Liver Endothelium Microenvironment Promotes HER3-mediated Cell Growth in Pancreatic ductal adenocarcinoma

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Abstract

~90% metastatic PDAC (mPDAC) are found in the liver, and 5-year survival rate for patients with mPDAC is only at 3%. Our previous studies have shown that liver endothelial cells (ECs) secreted soluble factors to promote the survival of colon cancer cells in a paracrine fashion. However, the influence of the liver EC microenvironment on mPDAC growth and survival has not been elucidated. In this study, we investigate the paracrine effects of liver ECs on the survival and growth profiles of PDAC and elucidate the involved mechanism(s). Primary liver ECs were isolated from non-neoplastic liver tissues to mimic the liver EC microenvironment. Conditioned medium (CM) from liver ECs were collected and then applied to PDAC cells, with CM from PDAC as control CM. Compared to PDAC CM, liver EC CM promoted proliferation in varied PDAC cells. We found that human epidermal growth factor receptor 3 (HER3, also known asERBB3) was only expressed and activated in BxPC-3 and CaPan2 cells (HER3+ve), in which the HER3-AKT signaling pathway was activated by EC CM. Furthermore, blocking HER3 activation with a humanized HER3 antibody, seribantumab, significantly blocked EC CM-induced AKT activation and cell proliferation. Moreover, depletion of neuregulin (NRG) from EC CM attenuated HER3-AKT activation and indicated that EC-secreted NRG might play a role in promoting PDAC growth. Our results demonstrated that liver EC-secreted factors promoted PDAC growth *in vitro* and *in vivo*, and HER3 was expressed in a subset of PDAC cells and mediated EC-induced proliferation. Blocking HER3 activation inhibited PDAC growth and survival. Our findings provide a potential strategy for treating patients with HER3+ve mPDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common cancer-causing pancreatic tumor of death. PDAC morbidity and mortality are nearly equal, and surgical resection is the only treatment that prolongs survival and may be curative, but its prognosis is particularly poor (1). Even after potentially curative resection, about 70% of patients experience recurrence within 2 years, and the 5-year survival rate is only about 8% (2). Furthermore, there is little evidence to support surgical resection of metastatic PDAC, especially liver metastases (3). In a phase III randomized study, FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin) effectively improved overall survival (OS) (10.7-11.1 months) (4). Compared with gemcitabine, it has obvious advantages.

As the largest solid organ, the liver is one of the most susceptible organs for PDAC tumor cells to metastasize (5). Liver sinusoidal endothelial cells (LSEC) play a key role in liver physiology and pathology. Previous studies have found that increased interaction and adhesion of circulating tumor cells (CTCs) with LSEC in the local microenvironment and LSEC-induced immune tolerance promote cancer liver metastasis (6). At the same time, our past research also found that liver sinusoidal endothelial cells can secrete small molecules to promote the growth and drug resistance of liver metastatic cancer cells.

The erythrocytic leukemia virus oncogene (ErbB)/human epidermal receptor (HER) receptor tyrosine kinase (RTK) family, consisting of HER1 (EGFR), HER2, HER3 and HER4, is a key regulator of cell growth and differentiation (6). HER3 is distinguished from other ErbB family members by two properties. First, HER3 lacks a functional kinase domain. Second, HER3 is a potent inducer of the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT) pathway, binding to the PI3K p85 subunit through six consensus phosphotyrosine sites on its C-terminal tail (7). This activates a variety of signals, including the AKT signaling pathway, which is extremely relevant to cell growth and survival. Our past studies have found that conditioned medium from hepatic sinusoidal endothelial cells (LSEC) can effectively activate the HER3/AKT signaling pathway to promote the growth and drug resistance of mCRC cells (8). Nonetheless, the role of liver sinusoidal endothelial cells (LSEC) in mPDAC has not been understood. In this study, we hope to investigate how EC affects the hepatic microenvironment to influence the growth and survival of mPDAC, and to find its underlying mechanisms and potential therapeutic approaches

Methods

Cell culture

The established PDAC cell lines BxPC3, CAPAN2, PANC1 and MIA-PaCa2 were purchased from ATCC (Manassas, VA, USA). Human liver parenchymal primary ECs (EC9) lines were isolated and established in our laboratory using MACS microbead-conjugated antibodies and separation columns (Miltenyi Biotec,

Bergisch Gladbach, Germany). ant-CD31 was used for sorting of ECs. All PDAC cells were cultured in DMEM (Sigma-aldrich, St. Louis, MO, USA) supplemented with 5% FBS (Atlanta Biologicals, Atlanta, GA), vitamins (1x), nonessential amino acids (1x), penicillin-streptomycin antibiotics (1x), 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) supplemented with 10% human serum (Atlanta Biologicals) and antibiotics-antimycotic (1x, Thermo Fisher Scientific/Gibco). For primary cell lines ECs established in our laboratory, genomic DNA from the original tissues was used for authentication. For cell lines from the ATCC, STR profiles of cell lines cultured in our laboratory were compared with the public CCSG Core Shared Resources database at University of Texas M.D. Anderson Cancer Center. All cell lines were tested for mycoplasma contamination for every 6 months.

Conditioned medium

3 x 10^5 of PDAC cells or ECs were seeded in T25 culture flasks for two days, and then cells were washed two times with 1X PBS and then cultured in 3ml DMEM or EC Growth Medium MV2 with 1% FBS (0.1x10^6 cells/mL) for 48 hours. Conditioned medium (CM) was harvested and centrifuged at 4,000 g for 10 minutes to remove cell debris. CM from each PDAC cell line was used as controls.

RTK assay

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 ×10^6 PDAC cells were incubated in 1% FBA medium overnight, and then treated with control or EC1 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.

MTT assay

PDAC cells were seeded at 2,000 cells/well in 96-well plates, cultured in 1% FBS overnight and then incubated in CM for 48 hours. Cells were pretreated with seribantumab (250 µg/mL) in 1% FBS medium overnight, and then cultured in CM with seribantumab for 48 hours. Cell proliferation was assessed by adding 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 added with 50 µL DMSO. The optical density of converted substrate was measured at 570 nm, and relative MTT was presented as percent of control groups with cells treated with PDAC CM only.

Pico green assay

PDAC cells were seeded at 2,000 cells/well in 96-well plates, cultured in 1% FBS overnight and then incubated in CM for 48 hours. When seribantumab (250 μg/ml) was used, cells were pretreated with seribantumab in 1% FBS medium overnight, and then cultured in CM with seribantumab for 48 hours. After treatment, culture medium was discarded and 100 μL molecular water was added to cells for 1 hour incubation and then added with 100 μL 1X Pico Green in molecular water for another 1 hour. Fluorescence was measured at 480 nM by microplate reader.

siRNA transfection

For each transfection, 2 x 10^5 ECs were transiently transfected with 1 μg siRNAs coding NRG1 plus 5 μL lipofatamin in 150 μL Opti medium for overnight. 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 48 hours for the MTT or Pico green assay.

Xenograft tumor models

3 x 10^6 BXPC3 cells were implanted subcutaneously (subQ) into the right flanks of athymic nude mice in an inoculation matrix (100 μl of 1:1 mix of growth-factor-reduced Matrigel and concentrated BxPC3 or EC CM). After implantation, mice were treated with concentrated CM by subQ injection adjacent to implanted tumors once a week. Seribantumab (5 mg/mouse) was IP injected to mice twice a week. Tumor volumes were measured with a caliper.

Immunohistology

Mouse tumors were fixed in 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin. Sections were cut (10 μm in thickness). Immunohistochemistry was done with antibodies against P-AKT (1:200; Cell Signaling Technology, 9271) (Supplemental Table 1), P-HER3 (1:100; Cell Signaling Technology, 2842), Ki67 (mouse) (1:200; Cell Signaling Technology, 12202T), Ki67 (human) (1:200, Cell Signaling Technology, 9449T). Isotype-matched immunoglobulin was used as a control. The secondary antibody included the HRP-conjugated anti-rabbit (Cell Signaling Technology, 8114p) antibody that were detected with the DAB substrate kit (Vector Laboratories, SK-4800). Stained sections were examined under a light microscope (Olympus BX51). To quantify staining intensity, images were analyzed by QuPath software (NIH).

Results

Liver EC CM promoted PDAC cell growth

MTT results showed that both EC1 and EC9 could significantly promote the growth of PDAC cells (Figure 1A). At the same time PICO GREEN ASSAY also showed similar results (Figure 1B). To further confirm, the colony assay was executed, and the result also showed that EC CM effectively promoted the growth of PDAC cells.

Liver EC CM activated HER3-AKT pathway to promote PDAC cell growth

To understand the mechanism by which EC CM promotes cell growth, unbiased RTK assay was used to test the effect of EC CM on cells. The results showed that HER3 was significantly activated by EC CM (Figure 2A top panel). Interestingly, not all PDAC cells express HER3 (Figure 2A bottom panel). To further confirm, protein expressions were measured by western blot and showed that EC CM could activate the HER3-AKT pathway of BXPC3 and CAPAN2 in PDAC cells, but in HER3 negative cells, EC CM could still activate AKT signaling (Figure 2B). Taken together, EC CM can activate the HER3-AKT pathway to promote the growth of HER3 positive PDAC cells.

NRG in EC CM played the critical role on EC CM induced cell growth

NRG has been reported to activate HER3 in many studies. To check this case, recombinant NRG was used to stimulate cells. Western blot results showed that NRG could activate the HER3-AKT signaling pathway in HER3-positive cells such as BXPC3 and CAPAN2, while in HER3-negative cells such as PANC-1 and MIA, NRG could still activate AKT signaling even without HER3 (Figure 3A). To further confirm the role of NRG in EC CM, we removed NRG from EC CM cells by IP, and then treated BXPC3 cells with NRG-removed EC CM. The results showed that removal of NRG effectively prevented EC CM from activating the HER3-AKT signaling pathway (Figure 3B). We also used siRNA to reduce NRG gene expression in EC cells, and then harvested CM from such cells to treat BXPC3 cells. It is clear that CM produced by EC cells with low NRG expression cannot effectively activate the HER3-AKT signaling pathway (Figure 3C). The same experiments were repeated on another HER3-positive PDAC cells CAPAN2, and the results were consistent with the findings on BXPC3 cells (Supplement Figure 1). To further confirm the role of NRG, NRG was used to treat BXPC3 and CAPAN2 cells, and cell proliferation results showed that recombinant NRG effectively promoted the growth of PDAC cells based on dose dependent manner (Figure 4A). Nonetheless, CM from EC cells with low NRG expression did not promote cell growth as effectively as control EC CM (Figure 4B). In summary, NRG in EC CM plays a critical role in EC CM-induced HER3-AKT signaling pathway.

HER3 antibody seribanzumab blocked EC CM induced PDAC cell growth either *in vitro* or *in vivo*

Serituzumab is the monoclonal antibody and was used to block HER3 activation (9). Seribantumab significantly inhibited EC CM activation of HER3/AKT signaling pathway (Figure 4A). Meanwhile, MTT results showed that seribantumab could effectively inhibit EC CM to promote cell growth (Figure 4B). However, on HER3-negative cells, seribantumab had no effect either on blocking AKT activation or cell proliferation (Supplementary Figure 2 A and B). Interestingly, when combined with seribantumab and trastuzumab to inhibit both HER3 and HER2 activation, there was no synthetic effect on the inhibition of EC CM-induced cell growth (Figure 4 C and D). Furthermore, inhibition of HER2 activation with trastuzumab alone was not very effective in inhibiting EC CM-induced cell growth. This result suggests that although HER2 and HER3 form a

heterodimer, inhibiting HER2 to inhibit the HER3/AKT signaling pathway is not as effective as directly inhibiting HER3 activation.

Blocking HER3 activation inhibited tumor growth *in vivo*

Xenograft results showed that EC CM can significantly promote tumor growth, but inhibition of HER3 activation with seribanzumab can effectively inhibit EC CM-induced tumor growth (Figure 5A and B). IHC results also confirmed that EC CM can promote HER3/ Activation of AKT increases the expression of growth factor in KI67 cells, while inhibition of HER3 can effectively attenuate the effect of EC CM (Figure 5C). These in vivo results are consistent with the in vitro results, further demonstrating that EC CM activates HER3/ AKT signaling pathway to promote the growth of PDAC.

Discussion

The regulation of cancer cell function by stromal cells in the tumor microenvironment has been reported in many studies (10, 11). Furthermore, studies by our group and others have shown that ECs promote cell survival in CRC and other types of cancer by secreting paracrine factors (8). However, the effect of EC on liver metastasis of pancreatic cancer has not been studied. In this study, we better understand how the hepatic EC microenvironment affects mPDAC growth and response to monoclonal antibodies after PDAC cell transfer to the liver. Using primary liver ECs, we determined that liver ECs promote PDAC cell growth mainly by secreting NRG to activate the HER3/AKT signaling pathway. Furthermore, we demonstrated that the HER3 antibody seribantumab effectively blocked hepatic EC-induced PDAC cell proliferation and tumor growth.

HER3 is a protein with weak RTK kinase activity and is considered a "kinase death" receptor (12). Dimerization of HER3 with its ligands and with other proteins with kinase activity is a typical mode of activation of HER3 in CRC and other cancer cells (13). To date, this neuregulin family protein is the only HER3 ligand identified. Neuregulin binding leads to dimerization of HER3 with HER2 and, to a lesser extent, other HER family receptors that can activate downstream targets, such as AKT (14). In this study, we also found that both HER2 and HER3 are activated by EC CM to activate AKT signaling, and this activation is mainly derived from the role of NRG in EC CM. More importantly, blocking HER3 completely blocked EC-induced AKT activation and PDAC cell survival.

More interestingly, in PDAC cells without HER3 expression, EC remained a client to activate AKT signaling and promote cell growth. This is most likely related to activation of other signals, most likely the activation of ERK signaling that we observed in HER3 negative PDAC cells (data not shown). Our laboratory is currently working to identify key EC-secreted factors that bind and activate AKT in HER3 negative PDAC cells. Seribantumab and several other HER3 antibodies/inhibitors have been studied in combination with paclitaxel or EGFR/HER2-targeted therapy alone or alone in patients with breast, lung, or ovarian cancer (15-18). In

2020, a phase II clinical trial was initiated to evaluate slibanumab as a monotherapy in patients with NRG-1 fusion solid tumors (CRESTONE, NCT04383210). However, such patients represent approximately 0.2% of all solid tumors. These studies suggest that seribantumab and other HER3-targeted therapies are only effective in patients with NRG-1 gene fusions. On the other hand, the effect of HER3 targets on PDAC therapy is still unclear, so the effect of HER3 antibody/inhibitor alone or in combination with chemotherapy in patients with PDAC needs to be further determined.

Taken together, the results of this study identify an important role for the hepatic EC microenvironment in activating HER3 and promoting PDAC cells. Furthermore, we also found that HER3 inhibition blocked EC-induced PDAC proliferation and PDAC tumor growth. Our findings suggest a potential therapeutic strategy using HER3 antibodies/inhibitors in combination with established chemotherapeutics for the treatment of PDAC patients.

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Figure Legends

Figure 1. Liver ECs promoted PDAC cells growth *in vitro*.

Figure 2. EC CM activated HER3-AKT pathway to promote PDAC cells growth.

Figure 3. NRG in EC CM played the major role in promotion of PDAC cells growth.

Figure 4. HER3 antibody seribantumab blocked liver EC-induced PDAC cells growth.

Figure 5. EC CM promoted PDAC tumor growth and HER3 antibody seribantumab blocked EC-induced tumor growth *in vivo*.

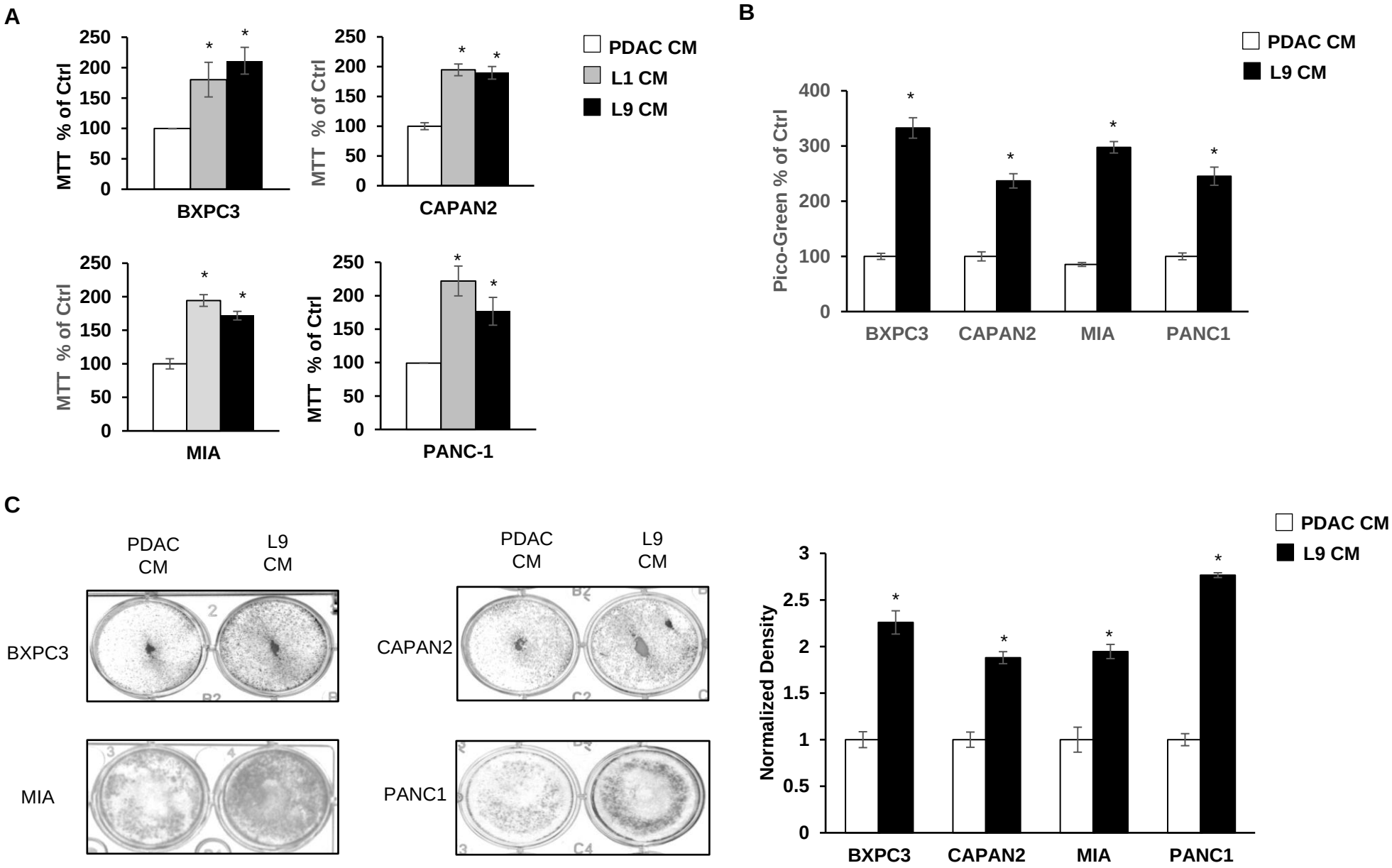
Supplementary Information

Supplementary Figure 1. NRG knock-down ECs were not able to activate HER3-AKT pathway in CAPAN-2 cells.

Supplementary Figure 2. HER3 antibody seribantumab had no any effects on EC-induced growth of HER3(-) PDAC cells

Supplementary Figure 3. EC CM promoted HER3(-) PDAC tumor growth *in vivo*.

Figure 1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CAPAN2
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Pico-Green % of Ctrl
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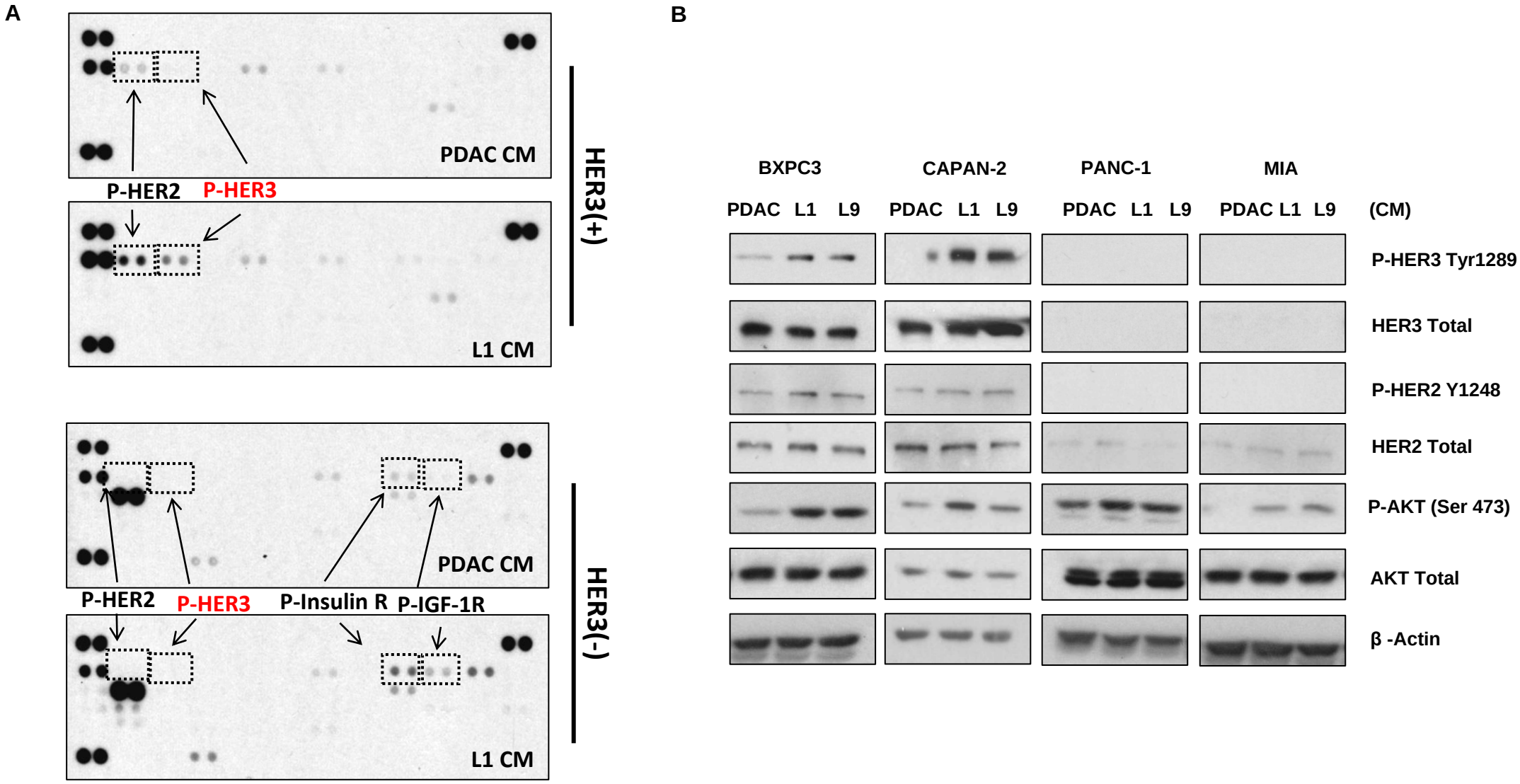
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PANC-1
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PDAC L1 L9
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PDAC L1 L9
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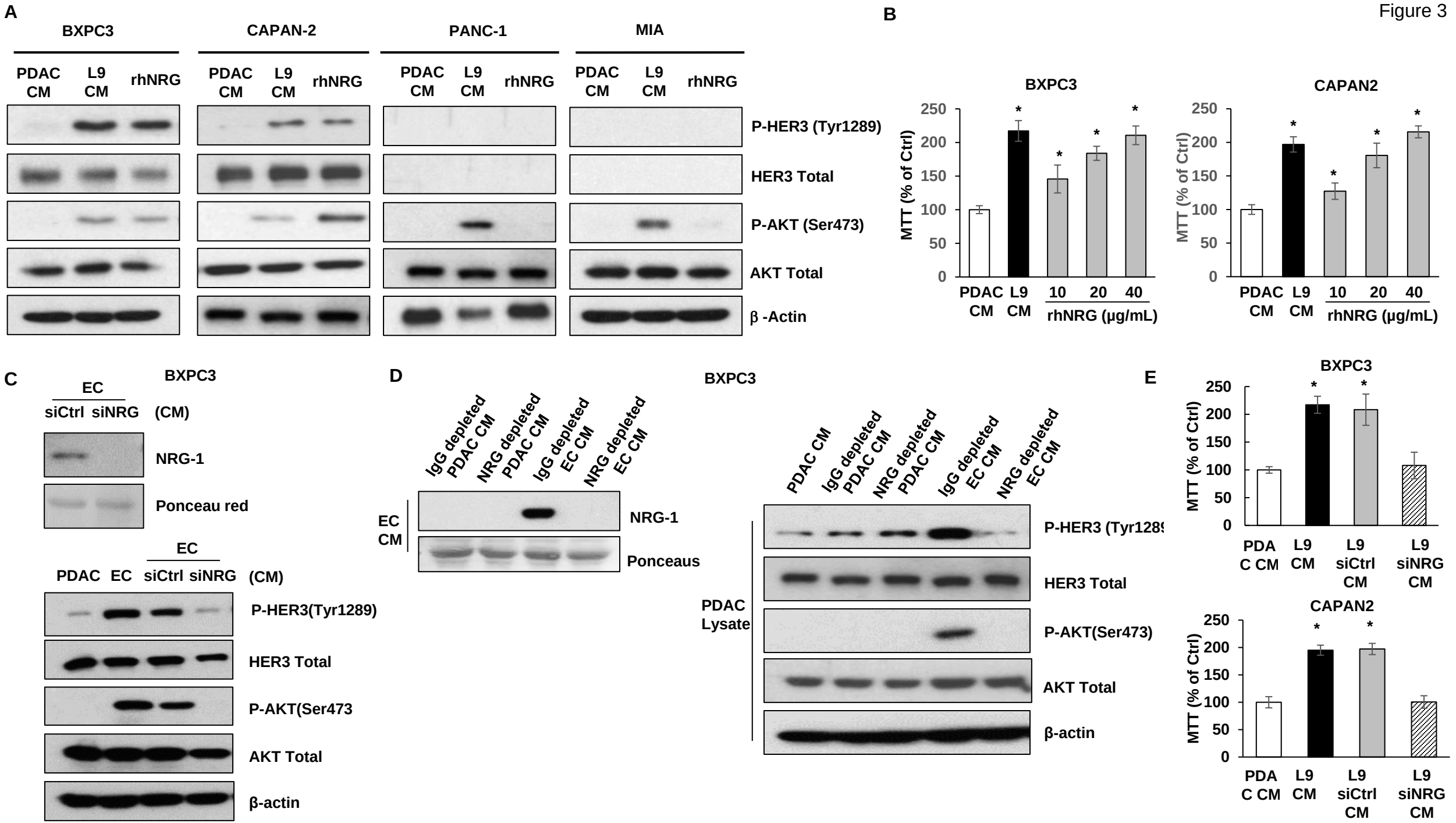
P-AKT (Ser 473)
AKT Total
β -Actin

Figure 3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AKT Total
β -Actin
B
BXPC3
CAPAN2
MTT (% of Ctrl)
PDAC CM
L9 CM
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40
rhNRG (μg/mL)
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BXPC3
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NRG-1
Ponceaus
BXPC3
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IgG depleted EC CM
NRG depleted EC CM
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P-HER3 (Tyr1289
HER3 Total
P-AKT(Ser473)
AKT Total
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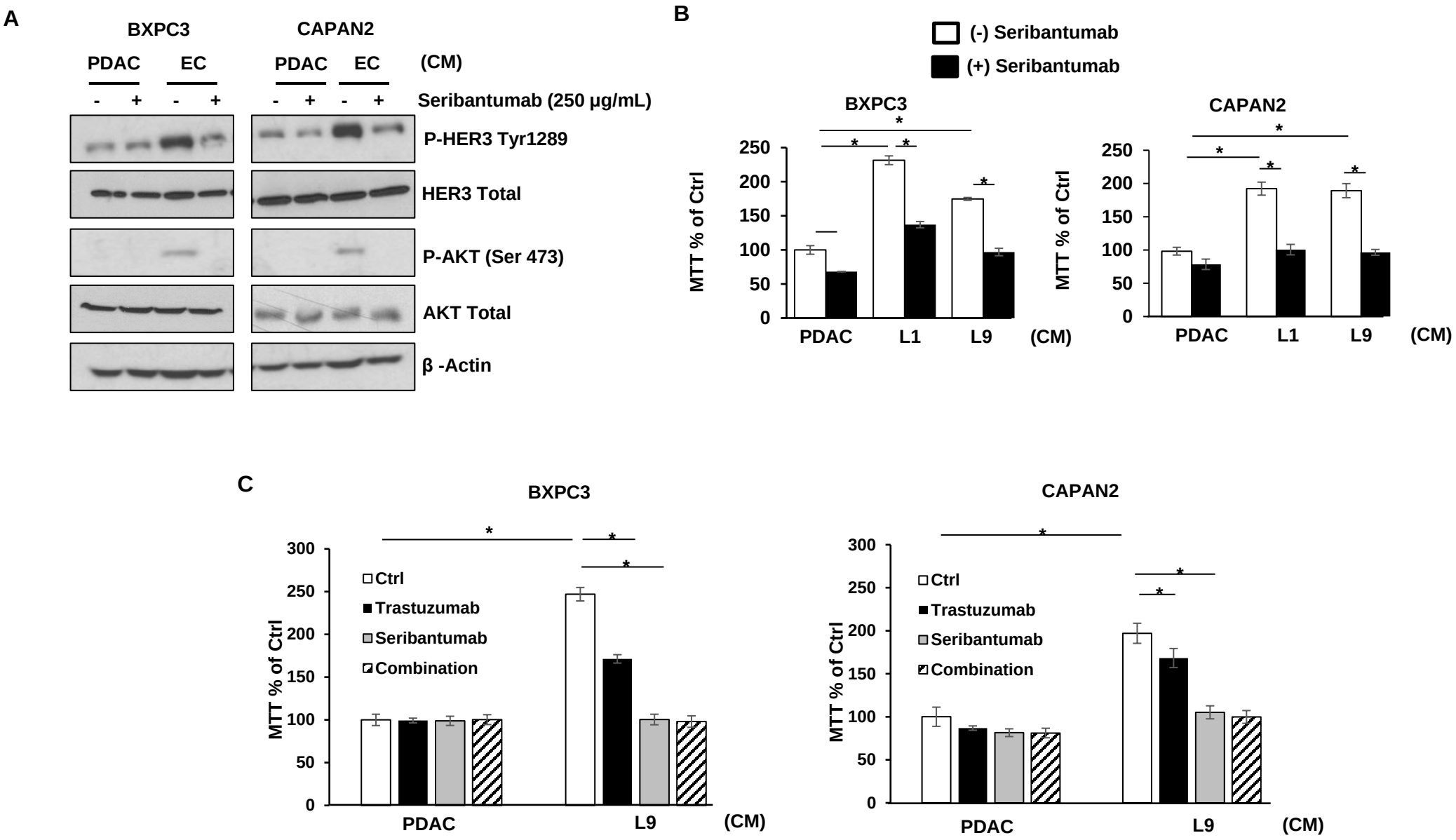
L9 siNRG CM

Figure 4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HER3 Total
P-AKT (Ser 473)
AKT Total
β -Actin
B
(-) Seribantumab
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BXPC3
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MTT % of Ctrl
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PDAC
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C
BXPC3
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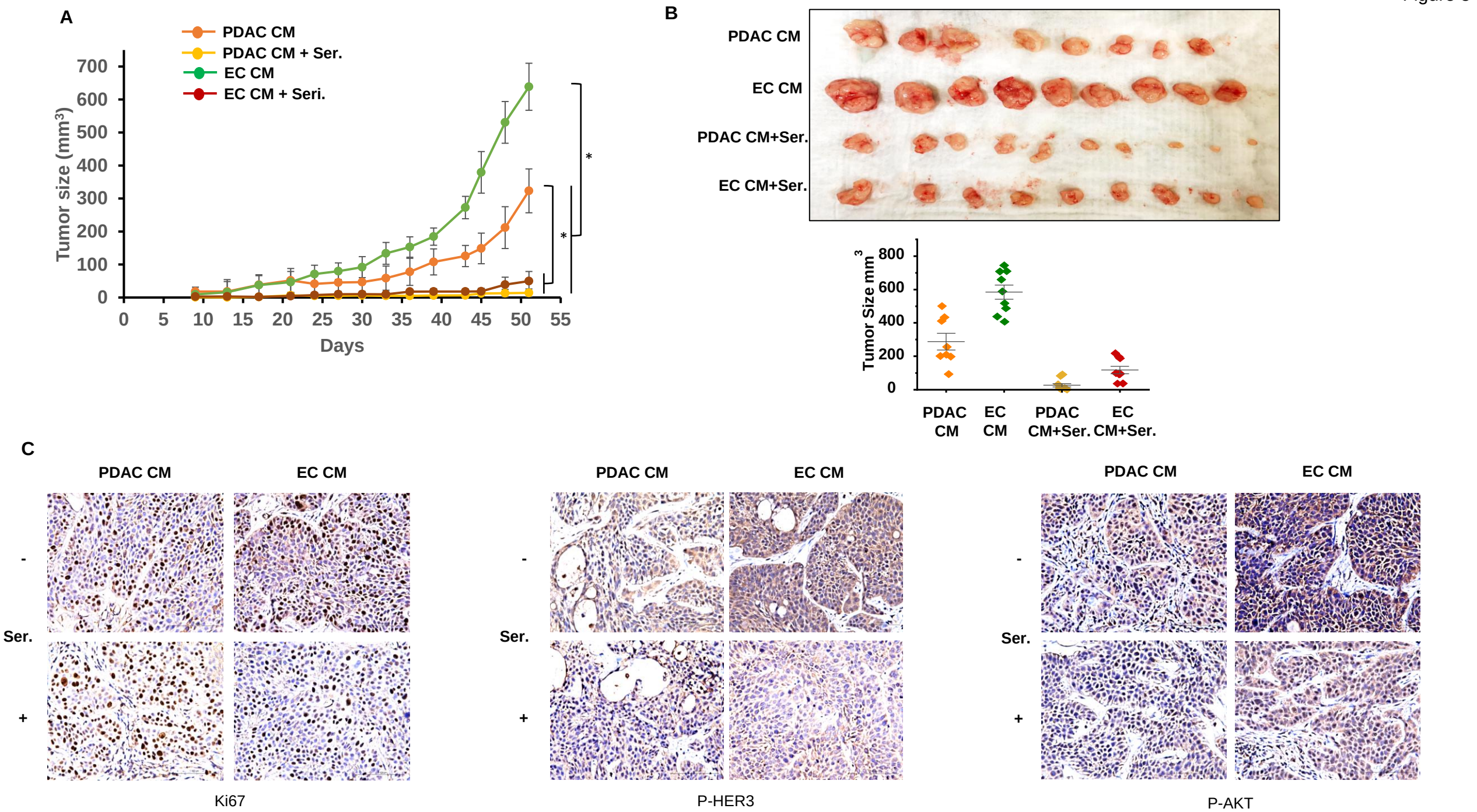
Figure 5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PDAC CM + Ser.
EC CM
EC CM + Seri.
Tumor size (mm³)
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600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55
Days
*
*
B
PDAC CM
EC CM
PDAC CM+Ser.
EC CM+Ser.
Tumor Size mm³
800
600
400
200
0
PDAC CM
EC CM
PDAC CM+Ser.
EC CM+Ser.
C
PDAC CM
EC CM
-
Ser.
+
Ki67
PDAC CM
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Ser.
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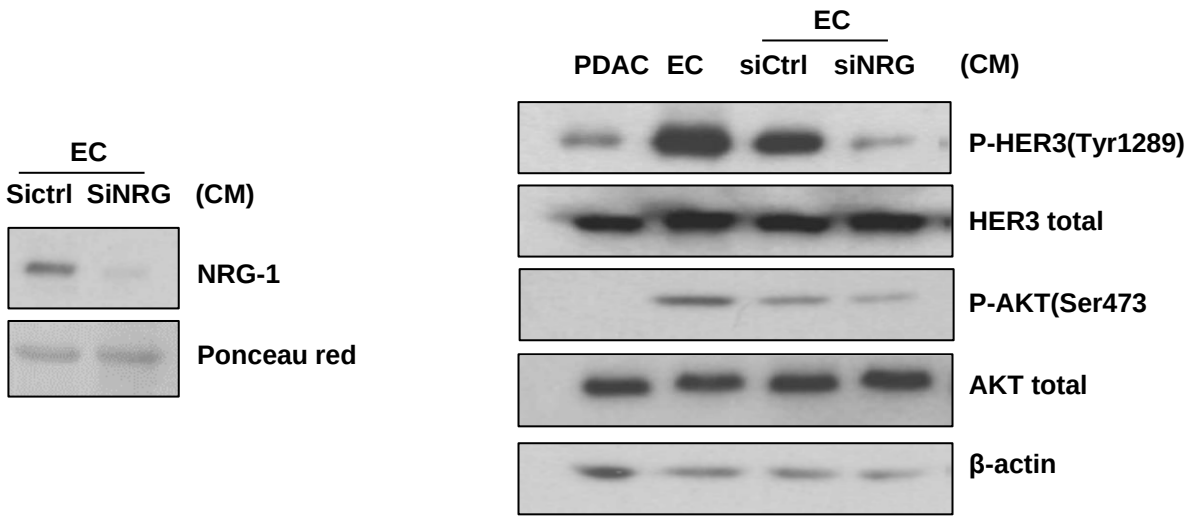
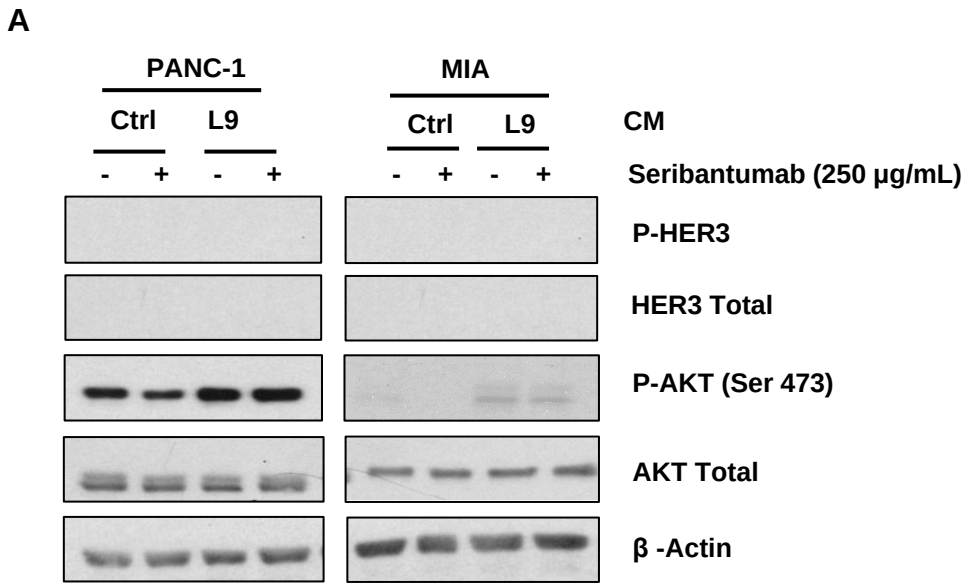
P-AKT

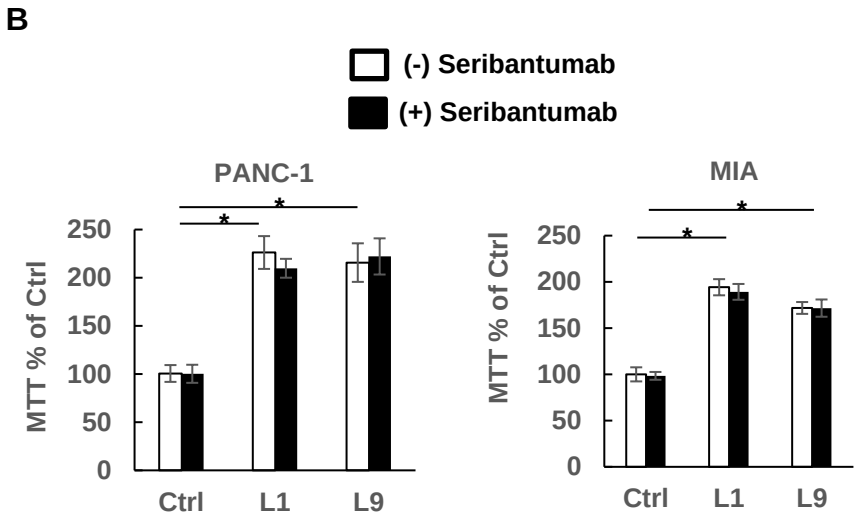
Supplementary Figure 1

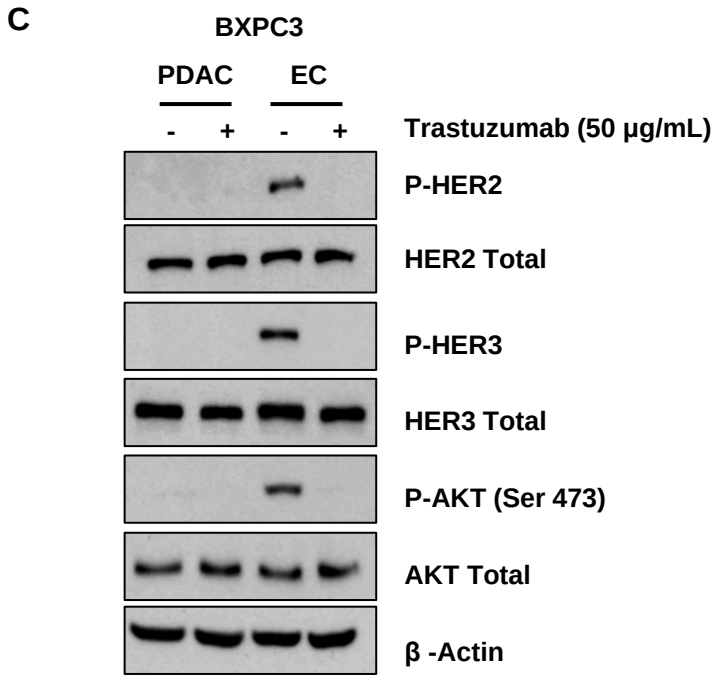
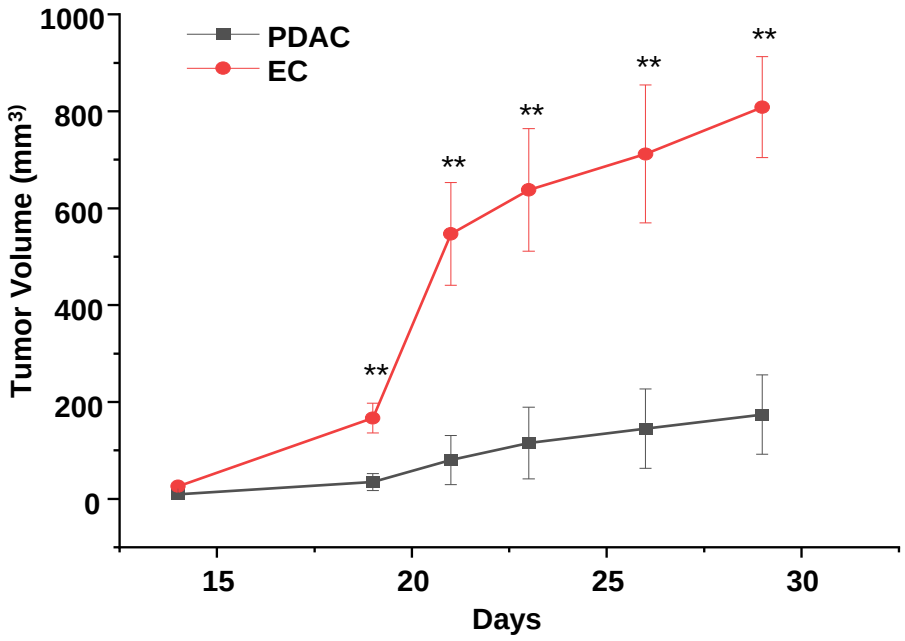
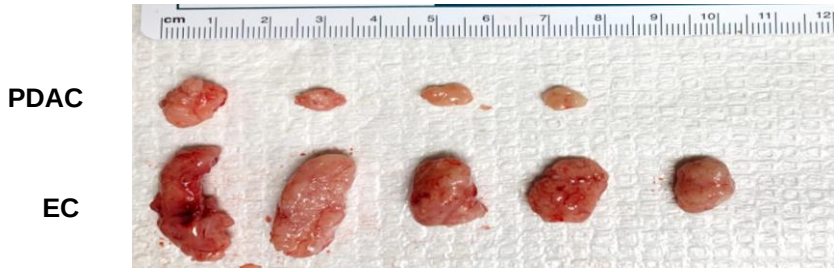
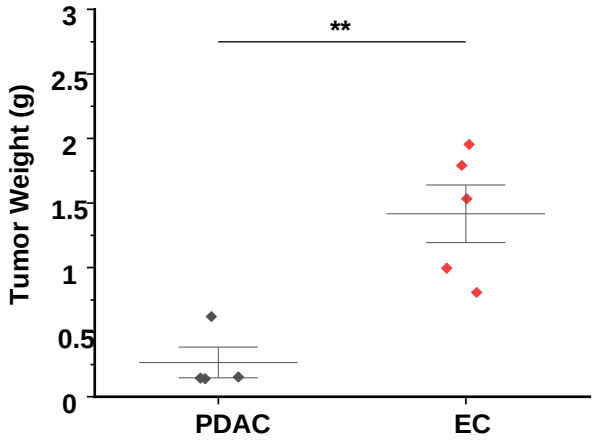
CAPAN2



Supplementary Figure 2







Supplement Figure 3