Small Molecule Therapeutics

Anti-miR182 Reduces Ovarian Cancer Burden, Invasion, and Metastasis: An In Vivo Study in Orthotopic Xenografts of Nude Mice

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Abstract

High-grade serous ovarian carcinoma (HGSOC) is a fatal disease, and its grave outcome is largely because of widespread metastasis at the time of diagnosis. Current chemotherapies reduce tumor burden, but they do not provide long-term benefits for patients with cancer. The aggressive tumor growth and metastatic behavior characteristic of these tumors demand novel treatment options such as anti-microRNA treatment, which is emerging as a potential modality for cancer therapy. MicroRNA-182 (miR182) overexpression contributes to aggressive ovarian cancer, largely by its negative regulation of multiple tumor suppressor genes involved in tumor growth, invasion, metastasis, and DNA instability. In this study, we examined the therapeutic potential of anti-miR182 utilizing the animal orthotopic model to mimic human ovarian cancer using ovarian cancer cells SKOV3 (intrabursal xenografts) and OVCAR3 (intraperitoneal injection). These models provide a valuable model system for the investigation of ovarian cancer therapy in vivo. Through a combination of imaging, histological, and molecular analyses, we found that anti-miR182 treatment can significantly reduce tumor burden (size), local invasion, and distant metastasis compared with its control in both models. The bases of anti-miR182 treatment are mainly through the restoration of miR182 target expression, including but not limited to BRCA1, FOXO3a, HMG2A, and MTSS1. Overall, our results strongly suggest that anti-miR182 can potentially be used as a therapeutic modality in treating HGSOC. Mol Cancer Ther; 13(7); 1729–39. ©2014 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy in women and is the fifth leading cause of cancer-related death. Despite great efforts and progress in surgery and chemotherapy, 5-year survival for advanced-stage ovarian cancer remains only 30%. High-grade serous ovarian carcinoma (HGSOC) is the most aggressive form of ovarian cancer, contributing to the majority of ovarian cancer fatalities because of early invasion and metastasis at the time of diagnosis. The tumor dissemination of HGSOC originates in the contralateral ovary, followed by widespread metastasis within the abdominal cavity, peritoneal organs, and regional lymph nodes (1). Emerging data show that some of the miRNA dysregulation may contribute to the aggressive metastasis of HGSOC, including microRNA-182 (miR182; refs. 2 and 3) and miR200 (4, 5) dysregulation. Currently, the targeted miRNA therapy for ovarian cancer invasion and metastasis has yet to be reported.

miR182, a member of the miR183-96-182 cluster, is overexpressed in HGSOC and associated with tumor growth and invasion in these tumors (2, 6). miR182-mediated aggressive growth is mostly contributed by the direct regulation of several genes associated with tumor invasion and metastasis (2, 7–9). Moreover, miR182 overexpression promotes the invasion and metastasis of several other human cancers (2, 7, 8). Therefore, anti-miR182 may provide a beneficial therapy to reduce the tumor burden and metastasis in those malignant neoplasms with miR182 overexpression. For example, Hernando’s group was the first to provide proof-of-principle of the antimetastatic potential of anti-miR182 in melanoma using a mouse model (10). Compared with other solitary carcinomas, ovarian cancer has its own unique features of tumor...
growth and metastasis that need to be further studied to develop a specialized therapeutic. Investigation of the therapeutic potential of anti-miR182 in a mouse model that mimics the corresponding human ovarian cancer tumors is one of the approaches to determine the value of miRNA-based gene therapy against human HGSOC.

In this study, we investigate the potential of anti-miR182 treatment as an anti-invasion therapeutic strategy for ovarian cancer. We selected 2 ovarian cancer cell lines overexpressing miR182 and prepared mouse xenografts by implanting cancer cells into intrabursally or intraperitoneally. Tumor growth, invasion, and metastasis were evaluated during anti-miR182 treatment by luciferase imaging [in vivo imaging analysis (IVIS) system] and histopathology, followed by thorough analysis of miR182 expression and target gene expression. We found that anti-miR182 treatment could significantly reduce ovarian cancer burden and metastasis with minimal toxicity. Our study provides a potential therapeutic modality that targets the aggressive tumor growth of HGSOC.

Materials and methods

Ovarian cancer cell line with stable miR182 and luciferase transfection

Human ovarian cancer cell lines, SKOV-3 and OVCAR3 were purchased from the ATCC and stored during early passage. No authentication was done after resuscitation. SKOV3 lines with stable miR182 overexpression were prepared off site and are described elsewhere (11). Human FUW-LucNeo (lentivirus) expressing luciferase was prepared in HEK293T cells packaged by pMD2G and psPAX2. Cultured cells (4 x 10⁴) were placed and replaced with 1 mL per well of Opti-MEM I reduced serum medium containing 12 μg/mL polybrene. Fifty microliters of concentrated lentiviral particles was added. Forty-eight hours later, fresh medium containing 300 μg/mL G418 was added. Fresh medium containing G418 was replaced every 3 to 4 days. Single colonies were obtained 4 weeks after G418 selection. SKOV3 cells were maintained in McCoy’s 5A medium plus 10% fetal bovine serum (FBS; USA Scientific), and OVCAR3 cells with high endogenous miR182 (12) were cultured in DMEM medium plus 20% McCoy’s 5A medium plus 10% fetal bovine serum (FBS; USA Scientific), and OVCAR3 cells with high endogenous miR182 and prepared mouse xenografts by implanting cancer cells into intrabursally or intraperitoneally. Tumor growth, invasion, and metastasis were evaluated during anti-miR182 treatment by luciferase imaging [in vivo imaging analysis (IVIS) system] and histopathology, followed by thorough analysis of miR182 expression and target gene expression. We found that anti-miR182 treatment could significantly reduce ovarian cancer burden and metastasis with minimal toxicity. Our study provides a potential therapeutic modality that targets the aggressive tumor growth of HGSOC.

Anti-miR182 transient transfection

The anti-miR182 and scramble control compounds were provided by Regulus Therapeutics (http://www.regulusrx.com/about-micrornas/). The efficacy of anti-miR182 in vitro was tested in serial dilutions of 20, 40, 60, and 100 nmol/L. In brief, cells were placed in a 6-well plate (2 x 10⁵ per well) in medium without antibiotics. At 70% confluence, cells were transfected with anti-miR182, scrambled control at a concentration of 100 nmol/L, using Lipofectamine 2000 according to the manufacturer’s protocol. After transfection, cells were harvested and analyzed at the indicated times.

Matrigel invasion and migration assays

A total of 2.5 x 10⁵ SKOV3 cells were seeded into Matrigel-coated upper chambers (BD Biosciences) in McCoy’s 5A medium containing 0.5% FBS. The lower chamber of the transwell was filled with culture media containing 10% FBS as a chemo-attractant. After 24 hours, the noninvading cells in the upper chamber were removed with a cotton swab. Cells on the lower surface of the membrane were fixed by 10% formalin, stained by 0.1% crystal violet for 30 minutes, and counted under a light microscope.

For the migration array, OVCAR3 cells (1.5 x 10⁵ cells) transfected with anti-miR182 or scramble were seeded into 6-well plate. When cells reached confluence, a scratch was made by a 10-μL tip. The scratches were then recorded at 0 and 48 hours, respectively.

Soft agar colony-formation assay

The cells (0.75 x 10⁵ cells) were suspended in 3 mL of culture medium containing 0.3% agar (USB Corporation) and seeded onto a base layer of 3 mL of a 0.6% agar bed in 60-mm tissue culture dishes. The media was changed twice a week. After 3 weeks, colonies were stained with 0.005% crystal violet and photographed. Colonies > 0.1 mm in diameter were counted, and the average numbers (n = 3) in each group were calculated.

Cellular proliferation assay

Cells (2 x 10⁴ cells per well) were seeded onto 96-well plates in triplicate. Cell proliferation was monitored by WST (Roche) at different times (0–5 days) according to manufacturer’s instructions. Briefly, 10 μL WST were added into each well. After incubation for 4 hours at 37°C, the absorbance of the samples was measured against a background control using a microplate reader at 450 nm. The cell numbers were calculated as OD value of different time point/OD0h value x 2,000.

Reverse transcription PCR and real-time PCR

Total RNA was extracted using the mirVanaTM RNA Isolation Kit following manufacturer’s instructions (Ambion). One microgram of total RNA was reverse-transcribed to cDNA using an Advantage RT for PCR Kit (Clontech). MirVana qRT-PCR primers were used to test the expression of miRNA and U6 was used as a control. For real-time PCR, cDNA was synthesized by SYBR Green real-time PCR master mix (Bio-Rad) using a MyiQ and iQ5 real-time PCR detection system with sequence specific miRNA primers (Supplementary Table S1).

Western blotting

The rabbit antihuman FOXO3 (EP1949Y) and mouse antihuman BRCA1 antibodies were obtained from EMD Millipore Corporation. The mouse antihuman MTSS1 antibody was obtained from Abnova. The rabbit antihuman HMGA2 polyclonal antibody was purchased from BioCheck, Inc. (Supplementary Table S2). Cultured cells were harvested and lysed on ice in a NP40 cell lysis buffer.
(Invitrogen) supplemented with protease inhibitor cocktail. Total protein (30 μg) was separated by SDS-PAGE and electrotransferred onto polyvinylidene fluoride membrane. The membrane was incubated with primary antibodies overnight at 4°C. The specific horseradish peroxidase–conjugated goat anti-rabbit or goat antimouse secondary antibody was used to blot the target proteins, and the secondary antibody was detected by an enhanced chemiluminescence ECL detection kit.

**Xenografts in nude mice**

Female athymic NCr-nu/nu mice (NCI-Frederick) were used. Mice were maintained in laminar flow rooms, maintaining consistent temperature and humidity and were given free access to water and a normal diet. Experiments were approved by the Institutional Animal Care and Use Committee of Northwestern University.

**Preparation of cell pellets.** SKOV3-miR182 cells with luciferase expression were washed twice with PBS. Cell count and viability were performed with the Countess analyzer (Invitrogen) using 0.4% Trypan Blue solution. Cells were suspended into rat type I collagen (6.8 mg/mL; BD Biosciences) at 10^6 per 10 μL. The cell–collagen mixture was then dropped onto a 6-well plate and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. The pellets were incubated overnight as floating cultures.

**Surgical procedure for implantation of xenografts in intravascular bursa.** Eight-week-old female nude mice were anesthetized by intraperitoneal injection of ketamine/xylazine (90/8 mg/kg) and disinfected with providone iodine prep pads. An incision about 1.0 cm in length was made in the skin just laterally to the midline of the lower back, and the ovary was visible under the microscope, and the cell pellet was grafted into the intravascular bursa.

**Intraperitoneal injection for OVCAR3.** OVCAR3 cells with luciferase expression were suspended in PBS at 1 × 10^7/mL. The 100 μL cell suspension was then injected intraperitoneally into nude mice using a 25 G needle.

**Anti-miR182 treatment.** Three days after the xenograft surgery, mice were divided into 2 groups randomly and administrated intraperitoneally with either anti-miR182 (25 mg/kg) or a scramble control with the same chemical modification pattern twice a week (Supplementary Fig. S2A). Ovary was put back in place, and bursa. The pellet was fixed because of the tension of bursa. Observation was made in the skin just laterally to the midline of the lower back, and the ovary was visible under the microscope, and the cell pellet was grafted into the intravascular bursa. The pellet was fixed because of the tension of bursa.

**IVIS monitoring.** The tumor growth was monitored using in vivo bioluminescence imaging (IVIS, Xenogen Corp./Caliper Life Science Hopkinton) according to the instructions once a week. Briefly, mice were injected intraperitoneally with firefly Luciferin (150 mg/kg; PerkinElmer). After 10 minutes, mice were anesthetized by 3% isoflurane, put in the IVIS imaging box, and imaged dorsally. Tumor size was measured by normalized luminescence acquisition (IVIS software).

**Necropsy and histologic evaluation**

Mice were sacrificed 8 weeks after the implantation of cancer cells. The blood was drawn via the heart 2–5 minutes after sacrifice, centrifuged at 2,000 × g at 4°C for 15 minutes to remove the cellular component, and then stored at −80°C for future use. The bilateral ovaries and uterus were then isolated, and tumors in ovaries were measured and photographed. Simultaneously, the other organs were dissected and collected, including spleen, kidneys, liver, intestine, pancreas, omentum, stomach, omentum, pelvic lymph nodes, diaphragm, lungs, and heart. All primary tumor tissue and organs were processed (fixed in 10% formalin, embedded in paraffin) and sectioned for histologic examination. Tumor histology, differentiation, invasion, and metastasis were examined by pathologists. Part of the tumor and liver tissue from each case was frozen at −80°C for molecular analysis.

**Statistical analysis**

All data are presented as means and standard errors of at least 3 independent experiments. The Student t test was used for comparisons between 2 groups of experiments, and one-way ANOVA analysis was used for comparisons among 3 or more groups. P < 0.05 was considered significant.

**Results**

miR182 is overexpressed in the majority of HGSOC (3), and it promotes tumor growth, invasion, and metastasis, mostly mediated by the repression of many tumor suppressors (2, 7). To test whether anti-miR182 treatment can be used in ovarian cancer therapy, in this study, we investigated the effects of anti-miR182 treatment on tumor growth, invasion, and metastasis of ovarian cancer cells in xenograft experiments using nude mice.

**Effects of anti-miR182 treatment on ovarian cancer cells in vitro**

To evaluate the efficacy of anti-miR182 in target gene expression and oncogenic properties in ovarian cancer, we selected 3 ovarian cancer cell lines (HEY, OVCAR3, and SKOV3) for in vitro analysis. Our results showed that OVCAR3 exhibited high endogenous miR182 expression (12) whereas HEY and SKOV3 had stable miR182 overexpression as the lines were established by lentiviral infection. For all of the tested cell lines, miR182 expression was significantly reduced from 70% to 90% when the cells were treated with 60 μmol/L anti-miR182 (Fig. 1A and B). Anti-miR182 treatment could significantly rescue BRCA1 (breast cancer 1), FOXO3 (forkhead box O3), and MTSS1 (metastasis suppressor 1) expression, thus inhibiting HMGA2 (high mobility group AT-hook 2) expression and oncogenic properties in ovarian cancer cells in xenograft experiments using nude mice.
Next, we examined the role of anti-miR182 treatment in reducing the miR182 mediated oncogenic properties in ovarian cancer cells. In SKOV3 cell lines, administration of anti-miR182 significantly reduced tumor cell invasion through Matrigel (Fig. 1C) and anchorage-independent tumor growth (Fig. 1D). These phenotypes could also be observed in ovarian cancer cell lines: HEY (Supplementary Fig. S1B) and OVCAR3 (Supplementary Fig. S1C).

Antiproliferation by anti-miR182 has not been previously reported. To further determine whether anti-miR182 inhibits cell proliferation, we examined the growth curves of normal and ovarian cancer cell lines and found that anti-miR182 constantly inhibited cell proliferation in vitro in all of the tested cells (Supplementary Fig. S1A). Tumor cell proliferation rate can be further inhibited by combined cisplatin and anti-miR182 treatments in both SKOV3-miR182 and OVCAR3 tumor cells (Fig. 1E). These findings support that anti-miR182 inhibits miR182 expression, restores its target genes' expression that reduces miR182-mediated tumor growth in vitro.

Intrabursal implantation of SKOV3 tumor cells and intraperitoneal injection of OVCAR3 in nude mice

In this in vivo study, we chose SKOV3 and OVCAR3 cell lines for the following reasons: (i) SKOV3 is commonly used in xenograft experiments of nude mice for ovarian cancer studies; (ii) miR182 overexpression in SKOV3 cells promotes metastasis in nude mice (2); (iii) OVCAR3 has high endogenous miR182 expression (Fig. 1B) and is a probable HGSOC cell model (13); and (iv) anti-miR182 can restore its target gene expression and function in both cell lines in vitro (Fig. 1A and B).

To produce a mouse model that mimics the human ovarian cancer microenvironment, we implanted tumor xenografts in the mouse ovarian intrabursal space. First, we prepared SKOV3 cells with stable miR182 and luciferase expression (see Materials and Methods). To avoid tumor cell leakage in the small space of the intrabursa, we prepared tumor spherical nodules of about 0.2 cm in diameter by embedding SKOV3 cells into collagen I.

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**Figure 1.** Anti-miR182 treatment rescues the miR182 target gene expression and restores tumor cell growth and invasion in ovarian cancer cell line in vitro. A and B, expression of miR182 and 4 target genes in SKOV3 (A) and OVCAR3 (B) with anti-miR182 treatment is presented by Western blot analysis and RT-PCR. β-Actin and U6 were used as protein and RNA loading controls. The average levels of proteins (open box, FOXO3; light gray, MTS51; dark gray, BRCA1; and black box, HMGA2) are shown below the blotting. C and D, SKOV3 cell invasion by Matrigel (C) and anchorage-independent tumor growth by soft agar (D) were significantly different between anti-miR182 and control. E and F, growth curve shows the treatment effects in SKOV3 (E) and OVCAR3 (F) cell proliferation in control, cisplatin, anti-miR182, and combined cisplatin and anti-miR182. *, P < 0.05; **, P < 0.01; ***. P < 0.001.

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matrix (Fig. 2A). The tumor nodules were then implanted into the left ovarian intrabursal space in 8-week-old mice (Fig. 2B). The detail for the intrabursal implantation of ovarian cancer is summarized in Fig 2B and Supplementary Fig. S2A.

As an alternative, the ovarian cancer cell line, OVCAR3 with luciferase overexpression was chosen for intraperitoneal injection (2.10⁷ cells per mouse) in nude mice. Intraperitoneal injection has been broadly used to mimic human ovarian cancer growth.

The luciferase expression was used as a tracer molecule for IVIS of the tumor status. After surgery, the baseline for the tumor cells was examined and documented by IVIS. As shown in Fig. 2C and D and Supplementary Fig. S2B and S2C, all tumor xenografts could be detected on the initial IVIS scanning and were monitored weekly by IVIS.

**Anti-miR182 treatment reduces tumor growth in vivo**

Forty mice receiving SKOV3 xenografts were randomly divided into 2 groups of 20 mice each: control and test. Out of all of the mice, 18 from the control and 19 from the test groups survived at the end of the experiments. A total of 14 mice received intraperitoneal injection of OVCAR3 tumor cells and were randomly divided (7 mice/group) into control and test groups. All of the mice survived at the experimental end point.

On the third day postsurgery, control mice were treated with scramble and test mice were treated with anti-miR182 (dose of 25 mg/kg body weight) via intraperitoneal injection twice weekly. Among 19 mice with SKOV3 xenografts from the test group, 4 mice were treated with anti-miR182 for 1 week (short term), and 15 mice were treated for 8 weeks (long term; Supplementary Fig. S3A).
Tumor growth in each mouse (Fig. 3A and Supplementary Fig. S3B) and average tumor size (Fig. 3B) was monitored by IVIS weekly for up to 8 weeks. No mouse body weight differences were noted between the control and treated groups (Supplementary Fig. S3C).

The mice with SKOV3 xenografts were euthanized at the end of 8 weeks, and the tumor sizes were further examined and measured in oophorectomy specimens (Fig. 3C and D) using the formula $4/3\pi (d/2)^2 (D/2)$, where $d$ is the minor tumor axis and $D$ is the major tumor axis (14). The tumor size and volume in the anti-miR182 treatment group ($5.42 \pm 1.00 \text{ mm, } 90.59 \pm 37.31 \text{ mm}^3$) was significantly smaller than that in the control ($8.07 \pm 1.74 \text{ mm, } 310.89 \pm 187.12 \text{ mm}^3$; $P < 0.001$). The treated tumors were reduced by 32.8% (Fig. 3D). The mouse serum level for CA125 was measured by ELISA in the control and test mice. We found that the CA125 level was lower in the test group than that in the control group ($P = 0.06$; Supplementary Fig. S4B).

The tumor growth rate for OVCAR3 cells in nude mice was detected and scored by IVIS from 0 to 8 weeks (Fig. 2D and F). As shown in Fig. 3E, at the end of the experiment, the tumor size and number of tumors were significantly higher in control group than in mice with anti-miR182 treatment ($P < 0.01$).

In this study, anti-miR182 treatment reduces the tumor growth rate and tumor size. Because several studies (7, 8, 15) showed inconsistent results for miR182-mediated tumor growth in breast cancer and melanoma, we want to know whether the reductions in tumor growth and burden by anti-miR182 treatment in ovarian cancer cell lines are target specific or a nonspecific genotoxic effect. As a result, we examined several regulators of cell cycle and cell death that are predicted target genes of miR182 (16). Real-time PCR analysis revealed that anti-miR182 treatment restored several cell-cycle genes, including CDKN1A, CDKN1B, CHEK2, CYLD, FOXO1, PDCD4, and RECK (Fig. 4).
Anti-miR182 overexpression promotes tumor metastasis (2, 8, 17). To test whether anti-miR182 treatment can prevent metastasis in ovarian cancer, we prepared the xenografts in the mouse ovarian intrabursa to mimic the human ovarian cancer environment, followed by anti-miR182 treatment to the mice. Although the IVIS system could be relatively reliable in monitoring tumor growth (Fig. 3), it is not sensitive enough to detect the micrometastatic tumors in living mice. For example, the incidence of metastatic tumors detected by IVIS at 8 weeks was 28% (5/18) in the control and 7% (1/15) in the test group (Supplementary Fig. S2C). We found that the metastases detected by IVIS were generally larger than 1 mm in diameter (Fig. 5C). To further evaluate the actual metastatic disease, we collected all of the peritoneal organs, including the liver, spleen, pancreas, gut, lymph nodes, omentum, kidneys, oviducts, and uterus as well as the lungs and heart and examined the tissue sections microscopically. In fact, many micrometastases with the tumor size of 0.2 to 0.3 mm could not be detected by the IVIS system (Fig. 5A and Supplementary Fig. S5). Overall, 77.8% (14/18) of the control mice and 33.3% (5/15) of the test mice had at least one metastatic lesion. A total of 31 metastases were found in the controls compared with 6 metastases in the treated mice (Supplementary Table S3). The difference in metastasis between the test and control mice was statistically significant ($P < 0.05$, Fig. 5B and Supplementary Table S3).

The most common sites of metastatic disease between control and test mice, respectively were the following: kidney [50% (9/18) and 0% (0/15)]; spleen [44% (8/18) and 20% (3/15)]; omentum [28% (5/18) and 13% (2/15)]; pancreas [22% (4/18) and 0% (0/15)]; and liver [11% (2/18) and 0% (0/15); Fig. 5B]. In addition, rare metastases in the diaphragm (1/18) and peritoneal lymph node (1/18) were noted. Notable observations were also the large metastases in the pancreas (Fig. 5C) and metastases in the kidney that was mostly confined to the renal capsule (Fig. 5D). Metastases in the spleen and omentum were summarized in Supplementary Fig. S5. As opposed to the human, metastasis to the contralateral ovary was rarely seen in the mice and only one metastasis was noted in the control group. No lung metastasis was found, which is similar to human ovarian cancer. Our findings demonstrate that blocking miR182 expression can significantly reduce the metastatic disease in ovarian cancer.
Molecular analysis of target gene alterations in xenografts treated by anti-miR182

To confirm that the reduced tumor burden and metastasis by anti-miR182 treatment is target gene specific in vivo, we collected the tumor xenografts for molecular analyses. Real-time RT-PCR analysis revealed that miR182 expression occurred in very low or nondetectable levels in all tumors treated with anti-miR182 in comparison to the control (Fig. 6A). There was more than a 90% reduction of miR182 expression. Next, we examined the polycistron cluster of the miR182 family, including miR96 and miR183. We found that anti-miR182 could significantly reduce miR183 expression, but not miR96 (Fig. 6B). Reduction of miR182 and miR183 was also found in the mouse serum samples (Fig. 6C).

Eight tumor xenografts from the control and test groups were randomly selected for miR182 target gene expression. Western blot (Fig. 6D and E) and qRT-PCR (Supplementary Fig. S6) analyses revealed that the miR182 target genes were restored by anti-miR182 treatment. These findings support that a single RNA treatment can correct or restore many dysregulated target genes in tumor cells.

miR182 also enhances HMGA2 expression (12). Because LIN28B or MYCN expression are closely associated with HMGA2 overexpression (18), we examined their expression in tumor xenografts with and without anti-miR182 treatment, and we found that MYCN expression was significantly reduced in tumors treated by anti-miR182 (Supplementary Fig. S4A) and no change of LIN28B was noted.

Discussion

Mouse models of ovarian cancer have been studied extensively (19), however the majority of the models using ovarian cancer xenografts utilize either subcutaneous (20) or intraperitoneal (21) injection. Recent development of
orthotopic mouse models of ovarian cancer intends to inject human ovarian cancer cells directly into the ovarian intrabursa (22) in order to mimic the optimal cancer environment. However, we feel that several technical issues may limit its application, such as the small space of the intrabursa to hold a sufficient cell suspension volume, variability in injected cell numbers among mice, or the potential for peritoneal seeding because of leakage. To improve the model, we prepared a solid tumor nodule/pellet rather than a loose cell suspension and then implanted it intrabursally (Fig. 2 and Supplementary Fig. S2A). To our knowledge, this is the first attempt to implant tumor cell pellets into the ovarian intrabursa.

Our system has several advantages: preparing the tumor nodule/pellet by embedding tumor cells into the collagen matrix, which allows for a consistent number of tumor cells into the small intrabursa (Fig. 2B). During our experiences, no leakage or tumor initiation spread outside of the intrabursa was noted. Furthermore, the ovarian intrabursa functions as a barrier and any tumor cell spread or metastasis requires either invasion through the bursa or the lymphovascular system. To maximize the detection of early and micrometastatic disease, we examined tumor metastasis and invasion not only by IVIS scan, but also by a thorough histologic examination of all peritoneal organs microscopically (Fig. 5).

miR182 overexpression promotes tumor cell invasion and metastasis in various cancer subtypes, including melanoma (7), colon cancer (23), breast cancer (8, 17), and ovarian cancer (2). Further analysis revealed that miR182 mediated tumor invasion and metastasis occurred through the regulation of many tumor suppressors and oncogenes, including MTSS1 (2, 8), and MITF (7), HMGA2 (2), and other genes (9, 24–26). MTSS1 and HMGA2 may play a central role in miR182-mediated tumor metastasis. MTSS1 inhibits cell motility and invasion of breast cancer cells, which are mediated by the regulation of RhoA to act in cytoskeleton rearrangement and invasion of cancer cells (8). MTSS1 also suppresses stress fiber (F-actin) formation, a critical event of cytoskeleton rearrangement during cancer cell migration and invasion (27). MTSS1 is a specific target gene of miR182 (2) and is significantly downregulated in HGSOC (6).

HMGA2 is a well-known oncogene, and its tumorigenic function in ovarian cancer was recently characterized (11). HMGA2 enhances ovarian cancer invasion and metastasis through promoting cell proliferation (28, 29), an epithelial to mesenchymal transition (11, 30). Another miR182 target gene, RECK, can be restored by anti-miR182 treatment (Fig. 4G). RECK is an inhibitor of the matrix metalloproteinase for invasion (17). A target analysis shows that miR182 can bind a large group of functional genes, and...
its overexpression may impair several normal cellular functions (16). Therefore, blocking miR182 expression by anti-miR182 treatment will not only rescue some well-known tumor suppressor genes, but also restores the critical biologic functions necessary for cell defenses.

The role of miR182 in the regulation of cell proliferation has not been well characterized. In this study, we observed that anti-miR182 treatment can consistently reduce tumor cell proliferation in vitro (Supplementary Fig. S1) and tumor growth in vivo (Fig. 3). We further confirmed that antiproliferation by anti-miR182 treatment was contributed by restoring several cell-cycle negative regulators (16), the miR182 target genes in xenograft tumors (Fig. 4). These findings suggest that anti-miR182 can reduce tumor burden in ovarian cancer.

miRNA target therapies have been emerging as a new treatment modality in several human cancers (10, 31). Toxicity of anti-miRNA treatment is a major concern as most miRNAs regulate numerous target genes simultaneously. As part of this study, we are also interested in the toxicity of anti-miR182 treatment in host mice. By histologic examination of organs, we did not see significant histologic and cytologic changes after 8 weeks of treatment in the liver, pancreas, gut, kidneys, spleen, and uterus. However, we did note an increase of monocytes in the paraovarian fat pad. Similar findings of an increase in Kupffer cells (modified monocytes) in the liver was also noted (10). The significance of monocytic aggregates remains unclear.

In summary, we investigate the therapeutic potential of anti-miR182 in treating ovarian cancer growth, invasion, and metastasis in the mouse model. We were able to evaluate the therapeutic effects at molecular, cellular, and anatomic levels. Our study demonstrates that anti-miR182 treatment can significantly reduce the tumor burden of primary tumors and inhibit tumor invasion and metastasis. The efficacy of anti-miR182 treatment seems largely mediated by restoring target gene expression. Future studies will focus on examining the therapeutic potential in treating primary HGSCC from patients’ derived xenografts or xenopatients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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