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Endothelial Cells Promote Colorectal Cancer Cell Survival by Activating the HER3-AKT Pathway in a Paracrine Fashion

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Running title: HER3 Activation in CRC by Endothelial Cells

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

ABBREVIATIONS

5-FU	5-fluorouracil	HCP-1	Human CRC primary cell line -1
CM	Conditioned medium	HER3	Human epidermal growth factor receptor 3
co-IP	Co-immunoprecipitation	IVIS	*In Vivo* imaging system
CRC	Colorectal cancer cell	LPEC	Liver parenchymal endothelial cell
CSC	Cancer stem cell	NRG-1	Nuregulin-1
EC	Endothelial cell	RTK	Receptor tyrosine kinase

EMT	Epithelial-mesenchymal transition	subQ	Subcutaneous
		FACS	Fluorescence-activated cell sorting

ABSTRACT

The regulation of colorectal cancer (CRC) cell survival pathways remains to be elucidated. Previously it was demonstrated that endothelial cells (ECs) from the liver (liver parenchymal ECs or LPECs), the most common site of CRC metastases, secrete soluble factors in the conditioned medium (CM) that, in turn, increase the cancer stem cell phenotype in CRC cells. However, the paracrine effects of LPECs on other CRC cellular functions have not been investigated. Here, results showed that CM from LPECs increased cell growth and chemoresistance by activating AKT in CRC cells *in vitro*. Using an unbiased Receptor Tyrosine Kinase (RTK) array, it was determined that human epidermal growth factor receptor 3 (ERBB3/HER3) was activated by CM from LPECs and it mediated AKT activation, cell growth and chemoresistance in CRC cells. Inhibition of HER3, either by an inhibitor AZD8931 or an antibody MM-121, blocked LPEC-induced HER3-AKT activation and cell survival in CRC cells. In addition, CM from LPECs increased *in vivo* tumor growth in a xenograft mouse model. Furthermore, inhibiting HER3 with AZD8931 significantly blocked tumor growth induced by EC CM. These results demonstrated a paracrine role of liver ECs in promoting cell growth and chemoresistance via activating HER3-AKT in CRC cells.

Implications: This study suggested a potential of treating patients with metastatic colorectal cancer with HER3 antibodies/inhibitors that are currently being assessed in clinical trials for various cancer types.

INTRODUCTION

Colorectal cancer (CRC) remains the second-leading cause of cancer-related death in the United States. Patients with early stage CRC (stages I-III) have 5-year survival rates between 53%-92% (1), of which the cancer is potentially curable by surgical resection and adjuvant therapy when appropriate. However, in patients with metastatic CRC (mCRC), the 5-year survival rate is <14% (1,2). More than 40% of these patients do not respond to systemic therapy (3), and those who respond to first-line therapy are likely to develop drug resistance within 1 year of treatment (4). Therefore, a better understanding of the regulation of CRC cell survival pathways is necessary in the development of new therapeutic strategies that will improve outcomes for patients with mCRC.

The effects of the microenvironment on cancer cell functions have been studied extensively. In the past decade, preclinical studies from several groups demonstrated that endothelial cells (ECs) promote cancer cell survival (including cell growth and chemoresistance) by secreting soluble factors in a paracrine fashion in glioblastoma (5), lung cancer (6) and other cancer types (7-9). Results from those studies showed that soluble factors secreted from ECs activated “cancer-promoting” signaling pathways such as AKT, NFκB, and epithelial-mesenchymal transition (EMT) pathways. In the past few years, our laboratory has isolated primary ECs from non-malignant liver and established an *in vitro* model using conditioned medium (CM) from these primary ECs to study their effects on CRC cells. With this model, we previously demonstrated that ECs secrete soluble factors in CM that, in turn, increase the cancer stem cell (CSC) phenotype in CRC cells in a paracrine fashion (10,11). In these prior studies, we

showed that incubation of CM from liver ECs activated CSC-associated pathways (such as NOTCH and NANOG) and induced CSC-associated functions (including sphere formation, resistance to chemotherapy, and potential to metastasize) in CRC cell. These findings suggested that inhibiting NOTCH and NANOG can be potential therapeutic strategies for treating patients with mCRC. However, clinical trials for NOTCH- or NANOG-targeted therapies did not deliver an impact in the clinic. Our unpublished data from unbiased cytokine array assay, together with studies of ECs in other cancer types mentioned above, suggest that ECs secrete a large number of factors and can activate a variety of pathways in adjacent cancer cells. Therefore, the EC-induced chemoresistance in CRC cells is likely to be mediated via multiple signaling pathways in addition to NOTCH and NANOG.

The aims of the current study were to 1) elucidate the paracrine role of liver ECs in mediating CRC cell growth, 2) validate the roles of liver ECs in mediating CRC cell chemoresistance, and 3) determine the mechanism involved. We demonstrated that CM from liver ECs significantly increased CRC cell growth and chemoresistance, and activated the AKT pathway in CRC cells *in vitro*. We then showed that human epidermal growth factor receptor 3 (HER3, also known as ERBB3) mediated the EC CM-induced cell survival and AKT activation in CRC cells. Furthermore, we used a proof-of-principle subcutaneous (subQ) xenograft tumor model to validate that CM from liver ECs promotes CRC tumor growth *in vivo* and inhibiting HER3, by the HER3 inhibitor AZD8931, blocked the EC CM-induced tumor growth. These findings demonstrated a

paracrine role of ECs in promoting cell growth and chemoresistance via activating the HER3-AKT signaling axis in CRC cells.

MATERIALS AND METHODS

Cell culture

The colorectal cancer (CRC) cell lines SW480, HT29, HCT116, RKO, SW48 and Caco2 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The Human CRC Primary cell line (HCP-1), luciferase-labeled HCP-1 cells, Liver Parenchymal Endothelial Cell (LPEC-1 and LPEC-6) lines, ECs from lung (lung ECs), and ECs from colon mucosa (colon ECs) were established in our laboratory (10,11). CRC cells were cultured in MEM supplemented with 5% FBS (Atlanta Biologicals, Atlanta, GA), vitamins (1x), nonessential amino acids (1x), penicillin-streptomycin antibiotics (1 x), sodium pyruvate (1x), and L-glutamine (1x), all from Invitrogen (Carlsbad, CA). ECs were cultured in Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany) supplemented with 10% human serum (Atlanta Biologicals). All cell lines were used within 10 passages, with approximately ~1 week per each massage. Authentication for all cell lines were done in every 6 months by short tandem repeat (STR) test at the Characterized Cell Line Core Facility at M.D. Anderson Cancer Center (Table 1 in Supplementary Data). For primary cell lines (HCP-1 and ECs) established in our laboratory, genomic DNA of the original tissues were used for authentication. For cell lines from ATCC, STR profiles of cell lines cultured in our laboratory were compared with the public CCSG Core Shared Resources database. Also, all cell lines were mycoplasma-free.

Reagents

HER3 inhibitor AZD8931 and MET inhibitor PHA-66752 were obtained from Selleck Chemicals (Houston, TX) for *in vitro* assays. For *in vivo* studies, large quantity of AZD8931 was obtained from MedChem Express (Monmouth Junction, NJ). Humanized HER3 antibody MM-121 was provided by Merrimack Pharmaceuticals (Cambridge, MA). Recombinant human neuregulin-1β was obtained from BioVision (Milpitas, CA). 5-fluorouracil (5-FU) and cetuximab were obtained from the pharmacy at The University of Texas MD Anderson Cancer Center. Human *ERBB3* (HER3) specific siRNAs (si-3: 5'-GCUGAGAACCAAUACCAGA, si-4: 5'-CCAAGGGCCCAAUCUACAA) and a validated control siRNA were obtained from Sigma-Aldrich (St. Louis, MO).

Conditioned medium (CM)

Equal numbers of CRC cells or ECs were seeded on culture plate. Cells were washed two times with 1X PBS and then cultured in growth medium with 1% FBS ($0.1x10^6$ cells/ml) for 48 hours. CM was harvested and centrifuged at 4,000 *g* for 5 minutes to remove cell debris. CM from each CRC cell line was used as controls.

Western blotting

Cell lysates were processed and run by SDS-PAGE gel electrophoresis as described previously (12,13). Antibodies used to detect α-tubulin, and HRP conjugated β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were from Cell Signaling (Beverly, MA). For each experiment, protein lysates were loaded to 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 α-tubulin

or β-actin as loading controls and a representative image was shown for each experiment. Each Western blot figure shows representative results of at least three independent experiments.

siRNA transfection

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

MTT assay

CRC cells were seeded at 3,000 cells/well in 96-well plates, cultured in 1% FBS overnight and then treated with CM for the indicated times. When the HER3 inhibitor AZD8931 (2 μM), HER3 antibody MM-121 (125 μg/ml), or 5-FU (2 μg/ml) were used, cells were pretreated with AZD8931 or MM-121in 1% FBS medium overnight, and then cultured with or without 5-FU and AZD8931 or MM-121 in CM for 72 hours. Cell viability was assessed by adding MTT substrate (0.25% in PBS, Sigma) in growth medium (1:5 dilution) to cells for 1 hour at 37 °C. Cells were washed with PBS and 50 μl DMSO was added. Optical density was measured at 570 nm and relative MTT was presented as % of control.

Cell apoptosis

CRC cells were cultured in 1% FBS overnight and then cultured with or without 5-FU with CM for 48 hours (HCP-1 cells) or 72 hours (HT29 and SW480). When the HER3

inhibitor AZD8931 was used, cells were pretreated with AZD8931 in 1% FBS medium overnight, and then cultured with or without 5-FU and AZD8931 in CM. Cell apoptosis was determined using the FITC Annexin V Apoptosis Detection Kit I from BD Biosciences (San Jose, CA) as described before (10). In brief, single suspended cells were double-stained with FITC Annexin V and propidium iodide and analyzed by fluorescence-activated cell sorting. Double-positive cells were counted as apoptotic cells and presented as a % of the total population.

Receptor tyrosine kinase (RTK) array

RTK array kit was purchased from R&D Systems (Minneapolis, NE) and the assay was performed according to the manufacturer's instructions. In brief, 0.5 x10^6 HCP-1 cells were incubated in 1% FBA medium overnight, and then treated with control or LPEC-1 CM for 30 minutes. Cell lysates were prepared with lysis buffer from the kit and 300 μg total proteins from each group were loaded to the membranes. Intensities of spots for P-EGFR, P-MET, and P-HER3 on the same film were measured and compared with the reference spots by ImageJ version 1.47.

Co-immunoprecipitation (co-IP) pull-down assay

HCP-1 cells were treated with either recombinant human neuregulin-1β in 1% FBS medium or CM for 30 minutes and then harvested for cell lysates with RIPA buffer in 10% glycerol. For each IP, 0.2 μg of HER2- (Cell Signaling #2165) or HER3- (Cell Signaling #12708) specific antibodies were added to 200 μl of 100 μg total proteins. After rocking at 4 °C for 2 hours, a 30 μl mixture of protein A/G PLUS-Agarose beads in buffer (Santa Cruz #SC-2003) was added to each IP and rocked at 4°C for 1 hour. Beads with pulled-

down proteins were directly boiled in 2x SDS-PAGE buffer for Western blotting. Also, 100 μl protein lysates before IP from each group were processed for Western blotting.

In vivo tumor growth

HCP-1 cells labeled with a CMV-driven luciferase reporter were pretreated with CM for 24 hours and then suspended in an inoculation matrix (1:1 mix of growth-factor-reduced Matrigel and concentrated HCP-1 or LPEC-1 CM) and injected subcutaneously into the right flanks of athymic/nude mice ($1x10^6$ cells in 100 μl/injection, n=10 mice/group). After injection, tumor burden was assessed by bioluminescence with the *In Vivo* Imaging System (IVIS) and D-Luciferin substrate (Xenogen, Alameda, CA) according to the manufacturer's instructions. Tumor volumes were measured with a caliper. Owning to Hurricane Harvey, we could measure bioluminescence with IVIS only on Day 4 and Day 15, and we measured tumor volumes with a caliper on Day 4, Day 11, and Day 15 after injection. AZD8931 was suspended in 1% (v/v) solution of polyoxyethylenesorbitan monooleate (Tween 80) in deionized water and gavaged once daily from Day 1 at 25mg/kg in 100μl per mouse, with 1% Tween 80 only for control groups. All mice were euthanized when 3 mice from any group had tumor size reached 1,000mm^3, and tumors were harvested for weighing. An IACUC protocol for this study was approved by the UT M.D. Anderson Cancer Center.

Statistical analysis

For *in vitro* assays, all quantitative data were reproduced in at least three independent experiments with multiple measures in each replicate. Groups were compared by two-tailed Student's t-test and data was expressed as means -/+ standard error of the mean

(S.E.M.) with significance of P<0.05. For *in vivo* assays, Wilcoxon rank-sum test was used for tumor volume and burden change over time, and one-way ANOVA was used for comparing tumor weight and size between groups after tumors were harvested. Data was expressed as means -/+ standard deviation (SD) with significance of P<0.05.

RESULTS

CM from liver ECs increased CRC cell survival and activated the AKT pathway

To determine the effects of ECs on CRC cell survival (including cell viability and chemoresistance), we used primary liver EC lines (LPEC-1 and LPEC-6) that our laboratory established from non-malignant liver tissues (10). CM containing EC-secreted factors was harvested and added to CRC cells (HCP-1, HT29 and SW480), with CM from CRC cells themselves serving as control CM. Our preliminary study determined that the proliferation rate of CRC cells and ECs are similar with doubling times of 48-72 hours. This finding suggests that CRC cells and ECs have similar metabolism rates, resulting in similar consumption of the nutrient in 1% FBS culture medium and should have similar amounts of nutrients remaining in the CM. Therefore, CM from CRC cells and ECs are expected to be different only in the contents of paracrine factors secreted by CRC cells vs ECs.

Compared with control CM, CM from LPECs significantly increased the relative number of viable CRC cells over time, leading to ~2-fold (HCP-1 and SW480) and ~4-fold increases (HT29) in cell viability at Day 4 (Fig. 1 A, C, E). Chemoresistance was assessed by measuring CRC cell apoptosis induced by 5-fluorouracil (5-FU, a widely

used chemotherapy for treating patients with CRC and other types of cancer). Fluorescence-activated cell sorting (FACS) with FITC Annexin V and propidium iodide double staining were used to measure CRC cell apoptosis. Cells were treated with 5-FU (2 μg/ml, a clinically relevant dose (14)) in either CRC or LPEC CM. When CRC cells were incubated in CRC CM, cell apoptosis was significantly increased in cells treated by 5-FU compared to those treated with solvent, as expected. However, 5-FU-induced apoptosis was significantly less in CRC cells incubated in LPEC CM compared to those in CRC CM (Fig. 1 B, D, F). These findings suggest that liver EC CM decreased 5-FU-induced apoptosis, therefore, increased chemoresistance in CRC cells.

After demonstrating that CM from ECs promoted CRC cell survival, we sought to determine whether the increase in cell survival was mediated by the known survival pathway, AKT. Compared with CM from CRC cells, CM from LPECs increased phosphorylation of AKT and several downstream targets of AKT (P70 (15) and S6 (16) kinases, eIF4B (17), and eEF2K (18)) in CRC cells (Fig. 2).

CM of ECs activated HER3 pathways in CRC cells

In order to activate the intracellular AKT pathway by the extracellular stimulation of EC CM, there is likely to be a membrane-bound receptor with kinase activity to initiate downstream signaling. Using a receptor tyrosine kinase (RTK) array we detected high levels of phosphorylation of EFGR, MET and HER3 (P-EFGR, P-MET, and P-HER3 respectively) in CRC cells, whereas there were low or undetectable levels of phosphorylation for other RTKs on the membrane (Fig. 3A). Among these three RTKs,

only P-HER3 levels were dramatically increased in CRC cells treated by LPEC-1 CM. Measurements of intensities of the spots for these three RKTs showed that only P-HER3 was significantly increased in LPEC-1 CM-treated CRC cells compared to CRC CM-treated cells (Fig. 3B). We detected a ~20% increase in the intensity of P-EGFR but the difference was not significant on statistical analysis. Also, MET was highly phosphorylated in cells from both groups and measurements from a film with much lighter exposure suggested that the P-MET levels were not altered by LPEC-1 CM. We then used Western blotting to validate that liver EC CM dramatically increased HER3 and AKT phosphorylation without affecting EGFR phosphorylation in CRC cells (Fig. 3C). We used additional CRC cell lines (HCT116, RKO, SW48, and Caco2) and additional primary EC lines from different organs (lung and colon mucosa) to confirm that CM from different EC lines activated HER3 and AKT and promoted cell growth in multiple CRC cell lines (Suppl. Fig. 1 and 2).

Furthermore, we validated that MET phosphorylation was not altered by EC CM, and demonstrated that blockade of MET (by PHA-66752) or EGFR (by cetuximab) did not affect HER3 and AKT phosphorylation in CRC cells and did not block LPEC-1 CM induced phosphorylation of HER3 and AKT (Suppl. Fig. 3).

We did not detect HER2 phosphorylation in the RTK array, and subsequent Western blotting confirmed that there was no detectable HER2 phosphorylation in CRC cells after LPEC-1 CM treatment. Moreover, we demonstrated that even though the canonical HER2-HER3 dimerization and phosphorylation of HER2 in CRC cells could

be induced by recombinant human neuregulin-1β (a well-characterized HER3 ligand (19)) (Suppl. Fig. 4A—C), LPEC-1 CM could induce HER3 phosphorylation in CRC cells in absence of detectable HER2 phosphorylation, or HER3 dimerization with either EGFR, HER2 or MET (Suppl. Fig. 4D—F). Also, we conducted dimerization assay with non-reducing Western blotting and confirmed that HER3-HER3 homo-dimmers were not detected in EC CM treated cells. These findings confirmed that EC CM-induced HER3 activation is independent of the canonical HER2-HER3 dimerization. In addition, we did not detect HER4 expression in CRC cells by Western blotting. These findings suggest that HER3 is likely to mediate EC CM-induced AKT activation and CRC cell survival and is independent of MET and other HER receptors (EGFR, HER2 and HER4).

HER3 mediated liver EC CM-induced AKT activation and cell survival in CRC cells

To determine whether HER3 mediated EC CM-induced AKT activation and cell survival, we used HER3 specific siRNAs to show that HER3 knockdown blocked AKT phosphorylation induced by LPEC-1 CM, and significantly inhibited EC CM-induced cell viability in CRC cells (Fig. 4A, B). Similar effects were observed with LPEC-6 CM. When we sought to determine the effects of knocking down HER3 by siRNAs on CRC cell apoptosis, we noted that transient transfection of either control or HER3-specific siRNAs dramatically increased apoptosis in CRC cells (up to 40%, compared to 1.5%-8% in un-transfected CRC cells) and treating siRNA-transfected cells with 5-FU did not further increase apoptosis. Therefore, we could not determine the effects of EC CM on 5-FU-induced cell apoptosis when CRC cells were transfected with siRNAs. As an alternative, the pan-HER inhibitor AZD8931 was used to block HER3 in CRC cells. Because we

determined that other HER receptors were not involved in EC CM activation of HER3 in CRC cells, the effects of the pan-HER inhibitor on EC CM-induced cell survival were mainly due to HER3 inhibition. AZD8931 nearly complete inhibition of HER3 phosphorylation in CRC cells (Fig. 4C).

Moreover, we used MTT assay to determine the effects of AZD8931 on CRC cell viability when cultured with LPEC CM and 5-FU (Fig 5A, C, E). The relative numbers of viable CRC cells were significantly increased by LPEC-1 CM compared to those with CRC CM, as expected. 5-FU or AZD8931 single agent treatments decreased CRC cell viability in both CRC CM and LPEC-1 CM. When CRC cells were treated in combination of 5-FU and AZD8931, the relative numbers of viable cells were as low as half of that from single agent treatment. We then used AZD8931 to determine the effects of blocking HER3 on chemoresistance in CRC cells (Fig. 5B, D, F). AZD8931 alone led to an insignificant change in apoptosis in CRC cells incubated in either CRC or LPEC-1 CM. 5-FU alone induced CRC cell apoptosis but to a less extent in LPEC-1 CM than in CRC CM. In contrast, levels of apoptosis were significantly higher in cells treated with 5-FU and AZD8931 than in those with 5-FU alone, even in cells incubated with LPEC-1 CM. This data suggest that inhibiting HER3 by AZD8931 blocked LPEC-1 CM-induced chemoresistance in CRC cells.

We further validated our findings with a HER3-specific humanized antibody, MM-121, that is currently in clinical trials for treating several types of cancers (20,21). Like AZD8931, MM-121 also blocked LPEC-1 CM-induced HER3 and AKT phosphorylation

in CRC cells (Suppl. Fig. 5A), and blocked LPEC-1 CM-induced CRC cell viability and chemoresistance assessed by MTT assay (Suppl. Fig. 5B—D). These findings validated that blockade of HER3 sensitized CRC cells to 5-FU, suggesting that inhibition of HER3 blocked LPEC-1 CM-induced chemoresistance.

HER3 inhibition blocked EC CM-induced CRC tumor *in vivo*

In order to validate the effects of EC CM on CRC cell growth *in vivo*, we used a proof-of-principle subQ xenograft tumor model with Luciferase-labeled HCP-1 cells. HCP-1 cells were pretreated with either CRC or LPEC-1 CM and then injected subQ in an inoculation mixture of concentrated CM and Matrigel. As a result, HCP-1 tumors injected with LPEC-1 CM had significantly greater tumor burden and volume over time (Suppl. Fig. 6A—C) compared to the control group injected with CRC CM. After tumors were harvested, HCP-1 tumors treated with LPEC-1 CM were significantly larger and weighed more than those treated with HCP-1 control CM (Suppl. Fig. 6D—F).

The effects of blocking HER3 on CRC tumor growth were further determined by the subQ xenograft tumor model with treatment of the HER3 inhibitor AZD8931. After HCP-1 cells were injected subQ in the mixture of CM and Matrigel as described above, mice were then treated with either vehicle or AZD8931 by gavage and the tumor growth was monitored over time (Fig. 6). Our results showed that LPEC-1 CM treated tumors led to significantly greater tumor growth, as expected. More importantly, AZD8931 significantly inhibited the tumor growth in both CRC CM and LPEC-1 CM-treated CRC tumors compared with tumors without AZD8931 treatment (Fig. 6B). In addition, CRC tumors

that were treated by AZD8931 had significantly lower tumor weight compared with tumors not treated with AZD8931, leading to ~2-fold decrease compared to CRC CM treated tumors, and > 4-fold decrease compared to LPEC-1 CM treated tumors (Fig. 6C).

DISCUSSION

Roles of the microenvironment in mediating tumor progression and responses to therapy have been discussed in depth in many types of cancers (22,23). This study sought to elucidate the role of ECs in mediating CRC cell growth and chemoresistance and determine the signaling pathway(s) involved using ECs from liver tissues to represent the liver EC microenvironment. We demonstrated that liver ECs promoted CRC cell growth and chemoresistance via activating AKT in a paracrine fashion. Furthermore, we determined that HER3 mediates the EC CM-induction of AKT activation and CRC cell survival.

Our previous studies reported that CM from ECs increased the CSC phenotype by activating NOTCH and NANOG pathways in CRC cells (10,11). As a result, chemoresistance, as one of the CSC-associated features, was increased in CRC cells. Like many cellular functions in cancer cells, chemoresistance is regulated by a network of extracellular and intracellular signaling pathways. In the present study, we validated our previous finding that EC CM promotes CRC cell chemoresistance using additional data from apoptosis assays with FACS and MTT assays. Furthermore, we identified another mechanism of EC CM promoting chemoresistance by activating the HER3-AKT

signaling axis in CRC cells. Since ECs secrete a large number of soluble factors and can affect diverse signaling pathways (24,25), our findings suggest that chemoresistance and potentially other cellular functions in CRC cells are mediated by various pathways that are independently triggered by different soluble factors secreted by ECs.

The role of HER3 in cancer cells has been studied primarily in breast cancer, gastric cancer, and a few other cancer types (26,27). HER3 has an extracellular domain for ligands binding and an intracellular domain with weak kinase activity (28). Therefore, even though it is a member of the HER family, HER3 is often considered as a kinase-dead RTK (29). As a result, upon binding of the ligand neuregulin (also called heregulin) to HER3, hetero-dimerization of HER3 and other receptors with kinase activity, most often with HER2 and to a less extent with other HER family receptors (30). This dimerization activates the coupled HER receptor, and the activated HER receptor then leads to HER3 trans-phosphorylation and activation (31,32). In many types of cancers, HER3 phosphorylation activates downstream targets, including AKT and other cell survival pathways (33). However, the role and regulatory mechanisms of HER3 in CRC cells remain unclear. Preclinical studies reported that HER3 is expressed in more than 75% of CRC primary and metastatic tumors (34,35), and its overexpression mediates CRC cell resistance to anti-EGFR therapy (36-38).

In this study, we found that the canonical neuregulin-triggered HER3 activation by other HER receptors does not occur in CRC cells treated with LPEC CM. We did not detect

HER2 phosphorylation, the most common mediator of neuregulin-triggered HER3 activation (39,40), when CRC cells were treated with EC CM. Even though strong EGFR and MET phosphorylation were detected and these two receptors have been reported to mediate HER3 activation (41,42), inhibition of these two receptors did not block EC CM-induced HER3 and AKT activation in CRC cells. Also, there was no detectable HER4 in CRC cells. Our results demonstrated a mechanism of HER3 activation that is independent of the known HER3 ligand neuregulin and does not involve activation of other HER receptors and MET. Studies in breast cancer showed that when EGFR or HER2 was inhibited by RTK inhibitors, HER3 overexpression and changes in cellular localization and phosphorylation of HER3 compensated for the EGFR/HER2 inhibition and led to activation of downstream survival pathways (such as AKT) (43,44). Together with these studies, our findings suggest that EC CM may trigger HER3 to dimerize with co-factor(s) that have not yet been identified to promote downstream AKT activation and cell survival in CRC cells. The identification of the soluble factors secreted by ECs that mediate HER3 activation and the possible co-factor(s) that interact with HER3 upon activation is currently under study.

In summary, our results demonstrated a paracrine role of liver ECs in promoting cell growth and chemoresistance via activating HER3-AKT in CRC cells. Also, we showed that blocking HER3 activity, with the HER3 inhibitor AZD8931, inhibited CRC tumor growth *in vivo*. This study suggests a potential strategy of treating mCRC patients with HER3 antibodies/inhibitors, including AZD8931 and MM-121 we used, that are already being assessed in clinical trials for various cancer types (45,46).

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FIGURE LEGENDS

Figure 1. CM of liver ECs increased CRC cell viability and chemoresistance.

CRC cells were cultured with control (CRC) or liver EC (LPEC-1 and LPEC-6) CM. **(A, C, E)** MTT assay showed that EC CM increased cell viability in CRC cells. **(B, D, F)** CRC cells were treated without (solvent) or with 5-FU in CM. The number of apoptotic cells was determined by FACS with Annexin V and propidium iodide double staining and were presented as a percentage of the total cells. Mean +/- SEM of 3 experiments, *p<0.05 t-test.

Figure 2. CM of liver ECs activated the AKT pathway in CRC cells.

CRC cells were treated with control (CRC) or liver ECs (LPEC-1 or LPEC-6) CM for 30 minutes. Western blotting showed increased levels of **(A)** AKT phosphorylation and **(B)** phosphorylation of AKT downstream targets when cells were incubated in EC CM. α-tubulin was used as the loading control. Data represents results of 3 experiments.

Figure 3. CM of ECs increased HER3 phosphorylation in CRC cells.

HCP-1 cells were treated with CM for 30 minutes. **(A)** RTK array showed phosphorylation of RTKs in HCP-1 CM or LPEC-1 CM treated CRC cells, with marked EGFR (P-EGFR), MET (P-MET), and HER3 (P-HER3). **(B)** Intensities of P-EGFR and P-HER3 spots on the membrane were measured and presented relative to the reference spots in the corners. Mean +/- SEM of 3 experiments, *p<0.05 t-test. **(C)** Western blotting showed that CM from ECs (LPEC-1 and LPEC-6) increased HER3 and

AKT phosphorylation, but not EGFR phosphorylation. β-actin was used as the loading control. Data represents results of 3 experiments.

Figure 4. HER3 blockade inhibited LPEC-1 CM-induced AKT activation and cell viability in CRC cells.

CRC cells were transfected with control (si-Ctrl) or HER3-specific (si-3 and si-4) siRNAs and treated with control (CRC) or LPEC-1 CM. **(A)** Western blotting showed that HER3 siRNAs decreased the protein levels of HER3, and blocked LPEC-1 CM induced HER3 and AKT phosphorylation. β-actin was used as the loading control. **(B)** MTT assay showed that HER3 siRNAs blocked CRC cell viability induced by LPEC-1 CM. CRC cells were transfected with control (si-Ctrl) or HER3 specific siRNAs (si-3 and si-4) and then treated with control (CRC) or LPEC-1 CM. Mean +/- SEM of 3 experiments, *$p<0.05$ t-test. **(C)** Western blotting showed that AZD8931 blocked LPEC-1 CM-induced HER3 and AKT phosphorylation. β-actin was used as the loading control. Data represents results of 3 experiments.

Figure 5. HER3 inhibitor AZD8931 blocked LPEC-1 CM-induced chemoresistance and cell viability in CRC cells.

CRC cells were pretreated with or without AZD8931 and then treated with or without 5-FU and AZD8931 in control (CRC) or LPEC-1 (L-1) CM. **(A, C, E)** Cell viability was determined by MTT assay. **(B, D, F)** The numbers of apoptotic cells were determined by FACS with Annexin V and propidium iodide double staining and were presented as a percentage of the total cells. Mean +/- SEM of 3 experiments, *$p<0.05$ t-test.

Figure 6. LPEC-1 CM increased CRC cell growth *in vivo*.

HCP-1 CRC cells were pretreated with control CM (CRC CM) or LPEC-1 CM for 24 hours and then injected subQ into athymic/nude mice in an inoculation matrix with either control or LPEC-1 CM. Mice were then gavaged once a day with either vehicle or the HER3 inhibitor AZD8931 starting from Day 1. **(A)** Tumors harvested on Day 13. **(B)** LPEC-1 CM treated tumors showed increased tumor volume after injection, and AZD8931 inhibited tumor growth. Mean +/- SD of 10 mice, *p<0.01 Wilcoxon rank-sum test for LPEC-1 CM treated group (black solid line) compared with CRC CM control group (black dash line), and for AZD8931 treated groups (red lines) compared with groups without AZD8931 (black lines). **(C)** Scatter plots show weights of tumors harvested on Day 13. Mean +/- SD, p <0.001 one-way ANOVA for LPEC-1 CM treated group compared with CRC CM control group, and for AZD8931 treated groups compared with first two groups without AZD8931.

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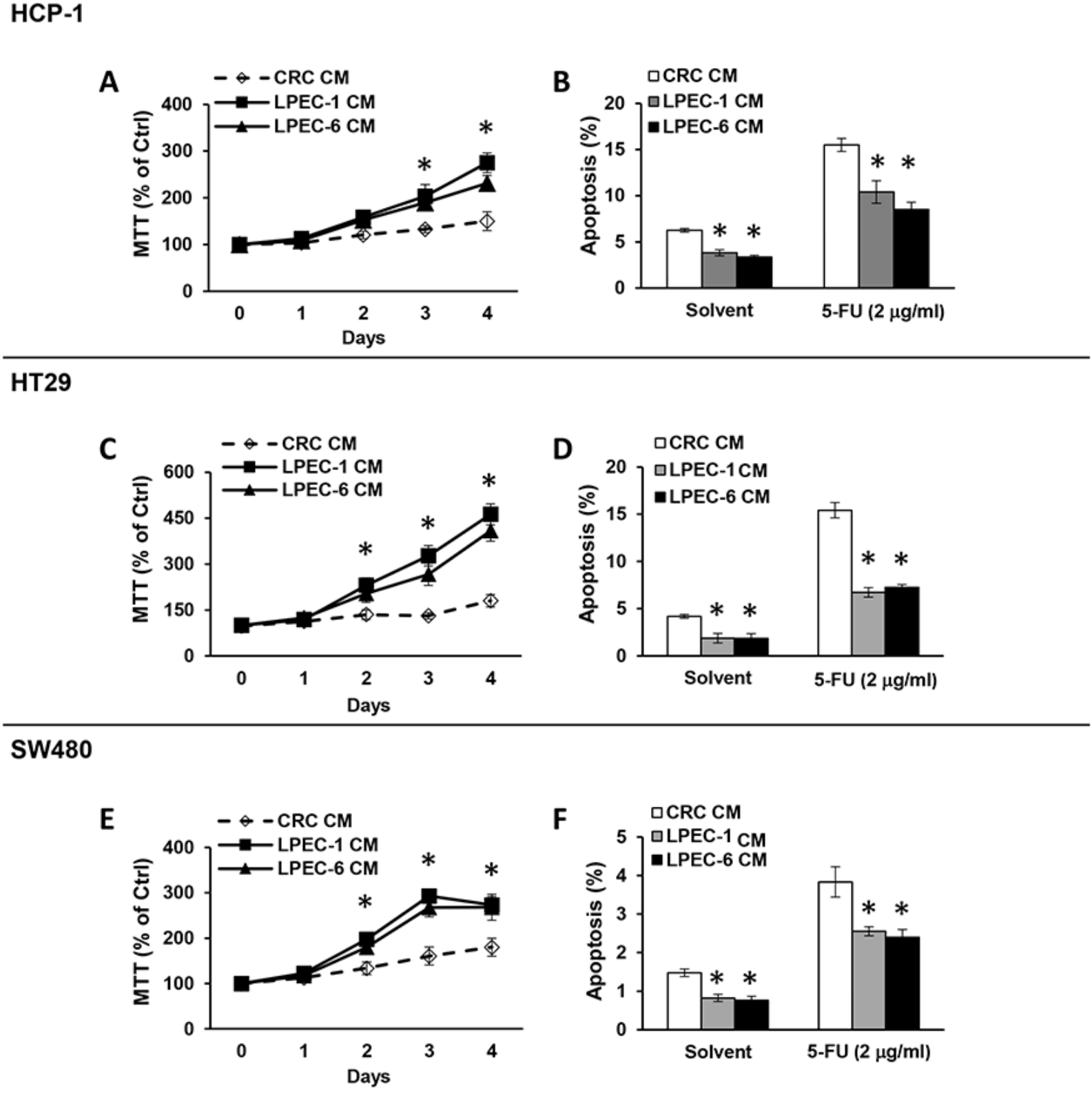

Figure 1

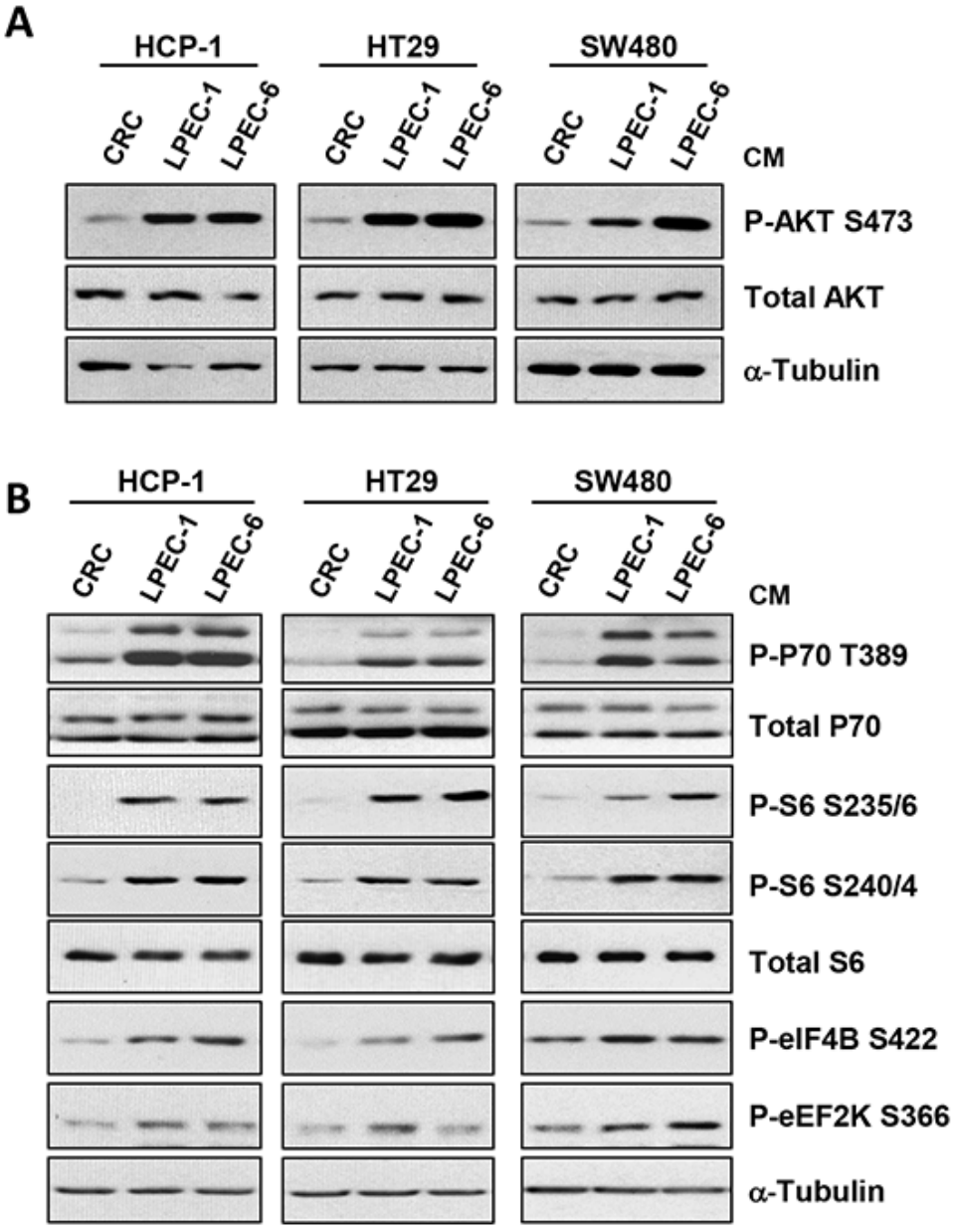

Figure 2

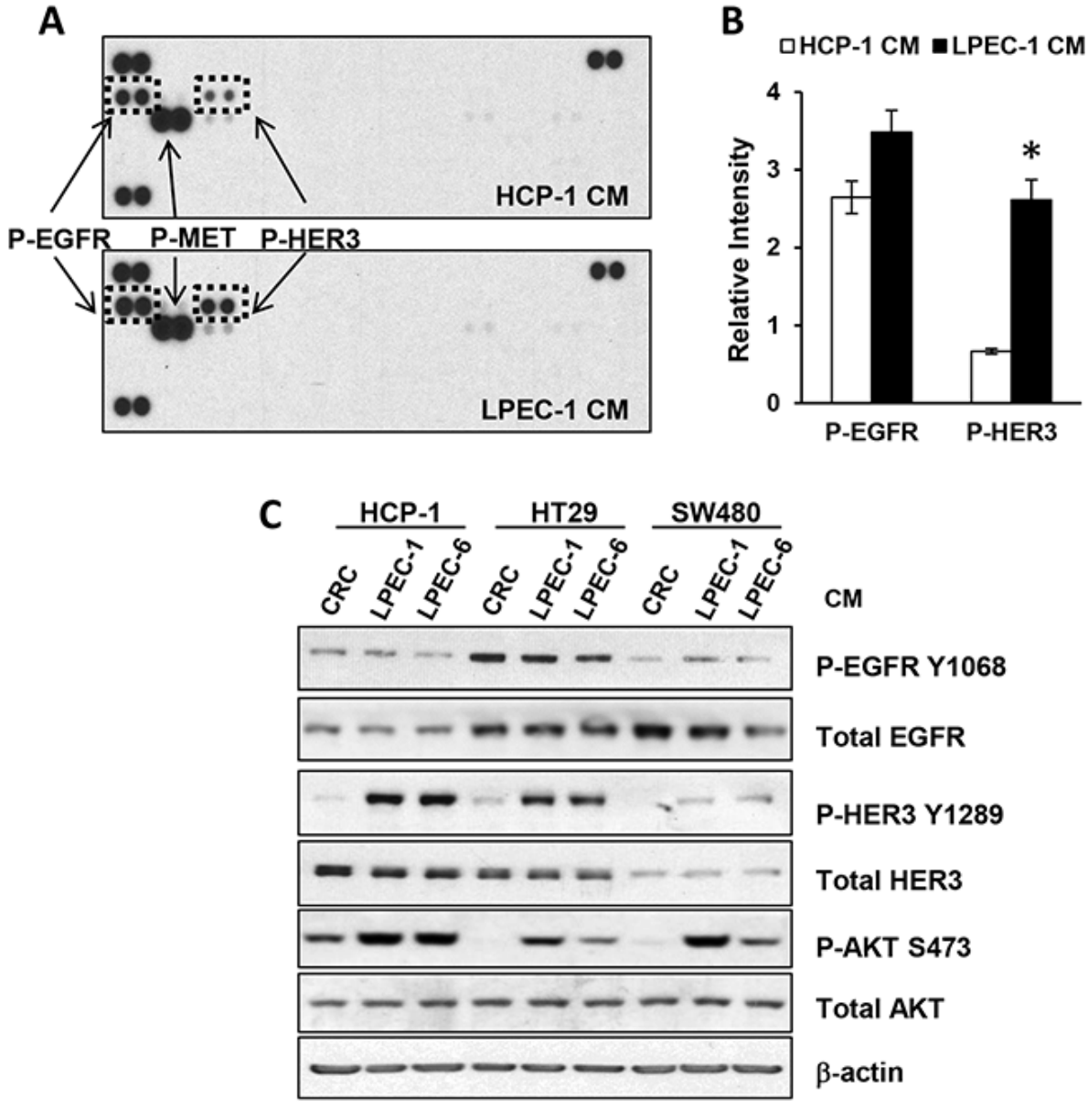

Figure 3

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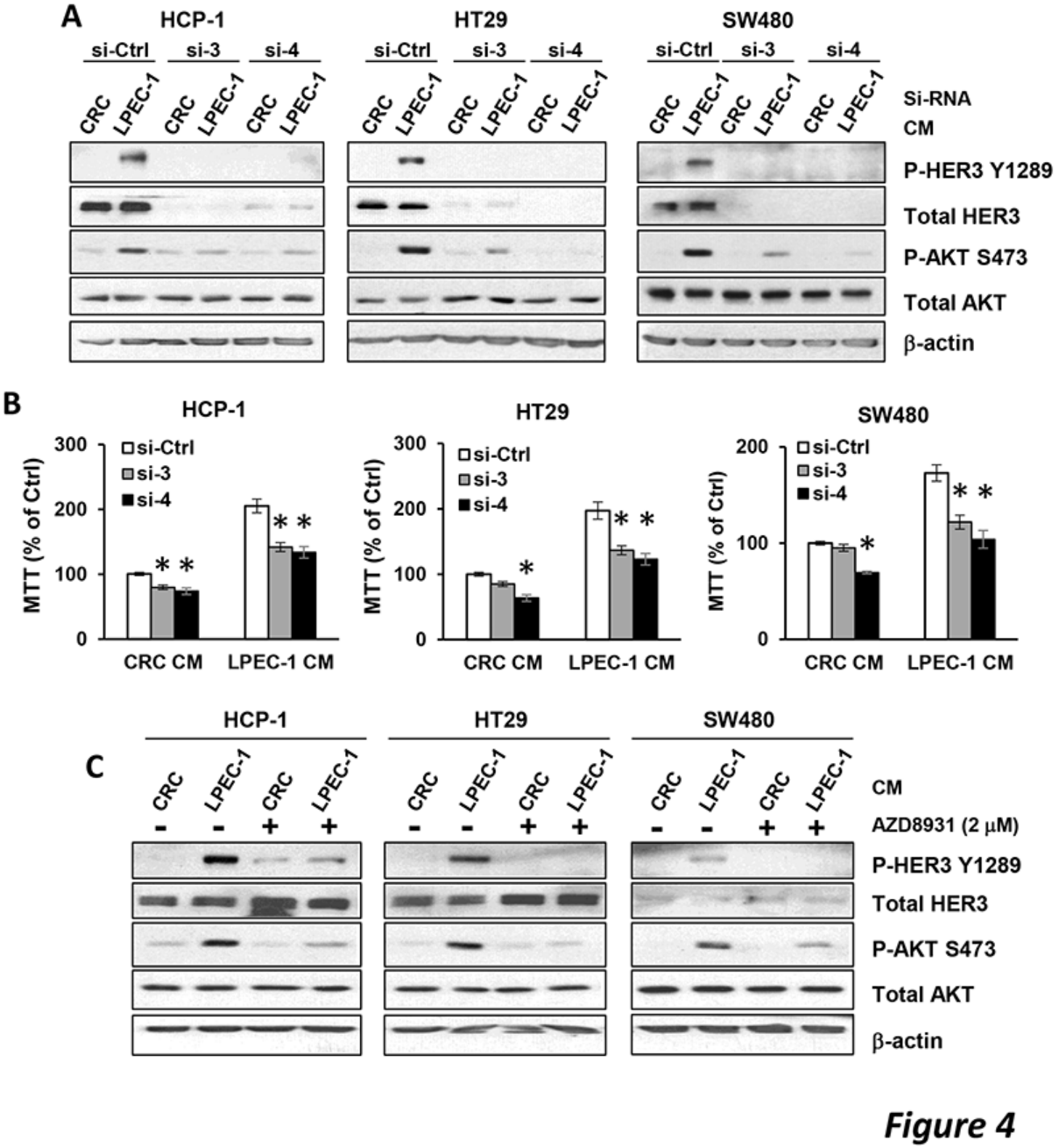

Figure 4

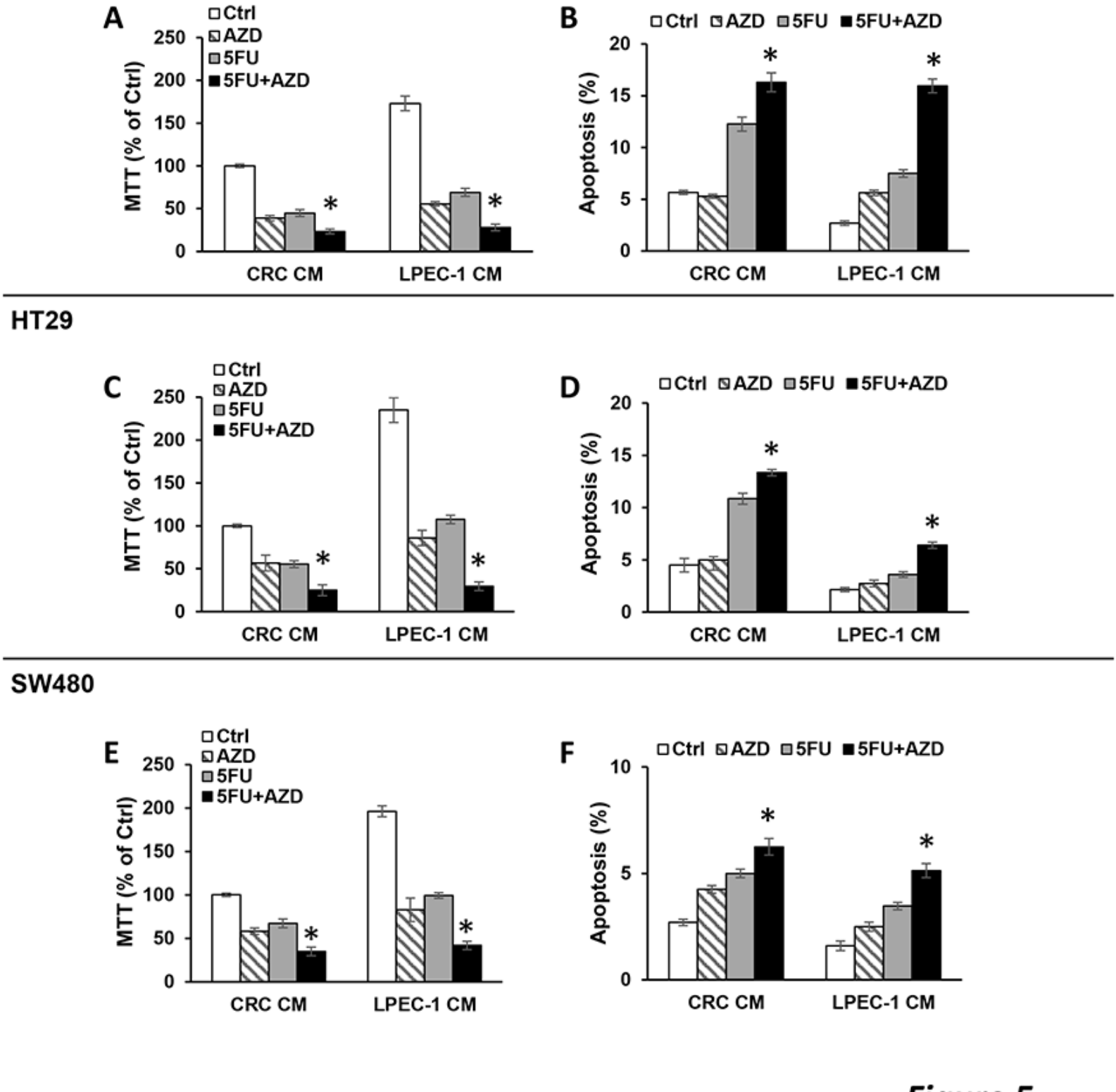

Figure 5

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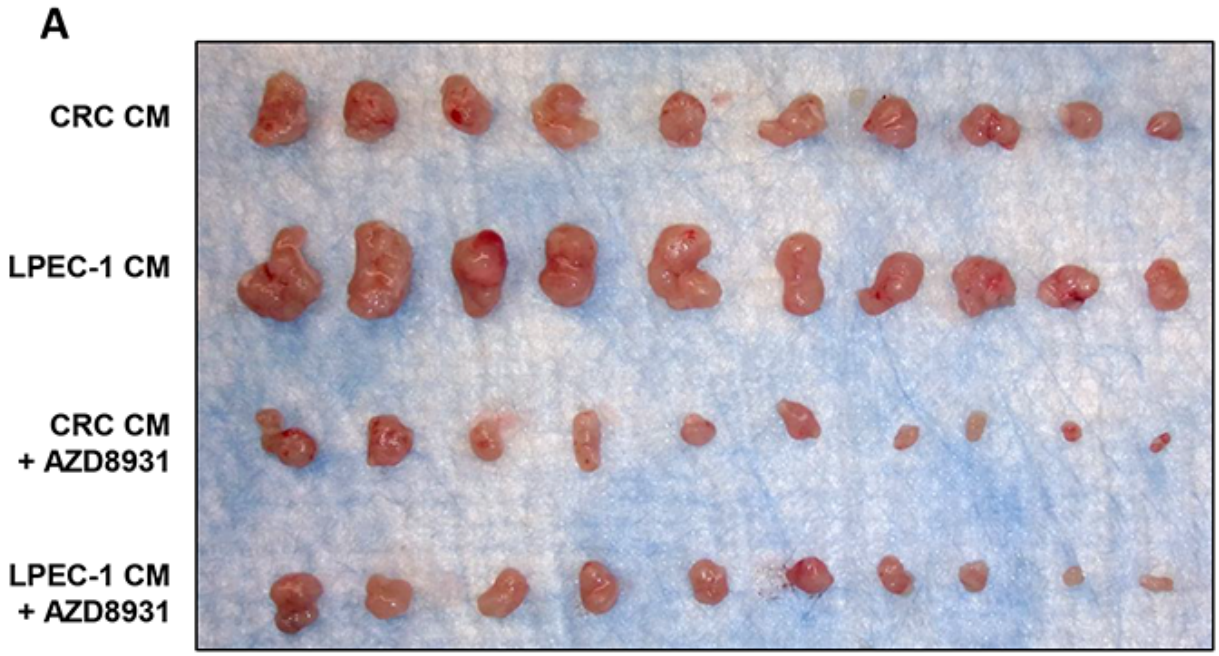

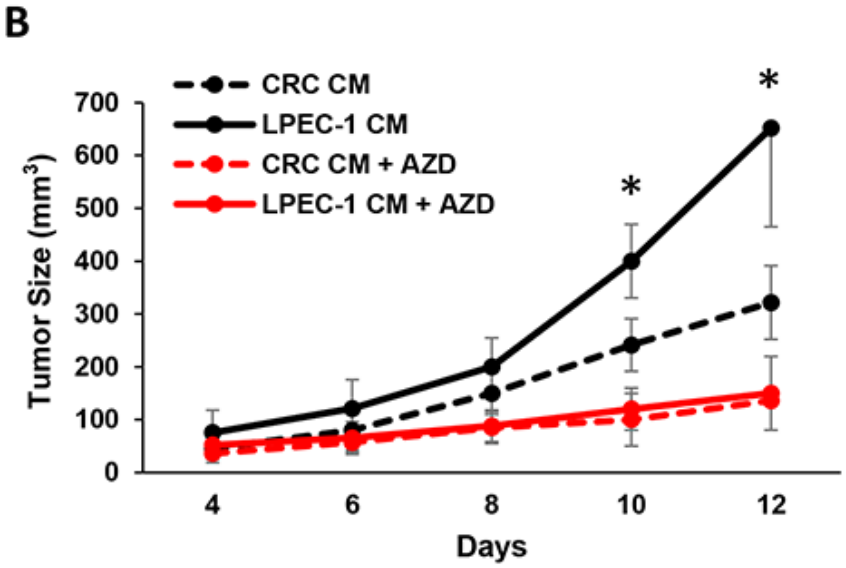

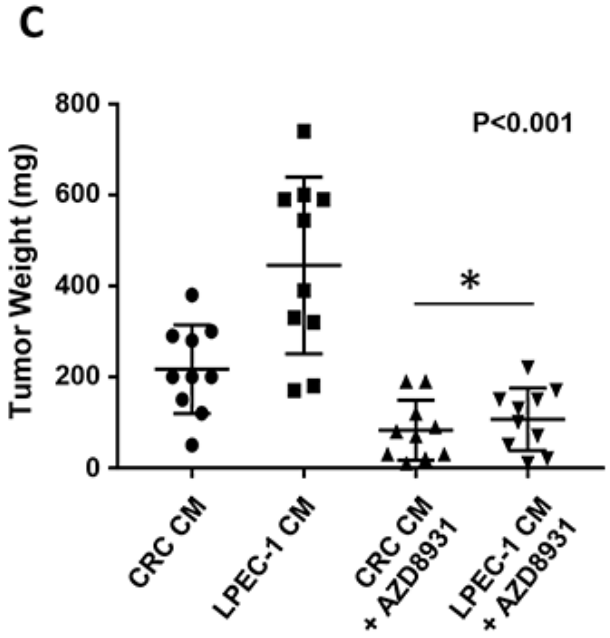

Figure 6

AACR American Association for Cancer Research

Molecular Cancer Research

Endothelial Cells Promote Colorectal Cancer Cell Survival by Activating the HER3-AKT Pathway in a Paracrine Fashion

Rui Wang, Rajat Bhattacharya, Xiangcang Ye, et al.

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