MicroRNA replacement therapy for miR-145 and miR-33a is efficacious in a model of colon carcinoma

Ahmed Fawzy Ibrahim1,*, Ulrike Weirauch1,*, Maren Thomas2, Arnold Grünweller2, Roland K. Hartmann2, and Achim Aigner1,3

1 Institute of Pharmacology, Faculty of Medicine, Philipps-University, Marburg, Germany
2 Institute of Pharmaceutical Chemistry, Philipps-University Marburg, Germany
3 Corresponding author

* These authors contributed equally to the work

Running title: Therapeutic PEI/miRNA delivery
Key words: RNAi, miRNA, miR-145, miR-33a, polyethylenimine, PEI, colon carcinoma, miRNA replacement therapy

Corresponding author:
Dr. 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
email: aigner@staff.uni-marburg.de
Abstract

MicroRNAs aberrantly expressed in tumors may offer novel therapeutic approaches to treatment. miR-145 is downregulated in various cancers including colon carcinoma where in vitro studies have established pro-apoptotic and anti-proliferative 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 non-viral delivery methods for in vivo administration. Here we report a method of miRNA delivery 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 anti-tumor 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 anti-tumor effects comparable to PEI/siRNA-mediated direct in vivo knockdown of Pim-1 in the model. Our findings demonstrate 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.

Précis

This study establishes a simple method for non-viral miRNA replacement therapy to treat cancer.
**Introduction**

MiRNAs are highly conserved, small 17 - 25 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 ~ 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 since 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 e.g., (4) for review). Crucial for miRNA specificity is the 'seed region' (nucleotides 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 >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 re-introduction of miR-26a in a liver cancer mouse model 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 NSCLC 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 anti-tumor effects upon application of miR-34 formulated with a lipid-based delivery reagent (8). The 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 anti-tumor 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. Since viral delivery may raise several issues including potential safety problems due to 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, non-viral 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 e.g. (12)), and previous \textit{in vitro} studies have established a pro-apoptotic and anti-proliferative 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 proto-oncogene 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 (PEIs) are linear or branched polymers which are partially protonated under physiological 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 non-modified miRNAs in miRNA replacement therapy and demonstrate anti-tumor 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, Ebersberg, Germany) or from Thermo Scientific (Schwerte, Germany). 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 (ATCC) and authenticated by the vendor. Cells were cultivated in a humidified incubator under standard conditions (37°C, 5% CO₂) in keratinocyte-SFM medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 20-30 mg/ml bovine pituitary extract, 0.1–0.2 ng/ml rEGF and 10% fetal calf serum (PAA, Coelbe, Germany) (1205LU cells) or IMDM / 10% FCS (all other cell lines). Athymic nude mice (nu/nu) were purchased from Harlan Winkelmann (Borchen, Germany) and kept at 23°C in a humidified atmosphere with food and water ad libitum. Animal studies were performed 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 mM HEPES / 150 mM NaCl, pH 7.4, and incubated for 10 min. 10 µl PEI F25-LMW (5 µg/µl) (19) was dissolved in 75 µl of the same buffer, and after 10 min 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 devided into aliquots and stored frozen (23). Prior to use, complexes were thawed and incubated for 1 h at room temperature (RT). jetPEI complexation was performed in 1 M HEPES / 150 mM 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 [³²P]-end-labeled at both strands using T4 polynucleotide kinase (New England Biolabs) and γ-[³²P]ATP (GE Healthcare), prior to purification by microspin columns (Bio-Rad, München, Germany) to remove unbound radioactivity and complexation as described above. 1.5 x 10⁶ 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 ~ 8 mm in diameter. The complexes, or non-complexed 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 h, 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 prior to blotting and autoradiography (Biomax, Eastman-Kodak, Rochester, NY). Quantitation was performed by phosphorimager analysis.

**PEI F25-LMW/miRNA treatment in subcutaneous tumor models**

Athymic nude mice (Hsd:Athymic Nude-Foxn1nu, 6-8 weeks of age) were used and kept in tight cages with standard rodent chow and water available ad libitum, and a 12-h light/dark cycle. 1.5 x 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 non-treatment groups (n=12 tumors per group). Treatment with PEI/miRNA or PEI/siRNA complexes was performed by intraperitoneal (i.p.) injection of 0.77 nmol (10 µg) or intratumoral (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 - 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 h prior to transfection in 96-well plates at 1000 (LS174T) or 300 (HCT-116) cells / well. Transfections were performed by the addition of the specific or non-specific PEI F25-LMW/miRNA or jetPEI/miRNA complexes corresponding to 10 pmol miRNA / well. At the time points indicated, the numbers of viable cells in eight wells were determined using a colorimetric assay according to the manufacturer's protocol (Cell Proliferation Reagent WST-1, Roche, Mannheim, Germany).

Anchorage-independent proliferation was studied in soft agar assays essentially as described previously (24). 1*10^5 cells / well were transfected in six-well plates with 100 nM PEI-complexed miRNAs or non-specific control siRNA and 24 h after transfection trypsinized and counted. 20,000 cells in 0.35% agar (Bacto Agar, Becton Dickinson, Heidelberg, Germany) 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 - 3 weeks of incubation, colonies more than 50 µm in diameter were counted by at least two independent blinded investigators.

**Caspase assay and FITC-annexin assay**

To test for apoptosis in vitro, a commercially available bioluminescent caspase-3/7 assay (Caspase-Glo 3/7 assay, Promega, Mannheim, Germany) was applied. The Caspase-Glo 3/7 assay was performed in 96-wells with cells transfected as described above, and luminescence was measured after 1 h incubation at 27°C in the dark using a Fluostar Optima reader (BMG Labtec, Jena, Germany).
normalize for differences in cell densities, a WST-1 assay was performed 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 FITC-annexin assay was performed (Becton Dickinson). LS174T cells were transfected with 100 nM miRNAs in 6-well plates using jet-PEI as described above. After 120 h, the cells were harvested by trypsinization, washed twice with cold PBS and resuspended in 1x Binding buffer (0.01 M Hepes (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 10⁶ cells/ml. 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 min at room temperature in the dark. Directly before measurement, 400 µl 1x Binding Buffer was added to the mixture and samples were measured by FACS (FACSCalibur, Becton-Dickinson) within 1 h.

RNA preparation and Quantitative RT-PCR for mRNA and miRNA detection

Total RNA from tumor cells was isolated using the Trizol reagent (Sigma, Deisenhofen, Germany) 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) using the RevertAid H Minus First Strand cDNA Synthesis Kit from Fermentas (St. Leon-Rot, Germany) and quantitative PCR in a LightCycler from Roche (Penzberg, Germany) using the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) were performed 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⁻ⁿ(CP(ERK5) / CP(Actin)) with CP = cycle number at the crossing point (0.3).

To isolate microRNA, the mirVana™ miRNA isolation kit was used according to the manufacturer’s protocol (Applied Biosystems, Darmstadt, Germany). Adherent cells from a six-well plate were trypsinized and washed in PBS, prior to adding 300-600 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 reverse transcription, 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 min followed by 55 cycles comprising 95°C for 10 s, 55°C for 10 s and 72°C for 10 s. Levels were determined as described above.

Western blots and immunohistochemistry

LS174T cells from 6-well plates were scraped at 96 h post transfection, lysed in 200 µl denaturing lysis buffer and sonicated for 20 s, prior to incubation on ice for 30 min and centrifugation at 13,000 rpm for 25 min. Tumor tissue was homogenized in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) using a Dounce homogenizer. After
centrifugation and determination of the protein concentration in the supernatant, samples were analysed by Western blotting (see Supplementary Methods for details).

Immunohistochemical staining of paraffin-embedded sections for PCNA was performed essentially as described previously (20); see Supplementary Methods for details). For the assessment of proliferation, the PCNA staining intensity after diaminobenzidine development was evaluated in at least five 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 performed 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 analysed per well.

**Statistics**

Statistical analyses were performed by Student's t-test, One-way ANOVA/Tukey's multiple comparison post-tests or Two-way ANOVA / Bonferroni post-tests using GraphPad Prism4, and significance levels are * = 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 to the non-tumor fibroblast cell line NIH/3T3 (Suppl. Figure 1). While our data confirm previous studies regarding the downregulation of miR-145 in tumors (12, 26, 27), we also demonstrate 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, while in PC-3 cells almost normal levels were observed. Likewise, 1205LU melanoma cells showed high miR-145 expression almost in the range of non-tumor cells. Notably, all seven 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 pathological 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 a > 10-fold increase in intracellular miR-145 levels, indicating that the intact miRNA is efficiently delivered into the cells (Figure 1A). JetPEI was used in these experiments, since 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 > 60% reduced cell proliferation as compared to non-transfected or negative control transfected cells (Figure 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 demonstrates 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 (Figure 1C). The comparison between non-transfected and negative control transfected cells also demonstrates the absence of non-specific PEI effects on proliferation (Figs. 1B,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 FACS-based FITC-annexin assay, a > 2-fold increase in early stage and late stage apoptotic cells was observed upon PEI/miR-145 treatment as compared to negative controls (Figure 1D). The parallel increase in caspase-3/7 activation (Figure 1E) indicated that this induction of apoptosis relied on a caspase-3/-7 dependent pathway. Additionally, 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 (Figure 1F). While 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 (Suppl. Figure 2, right). Anchorage-dependent proliferation, however, was not impaired by miR-143 (Suppl. Figure 2, left). Thus, while miR-143 exerts some tumor cell-inhibiting effects as well, our findings indicate differences between both miRNAs regarding biological effects and targeted pathways as suggested previously (27). Due to its more universal anti-tumor effects, miR-145 was selected for subsequent in vivo experiments.
Anti-tumor 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/non-specific RNA complexes as negative controls, rapid tumor growth was detected, with a ~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 (Figure 2A). Upon termination of the experiment, tumors (see representative examples in Figure 2A, right panel) were analysed for ERK5 which has been shown in vitro to be downregulated by miR-145 (see above). Indeed, a statistically significant ~50% decrease in ERK5 protein levels was detected in the tumors (Figure 2B, left), while, again comparable to the in vitro situation, ERK5 mRNA levels remained largely unchanged (data not shown). This indicates that PEI-delivered miR-145 exerts anti-tumor effects through inhibition of ERK5 translation. Strikingly, the PEI/miR-145 effects on c-Myc, another established target for miR-145 (30), were even more profound with ~80% decreased c-Myc protein levels as compared to negative controls (Figure 2B, center). Concomitantly, a ~50% reduction in tumor cell proliferation was observed in the specific treatment group (Figure 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 [32P]-labeled miRNA, total RNAs were prepared from tissues and analysed by gel electrophoresis and autoradiography. This revealed substantial uptake of miRNA in liver and kidney, among others, and to a lesser extent in spleen (Figure 2C, upper right panel). Particularly high levels of intact, full-length miRNA were observed in the tumor xenografts (Figure 2C, upper 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 (Figure 2C, upper right panel). The quantitation of miRNA bands and normalization for protein content confirmed the PEI-mediated miRNA delivery into the xenografts (Figure 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 (Figure 2C, lower 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 (Figure 2C, lower right panel), the i.v. injection also confirmed that the detection of intact miRNAs in a given tissue is dependent on PEI complexation.

Anti-tumor 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 anti-proliferative effects also in this cell line, as seen by a less profound, although still statistically significant ~25 – 30% decrease in colony growth in soft agar assays (Suppl. Fig 3). Again, the treatment three times per week was started after the establishment of tumor xenografts; however, since PEI complexes were applied locally, miRNA amounts were reduced to 4 µg. No unwanted side effects in terms of change in mouse body weight (Suppl. Fig. 4A, right), behavioural alterations or other signs of discomfort were observed. Untreated tumors showed a rapid growth with a ~ 8-fold increase over 2.5 weeks (Figure 3A). Reduced tumor growth was observed in the PEI/negative control RNA group, indicating some non-specific effects of the PEI complexes upon local injection (Figure 3A). A control experiment confirmed the non-specific 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 (Figure 3B). Notably, however, the intratumoral application of PEI/miR-145 complexes resulted in a very profound tumor growth inhibition, with tumor volumes in the treatment group being reduced to ~40% or ~60% of the untreated or negative control treated tumors, respectively (Figure 3A). After termination of the experiment, the immunohistochemical analysis of the tumors revealed a marked anti-proliferative effect of the PEI/miR-145 treatment (Figure 3C).

Anti-tumor 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 demonstrated 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 revealed a direct effect of miR-33a on the Pim-1 3′-UTR, as indicated by a ~50% inhibition of luciferase activity (Suppl. Fig. 5A, left panel). This effect was abolished upon miR-33a seed mutagenesis in the reporter construct (Suppl. Fig. 5A, 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 to PEI/siRNA-mediated Pim-1 knockdown (Suppl. Fig. 5B). Thus, this establishes an anti-proliferative role of miR-33a and makes it an attractive candidate miRNA for replacement therapy.

To assess the therapeutic anti-tumor 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 ~40% reduction of tumor growth as compared to negative control PEI/RNA-
treated mice (Figure 4A). Similar results were obtained upon PEI-mediated delivery of a Pim-1-specific siRNA (Figure 4B; see Figure 4C for representative mice). Concomitantly, the analysis of the tumors upon termination of the experiment revealed a ~40% downregulation of Pim-1 in both treatment groups (Figure 4D). The analysis of the mouse body weights during the whole treatment period revealed no changes (Suppl. 4A, left), and no other unwanted side effects in terms of behavioural 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 (Suppl. Fig. 4B). Likewise, no increase in the activity of the liver enzymes aspartate aminotransferase (AST, SGOT; Suppl. Fig. 4C, left) or alanine aminotransferase (ALT, SGPT; Suppl. Fig. 4C, right) were detected, thus confirming the absence of hepatotoxicity.

Discussion

With the discovery of 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 e.g. (3)). The functional relevance of miRNA dysregulation is supported by the fact that the repression or re-introduction 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 (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. While 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 anti-tumor 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 since 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. While miR-33a was previously shown to repress ABCA1, an important positive regulator of high-density lipoprotein (HDL) synthesis and reverse cholesterol transport (33, 34), we have recently demonstrated that miR-33a is able to repress the proto-oncogene Pim-1, thereby acting as a tumor suppressor (14). Here, we demonstrate anti-tumor 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 since we found that e.g. 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 e.g., (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. While certain PEIs have been used previously for the delivery of other nucleic acids including small RNA molecules in vitro and in vivo (see (41, 42) for review), this paper establishes for the first time a PEI-based miRNA replacement therapy. Notably, while 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 demonstrate 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' in order to indicate that it is in fact the naturally occurring miRNA employed here.

The bioactivity and specificity of PEI/miRNA complexes is demonstrated by their anti-tumor effects and their inhibition of specific targets (i.e., downregulation of c-Myc, ERK5, 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 anti-tumor 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 vs. PEI/siRNA-induced Pim-1 knockdown led to similar anti-tumor effects. This was true despite the fact that oncogenic lesions commonly result in the simultaneous repression of many miRNAs (e.g., (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 non-viral 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 analysing miRNA expression profiles in a given tumor prior to selectively combining miRNAs identified as being downregulated.

While miRNA replacement therapy appears to be rather mild and biocompatible, yet specific and efficient as a therapeutic strategy, it still requires the careful monitoring of unwanted specific or non-specific effects. Based on 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 since 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. While, in principal, non-specific 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 physiological 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 favourable 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 e.g. related to insertional mutagenesis upon viral or non-viral 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).

Acknowledgments
We are grateful to Andrea Wüstenhagen for expert technical assistance.

Grant Support
This work was supported by grants from the German Cancer Aid (Deutsche Krebshilfe, grants 106992 and 109260 to A.G., R.K.H. and A.A.) and the Deutsche Forschungsgemeinschaft (Forschergruppe 'Nanohale' AI 24/6-1 to A.A.), as well as by a Ph.D. fellowship grant from the Youssef Jameel Foundation to A.F.I.
References


**Figure Legends**

**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 demonstrated 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.** Anti-tumor 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 to untreated or PEI/RNA negative control treated mice. Right panel: 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 (center), 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 panels). Differences in the biodistribution are observed between i.p. (upper panel) and i.v. injection (lower panel), with particularly efficient miRNA delivery into tumors upon i.p. administration. The delivery of intact miRNA is dependent on PEI complexation since no signals are observed upon injection of naked [$^{32}$P]end-labeled miRNAs. Left panel: quantitation of miRNA levels, based on phosphoimaging, after i.p. injection.

**Figure 3.** Tumor-inhibitory effects of PEI/miR-145 complexes in s.c. HCT-116 colon carcinoma xenografts upon intratumoral injection. (A) The local administration leads to some non-specific effects of PEI/negative control RNA complexes as compared to untreated controls; more profound effects, however, are observed in the PEI/miR-145 treatment group. (B) Moderate non-specific 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 anti-tumor effects are reflected by reduced tumor cell proliferation in the xenografts as determined by immunohistochemical staining for PCNA (right).

**Figure 4.** Anti-tumor effects of systemic PEI/miR-33a treatment in LS174T tumor xenografts as compared to PEI/siRNA-mediated knockdown of Pim-1. (A) Systemically injected PEI/miR-33a complexes result in inhibition of tumor growth as compared to negative control-treated mice. (B)
Similar anti-tumor 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 to negative control treatment.
A

Tumor growth (×-fold over treatment start) ± SEM

Untreated PEI / miR-145

B

ERK5

β-actin

c-myc

β-actin

PCNA

C

Complexed miRNA (intraperitoneal injection)

Liver Kidney Spleen Tumor

Complexed miRNA (intravenous injection)

Liver Kidney Spleen Tumor

Ibrahim et al., Fig. 2 - revised

Downloaded from cancerres.aacrjournals.org on April 7, 2017. © 2011 American Association for Cancer Research.
A

B

C

Tumor volume (mm³ ± SEM)

PCNA score (arbit. units ± SEM)

untreated

PEI

miR-145

PEI / -ctrl. RNA

untreated

PEI / -ctrl. RNA

PEI / ctrl. RNA

PEI / miR-145

Ibrahim et al., Fig. 3 - revised
MiRNA replacement therapy through PEI-mediated in vivo delivery of miR-145 or miR-33a in colon carcinoma

Ahmed Fawzy Ibrahim, Ulrike Weirauch, Maren Thomas, et al.

Cancer Res  Published OnlineFirst June 20, 2011.