Improving vascular maturation using noncoding RNAs increases antitumor effect of chemotherapy

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Introduction

Although angiogenesis plays a critical role in cancer growth and metastasis, current antiangiogenic therapies have shown only modest efficacy (1). This is thought to occur, in part, because of the development of hypoxia with prolonged treatment (2, 3). The induction of hypoxia is not surprising given the pronounced reduction in the number of blood vessels with prolonged treatment (4). Targeting hypoxia pathways such as HIF1α (e.g., topotecan, temsirolimus) has resulted in improved efficacy of antiangiogenic drugs (5, 6). However, novel approaches to target the tumor vasculature are needed to maintain antitumor efficacy without increasing hypoxia and other deleterious effects.

MicroRNAs (miRs) have been shown to play a vital role in tumor development and angiogenic processes by modulating the expression of critical angiogenesis factors (7). Deregulation of miRs has been identified in many cancer types and is closely related to tumor progression and metastasis. However, the role of tumor endothelium–derived miRs in regulating tumor vascularization remains to be elucidated. To address this knowledge gap, we carried out systematic profiling of miRs in ovarian tumor ECs compared with normal ECs. We identified a set of miRs that regulate the integrity of tumor vasculature. In validation studies, we focused on miRs upregulated in tumor ECs and found that inhibiting these miRs reduced vas-
cular permeability and increased maturation without induction of hypoxia. Delivery of the miR inhibitors was achieved using nanoparticles (NPs) decorated with thiophosphate backbone–modified aptamers (thio-aptamers [TAs]) identified through cell-based SELEX (cell-SELEX) approaches with TAs.

Results

**Altered miRs in tumor vasculature.** To identify miRs with altered expression in tumor vasculature, we isolated ECs from human tissue samples of high-grade serous ovarian cancer (n = 3) and normal ovarian tissues (n = 3) and performed nanostring analysis (7). Array results showed that 5 miRs were markedly upregulated and 7 miRs were downregulated in ovarian tumor ECs compared with normal ECs (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/jci.insight.87754DS1). We selected the 3 most highly upregulated miRs (miR106b-5p, miR30c-5p, and miR141-3p) and validated the expression of these overexpressed miRs in tumor ECs using 3 independent patient samples. Consistent with our discovery data set, expression of these miRs was substantially higher in tumor ECs than in normal ECs (Figure 1A). Prior to carrying out functional experiments, we asked whether tumor-derived factors could induce expression of these miRs. Indeed, exposure to conditioned media (CM) from ovarian cancer cells resulted in increased levels of miR106b-5p, miR30c-5p, and miR141-3p in RF24 and G1S1 ECs. Similar results were observed when normal ECs were cocultured with SKOV3ip1 cancer cells (Figure 1B). Since VEGF is the dominant factor in blood vessel leakiness (1), we hypothesized that VEGF-induced leakiness could be promoted by upregulation of these miRs. We checked the expression of miR106b-5p and miR30c-5p in the presence of 10 ng VEGF. Expression of both miRs was substantially increased (2-fold) upon treatment of RF24 ECs with VEGF at 48 hours (Supplemental Figure 1A). Silencing of either VEGFR1 or VEGFR2 receptors using specific siRNAs (Supplemental Figure 1B) in VEGF-treated ECs resulted in decreased expression of both miR106b-5p and miR30c-5p compared with control cells (not exposed either to VEGF or CM), suggesting the involvement of VEGF-mediated upregulation of these miRs (Supplemental Figure 1C). Similar results were observed when normal ECs were exposed to CM after silencing of VEGFR1 and VEGFR2 receptors (Supplemental Figure 1C).

To determine the potential effects of deregulated miRs on angiogenesis, we used integrated pathway analysis and found that tight junction signaling was the pathway most affected by deregulated miRs (Supplemental Figure 2 and Supplemental Table 2). To confirm this finding, we analyzed the expression of several tight junction genes when these miRs were upregulated. Consistent with the pathway analysis results, exposure to tumor CM resulted in decreased expression of several genes that regulate tight junctions, including magi2, mpdz (MUPP1), myh11 (myosin), claudin 4, claudin 5, cdh5 (VE-cadherin), occludin, zo1, and zo2 in ECs (Figure 1C).

Because tight junction proteins play a crucial role in regulating the paracellular permeability of both epithelial cells and ECs, we next tested the effects of inhibiting miR106b-5p, miR30c-5p, and miR141-3p on these tight junction genes. In G1S1 cells, treatment with miR inhibitors resulted in 80%–85% lower miR levels at 36 hours (Supplemental Figure 3) and increased the expression of tight junction genes (Figure 1D). Because downregulation of tight junction proteins leads to increased permeability and angiogenesis, we analyzed the effect of miR silencing on endothelial permeability. Compared with untreated ECs, cells with silenced miR106b-5p, miR30c-5p, or miR141-3p showed a 57%, 47%, or 35% decrease in endothelial permeability, respectively (Figure 1E). We next asked whether these miR inhibitors could have direct effects on endothelial tube formation. None of the 3 miR inhibitors had a significant effect on endothelial tube formation in 3-dimensional assays (Figure 1F), indicating that their role is likely to be more important for the structural integrity of vessels rather than endothelial recruitment.

Based on these in vitro findings, we sought to determine the biological effects of these miRs on tumor growth and angiogenesis in vivo. Because miR106b-5p and miR30c-5p inhibition led to the greatest reduction in endothelial permeability, we tested these 2 miR inhibitors in vivo. For specific delivery of miR inhibitors to tumor vasculature, we first developed a method for targeted delivery using TAs.

**Targeted delivery to tumor vasculature.** To develop a delivery system to target tumor vasculature, we used a large TA library. Freshly isolated human ovarian tumor ECs from 10 patients with ovarian cancer were used as target cells. We started the selection by screening a 73-mer single-stranded DNA TA library, containing a 30-mer random region flanked with 21- and 23-mer primers. Before incubating the TA library with the target cells, we first depleted it of all TAs that bind to normal ovarian ECs in a counter-selection step. The correct size of the TA library was confirmed using polyacrylamide gel electrophoresis after eluting the TAs from target cells and sepa-
Figure 1. Upregulation of miRs in tumor endothelial cells decreases the expression of tight junction proteins. (A) Expression of miRs upregulated in patient tumor endothelial cells (ECs) compared with normal ovarian ECs. (B–E) ECs were incubated with conditioned medium (CM) from ovarian cancer SKOV3ip1 cells to mimic the tumor microenvironment. (B) Expression of miRs upregulated in tumor-associated ECs compared with untreated ECs. (C) Increased expression of miRs resulted in reduced levels of tight junction proteins. (D) miR silencing increased the expression of tight junction proteins. Fold changes in A–D represent the mean of triplicate experiments compared with normal or control or untreated cells. (E) miR silencing decreases the permeability in tumor-associated ECs. miR-inh, miRNA inhibitor. (F) Effect of miR silencing on angiogenesis (tube formation). All experiments were performed 3 times with triplicate samples (n = 3). Values are means ± SEM. *P < 0.05 (1-way ANOVA followed by a Tukey’s multiple comparison post-hoc test). NS, not significant.
**Figure 2. Selection and identification of thioaptamers that bind to human ovarian cancer vascular endothelial cells (ECs).** (A) Cell-SELEX procedure with thioaptamers (TAs). A library of ssDNA TAs was selected, and these TAs were incubated with purified human ovarian cancer ECs, washed, eluted, and amplified for 10 rounds. (B) Representative tumor sections showing binding of Endo28 to human ovarian tumor vasculature. Sections from normal ovary and tumor were incubated with Cy3-labeled Endo28 and anti-CD31 antibody to stain blood vessels. Nuclei are shown in blue, blood vessels in green, and Endo28 in red. Arrows denote colocalization of Endo28 (red) and CD31 (green). (C) Representative tumor sections showing expression of annexin A2 on human ovarian tumor vasculature. Sections from normal ovary and tumor were incubated with anti-annexin A2 and anti-CD31 antibodies. Nuclei are stained blue, annexin A2 green, and blood vessels red. Arrows denote colocalization of CD31 (red) and annexin A2 (green). (D) Annexin A2-positive human microvascular ECs (HMVECs) were detected by flow cytometry. (E) Representative immunofluorescence staining showing that silencing of annexin A2 resulted in reduced binding of Endo28 and Endo31 to HMVECs. Scale bars: 100 μm (C and E).
**Figure 3. Delivery of CH/Endo28-NPs to tumor vasculature.** Tumors and organs were harvested 6 hours after a single intravenous injection of CH-Endo28-NPs (150 pmol/mouse) into HeyA8 tumor-bearing mice and stained with anti-CD31 antibody to detect blood vessels. **(A)** Representative tumor sections showing binding of CH/Endo28-NPs to tumor vasculature. Nuclei are shown in blue, blood vessels in green, and CH-Endo28-NPs in red (Cy3). CH/Endo28-NPs, chitosan/Endo28-nanoparticles. **(B)** Representative tumor sections showing colocalization of CH-Endo28-NPs (red) with blood vessels (green). Arrows in **A** and **B** denote colocalization of Endo28 (red) and CD31 (green). **(C)** Representative tumor sections showing delivery of Alexa 488-conjugated siRNA using CH/Endo28-NPs into tumor cells (blue indicates nuclei, green indicates Alexa 488-conjugated siRNA, and red indicates CH-Endo28-NPs). CH/R4-NPs, chitosan/R4-nanoparticles. Arrows denote colocalization of CH/Endo28 (red) and Alexa 488 siRNA (green). **(D)** Representative tumor sections showing that annexin A2 silencing reduced delivery of CH/Endo28-NPs to the tumor vasculature. Mice were treated with either CH/control siRNA or CH/mouse annexin A2 (CH/m Annexin A2) siRNA for 48 hours before a single intravenous injection of CH/R4-NPs or CH/Endo28-NPs. Tumors were harvested 24 hours after NP injection and stained with anti-CD31 antibody to visualize blood vessels. Scale bars: 50 μm. **(E)** Distribution of CH/Endo28-NPs in tumor cells and organs, examined using IVIS imaging (see Supplemental Methods). Optical imaging of organs and tumors from HeyA8 tumor-bearing mice treated with CH-NPs or CH/R4-NPs or CH/Endo28-NPs shows fluorescence intensity overlaid on white light images of different mouse organs and tumor.

**Biological effects of miR106b-5p and miR30c-5p silencing.** To evaluate the in vivo biological effects of miR106b-5p and miR30c-5p silencing, we used a well-characterized mouse model. Because HeyA8 cancer cells have low expression of these 2 miRs (Supplemental Figure 10), we used this model to assess the influence of miRs on tumor vasculature. Treatment with CH/Endo28-miR106b-5p inhibitor resulted in 50% reduction in tumor growth, and treatment with CH/Endo28-miR30c-5p inhibitor resulted in ~25% reduction in tumor growth (Figure 4, A–C). Remarkably, the addition of paclitaxel to either the miR106b-5p inhibitor or the miR30c-5p inhibitor resulted in a substantially greater reduction in tumor growth (78%–90%; P < 0.005) compared with controls (Figure 4, A–C). Mean mouse body weights were similar regardless of the treatment and animal model (data not shown), suggesting that these combinations lack any overt toxicity.

To determine whether these miRs could have relevance for other tumor types, we examined the expression of annexin A2 in uterine (HEC1A) and breast (MDA-MB-231) tumor vasculature. Both tumors showed expression of annexin A2 (Supplemental Figure 11A). To evaluate the therapeutic efficacy of CH/Endo28-NPs, we next used an orthotopic breast cancer model (MDA-MB-231). Treatment with CH/Endo28-miR106b-5p inhibitor resulted in 51% (P < 0.001) reduction in tumor growth and a combination of paclitaxel and miR106b-5p silencing resulted in greater than 90% reduction in tumor growth (P < 0.0001) compared with the control miR inhibitor group (Supplemental Figure 11, B–D).

Next, we examined the vascular characteristics of the treated tumors. Pericyte coverage was markedly increased in tumors treated with miR inhibitors compared with those treated with control miR inhibitor (Figure 4, D and G). Moreover, vascular leakiness was also decreased after miR silencing, suggesting that silencing these miRs stabilized blood vessels by reducing the leakiness (Figure 4, E and G). The hypoxia level was decreased after miR silencing (Figure 4F), which is consistent with improved vascular function. Similar effects were observed in combined treatment with miR inhibitors and paclitaxel (Figure 4, D–G). Neither of these miR inhibitors resulted in significant reduction of blood vessel number (Figure 4H), which is consistent with their role in promoting vascular structure rather than EC viability. Expression of tight junction proteins such as zonula occludens 1/2 and claudin 5 was markedly increased in tumor endothelium after miR silencing (Figure 5, A–C). No significant reduction was observed in the number of blood vessels after miR silencing (Supplemental Figure 12A). Given the profound antitumor effects with paclitaxel despite the lack of reduction in microvessel density, we asked whether the improved vascular features in the tumor microenvironment could lead to increased drug delivery. Indeed, inhibition of either miR increased the delivery of paclitaxel into tumors. Tumor-bearing animals treated with the CH/Endo28-miR106b-5p inhibitor showed 4-times higher paclitaxel content; those treated with the CH/Endo28-miR30c-5p inhibitor showed 2.5-times higher paclitaxel content than those treated with the CH/Endo28-control miR inhibitor (Supplemental Figure 12B). Collectively, we propose a model showing that miR silencing leads to restoration of tight junction function and in turn decreases angiogenesis (Figure 6).

**Biosafety and efficacy of CH/Endo28-siRNA-NPs.** We next evaluated the effect of CH/Endo28-NPs on C57/BL6 mice after intravenous administration of a therapeutic dose of CH/Endo28-control siRNA (5 μg of siRNA). The blood chemistry results confirmed that the NPs did not cause any significant changes in any of the parameters tested (Supplemental Figure 13, A–F). Similar to the blood chemistry results, the values of the cytokines tested were not significantly higher in the mice injected with NPs than in control mice.
Discussion

In the current study, we developed a potentially new method of targeting tumor angiogenesis through 2 levels of targeting: (a) binding of TA-Endo28-decorated CH-NPs (CH/Endo28-NPs) to the tumor EC–specific membrane protein annexin A2, and (b) delivering miR inhibitors to the tumor vasculature and restoring tight junction function. We showed that this technique markedly improved the delivery of chemoth-erapeutic agents (i.e., paclitaxel) and reduced tumor growth in orthotopic ovarian cancer mouse models.

Despite promising results in preclinical models, approved antiangiogenic therapies using monoclonal antibodies have shown modest and transient efficacy in the clinic (13). Novel approaches to inhibit tumor angiogenesis in a specific and long-lasting manner are therefore critically needed. Targeting receptors specific to tumor ECs in combination with RNAi-mediated gene silencing provides a highly selective and effective new mode of antiangiogenic therapy. Several studies have shown that a combination of bevacizumab antibody and chemotheraphy is required to improve survival in patients with non–small cell lung cancer, metastatic colorectal cancer, and HER2-negative breast cancer (14–17). Huang et al. showed that treatment with low doses of anti-VEGFR2 antibody helps restore the function of tumor vasculature, reducing leakiness and interstitial pressure, and thereby allowing for improved drug delivery in animal models (18). However, the periodic use of antiangiogenic antibodies leads to treatment resistance and ultimately tumor relapse. The technique developed in the current study helps overcome these challenges by selectively delivering antitumor agents directly to the tumor vasculature.

The differences between normal and tumor vasculature have been conventionally studied using cell culture and animal models. However, the vascular characteristics in these models may not resemble those in human tumors (19). In the current study, ECs were extracted directly from patient tumor samples to obtain a more accurate molecular profile of the tumor vasculature in human disease. Many of the top dysregulated miRs are directed toward tight junction modulation. Unsurprisingly, vascular remodeling is a process associated with tumor growth and metastasis. Increases in vessel permeability can be characterized as loss of endothelial cell-cell adhesion, as reflected by downregulation of important tight junction proteins such as occludin, claudins, z1/2, and VE-cadherin. ECs are the first barrier that tumor cells must cross to enter the circulation for metastasis. Immature tumor vasculature and resultant high interstitial pressure contribute to an unfriendly microenvironment for chemotherapeutical drug delivery. Selective interference with these dysregulated miRs can globally restore endothelial tight junctions and enhance the effect of chemotherapy. A recent study has demonstrated that silencing of hypoxia-induced miR101 can reduce the number of capillaries in the ischemic hind limb and interfere with regional blood flow (20).

We also screened aptamers with high binding affinities for these patient-derived tumor ECs using the cell-SELEX method (10). The thio phosphatase modifications in the TAs enhance both nuclease resistance and higher affinity for target proteins (21). Aptamers can possess dual functions, acting both as agents that target cell surface receptors or ligands, and as vehicles to deliver siRNA/miR cargo to specific cells. For example, a polyethylene glycol–conjugated aptamer that binds to VEGF has been approved by the US FDA for treatment of age-related macular degeneration (22), and more aptamers are currently being evaluated in clinical trials (23, 24). As a targeting moieties, a prostate-specific membrane antigen–targeting (PSMA-targeting) aptamer
Figure 5. miR silencing increases the expression of tight junction proteins and chemotherapeutic drug delivery. Seven days following tumor cell injection, mice were randomly divided into 6 groups (9-10 mice per group) to receive 1 of the following 6 therapies: (i) CH/Endo28-control miR inhibitor, (ii) CH/Endo28-control miR inhibitor + paclitaxel, (iii) CH/Endo28-miR106b-5p inhibitor, (iv) CH/Endo28-miR106b-5p inhibitor + paclitaxel, (v) CH/Endo28-miR30c-5p inhibitor, or (vi) CH/Endo28-miR30c-5p inhibitor + paclitaxel. Mice were sacrificed when any animals in a control or treatment group became moribund (after 3 to 4 weeks of therapy). Tumor sections were stained with anti-zo1, -zo2 and –claudin 5 along with -CD31 antibodies. (A) Expression of zo1 (red indicates blood vessels, green indicates zo1). (B) Expression of zo2 (red indicates blood vessels, green indicates zo2). (C) Expression of claudin 5 (red indicates blood vessels, green indicates claudin 5). Staining was done on 5 sections per group (A-C). Scale bars: 100 μm. miR-inh, miRNA inhibitor.

has been conjugated to doxorubicin micelles, which led to an increased uptake by PSMA-positive CWR22R-nul prostate cancer cells and resulted in higher cytotoxicity than that achieved using nontargeting micelles (25). More recently, a similar strategy was used in constructing 225Ac-loaded liposomes to target and selectively kill PSMA-expressing cells such as LNCaP, Mat-Lu, and HUVEC using radiation (26). However, aptamers utilized in these studies were selected on the basis of binding to isolated surface markers or recombinant proteins, which might poorly reflect the native protein conformations owing to denaturing conditions during purification (27, 28). We used patient-derived ovarian tumor ECs to screen aptamers, and the annexin A2–targeted TA used in the current study offer the advantage of high binding affinity for surface markers in their native conformations. Although CH has not yet been approved by the FDA for drug delivery purposes, it has already been used in hemostasis products and has cleared many of the safety concerns about application in clinical settings (29–31). Therefore, our CH/TA-NPs constitute a uniquely safe and efficient delivery system for targeting tumor ECs and interfering with key angiogenic genes, providing a potentially new approach to selectively suppress tumor growth and angiogenesis. Furthermore, these methods of aptamer selection and NP assembly allow for rapid and highly specific development of new targeted therapies for cancer, paving the way for truly personalized cancer intervention.

Methods

Cell lines and culture. Maintenance of the human epithelial ovarian cancer cells and ECs is described in the supplemental methods (32).

TA selection and annexin A2 identification. In brief (see supplemental methods), we used an ssDNA-TA library and a cell-SELEX method to identify a few dozen putative TAs that target human ovarian cancer ECs. These TAs (50 nM) were tested for their relative binding affinity for ECs on ovarian cancer tissue sections. Annexin A2 protein was identified as the target of these selected TAs by cross-linking and mass spectrometry (12).

In vitro transfection. We silenced miRs in ECs using miR inhibitors and RNAfect reagent (Qiagen) as described in the supplemental methods. VEGFR1 and VEGFR2 were silenced in RF24 cells and annexin A2 in HMVECs was silenced with either control or target siRNAs using Lipofectamine 2000 reagent (Invitrogen) as described previously (33). The expression of annexin A2, miRs, and tight junction proteins was determined using quantitative real-time PCR and Western blot analyses as described previously (34, 35). After transfecting RF24 cells with miR inhibitors, we performed cell permeability and tube formation assays as described in the supplemental methods.

Synthesis and characterization of CH/Endo28-siRNA-NPs. The activation and conjugation of TAs (negative control R4 and targeted Endo28) on CH and the characterization of NPs are described in the supplemental methods. In brief, the grafting efficiency of Endo28 on CH-NPs and the size and zeta potential of the CH/Endo28-siRNA-NPs were measured. The grafting of Endo28 on CH was confirmed by proton NMR (1H NMR) and the morphology of the CH/Endo28-NPs was determined using atomic force microscopy. The intact binding of TA-Endo28 and siRNA with CH and the stability of NPs were confirmed by gel retardation and a stability assay using agarose gel electrophoresis. The internalization and endosomal escape of CH/Endo28-NPs in MOECs and RF24 ECs were analyzed using confocal microscopy.

TA binding to ovarian tumor vasculature and therapeutic efficacy of miRs in ovarian tumors. The binding of CH/Endo28-NPs to the tumor vasculature and the uptake of NPs by the tumor, organs, and macrophages are described in the supplemental methods. Cell permeability, microvascular density, pericyte coverage, and delivery of paclitaxel are also described in the supplemental methods.

Statistics. All statistical tests were performed using Excel and GraphPad Prism 6 software. Differences between groups were evaluated using 2-tailed Student’s t test or 1-way ANOVA, adjusting for multiple comparisons. Results are presented as the mean ± SEM. For animal experiments, 10 mice were assigned
per treatment group. This sample size gave 80% power to detect a 50% reduction in tumor weight with 95% confidence. Tumor weights and the number of tumor nodules for each group were compared using 1-way ANOVA followed by a Tukey’s multiple comparison post-hoc test. For all statistical analyses, $P < 0.05$ was considered statistically significant.

Study approval. All approvals for experiments depicted here were obtained from The University of Texas MD Anderson Cancer Center Institutional Review Board. All animal experiments were approved and supervised by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Author contributions
LSM and DJ conceived and designed the experiments, synthesized and characterized the nanoparticles, conducted in vitro and in vivo experiments, and prepared the manuscript. HW conceived and designed the experiments; conducted in vitro cell and tissue binding, selection, and aptamer pull-down experiments; and prepared the manuscript. SYW and XZ conducted in vivo experiments. WH conducted isolation of endothelial cells. AS, XL, and TT conducted cell-SELEX experiments. NB and LL conducted mass spectrometry. SNM and LJNC conducted nanostring analysis. DEV, LGR, and VT provided thioaptamers. XY,
provided 2′-OMe-PS2–modified anti-miR inhibitors. SP, CRA, EB, RB, ASN, RR, KMG, MHM, MH, HJC, RAP, and PLD assisted with in vivo experiments. HJC assisted in drawing the blood. KPR, GLB, DGG, and AKS conceived the project and provided guidance.

Acknowledgments

This work was supported by the Welch Foundation (AU-1296), National Institutes of Health (grants U54CA151668, CA109298, P50CA083639, P50CA098258, UH3TR000943, HHSN268201000037C), National Center for Advanced Translational Sciences Clinical and Translational Science Award (UL1 TR000371), the Ovarian Cancer Research Fund, Inc. (Program Project Development Grant), the Blanton-Davis Ovarian Cancer Research Program, the RGK Foundation, the Frank McGraw Memorial Chair in Cancer Research, the V-Foundation, and CPRIT grant RP110595.

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