA targets in Drosophila. Genome Biol 2003;5:R1.
27. Lehmann U, Hasemeier B, Christgen M, *et al.* Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol 2008;214:17–24.
28. Kristjansdottir K, Rudolph J. Cdc25 phosphatases and cancer. Chem Biol 2004;11:1043–1051.
29. Grutzmann R, Foerder M, Alldinger I, *et al.* Gene expression profiles of microdissected pancreatic ductal adenocarcinoma. Virchows Arch 2003;443:508–517.
30. Guo J, Kleeff J, Li J, *et al.* Expression and functional significance of CDC25B in human pancreatic ductal adenocarcinoma. Oncogene 2004;23:71–81.
31. Ito Y, Yoshida H, Uruno T, *et al.* Expression of cdc25A and cdc25B phosphatase in breast carcinoma. Breast Cancer 2004;11:295–300.
32. Ngan ES, Hashimoto Y, Ma ZQ, *et al.* Overexpression of Cdc25B, an androgen receptor coactivator, in prostate cancer. Oncogene 2003;22:734–739.
33. Takemasa I, Yamamoto H, Sekimoto M, *et al.* Overexpression of CDC25B phosphatase as a novel marker of poor prognosis of human colorectal carcinoma. Cancer Res 2000;60:3043–3050.
34. Hernandez S, Bessa X, Bea S, *et al.* Differential expression of cdc25 cell-cycle-activating phosphatases in human colorectal carcinoma. Lab Invest 2001;81:465–473.
35. Kudo Y, Yasui W, Ue T, *et al.* Overexpression of cyclin-dependent kinase-activating CDC25B phosphatase in human gastric carcinomas. Jpn J Cancer Res 1997;88:947–952.