# Early Epigenetic Downregulation of microRNA-192 Expression Promotes Pancreatic Cancer Progression

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## Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by very early metastasis, suggesting the hypothesis that metastasis-associated changes may occur prior to actual tumor formation. In this study, we identified miR-192 as an epigenetically regulated suppressor gene with predictive value in this disease. miR-192 was downregulated by promoter methylation in both PDAC and chronic pancreatitis, the latter of which is a major risk factor for the development of PDAC. Functional studies *in vitro* and *in vivo* in mouse models of PDAC showed that overexpression of miR-192 was sufficient to reduce cell proliferation and invasion. Mechanistic analyses correlated changes in miR-192 promoter methyl-ation and expression with epithelial–mesenchymal transition. Cell proliferation and invasion were linked to altered expression of the miR-192 target gene *SERPINE1* that is encoding the protein plasminogen activator inhibitor-1 (PAI-1), an established regulator of these properties in PDAC cells. Notably, our data suggested that invasive capacity was altered even before neoplastic transformation occurred, as triggered by miR-192 downregulation. Overall, our results highlighted a role for miR-192 in explaining the early metastatic behavior of PDAC and suggested its relevance as a target to develop for early diagnostics and therapy. *Cancer Res; 76(14); 4149–59. ©2016 AACR.*

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive tumors, with a 5-year survival rate of less than 5% (1). Most patients die within a few months after diagnosis. The primary reason for the bleak survival statistics is the early metastatic dissemination of PDAC originating from very small primary tumor lesions (2). Beyond developing novel therapies, the identification of early diagnostic markers with a good predictive value is among the most promising and realistic strategies to reduce mortality in PDAC patients. One way to address this problem is to identify PDAC-associated molecular changes in pancreatic diseases with a known predisposition to the development of pancreatic cancer. Chronic pancreatitis (CP) is an inflammation of the pancreas (3). Molecular alterations that occur in CP and persist in PDAC could be potential early markers for PDAC prognosis (4).

As a part of an effort to investigate early molecular events that occur in PDAC at various molecular levels, we had previously profiled microRNA (miRNA) expression in samples of healthy pancreas tissues (NP), CP, and PDAC patients (5). miRNAs are noncoding RNA molecules of 19 to 25 nucleotides in length and involved in regulating a variety of cellular processes, including development, differentiation, apoptosis, and proliferation (6). Some miRNAs are known to contribute to the multistep process of tumorigenesis either as oncogenes or as tumor suppressor genes (7). Emerging studies support that dysregulation of miRNA expression in cancer is also mediated by DNA methylation (8, 9). Interestingly, aberrant changes in DNA methylation and miRNA expression were shown to be early events in PDAC (10), as well as in other cancers (11) and have been linked to the initiation and progression of cancer. For example, miR-148a was reported to be downregulated in multiple cancers like colon, melanoma, lung cancer (12), and PDAC (13).

Based on our earlier expression data, we focused on miRNA molecules whose expression was regulated by DNA methylation of promoter regions. With this approach, we identified the microRNA miR-192 to be epigenetically downregulated in CP

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**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and PDAC. Using patient tissue material, *in vitro* cellular studies and tumor mouse models, we explored the possible underlying mechanisms of miR-192 function. We could show that it plays an important role in pancreatic cancer by regulating the expression of plasminogen activator inhibitor-1 (PAI-1) and by modulating the epithelial–mesenchymal transition (EMT). PAI-1 is a protein encoded by the gene *SERPINE1* and belongs to the class of serine protease inhibitors. It is the principal component of the plasminogen system, which is upregulated in inflammation and cancer (14). EMT facilitates cell migration and is central for tumor cell dissemination (15). We show that all this does not only happen in tumor cells but already in CP and thus prior to cell transformation and tumor development. While the process does not contribute to cell transformation, it nevertheless defines a process that becomes an integral part of tumor behavior and contributes to its high proliferation and invasion capacity.

## Materials and Methods

### miRNA and mRNA profiles

The analysis of miRNA expression in 128 pancreatic tissues was published earlier (5) and is publicly accessible at http://www.ncbi.nlm.nih.gov/projects/geo/, access code GSE24279. mRNA profiles from 298 pancreas tissues were produced on Sentrix Human-6v3 Whole Genome Expression BeadChips (Illumina). The 298 mRNA profiles are a subset of a larger study on 457 pancreatic samples (accessible at ArrayExpress: accession number: E-MTAB-1791; name: DKFZ_pancreatic cancer). The 457 mRNA profiles represent a variety of pancreatic sample types. The 298 used here were made from NP, CP, and PDAC samples, which are the ones relevant for this study. Further details are provided in the Supplementary Methods. The type of analysis performed on each of the various samples used in this study as well as relevant clinical annotations are listed in Supplementary Table S1. In total, 356 samples were used in the analyses. For 128 and 298 samples, profiles of their miRNA or mRNA, respectively, are available. For 70 samples, both miRNA and mRNA profiles exist. The DNA investigated for the methylation status was isolated from 30 samples, for which both miRNA and mRNA data are available.

### Methylation analysis of deregulated miRNAs in PDAC

The 10-kb genomic region upstream of the transcription start sites (TSS) of 13 miRNAs deregulated in PDAC was examined *in silico* for the presence of CpG islands (CpGI). Simultaneously, primers were designed for the region's amplification from a bisulfite treated DNA template using the methprimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi; Supplementary Table S2). DNA was isolated from patient samples (Supplementary Methods) that were collected during surgery. In all cases, written informed consent was obtained from the patients. Ethical approval was obtained from local ethics committees at the universities of Heidelberg and Liverpool. From the 70 samples, for which profiles of both miRNA and mRNA exist, 10 different patients of PDAC, CP, and NP, respectively, were selected. Equal amounts of DNA were pooled and subjected to bisulfite treatment. The CpGIs closest to the TSSs were selected and amplified from the bisulfite-treated DNA pools separately. Their methylation status was assessed by bisulfite sequencing using 454 GS Junior Next

Generation Sequencing (Roche). The methylation status of the obtained sequences was analyzed using BiQ Analyzer HT (http://biq-analyzer-ht.bioinf.mpi-inf.mpg.de/).

### Quantitative methylation by pyrosequencing

The differentially methylated part of CpGI-192 (Fig. 1A) was PCR amplified at an annealing temperature of 52°C. The primer sequences are listed in Supplementary Table S2. The PCR product was analyzed using the PyroMark Gold Q24 reagents (970802; Qiagen) in a PyroMark Q24 Pyrosequencing System according to the manufacturer's protocol. The amplification bias toward unmethylated alleles was corrected using the calibration data derived from a set of EpiTect control DNA samples of 0%, 20%, 40%, 50%, 60%, 80%, 100% methylation (Qiagen, 59695), and cubic polynomial regression as previously described (16).

### Analysis of the promoter activity of the CpGI located upstream of the miR-192 gene

To investigate the promoter activity of the CpGI located upstream of the miR-192 gene (CpGI-192), it was amplified by PCR (primers listed in Supplementary Table S2). The amplified sequence (Supplementary Fig. S1) was digested with *Bgl*II (R0144S; NEB) and *Nco*I (NEB, R0193S) and cloned upstream of the firefly luciferase gene in vector PCpGL (generously donated by Prof. Christoph Plass, DKFZ, Heidelberg). In order to interrogate the influence of methylation on promoter activity, the promoter construct was subjected to *in vitro* methylation using enzyme *M.SssI* (NEB, M0226S). *In vitro* methylation was verified by digesting the purified methylated and unmethylated constructs by restriction enzyme *Aci*I (NEB, R0551S; Supplementary Fig. S2). The empty PCpGL vector, its variant with the CpGI-192 insert (unmethylated) or its *in vitro*–methylated version, respectively, were individually cotransfected with the *Renilla* luciferase vector (for normalization) into CFPAC-1 cells. Luciferase activity was measured 24 hours after transfection using the Dual-Luciferase Reporter Assay System (E1960; Promega).

### Screening for appropriate cell lines for the *in vitro* studies

To establish a cell line system that facilitates both overexpression and knockdown studies, the miR-192 expression was assessed in five different pancreatic cancer cell lines: PANC-1, MIAPaCa-2, BxPC-3, CAPAN-1, and CFPAC-1. All of them had been obtained from biobanks or commercial providers prior to this study. Before being used in our experiments, the DKFZ in-house service or the service of Eurofins Medigenomix (Ebersberg, Germany) were used for cell line authentication by means of analyzing genetic characteristics. All were tested *mycoplasma* negative with the Venor GeM Mycoplasma Detection kit (Minerva Biolabs). Total RNA was isolated using the miRNeasy kit (Qiagen, 217004). The expression level of miR-192 was analyzed by qRT-PCR with the Taqman assay (000491; Life Technologies), using *RNU44* as a housekeeping gene (Life Technologies, 001094).

### Overexpression and inhibition of miR-192

miR-192 overexpression and inhibition were achieved by transient transfection of a miR-192 mimic (MC10456; Ambion) or a miR-192 inhibitor (Ambion, MH10456), respectively, at a final concentration of 30 nmol/L. The extent of overexpression

**Figure 1.**
Downregulation of miR-192 in CP and PDAC patient samples compared with NP is epigenetically regulated. A, pooled bisulfite sequencing of the CpGI located 3.5 kb upstream of miR-192 (CpGI-192) revealed an increase in its methylation status in PDAC and CP samples compared with the corresponding region in NP (boxed region in the heatmap). B, the hypermethylation inversely correlated with expression of miR-192 in CP and PDAC, suggesting a role of DNA methylation in its regulation. C–E, methylation levels in individual patients of CpGs 18, 19, and 20 that had been identified in the pool analysis. F, inverse correlation of cumulative methylation at CpGs 18, 19, and 20 with the expression of miR-192 in individual patients. G, treating pancreatic cells (MIAPaCa-2) with the DNA demethylating drug 5-azacytidine resulted in an increase of miR-192 expression, confirming the role of methylation in its regulation. Data represent the mean ± SD of triplicate qRT-PCR values from a representative experiment performed at least three times. H, the relevance of the CpGI-192 and the influence of methylation on modulating miR-192 expression were demonstrated by assessing the promoter activity via luciferase assay. Data are presented as average ± SEM of three experiments.
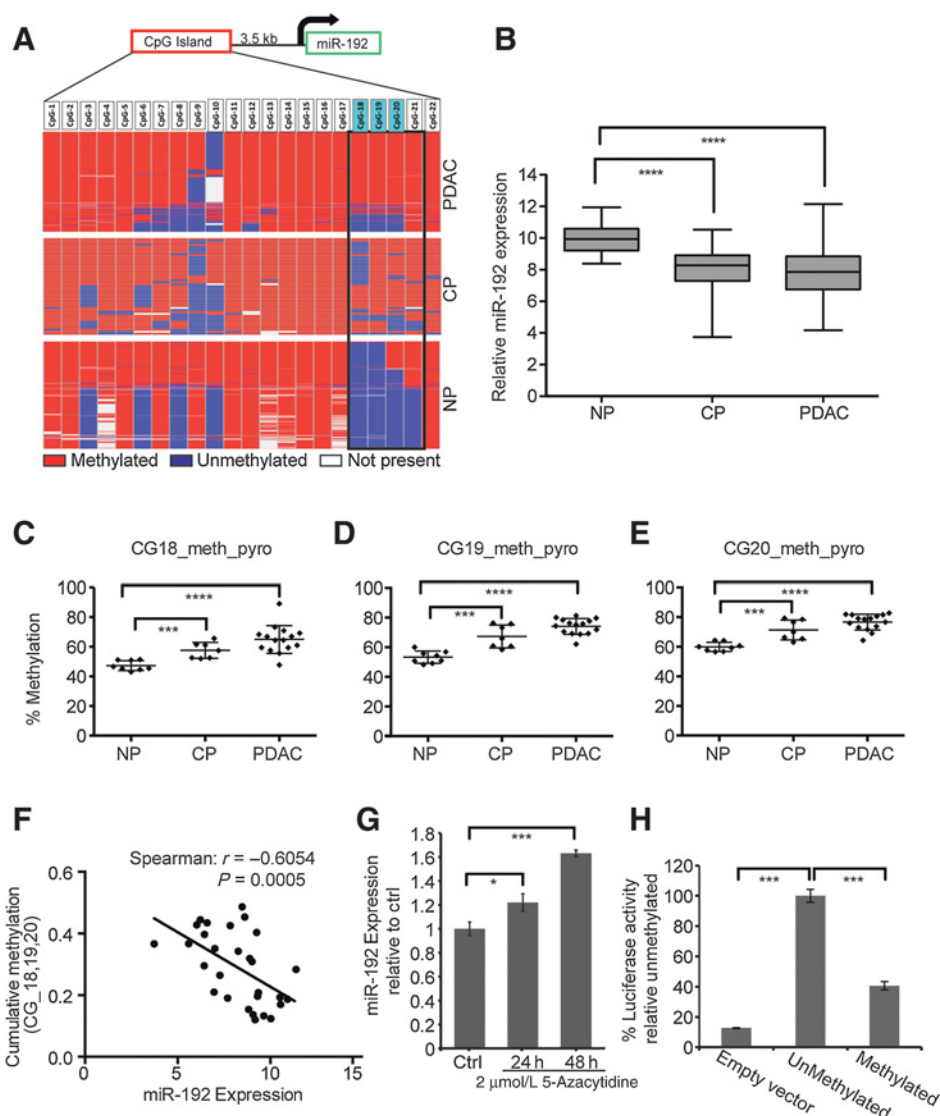
and inhibition was assessed relative to negative controls, mirVana miRNA Mimic Negative Control #1 (Ambion, 4464058) and mirVana miRNA Inhibitor Negative Control #1 (Ambion, 4464076).

## Establishing stable cell lines for miR-192 overexpression and knockdown

Lentiviral constructs for overexpressing miR-192 (PMIRH192PA-1-GVO-SBI; System Biosciences) and inhibiting its activity (System Biosciences, MZIP192-PA-1-GVO-SBI) were purchased. Lentiviruses for miR-192 overexpression and knockdown as well as controls containing scrambled sequences were produced in HEK293T cells using the lentiviral expression system (System Biosciences, LV500) following the manufacturer's protocol. MIAPaCa-2 and CFPAC-1 cells were transduced for stable overexpression and knockdown, respectively. Because the vectors also express green fluorescent protein (GFP), a pure cell population was obtained by FACS-sorting for GFP-positive cells 4 weeks after transduction. These stable cell lines were used for all *in vivo* experiments.

## 3′-UTR luciferase assay to establish *SERPINE1* mRNA as a direct target of miR-192

A 450-bp segment of the 3′UTR of the *SERPINE1* gene, which harbors the miR-192 binding site, was amplified (for primers, see Supplementary Table S2) and cloned into the psiCHECK-2 vector (Promega, C8021). A mutant construct of the miR-192 binding site was generated using the QuickChange II XL Site-Directed Mutagenesis Kit (200521; Agilent Technologies). Cotransfections of the wild-type *SERPINE1* 3′UTR or the mutated *SERPINE1* 3′UTR with the miR-192 mimic into the cells was accomplished with the Lipofectamine 2000 Transfection Reagent (Life Technologies, 11668019). Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, E1960).

## Tumor xenografts

All animal procedures were approved by the Regierungspräsidium Karlsruhe (protocol number: G-101/13). Tumor xenograft experiments were performed with nude CD1 mice purchased from the Jackson Laboratory. Per mouse, $1 \times 10^6$

CFPAC-1 or MIAPaCa-2 cells in 100 µL PBS were mixed with 100 µL of Matrigel (BD Biosciences) and subcutaneously injected into the left flank. Tumor sizes (length, width, depth) were measured using calipers and tumor volume was calculated $(0.52 \times l \times b \times h)$. Mice were sacrificed 27 days (CFPAC-1) or 38 days (MIAPaCa-2) after tumor inoculation. The final tumor volumes were 80.5 and 34.2 mm$^3$ for MIAPaCa-2 control and miR-192 overexpression, respectively. In case of CFPAC-1, control and knockdown tumor volumes were 96.5 and 330.7 mm$^3$, respectively. Primary tumors were resected, zinc-fixed, and paraffin-embedded for further analysis. H&E staining (Supplementary Methods) was performed and analyzed using an Olympus IX71 microscope. Both the axillary lymph nodes were harvested combined together, and RNA was extracted using the miRNeasy mini kit (Qiagen, 217004). Axillary lymph node metastasis was investigated by quantifying by qRT-PCR the human *B2M* endogenous control gene (Life Technologies, Hs00984230_m1), specific for the injected human tumor cell lines, relative to the mouse *β-actin* housekeeping gene (Life Technologies, Mm00607939_s1), specific for murine cells. The mouse *β-actin* Taqman assay could not detect any mRNA in the human cell line RNA (data not shown).

### Other experimental assays

Further routine assays performed with commercially available kits are described in the supplementary information: Western blot analysis; qRT-PCR; assays of proliferation, viability, apoptosis and invasion.

### Statistical analysis

For the patient data and *in vivo* tumor volume data, statistical significance was calculated using the Mann–Whitney *U* test. For patient survival analysis, log-rank test was applied. Correlation analysis between patient methylation and microRNA expression was tested for significance using the Spearman correlation coefficient. *In vitro* experiments were tested for significance using the Student *t* test. In all cases, *P* values were calculated; $P < 0.05$ was considered statistically significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. The significance in case of the Allred scoring of the immunohistochemical staining was calculated using the Mann–Whitney test. The statistical tests were performed using the Graph pad prism software (version 6) or Microsoft Excel.

## Results

### miR-192 is epigenetically downregulated in PDAC and CP

In an earlier analysis on 128 pancreatic tissue samples (5), we identified a distinct set of miRNAs with differential expression in PDAC or CP compared with NP (Supplementary Figs. S3 and S4; a three-way comparison is presented in Supplementary Fig. S5). A set of 13 miRNAs exhibited variations of a similar nature in both CP and PDAC and had CpGIs within 10 kb upstream their TSSs (Supplementary Table S3). The DNA methylation patterns of the CpGIs closest to the TSSs of these miRNA genes were examined by bisulfite sequencing. The CpGI located 3.5 kb upstream of the miR-192 gene (CpGI-192) exhibited an increase in methylation in the DNA of PDAC and CP patients (Fig. 1A). The methylation changed at the same position and to a very similar degree for both disease states, as opposed to results observed in other promoters (Supplementary Table S3).

The promoter of miR-181c, for example, exhibited a gradual decrease in methylation from normal, via CP to PDAC (Supplementary Fig. S6).

The hypermethylated status of CpGI-192 inversely correlated with the expression of miR-192 in PDAC and CP (Fig. 1B). To assess the correlation of methylation and miR-192 expression in individual patients, we quantified by pyrosequencing the methylation levels of the CpGs identified to be differentially methylated by the pooled bisulfite sequencing (CpGs 18, 19, 20; Fig. 1C–E). Subsequently, we performed a correlation analysis of the cumulative methylation at the three CpGs and miR192 expression, confirming also for individual patient samples a significant inverse correlation (Fig. 1F). Moreover, miR-192 expression increased upon treatment of the pancreatic cancer cell line MIAPaCa-2 with 5-azacytidine, a DNA-demethylating drug (Fig. 1G). The effect was also studied on the pancreatic cancer cell lines BxPC-3 and PANC-1, both originating from primary tumor. BxPC-3 showed the same effect as MIAPaCa-2. While PANC-1 exhibited the same trend, the changes were not as pronounced as for the others (Supplementary Fig. S7). Furthermore, CpGI-192 harbors the nanomer sequence d(GGGAGGAGG), which is the most common motif found in putative miRNA promoters in *Homo sapiens*, suggesting that it could be a putative promoter of miR-192. Also, the presence of two p53 binding sites located about 100 bp downstream of CpGI-192 (Supplementary Fig. S1) strengthened this view, because miR-192 expression is regulated by p53 (17). We therefore examined the promoter activity of CpGI-192 using a luciferase reporter assay (18). It showed that CpGI-192 acts as a promoter and its activity was reduced upon methylation (Fig. 1H).

### Effect of miR-192 expression on PDAC patient survival

CP is a chronic inflammation of the pancreas that increases the risk of developing PDAC by about 15-fold (19). Genetic or epigenetic changes in CP that persist in PDAC could be early events associated with PDAC behavior. We therefore probed the suitability of the miR-192 as a diagnostic and prognostic marker for CP and PDAC. Receiver operating characteristic (ROC) curve analysis showed that miR-192 expression distinguished between NP and CP patients with 90% accuracy (AUC = 0.90; Fig. 2A). Discrimination between NP and PDAC was similarly accurate with 90% (Fig. 2B). However, with a value of 54%, miR-192 expression could not distinguish between PDAC and CP (Supplementary Fig. S8). Also, there was no correlation of miR-192 expression levels and tumor grades (Supplementary Fig. S9). Importantly, however, survival was significantly extended in PDAC patients with high miR-192 expression (above median ± SEM expression in our cohort of samples) compared with patients with low miR-192 expression (below median ± SEM; Fig. 2C), establishing low miR-192 as a robust prognostic indicator of tumor progression.

### miR-192 is involved in the EMT

Because low miR-192 correlated with shorter survival times, which is often due to metastasis, we examined the role of miR-192 in PDAC metastasis focusing on EMT, a predominant phenotype of tumor progression and invasiveness (20, 21). To this end, we performed qRT-PCR analysis of miR-192 in five different pancreatic cancer cell lines of two different subtypes: cell lines that originated from primary pancreatic
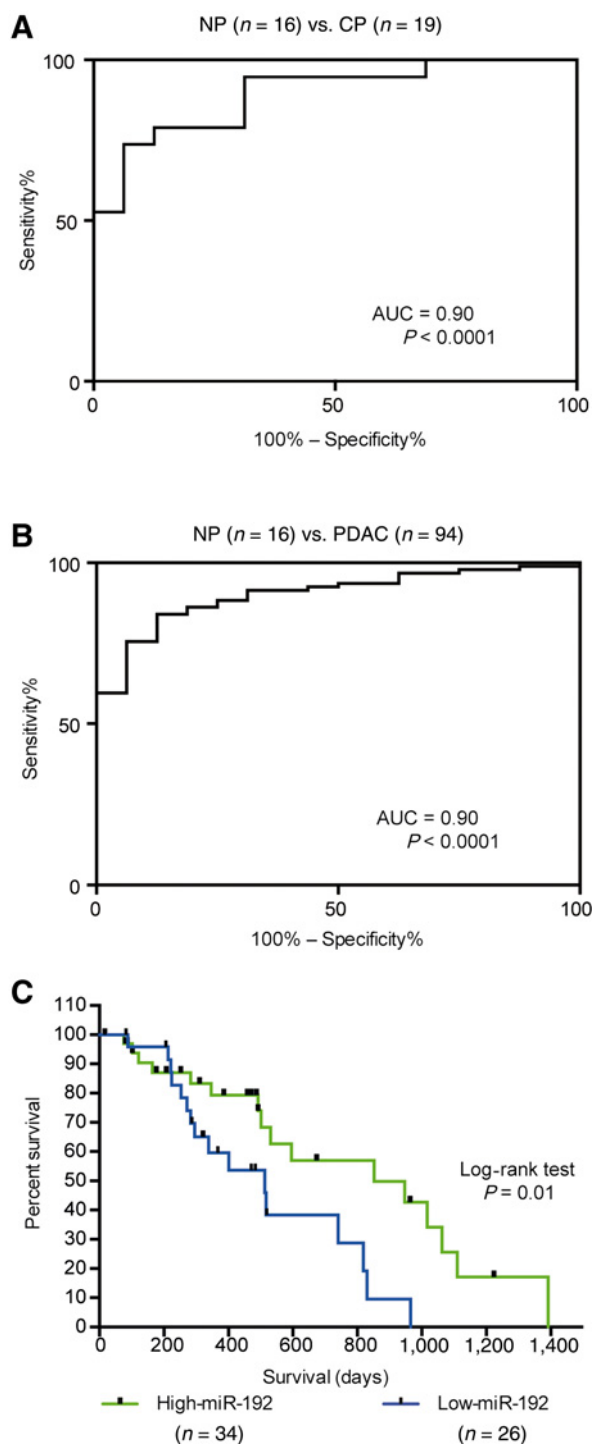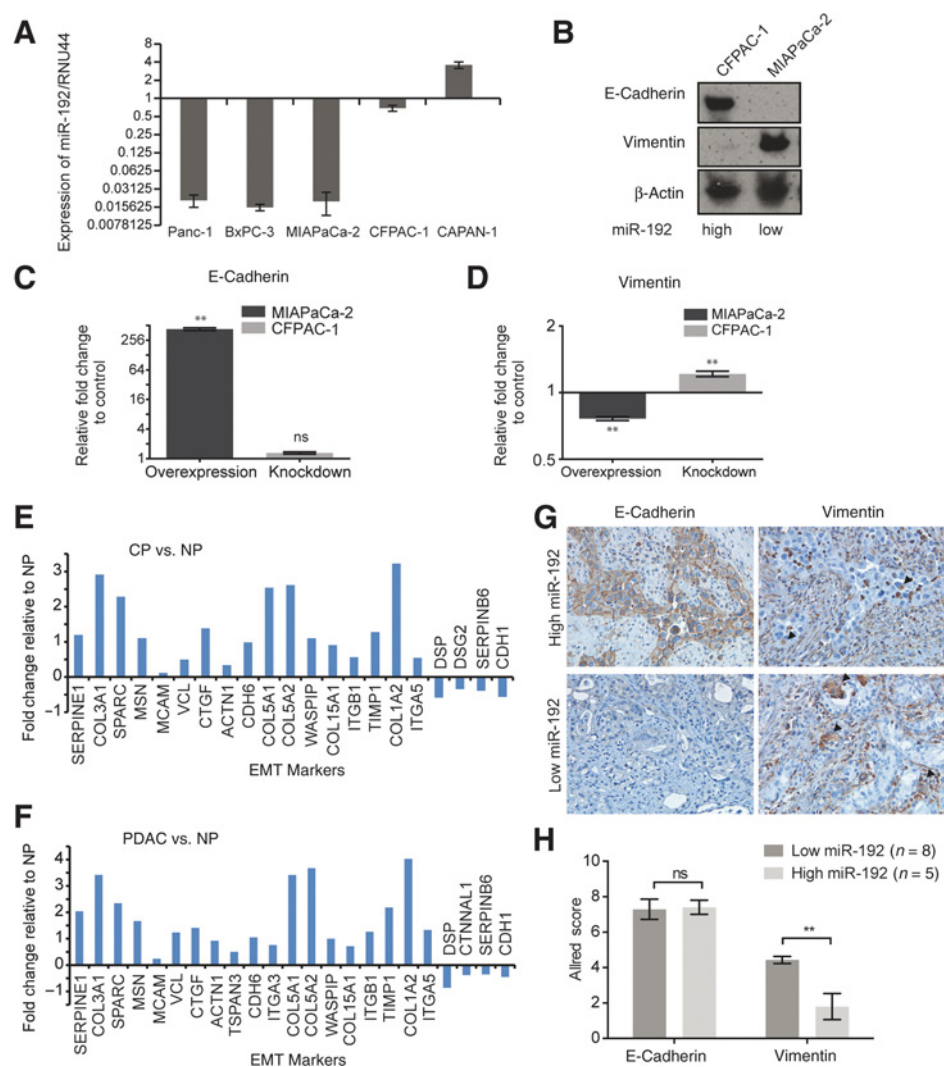
**Figure 2.**
Potential of miR-192 expression to serve as a biomarker of disease. Analysis by ROC curves demonstrated its utility to differentiate PDAC and CP from NP. A, for NP versus CP, an accuracy of 90% was achieved. B, discrimination between NP and PDAC was 90% accurate. C, in a Kaplan–Meier plot, the variation in life expectancy is shown between PDAC patients with high (above median ± SEM in our patient cohort) and low miR-192 expression (below median ± SEM). Patient survival was significantly increased with high miR-192 level. A P value for the Kaplan–Meier curve was obtained with a log-rank test.

tumors (PANC-1, MIAPaCa-2, and BxPC-3) and cells from liver metastases (CAPAN-1 and CFPAC-1; Fig. 3A). Remarkably, miR-192 expression differed strongly between the two cell line groups, while *KRAS* and *p53* mutations, which are highly associated with PDAC, could not differentiate between them (Table 1). Based on these observations, we defined two cell lines—MIAPaCa-2 (primary tumor and low miR-192 expression) and CFPAC-1 (metastatic tumor and high miR-192 expression)—as a suitable system to perform overexpression and knockdown studies, respectively.

We identified a strong inverse correlation between expression of miR-192 and that of EMT markers, such as E-CAD-HERIN and VIMENTIN (Fig. 3B). Overexpression of miR-192 in MIAPaCa-2 and knockdown in CFPAC-1 could reverse the expression of *E-CADHERIN* and *VIMENTIN* (Fig. 3C and D), suggesting that low miR-192 expression is needed for EMT. In addition, a panel of clearly EMT-associated genes that was reported before (22) was evaluated in our patient data. A strong EMT phenotype was observed in CP (Fig. 3E) and PDAC (Fig. 3F) compared with NP. For confirmation at protein level, tumor sections from high and low miR-192 expressing PDAC patients from the same patient group used for miRNA and mRNA profiling were costained with mutated p53 and either E-CADHERIN or VIMENTIN. This immuno-staining analysis enabled us to discriminate tumor cells from stromal cells and to examine the expression of the EMT markers in the epithelial compartment (e.g., Fig. 3G). For quantification, we scored the levels of E-CADHERIN and VIMENTIN in low ($n = 8$) and high ($n = 5$) miR-192 expressing patient samples using the Allred scoring system (Fig. 3H; ref. 23). As expected, VIMENTIN exhibited significant differences ($P = 0.0043$) showing an inverse correlation of miR-192 and VIMENTIN levels. The Allred scores were not significant for E-CADHERIN expression. Intriguingly, the staining documented an intratumor inconsistency; not all tumor cells within the lesion were homogeneous and strictly E-CADHERIN[+]/VIMENTIN[−], and vice versa. E-CADHERIN staining might not be sensitive enough to decipher changes in such a heterogeneous and complex system. Altogether, these findings indicate that low miR-192 expression, as seen in CP and PDAC, may contribute to either initiating or sustaining an EMT phenotype and thus promoting metastasis, which causes shorter patient survival times.

### The mRNA of *SERPINE1* is a target of miR-192

miRNAs execute their cellular function by negatively regulating mRNA targets. Therefore, we examined patient data for inverse correlations of mRNA and related miRNA expression. Target prediction was performed on the basis of an integrated database, which included miRecords, Tarbase, and TargetScan Human (www.ingenuity.com/products/ipa/microrna-target-filter). By this process, we identified a list of six putative miR-192 targets (Supplementary Table S4). For validation, MIAPaCa-2 and CFPAC-1 cells were transiently transfected with miR-192 mimic or miR-192 inhibitor, respectively. The efficacy of miR-192 overexpression or knockdown was subsequently assayed using a Taqman assay (Fig. 4A). Protein lysates were analyzed by Western blot. Among the shortlisted targets, PAI-1, the gene product of *SERPINE1*, exhibited a strong decrease or increase at the protein level upon overexpression or knockdown of miR-192, respectively (Fig. 4B), which was not observed for the

Figure 3.
The role of miR-192 in EMT. A, miR-192 profiling in five pancreatic cancer cell lines was performed. Data represent mean ± SD of triplicate qRT-PCR values from a representative experiment performed at least three times. B, low miR-192 expression positively correlated with EMT (low E-CADHERIN and high VIMENTIN levels) and vice versa in native cell lines as demonstrated by Western blot. C and D, overexpression of miR-192 in MIAPaCa-2 and knockdown in CFPAC-1 could reverse the expression of *E-CADHERIN* and *VIMENTIN*. Data represent mean ± SD of nine qRT-PCR values. In CP (E) and PDAC (F) patients, a panel of 22 EMT markers exhibited an expression pattern typical for an EMT phenotype, compared with NP samples. G, immunohistochemistry was performed on tumor sections from high and low miR-192–expressing PDAC patients costained with mutated p53 (blue) and E-CADHERIN or VIMENTIN (in brown). Arrowheads, regions with varied expression. H, stained tumor sections were scored for E-CADHERIN and VIMENTIN expression. While the increase of E-CADHERIN was not significant, there was clear downregulation of VIMENTIN upon high miR-192 levels.

other predicted targets (data not shown). The presence of a binding site for miR-192 in the 3'-untranslated region (3'-UTR) of *SERPINE1* suggested that it could regulate *SERPINE1* expression directly. Luciferase reporter vectors containing the 3'UTR of *SERPINE1* with the miR-192 target site in either its wild-type (Fig. 4C) or a mutated form (Fig. 4D) were coexpressed with miR-192 and the luciferase activity was measured (Fig. 4E). Minor yet significant changes were observed in the luciferase assay with the wild-type form but not with the mutant. This provided evidence for a direct role of miR-192 in regulating *SERPINE1* expression.

**Table 1.** Correlation of miR-192 expression and cell line origin

| Cell line | Tumor type | KRAS mutation | p53 mutation | miR-192 expression |
|-----------|-----------|---------------|--------------|--------------------|
| PANC-1 | Primary tumor | Yes | Yes | Low |
| BxPC-3 | Primary tumor | No | Yes | Low |
| MIAPaCa-2 | Primary tumor | Yes | Yes | Low |
| CFPAC-1 | Liver metastasis | Yes | Yes | High |
| Capan-1 | Liver metastasis | Yes | Yes | High |

NOTE: Differences in miR-192 expression correlate with cell line origin, but not with *KRAS* or *p53* mutational status.

### Overexpression of miR-192 reduces pancreatic tumor cell proliferation and viability, inducing apoptosis

Given the decreased expression of miR-192 in PDAC, we hypothesized that it exhibits tumor suppressor functions. To validate this hypothesis, miR-192 was transiently overexpressed in MIAPaCa-2. This resulted in reduced tumor cell proliferation and viability, but increased apoptosis (Fig. 5A–C). These findings were cross-validated by inhibiting miR-192 expression in CFPAC-1 tumor cells, which resulted in an increase in proliferation, viability and reduced apoptosis (Fig. 5D–F). Based on these cellular findings, we performed gain-of-function and loss-of-function tumor experiments *in vivo* using mouse tumor xenograft models. MIAPaCa-2 cells stably overexpressing miR-192 or CFPAC-1 cells with a stable knockdown of miR-192 were subcutaneously implanted into CD1 nude mice. Overexpression of miR-192 strongly inhibited tumor growth (Fig. 5G), whereas miR-192 knockdown had an opposite effect, leading to enhanced tumor growth (Fig. 5H) compared with respective controls of the two xenograft models. These data validated our hypothesis that miR-192 acts as a functional suppressor of tumor growth in PDAC. H&E staining

**Figure 4.**
miR-192 target identification. A, to validate the target genes, miR-192 was overexpressed and inhibited in MIAPaCa-2 and CFPAC-1 cells, respectively. The extent of miR-192 overexpression or knockdown was measured by qRT-PCR. Data represent mean ± SD of triplicate qRT-PCR values from a representative experiment performed at least three times. B, Western blot analysis demonstrated an increase or decrease of PAI-1 protein upon miR-192 knockdown or overexpression, respectively. C, sequence alignment showed that *SERPINE1* (region at base pairs 183 to 189 in the 3'UTR) harbors a binding site for miR-192. D, to demonstrate the specificity of the miR-192 binding site, two point mutations (G185A and C188A) were made in the putative binding region. E, luciferase activity was significantly reduced upon miR-192 overexpression in case of *SERPINE1* wild-type 3'UTR, but was not affected by mutant 3'UTR, indicating that miR-192 directly targets *SERPINE1*. The data are the average ± SEM of triplicate experiments.
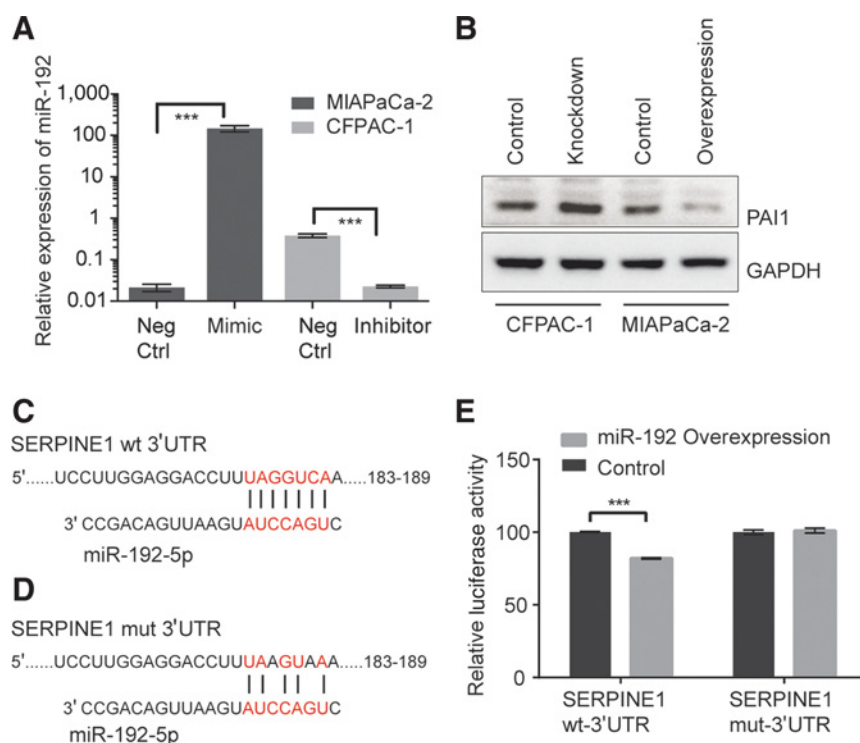
(Fig. 5I and J) did not show any substantial histological differences.

## Overexpression of miR-192 negatively regulates tumor cell invasion

Based on the observed EMT and *SERPINE1* regulation by miR-192, we studied the role of miR-192 in tumor cell invasion and metastasis. Invasion studies using the Matrigel assays revealed that miR-192 overexpression in MIAPaCa-2 cells significantly decreased tumor cell invasion (Fig. 6A). Conversely, knockdown in CFPAC-1 cells increased cell invasion (Fig. 6B). In the *in vivo* settings, a stable overexpression of miR-192 did not significantly affect axillary lymph node metastasis within 6 weeks of primary tumor implantation (Fig. 6C). However, a stable knockdown of miR-192 in CFPAC-1 cells exhibited a significant increase in axillary lymph node metastasis (Fig. 6D).

## Discussion

Pancreatic cancer exhibits a very early onset of metastasis, extraordinary tumor aggressiveness, and a high degree of resistance to therapy modalities that are currently applied upon detection of the tumor. In combination, this results in a particularly high mortality rate that is close to incidence. Despite intensive research on the disease, relatively little gain in treatment options and survival time has been achieved to date. In order to overcome this bleak prognosis, it is crucial to identify relevant molecular events that occur very early during tumor development, or possibly before, and gain a mechanistic insight into the aspects of early PDAC pathophysiology. In our analysis, we focused on miRNAs whose promoter sequences showed variations in DNA methylation, which is an alteration occurring early during carci-

nogenesis. In contrast to a typical search for specific PDAC markers, we concentrated on methylation and expression variations that occur in CP and are sustained in PDAC, because changes that happen already during inflammation may be crucial for the eventual tumor pathology, once other factors initiate tumor development.

miR-192 expression was found to be downregulated significantly enough to serve as an accurate diagnostic marker of PDAC and CP, in contrast to a recent report (24). Its abnormal expression in PDAC and CP was mediated by DNA methylation at the same genomic locus within the promoter region, which showed a significant inverse correlation with the miR-192 expression in individual patients of the cohort. This suggests that the low miR-192 level in PDAC might be inherited from earlier pancreatitis, given the stability and heritable nature of DNA methylation patterns. In order to inspect if miR-192 downregulation was functionally involved in cancer pathophysiology or a mere indicator of pancreatic abnormality, functional experiments were carried out both *in vitro* and *in vivo*, indicating a tumor suppressor role of miR-192 in PDAC. This finding is in agreement with earlier reports that have suggested a tumor suppressor function of miR-192 in other cancers, such as lung (25), breast (26), and epithelial ovarian cancer (27). Additionally, our observation that patients with high miR-192 levels in the tumor have significantly longer survival times indicates that miR-192 expression contributes to the invasive power of PDAC, as most PDAC-associated deaths are due to metastasis.

To clarify the role of miR-192 in metastasis, we investigated the correlation between its expression and EMT, an important cellular transformation that contributes to the invasive abilities of tumor cells and was shown to have an important role in PDAC metastasis (28). Low miR-192 expression was associated with low
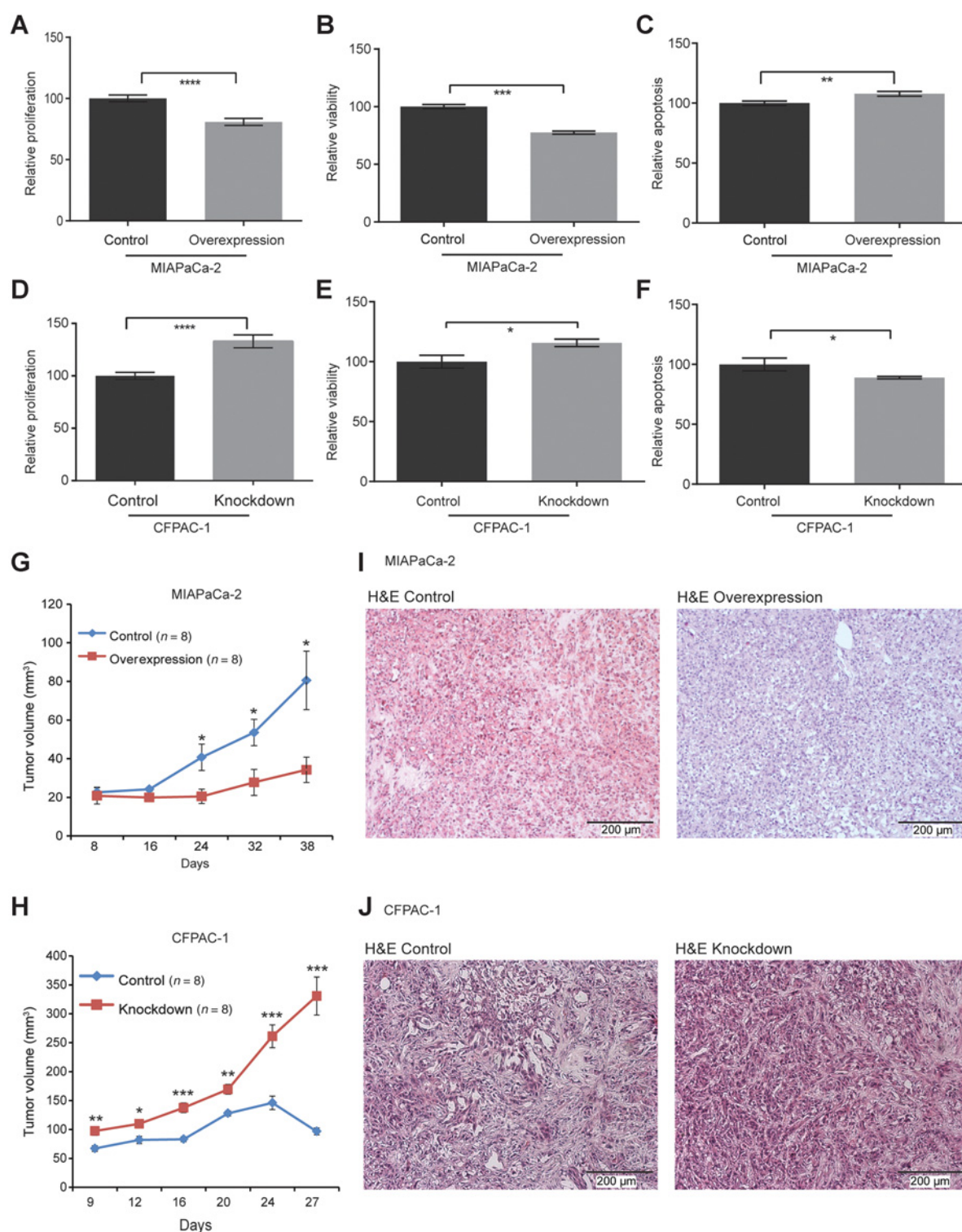
**Figure 5.**
Effect of miR-192 expression on pancreatic tumor cell proliferation, viability, and apoptosis, *in vitro* and *in vivo* analyses. A–C, miR-192 overexpression in MIAPaCa-2 reduced tumor cell proliferation, determined by the SRB assay, and reduced cell viability, as quantified by ATP measurement, but increased apoptosis. D–F, the opposite effects were observed when miR-192 was inhibited in CFPAC-1 cells; tumor cell proliferation and viability were increased while apoptosis was reduced. Data represent average ± SEM of triplicate experiments. G, stably miR-192 overexpressing MIAPaCa-2 tumor xenografts demonstrated a significant decrease in volume compared to control tumors. H, conversely, CFPAC-1 tumors having a stable knockdown of miR-192 demonstrated enhanced tumor volume. Each data point in G and H represents the average ± SEM of tumor volumes of 8 mice per group. I and J, H&E staining of tumor tissues did not reveal any histologic differences compared with respective controls.
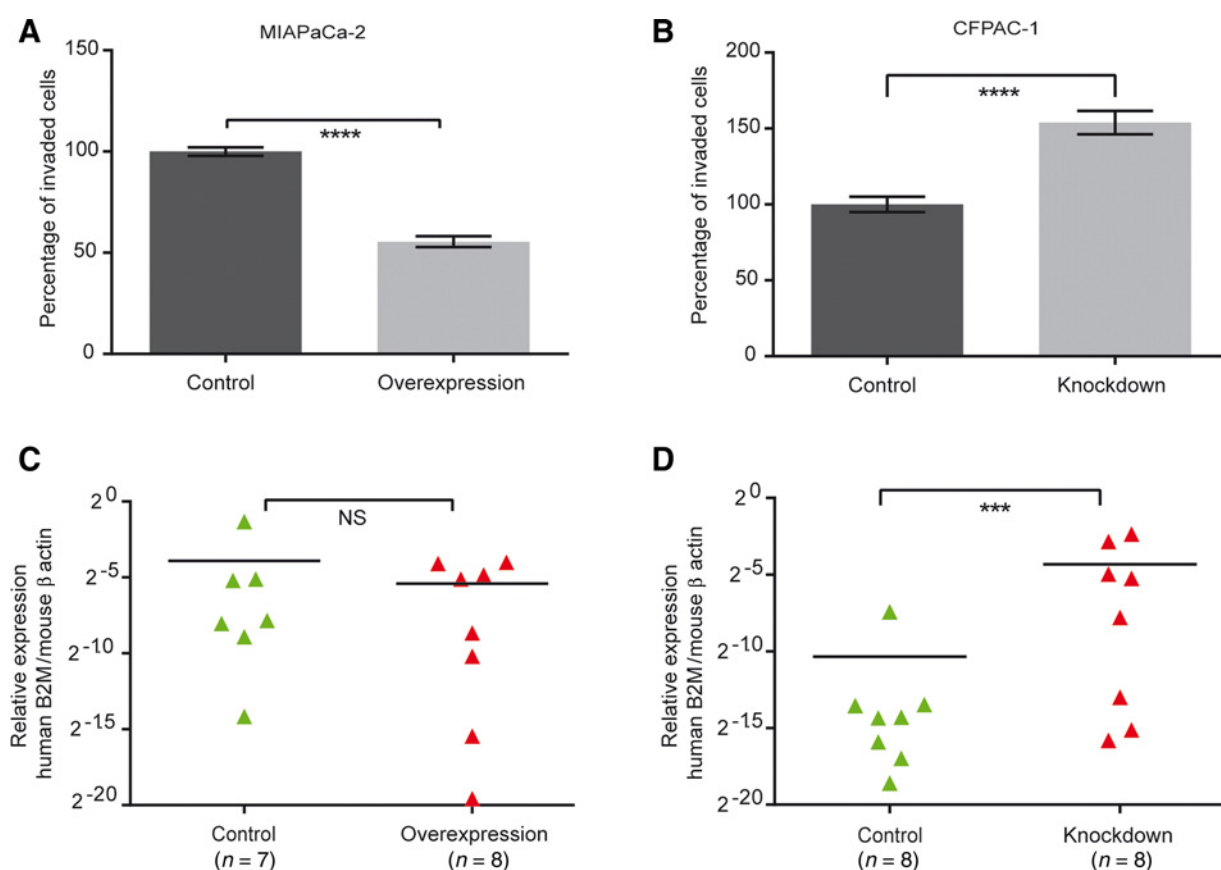
**Figure 6.**
Role of miR-192 in tumor cell invasion. A, miR-192 overexpressing MIAPaCa-2 cells showed a significant decrease in tumor cell invasion compared with control when subjected to a Matrigel invasion assay. B, conversely, miR-192 knockdown in CFPAC-1 cells significantly enhanced tumor cell invasion. Data show the average $\pm$ SEM of triplicate experiments. C, axillary lymph node metastasis in tumor xenograft models was investigated by quantifying the human *B2M* transcript relative to mouse *β-actin* gene using qRT-PCR. Mice bearing MIAPaCa-2 tumors showed no significant variation in lymph node metastasis compared with the respective control mice. D, however, mice having CFPAC-1 tumors exhibited a significant increase in lymph node metastasis.

*E-CADHERIN* and high *VIMENTIN* expression and thus EMT, while cells with high miR-192 levels exhibited only the epithelial marker. This interesting result was further strengthened by the expression pattern of miR-192 in different pancreatic cancer cell lines. Cancer cell lines that originate from primary tumors (PANC-1, MIAPaCa-2, and BxPC-3) expressed negligible levels of miR-192, while the pancreatic cancer cell lines that were isolated from liver metastases (CFPAC-1 and CAPAN-1) showed high levels of miR-192. These cells have undergone mesenchymal–epithelial transition (MET), the reverse process of EMT, that is required for tumor cells to colonize at the metastatic site. This is consistent with a study that reported the inverse correlation between miR-192 expression and the colon cancer cell metastatic potential (29). It was also shown that downregulation of miR-192 increased the expression of ZEB2, which in turn reduces *E-CADHERIN* expression, thus contributing to EMT (30). In our study, both CP and PDAC patients showed an EMT-associated gene expression pattern across a panel of EMT markers. In tumor tissue analysis, changes in expression of EMT markers can be influenced by the stromal compartment. However, our immunohistochemical studies done by costaining tumor cells with mutated p53 and EMT

markers in PDAC samples distinctly demonstrated EMT in tumor cells, which inversely correlated with miR-192 expression. Consistent with these findings, the epithelial cell lines used in this study also showed an inverse correlation between miR-192 expression and EMT. These findings corroborate the role of miR-192 in metastasis.

To obtain a mechanistic insight, mRNA targets of miR-192 were predicted by a cross-correlation analysis of patient mRNA and miRNA profiles. Predicted targets were validated, which led to the identification of *SERPINE1* (PAI-1) as a new functional target of miR-192. PAI-1 belongs to the class of serine protease inhibitors. It is a primary physiologic inhibitor of the tissue-type and the urokinase-type plasminogen activator (tPA and uPA; ref. 31), and is mainly involved in modulating cell migration and invasion (32). The process of metastasis requires proteolytic cleavage of the extracellular matrix and basement membrane, which constitutes stromal remodeling. This is brought about by uPA and PAI-1, which coregulate each other's expression (33). Previous studies have shown that low PAI-1 levels correlate with significantly reduced tumor progression (34), whereas high PAI-1 levels are associated with poor prognosis in many cancers (35, 36). Suppression of the uPAR–uPA system inhibits angiogenesis, *in vivo*

tumor development and invasion in experimental pancreatic cancer (37). In advanced pancreatic cancer, PAI-1 levels predict response to gemcitabine chemotherapy (38) and pharmacological inhibition of uPA in combination with gemcitabine is a promising new treatment option for pancreatic cancer (39). Additionally, overexpression of PAI-1 is a prominent feature of EMT (40). Therefore, upregulation of PAI-1 together with EMT resulting from low miR-192 levels could trigger cell migration and contribute to an invasive phenotype.

Using a lineage-tracing approach in a mouse model of pancreatic cancer, a recent study has shown that EMT and metastatic dissemination precede pancreatic tumor formation (41), suggesting that metastatic potential is attained prior to tumorigenesis. Along a similar line, we suggest that low miR-192 expression mediates changes in CP that provide a favorable environment for metastasis, once other factors have triggered the transformation of the cell into a tumor cell. Molecular variations needed for high proliferation and invasion capacity and therefore metastasis may actually occur before cell transformation. Low miR-192 expression in CP is not inducing transformation into PDAC, however.

In summary, methylation of a particular CpGI in the miR-192 promoter region results in low miRNA expression, which in turn contributes to decreased *E-CADHERIN* and increased PAI-1 levels in PDAC, thereby enhancing the metastatic potential of a cell via two different axes. This facilitates the process of pancreatic tumor cell proliferation and invasion through the plasminogen system, which eventually leads to metastasis and consequently poor patient survival.

## Disclosure of Potential Conflicts of Interest

J.P. Neoptolemos reports receiving a commercial research grant from Taiho Pharma (Japan), KAEL GemVax (Korea), and AstraZeneca; has received speakers bureau honoraria from Amgen; and is a consultant/advisory board member for Boehringer Ingelheim Pharma GmbH & Co. KG, Novartis Pharma AG, KAEL GemVax, and Astellas. No potential conflicts of interest were disclosed by the other authors.

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