

# Suppression of pancreatic cancer growth and metastasis by HMP19 identified through genome-wide shRNA screen

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Therapeutic effectiveness against metastatic or even locally advanced pancreatic ductal adenocarcinoma (PDAC) is dismal, with 5-year survival less than 5%. Even in patients who undergo potentially curative resection, most patients' tumors recur in the liver. Improving therapies targeting or preventing liver metastases is crucial for improving prognosis. To identify genes suppressing metastasis, a genome-wide shRNA screen was done using the human non-metastatic PDAC cell line, S2-028. After identification of candidates, functional validation was done using intrasplenic and orthotopic injections in athymic mice. HMP19 strongly inhibited metastasis but also partially attenuated tumor growth in the pancreas. Knockdown of HMP19 increased localization of activated ERK1/2 in the nucleus, corresponding to facilitated cell proliferation, decreased p27<sup>Kip1</sup> and increased cyclin E1. Over-expression of HMP19 exerted the opposite effects. Using a tissue microarray of 84 human PDAC, patients with low expression of HMP19 showed significantly higher incidence of liver metastasis ( $p = 0.0175$ ) and worse prognosis ( $p = 0.018$ ) after surgery. HMP19, a new metastasis/tumor suppressor in PDAC, appears to alter signaling that leads to cell proliferation and appears to offer prognostic value in human PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related mortalities.<sup>1,2</sup> Despite improvements in detection and management of PDAC, only ~4% of patients live 5 years post-diagnosis,<sup>3</sup> underscoring the need to develop more effective therapies. PDAC exhibits disproportionately high mortality because of its propensity to invade adjoin-

ing tissues and disseminate long before inducing symptoms and diagnosis.<sup>4</sup> The majority of PDAC are advanced at diagnosis (metastatic 50.5%, regional spread 25.9%, localized 8%, and unstaged 15.5%).<sup>5</sup> The most common sites of distant metastasis are liver, peritoneum and lung. More than half of patients who undergo potentially curative resection suffer from a local recurrence and liver metastasis.<sup>6</sup> Preventing metastasis in addition to curative resection of the primary tumor is essential to improve prognosis of patients with pancreatic cancer.

**Key words:** pancreatic ductal adenocarcinoma, metastasis, metastasis suppressor, shRNA, ERK, p27kip1, cyclin E, cell cycle

**Abbreviations:** CDK: cyclin dependent kinase; co-IP: Co-immunoprecipitation; ERK: extracellular signal-regulated kinase; IF: Immunofluorescence; MAPK: mitogen-activated protein kinase; p-ERK: phosphorylated-ERK

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Metastasis is determined by coordinated up-regulation of metastasis promoting and down-regulation of metastasis suppressing genes.<sup>7</sup> To date >30 metastasis suppressors have been discovered and functionally validated (reviewed in<sup>8,9</sup>) as being able to inhibit cancer metastasis without blockade of primary tumor growth. While a few anecdotal clinical studies described associations of metastasis suppressors and PDAC,<sup>10-15</sup> only KISS1 has been functionally validated to date.<sup>10</sup>

RNA interference has enabled interrogation of gene function by facilitating loss-of-function genetics in mammalian cells.<sup>16-19</sup> To identify genes that suppress PDAC liver (and potentially other) metastasis, we performed a genome-wide RNAi screen using a pooled short hairpin RNA (shRNA) in athymic mice. This approach led to the identification of HMP19.

## Materials and Methods

### Cell culture

Four human PDAC cell lines were initially chosen because of reported differential metastatic propensities. S2-007 cells and S2-028 cells are variants of the SUI2-2 PDAC<sup>20</sup> and were

**What's new?**

Pancreatic cancer is a frequently intractable disease, due in part to its late diagnosis and propensity to metastasize. Indeed, potentially curative resection fails in more than half of patients with pancreatic ductal adenocarcinoma (PDAC), owing to recurrence in the pancreas as well as to metastasis, particularly to the liver. Prognosis may be improved, however, by leveraging the inhibitory strength of novel metastasis suppressors. A promising candidate is HMP19, described in this study. In xenograft models, HMP19 overexpression significantly suppressed PDAC tumor growth and spread. Its elevated expression in clinical samples was associated with reduced liver metastasis and improved patient survival.

validated to be highly and non-metastatic, respectively.<sup>21</sup> MIAPaCa2 was moderately and BxPC3 was poorly metastatic. SUI2-derived S2-007 and S2-028 cells and MIAPaCa2 cells contain driver KRAS mutations while BxPC3 retains wild-type K-ras. All cells were routinely cultured as described in Supplemental Data.

PDAC cells received from Dr. Anthony Hollingsworth in the 1990s were cultured and immediately frozen. They were passaged fewer than 10 times before initiating experiments. All cells were most recently validated as human and having expected STR polymorphisms and K-Ras mutations. Following transduction, experiments were completed using cells within 5–10 passages; however, although results have been consistent for >20 passages (unpublished observations).

**Cell migration, growth in soft-agar and cell cycle analysis**

To measure migration, an *in vitro* wound healing (i.e., scratch) assay was performed. Cells were grown to near confluence (~90%;  $n = 6$ ) on 6-well plates. Sterile 200  $\mu\text{m}$  micropipet tips were used to scratch cell layers, which were then washed for several times with PBS to remove cell debris. Wounds were measured immediately and again 24 h later. The ratio was used to quantify relative motility.

To assess anchorage-independent cell growth, cells ( $1 \times 10^4$ ) were suspended in a solution of 0.3% agar in complete medium, layered atop a solution containing 0.6% agar previously hardened in 6-well plates ( $n = 4$ ). The plates were incubated at 37°C in a humidified chamber containing 5% CO<sub>2</sub> for 3 weeks. Colonies/cells were stained with crystal violet and counted. Cell cycle was analyzed with GFP-certified Nuclear-ID Red Cell Cycle Analysis Kit (Enzo, Farmingdale, NY). Cells were fixed with ice-cold 70% EtOH for at least 4 hr and resuspended in 500  $\mu\text{l}$  of DNA staining solution (10  $\mu\text{M}$  nuclear-ID Red dye). After 30 min, cells were analyzed by an Accuri C6 flow cytometer (BD Biosciences).

**In vivo studies**

Tumor growth was assessed following orthotopic injection as previously described<sup>22</sup> and elaborated in Supplemental Materials. For experimental metastasis, PDAC cells ( $5 \times 10^5$  cells/100  $\mu\text{l}$  HBSS) were injected directly into the tail of the spleen, which facilitates delivery of cells to the liver.<sup>23,24</sup> Mice were euthanized 4 weeks after injection and the percentage of mice injected with liver metastases and number of metastatic

foci per liver were determined. A minimum of 10 mice per experimental group were used for each experiment and each study was replicated at least twice independently.

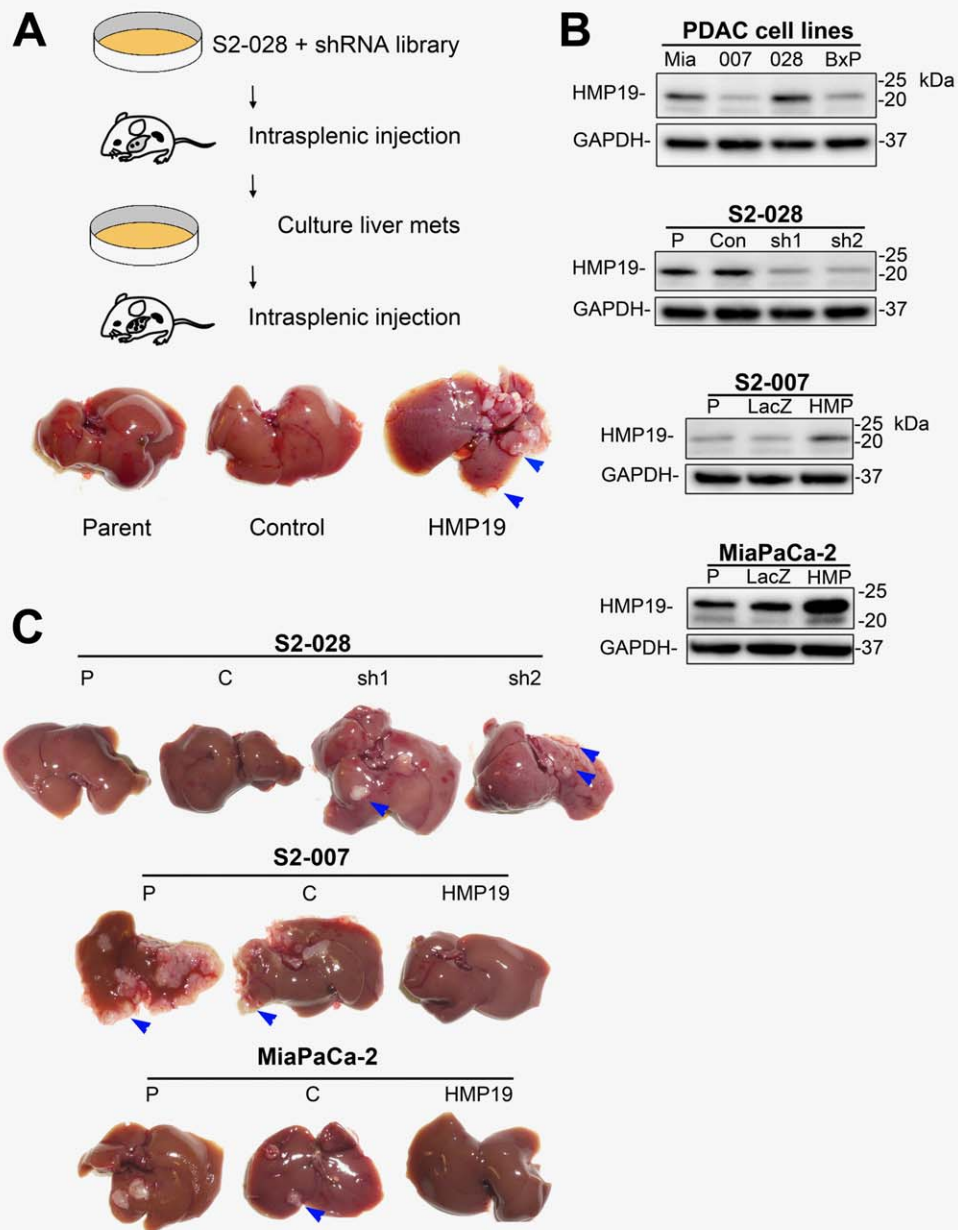
**shRNA library screen**

A near-confluent culture of non-metastatic S2-028 cells was infected with the pGIPZ lentiviral shRNAmir library or non-silencing control shRNAmir (S2-028 shCon) (Thermo Scientific Open Biosystems, Huntsville, AL). The library consisted of 74,468 shRNA directed against 21,416 genes and was transduced at a multiplicity of infection of 0.2 into  $1 \times 10^7$  to  $5 \times 10^8$  cells. Pooled transductants were injected intrasplenically (i.s.) to identify genes that suppress liver metastasis formation (i.e., knockdown increases metastasis). Mice were euthanized 4 weeks later and liver metastases cultured. Cultures derived from each liver metastasis were re-injected intrasplenically to reduce false positives. Following two intrasplenic injections, genomic DNA was isolated from each liver metastasis. Individual integrated shRNAmir were identified using primers flanking the coding region. Two candidates were identified and HMP19 was chosen for detailed characterization.

**HMP19 knockdown and overexpression**

To validate of screening results, PDAC cell lines were generated with reduced or over-expression of HMP19. Two different shRNA sequences (shHMP19(a) (5'-accagctgggagaattggtat-3') or shHMP19(b)) (5'-ccagttacaagacaactgta-3') targeting the 3' UTR were chosen. shHMP19(a) was the same sequence identified in the shRNAmir library screening. pGIPZ lentiviral and shCon were used to infect S2-028 cells [S2-028<sup>shHMP19(a)</sup>, S2-028<sup>shHMP19(b)</sup>, and S2-028<sup>shCon</sup>].

For over-expression, full-length, wild-type HMP19 cDNA was purchased from Origene (Rockville, MD) and cloned into pLenti6/V5-DEST lentiviral HMP19 vector or lacZ vector as a control (Invitrogen). For rescued expression of HMP19 in S2-028<sup>shHMP19(a)</sup> cells an HMP19 open reading frame cDNA (i.e., lacking 5' and 3' untranslated regions making it resistant to shHMP19(a)) was cloned into the pLenti6/V5-DEST lentiviral vector. The lentiviral vectors were used to infect S2-007 (designated S2-007<sup>HMP19</sup> and S2-007<sup>lacZ</sup>), MIAPaCa2 (designated MIAPaCa2<sup>HMP19</sup> and MIAPaCa2<sup>lacZ</sup>) or S2-028<sup>shHMP19(a)</sup> (designated rescue).

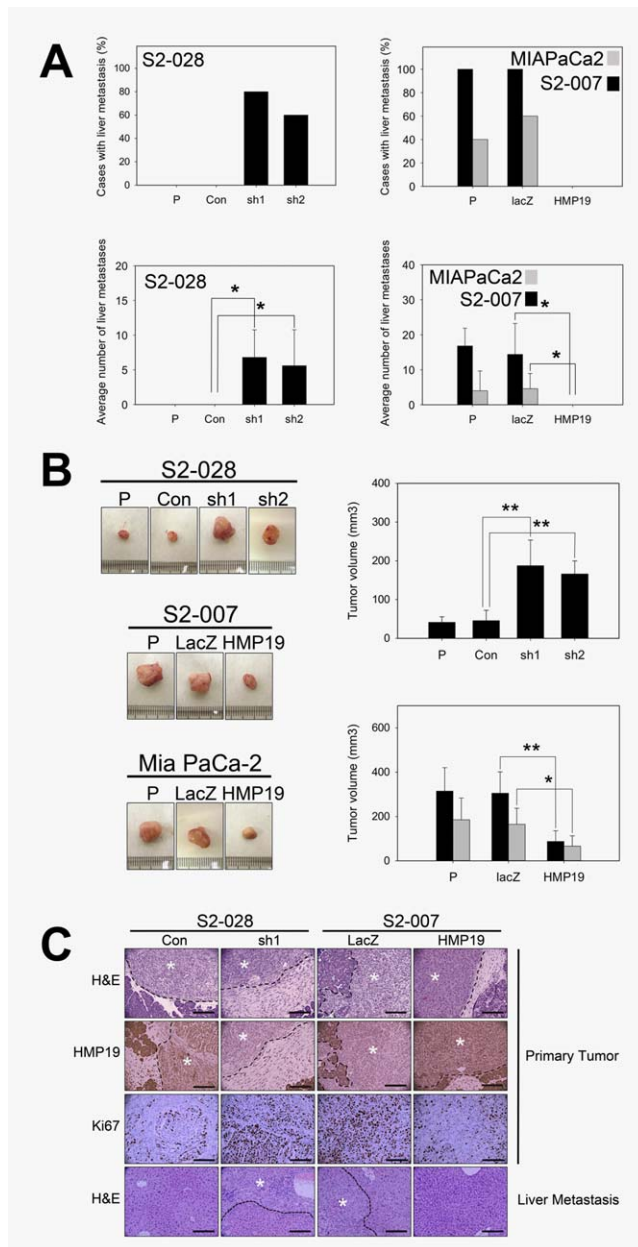


**Figure 1.** Experimental design to identify HMP19 and *in vivo* data showing HMP19 as a metastasis suppressor in PDAC. (a) Schematic diagram shows double selection of shRNA library in S2-028 PDAC cells using an intrasplenic model of experimental liver metastasis. Representative liver images show that parental S2-028 cells and S2-028<sup>shCon</sup> (control vector) cells did not form liver metastasis (arrows), but knockdown of HMP19 with the pGIPZ lentiviral shHMP19 vector did. (b, top panel 1) Western blot showing a comparison of HMP19 protein expression in MIAPaCa2 (Mia), S2-007 (007), S2-028 (028) and BxPC3 (BxP) PDAC cell lines. (b, panel 2) Immunoblot showing HMP19 expression in S2-028 (P), S2-028<sup>shCon</sup>, and two shRNA targeting HMP19 (sh1 and sh2). (b, panels 3 and 4) Western blot showing HMP19 expression in S2-007 or MIAPaCa2 parental (P) or pLenti6/V5-DEST lentiviral vector containing lacZ or HMP19 (HMP). (c) Representative images of livers following intrasplenic injection of the cell lines depicted in Panel b. Liver metastases are highlighted by blue arrowheads. Abbreviations: P, Parental; Con or C, Control (empty vector); sh1/sh2, different shRNA targeting HMP19; LacZ, vector containing only the LacZ gene. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

### Patients and immunohistochemistry

Surgical specimens were obtained from 84 patients (55 men and 29 women; 42 to 81 years (median, 66.4 years) with pancreatic cancer, who received surgical treatment at Kagoshima University Hospital between January 1990 and December

2006 (table in Fig. 6). All patients underwent macroscopically curative resection. No patients received preoperative chemotherapy or radiotherapy. All resected specimens were examined histologically by hematoxylin and eosin (H&E), using standard TNM classification system.<sup>25</sup> Pathologically, all



**Figure 2.** Liver metastases and orthotopic tumor growth are inhibited by restored expression of HMP19. (a) PDAC cells from Figure 1 were injected into the spleen and the proportion of mice developing liver metastases (top row) and number of liver metastases per mouse (bottom row) is shown for S2-028 cells in which HMP19 was knocked down (left column) or re-expression (right column) in S2-007 (black) or MIAPaCa2 (grey) cells. Results are mean  $\pm$  standard deviation. \* $p < 0.05$ . (b) Representative images of orthotopic tumors extracted from PDAC cells with graphical representation of tumor volume. Results are mean  $\pm$  standard deviation. \* $p < 0.05$  \*\* $p < 0.01$ . (c) Representative histologic images comparing PDAC primary tumors by hematoxylin and eosin, HMP19 and Ki67 staining and liver metastases (bottom row). Tumor is identified by white asterisk (\*) and delineated by dashed line. No tumor cells were detected in S2-028 parental or S2-007 cells over-expressing HMP19. Abbreviations: P, Parental; Con or C, Control (empty vector); sh1/sh2, different shRNA targeting HMP19; LacZ, vector containing only the LacZ gene. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

tumors were invasive PDAC. Thirty-three patients (39.3%) developed liver metastasis after surgery. This study was approved by the Institutional Review Board of Kagoshima University.

Details regarding staining are described in Supplemental Materials. Briefly, thin sections (3  $\mu$ m) from paraffin blocks were blocked for endogenous peroxidase activity before antigen retrieval and staining using anti-HMP19 (Proteintech, Chicago, IL; 1:50 overnight at 4°C). No significant staining was observed in negative controls. All tissue sections were assessed by two investigators (H.K., S.N) who were blinded to the patient clinicopathological details. HMP19 staining was considered as positive if at least 5% cancer cells were stained and was scored as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).

### Statistical analysis

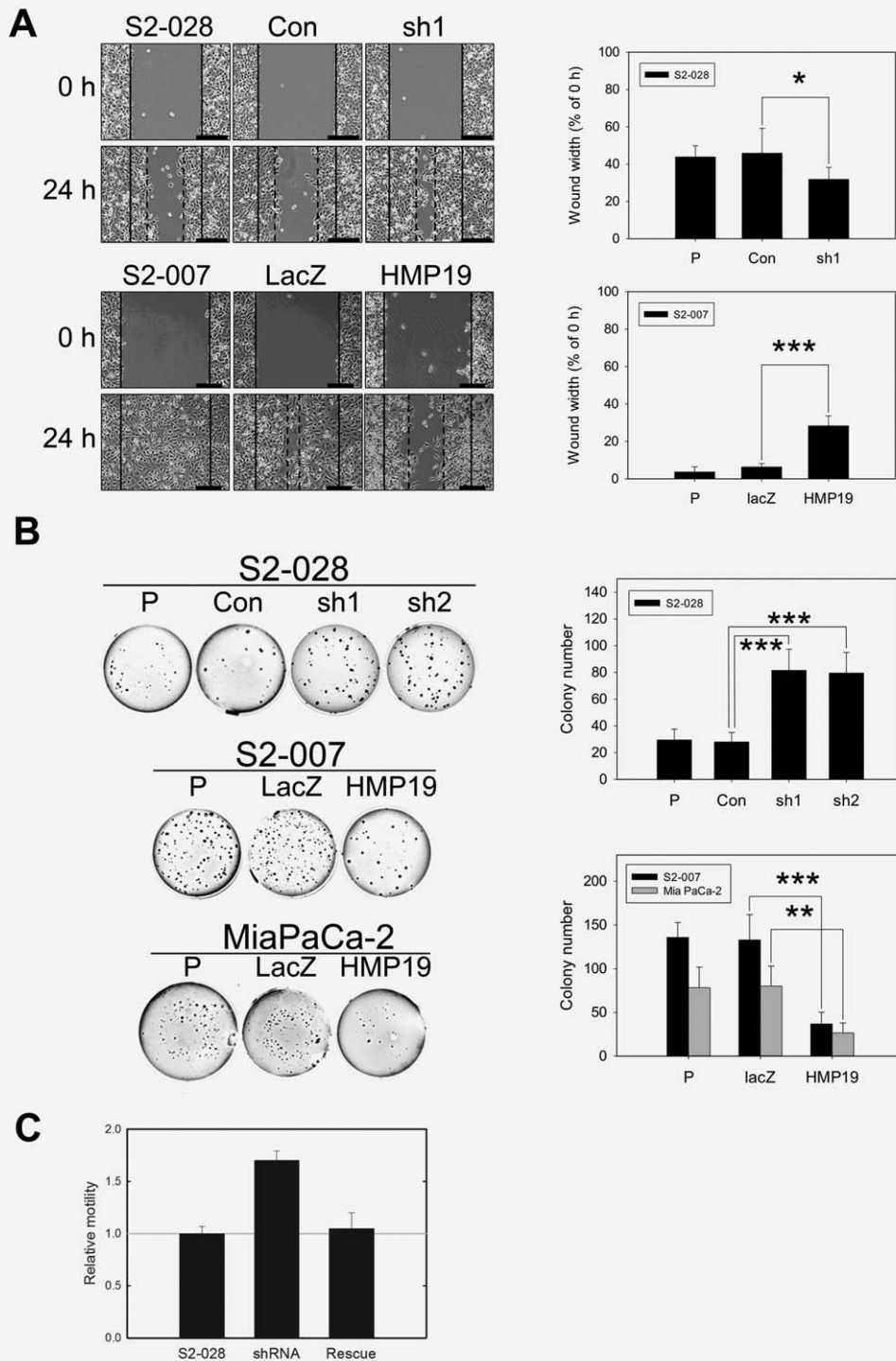
In vivo experiments were performed with at least 10 mice per group, unless otherwise stated, and were replicated at least twice independently. In vitro studies were performed at least twice in triplicate. Associations between different categorical variables were assessed using the  $X^2$  test. Continuous variables were compared between 2 groups using the Mann-Whitney  $U$  test or the paired Student's  $t$  test. Survival rates were calculated using the Kaplan-Meier method, and significant differences in survival were determined by the log-rank test.  $p$  values  $< 0.05$  were considered statistically significant.

## Results

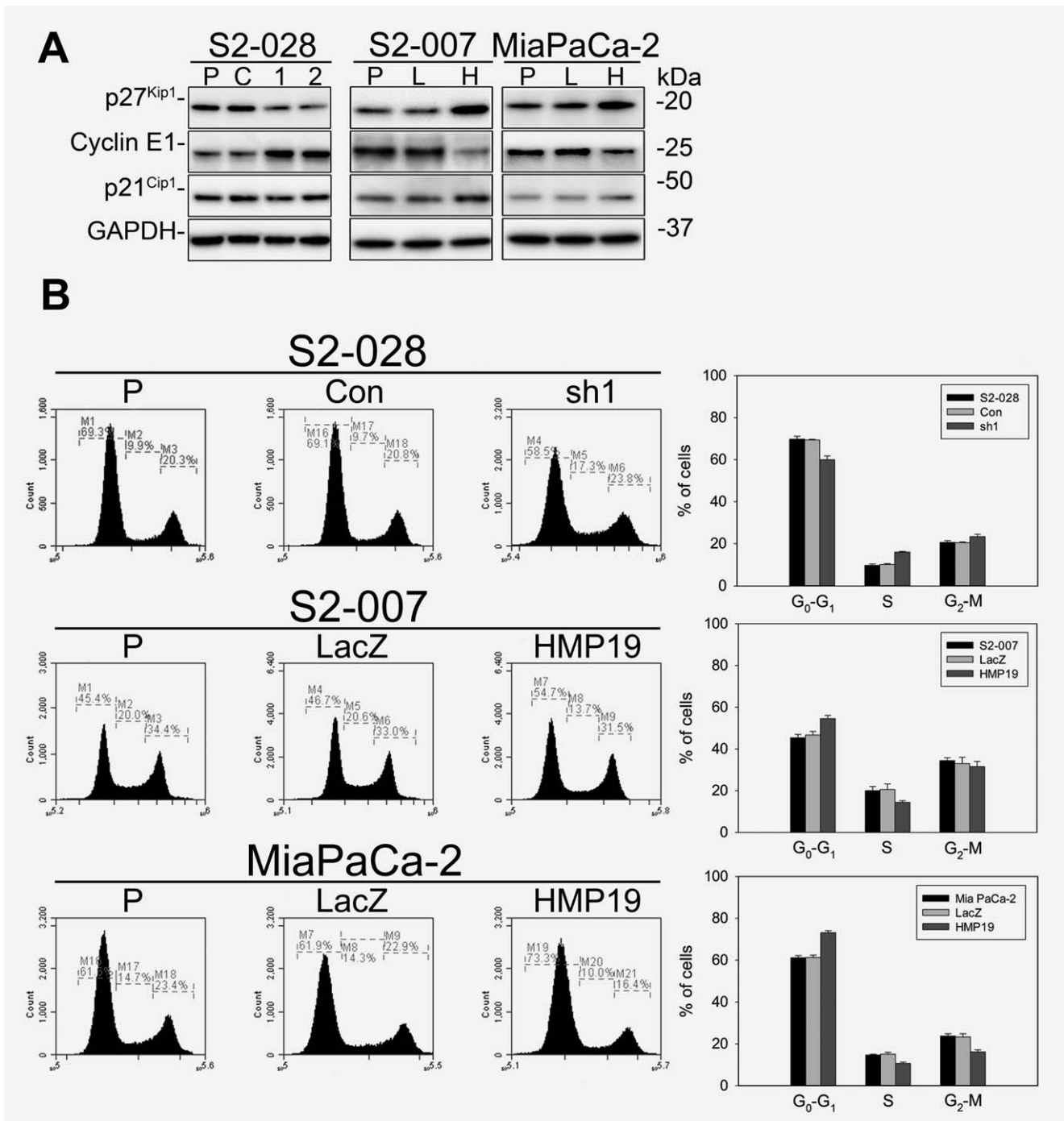
### In vivo screening identifies candidate genes capable of suppressing liver metastasis

Following the first i.s. injection of S2-028 shRNA library cells into 10 athymic mice, a total of 6 liver metastases were observed, versus no liver metastasis in mice injected with S2-028 or S2-028<sup>shCon</sup> cells. The liver metastases were isolated and established as individual cultures and then re-injected intrasplenically. Two of the 6 cultures formed 20 and 4 liver metastases, respectively. From those cultures, two shRNA were isolated using the sequences flanking the shRNAmiR – HMP19 (Fig. 1a) and ITIH5. Since the effect of HMP19 on metastasis was more robust (i.e., more metastases following knockdown), it was initially prioritized for further analysis. ITIH5 has also been validated as a metastasis suppressor in PDAC models. HMP19 and ITIH5 are distinct molecules and reside in different cellular compartments. Studies to better define ITIH5 mechanism of action are underway (K. Sasaki, H. Kurahara, S. Natsugoe, A. Ijichi, T. Iwakuma and D.R. Welch, manuscript in preparation).

Expression of HMP19 in a panel of human PDAC cell lines of varying metastatic propensity was then compared by immunoblot (Fig. 1b). Non-metastatic S2-028 cells exhibited the highest expression of HMP19 protein (Fig. 1b, top panel); whereas, highly metastatic S2-007 expressed the lowest amount. MIAPaCa2 and BxPC-3 cells showed intermediate expression. Although imperfect, the trends toward reduced



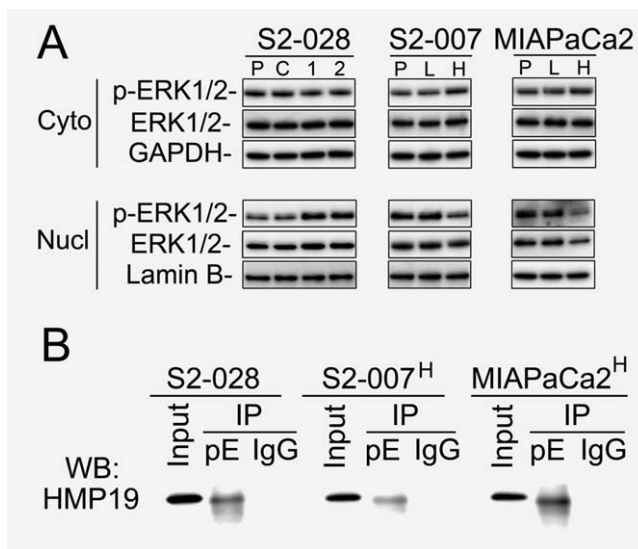
**Figure 3.** *In vitro* characterization of PDAC cells with altered HMP19 expression. (a) S2-028 or S2-007 PDAC cells with control (Con or LacZ) vector or knocked down (sh1) or over-expressed HMP19 were evaluated for migration using a scratch assay. Original edges are marked with a solid line and the average edge at 24 hr is depicted by the dashed lines. Data are quantified in the bar graphs to the right of original images. (b) Knockdown of HMP19 promoted colony formation of S2-028 cells in soft agar while over-expression of HMP19 inhibited colony formation of S2-007 cells and MIAPaCa2 cells. Data are quantified in the bar graphs to the right of original images. Results are mean  $\pm$  standard deviation. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . (c) Rescue of motility inhibition following re-expression of shRNA-resistant HMP19 demonstrates selectivity of shRNA to HMP19. Values shown are relative to parental S2-028 cells. Abbreviations: P, Parental; Con or C, Control (empty vector); sh1/sh2, different shRNA targeting HMP19; LacZ, vector containing only the LacZ gene.



**Figure 4.** HMP19 alters cell cycle distribution and cell cycle regulatory proteins. (a) Western blots (whole cell lysate) from PDAC cells (P = parental, C = shRNA control vector; L = LacZ control vector; 1 or 2 = shRNA targeting HMP19 or H = HMP19) differentially expressing HMP19 (compare with Figure 1). Expression of p27<sup>Kip1</sup>, cyclin E1 and p21<sup>Cip1</sup> are shown using GAPDH as a control. (b) Representative flow cytometry histograms showing cell cycle distribution of the cells in panel A. Cells were stained with GFP-certified nuclear-ID red cell cycle analysis kit (Enzo, Farmingdale, NY) and analyzed using an Accuri C6 flow cytometer. Data are quantified in the bar graphs to the right of original images. Cell cycle distributions were statistically different ( $p < 0.01$ ). Abbreviations: P, Parental; Con or C, Control (empty vector); sh1/sh2, different shRNA targeting HMP19; LacZ, vector containing only the LacZ gene.

expression with increased metastatic potential supported follow-up. As expected HMP19 expression was reduced when S2-028 cells were transduced with two different shRNA targeting HMP19 (Fig. 1b, second panel). S2-007 and

MIAPaCa2 cells were transduced with pLenti6/V5-DEST lentiviral HMP19 vector (Fig. 1b, bottom panels) and, as expected, HMP19 expression was higher than in parental cells or LacZ vector controls.



**Figure 5.** HMP19 interacts with phospho-ERK1/2 (p-ERK1/2) to change subcellular distribution. (a) Knockdown or overexpression of HMP19 in PDAC cell lines did not show distinct difference of p-ERK1/2 and total ERK1/2 in pancreatic cancer cells. However, knockdown of HMP19 in S2-028 cells increased, while overexpression of HMP19 in S2-007 cells or MIAPaCa2 cells reduced p-ERK1/2 localized in the nuclear fraction. (b) Interactions between HMP19 and p-ERK1/2 (pE) were observed by co-immunoprecipitation of p-ERK1/2 and detection of associated HMP19. Normal rabbit IgG served as control. Representative blots are presented, but all immunoblots were performed at least 2–3 times.

#### HMP19 levels inversely correlate with liver colonization in PDAC cells

PDAC cell lines with experimentally manipulated HMP19 expression were injected into the spleens of athymic mice and assessed for liver colonization. Figure 1c shows representative livers from S2-028, S2-007 and MIAPaCa2, HMP19 derivatives and vector controls.

S2-028 (parental) and S2-028<sup>shCon</sup> (shRNA control) cells did not form liver metastasis, while two different shRNA (designated sh1 and sh2) transductants colonized the liver (80% and 60% incidence, respectively, Fig. 1c and Fig. 2a). The number of liver metastases in the HMP19 knockdown cells were modest (5–6 metastases per liver), but the effects were statistically significant ( $p < 0.05$ ) and reproducible in 3–5 independent biological replicates.

Every liver from mice injected with S2-007 and S2-007<sup>LacZ</sup> developed large macroscopic liver metastases (Fig. 1c and Fig. 2a) while HMP19-expressing cells (S2-007<sup>HMP19</sup>) no longer formed hepatic metastases. As expected MIAPaCa2 parental and vector-control cells formed a small number of liver metastases (typically 2–5) in 40–60% of mice in which the cells were injected into the spleen. Metastasis suppression was as profound in the MIAPaCa2<sup>HMP19</sup> as it was in the S2-007<sup>HMP19</sup> ( $p < 0.05$ ).

To assess whether HMP19 affected orthotopic growth, PDAC cells were injected as a bolus directly into the pancreas. *In vitro* HMP19 over-expressing PDAC cells grew

more slowly than parental or vector-only transductant cells while knockdown resulted in faster growth *in vitro* (Supplemental Fig. 1). Similar shifts were observed *in vivo*. S2-028<sup>shHMP19</sup> cells formed dramatically larger tumors in the pancreas compared with S2-028<sup>shCon</sup> cells. Conversely, mice injected with S2-007<sup>HMP19</sup> and MIAPaCa2<sup>HMP19</sup> cells formed distinctly smaller pancreatic tumors compared to their respective controls (Fig. 2b). Immunohistochemical analysis of orthotopic tumors showed that tumors expressing HMP19 (e.g., S2-028<sup>shCon</sup> and S2-007<sup>HMP19</sup>) had reduced Ki67 staining (Fig. 2c,  $p < 0.001$ ).

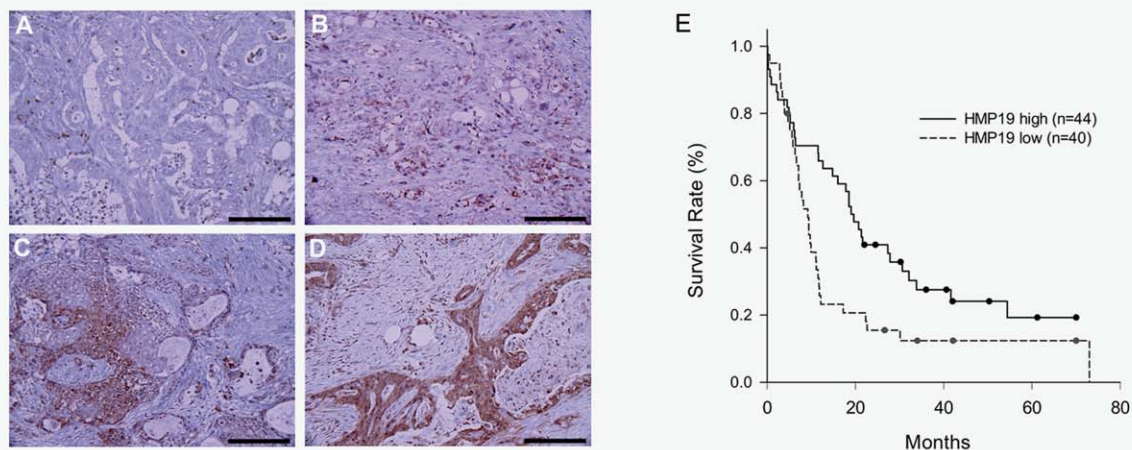
Spontaneous metastases from *in situ* pancreatic tumors were also measured. The efficiency of liver colonization was expectedly less than direct injection into the spleen (40% vs. >90%, respectively). Nonetheless, the same pattern of metastatic efficiency was observed. Cells expressing HMP19 (S2-028, S2-028<sup>shCon</sup>, MIAPaCa2, S2-007<sup>HMP19</sup> and MIAPaCa2<sup>HMP19</sup>) did not form macroscopic liver metastases, while cells with low to no HMP19 (S2-007, S2-007<sup>LacZ</sup>) did (Supplemental Fig. 2). Disseminated cells were not found in random sections in the liver from S2-028<sup>shCon</sup>, S2-007<sup>HMP19</sup> or MIAPaCa2<sup>HMP19</sup> cells (Supplemental Fig. 2). Representative sections are shown for S2-028<sup>shCon</sup> and S2-007<sup>HMP19</sup> in Figure 2c.

#### HMP19 inhibits tumor cell proliferation, migration and anchorage-independent growth *in vitro*

To begin exploring potential mechanisms responsible for metastasis suppression, several *in vitro* assays were done to explore metastasis-associated properties. As observed *in vivo*, expression of HMP19 corresponded with a reduction of growth rate (Supplemental Fig. 1), reduced migration (Fig. 3a) and inhibition of anchorage-independent cell growth (Fig. 3b). Knockdown of HMP19 promoted cell proliferation, migration, and growth in soft agar of S2-028 cells, while overexpression suppressed cell proliferation, migration, and anchorage-independent cell growth of S2-007 cells and MIAPaCa2 cells. When S2-028<sup>shHMP19</sup> cells were transduced with HMP19 cDNA that is not targeted by the shRNA, then migration effects are reversed (Fig. 3c), supporting the specific effects of HMP19 on the phenotype.

#### HMP19 inhibits G<sub>1</sub>/S transition

Because HMP19 slowed tumor growth *in vivo* and cell proliferation *in vitro*, assessment of cell cycle regulatory proteins<sup>26</sup> (e.g., p27<sup>kip1</sup>, p21<sup>cip1</sup>, cyclin E1) was done. p27<sup>kip1</sup>, which is associated with cell cycle inhibition, and cyclin E1, which is responsible for inducing cell division, were down-regulated and up-regulated, respectively, when HMP19 was knocked down in S2-028<sup>shHMP19</sup> cells (Fig. 4a). Correspondingly, protein levels of p27<sup>kip1</sup> and cyclin E1 changed in the opposite direction when HMP19 was over-expressed in S2-007<sup>HMP19</sup> and MIAPaCa2<sup>HMP19</sup> cells (Fig. 4a). Cell cycle distributions were consistently and significantly ( $p < 0.01$ ), altered with HMP19 expression (Fig. 4b). Flow cytometry measured



Clinicopathological factors		HMP19 high (n=41)	HMP19 low (n=43)	P value
Age (median)		67	68	0.710
Gender	male	26	29	0.874
	female	15	14	
Histology	well	16	15	0.867
	moderate/poor	25	28	
Primary tumor	T1, 2	6	1	<b>0.055</b>
	T3, 4	35	42	
Lymph node metastasis	negative	16	15	0.867
	positive	25	28	
Extrapancreatic plexus invasion	negative	34	20	<b>0.001</b>
	positive	7	23	
Liver metastasis	negative	31	20	<b>0.012</b>
	positive	10	23	

**Figure 6.** High HMP19 expression correlates with better prognosis. (a–d) Representative images of immunohistochemical staining of HMP19 showing no (A), 1+ (B), 2+ (C) and 3+ (D) expression in human pancreatic cancer specimens (Magnification  $\sim \times 200$ ). Bar = 200  $\mu\text{m}$ . (e) Kaplan–Meyer survival analysis comparing PDAC from high (2+/3+; solid line) versus low (0, 1+; dashed line) expression of HMP19. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

decreased  $G_0$ – $G_1$  and concomitant increased S and  $G_2$ –M phase populations when HMP19 was knocked down in S2-028<sup>shHMP19(a)</sup> compared with S2-028<sup>shCon</sup> cells. Conversely, cells over-expressing HMP19 (S2-007<sup>HMP19</sup> and MIAPaCa2<sup>HMP19</sup>) accumulated more in  $G_0$ – $G_1$  phase while cells actively undergoing DNA synthesis decreased (Fig. 4b).

#### HMP19 reduces nuclear translocation of p-ERK1/2

The changes in cell cycle distribution affected by HMP19 compelled examination of ERK pathways components for two reasons: (1) because ERK signaling is crucial in cell proliferation<sup>27,28</sup>; and, (2) because PDAC cell lines have frequent mutations of K-ras, which is upstream of ERK. Somewhat surprisingly, no obvious differences in ERK1/2 and p-ERK1/2 protein levels were observed in whole cell lysates (data not shown). However, when ERK1/2 and p-ERK1/2 were measured in cytoplasmic and nuclear fractions, subtle differences were noted. p-ERK1/2 in the nuclear

fraction was elevated in S2-028<sup>shHMP19</sup> cells compared with S2-028<sup>shCon</sup> cells (Fig. 5a). Correspondingly, nuclear localization of activated ERK1/2 was reduced in S2-007<sup>HMP19</sup> and MIAPaCa2<sup>HMP19</sup> cells compared to S2-007<sup>lacZ</sup> and MIAPaCa2<sup>lacZ</sup> cells (Fig. 5a). Suspecting that HMP19 might interact with p-ERK1/2, co-immunoprecipitation was done. HMP19 and p-ERK1/2 co-precipitated from S2-028, S2-007<sup>HMP19</sup>, and MIAPaCa2<sup>HMP19</sup> cells and could be detected by western blotting with anti-HMP19. The result suggested a potential interaction between HMP19 and p-ERK1/2 that determines subcellular distribution of activated ERK1/2 (Fig. 5b), but additional studies will be needed to validate this conclusion.

#### HMP19 expression correlates with improved prognosis

The promising correlations of HMP19 in experimental systems compelled examination of HMP19 expression in a tissue microarray of PDAC. Figs. 6a–6d are representative images of the 4 scores of HMP19 expression in human PDAC. Of



the 84 cases examined, HMP19 expression was negative (0,  $n = 7$ ), weak (1+,  $n = 36$ ), moderate (2+,  $n = 29$ ), or strong (3+,  $n = 12$ ), respectively. For further analyses, cases were assigned to two categories depending upon staining intensity: low (0 or 1+) or high (2+ and 3+).

Comparison of clinicopathologic features with HMP19 showed that low expression of HMP19 was significantly associated with invasion into the extrapancreatic plexus ( $p = 0.001$ ) and postoperative liver metastasis ( $p = 0.012$ ) (table in Fig. 6). Primary tumors expressing low HMP19 tended to be larger, but the correlations did not meet the level of statistical significance.

Kaplan-Meier survival analysis (Fig. 6e) shows that median survival post-surgery of patients with high expression of HMP19 and low expression of HMP19 was 20.7 months and 9.2 months, respectively. Patients with low expression of HMP had a significantly poorer prognosis compared with the patients with high expression of HMP19.

## Discussion

The prognosis for most PDAC patients is bad. Even with potentially curative surgery and chemotherapy, survival rates remain only in the single digits. The reasons for poor survival relate to the lack of symptoms until the tumors are large and already having invaded surrounding duodenum, stomach and peritoneal cavity as well as the propensity for the tumors to form liver metastases. There is obvious need to identify biomarkers that allow earlier detection, stratification, and/or better prediction of effectiveness of therapies. This study was undertaken in order to identify possible metastasis suppressors in PDAC.

Following transduction of a shRNA library into the non-metastatic S2-028 PDAC cell line, metastases were isolated and confirmed to be stably metastatic in experimental models. HMP19 was identified because knockdown in non-metastatic cells resulted in metastasis and over-expression in metastatic cell lines blocked metastasis. The effect on metastasis was not without effect on orthotopic primary tumor growth, however. Nonetheless, HMP19-expressing tumor cells still grow in the pancreas which justifies addition of HMP19 to the growing list of metastasis suppressors.<sup>8,9</sup>

We report, for the first time, that HMP19 intensely suppresses PDAC tumor growth and metastasis (mostly liver, but also peritoneal) in PDAC in experimental xenograft models. Moreover, high expression of HMP19 in clinical samples of PDAC is significantly associated with better survival and reduced development of liver metastasis. It is important to note that this report deals exclusively with pancreatic ductal adenocarcinomas, not neuroendocrine pancreas tumors. Therefore, both clinical and experimental models support the conclusion that HMP19 is a metastasis suppressor. Our observations that high HMP19 expression results in ~20% long-term survival may also portend that this biomarker could be a useful predictor of outcomes in patients undergoing pancreaticoduodenectomy. The Whipple procedure is

associated with challenging recovery and numerous morbidities and only 25–30% 5-year survival. It is interesting to speculate that HMP19 could spare some patients surgical complications that do not improve likelihood for long-term survival.

Remarkably little about HMP19 has been previously reported. HMP19 encodes a  $M_r \sim 19$  kDa protein that reportedly localizes to the Golgi apparatus in neural and neuroendocrine cells.<sup>29</sup> Malfunction of this protein has been implicated in Huntington's disease.<sup>30</sup> Roles of HMP19 in cancer development and/or progression have not been previously reported.

As a result of few structural and sequence domains that predict function and limited prior knowledge about HMP19, ascertaining a mechanism of action for metastasis suppression has been challenging. Contributing to the difficulties was also the lack of validated antibodies. The results reported herein do not include results from antibodies/antisera that did not pass rigorous validation assessments (i.e., reduced expression in shRNA-expressing cells; increased expression in constitutively expressing cells; appropriate band size). Correspondingly, clues regarding the step(s) in the metastatic cascade inhibited by HMP19 were initially few based upon the *in vivo* studies. When HMP19 was expressed and injected orthotopically, no HMP19-expressing PDAC cells were found in random histologic sections from liver. Those observations suggested that steps prior to seeding liver were most likely to be involved and became the focus of subsequent experiments.

*In vitro* assays confirmed that HMP-19 expressing PDAC cells were inhibited for motility and invasion as well as verifying slower proliferation rates. Since KRAS mutations, mostly at codon 12 but sometimes at codons 13 and 61, are nearly universal (>95%) in PDAC<sup>31,32</sup> and since the KRAS mutations result in a protein locked in a constitutively active state (i.e., independent of external signals), we reasoned that the resulting aberrant activation of downstream effector pathways, including ERK1/2,<sup>31</sup> could be, at least partially, responsible for the observed changes associated with HMP19 expression. Extracellular signal-regulated kinase (ERK1/2) signaling controls diverse cellular functions, such as proliferation, survival, migration, and chemotherapeutic drug resistance.<sup>27,28,33,34</sup> As a result, the ERK1/2 pathways are already recognized as crucial targets for therapeutic intervention. However, their roles in metastasis have often been considered secondary to regulating primary tumor growth. Yet, both *in vitro* and *in vivo* growth were significantly reduced when HMP19 was expressed; therefore, we decided to explore potential relationships further.

Previous studies, especially from the laboratory of Aguirre-Ghiso and colleagues, showed that the stoichiometry between pERK and phospho-p38 contribute to disseminated tumor cell dormancy.<sup>35–37</sup> It is possible that the actions of HMP19 may exert some effect on the ratio of these molecules or the complexes in which they reside. However, since we did not observe tumor cell seeding in the liver when HMP19

was re-expressed, experiments associated with earlier steps in metastasis were prioritized.

HMP19 expression did not appear to overtly change of total amount of activated ERK1/2 in any of the PDAC cell lines examined. However, knockdown of HMP19 increased the presence of activated ERK1/2 in the nucleus concomitant with down-regulation of p27<sup>kip1</sup> and up-regulation of cyclin E1. The opposite effects were seen when HMP19 was over-expressed (i.e., reduced p-ERK1/2 in the nucleus; higher p27<sup>kip1</sup>; lower cyclin E1). The net effect of HMP19 expression appears to be slowing of cell cycle progression, which is consistent with significantly ( $p < 0.001$ ) fewer Ki67 staining cancer cells in HMP19 expressing tumors.

Following activation, ERK1/2 translocates into the nucleus where it can phosphorylate over 100 possible substrates representing diverse functions, including cell cycle progression, proliferation, cytokinesis, transcription, metabolism, differentiation, migration, senescence, and apoptosis.<sup>28,38</sup> Repeated immunoblots consistently demonstrated that HMP19 expression corresponded with reduced nuclear levels of activated p-ERK1/2 compared to when HMP19 levels were low. These findings hint, but do not prove, that HMP19 limits p-ERK1/2 translocation into the nucleus, thereby attenuating activation of effectors downstream of ERK signaling. Activated nuclear ERK1/2 then mediates G1 to S phase cell cycle progression by controlling interactions between cyclin D, cyclin E, cyclin dependent kinase (CDK), and CDK inhibitors such as p21<sup>cip1</sup> and p27<sup>kip1</sup>.<sup>38–41</sup> Consistent with this activity, up-regulation of cyclin E and down-regulation of p21<sup>cip1</sup> and p27<sup>kip1</sup> are related tumor progression and poor prognosis in PDAC.<sup>41–43</sup> Reported here, p27<sup>kip1</sup> and cyclin E1 are significantly affected by HMP19 expression. Moreover, the proliferative changes observed as a result of HMP19 manipulation are consistent with known p-ERK1/2, cyclin E and p27<sup>kip1</sup> clinical correlations. Therefore, our current working hypothesis is that HMP19 alters subcellular localization of ERK1/2 in order to control tumor growth and metastasis. Data have yet to identify other pathways besides K-ras – Raf – MEK – ERK1/2 that might also be affected. Additional experiments exploring cell cycle machinery in orthotopic tumors, metastases and patient samples will be needed to refine the pathway(s) involved.

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Published yeast two-hybrid (Y2H) protein-protein interaction networks found that HMP19 interacts with several signaling proteins including C2orf18, APOC1, or SERPINH1,<sup>44</sup> some of which are implicated in PDAC invasion and progression.<sup>45–47</sup> The interrelationships between HMP19 and these proteins have not been further validated, however.

Although new molecular targets in combination with gemcitabine have been tested in PDAC randomized clinical trials, very little improvement in outcome has been achieved.<sup>48</sup> Among the recent promising agents to improve survival in advanced PDAC is erlotinib, an epidermal growth factor receptor inhibitor.<sup>48</sup> As suspected with colorectal cancer, high frequency of KRAS2 mutations in PDAC may limit the benefits of erlotinib.<sup>48</sup> Since HMP19 is involved in tumor growth and metastasis in PDAC cell lines with KRAS mutations and since HMP19 may regulate common signaling pathways downstream of EGFR, there is the possibility that the data reported here could help more accurately determine which patients would benefit most from the newer targeted therapies.

In summary, HMP19 represents a promising prognostic and perhaps predictive biomarker in PDAC, but more strongly powered clinical studies will be required to determine the significance. Importantly, in preclinical model systems, HMP19 exerts profound inhibition of PDAC metastasis, the most lethal attribute of the cancer cells. Further elucidation of the mechanisms by which HMP19 suppresses tumor growth and inhibits liver metastasis may uncover new therapeutic options for patients with pancreatic cancer.

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