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ORIGINAL ARTICLE

miR-219-1-3p is a negative regulator of the mucin MUC4 expression and is a tumor suppressor in pancreatic cancer

F Lahdaoui^{1,2,3}, Y Delpu^{4,5}, A Vincent^{1,2,3}, F Renaud^{1,2,3}, M Messenger^{1,2,3}, B Duchêne^{1,2,3}, E Leteurtre^{1,2,3}, C Mariette^{1,2,3}, J Torrisani^{4,5}, N Jonckheere^{1,2,3} and I Van Seuningen^{1,2,3}

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal cancers in the world with one of the worst outcome. The oncogenic mucin MUC4 has been identified as an actor of pancreatic carcinogenesis as it is involved in many processes regulating pancreatic cancer cell biology. MUC4 is not expressed in healthy pancreas whereas it is expressed very early in pancreatic carcinogenesis. Targeting *MUC4* in these early steps may thus appear as a promising strategy to slow-down pancreatic tumorigenesis. miRNA negative regulation of *MUC4* could be one mechanism to efficiently downregulate *MUC4* gene expression in early pancreatic neoplastic lesions. Using *in silico* studies, we found two putative binding sites for miR-219-1-3p in the 3'-UTR of *MUC4* and showed that miR-219-1-3p expression is downregulated both in PDAC-derived cell lines and human PDAC tissues compared with their normal counterparts. We then showed that miR-219-1-3p negatively regulates MUC4 mucin expression **via** its direct binding to *MUC4* 3'-UTR. MiR-219-1-3p overexpression (transient and stable) in pancreatic cancer cell lines induced a decrease of cell proliferation associated with a decrease of cyclin D1 and a decrease of Akt and Erk pathway activation. MiR-219-1-3p overexpression also decreased cell migration. Furthermore, miR-219-1-3p expression was found to be conversely correlated with Muc4 expression in early pancreatic intraepithelial neoplasia lesions of *Pdx1-Cre;LSL-Kras^{G12D}* mice. Most interestingly, *in vivo* studies showed that miR-219-1-3p injection in xenografted pancreatic tumors in mice decreased both tumor growth and MUC4 mucin expression. Altogether, these results identify miR-219-1-3p as a new negative regulator of MUC4 oncomucin that possesses tumor-suppressor activity in PDAC.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of death by cancer in western countries. With a survival rate of 6 months and a 5-year survival about 5%, pancreatic cancer is characterized by a poor prognosis due to a late diagnosis and the lack of efficient treatment.¹ Pancreatic ductal adenocarcinoma (PDAC) is by far the most frequent pancreatic cancer as it represents approximately 90% of pancreatic tumors. PDAC develops from non-invasive precursor lesions. Histologically three different types of precursor lesions have been described. Among them, pancreatic intraepithelial neoplasia (PanIN) lesions are the most common.²

The membrane-bound mucin MUC4, which is not expressed in healthy pancreas, is neoexpressed in early PanIN lesions. MUC4 is involved in pancreatic carcinogenesis (proliferation, migration, invasion and chemoresistance),^{3–5} its expression correlates with the disease advancement⁶ and it is the membrane partner of oncogenic receptor ErbB2.³ As such, this oncomucin appears as a good target to slow-down pancreatic tumorigenesis.

MUC4 gene is located on the chromosome 3q29, a chromosomal region characterized by a high CpG content. Our previous studies on *MUC4* epigenetic regulation have shown that *MUC4* 5'-UTR is hypermethylated in the human PDAC cell line Panc-1, which does not express MUC4 whereas this region is hypomethylated in the PDAC cell line Capan-1, which expresses a high level of MUC4.⁷ Furthermore, histone deacetylation is also involved in the

repression of *MUC4* in PDAC cells.^{7,8} From these results, a model for *MUC4* epigenetic regulation in pancreatic carcinogenesis was proposed.⁷

Next to epigenetic regulation by DNA methylation and histone modifications, gene regulation by microRNAs (miRNA) recently appeared as a new exciting strategy to control gene expression in cancers, which could possibly translate into clinical applications.^{9,10} miRNAs are small non-coding RNA of 18–25-nt length, which are conserved throughout evolution. They post-transcriptionally regulate gene expression by triggering either cleavage or translation inhibition of their target mRNAs.¹¹ miRNAs are involved in the regulation of cellular development, proliferation, differentiation, apoptosis and carcinogenesis. In many cancers, miRNAs are aberrantly expressed and either mediate the oncogenic process or display a tumor-suppressor effect depending on their mRNA target.¹²

Over the past years, advanced technologies used in miRNA expression studies have shown a general downregulation of miRNAs in tumors including pancreatic cancer.¹³ However, studies aiming at identifying miRNA regulating *MUC4* expression in PDAC remain scarce. In the only study published so far, *MUC4* 3'-UTR was shown to be directly targeted by miR-150 in HPAF, Panc10.05 and Colo357 PDAC cell lines.¹⁴ To our interest, as we were studying *MUC4* regulation by miR-219-1-3p in PDAC cells, Schultz *et al.*¹⁵ showed that miR-219-1-3p was strongly inhibited in pancreatic cancer **versus** normal tissue, confirming the real interest in

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2 studying the impact of this miRNA on pancreatic cancer cell biology.

In this report, we not only identify miR-219-1-3p as a new negative regulator of MUC4 mucin expression in the early steps of pancreatic carcinogenesis, but also show that miR-219-1-3p possesses tumor-suppressive activity. MiR-219-1-3p appears thus as a good target to slow-down pancreatic tumorigenesis.

RESULTS

Identification of two putative miR-219-1-3p binding sites in the *MUC4* 3'-UTR

Using bioinformatic algorithms, we searched miRNAs putative binding sites in human *MUC4* 3'-UTR. Several miRNAs came out and miR-219-1-3p became of particular interest as, at that time, no target gene had been experimentally validated for this miRNA and nothing was known about its biological role in pancreatic carcinogenesis. Two miR-219-1-3p putative binding sites were identified covering the nucleotides 19–40 (site #1) and 356–372 (site #2) of *MUC4* 3'-UTR (Figure 1a). Sequence alignment of human miR-219-1-3p among different species revealed that it is fully conserved across species (Figure 1a).

miR-219-1-3p is downregulated in human PDAC cell lines and tissues

To validate the potential of miR-219-1-3p in pancreatic cancer, its expression was studied both in PDAC cultured cells and human PDAC tissues by quantitative reverse transcriptase (qRT)-PCR. Our results indicate that miR-219-1-3p expression was reduced in all five PDAC cell lines (Capan-1, Capan-2, BxPC-3, Panc-1 and MIA PaCa-2) compared with normal human pancreatic ductal cells (Figure 1b). As expected, MUC4 mucin is expressed in Capan-1, Capan-2 and BxPC-3 cell lines^{8,16} (Figure 1b insert). Absence of MUC4 expression in Panc-1 was already shown as being due to epigenetic repression of *MUC4* gene.⁸ In *MIA PaCa-2*, *MUC4* gene is so epigenetically repressed by DNA methylation (data not shown). miR-219-1-3p expression in 22 paired human PDAC tissues and their corresponding nontumoral adjacent tissues indicated a significant downregulation of miR-219-1-3p in 19 out of 22 PDAC tissues (Wilcoxon signed-rank test, $P=0.0164$; Figure 1c). Interestingly, in the three human PDAC tissues in which miR-219-1-3p was overexpressed (patients #20–22), MUC4 expression was not observed (Figure 1c, insert).

MUC4 is a direct target of miR-219-1-3p

To study MUC4 regulation by miR-219-1-3p, Capan-1, Capan-2 and BxPC-3 cell lines were transiently transfected with miR-219-1-3p mimics or with a scrambled miRNA (Supplementary Figure S1A). Overexpression of miR-219-1-3p led to a marked reduction of MUC4 protein level in the three cell lines when compared with the scramble miRNA-transfected cells (Figure 2a, left panel). Conversely, inhibition of miR-219-1-3p in Capan-2 and BxPC-3 cells, the two cell lines showing the highest level of miR-219-1-3p expression, using anti-miR-219-1-3p oligonucleotides, led to an increase of MUC4 protein expression (Capan-2, 2.05-fold and BxPC-3, 1.64-fold) (Figure 2a, right panel).

To determine whether miR-219-1-3p regulates MUC4 expression by directly interacting with the *MUC4* mRNA, we then used an affinity-based approach in which biotinylated miR-219-1-3p or scramble miRNAs were transfected into Capan-2 cells. Using qRT-PCR, we show a significant enrichment of *MUC4* mRNA (5.28-fold, $P=0.0011$) in the miR-219-1-3p pull-down compared with the scramble miRNA transfected cells (Figure 2b).

To functionally validate this interaction, we cloned the 3'-UTR of human *MUC4* downstream of the luciferase reporter gene

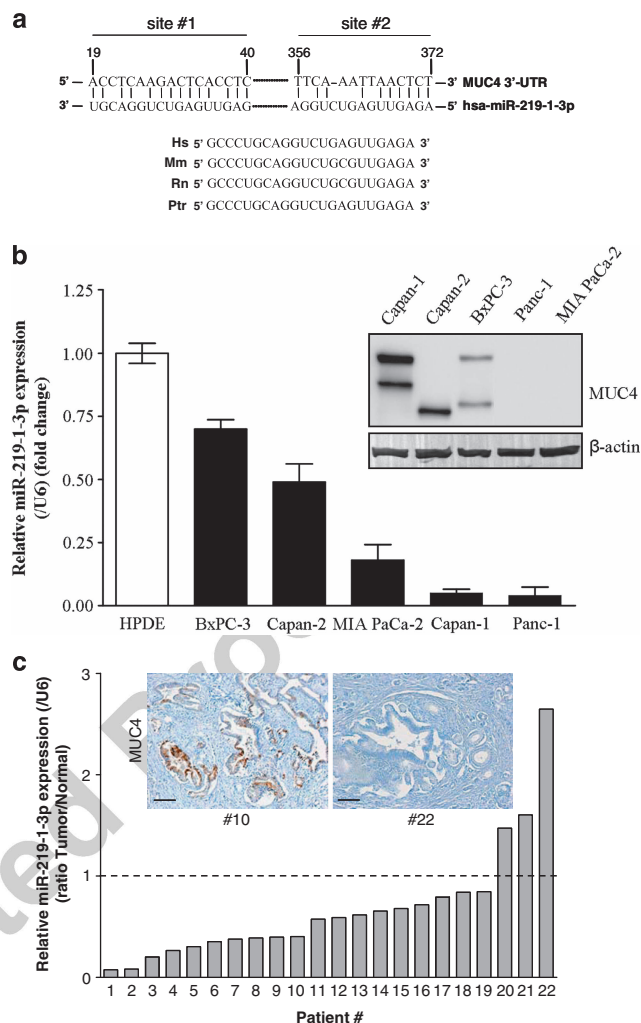


Figure 1. miR-219-1-3p is downregulated in pancreatic cancer cell lines and PDAC tissues. **(a)** Identification of two putative miR-219-1-3p binding sequences in the *MUC4* 3'-UTR at nucleotides 19–40 (site #1) and 356–372 (site #2). Alignment of the hsa-miR-219-1-3p seed region in different species (human Hs, mouse Mm, rat Rn and chimpanzee Ptr) demonstrates conservation across species. **(b)** MiR-219-1-3p expression was determined by qRT-PCR in Capan-1, Capan-2, BxPC-3, Panc-1 and MIA PaCa-2 pancreatic cancer cells and compared with the human pancreatic ductal cells (HPDE; arbitrarily set to 1). Western blotting analysis of MUC4 and β -actin expression (insert). **(c)** qRT-PCR analysis of miR-219-1-3p expression in 22 paired human pancreatic cancers and their corresponding healthy tissues. Results are expressed using $2^{-\Delta\Delta C_t}$ method ($\Delta C_t = C_t$ miR-219-1-3p – C_t U6). MiR-219-1-3p expression in PDAC tissues and nontumoral pancreatic tissues were compared using the Wilcoxon signed-rank test ($*P < 0.05$). Immunohistochemical staining of MUC4 in patients #10 (left) and #22 (right) (insert). Original magnification, $\times 20$. Scale bar = 50 μ m.

(pGL3-*MUC4*-3'-UTR). As shown in Figure 2c, miR-219-1-3p overexpression led to a significant decrease of pGL3-*MUC4*-3'-UTR luciferase activity both in Capan-1 ($P < 0.001$) and BxPC-3 ($P < 0.05$) cell lines compared with cells transfected with scramble miRNAs. Mutation of both miR-219-1-3p binding sites (site #1 mut or site #2 mut) restored normal luciferase activity of *MUC4* 3'-UTR (Figure 2c). When cells were transfected with anti-miR-219-1-3p, pGL3-*MUC4*-3'-UTR luciferase activity significantly increased ($P < 0.01$) in both cell lines compared with the scramble miRNA transfected cells. No effect was observed on mutated forms pGL3-*MUC4*-site#1_mut or pGL3-*MUC4*-site#2_mut (Figure 2d).

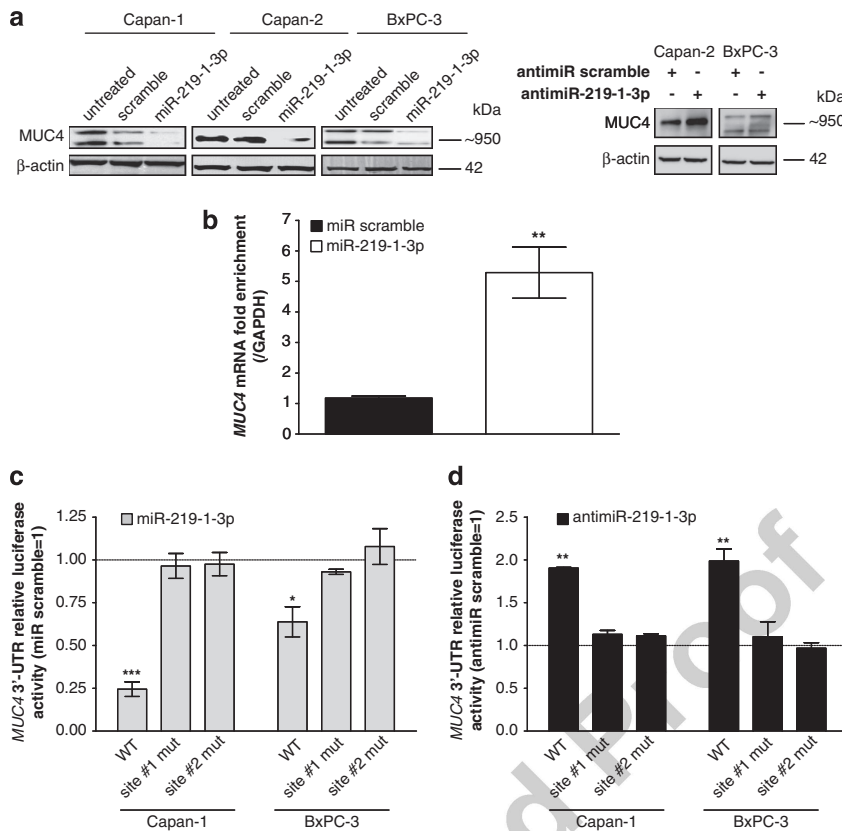


Figure 2. miR-219-1-3p directly inhibits the expression of MUC4 through its 3'-UTR. **(a)** Western blotting analysis of MUC4 and β-actin expression in Capan-1, Capan-2 and BxPC-3 cells not transfected (untreated) or transfected with either scramble pre-miRNA (scramble) or pre-miR-219-1-3p (miR-219-1-3p) (left panel) and Capan-2 and BxPC-3 cells transfected with anti-miR scramble or anti-miR-219-1-3p (right panel). **(b)** Biotinylated miR-219-1-3p or scramble miRNAs were transfected in Capan-2 cells. Enrichment of MUC4 mRNA was assessed by qRT-PCR. The MUC4/GAPDH ratio was calculated and normalized to the input. **(c, d)** Cells were co-transfected with a reporter plasmid containing either the wild-type (WT) or mutant versions in binding site #1 (site #1 mut) or binding site #2 (site #2 mut) of pGL3-MUC4-3'-UTR in the presence of pre-miR-219-1-3p or scramble pre-miRNA **(c)** or in the presence of anti-miR-219-1-3p or scramble anti-miRNA **(d)**. Luciferase activity in **(c)** scramble miR- or **(d)** scramble anti-miR-transfected cells was arbitrarily set to 1. Data are reported as mean ± s.e.m. for three independent experiments (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$).

Altogether, these results indicate that miR-219-1-3p inhibits MUC4 expression through a direct interaction within its 3'-UTR.

miR-219-1-3p inhibits proliferation and migration of PDAC cells *in vitro*

As MUC4 is directly targeted by miR-219-1-3p, we investigated whether miR-219-1-3p was also able to interfere with proliferative and migrating properties of pancreatic cancer cells. Capan-1 ($P < 0.001$), Capan-2 ($P < 0.05$) and BxPC-3 ($P < 0.01$) cell growth was significantly decreased 72-h post-transfection of miR-219-1-3p mimics compared with scramble miRNA (Figure 3a), whereas inhibition of endogenous miR-219-1-3p by anti-miR-219-1-3p led to a significant increase of cell proliferation both in Capan-2 ($P < 0.01$) and BxPC-3 ($P < 0.01$) cells at 48-h and 72-h post-transfection (Figure 3b). To further determine whether miR-219-1-3p expression could be associated with the progression of pancreatic cancer, we evaluated its effect on the cell migrating behavior. As shown in Figure 3c, miR-219-1-3p overexpression significantly reduced migration of Capan-1 and Capan-2 cells ($P < 0.01$) and of BxPC-3 to a lower extent. Taken together, these data show that miR-219-1-3p modulates biological properties of PDAC cells by decreasing both their proliferation and migration properties. Previous studies have shown that direct knock-down of MUC4 in Capan-2 cells led to a decrease of cell proliferation and migration.³ To test the hypothesis whether the decreased proliferation and migration observed in this report was due, at

least in part, to the ability of miR-219-1-3p to inhibit MUC4 expression, transient transfections with anti-miR-219-1-3p in Capan-2 MUC4-KD cells were carried out. The results show that proliferation and migration promoting effects of anti-miR-219-1-3p are strongly attenuated in Capan-2 MUC4-KD cells (Supplementary Figure S2) suggesting the implication of MUC4 in miR-219-1-3p-mediated reduced cell proliferation and migration.

miR-219-1-3p biological activities impair signaling pathways associated with cell cycle, cell proliferation and cell survival

To decipher the molecular mechanisms related to the decreased proliferation observed in PDAC cells transiently overexpressing miR-219-1-3p, we assessed the expression of cyclin D1 cell cycle marker and the activation of Akt and Erk signaling pathways as these were previously reported to be responsible for decreased proliferation mediated by MUC4.^{3,17} Our results indicate that miR-219-1-3p overexpression induces a strong decrease of cyclin D1 expression in the three cell lines (Figure 3d). Furthermore, the phosphorylation levels of Akt and Erk were also decreased in miR-219-1-3p-transfected cells compared with miRNA scramble-transfected cells. These results indicate that miR-219-1-3p reduces PDAC cell proliferation by inhibiting cell cycle progression and by reducing proliferation- and survival-associated signaling pathway activation.

To further assess the biological activities of miR-219-1-3p, we generated two pancreatic stable cell lines overexpressing

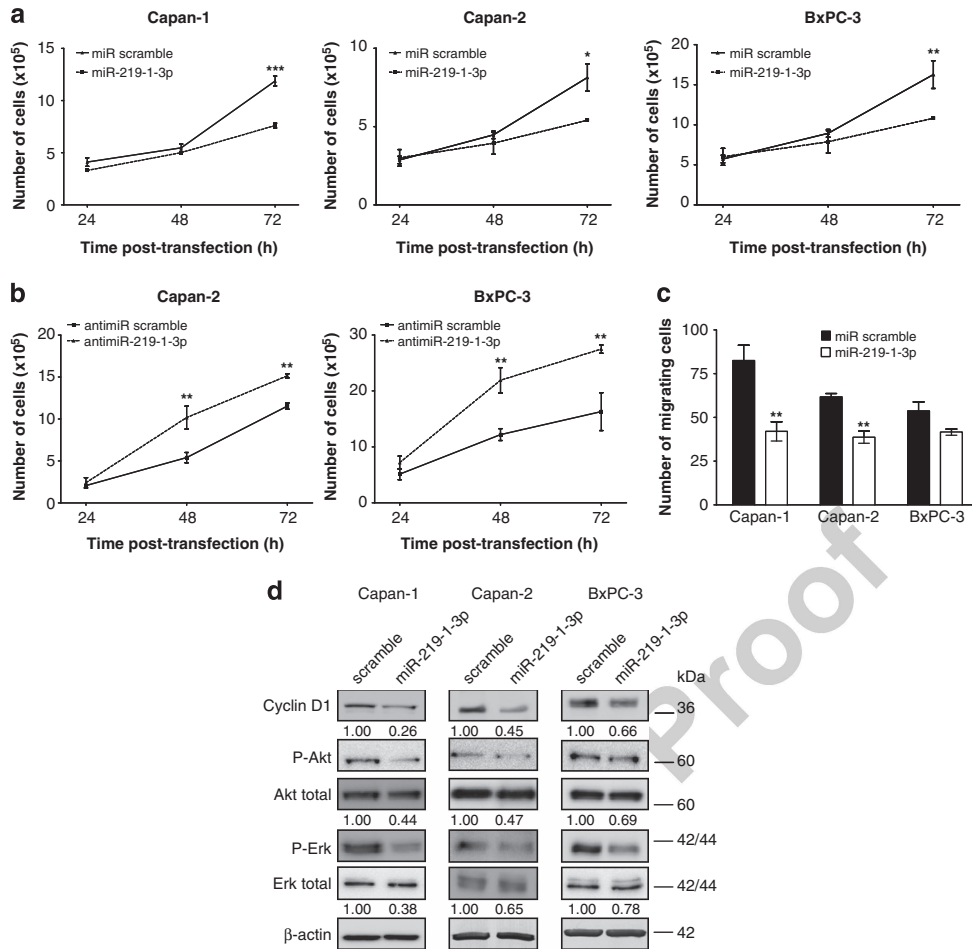


Figure 3. miR-219-1-3p inhibits pancreatic cancer cell proliferation and migration. **(a)** Overexpression of miR-219-1-3p inhibits proliferation of Capan-1, Capan-2 and BxPC-3 cells. **(b)** Inhibition of miR-219-1-3p increases proliferation of Capan-2 and BxPC-3 cells. **(c)** Capan-1, Capan-2 and BxPC-3 cells were transfected with pre-miR-219-1-3p or scramble pre-miRNA for 24 h. Results are expressed as average migrating cell number per field. Data are reported as mean \pm s.e.m. for three independent experiments (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$). **(d)** MiR-219-1-3p inhibits proliferation and signaling pathways downstream of MUC4. Western blotting analysis of cyclin D1, P-Akt, Akt, P-Erk and Erk levels in Capan-1, Capan-2 and BxPC-3 cells after miR-219-1-3p transfection. β -Actin serves as the loading control. Bands were quantified by densitometry.

miR-219-1-3p. Successful and significant overexpression of miR-219-1-3p in Capan-1 (Capan-1 LV miR-219, 6.34 fold, $P < 0.001$) and in Capan-2 (Capan-2 LV miR-219, 12.68 fold, $P < 0.001$) cells was confirmed by qRT-PCR (Supplementary Figure S1C). Overexpression of miR-219-1-3p (i) inhibited MUC4 protein expression by 60% in Capan-1 cells and by 80% in Capan-2 cells (Figure 4a), (ii) significantly reduced the proliferation rate of Capan-1 as early as 96h ($P < 0.01$) and of Capan-2 as early as 72 h ($P < 0.05$; Figure 4b) and (iii) reduced their migrating capacity (Capan-1, $P < 0.001$ and Capan-2 $P < 0.01$; Figure 4c). In Capan-2, stable overexpression of miR-219-1-3p led to a decrease of cyclin D1 expression and a decrease of both Erk and Akt activation. In Capan-1, the same effects were visualized except for Akt (Supplementary Figure S3). Taken together, these results indicate that stable overexpression of miR-219-1-3p in PDAC cells led to the same biological effects (decreased proliferation and decreased migration) as those observed in transiently transfected cells.

miR-219-1-3p inhibits pancreatic tumor progression *in vivo*

To further assess the tumor-suppressive activity of miR-219-1-3p *in vivo* and its possible value as a therapeutic tool, a xenograft tumor model was established with Capan-1, Capan-2 and MIA PaCa-2 cells in severe combined immunodeficient mice. As shown

in Figure 5a, intratumoral injection of miR-219-1-3p in both Capan-1 and Capan-2 led to a significant decrease ($P < 0.05$) of tumor growth 6 and 7 days post-injection, respectively. Tumor growth further decreased until the experimental end point was reached ($P < 0.05$). A significant decrease of tumor growth was also observed a week post-injection in MIA PaCa-2 cells compared with control mice (data not shown). Measurement of miR-219-1-3p and MUC4 expression in these tumors confirmed the overexpression of miR-219-1-3p (Figure 5b) conversely to MUC4 that was weakly (Capan-1) or no longer (Capan-2) expressed when compared with controls (Figure 5c). Decreased expression of Ki67 in miR-219-1-3p tumors confirmed miR-219-1-3p negative effect on proliferation. Thus, these results confirm (i) the tumor-suppressor activity of miR-219-1-3p on PDAC cell growth and (ii) the converse correlation between miR-219-1-3p and MUC4 expression in pancreatic tumors.

Muc4 expression is conversely correlated to miR-219-1-3p expression in PanIN lesions

To establish a correlation between MUC4 and miR-219-1-3p expression early in the development of pancreatic cancer, their expression was studied in the pancreas of the *Pdx1-Cre;LStopL-Kras^{G12D}* preclinical mouse model of pancreatic cancer. We found

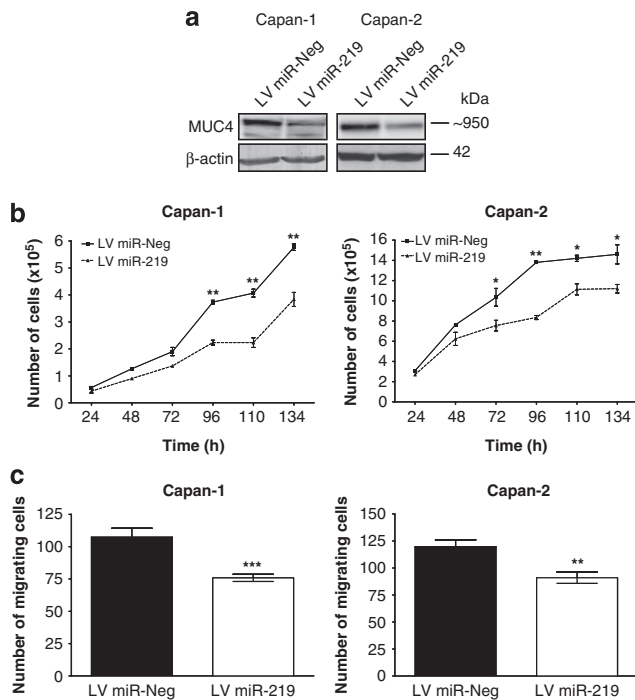


Figure 4. Characterization of stable miR-219-1-3p expressing cell lines. **(a)** Western blotting analysis of MUC4 and β -actin expression in Capan-1 LV miR-219, Capan-2 LV miR-219 and their controls Capan-1 LV miR-Neg and Capan-2 LV miR-Neg. **(b)** Proliferation and **(c)** migration properties of Capan-1 LV miR-219 and Capan-2 LV miR-219 cells compared with their controls (LV miR-Neg). Data are reported as mean \pm s.e.m. for three independent experiments (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$).

that miR-219-1-3p was strongly expressed in normal acinar cells ($Kras^{WT}$) (Figure 6a). In PanIN lesions ($Kras^{G12D}$), miR-219-1-3p expression was strongly decreased when compared with normal tissues. Its expression in ductal neoplastic cells was conversely correlated to Muc4 expression. Immunohistochemistry and qRT-PCR were then respectively used to measure Muc4 and miR-219-1-3p expression levels in PanIN lesions. In mouse PanIN lesions ($Kras^{G12D}$), miR-219-1-3p expression was significantly decreased ($P < 0.001$) when compared with normal pancreatic tissue ($Kras^{WT}$) (Figure 6b). To establish the converse correlation between miR-219-1-3p and Muc4 expression, a score for Muc4 was calculated. As shown in Figure 6c, a negative correlation between miR-219-1-3p and Muc4 expression was observed in PanIN tissues ($r = -0.5847$, $P = 0.0137$; Spearman's correlation). In conclusion, Muc4 overexpression in mouse PanIN lesions is associated with miR-219-1-3p loss of expression suggesting, in addition to the data obtained in xenograft studies, that the upregulation of Muc4 in early PanIN lesions may result from the repression of miR-219-1-3p.

DISCUSSION

In this study, we have identified miR-219-1-3p as a novel negative regulator of MUC4 mucin expression in PDAC cells as early as in PanIN-1/2 neoplastic lesions. Moreover, we show that miR-219-1-3p inhibits pancreatic cancer cell proliferation *in vitro* and *in vivo* and migration *in vitro*. MiR-219-1-3p appears thus as a good target to (1) reduce pancreatic cancer cell proliferation and (2) inhibit expression of oncogenic MUC4 mucin.

MUC4 is an actor of pancreatic tumorigenesis as it contributes to tumor progression by inducing cell proliferation, survival and migration.^{3,18,19} Moreover, neo-expression of MUC4 in the early stages of pancreatic carcinogenesis confers to this mucin a

potential interest as a therapeutic target in pancreatic cancer.^{6,20} Although molecular mechanisms responsible for the induction of MUC4 expression in cancer are not well defined, it is known that MUC4 may be regulated at the transcriptional level via the epidermal growth factor²¹ and transforming growth factor β ^{8,22} pathways among others²³ and at the epigenetic level via DNA methylation and histone modifications.⁷ Recently, miRNAs were shown to be involved in post-transcriptional regulation of MUC4.¹⁴ In this study, using complementary approaches (*in vitro* stable and transient transfections and *in vivo* models) we demonstrate that MUC4 is a functional target of miR-219-1-3p. We showed that miR-219-1-3p inhibits MUC4 expression at the protein level, it directly interacts with MUC4 mRNA and exerts its repressive effect by binding to the two nucleotidic sites in the MUC4 3'-UTR. We showed that overexpression of miR-219-1-3p inhibits cell proliferation and reduces migration of PDAC cells by targeting MUC4. MUC4 is known to be involved in Akt and mitogen-activated protein kinase survival signaling pathways and cell cycle progression regulation.³ Inhibition of cell proliferation by miR-219-1-3p prompted us to study MUC4 downstream survival and apoptotic signaling pathways in the context of its regulation by miR-219-1-3p. We found that cyclin D1 expression decreased concomitantly to the inhibition of activation of Akt and mitogen-activated protein kinase signaling pathways whereas apoptosis does not seem to be involved. More interestingly, increasing evidence of miRNA influence on cell behavior through the regulation of multiple target genes prompted us to search for putative targets of miR-219-1-3p using an *in silico* approach. CDK1 and ICAM-1 known to promote cell proliferation and migration were identified, however, pull-down experiments did not allow us to validate these targets in pancreatic cancer cells (data not shown). This indicates that miR-219-1-3p may act as a tumor suppressor in PDAC development mainly by targeting the oncogenic mucin MUC4 and by affecting associated downstream signaling pathways. Accordingly to this observation, we found that miR-219-1-3p expression was conversely correlated to Muc4 in mouse PanIN and that in the three human PDAC tissues overexpressing miR-219-1-3p, MUC4 was not observed. This suggests that the negative regulation of MUC4 by miR-219-1-3p involves a conserved mechanism between these two species.

MIR219-1 was initially described as a brain-specific miRNA as a clock and light regulated gene having a role in regulating the length of the circadian cycle²⁴ and as a key regulator of oligodendrocyte differentiation.^{25,26} Downregulation of miR-219-1-3p in PDAC cell lines and PDAC tissues, found in this report, suggests a new tumor-suppressive function for this miRNA in pancreatic cancer. To determine the mechanisms underlying miR-219-1-3p downregulation, we searched for the genomic region containing MIR219-1 gene using the UCSC Genome Browser and we observed that MIR219-1 gene is located within a CpG island. Preliminary experiments in pancreatic cancer cells to determine whether DNA hypermethylation could impair miR-219-1-3p expression were carried out. Inhibition of DNA methylation with 5-aza-2'-deoxycytidine treatment led to upregulation of miR-219-1-3p expression, suggesting that aberrant DNA methylation may be associated with miR-219-1-3p silencing in PDAC cells (data not shown). Another mechanism that could control miR-219-1-3p expression is genetic alteration. The majority of miRNAs are located in cancer-associated genomic regions such as common breakpoint regions, fragile sites, minimal regions of loss of heterozygosity and amplification.²⁷ By genomic sequence analysis Xu *et al.* mapped the MIR219-1 gene to chromosome 6p21.32.²⁸ Genome-wide copy number analysis in PDAC enabled the identification of the 6p region as a region of high-frequency loss^{29,30} suggesting that the chromosomal region in which lies MIR219-1 may be the subject of genetic alterations. Therefore, epigenetic and/or genetic mechanisms may also be responsible for MIR219-1 silencing in PDAC, which is another argument

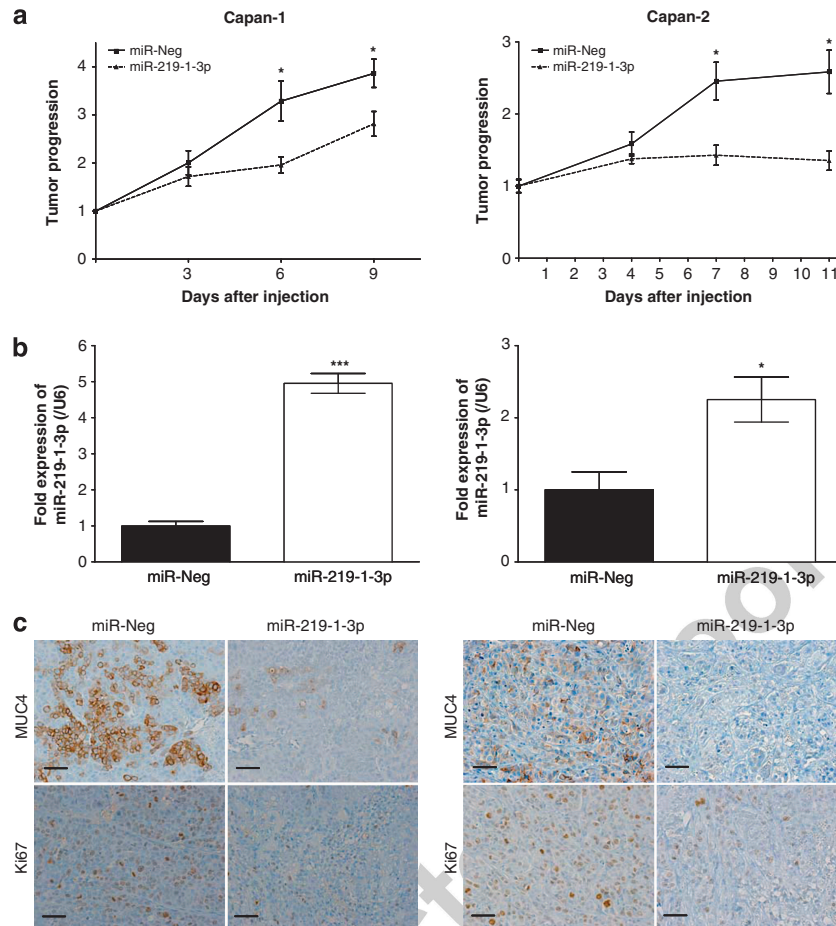


Figure 5. miR-219-1-3p inhibits pancreatic tumor growth *in vivo*. Left panel: Capan-1 and right panel: Capan-2 cells. **(a)** Cells were xenografted subcutaneously into severe combined immunodeficient mice and the established tumors were then treated with either pcDNA6.2-miR-neg or pcDNA6.2-miR-219 vectors. Measurement of tumor growth was performed following intratumoral injection of miR vectors and tumor volume was calculated ($*P < 0.05$). Results are expressed as the ratio of tumor progression relative to tumor volume on the day of miRNA injection (arbitrarily set to 1). **(b)** Expression of miR-219-1-3p in xenografted tumors was measured by qRT-PCR ($*P < 0.05$, $***P < 0.001$). **(c)** Expression of MUC4 and Ki67 in xenografted tumors was studied by immunohistochemistry. Original magnification, $\times 20$. Scale bar = 50 μm .

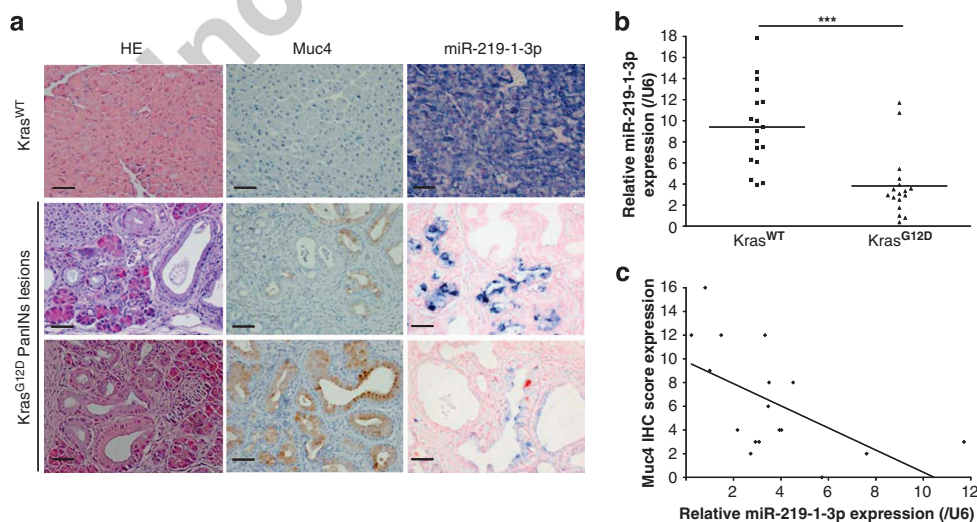


Figure 6. miR-219-1-3p expression is conversely correlated to Muc4 expression in early pancreatic PanIN lesions. **(a)** Hematoxylin and eosin staining (left panel), MUC4 immunohistochemical staining (middle panel) and miR-219-1-3p *in situ* hybridization (right panel) in mice tissues. Original magnification, $\times 20$. Scale bar = 50 μm . **(b)** Expression of miR-219-1-3p in PanINs ($Kras^{G12D}$) and normal pancreas ($Kras^{WT}$) by qRT-PCR. Data were normalized to U6 expression and expressed as $2^{-\Delta\text{Ct}}$ ($***P < 0.001$). **(c)** Correlation analysis between miR-219-1-3p expression and Muc4 expression. Statistical analyses were performed using Pearson's correlation coefficient ($r = -0.5847$, $P = 0.0137$).

to propose miR-219-1-3p as a promising tumor-suppressor candidate.

As we were studying MUC4 regulation by miR-219-1-3p, several studies proposed miRNAs signatures of pancreatic cancer.^{31–33} In the most recent study, Schultz *et al.* performed a miRNA profiling array using for the first time non micro-dissected human tissues in order to discover new miRNA differentially expressed in pancreatic tissues, including stromal and cancer cells, comparing PDAC with normal pancreas.¹⁵ Among the miRNAs that were down-regulated in PDAC compared with normal tissues, miR-219-1-3p emerged as one of the most relevant, confirming the interest of studying the biological role of this miRNA for which nothing was known in pancreatic carcinogenesis. However, to date, a very small number of studies explored miRNAs expression alterations in PanIN lesions.^{10,34,35} Our analysis of miR-219-1-3p expression in genetically engineered mice *Kras*^{G12D}-driven pancreatic cancer model allowed us to show that miR-219-1-3p downregulation is an early event of pancreatic carcinogenesis as miR-219-1-3p expression is lost in the PanINs pre-neoplastic lesions.

On the basis of these observations, the early miR-219-1-3p repression and the resulting increased expression of the transmembrane mucin MUC4 may represent two key events that favor pancreatic tumor progression. It is therefore tempting to speculate that induction of MUC4 expression in the early steps of pancreatic carcinogenesis is epigenetically regulated by DNA methylation at two levels (i) directly on its 5'-UTR⁷ and (ii) via repression of its inhibitor miR-219-1-3p (this report). Hence, identification of these two mechanisms regulating MUC4 expression in pancreatic cancer gives new insight to develop a novel therapeutic approach targeting MUC4 that would combine miRNA and methylated gene-specific reactivation using artificial transcription factors.³⁶

In summary, our findings indicate that miR-219-1-3p is a good tumor-suppressor candidate inhibiting both MUC4 expression and MUC4 downstream signaling pathways, highlighting the therapeutic potential of miR-219-1-3p in pancreatic cancer.

MATERIALS AND METHODS

Cell culture

Human pancreatic ductal epithelial cells were obtained from Dr MS Tsao (UHN, Toronto, Canada).^{37,38} BxPC-3 and MIA PaCa-2 pancreatic cancer cells were purchased from ATCC and were cultured as described before.³⁹ Capan-1, Capan-2 (ATCC) and Panc-1 (ECACC) pancreatic cancer cells were grown as previously described.²¹

Human samples

Paired human PDAC and their corresponding nontumoral tissues were collected from surgical resections of 22 patients with PDAC from 2006 to 2009 in Lille University Hospital. Signed informed consent was obtained from all patients.

Establishment of miR-219-1-3p overexpressing stable cell lines

Capan-1 LV-miR-Neg and Capan-2 LV-miR-219 stable cell lines were obtained as previously described³⁸ miR-219-1_F 5'-TGCTGAGAGTTGAG TCTGGACGTCCCGGTTTTGGCCACTGACTGACCGGGGACGTCCAGTCAACTCT-3' and miR-219-1_R 5'-CTGAGA GTTGACTGGACGTCCCGGTCAGTCAAGTGGCCAAAACCGGGACGTCCAGACTCAACTCTC-3' oligonucleotides were annealed and cloned into pcDNA6.2-GW/emGFP-miR vector (Life Technologies, Villeban sur Yvette, France) to obtain pcDNA6.2-GW/emGFP-miR-219 vector. pcDNA6.2-GW/emGFP-miR-Neg vector (Life Technologies) was used as control. Green fluorescent protein (GFP)-miR-219 and GFP-miR-Neg cassettes were cloned into pDONR221 (Life Technologies), using the BP Clonase reaction (Life Technologies), and subsequently transferred into pLenti4/TO/V5-DEST lentiviral vector (Life Technologies), using the LR Clonase reaction (Life Technologies) to create pLenti4/TO/GFP-miR-219 and pLenti4/TO/GFP-miR-Neg vectors, respectively. Lentiviral particles were produced in HEK-293FT cells with pLenti4/TO/GFP-miR-219 and pLenti4/TO/GFP-miR-Neg vectors and Virapower lentiviral packaging mix

(Life Technologies). Capan-1 and Capan-2 cells were incubated with the lentiviral particles (multiplicity of infection, 1) for 24 h and subsequently selected with Zeocin (Invivogen, Toulouse, France) at 100 µg/ml for 2 weeks. Efficiency of viral infection and selection was estimated by measuring the percentage of GFP-expressing cells by flow cytometric analysis. Overexpression of mature miR-219-1-3p was quantified by qRT-PCR as described below.

Pdx1-Cre;LSL-Kras^{G12D} mouse model

Pdx1-Cre mice were obtained from the Mouse Models of Human Cancer Consortium (MMHCC, USA). *LStopL-Kras*^{G12D} mice were obtained from Dr D Tuveson (Cambridge Research Institute, England, UK).⁴⁰ All procedures were in accordance with the guideline of animal care committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, #AF042008). *LSL-Kras*^{G12D} (*Kras*^{WT}) and *Pdx1-Cre* mice were maintained as heterozygous lines and crossed to obtain *Kras*^{G12D} (*Pdx1-Cre;LSL-Kras*^{G12D}) mice. Mice were killed at 9 months of age. Two pathologists scored independently the PanIN grade as PanIN-1 and PanIN-2. Tail snips, harvested from offsprings of *LSL-Kras*^{G12D} and *Pdx1-Cre* mice, were digested overnight, and genomic DNA was extracted using the Nucleospin Tissue kit (Macherey Nagel, Hoerd, Germany). The *Cre* and *Kras*^{G12D} alleles were identified by PCR.

miRNA target prediction

To identify miRNA potentially targeting MUC4 3'-UTR, Microcosm (www.ebi.ac.uk/enright-srv/microcosm) and MiRanda (<http://www.microrna.org>) databases were used. The mature miRNA sequence was confirmed with miRBase (<http://microrna.sanger.ac.uk/>).

pGL3-MUC4-3'-UTR cloning

The MUC4 3'-UTR was amplified from Capan-2 genomic DNA using the following primers: MUC4_3'-UTR_F: 5'-CGCTCTAGAGGGCCAGGTTCTCCTAT TTC-3' and MUC4_3'-UTR_R: 5'-CGCTCTAGAGGGCCACCAGAAGAAAATCAA-3'. The PCR product was digested with *Xba*I restriction enzyme and inserted into pGL3 Promoter vector (Promega, Charbonnières-les-Bains, France) downstream of the luciferase gene. To generate pGL3-MUC4-site#1_mut miR-219-1-3p binding site #1, sequence ACCTCAAGACTCACCTC was mutated to ACCTCAAGAAGAACCTC and the miR-219-1-3p binding site #2, sequence TTCAAATTAACTCT was mutated to TTCAAATGGCGAT (pGL3-MUC4-site#2_mut) using the Quick-Change XL Site-Directed Mutagenesis kit (Stratagene, Agilent Technologies, Massy, France).

Cell transfection and luciferase reporter assays

Cells were transfected with pre-miR-219-1-3p (9 nM) or anti-miR-219-1-3p (18 nM) by the reverse transfection method using the siPORT *NeoFX* transfection reagent (Ambion, Life Technologies, Villeban sur Yvette, France). Cells transfected with the siPORT *NeoFX* transfection reagent (untreated) or a scramble sequence were examined in parallel as controls. Cells were subjected to RNA or protein extraction 48-h post-transfection. Co-transfection was carried out in the presence of 1 µg of pGL3-MUC4-3'-UTR reporter plasmid and either pre-miR-219-1-3p precursor or anti-miR-219-1-3p (Ambion, Life Technologies). Luciferase activity and protein assays were performed as described in Skrypek *et al.*⁵

RNA isolation and qRT-PCR

Total RNA including miRNAs from cells and from formalin-fixed paraffin-embedded was extracted using miRNeasy Mini Kit (Qiagen, Courtaboeuf, France) and RecoverAll Total Nucleic Acid Isolation Kit (Ambion). One µg of RNA was reverse transcribed with the QuantiMiR Reverse Transcription Kit (System Biosciences, Ozyme, Saint Quentin Yvelines, France). qRT-PCR was carried out using *SsoFast EVA Green Supermix* (Bio-Rad, Marnes-la-Coquette, France) and *CFX96* Detection System (Bio-Rad). The forward primer was the same sequence as the mature miR-219-1-3p (5'-AGA GTTGAGTCTGGACGTCCCG-3'), and the universal reversed primer was provided in the QuantiMiR Reverse Transcription Kit (System Biosciences, Ozyme). Human and mouse U6 snRNA, used as controls, were amplified with primers provided from the QuantiMiR Reverse Transcription Kit (System Biosciences, Ozyme). Expression levels were calculated using the 2^{-ΔCt} method. The miR-219-1-3p expression level was normalized against U6 snRNA and was assayed in triplicate in three independent experiments.

Biotin pull-down assay

Capan-2 cells (1×10^6) were transfected in triplicate with 3'-biotinylated miR-219-1-3p or 3'-biotinylated scramble miRNA (Dharmacon, Thermo Scientific, Brebières, France) at 100 nM final as described above. Biotin pull-down assay was performed as previously described.⁴¹ RNA was directly extracted from the beads (pulled-down RNA) or from the input RNA using the miRNeasy Mini kit (Qiagen). Extracted RNA was subjected to complementary DNA synthesis as previously described.⁴² qPCR was performed using the SsoFast Evagreen Supermix kit (Bio-Rad) following manufacturer's protocol using the CFX96 real-time PCR system (Bio-Rad). Primers used were: MUC4_F, 5'-GCCCAAGCTACAGTGTGACTCA-3'; MUC4_R, 5'-ATGGTCCGGTTGTAATTTGTTGT-3'; GAPDH_F, 5'-CCACATCGCTCAGACACAT-3'; GAPDH_R, 5'-CCAGGCGCCAATACG-3'. The MUC4 mRNA levels were normalized to the mRNA level of GAPDH in the miR-219-1-3p pull-down and then normalized to the amount in the input to obtain the enrichment ratio. This was calculated using the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were carried out.

Cell proliferation and migration assays

Capan-1, Capan-2 and BxPC-3 cells were transfected with pre-miR-219-1-3p or scramble miRNA and then seeded at 10^5 cells per well in six-well plates. Cells were counted at 24, 48 and 72-h post-transfection using Countess Automated Cell Counter (Life Technologies). In stable cell lines, cells were counted until 134 h. Cells were transfected during 24 h, 5×10^4 cells were then plated into migration chambers (Pharming, BD Biosciences, Le Pont de Claix, France). Each experiment was assayed in triplicate in at least three independent experiments.

Subcutaneous xenografts

Capan-1, Capan-2 (2×10^6 cells in 100 μ l Matrigel) cells were injected subcutaneously into the flank of 8-week-old male severe combined immunodeficient mice (CB17, Charles Rivers, L'Arbresle, France) to generate a tumor. Once palpable tumors developed (250 mm³), 20 μ g of pcDNA6.2-miR-219 or pcDNA6.2-miR-neg vector complexed with ExGen 500 transfection reagent (Euromedex, Souffelweyersheim, France) in 5% (w/v) glucose were injected in tumors. Six mice were used per group. Tumor size was monitored by measuring length (*l*) and width (*l*) twice a week and tumor volume was calculated with the formula ($l^2 \times l$). All procedures were in accordance with the guideline of animal care committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, #122012).

Immunoblotting

Cell lysis, western-blotting, immunostaining and protein assay were carried out as described before.⁴³ The membranes were incubated with phospho-Erk1/2 (Thr202/Tyr204) (clone 20G11, 1/500), Erk1/2 (clone I37F5, 1/500), phospho-Akt (Ser473) (clone D9E, 1/1000), Akt (clone C67E7, dilution 1/1000), cyclin D1 (clone DCS6, dilution 1/500), all from Cell Signalling Technology, (Ozyme) MUC4 (clone 8G7, 1/500), Bax (clone N-20, 1/500) and α -tubulin (clone H-5, 1/500) from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) and β -actin (A5441, 1/5000) from Sigma-Aldrich (Saint Quentin Fallavier, France). Antibodies were diluted in 5% (w/v) non-fat dry milk in Tris-buffered saline Tween-20, except for MUC4 and β -actin diluted in Tris-buffered saline Tween-20, and incubated overnight at 4°C. The membranes were then incubated with peroxidase-conjugated secondary antibodies (Sigma-Aldrich). The signal was detected using LAS 4000 apparatus (Fujifilm, F.S.V.T., Courbevoie, France). Bands were quantified with the image analysis software Gel analyst (Clara Vision, Verrières-le-Buisson, France). Three independent experiments were performed.

Immunohistochemistry

Tumor tissues were fixed in formalin and embedded in paraffin. Tissue sections (5 μ m) were stained with hematoxylin and eosin. Immunostaining with primary antibodies for human MUC4 (8G7) or mouse Muc4 (1G8) (Santa Cruz Biotechnology Inc.) and human Ki67 (Leica Microsystems, Nanterre, France) were performed as described previously.^{44,45} The slides were scored independently by two pathologists. Intensity of staining was graded as weak (1), moderate (2) or strong (3). The percentage of ductal stained cells was graded as 1 (0–25%), 2 (25–50%), 3 (50–75%) and 4 (75–100%). Total score was calculated by multiplying the intensity score and percentage score.

miRNA *in situ* hybridization

In situ hybridization for miR-219-1-3p was performed in the formalin-fixed and paraffin-embedded mouse pancreatic tissue specimens by using the MiRCURY LNA microRNA ISH Optimization Kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's protocol. The miR-219-1-3p locked nucleic acid sequence is 5'-DIG- CGGGACGTCCAGACTCAACTCT-3'-DIG. The slides were then counterstained with nuclear fast red (Sigma-Aldrich) and mounted with mounting medium (VWR, Fontenay sous Bois, France).

Statistical analyses

All statistical analyses were carried out using the Graphpad Prism 4.0 software. Unless indicated, differences between groups were analyzed using the Student's *t*-test or analysis of variance test. Data were shown as mean \pm s.e.m. and considered significant when **P* < 0.05, ***P* < 0.01 or ****P* < 0.001.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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