GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Malnutrition in Pancreatic Ductal Adenocarcinoma (PDA)

Dietary Pancreatic Enzymes Improve Short-Term Health but Stimulate Tumor Growth

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Pancreatic ductal adenocarcinoma (PDA) is a deadly cancer that resists efforts to identify better chemotherapeutics. PDA is associated with chronic pancreatitis and acinar cell dedifferentiation. This reduces enzyme production by the exocrine pancreas, resulting in digestive insufficiencies. Malabsorption of partially digested food causes bloating, overfilled intestines, abdominal pain, excessive feces, steatorrhea, and malnutrition. These maladies affect quality of life and restrict treatment options for pancreatitis and PDA. Here, we characterize health benefits and risks of dietary pancreatic enzymes in three mouse models of PDA-KC, KCR8-16, and KIC. KC expresses oncogenic *Kras*^{G12D} in pancreatic tissue whereas KCR8-16 also has deletions of the *Rgs8* and *Rgs16* genes. Rgs proteins inhibit the release of digestive enzymes evoked by G-protein-coupled-receptor agonists. KC and KCR8-16 mice developed dedifferentiated exocrine pancreata within 2 months of age and became malnourished, underweight, hypoglycemic, and hypothermic. KC mice adapted but KCR8-16 mice rapidly transitioned to starvation after mild metabolic challenges. Dietary pancreatic enzyme supplements reversed these symptoms in KC and KCR8-16 animals, and extended survival. Therefore, we tested the benefits of pancreatic enzymes in an aggressive mouse model of PDA (KIC). Median survival improved with dietary pancreatic enzyme supplements and was extended further when combined with warfarin and gemcitabine chemotherapy. However, dietary pancreatic enzymes stimulated tumor growth in the terminal stages of disease progression in KIC mice. (*Am J Pathol* 2018; 188: 616–626; <https://doi.org/10.1016/j.ajpath.2017.11.014>)

Pancreatic ductal adenocarcinoma (PDA) is the most lethal among all major cancers in the United States and the number of individuals who succumb annually is now third among all cancers.¹ Disease symptoms tend to manifest at later stages. A major reason attributed to the high lethality is the lack of early detection for PDA.² The symptoms of PDA and associated pancreatitis in humans include maldigestion or malabsorption, causing steatorrhea and malnutrition with weight loss.^{3–8} Maldigestion results from the paucity of pancreatic digestive enzymes in the pancreatic juice released into the duodenum. Insufficiency

of digestive enzymes is caused by extensive acinar cell dedifferentiation, transformation, and/or tumor occlusion of the pancreatic duct. Dietary supplementation of pancreatic

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enzymes (PEs) is now commonly prescribed to alleviate malnutrition caused by pancreatic insufficiency before and after pancreatic resection.⁹ Although enhanced nutrition improves short-term quality of life in patients, the effects on tumor growth or relapse after surgery are unknown.

Studies of PDA initiation and progression in a preclinical mouse model have indicated that caloric restriction inhibits progression to PDA.^{10,11} This presents a potential contradiction to the current medical practice of prescribing PEs specifically to improve nutrition in patients because the long-term effects might promote tumor growth.

We investigated the question of potential benefits and risks of PEs to overall health and life expectancy in three mouse models of PDA. All of the models were derived from KC (*p48::Cre/+; Kras^{G12D/+}*) mice, which develop extensive pancreatic exocrine cell dedifferentiation and progress toward PDA.^{12–15} Treatment of KC mice with caerulein rapidly advances acinar cell dedifferentiation, acinar to ductal metaplasia (ADM), and tumor initiation and progression.^{15,16} Caerulein is a mimetic of cholecystokinin, a G-protein–coupled–receptor hormone that stimulates digestive enzyme release from acinar cells.^{17,18} The massive release of digestive enzymes contributes to inflammation and extensive ADM. Wild-type (WT) mice recover normal acinar cell morphology and function within 7 days of caerulein treatment, but expression of *Kras^{G12D}* throughout the pancreas in KC mice prevents recovery and stimulates progression to PDA.^{15,19–22}

Cholecystokinin and two other Gq-coupled G-protein–coupled–receptor agonists, bombesin and acetylcholine, stimulate intracellular Ca^{2+} signaling required for acinar cell secretion of digestive enzymes.^{23–26} Regulators of G-protein signaling *Rgs4* and *Rgs16*, among others, inhibit Ca^{2+} signaling evoked by Gq-coupled agonists.^{27–30} In previous studies, we showed that *Rgs8* and *Rgs16* (tandemly duplicated, functionally redundant genes³¹) modulate energy homeostasis,^{32,33} and are co-expressed in the earliest neoplastic lesions and throughout PDA progression.³⁴ To determine whether deficiency of *Rgs8-16* would exacerbate ADM and PDA initiation in KC mice, *Rgs8-16* double knockout (dKO) were crossed into KC (termed KCR8-16). Metabolic control was compromised further in KCR8-16 compared with KC mice, consistent with the function of *Rgs8* and *Rgs16* in maintaining energy homeostasis in the liver and inhibiting digestive enzyme release from pancreatic acinar cells. Dietary supplements of PE dramatically improved quality of life, as measured by temperature, body weight, blood glucose, and life expectancy in KCR8-16 mice. Therefore, the effects of PE were tested in KIC (*p48::Cre/+; Kras^{G12D/+}; Cdkn2a^{fl/fl}*) mice, which progress rapidly to PDA. PE improved the overall health in KIC mice during middle to end stages of PDA progression. However, PE only modestly extended survival, even when treated with the combined chemotherapy of warfarin and gemcitabine (WG), because PE stimulated tumor growth in the terminal stages of disease progression in KIC mice.

Materials and Methods

Ethical Considerations

All experimental procedures were reviewed and approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee (protocol: 2016-101480). All efforts were made to minimize animal suffering.

Animals

Both male and female adult mice were used for the experiments. The mouse models used were as follows: KC (*p48::Cre/+; Kras^{G12D/+}*), dKO (*Rgs8^{-/-}; Rgs16^{-/-}*), KCR8-16 (*p48::Cre/+; Kras^{G12D/+}; Rgs8^{-/-}; Rgs16^{-/-}*), and KIC (*p48::Cre/+; Kras^{G12D/+}; Cdkn2a^{fl/fl}*). KC and KIC mice had the *Rgs16::GFP* reporter gene and were generated by crossing with *Rgs16::GFP* transgenic mice as described previously.³⁵ *Rgs16::GFP* expression in KC and KIC mice were identified by blue light excitation of GFP in the brain of newborn pups or in the retina of adult mice. The genotype of all mice was confirmed by PCR. Mice were maintained on a 12-hour day:12-hour night cycle [Zeitgeber time (ZT); light phase 6 AM to 6 PM, ZT0 to ZT12; dark phase ZT12 to ZT24], on normal chow (NC) *ad libitum* (unless otherwise stated) according to the rules and standards of the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Genotyping

Mice were genotyped as previously described.³⁴ In short, tail clips were collected for genotyping. Primers used for KC, KCR8-16, and KIC were as follows: *p48^{Cre}* (forward: 5'-CCTGGAAAATGCTTCTGTCCG-3'; reverse: 5'-CAGGGTGTATAAGCAATCCC-3'; product: 392 bp), *LSL-Kras^{G12D}* (forward: 5'-CTAGCCACCATGGCTTGAGT-3'; reverse: 5'-TCCGAATTCAGTGACTACAGATG-3'; product: 327 bp), *Cdkn2a^{fl/fl}* (forward: 5'-TTGTGGCCAGGATGCCGACATC-3'; reverse: 5'-CCAAGTGTGCAACCCAGGCTCC-3'; product: 145 bp for wild-type, 179 bp for *loxP* inserted allele), *Rgs16* WT (forward: 5'-ACAGCTCTGGTGATTCTGTGGGAT-3'; reverse: 5'-TAGGGCTTGCAAGCATTCACTACT-3'; product: 237 bp), *Rgs16* KO (forward: 5'-ACAGCTCTGGTGATTCTGTGGGAT-3'; reverse: 5'-TAGGGCTTGCAAGCATTCACTACT-3'; product: 350 bp), *Rgs8* *Flox/WT* (forward: 5'-TGGGGAAGCAAACCTAGAACTCA-3'; reverse: 5'-CGCACATATGCGATGTGATGGGGCCTGCATTCTAC-3'; product: 331 bp), and *Rgs8* KO (forward: 5'-TGGGGAAGCAAACCTAGAACCTCA-3'; reverse: 5'-GCTAGCTTGGGCTGCAGGT-3'; product: 122 bp). All PCR programs consisted of a 10-minute genomic denaturation at 94°C, followed by 33 cycles of 30 seconds denaturation at 94°C, 1-minute annealing at 60°C, and 1-minute elongation at 72°C. PCR

products were run in a 1% agarose gel stained with 0.001% (vol/vol) ethidium bromide and DNA bands were visualized under UV light with 1-second exposure.

Animal Diet

To investigate if supplementation of PEs would help compensate for pancreatic insufficiency, mice were fed a PE-supplemented diet along with NC mouse diet as mentioned in [Results](#). Two different PE-supplemented diets were used for this study: bacon-flavored chow with PE and a liquid diet with PE. Bacon-flavored chow with PE contained 90% NC with 10% bacon-flavored chow. For bacon-flavored chow with PE, 720 g grinded NC (Harlan Teklad, Houston, TX) and 14.20 g Pancrezyme (Cpp Pet Care, Hayward, CA) (1.8% of the chow) were mixed with 300 mL of deionized water to make a NC dough. Afterward, 80 g of fine chopped bacon-flavored chow (Transgenic Dough Diet; Bio-Serv, Flemington, NJ) was mixed with the NC dough using a food blender (Shortcut food processor; Black & Decker, Towson, MD). The bacon-flavored chow with PE dough was wrapped in a paper towel to absorb excess moisture and kept at room temperature for an hour to let the enzyme act, and then stored in the refrigerator for up to 4 days. It was served to mice in small cylinder-shaped pieces *ad libitum*. Bacon-flavored chow was added to make the chow more palatable and thus preferable over NC. For the liquid diet with PE, 260 g rodent liquid diet powder (Lieber-DeCarli '82, Shake and Pour, Control; Bio-Serv) and 3.6 g of PE were mixed thoroughly in 890 mL water. Each cage received a 50-mL bottle of this freshly made liquid diet every day.

I.P. Glucose Tolerance Test

Adult mice (2 to 3 months old, male and female) were used for the intraperitoneal glucose tolerance test (GTT). Mice were fasted for 5 hours, starting at 8 AM. A glucose solution of 2 mg/g body weight was injected into the i.p. cavity after fasting. The blood glucose level was measured at the beginning of the fast (time = -5 hours), just before glucose injection (time = 0), and at intervals thereafter (time = 20, 60, 90, and 120 minutes) with a Contour TS (Bayer, Whippany, NJ) blood glucose meter.

Insulin Measurements

For plasma insulin levels, blood was sampled via tail bleed at 0 and 20 minutes, centrifuged at $15,600 \times g$ for 10 minutes, and plasma was collected and stored at -20°C . Insulin levels were assayed using the Ultra-Sensitive Mouse Insulin Enzyme-Linked Immunosorbent Assay Kit (Crystal Chem, Downers Grove, IL) following the manufacturer's protocol.

Survival Study

KIC and control mice were assigned randomly to different diet (NC or PE) and treatment groups. The NC group was fed

the same diet until moribund (sacrificed), whereas mice assigned to the PE group were switched to a diet supplemented with PE after 1 month of age. Both diets were given *ad libitum*. Animals in either diet group were divided further into drug-treated or untreated subgroups. Warfarin (W; 0.2 mg/kg $\times$ 5 days/week) and gemcitabine (G; 12.5 mg/kg $\times$ 3 days/week) (Eli Lilly, Clinton, IN) were injected intraperitoneally starting from postnatal day (P) 15 until the end point (WG group). WG* mice received warfarin injections, as for WG, but gemcitabine administration was withdrawn between 6 and 7 weeks of age. There was no difference in survival between WG and WG* mice. KIC mice not treated with drugs were controls for WG and WG* groups.

Food Consumption Measurement

Food was weighed before feeding, then remeasured 24 hours after *ad libitum* feeding.

Blood Glucose

Blood glucose levels were measured from approximately 3- μL tail-blood samples using a commercially available glucometer (Contour TS).

Body Temperature

Mice rectal temperatures were measured with a BAT-12 microprobe thermometer (Physitemp, Clifton, NJ).

Dissection and Tissue Preparation

Mice were euthanized with CO_2 , guts and pancreas were collected, pancreas and hepatopancreatic common duct were dissected, washed with phosphate-buffered saline (4°C), and fixed overnight in 10% formalin. Tissue samples were washed five times for 5 minutes in phosphate-buffered saline and stored in 70% EtOH (4°C) until embedded in paraffin, and then sectioned (5- μm slices).

Immunofluorescence Staining

For immunofluorescence staining, sections were deparaffinized with xylene, rehydrated, and subject to antigen retrieval in antigen unmasking solution (Vector Laboratories, Burlingame, CA). Nonspecific antibody binding was blocked with normal donkey serum. Sections were stained with primary antibodies against amylase [1:1000; a gift from Dr. Raymond MacDonald (University of Texas Southwestern Medical Center, Dallas, TX)³⁶] and E-cadherin (1:300; BD Transduction Laboratories, San Jose, CA), followed by fluorescent secondary antibodies (1:300, anti-rabbit and 1:300, anti-mouse; both Jackson ImmunoResearch, West Grove, PA). The sections then were mounted with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA).

Alcian Blue/Periodic Acid-Schiff Staining

Pancreatic sections were deparaffinized with xylene and rehydrated in deionized water, stained with Alcian blue (30 minutes), rinsed with tap water (2 minutes), counterstained in nuclear fast red (5 minutes), rinsed with water (1 minute), dehydrated through absolute ethanol, cleared in xylene, and mounted on coverslips with mounting medium.

Statistical Analysis

Graphs and their statistical comparisons were performed using GraphPad Prism software version 7 (GraphPad Software, Inc., La Jolla, CA). Between-subject analysis of variance was used to detect significant changes among multiple groups or conditions, and post hoc testing with simple effects tests and *t*-tests were performed wherever applicable.

Results

Rgs8-16 Is Required for Metabolic Adaptation to Malnutrition Caused by Pancreatic Insufficiency in KC Mice

Body weight, blood glucose, and temperature are indicators of overall health in mice. It was initially observed that body

weight and blood glucose levels of KC mice were lower compared with WT littermates or age-matched controls after approximately 2 months of age (Figure 1A). Because *Rgs8-16* regulate energy homeostasis³³ and are expressed in early pancreatic neoplasia,³⁴ we crossed the *Rgs8-16* dKO into KC mice (KCR8-16) and determined if metabolic control was compromised further in KCR8-16 compared with KC mice. A general survey of the colony showed that body weight and glucose levels were reduced similarly in male and female KCR8-16 mice compared with their corresponding age-/sex-matched dKO controls (Figure 1A). Body temperature was reduced most dramatically in KCR8-16, but the average temperature of KC mice also was lower than either WT or dKO mice (Figure 1B). Blood glucose and body temperature were reduced particularly in mice with the lowest body weight.

In KC and KCR8-16 mice, *Kras*^{G12D} expression in acinar cells promoted widespread acinar to ductal metaplasia (ADM) (Figure 1C).³⁷ Pancreatic enzyme production fails in the dedifferentiated exocrine pancreas,^{37,38} exemplified by extensive intraductal papillary mucinous neoplasms and an almost complete loss of amylase expression, causing pancreatic deficiency and malnutrition in KC and KCR8-16 mice (Figure 1C).

KCR8-16 mice died younger and more frequently than KC mice. KCR8-16 mice in the colony died as early as by day 50, whereas the earliest death in KC was day 86. A

Figure 1 KCR8-16 mice are hypersensitive to severe pancreas deficiency. **A:** Glucose values and body weights of wild-type (WT), double knockout (dKO), KC, and KCR8-16 mice on normal chow between ages 2 to 6 months *ad libitum* are compared. Linear regression line for each cohort depicts the general trend between body weight and glucose level (WT, dKO, KC, and KCR8-16, as indicated). **B:** Body temperature of WT, dKO, KC, and KCR8-16 mice on normal chow *ad libitum*. **C:** Adult [postnatal day (P) 100] pancreas sections of KC and KCR8-16 mice with alcian blue–periodic acid-Schiff (PAS) staining, and amylase (red)–E-cadherin (green) co-immunofluorescence. Red arrows mark an example of amylase overlap with E-cadherin. **D:** Kaplan-Meier curve of single-caged KC and KCR8-16 mice. Twelve KCR8-16 mice died (ages between P46 and P104) and the remaining 8 mice were sacrificed (ages between P104 and P176). In contrast, 1 KC mouse died (P237) and all other 34 mice were sacrificed at various times (ages between P111 and P476). Data are expressed as means $\pm$ SEM (**B**). $n = 27$ WT, 30 dKO, 26 KC, and 7 KCR8-16 mice (**A**); $n = 35$ KC and 20 KCR8-16 mice (**D**). $^{**}P < 0.01$, $^{****}P < 0.0001$ (calculated by one-way analysis of variance followed by Tukey's post hoc test).

dramatic manifestation of global *Rgs8-16* deficiency was shown when KCR8-16 mice were subjected to additional mild metabolic stress. Essentially all of the adult KCR8-16 mice (ages 8 to 12 weeks) died within a couple days (Figure 1D) after routine metabolic tests, including single-cage housing to determine 24-hour food intake and a 4- to 5-hour morning fast (standard for the GTT). By contrast, KC mice survived these metabolic assays and nearly all survived longer than 1 year, although they were not as robust as WT littermates. These results suggest that metabolic stress, caused by malnutrition resulting from pancreatic insufficiency, exacerbated by food restriction, was greater in KCR8-16 than KC mice.

Malnutrition Provokes Increased Food Intake in KC and KCR8-16 Mice

Food consumption of age-matched KC, KCR8-16, and their littermate controls was measured to determine whether pancreatic insufficiency was compensated by increased feeding. KC and KCR8-16 mice maintained on NC had increased food intake compared with their littermate controls (Figure 2A). Their intestines were engorged with poorly digested food and they developed steatorrhea (Figure 2B). An increase in daily feces production mirrored increased food consumption (Figure 2, C and D).

Dietary PEs Preferentially Improve Health of KCR8-16 Mice

Cohorts of age-matched KC and KCR8-16 mice were maintained on NC or switched to a diet supplemented

with PE after they started to show signs of malnutrition (about 8 weeks of age). In KCR8-16 mice on NC, body weight (Figure 3A), blood glucose (Figure 3C), and body temperature (Figure 3E) all decreased progressively after 10 weeks of age. By contrast, these health parameters dramatically improved 10, 20, 30, 60, and 80 days after adding dietary PE (Figure 3, A, C, and E). In KC mice, decreases in these parameters were less pronounced on NC and therefore dietary supplements of PE had a smaller restorative effect (Figure 3, B, D, and F). WT and dKO mice appeared similar on either diet (data not shown).

Hypoglycemia Is More Pronounced in KCR8-16

GTTs were conducted to further evaluate differences between KC and KCR8-16 mice, and their respective controls (WT and dKO). All mice were tested at P60, when the earliest signs of malnutrition start to manifest in KC and KCR8-16. Among the mice maintained on NC, dKO had significantly increased blood glucose levels with the GTT compared with WT, KC, and KCR8-16 mice (Figure 4, A and C). This dramatic difference can be attributed to increased hepatic fatty acid oxidation in fasted dKO mice.³³ By contrast, among the mice maintained on diet supplemented with carbohydrates and PE for 7 days before GTT, blood glucose levels increased transiently in WT, dKO, and KC mice, whereas it barely responded in KCR8-16 mice (Figure 4, B and D). Insulin levels were normal in all cases, negating the possibility that hyperinsulinemia drove low glucose levels in KCR8-16 mice (data not shown).

Figure 2 KC and KCR8-16 mice become hyperphagic with distended intestines, steatorrhea, and excessive feces. **A:** Consumption of normal chow (NC, overnight, single-caged, *ad libitum*) by wild-type (WT), double knockout (dKO), KC, and KCR8-16 mice. **B:** Small and large intestines of a KCR8-16 mouse and an age-matched dKO mouse (NC, *ad libitum*). **C:** Weight of feces produced in 24 hours by WT, dKO, KC, KCR8-16, and KIC mice. Ages of the mice are indicated for each group. **D:** Representative fresh feces of KCR8-16 and dKO mice [postnatal day (P) 42]; standard US nickel is for size comparison. Data are expressed as means $\pm$ SEM (**A**). Means are indicated by horizontal lines (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (one-way analysis of variance followed by Tukey's post hoc test).

Figure 3 Dietary pancreatic enzyme (PE) supplements improve body weight, hypoglycemia, and hypothermia in KCR8-16 mice. **A** and **B**: Body weights of KCR8-16 and KC mice on normal chow (NC)- or PE-supplemented diet *ad libitum*. **C–F**: Body temperature (**C** and **D**) and blood glucose levels (**E** and **F**) of KCR8-16 and KC mice on *ad libitum* NC or PE-supplemented diet. Data are expressed as means $\pm$ SEM. $n = 7$ KCR8-16 mice on NC, 24 KCR8-16 mice on PE, 7 KC mice on NC, and 11 KC mice on PE (**A** and **B**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (unpaired *t*-test). Glu, glucose.

KCR8-16 and to a lesser extent KC mice consumed (per day) more PE diet than WT and dKO controls to compensate for malnutrition caused by pancreatic insufficiency (Supplemental Figure S1). KCR8-16 mice showed the

greatest sensitivity to malnutrition, probably because *Rgs8-16* dKO (similar to *Rgs16* KO mice) do not modulate hepatic fatty acid oxidation and glucose production properly during chronic fasting conditions caused by pancreatic insufficiency.³³

Figure 4 KCR8-16 mice tend toward hypoglycemia despite dietary pancreatic enzyme (PE) supplements. **A** and **B**: The glucose tolerance test (GTT) was performed in wild-type (WT), double knockout (dKO), KC, and KCR8-16 mice at 6 to 8 week of age for 8 to 12 mice per group on a normal chow (NC)-supplemented (**A**) or PE-supplemented (**B**) diet (age-matched groups). GTTs were performed after 5 hours of fasting (Zeitgeber time, 7–12). Each test was performed at least twice. **C** and **D**: The symbol and number of mice in each genotype. Difference between blood glucose before *i.p.* injection of glucose and 20 minutes after, in mice fed NC (**C**) or PE (**D**). Error bars show SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (one-way analysis of variance followed by Tukey's post hoc test). Glu, glucose; LD.PE, liquid diet with PE; NB.PE, bacon-flavored chow with PE.

KIC Mice Show Signs of Malnutrition during PDA Progression

Similar to KC and KCR8-16 mice, because pancreatic failure progresses by 4 to 5 weeks of age, KIC mice also have reduced body weight and significantly lower blood glucose levels compared with WT controls (including littermates) (Figure 5A). At P42, an inverse correlation was noted between blood glucose levels and pancreas tumor size; blood glucose levels were normal if PDA was early stage, whereas mice were hypoglycemic if PDA was advanced (depicted by increased pancreas weight) (Supplemental Figure S2). The first PDA lesions initiate in KIC mice by P15. They can be larger than 1 cm by P29, and can overtake the pancreas entirely before the mice die at around P60.³⁴ At P40, KIC mice consume less chow than WT mice, and this difference was exacerbated over the next 13 days (Figure 5B). P40 to P50 is the age when KIC body weight becomes noticeably less than their WT littermates. Blood glucose levels also decrease during this interval, followed by a precipitous decrease in body temperature 2 or 3 days before they succumb to PDA. Because KC and KCR8-16 mice significantly benefited from a PE-supplemented diet, it was tested if KIC mice might benefit similarly from a PE-supplemented diet.

Figure 5 KIC mice are hypersensitive to severe pancreas deficiency. **A:** Blood glucose and body weights of KIC mice and age-matched wild-type (WT) siblings on normal chow (NC) *ad libitum*, at 4 weeks of age [postnatal day (P) 28]. **B:** Daily normal chow (*ad libitum*) intake of KIC mice and WT siblings from P40 to P53. $n = 25$ KIC mice and 12 WT siblings (**A**); $n = 4$ KIC mice at P40 to P47, 3 KIC mice at P48 to P50, 2 KIC mice at P51 to P53, and 4 WT siblings (**B**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (unpaired *t*-test). Glu, glucose.

Dietary PEs Improve Short-Term Health but Stimulate Tumor Growth

KIC mice have two simultaneously progressing pancreatic diseases: first, pancreatic deficiency caused by ADM (as in KC mice), and, second, the growth of spontaneous PDA that rapidly overtakes the pancreas from about P42. Because the overall health of KC and KCR8-16 mice is improved by dietary supplements of PE (Figure 3, A and B), the potential survival benefit of weaning and maintaining KIC mice on PE at P28 until P42 was tested. During this early stage, PE supplements did not have any benefit over NC (data not shown). A survival study showed PE supplements (started at P28) extended survival by approximately 1 week in the 60% of longest-lived mice (Figure 6A). Fat depots were increased by PE in KIC mice at end of life (Figure 6, B and C). In addition, KIC mice on PE maintained body temperature and blood glucose within normal ranges (Figure 6D–F).

Although tumor size at P42 was similar in KIC mice fed NC or PE-supplemented diet (Supplemental Figure S3), by P60 tumor size was greater in PE-supplemented than NC-fed KIC mice, even when normalized to body mass. Because body weight was significantly higher in males than in females in all treatment groups (Figure 7, D and G), pancreas weight in males (Figure 7, E and F) and females (Figure 7, H and I) was analyzed independently. The median survival was extended a few days in the PE-fed group (Figure 6A). Presumably, increased fat mass when the mice were younger helped them survive a few days longer during the late stages of aggressive PDA growth.

A combination of warfarin and gemcitabine inhibited metastases in murine models of PDA.³⁹ In KIC mice treated between P15 to P29, WG reduced PDA initiation and progression, and thus the incidence of large tumors at P29.³⁴ Here, WG extended median and overall survival by approximately 2 weeks for KIC mice fed NC (Figure 6A), and significantly reduced tumor mass compared with untreated KIC. PE supplements in WG-treated KIC mice initially improved body weight, blood glucose, and temperature (Figure 7, A–C), and extended median survival by 4 days (Figure 6A). Thus, the benefit of PE was similar in drug-treated and untreated mice, primarily improving overall health in middle to late stages, but not end-stage disease, because PE-supplemented KIC animals treated with WG had larger tumors than KIC on NC treated with WG (most pronounced in female mice) (Figure 7, H and I).

Discussion

PDA typically is diagnosed late in the course of disease, and only 20% of patients are eligible for surgical resection. PDA is associated with pancreas insufficiency. Weight loss and symptoms of poor digestion and malnutrition caused by pancreas insufficiency often are treated with dietary

Figure 6 Dietary pancreatic enzyme (PE) supplements improve survival and quality of life of untreated or chemotherapy-treated KIC mice. **A:** Kaplan-Meier curve of KIC mice on normal chow (NC) or liquid diet supplemented with PE is shown without chemotherapy or with warfarin and gemcitabine (WG) treatment. WT mice treated with drugs and put on PE served as control and were sacrificed at postnatal day (P) 162. The median survival (dashed line) of untreated KIC mice on NC and PE is compared with the drug treatment conditions of each diet. The Mantel-Cox test was used to calculate the statistical significance between untreated versus WG (NC or PE, $P < 0.0001$) and WT versus any KIC group (NC or PE, $P < 0.0001$). **Green arrow** indicates the start day of drug treatment. **B** and **C:** Abdominal dissection of age-matched untreated KIC mice on normal chow (KIC NC) or KIC mice fed with PE-supplemented liquid diet (KIC PE). **D–F:** Temperature (**D**), body weight (**E**), and blood sugar levels (**F**) in WG-treated, age-matched KIC on NC versus PE-supplemented diet. Data are expressed as means $\pm$ SEM (**D–F**). Median survival was P55 for 51 KIC mice on NC, P59 for 32 KIC mice on PE, P68 for 24 WG-treated mice on NC, and P72 for 30 WG-treated mice on PE. $n = 6$ WT control mice on WG-PE (100% survival) (**A**). * $P < 0.05$, ** $P < 0.01$ (unpaired t -test; **D–F**).

PEs.^{40–42} However, the effects of improved nutrition on tumor growth are not known in humans. One study in mice showed that caloric restriction slowed PDA progression,^{10,11} suggesting that PE may increase nutrient availability to the tumor, and shorten survival.

In this study, three mouse models of PDA were used to test the effects of PE on short-term health and survival. All three mouse models expressed *Kras*^{G12D} in the pancreas from fetal development onward. Two strains of mice progressed slowly to PDA, although most unchallenged KC lived longer (12 months) than KCR8-16 (7 months). By contrast, KIC is an aggressive model of PDA, with a median survival of 56 to 60 days.^{43,44}

KC and KCR8-16 mice were afflicted similarly, starting at approximately 2 months of age, with low body weight and hypoglycemia, and hypothermia in the most severe cases later in disease progression (Figure 1, A and B). Both strains of mice had extensive pancreatic failure by 3 months of age, and showed classic features of malnutrition, including hyperphagia, increased defecation, and steatorrhea (Figure 2). KCR8-16 mice, which also have whole-body deletions of the *Rgs8* and *Rgs16* genes, were affected more severely than KC. This is consistent with the role of *Rgs8-16* in conserving energy utilization during fasting.³³ Indeed, KCR8-16 mice were

hypersensitive to mild metabolic stresses, such as individual caging overnight, with a 5-hour fast during ZT2 to ZT6, immediately followed by the GTT (Figure 1D). Dietary PE normalized body weight and blood glucose in both KC and KCR8-16. The response of KCR8-16 to dietary PE was particularly dramatic (Figure 3), and even allowed KCR8-16 mice to survive GTT (Figure 4).

KIC mice have two pancreatic afflictions: the same loss of acinar cell function as in KC, and aggressive PDA initiation and progress. KIC mice showed the familiar features of reduced body weight and hypoglycemia, but started to appear between 30 and 40 days of age (P34) (Figure 5), much earlier than in KC and KCR8-16 mice. The growing pancreatic tumor(s) in KIC probably limited nutrition uptake in the digestive tract by blocking the release of pancreatic juice to the intestine and/or occluding food passing through the duodenum. Indeed, increased tumor size at this age correlated with decreased body weight and blood glucose levels (Supplemental Figure S2). Also in contrast to KC and KCR8-16 mice, KIC mice soon became hypophagic past P40, presumably because the growing pancreatic tumor(s) caused pain and discomfort. At end-stage disease in KIC mice fed NC, PE added to a liquid diet transiently improved health and extended life for up to a week in some animals. Mice in distress started feeding again, possibly

Figure 7 Dietary pancreatic enzyme (PE) supplements improve health parameters of KIC mice, and also increase the pancreatic tumor burden. **A–C:** Body weight (**A**), blood glucose (**B**), and body temperature (**C**) of KIC mice on two different diets: normal chow (NC) or PE supplements. All mice were treated with warfarin and gemcitabine (WG) and sacrificed at age intervals of postnatal day (P) 31 to P46 or P46 to P61. WG administration started at P15 and continued until death. **D–F:** Body weight (**D**), pancreas weight (**E**), and pancreas-to-body weight ratio (**F**) of male KIC mice at end point (terminal stage). **G–I:** Body weight (**G**), pancreas weight (**H**), and pancreas-to-body weight ratio (**I**) of female KIC mice at end point (terminal stage). Mice were given NC or PE-supplemented diet *ad libitum* without chemotherapy or with WG from P15. **D–I:** There was no difference in survival whether gemcitabine was administered until death or sacrificed between 6 and 7 weeks of age; warfarin treatment continued until death in all studies. Error bars are SEM (**A–C**). $n = 23$ mice on NC, 29 WG-treated mice on NC, 17 mice on PE, and 29 WG-treated mice on PE (**A–C**); $n = 23$ mice on NC, 19 WG-treated mice on NC, 16 mice on PE, and 6 WG-treated mice on PE (**D**); $n = 30$ mice on NC, 19 WG-treated mice on NC, 16 mice on PE, and 2 WG-treated mice on PE (**E–F**); $n = 6$ mice on NC, 14 WG-treated mice on NC, 5 mice on PE, and 7 WG-treated mice on PE (**G–I**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (one-way analysis of variance followed by Tukey's post hoc test); † $P < 0.01$ for NC WG and PE WG groups that were partitioned further (unpaired *t*-test followed by Welch correction). BW, body weight; PW, pancreas weight.

because the liquid diet is sweet and passes through the duodenum more easily than solid food.

In KIC mice continuously fed PE from P28, tumor size was approximately the same at 6 weeks of age as mice fed NC (Supplemental Figure S3). At ages older than P50, PE-fed KIC mice were healthier than those fed NC, including higher body weight, normalized blood glucose, improved appearance of the coat, increased activity, and alertness. However, PE only extended median survival by approximately 4 days (Figure 6). These KIC mice continuously fed PE from P28 abruptly stopped eating and rapidly declined in health because the pancreatic tumors at the end

of life were significantly larger than in KIC mice fed NC their entire lives (Figure 7, E, F, H, and I).

Combined chemotherapy of WG reduces tumor initiation and slows early stage progression of PDA in KIC mice.³⁴ In this study, WG prolonged survival by approximately 2 weeks (Figure 6). PE helped overcome retardation of body weight gain caused by WG in KIC mice. However, as in untreated mice fed NC, dietary PE only extended the median survival by 4 days beyond WG-treated mice fed NC.

Dietary PE transiently overcame the debilitating effects of malnutrition caused by pancreatic failure but increased nutrition-stimulated tumor growth. Our results in mice raise

questions about the tumor growth implications of dietary supplement use in human patients and points out the need for specific studies aimed at assessing their overall impact on disease progression during treatment. Future goals are to identify improved selective PDA therapeutics that remain effective in combination with dietary PE, so that better nutrition and short-term health do not come at the expense of accelerated tumor growth. Alternatively, specialized diets in combination with targeted therapies might limit tumor growth while maintaining overall body health.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.11.014>.

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