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14. ABSTRACT
Through this grant we have not only published 4 high-impact papers, we identified cancer stem cell's role in aggressive behavior of peritoneal carcinomatosis. We have developed new cell lines and animal models. We identified which oncogene (Yap1) drives peritoneal carcinomatosis and we could reduce the incidence of peritoneal metastases. We have extensively characterized PDX models and cell lines (these will be useful resources to the oncology community). We have accomplished single cell sequencing in 20 patients with unique results to be published in Nature Medicine. Discovered crosstalk between TRIM28 and Yap1 (potentially both can be exploited in the clinic). We have defined proteomics of peritoneal cells at the mass spect level (never been reported for gastric cancer) and defined mRNAs as well a unique long-non-coding RNA (CCAT2) that regulated chromosomal instability. We are now poised to take our discoveries to the next level.

15. SUBJECT TERMS
Non-codingRNAs, microRNAs, ultraconserved transcripts, pyknon transcripts, expression, Ascites; Cancer stem cell; Carcinomatosis; Exosome; Gastric Adenocarcinoma; Molecular profiling; Metastasis; Novel therapeutics; Peritoneal; Proteomics; RNA; Therapeutic target

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Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

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1A. Expand PC cells by in vitro conditional reprogramming of cells (CRCs) and cell line generation. 1B. Establish PC-PDX model (subcutaneous tumors forming after injection of PC cells) and spontaneous-PC-PDX model (SPC-PDX; subcutaneously injected PC cells migrate/prosper to the peritoneal cavity of mice), 1C. Study functional relevance of CSC pathways in in vitro/in vivo. This aim is focused on detail examination of selected CSC pathways in human PC, in vitro/in vivo functional validation (genetic manipulations) of these CSC pathway targets (including in PDX models).

Aim 2. Identification of additional novel therapeutic targets (CSC and non-CSC) in human PC cells through a multi-omics platform.
2A. RNA/Exome Seq profiling (n=40 pairs; primary/normal vs. PC),
2B. Proteomic/exosome characterization (MS) (n=40; segregated PC cells from stroma),
2C. Profiling miRNAs/lncRNAs (n=40 pairs; primary vs PC). An integrated analysis will be carried out. This aim is focused on an unbiased multiomics study of human PCs to facilitate integrated analysis that will yield top candidate targets for validation. Purified PC cells will be studied for genomics/proteomics (PC cells from malignant ascites and in primary GAC (FFPE) specimens, we are able to isolate DNA/RNA).

Aim 3. Preclinical evaluations (genetic and pharmacologic manipulations) of targets identified in Aim 2.
3A. Genetic modulations of the candidate targets in human PC cells emerging from Aim 2 in vitro to establish their functional relevance.
3B. In vitro studies of human PC cells with novel agents against CSC pathways and other novel targets emerging from Aim 2, 3C. in vivo studies with human PC PDX models (novel agents and genetic modulations of PC cells). This aim is focused on emerging targets (CSC or non-CSC) from Aim 2 and will carry out genetic manipulations and pharmacologic studies to validate exploitable therapeutic targets (in vivo and in vitro). Combinations will be emphasized. Novel agents will be identified for future clinical trials.

KEYWORDS:
Provide a brief list of keywords (limit to 20 words).

Non-codingRNAs, microRNAs, ultraconserved transcripts, pyknon transcripts, expression, Ascites; Cancer stem cell; Carcinomatosis; Exosome; Gastric Adenocarcinoma; Molecular profiling; Metastasis; Novel therapeutics; Peritoneal; Proteomics; RNA; Therapeutic target

ACCOMPLISHMENTS:
The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals of the project during the reporting period were to (1) optimize purification of tumor cells and exosomes from gastric adenocarcinoma ascites, (2) to initiate proteomic profiling of these disseminated GAC cells and exosomes, and (3) Identification of additional novel therapeutic non-codingRNAs targets (CSC and non-CSC) in human PC cells through a genome-wide profiling

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

CA160445 (PI: Dr.Jaffer Ajani)

Aim 1: Molecular profiling of CSC pathways from PC. 1A. Expand PC cells by in vitro conditional reprogramming of cells (CRCs) and cell line generation. 1B. Establish PC-PDX model (subcutaneous tumors forming after injection of PC cells) and spontaneous-PC-PDX model (SPC-PDX; subcutaneously injected PC cells migrate/prosper to the peritoneal cavity of mice),

1C. Study functional relevance of CSC pathways in in vitro/in vivo. This aim is focused on detail examination of selected CSC pathways in human PC, in vitro/in vivo functional validation (genetic manipulations) of these CSC pathway targets (including in PDX models).

1A. Development of patients derived PC cell lines and PDX and patient-derived metastatic orthotopic models from ascites cells of GAC patients. PC cells were collected from volunteering patients with GAC and malignant ascites. Three patients-derived PC cell lines-GA0518, GA0825 and GA0804 were established from either PDXs of two donor PC cells (IP-013 and IP-116) or the GA0804 cell line could be established directly from donor PC cells (patient IP-107-02) via culture in RPMI medium (7% FBS, 1% antibiotics) and expanding them for more than 10 passages. The three PC cell lines had different morphologies under the microscope (Figures 1A and 1B, upper panel). In the 2D culture system, most GA0518 cells grew adherent as a monolayer of epithelial-like cells and spindle-like cells with about 5% of cells remained in suspension. GA0825 cells grew adherent as a monolayer of spindle shape with a few cells remained in suspension. While GA0804 cells grew and organized differently showing cobblestone like shape with ~10% grew in suspension (Figure 1A). To visualize cell growth *in vitro* and *in vivo*, we stably transfected mCherry-Luciferase into each of the three cell lines to decipher the morphology more clearly and allow monitoring by BLI *in vivo* (Figure 1B) Doubling time was estimated during the exponential phase of growth. GA0518 cells had the shortest doubling time, 22 hours. GA0804 and GA0825 cells had similar doubling times, around 39 hours and 37 hours, respectively (Figure 1C). In addition, GA0518 cell line showed a high level of heterogeneity with several chromosomal abnormalities that matched the donor sample (IP-013) with more mutations. Their karyotype was heterogeneous as shown in Figure 1D for both GA0518 and GA0804.

Expression of CSC markers and oncogenic genes in these cell lines and donor PC samples

We further character these cell lines by performing Western blot using total cell lysates from the three PC cell lines and compared the results with those of the normal gastric epithelial cell line GES-1 and the

Figure 1. Characterization of the new GAC cell lines and *in vitro* growth features

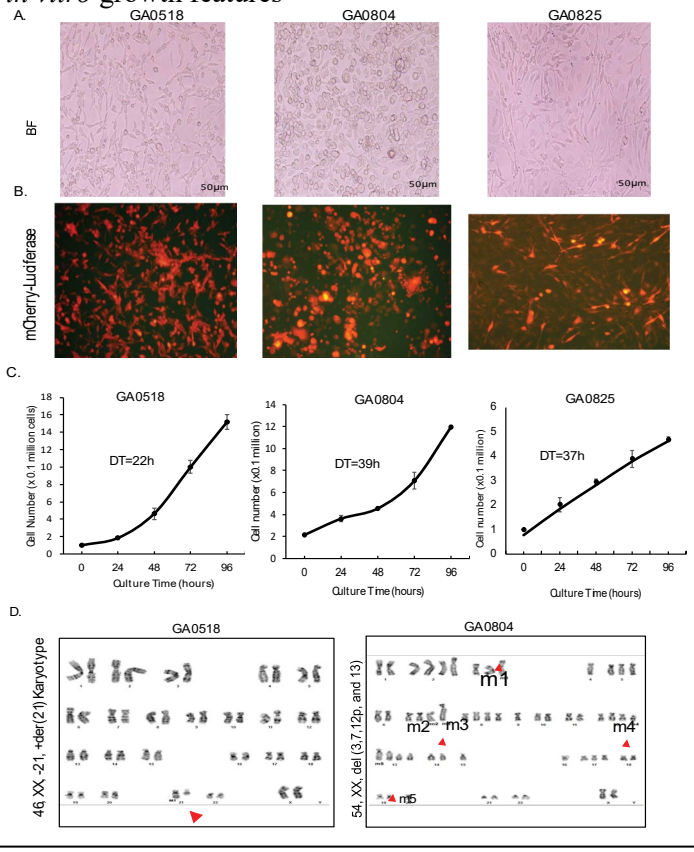
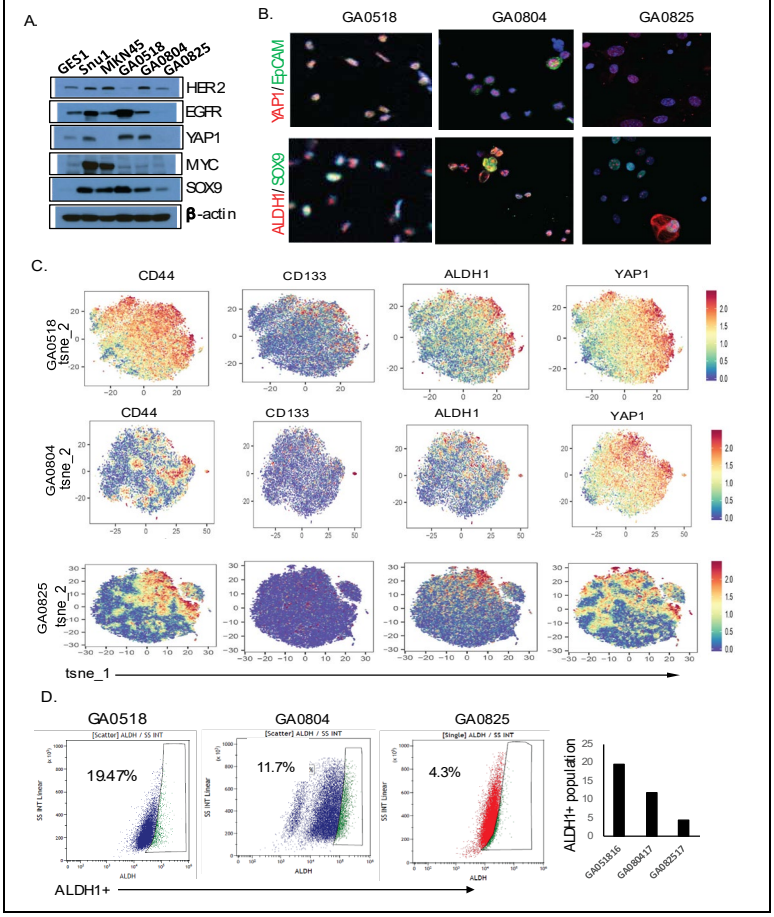


Figure 2. Expression of oncogenic and CSC markers in these cell lines.



commercial GAC cell lines, Snu-1 and MKN45. GA0518 and GA0804 cells had similar expression patterns for EGFR, YAP1, MYC, and SOX9, but with higher expression of these markers in GA0518 cells (Figure 2A). Immunofluorescence (IF) staining of several notable CSC makers (YAP1, EpCAM, ALDH1, and SOX9) in three cell lines (Fig.2B) showed expression of YAP1, EpCAM, ALDH1, and SOX9 was heterogenous and varied in three PC cell lines with highest of GA0518 cells (Figure 2B). Expression of GAC CSC markers including CD44, CD133, ALDH1 and YAP1 were also evaluated by CyTOF in three cell lines. As shown in Figure 2C, expression of these markers was heterogeneous among these cell lines. All four markers are relatively high in GA0518 cells, while relative lower in GA0825 cells which may reflect its tumorigenicity and metastatic potential.

Oncogenic activation and malignant behaviors of the three cell lines *in vitro* We determined the activation oncogenic pathways in three cell lines and the corresponding donor PC cell samples by CyTOF. As shown in Figure 3A, activation of oncogenic pathways such as pS6, p-AKT, p-FAK and mTOR and MYC were activated differently among three cell lines. GA0518 is the highest in expression of pS6, p-AKT, p-FAK and MYC (Figure 3A). The next is GA0804 and its donor PC cells IP-107-2 PC cells, while GA0825 cell and its donor IP-116 PC cells showed the lowest expression of these oncogenic markers (Fig.3A). We then performed functional *in vitro* assays in the three cell lines. The colony formation capability was highest in GA0518, followed by GA0804, and then GA0825(Figure 3B). GA0518 also had a higher tumor sphere forming capacity than GA0804 or GA0825 (Figure 3C). Both colony formation and tumor sphere formation corresponded to the ALDH1+ and CD44+ population levels with GA0518 the highest (Figure 2). The migration capacity also varied; GA0518 had the highest capacity for invasion, followed by GA0804, and then GA0825 (Figure 3D).

1B. Establish PC-PDX model (subcutaneous tumors forming after injection of PC cells) and spontaneous-PC-PDX model (SPC-PDX; subcutaneously injected PC cells migrate/prosper to the peritoneal cavity of mice)

Robust *in vivo* tumorigenicity in mouse xenografts To test tumorigenicity, we subcutaneously injected varying numbers of limiting diluted cells from each of the three cell lines into the back flanks of either nude mice or SCID mice (Figure 4A). All three lines formed xenograft tumors robustly, but GA0518 was more efficient than GA0804 and GA0825. GA0518 formed tumors in all nude mice injected, even with a low cell inoculation (1000 cells) (Figure 4B), while GA0804 formed tumors in only 87.5% (7/8) of SCID mice injected with 1×10^5 cells and 75% (6/8) of SCID mice injected with 1×10^4 cells (Figure 4C). GA0825 was able to produce tumors in 100% of SCID mice injected with 1×10^6 and 1×10^5 cells but was hard to grow when injected at 1×10^4 and 1×10^3 cells, although some BLI signals were visible (Figure 4D, Supplemental Figure 5). Furthermore, GA0518 grew faster *in vivo* than GA0804 and GA0825, which is consistent with the cell lines' doubling times *in vitro* and their ALDH1+ populations indicating that the GA0518 cell line was more aggressive both *in vitro* and *in vivo* (Figure 4E).

Figure 3. Activation of oncogenic pathways by CyTOF and malignant behaviors of three cell lines *in vitro*

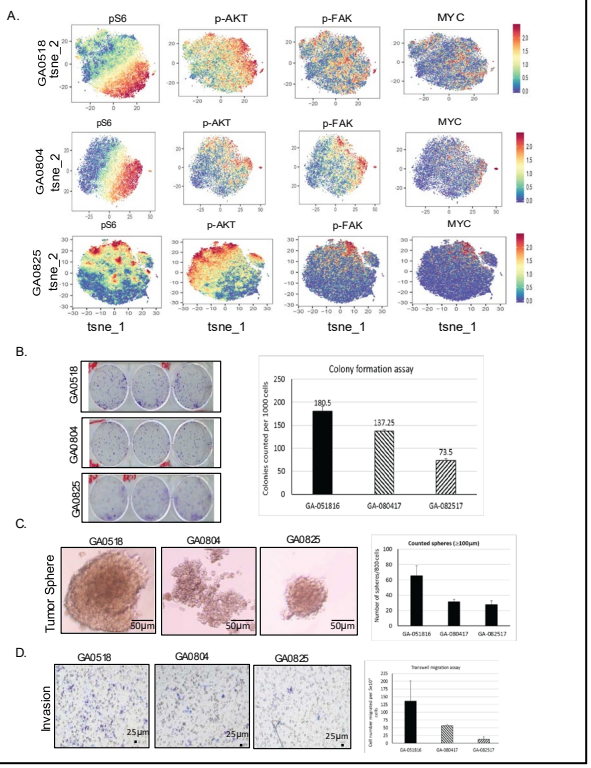
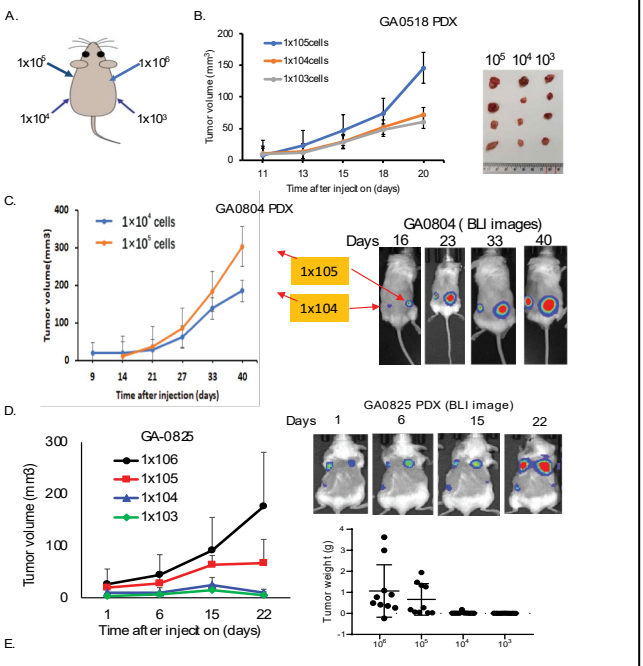


Figure 4. Robust *in vivo* tumorigenicity of three new PC cell lines in xenograft (PDX)



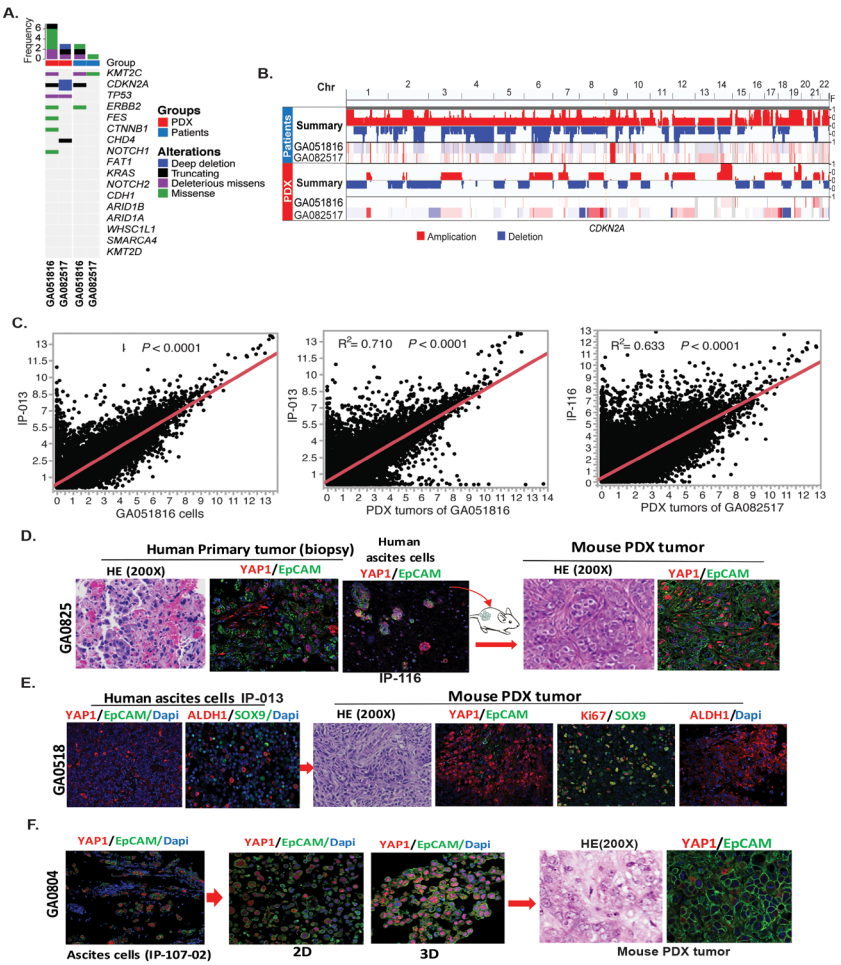
E.

Number of tumor-bearing mice/ Number of xenograft mice						
Cell Line	Cell Number Injected				Day post injection	Mouse strain
	10^6	10^5	10^4	10^3		
GA0518	10/10	10/10	10/10	10/10	23	SCID
GA0825	10/10	10/10	0/10	0/10	22	SCID
GA0804		7/8	6/8		26	SCID

Molecular features and gene expression were preserved in PDXs compared to donor PC cells We performed WES and RNA-seq of PDXs derived from GA0518 and GA0825 and of the corresponding donor PC cells directly from patients. As shown in Figure 5A, several key mutations such as *KMT2C, CDKN2A*, and *ERBB2* mutations were preserved in the GA0518 PDXs, while GA0518 and GA0825 PDXs displayed new mutations in *TP53, FES, CTNNB1, NOTCH1*, and *CHD4*, which we have reported in the corresponding PC cells[10]. In addition, there were shared copy-number variations between the PDXs and the donor PC cells, including gains in chromosomes 1q, 7q 10p, 14q, and 19q and losses at 7p and 18q (Figure 5B). Of note, there was a loss of *CDKN2A* in both PDXs and in the PC cells, suggesting that loss of *CDKN2A* may contribute to progression of GAC.

RNA-seq revealed a significant positive correlation in gene expression profile between PDXs from GA0518 and the corresponding donor PC cells (IP-013) and between the GA0518 cell line and IP-013 ($p<0.0001$ for each; Figure 5C, left and middle). Gene expression was also positively correlated between GA0825-derived PDXs and the corresponding donor PC cells, IP-116 ($p<0.0001$; Figure 5C, right), indicating that the gene signature in PDX tumors recapitulates that of the donor PC cells directly from patients. Further, we then compared phenotypes of PDXs and corresponding primary and PC cell samples from patients by hematoxylin/eosin staining and IF staining. As shown in Figure 5D, for GA0825, the only cell line with a primary tumor sample available, the morphology of PDXs from mouse on hematoxylin and eosin was similar to that of the primary tumor of the patient. Interestingly, on IF staining, the expression of CSC markers YAP1 and EpCAM were preserved in GA0825 PDX, recapitulating the expression in the corresponding donor PC cells (IP-116) and its primary tumor (Figure 5D). Similarly, expression of YAP1, EpCAM, ALDH1, and SOX9 were preserved in GA0518 PDX that recapitulate the expression in the corresponding donor IP-013 PC cells (Figure 5E). For GA0804, the expressions of YAP1 and EpCAM were preserved in 2D and 3D culture conditions as well as GA0804 PDX from the corresponding donor PC cells (IP-107-02) (Figure 5F). These results showed that the molecular features and gene expressions in the cell lines and PDXs has similar patterns to that of the donor PC cells from patients, suggesting that the cell lines and PDXs are potentially highly valuable to represent donor PC cells for preclinical studies.

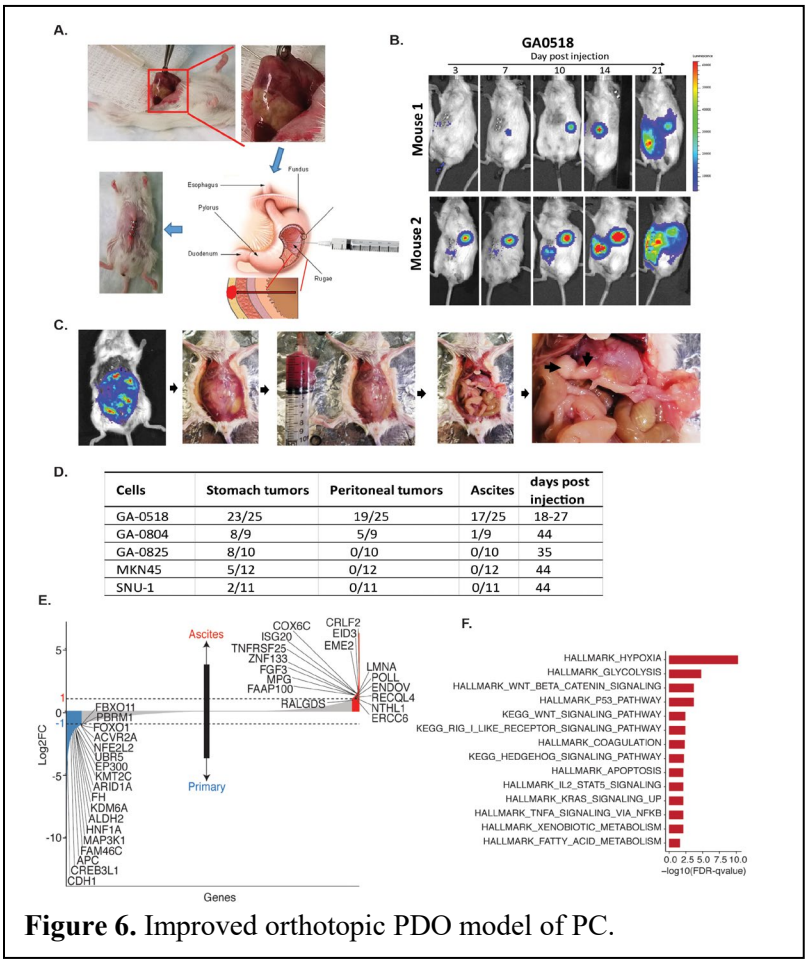
Figure 5. Molecular and gene expression features were preserved in PDXs compared with donor PC cells



Development of patient-derived metastatic orthotopic (PDO) models from ascites cells of GAC patients. We developed PDO model by improved method as follows: three PC cell lines and GAC cell lines injected into the serosa could leak into the peritoneal cavity. SCID mice inhaled anesthetic with isoflurane, and then the surgical site was disinfected and shaved (Figure 6A). An upper midline incision about 1-1.5 cm long was made from the xiphoid process. The stomach was exteriorized gently using ring forceps. The syringe was inserted with the right hand through the posterior wall to penetrate into the gastric cavity, and 50% Matrigel–phosphate-buffered saline containing 1×10^5 cells (30 µl volume) was injected into the anterior wall, each injection lasting 30 seconds. After the injection, an alcohol swab was used to sterilize the posterior wall. The stomach was repositioned into the abdominal cavity, and the wound was closed (Figure 5A). After injection of cells, the tumor growth was monitored by BLI imaging weekly (Figure 5B and 5C). In these PDO models, the three lines grew tumors in the stomach and formed PC with varying efficiency (Figures 6B-6D). GA0518 was the most aggressive, with 92% (23/25) of mice developing stomach tumors and 76% (19/25) of mice

developing PC in 4 weeks (Figure 6B-6D). With GA0804, 89% (8/9) of mice developed stomach tumors and 56% (5/9) of mice had PC, while with GA0825, 80% (8/10) of mice formed stomach tumors and none (0/10) of mice had PC in about 5 weeks (Figure 6D). PC did not develop from commercial GAC cell lines MKN45 or Snu-1, but 42% (5/12) of mice with MKN45 and 18% (2/11) of mice with Snu-1 grew stomach tumors in 6 weeks (Figure 6D).

Importantly, in the PDO model, 68% (17/25) of mice with GA0518 cells developed bloody ascites within 4 weeks (Figure 6B-6D). Further, RNA-seq of PC ascites cells and matching stomach tumors from the same GA0518 PDO mice revealed that many important oncogenes were significantly upregulated in PC cells compared with stomach tumor cells (Figure 6E). Gene Ontology analysis identified these genes as components of many important pathways, including hallmark of hypoxia, glycolysis, Wnt/□-catenin signaling, and TP53 pathways (Figure 6F). These data, taken together, indicate that the GA0518 line is a good cell model to elucidate molecular mechanisms of PC and may be used for target validation.



Cells	Stomach tumors	Peritoneal tumors	Ascites	days post injection
GA-0518	23/25	19/25	17/25	18-27
GA-0804	8/9	5/9	1/9	44
GA-0825	8/10	0/10	0/10	35
MKN45	5/12	0/12	0/12	44
SNU-1	2/11	0/11	0/11	44

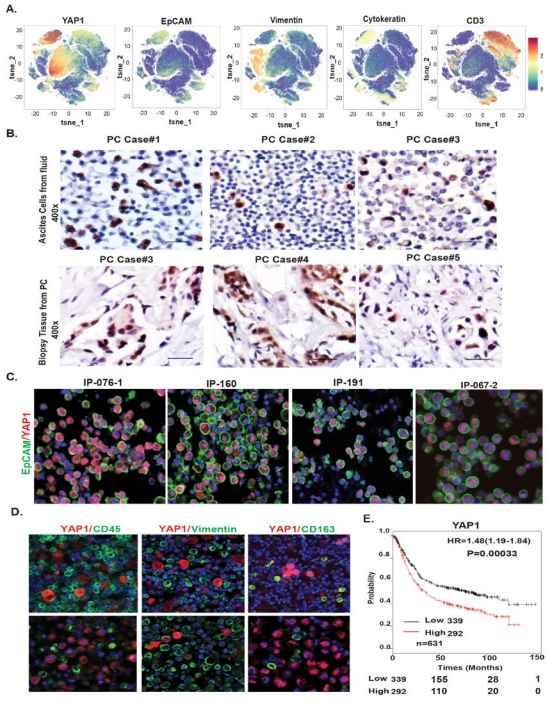
Figure 6. Improved orthotopic PDO model of PC.

1C. Study functional relevance of CSC pathways in *in vitro/in vivo*.

YAP1 is highly expressed only in PC malignant cells and peritoneal biopsy tissues Yes-associated protein 1 (YAP1) is a transcriptional coactivator that developmentally regulates organ size and proliferation. We and others have reported that YAP1 is frequently overexpressed in several cancer types and plays an oncogenic role in cancer progression and confer CSCs properties. YAP1 overexpression and its activation (nuclear localization) correlate with poor outcomes in several tumor types. Using CyTOF, we found that YAP1 is highly expressed in PC cells (Figure 7A). To elucidate the expression patterns of YAP1 in PC cells (from ascites and peritoneal biopsies), we stained PC specimens from patients using IHC/immunofluorescence. As shown in Fig. 7B, YAP1 was expressed mostly in tumor cells in PC specimens by IHC (ascites cells [top] and biopsies [bottom]). The expression patterns varied among cases but YAP1 was mostly in the nucleus and only weak staining was noted in the cytoplasm of tumor cells. As indicated in Figure 1C in representative PC cases, YAP1 expression was enriched in malignant tumor cells, while lower co-expression in CD45+ immune cells, vimentin+ stromal cells, or CD163+ macrophages. sc-RNA Seq in 20 PC specimens further Stratification of GAC patients by YAP1 expression revealed that YAP1 level is significantly associated with shorter survival in advanced GAC patients (Fig.1E) This suggests that YAP1 overexpression is a poor prognosticator and may play an important role in PC.

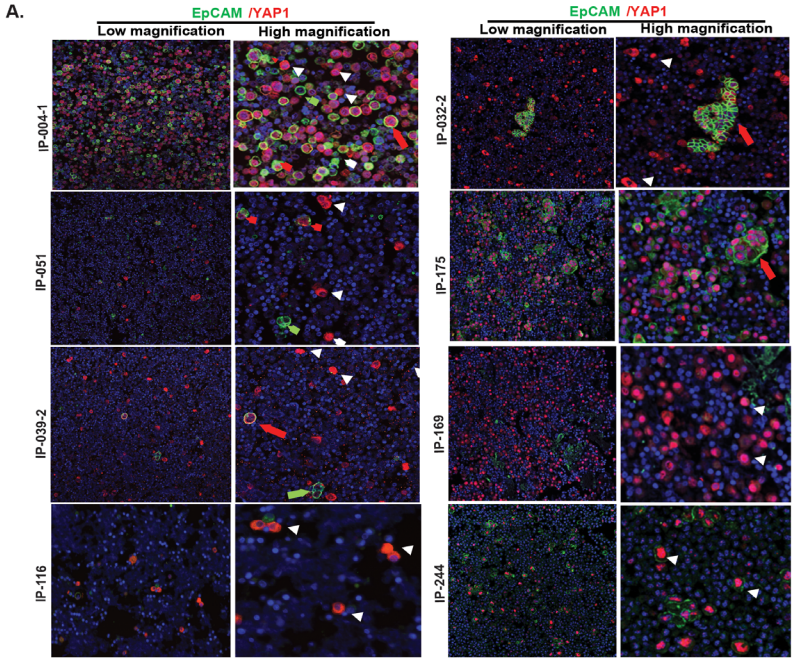
Heterogeneity of malignant PC cells by YAP1/EpCAM dual immunofluorescent staining and scRNA Seq EpCAM is an established diagnostic

Figure 7. YAP1 is highly expressed only in PC malignant cells and peritoneal biopsy tissues



biomarker for epithelial malignancies. We co-stained EpCAM and CD45 using immunofluorescence and found EpCAM had membranous staining in PC tumor cells, clearly distinguishable from CD45 stained immune cells. Then, we double-stained YAP1 and EpCAM in PC samples using dual-immunofluorescence and noted membranous EpCAM and nuclear YAP1 co-expressing in PC tumor cells in 55 cases (45%). Interestingly, EpCAM and YAP1 are not always co-expressed in PC tumor cells. Nearly, 55% of PC samples had discrepant expression of YAP1 and EpCAM. As shown in Fig. 8A, some PC tumor cells shared the membranous expression of EpCAM and nuclear YAP1(red arrow), while others expressed only nuclear YAP1 (white arrow) or membranous EpCAM (green arrow) indicating tumor cell heterogeneity within and among PC samples. The proportion of YAP1 and EpCAM shared expression or either alone is shown in Figure 8B. sc-RNA-Seq in additional 20 PC samples was performed to further demonstrate tumor heterogeneity. As shown in Fig. 8C, among tumor cell clusters (here we excluded all immune cells), expression of YAP1 and EpCAM was heterogeneous with some expressed both YAP1 and EpCAM, while some clusters expressed only EpCAM (green) or only YAP1(red). The status of EpCAM+ or YAP1+cells alone or shared cells from sc-RNA Seq is shown in Figure 8C (right). On the basis of these results, we hypothesized that YAP1 or/and EpCAM positive cells may represent different tumor cell states with varying metastatic potential.

Figure 8. Heterogeneity in PC cells by YAP1/EpCAM co-staining and sc-RNA Seq

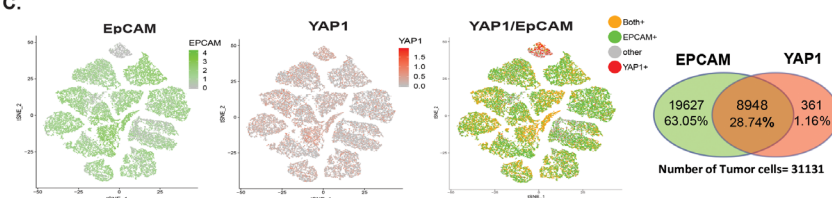


B.

Expression Status of YAP1/EpCAM in 123 PC cases

	Shared dual positive (n=101)			Either positive (n=22)		total
	YAP1 (high)	EpCAM (high)	YAP1+/EpCAM+ Equal	YAP1+ Only	EpCAM+ Only	
PC cases	39(32%)	15(12%)	55(45%)	20(16%)	2(1.6%)	123

C.



YAP1^{high} PC cells easily form PDXs in mice irrespective of PC tumor cell purity and Knockout YAP1 reduced tumorigenicity of YAP1^{high} PC cells in the PDX model We hypothesized that YAP1^{high} PC cells represent undifferentiated cells with CSC traits and have high tumorigenicity and metastatic potential. To test this hypothesis, we generated PDX tumors from PC specimens by subcutaneously injecting PC cells into nude/SCID mice (5×10^6 cells from one patient per mouse). Of the 66 cases, 15 (22.7%) generated PDXs successfully. Upon detailed analyses, we noted that PDX formation did not depend on tumor cell purity (tumor cell % of PC sample). However, PDX formation was associated with YAP1^{high} expression. Of the 15 mice that formed PDXs, 13 had YAP1^{high} expression regardless of tumor cell percentage and EpCAM expression; some had either low or no EpCAM expression. As shown in Fig. 9A, two representative PC samples, IP-116 and IP-013, with YAP1^{high} staining but low EpCAM expression (IP-116) or no EpCAM expression (IP-013), easily formed PDXs and even formed PC metastasis in nude mice (Fig. 9A, right). The morphology of these PDX tumors and the expression of YAP1 seen in the patient PC samples were preserved in these PDX tumors (Fig. 9A [bottom] and in more PDX tumors from additional PC samples). Altogether, these data suggest that YAP1 may govern CSC properties that drive PC. Successful knockout of YAP1 strikingly decreased tumor sphere formation and invasive capacity in two individual subclones of GA051816 as compared to parental cells (Figs. 9B & 9C). Knockout YAP1 significantly decreased CSCs ALDH1+ population (19.5% to 2.07%) (Fig. 9D). Further, serial dilutions of 1×10^5, 1×10^4, 1×10^3 cells from control and YAP1-knockout GA051816 cells were injected subcutaneously in nude mice. YAP1

knockout significantly delayed and reduced tumor growth at each dilution suggesting that YAP1 is critical for GAC progression.

To examine the mechanism through which YAP1 regulates the acquisition of stemness and tumorigenicity, RNA-Seq studies found depletion of YAP1 significantly decreased the expression of genes whose expression is associated with CSCs signaling—*SOX9*, *HES1*, *EGR3*, and *ALDH3A1*(red arrow) and EMT signaling-CDH11, DKK1, MFAP5 and IGFBP2 and IGFBP3 (green arrow) with FDR q-value between $3.86e^{-13}$ to $3.09e^{-5}$ (Supplemental Table 3). Most importantly, most YAP1 regulated CSCs and EMT genes are associated with poor survival in GC patients (Supplemental Fig.3) suggesting that YAP1 mediated stemness and PC in part through regulation of CSCs and EMT.

Aim 2. Identification of additional novel therapeutic targets (CSC and non-CSC) in human PC cells through a multi-omics platform.

2A. RNA/Exome Seq profiling (n=40 pairs; primary/normal vs. PC),

2B. Proteomic/exosome characterization (MS) (n=40; segregated PC cells from stroma)

2C. Profiling miRNAs/lncRNAs (n=40 pairs; primary vs PC). An integrated analysis will be carried out. This aim is focused on an unbiased multiomics study of human PCs to facilitate integrated analysis that will yield top candidate targets for validation. Purified PC cells will be studied for genomics/proteomics (PC cells from malignant ascites and in primary GAC (FFPE) specimens, we are able to isolate DNA/RNA).

Figure 9. YAP1^{high} PC cells easily form PDXs in mice irrespective of PC tumor cell purity or EpCAM expression and Knockout YAP1 reduced tumorigenicity of YAP1^{high} PC cells in the PDX model

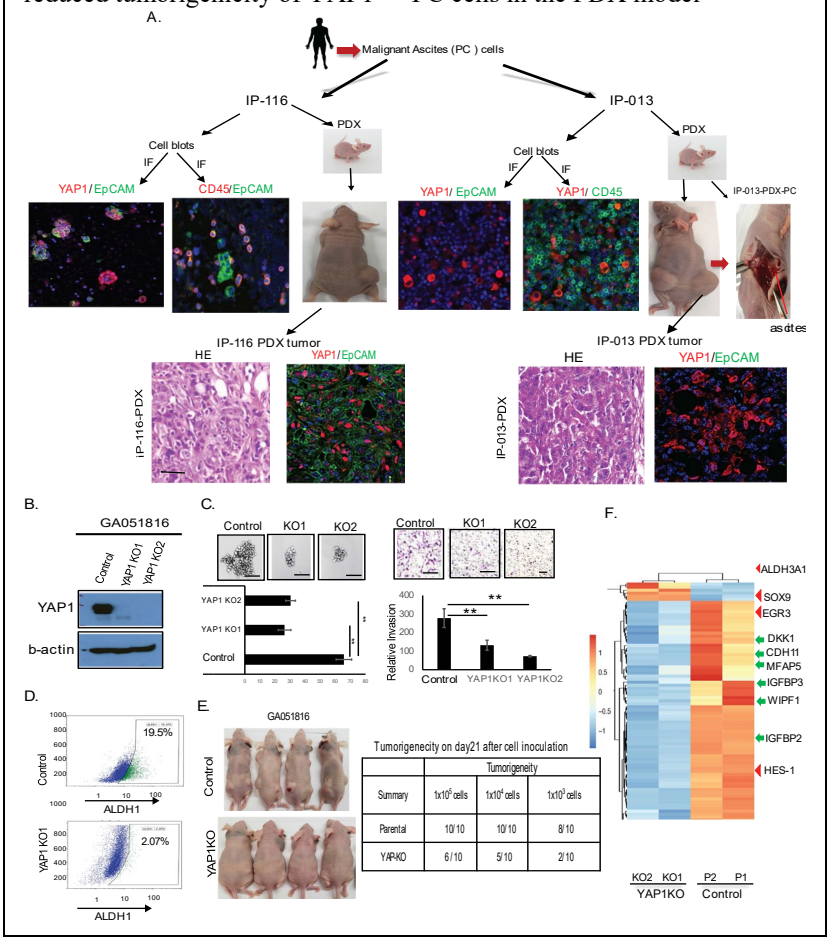
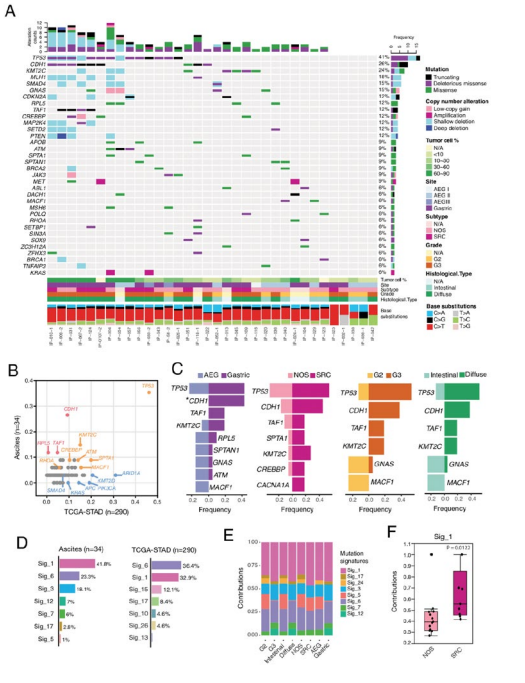
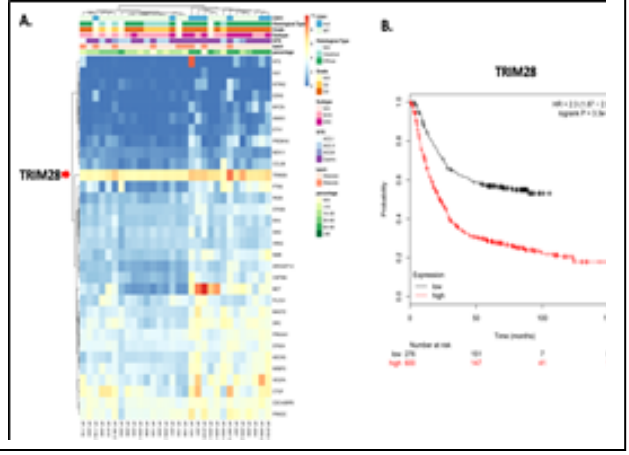


Figure 10. Somatic mutations and signatures of peritoneal carcinomatosis (PC) specimens



Whole Exome Seq (WES) showed more frequent *CDH1* alterations in PC cases than in (TCGA) primary GACs. We analyzed cytologically confirmed PC cells from GAC patients using WES. We found that the overall median mutation frequency was similar to TCGA primary GACs and esophageal carcinoma. Most altered genes in PC cells were *TP53 (41%)* and *CDH1* (26%) (**Fig. 10A**). *CDH1* was mutated at higher frequency in PC cases than in TCGA (26% vs. 9%; **Fig. 10B**). 8 out of 9 *CDH1* mutations were truncating or deleterious missense (by 12 prediction algorithms) and were in the cadherin domain (loss of function). *CDH1*

Figure 11. Bulk RNAseq data (A) show TRIM28 is highly expressed and prognostic (B) in large cohort.



mutations were exclusively observed in dGACs and 2.5 x higher in PC cells with signet ring cells (SRCs) (**Fig. 10C**). We identified targetable proteins when *CDH1* alterations(mutation/loss) were noted.

Figure 12. Single cell RNAseq shows TRIM28 enriched in tumor cell clusters (A) and highly expressed in GAC by MassSpec (B). TRIM24 not expressed in PC(C)

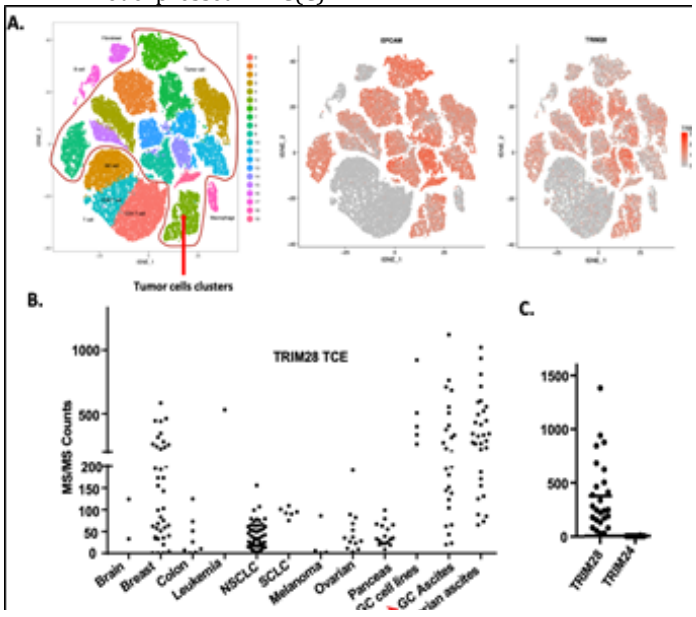
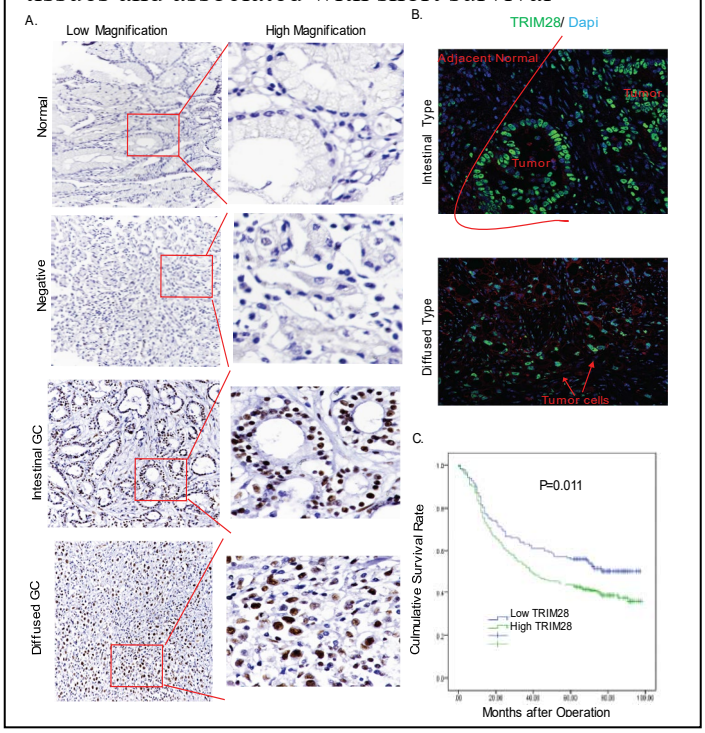


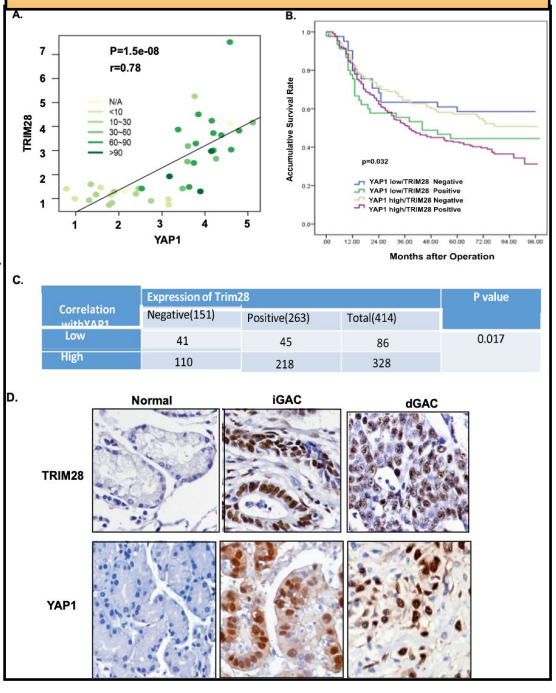
Fig. 13. TRIM28 is highly expressed in tumor tissues and associated with short survival



<u>scRNAseq and MS showed that TRIM28 was expressed mainly in tumor cells</u> Our scRNAseq analyses in 20 PC cases (containing tumor cells and other cells) found that TRIM28 was highly expressed in most tumor cell clusters but there was limited expression in stromal or immune cells (**Fig. 12A**). Through collaboration with Dr. Hanash, we performed MS on 26 PC cases and identified several potential protein targets that were highly enriched in PC-tumor cells. Among many proteins we noted that TRIM28 (**Fig. 12B**) but not TRIM24 (**Fig. 12C**) was enriched.

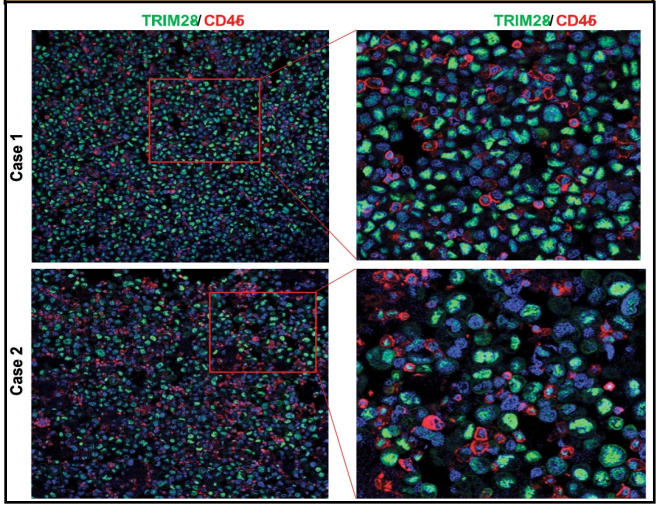
<u>TRIM28 is overexpressed in primary GACs by immunohistochemistry (IHC) and immunofluorescence (IF) and associated with poor outcome.</u> We stained TRIM28 by IHC using a >400-case GAC TMA and found that TRIM28 was significantly upregulated in both iGACs and dGACs and this was validated by IF; TRIM28 (green)(**Fig. 13**). TRIM28 localized in the nucleus of tumor cells and was significantly higher in the primary GACs vs. normal tissues. (**Figs. 13A&13B**). High expression of TRIM28 was associated with poor survival and aggressive phenotype (**Fig.13C**).

Fig. 15. TRIM28 expression correlates with YAP1 expression in PC samples and both high confer worse prognosis.



Correlation	Expression of Trim28			P value
	Negative(151)	Positive(263)	Total(414)	
Low	41	45	86	0.017
High	110	218	328	

Fig. 14. TRIM28 is highly expressed (nuclear) in GAC cells but not in immune cells.

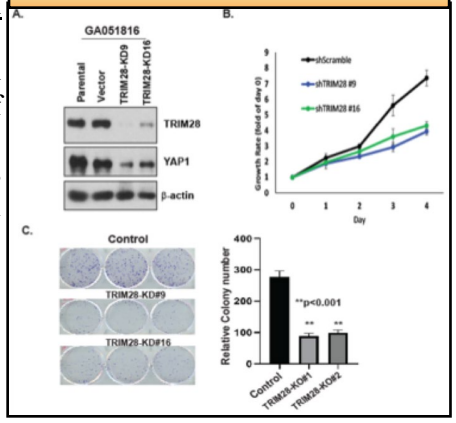


<u>TRIM28 significantly upregulated in PC samples.</u> To investigate if TRIM28 was also upregulated in mainly PC cells, we double stained TRIM28 (green) and CD45, an immune cell marker (red) by IF in >100 PC samples. As shown in **Fig. 14**,

TRIM28 was significantly over-expressed in tumor cells and but not in CD45 positive cells.

Positive correlation between TRIM28 and YAP1 in PC samples and combined expression conferred worse outcome. Analysis of RNAseq data from PC cases, showed that TRIM28 was highly associated with YAP1 expression (**Fig. 15A**) and expression of both was associated with worst prognosis (**Fig. 15B**). Expression of TRIM28 was significantly associated with YAP1 expression in this >400 GAC cohort (**Figs. 15C&15D**). Further, KD of TRIM28 in patient-derived cells dramatically decreased YAP1 expression and transcription along with reduced cell proliferation and colony formation (**Fig.16**).

Fig. 16. KD of TRIM28 reduced YAP1 activation/transcription (GAC).



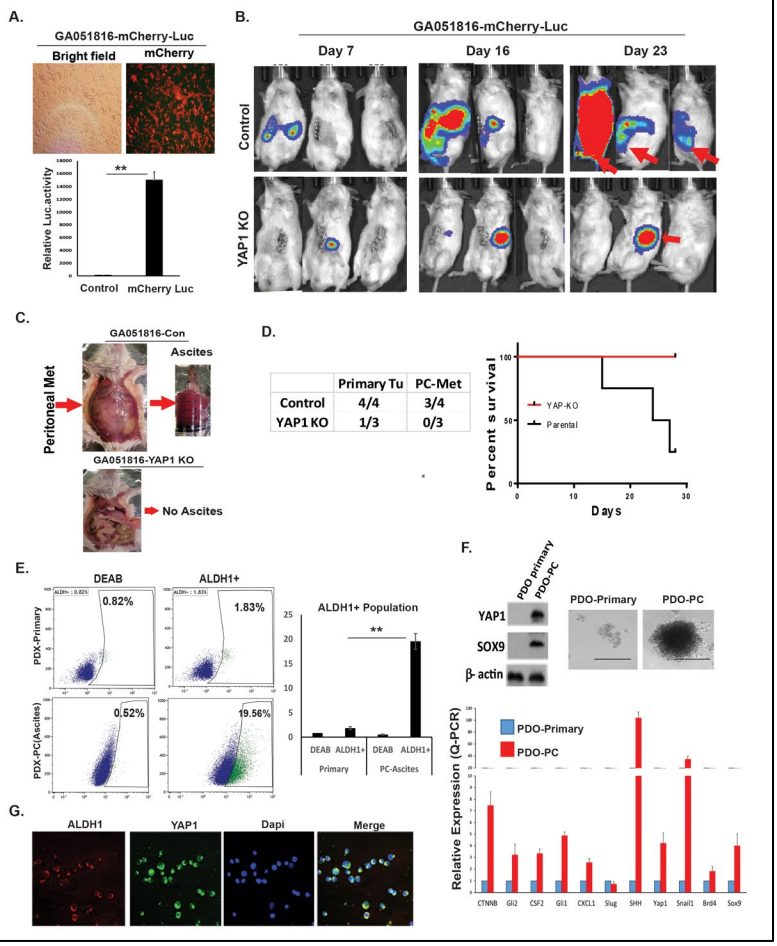
Aim 3. Preclinical evaluations (genetic and pharmacologic manipulations) of targets identified in Aim 2.

3A. Genetic modulations of the candidate targets in human PC cells emerging from Aim 2 in vitro to establish their functional relevance. 3B. In vitro studies of human PC cells with novel agents against CSC pathways and other novel targets emerging from Aim 2, 3C. in vivo studies with human PC PDX models (novel agents and genetic modulations of PC cells). This aim is focused on emerging targets (CSC or non-CSC) from Aim 2 and will carry out genetic manipulations and pharmacologic studies to validate exploitable therapeutic targets (in vivo and in vitro). Combinations will be emphasized. Novel agents will be identified for future clinical trials.

Knockout YAP1 reduced PC metastasis in the PDO model To further explore the role of YAP1 in PC, we transfected YAP1^{high} GA051816 cells with mCherry-Luciferase and confirmed high luciferase activity in mCherry-Luciferase GA051816 cells (Fig. 17A). mCherry-Luciferase GA051816 cells with or without depletion of YAP1 (1×10^5 cells) were mixed with Matrigel and injected into the stomach wall of NOD/SCID mice (PDO model). Tumor growth in the stomach wall and metastases to the peritoneal cavity or other organs were monitored by bioluminescence weekly. As shown in Figs. 17B-5D, among mice given control GA051816 cells, 100% (4/4) formed tumors in the stomach and 75% (3/4) had PC, while among mice given YAP1-knockout GA051816 cells, only 33% (1/3) formed a small tumor in the stomach, and none (0/3) had PC (Fig. 17D, left). Furthermore, YAP1 knockout significantly prolonged overall survival compared with that in mice with parental cells (Fig. 5D, right).

Further analyses of primary tumor cells with paired PC cells in the PDO models from YAP1^{high} control group, we found that the PDO-PC cells were enriched in CSCs with high YAP1 and SOX9 expression, formed larger tumor spheres, and had a high ALDH1+ population compared with the primary tumor cells (Figs. 17E&17F). The expression of YAP1 was strongly associated with that of ALDH1 (Fig. 17G). Similarly, other CSC genes were significantly upregulated in PC cells compared to primary tumor cells of the PDO model by Q-PCR (Fig. 17F, bottom).

Figure 17. YAP1 is essential for PC metastases in PDO model. **Knock out YAP1 reduced PC and increased survival**



Pharmacologic inhibition of YAP1 reduced CSC properties and PDX tumor growth

CA3, a novel specific YAP1 inhibitor, was reported to inhibit YAP1 in esophageal cancer. We aim to investigate whether pharmacologic inhibition of YAP1 by CA3 reduce CSC properties and PDX tumor growth in PC. We observed that CA3 suppressed YAP1 expression, tumor sphere formation, and ALDH1+ population in aggressive GA051816 cells (Fig.18A-6C). Three additional PC cells were also investigated. CA3 strongly suppressed YAP1 expression and reduced

tumor sphere formation in all three PC samples (Fig. 18D). Further, we noted that cells with YAP1^{high} expression were sensitive to CA3 at lower doses (0.25 μM and 0.5 μM), while two stable YAP1 knockout clones (YAP1 KO1 and YAP1 KO2) were resistant to CA3 (Fig.6E). In addition, CA3 effectively suppressed colony formation in YAP1^{high} GA051816 cells but not in MKN45 GC cells with no YAP1 (Fig. 18F). These data indicate that the activity of CA3 depends on YAP1 expression.

The specificity of CA3 targeting of YAP1 was also demonstrated *in vivo* PDX, in which CA3 at only 1 mg/kg three times per week significantly suppressed the growth of implanted YAP1^{high} GA051816 cells (Figs. 19A&19B) but had minimal effect in MKN45 (no YAP1) cells (Figs. 19D) without side effects (body weight reduction) in both GA0518 and MKN45 models (Fig.19C&19E). Moreover, when CA3 was combined with the commonly used docetaxel, the reduction in PDX growth was significantly amplified (Figs. 19F). Dual staining of YAP1/EpCAM and Ki67/SOX9 in these PDX tumors revealed that CA3 dramatically decreased the expression of YAP1 and SOX9 in CA3 group, while docetaxel was less effective on inhibition of YAP1 and SOX9 expression but more effective in reducing Ki67^{+} proliferating cells. The combination of CA3 and docetaxel utmost suppressed expression of both YAP1/SOX9 and Ki67+ population (Fig. 19G) suggesting a great value of targeting YAP1 in combination with cytotoxics in GAC patients with PC.

Sensitivity to cytotoxic agents and novel targeted agents *in vitro* and *in vivo* To evaluate the suitability of the cell lines and improved PDO model for drug testing, we first examined the sensitivity of the new cell lines to agents commonly used in the clinic by performing MTS cell survival assays (Figure 19A). In addition to the cytotoxic agents 5-fluorouracil and docetaxel, we tested the targeted agents erlotinib (EGFR inhibitor) and RAD001 (an FDA improved mTOR inhibitor). As shown in Figure 20A, the three cell lines had varied sensitivity to these four agents. GA0518 cells were highly sensitive to docetaxel, with a half maximal inhibitory concentration (IC_{50}) of 13.39 nM but were less sensitive to RAD001 and 5-fluorouracil, with IC_{50} values of 45.03 nM and 28.91 nM, respectively, and were resistant to erlotinib, at an IC_{50} of 261.9 nM. Similarly, GA0804 cells were sensitive to docetaxel, with an IC_{50} of 13.82 nM, but less sensitive to the other three drugs (Supplemental Figure 19B). Interestingly, GA0825 cells were highly sensitive to RAD001, with the lowest IC_{50} of 12.42 nM, and were sensitive to docetaxel, with an IC_{50} of 14.05

Figure 18. Pharmacologic inhibition of YAP1 specifically suppress patient-derived cells growth and colony formation in YAP1 high cells

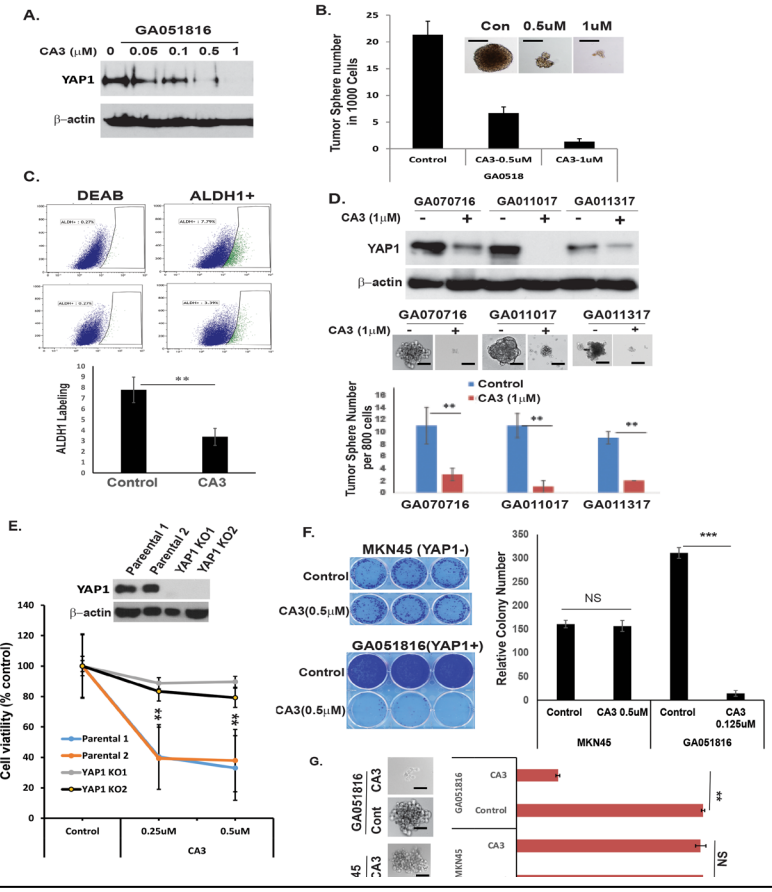
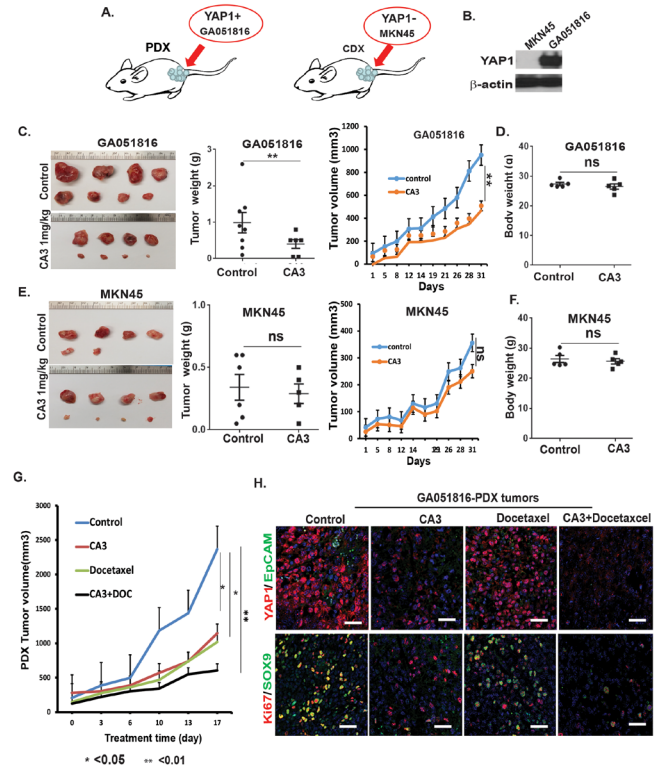
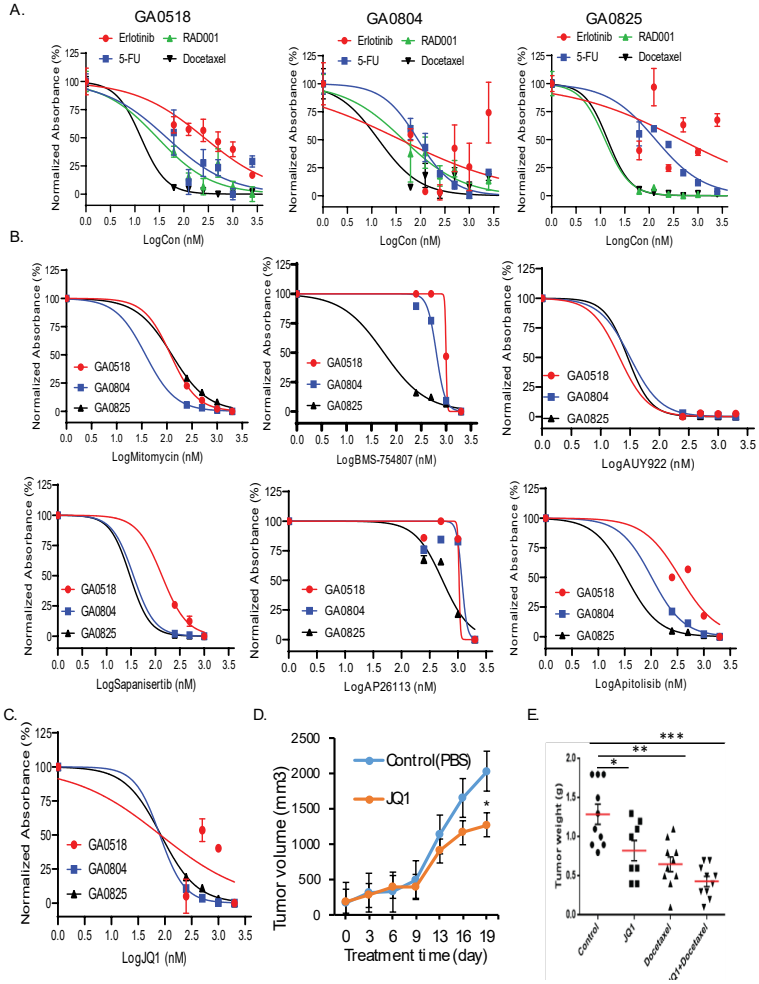


Figure 19. Pharmacologic inhibition of YAP1 significantly inhibit PDX tumor growth in YAP1 high PC cells *in Vivo*



nM, but were resistant to the other two drugs (Figure 7A). In addition, we tested seven additional cytotoxic and target inhibitors, including mitomycin, sapanisertib (mTOR inhibitor), apitolisib (PI3K inhibitor), BMS-754807 (IGF-1R inhibitor), AP26113 (EGFR inhibitor), AUY922 (HSP90 inhibitor), and JQ1 (inhibitor of BET family members including BRD2, BRD3, and BRD4). As shown in Figure 20B, although responses to the inhibitors varied widely, the cell lines were generally the most sensitive to AUY922 and mitomycin (Figure 20B). Cells were also relatively sensitive to JQ1 (Figure 20C). We then validated the *in vitro* results in the *in vivo* PDX model, studying GA0518 PDXs with JQ1 alone or in combination with docetaxel. As shown in Figure 19D, JQ1 at 10 mg/kg three times per week significantly suppressed tumor growth, and its anti-tumor activity was significantly amplified in combination with docetaxel (Figure 20E). These results suggest that JQ1 in combination with docetaxel could be a potentially effective strategy worth testing in the clinic and that GA0518 cells are a good model for drug testing *in vitro* and *in vivo*.

Figure 20. Sensitivity to cytotoxic agents and targeted agents *in vitro* and *in vivo*



CA160445P1: PI(Dr. Samir Hanash)

Team effort was primarily directed at Specific Aims 2 and 3, in which multi-omics profiling was applied for identification of novel therapeutic targets in human PC cells. Specifically, mass-spectrometry based proteomics was performed on 38 ascites-derived cell samples and 6 gastric cancer cell lines. These included in-depth profiling of total cell extracts, cell surfaceome and conditioned media and ascites-resident exosomes and extracellular vesicles (EVs) with the overall goal of delineating cell targetable as well as vesicle-associated protein markers and mediators of peritoneal metastasis.

Through these comprehensive analyses, identification of more than 15,000 gene products from total cell lysate and cell surface compartments was achieved. Profiling of GC cell line EVs resulted in identification of 4,600 protein features. These datasets have been archived within a searchable repository, providing a resource that continues to be mined and intersected with new orthogonal omic-datasets to provide additional targets as well as biological insights into the mechanisms underlying metastatic progression in gastric adenocarcinoma.

Within the scope of this project, focused exploration of these datasets was performed to uncover features that exhibited surface enrichment in both gastric cancer ascites cell samples and cell lines. This resulted in an initial panel of 49 candidate surface enriched proteins that were ultimately prioritized for further study with respect to potential therapeutic utility. Ingenuity Pathway Analyses (IPA) indicated (Figure X1) that these surface features are members of canonical pathways and functional networks converging on interferon gamma (IFNG) signaling, a known promoter of EMT transition and PD-L1 expression in many cancers.

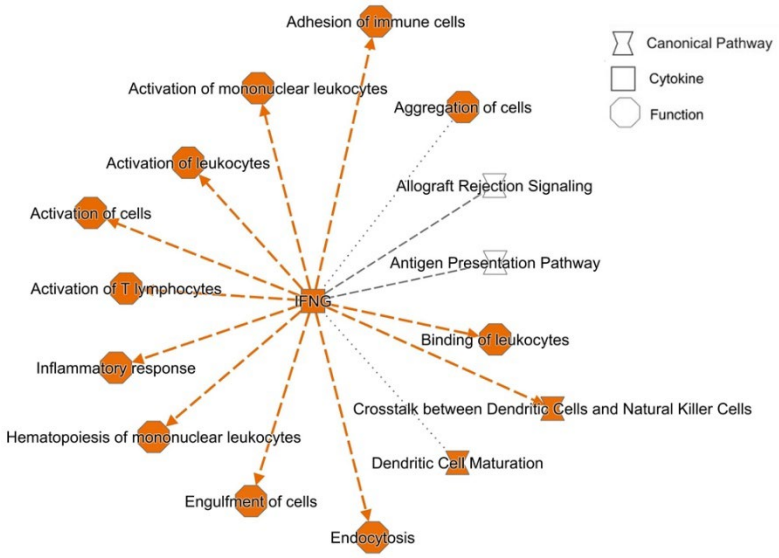


Figure X1. IPA Core Analysis Graphical Summary of surface-enriched features identified via proteomic profiling of ascites-derived GC cells and GC cell lines.

Significantly represented IPA Canonical Pathways in the GC cell surfacome include *Antigen Presentation*, *Immune Response*, Immune Cell *Signaling* and *Development*, and *PD-1, PD-L1 Cancer Immunotherapy* Pathways (*p*-value: 1.86E-08 – 7.41E-05). IPA interaction network analyses revealed relationships between the 49 candidate GC surface features annotated to functions including *Cell-To-Cell Signaling and Interaction*, *Cellular Function and Maintenance*, *Cellular Morphology* as well as *Immune Cell Trafficking* and *Hematological System Function* and *Development* (*Network scores*: 31 – 20).

This candidate surface-enriched feature panel includes membrane-localized proteins that i) were previously identified in tumor tissues but not normal tissues, ii) exhibited concordant elevations in mRNA expression based off analysis of TCGA datasets and iii) were associated with statistically significantly poor prognosis in diffuse type gastric cancer. This represents a major milestone accomplishment in characterizing the cancer cell surface in the context of gastric cancer and provides and abundance of novel points for therapeutic targeting towards potential interception of effectors involved in metastatic progression of GC. Full development of these candidates was beyond the means of this project; however they will continue to be investigated in future studies.

Workflows for high purity exosome isolation from ascites were established during the project and continue to be applied to provide comprehensive proteomic and metabolomic characterization of extracellular vesicles extant within the ascites milieu. Data from GC cell line and patient ascites EV campaigns indicates multiple candidates for mechanistic exploration and potential therapeutic targeting. Achievement of these standardized EV isolation and MS profiling workflows positions the group for continued exploration and provides a facile means for additional discovery.

Proteomic profiling of ascites-derived EV samples yielded identification of approximately 800 EV-associated protein features. Bioinformatic assessment of these features was performed. Gene Ontology (GO) analyses of the EV-associated protein datasets indicated GO Cellular Component annotations with the highest significance to be *extracellular region*, *organelle*, *vesicle* and *exosome* as well as *blood microparticle* (*p*-value: 3.97E-27 – 6.00E-18), additionally confirming that our sample processing workflow yields isolates highly enriched in EVs/exosomes. Ingenuity Pathway Upstream Regulator Analyses of the features indicated that the proteins conveyed by EVs into the ascites milieu were most significantly annotated as downstream effectors of known tumor and neuroendocrine pathways. The top five IPA upstream regulators were APP, PSEN1, MAPT, TGFB1 and TP53 (*p*-value: 3.98E-41 – 6.29E-29).

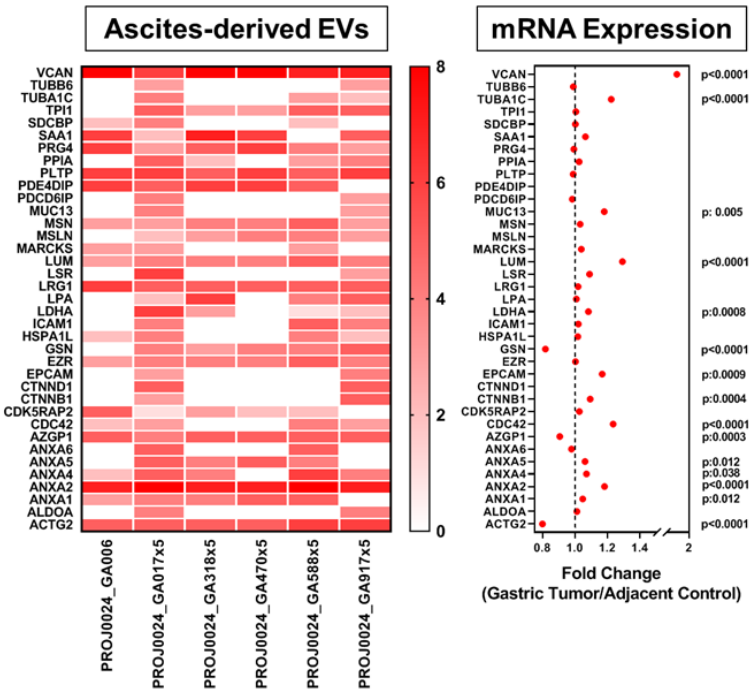


Figure X2. GC ascites-derived EV-associated GC features (l); Corresponding mRNA expression in the Cui Gastric Cancer Cohort (r).

Among the protein features identified in the EV compartment with high abundance and representation across multiple patient samples (Figure X2) are canonical EV markers SDCBP and PDCD6IP/ALIX as well as proteins with previously described in tumor progression, matrix remodeling, invasion and metastasis, onco-metabolism, immunomodulation and EMT / cancer stemness. These features were compared to mRNA expression data for the Cui Gastric Cohort (Oncomine Database). The dataset consisted of 80 gastric tumors and 80-matched adjacent control tissues. *P*-values were determined using 2-sided paired t-tests. Many of the EV-associated features were highly enriched in GC tumor relative to adjacent control tissues, indicative of cancer cell origin of EVs isolated from ascites and proteomically profiled. Profiling of ascites-derived EVs from additional samples and iterative data analyses continues and with the goal to develop a fully curated candidate list to additionally support mechanistic exploration of peritoneal carcinomatosis in the context of GAC.

Based on our proteomic profiling and bioinformatic evaluation efforts within this project, Protein Y was prioritized as a candidate for development as a therapeutic target. Focus was made on in vitro studies to determine the functional relevance of the protein in gastric cancer. A summary of the key accomplishments in this regard are as follows: (i) Binding partner proteins of Protein Y were identified based on co-immunoprecipitation and suggest and interaction of Protein Y with cell structural proteins; (ii) Knock-down of Protein Y was shown to induce a decrease in expression of cancer stem cell-associated proteins based on quantitative proteomics profiling; these studies also identified an

endocytosis-related protein signature; (iii) Small-molecule inhibitor targeting against Protein Y was demonstrated to suppress cell invasion, migration, and proliferation.

Precision therapeutic targeting of GC via surface Protein Y was also pursued via development of antibody mediated drug delivery. Antibody-internalization protocols and antibody-drug conjugate (ADC) constructs were developed and preliminary in vitro assays were performed test the capacity of Protein Y to serve as an ADC-target. Antibody-internalization was confirmed and evaluation of the IC_{50} of the Protein Y-ADC in multiple gastric cancer cell lines showed improved efficacy compared IgG control-ADC (Figure X3). In vivo ADC tumor targeting experiments were planned, but were unable to be completed due to mandatory institutional lab shutdowns. Testing of additional anti-Protein Y monoclonal antibody candidates with improved target avidity continues.

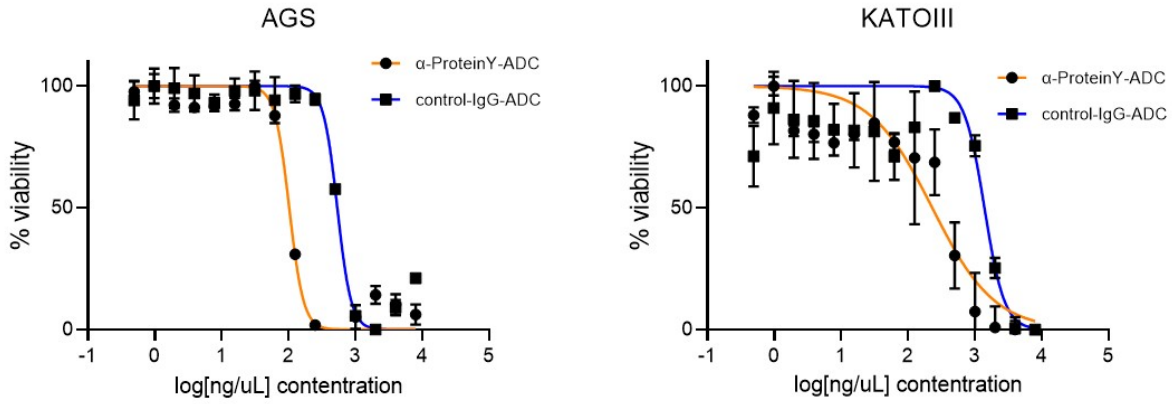


Figure X3. Antibody-drug conjugate targeting of surface Protein Y in gastric cancer cell lines.

CA160445 P2 (PI: Dr.George Calin)

Calin's laboratory contributed in the 3 years period of this project to each of the aims, mostly to the Aim 3 as followings:
Aim 2. Identification of additional novel therapeutic targets (CSC and non-CSC) in human PC cells through a multi-omics platform.
We performed a non-coding gene expression arrays using an in-house build array in collaboration with Agilent containing probes for all 482 ultraconserved genomic regions larger as 200 nucleotides, on patient samples comparing ten normal gastric cancer (GC) samples with ten ascites samples from peritoneal carcinomatosis of GC. One of the top up-regulated non-coding transcripts in peritoneal carcinomatosis was the highly conserved long non-codingRNA named CCAT2 (P=0.0115), that was previously cloned in Calin's laboratory (see Ling et al, Genome Res. 2013 Sep;23(9):1446-61. PMID: 23796952).

Aim 3. Preclinical evaluations (genetic and pharmacologic manipulations) of targets identified in Aim 2.
We found that overexpression of *CCAT2* in gastric cancer cells promotes CIN and carcinogenesis, by stabilizing and inducing expression of BOP1 an activator of AURKB.
In addition to the studies presented above for Aim 2, we further analyzed the *CCAT2* lncRNA expression in two pairs of GC patients derived xenografts (PDXs) and one GC patient derived organoid (PDO). In each of them *CCAT2* was up-regulated compared to parental cells, showing that successful engraftment, a marker of poor prognosis, is associated with high *CCAT2* levels. Next, we did chromosomal instability CINdex analysis using the GC TCGA cohort and identified that aurora family and PeBoW complex genes positively correlated with CIN at chromosome level. Additionally, we used the primary GC cells, AGS, with euploid chromosomal number and KATO III with a tetraploid chromosome number. The RNA expression levels of *CCAT2* and the ribosomal biogenesis factor *BOP1* and the protein levels of BOP1, a component of the PeBoW complex, and pAURKB were higher in KATO III compared to AGS. These data imply that the identified CCAT2-related mechanism relates to chromosomal instability (CIN) more generally. These data and many others were published in Chen B, et al, Gastroenterology 2020 PMID: 32805281.

We further presented the translational opportunities related to the study of ncRNAs in a high impact journal review, see Anfossi et al, Nature Reviews Clinical Oncology 2018. Due to their large numbers and stability, circulating ncRNAs have

the potential to represent suitable and non-invasive blood-based biomarkers which can provide information on the biology of tumor and the effects of treatments, such as targeted therapies and immunotherapies. Increasing evidences highlighted the abilities of ncRNAs to regulate gene expression outside the cell of origin through their transfer to recipient cells by extracellular vesicle-mediated cell-to-cell communication. Recently, it has been shown that xeno-miRNAs codified by non-human genomes and present in human body fluids can be used as biomarkers. We presented the latest developments in the use of circulating ncRNAs as prognostic and predictive biomarkers and discusses their role in cell-to-cell communication. We included a compendium of microRNAs and long ncRNAs reported in literature to be present in body fluids and to have the potential to be used as diagnostic and prognostic biomarkers for cancer patient's management.

The major accomplishement was the discovery of a new mechanism of chromosomal instability in gastric cancer that is common also to colon cancer and therefore can be targeted therapeutically for gastrointestinal cancers. The development of RNA therapeutics targeting the oncogenic CCAT2 is a major topic for the continuation of the research developed in this project.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The research projects supported by the Funding have provided training and professional development opportunities for Graduate student and post-doctoral fellows with both basic science and clinical background. They are able to develop their knowledge, skills and expertise in the designed research field. They made a tremendous academic growth through participating the studies in this proposal.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Interim results have been disseminated to communities of interest primarily at monthly team meeting, local and inter-instructional public seminars as well as scientific meetings. We had several publications associated with this funding have been in PUBMED. Final results will be published in peer-reviewed journals and presented at international scientific meetings. We have presented our findings in local, national meeting and international meeting. We will continue the analyses of the profiling data and functional data and will prepare for manuscripts and abstracts for meetings.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

N/A

IMPACT:

Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our RNA Seq and Single cell Seq data thus far generated some important tumor cell targets as well as novel immune checkpoint targets which led to a publication in GUT and two additional manuscripts were submitted and under revision and revision in Nature medicine and GUT respectively. The functional studies of CSCs/YAP1 led to a publication in GUT 2020. The proteomic data resulting from the project activities is a fundamental component in establishing a complete molecular signature of drivers of metastatic disease in the context of GAC. Initial analyses of total cell and surface compartments has yielded novel surface protein candidates that are currently being further validated. Importantly, the integrated molecular profiling efforts of this collaborative project will facilitate development of new and exploitable therapeutic targets for peritoneal carcinomatosis. The new patients derived cell lines and PDX/PDO models as well as novel targets-TRIM28 and GRK3 are being summarized or going to summarized for several more manuscripts for potential publications. The metastatic signature identified in this study could be applied to other tumor types on variety of metastatic sites.
Chromosomal instability (CIN) is a carcinogenesis event that promotes metastasis and resistance to therapy, by unclear mechanisms. Strategies to target this pathway might be developed for treatment of patients with microsatellite stable gastrointestinal (gastric and colorectal) tumors. Deciphering the CCAT2 involvement in the initiation of the CIN offers a new target for therapeutic development. This is important as CCAT2 is involved in the initiation of CIN and therefore its targeting can have therapeutic effects early in tumorigenesis.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Novel targets are applicable and provide strong rationale for potential clinical trials.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *Transfer of results to entities in government or industry;*
- *Instances where the research has led to the initiation of a start-up company; or*
- *Adoption of new practices.*

The results of the project are likely to be translatable into clinical practice to improve outcomes for patients with gastric adenocarcinoma or other cancers

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *Improving social, economic, civic, or environmental conditions.*

CHANGES/PROBLEMS:

The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipate

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

PRODUCTS:

List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Dragomir MP, Knutsen E, Calin GA. SnapShot: Unconventional miRNA Functions. Cell. 2018 Aug 9;174(4):1038-1038.e1. doi: 10.1016/j.cell.2018.07.040.
2. Wang R, Song S, Harada K, Ghazanfari Amlashi F, Badgwell B, Pizzi MP, Xu Y, Zhao W, Dong X, Jin J, Wang Y, Scott A, Ma L, Huo L, Vicente D, Blum Murphy M, Shanbhag N, Tatlonghari G, Thomas I, Rogers J, Kobayashi M, Vykoukal J, Estrella JS, Roy-Chowdhuri S, Han G, Zhang S, Mao X, Song X, Zhang J, Gu J, Johnson RL, Calin GA, Peng G, Lee JS, Hanash SM, Futreal A, Wang Z, Wang L, Ajani JA. Multiplex profiling of peritoneal metastases from gastric adenocarcinoma identified novel targets and molecular subtypes that predict treatment response. GUT, 2020 Jan;69 (1):18-31 PubMed PMID: 31171626. We have acknowledged the support from DOD.
3. Diverse Cell-of-Origin of Gastric Cancer Defines Intratumoral Heterogeneity. Ruiping Wang, Shumei Song2, Kazuto Harada2, Guangchun Han1, Melissa Pool Pizzi2, Meina Zhao2, Ghia Tatlonghari2, Shaojun Zhang, Yuanxin Wang, Shuangtao Zhao, Brian D. Badgwell, Mariela Blum Murphy, Namita Shanbhag, Jeannelyn S. Estrella, Sinchita Roy-Chowdhuri, Ahmed Adel Fouad Abdelhakeem, Guang Peng, George A. Calin, Samir Hanash, Alexander J. Lazar, Andrew Futreal, Jaffer A. Ajani*, Linghua Wang*. Nature Medicine. 2020, Accepted, in press.
4. Ajani JA, Xu Y, Huo L, Wang R, Li Y, Wang Y, Pizzi MP, Scott A, Harada K, Ma L, Yao X, Jin J, Zhao W, Dong X, Badgwell BD, Shanbhag N, Tatlonghari G, Estrella JS, Roy-Chowdhuri S, Kobayashi M, Vykoukal JV, Hanash SM, Calin GA, Peng G, Lee JS, Johnson RL, Wang Z, Wang L, **Song S*.** YAP1 mediates gastric adenocarcinoma peritoneal metastases that are attenuated by YAP1 inhibition. Gut. 2020 Apr 27:gutjnl-2019-319748. doi: 10.1136/gutjnl-2019-319748. Online ahead of print. PMID: 32345613; Affiliations: 1 Department of Gastrointestinal Medical Oncology, 3 Genomic Medicine, 4 Surgical Oncology, 5 Pathology, 6 Clinical Cancer Prevention, 7 Experimental Therapeutics, 8 Systems Biology, 9 Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. 2 Department of Surgical Oncology and General Surgery, First Hospital of China Medical University.
5. Xu Y, Song S, Wang Z, Ajani JA*. The role of hedgehog signaling in gastric cancer: molecular mechanisms, clinical potential, and perspective. Cell Commun Signal. 2019 Nov 27;17(1):157.
6. Xu Y, Li Y, Jin J, Han G, Sun C, Pizzi MP, Huo L, Scott A, Wang Y, Ma L, Lee JH, Bhutani MS, Weston B, Vellano C, Yang L, Lin C, Kim Y, MacLeod AR, Wang L, Wang Z, Song S, Ajani JA. LncRNA PVT1 up-regulation is a poor prognosticator and serves as a therapeutic target in esophageal adenocarcinoma. Mol Cancer. 2019 Oct 10;18(1):141.
7. Song S, Wang Z, Li Y, Ma L, Jin J, Scott AW, Xu Y, Estrella JS, Song Y, Liu B, Johnson RL, Ajani JA. PPARδ Interacts with the Hippo Coactivator YAP1 to Promote SOX9 Expression and Gastric Cancer Progression. Mol Cancer Res. 2020, 18(3):390-402
8. Jin J, Xu Y, Huo L, Ma L, Scott AW, Pizzi MP, Li Y, Wang Y, Yao X, Song S*, Ajani JA*. An improved strategy for CRISPR/Cas9 gene knockout and subsequent wildtype and mutant gene rescue. PLoS One. 2020;15(2):e0228910. eCollection 2020. PMID: 32053639
9. Song S, Li Y, Xu Y, Ma L, Pool Pizzi M, Jin J, Scott AW, Huo L, Wang Y, Lee JH, Bhutani MS, Weston B, Shanbhag ND, Johnson RL, Ajani JA*. Targeting Hippo coactivator YAP1 through BET bromodomain inhibition in esophageal adenocarcinoma. Mol Oncol. 2020;14(6):1410-1426. PMID: 32175692.
10. Yao X, Ajani JA and Song S. Molecular biology and immunology of gastric cancer peritoneal metastasis. Review article. In press. 2020 Translational Gastroenterology & Hepatology Accepted: 03 February 2020.
11. Jin J, Huo L, Song S and Ajani JA. CRISPR/Cas9 Technology Improvements and the RNA-Editing Trend. Journal of Molecular Biology and Biotechnology. 2020. Vol.5 No.01:02. In press. Received date: April 11, 2020; Accepted date: April 16, 2020; Published date: April 23, 2020.
12. Murphy B, Ikoma N, Wang X, Estrella J, Roy-Chowdhuri S, Das P, Minsky BD, **Song S**, Mansfield P, Ajani JA, Badgwell B. Phase I Trial of Hyperthermic Intraperitoneal Chemoperfusion (HIPEC) with Cisplatin, Mitomycin, and Paclitaxel in Patients with Gastric Adenocarcinoma and Associated Carcinomatosis or Positive Cytology. Ann Surg Oncol 2020. In press. Published online: Jan.23,2020.
13. Li Y, Zhen N, Ajani JA and **Song S**. Drug Resistance and Cancer Stem Cells. Review article. Cell Commun Signal. 2020. Accepted. In Press.
14. Chen B, Dragomir MP, Fabris L, Bayraktar R, Knutsen E, Liu X, Tang C, Li Y, Shimura T, Ivkovic TC, De Los Santos MC, Anfossi S, Shimizu M, Shah MY, Ling H, Shen P, Multani AS, Pardini B, Burks JK, Katayama H, Reineke LC, Huo L, Syed M, **Song S**, Ferracin M, Oki E, Fromm B, Ivan C, Bhuvaneshwar K, Gusev Y, Mimori K, Menter D, Sen S, Matsuyama T, Uetake H,Vasilescu C, Kopetz S, Parker-Thornburg J, Taguchi A, Hanash SM, Girnita L, Slaby O, Goel A,Varani G, Gagea M, Li C, Ajani JA, Calin GA. The Long Noncoding RNA CCAT2 induces chromosomal instability through BOP1 - AURKB signaling. Gastroenterology. 2020. Epub 2020/08/18. PubMed PMID: 32805281.
15. Chen B, Dragomir MP, Fabris L, Bayraktar R, Knutsen E, Liu X, Tang C, Li Y, Shimura T, Ivkovic TC, De Los Santos MC, Anfossi S, Shimizu M, Shah MY, Ling H, Shen P, Multani AS, Pardini B, Burks JK, Katayama H, Reineke LC, Huo L, Syed M, Song S, Ferracin M, Oki E, Fromm B, Ivan C, Bhuvaneshwar K, Gusev Y, Mimori K, Menter D, Sen S, Matsuyama T, Uetake H, Vasilescu C, Kopetz S, Parker-Thornburg J, Taguchi A, Hanash SM, Girnita L, Slaby O, Goel A, Varani G, Gagea M, Li C, Ajani JA, Calin GA. The Long Noncoding RNA CCAT2 induces chromosomal instability through BOP1 - AURKB signaling. Gastroenterology. 2020 Aug 14:S0016-5085(20)35057-5. doi: 10.1053/j.gastro.2020.08.018. Online ahead of print. PMID: 32805281
16. Anfossi S, Babayan A, Pantel K, Calin GA. Clinical utility of circulating non-coding RNAs - an update. Nat Rev Clin Oncol. 2018 Sep;15(9):541-563. doi: 10.1038/s41571-018-0035-x. PMID: 29784926

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

N/A

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

By CA160445

1. Harada K[*], Song S[*#], Xu Y, Badgwell B, Pizzi M, Jin J, Wang Y, Scott AW, Ma L, Amlashi F, Kobayashi
2. M, Vykoukal JV, Ivan C, Estrella JS, Chowdhuri SR, Calin GA, Hanash S, Lee JS, Liu B, Ajani JA# Genomic Profiling of Metastatic Gastric Adenocarcinoma AACR, Chicago, April 16-19, 2018;.
3. Pizzi M, Harada K, Ivan C, Jin J, Li Y, Scott AW, Wang Y, Ma L, Xu Y, Badgwell BD, Ray-Chowdhuri S, Wang ZN, Calin GA, Song S, Ajani JA. MicroRNA profiling of primary and peritoneal metastases of Gastric Adenocarcinoma identified miR650 that suppresses invasion. AACR, March29-April 3, 2019 Atlanta, Georgia.
4. Song S, Xu Y, Huo L, Li Y, Wang R, Pizzi M, Scott AW, Wang Y, Jin J, Ma L, Badgwell BD [3], Zhao W, Estrella JS, Ray-ChowdhuriS, Wang LH, Ajani JA. Development and Characterization of Patient-derived Aggressive Gastric Adenocarcinoma Cell Lines and Orthotopic Mouse Model from Peritoneal Carcinomatosis for Preclinical Research. AACR virtual meeting 2020.
5. Ajani JA, Xu Y, Huo L, Li Y, Wang R, Wang Y, Pizzi M, Scott AW, Harada K, Ma L, Yao X, Jin J, Zhao W, Dong X, Badgwell BD, Shanbhag ND, Estrella JS, Roy Chowdhuri S, Kobayashi M, Vykouka JV, Hanash S, Calin GA, Peng G, Lee JS, Johnson RL, Wang ZN, Wang LH& Song S. Hippo Coactivator YAP1 is Essential for Peritoneal Metastases in Gastric Adenocarcinoma and Targeting YAP1 is a Novel Therapeutic Strategy. AACR virtual meeting 2020.

Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

Technologies or techniques

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

Other Products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*

Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

For- CA160445P2.

Name: George A. Calin, MD, PhD
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 274270578
Nearest person month worked: 2
Contribution to Project: Oversee the full spectrum of experiments, specifically the anti-miR-10b in vitro and in vivo small molecules targeting experiments to understand the safety of this new type of therapy.
Funding Support:

Name: Cristina Ivan, PhD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Perform all data analyses and the statistical analyses necessary for the data interpretation, including analyze of CLIA laboratory date profiling from the clinical trial samples.
Funding Support:

Name: Simone Anfossi, PhD
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Responsible for medicinal synthesis and SAR studies for lead optimization, perform rational molecular design and establish SARs of the lead compound and derivatives.
Funding Support:

Name: Chunlai Li, PhD
Project Role: Instructor
Researcher Identifier (e.g. ORCID ID): 318979889
Nearest person month worked: 6
Contribution to Project: Responsible for the experiments related to the interaction of CCAT2 with the BOP1 and other proteins and also for the in vivo experiments performed on the carcinogenic model.
Funding Support:

Name: Meng Chen
Project Role: Research Assistant I
Researcher Identifier (e.g. ORCID ID): 224495904
Nearest person month worked: 3
Contribution to Project: Responsible for the statistical analyses of the data generated in Calin's laboratory and during the collaborations with the memebers of the Ajani's and Hanash's laboratories/
Funding Support:

For CA160445P1

Name:	*Dr. Samir Hanash*
Project Role:	*PI*
Nearest person month worked:	*1*
Contribution to Project:	Oversight of the project and coordinated activities with co-investigators
Name:	*Jody Vykoukal*
Project Role:	*Sr. Research Scientist*
Nearest person month worked:	*1*
Contribution to Project:	*Develop and apply novel, optimized workflows for the isolation of extracellular vesicles from ascites, serum, plasma, cell line conditioned media, and other biofluids, as well as companion procedures that enable molecular profiling of circulating vesicle-associated protein and nucleic acid markers in the context of gastric cancer.*
Name:	*Yihui Chen*
Project Role:	*Post Doc*
Nearest person month worked:	*1 person month*
Contribution to Project:	*Protein extraction from gastric cancer ascites fluid-derived tumor cells and sample prep for LCMS proteomics platform. Pickup novel therapeutic target for gastric cancer from LCMS data and perform candidate validation using proteomics, pathology, and molecular biology methods.*
Funding Support:	

For CA160445

Name: *Jaffer Ajani*
Project Role: *Principal Investigator*
Nearest person month worked: *.6*
Contribution to Project: *Oversight of the project and coordinated activities with co-investigators. Responsible for the overall administration and direction of the grant.*

Name: *Shumei Song*
Project Role: *Co-Investigator*
Nearest person month worked: *1.8*
Contribution to Project:. *Responsible for the proposed studies in in vitro and in vivo and the functional studies. She defines the novel biomarkers or targets from the related research. She is supervising the postdoctoral fellows and Research Investigators and other personnel for the studies in this proposal.*
Funding Support:

Name: *Ailing Scott*
Project Role: *Procurement Spec*
Nearest person month worked: *2.4*
Contribution to Project: *Responsible for general experiments such as cell culture, Western blotting and establishment of cell lines from animals and tumor tissues and perform functional studies including Cell growth inhibition (MTS), tumor sphere and tumorigenecity in vivo under the supervision of Dr. Song.*
Funding Support:

Name: *Jiankang Jin*
Project Role: *Research Investigator*
Nearest person month worked: *1.8*
Contribution to Project: *Responsible for tissue collection and RNA/DNA isolation and Q-PCR from cell lines and tissues. He also facilitates some of the clinical trial data coordination.*
Funding Support:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: N/A

APPENDICES:
N/A