The feasible role of soluble E-cadherin in spheroidogenesis of HCT116 colorectal cancer cells, a candidate biomarker for liquid biopsy

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Abstract. Although E-cadherin is known as a tumor suppressor via its effects on cell to cell adhesion, the effects of E-cadherin on malignant transformation have not yet been thoroughly investigated. In the present study, after malignant transformation was induced by spheroid formation in a fetal bovine serum-supplemented environment, the effects of soluble E-cadherin on the spheroidogenesis of colorectal cancer cells were investigated. E-cadherin knock-out (KO) was performed in HCT116 cells, targeting exon 3 of the CDH1 gene. A cell viability assay was performed to determine the proliferation and viability of wild type and CDH1 KO HCT116 cells after treatment with anticancer drugs. Spheroidogenesis was compared with or without exogenous E-cadherin, antibody against the ectodomain of E-cadherin (DECMA-1) and PD98059 treatment. In addition, morphometry, immunocytochemistry and western blotting were performed. Soluble E-cadherin in culture media was measured using an enzyme-linked immunosorbent assay. Firstly, CDH1 KO was confirmed by western blotting. Notably, the proliferation and viability of cells following treatment with 5-fluorouracil, epidermal growth factor receptor inhibitor and src kinase inhibitor were similar between the cell lines. Exogenous E-cadherin or DECMA-1 treatment did not affect spheroidogenesis, although long-term maintenance was slightly disturbed in CDH1 KO spheroids compared with that in wild type spheroids. In addition, E-cadherin was increased in spheroid culture as compared with that in conventional culture. Soluble E-cadherin was increased in a time-dependent manner, particularly in wild type HCT116 cells. PD98059 inhibited ERK activation and enhanced E-cadherin expression in conventional culture without affecting spheroidogenesis. These results suggested that soluble E-cadherin may be considered as a biomarker for colorectal cancer, although exogenous E-cadherin might not have a further role in malignant transformation.

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Introduction

Mortality caused by cancers including colorectal cancer (CRC) has continued to decline recently because of earlier detection and improved treatment options. Incidence rates, however, increased for CRC in young adults, which is first leading cause of cancer death in men and second in women (1). Despite continued overall declines, CRC is rapidly shifting to diagnosis at a younger age, in the left colon or rectum, and at a more advanced or metastatic stage (2). Chemotherapy regimens for metastatic CRC are classified as first-, second-, and third-line treatment such as 5-fluorouracil (5-FU), epidermal growth factor (EGF) receptor (EGFR) inhibitor, and src kinase inhibitor, respectively (3).

CDH1 gene located on chromosome 16q22.1 is composed of 16 exons. It encodes the E-cadherin protein. Frequent mutations of CDH1 gene have been reported mostly in gastric cancers (4,5), in which most variations have been identified in exons 4-12 encoding the extracellular domain. Based on cell-cell adhesion properties, E-cadherin is known as a tumor suppressor protein in experimental cancer researches. E-cadherin, however, might promote proliferation via interaction with EGFR, resulting in EGF-dependent ERK activation in CRC (6-8). Lack or dysfunction of the transmembrane domain of E-cadherin might trigger cell proliferation or epithelial-mesenchymal transition (EMT) by activating β-catenin. The loss or dysfunction on extracellular domain of E-cadherin might accelerate malignant transformation by ruining EGFR/E-cadherin complex (9,10).

A three-dimensional (3D) spheroid formation model is a basic tool for cancer research (11), because it resembles *in vivo* solid tumors by anchorage-independent EMT (12). In this experimental setting, cells initially aggregate and then form compact spheroids via E-cadherin (12), which is inversely correlated with EMT similar to malignant transformation (13).

Although E-cadherin is frequently downregulated with carcinogenesis, cell-cell adherence between cancer cells is disturbed after adding a soluble fragment of E-cadherin, leading to malignant transformation (14,15). Contrary to the well-known tumor suppressor role of E-cadherin (9,10), the extracellular domain of E-cadherin can act as EGF to EGFR. Therefore, the extracellular domain of E-cadherin has been suggested as a possible oncogene (14,16,17).

Growth factors or fetal bovine serum (FBS) could be supplemented in a spheroid formation model, although the efficiency could differ between cell lines (12,18,19). We have previously revealed that spheroid formation is related to soluble E-cadherin and pan-RAS in CRC cells, including 5-FU-resistance acquired cell line, but not related to any marker for cancer stem cells or drug resistance (19). Increased soluble E-cadherin during spheroid formation has only been observed in FBS-supplemented environment, not in growth factor-supplemented environment irrespective of 5-FU resistance. Therefore, application of exogenous E-cadherin in FBS-supplemented spheroid formation is needed to reveal whether soluble E-cadherin is a result or a cause of spheroidogenesis.

The extracellular domain of E-cadherin (around 80 kDa) is a soluble fragment. A commercial E-cadherin protein (around 64.4 kDa) could be collectively regarded as a soluble E-cadherin (19). Exogenous soluble E-cadherin (around 1 μg/ml) is known to contribute to ultraviolet-induced skin carcinogenesis via association with human epidermal growth factor receptor (14). Treatment with DECMA-1 (antibody against the ectodomain of E-cadherin) at 1 mg/kg can suppress breast carcinogenesis in an *in vivo* model (20). Although germline variants in CDH1 are associated with elevated risks of diffuse gastric cancer (21), whether they could increase the risk of CRC has not been reported yet. Effects of E-cadherin on FBS-supplemented spheroid formation and associated mechanisms have not been suggested either. Thus, this study aimed to investigate whether exogenous soluble E-cadherin or DECMA-1 could affect spheroidogenesis of CRC cells and determine possible mechanisms involved.

Materials and methods

Reagents and antibodies. Reagents and antibodies used in this study are summarized in Table I.

Cell culture. Wild-type (WT) and CDH1 knock-out (KO) HCT116 cells were purchased from SYNTHEGO (Synthego Engineered cells; Redwood city, CA, USA). CDH1 KO was performed using the CRISPR/Cas-9 system targeting the exon 3, corresponding to the precursor of CDH1. Guide RNA sequences were 'UUACAGUCAAAGGCCUCUA', 'CUU UCUGUAGGUGGAGUCCC', and 'AACAUACCUGAUGGG GCGGG'.

Cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 5% CO_2, 37˚C, and humidified atmosphere conditions. All cell culture reagents were obtained from Corning Inc. (Corning, NY, USA).

Cell viability assay. Cell proliferation was determined using MTT assay. Briefly, cells were plated in a 96-well plate ($2x10^3$ cells/well in 200 μl of media). Starting from the day of seeding (day 0), cell viability was checked for 4 days consecutively. Each day, 20 μl of MTT reagent was added to each well. After 3 h, formazan crystals were dissolved in dimethylsulfoxide. The absorbance was read at 595 and 620 nm using a VERSAmax microplate reader (Molecular Devices Korea LLC.; Seoul, Republic of Korea). Absorbance values of treated cells were compared to those of vehicle-treated cells, representing 100% cell viability.

Cells were seeded in triplicate wells of 96-well plates, and treated with 5-FU, erlotinib, saracatinib (0, 0.1, 1, 10, and 100 μM/each), E-cadherin (0, 0.1, 1, 10, and 100 ng/ml), DECMA-1 (0, 1, 10, 100, and 1,000 ng/ml), or PD98059 (0, 1, 10, 20, and 50 μM). After cells were incubated for 3 days, cell viability was assessed as described above.

Spheroid formation. Ultra-low attachment 96-well plates were purchased from Corning Inc. (Corning) to create an anchorage-independent environment. Cells were cultured with conventional culture media as described above. After adding E-cadherin (0, 1, 10, 100, and 1,000 ng/ml), DECMA-1 (0, 1, 10, 100, and 1,000 ng/ml), or PD98059 (0, 1, 10, 20, and 50 μM), cells were cultured in round bottom plates (Corning, #7007) at a density of $2x10^2$ cells/well. Long-term changes of spheroidogenesis were evaluated using flat bottom plates (Corning, #3474) at a density of $2x10^3$ cells/well. Spheroid formation was checked for morphometry on day 5 in round bottom plates or on days 7, 14, and 21 in flat bottom plates.

Western blot analysis. WT and CDH1 KO cells were cultured in conventional 2D monolayer for 3 days with or without PD98059 (20 μM) and in spheroid formation culture in flat bottom plates for 7, 14, and 21 days. Cells and spheroids were harvested in T-PER™ protein extraction reagent including 1% protease inhibitor cocktail, 0.5% phosphatase inhibitor cocktail I, and 0.5% phosphatase inhibitor cocktail II. Protein concentrations were assessed by BCA protein assay according to the manufacturer's instructions (19).

Cell lysates (30 μg protein/lane) were subjected to 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (#162-0176; Bio-Rad Laboratories, Hercules, CA, USA), and then blocked with 5% skimmed milk at room temperature for 1h. These membranes were incubated with primary antibodies (diluted 1:1,000; 1:2,000 for β-actin and GAPDH) at 4˚C overnight and appropriate secondary antibodies (diluted 1:2,000) at room temperature for 1h. Protein bands were detected using the Azure™ c300 and quantified using the AzureSpot analysis software (version 14.2; Azure Biosystems, Inc., Dublin, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to the manufacturer's instruction (19). Briefly, conventional 2D monolayer and spheroid formation culture media samples (50 μl/each) were added into each well to bind E-cadherin antibody cocktail (50 μl/each) followed by incubation at room temperature for 1 h. After wells were washed with a washing buffer, TMB development solution (100 μl/each) was added into each well followed by incubation for 10 min at room temperature. A stop solution (100 μl/each)

Table I. Reagents and antibodies used in the experiment.

A, Reagents

Name (cat. no.)	Company	Purpose/action
MTT (M6494)	Thermo Fisher Scientific	Cell viability
5-FU (F6627); erlotinib (S2156)	Sigma-Aldrich (Merck KGaA)	Anti-cancer drugs
Saracatinib (11497)	Cayman Chemical	
T-PER™ protein extraction reagent (78510)	Thermo Fisher Scientific	Preparation for western blotting
Protease inhibitor cocktail (ab201111); phosphatase inhibitor cocktail I (ab201112); phosphatase inhibitor cocktail II (ab201113)	Abcam	Preparation for western blotting
Pierce™ BCA protein assay kit (23227)	Thermo Fisher Scientific	Preparation for western blotting
PD98059 (9900)	Cell Signaling Tech	MEK-ERK inhibitor
Hematoxylin (H-3404)	Vector Laboratories	Counter-staining
Human E-cadherin protein (ab235682)	Abcam	Recombinant E-cadherin protein for spheroidogenesis
DECMA-1 (ab11512)	Abcam	Anti-E-cadherin antibody (intercellular junction marker) for spheroidogenesis
Human E-cadherin SimpleStep ELISA Kit (ab233611)	Abcam	Soluble E-cadherin detection

B, Antibodies

Name (cat. no.)	Company	Purpose/action
Phosphor-ERK (4370)	Cell Signaling Tech	Primary antibody
EGFR (ab52894)	Abcam	Primary antibody
ERK (sc-93); β-catenin (sc-7199); pan-RAS (sc-166691); β-actin (sc-47778); GAPDH (sc-47724)	Santa Cruz Biotechnology	Primary antibody
Anti-mouse IgG (PI-2000); anti-rabbit IgG (PI-1000)	Vector Laboratories	Secondary antibody
ImmPRESS Reagent kit (MP-7401)	Vector Laboratories	Immunocytochemistry

5-FU, 5-fluorouracil; phosphor, phosphorylated.

was then added and optical density was measured at 450 nm using a VERSAmax microplate reader.

Immunocytochemistry. One and three weeks after spheroid formation culture, formed spheres (>70 μm) were collected using a cell strainer with a pore size of 70 μm (Corning Inc., #CLS431751) and fixed with 4% paraformaldehyde for 24 h at 4˚C. The paraffin-embedded sections (4 μm-thick) were cut with a microtome and prepared for immunocytochemistry as previously described (19). These sections were then blocked with 10% normal horse serum for 1 h at room temperature. Incubation with an anti-E-cadherin antibody (diluted 1:100) was performed at 4˚C overnight and an anti-rabbit secondary antibodies (ready-to-use) for 1h at room temperature. The binding was visualized using 3,3'-diaimnobenzidine, and nuclei were counterstained with hematoxylin (#H-3404, Vector) for 1 min at room temperature. Immunolabelled images were directly captured using a DP22 digital camera and BX-51 light microscope (Olympus, Tokyo, Japan).

Statistical analysis. All data were compiled from a minimum of three replicate experiments. Data are expressed as mean values ± standard deviation. P<0.05 was considered to indicate a statistically significant difference as determined using Student's t-test or two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. MS Excel 2016 was used for statistical analysis.

Results

Characteristics of WT and CDH1 KO HCT116 cells. E-cadherin in CDH1 KO cells was decreased 0.48±0.05-fold (P<0.001) compared to that in WT HCT116 cells (Fig. 1A). WT and CDH1 KO HCT116 cells showed similar proliferation curves in a time-dependent manner (P<0.001), but no difference between cell types (P=0.760) (Fig. 1B).

Effects of anticancer drugs on cell viability were assessed using the MTT assay (Fig. 1C). Cell viability was decreased in a dose-dependent manner (P<0.001 for both) in both WT

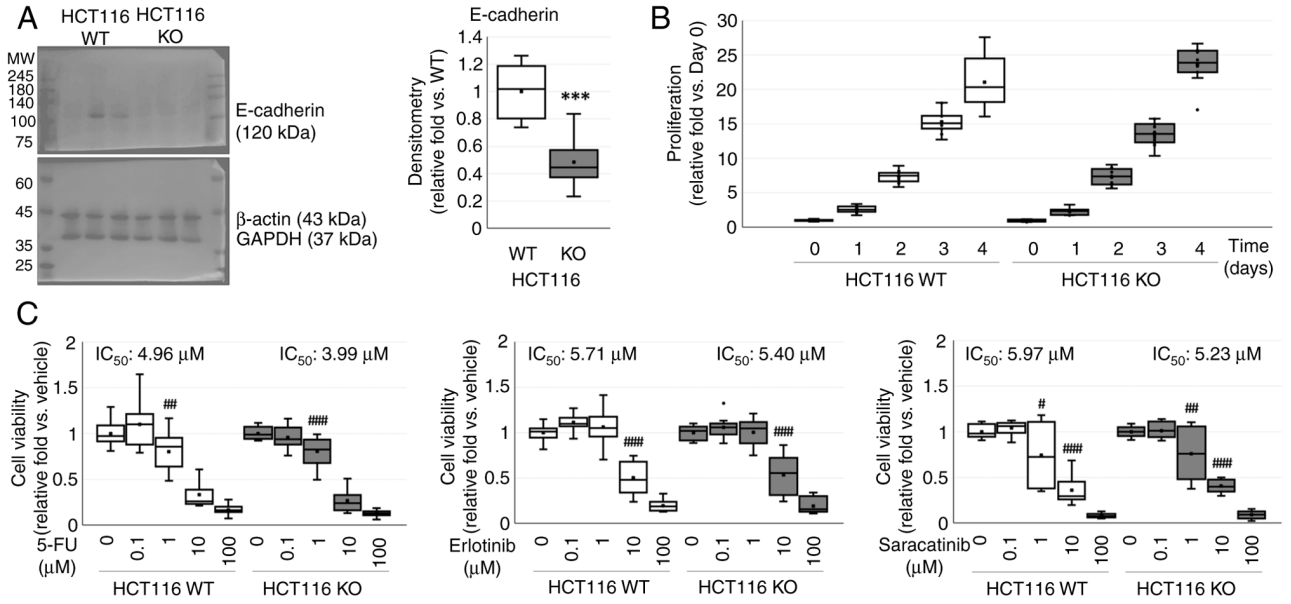

Figure 1. General characteristics of WT and CDH1 KO HCT116 cells. (A) CDH1 KO was confirmed by western blotting. (B) Proliferation was similar between two cell types. (C) Respective cell viability after treatment with an anticancer drug, 5-FU, epidermal growth factor receptor inhibitor (erlotinib) or src kinase inhibitor (saracatinib) was similar between two cell lines. ***P<0.001 vs. WT; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. vehicle. WT, wild-type; KO, knock-out; 5-FU, 5-fluorouracil.

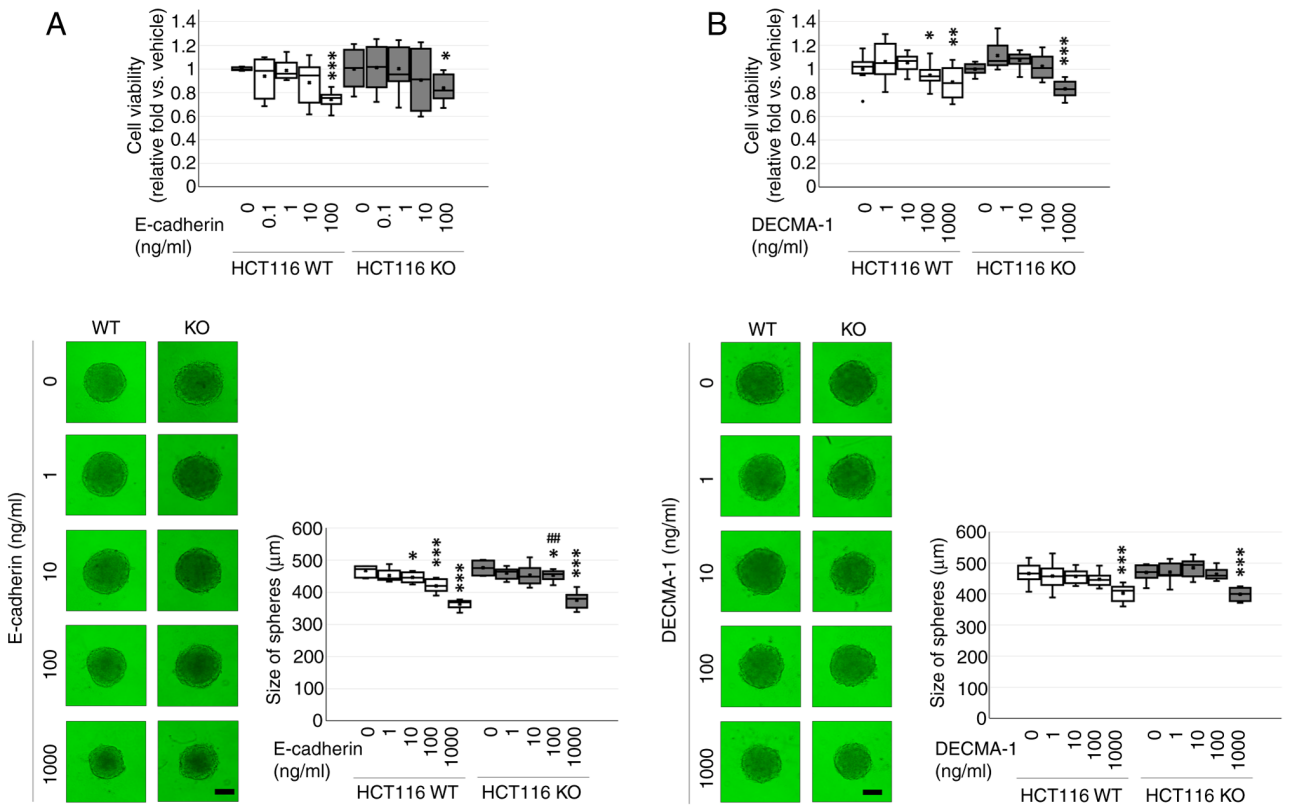

Figure 2. Effects of exogenous E-cadherin and DECMA-1 on WT and CDH1 KO HCT116 cells. (A) Although exogenous E-cadherin decreased viability and sizes of spheroids in both cells, a little but considerable changes was observed in CDH1 KO cells at higher dose (100 ng/ml). (B) Exogenous DECMA-1 significantly decreased viability and sizes of spheroids of both cells. Scale bar=200 μm. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle; $^{\#\#}$P<0.01 vs. WT at same concentration. WT, wild-type; KO, knock-out.

and CDH1 KO HCT116 cells with similar IC_{50} values, but no differences between cell types against 5-FU (P=0.065), erlotinib (P=0.516), and saracatinib (P=0.802).

Effects of exogenous E-cadherin and anti-E-cadherin antibody on WT and CDH1 KO HCT116 cells. Cell viability was decreased after exogenous E-cadherin treatment in a

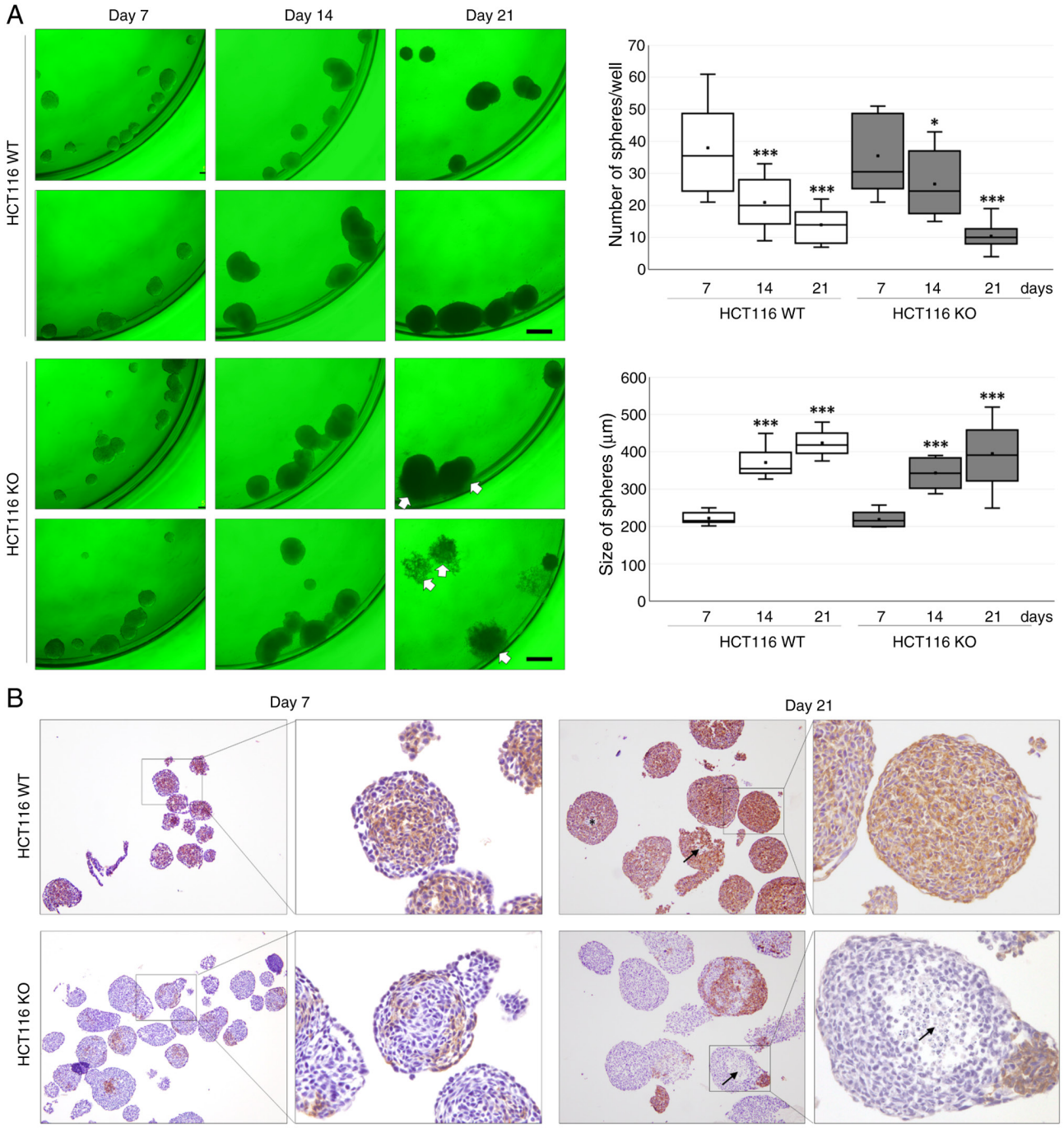

Figure 3. Long-term spheroidogenesis of WT and CDH1 KO HCT116 cells. (A) When spheroidogenesis was maintained for 21 days, the number of spheroids was decreased while sizes of spheroids were increased in a time-dependent manner in both cells. Interestingly, some spheroids from CDH1 KO cell line lost their morphological integrity (arrows). Scale bar=500 μm. (B) Immunocytochemistry (magnification, x4 and x20) using with an anti-E-cadherin antibody for spheroids revealing decreased E-cadherin in CDH1 KO cells. Arrows indicate necrosis. *P<0.05, ***P<0.001 vs. Day 7. WT, wild-type; KO, knock-out.

dose-dependent manner (P=0.001) in both WT and CDH1 KO HCT116 cell, but no differences between cell types (P=0.248). Exogenous E-cadherin at 100 ng/ml decreased cell viability of WT (0.7±0.03-fold, P<0.001) and CDH1 KO (0.84±0.04-fold; P=0.019) HCT116 cells. Exogenous E-cadherin inhibited spheroid formation in a dose-dependent manner (P<0.001) and between WT and CDH1 KO HCT116 cell types (P=0.003). Significant difference between cells was observed only at 100 ng/ml (P=0.002) of WT (0.90±0.02-fold) and CDH1 KO (0.95±0.01-fold) HCT116 cells (Fig. 2A).

Cell viability was decreased after exogenous DECMA-1 treatment in a dose-dependent manner (P<0.001) in both WT and CDH1 KO HCT116 cells, but no differences between cell types (P=0.427). Exogenous DECMA-1 at 100 ng/ml decreased viability of WT (0.87±0.03-fold; P=0.002) and CDH1 KO (0.82±0.03-fold; P=0.042) HCT116 cells without causing an inter-cellular difference. Exogenous DECMA-1 inhibited spheroid formation in a dose-dependent manner (P<0.001) in both WT and CDH1 KO HCT116 cells, but no differences between cell types (P=0.086). Exogenous DECMA-1 at 1000 ng/ml suppressed spheroidogenesis of WT

Table II. Densitometric comparison of spheroid formation between WT and CDH1 KO HCT116 colorectal cancer cells.

Protein	Cell type	2D	Spheroids		
			Day 7	Day 14	Day 21
E-cadherin	WT	1.00±0.17	4.01±0.73 (^{b}P=0.001)	4.34±0.85 (^{b}P=0.002)	4.24±0.74 (^{b}P=0.001)
	KO	0.49±0.08 (^{f}P<0.001)	1.05±0.21 (^{a}P=0.014; ^{e}P=0.001)	1.22±0.24 (^{b}P=0.008; ^{e}P=0.003)	0.88±0.10 (^{b}P=0.003; ^{f}P<0.001)
EGFR	WT	1.00±0.05	1.74±0.46 (^{b}P=0.006)	1.64±0.50 (^{a}P=0.013)	1.35±0.17 (^{b}P=0.001)
	KO	0.80±0.22	1.77±0.45 (^{c}P<0.001)	1.71±0.41 (^{c}P<0.001)	1.68±0.49 (^{b}P=0.001)
β-catenin	WT	1.00±0.05	2.19±0.46 (^{c}P<0.001)	2.31±0.53 (^{c}P<0.001)	1.81±0.35 (^{c}P<0.001)
	KO	0.83±0.29	1.75±0.36 (^{c}P<0.001)	2.68±0.27 (^{c}P<0.001)	2.28±0.65 (^{c}P<0.001)
Pan-RAS	WT	1.00±0.05	2.51±0.85 (^{c}P<0.001)	2.86 0.64 (^{c}P<0.001)	2.69±0.49 (^{c}P<0.001)
	KO	1.19±0.28	2.00±0.35 (^{c}P<0.001)	2.67±0.67 (^{c}P<0.001)	2.57±0.57 (^{c}P<0.001)
pERK/ERK	WT	1.00±0.27	1.62±0.55 (^{a}P=0.016)	1.62±0.62 (^{a}P=0.029)	2.32±0.72 (^{b}P=0.003)
	KO	1.16±0.35	2.40±0.45 (^{c}P<0.001; ^{d}P=0.012)	2.15±0.66 (^{b}P=0.005)	2.31±0.80 (^{b}P=0.007)

Results are expressed as relative fold as compared to 2D culture condition of WT cells. ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001 vs. 2D culture condition; ^{d}P<0.05, ^{e}P<0.01, ^{f}P<0.001 vs. WT. WT, wild-type; KO, knock-out; 2D, 2-dimensional.

(0.86±0.01-fold; P<0.001) and CDH1 KO (0.85±0.02-fold; P<0.001) HCT116 cells (Fig. 2B).

Long-term spheroidogenesis of WT and CDH1 KO HCT116 cells. Long-term spheroidogenesis was evaluated until day 21 in WT and CDH1 KO HCT116 cells. Although the number of spheroids was decreased in a time-dependent manner (P<0.001) in both WT and CDH1 KO HCT116 cells, there was no difference between cell types (P=0.970). The size of spheroids was increased in a time-dependent manner (P<0.001) in both WT and CDH1 KO HCT116 cells, but no differences between cell types (P=0.055). Compared to WT HCT116 cells, CDH1 KO HCT116 cells showed some scattering or inability to maintain the shape of spheroids (Fig. 3A).

Morphological features were evaluated by performing immunostaining of E-cadherin (Fig. 3B). E-cadherin was immunostained in spheroids of WT HCT116 cells from day 7 to day 21. In spheroids from CDH1 KO HCT116 cells, E-cadherin immunostaining was only observed for a few cells. Some central area was neither immunostained with E-cadherin nor stained with hematoxylin for nuclei, suggesting necrotic changes.

As compared with 2D monolayer culture, spheroid cultures showed significant increase of E-cadherin in a time-dependent manner (P<0.001) and between WT and CDH1 KO HCT116 cells (P<0.001). Although E-cadherin was increased by about 4 times in spheroids as compared to that in 2D culture of WT HCT116 cells, it was increased by about 2 times in spheroid cultures of CDH1 KO HCT116 cells (Fig. 4A and Table II).

Concentrations of E-cadherin in cultured media of WT and CDH1 KO HCT116 cells for 2D monolayer and spheroid cultures were estimated by ELISA (Fig. 4B). ELISA results showed significant increase of soluble E-cadherin in a time-dependent manner (P<0.001) and between WT and CDH1 KO HCT116 cell types (P<0.001). The ratio of soluble E-cadherin in WT HCT116 cells was found to be significantly increased at 14 (2.76±0.05-fold vs. 1.19±0.03-fold; P<0.001) and 21 (5.36±0.05-fold vs. 1.39±0.05-fold; P<0.001) days after incubation in WT and CDH1 KO HCT116 cells as compared with 2D monolayer culture condition.

Table III. Densitometric comparison of effects of PD98059 treatment on WT and CDH1 KO HCT116 colorectal cancer cells.

Protein	Cell type	Vehicle	PD98059
pERK/ERK	WT	1.00±0.11	0.61±0.16 (^{c}P<0.001)
	KO	1.14±0.40	0.66±0.18 (^{a}P=0.022)
E-cadherin	WT	1.00±0.12	2.15±1.26 (^{a}P=0.012)
	KO	0.55±0.16 (^{f}P<0.001)	0.76±0.18 (^{e}P=0.004)
EGFR	WT	1.00±0.16	1.14±0.22
	KO	1.13±0.16	1.12±0.13
β-catenin	WT	1.00±0.04	3.08±0.74 (^{c}P<0.001)
	KO	1.50±0.43	2.52±0.92 (^{b}P=0.005)
Pan-RAS	WT	1.00±0.02	1.58±0.24 (^{c}P<0.001)
	KO	1.18±0.17 (^{b}P=0.001)	1.45±0.28 (^{e}P=0.005)

Results are expressed as relative fold as compared to vehicle-treated condition of WT cells. ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001 vs. vehicle-treated condition; ^{e}P<0.01, ^{f}P<0.001 vs. WT. WT, wild-type; KO, knock-out.

E-cadherin related proteins such as EGFR, β-catenin, pan-RAS, and ERK were further checked in spheroidogenesis. The proteins examined were significantly increased in spheroids as compared to those under 2D culture conditions in a time-dependent manner (P<0.001 for both) in both WT and CDH1 KO HCT116 cells, but there was no difference between cell types on EGFR (P=0.618), β-catenin (P=0.597),

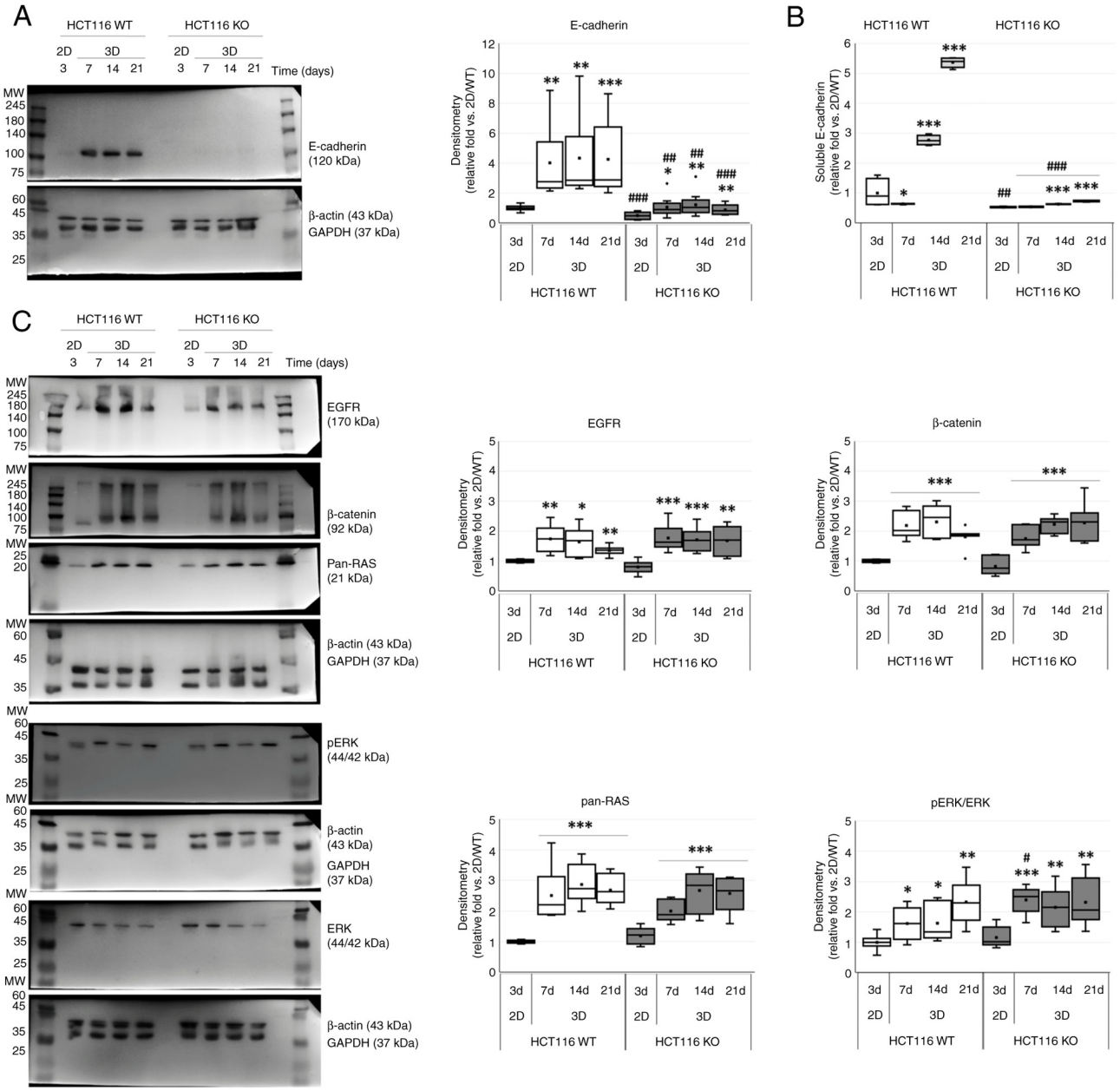

Figure 4. Characteristics of long-term-maintained spheroids of WT and KO HCT116 cells. (A) Western blotting showing significant increase of E-cadherin in spheroids (3D) as compared to conventional 2D culture in both cell lines. (B) ELISA on culture media showing time-dependent increases of soluble E-cadherin in spheroid culture, with such increases being prominent in WT HCT116 cells. (C) Western blotting showing significant increases of EGFR, β-catenin, pan-RAS, and ERK activation in spheroid culture as compared to 2D cultures in both cell lines. *P<0.05, **P<0.01, ***P<0.001 vs. 2D; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. WT at same time. WT, wild-type; KO, knock-out; 2D, 2-dimensional; 3D, 3-dimensional.

and pan-RAS (P=0.253), except ERK activation (P=0.037). ERK activation was significantly increased between cell types at 7 days after spheroidogenesis (P=0.012) (Fig. 4C; Table II).

PD98059-induced E-cadherin on spheroidogenesis of WT and CDH1 KO HCT116 cells. As a significant change on ERK activation was observed in spheroidogenesis, a MEK-ERK inhibitor (PD98059) was adopted. Cell viability was decreased after PD98059 treatment in a dose-dependent manner (P<0.001) and between WT and CDH1 KO HCT116 cells (P<0.001). Cell viability was significantly decreased by PD98059 at a concentration of 10 μM (0.80±0.02-fold vs. 0.68±0.03-fold; P<0.001 for both). Intercellular difference in viability was observed for groups treated with PD98059 at 10 (P=0.002), 20 (P=0.005), or 50 μM (P=0.008) (Fig. 5A).

There was no significant difference in ERK activation between WT and CDH1 KO (1.08±0.12-fold; P=0.283) HCT116 cells (Fig. 5A). ERK activation was significantly decreased after PD98059 (20 μM; P<0.001) treatment, but there was no difference between cell types (P=0.388). However, E-cadherin was significantly increased depend on PD98059 treatment (P=0.004) and between WT and CDH1 KO HCT116 cells (P<0.001). Although E-cadherin was lower in CDH1 KO HCT116 cells as compared to WT HCT116 cells, PD98059 considerably increased E-cadherin (Fig. 5B and Table II).

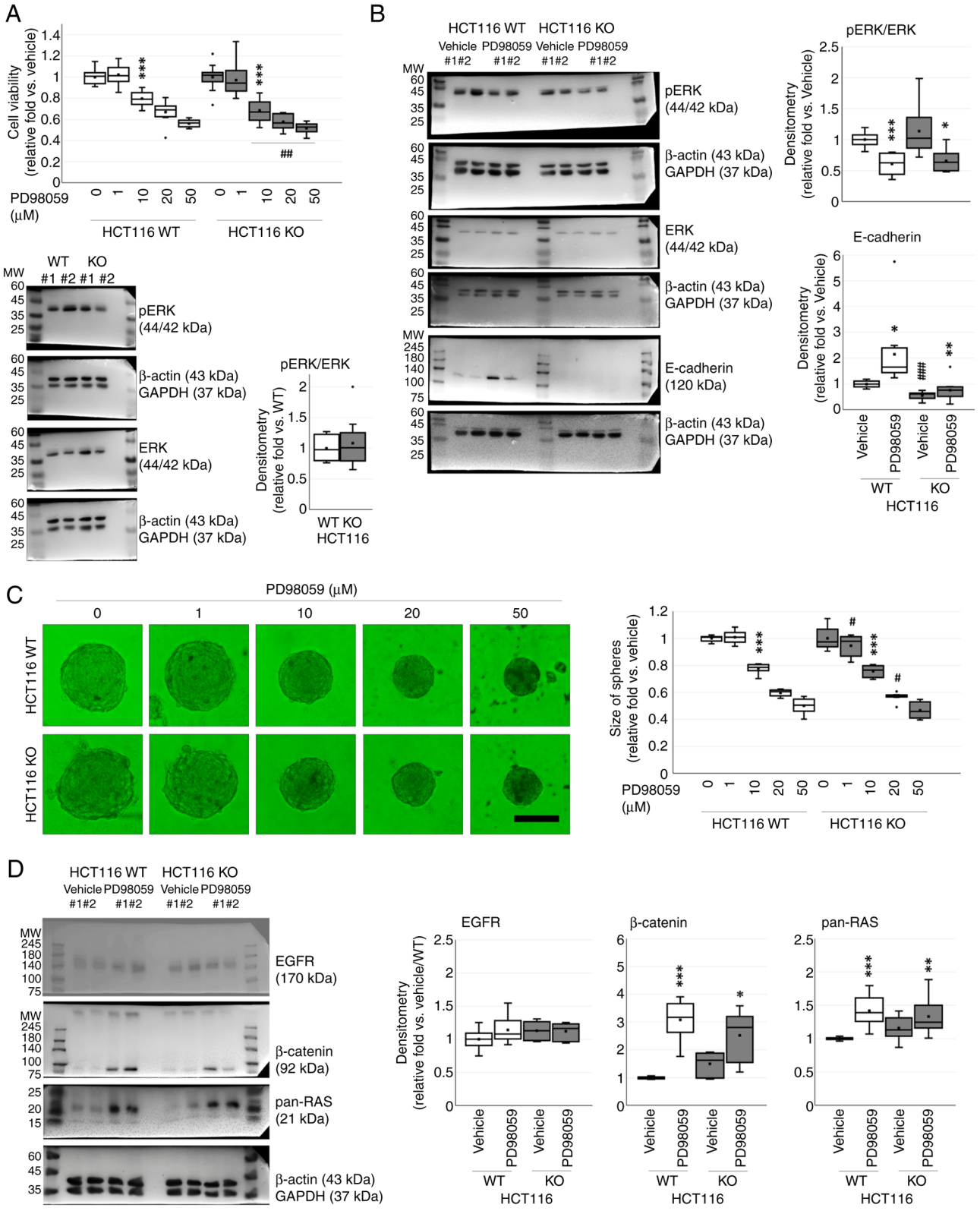

Figure 5. Effects of PD98059 on E-cadherin and spheroidogeneis of WT and CDH1 KO HCT116 cells. (A) PD98059 decreased cell viability in dose-dependent manner with a significant difference between WT and CDH1 KO HCT116 cells while ERK activation was similar. (B) PD98059 (20 μM) inhibited ERK activation in both cells, but increased E-cadherin expression. (C) PD98059 decreased sizes of spheroids in both cell lines. (D) PD98059 significantly increased the expression of β-catenin and pan-RAS without affecting the expression of EGFR. Scale bar=200 μm. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. WT. WT, wild-type; KO, knock-out.

PD98059 inhibited spheroid formation in a dose-dependent manner (P<0.001) and between WT and CDH1 KO HCT116 cells (P=0.014) At 10 μM, it significantly decreased spheroid formation in both cells (P<0.001 for both). Intercellular difference was observed at 1 μM (1.01±0.05-fold vs. 0.95±0.08-fold; P=0.034) and 20 μM

(0.60±0.01-fold vs. 0.57±0.01-fold; P=0.032) of PD98059 (Fig. 5C).

The related proteins suggested in spheroidogenesis were further checked. β-catenin and pan-RAS were significantly increased after PD98059 treatment (P<0.001 for both), but there was no difference between WT and CDH1 KO HCT116 cells on β-catenin (P=0.913) and pan-RAS (P=0.698). However, EGFR did not change after PD98059 treatment (P=0.346) or between cell types (P=0.451) (Fig. 5D; Table III).

Discussion

This study determined whether exogenous E-cadherin could affect spheroidogenesis of CRC cells in FBS-supplemented environment. First of all, we set CDH1 KO condition targeting the precursor exon 3 to compare general characteristics of HCT116 cells. CDH1 KO did not affect cell proliferation, chemotherapeutic responses against respective first- to third-line regimens, or spheroidogenesis as compared to WT HCT116 cells. The addition of exogenous soluble E-cadherin or DECMA-1 did not cause significant changes between WT and CDH1 KO HCT116 cells. Subtle differences in long-term maintenance of spheroids were observed. Some spheroids from CDH1 KO HCT116 cells did not maintain their shapes consistently at day 21. However, total number and mean size of spheroids were not considerably changed between WT and CDH1 KO HCT116 cells.

Controversy over E-cadherin on spheroidogenesis has been continued. Cells can aggregate and then form spheroids via E-cadherin during the initial spheroidogenesis in any environment (12). Although the expression of E-cadherin (120 kDa) was decreased during spheroid formation (22,23), E-cadherin might also induce EMT of CRC cells (24). Based on decreased E-cadherin and increased soluble E-cadherin in FBS-supplemented spheres (19), we have previously suggested that soluble E-cadherin might promote growth of spheroids and be an essential component of spheroidogenesis in FBS-supplemented environment. This hypothesis can be reinforced by previous studies showing that soluble E-cadherin could be an oncogene like EGF in spheroidogenesis (14-16) as well as conventional cultures (14). Contrary to expectations, the addition of E-cadherin protein or DECMA-1 did not show any significant effect on spheroidogenesis of HCT116 cells irrespective of CDH1 KO.

The activation of ERK was not different between WT and CDH1 KO HCT116 cells, although it was increased in spheroidogenesis. When ERK activity was inhibited by PD98059, proliferation of both WT and CDH1 KO HCT116 cells was also inhibited in a dose-dependent manner. While E-cadherin was significantly increased by PD98059 treatment, it did not lead to significant changes in spheroidogenesis of WT or CDH1 KO HCT116 cells. As compared to spheroidogenesis, PD98059 did not affect EGFR expression. EGF-dependent ERK activation (6-8) might be a relevant pathway in CRC. However, the possibility of soluble E-cadherin as an oncogene like EGF was not supported in this study.

ERK might promote EMT-like phenotype from E-cadherin to N-cadherin shift (25,26). The condition of an activated ERK with E-cadherin suppressed is known to promote proliferation and/or metastasis in CRC (27-33). Opposite results on ERK and E-cadherin have been reported (34-38), similar to results of the present study. When ERK activation was inhibited by PD98059, the proliferation of both WT and CDH1 KO HCT116 cells was inhibited with E-cadherin expression increased. Although ERK activation might have no effect on EMT in CRC (39), it decreased proliferation and inhibited invasion of CRC when E-cadherin was stabilized (40). In this context, overexpression of E-cadherin (41) or soluble E-cadherin (24) can stimulate EGFR and EGF-dependent ERK activation, resulting in cancer stem cell properties such as spheroid formation. However, when ERK activity was inhibited by PD98059, spheroidogenesis was also inhibited by PD98059 in a dose-dependent manner regardless whether E-cadherin expression was induced or CDH1 was knocked out.

To suggest details of prognostic factors for highly aggressive soft tissue sarcomas, inflammatory markers were investigated. Although clinical trial did not reveal significant differences between inflammatory markers of remission and non-remission cases (42), the tumor microenvironment would be different from tumors. E-cadherin might be involved in the pathogenesis of inflammatory breast cancer, but not that of non-inflammatory breast cancer, in which a positive association between E-cadherin loss and poor prognosis has been suggested (43,44). While reduced or loss of E-cadherin in localized tumor cells is correlated with proliferation, an increased level of soluble E-cadherin in circulation might be a marker for the extent of damaged skin caused by tumor and/or inflammation (45) or bacteria-induced diseases (46). Overexpression of soluble cell adhesion molecules, such as soluble E-cadherin, in body fluids can trigger inflammation and pro-carcinogenic programming leading to tumor induction and metastasis (46). Although the relationship among soluble E-cadherin, colon cancer and inflammation has not been thoroughly investigated, the potential value of E-cadherin for liquid biopsy in cancers has been suggested since soluble E-cadherin is significantly elevated in patients suffering from epithelial cancers including CRCs (47,48). Although soluble E-cadherin becomes hard to generalize the value due to lack of specificity and sensitivity as compared to existing tumor markers, such as carcinoembryonic antigen, it has been suggested as an alternative diagnostic biomarker for monitoring advanced CRC (49,50). Soluble E-cadherin can diffuse into the extracellular environment including blood and/or urine and act as a paracrine and/or autocrine signaling molecule (15). In this study, soluble E-cadherin was significantly increased in long-term maintained spheres with an FBS-supplemented environment, not in GF-supplemented environment as previously reported (19). This means that soluble E-cadherin cannot act as an oncogene like EGF. Although spheroidogenesis might not be closely related to CDH1 expression in HCT116 cells, the soluble E-cadherin showed significantly increases in a time-dependent manner. Therefore, it could be a biological marker for monitoring the progression of CRCs. However, this relationship of soluble E-cadherin with inflammation should further be investigated in CRC.

However, the present study has certain limitations. First of all, the possible role of soluble E-cadherin as on oncogene was suggested in SNU-C5 CRC cells (19), but there remained

the un-met issue using exogenous E-cadherin or DECMA-1 treatment for spheroidogenesis. To investigate the issue timely, the commercial HCT116 cell line, well-known for 3D culture and already prepared for CDH1 KO, were purchased from the company. In order to clarify the feasible role of soluble E-cadherin, additional CRC cell lines should be further included. Second, ERK activation should be more precisely interpreted while spheroidogenesis. As ERK activation was increased in spheroidogenesis regardless of E-cadherin level, an ERK inhibitor PD98059 was adopted. PD98059 treatment inhibited ERK activation and spheroidogenesis, but increased E-cadherin. Therefore, the decreased ERK rather than ERK activation might be more paid attention to interpret the present results. In addition, PD98059 treatment did not affect EGFR expression compared to spheroidogenesis, which might mean EGFR overexpression is an essential to spheroidogenesis. As EGF-dependent ERK activation (6-8) was suggested as a relevant pathway in CRC, the related signaling pathways on E-cadherin, EGFR, ERK, or β-catenin should be further investigated. Nevertheless, a candidate signaling pathway could not be specified based on current results. Finally, further experiments using animal models or human samples including blood or urines are needed to ensure the hypothesis, a biomarker for liquid biopsy for CRC.

In conclusion, E-cadherin did not affect proliferation, viability, or spheroidogenesis of WT or CDH1 KO HCT116 cells, although soluble E-cadherin was increased in a time-dependent manner, especially being prominent in WT. These results suggest that soluble E-cadherin could be a biomarker for colorectal cancer although exogenous E-cadherin might not have a further role like an oncogene in this experimental setting. Further studies should be focused on multiple signaling pathways that can be activated or inhibited by soluble E-cadherin.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

IYC and SPY conceived and designed the present study. IYC, HJB and JWH performed the experiments for data acquisition and analysis, and interpreted the experimental results. IYC and SPY confirm the authenticity of all the raw data. IYC wrote the original manuscript. SPY revised the manuscript. All authors read and approved the final version of the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflict of interest statement

The authors declare that they have no competing interests.

References

1. Siegel RL, Giaquinto AN and Jemal A: Cancer statistics, 2024. CA Cancer J Clin 74: 12-49, 2024.
2. Siegel RL, Wagle NS, Cercek A, Smith RA and Jemal A: Colorectal cancer statistics, 2023. CA Cancer J Clin 73: 233-254, 2023.
3. Hecht JR: Current and emerging therapies for metastatic colorectal cancer: Applying research findings to clinical practice. Am J Health Syst Pharm 65 (11 Suppl 4): S15-S21; quiz S22-4, 2008.
4. Berx G, Becker KF, Höfler H and van Roy F: Mutations of the human E-cadherin (CDH1) gene. Hum Mutat 12: 226-237, 1998.
5. Bustos-Carpinteyro AR, Oliveira C, Sousa A, Oliveira P, Pinheiro H, Carvalho J, Magaña-Torres MT, Flores-Miramontes MG, Aguilar-Lemarroy A, Jave-Suárez LF, *et al*: CDH1 somatic alterations in Mexican patients with diffuse and mixed sporadic gastric cancer. BMC Cancer 19: 69, 2019.
6. Bocca C, Bozzo F, Cannito S, Parola M and Miglietta A: Celecoxib inactivates epithelial-mesenchymal transition stimulated by hypoxia and/or epidermal growth factor in colon cancer cells. Mol Carcinog 51: 783-795, 2012.
7. Kim K, Kim KH, Roh K, Yoo BC, Ku JL, Shin YK, Cho JY, Kim M, Kwon MH, Goh SH, *et al*: Antitumor effects of calgranulin B internalized in human colon cancer cells. Oncotarget 7: 20368-20380, 2016.
8. Trinh NT, Nguyen TMN, Yook JI, Ahn SG and Kim SA: Quercetin and quercitrin from Agrimonia pilosa ledeb inhibit the migration and invasion of colon cancer cells through the JNK signaling pathway. Pharmaceuticals (Basel) 15: 364, 2022.
9. Hu MN, Hu SH, Zhang XW, Xiong SM and Deng H: Overview on new progress of hereditary diffuse gastric cancer with CDH1 variants. Tumori 106: 346-355, 2020.
10. Lee Y, Ko D, Yoon J, Lee Y and Kim S: TMEM52B suppression promotes cancer cell survival and invasion through modulating E-cadherin stability and EGFR activity. J Exp Clin Cancer Res 40: 58, 2021.
11. Manuel Iglesias J, Beloqui I, Garcia-Garcia F, Leis O, Vazquez-Martin A, Eguiara A, Cufi S, Pavon A, Menendez JA, Dopazo J and Martin AG: Mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin. PLoS One 8: e77281, 2013.
12. Han SJ, Kwon S and Kim KS: Challenges of applying multicellular tumor spheroids in preclinical phase. Cancer Cell Int 21: 152, 2021.
13. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES and Weinberg RA: Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 68: 3645-3654, 2008.
14. Brouxhon SM, Kyrkanides S, Teng X, Athar M, Ghazizadeh S, Simon M, O'Banion MK and Ma L: Soluble E-cadherin: A critical oncogene modulating receptor tyrosine kinases, MAPK and PI3K/Akt/mTOR signaling. Oncogene 33: 225-235, 2014.
15. Hu QP, Kuang JY, Yang QK, Bian XW and Yu SC: Beyond a tumor suppressor: Soluble E-cadherin promotes the progression of cancer. Int J Cancer 138: 2804-2812, 2016.

16. Patil PU, D'Ambrosio J, Inge LJ, Mason RW and Rajasekaran AK: Carcinoma cells induce lumen filling and EMT in epithelial cells through soluble E-cadherin-mediated activation of EGFR. J Cell Sci 128: 4366-4379, 2015.
17. Ramírez Moreno M and Bulgakova NA: The cross-talk between EGFR and E-cadherin. Front Cell Dev Biol 9: 828673, 2022.
18. Min SO, Lee SW, Bak SY and Kim KS: Ideal sphere-forming culture conditions to maintain pluripotency in a hepatocellular carcinoma cell lines. Cancer Cell Int 15: 95, 2015.
19. Chang IY and Yoon SP: Increased soluble E-cadherin of spheroid formation supplemented with fetal bovine serum in colorectal cancer cells. Oncol Lett 25: 207, 2023.
20. Brouxhon SM, Kyrkanides S, Teng X, Raja V, O'Banion MK, Clarke R, Byers S, Silberfeld A, Tornos C and Ma L: Monoclonal antibody against the ectodomain of E-cadherin (DECMA-1) suppresses breast carcinogenesis: Involvement of the HER/PI3K/Akt/mTOR and IAP pathways. Clin Cancer Res 19: 3234-3246, 2013.
21. Stanich PP, Elgindi D, Stoffel E, Koeppe E, Bansal A, Stetson R, Collins DL, Clark DF, Karloski E, Dudley B, *et al*: Colorectal neoplasia in CDH1 pathogenic variant carriers: A multicenter analysis. Am J Gastroenterol 117: 1877-1879, 2022.
22. Han XY, Wei B, Fang JF, Zhang S, Zhang FC, Zhang HB, Lan TY, Lu HQ and Wei HB: Epithelial-mesenchymal transition associates with maintenance of stemness in spheroid-derived stem-like colon cancer cells. PLoS One 8: e73341, 2013.
23. Zhang Z, Bu X, Chen H, Wang Q and Sha W: Bmi-1 promotes the invasion and migration of colon cancer stem cells through the downregulation of E-cadherin. Int J Mol Med 38: 1199-1207, 2016.
24. Qian Y, Wu X, Yokoyama Y, Okuzaki D, Taguchi M, Hirose H, Wang J, Hata T, Inoue A, Hiraki M, *et al*: E-cadherin-Fc chimera protein matrix enhances cancer stem-like properties and induces mesenchymal features in colon cancer cells. Cancer Sci 110: 3520-3532, 2019.
25. Tay PN, Tan P, Lan Y, Leung CH, Laban M, Tan TC, Ni H, Manikandan J, Rashid SB, Yan B, *et al*: Palladin, an actin-associated protein, is required for adherens junction formation and intercellular adhesion in HCT116 colorectal cancer cells. Int J Oncol 37: 909-926, 2010.
26. Rao C, Frodyma DE, Southekal S, Svoboda RA, Black AR, Guda C, Mizutani T, Clevers H, Johnson KR, Fisher KW and Lewis RE: KSR1- and ERK-dependent translational regulation of the epithelial-to-mesenchymal transition. Elife 10: e66608, 2021.
27. Díaz VM, Hurtado M, Kort EJ, Resnati M, Blasi F, Thomson T and Paciucci R: Requirement of the enzymatic and signaling activities of plasmin for phorbol-ester-induced scattering of colon cancer cells. Exp Cell Res 312: 2203-2213, 2006.
28. Gao S, Hu J, Wu X and Liang Z: PMA treated THP-1-derived-IL-6 promotes EMT of SW48 through STAT3/ERK-dependent activation of Wnt/β-catenin signaling pathway. Biomed Pharmacother 108: 618-624, 2018.
29. Park YR, Seo SY, Kim SL, Zhu SM, Chun S, Oh JM, Lee MR, Kim SH, Kim IH, Lee SO, *et al*: MiRNA-206 suppresses PGE2-induced colorectal cancer cell proliferation, migration, and invasion by targetting TM4SF1. Biosci Rep 38: BSR20180664, 2018.
30. Dong L, Ding C, Zheng T, Pu Y, Liu J, Zhang W, Xue F, Kang P, Ma Y, Wang X and Mao C: Extracellular vesicles from human umbilical cord mesenchymal stem cells treated with siRNA against ELFN1-AS1 suppress colon adenocarcinoma proliferation and migration. Am J Transl Res 11: 6989-6999, 2019.
31. Yang X, Zheng YT and Rong W: Sevoflurane induces apoptosis and inhibits the growth and motility of colon cancer in vitro and in vivo via inactivating Ras/Raf/MEK/ERK signaling. Life Sci 239: 116916, 2019.
32. Cheng B, Rong A, Zhou Q and Li W: LncRNA LINC00662 promotes colon cancer tumor growth and metastasis by competitively binding with miR-340-5p to regulate CLDN8/IL22 co-expression and activating ERK signaling pathway. J Exp Clin Cancer Res 39: 5, 2020.
33. Jun JH, Oh JE, Shim JK, Kwak YL and Cho JS: Effects of bisphenol A on the proliferation, migration, and tumor growth of colon cancer cells: In vitro and in vivo evaluation with mechanistic insights related to ERK and 5-HT3. Food Chem Toxicol 158: 112662, 2021.
34. Minelli R, Serpe L, Pettazzoni P, Minero V, Barrera G, Gigliotti C, Mesturini R, Rosa AC, Gasco P, Vivenza N, *et al*: Cholesteryl butyrate solid lipid nanoparticles inhibit the adhesion and migration of colon cancer cells. Br J Pharmacol 166: 587-601, 2012.
35. Satow R, Hirano T, Batori R, Nakamura T, Murayama Y and Fukami K: Phospholipase Cδ1 induces E-cadherin expression and suppresses malignancy in colorectal cancer cells. Proc Natl Acad Sci USA 111: 13505-13510, 2014.
36. Peng C, Li Z, Niu Z, Niu W, Xu Z, Gao H, Niu W, Wang J, He Z, Gao C, *et al*: Norcantharidin suppresses colon cancer cell epithelial-mesenchymal transition by inhibiting the αvβ6-ERK-Ets1 signaling pathway. Sci Rep 6: 20500, 2016.
37. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.
38. Chang YF, Wang HH, Shu CW, Tsai WL, Lee CH, Chen CL and Liu PF: TMEM211 promotes tumor progression and metastasis in colon cancer. Curr Issues Mol Biol 45: 4529-4543, 2023.
39. Li Y, Zhu G, Zhai H, Jia J, Yang W, Li X and Liu L: Simultaneous stimulation with tumor necrosis factor-α and transforming growth factor-β1 induces epithelial-mesenchymal transition in colon cancer cells via the NF-κB pathway. Oncol Lett 15: 6873-6880, 2018.
40. Lin MC, Wang FY, Kuo YH and Tang FY: Cancer chemopreventive effects of lycopene: Suppression of MMP-7 expression and cell invasion in human colon cancer cells. J Agric Food Chem 59: 11304-11318, 2011.
41. Bartucci M, Svensson S, Ricci-Vitiani L, Dattilo R, Biffoni M, Signore M, Ferla R, De Maria R and Surmacz E: Obesity hormone leptin induces growth and interferes with the cytotoxic effects of 5-fluorouracil in colorectal tumor stem cells. Endocr Relat Cancer 17: 823-833, 2010.
42. Hashimoto K, Nishimura S, Shinyashiki Y, Ito T and Akagi M: Characterizing inflammatory markers in highly aggressive soft tissue sarcomas. Medicine (Baltimore) 101: e30688, 2022.
43. Kleer CG, van Golen KL, Braun T and Merajver SD: Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol 14: 458-464, 2001.
44. Cohen EN, Gao H, Anfossi S, Mego M, Reddy NG, Debeb B, Giordano A, Tin S, Wu Q, Garza RJ, *et al*: Inflammation mediated metastasis: Immune induced epithelial-to-mesenchymal transition in inflammatory breast cancer cells. PLoS One 10: e0132710, 2015.
45. Shirahama S, Furukawa F, Wakita H and Takigawa M: E- and P-cadherin expression in tumor tissues and soluble E-cadherin levels in sera of patients with skin cancer. J Dermatol Sci 13: 30-36, 1996.
46. Devaux CA, Mezouar S and Mege JL: The E-cadherin cleavage associated to pathogenic bacteria infections can favor bacterial invasion and transmigration, dysregulation of the immune response and cancer induction in humans. Front Microbiol 10: 2598, 2019.
47. Katayama M, Hirai S, Yasumoto M, Nishikawa K, Nagata S, Otsuka M, Kamihagi K and Kato I: Soluble fragments of e-cadherin cell-adhesion molecule increase in urinary-excretion of cancer-patients, potentially indicating its shedding from epithelial tumor-cells. Int J Oncol 5: 1049-1057, 1994.
48. Repetto O, De Paoli P, De Re V, Canzonieri V and Cannizzaro R: Levels of soluble E-cadherin in breast, gastric, and colorectal cancers. Biomed Res Int 2014: 408047, 2014.
49. Weiss JV, Klein-Scory S, Kübler S, Reinacher-Schick A, Stricker I, Schmiegel W and Schwarte-Waldhoff I: Soluble E-cadherin as a serum biomarker candidate: Elevated levels in patients with late-stage colorectal carcinoma and FAP. Int J Cancer 128: 1384-1392, 2011.
50. Okugawa Y, Toiyama Y, Inoue Y, Iwata T, Fujikawa H, Saigusa S, Konishi N, Tanaka K, Uchida K and Kusunoki M: Clinical significance of serum soluble E-cadherin in colorectal carcinoma. J Surg Res 175: e67-e73, 2012.