

ORIGINAL ARTICLE

Polarity switching of ovarian cancer cell clusters via SRC family kinase is involved in the peritoneal dissemination

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Funding information

Japan Agency for Medical Research and Development, Grant/Award Number: 19cm0106203h0004 and 21am0401004h0003; Japan Society for the Promotion of Science, Grant/Award Number: 18H02648 and 21K07942; P-CREATE; Science and Technology Platform Program for Advanced Biological Medicine; Takeda Science Foundation

Abstract

Peritoneal dissemination is a predominant pattern of metastasis in patients with advanced ovarian cancer. Despite recent progress in the management strategy, peritoneal dissemination remains a determinant of poor ovarian cancer prognosis. Using various histological types of patient-derived ovarian cancer organoids, the roles of the apicobasal polarity of ovarian cancer cell clusters in peritoneal dissemination were studied. First, it was found that both ovarian cancer tissues and ovarian organoids showed apicobasal polarity, where zonula occludens-1 (ZO-1) and integrin beta 4 (ITGB4) served as markers for apical and basal sides, respectively. The organoids in suspension culture, as a model of cancer cell cluster floating in ascites, showed apical-out/basal-in polarity status, while once embedded in extracellular matrix (ECM), the organoids switched their polarity to apical-in/basal-out. This polarity switch was accompanied by the SRC kinase family (SFK) phosphorylation and was inhibited by SFK inhibitors. SFK inhibitors abrogated the adherence of the organoids onto the ECM-coated plastic surface. When the organoids were seeded on a mesothelial cell layer, they cleared and invaded mesothelial cells. In vivo, dasatinib, an SFK inhibitor, suppressed peritoneal dissemination of ovarian cancer organoids in immunodeficient mice. These results suggest SFK-mediated polarity switching is involved in peritoneal metastasis. Polarity switching would be a potential therapeutic target for suppressing peritoneal dissemination in ovarian cancer.

KEYWORDS

cell polarity, metastasis, organoids, ovarian cancer, SRC family kinase

Abbreviations: CTOS, cancer tissue-originated spheroids; ECM, extracellular matrix; ITGB4, integrin beta 4; OC, ovarian cancer; SFK, SRC family kinase; ZO-1, zonula occludens-1.

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1 | INTRODUCTION

Ovarian cancer (OC) is one of the most leading causes of gynecologic cancer-related death in women worldwide.¹ Epithelial OC comprises several histological subtypes that affect the selection of treatment and prognosis: high-grade and low-grade serous, clear cell, endometrioid, and mucinous subtypes. Owing to the anatomical location of the ovary, cancer cells detached from the original tumor mass easily disseminate into the peritoneal cavity. Therefore, peritoneal dissemination is a typical route of OC metastasis, which precedes hematogenic or lymphatic metastasis. Thus, peritoneal metastasis is a vital determinant of poor prognosis.² As most OC cases lack overt symptoms, only ~20% of patients are diagnosed at stage I, when cancer is still localized to the ovary and a high survival rate is expected, while the rest are diagnosed with intrapelvic, abdominal, or more distant metastasis.³ Despite recent advances in the development of treatment, including immune checkpoint inhibitors, further progress is needed.

Peritoneal metastasis is a multistep event that requires OC cell adaptation to the peritoneal, mesenteric, or omental surface.⁴⁻⁶ Integrin-mediated adhesion to the extracellular matrix (ECM) and evasion of peritoneal mesothelial cells by mechanical forces, a phenomenon termed "mesothelial clearance," are important steps for OC cells to settle under the mesothelial monolayer located at the peritoneum surface.⁶ Although several clinical trials target the molecules involved in the mechanisms of peritoneal metastasis, no such treatment has yet been approved.

Recently, 3D organoid cultures of various types of cancer cells, including OC, derived from patient tissue have been reported.⁷⁻⁹ A method for preparing and culturing organoids, the cancer tissue-originated spheroids (CTOS) method, for various types of cancer was reported.^{9,10} The organoids can be prepared using the CTOS method from patient tissue and from xenograft tumors with high purity, viability, and yield by retaining the cell-cell interaction throughout the procedures of preparation and culture.¹⁰ Such cancer organoids represent physiological features reflecting clinical characteristics of the original tumors more than conventional established 2D cell lines.^{9,10} Especially, some characteristics related to 3D morphology are observed only in the organoids. The apicobasal polarity of cancer tissue and cell clusters are well preserved in the organoids but lost in the spheroid of conventional cell lines.^{10,11} Using the organoid model, it was reported that the organoids of colorectal cancer exhibit polarity switching, a dynamic conversion of apicobasal polarity upon contact with ECM, which is related to cancer phenotype and metastasis.^{11,12} However, the polarity of the epithelial cellular compartment has been insufficiently described in OC tissue.

Even with the recent advances in understanding the molecular mechanisms of peritoneal dissemination of OC,⁶ the involvement of cell cluster polarity and polarity switching has not been examined. Therefore, in this study, the polarity status of OC cell clusters, including polarity, molecular mechanism of polarity switching, and the therapeutic potential of targeting polarity switching for managing peritoneal metastasis, was investigated.

2 | MATERIALS AND METHODS

2.1 | Patient samples and animal studies

The Institutional Ethics Committees at Osaka International Cancer Institute (1712225296, 1803125402), Osaka University (10182), Kyoto University (R1575, R2444), and Kaizuka City Hospital (224) approved this study. This study was performed in accordance with the Declaration of Helsinki.

The Institutional Animal Care and Use Committees of Kyoto University and Osaka International Cancer Institute approved the animal studies, and they were performed according to institutional guidelines.

2.2 | Generation and treatment of xenograft tumors

The organoids were injected into female NSG (Charles River Japan) or NOD/SCID mice (CLEA Japan) to generate subcutaneous xenograft tumors. For in vivo treatment studies, the organoids were either subcutaneously or intraperitoneally injected into NSG mice. Dasatinib (LC Laboratories) solution was prepared in 80mM citrate buffer (80mM citric acid, pH 3.1) and administered to mice daily using oral gavage at 10 or 50mg/kg or buffer alone. For the subcutaneous tumor model, the subcutaneous xenograft volume was calculated using the formula: volume = (width)² × (length)/2, and the treatment started when the volume exceeded 300mm³. For the peritoneal dissemination model, the mice were pretreated with dasatinib the day before peritoneal injection of Ov114 organoids. Dasatinib was orally administered daily for 4 weeks. The tumor number was counted for overt nodules larger than 2 mm in diameter.

2.3 | Quantitative analysis of polarity status

The organoids were immunostained with ZO-1 and ITGB4 and classified into three groups based on each marker's distribution^{11,12}: "out" (marker proteins located only at the outermost membrane of an organoid), "in" (marker proteins located only inside of an organoid), and "mix" (marker proteins located both at the outermost membrane and inside of an organoid). Fifty organoids were evaluated at the cross-section of the maximum diameter in each condition, and the proportion of each polarity status was calculated.

2.4 | Adhesion assay

Matrigel-coated plates were prepared by dispensing Matrigel-GFR into a 96-well plate and incubated at 37°C for 1 hour followed by rinsing. Approximately 30 organoids of 40-100µm diameter were plated in each well. After 2 days of culture, the organoids' area was measured using the Cell3iMager (SCREEN) before and after washing.

The ratio of adhering organoids was calculated by dividing the area after washing by the area before washing.

2.5 | Mesothelial clearance

A mesothelial clearance assay was performed as described previously.¹³ Briefly, mCherry expressing HOMC-B1 cells were pre-cultured in a 96-well plate to reach confluence. Then, GPI-GFP Ov114 organoids were placed on the HOMC-B1 cell layer and cultured for another 3 days. The clearance area was quantified for the adhered 30 organoids using LEICA Dmi8 microscope (Leica) and quantified using ImageJ software (U. S. National Institutes of Health).

Additional information for materials and methods are described in Appendix S1.

3 | RESULTS

3.1 | Ovarian cancer tissue and cell clusters exhibit apicobasal polarity

Firstly, the polarity status in OC tissues and cell clusters in ascites was evaluated using immunofluorescent imaging. ZO-1, a tight-junction protein, and integrin beta 4 (ITGB4) were used as the apical and basal markers, respectively.^{11,12} Unsurprisingly, in the OC tissue, ITGB4 was expressed on the membrane facing the stroma in the outermost cells of the cancer cell region (Figure 1A). Meanwhile, ZO-1 is typically localized in the lining of the surface facing the lumen inside the cancer cell region (apical-in; Figure 1A). The pattern was observed in eight cases, including all four major pathological subtypes; serous, mucinous, endometrioid, and clear cell carcinoma (Figure 1A, Figure S1, and Table S1). Next, the cellular polarity in cell clusters collected from the ascites of two patients with OC was evaluated. In both cases, there existed cancer cell clusters in the ascites (Figure 1B), which expressed ZO-1 at the outer surface of the cell cluster facing the peritoneal fluid, while ITGB4 was observed at the cellular boundaries (apical-out; Figure 1C). These results indicate that the OC cell clusters detached from the primary tumor mass retain the cellular polarity, although reversed when they are in the peritoneal cavity. To further investigate the regulatory mechanisms of cellular polarity in peritoneal metastasis, a three-dimensional (3D) organoid model was employed.^{9,10} In ovarian organoids, the polarity of OC cell clusters in floating culture conditions was preserved in the apical-out status. Thus, OC organoids were used for the model of the cell clusters in peritoneal fluid thereafter (Figure 1D).

3.2 | Polarity of the OC cell cluster exhibits dynamic change upon contact with ECM

Ovarian cancer organoids were embedded in ECM to investigate the dynamic change of the polarity, which was reported as a "polarity switching" in colorectal cancer cell clusters.^{11,12} Matrigel,

mainly composed of laminin, entactin, and type IV collagen,¹⁴ was used as the ECM. After 2 days of embedding in Matrigel, ZO-1 disappeared from the outer surface of the organoids and emerged at the intraorganoid lumen and cellular boundary (Figure 2A). Conversely, intercellular ITGB4 was diminished after 2 days in Matrigel, while ITGB4 expression at the outer surface of the organoids emerged (Figure 2A). Notably, polarity switching was also observed in cell clusters directly derived from ascites (Figure S2A). The result shows that the polarity status of the organoids was altered mainly after 2 days of culture in Matrigel (Figure 2B,C and Figure S2B,C), representing a polarity-switching event similar to the colorectal organoids. When the detailed period of the polarity change was evaluated in Ov114 organoids by ZO-1 and ITGB4, the change started from as early as 6 hours after being embedded in Matrigel and proceeded gradually (Figure 2D). A consistent result was observed with another method: GPI-anchored GFP (GPI-GFP), which preferentially localizes to the apical membrane,¹² was forced-expressed in Ov114 organoids to evaluate polarity status. The polarity change started from 6 hours after embedding in Matrigel and proceeded to completion in 48 hours (Figure 2E and Figure S3). This result shows that dynamic changes occur upon the contact to ECM in the apicobasal polarity of the OC cell clusters.

3.3 | Polarity switching of OC cell clusters occur via SRC family kinase (SKF) activation triggered by contact with ECM

Next, the role of SKF activation was investigated because our previous study revealed that SKF is the key molecule in the polarity switching of colorectal cancer.^{11,12} In Ov114 and Ov129 OC organoids 48 hours after embedding in Matrigel, SKF phosphorylation upregulation was induced (Figure 3A). This SKF activity upregulation was suppressed by SKF inhibitors, robustly by dasatinib and mildly with PP1 (Figure 3A) at the concentration below the toxic level (Figure S4). Following the suppressed SKF activity, polarity switching was inhibited in the OC organoids after embedding in Matrigel. Dasatinib efficiently suppressed the polarity switching in Ov101, 114, and 129 (Figure 3B). Additionally, PP1 suppressed polarity switching in these OC organoids, although not robustly (Figure S5). Dasatinib administered simultaneously with embedding in the ECM inhibits SKF activation from an early stage (Figure S6). Notably, when dasatinib was added 12 hours after embedding in Matrigel, the progression of polarity switching was halted but not reverted (Figure 3E), supporting the vital role of SKF activation in triggering but not maintaining the progression of polarity switching.

3.4 | Polarity switching is involved in the adhesion of cell clusters to matrices

At the initial step of peritoneal dissemination, cell clusters are not supposed to be embedded in the peritoneal wall at once. Instead, only a part of a cancer cell cluster would touch the peritoneal wall.

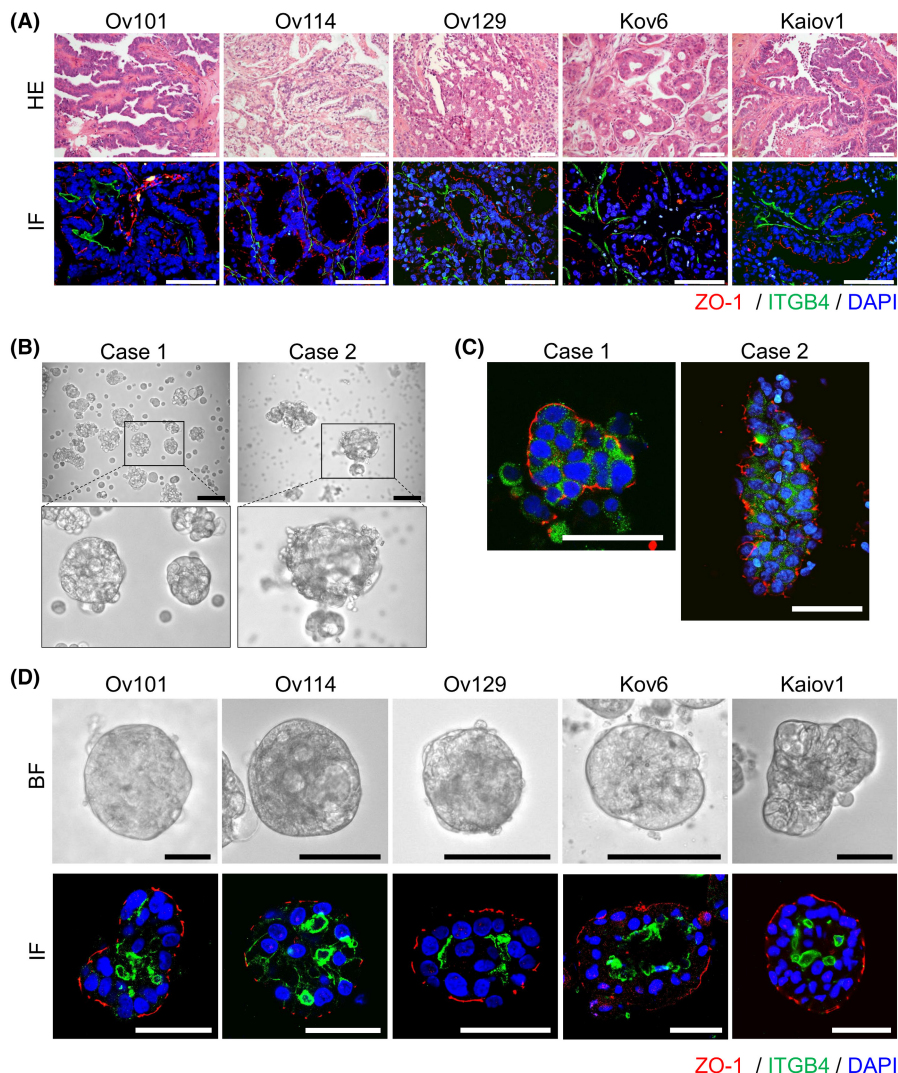


FIGURE 1 Ovarian cancer (OC) tissue and cell clusters exhibit apicobasal polarity. A, Hematoxylin and eosin staining (HE, upper panels) and immunofluorescent images (IF, lower panels) of OC organoid-derived xenograft tumors. Green, ITGB4; red, ZO-1; blue, DAPI; scale bars, 100 μ m. B, Bright-field images of cell clusters obtained from peritoneal fluid of patients with OC. Boxed areas in the upper panels are enlarged in the lower panels. Scale bars, 100 μ m. C, Immunofluorescent staining of OC cell clusters in peritoneal fluid. Green, ITGB4; red, Z-1; blue, DAPI; scale bars, 50 μ m. D, Bright-field images (BF) and immunofluorescent images (IF) of OC organoids derived from five different patients. Green, ITGB4; red, ZO-1; blue, DAPI; scale bars, 50 μ m

Therefore, the attachment and adhesion of the organoids to the Matrigel-coated dish was investigated to model the first encounter of organoids and peritoneum. Floating organoids were transferred to the plates coated with Matrigel. After 2 days of transfer, the organoids adhered to the Matrigel surface. Then, the organoids' polarity at the cell-matrix interface was evaluated using confocal fluorescence microscopy. Like the organoids embedded in Matrigel, ZO-1 expression disappeared from the cell-ECM interface, while ITGB4 emerged at the cell-ECM interface (Figure 4A). Of note, deposition of laminin, a ligand for the integrin $\alpha 6 \beta 4$, was detected in close overlapping with ITGB4 (Figure S7), indicating that ITGB4 is binding to laminin. This dynamic change of the polarity is almost completed 48 hours after contact with the ECM surface (Figure 4B).

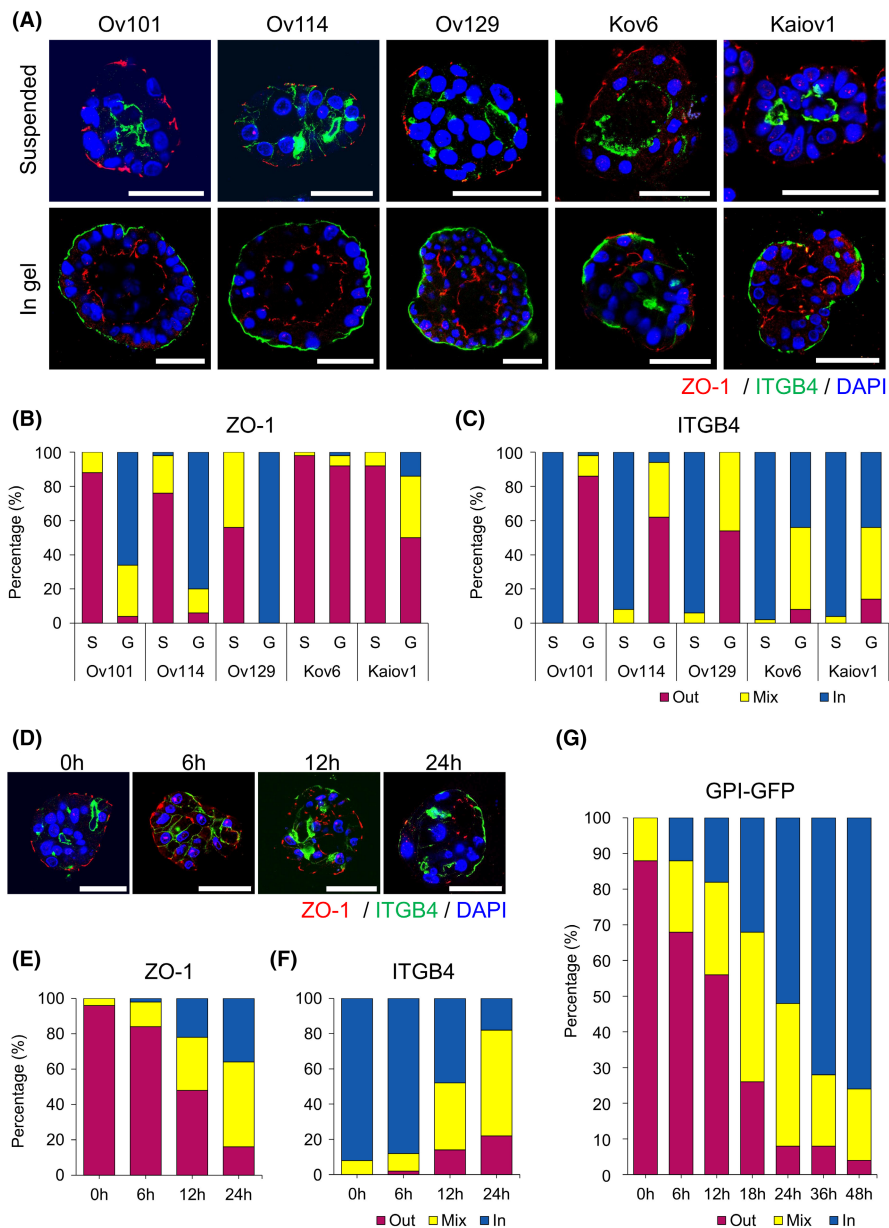
By shaking the culture plate, the retention of the organoids to the culture vessel surface after exposure to mechanical force was evaluated to quantify the strength of the adhesion of the organoids to the Matrigel-coated culture vessel surface. The adhesive organoids' area that remained on the culture vessel increased over time (Figure 4C). Interestingly, the increase in adhesion strength paralleled the progression of polarity switching in the organoids placed on Matrigel (Figure 4B), indicating the correlation between polarity switching and adhesion of the organoids to ECM. Next, the SFK inhibition effect on

the adhesion of the organoids to the Matrigel-coated culture vessel was evaluated. Unsurprisingly, dasatinib significantly suppressed the progression of adhesion over time (Figure 4D), similar to the polarity switching (Figure 3C,D). Dasatinib strongly inhibited the organoids' adhesion to Matrigel-coated plate in all five ovarian organoid lines tested (Figure 4D). Similarly, PP1 also inhibited these organoids' adhesion, although insignificantly (Figure S8). Notably, when dasatinib was added 24 hours after placing the organoids on the Matrigel-coated plates, the organoids were undetached, indicating adhesion was not reverted by dasatinib once established (Figure 4E). This is consistent with the previous result that the polarity switch was not reverted by dasatinib once established (Figure 3E). These results indicate that SFK-mediated polarity switching is involved in, and possibly triggers, the adhesion of OC clusters to ECM.

3.5 | Polarity switching occurs during the invasion of cell clusters through the mesothelial cell sheet

The experiments above model the adhesion of cell clusters to ECM. On the other hand, unless ECM is exposed, cancer cell clusters

FIGURE 2 The polarity of the ovarian cancer (OC) cell cluster exhibits dynamic changes upon contact with extracellular matrix (ECM). A, Immunofluorescent staining of OC organoids under suspended culture (upper panels) and Matrigel-embedded culture (lower panels). Histological subtypes for each case are described in Table S1. Green, ITGB4; red, ZO-1; blue, DAPI; scale bars, 50 μ m. B, C, Quantitative analysis of ZO-1 (B) and ITGB4 (C) localization at suspended culture condition (S) and Matrigel-embedded condition (G). $N = 50$ for each condition. D-F, Time course changes in the polarity status of Ov114 organoids after embedding in Matrigel. Representative immunofluorescent images of each time point (D) and quantitative analyses of ZO-1 (E) and ITGB4 (F) localization at each time point are shown. $N = 50$ for each condition. Green, ITGB4; red, ZO-1; blue, DAPI; scale bars, 50 μ m. G, Time course changes in the polarity status of GPI-GFP Ov114 organoids after embedding in Matrigel. Definition and representative images of each status are described in Figure S3



need to invade via the mesothelial cell layer after initial attachment to establish peritoneal metastatic foci. OC organoids were cocultured with a mesothelial cell line, HOMC-B1,¹⁵ to further evaluate whether SFK-mediated polarity switching is involved in the peritoneal metastasis. When OC organoids were seeded on the confluent mesothelial cell sheet, OC organoids adhered to the mesothelial cells, and the adhered organoids demonstrated a clearance of the mesothelial cell sheet (Figure 5A). The mesothelial cells' clearance area quantification revealed that the clearance proceeded over time (Figure 5B), indicating that the mesothelial layer clearance is not by an accidental encountering of the organoids to the region uncovered by mesothelium but an active and dynamic mesothelial layer extrusion. Confocal microscopic analysis showed that the surface of the organoids attached to the mesothelial cells demonstrated heterogeneous ITGB4 expression. In some parts, ITGB4 was expressed at the interface of the organoids and mesothelial cells, while in other

parts, ITGB4 was undetected at the interface (Figure 5C, panel i-iii). Conversely, in most parts where the organoids cleared the mesothelial cells, ITGB4 was expressed at the interface of the organoids and the cleared area (Figure 5C, panels iv-vi), consistent with the case in which the organoids attached to the Matrigel-coated plastic surface without mesothelial cells (Figure 4A). These results indicate that polarity switching is involved in cancer cluster invasion through the mesothelial cell layer, and the event might precede mesothelial clearance.

3.6 | SRC family kinase inhibition suppresses peritoneal metastasis of OC cell clusters in vivo

Next, the involvement of polarity switching in establishing peritoneal metastasis foci in vivo was investigated. The model was established

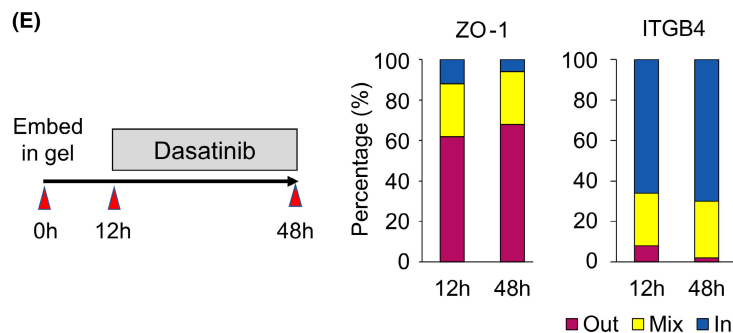
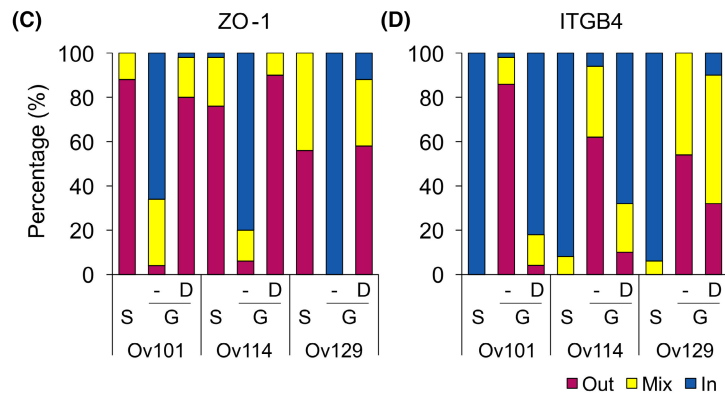
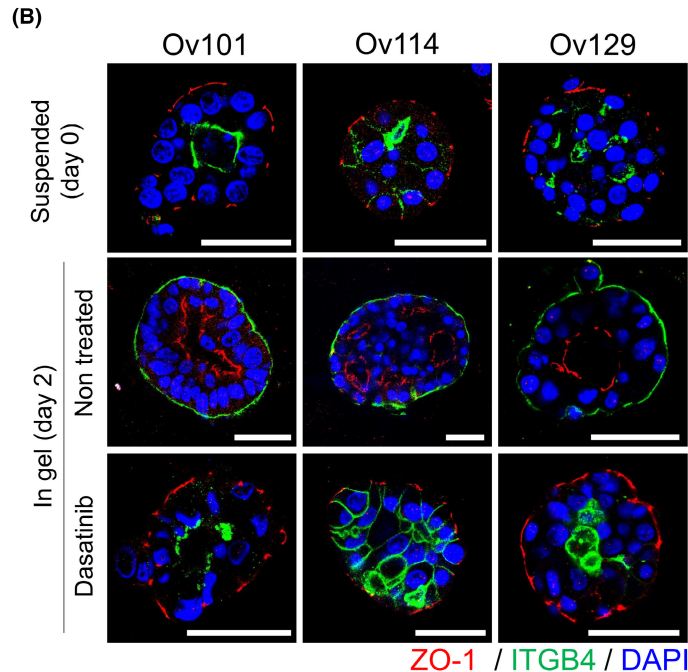
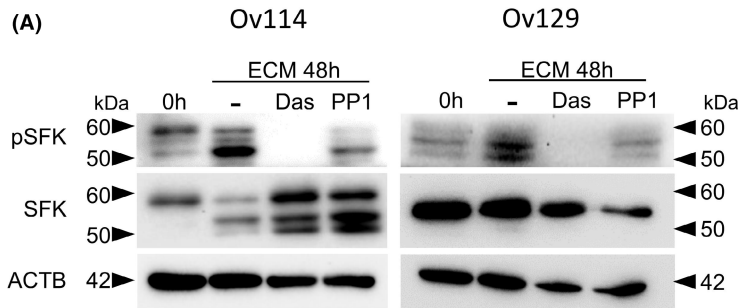


FIGURE 3 Polarity switching of ovarian cancer (OC) cell clusters occurs via SRC family kinase (SFK) activation triggered by contact with extracellular matrix (ECM). A, Western blot of Ov114 and Ov129 organoids before (0h) and after being embedded in Matrigel and cultured for 48 h (ECM 48 h) in the absence or presence of SFK inhibitors (500 nM dasatinib [Das] and 10 μ M PP1). Beta-actin (ACTB) was used for loading control. B, Immunofluorescent images of organoids embedded in Matrigel cultured with or without 500 nM dasatinib. Green, ITGB4; red, ZO-1; blue, DAPI; scale bars, 50 μ m. C, D, Quantitative analysis of ZO-1 (C) and ITGB4 (D) localization after embedding in Matrigel with dasatinib treatment. Polarity status was evaluated for suspended (S) and Matrigel-embedded condition (G), either nontreated (-) or with dasatinib (D). The data for suspended culture, as baseline control, is identical with Figure 2E,F. $N = 50$ for each condition. E, Illustrated protocol for the experiment (left panel). Dasatinib treatment started 12 h after embedding in Matrigel and was evaluated 48 h after embedding. Quantitative analysis of ZO-1 (middle panel) and ITGB4 (right panel) localization of Ov114 organoids. $N = 50$ for each condition

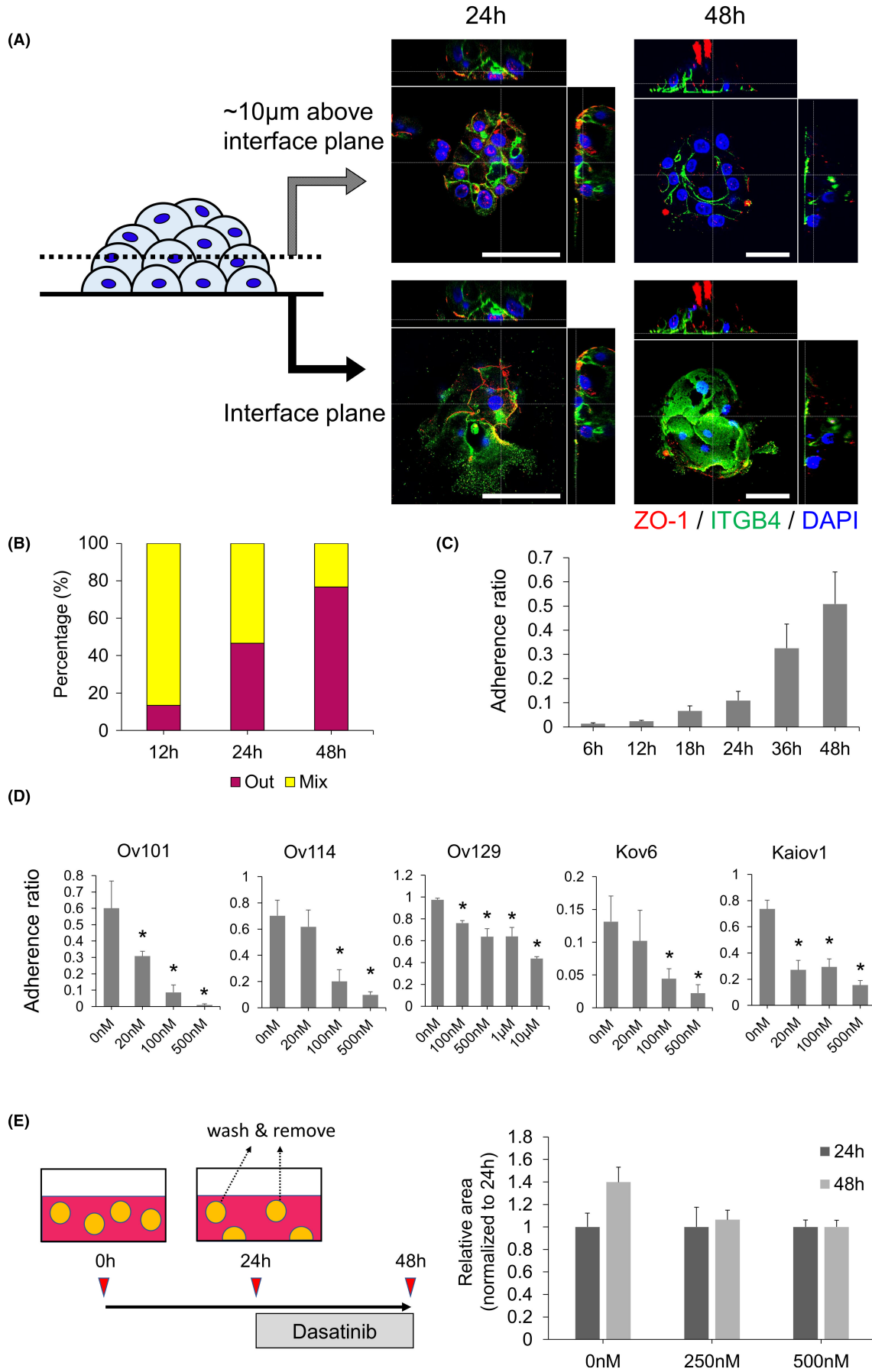


FIGURE 4 Legend on next page

FIGURE 4 Polarity switching is involved in the adhesion of cell clusters to matrices. A, Confocal immunofluorescent images of Ov114 organoid adhering on Matrigel-coated plastic surface at 24 and 48 h. Images are shown with orthogonal views, as illustrated in the left panel. Green, ITGB4; red, ZO-1; blue, DAPI; scale bars, 50 μ m. B, Time course quantitative analysis of ITGB4 expression at the adhering interface of Ov114 organoids and extracellular matrix (ECM) coated plastic surface. Organoids that failed to adhere to the plastic surface were excluded from the quantification. $N = 30$ for each time point. * $p < 0.05$, Dunnett's test. C, Time course quantitative analysis of Ov114 organoids tightly adhered to the Matrigel-coated plastic surface. After shaking the culture plate, the percentage of organoids persisting on the plastic surface was quantified. $N = 3$ for each time point. Data are presented as mean \pm SD. D, Quantitative analysis of the organoids tightly adhered to the Matrigel-coated plastic surface. Percentages of organoids treated with increasing doses of dasatinib were quantified for persistence on the plastic surface after shaking the culture plate. $N = 3$ for each dose. Data are presented as mean \pm SD, * $p < 0.05$, Dunnett's test. E, Illustrated protocol for the quantitative analysis of organoids adhering on the Matrigel-coated plastic surface (left panel). Dasatinib was added 24 h after placing organoids on the ECM-coated plates. Relative change of the area of the organoids persisting on the plastic surface is shown. $N = 3$ for each condition. Data are presented as mean \pm SD

by injecting Ov114 organoids maintained under suspension culture into the peritoneal cavity of female NSG mice. Peritoneal metastasis foci were observed after 4 weeks. Immunofluorescent analysis revealed that the metastatic foci expressed ITGB4 at most of the adhering interface of Ov114 cell clusters to the host, while ZO-1 was fairly visible inside of the clusters (Figure 6A), indicating that polarity switching occurred during the metastatic process. The effect of the polarity-switching inhibition *in vivo* was evaluated by treating the mice with dasatinib, which suppressed polarity switching without growth inhibition *in vitro* (Figure 4D and Figure S4). First, the dasatinib effect on xenograft tumor growth was assessed. Daily oral administration of 50 mg/kg dasatinib to the mice bearing subcutaneous Ov114 tumors did not exhibit any inhibitory effect on tumor growth (Figure 6B). Finally, the dasatinib effect on the establishment of peritoneal metastasis was evaluated. The mice were sacrificed 4 weeks after injection of the organoids, and the number of peritoneal metastatic foci was evaluated. Treatment with dasatinib significantly suppressed the number of peritoneal metastatic foci (Figure 6C) and the weight of the liver and diaphragm, indicating that the amount of the metastatic foci on these organs tended to be lighter in dasatinib-treated mice (Figure 6D). Conversely, the metastatic tumor size was not reduced in line with the previous experiments, showing that dasatinib did not inhibit the organoids' growth (Figure 4D and Figure S4) nor the established tumors (Figure 6B). These results indicate that SFK inhibition suppresses the incidence of peritoneal metastasis of OC cell clusters *in vivo*, possibly by suppressing polarity switching.

4 | DISCUSSION

In this study, it was reported that OC exhibits apicobasal polarity in primary cancer tissues. Cancer cell clusters in the peritoneal fluid and in the organoids derived from patients with OC preserved the polarity. Such apicobasal polarity in OC cell clusters altered dynamically upon attachment to Matrigel; apical-out for the cell clusters floating in peritoneal fluid or in culture medium, and basal-out for the cell clusters attaching to Matrigel. In addition, the polarity switching of the OC cell clusters was essential for the subsequent adhesion to Matrigel. Therefore, the polarity switching is a vital step for peritoneal metastasis, which is regulated by SFK activation, similar to our

previous report in colorectal cancer.¹¹ Treatment with dasatinib, an SFK inhibitor, suppressed the polarity switching *in vitro* and peritoneal metastasis *in vivo*.

Ovarian cancer peritoneal metastasis occurs via multiple biological processes in which cancer and various types of microenvironment cells, such as inflammatory cells, MSC, fibroblasts, adipocytes, and mesothelial cells, are involved.⁴⁻⁶ Additionally, investigations on these complex biological mechanisms have shown that cancer cell clusters detached from the original tumor mass, either as clusters or as single cells that later form spheroids in the peritoneal cavity, play essential roles in peritoneal metastasis.^{5,6} Previous studies have shown the advantages of forming cell clusters for the survival^{16,17} and establishment of metastasis^{18,19} as well as chemoresistance.^{20,21} A simple explanation for this is that cell clusters can avoid anoikis, a particular form of apoptosis induced by a loss of anchorage.^{16,17} However, in most of the biological studies conducted with established cell lines, the importance of the polarity in OC cell clusters has not been well studied or even documented. In this study, ZO-1 and ITGB4 were successfully used as apical and basal markers, respectively; ZO-1 is a tight-junction protein expressed at the apical side of epithelial cells, while ITGB4 is an adhesion molecule that interacts with ECM proteins. Integrins are proteins important for establishing peritoneal metastasis. It has been reported that integrins regulate the adhesion of OC cells to ECM^{22,23} and mesothelial cells.²⁴⁻²⁶ Importantly, our data revealed that OC cell clusters in the patient's peritoneal cavity are at apical-out status where ITGB4 was not exposed to the surface of cell clusters. This is recapitulated in the organoids but not in OC cell lines,²² suggesting the importance of choosing a model suitable for investigating cancer cell clusters. Considering the accumulated knowledge on the importance of integrins for the interaction between cancer cell clusters and ECM as well as mesothelial cell, and our findings that polarity switching is tightly linked with the relocation of integrins to the ECM interface, polarity switching is a critical step to establish adhesion to ECM. Indeed, polarity switching suppression by SFK inhibitor significantly reduced OC organoid adhesion to Matrigel. Notably, in the OC organoids, collagen type I only induced loss of polarity and did not complete the switch (Figure S9), differently from CRC organoids, in which both Matrigel and collagen type I induced polarity switching.¹¹ Such ECM selectivity in polarity switching might affect the organ selectivity in metastasis.

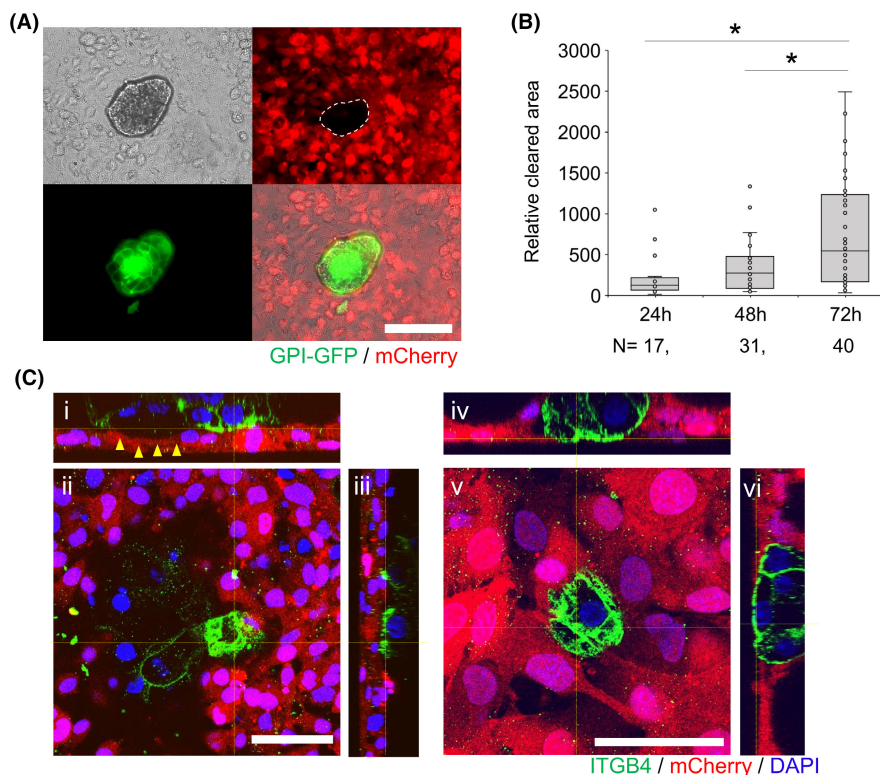


FIGURE 5 Polarity switching occurs during the invasion of OC cell clusters through mesothelial cell sheet. A, Images of Ov114 organoid invading HOMC-B1 mesothelial cell sheet. Bright-field image (upper left), mCherry-expressing mesothelial cells (red, upper right), GPI-GFP Ov114 organoid (green, lower left), and merged image (lower right) are shown. Dotted line indicates the area where HOMC-B1 cells were cleared. Scale bars, 100 μ m. B, Quantitative analysis of cleared areas in HOMC-B1 cell sheet by invading GPI-GFP Ov114 organoids. Data are presented as box plots. Each dot indicates a cleared spot. $N = 17, 31, \text{ and } 40$ for each time point. $*p < 0.05$, Steel-Dwass test. C, Confocal immunofluorescent images of Ov114 organoid attached on the mCherry (red)-expressing HOMC-B1 cells labeled for ITGB4 (green). Images of the XY plane (panels ii and v) are shown with orthogonal views (panels i, iii, iv, and vi). Yellow arrowheads indicate the interface of mesothelial cells and metastatic cancer cell clusters lacking ITGB4 expression. Green, ITGB4; red, mCherry; blue, DAPI; scale bars, 50 μ m

Some literature has implicated the efficacy of SFK inhibition in OC. In vitro and in vivo studies using OC cell lines showed that dasatinib combined with gefitinib suppressed growth and invasion of OC cells,²⁷ a specific SFK inhibitor PP2 suppressed anchorage-dependent and -independent growth of phospho-SFK-positive OC cells,²⁸ and dasatinib suppressed SFK activation induced by paclitaxel with a reduced tendency of invasion.²⁹ Conversely, clinical trials showed that dasatinib single-agent treatment did not have clinical efficacy,³⁰ and saracatinib, an SFK inhibitor, did not improve the effect of weekly paclitaxel in platinum-resistant OC.³¹ Using OC organoid, it was shown that dasatinib inhibited organoids' adhesion to ECM and suppressed the establishment of peritoneal metastasis, possibly through polarity-switching inhibition, while the tumor volume was not reduced once colonized and tumor formed, being consistent with the clinical trial. Thus, the effective clinical use of SFK inhibitor would be better focused on preventing peritoneal metastasis at appropriate timings. For example, perioperative peritoneal treatment might be a proper way, expecting the prevention of peritoneal metastasis induced by surgical stress,^{32,33} particularly on the surgical wound.^{34,35}

Mesothelial cells secrete wide ranges of ECM, including collagen types I, III, and IV; fibronectin; and laminin.³⁶ It has been reported

that ECM, such as fibronectin, is vital for the initial attachment of cancer cell clusters to mesothelial monolayer.^{6,37} However, this interaction of cancer cells with mesothelial cells is less firm than with the basement membrane.³⁸ Thus, mesothelial clearance and subsequent adherence to the underlying ECM is an important step for establishing peritoneal metastasis. OC cell clusters disrupt mesothelial cell-cell junctions and penetrate under the mesothelial layer, where integrin and myosin are involved in generating actomyosin-generated force to physically displace mesothelial cells.³⁹ In our experiments, OC organoid exhibited the ability to clear and invade the mesothelial layer, and polarity switching occurred during the invasion step through the HOMC-B1 mesothelial cell line. Further investigation is needed to elucidate whether the ECMs secreted by the mesothelial cells influence polarity switching.

In this study, the role of polarity switching during the steps of OC peritoneal metastasis mediated by SFK activation was described. The finding provided new insight into the understanding of peritoneal metastasis that integrins are not initially exposed at the surface of cell clusters and the polarity switching is a critical step that lies between mesothelial clearance and adhesion to ECM. Additionally, polarity switching can be a novel therapeutic target for suppressing peritoneal metastasis. Additional key molecules and pathways for

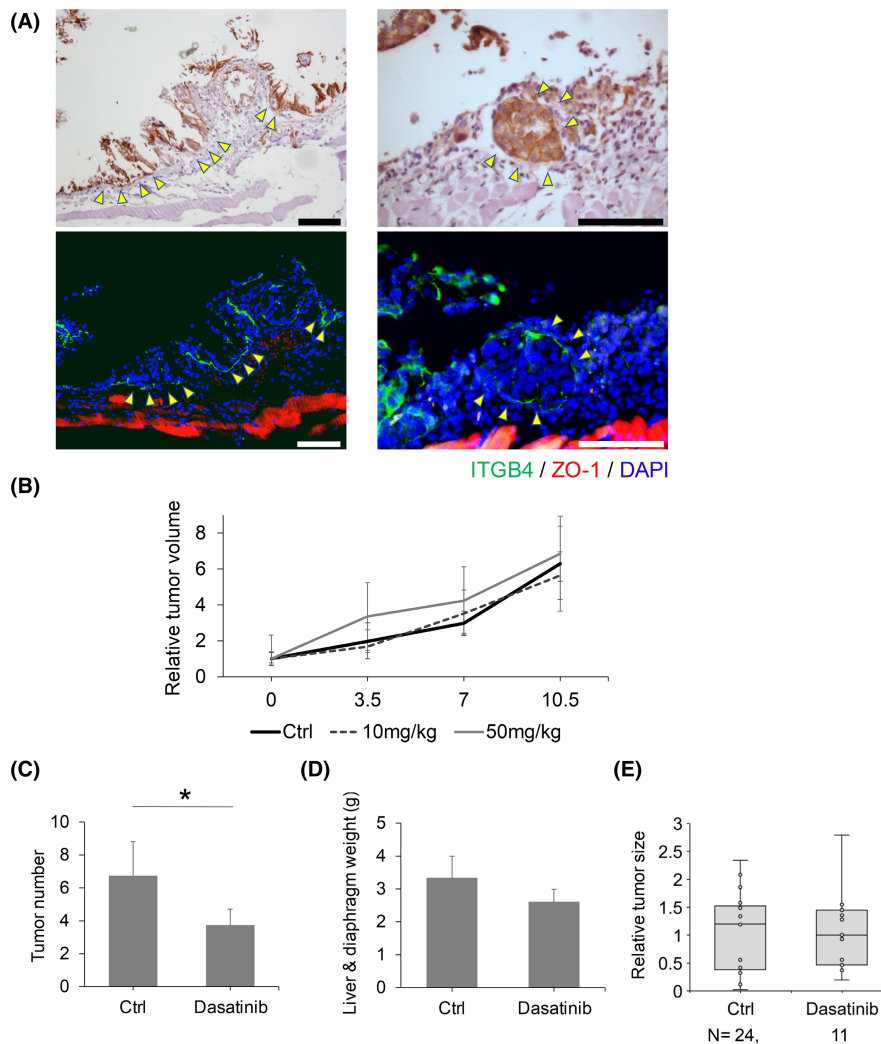


FIGURE 6 SRC family kinase (SFK) inhibition suppresses peritoneal metastasis of ovarian cancer (OC) cell clusters in vivo. **A**, Immunohistochemistry images for E-cadherin (upper panels) and immunofluorescent images (lower panels; green, ITGB4; red, ZO-1; blue, DAPI) of serial sections from peritoneal metastatic foci of Ov114 in NSG mouse. Two representative foci are shown. Yellow arrowheads indicate the border of the tumor and peritoneal wall in each focus. Scale bars, 100 μ m. **B**, Growth curves of subcutaneous xenograft tumors originating from Ov114 organoids with the oral treatment of dasatinib or buffer alone (Ctrl). Data are shown as mean \pm SD. $N = 6$ per group. One-way ANOVA detected no significance. **C**, Number of overt peritoneal metastatic foci of NSG mice 4 weeks after peritoneal Ov114 organoids injection, treated either with dasatinib or buffer alone (Ctrl). Data are presented as mean \pm SD. $N = 4$ per group. * $p < 0.05$, *t* test. **D**, Weight of the liver and the diaphragm of NSG mice 4 weeks after peritoneal Ov114 organoids injection, treated either with dasatinib or buffer alone (Ctrl). Data are presented as mean \pm SD. $N = 4$ per group. No significance was detected using *t* test. **E**, Relative tumor volume of overt peritoneal metastatic foci in NSG mice 4 weeks after peritoneal Ov114 organoids injection, treated either with dasatinib or buffer alone (Ctrl). Data are presented as box plots. $N = 24$ and 11 per group. No significance was detected using the Wilcoxon rank-sum test

polarity switching need to be elucidated. The organoid model can be used to screen effective reagents to suppress polarity switching and adhesion to the mesothelium and ECM.

AUTHOR CONTRIBUTIONS

M Kawata: Investigation, Methodology, Writing – Review & Editing, **J Kondo:** Conceptualization, Validation, Visualization, Writing – Original draft preparation, **K Onuma:** Methodology, **Y Ito:** Resources, **T Yokoi:** Resources, **J Hamanishi:** Resources, **M Mandai:** Resources, **T Kimura:** Writing – Review & Editing, **M Inoue:** Supervision, Validation, Writing – Review & Editing, Funding acquisition.

ACKNOWLEDGMENTS

The authors thank Dr. Y. Sekido for kindly providing HPMC-B1 cells, Dr. S. Kawahara for technical assistance, and M. Izutsu for secretarial assistance.

FUNDING INFORMATION

This study was supported by the Japan Society for the Promotion of Science grant-in-aid for scientific research (B) 18H02648 and 21K07942 (KO, MI); a grant-in-aid from P-CREATE, Japan Agency for Medical Research and Development 19cm0106203h0004 (JK, KO, MI) and 21am0401004h0003 (JK, KO, JH, MM, MI); a grant-in-aid

from Science and Technology Platform Program for Advanced Biological Medicine; and a grant-in-aid from Takeda Science Foundation (MI).

DISCLOSURE

KO and MI are members of the Department of Clinical Bio-resource Research and Development at Kyoto University, which is sponsored by KBBM, Inc. The other authors declare no conflict of interest associated with this manuscript.

ETHICS STATEMENT

The Institutional Ethics Committees at Osaka International Cancer Institute, Osaka University, Kyoto University, and Kaizuka City Hospital approved this study. Patient samples and information were collected under written informed consent. The Institutional Animal Care and Use Committees of Kyoto University and Osaka International Cancer Institute approved the animal studies, and they were performed according to institutional guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kawata M, Kondo J, Onuma K, et al. Polarity switching of ovarian cancer cell clusters via SRC family kinase is involved in the peritoneal dissemination. *Cancer Sci*. 2022;113:3437-3448. doi: [10.1111/cas.15493](https://doi.org/10.1111/cas.15493)