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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Involvement of *CD40* Targeting miR-224 and miR-486 on the Progression of Pancreatic Ductal Adenocarcinomas

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ABSTRACT

Background. Genetic and epigenetic alterations during development of pancreatic ductal adenocarcinomas (PDAC) are well known. Genetic and epigenetic data were correlated with tumor biology to find specific alterations responsible for invasion and metastasis in pancreatic ductal adenocarcinomas.

Methods. A total of 16 human PDAC cell lines were used in murine orthotopic PDAC models. By means of standardized dissemination scores, local invasion and metastatic spread were assessed. mRNA and microRNA expression were studied by microarray and TaqMan low-density array. Quantitative real-time–polymerase chain reaction and flow cytometry were used for expression validation.

Results. *CD40* was detected as a relevant target gene for differentially expressed miRNAs observed in highly invasive and metastatic PDAC only. A significant overexpression ($P < .05$) of *CD40*-related miRNAs miR-224 and miR-486 was detected in highly invasive and metastatic PDAC, whereas *CD40* mRNA expression was not significantly altered. Instead, *CD40* protein expression at cell surfaces of these highly invasive and metastatic PDAC was significantly reduced ($P < .01$).

Conclusions. Epigenetic alterations with upregulated *CD40*-targeting miR-224 and miR-486 are related to downregulated *CD40* protein expression at cell surfaces in highly invasive and metastatic PDAC. Thus, miRNA-regulated *CD40* expression seems to play an important role in progression of PDAC. These data suggest a diagnostic and therapeutic potential for *CD40* and/or its targeting miRNAs in PDAC.

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant tumors and has an unfavorable prognosis. The 5-year survival rate of all patients is <5%, and the median survival time after diagnosis is approximately 6 months.[1,2] The aggressive nature of this neoplasia, 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, as well as alterations in DNA methylation patterns of genes critical for tumor development and progression.[3,4]

Beside genetic alterations, recent studies demonstrated the effect of epigenetic changes for PDAC. Epigenetic mechanisms lead to modifications in gene expression that are controlled by heritable but potentially reversible changes in DNA methylation and/or chromatin structure. Recently, miRNAs have gained attention as another family of molecules involved in cancer development.[5,6] MicroRNAs (miRNA) 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 to 23 nucleotides, and they contribute to epigenetic alterations.[7] Single-stranded miRNAs bind messenger RNAs (mRNA) of potentially hundreds of genes at the 3′ untranslated region with perfect or near-perfect complementarities. Upon binding to their target mRNAs, miRNAs cause posttranscriptional 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 and some solid tumor entities.[10–12]

As a result of its specific tumor biology and failure of most therapeutic targets, PDAC is likely to have currently unknown regulatory mechanisms. Because alterations in developmental pathways can play critical roles in pancreatic cancer development, alterations in miRNA expression may be important contributors to the development and progression of this tumor entity.[13,14] Therefore, 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 more importantly may help us devise strategies for earlier diagnosis and identify novel therapeutic targets.

The aim of this study was to identify genetic and epigenetic alterations that contribute to the development and the progress of PDAC. Therefore, we evaluated the expression profiles of RNAs and miRNAs and identified specific alterations related to the process of local invasion and distant metastasis formation in PDAC.

MATERIALS AND METHODS

Cell Lines

A total of 16 human PDAC cell lines were analyzed (Fig. 1). 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) and were *Mycoplasma* negative. For culturing, they were incubated at 37°C in humidified air with 5% or 10% CO_2. The medium was replaced twice a week, and cells were maintained by serial passaging after trypsinization with .1% trypsin.

Laboratory Animals

Four-week-old male nude mice (Crl:NU/NU-nuBR) weighing 20 to 22 g were obtained from Charles River Laboratories. The animals were housed in cages with autoclaved bedding, food, and water. The mice were maintained on a daily 12-hour light, 12-hour dark cycle.

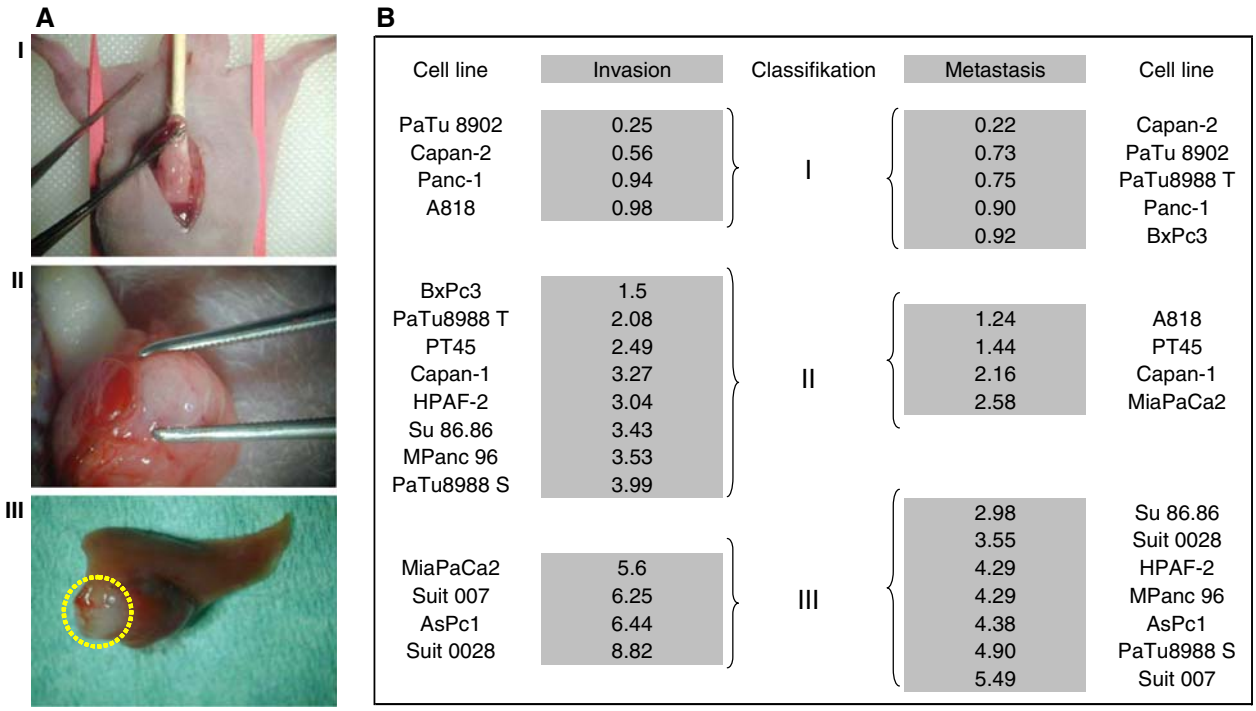


FIG. 1 Evaluation of 16 pancreatic ductal adenocarcinoma (PDAC) cell lines. **A** Animal model. Exploration of the pancreas, evaluation of the primary tumor, and semiquantitative assessment of metastasis (e.g., liver metastases) during the final exploration of the mice. **B** Biological classification of PDAC cell lines into three groups according to their invasive and metastatic properties. Cell lines were classified with the dissemination score that implicates tumor growth, local invasion, and metastasis

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 modified according to a previously described technique.[15] Donor nude mice were anesthetized by isoflurane (Forene; Abbott) inhalation. A total of 5×10^6 cells of each human PDAC cell line were injected subcutaneously into the flanks of the animals. The mice were humanely killed by a lethal dose of isoflurane after 3 to 4 weeks, when the subcutaneous tumors had reached a size of 1 cm in the largest diameter. The donor tumors were collected under strictly aseptic conditions and minced into small fragments 1 mm^3 in size. To avoid necrotic tissue from central tumor areas, only macroscopically viable tumor tissue from the outer part of the donor tumors was used for orthotopic implantation. Tumor recipient nude mice were anesthetized 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 two layers with 5-0 absorbable Vicryl sutures (Ethicon). Postoperative analgesia was performed by subcutaneous injection of carprofen (Rimadyl, Pfizer Animal Health; 5 mg/kg body weight) every 24 hours.

Biological 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 intraabdominal tumor growth (Fig. 1A), clinical signs of tumor burden, primary tumor volume, local infiltration, and patterns of local and systemic metastases were assessed with a modified, standardized dissemination score.[15] 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 evaluated as distant metastases.

Tumor dissemination was evaluated by crediting one point for every massive local infiltration, every metastasis, multiple metastatic lesions (more than one in liver, more than five on diaphragm, mesentery, retroperitoneum), and metastases >50 mm^3. Score values represented mean sums of the obtained credit points for all mice in the specific group/cell line (Fig. 1B). Consecutively, cell lines were classified into three 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 and miRNA isolation from tumor cell lines were performed with the RNeasy Mini Kit (Qiagen) and miRNeasy Mini Kit (Qiagen), respectively, according to the manufacturer's instructions. Purity and integrity of the isolated RNA and miRNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies).

For gene expression profiling of mRNA, low RNA Input Linear Amplification Kit PLUS, One-Color (Agilent Technolgies) 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 on microarrays were performed according to the instructions of Agilent Technologies. Gene expression profiling was performed by Agilent's Whole Human Genome Oligo Microarrays (4×44 k, each array with 45,220 features). For miRNA gene expression profiling, 100 ng of total RNA was dephosphorylated (calf intestinal alkaline phosphatase; GE Healthcare) and labeled by ligation (T4 RNA Ligase, New England Biolabs) with one cyanine 3–pCp molecule to the 3′ end of the RNA molecules with an miRNA labeling reagent and hybridization kit (Agilent Technolgies). Labeled miRNAs were desalted with Micro Bio-Spin chromatography columns (BioRad Laboratories) 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. Gene expression profiling was performed with Agilent's human miRNA microarrays (version 1.0, based on Sanger miRBase version 9.1), carrying 555 human miRNAs (http://microrna.sanger.ac.uk).[16]

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

Statistical and Bioinformatical Analysis of Microarrays

Data files from mRNA and miRNA microarrays were analyzed by GeneSpring GX 7.3.1 (Agilent Technolgies). 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 < .01 were set to .01, and (2) normalization per chip to the 50th percentile. The normalized data were filtered on expression. Only mRNAs/miRNAs that were detected in all compared samples were included into the following considerations. Genes 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 up- and downregulated mRNAs and miRNAs, analysis of variance (ANOVA) was carried out using a false-positive rate of .0001 for mRNA and .05 for miRNA. The Benjamini and Hochberg false-discovery rate was taken as the level of significance.[17] ANOVA mRNAs and miRNAs (significantly altered mRNAs and miRNAs) were used for hierarchical cluster analysis to determine expression signatures. Hierarchical clustering was performed by the complete linkage algorithm, Spearman correlation for mRNA, and Pearson correlation for miRNA as similarity measure. Comparisons of mRNA and miRNA expression across the different groups were performed by Venn diagrams.

TaqMan Low-Density Array (TLDA)

TLDAs based on Applied Biosystems' 7900HT Micro Fluidic Cards 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 miRBase database, and two endogenous controls, small nucleolar RNAs (snoRNAs) RNU48 (SNORD48) and RNU44 (SNORD44). TLDAs were performed in a two-step process: during the first step, total RNA was reverse transcribed with predefined RT primers that are specific for only the mature miRNA species. In the second step, each of the resulting eight RT pools containing cDNA template was diluted, mixed with TaqMan Universal PCR Master Mix, and loaded into each of the eight fill ports on the TaqMan array. The card was briefly centrifuged for 1 minute at 280 × *g* to distribute samples to the multiple wells on the array, 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). Comparative RT-PCR was performed in duplicate, including no template controls.

miRNA expression was calculated by the comparative $2^{-\Delta\Delta CT}$ method with RNU44 and RNU48 (Applied Biosystems) as endogenous controls.[18] Data were analyzed with Real-Time StatMiner (Integromics). Significance was considered at $P < .05$.

Target Genes and Complementarity of miRNAs

For identification of putative target genes of the expressed miRNAs, the Find miRNA Target Genes software (Agilent Technologies) and the miRGen database (http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html) were used.[19] The complementarity of miRNAs and their targets were analyzed by NCBI BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov).

Validation of Microarray and TLDA Data by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

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

Similarly, the TaqMan gene expression array was undertaken with primers and probes (predesigned, preoptimized) obtained from Applied Biosystems for *CD40* and *CD40L* expression determination. The assays used 150 ng of RNA per sample, and housekeeping genes *PPIB* and *HPRT1* were used to normalize all samples.

Flow Cytometric Analysis

Cell surface phenotype studies were performed as previously described.[20] Cells were fixed with 4% paraformaldehyde and then washed and resuspended in phosphate-buffered saline containing .5% bovine serum albumin. After this the cells were incubated for 45 minutes with phycoerythrin-conjugated *CD40* and *CD40L*

antibodies (Biozol Diagnostica). After washing, *CD40* and *CD40L* surface expression was measured with a FC500 flow cytometer (Beckman Coulter).

RESULTS

Tumor Biology of Pancreatic Cancer Cell Lines

By using the dissemination score to assess the invasive and metastatic potential, we classified the pancreatic cancer cell lines into three groups each. Cell lines with scores of < 1 were classified in group I (absent-low) for both criteria. For metastasis, cell lines with a dissemination score between 1 to 3 and mild liver metastasis and for invasion, cell lines with a dissemination score between 1 to 4 were classified in group II (medium). A dissemination score of >3 with pronounced lymph node and/or liver metastasis was defined as group III (high) in metastasizing cell lines. For invasion, a dissemination score of >4 was defined as group III (high). Group I and II cell lines were considered to be less invasive/metastatic; group III represented highly invasive/metastatic cell lines (Fig. 1).

Expression Profiles of microRNA

The expression of miRNAs was independently determined by miRNA microarrays and TLDA. To uncover potential miRNAs relevant to invasive or metastatic properties of PDAC, statistically significant differences of miRNA expression between the biological groups were defined (high vs. medium, high vs. absent-low, medium vs. absent-low).

The miRNA microarrays identified 26 and 43 miRNAs that were differentially expressed ($P < .0001$) between highly metastatic and absent-low/medium metastatic or invasive cell lines, respectively. A literature research revealed several of these miRNAs as related to *CD40* or *CD40L*. Out of the highly metastatic group, two miRNAs are targeting for *CD40* (miR-196b, miR-224), and one is targeting for *CD40L* (miR-210). Considering the invasive potential of the cells, four miRNAs are related to *CD40* (miR-196b, miR-224, miR-424, miR-584), whereas two miRNAs target for *CD40L* (miR-210, miR-532) (Figs. 2 and 3).

The analysis of TLDAs resulted in 54 and 74 miRNAs that were differentially expressed between highly metastatic and absent-low/medium metastatic and invasive cell lines, respectively. Highly metastatic cell lines revealed 19 miRNAs being upregulated and 35 being downregulated. Among the statistically significantly upregulated miRNAs in the highly metastatic cell lines, one *CD40*-related miRNA was detected (miR-224) (Fig. 2), whereas 5 *CD40L* targeting miRNAs were identified among the significantly downregulated miRNAs (miR-19b, miR-24, miR-146b, miR-210, miR-532). Regarding invasion, 8 miRNAs were upregulated and 66 were downregulated. Among those miRNAs, two upregulated miRNAs were detected as targeting for *CD40* (miR-224 and miR-486) and seven downregulated miRNAs as targeting for *CD40L* (miR-19b, miR-24, miR-146b, miR-210, miR-432, miR-484, miR-532) (Fig. 3).

To validate these results, qRT-PCR were performed for *CD40*- and *CD40L*-related miRNAs. *CD40*-related miRNAs miR-224 and miR-486 were confirmed to be significantly upregulated by qRT-PCR ($P < .05$). Although several *CD40L* targeting miRNAs were described to be significantly downregulated by miRNA microarray and TLDA, qRT-PCR only partially confirmed these alterations (Fig. 4).

mRNA Expression Profiling

Regarding metastasis grouping, the microarray-based gene expression analysis revealed a total of 29,177 (absent-low), 30,861 (medium), and 28,354 (high) detected genes. For invasion, 29,570, 34,682, and 31,500 genes were detected, respectively. ANOVA resulted in 217, 223, and 209 (metastasis) and 208, 246, and 214 (invasion) genes with statistically significant differential expression ($P < .0001$) in the three biological groups.

By using Gene Spring for target gene search of differentially expressed miRNAs, we found that highly invasive cell lines displayed *NFκB1* and *CD40* mRNA; we also found that highly metastatic PDAC *CD40* mRNA were relevant target genes that were expressed in group III (high) only (Fig. 5). Compared with cell lines with absent-low invasive potential (5.08 ± 3.11), those with medium (2.1 ± 2.05) and high (2.11 ± 1.66) invasion displayed significantly decreased expression ($P < .05$) of *CD40* mRNA (Fig. 6). In contrast, expression of *CD40* mRNA was not significantly different between the various metastatic groups. Furthermore, its ligand, *CD40L*, was not found in any gene list for differential expression.

The microarray-based *CD40* mRNA expression was validated by qRT-PCR. Although microarray experiments showed decreased *CD40* expression by medium and highly invasive cell lines, these differences could not be verified by qRT-PCR.

Flow Cytometric Analysis

By means of flow cytometry, we analyzed *CD40* and *CD40L* expression at cell surfaces of all PDAC cell lines. Cell lines with absent-low and medium metastatic or invasive potential had comparable *CD40* expression

FIG. 2 Dysregulated miRNA expression for highly invasive and metastatic cell lines detected by microarrays or TaqMan low-density arrays. *CD40* and *CD40L* targeting miRNAs are boldface and underlined

Significantly dysregulated miRNAs, expressed in highly invasive and metastatic cell lines

Upregulation		**Upregulation**		**Downregulation**	
Microarray		**TLDA**		**TLDA**	
Invasion	Metastasis	Invasion	Metastasis	Invasion	Metastasis
hsa-miR-10b	hsa-miR-10a	hsa-miR-135b	hsa-miR-10b	hsa-miR-504	hsa-miR-191
hsa-miR-23b	hsa-miR-10b	hsa-miR-192	hsa-miR-21	hsa-miR-17-5p	hsa-miR-24
hsa-miR-26a	hsa-miR-21	hsa-miR-194	hsa-miR-31	hsa-miR-614	hsa-miR-17-5p
hsa-miR-26b	hsa-miR-23b	**hsa-miR-224**	hsa-miR-135b	**hsa-miR-24**	hsa-miR-324-3p
hsa-miR-27b	hsa-miR-26b	**hsa-miR-486**	hsa-miR-141	hsa-miR-216	hsa-miR-338
hsa-miR-29c	hsa-miR-31	hsa-miR-545	hsa-miR-142-3p	hsa-miR-151	**hsa-miR-146b**
hsa-miR-30b	hsa-miR-100	hsa-miR-616	hsa-miR-182	hsa-miR-512-5p	hsa-miR-381
hsa-miR-30d	hsa-miR-125b	hsa-miR-642	hsa-miR-192	**hsa-miR-146b**	hsa-miR-19a
hsa-miR-34a	hsa-miR-126		hsa-miR-194	hsa-miR-575	hsa-miR-19b
hsa-miR-100	hsa-miR-140		hsa-miR-200a	hsa-miR-340	hsa-miR-20a
hsa-miR-125b	hsa-miR-146a		hsa-miR-200a	hsa-miR-551a	hsa-miR-503
hsa-miR-126	hsa-miR-192		hsa-miR-200b	hsa-miR-579	**hsa-miR-210**
hsa-miR-130a	hsa-miR-194		hsa-miR-200c	hsa-miR-365	hsa-miR-579
hsa-miR-140	hsa-miR-196a		hsa-miR-215	hsa-miR-450	hsa-miR-33
hsa-miR-141	**hsa-miR-196b**		**hsa-miR-224**	hsa-miR-20a	hsa-miR-103
hsa-miR-146a	hsa-miR-203		hsa-miR-374	hsa-miR-191	hsa-miR-425
hsa-miR-149	**hsa-miR-210**		hsa-miR-429	hsa-miR-425-5p	hsa-miR-485-5p
hsa-miR-181a	hsa-miR-215		hsa-miR-452	hsa-miR-519c	hsa-miR-501
hsa-miR-181b	**hsa-miR-224**		hsa-miR-642	hsa-miR-19a	hsa-miR-660
hsa-miR-181d	hsa-miR-320			hsa-miR-548d	**hsa-miR-532**
hsa-miR-188	hsa-miR-338			hsa-miR-422a	hsa-miR-139
hsa-miR-192	hsa-miR-374			hsa-miR-515-3p	hsa-miR-500
hsa-miR-193a	hsa-miR-424			hsa-miR-324-3p	hsa-miR-22
hsa-miR-194	hsa-miR-452			hsa-miR-19b	hsa-miR-195
hsa-miR-196a	hsa-miR-625			hsa-miR-660	hsa-miR-379
hsa-miR-196b	hsa-miR-652			hsa-miR-376a	hsa-miR-485-3p
hsa-miR-210				hsa-miR-214	hsa-miR-512-3p
hsa-miR-215				hsa-miR-542-5p	hsa-miR-424
hsa-miR-224				**hsa-miR-432**	hsa-miR-145
hsa-miR-320				hsa-miR-125a	hsa-miR-126
hsa-miR-324-5p				hsa-miR-526b	hsa-miR-376a
hsa-miR-370				hsa-miR-189	hsa-miR-362
hsa-miR-424				**hsa-miR-484**	hsa-miR-193a
hsa-miR-452				**hsa-miR-532**	hsa-miR-218
hsa-miR-452*				hsa-miR-449	hsa-miR-214
hsa-miR-513				hsa-miR-425	hsa-miR-99b
hsa-miR-532				hsa-miR-381	
hsa-miR-584				hsa-miR-520h	
hsa-miR-625				hsa-miR-98	
hsa-miR-630				hsa-miR-28	
hsa-miR-652				hsa-miR-181b	
hsa-miR-660				hsa-miR-195	
hsa-miR-801				hsa-miR-181d	
				hsa-miR-33	
				hsa-miR-22	
				hsa-miR-103	
				hsa-miR-145	
				hsa-miR-379	
				hsa-miR-193a	
				hsa-miR-500	
				hsa-miR-501	
				hsa-miR-518b	
				hsa-miR-485-3p	
				hsa-miR-656	
				hsa-miR-517b	
				hsa-miR-126	
				hsa-miR-218	
				hsa-miR-487b	
				hsa-miR-210	
				hsa-miR-512-3p	
				hsa-miR-433	
				hsa-miR-410	
				hsa-miR-16	
				hsa-miR-362	
				hsa-miR-187	
				hsa-miR-99b	
				hsa-miR-130a	

(86.43% ± 18.35% and 60.04% ± 19.99% *CD40*-positive cells, respectively). In contrast, highly metastatic and highly invasive cell lines (1.37% ± 1.18% *CD40*-positive cells) displayed significantly reduced and almost completely lost *CD40* expression ($P < .01$) (Fig. 7). The analysis of *CD40L* revealed very low expression of all cell lines without any marked differences between the groups (*CD40*-positive cells: absent-low .38% ± .46%; medium .99% ± .4%; high .96% ± .69%).

Role of miRNAs—Review of Literature

We performed a systematic Medline search to find published association between cancer or cancer-related alterations and *CD40* and/or *CD40L* targeting miRNAs of our study. Search terms included: miR, micro RNA, cancer, carcinoma, pancreatic carcinoma, and pancreatic cancer. Among the qRT-PCR-validated miRNAs, miR-224 and miR-486 were found to be related to *CD40* and

possibly involved in different malignancies.[21–25] However, none of the detected miRNAs from our investigation has been associated to PDAC so far.

TLDA: CD40/CD40L encoding miRNAs expressed in highly metastatic and invasive cell lines

Upregulated Invasion	Metastasis	**Downregulated** Invasion	Metastasis
miR-224 (2.93) miR-486 (28.84)	miR-224 (6.16)	miR-19b (0.37) miR-24 (0.51) miR-146b (0.49) miR-210 (0.17) miR-432 (0.35) miR-484 (0.32) miR-532 (0.32)	miR-19b (0.54) miR-24 (0.59) miR-146b (0.55) miR-210 (0.49) miR-532 (0.37)

FIG. 3 Expression of up- and downregulated miRNAs in highly metastatic and invasive cell lines by TaqMan low-density arrays. *CD40*-targeting miRNAs are significantly upregulated, and *CD40L*-targeting miRNAs are significantly downregulated (fold change)

DISCUSSION

The influence of genetic alterations in the development of pancreatic ductal adenocarcinomas (PDAC) is well known. Recently, epigenetics attracted attention, and with the discovery of miRNAs, a new class of epigenetic regulators has been identified, adding another layer of complexity to the understanding of gene expression control. Physiological 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.[5] Possibly, they negatively regulate their targets in one of two ways depending on the degree of complementarities between miRNA and their potential target sequences. As the first assumed mode of action, miRNAs bind with perfect or nearly perfect complementarity to protein coding mRNA sequences and subsequently induce the RNA-mediated interference pathway.[26] Briefly, mRNA

microRNA (Detection / Methods / Values)

hsa-miR	Encodes	Detection Taqman	Microarray	Taqman Fold ch Inv	Taqman Fold ch Met	Microarray Express Inv	Microarray Express Met	RT-PCR Fold ch Inv	RT-PCR Fold ch Met
486	CD40	Inv	nd	28.84	nd	nd	nd	11.96*	3.12*
224	CD40	Inv & Met	Inv & Met	2.93	6.16	+	++	2.67*	7.96*
19b	CD40L	Inv & Met	nd	0.37	0.54	nd	nd	0.94	0.11
210	CD40L	Inv & Met	Inv	0.17	0.49	++	nd	0.84	0.10
532	CD40L	Inv & Met	Inv	0.32	0.37	+	nd	0.13	0,09

CD40

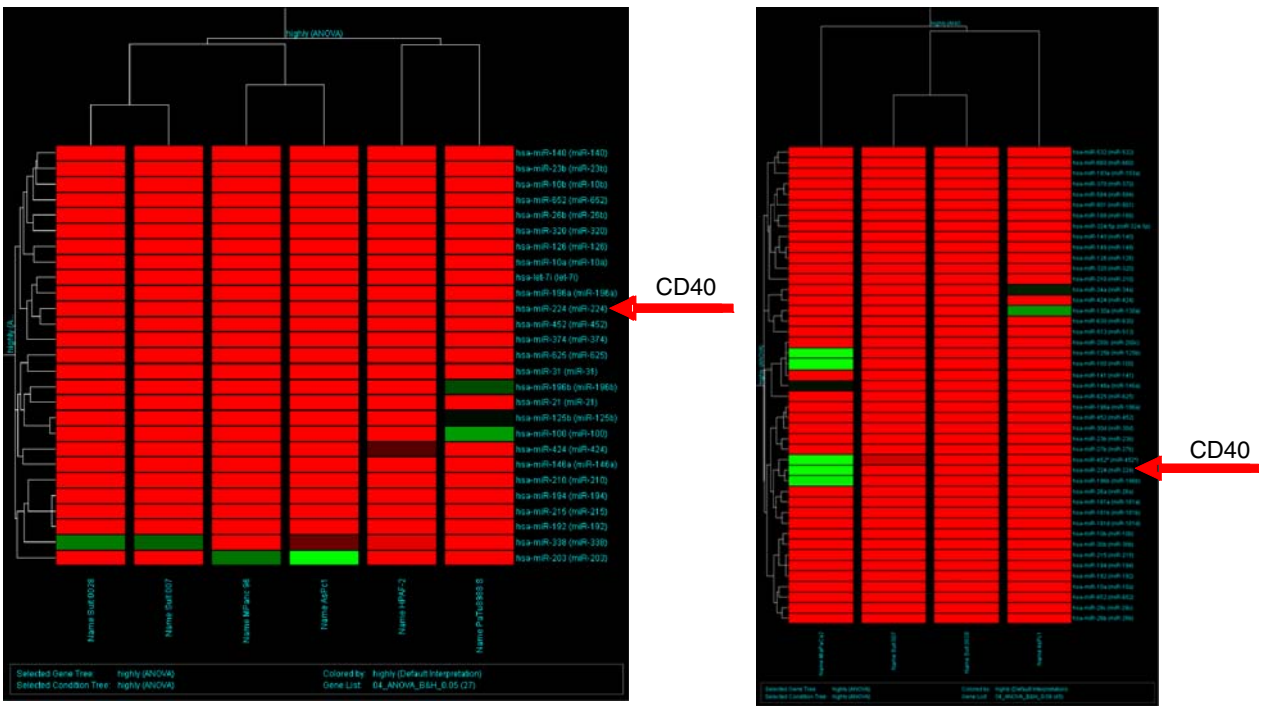
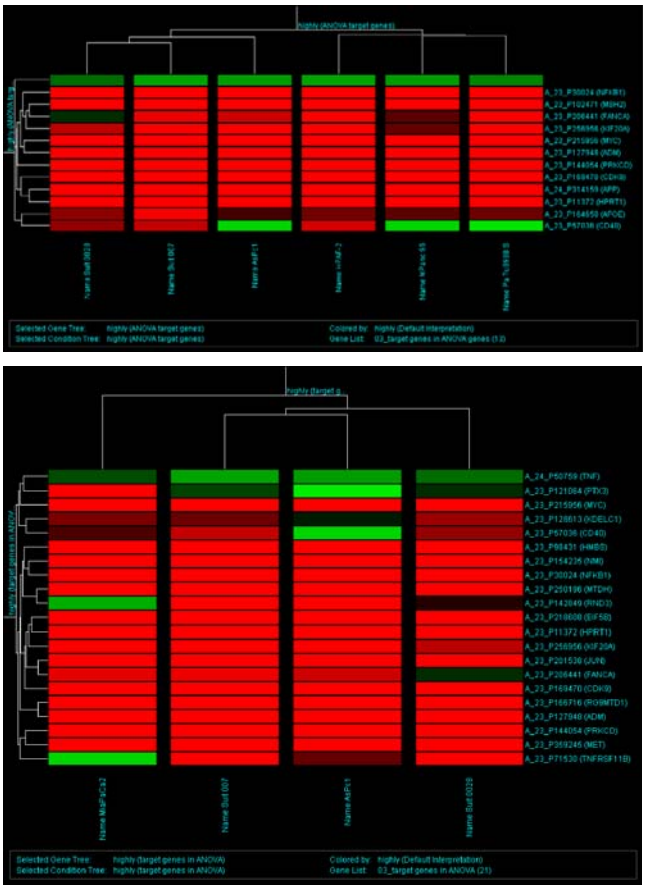


FIG. 4 Detection of upregulated miRNAs by different techniques (TaqMan, microarray, quantitative reverse transcriptase–polymerase chain reaction). * Significant change at $P > 0.05$. *Inv* invasion, *Met* metastasis. Microarray: *nd* nondetected; + moderate overexpression (>1); ++ strong overexpression (>10). *CD40* targeting miR-224 is marked by red arrow

A

Target genes of miRNAs, (highly metastatic and invasive cell lines)

Biological impact	Metastasis	Invasion
Genes	ADM	ADM
	APOE	
	APP	
	CD40	**CD40**
	CDK9	CDK9
		EIF5B
	FANCA	FANCA
		HMBS
	HPRT1	HPRT1
		JUN
		KDELC1
	KIF20A	KIF20A
	MSH2	
		MET
		MTDH
	MYC	MYC
	NFKB1	NFKB1
		NMI
	PRKCD	PRKCD
		PTX3
		RG9MTD1
		RND3
	TNF	TNF
		TNFRSF11B



B

Invasion			
Biological group	I	II	III
Metastatic potential	absent-low	medium	highly
Genes	E2F1	COL1A2	NFKB1
	CDK2		**CD40**
	DDX18		
	SMAD2		
	PARP1		
	VEGF		
	NOTCH1		
	PLA2G4A		
	LMO2		
	THBS1		

Metastasis			
Biological group	I	II	III
Metastatic potential	absent-low	medium	highly
Genes	FN1	ITGB1	**CD40**
		EXO1	

FIG. 5 Target genes of miRNAs expressed in highly metastatic and invasive cell lines. **A** Target genes of significantly expressed miRNAs in group III (high) for invasion and metastasis. Illustration of microarrays for metastasis (*top*) and invasion (*down*): *red* upregulated (expression >1), *green* downregulation (expression >1). **B** Target genes of significantly expressed miRNAs that are expressed in only one biological group (I absent-low, II medium, III high)

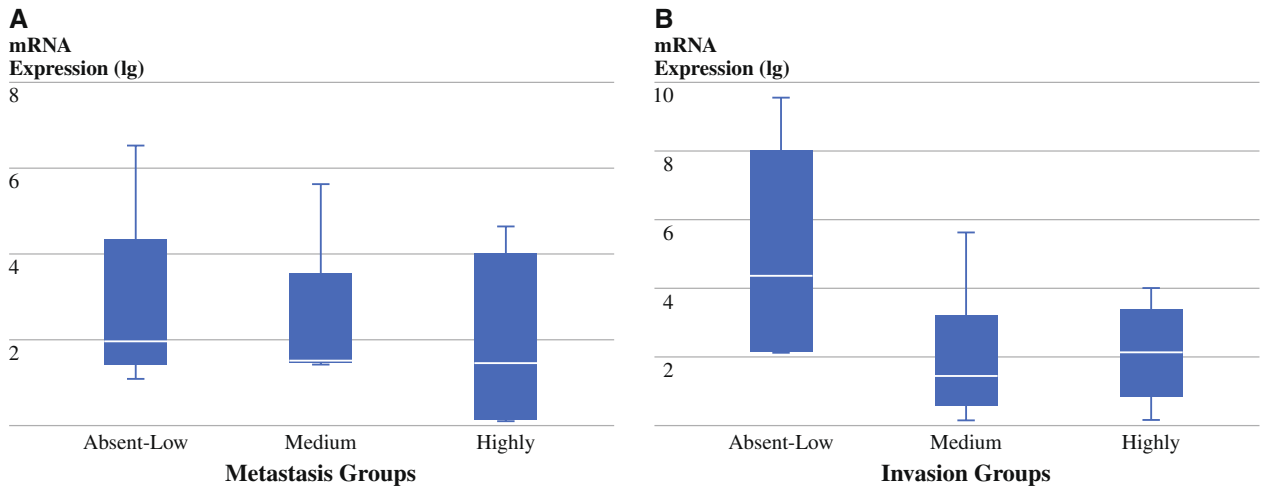


FIG. 6 Expression of *CD40* mRNA. *CD40* mRNA expression detected by microarrays [expression factor; lg]. **A** metastasis groups, no significances. **B** Invasion groups. Medium (2.1 ± 2.05) and highly (2.11 ± 1.66) invasive cell lines displayed significantly decreased expressions (* $P < 0.05$) of *CD40* compared with cell lines with absent-low invasive potential (5.08 ± 3.11)

transcripts are cleaved by ribonucleases in the miRNA-associated, miRNA-associated, and multiprotein RNA induced-silencing complex, which results in the degradation of target mRNAs.[27] A second mechanism of gene regulation without cleavage of mRNA targets has also been described, whereby regulatory effects of miRNAs occur by binding to imperfect complementary sites within the 3′ untranslated regions of their mRNA targets. miRNAs repress target-gene expression posttranscriptionally through a RNA induced-silencing complex, but the targets are not degraded.[28,29]

The present study identified several miRNAs with expression patterns distinctive for pancreatic cancer, such as miR-21 or miR-181b, that were also found by others.[30–32] When we correlated these expression profiles with progressive potential of the cells, we found that several miRNAs turned out to be differentially expressed in the biological groups. Target gene search of differentially expressed miRNAs in highly invasive cells displayed NFκB1 and *CD40* as potential target genes related to aggressive phenotypes, whereas in highly metastatic PDAC, only *CD40* was found. When we searched for *CD40*- and *CD40L*-targeting miRNAs in highly metastatic and highly invasive groups, we found miR-196b, miR-224, miR-424, miR-486, and miR-584 to be greatly upregulated. Interestingly, miR-224 was identified to be upregulated in highly metastatic and highly invasive cell lines only—a finding confirmed by all methods used (microarray, TLDA, qRT-PCR). In addition, our study detected five and seven greatly downregulated miRNAs as targeting for *CD40L* for metastasis and invasion, respectively.

miR-224 has been described to be greatly upregulated in various cancers, including thyroid, hepatocellular, and prostate cancer as well as acute myeloid leukemia.[21–25] Apoptosis inhibitor 5 is the likely in vivo target of this miR-224. The other specifically overexpressed miR-486 was reported to be upregulated in glioblastoma stem cells and bronchioalveolar stem cells.[33,34]

Because *CD40*-targeting miRNAs are upregulated and *CD40L*-targeting miRNAs are downregulated, a decreased expression of *CD40* mRNA would be expected. Contradictory results of *CD40* mRNA expression between microarrays and qRT-PCR suggest that regulatory effects of upregulated *CD40*-related miRNAs (miR-224, miR-486) mostly occur by binding to imperfect complementary sites of their mRNA targets without cleavage, but translational inhibition of these mRNA targets. This was further supported by an NCBI BLAST search that excluded full complementarities for these miRNAs and the *CD40* sequence (data not shown). Because highly metastatic and highly invasive cell lines displayed greatly reduced *CD40* protein expression compared with less aggressive cell lines, partial complementarities of miRNAs with their targets likely exert translational effects and reduce *CD40* expression at cell surfaces without required degradation of *CD40* mRNA.

CD40 is a membrane protein of the tumor necrosis factor receptor family and is expressed on antigen-presenting cells, including dendritic cells, B cells, activated macrophages, and follicular dendritic cells.[35] *CD40*/*CD40L* interactions play a pivotal role in activation of dedicated antigen-presenting cells. Interaction with its ligand (*CD40L*, a type II membrane protein of 33 kDa and a member of the tumor necrosis factor gene family) results in potent activation and survival stimuli: *CD40L* enhances antigen presentation of *CD40*-expressing antigen-presenting cells followed by the activation of effector T and NK cells and can initiate immune responses.[35–37]

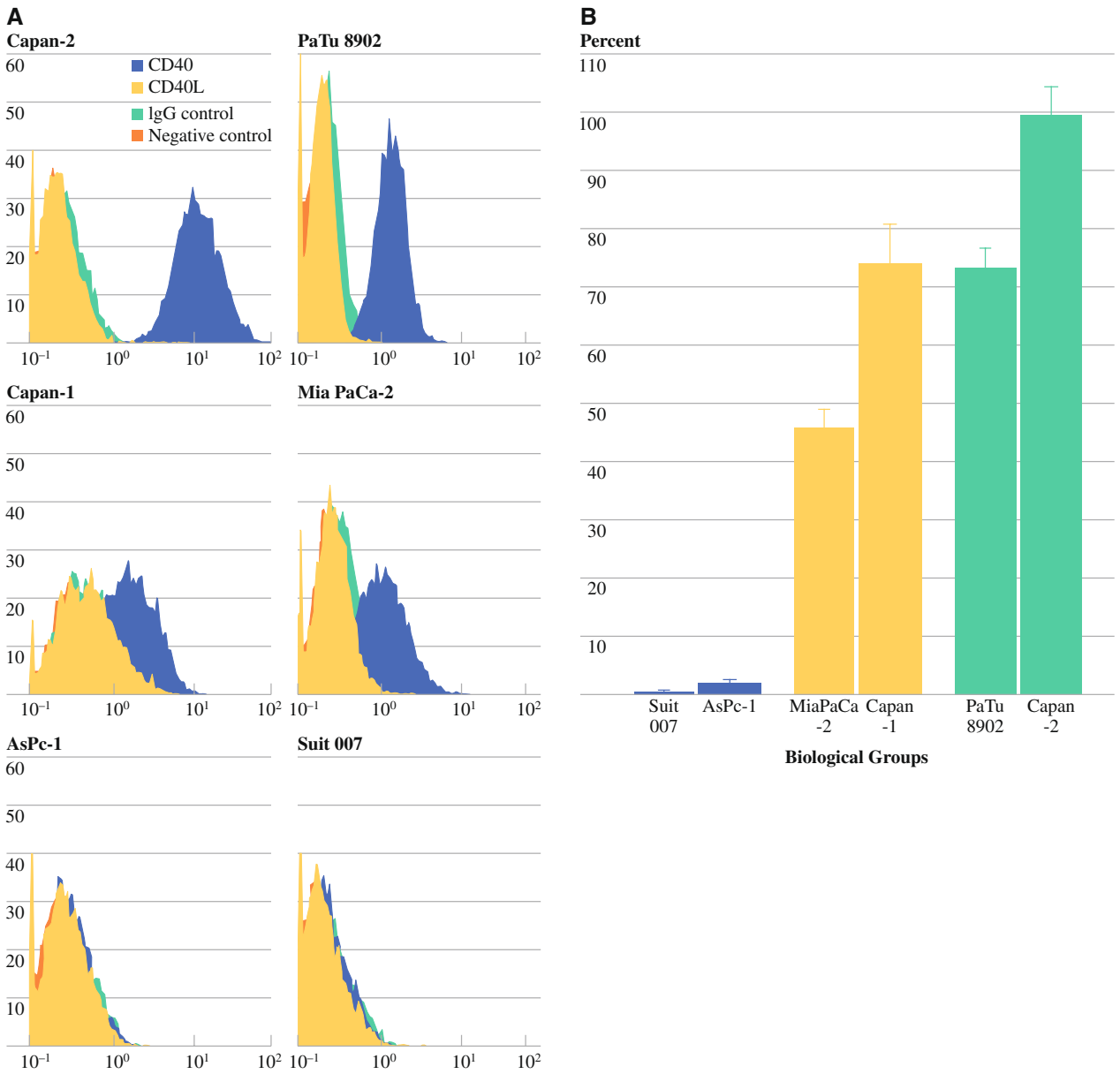


FIG. 7 Flow cytometry analyses of *CD40* cell surface expression on pancreatic ductal adenocarcinoma (PDAC) cells. Exemplary illustration of 2 cell lines for each biological group: *upper line*: group I, absent-low (PaTu 8902, Capan-2); *medium line* group II, medium (MiaPaCa2, Capan-1); *lower line*: group III, high (AsPc-1, Suit 007). **A** Flow cytometry. **B** *CD40* expression on cell surfaces (%), mean ± SD

CD40 is expressed by a variety of human carcinomas, including bladder, breast, and ovarian cancers, as well as on melanoma cells. In contrast to leucocytes, where the role of *CD40*/*CD40L* has been systematically investigated, their function in the biology of solid tumors remains surprisingly obscure, even though *CD40* was initially described in carcinomas.[38] However, recent data suggest that its role at tumor cells may differ from that ascribed to immune cells. We and others have demonstrated that expression of *CD40* (but not *CD40L*) can be identified at several carcinoma cell lines (bladder, breast pancreas, melanoma, erythroleukemia), whereas *CD40L* is absent at solid tumors and hematological malignant cells.[20,36] Most recently, Hamzah et al. demonstrated that triggering of *CD40* on endothelial cells by anti-*CD40* agonist antibodies induced an inflammatory response of the vessel wall, facilitated effector cell accumulation in the tumor parenchyma, and resulted in delayed tumor growth in a transgenic mouse model of islet cell carcinogenesis.[39] Impaired antitumor immune responses were associated with either reduced expression of *CD40L* at T cells or *CD40* at dendritic cells that has been related to tumor-

derived interleukin 10.[40–42] Although the functional changes in antitumor immune responses mediated by altered *CD40* or *CD40L* expression are not yet understood, treatment with activating *CD40* antibodies, mimicking *CD40L* binding, leads to marked reduction in tumor growth in an orthotopic hepatocellular carcinoma model of the rat and in a PDAC mice model via *CD40L* plasmid-mediated activation of $CD8^+$ cells.[43,44]

In conclusion, this study demonstrated an upregulation of *CD40* and downregulation of *CD40L* targeting miRNAs during the process of tumor metastasis and invasion in pancreatic cancer. This seems to result in altered translational processes leading to loss of *CD40* protein expression in the most aggressive PDAC cell lines. This tumor suppressor activity of *CD40* in PDAC may be induced by antitumor immune responses that require further analysis. The *CD40*/*CD40L* complex and specific *CD40*/*CD40L* targeting miRNAs seem to represent promising targets for diagnostic or therapeutic approaches of PDAC.

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