WNT/β-Catenin Signaling Inhibitor IC-2 Suppresses Sphere Formation and Sensitizes Colorectal Cancer Cells to 5-Fluorouracil

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Abstract. *Background/Aim: Colorectal cancer (CRC) is one of the most malignant types of cancer worldwide. Recent studies suggest that a small subpopulation of cells, so-called cancer stem cells (CSCs), promote the high metastasis and relapse associated with CRC. WNT/β-catenin signaling plays a critical role in CSC maintenance. Therefore, its inhibitor may suppress CSCs and improve therapeutic effects on CRC. Materials and Methods: The effects of a derivative of WNT/β-catenin signaling inhibitor, IC-2, which we recently developed, on the CRC cell line DLD-1, were examined by luciferase reporter assay, WST assay, western blot, and sphere assay. Results: The reporter assay showed that IC-2 reduced WNT/β-catenin transcriptional activity in DLD-1 cells. Notably, IC-2 reduced expression levels of CSC marker proteins, as well as sphere formation. In addition, IC-2 increasesd cytotoxicity of 5-fluorouracil (5-FU) in DLD-1 cells. Conclusion: These results suggest that the combination treatment of IC-2 and 5-FU can stimulate tumor-suppressive effects on CRC.*

Colorectal cancer (CRC) is one of the most common cancer types in the world and carries the second highest mortality rate (1). Although initial events in CRC are relatively well studied and treatment for early-stage disease has significantly improved over the past decades, the mechanisms of

metastasis and relapse, which are the main causes of death, remain poorly characterized (2). Currently, no effective therapy is available for advanced or metastatic disease and the survival rate at 5 years of follow-up is approximately 50% (3). Thus, a novel therapeutic strategy is urgently required for improving the clinical outcome of CRC.

Recent studies suggest that a small subpopulation of cells, so-called cancer stem cells (CSCs), possess high tumorigenicity (4-6). CSCs have the unique features of self-renewal and asymmetrical division, and are resistant to radio- and chemotherapy (7, 8). The CSCs remaining after conventional therapies may promote the high metastasis and relapse of CRC. Therefore, suppression of CSCs is considered a promising approach to improve therapeutic effects on many types of cancers including CRC.

Most CRCs carry somatic mutations in one of two genes, adenomatous polyposis coli (APC) and β-catenin (*CTNNB1*) (9-11). Defect of these genes activates the canonical wingless-type MMTV integration site family (WNT)/β-catenin signaling pathway. It has been shown that the WNT/β-catenin signaling regulates expression levels of CSC marker genes and is essential for maintenance of the undifferentiated status and self-renewal capability of CSCs (12-14). Since we recently developed a derivative of a WNT/β-catenin signaling inhibitor, IC-2, which efficiently suppresses WNT/β-catenin transcriptional activity and induces hepatic differentiation of human mesenchymal stem cells (MSCs) (15, 16), we explored its effect on colorectal cancer cells.

Materials and Methods

Small molecular compounds. We recently developed WNT/β-catenin signaling inhibitors. IC-2, a derivative of ICG-001, efficiently represses WNT/β-catenin signaling and most effectively induces hepatic differentiation of human MSCs (16). The DNA/RNA synthesis inhibitor, 5-fluorouracil (5-FU) was purchased from Nacalai tesque inc. (Kyoto, Japan). The compounds were

dissolved in dimethylsulfoxide (DMSO), of which the final concentration was 1%, and used in the experiments.

Cells and culture. Human CRC cell line DLD-1 was provided by Tohoku Univ. Cell Resource Center (Sendai, Japan). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin, 2 mM L-glutamine, 0.2% $NaHCO_3$ and 3.5 g/l D-glucose with or without chemical compounds (DMSO, 5-FU and IC-2).

Luciferase reporter assay. DLD-1 cells were seeded onto a 24-well plate at a density of 5×10^4 cells/well. After overnight incubation, the cells were transfected with pTCF4-CMVpro-Fluc plasmid to measure TCF4/beta-catenin transcriptional activity with pRL-CMV-Rluc plasmids (18) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 4 h, and then treated with different concentrations of compounds (DMSO, 5-FU and IC-2) for 48 h. The luciferase activity was measured by Dual-LuciferaseR Reporter assay system (Promega, Madison, WI, USA) and Mini-Lumat LB 9506 (Berthold Technologies, Bad Wildbad, Germany).

WST assay. DLD-1 cells were seeded onto a 96-well plate at a density of 5.0×10^3 cells/well. After overnight incubation, the cells were treated with different concentrations of compounds (DMSO, 5-FU and IC-2). Cell viability was analyzed by WST assay using Cell Counting kit-8 (Dojindo, Kumamoto, Japan) and Micro plate reader (Tecan, Mannedorf, Switzerland). The 50% cell growth-inhibitory concentration (IC_{50}) for each compound was obtained from the following equation: $IC_{50}=10^{\wedge}[LOG(A/B) \times (50-C)/(D-C) + LOG(B)]$, where A is the higher concentration of two values that sandwich the IC_{50}; B is the lower concentration of two values that sandwich the IC_{50}, C is cell viability (%) at B and D is a cell viability (%) at A.

Western blot analysis. Proteins from cell lysis (20 µg) were subjected to western blot assay. Mouse monoclonal antibody against CD44 (Cell Signaling Technology, Danvers, MA, USA), mouse antibody against CD133 (Miltenyi Biotec, Auburn, CA, USA), rabbit monoclonal antibody against leucine rich repeat containing G protein-coupled receptor 5 (LGR5; Abcam Ltd., Cambridge, UK), mouse monoclonal antibody against Nanog homeobox (NANOG; Cell Signaling Technology), rabbit monoclonal antibody against octamer-binding transcription factor 3/4 (OCT3/4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat polyclonal antibody against actin (Santa Cruz Biotechnology) were used. Actin served as an internal control.

Sphere formation assay. To examine sphere formation, 5×10^3 DLD-1 cells were incubated in serum-free media composed of DMEM/F12 (Sigma-Aldrich Corp., St. Louis, MO, USA) with 0.6% methylcellulose in ultra-low attachment plate (Corning Inc., Corning, NY, USA). The medium was supplemented with 20 ng/ml recombinant human epidermal growth factor, 20 ng/ml recombinant human basic fibroblast growth factor, 1,000× leukemia inhibitory factor (Wako Pure Chemical Industries Ltd., Osaka, Japan), 4IU/ l Insulin(Biosource), 200 mmol/l L-glutamine (Thermo Fisher Scientific Inc., Madison, MA, USA), and 100 Units/ml penicillin and 100 µg/ml streptomycin (Nacalai tesque, inc., Kyoto, Japan). The images were captured a Keyence microscope (×4) after 7 days'incubation. The number of spheres (>50 µm) were

analyzed using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA.).

Statistical analysis. All values are the expressed as mean±SD. The differences between two groups were analyzed by an unpaired two-tailed Student's *t*-test. A *p*-value of less than 0.05 was considered to be significant.

Results

IC-2 inhibits TCF4/β-catenin transcriptional activity of CRC DLD-1 cells. In order to examine the effect of derivative of WNT/β-catenin signal inhibitor IC-2 on TCF4/β-catenin transcriptional activity in CRC cells, a luciferase reporter assay was performed using DLD-1 cells. The cells were treated with different concentrations of IC-2 or 5-FU for 48 h (Figure 1A). The reporter assay shows that IC-2 clearly suppressed the luciferase activity in a dose-dependent manner, whereas 5-FU had little effect, indicating that IC-2 has an inhibitory effect on WNT/β-catenin signal in DLD-1 cells (Figure 1A). Next, we investigated the effect of IC-2 and 5-FU on DLD-1 cell proliferation by WST assay. The cells were treated with different concentrations of agents for 0, 24, 48, and 72 h (Figure 1B). As previously reported (19), 5-FU reduced cell viability in a dose-dependent manner (Figure 1C). IC-2 did not affect viability at concentrations below 10 µM, whereas it reduced viability at higher concentrations (Figure 1C).

IC-2 reduces expression of CSC marker proteins. It has been reported that CSCs are highly resistant to conventional therapies and are responsible for recurrence and metastasis, suggesting that suppression of CSCs is important for cancer therapy. In order to examine the effect of WNT/β-catenin inhibitor on CSCs, we analyzed expression levels of CSC marker proteins CD44, CD133, OCT3/4, NANOG and LGR5. As shown in Figure 2, IC-2 reduced the levels of these CSC markers, suggesting that IC-2 may suppress CSCs in CRC cells.

IC-2 suppresses sphere formation activity of DLD-1 cells. To further investigate whether IC-2 suppresses CSCs in CRC cells, we next performed sphere formation assays by culturing cells for 7 days. To do this assay, we first determined the IC_{50} by WST assay. The IC_{50} of 5-FU and IC-2 were 2.2 µM and 21.5 µM, respectively (Figure 3A). Using these concentrations, we carried out the sphere assay. 5-FU reduced the number of sphere to about 60% compared to DMSO control (Figure 3B). Notably, IC-2 more greatly reduceded the sphere number compared to DMSO and 5-FU (Figure 3B), suggesting that IC-2 suppresses CSCs in CRC cells.

Next, we asked whether IC-2 could selectively target CSCs through WNT/β-catenin signaling inhibition. We
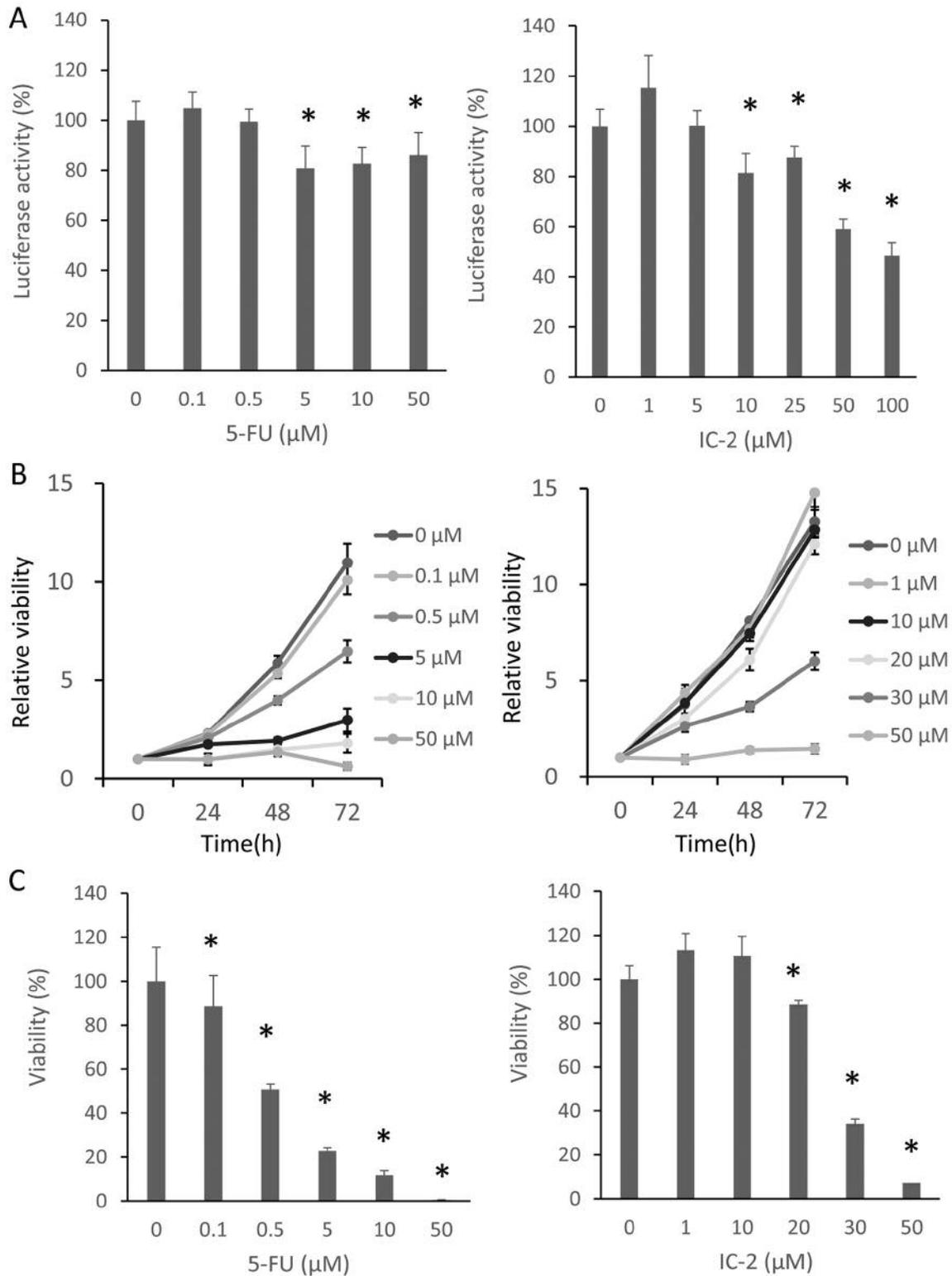
Figure 1. *Small-molecule compound, IC-2, inhibits transcription factor 4 (TCF4)/β-catenin transcriptional activity and cell viability of DLD-1 colorectal cancer cells. A: TCF4/β-catenin transcriptional activity was examined by luciferase reporter assay in DLD-1 cells under treatment with 5-fluorouracil (5-FU: 0-50 μM) and IC-2 (0-100 μM). Cell viability of DLD-1 cells analyzed by WST assay. Cells were treated with increasing concentrations of 5-FU and IC-2 for 0, 24, 48, and 72 h (B) or 48 h (C). Data are shown as mean±SD of three experiments. *p<0.05, compared to untreated cells (0 μM) by unpaired the Student's t-test.*
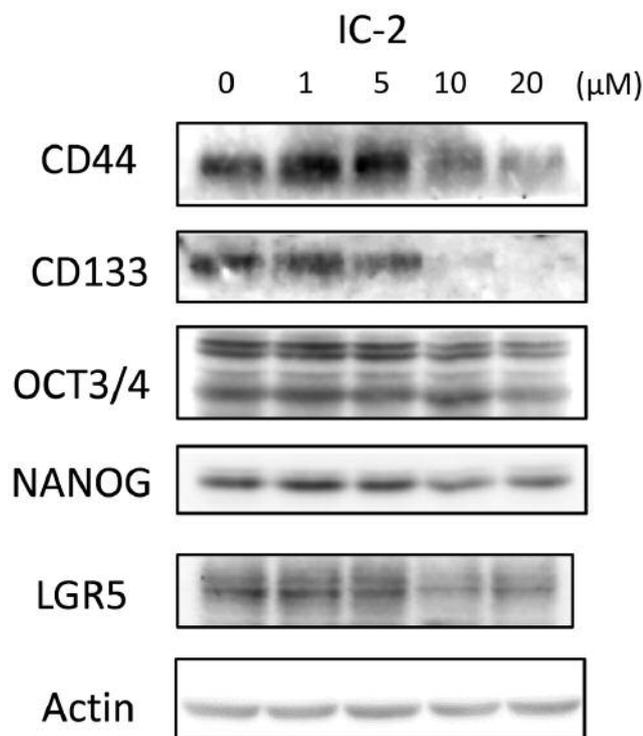
Figure 2. *IC-2 reduces expression of cancer stem cell's marker proteins. Western blot analysis of DLD-1 cells treated with several concentrations of IC-2 for 7 days using antibodies to CD44, CD133, octamer-binding transcription factor 3/4(OCT3/4), Nanog homeobox (NANOG) and leucine rich repeat containing G protein-coupled receptor 5 (LGR5). Actin was used as an internal control.*

isolated CSC marker CD44^{high} and CD44^{low} DLD-1 cells by cell sorting analysis and performed sphere assay with treatment with IC-2 or 5-FU. IC-2 reduced sphere numbers of CD44^{high} cells to a greater extent compared with CD44^{low} cells (Figure 3C), suggesting that IC-2 preferentially suppresses CSCs.

IC-2 enhances the cytotoxicity of CRC cells to 5-FU. Suppression of CSCs by IC-2 treatment raised the possibility that IC-2 increases the cytotoxicity of conventional anticancer drugs, such as 5-FU. In order to test the effect of the combination treatment, DLD-1 cells were treated with 0 to 5 μM 5-FU alone and in combination with IC-2 (Figure 4). IC-2 was used at 10 μM, a concentration which showed no inhibitory effect on DLD-1 proliferation but did reduce expression of CSC markers (Figure 1A and 2). As shown in Figure 4, IC-2 moderately but significantly enhanced the cytotoxicity of 5-FU at concentrations above 2 μM (Figure 4). Together, these results suggest that IC-2 sensitizes CRC cells to 5-FU through suppression of CSCs.

Discussion

CSCs are involved in resistance to chemotherapy, metastasis formation, and cancer relapse (20). Therefore, targeting the CSCs is considered critical for improving therapeutic effect on cancers. Since WNT/β-catenin signaling plays a crucial role in CSC maintenance, it is an important target to pursue in the development of new therapeutic strategies. Herein, we show that the small molecule compound IC-2 represses WNT/β-catenin signaling in CRC cells. In addition, IC-2 reduces protein levels of CSC markers as well as sphere formation, suggesting that IC-2 has an inhibitory impact on CSCs. Furthermore, IC-2 sensitizes CRC cells to 5-FU, suggesting that the combination treatment of IC-2 and 5-FU can stimulate tumor-suppressive effects on CRC.

Using the IC_{50} value, IC-2 significantly reduced sphere forming ability to 20% compared to the control (Figure 3B). We have previously shown that IC-2 efficiently induces hepatic differentiation of human MSCs (15,16), thus IC-2 may also enhance differentiation of CSCs, which could contribute to inhibition of sphere formation. Since CSCs are relatively resistant to chemotherapy including 5-FU (21), if CSC differentiation is stimulated by IC-2, it may potentiate the sensitivity of CRC to 5-FU. Indeed, 10 μM IC-2, which did not affect proliferation but reduced expression levels of CSC marker proteins, significantly reduced cell viability in combination with 5-FU (Figure 4). These results suggest that IC-2 sensitizes CRC cells to 5-FU, at least in part, through enhancement of CSC differentiation (Figure 5).

In the present study, the factors that IC-2 interacts with remain elusive. IC-2 is a derivative of ICG-001 (16), which suppresses WNT/β-catenin signaling by binding to CREB-binding protein (22), suggesting that IC-2 may also interact with this protein. Therefore, the next challenge is to clarify the proteins that IC-2 interacts with and to uncover the molecular mechanism of WNT/β-catenin signaling inhibition.

Besides ICG-001, several approaches for targeting the components of the WNT/β-catenin signaling pathway, such as tankyrases [XAV939 (23) and JW55 (24)], frizzled receptors[OMP-18R5 (25) and OMP-54F28 (26)], porcupine [LGK974 (27)], and CREB-binding protein [PRI-724 (28) and HC-1 (14)], have been developed. Notably, some of them are under evaluation in early-phase clinical trials. Therefore, it is important to test whether IC-2 is clinically safe in future study.
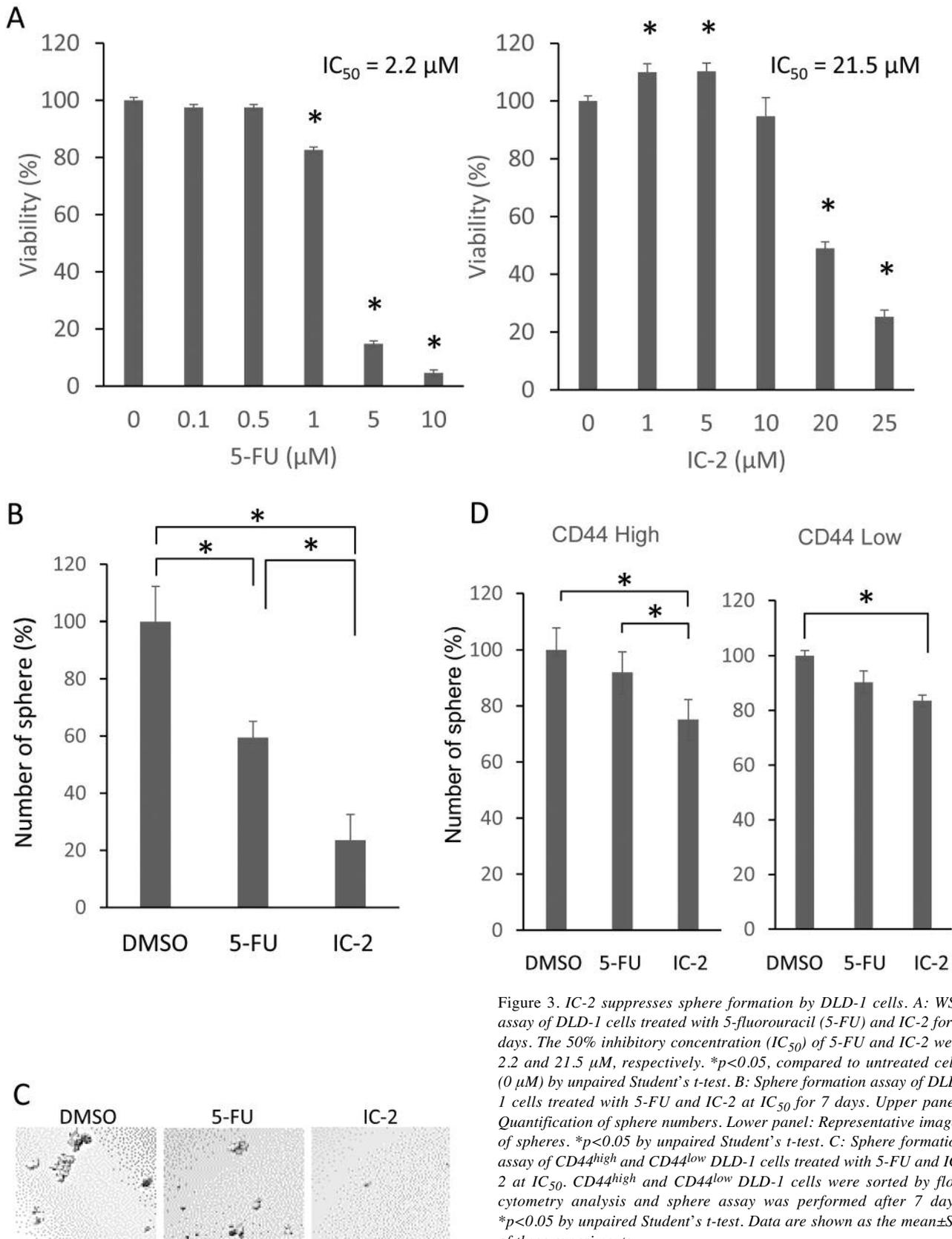
Acknowledgements

Figure 3. *IC-2 suppresses sphere formation by DLD-1 cells. A: WST assay of DLD-1 cells treated with 5-fluorouracil (5-FU) and IC-2 for 7 days. The 50% inhibitory concentration (IC_{50}) of 5-FU and IC-2 were 2.2 and 21.5 μM, respectively. *p<0.05, compared to untreated cells (0 μM) by unpaired Student's t-test. B: Sphere formation assay of DLD-1 cells treated with 5-FU and IC-2 at IC_{50} for 7 days. Upper panel: Quantification of sphere numbers. Lower panel: Representative images of spheres. *p<0.05 by unpaired Student's t-test. C: Sphere formation assay of CD44^high and CD44^low DLD-1 cells treated with 5-FU and IC-2 at IC_{50}. CD44^high and CD44^low DLD-1 cells were sorted by flow cytometry analysis and sphere assay was performed after 7 days. *p<0.05 by unpaired Student's t-test. Data are shown as the mean±SD of three experiments.*
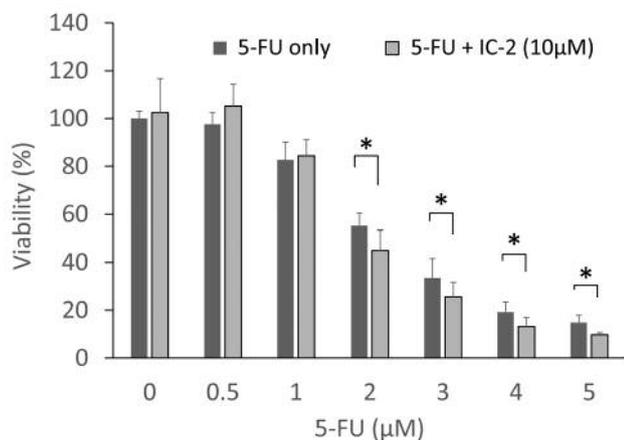
Figure 4. *IC-2 enhances the cytotoxicity of colorectal cancer cells to 5-fluorouracil (5-FU). A: Cells were treated with increasing concentrations of 5-FU alone or in combination with 10 μM IC-2 for 7 days. Cell viability was analyzed by WST assay. *p<0.05 by unpaired Student's t-test. Data are shown as the mean±SD of three experiments.*
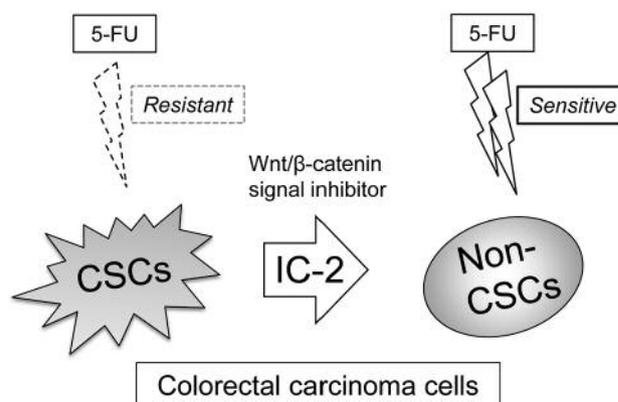

Figure 5. *Model of the chemotherapeutic effect of combined treatment with IC-2 and 5-fluorouracil (5-FU) on colorectal cancer cells. Our data suggest that IC-2 enhances differentiation of cancer stem cells (CSCs) into non-CSCs and sensitizes DLD-1 cells to 5-FU.*

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