Liver endothelium promotes HER3-mediated cell survival in KRAS wild-type and mutant colorectal cancer cells

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Abstract

We previously showed that human epidermal growth factor receptor 3 (HER3, also known as ERBB3) is a key mediator in liver endothelial cell (EC) promoting colorectal cancer (CRC) growth and chemoresistance, and suggested HER3-targeted therapy as a strategy for treating patients with metastatic CRC (mCRC) in the liver. Meanwhile, *KRAS* mutations occur in 40–50% of mCRC and render CRC resistant to therapies targeting the other HER family protein epidermal growth factor receptor (EGFR). It is necessary to elucidate the roles of *KRAS* mutation status in HER3-mediated cell survival and CRC response to HER3 inhibition. In the present study, we demonstrated that liver EC-secreted factors activated HER3 and promoted cell survival in *KRAS* wild-type and mutant CRC cells and tumors, and that blocking HER3 with an antibody, seribantumab, blocked EC-induced CRC survival. Our findings highlight a potential of utilizing HER3-targeted therapies for treating patients with mCRC regardless of *KRAS* mutation status.

Introduction

In the United States, colorectal cancer (CRC) is the second-leading cause of cancer-related deaths, with an estimated death of > 50,000 patients per year. Close to 25% of CRC cases are metastatic (mCRC) at the time of diagnosis and up to 50% of all patients with CRC will eventually develop metastases [1,2]. With the exception of immune checkpoint inhibitors for patients with microsatellite instability-high (MSI-H) tumors, nearly 50% of patients with mCRC do not have durable responses to systemic therapies, with the duration of response being less than one year [3,4]. As a result, the 5-year survival rate of patients with mCRC is only at 14.2% [1]. Even with the intensified regimen bevacizumab + FOLFOXIRI (fluorouracil, leucovorin, oxaliplatin, and irinotecan) that causes significant adverse effects, the 5-year survival rate of patients with unresectable mCRC is only 22% at best [5]. Therefore, a better understanding of the regulation of CRC cell survival pathways is urgently needed to develop novel therapeutic strategies for patients with mCRC.

Over 80% of patients with mCRC develop metastases in the liver [6]. Therefore, our laboratory aims to elucidate the potential roles of the liver microenvironment in CRC survival pathways after CRC cells metastasized to the liver. We specifically focus on the liver endothelial cells (ECs) as ECs comprise more than 50% of all of the stromal cells in the liver [7]. Moreover, preclinical studies from other groups in gastric, liver, and other types of cancer showed that ECs secreted soluble factors and activated cancer-promoting signaling pathways (such as AKT and NFκB) in cancer cells [8–11], known as angiocrine signaling. However, due to technical and resource limitations, most discoveries from those studies were made by using established human umbilical vein ECs that have been cultured extensively. To better recapitulate the liver EC microenvironment, our laboratory isolated primary ECs from non-neoplastic liver tissues. Using these primary liver ECs, we demonstrated that liver ECs secreted soluble factors that activated human epidermal growth factor receptor 3 (HER3, also known as ERBB3), a member of the HER family receptor tyrosine kinase (RTK) proteins, and its downstream target AKT. As a result, CRC cells had increased proliferation and became resistant to 5-fluorouracil (5-FU) induced apoptosis [12]. This line of work identified HER3 as a key mediator of liver EC microenvironment-induced CRC survival, and

suggested a potential of blocking HER3 with antibodies/inhibitors for treating patients with mCRC. Meanwhile, preclinical and clinical studies demonstrated that anti-RTK targeted therapies have limited effects on patients with cancers harboring mutations in *RAS* genes, which lead to the constitutive activation of RTK downstream pathways [13–15]. For example, *RAS* mutations occur in ~ 50% of patients with CRC [15]. Targeted therapies against epidermal growth factor receptor (EGFR), another RTK in the HER protein family, failed to improve the outcomes of patients with *RAS* mutant mCRC [16–20]. As a result, *RAS* mutations are approved by the FDA as resistance markers for patients with CRC response to EGFR-targeted therapies. On the other hand, the effects of HER3-targeted therapies on CRC and other types of cancer with *RAS* mutations have not been elucidated. As over 90% of all *RAS* mutations in mCRC occur in *KRAS* [15,20,21], we aim to determine the effects of blocking HER3 on cell survival in CRC cells with wild-type and mutant *KRAS* genes.

In this present study, we first used a patient-derived xenograft (PDX) tumor model and CRC cell lines and determined that conditioned medium (CM) from liver ECs increased CRC survival in CRC PDXs and cells with either wild-type or mutant *KRAS* genes. Then we used CRC cell lines and demonstrated that liver ECs activated the HER3-AKT signaling pathway and increased CRC cells growth and resistance to 5-FU regardless of the *KRAS* mutation status. Moreover, the EC-induced pro-survival effects on CRC cells were completely blocked by HER3 siRNA knockdown, or with the HER3 antibody seribantumab that was used in preclinical and clinical studies [22–24]. Lastly, we used an orthotopic liver injection xenograft model and determined that blocking HER3 with seribantumab decreased CRC tumor growth and sensitized CRC to 5-FU chemotherapy. Overall, our results demonstrated that liver EC-induced HER3 activation and CRC survival were independent of *KRAS* mutations, and that blocking HER3 with the antibody seribantumab effectively blocked CRC growth and chemoresistance in both *KRAS* wild-type and mutant CRC cells. As seribantumab is currently being assessed in a phase II clinical trial for treating patients with solid tumors harboring NRG-1 gene fusions (NCT04383210) [25], this study highlighted the potential for a rapid translation of treating patients with *KRAS* wild-type and *KRAS* mutant mCRC with the combination of 5-FU based chemotherapies and seribantumab, and potentially other HER3 antibodies/inhibitors.

Results

CM from liver ECs promoted the growth of CRC PDX tumors with wild-type or mutant *KRAS*

We used a proof-of-principle xenograft model with CRC PDXs to determine the effects of liver EC-secreted factors on CRC growth. CRC PDXs harboring either wild-type (*KRAS* WT) or mutant *KRAS* (*KRAS* mut) genes were implanted subcutaneously (subQ) into athymic nude mice in an inoculation mixture of Matrigel and either control CM (CM from HCP-1 CRC cells) or CM from human primary liver ECs (EC-1). To maintain the effects of CM on the PDXs during the experiment, CM from HCP-1 or EC-1 cells were injected subQ into the spaces between the tumors and skin tissues once a week throughout the experiment. Our results showed that, compared to CRC CM, liver EC CM resulted in over 4-fold increases in tumor sizes and 2-fold increases in tumor weights in both *KRAS* WT and *KRAS* mut PDXs (Fig. 1).

CM from liver ECs activated the HER3-AKT pathway and increased cell survival in CRC cells with wild-type or mutant KRAS

To further elucidate the effects of liver EC CM on CRC cell survival and the involved signaling pathways, we used two primary liver EC lines (EC-1 and EC-6) and multiple CRC cell lines that have either wild-type (SW48 and Caco2) or mutant (HCP-1, HCT116, and DLD-1) *KRAS* genes. Moreover, we used modified HCT116 and DLD-1 cells that have the heterozygous *KRAS* mutant alleles knocked out (*KRAS* mut KO) [26]. As a result, these modified cells only have the wild-type *KRAS* alleles and express wild-type *KRAS* (Suppl. Table 1). We treated CRC cells with either liver EC CM or CM from CRC cells themselves as control CM. Compared to the control CM, liver EC CM dramatically increased the levels of phosphorylation in HER3 and its downstream target AKT, demonstrating activation of the HER3-AKT survival pathway in both *KRAS* WT and *KRAS* mut CRC cells (Fig. 2). Moreover, we found that the modified HCT116 and DLD-1 cells (*KRAS* mut KO) that only have wild-type *KRAS* alleles responded to EC CM similarly to the unmodified parental HCT116 and DLD-1 cells with mutant *KRAS*. EC CM increased phosphorylation in HER3 and AKT in both the parental and modified cells (Fig. 2b). We noticed that under the control conditions, HCP-1 cells and modified HCT116 cells had higher basal levels of phosphorylation in HER3 and AKT than other cells. However, similar non-detectable basal levels of HER3 and AKT phosphorylation were detected in other *KRAS* mut cells (HCT116 and DLD-1) and wild-type *KRAS* expressing cells (SW48, Caco2, and modified DLD-1). The high basal levels of HER3 and AKT phosphorylation are likely to be cell line-specific and not related to the *KRAS* mutation status.

We then sought to determine the effects of liver ECs on CRC cell proliferation and response to chemotherapy. CRC cells were incubated in the control CM or liver EC CM and then treated without or with 5-FU, a widely used cytotoxic chemotherapy for treating patients with CRC. The MTT assay was used to determine cell viability under different conditions and data were presented as percent viability relative to cells in the CRC control CM without the 5-FU treatment. We found that in the absence of 5-FU, CM from liver ECs significantly increased the viability in CRC cells, an indication of increased proliferation, compared to CRC CM (Fig. 3). Interestingly, *KRAS* WT CRC cells (SW48 and Caco2) had less than 2-fold increases in cell viability, whereas KRAS mut cells (HCP-1, HCT116 and DLD-1) exhibited greater ~ 2.7–3.5 fold increases in cell viability. We also compared the effects of EC CM between HCT116 and DLD-1 parental and modified cells. We found that EC CM caused a 3.5-fold increase in the cell viability of HCT116 parental cells, compared to a 2.3-fold increase in that of HCT116 *KRAS* mut KO cells. We also found that EC CM caused a 2.8-fold increase in the cell viability of DLD-1 parental cells, compared to a 2.2-fold increase in that of DLD-1 *KRAS* mut KO cells (Fig. 3b, c). These findings confirmed that when stimulated by EC CM, CRC cells expressing mutant *KRAS* had greater cell viability than cells with wild-type *KRAS*.

To determine the effects of EC CM on CRC cells response to chemotherapy, we treated CRC cells with a clinically relevant dose of 5-FU (2 μg per ml) [27], which increased the levels of apoptotic markers (cleaved PARP and cleaved Caspase 3) and led to significant levels of apoptosis in CRC cells in our previous studies [12,28,29]. We confirmed that when treated with the control CRC CM, 5-FU decreased CRC cell

viability to ~ 60% relative to control groups without 5-FU treatment. In contrast, in both *KRAS* WT and *KRAS* mut cells, 5-FU had limited effects on CRC cells in the presence of EC CM and led to 100–150% cell viabilities relative to control groups with CRC CM and no 5-FU. These findings suggested that EC CM blocked the cytotoxic effects of 5-FU on CRC cells regardless of the *KRAS* mutation status, and therefore, CRC cells became more resistant to 5-FU when incubated in EC CM.

HER3 mediated liver EC-induced AKT activation and cell survival in CRC cells with wild-type or mutant *KRAS*

To determine the role of HER3 in mediating EC CM-induced AKT activation and CRC cell survival, we used siRNAs to knock down the expressions of HER3 in CRC cells. We found that EC CM failed to induce AKT phosphorylation without HER3 expression in both *KRAS* WT and *KRAS* mut CRC cells (Fig. 4). This observation was further validated by blocking HER3 with seribantumab, a humanized HER3-specific antibody that demonstrated significant HER3 inhibition in previous studies [22,23,30]. We found that seribantumab completely blocked EC-induced HER3 and AKT phosphorylation in all cell lines we used (Fig. 5). Then we used the MTT assay to determine the effects of blocking HER3 on EC-induced CRC cell proliferation and resistance to 5-FU treatment. First, we confirmed that seribantumab significantly blocked EC-induced cell viability, suggesting inhibition of proliferation, in CRC cells with different *KRAS* mutation status (Suppl. Figure 1). Subsequently, we incubated CRC cells in CM with 5-FU either alone or in combination with seribantumab. As expected, seribantumab blocked EC-induced cell viability, and 5-FU decreased cell viability in cells in CRC CM and, to a lesser extent, in EC CM (Fig. 6). In contrast, when we treated CRC cells with the combination of seribantumab and 5-FU, EC-induced cell resistance to 5-FU was completely blocked by seribantumab. As a result, CRC cells with the combination treatment had significantly lower cell viabilities (~ 40% in both CRC and EC CM treated groups) than those treated by either 5-FU or seribantumab alone (65–96% in CRC CM treated groups, and 85–120% in EC CM treated groups). Similar results were found in HCT116 and DLD-1 CRC cells with or without *KRAS* mutant alleles (Fig. 6b, c). Taken together, our findings suggested that HER3 mediated EC-induced CRC cell survival independent of the *KRAS* mutation status. Moreover, we demonstrated that blocking HER3 with seribantumab blocked EC-induced cell proliferation and sensitized cells to 5-FU treatment in both *KRAS* WT and *KRAS* mut CRC cells.

HER3 inhibition blocked KRAS mutant CRC tumor growth and sensitized tumors to 5-FU *in vivo*

The present study used multiple cell lines and demonstrated that blocking HER3 with the HER3 antibody seribantumab blocked liver EC-induced cell proliferation and resistance to 5-FU in both *KRAS* WT and *KRAS* mut CRC cells. These findings suggested that HER3 antibodies/inhibitors can potentially be used either alone or in combination with 5-FU-based chemotherapies for treating patients with either *KRAS* WT or *KRAS* mut mCRC. Compared to patients with *KRAS* WT mCRC that are eligible to and often benefit from existing EGFR targeted therapies, patients with *KRAS* mut mCRC are excluded from anti-EGFR regiments. It is necessary to develop and validate targeted therapies that are effective in patients with *KRAS* mut mCRC. Therefore, we focused on determining the effects of blocking HER3 on *KRAS* mut mCRC in a liver

injection orthotopic xenograft model. Prior to the experiment, we isolated murine primary liver ECs from athymic nude mice and confirmed that secreted factors from the murine liver ECs activated HER3-AKT and promoted cell growth in HCP-1 human CRC cells (*KRAS* mut) (Suppl. Figure 2). We then injected luciferase-labeled HCP-1 cells into the livers of athymic nude mice to replicate CRC liver metastases in patients with mCRC. After confirming tumor burden by bioluminescence, mice were randomized and then treated with seribantumab (20 mg per kg) either alone or with 5-FU (20 mg per kg) in saline in every three days. Saline with human IgG (20 mg per kg) was used as the control treatment. Compared to the control treatment, monotherapies with either seribantumab or 5-FU caused significant, but modest, decreases in tumor growth and resulted in 25–40% decreases in tumor burden at the end of the experiment (Fig. 7a). In contrast, the combination of seribantumab and 5-FU dramatically decreased tumor growth and resulted in an over 70% decrease in tumor burden compared to the control group at the end of the study. Figure 7b showed a picture of the livers harvested from all four groups at the end of the experiment and two enlarged images of the livers from either the control or the combination treatment group. Each liver from the control group had over 80% the liver occupied by CRC tumor tissues (T, pale light pink tissues), and only had a small fraction of the liver remained normal/non-neoplastic (N, dark red tissues). In contrast, the livers from the combination treatment group had less tumor tissues and more normal liver tissues. Specifically, each liver from the combination treatment group had only ~ 40–50% of the liver occupied by CRC tumors. The experiment ended 21 days after CRC cell implantation due to fast tumor growth. As a result, tumor-bearing mice only received four treatments in a period of 10 days, which could limit the effects of seribantumab on CRC. Additional *in vivo* models that have a longer window of opportunity for anti-HER3 therapies, such as transgenic mice for developing spontaneous mCRC liver metastases, can be used in future studies to further determine the anti-tumor effects of HER3 antibodies/inhibitors on mCRC. Taken together, our results showed that blocking HER3 with seribantumab decreased CRC tumor growth in the liver. More importantly, we demonstrated that blocking HER3 sensitized CRC tumors to 5-FU treatment and that the combination of seribantumab and 5-FU had the most significant inhibition on CRC tumor growth compared to monotherapies.

Discussion

Previous studies in different types of cancer have demonstrated the critical roles of stromal cells in the tumor microenvironment in regulating cancer cell functions [31–34]. Moreover, studies from our group and others suggested that ECs, a critical but understudied component of the microenvironment, promoted cell survival in CRC and few other types of cancer by secreting paracrine factors (angiocrine) [12,28,35,36]. The present study sought to better understand how the liver EC microenvironment affects mCRC growth and response to chemotherapy after CRC cells metastasized to the liver. Using primary liver ECs, we determined that liver ECs promoted CRC cell survival by increasing proliferation and chemoresistance independent of *KRAS* mutations status and HER3-AKT was the mediating signaling pathway. Moreover, we demonstrated that HER3 inhibition blocked liver EC-induced CRC survival in both *KRAS* WT and *KRAS* mut CRC cells, and that the HER3 antibody seribantumab blocked CRC growth and chemoresistance in *KRAS* mut CRC tumors in the liver.

The roles of HER3 in mediating cancer cell survival have been studied in different types of cancer such as breast, lung, and ovarian cancer [37,38]. In CRC, HER3 is expressed in ~ 75% of primary and metastatic tumors [39,40], and HER3 overexpression is associated with poor prognoses in patients with CRC [41,42]. Because HER3 is a RTK protein with weak kinase activity and is considered as a “kinase-dead” receptor [43], the canonical activation of HER3 in CRC and other cancer cells involves HER3 binding to its ligands and dimerizing with other proteins that have kinase activities. To date, neuregulin family proteins have been the only identified ligands for HER3 [38,44]. Neuregulin binding leads to HER3 dimerize with HER2 and, to a lesser extent, with other HER family receptors to activate downstream targets including AKT [44,45]. Similar to other RTK pathways, HER3-mediated AKT activation and cell survival involves PI3K and/or MAPK pathways [38,46]. When gain-of-function mutations occur in key factors in these pathways, such as *KRAS* mutations, HER3-mediated cell survival pathways are expected to be highly active and blocking the upstream HER3 activation should not affect downstream AKT activation and cancer cell functions. To our surprise, the HER3-AKT pathway was activated by liver EC-secreted factors in *KRAS* mut CRC cells. More importantly, blocking HER3 completely blocked EC-induced AKT activation and CRC survival in both *KRAS* WT and *KRAS* mut cells. It is possible that the HER3-AKT activation we observed was mediated by other signaling pathways. Indeed, our previous studies demonstrated that EC-induced HER3-AKT activation in CRC cells was independent of HER3 dimerization with HER2 or EGFR, and was also independent of the HER3 ligand neuregulins [12]. In this study, we also confirmed that EC CM did not activate EGFR or HER2, and blocking EGFR or HER2 did not affected EC-induced HER3 and AKT activation in CRC cell lines with different *KRAS* mutations status (data not shown). These findings strongly suggest that EC-secreted factors activate HER3 via a mechanism that has not been reported before and is independent of the *KRAS* mutation status. Our laboratory is currently working on identifying the key EC-secreted factor(s) that bind to and activate HER3, and determining the possible co-factor(s) that dimerize with HER3 for activating downstream pathways. Both studies will help us to better understand the regulatory mechanisms of the pro-survival HER3 pathway in CRC but are beyond the scope of this study.

The present study focused on comparing the effects of liver ECs and HER3 inhibition on *KRAS* WT and *KRAS* mut CRC cells. Meanwhile, mutations in other key oncogenic genes also affect cancer cell functions and response to therapies. For example, mutations in *PIK3CA* and *TP53* occur often in CRC and are also suggested to render mCRC resistant to EGFR targeted therapies [47–50]. Specifically, *PIK3CA* mutations lead to activation of PI3K, another key mediator in RTK signaling pathways including HER3 [49,51]. The cell lines we used in this study also harbor mutations in *PIK3CA* and/or *TP53* genes (Suppl. Table 1). Our results showed that liver ECs induced HER3-AKT activation and cell survival, and HER3 inhibition blocked EC-induced survival in all the cell lines we used. These findings suggest that the pro-survival effects of liver ECs are also independent of mutations in these genes and, more importantly, that HER3 targeted therapies can potentially be used for treating patients with mCRC with *PIK3CA* and *TP53* alterations.

Seribantumab and several other HER3 antibodies/inhibitors have been assessed in clinical studies for treating patients with breast, lung, or ovarian cancer, either alone or in combination with paclitaxel or EGFR/HER2 targeted therapies [52–57]. These studies showed that seribantumab and other HER3 targeted therapy agents were only effective in patients with NRG-1 gene fusions, which occur in ~ 0.2% of all solid tumors [56,58]. As a result, a phase II clinical trial was initiated in 2020 to assess the effects of seribantumab as a monotherapy in treating patients with NRG-1 fusion solid tumors (CRESTONE, NCT04383210) [59]. On the other hand, the effects of HER3 target therapies on CRC remain unclear and the effects on *KRAS* mut CRC is completely unknown. Three clinical studies using seribantumab or a HER3 inhibitor (AZD8931) collectively showed that HER3 inhibition led to stable diseases in few patients with *KRAS* WT CRC but did not improve the cytotoxic effects of irinotecan [60–62]. Considering the small number of patients enrolled in these studies and that irinotecan is rarely being used as a single agent chemotherapy for treating patients with CRC, the effects of HER3 antibodies/inhibitors either alone or in combination with chemotherapy in patients with CRC need to be further determined. In July 2020, another phase II clinical trial was initiated to use an antibody-drug conjugate agent with a HER3 antibody and an irinotecan derivative compound for treating patients with mCRC (NCT04479436). Although there is no data available from this study yet, it highlighted a need of assessing HER3 targeted therapies in additional clinical studies for treating patients with mCRC. Our previous and current findings not only determined that blocking HER3 effectively blocked cell survival in both *KRAS* WT and *KRAS* mut CRC cells, but also demonstrated that combining HER3 with 5-FU significantly improved 5-FU cytotoxic effects on CRC cells and CRC tumors in the liver. As 5-FU based regiments are the most commonly used chemotherapies in treating patients with mCRC, our findings strongly support the notion of using seribantumab, and potentially other HER3 antibodies/inhibitors, in combination with 5-FU based therapies for treating patients with mCRC regardless of *KRAS* mutation status.

In summary, findings from the present study determined that the liver EC microenvironment has an important role in activating HER3 and promoting CRC cell survival independent of *KRAS* mutations, and potentially other key oncogenic mutations. Furthermore, we showed that HER3 inhibition blocked EC-induced CRC proliferation and chemoresistance in *KRAS* WT and *KRAS* mut CRC cells, and the HER3 antibody seribantumab blocked *KRAS* mut CRC tumor growth and sensitized CRC tumors to 5-FU. Our findings suggest a potential therapeutic strategy of using HER3 antibodies/inhibitors in combination with established chemotherapy agents for treating patients with either *KRAS* WT or *KRAS* mut mCRC.

Methods

Cell lines and tissue culturing

The established CRC cell lines SW48 and Caco2 were purchased from ATCC (Manassas, VA, USA). The parental and *KRAS* mut allele-deleted HCT116 and DLD-1 cells were previously described [26].The human CRC primary cell line (HCP-1) and human liver parenchymal primary EC lines (EC-1 and EC-6, also known as LPEC-1 and − 6 in previous studies) lines were isolated and established using MACS microbead-

conjugated antibodies (anti-EpCAM for HCP-1, and ant-CD31 for liver ECs) and separation columns[12,28,29,36], all from Miltenyi Biotec, Bergisch Gladbach, Germany. All CRC cells were cultured in MEM (Sigma-aldrich, St. Louis, MO, USA) and supplemented with 5% FBS (Bio-Techne, R&D Systems, Minneapolis, MN, USA), vitamins (1x), nonessential amino acids (1x), penicillin-streptomycin antibiotics (1 x), sodium pyruvate (1x), and L-glutamine (1x), all from Thermo Fisher Scientific/Gibco (Grand Island, NY, USA). Human liver primary ECs were cultured in Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany) and supplemented with 10% human serum (Bio-Techne, R&D Systems) and antibiotics-antimycotic (1x, Thermo Fisher Scientific/Gibco). Murine liver primary ECs were isolated from athymic nude mice also using MACS microbead-conjugated antibodies for murine CD31 (Miltenyi Biotec) and cultured in MV2 EC culture medium as described above. HCP-1 cells, human and murine liver ECs were used within 10 passages, with approximately 1 week per passage.

Cell line mutation statuses were determined in previous studies[36,63–66], or from public databases including the Cancer Cell Line Encyclopedia (CCLE) and the Catalogue of Somatic Mutations in Cancer (COSMIC). Authentication for all cell lines were done in every 6 months by short tandem repeat (STR) tests. For the primary cell lines (HCP-1 and ECs), genomic DNA samples from the original tissues were used for the STR authentication. All cell lines were also tested for mycoplasma contamination every 6 months.

Reagents

The fully humanized IgG2 anti-HER3 antibody seribantumab (previously known as MM-121) was provided by Merrimack Pharmaceuticals (Cambridge, MA, USA), and is now owned by Elevation Oncology Inc. (New York, NY, USA). The control human IgG antibody for *in vitro* and *in vivo* studies was from Invitrogen (Carlsbad, CA, USA). Pharmaceutical grade 5-fluorouracil (5-FU) was obtained from Sigma-aldrich and was dissolved in DMSO for storage. 200 µg per ml seribantumab and 2 µg per ml 5-FU were used for all *in vitro* studies,. Validated control siRNAs and human *ERBB3* (HER3) specific siRNAs (si-HER3-a: 5'-GCUGAGAACCAAUACCAGA, si-HER3-b: 5'-CCAAGGGCCCAAUCUACAA) were obtained from Sigma-Aldrich.

Conditioned medium (CM)

$3x10^5$ CRC cells or ECs were seeded in T25 culture flasks. The next day, the cells were washed with 1X PBS and then cultured for 48 hours in 3 ml MEM medium with 1% FBS ($1x10^5$ cells per ml). CM were harvested and centrifuged at 4,000 *g* for 10 minutes to remove cell debris. CM from each CRC cell line were used as controls.

siRNA transfection

For each transfection, 1×10^6 CRC cells were transiently transfected with 400 pmol siRNAs via electroporation using the Neon Transfection System (Invitrogen, Carlsbad, CA, USA) with 3 pulses of 10 milliseconds at 1,600 V according to the manufacturer's instructions. Cells were recovered in 5% FBS for 24–48 hours, cultured in 1% FBS overnight, and then incubated in CM for 30 minutes for Western blotting, or up to 72 hours for the MTT assay.

Western blotting

CRC cells were treated with CM and/or seribantumab for 30 minutes. Cell lysates were processed and run through SDS-PAGE gel electrophoresis as described previously [29,67]. An HRP-conjugated β-actin antibody was obtained from Santa Cruz Biotechnology and was used in 1:8000 dilution (Santa Cruz, CA, USA). All other antibodies were from Cell Signaling Technology and were used in 1:1000 dilutions (Beverly, MA, USA). For each experiment, protein lysates were loaded into two gels and processed at the same time for separately probing for antibodies specific to phosphorylated proteins and total proteins. All membranes were probed with β-actin as loading controls. Each Western blotting figure shows representative results from one experiment of at least three independent experiments.

MTT assay

CRC cells were seeded at 3,000 cells/well in 96-well plates, cultured in 1% FBS overnight, and incubated in CM for 72 hours without or with 5-FU (2 μg per ml). When seribantumab (200 μg per ml) were used, cells were pretreated with seribantumab in 1% FBS medium for one hour, and then cultured with seribantumab without or with 5-FU, seribantumab, or both in CM for 72 hours. Cell viability was assessed by adding the MTT substrate (0.25% in PBS, Sigma-Aldrich) in growth medium (1:5 dilution) for 1 hour at 37°C. Cells were washed with 1x PBS and then incubated with 50 μl DMSO. The optical density of converted substrate was measured at 570 nm, and the relative MTT was presented as percent viability relative to cells in the CRC control CM without any drug treatment.

Xenograft tumor models

The CRC PDXs were established previously [68]. Frozen PDX tumors were expanded in athymic nude mice, sliced into ~ 5mm^3 pieces, and then implanted subcutaneously (subQ) into the right flanks of the athymic nude mice in an inoculation matrix (100 μl of 1:1 mix of growth-factor-reduced Matrigel and HCP-1 or EC-1 CM). After PDX implantation, mice were treated with CM once a week by injecting CM subQ into the spaces between PDXs and skin tissues. Tumor volumes were measured with a caliper.

For liver injection orthotropic xenografts, CMV-driven luciferase reporter-labeled HCP-1 CRC cells were suspended in an inoculation matrix (1:1 mix of growth factor-reduced Matrigel and serum-free MEM medium) and injected into the left lobe of the livers in athymic nude mice (1x10^6 cells in 50 μl per injection). After injection, the tumor burdens were assessed by bioluminescence with the *In Vivo* Imaging System (IVIS) and D-Luciferin substrate (Xenogen, Alameda, CA, USA) according to the manufacturer's instructions. When the tumor burdens were confirmed (on Day 10 after implantation), the mice were randomized into four groups with equal average tumor burdens (n = 10 per group) and were treated with control IgG (20 mg per kg), 5-FU (20 mg per kg), or seribantumab (20 mg per kg) in 100 μl saline by intraperitoneal injection in every three days (on Days 11, 14, 17, and 20 after implantation).

For all xenograft studies, mice were euthanized when three mice in any group became moribund or their tumor sizes reached 1,000mm^3.

Statistical analysis

For *in vitro* assays, all quantitative data were reproduced in at least three independent experiments and multiple replicates in each experiment. Groups were compared by two-tailed Student's *t*-test and data were expressed as means -/+ standard error of the mean (SEM) with significance of $P < 0.05$. For *in vivo* studies, Wilcoxon rank-sum test was used for tumor volume and burden changes over time, and one-way ANOVA was used to compare tumor weights and sizes between groups after tumor harvest in PDX studies. Data were presented as means -/+ standard deviation (SD) with significance of $P < 0.05$.

Declarations

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AUTHOR CONTRIBUTIONS

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Jordan Winter	Data interpretation, manuscript editing
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Joseph Willis	Resource sharing
Lee M. Ellis	Conception and design, data analysis and interpretation, financial support,
Rui Wang	Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript

COMPETING INTERESTS

The authors declare no competing interests.

I, Rui Wang, PhD, confirm that all non-human vertebrate studies conducted in the submitted manuscript entitled "Liver endothelium promotes HER3-mediated cell survival in KRAS wild-type and mutant colorectal cancer cells" were approved by Case Western Reserve University Institutional Animal Care and Use Committee (IACUC), which provided oversight and support for animal care and ethics.

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Figures

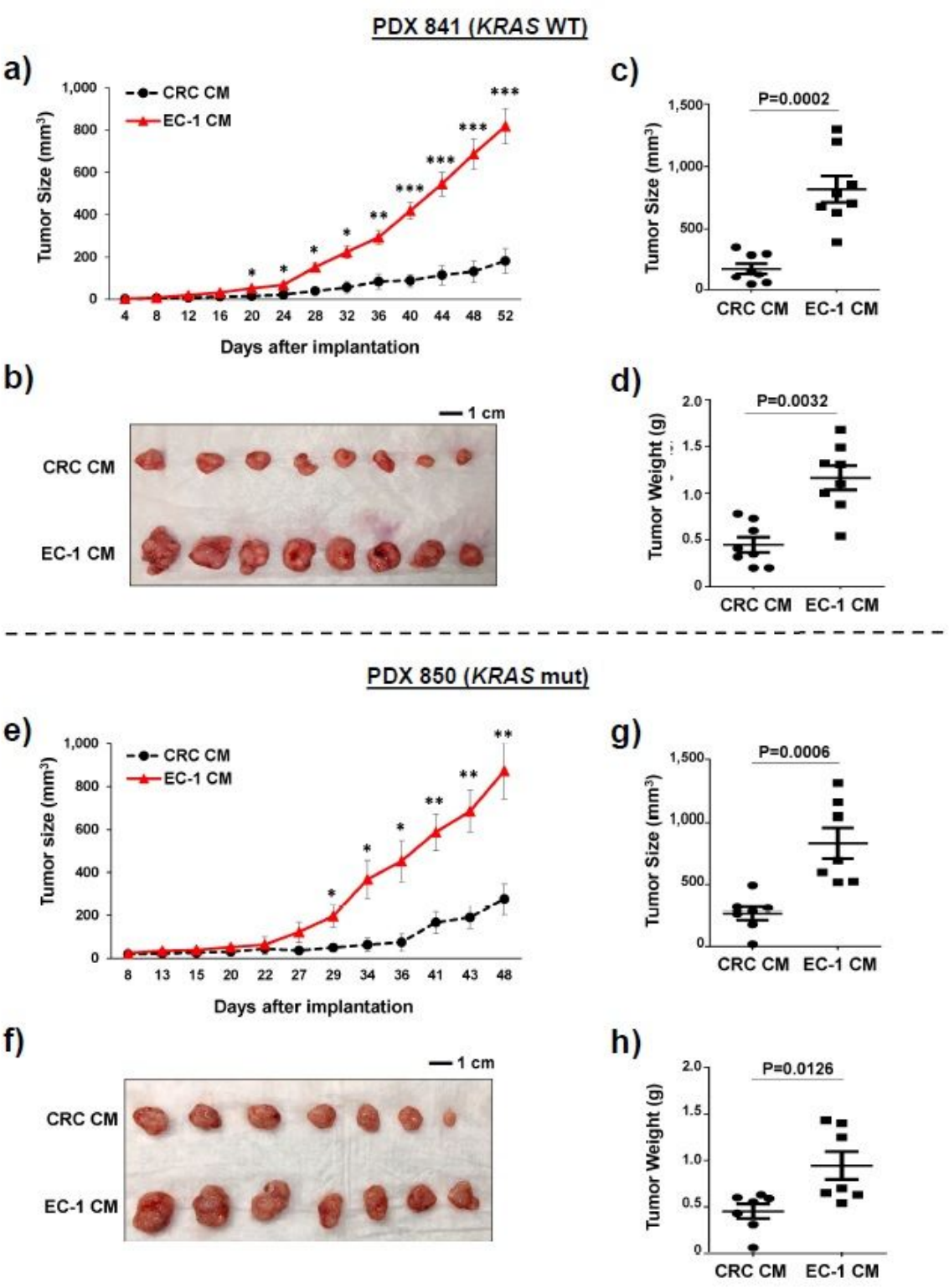

Figure 1

CM from liver ECs promoted tumor growth in KRAS wild-type and mutant PDXs in vivo. CRC PDXs with wild-type (KRAS WT) or mutant (KRAS mut) KRAS genes were implanted subQ with CM from HCP-1 cells (CRC CM) or primary liver EC (EC-1 CM) (EC-1 CM). (a, e) Tumor size measurements over time showed that EC-1 CM promoted PDX tumor growth. Mean -/+ SD, *P<0.01, **P<0.001, ***P<0.0001 Wilcoxon rank-sum test. (b, f) Pictures of tumors harvested from each group. Scale bars represent 1 cm. (c, d, g, h)

Scatter plots of tumor sizes and weights measured immediately after tissue harvest. Mean +/- SD, P values by one-way ANOVA.

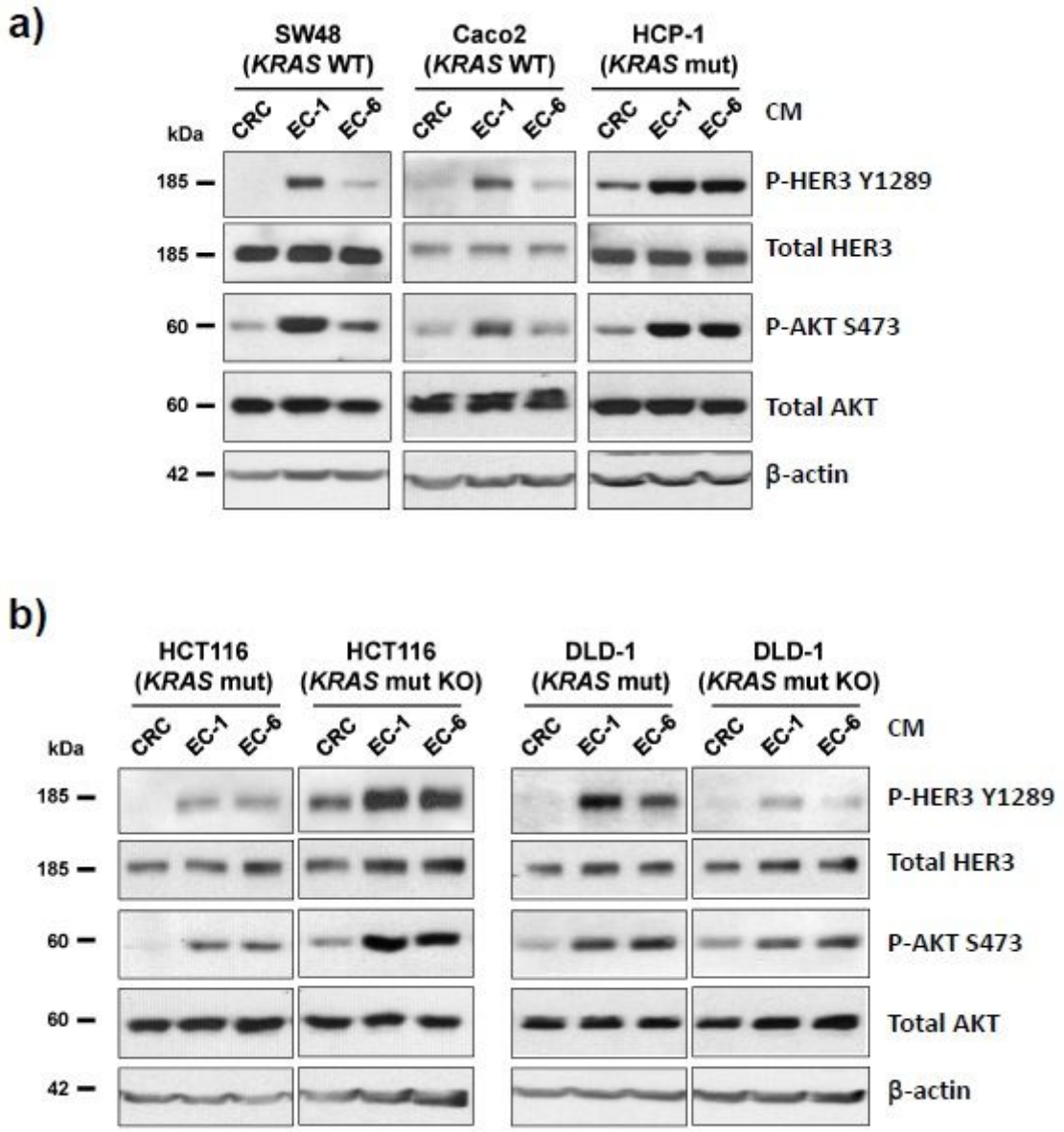

Figure 2

CM from liver ECs activated HER3-AKT in CRC cells expressing either wild-type or mutant KRAS genes. CRC cells were incubated in control CRC CM or CM from different primary liver ECs (EC-1 and EC-6) for 30 minutes. Western blotting showed that EC CM increased the levels of HER3 and AKT phosphorylation in (a) SW48 and Caco2 cells with wild-type KRAS (KRAS WT) and HCP-1 cells with mutant KRAS (KRAS mut), and (b) parental HCT116 and DLD-1 cells with mutant KRAS (KRAS mut) and their modified sub-clones with the mutant KRAS alleles knocked out (KRAS mut KO). Total levels of HER3, AKT, and β-actin were used as loading controls. Data represent the results of at least three independent experiments.

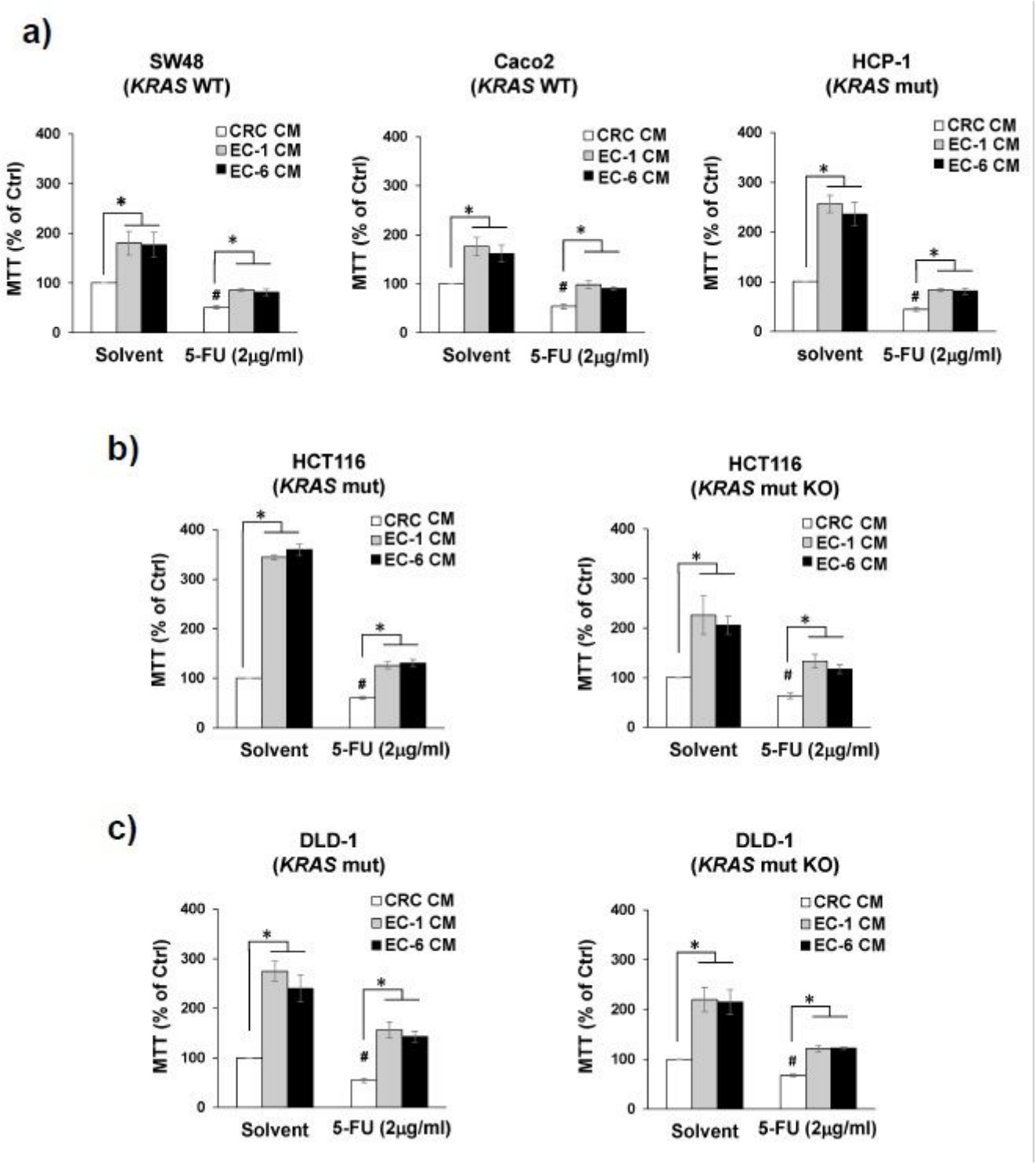

Figure 3

CM from liver ECs increased cell viability and resistance to chemotherapy in CRC cells expressing either wild-type or mutant KRAS genes. CRC cells were incubated with control CRC CM or CM from different primary liver ECs (EC-1 and EC-6) and treated without (Solvent) or with 5-FU in CM for 72 hours. The MTT assay showed that the CM from liver ECs increased the cell viability in (a) SW48 and Caco2 cells (KRAS WT) and HCP-1 cells (KRAS mut), and in (b, c) parental HCT116 and DLD-1 cells (KRAS mut) and their modified sub-clones with the mutant KRAS alleles knocked out (KRAS mut KO). Cell viabilities were

presented as % control relative to control groups with CRC CM and solvent. Mean +/- SEM of at least three experiments, *p<0.01 t-test compared to adjacent groups, #p<0.01 t-test compared to control groups with CRC CM and solvent.

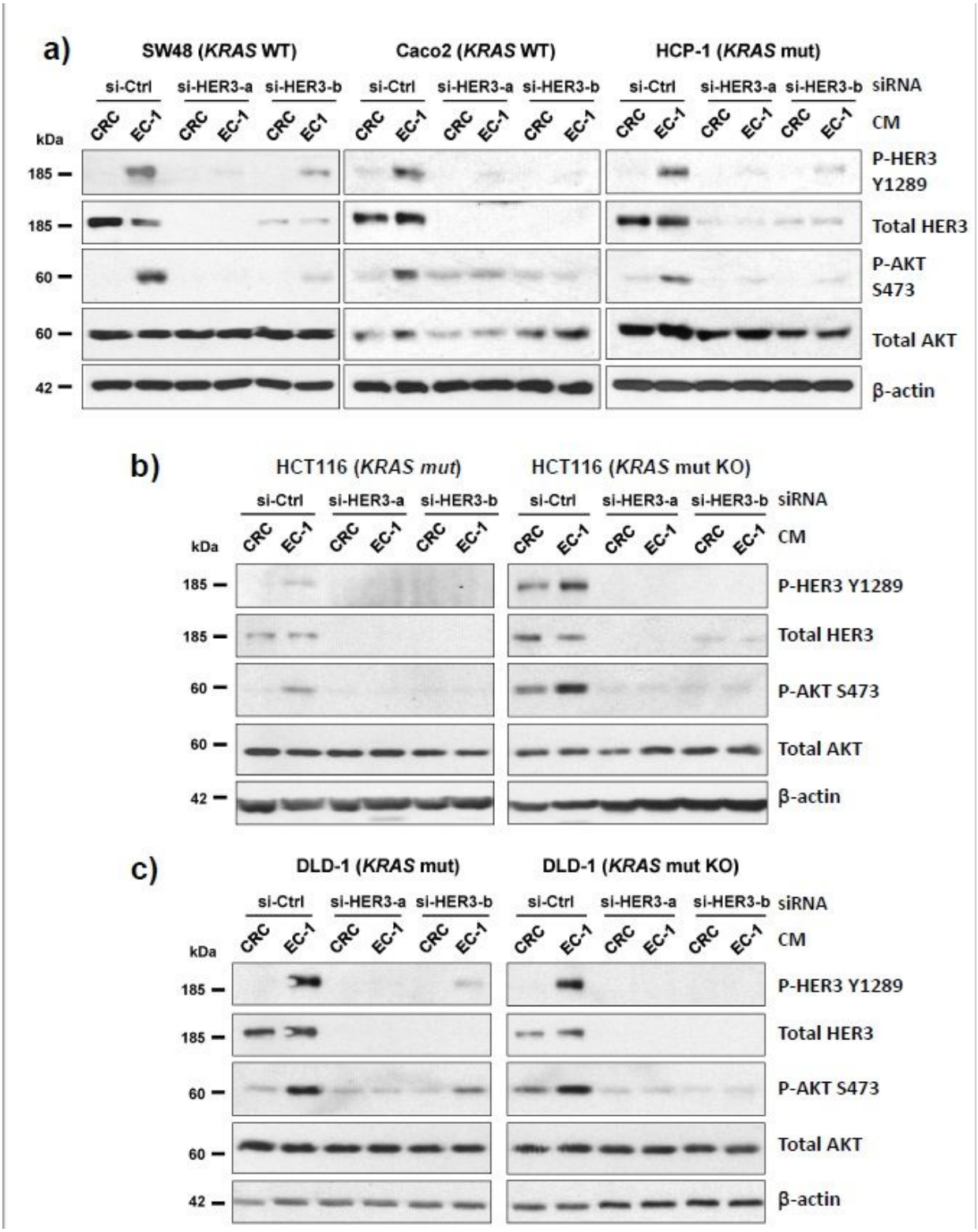

Figure 4

HER3 knockdown by siRNAs inhibited liver EC-induced HER3-AKT activation in CRC cells expressing either wild-type or mutant KRAS genes. CRC cells were transfected with control (si-Ctrl) or HER3-specific siRNAs

(si-HER3-a and si-HER3-b) and then incubated with control CRC CM or CM from different primary liver ECs (EC-1 and EC-6). The Western blotting showed that HER3 siRNAs decreased HER3 protein levels, and blocked liver EC CM-induced AKT phosphorylation in (a) SW48 and Caco2 cells (KRAS WT) and HCP-1 cells (KRAS mut), and (b, c) parental HCT116 and DLD-1 cells (KRAS mut) and their modified sub-clones with the mutant KRAS alleles knocked out (KRAS mut KO). Total levels of HER3, ATK and β-actin were used as loading controls. Data represent the results of at least 3 independent experiments.

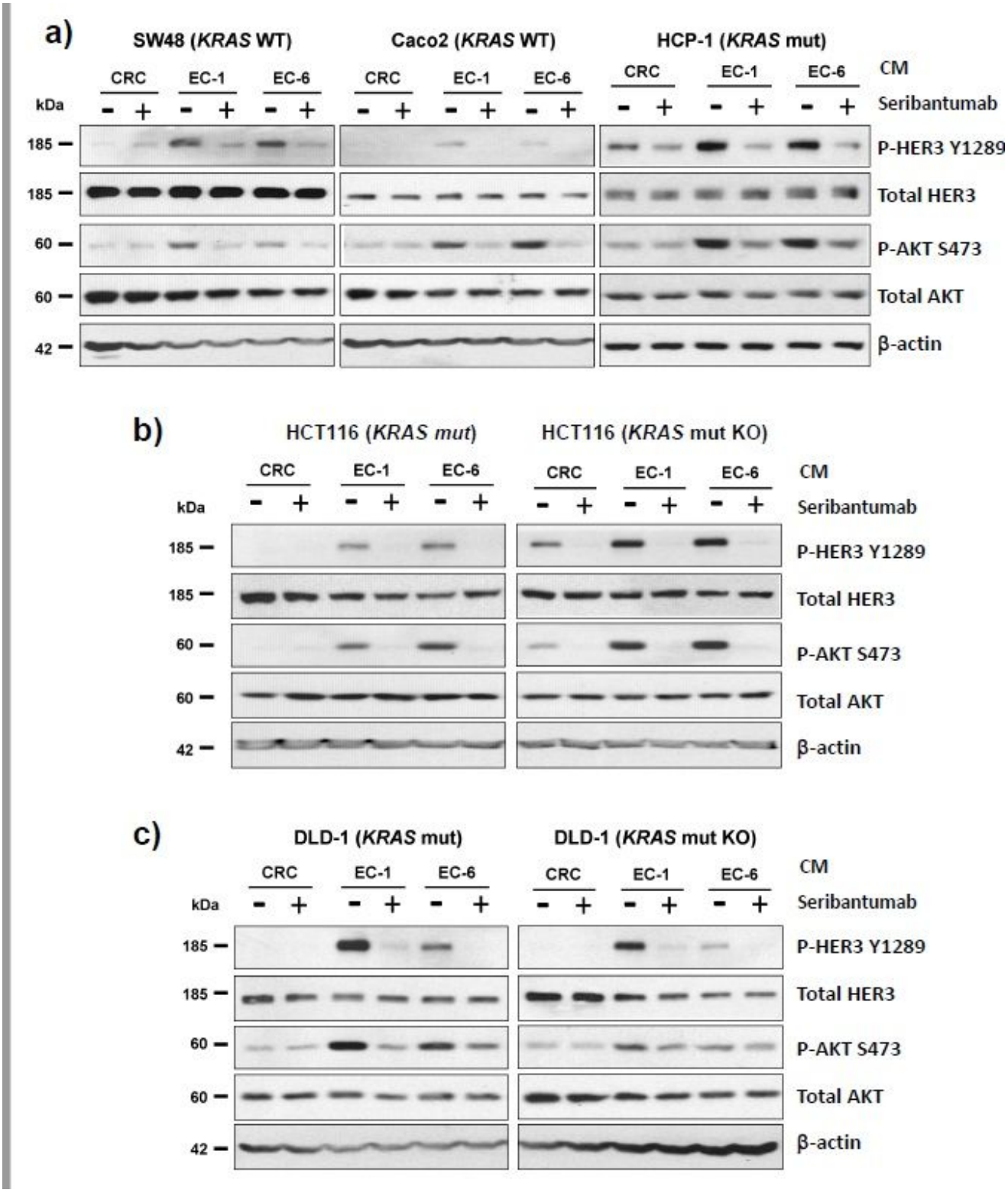

Figure 5

HER3 antibody seribantumab blocked liver EC-induced HER3-AKT activation in CRC cells expressing either wild-type or mutant KRAS genes. CRC cells were incubated in control CRC CM or CM from different primary liver ECs (EC-1 and EC-6) either in the presence or the absence of the HER3 antibody seribantumab (200 □g per ml) for 30 minutes. The Western blotting showed that seribantumab blocked liver EC CM-induced HER3 and AKT phosphorylation in in (a) SW48 and Caco2 cells (KRAS WT) and HCP-1 cells (KRAS mut), and (b, c) parental HCT116 and DLD-1 cells (KRAS mut) and their modified sub-clones with the mutant KRAS alleles knocked out (KRAS mut KO). Total levels of HER3, ATK and □-actin were used as loading controls. Data represent the results of at least 3 independent experiments.

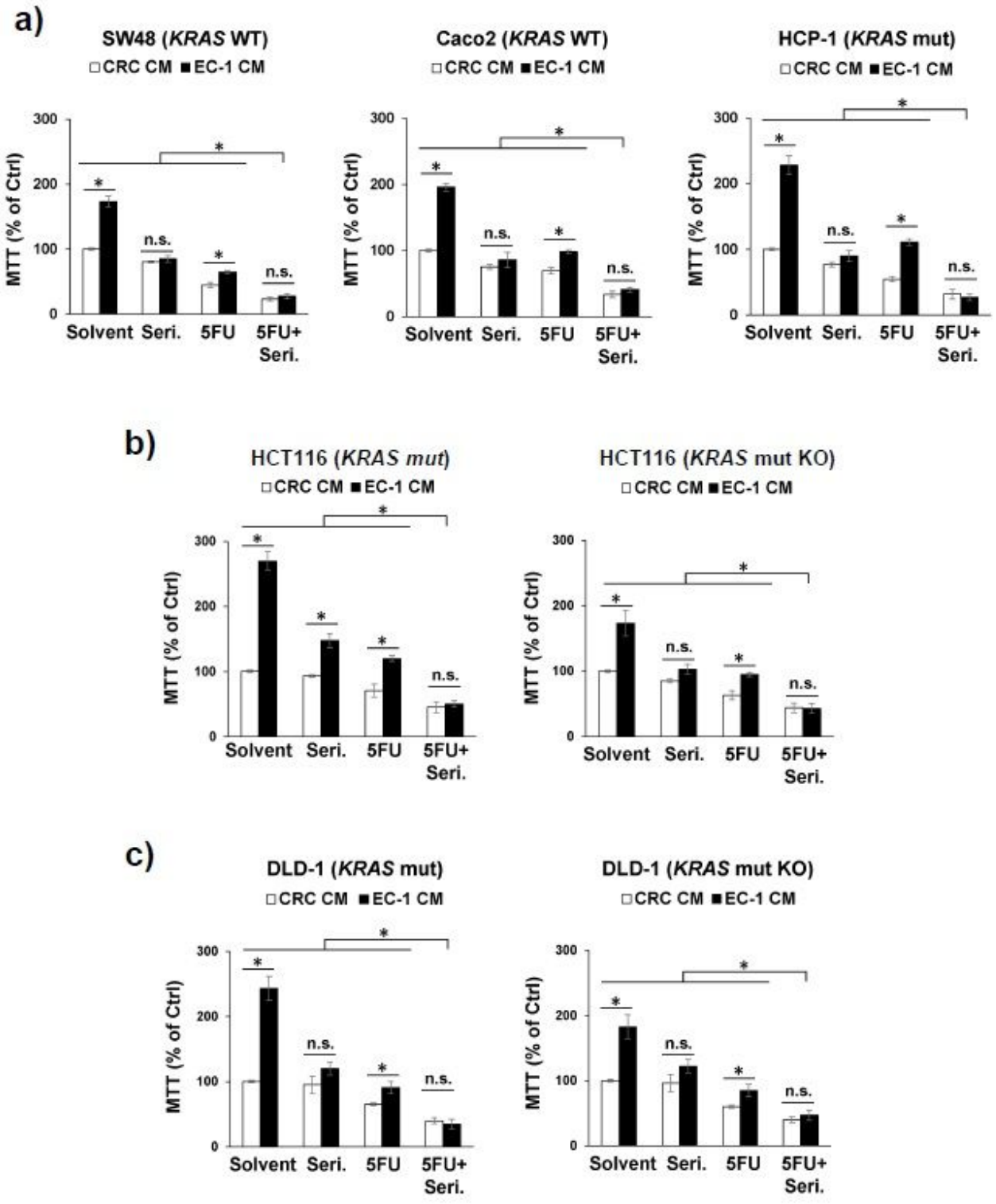

Figure 6

HER3 antibody seribantumab blocked liver EC-induced CRC cell viability and chemoresistance in CRC cells expressing either wild-type or mutant KRAS genes. CRC cells were incubated in control CRC CM or CM from primary liver ECs (EC-1) and treated with the HER3 antibody seribantumab (Seri., 200 □g per ml) or/and 5-FU (2 □g per ml) for 72 hours. The MTT assay showed that seribantumab or 5-FU decreased EC CM-induced CRC cell viability, and the combination of seribantumab and 5-FU further decreased cell

viability in in (a) SW48 and Caco2 cells (KRAS WT) and HCP-1 cells (KRAS mut), and (b, c) parental HCT116 and DLD-1 cells (KRAS mut) and their modified sub-clones with the mutant KRAS alleles knocked out (KRAS mut KO). Cell viabilities were presented as % control relative to control groups with CRC CM and solvent. Mean +/- SEM. *p<0.01 t-test. n.s., not significant by t-test.

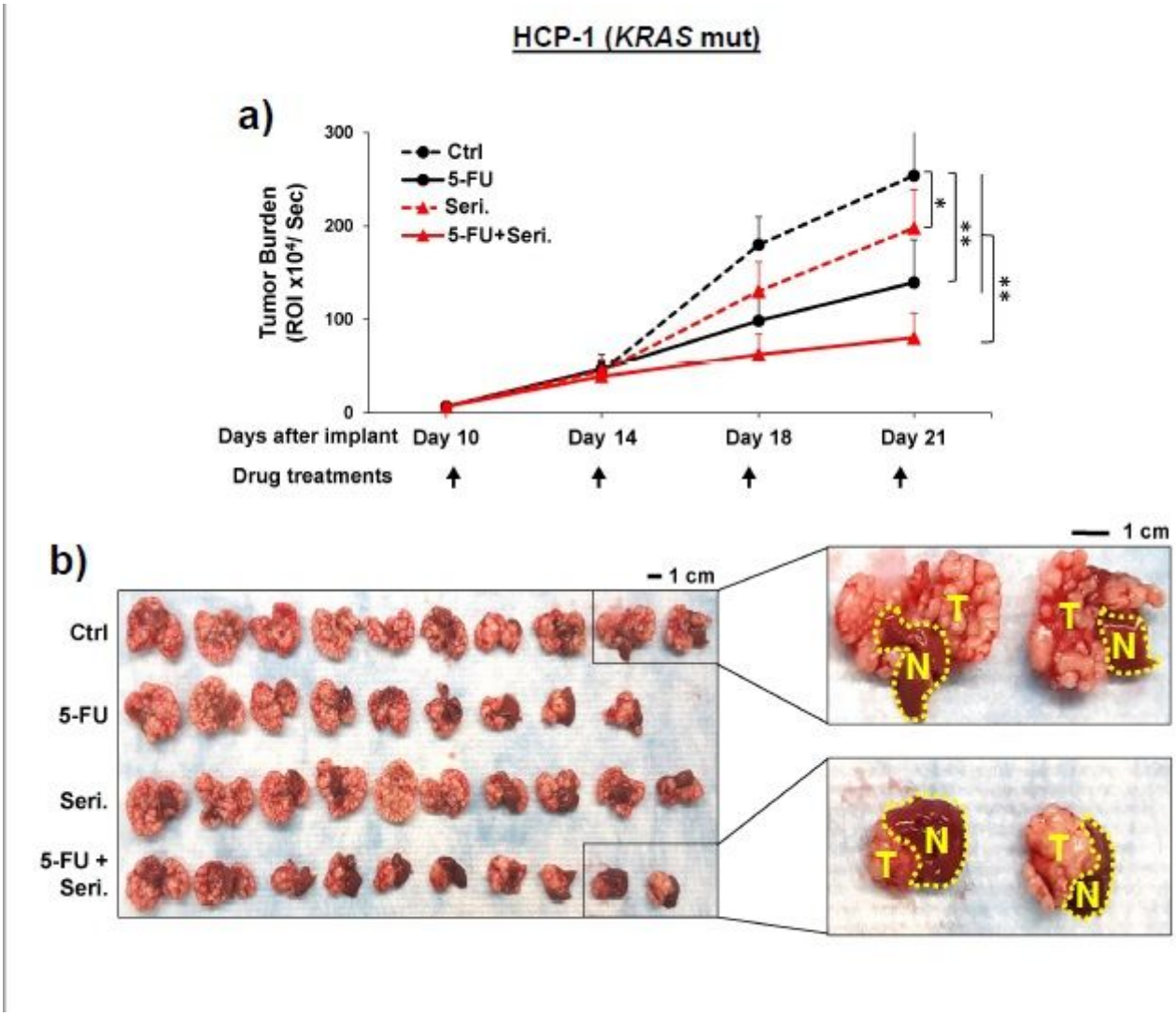

Figure 7

HER3 antibody seribantumab sensitized KRAS mutant CRC tumors to chemotherapy in vivo. Luciferase reporter-labeled HCP-1 CRC cells (KRAS mut) were injected into the livers of athymic nude mice. Once tumor burdens were confirmed by bioluminescence on Day 10, mice were randomized and treated with control IgG (Ctrl) alone, 5-FU alone, seribantumab alone (Seri.), or a combination of 5FU and seribantumab (5FU+Seri.) every three days (black arrow). (a) Tumor burdens measured by bioluminescence with IVIS over time. Mean -/+ SD, *P<0.02, **P<0.001 Wilcoxon rank-sum test between groups on Days 18 and 21. (b) A picture of the tumor-bearing livers harvested from each group (left), and enlarged images of two livers from Ctrl or 5-FU+Seri. groups (right). Tumor tissues (T, pale light red regions) and non-neoplastic normal liver tissues (N, dark red regions) were labeled. Scale bars represent 1 cm.

Supplementary Files

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