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Solange Tréhoux, Fatima Lahdaoui, Yannick Delpu, Florence Renaud, Emmanuelle Leteurtre, et al.. Micro-RNAs miR-29a and miR-330-5p function as tumor suppressors by targeting the MUC1 mucin in pancreatic cancer cells. *Biochimica et Biophysica Acta - Molecular Cell Research*, 2015, <10.1016/j.bbamcr.2015.05.033>. <hal-02905657>

HAL Id: hal-02905657

<https://hal.science/hal-02905657v1>

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Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Q4 Micro-RNAs miR-29a and miR-330-5p function as tumor suppressors by targeting the MUC1 mucin in pancreatic cancer cells

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10 ARTICLE INFO

Article history:

Received 26 January 2015

Received in revised form 11 May 2015

Accepted 28 May 2015

Available online xxxx

Keywords:

MUC1 mucin

Pancreas

Cancer

MiRNA

Chemoresistance

ABSTRACT

MUC1 is an oncogenic mucin overexpressed in several epithelial cancers, including pancreatic ductal adenocarcinoma, and is considered as a potent target for cancer therapy. To control cancer progression, miRNAs became very recently, major targets and tools to inhibit oncogene expression. Inhibiting MUC1 using miRNAs appears thus as an attractive strategy to reduce cancer progression. However, potent miRNAs and associated mechanisms regulating MUC1 expression remain to be identified. To this aim, we undertook to study MUC1 regulation by miRNAs in pancreatic cancer cells and identify those with tumor suppressive activity. MiRNAs potentially targeting the 3'-UTR, the coding region, or the 5'-UTR of MUC1 were selected using an in silico approach. Our in vitro and in vivo experiments indicate that miR-29a and miR-330-5p are strong inhibitors of MUC1 expression in pancreatic cancer cells through direct binding to MUC1 3'-UTR. MUC1 regulation by the other selected miRNAs (miR-183, miR-200a, miR-876-3p and miR-939) was found to be indirect. MiR-29a and miR-330-5p are also deregulated in human pancreatic cancer cell lines and tissues and in pancreatic tissues of Kras^{G12D} mice. In vitro, miR-29a and miR-330-5p inhibit cell proliferation, cell migration, cell invasion and sensitize pancreatic cancer cells to gemcitabine. In vivo intra-tumoral injection of these two miRNAs in xenografted pancreatic tumors led to reduced tumor growth. Altogether, we have identified miR-29a and miR-330-5p as two new tumor suppressive miRNAs that inhibit the expression of MUC1 oncogenic mucin in pancreatic cancer cells.

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1. Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth leading cause of death by cancer in Western countries and has a very poor prognosis due to a late diagnosis and a lack of efficient treatment. The five year survival rate is lower than 5% [1] and less than 20% of patients are entitled to surgical resection [2]. The remaining 80% of patients present a locally advanced metastatic PDAC and may benefit from palliative chemotherapy based either on gemcitabine or FOLFIRINOX [3]. Despite this, PDAC prognosis remains very poor and is highly resistant to chemotherapeutic treatments. It is thus mandatory to find early biomarkers, to better understand the molecular mechanisms underlying the disease and to identify

new therapeutic targets/tools to allow better disease management and reduce pancreatic cancer progression.

The mucin MUC1 is a membrane-bound glycoprotein expressed at the apical pole of normal polarized epithelial cells. In most epithelial cancers including PDAC [4,5], MUC1 becomes oncogenic as it is overexpressed, circumferentially delocalized around the tumor cell surface and involved in several oncogenic pathways [6,7]. These properties are the consequence of MUC1 interaction with the epidermal growth factor receptor (EGFR) [8] which leads to the activation of several oncogenic pathways (MAPK, Wnt/β-catenin) and increased cell proliferation and survival [5,9,10]. We recently showed that MUC1 regulates human PDAC cell proliferation via p42–44 MAPK and β-catenin pathways, decreases cell migration and invasion via MMP13 and sensitizes PDAC cells to 5-fluoro-uracil (5-Fu) and gemcitabine [5]. Others showed that MUC1 may also regulate PDAC cell invasion through activation of Stat3 [11,12] or PDGFR-β [13]. MUC1 also induces epithelial mesenchymal transition (EMT) [14] and increases resistance to gemcitabine via mechanisms dependent or not of Akt [15]. Finally, MUC1 was found as one robust predictive marker of PDAC survival [16]. For all these

Abbreviations: IC₅₀, concentration giving half-maximal inhibition; miRNA, microRNA; PDAC, pancreatic ductal adenocarcinoma; UTR, untranslated region

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<http://dx.doi.org/10.1016/j.bbamcr.2015.05.033>

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Please cite this article as: S. Tréhoux, et al., Micro-RNAs miR-29a and miR-330-5p function as tumor suppressors by targeting the MUC1 mucin in pancreatic cancer cells, Biochim. Biophys. Acta (2015), <http://dx.doi.org/10.1016/j.bbamcr.2015.05.033>

reasons, MUC1 appears as an attractive target to slow-down pancreatic tumorigenesis.

MUC1 is also translocated to the nucleus and associated with co-factors and/or transcription factors on gene promoters to modulate their expression. It was recently shown that MUC1 regulates miRNA expression by binding with ZEB1 to the promoter of miR-200c resulting in its inhibition and increased cell invasion and EMT [17]. Since miRNAs regulate a large number of human genes, targeting regulatory mechanisms mediated by miRNAs appears as an attractive strategy to inhibit oncogenic proteins and propose new therapeutic approaches [18,19]. MiRNAs are 18 to 25 nucleotide long small non-coding RNAs which regulate gene expression by binding to the 3'-UTR, 5'-UTR or coding regions of their target mRNAs [20]. MiRNAs can also inhibit the translation of their target or lead to the degradation of the mRNA and thereby protein expression [21]. MiRNAs are frequently deregulated in cancer, including PDAC. Several studies have investigated the expression profile of miRNAs in PDAC but few of them have studied their biological role and/or biological relevance [22,23]. We recently showed that miRNA can effectively inhibit oncogenic mucin MUC4 in PDAC through the overexpression of tumor suppressive miRNA [23] and the mucin MUC13 was also shown to be directly regulated by miRNA in PDAC [24]. This highlights the interest in targeting oncogenic mucins in PDAC which are known to mediate PDAC progression but so far no miRNAs have been identified as targeting the oncogenic mucin MUC1 in PDAC.

In this report, we undertook to identify miRNAs directly binding to MUC1 regulatory regions including the 5'-UTR, the coding region and the 3'-UTR. Using in vitro and in vivo approaches we show that miR-29a and miR-330-5p are direct negative regulators of MUC1 expression with tumor suppressive activity in pancreatic cancer. These two miRNAs appear thus as potential new actors to reduce pancreatic cancer progression.

2. Material and methods

2.1. Cell culture

Capan-2, Capan-1, MiaPaCa-2 and BxPC-3 cell lines were purchased from ATCC and Panc-1 from ECACC. PDAC cell lines were cultured as described previously [5,23,25,26]. HPDE (Human Pancreatic Duct Epithelial) cells were obtained from Dr M.S. Tsao (UHN, Toronto, Canada) and were cultured as described previously [23].

2.2. Establishment of stable cell lines

MiaPaCa-2 stable cell lines overexpressing miR-29a, miR-330-5p or miR-neg (Table 1) were generated using lentiviral particles and miR-330-5p, miR-29a or miR-Neg pLenti4/TO/GFP-vectors and ViraPower lentiviral packaging mix (Life Technologies) as described before [22,23].

2.3. Transient cell transfections

Transfections of pre-miRTM (30 nM) miRNA precursors (Ambion) in the cell lines were performed in 6-well plates during 48 h using siPORTTM NeoFXTM Transfection Agent (Ambion) and OptiMEM[®] (Life Technologies) according to the manufacturer's instructions. Pre-miRTM miRNA precursor Negative Control #1 (Ambion) was used as control. Total RNAs were extracted using miRNeasy Mini Kit with Qiazol[®] (Qiagen). Total proteins were extracted as above. Three independent experiments were performed in triplicate. For loss-of-function studies in stable cell lines overexpressing miR-29a or miR-330-5p, transfection of anti-miRTM (60 nM) miRNA inhibitors (Ambion) was performed as indicated above. Anti-miRTM miRNA Inhibitor Negative Control #1 (Ambion) was used as control.

2.4. Cell proliferation, migration and invasion assays

Transfected cells or stable cell lines were seeded at 10^5 cells per well in 6-well plates. Cells were counted every day using a Malassez counting chamber using Trypan Blue exclusion dye (Life Technologies) during 72 h for transfected cells and during 96 h for stable cell lines [5]. Invasion and migration assays were respectively performed using 24-well Boyden chambers (8 μ m pores) coated or not with MatrigelTM (Pharmingen, BD Biosciences). Briefly, 5×10^4 to 10^5 cells were seeded on the top chamber and FBS 10% (v/v) was used as a chemoattractant in the bottom chamber for 24 h to 48 h [5]. Three independent experiments were performed in triplicate.

2.5. Cytotoxicity assay

10^4 cells were seeded in 96-well plates during 24 h. Medium was refreshed with gemcitabine, 5-Fu, oxaliplatin or SN-38 for 72 h. The viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay (MTT, Sigma-Aldrich, Saint Quentin Fallavier, France) as previously described [5]. Formazan crystals were solubilized in dimethylsulfoxide (Sigma-Aldrich) and analyzed at 570 nm with a microplate reader (Bio-Rad).

2.6. Human pancreatic tissues

Human PDAC samples were obtained from the Lille University Hospital with the approval of the Institutional Review Board. No patient received either chemotherapy or radiotherapy before the surgical resection except one patient.

2.7. Pdx-1-Cre; LStopL-Kras^{G12D} mouse model of early pancreatic carcinogenesis

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). All procedures are in accordance with the guidelines of animal care ethical committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, #AF042008) [23].

2.8. Subcutaneous xenografts

Subcutaneous (SC) xenografts (2×10^6 cells in 100 μ l of RPMI 1640 or DMEM media) of miR-neg, miR-29a or miR-330-5p cell lines were carried out with 100 μ l of MatrigelTM (BD Biosciences) into the flank of severe-combined immunodeficient (SCID) male mice (CB-17, Charles Rivers) that were bred and maintained under pathogen-free conditions (6 mice/group). Tumor development was followed weekly. The tumor volume (mm^3) was determined by calculating $V = W^2 \times L$ in which W corresponds to the width (mm) and L to the tumor length (mm). Mice were sacrificed 28 days after inoculation. All procedures were performed in accordance with the guidelines and approved by the animal care ethical committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, CEEA #122012) [5]. For intra-tumoral injection of miRNAs, 2×10^6 cells for Capan-2 and 3×10^6 cells for MiaPaCa-2 were xenografted. After 10 days, 20 μ g of miRNAs or miR-neg cloned into the pCDNA6.2emGFP vector was injected into the SC tumors ($\sim 300 \text{ mm}^3$) using Exgen 500 (Euromedex) reagent and glucose 5% (v/v) as described by the manufacturer's instructions. Tumors were followed twice a week and mice were sacrificed 20 days after miRNA injection.

2.9. Cloning of MUC1 3'-UTR, MUC1 5'-UTR/first exon and luciferase assays

Human MUC1 3'-untranslated region (UTR) or MUC1 first exon containing the 5'-UTR was cloned into the pGL3-promoter vector 183

Table 1

Characteristics of the primers used for qRT-PCR, cloning and site-directed mutagenesis.

Gene	Forward/reverse	Sequences 5' → 3'
<i>MUC1</i>	Forward	TGCCGCCGAAAGAAGTACG
	Reverse	TGGGGTACTCGCTCATAGGAT
<i>GAPDH</i>	Forward	CCACATCGCTCAGACACCAT
	Reverse	CCAGGCGCCCAATACG
<i>U6</i>	Forward	CGCAAGGATGACACGCAA
<i>u6</i>	Forward	TGGCCCTGCGCAAGGATG
<i>hsa-miR-330-5p</i>	Forward	TCTCTGGGCTGTGTCTTAGGC
<i>hsa-miR-29a</i>	Forward	TAGCACCATCTGAAATCGGTTA
<i>hsa-miR-183</i>	Forward	TATGGCACTGGTAGAATTCAT
<i>hsa-miR-200a</i>	Forward	TAACACTGTCTGGTAACGATGT
<i>hsa-miR-876-3p</i>	Forward	TGGTGGTTTACAAAGTAATTC
<i>hsa-miR-939</i>	Forward	TGGGGAGCTGAGGCTCTGGGGGTG
<i>MUC1</i> 3'-UTR <i>XbaI</i>	Forward	CGCTCTAGATCTGCCAATTGTAG
	Reverse	CGCTCTAGATTGGCGCAGTGGGAGA
<i>MUC1</i> 3'-UTR mut miR-330-5p #1	Forward	CCCAGGAGGACTGAAGCAACAAGCCCTGAGATAGC
	Reverse	GCTATCTCAGGGCTTGTGCTTCAGTCTCTCTGGG
<i>MUC1</i> 3'-UTR mut miR-29a	Forward	CTGTTTGGGCTGGTGAAGTCTGAGTGGGCAATCAGACCTCTT
	Reverse	AAGGAGGCTGTGATTGGCCACCGTACTCCAGCTCACCAGCCCAACAG
<i>MUC1</i> 3'-UTR mut miR-330-5p #2	Forward	CTCAGGTTCTCAGGGCGCCGCCCTGCACCCTGTTT
	Reverse	AAACAGGGTGCAGGGGCGCGCCCTGAAGAACCTGAG
<i>MUC1</i> 5'UTR <i>HindIII</i>	Forward	CGAAGCTTCCACCTCTCAAGCAGCCA
	Reverse	CGCAAGCTTCTGAAGCACTGTGAGGAGC
<i>MUC1</i> 5'-UTR mut miR-876-3p	Forward	CCTCCCCACCATTTCACTCTAACCATGACACCGGGCACC
	Reverse	GGTGGCCGGTGTATGTTAGTGAATGGTGGGGAGG
<i>MUC1</i> 5'-UTR mut miR-939	Forward	GTGGTGGTGAATGGTTATAAGGGGGCAGAACAGATTACGGCAG
	Reverse	CTGCCTGAATCTGTTCTGCCCCCTATAACCCATTTCACACAC
<i>LV-hsa-miR-29a</i>	Forward	TGCTGTAGCACCCTGAAATCGGTTAGTTTGGCCACTGACTACTAACCGATTTCATGGTGCTA
	Reverse	CCTGTAGCACCCTGAAATCGGTTAGTCTAGTCACTGGCCAAAACCTAACCGATTTC
<i>LV-hsa-miR-330-5p</i>	Forward	TGCTGTCTCTGGGCTGTGTCTTAGGCGTTTGGCCACTGACTGACGCTAAGACACACCCAGAGA
	Reverse	CCTGTCTCTGGGCTGTGTCTTAGGCGTCACTGAGGCGCAAAACGCTAAGACACAGGCCAGAGAC
<i>LV-hsa-miR-neg</i>	Forward	AAATGTACTGCGGTGAGACGTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
	Reverse	AAATGTACTGCGGTGAGACGTCAGTCACTGAGGCGCAAAACGCTCCACGCAGTACATTT

(Promega) using *XbaI* or *HindIII* restriction sites (Biolabs) (Table 1). Mutations were performed using the QuickChange® XL site-directed mutagenesis kit (Stratagene, Agilent Technologies) (Table 1). Luciferase activity and protein assays were performed as previously described [23,26]. Luciferase assays were carried out three times in triplicate.

2.10. Biotin pull-down assay

10⁶ cells were transfected with miR-29a, miR-330-5p or scramble 3'-biotinylated miRNA (100 nM) (Dharmacon, Thermo Scientific) as described above. Biotin pull-down assays were performed in Capan-1, Capan-2 and MiaPaCa-2 cell lines in triplicate in three independent series of experiments as previously described [23,27]. Pulled-down RNA was directly extracted from the beads or from the input RNA using the miRNeasy Mini kit (Qiagen). Reverse transcription and qRT-PCR were then performed.

2.11. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA including miRNAs from cells and formaldehyde-fixed paraffin-embedded tissues was extracted using miRNeasy Mini Kit with Qiazol® (Qiagen) and RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Life Technologies) according to the manufacturer's instructions. RNA (1 µg) was reverse transcribed with the QuantiMiR Reverse Transcription Kit (System Biosciences, Ozyme). For mRNA, cDNA was prepared as previously described [5,26]. qPCR was performed using SsoFast Evagreen® Supermix (Bio-Rad) and the CFX96 real time PCR system (Bio-Rad). Expression levels of *MUC1* and miRNAs of interest were respectively normalized to *GAPDH* and human *U6*/mouse *u6* (Table 1). MiRNA universal primers were provided with the

quantiMiR™ kit (System Bioscience, Ozyme). Expression levels were calculated using the 2^{-ΔΔCt} method. Three independent experiments were performed.

2.12. Protein extraction and Western-blot analysis

Total proteins were extracted, electro-transferred, immunostained, and visualized as described before [5,23,26,28]. Antibodies used are MUC1 (M8, 1/250, from Pr D. Swallow); β-actin (A5441 AC15, 1/5000) from Sigma-Aldrich; cyclin D1 (sc-718, 1/250) and CDK6 (sc-177, 1/250) from Santa Cruz; and EGFR (#4267s, 1/500), Bcl-2 (#2872, 1/250), phospho P53 (#9284, 1/500), P53 (#9282, 1/500), phospho p42-44 MAPK (#9101, 1/500), p42-44 MAPK (#9102, 1/500), phospho Stat3 (#9145, 1/250), Stat3 (#9139, 1/500), β-catenin (#8480s, 1/1000), phospho AKT (#4060, 1/500), and AKT (#4691s, 1/500) from Cell Signaling, Ozyme. The signal was detected using LAS 4000 apparatus (Fujifilm). Densities of bands were integrated using image J analysis software and represented as histograms.

2.13. Immunohistochemistry

Human pancreatic tissues, tumor xenografts and mouse tissues were fixed in 10% (w/v) buffered formaldehyde, paraffin-embedded, cut at 4 µm thickness and applied on SuperFrost® slides (Menzel-Glaser, Thermo Scientific). Histology was assessed by staining tissues with Hematoxylin-Eosin. Manual immunohistochemistry (IHC) was carried out as described [30] and automatic IHC was performed with an automated immunostainer (ES, Ventana Medical System, Strasbourg, France) as described [5,31]. Detection of MUC1 in human tissues and xenografts was carried out with anti-MUC1 M8 Mab (1/50) [5] and in mouse tissues

with anti-MUC1 Ab5 (MH1, 1/200) (HM-1630P1, Neomarkers Labvision, Thermo Scientific).

2.14. Statistical analyses

Statistical analyses were performed using Graphpad Prism 4.0 software (Graphpad softwares Inc.). Data are presented as mean \pm SD or \pm SEM. Differences in the mean of samples were analyzed by the student's t test or one way ANOVA test with selected comparison using Tukey's HSD post-hoc test or Wilcoxon signed-rank test. Differences less than 0.05 were considered significant and were indicated with an "****". ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

3. Results

3.1. In vitro regulation of MUC1 by miRNAs in PDAC cell lines

Using miRanda, miRWalk and mirBase softwares, we found one potential binding site for miR-29a and two potential binding sites for miR-330-5p on MUC1 3'-UTR (Fig. 1A). Additionally, we found one binding site for miR-876-3p and miR-939 on MUC1 5'-UTR and one binding site in the 3'-UTR and the first exon of MUC1 for miR-183 and miR-200a. Overexpression of these miRNAs in PDAC cells induced a strong decrease of MUC1 expression compared to miR-neg (Fig. 1B). Co-transfection of MUC1 3'-UTR and the miRNAs of interest led to a significant decrease of luciferase activity in the presence of miR-29a or miR-330-5p (Fig. 1C). Mutation of miR-29a or miR-330-5p binding sites into MUC1 3'-UTR led to luciferase activity restoration for miR-29a and miR-330-5p site #2 but not for miR-330-5p site #1 (Fig. 1C). No significant variation of luciferase activity was found with any other miRNAs. To further validate the direct interaction between miR-29a, miR-330-5p and MUC1 mRNA we used an affinity-based approach with biotinylated miRNAs. In Capan-2 cells, we show a 1.5 fold enrichment of MUC1 mRNA for both miR-29a and miR-330-5p pulldown (Fig. 1D). The same fold enrichments were found in Capan-1 cell line (1.8 for miR-29a pulldown and 1.5 for miR-330-5p pulldown, not shown). In MiaPaCa-2 cell line, enrichments were stronger with a 2.5-fold enrichment of MUC1 mRNA for miR-29a pulldown and 4.4-fold for miR-330-5p pulldown (Fig. 1D). Altogether these results demonstrate that miR-29a and miR-330-5p (site #2) negatively regulate MUC1 expression by interacting directly with its 3'-UTR.

3.2. Expression of miR-29a, miR-330-5p and MUC1 in human and mouse PDAC cells and tissues

Having shown in vitro that miR-29a and miR-330-5p are direct negative regulators of MUC1 expression in PDAC cells, we undertook to study their expression in human PDAC cell lines and ex vivo in human and mouse pancreatic tissues. A decreased expression of miR-29a was found in the five PDAC cell lines (MiaPaCa-2, Capan-1, Panc-1, BxPC-3, and Capan-2) compared to normal HPDE cells (Fig. 2A). MiR-330-5p expression was decreased in MiaPaCa-2 and Capan-1 cells (Fig. 2A). Expression of MUC1 mucin in the different cell lines is also shown (Fig. 2A, insert). Pancreatic expression was then studied in a pre-clinical mouse model of pancreatic carcinogenesis (Kras^{G12D}) developing PanIN preneoplastic lesions in which we found a mild decreased of miR-29a and miR-330-5p expression compared to control mice (Kras^{WT}) (Fig. 2B) whereas Muc1 was overexpressed (Fig. 2C). In human PDAC samples, a significant decreased expression of miR-29a and miR-330-5p was found compared to non-tumoral adjacent tissues (Fig. 2D). This was correlated to overexpression and cytoplasmic delocalization of MUC1 (Fig. 2E). Altogether these results indicate a decreased expression of miR-29a or miR-330-5p, associated with MUC1 overexpression in human and mouse PDAC tissues.

3.3. Biological properties of PDAC cells transiently overexpressing miR-29a and miR-330-5p

To further study the functional activity of miR-29a and miR-330-5p, transient overexpression of these miRNAs was realized in Capan-2, MiaPaCa-2 and Capan-1 cell lines (Fig. 3A). MiRNA overexpression led to a significant decreased expression of both MUC1 mRNA (Fig. 3B) and protein (Figs. 1B and 3C) levels in all cell lines. Simultaneous overexpression of both miR-29a and miR-330-5p did not potentiate the effects. Overexpression of miR-29a or miR-330-5p in cells alone or in combination induced a significant decrease of cell proliferation (Fig. 3D), cell migration (Fig. 3E) and cell invasion (Fig. 3F). Altogether these results indicate that both miR-29a and miR-330-5p alter PDAC cell proliferation, migration and invasion.

3.4. Biological properties of PDAC cells stably overexpressing miR-29a and miR-330-5p

Having shown that miR-29a, miR-330-5p and MUC1 had an inverse profile of expression in PDAC, and that these two miRNAs altered biological properties of PDAC cells, we established stable cell lines overexpressing either miR-29a or miR-330-5p in MiaPaCa-2 cells in order to further assess their biological effects (Fig. 4A). A strong decrease of MUC1 mRNA and protein expression was observed in stable cell lines compared to control cells (Fig. 4B & C). Cells overexpressing miR-29a or miR-330-5p showed a statistically significant decrease of cell proliferation (Fig. 4D), migration and invasion (Fig. 4E). Accordingly to the decreased of cell proliferation, we observed a decrease of phospho p42–44 MAPK, cyclin D1 and β -catenin expression in the two cell lines and a decrease of CDK6 and EGFR in cells overexpressing miR-29a (Fig. 4C). Finally, we performed loss-of-function studies using anti-miRNAs and measured impact on cell proliferation (Fig. 4F) and cell migration/invasion (Fig. 4G). The results indicate a statistically significant increase of cell proliferation (Fig. 4F), migration and invasion (Fig. 4G) for both anti-miRNAs. Altogether, these results indicate that PDAC cells overexpressing transiently or stably miR-29a or miR-330-5p harbor an altered PDAC cell behavior that is less tumorigenic.

3.5. Effect of miR29a and miR-330-5p on PDAC cell chemosensitivity, survival and apoptosis

PDAC being an extremely chemoresistant cancer, we undertook to evaluate miR-29a and miR-330-5p effects on PDAC cell chemosensitivity. Measurement of cell survival following drug treatment indicated that overexpression of miR-29a or miR-330-5p led to a significant decrease of cell viability following treatment with gemcitabine whereas no significant variation was found with drugs of the FOLFIRINOX protocol (oxaliplatin, 5-Fu or SN-38) (Fig. 5A). IC₅₀ determination also indicated that stable cell lines overexpressing miR-29a (~383 nM) or miR-330-5p (~239 nM) were more sensitive to gemcitabine than miR-neg cells (~646 nM) (Fig. 5B). We then measured the activation of major signaling markers/pathways involved in cell survival or cell apoptosis in these cellular models. We found an increased level of apoptotic marker phospho-P53 and a decrease of anti-apoptotic marker Bcl-2 together with a decrease of phospho-Akt and phospho-Stat3 (Fig. 5C). Altogether, these results indicate that PDAC cells overexpressing miR-29a or miR-330-5p are more sensitive to gemcitabine, survival less and are more apoptotic.

3.6. In vivo biological effects of miR-29a and miR-330-5p on PDAC tumor properties

Having shown in vitro that miR-29a and miR-330-5p possessed tumor suppressive activities, we undertook to evaluate their activity in vivo. To this aim, subcutaneous (SC) xenografts of Capan-2 and

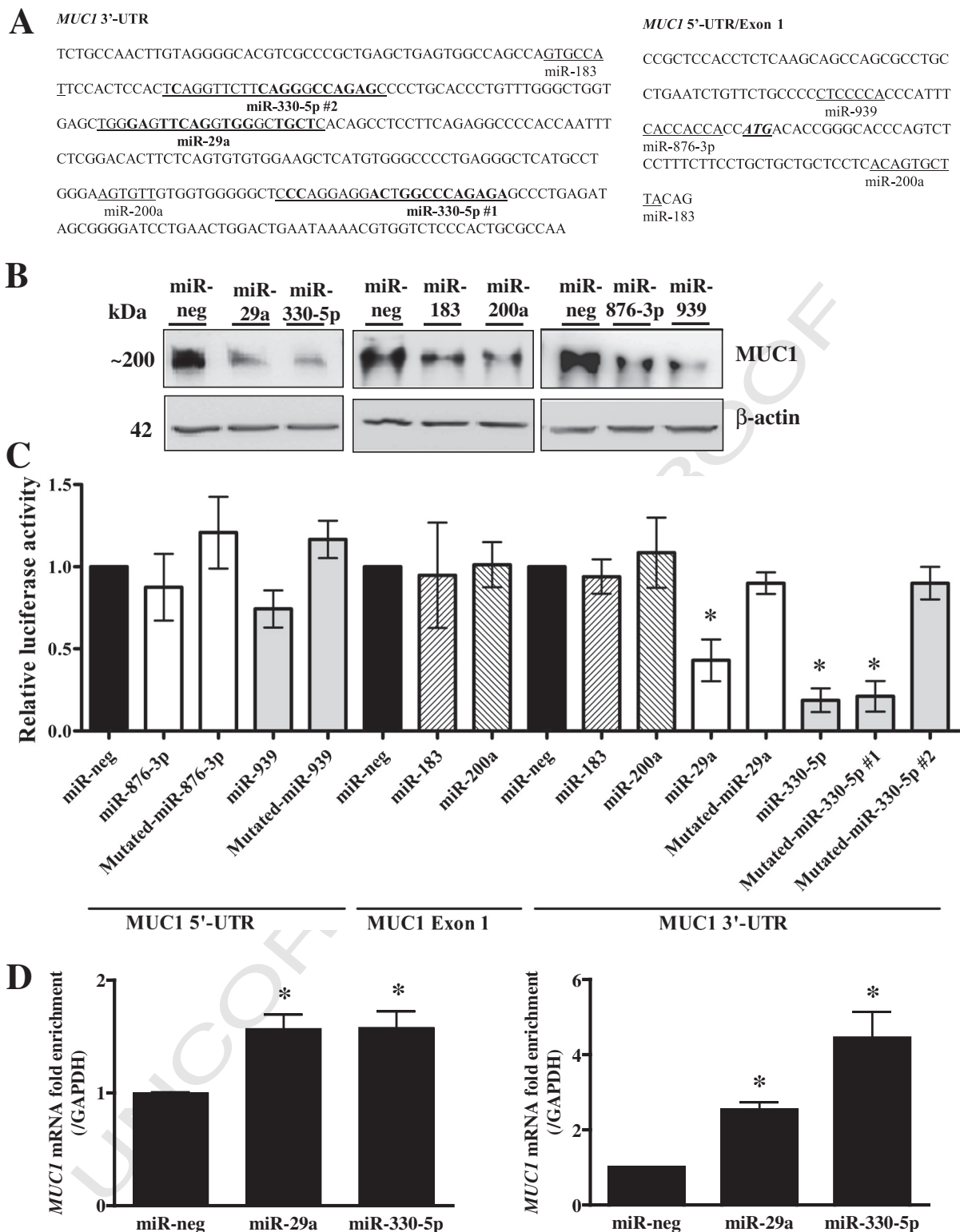


Fig. 1. *MUC1* regulation by miRNAs in PDAC cell lines. (A) Sequence of *MUC1* 3'-UTR and position of miR-29a, miR-330-5p, miR-183, and miR-200a potential binding sites (left) and sequence of *MUC1* first exon containing its 5'-UTR and position of miR-876-3p, miR-939, miR-183, and miR-200a potential binding sites (right). (B) Study of the expression of *MUC1* in Capan-2 cells transfected with the miRNA of interest or miR-neg by Western-blot. (C) Measurement of luciferase activity in Capan-2 cells co-transfected with miRNAs of interest and *MUC1* 3'-UTR or *MUC1* Exon1 containing the 5'-UTR cloned into pGL3 promoter luciferase-reporter vector. The miR-neg luciferase activity is arbitrarily set to 1. (D) Biotinylated miR-29a or miR-330-5p or scramble miRNAs (miR-neg) were transfected in Capan-2 (left panel) and MiaPaCa-2 (right panel) cells. Enrichment of *MUC1* mRNA in miRNA pulldown was assessed by qRT-PCR. The *MUC1*/GAPDH ratio was calculated and normalized to the input.

MiaPaCa-2 cells were performed in SCID mice followed by an intra-tumoral injection of the miRNA of interest. A significant decrease of Capan-2 ($p < 0.05$) and MiaPaCa-2 ($p < 0.001$) tumor growth was

found when compared to control miR-neg (Fig. 6A). Tumor weights were also significantly decreased when injected with miR-29a in Capan-2 ($p < 0.05$) or both miRNAs in MiaPaCa-2 ($p < 0.05$) (Fig. 6B).

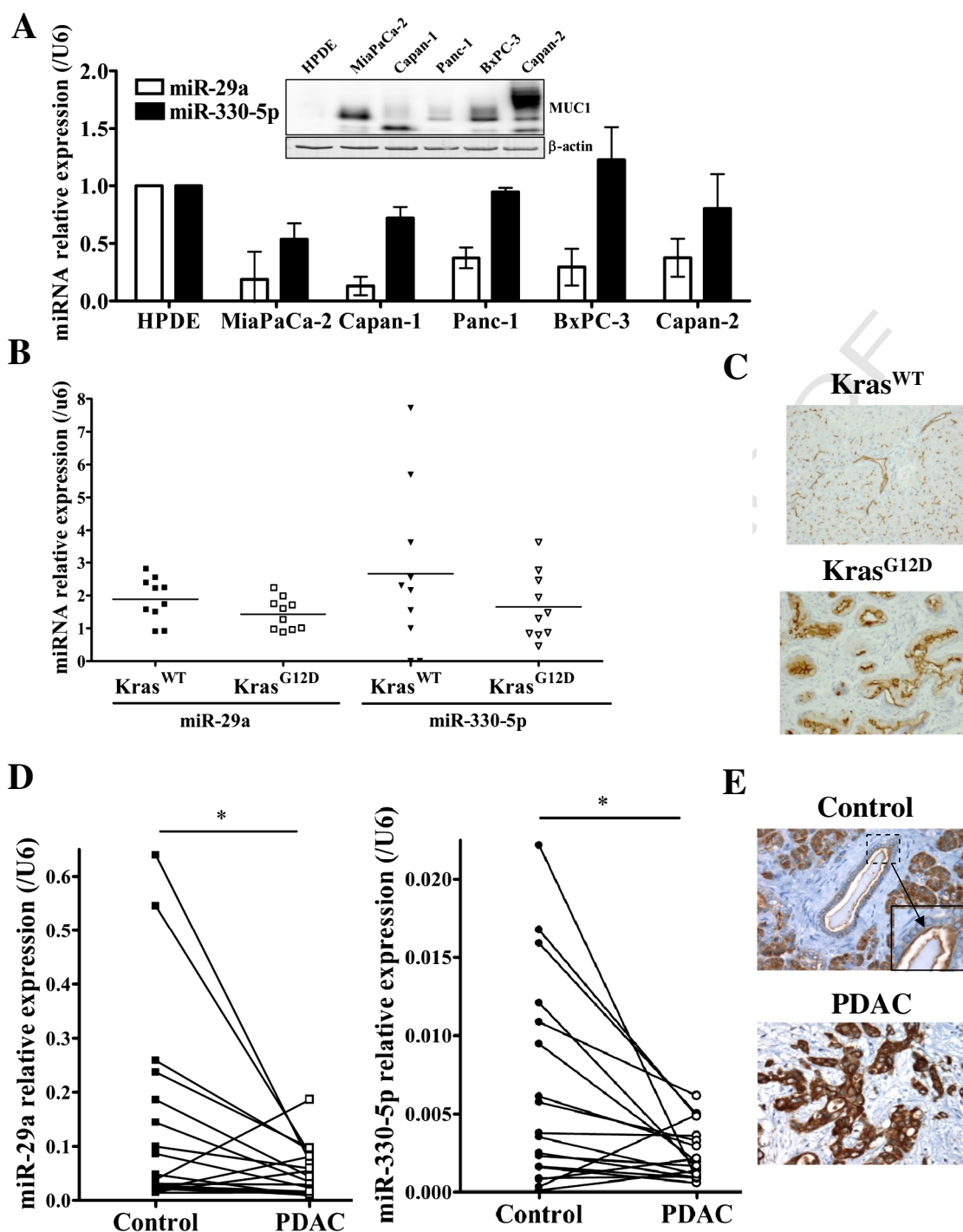


Fig. 2. MiRNA expression in PDAC cells and tissues. (A) Expression of miRNAs in HPDE, MiaPaCa-2, Capan-1, Panc-1, BxPC-3, and Capan-2 cell lines was studied by qRT-PCR. MiRNA expression was normalized to control U6 RNA. MiRNA level in normal pancreatic ductal cells HPDE was arbitrarily set to 1. Insert: expression of MUC1 mucin by Western-blotting. (B) MiRNA expression in control Kras^{WT} and Kras^{G12D} mice studied by qRT-PCR. MiRNA expression was normalized to control u6 RNA. Expression in Kras^{WT} mice was arbitrarily set to 1. (C) MUC1 expression by IHC in control Kras^{WT} and in Kras^{G12D} mice. (D) MiR-29a and miR-330-5p expression in a cohort of 18 patients with PDAC by qRT-PCR. MiRNA expression was normalized to control U6 RNA and expressed as a ratio of PDAC/non-tumoral adjacent tissues (control). (E) MUC1 expression by IHC in non-tumoral adjacent tissues (control) and in tumors (PDAC). As an illustration, results with patient #1 are shown.

SC xenografts of the MiaPaCa-2 cells stably overexpressing miR-29a or miR-330-5p were also carried out, in which a significant decrease of tumor growth was observed ($p < 0.01$) (Fig. 6C). This was correlated to a strong decrease of MUC1 expression (Fig. 6D). Altogether these results indicate that in vivo miR-29a and miR-330-5p possess tumor suppressive activities on PDAC tumors.

4. Discussion

Pancreatic Ductal Adenocarcinoma suffers from a very poor prognosis and absence of early detection and efficient therapy [2,19]. Recently, miRNAs have emerged as very attractive molecules to target cancer since they regulate many genes encoding oncogenic proteins [20]. In

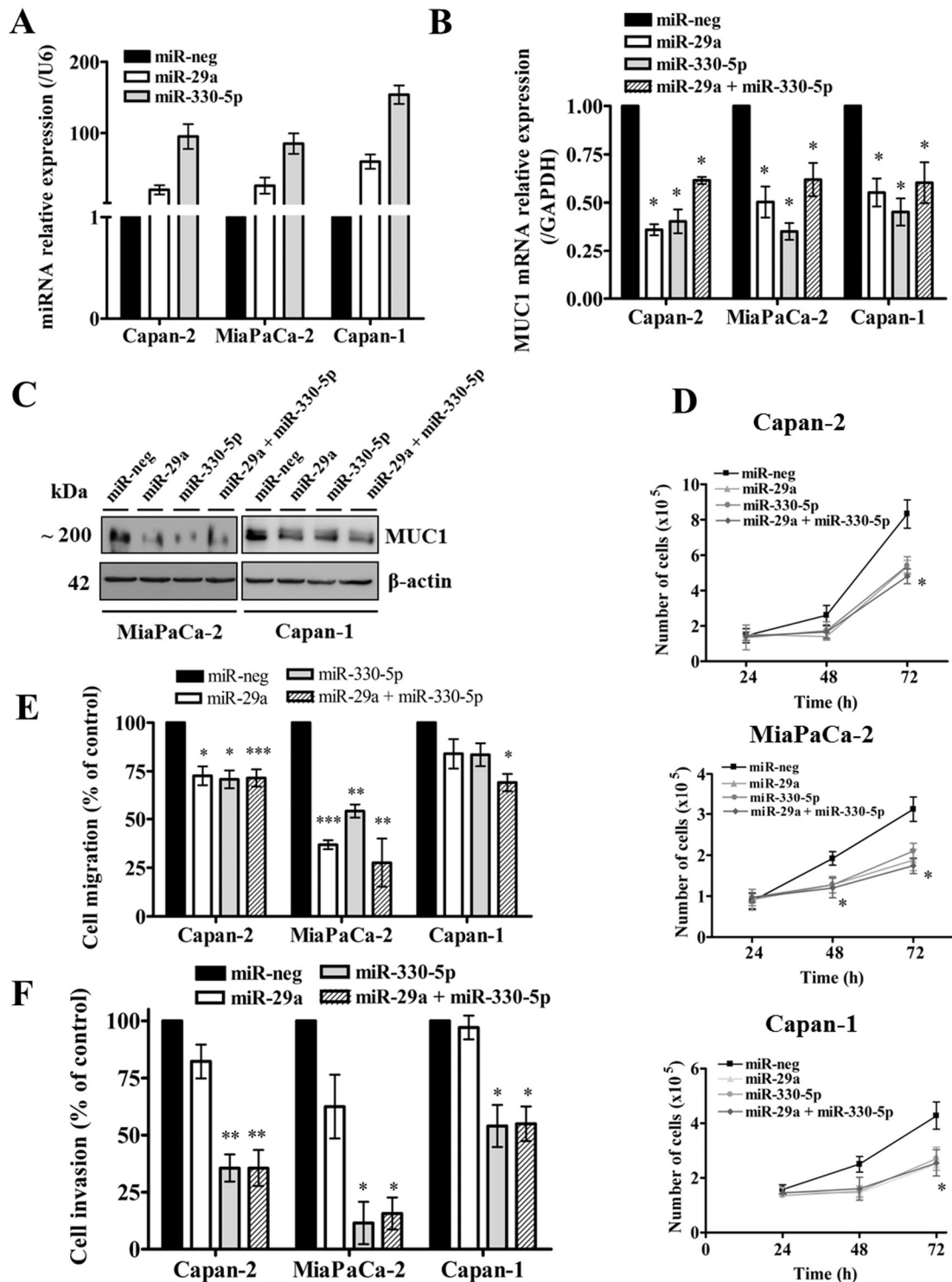
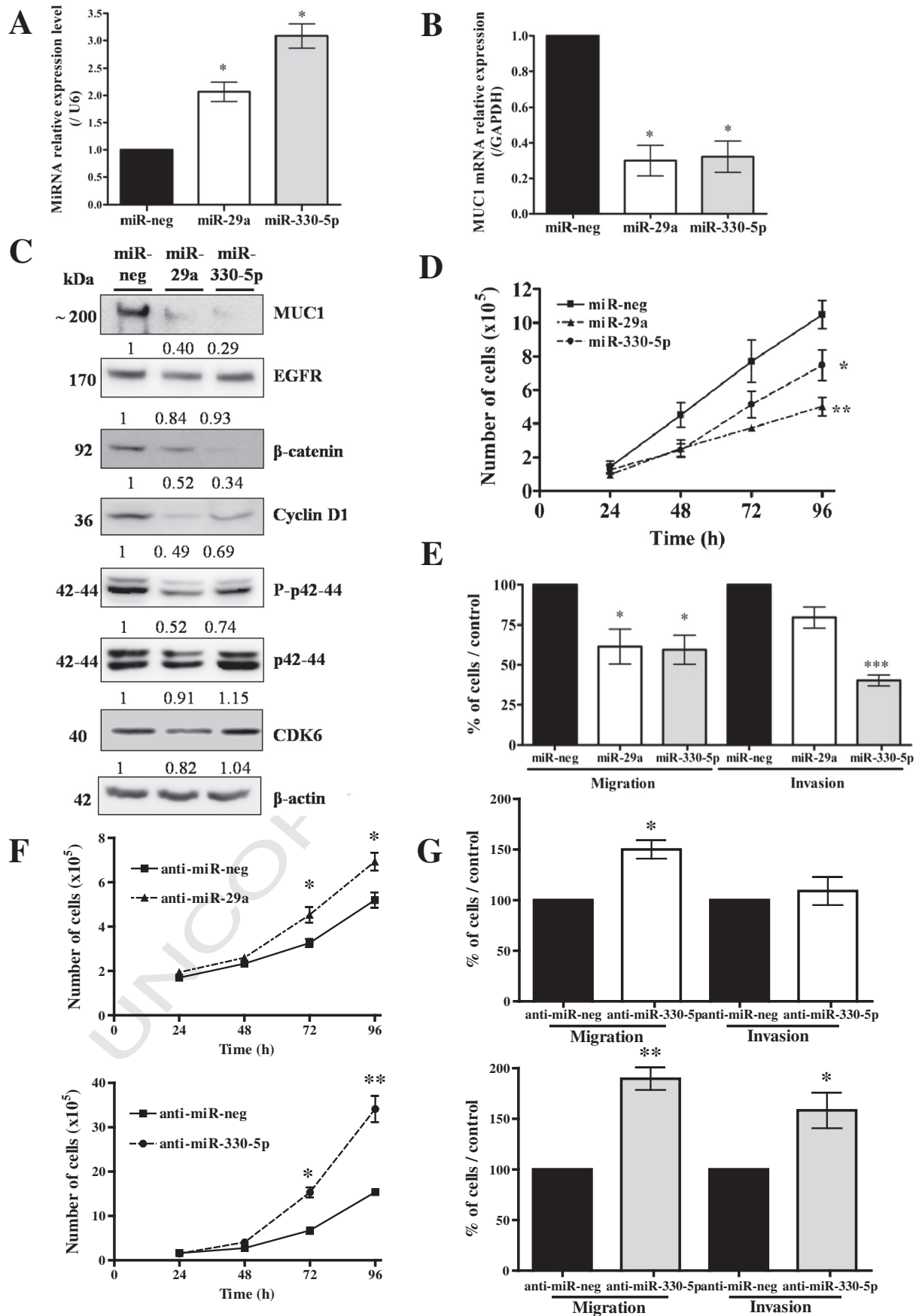


Fig. 3. Biological properties of PDAC cells transiently overexpressing miR-29a or miR-330-5p. (A) Expression of miR-29a and miR-330-5p was measured after transient overexpression in Capan-2, MiaPaCa-2 and Capan-1 cells by qRT-PCR. MiRNA expression was normalized to control U6 RNA. The miR-neg expression was arbitrarily set to 1. (B) Analysis of MUC1 mRNA expression in Capan-2, MiaPaCa-2 and Capan-1 cells transfected with pre-miRNAs of interest or miR-neg by qRT-PCR. MUC1 expression was normalized to control GAPDH. The miR-neg expression was arbitrarily set to 1. (C) Analysis of MUC1 protein expression in MiaPaCa-2 and Capan-1 cells transfected with pre-miRNAs of interest or miR-neg by Western-blot. (D) Cell proliferation, (E) cell migration and (F) cell invasion were measured in Capan-2, MiaPaCa-2 and Capan-1 cells transfected with pre-miRNAs. (E, F) Results are expressed as a percentage of migration or invasion in miR-transfected cells compared to the control miR-neg (100%).

PDAC, several studies have described the pattern of expression of miRNAs but their exact biological role and the identification of their targets remain largely unknown [22,23]. In this report, we have identified miR-29a and miR-330-5p as two new miRNAs directly targeting the 3' UTR of MUC1 oncogenic mucin in PDAC cells and demonstrate that these two miRNAs display tumor suppressive activities in PDAC both

in vitro and in vivo. We thus propose miR-29a and miR-330-5p as appropriate targets to inhibit MUC1 expression in order to slow-down pancreatic carcinogenesis and develop new potential therapeutic tools. MiR-29a and miR-330-5p were shown to inhibit MUC1 expression by directly binding to its 3'-UTR but interestingly we have also identified additional potential binding sites for miR-29a and miR-330-5p



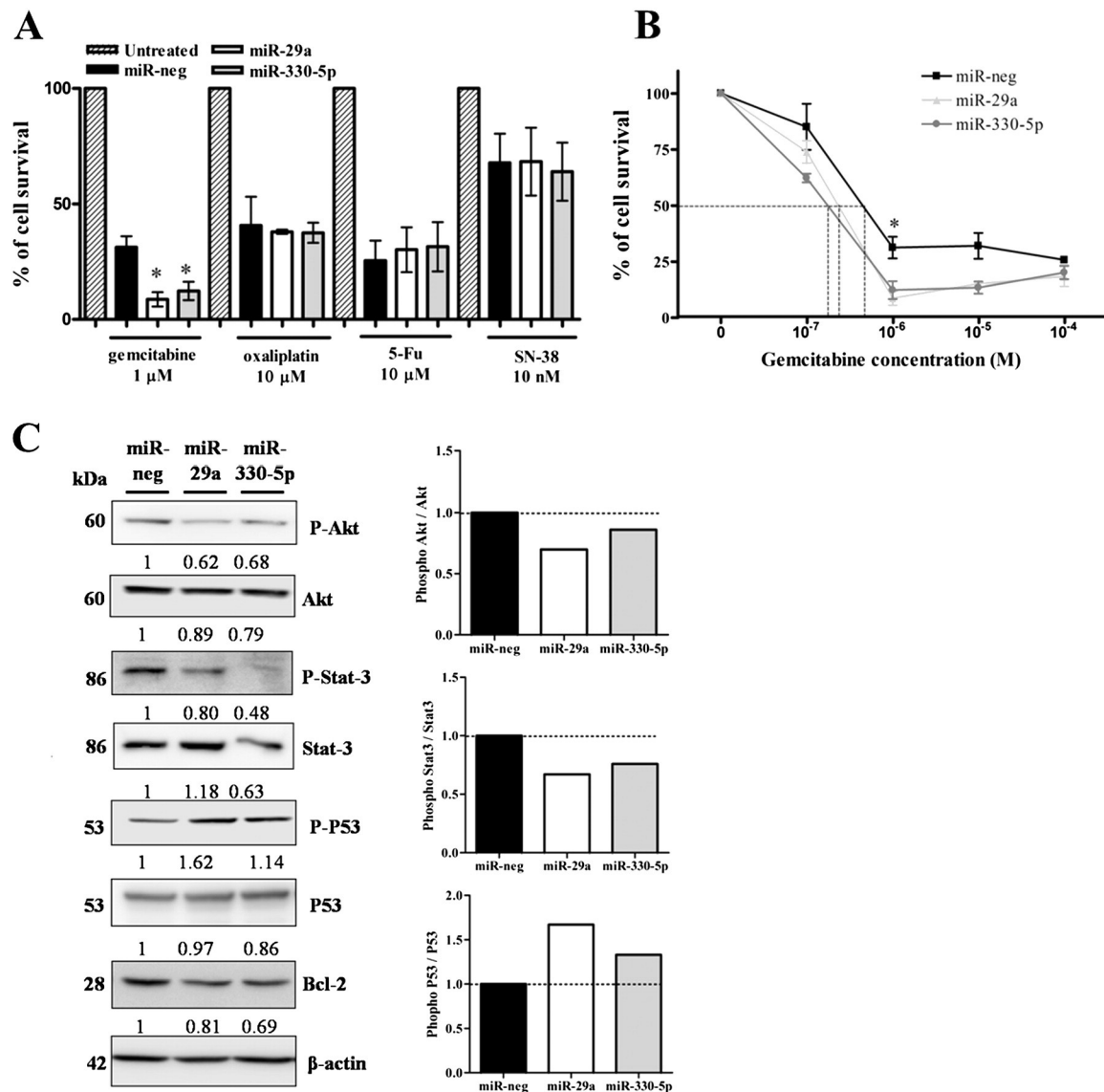


Fig. 5. Chemosensitivity of PDAC cells overexpressing miR-29a or miR-330-5p. (A) Cells were treated for 72 h with different concentrations of gemcitabine, 5-Fu, oxaliplatin or SN-38 before cell survival measurement. (B) IC_{50} rates after 72 h of gemcitabine treatment. (C) Analysis of phospho-Akt, Akt, phospho Stat3, Stat3, phospho-P53, P53 and Bcl-2 expression by Western-blot. Density of each marker was measured and protein of interest/ β -actin ratio is indicated. Expression in miR-Neg cells was arbitrarily set to 1. Phospho Akt/Akt, phospho Stat3/Stat3, and phospho P53/P53 ratios were determined and represented as histograms.

into *MUC1* coding regions suggesting that the inhibition of *MUC1* could also involve the binding of these two miRNAs to additional regions. The validation of these sites will be of particular interest especially because it was shown that *MUC4* and *MUC16* oncogenic membrane-bound mucins are regulated by miRNAs through their coding regions [32]. This could thus represent a general mechanism of mucin gene regulation by miRNAs.

In breast cancer cells, *MUC1* is regulated by miR-145 [33], miR-1226 [34] and miR-125b [35]. In this report we show that, *MUC1* is directly regulated by miR-29a and miR-330-5p and indirectly regulated by miR-876-3p, miR-939, miR-183 and miR-200a in PDAC cells. MiR-183 and miR-200 families are EMT inhibitors and favor epithelial

differentiation [36] by inhibiting the Wnt/ β -catenin signaling pathway [37,38]. The membrane-bound mucin *MUC1* is an epithelial marker of polarized epithelial cells [4,6,7] but aberrant overexpression of *MUC1* in cancer cells is correlated with an induction of EMT through the Wnt/ β -catenin signaling pathway [14]. The indirect inhibition of *MUC1* by miR-183 and miR-200a could be an additional mechanism to inhibit EMT in PDAC. The role of miR-876-3p and miR-939 is hardly known in cancer. The indirect mechanisms leading to *MUC1* inhibition will have to be identified in future studies. The identification of predicted partners such as EGFR, cyclin D1, and Stat3, indirectly regulating *MUC1*, that are potential targets of miR-876-3p or miR-939 (personal communication) suggests that targeting these miRNAs could represent

Fig. 4. Biological properties of PDAC cells stably overexpressing miR-29a or miR-330-5p. (A) Expression of miR-29a and miR-330-5p in stable cell lines was measured by qRT-PCR. MiRNA expression was normalized to control *U6* RNA. The miR-neg expression was arbitrarily set to 1. (B) Analysis of *MUC1* mRNA expression by qRT-PCR. *MUC1* expression was normalized to control *GAPDH*. The miR-neg expression was arbitrarily set to 1. (C) Analysis of *MUC1*, EGFR, β -catenin, cyclin D1, phospho-p42-44 MAPK, p42-44 MAPK, and CDK6 expression by Western-blot. Density of each marker was measured and protein of interest/ β -actin ratio is indicated. Expression in miR-Neg cells was arbitrarily set to 1. (D) Measurement of cell proliferation and (E) cell migration and invasion. (E) Results are expressed as a percentage of migration or invasion of cells compared to the control miR-neg or Mock cells (100%). Measurement of cell proliferation (F) and cell migration/invasion (G) in MiaPaCa-2 stable cell lines overexpressing miR-29 (top panel) or miR-330-5p (bottom panel) treated with anti-miR-neg, anti-miR-29a or anti-miR-330-5p at 60 nM.

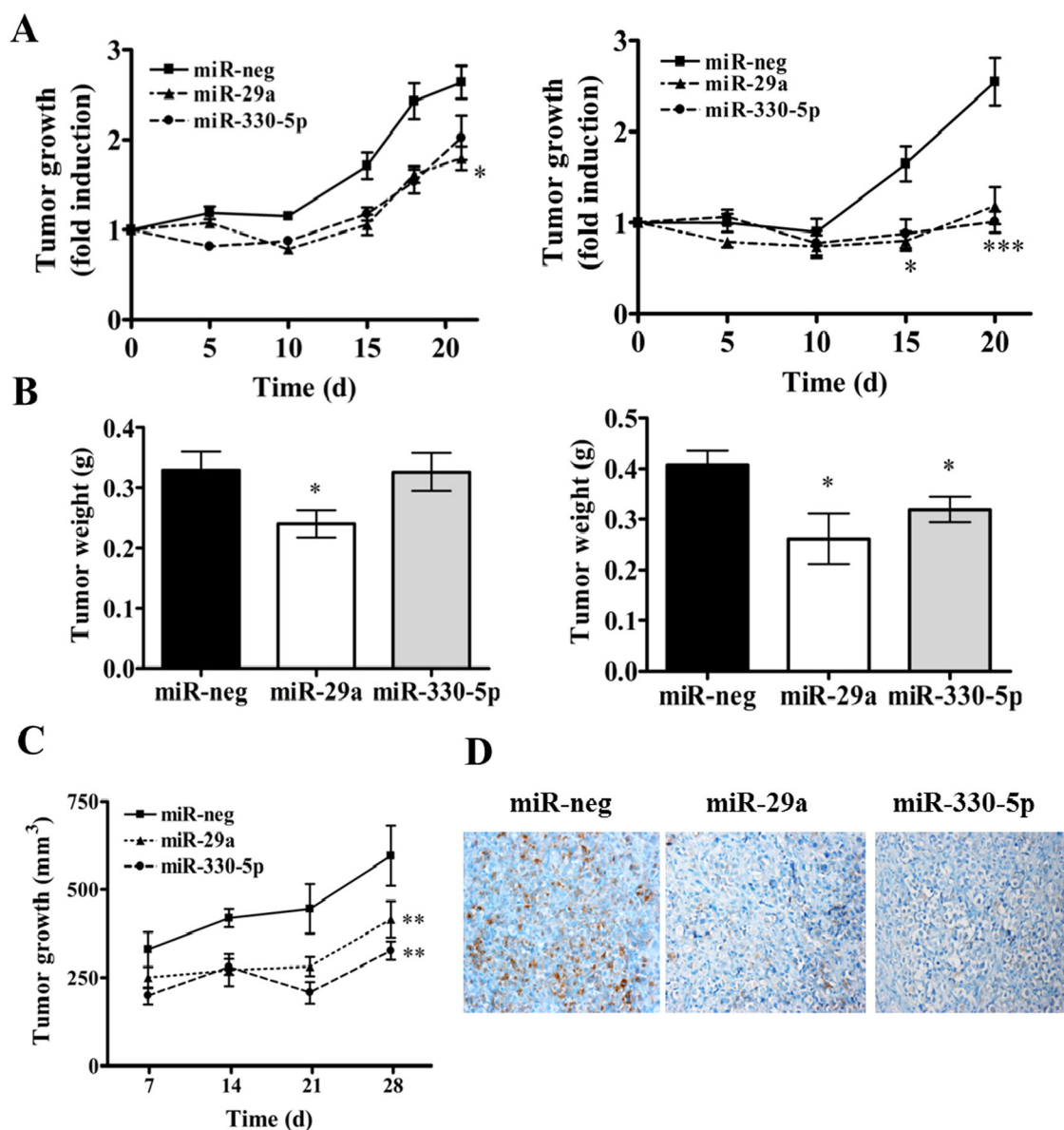


Fig. 6. In vivo effects of miR-29a and miR-330-5p on PDAC tumor properties. Subcutaneous (SC) xenografts of Capan-2 (left panel) and MiaPaCa-2 (right panel) cells in SCID mice. Intratumoral injection of miR-neg, miR-29a or miR-330-5p was performed 10 days post-xenografting (~300 mm³). (A) Tumor growth (mm³) was measured for 20 days after miRNA injection. (B) Tumor weight (g) was measured at the sacrifice in Capan-2 (left panel) and MiaPaCa-2 (right panel) tumors injected with miR-neg, miR-29a or miR-330-5p. (C) SC xenografts of MiaPaCa-2 cells stably overexpressing either miR-neg, miR-29a or miR-330-5p in SCID mice. Tumor growth (mm³) was measured during 20 days. (D) MUC1 protein expression by IHC in SC tumors.

an interesting approach to slow-down carcinogenesis. We cannot also exclude that miR-145, miR-1226 and miR-125b known to regulate MUC1 in breast cancer cell lines may also be MUC1 regulators in pancreatic cancer cells. Some preliminary data obtained in our laboratory seem to indicate that these miRNAs also downregulate MUC1 in pancreatic cancer cells (personal communication).

We also demonstrate that miR-29a and miR-330-5p inhibit MUC1 expression and deregulate signaling pathways as well as PDAC cell proliferation, migration, invasion and tumor growth. It is well-established that MUC1 is involved in these pathways [5,39]. These mechanisms may involve p42–44 MAPK, cyclin D1 and β -catenin as we recently showed that inhibiting MUC1 in PDAC cells altered these pathways [5]. It was also proposed that the overexpression of miR-29a decreased β -catenin expression and cell proliferation in non-small cell lung cancer [40]. The same effect was observed in miR-29a overexpressing PDAC cells and in PDAC cells lacking MUC1 [5]. The overexpression of miR-330-5p in colorectal cancer cells was previously shown to decrease

cell proliferation through direct regulation of Cdc42 and decrease expression of cyclin-D1 [41]. The same effect was observed in PDAC cells overexpressing miR-330-5p. Moreover, the fact that miR-29a alters proliferation more profoundly than miR-330-5p suggests that both miRNAs have additional targets. In that matter, we observed the decreased expression of CDK6 and Bcl-2 two known targets of miR-29a when it was overexpressed [42,43]. Altogether these data are in favor of a tumor suppressor activity for these two miRNAs in PDAC cells.

Contrarily to miR-330-5p, overexpression of miR-29a had a modest effect on PDAC cell invasion. This could result from the inhibition of CDK6 that we observed in these cells, loss of CDK6 being a marker of several PDAC cell line invasiveness [44]. This may also be due to other properties of miR-29a that depend on the cellular context: increased invasion through MMP2, E-cadherin and KLF4 in colorectal cancer cells [45], decreased invasion in gastric [46], lung and pancreatic cancer cells [47], or decreased invasion and metastasis probably through the inhibition of CEACAM6 in pancreatic cancer [48].

Additionally, we found that overexpression of miR-29a or miR-330-5p sensitizes PDAC cells to gemcitabine and induces a decrease of Akt pathway activation. Interestingly, MUC1 is known to mediate PDAC cell chemoresistance to gemcitabine by regulating *MRP1* gene expression through the PI3K/Akt pathway [5,15]. Although, miR-29a overexpression was previously shown to induce gemcitabine resistance in other PDAC cells (BxPC-3 and Panc-1) through the Wnt/ β -catenin signaling pathway [49], other results in lung cancer cells indicate that overexpression of miR-29a inhibits Wnt/ β -catenin signaling and β -catenin expression [40]. The same effects were described in PDAC cells lacking MUC1 [5]. Interestingly, it was also shown that miR-29a inhibits Akt expression [50] and sensitizes ovarian cancer cells to cisplatin [51], and that miR-330-5p overexpression decreased Akt phosphorylation in colorectal cancer [41]. The decrease of Akt expression suggests a MUC1/Akt dependent mechanism mediating miR-29a/miR-330-5p effects on MiaPaCa-2 cell sensitivity to gemcitabine. Altogether, these data highlight the complex role of these miRNAs in chemoresistance processes triggered by cancer cells that most likely are cell-specific. Further investigation will be needed to decipher the mechanisms underlying the sensitivity of PDAC cells to chemotherapeutic drugs in relation with MUC1, miRNA and Akt expression. Moreover, we also showed that overexpression of miR-29a or miR-330-5p increases P53 activity as previously proposed for miR-29a [52] and decreases Bcl-2 expression like it was previously proposed in PDAC cells lacking MUC1 [5] suggesting an involvement of these two miRNAs in cell apoptosis.

We also show that miR-29a and miR-330-5p are deregulated in human PDAC cell lines and tissues, and in the *Kras*^{G12D} pre-clinical mouse model of pancreatic cancer. Expression studies in a cohort of 18 human PDAC tissues showed inhibition of miR-29a and miR-330-5p which is in accordance with previous studies showing that miR-29a is frequently deregulated in cancerous tissues. This confirms the potential of miR-29a as a target for cancer therapy as it was previously proposed [53] and highlights the interest for miR-330-5p in PDAC.

5. Conclusion

In conclusion, we show (i) that MUC1 is regulated by miRNAs in PDAC cells by direct and indirect mechanisms, (ii) that miR-29a and miR-330-5p are deregulated in PDAC and that PDAC cells overexpressing transiently or stably either miR-29a or miR-330-5p possess similar biological properties: in vitro decreased proliferative, migrating, and invasive properties and in vivo decreased tumor growth, and (iii) that miR-29a and miR-330-5p sensitize PDAC cells to gemcitabine. Based on that, we propose that tumor suppressive miR-29a and miR-330-5p may represent attractive targets to inhibit the expression of the oncogenic MUC1 mucin and decrease pancreatic cancer progression.

Author contribution

Solange Tréhoux conceived and designed the project, performed experiments, data analysis and interpretation, and wrote the paper. Fatima Lahdaoui performed in vitro experiments, data analysis, interpretation, and revised the manuscript. Isabelle Van Seuningen conceived and designed the project, provided financial support, data analysis and interpretation and wrote the paper. Nicolas Jonckheere performed in vivo experiments in mice models, data analysis and interpretation, and revised the manuscript. Florence Renaud and Emmanuelle Leteur performed anatomopathological analyses and revised the manuscript. Yannick Delpu and Jérôme Torrisani raised stable cell lines overexpressing miR-29a and miR-330-5p and revised the manuscript.

Conflicts of interest

Authors declare no conflict of interest.

Uncited reference

[29]

Acknowledgments

We thank D. Tuveson (Cambridge Research Institute, England) for the kind gift of *LStopL-Kras*^{G12D} mouse model; M. S. Tsao (University Health Network, Toronto, Canada) for the kind gift of HPDE cells; D. Swallow (MRC, London, UK) for providing MUC1 M8 antibody, and A. Lansiaux and S. Meignan (Centre Oscar Lambret and Inserm UMR837, team 3) for the chemotherapeutic drugs. We are grateful to B. Barbot and B. Duchêne (Inserm UMR837, team 5) and M.H. Gevaert and R. Siminsky (Department of Histology, Faculty of Medicine, University of Lille 2) for their excellent technical help. We thank the IFR114/IMPRT (University of Lille 2) facilities for luciferase measurements (A.S. Drucbert) and animal experimentation (D. Taillieu).

Funding: Solange Tréhoux is a recipient of a PhD fellowship of the University of Lille 2. Isabelle Van Seuningen is the recipient of a “Contrat Hospitalier de Recherche Translationnelle” (AVIESAN/CHRT 2010). Fatima Lahdaoui is a recipient of a SIRIC ONCOLille fellowship (Grant INCA-DGOS-Inserm 6041 (IVS)). This work was supported by grants from la Ligue Nationale Contre le Cancer (Equipe Labellisée Ligue 2011–2013, IVS), from SIRIC ONCOLille, Grant INCA-DGOS-Inserm 6041 (IVS) and from “Contrat de Plan Etat Région” CPER Cancer 2007–2013 (IVS).

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