UICC
global cancer control

IJC
International Journal of Cancer

The activated transforming growth factor-beta signaling pathway in peritoneal metastases is a potential therapeutic target in ovarian cancer

Shogo Yamamura[1], Noriomi Matsumura[1], Masaki Mandai[1], Zhiqing Huang[2], Tomonori Oura[3], Tsukasa Baba[1], Junzo Hamanishi[1], Ken Yamaguchi[1,2], Hyun Sook Kang[1], Takako Okamoto[1], Kaoru Abiko[1], Seiichi Mori[4], Susan K. Murphy[2] and Ikuo Konishi[1]

[1] Department of Gynecology and Obstetrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[2] Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC
[3] Department of Biostatistics, Graduate School of Public Health, Kyoto University, Kyoto, Japan
[4] Genomic Oncology Programme, Cancer Science Institute Singapore, National University of Singapore, Singapore

Peritoneal dissemination including omental metastasis is the most frequent route of metastasis and an important prognostic factor in advanced ovarian cancer. We analyzed the publicly available microarray dataset (GSE2109) using binary regression and found that the transforming growth factor (TGF)-beta signaling pathway was activated in omental metastases as compared to primary sites of disease. Immunohistochemical analysis of TGF-beta receptor type 2 and phosphorylated SMAD2 indicated that both were upregulated in omental metastases as compared to primary disease sites. Treatment of the mouse ovarian cancer cell line HM-1 with recombinant TGF-β1 promoted invasiveness, cell motility and cell attachment while these were suppressed by treatment with A-83-01, an inhibitor of the TGF-β signaling pathway. Microarray analysis of HM-1 cells treated with TGF-β1 and/or A-83-01 revealed that A-83-01 efficiently inhibited transcriptional changes that are induced by TGF-β1. Using gene set enrichment analysis, we found that genes upregulated by TGF-β1 in HM-1 cells were also significantly upregulated in omental metastases compared to primary sites in the human ovarian cancer dataset, GSE2109 (false discovery rate (FDR) $q = 0.086$). Therapeutic effects of A-83-01 in a mouse model of peritoneal dissemination were examined. Intraperitoneal injection of A-83-01 (150 μg given three times weekly) significantly improved survival ($p = 0.015$). In summary, these results show that the activated TGF-β signaling pathway in peritoneal metastases is a potential therapeutic target in ovarian cancer.

Ovarian cancer is the most lethal gynecologic cancer in the Western world. Because of its absence of obvious symptoms, the diagnosis of ovarian cancer is often made when the disease is at an advanced stage, resulting in a survival rate of only 20–30%.[1] Peritoneal dissemination is the most frequent route of spread and is the most significant prognostic factor in ovarian cancer.[2] Although many efforts have been made to treat peritoneal dissemination of ovarian cancer, such as debulking surgery and systemic or intraperitoneal chemotherapy, effective eradication of peritoneal dissemination remains challenging. Therefore, there is an urgent need to develop new treatment modalities, especially targeted molecular therapies, through the study of basic biology underlying peritoneal disease spread.

Recently, the roles of the transforming growth factor (TGF)-beta signaling pathway in cancer have been studied in great depth, particularly in the progression of cancer and metastasis.[3,4] In the early phases of cancer development, TGF-β is thought to suppress the proliferation of tumor cells. In the advanced phases, however, TGF-β may promote cancer progression. Inhibition of TGF-β2 production by antisense oligonucleotides (AP12009) is a promising therapeutic strategy against glioma[5] and a phase III clinical trial is currently underway. However, little is known about the role of the TGF-β signaling pathway in ovarian cancer.

In our study, we performed gene expression microarray analysis and revealed that the TGF-β signaling pathway is activated in omental metastases of ovarian cancer. Activated TGF-β signaling promoted metastatic properties of ovarian cancer cells, including adhesion to extracellular matrix, cell motility and invasiveness. In addition, we examined the potential efficacy of A-83-01, a small molecule inhibitor of TGFBR1 kinase activity, in the treatment of ovarian cancer

Key words: ovarian cancer, metastasis, TGF β
Additional Supporting Information may be available in the online version of this article.
Grant sponsor: Japan Society for the Promotion of Science; **Grant numbers:** 21791551, 19890114, 21390452; **Grant sponsor:** Department of Defense CDMRP Ovarian Cancer Research Program; **Grant number:** W81XWH-05-1-0053
DOI: 10.1002/ijc.25961
History: Received 25 May 2010; Accepted 20 Dec 2010; Online 3 Feb 2011
Correspondence to: Masaki Mandai, Department of Gynecology and Obstetrics, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan, Tel: +81-75-751-3269; Fax: +81-75-761-3967, E-mail: mandai@kuhp.kyoto-u.ac.jp

metastases, both *in vitro* and *in vivo*. This report describes for the first time that the activated TGF-β signaling pathway in omental metastases of ovarian cancer is a potential therapeutic target.

Material and Methods

Cell line

The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line, purchased from the RIKEN BioResource Center (Tsukuba, Japan), was cultured as previously described.[6] The culture medium consists of minimum essential medium (MEM) alpha (Invitrogen, Carlsbad) supplemented with 10% heat-inactivated fetal bovine serum (v/v; Biowest, France) and penicillin–streptomycin (100 IU/ml penicillin, 100 μg/ml streptomycin; Nacalai Tesque, Kyoto, Japan). SK-OV-3 human ovarian cancer cell line was purchased from the ATCC (Rockville, MD) and cultured in RPMI1640 (Nikken Biomedical Laboratory, Kyoto, Japan) supplemented with the same reagents as MEM alpha.

Reagents

A-83-01, a small molecule inhibitor of TGF-β receptor type I kinase activity, was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF-β1 was purchased from Peprotech (Rock Hill, NJ).

Microarray analysis

HM-1 cells were treated with A-83-01 (1 μM) or vehicle dimethyl sulfoxide (DMSO) for 2 hr followed by the addition of TGF-β1 (1 ng/ml) or vehicle phosphate buffered saline (PBS). Four hours later, HM-1 cells were harvested, and RNA was extracted using the RNeasy Mini Kit (Qiagen). Gene expression data were generated at Singapore University as previously described.[7] We used 200 ng total RNA per sample on Affymetrix Mouse ST v1.0 GeneChips containing probes for over 28,000 well-annotated genes.

Microarray datasets were obtained from the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). Serous ovarian cancer samples (primary; $n = 88$, omental metastasis; $n = 38$) in GSE2109 were used for the analysis. Of these, 15 cases had received chemotherapy prior to the operation. Samples used in the study are listed in Supporting Information Table 1.

Bioinformatics analyses

Normalization. Data with the MAS5 format were used for binary regression analyses, whereas other analyses were performed using robust multi-array average (RMA)-normalized data. *R* version 2.8.2 was used for the normalization.[8]

Genes differentially expressed between control and TGF-β1-treated samples were identified using significance analysis of microarrays (SAM).[9] Gene probes with FDR $q < 0.25$ were selected for further analyses.

Binary regression was performed as previously described.[10–12] This is a tool to determine the probability of gene signature for individual samples to examine pathway activity. Training set had been developed by overexpression of oncogenes (Src, Myc, Ras, β-catenin and E2F3) or addition of cytokines (tumor necrosis factor (TNF)-alpha and TGF-β1) onto cultured human cells. Differentially expressed genes by the treatment were identified and designated as the gene signature. Probabilities of the gene signature can be determined for individual samples in external datasets. In this research, we determined gene signature probabilities for the serous ovarian cancer samples (Figs. 1 and 2*a*).

Gene set enrichment analysis (GSEA) was conducted as previously reported.[13] We used C2 curated gene sets of the MSigDB (http://www.broadinstitute.org/gsea/msigdb/) to determine the enriched pathways in omental metastases of ovarian cancer. Briefly, we performed the GSEA analysis to compare between primary sites ($n = 88$) *versus* omental metastases ($n = 38$) of GSE2109. At first, we applied all the C2 curated gene sets ($n = 1892$) with the following parameters; "Number of permutations = 10" and "Permutation type = phenotype." All other parameters were default. As a result, 657 gene sets showed statistically significant (FDR q value <0.25) enrichment among the omental metastases. To analyze selected gene sets relevant to the TGF-β pathway more accurately, we downloaded several gene sets (that appears in the Results section) from the MSigDB and analyzed individually with the following parameters; "Number of permutations = 1000" and "Permutation type = phenotype". Genes upregulated by TGF-β1 ($n = 173$) in HM-1 cells (Supporting Information Table 2) were also used to generate a gene set for a GSEA analysis. Genes downregulated by TGF-β1 ($n = 12$) were not used for this purpose because this gene set did not meet the gene set size threshold (≥ 15) to run the GSEA software.

An average-linkage hierarchical clustering analysis was performed using Cluster 3.0 available from http://rana.lbl.gov/EisenSoftware.htm. Java TreeView (http://jtreeview.sourceforge.net/) was used to visualize the heat map.

Immunohistochemical staining

Ovarian cancer specimens taken from women who received primary tumor resection at the Department of Gynecology and Obstetrics of Kyoto University Hospital from 2003 through 2006 were used for immunohistochemical staining. Prior written informed consent from all the patients was received, and approval was given by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee. For our study, we used only serous ovarian cancer samples with no prior treatment. Immunohistochemical staining of TGFBR2 was carried out using the streptavidin–biotin–peroxidase method. Briefly, formalin-fixed paraffin-embedded tissue sections were deparaffinized and antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at 120°C for 5 min. The slides were incubated with primary anti-TGFBR2 antibody at a 1:100 dilution (Abcam, Cambridge, MA) overnight at 4°C, followed by incubation with biotinylated goat anti-rabbit secondary antibodies (Nichirei). For staining of phosphorylated SMAD2 (pSMAD2), anti-pSmad2 antibody (Novus Bio, Littleton, CO) was used at a 1:100

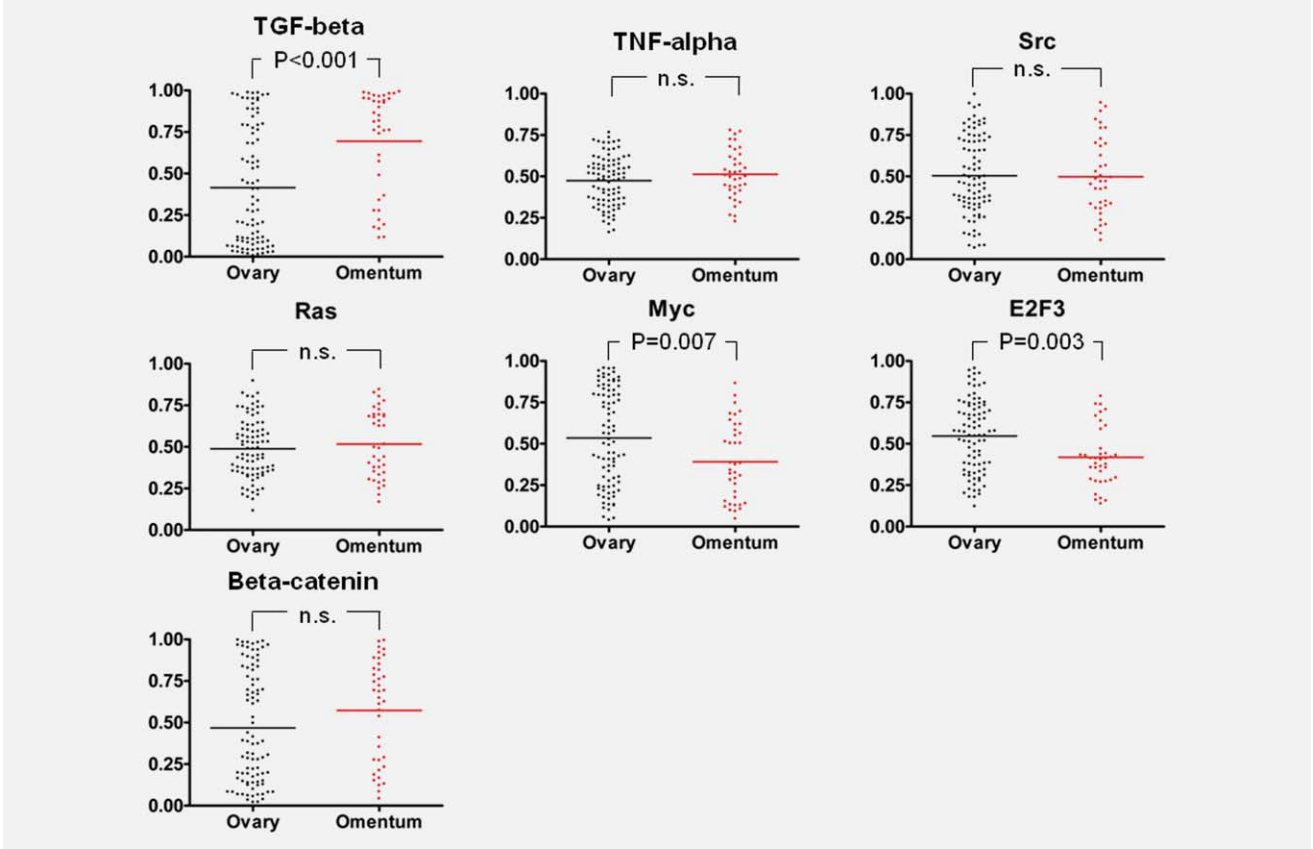

Figure 1. Activities of signaling pathways in primary sites and metastatic sites of ovarian cancer. Activities of seven signaling pathways including TGF-β, TNF-alpha, Src, Ras, Myc, E2F3 and β-catenin were analyzed using binary regression in the GSE2109 dataset. In each scatter diagram, individual dots show the activity of the signaling pathway for 88 primary sites (left) and 38 omental metastases (right) of ovarian cancers. *Y* axis; signature probabilities. n.s.; not significant.

dilution with the same subsequent procedure as TGFBR2. Two independent gynecological pathologists examined the immunohistochemistry slides without any prior information about the clinical history of the patients. TGFBR2 and pSMAD2 expression was evaluated according to staining intensity and was scored as follows: 0, negative, no staining in cancer cells (same or weaker than the cancer stroma); 0.5, weakly positive (staining of the cancer cells is slightly stronger than that of the cancer stroma); 1, positive (staining of the cancer cells is stronger than that of the cancer stroma); 2, strongly positive (staining of the cancer cells is much stronger than that of the cancer stroma).

Western blot analysis

A-83-01 (1 or 10 μM) or vehicle (DMSO) control were added to HM-1 cells followed by TGF-β1 (1 or 10 ng/ml) or vehicle control 30 min later. Cells were harvested 60 min later and lysed in radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA) with a protease inhibitor cocktail (EMD, Madison, WI) and a phosphatase inhibitor cocktail (Nacalai Tesque). Protein was quantified using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). Twenty micrograms of sodium dodecyl sulfate (SDS)-treated protein was loaded onto a 10% SDS-PAGE (polyacrylamide gel electrophoresis) gel (Ready Gels J, Bio-Rad). Gels were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Nonspecific binding of the antibody was blocked by incubating for 30 min at room temperature in Blocking One-P (Nacalai Tesque). The membranes were incubated overnight at 4°C with antiphosphorylated Smad3 antibody (1:1,000, pSmad3 Rabbit mAb, Immuno-Biology Laboratories, Japan), which reacts with both human and mouse protein. After washing in tris-buffered saline (TBS)-T, the blots were incubated with the appropriate peroxidase-coupled secondary antibody (1:10,000; Anti-rabbit HRP, GE Healthcare Life Sciences, Uppsala, Sweden). An anti-human β-actin antibody (1:5,000; Rabbit mAb, Abcam) were used for the endogenous controls. Specific proteins were detected using ECL Plus Western Blotting Reagent (GE Healthcare Life Sciences).

Cell proliferation assay

HM-1 cells were seeded into a 96-well plate (Asahi Glass, Japan) and were incubated for 18 hr. A-83-01 (1 μM) or vehicle were then added for 12 hr followed by the addition of

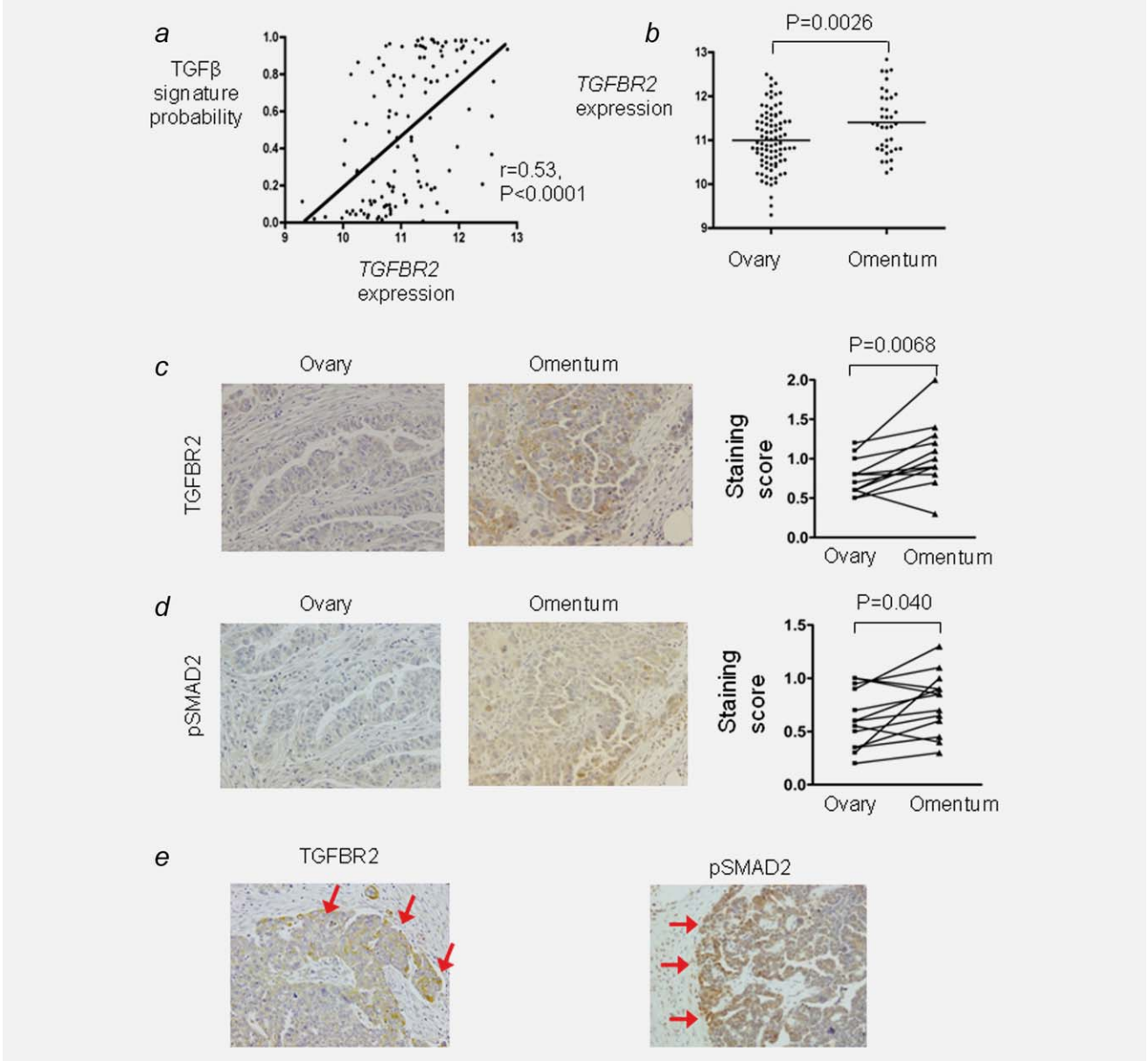

Figure 2. TGFBR2 expression and TGF-β pathway activity in primary ovarian carcinomas and omental metastases. (*a*) Expression of *TGFBR2* (208944_at) is positively correlated with TGF-β signature probabilities in the GSE2109 dataset. TGF-β signature probability for the individual tumor sample was determined as previously described.[12] (*b*) Expression of *TGFBR2* (208944_at) was significantly higher among the omental metastases in the GSE2109 dataset. (*c*) Immunohistochemical expression of TGFBR2. Scatter diagram shows staining scores of the paired samples. (*d*) Immunohistochemical expression and scatter diagram of pSMAD2. The sections of TGFBR2 and pSMAD2 are serial sections. (*e*) Representative figures of TGFBR2 and pSMAD2 expression in the invasive front (red arrows).

TGF-β1 (1 ng/ml) or vehicle for 60 hr. The number of viable cells in each well was examined using the WST-1 assay (Takara Bio) following the manufacturer's instructions.

Cell adhesion assay

HM-1 cells were treated with A-83-01 (1 μM) or vehicle for 12 hr followed by treatment with TGF-β1 (1 ng/ml) or vehicle for 8 hr. Next, 10^5 cells were seeded into each well of collagen IV-coated 96-well plates (Asahi Glass) and incubated for 2 hr. After that, wells were washed twice with PBS, and the number of attached cells was evaluated using the WST-1 assay.

Cell migration assay

HM-1 cells were seeded onto 10 cm tissue culture dishes (Greiner bio-one, Monroe, NC) and incubated until cells were confluent. Next, scratch lines (1000–1200 μm width) were introduced using a plastic tip and A-83-01 (1 μM) or vehicle was added. Six hours later, TGF-β1 (1 ng/ml) or

vehicle was added. The widths of the gaps in the cell monolayers were measured 20 hr after scratch introduction.

Cell invasion assay

HM-1 cells were treated with 1 μM of A-83-01 for 12 hr followed by treatment with 1 ng/ml of TGF-β1 for 8 hr. Next, 10^5 cells were seeded into Boyden chambers with 8.0 μm pore PET membranes (Becton Dickinson, Franklin Lakes, NJ) coated with Matrigel. After 20 hr, membranes of the Boyden chambers were fixed with 99% methanol and stained in hematoxylin. The number of cells that invaded through the membrane were visually counted in five high power fields (200×) and averaged. To confirm the effect of TGF-β1 and A83-01 on human ovarian cancer cell lines, we did invasion assay with SK-OV-3 cells.

ELISA assay of TGF-β1

Ascites were retrieved from the abdominal cavity of mice 10 days after inoculation with 1×10^6 HM-1 cells. The concentration of TGF-β1 in ascites was determined with the Quantikine human TGF-β1 immunoassay kit (R&D Systems, Minneapolis, MN) following manufacturer's instructions. The optical density of each well was read with an Emax microplate reader (Molecular Devices, Silicon Valley, CA).

In vivo experiments

Female B6C3F1 mice used for the *in vivo* studies were purchased from CLEA Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions. All animal experiments were approved by the Kyoto University Animal Research Committee. To evaluate the effect of A-83-01 on the survival of mice bearing peritoneal dissemination, HM-1 cells (1×10^6) were injected into the abdominal cavity *via* the left flank of the mouse. Starting the next day, A-83-01 (150 μg/body) or vehicles (PBS with 0.5% DMSO) were injected into the abdominal cavity three times per week. Mice were euthanized before reaching the moribund state.

Statistical analysis

All statistical analyses were performed using Prism 4.0b. Student's *t*-test was used unless indicated in the text. p value < 0.05 was considered statistically significant.

Results

Identification of the TGF-β activated signaling pathway in the omental metastases of ovarian cancer by gene expression profiling analysis

To investigate signaling pathways that may differ between the primary site and the omental metastases of ovarian cancer, we conducted gene expression microarray analysis using the publically available dataset GSE2109. Based on the previously reported gene signatures of seven signaling pathways (Src, Myc, Ras, TNF-alpha, β-catenin, TGF-β and E2F3),[10–12] we evaluated signature probabilities on behalf of each pathway in this data. Among the seven pathways, only the TGF-β signaling pathway was more active in the omental metastases than the primary sites (Fig. 1, $p < 0.0001$). In contrast, the Myc and E2F3 pathways were more activated in the primary sites ($p < 0.007$ and $p < 0.003$, respectively).

Consistent with the result from the binary regression, GSEA revealed that "TGF_BETA_SIGNALING_PATHWAY" was enriched in the omental metastases (FDR q value = 0.03). Furthermore, the characteristic *in vitro* effects of TGF-β on cancer cells[14–16] such as "CELL_MOTILITY," (FDR $q < 0.001$) "CELL_ADHESION" (FDR $q < 0.001$) and "MATRIX_METALLOPROTEINASES" (FDR $q = 0.006$) were also enriched in the omental metastases (data not shown).

Comparison of TGFBR2 and pSMAD2 expression in paired samples of primary site and omental metastases by immunohistochemistry

Among the core components of the TGF-β signaling pathway, *TGFBR2* (208944_at) showed a significant positive correlation with TGF-β signature probability scores ($r = 0.53$, $p < 0.0001$, Pearson's correlation) (Fig. 2*a*). In addition, *TGFBR2* expression was significantly upregulated in the omental metastases compared to primary sites ($p = 0.0026$; Fig. 2*b*).

We next examined expression of TGFBR2 and phosphorylated SMAD2 (pSMAD2) in ovarian cancer tissue samples by immunohistochemistry. We used samples for which both primary and omental metastasis sites were available ($n = 14$), and we compared their expression using paired analysis. Both TGFBR2 and pSMAD2 were upregulated in the omental metastases ($p = 0.0068$ and $p = 0.040$, respectively, Figs. 2*c* and 2*d*). In addition, among the cancer tissues, expression of both TGFBR2 and pSMAD2 tended to be stronger at the invasive front and in the small cancer nests rather than the center of tumor nodules (Fig. 2*e*).

Effect of TGF-β1 and the TGF-β pathway inhibitor A-83-01 on the in vitro metastatic properties of ovarian cancer cells

The *in vitro* effects of recombinant TGF-β1 or the TGF-β pathway inhibitor A-83-01 were examined using the HM-1 mouse ovarian cancer cell line.[6] First, we examined the expression of phosphorylated Smad3 (pSmad3) by Western blotting. As expected, expression of pSmad3 was increased by the addition of TGF-β1, and the effects of TGF-β1 were inhibited by A-83-01 (Fig. 3).

Addition of TGF-β1 or A-83-01 did not alter proliferation of HM-1 cells (Fig. 4*a*). Thus, we next examined the influence of TGF-β1 or A-83-01 on cell motility, adhesion and invasion. TGF-β1 increased cell motility, adhesion and invasion, while A-83-01 decreased these behaviors (Figs. 4*b*, *c* and *d*). When only A-83-01 was added, cell motility, adhesion and invasion were decreased (Figs. 4*b*, *c* and *d*). This appears to be a consequence of inhibition of autocrine/paracrine TGF-β signaling in HM-1 cells because the concentration of TGF-β1 in the culture medium (1,260 ± 17 pg/ml) increased after HM-1 cells were cultured for 18 hr (3,395 ± 15 pg/ml; data not shown).

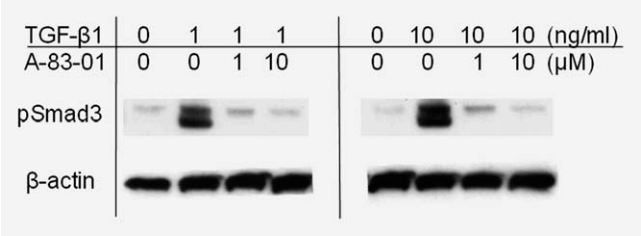

Figure 3. Transcriptional changes of HM-1 cells with TGF-β1 and/or A-83-01. Western blotting for pSMAD3 of HM-1 cells in reaction to TGF-β1 (1 or 10 ng/ml) and/or A-83-01(1 or 10 μM).

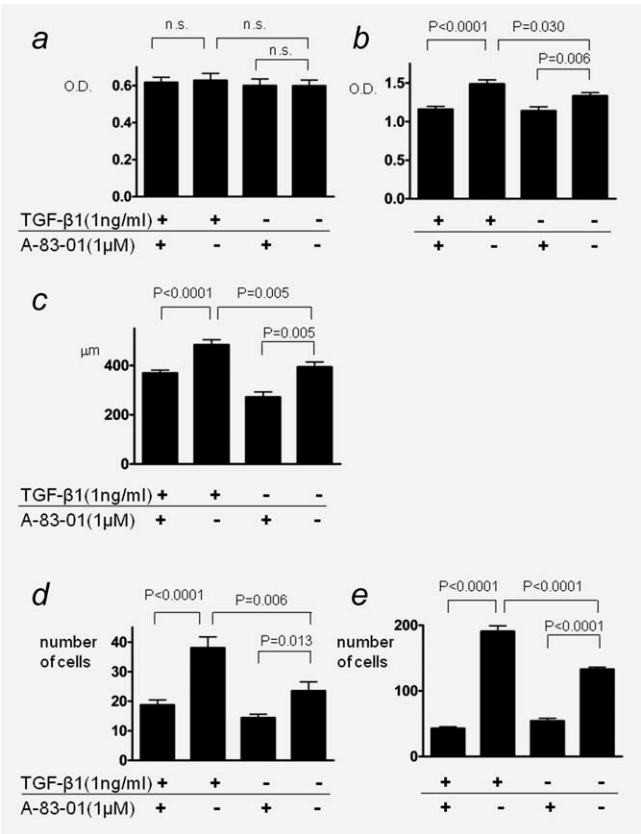

Figure 4. *In vitro* treatment of HM-1 cells and SK-OV-3 cells with TGF-β1 and/or A-83-01. (*a*) WST-1 assay of HM-1 cells to analyze proliferation of cells 60 hr after treatment ($n = 12$ each). (*b*) WST-1 assay of HM-1 cells to analyze the adhesion of cells onto collagen type IV-coated dishes, 150 min post-treatment ($n = 20$ each). (*c*) Wound healing assay of HM-1 cells to analyze cell motility ($n = 12$ each). The *y* axis indicates the distance of migration 20 hr post-treatment. (*d*) Boyden chamber assay of HM-1 cells to analyze cell invasion. Invaded cells were counted 20 hr post-treatment ($n = 12$ each). *Y* axis indicates the average of invading cell numbers in high power fields (200×). (*e*) Boyden chamber assay of SK-OV-3 cells to analyze cell invasion.

As to invasion assay, SK-OV-3 cells, human ovarian cancer cells, showed same tendency as HM-1 cells with addition of TGF-β1 or A-83-01 (Fig. 4*e*).

Identification of genes that are regulated by TGF-β

We next conducted microarray analysis using HM-1 cells to compare gene expression among vehicle ($n = 3$), TGF-β1 ($n = 3$), TGF-β1 + A-83-01 ($n = 3$) or A-83-01 treatments ($n = 1$). Differentially expressed genes between vehicle and TGF-β1-treated samples were identified using SAM. A cut-off of FDR $q < 0.25$ identified 241 probes, including 218 probes (173 genes) upregulated and 23 probes (12 genes) downregulated by TGF-β1 (Supporting Information Table 1).

An average-linkage hierarchical analysis of the 10 HM-1 samples using the above 241 probes was performed. As expected, these genes were found to distinguish TGF-β1-treated samples from the other samples (Fig. 5). The similarity of the TGF-β1 + A-83-01 group with vehicle and not the TGF-β1 group indicates that A-83-01 efficiently reversed gene expression changes induced by TGF-β1.

The 173 upregulated genes were used in a GSEA analysis to determine if they were differentially expressed between primary sites and the omental metastases of ovarian cancer in the GSE2109 dataset. These genes were significantly enriched among the omental metastases (FDR *q* value = 0.086 <0.25).

Therapeutic effects of A-83-01 in a mouse model of peritoneal dissemination of ovarian cancer

We first examined side effects of A-83-01 in B6C3F1 mice. Peritoneal injection of A-83-01 twice a week for 4 weeks at doses of 50, 150 and 500 μg did not influence body weight or neurobehavioral appearances. No change was found in the histolopathological appearance of lung, heart, liver or kidney (data not shown).

We then investigated the therapeutic potential of A-83-01 in a mouse model of peritoneal dissemination of ovarian cancer. Intraperitoneal injection of 1×10^6 HM-1 cells into B6C3F1 mice led to ascites accumulation with diffuse disseminated tumors on the peritoneum within 2 weeks (Fig. 6*a*). The ascites contained abundant TGF-β (26.993 ± 0.068 ng/ml, data not shown). A-83-01 was injected into the peritoneal cavity (150 μg/mouse × 3 times/week) starting 1 day postinjection of HM-1 cells. Formation of ascites tended to be slower in the A-83-01-treated group (Supporting Information Fig. 1), and A-83-01 significantly improved survival of the mice (Fig. 6*b*, $p = 0.0148$).

Discussion

Ovarian cancer is the most lethal malignancy among the gynecological cancers because most cases are diagnosed only after the disease has reached an advanced stage. Peritoneal dissemination, which frequently involves the omentum, is the most typical mode of spread of ovarian cancer.[2] To clarify the molecular mechanisms underlying the metastasis of ovarian cancer, we compared the primary site and omental metastases of ovarian cancer. We have recently used bioinformatics approaches such as binary regression to elucidate

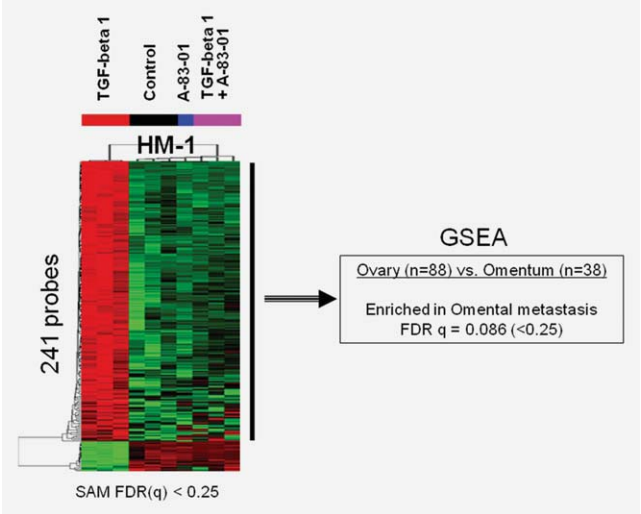

Figure 5. Identification of genes regulated by TGF-β1 in HM-1 cells. Microarray analysis of HM-1 cells for the four treatment groups; control ($n = 3$), TGF-β1 ($n = 3$), TGF-β1 + A-83-01 ($n = 3$) and A-83-01 only ($n = 1$). Average-linkage hierarchical analysis was performed using the 241 differentially expressed probes (FDR $q <$ 0.25) between the control and TGF-β1-treated cells. Upregulated genes were used for GSEA analysis to examine relevance with the clinical cancer dataset GSE2109.

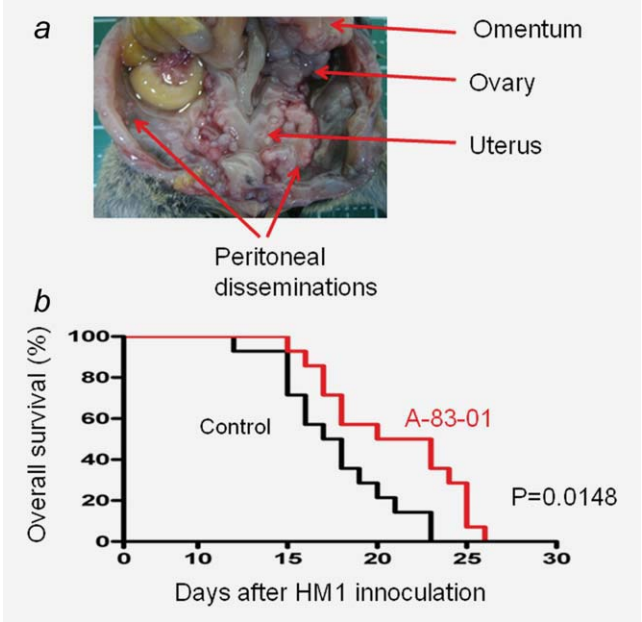

Figure 6. Mouse model of intraperitoneal dissemination using HM-1 cells and treatment with A8301. (*a*) Intraperitoneal findings after 10 days of HM-1 cells inoculation. Disseminated nodules can be seen on the peritoneum and abdominal organs, especially omentum, mesentery, uterus and ovary. (*b*) Survival curves of the A-83-01 treated group and the control group ($n = 14$ each).

activated signaling pathways in cancer.[10–12,17,18] In our study, we found that the TGF-β pathway activity was upregulated in the omental metastases compared to primary sites (Fig. 1). GSEA also demonstrated enrichment of gene sets relevant to the TGF-β signaling pathway. Based on the results from the computational screening, we further examined expression of pSMAD2, a representative molecule indicating activated TGF-β signaling.[19] Immunohistochemical analysis revealed significant upregulation of pSMAD2 expression in the omental metastases (Fig. 2*b*). These results demonstrated that the TGF-β signaling pathway is activated in ovarian cancer metastases. Furthermore, pSMAD2 expression was stronger in the invasive front of cancer nests (Fig. 2*d*). Localized TGF-β signaling within cancer tissue may be attributed to the inherent heterogeneity of the tumor. Here, we found higher expression of pSMAD2 in invasive fronts and small cancer nests, and this would result in higher expression overall in metastases because invasive fronts tended to be more abundant in omental metastasis than in primary sites (data not shown). Nonetheless, activation of the TGF-β signaling pathway suggests a role in the metastatic process of ovarian cancer.

Expression of *TGFBR2*, a core component of the TGF-β signaling pathway, showed a significant positive correlation with TGF-β signature probability scores (Fig. 2*a*), and TGFBR2 was upregulated in metastases similar to pSMAD2 (Figs. 2*c*, 2*e*). Thus, transcriptional regulation of *TGFBR2* could play a crucial role in the regulation of TGF-β signaling. Although TGF-β signaling leads to cell cycle arrest and/or apoptosis of normal epithelial cells, this "tumor-suppressive function" of TGF-β signaling is inhibited through genetic or epigenetic changes in pathway components in virtually all types of cancers.[20] Lynch et al.[21] reported that in ovarian cancer, expression of TGFBR2 is lost by mutations in the coding regions of the *TGFBR2* gene. However, there have been several reports that challenge this observation. Another group showed that the frequency of *TGFBR2* mutation is quite rare in ovarian cancer.[22] In most cases of ovarian cancer, addition of TGF-β to primary cancer cells leads to activation of internal signals.[23,24] In our study, given the specific expression of pSMAD2 (Fig. 2*d*) in tumor cells, loss of internal signaling by receptor mutations is unlikely. Epigenetic regulation, however, could be one possible mechanism that alters transcriptional regulation of *TGFBR2*. We recently found that promoter DNA methylation of the *TGFBR2* gene is often found in ovarian cancer and its status negatively correlates with *TGFBR2* expression (submitted for publication). However, further studies are necessary to clarify the precise mechanisms of activation of the TGF-β signaling pathway in omental metastases.

Next, to reveal the functional role of the TGF-β signaling pathway in ovarian cancer, we conducted *in vitro* assays using the HM-1 mouse ovarian cancer cell line.[6] In our preliminary experiments, HM-1 cells expressed *Muc16* (data not shown), an ortholog of human *MUC16*, which encodes CA125, a specific biomarker of human epithelial ovarian cancer. The addition of TGF-β1 increased expression of pSmad3 in HM-1 cells (Fig. 3), indicating that the signaling pathway is not disrupted at the receptor level. In *in vitro* experiments,

addition of TGF-β1 increased cell motility, invasiveness and attachment of HM-1 cells (Figs. 4*b*, *c* and *d*), and these effects were inhibited by the addition of A-83-01. We also conducted similar experiment using SK-OV-3, human serous ovarian cancer cell line, which showed the same tendency as HM-1 cells in reaction to TGF-β1 and A-83-01. These effects of TGF-β1 might promote detached cancer cells to attach to the extracellular matrix and promote invasion into the peritoneal membrane, resulting in peritoneal dissemination. A-83-01 is likely to block TGF-β1-induced metastatic properties efficiently through disrupting kinase activity of TGF-β type 1receptor, although its inhibitory effects through other type 1 receptor of TGF-β superfamily such as ALK4 and ALK7 might not be excluded.[25] This prominent inhibitory effect of A-83-01 suggests the potential utility of A-83-01 in the treatment of ovarian cancer. In contrast, TGF-β1 or A-83-01 did not affect the proliferation of HM-1 cells (Fig. 4*a*), indicating that the "tumor suppressive" activity is not functional, as in many cancer cells. Interestingly, GSEA analysis to clarify the differences between primary sites and omental metastases of ovarian cancers indicated that gene sets relevant to cell motility, adhesion and invasion were enriched among the omental metastases, suggesting an *in vivo* role of the TGF-β pathway in ovarian cancer.

We next examined transcriptional changes caused by TGF-β1 and/or A-83-01 in HM-1 cells and found that A-83-01 reversed the effects of TGF-β1 (Fig. 3). From GSEA analysis, genes upregulated by TGF-β1 in HM-1 cells were highly expressed in omental metastases of ovarian cancer. These results indicated that the TGF-β signaling pathway in HM-1 cells bears relevance to omental metastasis in human ovarian cancer. Genes upregulated by TGF-β1 in HM-1 cells included several "metastasis promoter genes", such as acylglycerol kinase (*AGK*), granulin (*GRN*), heparanase (*HPSE*), oncostatin M (*OSM*), *slingshot homolog2 (SSH2)* and TGF-β-induced (*TGFBI*) (Supporting Information Table 1). AGK increases formation and secretion of lipoprotein(a) LPA, resulting in increased migration of prostate cancer cells.[26] HPSE increases metastatic potential by remodeling extracellular matrix in many types of cancers.[27] GRN increases the motility and invasion of ovarian cancer cells[28] and in hepatocellular carcinoma.[29] OSM increases the invasive capacity of breast cancer cells.[30] SSH2-mediated dephosphorylation of cofilin/ADF (actin depolymerizing factor) increases the migration and invasion of hepatoma cells.[31] TGFBI promotes metastasis of colon cancer cells by accelerating cell extravasation.[32] Although further investigation is necessary, increased metastatic properties resulting from TGF-β signaling in ovarian cancer may be attributed to upregulation of multiple metastasis-promoting genes.

Finally, we analyzed the *in vivo* therapeutic effects of A-83-01. As A-83-01 has never been used for *in vivo* experiments, we first examined side effects of A-83-01 in mice; no significant side effects were observed as the previous reports with the other TGF-β inhibitors.[14,15] It is known that considerable amounts of TGF-β1 exist in the ascitic fluid of ovarian cancer patients.[33] Similarly, the concentrations of TGF-β1 were high in the ascitic fluid associated with peritoneal dissemination of HM-1. Therefore, this mouse model is appropriate to examine the *in vivo* therapeutic effects of A-83-01. Accumulation of ascitic fluid in A-83-01-treated mice tended to be slower than the control group (Supporting Information Fig. 1). In addition, A-83-01 significantly improved survival (Fig. 6*b*). Recently, inhibition of the TGF-β pathway has been shown to be a promising treatment modality against cancers. AP12009, an antisense oligonucleotide against TGF-β2, was found to be very effective against recurrent glioma in a phase I/II clinical trial,[5] and a phase III clinical trial is now underway. Efficacy of TGFBR kinase inhibitors, such as A-77, Ki26894 and LY2109761 was demonstrated in preclinical studies of gastric cancer, breast cancer and pancreatic cancer.[14,15,34] Although there may be a risk of carcinogenesis[20] with these drugs, this has never been reported in clinical and preclinical studies for TGF-β inhibitors. Therefore, A-83-01 may be a useful drug in the treatment of advanced ovarian cancer.

In summary, starting from gene expression profiling analysis, we found that the TGF-β signaling pathway is activated in omental metastases of ovarian cancers. We then demonstrated the *in vitro* and *in vivo* efficacy of A-83-01, a TGFBR1 kinase inhibitor. This report shows for the first time that inhibition of the TGF-β signaling pathway by a small molecule is a promising strategy in the treatment of ovarian cancer.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Ms. Maki Sakaeyama. The Japan Society for the Promotion of Science to N.M. (21791551), N.M. (19890114) and I.K. (21390452); The Department of Defense CDMRP Ovarian Cancer Research Program (W81XWH-05-1-0053) to S.K.M are also acknowledged.

References

1. Roett MA, Evans P. Ovarian cancer: an overview. *Am Fam Physician* 2009;80:609–16.
2. Tan DS, Agarwal R, Kaye SB. Mechanisms of transcoelomic metastasis in ovarian cancer. *Lancet Oncol* 2006;7:925–34.
3. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–29.
4. Padua D, Massague J. Roles of TGF beta in metastasis. *Cell Res* 2009;19:89–102.
5. Hau P, Jachimczak P, Schlingensiepen R, Schulmeyer F, Jauch T, Steinbrecher A, Brawanski A, Proescholdt M, Schlaier J, Buchroithner J, Pichler J, Wurm G, et al. Inhibition of TGF-beta2 with AP 12009 in recurrent malignant gliomas: from preclinical to phase I/II studies. *Oligonucleotides* 2007;17:201–12.
6. Hamanishi J, Mandai M, Matsumura N, Baba T, Yamaguchi K, Fujii S, Konishi I. Activated local immunity by CC chemokine ligand 19-transduced embryonic endothelial progenitor cells suppresses metastasis of murine ovarian cancer. *Stem Cells (Dayton, Ohio)* 2010;28:164–73.

7. Juul SE, Beyer RP, Bammler TK, McPherson RJ, Wilkerson J, Farin FM. Microarray analysis of high-dose recombinant erythropoietin treatment of unilateral brain injury in neonatal mouse hippocampus. *Pediatr Res* 2009;65:485–92.
8. R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org
9. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001;98:5116–21.
10. Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D, Joshi MB, Harpole D, Lancaster JM, Berchuck A, Olson JA, Jr., Marks JR, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006;439:353–7.
11. Mori S, Rempel RE, Chang JT, Yao G, Lagoo AS, Potti A, Bild A, Nevins JR. Utilization of pathway signatures to reveal distinct types of B lymphoma in the Emicro-myc model and human diffuse large B-cell lymphoma. *Cancer Res* 2008;68:8525–34.
12. Andrechek ER, Mori S, Rempel RE, Chang JT, Nevins JR. Patterns of cell signaling pathway activation that characterize mammary development. *Development (Cambridge, England)* 2008;135:2403–13.
13. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005;102:15545–50.
14. Kawajiri H, Yashiro M, Shinto O, Nakamura K, Tendo M, Takemura S, Node M, Hamashima Y, Kajimoto T, Sawada T, Ohira M, Hirakawa K. A novel transforming growth factor beta receptor kinase inhibitor, A-77, prevents the peritoneal dissemination of scirrhous gastric carcinoma. *Clin Cancer Res* 2008;14:2850–60.
15. Ehata S, Hanyu A, Fujime M, Katsuno Y, Fukunaga E, Goto K, Ishikawa Y, Nomura K, Yokoo H, Shimizu T, Ogata E, Miyazono K, et al. Ki26894, a novel transforming growth factor-beta type I receptor kinase inhibitor, inhibits in vitro invasion and in vivo bone metastasis of a human breast cancer cell line. *Cancer Sci* 2007;98:127–33.
16. Halder SK, Beauchamp RD, Datta PK. A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 2005;7:509–21.
17. Matsumura N, Huang Z, Baba T, Lee PS, Barnett JC, Mori S, Chang JT, Kuo WL, Gusberg AH, Whitaker RS, Gray JW, Fujii S, et al. Yin yang 1 modulates taxane response in epithelial ovarian cancer. *Mol Cancer Res* 2009;7:210–20.
18. Mori S, Chang JT, Andrechek ER, Matsumura N, Baba T, Yao G, Kim JW, Gatza M, Murphy S, Nevins JR. Anchorage-independent cell growth signature identifies tumors with metastatic potential. *Oncogene* 2009;28:2796–805.
19. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells* 2002;7:1191–204.
20. Massague J. TGF beta in Cancer. *Cell* 2008;134:215–30.
21. Lynch MA, Nakashima R, Song H, DeGroff VL, Wang D, Enomoto T, Weghorst CM. Mutational analysis of the transforming growth factor beta receptor type II gene in human ovarian carcinoma. *Cancer Res* 1998;58:4227–32.
22. Alvi AJ, Rader JS, Broggini M, Latif F, Maher ER. Microsatellite instability and mutational analysis of transforming growth factor beta receptor type II gene (TGFBR2) in sporadic ovarian cancer. *Mol Pathol* 2001;54:240–3.
23. Lin SW, Lee MT, Ke FC, Lee PP, Huang CJ, Ip MM, Chen L, Hwang JJ. TGF beta1 stimulates the secretion of matrix metalloproteinase 2 (MMP2) and the invasive behavior in human ovarian cancer cells, which is suppressed by MMP inhibitor BB3103. *Clin Exp Metastasis* 2000;18:493–9.
24. Rodriguez GC, Haisley C, Hurteau J, Moser TL, Whitaker R, Bast RC, Jr.Stack MS. Regulation of invasion of epithelial ovarian cancer by transforming growth factor-beta. *Gynecol Oncol* 2001;80:245–53.
25. Tojo M, Hamashima Y, Hanyu A, Kajimoto T, Saitoh M, Miyazono K, Node M, Imamura T. The ALK-5 inhibitor A-83–01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* 2005;96:791–800.
26. Bektas M, Payne SG, Liu H, Goparaju S, Milstien S, Spiegel S. A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells. *J Cell Biol* 2005;169:801–11.
27. Vlodavsky I, Friedmann Y. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *J Clin Investig* 2001;108:341–7.
28. Pizarro GO, Zhou XC, Koch A, Gharib M, Raval S, Bible K, Jones MB. Prosurvival function of the granulin–epithelin precursor is important in tumor progression and chemoresponse. *Int J Cancer* 2007;120:2339–43.
29. Cheung ST, Wong SY, Leung KL, Chen X, So S, Ng IO, Fan ST. Granulin–epithelin precursor overexpression promotes growth and invasion of hepatocellular carcinoma. *Clin Cancer Res* 2004;10:7629–36.
30. Queen MM, Ryan RE, Holzer RG, Keller-Peck CR, Jorcyk CL. Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. *Cancer Res* 2005;65:8896–904.
31. Horita Y, Ohashi K, Mukai M, Inoue M, Mizuno K. Suppression of the invasive capacity of rat ascites hepatoma cells by knockdown of Slingshot or LIM kinase. *J Biol Chem* 2008;283:6013–21.
32. Ma C, Rong Y, Radiloff DR, Datto MB, Centeno B, Bao S, Cheng AW, Lin F, Jiang S, Yeatman TJ, Wang XF. Extracellular matrix protein betaig-h3/TGFBI promotes metastasis of colon cancer by enhancing cell extravasation. *Genes Dev* 2008;22:308–21.
33. Abendstein B, Stadlmann S, Knabbe C, Buck M, Muller-Holzner E, Zeimet AG, Marth C, Obrist P, Krugmann J, Offner FA. Regulation of transforming growth factor-beta secretion by human peritoneal mesothelial and ovarian carcinoma cells. *Cytokine* 2000;12:1115–9.
34. Melisi D, Ishiyama S, Sclabas GM, Fleming JB, Xia Q, Tortora G, Abbruzzese JL, Chiao PJ. LY2109761, a novel transforming growth factor beta receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. *Mol Cancer Ther* 2008;7:829–40.