Cell signaling pathways associated with a reduction in mammmary cancer burden by dietary common bean (\textit{Phaseolus vulgaris} L.)


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Emerging evidence indicates that common bean (\textit{Phaseolus vulgaris} L.) is associated with reduced cancer risk in human populations and rodent carcinogenesis models. This study sought to identify cancer-associated molecular targets that mediate the effects of bean on cancer burden in a chemically induced rat model for breast cancer. Initial experiments were conducted using a high dietary concentration of bean (60% wt/wt) where carcinoma burden in bean-fed rats was reduced 62.2% (\(P < 0.001\)) and histological and western blot analyses revealed that the dominant cellular process associated with reduced burden was induction of apoptosis. Further analysis of mammary carcinomas revealed changes in the phosphorylation states of mammalian target of rapamycin (mTOR) substrates (4E-binding protein 1 and p70S6 kinase) and mTOR regulators adenosine monophosphate-activated protein kinase (AMPK) and protein kinase B (Akt) (\(P < 0.001\)). Effects on mTOR signaling in carcinomas were also found at lower dietary concentrations of bean (7.5–30% wt/wt). Liquid chromatography–time of flight–mass spectrometry analysis of plasma provided evidence of altered lipid metabolism consistent with reduced mTOR network activity in the liver (\(P < 0.001\)). Plasma concentrations of insulin and insulin-like growth factor-I were reduced (\(P < 0.001\), identifying a link to Akt regulation. Plasma C-reactive protein, a prognostic marker for long-term survival in breast cancer patients, was reduced by 23% (\(P < 0.001\)) in bean-fed rats. Identification of a role for the mTOR signaling network in the reduction of cancer burden by dietary bean is highly relevant given that this pathway is deregulated in the majority of human breast cancers.

Materials and methods

**Chemicals**

Primary antibodies used in this study were anti-cyclin D1, anti-EZF-1 and anti-p27kip1 from Thermo Fisher Scientific (Fremont, CA); anti-retinoblastoma (Rb), anti-B-cell lymphoma 2 (Bcl-2), anti-X-linked inhibitor of apoptosis protein and anti-Bcl-2 associated X protein (Bax) from BD Biosciences (San Diego, CA); anti-apoptosis protease-activating factor-1 from Millipore (Billerica, MA); anti-pAMPK/adenosine monophosphate-activated protein kinase (AMPK), anti-phospho-acetyl-CoA carboxylase (pACC)/ACC, anti-pAkt/Akt, anti-pp70S6K/p70S6K, anti-p4E-BP1/4E-BP1, anti-pRaptor/Raptor, anti-pp70S6K/4E-BP1, anti-pRaptor/Raptor, anti-ppRAS40/RAS40, anti-rabbit immunoglobulin-g-horseradish peroxidase-conjugated secondary antibody and LumiGLO reagent with peroxide were purchased from Cell Signaling Technology (Beverly, MA); anti-p21\(^{CIP1}\) and anti-mouse immunoglobulin-g-horseradish peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-β-actin primary antibody was obtained from Sigma–Aldrich (St Louis, MO) rabbit anti-Ki-67, clone SP6, was obtained from Labvision (Fremont, CA). Biotinylated donkey anti-rabbit and normal donkey serum were obtained from Jackson ImmunoResearch (West Grove, PA); horseradish peroxidase-conjugated streptavidin was obtained from Dako (Carpinteria, CA) and Stable DAB was obtained from Invitrogen (Carlsbad, CA). The following chemicals for metabolite extraction and liquid chromatography–mass spectrometry were used as received: ethanol (HPLC grade; Fisher, Pittsburgh, PA); methanol (Optima LC-MS grade; Fisher) and formic acid (LC-MS grade; Fluka, St Louis, MO).

Introduction

Common bean (\textit{Phaseolus vulgaris} L.), also referred to as dry bean, is known as a staple food because of its contribution to daily caloric intake in many populations. It is widely available and affordable (1). In many areas of the world, average consumption of common bean can reach 150–200 g (dry weight) per day (2), but typical USA intake (7.5–10 g/ day, dry weight) (3) is well below recommended levels in part the result of the use of animal products rather than legumes as a source of dietary protein (4). Given evidence that bean consumption is inversely associated with cancer risk, current consumption patterns in the USA suggest that bean is an under-utilized food for cancer prevention and control.

Epidemiological studies, such as the Nurses’ Health Study II, have found intake of common beans and lentils to be associated with reduced breast cancer risk (relative risk = 0.76, \(P < 0.03\)) (5). In the Four-Corners Breast Cancer Study, a relationship between bean consumption and reduced breast cancer risk was reported in which breast cancer incidence in Hispanic women who consumed a native Mexican diet (characterized by higher pulse consumption, such as common bean) was two-thirds that of the non-Hispanic white population whose diet was characterized as high in red meat, sugar and processed foods (6). Additional epidemiological and preclinical studies evaluating colon cancer (7–10) and prostate cancer (11,12) have lent further support for an inverse relationship between bean consumption and the development of cancer. Our laboratory has shown significant inhibition of the post-initiation stage of chemically induced mammary carcinogenesis in the rat by common bean (13). However, little is known about the cell signaling pathways by which bean exerts its effect.

In cancer, host systemic factors, such as insulin and insulin-like growth factor-1 (IGF-1), are known to contribute to the survival and proliferation of cancer cells through the activation of intracellular signaling pathways that can also act autonomously of growth factors during the progression of cancer (14). Therefore, our investigations of dietary bean consumption have centered: (i) on systemic factors (e.g. glucose-dependent growth factor signaling, inflammatory pathways), (ii) on cell autonomous mechanisms (e.g. cellular energy and nutrient-sensing networks) such as the mammalian target of rapamycin (mTOR) network and (iii) on signaling pathways through which systemic factors regulate cell proliferation and apoptosis. Given the emerging evidence that the mTOR network is deregulated in cardiovascular disease, type-2 diabetes, and in cancer, including breast cancer (15–18), and that little is currently known about the deregulation of mTOR components during early stages of carcinogenesis, the focus of our analysis was on mechanisms operative in mammary carcinomas.

Information gleaned from the analysis of carcinomas has strong implications for cancer control, i.e. for individuals undergoing cancer treatment and for cancer survivors.
Animals and experimental design
The plasma and tissue evaluated in this study were obtained from two previously reported experiments (13,19). Briefly, female Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY at 20 days of age. Animal rooms were maintained at 22 ± 2°C with 50% relative humidity and a 12 h light/12 h dark cycle. During the experiment, rats were weighed three times per week. At 21 da of age, rats were injected subcutaneously with 1-methyl-1-nitrosourea (50 mg/kg body wt, intraperitoneally), as described previously (20). For the first week of the study, rats were housed three per cage in solid-bottomed polycarbonate cages equipped with a food cup; they were given free access to AIN-93G control diet. Seven days following carcinogen injection, all rats were randomized to diet groups based on body weight. Bean was incorporated at 60% wt/wt or for the dose response, 7.5, 15, 30 and 60% wt/wt. Rats were fed their assigned diets ad libitum until the end of the study at 46 days post-carcinogen. The post-initiation design of this experiment simulates the progression and prevention events of the disease process, which are highly relevant to women at increased risk for breast cancer and to breast cancer survivors. The work followed guidelines approved by the Colorado State University Animal Care and Use Committee.

Composition of diets
Beans were kindly provided by Archer Daniels Midland Company (Decatur, IL), a commercial bean processor, as seed. Seed was grown at multiple locations and mixed; therefore, it was representative of the variation in environmental and genetic differences within beans typically consumed in the USA. Bean seed was sent to Bush Brothers and Company (Chestnut Hill, TN) for canning and all material was processed according to industry standard methods. Cooked beans were packed in standard brine without the incorporation of any additives. Beans were then sent to Van Drunen Farms (Momence, IL) where the beans were removed from the cans, drained and then immediately freeze-dried. The freeze-dried product was milled into a homogenous powder and sent to Colorado State University where bean powders were stored at −20°C until incorporated into diets. Diets were formulated using specific guidelines and adjusted using data from proximate analysis (Warren Analytical, Greeley, CO). The diets were formulated to match macronutrient levels (i.e., protein, carbohydrate and crude fiber) across the diet groups. The differences in macronutrient composition were balanced with purified diet components. Diet formulations are reported in (13,19). Diets were stored at −20°C until fed to animals.

Necropsy
Following an overnight fast, rats were euthanized over a 3 h time interval via inhalation of gaseous carbon dioxide. The sequence in which rats were euthanized was stratified across groups so as to minimize the likelihood that order effects would masquerade as treatment effects. After the rats lost consciousness, blood was directly obtained from the retro-orbital sinus and gravity fed into collection tubes (Becton Dickinson, Franklin Lakes, NJ) to obtain plasma. The bleeding procedure took ~1 min/rat. Plasma was isolated by centrifugation at 1000g for 10 min at room temperature (22 ± 2°C). Following blood collection and cervical dislocation, rats were then skinned and the skin to which the mammary gland chains were attached was examined under translucent light for detectable mammary pathologies. All grossly detectable mammary gland pathologies were excised, weighed and a section was fixed in neutral buffered formalin; the remainder of each lesion was snap frozen in liquid nitrogen. Mammary pathologies were histopathologically classified following routine hematoxylin and eosin staining as previously reported (21). Cancer incidence, multiplicity and tumor burden were based on histologically confirmed mammary adenocarcinomas.

Measurement of plasma glucose, insulin, IGF-1, interleukin-6 and C-reactive protein levels
Glucose was determined using a kit obtained from Thermo Fisher Scientific (Waltham, MA). Insulin was determined by commercial enzyme-linked immunosorbent assay kit from Millipore. IGF-1 was determined using a commercial rat enzyme immunoassay kit from Diagnostic Systems Laboratories (Webster, TX). Interleukin-6 (IL-6) and C-reactive protein (CRP) were determined using enzyme-linked immunosorbent assay kits from BD Biosciences. All analyses were performed according to manufacturer’s instructions.

Cell proliferation and apoptosis
Ki-67 immunohistochemical staining was used as an index of tumor growth fraction and was determined as described previously (22). Ki-67-stained sections were analyzed using a CAS-200 image analysis system (Bacus Labs, Lombard, IL). Apoptosis was quantified using the criteria developed by Kerr for its detection (23,24); images of corresponding hematoxylin- and eosin-stained serial sections were acquired using a Zeiss Axioskop II (Carl Zeiss, Thornwood, NY) at a magnification of ×400. Apoptotic and normal cells were marked and counted using the manual tag tools in Image Pro Plus 4.5 (Media Cybernetics, Bethesda, MD).

Western blotting
Liver and mammary carcinomas (seven to twenty per group) were homogenized in lysis buffer [40 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.25 M sucrose, 3 mM ethyleneglycol-bis(aminomethyl)tetraacetic acid, 50 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Calbiochem, San Diego, CA)]. The lysates were centrifuged at 7500g for 10 min at 4°C and supernatants fractions collected and stored at −80°C. Supernatant protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Western blotting was performed as described previously (25). Briefly, 40 μg of protein lysate per sample was subjected to 8–16% sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis after being denatured by boiling with sodium dodecyl sulfate sample buffer [63 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol and 0.01% bromphenol blue] for 5 min. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The levels of cytokine (C5, E2F1, Rb, p21Cip1, p27Kip1, pp53Rb/Rb, Bcl-2, X-linked inhibitor of apoptosis protein, Bax, apoptosis protease-activating factor-1, VEGF, pAMPK, AMPK, pACC, pAkt, Akt, p70S6K, p70S6K, pE3-Binding protein 1, 4E-binding protein, pRaptor, Raptor, pPRAS40, PRAS40 and β-actin) were determined using specific primary antibodies, followed by treatment with the appropriate peroxidase-conjugated secondary antibodies and visualized by LumiGLO reagent. Western blotting detection system. The chemiluminescence signal was captured using a ChemiDoc densitometer (Bio-Rad) that was equipped with a CCD camera having a resolution of 1300 × 1030. Quantity One software (Bio-Rad) was used in the analysis. The actin-normalized scanning density data were used for analysis.

Extraction and analysis of plasma metabolites using liquid chromatography–mass spectrometry
Plasma samples from rats (100 μl) were extracted in 65% ethanol (750 μl), centrifuged (1000g, 5 min), supernatant was transferred to a clean 1.5 ml microcentrifuge tube and dried using a rotatory evaporator without heat. Dry samples were resuspended in eluent prior to liquid chromatography–mass spectrometry. An Acquity UPLC controlled with MassLynx software, version 4.1 (Waters, Millford, MA) equipped with a 1.0 × 100 mm Waters Acquity UPLC BEH C18 column with 1.7 μm particle size was used for sample separation. Injection volume was 5 μl. Separation was performed by reverse phase chromatography at a flow rate of 0.14 ml/min. In order to prevent evaporation, samples were held at 10°C in a sample manager during the analysis. The complete sample set was randomized and profiled in triplicate. One microfilter sample injections were made from 100 μl sample volumes. The eluent consisted of water and methanol (Optima LC-MS grade; Fisher) and formic acid (LC-MS grade; Fluka) in the following proportions: Solvent A = 95.5% water:methanol + 0.1% formic acid; Solvent B = 100% methanol + 0.1% formic acid. The separation method is described as follows (58 min total): 3 min hold at 100% A, 30 min linear gradient to 100% B, 12 min hold at 100% B, 3 min linear gradient to 100% A and 10 min hold at 100% B for equilibration. A Q-TOF Micro quadrupole orthogonal acceleration time-offlight mass spectrometer (Waters/Micromass) using positive ion mode electrospray ionization (ESI+) was used to collect mass spectral data at a rate of two scans per second between 50 and 1000 mass-to-charge ratio of ion (m/z). The voltage and temperature parameters were tuned for general profiling as follows: capillary = 3000 V; sample cone = 30 V; extraction cone = 20 V; desolvation temperature = 250°C; source temperature = 130°C. Desolvation and cone nitrogen flows were set to 400 and 50 l/h, respectively. Leucine enkephalin was infused via a separate orthogonal ESI spray and baffle system (LockMass) which allowed ions to be detected for a single half-second scan every 10 s in an independent data collection channel. The standard mass was averaged across 10 scans providing a continuous reference for mass correction of analyte data. Mass spectrometric scans were centered in real time producing centroid data using MassLynx software (Waters).

Statistical analyses
Cancer burden, rates of apoptosis and Ki-67 staining and actin-normalized western blot data were initially evaluated by the Kruskal–Wallis Test with Dwass–Steel–Chrichtlow–Fligner Test for pairwise comparisons. Differences among groups in plasma molecules were analyzed by analysis of variance. Test for linear trends was done by regression analysis on dietary concentration of bean. Data were evaluated within SAS v 9.1.3 (Cary, NC), STATA (StataCorp, College Station, TX).

Mammary cancer burden and common bean
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Corporation, College Station, TX) or Systat statistical analysis software. Version 12.02. All P values are two sided and statistical significance was set at P < 0.05.

Liquid chromatography–time of flight–mass spectrometry data

Centroid and integrated data were detected, extracted and aligned using MarkerLynx software (Waters). Chromatographic peaks were extracted from 0 to 35 min with a retention time error window of 0.1 min and mass spectral peaks detected from 50 to 1000 m/z with a mass error window of 0.07 m/z generating a data matrix consisting of retention time, m/z and peak intensity for all features as determined by peak area. Data were mean centered and normalized with Pareto scaling for principal components analysis, variable importance in the projection (VIP) and orthogonal partial least squares to latent structures discriminant analysis using SIMCA-P+ v12 (Umea, Sweden). Lists of potential markers for both differences in plasma obtained from bean-fed versus control-fed animals were generated using VIP with a threshold of 5 and p (corr)[1] < 0.5 or p (corr)[1] > 0.5 obtained from orthogonal partial least squares to latent structures discriminant analysis. Corresponding S-plots and principal components analysis score plots were generated using SIMCA-P+.

Metabolic pathway analysis

MassTRIX: Mass Translator into Pathways (www.masstrix.org) was used to identify preliminary ions and Kyoto Encyclopedia of Genes and Genomes pathways, using the Rattus norvegicus database, which distinguish plasma from control rats and bean-fed rats. Proton and sodium corrections were applied in MassTRIX using a mass error of 7 p.p.m.

Results

Tumor burden and mechanisms of its reduction

For the initial mechanistic experiments reported in this paper, analyses focused on the 60% wt/wt concentration of dietary bean in order to enhance sensitivity to detect treatment effects and because of other previously reported rodent carcinogenesis experiments using similarly high concentrations (7,9). The mass of mammary carcinoma per rat was previously reported rodent carcinogenesis experiments using similarly high concentrations (7,9). The mass of mammary carcinoma per rat was markedly reduced (62.2%) in bean-fed rats (Table I). Also shown in the table are the effects of dietary bean on the Ki-67 labeling index, a measurement of cell growth fraction, i.e. the number of cells in the cell cycle, and the apoptotic index, measured histologically using the criteria initially developed by Kerr (23). For Ki-67, a small numerical reduction was observed in carcinomas from bean-fed rats, but the effect was not statistically significant. On the other hand, the apoptotic index was increased 3-fold (P < 0.001) in the carcinomas from bean-fed rats.

Molecular markers of cell proliferation. To extend the immunohistochemical findings on cell growth fraction determined by Ki-67 staining to the molecular machinery underlying cell proliferation, western blotting of mammary carcinomas was carried out for the cell cycle regulatory proteins cyclin D1, E2F-1, p21, p27 and pRb (Table I). Levels of cyclin D1 (P < 0.001) and E2F-1 (P < 0.001) were significantly lower in carcinomas from bean-fed animals, consistent with the finding that the ratio of hyper-phosphorylated Rb to hypo-phosphorylated Rb (ppRb/pRb) was decreased (P < 0.001). Cyclin-dependent kinase inhibitors p21 (P < 0.001) and p27 (P < 0.001) were increased in bean-fed animal carcinomas. Representative western blots for proteins involved in cell proliferation are shown (Figure 1A).

Molecular markers of apoptosis. In order to assess candidate pathways of apoptosis induction, mammary carcinomas were western blotted to determine levels of anti-apoptotic and pro-apoptotic factors. Anti-apoptotic factors Bcl-2 (P < 0.001) and X-linked inhibitor of apoptosis protein (P < 0.001) were decreased in mammary carcinomas from bean-fed rats; whereas pro-apoptotic factors Bax (P < 0.001) and apoptosis protease-activating factor-1 (P < 0.001) increased with bean consumption. The ratio of Bax to Bcl-2, an overall indicator of the apoptotic potential of the intracellular environment, was significantly higher (2.9-fold, P < 0.001) in carcinomas from bean-fed rats, a finding consistent with an elevated rate of apoptosis and reduced carcinoma burden. Representative western blots for proteins involved in apoptosis are shown (Figure 1A).

Systemic factors. The effects of feeding bean on plasma concentrations of glucose, insulin, IGF-1, CRP and IL-6 are shown (Table II). The concentrations of each analyte were reduced in bean-fed rats versus control: glucose (27.0% reduction, P < 0.001), insulin (36.3% reduction, P < 0.001), IGF-1 (38.8% reduction, P < 0.001), CRP (23.1% reduction, P < 0.001) and IL-6 (24.4%, P < 0.001).

mTOR and mammary carcinomas. A selected number of protein targets upstream or downstream of mTOR were investigated in mammary carcinomas to test the hypothesis that activation of AMPK, downregulation of protein kinase B (Akt) and/or suppression of mTOR signaling are candidate pathways for carcinoma burden reduction by dietary bean (60% wt/wt). The activity of upstream mTOR regulator AMPK was significantly increased (P < 0.001) while Akt was significantly decreased (P < 0.001) in bean-fed rats compared with control (Table II). The activity of ACC, a direct target of activated AMPK that regulates lipid biosynthesis, was also evaluated. Phosphorylation of ACC was increased in carcinomas of bean-fed rats (P < 0.001). Lastly, the activity of mTOR in carcinomas from bean-fed rats was evaluated using p70S6K and 4EBP-1, two downstream targets of mTOR. Decreased phosphorylation of both proteins in the carcinoma was observed in bean-fed rats, consistent with reduced mTOR activity and cancer burden. Representative western blots are shown (Figure 1B).

Liver mTOR. The analysis of mTOR activity was extended to the liver. Hepatic mTOR response to dietary treatment is shown (Table II), with upstream mTOR regulators AMPK and Akt being up (30.7% increase, P = 0.011) and downregulated (29.2% decrease, P = 0.001), respectively. Direct regulator of mTOR activity, Raptor, a target of activated AMPK, and the regulator PRAS40, a target of activated Akt, were also analyzed in liver. The AMPK phosphorylation site on Raptor was modestly increased (14.3%, P = 0.037) by bean feeding; whereas a decrease in phosphorylation of PRAS40, predicted by the lower levels of activated Akt in bean-fed rats, was more apparent (23.3% reduction, P < 0.001). p70S6K and 4EBP-1, downstream targets of activated mTOR, were also assessed. The phosphorylation of both proteins was lower in livers of bean-fed rats (p70S6K = 0.037 and 4EBP-1 = 0.001). Overall, effects of bean feeding are consistent with a reduction in mTOR activity in the liver. Representative western blots are shown (Figure 1C).

Plasma metabolome. Plasma samples from control and bean-fed rats were subjected to alcohol extraction and analysis by liquid
chromatography–time of flight–mass spectrometry to determine if bean consumption affected the plasma metabolome. Principal components analysis was performed on the resulting dataset and revealed separation of treatment groups with 45.59% of the total variance explained by the first three principal components (PC 1: 22.7%, PC 2: 14.99%, PC 3: 7.9%) (Figure 1D). The plasma metabolite profiles were further analyzed using VIP and orthogonal partial least squares to latent structures discriminant analysis as described in the Materials and Methods. Features indicated by circles on the S-plot (Supplementary Figure 1 is available at Carcinogenesis Online) were those which passed the VIP threshold of 1 and p(corr) > 0.5 when comparing control to bean-fed animals. An inclusion list of ions contributing to the differences between bean-fed and control animals is shown (Supplementary Table 1 is available at Carcinogenesis Online). All features were evaluated using MassTRIX to determine possible ion identities and candidate pathways differentially regulated in bean-fed animals versus control. Bioinformatic pathway analysis indicated fatty acid biosynthesis, steroid metabolism and liver bile acid metabolism were affected by feeding bean, consistent with the effect of dietary bean on the mTOR network.

Dose-dependent effects of dietary bean on cancer burden, induction of apoptosis, systemic factors and mTOR signaling
Analysis of the mTOR network was extended to mammary carcinomas from rats fed 7.5, 15 or 30% (wt/wt) common bean, dietary concentrations that correspond to amounts of bean consumed by various populations around the world. Effects on regulators of mTOR were dose dependent (Table III and Figure 2). While AMPK activation was markedly induced only at the highest dietary concentration, the phosphorylation of Akt was reduced across the dose response (P = 0.008). Decreased phosphorylation of p70S6K (P = 0.037) and 4E-binding protein 1 (P = 0.001) indicated a dietary dose-dependent downregulation of mTOR activity resulting from bean feeding.

Discussion
Tumor burden and mechanisms of its reduction
Evidence presented in this paper and previously (13,19) has demonstrated that rats fed common bean have reduced mammary carcinoma burden, implicating processes that regulate the proliferation and...
Survival of cancer cells. Summarizing this evidence, mammary cancer burden as well as the average number of cancers per rat, is reduced dose dependently over a range of dietary bean concentrations (Figure 3A). Data also indicate that induction of apoptosis is dose dependent and associated with reduced cancer burden and that death induction is primarily through the intrinsic mitochondrial pathways indicated by the ratio of Bax to Bcl-2 (Figure 3B). Unexpectedly, there is little evidence that the fraction of cells in the proliferative pool (Ki-67 staining index, Figure 3B) is affected by bean consumption despite effects on cell cycle machinery (Table I). This is possibly because these effects are secondary to induction of a pro-apoptotic environment within carcinomas. Apoptosis being a dominant mechanism is consistent with the argument that dietary bean may be beneficial in the cancer control context where agents that induce apoptosis have been reported to have greater clinical efficacy (26), an argument that also is supported by the dose-dependent reduction in CRP with increasing dietary bean (Figure 3C). CRP, the synthesis of which occurs in the liver and responds to circulating levels of IL-6, has recently been shown to have prognostic value for long-term survival following treatment for breast cancer (27).

**Table II. Effect of feeding bean-containing diet on systemic factors and mTOR network components in mammary carcinomas and liver**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Units</th>
<th>Control</th>
<th>Bean-fed</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPK Ratio</td>
<td>2.43 ± 0.28</td>
<td>3.18 ± 0.20</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>ACC Ratio</td>
<td>3.23 ± 0.26</td>
<td>4.15 ± 0.14</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Akt Ratio</td>
<td>0.65 ± 0.06</td>
<td>0.46 ± 0.03</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>p70S6K Ratio</td>
<td>0.33 ± 0.04</td>
<td>0.23 ± 0.02</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>4E-BP1 Ratio</td>
<td>4.31 ± 0.42</td>
<td>2.71 ± 0.10</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose mg/dl</td>
<td>125.3 ± 5.4</td>
<td>91.5 ± 2.6</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Insulin ng/ml</td>
<td>2.73 ± 0.1</td>
<td>1.74 ± 0.05</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>IGF-1 ng/ml</td>
<td>1586 ± 54</td>
<td>969 ± 31</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>334 ± 9</td>
<td>257 ± 5</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>IL-6 pg/ml</td>
<td>48.8 ± 1.2</td>
<td>36.9 ± 1.3</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPK Ratio</td>
<td>3.15 ± 0.10</td>
<td>3.65 ± 0.10</td>
<td>0.11</td>
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<tr>
<td>Raptor Ratio</td>
<td>1.40 ± 0.10</td>
<td>1.60 ± 0.10</td>
<td>0.037</td>
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<tr>
<td>Akt Ratio</td>
<td>0.34 ± 0.12</td>
<td>0.26 ± 0.01</td>
<td>&lt;0.001</td>
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<tr>
<td>PRAS-40 Ratio</td>
<td>1.12 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.037</td>
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<tr>
<td>p70S6K Ratio</td>
<td>3.23 ± 0.29</td>
<td>3.02 ± 0.22</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Values are means ± SEM, (control, n = 9; bean, n = 20). Actin-normalized western blot data were analyzed by the Kruskal–Wallis test. Plasma analyte data were analyzed by analysis of variance. Ratio, the ratio of phospho-protein (arbitrary units of optical density) to total protein (arbitrary units of optical density); 4E-BP1, 4E-binding protein 1.</td>
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</tbody>
</table>

**Table III. Effect of feeding bean-containing diets on the mTOR signaling network in mammary carcinomas**

<table>
<thead>
<tr>
<th>Diet</th>
<th>AMPK Ratio</th>
<th>ACC Ratio</th>
<th>Akt Ratio</th>
<th>p70S6K Ratio</th>
<th>4E-BP1 Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.43 ± 0.28</td>
<td>3.23 ± 0.26^a</td>
<td>0.65 ± 0.06</td>
<td>0.33 ± 0.04^b</td>
<td>4.31 ± 0.42^a</td>
<td></td>
</tr>
<tr>
<td>7.5% Beans</td>
<td>2.47 ± 0.12</td>
<td>3.84 ± 0.17^a</td>
<td>0.50 ± 0.07^b</td>
<td>0.28 ± 0.05^b</td>
<td>4.36 ± 0.47^b</td>
<td></td>
</tr>
<tr>
<td>15% Beans</td>
<td>2.65 ± 0.23</td>
<td>3.97 ± 0.23^a</td>
<td>0.51 ± 0.05^b</td>
<td>0.27 ± 0.05^b</td>
<td>4.18 ± 0.18^a</td>
<td></td>
</tr>
<tr>
<td>30% Beans</td>
<td>2.37 ± 0.28</td>
<td>3.99 ± 0.44^a</td>
<td>0.52 ± 0.05^b</td>
<td>0.26 ± 0.03^b</td>
<td>3.27 ± 0.40^b</td>
<td></td>
</tr>
<tr>
<td>60% Beans</td>
<td>3.18 ± 0.20</td>
<td>4.15 ± 0.14^a</td>
<td>0.46 ± 0.03^c</td>
<td>0.23 ± 0.02^c</td>
<td>2.71 ± 0.10^c</td>
<td></td>
</tr>
<tr>
<td>P-linear trend</td>
<td>0.011</td>
<td>0.006</td>
<td>0.008</td>
<td>0.037</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM, (control, n = 9; 7.5% bean, n = 8; 15% beans, n = 9; 30% beans, n = 7; 60% bean, n = 20). Data were analyzed by the Kruskal–Wallis test with Dwass–Steel–Chritchlow–Fligner test for pairwise comparisons as implemented in Systat Statistical Analysis Software. P-value for linear trend was determined by regression analysis. Values within a column with different superscripts (a, b, c) are statistically different from each other (P < 0.05). 4E-BP1, 4E-binding protein 1.

**Fig. 2. Dose-dependent effects of bean feeding on mTOR signaling components.** The images shown are those directly acquired from the ChemiDoc work station that is equipped with a CCD camera having a resolution of 1300 × 1030. A composite image of representative western blots of lysates of carcinomas from control (CTRL) rats and from rats fed 7.5, 15, 30 or 60% (wt/wt) bean in the diet. Images are for components of the AMPK-Akt-mTOR signaling network, phosphorylated and total: AMPK, ACC, Akt, p70S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1). mTOR and mammary carcinomas

mTOR is an evolutionarily conserved serine/threonine kinase that integrates external cellular stimuli with intracellular energy and nutrient-sensing pathways to regulate cellular metabolism and growth (28). Two upstream regulators were investigated. AMPK was chosen because it is the target of the widely used diabetes management drug, metformin, which is a nitrogen containing phytochemical derivative, and common bean is a rich source of small nitrogen containing compounds (29). Moreover, AMPK is a key regulator of lipid metabolism directly via effects on ACC, one of its downstream targets (30) and dietary bean affects altered lipid metabolism in cardiovascular disease (31). At the highest dietary concentration of bean, AMPK was activated and ACC was downregulated. This finding is noteworthy because AMPK-mediated suppression of ACC activity limits the ability of the cell to carry out robust lipid synthesis required for tumor growth (32–34). It is not clear whether activation of ACC was due to a specific perturbation of energy metabolism related to signaling through the IGF-1 receptor, to the effects on plasma cytokines, such as adiponectin which is known to regulate the activity of AMPK (35), or to other factors. However, since consistent activation of AMPK was
not observed at lower dietary concentrations, despite evidence of increased phosphorylation of ACC, other mechanisms are likely to be involved in mTOR regulation in mammary carcinomas. The second upstream regulator of the mTOR network that was investigated was protein kinase B (Akt). It was selected for analysis because it is a downstream effector of the insulin and IGF-1 receptors and both plasma insulin and IGF-1 were reduced dose dependently with increasing dietary concentration of bean (Figure 3C). Reduced activation of Akt was observed at lower concentrations of bean, a finding consistent with the dose-dependent effects of dietary bean on insulin and IGF-1 and the induction of apoptosis (36).

Two downstream targets of mTOR are generally evaluated to assess mTOR activity. They are 4E-binding protein 1 and p70S6 kinase (37,38). In carcinomas, the phosphorylation of both proteins was dose dependently reduced, consistent with downregulation of mTOR (Table III and Figure 3D). Through mTOR-mediated phosphorylation, 4E-binding protein 1 is de-repressed which enhances ribosomal biogenesis, and phosphorylation of p70S6 kinase by mTOR stimulates cap-dependent protein synthesis. The decreased level of phosphorylation of both proteins is consistent with reduced cancer burden and induction of apoptosis via the mitochondrial pathway (36).

In addition to mammary carcinomas, the liver was investigated because of its central role in sensing the availability to the organism of energy and nutrients and in turn controlling metabolism and the homeostatic regulation of size in peripheral tissues like the breast. mTOR activity was downregulated in the liver providing evidence that feeding bean in the diet was exerting a systemic effect on the host and as noted above, evidence of these effects was observed not only in the dose-dependent suppression of plasma growth factors, hormones and inflammatory factors but also in lower concentrations of fasting blood glucose (Table II). Moreover, the effects of bean on various nodes in the mTOR signaling network is consistent with the altered patterns of plasma lipids detected by metabolomics analysis (Figure 1D; Supplementary Table 1 is available at Carcinogenesis Online). It is noteworthy that mTOR itself has direct effects on lipid biosynthesis that are mediated via sterol response element-binding protein (38). Therefore, alterations in hepatic mTOR activity are consistent with the evidence that dietary bean impacts lipid profiles in a number of chronic disease states (31). In cancer, the possible alteration of lipid biosynthesis within carcinomas would suggest an additional mechanism of suppressed tumor burden given the reliance of tumor growth on de novo lipid biosynthesis (32–34).

Limitations
These findings provide an important foundation for new experiments to further investigate the role of dietary bean in cancer control as well as prevention. Nonetheless, the design of the current experiments necessitates that the reported data be interpreted with appropriate caution. In terms of effects on molecular pathways that regulate tissue size
homeostasis, carcinomas from untreated rats may respond differently to dietary bean than carcinomas that have emerged in bean-fed rats.

Concluding comments
Collectively, common bean appears to reduce mammary cancer burden primarily through inducing apoptosis and modifying key metabolic signaling networks linked to cell growth and survival. Part of this effect on cellular pathways is attributed to alterations in levels of systemic factors linked to mTOR-associated intracellular signaling. Understanding how dietary bean influences the interaction of systemic factors and cellular pathways, particularly its dose dependence, will be especially beneficial in guiding public health decisions regarding bean consumption for cancer control in the USA where consumption of common bean is markedly below recommended levels.

Supplementary material
Supplementary Figure 1 and Table 1 can be found at http://carcin.oxfordjournals.org/

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