

EP300—a miRNA-regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas

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Genetic and epigenetic alterations during development of pancreatic ductal adenocarcinomas (PDACs) are well known. This study investigates genetic and epigenetic data together with tumor biology to find specific alterations responsible for metastasis formation. Using 16 human PDAC cell lines in a murine orthotopic PDAC model, local infiltration and metastatic spread were assessed by standardized dissemination scores. The cell lines were further classified into 3 hierarchical groups according to their metastatic potential. Their mRNA and microRNA (miRNA) expression was profiled *via* mRNA-microarray as well as Taqman Low Density Array, and validated by single quantitative RT-PCR and Western blotting. In the highly metastatic group, a significant induction of EP300 targeting miRNAs miR-194 (fold change: 26.88), miR-200b (fold change: 61.65), miR-200c (fold change: 19.44) and miR-429 (fold change: 21.67) ($p < 0.05$) was detected. Corresponding to this, decreased expression of EP300 mRNA ($p < 0.0001$) and protein ($p < 0.05$) were detected in the highly metastatic PDAC cell lines with liver metastases compared to the nonmetastatic or marginally metastatic cell lines, while no correlation with local tumor growth was found. In conclusion, epigenetic alterations with upregulated EP300 targeting miRNAs miR-194, miR-200b, miR-200c and miR-429 are related to reduced EP300 mRNA and protein in PDAC. These results demonstrate that miRNAs might be able to modulate the expression of metastasis-specific suppressor genes and metastatic behavior in PDAC, suggesting diagnostic and therapeutic opportunities for EP300 and its targeting miRNAs in PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is among the most malignant tumors with a highly unfavorable prognosis. The 5-year survival rate of all patients is below 5%, and the median survival time after diagnosis is ~6 months. Even af-

Key words: pancreatic cancer, metastasis, metastasis suppressor genes, epigenetics, microRNA

Abbreviations: ANOVA: analysis of variance; CDKN2a: cyclin-dependent kinase 2a; EP300: E1A binding protein p300; miRNA: micro ribonucleic acid; miRISC: miRNA-associated, multiprotein RNA-induced silencing complex; PDAC: pancreatic ductal adenocarcinoma; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; RNAi: RNA-mediated interference; SERPIND1: serpin peptidase inhibitor, clade D, member 1; TLDA: Taqman low density array

Grant sponsor: Foerdereverein Peter Geiger, Beilstein, Germany
Results presented at the Digestive Disease Week, May 30th-June 04th, 2009, Chicago

DOI: 10.1002/ijc.24695

History: Received 11 Feb 2009; Accepted 9 Jun 2009; Online 30 Jun 2009

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ter operation with curative intention, the 5-year survival rate in specialized centers is below 15%, increasing to only 25% when adjuvant chemotherapy is applied.^{1,2} The cancer's aggressive nature, the lack of methods for early detection and the limited response to available treatments contribute to its high mortality rate. Pancreatic cancer is characterized by modifications in gene expression due to mutations, deletions and amplifications of genes critical for tumor development and progression. Although these alterations are purely genetic, epigenetic mechanisms such as shifts in DNA methylation patterns can also contribute to the induction and maintenance of pancreatic cancer.^{3,4} Epigenetic mechanisms are modifiers of gene expression that are heritable but potentially reversible and do not involve changes in the DNA sequence. At the post-transcriptional level, epigenetic regulation can take place *via* microRNAs (miRNAs). miRNAs are small noncoding RNAs that are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors in the cytoplasm by RNaseIII Dicer into their mature form of 18–23 nucleotides.⁵ Single-stranded miRNAs bind messenger RNAs (mRNA) of potentially hundreds of genes at the 3'un-translated region with perfect or near-perfect complementarity, resulting in degradation or inhibition of the respective target mRNA. Recently, miRNAs have gained attention as another family of molecules involved in cancer development.^{6,7} Upon binding to their target mRNAs, miRNAs initiate post-transcriptional

gene silencing by either cleaving the target mRNA or by inhibiting the translational process.⁸ Regulated miRNA expression has been demonstrated in a variety of eukaryotic organisms to be a key player in developmental, cell growth and differentiation processes.⁹ It was therefore predicted early that dysregulation of this highly conserved class of genes would likely play a role in cancer development. This hypothesis has been supported by the demonstration of altered miRNA expression in a variety of hematological as well as some solid tumor entities.^{10–12}

Detailed knowledge of genetic and epigenetic alterations and associated molecular mechanisms during pancreatic cancer development and metastasis not only broadens our biological understanding of the disease, but also more importantly may help devising strategies for earlier diagnosis and identifying novel therapeutic targets. Therefore, the aim of this study was to identify genetic and epigenetic alterations that contribute to the development and the progress of PDAC. We have established an orthotopic model for PDAC with 16 cell lines of differing origin and differentiation to define the tumor biology (growth, infiltration and metastasis) of this tumor entity. Based on this characterization, we evaluated mRNA and miRNA expression profiles to identify specific alterations correlating with the process of distant metastasis formation in PDAC.

Material and Methods

Cell lines

A total of 16 human PDAC cell lines were analyzed (Fig. 1c). Based on a review of literature (Pubmed, American Type Culture Collection), all cell lines were previously classified as well, moderate, or poorly differentiated according to the tumor from which they were derived and by their histological appearance in culture. Cells were maintained in recommended growth media and all media were supplemented with 10% heat inactivated fetal bovine serum (Gibco, Karlsruhe, Germany) and were Mycoplasma negative. For culturing, they were incubated at 37°C in humidified air with 5 or 10% CO₂. The medium was replaced twice weekly, and cells were maintained by serial passaging after trypsinization with 0.1% trypsin.

Laboratory animals

Four-week-old male nude mice (CrI:NU/NU-nuBR) weighing 20–22 g were obtained from Charles River Laboratories (Sulzfeld, Germany). The animals were housed in cages with autoclaved bedding, food and water. The mice were maintained on a daily 12-hr light, 12-hr dark cycle. All experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals, and the experimental protocol was approved by the state agency for animal welfare of North Rhine-Westphalia (LANUV NRW, Germany).

Orthotopic implantation technique

Tumor models were carried out as described previously.¹³ Briefly, animals were anaesthetized using isoflurane (Forene[®],

Abbott, Wiesbaden, Germany) and 5×10^6 cells were injected subcutaneously into the flanks of the animals for each human PDAC cell line. The mice were humanely killed by a lethal dose of isoflurane when the subcutaneous tumors had reached a size of 10 mm in the largest diameter. The donor tumors were minced into small fragments of 1 mm³ in size, and only macroscopically viable tumor tissue from the outer part of the donor tumors was used for orthotopic implantation. Tumor recipient nude mice were anaesthetized with isoflurane (Forene, Abbott). The animals' abdomens were opened by a midline incision, and the pancreatic tail with the spleen was gently exteriorized. Two small tissue pockets were prepared in the pancreatic parenchyma as an implantation bed. One donor tumor fragment was placed into each pancreatic tissue pocket in a way that the tumor tissue was completely surrounded by pancreatic parenchyma. No sutures or fibrin glue were used to fix the tumor fragments to the recipient pancreas. The pancreas was relocated into the abdominal cavity, which was then closed in 2 layers with 5-0 absorbable Vicryl sutures (Vicryl[®], Ethicon, Norderstedt, Germany). Postoperative analgesia was performed by subcutaneous injection of carprofen (Rimadyl, Pfizer Animal Health, New York, NY; 5 mg/kg body weight) every 24 hr.

Classification of cell lines

Clinical and pathological tumor biology has been analyzed for all 16 PDAC cell lines (10 mice per cell line). After 12 weeks of intra-abdominal tumor growth (Fig. 1a), clinical signs of tumor burden, primary tumor volume, local infiltration and patterns of local and systemic metastases were assessed using a modified, standardized dissemination score.¹³ Local infiltration was determined at the abdominal organs (*e.g.*, spleen, stomach, liver, kidney, diaphragm, mesentery, bowel loops), the retroperitoneum and the abdominal wall. Distant metastasis was determined at the following sites: liver, lymph nodes in the upper abdomen, diaphragm, mesentery and retroperitoneum; isolated tumor nodules with no anatomic connection to the primary tumor were considered as distant metastases.

Tumor dissemination was evaluated by crediting one point for every massive local infiltration, every metastasis, multiple metastatic lesions (>1 in liver, >5 for diaphragm, mesentery, retroperitoneum) and metastases >50 mm³. Score values represented mean sums of the obtained credit points for all mice in the specific group/cell line. Consecutively, cell lines were classified into 3 groups with a hierarchical order representing the parameters infiltration and metastasis: Group I (absent-low), Group II (medium) and Group III (high). Specimens from each tumor localization were collected, snap frozen and stored at –80°C.

mRNA and miRNA microarray

RNA isolation from tumor cell lines was performed according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany) for mRNA and miRNeasy Mini Kit for miRNA (Qiagen). Purity and integrity of the

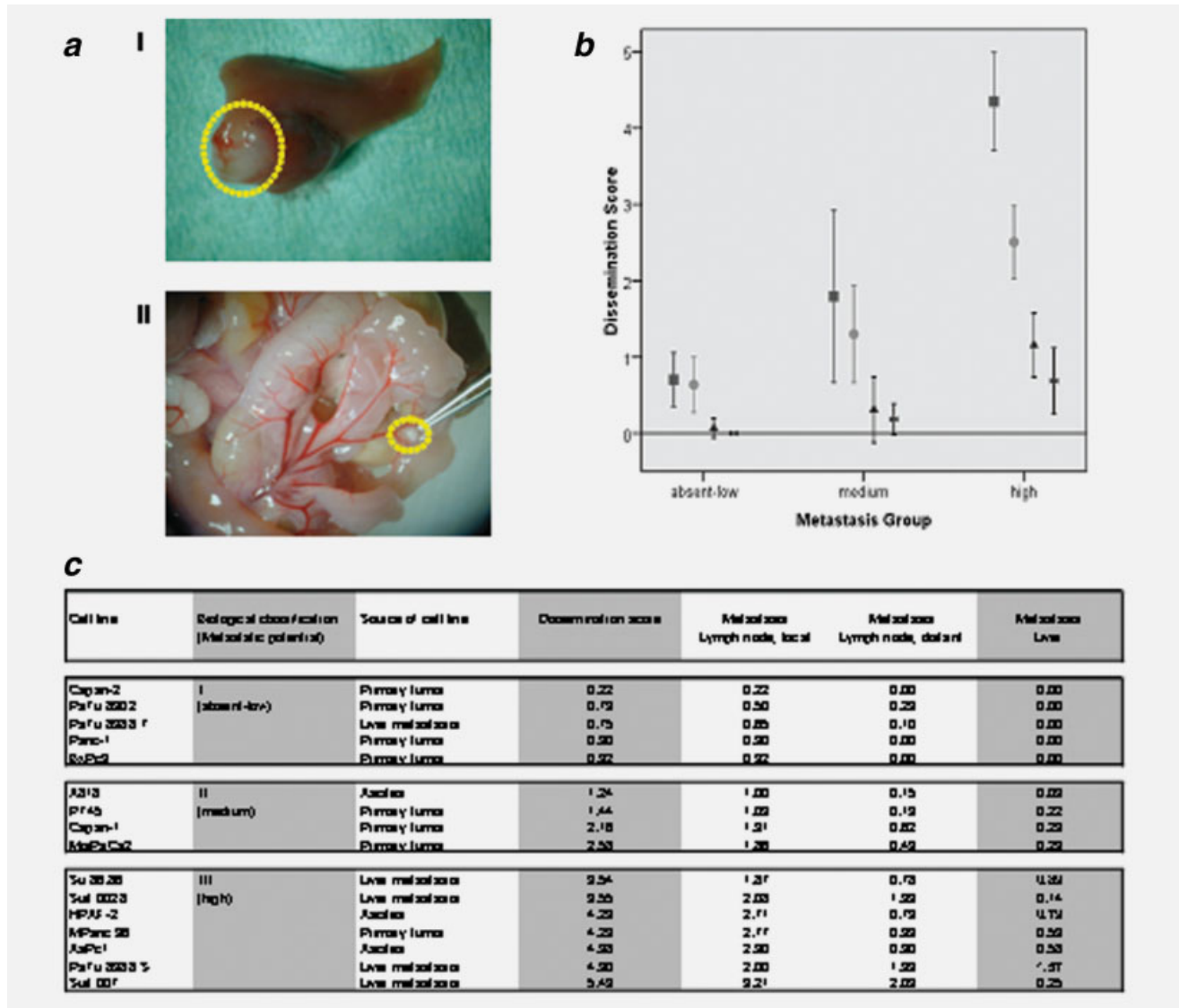


Figure 1. Evaluation of metastasis in 16 PDAC cell lines. (a) Evaluation of metastasis during the final exploration of the mice with detection of liver and lymph node metastases; (b) Extent of metastasis in the different biological groups. Data are represented as mean ± SEM. Dissemination score: ■ total value; ● local lymph node metastasis; ▲ distant lymph node metastasis; ►◄ liver metastasis.; (c) Biological classification of PDCA cell lines into 3 groups according to their metastatic properties. Cell lines were classified using the dissemination score that implicates local and distant lymph node metastasis and liver metastasis. Cell lines with pronounced liver metastasis and downregulated mRNA of EP300 printed bold-faced.

isolated RNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip[®] reagent set (Agilent Technologies, Boeblingen, Germany).

For gene expression profiling of mRNA, low RNA Input Linear Amplification Kit PLUS, One-Color (Agilent Technologies) was used to amplify and label target mRNA (400 ng of total RNA) and to generate complementary RNA. Hybridization, microarray washing and detection of the labeled RNA by microarray were performed according to the instructions of Agilent Technologies. Gene expression profiling was performed using Agilent's Whole Human Genome Oligo Microarrays (4 × 44k, each array with 45,220 features). For

miRNA expression profiling, 100 ng of total RNA were dephosphorylated (Calf Intestinal Alkaline Phosphatase, GE Healthcare, Munich, Germany) and labeled by ligation (T4 RNA Ligase, NEB Biolabs, Frankfurt, Germany) with one Cyanine 3-pCp molecule to the 3' end of the RNA molecules using Agilent's miRNA Labeling Reagent and Hybridization-Kit (Agilent Technologies). Labeled miRNAs were desalted with Micro Bio-Spin[™] Chromatography Columns (BioRad Laboratories, Munich, Germany) as described by Agilent Technologies. Hybridization, microarray washing and detection of the labeled miRNA on the microarray were performed according to the instructions of Agilent Technologies.

MiRNA expression profiling was performed using Agilent's Human miRNA Microarrays (version 1.0, based on Sanger miRBase version 9.1), carrying 555 human miRNAs (Ref. 14, <http://microrna.sanger.ac.uk>).

Array image acquisition and feature extraction was performed using the Agilent G2505B Microarray Scanner and Feature Extraction software version 9.5 (Agilent Technologies).

Statistical and bioinformatical analysis of microarrays

Data files from mRNA- and miRNA-microarrays were analyzed using GeneSpring GX 7.3.1 (Agilent Technologies). Average values of the replicate spots of each mRNA and miRNA were background subtracted, normalized and further analyzed. Normalization was performed in accordance with the following terms: (1) data transformation measurements less than 0.01 were set to 0.01 and (2) normalization per chip to the 50th percentile. The normalized data were filtered on expression. Only mRNAs/miRNAs detected in all samples were included into the following considerations. MRNAs/miRNAs, whose expression did not change across the experiment, were eliminated by Venn Diagram and the remaining, changing mRNAs/miRNAs were used for further statistical analysis. To identify upregulated and downregulated mRNAs/miRNAs, analysis of variance (ANOVA) was carried out using a false positive rate of 0.0001 for mRNA and 0.05 for miRNA. Benjamini and Hochberg False Discovery Rate¹⁵ was taken as the level of significance. ANOVA mRNAs and miRNAs (significantly altered mRNAs and miRNAs) were used for hierarchical cluster analysis to determine expression signatures. Hierarchical clustering was performed using Complete Linkage Algorithm, Spearman Correlation for mRNA and Pearson Correlation for miRNA as similarity measure. Comparisons of mRNA/miRNA expression across the different groups were performed using Venn Diagrams.

TaqMan low density arrays (TLDA)

TaqMan[®] Low Density Arrays based on an Applied Biosystems 7900HT Micro Fluidic Cards (Applied Biosystems, Darmstadt, Germany) were used to detect and quantify mature miRNAs in accordance with the manufacturer's instructions.

Each TLDA Human MicroRNA Panel v1.0 card contains 365 preloaded human miRNA targets; all cataloged in the miRNA database miRBase, and 2 endogenous controls, small nucleolar RNAs (snoRNAs) RNU48 (SNORD48) and RNU44 (SNORD44). TLDA were performed in a 2-step process: during the first step, total RNA was reverse transcribed in a total of 8 multiplex reactions using predefined RT primers that are specific for only the mature miRNA species. In the second step, each of the resulting 8 RT pools containing cDNA template was diluted, mixed with TaqMan Universal PCR Master Mix and loaded into 1 of the 8 fill ports on the TaqMan Array. The card was briefly centrifuged for 1 min at

280g to distribute the samples to the array's wells, and then sealed to prevent well-to-well contamination.

Finally, cards were processed and analyzed in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Reactions were performed in duplicate, including no template controls.

MiRNA expression was calculated using the comparative $2^{-\Delta\Delta CT}$ Method¹⁶ with RNU44 and RNU48 (Applied Biosystems) as endogenous controls. Data were analyzed with Real-Time StatMiner (Integromics, Madrid, Spain). Significance was considered for $p < 0.05$.

Target genes and complementarity of miRNAs

For identification of putative target genes of the expressed miRNAs "Find miRNA Target Genes" software (Agilent Technologies) and the miRGen database [<http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html>¹⁷] were used. The complementarity of miRNAs and their targets were analyzed using NCBI BLAST (Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov>).

Quantitative reverse transcriptase PCR

To verify the accuracy of our microarray and TLDA data, we performed single quantitative real time reverse transcriptase PCR (qRT-PCR) for representative miRNAs using TaqMan miRNA Assays (Applied Biosystems) in accordance with the manufacturer's instructions. Normalization was performed with the small nuclear RNAs RNU48 and RNU44 (Applied Biosystems). All reverse transcriptase reactions, including no-template controls and real-time minus controls, were run in a mastercycler gradient[®] (Eppendorf, Germany). mRNA and miRNA expression levels were quantified using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). qRT PCR was performed in triplicate, including no template controls. Relative expression was calculated using the $2^{\Delta\Delta CT}$ Method and data were analyzed with Real-Time StatMiner (Integromics).

Similarly, the TaqMan Gene Expression Assay was undertaken using primers and probes (predesigned, preoptimized) obtained from Applied Biosystems for EP300 expression determination. The assays used 150 ng of RNA per sample and house keeping genes PPIB and HPRT1 were used to normalize all samples.

Western blot analysis

Cells were washed twice with ice cold PBS and lysed with 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA and 1 μ l inhibitor cocktail (Sigma, Deisenhofen, Germany) per 1 ml lysis puffer. Lysates were clarified by centrifugation at 14.000g for 5 min and stored at -80°C . Protein was quantitated and 15 μ g (1 μ g/ μ l, RIPA buffer) were added to 5 μ l 4 \times Laemmli sample buffer and boiled for 10 min at 95°C . The lysates were loaded on 10% polyacrylamid gels, then transferred to PVDF

membranes and EP300 detected with mouse anti-EP300 antibody (Abnova, Heidelberg, Germany; 1:1,000, 1 µg/ml).

Fresh frozen PDAC tissue (FFT) specimens from 3 patients were retrieved from the files of the Gerhard-Domagk Institute of Pathology of the University Hospital, Muenster. All samples were reviewed for confirmation of diagnosis and adequacy of material by a pathologist. EP300 bands were visualized with enhanced chemiluminescence (Millipore, Schwalbach, Germany). Quantitative densitometric analysis was performed using ImageJ densitometry software (version 1.6, NIH, Bethesda, MD) and selected bands were quantified based on their relative intensities.¹⁸

Results

Characterization of PDAC cell lines

By using the dissemination score to assess the metastatic potential, we classified the PDAC cell lines into 3 groups with different metastatic behavior: cell lines with a dissemination score <1 (only sporadic lymph node metastases, *i.e.*, <1 metastase/mouse) and without liver metastasis were classified in Group I (absent-low). Group II (medium) showed a dissemination score from 1 to 3 and mild liver metastasis, meaning at least 1 or more lymph node metastases and sporadic liver metastasis (0–0.3 metastases/mouse). In Group III (high), mice showed a dissemination score >3 with consistent lymph node and liver metastasis: 2–5 lymph node metastases and a constant occurrence of liver metastases (0.14–1.57 metastases/mouse) (Figs. 1a–1c). Cell lines from Group III with pronounced liver metastasis (0.79–1.57 metastases/mouse; HPAF-2, SU 86.86 and PaTu 8988 S), meaning almost every mouse showed 1 or more liver metastases, were defined as a liver-metastasizing subgroup (Figs. 1c and 2b).

Primary results: Screening

MiRNA expression profiling. MiRNA expression profiles were independently determined by miRNA-microarrays and TLDA. To uncover miRNAs relevant to metastatic progression, we evaluated the expression profiles of the priorly defined groups (absent-low, medium, high) for differential miRNA expression.

MiRNA-microarray. The miRNA-microarray identified 28 miRNAs that were differentially expressed in Group III (high) *vs.* Groups I (absent-low) and II (medium). All 28 miRNAs were significantly upregulated in Group III ($p < 0.0001$) (Fig. 2c) and of these, 9 miRNAs presented extraordinarily high expression factors [expression factor >800] (Fig. 2c).

TLDA. MiRNA expression profiling by TLDA uncovered 54 differentially expressed miRNAs in Group III (high) *vs.* Group I (absent-low) and Group II (medium), with 19 miRNAs being upregulated and 35 downregulated in Group III ($p < 0.05$) (Fig. 2c). Eight miRNAs showed high fold changes

>10 (miR-135b, miR-141, miR-192, miR-194, miR-200a, miR-200b, miR-200c, miR-429).

mRNA expression profiling. mRNA expression profiles of the PDAC cell lines were investigated by microarray only as the overwhelming number of targets renders PCR-based approaches unfeasible. Here, as with the miRNA expression profiling, our goal was to uncover RNAs relevant to metastatic progression, and so we compared the expression profiles of the priorly defined groups (absent-low, medium, high) for differential mRNA expression.

Primary gene expression analysis detected 29,177 expressed genes in Group I (absent-low), 30,861 expressed genes in Group II (medium) and 28,354 expressed genes in Group III (highly). Further analysis using ANOVA showed 217 genes to be expressed in Group I, 223 in Group II and 209 in Group III ($p < 0.0001$). Significantly, differentially expressed genes in 1 group *vs.* the remaining 2 were identified: 8 genes in Group I *vs.* Groups II and III, 12 genes in Group II *vs.* Groups I and III and 3 genes in Group III *vs.* Groups I and II (Fig. 2a). The highly metastatic cell lines (Group III) displayed a significant downregulation of the genes EP300 (0.71 ± 0.32) and SERPIND1 (0.33 ± 0.18) and significant upregulation of CDKN2a (14.6 ± 12.11) ($p < 0.05$, Fig. 2d). In contrast, these 3 genes were not identified as differentially expressed in cell lines with high local infiltrative potential (data not shown).

Consecutive analysis: Data correlation

MiRNA. We further analyzed the expression profiling data generated by the miRNA microarrays and TLDA by searching for common putative targets among the identified miRNAs. Because of our objective in this study, our focus was placed on tumor relevant genes.

MiRNA-microarray. When reviewing the 9 highly differentially expressed miRNAs identified by miRNA-microarray in the miRGen database¹⁹ and the literature, tumor suppressor genes EP300 and PTEN were revealed as putative targets for 5 of the 9 miRNAs: miR-21 (PTEN²⁰), miR-26b (EP300, PTEN), miR-194 (EP300²¹), miR-320 (PTEN) and miR-374 (EP300) (Fig. 2c).

TLDA. Among the 19 TLDA-identified significantly upregulated miRNAs in the highly metastatic cell lines, 6 EP300 related miRNAs (miR-194, fold change: 49.34; miR-200b, fold change: 28.36; miR-200c, fold change: 17.39; miR-429, fold change: 15.23; miR-182, fold change: 1.87; miR-374, fold change: 1.74) were detected. Regarding miRNAs targeting PTEN, only miR-21 with a fold change of 3.31 was shown to be significantly upregulated by TLDA as opposed to miR-21, -26b and -320 shown to be upregulated in the microarray analysis (Figs. 2c and 3).

When investigating cell lines according to their local infiltrative potential, EP300 and PTEN targeting miRNAs were not found to be significantly differentially expressed between groups with the exception of miR-194 (data not shown).

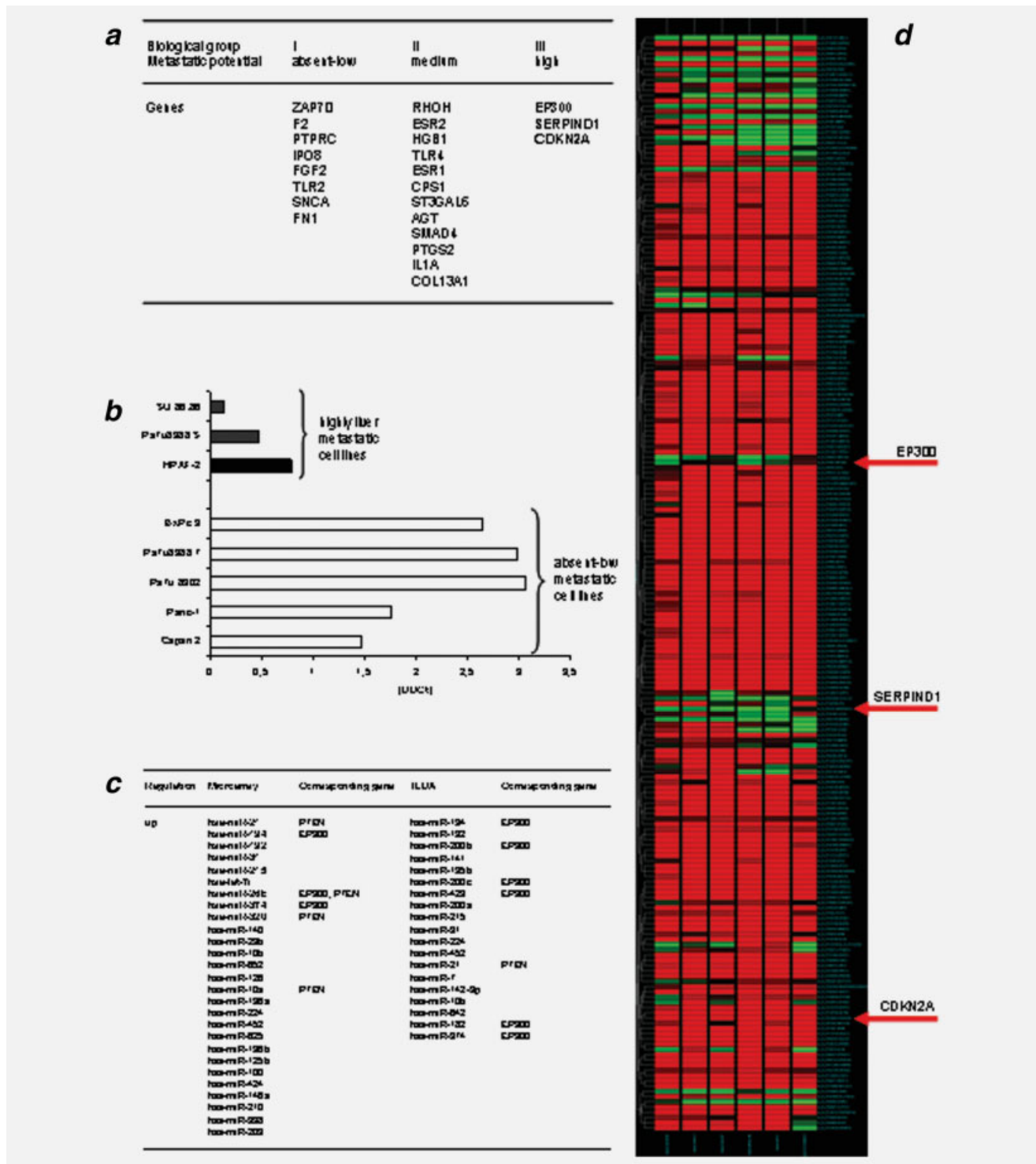


Figure 2. Expression profiling of mRNA and miRNA. (a) Gene expression profiling uncovered significantly differentially expressed genes, which were detected in only 1 of the 3 groups; (b) Quantitative RT-PCR revealed a significant downregulation ($p < 0.05$) of EP300 in highly metastatic cell lines with pronounced liver metastasis compared to medium and absent-low metastatic cell lines; (c) Analysis of microarray and TLDA detected miRNAs in highly metastatic cell lines. Significantly overexpressed miRNAs and their corresponding target genes in highly metastatic cell lines compared to absent-low or medium metastatic cell lines. Significantly upregulated miRNAs with extraordinary high expression factors detected by microarray analyses are printed bold-faced (lg expression factor >800). (d) Hierarchical cluster analysis of highly metastatic cell lines. Compared to cell lines with absent-low or medium metastatic potential highly metastatic cell lines displayed significant different expressions of the genes EP300 (top), SERPIND1 (middle) and CDKN2a (bottom); marked by red arrows. Color coding: red = significant upregulation (<1), green = significant downregulation (>1).

Validation

miRNA. MiRNA expression profiling showed miRNAs with EP300 and PTEN as putative targets to be differentially expressed between the investigated groups in both TLDA and miRNA-microarray. To validate these results, qRT-PCR experiments were performed for the EP300 and PTEN related miRNAs (EP300: miR-26b, -194, -200b, -200c, -429, -182, -374; PTEN: miR-21, -26b, -194, -320). EP300 related miRNAs detected by TLDA with a fold change >10 (miR-194, -200a, -200b, -429) were confirmed to be significantly upregulated by qRT-PCR ($p < 0.05$). Although miR-26b (with both EP300 and PTEN as putative targets) was detected as significantly upregulated in the miRNA-microarray, qRT-PCR results did not confirm this alteration. The PTEN targeting miRNAs miR-21, -26b and -320 detected to be overexpressed by both TLDA and miRNA-microarray could not be validated as such by qRT-PCR (Fig. 4a).

MRNA. MRNA expression profiling *via* microarray showed EP300 mRNA levels to be lowered in the highly metastatic group and in accordance with EP300 related miRNA expression. Therefore, we validated EP300 mRNA expression by quantitative reverse transcriptase PCR (qRT-PCR). Although mRNA analysis did not show significant differential EP300 mRNA expression in Group III (high) vs. Groups I (absent-low) and II (medium), we found a significant downregulation ($p < 0.05$) of EP300 in the liver-metastasizing subgroup of highly metastatic cell lines (0.46 ± 0.32) compared to medium (1.94 ± 0.96) and absent-low (2.38 ± 0.73) metastatic cell lines (Fig. 2b).

PTEN mRNA expression did not show significant differences in the microarray analysis. QRT-PCR validation of PTEN mRNA expression confirmed the microarray results: significant differences between high (1.71 ± 0.73), medium (1.91 ± 0.48) and absent-low (1.74 ± 0.98) metastatic cell lines were not found.

Protein expression

Using Western blot analysis, we compared protein expression of EP300 between all groups. Highly metastatic cell lines displayed a significantly reduced protein expression of EP300 compared to absent-low and medium metastatic cell lines ($p < 0.05$). Densitometric quantification showed mean gray intensities for Group I of 15.96×10^3 (95%CI: $15.3\text{--}16.47 \times 10^3$), Group II of 17.21 (95%CI: $16.39\text{--}18.63 \times 10^3$) and Group III of 13.67 (95%CI: $11.45\text{--}14.75 \times 10^3$) (Fig. 4b). An analysis of fresh frozen tissue from human PDAC revealed marginal expression levels of p300 in all samples ($n = 3$; $5.24 \pm 0.26 \times 10^3$; Fig. 4).

Discussion

The influence of genetic and epigenetic alterations in the development of PDACs is well known, and the inactivation of tumor suppressor genes is recognized as one of the most common oncogenic events in PDAC.^{29,30} Although genetic

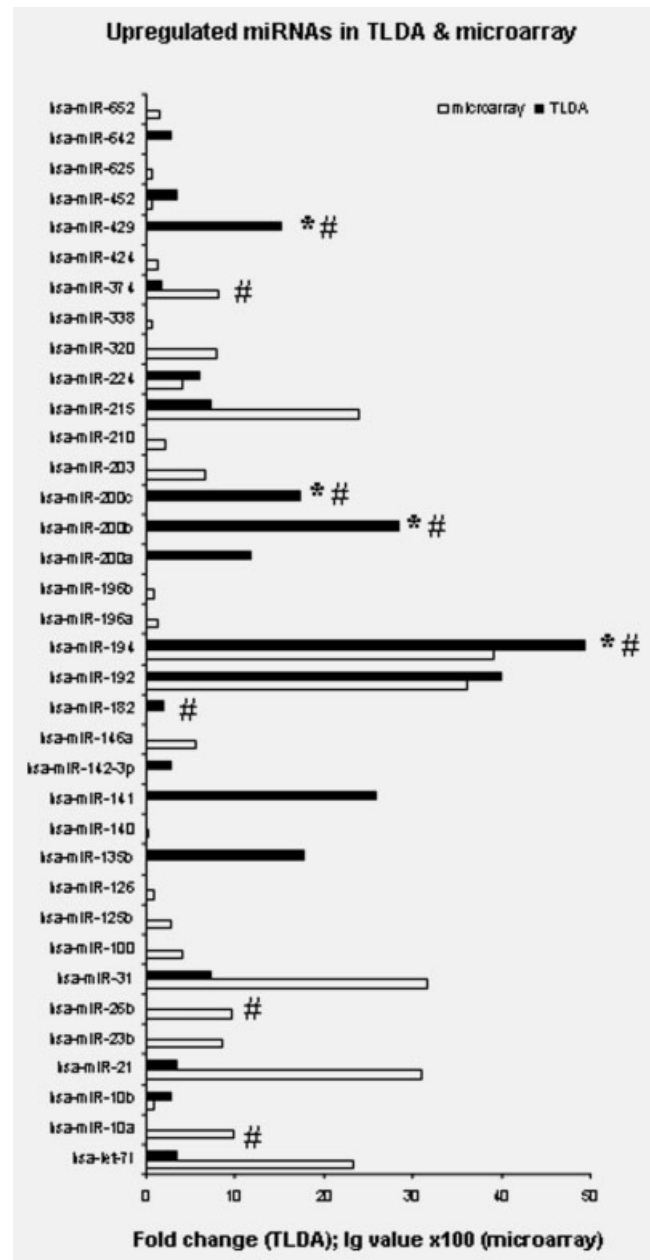


Figure 3. miRNA expression profiling *via* TLDA and microarray. Detection of upregulated miRNAs using different techniques (■ TLDA, □ microarray). EP300 targeting miRNAs (#) were validated using qRT-PCR as a third technique (significant changes: *, $p > 0.05$). To provide comparable visualization results of TLDA was presented as fold changes and results of microarrays as lg values $\times 100$.

alterations and their consequences have been the focus of many studies, the influence of epigenetic mechanisms on the progression of PDAC has remained underinvestigated thus far.

In the present study, we have performed detailed genetic and epigenetic expression profilings to identify specific

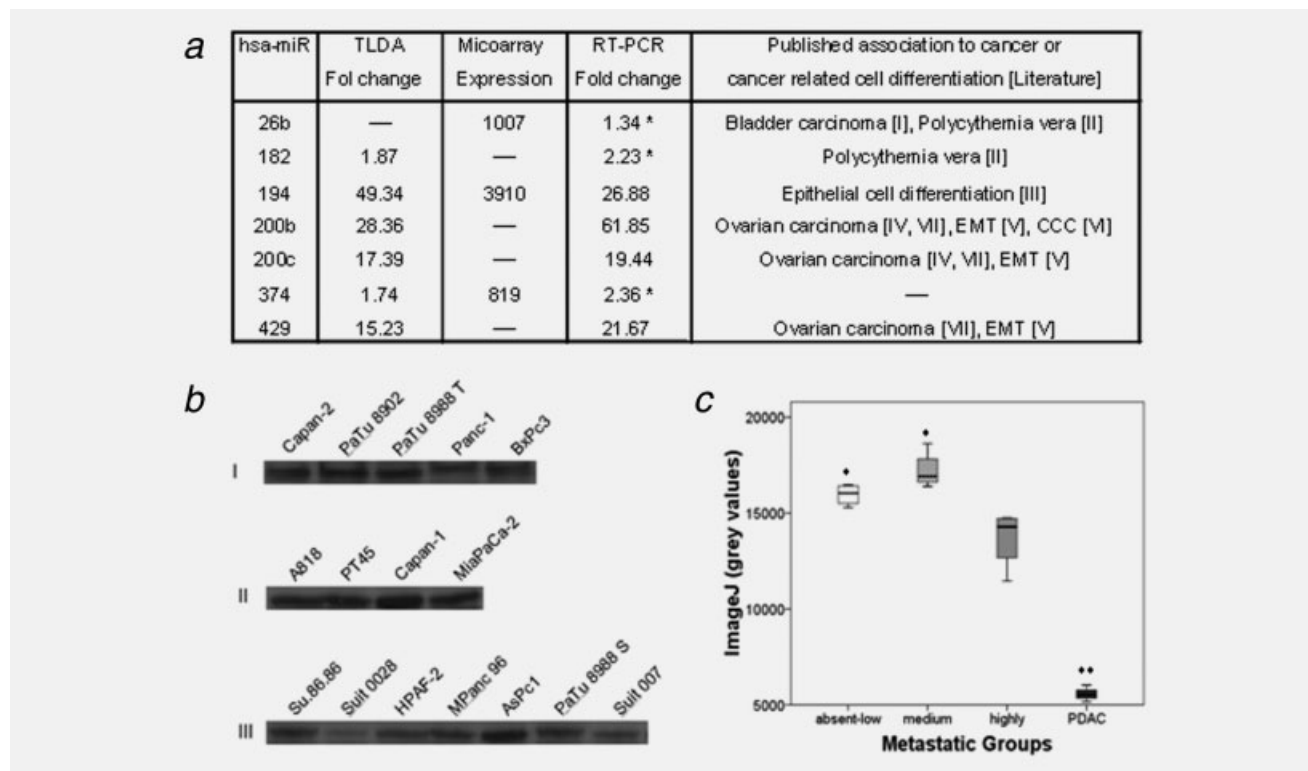


Figure 4. Protein analysis and role of miRNAs. (a) Comparison of microarray, TLDA and qRT-PCR results regarding EP300 targeting miRNA expression. MiRNA expressions that were not validated by RT-PCR are signed by *. Published associations to cancer or cancer-related cell differentiation of these miRNAs were provided [I = Ref. 22, II = Ref. 23, III = Ref. 24, IV = Ref. 25, V = Ref. 26, VI = Ref. 27, VII = Ref. 28]. (b) Enhanced ChemiLuminescence (ECL) images of the Western blots. I = Group I (absent-low), II = Group II (medium) and III = Group III (highly). (c) Protein expression of EP300 using Western blot analysis showed significantly reduced protein p300 expression in highly metastatic cell lines ($p < 0.05$; *) and in human PDAC tissue ($p < 0.05$; **). Densitometric quantification was performed using ImageJ densitometry and presented as optical density (□ Group I, /// Group II, ■ Group III).

alterations responsible for the process of metastasis formation in PDAC.

With the recent discovery of miRNAs, a new class of gene regulators has been identified, adding another layer of complexity to the understanding of gene expression control. Physiologic expression of miRNAs can affect a variety of cellular processes including cell growth, differentiation and apoptosis. MiRNAs are believed to function primarily as negative regulators of gene expression,⁶ regulating their targets depending on the degree of complementarity between miRNA and target. MiRNAs can bind with perfect or near perfect complementarity to protein coding mRNA sequences and subsequently induce the RNA-mediated interference (RNAi) pathway.³¹ Briefly, mRNA transcripts are cleaved by ribonucleases in the miRNA-associated, multiprotein RNA-induced silencing complex (miRISC), which results in the degradation of target mRNAs. A second mechanism of gene regulation without cleavage of mRNA targets has been described, in which the regulatory effects of miRNAs occur by binding to imperfect complementary sites of their mRNA targets.^{32–34} Alterations in developmental pathways can play a critical role in pancreatic cancer development^{35,36} and

modifications in miRNA expression may be important contributors to the development and progression of this tumor entity.

We identified several miRNAs with expression patterns distinctive for pancreatic cancer, such as miR-21 or miR-181b, as has been described in recent studies.^{37–39} Further, several miRNAs turned out to be differentially expressed between the groups with varying metastatic potential that were investigated in this study. Interestingly, the analysis of the most significantly differentially regulated miRNAs in the highly metastatic group revealed 6 EP300 targeting miRNAs to be significantly upregulated (miR-194, -200b, -200c, -429, -182 and -374). The expression of miR-194, -200b, -200c and -429 in highly metastasizing PDAC was validated by single qRT PCR with fold changes from 19-fold to 62-fold, confirming the association between overexpression of EP300 targeting miRNA and increasing propensity to metastasize.

The current literature revealed associations between cancer or cancer-related cell differentiation and the differentially regulated miRNAs targeting EP300 identified in our study. With the exception of miR-374, all other significantly differentially expressed miRNAs were found to be malignancy

associated.^{20,24,26,28,40} However, none of the detected miRNAs from our investigation has so far been implicated in PDAC (Fig. 4a).

In addition to the miRNA expression profiling, we analyzed mRNA expression in PDAC. Comparing the gene expression profiles of the biological groups with different metastatic potential, metastasis-specific alterations were identified. In Group III (high), only 3 genes showed significant differential expression compared to Groups I (absent-low) and II (medium) (Fig. 2a): the serine proteinase inhibitor SERPIND1 and the tumor suppressor gene EP300 (E1A binding protein p300) were detected to be significantly down-regulated, while CDKN2a (cyclin dependent kinase 2a) was significantly upregulated. In an alignment of the epigenetic and genetic expression profiling, we identified the tumor suppressor gene EP300 as an important target of several significantly upregulated miRNAs, while miRNAs with putative targets for SERPIND1 or CDKN2a were not found.

EP300 is located at chromosome 22q and codes for a histone acetyltransferase and belongs to a group of proteins that regulate transcription *via* chromatin remodeling,⁴¹ functions as a transcriptional coactivator⁴² and acts as intrinsic factor of TP53 acetylation in apoptotic response to DNA damage.⁴³ EP300 has also been implicated in the control of cell cycle arrest, in growth- and differentiation-signaling^{44,45} and has been described to be involved in tumorigenesis.^{46,47} The EP300 protein is a target for the adenoviral E1A oncoprotein associated with malignant transformation, suggesting EP300 as a putative tumor suppressor gene.⁴⁸ However, in different tumor entities the various cellular functions of EP300 appear to be involved at different levels of tumor progression. For example, in colon and gastric cancers EP300 has been found to be altered in both alleles, suggesting that inactivation of this gene is involved in the development of gastrointestinal carcinomas.⁴⁹ These mutations were predominantly truncations predicted to have lost histone acetylase activity and were accompanied by a lack of wild-type EP300 expression.⁵⁰ Furthermore, repression of p53 by E1A binding can be reversed by overexpression of p300.⁵¹ These studies provided first evidence that EP300 has characteristics of a classical tumor suppressor, but the low frequency of EP300 mutations (~3%) in pancreatic and other epithelial cancers does not support specific importance of genetic alterations of EP300 in carcinomas.

Our epigenetic studies showed significant upregulation of EP300 targeting miRNAs in the highly metastatic cell lines. MRNA analysis did not show significant differential EP300 mRNA expression in Group III (high) *vs.* Groups I (absent-low) and II (medium), but a significant downregulation of EP300 in the subgroup of liver-metastasizing cell lines within Group III compared to cell lines with absent-low or medium metastatic potential was detected. Therefore, EP300 could present a miRNA-regulated marker for metastatic potential with likely organ-specificity for liver metastasis as suggested by mRNA expression profiling.

Investigation of the EP300 protein showed a significantly reduced p300 expression in the highly metastatic group compared to absent-low and medium metastatic PDAC. Regulatory effects of miRNAs mainly occur by their binding to perfectly complementary sites of their mRNA targets and the target protein expression subsequently being repressed. However, recent findings indicate that miRNAs sharing only partial complementarity with their targets can also induce mRNA degradation, but it is unclear if translational inhibition precedes destabilization of the gene targets in these cases.⁵² Using NCBI BLAST, we investigated the complementarity of the differentially expressed EP300 targeting miRNAs identified in our study (miR-194, -200b, -200c, -429, -182 and -374) to the EP300 mRNA sequence: no perfect matches were found. Therefore, in the case of EP300, partial complementarity seems to exert a translational effect and reduce EP300 protein expression. Consequently, upregulation of EP300 targeting miRNAs is likely to cause downregulation of EP300 mRNA and reduced EP300 protein expression in highly metastatic PDAC cell lines.

In addition, Western blots of human PDAC samples revealed hardly detectable EP300 expression. A qRT-PCR analysis of the EP300 mRNA expression of our human PDAC specimens was not possible due to very poor mRNA quality. We assume that the ischemia time from ligation of the duodenal/pancreatic vessels to the withdrawal of PDAC tissue for analysis (backtable procedure) is too long and causes massive mRNA degradation in the PDAC tissue. Since this ischemia time is an unalterable component of the operation, we consider mRNA analyses of PDAC tissues to be a very difficult procedure.

In addition to the metastatic classification of the cell lines, the evaluation also contained criteria of local tumor growth, such as local infiltration of the tumors into neighboring tissues. Similar analyses as shown for the metastatic dissemination were done using these local infiltration scores (Data not shown). In contrast to the correlation with metastatic patterns, EP300 mRNA and EP300 targeting miRNAs did not show any correlation to tumor growth or infiltration. Taken together, these observations suggest that changes in EP300 regulation and expression are specific for the process of metastasis and therefore EP300 might be considered a metastasis suppressor gene in PDAC.

In conclusion, this study demonstrates a significant upregulation of EP300 targeting miRNAs during the process of metastasis formation and a significant downregulation of the corresponding tumor suppressor gene EP300 in liver-metastasizing PDAC at the mRNA and protein level. Since EP300 seems to play an important role during metastasis formation without influencing tumor growth or invasion of PDAC at the primary sites, the role of EP300 as a metastasis suppressor gene should be considered. Therefore, EP300 and specific EP300 targeting miRNAs seem to represent promising targets for diagnostic or therapeutic approaches.

Acknowledgements

Microarray hybridization and bioinformatical analysis were carried out at arrows biomedical Deutschland GmbH, Muenster, Germany. Taqman Low Density Arrays, real time PCR and analysis were performed with support of the Integrated Functional Genomics (IFG) facilities of the IZKF, Muenster, Germany. The authors thank Ms. F. Spiecker (Dept. of General and Visceral

Surgery) and Ms. H. Stegemann (Dept. of Bioinformatics) for support and expert technical assistance. Furthermore, they thank Prof. H. Kalthoff (Dept. of Surgery, Univ. Hosp. Kiel, Germany) for the cell-lines A818 and PT45, and Dr. H. and Ms. B. Hotz (Dept. of Surgery, Charite CBF, Univ. Hosp. Berlin, Germany) for the cell-lines HPAF-2 and MiaPaCa2 and advice with the murine model.

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