SEX STEROID AND GROWTH FACTOR EXPRESSION IN PROSTATES OF TRANSGENIC MICE EXPOSED TO DIETARY POLYPHENOLS

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SEX STEROID AND GROWTH FACTOR EXPRESSION IN PROSTATES OF TRANSGENIC MICE EXPOSED TO DIETARY POLYPHENOLS

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5TH YEAR M.S. IN BIOLOGY PROGRAM

Prostate cancer is the second leading cause of cancer death in men. Because prostate cancer often progresses towards androgen-independence to become a malignant disease, chemoprevention is an attractive method of dealing with this disease. Several factors, including diet can contribute to risk of prostate cancer. Epigallocatechin-3-gallate (EGCG), genistein, and resveratrol are dietary polyphenols that have been demonstrated to be chemopreventive of various cancers, including prostate cancer. However, few studies have been devoted to the chemopreventive abilities of these polyphenols in combination. The goal of this investigation was to determine the ability of EGCG, resveratrol, and genistein in combination of twos to inhibit prostate cancer through regulation of sex steroid and growth factor signaling. For these studies, we have employed a transgenic mouse model that spontaneously develops prostate cancer (Transgenic Adenocarcinoma Mouse Prostate (TRAMP)). TRAMP mice exhibit the progressive stages of prostatic carcinoma. The treatments were 1) control phytoestrogen-free AIN-76A, 2) resveratrol and genistein combination, 3) resveratrol and EGCG combination, and 4) genistein and EGCG combination diet. Non-transgenic C57BL/6 mice were also generated for comparison to TRAMP controls. In the dorsolateral prostates (DLP) and ventral prostates (VP) of genistein and EGCG combination-fed TRAMP mice, insulin-like growth factor 1
(IGF-1) and IGF-1R beta were significantly down-regulated while total extracellular signaling kinases (total-ERKs) were significantly up-regulated in the DLP. In addition, genistein and EGCG in combination down-regulated cell proliferation and up-regulated apoptosis in the DLP of TRAMP mice. Resveratrol and genistein in combination significantly up-regulated apoptosis in the DLP and VP of TRAMP mice, yet resveratrol and EGCG in combination did not significantly alter growth factor signaling, cell proliferation, or apoptosis. The results provide mechanistic basis for EGCG, resveratrol, and genistein in combinations being chemopreventive against prostate cancer development.
DEDICATION

I would like to dedicate this work to my mother, Anna C. Cook, for all of her support and encouragement throughout the years.
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First, I would like to thank my mentor, Dr. Coral Lamartiniere, for allowing me to do research in his lab. He has truly been a great mentor, always encouraging me to do my best. Dr. Lamartiniere has greatly contributed to my understanding and appreciation for science and, for that, I will always be grateful. Working in his laboratory has been a monumental experience in my life that I will never forget and I hope that the research I have contributed will be an asset to him.

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Next, I’d like to thank the other members of the prostate team in Dr. Lamartiniere’s lab, Dr. Jun Wang and BJ Patel, both of whom contributed to my research as well as my understanding of prostate cancer.

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PI3K................................................................................................. Phosphatidylinositol 3-kinase
PTEN.................................................................................... Phosphatase and Tensin Homolog
SV .............................................................................................................. Seminal Vesicle
SV-40 ........................................................................................................ Simian Virus 40
TERK .......................................................................................... Total Extracellular Signaling Kinase
TRAMP .............................................................. Transgenic Adenocarcinoma Mouse Prostate
VP ............................................................................................................... Ventral Prostate
INTRODUCTION

Prostate Cancer

Cancer is a disease characterized by uncontrolled cell growth and spread of abnormal cells. Prostate cancer is the leading cause of cancer death among men. It develops in one of every six American men. It was estimated that nearly 218,890 men in the U.S. will be diagnosed with prostate cancer in 2007. Over 27,000 men were estimated to die of prostate cancer in 2007 [1]. Contributing to the cause of prostate cancer are endogenous factors (hormones, and inherited and metabolism-derived mutations), as well as exogenous agents including environmental exposures such as smoking and dietary factors.

Treatments and Chemoprevention

Several treatments for prostate cancer include radical prostatectomy, castration, and hormonal therapy. Although prostatectomy and castration can be used to treat localized prostate cancer, studies demonstrate a high incidence of disease recurrence. Castration has been shown to increase incidence of osteoporosis, and hormonal therapy can result in gynaecomastia [2]. Due to the emergence of androgen-independence, prostate cancer is mostly unpredictable. However, since the disease exhibits slow metastasis and progression to malignancy, chemoprevention has become an innovative method to com-
bat prostate cancer. Chemoprevention is characterized by the use of natural products and pharmaceutical agents to inhibit one or more steps in the initiation, development, and progression of disease.

Anatomy of the Prostate

The prostate, a gland of the male reproductive system, is responsible for fluid production in the semen that nourishes and protects sperm. In humans, the prostate lacks lobular organization and has been characterized as having three morphological regions or zones: the peripheral zone, the transition zone, and the central zone [3]. Prostate carcinoma arises primarily in the peripheral zone.

In contrast with humans, rodent prostates are divided into four lobes: the anterior, dorsal, lateral, and ventral lobes. The combined dorsolateral lobe has been linked to being morphologically similar to the peripheral zone of the human prostate [3]. Furthermore, Berquin et al. demonstrated that, in a comparison of genes of mouse prostate lobes and the human prostate, gene expression patterns were found to be closest between the dorsolateral lobe of the mouse prostate and the peripheral zone of the human prostate [4].

The TRAMP Model

In 1995, Greenberg et al. developed the Transgenic Adenocarcinoma Mouse Prostate (TRAMP) model. Generation of the TRAMP model, a system based on prostate-specific transgene expression, involves ligation of the prostate-specific rat probasin promoter and simian virus-40 (SV-40) large T antigen. SV-40 large T antigen acts as an on-
coprotein through interactions with the retinoblastoma and p53 tumor suppressor gene products. TRAMP mice exhibit mild to severe epithelial hyperplasia at ten weeks of age in the dorsolateral prostate (DLP) and, as seen in human prostatic carcinoma, show signs of invasive adenocarcinoma (by eighteen weeks of age). Metastasis of prostate cancer in these mice closely mimics that seen in human prostate cancer [5].

**Sex Steroid and Growth Factor Signaling**

*Androgens*

Androgens are required for development, normal function, and growth of the prostate. The ligand acts through the androgen receptor. Transcriptional activity is initiated by the binding of testosterone and DHT, the most potent androgen, to a functional androgen receptor (AR). Androgen-induced transcriptional activity of AR is regulated by the phosphorylation of AR and its interaction with AR coregulators in response to growth factors [6]. Once bound to AR, the androgen-AR complex can then translocate into the nucleus and initiate gene transcription. Through AR activation, androgen regulates proliferation and cell growth in prostate cancer cells [7]. The majority of AR is found in the secretory epithelial cells [8]. Although most prostate cancer patients respond favorably to androgen ablation and anti-androgen therapy, most relapse into clinically defined androgen-independent progression [9].

*Estrogens*

Although estrogens were originally believed to be solely associated with the female reproductive system, they also play a role in the male reproductive axis. Estrogens
affect the hypothalamic-pituitary-gonadal axis through its regulation of androgen secretion. They can also directly affect the testes by stimulating the production of luteinizing hormone (LH), a hormone required for testosterone production [10]. Estrogens act through the estrogen receptor (ER), which has two subtypes, alpha and beta. ER-beta is found in human and rodent prostates and is mostly localized to the epithelial cells but can also be found in stromal cells. ER-alpha is mainly found in stromal cells [10]. Although definitive roles of ER-alpha and ER-beta remain unclear, studies give indication of their actions through the use of knockout models. In 2001, Prins demonstrated in ER-alpha knockout mice that ER-alpha may be the mediator for estrogen responses contributing to prostatic lesions. ER-beta knockout mice exhibit differentiation defects [11], hence ER-beta appears to be a marker for cell differentiation.

**Insulin-like Growth Factor axis**

**IGFs.** Insulin-like growth factors (IGFs) play a role in cell proliferation, differentiation, and apoptosis. IGF-1 and IGF-II both have mitogenic and apoptotic actions. Studies indicate that IGF-II plays a significant role during embryonic and fetal growth. After birth, the role of IGF-II is gradually taken over by IGF-1 [12]. A majority of circulating IGF-1 is produced by the liver, although various tissues including: brain, smooth muscle, and the prostate are capable of IGF-1 synthesis [13-15]. IGF-1 has a long term impact on cellular activities. Through mediation of its actions by IGF-1 receptor (IGF-1R), located in the cell membrane, IGF-1 increases DNA synthesis and stimulates cyclin D1 expression, accelerating progression of the cell cycle from G1 to S phase [16]. IGF-1R has two subunits: alpha and beta. IGF-1 binds to the alpha subunit of IGF-1R which protrudes from
the cell membrane. Binding of IGF-1 to the alpha subunit, activates the beta subunit, embedded within the cell membrane with its opposite end extending into the intracellular matrix, hence initiating signal transduction within the cell (Figure 1) [17]. IGF-1 action can be regulated by several hormones and growth factors including growth hormone (GH), follicle-stimulating hormone (FSH), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) as well as ER alpha [18].

Insulin-like growth factor binding proteins. There are six insulin-like growth factor binding proteins. IGF Binding Protein 3 (IGFBP-3) is synthesized in the liver as well as other tissues and is, therefore, available in the extracellular fluid to regulate the actions of IGF-1 [19]. An excess of IGFBP-3, the main carrier of IGF-1 in the serum, has been shown to significantly inhibit IGF-1 stimulated DNA synthesis [20]. The mechanism through which IGFBP-3 inhibits IGF-1 action involves IGF-1R. Although the IGF-1/IGFBP-3 complex can interact with the cell surface, no investigations to date have reported interaction of this complex with the IGF-1 receptor. This evidence suggests that IGFBP-3 has a higher binding affinity for IGF-1 than does the IGF-1 receptor hence IGF-BP3 affects IGF-1 bioavailability.

Mitogen-activated protein kinases. IGF-1 acts through the mitogen-activated protein kinases (MAPKs) to initiate transcription of genes associated with increased cell proliferation, growth, and anti-differentiation. Binding of IGF-1 to its receptor activates the Ras-Raf system which involves activation of the MAPKs, including total extra-cellular regulating kinases 1 & 2 (total-ERKs 1/2) and its activated form phospho-extracellular...
regulating kinases 1 and 2 (phospho-ERKs 1 and 2), which act as transcription factors to initiate transcription (Figure 1) [21].

**PTEN/ phospho-Akt Signaling Pathways.**

PTEN, a tumor suppressor protein, is a novel cell growth modulator that inhibits the actions of oncogenes that encode for the genes of phosphatidylinositol 3-kinase (PI3K) and phospho-protein kinase B (pAkt). Mutations of PTEN are extremely common in several melanoma cell lines [22], endometrial carcinomas [23], a significant percentage of breast cancer cell lines [24], and advanced prostate cancers [25]. PTEN can also dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP₃), a signal transduction molecule known for activating Akt (Figure 1) [26]. In general, PTEN is one of the most common mutations found in human cancers.

PI3K plays a significant role in intracellular signaling by the activation of the Akt pathway. Although other signaling pathways are activated downstream of PI3K, Akt has caught much attention because of its role in cell survival. Akt has been shown to induce phosphorylation of multiple targets, including Bcl2-Antagonist of Cell Death (BAD). In the absence of this phosphorylation, BAD induces apoptosis in the cell. Through phosphorylation, Akt inhibits the activity of BAD [27].

Several investigations now establish a correlation between activation of the PI3K/Akt pathway and human cancers via defects in PTEN. Normally, Akt is low in the absence of growth factor stimulation. However, tumors derived from PTEN deficient
mice exhibit increased phosphorylation of Akt [28] as well as growth factor induced Akt phosphorylation. Reconstitution of PTEN through retroviral expression restores normal Akt regulation. Yet in normal PTEN cells, growth factors remain able to activate Akt via a PI3K dependent pathway suggesting that PTEN is possibly activated by growth factor depletion [29].

**Figure 1.** Growth factor signaling pathway
**TRAMP Mice and Signaling Pathways**

Wang et al. demonstrated several changes in sex steroid and growth factor signaling regulation in the dorsolateral prostates of TRAMP mice. TRAMP mice and non-transgenic C57BL/6 were generated, weaned at five weeks of age, and sacrificed at 12 weeks. Both groups were placed on a phytoestrogen free powder diet. Immunoblot analysis of dorsolateral prostate showed a 26-fold increase in AR expression in prostates of TRAMP mice when compared to the C57BL/6 mice. ER-alpha was significantly increased five-fold in TRAMP mice; however, there was no change in ER-beta expression. There were also significant differences in expression of various components of the IGF-1 pathway in prostates of TRAMP mice. Expression of IGF-1R, ERK-1, and ERK-2 was significantly higher in dorsolateral prostates of TRAMP mice when compared to those of C57BL/6 mice [30].

**Polyphenols and Prostate Cancer Chemoprevention**

*Green Tea Consumption and EGCG*

Long term consumption of green tea has been shown to lower the incidence of various cancers such as colon, gastric, and breast cancers. A recent epidemiological study performed in southeast China demonstrated a significant correlation between green tea consumption and incidence of prostate cancer. The risk of prostate cancer tended to decline with increasing frequency and duration of tea drinking. Also, increasing the
number of new batches of tea brewed per day to 2 or more was associated with a 76% reduced risk of prostate cancer [31]. Despite several investigations demonstrating the chemopreventive effects of tea, the relationship between green tea consumption and prostate cancer risk remains unclear.

Epigallocatechin-3-gallate (EGCG) (Figure 2) is the most abundant of the four catechins found in green tea, making up 62% of its composition and may be a mechanism of action for the chemopreventive effects of green tea.

![Structure of epigallocatechin-3-gallate (EGCG)](image)

Figure 2. Structure of epigallocatechin-3-gallate (EGCG)

EGCG has been shown to inhibit phosphorylation of three distinct MAPKs including ERKs 1 and 2 [32]. Also, EGCG has been reported to have inhibitory effects on PI3K in mouse epithelial JB6 cells, therefore inhibiting Akt signaling [33].

Gupta et al. demonstrated that administration of green tea polyphenols to TRAMP mice significantly lowered serum levels of IGF-1 and significantly increased IGF-BP3 serum levels [34]. In the DLP of TRAMP mice, EGCG significantly reduced IGF-1 ex-
pression when compared to control-fed TRAMP mice. In the VP of EGCG-fed TRAMP mice, IGF-1, IGF-1R alpha, phospho-ERK 1, and phospho-ERK 2 were significantly reduced [64 and Appendix A]. Although some investigations waver on the issue of green tea intake decreasing the risk of prostate cancer, EGCG has been shown to regulate some of the key pathways associated with prostate cancer.

**Genistein**

Incidence of prostate cancer can vary considerably between continents with as much as a 90-fold difference. The lowest rates are seen in Asia with only two Asian men out of 100,000 men developing clinical prostate cancer [35]. In comparison with Western nations, Asians have a greater per capita consumption of soy products (and green tea). Several studies have demonstrated soy intake as a method of lowering prostate cancer risk by as much as 30% [36]. Epidemiological studies have represented an inverse correlation between soy consumption and prostate cancer mortality [37].

Genistein is a major component of soybeans and other legumes. Its structural resemblance to estrogen (Figure 3) allows it to bind to estrogen receptors and exert weak estrogenic effects [38]. Asian women that consume large amounts of soy have a low incidence of breast cancer [39]. This evidence suggests that genistein may be a chemopreventive agent acting as an anti-estrogen to inhibit signaling pathways associated with breast cancer.
In addition to reducing breast cancer risk, genistein has become a novel method for prostate cancer chemoprevention. Lamartiniere et al. investigated the ability of genistein to protect against prostate cancer in Lobund-Wistar rats and TRAMP mice. Their studies revealed that lifetime administration of dietary genistein significantly reduced the incidence of prostate tumors classified as invasive adenocarcinomas in both rats and mice [40].

Genistein has also been shown to regulate sex steroid and growth factor signaling in the prostate. Treatment of androgen-sensitive LnCaP cells with genistein results in a dose-dependent decrease of AR [41] and TRAMP mice administered dietary genistein for 12 weeks showed significant signaling regulation. Epidermal growth factor (EGFR), IGF-1R and ERKs 1 and 2 were significantly reduced. Although there was no significant change in AR expression, cell proliferation was significantly reduced by 47% [20] in the prostates of genistein-fed TRAMP mice when compared to TRAMP controls. Although Asian diets include large amounts of soy consumption as well as green tea, few studies have investigated the effects of genistein and EGCG in combination as a means of prostate cancer chemoprevention.
Resveratrol

Several epidemiological studies have linked nutrition to decreased risk of cancer. More specifically, diets rich in soy, green tea, tomatoes, and other isoflavones have been shown to decrease the risk of prostate cancer [42]. However, the French diet seems contradictory to these studies. Although an average diet of a French person is high in saturated fats, it has been proposed that their high intake of red wine has led to a decreased risk of mortality due to coronary heart disease, a phenomenon known as the “French Paradox” [43].

Resveratrol is a phytoalexin (Figure 4) released from the skin of red grapes under stressful conditions caused by fungal infections and cold temperature [44]. Although resveratrol can be derived from various sources (peanuts, red grapes, and pines), the fermentation process of red wine plays a role in resveratrol concentrations extracted from red grape skins [45]. In the early 1990s, scientists began to investigate resveratrol as a possible method of chemoprevention.

![Figure 4. Structure of resveratrol](image-url)
Resveratrol has been shown to inhibit cellular events associated with tumor initiation, promotion, and progression. Resveratrol inhibited tumor initiation in human HL-60 leukemia cells when administered in a dose-dependent manner [46]. In a mouse mammary gland culture model of carcinogenesis, resveratrol inhibited the development of DMBA-induced preneoplastic lesions [46].

Resveratrol has also been linked to prostate cancer chemoprevention. Resveratrol was found to inhibit the growth of androgen-sensitive LnCaP cells in a concentration dependent manner by inhibition of androgen receptor function [47]. Harper et al. demonstrated the ability of resveratrol to reduce the number of poorly differentiated tumors in TRAMP mice [63 and Appendix A]. Their evidence suggests that resveratrol inhibits prostate tumor promotion.

It has been demonstrated that resveratrol can bind to both ER-alpha and ER-beta. Although resveratrol appears to be an agonist for ER-beta and an antagonist for ER-alpha in a number of models and investigations, some studies show it to be a weak agonist for ER-alpha and its effects on estrogen modulation still remain unclear [48].

In the DLP of resveratrol-fed TRAMP mice, ER-beta was significantly increased, IGF-1 was significantly reduced, and phospho-ERK 1 was significantly reduced when compared to the DLP of TRAMP controls. In comparison to the VP of TRAMP controls, IGF-1R alpha was significantly down-regulated, phospho-ERK 1 and phospho-ERK 2 were significantly down-regulated [63 and Appendix A]. Although resveratrol in solitary
doses has been shown to be chemopreventive in prostates of TRAMP mice, few studies have investigated the ability of resveratrol in combination with other polyphenols to possibly prevent cancer.

**Goal and Hypothesis**

The goal of this study was to investigate the potential of combinations of genistein, EGCG, and resveratrol to regulate sex steroid and growth factor protein expression in the prostates of TRAMP mice. We hypothesized that nutritional polyphenols can regulate sex steroid and growth factor signaling to suppress prostate cancer development. Understanding mechanisms of action of these polyphenols will assist in understanding how chemical agents can protect against cancer.
MATERIALS AND METHODS

Animals and Treatments

All animal studies were carried out via the approval of the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC) in conjunction with the UAB Animals Resources Program. Animals were fed phytoestrogen-free pelleted AIN-76A diet. The facility room was held at a constant temperature of 76 °F, with a 12-hour day/night light cycle. Females heterozygous for the SV-40 transgene were bred with C57BL/6 males in a 2:1 ratio. Females were then separated from the breeding cage upon pregnancy. At the age of three weeks, males and females of each litter were tail clipped (0.5 cm), weaned, and animal ears were punched for identification purposes. DNA was extracted from tail clippings using the DNEasy Extraction Kit (Qiagen, Valencia, CA) and used in a Polymerase Chain Reaction (PCR) for qualitative probing of the target transgene [3].

PCR

For each sample, a PCR Mastermix was prepared based on requirements for the use of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA): 5 µL 10X PCR Buffer (Invitrogen), 1 µL 10 mM dNTP mixture, 1.5 µL 50mM MgCl₂, 1 µL each of a forward and reverse primer, 4.2 µL template DNA, and 0.2 µL Platinum Taq DNA Polymerase. The Mastermix was aliquotted into 25 µL tubes and 150 ng of each DNA sample was
added to the individual tubes. The tubes were placed into a thermocycler (MJ Research PTC-200, Ramsey, MN) and PCR was ran under the following conditions: 1) 95 °C for 3 minutes, 2) 94 °C for 1 minute, 3) 60 °C for 1 minute, 4) 72 °C for 1 minute, steps 2, 3, and 4 were repeated 35 times, 5) 72 °C for 10 minutes. Sample buffer (7 µL) (BioRad, Hercules, CA) was added to each tube of PCR product and 7 µL of the mixture was loaded into an agarose gel with 3% ethidium bromide. Bands were detected by UV light.

Treatments

Approximately 160 TRAMP animals were generated and placed on one of five treatment groups: 1) control (AIN 76-A powdered diet), 2) 625 mg resveratrol + 250 mg genistein/kg diet, 3) 625 mg resveratrol/kg diet + 0.06 % EGCG in water, 4) 250 mg genistein/kg diet + 0.06 % EGCG in water. Polyphenol concentrations were based on concentrations shown to be chemopreventive in previous studies of our laboratory. All polyphenols were determined to be ≥ 99% by HPLC and mass spectrometry.

Approximately sixty non-transgenic C57BL/6 mice were placed on phytoestrogen-free AIN 76-A powdered diet. All animals were sacrificed at 12 weeks of age, when TRAMP males develop prostate cancer Grade 4 (well-differentiated lesion) [49]. At necropsy, body weights were recorded and blood was collected for serum preparation. DLP and VP were weighed and flash frozen via liquid nitrogen. Eight DLP and VP of each treatment group, including C57BL/6 mice, were blocked in cassettes and solidified in paraffin for tissue slicing. Both lobes of the prostate were studied in order to determine
differences in mechanisms within the lobes of the prostate. Testes and seminal vesicle weights were also recorded at necropsy.

**Immunoblot Analysis**

When possible, protein expression levels of sex steroid and growth factor receptors and their ligands were measured by western blot analysis. Briefly, tissues were homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) and protease/phosphatase inhibitors (Sigma, St. Louis, MO). Protein concentrations for each sample were determined using the Bradford Protein assay (Pierce, Rockford, IL). Protein supernatant from each sample was added at a ratio of 1:1 to sample buffer containing the following: 0.5 M Tris pH 8.8, glycerol, 10% Sodium Dodecyl Sulfate (SDS), 1% bromophenol blue, and β-mercaptoethanol.

Prior to the analysis of the samples, antibody conditions were optimized using mouse prostate tissue and positive and negative controls in order to determine required amount of tissue and to correctly identify the band of interest. Equal concentrations of protein were electrophoresed hence changes. Gel electrophoresis, using a 10-20% gradient acrylamide gel, was performed for three hours at 120 V. Each gel contained 8 samples per group (24 total samples) and two molecular weight ladders (Kaleidoscope and Pre-stained Broad Range, BioRad Hercules, CA). The gel was blotted to a nitrocellulose membrane at 4 °C. The membranes were blocked with 5% non-fat milk and incubated overnight with the appropriate primary antibodies. After a series of washes, the proper secondary antibody conjugated with horseradish peroxidase was applied. After an addi-
tional series of washes, SuperSignal West Dura Chemiluminescence (Pierce, Rockford, IL) was applied to detect the proteins of interest. Antibodies were purchased from commercial sources and detailed as follows: AR (Santa Cruz Biotechnology, Santa Cruz, CA, SC-816), ER-α (Santa Cruz, SC-7207), ER-β (Santa Cruz, SC-8947), insulin-like growth factor-1 receptor alpha (IGF-1R-α) (Santa Cruz, SC-712), insulin-like growth factor-1 receptor beta (IGF-1R-β) (Cell Signaling Technology, Danvers, MA, #3027) insulin-like growth factor-binding protein 3 (IGF-BP3) (Santa Cruz, SC-9028), phospho-extracellular regulating kinases 1 and 2 (phospho-ERKs 1 and 2) (Cell Signaling, #9101S), total-extracellular regulating kinases 1 and 2 (total-ERKs 1 and 2) (Cell Signaling, #9102), PTEN (Cell Signaling, #9554), pAkt 1/2/3 (Santa Cruz, SC-7985), beta actin (Santa Cruz, SC-47778), SV-40 (Santa Cruz, SC-147). Positive protein controls were purchased from the supplier of the corresponding antibodies and the use of Kaleidoscope Precision Plus Protein and Pre-stained SDS-PAGE Broad Range standards (BioRad) were employed in order to identify the protein of interest. The relative intensity of the bands was measured using BioMax Imaging Software (Biomax Informatics, Martinsried, Germany). Intensity measurements were determined to be sufficient for quantitation of protein expression by performing preliminary optimizing steps which demonstrated that increasing protein concentrations exhibited increased band intensities. Since equal concentrations of protein for each sample were loaded in the gel, differences in band intensity reflect differences in protein expression between samples.
Enzyme-linked Immunoabsorbant Assay (ELISA)

IGF-1 protein levels were quantified in the prostate by indirect ELISA as described by Crowther et al. [50]. Prior to analysis, kinetic curves were set up to establish zero order kinetics. A 96-well plate (Fisher Scientific, Pittsburgh, PA) was coated with 1 ng of protein from each sample in duplicates and incubated at room temperature overnight. Protein was expelled from the plate and washed three times with 200 µL of wash buffer (0.05 % Tween in 1X PBS (BioRad)). A blocking solution (100 µL of 1% Bovine Serum Albumin) was added to each well and allowed to incubate at 37 °C for 1 hour. Blocking solution was expelled and the plate was washed with wash buffer three times. Appropriate primary antibody (100 µL of IGF-1, Santa Cruz, SC-9028) was added to each well and allowed to incubate for two hours at 37 °C. Primary antibody was expelled and the plate was washed three times. Appropriate secondary antibody (100 µL) (goat anti-rabbit (SC-2054) was added to each well and allowed to incubate for two hours at 37 °C. Secondary antibody was expelled and the plate was washed three times. A TMB substrate solution (100 µL) (Sigma, St. Louis, MO) was added to each well and allowed to incubate for 20 minutes. A stopping solution (100 µL of 1N H₂SO₄) was added to each well and allowed to incubate for five minutes. Absorbance was read at 450 nm on the Versa Max plate reader (Molecular Devices, Sunnyvale, CA).

IGF-1 Serum Analysis

Serum levels of IGF-1 were measured using the DSL Mouse/Rat IGF-1 Enzyme Immunoassay Kit (Diagnostic Systems Laboratories, Webster, TX). Serum samples were pretreated with provided sample buffers. Ten µL of each sample were added to a 12 x 75
polypropylene tube along with 140 µL of Sample Buffer I. Each solution was then vortexed and incubated at room temperature for 30 minutes. Sample Buffer II (150 µL) was then added to each tube and the pretreated samples were stored at -20 °C. All samples and kit reagents were allowed to reach room temperature before usage. A 96-well plate was coated with 50 µL of the pretreated samples, standards, and controls in duplicates. The mouse/rat biotin conjugate solution (100 µL of a 1:2000 dilution of mouse/rat Biotin Conjugate concentrate in the Biotin Conjugate Diluent) was then added to each well along with 100 µL of mouse/rat IGF-1 antiserum. The plate was incubated on an orbital microplate shaker for 1 hour while shaking at a speed of 300 rpm. Each well was aspirated and washed five times with 350 µL of Wash Solution (60 mL Wash Concentrate and 1500 mL deionized water). Excess wash solution was expelled by blotting the plate on absorbent material.

The Streptavidin-Enzyme Conjugate Diluent was added to the Streptavidin-Enzyme Conjugate Concentrate (1:50 ratio) just before adding 200 µL of the solution to each well. The plate was incubated on an orbital shaker for 30 minutes while shaking at 300 rpm. Each well was again aspirated and washed five times with 350 µL of Wash Solution and blotted dry. TMB Chromogen Solution (100 µL) was added to each well and the plate was incubated on an orbital microplate shaker for 30 minutes while shaking at 300 rpm. A Stopping Solution (100 µL) was added to each well and hand shaken for 5 seconds. Absorbance of the solution in the wells was measured using the VersaMax microplate reader set to 450 nm. Concentration (ng/mL) of samples and controls were de-
termined by plotting the mean absorbance readings of the controls and unknowns against the mean absorbance readings of all standards in a four-parameter curve fit.

**Ki67 Proliferation Assay**

Ki67 is a biomarker for cell proliferation. It is a protein expressed when the cell cycle progresses from G1 to the S phase. The assay was performed using paraffin-embedded prostate tissue that was sliced with 0.5 µm thickness and fixed on glass slides. All slide preparations were performed by the UAB Comparative Pathology Laboratory. Each slide was de-waxed and rehydrated in gradient alcohols in the following steps at room temperature: 1) xylene for five minutes three times, 2) 100% ethanol for three minutes, 3) 100% ethanol for three minutes, 4) 95% ethanol for three minutes, 5) 95% ethanol for three minutes, 6) 70% ethanol for three minutes, 7) 50% ethanol for three minutes, 8) dH$_2$O for three minutes, 9) dH$_2$O for three minutes and 12) 1X PBS for three minutes.

Tissue slides were boiled in a diluted (1:30) antigen unmasking solution (Vector, Burlingame, CA) for 15 minutes and allowed to cool at room temperature for 20 minutes. Tissue specimens were encircled using a hydrophobic pen and slides were incubated in 3% H$_2$O$_2$ for 10 minutes to block peroxidase activity. All slides were rinsed with 1X PBS and incubated in 1X PBS for five minutes. They were rinsed again with 1X PBS and again allowed to incubate in 1X PBS for five minutes. Slides were incubated in a horse serum blocking solution (ImmPRESS anti-Mouse Kit, Vector) for one hour. Primary antibody for Ki67 (Clone MM1 Antigen, VP-K452, Vector) was diluted in a 1:100 ratio in 1% BSA and added to each slide. The slides were incubated at room temperature
in a hydration chamber for one hour. Slides were then washed twice in 1X PBS for five minutes. Appropriate secondary antibody (ImmPRESS kit, Vector) was applied to each slide and incubated at room temperature for 30 minutes. DAB staining solution (DAB Substrate Kit for Peroxidase, SK-4100, Vector) was prepared according to kit instructions immediately prior to application to the tissue. DAB was added to each slide and incubated for three minutes. Slides were then rinsed in tap water for five minutes.

Each slide was incubated with hematoxylin counterstain (Vector) for 90 seconds and dipped in distilled water for 10 seconds. Tissue slides were cleared and dehydrated in gradient alcohols in the following steps: 1) 50% ethanol for 20 seconds, 2) 70% ethanol for 20 seconds, 3) 95% ethanol for 20 seconds, 4) 95% ethanol for 20 seconds, 5) 100% ethanol for 20 seconds, 6) 100% ethanol for 20 seconds, 7) xylene for 20 seconds, 8) xylene for two minutes or until ready to coverslip. Coverslips were mounted on the tissue slides using Vector Mounting Media. Cells positive for Ki67 were stained brown and total proliferation per animal was calculated as a percentage of positive per total amount of cells. At least 750 cells were counted for each prostate sample (DLP & VP) for a total of six animals per treatment group.

**Apoptosis**

Dorsolateral prostate and ventral prostate tissues were blocked in a cassette and embedded in paraffin. Tissues were sliced with 0.5 µm thickness and fixed on glass slides by the UAB Comparative Pathology Laboratory. Tissues were then H/E stained and mounted with coverslips. Apoptotic bodies were counted and apoptosis was calcu-
lated as a percentage of total number of apoptotic bodies per total amount of cells. At least 750 cells were counted for each prostate sample (DLP & VP) for a total of six animals per treatment group. Identification of apoptotic bodies was confirmed by the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA). The ApopTag kit reveals focal staining inside early apoptotic nuclei and apoptotic bodies.

*ApopTag Apoptosis Detection Kit*

Fixed tissues on glass slides were deparaffinized in the following steps at room temperature: 1) xylene for five minutes three times, 2) 100% ethanol for five minutes, 3) 100% ethanol for five minutes, 4) 90% ethanol for three minutes, 5) 80% ethanol for three minutes, 6) 70% ethanol for three minutes, 7) 1X PBS for five minutes. Tissue specimens were then encircled using a hydrophobic pen and incubated in Proteinase K (Qiagen, Valencia, CA) for 15 minutes at room temperature. Slides were washed twice in distilled water for two minutes. All tissue slides were then incubated in 3% H$_2$O$_2$ in PBS for five minutes to quench endogenous peroxidases. Slides were rinsed twice with 1X PBS for five minutes.

Slides were then incubated in equilibration buffer (provided in kit) for at least 10 seconds. Working strength TdT enzyme was applied to each specimen, covered with a plastic coverslip, and the slides were incubated at 37 °C for one hour. Stop/wash buffer was diluted according to kit instructions and, after removing the plastic coverslips, was applied to each slide. Tissue slides were incubated in stop/wash buffer for 10 minutes.
and washed three times for one minute in 1X PBS. Anti-Digoxigenin Conjugate was added and all slides were incubated at room temperature for 30 minutes in a hydration chamber.

Specimens were washed twice in PBS for four minutes and incubated in DAB peroxidase substrate (provided in kit) for approximately three minutes. Slides were washed in distilled water three times for one minute and incubated in distilled water for five minutes. Methyl green counterstain was added to each specimen and allowed to incubate for 10 minutes at room temperature. Tissue slides were then washed in three changes of distilled water for approximately one minute. Slides were then washed in three changes of 100% n-Butanol for approximately one minute. Lastly, specimens were incubated in xylene three times for two minutes each time and mounted under a glass coverslip using Vector Mounting Medium. Apoptotic bodies stained brown.

**Statistical Analysis**

All statistics were performed using two-sample t-test using unequal variances, ANOVA (Analysis of Variance) test, and the Tukey test. $P<0.05$ is considered statistically significant.
RESULTS

In comparison to non-transgenic C57BL/6 mice, body weights of AIN-76A-fed TRAMP mice were significantly lower. Total prostate weights, DLP, and SV were significantly larger in TRAMP controls, with no significant difference in TRAMP VP sizes (Table 1). There were no significant differences in body weights, prostate weights, or seminal vesicle weights between TRAMP controls and polyphenol-fed TRAMP mice (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Total Prostate (mg)</th>
<th>DLP (mg)</th>
<th>VP(mg)</th>
<th>SV(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td>59</td>
<td>30.4 ± 0.5</td>
<td>22.1 ± 0.6</td>
<td>11.4 ± 0.4</td>
<td>10.7 ± 0.4</td>
<td>291.4 ± 6.7</td>
</tr>
<tr>
<td>TRAMP Controls</td>
<td>55</td>
<td>28.6 ± 0.4**</td>
<td>29.5 ± 0.8**</td>
<td>18.8 ± 0.7**</td>
<td>10.7 ± 0.5</td>
<td>422.8 ± 3.4</td>
</tr>
<tr>
<td>TRAMP Resveratrol and Genistein</td>
<td>35</td>
<td>28.5 ± 0.5</td>
<td>28.2 ± 0.9</td>
<td>18.6 ± 0.8</td>
<td>9.6 ± 0.5</td>
<td>407.5 ± 1.6</td>
</tr>
<tr>
<td>TRAMP Resveratrol and EGCG</td>
<td>35</td>
<td>27.5 ± 0.4</td>
<td>29.2 ± 1.2</td>
<td>18.2 ± 0.8</td>
<td>11.0 ± 0.7</td>
<td>426.3 ± 4.5</td>
</tr>
<tr>
<td>TRAMP Genistein and EGCG</td>
<td>37</td>
<td>28.1 ± 0.6</td>
<td>28.6 ± 0.6</td>
<td>18.0 ± 0.9</td>
<td>10.7 ± 0.8</td>
<td>386.9 ± 3.1</td>
</tr>
</tbody>
</table>

C57BL/6 and TRAMP mice were provided either AIN-76A powdered diet or one of three combination diets (625 mg resveratrol + 250 mg genistein/kg diet, 625 mg resveratrol/kg diet + 0.06% EGCG, 250 mg genistein/kg diet + 0.06% EGCG) from birth until 12 weeks of age. Values represent means ± SEM. * \(P < 0.05\) and ** \(P < 0.01\) when TRAMP controls were compared to C57BL/6 mice.
Sex Steroid and Growth Factor Protein Expression in Non-transgenic and Transgenic Mice

When compared to non-transgenic C57BL/6 mice, AR expression was significantly higher in the DLP of TRAMP controls. However, there was no significant difference in AR expression in VP tissue (Figure 5). IGF-1 expression was significantly higher in the DLP, but not in the VP, of TRAMP controls compared to C57BL/6 mice(Figure 6). IGF-1R beta expression was significantly lower in the TRAMP VP but not significantly different in the DLP compared to C57BL/6 VP tissue (Figure 7). Expression of phospho-Akt was significantly reduced in TRAMP DLP with no significant difference in VP levels compared to C57BL/6 tissues (Figure 8). On the other hand, PTEN expression was significantly reduced in the TRAMP VP but not in the DLP in comparison to C57BL/6 prostates (Figure 9).

Sex Steroid and Growth Factor Protein Expression in TRAMP Mice Treated with Polyphenols

Resveratrol and Genistein. In comparison to prostates of TRAMP controls, IGF-1 was significantly down-regulated (10%) in the DLP of combination resveratrol and genistein-fed TRAMP mice with no significant change found in the VP (Figure 10). Furthermore, resveratrol and genistein did not significantly regulate AR, ER-alpha and -beta, IGF-1R-alpha and -beta, or IGF-BP3 in the DLP and VP of TRAMP mice. (data not shown).
**Resveratrol and EGCG.** In comparison to prostates of TRAMP controls, IGF-1 expression was significantly down-regulated (10%) in the DLP of combination resveratrol and EGCG-fed TRAMP mice with no significant change found in the VP (Figure 10). Furthermore, resveratrol and EGCG did not significantly regulate AR, ER-alpha and -beta, IGF-1R-alpha and -beta, or IGF-BP3 in the DLP and VP of TRAMP mice (data not shown).

**Genistein and EGCG.** IGF-1 expression was significantly down-regulated in the DLP (16%) and VP (5%) of combination genistein and EGCG-fed TRAMP mice when compared to prostates of TRAMP controls (Figure 10). Western blot analysis showed that expression of IGF-1R beta was significantly reduced in both the DLP and VP of genistein and EGCG TRAMP-fed mice (Figures 11 & 12); there was no significant regulation of IGF-R alpha in the prostates of genistein and EGCG TRAMP mice (data not shown). Although total-ERK 1 and 2 expressions were significantly increased in DLP of genistein and EGCG-fed TRAMP mice (Figure 13), there was no significant regulation of phospho-ERKs 1 and 2 when compared to TRAMP controls (data not shown). Also, there was no significant difference in the total-ERK to phospho-ERK expression ratio in DLP tissue of the combination-fed TRAMP mice when compared to TRAMP controls (data not shown). EGFR expression was increased by 28%, although not significantly, in the ventral prostate of genistein and EGCG-fed TRAMP mice (data not shown). Genistein and EGCG did not significantly regulate AR, ER-alpha and -beta, or IGF-BP3 (data not shown).
There was no significant difference in serum IGF-1 levels among the TRAMP combination groups when compared to TRAMP controls (Figure 14).

**Proliferation and Apoptosis**

Cell proliferation was significantly increased in both the DLP and VP of TRAMP controls in comparison to prostates of non-transgenic C57BL/6 mice (Table 2 and Figure 15, A-D). On the other hand, the percentage of apoptotic bodies was significantly lower in both the DLP and VP of TRAMP controls in comparison to prostates of C57BL/6 mice (Table 2 and Figure 16, A-D).

In TRAMP mice fed resveratrol and genistein in combination, cell proliferation was not significantly altered in the DLP and VP, while apoptotic bodies were significantly increased in both the DLP (167%) and VP (144%) (Figure 17, C and D).

In prostates of combination resveratrol and EGCG-fed TRAMP mice, there was no significant change in cell proliferation found in the DLP and VP when compared to prostates of TRAMP controls (Table 2 and Figure 17, D). In addition, there was no significant change in apoptosis in the DLP (102%) and VP (70%) of resveratrol and EGCG-fed TRAMP (Figure 18, F and Table 2).

In combination genistein and EGCG-fed TRAMP mice, cellular proliferation was significantly reduced in the DLP (58%), but not in the VP (38%) compared to TRAMP controls (Table 2 and Figure 17, C). Apoptotic bodies were significantly increased in the DLP (178%), but not the VP of genistein and EGCG-fed TRAMP mice (Figure 18, E and Table 2).
Table 2. Prostate Cell Proliferation and Apoptosis Indices of 12 Week Old Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation Index</th>
<th>Apoptosis Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLP</td>
<td>VP</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>TRAMP Controls</td>
<td>19 ± 4**</td>
<td>13 ± 2**</td>
</tr>
<tr>
<td>TRAMP Resveratrol and Genistein</td>
<td>13 ± 1</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>TRAMP Resveratrol and EGCG</td>
<td>10 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>TRAMP Genistein and EGCG</td>
<td>8 ± 2*</td>
<td>8 ± 0</td>
</tr>
</tbody>
</table>

TRAMP and C57BL/6 mice were exposed to AIN-76A powdered diet alone or one of three combination diets (625 mg resveratrol + 250 mg genistein/kg diet, 625 mg resveratrol/kg diet + 0.06% EGCG, 250 mg genistein/kg diet + 0.06% EGCG) from birth until 12 weeks of age. Cell proliferation and apoptosis indices were calculated as the percentage of epithelial cells staining positive for Ki67 protein or apoptotic bodies per total epithelial cells, respectively. Values represent means ± SEM. * P < 0.05 and ** P < 0.01 compared to C57BL/6 mice or TRAMP controls.
Figure 5. Western blot analysis of AR in DLP and VP of TRAMP controls compared to C57BL/6 mice. Each group contained six samples. Densitometric values of AR in the DLP and VP of C57BL/6 mice were set to 100. Values represent mean ± SEM. **P<0.01 when compared to C57BL/6 mice. Representative western blots are shown at the top: T-D = TRAMP DLP, T-V = TRAMP VP, N-D = C57BL/6 DLP, N-V = C57BL/6 VP. N.S. = values were not significant.
Insulin-like Growth Factor 1 Expression
in the DLP and VP of TRAMP and Non-transgenic Mice

**Figure 6.** Enzyme-linked Immunoabsorbant Assay (ELISA) analysis of IGF-1 in DLP and VP of TRAMP controls compared to C57BL/6 mice. Each group contained eight samples. Absorbance values of IGF-1 in the DLP and VP of C57BL/6 mice were set to 100. Values represent mean ± SEM. *P<0.05 when compared to C57BL/6 mice. N.S. = values were not significant.
Figure 7. Western blot analysis of IGF-1R beta in DLP and VP of TRAMP controls compared to C57BL/6 mice. Each group contained six samples. Densitometric values of IGF-1R Beta in the DLP and VP of C57BL/6 mice were set to 100. Values represent mean ± SEM. *P<0.05 when compared to C57BL/6 mice. Representative western blots are shown at the top: T-D = TRAMP DLP, T-V = TRAMP VP, N-D = C57BL/6 DLP, N-V = C57BL/6 VP. N.S. = values were not significant.
Figure 8. Western blot analysis of phospho-Akt in DLP and VP of TRAMP controls compared to C57BL/6 mice. Each group contained six samples. Densitometric values of pAkt in the DLP and VP of C57BL/6 mice were set to 100. Values represent mean ± SEM. *P<0.05 when compared to C57BL/6 mice. Representative western blots are shown at the top: T-D = TRAMP DLP, T-V = TRAMP VP, N-D = C57BL/6 DLP, N-V = C57BL/6 VP. N.S. = values were not significant.
Figure 9. Western blot analysis of phosphatase and tensin homolog (PTEN) in DLP and VP of TRAMP controls compared to C57BL/6 mice. Each group contained six samples. Densitometric values of PTEN in the DLP and VP of C57BL/6 mice were set to 100. Values represent mean ± SEM. **P<0.01 when compared to C57BL/6 mice. Representative western blots are shown at the top: T-D = TRAMP DLP, T-V = TRAMP VP, N-D = C57BL/6 DLP, N-V = C57BL/6 VP. N.S. = values were not significant.
Figure 10. Enzyme-linked Immunoabsorbant Assay (ELISA) analysis of insulin-like growth factor 1 in dorsolateral prostates (DLP) and ventral prostates (VP) of combination-fed TRAMP compared to TRAMP controls. Each group contained eight samples. Absorbance values of IGF-1 in the DLP and VP of TRAMP controls were set to 100. Values represent mean ± SEM. *P<0.05 when compared to TRAMP controls. N.S. = values were not significant.
Figure 11. Western blot analysis of IGF-1R beta in DLP of combination-fed TRAMP compared to TRAMP controls. Each group contained eight samples. Densitometric values of IGF-1R beta in the DLP of TRAMP controls were set to 100. Values represent mean ± SEM. *P<0.05 when compared to TRAMP controls. **P<0.01 when compared to TRAMP controls. Representative western blots are shown at the top: C = control, RG = resveratrol + genistein, RE = resveratrol + EGCG, GE = genistein + EGCG. N.S. = values were not significant.
Figure 12. Western blot analysis of IGF-1R beta in VP of combination-fed TRAMP compared to TRAMP controls. Each group contained eight samples. Densitometric values of IGF-1R beta in the DLP of TRAMP controls were set to 100. Values represent mean ± SEM. *P<0.05 when compared to TRAMP controls. Representative western blots are shown at the top: C = control, RG = resveratrol + genistein, RE = resveratrol + EGCG, GE = genistein + EGCG. N.S. = values were not significant.
Figure 13. Western blot analysis of total extracellular signaling kinases 1 and 2 (TERK 1 and 2) in DLP of combination-fed TRAMP compared to TRAMP controls. Each group contained eight samples. Densitometric values of TERKs 1 and 2 in the DLP of TRAMP controls were set to 100. Values represent mean ± SEM. **P<0.01 when compared to TRAMP controls. Representative western blots are shown at the top: C = control, RG = resveratrol + genistein, RE = resveratrol + EGCG, GE = genistein + EGCG. N.S. = values were not significant.
Figure 14. Serum analysis of insulin-like growth factor 1 in combination-fed TRAMP compared to TRAMP controls. Each group contained eight samples. Absorbance values of IGF-1 of TRAMP controls were set to 100. Values represent mean ± SEM. N.S. = values were not significant.
Figures 15 A-D. Cell proliferation in prostates of 12 Week Old C57BL/6 and TRAMP mice. C57BL/6 and TRAMP mice were fed AIN-76A powdered diet from birth until 12 weeks of age. DAB staining (brown) for Ki67 was a positive indicator for cell proliferation. TRAMP controls were compared to non-transgenic C57BL/6 mice. There was a significantly greater amount of proliferation in both the DLP and VP of TRAMP mice compared to C57BL/6 mice. Pictures were taken at 200x and 400x magnification, respectively.
Figures 16 A-D. Apoptosis in prostates of 12 week old C57BL/6 and TRAMP mice. C57BL/6 mice were fed AIN-76A powdered diet. Apoptotic bodies were counted and total apoptosis was calculated as the percentage of apoptotic bodies per total cells. TRAMP controls were compared to non-transgenic C57BL/6 mice. There was a significantly lower amount of apoptosis in both the DLP and VP of TRAMP mice compared to C57BL/6 mice. Pictures were taken at 400x magnification.
Figures 17 A-D. Cell proliferation in prostates of 12 week old TRAMP mice. TRAMP mice were exposed to AIN-76A powdered diet alone or one of three combination diets (resveratrol + genistein, resveratrol + EGCG, genistein + EGCG) from birth until 12 weeks of age. DAB staining (brown) for Ki67 was counted as a positive indicator for cell proliferation. Cell proliferation was calculated as the percentage of epithelial cells staining positive for Ki67 protein per total epithelial cells. Combination-fed TRAMP mice were compared to TRAMP controls. There was a significant down-regulation of proliferation in the DLP of genistein and EGCG-fed TRAMP mice and in the VP of resveratrol and EGCG-fed TRAMP. Since proliferation was not significantly altered in DLP or VP of combination genistein and EGCG-fed TRAMP mice, VP of combination genistein and EGCG-fed TRAMP mice, or DLP of combination resveratrol and EGCG-fed TRAMP mice, representative figures were not provided. Pictures were taken at 200x and 400x magnification, respectively.
Figures 18 A-F. Apoptosis in prostates of 12 Week Old TRAMP mice. TRAMP mice were exposed to AIN-76A powdered diet alone or one of three combination diets (resveratrol + genistein, resveratrol + EGCG, genistein + EGCG) from birth until 12 weeks of age. Apoptotic indices were calculated as the percentage of apoptotic bodies per total epithelial cells, respectively. Combination-fed TRAMP mice were compared to TRAMP controls. There was a significant up-regulation of apoptosis in both the DLP and VP of combination resveratrol and genistein-fed TRAMP and in the DLP of combination genistein and EGCG-fed TRAMP. Pictures were taken at 400x magnification. Since apop-
tosis was not significantly changed in the VP of genistein and EGCG combination and resveratrol and EGCG combination-fed TRAMP mice, representative figures were not provided.
Many factors, including diet, can directly contribute to the risk of prostate cancer development. The TRAMP model displays benign hyperplasia of the DLP at 10 weeks of age which develops into invasive adenocarcinoma by 18 weeks [51]. Because the mouse DLP has been shown to morphologically resemble the peripheral zone of the human prostate, the zone in which human prostate cancer arises [3,52], the TRAMP model has been used in prostate cancer chemoprevention studies. Several investigations have demonstrated the possibly chemopreventive effects of the dietary polyphenols: resveratrol, EGCG, and genistein [40,46,53]. However, few studies have investigated combinations of these agents and their abilities to inhibit prostate cancer development and progression. In this investigation, TRAMP mice were fed several combinations of resveratrol, EGCG, and genistein in order to determine their abilities to regulate sex steroid and growth factor signaling in the prostate. Sex steroid and growth factor signaling was analyzed in both lobes of the prostate in order to investigate possible differences in the DLP and the VP.

TRAMP mice have been shown to mimic the stages of prostate cancer found in humans [51]. Adult humans with the later stages of prostate cancer tend to exhibit weight loss related to metastasis [54]. At 12 weeks of age, TRAMP controls exhibited statisti-
cally smaller body weights when compared to non-transgenic C57BL/6 mice. Our data is consistent with the report of Gingrich et al. who reported that, between the period of 8-12 weeks of age, TRAMP mice exhibit carcinoma resembling the progressive stages of prostate carcinoma in humans and 12 weeks may be an age just prior to metastasis of prostate cancer in the TRAMP mice, an observation consistent with the report of Gingrich et al. [55]. On the other hand, there was no significant difference in body weights of polyphenol-fed TRAMP mice when compared to TRAMP controls, which shows that the combination diets were not toxic to the mice.

Androgen activity is one of the key factors necessary for prostate growth. AR defects have been related to development of prostate cancer. Likewise, IGF-1 signaling pathway plays a large role in prostate growth. Increased expression of IGF-1 and IGF-1R can promote development and progression of prostate cancer [19]. In our study, TRAMP mice had significantly higher AR and IGF-1 expressions in the DLP compared to the DLP of C57BL/6 mice. This is consistent with the report of Wang et al. which demonstrated increased AR and IGF-1 expression in the dorsolateral prostates of 12-week old TRAMP mice in comparison to C57BL/6 mice [30]. In our study, total prostate, dorsolateral prostate, and seminal vesicle weights were significantly increased in TRAMP mice when compared to C57BL/6 mice, confirming hyperplasia and eventually tumorigenesis. Likewise, increased AR and IGF-1 expressions have been associated with increased growth and proliferation in the prostate.
Mutations in PTEN, the tumor suppressor gene, have been related to increased risk of various cancers [24]. Desai et al. demonstrated that castrated Sprague-Dawley rats exhibit a significant up-regulation of PTEN in the VP [52]. Our results showed PTEN to be down-regulated in the VP of TRAMP controls when compared to C57BL/6 mice, an effect consistent with increased prostate cancer. This trend was also demonstrated in the investigations of Li et al. [56] who determined an antagonistic relationship between AR and PTEN in prostate cancer cells. Since PTEN expression is increased with androgen withdrawal, our results indicate that, when AR/androgen activity is increased, PTEN expression is reduced and apoptosis also declines. Although pAkt expression was significantly lower in the DLP of TRAMP controls when compared to C57BL/6, this evidence may indicate that the mechanism of growth within the prostate of TRAMP mice may be due to increased androgen and IGF signaling without the involvement of pAkt activation.

Increased activation of the insulin-like growth factor axis has been associated with increased risk of prostate cancer. IGF-1 promotes cell proliferation, growth, and anti-apoptotic effects within the cell [19]. IGF-1R, the key regulator of IGF-1 activity, is responsible for activation of tyrosine kinase signaling within the cell and initiates proliferative actions through activation of the ERKs [57]. The IGF-1R has two subunits, the alpha and beta subunit. Binding of IGF-1 to the alpha subunit activates the beta subunit, embedded within the membrane and intracellular matrix, which activates signaling of the IGF-1 axis [17].
In the DLP, genistein alone has been shown to significantly reduce IGF-1 (~30%) and IGF-1R alpha (~30%) in 12 week-old TRAMP mice [30]. EGCG, the most abundant catechin found in green tea, has been shown to impede activation of the IGF-1 receptor in human colon cancer cells [58] as well as reducing IGF-1 (~25%) expression in the DLP of 12-week old TRAMP mice [64 and Appendix A]. Our results are consistent with previous investigations demonstrating the abilities of genistein and EGCG to negatively modulate various components of the IGF-1 axis [30,53]. Although TRAMP mice fed a combination diet of genistein and EGCG exhibited decreased IGF-1 expression (~15%) in the DLP, the lowered percentage of IGF-1 reduction by the combination diet indicates that there may be no additive or synergistic effect on IGF-1 expression. Genistein alone reduced IGF-R alpha expression in the DLP of 12 week-old TRAMP mice by approximately 30% [30] and EGCG increased IGF-1R alpha in the DLP of TRAMP mice by approximately 20%, though not significantly [64 and Appendix A]. However, when genistein and EGCG were given in combination via the diet, there was no significant change in IGF-1R alpha expression in the DLP of TRAMP mice. One seems to have canceled the action of the other. IGF-1R beta was significantly down-regulated by 40% in the DLP of combination-fed TRAMP mice. IGF-1R beta expression was not measured in prostates of TRAMP mice fed genistein and EGCG in solitary doses; however, our results indicate that genistein and EGCG in combination may be playing a large role in the regulation of IGF activity in the DLP by down-regulating IGF-1R beta, hence significantly reducing activation of the IGF-1 axis.
In the VP, we previously observed that EGCG alone significantly reduced IGF-1 expression (30%) [64 and Appendix A]. However, genistein alone did not alter IGF-1 expression in the VP. In this study, we found that IGF-1 was significantly reduced by 5% in the VP of genistein and EGCG combination-fed TRAMP although, as seen in the DLP, the combination was not as effective as the solitary treatments in reducing IGF-1 expression and, when added to EGCG, genistein appears to be hindering the ability of EGCG to down-regulate IGF-1 expression. IGF-1R beta was significantly reduced in the VP of genistein and EGCG combination-fed TRAMP mice by 56% indicating that, although, genistein and EGCG in combination are not as effective as the single agents in regulating IGF-1 ligand expression, the combination significantly reduces activation of the IGF pathway through IGF-1R beta inhibition and may a play a role in inhibiting progression of prostate cancer in both the DLP and VP of TRAMP mice.

Activation of the ERKs is a down-stream mechanism of IGF-1 to activate transcription of genes that promote cell proliferation and anti-apoptosis [59]. ER alpha has been shown to activate IGF signaling by binding to IGF-1R beta from within the cell [18]. Like IGF-1, ER alpha has the ability to activate the ERKs 1 and 2 via tyrosine kinase signaling. Although there was no significant change in total ERKs 1 and 2 or phospho-ERKs 1 and 2 expression in the DLP of genistein-fed TRAMP and EGCG-fed TRAMP [30, 64, and Appendix A], there was a significant increase of total-ERKs 1 and 2 expression in the DLP of genistein and EGCG combination-fed TRAMP mice when compared to TRAMP controls. Although total-ERK expression was increased, phospho-ERK expression was not changed. ER alpha expression in the DLP of combination-fed
TRAMP mice was increased by 26 % (though not significantly), a trend also seen in the DLP of EGCG-fed TRAMP mice. Due to reduced activation of the IGF-1 axis in the DLP of genistein and EGCG combination-fed TRAMP mice, total-ERK expression may have been up-regulated by the binding of ER alpha to IGF-1R in a compensatory mechanism to advance tyrosine kinase signaling within the cell. However, the effect was not strong enough to increase phosphorylation of the ERKs and may be negligible in promoting transcription.

In the VP, EGCG-fed TRAMP mice exhibited significantly reduced phospho-ERK 1 and 2 expressions [64 and Appendix A], a trend that was not seen in the VP of genistein and EGCG combination-fed TRAMP. There was no regulation of total ERKs 1 and 2 or phospho-ERKs 1 and 2 in the VP of genistein and EGCG combination-fed TRAMP mice indicating that the combination diet may be less effective in effecting downstream IGF signaling in the VP than the agents in solitary doses.

Resveratrol has been shown to inhibit tumor initiation in human leukemia cells [46] and incidence of poorly undifferentiated tumors in TRAMP mice [63 and Appendix A]. IGF-1 expression was significantly down-regulated in the DLP of 12-week old resveratrol-fed TRAMP mice by 52%. However, combination resveratrol and genistein-fed TRAMP and combined resveratrol and EGCG-fed TRAMP only exhibited a 10% reduction in IGF-1 expression in the DLP. As seen with IGF-1 ligand regulation in the prostates of genistein and EGCG combination-fed TRAMP, resveratrol in combination with other polyphenols was less effective in reducing IGF-1 expression and therefore demon-
Stratified no additive or synergistic effect. No IGF-1 regulation was seen in the VP of combination resveratrol and genistein-fed TRAMP mice or combination resveratrol and EGCG-fed TRAMP mice, a trend also seen in the VP of resveratrol alone-fed TRAMP mice.

Cell proliferation was significantly increased in both the DLP and VP of TRAMP controls when compared to the prostates of C57BL/6 mice. These results are also consistent with the report of Wang et al. [1] who reported increased cell proliferation in the DLP of TRAMP mice. IGF-1 acts through its receptor to activate gene expression of various genes that promote cell proliferation and cell growth [19]. In our investigation, TRAMP controls exhibited increased expression of both IGF-1 and IGF-1R which likely supports our findings of increased cell proliferation in comparison to non-transgenic C57BL/6 mice.

Apoptosis is a cellular mechanism initiated by the cell when DNA damage has been detected. Because TRAMP mice exhibit mutated DNA due to oncogenic activities of the SV-40 transgene, intracellular mechanisms involving initiation of apoptosis may be inhibited or even ceased [55]. Reduced apoptosis is an indication of inhibited damage recognition hence anti-apoptotic effects are being initiated within the prostate. The number of apoptotic bodies was significantly lower in TRAMP controls in comparison to non-transgenic C57BL/6 mice. The data from our investigation is consistent with the report of Elgavish et al. who demonstrated that transgenic mice with mutant p53 exhibited reduced apoptosis, a characteristic of prostate tumor cells [60].
Cell proliferation was significantly reduced by 47% in the DLP of genistein-fed TRAMP mice [29] and by 53% in the DLP of EGCG-fed TRAMP mice [64], also a significant reduction. Combination genistein and EGCG significantly reduced cell proliferation by 58% in the DLP of combination-fed TRAMP mice when compared to TRAMP controls. This evidence indicates that genistein and EGCG in combination may be reducing prostate carcinogenesis promotion by inhibiting the IGF axis and, in effect, reducing cell proliferation.

Both EGCG and genistein administered in solitary doses have been shown to induce apoptosis in human prostate cancer cells [61,62]. Although apoptosis was significantly increased in the VP of EGCG-fed TRAMP [64] mice by 210 % with no significant change seen in the DLP, combination genistein and EGCG-fed TRAMP exhibited a statistically significant increase (177%) in apoptosis in the DLP with no significant change seen in the VP in comparison to TRAMP controls. Although protein regulation, cell proliferation, and apoptosis trends seem to differ in the prostates of EGCG-fed TRAMP mice and genistein-fed TRAMP mice, the two polyphenols, when combined, seem to work together to inhibit prostatic carcinoma by reducing cell proliferation and increasing apoptosis within the DLP of TRAMP mice.

Although resveratrol-fed TRAMP demonstrated no significant change in apoptosis in the DLP (120%) or the VP (27%) [63], in comparison to the other combination diets, resveratrol and genistein in combination demonstrated the greatest ability to induce
apoptosis in both the DLP (167%) and the VP (144%) of TRAMP mice. In the VP of combination resveratrol and genistein-fed TRAMP mice, the amount of apoptosis seen was 5% higher than that seen in VP of non-transgenic C57BL/6 mice, a remarkable finding suggesting that resveratrol and genistein, when combined, may have a synergistic effect on apoptosis and may be chemopreventive. Although genistein has the ability to reduce cell proliferation, there was no significant change in either the DLP or VP of animals treated with resveratrol and genistein in combination.

While cell proliferation was significantly increased in the DLP and VP resveratrol only-fed [63] and in the VP of EGCG only-fed TRAMP mice [64], there was no significant change in proliferation found in the DLP or VP of resveratrol and EGCG combination-fed TRAMP mice when compared to TRAMP controls. Although EGCG significantly increased apoptosis in the VP of TRAMP mice by 160%, resveratrol alone did not significantly alter apoptosis in the DLP and VP of TRAMP mice [63, 64]. Apoptosis was increased, though not significantly, in both the DLP (102%) and VP (70%) of resveratrol and EGCG combination-fed TRAMP mice. This evidence suggests that, although resveratrol and EGCG alone reduced cell proliferation in the VP and increased apoptosis, when combined, the two polyphenols seem to be antagonists and the combination may not be a possible method for chemoprevention.

There was no significant regulation of the sex steroid receptors: AR, ER-alpha, and ER-beta in the prostates of the combination-fed TRAMP when compared to TRAMP controls. Although the solitary doses of polyphenols modulated the sex steroid receptors,
when combined, resveratrol, genistein, and EGCG did not affect the sex steroid pathways and seem to be working through the IGF-1 axis or other apoptotic mechanisms such as BAD signaling.

Several investigations have demonstrated that resveratrol, genistein, and EGCG given in solitary doses can modulate key regulating pathways associated with increased risk of numerous cancers such as: colon, gastric, breast, and prostate cancers. Few studies have investigated the cellular mechanisms of these polyphenols when fed in combination. From our investigation, we conclude that the combination of genistein and EGCG significantly reduced cell proliferation through inhibition of IGF-1 expression. Genistein and EGCG in combination seem to work together to inhibit prostate cancer progression. This data is consistent with reports that demonstrate reduced risk of clinical prostate cancer with consumption of both soy and green tea [53], a trend seen among Asian men. The combination of resveratrol and genistein greatly increased apoptosis which indicates that the combination treatment may reduce the incidence of tumor initiation possibly proving to be chemopreventive. The combination of resveratrol and EGCG demonstrated no significant modulation in any of the investigated mechanisms contributing to the development and promotion of prostate cancer. Finally, the results of this study indicate the importance of understanding mechanisms of action of dietary polyphenols in developing a regimen for suppressing prostate cancer development.
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APPENDIX A

SEX STEROID AND GROWTH FACTOR EXPRESSION IN PROSTATES OF TRANSGENIC MICE EXPOSED TO DIETARY POLYPHENOLS
Sex Steroid and Growth Factor Protein Expression in TRAMP Mice Exposed to Genistein, Resveratrol or EGCG in the Diet

<table>
<thead>
<tr>
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<th>Control</th>
<th>Genistein</th>
<th>Resveratrol</th>
<th>EGCG</th>
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</thead>
<tbody>
<tr>
<td><strong>Dorsolateral Prostate</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>AR</td>
<td>100 ± 7</td>
<td>86 ± 8*</td>
<td>260 ± 37*</td>
<td>121 ± 16</td>
</tr>
<tr>
<td>ER-α</td>
<td>100 ± 15</td>
<td>93 ± 7</td>
<td>139 ± 21</td>
<td>119 ± 24</td>
</tr>
<tr>
<td>ER-β</td>
<td>100 ± 13</td>
<td>66 ± 9*</td>
<td>165 ± 18*</td>
<td>129 ± 16</td>
</tr>
<tr>
<td>IGF-1</td>
<td>100 ± 8</td>
<td>62 ± 15*</td>
<td>48 ± 10*</td>
<td>69 ± 7*</td>
</tr>
<tr>
<td>IGF-1R α</td>
<td>100 ± 8</td>
<td>62 ± 6*</td>
<td>132 ± 8</td>
<td>121 ± 10</td>
</tr>
<tr>
<td>IGF-1R β</td>
<td>---</td>
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<td>---</td>
<td>---</td>
</tr>
<tr>
<td>IGF-BP3</td>
<td>100 ± 8</td>
<td>38 ± 14*</td>
<td>107 ± 4</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>T-ERK-1</td>
<td>100 ± 5</td>
<td>93 ± 17</td>
<td>100 ± 7</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>T-ERK-2</td>
<td>100 ± 9</td>
<td>77 ± 8</td>
<td>99 ± 7</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>P-ERK-1</td>
<td>100 ± 16</td>
<td>104 ± 14</td>
<td>49 ± 8*</td>
<td>92 ± 15</td>
</tr>
<tr>
<td>P-ERK-2</td>
<td>100 ± 13</td>
<td>81 ± 11</td>
<td>92 ± 12</td>
<td>93 ± 10</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Genistein</th>
<th>Resveratrol</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ventral Prostate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>100 ± 14</td>
<td>66 ± 8*</td>
<td>84 ± 14</td>
<td>41 ± 8*</td>
</tr>
<tr>
<td>ER-α</td>
<td>100 ± 3</td>
<td>---</td>
<td>97 ± 3</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>ER-β</td>
<td>---</td>
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<td>---</td>
<td>---</td>
</tr>
<tr>
<td>IGF-1</td>
<td>100 ± 5</td>
<td>100 ± 9</td>
<td>98 ± 5</td>
<td>70 ± 5*</td>
</tr>
<tr>
<td>IGF-1R α</td>
<td>100 ± 11</td>
<td>89 ± 17</td>
<td>66 ± 9*</td>
<td>58 ± 4*</td>
</tr>
<tr>
<td>IGF-1R β</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>IGF-BP3</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>T-ERK-1</td>
<td>100 ± 17</td>
<td>185 ± 105</td>
<td>63 ± 9</td>
<td>74 ± 17</td>
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<tr>
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<td>100 ± 27</td>
<td>350 ± 149</td>
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<tr>
<td>P-ERK-1</td>
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<td>64 ± 9*</td>
<td>66 ± 9*</td>
<td>55 ± 7*</td>
</tr>
<tr>
<td>P-ERK-2</td>
<td>100 ± 11</td>
<td>65 ± 16</td>
<td>58 ± 10*</td>
<td>44 ± 5*</td>
</tr>
</tbody>
</table>

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APPENDIX B

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

63
MEMORANDUM

DATE: January 3, 2007

TO: Coral A. Lamartine, Ph.D.
   VH-124 0019
   FAX: 934-8240

FROM: Judith A. Kopp, Ph.D., Chair
   Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 27, 2006.

Title of Application: Polyphenols and Prostate Cancer Chemoprevention

Fund Source: Department of Defense

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A1255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

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