GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Dynamic Change of Polarity in Primary Cultured Spheroids of Human Colorectal Adenocarcinoma and Its Role in Metastasis

Hiroaki Okuyama,^{*} Jumpei Kondo,^{*} Yumi Sato,^{*} Hiroko Endo,^{*} Aya Nakajima,^{*} Jose M. Piulats,^{*} Yasuhiko Tomita,[†] Takeshi Fujiwara,[‡] Yu Itoh,[‡] Akira Mizoguchi,[‡] Masayuki Ohue,[§] and Masahiro Inoue^{*}

From the Departments of Biochemistry,^{*} Pathology,[†] and Surgery,[§] Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; and the Department of Anatomy,[‡] Faculty of Medicine, Mie University, Tsu, Japan

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Address correspondence to
Masahiro Inoue, M.D., Ph.D.,
Department of Biochemistry,
Osaka Medical Center for
Cancer and Cardiovascular
Diseases, 1-3-3 Nakamichi,
Higashinari-ku, Osaka
537-8511, Japan. E-mail:
inoue-ma2@mc.pref.osaka.jp.

Intestinal epithelial cells possess apical-basal polarity, which governs the exchange of nutrients and waste. Perturbation of cell polarity appears to be a general feature of cancers, although most colorectal cancers are differentiated adenocarcinomas, in which polarity is maintained to some extent. Little is known about the role of dysregulated polarity in cancer. The cancer tissue—originated spheroid method was applied to the preparation and culture of spheroids. Spheroids were cultured in suspension or in type I collagen gel. Polarity was assessed by IHC of apical markers and electron microscopy. Two types of polarity status in spheroids were observed: apical-in, with apical membrane located at cavities inside the spheroids in type I collagen gel; and apical-out, with apical membrane located at the outermost layer of spheroids in suspension. These polarities were highly interchangeable. Inhibitors of Src and dynamin attenuated the polarity switch. In patients, clusters of cancer cells that invaded vessels had both apical-in and apical-out morphologic features, whereas primary and metastatic tumors had apical-in features. In a mouse liver metastasis model, apical-out spheroids injected into the portal vein became apical-in spheroids in the liver within a few days. Inhibitors of Src and dynamin significantly decreased liver metastasis. Polarity switching was observed in spheroids and human cancer. The polarity switch was critical in an experimental liver metastasis model. (*Am J Pathol* 2016, 186: 899–911; <http://dx.doi.org/10.1016/j.ajpath.2015.12.011>)

Intestinal epithelial cells are organized into adherent groups of cells that separate and protect the organism from the external environment. A closed epithelium regulates the exchange of nutrients and waste between the internal and external environments. The direction of the exchange is determined by the epithelial cells' apical-basal polarity. The molecular mechanism of forming cell polarity is based on evolutionarily conserved partition-defective (PAR)/atypical protein kinase C (aPKC) system.^{1,2} Asymmetrically localized components of the PAR-aPKC system are linked with other cellular machinery, such as junctional structure formation and actin polymerization. The perturbation of cell polarity is a general feature in cancer. It is becoming evident that Par proteins are directly involved in the loss of cell and tissue architecture in carcinoma.^{3,4} For example, the loss of Par3 is directly linked to tumor invasion and metastasis in breast cancer.^{5,6}

Because >90% of colorectal cancers (CRCs) are differentiated adenocarcinoma, polarity in most CRCs is disorganized but not completely lost. For most histologic types of CRC, the pathologic grades are independent of disease progression; even differentiated CRCs have malignant features.⁷ The status and role of disorganized polarity in

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Current address of J.K., Departments of Medicine and Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN; of A.N., Department of Radiation Oncology, Shiga Medical Center for Adults, Shiga, Japan; of J.M.P., Department of Medical Oncology, Institut Català d'Oncologia, Gran Via de l'Hospitalet, Barcelona, Spain.

differentiated cancer are poorly understood because most established CRC cell lines barely retain polarity as clusters of cells *in vitro*, with the exception of a few lines.^{8,9}

We recently developed a method of preparing and culturing cancer cells from patient tumor samples or mouse xenografts: the cancer tissue–originated spheroid (CTOS) method.^{10–12} The principle of the CTOS method is to maintain cell-cell contact throughout the process of preparation and culture. A CTOS is a spheroid that consists of pure cancer cells; it retains characteristics of original tumors and mouse xenograft tumors *in vitro*, particularly the formation of gland-like structures.

It has been widely accepted that metastasis originates from a single cell. In theory, multiple distinct steps are involved: a cancer cell detaches from the original tumor, migrates to the blood or lymphatic vessels, penetrates the lumen, circulates to distant organs, attaches to the vessels of distant organs, invades into the parenchyma, and finally forms tumors.¹³ However, it was recently proposed that cancer cells that originate from solid tumors migrate not only as single cells but also as cell clusters in a process referred to as collective migration.¹⁴ Clusters that maintain E-cadherin–based adhesions migrate in Matrigel synthetic matrix *in vitro*.⁵ In clinical pathology, microvascular invasion in a solid tumor is diagnosed as a tumor mass surrounded by blood or lymphatic vessels. Microvascular invasion correlates strongly with the risk of metastasis.¹⁵ In addition, circulating tumor clusters in the bloodstream of patients with breast cancer have been linked with disease progression.¹⁶ Thus, clusters of cancer cells may also be an origin of metastasis.

We found that a dynamic change of polarity status occurs in CRC CTOS *in vitro* and *in vivo*. We also investigated the role of polarity in the metastasis of CTOS as clusters of cancer cells.

Materials and Methods

Ethics Statement

The institutional ethics committees at the Osaka Medical Center for Cancer and Cardiovascular Diseases (OMCCCD) approved this study. CRC specimens were collected from patients treated at the Department of Surgery, OMCCCD. Animal studies were approved by the OMCCCD Institutional Animal Care and Use Committee and performed in compliance with the institutional guidelines.

Isolation and Culture of CTOS from Xenograft and Primary Tumors

Primary cultures of human CRC cells were prepared according to the CTOS method.¹⁰ Briefly, surgically resected tumor samples or xenograft tumors were mechanically dissociated, partially digested with liberase DH (Roche, Mannheim, Germany), and filtered through cell strainers.

Cell clusters were collected on 100- μ m or 40- μ m cell strainers (BD Falcon, Franklin Lakes, NJ). CTOSs were cultured in StemPro hESC (Invitrogen, Carlsbad, CA). Institutional pathologists examined the colorectal tumors. CTOSs in gel were embedded in CellMatrix I-A (Nitta Gelatin, Osaka, Japan) on 35-mm dishes and cultured in StemPro medium. CTOS in suspension were cultured with the CTOS method in noncoated 10-cm dishes in StemPro medium.

Immunostaining

CTOSs in suspension were fixed for 5 minutes with 10% formalin, embedded in CellMatrix I-A (Nitta Gelatin) to condense the CTOSs and fixed for 1 additional hour. CTOSs in gel were also fixed for 1 hour. Next, gel droplets that contained CTOSs were stained with Sirius red to facilitate further manipulation. Then, the gel was embedded in paraffin. The paraffin blocks were cut into 4- μ m sections and subjected to immunostaining. Immunohistochemistry (IHC) was performed as previously described.¹⁰ The following primary antibodies were used: E-cadherin (BD Biosciences, San Jose, CA), PARD3 (Sigma-Aldrich, St Louis, MO), D2-40 (Nichirei Biosciences, Tokyo, Japan), CD31 (Dako, Glostrup, Denmark), villin and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), and ezrin and ZO-1 (Invitrogen). LMO7 was a gift from Dr. Jun Miyoshi (OMCCCD, Osaka, Japan). For whole-mount staining of CTOSs, the CTOSs in gel were extracted by digesting the gel with 2 mg/mL of collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) for 10 minutes. The extracted CTOSs in gel or in suspension were fixed and permeabilized with 4% paraformaldehyde/phosphate-buffered saline containing 1% Triton X-100 at 4°C for 1 hour. After blocking with 5% bovine serum albumin (Sigma-Aldrich) for 1 hour, CTOSs were incubated with anti-E-cadherin antibody (BD Biosciences) overnight at 4°C. Next, they were incubated with secondary antibodies conjugated with Alexa-488 or Alexa-555 (Life Technologies, Carlsbad, CA) or Rhodamine Phalloidin with Hoechst33342 (Life Technologies) overnight. Cells were mounted with FluorSave Reagent (Calbiochem, San Diego, CA). Fluorescence images were obtained using confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Quantitative Analysis of Polarity Status

The CTOSs were immunostained with villin. The polarity status of the CTOSs was classified into 4 groups: apical-out (apical proteins located only at the outermost membrane of a CTOS), apical-in (apical proteins located only at multiple lumens inside a CTOS), mixed (apical proteins located both at the outermost membrane and at multiple lumens inside a CTOS), and none (apical proteins not detected). Multiple CTOSs were analyzed and categorized, and the proportion of each polarity status was calculated. The number of CTOSs

examined in each experiment is given in [Supplemental Table S1](#).

Cell Death Assay

Apoptosis was detected by cleaved caspase-3 staining. The rate of apoptosis in each CTOS or liver focus of cancer cells was calculated by dividing all the nuclei that stained positive for cleaved caspase-3 by the total number of DAPI-stained nuclei. Multiple CTOSs or liver foci were analyzed and subjected to statistical analysis.

Electron Microscopy

CTOSs cultured in suspension and type I collagen gel were fixed with a cacodylate-buffered solution (0.15 mol/L, pH 7.4) that contained freshly prepared 2% paraformaldehyde, 2% glutaraldehyde, and 2 mmol/L CaCl_2 at room temperature for 12 hours. CTOSs were postfixed with 1.0% OsO_4 in cacodylate buffer (0.15 mol/L, pH 7.4) that contained 2 mmol/L CaCl_2 for 90 minutes at 4°C. The following electron microscopic procedures were performed as previously described.¹⁷

RNA Interference Experiments

The Src-specific RNA interference (RNAi) and control RNAi were from the siGENOME Human siRNA-SMART pool (Thermo Scientific Dharmacon, Lafayette, CO). C45-2D cells were established from the C45 CTOS line. Briefly, C45 CTOS were cultured on a type I collagen (Cellmatrix I-C, Nitta Gelatin)—coated dish with DMEM/F12 (Invitrogen) and 10% fetal bovine serum. The CTOSs that grew on the dish were passaged once each week. After several passages, C45-2D cells were established. RNAi was transfected to C45-2D cells by lipofection using Lipofectamine RNAiMAX (Life Technologies).

Quantitative RT-PCR

Total RNA was extracted from CTOSs or the HT29 cell line using Trizol (Invitrogen). The RT-PCR reactions were performed with 1 µg of total RNA using Superscript III Reverse Transcriptase (Invitrogen). The primers for PCR were as follows^{18,19}: FYN-forward, 5'-TGTGACCTCCATCCCAACT-3'; FYN-reverse, 5'-AACTCAGGTCATCTTCTGTCCGT-3'; LYN-forward, 5'-GAGGCTCTACGCTGTGGTCA-3'; LYN-reverse, 5'-GACTCGGAGACCAGAACATTAGC-3'; SRC-forward, 5'-CAGTGTCTGACTTCGACAACGC-3'; SRC-reverse, 5'-CCATCGGCGTGT-TGGAGTA-3'; YES-forward, 5'-GCTGCACTGTATGGTCGGTT-3'; YES-reverse, 5'-AGGGCACGGCATCCTGTATC-3'; DNM2-forward, 5'-ACACGGAGCAGAGAAACGTCTACA-3'; DNM2-reverse, 5'-GGTGCATGATGTCTTTGGCATGA-3'; 18s rRNA-forward, 5'-AAACGGCTACCACATCCAAG-3'; and 18s rRNA-reverse, 5'-CCTCCAATGGATCCTCGTTA-3'. The 18s rRNA

expression was used as an internal control. For real-time PCR analysis, the mRNA expression of human SRC and DNM2 was quantified using SYBR Green. Real-time PCR was performed in triplicate for each gene. Each SYBR Green assay was performed in a 10-µL reaction volume, which included 5 µL of Fast SYBR Green master mix (Applied Biosystems, Foster City, CA) with 200 nmol/L of each primer. Assays were performed on StepOne Real-Time PCR System (Applied Biosystems) under fast thermal cycling: 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds, followed by melting curve analysis. Data were analyzed by the StepOne software version 2.1 (Applied Biosystems). Fold difference of gene expression was determined by the $\Delta\Delta\text{CT}$ method.

Western Blots

Western blotting was performed as previously described.¹² The CTOSs in gel were digested by 2 mg/mL of collagenase IV (Worthington Biochemical Corporation) for 10 minutes, rinsed, and lyzed in the same way as the CTOSs in suspension. Primary antibodies against the phospho-Src family (Tyr416) and Src (36D10) were obtained from Cell Signaling Technology.

Luciferase Vector Construction and Gene Transfer

To generate pPB/Luc, the BglII-EagI fragment, including a Luciferase gene, was transferred to the pPB-UBC/ires/Puro vector.²⁰ CTOSs were prepared from xenograft tumors originated from C45 CTOSs. To prepare for electroporation, C45 CTOSs were pretreated with 5 mmol/L EDTA/phosphate-buffered saline for 30 minutes at room temperature, then mixed with pPB/Luc and the transposase expression vector pCMV-hyPBBase.²⁰ Electroporation was performed in 2-mm gap cuvettes at 150 V for 5 ms using a Type II NEPA21 electroporator (Nepa Gene, Chiba, Japan). C45Luc CTOSs were established by puromycin selection. C45Luc CTOSs were subcutaneously inoculated into a NOD/SCID mouse to generate a xenograft tumor. C45 Luc CTOSs were prepared from the xenograft tumor and further selected by culture with puromycin. Then, C45Luc CTOSs were subjected to the *in vivo* liver metastasis model.

Liver Metastasis Model with CTOSs

The C45 CTOS line was used for liver metastasis model.¹⁰ The C45Luc CTOSs (40 to 100 µm) were collected into 1.5-mL tubes (100 CTOSs per tube). With the mice under anesthesia, the CTOSs were injected into the portal vein of nude mice using a 30-G needle. The volume of the vehicle was 300 µL. Mice were anesthetized by three types of mixed agents, including 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. The anesthetics were administered to male nude mice by i.p. injection. Four weeks later, mice were sacrificed and livers were collected. For *in vivo* bioluminescence imaging, 2 mg

Figure 1 Characteristics of polarity status in cancer tissue-originated spheroids (CTOSs) derived from a xenograft of human colorectal cancer. **A:** Immunofluorescence staining of CTOSs in suspension (suspended) and CTOSs embedded in type I collagen gel and cultured for 48 hours (in gel). Red indicates villin; green, E-cadherin; blue, DAPI. **B:** Immunofluorescence staining of CTOSs in suspension and CTOSs in gel using antibodies against indicated molecules. CTOSs were stained with phalloidin using whole-mount staining. **C–H:** Electron microscopy images of CTOSs in suspension and in gel. Microvilli (MV), tight junctions (TJ), adherence junctions (AJ), and desmosomes (DS) are indicated. The **arrowhead** indicates basement membrane-like structure. All experiments were performed with CTOSs from xenograft tumors originated from C45 CTOSs. Scale bars: 50 μ m (**A** and **B**); 1 μ m (**C–H**).

of D-luciferin (VivoGlo luciferin; Promega, Madison, WI) was injected i.p. into anesthetized tumor-bearing mice 10 minutes before imaging. Bioluminescence was measured using the IVIS system (Xenogen Corporation, Alameda, CA). The mice were sacrificed immediately, livers were removed, and *ex vivo* imaging was performed using the same system. We visually counted the number of nodules on the surface of each liver. Next, the livers were fixed in 10% neutral buffered formalin at 4°C for 3 hours. The livers were cut into 2-mm sections, and all sections were placed in 10% neutral buffered formalin again at 4°C overnight. After paraffinization, the tissue samples were cut into 4 μ m sections and subjected to IHC.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Data are

expressed as means $\pm$ SD. Statistical significance was tested using an unpaired *t*-test for single comparison and a one-way analysis of variance followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Polarity Status in CTOSs and Patient Tumors of CRC

First, CTOSs can be stably cultured not only in type I collagen gel but also in suspension.¹⁰ In contrast, a suspension culture of spheroids from normal crypt cells has not been reported.²¹ When C45 CTOSs were cultured in suspension, the apical membrane detected by villin formed on the outside surface (apical-out) (Figure 1A). As previously reported,¹⁰ when cultured in type I collagen gel, the formed

apical lined the surface of multiple lumens inside the CTOSs (apical-in) (Figure 1A). This orientation was confirmed by staining with multiple markers of the apical membrane, including villin, PARD3,²² LMO7,²³ ZO-1, and phalloidin (Figure 1B).

Electron microscopic analysis revealed that microvilli were formed on the outside surface membrane of CTOSs cultured in suspension (Figure 1C). Junctional structures, including tight junctions, adherens junctions, and desmosomes, were observed between the outermost cells of CTOSs (Figure 1, D and E). No inside lumen with microvilli was observed in the CTOSs in suspension. In contrast, microvilli were not formed on the outside-surface membrane of CTOSs cultured in collagen gel (Figure 1F). Instead, basement membrane-like structures were observed near the outside-surface membrane of the outermost cells. The surfaces of the small lumens inside CTOSs were covered by microvilli and sealed with junctional structures (Figure 1, G and H).

We investigated whether these two types of polarity exist in CRC patient tumors. Although most cancer cell nests in differentiated adenocarcinoma were apical-in, both types of polarity were observed in regions of lymphovascular invasion (Figure 2). When a cluster of cancer cells occupied the entire lumen, its polarity status was mainly apical-in (Figure 2, A and B). However, when clusters inside the vessel lumen were apart from surrounding endothelial cells, the polarity status was mainly apical-out (Figure 2, A

and B). The apical-out phenotype was also observed in a case of gastric cancer (Supplemental Figure S1A).

Dynamic Change of Polarity Status in CTOSs

Next, we examined time courses of the change of polarity status. When the culture condition was changed, the polarity status changed from apical-out to apical-in within 8 to 18 hours and vice versa (Figure 3, A and B). The dynamic change of polarity status is referred to as polarity switching. We investigated various extracellular matrices (ECMs) as a trigger of polarity switching and found that type I collagen and Matrigel (which mainly consists of type IV collagen and laminin) potentially induced polarity switching. None of the other matrices examined (eg, fibronectin, vitronectin, hyaluronic acid) affected the polarity status (Figure 3C).

We also assessed the polarity status in 10 CTOS lines, including the C45 CTOS line, derived from nine patients. All 10 CTOS lines exhibited the apical-out phenotype in suspension; however, six of 10 CTOS lines exhibited polarity switching (Figure 3D). When we assessed polarity status in 15 primary CTOS tumors from 12 patients, we observed that all CTOSs exhibited the apical-out phenotype in suspension, and 10 of 15 CTOSs exhibited polarity switching (Supplemental Figure S1B). Thus, polarity switching is a common phenomenon in CRC CTOS.

On the other hand, established cell lines of CRC rarely adopt polarity.^{8,9} We examined three relatively

Figure 2 Both apical-out and apical-in polarity statuses are observed in cancer cell clusters inside the vessels in patient's colorectal cancers. **A:** Immunofluorescence staining images of lymphatic vessel invasion in human colorectal cancer. Green indicates D2-40 (lymphatic endothelial cells); red, LMO7 (apical membrane of cancer cells); blue, DAPI. **B:** Immunofluorescence staining images of blood vessel invasion in human colorectal cancer. Green indicates CD31 (endothelial cells). Patients' clinical characteristics are listed in Supplemental Table S2. Immunohistochemistry was performed with surgically resected tumor samples from patients. Scale bar = 50 μm (A and B).

To further investigate the characters of CTOSs in the none group, which lacked villin staining, we examined another apical marker, ezrin. Double staining of villin and ezrin in CTOSs revealed that these apical markers colocalized both in apical-in and apical-out CTOSs (Supplemental Figure S2, A and C). Cultured in gel, the percentage of CTOSs in the no villin group was almost the same with that in the no ezrin group (Supplemental Figure S2, B and E). In contrast, cultured in floating

Next, we investigated the molecular mechanism of polarity switching in CTOSs. Integrin $\beta 1$ is reportedly involved in polarity formation in Madin-Darby canine kidney (MDCK) cells and primary mammary epithelia.^{27,28} We assessed the role of integrin $\beta 1$ in polarity switching. When treated with a neutralizing antibody of integrin $\beta 1$, the polarity switch from apical-out to apical-in was partially inhibited

Figure 4 Src family kinases (SFKs) and dynamin regulate polarity switching in cancer tissue-originated spheroids (CTOSs). **A:** Quantitative analysis of polarity status to evaluate the effect of a neutralizing antibody against 10 $\mu\text{g}/\text{mL}$ of integrin $\beta 1$ (ITGB1). **B:** Effect of 100 nmol/L focal adhesion kinase (FAK) II; 1 $\mu\text{mol}/\text{L}$ integrin-linked kinase (ILK) inhibitor Cpd22; and SFK inhibitors, including 10 $\mu\text{mol}/\text{L}$ PP1 and 10 $\mu\text{mol}/\text{L}$ dasatinib, on polarity switching *in vitro*. Representative images of villin staining in CTOSs treated with 10 $\mu\text{mol}/\text{L}$ PP1 and 10 $\mu\text{mol}/\text{L}$ dasatinib. **C:** Time course of SFK phosphorylation detected by Western blot in CTOSs embedded in gel and cultured for 24 hours at 50 kDa (white arrowhead) and 75 kDa (black arrowhead). **D:** Western blot shows phosphorylated SFK (p-SFK) and total Src levels from cell lysates of CTOSs treated with dimethyl sulfoxide (DMSO), 10 $\mu\text{mol}/\text{L}$ PPI, or 10 $\mu\text{mol}/\text{L}$ dasatinib in suspension and in gel. **E:** The effect of microtubule inhibitors (1 $\mu\text{mol}/\text{L}$ nocodazole and 100 nmol/L paclitaxel) and dynamin inhibitors (40 $\mu\text{mol}/\text{L}$ MITMAB and 100 $\mu\text{mol}/\text{L}$ dynasore) on polarity switching in CTOSs. Representative images of villin staining in CTOSs treated with 100 $\mu\text{mol}/\text{L}$ dynasore. All experiments were performed with CTOSs from xenograft tumors originated from C45 CTOSs.

(Figure 4A). We also assessed the role of downstream molecules of integrin $\beta 1$ signaling: integrin-linked kinase (ILK), focal adhesion kinase (FAK), and Src.^{29,30} Administration of an FAK inhibitor (FAKII) partially inhibited polarity switching (Figure 4B). An ILK inhibitor (Cpd22) inhibited polarity switching more prominently (Figure 4B). The Src inhibitors PP1 and PP2 robustly inhibited polarity switching (Figure 4B and Supplemental Figure S3, A and B). Polarity switching was completely blocked by dasatinib (Figure 4B and Supplemental Figure S3, A and B), whereas imatinib had no effect (Supplemental Figure S3, A and B). A similar effect of dasatinib was observed in a primary CTOS, C241 (Supplemental Figure S3C).

Inhibition of Src Suppresses Polarity Switching

We further examined Src in the phenomenon of polarity switching. We assessed the change in phosphorylation of the Src family kinase (SFK) by Western blotting in C45 CTOSs. Phosphorylation of SFK was observed in suspension and transiently and marginally increased after cells

were embedded in type I collagen gel (Figure 4C). Both dasatinib and PP1 inhibited phosphorylation (Figure 4D). Dasatinib inhibited even basal levels of phosphorylation in suspension. A similar effect of dasatinib was observed in the C97 CTOS line (Supplemental Figure S3, D and E). Next, we used siRNA methods to suppress SRC gene expression. Effective suppression of total SRC expression by siRNA marginally suppressed polarity switching (Supplemental Figure S3, F and G).

Inhibition of Dynamin Suppresses Polarity Switching

Dynamic microtubule changes and endocytosis are reportedly involved in the formation of cell polarity and lumen formation in normal epithelial cells.³¹ Nocodazole and paclitaxel, inhibitors of microtubule reformation, each had a marginal effect on polarity switching (Figure 4E). Dynamin (DNM), a GTPase, regulates the endocytic trafficking required during apical membrane removal.³¹ The DNM inhibitors MITMAB and dynasore remarkably suppressed polarity switching (Figure 4E). Next, we suppressed DNM2

gene expression by siRNA (Supplemental Figure S3F). Suppression of DNM2 expression marginally suppressed polarity switching (Supplemental Figure S3G).

Polarity Switching *in Vivo*

The tumor from which the C45 CTOS line originated was a moderately differentiated adenocarcinoma. The gland-like structure in the original tumor was apical-in (Supplemental Figure S4A). When it was prepared as a CTOS, the spheroid exhibited apical-out polarity in suspension (Supplemental Figure S4A). When the CTOSs in suspension were s.c. injected into mice, the consequent tumors formed gland-like structures that had regained apical-in polarity (Supplemental Figure S4A). Thus, polarity switching must happen *in vivo* during tumor formation. Next, we assessed the time course of polarity switching *in vivo*. To follow the fate of a single CTOS, we injected CTOSs into the mouse portal vein and examined the morphologic features of CTOSs implanted in the liver at each time point after injection. The

CTOSs mostly remained apical-out at 1 hour within the peripheral portal vein (Supplemental Figure S4B). At day 2, the apical marker was partly lost at the outermost membrane of the cancer cell clusters, whereas lumens lined by apical membrane appeared inside the clusters. At day 5, almost all CTOSs were apical-in (Supplemental Figure S4B). At 4 weeks, all CTOSs were apical-in and featured large lumens lined with apical membrane.

Dasatinib and Dynasore Suppress Formation of Liver Metastases

Portal injection of C45 CTOSs efficiently generated foci of liver metastasis. Using the liver metastasis model, we examined the effects of dasatinib and dynasore. Dasatinib pretreatment alone had no significant effect on the number of liver metastasis foci (Supplemental Figure S5A). Dasatinib treatment of the mice without pretreatment also had no effect on the number of liver metastasis foci (Supplemental Figure S5, B and C). Therefore, in addition to the

Figure 5 Pretreatment with dasatinib and dynasore attenuates metastatic colonization of cancer tissue—originated spheroids (CTOSs) in the liver of nude mice. **A** and **B**: The number of metastatic nodules (**A**) and photon counts (**B**) in mouse livers 28 days after injection of C45/Luc CTOSs. CTOSs were pretreated *in vitro* with dimethyl sulfoxide (DMSO) or dasatinib for 24 hours. After injecting CTOSs into the portal vein, vehicle or dasatinib was administered orally for 28 days. **C**: Representative images of livers in **A** and **B**. Macroscopic and bioluminescent images are shown. **D** and **E**: The number of metastatic nodules (**D**) and photon counts (**E**) in mouse livers 28 days after the injection of C45/Luc CTOSs. CTOSs were pretreated *in vitro* with DMSO or dynasore for 48 hours. The mice were not treated with any drug after CTOS injection. **F**: Representative images of livers in **D** and **E**. Macroscopic and bioluminescent images are shown. Data are expressed as means ± SD (**A**, **B**, **D**, and **E**). $n = 7$ to 8 (**A** and **B**); $n = 14$ to 16 (**D** and **E**). * $P < 0.05$, ** $P < 0.01$.

pretreatment of CTOSs, we treated the mice orally with dasatinib after the injection of CTOSs. Treatment of CTOSs and mice with dasatinib significantly suppressed the number of liver metastasis foci compared with nontreated CTOSs (Figure 5, A–C). Meanwhile, pretreatment alone with dynasore significantly suppressed the number of liver metastasis foci compared with nontreated CTOSs (Figure 5, D–F).

Treatment with Dasatinib or Pretreatment with Dynasore Delays Polarity Switching *in Vitro* and *in Vivo*, Accompanied by Remarkable Cell Death Only *in Vivo*

We investigated the role of polarity switching in the process of forming liver metastases *in vivo*. We examined the effects of pretreatment with dasatinib or dynasore on CTOSs *in vitro*. CTOSs were cultured in medium that contained dasatinib for 24 hours or dynasore for 48 hours in suspension. After washing out the drugs, CTOSs were cultured in gel *in vitro*. Delay of the polarity switch from apical-out to apical-in was still observed in both cases at 24 hours (Supplemental Figure S5D). The growth rate of dasatinib-treated CTOSs *in vitro* did not differ from that of control at any time point (Figure 6A). In contrast, the growth rate of dynasore-treated CTOSs *in vitro* was slightly lower than that of control around day 7 but eventually caught up with control (Figure 6D). Neither treatment induced cell death (Supplemental Figure S5E). Time course analysis of polarity status in the liver after the injection of CTOSs revealed a remarkable delay of polarity switching in the dasatinib-treated CTOSs (Figure 6B and Supplemental Figure S6A) and the dynasore-pretreated CTOSs (Figure 6E and Supplemental Figure S6B). In cancer cell clusters in the liver at day 5 after CTOS injection into the portal vein, we observed disorganized structure and a remarkable increase of cell death with both dasatinib treatment (Figure 6C) and dynasore pretreatment (Figure 6F).

Discussion

The function of epithelial plasticity, the ability of epithelial cells to reversibly alter their phenotype, is attracting increasing attention in cancer metastasis.³² When a tumor metastasizes, its cells reportedly undergo epithelial-to-mesenchymal transition (EMT) and acquire a migratory and invasive phenotype. Meanwhile, the reversion of EMT, the mesenchymal-to-epithelial transition (MET), is essential for metastasized cells to settle in the target organ.³³ We found that CTOSs can form metastases, even though they retain cell-cell contact with the ultrastructure of the epithelial cell-cell junction and membranous localization of E-cadherin in both gel and suspension culture conditions. Thus, the metastasis of cancer cell clusters might not necessarily be accompanied by the EMT/MET that happens in single cell–originated metastasis. Polarity switching might

be a key event for epithelial plasticity in differentiated CRC (Figure 7). Spread and the development of metastasis are intimately dependent on the interaction between the tumor cells and the surrounding tumor microenvironment.³⁴ Interaction between single cells and the microenvironment has been intensively studied. In this study, we found that microenvironment is also a critical cue for polarity switching and possibly subsequent metastasis in CTOSs because CTOSs had an apical-in phenotype within type I collagen gel, whereas floating CTOSs had an apical-out phenotype. Interplay of tumor cell clusters with the microenvironment should be elucidated more in detail in future.

The extent of polarity switching varied among patients (Figure 3D and Supplemental Figure S1B). We previously reported that the status of *KRAS* mutation in primary tumors is mostly preserved in CTOSs.¹⁰ We did not find any correlation between *KRAS* mutation and extent of polarity switching. The viability of primary CTOSs in culture affected their ability to switch polarities: all the CTOSs with poor growth (four of eight) also exhibited poor polarity switching. Thus, poor switching of polarity in CTOSs might be, at least in part, an artifact of *in vitro* culture conditions. In any case, polarity switching is a general characteristic of CRC-derived CTOSs.

The discrepancy between villin and ezrin expression in floating CTOSs might be due to different levels of maturity in apical membrane; villin might be expressed only in the matured apical membrane in CTOSs. Indeed, electron microscopic analysis revealed that the length of microvilli on the outermost cells on floating CTOSs was lower than that on the lumens inside CTOSs in gel (Figure 1, C–H), indicating that apical membrane on floating CTOSs might be more immature than that on CTOSs in gel. Ashley et al⁹ reported that the C80 and SW1222 cell lines, which were relatively differentiated CRC cell lines, expressed both villin and ezrin in lumens inside cell clusters. In contrast, the HCT116 cell line, which was an undifferentiated line, did not express villin but strongly expressed ezrin, although in a nonpolarized manner.

How cell polarity is oriented has been intensively studied with MDCK cells and with mammary epithelial cells grown as hollow cysts.^{31,35} *De novo* lumen formation is accomplished by the initial adherence of cells lacking a luminal space on 3D Matrigel, exocytosis of vesicles to a central luminal region, and then the generation of apical-basal polarization.³⁶ Many laboratories have examined the molecular mechanisms that regulate *de novo* lumen formation or the reorientation of polarity. When cells interact with the ECM through integrins, downstream signals (eg, Rac1)^{27,35} are activated and RhoA is inactivated.³⁷ In mammary epithelia, an ILK-microtubule pathway is used instead of Rac1.³¹ Apical proteins undergo endocytosis from the membrane that directly interacts with ECM and transcytosis to the opposite apical membrane.³¹ A new apical face is established in opposition to the basement membrane, which subsequently leads to lumen formation.

Figure 6 Delayed polarity switching and increased apoptosis *in vivo* after pretreatment with dasatinib or dynasore. **A:** Growth of cancer tissue—originated spheroids (CTOSs) after pretreatment with dasatinib or dynasore. After pretreatment, CTOSs were washed, embedded in gel, and cultured in StemPro medium. Fold-change relative to day 0 is shown. **B:** Quantitative analysis of polarity status in the liver after injection of CTOSs into the portal vein. CTOSs were pretreated *in vitro* with dimethyl sulfoxide (DMSO) (upper row) or dasatinib (lower row) for 24 hours. After injection of the CTOSs into the portal vein, vehicle or dasatinib was administered orally to mice every day. Each time point after injection is indicated. **C:** Representative images of cleaved caspase 3 staining (green) in **B**. Each time point after injection is indicated. Percentage of cleaved caspase 3—positive cells as a percentage of all cells in the tumors in **B**. Each dot represents one focus of the tumor. **D:** Growth of CTOSs after pretreatment with dynasore. After pretreatment, CTOSs were washed, embedded in gel, and cultured in StemPro medium. Fold-change relative to day 0 is shown. **E:** Quantitative analysis of polarity status in the liver after injection of CTOS into the portal vein. CTOSs were pretreated *in vitro* with DMSO or dynasore for 48 hours. The mice were not treated with any drug after CTOS injection. **F:** Representative images of cleaved caspase 3 staining (green) in **E**. Each time point after injection is indicated. Percentage of cleaved caspase 3—positive cells as a percentage of all cells in the tumors in **E**. Each dot represents one focus of the tumor. All experiments were performed with CTOSs from xenograft tumors originated from C45 CTOSs. Data are expressed as means $\pm$ SD. $n = 7$ to 23 (**C**); $n = 15$ to 29 (**F**). * $P < 0.05$, ** $P < 0.0001$, one-way analysis of variance. Scale bar = 50 μ m (**C** and **F**).

Polarity switching in CRC CTOSs is much faster than lumen formation or polarity reorientation in normal cells. It takes approximately 18 hours for polarity to switch in CRC CTOSs; in comparison, polarity reorientation in MDCK cells or primary mammary epithelium takes several days.^{31,38} In addition, CTOSs can survive and even grow in suspension, enabling the switch from apical-in to apical-out; however, suspension culture of spheroids from normal crypt has not been reported.²¹ As with lumen formation in normal cells, polarity switching in CTOS was also triggered by

attachment to or detachment from the ECM, which was partially inhibited by an integrin $\beta 1$ -blocking antibody. In contrast to normal lumen formation, polarity switching in CTOSs was suppressed by neither inhibitors of FAK or ILK nor destabilizers of microtubules. In contrast, inhibitors of Src and DNM efficiently suppressed polarity switching. Altered intracellular signaling or membrane trafficking systems in cancer cells might cause this discrepancy. Indeed, we found that Src activity is critical for polarity switching of CTOSs, a fact that has not been reported in

Figure 7 A schematic representation of polarity switching in liver metastasis of colorectal cancer (CRC) clusters. **A:** At a primary site, original tumor nests display apical-in polarity. When tumor cell clusters invade blood or lymphatic vessels, clusters of cancer cells inside the vessel lumen exhibit apical-out polarity. These clusters may grow and fill the vessel lumen to form apical-in nests or may be carried away by the flow of blood or lymph. **B:** At a distant metastatic site, tumor clusters with apical-out polarity attach to the vessel walls, extravasate from the vessel lumen, and form metastatic foci, which in turn come to exhibit apical-in polarity. If tumor clusters retain the apical-out phenotype inside the vessel lumen, they are eliminated from the bloodstream through an unknown mechanism.

studies of lumen formation in normal cells. The Src molecule is downstream of integrin signals,³⁹ and Src expression is generally stronger in CRC specimens than in normal colonic epithelium.⁴⁰ Increased Src activity is reportedly an independent indicator of poor clinical prognosis at all stages of colon cancer.⁴¹ High levels of Src activity in CRC might contribute to the rapid switching of polarity in CTOS compared with normal epithelial cells or MDCK cells.

The Src inhibitor dasatinib and the DNM inhibitor dynasore were each able to suppress polarity switching. Dasatinib is a multi-tyrosine kinase inhibitor that targets Src family kinases and receptor tyrosine kinases (c-Kit, platelet-derived growth factor receptor [PDGFR] α and β , DDR1, c-FMS, ephrin receptors, and epidermal growth factor receptor [EGFR]) and nonreceptor tyrosine kinases (FRK, BRK, and ACK).^{42,43} Dasatinib is also a BCR/Abl inhibitor approved for use in patients with chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Given that other Src inhibitors and siRNA of Src also suppressed polarity switching, we conclude that Src is a target of dasatinib. However, because dasatinib had the strongest effect among the Src inhibitors tested, it is possible that other targets of dasatinib were also involved in polarity switching because dasatinib targets a broad spectrum of kinases. We examined the mRNA expression of target kinases of dasatinib in the CTOSs by RT-PCR and found that C45 CTOSs expressed a substantial amount of not only SRC but also LYN and YES (Supplemental Figures S7 and S8, A and B). These Src kinase families might also play a role in polarity switching. Because imatinib had no effect on polarity switching, ABL, c-Kit, and PDGF are not likely to be key regulators of polarity switching (Supplemental Figure S3A).

Dynasore is an inhibitor of DNMs (including DNM-1 and DNM-2), large GTPases that are essential for endocytosis and membrane trafficking.⁴⁴ Dynasore suppresses lumen

formation in mammary epithelial cells.³¹ DNM-2 activates Rac1,⁴⁵ which in turn is involved in lumen formation in MDCK cells.³⁵ Dynasore might suppress polarity switching through the inactivation of RAC1 in CTOSs. Because DNM-2 is reportedly a substrate of Src kinase,^{46,47} it might be an effector of Src activation in CTOSs.

It has been reported that low expression of DNM2 is correlated with shorter overall survival of the patients with hepatocellular carcinoma, suggesting that low levels of DNM2 may rather promote metastasis in hepatocellular carcinoma.⁴⁸ In our study, RT-PCR revealed that the mRNA expression levels of DNM2 were high enough to be easily detected in all CTOSs examined (Supplemental Figure S8A). We did not find CTOSs with low expression levels of DNM2; therefore, polarity switching in such CTOSs is unknown. Among these DNM2-expressing CTOSs, DNM2 levels did not correlate with the ability of polarity switching (Supplemental Figure S8C). Thus, inhibition of DNM2 suppressed polarity switching, but steady-state levels of DNM2 did not correlate with polarity switching. It is also reported that inhibition of DNM2 suppresses invasion of human osteosarcoma cell line.⁴⁹ Function of DNM2 might depend on the cell context. Unlike with dynasore, pretreatment alone with dasatinib did not suppress liver metastasis; additional posttreatment with dasatinib was required. Treatment with dasatinib affected neither growth nor death *in vitro*. The time course of polarity switching after pretreatment with dasatinib or dynasore exhibited a similar pattern. Thus, the reason why posttreatment with dasatinib is required must still be clarified.

We found that dasatinib and dynasore each inhibited liver implantation of CRC CTOSs *in vivo*. Dasatinib and dynasore each reportedly suppress the migration of cancer cells,^{49,50} but the cell death in injected CTOSs happened before cancer cells migrated into the liver parenchyma. Pretreatment of CTOSs alone was sufficient for dynasore to suppress liver implantation of the CTOSs. On the other

hand, additional *in vivo* treatment was needed for dasatinib to have a suppressive effect. In both cases, pretreatment induced neither remarkable growth retardation nor cell death *in vitro*, suggesting that the effect of dasatinib or dynasore *in vivo* might be due to inhibition of polarity switching rather than direct inhibition of apoptosis and proliferation. In contrast, a significant increase of cell death was observed in cancer cell clusters in the liver *in vivo*. Because the delay of polarity switching is a common feature of CTOS pretreated by two different drugs *in vitro* and *in vivo*, this delay is likely related to cell death *in vivo*.

Because the presence of epithelial apical membrane in the bloodstream is abnormal, it is reasonable to speculate the existence of some unknown mechanism to eliminate cells with abnormal cellular components, such as apical membrane, from the bloodstream (Figure 7). We found that injected CTOSs underwent massive cell death even in immune-compromised mice when polarity switching was impaired, suggesting that acquired immunity is not necessary. Mochizuki et al⁵¹ reported that von Willebrand factor is a serum factor that induces apoptosis of cancer cells in lung blood vessels. Alternatively, interaction between hepatic sinusoidal endothelial cells and cancer cells might cause apoptosis in apical-out CTOSs in the liver.⁵² In any case, quick polarity switching and subsequent adhesion might be necessary for cancer cell clusters to survive in the bloodstream (Figure 7).

There was no remarkable correlation between polarity switching, SRC or DNM2 expression, and the clinical stage in nine CTOS lines and 12 primary CTOSs (Figure 3D, Supplemental Figure S1B Supplemental Figure S8, A–C, and Supplemental Table S2), although a larger number of patient samples have to be evaluated to assess clinical relevancy to polarity switching in future.

As for the functional role of polarity switching in patient tumor, we propose that polarity switching is not a simple observational phenomenon but is involved in liver metastasis of CRC because i) polarity switching is a rapid and bidirectional phenomenon; ii) polarity switching is a common ability of CRC cell clusters; iii) apical-out phenotype was observed in the microvessel invasion in patient tumors, whereas both original tumor and metastatic foci were all apical-in phenotype, except for micropapillary phenotype; and iv) inhibitors of Src and DNM, even with pretreatment in case of a DNM inhibitor, suppressed polarity switching both *in vitro* and *in vivo* and suppressed formation of metastatic foci in the liver when injected into portal vein. Nonetheless, because both Src and DNM have broad range of targets, it is hard to exclude the possibility that those inhibitors also affected the pathway irrelevant to polarity switching. It would be clarified when the molecules that are critical and specific to polarity switching are elucidated in future.

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Supplemental Data

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References

1. Suzuki A, Ohno S: The PAR-aPKC system: lessons in polarity. *J Cell Sci* 2006, 119:979–987
2. Goldstein B, Macara IG: The PAR proteins: fundamental players in animal cell polarization. *Dev Cell* 2007, 13:609–622
3. Mellman I, Nelson WJ: Coordinated protein sorting, targeting and distribution in polarized cells. *Nat Rev Mol Cell Biol* 2008, 9: 833–845
4. Lee HS, Nishanian TG, Mood K, Bong YS, Daar IO: EphrinB1 controls cell-cell junctions through the Par polarity complex. *Nat Cell Biol* 2008, 10:979–986
5. McCaffrey LM, Montalbano J, Mihai C, Macara IG: Loss of the Par3 polarity protein promotes breast tumorigenesis and metastasis. *Cancer Cell* 2012, 22:601–614
6. Xue B, Krishnamurthy K, Allred DC, Muthuswamy SK: Loss of Par3 promotes breast cancer metastasis by compromising cell-cell cohesion. *Nat Cell Biol* 2013, 15:189–200
7. Compton CC: Colorectal carcinoma: diagnostic, prognostic, and molecular features. *Mod Pathol* 2003, 16:376–388
8. Whitehead RH, Jones JK, Gabriel A, Lukies RE: A new colon carcinoma cell line (LIM1863) that grows as organoids with spontaneous differentiation into crypt-like structures in vitro. *Cancer Res* 1987, 47: 2683–2689
9. Ashley N, Yeung TM, Bodmer WF: Stem cell differentiation and lumen formation in colorectal cancer cell lines and primary tumors. *Cancer Res* 2013, 73:5798–5809
10. Kondo J, Endo H, Okuyama H, Ishikawa O, Iishi H, Tsujii M, Ohue M, Inoue M: Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer. *Proc Natl Acad Sci U S A* 2011, 108:6235–6240
11. Endo H, Okami J, Okuyama H, Kumagai T, Uchida J, Kondo J, Takehara T, Nishizawa Y, Imamura F, Higashiyama M, Inoue M: Spheroid culture of primary lung cancer cells with neuregulin 1/HER3 pathway activation. *J Thorac Oncol* 2013, 8:131–139
12. Okuyama H, Yoshida T, Endo H, Nakayama M, Nonomura N, Nishimura K, Inoue M: Involvement of heregulin/HER3 in the primary culture of human urothelial cancer. *J Urol* 2013, 190:302–310
13. Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009, 119:1420–1428
14. Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Rothenberg SM, Shah AM, Smas ME, Korir GK, Floyd FP Jr, Gilman AJ, Lord JB, Winokur D, Springer S, Irimia D, Nagrath S, Sequist LV, Lee RJ, Isselbacher KJ, Maheswaran S, Haber DA, Toner M: Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc Natl Acad Sci U S A* 2010, 107: 18392–18397
15. Rodriguez-Peralvarez M, Luong TV, Andreana L, Meyer T, Dhillon AP, Burroughs AK: A systematic review of microvascular invasion in hepatocellular carcinoma: diagnostic and prognostic variability. *Ann Surg Oncol* 2013, 20:325–339
16. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Sequist LV, Brachtel E, Sgroi D, Baselga J, Ramaswamy S, Toner M, Haber DA, Maheswaran S: Circulating

- breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* 2013, 339:580–584
17. Honda T, Sakisaka T, Yamada T, Kumazawa N, Hoshino T, Kajita M, Kayahara T, Ishizaki H, Tanaka-Okamoto M, Mizoguchi A, Manabe T, Miyoshi J, Takai Y: Involvement of nectins in the formation of puncta adherentia junctions and the mossy fiber trajectory in the mouse hippocampus. *Mol Cell Neurosci* 2006, 31:315–325
 18. Daoud G, Rassart E, Masse A, Lafond J: Src family kinases play multiple roles in differentiation of trophoblasts from human term placenta. *J Physiol* 2006, 571:537–553
 19. Gibbs EM, Davidson AE, Trickey-Glassman A, Backus C, Hong Y, Sakowski SA, Dowling JJ, Feldman EL: Two dynamin-2 genes are required for normal zebrafish development. *PLoS One* 2013, 8: e55888
 20. Yusa K, Zhou L, Li MA, Bradley A, Craig NL: A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A* 2011, 108:1531–1536
 21. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD, Clevers H: Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011, 141:1762–1772
 22. Mojallal M, Zheng Y, Hultin S, Audebert S, van Harn T, Johnsson P, Lenander C, Fritz N, Mieth C, Corcoran M, Lembo F, Hallstrom M, Hartman J, Mazure NM, Weide T, Grandner D, Borg JP, Uhlen P, Holmgren L: AmotL2 disrupts apical-basal cell polarity and promotes tumour invasion. *Nat Commun* 2014, 5:4557
 23. Tanaka-Okamoto M, Hori K, Ishizaki H, Hosoi A, Itoh Y, Wei M, Wanibuchi H, Mizoguchi A, Nakamura H, Miyoshi J: Increased susceptibility to spontaneous lung cancer in mice lacking LIM-domain only 7. *Cancer Sci* 2009, 100:608–616
 24. Cohen E, Ophir I, Shaul YB: Induced differentiation in HT29, a human colon adenocarcinoma cell line. *J Cell Sci* 1999, 112(Pt 16): 2657–2666
 25. Jordinson M, El-Hariry I, Calnan D, Calam J, Pignatelli M: Vicia faba agglutinin, the lectin present in broad beans, stimulates differentiation of undifferentiated colon cancer cells. *Gut* 1999, 44:709–714
 26. Murakami H, Masui H: Hormonal control of human colon carcinoma cell growth in serum-free medium. *Proc Natl Acad Sci U S A* 1980, 77:3464–3468
 27. Yu W, Datta A, Leroy P, O'Brien LE, Mak G, Jou TS, Matlin KS, Mostov KE, Zegers MM: Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Mol Biol Cell* 2005, 16:433–445
 28. Naylor MJ, Li N, Cheung J, Lowe ET, Lambert E, Marlow R, Wang P, Schatzmann F, Wintermantel T, Schuetz G, Clarke AR, Mueller U, Hynes NE, Streuli CH: Ablation of beta1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *J Cell Biol* 2005, 171:717–728
 29. Playford MP, Schaller MD: The interplay between Src and integrins in normal and tumor biology. *Oncogene* 2004, 23:7928–7946
 30. Wu C, Dedhar S: Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J Cell Biol* 2001, 155: 505–510
 31. Akhtar N, Streuli CH: An integrin-ILK-microtubule network orients cell polarity and lumen formation in glandular epithelium. *Nat Cell Biol* 2013, 15:17–27
 32. Nieto MA: Epithelial plasticity: a common theme in embryonic and cancer cells. *Science* 2013, 342:1234850
 33. Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J: Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell* 2012, 22:725–736
 34. Joffe C, Barrow R, Menard L, Calleja V, Hart IR, Kermorgant S: A direct role for Met endocytosis in tumorigenesis. *Nat Cell Biol* 2011, 13:827–837
 35. O'Brien LE, Jou TS, Pollack AL, Zhang Q, Hansen SH, Yurchenco P, Mostov KE: Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat Cell Biol* 2001, 3:831–838
 36. Sigurbjornsdottir S, Mathew R, Leptin M: Molecular mechanisms of de novo lumen formation. *Nat Rev Mol Cell Biol* 2014, 15:665–676
 37. Yu W, Shewan AM, Brakeman P, Eastburn DJ, Datta A, Bryant DM, Fan QW, Weiss WA, Zegers MM, Mostov KE: Involvement of RhoA, ROCK I and myosin II in inverted orientation of epithelial polarity. *EMBO Rep* 2008, 9:923–929
 38. Peng J, Awad A, Sar S, Hamze Komaiha O, Moyano R, Rayal A, Samuel D, Shewan A, Vanhaesebroeck B, Mostov K, Gassama-Diagne A: Phosphoinositide 3-kinase p110delta promotes lumen formation through the enhancement of apico-basal polarity and basal membrane organization. *Nat Commun* 2015, 6:5937
 39. Huveneers S, Danen EH: Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci* 2009, 122:1059–1069
 40. Cartwright CA, Kamps MP, Meisler AI, Pipas JM, Eckhart W: pp60c-src activation in human colon carcinoma. *J Clin Invest* 1989, 83:2025–2033
 41. Aligayer H, Boyd DD, Heiss MM, Abdalla EK, Curley SA, Gallick GE: Activation of Src kinase in primary colorectal carcinoma: an indicator of poor clinical prognosis. *Cancer* 2002, 94:344–351
 42. Montero JC, Seoane S, Ocana A, Pandiella A: Inhibition of SRC family kinases and receptor tyrosine kinases by dasatinib: possible combinations in solid tumors. *Clin Cancer Res* 2011, 17:5546–5552
 43. Li J, Rix U, Fang B, Bai Y, Edwards A, Colinge J, Bennett KL, Gao J, Song L, Eschrich S, Superti-Furga G, Koomen J, Haura EB: A chemical and phosphoproteomic characterization of dasatinib action in lung cancer. *Nat Chem Biol* 2010, 6:291–299
 44. Kirchhausen T, Macia E, Pelish HE: Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis. *Methods Enzymol* 2008, 438:77–93
 45. Razidlo GL, Wang Y, Chen J, Krueger EW, Billadeau DD, McNiven MA: Dynamin 2 potentiates invasive migration of pancreatic tumor cells through stabilization of the Rac1 GEF Vav1. *Dev Cell* 2013, 24:573–585
 46. Weller SG, Capitani M, Cao H, Micaroni M, Luini A, Salles M, McNiven MA: Src kinase regulates the integrity and function of the Golgi apparatus via activation of dynamin 2. *Proc Natl Acad Sci U S A* 2010, 107:5863–5868
 47. Feng H, Liu KW, Guo P, Zhang P, Cheng T, McNiven MA, Johnson GR, Hu B, Cheng SY: Dynamin 2 mediates PDGFRalpha-SHP-2-promoted glioblastoma growth and invasion. *Oncogene* 2012, 31:2691–2702
 48. Gong C, Zhang J, Zhang L, Wang Y, Ma H, Wu W, Cui J, Wang Y, Ren Z: Dynamin2 downregulation delays EGFR endocytic trafficking and promotes EGFR signaling and invasion in hepatocellular carcinoma. *Am J Cancer Res* 2015, 5:702–713
 49. Yamada H, Abe T, Li SA, Masuoka Y, Isoda M, Watanabe M, Nasu Y, Kumon H, Asai A, Takei K: Dynasore, a dynamin inhibitor, suppresses lamellipodia formation and cancer cell invasion by destabilizing actin filaments. *Biochem Biophys Res Commun* 2009, 390:1142–1148
 50. Pichot CS, Hartig SM, Xia L, Arvanitis C, Monisvais D, Lee FY, Frost JA, Corey SJ: Dasatinib synergizes with doxorubicin to block growth, migration, and invasion of breast cancer cells. *Br J Cancer* 2009, 101:38–47
 51. Mochizuki S, Soejima K, Shimoda M, Abe H, Sasaki A, Okano HJ, Okano H, Okada Y: Effect of ADAM28 on carcinoma cell metastasis by cleavage of von Willebrand factor. *J Natl Cancer Inst* 2012, 104: 906–922
 52. Vekemans K, Braet F, Muyliaert D, Wisse E: Nitric oxide from rat liver sinusoidal endothelial cells induces apoptosis in IFN gamma-sensitized CC531s colon carcinoma cells. *J Hepatol* 2004, 41: 11–18