Metastatic seeding of human colon cancer cell clusters expressing the hybrid epithelial/mesenchymal state

Kosuke Mizukoshi1,2*, Yu Okazawa1,2*, Hiroshi Haeno4,4, Yu Koyama2,5, Kaidiliayi Sulidan2,6, Hiromitsu Komiyama1, Harumi Saeki7, Naomi Ohtsuji1, Yasuhiko Ito2, Yutaka Kojima3, Michitoshi Goto1, Sonoko Habu7, Okio Hino2, Kazuhiro Sakamoto1 and Akira Orimo2

1Department of Coloproctological Surgery, Juntendo University Faculty of Medicine, Tokyo, Japan
2Department of Molecular Pathogenesis, Juntendo University Faculty of Medicine, Tokyo, Japan
3Division of Translational Genomics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Chiba, Japan
4Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan
5Department of Oral Pathobiological Science and Surgery, Tokyo Dental College, Tokyo, Japan
6Department of Obstetrics and Gynecology, Graduate School of Medicine, Juntendo University, Tokyo, Japan
7Atopy Research Center, Juntendo University Faculty of Medicine, Tokyo, Japan

Emerging evidence supports the theory that tumor cell clusters efficiently metastasize to distant organs. However, the roles of epithelial-to-mesenchymal transition (EMT) in metastasizing tumor cell clusters have not yet been fully elucidated. To investigate this issue, tumor fragments were dissected from 40 colorectal cancer (CRC) patients and implanted subcutaneously into immunodeficient mice. We observed that tumors developed from the tumor fragments obtained from 28 of the 40 CRC patients. The tumors were then dissociated into cell suspensions to be orthotopically injected into secondary mice. The tumors from 13 of the 28 patients progressed. Furthermore, metastases formed spontaneously in the liver and lungs from the tumor fragments obtained from 8 of these 13 patients. Moreover, employing a mathematical analysis, we showed that tumor cell clusters seeded these metastases significantly more often than did single tumor cells. Membrane E-cadherin- and nuclear ZEB1-positive tumor cells indicating the hybrid epithelial/mesenchymal state were also detected in primary tumors of various CRC patients, and in the corresponding patient-derived xenografts (PDXs) and circulating tumor cell clusters in the bloodstream of mice. In contrast, ZEB1 staining was barely detectable in the patient-matched liver metastases presumably developing through mesenchymal-to-epithelial transition. Inhibition of E-cadherin or ZEB1 expression by shRNA notably

*K.M. and Y.O. contributed equally to this work


Additional Supporting Information may be found in the online version of this article.

Key words: patient-derived tumor xenografts, human colon tumor organoids, epithelial-to-mesenchymal plasticity, partial EMT, metastasis

Abbreviations: CEA: carcinoembryonic antigen; cPARP: cleaved poly ADP-ribose polymerase; CRC: colorectal cancer; CTC: circulating tumor cell; E/M: epithelial/mesenchymal; E-cad: E-cadherin; EDTA: ethylenediaminetetraacetic acid; EMT: epithelial-to-mesenchymal transition; FBS: fetal bovine serum; FN: fibronectin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MET: mesenchymal-to-epithelial transition; NOG: NOD/Shi-scid IL2R null; pan-CK: pan-cytokeratin; PBS: phosphate buffered saline; PDXs: patient-derived xenografts; vim: vimentin

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Correspondence to: Akira Orimo, Department of Molecular Pathogenesis, Juntendo University Faculty of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, Tel.: +81-3-5802-1039, Fax: +81-3-35684-1646, E-mail: aorimo@juntendo.ac.jp; or Kazuhiro Sakamoto, Department of Coloproctological Surgery, Juntendo University Faculty of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, E-mail: kazuaka@juntendo.ac.jp

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What’s new?
Coherent sheets of tumor cells known as tumor cell clusters exhibit a high degree of competency when colonizing distant organs during metastasis. Whether tumor cell clusters undergoing metastasis are influenced by epithelial-to-mesenchymal transition, however, remains unknown. Here, in patient-derived tumor xenograft murine models of colorectal cancer (CRC), hybrid epithelial/mesenchymal plasticity in tumor cell clusters was found to drive metastatic seeding. Human CRC cell clusters seeded spontaneous metastases more often than single tumor cells. In addition, E-cad- and ZEB1-expressing hybrid tumor cells were detectable in CRC cell clusters. Inhibition of these factors attenuated liver metastasis in mice following intrasplenic CRC cell organoid injection.

Introduction
Colorectal cancer (CRC) is the third most commonly diagnosed tumor and the fourth leading cause of cancer-related mortality worldwide. Metastatic spread is a major cause of death in affected patients and CRC often metastasizes to the liver and lungs after curative resection of the primary tumor plus adjuvant therapies. In order to improve current cancer treatment strategies and develop effective new, possibly radical, therapies, a precise understanding of tumor metastasis biology is essential.

Epithelial-to-mesenchymal transition (EMT) is widely accepted as a main driver of tumor progression. Single cancer cells detaching from the epithelial sheet via EMT are believed to intravasate, survive in the circulation, extravasate and ultimately colonize distant organs. The last step of the metastatic process, colonization of distant organs is believed to be mediated via mesenchymal-to-epithelial transition (MET) that facilitates the dissemination of carcinoma cells to form micro- and macro-metastases. The requirement for EMT involvement in metastasis has, however, long been debated because of difficulty in identifying cancer cells showing EMT histopathologically in tumors from patients. In contrast, a recent emerging view supports the importance of epithelial-mesenchymal plasticity, as exemplified by partial EMT frequently observed in various human carcinomas; the hybrid epithelial/mesenchymal (E/M) state plays crucial roles in both invasion and metastasis.

A group of carcinoma cells that express E-cadherin (E-cad) and thus maintain their cell–cell adhesions collectively migrate as coherent sheets to seed metastasis, a process known as “collective cell migration.” These cells, constituting a so-called “tumor cell cluster or embolus” have, in fact, often been observed histologically within the tumor-associated vasculature in various cancers. This raises the possibility of tumor cell clusters being passively shed from tumor islands to invade blood vessels and become trapped on the vascular endothelium. Moreover, multicellular circulating tumor cell (CTC) clusters were characterized based on the peripheral blood circulation of patients suffering from various carcinomas including those of the colon. These CTC clusters were notably shown to be more competent at colonizing distant organs than single CTCs. Furthermore, CTC clusters tended to be highly detected in the advanced stages of cancer and correlated with poorer patient outcomes. Collectively, these findings support the notion that the formation of tumor cell clusters, maintaining their cell-to-cell adhesions, contributes significantly to the seeding of metastasis. Moreover, partial EMT contributes to the formation of tumor cell clusters and thereby allows their collective invasion and dissemination.

However, roles of EMT in the tumor cell clusters involved in metastasis formation remain unknown. Studies focusing on collective cancer cell migration have also depended mostly on specific cultured cell lines and experimental murine tumors, while investigations using human clinical materials are rare. The cellular and molecular mechanisms, by which tumor cell clusters invade and seed metastases via EMT, also have not as yet been sufficiently examined using human materials.

In our study, fresh tumor tissues dissected surgically from 40 CRC patients in total were implanted subcutaneously into immunodeficient mice, followed by their subsequent orthotopic transplantation into secondary recipient mice. To explore the possible roles of E/M plasticity in metastatic seeding of CRC cell clusters, we have established eight transplantable patient-derived xenografts (PDXs), which have been shown to metastasize spontaneously to distant organs, and the corresponding tumor organoids were examined in culture.

Materials and Methods
Tissue collection and patient information
All patients provided written informed consent and our study was approved by the Research Ethics Committee of the Juntendo University Faculty of Medicine. CRC patients with no history of preoperative therapies were enrolled. Primary CRC tissues were obtained as soon as possible after surgical resection, and sections for immunohistochemistry were obtained from formalin-fixed paraffin-embedded tissue blocks. Detailed clinicopathological information, including tumor location and TNM status, was summarized for each case (Supplementary Tables S1 and S2a).
Animals
Male NOD/Shi-scid IL2Rγ null (NOG) mice at 6 weeks of age were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). Mice were bred under germ-free and specific pathogen-free conditions, and the experiments were approved by the Animal Research Ethics Committee of the Juntendo Faculty of Medicine.

Establishment of the CRC PDX mouse models by subcutaneous and orthotopic implantation
Fresh tumor specimens surgically dissected from CRC patients were implanted subcutaneously into NOG mice, as previously described. Tumor xenografts that developed, to ~1 cm³ in size, were also resected and grossly necrotic areas were removed. The tumors were then minced mechanically with a razor blade and transferred into a 15 ml tube containing DMEM/F-12 with GlutaMAX™ supplemented (Thermo Fisher Scientific, Waltham, MA) with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), penicillin (100 U/ml)-streptomycin (100 μg/ml, Thermo Fisher Scientific) and collagenase Type I (1.5 mg/ml; Sigma-Aldrich) followed by slow agitation for 2 hr at 37°C. The resulting solution with enzymatically digested tissues was filtrated twice using a cell strainer with a 40 μm pore size (Corning, NY). The resulting tumor cell suspension (50 μl) including 1 × 10⁶ PDX-dissociated cells with 50% Matrigel (Corning, NY) was then injected orthotopically into the rectal submucosa of NOG mice using a Hamilton syringe (needle size: 22 gauge, Tokyo Science, Tokyo, Japan). Mice with xenografts that grew orthotopically were then sacrificed to assess metastases that had grown spontaneously.

Establishment of experimental tumor and metastasis models of CRC tumor organoids
Tumor cell organoids were prepared from CRC PDXs prior to in vitro culture, as described previously. These tumor organoids were also infected with or without lentiviral particles expressing GFP and different shRNAs. To generate primary tumors, the CRC organoid cell suspension (50 μl) including 5 × 10⁶ cells with 50% Matrigel (Corning, NY) was injected orthotopically into the rectal submucosa of NOG mice using a Hamilton syringe (needle size: 22 gauge, Tokyo Science) (Figs. 2c–2e and 3; Supplementary Figs. S2b–S2d and S6a), as described previously. To generate liver metastases, the CRC organoid cell suspension (50 μl) including 4 × 10⁴ cells in phosphate buffered saline (PBS) was injected intrasplenically into NOG mice using a Hamilton syringe (needle size: 22 gauge, Tokyo Science) (Figs. 2f–2h, 4b, 4c, 5c, and 5d; Supplementary Figs. S2c–S2g), as described previously.

Retroviral and lentiviral infections
Retroviral and lentiviral infections were performed as described previously. After infection, human colon fibroblasts (pBabe-hTERT-puro vector) and tumor organoids (PLKO-1-shRNA-hygro vectors) were cultured for 4–6 days in the presence of the appropriate antibiotic for each plasmid; puromycin (1 μg/ml) or hygromycin (50 μg/ml). Tumor organoids were also infected with nearly 90% infection efficiency by a PRRL-GFP virus enriched by ultracentrifugation.

Cell culture of human colon fibroblasts
Primary fibroblasts were extracted from a non-tumor-associated region of human colon tissues as described previously. The retroviral pBabe-puro vector expressing hTERT cDNA was introduced to achieve their immortalization and cultured in DMEM/F-12 medium with GlutaMAX™ supplement (Thermo Fisher Scientific) containing 10% FBS (Supplementary Fig. S4b).

Preparation of blood smear using cytospin centrifuge for detection of CTCs
Peripheral blood (~500 μl) was collected from the submandibular vein of a mouse bearing 1- to 2-month-old tumors raised by implantation of CRC PDXs and tumor organoid cells (Figs. 1e and 2d). Blood sampling was performed from 4 to 6 mice for each group and was repeated on a weekly basis until the objective achievement. Samples transferred into ethylenediaminetetraacetic acid (EDTA)-containing tubes were incubated for 10 min with lysis buffer (150 mM NH₄Cl, 40 mM KHCO₃ and 0.1 mM EDTA) to eliminate red blood cells. The resulting cells were then centrifuged at 4500 rpm for 5 min and washed with PBS and centrifuged twice again. The pellets were dissolved in 500 μl of 1% FBS in PBS. Then, 100 μl aliquots of the cell suspensions were pasted to a silane-coated slide at 800 rpm for 3 min using a Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific) to prepare five slides in total per mouse. The slides were dried in air for at least 20 min and stored at ~80°C.

Mathematical estimation of the number of cancer cells in a disseminating tumor cell cluster during metastatic colonization
The P10 CRC organoids were infected with GFP lentiviral particles, allowing generation of GFP-positive and -negative tumor cells prior to orthotopic injection of 5 × 10⁵ cells into three NOG mice (mice 1–3). Orthotopic tumors, the liver and lungs were dissected at 74–85 days after injection. The sections were also prepared from these tissues to be stained with anti-GFP-antibody. Both GFP-positive and -negative cancer cells were then counted in each of 10 fields in orthotopic tumor sections at 400× magnification (Supplementary Fig. S3a and Table S3A). The GFP-positive tumor cells were not uniformly distributed and the frequency varied among locations within tumors. In this case, the local frequency of GFP-positive tumor cells, which is a random variable, ranges from zero to one and the distribution pattern cannot readily be discerned. Therefore, we assumed that the beta distribution is applicable to predicting the local frequencies of GFP-positive cells in orthotopic tumors (Supplementary Fig. S3a). Then, the probability that the frequency of GFP-positive cells in some regions of tumors becomes x is given by
\[ p(x; \alpha, \beta) = \frac{x^\alpha (1-x)^\beta}{B(\alpha, \beta)}. \] (1)

Here, \( B(\alpha, \beta) \) represents the beta function. From the experimental data showing that the total tumor cell counts were 165, 183, and 154 in tumors raised in mice 1–3 (Supplementary Fig. S3a), we calculated the mean and the variance of the frequency of GFP-positive cells to be 0.373 (mean) and 0.120 (variance) for mouse 1, 0.402 and 0.054 for mouse 2, and 0.189 and 0.044 for mouse 3, respectively. Applying these values, we obtained \( \alpha = 0.354 \) and \( \beta = 0.594 \) for mouse 1, \( \alpha = 1.388 \) and \( \beta = 2.062 \) for mouse 2 and \( \alpha = 0.472 \) and \( \beta = 2.030 \) for mouse 3. We thus confirmed that the beta functions for mice 1–3 fit the data very well (Supplementary Fig. S3b).
Next, we anticipated the chimeric patterns of GFP-positive cells in metastases after dissemination (Fig. 3a). By predicting the number of tumor cells in a disseminating tumor cell cluster during seeding to be “n,” the probability that “y” GFP-positive cells are involved in the cluster follows a beta-binomial distribution and is given by

$$P(y) = \frac{\binom{n}{y} \binom{n-y}{P_0} (1-P_0)^{n-y}}{\binom{n}{y}} \frac{\sum_{x=0}^y \binom{y}{x} \binom{n-y-x}{P_{100}} (1-P_{100})^{n-y-x}}{\binom{n}{y}} \frac{\sum_{x=0}^y \binom{y}{x} \binom{n-y-x}{P_{mix}} (1-P_{mix})^{n-y-x}}{\binom{n}{y}}$$

(2)

By substituting “0” and “n” for “y” in equation (2), we obtained $P(0)$ and $P(n)$ as follows:

$$P(0) = \frac{B(\alpha+n,\beta)}{B(\alpha,\beta)}$$

(3)

and

$$P(n) = \frac{B(\alpha+n,\beta)}{B(\alpha,\beta)}$$

(4)

Here, $P(0)$ represents the probability that there are no GFP-positive cells comprising the tumor cell clusters, which would lead to all metastases being GFP-negative, whereas $P(n)$ represents the probability that all cells in the tumor cell clusters are GFP-positive, which would lead to all metastases being GFP-positive. The probability that the metastases contain a mixture of GFP-positive and -negative cells is given by $1 - P(0) - P(n)$.

We also obtained experimental data on the chimeric patterns of GFP-positive cells using immunohistochemistry with anti-GFP antibody. The ratios of the number of metastatic colonies, which are all GFP-positive ($d_{100}$), all GFP-negative ($d_0$) or a mixture of GFP-positive and -negative ($d_{mix}$) relative to the total number of colonies, were calculated in the liver and lungs as the frequency (Fig. 3a and Supplementary Table S3B). Then, the residual sum of squares between the theoretical equations and experimental data is given by

$$D = (P_0 - d_0)^2 + (P_{100} - d_{100})^2 + (P_{mix} - d_{mix})^2$$

(5)

Here, $P_0 = P(0)$, $P_{100} = P(n)$ and $P_{mix} = 1 - P(0) - P(n)$. By minimizing the residual sum of squares, we estimated the number of cancer cells in a disseminating cluster during metastatic seeding (Fig. 3b).

**Immunohistochemistry of blood smear, CRC organoids and tumor tissues**

Air-dried slides prepared from peripheral blood taken from mice bearing PDXs were fixed with 4% paraformaldehyde for 10 min and washed with PBS. Endogenous peroxidase was blocked with 0.3% H$_2$O$_2$ and sections were washed with PBS. Slides were incubated with primary antibodies at 4°C overnight. Sections were subsequently treated with secondary antibodies for 1 hr. Diaminobenzidine was used as the chromogen followed by hematoxylin counterstaining.

Matrigel with tumor organoids was solidified by iPGeLt® (Funakoshi, Tokyo, Japan) and then fixed with 10% neutral buffered formalin. Tumor organoids, PDXs and tumors dissected directly from CRC patients were immunostained according to standard procedures. The conditions and antibodies used are listed (Supplementary Table S4).

**Quantification of ZEB1-positive tumor cells in primary tumors and metastases**

Quantification of ZEB1-positive tumor cells was performed as follows. Ten different fields per each of the primary tumors and metastases were captured per slide using 400x magnification under a microscope (Figs. 1b and 1c). At a maximum,

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Figure 1. Development of tumors and metastases in mice bearing PDXs from 40 CRC patients. (a) Schematic representation of the generation of primary tumors and metastases in the CRC PDX mouse model. The number of mice developing subcutaneous (s.c.) tumors, orthotopic (o.t.) tumors (CRC, lymphoma and no growth) and spontaneous metastases (Spon. meta.) is shown as the numerator of a fraction within each bracket. The number of all examined mice is also included in the denominator. Liver metastatic nodules (yellow arrows) are generated spontaneously in mice bearing PDXs from the P10 patient. imp., implantation; inj., injection. Scale bar, 5 mm. (b) HE staining and immunohistochemistry of the primary CRC tumor dissected from the P10 patient and the corresponding PDXs orthotopically generated in mice (o.t. tumor) using anti-E-cadherin (E-cad), ZEB1, fibronectin (FN) and -vimentin (Vim) antibodies. Positive staining is indicated by the brown color. Dashed-line circles indicate small metastatic nodules. ZEB1-positive carcinoma cells are also indicated by arrows and ZEB1-, FN- and vim-positive stromal cells are depicted by arrowheads. Scale bar, 50 μm. (c) Immunohistochemistry of primary tumor and liver metastasis sections prepared from the same CRC patient (PL-P15) using anti-ZEB1 antibody (upper). Immunofluorescence of the primary tumor using anti-E-cad and -ZEB1 antibodies (lower). ZEB1-positive tumor cells (arrows) are shown (upper). E-cad$^{+/+}$ZEB1$^+$ (E/M) tumor cells (arrows) are shown (lower). ZEB1-positive stromal cells (arrowheads) are also shown (upper and lower). Upper: immunohistochemistry, scale bar, 50 μm. Lower: immunofluorescence, scale bar, 10 μm. (d) Immunofluorescence of sections prepared from primary tumors of the indicated CRC patients and the corresponding PDXs orthotopically generated in mice using anti-E-cad and -ZEB1 antibodies. E-cad$^{+/+}$ZEB1$^+$ (E/M) tumor cells (arrows) are shown in ZEB1-positive stromal cells (arrowhead) are also shown. Scale bar, 10 μm. (e) Immunohistochemistry of the blood smears prepared from mice bearing PDXs from the indicated patients using anti-carcinoembryonic antigen (CEA), -pan-CK, -E-cad and -ZEB1 antibodies. Positive staining is shown as brown. Nuclear ZEB1 staining (arrowheads) is also indicated. Nuclei of carcinoma cells in multicellular CTC clusters (simple arrows) and peripheral blood mononuclear cells (triangular arrows) are stained with hematoxylin. Scale bars, 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]
10 different metastatic nodules of the liver sections were evaluated per slide at 400× magnification under a microscope (Fig. 2h and Supplementary Fig. S2g). ZEB1-positive tumor cell proportions (%) were calculated as the ratio of the number of ZEB1-positive tumor cells relative to that of all tumor cells.

**Quantification of apoptotic CRCs in liver metastases**

To quantify the apoptotic tumor cell numbers, 40 random images at 400× magnification were taken from liver metastasis sections stained with anti-cleaved poly ADP-ribose polymerase (c-PARP) antibody in four different mice per group in Figure 4b. Images of the necrotic tissue areas were removed.
The proportions of c-PARP-positive cancer cells versus total carcinoma cells per field were evaluated.

Quantification of GFP-positive CRC organoids that metastasized to the lungs and liver
To evaluate metastases of GFP-positive CRC organoids to the lungs and liver, as shown in Figures 4b and 5c, the total intensity of GFP was quantified using Cell Profiler cell image analysis software (http://cellprofiler.org/).

Real-time RT-PCR analysis
Total RNA was extracted using NucleoSpin® RNA II (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer’s protocol. SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) was used to synthesize cDNA. Real-time RT-PCR analysis was performed as previously described. Data for each sample were normalized relative to the expression level of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Primers used for RT-PCR analyses are described below.

PCR primers for real-time PCR
E-cad
FW: 5'-TGCCAGAATGAAAAAGG-3'
RV: 5'-GTATGTGGCAATGCGTT-3'
ZEB1
FW: 5'-AGCAGTGGAGAGGAAATGC-3'
RV: 5'-GGTCTCTCTAGGCTCACG-3'
GAPDH
FW: 5'-ACCAGAGCTGGATGG-3'
RV: 5'-CTACAGCCGAGGCAGT-3'

shRNA target sequences
Control (non-mammalian sequence) shRNA; 5'-CCTAAGGT TAATGCCTCG-3'
E-cad-1 shRNA; 5'-ACCGATCAGAATGCAACCAAG-3'
E-cad-2 shRNA; 5'-CGGGAACAGTTTATTACAT-3'
ZEB1 shRNA; 5'-GCTGTGTCTTGCAACAGTT-3'

Statistical analysis
Data distribution and the significance of differences between groups were analyzed by the Mann–Whitney U-test and by Student’s t-test. p < 0.05 was considered to indicate a statistically significant difference.

Data availability
The data that support the findings of our study are available from the corresponding author upon reasonable request.

Results
Establishment of an orthotopically transplantable CRC PDX model
Tumors were surgically dissected from 40 CRC patients prior to anti-cancer treatments (Supplementary Tables S1 and S2A). Small tumor fragments were then implanted subcutaneously into the flanks of highly immunodeficient NOG mice for simplicity and ease of access, thereby facilitating the initial implantation. The tumors progressed in mice with 28 of the 40 samples, for 6 months (Figs. 1a–s.c. imp.). The PDXs corresponding to these 28 patients were also dissected and enzymatically dissociated into cell suspensions prior to orthotopic injection into the rectal submucosa of secondary recipient NOG mice (Fig. 1a-o.t. inj.). In summary, subcutaneous PDXs from 13 of the 28 patients formed CRCs (~1 cm³) orthotopically in recipient mice (Fig. 1a-o.t. inj. for CRC). Human CD20-positive B cell lymphomas that had transformed asymptptomatically in six human subjects (P7, P11, P14, P17, P18 and P22) grew in mice due to their immunodeficient conditions (Fig. 1a-lymphoma and Supplementary
Fig. S1a), observations consistent with those in the previous literature. The tumors from nine patients showed no growth in recipient mice (Fig. 1a-no growth).

We next sought to determine, by examining PDXs, how frequently CRC cells metastasize. Of note, tumor cells spread spontaneously from orthotopic tumors to the most common metastatic sites of human CRC in the PDXs from 8 of the 13 patients (62%) (Fig. 1a-o.t. inj. for Spon. meta. and Table 1). This included liver metastases associated with three PDXs (P10, P12 and P25) and lung metastases from eight PDXs (P2, P4, P10, P12, P21, P25, P26 and P38; Table 1). We attributed the higher frequency of lung metastases to spread of tumor cells from the lower rectum via the inferior vena cava. This observation is consistent with previous studies indicating the ability of CRC cells to metastasize in some PDX mouse models. Epithelial structures in primary and metastatic tumor sites of different PDXs were similar to those of tumors obtained from all CRC patients examined, indicating maintenance of their inherent epithelial nature (Fig. 1b and Supplementary Figs. S1b–S1i).
The hybrid E/M trait is present in primary tumors but not in distant metastases

To examine the E/M plasticity of tumor cells, tumor sections were stained with epithelial and mesenchymal markers. The tumor cells in the primary tumors of CRC patients, as exemplified by P10, and the corresponding PDXs stained positive for carcinoembryonic antigen (CEA), a marker for human CRC cells, and Ki-67, a marker of cellular proliferation (Supplementary Figs. S1b and S1c). The CRC cells also stained positive for pan-cytokeratin (pan-CK) and E-cad, both of which are epithelial cell markers, but negative for human fibronectin (FN) and human vimentin (vim), both of which are highly mesenchymal cell markers detected by...
human-specific antibodies (Fig. 1b and Supplementary Figs. S1b–S1i). Human FN- and vim-positive stroma that had been present in the primary tumors of these patients were largely replaced in PDXs by murine stromal cells negative for both human FN and vim (Fig. 1b and Supplementary Figs. S1c–S1i), as previously reported.37 However, we observed considerable proportions of the tumor cells to show positive staining for nuclear ZEB1, a marker of partial EMT,6,7 in primary tumors (27.7%) from the P10 CRC patient (Fig. 1b). ZEB1 staining was also detected in murine stromal cells in these tumors, consistent with the previous literature.8 Moreover, larger ZEB1-positive tumor cell proportions were found in the corresponding P10 PDXs orthotopically generated in mice, indicating increased ZEB1-expressing tumor cell proportions during in vivo pas-saging of PDXs. Importantly, there were fewer ZEB1-positive cancer cells in the liver (38.3%) and lung (18.1%) metastases than in the primary tumors in mice (Fig. 1b).

To further confirm this finding, we employed primary tumors and metastases simultaneously dissected from the same CRC patients suffering from liver metastases. The tissue sections were then prepared for immunohistochemistry using anti-E-cad and -ZEB1 antibodies. We found considerable numbers of ZEB1-positive carcinoma cells in primary tumors of five CRC patients (PL-P1, -P15, -P25, -P30 and -P31), whereas few ZEB1-positive cancer cells were observed in the patient-matched liver metastases which had presumably developed via MET (Fig. 1c, Supplementary Fig. S1j and Table S2B). Indeed, ZEB1-positive tumor cell proportions were 24.4% and 2.6% in the primary tumor and liver metastasis, respectively, of a colon cancer patient (PL-P15) (Fig. 1c). In primary tumors, nuclear ZEB1 staining was also detected in cancer cells weakly expressing membrane E-cad by immunofluorescence (Fig. 1c), indicating E-cadlowZEB1+ (E/M) human colon cancer cells. This finding is consistent with a previous report, indicating both E-cad- and ZEB1-positive tumor cells to be detected in various types of human carcinomas.8 We also sought to confirm the presence of E/M tumor cells in different human CRCs employing immunohistochemistry with anti-E-cad and anti-ZEB1 antibodies. We thus examined primary tumor samples from the aforementioned 8 of the 13 CRC patients and their corresponding PDXs that spontaneously formed metastases in distant organs of mice (Fig. 1a-o.t. inj. for Spon. meta. and Table 1). E/M tumor cells were detected in all tumor samples studied (Fig. 1d). However, a more marked E/M state was observed in moderately and poorly differentiated CRCs (P4, P10, P12, P21, P25, P26 and P38) than in the highly differentiated tumor (P2) (Fig. 1d and Table 1). Taken together, these findings indicate that, in CRC patients, the hybrid E/M state is often present in the tumor cells in primary tumors but not those in their metastases.

We also examined whether the E/M tumor cell proportions correlated with their metastatic ability. To investigate this possibility, we additionally prepared primary tumor samples from

<table>
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<tr>
<th>Patients</th>
<th>No.</th>
<th>Tumor differentiation</th>
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<td>P26</td>
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<td>P38</td>
<td>MUC » M</td>
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The gray indicates the PDXs with metastases. Numbers of mice developing tumors and/or metastases and of all mice examined are the numerator and denominator of the fraction, respectively. Abbreviations: M, moderately differentiated adenocarcinoma; MUC, mucinous adenocarcinoma; o.t. inj., orthotopic injection; P, poorly differentiated adenocarcinoma; W, well-differentiated adenocarcinoma.
5 of the 13 CRC patients and their corresponding PDXs that did not form spontaneous metastases in distant organs of mice (Table 1). E/M tumor cells were common in some of the PDXs without metastasis-forming ability (P6, P9, P32 and P99), based on immunofluorescence using anti-E-cad and -ZEB1 antibodies (Supplementary Fig. S1k). These data thus indicate that there is a slight correlation between the E/M tumor cell proportions and their metastatic ability. Acquisition of the E/M state would be required for colon tumor cells in primary tumors to metastasize, but this state is not sufficient to induce metastasis without the actions of metastasis-promoting epi/genetic alterations harbored in tumor cells.

CTC clusters detected in the bloodstream of mice bearing CRC PDXs

CTC clusters and single CTCs were present in the bloodstream of patients.17–19 To investigate these CTCs in our PDX model, 150 slides with blood smears were prepared from 4 to 6 mice bearing 1-month-old subcutaneous PDXs, from each of the patients, using a cytopsin centrifuge. We observed different sizes of CTC clusters positive for CEA, pan-CK, E-cad and ZEB1 in mice bearing PDXs from CRC patients (P10 and P12) (Fig. 1c). Importantly, positivity against human CEA, which is specific to human cancer cells but not murine cells due to absence of the human homolog from the mouse genome,38 confirmed the PDX-derived origin of CTC clusters. Our data are also consistent with previous reports indicating that mesenchymal traits are present on CTC clusters from cancer patients.17,18 In contrast, such CTC clusters were barely detectable in non-tumor-bearing mice.

Single CTC cells were also virtually absent from PDX-bearing mice, although both single CTC and CTC clusters were reportedly detected using magnetic separation of cytokeratin-positive tumor cells in circulating blood obtained from human colon cancer patients.39 There is a possibility that our assay was not sufficient for detecting single CTCs that might be present in circulating blood of our PDX murine models and human colon cancer patients. Taken together, these findings indicate that CTC clusters, presumably with the E/M trait, are present in mice bearing CRC PDXs.

Establishment of CRC PDX-derived tumor organoids with the hybrid E/M trait

To further investigate biological features of CRC cell clusters, tumor organoids were established from different CRC PDXs in culture and examined immunohistochemically. The P10 and P4 PDX-derived tumor organoids expressed remarkably high levels of CEA and epithelial markers (pan-CK, p120-catenin, α-catenin, β-catenin, γ-catenin and E-cad) (Fig. 2a and Supplementary Fig. S2a). The mesenchymal marker ZEB1, but neither FN nor vim, was also detected in these tumor organoids (Fig. 2a and Supplementary Fig. S2a). Moreover, greater proportions of E/M tumor cells were observed in the moderately differentiated P4 and P10 tumor organoids than in the highly differentiated P2 tumor organoids (Fig. 2b and Table 1). These findings suggest that the E/M state in CRCs of patients is retained in the cultured PDX-derived tumor organoids during in vitro propagation.

To next examine whether the CRC PDX-derived organoids are capable of forming tumors in vivo, the P2, P4 and P10 tumor organoid cells were injected orthotopically into recipient mice. These cells developed tumors expressing CEA, Ki-67, pan-CK, E-cad and ZEB1 (Fig. 2c and Supplementary Fig. S2b). The E/M tumor cell proportions were also greater in tumors raised by the P4 and P10 tumor organoids than in those of P2 (Fig. 2c).

We next determined whether highly mesenchymal markers, such as vim and FN, are also expressed in the E/M tumor cells by employing immunohistochemistry. We observed small proportions of vim-positive tumor cells expressing E-cad in PDXs generated by orthotopic injection of P10 colon tumor organoids into mice (Supplementary Figs. S2b and S2c). However, such vim- and E-cad-positive tumor cells were extremely rare in other PDXs generated by P2 and P4 colon tumor organoids (Supplementary Fig. S2b). Likewise, there were no vim-positive tumor cells in the primary tumors of human colon cancer patients or in their corresponding orthotopically generated PDXs of P2, P4 and P10 (Fig. 1b, Supplementary Fig. S1c and S1d), indicating the highly mesenchymal traits to have been induced only in P10 organoid-derived tumors. No FN-positive tumor cells were detected in any tumor cells of PDXs or in any of the primary tumors of patients (Fig. 1b, Supplementary Figs. S1c and S2b). Taken together, these findings indicate that human colon cancer cells with the E/M state often become positive for ZEB1, a partial EMT marker, while showing minimal positivity for vim and FN, which are exclusively EMT markers.

Moreover, CEA-, pan-CK, E-cad- and ZEB1-expressing CTC clusters were detected in the bloodstream of mice bearing orthotopic tumors generated from the P2, P4 and P10 tumor organoids (Fig. 2d). We also observed tumor emboli of different sizes within the pulmonary vasculature and in the vascular media layer in the lungs of mice at 60 days after orthotopic injection of the P10 tumor organoids (Fig. 2e). Ki-67, pan-CK, E-cad and ZEB1 were expressed in such tumor emboli, whereas neither FN nor vim was detected (Fig. 2e and Supplementary Fig. S2d). These observations thus support the notion that CTC clusters proliferate within blood vessels, potentially leading to vascular rupture, extravasate and then grow in surrounding tissues, as previously described.40

Metastatic colonization of CRC organoids with the E/M state via MET

To further investigate the E/M plasticity in CRC cell clusters during metastatic colonization, the GFP-labeled P4 and P2 tumor organoids were intrasplenically injected into NOG mice prior to dissection of the liver for immunohistochemistry using different antibodies. We observed progressive growth of GFP-positive colonies expressing CEA, Ki-67, pan-CK and E-cad, but neither FN nor vim, during metastatic colonization, indicating proliferation of the PDX-derived tumor organoids with epithelial...
traits in the liver (Fig. 2f and Supplementary Fig. S2e). In contrast, proportions of ZEB1-expressing tumor cells in metastatic foci appeared to gradually decrease during metastatic colonization (Fig. 2f and Supplementary Fig. S2e). The ZEB1-positive tumor cell proportions (31.4%) in metastatic nodules of P4 organoids at 6 hr after injection showed progressive attenuation until Day 27 (0.92%) (Figs. 2g and 2h). The ZEB1-positive tumor cell proportions (30.5%) in metastatic nodules of P2 organoids at 6 hr after injection also showed increasing attenuation until Day 27 (0.9%) (Supplementary Fig. S2f and S2g). Collectively, these results indicate that the partial mesenchymal trait mediated by ZEB1 expression is attenuated in CRC cell clusters, presumably via MET, during metastatic colonization of the liver.

Metastatic seeding arises from tumor cell clusters rather than single cancer cells

Given the presence of circulating CTC clusters in mice bearing CRC PDXs and tumor organoids, we next sought to determine whether disseminating tumor cell clusters do indeed contribute to the formation of spontaneous metastases. Multicolor lineage-tracing assays previously conducted by others indicated a greater metastatic seeding of mammary and pancreatic tumor cell clusters than of single cells. Since human tumor cell lines and mouse-derived tumors were employed in these studies, we investigated this possibility using clinical materials. The PDX-derived P10 CRC organoids were infected employing a GFP lentiviral particle. The resulting tumor organoids including both GFP-positive and -negative tumor cells were orthotopically injected into three recipient mice to generate orthotopic tumors and metastases in distant organs. The chimeric patterns of GFP in the orthotopic tumors comprised of GFP-positive or -negative cells were then examined by immunostaining with anti-GFP antibody (see Materials and Methods section, Supplementary Fig. S3a and Table S3A). The results were fitted to beta distributions allowing assumption of the local frequencies of GFP-positive cells in orthotopic tumors (Supplementary Fig. S3b).

We next calculated the probabilities of metastatic nodules that would become all GFP-positive (P_{100}), all GFP-negative (P_{0}) or a mixture of GFP-positive and -negative (P_{mix}) in the liver and lungs, by assuming the number of cancer cells in a disseminating tumor cell cluster to be “n” cells (see Materials and Methods section and Fig. 3a). The chimeric patterns of GFP were also investigated in liver and lung metastases by immunohistochemistry using anti-GFP antibody. The frequencies of the metastatic nodules, that is, all GFP-positive (d_{100}), all GFP-negative (d_{0}), and a mixture of GFP-positive and -negative (d_{mix}), were quantified (Fig. 3a and Supplementary Table S3B).

To evaluate the differences between the predicted values and the experimental data, denoted by D, we minimized D by examining different numbers of cancer cells in disseminating tumor cell clusters, n. The evaluations of 2–3 and 3–35 cancer cells, both of which were deemed to represent “minimal D,” showed the highest predictive values for chimeric patterns of GFP in liver and lung metastases, respectively (Fig. 3b). In contrast, lower predictive values were revealed by the estimations made for a single cell (n = 1) and larger clusters (Fig. 3b), indicating that tumor cell clusters of a certain size have greater ability to contribute to metastatic seeding. Collectively, these findings support the likelihood of metastatic colonization of multicellular clusters of CRC cells rather than of single tumor cells.

Role of the E-cad/catenin complex in the formation of CRC cell clusters

E-cad plays central roles in maintaining epithelial integrity and cell–cell adhesions by forming a complex with p120-catenin, α-catenin, β-catenin and γ-catenin in adherens junctions. To elucidate the roles of E-cad expression in CRC cell cluster formation, we generated two independent shRNAs (1 and 2) against E-cad, both of which significantly suppressed E-cad expression in P4 and P10 tumor organoids, as compared to the control shRNA (Fig. 4a and Supplementary Fig. S4). Expression levels of the E-cad-associated catenin proteins were also downregulated in organoids expressing each of the E-cad-shRNAs, resulting in the formation of tumor cell clusters with diminished cell–cell adhesions. Furthermore, FN and vim expressions remained undetectable in all of the organoids, while ZEB1 expression was detected regardless of the presence or the absence of E-cad-shRNAs (Fig. 4a and Supplementary Fig. S4). Taken together, these findings indicate that E-cad expression is required for maintenance of the E-cad/catenin complex that mediates the intact cell–cell adhesions and thus CRC cell cluster formation.

Roles of E-cad expression in CRC cell clusters in the formation of metastasis

E-cad has been recognized to act as a metastasis suppressor in various carcinomas, presumably by inhibiting EMT. In contrast, metastasis-promoting roles of E-cad expression have also been demonstrated, as exemplified by MET being required for metastatic colonization by carcinoma cells. Oncogenic roles of the E-cad expression that is required for tumor cell cluster formation, collective cell invasion and metastatic dissemination have also reportedly been demonstrated. To determine the effects of E-cad expression on human CRC clusters in metastasis, GFP-labeled P4 and P10 tumor organoids expressing the E-cad-shRNA were intrasplenically injected into recipient mice. GFP-positive liver metastases were significantly attenuated by inhibition of the E-cad expression by shRNA, as compared to the effect of the control shRNA (Figs. 4b and 4c).

Moreover, the apoptotic cancer cell proportions positive for cPARP were significantly increased in E-cad-shRNA-expressing groups (Figs. 4b and 4c). Increased cPARP expression was also evident in E-cad-shRNA-expressing tumor cell
populations with reduced E-cad expression (Fig. 4c). These data therefore indicate E-cad expression to be required for metastatic colonization and growth of CRC cell clusters. E-cad expression is also necessary for the maintenance of cell–cell adhesion and thus exerts an anti-apoptotic effect in distant organs.

**ZEB1 expression is required for CRC cell clusters to generate metastasis**

Although ZEB1 expression mediates the E/M state in tumor cell clusters of various CRC patients, roles of ZEB1 expression in metastasis formation remain uncertain. To investigate this issue, we generated ZEB1-shRNA, which significantly
suppressed ZEB1 expression in P10 tumor organoids as compared to the control shRNA (Figs. 5a and 5b). Inhibition of ZEB1 expression by shRNA also upregulated E-cad expression in P10 tumor organoids (Fig. 5a and Supplementary Fig. S5a), indicating E-cad expression to be downregulated by ZEB1, a well-known E-cad repressor, in the E/M colon cancer cells.

GFP-labeled P4 and P10 tumor organoids expressing the control shRNA or ZEB1-shRNA were then injected intrasplenically into recipient mice. Inhibition of ZEB1 expression by the shRNA markedly attenuated liver metastases as compared to the effect of the control shRNA (Figs. 5c and 5d), indicating ZEB1 expression in CRC organoids to be required for metastatic colonization. This observation is consistent with the results of previous studies showing crucial roles of ZEB1 expression in metastatic colonization of cancer cells due to stemness/metastasis-initiating and cellular plasticity abilities.7,49 Taken together with earlier findings, our present observations indicate that the E/M state mediated by E-cad and ZEB1 expressions is crucial for the metastatic colonization of CRC cell clusters in distant organs.

**Discussion**

**Human CRC cell clusters with the E/M state contribute to metastatic seeding**

Solitary mesenchymal tumor cells were often induced by loss of E-cad expression via EMT in culture, acquiring locally invasive and tumor-initiating properties.2,6,50 However, a previous study, using intravital imaging of E-cad-GFP-expressing tumor cells in a genetically engineered murine pancreatic tumor model, indicated membrane E-cad expression to be retained on tumor cell clusters during invasion and metastasis.51 Tumor buds weakly expressing membrane E-cad expression are also positive for partial EMT markers, as exemplified by ZEB1 at the invasive front of human pancreatic tumors.52 Furthermore, E-cad-expressing tumor emboli and CTC clusters with mesenchymal traits have been detected in circulating peripheral blood from various cancer patients.17,18,53 The partial EMT program also reportedly plays roles in regulating collective invasion and the dissemination of various carcinoma cell clusters.2,23,25,26 Collectively, these series of observations indicate important associations between the E/M state in the tumor cell cluster and the early steps of the invasion-metastasis cascade, although the functional roles of E/M plasticity in the tumor cell cluster in metastatic colonization have yet to be fully investigated. Previous studies also employed established cultured cancer cell lines and experimental murine tumors, but not human clinical materials.23,25,26

Herein, using various PDXs and their corresponding tumor cell organoids, we obtained previously unrecognized findings that the hybrid E/M state in human CRC cell clusters does indeed drive their metastatic seeding (Fig. 5e). (i) Metastases formed spontaneously in the liver and lungs in mice bearing orthotopically implanted PDXs obtained from eight CRC patients. (ii) Human CRC cell clusters seeded these spontaneous metastases significantly more often than did single cancer cells. (iii) Membrane E-cad- and nuclear ZEB1-expressing E/M tumor cells are detectable in primary tumors of CRC patients, and in the corresponding PDXs and presumably CTC clusters in the bloodstream of mice. (iv) In contrast, ZEB1 expression was found to be attenuated in disseminating tumor cell clusters during metastatic colonization via MET. (v) Inhibition of E-cad or ZEB1 expression in CRC cell organoids attenuated metastasis in the liver, when intrasplenically injected into mice, indicating E-cad and ZEB1 expressions to be required for these tumor cell organoids to colonize the liver. Taken together, these data suggest that the E/M state mediates the metastatic seeding of human CRC cell clusters into distant organs.

We observed ZEB1-positive cancer cells at the stromal interface in primary tumors of colon cancer patients (Fig. 1c), consistent with a previous report indicating partial EMT induced in the breast, head and neck cancer cells to be associated with the tumor stroma.27,54 However, ZEB1 staining was also detected in tumor cells that are not adjacent to the stroma in some PDXs (P10, P21 and P38) with abundant ZEB1 expression (Fig. 1d). As noted above, during *in vivo* pasaging of PDXs in mice ZEB1 expression tends to be induced in colon tumor cells (Fig. 1d), presumably through interactions with host murine stromal cells. These ZEB1-expressing tumor cells may become independent of signaling from the stroma, thereby allowing them to maintain the increased ZEB1 expression.

ZEB1 expression is reportedly induced by β-catenin signaling in human colon cancer cells.55 Nuclear β-catenin staining is also detected in tumor budding cells at the invasive front associated with the stroma of human colon carcinomas.56 Thus, we speculated that ZEB1 expression might be induced by activation of β-catenin signaling in the E/M colon cancer cells. To assess this possibility, sections prepared from P10 PDXs that had developed at orthotopic sites of recipient mice were stained with anti-non-phosphorylated (active) β-catenin antibody. We observed cytoplasmic and membrane β-catenin staining in a majority of tumor budding cells (left, Supplementary Fig. S6a). However, nuclear β-catenin staining, indicative of activated β-catenin signaling, was minimal. A number of nuclear ZEB1-expressing tumor cells also showed cytoplasmic and membrane, but not nuclear, β-catenin staining by immunofluorescence using anti-ZEB1 and -β-catenin antibodies (right, Supplementary Fig. S6a). These observations suggest that ZEB1 expression may be regulated independently of activated β-catenin signaling in the colon tumor budding cells of our PDX models. The precise molecular mechanisms underlying ZEB1 expression induced in colon carcinoma cells have yet to be fully investigated and elucidation of the details awaits a future study.

Various cell types undergo collective migration *in vivo* during morphogenesis and tissue renewal.11,12 Leader cells, generally localized at the front of cell groups, communicate with follower cells to drive their migration with proper and coordinated cell movement.12,57 A previous report described leader cells in melanoma as acquiring a more mesenchymal phenotype with cell protrusions and activation of Rac1, features which are necessary for spreading.12 Mesenchymal breast cancer cell populations, termed
trailblazer cells, are also reportedly defined as leader cells that are associated with follower non-trailblazer cells expressing the epithelial trait in tumor cell clusters, leading to their collective invasion. Moreover, E-cad expression mediating cell–cell adhesion between leader and follower cells is required for the appropriate collective cell migrations occurring in a wide variety of cell types including carcinoma cells. These findings raise the possibility that mesenchymal tumor cell populations serve as leader cells directing the multicellular CTCs toward chemoattractants in the bloodstream. However, the roles of the E/M state in CRC clusters as leader cells await further clarification.

Considering the significant dependence of metastatic seeding of multicellular CRCs on the E/M state in distant organs, future investigations focused on signals regulating the E/M state and tumor cell cluster formation might reveal potential therapeutic targets involving CRC cell clusters during metastatic dissemination.

References


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