TUMORIGENESIS AND NEOPLASTIC PROGRESSION

miR-92a Inhibits Peritoneal Dissemination of Ovarian Cancer Cells by Inhibiting Integrin α5 Expression


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Ovarian cancer is characterized by widespread peritoneal dissemination and ascites and has a cure rate of only 30%. As has been previously reported, integrin α5 plays a key role in the peritoneal dissemination of ovarian cancer. Our aim was to identify a new miRNA that regulates integrin α5 expression and analyze the therapeutic potential of targeting this miRNA. By using an IHC analysis, we proved that high integrin α5 expression correlates with a poor prognosis in Japanese patients with International Federation of Gynecology and Obstetrics stage III ovarian cancer. Based on an miRNA algorithm search, we identified hsa-mir-92a (miR-92a) as a candidate. The level of miR-92a expression was significantly inversely correlated with ITGA5 expression in various cancer cells. Transfection of precursor miR-92a reduced integrin α5 expression in ovarian cancer cells, which was accompanied by the inhibition of cancer cell adhesion, invasion, and proliferation. miR-92a overexpression reduced the luciferase activity of the ITGA5 3′-untranslated region, suggesting that ITGA5 mRNA is a direct target of miR-92a. In vivo ovarian cancer xenografts, the enforced expression of miR-92a in HeyA-8 cells suppressed peritoneal dissemination. Although we still have a long way to go before an effective and nontoxic miRNA-based cancer therapy can be introduced into the clinic, the inhibition of integrin α5 expression by targeting miR-92a needs to be explored further for future applications in ovarian cancer treatment. (Am J Pathol 2013, 182: 1876–1889; http://dx.doi.org/10.1016/j.ajpath.2013.01.039)

Ovarian cancer, a highly metastatic disease characterized by widespread peritoneal dissemination and ascites, is the leading cause of death from gynecologic malignancies in industrialized countries. Because it is often diagnosed at a late stage, 1 63% of all patients with ovarian carcinoma will succumb to their disease, making it the fifth leading cause of cancer death among US women. 2 Treatment strategies for advanced ovarian carcinoma consist of aggressive cytoreductive or tumor debulking surgery, followed by a combination of platinum and taxane-based chemotherapy. Maximal surgical cytoreduction is one of the most critical prognostic factors in ovarian cancer treatment. 3 This is indicative of a dramatic difference in the biological behavior of ovarian cancer compared with other malignancies, because in most other cancers, the removal of metastatic tumors does not necessarily lead to improved survival. 4 One of the main reasons for this difference is that ovarian cancer metastasis is generally confined within the abdominal cavity and, unlike other malignancies, rarely disseminates through the vasculature, although metastasis in pelvic and/or para-aortic lymph nodes is occasionally found. 5 Until recently, most epithelial ovarian cancers had been thought to arise from the ovarian surface epithelium, a single continuous layer of flat-to-cuboidal mesothelial cells covering the ovary. 6,7 However, supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid for Scientific Research 21791555 and 23592447 (K.S.), 22390308 (H.K.), and 24249080 (T.K.), and partly by the Uehara Memorial Foundation, the Kanae Foundation for the Promotion of Medical Science, and the Sagawa Foundation for Promotion of Cancer Research (K.S.).
recent morphological, immunohistochemical (IHC), and molecular genetic studies have led to a new paradigm for the pathogenesis and origin of ovarian cancer. These observations strongly suggest that fallopian tubal epithelium that implants on the ovary is the source of high-grade serous carcinoma rather than ovarian surface epithelium. Similarly, it is widely accepted that endometriosis is the precursor of endometrioid and clear-cell carcinomas (CCCs). Kindelberger et al reported that serous tubal intraepithelial carcinomas were observed in >70% of sporadic (nonhereditary) ovarian and peritoneal high-grade serous carcinomas. Given that the first key step of ovarian cancer metastasis is when cancer cells, implanted from the fallopian tubes, attach to the layer of mesothelial cells that line the inner surface of the peritoneum, inhibiting the initial attachment of cancer cells could be a means of preventing ovarian cancer progression. Several integrins have been identified as important mediators of ovarian carcinoma metastasis to the mesothelium. Of these, integrin α5 is of particular interest in ovarian cancer biological characteristics.

Integrin α5 predominantly binds with integrin β1. Integrin α5β1 recognizes the arginine-glycine-aspartic acid (RGD) sequence on its respective ligand, fibronectin, which is one of the most abundant proteins in the extracellular matrix (ECM) of the omentum and the peritoneum. Once ovarian cancer cells have detached from the primary site, they float in the ascites as single cells or as multicellular spheroids. Integrin α5β1 and fibronectin mediate the formation of ovarian carcinoma spheroids and their adhesion to ECMs at the secondary tumor growth sites. Ovarian cancer metastasis starts from the attachment of cancer cells onto the layer of mesothelial cells that cover the peritoneal cavity. Blocking antibodies against α5 and β1-integrin and RGD peptide inhibits the binding of ovarian cancer cells to mesothelial cells, suggesting that integrin α5β1 is the major receptor responsible for fibronectin-mediated ovarian cancer binding to the mesothelium. Recently, we reported that the epithelial-mesenchymal transition of ovarian cancer cells induces the up-regulation of integrin α5, which promotes the adhesion of ovarian cancer cells to secondary metastasis sites in the peritoneal cavity. These cumulative results strongly suggest that a new therapeutic target could be the inhibition of this integrin’s function, at least for a subset of patients with ovarian cancer.

miRNAs are an evolutionarily conserved class of small noncoding RNAs that control a wide range of physiological and pathophysiological processes. They do so by regulating gene expression through matching to target genes, either completely or partially, at the 3′-untranslated region (UTR), causing the suppression of protein translation or mRNA degradation. More than 1500 different miRNAs have been identified in human cells, and these have the potential to regulate up to 30% of human genes or genetic pathways. More than 50% of these miRNA genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRNAs are deeply involved in the pathogenesis of cancers, including cancer metastasis. A handful of miRNAs have been identified as potential therapeutic targets in various human cancers. Inhibition or overexpression of these oncomirs might regulate the expression of their associated genes, therefore repressing cancer proliferation or metastasis.

We attempted to identify the miRNAs that regulate integrin α5 expression, which is known to play a pivotal role in ovarian cancer dissemination, and to analyze the therapeutic potential of targeting this miRNA. In the present study, we identified hsa-mir-92a (miR-92a) as a candidate. miR-92a is expressed at low levels in ovarian cancer cells, which express high levels of integrin α5. However, it is highly expressed in RMUG-S cells, which express a low level of integrin α5. Transfection of precursor miR-92a reduced integrin α5 expression in ovarian cancer cells, accompanied by the inhibition of cell adhesion, invasion, and proliferation. miR-92a overexpression reduced luciferase activity of ITGA5 3′-UTR, suggesting that ITGA5 mRNA is a direct target of miR-92a. In vivo ovarian cancer xenografts, lentiviral transfection of miR-92a into HeyA-8 ovarian cancer cells reduced peritoneal dissemination. These results suggested that targeting miR-92a may prove to be a novel and effective gene therapy for patients with ovarian cancer. Further research will be needed to develop future clinical applications.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM) was purchased (product 08458-45) from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). An antibody against focal adhesion kinase (FAK; 610087, monoclonal), growth factor-reduced basement membrane proteins (Matrigel), fibronectin, collagen type 1, and 24-transwell chambers were purchased from BD Biosciences (Bedford, MA). Bovine serum albumin and antibodies against FAK (polyclonal) and phosphorylated-FAK (pTyr397) (F9176, polyclonal) were purchased from Sigma Aldrich Corp (St. Louis, MO). Antibodies against α1-integrin (R-164, sc-10728), α5-integrin (H-104, sc-10729), and matrix metalloproteinase (MMP)-2 (H-76, sc-10736) were from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 and TRIZol were obtained from Invitrogen (Carlsbad, CA). An Integrins Sampler Kit (611435, including integrin α2, α3, αV, β1, β3, and β4 monoclonal antibodies) was purchased from BD Biosciences.

TMA Preparation and IHC

Ovarian carcinoma samples were collected from 148 patients treated at Osaka Medical Center for Cancer and Cardiovascular Disease (Osaka, Japan) between 2000 and 2005 and used to construct the tissue microarray (TMA) slides. Briefly, resected specimens were fixed in 10% buffered formalin, and
representative regions were processed for paraffin embedding. Slices cut (2 μm thick) were mounted on microscope slides and stained with H&E. All of the slides were reviewed, and areas containing both tumorous and nontumorous regions without massive necrosis were chosen. From the corresponding regions in paraffin blocks, tissue cores (diameter, 3 mm) were removed using a hollow needle. These tissue cores were arrayed in paraffin blocks (TMA) and sliced (4 μm thick) onto slides. Institutional Review Board approval was obtained from the Institute Osaka Medical Center for Cancer and Cardiovascular Disease. Satisfactory tissue cores were finally obtained from 114 patients, and the corresponding clinical data were collected. The TMA slides were deparaffinized in xylene and dehydrated with 100% ethanol before antigen unmasking was performed by boiling the slides in Target Retrieval Solution (pH 9.0) (Dako, Glostrup, Denmark). After being placed in 3% H2O2 and being blocked with blocking solution (Dako), they were incubated with the primary integrin α5 antibody at 1:200 for 18 hours at 4°C. After washing with PBS, they were stained using the Envision system (Dako) and then counterstained with Mayer’s hematoxylin. Slides were intensively examined by two independent qualified pathologists (Y.T. and S.N.), without knowledge of clinical outcomes, and each sample was scored based on the percentage of positive cells (0, ≤10%; 1, 10% to 25%; 2, 25% to 50%; and 3, ≥50%) and the intensity of the staining (0, none; 1, very weak; 2, weak; and 3, strong). The definition of high integrin α5 expression was a total score of density and intensity of five or more. Low expression was defined as a total score of density and intensity of four or less.

Cell Culture

The ovarian cancer cell lines, A2780 and CaOV3, were purchased from ATCC (Rockville, MD). RMUG-S, OVISE, and RMG-1 cell lines were obtained from the Health Science Research Resources Bank (Osaka). SKOV3ip1 cells were from University of Chicago (Chicago, IL), and HeyA-8 cells were kindly provided by Dr. Andi Sood (MD Anderson Cancer Center, Houston, TX). Cells were cultured in DMEM supplemented with 10% FBS and 1000 U/mL penicillin/streptomycin, incubated in 95% air/5% CO2 at 37°C. A2780, CaOV3, HeyA-8, and SKOV3ip1 cells were established from serous papillary adenocarcinomas of human ovary. RMUG-S cells were from a human mucinous cystadenocarcinoma. OVISE and RMG-1 cells were from CCCs of human ovary.

Real-Time RT-PCR Analysis of miR-92a

Real-time quantitative RT-PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). RNA was extracted using TRIZol and was transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or a TaqMan MicroRNA RT Kit (Applied Biosystems). Accurate quantitation of mature miR-92a was obtained using the TaqMan MicroRNA Assays (hsa-miR-92a; PN, 000431). TaqMan endogenous control (RNU6B; N/P, 4373381) was used to normalize miRNA expression levels. Relative levels of miR-92a expression were calculated using the 2−ΔΔCT method, as previously described.22

NCI60 miRNA and mRNA Correlation Analysis

Normalized Agilent miRNA and mRNA data sets from NCI60 cell lines were obtained from Cellminer (http://discover.nci.nih.gov/cellminer, last accessed March 28, 2012). The Pearson correlation and associated P value were calculated between the expression levels of hsa-mir-92a (MI0000093) and the ITGA5 probe set (A_23_P36562).

**Table 1** Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>No. of patients</td>
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</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Observation time of patients alive (months)</td>
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<tr>
<td>FIGO stage</td>
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</tr>
<tr>
<td>I</td>
<td>63 (55.3)</td>
</tr>
<tr>
<td>II</td>
<td>6 (5.3)</td>
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<tr>
<td>III</td>
<td>30 (26.3)</td>
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<tr>
<td>IV</td>
<td>15 (13.2)</td>
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<td>Disease</td>
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<tr>
<td>Ovarian cancer</td>
<td>108 (94.7)</td>
</tr>
<tr>
<td>Primary peritoneal cancer</td>
<td>5 (4.4)</td>
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<tr>
<td>Primary fallopian tube</td>
<td>1 (0.9)</td>
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<tr>
<td>Histological feature</td>
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<td>Serous papillary adenocarcinoma</td>
<td>31 (27.2)</td>
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<tr>
<td>Endometrioid adenocarcinoma</td>
<td>13 (11.4)</td>
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<tr>
<td>Clear-cell adenocarcinoma</td>
<td>17 (14.9)</td>
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<tr>
<td>Mucinous adenocarcinoma</td>
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<tr>
<td>Low potential malignancy</td>
<td>23 (20.2)</td>
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<tr>
<td>TXL-CDGP</td>
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<tr>
<td>CPT-11-CDDP</td>
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<tr>
<td>BEP</td>
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<tr>
<td>Ifosfamide</td>
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<tr>
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<tr>
<td>Unknown</td>
<td>5 (4.4)</td>
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</table>

Data are given as number (percentage) of patients unless otherwise indicated.

*Data are given as median (range). BEP, bleomycin, etoposide and cisplatin; CPT-11-CDDP, irinotecan-cisplatin; FIGO, International Federation of Gynecology and Obstetrics; TC, paclitaxel-carboplatin; TXL-CDGP, paclitaxel-nedaplatin.
carboxyfluorescein (FAM)-labeled Negative Control 1 miRNA (AM17121; Invitrogen). Oligonucleotide transfection was performed with Lipofectamine 2000 in accordance with the manufacturer’s instructions. Transfection efficiency was confirmed by detecting FAM-labeled cells using FV1000-D Laser Scanning Confocal microscopy (Olympus, Tokyo, Japan). At 48 hours after transfection, samples were collected for subsequent analysis.

Western Blot Analysis
A total of $5 \times 10^5$ cells were plated onto 6-well plates and lysed with 1× Cell Lysis buffer (Cell Signaling, Beverly, MA) [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na$_2$EDTA, 1 mmol/L EGTA, 1% Triton-X 100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na$_3$VO$_4$, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride]. Lysates (15 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes, followed by incubation with the primary antibodies (integrin α5, 1:2000 in 5% bovine serum albumin; actin, 1:10,000 in 5% milk) and then with a corresponding secondary horseradish peroxidase–conjugated IgG. The proteins were visualized with the electrochemiluminescent system (PerkinElmer Life Science, Boston, MA).

**In Vitro Adhesion Assay to ECM Components**
At 48 hours after the transfection, $5 \times 10^4$ ovarian cancer cells were plated in a 96-well plate precoated with 50 μg/mL fibronectin or 50 μg/mL collagen type 1. After incubation for 1 hour at 37°C, cells were washed three times with PBS, fixed with methanol, and stained with Giemsa solution. The number of adhesive cells was quantified by adding 100 μL of 0.2% Triton X-100 to lyse cells and measure the absorbance at 560 nm with a microplate absorbance reader (Bio-Rad, Hercules, CA).

**Matrigel Invasion Assay**
*In vitro* cellular invasion was assayed by determining the ability of cells to invade through a synthetic basement membrane. Briefly, pore size polycarbonate filters (8 μm thick), coated with

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**Figure 1** Integrin α5 overexpression is a prognostic marker in advanced ovarian cancer. IHC staining of a TMA with malignant ovarian tissue sections. Representative sections of four different ovarian cancers stained with an antibody against human integrin α5 are shown and scored as negative (A), low (B), and overexpression (C–F). Arrows in A show blood vessels, which served as an internal control. Original magnification, ×200 (A–F). G and H: Kaplan-Meier curves of OAS (G) and PFS (H) of 30 patients with International Federation of Gynecology and Obstetrics stage III ovarian cancer, treated at Osaka Medical Center for Cancer and Cardiovascular Disease (Osaka, Japan), between 2000 and 2005.
25 μg Matrigel (BD Biosciences), were placed in a modified Boyden chamber. At 48 hours after the transfection, 1 × 10^5 ovarian cancer cells per well were plated onto the top chamber in serum-free medium and incubated with 10% FBS-containing medium as a chemoattractant in the bottom chamber. After the cells were allowed to invade through the Matrigel barrier for 48 hours, the filter was fixed and stained with Giemsa solution. Noninvading cells were removed using a cotton swab, and invading cells on the underside of the filter were enumerated using an inverted microscope.

**In Vitro Cell Proliferation Assay**

After the miRNA transfection, 5 × 10^3 SKOV3ip1 cells were seeded in 96-well plates precoated with 50 μg/mL fibronectin or 50 μg/mL collagen type 1 and cultured in DMEM supplemented with 2% FBS. At the indicated time points, cells were washed with serum-free medium and frozen at −80°C. Cell proliferation was measured using the CyQuan cell proliferation assay kit (Molecular Probes, Eugene, OR), in accordance with the manufacturer’s instructions. Briefly, cells were lysed with a buffer containing the fluorescent dye, and fluorescence was measured using a multiwell fluorescence plate reader with an excitation at 480 nm and an emission at 520 nm.

**Luciferase Activity Assay**

pMIR-REPORT Luciferase miRNA expression reporter vectors (Invitrogen) bearing 4 × integrin α5 3′-UTR sequences, which are predicted to bind hsa-miR-92a or a mutated sequence, were kindly provided by Dr. Angelika Bonauer (Goethe University Hospital, Frankfurt, Germany). After 3 × 10^5 cancer cells were seeded in 6-well plates, 40 nmol/L of precursor miRNA or control miRNA was transfected with Lipofectamine 2000. At 24 hours after the initial transfection, 2 μg of the pMIR-REPORT vectors were further transfected with 0.2 μg of pRL-TK Renilla luciferase vector. After 24 hours, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), according to the manufacturer’s instructions. Firefly luciferase values were normalized to Renilla luciferase values.

**MTS Assay**

Cell viability was assessed by an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay using a CellTiter 96 AQueousOne Solution Cell Proliferation Assay (Promega). Briefly, 3 × 10^3 ovarian cancer cells per well were plated in 96-well plates and allowed to attach overnight, and then the cells were transfected with 40 nmol/L of precursor miRNA or FAM-labeled negative control miRNA using Lipofectamine 2000. At 48 hours after the transfection, the number of surviving cells was determined by measuring the absorbance at 590 nm of the dissolved formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt for 1 hour, as described by the manufacturer. The experiments were performed in triplicate.

**Immunofluorescent Analysis**

The transfected cells were plated on 8-well chamber slides and allowed to attach overnight. After incubation, the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and stained with rabbit anti-human integrin α5 [polyclonal (R-164, 1:50); Santa Cruz Biotechnology] at room temperature for 1 hour. After washing, samples were incubated with 1:100 Alexa Fluor 555—labeled goat anti-rabbit IgG (A-21429; Invitrogen) and finally stained with DAPI. The samples were observed using an FV1000-D Laser Scanning Confocal microscope (Olympus).

**Figure 2** Inverse correlation between integrin α5 and miR-92a in ovarian cancer cell lines. A: Western blot analysis. Cell lysates from seven different ovarian cancer cells were resolved by SDS-PAGE and immunoblotted with an antibody against integrin α5. β-Actin was used as a loading control. Blots are representative of three experiments. B: miRNA RT-PCR. Top panel: Total RNA was collected from ovarian cancer cell lines using TRizol and subjected to miRNA RT-PCR. The 2−ΔΔCt method was used to calculate the relative abundance of miR-92a with respect to RNU6B expression. Relative fold differences with respect to RMUG-S cells are presented. Bottom panel: The average fold differences from three independent experiments are shown. C: Correlation plot extracted from NCI60 microarray data sets (R = −0.323, P = 0.012 by Pearson’s test).
Lentivirus Transduction

The lentivirus vector, hsa-mir-92a-1 lentivirus or mir-control lentivirus, was obtained from Biosettia (San Diego, CA) with the titer of 10^7 IU/mL. A total of 1 x 10^5 HeyA-8 cells were plated in 6-well plates overnight, and 20 μL of the lentivirus diluted in 2 mL of fresh DMEM with 10% FBS was treated in the presence of 5 μg/mL of polybrene (TR-1003-G; EMD Millipore, Billerica, MA). After 24 hours, the culture medium was replaced by fresh medium and the transduced cells were positively selected by continuous exposure to 5 μg/mL puromycin (anti-pr-1; Invivo-Gen, San Diego, CA). At 14 days after the selection, >90% of the cells displayed red fluorescence at excitation/emission wavelengths of 587/610 nm. Real-time RT-PCR assays were used to detect the expression of miR-92a in these stable cell lines.

Animal Experiments

Female athymic BABL/c nude mice (aged 4 to 5 weeks) were purchased from Charles River Japan (Yokohama) and were bred in aseptic conditions and kept at constant humidity and temperature (25°C to 28°C). All animal studies were approved by the Institutional Animal Care and Usage Committee of Osaka University (Osaka), in accordance with institutional and NIH guidelines. HeyA-8 cells, stably expressing either miR-92a or control miRNA, were suspended as single cells in a volume of 500 μL of PBS and injected i.p. into female athymic nude mice. Mice were assessed daily for general health and development of ascites, and their weight was measured and their abdominal circumference was determined twice weekly. Two weeks after the inoculation, they were sacrificed. The number of metastases in each mouse was counted, they were carefully dissected, and the removed tumors were weighed.

Tumor tissues were immediately fixed in 10% PBS-buffered formalin and embedded in paraffin. The slides were prepared, as previously described, and incubated with the polyclonal integrin α5 antibody at 1:300 for 1 hour; a polyclonal phospho-FAK (Tyrr861) antibody at 1:200 for 30 minutes, and a monoclonal Ki-67 antibody (Sp6; LabVision, Fremont, CA) at 1:300 for 30 minutes at room temperature. After washing with PBS, they were stained using N-Histo Simple Stain MAX PO (424141; Nichirei Biosciences, Tokyo) and then counterstained with Mayer’s hematoxylin. For Western blot analysis, tumor tissues were snap frozen by liquid nitrogen, ground, and lysed with ice-cold radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L NaVO₄, and 1:1000 protease inhibitor cocktail; Nacalai Tesque]. An equal amount (15 μg) of cell extracts was separated by SDS-PAGE. Proteins were visualized with enhanced chemiluminescence.

Statistical Analysis

Statcel version 3 (OMS-Publishing Inc., Saitama, Japan) and JMP version 9.02 (SAS Institute Japan Inc., Tokyo) were used for statistical analyses. Data are expressed as means ± SD. Differences were analyzed using the Student’s t-test or one-way analysis of variance for multiple comparisons. Survival estimates were computed using the Kaplan-Meier method, and comparisons between groups were analyzed using the log-rank test. Multivariable analysis was performed using a Cox proportional hazards regression model. Differences were considered statistically significant at P < 0.05.

Results

High Integrin α5 Expression Is an Independent Prognostic Marker for Patients with Advanced Stage Ovarian Cancer

First, we established TMA slides from Japanese patients with ovarian cancer, all of whom underwent primary debulking surgery at Osaka Medical Center for Cancer and Cardiovascular Disease (Osaka, Japan) between 2000 and 2005. Satisfactory spots were finally obtained from 114 patients, and the corresponding clinical data were analyzed. The characteristics of patients are summarized in Table 1. Integrin α5 protein expression was evaluated by IHC, and each sample was scored based on the percentage of positive cells and the intensity of the staining. Typically, clear membranous staining was seen in the cases of high integrin α5 expression (Figure 1, A–F). Of 114 patients, 35 (30.7%) had high integrin α5 expression, including 7 (22.6%) of 31 serous, 2 (16.7%) of 12 mucinous, 1 (7.7%) of 13 endometrioid, 13 (76.5%) of 17 clear cell, 7 (30.4%) of 23 low potential malignant, and 5 (27.8%) of 18 other ovarian carcinomas. To determine whether high integrin α5 expression occurs during early or late ovarian cancer progression, we compared the percentage of cases of high integrin α5 expression in early (stage III) and advanced (stage IV) disease. Of 69 patients in early stages, 24 (34.8%) had high integrin α5 expression, whereas 11 (24.4%) of 45 patients in advanced stages showed high integrin α5 expression, suggesting that integrin α5 expression occurs early in ovarian cancer development. Among the patients with stage III ovarian cancer, whose tumors had spread throughout the peritoneal cavity (n = 30), those who had high integrin α5 expression showed significantly worse overall survival (OAS) and progression-free survival (PFS) than those who had low expression [OAS, 10.8 versus 40.4 months (P = 0.002); PFS, 0 versus 13.0 months (P = 0.02)] (Figure 1, G and H, respectively). In patients with early stage (III) ovarian cancer whose tumors were confined within the reproductive organs, high integrin α5 expression was not associated with a clinical impact on PFS or OAS (Supplemental Figure S1). Therefore, integrin α5 expression is likely to affect the prognosis only when tumors extend and disseminate throughout the peritoneal cavity. For a multivariate analysis, a backwards elimination approach was used to select a model for survival with multiple predictors. Grading, the size of residual tumor, age, and high integrin α5 expression were entered in this model. The final model included high integrin α5 expression as a significant predictor for reduced OAS in patients with stage III disease (P = 0.006).
Prediction of Putative miRNAs Interacting with Integrin α5

Because integrin α5 overexpression significantly affects the prognoses of patients with disseminated tumors in their peritoneal cavities, we were motivated to identify miRNAs that regulate integrin α5 expression. Possible candidate miRNAs were found using the Diana-micro T, version 3.0 (Athens, Greece),24,25 (Supplemental Table S1) and TargetScan, version 6.2 (Cambridge, MA),26–29 (Supplemental Table S2). Hsa-miR-92a (miR-92a) and hsa-miR-92b showed the highest score in both integrated databases of miRNA targets. Because
Bonauer et al previously revealed that forced overexpression of miR-92a in human endothelial cells blocks angiogenesis by selectively inhibiting integrin α5 expression, we chose to investigate miR-92a in the present study.

Expression Level of miR-92a Inversely Correlates with Integrin α5 Expression in Ovarian Cancer Cell Lines

To determine whether the expression of miR-92a and integrin α5 are correlated, we measured integrin α5 expression by using Western blot analysis (Figure 2A) and miR-92a expression by miRNA RT-PCR (Figure 2B) in seven different ovarian cancer cell lines. Six of seven cell lines (CaOV3, RMG1, OVISE, A2780, SKOV3ip1, and HeyA-8) expressed high levels of integrin α5, whereas RMUG-S (mucinous) expressed a low level of integrin α5. In contrast, miR-92a relative expression levels were found to be significantly lower (0.002 to 0.079) for all six ovarian cancer cell lines that expressed high levels of integrin α5 compared with RMUG-S cells. The US National Cancer Institute’s NCI60 database, which contains a panel of 60 diverse human cancer cell lines, including five ovarian cancer cell lines, was evaluated to analyze the correlation between miR-92a and integrin α5 expression in different types of cancer cell lines. The miR-92a expression level was significantly inversely correlated with ITGA5 expression ($R = -0.323, P = 0.012$) (Figure 2C), strongly suggesting that miR-92a is one of the key regulators of integrin α5 in cancer cells.

Integrin α5 Is a Direct Target of miR-92a

To show that miR-92a directly participates in the regulation of integrin α5 expression, we performed an in vitro functional analysis by either restoring or silencing miR-92a. SKOV3ip1 (serous), A2780 (serous), and OVISE (CCC) cell lines were chosen for the enforced expression of miR-92a because they display low constitutive levels of this miRNA and high integrin α5 protein expression (Figure 2, A and B). High transfection efficiency (approximately 80%) was confirmed by FAM-labeled control miRNA without impairing the cell viability (Supplemental Figure S2, A and B). The enforced expression of miR-92a in these ovarian cancer cell lines caused a significant decrease in protein levels of integrin α5, as determined by using Western blot analysis (Figure 3A and B). The reduction of integrin α5 expression in miR-92a transfected cells was also confirmed by immunofluorescent analysis (Supplemental Figure S3, A and B). The ITGA5 mRNA level was measured by real-time RT-PCR. When miR-92a expression was enforced in ovarian cancer cells, ITGA5 mRNA showed a 40% to 60% decrease when compared with the control group (SKOV3, 54%; A2780, 46%; OVISE, 62%) (Figure 3C). Because it is known that the extensive complementarity in the coding region of 3′-UTR leads to a direct mRNA cleavage, the enforced expression of miR-92a appears to result in ITGA5 mRNA degradation.
However RMUG-S cells were chosen for miR-92a inhibition because they express the highest miR-92a basal levels associated with a low integrin α5 protein level. The inhibition of miR-92a by the transfection of the specific antagomir revealed an increase of integrin α5 protein (Figure 3, D and E) and a 3.9-fold increase of ITGA5 mRNA (Figure 3F). To determine whether the enforced expression of miR-92a might affect other integrins, the expression of various integrins was examined by using Western blot analysis (Supplemental Figure S4). The enforced expression of miR-92a in SKOV3ip1 and OVISE cells did not alter the expression levels of the integrins tested (integrins α1, α2, α3, αV, β1, β3, and β4).

To further investigate whether miR-92a directly targets the 3′-UTR of ITGA5 mRNA, we performed a dual luciferase reporter assay. The 3′-UTR of the ITGA5 mRNA contains one conserved predicted binding site for miR-92a (Figure 4A). A significant decrease in relative luciferase
activity was observed in ovarian cancer cells (SKOV3ip1 and HeyA-8) transfected with pre-miR-92a when compared with control miRNA (Figure 4B). The site-specific mutation of the target sequence prevented down-regulation of luciferase activity induced by miR-92a transfection, indicating a direct interaction of miR-92a with the 3′-UTR of ITGA5 mRNA. Collectively, these data showed a post-transcriptional regulation of integrin α5 directly regulated by miR-92a in ovarian cancer cell lines.

miR-92a Inhibits Adhesion, Invasion, and Proliferation of Ovarian Cancer Cells

Because integrin α5 has been shown to play a pivotal role in ovarian cancer progression by several groups, including ours,13,15 we enforced miR-92a expression in ovarian cancer cells and evaluated the effects on cell adhesion, invasion, and proliferation. Transfection of precursor miR-92a significantly inhibited the adhesion onto fibronectin of three ovarian cancer cell lines by 44% to 82% (SKOV3ip1, 44%; A2780, 82%; OVISE, 55%) (Figure 5A), whereas miR-92a transduction did not affect the adhesion onto collagen type 1 (Figure 5B). Given that integrin α5 predominantly binds to the RGD motif of fibronectin and much less efficiently to that of collagen,32 it is likely that the inhibitory effect of miR-92a on cell adhesion is primarily mediated via integrin α5 expression. Because adhesion is followed by invasion, we then determined whether miR-92a overexpression also affects the invasive activities of cancer cells. Enforced expression of miR-92a significantly impaired the invasion of SKOV3ip1 and A2780 cells (SKOV3ip1, 77%; A2780, 87%) (Figure 5C). Cell proliferation was assessed by culturing cancer cells onto two representative ECMs, fibronectin and collagen type 1. Enforced expression of miR-92a only inhibited the proliferation of SKOV3ip1 and OVISE cells when they were plated onto fibronectin (Figure 5, D and E). Remodeling cell-ECM contacts via integrins generates a cascade of phosphorylation of signal transduction molecules at focal adhesions. Tyrosine phosphorylation of FAK is known to be especially critical in integrin-mediated outside-in signal transduction pathways, which regulate gene expression or cell behavior, such as cell spreading and migration.33,34 Enforced expression of miR-92a significantly inhibited integrin α5 expression and the tyrosine phosphorylation of FAK, followed by the down-regulation of MMP-2, which is known to play a major role in ovarian cancer invasion (Figure 5, F and G).

miR-92a Inhibits Peritoneal Metastasis in an Ovarian Cancer Xenograft Model

Given that enforced expression of miR-92a inhibited the adhesion, invasion, and proliferation of ovarian cancer cells by inhibiting integrin α5 expression, we examined the therapeutic potential of enforced expression of miR-92a in an ovarian cancer xenograft model. Lentiviruses containing the precursor of miR-92a (Figure 6A) or scrambled miRNA (control miRNA) were obtained and stably transduced into HeyA-8 cells. High and stable transduction efficiency (>90%) was confirmed by the red fluorescent puromycin-N-acetyltransferase from the Lenti-miRNA-vector (Biosetia) (Figure 6B). The enforced expression of miR-92a (Figure 6C) and the subsequent down-regulation of integrin α5 expression (Figure 6D) were confirmed. In vitro cell proliferation was assessed by culturing transduced HeyA-8 cells onto fibronectin and collagen type 1. Enforced expression of miR-92a inhibited the cell proliferation of HeyA-8 cells when plated onto fibronectin (Figure 6E). Two weeks after the i.p. inoculation of HeyA-8 cells, mice showed multiple tumors on the peritoneal surface, omentum, small-bowel mesentery, and both ovaries. Integrin α5 expression and the tyrosine phosphorylation of FAK (Tyr 861) were inhibited in the tumor nodules of the mice inoculated with cells overexpressing miR-92a compared with those inoculated with control (Figure 6F). Western blot analysis of the lysates from the inoculated nodules revealed that a 65% reduction of integrin α5 expression was induced by miR-92a overexpression (Figure 6G). The number of metastases and the tumor burden were significantly inhibited in mice inoculated with cells that overexpressed miR92a compared with controls (number of metastases: miR-92a, 54.0 ± 23.0; control, 1885.

Figure 5 Overexpression of miR-92a causes significant inhibition of adhesion, invasion, and proliferation of ovarian cancer cell lines. A: In vitro adhesion assay. Ovarian cancer cells that were transfected with either pre-miR-92a or scrambled miRNA (control) were plated onto 50 µg/mL fibronectin-coated 96-well plates. One hour after the incubation, plates were washed and stained with Giemsa solution. The number of adhesive cells was quantified by adding 100 µL of 0.2% Triton X-100 to lyse cells and measuring the absorbance at 560 nm. Representative images are shown (bottom panel). B: miR-92a does not inhibit the adhesion of ovarian cancer cells onto collagen. OVISE cells transfected with either pre-miR-92a or scrambled miRNA (control) were plated onto 50 µg/mL collagen type 1—coated, 96-well plates. One hour after the incubation, plates were washed and stained. The number of adherent cells was quantified as previously mentioned. Representative images are shown (bottom panel). C: Matrigel invasion assay. Ovarian cancer cells transfected with either pre-miR-92a or scrambled miRNA (control) were plated in a 25-µg Matrigel-coated Boyden chamber. At 48 hours after the incubation, noninvasive cells were removed using a cotton swab, and invading cells on the underside of the filter were enumerated. Representative images are shown (bottom panel). D: In vitro proliferation assay. SKOV3ip1 cells that were transfected with either pre-miR-92a or scrambled miRNA (control) were plated onto 50 µg/mL fibronectin (left panel) or 50 µg/mL collagen type 1—coated, 96-well plates and cultured in DMEM containing 2% FBS. Cell proliferation was measured using a fluorescent dye that incorporates into nucleic acids. E: In vitro proliferation assay. OVISE cells that were transfected with either pre-miR-92a or scrambled miRNA (control) were plated onto 50 µg/mL fibronectin (left panel) or 50 µg/mL collagen type 1—coated, 96-well plates and cultured in DMEM containing 2% FBS. Cell proliferation was measured as previously described. F: Western blot analysis. At 48 hours after the transfection of pre-miR-92a or scrambled miRNA (control), the cell lysates from OVISE cells were collected and resolved by SDS-PAGE and immunoblotted with antibodies against integrin α5, phospho-FAK, total-FAK, and MMP-2. β-Actin was used as a loading control. Blots are representative of three experiments. G: The relative expression levels of integrin α5, FAK phosphorylation, and MMP-2 in OVISE cells transfected with pre-miR-92a or scrambled miRNA (control), β-Actin was used as a loading control for integrin α5 and MMP-2, and total FAK was used for FAK phosphorylation. The relative density of each band was densitometrically quantified. Data are means ± SD of three experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.
126.0 ± 20.4; tumor weight: miR-92a, 0.46 ± 0.21 g; control, 1.42 ± 0.31 g) (P < 0.001) (Figure 6H). The inhibition rate induced by enforced expression of miR-92a tended to be higher in tumor weight (68.4%) than in metastatic nodule number (57.1%). The tumor nodules generated by miR-92a-overexpressing cells also did not grow as large as those generated by control cells, suggesting that miR-92a might have an antiproliferative effect in the in vivo xenograft model. Indeed, staining of i.p. tumor nodules with the proliferation marker Ki-67 showed significant differences between miR-92a overexpression and control tumors (7.8% versus 4.1% Ki-67-positive cells; P < 0.001) (Figure 6I).

These in vivo data mirror the inhibitory effect of miR-92a on the proliferation of ovarian cancer cells in the in vitro study shown in Figure 6E.

**Discussion**

Increasing evidence has led to the view that miRNAs play important roles in human cancer and that they are promising therapeutic targets. The expression of miRNAs is remarkably deregulated in ovarian cancer, strongly suggesting that they are involved in the initiation and progression of this disease. In this study, we identified miR-92a as...
a potential target, because it causes the inhibition of integrin α5, which plays a critical role in ovarian cancer dissemination.

By using the TMA slides of Japanese patients with ovarian cancer, all of whom were Asian, we identified integrin α5 as a prognostic marker of aggressive ovarian cancer. Of 114 patients, 35 (30%) showed high α5 expression, regardless of clinical stage. In the previous report from the University of Chicago, 10 (9%) of 107 stage II to IV ovarian cancer tissues had α5-integrin overexpression and 45 (39%) had some level of α5-integrin expression. Herein, each sample was scored based on the percentage of positive cells (0, 0% to 10%; 1, 10% to 25%; 2, 25% to 50%; 3, 50% to 100%) and the intensity of the staining (0, none; 1, weak; 2, strong). Only the samples that had strong staining of integrin α5 (intensity, 2) in 50% of tumor cells (score, 3) were considered to be tumors overexpressing integrin α5. Although it is difficult to directly compare our present work with that previously reported, we found that only a score of 6 in our data meets the criteria set for tumors overexpressing integrin α5, as previously reported. This would be one of the reasons that our clinical data appear to show more cases with high integrin α5 expression.

Analysis of the clinical data revealed that elevated expression of integrin α5 is linked to significantly reduced survival in patients with stage III advanced ovarian cancer, whose tumors had spread throughout the peritoneal cavity. This strongly indicates that integrin α5 plays a pivotal role in ovarian cancer dissemination, as is suggested by several previous in vitro studies. Among the ovarian cancer subtypes, approximately 75% (13 of 17) of CCCs showed high integrin α5 expression. However, because ovarian CCC tends to present at earlier stages because the tumors often form a large pelvic mass, most cases (14 of 17) in our clinical samples were diagnosed as stage I or II and only three cases were found in the advanced stage IIIc. The prognoses of the patients in the early stages were generally good, and we could not find any significant differences in either OAS or DFS between the high and low integrin α5 expression groups. Further analysis with a larger population would be required to conclude whether high integrin α5 expression is an independent prognostic factor for patients with ovarian CCC.

Recognition of the need for cytoreduction, the evolution of surgical techniques, and the establishment of chemotherapy regimens allows most patients with ovarian cancer, even in advanced stages, to achieve temporary disease-free status after initial treatment. However, one of the major disappointments with current ovarian cancer treatments is failure to achieve a complete cure, even in optimally debulked or chemosensitive patients. Therefore, the development of new efficacious therapies to prevent ovarian cancer cells from disseminating after the initial treatment could greatly improve the dismal prognosis for ovarian cancer. In that sense, the inhibitors against integrin α5 obviously have the potential to be developed for clinical use. Targeting integrin α5 can affect diverse functions of tumor cells, including adhesion, migration, invasion, proliferation, and survival, and could affect the tumor microenvironment, especially the angiogenic endothelial cells. A chimeric monoclonal antibody directed against integrin α5β1 (velociximab) and a non–RGD-based pentapeptide binding to integrin α5β1 and zVFP3 were recently developed and tested in preclinical phase I or 2 trials with patients with recurrent ovarian cancer. However, they did not show sufficient efficacy to warrant further clinical investigation, at least as monotherapies. Therefore, alternative or more powerful approaches to the inhibition of this integrin need to be explored.

The inverse relationship of miR-92a and integrin α5 expression is shown in Figure 2. Although we intended to identify a similar inverse correlation in human ovarian cancer clinical samples to confirm the relevance of our in vitro findings, the Institutional Review Board of our institute did not allow us to perform the genomic analyses. Instead, we compared the expression levels of miR-92a and ITGA5 in NCI60 cell lines and showed the significant inverse relationship of these two genes, suggesting that miR-92a regulates integrin α5 expression in various types of cells. Indeed, in mouse models of limb ischemia and myocardial infarction, miR-92a has been reported to repress expression of integrin α5 and, thus, block the potential angiogenesis of endothelial cells. miR-92a belongs to the miR-17-92 cluster and is located on chromosome 13q32-33. This conserved miR-17–92 cluster consists of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a Inhibits Peritoneal Dissemination

Figure 6 miR-92a inhibits peritoneal metastasis in an ovarian cancer xenograft model. A: Diagram of a lentivirus construct expressing two hairpin structures of pre–miR-92a and a puromycin-resistance gene (rPuro). B: HeyA-8 cells were infected with miR-92a lentivirus (miR-92a) or control lentivirus (control) and positively selected by continuous exposure to 5 μg/mL puromycin. Stably transfected HeyA-8 cells were examined by phase-contrast microscopy (left panel) and fluorescent microscopy (right panel). C: miRNA RT-PCR. HeyA-8 cells were infected with miR-92a lentivirus or control lentivirus and positively selected by continuous exposure to 5 μg/mL puromycin. Stably transfected HeyA-8 cells were collected, and the total RNA was extracted. Relative expression of miR-92a with respect to RNU6B was measured using TaqMan miRNA RT-PCR. D: miR-92a overexpression inhibits integrin α5 expression of HeyA-8 cells (Western blot analysis). The cell lysates were collected and resolved by SDS-PAGE and immunoblotted with an antibody against integrin α5. β-Actin was used as a loading control. E: In vitro proliferation assay. HeyA-8 cells transduced with miR-92a lentivirus or control lentivirus were plated onto 50 μg/mL fibronectin (left panel) or 50 μg/mL collagen type I–coated, 96-well plates and cultured in DMEM containing 2% FBS. Cell proliferation was measured using a fluorescent dye that incorporates into nucleic acids. F: Representative tumor areas were stained with H&E, antibodies against integrin α5, phospho-FAK (Y498), and the proliferation marker Ki-67. A chimeric monoclonal antibody directed against integrin α5β1 (velociximab) and a non–RGD-based pentapeptide binding to integrin α5β1 and zVFP3 were recently developed and tested in preclinical phase I or 2 trials with patients with recurrent ovarian cancer.
and miR-92a. Various studies have reported contrasting roles in tumorigenesis for these miRNAs as a cluster and as individual miRNAs. miR-92a is the least characterized member of the cluster. Initially, miR-92a was reported to be frequently amplified in B-cell lymphoma. Reports that it was amplified in colorectal cancer, hepatocellular carcinoma, and esophageal cancer followed. In contrast, in the genomewide miRNA DNA copy number analysis using 227 cancer samples, the miR17-92a cluster was deleted in 21.9% of analyzed breast cancer samples. Recently, Nilsson et al proved that miR-92a expression was inversely correlated to recurrence-free survival of patients with breast cancer and the down-regulation of miR-92a was associated with increased tumor macrophage infiltration, indicating that miRNA alternations differ depending on cancer type. The role of miR-92a in ovarian cancer has not been clarified thus far. In the public microarray gene expression data offered by the European Bioinformatics Institute, E-TABM-343 investigated the expression pattern of miR-92a in ovarian cancer samples. In that study, miRNA profiling was compared between 15 normal ovarian tissue sections and 69 ovarian epithelial carcinomas, and miR-92a expression was significantly down-regulated in ovarian malignant tissues, suggesting that miR-92a down-regulation might be involved in the tumorigenesis or progression of ovarian cancer. Given that the expression pattern of miRNA is complex and seems to be cell or tissue specific and to vary in different malignancies, it is important to carefully clarify the functional role of each miRNA in each tumor type separately. Our current data suggested that the restoration of miR-92a function may serve as a potent suppressor of peritoneal dissemination of ovarian cancer cells by inhibiting integrin a5 expression.

miRNAs as tumor suppressors represent a major opportunity for the future treatment of patients with cancer. Although it is technically easy to synthesize these naturally occurring oligonucleotides, the lack of effective and nontoxic delivery technologies remains a major obstacle to clinical application. However, rapid advances in systemic drug delivery systems have led to the emergence of successful miRNA-based therapeutics. Wiggins et al demonstrated that a therapeutic formulation using chemically synthesized miR-34a with a lipid-based delivery system blocked tumor growth in mouse models of non—small-cell lung cancer without inducing an immune response. A locked nucleic acid—modified anti—miR-122 (Miravirsen; Santaris Pharma, San Diego) effectively treated chimpanzees infected with hepatitis C virus without any observable resistance or physiological adverse effects. These approaches may facilitate a rapid route for miRNA replacement therapy in the clinical setting in the near future.

Collectively, in the present study, we identified miR-92a as a suppressor of ovarian cancer peritoneal dissemination through the inhibition of integrin a5 expression. Transfection of precursor miR-92a reduced integrin a5 expression in ovarian cancer cells, accompanied by the inhibition of cell adhesion, invasion, and proliferation. In vivo ovarian cancer xenografts, lentiviral transfection of miR-92a into HeyA-8 ovarian cancer cells injected i.p. reduced peritoneal metastases. Although there is still a long way to go before the advent of an effective and nontoxic miRNA-based cancer therapy, targeting miR-92a generates new possibilities for ovarian cancer treatment through the inhibition of integrin a5 expression. We believe that it needs to be explored further for future clinical application.

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Supplemental Data

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