Cholinergic Signaling via Muscarinic Receptors Directly and Indirectly Suppresses Pancreatic Tumorigenesis and Cancer Stemness

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INTRODUCTION

Extensive efforts in cancer research over recent decades have led to a steady increase in survival for most cancer types, but less so for pancreatic ductal adenocarcinoma (PDAC), which continues to have the lowest 5-year survival rate for cancers, at 8% (1). With few specific symptoms and no reliable test for early detection, PDAC is typically diagnosed at a locally advanced or distant stage (2). After careful staging, only 15% to 20% of patients are eligible for up-front resection (3). Even after potential curative resection, most patients will eventually have recurrence, and 5-year survival of completely resected patients is only 26% (3). In the metastatic setting, FOLFIRINOX and nab-paclitaxel–gemcitabine (GEM) are standard treatment options in patients with good performance status and have shown benefit over previously standard GEM monotherapy, but even in those highly selected patients, overall survival remains poor (3).

The resistance of PDAC to treatment has been attributed in part to the tumor microenvironment and the complex desmoplastic stroma (4), which includes immune cells, endothelial cells, stellate cells, matrix proteins, and nerves (5, 6). In this regard, the nervous system is increasingly recognized as a central regulator of both normal stem-cell function and cancer growth across a range of tissues (7). In a pathogenic context, neurons are emerging as a critical microenvironmental element in a variety of cancers, i.e., prostate (8, 9), skin (10), gastric (11–13), and pancreatic (14–17) cancers. Furthermore, growing evidence suggests that there is increased cross-talk between tumor cells and nerves, with tumors able to induce active axonogenesis (13, 17–19).

In many solid tumors, parasympathetic input is provided by the vagus nerve, which has been shown to modulate tumor growth. However, whether cholinergic signaling directly regulates progression of pancreatic ductal adenocarcinoma (PDAC) has not been defined. Here, we found that subdiaphragmatic vagotomy in LSL-Kras\(^{+/G12D}\)/Pdx1-Cre (KC) mice accelerated PDAC development, whereas treatment with the systemic muscarinic agonist bethanechol restored the normal KC phenotype, thereby suppressing the accelerated tumorigenesis caused by vagotomy. In LSL-Kras\(^{+/G12D}\)/LSL-Trp53\(^{-/R172H}\)/Pdx1-Cre mice with established PDAC, bethanechol significantly extended survival. These effects were mediated in part through CHRM1, which inhibited downstream MAPK/EGFR and PI3K/AKT pathways in PDAC cells. Enhanced cholinergic signaling led to a suppression of the cancer stem cell (CSC) compartment, CD11b\(^{+}\) myeloid cells, TNF\(\alpha\) levels, and metastatic growth in the liver. Therefore, these data suggest that cholinergic signaling directly and indirectly suppresses growth of PDAC cells, and therapies that stimulate muscarinic receptors may be useful in the treatment of PDAC.

SIGNIFICANCE: Subdiaphragmatic vagotomy or Chrm1 knockout accelerates pancreatic tumorigenesis, in part via expansion of the CSC compartment. Systemic administration of a muscarinic agonist suppresses tumorigenesis through MAPK and PI3K/AKT signaling, in early stages of tumor growth and in more advanced, metastatic disease. Therefore, CHRM1 may represent a potentially attractive therapeutic target.

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parasympathetic nervous system, with the vagus nerve strongly promoting epithelial proliferation, stem cell activity, and tumorigenesis. Thus, in the case of gastric cancer, abrogation of cholinergic input by vagotomy or chemical denervation inhibits the growth of gastric cancer (11, 12). In addition to directly regulating the epithelium, nerves have been shown to act indirectly through effects on the tumor stroma or microenvironment (8, 9). In mouse models of prostate cancer, for example, cholinergic signals transduced in the tumor stroma by the muscarinic type 1 receptor (CHRM1) promote tumor invasion (9).

Similar to many other solid organs, the pancreas is innervated by both sympathetic and parasympathetic nerves (20). In the pancreas, the autonomic nervous system regulates both exocrine and endocrine functions, and influences normal pancreatic development (21). In addition, the vagus nerve has been shown to stimulate proliferation of the normal exocrine pancreas, such that ventromedial hypothalamic lesions that induce vagal hyperactivity stimulate pancreatic proliferation and lead to pancreatic hypertrophy, whereas vagotomy leads to decreased pancreatic acinar growth (22). On the other hand, clinical studies have suggested that vagus nerve signaling may actually slow pancreatic tumor progression (23). In this regard, a higher vagal nerve activity as expressed by a higher heart rate variability was reported to correlate significantly with lower risk of death in metastatic PDAC. These results are in line with possible vagal nerve protection in this fatal cancer (23). Furthermore, a higher incidence of PDAC was found in patients who underwent vagotomy for gastric ulcer disease in the past (24). Similarly, a study in orthotopic and syngeneic PDAC mouse models demonstrated that vagotomy promoted tumor growth and shortened overall survival, although this was largely attributed to indirect effects on tumor-associated macrophages and elevated TNFα levels (16). In addition, tumor-bearing animals that underwent chemical or surgical vagotomy showed enhanced metastasis of breast cancer cells (25).

Nevertheless, although these earlier studies suggested that vagal signaling could be suppressive in some cancer models, a direct role for cholinergic signaling in genetically engineered mouse models (GEMM) and metastatic models of PDAC has not been demonstrated, nor has the mechanism been elucidated. Consequently, we investigated the direct contribution of the vagus nerve, cholinergic signaling, and muscarinic receptors to PDAC development and progression in GEMMs LSL-KrasG12D;Pdx1-Cre (KC), LSL-KrasG12D;LSL-Trp53R172H;Pdx1-Cre (KPC), which phenocopy human PDAC (26), and metastatic models.

RESULTS
Subdiaphragmatic Vagotomy Promotes Pancreatic Tumorigenesis

We first investigated the specific effects of parasympathetic denervation on PDAC development. Genetically engineered KC mice underwent a subdiaphragmatic vagotomy with a subsequent pyloroplasty (KC + VxPP) at 8 weeks of age. When analyzed at 20 weeks (Supplementary Fig. S1A), KC + VxPP mice showed a similar ratio of pancreas weight to body weight (PW/BW) compared with control KC mice, which received pyloroplasty only (KC + PP; Supplementary Fig. S1B). Analysis of mRNA expression revealed significantly increased pancreatic expression of the Chrm1 (P < 0.05), whereas expression levels of other muscarinic receptors did not differ when compared with the control group (KC + PP) at 20 weeks (Fig. 1A). This finding was confirmed by immunofluorescent staining (IHC-F), where CHRM1 was more highly expressed in pancreatic acinar and tumor epithelial cells in KC + VxPP mice (n = 13) compared with KC + PP mice (n = 10; Fig. 1B and C). Morphometric quantification of pancreatic intraepithelial neoplasia (PanIN) lesions in pancreata from KC + VxPP mice at 20 weeks revealed a significantly larger area affected by PanINs than in KC + PP mice (P < 0.01; Fig. 1D–F). Importantly, PDAC lesions, not observed in KC + PP mice, were present in 40% of the KC + VxPP mice (Fig. 1E and G).

Given that subdiaphragmatic vagotomy appeared to accelerate PDAC development and upregulate expression of CHRM1 on epithelial cells, we sought to determine whether systemic muscarinic stimulation could rescue the regular KC phenotype and suppress PDAC development. Thus, we examined the effect of systemically administered bethanechol, a broad muscarinic agonist, on KC mice that had undergone vagotomy. Bethanechol treatment was initiated in KC mice at 8 weeks, immediately after the mice had undergone surgery (Vx + PP), and continued until 20 weeks of age (Supplementary Fig. S1C). Administration of bethanechol led to a significant reduction in PanIN area and pancreatic tumor incidence in KC + VxPP mice (n = 14; P < 0.01 and P < 0.05, respectively) compared with untreated KC + VxPP mice (n = 13; Fig. 1E–H).

Because CD44 expression is known to mark a subpopulation of PDAC cells that harbor a higher grade of plasticity and greater resistance to treatment (27), we investigated expression of CD44 in the pancreas of these mice. Herein, we found increased levels of CD44 in pancreata of KC + VxPP versus KC + PP mice (P < 0.05) at 20 weeks (Supplementary Fig. S1D). In contrast, bethanechol-treated KC + VxPP mice showed significantly reduced pancreatic CD44 expression compared with KC + VxPP mice (Supplementary Fig. S1D).

The pancreas has multiple sources of innervation, such that subdiaphragmatic vagotomy typically would not be expected to lead to a significant reduction in overall nerve density (28). Nevertheless, we sought to confirm the efficacy of vagotomy procedure. First, we performed immunohistochemical (IHC) studies of pancreatic sections with an antibody recognizing β-Tubulin III, thereby staining neuronal fibers. As expected, we found only minimal alteration in neuronal density in KC + PP versus KC + VxPP mice (Fig. 1I). However, when we analyzed the density of cholinergic fibers, which were stained with an antibody against the vesicular acetylcholine transporter (VACHT) in pancreata of wild-type C57BL/6 (WT) + PP versus WT + VxPP mice, a significant reduction in VACHT-positive structure was detected (Supplementary Fig. S1E and S1F).

As an effective subdiaphragmatic vagotomy should result in gastric distention through the inability of pyloric musculature relaxation (29), we measured in our study animals the gastric diameter as the distance from the mid of the lesser curvature to the greater curvature in a perpendicular way. An increase of more than 1.5-fold was used as a cutoff for indicating a successful surgical procedure, which was documented in all study animals (Supplementary Fig. S1G and S1H). In addition, the completeness of vagotomy was verified...
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**Figure 1.** Subdiaphragmatic vagotomy promotes pancreatic tumorigenesis. A, Relative quantification of mRNA expression of Chrm1 to Chrm5 in KC + PP mice pancreata compared with KC + VxPP mice pancreata at 20 weeks (n = 3, each group). B, Representative image of Chrm1 IHC-F of pancreata from KC + PP at 20 weeks. White arrowheads indicate Chrm1-positive cells in PanIN lesions (CHRM1, green; DAPI, white). C, Representative image of CHRM1 IHC-F of pancreata from KC + PP at 20 weeks. White arrowheads indicate CHRM1-positive cells in PanIN lesions (CHRM1, green; DAPI, white). D, Representative image of CHRM1 IHC-F of pancreata from KC + PP mice at 20 weeks showing low-grade PanIN lesions. E, Representative image of hematoxylin and eosin (H&E)-stained pancreatic section from KC + PP mice at 20 weeks showing low-grade PanIN lesions. F, Representative image of H&E-stained pancreata from KC + VxPP mice at 20 weeks showing high-grade PanIN/PDAC lesions. G, Percentage of PanIN area in higher power fields in pancreata from KC + VxPP mice (n = 12), KC + PP mice (n = 10), and KC + PP + bethanechol (n = 14) at 20 weeks. H, Percentage of PanIN area in higher power fields in pancreata from KC + VxPP mice (n = 13) compared with KC + PP mice (n = 10) and KC + PP + bethanechol mice (n = 14) that developed pancreatic cancer at 20 weeks. I, Representative image of β-tubulin III–stained area/field ratio (%) in pancreata from KC + PP and KC + VxPP mice at 20 weeks (n = 4, each group). Bar graph showing quantification of β-tubulin III–stained area in pancreata from KC + PP and KC + VxPP mice. J, Representative images of pancreatic IHC for CD11b in KC + PP, KC + VxPP, and KC + VxPP + bethanechol mice at 20 weeks. Bar graph showing quantification of CD11b–stained area in pancreata from KC + PP, KC + VxPP, and KC + VxPP + bethanechol mice at 20 weeks. K, Representative images of pancreatic IHC for F4/80 in KC + PP, KC + VxPP, and KC + VxPP + bethanechol mice at 20 weeks. Bar graph showing quantification of F4/80–stained area in pancreata from KC + PP, KC + VxPP, and KC + VxPP + bethanechol mice (n = 3, each group). *, P < 0.05; **, P < 0.01; mean ± SD; scale bars, 100 μm.
During postmortem inspection of vagal nerve endlings using microscopic inspection.

Because the biological effects of vagotomy are complex, and thus could modulate cancer development indirectly through effects on the tumor microenvironment, we analyzed effects of vagal transection in KC + VxPP versus KC + PP mice on the stromal compartment. As demonstrated in the past (30), sub-diaphragmatic vagotomy resulted in an increase in systemic and splenic levels of TNFα in KC + VxPP mice compared with the KC + PP mice (Supplementary Fig. S1I–S1K). Surprisingly, treatment with bethanechol resulted in suppression of TNFα levels, both in the spleen and in the circulation (Supplementary Fig. S1I–S1K). Further analysis of the immune cell compartment showed increased levels of CD11b+ myeloid cells (Fig. 1I) and F4/80+ cells (Fig. 1K). Flow-cytometric analysis of the spleen revealed differences only in F4/80+ cells (Supplementary Fig. S1L). In contrast, we found no major differences in the levels of T (CD3) or B (B220) lymphoid cells in the spleen (Supplementary Fig. S1M and S1N). Furthermore, IHC analysis of CD3+ cells, B220+ cells, myofibroblasts (α-SMA+), endocrine cells (CgA+), and endothelial cells (CD31+) showed no significant changes in these stromal lineages in the pancreas of KC + VxPP mice compared with control KC + PP mice (Supplementary Fig. S1O–S1S). Taken together, these data suggest that vagal denervation in the presence of an oncogenic Kras mutation promotes pancreatic tumorigenesis through an expansion of CD44+ epithelial cells and potentially through enhanced inflammation.

**Muscarnic Stimulation Suppresses Pancreatic Tumorigenesis and Extends Overall Survival in KPC Mice**

Next, we investigated the role of cholinergic signaling in established PDAC, using the KPC mouse model, which develops frank PDAC at a median age of 17 to 19 weeks (31). KPC mice with pancreatic tumors that measured 3 to 5 mm in diameter on ultrasound were randomized to treatment with GEM alone, or GEM + bethanechol (Fig. 2A). Treatment with GEM + bethanechol extended the overall survival of KPC mice treated with GEM alone from 29 to 48 days (P < 0.001; Fig. 2B). Pancreatic tumor sections stained by hematoxylin and eosin (H&E) at the time of necropsy did not reveal obvious differences in histology between the two groups (Fig. 2C and D). However, treatment with GEM + bethanechol led to a significant reduction in pancreatic CD44 expression compared with GEM alone (P < 0.05; Fig. 2E).

To further characterize this CD44+ population, we examined the expression of markers used to isolate cancer stem cells (CSC) in human tumors (32), as specific CSC markers for murine PDAC have been less well validated. In this regard, the CD44+CD133+ population was described earlier as putative murine CSCs (33, 34). Intriguingly, this double-positive population was significantly reduced in the GEM + bethanechol group compared with control (P < 0.05; Fig. 2F and G). In addition, analysis of the CD44+CD24+EPCAM+ population also revealed a trend toward suppression of the CD44+CD24+EPCAM+ population in tumors of KPC mice treated with GEM + bethanechol versus GEM-only treatment in KPC mice (Supplementary Fig. S2A and S2B). Taken together, these data suggest that muscarinic agonists can suppress pancreatic tumorigenesis in part by suppression of the CSC compartment.

**Cholinergic Signaling Directly Promotes Cell Proliferation in Kras-Mutant Spheres via CHRM1 and Regulates Cancer Stemness**

To determine whether cholinergic agonists suppress tumor development in part in direct stimulation of muscarinic receptors on pancreatic epithelial cells, we assessed the sphere-forming capacity of KrasG12D pancreatic acinar cells in an established three-dimensional (3-D) Matrigel culture system. These cultures are known to mimic acinar to ductal metaplasia (35), the proposed first step of PDAC tumorigenesis (36). KrasG12D-mutant spheres were generated from LSL-KrasG12D mice by delivery of adeno-viral-Cre (Adeno-Cre) and then treated with the nonselective muscarinic agonist pilocarpine, the nonselective muscarinic antagonist scopolamine, the CHRM1-selective agonist McN-34A, or the CHRM1-selective antagonist pirenzepine. Pilocarpine- or McN-34A-treated Kras-mutant acinar cell cultures formed significantly fewer and smaller spheres, whereas acinar cultures treated with scopolamine or pirenzepine formed significantly more and larger spheres (Fig. 3A–J). In addition, we studied the effects of muscarinic agonism in 3-D primary human PDAC organoids (37). After 6 days of treatment with increasing doses of pilocarpine (6.25–400 μmol/L), we observed fewer viable spheres in these cultures in a dose-dependent manner (Fig. 3K–M).

Analysis of muscarinic receptor expression in human (Panc1 and MiaPaca2) and murine (K8282 and Panc02) PDAC cell lines revealed higher CHRM1 and Chrm1 expression after scopolamine treatment, respectively, and lower CHRM1 and Chrm1 expression with pilocarpine treatment (Supplementary Fig. S3A–S3D) compared with control. In addition to CHRM1 expression, Chrm3 and Chrm4 were highly expressed in human pancreatic cancer cell lines, although they did not show significant changes with drug treatment. Moreover, MTT assays demonstrated that pilocarpine decreased cell viability of a human (Panc1) cancer cell line in a dose-dependent manner (Supplementary Fig. S3E). Muscarinic agonists decreased cell viability of human (Panc1) and murine (K8282) cancer cell lines, whereas muscarinic antagonists (scopolamine and pirenzepine) had the opposite effect (Supplementary Fig. S3F and S3G). These results fueled the hypothesis that muscarinic receptors and CHRM1 in particular were largely responsible for modulating the anti-proliferative effects of muscarinic agonists. Furthermore, we also studied the effects of muscarinic agonism on nontumorigenic, HPV-16E6E7–immortalized human pancreatic ductal epithelial (HPDE-E6E7) cells (38). Interestingly, pilocarpine in doses up to 100 μmol/L had no effect on the viability of these cells. In contrast, the expression of mutant KRASG12D in HPDE-E6E7 cells by retroviral transduction resulted in a significant reduction in viability at doses as low as 100 μmol/L of pilocarpine (Supplementary Fig. S3H). Similarly, we observed that pilocarpine suppresses the growth of Kras-mutant pancreatic spheroids, but stimulates the growth of WT pancreatic
Figure 2. Muscarinic stimulation suppresses pancreatic tumorigenesis and extends overall survival in KPC mice. A, Experimental setup: KPC mice were enrolled with tumors of 3 to 5 mm, confirmed by high-resolution ultrasound, and treated with GEM (100 mg/kg) biweekly (n = 15) or GEM + bethanechol in the drinking water (400 μg/mL; n = 10) until they became moribund and needed to be sacrificed. B, Kaplan-Meier curve comparing overall survival of KPC mice treated with GEM (n = 15) or GEM + bethanechol (n = 10) after initiation of the respective therapy. C, Representative images of H&E-stained PDAC from GEM-treated KPC mice. D, Representative images of H&E-stained PDAC from GEM + bethanechol-treated KPC mice. E, Representative images of IHC for CD44 in tumors from KPC mice treated by GEM or GEM + bethanechol. Bar graph showing quantification of the area stained positive for CD44 in PDAC from KPC mice treated with GEM (n = 15) or GEM + bethanechol (n = 10). F, Representative flow cytometric plot of CD44+CD133+ cells in PDAC from KPC mice treated with GEM or GEM + bethanechol. Numbers are showing ratio of CD44+CD133+ cells to viable cells. G, Bar graph shows quantification of CD44+CD133+ cells in PDAC from KPC mice treated with GEM (n = 3) or GEM + bethanechol (n = 4). Scale bars, 100 μm. Mean ± SE in E and mean ± SD in G. *, P < 0.05; ***, P < 0.001.

spheroids. This study was performed using spheres that were generated from LSL-KrasG12D pancreata recombined by Adeno-Cre recombinase (Kras mutant) or without treatment of Adeno-Cre (Kras WT; Supplementary Fig. S3I and S3J).

To further address the potential effects of muscarinic agonists on the CSC compartment, we studied the effects on a human pancreatic cancer cell line (Panc1) and a murine cancer cell line (K8282) in anchorage-independent soft-agar cultures. These experiments revealed reduced sphere-forming capability after pilocarpine treatment, suggesting suppression of the CSC compartment in human and murine cancer cell lines (Fig. 3N–Q). To elaborate this observation...
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To determine potential mechanisms by which muscarinic agonists are able to suppress progression of PDAC, we analyzed Panc1 cells following pilocarpine treatment using RNA sequencing (RNA-seq; Supplementary Fig. S4A–S4C). Growth-related genes that were suppressed by pilocarpine included EGER and PIK3CA (Supplementary Fig. S4A). A more detailed analysis of differentially expressed genes in terms of Kyoto Encyclopedia of Genes and Genomes pathways using iPathwayGuide suggested a possible mechanism of tumor suppression through the PI3K–AKT and MAPK pathways (Supplementary Fig. S4B and S4C). The RNA-seq data are consistent with inhibition of pilocarpine by the PI3K/AKT pathway (Supplementary Fig. S4B; ref. 39). PIK3CA (PI3K) is activated by EGER, KDR (RTK), and ITGAV (ITGA), all of which were downregulated by pilocarpine, so that PIK3CA (PI3K) is inhibited at the signaling level as well. PIK3CA (PI3K) is an activator of AKT, so that inhibition of PIK3CA (PI3K) leads to inhibition of AKT, thus reducing proliferative signals. In the classic MAPK signaling pathway (Supplementary Fig. S4C), NTRK2 (TRKB) and BRF (RAFB), which are also proproliferative (40), were also downregulated according to the RNA-seq results.

To test key aspects of the above model, we examined the expression of phosphorylated forms of EGFR, PI3K, and ERK1/2 (pEGFR, pPI3K, and pERK1/2) by IHC staining on sections from mice with different treatments (Fig. 3). To assess the biological significance of these observations, we treated first with pilocarpine, and then with the MEK inhibitor selumetinib (AZD6244). We found that administration of the MEK inhibitor significantly reduced PanIN progression that could be attributed to vagotomy (KC + VxPP; Fig. 4D). Taken together, muscarinic receptors appear to suppress signaling through MAPK and other downstream pathways that likely contribute to pancreatic tumorigenesis.

Figure 3. Cholinergic signaling directly promotes cell proliferation in Kras-mutant spheres via CRHMR1 and regulates cancer stemness. A–C, Representative images of pancreatic spheres isolated from LSL-Kras<sup>G12D</sup> mice and treated with an Adeno-Cre virus (A) or an Adeno-Cre virus and pilocarpine (B) or an Adeno-Cre virus and scopolamine (C), D and E, Number of spheres per well (D) and size of spheres (E) isolated from LSL-Kras<sup>G12D</sup> mice and cultured in the presence of an Adeno-Cre virus, which are untreated, treated with pilocarpine, or treated with scopolamine (n = 3, each group). F–H, Representative images of spheres isolated from LSL-Kras<sup>G12D</sup> mice and treated with an Adeno-Cre virus (F) or an Adeno-Cre virus and McriKO<sup>G12D</sup> (G) or Adeno-Cre virus and pirenzepine (H). I and J, Number of spheres per well (I) and size of spheres (J) isolated from LSL-Kras<sup>G12D</sup> mice and cultured in the presence of Adeno-Cre virus, which are untreated, treated with Mcn-34A, or treated with pirenzepine (n = 3, each group). K, Representative image of organoids generated from primary resected human PDAC specimen without treatment. L, Representative image of organoids generated from primary resected human PDAC specimen on pilocarpine treatment. M, Representative images of spheres isolated from LSL-Kras<sup>G12D</sup> mice and treated with an Adeno-Cre virus (N) or an Adeno-Cre virus and pilocarpine (O) or an Adeno-Cre virus and scopolamine (P) at day 14. Q, Bar graph showing quantification of numbers of spheres from Kras<sup>G12D</sup> mice plated in soft agar which are untreated, treated by pilocarpine, or treated by scopolamine and pilocarpine at day 14 (n = 3, each group). R, Flow cytometric analysis of CD44<sup>+</sup>/CD24<sup>+</sup>/EPCAM<sup>+</sup> cells in Panc1 cells that are untreated, treated by pilocarpine, or treated by scopolamine and pilocarpine at different time points (n = 3). S, Percentage of CD44<sup>+</sup>/CD24<sup>+</sup>/EPCAM<sup>+</sup> cells in Panc1 cells that are untreated, treated by pilocarpine, or treated by scopolamine and pilocarpine 9 days after injection of 25,000 injected Panc1 cells with and without pretreatment with pilocarpine (n = 10, each group). Scale bars, 100 μm in A–C and F–H. 500 μm in K, L, N, and O, and 0.5 μm ± SD in D, E, I, J, M, and T, mean ± SEM in P, Q, and S, *, P < 0.05; **, P < 0.01.
Figure 4. Muscarinic signaling inhibits downstream EGFR/MAPK and PI3K/AKT signaling in PDAC cells. A–C, Representative images of IHC for pEGFR (A), pPI3K (B), and pERK1/2 (C) in pancreatic sections from KC + PP mice, KC + VxPP mice, and KC + VxPP + bethanechol mice. Bar graphs show quantification of pEGFR, pPI3K, or pERK1/2 staining in pancreata from KC + PP mice, KC + VxPP mice, and KC + VxPP + bethanechol mice (n = 3, each group). D, Representative western blot showing pERK1/2 and ERK1/2 relative to β-actin in Panc1 cells after treatment with indicated dosages of scopolamine and selumetinib. E, Representative H&E-stained images of pancreatic sections from KC + PP mice, KC + VxPP mice, and KC + VxPP + selumetinib mice. Bar graph shows quantification of PanIN area in pancreata from KC + PP mice, KC + VxPP mice, and KC + VxPP + selumetinib mice (n = 3, each group). Scale bars, 100 μm; mean ± SD. *, P < 0.05; **, P < 0.01.
Knockout of CHRM1 Results in Larger PanIN Area and Tumor Incidence in KC Mice and Shorter Overall Survival in KPC Mice

Given that unselective and selective CHRM1 agonists and antagonists modulated pancreatic tumorigenesis most likely via MAPK and PI3K–AKT signaling, we sought to confirm the role of CHRM1 in pancreatic tumor development by reducing receptor expression through genetic deletion. Therefore, we crossed KC mice to Chrm1-KO mice and generated KC/Chrm1-KO (KCM) mice (n = 11). We confirmed the absence of Chrm1 expression in KC mice by qRT-PCR and protein expression by IHC (Fig. 5A–C). Similar to vagotomized KC mice, KC mice showed more advanced PanINs, including PDAC, which was absent in KC mice (Fig. 5D–G). Morphometric analysis revealed a significantly larger PanIN area in KC mice compared with KC mice (n = 10; P < 0.01; Fig. 5H). Moreover, the PanIN incidence in KC mice was significantly higher compared with control KC mice (P < 0.05, 36.7% vs. 0%; Fig. 5I). Furthermore, IHC revealed significantly increased expression of CD44 (Fig. 5J), pEGFR (Fig. 5K), pPI3K (Fig. 5L), and pERK1/2 (Fig. 5M) in KC mice compared with KC mice (P < 0.05, respectively; Fig. 5N).

Next, we assessed the sphere-forming capacity of LSL-KrasG12Dmutant spheres versus LSL-KrasG12D-Chrm1-KO mutant spheres in a 3-D Matrigel culture system. The Kras mutation was activated in pancreatic spheres by infection with Adenoviral-Cre. As expected, the LSL-KrasG12D-Chrm1-KO sphere cultures generated more spheres compared with LSL-KrasG12D control spheres. Consistent with an absence of CHRM1 signaling, after pilocarpine or scopolamine treatment, LSL-KrasG12D-Chrm1-KO spheres showed a similar size and number compared with the untreated controls, whereas LSL-KrasG12D spheres showed a significant decrease or increase, respectively (Supplementary Fig. S5A–S5D).

Finally, we crossed KPC mice to Chrm1-KO mice, generating KPC/Chrm1-KO (KPCM) mice (n = 13). The median overall survival in KPCM mice was significantly decreased compared with KPC mice (n = 18; P < 0.001; Fig. 5O). Furthermore, IHC showed significantly increased expression of CD44, pEGFR, pPI3K, and pERK1/2 in tumors of KPCM compared with KC mice (P < 0.05, respectively; Supplementary Fig. S5E–S5H). Thus, these data suggest that an absence of CHRM1 signaling leads to enhanced EGFR/MAPK and PI3K/AKT signaling, increased CD44 expression, and accelerated PDAC progression.

Parasympathetic Signaling Influences Survival in a Model of Hepatic Metastasis

Our studies in KC and KPC mice suggest that signaling through CHRM1 inhibits growth of primary pancreatic tumors through downregulation of growth factor pathways. However, given that many patients with PDAC eventually die with liver metastases, we examined the effect of parasympathetic signaling on the growth of hepatic lesions, utilizing a well-established syngeneic model of metastatic PDAC (41). The surgical procedures used in this study are shown in Fig. 6A and Supplementary Fig. S6A–S6D. At 30 days after injection of murine PDAC cells (Panc02) into the spleen, cancer cells appear as large pale nodules replacing macroscopically normal liver tissue (Fig. 6B–D). To increase the detection of small metastases, Panc02 cells were stably transfected with a GFP-expressing construct, which was readily detected following splenic injection (Supplementary Fig. S6E and S6F). WT C57BL/6 mice received splenic injections of 2 × 10^5 GFP-labeled Panc02 cells and were then divided into 3 groups: untreated controls (n = 10), bethanechol treated (n = 10), and selective parasympathetic liver denervation by transection of the hepatic branch of the vagus (n = 11). Mice treated with bethanechol showed significantly longer survival (P < 0.001), whereas the group pretreated with selective hepatic vagotomy (SHVs) showed significantly shorter survival (P < 0.01; Fig. 6E). As shown by quantification of number of metastases and maximum volume of metastases (Fig. 6F and G), metastatic growth was expanded by SHVs (P < 0.05), whereas growth was inhibited by bethanechol (P < 0.05). Moreover, to confirm that the parasympathetic pathway directly regulated tumor cells at the liver metastatic site, expressions of Ki-67 (Fig. 6H), pEGFR (Fig. 6I), and CD44 (Fig. 6J) were analyzed immunohistochemically. As expected, quantitative scoring indicated that their expression was significantly decreased in the bethanechol group (P < 0.05) and significantly increased in the selective vagotomy group (P < 0.05). Therefore, these data suggest that parasympathetic signaling not only suppresses the growth of the primary tumor, but also inhibits the growth of metastatic hepatic lesions.

DISCUSSION

Accumulating evidence has revealed a key role for the autonomic nervous system in the development of cancer (7, 42). Here, we have shown that subdiaphragmatic vagotomy accelerates PDAC progression in KC mice. Importantly, systemic administration of a broad muscarinic agonist (bethanechol) rescued the regular KC phenotype (i.e., early PanINs) in vagotomized KC mice and significantly extended the survival of KC mice with established PDAC. In vivo studies revealed that bethanechol suppressed the CD44+/CD133+ and CD44+/CD24−/EPCAM+ putative CSC populations and increased levels of circulating TNFα levels along with CD11b+ myeloid cells within the pancreas. In vitro studies confirmed that muscarinic agonists showed antiproliferative effects on Kras-mutant pancreatic spheres and suppressed the CD44+/CD133+ and CD44+/CD24−/EPCAM+ cell fraction in PDAC cell lines. We identified CHRM1 as the receptor largely responsible for cholinergic suppressive effects in mice. Further analysis of downstream signaling pathways indicated that CHRM1 signaling is able to suppress the EGFR/MAPK/PI3K/AKT pathway. Finally, we found that bethanechol treatment alone was sufficient to extend the survival of mice with hepatic metastases. Taken together, these data reveal that activation of muscarinic signaling via CHRM1 can directly and indirectly suppress the initiation and progression of PDAC (Supplementary Fig. S6G).

The nervous system in theory has the potential to influence the progression of cancer indirectly by modulating the immune system, tumor metabolism, angiogenesis, and tumor stroma cross-talk, or through direct effects on tumor cells (9, 12, 17). In prostate cancer, both sympathetic and parasympathetic nerves are significantly involved in promoting all phases of murine cancer development (8, 9). In gastric cancer, cholinergic signaling supports stem cell growth directly via the M3...
Figure 5. Knockout of CHRM1 results in larger PanIN area and tumor incidence in KC mice and shorter overall survival in KPC mice. A and B, Representative images of CHRM1 immunofluorescence in pancreatic sections from KC (A) and KCM (B) mice at 20 weeks (CHRM1, green; DAPI, white). C, Relative quantification of mRNA expression of Chrm1–5 in KC compared with KCM mice at 20 weeks (n = 3, each group). D and E, Representative images of H&E-stained pancreatic sections from KC mice at 20 weeks showing low-grade PanIN lesions in low (D) and high (E) power magnification. F and G, Representative images of H&E-stained pancreatic sections from KCM mice at 20 weeks showing high-grade PanIN lesions/PDAC in low (F) and high (G) power magnification. H, Quantification of PanIN area in pancreatic sections from KC and KCM mice at 20 weeks (n = 5, each group). I, Percentage of KC and KCM mice with PDAC development at 20 weeks (n = 10, each group). J–M, Representative images of pancreatic IHC for CD44 (J), pEGFR (K), pPI3K (L), and pERK1/2 (M) in KC and KCM mice at 20 weeks. N, Bar graph shows quantification of CD44, pEGFR, pPI3K, and pERK staining in pancreata from KC and KCM mice at 20 weeks (n = 3, each group). O, Kaplan-Meier curve comparing overall survival of KPC (n = 19) and KPCM (n = 13) mice. Scale bars, 100 μm; mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 6. Parasympathetic signaling influences survival in a model of hepatic metastasis. A, Experimental setup for the studies depicted in A to J. WT C57BL/6 mice received splenic injections of 2 × 10^6 GFP-labeled Panc02 cells and were then divided into 3 groups: untreated controls (n = 10), bethanechol-treated (n = 10), and selective hepatic vagotomy by transection of the hepatic branch of the vagus nerve (SHVx; n = 11). Mice were observed until they became moribund and needed to be sacrificed. B, Representative images at the time of necropsy. Mouse shows massive bloody ascites. C and D, Representative images showing cancer cells replacing the normal liver tissue as large pale nodules. E, Kaplan–Meier curve comparing overall survival after splenic injection of GFP-labeled Panc02 cells in control mice (black), mice with SHVx (red), or mice treated with bethanechol (blue). F, Tumor number at necropsy in mice that received SHVx (red) and bethanechol (blue), compared with untreated control (black). G, Tumor volume in mice that received SHVx (red) and bethanechol (blue) compared with untreated control (black). H–J, Representative images of immunofluorescent staining of liver metastases from untreated control, bethanechol-treated, and SHVx mice for Ki-67 (Ki-67, red; H), pEGFR (pEGFR, red; I), and CD44 (CD44, red; J). GFP, green; DAPI, blue; white arrowheads, red-positive cells. Bar graphs show quantitative analysis of positive cells for each staining in untreated control, bethanechol-treated, and SHVx mice (n = 3, each group). Scale bars, 100 μm; mean ± SD. *, P < 0.05; ***, P < 0.001.
muscarinic receptor (CHRM3) through WNT signaling, and denervation of the mouse stomach markedly reduces tumor incidence and progression of gastric cancer (11).

The current study demonstrates that vagal nerve signaling can have an opposite and thus inhibitory effect on tumorigenesis in the Kras-mutated pancreas. In KPC, KPC, and metastatic mouse models, pancreatic cancer development was accelerated by vagotomy and inhibited by cholinergic muscarinic agonists. Herein, the vagus nerve acts to directly suppress tumorigenesis in part through inhibition of CD11b\(^+\) myeloid cells, suppression of CSCs, and downregulation of the MAPK pathway (Supplementary Fig. S6G). These observations are consistent with clinical findings suggesting a protective role of the vague nerve in PDAC and specifically in the metastatic stage (23).

Interestingly, a retrospective cohort study comprising 27,930 patients with PDAC revealed that body/tail tumors have a much better prognosis after surgical resection than pancreatic head tumors (43). It is tempting to speculate that the transection of the vagus associated with pancreatic head (but not tail) resections may partly explain these findings.

The innervation of the pancreas is indeed quite complex, as it contains nerves derived from spinal cord as well as enteropancreatic nerves from the myenteric plexus of the pyloric stomach and the duodenum (44, 45). In addition to sympathetic and parasympathetic nerves, the pancreas contains abundant sensory nerves. Previous reports have noted that up to 80% of vagal fibers are sensory (46), suggesting that vagotomy may also reduce pancreatic input from sensory neurons. Nevertheless, given the strong evidence that sensory neurons in general strongly promote pancreatic tumorigenesis (15), this argues even more for a distinct suppressive effect by the vagal cholinergic fibers, which comprise only a minor subset of nerve fibers in the vagus.

Previous studies have established the ability of parasympathetic signaling to modulate TNF\(\alpha\) expression in the spleen as part of the neural inflammatory reflex (47). Recently, we extended this paradigm by showing that the neural inflammatory reflex inhibits colon cancer development in part through arresting myeloid cell expansion (48). In addition, studies using orthotopic models of pancreatic cancer have suggested that interruption of this pathway by surgical vagotomy promotes cancer growth, possibly due to upregulation of TNF\(\alpha\) (16). However, although cholinergic signaling mediated by the vagus nerve suppresses inflammatory responses, it is thought to do so through binding of acetylcholine to the \(\alpha7\) subunit of the nicotinic acetylcholine receptor (\(\alpha7\) nAChR). Thus, nicotinic and not muscarinic receptors were shown to mediate the vagal inhibitory effect on macrophage activation and TNF\(\alpha\) synthesis in cancer and sepsis (47, 49).

In this study, we confirmed that subdiaphragmatic vagotomy in KC mice leads to increased TNF\(\alpha\) levels in the spleen and circulation, demonstrating the effectiveness of the surgical procedure. In addition, analysis of pancreatic stroma in KC + VxPP mice revealed increased levels of CD11b\(^+\) myeloid cells, along with increased levels of TNF\(\alpha\) in the spleen and circulation, consistent with greater inflammation. Surprisingly, treatment with a muscarinic agonist led to significant suppression of TNF\(\alpha\) levels in the spleen and circulation, indicating a role for muscarinic receptors in this setting. Indeed, muscarinic receptors are expressed in many cell types, including cells of the central nervous system, diverse immune (myeloid and lymphoid) cells, and other stromal cells (50). It has to be stated that muscarinic agonists clearly had direct effects on pancreatic epithelial cells, suppressing signaling and growth in both PDAC cell lines and Kras-mutant spheres. However, we cannot exclude a role for suppression of inflammation by muscarinic agonists in the inhibition of PDAC development, which will need to be elucidated in future studies.

Interestingly, pilocarpine had no effect on the viability of non-tumorigenic HPDE cells with wild-type Kras, whereas in contrast, pilocarpine treatment of HPDE cells expressing mutant Kras\((\text{G12D})\) resulted in a significant reduction in viability. Furthermore, pilocarpine also suppressed the growth of Kras-mutant pancreatic spheroids, but stimulated the growth of WT pancreatic spheroids. These data suggest that Kras mutation changes the effect of transmitted signals from muscarinic receptors from a stimulatory to an inhibitory pathway. This potential mechanism requires further study, including the potential impact of mutant Kras signaling on the allosteric modulation on receptor regulatory mechanisms such as \(\beta\)-arrestin recruitment, receptor internalization, or endocytic trafficking. However, it is tempting to speculate that previously described distinct binding modes of \(\beta\)-arrestin to CHRM1 (transient or stable; ref. 51), which can induce opposite effects on ERK activation, might explain in part the suppression of signaling by muscarinic receptors.

CD44 expression was markedly increased in vagotomized KC mice and suppressed by bethanechol to similar levels as in controls. Beyond serving as a marker of CSCs in pancreatic cancer, CD44 expression level correlates with cell plasticity and invasiveness in PDAC (27). As CSC markers in murine PDAC are less validated, we analyzed double-positive (CD44\(^+\)/CD133\(^+\)) and triple-positive (CD44\(^+\)/CD24\(^+\)/EPCAM\(^+\)) putative CSCs in KPC tumors as well as murine and human PDAC cell lines. We found significant decreases in CD44\(^+\)/CD133\(^+\) CSCs in KPC tumors treated with a muscarinic agonist with a trend of suppressed CD44\(^+\)/CD24\(^+\)/EPCAM\(^+\) cells possibly owing to an insufficient number of animals. Importantly, in murine and human PDAC cell lines, the triple-positive population was significantly decreased even with doses as low as 100 \(\mu\)mol/L of pilocarpine. Interestingly, EGFR signaling, which was suppressed by muscarinic agonists, contributes to the acquisition of cancer stem-like properties, including the enrichment of the CD44\(^+\) population of cancer cells in breast cancer (52). CD44 also plays an important role in tumorigenesis and tumor progression by promoting cell proliferation and migration via several signaling pathways/networks, including pAKT or pERK (53, 54).

Multiple lines of evidence indicated that the suppression of pancreatic tumorigenesis by muscarinic signaling was mediated by CHRM1. Only \(\text{Chrm1}\) expression was upregulated in the murine pancreas following surgical denervation, and the growth of pancreas spheres and PDAC cell lines was suppressed by CHRM1-selective agonists (McN-343) and stimulated by CHRM1-selective antagonists (pirenzepine). Finally, genetic ablation of \(\text{Chrm1}\) resulted in the same accelerated PDAC phenotype as seen with subdiaphragmatic vagotomy. Interestingly, CHRM1 is also expressed at high levels in the healthy prostate gland of mice, but in prostate cancer, \(\text{Chrm1}\)-KO reduced progression in an orthotopic mouse model (8), whereas \(\text{Chrm1}\)-KO accelerated PDAC. CHRM1 is a G protein–coupled receptor (GPCR), associated with both \(\text{Gq}-\) and \(\text{arrestin-dependent}\)
pathways, and has been shown to enhance EGFR and ERK activation and stimulate cell proliferation (55). Indeed, most GPCRs have generally been considered to be protumorigenic, overexpressed, or activated to drive cancer progression. Nevertheless, it is also clear that some GPCRs can play tumor-suppressive roles, including the melanocortin 1 receptor (56), GPR55 (57), and the cannabinoid receptors CB1 and CB2 (58). CB2, for example, suppresses EGFR/ERK and AKT signaling in breast cancer cells, although the mechanisms have not been well defined (58). Possible mechanisms could include modulation of receptor cross-talk through heterodimerization, and altered β-arrestin interactions. Furthermore, it is known that GPCRs possess two different conformations, active and inactive, and that they spontaneously alternate between the two in the absence of ligands (59), possibly explaining the increased sphere-forming capacity of Chrml1-KO;LSL-KrasG12D sphere cultures compared with LSL-KrasG12D spheres. In addition, it is important to note that roles of other potential inhibitory muscarinic receptors have not been addressed in this study. Notably, we have shown that CHRM4 is the predominant receptor expressed in human pancreatic cancer cell lines (Supplementary Fig. S3A and S3B). Further studies are needed to elucidate the roles of the other muscarinic receptors in regulating pancreatic cancer growth. In any case, our findings that CHRM1 signaling has the potential to suppress numerous growth factor pathways broaden our view regarding the role of GPCRs in cancer.

In summary, we have demonstrated that muscarinic signaling via CHRM1 can directly suppress pancreatic tumor development through downregulation of the MAPK and PI3K/AKT signaling pathway in cancer cells, as well as potentially indirectly through suppression of myeloid cells. Although the role of muscarinic signaling in modulating cross-talk between pancreatic tumor cells and the microenvironment needs to be further investigated, the findings reveal a surprising inhibitory role for parasympathetic signaling in PDAC and point to cholinergic agonists as potentially useful adjunctive therapies in the treatment of PDAC in early and late stages.

METHODS

Human Tissue Samples

Human pancreatic cancer samples for organoid cultures were obtained from the Technische Universität München (Dr. Maximilian Reichert, Department of Medicine II). All samples were anonymized. All protocols using human materials were approved by the ethics committee of the University of Munich or Technische Universität München. Written informed consent was obtained from all patients.

Animals

KC and KPC mice were described previously (26, 31) and provided by Dr. Kenneth P. Olive. Chrml1-KO mice (C57BL/6-Chrml1tm1Stl/J) (M1RKO; ref. 60) were purchased from the Jackson Laboratory. KC mice and KPC mice were crossed to M1RKO mice. In the vagotomy experiments, KC mice received pyloroplasty with or without vagotomy at 8 weeks. Bethanechol was administered at 400 µg/mL in drinking water (Sigma-Aldrich) and selumetinib (AZD6244) by s.c. injection of 1% Triton X-100 in PBS, rinsed, and blocked for 30 minutes with 2% BSA or 10% serum. Primary antibodies and biotinylated secondary antibodies (Jackson Immunoresearch) were diluted in PBS with 2% BSA or 10% serum. Primary antibodies were used: pEGFR (1:200; Abcam), CD44 (1:200; Bio-Rad), p-pERK1/2 (1:200; Cell Signaling Technology), oSMA (1:500; Abcam), α-SMA (1:500; Abcam), CgA (1:400; Abcam), and CD31 (1:500; Abcam). Subsequently, slides were incubated with peroxidase-conjugated streptavidin (Vector Laboratories) and 3,3′-diaminobenzidine (Dako) as chromogens, respectively. Slides were counterstained with hematoxylin and mounted for viewing. Bright field and fluorescence images were acquired using an Eclipse TI2000-U microscope (Nikon) connected to a cooled color CCD camera (RTK Diagnostic Instruments) using SPOT software (Spotimaging).

RNA-seq Experiments

Panc1 cells were treated with or without 1 mmol/L pilocarpine (Sigma-Aldrich) in RPMI-1640 with 0.5% FBS and 1% antibiotics for 72 hours. Panc1 cells were lysed in RNA lysis buffer supplied in ARCTURUS PicoPure RNA isolation kit (Life Technologies, n = 4). Total RNA was isolated in accordance with the manufacturer’s protocol. cDNA was amplified, and libraries were constructed using the SMARTer Ultra Low Input RNA Kit (Clontech Laboratories) and Nextera XT DNA Library Preparation Kit (Illumina) according to the respective manufacturer’s instructions. Sequencing was performed with Hiseq 2500 (Illumina; 30 M 100 bp single end reads per sample).

Accession Number

The full RNA-seq data have been deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession Code GEO: GSE102880.
Three-dimensional spheroids were isolated from mouse pancreas and cultured as previously described (37). Tissue was digested as described in the flow cytometry section with a shorter incubation (20 minutes). Cells were resuspended in Matrigel (Corning) with or without Adeno-Cre virus (provided by the University of Iowa), and 6.5 × 10^5 cells/well were seeded into a prewarmed 24-well plate. Cells were cultured in RPMI-1640 containing B27 and N2 supplements (Gibco), 5% Nu-Serum IV (Corning), 100 μg/mL trypsin inhibitor, and 100 ng/mL Cholera Toxin (Sigma-Aldrich). Pilocarpine, scopolamine, McN-34A, or pirenzepine (all Sigma-Aldrich) was added to the medium, and the medium was replaced every day. Sphere size and number were analyzed using ImageJ software at day 5.

Human patient-derived organoids (PDO) were isolated as previously described (37). Cultured PDOs were mechanically broken and furthermore enzymatically dissociated into single cells. Cell pellets were counted, and 1,000 cells in a mixture of Matrigel and medium were seeded in each well. After 24 hours, 10 μL of human organoid medium containing the drug was added in each well. After 72 hours, the viability was measured with the CellTiter-Glo 3D Cell Viability Assay (Promega).

**Morphometric Analysis of Murine PanIN and PDAC**

Murine PanIN was diagnosed based on the degree of cytologic atypia and epithelial proliferation. PanIN area was measured by ImageJ software. Microcinarcoma appeared as nodular clusters of coalescing small ducts expanded by solid neoplastic epithelial proliferation. These microcinarcomas showed foci of microinvasion and were also seen adjacent to larger poorly differentiated carcinomas. The existence of PDAC was assessed on full-face sections of the entire mouse pancreas.

**Disclosure of Potential Conflicts of Interest**

C.B. Westphalen has received honoraria from the speakers bureaus of Roche and Ipsen, and is a consultant/advisory board member for Roche, Celgene, Shire, and RedHill. No potential conflicts of interest were disclosed by the other authors.

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Cholinergic Signaling Suppresses Pancreatic Tumorigenesis


Cholinergic Signaling via Muscarinic Receptors Directly and Indirectly Suppresses Pancreatic Tumorigenesis and Cancer Stemness

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