# Effect of Low-Fat Diet on Development of Prostate Cancer and Akt Phosphorylation in the Hi-Myc Transgenic Mouse Model

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## Abstract

This study evaluated the effect of dietary fat on prostate cancer development by using the Hi-Myc mouse transgenic prostate cancer model. Hi-Myc mice develop murine prostatic intraepithelial neoplasia (mPIN) as early as 2 to 4 weeks and invasive adenocarcinoma between 6 and 9 months due to the overexpression of human c-Myc in the mouse prostate. Three-week-old male Hi-Myc mice were placed on high-fat (HF; 42% Kcal) or low-fat (LF; 12% Kcal) diets, and equal caloric intake was maintained until euthanasia at 7 months. The number of mice that developed invasive adenocarcinoma at 7 months was 27% less in the LF diet group (12/28) compared with the HF diet group (23/33, $P < 0.05$). Epithelial cells in mPIN lesions in the LF group had a significantly lower proliferative index compared with epithelial cells in the HF group (21.7% versus 28.9%, $P < 0.05$). During the mPIN phase of carcinogenesis (4 months), the LF group had higher serum insulin-like growth factor (IGF) binding protein-1 levels (21.0 ± 8.9 ng/mL versus 3.2 ± 0.8 ng/mL, $P < 0.05$) relative to the HF group. Akt (Ser[473]) phosphorylation, Akt kinase activity, and phosphorylation of downstream targets of Akt in prostates were significantly reduced in the LF diet group compared with the HF group. We conclude that dietary fat reduction delays transition from mPIN to invasive cancer in this Myc-driven transgenic mouse model, possibly through suppression of the IGF-Akt pathway and decreased proliferation of mPIN epithelial cells. [Cancer Res 2008;68(8):3066–73]

## Introduction

Epidemiologic studies suggest that environmental factors associated with Western life-style may promote the development of clinical prostate cancer. One such factor that has been implicated is dietary fat. A number of case-controlled and cohort studies found that increased total fat intake, increased intake of saturated and monounsaturated fat, and increased intake of linolenic acid were associated with a higher risk of developing prostate cancer (1–3), whereas other studies showed no association between dietary fat intake and prostate cancer risk (4, 5). Few studies have shown an association between ω-6 polyunsaturated fat intake and prostate cancer risk (3, 6). The mechanisms through which dietary fat may affect the development of prostate cancer remain to be defined and include effects on the insulin-like growth factor (IGF) system, sex hormone metabolism, free radical damage, and fatty acid metabolic pathways (7). Whereas animal feeding studies have shown dietary fat reduction slowed prostate cancer xenograft growth, few studies have evaluated the role of dietary fat in prostate cancer development (8, 9). Reduction of dietary fat intake lowered the incidence of prostate cancer in two rat models of prostate cancer, but these studies did not incorporate controlled feeding protocols to ensure equal caloric intake between feeding groups (10, 11). Several other studies using rat chemoprevention models found that dietary fat modification did not affect the development of prostate cancer (12–14).

Epidemiologic studies have shown that lower serum IGF-I levels and increased IGF binding protein-3 (IGFBP-3) levels are associated with decreased prostate cancer risk, but no link has yet been established between serum IGFBP-1 and prostate cancer risk (15). There is a paucity of clinical studies evaluating IGFBP-1 and prostate cancer risk, in part due to the fact that IGFBP-1 is nutritionally regulated and serum samples must be obtained in the fasting state. In human studies, dietary fat reduction combined with exercise was associated with increased serum IGFBP-1 and decreased IGF-I levels and was causally linked to decreased mitogenic effects of human serum on LNCaP cells (16). Likewise, in controlled feeding studies in mice, fat reduction was associated with increased IGFBP-1 levels, decreased IGF-I levels, and decreased prostate cancer xenograft growth (9). To date, however, no controlled feeding studies have evaluated the effects of dietary fat intake on prostate cancer prevention and IGF-axis variables. To study the chemopreventive role of dietary fat reduction in prostate cancer, we used a transgenic mouse model that overexpresses the human c-*Myc* oncogene in a prostate-specific manner from the ARR$_2$/probasin promoter (17). These transgenic mice (Hi-Myc mice) develop murine prostatic intraepithelial neoplasia (mPIN) as early as 2 to 4 weeks of age and invasive adenocarcinoma of the prostate between 6 and 9 months. The Myc mouse prostate gene expression signatures share features seen in human prostate cancer development and progression (17). The primary aim of the present study was to evaluate the calorie-independent effects of dietary fat reduction on chemoprevention of prostate cancer.

## Materials and Methods

**Animal husbandry and feeding protocol.** The experimental protocol was approved by the University of California at Los Angeles Chancellor's Animal Research Committee, and the animals were cared for in accordance with institutional guidelines. The transgenic mice used in this study (Hi-Myc

mice), in which the prostate specific expression of human c-*Myc* is driven by the rat probasin promoter with two androgen response elements (ARR$_2$/probasin promoter), were a generous gift from Katharine Ellwood-Yen and Charles L. Sawyers (17). Mice were weaned at 21 d postbirth and randomly assigned to the low-fat (LF) or the high-fat (HF) diet and housed one mouse per cage to accurately monitor and control caloric intake. The diet was prepared by DYETS, Inc. The compositions of the diets are listed in Table 1. Initially a palatability study with *ad libitum* intake showed that the LF group consumed fewer calories than the HF group. Thus, the LF mice were fed *ad libitum* throughout the experiment, and the average daily caloric intake of the LF group was determined once per week. The HF group was given HF food to match the average daily caloric intake of the LF group thrice per week (Monday, Wednesday, and Friday). This modified pair feeding technique has provided equal caloric intake in our previous isocaloric feeding study (9). Body weight was measured weekly.

**Blood and prostate tissue collection.** Blood was collected from the retroorbital vein in all mice at 4 mo of age after a 14-h fast. The serum was separated, frozen, and stored at $-80°C$. All mice were euthanized at 7 mo (after a 14-h fast). Blood was collected, and urogenital organs were harvested en block, immediately rinsed, and placed in ice-cold PBS. Using a dissecting microscope, the ventral, dorsal, lateral, and anterior prostate lobes were dissected from one side and frozen in liquid nitrogen. The remainder of each prostate was fixed in 10% buffered formalin for 12 h, washed in running water, and transferred to 50% ethanol before embedding in paraffin blocks.

**Pathology.** Sections (4 μm) were obtained from the paraffin-embedded blocks and stained with H&E. Histopathologic analysis to determine presence or absence of PIN and presence of invasive adenocarcinoma was performed in a blinded fashion by a single pathologist (J.S.). Laminin and smooth muscle actin immunostaining were used to define invasion through the basement membrane and fibromuscular layer using the antibodies obtained from Dako Corporation. We used criteria for distinguishing normal, mPIN, and cancer, as defined by the consensus report from the Bar Harbor Meeting of the Mouse Models of Human Cancer Consortium (18). The mouse prostate lobes (dorsal, lateral, ventral, and anterior lobes) were identified histologically in H&E-fixed sections using published criteria (18, 19). Neoplastic proliferation of premalignant potential or mPIN was defined as a proliferation or stratification of atypical epithelial cells showing nuclear atypia within preexisting glands usually with tufting, micropapillary, or cribriform growth pattern. Nuclear atypia was identified by the presence of nuclear hyperchromasia with chromatin clumping and presence of nucleoli. Microinvasive carcinoma was the earliest form of carcinoma, recognized by the presence of malignant glands penetrating through the basement membranes into the surrounding stroma. Invasive carcinoma was defined by the presence of malignant glands showing destructive growth in the prostate parenchyma. Both microinvasive and invasive cancer lesions were classified as prostate cancer for statistical analyses. The carcinomas were well differentiated with formation of discrete, well-formed glands and showed glandular differentiation classifiable as adenocarcinoma in all cases.

**Immunohistochemistry.** Ki-67 immunostaining was performed as previously described (20). A total of 400 cells, 100 cells per field, were counted for each mouse prostate, and the number of Ki-67–positive cells was scored by a single pathologist. Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assays were performed, as previously described (20), using the Apop Tag Peroxidase *In situ* Apoptosis Detection kit from Chemicon. A total of 800 cells were counted from four fields for each mouse prostate, and the number of positive nuclei was expressed as a percentage by dividing the number of positive staining nuclei by the total number of cells counted.

***Ex vivo* bioassay.** LNCaP cells were obtained from American Type Culture Collection and grown in RPMI medium without phenol red (Omega Scientific) supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin, 100 μg/mL streptomycin, and 4 mmol/L L-glutamine (Omega Scientific). Myc-CaP cells (21) were a generous gift of Phil Watson and Katharine Ellwood-Yen and grown in DMEM supplemented with 10% FBS (Omega Scientific). LNCaP and Myc-Cap cultures were maintained at 37°C and supplemented with 5% CO$_2$ in a humidified incubator. The mitogenic

**Table 1.** Ingredients of experimental diets

| Ingredient | LF diet | | HF diet | |
|---|---|---|---|---|
| | g | % energy | g | % energy |
| Corn oil | 50 | 12.38 | 175 | 43.32 |
| Casein (sterile)-GI | 200 | 20.35 | 200 | 20.35 |
| L-Cystine | 3 | 0.33 | 3 | 0.33 |
| L-Methionine | 1.6 | 0.18 | 1.6 | 0.18 |
| Sucrose | 0 | 0.00 | 0 | 0.00 |
| Maltose dextrin | 30 | 3.14 | 30 | 3.14 |
| Cornstarch | 502.9 | 49.79 | 273.2 | 27.05 |
| Dyetrose | 105 | 10.97 | 25 | 2.61 |
| Cellulose | 50 | 0.00 | 50 | 0.00 |
| AIN-93 G mineral mix* | 35 | 0.87 | 35 | 0.87 |
| AIN-93 vitamin mix* | 10 | 1.07 | 10 | 1.07 |
| Vitamin B12 supplement* | 10 | 1.10 | 10 | 1.10 |
| Choline bitartrate | 2.5 | 0.00 | 2.5 | 0.00 |
| Total | 1000 | 100.00 | 815.3 | 100.00 |

*See ref. 50.

effect of mouse serum on LNCaP and Myc-CaP proliferation was studied using an in-house bioassay. The cells were plated at $5 × 10^3$ cells per well in 96-well plate and incubated for 24 h before changing to fresh media containing 10% mouse serum or 10% FBS (control). Each serum sample from individual mouse was tested in triplicate. The cell proliferation in media containing mouse serum was measured by using the CellTiter 96 Aqueous One solution Cell Proliferation Assay (Promega Corporation) as previously described (9, 20) after 48 h of incubation for LNCaP and 24 h for Myc-CaP at 37°C. The data were expressed as percentage of the proliferating cells grown in media containing 10% FBS. The interassay and intraassay coefficient of variation for the *ex vivo* bioassay is 6.95 and 2.98, respectively.

**Measurement of mouse serum IGF-I, IGFBPs, and free and total testosterone levels.** The levels of murine IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were measured using in-house mouse-specific ELISAs that have been described previously (22, 23) using mouse-specific antibodies and recombinant mouse IGF and IGFBP standards. The mouse IGF-I assay has a sensitivity of 0.1 ng/mL and no cross-reactivity with mouse IGF-II or human IGF-I. The intraassay and interassay coefficient of variations were <10% in the range from 1 to 10 ng/mL. The mouse IGFBP-1, IGFBP-2, and IGFBP-3 assays have sensitivities of 0.2 ng/mL and no cross-reactivity with other IGFBPs or the human homologues. The intraassay and interassay coefficient of variations were <6% and<8%, respectively, in the range from 1 to 6 ng/mL.

Serum testosterone levels were measured at 4 mo (pooled serum) and 7 mo (serum from individual mice; $n = 8$ per group) using total testosterone ELISA from Calbiotech and free-testosterone ELISA from Immuno-Biological Laboratories, Inc.

***In vitro* Akt kinase assay.** Frozen mouse prostate tissues (ventral, dorsal, and lateral lobes) were homogenized in cell lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium PPi, 1 mmol/L β-glycerophosphate, 1 mmol/L Na$_3$VO$_4$, 1 μg/mL leupeptin] with 1 μmol/L phenylmethylsulfonyl fluoride followed by 20-s sonication (50% power) on ice. The protein concentration of the cleared lysate was determined with the bicinchoninic acid protein assay kit (Pierce) and adjusted to be the same in all samples. This lysate was used for immunoprecipitation with immobilized Akt monoclonal antibody supplied in the Akt kinase assay kit from Cell Signaling Technology. The Akt kinase assay was performed according to the manufacture's protocol using GSK-3 fusion protein as a substrate, and phosphorylation of GSK-3 was measured by Western blotting with anti-phospho-GSK-3α/β (Ser[21/9]) antibody.

**Western blot analysis.** Mouse prostate lysate (50 μg) was subjected to SDS-PAGE followed by Western blot analysis and densitomeric quantification, as previously described (20). Antibodies for Akt, phosphorylated Akt (Ser[473]), p70 S6 kinase, phosphorylated p70 S6 kinase (Thr[389]), phosphorylated p70 S6 kinase (Thr[421]/Ser[424]), and phosphorylated GSK-3α/β (Ser[21/9]) were from Cell Signaling Technology and used at 1:1,000 dilution. Monoclonal antibody 9E10 was from Abgent and used to detect Myc protein. The secondary horseradish peroxidase–linked antibody was used at 1:2,000 dilutions. Personal Densitometer SI (Molecular Dynamics) and Image Quant (Amersham Biosciences) were used to quantify the Western signals.

**Statistical analysis.** Quantitative measures were compared between the groups (LF versus HF) using two-tailed Student's $t$ test calculated by Prism 3.0 software. The $P$ values <0.05 were considered significant. The data are presented as mean ± SE. The proportions of mice with prostate cancer versus mice with mPIN and benign prostate were compared between the diet groups with Fisher's exact test (two-sided; Splus, version 6).

## Results

**Slower transition from mPIN to cancer with dietary fat reduction.** Throughout the 7-month study, there was no significant difference in caloric intakes or mean body weights between the LF and HF diet groups (Supplementary Fig. S1). There was a 27% reduction in the incidence of prostate cancer in the LF group compared with the HF group at the age of 7 months (12 of 28, 42.8% versus 23 of 33, 69.7%; $P$ = 0.042; Fig. 1*A*). mPIN and cancerous lesions were multifocal and most prominent in the lateral prostate lobes but were also variably present in the dorsal, ventral, and anterior lobes. mPIN lesions generally comprised 15% to 30% of the affected prostate lobes, and cancer comprised ∼20% to 40% of the affected lobes. Cancerous lesions extended directly from mPIN lesions, thus demonstrating a clear transition from mPIN to cancer.

The histologic appearance of mPIN and invasive prostate cancer are shown in Fig. 1*B* and *C*.

**Reduced prostate cell proliferation in LF diet group mPIN.** Given that a central mechanism in *Myc*-induced conversion from mPIN to invasive prostate cancer may involve cell proliferation, we investigated whether dietary fat modification affected Hi-Myc mouse prostate epithelial cell proliferation by Ki-67 immunostaining (Fig. 2*A* and *B*). Epithelial cells in mPIN lesions in the LF group had a significantly lower proliferative index compared with epithelial cells in the HF group (21.7% versus 28.9%, $P$ = 0.05; Fig. 2*B*). Likewise, the proliferative index of invasive cancer epithelial cells was also lower in the LF group than the HF group (25.4% versus 31.8%, $P$ = 0.07), although the results did not reach statistical significance.

The percentage of apoptotic cells (by TUNEL staining) in mPIN lesions was similar between the LF and HF diet groups (1.3% versus 1.7%; Fig. 2*C*). However, in the cancer lesions the apoptotic index was lower in the LF diet group relative to HF group (2.7% versus 5.3%, $P$ = 0.015; Fig. 2*C*). Prostate cancer lesions had higher proliferation and apoptosis levels relative to mPIN lesions in both diet groups (Fig. 2*B* and *C*).

**Lower mitogenicity of LF group serum.** The mitogenicity of LF and HF mouse serum was evaluated by measuring the cell growth of androgen-dependent cell lines, LNCaP and Myc mouse–derived Myc-CaP (21), in media containing 10% mouse serum from 7-month-old Hi-Myc mice. LNCaP and Myc-CaP cell lines had significantly lower proliferation in media containing the LF serum compared with the growth in the media containing the HF serum (LNCaP, 32% reduction, $P$ = 0.01 and Myc-CaP, 8% reduction, $P$ = 0.05; Fig. 3*A* and *B*).

**Increased serum IGFBP-1 in LF group during the mPIN phase of cancer development.** To determine if dietary fat affects
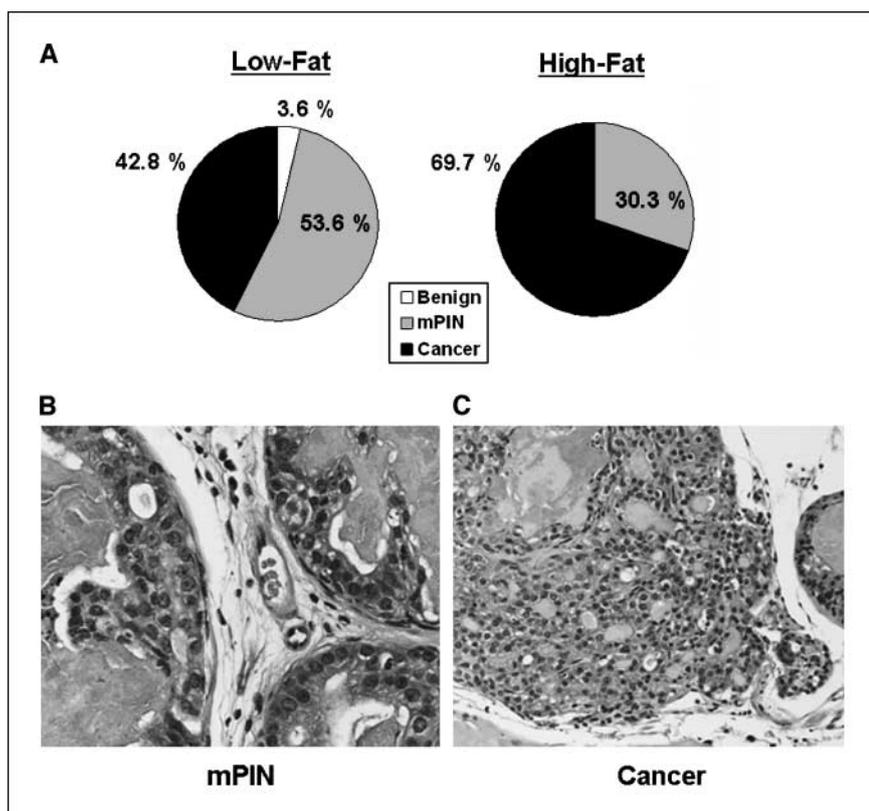
**Figure 1.** Effect of dietary fat reduction on transition from mPIN to cancer in Hi-Myc mouse prostate. *A,* reduced incidence of prostate cancer in LF diet group compared with the HF diet group. The prostates dissected from 7-mo-old Hi-Myc mice were analyzed in H&E–stained histologic sections at two different depths by a pathologist blinded to the treatment groups. $n$ = 28 for LF group and $n$ = 33 for HF group. Statistical analyses were performed comparing the presence or absence of prostate cancer phenotype in the LF and HF group. The Fisher's exact test had a $P$ value of 0.042 (two-sided). *B,* mPIN involving the lateral lobe of the prostate that was characterized by tufted proliferation of epithelial cells with enlarged nuclei and prominent nucleoli. A peripheral basal layer of flattened epithelial cells is evident. The amorphous particulate and eosinophilic secretions are characteristic of the lateral lobe of the mouse prostate (magnification, 300×). *C,* well-differentiated carcinoma consisting of increased numbers of small neoplastic glands with back-to-back configuration. Neoplastic glands infiltrate outside of the prostate lobe and into adjacent fibrous tissue (magnification, 200×).

**Figure 2.** Effect of dietary fat content on Hi-Myc mouse prostate cell proliferation. *A,* Ki-67 immunostaining of mPIN and cancer lesions of mouse prostates from the LF and HF groups. *Arrows,* positive cells. *B,* Ki-67 immunohistochemistry showed decreased cell proliferation for the LF group in both mPIN and cancer lesions. To determine the Ki67 score, 100 cells were counted in four separate high-power fields per sample (400 cells per sample), and the average Ki67-positive cells was expressed as a percentage of total cells. *Columns;* mean; *bar,* SE. mPIN, *n* = 15 for LF group and 13 for HF group. Cancer, *n* = 10 for LF group and 11 for HF group. *, *P* = 0.05 by Student's *t* test. *C,* apoptosis determined by TUNEL labeling. A total of 800 cells were counted from four fields for each sample, and the number of apoptotic cells was expressed as a percentage of the total number of cells counted. MPIN, *n* = 8 for LF group and 10 for HF group. Cancer, *n* = 8 for LF group and 14 for HF group. *, *P* = 0.015.
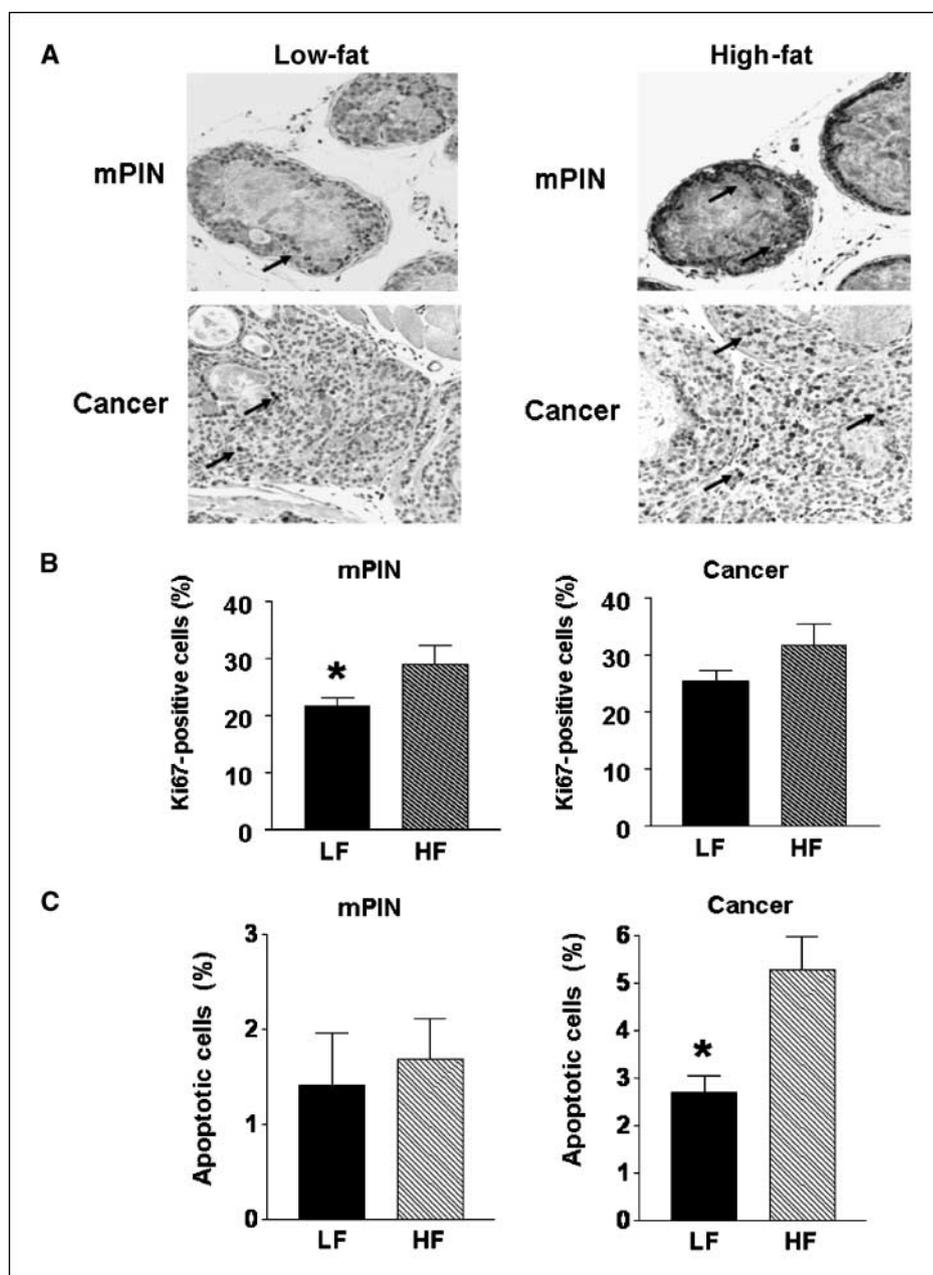
serum levels of IGF-I and IGFBP's during the mPIN and invasive cancer phases of carcinogenesis, mouse IGF axis levels were measured at 4 months (mPIN phase) and 7 months of age (Table 2). Hi-Myc mice in the LF group had significantly higher fasting serum IGFBP-1 levels relative to the HF group at 4 months (21.0 ± 8.9 versus 3.2 ± 0.8 ng/mL; *P* = 0.05) and at 7 months (6.2 ± 1.0 versus 2.0 ± 0.5 ng/mL; *P* = 0.002; Table 2). Fasting serum IGF-I levels were lower in the LF group relative to the HF group at 4 months (290.2 ± 35.0 versus 322.8 ± 33.0 ng/mL; *P* = 0.19), although the difference was not statistically significant. Fasting serum, IGFBP-2, IGFBP-3, and IGF-I levels were similar in the LF and HF groups at 7 months of age.

**Unchanged serum testosterone levels in response to fat modification.** Given the potential for the LF diet to lower testosterone levels and affect cancer development, we measured free and total testosterone levels in serum from the 4-month-old

and 7-month-old mice. At 4 months, the free and total serum testosterone levels were similar between the LF and HF groups (free testosterone, LF 3.8 pg/mL, HF 2.7 pg/mL; total testosterone, LF 1.3 ng/mL, HF 0.7 ng/mL). At 7 months, serum total testosterone levels were not significantly different between the LF and HF diet groups (LF 0.31 ± 0.13 ng/mL; HF 0.22 ± 0.049 ng/mL; *P* = 0.48). Free testosterone levels at 7 months were below the level of detection of the ELISA assay in both diet groups. Given that androgen levels affect *Myc* transgene expression, we examined Myc protein levels in the mouse prostates by Western blot analysis and found similar levels of Myc protein expression in the LF and HF diet groups (data not shown), consistent with our finding of no significant differences in testosterone levels between the two diet groups.

**Down-regulation of Akt pathway in LF group prostate tissue.** Binding of growth factors, such as IGF-I, to their tyrosine kinase
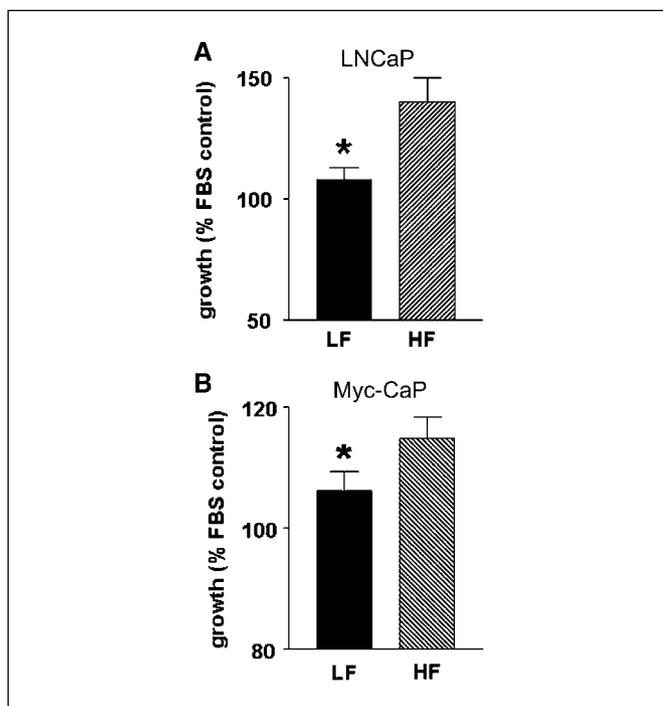
**Figure 3.** LNCaP and Myc-CaP proliferation in media containing HF and LF serum. *A,* LNCaP cells had less proliferation in media containing the LF mouse serum compared with the cells grown in media containing the HF mouse serum from Hi-Myc mice. The cell proliferation was measured after 48 h incubation in the media containing 10% mouse serum. *n* = 8 for each diet group. All experiments were performed in triplicate with the serum from individual mice (not pooled). Data is expressed as a percentage of the cell growth in media containing 10% FBS as a control. *Columns,* means; *bars,* SE. *, *P* < 0.05. Proliferation was determined by measuring colorimetric changes of MTS tetrazolium compound into colored formazan product by NADPH or NADH produced by active cells. *B,* Myc-CaP cells grown in media containing 10% Myc mouse serum had less proliferation with the LF group serum compared with the serum from HF diet group after 24 h incubation. *n* = 5 for each group. *, *P* < 0.05.

receptors leads to autophosphorylation of the receptors and activation of the serine-threonine Akt kinase (protein kinase B) via phosphoinositide 3-kinase. Western blot analyses of the mouse prostate protein showed that the LF group tissue contained 62% less phosphorylated Akt (Ser$^{473}$) relative to the HF group (*P* = 0.016; Fig. 4*A*). Likewise, there was a 65% reduction in Akt kinase activity in the LF group prostate relative to the HF group as measured by an *in vitro* kinase assay (*P* = 0.045; Fig. 4*B*). Phosphorylation of

downstream targets of Akt were also reduced in the LF group prostate relative to the HF group with a 58% reduction (*P* = 0.005) in GSK-3 $\alpha/\beta$ (Ser$^{21/9}$) phosphorylation and a 90% reduction (*P* = 0.0007) in p70S6K (Thr$^{389/421/424}$) phosphorylation (Fig. 5*A* and *B*).

## Discussion

In the present study, dietary fat reduction resulted in increased serum IGFBP-1 levels, down-regulation of the Akt-mTOR pathway in prostate, and delayed development of invasive murine prostate cancer. Previously, we reported that decreasing dietary fat intake slowed growth of LAPC-4 human prostate cancer xenografts in SCID mice and modulated the circulating IGF axis (9). Xenograft models are derived from late-stage or metastatic samples of human prostate cancer. Transgenic mouse models are more appropriate to study the efficacy of chemopreventive agents and mechanisms related to early tumor development. Hi-Myc transgenic mice represent a suitable model for human prostate cancer development. *Myc* gene amplification and overexpression are seen in ~30% of human prostate cancer (24). As well, Hi-Myc mice do not develop neuroendocrine features seen in mouse prostate cancer carcinogenesis driven by the SV40 T-antigen as in TRAMP and LADY mice (19, 25).

Mice were housed one mouse per cage, and equal caloric intake was maintained throughout the study, resulting in similar body weights between the LF and HF diet groups. Whereas two prior feeding studies using rat models of prostate cancer showed that dietary fat reduction lowered the incidence of prostate cancer, the animals in these studies were fed *ad libitum*, and therefore, dietary fat intake could not be differentiated from calorie intake (8, 11). In these prior studies, animals in the HF groups had higher calorie intake and increased body weight. Whereas calorie restriction and calorie excess are known to affect cancer progression, our study excluded these variables and more clearly isolated the effect of dietary fat modification on the development of prostate cancer.

IGF-I is a known mitogen to prostate cancer cells, and epidemiologic studies have linked elevated serum levels of IGF-I in young adulthood with an increased incidence of clinical prostate cancer (26–28). Of interest, a recent a case-controlled study showed that high-serum IGF-I levels at the time of prostate biopsy were associated with the presence of high-grade PIN in biopsy specimens (29). Reduction in IGFBP-1 levels has also been causally linked with prostate cancer progression in experimental systems, although this link has not been established in epidemiologic

**Table 2.** Fasting serum IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations of Hi-Myc mice on LF or HF diet

| Age (mo) | (ng/mL) | LF | HF | *P* |
|---|---|---|---|---|
| 4 | IGF-I | 290.2 ± 35.0 (*n* = 6) | 322.8 ± 33.0 (*n* = 6) | >0.05 |
| 4 | IGFBP-1 | 21.0 ± 8.9 (*n* = 7) | 3.24 ± 0.8 (*n* = 8) | 0.05* |
| 7 | IGF-I | 199.8 ± 17.4 (*n* = 8) | 204.8 ± 10.4 (*n* = 8) | >0.05 |
| 7 | IGFBP-1 | 6.2 ± 1.0 (*n* = 8) | 2.0 ± 0.5 (*n* = 8) | 0.002* |
| 7 | IGFBP-2 | 245.3 ± 6.1 (*n* = 8) | 211.0 ± 32.7 (*n* = 8) | >0.05 |
| 7 | IGFBP-3 | 321.8 ± 23.3 (*n* = 8) | 322.9 ± 24.1 (*n* = 8) | >0.05 |

NOTE: The average values are presented with ±SE.
*Indicates statistically significant difference between the two diet groups by Student's *t* test (two-tailed).
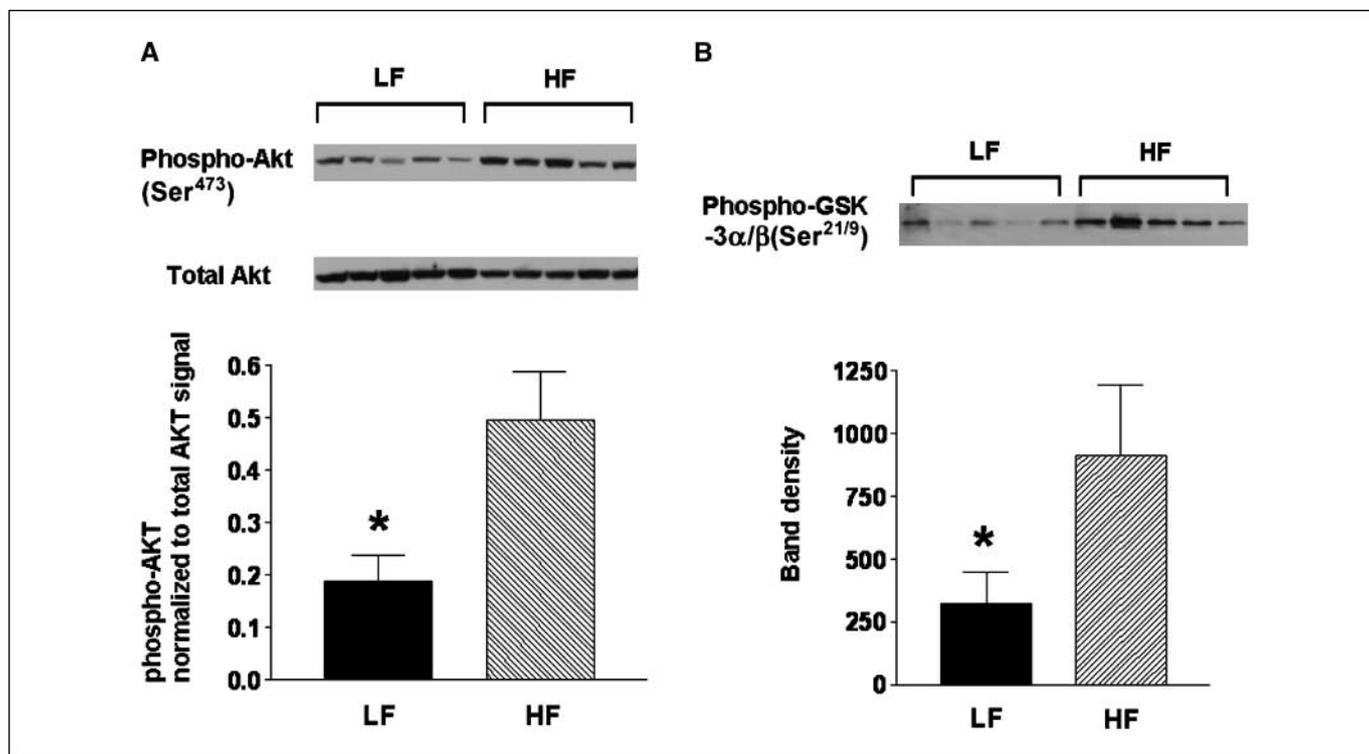
**Figure 4.** Phosphorylated Akt levels and Akt kinase activity in HF and LF Hi-Myc mouse prostate tissue. *A,* Western blot analysis of prostate lysate showed marked decrease of phosphorylated Akt (Ser$^{473}$) for the LF group compared with the HF group. The bands on the Western blots were scanned by a densitometer, and the phosphorylated Akt signal was normalized to total Akt bands. Values are relative densitometric units with SE; $n = 6$ mice for each diet group. *, $P = 0.016$ by Student's $t$ test. *B,* in vitro Akt kinase assay. Total Akt was immunoprecipitated from mouse prostate lysate and tested for kinase activity using GSK-3 subunit as a substrate. The resulting phosphorylated GSK-3 was detected by Western blot. The bands on the Western blots were densitometrically quantitated. $n = 5$ for each group. *, $P = 0.045$.

studies, possibly due to the fact that IGFBP-1 is nutritionally regulated and fasting serum specimens are required for analyses. The present study found that the LF group had increased IGFBP-1 levels (relative to the HF group) at 4 months of age during the premalignant stage of tumor development. In addition, the LF mouse serum had reduced mitogenicity on LNCaP and Myc-CaP cells *in vitro* compared with the HF serum. Likewise, relative to the HF group, epithelial cells of mPIN lesions in the LF group had reduced proliferation, as shown by Ki-67 immunostaining. Taken together, these data suggest that dietary fat reduction may attenuate proliferation of prostate epithelial cells in mPIN lesions via mechanisms involving the IGF axis and ultimately delay the progression to invasive prostate cancer. Given that serum IGFBP-1 levels are modifiable by dietary changes and may affect bioavailable IGF-I, serum levels of IGFBP-1 may potentially serve as a tool for achieving and monitoring therapeutic and prevention goals. Dietary recommendations for the purpose of cancer prevention would be dramatically improved if a serum marker could be followed to assess patient compliance and responsiveness to diet intervention, much like the role of LDL cholesterol for prevention of coronary disease. Future large-scale clinical studies will determine to what degree fasting serum IGFBP-1 may serve in such a role.

The LF Myc mouse prostate tissue contained lower levels of phosphorylated Akt and Akt kinase activity compared with the HF group. Overexpression of Akt in the prostate was previously shown to promote mPIN in transgenic mice (30). Also, Akt1 deficiency markedly inhibited development of mPIN in PTEN$^{+/-}$

mice (31). Studies with human prostate cancer specimens showed increased immunohistochemical staining of Akt (p-S$^{473}$) in malignant and PIN cells relative to benign epithelial cells, and staining intensity positively correlated with PSA levels, Gleason grade, and biochemical recurrence after radical prostatectomy (32–34). Akt promotes cell growth and survival by affecting the activity of various factors, such as GSK3, mTOR (TSC2), BAD, and FoxO. We report that the LF Hi-Myc mouse prostate tissue contained lower levels of phophorylated GSK3 and phosphoryalated p70S6K than the HF prostates. P70S6K is a downstream target of mTOR and regulates cap-dependent protein synthesis. Thus reduction of dietary fat may inhibit the transition from mPIN to invasive cancer by affecting cell proliferation via the Akt-mTOR pathway. In the present study, whole prostate tissue from dorsolateral and ventral lobes was used to prepare the lysate and the benign, mPIN, and cancer lesions were not separately analyzed. Therefore, we cannot differentiate the possibility that the Akt-mTOR pathway was modified in the benign, mPIN, and/or cancer cells. Further studies are required to delineate the effect of dietary fat intake on Akt activation in different stages of prostate carcinogenesis in Hi-Myc mice.

Conflicting reports exist on whether dietary fat alteration affects serum testosterone levels (8, 35). We measured free and total testosterone levels in mouse serum by ELISA and found no significant differences between the LF and HF groups. Likewise, given the potential for dietary fat to affect androgen levels and affect *Myc* transgene expression directly, we examined Myc protein levels in Myc mouse prostates by Western blots and found similar

amounts of Myc protein for the LF and HF diet groups. Further studies are warranted, evaluating potential interactions between dietary fat modification and androgen pathways related to prostate carcinogenesis.

A number of factors that are implicated in c-*Myc*–mediated transformation are regulated by downstream targets of Akt and therefore may be affected by dietary fat modification. For example, hypophosphorylation of 4EBP-1 by Akt and mTOR leads to the release of eIF4E (mRNA-cap-binding protein) that is rate-limiting for $G_1$ progression and a target of c-*Myc* (36). Also *Myc*-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO protein (37). Activated Akt phosphorylates FoxO proteins resulting in exclusion of FoxO proteins from nuclei and subsequent degradation. In a nonphosphorylated state, FoxO factors inhibit induction of multiple Myc target genes and Myc-induced cell proliferation (37–39). The genes down-regulated by FoxO include cyclin D1, of which coexpression with Ki-67 has been reported (40–42).

Myc not only promotes cell proliferation but also sensitizes cells to various apoptotic signals, such as hypoxia, DNA damage, glucose starvation, inhibition of translation and transcription, heat shock, and chemotoxin (43, 44). The apoptotic index increases as tumors progress from mPIN to invasive cancer in Myc mice (17), in other animal models of prostate cancer (45), and in human prostate cancer (40, 46). In the present report, there was no difference in the apoptotic index in mPIN lesions in the LF and HF group, suggesting dietary fat did not significantly affect apoptosis during the mPIN phase of prostate carcinogenesis. However, of interest, prostate cancer lesions in the LF group had a lower apoptotic index relative to the cancer in the HF group. This finding was the opposite of what we expected to see, as anticancer therapies generally are associated with increased apoptosis. Prior xenograft and human serum studies showed increased apoptosis of prostate cancer cells in response to dietary fat reduction (9, 16). Data in the literature is

conflicting with regards to the biological significance of apoptosis in prostate cancer. A prior retrospective clinical study found that higher apoptotic activity in prostate cancer positively correlated with higher proliferation rates, positive surgical margins, and increased risk of death from prostate cancer (40). Similar observations were made in rat models (45). Further studies are required to determine the mechanism through which fat reduction resulted in lower rates of apoptosis in prostate cancer in the Hi-Myc mouse model.

The fat used in the diets in the present study was from corn oil, which is primarily composed of linoleic acid, an ω-6 polyunsaturated fatty acid. Membrane arachidonic acid (ω-6) derived from linoleic acid is converted by the cyclooxygenase and lipoxygenase pathways to eicosanoids, such as prostaglandin E2, leukotrienes, and hydroxyl derivatives of fatty acids. These eicosanoids have been implicated in the pathogenesis of cancer and are believed to play important roles in tumor promotion, progression, and metastasis (47, 48). Whereas the present study focused on the preventative effects of lowering dietary fat in the form of corn oil on the IGF-I–Akt pathway, other factors may also play a role in the preventive effects of dietary fat modification, such as the ratio of ω-6 and ω-3 fatty acids (49). Likewise, further studies should also address if other forms of dietary fat typically found in the Western diet, including saturated fat, affect the development of prostate cancer.

In summary, our study found that lowering dietary fat (corn oil) resulted in (*a*) higher serum IGFBP-1 and reduced serum mitogenicity, (*b*) lower proliferation index of prostate epithelial cells in mPIN and invasive prostate cancer, (*c*) lower phosphorylated Akt protein, Akt kinase activity, and lower levels of phosphorylated GSK3 and p70S6K in murine prostate, and (*d*) reduced transition from mPIN to cancer. Taken together, these studies suggest that reducing intake of dietary fat from corn oil may play a role in prostate cancer prevention.
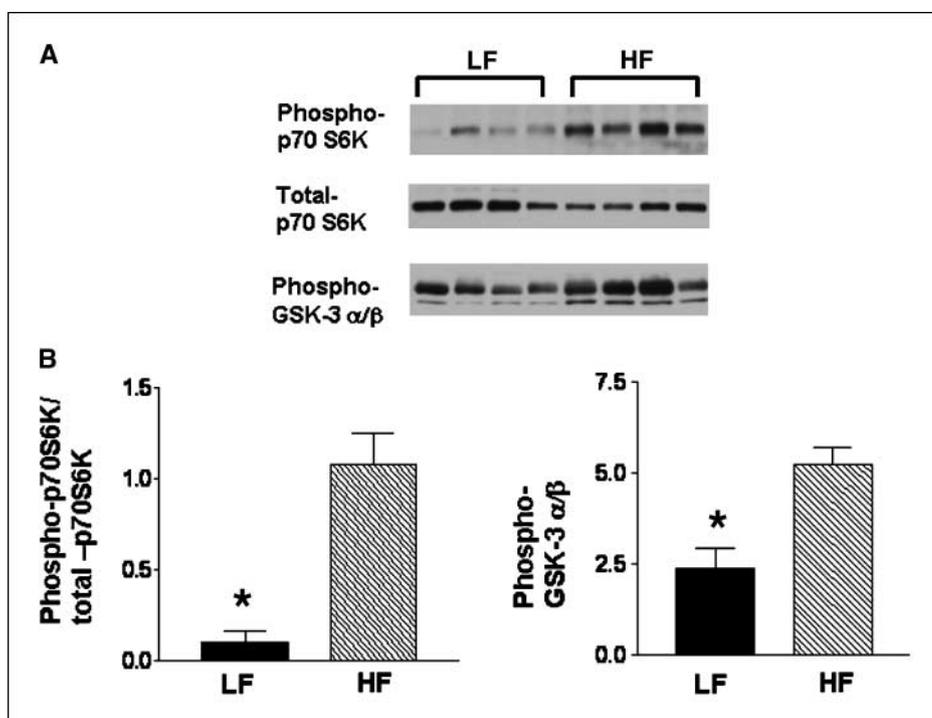
**Figure 5.** LF diet prostate contained decreased levels of phosphorylated Akt downstream targets. *A,* Western blot analysis of Hi-Myc mouse prostate homogenate for phosphorylated p70S6K (Thr$^{389/421/424}$) and phosphorylated GSK-3(Ser$^{21/9}$). *B,* the bands on the Western blots were densitometrically quantitated. The phosphorylated p70S6K bands were normalized to total p70S6K and phosphorylated GSK-3 was normalized to β-actin and shown as a histogram (values are relative densitometric units with SE). $n = 5$ for each group. *, $P = 0.0007$ for phosphorylated p70S6K and 0.005 for phosphorylated GSK3 α/β.

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