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Chemopreventive Effects of Dietary Selenium and Soy Isoflavones in a Mouse Model of Prostate Cancer

Trevor Elisha Quiner

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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Brigham Young University

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ABSTRACT

Chemopreventive Effects Of Dietary Selenium and Soy Isoflavones in a Mouse Model of Prostate Cancer

Trevor Elisha Quiner

Department of Nutrition, Dietetics, & Food Science

Master of Science

Prostate cancer is the most commonly diagnosed non-skin cancer in men and the second leading cause of cancer death in the United States. Prostate cancer, like many cancers, is a disease that generally requires a long period of time to develop and grow before it becomes detectable. This long period of latency makes prostate cancer a candidate for dietary chemoprevention. Soy and selenium (Se), are associated with a decreased risk of prostate cancer.

We previously showed that high dietary intake of selenium (Se) and soy isoflavones decreased the expression of the androgen receptor (AR) and AR-regulated genes in the prostates of healthy rats. In this study we hypothesized that the downregulation of AR and AR-regulated genes would inhibit tumorigenesis in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse.

Mice were fed one of two stock diets with or without a supplement of Se in a 2 X 2 factorial design. The stock diets provided high or low dietary isoflavones. Mice were exposed to the diets from conception and sacrificed at 18 or 24 weeks of age. Prostate histopathology, urogenital tract (UGT) weight, serum IGF-1 levels, and the expression of AR and AR-regulated genes in the dorsolateral prostate was examined using quantitative PCR and Western blotting.

Urogenital tract (UGT) weight was reduced compared to control in all dietary groups containing high Se, isoflavones, or both at 24 weeks ($p<0.005$). Dietary isoflavones delayed tumor progression and downregulated protein levels of AR, AR-regulated genes, and upregulated the protective FOXO1 and FOXO3a transcription factors. High dietary isoflavones also decreased the phosphorylation of the IGF-1R. The only main effect of Se was the upregulation of AKR1C14 the enzyme that deactivates 5α-DHT.

This study identifies a previously unknown effect of isoflavones in the upregulation of FOXO expression and confirms previous studies of isoflavones'anticancer effects. Further

research is needed to find a protective dose or form of Se and to elucidate the mechanism of isoflavones.

Keywords: androgen receptor, AR, FOXO, aldo-keto reductase, IGF-1, TRAMP

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Abstract

Background

We previously showed that high dietary selenium (Se) and soy isoflavones decreased the expression of the androgen receptor (AR) and AR-regulated genes in prostatic tissue of healthy rats. In this study we hypothesized that the downregulation of AR and AR-regulated genes would inhibit tumorigenesis in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse.

Methods

Mice were fed one of two stock diets with or without a supplement of Se in a 2 X 2 factorial design. The stock diets provided high or low dietary isoflavones. Mice were exposed to the diets from conception and sacrificed at 18 or 24 weeks of age. Prostate histopathology, urogenital tract (UGT) weight, serum IGF-1 levels, and the expression of AR and AR-regulated genes in the dorsolateral prostate was examined using quantitative PCR and Western blotting.

Results

Urogenital tract (UGT) weight was reduced compared to control in all dietary groups containing high Se, isoflavones, or both at 24 weeks ($p<0.005$). Dietary isoflavones delayed tumor progression and downregulated protein levels of AR, AR-regulated genes, and upregulated the protective FOXO1 and FOXO3a transcription factors. High dietary isoflavones also decreased the phosphorylation of the IGF-1R. The only main effect of Se was the upregulation of AKR1C14 the enzyme that converts 5α-DHT to a weaker androgen; thus potentially decreasing androgen hormone action.

Conclusions

This study identifies a previously unknown effect of isoflavones in the upregulation of FOXO expression and confirms previous studies of isoflavones' anticancer effects. Further research is needed to find a protective dose or form of Se and to elucidate isoflavones' mechanism for improved prostate health.

Introduction

Prostate cancer (PC) is the most commonly diagnosed non-skin cancer in men and the second leading cause of cancer death in the United States. It is estimated that in 2010 approximately 217,730 new cases of prostate cancer will be diagnosed and an estimated 32,050 men will die from this disease (1). Approximately 1 in 5 American males will develop prostate cancer during their lifetime (1). Prostate cancer, like many cancers, is a disease that generally requires a long period of time to develop and grow before it becomes detectable. This long period of latency makes prostate cancer a candidate for dietary chemoprevention. There are many dietary components that are associated with prostate cancer risk. Some, such as processed meats and high intake of animal fat, are associated with increased risk and others, such as soy and selenium (Se), are associated with decreased risk (2-3).

Despite the failure of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) to demonstrate a protective effect of supplemental Se on prostate cancer (4), a failure agreed upon by many to be the fault of poor study design, there is still a great deal of evidence that methylated-Se compounds, other than the selenomethionine used in SELECT, have chemopreventive effects *in vitro* and *in vivo* (5-8). Recent studies have shown that Se-methylselenocysteine (SMSC) and methylseleninic acid (MSA) downregulate the androgen receptor (AR) and many AR-regulated, prostate cancer-associated genes (9-12). These effects have been documented in cultured prostate cancer cells, healthy animal models, and in a mouse model of prostate cancer model (8).

The AR is central to prostate cancer growth and promotion. Even castration-resistant prostate cancer (CRPC), which is unaffected by androgen ablation, requires AR induction of

transcription for growth and survival (13-14). In some cases CRPC overcomes androgen ablation through localized production of androgen, by mutation of AR which renders it constitutively active, or by promiscuously binding to a variety of ligands (14). In all of these cases AR remains a significant determinate of tumor growth. Therefore any pharmaceutical or nutritional intervention that inhibits AR directly could be therapeutic in localized and advanced prostate cancer.

Soy isoflavones have also been shown to inhibit the expression of AR in cell culture and animal models (15-16). This is likely one of the mechanisms that accounts for the epidemiological studies of high soy consumption and very low rates of prostate cancer incidence (2,17). Many studies have demonstrated additional anti-cancer effects of individual soy isoflavones such as genistein (18-20).

We have previously shown changes, consistent with chemoprevention, in AR and AR-regulated gene expression in healthy rat prostates due to the combination of high dietary intake of Se and soy isoflavones (12). In this study we examined the same diets for their effects on prostate cancer development and progression in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model. Only one previous study (Wang et al. (7)) has examined effects of Se in TRAMP mice. In that work MSA and SMSC were given by gavage at doses that far exceeded the concentrations provided by the Se-supplemented diets in this study.

Materials and Methods

Animals

All procedures related to animal care and use were approved by the Institutional Animal Care and Use Committee of Brigham Young University.

Male and female heterozygous C57BL/6 transgenic TRAMP mice, and male and female FVB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The TRAMP mice were crossed to generate homozygous C57BL/6 TRAMP mouse breeders. Homozygous TRAMP breeders were crossed with wild-type FVB breeders to produce the heterozygous transgenic male pups (F1) used in these studies. Throughout the experiment, the animals were housed in cages with wood chip bedding in a temperature-controlled room (69–72°F) with a 12-h light-dark cycle.

Diets

Mice were fed one of two stock diets with or without a supplement of Se in a 2 X 2 factorial design. One stock diet was the Zeigler Rodent Phytoestrogen Reduced I formulation which provides approximately 10 ppm isoflavones (21) and 0.37 ppm Se. The other diet was the Harlan-Teklad 8604 formulation which provides approximately 600 ppm isoflavones (22) and 0.33 ppm Se. Half of the animals received diets that were supplemented with 3.0 ppm Se as Se-methylselenocysteine (Kelatron Corp., Ogden, UT), which was added to the basal formulations at the time of production.

Study Design

To ensure that F1 pups used as subjects in this study would be exposed to their respective dietary treatments from conception, breeders were fed their respective diets for 30 days prior to mating. Dams continued to receive their respective diets during lactation and pups were weaned to the same diets consumed by their parents. Pups continued to consume their respective diets until they were killed at 18 or 24 weeks of age by decapitation. Urogenital tracts (UGT), including bladder, seminal vesicles, and prostate lobes, were removed and weighed. Dorsolateral prostate (DLP) lobes were dissected and either snap-frozen in liquid nitrogen for RNA or protein

isolation, or fixed in 4%-paraformaldehyde in PBS for 24 hours prior to histological processing. Blood was collected and serum was isolated for use with ELISAs. Livers were dissected and flash-frozen in liquid nitrogen and stored until subsequent enzyme activity assays. Frozen samples were stored at -80°C until analyzed.

Glutathione Peroxidase Activity

Liver samples (approximately 0.5 g) were homogenized in three volumes of ice-cold 0.25 mol/L sucrose buffer, prepared according to the method of Lawrence and Burk (23). Homogenates were centrifuged at 105,000 x g for 1 h and the supernatant was collected as cytosol. Cytosolic Se-dependant glutathione peroxidase (GPx) activity was assayed by the coupled method of Lawrence and Burk (1976) using 2.0 mmol/L reduced glutathione, and 0.25 mmol/L H_2O_2 as substrate. Protein concentration was determined by the Coomassie Plus (Bradford) Protein Assay (Thermo Scientific, Rockford, IL). One unit of enzyme activity was defined as 1 μmol NADPH oxidized/min.

Histopathology

After fixing for 24 hours in 4% paraformaldehyde samples were transferred to 70% ethanol until processed. At processing, the fixed tissues were dehydrated in ascending grades of ethanol and xylene, before being embedded in paraffin. Sections (5 μm) were cut with a microtome and mounted on microscope slides. Tissues sections were processed and stained with hematoxylin and eosin (H&E) for routine histopathologic evaluation. Slides were examined by pathologist a board-certified pathologist (PMU) blind to the treatments and by a trained associate (TQ) and classified according to the grading scheme developed by Suttie A et al. (24). Slides are ranked from 1-6 according to the severity of the lesions present in the DLP: 1- normal gland

structure with minimal hyperplasia, 2- papillary structures and some piling of cells with mostly normal gland structure (low-grade prostatic intraepithelial neoplasia (PIN) , 3- hyperplasia and cribiform forms fill much of the lumen but the shape of the gland is not compromised (high grade PIN), 4-hyperplasia completely fills the lumen and the gland structure (well-differentiated prostate cancer), 5-epithelial cells expand the lumen and/or are in a well-defined mass with beginnings of invasion into basement membrane (moderately-differentiated prostate cancer), 6-Poorly differentiated epithelial tissue, local invasion, or distant metastasis.

Gene selection process

Genes selected for analysis in this study were those identified by Zhang et al. (10)that met three criteria: 1) they are dysregulated in human prostate cancer, 2) they are AR-regulated and 3) the androgen effect is opposed by Se. By microarray analysis, they identified 422 AR-regulated genes in LNCaP human prostate cancer cells, and over 1000 Se-regulated genes in the same cell line. Comparison of the two lists revealed 92 genes regulated by both Se and androgen, of which 37 were reciprocally regulated. These authors also reported differences in gene expression found in three independently published microarray analyses of gene expression in human prostate tumors compared to normal human prostate tissue. Over 1000 genes appeared in all three reports. Of the 37 genes reciprocally regulated by androgen and Se in LNCaP cells, 6 were among the genes dysregulated in prostate cancer: FACL3 (fatty acid CoA ligase 3; also known as ACSL3), GUCY1A3 (guanylate cyclase alpha 3), DHCR24 (24-dehydrocholesterol reductase), ABCC4 (ATP-binding cassette sub- family C member 4), human kallikrein 2 and kallikrein 3. Kallikrein 2 and kallikrein 3 have no homologs in rodents. In addition to these AR- and Se-regulated genes relevant in prostate cancer, we examined expression of AR itself and of AK1C14 - the gene for the enzyme which can catalyze the reduction of dihydrotestosterone to

the less active 3α-androstenediol. These are the same genes studied by Legg et al. in our previous work exploring Se and isoflavone effects in healthy rat prostate tissue (12).

Zhang et al. also identified forkhead box proteins dysregulated in prostate cancer, the regulation of which is reversed by high Se treatment. Interestingly these Se-regulated forkhead box proteins interact with AR, affecting the expression of AR-regulated genes. Accordingly, we determined to assess the effects of Se and isoflavones on expression of FOXA1, FOXO1a, and FOXO3a. Finally, we sought to confirm the report of Wang et al. (8) concerning the effects of Se on the IGF-1 axis in the TRAMP model by measuring the expression of IGF-1 in the prostate as well as IGF binding protein 3 (IGFBP3), an important regulator of IGF-1 activity. The genes examined in this study and the role each plays in metabolism are summarized in Table 1.

Steady state mRNA quantitation - qPCR

RNA was isolated from mouse dorsolateral prostates using the RNeasy kit (QIAGEN, Germantown, MD). Concentration and purity of RNA were determined spectrophotometrically (Nanodrop,Thermo Scientific, Rockford, IL), and RNA integrity was verified using Experion RNA StdSens Analysis chips in the Experion system (Biorad, Hercules, CA). Equal quantities of RNA from each of five individual DLP RNA isolations within each dietary group were combined to form a total RNA pool for that group. Total RNA pools were reverse transcribed using random hexamers as primers. PCR Primers (Table 2) were designed using NCBI's "Primer-BLAST" website. Optimum temperatures for primer annealing were determined experimentally for each primer pair using a range of annealing temperatures (RoboCycler, Stratagene, La Jolla, CA) followed by gel electrophoresis to confirm amplification of a single band of the expected size. First strand cDNA was used as a template in quantitative PCR analysis

(LightCycler, Roche, Mannheim, Germany). For each gene, at least three LightCycler runs were performed. Each run included three replicates for each dietary group. Steady state mRNA levels for the invariant genes HPRT and TBP (25) were also quantified and used for normalization. Concentration for each sample was calculated based on an internal standard curve using the second derivative maximum of the amplification curve as calculated by LightCycler software v2.0 (Roche). Each sample was normalized using the average of the concentrations of HPRT and TBP.

Immunoblot analysis of DLP lysates

Equal quantities of protein from each of four or five DLP lysates within each dietary group were combined to form a total protein lysate pool for Western blotting in each dietary group for both 18 and 24 week subjects. The tissues were homogenized in modified radioimmunoprecipitation (RIPA) buffer (50 mmol/L Tris-HCl(pH 7.4),150 mmol/L NaCl, 1% NP-40, 1 mmol/L EDTA, 1 mmol/L PMSF, 0.25% sodium deoxycholate, 0.5% NP40, 1ug/mL aprotinin/pepstatin/leupeptin) with dissolved PhoSTOP, phosphatase inhibitors (Roche, Mannheim, Germany). Protein concentration in lysates was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). For immunoblot analyses, 50 μg of protein per pool were denatured in LDS Sample Preparation Buffer and subjected to electrophoresis on 4-12% Bis-Tris gels (Invitrogen, US). The separated proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) followed by blocking with 5% non-fat milk powder (w/v) in PBST (1XPBS, 2% Tween 20). The membranes were probed with primary antibodies for AR (Novus Biologicals, Littleton, CO), Gucy1a3, Acsl3, FoxO1, FoxO3a, FoxA1 pIGF1R, IGFR1 (Abcam, Cambridge, MA), Dhcr24, and Akr1c14 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The membranes were then probed with the appropriate peroxidase-conjugated secondary

antibodies (Novus Biologicals and Abcam) and detected by enhanced chemofluorescence (Thermo, Rockford. IL). Band density was measured using ImageJ software (NIH-add url).

Serum IGF-1 Analysis

Serum IGF-1 levels were measured using a commercially available kit (R&D Diagnostics, Greece) according to the manufacturer's instructions.

Statistical analysis

For each gene, statistical analysis was performed on the nine normalized replicates for each dietary treatment using ANOVA, followed by Fisher's pairwise comparison to determine significance of differences between dietary groups (Minitab, State College, PA). In the process, a normalized mean value was calculated for each dietary group. Finally, to compare relative expression among the four dietary groups for each gene, the HPRT/TBP-normalized mean for each of the other dietary groups was divided by the HPRT/TBP-normalized mean of the adequate Se/low isoflavone group. All bar graphs (Figure 1, 4-7) show the mean for each group with error bars showing the standard error.

Due to the non-normal distribution of the UGT weights for each diet group ANOVA was not appropriate. Instead the Kruskal-Wallis test, which uses rank order of samples to find statistical differences in non-normally distributed data sets, was used. Pairwise comparisons were made using Bonferroni's correction for a 95% CI (Minitab, State College, PA).

Results

GPx1 Activity

There were no significant differences due to diet in the activity of hepatic GPX1 (Figure 1). This was expected as all the diets provided a concentration of Se higher than needed to maximize the activity of GPx1 in mouse liver. The mean values obtained for activity 400-600 mU/mg total protein are consistent with previously reported levels of GPx1 activity in healthy rodent liver (26).

Measurements of Prostate Tumor Burden and Progression

Urogenital Tract Weight

The presence of one or more exceptionally large tumors in most groups at both 18 and 24 weeks of age resulted in a non-normal distribution in data for UGT weights. ANOVA is inappropriate for use with data with non-normal distribution. Instead the non-parametric, rank-sum based Kruskal-Wallis test was used to analyze differences between groups. At 18 weeks (Figure 2A) only the mice fed the adequate Se/ high isoflavone diet had significantly lower UGT weight compared to the mice fed the control diet (adequate Se/ low isoflavone). However, at 24 weeks (Figure 2B) mice fed any combination of high Se and/or high isoflavones had significantly lower UGT weights than the adequate Se/ low isoflavone dietary group.

Histopathology

As shown in Fig. 3A the proportion of mice with higher grade carcinomas (4-6) to lower grade PIN lesions (1-3) at 18 weeks was lower in the mice fed high isoflavone diets. This suggests a protective role of isoflavones in slowing the progression from PIN to carcinoma even in the fast progressing TRAMP model.

DLP Gene Expression

The control group, used as the reference for all measures of relative gene expression, was comprised of animals fed the adequate Se/ low isoflavone diet. Western blot lanes are all from the same blot but have been rearranged to present data in terms of high or low isoflavone diets.

Androgen Receptor

At 18 weeks mice fed the high Se/ high isoflavone diet had significantly higher levels of AR mRNA compared to control (Figure 4A) while the high Se/low isoflavone dietary group had significantly lower levels of AR mRNA (Figure 4A). Western blotting revealed a significant decrease in AR protein levels in animals fed the high isoflavone diets regardless of Se levels (Figure 4B). There was also a significant increase in AR protein levels in the high Se/high isoflavone diet. At the 18 week data collection, in general, there was a lack of correspondence between mRNA and protein levels that will be discussed subsequently.

At 24 weeks of age AR mRNA levels were mostly equal between diet groups except for a significantly higher level in the high Se/ low Isoflavone diet (Figure 4A) dietary group. The high Se/ low Isoflavone and adequate Se/ high isoflavone dietary groups had increased AR protein levels compared to the control dietary group (Figure 4B). For this time interval there was correspondence between mRNA and protein levels.

AR-Regulated Genes

GUCY1A3

At 18 weeks all diets had significantly lower levels of GUCY1A3 mRNA compared to the adequate Se/ low isoflavone diet (Figure 5A). However, GUCY1A3 protein levels were significantly lower in the high isoflavone diets and supplemental Se slightly increased protein

levels (Figure 5F). At 24 weeks both GUCY1A3 mRNA and protein levels were significantly lower only in the mice fed high isoflavone diets (Figure 5A and F).

DHCR24

At 18 weeks the main effect of isoflavones was significant in the higher levels of DHCR24 mRNA compared to control. At 24 weeks the main effects of Se in increasing mRNA levels of DHCR24 was significant (Figure 5B) Western blot results revealed a somewhat different pattern—at 18 weeks the main effects of isoflavones were significant in reducing levels of DHCR24 protein levels. At 24 weeks protein levels were not significantly different between diet groups (Figure 5F).

ACSL3

At 18 weeks the main effect of high isoflavone diet in reducing ACSL3 mRNA and protein levels was significantly (Figure 5D and F). At 24 weeks mice fed the high Se/ high isoflavone diet still had significantly lower levels of ACS3 mRNA (Figure 5D).

ABCC4

At 18 weeks all diets had significantly higher levels of ABCC4 mRNA compared to the adequate Se/ low isoflavone diet (Figure 5E).

AKR1C14

At 18 weeks the effects of Se were highly significant in increased levels of AKR1C14 mRNA and protein levels. At 24 weeks mice fed the adequate Se/ high isoflavones had significantly higher levels of AKR1C14 mRNA and protein (Figure 5C and F).

AR-Associated Forkhead Box Transcription Factors

FOXA1

At 18 weeks the main effect of isoflavones was significant in increased levels of FOXA1 mRNA (Figure 6A). At 24 weeks the main effect of Se in increasing mRNA levels of FOXA1 was significant. Western blot results revealed a somewhat different pattern—at 18 weeks the main effect of isoflavones was significant in reducing levels of FOXA1 protein levels (Figure 6D). At 24 weeks the FOXA1 protein level of the high Se/ high isoflavone dietary group remained significantly lower than other dietary groups (Figure 6A).

FOXO1a and FOXO3a

The main effects of isoflavones at 18 and 24 weeks were highly significant in increased mRNA and protein levels of FOXO1a and FOXO3a (Figure 6B, C, and D). The high Se/ high isoflavone diet had significantly higher mRNA and levels at 18 weeks for both genes.

IGF-1 Signaling

There were no significant differences in serum IGF-1 between diets (data not shown). Analysis of DLP IGF-1 mRNA levels showed a significant main effect of both Se and isoflavone in reducing IGF-1 mRNA levels at 18 weeks (Figure 7A). There were significant main effects of both isoflavones and Se in increasing IGF-1 mRNA levels at 24 weeks (Figure 7A). The expression of IGFBP3 as shown in Figure 7B is consistent with the decreased IGF-1 levels suggested by the mRNA levels. Probing of Western blots for phosphorylated IGF1R showed a decrease in phosphorylation in mice fed high isoflavone diets (Figure 7C).

Discussion

Our previous study in which healthy rats were fed diets high in Se and isoflavones showed effects in the prostate consistent with prevention of cancer (12). The purpose of this study was to determine if the same dietary treatments would reduce tumorigenesis in an animal model of prostate cancer (27), and to identify possible mechanisms for this effect. In this study high dietary intake of soy isoflavones decreased tumor burden and progression as indicated by reduced UGT weight (Figure 2) and histopathology (Figure 3A). The changes in gene expression observed in the high isoflavone dietary groups are consistent with those we and others have previously reported (12,16,28).

In contrast, beneficial effects of high supplemental SMSC were confined to UGT weights (Figure 4). Wang et al. also reported a reduction in UGT weights in TRAMP mice given supplemental Se. However, in their study that difference was statistically significant at 18 weeks while in this work statistically significant effects of Se were not seen until 24 weeks of age. They also reported reductions in circulating IGF-1 levels and in phosphorylation of IGF-1R which were not seen in this work. Differences between the two studies include the composition of the basal diet, and the method and level of Se dosing. Wang et al. fed TRAMP mice the purified AIN-93M formulation containing negligible amounts of isoflavones and administered supplemental SMSC and SMA by bolus oral dosing of 3 mg Se per kilogram of body weight five days a week. In contrast, mice in this study received stock diets in which supplemental Se was included in the formulation. Assuming that a 20 g mouse consumes 5 g of diet/day containing a supplement of 3.0 mg Se/kg diet as SMSC, that mouse would consume a supplement of 0.75 mg Se/ kg BW/day, or only 25% of the dose given to mice in the study of Wang et al. The level of Se supplementation in this study was chosen based on the report of Medina et al. showing that

the maximum tolerable dose of dietary SMSC in rats is 5.0 ppm Se (29).This level of supplementation resulted in significant differences in several parameters in our previous study of healthy rats which were not seen in this experiment with TRAMP mice. This suggests that a higher dose of Se may be required in mice than in rats to observe potentially protective effects against cancer. This difference has not been investigated but in terms of pharmaceutical studies there are differences between species (30).

There have been many studies done on the effects of purified isoflavones in cancer models, including a number of studies in the TRAMP model with supplemental genistein (18,31). While supplemental genistein was shown to be protective against poorly differentiated tumor development when supplementation began before maturity, a later study by the same group showed that a moderate dose of genistein begun later in life actually induced a more aggressive prostate cancer with increased incidence of poorly differentiated tumors and metastasis (32). Other data show the superiority of a combination of genistein and daidzein, or other isoflavone combinations compared to supplementation of genistein alone (33). This study makes use of a soy-based stock diet that is naturally high in isoflavones and more closely mimics the high soy diets in Asia that have been associated with a decreased risk for prostate cancer (22). Notably, in brief, consumption of soy-containing diets in rodents results in high equol levels (representing 70-90% of the total circulating isoflavone levels. Equol, an intestinal metabolite of daidzein, binds ER beta with high affinity that is known to down regulate AR expression in the prostate (16). This notion is supported by the observed significant decrease in AR protein levels at 18 weeks of high isoflavone consumption regardless of Se supplementation. This concept of ER subtypes involvement in regulatory androgen hormone action via AR protein expression is supported by soy dietary consumption in the present study at the 18 week treatment interval

(figure 4). On the other hand, this effect is not seen at 24 weeks, at which time point the TRAMP mouse is characterized by invasive, poorly differentiated tumors and metastases (27). However, it is likely that the downregulation of the AR and AR-regulated genes seen at 18 weeks is at least partially responsible for the decreased tumor size at 24 weeks. There is a difference in the pattern of mRNA and protein level expression. This data suggests a post-transcriptional regulation of AR by isoflavones possibly involving the FOXO proteins.

GUCY1A3 is a subunit of an enzyme that catalyzes the production of cyclic guanosine 3′,5′-monophosphate (cGMP) in response to NO signaling (34); cGMP regulates kinases, ion channels, and phosphodiesterases (35). The silencing of GUCY1A3 with siRNA in glioma cells revealed that it is an upstream regulator of VEGF, and as such when these cells were injected into a nude mouse their ability to form a tumor was severely decreased (36). The downregulation of GUCY1A3 by isoflavones in the DLP is consistent with chemoprevention.

Acyl-CoA synthetase long-chain family member 3 (ACSL3) catalyzes the production of long-chain fatty acyl-CoA, which inhibits the machinery of *de novo* fatty acid synthesis including the vitally important fatty acid synthase (FAS) (37). Inhibition of FAS has been shown to induce senescence or apoptosis in normal and cancer cells through various mechanisms such as the toxic build-up of malonyl-CoA, inhibition of β-oxidation (38) , the starvation of phospholipids and other membrane components synthesized by FAS-dependent processes (39-40), and the inhibition of DNA replication (40). The high expression of FAS in breast and prostate tissue associated with advanced tumors is being evaluated as a potential marker for tumor progression and prognosis (38,41). Current research is focusing on FAS inhibition for drug development and thus any dietary element that induces ACLS3 would be expected to be protective, while any effect that decreases its activity could be considered non-protective in

cancer (41). Isoflavones did decrease the expression of ACSL3 at 18 weeks but the decrease was not as dramatic as with GUCY1A3 (Figure 5D).

MRP4/ABCC4 is a member of the ATP-binding cassette transporter family. These proteins are responsible for the ATP driven efflux of pharmaceuticals and other molecules across epithelial membranes (42-43). ABCC4 is expressed mostly in the prostate and has been found to be expressed up to three times higher in prostate tumor tissue samples (42). The efflux of prostaglandins by ABCC4 may be an important part of PC development as this efflux may induce inflammation and which could help promote hyperplasia of prostate epithelial cells (44-46). Since the upregulation of ABCC4 appears to be an important part of prostate carcinogenesis, and the inhibition of ABCC4 may lead to greater drug sensitivity the antagonism of this gene may be protective in prostate cancer and a potential drug target. ABCC4 mRNA was upregulated by all intervention diets at 18 weeks (Figure 5E). The increase in mRNA levels in high isoflavone diets may be due to the increased need to flux isoflavones from the prostate; but there is no data supporting this in the literature but it is possible.

DHCR24 or seladin-1 is an enzyme with several different functions that was originally described as an integral enzyme in the synthesis of cholesterol from desmosterol (47). DHCR24 is expressed ubiquitously and is AR-regulated in the prostate (48-52) . Closer study of DHCR24 has revealed its ability to resist the effects of β-amyloid/oxidative stress and prevent apoptosis by inhibiting caspase-3 and acting as a ROS-scavenger (53). An interesting phenomenon occurs with the prostate expression of DHCR24 during prostate cancer progression. In low-grade, androgen-sensitive, prostate tumors DHCR24 is highly expressed. However, as the tumors lose androgen sensitivity the DHCR24 expression drops drastically (51,54-55). This effect may be explained by the observation that while DHCR24 does inhibit apoptosis, it also induces

senescence and is pro-differentiation (54). The loss of DHCR24 expression in CRPC tumors may contribute to the increased pace of proliferation and invasion. It has been shown with human prostate cancer tissue that androgen-dependent tumors have significantly higher expression of DHCR24 compared to metastatic androgen insensitive tumors even before these phenotypes are observed, suggesting that tumor levels of DHCR24 may be diagnostic (54-55). High isoflavones decrease protein levels of DHCR24 at 18 weeks which may sensitize DLP cells to apoptosis when challenged with oxidative stress.

Members of the AKR1C family in humans are reductases that have high specificity for steroid hormones including 5α-DHT. These AKRs are an important component of regulation of ligand availability for AR (48-50). While there is some question as to the homology of human AKR1C proteins and their rat/mouse analogs in terms of expression patterns and function, AKR1C14 is recognized to be a 3α-hydroxysteriod dehydrogenase (3α-HSD) and have the ability to convert 5α-DHT to the less active 3α-androstanediol (56). In this context increased AKR1C14 expression seems protective against prostate cancer in that it can inactivate 5α-DHT. The most significant effect of Se in this study was the upregulation of the AKR1C14 gene (Figure 5C). Whether this served to decrease the activity of androgen it was not determined.

FOXA1 belongs to the family of forkhead box proteins originally described as being essential in endoderm development (57-58). FOXA1 has been described as working together with AR, binding near AREs, facilitating AR binding, and making transcription possible for AR-regulated genes in humans and mice (58-59). FOXA1 is upregulated in prostate cancer and is even more abundant in CRPC. Inhibition of FOXA1 protein levels by high isoflavone diets may be one of the mechanisms by which isoflavones decrease the expression of AR-regulated genes such as GUCY1A3, ACSL3, and DHCR24 (59).

FOXO1 and FOXO3a are both members of the forkhead box O family transcription factors. These proteins were originally found at the site of chromosomal translocations in tumors which suggested that they may be tumor suppressors (60). FOXO proteins induce the expression of FAS ligand, TRAIL, BIM, $p27^{kip1}$, MnSOD, and IGFBP1 and thus are integral in the regulation of cell proliferation and apoptosis (61). FOXO1 has been shown to bind directly to the N-terminus domain of AR and induce its nuclear export and inactivation (62-63). Activation of Akt/PKB induces the phosphorylation and cytoplasmic localization of FOXO proteins and inhibition of activity. Decreased protein levels of FOXO1 and FOXO3a are seen in localized prostate cancer and even more so in CRPC (61). Isoflavones' maintenance/induction of high FOXO1 and FOXO3a levels is a novel observation and provides insight as to a possible mechanism for isoflavone down regulation of AR.

The IGF-1 signaling pathway is an active area of research in prostate cancer work. There is evidence that increased levels of serum IGF-1 are associated with prostate cancer (64-66), and that prostate stromal cells paracrine signaling using IGF-1 can play a role in CRPC (67). While there was no evidence of different serum levels of IGF-1 in the mice there was a significant decrease in prostate IGF-1 mRNA levels at 18 weeks (Figure 7A) with both high Se and isoflavones. Added to this observation are the Western blots that revealed that high isoflavone diets significantly reduced the phosphorylation of the IGF-1R which may correlate with decreased Akt/PKB activation (Figure 7C). This possible inactivation of the IGF-1R/Akt pathway may explain the increased levels of the FOXO proteins which are downregulated by Akt (61-62). Furthermore, as expected with decreased IGF-1 levels, prostate IGFBP3 mRNA levels decreased at 18 weeks in each intervention dietary group.

While the chemopreventive effects of Se in this study were far from dramatic there is some evidence for an interaction between high dietary Se and the isoflavone content of the diet that provided additional protection in some cases. While high Se increased expression of FOXA1 when added to the low isoflavone diet, it decreased expression when added to the high isoflavone background and, unlike the other diets. Those decreased expression persisted even at 24 weeks (Figure 6A). When added to the low isoflavone diet high Se decreased FOXO1 and FOXO3a levels at 18 weeks (Figure 6B-D), but when added to the high isoflavone diets it increased protein levels modestly compared to the high isoflavone diet alone. Those increased protein levels persisted to 24 weeks only in the high Se/ high isoflavone dietary for FOXO3a. These interactions suggest that the effects of Se in prostate chemoprevention may in part be dependent on the content of the diet to which the Se is added.

Conclusion

This study is the first to provide evidence of isoflavones influencing the expression of the forkhead box O proteins as a possible mechanism for isoflavones' well documented inhibition of AR. While this study did not examine the effects of isoflavone on estrogen receptor (ER) expression signaling it is almost certain that many of the effects observed in the high isoflavone diets are mediated through ERβ and/or ER subtypes via mechanisms that are currently unknown. However, further research is warranted to determine whether these isoflavone effects are ER mediated or otherwise and which ER subtypes are activated. To better understand Se's role in chemoprevention a better understanding of effective and toxic dosages for all species must be obtained. Finally, possible molecular and biochemical mechanisms for interactions between Se and isoflavones need to be better explored In order to pursue better treatments to improve prostate health.

Tables and Figures

Gene Name	Protein	Description
AR	Androgen Receptor	Nuclear hormone receptor that binds to androgen and induces transcription of AR-regulated genes.
GUCY1A3	Guanylate cyclase 1 subunit alpha	Catalyzes the production of cGMP in response to NO signaling, can induce VEGF expression
ACSL3	Acyl-CoA synthetase long-chain family member 3	Catalyzes the production of long-chain fatty acyl-CoA; inhibits anti-apoptotic fatty acid synthase (FAS).
DHCR24	24-dehydrocholesterol reductase/ seladin-1	Synthesis of cholesterol from desmosterol; inhibition of apoptosis-inducing caspase 3.
ABCC4	ATP-binding cassette, sub-family C4	Efflux of prostaglandins and some drugs from the cell.
AKR1C14	Aldo-keto reductase family 1 member C14	Conversion of 5α-DHT to 3α-androstendiol and androstenedione into testosterone.
FOXA1	Forkhead box A1	Transcription factor; involved in AR mediated expression.
FOXO1	Forkhead box O1	Transcription factor; involved in upregulation of p27, FasL, TRAIL etc. Also interacts with AR.
FOXO3a	Forkhead box O3a	Transcription factor; similar to FOXO1 in function.
IGF-1	Insulin-like Growth Factor	Important growth factor; associated with increase cancer risk.
IGFBP3	IGF Binding Protein 3	Binds to IGF-1 outside of the cell and prevents its activity.

Table I Genes examined with a brief description of function.

Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')
AR	CTGGGAAGGGTCTACCCAC	GGTGCTATGTTAGCGGCCTC
GUCY1A3	CCCCTGGTCAGGTTCCTAAG	GGAGACTCCCTTCTGCATTCT
ACSL3	AACCACGTATCTTCAACACCATC	AGTCCGGTTTGGAACTGACAG
AKR1C14	GTGTGGTACTAAACGATGGTCAC	CAAATAAGCGGAGTCAAAATGGC
DHCR24	CTCTGGGTGCGAGTGAAGG	TTCCCGGACCTGTTTCTGGAT
ABCC4	GCTCGAGCATCCTCACCCGC	CGGGTTGAGCCACCAGAAGAACA
FOXA1	TACTGGACGCTGCACCCGGA	TGCGACTTTCTGGGCCCCCT
FOXO1	CCCAGGCCGGAGTTTAACC	GTTGCTCATAAAGTCGGTGCT
FOXO3A	CTGGGGGAACCTGTCCTATG	TCATTCTGAACGCGCATGAAG
IGF-1	GCTCCAGCATTCGGAGGGCA	ACGGGGACTTCTGAGTCTTGGGC
IGFBP3	GAAGCAGTGCCGCCCTTCCA	GGCTCTGCACGCTGAGGCAA
DIO1	AGGGCACTCACTCACATGCTTG	AGCAGAACATGCCTGCCTCTTG

Table II Primers used in qPCR.

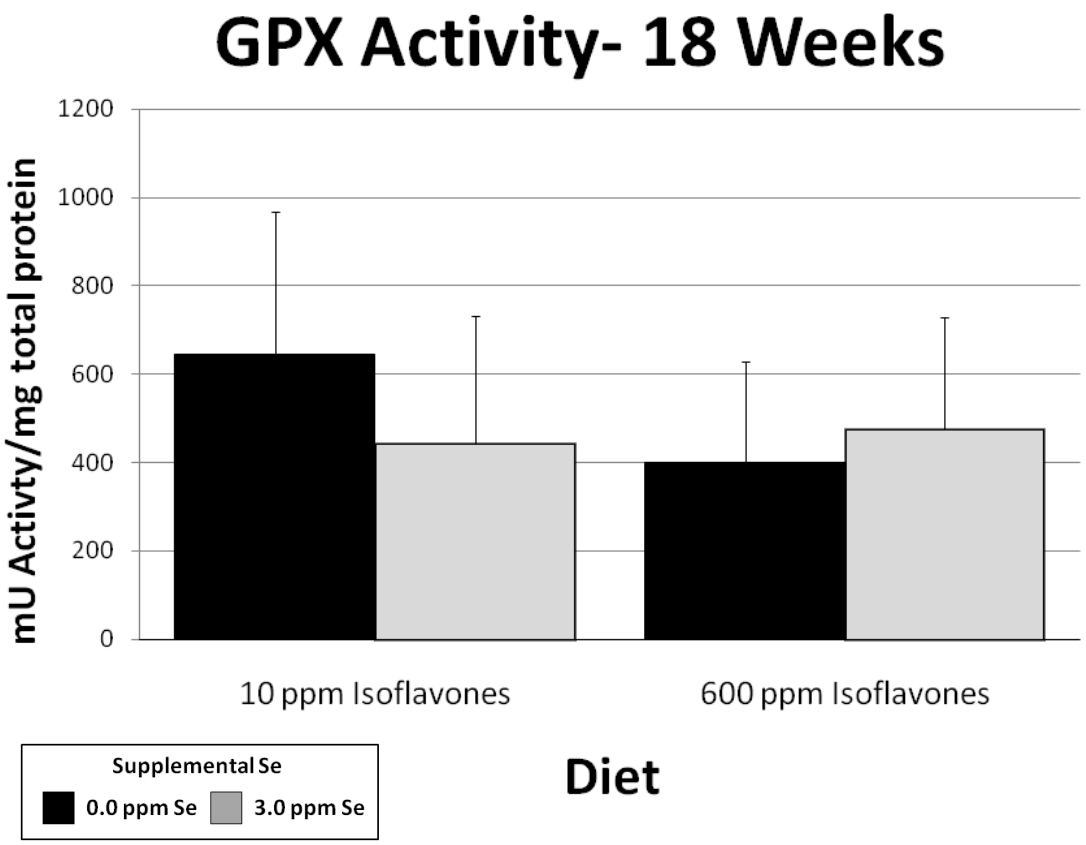

Figure 1 GPx activity is unchanged by supplemental Se.

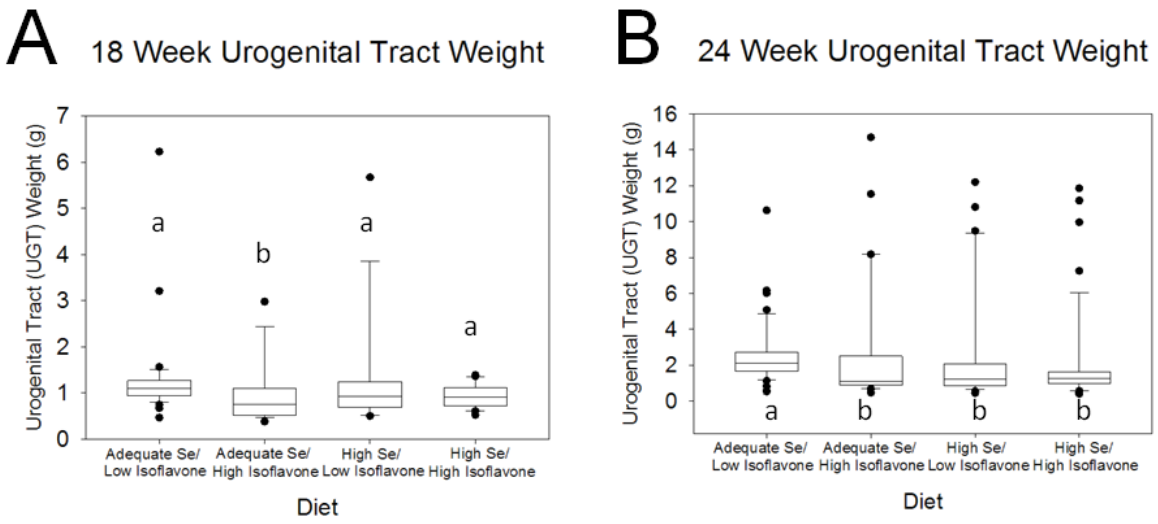

Figure 2 Dietary isoflavones and supplemental Se decrease UGT weight.

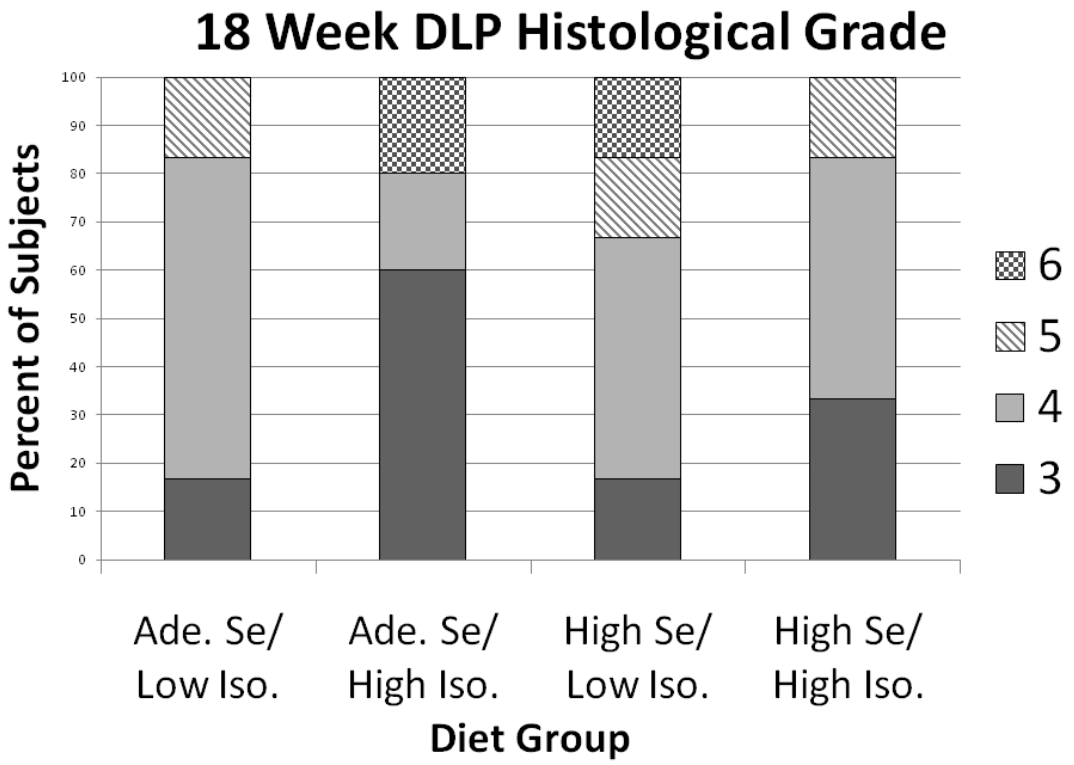

Figure 3 Dietary isoflavones inhibit tumor progression.

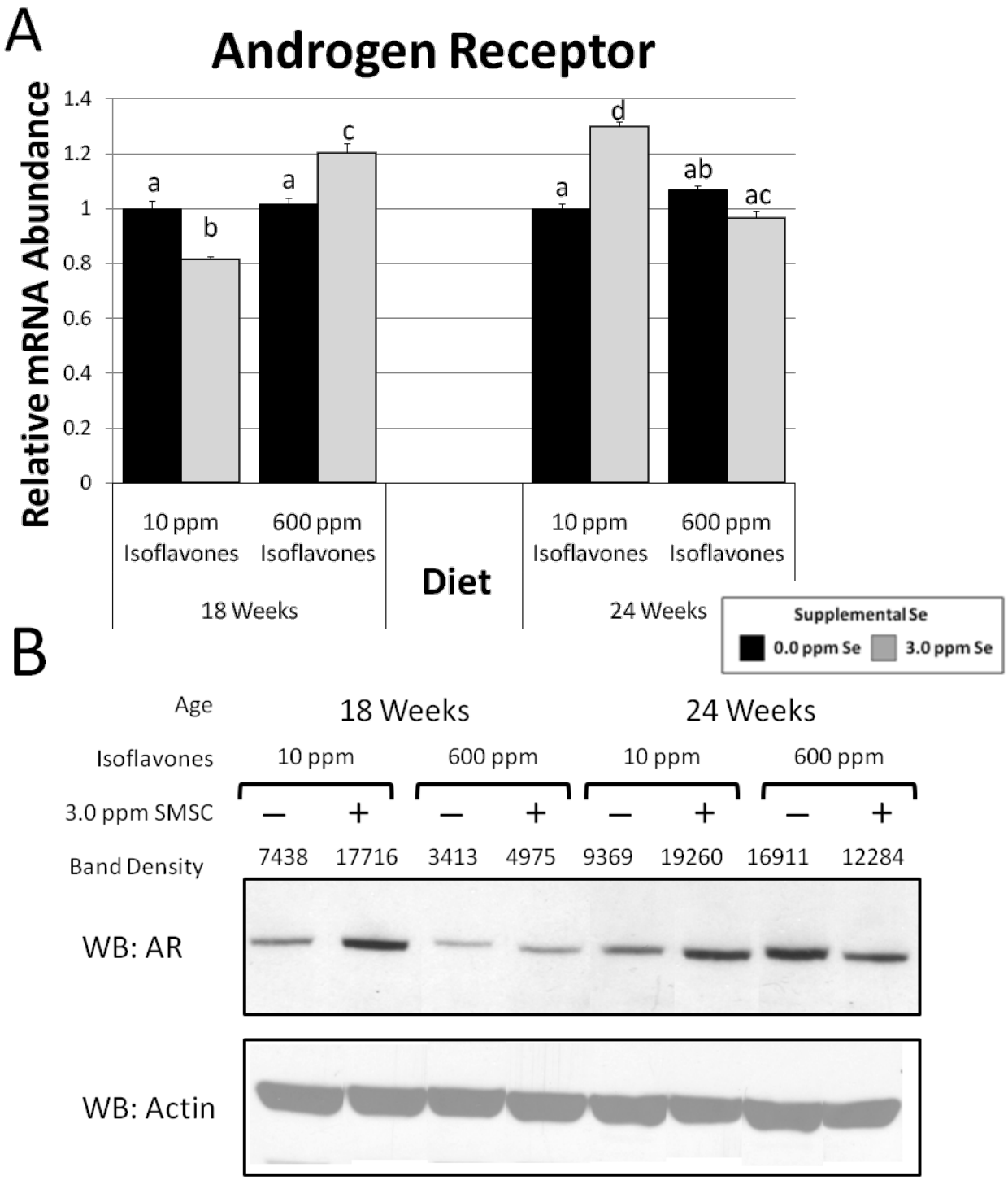

Figure 4 High dietary isoflavones decrease AR, high dietary Se increases AR.

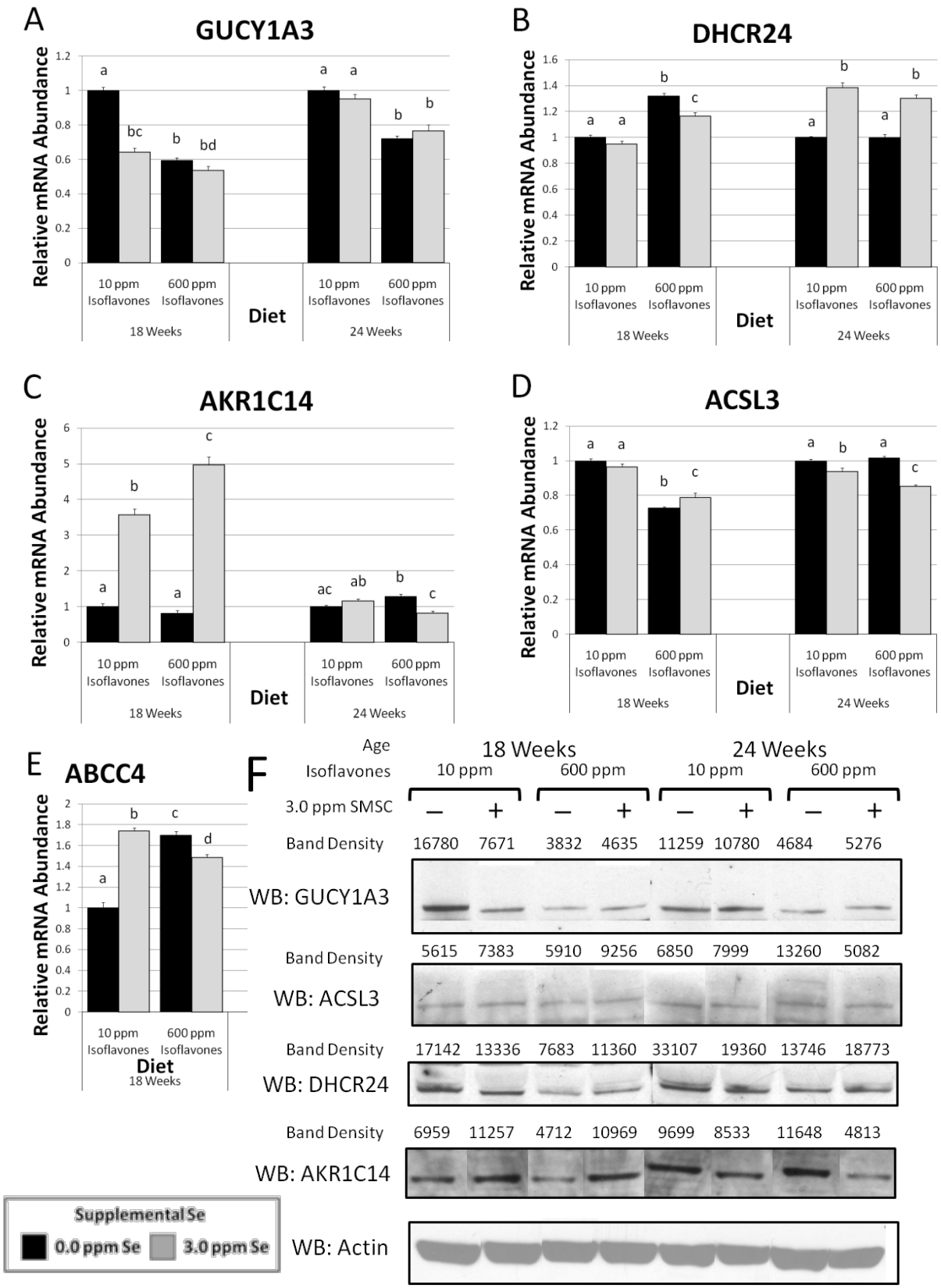

Figure 5 Dietary isoflavones and Se regulate the expression of some AR-regulated genes.

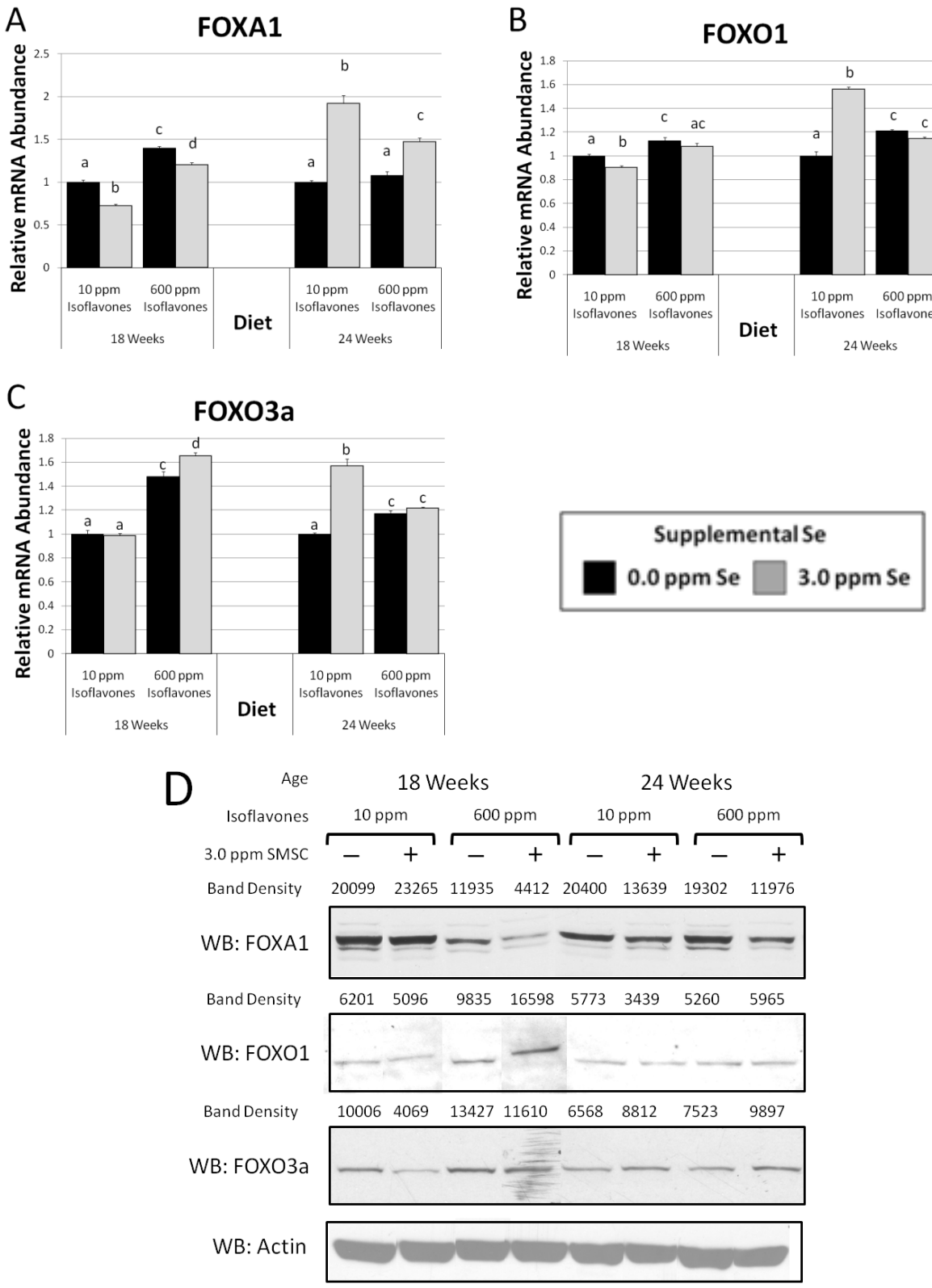

Figure 6 Dietary isoflavones induce a pattern of forkhead box protein expression consistent with chemoprevention.

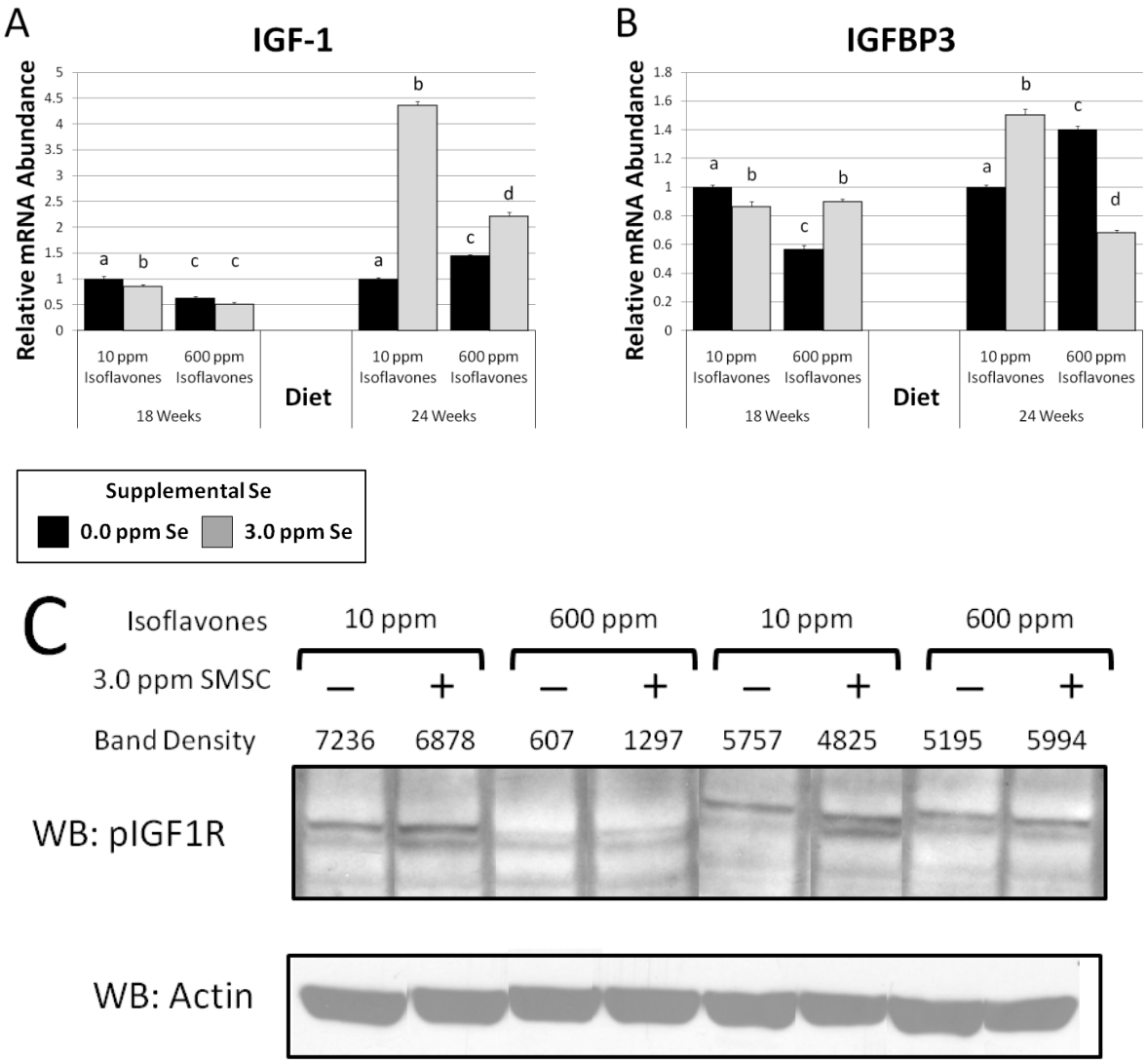

Figure 7 IGF-1 signaling is affected by isoflavones/Se.

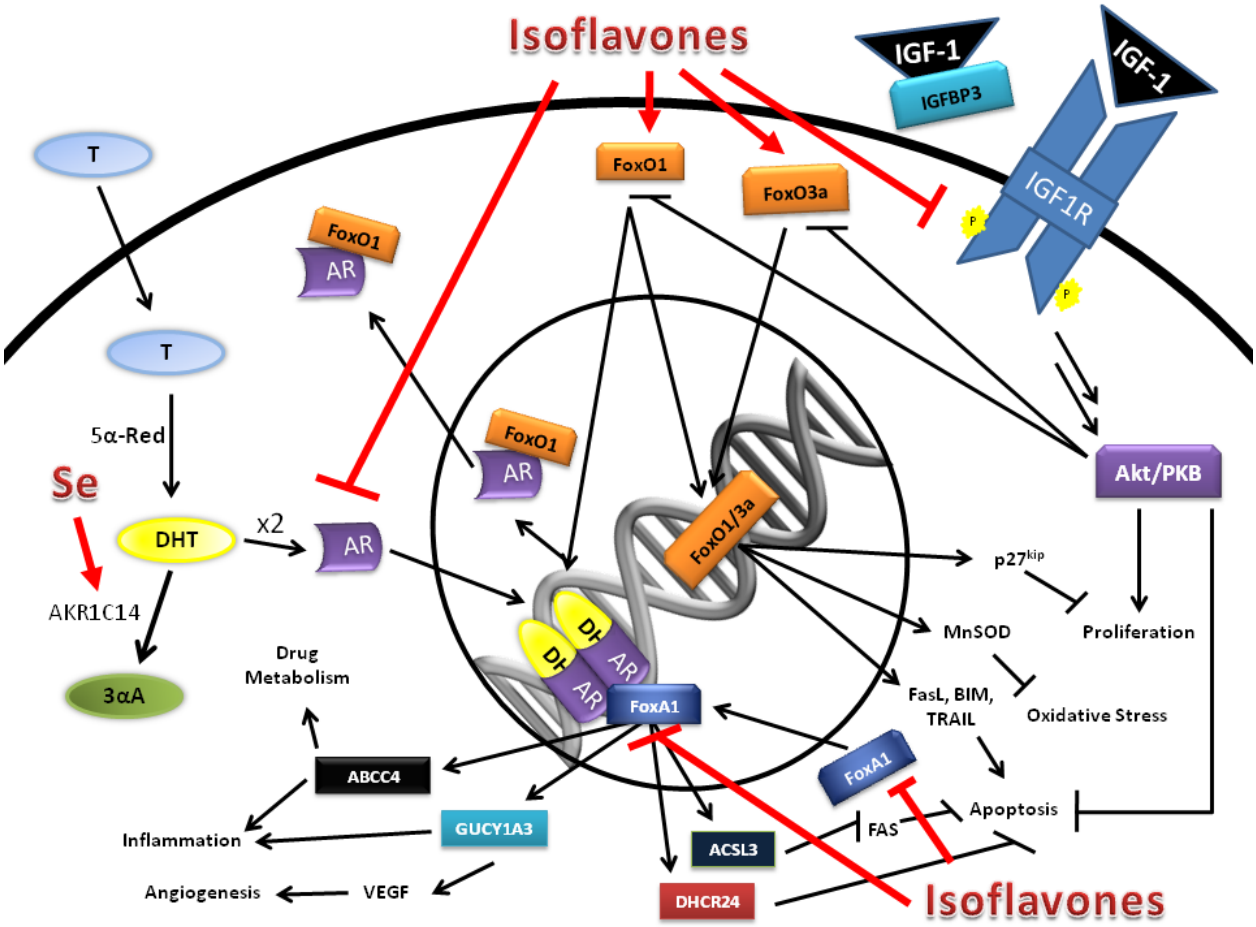

Figure 8 Proposed model and summary.

Figure 1. GPx activity is unchanged by supplemental Se. UGT weight was measured at sacrifice as a surrogate for tumor volume. (A) At 18 weeks the UGT weight of mice fed the adequate Se/ high isoflavone diet was significantly lower (p=0.009) than the control diet (adequate Se/ low isoflavone). n=16-36 (B) At 24 weeks UGT weights in all animals fed experimental diets were significantly smaller than control. Ade Se/ Low Iso (p=0.0043), High Se/ Low Iso (p=.0041), High Se/High Iso (p=0.0002). n=31-59 per treatment group. The comparison of dietary groups without a common superscript are statistically significant.

Figure 2. Dietary isoflavones and supplemental Se decrease UGT weight. UGT weight was measured at sacrifice as a surrogate for tumor volume. (A) At 18 weeks the UGT weight of mice fed the adequate Se/ high isoflavone diet was significantly lower (p=0.009) than the control diet (adequate Se/ low isoflavone). n=16-36 (B) At 24 weeks UGT weights in all animals fed experimental diets were significantly smaller than control. Ade Se/ Low Iso (p=0.0043), High Se/ Low Iso (p=.0041), High Se/High Iso (p=0.0002). n=31-59 per treatment group. The comparison of dietary groups without a common superscript are statistically significant.

Figure 3. Dietary isoflavone inhibit tumor progression. A greater percentage of animals fed diets high in isoflavones had lower grade prostate lesions compared to the low isoflavone diets. 3 = high grade PIN, 4 = well differentiated prostate cancer, 5 = moderately differentiated prostate cancer, 6 = poorly differentiated, invasive prostate cancer. n=5-6 per treatment group.

Figure 4. High dietary isoflavones decrease AR, high dietary Se increases AR. (A) There was a main effect of isoflavones increasing AR mRNA at 18 weeks (p=0.001). (B) Protein levels were different than the mRNA levels possibly due to post-transcriptional regulation of AR by

isoflavones. In protein levels there is a main effect of isoflavones. The comparisons of dietary groups without a common superscript are statistically significant.

Figure 5. Dietary isoflavones and Se regulate the expression of some AR-regulated genes. (A) 18 weeks both Se and isoflavone main effects are significant in reducing GUCY1A3 mRNA ($p<0.0001$). At 24 wk isoflavone main effects are also significant ($p<0.0001$). (B) AT 18 weeks the main effect of isoflavones increasing DHCR24 was significant ($p<0.0001$) and at 24 weeks Se was significant increasing expression ($p<0.0001$) (C) Se had a significant main effect at 18 weeks in the upregulation of AKR1C14 ($p<0.0001$). (D)At 18 weeks isoflavones significantly decreased expression ($p<0.0001$) and at 24 weeks Se decreased expression ($P<0.0001$). (E) No main effects. (F) DHCR24 mRNA and protein levels do not have the same pattern suggesting a post-transcriptional mechanism. The comparisons of dietary groups without a common superscript are statistically significant.

Figure 6. Dietary isoflavones induce a pattern of forkhead box protein expression consistent with chemoprevention. (A) Both Se ($p=0.007$) and isoflavones ($p<0.0001$) increased FOXA1 mRNA with significant levels at 18 weeks. At 24 weeks the main effect of Se in increasing FOXA1 mRNA was significant ($p<0.0001$). (B) The main effects of isoflavones in increasing FOXO1 and FOXO3a (C) was significant ($P<0.0001$). At 24 weeks there was as significant effect of Se in increasing mRNA levels ($p<0.0001$). (D) Protein levels of FOXA1 are reverse what the mRNA levels show. Se increased FOXO1 and FOXO3a when added to a high isoflavone diet but not otherwise. The comparisons of dietary groups without a common superscript are statistically significant.

Figure 7. IGF-1 signaling is affected by isoflavones/Se. (A) The main effects of Se ($p=0.041$) and isoflavones ($p<0.0001$) were significant in reducing IGF-1 mRNA levels at 18 weeks. (B) Decreased IGF-1 downregulated IGFBP3. (C) Decreased phosphorylation of IGF1R is significant in the high isoflavone diets.

Figure 8. Proposed model and summary. The statistically significant main effects of Se and isoflavones on gene expression are shown. The model shows the role of each gene product in metabolic pathways to one another. Elucidation of effects on gene expression in this study suggests molecular mechanisms for the individual effects chemopreventive Se and isoflavones individually, and the potential for and enhanced protective effect by combining the two dietary treatments.

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Appendix A: Summary of data presented at Experimental Biology 2010 meeting.

BASAL DIET COMPOSITION DETERMINES THE EFFECT OF SUPPLEMENTAL SELENIUM.

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Abstract

The effects of supplemental selenium (Se) in animal models of prostate cancer and diabetes may depend upon the composition of the basal diet to which Se is added. In this study, we examined effects of dietary Se and isoflavones, alone and in combination, on body weight, fat mass, and serum T3 levels. Non-transgenic male littermates of TRAMP mice, produced for a study of tumorigenesis, were weaned to one of two Se-adequate stock diets, either low (Zeigler Bros. Phytoestrogen-Red. Diet I) or high (Harlan Teklad 8604) in isoflavones, or the same diets supplemented with 3.0 ppm Se as Se-methylselenocysteine. Mice were killed at 18 weeks of age. Serum levels of T3 were measured using ELISA kits. Hepatic expression of the iodothyronine deiodinase I gene was determined by real time RT-PCR. These data show a significant interaction between isoflavones and Se, and demonstrate opposite effects of supplemental Se based on the isoflavone content of the basal diet.

Introduction

There have been many studies in animal models investigating the effects of supplemental Se in the development and progression of prostate cancer. The results of these studies are often conflicting—some showing dramatic protective effects (e.g. Wang L. et al 2009) and others showing no effects or harm (e.g. Ozten N et al 2010). A possible source of these discrepancies is the different basal diets used. Many standard stock diets contain high levels of soy isoflavones which include have been shown to have chemopreventive effects in animal models (e.g. Wang J. et al 2007). The isoflavone content of the basal diets may decrease the ability to notice differences with Se supplementation or the sensitivity to determine significance. Furthermore, there may be interaction effects that could either enhance or decrease the chemopreventive effects of either compound. This research explores some of the interactions between dietary soy isoflavones and supplemental Se.

Material and Methods

All procedures involving animals were approved by the BYU Institutional Animal Care and Use Committee. The subjects were wild-type litter mates to C57BL6/FVB TRAMP mice bred for a study investigating Se and isoflavones effects in prostate cancer. Breeder mice were fed diets low (Zeigler Phytoestrogen Reduced Diet I, 0 ppm), and high (Harlan Teklad 8604, 600 ppm) in soy isoflavones. Basal stock diets provided 0.34-0.37 ppm Se, and some of each diet was supplemented with 3.0 ppm Se as Se-methylselenocysteine (SMSC). Breeders (males and females) received their assigned diets for 28 days before breeding. Pregnant and subsequently nursing dams were fed their assigned diet, and their male pups were weaned to those same diets, which they continued to receive to 18 weeks of age. Measurements were made of body weight,

abdominal fat, serum free T_3 and free T_4 levels using ELISA kits (F4107T, F3106T Calbiotech), and liver gene expression (using real time qPCR of mRNA levels). Statistical analysis of weights, hormone levels, and gene expression was by ANOVA.

Discussion

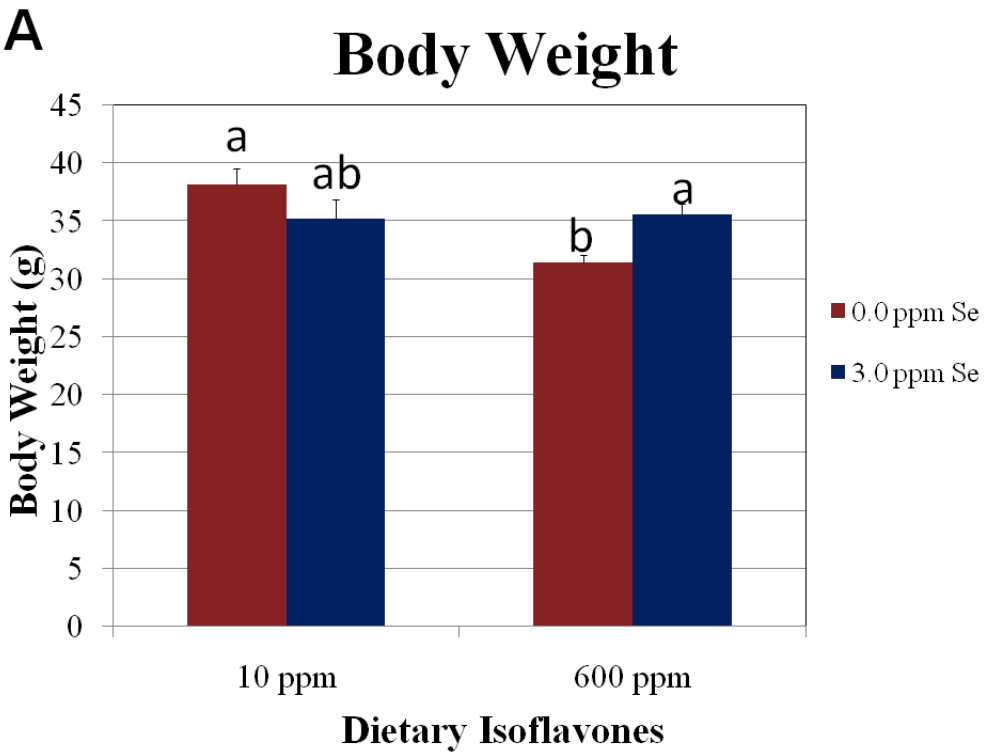
The differences in weight and abdominal fat pad mass demonstrate an interaction effect between Se and isoflavones. While mice eating the low isoflavone diet had decreased weight and abdominal fat when given supplemental Se, the mice on the high isoflavone diet that received the supplemental Se had significantly increased weight and abdominal fat compared to those mice that did not. The levels of triiodothyronine (T_3) also demonstrated this interaction effect–mice receiving supplemental Se showed increased T_3 when on the low isoflavone diet and decreased T_3 when on the high isoflavone diet. The levels of T_3 match closely with the body weight and abdominal fat levels measured in each diet group. As T_3 is an important regulator of body metabolism, the different levels of T_3 could offer a partial explanation for the difference in anthropometric measurements.

In a preliminary exploration of possible mechanisms for these differences in hormone and fat levels the expression of two related genes were examined. The selenoprotein iodothyronine deiodinase I (DIO1) is one of three deiodinases that regulate the conversion of T_4 to T_3. The differential expression of DIO1 between dietary groups does not, by itself, fully explain the differences in T_3 levels. Further research focusing on other deiodinases and thyroid hormone-regulating mechanisms will be necessary to understand diet's effects on blood T_3 concentrations. The decrease in IGF-1 expression with supplemental Se is consistent with our observation that

Se-supplemented mice fed the high isoflavone diet had increased body fat. Lower levels of IGF-1 are associated with insulin resistance and obesity.

Conclusions

- The isoflavone content of rodent basal diets can modify the effects of supplemental Se.
- The interaction of Se and isoflavones may explain, in part, previous inconsistencies in studies of Se and prostate cancer.
- Researchers must take into consideration the isoflavone content of the basal diet when conducting Se supplementation studies.


A
Body Weight
Body Weight (g)
45
40
35
30
25
20
15
10
5
0
a
ab
b
a
10 ppm
600 ppm
Dietary Isoflavones
0.0 ppm Se
3.0 ppm Se

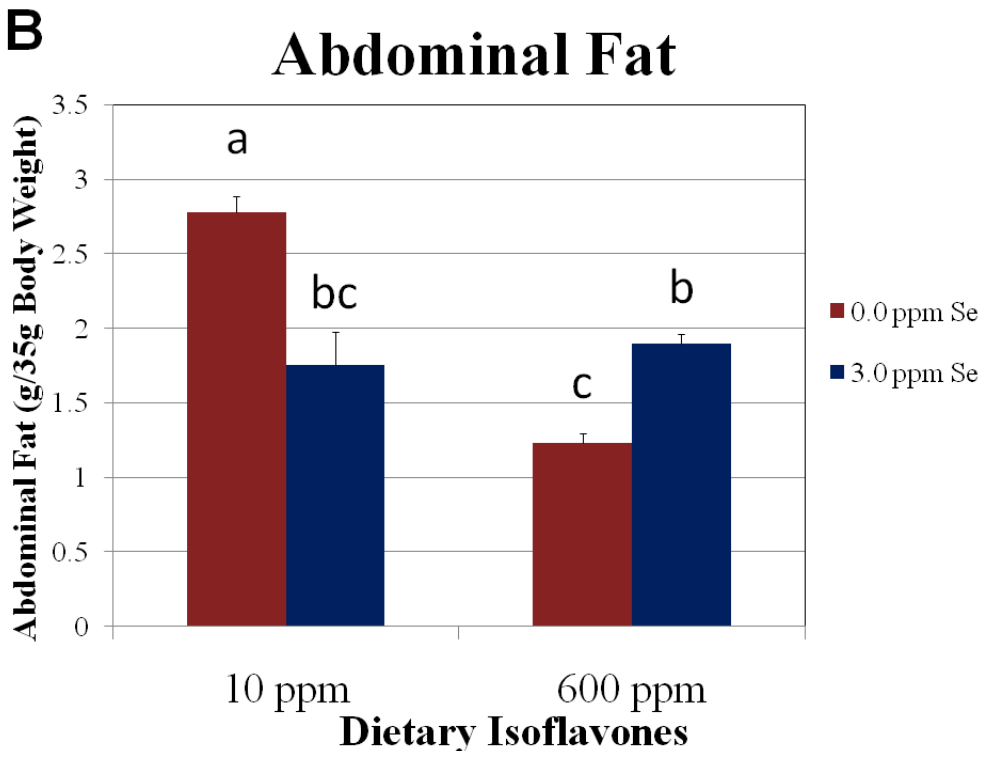
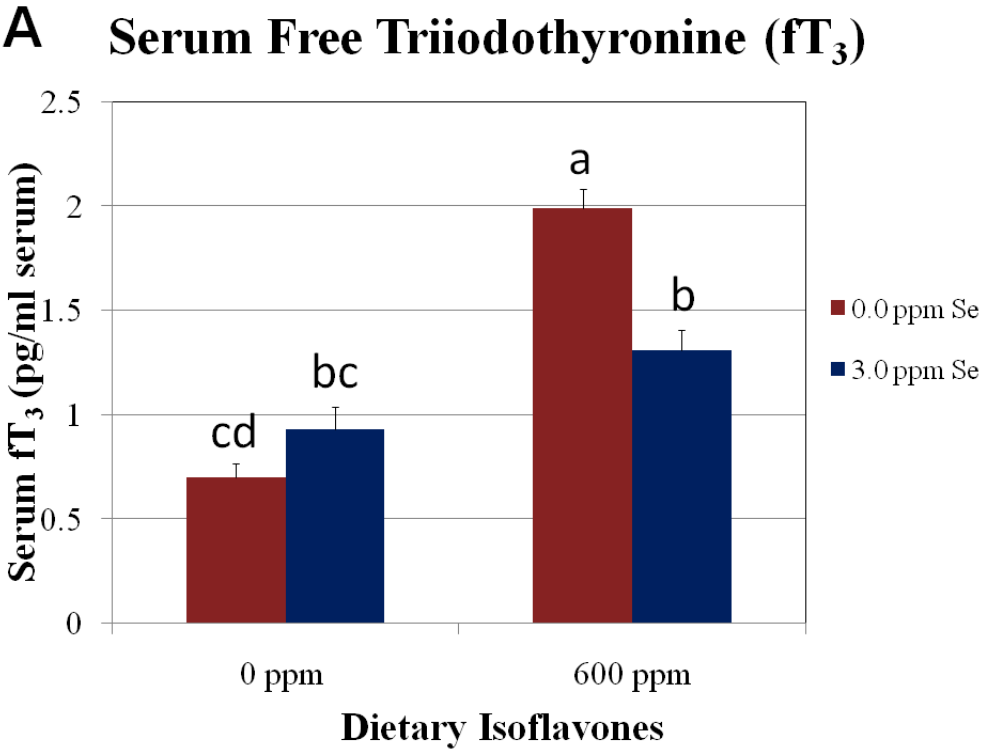
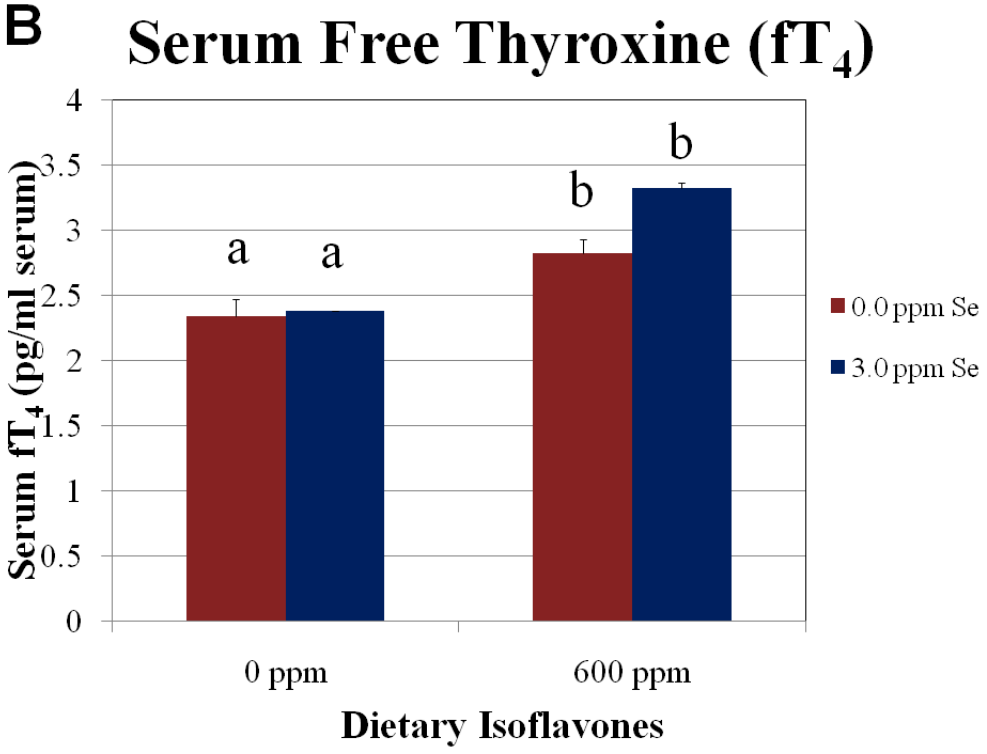

B
Abdominal Fat
Abdominal Fat (g/35g Body Weight)
3.5
3
2.5
2
1.5
1
0.5
0
a
bc
c
b
10 ppm
600 ppm
Dietary Isoflavones
0.0 ppm Se
3.0 ppm Se

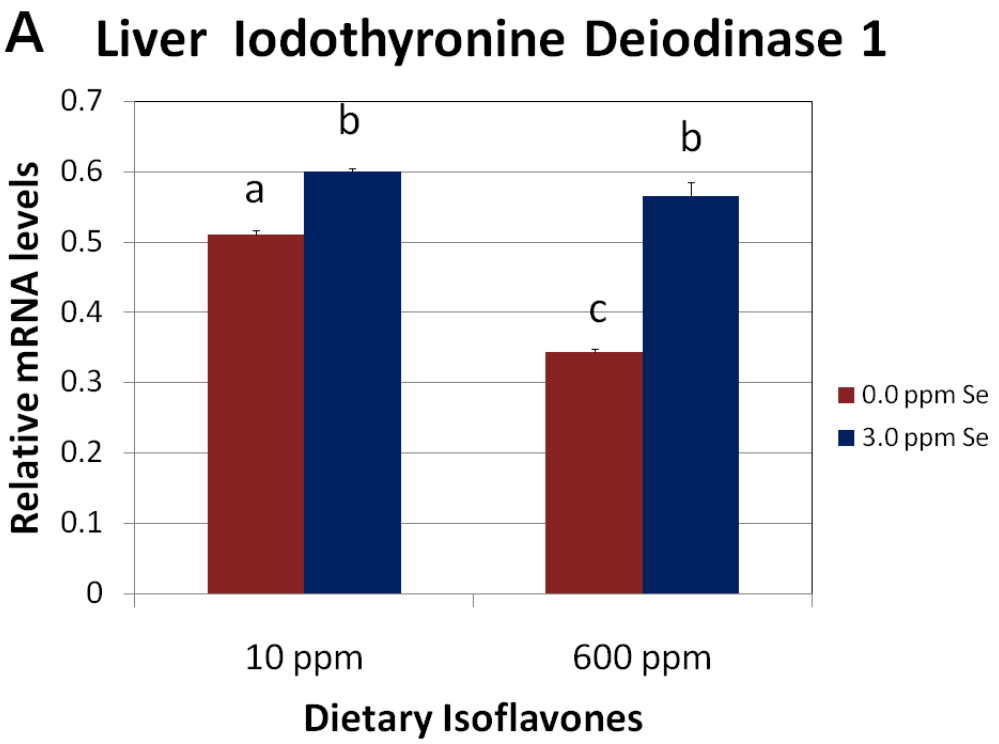
Figure 9

A


Serum Free Triiodothyronine (fT_3)
Serum fT_3 (pg/ml serum)
2.5
2
1.5
1
0.5
0
a
b
bc
cd
0 ppm
600 ppm
Dietary Isoflavones
0.0 ppm Se
3.0 ppm Se

B


Serum Free Thyroxine (fT_4)
Serum fT_4 (pg/ml serum)
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
b
b
0 ppm
600 ppm
Dietary Isoflavones
0.0 ppm Se
3.0 ppm Se


A
Liver Iodothyronine Deiodinase 1
Relative mRNA levels
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
b
10 ppm
600 ppm
Dietary Isoflavones
0.0 ppm Se
3.0 ppm Se

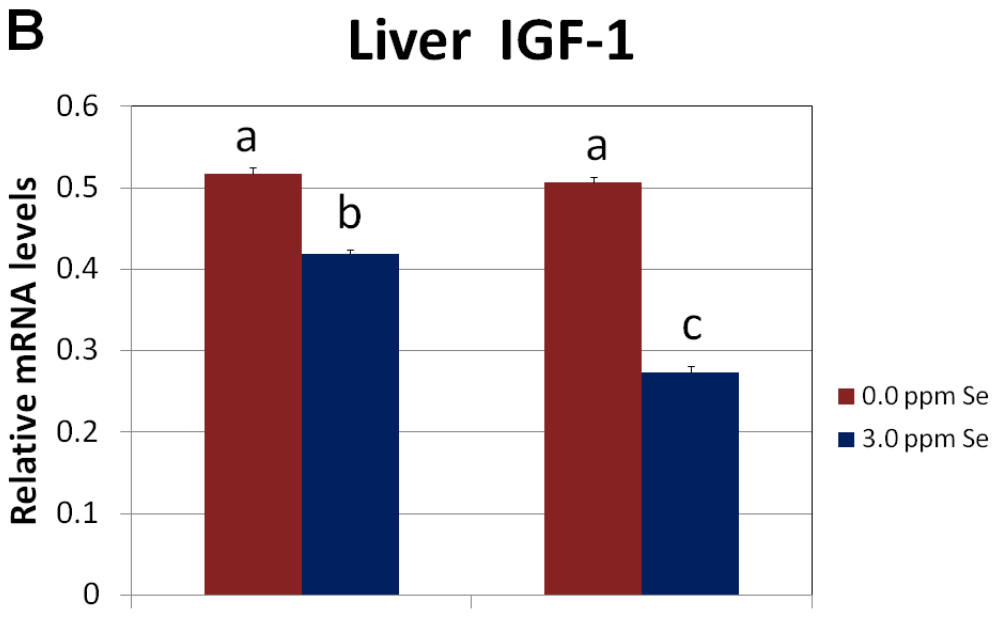

B
Liver IGF-1
Relative mRNA levels
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
a
c
0.0 ppm Se
3.0 ppm Se

Figure 11

Figure 1. A. The body weight of 18 week old mice. The main effect of isoflavones is significant ($p<0.05$). Means ± SD of 7-17 mice. B. The abdominal fat of 18 week old mice. The main effect of isoflavones is significant ($p<0.05$). Means ± SE of 4-8 mice. In each graph bars with different superscripts are significantly different by Fisher's pairwise comparisons.

Figure 2. A. The free triiodothyronine in serum of 18 week old mice. The main effect of isoflavones is significant ($p<0.05$). Means ± SD of 4-8 mice. B. The abdominal fat of 18 week old mice. The main effect of isoflavones is significant ($p<0.05$). Means ± SD of 4-8 mice. In each graph bars with different superscripts are significantly different by Fisher's pairwise comparisons.

Figure 3. A. Relative expression of the liver iodothyronine deiodinase I gene in liver of 18 week old mice. The main effects of Se ($p<0.0001$) and isoflavones ($p=.007$) were significant. B. Relative expression of the insulin-like growth factor gene in liver of 18 week old mice. The main effects of Se ($p<0.0001$) and isoflavones ($p=.026$) were significant. In each graph values were normalized to the expression of the 0 ppm supplemental Se0 ppm isoflavone group. Bars with different superscripts are significantly different ($p<.05$) by Fisher's pairwise comparisons.

Appendix B: Literature Review

Selenium and Nutrition

Selenium (Se) is the nonmetal element with the atomic number 34 that has chemical properties related to sulfur and tellurium. Recognition of Se's important role in animal health began with the recognition of Se's toxicity in animals in 1933 (1-2). It wasn't until 1957 when Schwarz et al. identified Se as the key component of factor 3, which was protective against liver necrosis in rats, in that Se began to be known as essential to animal physiology(1-2). The discovery that Se is an essential component of the antioxidant enzyme glutathione peroxidase revealed a mechanism for the previously described need for dietary Se and the first selenoprotein (2-4). Further research revealed that Se is incorporated into proteins in the form of selenocysteine (Sec) in which Se takes the place of the sulfur atom in cysteine (5). It was also learned that both the creation of Sec and its incorporation into proteins is not random but carefully controlled by genes and the protein synthesis machinery (6). In fact, Sec incorporation is coded for by a UGA (STOP) codon in the open reading frame and an intricate RNA-loop and protein structure called a SECIS(7). There have been twenty-five Sec containing selenoproteins discovered in humans with functions including antioxidant activity, control of the cellular redox state, and the regulation of thyroid hormone activation (8). There is a rigid hierarchy for Se allocation into selenoproteins during periods of marginally deficient Se intake (9-11). The recommended dietary allowance (RDA) for Se has been set at 55μg/day for adults >14 years old based on intake levels required to maximize the activity of serum glutathione peroxidase (12). The dietary need for Se is met through a variety of both organic (e.g. selenocysteine and selenomethionine) and inorganic compounds (e.g. selenite and selenate) found in plant and animal foods. In areas where soil is deficient in Se the plants are also low in Se and deficiency can develop (13). Deficiency in Se can contribute to a deficient antioxidant system, impaired

thyroid hormone (TH) synthesis, and developmental problems including Keshan's disease, an endemic cardiomyopathy that can develop only in Se deficiency (14). Se nutrition is an important area of medical research not only in terms of deficiency but in with regards to supranutritional levels of Se intake and the evidence of Se's protection/antagonism against several chronic diseases including cancer, diabetes, and heart disease.

Soy Isoflavones

Isoflavones are a class of biologically active polyphenol compounds found many plants but found at the highest levels in certain legumes (15). Soybeans are especially rich in isoflavones such as genistein and daidzein. Soy isoflavones can act as phytoestrogens, plant polyphenols with estrogenic activity (15). Though isoflavones affinity for the estrogen receptors is 10^2-10^3 times weaker that 17β-estradiol, isoflavones can bind to both alpha and beta estrogen receptors (ERα, ERβ) but some isoflavones, including genistein, shows preferential binding (20-30x) to ERβ (16). While ER binding affinity is lower for isoflavones, there is a much higher concentration, up to 100,000 times higher, of phytoestrogens in the plasma than endogenous estradiol thus allowing isoflavones their hormonal effects (17). Thus many of isoflavones' actions are mediated through the estrogen receptors. Other demonstrated functions of isoflavones include antioxidant activity (18), topoisomerase inhibition(19), and protein kinase inhibition (genistein)(20). There are many health benefits with epidemiological association to high soy consumption including reduced type II diabetes, reduced cancer risk, and less osteoporosis.

Prostate Cancer

The prostate is a male secondary sex organ responsible for the production of an alkaline fluid that makes up approximately 25% of seminal fluid and important smooth muscle

contractions that aid in ejaculation and urine regulation. The growth and maturation of the prostate is largely regulated by circulating androgen mediated by local androgen activation (conversion to DHT) and actions of the androgen receptor. The pro-proliferation and pro-survival genes induced by androgen contribute to the development of prostatic disease including benign prostatic hyperplasia (BPH) and prostate cancer (PC).

Prostate cancer is the most commonly diagnosed cancer (other than skin basal cell carcinoma) in men and the second leading cause of cancer death in the United States. In 2010 approximately 217,730 new cases of prostate cancer will be diagnosed and an estimated 32,050 men will die from prostate cancer. Approximately 1 in 5 American males will develop prostate cancer during their lifetime (21). There are several genetic changes that are common to many PC tumors; PTEN is often silenced by deletion, mutation, or methylation (22-23), IGF-1R is often upregulated (24), and NKX3.1 expression is usually lost (25). No matter the genetic insult that initiates prostate cancer development, androgen provides essential growth promotion and survival signaling for tumor development(26). Because early prostate cancer is so dependant on androgen, upon diagnosis of prostate cancer the most common treatment is androgen deprivation through surgical or pharmacological castration (26-27). Androgen ablation induces dramatic tumor size reduction and prevents further tumor progression; however, in most cases of advanced PC and some case of low-grade cancer, the effects of androgen ablation are transient and the tumor begins to grow again, usually more virulently(26). These recurrent tumors are no longer sensitive to androgen ablation, are often metastatic, and account for most prostate cancer deaths; these tumors are referred to as castration resistant prostate cancer (CRPC)(26). The mechanisms for this transformation are not entirely characterized, but it is known that AR is pivotal.

Tumors often find mechanisms to induce growth via AR despite low levels of circulating androgen. This can be accomplished through mutation of AR to be ultra-sensitive to even low levels of androgen, to become ligand independent and thus constitutively active, or to bind promiscuously to ligands other than androgen. Because AR remains pivotal in all stages of prostate cancer development, any dietary or pharmacological intervention that can inhibit the AR is potentially therapeutic.

Androgen metabolism and the Androgen Receptor

The androgen receptor is a classic nuclear hormone receptor with a ligand binding domain (LBD) on the C-terminus, a DNA binding domain (DBD) with two zinc fingers and a hinge domain with a nuclear localization signal (NLS), and the N-terminus with sites for dimerization and association with the cellular machinery required to initiate transcription (28). Without ligand-binding the AR is a monomer associated with heat shock proteins (HSP) that mask its NLS and prevent dimerization, when ligand binds to the AR the confirmation changes and the HSPs disassociate allowing AR to dimerize and enter the nucleus where it binds to specific androgen response elements (AREs), recruits the required transcriptional coactivators and polymerase and initiates transcription of AR-regulated genes including genes necessary for the development of secondary sex-characteristics (28).

When stimulated by luteinizing hormone produced by the pituitary, the Leydig cells of the testes produce testosterone and release it into circulation (29). In peripheral tissues testosterone binds to the androgen receptor and initiates AR-regulated gene transcription. In the prostate the testosterone that enters the cell is converted to dihydrotestosterone (DHT) by one of

two isoforms of the 5α-reductase enzyme(28). DHT is a more potent inducer of gene expression than testosterone due to its higher affinity (3X) for AR (30).

Selenium and Prostate Cancer

Selenium's role in antioxidant defense and the role of oxidative stress in carcinogenesis suggest an important role for Se in chemoprevention. In 1969 Shamberger and Frost reported that the Se content of local crops was inversely correlated with total cancer death in the same areas (31). Many case-control studies(32-33) and several meta-analysis of Se intake (34)and body Se levels (35)have shown inverse relationships between higher serum Se levels and higher intake of Se and reduced incidence of cancer and cancer death including prostate cancer.

In 1996 Clark et al. reported the results of the Nutritional Prevention of Cancer Trial, a randomized controlled trial, where subjects received 200μg of supplemental selenium as selenized yeast or placebo. Although the hypothesis of this study was that Se supplementation would reduce incidence of skin cancer there was no decrease in skin cancer, but, even after careful correction for other factors, analysis of secondary endpoints revealed that there was 65 percent decrease in relative risk for prostate cancer incidence in patients receiving Se compared to the placebo (36). Since all subjects had an initial serum Se level above that needed to maximize plasma glutathione peroxidase (GPx), the accepted biomarker for replete Se status, the effects of Se in NPC are attributed to supranutritional levels suggesting that the mechanisms relied not on selenoproteins, whose expression was already maximized, but on the effects of low-molecular-weight selenometabolites that increase with supranutritional Se supplementation (37-38).

After these dramatic results some small prospective studies showed a decrease prostate cancer risk with supplemental Se(39), however the recent Selenium and Vitamin E Cancer Prevention Trial (SELECT) in which 35,533 subjects participated failed to show any protection after seven years of supplementation with 200µg of supplemental selenium as selenomethionine(Se-Met) (40). While the SELECT trial was a very large study it's negative results do not preclude Se from having a chemopreventive effect there are several factors that require consideration. Importantly the initial plasma levels of Se in SELECT subjects are much higher than the subjects of NPC; 135ng/mL average serum Se in SELECT versus 113ng/mL average serum Se in NPC(36,40). This is of importance because further analysis of NPC data shows that the dramatic treatment effect was only found in individuals with lower initial Se levels (37-38). This demonstrates that, while most Americans (who have generally high serum Se levels) may not benefit measurably from Se supplementation, a subset of the American population and much of the population of Europe, which has a much lower average serum Se, may benefit greatly from Se supplementation in the prevention of prostate cancer (41). While the form of Se used in SELECT, selenomethionine (Se-Met), is the most abundant form of Se present in the selenized yeast used for supplementation in NPC (36)there is evidence that, compared to other organic selenium compounds found in selenized yeast, selenomethionine is not as chemopreventive(42-43). Several studies have shown that mono-methylated Se compounds such as the metabolites methyl selenic acid (MSA) and Se-methylselenocysteine (SMSC) are much more important in chemoprevention (44-46).Furthermore, the selenomethionine in the selenized yeast is protein bound and possibly more available than the synthetic Se-Met given subjects in SELECT (47).

There is some data to suggest that Se only has protective effects in a subset of the population that have specific polymorphisms; one study found a significant PC protective effect between the highest and lowest quartile of serum Se levels, 0.3 relative risk (RR) for all PC and 0.2RR for advanced prostate cancer, but only in men with two copies of the manganese superoxide dismutase with the same rare single nucleotide polymorphism (48).

There is substantial cell and some animal model data that supports specific mechanisms for Se's protective effects in prostate cancer. The most promising of these mechanisms are the downregulation of mitogenic signaling pathways, the interruption of cell survival signaling, the induction of apoptosis, the inhibition of the cell cycle, and the downregulation of genes essential in angiogenesis and invasion/metastasis.

Mitogenic signaling:

Se's effects on AR are central to its role in prostate cancer and will be discussed in more detail further on in this document. Wang et al. demonstrated that 3.0mg/kg of daily SMSC resulted in decreased circulating IGF-1 hormone levels, and decreased IGF-1R phosphorylation all associated with improved survival in a transgenic mouse model of PC (49). Se-Met administration has been shown to induce IGFBP3 (50) in rats.

Cell Cycle inhibition:

Se administration in PC cell lines decreased the expression of Cyclin A, CDK1, CDK2, CDK4, genes responsible for the progression of the cell cycle, and increased the expression of cell cycle inhibitors such as GADD153, p19, p21, and RXR(51-52). These effects on gene expression correlated with LNCaP cells being forced out of the cell cycle or experiencing cell cycle arrest at the G_1/S and G_2/M checkpoints(53-54).

Inhibition of cell survival:

NFκB is a potent downstream effector of many cytokines and growth factors that promotes cell survival and inhibits apoptosis; it is often deregulated in CRPC(55-57). Se administration to PC cell lines resulted in a drop in NFκB activity and the expression of NFκB-regulated genes (58-59).

Induction of Apoptosis:

Both selenite, through a p53 mediated pathway, and MSeC induce apoptosis of PC tumor cells and in xenograft models by the induction of caspase-9 (57,60-63).

Angiogenesis/ metastasis:

Administration of monomethylated Se-compounds reduced the expression of VEGF in PC cells and reduced tumor microvessel density (57,64-65). MSeA has also been shown to decrease the expression of MMP-2 an important mediator of cellular migration and often upregulated in invasive PC (64-65).

Se and AR

It is well accepted that Se does not affect levels of circulating androgen (66-67). In 2004 Dong and colleagues showed that administration of MSeA was able to decrease levels of AR mRNA and protein (68-69). As additional evidence of MSeA's interference in AR signaling, the expression of the AR-regulated prostate specific antigen (PSA) was also dramatically reduced in MSeA treated prostate cancer cells (68-69). Later work by the same group showed that 10μM MSeA prevented the recruitment of co-activators of AR-regulated expression while simultaneously increasing the recruitment of repressors of AR-regulated genes(70). Se's actions

to decrease AR stability, increase AR degradation, prevent AR nuclear localization, and inhibit the recruitment of coactivators translated into a dose-dependant decrease in proliferation in PC cells (70-72). In fact, Dong et al. demonstrated the extent to which Se can reduce proliferation in PC cells is dependent on the level of AR-signaling activity in that cell line (73). The same year this group published a data-mining micro-array paper investigating Se and its effects on AR-regulated genes associated with prostate cancer. In this paper Zhang et al identified eight genes related to prostate carcinogenesis that are AR-regulated and that showed changes in expression opposite to their expression in PC when cells were treated with Se. These genes are FOXA1, ABCC4, FACL3, GUCAY1A3, DPYSL2, KLK2 and 3, and DHCR24. Furthermore, they identified that Se induced the expression of the potential protective FOXO1 transcription factor (74). So far the only *in vivo* data supporting this PC cell data is the work of Legg et al. (75). In this study healthy rats were fed diet containing 3.0ppm SMSC for 200 days; Legg reported that the animals fed the supplemental Se diet had significantly reduced mRNA levels and protein levels of AR. In accordance with this decrease in AR, there was a decrease in the expression of some AR-regulated genes (75).

Soy Isoflavones and Prostate Cancer

The epidemiological data relation soy consumption and prostate cancer is conflicting and insufficient. Some studies show a clear protective effect of high soy consumption for prostate cancer like a study of soy milk intake among Seventh-day Adventists where frequent soy milk intake was associated with a significant 70% decreased risk for PC(76). Most studies have shown some protective effect both in Asia and the United States (77-79)—though in some cases the protective effects were limited to specific groups as in a large prospective Japanese study where there was a dose-dependent protective effect but only in men >60 years old and the ~50%

decreased risk was only for localized prostate cancer (78). Some other studies find a difference in ethnicity and the protection offered by high soy consumption(79). However, some studies have found no relation between soy milk or legume intake and protection against prostate cancer (80-83). There are several possible explanations as to why the results are not more clear—one such possible explanation lies in the fact that not all humans have the intestinal bacteria culture that can convert daidzein to equol, a more potent phytoestrogen. Different races and cultures/societies with different legume consumption patterns have different microflora profiles (84-86). Even taking this into account and measuring serum isoflavinoid concentration in one study did not show a clear protective effect against prostate carcinogenesis—instead the prostate cancer patients had the highest serum isoflavone concentration(85). One study did show decreased risk of overall cancer associated with higher serum equol levels (86). There is also evidence that polymorphisms in estrogen related genes may influence the potency of isoflavones against PC (87).

The laboratory data for isoflavones protective effects against prostate cancer is very positive. *In vitro* studies have shown that genistein can induce the expression of protective genes(88-89), inhibit the activity of NFκB (90-91), inhibit protein-tyrosine kinases (92), and inhibit angiogenesis and invasion (93-94). *In vivo* studies involving isoflavone intake's effects on xenograft and chemically induced cancer in rats showed decreased metastasis (95-96). In the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model dietary genistein given throughout life decreased the incidence of poorly differentiated PC by 50% in castrated mice (97). Interestingly, when a slightly lower dose of genistein is given to the same mice but given at 12 weeks of age it promotes the development of advanced prostate cancer (98).

Soy Isoflavones and AR axis

It is well established in PC cell line *in vitro studies* and *in vivo* animal studies that high isoflavone intake can decrease the expression of the AR and the levels of circulating androgen (96-101). Some evidence shows that isoflavones also inhibit the actions of the 5α-reductase enzyme, which converts the less-active testosterone to 5α-DHT (75,102), while in some studies, including one with human subjects, increased blood isoflavones are associated with no change or an increase in serum testosterone levels and 5α-reductase activity (103-105). There is also yet to be published data showing equol binding to and preventing the action of 5α-DHT (Lephart, unpublished). It has been shown that the binding of equol to ERβ results in a downregulation of AR (101). This mechanism is not completely understood but there is some evidence that genistein inhibits the activity of HDAC6-Hsp90 which is an essential deacetylase that works with AR in transcription, its inactivation leads to the destruction of AR (99).

24-dehydrocholesterol reductase (DHCR24)

DHCR24 is an enzyme with several different functions, but it was originally described as an integral enzyme in the synthesis of cholesterol from desmosterol (106). DHCR24 expressed ubiquitously and is AR regulated in the prostate(107-108). DHCR24 has also been named seladin-1 (for SELective Alzheimer Disease INdicator 1) because it was found to be decreased in especially vulnerable parts of the brain in Alzheimer's disease (109). Closer study of DHCR24 has revealed its ability to resist the effects of β-amyloid/oxidative stress and prevent apoptosis by inhibiting caspase-3 and acting as a ROS-scavenger (109). In some cancers the ability to resist apoptosis through DHCR24 may be a reason for its upregulation in some cancers. An interesting

phenomena occurs with the prostate expression of DHCR24 during PC progression. In low-grade, androgen-sensitive, prostate tumors DHCR24 is highly expressed; however as the tumors loose androgen sensitivity the DHCR24 expression drops drastically (107,110-111). This makes more sense when it is understood that while DHCR24 does inhibit apoptosis, it also induces senescence and is pro-differentiation (110). The loss of DHCR24 expression in CRPC tumors may contribute to the increased pace of proliferation and invasion, it has even been shown with human PC tissue that androgen-dependent tumors have significantly higher expression of DHCR24 compared to metastatic androgen insensitive tumors even before these phenotypes are observed, suggesting that DHCR24 levels may be diagnostic (110-111).

In a culture of fetal neuroepithelial cells administration of estrogen, tamoxifene, and genistein all induced the expression of DHCR24, and it was confirmed that there is a half-palindromic estrogen response element (112). Russ et al. saw no modulation of DHCR24 expression in rat prostates with high dietary isoflavones (75). In addition to the micro-array data showing a decrease in DHCR24 expression with MSeA administration (74), the same group published another paper showing that the decrease in DHCR24 could be reversed by overexpression of AR (68). Russ et al. showed a significant decrease in DHCR24 expression with a high SMSC diet (75).

Guanylate Cyclase 1 alpha 3 (GUCY1A3)

Gucy1a3 is a subunit of an enzyme that catalyzes the production of cGMP in response to NO signaling (113). The silencing of GUCY1A3 with iRNA in glioma cells revealed that it is an upstream regulator of VEGF, as such when these cells were injected into a nude mouse their ability to form a tumor was severely decreased(114). Gucy1a3 is AR regulated and found to be

decreased with MSeA administration by Dong et al(68). However, Legg et al did not observe the same Se effect in healthy rats. In Legg's work high dietary Se reduced Gucy1a3 expression (75). Gucy1a3's role in mediating inflammation may share some insight into PC development and inflammation.

Acyl-CoA synthetase long-chain family member 3 (ACSL3)

In the prostate, ACSL3 is the most highly expressed member of this family of proteins that activate fatty acids to acyl-CoA before they can be oxidized in the mitochondria (115). When there are enough dietary fatty acids to meet the cell's needs, the excess of long-chain fatty acyl-CoA, which is produced by ACSL3, inhibits the machinery of *de novo* fatty acid synthase including the vitally important fatty acid synthase (FAS) (116). Inhibition of fatty-acid synthase has been shown to induce senescence or apoptosis in normal and cancer cells through various mechanisms such as the toxic build-up of FAS's ligand, malonyl-CoA, inhibiting β-oxidation and inducing ceramide mediated apoptosis(117) , through the starvation of phospholipids and other membrane components synthesized by FAS-dependent processes (118-119), and inhibition of DNA replication(119). Recently, the high expression of FAS in breast and prostate tissue associated with advanced tumors is being evaluated for its potential marker for tumor progression . Administration of $1,25(OH)_2$ D_3 increased the activity of abundance ACSL3 through an AR-mediated upregulation of its expression (115). The inhibition of FAS by ACSL3 results in cancer cells leaving the cell cycle and an increase in apoptosis. From the data-mining results of Zhang et al we learn that Se can decrease expression of ACLS3, a potentially undesirable effect (74). However, Legg et al reported that dietary isoflavones, but not Se

supplementation, reduced ACSL3 expression in rat prostate (75). Current research is focusing on FAS inhibition for drug development and thus any dietary element (vitamin D) that induces ACLS3 would be expected to be protective, while any effect that decreases its activity could be considered detrimental in cancer (120).

Multidrug Resistance-associated Protein 4 (MRP4/ABCC4)

MRP4/ABCC4 is a member of the ATP-binding cassette transporter family; these proteins are responsible for the ATP driven efflux of pharmaceuticals and other molecules across epithelial membranes (121-122). ABCC4 is expressed mostly in the prostate and has been found to be expressed up to three times higher in prostate tumor tissue samples (121). Although there is no androgen response element (ARE) in the promoter region of ABCC4 (121), it is regulated by AR (121,123), perhaps through the NFκB pathway, and is known to bind and efflux steroid hormones including androgen, estrogens, and other steroids that may antagonize AR (122,124-125). While the ABC family of proteins, as a whole, has been implicated in the development of drug resistance by tumors, including drugs used in chemotherapy, ABCC4 has not been shown to efflux Doxorubicin, Docetaxel or Mitoxantrone, three chemotherapy agents commonly administered in PC treatment. The efflux of prostaglandins by ABCC4 may be an important part of PC development as this efflux may induce inflammation and which could help promote hyperplasia of prostate epithelial cells (126-128). Since the upregulation of ABCC4 appears to be an important part of prostate carcinogenesis, and the inhibition of ABCC4 may lead to greater drug sensitivity the antagonism of this gene may be protective in prostate cancer and a potential drug target. Dong et al. and Legg et al both found that Se decreased expression of ABCC4 in PC cell lines and in rats (68,75).

3α-hydroxysteroid dehydrogenase (HDS) (AKR1c14)

Members of the AKR1C family in humans are reductases that have high specificity for steroid hormones including 5α-DHT. These AKRs are an important component of regulation of ligand availability for AR (129-131). While there is some question as to the homology of human AKR1C proteins and their rat/mouse analogs in terms of expression patterns and function, AKR1C14 is recognized to be a 3α-hydroxysteriod dehydrogenase (3α-HSD) and have the ability to convert 5α-DHT to the less active 3α-androstanediol (132). In this context Akr1c14 expression seems protective against PROSTATE CANCER in that it can inactivate 5α-DHT. However, Akr1c14 is most homologous to the human AKR1C3 which, in addition to converting 5α-DHT to the inactive 3α -androstanediol, can convert delta-*4-androstene-3*, *17-dione* into testosterone and estrone to 17β-estradiol (131). AKR1C3 has been found to be abundantly expressed in prostate cancer cell lines and samples and to be upregulated in CRPC samples (131). It is postulated that AKR1C3 may be a key enzyme in the local production of androgen that allows CRPC tumors to overcome androgen ablation. Down regulation, therefore, may be the most protective for AKR1C14.

Forkhead box A1 (FOXA1)

FOXA1 belongs to the family of forkhead box proteins originally described as being essential in endoderm development (133-134). FOXA1 has been described as working together with AR, binding near AREs, facilitating AR binding, and making transcription possible for AR-regulated genes in humans and mice (134-135). FOXA1 is upregulated in prostate cancer and is even more abundant in CRPC. Inhibition of FOXA1 protein levels by high isoflavone diets may be one of the mechanisms by which isoflavones decrease the expression of AR-regulated genes such as GUCY1A3, ACSL3, and DHCR24 (135).

Forkhead box O Transcription Factors

FOXO1 and FOXO3a are both members of the forkhead box O family transcription factors. These proteins were originally found at the site of chromosomal translocations in tumors which suggested that they may be tumor suppressors (136). FOXO proteins induce the expression of FAS ligand, TRAIL, BIM, $p27^{kip1}$, MnSOD, and IGFBP1 and thus are integral in the regulation of cell proliferation and apoptosis (137). FOXO1 has been shown to bind directly to the N-terminus domain of AR and induce its nuclear export and inactivation (138-139). Activation of Akt/PKB induces the phosphorylation and cytoplasmic localization of FOXO proteins and inhibition of activity. Decreased protein levels of FOXO1 and FOXO3a are seen in localized prostate cancer and even more so in CRPC (137).

IGF-1 and Prostate Cancer

The IGF-1 signaling pathway is an active area of research in prostate cancer work. There is evidence that increased levels of serum IGF-1 are associated with prostate cancer (24,140-141), and that prostate stromal cells paracrine signaling using IGF-1 can play a role in CRPC (142).

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Appendix C: Materials and Methods

RNA Extraction

RNA extraction/purification was done using the RNeasy Kit from QIAGEN. The manufacturer's printed instructions were followed.

Reverse transcription

Label PCR tubes according to the labeled RNA you will use.

Assemble the following into an ice bucket:

- Random Hexamers (50 µM) or random primers
- dNTP mix (10 µM) or PCR nucleotides
- RNA-appropriate volume for 5 mg RNA
- Rnase-free H20
- First Strand Buffer
- 0.1 M DTT
- Rnase Out
- Superscript II (SSII)
- Rnase H (2u/ µl)

Protocol

1. Combine the following in a 0.5 mL tube:

   1 µl 50 µM random hexamers

   1 µl 10 mM dNTP mix

   appropriate volume for 5 µg RNA

   Rnase-free H_2O to 12 µl

   12 µl total volume

   Add 2 µl of mineral oil to each tube

2. Heat @ 65°C for 5 min (Program 22). (PUSH HOLD) Quickly place on ice for 2-3 min.

3. Centrifuge briefly and add:

    4 µl first strand buffer

    2 µl 0.1 M DTT

    1 µl Rnase OUT

    19 µl Total Volume

4. Tap tube gently, centrifuge briefly (RESUME) and incubate @ 42°C for 2 min (PUSH HOLD).

5. Add the following to each tube:

    1 µl SSII

    20 µl Total Volume

    Mix by pipeting gently up and down.

6. (RESUME) Incubate @ 42°C for 50 min.

7. Inactivate the reaction by heating to 70C for 15 min (PUSH HOLD).

8. Add the following to each tube:

    1 µl RnaseH (2u/µl).

    21 µl Total Volume

9. (RESUME) Incubate @ 37°C for 20 min.

10. Run Qiagen PCR purification kit columns. (All centrifugations @ 13,000 RPM.)

    (1) Combine 5 volumes of Buffer PB with 1 volume of the PCR sample and mix. (105 µl Buffer PB to 21 µl sample.)

    (2) Place QIAquick spin column in a provided 2 mL collection tube.

(3) Apply the sample to the column. Centrifuge 1 min.

(4) Discard flow through and place the QIAquick column back in the same tube.

(5) To wash, add 750 µl Buffer PE to the column and centrifuge 1 min.

(6) Discard flow through and place the column back in the same tube. Centrifuge the column for an additional 1 min.

(7) Place QIAquick column in a clean 1.5 mL centrifuge tube.

(8) Elute DNA by adding 30 µl EB buffer 9110 mM Tris-HCl, pH 8.5) to the center of the QIAquick membrane and incubate for 1 min.

(9) Centrifuge for 1 min.

(10) Add an additional 30 µl of EB buffer and incubate 1 min.

(11) Centrifuge for 1 min.

(12) Store cDNA at –20°C until ready for use.

Light cycler run

Assemble the following into an ice bucket:

- ✓Taq polymerase
- ✓10 X buffer
- ✓dNTP mix (10µM) or PCR nucleotides
- ✓cDNA:
  - 1:1 pool
  - 1:2 pool
  - 1:4 pool
  - 1:8 pool
  - 1:16 pool
  - 1:6 Dilutions of each diet group
- ✓SYBR green 1:50 dilute (add 2 µl of this to 78 µl H_20 for 1:2000 dilution)
- ✓Primers (10µM or 25 µM each)
- ✓$MgCl_2$
- ✓BSA
- ✓Purified H_2O

Protocol:

1. Label a 1.5 ml tube MM for **Master Mix**

2. Add each reagent according to the table below starting with the smallest amount to the largest. With the exception of the SYBR green which is to be diluted and added in after the H^2O and the cDNA which will be added individually at the end.

3. Vortex each reagent before adding it to the master mix, *with the exception of the Taq enzyme*. After adding each ingredient, pipet the master mix up and down gently.

	1 Rxn	31 Rxn
Taq	0.16µl	4.96 µl
dNTP mix	0.40µl	12.4 µl
Primers (10µM each)	1.00µl (10 µM) or 0.4 µl (25 µM)	31 µl
10 X buffer	2.00µl	62 µl
$MgCl^2$	3.20µl	99.2 µl
BSA	5.00µl	155 µl
H^2O	5.10µl or 5.70 µl	158 µl
SYBR green (1:2000 dilute)	1.14µl	35.3 µl
	20µl per capillary	557.86 =18 µl per cap.

4. Once master mix is complete, centrifuge for 5 seconds.

5. Place 33 capillaries in the LightCycler centrifuge adaptors.

6. Dispense 18 µl of master mix into each capillary.

7. Dispense 2 µl of cDNA into each capillary

8. Centrifuge capillaries in their adaptors for 10 sec at 2500 rev/min.

10. Cap each capillary with white cap.

11. Gently twist capillaries into LightCycler capillary holders. THEY EASILY BREAK!!

To Run LightCycler:

1. Double click on NEW LIGHT CYCLER SOFTWARE 3.5.3

2. Double click RUN on menu

3. Allow machine to self test, once finished click OK.

4. Go to file and click open. Select program *Mouse Experiment.exp* and click OPEN.

5. Under Experiment, click on Amplification. Adjust the middle Target Temperature to desired annealing temperature - 68°C.

6. Click on "Edit Samples" and enter names of samples loaded in capillaries, **being careful to note if you skipped any capillary positions!** Click on "Done".

7. Click RUN and save experiment. Click on "Done" again.

8. Once the flow reaches about 100 or the graph starts to slope off click END PROGRAM and allow machine to go to next program, melting curve analysis.

Average run time: 45 minutes

Quantification:

Once experiment has finished Data Analysis will automatically open.

1. At the top left click on "Select a Program" and choose "Melting Curve Analysis…"

2. Click on "Melting Curve" on the top of the screen. Check to make sure all DNA denatured at the same degree and that there is only one peak on the bottom graph.

3. Close that screen and click on "Select a Program" again. Click on "Amplification is a…"

4. On the top of the screen click "Quantification."

5. Select “Second Derivative Maximum”.

6. Data is exported to Excel for analysis.

Western Blots

The following protocol uses NuPAGE® Novel Bis-Tris Gels and the XCell *Surelock*™ Mini-Cell from Invitrogen and is adapted from the NuPAGE® electrophoresis system protocol.

Preparing Samples

Materials needed:

- Protein samples
- Novex® Sharp Protein Standard (in -20°C freezer)
- NuPAGE® LDS Sample Buffer (4X)
- Deionized H_2O

1. Thaw the protein samples and the Novex® Sharp Protein Standard and gently vortex to ensure homogeneity. Keep the samples on ice when they are not in use.
2. To make 10 µl of sample for electrophoresis add the following to each tube:

Reagent	Volume
Protein Sample	x µl
NuPAGE® LDS Sample Buffer (4X)	2.5 µl
Deionized H_2O	Up to 7.5 µl

   a) The amount of sample used is determined by the protein concentration of each sample. Typically, you need between 10-20 µg of total protein in each sample used for electrophoresis.

b) The maximum loading volume for each well of the 1.0 mm X 10 well gels is 25 µl, so more of each of the above reagents may be used if the sample protein concentrations are low.

3. Centrifuge the samples briefly.
4. Heat the samples at 70°C for 10 minutes (use the ERICOMP Power Block).
5. While the samples are being heated, you can prepare the gel and Mini-Cell for electrophoresis. See below for instructions.
6. When heating is finished, briefly centrifuge the samples again.

Gel Electrophoresis

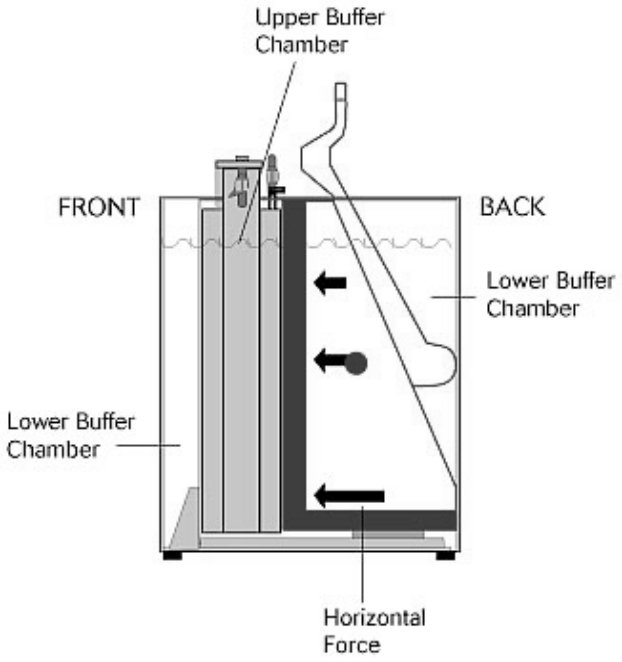
Materials needed:

- NuPAGE® Novex Bis-Tris Gel
- 1X NuPAGE® SDS Running Buffer (recipe on p. 6)
- Deionized H_2O
- Prepared protein samples
- Novex® Sharp Protein Standard

1. Remove the NuPAGE® Gel from the pouch.
2. Rinse the gel cassette with deionized H_2O. Peel off the tape from the bottom of the cassette.

3. In one smooth motion, gently pull the comb out of the cassette.

4. Rinse the wells with 1X NuPAGE® SDS Running Buffer. Invert the gel and shake to remove the buffer. Repeat two more times.



5. Orient the gel in the Mini-Cell so that the notched "well" side of the cassette faces inwards toward the buffer core. Insert the plastic buffer dam on the opposite side of the buffer core. Seat the gel and buffer dam on the bottom of the Mini-Cell and lock into place with the gel tension wedge.

   a) When using two gels, the second gel replaces the buffer dam.

6. To check for tightness of seal fill the upper buffer chamber with a small amount of running buffer (see figure). If there is a leak from the upper to the lower buffer chamber, discard the buffer, reseal the chamber, and refill.

7. Fill the upper buffer chamber with enough running buffer to exceed the level of the wells.

8. Load 10 µl of Novex® Sharp Protein Standard into the first well.

9. Load the appropriate volume of sample at the desired protein concentration into each well.

10. Fill the lower buffer chamber with ~600 ml of running buffer.

11. Run the gel at 200 V for approximately 35 minutes.

12. While the gel is running, begin preparations for Western blotting.

Western Blotting

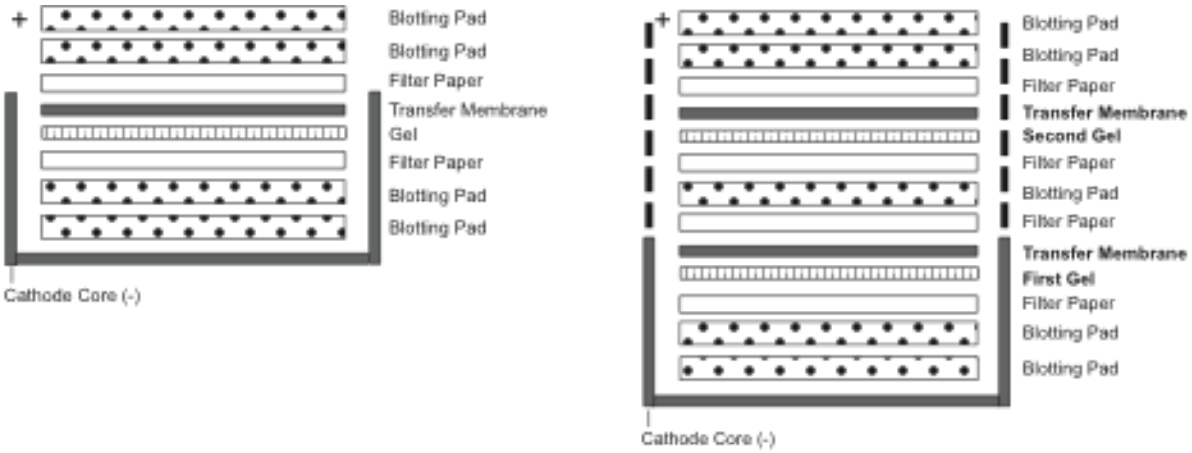
Materials needed:

- 1X NuPAGE® Transfer Buffer (recipe on p. 6)
- Blotting pads
- PVDF membrane (cut to appropriate size)
- Filter paper
- Deionized H_2O

Transferring One Gel

1. Soak the blotting pads in 1X transfer buffer until they are saturated. Remove air bubbles by squeezing the pads while submerged in buffer.
2. Prepare the PVDF membrane by cutting it to the appropriate size and by making a notch on one of the corners (this makes it easy to remember which side contains the proteins). Pre-wet the membrane for 30 seconds in methanol. Rinse the membrane briefly in deionized H_2O, then soak it in transfer buffer for several minutes.
3. Soak the filter paper in transfer buffer immediately prior to use.
4. After electrophoresis is complete, shut off the power supply, disconnect electrodes, and remove the gel from the Mini-Cell.
5. Separate each of the three bonded sides of the of the gel cassette by inserting a knife or spatula into the gap between the cassette's two plates. The notched ("well") side of the cassette should face up.

6. Push down gently on the knife handle to separate the plates. Use caution while inserting the knife between the two plates to avoid excessive pressure towards the gel.
7. Carefully remove and discard the top plate, allowing the gel to remain on the bottom (slotted) plate.
8. Remove the wells by cutting them with the knife.
9. Place a piece of pre-soaked filter paper on top of the gel, and lay just above the slot in the bottom of the cassette, leaving the "foot" of the gel uncovered. Keep the filter paper saturated with the transfer buffer and remove all trapped air bubbles by rolling a pipette over the surface.
10. Turn the plate over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface.
11. Use a knife to push the foot out of the slot in the plate and the gel will fall off.
12. When the gel is on a flat surface, cut the foot off the gel with the knife.
13. Wet the surface of the gel with transfer buffer and position the pre-soaked transfer membrane on the gel, ensuring all air bubbles have been removed.
14. Place another pre-soaked filter paper on top of the membrane and remove any trapped air bubbles.
15. Place two soaked blotting pads and gel assembly into the cathode (-) core of the blot module.
16. See figure below for the arrangement of the gel/membrane/blotting pad sandwich.



17. Add enough pre-soaked blotting pads to rise to 0.5 cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The gel/membrane assembly should be held securely between the two halves of the blot module.

18. Position the gel/membrane assembly and blotting pads in the cathode core of the blot module to fit horizontally across the bottom of the unit. There should be a gap of approximately 1 cm at the top of the electrodes when the pads and assembly are in place.

19. Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module will only fit into the unit one way, so the (+) sign can be seen in the upper left hand corner of the blot module. Properly placed, the inverted gold post on the right hand side of the blot module will fit into the hole next to the upright gold post on the right side of the lower buffer chamber.

20. Place the gel tension wedge so that its vertical face is against the blot module. Lock the gel tension wedge by pulling the lever forward.

21. Fill the blot module with 1X transfer buffer until the gel/membrane assembly is covered. Do not fill all the way to the top (this will only generate extra conductivity and heat).

22. Fill the outer buffer chamber with deionized water (approximately 650 ml). The water level should be about 2 cm below the top of the lower buffer chamber. This serves to dissipate heat produced during the run.

23. Place the lid on top of the unit. With the power turned off, plug the leads into the power supply.

24. Run the transfer at 30 V for 1 hour.

25. While the transfer is taking place, prepare blocking solution.

Blocking and Probing

Materials needed:

- 1X PBS-Tween (recipe on p. 7)
- Blocking buffer (recipe on p. 7)
- Nonfat dried milk
- Primary antibody
- Secondary antibody
- Large weigh boats

1. Immediately following the transfer, rinse the membrane with PBST.
2. Block the membrane by placing it in a large weigh boat filled with 50 ml of 5% blocking buffer for one hour on the orbital shaker (~50 rpm) or overnight at 4°C. You should still place the membrane on the orbital shaker for at least 15 minutes even if it is left to block overnight.

3. Quickly rinse the membrane and wash it in PBST (25-50 ml) for about 5 minutes on the orbital shaker.
4. Prepare the primary antibody by diluting it in PBST with 5% milk. The optimum dilution depends on several factors (the antibody, the samples, etc.) and must be determined experimentally. A typical starting range is 1:1000 to 1:5000.
5. Place the membrane in the primary antibody solution and incubate in the orbital shaker (~50 rpm) for 1 hour.
6. Quickly rinse the membrane and wash it in PBST 3 times for 5 minutes.
    a) Increasing the length or the number of washes or the amount of PBST may help to reduce background, but doing so could also wash the sample proteins off the membrane.
7. Dilute the secondary antibody in PBST. Again, the optimal dilution must be determined experimentally.
8. Incubate the membrane in the secondary antibody solution for 1 hour in the orbital shaker (~50 rpm).
9. Quickly rinse the membrane and wash it in PBST 3 times for 5 minutes.
10. Prepare for immunodetection.

Immunodetection – Electrochemical Luminescence

Materials needed:

- Pierce ECL Detection Reagents
- Plastic wrap
- Kim wipes

- X-ray film
- Tape

1. Go to the dark room and turn the “Wash” knob from “Open” to “Closed” on the film developer. Turn the developer on and allow it to warm up for at least a few minutes.
2. Run the large blank sheet of film through the developing machine.
3. Prepare the detection solution immediately before detection by combining 2 ml of solution A with 2 ml of solution B.
4. Place the membrane protein side up on a piece of plastic wrap.
5. Pipette the detection solution onto the membrane and allow it to incubate for 1 minute.
6. Using a Kim wipe, dab off excess detection solution.
7. Place the membrane protein side down onto a new piece of plastic wrap, and fold the plastic wrap over. Remove air bubbles by rolling a pipette over the membrane.
8. Place the membrane protein side up in the film cassette. Tape one edge of the membrane down. (This makes it easy to determine the orientation of the film once it is developed.)
9. Turn on the red light and turn off the white light.
10. Place a sheet of film on top of the membrane and close the cassette. The exposure time may vary from 10 seconds to 1 minute.
11. Following exposure, place the film in the developing machine and wait for it to be processed.
    a) When the developing machine beeps, it is safe to turn on the white light. Remember to put the film in its box before turning on the light.

12. If the picture on the film is not good, try changing the exposure time.

Membrane Stripping

Completely removes primary and secondary antibodies (may be performed several times).

Materials needed:

- Stripping buffer (recipe on p. 7)
- 1X PBST (recipe on p. 7)

1. Submerge membrane in stripping buffer and incubate at 50°C for 30 minutes on the orbital shaker (~50 rpm).
2. Rinse the membrane briefly and wash it in PBST 3 times for 10 minutes (at room temperature).
3. If desired, you may perform an immunodetection to determine if all of the antibodies were remove. This step is not necessary, however.
4. Block the membrane following the protocol above and proceed with probing and immunodetection.

Membrane Storage – PVDF

PVDF membranes may be stored indefinitely after transfer. Allow the membrane to air dry, wrap it in plastic wrap, and store it at 4°C. I'm not sure, but I think that you must first rinse the stored membrane with methanol before you use it again.

Western Blotting Solution Recipes

1X NuPAGE® MES SDS Running Buffer (1 L)

- Mix the following reagents:
    - 50 ml – NuPAGE® MES SDS Running Buffer (20X)
    - 950 ml – Deionized H_2O
- Store the diluted buffer at 4°C.

1X NuPAGE® Transfer Buffer (1 L)

- Mix the following reagents:
    - 50 ml – NuPAGE® Transfer Buffer (20X)
    - 100 ml – Methanol
    - 850 ml – Deionized H_2O
- When transferring two gels, use 200 ml of methanol to ensure efficient transfer of both gels.
- Store the diluted buffer at 4°C.

1X PBST (2 L)

- Mix the following reagents:
    - 200 ml – 10X PBS
    - 2.0 ml – Tween 20
    - 1.8 L – Deionized H_2O
- Store at 4°C.

5% Blocking Buffer (50 ml)

- Mix the following reagents and stir until dissolved:
    - 2.5 g – Nonfat dried milk
    - 50 ml – 1X PBS-Tween
- Adjust pH to 7.4 with a few drops of 5N NaOH.
- Do not store the blocking buffer; make it fresh for each use.

Stripping Buffer (200 ml)

- Mix the following reagents:
    - 4.0 g – SDS (sodium dodecyl sulfate)
    - 1.97 g – Tris-HCl
    - 1402 µl – 2-mercaptoethanol
    - 200 ml – Deionized H_2O
- Add the 2-mercaptoethanol under the hood just before using the buffer.
- Store at 4°C.

Bradford protocol (Coomassie plus kit)

Before beginning remember these pointers.

1. *Pipette like a champion! These concentrations need to be very accurate. Use a new pipette tip each time you draw something up. Wet the pipette tip before you draw anything up by pipetting up the exact amount, then dispensing it back into the solution. Then you are ready to draw up your actual sample.*
2. *When dispensing a sample or standard into the well, make sure every last drop is out of the pipette tip. If you have a small droplet left in the tip, remove the tip. Then put the tip back on the pipette, and push the dispense button again. This added pressure hopefully will expel that last stubborn drop.*
3. *This process usually seems to work best if one person does all the pipetting. This eliminates some error because the plate reader is very sensitive.*
4. *Always use the smallest pipette for your concentration. For example, if you need to draw up 200 ul, use the 20-200ul pipette, not the 10-1000ul pipette.*

A. Preparation of Standards and Assay Reagent

Note: We dilute our standards in the same sucrose solution that we dilute our samples. Make sure that the concentration of the dilutent is the same for both samples and standards. For example, if you've diluted the concentration for the samples to 10%, make sure the concentration for the standards is also 10%.

1. Standards:

Separate the contents of one 1ml Albumin Standard (BSA) ampule into three 1ml centrifuge tubes. Each tube should have 300(A), 375(B) and 325(C) ul. (There will be a little bit left over) This is our stock solution from which we will make all of our dilutions. Its concentration is 2000ug/ml. Use Table 1 as a guide to prepare the protein standards from here.

Table 1. *Preparation of Diluted Albumin (BSA) standards*

Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	0	300 ul of stock	2000 ug/ml
B	125 ul	375 ul of stock	1500 ug/ml
C	325 ul	325 ul of stock	1000 ug/ml
D	175 ul	175 ul of vial B	750 ug/ml
E	325 ul	325 ul of vial C	500 ug/ml
F	325 ul	325 ul of vial E	250 ug/ml
G	325 ul	325 ul of vial F	125 ug/ml
H	400 ul	100 ul of vial G	25 ug/ml
I	400 ul	0	0 ug/ml=blank

Remember to mix each dilution thoroughly by vortexing for 5 seconds, especially the ones from which another concentration will be derived. After making these dilutions, you should have three tubes of each concentration. These dilutions can be frozen and kept for future assays.

2. **Coomassie Plus Reagent**

   Mix the Coommassie Plus reagent solution immediately before use by gently inverting the bottle several times. (Do not shake!) Remove the amount of reagent needed and equilibrate it to room temperature before use, place the reagent to be used in a drawer out of the light. Immediately replace the reagent bottle to the fridge.

   Note: Aggregates may form in this solution. To disperse them, gently shake and they should dissolve.

B. Assay Procedure (working range 100-1500 ug/ml)

   1. Pipette 15 ul of each standard or unknown sample into the appropriate microplate wells. Each sample or standard should have one duplicate.
   2. Add 300 ul of the Coomassie Plus reagent to each well and mix with plate shaker for 30 seconds.
   3. Remove the plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature.
   4. Measure the absorbance at or near 595 nm with a plate reader.
   5. Subtract the average 595 nm measurement for the blank replicates from the 595 nm measurement for each BSA standard vs. its concentration in ug/ml. Use the standard curve to determine the protein concentration of each unknown sample.
   6. Prepare a standard curve by plotting the average blank –corrected 595 nm measurement for each BSA standard vs. its concentration in ug/ml. Us the standard curve to determine the protein concentration of each unknown sample.

Appendix D: Raw Data

Sample ID	units/min	BLANK	Conc.	Units/min/mg
w167	-1.66E-01	-8.00E-04	2.85E-01	-5.83E-01
w168	-1.43E-01	-8.00E-04	2.30E-01	-6.23E-01
y122	-1.51E-01	-8.00E-04	3.50E-01	-4.33E-01
y126	-1.35E-01		3.07E-01	-4.40E-01
y186	-1.78E-01		3.61E-01	-4.94E-01
v180	-1.14E-01		2.35E-01	-4.85E-01
v188	-1.64E-01		2.63E-01	-6.23E-01
r109	-1.52E-01		2.35E-01	-6.48E-01
r111	-1.46E-01		1.99E-01	-7.32E-01
r113	-1.48E-01		2.32E-01	-6.39E-01
w55	-1.86E-01	3.24E-03	2.38E-01	-7.82E-01
w160	-1.89E-01	3.56E-03	1.36E-01	-1.39E+00
w162	-1.13E-01	3.40E-03	1.40E-01	-8.04E-01
y89	-7.09E-02		2.10E-01	-3.38E-01
y161	-7.01E-02		1.38E-01	-5.08E-01
r117	-5.87E-02		2.77E-01	-2.12E-01
r120	-4.97E-02		1.96E-01	-2.53E-01
v183	-5.05E-02		1.01E-01	-5.00E-01
v185	-3.04E-01		3.10E-01	-9.80E-01
v189	-7.91E-02		1.54E-01	-5.14E-01
v182	-2.25E-01	-4.10E-02	2.75E-01	-8.20E-01
v201	-2.34E-01	-5.40E-02	3.63E-01	-6.44E-01
w170	-2.09E-01	-4.75E-02	2.45E-01	-8.54E-01
w155	-8.66E-02		3.51E-01	-2.47E-01
w169	-1.90E-01		2.62E-01	-7.27E-01
r118	-1.96E-02		2.17E-01	-9.04E-02

r106	-1.31E-02		2.31E-01	-5.66E-02
r105	3.84E-02		1.32E-01	2.91E-01
y165	-2.02E-01		2.18E-01	-9.26E-01
y159	-5.39E-02		1.95E-01	-2.77E-01
w127	-2.01E-01	-4.10E-02	2.71E-01	-7.42E-01
v179	-6.37E-02	-5.40E-02	2.25E-01	-2.83E-01
r125	-2.71E-01	-4.75E-02	3.34E-01	-8.12E-01
r189	-3.03E-01		3.45E-01	-8.79E-01
y136	-1.28E-01		2.47E-01	-5.19E-01
v184	-7.76E-02		2.71E-01	-2.86E-01
y131	-2.02E-01		2.86E-01	-7.06E-01
w159	-1.97E-01		3.15E-01	-6.25E-01
y140	-1.83E-01		3.02E-01	-6.06E-01
w161	-1.44E-01		2.49E-01	-5.77E-01
y108	-6.32E-02	-3.46E-02	4.45E-01	-1.42E-01
r203	-3.71E-02	-4.40E-02	3.92E-01	-9.46E-02
y109	-9.02E-02	-3.93E-02	4.28E-01	-2.11E-01
v3	-3.46E-02		4.16E-01	-8.33E-02
v31	7.03E-03		4.08E-01	1.72E-02
y52	-3.79E-02		2.37E-01	-1.60E-01
w139	-9.97E-02	-4.40E-02	2.62E-01	-3.80E-01
y85	-2.70E-02	-2.80E-02	2.44E-01	-1.10E-01
v79	-1.67E-01	-3.60E-02	4.44E-01	-3.77E-01
v11	-1.68E-01		3.52E-01	-4.78E-01
r77	-1.64E-01		3.03E-01	-5.42E-01
v15	-2.04E-02		3.08E-01	-6.63E-02
r198	-1.00E-01		2.19E-01	-4.59E-01

w144	-2.53E-02		3.74E-01	-6.77E-02
r170	-1.14E-01		3.24E-01	-3.51E-01
y45	-9.31E-02		2.55E-01	-3.65E-01

GPx Activity Assay -18 week TRAMP

Quantitative PCR Data (Normalized to (HPRT + TBP/2)

AR							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1782	0.1850	0.1505	0.2030	0.1713	0.1808	0.2346	0.1698
0.1729	0.1792	0.1472	0.2148	0.1736	0.1888	0.2323	0.1558
0.1670	0.1717	0.1484	0.2014	0.1870	0.1945	0.2330	0.1822
0.1933	0.2000	0.1549	0.2386	0.1854	0.2006	0.2335	0.1655
0.1987	0.1828	0.1432	0.2278	0.1791	0.1965	0.2379	0.1727
0.1836	0.1918		0.2317	0.2012	0.2052	0.2473	0.1764
				0.1881	0.2068	0.2364	0.2035
				0.1890	0.1997	0.2620	0.1882
				0.1891	0.2053	0.2447	0.1926
IGF-1							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1480	0.1030	0.1731	0.0938	0.0807	0.1230	0.4109	0.1905
0.1917	0.1161	0.1460	0.0788	0.0834	0.1239	0.4079	0.2066
0.2144	0.1329	0.1409	0.0805	0.0828	0.1270	0.3618	0.1793
0.2203	0.1132	0.1458	0.1293	0.0935	0.1260	0.3600	0.1797
0.1897	0.1539	0.1584	0.1219	0.0881	0.1158	0.3792	0.1816
0.2120	0.1221	0.1615	0.1077	0.0893	0.1286	0.3761	0.2281
0.2248	0.1177	0.2026	0.1015	0.0845	0.1321	0.3831	0.2113
0.1557		0.1964	0.0990	0.0850	0.1326	0.3907	0.1832
0.1985		0.1734	0.0882	0.1075	0.1404	0.4023	0.2042
FOXO3a							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1563	0.1954	0.1557	0.2465	0.1566	0.1873	0.2515	0.2007
0.1462	0.2307	0.1450	0.2310	0.1546	0.1884	0.2544	0.1992
0.1628	0.2311	0.1456	0.2420	0.1634	0.2155	0.2373	0.2003
0.1444	0.2161	0.1520	0.2538	0.1631	0.1894	0.2209	0.1961
0.1424	0.2294	0.1427	0.2516	0.1725	0.1855	0.2520	0.1955
0.1223	0.1828	0.1321	0.2489	0.1643	0.1867	0.3228	0.1976
0.1322	0.2089	0.1445	0.2331	0.1560	0.1861	0.2336	0.1955
0.1512	0.2296		0.2232	0.1649	0.1955	0.2533	0.1953
0.1661	0.2341		0.2612	0.1580	0.1705	0.2545	0.1909

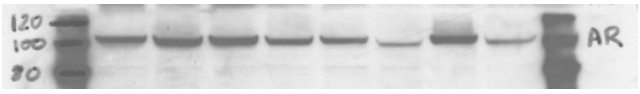
DHCR24							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1721	0.2300	0.1395	0.1659	0.1546	0.1787	0.2053	0.2139
0.1611	0.2201	0.1488	0.1916	0.1584	0.1388	0.2125	0.1960
0.1697	0.2179	0.1633	0.1842	0.1559	0.1562	0.1786	0.2099
0.1646	0.2169	0.1617	0.1934	0.1488	0.1514	0.2156	0.1981
0.1529	0.2281	0.1661	0.2048	0.1499	0.1473	0.2195	0.1857
0.1689	0.2308	0.1552	0.2003	0.1527	0.1538	0.2330	0.1865
0.1808	0.1939	0.1736	0.2141	0.1594	0.1527	0.2289	0.2129
	0.2287		0.1929	0.1571	0.1427	0.2200	0.2147
	0.2183		0.2059	0.1541	0.1640		0.1939
FOXA1							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1620	0.2375	0.1245	0.2070	0.1580	0.2028	0.3665	0.2970
0.1788	0.2513	0.1224	0.2258	0.1678	0.2080	0.4120	0.2536
0.1925	0.2506	0.1278	0.2131	0.1938	0.2139	0.3550	0.2610
0.1562	0.2336	0.1135	0.2075	0.1644	0.1601	0.3036	0.2659
0.1785	0.2403	0.1225	0.1897	0.1719	0.1648	0.3042	0.2488
0.1828	0.2463	0.1390	0.2168	0.1647	0.1659	0.3136	0.2451
0.1535	0.2366	0.1286	0.2182	0.1744	0.2061	0.3247	0.2207
0.1694	0.2313	0.1330	0.1952	0.1626	0.1609	0.2477	0.2193
0.1878	0.2589				0.1600	0.3057	0.2361
IGFBP3							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1935	0.1125	0.1673	0.1811	0.1590	0.2339	0.2436	0.1129
0.1960	0.1155	0.1733	0.1739	0.1495	0.2265	0.2460	0.1133
0.1866	0.1061	0.1530	0.1733	0.1562	0.1983	0.2352	0.1053
0.2054	0.1152	0.1653	0.1892	0.1646	0.2175	0.2555	0.1129
0.2076	0.1452	0.1952	0.1787	0.1692	0.2268	0.2541	0.1179
0.2076	0.1301	0.1867	0.1757	0.1690	0.2325	0.2469	0.1094
0.1892	0.0966	0.1667	0.1728	0.1485	0.2171	0.2373	0.0957
0.2053	0.0918	0.1401	0.1982	0.1540	0.2310	0.1906	0.1106
	0.0951	0.1988	0.1672	0.1612	0.2208	0.2445	0.1017

ABCC4							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.3835	0.5899	0.6077	0.5397				
0.3736	0.6106	0.6203	0.5565				
0.3085	0.6306	0.6552	0.5662				
0.4006	0.5839	0.6466	0.5174				
	0.6418	0.6641					
	0.6777						
AKR1C14							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1033	0.0828	0.2556	0.4697	0.1757	0.2271	0.2439	0.1351
0.1000	0.0583	0.3108	0.4344	0.1900	0.2219	0.2402	0.1647
0.0875	0.0909	0.3222	0.3922	0.1775	0.2704	0.2222	0.1553
0.0701	0.0630	0.2684	0.3583	0.1781	0.2362	0.2328	0.1317
0.0749	0.0454	0.3257	0.4498	0.2098	0.2841	0.1688	0.2100
0.0637	0.0651		0.3768	0.2154	0.2379	0.2185	0.1459
ACSL3							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1937	0.1347	0.1833	0.1641	0.2117	0.2111	0.1854	0.1761
0.1994	0.1384	0.1843	0.1488	0.2086	0.2100	0.2006	0.1663
0.2011	0.1479	0.2049	0.1584	0.2021	0.2148	0.1946	0.1758
0.1935	0.1400	0.1782	0.1457	0.2147	0.2168	0.1666	0.1825
0.1804	0.1343	0.1925	0.1593	0.1960	0.2059	0.2039	0.1822
0.1920	0.1446	0.1908	0.1320	0.2037	0.2048	0.2023	0.1669
0.1849	0.1411	0.1767	0.1410	0.2020	0.2039	0.1794	0.1703
0.1988	0.1360	0.1921	0.1397	0.2040	0.2029	0.2018	0.1761
0.1929	0.1465	0.1715	0.1795	0.1970	0.2020	0.1931	0.1737

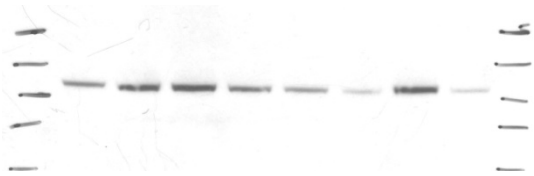
SV40 Large T Antigen							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1701	0.1755	0.1240	0.1346	0.1807	0.2246	0.1358	0.1670
0.1689	0.1716	0.1495	0.1370	0.1769	0.2122	0.1494	0.1589
0.1829	0.1729	0.1713	0.1352	0.1651	0.2190	0.1587	0.1575
0.1854	0.1767	0.1654	0.1303	0.1871	0.2076	0.1307	0.1718
0.1870	0.1597	0.1769	0.1275	0.1559	0.2102	0.1691	0.1505
0.1829	0.1797	0.1515	0.1272	0.1728	0.2059	0.1600	0.1588
				0.1687	0.2185	0.1520	0.1668
				0.1800	0.2192	0.1638	0.1542
				0.1856	0.2226	0.1600	0.1692
FOXO1							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.1617	0.1705	0.1212	0.1533	0.1662	0.1788	0.2545	0.1762
0.1547	0.1531	0.1393	0.1453	0.1110	0.1839	0.2449	0.1666
0.1542	0.1718	0.1314	0.1512	0.1609	0.1879	0.2401	0.1771
0.1518	0.1841	0.1398	0.1803	0.1625	0.1957	0.2398	0.1847
0.1473	0.1593	0.1323	0.1533	0.1561	0.1842	0.2432	0.1872
0.1502	0.1734	0.1472	0.1799	0.1568	0.1794	0.2343	0.1849
0.1497	0.1966	0.1383	0.1706	0.1594	0.1935	0.2247	0.1691
0.1505	0.1555	0.1341	0.1657	0.1607	0.1844	0.2399	0.1698
0.1405	0.1671	0.1433	0.1690	0.1542	0.1909	0.2440	0.1734
GUCY1A3							
18 weeks				24 weeks			
Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/ Low Iso	High Se/ High Iso
0.2790	0.1566	0.1417	0.1393	0.1977	0.1577	0.2350	0.1765
0.2424	0.1457	0.1675	0.1260	0.2246	0.1376	0.1950	0.1543
0.2476	0.1690	0.1798	0.1282	0.1969	0.1579	0.1772	0.1195
0.2433	0.1483	0.1539	0.1127	0.2260	0.1664	0.1946	0.1887
0.2628	0.1494	0.1854	0.1305	0.2314	0.1686	0.2028	0.1785
0.2453	0.1238	0.1569	0.1588	0.2260	0.1498	0.2213	0.1507
0.2369	0.1364	0.1276	0.1189	0.2020	0.1469	0.1985	0.1520
0.2317	0.1497	0.1665	0.1222	0.1985	0.1589	0.2029	0.1828
0.2454	0.1467	0.1565	0.1624	0.2179	0.1413	0.2009	0.1705

Western Blot Data

AR



BLOT 1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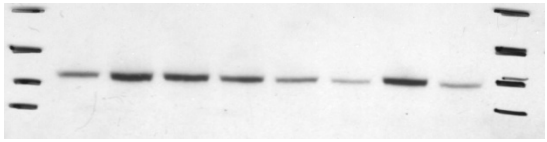
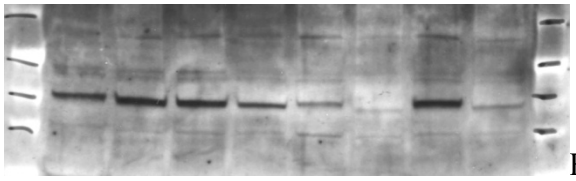

BLOT 3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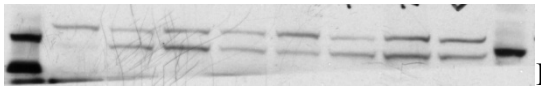
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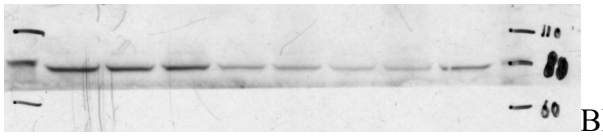

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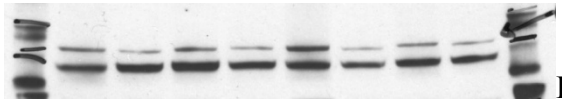
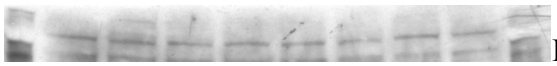

BLOT 6

ACSL3


BLOT 12


BLOT 13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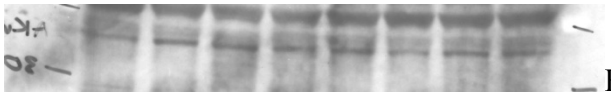
BLOT 10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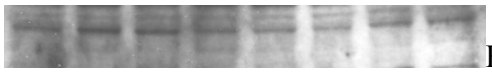
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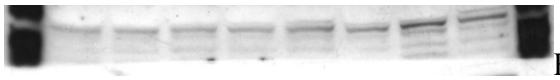
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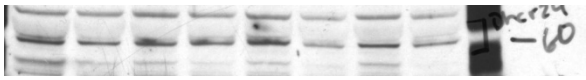
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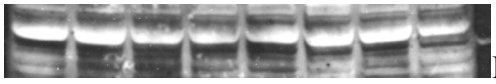
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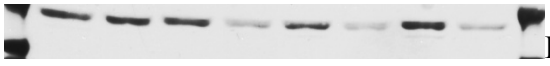
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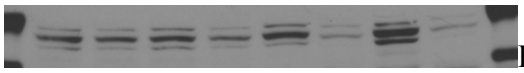
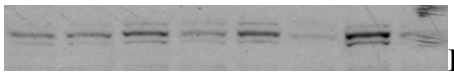

BLOT 5


BLOT 6

BLOT 7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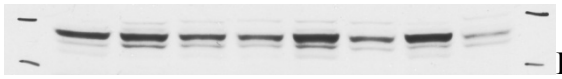
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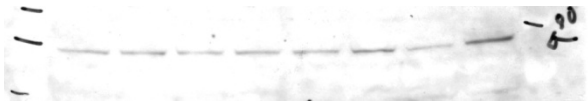

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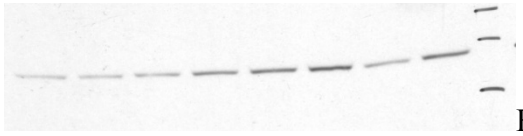
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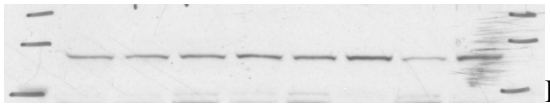
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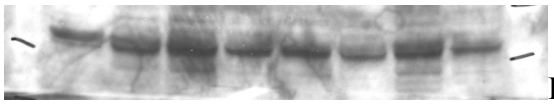
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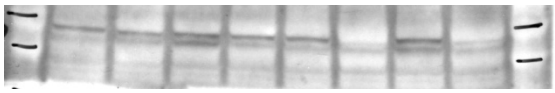
BLOT 14

UGT Weight Chart

	24 Week UGT				18 Week UGT			
Diet	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/Low Iso	High Se/High Iso	Ade Se/ Low Iso	Ade Se/ High Iso	High Se/Low Iso	High Se/High Iso
	0.52424	0.4644	0.4378	0.39718	1.22596	0.51616	0.52785	0.6344
	0.57127	0.6579	0.5447	0.46688	0.96164	0.66447	0.6597	0.80976
	0.81244	0.6804	0.5533	0.631	1.15275	0.70096	0.79845	0.80976
	1.12846	0.7593	0.66372	0.78496	1.25631	0.7460	0.8459	0.8197
	1.173	0.7738	0.6826	0.78735	1.117	0.76453	0.98817	0.88375
	1.262	0.8353	0.7306	0.80355	1.37615	0.79057	1.303	1.0712
	1.337	0.8877	0.7404	0.83164	1.2963	0.81535	1.701	1.0712
	1.37078	0.8928	0.7981	0.87288	6.22769	0.94965	0.6435	1.3289
	1.40233	0.9509	0.8397	0.902	0.91428	1.1013	1.0647	1.3289
	1.688	0.96	0.8403	0.93027	1.31791	1.15729	3.0842	1.3854
	1.7743	0.999	0.8717	0.95981	1.27475	1.8858	1.01387	1.08463
	1.83603	1.0044	0.9642	0.97049	1.07569	0.48457	0.49838	0.9387
	1.8842	1.073	0.9893	0.98324	1.13023	2.4321	0.8809	1.35909
	1.93155	1.103	0.999	0.99555	3.207	0.3762	0.777	0.60474
	1.9734	1.1053	1.10025	1.00927	1.13899	0.73193	0.9525	1.13082
	2.11137	1.1571	1.1037	1.01021	1.56124	0.70895	5.6708	0.84758
	2.1134	1.1829	1.183	1.01143	1.04832	2.9758		0.93563
	2.2292	1.229	1.22425	1.03203	0.926121	0.51939		0.69927
	2.2679	1.3571	1.30031	1.07	0.95972	0.47045		0.52086
	2.41686	2.1925	1.37707	1.07927	0.96962			0.69894
	2.41894	2.4954	1.46427	1.10822	0.97323			

	2.5949	7.1513	1.5032	1.14438	0.95309			
	2.64505	8.1194	1.69626	1.24131	1.18901			
	2.7728	8.1765	1.70143	1.2522	0.93524			
	6.01186	11.531	1.82765	1.25325	0.66832			
	1.88606	14.695	1.83193	1.267	0.93201			
	6.1603	0.8366	1.98035	1.2693	1.4765			
	5.08313	7.3743	2.30221	1.2916	0.83838			
	2.6371	1.6824	2.7209	1.2916	1.16173			
	2.60754	6.3517	3.04287	1.34455	1.14152			
	10.62558	0.9623	4.4038	1.37914	1.0027			
	3.4		9.34339	1.39443	1.26451			
	4.0323		9.488	1.4023	0.4602			
	1.648543		10.81	1.42587	0.99924			
	1.648982		12.198	1.439	0.74181			
	1.80454		4.0652	1.5294	0.81314			
	2.014		1.04958	1.57				
	1.36765		1.06541	1.58249				
	4.7469			1.62434				
	2.61069			1.73155				
	3.05			1.763				
	2.11825			1.79465				
	3.22345			1.84267				
	2.26327			2.29528				
	3.04025			3.53101				
				3.6879				
				6.04383				
				7.255				

				9.9678				
				11.16				
				11.174				
				11.8515				
				0.562015				
				0.49743				
				1.19206				
				0.51505				
				0.56414				
				1.18913				
				1.8412				