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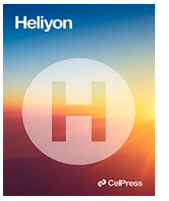

Dasatinib suppresses collective cell migration through the coordination of focal adhesion and *E*-cadherin in colon cancer cells

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ABSTRACT

Collective cell migration is an important process in cancer metastasis. Unlike single-cell migration, collective cell migration requires *E*-cadherin expression in the cell cohort. However, the mechanisms underlying cellular contact and focal adhesions remain unclear. In this study, Src was hypothesized to coordinate focal adhesion and Rab11-mediated *E*-cadherin distribution during collective cell migration. This study primarily used confocal microscopy to visualize the 3D structure of cell-cell contacts with associated molecules. These results demonstrate that the clinical Src inhibitor dasatinib was less toxic to HT-29 colon cancer cells; instead, the cells aggregated. 3D immunofluorescence imaging showed that Rab11 was localized with *E*-cadherin at the adherens junctions of the apical cell-cell contacts. In the transwell assay, Rab11 colocalized with a broad range of *E*-cadherin proteins in collectively migrated cells, and dasatinib treatment significantly suppressed collective cell migration. Transmission electron microscopy demonstrated that dasatinib treatment increased cell membrane protrusion contacts and generated spaces between cells, which may allow epidermal growth factor receptor activity at the cell-cell contacts. This study suggests that dasatinib treatment does not inhibit cell survival but targets Src at different cellular compartments in the coordination of focal adhesions and cell-cell contacts in collective cell migration through *E*-cadherin dynamics in colon cancer cells.

1. Introduction

Colorectal carcinoma is the leading cause of death worldwide, and epithelial-mesenchymal transition (EMT) is thought to be a sign of tumor progression and metastasis in epithelium-based cancers, including colorectal cancer [1]. Normal epithelial cells express *E*-cadherin, catenin, and other junctional adhesion proteins in areas of cell-cell contact; however, tumor cells that express mesenchymal markers have a greater tendency to invade and metastasize [2]. In the process of EMT, epithelial cells lose cell-cell contacts by downregulating *E*-cadherin and β-catenin in the cell membrane. When cells exhibit a fibroblast-like appearance during transformation, they upregulate mesenchymal markers such as vimentin which is associated with highly invasive and metastatic properties. EMT is regulated by the Snail, Slug, and Twist transcription factors [3–6]. Therefore, *E*-cadherin is considered a "tumor suppressor."

The loss of *E*-cadherin is necessary for cell transformation and subsequent single-cell migration; however, in collective cell

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migration, *E*-cadherin expression is required to maintain cellular connections of. Therefore, *E*-cadherin has a dual role in single-cell migration and collective cell migration [7–9]. Moderate *E*-cadherin expression indicates that its dynamics are related to collective cell migration [10–13]. Although some EMT-associated proteins, such as Snail, Slug, and ZEB1, can target the *E*-cadherin promoter [14], our previous work showed that ZEB1 expression is not correlated with *E*-cadherin expression in patients [15] and that Rab11, which is a recycling endosomal protein, can regulate *E*-cadherin during collective cell migration in colon cancer cells [16].

Vesicle trafficking systems, including endocytosis and exocytosis, are comprised by the cellular vesicles responsible for protein delivery and localization to different cell compartments. Rabs are GTPase proteins involved in vesicle trafficking [17]. Rab11 has been shown to facilitate membrane integrin recycling for focal adhesion turnover and cell motility [18–20] and has also been demonstrated to play an important role in *E*-cadherin transport in epithelial cell morphogenesis [21]. Although the role of *E*-cadherin in collective cell motility has recently emerged, the detailed mechanism of coordination between focal adhesions and cell-cell contact and its relationship to Rab11 is still under investigation.

In an earlier study, Src was found to be associated with collective cell migration in vivo [22]; however, the underlying molecular mechanism remains unknown. Src is a non-receptor tyrosine kinase discovered several decades ago as an oncoprotein. Src plays many important roles in cancer progression, especially in the activation of focal adhesion kinase (FAK) for cell migration [23]; however, few studies have focused on the role of Src in collective cell migration. Src has been identified to physically interacts with *E*-cadherin in head and neck squamous carcinoma cells, and elevated Src expression is related to *E*-cadherin expression. Src inhibitors can suppress collective cell migration [24]; however, the dynamics of *E*-cadherin and its association with focal adhesions remain unclear. Src activation occurs at a high frequency during colorectal cancer development [25], and it has also been reported that Src is activated during colorectal tumor development in highly malignant polyps, but not in benign polyps [26]. Recently, Src has been considered for therapeutic applications in RAS/RAF-mutated colon cancer with limited treatments [27,28]; however, the mechanism of its efficacy in colon cancer remains unclear and needs to be investigated. Dasatinib is an FDA-approved Src inhibitor with multiple targets for the treatment of chronic myeloid leukemia. Its application in other solid tumors, including colon cancer [29–31], has also been studied; however, its underlying mechanism in epithelial cells remains unclear. Therefore, this study demonstrates the application of dasatinib in colon cancer cells to decipher the role of Src in coordinating focal adhesions and *E*-cadherin in collective colon cell migration.

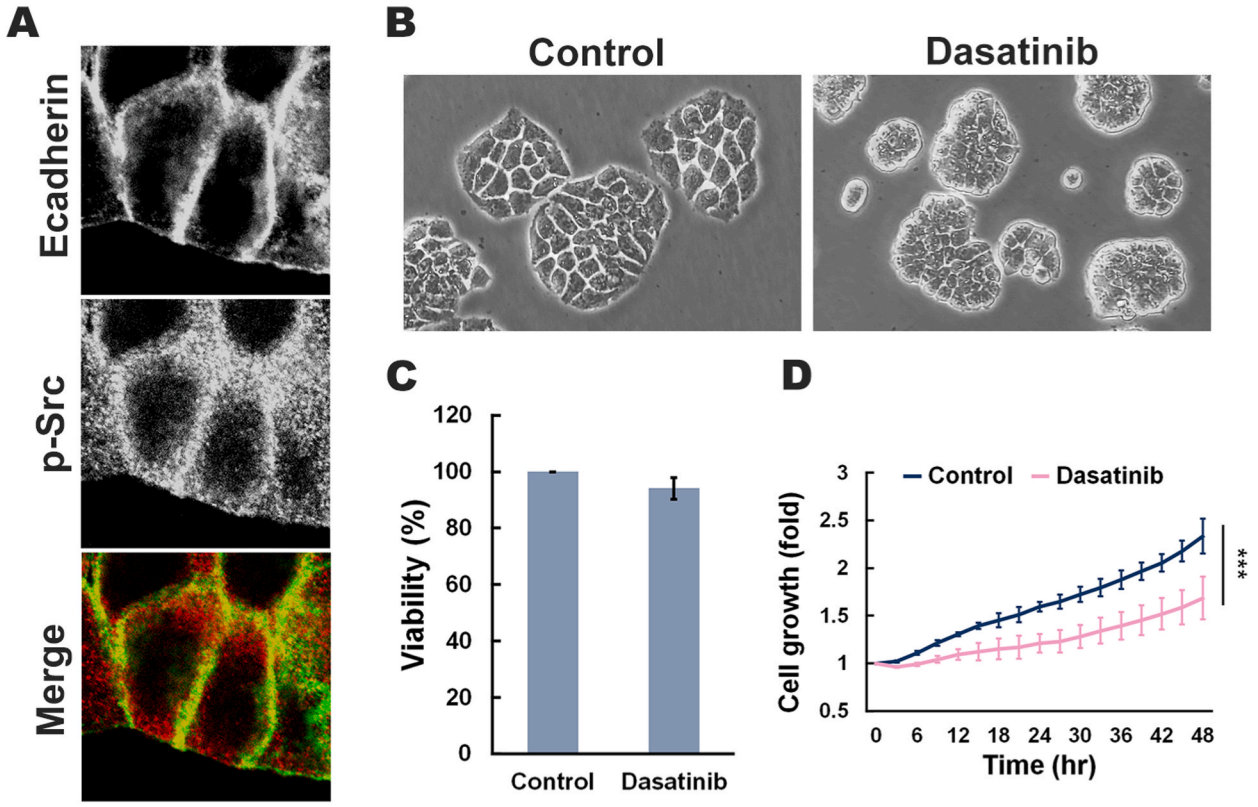

Fig. 1. Src inhibition induced the formation of cell clusters. (A) Immunoconfocal microscopy showed that *E*-cadherin and *p*-Src colocalized in the cell-cell contacts (scale bar = 10 μm). (B) Phase contrast image of HT-29 cells treated with dasatinib (scale bar = 50 μm). (C) HT-29 cells were treated with 20 nM dasatinib for 24 h, and cell viability was determined by MTT assay. The quantification shows no significant difference (value = mean ± SD, at least three independent experiments were performed). (D) Cell growth was determined by Incucyte imaging, which measured the cell area to calculate the cell growth rate (value = mean ± SD, ***$p < 0.001$. At least three independent experiments were performed).

2. Results

2.1. Src inhibition has no major effect on cell toxicity

Src is a well-known non-receptor tyrosine kinase that regulates FAK; however, in this study, active Src was also localized in cell-cell contacts and co-distributed with *E*-cadherin in HT-29 cells (Fig. 1A). HT-29 cells are colon cancer cells with an ideal epithelial phenotype and harbor *BRAF* mutations that can be used studying cell-cell junctions and drug resistance, as described in Materials and Methods. When cells were treated with 20 nM of the Src inhibitor dasatinib, the cell morphology shrank and the cells aggregated as cell clusters (Fig. 1B). Src inhibition suppresses cell growth In the MTT assay, dasatinib treatment had no major effect on cell viability (Fig. 1C). However, when cell growth was monitored by cell area, the results demonstrated a significant inhibition of cell growth (Fig. 1D).

These results suggest that the non-receptor tyrosine kinase Src is not critical for cell survival in HT-29 colon cancer cells; instead, Src localized in the cell-cell contacts may play a role in *E*-cadherin dynamics to alter the morphology of the cell cohort. *E*-cadherin is required as an adherens junction protein for epithelial cell––cell contact. Our results showed that Src inhibition had no toxic effect on cell survival. However, Src inhibition induced cell aggregation (Fig. 1B), and the shrinkage of the cluster area (Fig. 1D) may be associated with Src function in focal adhesions and the cell-cell contacts.

2.2. Src inhibition restores E-cadherin localization

E-cadherin is a major component of adherens junctions. In HT-29 cells, *E*-cadherin localized between neighboring cells (Fig. 2A). When the cells were treated with dasatinib, Most of the *E*-cadherin distribution lost polarity and was localized at the cell leading edge (Fig. 2A and B). This result demonstrates that Src may play a role in *E*-cadherin orientation in the cell-cell contact region. *E*-cadherin has been shown to play a role in the cell-cell contact inhibition of cell growth [32]. In this study, when the cells were stained with Ki67, a proliferation marker, although dasatinib treatment restored *E*-cadherin distribution, Ki67 expression did not differ (Fig. 2C). These results indicate that the inhibition of Src activity by dasatinib can induce *E*-cadherin redistribution in cells, and that the redistribution of *E*-cadherin has no effect on cell-cell contact inhibition, which can suppress cell proliferation.

For the phenomenon of cell-cell contact inhibition, cells divide and grow as a monolayer, and cells stop dividing when they are confluent and in contact with neighboring cells. *E*-cadherin is important for cell-cell contact and has also been demonstrated to be related to cancer progression [33]. In this study, dasatinib treatment altered the morphology of the cell cohort and was accompanied by *E*-cadherin reorientation; however, it had less of an effect on cell division.

2.3. Rab11 is responsible for E-cadherin orientation at adherens junctions

Since *E*-cadherin was redistributed after dasatinib treatment in our study and in other studies demonstrating that the recycling transport protein Rab11 played a role in *E*-cadherin turnover [15,21], the relocation of *E*-cadherin might be due to the effect of Rab11. When cells were treated with dasatinib, *E*-cadherin and Rab11 were still colocalized in the cell-cell contacts, but not in the leading cell edges (Fig. 3A). The line scan profile indicated that Rab11 was located in the cytosol near the leading edge of *E*-cadherin; however, the signals did not overlap. In the Z-scanning confocal images, Rab11 was localized to *E*-cadherin in the apical region of the cell-cell contacts (Fig. 3B and C); however, when cells were treated with dasatinib, Rab11 was distributed along the cell-cell contact from basal to apical. When the cellular protein (immunoprecipitation input) and mRNA expression levels were analyzed, *E*-cadherin and

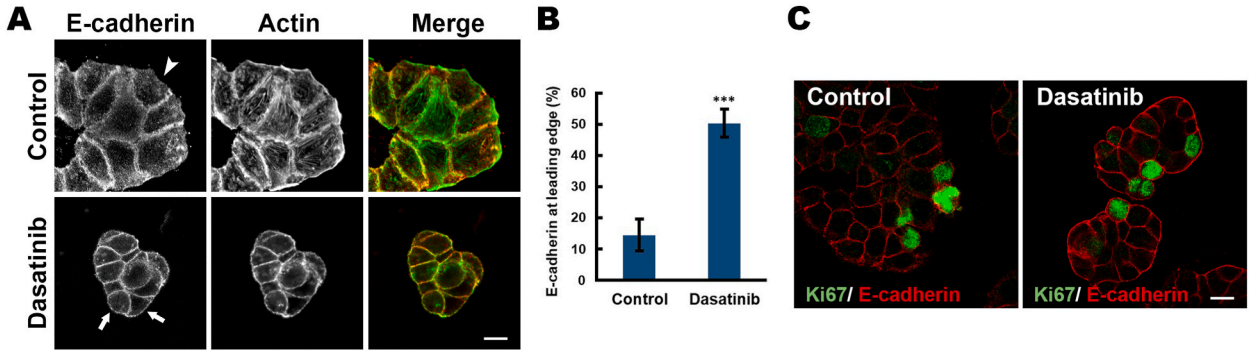

Fig. 2. Src inhibition restored *E*-cadherin localization. (A) HT-29 cells treated with 20 nM dasatinib were immunostained with *E*-cadherin and actin antibodies and visualized by confocal microscopy. In control cells, *E*-cadherin was not distributed in the leading edge (arrowhead). In dasatinib-treated cells, *E*-cadherin was observed in the cell leading edge (arrow) (scale bar = 20 μm). (B) The quantification of *E*-cadherin localized at cell leading edge after dasatinib treatment, (value = mean ± SD, ***p < 0.001. At least three independent experiments were performed for quantification). (C) Lower magnification images of HT-29 cells treated with 20 nM dasatinib were immunostained with *E*-cadherin (red) and Ki67 (green) antibodies. The immunofluorescence images showed that dasatinib treatment resulted in the redistribution of *E*-cadherin and had no influence on cell proliferation (scale bar = 100 μm).

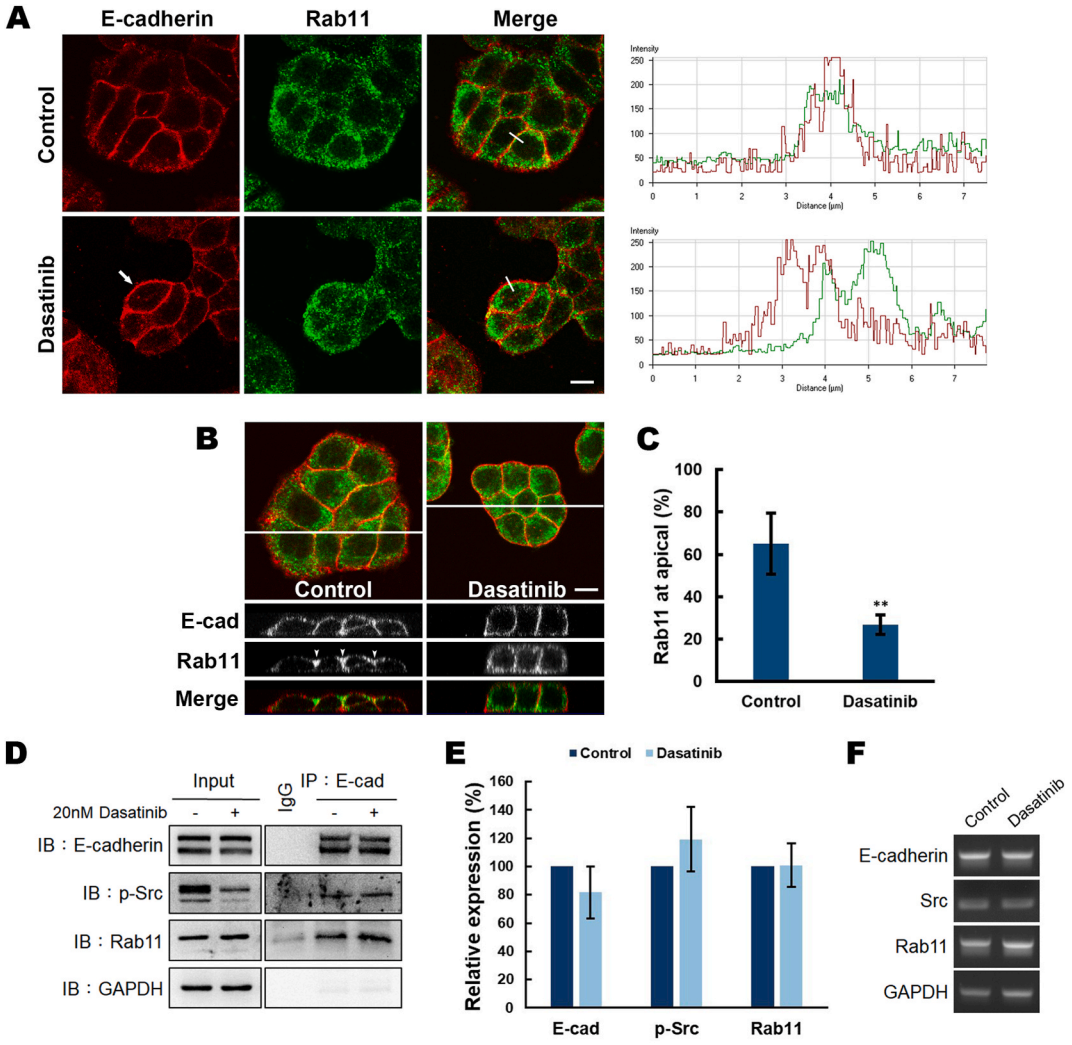

Fig. 3. Rab11 is responsible for *E*-cadherin orientation at adherens junctions. (A) HT-29 cells treated with 20 nM dasatinib were immunostained with *E*-cadherin and Rab11 antibodies and analyzed by confocal microscopy. In control cells, intense *E*-cadherin and Rab11 staining were revealed majorly colocalized at the cell-cell contact region, diffused-like cytosolic *E*-cadherin distribution can be observed at the leading edge. When HT-29 cells were treated with dasatinib, intensive *E*-cadherin was distributed in the leading edges (arrow) as described previously. The line scan profile of the indicated region (white line in the merged images) demonstrated that *E*-cadherin and Rab11 signals overlapped at the cell-cell contact sites; however, in the leading edges of dasatinib-treated cells, the Rab11 signal did not overlap with the *E*-cadherin signal (scale bar = 20 μm). (B) Cells were Z-scanned with sections for 3D construction, the side view of the indicated lines are shown, and the arrowhead shows Rab11 accumulated at the apical region of the cell-cell contacts in the control cells (Scale bar = 20 μm). (C) Quantification of Rab11 localization at the apical region, (value = mean ± SD, **p < 0.01. At least three independent experiments were performed for quantification). (D) Cell lysates of HT-29 cells treated with and without dasatinib were collected and subjected to immunoprecipitation for *E*-cadherin interaction with *p*-Src and Rab11. GAPDH was used as a control. The original images are shown in supplementary Figure S1 (E) Quantification of immunoprecipitation result of *E*-cadherin, pSrc and Rab11 expressions, (value = mean ± SD. At least three independent experiments were performed for quantification). (F) Cellular mRNA was extracted, and the expression levels of *E-cadherin*, *Src*, *Rab11*, and *GAPDH* were determined by RT-polymerase chain reaction (PCR). The original images are shown in Supplementary Fig. S2. (E-cad: *E*-cadherin).

Rab11 expression levels did not change after dasatinib treatment (Fig. 3D–F). In the immunoprecipitation results, dasatinib treatment did not inhibit the interaction of *E*-cadherin with Rab11 and *p*-Src (Fig. 3D); however, *p*-Src activation was decreased in the input due to dasatinib treatment. These results demonstrate that Src plays a role in the regulation of Rab11-mediated *E*-cadherin transport to cell-cell contacts in the apical region of adherens junctions. Inhibition of Src activity had less of an effect on the physical interaction between Src and *E*-cadherin in the cell-cell contact area; however, the apical distribution of Rab11 was disrupted after dasatinib treatment.

Adherens junctions, which are localized in the apical region of cell-cell membrane contacts, play an important role in maintaining epithelial cell polarity and flexibility. *E*-cadherin is the major transmembrane protein of adherens junctions and not only has a

structural function, but is also involved in signal transduction. These results suggest that Src phosphorylation is critical for Rab11-mediated *E*-cadherin localization at the apical region of cell-cell contacts and maintains the flexibility of adherens junctions. Dasatinib treatment can inhibit Src activation and affect Rab11 distribution, but not the affinity of the interaction between Src, *E*-cadherin, and Rab11, which may affect the dynamics of *E*-cadherin turnover on the membrane at cell-cell contacts.

2.4. Dasatinib inhibits collective cell migration by suppressing FAK activity

Focal adhesion is an important process in the adhesion and migration of mesenchymal cells as the mechanism of single cell migration. FAK is a major component of the focal adhesion complex and is regulated by Src kinase. In this study, HT-29 cells demonstrated FAK activity in the peripheral region of focal adhesion sites in an epithelial cell cohort (Fig. 4A). When dasatinib was applied, FAK activity in the peripheral region decreased; however, without FAK activity, the cells remained attached and connected by *E*-cadherin, as mentioned previously. Since FAK is associated with cell migration, the cell migration ability was examined using transwell assays. The results showed that dasatinib treatment inhibited HT-29 cell migration and collective cell migration, which is the movement of cell clusters (Fig. 4B). Quantification results are shown in Fig. 4C. The area of the migrated cell cluster was also examined, and dasatinib treatment resulted in 83 % single cluster size and 45 % total cluster size compared to the control group, indicating that dasatinib treatment inhibited both single and collective cell migration, but primarily suppressed the number of migrated clusters.

The distribution of *E*-cadherin and Rab11 was revealed in migrated cells through transwell assays, which demonstrated a broad range of *E*-cadherin and Rab11 in cell-cell contacts in collectively migrated cells (Fig. 4D). These results suggest that Src activity in focal adhesions is important for single-cell and collective cell migration and that the loose distribution of *E*-cadherin is critical for the movement of the cell cohort.

FAK and *E*-cadherin are localized in the focal adhesion of the leading edge and adherens junction in different cell areas; however, in collective cell migration, the movement of the cell cohort requires both turnover of FAK and *E*-cadherin. This study suggests that Src localized in different cellular compartments, such as focal adhesions and adherens junctions, has different drug sensitivities to Src inhibition and affects collective cell migration. Therefore, we assumed that the local distribution of Src plays a role in coordinating FAK and *E*-cadherin turnover for collective cell migration.

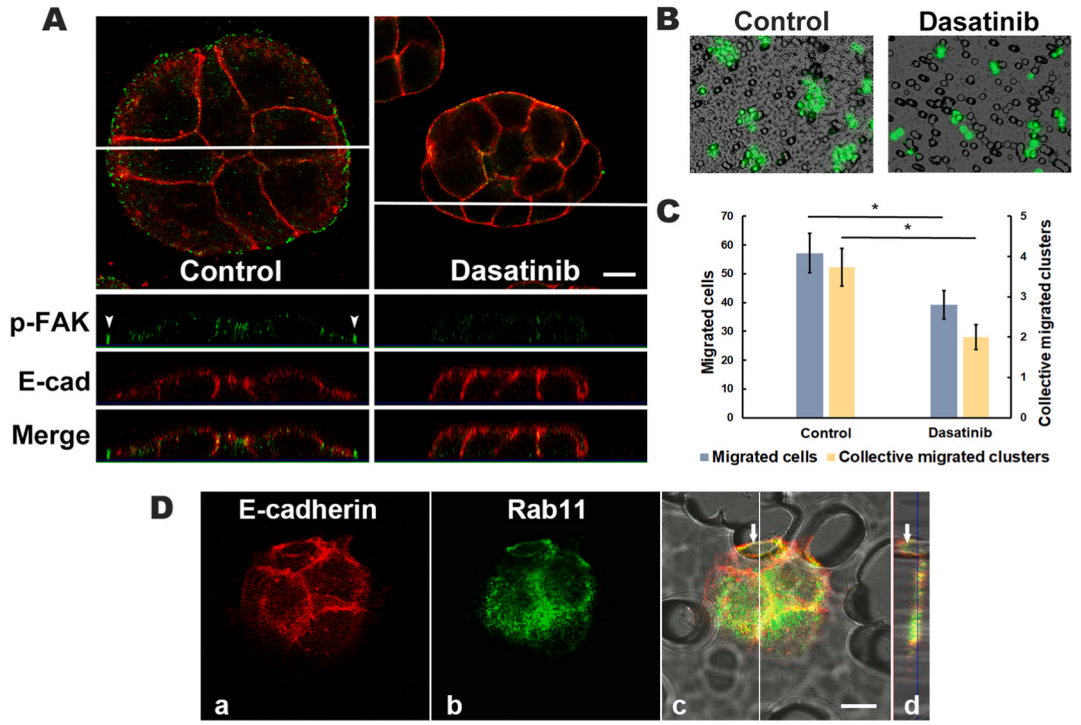

Fig. 4. Dasatinib inhibited collective cell migration by suppressing FAK activity. (A) HT-29 cells treated with and without dasatinib were immunostained with *E*-cadherin and *p*-FAK antibodies and revealed by confocal microscopy. Z-scanning with sections was composed for a side view of the indicated lines. Arrowheads indicate the region of *p*-FAK. (Scale bar = 20 μm). (B) Transwell assays determined the migration ability after Src inhibition. DAPI was used to stain migrated cells (pseudocolored as green). More than five cells aggregated as clusters were identified as collective migrated clusters. (C) The quantification of migrated cells and collectively migrated clusters (value = mean ± SD, *p < 0.05. At least three independent experiments were performed for quantification). (D) Migrated cells on the transwell membrane were stained with *E*-cadherin (a) and Rab11 (b) antibodies and Z-scanning for 3D images. The arrow shows (c and d) that the cells were migrating out of the membrane to the other side of the transwell (d) (Scale bar = 20 μm). (E-cad: *E*-cadherin)

2.5. Src inhibition remodels cell-cell contact structure

When cells were stained with the phospho-epidermal growth factor receptor (EGFR) antibody, confocal images showed that dasatinib treatment did not inhibit EGFR activation at the cell-cell contact (Fig. 5A and B). To investigate the ultrastructure of the cell-cell contacts, transmission electron microscopy (TEM) was performed to observe the HT-29 cell cohort after dasatinib treatment. In normal HT-29 epithelial cells, the average space between cells was approximately 1.13 μm (Fig. 5C and D). In dasatinib-treated cells, the average space between cells was approximately 0.59 μm, and more membrane protrusion contacts were observed. Although dasatinib treatment reduced the space between cells, membrane protrusion contacts prevented whole-cell membrane contacts and may leave space for the stimulation of other receptors, such as EGFR, which is the major signal upstream of cell growth.

Taken together, these results indicate that Src activation regulates collective cell migration by coordinating Rab11-mediated FAK and *E*-cadherin dynamics in focal adhesions and adherens junctions. *E*-cadherin dynamics, regulated by Src activity, may also be an important mechanism for preventing cell-cell contact inhibition in cell clusters during collective cell migration (Fig. 5E).

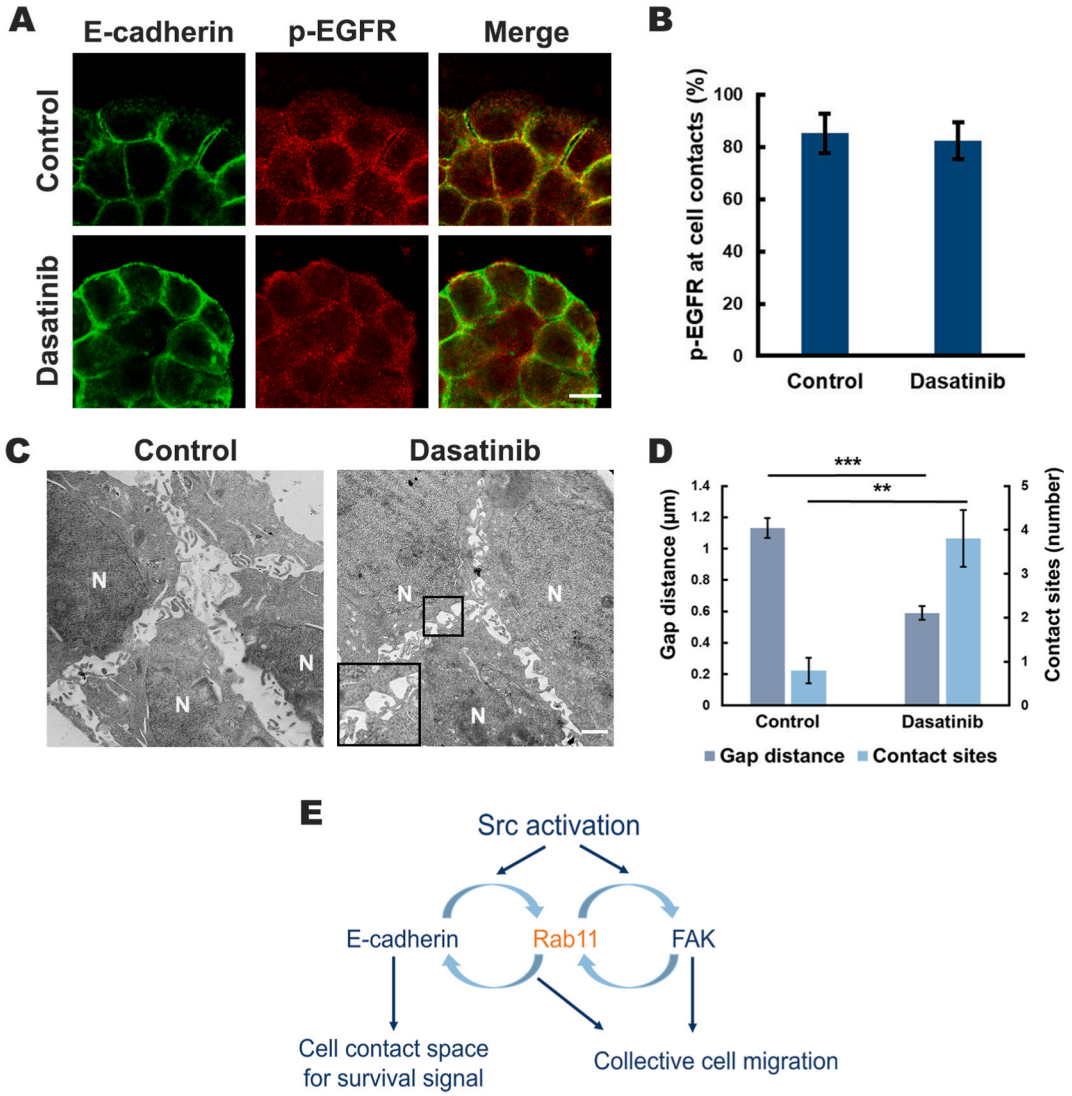

Fig. 5. Src inhibition remodels the cell-cell contact structure. (A) HT-29 cells treated with dasatinib were immunostained with *E*-cadherin and *p*-EGFR antibodies and observed by confocal microscopy. Phospho-EGFR was revealed at the cell-cell contacts after dasatinib treatment (scale bar = 20 μm). (B) Quantification of pEGFR distribution at the cell contacts after dasatinib treatment, (value = mean ± SD. At least three independent experiments were performed for quantification). (C) HT-29 cells treated with dasatinib were subjected to TEM. The micrographs show the region of cell-cell contacts (N, nucleus), and the square area is enlarged in the image (scale bar = 650 nm). (D) Quantification of gap distance and the number of contact sites from (C), (value = mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$. At least three independent experiments were performed for quantification). (E) Working model of Src activation coordinates *p*-FAK and *E*-cadherin dynamics in collective cell migration.

3. Discussion

E-cadherin dynamics at the cell membrane have been shown to be regulated by Rab11 [16,21]. This study is the first to demonstrate that Rab11 interacts with *E*-cadherin at the apical region of epithelial cells and that dasatinib treatment can alter Rab11 distribution and accumulation in cell-cell contacts, which interferes with collective cell migration. *E*-cadherin is an important cell-cell contact protein that acts as an adherens junction, and the loss of *E*-cadherin is necessary for cell transformation for single-cell migration. However, in collective cell migration, *E*-cadherin expression is required to maintain the connection between cells; therefore, *E*-cadherin plays a dual role in single-cell and collective cell migration [7,34]. Moderate expression of *E*-cadherin promotes breast cancer invasion, especially migration toward stromal cells [34]. Our previous study showed that elevated *E*-cadherin expression promoted collective cell migration in colon cancer cells [16]. Loosening *E*-cadherin with high dynamics leads to collective cell migration; adherens junctions act as "elastic bands" for the migrated cell cohort. This study demonstrates that Src inhibition can suppress the flexibility of adherens junctions during collective cell migration.

The location of *E*-cadherin in the cell-cell contacts was associated with epithelial cell polarity, and our results demonstrated that *E*-cadherin reorientation was accompanied by a decrease in FAK which is required for cell migration. The dynamics of *E*-cadherin at the adherens junction are also important for regulating physical tension and tracking forces during cell migration [35]; therefore, the different roles of *E*-cadherin are required for collective cell migration.

Vesicle trafficking is complicated and is regulated by various signals for different targets. During cell migration, the non-receptor tyrosine kinase Src can coordinate EGFR and integrin signals for cell migration processes [36,37]. Src regulates endosomal transport. During EMT, Src activation promotes the internalization of *E*-cadherin into lysosomes for degradation [38]. The inhibition of Src activity can decrease *E*-cadherin endocytosis and increase membrane stability [39]. In another study, Src underwent rapid exchange between the plasma membrane and lysosomes, which was blocked by the dominant-negative Rab11 [40]. These studies suggest that Src may be an important regulator of a variety of endosome transporters for *E*-cadherin turnover, resulting in the loosening of adherens junctions during collective cell migration. In our study, the migrated cells in the transwell assay demonstrated a wide *E*-cadherin distribution pattern, and TEM imaging demonstrated that the cells maintained space in cell-cell contacts for EGFR phosphorylation. These results support the conclusion that *E*-cadherin dynamics affect collective cell migration and cell growth.

An important function of Src is the regulation of focal adhesion dynamics. In the focal adhesion complex, integrin receptors and FAKs are regulated by Rab11 [18]. Our previous study also demonstrated that Rab11 directly interacts with FAKs to promote sarcoma cell migration [41]. This study showed that dasatinib treatment decreased FAK activity, resulted in the accumulation of Rab11 in the cytosol near the leading edge. Rab11 has been shown to colocalize with *E*-cadherin along −cell-cell contacts, and the response of Src activity in the FAK and *E*-cadherin machinery may be different. Our results demonstrated that dasatinib treatment inhibited Src phosphorylation and did not change Src transcription. However, in the immunoprecipitation results, dasatinib treatment did not reduce *E*-cadherin levels or the affinity of *E*-cadherin for phosphorylated Src. In previous studies, Src has been shown to promote EMT. Silencing of Src with siRNA increases *E*-cadherin expression in breast cancer cells [42]. Dosch et al. demonstrated that in pancreatic cancer cells, Src is a negative regulator of *E*-cadherin expression, which is important for EMT during single-cell migration, and that Src-mediated Slug translocation into the nucleus is responsible for dasatinib sensitivity in cells [43]. Our study demonstrated that Src inhibition was less toxic to HT-29 colon cancer cells and that *E*-cadherin expression was not affected by dasatinib treatment. These properties may be due to dasatinib resistance, according to Dosch et al. Furthermore, the loss of *E*-cadherin polarity in HT-29 cells after dasatinib treatment was associated with Rab11 expression and correlated with collective cell migration.

E-cadherin has been reported to activate Src during −cell contact to support its function. In our study, although Src activity was suppressed by dasatinib treatment, *E*-cadherin still recruited and activated Src [44]. These results indicated that an appropriate concentration of dasatinib may alter Src activity in focal adhesions and affect the polarized distribution of Rab11-mediated *E*-cadherin in collective cell migration. Our previous study on HT-29 cells also showed that Rab11 depletion with shRNA can inhibit *E*-cadherin expression and suppressed collective cell migration [16]. Therefore, the Rab11 vesicle protein is necessary for *E*-cadherin transport, however, the mechanism of signal regulations can further affect collective cell migration.

For collective cell migration, although Src and FAK form a signal complex in focal adhesion for cell migration, other study also indicated Src activity has different required in cancer cell motility modes [45]. Recent study in drosophila revealed Src 42 A mutation is required for *E*-cadherin dynamics at cell junctions [46], and the role of *E*-cadherin in border cell collective migration has also been well observed in drosophila [9]. The kinase activity of Src and the protein interaction between Src, *E*-cadherin and Rab11 are complicated. In this study, the mRNA expression and protein interactions of pSrc, *E*-cadherin and Rab11 were not affected by dasatinib treatment, but the localization has been modulated. Instead of transcriptional control, the regulation with the coordination of focal adhesion, adherens junction, signal transductions and vesicle trafficking system still need more investigations for understand the molecular networks.

Src is highly expressed in colon cancer and can be a potential therapeutic target; however, because of the multiple cellular functions of Src, the application of Src inhibitors still requires a specific mechanism to clarify precise drug efficacy. This study provides a novel mechanism for Src in collective cell migration. Collective cell migration is an important phenomenon in metastasis which lacks a treatment option. This study demonstrated that the clinical Src inhibitor, dasatinib, can be considered a treatment strategy to suppress collective cell migration in RAS/RAF-mutated colon cancer.

4. Materials and Methods

4.1. Cell culture and drug treatment

In this study, the HT-29 colon cancer cell line with a *BRAF* mutation (ATCC, Manassas, VA, USA) was used. HT-29 cells have an ideal epithelial phenotype for studying changes in cell polarity [47], and *E*-cadherin, the major component of adherens junctions, is well organized. HT-29 cells have been used in studies on *E*-cadherin function in colon cancer [48–50]. HT-29 cells with *BRAF* mutations are resistant to EGFR inhibitors [51]; therefore, HT-29 cells are an ideal cell line to investigate alternative drug treatments with *E*-cadherin function. HT-29 cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10 % fetal bovine serum (Gibco, Thermo Fisher Scientific, MA, USA) and penicillin-streptomycin antibiotics (TOKU-E, Bellingham, WA, USA) and maintained in a 37 °C incubator containing 5 % CO_2. HT-29 cells were treated with dasatinib (20 nM) for 24 or 48 h.

4.2. Immunofluorescence confocal microscopy

HT-29 cells were grown on coverslips for 24 h and treated with 20 nM dasatinib for 24 h. The cells were fixed with 3.7 % formaldehyde for 20 min and permeabilized with 0.1 % Triton X-100 for 1 min. The fixed cells were incubated with mouse *anti*-E-cadherin (BD Biosciences), rabbit *anti-p*-Src (Cell Signaling Technology), rabbit anti-actin (GeneTex), rabbit *anti*-Rab11 (Invitrogen), rabbit *anti*-pEGFR (Invitrogen) and rabbit *anti-p*-FAK (Cell Signaling Technology) for 1 h at room temperature, followed by incubation with secondary antibodies conjugated to Alexa Fluor 488 or 594 (Jackson ImmunoResearch) for 1 h at room temperature. After washing with PBS, the coverslips were mounted with Fluoromount (Merck-Sigma, MA, USA), and images were acquired using a Zeiss LSM 510 META confocal system with a 63*X* objective (1.4 oil). Z-scanning sections were taken for 3D imaging, and line scans and side views were analyzed using Zeiss LSM image software. For quantification of *E*-cadherin, Rab11 and pEGFR distribution at leading edge and the cell-cell contacts, more than 100 cells of each independent experiment were counted, the positive counts were divided by the total cell-cell contact numbers.

4.3. Transwell migration assay

Transwell chambers were used for the cell migration assays. A transwell chamber with a larger pore size (12 μm pore size) was used for collective cell migration. Cells (5×10^4) were seeded in the upper chamber with normal medium and serum-free medium was placed in the lower chamber. The cells were allowed to attach for 24 h. Medium in the upper chamber was then replaced with serum-free medium, and the lower chamber was incubated with normal medium to allow the cells to migrate. The culture dish was incubated for 24 h at 37 °C, nonmigrating cells were then removed from the upper side of the chamber with cotton swabs, and the remaining cells on the bottom side of the membrane were fixed for 20 min in 3.7 % formaldehyde/0.1 % Triton X-100 in PBS. Migrated cells were stained with DAPI to visualize the nuclei, and antibodies against *E*-cadherin and Rab11 were applied, followed by immunofluorescence staining. DAPI images were captured using a Zeiss AxioVision inverted microscope, and *E*-cadherin and Rab11 localization were observed using a Zeiss LSM 510 META confocal microscope, as described for immunofluorescence confocal microscopy.

4.4. Western blot and immunoprecipitation

Cells were lysed in lysis buffer that was prepared with RIPA buffer containing 20 μl/ml protease inhibitor (cOmplete, Roche, Germany), 100 mM Na_3VO_4, 100 mM PMSF, and phosphatase inhibitor (PhosSTOP, Roche. Germany) using an ice tray. Equal amounts of protein samples were separated by SDS-PAGE and then transferred to PVDF membranes (PerkinElmer, MA, USA) by eBlot® L1 transfer device (GenScript, NJ, USA). After blocking the PVDF membrane with 5 % low-lipid milk for 1 h, primary antibodies were applied to detect specific protein expression at 4 °C overnight and then incubated with the appropriate HRP-conjugated secondary antibodies. Protein signals were developed using an ECL reagent (PerkinElmer, MA, USA) and detected using a Multigel-21 digital system (Hung Chong, Taiwan). Relative intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The following antibodies were used: mouse *anti*-E-cadherin, rabbit *anti-p*-Src, and rabbit *anti*-Rab11. Mouse *anti*-GAPDH antibody (Santa Cruz Biotechnology) was used as a loading control.

For immunoprecipitation, cell lysates with 250 μg of protein were incubated with 2 μg of *anti*-E-cadherin antibody at 4 °C overnight. The protein-antibody mixture was incubated with protein A/G agarose (Pierce Biotechnology/Thermo Scientific, Waltham, IL, USA) at room temperature for 2 h. The protein-A/G-antibody complex was centrifuged and subjected to SDS-PAGE, followed by western blotting with *anti*-E-cadherin, *anti*-Rab11, *anti-p*-Src, and *anti*-GAPDH antibodies.

4.5. RT-PCR for mRNA detection

RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and isopropanol. First-strand cDNA was synthesized from 5 μg of total RNA using a Superscript IV kit (Invitrogen, MA, USA). mRNA expression was amplified from cDNA by PCR using *E*-cadherin, Src, Rab11, and GAPDH primers. The primer sequences were as follows (all written in the 5°' to 3' °direction): *GAPDH* forward, TCTGCTGATGCCCCCATGTTCGTC; reverse, TTCTTGATGTCATCATATTTGGCA. *E-cadherin*: forward, ATAGAGAACGCATTGCCACATAC; reverse, GTGTGGTGATTACGACGTTAGCC. *Src*, forward: TGCTCAGATCGCCTCAG; reverse: CGCCGTGTACTCATTGT. *Rab11* forward: CGACGAGTACGACTACCTCTTTA; reverse: CTTAGATGTTCTGACAGCACTGC; The PCR

product, which represents the mRNA expression, was visualized by ethidium bromide staining after 1.5 % agarose gel electrophoresis. *GAPDH* was used as an internal control.

4.6. Transmission electron microscopy

HT-29 cells were treated with 20 nM dasatinib and incubated for 48 h. Cells were fixed in 4 % formaldehyde and 5 % glutaraldehyde with 0.1 M sodium cacodylate buffer for 1 h and postfixed with 1 % osmium tetroxide for 30 min at room temperature. The cells were dehydrated using a graded ethanol series and embedded in LX112 (EMS). Sections of 0.6 μm thickness were stained with toluidine blue for light microscopy. Thin sections (80 nm) were stained with uranyl acetate and lead citrate and viewed using a Hitachi HT-7700 transmission electron microscope. For quantification of cell-cell contact distance, three gaps per cell were randomly chosen to measure the distance with "Gatan Digital Micrograph software." Cell-cell contact points were counted and 15 cells were counted for quantification.

4.7. Statistics

All experiments were performed independently at least three times. Cell viability, growth, migration, confocal and TEM images were quantified. Student's t-test was used for statistical analysis, and differences at the $p < 0.05$ level were considered statistically significant.

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Data availability statement

Data included in article/supp. Material in article. Data also available in the following link: https://papers.ssrn.com/sol3/papers.cfm?abstract_id=4441251.

CRediT authorship contribution statement

Yi-Wen Lu: Writing – original draft, Investigation, Formal analysis, Data curation. **Xiang-Ling Hou:** Formal analysis, Data curation. **Hui-Min Koo:** Formal analysis, Data curation. **Wei-Ting Chao:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23501.

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