Published in final edited form as:

Oncogene. 2019 August ; 38(33): 6095–6108. doi:10.1038/s41388-019-0862-y.

Identifying and targeting angiogenesis-related microRNAs in ovarian cancer

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Abstract

Current anti-angiogenic therapy for cancer is based mainly on inhibition of the vascular endothelial growth factor pathway. However, due to the transient and only modest benefit from such therapy, additional approaches are needed. Deregulation of microRNAs (miRNAs) has been demonstrated to be involved in tumor angiogenesis and offers opportunities for a new therapeutic approach. However, effective miRNA-delivery systems are needed for such approaches to be successful. In this study, miRNA profiling of patient data sets, along with in vitro and in vivo experiments, revealed that miR-204-5p could promote angiogenesis in ovarian tumors through THBS1. By binding with scavenger receptor class B type 1 (SCARB1), reconstituted high-density lipoprotein–nanoparticles (rHDL–NPs) were effective in delivering miR-204-5p inhibitor

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Supplementary information The online version of this article (<http://doi.org/10.1038/s41388-019-0862-y>) contains supplementary material, which is available to authorized users.

Conflict of interest The authors declare that they have no conflict of interest.

(miR-204-5p-inh) to tumor sites to suppress tumor growth. These results offer a new understanding of miR-204-5p in regulating tumor angiogenesis.

Introduction

Angiogenesis is known to be essential for tumor growth and progression of cancer, playing a critical role in tumor growth and metastasis [1]. Numerous therapies targeting angiogenesis have been developed and have shown robust anti-tumor activity in preclinical animal models [2]. The vascular endothelial growth factor (VEGF) signaling pathway is an important regulator of angiogenesis. VEGF-targeted drugs have been developed against various types of solid tumors [3], and have been approved by the U.S. Food and Drug Administration (FDA) for clinical applications (either as a single treatment or in combination with chemotherapy or radiotherapy) [4]. However, not all types of cancer respond well; anti-angiogenic therapies have shown only transient effects and modest benefit in most patients, mainly because of inherent or acquired resistance [5, 6]. Therefore, new strategies are needed to improve the efficacy of anti-angiogenic therapies.

miRNAs are a family of small noncoding RNAs (ncRNAs) that regulate a variety of biological processes [7]. Deregulation of miRNAs has been demonstrated in many types of human malignancies, and is closely related to tumorigenesis and disease progression [8]. One mechanism by which miRNAs influence the progression of cancer is by regulating angiogenesis, as critical angiogenic factors can be modulated by deregulated miRNAs [9–11]. Hence, targeting deregulated miRNAs could be a powerful approach to achieve anti-angiogenic effects. Unlike small interfering RNAs (siRNAs), a single miRNA can bind to hundreds of mRNA sequences [12]. However, there are barriers and challenges to the use of siRNAs and miRNAs in clinical applications, such as poor stability and off-target effects [5]. Consequently, effective delivery systems to transport miRNAs selectively to tumor sites must be developed.

High-density lipoproteins (HDLs) are nature's multifunctional nanoparticles (NPs) [13]. Besides transporting essential cellular lipid constituents, HDLs also transport endogenous proteins, hormones, vitamins [14], and endogenous miRNAs [15] to various organs. The properties of HDLs, including their ultra-small size, long circulation half-life, low toxicity, and intrinsic targeting to recipient cells, make them an attractive candidate vehicle for drug delivery [16]. HDL binds to SCARB1 with high affinity. SCARB1 is expressed primarily in the liver and frequently overexpressed in tumor cells; thus, it can be used for targeted delivery of drugs to tumor sites [17, 18]. Reconstituted HDL (rHDL), the synthetic form of HDL, exhibits similar properties to those of endogenous HDL, but has a tailorable structure and can be scaled up for pharmaceutical applications [19]. We have previously demonstrated that rHDL–NPs facilitated the systemic delivery of siRNAs, with minimal toxicity [20]. In this study, we examined the anti-angiogenic effects of delivering miR-204-5p-inh to tumor sites using rHDL–NPs.

Results

Effect of miR-204-5p on ovarian tumor angiogenesis

To identify miRNAs that were correlated with tumor angiogenesis, we utilized a microRNA data set generated from high-grade serous ovarian cancers using Nanostring technology [21]. Here, we focused on miRNAs with high endogenous levels that were positively correlated with high tumor microvessel density (MVD). We found that the expression of six miRNAs (hsa-miR-125b-5p, hsa-miR-199a-5p, hsa-miR-218-5p, hsa-miR-199b-5p, hsa-miR-204-5p, and hsa-miR-497-5p) was increased more than four fold in tumors with high-angiogenesis compared with those with low-angiogenesis (Fig. 1a; Supplementary Fig. 1a).

We next assessed the correlation between the expression of these miRNAs and patient survival in The Cancer Genome Atlas (TCGA) ovarian cancer data set ($n = 559$). High expression of miR-218-5p was found to be associated with poor disease-free survival (DFS), but not overall survival (OS). Patients with expression of miR-204-5p in the highest quartile had significantly poorer prognoses (OS and DFS) than did patients with expression in the lowest quartile (OS: HR = 1.25, CI [95%] = [1.01, 1.55], p -value [log-rank test] = 0.01; DFS: HR = 1.32, CI [95%] = [1.07, 1.63], p -value [log-rank test] = 0.01) (Fig. 1b; Supplementary Table 1). No other identified miRNAs showed a significant association with survival. Therefore, we considered miR-204-5p for further study.

Next, to determine the biological functions of miR-204-5p in tumor angiogenesis, we silenced miR-204-5p in RF24 endothelial cells using miR-204-5p-inh and assessed the ability of these cells to form tubes. First, we checked the expression of miR-204-5p in ovarian and breast cancer cells. HeyA8-MDR and MDA-MB-231 showed relatively high endogenous expression of miR-204-5p among different cell lines (Fig. 1c). Silencing of miR-204-5p using a specific miR inhibitor significantly reduced its expression ($p < 0.001$) (Supplementary Fig. 1b). Next, we assessed the effect of miR-204-5p silencing on angiogenesis. As shown in Fig. 1d, no significant difference in RF24 endothelial cell tube formation was observed between the control-miR-inh and miR-204-5p-inh-treated groups. We hypothesized that the effect of miR-204-5p on tumor angiogenesis is indirect, and is due to a paracrine effect from tumor cells. To test this hypothesis, we silenced miR-204-5p in HeyA8-MDR ovarian cancer cells. After silencing miR-204-5p for 36 h, conditioned medium from HeyA8-MDR cells was collected and applied to RF24 endothelial cells. The RF24 cells were cultured in the conditioned medium for 24 h, and then subjected to a tube-formation assay. Compared with control-miR-inh medium treated RF24 cells, miR-204-5p-inh medium-treated cells showed 70% reduction ($p = 0.0072$) in tube formation (Fig. 1e) and 50% reduction ($p = 0.0017$) in migration (Supplementary Fig. 1c). We also checked whether miR-204-5p overexpression would promote tube formation of endothelial cells. We overexpressed miR-204-5p in SKOV3ip1 cells using a miR-204-5p-mimic and treated RF24 cells with SKOV3ip1-conditioned medium and assessed tube formation. As shown in Supplementary Fig. 1d, RF24 cells treated with conditioned medium from miR-204-5p overexpressing cells showed a significant increase in tube formation compared with cells treated with conditioned medium from cont-miR-mimic treated cells.

A similar effect of miR-204-5p silencing on angiogenesis was observed in RF24 cells ($p = 0.0023$) treated with conditioned medium from miR-204-5p silenced MDA-MB-231 breast cancer cells (Fig. 1f). Based on our in vitro findings, we next asked whether miR-204-5p gene silencing would affect tumor growth. To achieve systemic delivery of miR-204-5p-inh into tumors, we used rHDL nano vector as a delivery tool (Fig. 2a). The nucleic acids reside in the core surrounded by a monolayer shell of phospholipid and free cholesterol. The protein component, Apo-A1 (inserted into the phospholipid shell) provides structure to the lipoprotein and targeting through its receptor, SCARB1. Figure 2b shows a representative example of the hydrodynamic diameter of the rHDL-miR-inh, measured by dynamic light scattering. The hydrodynamic diameter appears to be homogeneous, although larger than native HDL. However, synthetic HDL NPs often have larger diameters than native HDL [13, 22]. The native HDL has a “selective uptake” mechanism whereby HDL docks to the SCARB1 receptor and delivers its content into cells without internalization, presumably via a hydrophobic channel formed in the cell membrane [23]. Herein, we studied the interaction of rHDL and SCARB1 on MDA-MB-231 breast cancer cells, to determine whether synthetic HDL NPs use a similar mechanism of payload delivery. Two approaches were used to confirm the endosomal escape of ncRNA. As shown in Fig. 2c, ncRNA (labeled with green Alexa 488) delivered via rHDL in the cytoplasm was not associated with lysosomes (stained using LysoTracker Red dye), indicating that ncRNA can be delivered into the cells without passing through the endosome-lysosome pathway which tends to degrade therapeutic RNA and render it pharmacologically ineffective. On the other hand, we also confirmed the presence of rHDL-NPs outside the cell with respect to ncRNA delivered inside the cell cytoplasm. As shown in Fig. 2d, Alexa 647-labeled rHDL-NPs tended to accumulate near the cell membrane, dock to SCARB1 and deliver their contents into cells. These results suggest that nucleic acids can be delivered to the cytosol via rHDL while escaping the cell’s endosomal pathway.

Biological effects of miR-204-5p silencing on the tumor microenvironment

To evaluate the biological effects of miR-204-5p silencing in vivo, we utilized a well-characterized orthotopic mouse model of ovarian carcinoma. Seven days after injection of HeyA8-MDR ovarian cancer cells into the peritoneal cavity, mice were randomized to four groups to receive: (i) rHDL-cont-miR-inh, (ii) rHDL-miR-204-5p-inh, (iii) rHDL-cont-miR-inh plus paclitaxel, and (iv) rHDL-miR-204-5p-inh plus paclitaxel ($n = 10$ per group). Mice were killed when animals in any group became moribund due to significant tumor burden (4–5 weeks after cell injection). Compared with rHDL-cont-miR-inh, treatment with rHDL-miR-204-5p-inh alone ($p = 0.0001$) as well as combination with paclitaxel ($p = 0.0038$) resulted in a 76.7% reduction in tumor volume (Fig. 3a, b), and a 49.3% reduction in tumor nodules (Fig. 3c). HeyA8-MDR is a multi-drug-resistant ovarian cancer cell line; thus, the mouse model generated using this cell type was not sensitive to chemotherapy. To determine whether rHDL-miR-204-5p-inh NPs can combine with chemotherapy drugs to improve treatment outcomes, we administered rHDL-miR-204-5p-inh NPs, in combination with paclitaxel to HeyA8-MDR-tumor bearing mice. However, no significant difference was observed in combination treatment of miR-204-5p-inh and paclitaxel, compared with the miR-204-5p only treatment (Fig. 3a, b). Significant reduction of miR-204-5p expression (46.5%, $p = 0.0002$) was observed in mice treated with rHDL-miR-204-5p-inh compared

with the control-miR-inh treated group (Fig. 3d). To identify potential mechanisms underlying the efficacy of miR-204-5p silencing on ovarian tumors, we examined the effect of miR-204-5p silencing on biological endpoints, including cell proliferation (ki67) and MVD. No significant difference was observed in proliferation between the rHDL-control-inh and rHDL-miR-204-5p-inh treated groups (Supplementary Fig. 2a). However, a 65% decrease in MVD ($p = 0.0002$) was observed in the rHDL-miR-204-5p-inh treated group compared with the control-inh treated group (Supplementary Fig. 2b). These results are consistent with those of the in vitro functional assay.

To determine the effect of rHDL-miR-204-5p-inh NPs in other types of cancer, we checked their therapeutic effect in a well-characterized mouse model of breast cancer. MDA-MB-231 cells, which had relatively higher baseline miR-204-5p expression in a breast cancer cell panel (Fig. 1c), were injected into mammary pad and treated with either rHDL-control-miR-inh or rHDL-miR-204-5p-inh twice per week ($n = 10$ per group). Treatment with rHDL-miR-204-5p-inh significantly reduced tumor volume (Fig. 3e), as well as tumor nodules ($p = 0.0011$; Fig. 3f). In addition, miR-204-5p-inh treated tumors showed a 29% reduction in miR-204-5p expression compared with control-miR-inh treated tumors ($p = 0.0002$; Fig. 3g).

It has been well demonstrated that SCARB1 is highly expressed by tumor cells [20]. As shown in Supplementary Fig. 2c, both ovarian and breast cancer cells have increased expression of SCARB1. Since the liver had the highest expression of SCARB1 protein among eight tested organs in nontreated mice (Supplementary Fig. 2d), it is the most likely site of miR-204-5p-inh delivery besides tumor sites. However, the baseline expression of miR-204-5p was lowest in the liver compared with other organs (Supplementary Fig. 2e), which could minimize potential treatment-associated side effects at nontumor sites.

Thrombospondin1 (THBS1) is involved in the miR-204-mediated regulation of tumor angiogenesis

To identify potential molecular mechanisms by which miR-204-5p exerts its pro-angiogenic effects, we performed a gene expression microarray of HeyA8-MDR cells following treatment with miR-204-5p-inh. To identify potential downstream targets of miR-204-5p that regulate angiogenesis, we focused on the protein-coding genes that were upregulated (> 1.5 -fold increase in expression) after silencing of miR-204-5p ($p < 0.05$) (Fig. 4a). Forty-one of these genes were predicted to be the direct targets of miR-204-5p by four different databases from miRWalk (Supplementary Table 2). Three of them (thioredoxin interacting protein (*TXNIP*), pentraxin 3 (*PTX3*), and *THBS1*) were demonstrated to have anti-angiogenic effect in previously published studies [24–26]. We also confirmed that *THBS1* gene is the direct target of miR-204-5p by luciferase reporter assay. Co-transfection of hsa-miR-204-5p-mimic and *THBS1* 3'UTR vector significantly repressed luciferase activity compared with random 3'UTR control in HEK cells ($p = 0.0002$) (Supplementary Fig. 3a) and the other two genes did not show significant changes in luciferase activity (data not shown).

To validate the microarray findings, we determined *TXNIP*, *PTX3*, and *THBS1* expression levels by qRT-PCR after silencing and overexpressing miR-204-5p. Silencing of miR-204-5p using its specific inhibitor significantly reduced the levels of this miRNA in HeyA8-MDR

cells ($p = 0.0006$; Supplementary Fig. 3b) and increased the expression of all three genes ($p < 0.05$; Fig. 4b). To confirm that these genes are the downstream targets of miR-204-5p, we assessed the expression of these genes after miR-204-5p overexpression by introducing a miR-204-5p mimic into SKOV3ip1 cells, which showed the lowest expression of miR-204-5p in an ovarian cancer cell panel (Fig. 1c).

Overexpression of miR-204-5p resulted in a significant increase of miR-204-5p expression ($p = 0.001$) in SKOV3ip1 cells (Supplementary Fig. 3b). As shown in Fig. 4b, overexpression of miR-204-5p significantly reduced the expression of *TXNIP*, *PTX3*, and *THBS1*. To further validate these genes in a biological system, we determined their expression in tissue samples collected from the HeyA8-MDR tumor model. Compared with rHDL-cont-miR-inh, treatment with rHDL-miR-204-5p-inh increased the expression of *THBS1* (1.5-fold; $p < 0.01$) and did not change the expression of the other two genes (Fig. 4c). Therefore, we focused on *THBS1* for further study. According to the TCGA ovarian cancer data set, there was an inverse correlation between *THBS1* and miR-204-5p expression in the samples with the highest miR-204-5p expression (sextile) ($p < 0.05$) (Fig. 4d; Supplementary Table 3). We thus reasoned that *THBS1* is involved in the miR-204-5p-mediated regulation of tumor angiogenesis in ovarian cancer.

To test the hypothesis that *THBS1* is one of the downstream targets of miR-204-5p in the regulation of tumor angiogenesis, we co-transfected HeyA8-MDR cells with miR-204-5p-inh and *THBS1* silencing siRNA (siTHBS1). Silencing of miR-204-5p showed significant increase ($p < 0.001$) in the *THBS1* mRNA level (Supplementary Fig. 3c). The *THBS1* protein level in the conditioned medium was detected by ELISA. MiR-204-5p silencing using inhibitor resulted in significant increase of *THBS1* ($p = 0.047$), and silencing of *THBS1* using representative siRNA showed significant decrease ($p = 0.0137$) in the *THBS1* protein level (Fig. 4e). No change was observed between the groups treated with siTHBS1 alone or with both miR-204-5p-inh and siTHBS1 (Fig. 4e). We also checked the expression of *THBS1* protein after miR-204-5p silencing in HeyA8-MDR cells by western blotting. As shown in Supplementary Fig. 4a, silencing of miR-204-5p increased the expression of *THBS1* protein compared with cont-miR-inh treated cells. To check whether miR-204-5p silencing would decrease VEGF secretion in HeyA8-MDR tumor cells, we measured VEGF protein levels in conditioned medium. As shown in Supplementary Fig. 4b, miR-204-5p silencing resulted in a significant decrease in VEGF levels compared with cont-miR-inh treated ones.

Finally, we evaluated the paracrine effect of tumor cells subjected to miR-204-5p and *THBS1* silencing on endothelial cell tube formation. RF24 cells (after they reached 80% confluence) were serum starved overnight, followed by incubation with the conditioned medium collected from HeyA8-MDR cells 96 h post co-transfection with miR-204-5p-inh and siTHBS1. *THBS1* silencing significantly increased tube formation ($p = 0.0014$) compared with cont-miR-inh and sicontrol treatments (Fig. 4f), which confirmed the function of *THBS1* as an anti-angiogenic factor. Silencing of miR-204-5p resulted in a significant reduction in tube formation ($p = 0.002$). Silencing of both miR-204-5p as well as *THBS1* resulted in a 35.2% increase in tube formation ($p = 0.011$) compared with cont-miR-inh and sicontrol treatments, but no difference was seen between *THBS1* silencing alone or

silencing of both THBS1 and miR-204-5p together (Fig. 4f). These results show that the effect of miR-204-5p-inh on angiogenesis can be reversed by silencing *THBS1*.

Discussion

The key finding of this study was that miR inhibitors could be delivered by rHDL-NPs to silence the expression of the oncomiR miR-204-5p at tumor sites. Deregulated miR-204-5p promotes ovarian and breast cancer progression by enhancing angiogenesis and *THBS1* is one of the downstream targets of miR-204-5p that is engaged in this angiogenesis regulation (Fig. 5).

MiR-204-5p has been shown to be a key player in the regulation of the development of retinal pigment epithelium and adipose tissue [27, 28]. Its function has been observed in multiple pathologic conditions, such as pulmonary arterial hypertension, pancreatic islet beta cell dysfunction, and hippocampal sclerosis [29–31]. It has also been found to be involved in the carcinogenesis and progression of various types of cancer. Studies have demonstrated that miR-204-5p serves a dual function as both a tumor suppressor and an oncomiR [32–35]. In most cases, miR-204-5p has been found to function as a tumor suppressor by regulating apoptosis, proliferation, and epithelial–mesenchymal transition [32–35]. On the other hand, miR-204-5p can promote or suppress tumor progression in breast cancer, prostate cancer, and glioma [36–38]. Some studies have revealed that miR-204-5p can suppress tumor angiogenesis by regulating pro-angiogenic genes, such as *ANGPT1* and VEGF family members [39, 40]. However, no previous studies have investigated the regulatory role of miR-204-5p in angiogenesis in an ovarian cancer animal model.

RNA interference is a highly specific approach of gene silencing and holds tremendous potential for the treatment of various pathologic conditions [41]. Although gene silencing is widely used in preclinical models, its clinical application is challenging, primarily because of the difficulty of achieving efficient systemic delivery. “Smart” delivery systems are essential to enable the full therapeutic potential of RNA interference. Targeted delivery can reduce off-target effects and increase the bioavailability of the therapeutic agent at the target sites [42]. Over the past decade, great efforts have been undertaken to develop targeted delivery systems for RNA interference. RHDL, as a delivery vehicle for hydrophobic biomolecules, has the unique advantage of a high degree of tunability. It is an efficient platform for delivering various kinds of cargo into target cells and tissues [43]. Studies over the years have investigated the possibility of using rHDL as a carrier to deliver the anticancer drugs, and found that agents can be selectively delivered by rHDL with high efficiency for therapeutic applications [44, 45]. Intraperitoneal delivery could be of interest, and such an approach has been used in an adjuvant setting for chemotherapy [46]. However, the intraperitoneal route is thought to be more effective with minimal tumor burden. In the context of treating bulkier disease, the intravenous route would be more effective for delivering drug to the tumor. Our study demonstrated that rHDL NPs could deliver the miR inhibitors to the tumor sites in a highly efficient manner. Importantly, although miR-204-5p-inh reduced miR-204-5p expression by nearly 50%, the combination of rHDL-miR-204-5p-inh NPs and paclitaxel did not provide significant therapeutic benefit, this result may be due to the fact that HeyA8-MDR is a multi-drug-resistant cell line that is not sensitive to

chemotherapeutic drugs. For the upstream regulation of miR-204-5p, further study is needed to investigate the biogenesis of miR-204-5p in ovarian tumors with high levels of angiogenesis.

THBS1, downstream target of miR-204-5p, is a potent endogenous inhibitor of angiogenesis [47], regulating through multiple pathways [48]. Using a gene array analysis followed by multiple validations (in vitro and in vivo), we identified this gene as a direct target of miR-204-5p in regulating ovarian cancer angiogenesis. We confirmed this hypothesis by performing a functional assay of endothelial cells that were treated with the conditioned medium collected from HeyA8-MDR cells co-transfected with miR-204-5p-inh and siTHBS1. However, THBS1 may not be the only downstream target of miR-204-5p, but it is one of them in the network in which miR-204-5p plays a pro-angiogenic role. In further investigations, we will perform a broader high-throughput pathway analysis to determine the mechanism by which miR-204-5p regulates tumor angiogenesis.

In summary, our work provides important evidence of the potential of rHDL-NPs as a systemic delivery vehicle to selectively introduce miR-204-5p-inh into tumor sites; this treatment could function as an anti-tumor therapeutic by suppressing angiogenesis. Our findings also provide a new understanding of the role of miR-204-5p in regulating angiogenesis in ovarian and breast cancer, which could open rational avenues for solving issues in anti-angiogenic therapy.

Methods

Cell lines and culture

We obtained all cell lines from the MD Anderson Characterized Cell Line Core Facility, which supplies authenticated cell lines. The cell lines were subjected to routine testing to confirm the absence of mycoplasma, and all experiments were performed with cell lines at 60–80% confluence. Short tandem-repeat (STR) DNA fingerprinting was performed by the Cell Line Core. The human epithelial ovarian cancer cell line SKOV3ip1 was maintained in the RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Thermo Fisher Scientific). HeyA8-MDR cells were maintained in the RPMI 1640 medium supplemented with 15% FBS, 300 ng/ml paclitaxel, and 0.1% gentamicin sulfate. MDA-MB-231 cells were maintained in the DMEM/F-12 medium supplemented with 15% FBS and 0.1% gentamicin sulfate. RF24 human immortalized umbilical endothelial cells were cultured in the MEM supplemented with 15% FBS, 1% sodium pyruvate, 1% MEM nonessential amino acid, 1% multi-vitamin, and 1% penicillin–streptomycin. All cells were grown at 37 °C in a humidified chamber with 5% CO₂.

Clariom S Affymetrix data analysis

The CEL files generated were processed through Transcriptome Analysis Console 4.0, which normalizes (and applies the log₂ function to) array signals using a robust multiarray averaging (RMA) algorithm. Differential expression between miR-204-inhibitor and control-miR-inhibitor was determined by a fold change in absolute value equal or greater to 1.1 and a *p*-value obtained from the moderated *t*-statistic from LIMMA package less than 0.05. Gene

Level Differential Expression Analysis was performed with Transcriptome Analysis Console 4.0. To support visual data exploration a heatmap was generated using the heatmap.2 function of gplots library.

TCGA data analysis

We downloaded patient overall survival information for the TCGA patients with ovarian serous cystadenocarcinoma from cBioPortal (<http://www.cbioportal.org/>). We downloaded miRNA expression profiled from Agilent 8 × 15K Human miRNA-specific microarray (H-miRNA_8 × 15K) for miR-204-5p from Broad GDAC Firehose (<https://gdac.broadinstitute.org/>). Also, the miRNA-Seq data were acquired. We derived the ‘reads_per_million_miRNA_mapped’ values for the mature form miR-204-5p from the files “isoform expression” downloaded from the TCGA portal <https://portal.gdc.cancer.gov/>.

Survival analysis

A Cox regression analysis revealed that patients with expression of miR-204-5p in the last quartile have significant worse prognosis (overall and disease-free) compared to the patients with miR-204 in the first quartile (overall survival: HR = 1.25, CI[95%] = [1.01, 1.55], *p*-value (log-rank test) = 0.01; disease-free survival: HR = 1.32, CI[95%] = [1.07, 1.63], *p*-value (log-rank test) = 0.01). The Kaplan–Meier plots were generated for the two cohorts. The numbers of patients at risk in low and high miR-204-5p groups at different time points are presented at the bottom of the graph.

Correlation analysis

The Spearman’s rank-order correlation test was applied to measure the strength of the association between THB1 and miR-204-5p expression (data generated by the Agilent microarray technology). Statistical analysis was done using R (version 3.0.1) (<http://www.r-project.org/>) and the statistical significance was defined as a *p*-value less 0.05. We imposed also a cut-off of functional relevance on the Spearman correlation coefficient in absolute value of 0.2 based on the method published previously [49].

Prediction of miRNA–mRNA interactions

Information on miRNA-target possible interactions was retrieved from miRWalk2.0 (<http://zmf.umm.uniheidelberg.de/apps/zmf/mirwalk2/>) that hosts miRNA-target predictions from 12 programs.

In vitro transfection

All transfections were performed using RNAiMAX reagent (Invitrogen, Carlsbad, CA) following the reverse transfection protocol from the manufacturer. In brief, cells were transfected with 30pmol siRNA or 100pmol miRNA inhibitor and RNAiMAX reagent in serum-free medium. To minimize toxicity, the medium was changed to fresh complete medium 4–6 h after transfection. Gene silencing was assessed using a qRT-PCR analysis. All the siRNAs and miR inhibitors were obtained from Sigma-Aldrich, and miRNA mimics were obtained from Life Technologies. Experiments were performed in duplicate and repeated three times.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted from cells or tumors using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, USA) according to the manufacturer's standard protocol. cDNA was synthesized from 1 µg of the total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Sugar Land, TX) as per the manufacturer's instructions, and qRT-PCR was performed using SYBR Green Master Mix on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). 18S was used as the normalizing control along with other indicated primer sequences (Supplementary Table 4). For miRNA detection, Ambion assay probe sets (Life Technologies, Carlsbad, GA) were used according to the manufacturer's protocol. *RNU6B* was used as the normalizing control, and the data were analyzed using the comparative Ct method. The data for in vivo samples were analyzed as described previously [50]. Experiments were performed in triplicate and repeated three times.

Gene expression microarray and analysis

The total RNA from cont-miR-inh or miR-204-5p-inh-treated HeyA8-MDR cells was collected to assess global gene changes following miR-204-5p loss by gene expression array. The array was analyzed using the Affymetrix Human Clariom S Assay. The CEL files generated were processed through Transcriptome Analysis Console version 4.0 (Thermo Scientific, Waltham, MA), which was normalized (and applied the log2 function to) array signals using a robust multiarray averaging algorithm. Differential expression between the miR-204-5p-inh and cont-miR-inh was determined by a fold change in the absolute value that was equal to or greater to 1.1 and a *p*-value obtained from the moderated t-statistic from the LIMMA package that was less than 0.05. The gene-level differential expression analysis was performed using Transcriptome Analysis Console version 4.0. The array data were submitted to GEO under the accession number GSE120585.

Western blot analysis

Protein lysates from cultured cells were prepared using modified-radioimmunoprecipitation (RIPA) assay buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL). The lysates were loaded and separated on 10% SDS-polyacrylamide gels. Nitrocellulose membranes were subsequently probed with primary antibody against SCARB1 and THBS1 (1:1000; Cell Signaling Technology, catalog numbers 4153 and 37879) in 5% BSA in TBS-T at 4°C overnight. After being washed with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, GE Healthcare, catalog numbers NA931V and NA934V) for 1 h. Horseradish peroxidase was visualized using an enhanced chemiluminescence detection kit (PerkinElmer, catalog number NEL104001EA). Vinculin and β-actin were probed as the loading control (1:3000, Sigma, catalog number V9131 and A5441). Experiments were performed three times.

ELISA

To quantitatively determine THBS1 and VEGF concentrations in cell-conditioned medium, we used the quantitative sandwich ELISA technique with a commercially available human

THBS1 and VEGF-specific immunoassay kits (R&D Systems, Minneapolis, MN). The minimum detectable dose of THBS1 was less than 7.81 ng/ml. The assay was performed in triplicate according to the manufacturer's instructions. The THBS1 and VEGF concentration was determined from a standard curve. Experiments were performed in duplicate and repeated twice.

Tube-formation assay

After serum starvation overnight, 70–80% confluent RF24 cells were cultured with conditioned medium collected from miR-204-5p-inh or siRNA-treated HeyA8-MDR or miR-204-5p mimic from SKOV3ip1 cells for 24 h. The cells were trypsinized and plated onto 15-well plates that had been pre-coated with growth factor-reduced Matrigel matrix (10 μ L per well; BD Biosciences) and then were incubated at 37 °C for 6 h. Photographs of the tubular network in the wells were obtained using an Olympus IX81 inverted microscope. The number of tubes (defined as enclosed polygons) per well was quantified. Experiments were performed in triplicate and repeated twice.

Migration assay

RF24 cells (7.5×10^4) that had been cultured with conditioned medium collected from miR-204-5p-inh or siRNA-treated HeyA8-MDR cells were suspended in 100 μ L of serum-free medium and added to the upper compartments of modified Boyden chambers (Coster, Boston, MA) pre-coated with 0.1% gelatin. The serum-containing conditioned medium that was used to pretreat RF24 cells (500 μ L) was added to the bottom compartments as a chemo-attractant for cells. Chambers were incubated at 37 °C in 5% CO₂ for 6 h. After incubation, the cells in the upper compartments were removed with cotton swabs. Cells were fixed, stained, and counted using light microscopy. Cells from five random fields were counted. Experiments were performed in triplicate and repeated twice.

Luciferase reporter assay for miR-204-5p target gene binding

HEK293 cells (1×10^4 /well) were plated in each well of a white 96-well TC plate 24 h before transfection. The cells were co-transfected with 50 ng of the *THBS1* 3'UTR construct, *PTX3* 3'UTR construct, *TXNIP* 3'UTR construct, or empty 3'UTR construct (Switchgear Genomics, Carlsbad, CA) with 30 ng of the Cypridina TK control construct (Switchgear Genomics) and 50 nmol/L miR-204-5p mimic or cont-miR-mimic using FuGENE® HD Transfection Reagent. Luciferase activity was measured 48 h after transfection by The LightSwitch™ Dual Assay System (Switchgear Genomics, LightSwitch Dual Assay kit, DA010). Firefly luciferase activity was analyzed based on ratio of Renilla/Firefly. Experiments were performed in triplicate and repeated twice.

Assembly and characterization of rHDL-miR inhibitor NPs

All chemicals, unless otherwise indicated were from Sigma-Aldrich, St Louis, MO. Mixing of rHDL-miR-inh components was facilitated with the use of a microfluidics instrument, the NanoAssemblr Benchtop system (Precision Nanosystems, Vancouver, British Columbia). MiR-204-5p-inh (miR-inh) was heated at 90 °C for 1 min and subsequently placed on ice. A 50 μ g/mL of miR-inh was mixed with poly-L-lysine hydrobromide (MW 1000–5000), in a

ratio of 1:5 (w/w) and incubated at 37 °C for 30 min. Subsequently 5 mg of egg yolk phosphatidylcholine, 0.05 mg of cholesteryl oleate, 0.12 mg of unesterified cholesterol, and 0.5 mg of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], ammonium salt (Avanti Polar Lipids, Inc., Alabaster, AL) were dissolved in chloroform and dried to a thin film under N₂. This thin lipid film was rehydrated with 10 mM Tris, 0.1 M KCl, 1 mM EDTA, pH 8.0, and mixed with the miR-inh/poly-L-lysine complex. Upon adding 1.67 mg Apo-A1 (MC Lab South, San Francisco, CA), the mixture was subsequently dialyzed (using MWCO 12,000–14,000 dialysis tubing treated with RNase Zap and rinsed with RNase-free water) against 1× PBS (made from Fisher 10× PBS and RNase-free water) for 24 h. After removal from the dialysis tubing, 50% D-(+)-Trehalose dihydrate was added to the preparation as a cryoprotectant to a final concentration of 10%. The preparation was then passed through a 0.45 -µm PVDF sterile syringe filter. The incorporation of the miR-inh was quantified with the Quant-It™ RiboGreen RNA assay (Invitrogen ThermoFisher, Eugene, OR). The molecular size of the rHDL-miR-inh formulation was estimated as the hydrodynamic diameter of the NPs by dynamic laser scattering with a Delsa Nanoparticle Size Analyzer (Beckman Coulter Inc, Fullerton, CA). A mean of 70 runs was captured by the instrument and average particle size/diameter was calculated.

Internalization of rHDL-miR inhibitor NPs into MD-MB231 cells

Two separate experiments were carried out to demonstrate the endosomal escape of fluorescent-labeled ncRNA inside cells: (1) The first experiment was carried out to determine relative positions of ncRNA and lysosomes. MDA-MB231 cells were incubated with rHDL-NPs loaded with Alexa 488-labeled ncRNA. Lysosomes were stained by LysoTracker Red dye (Molecular Probes) separately by incubating the cells for 30 min at 37 °C. After incubation, cells were washed three times with sterile PBS to remove free lysoTracker and rHDL-NPs. The cells were then stained with the nuclear stain DAPI for 20 min and imaged using Biotek Cytation 3 (20X). Green fluorescence from Alexa 488 was imaged using FITC ex/em filter set, while red fluorescence from LysoTracker dye was imaged using Texas red ex/em filter set.

(2) The second experiment was carried out to evaluate the relative positions of ncRNA and rHDL-NPs during and after cell-surface interaction. For these studies, the Apo-A1 protein of the rHDL-NPs was labeled with Alexa 647 and purified by passage through Sephadex PD10. rHDL-NPs prepared with Alexa 488-labeled ncRNA and Alexa 647-labeled Apo-A1. These NPs were then incubated with MDA-MB231 cells at 37 °C for 30 min and stained with DAPI-nuclear stain. Green fluorescence from Alexa 488 was imaged using FITC ex/em filter set, while red fluorescence from an Alexa 647-labeled rHDL-NP was imaged using a Texas Red ex/em filter set. Multiple images were collected for each experiment.

Orthotopic ovarian and breast cancer mouse model

Female athymic NCr-nude mice were obtained from the Taconic Biosciences (Rensselaer, NY). Animals were cared for according to guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care and by the US Public Health Service policy on Humane Care and Use of Laboratory Animals. All mouse studies were

approved and supervised by the Institutional Animal Care and Use Committee at MD Anderson. In all, 8–12 weeks old female mice (at the time of injection) were used for therapy experiments. To establish the ovarian tumor model, we injected HeyA8-MDR cells (1×10^6 cells/mouse in 200 μ L of HBSS) intraperitoneally. Seven days after cell injection, mice were randomly allocated to different groups and treated with intravenous injections of rHDL-miR-inh NPs (5 μ g/mouse in 100 μ L of PBS) twice per week and paclitaxel (100 μ g/mouse) once per week.

To determine whether miR-204-5p silencing could have an effect in other tumor types, we established a breast cancer model by injecting MDA-MB-231 cells (2×10^6 cells/mouse in 50 μ L of HBSS containing 20% Matrigel) into the mammary fat pads of mice. Mice were treated twice per week, starting on the eighth post injection day, with intravenous injections of rHDL-miR-inh NPs. The mice were monitored for adverse effects, and all mice were killed by cervical dislocation and necropsied when mice in any group became moribund. Tumors were harvested, and weight along with the number and distribution of tumor nodules was recorded. Tissue samples (tumor and normal) were fixed in formalin for paraffin embedding and frozen in optimal cutting temperature medium. During the course of the experiment, the primary investigator was not blinded to the group allocation. At time of necropsy, all investigators were blinded to the group allocation to assess the outcome of the experiment.

Immunostaining

Immunostaining was performed on formalin-fixed, paraffin-embedded, 8- μ m-thick tumor sections or optimal cutting temperature-embedded frozen tissue sections. After deparaffinization, rehydration, and antigen retrieval or fixation, 3% H_2O_2 was used to block endogenous peroxidase activity for 10 min. Protein blocking of non-specific epitopes was performed using 5% normal horse serum, 1% normal goat serum, or 2.8% fish gelatin in PBS or TBS-T for 20 min. Slides were incubated with primary antibody for ki67 (1:200; RB-9043-P1, Thermo Fisher Scientific) and CD31 primary antibody (1:800, BD Pharmingen, San Diego, CA) at 4 °C overnight. For the immunohistochemical analysis, after the primary antibody had been washed with PBS, the appropriate amount of horseradish peroxidase-conjugated secondary antibody was added, visualized with 3,3'-diaminobenzidine chromogen, and counterstained with Gill's hematoxylin #3. For immunofluorescence, secondary antibody staining was performed with either Alexa 594 (Molecular Probes) or DyLight (Jackson ImmunoResearch Labs, West Grove, PA). Nuclear staining was performed with Hoechst 33342 (1:10,000; Molecular Probe H3570). Images were obtained using a Leica DM4000 B LED microscope and Leica DFC 310 digital camera.

To quantify microvessel density, we examined 5–10 random fields at $\times 100$ magnification for each tumor (five tumors per group) and counted the microvessels within those fields, as previously described [51]. A vessel was defined as an open lumen with at least one adjacent CD31-positive cell. Multiple positive cells beside a single lumen were counted as one vessel. Quantification was performed by two investigators in a blinded fashion. Proliferation indices

were determined from five representative fields at $\times 200$ magnification for each tumor (five tumors per group). All Ki67-positive cells per high-powered field were enumerated [52, 53].

Statistical analyses

Statistical analyses for TCGA and gene array data were performed as described above. For in vivo study, ten mice were assigned to each group and this sample size would give an effective size of 1.3 with 80% power to detect a 50% reduction in tumor weight with 95% confidence. For other assays, Student test was performed (GraphPad Prism version 7.03) to determine difference between control and treatment groups. A p -value less than 0.05 was deemed statistically significant. All statistical tests were two sided. The results are presented as mean $\pm$ SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work is supported, in part, by the National Institutes of Health (CA016672, UH3TR000943, P50 CA217685, P50 CA098258, R35 CA209904), Ovarian Cancer Research Fund, Inc. (Program Project Development Grant), Mr. and Mrs. Daniel P. Gordon, The Blanton-Davis Ovarian Cancer Research Program, the American Cancer Society Research Professor Award, and the Frank McGraw Memorial Chair in Cancer Research (AKS) and CPRIT (DP150091; LSM). SKD was supported by Foundation for Women's Cancer Grant, CRA was supported by the P50 CA217685 from the SPORE in Ovarian Cancer at MD Anderson.

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Fig. 1.

Deregulated miR-204-5p promotes tumor growth by regulating angiogenesis. **a** miRNA profiling in tumors with very high or low microvessel density (MVD). MVD was assessed in high-grade serous carcinoma (HGSCs) using CD31 staining. **b** Correlation between expression of the indicated miRNAs and patient survival. The analysis was performed using the TCGA ovarian cancer data set. The long-rank test was used to determine the association between miRNA expression and patient survival, including disease-free survival (DFS) and overall survival (OS). The number of patients at risk in the low and high miRNA expression

groups at different time points are presented at the bottom of the graph. **c** Relative expression of miR-204-5p in ovarian and breast cancer cell lines quantified by qRT-PCR. HIO180 and MCF10A were used for normalization. **d–f** Effect of miR-204-5p silencing on angiogenesis. **d** Effect of miR-204-5p silencing on tube formation of RF24 endothelial cells. **e, f** Silencing of miR-204-5p in cancer cells decreased the tube formation of endothelial cells. Tube formation was decreased in RF24 cells treated with conditioned medium collected from miR-204-5p silenced ovarian cancer HeyA8-MDR and breast cancer MDA-MB231 cells. The effects were assessed 6 h after incubation. **c–f** Values are the mean $\pm$ SEM ($n = 3$, Student *t* test), ns = not significant, $**p < 0.01$ vs. cont-miR-inh

Fig. 2. Characterization of rHDL-miR inhibitor NPs and internalization of the NPs. Schematic diagram (**a**) of the rHDL-miR inhibitor NPs. **b** Estimation of molecular diameter via dynamic light scattering. **c** Internalization of the payload from the rHDL-NPs by MDA-MB231 cells. ncRNA was labeled with Alexa 488, while the lysosomes were labeled with LysoTracker Red. (A) DAPI-nuclear stain, (B) Alexa 488-siRNA, (C) LysoTracker Red, (D) Overlap, and (E) insets from (D). **d** Internalization of the payload from the rHDL-NPs by MDA-MB231 cells. ncRNA was labeled with Alexa 488, Apo-A1 labeled with Alexa 647.

(A) DAPI-nuclear stain, (B) Alexa 488-ncRNA, (C) Apo-A1 (the protein component of the rHDL-NPs) was labeled with Alexa 647, (D) Overlap, and (E) inset from **d** (scale bar: 200 μm)

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Fig. 3.

MiR-204-5p silencing reduces tumor growth in ovarian and breast cancer mice models. Seven days following tumor cell injection, mice were randomly divided into four groups (ten mice per group) to receive one of the following therapies: (i) rHDL-cont-miR-inh, (ii) rHDL-miR-204-5p-inh, (iii) rHDL-cont-miR-inh + paclitaxel, or (iv) rHDL-miR-204-5p-inh + paclitaxel for HeyA8-MDR model and (i) and (ii) groups for MDA-MB231 model. **a** Representative images of tumor burden from at least five HeyA8-MDR tumor-bearing mice per group. **b** Effect of miR-204-5p silencing on tumor weight and (c) tumor nodules. **d** miR-204-5p silencing reduces the mRNA expression in HeyA8-MDR tumors. **e** Effect of

miR-204-5p silencing on weight and (f) nodules, and (g) mRNA expression of MDA-MB-231 tumors. Ten animals per group were used for the data generated in panels **b**, **c**, **e**, and **f**. Knockdown efficiency was checked in five tumors (**d**, **g**). Values are the mean $\pm$ SEM. ns not significant, ** $p < 0.01$, **** $p < 0.0001$ vs. rHDL-cont-miR-inh (one-way ANOVA followed by a Tukey's multiple comparison post hoc test for **b**, **C**, **e**, and **f**) and (Student's t test for **d** and **g**)

Fig. 4. MiR-204-5p mediates its pro-angiogenic effects by regulating THBS1. **a** Identification and prediction of the downstream targets of miR-204-5p in regulating tumor angiogenesis. **b** Validation of microarray findings by silencing and overexpressing miR-204-5p in vitro. **c** In HeyA8-MDR tumor samples. **d** Correlation between the expression of THBS1 and miR-204-5p from the TCGA data set. **e** The concentration of THBS1 in the cell culture medium of HeyA8-MDR cells 96 h after transfection with miRNA inhibitor and siRNA using ELISA. **f** Tube-formation assay of RF24 cells following incubation with the

conditioned medium from HeyA8-MDR cells after co-transfection with miR-204-5p-inh and THBS1 siRNA. The effects were assessed 6 h after incubation. Values are the mean $\pm$ SEM ($n = 3$, Student's t test), ns not significant, * $p < 0.05$, ** $p < 0.01$

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Fig. 5.

Schematic representation of miR-204-5p angiogenic function. Deregulated miR-204-5p in tumor cells promotes angiogenesis by suppressing anti-angiogenic gene *THBS1*. Silencing of miR-204-5p using rHDL-NPs leads to elevated THBS1 levels, which in turn suppresses angiogenesis and tumor growth