The Report Committee for Drew Hays
Certifies that this is the approved version of the following report:

Energy Balance Modulation and Pancreatic Tumor Growth: The Role of NF-kB

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor:

Stephen D. Hursting

Laura Lashinger

Energy Balance Modulation and Pancreatic Tumor Growth: The Role of NF-kB

by

Drew Hays, B.S. Ntr.

Report

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

The University of Texas at Austin

December, 2012

Acknowledgements

I would like to first thank Dr Stephen D. Hursting for his support throughout my graduate studies. His encouragement, patience, and understanding have allowed me to find a career path in which I can use all of my skills and training, and for this I will forever be grateful.

I would also like to thank Dr. Laura Lashinger for her timeless commitment, and unwavering support. Her encouragement, fortitude, and occasional comic relief inspired me to continue when I felt lost or discouraged. Her scientific knowledge paired with her approachable mentoring style allowed me to grow immensely throughout my graduate studies. I would also like to express my gratitude to Dr. Sarah Dunlap for her support, sense of humor, scientific advice, and for pushing me to be a better graduate student and scientist, and Dr. Nikki Ford for being my nutritional confidant.

I would also like to sincerely thank Audrey Jean Rassmussen, and Lauren Malone Harrison as they were invaluable to my research. I am extremely grateful for their dedication to the studies, never-ending helpfulness, as well as their extraordinary attitudes, which made work quite enjoyable.

Finally, I am truly appreciative of the support network and collaborative spirit of the Hursting lab, as each member has impacted me personally and contributed to my current path.

Abstract

Energy Balance Modulation and Pancreatic Tumor Growth: The Role of NF-kB

Drew Hays, MA

The University of Texas at Austin, 2012

Supervisor: Stephen D. Hursting

Obesity is a known risk factor for many types of cancer including pancreatic. Calorie restriction (CR), an anti-obesity diet regimen, has potent anticancer effects that may be mediated through its ability to reduce serum metabolic hormones and protumorigenic cytokines such as insulin-like growth factor (IGF)-1. IGF-1 is a metabolic hormone responsive to nutrient status that activates the inflammatory, cancer-related pathway, nuclear factor (NF)-κB. For this report, we tested the hypothesis that CR, via regulation of IGF-1, inhibits pancreatic tumor cell growth through modulation of NF-kB activation and protumorigenic gene expression. Male athymic nude mice were randomized to either a control diet consumed ad libitum (n=15) or a 30% CR diet (n=15) for 17 weeks, at which time, mice were injected with human pancreatic cancer cells (MiaPaca) and tumor growth was monitored for 6 weeks. Translocation of p65, a regulatory element of NF-kB, and expression of its downstream gene targets were analyzed in excised tumors. CR mice

weighed less, ($p<0.05$), and had smaller tumors ($p=0.022$) relative to controls. Tumors from CR mice, relative to controls, demonstrated significant decreases in NF-kB downstream genes CCND1, RELA, Survivin, VEGF, and XIAP. These findings parallel our previous studies in pancreatic tumors from mouse origin, and suggest that the inhibitory effects of CR on MiaPaca pancreatic tumor growth are associated with decreased NF-kB activation.

Table of Contents

List of Figures

Chapter 1: INTRODUCTION

Pancreatic ductal adenocarcinoma is the 4th leading cause of cancer mortality in the U.S [1]. Only 4% of diagnosed individuals survive beyond 5 years. Because pancreatic cancer is generally diagnosed in an advanced stage, as a result of minimal symptoms and lack of palpable tumor, survival rate is extremely low. Although little is known regarding the etiology of pancreatic cancer, epidemiologic evidence has recently established a significant link to obesity [2]. Obesity is particularly problematic in that it creates an ideal environment for tumor development and progression, including,increased blood glucose and insulin resistance which in turn lead to dysregulation of the hormones insulin, leptin, and IGF-1 [3, 4]. Additionally, obesity induces a state of chronic inflammation, which is associated with cancer promoting gene expression [5]. The prevalence of obesity is rapidly increasing in the U.S. Currently, 33.8% of all American adults are obese, and 68% are overweight [6]. Overweight and obese individuals are at increased risk for a number of health issues including insulin resistance, leptin resistance, heart disease, stroke and some cancers [7]. It is estimated that 90,000 cancer-related deaths could be prevented per year with the maintenance of normal body weight [1]. Additionally, both men and women are at greater risk or mortality from all cancers when compared to those of normal weight despite whether or not they smoke cigarettes [8, 9]. Calle et al. stress the strong link between obesity and cancer risk, but the underlying mechanisms are not well understood.

As mentioned, obesity promotes the dyeregulation of cancer related hormones, specifically leptin, adiponectin, insulin, and IGF-1 [10]. Insulin is produced by the beta cells of the pancreas in response to a raise in blood glucose. Elevated circulating insulin due to feeding results in increased glucose uptake in tissues such as adipocytes and muscle and enhances energy production or storage. The peptide hormone, leptin, is secreted by the adipocytes in response to insulin, and acts as an indicator of peripheral fat mass which is crucial for the regulation of satiety. In an obese state, tissues become less responsive, or resistant to these hormones. Similarly, circulating free IGF-1 levels are positively correlated with body mass index (BMI). High IGF-1 levels are observed in prostate, breast, colon and pancreatic cancers [11], and treatment with IGF-1 results in cancer promoting events such as proliferation and increased cell survival in multiple cell lines in vitro [12-14].

Not only does obesity promote these hormonal responses, it also produces a state of chronic low-grade inflammation [3]. Links between chronic inflammation and cancer are effectively demonstrated by the 53 percent increased risk of pancreatic cancer development in individuals who suffer from chronic hereditary pancreatitis [11, 15, 16]. Transcription factor, NK-kB is activated by inflammation through transcriptional regulation of various cancer promoting genes including those responsible for angiogenesis, cell proliferation, cell cycle regulation, apoptosis, and inflammation. NF-kB is commonly upregulated in many cancers, including 70 percent of pancreatic cancer cell lines. Harvey et al. found that NF-kB is modulated in response to elevated IGF-1 in vitro as well as in an in vivo transplant model of colon cancer [12]. Proinflammatory

stimuli, such as increased IGF-1 levels resulting from obesity, can activate NF-kB by targeting the inhibitory subunit IkBα for ubiquitination, thus freeing NF-kB to translocate to the nucleus where it can transcribe these cancer promoting genes [17, 18].

Calorie Restriction (CR) has been shown to exert substantial anticancer effects in many models of cancer, in part through its regulation of circulating levels of insulin, leptin, IGF-1, and adiponectin. From our lab, Lashinger et al. and Harvey et al. showed that CR significantly decreased pancreatic tumor growth in both syngeneic transplant and transgenic models of pancreatic cancer [10, 12, 19]. It is established that CR works through a variety of ways [20]. Lashinger et al. suggested that CR is effective through reductions in IGF-1 [10], and furthermore, Harvey et al. suggest that manipulations of IGF-1 work partially through modulation of NF-kB. Harvey et al. additionally demonstrated significantly lower levels of p65 (NF-kB subunit) staining in colon cancer tumor sections of mice on a CR regimen, as well as decreased levels of macrophage markers, and NF−κB downstream genes [12]. This has not, however, been demonstrated in the MiaPaca human pancreatic cancer cell line. Thus we hypothesized that CR will inhibit pancreatic tumor cell growth as a result of diminished NF-kB activity leading to decreased expression of cancer promoting gene expression.

Chapter 2: MATERIALS AND METHODS

2.1 Animals and Diets

Male Athymic Nude mice were purchased from Jackson Laboratories, (Bar Harbor, ME) and placed on chow diet for one week following arrival. Mice, (n=15/group) were randomized to receive either a control diet (#D0302702, Research Diets, New Brunswick, NJ) or an isonutrient 30% CR diet (#D03022702), administered as a daily aliquot of 70% of the daily amount consumed by the control group, resulting in a lean phenotype. Animals were singly housed and received diet beginning at 7 weeks of age for 23 weeks. Food intake and body weights were recorded weekly throughout the duration of the study. The same protocol was followed for the preliminary Panc02 study.

2.2 MiaPAca Tumor Cell injections

At week 17 of diet treatment, mice were subcutaneously injected with 4,000,000 human pancreatic cancer cells (MiaPaca) in the right flank. Diet regimens were continued until sacrifice at week 23. Following tumor implantation, tumor dimensions were recorded following palpation with Vermeer calipers bi-weekly. Following 6 weeks of tumor growth, mice were sacrificed when the majority of the tumors reached 1.5 cm in diameter. Sacrifice was executed by CO_2 asphyxiation followed by cervical dislocation. Final tumor volume was calculated from 3 dimensions measured on excised tumors using the formula $4/3\pi((r1)(r2)(r3))$. Tumors were then flash frozen in liquid nitrogen, and stored at -80°C or fixed in 10% neutral-buffered formalin overnight then switched to ethanol until paraffin embedding. Prior to injection MiaPaca cells were maintained in incubation conditions as previously described in media solution of DMEM (Invitrogen,

Carlesbad CA), 10,000 U/mL penicillin, 10,000U/mL of streptomycin, 1X Non Essential Amino Acids, 10mM HEPES buffer, and 1mM sodium pyruvate. Injections were on the left flank of each animal. The same protocol was followed for the preliminary Panc02 study, but with male C57B6 mice injected with 100,000 cells.

2.3 Real-time PCR

Gene expression of RELA, VEGFA, Cyclin D1, XIAP, and Survivin were determined through PCR using TaqMan primer-probes (Ambion, Austin TX), and normalized to β-actin in MiaPaca tumors following RNA extraction and reverse transcription. Total RNA was extracted using the Qiagen RNeasy Mini Kit following the Qiagen protocol (Qaigen CA). 2 ug of RNA was reverse transcribed using a high capacity cDNA Reverse Transcription kit (Ambion, Austin TX). Analysis was performed using the delta-delta CT method.

2.4 Immunohistochemimical (IHC) Staining of phospho p65

Tissue blocks were deparaffinized in xylene and then rehydrated in ethanol to water for IHC analysis. Endogoenous peroxidase activity was blocked with 3% H_20_2 in water for 10 minutes, and then washed. Antigen retrieval was performed with 10mM citrate buffer for 3 minutes at 100% power, and 15 minutes at 50% power in a microwave, followed by cooling for 20 minutes. Biocare blocking reagent (Biocare, catalog #BS977M, BS966M, Concord, CA) was incubated with slides for inhibition of non-specific antibody. Slides were then incubated with primary phospo p65 antibody (#3033, Cell Signagling, Boston, MA, #sc 101749, Santa Cruz Biotechnology, Santa Cruz, CA (human)) at 1:100 dilution for 8 hours. Slides were incubated with Dako

EnVision labeled polymer, anti-rabbit-HRP (catalog #K400, Carpinteria, CA), Dako diaminobexidine and counterstained with hematoxylin. Assessment of nuclear versus cytoplasmic staining was conducted by 2 independent blinded observers.

2.5 Statistics

All values are shown as $\pm$ standard error of the mean unless otherwise noted. Statistical Package for the Social Sciences (SPSS) was used for statistical analysis. Differences in body weight over time between CR and control groups were assessed using repeated measured; differences in final tumor measures and gene expression were determined by independent t tests. $P<0.05$ is considered statistically significant.

Chapter 3: RESULTS

3.1 Preliminary Panc02 Data

Diet significantly affected pancreatic tumor growth in C57BL/6 mice. Measurements taken 4 weeks after injection showed that the CR group had smaller tumors compared to control (Figure 1A). This finding was confirmed by ex vivo measurements of final tumor volume (Figure 1B). Analysis of inflammatory gene expression showed tumors from CR mice, relative to controls (n=3 mice/group), displayed up to a 71% decrease in the inflammatory markers, S100a9 and F4/80 ($p<0.05$ for each; Figure 1C) and a 56% decrease in the macrophage chemoattractant Ccl2 (gene that codes for the protein, MCP-1) ($p<0.05$; Figure 1C).

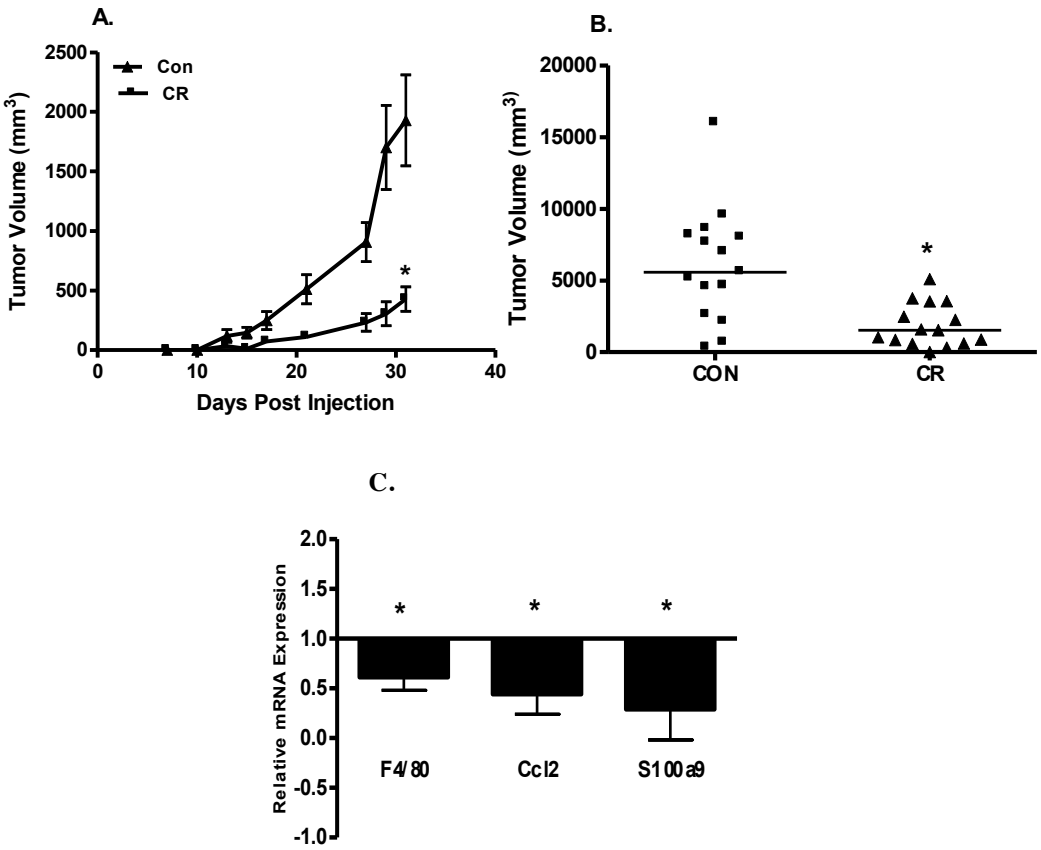

Figure 1: Effect of diet on Panc 02 tumor volume, and proinflammatory gene expression (Harvey dissertation, 2011)

Calorie restriction (CR) decreased tumor growth and overall median tumor volume compared to control-fed (CON) mice. (**A**) Average tumor volume in vivo, data shown mean $\pm$ SE, significance ($p<0.05$) between groups; (**B**) Median final tumor volume ex vivo, significance ($p<0.003$) between groups. (**C**) Calorie restriction (CR) modulates proinflammatory gene expression within the tumor microenvironment compared to control (CON) mice. All data represent CR gene expression relative to CON. Values are mean $\pm$ SE (n=3/group). Significance ($p<0.05$) between groups is denoted by *.

3.2 CR reduced body weight and tumor volume of male athymic mice

CR mice weighed significantly less than mice on control diet beginning at week three. An average 13g difference remained throughout the study ($p<0.05$; Fig. 8) (for clarity purposes body weight was graphed every 4 weeks).

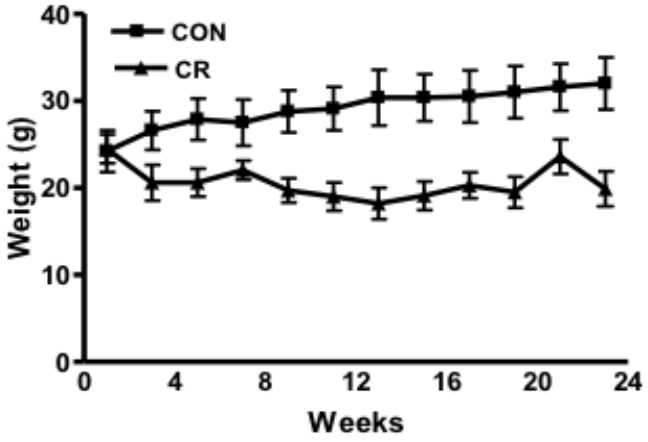

Figure 2: Effect of diet on body weight in male athymic nude mice

Calorie restriction (CR) decreased body weight and percent body fat relative to control (CON) ad libitum fed diet. Average body weight of mice fed a CR or CON diet (body weight graphed every 4 weeks for clarity purposes) All data shown are mean $\pm$ SD (n=15/group). Significance ($p<0.05$) is denoted by *.

3.3 CR Reduced MiaPaca Pancreatic Tumor Volume

Diet significantly affected pancreatic tumor growth in athymic nude mice. CR reduced tumor surface area as compared to control (data not shown). CR significantly reduced final tumor volume, measured ex vivo, relative to control mice (83 and 53 mm^3, respectively, $p=0.022$; Fig. 2).

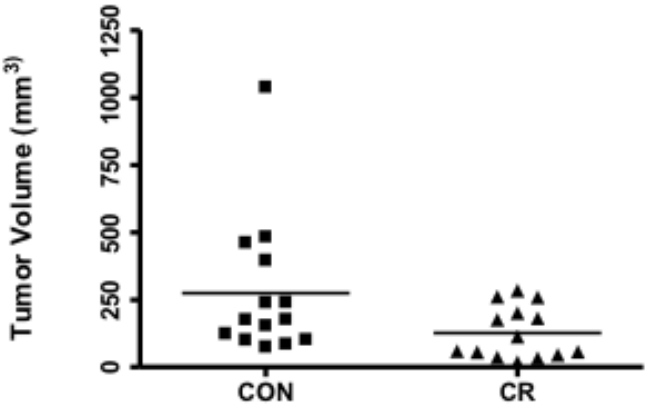

Figure 3: Effect of diet on MiaPaca tumor volume

The median final tumor volume ex vivo, was significantly smaller in the CR group compared to control (significance ($p<0.022$) between groups).

3.4 CR reduced p65 and inflammatory gene expression in MiaPaca tumors

Histological analysis of the tumors harvested from CR (n=8) and control (n=10) (samples limited to tissue availability) demonstrated a trend toward lesser nuclear p65 staining in control compared to CR tumors**,** but values were not statistically significant (p=.07)**.** The profile of inflammatory gene expression demonstrated that tumors from CR mice, relative to controls (n=6 mice/group), had significantly lower expression of NF-kB downstream genes Ccnd1 (1.8 fold decrease), Birc5 (8 fold decrease), VegfA (10 fold decrease), and Xiap (50 fold decrease). A Significant decrease in RelA (6 fold decrease),

the gene encoding for the p65 subunit was also observed. ($p<0.05$ for each; Fig. 3)

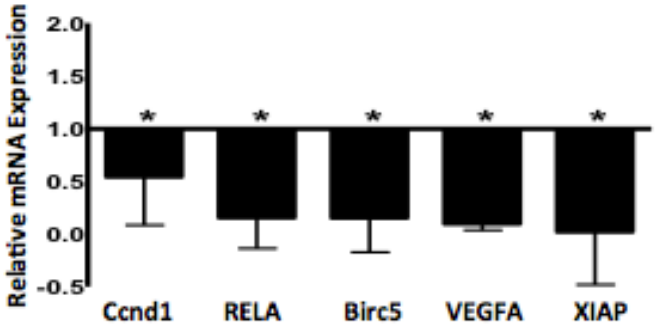

Figure 4: Effect of diet on NF-kB downstream genes in MiaPaca Tumors

Calorie restriction (CR) modulated downstream gene expression within the tumor microenvironment compared to control (CON) mice. All data represents CR gene expression relative to CON. Values are mean $\pm$ SE (n=6/group). Significance ($p<0.05$) between groups is denoted by *.

Chapter 4: DISCUSSION

It is well established that CR can effectively decrease inflammatory signals. Moreover, the links between CR, systemic hormones, and NF-kB, have been demonstrated in our lab in a transplant model of colon cancer. Tumors from CR mice demonstrated decreased expression of the proinflammatory cytokines, IL-6 and IL-1B and TNF-a, and the inflammatory enzyme COX-2; and increased the antiinflammatory enzyme Hpgd. Nuclear localization of the p65 subunit was also elevated following treatment with IGF-1 [14]. In preliminary studies in the mouse pancreatic cancer cell line Panc02, it was found that CR, relative to control fed mice, significantly reduced body weight, body fat, and effectively modulated serum hormones. Additionally, treatment with IGF-1 increased cell viability and nuclear translocation of NF-kB. On the other hand, silencing NF-kB activity with siRNA led to decreased expression of Cyclin D1, VegfA, Birc5, and Cox-2. Though CR has been shown to inhibit the expression of inflammatory markers [3, 21, 22], body weight, and tumor volume and growth, this had not previously been demonstrated using pancreatic cancer models.

In order to determine that the observed effects of CR are maintained in a human pancreatic cancer cell line, the MiaPaca cell line was used for this study. Paralleling our previous study of Panc02 transplant tumors, CR indeed led to lower body weight, and final tumor volume compared to mice on control diet. Similarly, decreased expression of the p65 subunit and NF-kB downstream genes was observed in tumor sections excised from these mice. NF-kB subunit, p65 is phosphorylated when fully activated, and analysis of tumor sections revealed a trend of less phospho-p65 in the nucleus in CR

tumor sections compared to sections taken from CR tumors. Though not statistically significant, these findings suggest that the CR decreases nuclear translocation of the p65 subunit, and thus minimizes NF-kB-induced gene transcription of proinflammatory mediators.

This research further defined our lab's investigation of the mechanisms through which CR reduces tumor burden [20]. We have demonstrated this in transgenic as well as several transplant models in the breast and the pancreas [10, 23-25]. Calorie restriction is a multifaceted intervention, and as low-grade, chronic inflammation becomes a better-understood player in tumorgenesis and progression, this research becomes increasingly important in narrowing our understanding. Pancreatitis is an established risk factor for pancreatic cancer, and highlights the need for development of early intervention targeting the inflammatory state for prevention [26-28]. In conclusion, this study shows that CR, and subsequent reductions in IGF-1, inhibits growth in both human and mouse pancreatic tumors, in part, though reduction in NF-κB-induced proinflammatory gene expression (Figure 5). Future studies will investigate the effects of obesity reversal, and the effects on final tumor burden in comparison to animals maintained on consistent diet intervention.

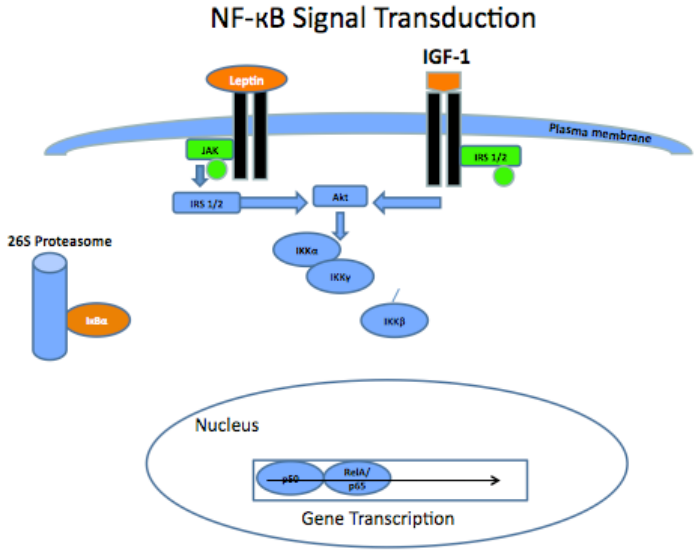

Figure 5: Model of IGF-1 induced chances in NF-kB signaling

In response to stimuli, such as elevated circulating IGF-1, IKKb activation targets 26S proteasomal degradation of IkBa though phosphorylation of IkBa. Degradation of IkBa relieves the cytosolic sequestration of NF-kB subunits and allows for nuclear translocation and subsequent NF-kB-induced gene transcription.

Chapter 5: BIBLIOGRAPHY

1. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. *New England Journal of Medicine* 2003, 348(17):1625-1638.
2. Raimondi S, Maisonneuve P, Lowenfels AB: Epidemiology of pancreatic cancer: an overview. *Nat Rev Gastroenterol Hepatol* 2009, 6(12):699-708.
3. Harvey AE, Lashinger LM, Hursting SD: The growing challenge of obesity and cancer: an inflammatory issue. *Ann N Y Acad Sci* 2011, 1229:45-52.
4. Hursting SD, Lashinger LM, Colbert LH, Rogers CJ, Wheatley KW, Nunez NP, Mahabir S, Barrett JC, Forman MR, Perkins SN: Energy balance and carcinogenesis: underlying pathways and targets for intervention. *Curr Cancer Drug Targets* 2007, 7(5):484-491.
5. Ye J, Keller JN: Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction. *Aging (Albany NY)* 2010, 2(6):361-368.
6. Flegal KM, Carroll MD, Kit BK, Ogden CL: Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA* 2012, 307(5):491-497.
7. Flegal KM: Excess deaths associated with obesity: cause and effect. *Int J Obes (Lond)* 2006, 30(8):1171-1172.
8. Raimondi S, Maisonneuve P, Lohr JM, Lowenfels AB: Early onset pancreatic cancer: evidence of a major role for smoking and genetic factors. *Cancer Epidemiol Biomarkers Prev* 2007, 16(9):1894-1897.
9. Flegal KM: The effects of changes in smoking prevalence on obesity prevalence in the United States. *Am J Public Health* 2007, 97(8):1510-1514.
10. Lashinger LM, Malone LM, McArthur MJ, Goldberg JA, Daniels EA, Pavone A, Colby JK, Smith NC, Perkins SN, Fischer SM *et al*: Genetic reduction of insulin-like growth factor-1 mimics the anticancer effects of calorie restriction on cyclooxygenase-2-driven pancreatic neoplasia. *Cancer Prev Res (Phila)* 2011, 4(7):1030-1040.
11. Coussens LM, Werb Z: Inflammation and cancer. *Nature* 2002, 420(6917):860-867.
12. Harvey AE, Lashinger LM, Otto G, Nunez NP, Hursting SD: Decreased systemic IGF-1 in response to calorie restriction modulates murine tumor cell growth, nuclear factor-kappaB activation, and inflammation-related gene expression. *Mol Carcinog* 2012.
13. Suganthi M, Sangeetha G, Benson CS, Babu SD, Sathyavathy A, Ramadoss S, Ravi Sankar B: In vitro mechanisms involved in the regulation of cell survival by lithium chloride and IGF-1 in human hormone-dependent breast cancer cells (MCF-7). *Toxicol Lett* 2012, 214(2):182-191.

14. Roberts CT, Jr.: IGF-1 and prostate cancer. *Novartis Found Symp* 2004, 262:193-199; discussion 199-204, 265-198.
15. Guerra C, Collado M, Navas C, Schuhmacher AJ, Hernandez-Porras I, Canamero M, Rodriguez-Justo M, Serrano M, Barbacid M: Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence. *Cancer Cell* 2011, 19(6):728-739.
16. Dite P, Hermanova M, Trna J, Novotny I, Ruzicka M, Liberda M, Bartkova A: The role of chronic inflammation: chronic pancreatitis as a risk factor of pancreatic cancer. *Dig Dis* 2012, 30(3):277-283.
17. Baker RG, Hayden MS, Ghosh S: NF-kappaB, inflammation, and metabolic disease. *Cell Metab* 2011, 13(1):11-22.
18. Basseres DS, Baldwin AS: Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* 2006, 25(51):6817-6830.
19. Lashinger LM, Malone LM, Brown GW, Daniels EA, Goldberg JA, Otto G, Fischer SM, Hursting SD: Rapamycin partially mimics the anticancer effects of calorie restriction in a murine model of pancreatic cancer. *Cancer Prev Res (Phila)* 2011, 4(7):1041-1051.
20. Hursting SD, Smith SM, Lashinger LM, Harvey AE, Perkins SN: Calories and carcinogenesis: lessons learned from 30 years of calorie restriction research. *Carcinogenesis* 2010, 31(1):83-89.
21. Vazzana N, Riondino S, Toto V, Guadagni F, Roselli M, Davi G, Ferroni P: Obesity-driven inflammation and colorectal cancer. *Curr Med Chem* 2012.
22. Maccio A, Madeddu C: Obesity, inflammation, and postmenopausal breast cancer: therapeutic implications. *ScientificWorldJournal* 2011, 11:2020-2036.
23. Perkins SN, Hursting SD, Phang JM, Haines DC: Calorie restriction reduces ulcerative dermatitis and infection-related mortality in p53-deficient and wild-type mice. *J Invest Dermatol* 1998, 111(2):292-296.
24. Hursting SD, Perkins SN, Brown CC, Haines DC, Phang JM: Calorie restriction induces a p53-independent delay of spontaneous carcinogenesis in p53-deficient and wild-type mice. *Cancer research* 1997, 57(14):2843-2846.
25. Hursting SD, Perkins SN, Phang JM: Calorie restriction delays spontaneous tumorigenesis in p53-knockout transgenic mice. *Proc Natl Acad Sci U S A* 1994, 91(15):7036-7040.
26. Lowenfels AB, Maisonneuve P, Lankisch PG: Chronic pancreatitis and other risk factors for pancreatic cancer. *Gastroenterol Clin North Am* 1999, 28(3):673-685, x.
27. Talamini G, Bassi C, Falconi M, Sartori N, Salvia R, Rigo L, Castagnini A, Di Francesco V, Frulloni L, Bovo P *et al*: Alcohol and smoking as risk factors in chronic pancreatitis and pancreatic cancer. *Dig Dis Sci* 1999, 44(7):1303-1311.
28. Gullo L, Pezzilli R: Pancreatitis and the risk of pancreatic cancer. *Pancreas* 1997, 14(1):105-107.