Restitution of Tumor Suppressor MicroRNAs Using a Systemic Nanovector Inhibits Pancreatic Cancer Growth in Mice

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Abstract

Mis-expression of microRNAs (miRNA) is widespread in human cancers, including in pancreatic cancer. Aberrations of miRNA include overexpression of oncogenic miRs (Onco-miRs) or downregulation of so-called tumor suppressor TSG-miRs. Restitution of TSG-miRs in cancer cells through systemic delivery is a promising avenue for pancreatic cancer therapy. We have synthesized a lipid-based nanoparticle for systemic delivery of miRNA expression vectors to cancer cells (nanovector). The plasmid DNA–complexed nanovector is approximately 100 nm in diameter and shows no apparent histopathologic or biochemical evidence of toxicity upon intravenous injection. Two miRNA candidates known to be downregulated in the majority of pancreatic cancers were selected for nanovector delivery: miR-34a, which is a component of the p53 transcriptional network and regulates cancer stem cell survival, and the miR-143/145 cluster, which together repress the expression of KRAS2 and its downstream effector Ras-responsive element binding protein-1 (RREB1). Systemic intravenous delivery with either miR-34a or miR-143/145 nanovectors inhibited the growth of MiaPaCa-2 subcutaneous xenografts ($P < 0.01$ for miR-34a; $P < 0.05$ for miR-143/145); the effects were even more pronounced in the orthotopic (intrapancreatic) setting ($P < 0.0005$ for either nanovector) when compared with vehicle or mock nanovector delivering an empty plasmid. Tumor growth inhibition was accompanied by increased apoptosis and decreased proliferation. The miRNA restitution was confirmed in treated xenografts by significant upregulation of the corresponding miRNA and significant decreases in specific miRNA targets (SIRT1, CD44, and aldehyde dehydrogenase for miR34a, and KRAS2 and RREB1 for miR-143/145). The nanovector is a platform with potential broad applicability in systemic miRNA delivery to cancer cells. Mol Cancer Ther; 10(8): 1470–80. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (i.e., pancreatic cancer) is the fourth leading cause of cancer-related death, accounting for approximately 38,000 deaths each year in the United States (1). The overwhelming majority of patients present with locally advanced or distant metastatic disease, rendering their cancers inoperable. In spite of advances in chemotherapy and radiation therapy, the 5-year survival rate is still less than 5%, indicating the ineffectiveness of current approaches to treatment. The recent sequencing of the pancreatic cancer genome has underscored the considerable heterogeneity of somatic DNA alterations between individual tumors (2) and the challenges for molecularly targeted therapies in this neoplasm.

MicroRNAs (miRNA) are small non-coding RNAs consisting of 18 to 24 nucleotides that regulate the expression of coding genes by binding imperfectly with their 3′-untranslated region (UTR; ref. 3). The effects of miRNAs in regulating eukaryotic transcript expression and physiologic processes, such as cell proliferation, differentiation, and apoptosis, have gained widespread interest in the last decade (4). More recently, the role of miRNAs in cancer pathogenesis has been studied extensively, with abnormal miRNA expression levels found in nearly all human cancers (5). Mis-expression of miRNAs is not merely an epiphenomenon of the neoplastic process, but deregulated miRNAs directly contribute to altered physiologic states in tumor cells. Analogous to coding genes, miRNAs are also composed of subsets that can promote tumorigenesis (Onco-miRs) or those that inhibit neoplastic transformation (tumor suppressor; TSG-miRs; refs. 6, 7). Onco-miRs are typically overexpressed in cancer cells, whereas TSG-miRs are downregulated compared with related non-neoplastic cell types, thus...
mimicking the pattern observed with most coding transcripts.

Not surprisingly, several groups (8–11), including ours (12), have catalogued miRNA abnormalities in pancreatic cancer. These studies have elucidated that, similar to other solid tumors, subsets of individual miRNAs (or corresponding miRNA clusters) are either overexpressed (e.g., miR-21, miR-17-92, miR-196a, miR-200a/b, miR-221) or downregulated (e.g., miR-34a, miR-143/145, let-7 family) in pancreatic cancer. Identification of aberrant miRNAs in pancreatic cancer not only provides biological insights into the pathogenesis of this neoplasm (13, 14) but also forms a seedbed for establishing promising biomarkers for early detection in clinical samples (15, 16).

In light of the widespread abnormalities of miRNA expression in human cancers, modulations of deregulated miRNAs in cancer cells has also emerged as a promising therapeutic strategy (17). This concept centers about either the inhibition of overexpressed Onco-miRs or the restitution of downregulated TSG-miRs in cancer cells. For example, specific chemically modified miRNA inhibitors known as antagonimics have been used to suppress the function of Onco-miRs in vitro and in vivo, resulting in the inhibition of tumor growth (18–20). On the contrary, restitution of TSG-miR function has also been used in recent preclinical studies with considerable success (21–25). Thus, one of the co-authors (J.T. Mendell) recently showed that adeno-associated virus–mediated delivery of miR-26a, whose expression is suppressed in hepatocellular carcinoma, leads to dramatic reversal of progression in a murine model of the disease (21). Similarly, 2 groups have shown the therapeutic efficacy of virally administered let-7 in attenuating mutant Kras-induced lung cancer progression in both xenograft and autochthonous mouse models (22, 23). In addition to adeno- and lentiviral vectors, nonviral lipid-based strategies have recently been developed for systemic miRNA delivery and have been applied successfully to lung and prostate cancer xenograft models (24, 25).

The objective of this current study was to establish the feasibility of systemic miRNA delivery to pancreatic cancer, specifically that of TSG-miRs that are commonly downregulated in this disease. We selected 2 candidates, miR-34a and the miR-143/145 cluster, whose expression is lost in the majority of pancreatic cancer samples (12, 26, 27). Of note, expression of both miR-34a and the miR-143/145 cluster is also attenuated in the most common subtypes of human cancer, such as non–small-cell, prostate, and colorectal cancers, among others (24, 25, 26), thus broadening the applicability of this approach beyond pancreatic cancer. We have synthesized a lipid-based nanoparticle for systemic delivery of miRNA expression vectors to cancer cells (nanovector) and have shown the significant therapeutic efficacy of restituting either miR-34a or miR-143/145 expression in subcutaneous and orthotopic pancreatic cancer xenograft models. Furthermore, we confirm the lack of demonstrable toxicity in mice from the nanovector alone. Our nonviral gene delivery platform provides a prototype for safe and efficient delivery of miRNA expression vectors to cancer cells and could be widely used in the preclinical and eventually, clinical arenas.

Materials and Methods

Reagents and cell lines

The reagents 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt; DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine–N-[methoxy(polyethylene glycol)-2000] (ammonium salt; DSPE-PEG-OMe) were purchased from Avanti Polar Lipids. Cell culture–grade cholesterol was purchased from Sigma-Aldrich. The MiaPaCa-2 cell line was obtained from the American Type Culture Collection and cells were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin. The American Type Culture Collection uses DNA fingerprinting (microsatellite analysis) for cell line authentication. CD-1 male athymic nu/nu mice (4 to 6 weeks old) were procured from Harlan.

miRNA expression constructs

The miR-143/145 cluster and miR-34a were amplified from genomic DNA using Pfu polymerase and cloned into the Xhol site in the pMCSV-puro expression construct (Clontech Laboratories). The primers for the miR-143/145 and miR-34a sequences have been previously described (26, 27). The sequences of the amplified products were confirmed by sequencing.

Preparation of lipid-based nanovector for systemic miRNA delivery

Liposomal nanoparticles were prepared by dissolving cationic amphiphile (DOTAP) and co-lipids (cholesterol and DSPE-PEG-OMe) in a 1:1:0.2 ratio, respectively, in a mixture of chloroform and methanol in a glass vial. The organic solvent was removed with a gentle flow of moisture-free nitrogen and the remaining dried film of lipid was then kept under high vacuum for 8 hours. Distilled water (in vitro) or 5% glucose (in vivo) was added to the vacuum-dried lipid film and the mixture was allowed to hydrate overnight. The vial was vortexed for 2 to 3 minutes at room temperature and occasionally shaken in a 45°C water bath to produce multilamellar vesicles. Small unilamellar vesicles were prepared by sonication of the multilamellar vesicles in an ice bath for 3 to 4 minutes until clarity, using a Branson 450 sonifier (Danbury) at 100% duty cycle and 25-W output power. The nanovector represents an electrostatic complex of positively charged liposomal nanoparticle and negatively charged plasmid DNA and was prepared by mixing pMCSV-puro vectors expressing corresponding miRNAs and liposome on a charge ratio basis. Two independent miRNA nanovectors were engineered: the first delivering miR-34a and the second...
delivering miR-143/145. For in vitro experiments, each mouse received 50 μg of DNA complexed with liposome at a 4:1 lipid/DNA charge ratio via tail-vein injection.

**In vitro transfection efficiency assay**

The in vitro transfection efficiency of the nanovector platform was assessed using a firefly luciferase expression vector on a plasmid backbone. MiaPaCa-2 cells were seeded at a density of 12,000 per well in a 96-well plate 18 to 24 hours before transfection. Plasmid DNA (0.3 μg, 0.9 nmol) was complexed with varying amounts of lipids (1–8 nmol) in serum-free medium (total volume up to 100 μL) for 30 minutes. The charge ratios were varied from 1:1 to 8:1 over these ranges of lipid concentration. Immediately before transfection, cells plated in the 96-well plate were washed with PBS (2 × 100 μL) followed by the addition of nanovector. After 4 hours of incubation, the medium was replaced with fresh complete medium containing 10% FBS. The luciferase reporter gene activity was estimated after 48 hours. The cells were washed twice with PBS (100 μL each) and lysed in lysis buffer (50 μL). Firefly luciferase assay was conducted in a Wallac Victor 2 plate reader (Perkin Elmer) using 25-μL injections. Total protein concentration in each well was determined by the modified Lowry method and the luciferase activity was expressed as the relative light unit per milligram of the protein. Lipofectamine 2000 (Invitrogen) was used as a positive control for transfection in this experiment. Each transfection experiment was repeated twice on 2 different days and reported as mean ± SD obtained for the entire series.

**In vitro green fluorescent protein transfection**

MiaPaCa-2 cells were seeded at a density of 48,000 per well in a 24-well plate 48 hours before transfection. Plasmid DNA (1 μg/well; pEGFP-C1; Clontech, catalogue no. 6084-1) was complexed with nanovector (1:1 charge ratio) in opti-MEM (Invitrogen). The resultant pEGFP-nanovector was added to MiaPaCa-2 cells growing in complete media. After 4 hours, growth media was aspirated and replaced with fresh complete media. Cells were allowed to grow for 48 hours, at which point, fluorescence images were taken on the fluorescent isothiocyanate channel.

**Nanovector toxicity study**

Male CD-1 mice (from the Harlan Laboratory) were injected intravenously with either 5% glucose as vehicle or miR-143/145 nanovector 3 times per week for 2 weeks. Blood was collected at necropsy via cardiac puncture. Blood chemistry and renal and liver function parameters were measured by the Johns Hopkins Phenotyping Core facility (http://www.hopkinsmedicine.org/mcp/PHENOCORE/).

**Xenograft studies**

All small-animal (CD-1 athymic mice) experiments described conformed to the guidelines of the Animal Care and Use Committee of the Johns Hopkins University. Mice were maintained in accordance with the guidelines of the American Association of Laboratory Animal Care.

**Systemic delivery of miRNA nanovectors to subcutaneous pancreatic cancer xenografts**

As stated above, we generated 2 independent miRNA nanovectors, with the first delivering miR-34a and the second delivering miR-143/145, and each was tested independently in subcutaneous and orthotopic pancreatic cancer xenograft models. To generate subcutaneous xenografts, 5 × 10⁶ MiaPaCa-2 cells suspended in a total volume of 200 μL [PBS/MatrixGel (Becton Dickinson Biosciences), 1:1 (v/v), prechilled to 4°C] were injected into the flanks of 5- to 6-week-old male nu/nu mice. One week after the injection of tumor cells, subcutaneous tumor volumes (V) were measured with digital calipers (Fisher Scientific) and calculated using the formula \(V = \frac{a b^2}{2}\), where \(a\) is the biggest and \(b\) is the smallest orthogonal tumor diameter (29). Fifteen mice with successfully engrafted MiaPaCa-2 xenografts were then randomized into 3 cohorts of 5 animals each and administered one of the following regimens via tail-vein injection: (i) vehicle, (ii) mock nanovector (nanovector complexed with pMSCV alone), and (iii) miR-34a nanovector, with each regimen administered 3 times per week for 3 weeks. A second series of experiments was conducted using subcutaneous MiaPaCa-2 xenografts administered either (i) vehicle or (ii) miR-143/145 nanovector (5 mice per arm), with the identical dosing schedule (the mock nanovector arm was not included on the basis of results from the miR-34a study; described in the following). Tumor size and body weight were measured once weekly. At the culmination of treatment, visceral organs and tumor tissues were harvested and either preserved in 10% neutral buffered formalin for histology and immunohistochemical studies or snap frozen for nucleic acid analysis.

**Systemic delivery of miRNA nanovectors to orthotopic pancreatic cancer xenografts**

The generation of orthotopic human pancreatic cancer xenografts by surgical implantation in athymic mice has been described previously by our group (29, 30). Briefly, freshly harvested subcutaneous MiaPaCa-2 xenografts were minced into 1-mm³ cubes under sterile conditions for orthotopic implantation. A small pocket was prepared inside the pancreas, into which one of the previously prepared fresh tumor chunks was inserted. Two weeks after surgical orthotopic implantation, the presence of primary tumors was confirmed by ultrasonography (Vevo660, VisualSonics) and measured in 3 orthogonal axes, \(a\), \(b\), and \(c\); tumor volumes were determined as \(V = \frac{abc}{2}\), as described previously (29, 30). Twenty mice with successfully engrafted MiaPaCa-2 xenografts were then randomized into 4 cohorts of 5 animals each and administered one of the following regimens by tail-vein injection: (i) 5% glucose as vehicle,
(ii) void nanovector, (iii) miR-34a nanovector, and (iv) miR-143/145 nanovector, each at 3 times per week for 3 weeks. At the culmination of therapy, visceral organs and tumor tissues were harvested and preserved in 10% neutral buffered formalin for histologic studies. In addition, tumor tissues were snap-frozen for nucleic acid analysis.

**Quantitative assessment of miRNA restitution upon nanovector therapy**

Total miRNA from snap-frozen MiaPaCa-2 xenografts was isolated using the mirVANA PARIS RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. Thereafter, 10 ng of RNA was reverse-transcribed with a miRNA Reverse Transcription Kit (Applied Biosystems) using miR-34a-, miR-143-, and miR-145–specific RT-primers (TaqMan miRNA Assay; Applied Biosystems) as described previously (12, 26, 27). Quantitative PCR was carried out using RNU6B or RNU44 as housekeeping control, and relative expression levels were calculated using the 2^ΔΔCt method (31).

**Quantitative assessment of miRNA targets upon nanovector therapy**

Total RNA (>200 basepairs) was isolated from snap-frozen MiaPaCa-2 xenografts using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Thereafter, cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. For the miR-143/145 nanovector-treated xenografts, quantitative PCR for expression of K Ras2 and RREB1 transcripts was carried out, as previously described (26). Recent data have also shown that miR-34a re-expression depletes tumor-initiating cells in pancreatic and prostate cancers (25, 32); therefore, expression of 2 credentialed markers of tumor-initiating cells in pancreatic cancer—CD44 and aldehyde dehydrogenase (ALDH; refs. 33, 34)—was determined by quantitative real-time PCR (qRT-PCR) in the miR-34a nanovector–treated xenografts.

**Western blot for SIRT1 expression**

Tissues were harvested with radioimmunoprecipitation assay buffer, and protein (50 μg) was loaded and separated on 4% to 20% SDS-polyacrylamide gradient gel. Proteins were transferred onto nitrocellulose membrane (Hybond-ECL; GE Healthcare) and blocked for 1 hour with 5% non-fat milk in PBS containing 0.5% Tween-20 (PBS-T). Blots were then incubated with anti-silent information regulator-1 (SIRT1) antibody (1:500 dilution) for 2 hours at room temperature, followed by incubation with secondary antibody conjugated to horseradish peroxidase at a 1:5,000 dilution for 1 hour. After washing with PBS-T (3 × 10 mL) for 5 minutes each, the chemiluminescence film was developed after addition of the substrate. Anti-tubulin antibody (dilution of 1:2,000) was used as an internal control for protein loading.

**Immunohistochemical analysis of Ras-responsive element binding protein-1 expression**

Immunohistochemistry for Ras-responsive element binding protein-1 (RREB1) was conducted on formalin-fixed paraffin-embedded xenograft tissues from 3 independent tumor samples in each cohort (control vs. treatment), using a standard technique (29, 30). Briefly, tissues were deparaffinized in xylenes and hydrated by a graded series of ethanol washes and pure water. Slides were incubated in 0.3% H2O2 in MeOH for 20 minutes, followed by antigen retrieval in a steamer in EDTA buffer (pH 8.0) at 90°C for 35 minutes. Sections were blocked with Dako Protein Block Serum-Free (Dako) for 10 minutes at room temperature. Slides were incubated with RREB1 antibody (1:200 dilution; Abcam; rabbit polyclonal antibody, catalogue no. ab64168) in Normal Antibody Diluent (ScyTek; phosphate buffered) for 1 hour at room temperature. Slides were then post-blocked in PowerVision Post-Blocking (Leica Microsystems) for 10 minutes at room temperature and incubated with appropriate PowerVision+ Poly-HRP conjugated antibody (Leica Microsystems) for 20 minutes at room temperature. The reaction was developed using DAB+ (Leica Microsystems; catalogue no. PV6126) and counterstained with hematoxylin. Relative intensity of staining was evaluated by a pathologist (A. Maitra) blinded to the arms of the study.

**Assessment of proliferation and apoptosis**

Immunohistochemistry for proliferation (Ki-67 antigen) was done on formalin-fixed paraffin-embedded xenograft tissues, as previously described (29, 30), using an anti-MIB-1 (Ki-67) antibody (clone K2; dilution 1:100; Ventana Medical Systems). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay for apoptosis was conducted according to the manufacturer's instructions (DeadEnd Fluorometric TUNEL System; Promega).

**Results**

**In vitro and in vivo characterization of nanovectors for systemic miRNA delivery**

First, we synthesized a liposomal nanoparticle through rehydration of a thin layer of DOTAP, cholesterol, and DSPE-PEG-O,e, in a 1:1:0.02 molar ratio. The resultant aqueous suspension of cationic liposomes could undergo complex formation with plasmid DNA to form a nanovector. The use of such a liposomal nanovector was decided on the basis of the established safety and efficacy of such formulations and the enhanced circulation stability afforded by the presence of polyethylene glycol (35). When evaluated by transmission electron microscopy, the nanovector with therapeutic cargo was found to have an average diameter in the range of approximately 100 nm (Fig. 1A). Next, we evaluated the in vitro transfection efficiency of the nanovector in MiaPaCa-2 cells, using a firefly luciferase reporter assay and enhanced GFP...
(EGFP) fluorescence microscopy. The transfection efficiency of the nanovector with a 4:1 lipid/DNA charge ratio was comparable with that of Lipofectamine 2000 (Invitrogen) as a positive control (Fig. 1B). In addition, visualization of cells transfected with EFGP-expressing vector indicated effective localization of the DNA cargo to cells in vitro (Fig. 1C). Thereafter, we evaluated the potential for adverse effects upon systemic nanovector therapy by measuring a panel of hematologic and biochemical parameters in non–tumor-bearing CD-1 mice that had received either buffer (5% glucose) or miR-143/145 nanovector by tail-vein injection, 3 times weekly for 2 weeks. Compared with mice treated intravenously with buffer, we observed no significant differences in any of the examined laboratory parameters, including hematology, and liver and renal function tests (Supplementary Fig. S1), underscoring the relative safety of this delivery platform.

Systemic miRNA delivery with nanovectors inhibits the growth of subcutaneous pancreatic cancer xenografts

We next moved to evaluating whether restitution of miRNA could inhibit pancreatic cancer growth in a subcutaneous xenograft model. Successfully engrafted MiaPaCa-2 xenografts were treated via tail-vein injection with either vehicle, mock nanovector, miR-34a nanovector, or miR-143/145 nanovector 3 times weekly, for 3 weeks. Significant tumor growth inhibition was observed when mice were treated with either miR-34a or miR-143/145 nanovectors, compared with mock and vehicle controls (Fig. 2A and B). Notably, the effects of the mock nanovector were essentially identical to vehicle-only treated xenografts, indicating that the growth-inhibitory effects were caused by the expression of the target miRNAs. Examination of hematoxylin and eosin (H&E)-stained sections of treated xenografts showed confluent sheets of necrosis in both miR-34a and miR-143/145 arms, compared with the vehicle and mock nanovector cohorts (Fig. 2C). This observation was confirmed by TUNEL assessment for fragmented DNA (Fig. 2D), which indicated more widespread apoptosis in treated tumors than in the vehicle and mock controls.

Systemic miRNA delivery with nanovectors inhibits the growth of orthotopic pancreatic cancer xenografts

Although such subcutaneous xenografts provided a preliminary indication of the efficacy of miRNA restitution, they fail to recapitulate many key aspects of the tumor microenvironment. To overcome some of these limitations, including drug distribution kinetics observed in the pancreas, an intrapancreatic orthotopic xenograft is considered to be more biologically relevant (36, 37). We, therefore, investigated the efficacy of restitution of miR-34a and miR-143/145 in an orthotopic MiaPaCa-2 pancreatic cancer xenograft model. As shown in Figure 3A, the growth of orthotopic xenografts was significantly inhibited by restitution of either miR-34a or miR-143/145 through systemic nanovector delivery, compared

Figure 1. Size determination and transfection efficiency of DNA-loaded nanovector. A, transmission electron microscopy image of DNA-loaded nanovector, measuring an average 81 ± 12 nmol/L in diameter. B, MiaPaCa-2 cells were transfected with nanovectors containing firefly luciferase expression vector. Relative luciferase units (RLU) are compared against those obtained using Lipofectamine 2000 as a positive control. C, in vitro transfection of pEGFP-C1 plasmid DNA in MiaPaCa-2 cells using the nanovector delivery system.

1474 Mol Cancer Ther; 10(8) August 2011 Molecular Cancer Therapeutics

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with vehicle and mock nanovector arms. Subsequent histologic analysis confirmed confluent sheets of necrosis in the miRNA nanovector arms, similar to what was observed in the subcutaneous milieu (Fig. 3B), a trend reiterated by TUNEL staining for apoptosis (Fig. 3C). Furthermore, to measure whether miRNA restitution impacted proliferation, we carried out immunohistochemical staining for Ki-67 and observed strikingly reduced nuclear Ki-67 levels in both treatment groups (Fig. 3D).

**Systemic nanovector therapy modulates miRNA targets in pancreatic cancer xenografts**

To confirm that the miRNA-delivering nanovectors were successfully reaching the tumor tissue, we measured the levels of mature miRNA in subcutaneous MiaPaCa-2 xenografts by qRT-PCR. Using miRNA-specific primers, we established significant increases in the expression levels of respective miRNAs in xenografts treated with either of the 2 nanovectors, compared with vehicle control (Fig. 4A; $P = 0.04$ for miR-34a; $P = 0.03$ for miR-143; and $P = 0.001$ for miR-145). To validate the effects of miRNA restitution, we analyzed expression levels of key targets of miR34a, miR-143, and miR145 after systemic therapy. For example, SIRT1 is a class III histone deacetylase that regulates apoptosis in response to various oxidative and genotoxic stress (38). SIRT1 expression is repressed by miR-34a binding to its 3’-UTR seed sequence, establishing SIRT1 as a direct target of miR-34a (39). We observed a
marked decrease in SIRT1 levels in miR-34a nanovector–treated xenografts as compared with vehicle control (Fig. 4B), indicating a tangible pharmacodynamic readout of miRNA delivery. Furthermore, multiple lines of evidence point to the role of miR-34a repression in the expansion of tumor-initiating cells (i.e., cancer stem cells) and that restitution of miR-34a expression depletes this subpopulation in cancers (25, 32). Therefore, expression of 2 credentialed markers of tumor-initiating cells in pancreatic cancer—CD44 and ALDH (33, 34)—was determined by qRT-PCR in the miR-34a nanovector–treated xenografts, with both showing a significant reduction (Fig. 4C; \( P = 0.01 \) for ALDH and \( P = 0.05 \) for CD44).

Recently, the repression of miR-143 and miR-145 by oncogenic Ras was identified by our group as a new tumor-promoting feed-forward pathway (26). Kras, through its downstream effector RREB1, represses the transcription of the miR-143/145 cluster, with RREB1 binding to the promoter element of the miRNA primary transcript. In turn, mature miR-143 and miR-145 repress KRAS2 and RREB1, respectively, effectively completing the loop. Therefore, restitution of miR-143/145 expression in MiaPaCa-2 xenografts should attenuate expression of...
both KRAS2 and RREB1. Expectedly, we observed significant downregulation of both transcripts ($P = 0.001$) in xenografts treated with miR-143/145 nanovector compared with controls (Fig. 4D; left). In addition, resultant downregulation of RREB1 protein expression could be observed through immunohistochemical staining of tumor sections from both miR-143/145 nanovector-treated and vehicle control xenografts (Fig. 4D; right). This serves as important in vivo confirmation of the aforementioned feed-forward pathway and...
indicates that treatment with the systemic miR-143/145 nanovector results in restitution of mature miR-143 and miR-145, with downregulation of KRAS2/RREB1 targets accompanying the observed tumor growth inhibition.

Discussion

In this study, we have shown that systemic delivery of TSG-miRs using a nanovector delivery platform can inhibit the growth of pancreatic cancer xenografts in both subcutaneous and orthotopic milieus. Most molecularly targeted therapeutic approaches are directed toward blocking aberrant hyperfunction of oncogenic components; however, the current miRNA delivery strategy is geared toward regaining function lost specifically in cancer cells. We selected 2 miRNA candidates for systemic delivery in pancreatic cancer models: miR-34a and the miR-143/145 cluster. As data from our group and others have established (8, 10, 12, 26, 27), these miRs are downregulated (or completely absent) in the majority of pancreatic cancers. Functionally, miR-34a is a component of the p53 transcriptional network and its loss in cancer cells is associated with resistance to apoptosis induced by p53-activating agents (27, 40, 41). In cancer cells with wild-type p53, ectopic expression of miR-34a levels can restore p53 function by repressing the deacetylase SIRT1, thereby enhancing levels of active (acetylated) p53 (42). The repertoire of miR-34a targets are expectedly quite diverse and include molecules involved in promoting cellular proliferation (cyclin D1 and cyclin-dependent kinases CDK4 and 6) and blocking apoptosis (Bcl-2; refs. 43–45). Thus, restoring miR-34a function in cancer cells is expected to have both proapoptotic and antiproliferative effects, as was observed in the miR-34a nanovector–treated xenografts by TUNEL and Ki-67 labeling, respectively. Recently, miR-34a has also been implicated in regulating the number and function of tumor-initiating cells (i.e., cancer stem cells) in solid cancers (25, 32). Specifically, studies in pancreatic and prostate cancer models have shown that cancer stem cells in these cancer types harbor low miR-34a levels whereas re-expression of this miRNA significantly decreases cancer stem cell clonogenicity and survival and tumor engraftment capacity in vivo. In pancreatic cancer, these profound deleterious effects on cancer stem cells are observed irrespective of p53 functional status (32), underscoring the applicability of therapeutic miR-34a restitution to a disease that harbors TP53 mutations in approximately 70% of cases (2). Indeed, using 2 credentialed surrogate measures of pancreatic cancer stem cells, ALDH and CD44 (33, 34), we showed a significant downregulation in expression of both transcripts in miR-34a nanovector–treated xenografts. Finally, given the widespread loss of expression of miR-34a in many other solid cancers (39, 44, 46), there is also compelling rationale to test the efficacy of the nanovector platform in corresponding preclinical disease models.

The second candidate we tested using nanovector delivery is a cluster of 2 co-transcribed miRNAs, miR-143/145, whose expression is also frequently lost in many solid and hematologic malignancies, including colorectal cancers where a consistent downregulation was first identified (47). Our recent work has identified the existence of a feed-forward loop in pancreatic cancer cells, wherein the Ras effector protein RREB1 directly represses the expression of miR-143/145, thereby relieving the miRNA-mediated repression of KRAS2 and RREB1 transcripts (26). Not unexpectedly, the robust tumor growth inhibition observed in vivo with miR-143/145 nanovector therapy is accompanied by significant downregulation in KRAS2 and RREB1 transcripts, as well as decreased RREB1 protein expression by immunohistochemistry. The seminal importance of the KRAS2 oncogene to pancreatic cancer cannot be overstated—somatic activating mutations are found in greater than 90% of cases, and Ras is implicated in both tumor initiation and tumor maintenance (1, 48). Nonetheless, pharmacologic blockade of this small GTPase protein has been challenging, as small-molecule inhibitors of Ras farnesylation have failed to improve median survival in clinical trials (49). The miR-143/145 nanovector represents a tangible genetic approach toward direct inhibition of KRAS2 in pancreatic cancer, and future studies in the Ras-driven genetically engineered models of pancreatic cancer (50) will provide additional insights on the therapeutic potential of this modality in an autochthonous setting.

We conducted 2 independent experiments using TSG-miRs (i.e., miRNA monotherapy), each of which showed significant and comparable tumor growth inhibition in vivo. We are currently developing delivery methods for concurrent restitution of 2 or more diverse TSG miRs targeting nonoverlapping coding genes (i.e., miRNA combination therapy) with the intent of achieving therapeutic synergy. This approach is based on either the concurrent administration of 2 independent nanovec- tors (e.g., miR-34a or miR-143/145 nanovectors illustrated in this study) or the generation of a single nanovector capable of delivering dual therapeutic cargo (e.g., a bi-cistronic vector expressing 2 miRNAs simultaneously). In addition, combination therapy with other traditional chemotherapy agents (e.g., gemcitabine) may yield improved effects in pancreatic cancer. The lack of demonstrable adverse effects at the histologic or biochemical level is encouraging and is probably because saturation of endogenous levels of miR-34a and miR-143/145 are already achieved in normal cells. Thus, in conclusion, the nanovector platform we have designed can be used for systemic delivery of TSG miRNAs to cancer cells. Although the proof-of-principle studies presented in this article use pancreatic cancer as a disease model, it is conceivable that this approach will be broadly applicable across other tumor types delivering potentially any TSG miR that is a candidate for restitution in cancer cells.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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