Expression of the miR200 Family of microRNAs in Mesothelial Cells Suppresses the Dissemination of Ovarian Cancer Cells

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Abstract

The TGFβ-mediated alteration of the tumor microenvironment plays a crucial role in tumor progression. Mesothelial cells are the primary components of the tumor microenvironment for ovarian cancer cells; however, the exact role of TGFβ-stimulated mesothelial cells in ovarian cancer progression remains uncertain. In this report, we examined the effects of TGFβ-treated mesothelial cells on ovarian cancer progression. We show that TGFβ-stimulated human primary mesothelial cells (HPMC) are able to promote cancer cell attachment and proliferation and the activation of the promoter activities of MMP-2 and MMP-9, which are metalloproteinases necessary for tumor invasion. Expression of the miR200 family was downregulated in HPMCs by TGFβ stimulation, and restoration of the expression of miR200 family members in HPMCs suppressed cancer cell attachment and proliferation. Downregulation of the miR200 family by TGFβ induced fibronectin 1 production, which promoted cancer cell attachment to HPMCs. Finally, we demonstrated that the delivery of the miR200s to mesothelial cells in mice inhibited ovarian cancer cell implantation and dissemination. Our results suggest that alteration of the tumor microenvironment by the miR200 family could be a novel therapeutic strategy for ovarian cancer treatment. Mol Cancer Ther; 13(8); 2081–91. © 2014 AACR.

Introduction

Ovarian cancer has the highest mortality rate of all gynecologic tumors. More than 70% of patients with ovarian cancer are diagnosed at the advanced stage after the cancer cells have already disseminated to a regional or distant area. Despite the recent progress in cytotoxic therapy for numerous types of cancer, most patients with ovarian cancer succumb to the disease within 5 years (1, 2). Therefore, greater understanding of the mechanisms involved in ovarian cancer progression is needed to develop new therapeutic agents.

One of the prominent characteristics of ovarian cancer is the rapid dissemination of cancer cells within the abdominal cavity (3). A crucial step in the dissemination of ovarian cancer cells is the adhesion and implantation of tumor cells onto mesothelial cells, and both steps are mediated in part by integrins and CD44, a cell surface receptor for hyaluronic acid (4–6). Mesothelial cells are the primary components of the microenvironment of ovarian cancer cells. A healthy, intact mesothelial cell layer is known to inhibit the attachment and invasion of cancer cells (7, 8); however, once the integrity of the mesothelial cells is disrupted by tumor cells or oxidative stress, the mesothelial cells acquire the ability to promote cancer cell invasion, metastasis, and drug resistance (9, 10). Transforming growth factor β (TGFβ) is one of the crucial factors that alter the architecture of the tumor microenvironment to allow tumor progression (11, 12). Stimulation of mesothelial cells by TGFβ induces epithelial-to-mesenchymal transition (EMT; refs. 13 and 14), which is a morphological conversion of epithelial cells (15). Although TGFβ-stimulated mesothelial cells have been reported to have tumor-promoting functions (16, 17), further detailed analysis is needed to determine the exact functions of activated mesothelial cells in ovarian cancer progression.

MicroRNAs (miRNA) are 18- to 25-nucleotide noncoding RNAs that regulate gene expression through either translational repression or the degradation of target mRNAs (18). A number of miRNAs that are associated with tumor suppression or tumor progression have been identified (19). The miR200 family of miRNAs is well known to play a pivotal role in the regulation of EMT during cancer progression and metastasis (20–22). Although the increase of miR200 expression is reported in several types of tumors (23–25), the downregulation of miR200 expression is correlated with the mesenchymal and drug-resistant phenotypes of cancer cells (26). The expression of the miR200s has been shown to inhibit cancer progression, promote apoptosis, and increase sensitivities to different types of drugs (27). These results...
have indicated that the miR200 family comprises tumor-suppressive miRNAs and raises potential interest in their use as a tool to treat cancer.

In this study, we examined the effects of the TGFβ treatment of mesothelial cells on ovarian cancer cells. We show here that the downregulation of the miR200 family in mesothelial cells by TGFβ is critical for cancer cell attachment and proliferation as well as activation of the MMP-2 and MMP-9 promoters. We also show that administration of the miR200s to mesothelial cells in mice suppresses the attachment and implantation of ovarian cancer cells. Our results define the novel interaction of mesothelial cells and ovarian cancer cells in the presence of TGFβ for the promotion of cancer progression.

Materials and Methods

The sequences of the primers and siRNAs used for the experiments are listed in Supplementary Table S1.

Cells

The ES-2, SKOV3, A2780, OVCAR3, and Caov3 cell lines were maintained in RPMI-1640 medium with 10% FBS and penicillin/streptomycin. These cell lines were obtained from the American Type Culture Collection in 2012 to 2013. Human primary mesothelial cells (HPMC) were isolated from the omentum of patients undergoing surgery. HPMCs were cultured in medium composed of DMEM and Hams F12 (1:1) with 10% FBS and 20 ng/mL of EGF (Peprotech). To generate stable cell lines that expressed GFP or luciferase, a recombinant retrovirus was used. 293T cells were transfected with the pQCXIP vector encoding each gene as well as the pVPack-GP and pVPack-Ampho vectors (Stratagene). The culture supernatant was collected 48 hours later and applied to cells with 2 µg/mL polybrene (Sigma). Cells were cultured for 24 hours, and then 1 µg/mL puromycin (Sigma) was added to select for infected cells.

Transfection

Cells were transfected with 50 nmol/L siRNA or 20 nmol/L mirVana miRNA mimics (hsa-miR200a: MC10991, hsa-miR200b:MH10492, hsa-miR429:MC10221; Ambion) using 0.25% HiPerFect (Qiagen Venlo) according to the manufacturer’s protocol.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. To determine levels of miRNA, mature miRNAs were reverse transcribed and subjected to real-time PCR analysis using TaqMan miRNA assays (Applied Biosystems). miRNA expression was normalized to U6 snRNA expression. For mRNA analysis, cDNA was produced from 1 µg of total RNA with random primers using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR reactions were performed using SYBR Premix Ex Taq II (TAKARA), and an Mx3005P machine (Agilent Technologies) was used for analysis. Expression levels were normalized to GAPDH expression.

Immunofluorescence

HPMCs were seeded onto type I collagen-coated glass coverslips and placed in 12-well plates. Cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, and blocked with PBS containing 10% FBS for 1 hour at room temperature. Cells were incubated with anti-FN1 antibody (Santa Cruz) for 1 hour, followed by incubation with an Alexa Fluor 488-labeled secondary antibody for 1 hour. Cells were stained with rhodamine-conjugated phalloidin (Invitrogen) and Hoechst and were observed using a fluorescence microscope (BX60; Olympus).

Adhesion assay and cell growth assay

Confluent monolayers of HPMCs were incubated with or without TGFβ (10 ng/mL) for 2 days, and cancer cells that constitutively expressed luciferase were then seeded. The cells were incubated for 20 minutes, washed 3 times with PBS and then subjected to a luciferase assay. To evaluate cell growth, cancer cells were seeded onto monolayers of HPMCs, and luciferase activities were measured 24, 48, and 72 hours later.

5-Ethynyl-2′-deoxyuridine incorporation assay

The thymidine nucleotide analog 5-ethyl-2′-deoxyuridine (EdUrd) was used for the in vitro labeling of the nuclei of dividing cancer cells on HPMCs. Cancer cells that constitutively expressed GFP were seeded onto confluent monolayers of HPMCs, and 24 hours later, EdUrd incorporation assays were performed. Briefly, half of the media was replaced with fresh media containing 20 µmol/L EdUrd and incubated for another 24 hours. Cells were fixed with formaldehyde, permeabilized with 0.5% Triton X-100, and stained with a reaction cocktail prepared according to the manufacturer’s instructions and Hoechst. Cells were imaged by fluorescence microscopy, and the percentage of EdUrd-positive cells among all GFP-expressing cells was evaluated.

MMP promoter assay

The previously reported promoter regions of MMP-2 (−683/+67) and MMP-9 (−710/0) were cloned into the pGL4 vector. Ovarian cancer cells were transfected with pGL4-MMP-2/promoter or pGL4-MMP-9/promoter together with pRLTK-luc to normalize transfection efficiency. Cells were harvested 24 hours later and then seeded onto monolayers of HPMCs that had been incubated with or without TGFβ for 48 hours. The cells were incubated for additional 24 hours, and the activities of Firefly luciferase and Renilla luciferase were measured using the Dual Luciferase Reporter Assay System (Promega).

Generation of recombinant adenovirus

The genomic regions of the pri-miRNA sequences of the miR200b/a/429 cluster were amplified by PCR and
cloned into the pacAd5 shuttle vector (Ad-miR200b/a/429). 293AD cells were transfected with linearized Ad-miR200b/a/429 vector and linearized pacAd5 9.2-100 Ad backbone plasmid (CELL BIOLABS), and recombinant virus encoding miR200s was generated (rAd-miR200). Control adenovirus was produced by transfecting an empty pacAd5 shuttle vector into 293AD cells (rAd-Ctrl). Recombinant adenovirus was amplified according to the manufacturer’s protocol and purified using AdEasy Virus Purification Kits (Agilent Technologies).

3’UTR luciferase reporter assay

The 3’UTR of FN1 was PCR amplified and cloned downstream of the Firefly luciferase-coding region of the pmirGLO vector (Promega). Cells were transfected with the vector together with miR200s or control RNA. To determine whether rAd-miR200 produced functional miR200s, recombinant adenovirus encoding luciferase fused to the target sequence of miR200b or miR429 was produced. HPMCs were infected with rAd-miR200 together with recombinant adenovirus encoding luciferase, and 48 hours later, cell were lysed to measure luciferase activity.

In vivo study

All procedures involving mice and experimental protocols were approved by the Animal Experimental Committee of the Graduate School of Medicine, Nagoya University, Nagoya, Japan. Six-week-old female BALB/c nude mice (Japan SLC, Nagoya, Japan) were intraperitoneally injected with Ad-Ctrl (1 × 10^9 pfu per 4 mL) or Ad-miR200 (1 × 10^9 pfu per 4 mL). After 2 days, 1 × 10^6
luciferase-expressing ES-2 cells were injected into the peritoneal cavity of mice. Anesthetized mice were retro-orbitally injected with 75 mg/kg p-luciferin to acquire images using a Xenogen IVIS 200 Imaging System (Caliper Life Sciences). Analysis was performed with Living Image software (Caliper Life Science) by measuring the photon flux of the isolated tissues and the abdominal area of mice in a supine position.

**Statistical analysis**

All data are expressed as the mean ± SD. Data were calculated from at least three independent experiments. The statistical significance of differences was analyzed by Student t test. A value of *P* < 0.05 was considered to be statistically significant. Kaplan–Meier metastasis-free survival curves were generated using GraphPad Prism 5 software (GraphPad Software).

**Results**

**TGFβ-treated HPMCs promote cell attachment and proliferation**

To determine the role of TGFβ-treated HPMCs in ovarian cancer progression, we first examined the attachment of cancer cells to HPMCs. Cancer cell adhesion to HPMCs is a critical step for the dissemination of ovarian cancer. A confluent monolayer of HPMCs was incubated with or without TGFβ (10 ng/mL) for 48 hours, and suspended ovarian cancer cells that constitutively expressed luciferase were then seeded onto the HPMCs. Twenty minutes later, the cells were washed with PBS 3 times to remove any unattached cells. The cells were lysed, and luciferase activity was measured to evaluate the attached cells. All the ovarian cancer cell lines used were more adhesive to TGFβ-treated HPMCs than untreated HPMCs (Fig. 1A).

We next examined the proliferation of cells cultured on HPMCs incubated with or without TGFβ. ES-2 and SKOV3 cells that constitutively expressed luciferase were cultured on HPMC monolayers, and the cells were lysed to measure luciferase activity to determine the proliferation rate. TGFβ-treated HPMCs were more effective than untreated HPMCs in stimulating the growth of ES-2 cells (Fig. 1B). Although SKOV3 cells did not grow on the untreated HPMCs, they were able to proliferate on the TGFβ-treated HPMCs (Fig. 1B). The addition of an inhibitor of TGFβ receptor-mediated signaling, SB431542, to the HPMCs suppressed the proliferation of both cancer cell lines on the TGFβ-treated HPMCs (Fig. 1B).

Degradation of the extracellular matrix by proteinases such as MMP-2 and MMP-9 is a critical step in cancer cell invasion. To determine the effect of the TGFβ treatment of HPMCs on cancer cell invasion, we examined the promoter activities of MMP-2 and MMP-9. ES-2 and SKOV3 cells were transfected with plasmids that expressed luciferase regulated by either the MMP-2 or MMP-9 promoter (pGL4-MMP-2/promoter or pGL4-MMP-9/promoter). After 24 hours, both cell lines were seeded onto monolayers of HPMCs treated or untreated with TGFβ, and luciferase activity was measured. The TGFβ treatment of HPMCs increased the transcriptional activities of both the MMP-2 and MMP-9 promoters when compared with untreated HPMCs (Fig. 1C). These results show that TGFβ-treated HPMCs can promote ovarian cancer progression.

**miR200 expression suppresses the effects of TGFβ-treated HPMCs on cancer cell proliferation**

TGFβ stimulation induces dynamic changes in the cellular gene expression profile. Previous studies have shown that the expression of miR200 family members is downregulated by TGFβ stimulation (28). We tested whether the suppression of miR200s is associated with the promotion of cancer cell proliferation by TGFβ-treated HPMCs. The miR200 family is composed of 2 polycistronic clusters, miR200c and miR141 on chromosome 12 and miR200a, miR200b and miR429 on chromosome 1. The TGFβ stimulation of HPMCs significantly suppressed the levels of miR200a, miR200b, and miR429, which are clustered on chromosome 1 (Fig. 2A). Although miR200c was suppressed to approximately 50%, the reduction in miR141 expression was minor. We next examined the effects of miR200b expression in HPMCs on cancer cell adhesion, proliferation and invasion. HPMCs were transfected with miR200b or control RNA, and 24 hours later, the cells were incubated with or without TGFβ for 48 hours. SKOV3 and ES-2 cells that constitutively expressed luciferase were seeded onto monolayers of pretreated HPMCs, and unattached cells were removed by washing with PBS 20 minutes later. The cells were lysed, and luciferase activity was measured to evaluate the cells attached to the HPMCs. As shown in Fig. 2B, the introduction of miR200b to HPMCs significantly reduced the attachment of both ES-2 and SKOV3 cells to the TGFβ-treated HPMCs. We tested whether miR200b expression in HPMCs affects cancer cell growth. Luciferase-expressing ES-2 and SKOV3 cells were cultured on HPMCs with...
different pretreatments, and luciferase activity was measured. The expression of miR200b suppressed the ability of the TGFβ-treated HPMCs to promote cancer cell proliferation (Fig. 2C). We also performed an EdUrd-incorporation assay to evaluate cancer cell proliferation. GFP-expressing ES-2 or SKOV3 cells grown on pretreated HPMCs were cultured with EdUrd, and 24 hours later, EdUrd-positive cells were counted. Nearly 70% to 80% of GFP-expressing cancer cells stained positively for EdUrd when cultured on TGFβ-treated HPMCs, whereas the ratio of positive cells was reduced to 30% to 40% when the TGFβ-treated HPMCs had been transfected with miR200b RNA (Fig. 2D). Finally, we examined the effect of miR200b expression on the stimulation of MMP-2 and MMP-9 promoter activities. As shown in Fig. 2E, miR200b expression in HPMCs suppressed the activation of the MMP-2 and MMP-9 promoters by TGFβ-treated HPMCs.

Production of fibronectin 1 by TGFβ is mediated by the suppression of miR200 family expression

To obtain insight into the molecular mechanisms of how miR200 family expression in HPMCs suppresses cancer cell adhesion, we searched for target miRNAs of miR200b using TargetScan. Among the predicted targets, we focused on fibronectin 1 (FN1) because of its important functions in cell adhesion and proliferation. In addition, previous study reported that miR200b repressed FN1 expression in kidney proximal tubular cells (29). Sequence analysis shows that the 3'UTR of FN1 mRNA has 2 putative regions targeted by the miR200 family (Fig. 3A). To confirm that FN1 is a direct target of miR200b, we created a vector that contained the 3'UTR of FN1 cDNA fused to the C-terminus of luciferase (FN-luciferase). 293T cells were transfected with the FN-luciferase vector together with control, miR200a, miR200b or miR429 RNA, and luciferase activity was measured 24 hours later. The expression of either miR200b or miR429 significantly reduced luciferase activity (Fig. 3B). However, miR200a did not reduce luciferase activity. miR200a has cytosine instead of uracil in the target region as depicted in Fig. 3A, thus, miR200a may not target FN1 3'UTR. Neither miR200b nor miR429 suppressed luciferase activity if mutations were introduced in the first target region of the FN1 3'UTR (Fig. 3B). We next examined FN1 expression in the presence of exogenous miR200a, miR200b, or miR429. HPMCs were transfected with control or miRNAs, stimulated with TGFβ, and then immunostained for FN1. Although TGFβ stimulation significantly promoted the production of FN1, the expression of either miR200b or miR429 suppressed FN1 expression (Fig. 3C). Consistent with the result that miR200a did not target 3'UTR of FN1, FN1 expression was not reduced by miR200a (Fig. 3C). Immunoblot analysis was also performed to examine FN1 expression. As shown in Fig. 3D, FN1 expression was profoundly suppressed by the expression of miR200b or miR429, but miR200a did not reduce FN1 expression. ZEB1 is a crucial factor in the promotion of EMT by TGFβ stimulation and suppresses transcription of miR200 family members (21). We tested whether the induction of FN1 by TGFβ was dependent on the expression of ZEB1. However, silencing of ZEB1 did not suppress FN1 production by TGFβ stimulation (Fig. 3E and 3F). In addition, ZEB1 depletion did not affect expression of miR200 families induced by TGFβ stimulation (Fig. 3G), indicating that the reduction of miR200s by TGFβ is independent of ZEB1. These results show the critical role of miR200b and miR429 in the promotion of FN1 production following TGFβ stimulation.

Fibronectin 1 is essential for cancer cell attachment to TGFβ-stimulated HPMCs

We next examined the effects of FN1 expression in HPMCs on ovarian cancer cells. HPMCs were transfected with FN1 siRNAs, and 24 hours later, the cells were incubated with or without TGFβ for 48 hours. The reduction of FN1 production was confirmed by immunoblotting and immunofluorescence analysis (Fig. 4A). The attachment of ES-2 and SKOV3 cells to pretreated HPMCs was also evaluated. As shown in Fig. 4B, the attachment of both cell types to HPMCs was reduced by FN1 depletion. miR200a, which did not reduce FN1 expression, did not suppress the attachment of cancer cells to TGFβ-stimulated HPMCs (Fig. 4C). We also examined cancer cell proliferation and the activation of the MMP-2 and MMP-9 promoters in the absence of FN1 expression in HPMCs. However, the depletion of FN1 in HPMCs did not affect cancer cell proliferation or the promoter activities (Supplementary Fig. S1). These results indicate that FN1 expression is critical for cancer cell attachment to TGFβ-stimulated HPMCs.

Administration of the miR200s suppresses cancer cell implantation in mice

We next examined whether the exogenous expression of the miR200s in mesothelial cells can suppress cancer cell dissemination in mice. To express the miR200s, we created a recombinant adenovirus that contained the cluster of miR200b, miR200a, and miR429 (rAd-miR200) as well as a control virus (rAd-Ctrl). Infection of HPMCs with rAd-miR200 significantly increased the expression of the miR200 family (Fig. 5A). To further confirm that the virus encoded functional miRNAs, we created a recombinant adenovirus that had the miR200b target sequence, the miR429 target sequence or 3'UTR of FN1 fused to luciferase. Each of these adenoviruses was infected to HPMCs together with rAd-miR200 and luciferase activity was measured. As shown in Fig. 5B, luciferase activity was significantly reduced by rAd-miR200 infection. In addition, the expression of endogenous FN1 in HPMCs was suppressed by rAd-miR200 infection (Fig. 5C). These results indicate that rAd-miR200 produces functional miRNAs.

We next tested whether the introduction of the miR200s into mesothelial cells in mice can inhibit cancer cell dissemination in the peritoneal cavity. We first evaluated the infection efficiency of the recombinant adenovirus in
Figure 3. FN1 expression is downregulated by miR200s. A, sequences of the target regions of the FN1 3′ UTR and members of the miR200 family. miR200a and miR141 have cytosine instead of uracil in the target region (the cytosine is underlined). Mutations introduced to the 3′ UTR of FN1 mRNA are indicated with asterisks. B, 293T cells were transfected with a plasmid encoding luciferase fused to the 3′ UTR of FN1 together with control or miR200 RNAs, and then luciferase activity was measured (**, P < 0.01). MUT1 has mutations in 245 to 251 and MUT2 in 530 to 536 of the 3′ UTR of FN1. C, HPMCs were transfected with control, miR200a, miR200b, or miR429 RNA, and, 24 hours later, the cells were treated with TGFβ for an additional 48 hours. The cells were fixed and immunostained with anti-FN1 antibody and phalloidin. D, HPMCs were transfected with the indicated miRNAs, and, 24 hours later, the cells were treated with TGFβ for an additional 48 hours. The expression of FN1 was examined by immunoblotting. E, HPMCs were transfected with the indicated siRNAs, and, 24 hours later, the cells were treated with TGFβ for additional 48 hours. The expression of FN1 was examined by immunoblotting. F, HPMCs were transfected with the indicated siRNAs, and, 24 hours later, the cells were treated with TGFβ for additional 48 hours. The expression of FN1 was determined by RT-PCR (**, P < 0.01; n.s., not significant). G, HPMCs were transfected with the indicated siRNAs, and, 24 hours later, the cells were treated with TGFβ for additional 48 hours. The expression of FN1 was determined by RT-PCR.
mesothelial cells following intraperitoneal injection. Different amounts of recombinant adenovirus were injected into the peritoneum, and 2 days later, the mice were sacrificed and stained with X-gal. We found that injection of 4 mL of 1 × 10^9 pfu of recombinant adenovirus was quite sufficient for the infection of mesothelial cells in the peritoneal cavity (Supplementary Fig. S2). Therefore, mice were intraperitoneally injected with 4 mL of 1 × 10^9 pfu of recombinant virus to transduce miR200s to mesothelial cells, and 2 days later, ES-2 cells were injected into the mouse peritoneum. Three days later, the implantation of ES-2 cells in the peritoneum, omentum, and organs was evaluated. Expression of the miR200 family in mesothelial cells significantly suppressed cancer cell implantation in the omentum and mesenterium (Fig. 5D and E). FN1 production was also suppressed by rAd-miR200 infection (Supplementary Fig. S3). These results indicate that the administration of miR200 family members to mesothelial cells can suppress cancer cell dissemination in the peritoneal cavity. We also examined the survival of cancer cell-injected mice. As shown in Fig. 5F, mice preinjected with rAd-miR200 showed significantly longer survival than rAd-Ctrl–injected mice.

Discussion

Accumulating evidence indicates that TGFβ is associated with ovarian cancer progression. Clinical studies have provided evidence that TGFβ is overexpressed in malignant ovarian tumors and elevated in the peritoneal fluid of patients with ovarian cancer (30, 31). TGFβ promotes ovarian cancer cell invasion by inducing MMP secretion and EMT (32, 33). In addition to the direct effect of TGFβ on cancer cells, recent studies have indicated that the TGFβ-mediated progression of ovarian cancer is partly mediated by nonmalignant cells in the tumor microenvironment. One of the critical nonmalignant cells that control TGFβ-dependent ovarian cancer progression is the cancer-associated fibroblast (CAF; ref. 34). TGFβ-stimulated CAFs secrete factors such as VCAN and promote tumor invasion and angiogenesis (35). In this study, we presented evidence indicating that TGFβ-stimulated mesothelial cells have the potential to promote cancer cell proliferation and invasion. The concentration of TGFβ we used (10 ng/ml) is higher than that of TGFβ in the ascites of patients with ovarian cancer (31). However, the concentration of TGFβ at the microenvironment of the tumor...
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Administration of miR200s to mesothelial cells inhibits cancer cell dissemination in mice. A, HPMCs were infected with rAd-Ctrl or rAd-miR200, and then the expression levels of miR200a, miR200b, and miR429 were determined by RT-PCR (**, P < 0.01). B, HPMCs were infected with rAd-miR200 together with a recombinant adenovirus encoding luciferase fused to the target sequence of miR200b, miR429, or FN1-3 UTR. Forty-eight hours later, the cells were lysed, and luciferase assay was measured. The miR200b mutant and the miR429 mutant have mutations in the target sequence (**, P < 0.01). C, HPMCs were infected with rAd-Ctrl or rAd-miR200, and the expression of FN1 was examined by immunoblotting. D, rAd-Ctrl or rAd-miR200 was injected into the peritoneum of mice, and 2 days later, ES-2 cells that constitutively expressed luciferase were implanted. Three days after tumor injection, tumor implantation was imaged using a Xenogen IVIS 200. Mice were also sacrificed, and tumor dissemination to the omentum and mesenterium was examined. E, tumor dissemination to the omentum and mesenterium was evaluated by imaging was plotted and is presented (**, P < 0.01; n.s., not significant). F, survival curves of mice treated with rAd-Ctrl or rAd-miR200 and injected with ES-2 cells.

Figure 5. Administration of miR200s to mesothelial cells inhibits cancer cell dissemination in mice. A, HPMCs were infected with rAd-Ctrl or rAd-miR200, and then the expression levels of miR200a, miR200b, and miR429 were determined by RT-PCR (**, P < 0.01). B, HPMCs were infected with rAd-miR200 together with a recombinant adenovirus encoding luciferase fused to the target sequence of miR200b, miR429, or FN1-3 UTR. Forty-eight hours later, the cells were lysed, and luciferase assay was measured. The miR200b mutant and the miR429 mutant have mutations in the target sequence (**, P < 0.01). C, HPMCs were infected with rAd-Ctrl or rAd-miR200, and the expression of FN1 was examined by immunoblotting. D, rAd-Ctrl or rAd-miR200 was injected into the peritoneum of mice, and 2 days later, ES-2 cells that constitutively expressed luciferase were implanted. Three days after tumor injection, tumor implantation was imaged using a Xenogen IVIS 200. Mice were also sacrificed, and tumor dissemination to the omentum and mesenterium was examined. E, tumor dissemination to the omentum and mesenterium was evaluated by imaging was plotted and is presented (**, P < 0.01; n.s., not significant). F, survival curves of mice treated with rAd-Ctrl or rAd-miR200 and injected with ES-2 cells.

may become high enough to promote cancer cell adhesion to the mesothelial cells. Our results indicate that TGFβ can regulate ovarian tumor progression via multiple nonmalignant cells in the tumor microenvironment.

The tumor microenvironment, which is composed of stromal cells and extracellular matrix, is a major determinant of tumor progression. Accumulating evidence suggests that tumor progression mediated by TGFβ is dependent on alteration of the tumor microenvironment (12). TGFβ-stimulated nonmalignant cells produce numerous cytokines as well as extracellular matrix proteins to reconstruct the microenvironment for tumor progression. Recent studies have revealed that the regulation of miRNA expression in nonmalignant cells by TGFβ is
associated with reorganization of the tumor microenvironment and tumor progression (36). For example, miR94 is induced by TGFβ stimulation in myeloid-derived suppressor cells (MDSC), which regulate tumor angiogenesis and metastasis. The increase in miR94 expression in MDSCs consequently upregulates MMPs and facilitates tumor invasion and metastasis (37). We showed that downregulation of the miR200 family by TGFβ played a central role in cancer cell attachment and proliferation on TGFβ-stimulated HPMCs. Interestingly, TGFβ stimulation specifically reduced levels of miR200a, miR200b, and miR429, which were clustered on chromosome 1. Although further analysis is needed, the promoter of miR200 families on chromosome 1 may be more sensitive to TGFβ stimulation, or epigenetic modifications may result in the specific downregulation in HPMCs. Our results indicate that the miR200 family is critical for the TGFβ-mediated remodeling of the tumor microenvironment for ovarian cancer progression. To determine the effect of miR200-mediated remodeling of the tumor microenvironment on tumor progression, we delivered miR200s to mesothelial cells in mice. The expression of the miR200 family in mesothelial cells significantly suppressed ovarian cancer implantation and prolonged survival after tumor injection. This result suggests that modification of the tumor microenvironment by the miR200s can be used as a novel therapeutic strategy for ovarian cancer. A previous study has reported that the expression of miR200c in ovarian cancer cells can inhibit tumor growth in mice (38); thus, administration of the miR200s to both cancer cells and mesothelial cells may have synergistic effects on the inhibition of ovarian cancer progression.

Fibronectin 1 (FN1) activates various signaling pathways to promote the migration and invasion of cancer cells. Fragments of FN1 are abundant in the ascites of patients with ovarian cancer, indicating a robust production of FN1 by tumor cells or peritoneal tissues (39). FN1 can stimulate ovarian cancer cell motility and invasion by promoting the secretion of MMP-9 in a focal adhesion kinase (FAK)- and Ras-dependent manner (40). In addition, FN1 has been reported to protect ovarian cancer cells from apoptosis induced by therapeutic agents (41). We speculated that FN1 was critical for the progression of ovarian cancer cells on TGFβ-stimulated HPMCs. However, only cancer cell attachment was suppressed when FN1 expression in HPMCs was knocked down. The expression of the miR200 family in HPMCs clearly inhibited cancer cell proliferation and the activation of the MMP-2 and MMP-9 promoters. Thus, the other factors such as cytokines, growth factors, and extracellular matrix whose expression is regulated by the miR200 family are necessary for cancer cell proliferation and invasion in the presence of TGFβ-stimulated HPMCs.

In summary, we have shown that TGFβ downregulates the expression of the miR200 family in HPMCs, which subsequently promotes cancer cell attachment and proliferation. In addition, we showed that the administration of the miR200s to mesothelial cells could inhibit tumor implantation. Data are increasingly providing evidence indicating that the tumor microenvironment plays a crucial role in ovarian cancer invasion and metastasis. Elucidation of the molecular mechanisms underlying how mesothelial cells regulate tumor progression will be beneficial for the development of novel strategies for ovarian cancer treatment.

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

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Conception and design: K. Sugiyama, H. Kajiyama, K. Shihata
Development of methodology: K. Sugiyama, H. Kajiyama, H. Yuan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Sugiyama, H. Kajiyama
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