Original Research

Calorie restriction delays the progression of lesions to pancreatic cancer in the LSL-Kras^{G12D}; Pdx-1/Cre mouse model of pancreatic cancer

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Abstract

Since pancreatic cancer is a lethal disease, developing prevention strategies is an important goal. We determined whether calorie restriction would prevent the development and delay progression of pancreatic intraepithelial neoplasms to pancreatic ductal adenocarcinoma (PDA) in LSL-Kras$^{G12D/+}$; Pdx-1/Cre mice that develop all the precursor lesions that progress to PDA. Eight-week-old LSL-Kras^{G12D}; Pdx-1/Cre mice were assigned to three groups: (1) *ad libitum* (AL) fed the AIN93M diet or (2) intermittently calorie restricted (ICR) a modified AIN93M at 50% of AL intake followed by one week intervals at 100% of AL intake, or (3) chronically calorie restricted (CCR) an AIN93M diet at 75% of AL intake. AL fed mice had a greater percentage of pancreatic ducts with PanIN-2 (13.6%) than did the ICR (1.0%) and CCR groups (1.6%), $P < 0.0001$. Calorie restriction (ICR [0%] and CCR [0.7%]) reduced the percentage of ducts with PanIN-3 lesions compared to the AL group (7.0%), $P < 0.0001$. The incidence of PanIN-2 or more lesions was significantly reduced in both ICR (27%; $n = 16$) and CCR (40%) mice ($n = 15$; $P < 0.001$) compared to AL (70%) fed mice ($n = 11$). The delayed progression of lesions in ICR and CCR mice was associated with reduced proliferation measured by proliferating cell nuclear antigen staining, reduced protein expression of Glut1, increased protein expression of Sirt1, increased serum adiponectin, and decreased serum leptin. CCR resulted in decreased phosphorylated mammalian target of rapamycin and decreased serum insulin-like growth factor-1. In summary, this is the first study to show in LSL-Kras^{G12D}; Pdx-1/Cre mice that ICR and CCR delay the progression of lesions to PDA.

Keywords: pancreatic cancer, LSL-Kras^{G12D}; Pdx-1/Cre mice, calorie restriction, intermittent calorie restriction, Glut1, mammalian target of rapamycin (mTOR)

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Introduction

Pancreatic cancer is a deadly disease with only 6% of the patients living longer than five years. Even if the disease is diagnosed when it is localized, the five-year survival rate is approximately 19%.[1] Presently, there is no way to detect the early stages of pancreatic cancer and when symptoms do occur the disease has often metastasized. Thus, there is an urgent need to identify strategies to prevent or treat this disease. Recent statistics also indicate that the annual incidence of pancreatic cancer is increasing.[1] There are known risk factors including smoking, obesity, increased alcohol consumption, diabetes, increased age, and family history that promote pancreatic cancer. There is mounting evidence that changes in energy metabolism resulting in increased body mass index, obesity, and abnormal glucose utilization are also risk factors for pancreatic cancer. Considering the increased incidence of pancreatic cancer and the increase in obesity, it is especially important to investigate intervention strategies to decrease risk.

In a wide range of species, calorie restriction resulting in lower body weight is well-recognized for extending life span and reducing incidence of many chronic diseases including several types of cancers.[2–8] To date, there have been no prevention studies investigating the protective

effects of calorie restriction in delaying the progression of early preneoplastic lesions to pancreatic cancer in a transgenic mouse model. It is generally recognized that pancreatic cancer develops from a progression of graded preneoplastic lesions known as pancreatic intraepithelial neoplasias (PanINs) to pancreatic ductal adenocarcinoma (PDA). With the development of the LSL-Kras^{G12D}; Pdx-1/Cre mice, researchers are now able to conduct long-term prevention studies in a mouse model that exhibits all the stages of human pancreatic cancer.[9,10]

Calorie restriction refers to decreased caloric intake while maintaining nutrient requirements. The anti-tumour benefits of life-long calorie restriction have been reported for breast, colon, liver, skin, and lung tumours in rodents.[5–8,11] Recently, animal studies have demonstrated that the benefits of calorie restriction can also be achieved by restricting calories intermittently followed by periods of *ad libitum* (AL) intakes. In an animal model of postmenopausal breast cancer, intermittent calorie restriction (ICR) at 50% of AL for three weeks followed by three weeks of eating 100% of AL intake (refeeding) was found to be even more protective than life-long, chronic calorie restriction (CCR) in reducing the incidence of mammary tumours in transgenic MMTV-TGF-α female mice.[12,13] In the TRAMP mouse model of prostate cancer, ICR in two-week cycles also at 50% restriction delayed the onset of tumours and extended survival.[14] In another study using a prostate cancer cell line grown as a xenograft, several different fasting/refeeding protocols exhibited non-significant trends toward improved survival.[15] In contrast to this study,[15] ICR was started prior to tumour development in the TRAMP mice, which suggests that the protective effect of calorie restriction takes place before or during transition of normal cells to cancer cells. Even in adult mice an intermittent protocol of one day/week fasting significantly reduced the incidence of lymphoma in p53-deficient mice compared to AL-fed mice and mice chronically restricted 40%.[16] These studies suggest that many cycles of calorie restriction followed by refeeding rather than the length of the cycles have an effect on tumour development and progression.

It is well known that calorie restriction is effective in reducing the incidence of cancer and delaying its progression in many animal models. However, the potential benefits of calorie restriction in pancreatic cancer are not well studied. Considering that the pancreas is a major regulator of metabolic function, it is susceptible to energy manipulation. In pancreatic cancer, Ras/MEK/ERK/PI3K/mTOR signalling and the insulin/IGF-1 pathways are deregulated to provide for the increasing nutrient demands of the growing cancer cells. Since calorie restriction alters these signalling pathways, we expect that restricting calories will disrupt the progression of preneoplastic lesions to PDA. The hypothesis of this study is that calorie restriction reduces the incidence and delays the development of high-grade lesions in the LSL-Kras^{G12D}; Pdx-1/Cre mouse model. We also determined whether an intermittent pattern of calorie restriction would offer additional protection greater than a life-long CCR. Since proliferation of cancer cells requires nutrients and energy, we investigated whether calorie restriction modified several proteins known to be involved in cell growth and energy metabolism. This study is the first to provide evidence in the LSL-Kras^{G12D}; Pdx-1/Cre transgenic mouse model that calorie restriction protects against the development and progression of lesions to PDA and to identify biomarkers associated with the benefits of calorie restriction.

Methods

Breeding and genotyping

All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. We used the LSL-Kras^{G12D}; Pdx-1/Cre mouse model of ductal pancreatic carcinoma for this study. Similar to human pancreatic cancer, the LSL-Kras$^{G12D/+}$; Pdx-1/Cre mice develop pancreatic precursor lesions PanIN-1A and PanIn-1B, intermediate lesions PanIN-2, and advanced PanIN-3 lesions, which progress to PDA.[9] The slow, progressive development of the invasive disease in older LSL-Kras^{G12D}; Pdx-1/Cre mice confirms that the lesions predispose them to pancreatic cancer. This model is suitable for prevention studies because it provides an extended period of time for intervention during the preinvasive state.

LSL-Kras^{G12D} and Pdx-1/Cre mice strains were interbred to obtain LSL-Kras^{G12D}; Pdx-1/Cre mice on a mixed genetic background. We confirmed the genotype of each pup by extracting genomic DNA from each tail cut using the REDExtract-N-Amp^{TM} Tissue PCR Kit (Sigma, St. Louis, MO, USA) according to previously described protocols.[9]

Experimental design and animal diets

Six-week-old LSL-Kras^{G12D}; Pdx-1/Cre mice were randomized to the following three groups: AL control ($n = 31$), chronic calorie restricted ([CCR] $n = 31$), and intermittent calorie restricted ([ICR] $n = 31$). The mice were fed modified AIN-93 diets based upon those described by Cleary *et al.*[13]; all diets were purchased from Harlan Laboratories (Madison, WI, USA).

Mice in each group were housed one mouse per cage. Mice were distributed so that there were an equal number of males and females of the same age in each group. Food intake was measured daily and body weights were recorded weekly. The mice in the AL group had free access to the control diet (AIN-93M). The AL control mice were started one week before the ICR mice and the CCR mice. The ICR group was fed a 50% calorie restriction diet during the one-week restriction period followed by one week of feeding at 100% of AL intake during the refeeding period. The ICR diet was adjusted to have a twofold increase in protein, fat, vitamin, and mineral content and was isocaloric with the AIN-93M diet. The CCR group was fed the AIN 93-M diet formulated to be isocaloric with the control diet with 25% increases in protein, vitamins, minerals, and fat content. This diet was given at 75% of the age-matched AL consumption. Thus, over the entire experimental period both CCR and ICR groups were restricted by 25% of their AL intake. All mice were euthanized at 44 weeks of age. At this time point, the mice in the ICR mice were euthanized at

the end of the restriction period. Blood was obtained by retroorbital bleeding and allowed to coagulate at room temperature for 15 min and then centrifuged at $10{,}000 \times g$ to obtain serum that was stored at −80°C. Pancreas, pancreatic tumours, spleen, and liver were removed and weighed. One aliquot of pancreas was prepared for histological analysis to determine the distribution of lesions, and the other was frozen in liquid nitrogen for determination of protein by Western analysis.

Histological evaluation

Sections (4 μm thick) from formalin-fixed, paraffin-embedded tissues were stained with haematoxylin and eosin (H&E). The pathologist evaluated several sections of each pancreas blinded to the experimental groups. Lesions and invasive carcinomas were classified by established histopathological criteria[9,17] as PanIN-1A/1B, PanIN-2, and PanIN-3. About 100 ducts were analysed for each section of the pancreas. To quantify the progression of the lesions, the relative proportion of each lesion to the total number of analysed ducts was calculated. In some mice, invasive carcinoma was present and these mice were classified as having pancreatic cancer or not.

Immunohistochemical (IHC) analysis

Tissue sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in phosphate-buffered saline (PBS). Antigen unmasking solution (Vector Lab, Burlington, CA, USA) was used to retrieve antigens. The tissue sections were quenched with 3% hydrogen peroxidase and blocked with 5% normal serum. Non-specific binding sites were blocked with AffiniPure Fab Fragment Donkey of anti-Mouse IgG (H + L) blocking reagent (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Sections were incubated with primary antibody overnight at 4°C. After washing in PBS, slides were incubated with secondary antibody at room temperature for 1 h and then incubated with ABC reagent. Sections were subsequently incubated with diaminobenzidine peroxidase, rinsed, and counterstained with haematoxylin and mounted on coverslips. Images were captured using a Nikon Eclipse 80i microscope.

Western-blot analysis

The pancreas was homogenized and lysed in ice-cold 1% Triton lysis buffer and protease inhibitor cocktail with phosphatase inhibitor. After vortexing, the lysates were separated by centrifugation at $12{,}000 \times g$ for 15 min at 4°C. Protein concentrations were measured with a BioRad DC (BioRad Laboratories, Hercules, CA, USA) protein assay with bovine serum albumin as the protein standard. Proteins were denatured by heat at 95°C. Equal amounts of protein for each sample were loaded and separated on NuPAGE gel and transferred to a nitrocellulose membrane. Non-specific binding was blocked with 5% dry milk in TBST (40 mmol/L Tris-Cl, pH 7.6, 150 mmol/L, NaCl, 0.2% Tween-20) for 1 h at room temperature. Membranes were probed for Glut 1 (Abcam, Cambridge, MA, USA), p-Akt, Akt, phospho-mammalian target of rapamycin (p-mTOR), mTOR, phospho-adenosine monophosphate-activated protein kinase (p-AMPK), AMPK (Cell Signaling, Beverly, CA, USA), proliferating cell nuclear antigen (PCNA), Sirt1 (Millipore, Billerica, MA, USA) and incubated overnight at 4°C. All antibodies were used at a 1 : 2000 dilution except Glut 1, which was used at 1 : 5000 dilution. After washing with TBST three times, the membranes were incubated with secondary antibodies at room temperature for 1 h with constant shaking. The expression of the targeted proteins was detected using an ECL kit (Amersham Biosciences, Piscataway, NJ, USA) following manufacturer's instructions and visualized by autoradiography with Hyperfilm. Then the blot was scanned; protein bands were quantitated by Image J software and expressed as arbitrary units relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Analysis of serum glucose, insulin-like growth factor (IGF-1), adiponectin, and leptin

Serum glucose was measured using an enzyme-linked immunosorbent assay ([ELISA] (Glucose Assay kit, Eton Bioscience, Cambridge, MA, USA). IGF-1 was measured by an ELISA assay (R & D Systems, Minneapolis, MN, USA). We measured serum concentrations of adiponectin and leptin by ELISA kits (R & D Systems and Millipore, respectively).

Statistical analysis

The values are expressed as mean ± standard deviation (SD), unless stated otherwise. Differences in food intake, spleen and pancreas weights, serum parameters, and Western-blot densitometry were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analysis. Exact Poisson regression analysis was used to test for differences in diet groups with respect to rate of preneoplasic lesion formation (number of ducts with PanIN-2/total number of ducts examined and number of ducts with PanIN-3/total number of ducts examined). Statistical analysis was conducted using SAS 9.3 software package (SAS Institute, Cary, NC, USA). Logistic regression analysis was used to compare groups with respect to percent positive PCNA staining. Generalized estimating equation methods were used to account for correlation of multiple samples from each mouse.

Results

Effect of ICR and CCR on body weights over the experimental period

AL control mice gained weight consistently over the entire experimental period while the weight gain of the calorie-restricted mice was slower (Figure 1). ICR mice exhibited a cyclic pattern of weight gain/loss. The fluctuations in body weights of the ICR mice reflected the feeding pattern of one week of refeeding followed by one week of 50% calorie restriction. During the refeeding period, the ICR mice were fed the amount of food consumed by the AL group during the previous one-week interval (Table 1). At the

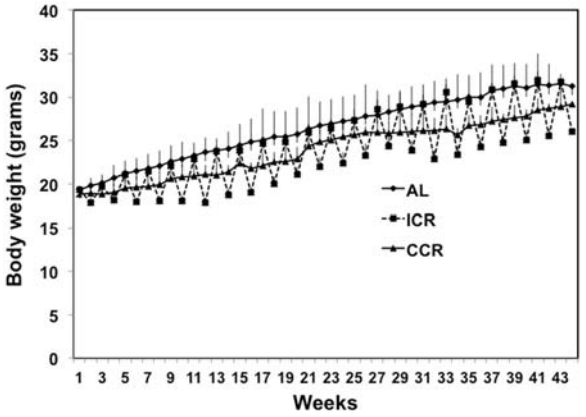

Figure 1 Body weights over the experimental period. Body weights were measured weekly for *ad libitum* (AL) control ($n = 31$), intermittent calorie-restricted ([ICR] $n = 31$), and chronic calorie-restricted ([CCR] $n = 31$) mice for 44 weeks. The body weights of the AL LSL-Kras^{G12D}; Pdx-1/Cre mice were significantly different from the restricted groups ($P < 0.001$, ANOVA) but the restricted groups were not significantly different from each other. Values are ± SEM

termination of the experiment (44 weeks of age) body weights of the ICR and CCR mice (21.7 ± 0.39 g, 21.0 ± 0.46 g, mean ± SD) were significantly lower than those of the AL mice (29.6 ± 1.4 g) but there was no difference in body weights between ICR and CCR mice (Table 2).

Calorie restriction reduces the number of PanIN-2 and PanIN-3 lesions

Similar to the human disease, LSL-Kras^{G12D}; Pdx-1/Cre mice display the entire spectrum of lesions that progress to PDA.[9,10] The full continuum of pancreatic ductal proliferations has been shown to be neoplastic based on clonal *K-ras* mutations.[17] Lesions are described as progressing from tall mucinous cells without cytologic atypia or papillary architecture (1A); to the same cells forming papillary tufts (1B); to cells with increasing cytologic atypia in the form of nuclear stratification, loss of polarity, and mild atypia (PanIN-2); to lesions with significant cytologic atypia, loss of nuclear polarity, tufting of cells into the lumen, mitoses, and necrosis (PanIN-3, carcinoma *in situ*). PanINs differs from invasive adenocarcinoma in that the latter shows invasion beyond the basement membrane of ducts with attendant stromal desmoplasia, variable perineural invasion, and necrosis. This model is suitable for prevention studies because it provides an extended period of time for intervention during the pre-malignant state. Figure 2(a) shows representative grades of lesions that were observed in LSL-Kras^{G12D}; Pdx-1/Cre mice along with a representative pancreatic tumour that was observed in the AL fed mice. Figure 2(b) indicates that AL fed mice had a greater percentage of pancreatic ducts with PanIN-2 (13.6%) than the ICR (1.0%) and CCR groups (1.6%), $P < 0.0001$. PanIN-3 lesions were present in the AL group (7.0%) and the CCR (0.7%); however, no PanIN-3 lesions were found in the ICR group. In comparison to CCR, ICR significantly delayed the formation of PanIN-3 lesions, $P < 0.0094$ (Figure 2b).

The weights of the pancreas are often increased in pancreatic cancer due to the growing tumour burden, which results in obstruction of the pancreatic duct. Splenomegaly is also present and may be caused by obstruction of the splenic vein. In calorie-restricted mice, the weights of the pancreas and spleens were significantly less than the AL fed LSL-Kras^{G12D}; Pdx-1/Cre mice. The spleens from the ICR mice weighed significantly less than those from the CCR. The effect of calorie restriction was maintained when weights of the pancreas and spleen were normalized to body weights (Table 1).

Figure 2(c) shows that the AL feeding led to higher incidence (percentage of mice) of PanIN-2 or greater lesions (70%) than the ICR mice (27%) or CCR (40%; $P < 0.05$). The percentage of ICR mice with PanIN-2 or greater lesions was significantly lower than the CCR ($P < 0.05$). PDA was present in 27% of the AL but not in the calorie-restricted groups (Figure 2a).

Calorie restriction reduced proliferation as evaluated by PCNA staining

PCNA is expressed in the nuclei of cells and is synthesized during the late G1 and reaches its maximum in the S phase of the cell cycle. PCNA is an established marker of DNA synthesis in cancer and was used in this study to examine the effect of calorie restriction on proliferation. Using IHC analysis, the percentage of PCNA positive ductal cells in the proliferative pool was compared between AL, ICR, and CCR LSL-Kras^{G12D}; Pdx-1/Cre mice. The PCNA positive cells were counted in five fields with ×20 magnification in pancreatic sections from each mouse (3 mice per group). All PCNA-stained ductal cells were counted regardless of the stage of lesions. PCNA proliferation index was calculated by dividing the number of PCNA stained ductal cells by the total number of ductal cells counted and expressed as a percent. The PCNA measurement represents the proportion of cells in the proliferating pool. The semi-quantitative analysis of PCNA-stained sections of the pancreas showed that ICR and CCR significantly reduced the percentage of PCNA positive ductal cells in comparison to AL feeding, $P < 0.05$. Figure 3(a) shows that pancreas from AL fed LSL-Kras^{G12D}; Pdx-1/Cre mice (AL, 61%) had extensive ductal cell proliferation relative to ICR and CCR LSL-Kras^{G12D}; Pdx-1/Cre mice (ICR, 21.3%; CCR, 23%); $P < 0.0001$ by logistic regression analysis with generalized estimated equations. Western analysis confirmed that both types of calorie restriction decreased protein expression of PCNA in pancreas tissue; there were no significant differences between ICR and CCR (Figure 3b).

ICR and CCR produced diverse effects on signalling pathways related to proliferation and survival

The Ras signalling pathway is activated in LSL-Kras^{G12D}; Pdx-1/Cre mice, which leads to downstream activation of Akt. In Figure 4(a), we show that neither CCR nor ICR altered protein expression phosphorylation of Akt or basal levels of Akt. Akt regulates cell proliferation through its effects on mTOR and AMPK. Western-blot analysis of pancreas tissue indicated that phosphorylation of AMPK

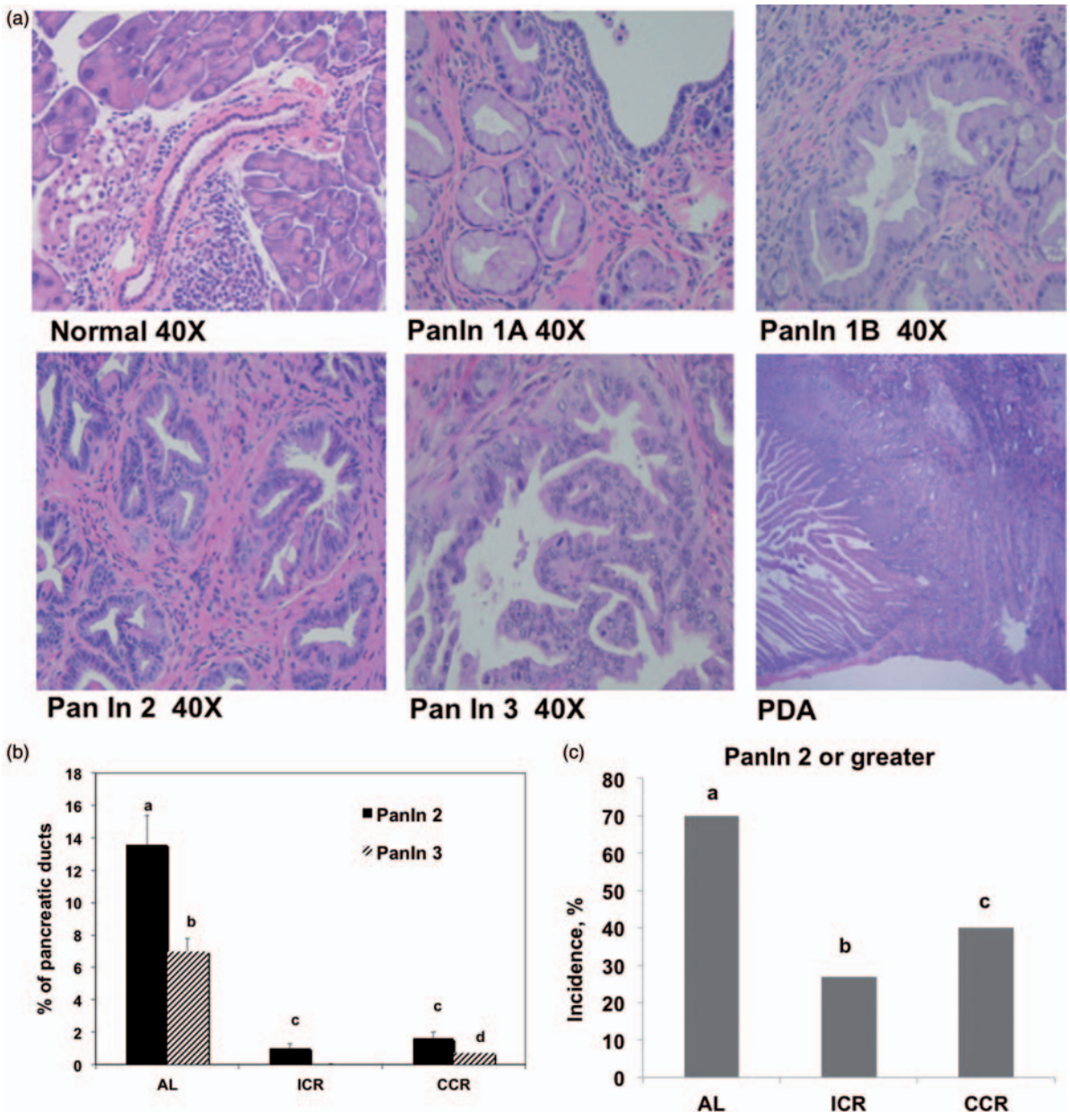

Figure 2 Pancreatic histopathology and assessment of lesions in $Kras^{G12D/+}$; Pdx-1Cre mice at 44 weeks of age. (a) Pathology: representative images show the progression of lesions from PanIN-1A to pancreatic ductal adenocarcinoma (PDA). (b) Quantitative analysis of PanIN lesions: The relative proportion of each lesion to the total number of analysed ducts was recorded for each mouse. Exact Poisson regression indicated that the AL group ($n = 11$) had a significantly higher percentage of PanIN-2 lesions than the restricted groups (ICR, $n = 16$ and CCR, $n = 15$), $P < 0.0001$ and there were no differences in the restricted group; for PanIN-3 lesions, all pairwise comparisons were significant, $P < 0.0001$ for AL vs. ICR and CCR and $P < 0.0094$ for ICR vs. CCR. (c) Incidence of PanIN-2 or greater lesions. The evaluation of lesions in AL, ICR, and CCR mice was based on H&E stained pancreas

was increased in the CCR-restricted mice but did not reach significance (Figure 4b). CCR decreased phosphorylation of mTOR in comparison to ICR and AL feeding ($P < 0.01$, Figure 4c). ICR did not alter phosphorylation of mTOR. Tumours cells increase glucose uptake by up-regulating Glut1 protein, which allows the energy-independent transport of glucose across the cell membrane. Figure 4(d) shows that CCR and ICR decreased protein expression of Glut-1 in the pancreas compared with AL fed mice as determined by Western analysis. Glut-1 protein expression relative to GAPDH decreased by 48% in the pancreas of ICR mice and 52% in the CCR mice in comparison to AL mice, $P < 0.05$. The silent regulator T1 (Sirt1) is induced by calorie restriction and may have a protective role in the anticancer effects of calorie restriction. We observed that both ICR and CCR increased protein expression of Sirt1 in the pancreas of $LSL\text{-}Kras^{G12D}$; Pdx-1/Cre mice compared with AL ($P < 0.05$). Sirt1 levels were significantly higher ($P > 0.05$) in the pancreas from ICR mice compared with those from the CCR-restricted mice (Figure 5).

CCR reduced serum IGF-1

A recent study has demonstrated that IGF-1 signalling mediates the protective effects of calorie restriction in the Br5.COX overexpression mouse model of inflammatory

Table 1 Average food intake (g/day) for AL-fed, ICR, and CCR$^{KrasG12D/+}$; Pdx-1Cre mice.[a]

Cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
AL	9.2 ± 0.3	9.0 ± 0.1	9.4 ± 0.3	9.2 ± 0.6	7.7 ± 0.3	8.3 ± 0.4	9.1 ± 0.1	9.4 ± 0.4	8.8 ± 0.5	8.4 ± 0.3	8.6 ± 0.5	8.5 ± 0.3	7.0 ± 0.2	9.3 ± 0.4	9.2 ± 0.3	8.5 ± 0.2	7.6 ± 0.3	8.8 ± 0.4	7.7 ± 0.1	8.0 ± 0.5	8.4 ± 0.2	8.5 ± 0.3
ICR	6.9 ± 0.5	7.0 ± 0.6	6.9 ± 0.5	6.9 ± 0.5	6.1 ± 0.5	5.9 ± 0.4	6.8 ± 0.3	7.0 ± 0.4	6.5 ± 0.3	6.1 ± 0.2	6.1 ± 0.4	6.4 ± 0.3	5.2 ± 0.3	6.8 ± 0.3	6.0 ± 0.2	6.4 ± 0.3	5.6 ± 0.3	6.6 ± 0.4	5.7 ± 0.2	6.0 ± 0.2	6.1 ± 0.2	6.4 ± 0.4
CCR	6.6 ± 0.2	6.9 ± 0.3	7.0 ± 0.3	6.9 ± 0.3	5.7 ± 0.1	6.1 ± 0.4	6.7 ± 0.3	6.9 ± 0.2	6.5 ± 0.2	6.1 ± 0.4	6.3 ± 0.4	6.3 ± 0.5	5.1 ± 0.5	6.7 ± 0.5	6.0 ± 0.4	6.3 ± 0.3	5.7 ± 0.4	6.4 ± 0.3	5.6 ± 0.6	5.9 ± 0.4	6.2 ± 0.4	6.3 ± 0.2

AL: ad libitum; ICR: intermittent calorie restriction; CCR: chronic calorie restriction.
[a]Values are presented as mean ±SEM. Data were analysed by ANOVA with Bonferroni-Dunn *post hoc* test. In each column AL group is significantly different from ICR and CCR ($P < 0.05$).

pancreatitis and dysplasia.[18] We demonstrated that CCR, but not ICR, significantly decreased serum levels of IGF-1, $P < 0.05$ (Table 3).

Calorie restriction decreased serum concentrations of glucose and leptin and increased adiponectin

Both types of calorie restriction significantly decreased circulating glucose concentrations compared with AL feeding, $P < 0.05$ (Table 3). Serum adiponectin and leptin are often deregulated in cancer; low circulating adiponectin and high serum leptin concentrations are associated with a poor prognosis.[19] ICR and CCR increased serum adiponectin concentrations and decreased serum leptin compared with the AL feeding, $P < 0.05$. CCR led to a greater reduction in serum leptin than ICR, $P < 0.01$ (Table 3).

Discussion

Earlier studies have demonstrated a protective effect of calorie restriction against the development of pancreatic tumours in chemically induced animal models[20–22] and in a transgenic mouse model of inflammatory pancreatitis.[18] However, this is the first study to demonstrate in LSL-Kras^{G12D}; Pdx-1/Cre transgenic mice that moderate calorie restriction prevents the development and progression of precursor lesions to PDA. This mouse model is clinically relevant because it simulates the slow progressive genetic and histological changes observed in human pancreatic cancer.[9] Importantly, this is the first study to demonstrate the protective effects of ICR in a mouse model of pancreatic cancer. We show that ICR was significantly more effective than CCR in reducing PanIN-2 and PanIN-3 lesions as well as decreasing the incidence of these advanced lesions. The pattern of weight loss of ICR mice differed from the pattern reported for transgenic mouse models of breast cancer,[12,13] which may be due to the use of both male and female mice in this study in contrast to the use of only female mice in the previous studies.[12,13] However, the protective effect was achieved in those studies and in ours, which suggests that multiple cycles of restriction/refeeding are important for achieving the protective effect. In this study, we also observed that calorie restriction reduced tumour formation compared with AL fed mice. In the initial paper that characterizes the K-ras; Pdx-1Cre mice, the investigators found that by 6.25 months of age over 3% of the mice displayed PDA with a mean survival of 15 months of age.[10] Since the authors of original manuscript indicate that there is variability in tumour burden in these mice,[9] it is reasonable that we found PDA in the 27% of AL fed K-ras; Pdx-1Cre mice at 11 months of age. A recent study showed that a 30% calorie restriction for 14 weeks decreased the extent of high-grade ductal lesions and reduced composite pathology scores in the Br5.COX overexpression mouse model of inflammatory pancreatitis and dysplasia.[18] This model differs from the K-ras; Pdx-1Cre mice used in our study in that lesions and PDA were not well defined and there was no evidence of K-ras mutations, which are found in over 90% of pancreatic tumours.[23,24] The latency time to tumour development also appears to be shorter (6–8 months) than that of the K-ras; Pdx-1Cre mice.[25] Calorie restriction also reduced tumour

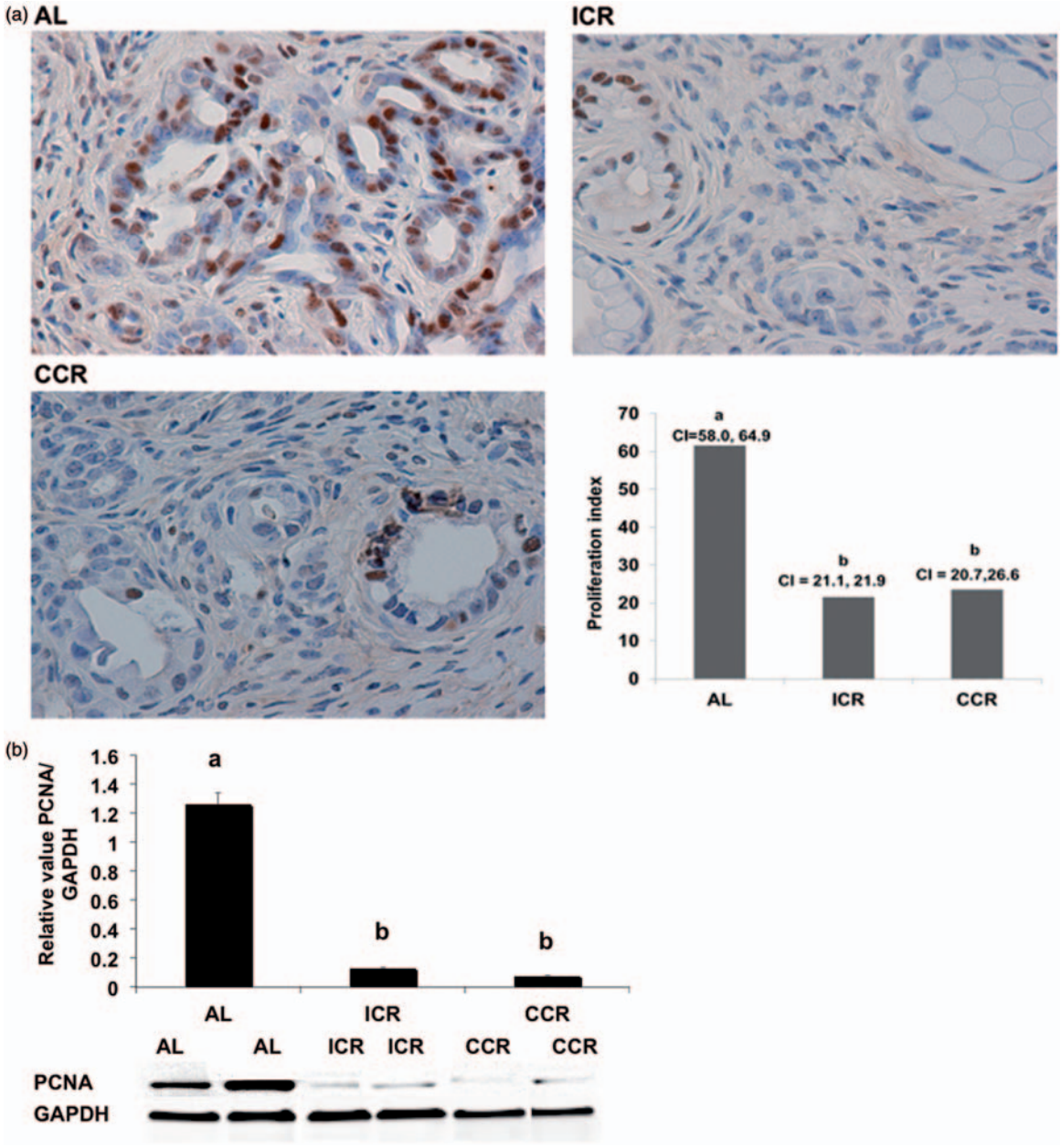

Figure 3 Calorie restriction decreased the ductal cellular proliferation in pancreas of LSL-Kras^{G12D}; Pdx-1/Cre mice. (a) Immunohistochemistry of PCNA staining in sections of pancreas tissue as described in the methods. Graph represents semi-quantitative analysis of PCNA staining with bars representing the means with confidence intervals (CI) for PCNA proliferation index that was calculated by dividing the number of PCNA stained ductal cells by the total number of ductal cells counted and expressed as a percent. Bars with different letters are significantly different, $P < 0.05$. (b) Representative Western blot of pancreas tissue lysates was immunoblotted for PCNA. Blots were reprobed with antibody to GAPDH to verify equal loading of protein. Blot data are from a single experiment that is representative of three independent experiments; three samples per group were analysed for each experiment. Bars are means ± SD of the scanning units derived from ImageJ analysis that were normalized to GAPDH. Bars ± SD with different letters are significantly different, $P < 0.05$

growth in a transplant model using cells derived the Br5.COX mice.[18] Earlier studies evaluated the effect of calorie restriction in animal models of chemically induced pancreatic cancer. One study conducted in 1981, found that CCR reduced the incidence of azaserine-induced pancreatic cancer in the rat.[20] In a subsequent study, this group found that meal feeding, resulting in 10–15% reduction in caloric intake, was also effective in reducing pancreatic cancer.[21] Another study reported that calorie restriction initiated after *N*-nitrosobis-2-(oxopropyl)amine (BOP) treatment did not alter incidence or tumour latency of BOP-induced pancreatic tumours in hamsters.[22] It was difficult to assess the effect of calorie restriction on incidence and latency in BOP-induced pancreatic tumours because of the low induction rate of pancreatic tumours.[22] Another study followed the effects of several levels of chronic calorie restriction over a two-year period on age-related changes in the rat pancreas.[26] CCR delayed the onset and decreased

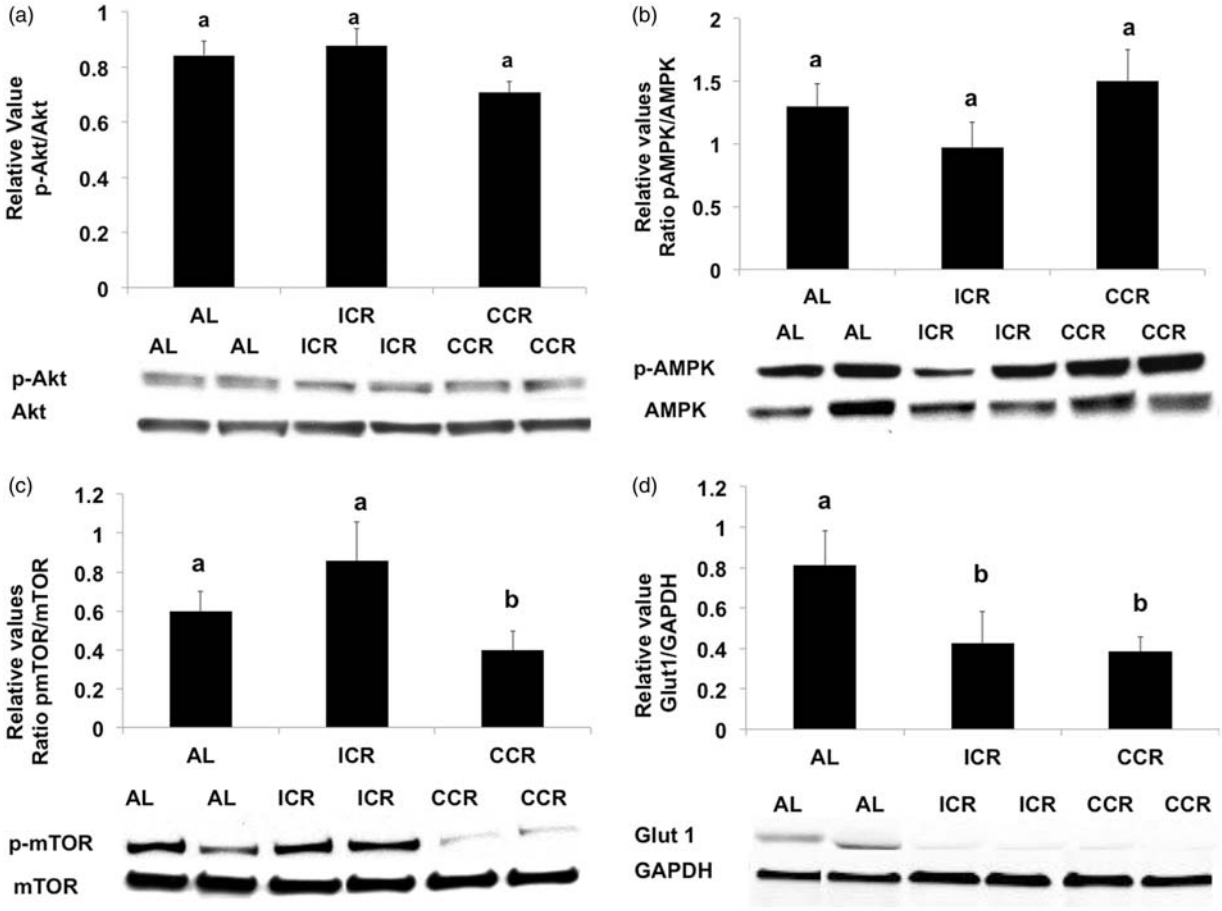

Figure 4 Effects of AL, ICR, and CCR on phosphorylation of AMPK, mTOR, Glut1, and Akt in pancreas of LSL-Kras^{G12D}; Pdx-1/Cre mice at 44 weeks of age. Levels of (a) Akt phosphorylation, (b) AMPK phosphorylation, (c) mTOR phosphorylation, and (d) Glut1. Pancreas tissue lysates from AL ($n = 11$), ICR ($n = 16$), and CCR ($n = 15$) Kras$^{G12D/+}$; Pdx-1Cre mice were subject to Western-blot analysis with antibodies to p-Akt, Akt, AMPK, AMPK, mTOR, p-mTOR, and Glut1. Blots were reprobed with antibody to GAPDH to verify equal loading of protein. Blot data are from a single experiment that is representative of three independent experiments. Graph represents semi-quantitative estimates of the amount of specific protein in the pancreas tissue. Bars are means ± SD of the scanning units derived from ImageJ analysis that were normalized to the unphosphorylated antibody. Bars ± SD with different letters are significantly different, $P < 0.05$

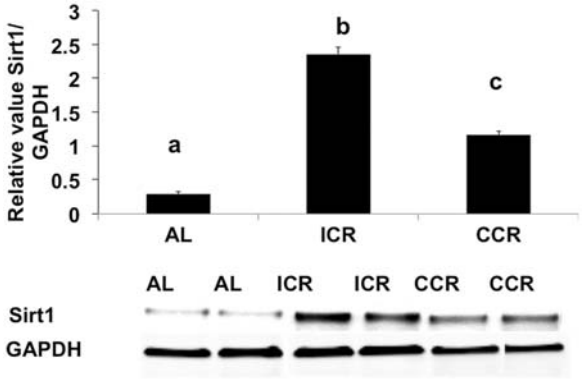

Figure 5 Calorie restriction decreases Sirt1 protein expression in the pancreas from LSL-Kras^{G12D}; Pdx-1/Cre mice. Pancreas tissue lysates were subject to Western-blot analysis with an antibody to Sirt1. Blots were reprobed with antibody to GAPDH to verify equal loading of protein. The blots are from a single experiment that is representative of three independent experiments. The graph represents semi-quantitative estimates of the amount of specific protein in the pancreas tissue. Bars are means ± SD of the scanning units derived from ImageJ analysis that were normalized to the GAPDH. Bars with different letters are significantly different, $P < 0.05$

the incidence and severity of degenerative changes in pancreatic islet tumours. Severe calorie restriction (50% of calories consumed during AL feeding) prevented these changes while moderate restriction (25% of AL caloric intake) delayed the onset of islet tumours. All of these studies are limited to observations in carcinogen-induced tumours.[26] With the availability of the K-ras; Pdx-1Cre transgenic mice we now have the opportunity to examine, in a clinically relevant model, the effects of calorie restriction on the progression of preneoplastic lesions to pancreatic tumours.

Mutations in K-ras result in continuous activation of downstream signalling proteins leading to increase in cell proliferation. Our observations show that calorie restriction decreased the PCNA proliferative index, which suggests that calorie restriction prevents the progression of 1A/1B lesions to advanced by inhibiting cell growth.

Serum IGF-1 is elevated in pancreatic cancer patients[27] and IGF-1 expression is increased in pancreatic tumours, which suggests that it is an important biomarker for increased risk.[28,29] A previous study observed that the

Table 2 Effects of ICR and CCR on body weights (BWs) and weights of pancreas and spleen

Dietary treatment	*N*	Body weights (g)	Pancreas weight (g)	Pancreas/BW (g)[a]	Spleen weight (g)	Spleen/BW (g)
AL	11	29.6 ± 1.4^{b}	0.66 ± 0.14^{b}	0.023 ± 0.007^{b}	0.38 ± 0.09^{b}	0.014 ± 0.004^{b}
ICR	15	21.7 ± 0.4^{b}	0.39 ± 0.05^{c}	0.018 ± 0.002^{c}	0.15 ± 0.04^{c}	0.006 ± 0.001^{c}
CCR	15	21.0 ± 0.5^{b}	0.36 ± 0.03^{c}	0.019 ± 0.002^{c}	0.24 ± 0.07^{d}	0.011 ± 0.002^{d}

AL: ad libitum; ICR: intermittent calorie restriction; CCR: chronic calorie restriction.
[a]Ratio of organ weight to body weight (BW). Values are means ± SD ($n = 15$).
[b]Superscripts with different letter are significantly different.
[c]Significant difference, $P < 0.05$.
[d]Significant difference, $P < 0.01$, ANOVA.

Table 3 Effect of calorie restriction on serum IGF-1, glucose, and adipokines[a]

	AL control	ICR	CCR
Glucose metabolism			
IGF-1, ng/mL	318.0 ± 16.7^{b}	284.1 ± 17.9^{b}	226.4 ± 12.2^{c}
Glucose, mmol/L	10.8 ± 0.9^{b}	7.1 ± 0.5^{c}	6.6 ± 0.6^{c}
Adipokines			
Adiponectin, μg/mL	7.1 ± 0.8^{b}	9.9 ± 0.6^{c}	9.7 ± 0.6^{c}
Leptin, ng/mL	3.7 ± 0.9^{b}	1.2 ± 0.2^{c}	0.9 ± 0.2^{d}

AL: ad libitum; ICR: intermittent calorie restriction; CCR: chronic calorie restriction.
[a]Data were analysed by one-way ANOVA with Bonferroni *post hoc* test. Values are means ± SEM, $n = 15$.
[b]Superscripts with different letter are significantly different.
[c]Significant difference, $P < 0.05$.
[d]Significant difference, $P < 0.01$.

protective effects of calorie restriction in the BK5.COX-2 mouse model were mediated, in part, by IGF-1. Findings from their study demonstrated that decreasing circulating IGF-1 by genetic reduction of IGF-1 resulted in reduced growth of pancreatic tumours.[18] Our data showing that CCR lowered serum IGF-1 concentration is consistent with the previous findings in CCR BK5. COX-2 mouse model. In contrast, we did not observe a change in circulating IGF-1 in the ICR K-ras; Pdx-1Cre mice, which is in contrast to previous studies showing that ICR reduced IGF-1 in mouse models of breast[30,31] and prostate cancer.[14]

The reduction in circulating IGF-1 in CCR was associated with decreased mTOR phosphorylation, which does not appear to be regulated by Akt as Akt phosphorylation was not altered by CCR or ICR (data not shown). In contrast to CCR, ICR did not alter serum concentrations of IGF-1, mTOR, or AMPK signalling, which indicates that these signalling proteins may not be mediators of the protective effects of ICR in reducing PanIN-2 and PanIN-3 lesions.

This is the first study to show that the reduction in proliferation and progression of PanIN lesions to PDA observed with ICR and CCR were associated with reduced protein expression of Glut1 in the pancreas. Glut1 expression is elevated in pancreatic cancer and has been shown to be associated with pancreatic invasiveness,[32] histological grade, and tumour size.[33] Glut1 accounts for the high uptake of glucose by malignant cells. Reductions in Glut1 protein expression by calorie restriction would decrease glucose availability and ultimately may result in decreased proliferation leading to delayed progression of lesions to PDA. Targeting Glut1 impaired growth of mouse mammary tumours[34] and mouse renal tumours.[35] A recent study demonstrated that silencing Glut1 inhibited cellular invasiveness and metastasis in pancreatic cancer cell lines grown as a xenograft models.[32] These findings suggest that Glut1 may be a target for the protective effect of both CCR and ICR against pancreatic cancer.

The role of Sirt1 in cancer is not well understood. Sirt1 is overexpressed in many cancers including prostate[36] and colon cancer.[37] We found that Sirt1 protein expression was increased in the pancreas from calorie-restricted mice with expression higher in the ICR mice than in the CCR mice. A key question is whether the increased Sirt1 protein expression has a role in the protective effects of calorie restriction against pancreatic cancer. A study using the Sirt1 transgenic mouse, with overexpression of Sirt1, demonstrated that Sirt1 protects against the development of intestinal tumours[37,38] and sarcomas and lymphoma.[39] Sirt1 transgenic mice were also protected against diabetes in diet-induced obesity[40,41] and hepatic steatosis.[41]

Serum adiponectin and leptin concentrations reflect the body's energy status and may have a role in the development of cancer. Circulating adiponectin is reduced in many cancers including pancreatic and is thought to be a predictive marker for increased risk.[19] Adiponectin levels have not been studied previously in calorie-restricted mouse models of pancreatic cancer. We show that the protective effects of CCR and ICR in reducing advanced lesions was associated with increased serum adiponectin in LSL-Kras^{G12D}; Pdx-1/Cre mice. Epidemiological studies indicate a correlation between decreased circulating leptin and increased prevalence of cancer.[42] We found that CCR and ICR reduced serum leptin levels. Leptin levels were also reduced in calorie-restricted mice bearing pancreatic tumour cells derived from the Br5.COX mice.[18,43] The increase in levels of adiponectin and decreased levels of leptin in the calorie-restricted LSL-Kras^{G12D}; Pdx-1/Cre mice were associated with a significant decrease in proliferation and advanced (PanIN-2 and PanIN-3) lesions. The decreased serum leptin levels observed in our study were also observed in the calorie restricted, BK5.COX-2[18] and the MMTV-TGF-α mouse model of breast cancer.[30,31]

Taken together, our findings show that both ICR and CCR were effective in delaying the progression of PanIN lesions to PDA and in reducing the incidence of PanIN-2 or greater lesions in LSL-Kras^{G12D}; Pdx-1/Cre mice.

Importantly, ICR had a stronger protective effect on the development of lesions in the LSL-Kras^{G12D}; Pdx-1/Cre mice than did CCR by completely preventing PanIN-3 lesions. Consistent with the delay in progression, PDA was not observed in either ICR or CCR LSL-Kras^{G12D}; Pdx-1/Cre mice whereas 27% (3/11) of the AL fed LSL-Kras^{G12D}; Pdx-1/Cre mice developed PDA. The reduction in PanIN-2 and PanIN-3 lesions observed in CCR and ICR mice was associated with decreased proliferation as measured by PCNA staining. This protective effect of both CCR and ICR led to decreased Glut1, increased Sirt1, increased serum levels of adiponectin, and reduced serum levels of leptin. This is the first report that calorie restriction alters Glut1, Sirt1, and adiponectin in an animal model of pancreatic cancer. The role of Glut1, Sirt1, and these adipokines in mediating the benefits of calorie restriction in pancreatic cancer needs to be explored. IGF-1 and mTOR signalling were altered by CCR but not ICR, which suggest that ICR is protecting against the development of advanced lesions by different mechanisms than CCR. In conclusion, our results demonstrate that CCR and ICR are effective strategies for slowing the progression of preneoplastic lesions to pancreatic neoplasia. These findings showing calorie restriction protects against pancreatic cancer now adds to the growing list of cancer types for which calorie restriction is protective.

Author contributions: All authors contributed to the design, interpretation of the studies, and analyses and review of the manuscript. GY, CL, and RH conducted the experiments, GR provided the breeders of the LSL-Kras^{G12D}; Pdx-1/Cre mice, JB conducted the pathological analyses.

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