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IQGAP1 Maintains Pancreatic Ductal Adenocarcinoma Clonogenic Growth and Metastasis

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Abstract

Objectives: IQ motif containing GTPase activating protein 1 (IQGAP1) acts as a scaffold for aberrant MAPK signaling driven by *KRAS* mutations in pancreatic ductal adenocarcinoma (PDAC). We determined the role of IQGAP1 in clonogenic growth and metastasis in PDAC.

Methods: We inhibited IQGAP1 expression using shRNA and assessed clonogenic growth, cell migration, and MAPK signaling *in vitro* and tumor initiation and metastasis *in vivo*. The efficacy of a peptide mimicking the IQGAP1 WW domain that binds and inhibits ERK1/2 was determined *in vitro* and *in vivo*.

Results: IQGAP1 loss inhibited clonogenic growth and migration of *KRAS*-dependent PDAC cells by disrupting MAPK signaling. In mice, IQGAP1 knockdown decreased tumor initiating cell frequency and metastasis. WW peptide treatment inhibited clonogenic growth and *in vivo* tumor growth.

Conclusions: Pancreatic ductal adenocarcinoma clonogenic growth, metastasis, and tumor initiation are dependent on MAPK signaling via IQGAP1. Treatment with a WW peptide disrupts IQGAP1 function and represents a novel targeting strategy for PDAC.

Keywords

IQGAP1; KRAS signaling; pancreatic cancer

Introduction

The prognosis of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) is poor with 5-year survival rates of <10%.¹ Surgery is potentially curative in early stage disease, but most patients experience local or metastatic recurrence following resection. Therefore, treatments that inhibit tumor relapse and dissemination may improve clinical outcomes. Tumor regrowth requires long-term maintenance of clonogenic growth, and in many normal

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Disclosure

The authors declare no conflict of interest.

and malignant tissues, the signaling pathways regulating self-renewal are distinct from those involved in cell proliferation and survival. Although many factors affecting PDAC cell proliferation have been identified, the processes regulating self-renewal are not fully understood.

Mutations in members of the RAS family of small GTPase proteins are found in a wide range of human cancers, and *KRAS* mutations are present in >90% of PDAC cases.² *KRAS* mutations are thought to be initiating events in PDAC as they are found in pancreatic intraepithelial neoplasia (PanIN) lesions that are carcinoma precursors and can induce PDAC in genetically engineered mouse models.^{3,4} In leukemia, multiple myeloma, and skin cancers, *RAS* mutations can lead to aberrant self-renewal,^{5–7} but their impact in PDAC is unclear. *KRAS* mutations activate downstream pathways including MAP kinase (MAPK) signaling to promote cell proliferation and survival.⁸ MEK inhibitors block MAPK signaling and are clinically effective in *BRAF*-mutant melanoma.^{9–11} However, resistance to MEK inhibition is common,¹² and targeting downstream pathway components, such as ERK1/2, may be more efficacious.

MAPK signaling requires intracellular scaffold proteins, including IQ motif containing GTPase activating protein 1 (IQGAP1), to bring RAF, MEK, and ERK1/2 into close proximity.¹³ The WW domain of IQGAP1 consists of amino acids 643–744 and specifically binds to ERK1/2.¹⁴ The loss of IQGAP1 can inhibit MAPK signaling by preventing the interaction of ERK1/2 with other MAPK pathway components.¹⁴ Furthermore, peptides mimicking the WW domain of IQGAP1 can sequester ERK1/2 to distally inhibit the MAPK pathway and slow the growth of PDAC in mutant *KRAS* mouse models.¹⁵

We studied IQGAP1 in PDAC and found that downregulation of its expression inhibited the clonogenic growth and invasion of *KRAS*-dependent PDAC *in vitro* as well as tumor initiation and metastasis *in vivo*. In addition, treatment with a peptide mimicking the IQGAP1 WW domain (WW peptide) inhibited tumor colony formation and growth. Therefore, MAPK signaling mediates clonogenic PDAC growth, and treatment with the WW peptide can disrupt IQGAP1 function and represents a novel approach to this disease.

MATERIALS AND METHODS

Cell Lines and Xenografts

Capan-1 and PANC-1 PDAC cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine. Low passage patient-derived xenografts (PDXs) generated from primary surgical specimens and the single cell dissociation of PDXs have been previously described.^{16,17}

Lentivirus Production and Transduction

Lentiviral particles containing an inducible shRNA vector containing *IQGAP1* targeting sequences (5'-GCCACATTGTGCCTTTATTCTC-3', control 5'-CCTAAGGTTAAGTCGCCCTCGCTC-3') or a scrambled control sequence cloned into the pLKO.Tet.On shRNA lentivector (Addgene, Cambridge, Mass) were generated as previously

described.⁶ Transduced PDAC cells were selected with 2 ug/mL puromycin and shRNAs were induced with 25 ng/mL (Capan-1) or 100 ng/mL (PANC-1) doxycycline (Sigma, St. Louis, Mo) for 3 or 7 days.

Colony Formation

Colony formation in methylcellulose was performed as previously described.^{16,17} Prior to plating, control and IQGAP1 directed shRNA were induced with doxycycline for 3 days or cells were treated with 1 uM scrambled control or WW peptide for 7 days.¹⁵ Peptide treatments were carried out on days 1 and 4. For secondary plating, colonies were digested with trypsin, washed, and replated in methylcellulose.

Transwell Migration

Following treatment with doxycycline or peptides as above, cells were seeded in tissue culture inserts with 8.0 µm pores (Corning, Corning, NY) in DMEM and placed in wells containing DMEM supplemented with 10% FBS for 24 hours. Membranes were fixed with the ProLong Gold Antifade Reagent with DAPI (Thermo Fisher, Waltham, Mass), and cells were counted using a fluorescence microscope (Nikon Instruments, Melville, NY).

Animal Studies

All mouse experiments were approved by the Johns Hopkins University Animal Care and Use Committee. To calculate tumor-initiating cell (TIC) frequency, control or IQGAP1 shRNA Capan-1 cells (1×10^6) were injected subcutaneously into NOD-*scid*IL2Rgamma^{null} (NSG) mice and allowed to form palpable tumors. Animals were treated for 7 days with water containing 2 mg/mL doxycycline, then tumors were harvested, dissociated into single cells, and injected into naïve secondary recipients (10^3 – 10^5 cells). After 5 weeks, TIC frequency was calculated using extreme limiting dilution analysis (ELDA).¹⁸ For evaluation of metastatic potential, Capan-1 control or IQGAP1 shRNA cells (2.5×10^5) were orthotopically injected into the pancreas of NSG mice. After 14 days, one mouse from each group was euthanized to macroscopically confirm primary tumor formation, and the remaining 4 mice were treated with water containing 2 mg/mL doxycycline for 25 days. Mice were euthanized, and metastases were identified by necropsy and hematoxylin and eosin (H & E) stained histological analysis. For WW peptide treatment, athymic nude mice were injected subcutaneously with Capan-1 cells or low-passage PDAC PDXs, and following the development of palpable tumors, mice were treated with 50 mg/kg scrambled control or WW peptide by intraperitoneal injection twice a day for 21 days. Tumors were measured twice a week and tumor volume was calculated using the formula $V = (L \times W^2)/2$.

qRT-PCR Analysis

mRNA was purified using the RNeasy Mini kit (Qiagen, Germantown, Md) and cDNA was synthesized using SuperScript III First Strand Synthesis System (Thermo Fisher, Waltham, Mass). Relative mRNA expression was determined using Taqman probes (*IQGAP1* probe #: hs00896595_m1, *GAPDH* probe #: hs99999905_m1) (Thermo Fisher, Waltham, Mass) and normalized to *GAPDH* expression.

Western Blot Analysis

Protein was extracted using Mammalian Protein Extraction Reagent (Thermo Fisher, Waltham, Mass) with protease and phosphatase inhibitors and electrophoresed on NuPage Novex 4–12% Bis-Tris Protein Gels, transferred to PVDF membranes, and blocked for one hour with 5% w/v BSA or 5% nonfat milk in 1X TBS and 0.1% Tween-20. Immunoblots were performed using rabbit anti-IQGAP1 (Abcam ab86064, Abcam, Cambridge, Mass), anti-pERK1/2 (Cell Signaling CST9101S, Cell Signaling, Danvers, Mass), and anti-ERK1/2 (Cell Signaling CST9102) polyclonal, and mouse anti-GAPDH monoclonal (Abcam ab8245) antibodies. Proteins were detected using the Amersham ECL reagent (GE Healthcare Life Sciences, Pittsburgh, Pa) on HyBlot CL film (Denville Scientific, Holliston, Mass). Densitometric analysis of protein expression was performed using ImageLab software (BioRad, Hercules, Calif).

Flow Cytometry

Aldehyde dehydrogenase (ALDH) staining was carried out using the Aldefluor reagent (Stemcell Technologies, Cambridge, Mass) according to the manufacturer's protocol. ALDH⁺ cells were identified by generating a control gate based on cells stained with Aldefluor in the presence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Analysis was performed using FlowJo software 8.7 (BD Biosciences, Franklin Lake, NJ).

Data Analysis

Statistical differences were analyzed using two-tailed, unpaired Student's *t* test with Welch's correction in Graphpad Prism 6 software (La Jolla, Calif.) for Mac OS. *P* values less than 0.05 were considered significant.

RESULTS

IQGAP1 is Required for *In Vitro* PDAC Clonogenic Growth and Self-renewal

Local and metastatic relapse are dependent on the maintenance of long-term clonogenic growth potential, and activating mutations in RAS family members can induce aberrant self-renewal in multiple systems, including multiple myeloma and epithelial cells.^{6,19,20} IQGAP1 facilitates MAPK signaling downstream of KRAS, and to determine its role in PDAC, we generated stable cell lines with doxycycline-inducible shRNA constructs (Supplemental Fig. 1A). As expected, the loss of IQGAP1 expression was associated with the inhibition of MAPK signaling in Capan-1 and PANC-1 PDAC cells as evidenced by decreased levels of pERK after 3 and 7 days of doxycycline exposure (Fig. 1A). We examined clonogenic growth and found that IQGAP1 knock-down significantly inhibited Capan-1 tumor cell colony formation by approximately 50% compared to cells with a scrambled control shRNA construct (Fig. 1B, *P* < 0.001). This inhibition was not due to the induction of cell death, as the number of viable cells did not significantly change following IQGAP1 knock-down (Supplemental Fig. 1B). In addition, the loss of IQGAP1 expression significantly inhibited self-renewal as the loss of clonogenic growth was maintained during serial colony formation despite the lack of further treatment with doxycycline (Fig. 1B, *P* < 0.05). We also examined the impact of IQGAP1 knock-down on PANC-1 cells that are not dependent on mutant

KRAS for cell proliferation or survival²¹ and found that primary and secondary colony formation was increased, rather than decreased as in *KRAS*-dependent Capan-1 cells, compared to cells with control shRNA (Supplementary Fig. 1C). In many diseases, self-renewal is enriched in rare populations of cancer stem cells (CSCs),²² and in PDAC, we previously demonstrated that CSCs express higher levels of ALDH.²³ We quantified PDAC CSCs and found that IQGAP1 knock-down did not significantly change the frequency of Capan-1 or PANC-1 ALDH+ cells (Supplementary Fig. 1D). Therefore, IQGAP1-mediated MAPK signaling impacts clonogenic growth and self-renewal in *KRAS*-dependent, but not independent, PDAC cells without significantly changing the frequency of phenotypically defined CSCs.

Loss of IQGAP1 Decreases *In Vivo* TIC Frequency

We examined the impact of IQGAP1 on PDAC tumor initiation *in vivo* and injected immunodeficient NSG mice with IQGAP1 or control shRNA Capan-1 cells to form subcutaneous tumors (Supplementary Fig. 2A). We then treated mice with doxycycline for 7 days, harvested tumors, verified IQGAP1 knockdown, and injected tumor cells in limiting dilution into naïve recipients (Supplementary Figs. 2A, B). Although tumor volumes did not significantly differ between the groups at the end of the 7 day treatment period (data not shown), the TIC frequency significantly decreased from approximately 1 in 300 to 1 in 18,000 in control and IQGAP1 shRNA cells, respectively (Table 1 and Supplemental Fig. 2A, $P < 0.001$). Therefore, IQGAP1 impacts the tumor-initiating potential of PDAC.

Loss of IQGAP1 Decreases Cell Migration and Metastasis

The development of metastatic disease requires cell invasion and migration in addition to clonogenic growth. We examined cell migration *in vitro* and found that these properties were significantly inhibited in Capan-1, but not PANC-1, cells following IQGAP1 knock-down compared to cells with the control shRNA (Fig. 2A, $P < 0.05$). To assess the role of IQGAP1 in metastasis, we orthotopically injected IQGAP1 or control shRNA Capan-1 cells into the pancreas of NSG mice and allowed primary tumors to form (Fig. 2B). Following 25 days of doxycycline treatment, primary tumors were identified in all mice (Figs. 2C, D). Liver metastases were identified in all mice injected with control shRNA cells, and additional metastases were present in the spleen (75%), intestine (25%), and mesentery (25%) of animals. In contrast, liver metastases were limited to 25% and splenic lesions to 50% of the animals receiving IQGAP1 shRNA cells. Therefore, IQGAP1 impacts PDAC cell migration *in vitro* and metastasis *in vivo*.

WW Peptide Inhibits Clonogenic Growth and Migration

A peptide mimicking the WW domain of IQGAP1 can disrupt IQGAP1-ERK interactions and extend then survival of PDAC mouse models.¹⁵ To determine whether the WW peptide could limit PDAC clonogenic growth, we treated Capan-1 cells with the WW peptide and found that it inhibited ERK activation, tumor colony formation, and cell migration compared to a scrambled sequence control peptide (Figs. 3A, B and Supplementary Fig. 3A). *In vivo* WW peptide treatment significantly decreased the growth of subcutaneous Capan-1 tumors and 1 of 2 PDXs (Figs. 3C, D, and Supplementary Fig. 3B). Therefore, the WW peptide inhibits clonogenic growth in PDAC and may represent a novel treatment strategy.

DISCUSSION

The poor prognosis of PDAC patients is driven by high rates of tumor relapse and metastatic dissemination. Self-renewal maintains clonogenic growth over time and is required for both local tumor regrowth and the formation of metastatic lesions at distal sites. Mutant *KRAS* signaling has been shown to impact self-renewal and metastasis in several solid tumors,^{19,20,24} and IQGAP1 is over-expressed in CD133+ glioblastoma TICs.²⁵ Here we demonstrate that mutant *KRAS*-dependent PDAC is dependent on IQGAP1 for clonogenic growth, self-renewal, tumor-initiation, and metastasis.

Our data also suggest that the effects of IQGAP1 knock-down are due to the disruption of MAPK signaling, similar to other studies.^{6,15} Recently, the interaction between the IQGAP1 and ERK2 has been found to be mediated by the IQ, rather than WW, domains.²⁶ Although the loss of total IQGAP1 expression would not distinguish between these possibilities, we found that the WW peptide decreased ERK phosphorylation and clonogenic growth suggesting that the WW domain is important in PDAC. However, it is possible that the effects of IQGAP1 loss in PDAC are mediated by its scaffold function for an alternative pathway, such as the interaction between Cdc42 and Rac1,²⁷ or another functional property.

CSCs have been identified in many diseases and are enriched for tumor-initiating and self-renewal potential. In solid tumors, CSCs have also been associated with metastatic disease.^{23,28–31} Despite the inhibition of clonogenic growth and self-renewal, we did not detect a change in the frequency of ALDH+ PDAC CSCs following the knock-down of IQGAP1. It is possible that the loss of IQGAP1 expression impacts both CSCs and non-CSCs equally so that the TIC frequency remains relatively constant. It is also possible that IQGAP1 knock-down uncouples the functional features of CSCs from their phenotype or that the changes we observed are attributable to ALDH-negative TICs.

The identification of molecular targets to inhibit aberrant self-renewal has been limited in most diseases, but blocking several potential pathways, such as developmental signaling pathways, may impact normal stem cells. Since *KRAS* mutations are typically limited to cancer cells, targeting aberrant RAS signaling may improve relapse rates and the development of metastatic disease without inhibiting normal tissue homeostasis. Similar to previous studies,^{6,15} the administration of the WW peptide did not appear to impact the health of mice, suggesting that this approach may be cancer specific.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

IQGAP1 knock-down inhibits PDAC clonogenic growth and self-renewal. A, Western blot of doxycycline treated Control and IQGAP1 shRNA cells. For quantification, IQGAP1 and pERK band density was normalized to GAPDH. B, Primary and secondary colony formation of shIQGAP1 and shControl Capan-1 cells. Data represent mean and standard deviation of 3 independent experiments.

FIGURE 2.

IQGAP1 loss decreases cell migration and metastasis. A, Transwell migration of doxycycline treated control and IQGAP1 shRNA Capan-1 cells. Data represent mean and standard deviation of 3 independent replicates. B, Experimental schema for *in vivo* metastasis formation assay. C, Quantification of metastases identified during necropsy in animals bearing control and IQGAP1 shRNA Capan-1 tumors. Four animals were analyzed in each group. D, Representative images of H & E stained liver and pancreas tumors derived from control or IQGAP1 shRNA Capan-1.

FIGURE 3.

WW peptide treatment inhibits clonogenic growth. A, Colony formation of Capan-1 cells treated with scrambled control or WW peptide. Data represent mean and standard deviation of 3 independent experiments. B, Transwell migration assays of Capan-1 cells treated with scrambled control or WW peptide. Data represent mean and standard deviation of 3 independent experiments. C, Relative tumor volume of Capan-1 tumors in mice treated with scrambled control or WW peptides. Data represent the mean and standard deviation of 4 individual tumors in each group. D, Relative tumor volume of PDX tumors in mice treated with scrambled control or WW peptides. Data represent the mean and standard deviation of 4 individual tumors in each group.

TABLE 1.**IQGAP1 Regulates TIC Frequency in PDAC**

No. Cells Injected	Tumor Incidence	
	shCtrl	shIQGAP1
10,000	4/4	4/4
1000	4/6	0/6
100	8/10	0/10
TIC frequency	1 in 303	1 in 18,040
TIC (95% CI)	1/711–1/129	1/71,205–1/4571
<i>P</i>	<0.001	

TIC frequency calculated using ELDA software.¹⁸