Published in final edited form as:

Biochem Biophys Res Commun. 2011 March 25; 406(4): 518–523. doi:10.1016/j.bbrc.2011.02.065.

miR-132 and miR-212 are increased in pancreatic cancer and target the retinoblastoma tumor suppressor

Jong-Kook Park¹, Jon C. Henry², Jinmai Jiang¹, Christine Esau³, Yuriy Gusev⁴, Megan R. Lerner⁵, Russell G. Postier⁶, Daniel J. Brackett⁵, and Thomas D. Schmittgen^{1,*}

¹College of Pharmacy, Ohio State University, Columbus, Ohio

²Department of Surgery, Ohio State University, Columbus, Ohio

³Regulus Therapeutics, Carlsbad, California

⁴Lombardi Cancer Center, Georgetown University, Washington, D.C.

⁵Veterans Affairs Medical Center, Oklahoma City, Oklahoma

⁶Department of Surgery, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Abstract

Numerous microRNAs (miRNAs) are reported as differentially expressed in cancer, however the consequence of miRNA deregulation in cancer is unknown for many miRNAs. We report that two miRNAs located on chromosome 17p13, miR-132 and miR-212, are over expressed in pancreatic adenocarcinoma (PDAC) tissues. Both miRNAs are predicted to target the retinoblastoma tumor suppressor, Rb1. Validation of this interaction was confirmed by luciferase reporter assay and western blot in a pancreatic cancer cell line transfected with pre-miR-212 and pre-miR-132 oligos. Cell proliferation was enhanced in Panc-1 cells transfected with pre-miR-132/-212 oligos. Conversely, antisense oligos to miR-132/-212 reduced cell proliferation and caused a G₂/M cell cycle arrest. The mRNA of a number of E2F transcriptional targets were increased in cells over expressing miR-132/-212. Exposing Panc-1 cells to the β 2 adrenergic receptor agonist, terbutaline, increased the miR-132 and miR-212 expression by 2 to 4 fold. We report that over expression of miR-132 and miR-212 result in reduced pRb protein in pancreatic cancer cells and that the increase in cell proliferation from over expression of these miRNAs is likely due to increased expression of several E2F target genes. The β 2 adrenergic pathway may play an important role in this novel mechanism.

Keywords

microRNA; retinoblastoma; β 2 adrenergic receptor

© 2011 Elsevier Inc. All rights reserved.

*To whom requests for reprints should be addressed. College of Pharmacy, Ohio State University, Columbus, Ohio 43210. Schmittgen.2@osu.edu, 614-292-3456, 614-292-7766 (FAX).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Pancreatic adenocarcinoma (PDAC) cancer continues to be a lethal diagnosis for most patients with 43,140 new diagnoses and 36,800 deaths predicted for 2010 in the United States alone [1]. Despite much research on this disease over the past several decades, new and effective treatment regimens are lacking. Chemotherapy and radiation therapy remain mostly ineffective [2]. Therefore surgery remains the only attempt at a curative resection, but only 15–20% pancreatic cancer patients are eligible for surgery at the time of presentation and of those who undergo successful surgical resection the 5-year survival rate is a dismal 15–23% [3]. Novel treatment strategies are needed to combat this disease, however a better understanding of the molecular pathogenesis of PDAC must be achieved before new therapies may be developed.

microRNAs (miRNAs) are small, non-coding RNAs that post transcriptionally regulate protein levels by binding to the 3' UTR of the mRNA [4]. miRNAs are differentially expressed in many solid tumors and often create a unique signature for each tumor type [5]. Our lab and others have demonstrated that miRNAs are linked to PDAC [6–9]. miR-212 was among the top differentially expressed miRNA precursors in PDAC with a 22-fold increased expression in the tumors [6]. miR-212 is located on chromosome 17p13.3, approximately 260 bp from a closely related miRNA, miR-132. Both miR-132 and miR-212 share the identical 5' seed sequence, thus, would be expected to regulate the identical target genes.

miR-132 expression is increased in lung cancer [5,10], endocrine pancreatic tumors [5], squamous cell carcinoma of the tongue [11], breast cancer [12], and colorectal carcinoma [13,14]. Increased miR-212 expression was reported in colorectal carcinoma [13]. Decreased miR-132 expression is seen in osteosarcoma [15] while miR-212 has been shown to be down regulated in gastric cancers [16,17], non-small cell lung cancers [18], and head and neck squamous cell carcinoma [19]. The different levels of expression of these miRNAs in various cancers, highlight their diverse function in cells and the difficult task of determining their regulation and potential targets. To our knowledge, no one has taken an in depth look into the role of miR-132/-212 in PDAC.

Several cancer-related targets of miR-132/-212 have been validated including c-Myc [16] and MECP2 [17] in gastric cancer, heparin binding EGF in head and neck carcinoma [19] and the antiapoptotic protein PED/PEA-15 in small cell lung cancer [18]. The only tumor suppressive target of miR-132/-212 to our knowledge is the pro angiogenic p120RasGAP that is decreased in endothelial cells of breast cancer due to upregulated miR-132 [12]. We report here that an important tumor suppressor, Rb1, is a target of miR-132/-212 in PDAC. Down regulation of pRb by miR-132/-212 leads to increased cell proliferation and cell cycle progression in Panc-1 cells. miR-132/-212 expression is increased by a β 2 adrenergic receptor agonist, suggesting a novel mechanism for pancreatic cancer progression.

2. Materials and methods

2.1. Tissue procurement and isolation of protein and RNA

The tissue samples analyzed in this study were derived from patients undergoing a surgical procedure to remove a portion of the pancreas at the University of Oklahoma Health Sciences Center. The collection of samples conformed to the policies and practices of the facility's Institutional Review Board. Sections from each specimen were examined by a pathologist and graded histologically. RNA or protein was extracted from the tissues following pulverization in a cold mortar and pestle. Total RNA was isolated from the tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer. An RNA integrity number (RIN) of 6 or

higher was used as the cutoff. For the protein samples, pulverized tissues were digested using Cellytic™ M (Sigma-Aldrich, St. Louis, MO) with protease and phosphatase Inhibitor (Pierce, Rockford, IL) according to the manufacturer's guidelines.

2.2. Cell line

The human pancreatic cancer cell line Panc-1 was purchased from American Type Tissue Collection (Manassas, VA). Cells were grown in Dulbecco's Modified Eagle Media (Invitrogen, Carlsbad, Calif) with L-glutamine (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS, HyClone). The cells were incubated at 37°C under a humidified atmosphere with 5% carbon dioxide.

2.3. Cell proliferation assay

pre-miR-132 and -212 mimics and control oligonucleotide were purchased from Ambion (Austin, TX). Cells (1500/well) were plated 24 hours prior to transfection with Lipofectamine 2000 (Invitrogen). Transfection with antisense oligonucleotides to miR-132 or miR-212 was conducted in the same manner. The cell proliferation assay was performed using the reagent WST-1 (Roche, Indianapolis, IN). All experiments were performed at least in triplicate. ASOs were chemically modified (100%) with a 2'-O-methoxyethyl and phosphorothioate backbone (2'-O-MOE-PS) and were provided by Regulus Therapeutics (Carlsbad, CA).

2.4. qPCR for miRNA and mRNA expression

One hundred ng of total RNA was primed using gene specific looped primers to miR-132 and miR-212. cDNA was then quantified with TaqMan miRNA Assays (Applied Biosystems, Foster City, CA). For mRNA expression analysis, cDNA was synthesized from 1 µg of total RNA using random primers. Gene expression analysis was performed by qPCR using the SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. For qPCR of both miRNA and mRNA, 18S rRNA was used as the reference gene and data were analyzed using the comparative C_T method. Primers used in this study are summarized in the Supplementary Information.

2.5. Luciferase reporter analysis

The full length Rb1 3' UTR was cloned into the psiCHECK-2 Vector (Promega, Madison, WI) using standard techniques. The resulting recombinant plasmid of Rb1 was designated psiC2-Rb1. The mutant reporter construct Rb1-MUT was generated using QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Reporter vectors were assayed for luciferase expression using the Dual Luciferase Report Assay System (Promega) following the manufacturer's instructions. Twenty-four h after transfection, relative luciferase activity was obtained by normalizing the renilla luciferase activity to the firefly luciferase activity. Primers used in this study were summarized in the Supplementary Information.

2.6. Protein extraction and immunoblotting

Protein was harvested using Cellytic™ M (Sigma-Aldrich) and 1 protease and phosphatase inhibitor (Pierce). Protein concentration was measured using the BCA Protein Assay Kit (Pierce). Thirty micrograms of total protein extract was separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Blotting was performed for Rb1 (BD Pharmingen, San Diego, CA), cyclin A2, and cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin (Abcam, Cambridge, MA) was used as a loading control. Secondary horseradish peroxidase antibody was detected using ECL Western Blotting Analysis System (Amersham Biosciences, Piscataway, NJ).

2.7. BrdU cell proliferation assay

Non-isotopic bromodeoxyuridine (BrdU) incorporation assay (EMD chemicals, Gibbstown, NJ) was performed according to the manufacturer's instructions.

2.8. Cell cycle analysis

Antisense oligonucleotide effects on cell cycle were performed as previously described [20]. Data were analyzed using flow cytometry (FACS Calibur; Becton Dickinson), and cell cycle analysis software (Modfit; Verity, Topsham, Me). For each sample, 20,000 events were acquired.

3. Results

3.1. miR-132/-212 are increased in pancreatic cancer and target Rb1

We previously reported that 100 miRNA precursors were aberrantly expressed in pancreatic cancer [6]. As miR-212 was one of the most highly differentially expressed miRNAs in our prior study, we wished to further investigate its' role in PDAC. qPCR was used to quantify the expression of mature miR-132/-212 in 21 pancreas specimens (4 normal and 6 adjacent benign pancreas and 11 pancreatic adenocarcinomas). Both miR-132 and miR-212 were significantly up-regulated in each of the 11 pancreatic adenocarcinomas compared to the normal and benign tissues (Fig. 1A).

The TargetScan algorithm was used to look for potential targets of miR-132/-212. The human Rb1 3' UTR has a miRNA binding sequence to both miR-132 and miR-212 (Supplementary Fig. 1A). To validate the interaction between the Rb1 3' UTR and miR-132/-212, Panc-1 cells were co-transfected with psiC2-Rb1 vector and pre-miR-132, pre-miR-212 or control oligo. Luciferase expression was significantly decreased with pre-miR-132 and pre-miR-212 compared to the negative control (Fig. 1B). No significant modulation of luciferase activity was observed when cells were co-transfected with the Rb1-MUT vector and the pre-miR oligos (Fig. 1B). To further verify that miR-132/-212 directly regulates Rb1, the expression of Rb1 protein was assayed in Panc-1 cells transfected with pre-miR-132, pre-miR-212 or control oligos. The hypo-phosphorylated and hyper-phosphorylated Rb1 levels were reduced by 58% compared with cells transfected with control oligo (Fig. 1C). To determine if miR-132/-212 affects Rb1 mRNA, qPCR was used to assay Rb1 mRNA levels in Panc-1 cells transfected with pre-miR-132 or pre-miR-212 oligo. Rb1 mRNA was decreased about 30% in Panc-1 cells (Supplementary Fig. 1B) demonstrating that miR-132/-212 down regulates Rb1 protein by both mRNA degradation and translational repression. Finally, to determine if the relationship between miR-132/-212 and pRb in the Panc-1 cells correlates with the tissues, the pRb protein levels were assayed in 4 pairs of pancreatic cancer and adjacent benign pancreas. pRb was reduced in 3 of the 4 pairs of pancreas tumors (Fig. 1D).

3.2. miR-132/-212 over-expression facilitates the proliferation of Panc-1 cells

Reduced pRb levels due to over-expression of miR-132/-212 should enhance proliferation by increasing the expression of transcriptional targets of E2F. To test this hypothesis, we first examined the proliferation of Panc-1 cells transfected with pre-miR-132 and pre-miR-212 oligos. miR-132/-212 over-expression significantly increased the proliferation rate of Panc-1 cells (Fig. 2A). qPCR was used to examine the consequence of miR-132/-212 on the expression of well-documented E2F transcriptional target genes. Expression of E2F target genes such as minichromosome maintenance complex component (MCM), proliferating cell nuclear antigen (PCNA), and cyclin A2 was significantly increased by over-expression of miR-132/-212 (Fig. 2B) ($P < 0.05$). Immunoblotting confirmed that the protein expression of two selected E2F transcriptional targets, cyclin A2 (CCNA2) and

cyclin B1 (CCNB1), was increased in pre-miR-132/-212 transfected Panc-1 cells (Fig. 2C). Finally, analyses of DNA synthesis by BrdU incorporation indicated that the portion of cells undergoing proliferation was higher in miR-132/-212 over-expression cells than in each of the control cells (Fig. 2D).

3.3. miR-132/-212 knockdown inhibits the growth of Panc-1 cells

The converse experiment was then performed in which the proliferation of pancreas cancer cells was studied following inhibition of mature miR-132/-212 with antisense. Representative dose response curves showed that nM concentrations of miR-132/-212 antisense significantly decreased the proliferation of Panc-1 cells compared to cells transfected with the scrambled control oligo (Fig. 3A). Antiproliferative activity parameters are summarized in Supplementary Table 1. Interestingly, we found that miR-132/-212 inhibition resulted in G₂/M phase cell cycle arrest in Panc-1 cells at 48 h exposure. The G₂/M phase was increased from 19.9 to 44% in Panc-1 cells following inhibition of miR-132/-212 (Fig. 3B). The G₁ and S phases of the cell cycle were reduced by 18.9 and 5.2%, respectively, following inhibition of the miRNAs with antisense. There was no difference of cell cycle distributions between lipofectamine alone and scrambled control (data now shown).

3.4. β 2 adrenergic receptor agonist increases mature miR-132-212 expression

Previous work has shown a link between β adrenergic receptors and pancreas cancer [21]. We wanted to further study this relationship and see if β 2 adrenergic receptors (B2AR) could modulate miR-132/-212 levels in pancreas cancer cells. The rationale for this relationship is that B2AR agonists activate cAMP response element binding protein (CREB) [22] and CREB activates miR-132/-212 transcription [23]. The B2AR mRNA was measured in clinical specimens of pancreas cancer and adjacent benign pancreas. B2AR mRNA was increased in the tumors compared to the normal and adjacent benign pancreas (8.2 and 16.5 fold average increase comparing to normal and benign, respectively) (Fig. 4A). Treatment of 1 μ M terbutaline, a B2AR agonist, had a pronounced effect on the miR-132/-212 levels in B2AR positive Panc-1 cells increasing the miRNA levels up to 4-fold (Fig. 4B).

4. Discussion

Down-regulation of tumor suppressors by oncogenic miRNAs could contribute to the malignant progression of cancer. We show here that miR-132/-212, two miRNAs that are significantly increased in PDAC, target the tumor suppressor Rb1. Rb1 mutations have been discovered in many cancers, but are not common in pancreatic cancer. In Barton et al.'s study [24], only 6% of the pancreatic cancer specimens had homozygous mutated Rb1 genes. Likewise, Huang, et al showed no mutations in the Rb1 promoter and used single-strand conformational polymorphism to conclude that Rb1 was infrequently mutated in PDAC [25]. Using immunohistochemistry, both reports showed variable (25–100%) pRb expression in the individual cells of pancreas tumors compared to adjacent pancreas [24,25]. We report significant reduction in pRb protein expression in 3 of the 4 pairs of tissues studied (Fig. 1D). While it is possible for pRb to be regulated by other miRNAs, our data suggest that reduced pRb protein in PDAC results from increased miR-132/-212 expression.

The protein product of the gene Rb1, pRb, binds to and sequesters transcription factors of the E2F family. Suppression of pRb by miR-132/-212 over-expression should result in increased unsequestered E2F and therefore increased expression of transcriptional targets of cell cycle-related genes such as cyclins A1, A2, B1 and E2. Several genes that are regulated by E2F, including cyclin A2 and cyclin B1, were increased in pancreas cancer cells following miR-132/-212 over-expression (Fig. 2B). In fact many of the genes that are

increased by miR-132/-212 over expression were also increased by siRNA knockdown of Rb1 [26]. This demonstrates that knockdown of pRb either by miRNA or siRNA would be expected to facilitate tumor cells growth by increasing the expression of cell cycle regulated genes.

We interestingly found that knockdown of miR-132/-212 induced G₂/M arrest. pRb can affect both G₁/S and G₂/M transition of the cell cycle [26], however, the predominate effect of pRb is a G₁ phase arrest. This effect can be attributed to pRb's binding to and inactivation of E2F proteins. The E2F proteins are well documented at inducing cells to enter the S-phase [27]. The Rb1/E2F interaction also regulates the G₂/M transition [26,28]. So the G₂/M arrest observed here may be due to miR-132/-212 targeting of Rb1 and likely other genes causing a heightened G₂/M arrest in comparison to the G₁ arrest that is usually more pronounced with Rb1. miR-132/-212 are predicted to target ATM and ATR, two genes that are involved in DNA damage and cell cycle arrest. Also, our luciferase assay screening data showed that miR-132/-212 potentially targets cyclin G1 (unpublished), which regulates the G₂/M transition [29]. Therefore, there are multiple avenues of cell cycle control imposed by miR-132/-212 that likely includes regulation of Rb1 and other target genes.

The cause of the miR-132/-212 up-regulation in pancreatic adenocarcinoma is unknown. We show that the B2AR agonist terbutaline increases miR-132/-212 expression (Fig. 4). G protein-coupled receptors such as the B2AR induce CREB trans-activation through the cAMP-protein kinase A (PKA) pathway. Transcription is induced following phosphorylation of CREB by PKA and recruitment of the coactivator CREB-binding protein (CBP) or its paralog, p300. miR-132 was transcriptionally activated by CREB in neurons [30] and more recently miR-132 was shown to be part of a feedback loop during antiviral innate immunity in which p300 induces the expression of miR-132 and miR-132 suppresses p300 [31]. DNA synthesis of pancreatic cell lines, Panc-1 and BXPC-3, was stimulated by β 2 adrenergic signaling pathway [21]. B2AR agonists increase pancreatic cancer cell proliferation by activating PKA, arachidonic acid and MAPK pathways [21,32] and B2AR antagonists inhibited activation of CREB [33]. We propose that stimulation of B2AR increase miR-132/-212 expression following activation of CREB, which in turn suppress pRb and cause an increase in proliferation.

We report here that miR-132/-212 are over-expressed in PDAC and target the tumor suppressor Rb1. The consequence of this interaction includes increased cell proliferation which results from an increased expression of many cell cycle-related transcriptional targets of E2F. Since stimulation of the adrenergic pathway by catecholamines released in the tumor microenvironment by known PDAC risk factors such as smoking, high fat diet and chronic stress may contribute to PDAC development, our findings suggest a novel mechanism for the malignant progression of PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grant R33 CA114304. J.K.P. is supported by a Provost's Targeted Investment in Excellence (TIE) grant from the Ohio State University. J.C.H. is supported by Training Grant 7T32CA009338-32.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]

2. Neoptolemos JP, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, Beger H, Fernandez-Cruz L, Dervenis C, Lacaine F, Falconi M, Pederzoli P, Pap A, Spooner D, Kerr DJ, Buchler MW. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med*. 2004; 350:1200–1210. [PubMed: 15028824]
3. Simianu VV, Zyromski NJ, Nakeeb A, Lillemoe KD. Pancreatic cancer: progress made. *Acta Oncol*. 2010; 49:407–417. [PubMed: 20059311]
4. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009; 136:215–233. [PubMed: 19167326]
5. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006; 103:2257–2261. [PubMed: 16461460]
6. Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*. 2007; 120:1046–1054. [PubMed: 17149698]
7. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA*. 2007; 297:1901–1908. [PubMed: 17473300]
8. Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, Labourier E, Hahn SA. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*. 2007; 26:4442–4452. [PubMed: 17237814]
9. Zhang Y, Li M, Wang H, Fisher WE, Lin PH, Yao Q, Chen C. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg*. 2009; 33:698–709. [PubMed: 19030927]
10. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*. 2006; 9:189–198. [PubMed: 16530703]
11. Wong TS, Liu XB, Chung-Wai Ho A, Po-Wing Yuen A, Wai-Man Ng R, Ignace Wei W. Identification of pyruvate kinase type M2 as potential oncoprotein in squamous cell carcinoma of tongue through microRNA profiling. *Int J Cancer*. 2008; 123:251–257. [PubMed: 18464261]
12. Anand S, Majeti BK, Acevedo LM, Murphy EA, Mukthavaram R, Schepke L, Huang M, Shields DJ, Lindquist JN, Lapinski PE, King PD, Weis SM, Cheres DA. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. *Nat Med*. 2010; 16:909–914. [PubMed: 20676106]
13. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*. 2008; 299:425–436. [PubMed: 18230780]
14. Yang L, Belaguli N, Berger DH. MicroRNA and colorectal cancer. *World J Surg*. 2009; 33:638–646. [PubMed: 19123024]
15. Gougelet A, Pissaloux D, Besse A, Perez J, Duc A, Dutour A, Blay JY, Alberti L. Micro-RNA profiles in osteosarcoma as a predictive tool for ifosfamide response. *Int J Cancer*. 2010
16. Xu L, Wang F, Xu XF, Mo WH, Xia YJ, Wan R, Wang XP, Guo CY. Down-regulation of miR-212 expression by DNA hypermethylation in human gastric cancer cells. *Med Oncol*. 2010
17. Wada R, Akiyama Y, Hashimoto Y, Fukamachi H, Yuasa Y. miR-212 is downregulated and suppresses methyl-CpG-binding protein MeCP2 in human gastric cancer. *Int J Cancer*. 2010; 127:1106–1114. [PubMed: 20020497]
18. Incoronato M, Garofalo M, Urso L, Romano G, Quintavalle C, Zanca C, Iaboni M, Nuovo G, Croce CM, Condorelli G. miR-212 increases tumor necrosis factor-related apoptosis-inducing ligand sensitivity in non-small cell lung cancer by targeting the antiapoptotic protein PED. *Cancer Res*. 2010; 70:3638–3646. [PubMed: 20388802]
19. Hatakeyama H, Cheng H, Wirth P, Counsell A, Marcrom SR, Wood CB, Pohlmann PR, Gilbert J, Murphy B, Yarbrough WG, Wheeler DL, Harari PM, Guo Y, Shyr Y, Slebos RJ, Chung CH.

- Regulation of heparin-binding EGF-like growth factor by miR-212 and acquired cetuximab-resistance in head and neck squamous cell carcinoma. *PLoS One*. 2010; 5:e12702. [PubMed: 20856931]
20. Park JK, Lee EJ, Esau C, Schmittgen TD. Antisense inhibition of microRNA- 21 or -221 arrests cell cycle, induces apoptosis, and sensitizes the effects of gemcitabine in pancreatic adenocarcinoma. *Pancreas*. 2009; 38:e190–e199. [PubMed: 19730150]
 21. Weddle DL, Tithoff P, Williams M, Schuller HM. Beta-adrenergic growth regulation of human cancer cell lines derived from pancreatic ductal carcinomas. *Carcinogenesis*. 2001; 22:473–479. [PubMed: 11238189]
 22. Baker JG, Hall IP, Hill SJ. Temporal characteristics of cAMP response element-mediated gene transcription: requirement for sustained cAMP production. *Mol Pharmacol*. 2004; 65:986–998. [PubMed: 15044629]
 23. Remenyi J, Hunter CJ, Cole C, Ando H, Impey S, Monk CE, Martin KJ, Barton GJ, Hutvagner G, Arthur JS. Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins. *Biochem J*. 2010; 428:281–291. [PubMed: 20307261]
 24. Barton CM, McKie AB, Hogg A, Bia B, Elia G, Phillips SM, Ding SF, Lemoine NR. Abnormalities of the RB1 and DCC tumor suppressor genes: uncommon in human pancreatic adenocarcinoma. *Mol Carcinog*. 1995; 13:61–69. [PubMed: 7605581]
 25. Huang L, Lang D, Geradts J, Obara T, Klein-Szanto AJ, Lynch HT, Ruggeri BA. Molecular and immunochemical analyses of RB1 and cyclin D1 in human ductal pancreatic carcinomas and cell lines. *Mol Carcinog*. 1996; 15:85–95. [PubMed: 8599583]
 26. Semizarov D, Kroeger P, Fesik S. siRNA-mediated gene silencing: a global genome view. *Nucleic Acids Res*. 2004; 32:3836–3845. [PubMed: 15272085]
 27. Dyson N. The regulation of E2F by pRB-family proteins. *Genes Dev*. 1998; 12:2245–2262. [PubMed: 9694791]
 28. Naderi S, Hunton IC, Wang JY. Radiation dose-dependent maintenance of G(2) arrest requires retinoblastoma protein. *Cell Cycle*. 2002; 1:193–200. [PubMed: 12429933]
 29. Kimura SH, Ikawa M, Ito A, Okabe M, Nojima H. Cyclin G1 is involved in G2/M arrest in response to DNA damage and in growth control after damage recovery. *Oncogene*. 2001; 20:3290–3300. [PubMed: 11423978]
 30. Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, Goodman RH, Impey S. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proc Natl Acad Sci U S A*. 2005; 102:16426–16431. [PubMed: 16260724]
 31. Lagos D, Pollara G, Henderson S, Gratrix F, Fabani M, Milne RS, Gotch F, Boshoff C. miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. *Nat Cell Biol*. 2010; 12:513–519. [PubMed: 20418869]
 32. Schuller HM. Mechanisms of smoking-related lung and pancreatic adenocarcinoma development. *Nat Rev Cancer*. 2002; 2:455–463. [PubMed: 12189387]
 33. Zhang D, Ma QY, Hu HT, Zhang M. beta2-adrenergic antagonists suppress pancreatic cancer cell invasion by inhibiting CREB, NFkappaB and AP-1. *Cancer Biol Ther*. 2010; 10:19–29. [PubMed: 20424515]

Fig. 1. miR-132 and miR-212 are over-expressed in pancreatic tumor tissues and target Rb1. (A) Expression patterns of miR-132 and miR-212 in normal pancreas (N), adjacent benign pancreas (BE) and pancreatic adenocarcinomas (T). (B) Luciferase reporter plasmids carrying a wild-type (psiC2-Rb1) or mutant binding sequences in 3' UTR of Rb1 (Rb1-MUT) was transiently cotransfected in Panc-1 cells with the negative control of pre-miR precursor (pre-miR-nega), the miR-132 precursor (pre-miR-132), or the miR-212 precursor (pre-miR-212) at 50 nM concentration. Luciferase activity was measured 24 h after transfection. The data are the mean $\pm$ s. d. of at least 3 independent transfections. (C) Rb1 protein was measured by immunoblotting in Panc-1 cells transfected with pre-miR-nega

control (60 nM) or both pre-miR-132 and pre-miR-212 (30 nM of each). β -actin served as a loading control. Relative expressions are indicated. (D) Western blot analysis of Rb1 in paired normal/adjacent benign samples.

Fig. 2.

Over-expression of miR-132 and miR-212 increases the proliferation of Panc-1 cells. (A) Proliferation of Panc-1 cells transfected with 60 nM of both pre-miR-132 and pre-miR-212 (30 nM of each) or pre-miR control oligos (60 nM). (B) qPCR verification of relative expression levels of 17 genes regulated by the E2F family of transcription factors. Individual mRNA expression levels were calculated relative to 18S rRNA and the data are expressed as fold-increase under input control, which was assigned a value of "1". (C) Lysates were immunoblotted for expression levels of cyclin A2 and B1 in Panc-1 cells, respectively. Normalized expression to β-actin is included. (D) Panc-1 cells were assayed for BrdU

incorporation following transfection of pre-miR-132/212 or pre-miR-nega (control) (60 nM, 48 h). The data are the mean $\pm$ s.d. for three independent experiments. **P<0.01.

Fig. 3.

Anti-proliferative effects of miR-132/-212 antisense in Panc-1 cells. (A) Representative dose-response curves of Panc-1 cells transfected with various concentrations of miR-132 and miR-212 antisense (open circles) or control oligonucleotide (closed circles). Cell viability was assessed following a 96 h continuous exposure. (B) Cell cycle distribution of Panc-1 cells transfected with miR-132 and miR-212 antisense (IC₅₀ for 48 h) as determined by flow cytometry analysis.

Fig. 4.

Increased expression of miR-132 and miR-212 by β_2 adrenergic receptor agonist. (A) qPCR analyses of β_2 adrenergic receptor mRNA expression levels in normal pancreas (N), adjacent benign pancreas (BE) and pancreatic adenocarcinomas (T), respectively (B) Panc-1 cells were serum starved (0.5% FBS) for 48 h and then treated with 1 μ M terbutaline for up to 72 h. Expression of mature miR-132 and miR-212 was determined by qPCR, respectively. Expression levels were calculated relative to 18S rRNA and the data are expressed as fold-increase under input control, which was assigned a value of "1". ** $P < 0.01$. The data are the mean $\pm$ s.d. for three independent experiments.