Published in final edited form as:

Cancer Prev Res (Phila). 2011 December ; 4(12): 2002–2014. doi:10.1158/1940-6207.CAPR-11-0182.

Dietary Energy Balance Modulates Prostate Cancer Progression in Hi-Myc Mice

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Abstract

Male Hi-Myc mice were placed on three dietary regimens [30% calorie restriction (CR), overweight control (modified AIN76A with 10kcal% fat), and a diet-induced obesity regimen (DIO) 60kcal% fat]. All diet groups had approximately similar incidence of hyperplasia and low grade PIN in the ventral prostate at 3 and 6 months of age. However, 30% CR significantly reduced the incidence of in situ adenocarcinomas at 3 months compared to the DIO group and at 6 months compared to both the overweight control and DIO groups. Furthermore, the DIO regimen significantly increased the incidence of adenocarcinoma with aggressive stromal invasion, as compared to the overweight control group (96% vs. 65% respectively, $p=0.02$) at the 6 month time point. Additionally, at both 3 and 6 months, only in situ carcinomas were observed in mice maintained on the 30% CR diet. Relative to overweight control, DIO increased, while 30% CR reduced activation of Akt, mTORC1, Stat3 and NF κ B (p65) in ventral prostate. DIO also significantly increased (and 30% CR decreased) numbers of T-lymphocytes and macrophages in the ventral prostate compared to overweight control. The mRNA levels for IL1 α , IL1 β , IL6, IL7, IL23, IL27, NF κ B1 (p50), TNF α and VEGF family members were significantly increased in the ventral prostate of the DIO group compared to both the overweight control and 30% CR diet groups. Collectively, these findings suggest that enhanced growth factor (Akt/mTORC1 and Stat3) and inflammatory (NF κ B, cytokines) signaling may play a role in dietary energy balance effects on prostate cancer progression in Hi-Myc mice.

Introduction

Obesity is a long-term consequence of energy imbalance that occurs when energy intake exceeds expenditure. Although data associating obesity and prostate cancer risk have been

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inconclusive (1, 2), studies have shown increased risk of biochemical failure and metastasis, as well as poorer survival among obese prostate cancer patients with androgen-dependent tumors, especially those who experienced rapid weight gain (3). Additionally, obesity has been shown to be associated with more aggressive tumors and adverse outcome, including mortality (1, 4–7). Waist-to-hip ratio (an indicator of body fat distribution) has also been positively correlated with other hormone-dependent cancers (8, 9). Men with low-volume prostate cancer have a lower BMI, less body fat, and a smaller waist-to-hip ratio than men with high-volume prostate cancer, which agrees with other reported findings (10, 11). Several biologic mechanisms have been postulated to explain the association between obesity and aggressive disease, including increases in circulating levels of growth factors (i.e., IGF-1 and leptin), hyperinsulinemia, and increased systemic and tissue inflammation (12–14)

Epidemiologic evaluations of the independent role of energy intake in prostate cancer incidence, disease progression or mortality are more limited. High total energy intake, independent of BMI, was associated with a statistically significant increased risk of fatal prostate cancer in the Health Professionals Follow-Up Study cohort (15). Similarly, energy intake was significantly higher among cases than controls in the UK multicenter, population-based case control study of incident prostate cancer; however, risk analyses were not computed (16). Fat, the most calorically dense component of total energy intake, has been the focus of most prostate cancer dietary epidemiologic investigations. High consumption of fat, especially saturated fat (17–19) is associated with advanced stage prostate cancer and mortality (17, 20). Several biologic mechanisms, similar to those of obesity per se, have been postulated to explain the role of energy intake and/or fat consumption in prostate cancer progression.

In the current study, we have used the Hi-Myc transgenic mouse model (21) of prostate cancer to explore the effects of dietary energy balance manipulation, including a standard diet-induced obesity (DIO) regimen, a control diet regimen that results in an overweight phenotype, and a calorie restriction (CR) regimen that leads to a lean phenotype, on prostate cancer progression. Given that approximately 68% of the adult population in the US is currently overweight (22), a comparison of the effects and underlying mechanisms of a lean, overweight and obese phenotype on prostate cancer progression was therefore evaluated. In the Hi-Myc transgenic mouse model, over-expression of c-Myc in the prostate is directed via the ARR₂Pb probasin promoter (21). Prostatic epithelial expression of c-Myc in the dorsolateral prostate (DLP), ventral prostate (VP), and anterior prostate (AP) lobes reportedly results in complete penetrance of prostatic intraepithelial neoplasia (PIN) as early as 2 to 4 weeks of age, which progressed to locally invasive adenocarcinomas within 6 to 12 months of age (21). Recently, Kobayashi *et al.* (23) showed that if Hi-Myc mice were maintained on a low fat (LF, 12 Kcal%) versus a high fat (42 Kcal% fat) isocaloric diet, the transition from PIN to prostate cancer was delayed. This delay was associated with a decrease in prostatic Akt activity as well as a decrease in phosphorylation of the downstream targets p70S6K and GSK3 β . More recently, increased NF κ B signaling also was reported to play a possible role in prostate cancer progression in the Hi-Myc model (24). The current study demonstrates that, relative to a control diet regimen that results in overweight mice, a

DIO regimen significantly enhances the progression of prostate cancer in Hi-Myc mice whereas a 30% CR regimen significantly delays prostate cancer progression. Further studies indicated that the modulating effects of both DIO and CR (relative to the overweight control diet regimen) on prostate cancer progression in Hi-Myc mice were associated with alterations in Akt/mTORC1, Stat3 and NF κ B signaling pathways as well as downstream inflammation associated signaling pathways. The current data in HiMyc mice support the hypothesis that dietary energy balance modulates signaling through multiple pathways leading to altered prostate cancer progression.

Materials and Methods

Study Design

Hi-Myc mice were obtained from the NIH MMRRRC on a FVB/N genetic background. All mice for the current experiments were bred in house. All diets were purchased from Research Diets, Inc (New Brunswick, NJ). Mice were placed on a modified AIN-76A semipurified diet, catalog #D12450B, fed ad libitum) at 5–7 weeks of age for a 1-week equilibration period and then randomized into three dietary groups for the duration of the study: (a) 30% CR diet (D03020702); (b) overweight control diet (continued on the modified AIN76A diet, # D12450B, 10kcal% fat), fed ad libitum; and (c) DIO diet (60kcal% fat; #D12492), fed ad libitum as previously described (25). The diet composition is shown in Supplemental Table 1. When fed in aliquots equivalent to 70% of the overweight control group's average daily intake, the CR diet (which was 14kcal% fat, 29kcal% protein and 57 kcal% carbohydrate and supplemented with additional vitamins and minerals), provided 100% of the vitamins, minerals, fatty acids, and amino acids but only 70% of the carbohydrate calories relative to the overweight control group (which is 10kcal% fat and 70 kcal% carbohydrate). The overweight control, 30% CR and DIO diets are commonly used to induce a range of body size phenotypes in rodents (26–28).

Average body mass and food consumption were determined bi-monthly for each dietary group. Groups of mice were terminated by CO₂ asphyxiation and the complete urinary tract was taken at 3 and 6 months of age for histopathological diagnosis and immunohistochemical analyses. An additional set of mice from each group was used for protein and RNA analyses (6 month time point only). For all the studies, mice were housed in suspended polycarbonate cages or individually ventilated cages (Lab Products, Maywood, NJ) on autoclaved hardwood bedding at room temperatures of 20–22°C, relative humidity of 60–70% and 14/10-hour light/dark cycle.

Histological and immunohistochemical (IHC) analyses

For histological analyses, the male reproductive tract was removed intact from Hi-Myc mice at 3 and 6 months of age, fixed in 10% formalin, embedded in paraffin and transversely sectioned. Sections of 4- μ m were stained with hematoxylin and eosin (H&E) for histopathologic diagnosis. All histopathologic diagnoses of prostate lesions were based on published criteria (29–31). Hi-Myc transgenic mice used for the current study develop prostate lesions starting from PIN at three months of age or younger which progresses to invasive tumors by 6 months of age (21). Under our experimental conditions, Hi-Myc mice

developed lesions primarily in the ventral and the dorsolateral prostate, with fewer lesions in the anterior prostate. Furthermore, at six months of age more than 90% of the mice developed invasive tumors primarily in the ventral prostate. In contrast, at the same time point, the number of invasive tumors in the dorsolateral and anterior prostate was significantly lower. Lesions in the dorsolateral prostate showed a more variable consistency in the number of hyperplasia and PINs as well. Thus, we focused our analyses of diet effects on the the ventral prostate gland which displayed a more homogeneous and consistent development of the lesions from hyperplasia to invasive adenocarcinoma within the 6 months time frame of our experiments.

IHC analyses were performed on paraffin-embedded prostate tissue sections. Primary antibodies (all from Cell Signaling Technology, Danvers, MA) were used to detect phospho-Akt (Ser⁴⁷³, 1:50), phospho-mTOR (Ser²⁴⁴⁸, 1:50), phospho-S6 ribosomal (Ser^{235/236}), phospho-Stat3 (Tyr⁷⁰⁵), phospho-NFκB (p65, Ser²⁷⁶, 1:100), Cyclin D1 (DCS6, 1:100). Additional antibodies were used to detect the following markers: CD31 (1:100, Pharmingen Clone MEC 13.3), Ki67 (1:50, Santa Cruz), Macrophages (RM0029-11H3, 1:200, Santa Cruz) and T-Lymphocytes, (CD3, 1:100, Serotec). Antibodies were detected with biotinylated secondary antibodies, followed by peroxidase-conjugated avidin/biotin (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and DAB substrate (Dako, Carpinteria, CA). All IHC slides were scanned and digitalized using a scanscope system (Scanscope XT, Aperio Technologies, Vista, CA) and quantitative analyses of IHC staining was performed using the image analysis software provided (ImageScope).

Western blot analyses

Individual prostate lobes were excised and homogenized in lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton x-100, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. Fifty μg of cell lysates were electrophoretically separated on a 7–10% SDS-PAGE gel and transferred onto nitrocellulose membrane (BioRad Laboratories, Hercules, CA). Transfer was performed at 20V overnight. After blocking with 5% skim milk in PBS containing 0.1% Tween, the membranes were incubated with antibodies as follows: Akt (1:1000), phospho-Akt (1:500 Thr³⁰⁸ and Ser⁴⁷³), mTOR (1:1000), phospho mTOR (Ser²⁴⁴⁸, 1:1000), phospho-S6 ribosomal protein (Ser^{235/236}, Ser^{240/244}, 1:1000) 4EB-P1 (1:1000), phospho-4EB-P1 (Thr^{37/46}, 1:1000), phospho-GSK3β (Ser⁹, 1:1000), phospho-FOXO3a (Ser²³⁵, 1:1000), Cyclin D1 (1:1000), c-Myc (1:1000), Stat3 (1:1000) and phospho-Stat3 (Tyr⁷⁰⁵, 1:1000). All of these antibodies for Western blot analyses were obtained from Cell Signaling Technology (Beverly, MA). β-actin was used as a loading control (mouse monoclonal, 1:4000, Sigma-Aldrich). Protein bands were detected using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL). Quantitation was performed with a G-Box system (Syngene, Frederick, MD) using the Genetool quantitation software.

The relative density of each protein band was normalized to the density of the corresponding β-actin band and where possible, phosphorylated proteins were presented as the ratio of phosphorylated/total protein.

Serum analyses

Blood was collected by cardiac puncture immediately following CO₂ asphyxiation (12 mice per diet) and spun at 7,500 rpm for 7 min. Serum was then collected, flash frozen in liquid nitrogen and stored at -80°C until analysis. Total mouse serum IGF-1 concentration was then measured using a 25 µL sample with a RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX). Levels of insulin, leptin and resistin were measured using a 10 µL sample with a Milliplex MAP Mouse Serum Adipokine panel multiplexing Luminex assay (Millipore, Billerica, MA). Adiponectin levels were determined using a 10 µL sample with a Milliplex MAP Mouse Adiponectin single plex Luminex assay (Millipore).

RNA isolation

Total RNA from the ventral prostate was extracted using a Qiagen RNeasy Protect Mini kit according to the manufacturer's protocol. The RNA was eluted in 100 µL of nuclease free water and stored at -80°C until further use. The RNA concentration was determined from the absorbance at a wavelength of 260 nm (by using an OD₂₆₀ unit equivalent to 40 µg/mL of RNA).

Reverse transcriptase reaction

RNA was analyzed for integrity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA). Total RNA (1 µg) was then used as template to synthesize cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The resultant cDNA was stored at -80°C until further use. qPCR was subsequently performed on the ABI 7900HT Fast Real Time PCR System (ABI) with assays on demand (AODs) (specific to the relevant genes of interest). RNA levels were normalized to the endogenous control gene, GAPDH, by using the ABI Mouse GAPDH Endogenous Control kit (ABI, cat. no. 4352339E). Data analysis was performed using Sequence Detection System software from ABI, version 2.2.2. The experimental Ct (cycle threshold) was calibrated against the GAPDH control product. All amplifications were performed in duplicate. The ddCt method was used to determine the amount of product relative to that expressed by (calibrator sample)-derived RNA (1-fold, 100%).

Statistical Analyses

Differences in levels of serum markers (Table 1) were analyzed using the Wilcoxon rank sum test. Difference in the incidence of prostate lesions (Supplemental Table 1 and Figure 2) were analyzed using Fisher's exact test. Differences in sub-classification of the adenocarcinomas (Supplemental Table 2 and Figure 2) were also analyzed using Fisher's exact test. Quantitative differences in phosphorylation and mRNA levels were also evaluated using the Wilcoxon Rank Sum test. Statistical analyses on the quantification of the IHC (Supplemental Figure, 1 panels A and B), was performed using Wilcoxon Rank Sum test. Significance was set in all cases at P 0.05.

Results

Body weights, body fat and serum levels of selected hormones and adipokines

Table 1 shows the average body weights, percent body fat and serum profile of Hi-myc mice maintained on a 30% CR, overweight control or DIO diet for 6 months. Weight distribution did not differ at the start of the study; however, the differences in body weight (i.e., body mass) among the three groups at the end of the study were statistically significant ($p < 0.05$). These values directly correlate with our previously published data obtained from FVB/N mice maintained on the same dietary regimens (25). In addition, previous studies using the same diet composition and feeding regimen used here have classified mice with body fat content values that correspond to those reported in Table 1 as lean, overweight and obese (32, 33). Thus, Hi-Myc (FVB/N genetic background) mice effectively respond to these dietary manipulations, producing similar body phenotypes as has been observed in previous studies.

Feed and calorie consumption was significantly reduced in the 30% CR group. In addition, caloric consumption was significantly higher in the DIO group, as compared to the overweight control diet group (147.2 kcal/week versus 114.7 kcal/week, respectively). The levels of circulating insulin, IGF-1, and resistin were significantly different among all three diet groups ($p < 0.05$). The levels of leptin were similar between the overweight control and DIO diet groups but significantly reduced ($p < 0.05$) in the 30% CR group. Finally, a significant increase in adiponectin level was observed in the 30% CR group relative to both the overweight control and DIO diet groups ($p < 0.05$).

Impact of dietary energy balance on prostate cancer progression in Hi-Myc mice

The effects of the three diets varying in caloric density on prostate cancer progression were assessed in Hi-Myc mice at 3 and 6 months of age. For these experiments, male mice (approximately 5–7 weeks of age) were maintained on the previously described dietary regimens for either 8 or 25 weeks. As noted in the Materials and Methods section, dietary energy balance effects were evaluated in the ventral prostate due to the greater variability and lower penetrance of lesions in the dorsolateral and anterior prostate lobes of Hi-Myc under the current experimental conditions. Histopathologic evaluations revealed that a variety of lesions were present at both 3 and 6 months of age including hyperplasia, PIN, and adenocarcinomas (Figure 1). In this regard, all diet groups had approximately similar incidence of hyperplasia and low grade PIN (lgPIN) at both 3 and 6 months of age (Figures 2A and 2B and Supplemental Table 2). Dietary energy balance, however, affected the progression of premalignant lesions to malignant lesions in the ventral prostate. In this regard, 30% CR significantly reduced the incidence of in situ adenocarcinomas at 3 months of age compared to Hi-Myc mice maintained on the DIO diet. Furthermore, the incidence of in situ adenocarcinomas at 6 months of age was significantly reduced by 30% CR compared to both the overweight control and DIO groups. Additionally, the incidence of adenocarcinomas with aggressive stromal invasion was increased in the DIO group relative to the overweight control group at both the 3 and 6 month timepoints; however, statistically significant increases were only observed at the 6 month timepoint (96% vs. 65% respectively, $p = 0.02$; Figure 2B). Notably, 30% CR completely suppressed the formation of

invasive adenocarcinomas at both 3 and 6 months (see Figures 2A, 2B and Supplemental Table 2).

The severity of invasive adenocarcinomas was also evaluated based on differentiation characteristics at both 3 and 6 months using established criteria (31). These results are summarized in Figure 2 and Supplemental Table 3. At 3 months of age, mice on the overweight control diet developed only well differentiated adenocarcinomas. In contrast, mice on the DIO diet developed a significant number of moderately differentiated adenocarcinomas. Furthermore, at 6 months of age, both the overweight control and DIO diet groups had poorly differentiated adenocarcinomas, with a significantly greater ($p=0.02$) incidence observed in the DIO diet group compared to the overweight control diet group. Notably, there were no invasive adenocarcinomas in the 30% CR diet group. Thus, dietary energy balance significantly affected the incidence and severity of the adenocarcinomas that developed at both the 3 and 6 month time points examined in the current study.

IHC analyses

IHC analyses were performed on sections of the ventral prostate from 6 month old Hi-Myc mice to evaluate potential diet-dependent differences in staining of phosphorylated levels of Akt, mTOR, S6 ribosomal protein, Stat3 and NF κ B (p65). Proliferation (Ki67 and Cyclin D1 staining), angiogenesis (CD31 staining), and inflammation (staining for macrophages and T-lymphocytes) were also assessed by IHC. Relative to the overweight control group, DIO increased, while 30% CR reduced staining for phospho-Akt (Ser⁴⁷³), phospho-mTOR (Ser²⁴⁴⁸) and phospho-S6-ribosomal protein (Ser^{235/236}) (Figure 3). Staining for both phospho-mTOR and phospho-S6-ribosomal protein was primarily cytoplasmic in all types of lesions. In contrast, staining for phospho-Akt was seen in cytoplasm, membrane and nucleus with some areas exhibiting more intense nuclear staining as shown in Figure 3. Similar diet-induced effects were observed for levels of phospho-Stat3, Ki67 and cyclin D1 (Figures 3 and 4). CD31 staining was significantly increased in ventral prostate of Hi-Myc mice receiving the DIO regimen, indicating a substantial increase in vascularization (see Figure 4). Changes in vascularization were further quantified as changes in microvessel density (MVD) and mean vessel area (MVA). Significant differences were seen in both MVD and MVA across the range of dietary energy balance (see Supplemental Figure 1). Furthermore, DIO increased, while 30% CR reduced the level of nuclear phospho-NF κ B (p65). Staining for macrophages and T-lymphocytes also revealed a significant increase with DIO compared to both the overweight control and 30% CR groups. Macrophages were observed primarily in the stromal compartment surrounding adenocarcinomas, whereas T-lymphocytes were found both in the stroma as well as infiltrating the adenocarcinomas. Quantitative analyses of the results from these IHC analyses is shown in supplemental Figure 1.

Western blot analyses of phosphoproteins in the ventral prostate of Hi-myc mice

Western blot analyses were performed on protein lysates from ventral prostate of Hi-Myc mice (6 months of age) maintained on the different diets to further analyze diet-induced changes in phosphorylation status of Akt, mTOR and several Akt and mTORC1 downstream targets. In addition, the status of Stat3 tyrosine phosphorylation was evaluated. As shown in

Figure 5, phosphorylation of Akt (Thr³⁰⁸), mTOR (Ser²⁴⁴⁸) and S6 ribosomal protein (Ser^{235/236}) was significantly different ($p<0.05$) across the three diet groups. Specifically, 30% CR reduced phosphorylation of these signaling molecules, while both the overweight control and DIO diets led to significant increases in phosphorylation of Akt, mTOR and S6 ribosomal protein. Furthermore, 30% CR significantly reduced phosphorylation of Akt (Ser⁴⁷³), GSK3 β (Ser⁹), Foxo3a (Ser²⁵³), 4E-BP1 (Thr³⁷), S6-ribosomal protein (Ser^{240/244}) and Stat3 (Tyr⁷⁰⁵) compared to both the overweight control and DIO diet groups ($p<0.05$); however, no significant differences were observed between the overweight control and DIO diet groups. These Western blot data are consistent with the IHC analyses presented in Figure 3 showing that the greatest differences in levels of phosphorylation occurred between the dietary energy balance extremes in this study.

Expression of selected genes associated with inflammation and angiogenesis

In light of the data in Figure 4 showing diet-dependent differences in CD31 staining and staining for inflammatory cells (macrophages and T-lymphocytes), we isolated RNA from the ventral prostate of Hi-Myc mice on the different diets at 6 months of age. Expression of a panel of selected genes associated with inflammation was analyzed via qPCR. As shown in Figure 6A, mRNA levels of IL1 α , IL7, IL23, IL27, NF κ B1 (p50) and TNF α mRNA were significantly increased in the ventral prostate of mice maintained on the DIO diet relative to both the overweight control and 30% CR groups. IL-1 β mRNA levels were also increased in the DIO diet group but slightly lower than the overweight control group. Regardless, IL-1 β mRNA levels were elevated in both of these diet groups relative to the 30% CR diet group. The differences in mRNA levels in the DIO diet group for all the other genes evaluated were significantly different ($p<0.05$) compared to both the overweight control and 30% CR diet groups.

In light of the dramatic diet-dependent differences seen in angiogenesis in ventral prostate of Hi-Myc mice, we also analyzed mRNA levels of VEGF family members. For these analyses, we quantified mRNA levels for VEGF A, B, C, D and PLGF (Placental Growth Factor). As shown in Figure 6B, mRNA levels for all VEGF family members were significantly ($p<0.05$) upregulated in ventral prostate of Hi-Myc mice on the DIO diet compared to mice on both the overweight control and 30% CR diets. In addition, differences seen in the mRNA levels between the overweight control and 30% CR diet groups for all VEGF family members were also significantly different ($p<0.05$). As shown in the figure, the difference between the DIO and 30% CR diet groups were particularly striking.

Discussion

The present study was designed to investigate the impact and to identify potential mechanisms for dietary energy balance effects on prostate cancer progression in an established mouse model of prostate cancer (i.e., Hi-Myc mice) (21). Diets of varying caloric density, which have been utilized in numerous studies to evaluate the effects of dietary energy balance on chronic disease (including cancer), were used and the resulting body weights and adiposity levels corresponded with previously published data using the same diets (32, 33). These previous reports classified mouse adiposity levels and provide a

corresponding body phenotype, as a function of both human body fat content and BMI classification. As such, the mice in the current study correspond to lean, overweight and obese phenotypes. The DIO regimen utilized has consistently been shown to effectively induce an obese state in mice, enabling evaluation of the direct effects of obesity on prostate cancer as well as comparisons among obese, overweight and lean phenotypes (26). While 30% CR significantly reduced body weight, adiposity and levels of globally active circulatory proteins, differences in body composition and serum profiles between the overweight control and DIO diet groups were not as dramatic (although still statistically significant). Thus, the current study evaluated a range of body weight and adiposity phenotypes with the biggest differences occurring between the 30% CR and DIO diet groups. The data clearly demonstrate an effect of dietary energy balance on prostate cancer progression through changes in both growth factor and inflammation signaling pathways.

In the current study, a spectrum of prostate lesions was observed, most consistently in the ventral prostate of Hi-Myc mice across the 30% CR, overweight control and DIO diet groups. The incidence of hyperplasia and IgPIN was similar across this spectrum of dietary energy balance in Hi-Myc mice at both 3 and 6 months of age. In contrast, dietary energy balance manipulation significantly affected the progression of premalignant lesions to malignant lesions. In this regard, although in situ carcinomas were observed in all diet groups, regardless of caloric consumption, the incidence of these lesions was significantly reduced ($p < 0.05$) in the 30% CR group relative to the overweight control at both 3 and 6 months of age. Furthermore, 30% CR completely suppressed the formation of invasive adenocarcinomas at both time points. DIO also significantly increased the incidence of adenocarcinomas with aggressive stromal invasion compared to the overweight control diet group at 6 months (96% vs. 65% respectively, $p = 0.02$). The severity of invasive adenocarcinomas was also significantly affected by the different diets with more severe lesions (i.e., less differentiated lesions) observed in the DIO diet group compared to the overweight control diet group at both the 3 and 6 month time points. Both IHC and Western blot analyses revealed that, relative to overweight control, DIO increased, while 30% CR reduced activation of signaling through both Akt and mTORC1 as well as Stat3. Markers for proliferation (cyclin D1, Ki67) and angiogenesis (CD3) also showed increased staining in tumors from the DIO group compared to the overweight control and 30% CR diet groups. Further analyses revealed a significant difference in inflammatory cell (macrophages and T-lymphocytes) infiltration in the ventral prostate of Hi-Myc mice maintained on the DIO diet compared to both overweight and 30% CR groups. Finally, significant elevations in inflammatory cytokine and VEGF family member gene expression were observed in the ventral prostate of Hi-Myc mice maintained on the DIO diet compared to both overweight and 30% CR diets. These changes were correlated with changes in expression of NF κ B1 and increased expression of nuclear phospho-NF κ B (p65). Collectively, the current data demonstrate that DIO enhances while 30% CR suppresses prostate cancer progression in Hi-Myc mice. Potential mechanisms for these effects involve altered growth factor signaling (Akt/mTOR and Stat3) accompanied by significant changes in inflammatory signaling, tissue inflammation and angiogenesis.

A number of potential cellular and molecular mechanisms are known to contribute to prostate cancer progression [reviewed in (34)]. One mechanism important to the current

study involves activation of Akt signaling. Tumor suppressor gene phosphatase and tensin homolog (PTEN), a lipid and protein phosphatase acts as a negative regulator of Akt and loss of PTEN function leads to constitutive activation of Akt [reviewed in(35)]. Loss of one PTEN allele is a high-frequency event in prostate cancer, observed in up to 70% to 80% of primary tumors (36–39). Homozygous inactivation of PTEN is associated with advanced disease and metastasis (40, 41). PTEN heterozygous null mice (PTEN^{+/-} mice) develop PIN with a variable penetrance varying from 40% to 50% (42–44) to 90% (45) depending on genetic background. Lesions develop mainly in the anterior and dorsolateral prostate but are also observed in the ventral prostate. Progression to adenocarcinoma is normally not observed in PTEN^{+/-} mice, possibly related to age-dependent morbidity due to the high incidence of thymic lymphomas that occur in these mice. In contrast, conditional PTEN knockout mice with complete loss of PTEN in the prostate develop invasive prostatic carcinoma but with an extended latent period (45, 46). Inactivation of PTEN in combination with other mutations can promote prostate cancer progression in transgenic mice (47–50). As shown in Figures 3, 5 and Supplemental Figure 1 of the current study, dietary energy balance modulated Akt and mTOR activity (i.e., phosphorylation) and downstream mTORC1 signaling in ventral prostate of the Hi-Myc mice that directly correlated with the diet-induced changes seen in prostate cancer progression in the Hi-Myc mice (highest with DIO and lowest with 30% CR). Furthermore, recent studies have shown that Akt activation can lead to activation of NFκB signaling via stimulation of IKKα (51). Studies in both mouse models and human prostate cancer have implicated deregulated NFκB signaling in mediating responsiveness to androgens, metastasis, and disease outcome (52–56). Jin et al (24) also recently reported that upregulation of NFκB activity in Hi-Myc mice (via crosses with IKBα^{+/-} mice) led to androgen-independent growth suggesting that NFκB signaling may regulate (at least in part) prostate cancer progression. Thus, differential activation of the Akt/mTOR signaling pathways to downstream effectors, including NFκB may have contributed to the effect of dietary energy balance on prostate cancer progression seen in Hi-Myc mice in our current study.

Recently, Kobayashi *et al.* (23) showed that the Hi-Myc prostate phenotype was responsive to dietary manipulation. They examined the effect of an increase in dietary fat on the development of prostate lesions. A modified pair-feeding regimen was used to ensure that the caloric intake was equivalent for the two groups. It is important to note that there were no differences in body weight between the two diet groups in this study. These investigators demonstrated that if the Hi-Myc mice were maintained on a low-fat (LF, 12 kcal% fat) diet versus a HF (42 kcal% fat) diet, the transition from PIN to prostate cancer was delayed and there was a decrease in prostatic Akt activity as well as a decrease in phosphorylation of the downstream targets p-70S6K and GSK3β. Although we cannot rule out a contribution of increased fat intake in the DIO diet (60kcal% fat), the fact that the 30% CR mice had such a striking reduction in prostate cancer phenotype despite consuming a diet with a higher fat composition (14 kcal % fat) than the overweight control group (10 kcal% fat) argues against fat alone as the major contributor in our studies. Furthermore, previous work by us and others showing that genetically obese mice or rats consuming the same AIN76A based diet (i.e., greater consumption of the diet due to hyperphagia) have much higher tumor development than non-obese mice. Additionally, studies of energy restriction accomplished

by carbohydrate restriction (as is done here), protein restriction or fat restriction, or total diet restriction all have similar effects in reducing tumor development in multiple models, suggesting total energy, independent of the macronutrient manipulated, is the major determining factor in the anticancer effects of such restricted diets (57–60). For example, we previously compared the same 30% CR regimen used here with a low carbohydrate/high fat diet in a colon cancer model in C57BL/6 mice. Only the 30% CR diet, and not the low carb/high fat diet (which mimicked the induction phase of the Atkins diet) reduced adiposity and impacted colon tumor progression in that model (60). Taken together, these findings suggest that energy balance may be a stronger determinant of tumor progression than fat or carbohydrate intake.

Despite no changes in body weight in the study by Kobayashi and coworkers (23), their findings further illustrate the importance of the Akt/mTOR signaling pathway in prostate tumor formation and progression in the Hi-Myc model. They also support the role of fat, per se, independent of energy balance status, in modulating prostate cancer progression in this model. We previously showed that dietary energy balance manipulation modulated the activity of the Akt and mTORC1 signaling pathways in several normal tissues, including the prostate, in wild-type FVB/N and C57BL/6 mice (25). In these studies, we found that, relative to same overweight control diet used here, 30% CR resulted in a reduction, whereas DIO increased steady-state signaling through these two important signaling pathways in the prostate. These effects appeared to be due primarily to alterations in upstream signaling through the IGF-1R and not through alterations in nutrient-sensing pathways such as AMPK(25). Thus, changes in Akt/mTOR signaling, as a result of dietary energy balance manipulation, would be expected to be present in ventral prostate of Hi-Myc mice both before and during prostate tumor development and tumor progression.

Evidence also points to activation of Stat3 in prostate cancer progression. In prostate cancer cell lines, Stat3 activation correlated with malignancy (61, 62). Inhibition of Stat3 in DU145 cells by antisense Stat3 oligonucleotides resulted in growth inhibition and apoptosis (61). Analysis of prostate adenocarcinoma specimens also revealed elevated levels of activated Stat3 that were positively correlated with a more advanced stage of tumors exhibiting higher Gleason scores (61). Thus, Stat3 seems to be involved in both proliferation and survival of prostate cancer cells as well as prostate cancer progression. We recently reported the development of a new mouse model of prostate cancer based on over-expression of a constitutively active form of Stat3 (Stat3C) coupled with PTEN loss in prostate epithelial cells (63). The combination of constitutive Stat3 activation and elevated Akt activity led to progression of prostate tumors and further elevations in endogenous Stat3 activity in these mice. Notably, tumor progression in these mice was also accompanied by a dramatic increase in nuclear staining for phospho-NF κ B (phospho-p65). The data with this new mouse model suggest that Akt/mTOR signaling cooperates with Stat3 signaling in prostate cancer progression. Thus, the diet-induced changes in Stat3 activation in ventral prostate of Hi-Myc mice seen in our current study, in concert with the changes seen in Akt/mTOR signaling, may cooperate in a similar manner in the effects of dietary energy balance on prostate cancer progression.

In this study, we also found significant diet-associated changes in tissue inflammation both at the cellular level (altered inflammatory cell infiltration and angiogenesis) and at the level of altered inflammatory gene expression that correlated with the progression of prostate cancer in the Hi-Myc mice. The links between obesity and inflammation and between chronic inflammation and cancer have been well described (64, 65). Chronic, low-grade systemic inflammation typically accompanying obesity is associated with two- to three-fold increases in the circulating levels of the cytokines TNF α , soluble TNF receptor, IL-1 β , IL-6, IL-1 receptor antagonist, and C-reactive protein (64). The source of these elevated cytokines may be adipocytes or immune cells such as macrophages (64). The diet-induced changes in tissue inflammation seen in Hi-Myc mice could be due to changes in both circulating levels of inflammatory mediators as well as increased local production by infiltrating macrophages and T-lymphocytes as well as prostate epithelial cells and prostate tumor cells. The fact that both Stat3 and NF κ B were activated to a greater extent in ventral prostate of Hi-Myc mice maintained on the DIO diet compared to the overweight control and 30% CR diet groups suggests that local production of inflammatory cytokines is likely an important driving force for obesity-induced tumor progression. Both Stat3 and NF κ B regulate expression of a number of inflammatory cytokines as well as VEGF family members (66). The inflammatory genes that we analyzed in this study include a subset of genes that are regulated by both Stat3 and NF κ B (66). Since both activated Stat3 and activated NF κ B were observed in the epithelial cells of the ventral prostate, including adenocarcinoma cells (Figures 3 and 4) it is likely that some of the tissue inflammation seen in ventral prostate is due to increased production of inflammatory cytokines from these cells. Recruitment and activation of macrophages and subsets of T-lymphocytes as well as increased numbers of adipocytes in the prostate gland that also produce a battery of pro-inflammatory cytokines would create a positive pro-inflammatory loop that may have facilitated prostate cancer progression. Overall, the data we have generated in this study suggest that the significant tissue inflammatory response seen in the DIO group relative to the overweight control and 30% CR diet groups may play an important role in the effects of dietary energy balance on prostate cancer progression in the Hi-Myc mouse model. Further, ongoing work in this area is aimed at identifying which inflammatory mediators might be most important in this regard.

In conclusion, we have shown that dietary energy balance modulates prostate tumor progression in an established mouse model of prostate cancer based on overexpression of c-myc in prostate luminal cells. DIO enhanced whereas 30% CR reduced prostate cancer progression. While the exact mechanism(s) for these effects of dietary energy balance on prostate cancer progression in Hi-Myc mice remain to be fully elucidated several possibilities were discovered including altered Akt/mTOR, Stat3 and NF κ B signaling. Some or all of these changes may have contributed to the dramatic changes in tissue inflammation in the ventral prostate of Hi-Myc mice. The ultimate goal of our studies is to interrogate the impact of dietary energy balance manipulation in the Hi-Myc mouse model (and possibly other mouse models of prostate cancer) at different stages of carcinogenesis to illuminate key signaling pathways that may ultimately represent targets for reversing the effects of obesity on prostate cancer progression in humans. Ultimately, it is hoped that studies in mouse models of prostate cancer will accelerate our knowledge of the effectiveness of

lifestyle and/or pharmacologic interventions that may offset the effects of obesity on prostate cancer progression. In this way, we hope to rapidly translate findings from the bench to bedside.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

To Nancy Otto and John Repass for their outstanding technical assistance in the IHC analyses and qPCR analyses, respectively. This study was supported by National Institutes of Health grant P50 CA140388, Start-up funds from the University of Texas at Austin, NIEHS Center grant ES-07784 and University of Texas MD Anderson Cancer Center Support Grant CA16672.

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Figure 1.

Representative lesions observed in the ventral prostate of Hi-Myc mice on the different diets at 3 (**panels A to F**) and 6 (**panels G to L**) months of age. Representative lesions in mice from the 30% CR diet group at 3 months [**Panel A**, hyperplasia and lgPIN; **Panel D**, in situ adenocarcinoma (AC)]. Representative lesions in mice from the 30% CR diet group at 6 months of age are shown in **Panels G** (hgPIN) and **J** (hgPIN and in situ AC). Representative lesions from mice from the overweight control diet group at 3 months of age are shown in **Panels B** (hgPIN) and **E** (well differentiated invasive AC) and at 6 months of age in **Panels**

H (lgPIN and hgPIN) and **K** (moderately differentiated invasive AC). Representative lesions in mice from the DIO diet group at 3 months of age are shown in **Panels C** (hgPIN) and **F** (moderately differentiated invasive AC) and at 6 months of age in **Panels I** (hgPIN) and **L** (poorly differentiated invasive AC). Arrows in the panels are as follows: $\Rightarrow$, hyperplasia; $\triangleleft$, lgPIN; $\blacktriangleright$, hgPIN; $\blacksquare$, in situ adenocarcinoma. Dotted black lines demarcate areas of well, moderate or poorly differentiated invasive adenocarcinomas as indicated.

Figure 2.

Summary of dietary energy balance effects on incidence of prostate lesions and severity of the invasive adenocarcinomas in Hi-Myc mice at 3 months (A) and 6 months of age (B). Total number of animals analyzed for each group at both timepoints is shown in Supplemental Tables 1 and 2. **Panel A**, a, 30% CR diet group significantly different than DIO diet group ($P=0.0002$); b, 30% CR diet group significantly different than DIO diet group ($p=0.015$). **Panel B**, a, overweight control diet group significantly different than DIO diet group ($p=0.0221$); b, overweight control diet group significantly different than 30% CR

group ($P=0.0001$); c, 30% CR diet group significantly different than overweight control and DIO diet groups ($p=0.0001$); d, 30% CR group significantly different than overweight control and DIO diet groups ($p=0.0113$); *, overweight control diet group statistically different than DIO diet group in the number of poorly differentiated invasive ACs ($p=0.02$). Fisher's exact test, two-tailed, was used for all comparisons with significance set at $p = 0.05$.

Figure 3.

IHC analyses of levels and/or phosphorylation status of selected proteins from representative sections of ventral prostate of Hi-Myc mice. Stained sections are from adenocarcinomas in the different diet groups at 6 months of age. Representative pictures from mice in the 30% CR diet group (left column) show in situ adenocarcinomas, and representative pictures from mice in overweight control and DIO diet groups show a mix of in situ and invasive adenocarcinomas. Staining is shown for phosphorylated Akt (Ser⁴⁷³), mTOR (Ser²⁴⁴⁸), S6

ribosomal protein (Ser^{235/236}) and Stat3 (Tyr⁷⁰⁵). Also shown is staining for the proliferation associated markers Ki67 and Cyclin D1.

Figure 4.

IHC analyses of phospho-NF κ B (phospho-p65), CD31 and staining for macrophages and T-lymphocytes from representative sections of ventral prostate of Hi-Myc mice. Stained sections are from adenocarcinomas in mice from the different diet groups at 6 months of age. Representative pictures from mice in the 30% CR diet group (left column) show in situ adenocarcinomas, and representative pictures from mice in the overweight control and DIO diet groups show a mix of in situ and invasive adenocarcinomas. Staining is shown for the

angiogenesis marker CD31, phosphorylated NF κ B (p65), and for macrophages (RM0029-11H3) and T-lymphocytes (CD3).

Figure 5.

Western blot analyses of phosphorylation status of selected signaling molecules. Ventral prostates (n=6–7) from animals from each of the dietary groups were collected at 6 months of age and Western blot analyses were performed to identify dietary energy balance related changes in Akt (Thr³⁰⁸ and Ser⁴⁷³), mTOR (Ser²⁴⁴⁸), 4E-BP1 (Thr^{37/46}), GSK3β (Ser⁹), FOXO3a (Ser²⁵³), S6 ribosomal protein (Ser^{235/236} and Ser^{240/244}) and Stat3(Tyr⁷⁰⁵) phosphorylation. The left panel shows representative Western blots, while the right panel shows quantitative results from three independent studies. Phosphorylation levels of Akt,

mTOR, 4E-BP1 and Stat3 were normalized to both total protein and actin, while all other phospho-proteins were normalized to actin alone. *, 30% CR group significantly different than overweight control and DIO diet groups ($p < 0.05$). **, all groups significantly different from one another ($p < 0.05$). The Wilcoxon Rank Sum test was used for comparisons with significance set at $p = 0.05$.

Figure 6. mRNA expression in ventral prostate for selected genes associated with inflammation. RNA was isolated from ventral prostate of Hi-Myc mice at 6 months of age. A, expression of IL1 α , IL1 β , IL6, IL7, IL23, IL27, NF κ B1 (p50) and TNF α . B, relative mRNA levels of VEGF family members (VEGF A, B, C, D and PLGF). The Wilcoxon Rank Sum test was used for comparisons with significance set at $p < 0.05$.

Table 1

Effect of dietary energy balance manipulation on body mass, body fat content and circulating growth factors/adipokines in Hi-Myc mice.^a

	30% CR	Overweight control	DIO
Initial mass (g)	25.9 ± 0.9	24.1 ± 0.96	22.3 ± 0.88
Final mass (g)^b	23.9 ± 1.2	40.1 ± 1.2	44.9 ± 1.6
Body fat (%)^b	21.1 ± 0.84	38.0 ± 0.87	41.2 ± 1.2
Feed consumption (g/wk)^b	21.2 ± 0.04	29.8 ± 0.43	28.1 ± 0.8
IGF-1 (ng/mL)^b	383.7 ± 47.1	550 ± 56.8	893. ± 90.9
Insulin (pg/mL)^b	2624.6 ± 235.9	3934.2 ± 557.9	5682.9 ± 557.5
Leptin (pg/mL)^c	801.6 ± 293.9	10385.9 ± 960.3	10392.1 ± 608.9
Adiponectin (ng/mL)^c	12205.7 ± 898.5	9417.3 ± 666.9	9654.7 ± 568.2
Resistin (pg/mL)^b	1516.1 ± 84.7	2003.7 ± 213.4	2538.5 ± 167.3

^aN=12 mice/group; values are means ± SEM. Serum analyses were performed at study's end.

^bValue for each diet group differed significantly (p<0.05, Wilcoxon rank sum) from that in the other groups for this parameter.

^cValue differed significantly (p<0.05, Wilcoxon rank sum) in the 30% CR group for this parameter relative to the other two groups.