Therapeutics, Targets, and Chemical Biology

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MicroRNA Replacement Therapy for miR-145 and miR-33a Is Efficacious in a Model of Colon Carcinoma

Ahmed Fawzy Ibrahim[1], Ulrike Weirauch[1], Maren Thomas[2], Arnold Grünweller[2], Roland K. Hartmann[2], and Achim Aigner[1]

Abstract

MicroRNAs (miRNA) aberrantly expressed in tumors may offer novel therapeutic approaches to treatment. miR-145 is downregulated in various cancers including colon carcinoma in which *in vitro* studies have established proapoptotic and antiproliferative roles. miR-33a was connected recently to cancer through its capacity to downregulate the oncogenic kinase Pim-1. To date, miRNA replacement therapy has been hampered by the lack of robust nonviral delivery methods for *in vivo* administration. Here we report a method of miRNA delivery by using polyethylenimine (PEI)-mediated delivery of unmodified miRNAs, using miR-145 and miR-33a to preclinically validate the method in a mouse model of colon carcinoma. After systemic or local application of low molecular weight PEI/miRNA complexes, intact miRNA molecules were delivered into mouse xenograft tumors, where they caused profound antitumor effects. miR-145 delivery reduced tumor proliferation and increased apoptosis, with concomitant repression of c-Myc and ERK5 as novel regulatory target of miR-145. Similarly, systemic injection of PEI-complexed miR-33a was validated as a novel therapeutic targeting method for Pim-1, with antitumor effects comparable with PEI/siRNA-mediated direct *in vivo* knockdown of Pim-1 in the model. Our findings show that chemically unmodified miRNAs complexed with PEI can be used in an efficient and biocompatible strategy of miRNA replacement therapy, as illustrated by efficacious delivery of PEI/miR-145 and PEI/miR-33a complexes in colon carcinoma. *Cancer Res; 71(15); 5214–24. ©2011 AACR.*

Introduction

MicroRNAs (miRNA) are highly conserved, small 17 to 25 nucleotide (nt) noncoding RNA molecules which specifically interact with target mRNAs, thus inhibiting translation or leading to mRNA cleavage and degradation (1). It is estimated from *in silico* analyses that miRNAs can control the expression of approximately 30% of all proteins in humans (2), and it has become clear that miRNAs regulate various cellular processes. Many miRNAs are aberrantly expressed in pathologies such as cancer, leading to the identification of "miRNA signatures" characteristic of certain tumors (3). Tumor-specific miRNA expression profiles are also functionally relevant because many miRNAs act as tumor suppressors or, to the contrary, as oncogenes (oncomiRs). For many miRNAs, target genes have been identified which are relevant in tumorigenesis, tumor growth, tumor angiogenesis, and metastasis (see ref. 4 for review). Crucial for miRNA specificity is the "seed region" (nt 2–8); however, owing to this limited sequence, complementarity miRNAs are capable of simultaneously targeting different genes. In fact, it is estimated that some miRNAs may have more than 100 target genes. This translates into a complex pattern of control of gene expression and suggests potentially increased efficacies of miRNAs by acting through different genes and pathways.

The observation of pathologically decreased levels of certain miRNAs acting as tumor suppressors has led to the concept of miRNA replacement therapy. Indeed, the reintroduction of miR-26a in a liver cancer mouse model by using an adeno-associated viral vector resulted in the inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and protection from disease progression (5). In line with this, the adenovirus-mediated delivery of a plasmid coding for miRNA let-7 reduced lung tumor formation in an orthotopic lung cancer mouse model (6). Likewise, intranasal administration of a lentiviral let-7 miRNA construct led to reduced tumor burden in an autochthonous non–small cell lung carcinoma tumor mouse model (7). This study also explored synthetic, formulated let-7 oligonucleotides which, upon intratumoral (i.t.) injection, led to reduced tumor growth (7). Local and/or systemic delivery in non–small lung cancer xenografts showed antitumor effects upon application of miR-34 formulated with a lipid-based delivery reagent (8). The

Authors' Affiliations: [1]Institute of Pharmacology, Faculty of Medicine; and [2]Institute of Pharmaceutical Chemistry, Philipps-University, Marburg, Germany

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A.F. Ibrahim and U. Weirauch contributed equally to the work.

Corresponding Author: Achim Aigner, Institute of Pharmacology, Faculty of Medicine, Philipps-University Marburg, Karl-von-Frisch-Strasse 1, D–35032 Marburg, Germany. Phone: 49-6421-286-2262; Fax: 49-6421-286-5600; E-mail: aigner@staff.uni-marburg.de

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same miRNA exhibited survivin downregulation and reduced metastatic tumor load in the lung when delivered in a liposomal nanoparticle formulation (9). In prostate tumors initiated in the bones of mice, intravenously (i.v.) injected atelocollagen-complexed miR-16 mimics led to reduced tumor growth (10). Finally, in colorectal carcinoma xenografts, i.t. or i.v. injection of miR-143 formulated in cationic liposomes resulted in antitumor effects in the case of modified (for increased nuclease resistance) miR-143, but not unmodified miR-145 (11). This so far limited number of studies that are not based on viruses indicates that applications aiming at miRNA replacement therapy critically depend on the development of miRNA delivery tools. Because viral delivery may raise several issues including potential safety problems because of the application of recombinant DNA, induction of toxic immune responses, and possible changes in gene expression upon random integration of DNA in the host genome, nonviral strategies would be preferable.

Colon carcinoma is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. Despite a favorable prognosis when detected at early stages, it is associated with limited survival when metastatic disease is present, which indicates the need for novel treatment strategies. In colon carcinoma, decreased levels of miRNA-145 are observed (see ref. 12), and previous *in vitro* studies have established a proapoptotic and antiproliferative role of miR-145 (13). In contrast, the functional relevance of miR-33a in cancer has only recently been established. It was shown that miR-33a can repress the protooncogene Pim-1 to act as a tumor suppressor inhibiting cell-cycle progression (14). Thus, both miRNA-145 and miR-33a may represent attractive candidates for miRNA replacement therapy that have not been explored so far.

Polyethylenimines (PEI) are linear or branched polymers which are partially protonated under physiologic conditions, thus allowing the formation of complexes with nucleic acids (15). PEIs have been used previously for the delivery of DNA plasmids (15) and other DNA or RNA molecules including ribozymes (16) and siRNAs (17). PEI-based complexes (polyplexes) are able to enter the cells via caveolae- or clathrin-dependent routes and, once internalized, the high efficiency of PEI polyplexes is governed by their facilitated release from endosomes due to the so called "proton sponge effect" (18).

This is the first study to explore the therapeutic effects of PEI/miRNA complexes. We introduce the low molecular weight branched PEI F25-LMW (19, 20) as a delivery platform for nonmodified miRNAs in miRNA replacement therapy and show antitumor effects of PEI/miRNA complexes upon their local or systemic application.

Materials and Methods

miRNAs, siRNAs, tissue culture, and animals

Chemically synthesized miRNAs and siRNAs without modifications were purchased from MWG (Eurofins MWG Operon) or from Thermo Scientific. The used miRNAs represent the double-stranded Dicer processing product as published in miRBase/release14 (21), but lacking the 5′-terminal phosphates. Additional details and sequences can be found in the Supplementary Methods.

All cell lines were obtained from the American type culture collection and authenticated by the vendor. Cells were cultivated in a humidified incubator under standard conditions (37°C, 5% CO_2) in keratinocyte-SFM medium (Invitrogen GmbH) supplemented with 20 to 30 mg/mL bovine pituitary extract, 0.1 to 0.2 ng/mL rEGF, and 10% fetal calf serum (FCS; PAA; 1205LU cells) or IMDM/10% FCS (all other cell lines). Athymic nude mice (nu/nu) were purchased from Harlan Winkelmann and kept at 23°C in a humidified atmosphere with food and water *ad libitum*. Animal studies were done according to the national regulations and approved by the Regierungspräsidium Giessen, Germany.

PEI complexation of RNAs

PEI F25-LMW/miRNA complexes were prepared essentially as described previously for siRNAs (20, 22). Briefly, 10 μg miRNA was dissolved in 75 μL 10 mmol/L HEPES/150 mmol/L NaCl, pH 7.4, and incubated for 10 minutes. Ten microliters of PEI F25-LMW (5 μg/μL) (19) was dissolved in 75 μL of the same buffer, and after 10 minutes, pipetted to the miRNA solution. This resulted in an N/P ratio = 33 which had been determined as optimal for siRNAs (20). For *in vivo* experiments, the mixture was divided into aliquots and stored frozen (23). Prior to use, complexes were thawed and incubated for 1 hour at room temperature. jetPEI complexation was done in 1 mol/L HEPES/150 mmol/L NaCl, pH 7.4 at N/P = 5 as described previously (22).

miRNA tissue uptake *in vivo*

For the radioactive determination of miRNA tissue distribution, 0.6 μg (0.05 nmol) miRNA were [^{32}P] end–labeled at both strands by using T4 polynucleotide kinase (New England Biolabs) and γ-[^{32}P] ATP (GE Healthcare), prior to purification by microspin columns (Bio-Rad) to remove unbound radioactivity and complexation as described above. A total of 1.5×10^6 LS174T cells in 150 μL PBS were injected s.c. into both flanks of athymic nude mice (nu/nu) and grown until they reached a size of approximately 8 mm in diameter. The complexes, or noncomplexed labeled miRNAs as negative control, were dissolved in 200 μL or 100 μL PBS for intraperitoneal (i.p.) or i.v. injection, respectively. After 4 hours, mice were sacrificed, organs were removed, and subjected to RNA preparation as described above. The total RNA was dissolved in 200 μL DEPC-treated water, and 10-μL samples were mixed with loading buffer, heat denatured, and subjected to agarose gel electrophoresis before blotting and autoradiography (Biomax; Eastman-Kodak). Quantitation was done by phosphorimager analysis.

PEI F25-LMW/miRNA treatment in subcutaneous tumor models

Athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu}, 6–8 weeks of age) were used and kept in tight cages with standard rodent chow and water available *ad libitum*, and a 12-hour light/dark cycle. A total of 1.5×10^6 LS174T or HCT116 cells in 150 μL PBS were injected s.c. into both flanks of the mice. When solid

tumors were established, mice were randomized into specific treatment, negative control treatment, and nontreatment groups ($n = 12$ tumors per group). Treatment with PEI/miRNA or PEI/siRNA complexes was done by i.p. injection of 0.77 nmol (10 μg) or i.t. injection of 0.3 nmol (4 μg) PEI F25-LMW-complexed specific or nonspecific siRNA at the time points indicated in the figures. Tumor volumes were monitored every 2 to 3 days as indicated in the figures and, upon termination of the experiment, mice were sacrificed and tumors removed. Pieces of each s.c. tumor were immediately fixed in 10% paraformaldehyde for paraffin embedding or snap-frozen for RNA preparation or Western blot analysis.

Proliferation and soft agar assays

For the assessment of anchorage-dependent proliferation, cells were seeded 24 hours prior to transfection in 96-well plates at 1,000 (LS174T) or 300 (HCT-116) cells per well. Transfections were done by the addition of the specific or nonspecific PEI F25-LMW/miRNA or jetPEI/miRNA complexes corresponding to 10 pmol miRNA per well. At the time points indicated, the numbers of viable cells in 8 wells were determined by using a colorimetric assay according to the manufacturer's protocol (Cell Proliferation Reagent WST-1; Roche).

Anchorage-independent proliferation was studied in soft agar assays essentially as described previously (24). A total of 1×10^5 cells per well were transfected in 6-well plates with 100 nmol/L PEI-complexed miRNAs or nonspecific control siRNA and 24 hours after transfection, trypsinized and counted. A total of 20,000 cells in 0.35% agar (Bacto Agar; Becton Dickinson) were layered on top of 1 mL of a solidified 0.6% agar layer in a 35-mm dish. Growth media with 2% FCS were included in both layers. After 2 to 3 weeks of incubation, colonies more than 50 μm in diameter were counted by at least 2 independent blinded investigators.

Caspase assay and fluorescein isothiocyanate–Annexin assay

To test for apoptosis *in vitro*, a commercially available bioluminescent caspase-3/7 assay (Caspase-Glo 3/7 assay; Promega) was applied. The Caspase-Glo 3/7 assay was conducted in 96-wells with cells transfected as described above, and luminescence was measured after 1 hour incubation at 27°C in the dark by using a Fluostar Optima reader (BMG Labtec). To normalize for differences in cell densities, a WST-1 assay was carried out in parallel on the same plate, and the results of caspase activity were adjusted to cell numbers of the different cell lines.

To distinguish between early and late apoptosis, a fluorescein isothiocyanate (FITC)–Annexin assay was done (Becton Dickinson). LS174T cells were transfected with 100 nmol/L miRNAs in 6-well plates by using jetPEI as described above. After 120 hours, the cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in 1× binding buffer (0.01 mol/L HEPES (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$) at a concentration of 10^6 cells/mL. A total of 100 μL of the cell suspension were transferred to a new Eppendorf tube, 5 μL FITC–Annexin and then 5 μL propidium iodide were added, the mixture was gently vortexed and incubated for 15 minutes at room temperature in the dark. Directly before measurement, 400 μL 1× binding buffer was added to the mixture and samples were measured by FACS (FACSCalibur; Becton-Dickinson) within 1 hour.

RNA preparation and quantitative RT-PCR for mRNA and miRNA dectection

Total RNA from tumor cells was isolated by using the Trizol reagent (Sigma) according to the manufacturer's protocol. For tissue homogenization prior to RNA preparation, tissues were mixed with 200 μL Trizol reagent and homogenized. Reverse transcription (RT) by using the RevertAid H Minus First Strand cDNA Synthesis Kit from Fermentas and quantitative PCR in a LightCycler from Roche by using the QuantiTect SYBR Green PCR kit (Qiagen) were done according to the manufacturers' protocols and as described previously (20). To normalize for equal cDNA amounts, PCR reactions with ERK5-specific and with actin-specific primer sets were run in parallel for each sample, and ERK5 levels were determined by the formula $2^{CP(actin)}/2^{CP(ERK5)}$ with CP = cycle number at the crossing point (0.3).

To isolate miRNA, the mirVana miRNA isolation kit was used according to the manufacturer's protocol (Applied Biosystems). Adherent cells from a 6-well plate were trypsinized and washed in PBS, prior to adding 300 to 600 μL lysis binding buffer and vigorous vortexing. In case of tumor tissue, the lysis binding buffer was added as 10 volumes of the tumor weight. For miRNA RT, specific stem-looped RT primers were used as described previously (25). The RT product was diluted 1:10 in nuclease-free water and used as a template for quantitative real-time PCR (see Supplementary Methods for primer sequences) under the following conditions: 95°C for 15 minutes followed by 55 cycles comprising 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 10 seconds. Levels were determined as described above.

Western blots and immunohistochemistry

LS174T cells from 6-well plates were scraped at 96 hours posttransfection, lysed in 200 μL denaturating lysis buffer, and sonicated for 20 seconds, prior to incubation on ice for 30 minutes and centrifugation at 13,000 rpm for 25 minutes. Tumor tissue was homogenized in radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] by using a Dounce homogenizer. After centrifugation and determination of the protein concentration in the supernatant, samples were analyzed by Western blotting (see Supplementary Methods for details).

Immunohistochemical staining of paraffin-embedded sections for proliferating cell nuclear antigen (PCNA) was done essentially as described previously (ref. 20; see Supplementary Methods for details). For the assessment of proliferation, the PCNA staining intensity after diaminobenzidine development was evaluated in at least 5 fields per section by rating as 0 (no staining), 1 (weak staining), 2 (intermediate staining), and 3 (strong staining), and expressed as PCNA score.

ELISA

For the quantitation of Pim-1 by sandwich ELISA, the "Pim-1 Total Antibody Pair" and buffers (Invitrogen) were used. The procedure was done according to manufacturer's protocol (see Supplementary Methods), with 100 μL supernatant from a tumor sample (homogenized in PBS and centrifuged at 13,000 rpm) being analyzed per well.

Statistics

Statistical analyses were done by Student's *t* test, 1-way ANOVA/Tukey's multiple comparison posttests or 2-way ANOVA/Bonferroni posttests by using GraphPad Prism4, and significance levels were *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, #, not significant.

Results

Analysis of miR-145 levels in tumor cells

Levels of miR-145 were assessed in various tumor cell lines and compared with the nontumor fibroblast cell line NIH/3T3 (Supplementary Fig. S1). Although our data confirm previous studies about the downregulation of miR-145 in tumors (12, 26, 27), we also show that miR-145 levels depend on the cell line. This is particularly true for prostate carcinoma cells with levels being very low in DU-145 cells, whereas in PC-3 cells, almost normal levels were observed. Likewise, 1205LU melanoma cells showed high miR-145 expression almost in the range of nontumor cells. Notably, all 7 colon carcinoma cell lines tested displayed low miR-145 levels, indicating that miR-145 downregulation is a general feature in colon carcinoma and relevant to the pathologic state of such tumors. For subsequent experiments, the tumorigenic colon carcinoma cell line LS174T and the particularly aggressive cell line HCT-116 were selected.

PEI-mediated miRNA delivery *in vitro*

To explore whether PEI is able to deliver miRNAs into cells, PEI complexes based on linear jetPEI or branched PEI F25-LMW were prepared. Transfection of LS174T cells with PEI/miRNA complexes led to more than 10-fold increase in intracellular miR-145 levels, indicating that the intact miRNA is efficiently delivered into the cells (Fig. 1A). JetPEI was used in these experiments, because *in vitro*, it displays higher transfection efficacy in this cell line than the branched PEI F25-LMW (data not shown). More importantly, PEI-mediated miRNA transfection led to markedly more than 60% reduced cell proliferation as compared with nontransfected or negative control transfected cells (Fig. 1B). As negative control, a small dsRNA molecule with length, structure, and GC content similar to that of the miRNAs studied here and directed against an irrelevant gene (luciferase) was employed, and complexed with PEI under identical conditions. The specific inhibition of cell proliferation confirms the biological relevance of miR-145 in colon carcinoma cells *in vitro* and shows that PEI-complexed miRNAs are functionally active. Results were confirmed in soft agar assays which monitor anchorage-independent cell growth. Again, a marked reduction in cell proliferation, as indicated by fewer and smaller colonies, was observed upon PEI-mediated miRNA delivery (Fig. 1C). The comparison between nontransfected and negative control transfected cells also shows the absence of nonspecific PEI effects on proliferation (Fig. 1B and C).

The tumor cell inhibition resulting from the PEI-mediated miR-145 delivery was studied in more detail on the cellular and molecular level. In a fluorescence-activated cell sorting (FACS)-based FITC–Annexin assay, a more than 2-fold increase in early-stage and late-stage apoptotic cells was observed upon PEI/miR-145 treatment as compared with negative controls (Fig. 1D). The parallel increase in caspase-3/-7 activation (Fig. 1E) indicated that this induction of apoptosis relied on a caspase-3/-7–dependent pathway. In addition, PEI/miR-145 treatment interfered with downstream signal transduction relevant to proliferation. More specifically, a decrease in ERK5 protein expression was observed; a parallel reduction in ERK5 mRNA levels was less pronounced and lacked statistical significance (Fig. 1F). Although miR-143 (see below) has been shown previously to directly target ERK5 (28), this establishes for the first time that ERK5 is modulated by miR-145 as well.

miR-143 belongs to the same miRNA family as miR-145 and has been shown to be downregulated along with miR-145 in tumors including colon carcinoma (29). Indeed, when monitoring anchorage-independent growth in soft agar assays, PEI-mediated delivery of miR-143 inhibited colony formation similar to miR-145 (Supplementary Fig. S2, right). Anchorage-dependent proliferation, however, was not impaired by miR-143 (Supplementary Fig. S2, left). Thus, whereas miR-143 exerts some tumor cell–inhibiting effects as well, our findings indicate differences between both miRNAs with regard to biological effects and targeted pathways as suggested previously (27). Because of its more universal antitumor effects, miR-145 was selected for subsequent *in vivo* experiments.

Antitumor effects upon systemic application of PEI/miR-145 complexes

LS174T colon carcinoma cells were s.c. injected into athymic nude mice, and upon establishment of tumor xenografts, mice were randomized into treatment and negative control groups. In untreated controls and in mice which were i.p. injected with PEI/nonspecific RNA complexes as negative controls, rapid tumor growth was detected, with an approximately 15-fold increase in tumor volume over 23 days. In contrast, i.p. injection of 10 μg PEI-complexed miR-145 three times per week resulted in a statistically significant, almost 50% decrease in tumor growth (Fig. 2A). Upon termination of the experiment, tumors (see representative examples in Fig. 2A, right panel) were analyzed for ERK5, which has been shown *in vitro* to be downregulated by miR-145 (see above). Indeed, a statistically significant, that is, approximately 50% decrease in ERK5 protein levels was detected in the tumors (Fig. 2B, left), whereas, again comparable with the *in vitro* situation, ERK5 mRNA levels remained largely unchanged (data not shown). This indicates that PEI-delivered miR-145 exerts antitumor effects through inhibition of ERK5 translation. Strikingly, the PEI/miR-145 effects on c-Myc, another

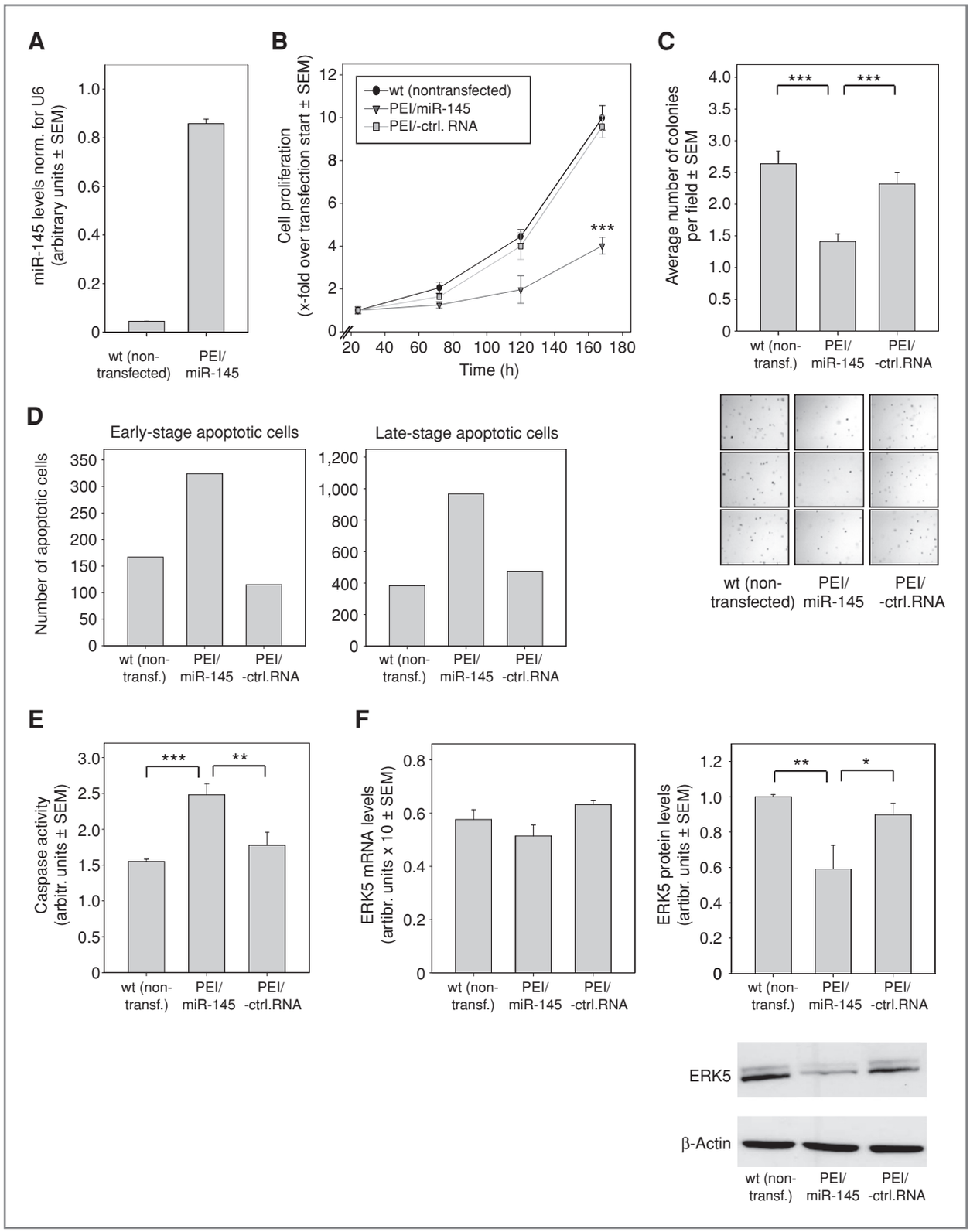

Figure 1. *In vitro* effects of PEI-mediated delivery of miR-145 in LS174T colon carcinoma cells. A, miR-145 levels are markedly upregulated upon PEI/miR-145 transfection as shown by qRT-PCR. PEI/miR-145 transfection reduces anchorage-dependent growth (B) and anchorage-independent soft agar colony formation (C). Increased apoptosis upon PEI-mediated miR-145 delivery is observed, as indicated by early-stage (D) and late-stage (E) apoptotic cells, and by caspase-3/-7 activation (E). ERK5 is established as novel target for miR-145, being downregulated on protein (F, right), but not on mRNA levels (F, left).

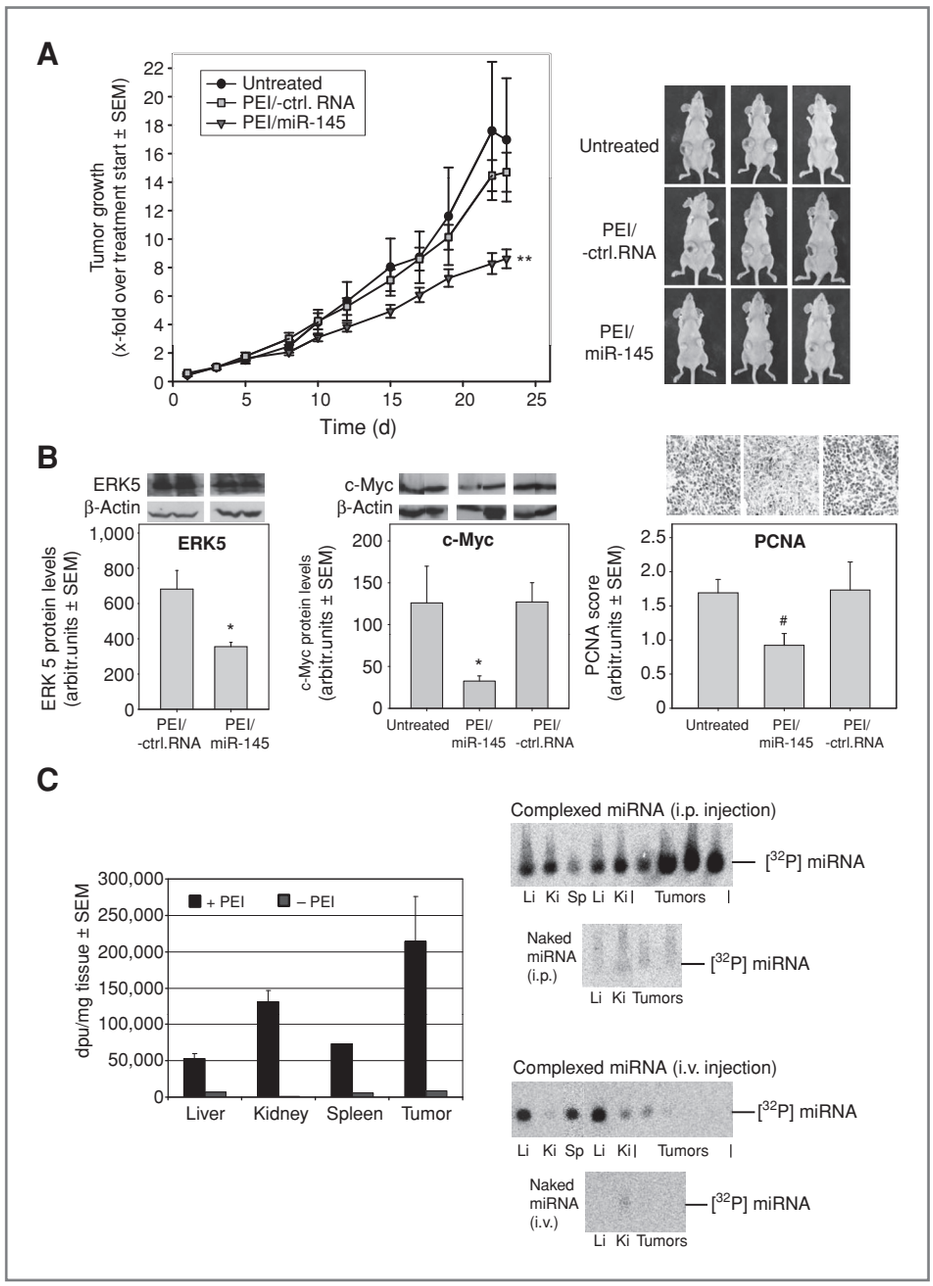

Figure 2. Antitumor effects of PEI/miR-145 treatment in s.c. LS174T colon carcinoma xenograft mouse models. A, systemic injection of PEI/miR-145 complexes results in reduced tumor growth as compared with untreated or PEI/RNA negative control–treated mice. Right: representative examples of mice after termination of the experiment. B, tumor-inhibitory effects of PEI/miR-145 complexes are mirrored by reduced protein levels of ERK5 (left) and c-Myc (middle), and by decreased cell proliferation in the tumor tissue as determined by immunohistochemical staining for PCNA (right). Representative examples of the Western blot data and of the immunohistochemical pictures are given. C, gel electrophoresis and autoradiography of [^{32}P] end–labeled miRNA reveals the delivery of intact full-length miRNA molecules to various tissues including liver (Li), kidney (Ki), spleen (Sp), and tumors (right). Differences in the biodistribution are observed between i.p. (top) and i.v. injection (bottom), with particularly efficient miRNA delivery into tumors upon i.p. administration. The delivery of intact miRNA is dependent on PEI complexation because no signals are observed upon injection of naked [^{32}P] end–labeled miRNAs. Left: quantitation of miRNA levels, on the basis of phosphoimaging, after i.p. injection.

established target for miR-145 (30), were even more profound with approximately 80% decreased c-Myc protein levels as compared with negative controls (Fig. 2B, middle). Concomitantly, an approximately 50% reduction in tumor cell proliferation was observed in the specific treatment group (Fig. 2B, right).

Radioactive biodistribution experiments confirmed that the observed effects were indeed based on the PEI-mediated delivery of intact miRNA molecules. Upon i.p. injection of PEI complexes containing [^{32}P]-labeled miRNA, total RNAs were prepared from tissues and analyzed by gel electrophoresis and autoradiography. This revealed substantial uptake of miRNA in liver and kidney, among others, and to a lesser extent in spleen (Fig. 2C, top right panel). Particularly high levels of intact, full-length miRNA were observed in the tumor xenografts (Fig. 2C, top right panel). In contrast, naked miRNA was detectable, though, at very low levels in the tested tissues, indicating the necessity for miRNA formulation with PEI (Fig. 2C, top right panel). The quantitation of miRNA bands and normalization for protein content confirmed the PEI-mediated miRNA delivery into the xenografts (Fig. 2C, left). To the contrary, i.v. injection resulted in strong signals in liver and spleen, but not in s.c. tumor xenografts, and was thus not employed in our experiments (Fig. 2C, bottom right

panel). This also indicates that the mode of administration introduces at least some tissue preference of the complexes, even in the absence of an active ligand-mediated targeting. By comparing the data with naked miRNAs (Fig. 2C, bottom right panel), the i.v. injection also confirmed that the detection of intact miRNAs in a given tissue is dependent on PEI complexation.

Antitumor effects upon local application of PEI/miR-145 complexes

To further explore the therapeutic applicability of PEI/miR-145 complexes, a local treatment regimen was tested next. In these experiments, the particularly tumorigenic colon carcinoma cell line HCT-116 was selected. Pre-experiments *in vitro* confirmed that PEI-mediated delivery of miR-145 exerts antiproliferative effects also in this cell line, as seen by a less profound, although still statistically significant approximately 25% to 30% decrease in colony growth in soft agar assays (Supplementary Fig. S3). Again, the treatment 3 times per week was started after the establishment of tumor xenografts; however, because PEI complexes were applied locally, miRNA amounts were reduced to 4 μg. No unwanted side effects in terms of change in mouse body weight (Supplementary Fig. S4A, right), behavioral alterations or other signs of discomfort were observed. Untreated tumors showed a rapid growth with an approximately 8-fold increase over 2.5 weeks (Fig. 3A). Reduced tumor growth was observed in the PEI/negative control RNA group, indicating some nonspecific effects of the PEI complexes upon local injection (Fig. 3A). A control experiment confirmed the nonspecific reduction of tumor growth also upon injection of naked negative control RNA or PEI alone, indicating the possibility of some local effects of the components of the PEI complexes (Fig. 3B). Notably, however, the i.t. application of

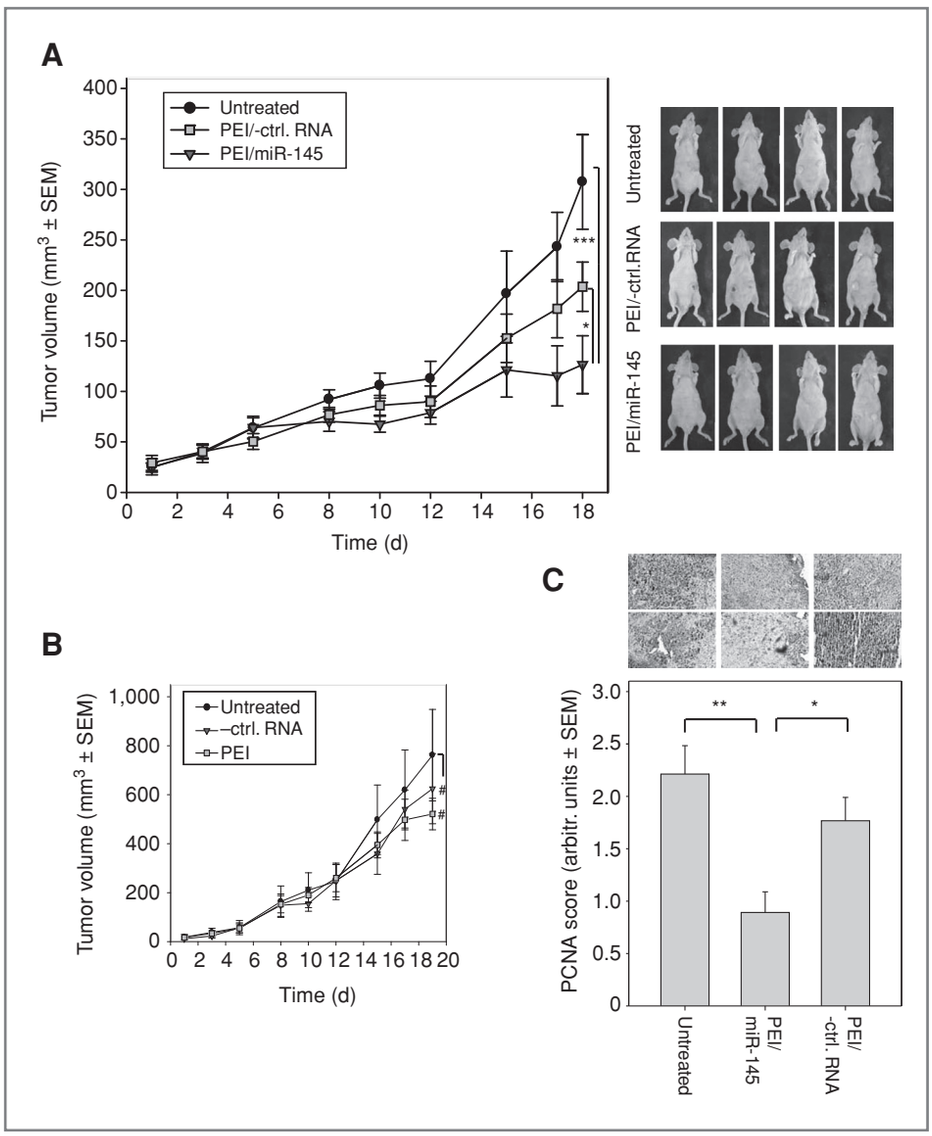

Figure 3. Tumor-inhibitory effects of PEI/miR-145 complexes in s.c. HCT-116 colon carcinoma xenografts upon i.t. injection. A, the local administration leads to some nonspecific effects of PEI/negative control RNA complexes as compared with untreated controls; more profound effects, however, are observed in the PEI/miR-145 treatment group. B, moderate nonspecific reduction of tumor growth upon injection of naked negative control RNA or PEI alone, indicating the possibility of some local effects of the components of the PEI complexes. C, specific antitumor effects are reflected by reduced tumor cell proliferation in the xenografts as determined by immunohistochemical staining for PCNA (right).

PEI/miR-145 complexes resulted in a very profound tumor growth inhibition, with tumor volumes in the treatment group being reduced to approximately 40% or 60% of the untreated or negative control–treated tumors, respectively (Fig. 3A). After termination of the experiment, the immunohistochemical analysis of the tumors revealed a marked antiproliferative effect of the PEI/miR-145 treatment (Fig. 3C).

Antitumor effects by systemic administration of PEI/miR-33a complexes

To further substantiate the concept of PEI-mediated miRNA replacement therapy, we selected a second miRNA, miR-33a, which we showed more recently to be relevant in the context of the oncogenic kinase Pim-1 (14). Pim-1 is upregulated in various cancers including lymphoma and colon carcinoma, is a marker of poor prognosis in prostate carcinomas, and has been implicated in early transformation and tumor progression (31, 32). Importantly, Pim-1 is negatively regulated by miR-33a. Luciferase assays based on reporter constructs comprising the luciferase gene and the Pim-1 3′-UTR (untranslated region) revealed a direct effect of miR-33a on the Pim-1 3′-UTR, as indicated by a approximately 50% inhibition of luciferase activity (Supplementary Fig. S5A, left panel). This effect was abolished upon miR-33a seed mutagenesis in the reporter construct (Supplementary Fig. S5A, right panel), confirming the specificity of this effect. So far, miR-33a has only been implicated with posttranscriptional repression of the ATP-binding cassette transporter A1 (ABCA1) resulting in inhibition of cholesterol efflux from macrophages and maintenance of cholesterol homeostasis (33, 34). However, PEI-based *in vitro* transfection of miR-33a resulted in decreased cell proliferation in colon carcinoma cells, comparable with PEI/siRNA-mediated Pim-1 knockdown (Supplementary Fig. S5B). Thus, this establishes an antiproliferative role of miR-33a and makes it an attractive candidate miRNA for replacement therapy.

To assess the therapeutic antitumor activity of miR-33a *in vivo*, mice bearing s.c. LS174T tumor xenografts were treated by i.p. injection of PEI/miR-33a complexes. The systemic PEI/miR-33a treatment starting upon establishment of the tumors at day 7 after cell injection resulted in a statistically significant, that is, approximately 40% reduction of tumor growth as compared with negative control PEI/RNA-treated mice (Fig. 4A). Similar results were obtained upon PEI-mediated delivery of a Pim-1–specific siRNA (Fig. 4B; see Fig. 4C for representative mice). Concomitantly, the analysis of the tumors upon termination of the experiment revealed an approximately 40% downregulation of Pim-1 in both treatment groups (Fig. 4D). The analysis of the mouse body weights during the whole treatment period revealed no changes (Supplementary S4A, left), and no other unwanted side effects in terms of behavioral alterations or other signs of discomfort were observed. Upon termination of the experiment, the determination of TNFα serum levels showed no induction of TNFα, indicating the absence of an immune response upon PEI/miRNA treatment (Supplementary Fig. S4B). Likewise, no increase in the activity of the liver enzymes aspartate aminotransferase (AST; Supplementary Fig. S4C, left) or alanine aminotransferase (ALT; Supplementary Fig. S4C, right) were detected, thus confirming the absence of hepatotoxicity.

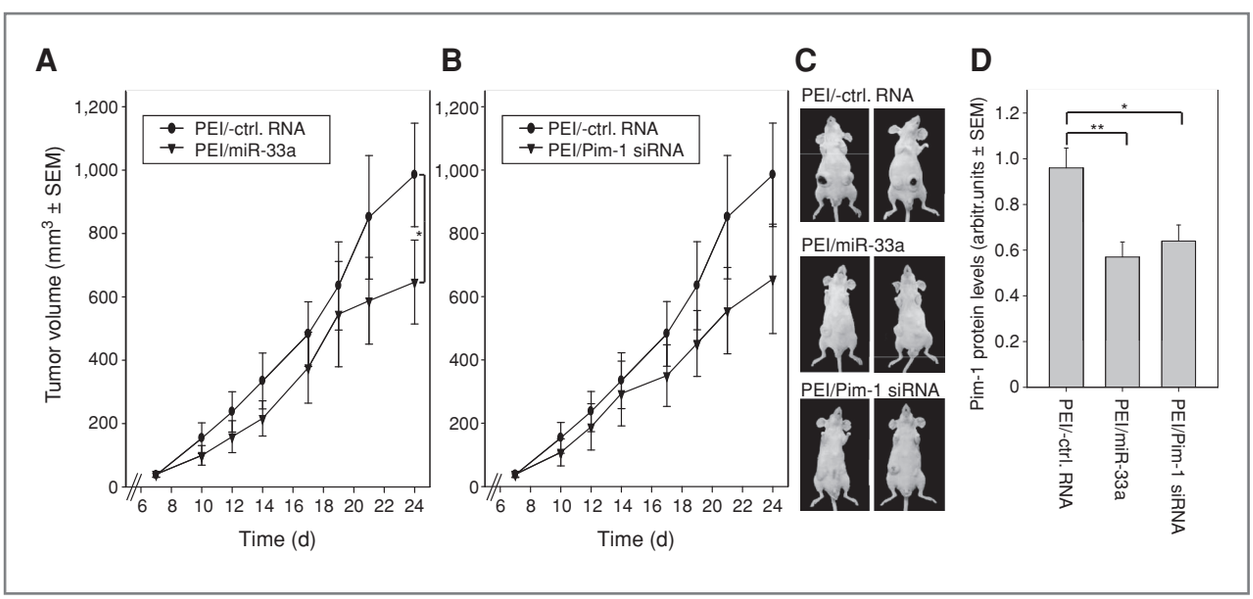

Figure 4. Antitumor effects of systemic PEI/miR-33a treatment in LS174T tumor xenografts as compared with PEI/siRNA-mediated knockdown of Pim-1. A, systemically injected PEI/miR-33a complexes result in inhibition of tumor growth as compared with negative control–treated mice. B, similar antitumor effects are observed upon PEI/siRNA treatment for Pim-1 knockdown. C, representative examples of mice. D, analysis of the tumor xenografts upon termination of the experiment reveals a comparable reduction of Pim-1 levels upon PEI/miR-33a or PEI/Pim-1 siRNA treatment as compared with negative control treatment.

Discussion

With the discovery of RNA interference (RNAi), knockdown strategies have gained increasing interest for the specific inhibition of pathologically upregulated genes. More recently, particular miRNAs were found to be aberrantly expressed in several tumors, favoring tumor development (see ref. 3). The functional relevance of miRNA dysregulation is supported by the fact that the repression or reintroduction of a single miRNA is able to substantially contribute to tumorigenesis, tumor progression, and/or tumor metastasis, and can correlate with tumor differentiation, stage, and prognosis (for review, see refs. 4, 35). Thus, in the case of upregulation, miRNAs represent interesting target molecules for inhibition through miRNA antagonists such as antagomiRs or antimiRs (36, 37). However, miRNAs are more frequently downregulated in tumors (38). Although this indicates the usefulness of miRNA replacement therapy, only a very few studies have explored this strategy so far. Although the literature on miRNA functionality suggests that miRNA effects on a given gene may be less pronounced than siRNA-mediated knockdown through RNAi, the major advantage may lie in the fact that a given miRNA targets many, possibly hundreds of genes, and consequently induces more widespread changes in protein expression (39). In fact, it is the inherent mechanism of miRNAs to repress a large number of mRNAs, also in the case of less than 100% complementarity to the mRNA with high specificity. Thus, the therapeutic application of a tumor suppressor miRNA, which is downregulated in tumors, may indeed entail multiple antitumor effects by specifically interfering with several oncogenic pathways, thus making it more difficult for tumor cells to activate escape mechanisms. One feature of this interference is the rather modest effects on any given single gene, which may actually be an advantage because the subtle regulation of genes is the natural mechanism and therefore particularly attractive also from a therapeutic viewpoint.

Our results establish, for the first time, miR-33a as tumor-relevant miRNA suited for miRNA replacement therapy. Although miR-33a was previously shown to repress ABCA1, an important positive regulator of high-density lipoprotein synthesis and reverse cholesterol transport (33, 34), we have recently shown that it is able to repress the proto-oncogene Pim-1, thereby acting as a tumor suppressor (14). Here, we show antitumor effects of miR-33a delivery *in vivo*; however, it will have to be seen if a prolonged treatment may lead to unwanted other effects related to the other functions of miR-33a. Future studies with the goal to identify and verify other miR-33a target genes will help to further scrutinize the suitability of miR-33a for miRNA replacement therapy. To this end, *in silico* predictions are insufficient because we found that, for example Cdk6, although identified by TargetScan 5.1 as another high probability target, is not regulated by miR-33a (14). Consequently, experimental confirmation is always needed to firmly establish any regulatory mechanism of miRNAs. This is also true for miR-145, although several target genes have already been confirmed (see ref. 13), including c-Myc. Notably, we newly identify ERK5, which so far had only been reported as a target for the related miRNA miR-143 (40), as being directly or indirectly regulated by miR-145.

Still, major limitations of siRNA-mediated therapeutic knockdown strategies also apply to miRNAs: their protection upon systemic injection *in vivo*, efficient delivery to the target organ, cellular uptake, and intracellular release into the right compartment. Although certain PEIs have been used previously for the delivery of other nucleic acids including small RNA molecules *in vitro* and *in vivo* (see refs. 41, 42 for review), this article establishes for the first time a PEI-based miRNA replacement therapy. Notably, although several, if not all PEIs are able to complex and protect nucleic acids, efficient PEI/miRNA-mediated gene targeting *in vivo* relies on the sufficient stability of the complexes as well as favorable pharmacokinetic properties and high biocompatibility. Our biodistribution data show the PEI-dependent delivery of unmodified miRNAs into the tumors, where intact full-length miRNAs accumulate. No chemical modifications of the miRNAs are necessary and thus we avoid the term "miRNA mimics" to indicate that it is in fact the naturally occurring miRNA employed here.

The bioactivity and specificity of PEI/miRNA complexes is shown by their antitumor effects and their inhibition of specific targets (i.e., downregulation of c-Myc, ERK5, and Pim-1). The previously observed biocompatibility of PEI F25-LMW–based complexes (20), even upon repeated injection, was confirmed in this study also for PEI/miRNA complexes. Notably, they can be stored frozen for a prolonged time, thus allowing their standardized preparation and injection as efficient and stable ready-to-use formulations without the need of processing prior to administration.

The observed antitumor effects and the fact that no unwanted side effects were detected indicate that the clinically more relevant systemic delivery rather than an only local delivery (i.t. injection) can be pursued.

Interestingly, we observed that PEI-mediated miR-33a delivery versus PEI/siRNA-induced Pim-1 knockdown led to similar antitumor effects. This was true despite the fact that oncogenic lesions commonly result in the simultaneous repression of many miRNAs (ref. 43), suggesting that more than one miRNA might be needed for a successful therapeutic intervention. However, previous studies indicated as well that the delivery of just one miRNA already exerts tumor-inhibitory effects (5–8, 10, 11). Nevertheless, it should be noted that the nonviral delivery of miRNAs through PEI complexes easily allows the combination of several miRNAs, which may result in additive or synergistic effects. This may well lead to the development of more personalized treatment strategies by precisely analyzing miRNA expression profiles in a given tumor prior to selectively combining miRNAs identified as being downregulated.

Although miRNA replacement therapy seems to be rather mild and biocompatible, yet specific and efficient as a therapeutic strategy, it still requires the careful monitoring of unwanted specific or nonspecific effects. On the basis of the rather highly abundant delivery of miRNAs, any miRNA replacement therapy will have to be tested for RISC saturation, competition with other endogenous miRNAs, or

overstimulation of a given pathway. Notably, however, the saturation of various cellular factors, which are required for the processing of small RNAs and whose saturation can result in lethal effects (44), is not an issue here because chemically synthesized mature miRNAs do not require cellular processing prior to RISC incorporation. Thus, miRNAs may provide better tools than DNA-based expression systems, as seen already in the case of small RNAs for gene knockdown (45). Still, any off-target effects of miRNA delivery will have to be monitored in preclinical studies. Although, in principal, nonspecific effects are rather unlikely because the applied miRNA is identical to the physiologically occurring one and is thus targeting the same mRNAs (as opposed to siRNAs which do not have natural counterparts), it has also been shown that off-target effects induced by certain sequence motifs of synthetic RNAs can be dose dependent (46). However, the literature on miRNA replacement approaches so far (5, 6, 8, 10), as well as our results even after prolonged treatment, do not support adverse effects. The fact that miRNAs exert only moderate effects on their target genes and are already present and functional under normal physiologic conditions may account for that. This may be particularly true for unmodified miRNAs, which are preferable because they preclude any potentially adverse effects resulting from chemical modification. Even more, however, this requires the development of powerful delivery techniques like PEI complexation which work for unmodified miRNAs.

Taken together, the PEI-based therapeutic application of unmodified miRNAs without any chemical modifications combines several favorable properties: (i) the miRNAs are identical to their naturally occurring counterparts (i.e., they are not just "biosimilars," but "bioidenticals") and, simultaneously, (ii) they are easy to produce via chemical synthesis. Previous studies (iii) have established the biocompatibility of low molecular weight PEIs, which in their linear form are already employed in clinical studies. The fact that (iv) no DNA-based expression constructs are employed, avoids safety issues, for example, related to insertional mutagenesis upon viral or nonviral delivery of DNAs or overloading of the cellular dsRNA processing system, and (v) the ability of miRNAs to target multiple oncogenic pathways predestines them for the therapy of cancer as a "pathway disease" (47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
2. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15–20.
3. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.
4. Aigner A. MicroRNAs (miRNAs) in cancer invasion and metastasis: therapeutic approaches based on metastasis-related miRNAs. J Mol Med 2011;89:445–57.
5. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 2009;137:1005–17.
6. Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. Cell cycle 2008;7:759–64.
7. Trang P, Medina PP, Wiggins JF, Ruffino L, Kelnar K, Omotola M, et al. Regression of murine lung tumors by the let-7 microRNA. Oncogene 2010;29:1580–7.
8. Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D, et al. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. Cancer Res 2010;70:5923–30.
9. Chen Y, Zhu X, Zhang X, Liu B, Huang L. Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. Mol Ther 2010;18:1650–6.
10. Takeshita F, Patrawala L, Osaki M, Takahashi RU, Yamamoto Y, Kosaka N, et al. Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. Mol Ther 2010;18:181–7.
11. Akao Y, Nakagawa Y, Hirata I, Iio A, Itoh T, Kojima K, et al. Role of anti-oncomirs miR-143 and -145 in human colorectal tumors. Cancer Gene Ther 2010;17:398–408.
12. Slaby O, Svoboda M, Fabian P, Smerdova T, Knoflickova D, Bednarikova M, et al. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. Oncology 2007;72:397–402.
13. Sachdeva M, Mo YY. miR-145-mediated suppression of cell growth, invasion and metastasis. Am J Transl Res 2010;2:170–80.
14. Thomas M, Lange-Grünweller K, Weirauch U, Gutsch D, Aigner A, Grünweller A, et al. The proto-oncogene Pim-1 is a target of miR-33a. Oncogene 2011 (in press).
15. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc Natl Acad Sci U S A 1995;92:7297–301.
16. Aigner A, Fischer D, Merdan T, Brus C, Kissel T, Czubayko F. Delivery of unmodified bioactive ribozymes by an RNA-stabilizing polyethylenimine (LMW-PEI) efficiently down-regulates gene expression. Gene Ther 2002;9:1700–7.
17. Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. Gene Ther 2005;12:461–6.
18. Behr JP. The proton sponge: a trick to enter cells the viruses did not exploit. Chimia 1997;51:34–6.
19. Werth S, Urban-Klein B, Dai L, Hobel S, Grzelinski M, Bakowsky U, et al. A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes. J Control Release 2006;112:257–70.

20. Hobel S, Koburger I, John M, Czubayko F, Hadwiger P, Vornlocher HP, et al. Polyethylenimine/small interfering RNA-mediated knockdown of vascular endothelial growth factor *in vivo* exerts anti-tumor effects synergistically with Bevacizumab. J Gene Med 2010;12: 287–300.
21. www.mirbase.org.
22. Grzelinski M, Urban-Klein B, Martens T, Lamszus K, Bakowsky U, Hobel S, et al. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs *in vivo* exerts antitumoral effects in glioblastoma xenografts. Hum Gene Ther 2006;17:751–66.
23. Hobel S, Prinz R, Malek A, Urban-Klein B, Sitterberg J, Bakowsky U, et al. Polyethylenimine PEI F25-LMW allows the long-term storage of frozen complexes as fully active reagents in siRNA-mediated gene targeting and DNA delivery. Eur J Pharm Biopharm 2008;70:29–41.
24. Wellstein A, Lupu R, Zugmaier G, Flamm SL, Cheville AL, Delli Bovi P, et al. Autocrine growth stimulation by secreted Kaposi's fibroblast growth factor but not by endogenous basic fibroblast growth factor. Cell Growth Differ 1990;1:63–71.
25. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
26. Motoyama K, Inoue H, Takatsuno Y, Tanaka F, Mimori K, Uetake H, et al. Over- and under-expressed microRNAs in human colorectal cancer. IntJ Oncol 2009;34:1069–75.
27. Arndt GM, Dossey L, Cullen LM, Lai A, Druker R, Eisbacher M, et al. Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. BMC cancer 2009;9:374.
28. Clape C, Fritz V, Henriquet C, Apparailly F, Fernandez PL, Iborra F, et al. miR-143 interferes with ERK5 signaling, and abrogates prostate cancer progression in mice. PLoS One 2009;4:e7542.
29. Iio A, Nakagawa Y, Hirata I, Naoe T, Akao Y. Identification of non-coding RNAs embracing microRNA-143/145 cluster. Mol Cancer 2010;9:136.
30. Sachdeva M, Zhu S, Wu F, Wu H, Walia V, Kumar S, et al. p53 represses c-Myc through induction of the tumor suppressor miR-145. Proc Natl Acad Sci U S A 2009;106:3207–12.
31. Bachmann M, Moroy T. The serine/threonine kinase Pim-1. Int J Biochem Cell Biol 2005;37:726–30.
32. Shah N, Pang B, Yeoh KG, Thorn S, Chen CS, Lilly MB, et al. Potential roles for the PIM1 kinase in human cancer - a molecular and therapeutic appraisal. Eur J Cancer 2008;44:2144–51.
33. Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, Gerszten RE, et al. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science 2010;328:1566–9.
34. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N, et al. MiR-33 contributes to the regulation of cholesterol homeostasis. Science 2010;328:1570–3.
35. Lee YS, Dutta A. MicroRNAs in cancer. Annu Rev Pathol 2009;4: 199–227.
36. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, et al. LNA-mediated microRNA silencing in non-human primates. Nature 2008;452:896–9.
37. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs *in vivo* with 'antagomirs'. Nature 2005;438:685–9.
38. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005; 435:834–8.
39. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature 2008;455:58–63.
40. Akao Y, Nakagawa Y, Iio A, Naoe T. Role of microRNA-143 in Fas-mediated apoptosis in human T-cell leukemia Jurkat cells. Leuk Res 2009;33:1530–8.
41. Aigner A. Nonviral *in vivo* delivery of therapeutic small interfering RNAs. Curr Opin Mol Ther 2007;9:345–52.
42. Gunther M, Lipka J, Malek A, Gutsch D, Kreyling W, Aigner A. Polyethylenimines for RNAi-mediated gene targeting *in vivo* and siRNA delivery to the lung. Eur J Pharm Biopharm 2011;77:438–49.
43. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 2008;40:43–50.
44. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 2006;441:537–41.
45. McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I, et al. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. Proc Natl Acad Sci U S A 2008;105:5868–73.
46. Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol 2005;23:457–62.
47. Bader AG, Brown D, Winkler M. The promise of microRNA replacement therapy. Cancer Res 2010;70:7027–30.