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Dietary Energy Balance Modulation of Kras⁻ and Ink4a/Arf^{+/-}-Driven Pancreatic Cancer: The Role of Insulin-like Growth Factor-1

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Abstract

New molecular targets and intervention strategies for breaking the obesity-pancreatic cancer link are urgently needed. Using relevant spontaneous and orthotopically transplanted murine models of pancreatic cancer, we tested the hypothesis that dietary energy balance modulation impacts pancreatic cancer development and progression through an insulin-like growth factor (IGF)-1-dependent mechanism. In LSL-Kras^{G12D}/Pdx-1-Cre/Ink4a/Arf^{lox/+} mice, calorie restriction, versus overweight- or obesity-inducing diet regimens, decreased serum IGF-1, tumoral Akt/mammalian target of rapamycin (mTOR) signaling, pancreatic desmoplasia, and progression to pancreatic ductal adenocarcinoma (PDAC); and increased pancreatic tumor-free survival. Serum IGF-1, Akt/mTOR signaling, and orthotopically transplanted PDAC growth were decreased in liver-specific IGF-1-deficient mice (versus wild-type mice), and rescued with IGF-1 infusion. Thus, dietary energy balance modulation impacts spontaneous pancreatic tumorigenesis induced by mutant Kras and Ink4a deficiency, the most common genetic alterations in human pancreatic cancer. Furthermore, IGF-1 and components of its downstream signaling pathway are promising mechanistic targets for breaking the obesity-pancreatic cancer link.

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Keywords

Pancreatic cancer; obesity; calorie restriction; Kras; Ink4a/Arf; Insulin-like growth factor-1

Introduction

Pancreatic cancer is currently the fourth leading cause of cancer death in the United States (US), and is one of the deadliest malignancies worldwide, with a 5-year survival rate less than 5% (1). The most effective therapy is surgical excision; however, only 10-15% of patients have localized disease amenable to curative resection (2). Although little is known about the etiology of pancreatic cancer, an early event that occurs in virtually all human pancreatic tumors is an activating mutation in the KRAS gene (3), usually in combination with another common genetic anomaly such as an INK4A deficiency (3). In mice, Kras mutation combined with Ink4a/Arf deficiency enables the formation of pancreatic intraepithelial neoplasia (PanIN) lesions and their progression to invasive pancreatic ductal adenocarcinoma (PDAC) (4). Epidemiologic studies implicate obesity as a significant risk factor for pancreatic cancer (5-9). With obesity prevalence (10) and pancreatic cancer incidence and mortality all rising in the US (and many parts of the world), and the prediction that pancreatic cancer will be the second leading cause of cancer death by 2015 (11), the identification of molecular targets and intervention strategies for breaking the obesity-pancreatic cancer link is urgently needed.

We and others have shown in preclinical models that diet-induced obesity (DIO) enhances the development and progression of several cancer types in association with increased insulin-like growth factor (IGF)-1 and activation of the Akt/mammalian target of rapamycin (mTOR) pathway (12-15). However, to our knowledge, the effects of DIO have not yet been reported in a preclinical model of spontaneous PDAC. A chronic positive energy balance state, DIO is typically accomplished by feeding a high-fat, high-calorie semipurified diet ad libitum that provides approximately 30% more total energy, with 100% of vitamins, minerals, and amino acids, relative to a control diet (16). There are limited reports of the enhancing effects of obesity on carcinogen-induced (17) and transplanted pancreatic cancer models (18, 19), but the underlying molecular alterations in the carcinogen-induced models are unknown, and transplant models can address tumor progression but not tumorigenesis. In a transgenic mouse model (p48^{Cre}-Kras^{LSL-G12D}), consumption of a high-fat diet, relative to standard chow, significantly increased the development and severity of PanIN lesions; however, the effect of obesity on PDAC development was not evaluated because this model develops only precancerous lesions (20).

An effective dietary strategy to induce negative energy balance, prevent obesity, inhibit several types of cancer (at least in animal models), and modulate energy balance-responsive growth factors such as IGF-1 is calorie restriction (CR) (21). However, the effects and underlying mechanisms of CR on pancreatic cancer are poorly characterized. CR regimens induce a lean phenotype in mice and typically involve 30% reductions in total energy intake, with 100% of vitamins, minerals, fatty acids and amino acids relative to ad libitum-fed control mice, which have an overweight phenotype (16). Recently, we reported that CR, relative to control diet, reduces tumor growth in a mouse transplant model of pancreatic cancer (Panc02 cells) and inhibits lesion development in a cyclooxygenase-2-driven transgenic mouse model of pancreatitis and high-grade pancreatic dysplasia (22, 23). In both models, CR also decreases circulating IGF-1 and signaling through the Akt/mTOR pathway, which lies at the convergence of extracellular growth factor and intracellular energy status signaling.

We hypothesized that the development and progression of PanIN lesions and PDAC, driven by Kras mutation and Ink4a/Arf deficiency, is responsive to dietary energy balance modulation in an IGF-1–dependent fashion. To test this hypothesis, we assessed the effects of CR (lean), control (overweight), and DIO diet regimens in LSL-Kras^{G12D}/Pdx-1-Cre/Ink4a/Arf^{lox/+} (hereafter referred to as Kras Ink4a^{+/-}) transgenic mice that develop pathological hallmarks of PanIN and PDAC progression modeling human pancreatic tumorigenesis. We also used liver-specific IGF-1 deficient (LID) mice, in combination with orthotopically transplanted pancreatic cancer cells derived from a spontaneous PDAC of a Kras Ink4a^{+/-} mouse, to explore causal links between serum IGF-1 levels and pancreatic cancer.

Materials and Methods

Mice, diets, and study design

All mice were singly housed in a semibarrier facility at the University of Texas at Austin Animal Resource Center. All experimentation was approved by the Institutional Animal Care and Use Committee at the University of Texas.

Spontaneous pancreatic tumor study—LSL-Kras^{G12D}/Pdx-1-Cre/Ink4a/Arf^{lox/+} (referred to as Kras Ink4a^{+/-}) male mice (breeding pairs provided by C.D.L.) were generated at the Animal Resource Center of the University of Texas (24). Mice were randomized (based on a single sequence of assignments from a random number generator) to receive one of the following three diets (all purchased from Research Diets, Inc., New Brunswick, NJ) beginning at 6–9 weeks of age: 1) control diet (D12450B, consumed ad libitum, n=42); 2) CR diet (D03020702; n=43), a modified AIN-76A semipurified diet fed in daily aliquots to provide 30% less total energy and 100% of all vitamins, minerals, fatty acids and amino acids relative to the control group; or 3) DIO diet (D12492, consumed ad libitum, n=39), a modified (60 kcal% fat) AIN-76A semipurified diet providing ~30% more total energy, with 100% of vitamins, minerals, and amino acids, relative to the control diet. Body weight and food intake were recorded weekly. Mice were killed following either 10 weeks (short-term substudy, n=15 mice/diet) or 56 weeks (long-term substudy, n=24–28 mice/diet) of dietary energy balance modulation, or when signs of moribundity occurred. Signs of moribundity included: 1) tumor size >1 cm in diameter; 2) distended abdomen; 3) hunched posture or loss of ambulation; and 4) significant loss of appetite and/or >10% weight loss.

In mice randomly selected for the short-term substudy (n=15/diet), quantitative magnetic resonance imaging (Echo Medical Systems, Houston, TX) was performed during the eighth week of diet administration. At 10 weeks, mice were fasted for 12 hours, anesthetized with CO₂ for blood collection by tail clip (for fasting glucose measurements) and by cardiac puncture (for serum hormone measurements), and then subsequently killed by cervical dislocation. Whole pancreata were harvested, weighed, sliced longitudinally, and either snap frozen in liquid nitrogen and stored in -80°C; or fixed in 10% neutral-buffered formalin overnight, transferred to 70% ethanol, and then embedded in paraffin. After coagulating for 30 minutes at room temperature (RT), blood samples were centrifuged at 9,300 × g for 5 min to obtain serum aliquots that were stored at -80°C.

In the long-term substudy, the same procedures for euthanasia and pancreata collection and storage were used when mice became moribund or completed 56 weeks of study.

Transplanted pancreatic tumor study—Liver-specific IGF-1-deficient (LID) mice were generated at the Animal Resource Center of the University of Texas by crossing albumin-cre transgenic FVB/N mice with FVB/N mice homozygotic for *igf1* flanked by *loxP* sites, as previously described (25). The original breeding pairs were a generous gift

from Dr. Derek LeRoith (Mt. Sinai School of Medicine, New York, NY). Wild-type FVB/N mice (WT) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were provided ad libitum-fed access to a chow diet (Purina Mills Lab Diet; Lab Supply, Fort Worth, TX).

Syngeneic mouse pancreatic cancer cells derived from an LSL-Kras^{G12D}/Pdx-1-Cre/*Ink4a*/*Arf*^{lox/+} mouse tumor (NB508 cells, generously provided by Dr. Nabeel Bardeesy, Harvard Medical School, Boston, MA) were orthotopically injected into LID mice (n=16) and WT mice (n=10). Prior to injection, these cells were cultured under an atmosphere of 5% CO₂ in a 37°C incubator with RPMI media (HyClone) supplemented with 10% fetal bovine serum (HyClone), penicillin/streptomycin, glutamine, non-essential amino acids, sodium pyruvate, and HEPES. Cells were trypsinized, washed in Hanks' Buffered Saline Solution (HBSS), and centrifuged. NB508 cells (1×10⁵) were resuspended in 50 µL of HBSS for intrapancreatic injection. Each mouse was anesthetized with ketamine-xylazine, a 2-cm dorsal incision was made below the lowest rib, and the pancreas wall was retracted. Tumor cells were injected using a 27-gauge needle into the pancreas tail, and the incision was closed using 9-mm wound clips. Species identification and karyotyping were conducted on the NB508 cell line at the T.C. Shu Molecular Cytogenetics Core at the University of Texas M.D. Anderson Cancer Center (Houston, TX).

Immediately following injections, an ALZET miniature osmotic pump (ALZET Osmotic Pumps, Cupertino, CA; #2004) was implanted subcutaneously through a small incision in the subscapular region, and the incision was closed using a wound clip. Carprofen was administered subcutaneously for postoperative analgesia. WT mice received continuous minipump infusion of vehicle (WT+vehicle, n=10), purchased from Tercica, Inc., (Brisbane, CA). LID mice were randomized (based on a single sequence of random assignments) to receive either a continuous infusion of vehicle (LID+vehicle, n=8) or 1 µg/hr of Increlex® (Tercica, Inc.) recombinant human IGF-1 (LID+IGF-1, n=8). After 28 days, mice were fasted for 12 hours and anesthetized with CO₂ for collection of blood, then killed and pancreatic tumors collected, weighed, and processed as previously described for the spontaneous pancreatic tumor study.

Blood glucose and serum energy balance-related hormone analyses

Blood glucose was analyzed using a Contour glucometer with Contour glucose test strips (Bayer HealthCare LLC, Mishawaka, IN). Serum levels of human and murine IGF-1 were determined by ELISA (Quantikine ELISA kit; R&D Systems, Inc., Minneapolis, MN) and RIA (DSL-2900 kit; DSL/Beckman Coulter Laboratories, Webster, TX), respectively, according to manufacturer's instructions. Serum levels of murine insulin, leptin, and adiponectin were measured using Lincoplex® bead-based assays (Millipore Corporation, Billerica, MA) on a BioRad Bioplex® analyzer (BioRad, Hercules, CA) according to manufacturer's directions.

Histopathologic and immunohistochemical analyses

Pancreatic tissues fixed in formalin and embedded in paraffin were cut into 4-µm thick sections, placed on glass slides (one section/slide) and processed for either hematoxylin and eosin (H&E) or immunohistochemical (IHC) staining. Slides were deparaffinized in xylene and sequentially hydrated in ethanol to water. Antigen retrieval required microwaving slides for 10 min with 10 mM citrate buffer. Endogenous peroxidase activity was quenched by exposing slides to 3% hydrogen peroxide for 10 min. Nonspecific binding was minimized by incubation with Biocare blocking reagent (Biocare Medical, Concord, CA) for 30 min at RT. Blocking was followed by incubation with primary antibody diluted in the same blocking buffer as follows: Ki-67 (Dako, Carpinteria, CA; 1:200, 4°C overnight); pAktS473

(Santa Cruz Biotechnology, Santa Cruz, CA; 1:50, 1 h RT); pmTOR_{Ser2448} (Cell Signaling, Danvers, MA; 1:50, 4°C overnight); pS6_(S235/236) ribosomal protein (Cell Signaling; 1:50, 1 h RT). After slides underwent two washes with PBS, secondary antibody was applied for 30 min at RT, followed by another three washes with PBS. Diaminobenzidine was used to develop the antibody stain followed by a hematoxylin counterstain to visualize nuclei. For each IHC marker, randomly selected slides (from 7 mice/diet for spontaneous pancreatic tumor study, and 4 mice/group transplanted pancreatic tumor study; using a single sequence of random assignments) were scanned and digitized using the Aperio Scanscope System (Scanscope XT; Aperio Technologies, Vista, CA). Quantitative analyses were performed on 4 fields per slide using the standard ImageScope algorithms for the percentage of cells with positive nuclear immunostaining of Ki-67 or positive cytoplasmic staining (and intensity of staining) of pAkt, pmTOR and pS6 ribosomal protein. Quantification of pAkt, pmTOR, and pS6 staining used the membrane/cytoplasmic-specific algorithm in order to exclude artifactual nuclear staining. Fields chosen for analysis of phosphorylated proteins included only cancerous regions of pancreatic tissue.

A certified veterinary pathologist (M.J.M.) histologically assessed (in a blinded manner) pancreatic sections from the Kras Ink4a^{+/-} mice for pancreatic lesions (e.g., PanIN 1-3 and PDAC) and liver and lung sections for evidence of metastatic lesions. The percentage of Kras Ink4a^{+/-} mice with high-grade (involving >25% of the tissue) pancreatic fibrosis or inflammatory cell infiltration was also determined.

Data analyses

Summarized data are reported as mean $\pm$ standard deviation (SD), with exception of serum markers and IHC quantification, which are reported as mean $\pm$ standard error of the mean (SEM), and transplanted pancreatic tumor weights, which are reported as medians. Comparisons between diet groups or genotypes with respect to final body weight and caloric intake, body fat, fasting glucose, serum markers, immunohistochemical markers, and tumor burden were performed using one-way ANOVA followed by Tukey's post-hoc test of significance. Data not meeting assumptions of normality were transformed by natural log prior to statistical analysis. One LID+IGF-1 mouse was excluded from serum hormone analyses (but not tumor analysis) due to minipump failure on the day before termination. The frequency distribution of spontaneous pancreatic lesions (no lesion, PanIN-1, PanIN-2, PanIN-3, or PDAC), was assessed wherein each mouse was counted once based on the most progressive lesion in their pancreata. The proportions of Kras Ink4a^{+/-} mice with versus without PDAC, with versus without high-grade fibrosis or inflammatory cell infiltration, and with versus without liver or lung metastasis were compared using Fisher's exact test. Kaplan-Meier survival curves for CR-, control- and DIO-fed Kras Ink4a^{+/-} mice were compared using the log-rank test. Mice were censored for the following criteria: i) death occurred before 9 weeks on study (CR, n=2; DIO, n=3); ii) generalized, nontumor-related death (CR, n=6; control, n=6); and iii) nonpancreatic tumor-related death (one mouse in each group died of lung tumor with no evidence of pancreatic tumor). Sample sizes after censoring were 19 for CR, 20 for control, and 20 for DIO. Statistical analyses were conducted using SPSS (Apache Software Foundation, Wilmington, DE). All tests were two-tailed, and results were considered significant if $P < 0.05$.

Results

Body composition, blood glucose and metabolic hormones in Kras Ink4a^{+/-} mice following short-term dietary energy balance modulation

A total of 124 Kras Ink4a^{+/-} mice were fed a CR, control, or DIO diet regimen and then killed following either 10 weeks (short-term substudy, n=15 mice/diet) or 56 weeks (long-

term substudy, n=24-28 mice/diet) of dietary energy balance modulation, or when signs of moribundity occurred. No mice in the short-term substudy were moribund or died prior to euthanasia.

Within 10 weeks, the diet regimens induced three different body size and metabolic phenotypes, from lean (CR: ~12% body fat, lowest IGF-1) to overweight (control: ~24% body fat, intermediate IGF-1) to obese (DIO: ~32% body fat, highest IGF-1). Specifically, CR diet, relative to control diet, fed to *Kras Ink4a*^{+/-} mice for 10 weeks resulted in significantly reduced caloric intake ($P<0.001$; Fig. 1A), body weight ($P<0.001$; Fig. 1B), percent body fat (measured at 8 weeks of diet; $P<0.001$; Fig. 1C), fasting blood glucose ($P=0.019$; Fig. 1D), serum IGF-1 ($P=0.041$; Fig. 1E), serum insulin ($P<0.001$; Fig. 1G), and serum leptin ($P<0.001$; Fig. 1H); and significantly increased serum adiponectin ($P<0.001$; Fig. 1F). The DIO diet, relative to control diet, produced significantly increased caloric intake ($P=0.001$; Fig. 1A), body weight ($P=0.002$; Fig. 1B), percent body fat (measured at 8 weeks of diet; $P<0.001$; Fig. 1C), and serum IGF-1 ($P=0.002$; Fig. 1E). Mean levels of fasting blood glucose (Fig. 1D), serum adiponectin (Fig. 1F), serum insulin (Fig. 1G), and serum leptin (Fig. 1H) were comparable between DIO and control mice. DIO, relative to CR, resulted in significantly higher calorie intake, body weight, percent body fat, blood glucose, serum IGF-1, serum insulin, and serum leptin; and significantly lower serum adiponectin (Fig. 1A-F; $P=0.001$ for each).

Pancreatic lesions in *Kras Ink4a*^{+/-} mice following short-term dietary energy balance modulation

After 10 weeks of dietary energy balance modulation in *Kras Ink4a*^{+/-} mice, the incidence of PDAC was 0% (0 of 15 mice) for CR, 20% (3 of 15 mice) for control, and 47% (7 of 15 mice) for DIO (Fig. 2A&B), and was significantly different between CR and DIO mice ($P=0.006$), but not control. Among mice without PDAC, all but 1 in each dietary energy balance group had PanIN lesions, and the frequency distributions of PanIN lesions were comparable between groups (Fig. 2B).

Pancreata from CR mice had a more normal architecture (based on the extent of histologically normal acini and islets) as compared with pancreata from control, and to a greater extent, DIO mice (Fig. 2A). Pancreata from DIO mice weighed significantly more than pancreata from CR mice or control mice ($P<0.001$, each; Fig. 2C). High-grade (involving >25% of the tissue) pancreatic fibrosis was present less often in CR mice (13%, 2/15) than control mice (27%, 4/15), and less often in control mice than DIO mice (60%, 9/15); the difference in frequency between CR and DIO was significant ($P=0.021$; Fig. 2D). High-grade inflammatory cell infiltration occurred in the pancreata of 1 (7%) CR mouse, 2 (13%) control mice, and 6 (40%) DIO mice, with the difference for CR versus DIO approaching statistical significance ($P=0.080$; Fig. 2E).

Pancreatic Akt/mTOR signaling following short-term dietary energy balance modulation in *Kras Ink4a*^{+/-} mice

Pancreatic protein expression of phosphorylated (p) Akt, pmTOR, and pS6 ribosomal protein, determined by immunochemistry, was consistently lowest for CR mice, intermediate for control mice, and highest for DIO mice (Fig. 3). Differences were statistically significant between CR and DIO mice in regards to overall and/or high-intensity staining of the pancreata for pAkt ($P=0.047$ and $P=0.089$, respectively), pmTOR ($P=0.049$ and $P=0.004$, respectively), and pS6 ribosomal protein ($P=0.001$ and $P=0.004$; Fig. 3).

Tumor-free survival and pancreatic tumor metastases in *Kras Ink4a*^{+/-} mice following long-term (up to 56 weeks) dietary energy balance modulation

CR (n=28), compared with control (n=27), significantly extended (by 50%) the lifespan of *Kras Ink4a*^{+/-} mice (median survival of 30.0 weeks versus 20.0 weeks, $P<0.001$; Fig. 4A). Median survival of DIO mice (n=24) was 20.5 weeks, which was significantly shorter than CR mice ($P<0.001$) and comparable with control. Mice were censored at the time of nonpancreatic tumor death (CR, n=9; control, n=7; DIO, n=4). All noncensored control mice and DIO mice showed signs of moribundity and were killed (or died) by 38 and 34 weeks, respectively. Three CR mice remained healthy and tumor-free throughout the 56-week study.

Four mice (CR, n=1; DIO, n=3) with pancreatic tumors were found dead, and hemolysis of their tissues precluded evaluation of metastasis. Among mice evaluable for metastasis, CR mice had a lower incidence of liver and lung metastases (28% and 22%, respectively) than the control (50% and 30%, respectively) or DIO groups (47% and 35%, respectively), although these differences were not statistically significant (Fig. 4B).

Serum hormones, orthotopically transplanted pancreatic tumor growth, and tumoral Akt/mTOR signaling in LID mice with and without IGF-1 infusion

LID mice and wild-type (WT) FVB/N mice (the background strain of the LID mice) were intrapancreatically injected with NB508 pancreatic tumor cells derived from a spontaneous *Kras Ink4a*^{+/-} tumor. Osmotic minipumps were subcutaneously implanted to continuously deliver vehicle to WT mice (n=10), vehicle to LID mice (n=8), or recombinant human (rh) IGF-1 to LID mice (n=8). After 28 days, mice were killed and serum and tumors were collected for analysis. Each mouse developed a single tumor.

Serum analysis confirmed lower IGF-1 levels in LID+vehicle mice, relative to WT+vehicle mice (~70% lower, $P<0.001$; Fig. 5A), and IGF-1 infusion in LID mice reversed the IGF-1 deficiency to levels exceeding the other 2 groups ($P<0.001$ each, Fig. 5A). Serum insulin levels were lowest in WT+vehicle mice, intermediate in LID+IGF-1 mice, and highest in LID+vehicle mice (Fig. 5B), with a significant between-group difference for WT+vehicle versus LID+vehicle mice ($P<0.001$) and LID+IGF-1 ($P=0.043$). Serum adiponectin and leptin levels were comparable among groups (Fig. 5C&D, respectively).

Pancreatic tumor weight was reduced in LID+vehicle mice relative to WT+vehicle mice ($P=0.014$) and LID+IGF-1 mice ($P<0.001$, Fig. 5E). Tumor weights were comparable between WT+vehicle and LID+IGF-1 mice (Fig. 5E). Proliferation, as measured by Ki-67 positivity, was reduced in tumors from LID+vehicle mice relative to WT+vehicle mice ($P=0.025$) or LID+IGF-1 mice ($P=0.026$), and was comparable in tumors from WT+vehicle and LID+IGF-1 mice (Fig. 5F).

Overall and high-intensity tumoral staining for pAkt and pS6 ribosomal protein was significantly lower for LID+vehicle mice, as compared with WT+vehicle or LID+IGF-1 mice ($P=0.010$ for each comparison, Fig. 6). High-intensity tumoral pmTOR staining was significantly lower in the LID+vehicle mice, relative to the other two groups ($P<0.001$, each group), despite no significant differences in overall positivity. Tumoral levels of pAkt, pS6 ribosomal protein and pmTOR were comparable between WT+vehicle and LID+IGF-1 mice (Fig. 6).

Discussion

Using murine spontaneous and orthotopically transplanted models of pancreatic cancer with genetic lesions (mutant *Kras*, and *Ink4a/Arf* deficiency) common to human pancreatic

tumors, we established that dietary energy balance modulation impacts pancreatic tumorigenesis in association with changes in circulating IGF-1. Specifically we showed that: 1) CR, relative to higher calorie control or DIO diets, delays the progression of PanIN lesions to PDAC, decreases pancreatic desmoplasia and metastases, and extends pancreatic tumor-free survival in *Kras Ink4a^{+/-}* mice in association with decreased serum IGF-1 and Akt/mTOR pathway signaling; and 2) transplanted pancreatic tumor growth and tumoral Akt/mTOR signaling are decreased in mice with genetically reduced serum IGF-1 and are rescued by IGF-1 infusion. Thus, IGF-1 and components of its downstream signaling pathway represent promising mechanistic targets for breaking the obesity-pancreatic cancer link.

Given that approximately one third of US adults are obese, one third are overweight, and one third are lean/normoweight (10), and that >90% of human pancreatic tumors have KRAS mutations (26), comparisons of pancreatic tumorigenesis among lean (CR), overweight (control) and DIO *Kras*-mutant mice are highly relevant to the human situation. Mice with *Kras* mutation alone develop PanIN lesions, but PDAC is rare in these mice without a collaborating genetic alteration, such as loss of the tumor suppressor *Ink4a/Arf* (24). Mice with *Kras* mutation and *Ink4a/Arf* homozygous deletion die from spontaneous PDAC early in life, and this limits their usefulness for diet studies (24). We therefore used mice with *Kras* mutation and *Ink4a/Arf* heterozygous deletion (*Kras Ink4a^{+/-}* mice) to assess the impact and underlying mechanisms of dietary energy balance modulation on pancreatic tumorigenesis. We showed that relative to DIO and control, CR decreases serum IGF-1, the progression of PanIN 1-3 lesions to PDAC after short-term (10 weeks) diet treatment, and liver and lung metastases and tumor-free survival after long-term (up to 56 weeks) diet treatment. Additional findings in this model were that CR, relative to DIO, decreases pancreatic levels of activated (phosphorylated) Akt, mTOR and S6 ribosomal protein, and decreases the development of pancreatic desmoplasia, a hallmark of PDAC that is characterized by a proliferative, reactive stroma with fibrosis and infiltrating immune cells.

To our knowledge, this is the first report of dietary energy balance (CR and DIO) effects on spontaneous PDAC development and progression. Previously, in a N-nitrosobis(2-oxopropyl)amine-induced hamster model of PDAC, consumption of a high-fat, high-calorie chow diet significantly shortened tumor latency and enhanced tumor multiplicity, as compared with standard rodent chow (17). Subcutaneous transplantation of mouse Panc02 cells into the flanks of obese mice, relative to lean mice, resulted in larger tumors (18, 19). In addition, a high-fat diet, relative to standard chow, significantly increased the development and severity of PanIN lesions in *p48^{Cre}-Kras^{LSL-G12D}* mice (20). In mice subcutaneously injected with Panc02 PDAC cells, CR, relative to control diet, suppressed PDAC progression, reduced serum IGF-1, and decreased signaling through the Akt/mTOR pathway (22). Similarly, CR decreased serum IGF-1, Akt/mTOR signaling, pancreatitis, and pancreatic dysplastic lesions in a cyclooxygenase-2 transgenic mouse model (23). Thus, our current findings reinforce and augment previously reported effects of dietary energy balance modulation in carcinogen-induced or subcutaneously transplanted models of PDAC and in spontaneous models of pancreatic preneoplasia.

We, and others, have previously shown in several nonpancreatic tumor models that circulating IGF-1 levels are associated with the energy balance–cancer link (12, 13, 27-29). However, the association between IGF-1 and PDAC has not been well established. In the current study, the diet-induced changes in levels of IGF-1, as compared with the other energy balance-related hormones measured (adiponectin, leptin and insulin), most closely mirrored the diet-dependent changes in body fat levels and PDAC development in *Kras Ink4a^{+/-}* mice (ie, CR lowest, control intermediate, DIO highest). Serum adiponectin, leptin and insulin levels also differed between CR and DIO mice; however, the relative

contributions of systemic IGF-1, insulin, leptin or adiponectin in the energy balance-pancreatic cancer link are unclear.

To determine if the observed association between serum IGF-1 and pancreatic tumor progression could be causal, we used LID mice, with or without rhIGF-1 infusion using a well-established IGF-1 addback protocol to increase serum IGF-1 in LID mice to levels between control and diet-induced obese mice (28-30). The LID mice were then injected with NB508 PDAC cells derived from a spontaneous tumor of a Kras Ink4a^{+/-} mouse. Previously, we used this model to establish that the effects of CR and obesity on tumor suppression or progression, respectively, were largely dependent upon modulation of circulating IGF-1 (15). In the LID mice, CR did not reduce mammary tumor growth or circulating levels of IGF-1 relative to the control diet. Although DIO LID mice displayed some IGF-independent effects, these were not able to overcome the prevailing impact of low circulating IGF-1 on tumor growth (15). Consistent with a causal relationship between IGF-1 and PDAC, LID mice, relative to WT mice, had significantly reduced serum IGF-1 levels, PDAC growth, tumoral proliferation (as assessed by Ki-67 staining), and tumoral Akt/mTOR signaling; each of which was rescued by IGF-1 infusion. The dose of rhIGF-1 used in this experiment increased total serum IGF-1 (murine plus human) in the LID+IGF-1 mice to levels two-fold higher than those of WT mice, but tumor growth was comparable. The possibility that a threshold exists above which increased serum IGF-1 has less effect on PDAC is under investigation. Pancreatic tumor growth and Akt/mTOR signaling had no association with serum leptin and adiponectin levels, and had a biologically implausible inverse association with insulin levels (31), suggesting that these three hormones may be lower priority targets than IGF-1 for breaking the obesity-pancreatic cancer link.

In conclusion, CR suppresses, while DIO enhances, spontaneous pancreatic tumorigenesis induced by altered Kras and Ink4a, the most commonly mutated genes in human pancreatic cancer. Furthermore, IGF-1 and components of its downstream signaling pathway are promising mechanistic targets for mimicking the anticancer effects of CR and breaking the obesity-pancreatic cancer link.

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Figure 1.

Body composition parameters, blood glucose and serum hormones in *Kras Ink4a^{+/-}* mice following short-term (10 weeks) dietary energy balance modulation (n=15 mice/diet group, unless otherwise noted). A, Caloric intake, by week (through ninth week) and group. B, Body weight, by week and group. C, Percentage of body fat (measured using quantitative magnetic resonance imaging at 8 weeks), by group. At study termination (10 weeks on diet) D, fasting blood glucose (n=12/group); E, serum IGF-1; F, serum adiponectin; G, serum insulin; and H, serum leptin were collected. Values are mean±SD (A-D) or mean±SEM (E-H). Different letters denote significant differences between groups. CR=30% calorie restriction, CON=control, DIO=diet-induced obesity, IGF-1=insulin-like growth factor-1.

Figure 2.

Characteristics of pancreatic tissue from *Kras Ink4a*^{+/-} mice after 10 weeks of dietary energy balance modulation (n=15 mice/diet group). A, Representative photomicrographs of H&E-stained pancreatic sections at 20× (scale bar=100 μm), by group (CR: single PanIN-3 lesion with the majority of section composed of normal acini; CON: mixed PanIN 1-3 lesions and fibrosis with some normal pancreatic parenchyma; DIO: mixed PanIN 1-3 lesions and PDAC with limited normal parenchyma). B, Frequency distribution of mice by their highest-grade pancreatic lesion, by group. C, Mean±SD gross pancreatic weight, by group. D, By-group percentages of mice with high-grade (>25% of tissue) pancreata. E, By-group percentages of mice with high-grade inflammatory cell infiltration. Different letters denote significant differences between groups. CR=30% calorie restriction, CON=control, DIO=diet-induced obesity, PanIN=pancreatic intraepithelial neoplasia, PDAC=pancreatic ductal adenocarcinoma.

Figure 3.

Immunohistochemical analysis of energy-responsive signaling intermediates in pancreatic tissue from *Kras Ink4a^{+/-}* mice after 10 weeks of dietary energy balance modulation. Representative photomicrographs of pancreatic sections captured with 20× objective (scale bar=100 μm), by diet group, stained for pAkt, pmTOR, and pS6 ribosomal protein, with associated stacked bar graphs showing the mean±SEM percentage of positively stained cells (filled bar: low-intensity staining; open bar: high-intensity staining; n=7 mice/diet). Different letters denote significant differences between groups in the overall percentage of positively stained cells. CR=30% calorie restriction, CON=control, DIO=diet-induced obesity.

B

Diet Group	Liver Metastasis	Lung Metastasis
CR	5/18 (28%)	4/18 (22%)
Control	10/20 (50%)	6/20 (30%)
DIO	8/17 (47%)	6/17 (35%)

Figure 4.

Long-term dietary energy balance modulation effects on tumor-free survival and metastases. A, Kaplan-Meier pancreatic tumor-free survival curve of *Kras Ink4a*^{+/-} mice fed a CR (n=28), CON (n=27), or DIO (n=24) diet. Mice were censored at the time of nonpancreatic tumor death (CR, n=9; CON, n=7; DIO, n=4). B, Percentage of mice, by diet group, with pancreatic cancer metastasis to liver or lungs. Asterisk denotes significant differences between CR and both CON and DIO. CR=30% calorie restriction, CON=control, DIO=diet-induced obesity.

Figure 5.

Energy balance-related hormones and orthotopically transplanted pancreatic tumor growth in liver IGF-1-deficient (LID) mice, with and without IGF-1 infusion. Mean±SEM fasting serum levels of A, IGF-1, B, adiponectin, C, insulin, and D, leptin collected 28 days following NB508 tumor cell injection into the pancreas of either wild-type+vehicle (WT+Veh) mice (n=10), LID+vehicle (LID+Veh) mice (n=8), or LID+recombinant human IGF-1 (LID+IGF-1) mice (n=8, except for serum analyses, n=7). E, Scatter plot of pancreatic tumor weights (horizontal line denotes median) measured after 28 days of intrapancreatic growth, by treatment. F, Representative photomicrographs (20×) showing Ki-67 immunostaining in pancreatic tumors from WT+Veh, LID+Veh and LID+IGF-1 mice (n=4/group; inset: mean±SEM percentage of positive cells). Different letters denote significant differences between groups.

Figure 6. Immunohistochemical analysis of Akt/mTOR pathway components in transplanted pancreatic tumors from LID mice with and without IGF-1 infusion. Representative photomicrographs of NB508 pancreatic tumor sections from wild-type+vehicle (WT+Veh) mice, LID+vehicle (LID+Veh) mice, or LID+recombinant human IGF-1 (LID+IGF-1) mice captured with 20× objective (scale bar=100 μ m), stained for pAkt, pmTOR, and pS6 ribosomal protein, with associated stacked bar graphs showing the mean $\pm$ SEM percentage of positively stained cells (filled bar: low-intensity staining; open bar: high-intensity staining). Different letters denote significant differences between groups.