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Q4 Micro-RNAs miR-29a and miR-330-5p function as tumor suppressors by 2 targeting the MUC1 mucin in pancreatic cancer cells

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A B S T R A C T

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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42 1. Introduction

43 Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth leading cause
44 of death by cancer in Western countries and has a very poor prognosis
45 due to a late diagnosis and a lack of efficient treatment. The five year sur-
46 vival rate is lower than 5% [1] and less than 20% of patients are entitled to
47 surgical resection [2]. The remaining 80% of patients present a locally ad-
48 vanced metastatic PDAC and may benefit from palliative chemotherapy
49 based either on gemcitabine or FOLFIRINOX [3]. Despite this, PDAC prog-
50 nosis remains very poor and is highly resistant to chemotherapeutic
51 treatments. It is thus mandatory to find early biomarkers, to better under-
52 stand 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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72 reasons, MUC1 appears as an attractive target to slow-down pancreatic
73 tumorigenesis.

74 MUC1 is also translocated to the nucleus and associated with co-
75 factors and/or transcription factors on gene promoters to modulate
76 their expression. It was recently shown that MUC1 regulates miRNA ex-
77 pression by binding with ZEB1 to the promoter of miR-200c resulting in
78 its inhibition and increased cell invasion and EMT [17]. Since miRNAs
79 regulate a large number of human genes, targeting regulatory mecha-
80 nisms mediated by miRNAs appears as an attractive strategy to inhibit
81 oncogenic proteins and propose new therapeutic approaches [18,19].
82 MiRNAs are 18 to 25 nucleotide long small non-coding RNAs which
83 regulate gene expression by binding to the 3'-UTR, 5'-UTR or coding re-
84 gions of their target mRNAs [20]. MiRNAs can also inhibit the translation
85 of their target or lead to the degradation of the mRNA and thereby pro-
86 tein expression [21]. MiRNAs are frequently deregulated in cancer, in-
87 cluding PDAC. Several studies have investigated the expression profile
88 of miRNAs in PDAC but few of them have studied their biological role
89 and/or biological relevance [22,23]. We recently showed that miRNA
90 can effectively inhibit oncogenic mucin MUC4 in PDAC through the
91 overexpression of tumor suppressive miRNA [23] and the mucin
92 MUC13 was also shown to be directly regulated by miRNA in PDAC
93 [24]. This highlights the interest in targeting oncogenic mucins in
94 PDAC which are known to mediate PDAC progression but so far no
95 miRNAs have been identified as targeting the oncogenic mucin MUC1
96 in PDAC.

97 In this report, we undertook to identify miRNAs directly binding to
98 MUC1 regulatory regions including the 5'-UTR, the coding region and
99 the 3'-UTR. Using in vitro and in vivo approaches we show that miR-
100 29a and miR-330-5p are direct negative regulators of MUC1 expression
101 with tumor suppressive activity in pancreatic cancer. These two miRNAs
102 appear thus as potential new actors to reduce pancreatic cancer
103 progression.

104 2. Material and methods

105 2.1. Cell culture

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

111 2.2. Establishment of stable cell lines

112 MiaPaCa-2 stable cell lines overexpressing miR-29a, miR-330-5p or
113 miR-neg (Table 1) were generated using lentiviral particles and miR-
114 330-5p, miR-29a or miR-Neg pLenti4/TO/GFP-vectors and ViraPower
115 lentiviral packaging mix (Life Technologies) as described before [22,23].

116 2.3. Transient cell transfections

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

2.4. Cell proliferation, migration and invasion assays

129 Transfected cells or stable cell lines were seeded at 10^5 cells per well
130 in 6-well plates. Cells were counted every day using a Malassez
131 counting chamber using Trypan Blue exclusion dye (Life Technologies)
132 during 72 h for transfected cells and during 96 h for stable cell lines [5].
133 Invasion and migration assays were respectively performed using 24-
134 well Boyden chambers (8 μ m pores) coated or not with MatrigelTM
135 (Pharmingen, BD Biosciences). Briefly, 5×10^4 to 10^5 cells were seeded
136 on the top chamber and FBS 10% (v/v) was used as a chemoattractant in
137 the bottom chamber for 24 h to 48 h [5]. Three independent experi-
138 ments were performed in triplicate. 139

2.5. Cytotoxicity assay

140 10^4 cells were seeded in 96-well plates during 24 h. Medium was
141 refreshed with gemcitabine, 5-Fu, oxaliplatin or SN-38 for 72 h. The vi-
142 ability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-
143 diphenyltetrazoliumbromide assay (MTT, Sigma-Aldrich, Saint Quentin
144 Fallavier, France) as previously described [5]. Formazan crystals were
145 solubilized in dimethylsulfoxide (Sigma-Aldrich) and analyzed at
146 570 nm with a microplate reader (Bio-Rad). 147

2.6. Human pancreatic tissues

148 Human PDAC samples were obtained from the Lille University Hos-
149 pital with the approval of the Institutional Review Board. No patient re-
150 ceived either chemotherapy or radiotherapy before the surgical
151 resection except one patient. 152

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

153 Pdx1-Cre mice were obtained from the Mouse Models of Human
154 Cancer Consortium (MMHCC, USA). LStopL-Kras^{G12D} mice were obtain-
155 ed from Dr D. Tuveson (Cambridge Research Institute, England). All pro-
156 cedures are in accordance with the guidelines of animal care ethical
157 committee (Comité Ethique Expérimentation Animale Nord Pas-de-
158 Calais, #AF042008) [23]. 159

2.8. Subcutaneous xenografts

160 Subcutaneous (SC) xenografts (2×10^6 cells in 100 μ l of RPMI 1640
161 or DMEM media) of miR-neg, miR-29a or miR-330-5p cell lines were
162 carried out with 100 μ l of MatrigelTM (BD Biosciences) into the flank of
163 severe-combined immunodeficient (SCID) male mice (CB-17, Charles
164 Rivers) that were bred and maintained under pathogen-free conditions
165 (6 mice/group). Tumor development was followed weekly. The tumor
166 volume (mm³) was determined by calculating $V = W^2 \times L$ in which W
167 corresponds to the width (mm) and L to the tumor length (mm). Mice
168 were sacrificed 28 days after inoculation. All procedures were per-
169 formed in accordance with the guidelines and approved by the animal
170 care ethical committee (Comité Ethique Expérimentation Animale
171 Nord Pas-de-Calais, CEEA #122012) [5]. For intra-tumoral injection of
172 miRNAs, 2×10^6 cells for Capan-2 and 3×10^6 cells for MiaPaCa-2
173 were xenografted. After 10 days, 20 μ g of miRNAs or miR-neg cloned
174 into the pCDNA6.2emGFP vector was injected into the SC tumors
175 (~300 mm³) using Exgen 500 (Euromedex) reagent and glucose 5%
176 (v/v) as described by the manufacturer's instructions. Tumors were
177 followed twice a week and mice were sacrificed 20 days after miRNA
178 injection. 179

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

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

t1.1 **Table 1**
t 1.3 Characteristics of the primers used for qRT-PCR, cloning and site-directed mutagenesis.

t1.3	Gene	Forward/reverse	Sequences 5' → 3'
t1.4	MUC1	Forward	TGCCGCCGAAAGAACTACG
t1.5		Reverse	TGGGGTACTCGTTCATAGGAT
t1.6	GAPDH	Forward	CCACATCGCTCAGACACCAT
t1.7		Reverse	CCAGGCGCCCAATACG
t1.8	U6	Forward	CGAAGGATGACACGCAA
t1.9	u6	Forward	TGGCCCTGCGCAAGGATG
t1.10	hsa-miR-330-5p	Forward	TCTCTGGCCCTGTGTCTTAGGC
t1.11	hsa-miR-29a	Forward	TAGCACCATCTGAAATCGGTTA
t1.12	hsa-miR-183	Forward	TATGGCACTGGTAGAATTCAT
t1.13	hsa-miR-200a	Forward	TAACACTGTCTGGTAACGATGT
t1.14	hsa-miR-876-3p	Forward	TGGTGGTTTACAAGTAATTC
t1.15	hsa-miR-939	Forward	TGGGGAGCTGAGGCTCTGGGGGTG
t1.16	MUC1 3'-UTR XbaI	Forward	CGCTCTAGATCTGCCAATTGTAG
t1.17		Reverse	CGCTCTAGATTGGCGCAGTGGGAGA
t1.18	MUC1 3'-UTR mut miR-330-5p #1	Forward	CCCAGGAGGACTGAAGCAACAAGCCCTGAGATAGC
t1.19		Reverse	GCTATCTCAGGGCTTGTGCTTCAGTCTCCTCGGG
t1.20	MUC1 3'-UTR mut miR-29a	Forward	CTGTTTGGGCTGGTGGAGCTGGGAGTACGGTGGCCAATCACAGCTCCTT
t1.21		Reverse	AAGGAGGCTGTGATTTGGCCACCGTACTCCAGTCCACCAGCCCAACAC
t1.22	MUC1 3'-UTR mut miR-330-5p #2	Forward	CTCAGGTTCTCAGGGCGCCGCCCTGCACCCTGTTT
t1.23		Reverse	AAACAGGGTGCAGGGGCCGCGCCCTGAAGAACCTGAG
t1.24	MUC1 5'UTR HindIII	Forward	CGAAGCTTCCACTCTCAAGCAGCCA
t1.25		Reverse	CGAAGCTTCTGTAAGCACTGTGAGGAGC
t1.26	MUC1 5'-UTR mut miR-876-3p	Forward	CCTCCCCACCATTTCACTCTAACCATGACACCCGGGCACC
t1.27		Reverse	GGTGGCCGGTGTATGTTAGAGTGAATGGTGGGGGAGG
t1.28	MUC1 5'-UTR mut miR-939	Forward	GTGGTGGTGAATGGGTTATAAGGGGGCAGAACAGATTACGGCAGC
t1.29		Reverse	CTGCCTGAATCTGTTCTGCCCCCTATAACCCATTTACACACAC
t1.30	LV-hsa-miR-29a	Forward	TGCTGTAGCACCATCTGAAATCGGTTAGTTTTGGCCACTGACTACTAACCGATTTTCATGGTGTCTA
t1.31		Reverse	CCTGTAGCACCATGAAATCGGTTAGTCACTGAGTGGCCAAAACCTAACCGATTTCA
t1.32	LV-hsa-miR-330-5p	Forward	TGCTGTCTCTGGCCCTGTGTCTTAGCCGTTTTGGCCACTGACTGACGCCTAAGACACACCCAGAGCA
t1.33		Reverse	CCTGTCTCTGGGTTGTCTTAGGGCTCAGTCACTGAGCCAAAACGCCTAAGACACAGGCCACAGAGAC
t1.34	LV-hsa-miR-neg	Forward	AAATGTACTGCGGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
t1.35		Reverse	AAATGTACTGCGGTGGAGACGTCAGTCACTGAGCCAAAACGTTCCACGCAGTACATTT

184 (Promega) using *XbaI* or *HindIII* restriction sites (Biolabs) (Table 1). Mu-
185 tations were performed using the QuickChange® XL site-directed muta-
186 genesis kit (Stratagene, Agilent Technologies) (Table 1). Luciferase
187 activity and protein assays were performed as previously described
188 [23,26]. Luciferase assays were carried out three times in triplicate.

189 2.10. Biotin pull-down assay

190 10^6 cells were transfected with miR-29a, miR-330-5p or scramble
191 3'-biotinylated miRNA (100 nM) (Dharmacon, Thermo Scientific) as de-
192 scribed above. Biotin pull-down assays were performed in Capan-1,
193 Capan-2 and MiaPaCa-2 cell lines in triplicate in three independent se-
194 ries of experiments as previously described [23,27]. Pulled-down RNA
195 was directly extracted from the beads or from the input RNA using the
196 miRNeasy Mini kit (Qiagen). Reverse transcription and qRT-PCR were
197 then performed.

198 2.11. Quantitative reverse transcription-polymerase chain reaction 199 (qRT-PCR)

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

211 quantiMiR™ kit (System Bioscience, Ozyme). Expression levels were
212 calculated using the $2^{-\Delta\Delta Ct}$ method. Three independent experiments
213 were performed.

214 2.12. Protein extraction and Western-blot analysis

215 Total proteins were extracted, electro-transferred, immunostained,
216 and visualized as described before [5,23,26,28]. Antibodies used are
217 MUC1 (M8, 1/250, from Pr D. Swallow); β -actin (A5441 AC15, 1/5000)
218 from Sigma-Aldrich; cyclin D1 (sc-718, 1/250) and CDK6 (sc-177, 1/
219 250) from Santa Cruz; and EGFR (#4267s, 1/500), Bcl-2 (#2872, 1/250),
220 phospho P53 (#9284, 1/500), P53 (#9282, 1/500), phospho p42-44
221 MAPK (#9101, 1/500), p42-44 MAPK (#9102, 1/500), phospho Stat3
222 (#9145, 1/250), Stat3 (#9139, 1/500), β -catenin (#8480s, 1/1000),
223 phospho AKT (#4060, 1/500), and AKT (#4691s, 1/500) from Cell Signal-
224 ing, Ozyme. The signal was detected using LAS 4000 apparatus (Fujifilm).
225 Densities of bands were integrated using image J analysis software and
226 represented as histograms.

227 2.13. Immunohistochemistry

228 Human pancreatic tissues, tumor xenografts and mouse tissues were
229 fixed in 10% (w/v) buffered formaldehyde, paraffin-embedded, cut at
230 4 µm thickness and applied on SuperFrost® slides (Menzel-Glaser, Ther-
231 mo Scientific). Histology was assessed by staining tissues with Hematox-
232 ylin-Eosin. Manual immunohistochemistry (IHC) was carried out as
233 described [30] and automatic IHC was performed with an automated
234 immunostainer (ES, Ventana Medical System, Strasbourg, France) as de-
235 scribed [5,31]. Detection of MUC1 in human tissues and xenografts was
236 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

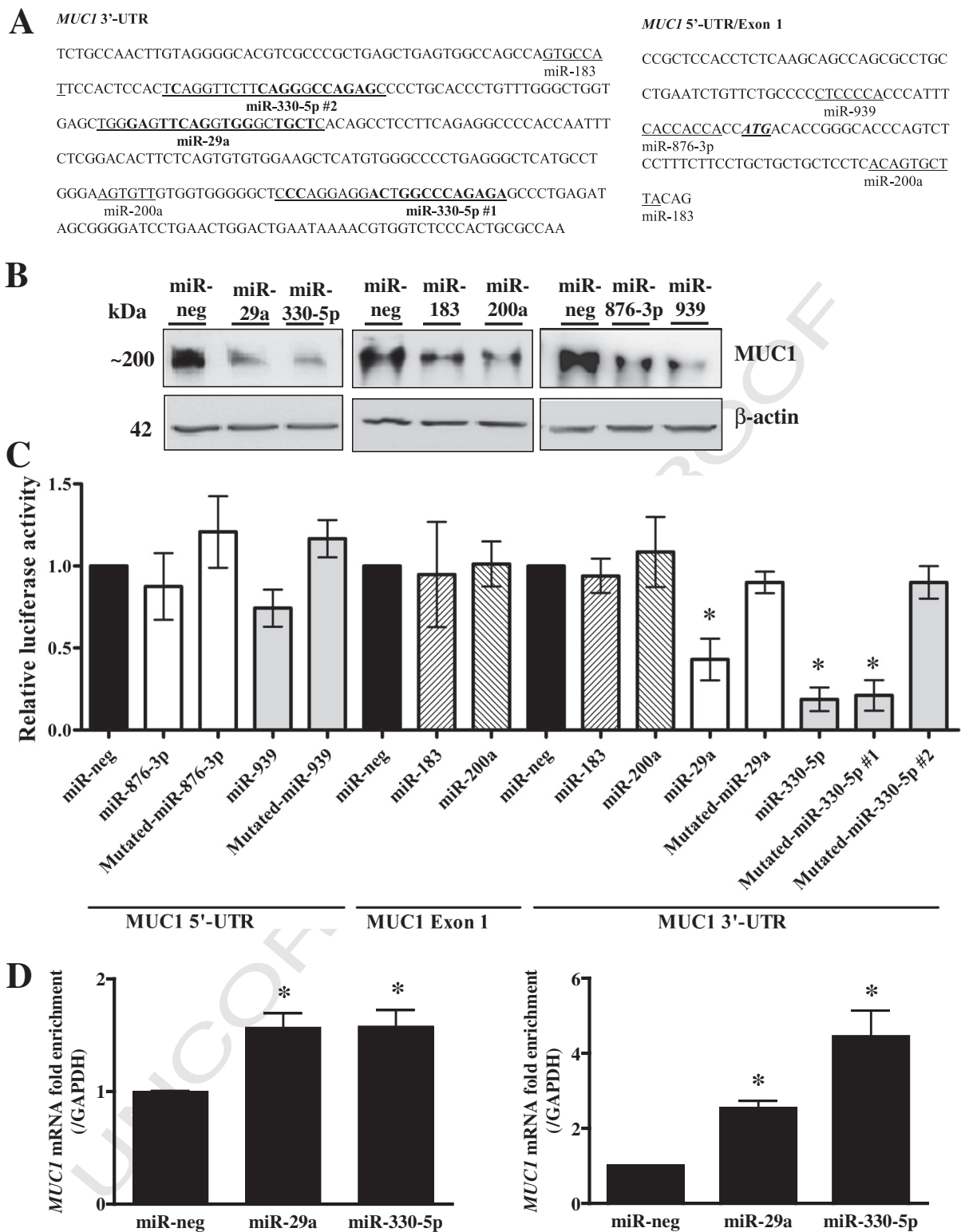
Having shown that miR-29a, miR-330-5p and MUC1 had an inverse profile of expression in PDAC, and that these two miRNAs altered biological properties of PDAC cells, we established stable cell lines overexpressing either miR-29a or miR-330-5p in MiaPaCa-2 cells in order to further assess their biological 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



Q2 Q1 **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 pull-down was assessed by qRT-PCR. The *MUC1*/*GAPDH* ratio was calculated and normalized to the input.

354 MiaPaCa-2 cells were performed in SCID mice followed by an intra-
 355 tumoral injection of the miRNA of interest. A significant decrease of
 356 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).
 359

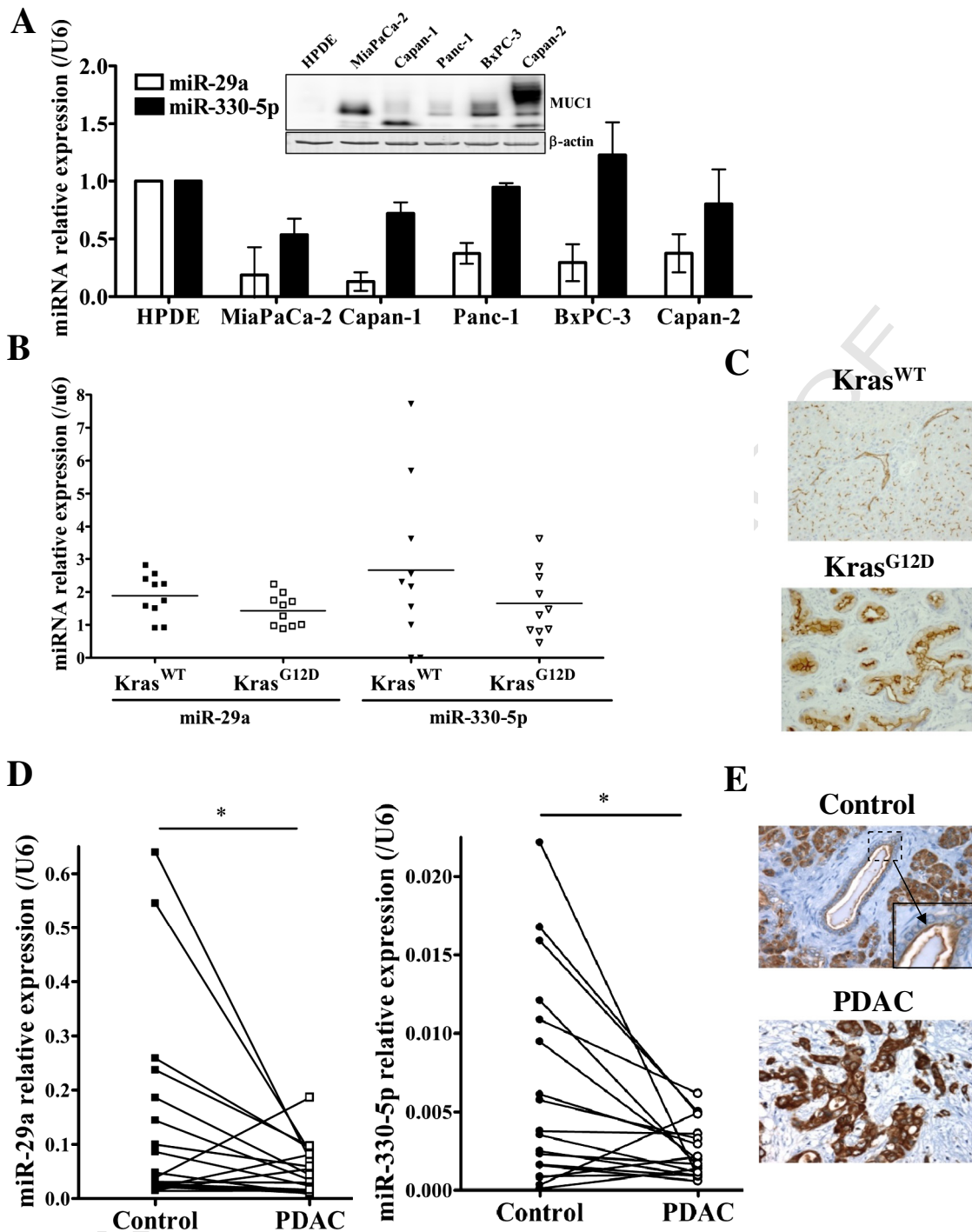


Fig. 2. MiRNA expression in PDAC cells and tissues. (A) Expression of miRNAs in HPDE, MiaPaCa-2, Capan-1, Panc-1, BxPC-3, and Capan-2 cell lines was studied by qRT-PCR. MiRNA expression was normalized to control U6 RNA. MiRNA level in normal pancreatic ductal cells HPDE was arbitrarily set to 1. Insert: 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.

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

4. Discussion

366
367 Pancreatic Ductal Adenocarcinoma suffers from a very poor prognos-
368 is and absence of early detection and efficient therapy [2,19]. Recently,
369 miRNAs have emerged as very attractive molecules to target cancer
370 since they regulate many genes encoding oncogenic proteins [20]. In 370

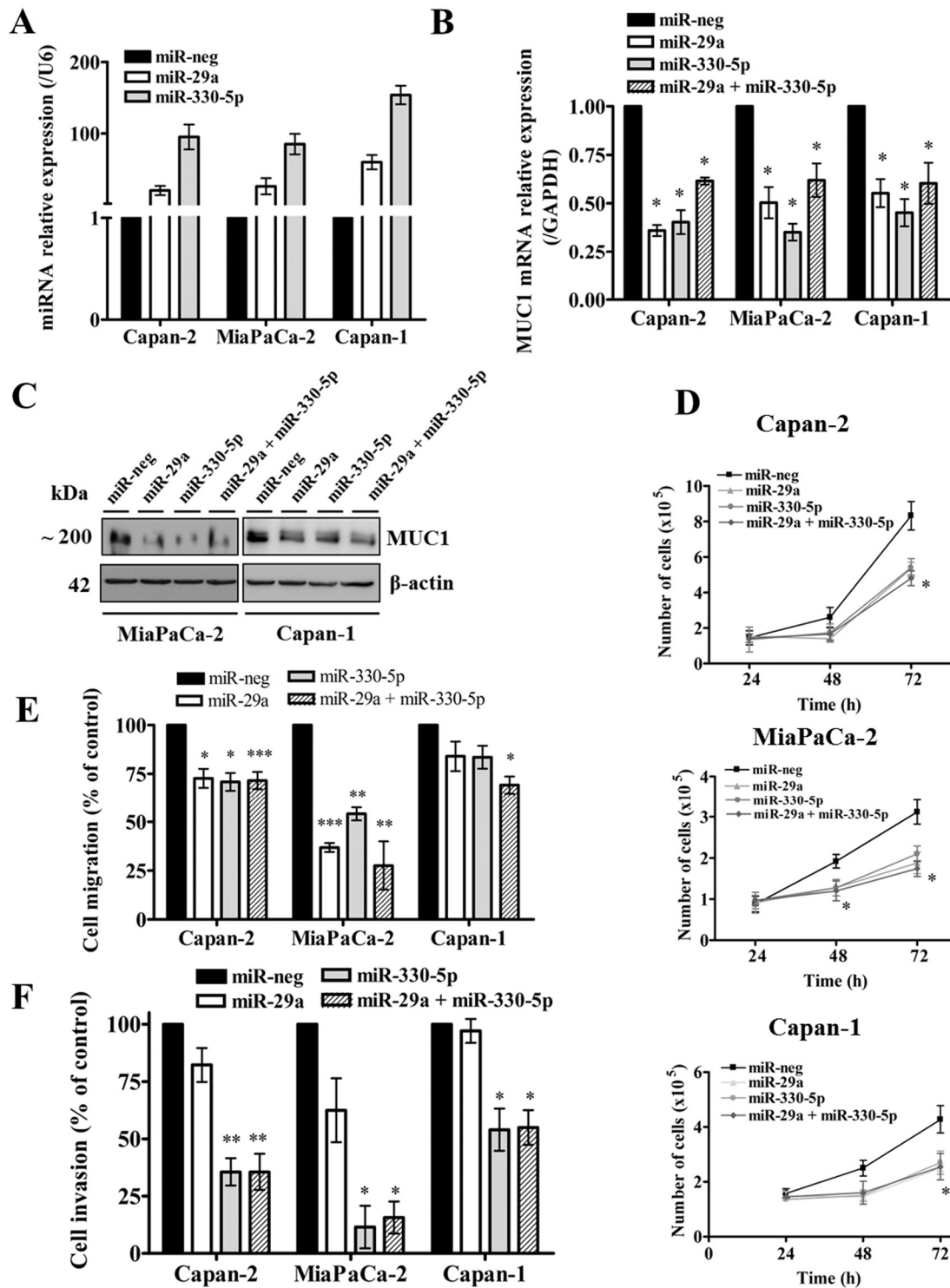
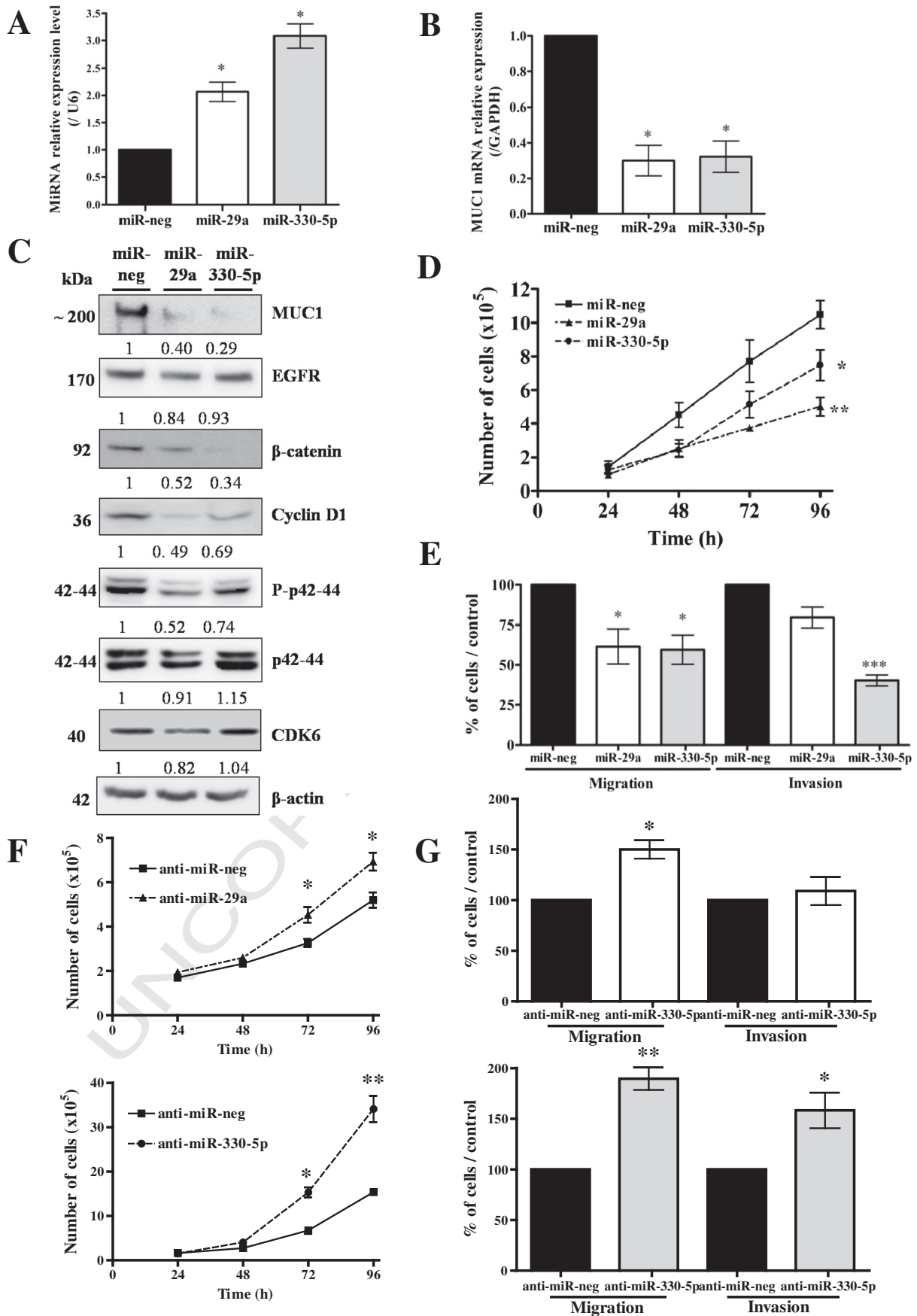


Fig. 3. Biological properties of PDAC cells transiently overexpressing miR-29a or miR-330-5p. (A) Expression of miR-29a and miR-330-5p was measured after transient overexpression in Capan-2, MiaPaCa-2 and Capan-1 cells by qRT-PCR. MiRNA expression was normalized to control U6 RNA. The miR-neg expression 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%).

371 PDAC, several studies have described the pattern of expression of
 372 miRNAs but their exact biological role and the identification of their tar-
 373 gets remain largely unknown [22,23]. In this report, we have identified
 374 miR-29a and miR-330-5p as two new miRNAs directly targeting the 3'
 375 UTR of MUC1 oncogenic mucin in PDAC cells and demonstrate that
 376 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 ap- 377
 appropriate targets to inhibit MUC1 expression in order to slow-down 378
 pancreatic carcinogenesis and develop new potential therapeutic tools. 379
 MiR-29a and miR-330-5p were shown to inhibit MUC1 expression 380
 by directly binding to its 3'-UTR but interestingly we have also identi- 381
 fied additional potential binding sites for miR-29a and miR-330-5p 382



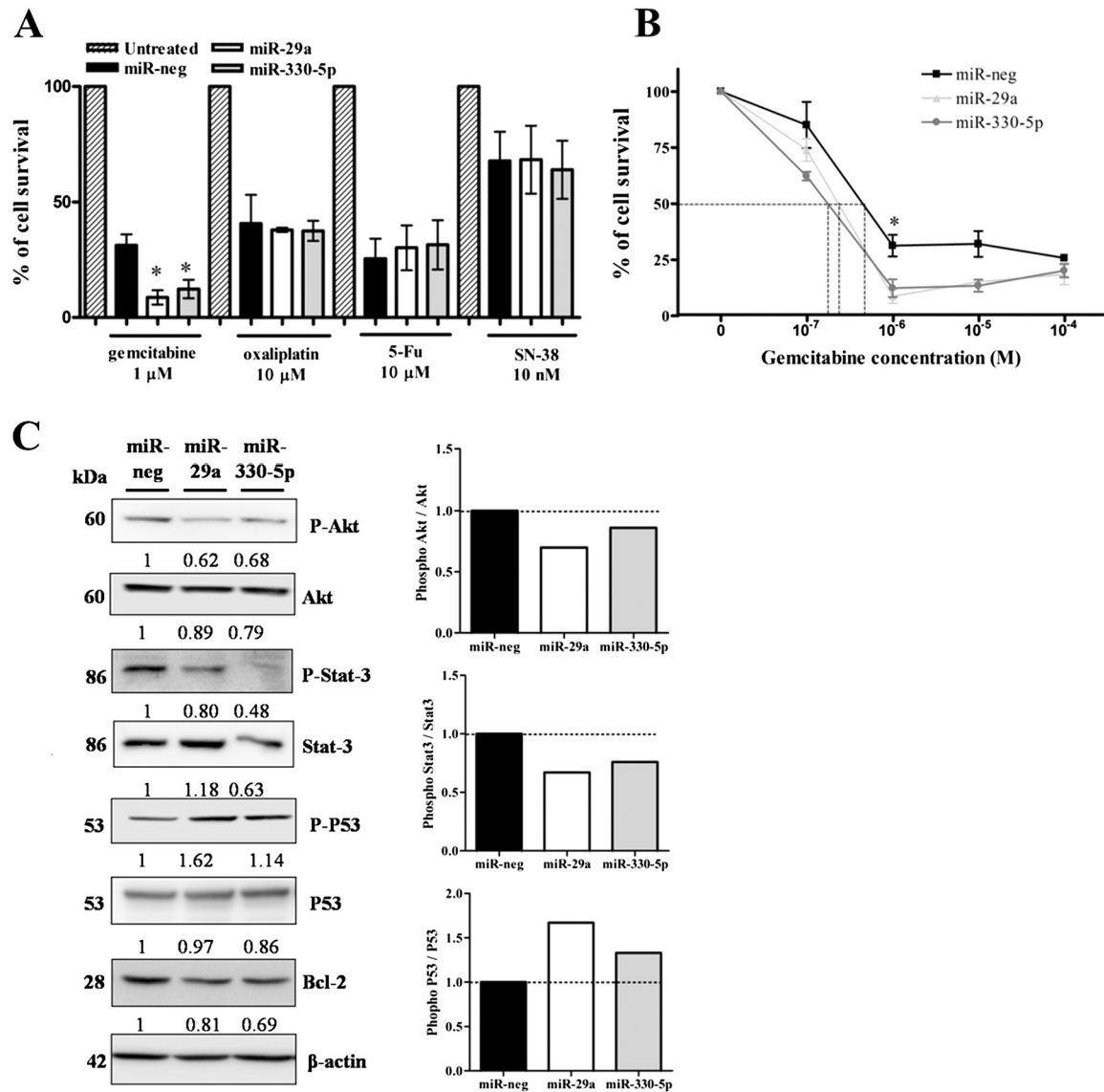


Fig. 5. Chemosensitivity of PDAC cells overexpressing miR-29a or miR-330-5p. (A) Cells were treated for 72 h with different concentrations of gemcitabine, 5-Fu, oxaliplatin or SN-38 before cell survival measurement. (B) IC₅₀ rates after 72 h of gemcitabine treatment. (C) Analysis of phospho-AKT, AKT, 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.

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

390 In breast cancer cells, *MUC1* is regulated by miR-145 [33], miR-1226
 391 [34] and miR-125b [35]. In this report we show that, *MUC1* is directly
 392 regulated by miR-29a and miR-330-5p and indirectly regulated by
 393 miR-876-3p, miR-939, miR-183 and miR-200a in PDAC cells. MiR-183
 394 and miR-200 families are EMT inhibitors and favor epithelial

395 differentiation [36] by inhibiting the Wnt/β-catenin signaling pathway
 396 [37,38]. The membrane-bound mucin *MUC1* is an epithelial marker of
 397 polarized epithelial cells [4,6,7] but aberrant overexpression of *MUC1*
 398 in cancer cells is correlated with an induction of EMT through the
 399 Wnt/β-catenin signaling pathway [14]. The indirect inhibition of
 400 *MUC1* by miR-183 and miR-200a could be an additional mechanism
 401 to inhibit EMT in PDAC. The role of miR-876-3p and miR-939 is hardly
 402 known in cancer. The indirect mechanisms leading to *MUC1* inhibition
 403 will have to be identified in future studies. The identification of predicted
 404 partners such as EGFR, cyclin D1, and Stat3, indirectly regulating
 405 *MUC1*, that are potential targets of miR-876-3p or miR-939 (personal
 406 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.

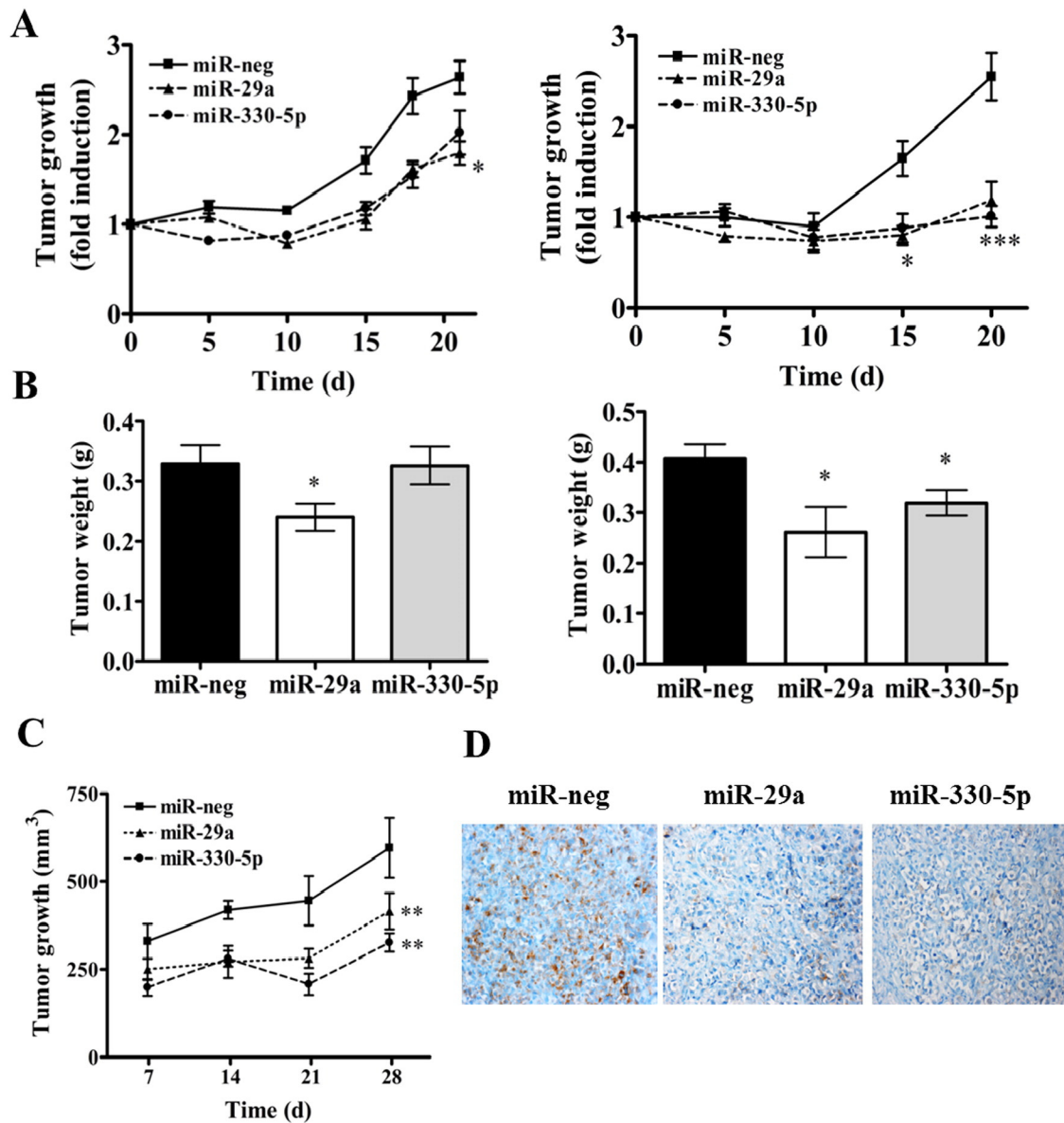


Fig. 6. In vivo effects of miR-29a and miR-330-5p on PDAC tumor properties. Subcutaneous (SC) xenografts of Capan-2 (left panel) and MiaPaCa-2 (right panel) cells in SCID mice. Intratumoral injection of miR-neg, miR-29a or miR-330-5p was performed 10 days post-xenografting ($\sim 300 \text{ mm}^3$). (A) Tumor growth (mm^3) 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 (mm^3) 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].

441 Additionally, we found that overexpression of miR-29a or miR-330-
 442 5p sensitizes PDAC cells to gemcitabine and induces a decrease of Akt
 443 pathway activation. Interestingly, MUC1 is known to mediate PDAC cell
 444 chemoresistance to gemcitabine by regulating *MRP1* gene expression
 445 through the PI3K/Akt pathway [5,15]. Although, miR-29a overexpression
 446 was previously shown to induce gemcitabine resistance in other PDAC
 447 cells (BxPC-3 and Panc-1) through the Wnt/ β -catenin signaling pathway
 448 [49], other results in lung cancer cells indicate that overexpression of
 449 miR-29a inhibits Wnt/ β -catenin signaling and β -catenin expression [40].
 450 The same effects were described in PDAC cells lacking MUC1 [5]. Interest-
 451 **Q16** ingly, it was also shown that miR-29a inhibits Akt expression [50] and
 452 sensitizes ovarian cancer cells to cisplatin [51], and that miR-330-5p
 453 overexpression decreased Akt phosphorylation in colorectal cancer [41].
 454 The decrease of Akt expression suggests a MUC1/Akt dependent mecha-
 455 nism mediating miR-29a/miR-330-5p effects on MiaPaCa-2 cell sensitiv-
 456 ity to gemcitabine. Altogether, these data highlight the complex role of
 457 these miRNAs in chemoresistance processes triggered by cancer cells
 458 that most likely are cell-specific. Further investigation will be needed to
 459 decipher the mechanisms underlying the sensitivity of PDAC cells to che-
 460 **Q17** motherapeutic drugs in relation with MUC1, miRNA and Akt expression.
 461 Moreover, we also showed that overexpression of miR-29a or miR-330-
 462 5p increases P53 activity as previously proposed for miR-29a [52] and de-
 463 creases Bcl-2 expression like it was previously proposed in PDAC cells
 464 lacking MUC1 [5] suggesting an involvement of these two miRNAs in
 465 cell apoptosis.

466 We also show that miR-29a and miR-330-5p are deregulated in
 467 human PDAC cell lines and tissues, and in the *Kras*^{G12D} pre-clinical
 468 mouse model of pancreatic cancer. Expression studies in a cohort of 18
 469 human PDAC tissues showed inhibition of miR-29a and miR-330-5p
 470 which is in accordance with previous studies showing that miR-29a is fre-
 471 quently deregulated in cancerous tissues. This confirms the potential of
 472 miR-29a as a target for cancer therapy as it was previously proposed
 473 [53] and highlights the interest for miR-330-5p in PDAC.

474 5. Conclusion

475 In conclusion, we show (i) that MUC1 is regulated by miRNAs in
 476 PDAC cells by direct and indirect mechanisms, (ii) that miR-29a and
 477 miR-330-5p are deregulated in PDAC and that PDAC cells overexpress-
 478 ing transiently or stably either miR-29a or miR-330-5p possess similar
 479 biological properties: in vitro decreased proliferative, migrating, and in-
 480 vasive properties and in vivo decreased tumor growth, and (iii) that
 481 miR-29a and miR-330-5p sensitize PDAC cells to gemcitabine. Based
 482 on that, we propose that tumor suppressive miR-29a and miR-330-5p
 483 may represent attractive targets to inhibit the expression of the onco-
 484 genic MUC1 mucin and decrease pancreatic cancer progression.

485 Author contribution

486 Solange Tréhoux conceived and designed the project, performed ex-
 487 periments, data analysis and interpretation, and wrote the paper. Fatima
 488 Lahdaoui performed in vitro experiments, data analysis, interpretation,
 489 and revised the manuscript. Isabelle Van Seuningen conceived and de-
 490 signed the project, provided financial support, data analysis and interpre-
 491 tation and wrote the paper. Nicolas Jonckheere performed in vivo
 492 experiments in mice models, data analysis and interpretation, and re-
 493 vised the manuscript. Florence Renaud and Emmanuelle Leteurtre per-
 494 formed anatomopathological analyses and revised the manuscript.
 495 Yannick Delpu and Jérôme Torrisani raised stable cell lines overexpress-
 496 ing miR-29a and miR-330-5p and revised the manuscript.

497 Conflicts of interest

498 Authors declare no conflict of interest.

Uncited reference

[29]

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