Dietary Chemoprevention Studies in Preclinical Models of Prostate Cancer: Bioactive Lipids and Vitamin D.

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2010

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ABSTRACT

Epidemiological evidence suggests that prostate cancer can be modulated by diet. Data exists that implicate both bioactive lipids and the vitamin D/calcium axis in prostate cancer pathogenesis. These data suggest that bioactive lipids such as omega-3 fatty acids protect against prostate cancer, whereas omega-6 fatty acids promote prostate cancer. Population studies also indicate that a diet high in vitamin D decreases prostate cancer risk while calcium increases risk for prostate cancer, possibly by lowering circulating levels of protective vitamin D. In contrast, recent epidemiological studies indicate that vitamin D does not protect against prostate cancer, indicating that the role of vitamin D and calcium in this disease process is far from established. Importantly, crucial animal experiments are lacking that may serve to provide mechanistic data for the roles of these nutrients.

The objective of the first study conducted in this dissertation was to evaluate and compare prostate carcinogenesis in murine models that utilize the SV40 Tag transformation system- TRAMP and Apt121/Rbf. These are commonly used models in prostate cancer chemoprevention studies. We characterized these models with respect to time to tumor development, tumor size and metastasis. We evaluated prostate lesions using established histopathological criteria and assessed the expression of biomarkers that are related to the hallmarks of cancer. From these studies we can
conclude that the TRAMP model is very aggressive and that the entire carcinogenic
cascade is not represented, making dietary prevention interventions difficult to evaluate
in this model. However, the Apt121/Rbf is a model of early prostate carcinogenesis and
more appropriate for nutritional studies. These data provide a basis for investigating the
effects of nutritional interventions against prostate cancer.

The second study investigated the effects of dietary bioactive lipids in the
TRAMP murine model of prostate cancer. Male TRAMP mice were fed high fat diets
that utilized fish oil or corn oil as the primary lipid source. The effects of bioactive lipids
on TRAMP carcinogenesis were assessed using parameters related to prostate
carcinogenesis. These included time to tumor development, tumor size and
histopathologic grade/classification and presence of metastasis. Markers of lipid
metabolism, including tissue prostaglandin and fatty acid profiles, were utilized to ensure
bioavailability to the prostate. We found that TRAMP mice were resistant to the
protective effects of omega-3 fatty acids from the fish oil diet. This was due in part to
genetic defects in lipid metabolism inherent in TRAMP mice and inability to overcome
the procarcinogenic effects of a high fat diet. These data indicate that the TRAMP mouse
may not be ideal for studying the effects of bioactive lipids on prostate carcinogenesis.

The final study examined the relationship between dietary calcium and vitamin D
intake and the protective role of serum 25-OHD and 1,25(OH)2D against prostate cancer
and in bone health. Serum vitamin D metabolite levels were modulated by manipulating
dietary calcium and vitamin D intake (3x3 factorial design) in male Apt121/Rbf mice in a
28 week study that measured prostate carcinogenesis parameters, serum markers and
bone density. We found that serum concentrations of the storage form of vitamin D were
responsive to levels of dietary vitamin D. Conversely, vitamin D activation was controlled by dietary calcium levels. Prostate carcinogenesis was reduced by increased dietary vitamin D, while dietary calcium only had a modest procarcinogenic effect. In contrast, bone health was more responsive to changes dietary calcium, with the highest calcium levels promoting optimal bone health. Moreover, in a high calcium setting, which is beneficial for bone health, vitamin D offered protection against prostate cancer.
DEDICATION

To my wife and best friend, Kristen-
Thank you for your patience, understanding and support throughout this unique experience. Never once have I felt that I was in this alone. You are truly the love of my life.

To my daughter, Madelyn-
Your bright eyes and beautiful smile give me a reason to want to be better and to work hard in all that I do.

To my parents, Bruce and Valerie Smolinski-
Thank you for pushing me to reach for the stars and instilling in me the faith in myself and what I can achieve.

In Memoriam- Dr. Russell Klein (1962-2006)
Thank you for starting me off on this journey. Your spirit has remained close through its completion.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Steven Clinton, for all of his efforts through this experience. Dr. Clinton offered to take me into his laboratory to finish the remainder of my research after the passing of my original advisor, Dr. Russell Klein. Not only was I given a new “home”, I was given an immense amount of guidance and support at such a trying time. He has challenged my thought process and has broadened my thinking about how I interpret scientific data and really understanding the big picture of what we do.

I would also like to thank Dr. Mark Failla for his support during Dr. Klein’s illness and death. He offered an ear to listen on many a weekend when our laboratory was in need of direction. Through many classes and meetings, Dr. Failla challenged my intellect and showed me that no matter how much you think you know, you are always a student.

I am grateful for the time commitment and assistance from my other committee members, Dr. Kichoon Lee and Dr. Charles Brooks. Their efforts truly gave me a more interdisciplinary approach to how I conducted and interpreted my research.

Last, but not least, I would like to offer my sincere gratitude to all of the past and present members of the Klein and Clinton laboratories who have been a part of this experience- Dr. Jun-Ge Yu, Dr. Sanjay Bhave, Dr. Jennifer Thomas-Ahner, Robert Rengel, Valerie DeGroff, Kimberly Carter, Besma Abbaoui, Colleen Spees, Lei Wan and Hseuh-Li Tan. Their support enabled me to reach this finish line.
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LIST OF ABBREVIATIONS

1,25(OH)2D  1,25-dihydroxy vitamin D
25-OHD  25-hydroxy vitamin D
α  alpha
ALA  Alpha Linolenic Acid
ANOVA  Analysys of Variance
AR  Androgen Receptor
AP  Anterior Prostate
Bcl2  B-Cell Lymphoma 2
BMP  Bone Morphogenen Protein
CO  Corn Oil
COX  Cyclooxygenase
DHT  Dihydrotestosterone
DHA  Docosahexaenoic Acid
DP  Dorsal Prostate
EPA  Eicosapentaenoic Acid
EGFR  Epidermal Growth Factor Receptor
FGF  Fibroblast Growth Factor
FO  Fish Oil
FSH  Follicle Stimulating Hormone
FOX  Forkhead Box
GnRH  Gonadotropin Releasing Hormone
GH  Growth Hormone
H&E  Hematoxylin and Eosin
HOX  Homeobox
HEPE  Hydroxyeicosapentaenoic Acid
HODE  Hydroxyoctadecaenoic Acid
IGF-1  Insulin-like Growth Factor-1
LP  Lateral Prostate
LOX  Lypoxygenase
MMP  Matrix Metalloproteinase
MD  Moderately Differentiated
NMU  N-Nitroso N-Methylurea
OR  Odds Ratio
PBS  Phosphate-Buffered Saline
PTEN  Phosphatase and Tensin Homologue on Chromosome 10
Phyl  Phylloides-Like
PUFA  Polyunsaturated Fatty Acids
PD  Poorly Differentiated
PGE  Prostaglandin E
PIN  Prostatic Intraepithelial Neoplasia
PSA  Prostate Specific Antigen
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<tr>
<td>RR</td>
<td>Relative Risk</td>
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<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
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<td>RAR</td>
<td>Retinoic Acid Receptor</td>
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<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
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<td>SV40</td>
<td>Simian Virus 40</td>
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<td>Shh</td>
<td>Sonic Hedgehog</td>
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<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>TRAMP</td>
<td>Transgenic Adenocarcinoma of Mouse Prostate</td>
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<tr>
<td>UGT</td>
<td>Urogenital Tract</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VP</td>
<td>Ventral Prostate</td>
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<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
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<tr>
<td>WD</td>
<td>Well Differentiated</td>
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<td>Wnt</td>
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CHAPTER 1

INTRODUCTION

Prostate cancer is the most common visceral malignancy among American men and the second leading cause of their cancer mortality [1]. With almost 25% of all new cancer cases in the United States being attributed to prostate cancer, it is a major public health problem. Several lines of evidence suggest that prostate cancer risk can be modulated by diet. However, little conclusive data has been reported that provides a direct association between any dietary factor and prostate cancer. Although prostate cancer is most prevalent in men >60 years old, preneoplastic lesions have been observed in men as young as 20. This suggests that there is a long latency period for cancer development, thus presenting an excellent opportunity for the use of nutritional and other interventional prevention strategies. Therefore, it is imperative that safe and effective prevention strategies are identified and implemented.

In order to establish effective interventions, we must conduct the critical preclinical and human studies to generate the evidence to warrant implementation of carefully formulated preventive strategies. This project focuses upon the laboratory evaluation of mechanisms whereby dietary factors will influence prostate carcinogenesis. The goal of these efforts is to better understand the process, but most importantly, to
define molecular biomarkers that can be used in subsequent human studies examining the relationship between diet and prostate cancer.

Dietary lipid consumption patterns have long been suspected to influence prostate carcinogenesis yet definitive data regarding a role for specific fatty acid profiles, cholesterol, lipid sources, or total fat have not emerged. Population studies suggest that a diet high in fish consumption reduces prostate cancer risk while a diet high in meat consumption increases risk [2]. Fish based diets contain high levels of omega-3 fatty acids while meat based diets contain high levels of omega-6 fatty acids. Omega-6 fatty acids promote while omega-3 fatty acids inhibit prostate cancer growth in cell culture and animal models [3, 4]. However, few studies have been conducted in transgenic prostate cancer models. Moreover, no studies have examined the effects of lipid source in the context of a high fat diet.

Epidemiologic studies suggest that both increased dietary calcium and low vitamin D status are associated with an increase in prostate cancer risk [5]. Low vitamin D status is increasingly more common in U.S. populations including African-Americans and the elderly [6]. High dietary calcium intake lowers serum levels of the biologically active form of vitamin D, 1,25 dihydroxyvitamin D (1,25(OH)2D), by suppressing conversion of 25 hydroxyvitamin D (25-OH D) to 1,25(OH)2D. It is plausible that excessive dietary calcium is another critical factor that limits or reduces a protective impact of vitamin D on prostate carcinogenesis. Cell culture studies confirm the protective effects of vitamin D [7]. However, dietary vitamin D consumption has received very little attention in animal models. The effects of calcium in cell culture and animal models are less well studied. Therefore, the controversy regarding calcium and
prostate cancer risk, and how vitamin D may impact this relationship, has not been resolved.

1.1 Hypotheses

1.1.1 The Apt121/Rbf is a model of early prostate carcinogenesis and more appropriate for nutritional studies.

1.1.2 An omega-3 fatty acid enriched diet will inhibit, whereas an omega-6 fatty acid enriched diet will exacerbate carcinogenesis.

1.1.3 High calcium diets reduce serum 1,25(OH)₂D levels and suppress the anti-carcinogenic effect of 1,25(OH)₂D but not of elevated 25-OH D. Accordingly, diets high in vitamin D will overcome the cancer promoting effects of high calcium diets.

1.2 Specific Aims

1.2.1 Evaluate and compare prostate carcinogenesis in murine models that utilize the SV40 TAg transformation system- TRAMP and Apt121/Rbf.

1.2.1.1 Establish a time course for both the TRAMP and Apt121/Rbf murine models of prostate carcinogenesis.

1.2.1.2 Perform an in-depth histopathological analysis of prostate tissues and characterize lesions at each time point with respect to established histopathological criteria.
1.2.1.3 Utilize immunohistochemical techniques to assess the expression of biomarkers related to the hallmarks of cancer and characterize biomarker expression as a function of histological grade.

1.2.2 Investigate the effects of dietary bioactive lipids in the TRAMP murine model of prostate cancer.

   1.2.2.1 Examine the effects of bioactive lipids on TRAMP carcinogenesis by assessing endpoints related to prostate carcinogenesis and assess the presence of metastasis to distant organs like the liver, lungs and lymph nodes.

   1.2.2.2 Examine markers of lipid metabolism to verify that our dietary treatment is in fact, bioavailable to the prostate by assessing prostatic prostaglandin levels using LC/MS/MS and prostate tissue fatty acid profiles using gas chromatography.

1.2.3 Establish the relationship between dietary calcium and vitamin D intake and the protective role of serum 25-OHD and 1,25(OH)2D against prostate cancer in vivo.

   1.2.3.1 Modulate serum vitamin D metabolite levels by manipulating dietary calcium and vitamin D intake (3x3 factorial design) in male Apt121/Rbf mice in a 30 week study measuring prostate carcinogenesis endpoints (e.g. PIN, adenocarcinoma, tumor number, lesion score, tumor location, and stage).

   1.2.3.2 Investigate the interactions between calcium and vitamin D on parameters specific to vitamin D/calcium metabolism.

   1.2.3.3 Establish the relationship between dietary calcium and vitamin D intake and bone density in vivo.
CHAPTER 2

LITERATURE REVIEW

2.1 Prostate Cancer

2.1.1 Prostate Development

In humans, prostate development begins in the second trimester and initial development is completed in the third trimester. In mice, prostate development and branching morphogenesis begins around the 16th day of a 19 day gestational strain [8].

The prostate originates from the urogenital sinus, an outgrowth of the cloaca. An additional surge in prostate size and final maturation occurs in puberty where the prostate grows to the size of a walnut. The function of the prostate is to produce secreted seminal fluid.

2.1.2 Hormonal Events in Prostate Development

Initial prostate development and eventual prostate maturation is triggered by the presence of androgens. The process begins in the hypothalamus and pituitary where GnRH causes the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). This stimulates the Leydig cells of the testes to produce androgens. The primary acting androgen, testosterone is produced by the testes where it is transported through the
bloodstream bound to sex hormone binding globulin and to a lesser extent, albumin. It is then delivered to the target tissues where it is converted to dihidrotestosterone (DHT) by the 5-alpha reductase enzyme, which is present in the cytoplasm of prostate epithelial cells. 5-alpha reductase deficiency or inhibition through chemical means results in lack of prostate development [9]. Both testosterone and DHT bind to the nuclear Androgen Receptor (AR). However, DHT binds to the AR with much greater affinity and is a more potent stimulator of androgen receptor mediated gene transcription. Hence, it is considered the “active” form of the hormone. Binding of androgens to the androgen receptor leads to the transcription of key genes containing androgen response elements, such as PSA, that are involved in prostate development and function which will be subsequently discussed.

Other hormones and peptide growth factors play a role in normal prostate development. Growth Hormone (GH), Insulin-like Growth Factor 1 (IGF-1) Prolactin and Luteinizing Hormone (LH) all have important roles in normal prostate development. GH produced by the pituitary causes the liver to produce IGF-1. Some of the effects of GH are therefore thought to be mediated by IGF-1. However, receptors for both are found in the developing and mature prostate and autocrine and paracrine production of IGF-1 by the prostate is well documented [10]. Mice deficient for either receptor develop small prostates. This is thought to be due in part to perturbed epithelial-mesenchymal communication, much like in the case of AR. Prolactin stimulates proliferation, differentiation and gene expression in the prostate gland[11].

2.1.3 Epithelial-Mesenchymal Interactions in Prostate Development
There is an elegantly orchestrated interplay between prostatic mesenchyme and epithelium that is essential for proper prostatic growth and function. This was demonstrated by classic tissue recombination and grafting experiments performed by Cunha and colleagues [12], using testicular feminized mice (Tfm) that lack the AR.

These tissue recombination experiments showed that mesenchyme expressing AR could be combined with epithelium not expressing AR and form prostate glandular tissue. Conversely, combining mesenchyme that did not express AR with epithelium expressing AR did not result in prostate glandular tissue. This indicates that mesenchymal AR is critical for prostate gland formation and that there is crosstalk between epithelium and mesenchyme in prostate development and maintenance. Similar effects were observed in human epithelial-mesenchymal recombination experiments. Additional experiments showed that epithelial tissue could be removed after the initial exposure to androgens and would form prostate glands that could be maintained in the absence of androgens[13]. Prostate epithelium collected before androgen exposure could not form prostate gland. Interestingly, prostatic mesenchyme can be combined with committed adult bladder epithelium which originates from the urogenital sinus as well, to form prostate glandular tissue. However, developmental origin is imperative as prostate can not form from combination with lung or other tissues. Further studies demonstrated that although prostate glands can form after removal of the initial androgen stimulus, optimal ductal branching and differentiation requires sustained androgen exposure [14].

Epithelial AR is also involved in regulation of mesenchymal proliferation and maturation. It is required for mesenchymal cell expansion, layering and terminal differentiation [15]. The AR is expressed initially at higher levels in the mesenchyme,
further implicating mesenchymal AR signaling as an earlier event. There is also interplay between epithelial and mesenchymal secreted factors and other molecular events but this will be discussed in following sections.

2.1.4 Cellular and Molecular Biology of Prostate Development

Although the importance of androgens in prostate development can not be understated, the ensuing cellular and molecular events resulting from androgen and growth factor stimulation are important for proper prostate function and optimal development. As previously stated, AR is initially expressed at higher levels in the developing mesenchyme and levels increase in the epithelium. Retinoic Acid Receptors (RARs), Retinoid X Receptors (RXRs), and the Estrogen Receptor (ER) are key developmental mediators as well and play a role in determination of cells to form prostate tissue [16]. The interactions between mesenchyme and epithelium suggested the presence of “andromedins” or factors that served as androgen regulated crosstalking molecules that can be summarized in Figure 2.1

Upon androgen exposure, one of the earliest factors to be expressed in Sonic Hedgehog (Shh) [17, 18]. This is one of the earliest developmental events and precedes the surge in epithelial AR expression. Shh binds to the patched receptor on adjacent cells. Shh is expressed in high levels in epithelium at the epithelial-mesenchymal interface, suggesting a role in ductal expansion. It is expressed at high levels in the initial stages of prostate branching and development and declines thereafter. Expression of Shh leads to epithelial Nkx3.1 expression [19]. Nkx3.1 is a homeobox gene that is essential for proper prostate branching morphogenesis. Knocking out Shh in the prostate prevents
the expression of Nkx3.1 and leads to improper prostate branching. Nkx3.1 expression is high when prostate development and maturation begins but declines slowly through adult life. However, Nkx3.1 has been shown to be downregulated in human prostate cancer and knocking the gene out in mice causes prostate cancer later in the lifespan (after defects in initial prostate development are seen) [20]. This suggests that maintenance of Nkx3.1, even at low levels, is required for proper prostate function throughout the lifespan.

Androgens also induce several HOX homeobox genes. The roles of these genes are better studied in mouse models than in humans but the little existing human evidence generally corresponds with what is seen in mice. In response to androgens, Hoxa13 and Hoxd13 are expressed in prostate mesenchyme in high levels in initial prostate development and decline sharply after [21]. In contrast, Hoxb13 is expressed in prostate epithelium in the puberty stage [22]. Knocking out any of the HOX factors in rodents results in small prostates, improper prostate branching and formation, and impaired secretory function [21]. Evidence in humans suggests that several of these genes are dysregulated in prostate cancer [23].

Forkhead box (Fox) transcription factors are other important factors implicated in prostate development. FoxA1 is expressed in the epithelium early in the developmental process [24]. Its expression is maintained throughout the lifespan and continued expression is critical for proper prostate maturation and secretory function. Loss of FoxA1 impairs probasin secretion in rodents and Prostate Specific Antigen (PSA) secretion in humans [24]. FoxA2 is only expressed early in developing epithelium and levels are not sustained beyond the initial development stages [25].
There are additional secreted molecules that mediate the effects of androgens. These include FGFs, Wnts, Notch and the Tgf/BMP family. Fibroblast growth factor (FGF) 10 is implicated in proper prostate branching as well [26]. The absolute androgen requirement for FGF expression has been questioned and is unclear. Nevertheless, FGF10 is expressed in the epithelium and is thought to be a chemoattractant that leads to the cellular recruitment and expansion of prostatic branching ducts [26]. The Wnt family of proteins is involved in prostate development as well but more evidence exists in mice and the role of this family is better studied in prostate cancer. However, the expression of canonical Wnts (that signal through the classical b-catenin signaling pathway) and non-canonical Wnts (that do not signal through b-catenin) has been somewhat studied [27]. These secreted proteins are paracrine mediators that are expressed in high levels at initial developmental stages and decrease thereafter [28]. Notch is also expressed in the developing prostate epithelium at high levels during the initial stages of growth and decline. Epithelial secreted Notch binds to the jagged receptor on adjacent stromal cells to stimulate the transcription of genes involved in differentiation [29]. The Transforming Growth Factor (Tgf)/Bone Morphogenic Protein (BMP) family of proteins is also involved in prostate development [30]. They are produced in both mesenchyme and epithelium but it appears that mesenchymal have the greatest developmental effects. In contrast to the previously discussed factors, this family is generally growth inhibitory. Tgfb inhibits FGF10 production and upregulates the expression of several cell cycle inhibitory proteins. BMPs also inhibit several of the factors associated with ductal proliferation. It appears that Tgfb is important for arresting cell growth and triggering epithelial cell differentiation [30].
2.1.5 The Mature, Developed Prostate

Following the initial developmental processes, a mature prostate is formed and cells differentiate into their respective functional state. In both humans and mice, the prostate consists of an epithelial and stromal component although the stromal compartment is more extensive in humans. The epithelium consists of neuroendocrine cells, basal cells, luminal cells and stem cells [31]. The luminal cells comprise the secretory and thus functional component of the prostate.

2.2 Molecular Biology of Cancer and Prostate Cancer

Hanahan and Weinberg elegantly summarized the characteristics of cancer in 2004 [32]. These are self-sufficiency in growth signals/factors, insensitivity to anti-growth factors, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis and sustained angiogenesis as represented in Figure 2.2.

Cancer cells do not rely on exogenously produced growth factors for survival. Even though the host may produce growth factors, these are not essential to the cancer cell. Cancer cells can circumvent the need for outside growth factors in several ways. They can produce their own growth factors. An example is autocrine/paracrine production of IGF-1 by prostate cancer cells [33]. Prostate cancer stromal cells produce IGF-1 and the IGF-1 receptor is expressed on adjacent epithelium. Additionally, cancer cells can harbor mutations that result in constitutively active growth factor and growth factor receptor pathways. This eliminates the need for binding of the growth factor ligand to the receptor to initiate the signaling cascade. The result is cell growth in the
absence of ligand. Mutations in the ras and Epidermal Growth Factor Receptors (EGFRs) are seen in prostate cancer [34]. Cancer cells can also overexpress growth factor receptors so that normal physiological levels of a growth factor result in hypersensitivity of that pathway. Prostate cancer cells commonly overexpress HER2-neu and the androgen receptor [35]. AR regulated gene expression can occur in the absence of androgen or small amounts of androgen that can lead to large increases in the expression of pro-carcinogenic genes.

Normal cell growth is the result of a balance between growth and anti-growth signaling. In order for cells to differentiate, they must halt the growth program and initiate the differentiation program. Cancer cells are typically less differentiated than normal cells, the result of unimpeded growth. A tumor is a collection of a mass of rapidly proliferating, undifferentiated cells. This process is dysregulated in prostate cancer development as seen in the case of TGFb. TGFb is an anti-growth factor under normal conditions [30]. It is thought to stimulate prostate epithelial cells to a more terminally differentiated state, the opposite of a cancer cell. TGF-b signals through Rb, presumably by causing an increase in cell cycle inhibitors like p21. Rb is commonly lost in prostate cancer, thus rendering the pro-differentiation, anti-proliferation effects of TGF-b null [36].

When a cell is damaged, processes are activated to signal the cell to initiate a cell death program. The body tries to eliminate damaged cells that do not function properly. It signals damaged cells to undergo apoptosis. Tumors are the result of proliferation of damaged cells that harbor many mutations that enable them to survive. These cells may produce factors that allow them to escape apoptotic cues. Bcl2 is an anti-apoptotic
protein and its expression is indicative of a cell that does not respond to apoptotic cues. Prostate cancer cells produce high levels of Bcl2 [37], rendering them resistant to apoptosis.

Normal cells typically replicate a few times over the course of their natural lifespan. Cancer cells can replicate many more times, possibly even 100. Prostate cancer cell cultures can survive for 20-30 passages. Although some of this might be due to the supraphysiological ideal conditions of cell culture, there are inherent defects in prostate cancer cells that enable this phenomena. In many cancer cell types, this is due to dysregulated telomere maintenance. Telomeres are small fragments of DNA at the ends of chromosomes [38]. After a cell undergoes replication, the telomere is shortened. Normally, after these telomeres are shortened to a designated length, this signals a cell to stop replicating. Cancer cells exhibit high levels of the enzyme telomerase, which maintains telomere length, thus allowing cancer cells to replicate beyond their normal programmed number of replications [38]. In fact many researchers simply introduce telomerase into cells to create an immortalized cell line. Other cell signaling pathways can converge on this pathway by influencing levels of cell cycle regulating proteins. This is seen in prostate cancer through losses of p21 or p27 [39] which are cell cycle inhibitors.

Generally speaking, death from cancer occurs as a result of metastasis of the original tumor. Cancer cells must metastasize to further survive, in a parasitic manner. The tissue of origin can only support so much tumor burden. Additionally, a tumor will only grow to a certain size because nutrients and oxygen can only penetrate a tissue so far, even in the presence of increased angiogenesis [40]. In order for a cancer cell to
metastasize, it must be able to degrade the basement membrane in the tissue of origin, penetrate the bloodstream (or lymph), survive in the bloodstream and then penetrate into the tissue of destination where it can invade and colonize. Only a few cells actually survive through all of these steps of the metastatic cascade. In prostate cancer, the loss of E-Cadherin is seen [41]. E-Cadherin is involved in tethering cells together and to the basement membrane and maintaining epithelial integrity. Essentially, its loss signifies that a cancer cell has acquired the ability to leave the prostate. Additionally, levels of matrix metalloproteases (MMPs) are increased in prostate cancer [42]. MMPs degrade stromal and basement membrane components, enabling cancer cells to escape the tissue of origin.

Cancer cells require additional vasculature to grow beyond a certain small size. Pioneering work by Judah Folkman revealed the absolute requirement for sustained angiogenesis in cancer [40]. The need for this additional vasculature is triggered by heightened requirements for oxygen supply, nutrient supply and waste removal. Additional angiogenesis and lymphangiogenesis may also be required for metastasis as well. Cancer cells proliferate rapidly so they require additional nutrients and oxygen and produce large amounts of waste. They produce factors that recruit endothelial progenitor cells and epithelial cells can even form primitive vascular channels. In prostate cancer, as with many other cancers, vascular endothelial growth factor (VEGF) production is elevated [43]. This paracrine factor stimulates nearby endothelial cells and even epithelial cells to form the small, irregular vasculature associated with tumorigenesis.

2.3 Hormones and Prostate Cancer
The observation of the initial dependence of the prostate gland on androgens led Nobel Laureate Charles Huggins to the idea of androgen removal as a plausible means for prostate cancer treatment. Huggins observed that castration of dogs caused shrinkage of prostate tumors, a phenomenon that was later reproduced in men [44]. The principle behind androgen deprivation therapy is that removal of a crucial prostate survival factor, testosterone, will lead to prostate cancer cell death. Prostate cancer cells, like normal prostate cells, are initially androgen dependent [44] and the androgen receptor functions normally (e.g., AR only responds to androgen, AR is one of the key players in prostate growth and maintenance and early prostate cancer cells respond to androgen removal and addition the way normal cells do). Androgen removal can be carried out through several avenues. The decision as to which method to use depends on the severity of the cancer, as the side effects can vary. Several will be discussed here. One of the most commonly used methods is 5α-reductase inhibition, decreasing levels of the active hormone DHT. This is generally used for early cancers and has mild side effects. In fact, finasteride, a 5α-reductase inhibitor, has been demonstrated to be a successful chemopreventive agent. Men taking 5mg of finasteride in the Prostate Cancer Prevention Trial (PCPT) were 25% less likely to develop prostate cancer by the end of the study [45]. Concerns were raised about finasteride causing more severe cancers but this has since been refuted. Androgen action can also be blocked at the receptor level by AR antagonists [46]. These antagonists can prevent any androgens, whether testicular or adrenally produced, from binding to the androgen receptor and initiating gene transcription. Testicular androgen production can be blocked by LH inhibition as well [47]. Combined androgen blockade, using a combination of the previously mentioned methods, blocks androgen production
and action and is used for more aggressive cancers [46]. Initially, most cancers respond well to androgen deprivation. However, prostate cancer cells develop survival mechanisms involving the androgen receptor that can render these treatments ineffective and catalyzes the progression from androgen sensitive to androgen insensitive disease.

The AR plays a pivotal role in androgen insensitive prostate cancer progression. It can develop abnormal functions that are not seen in normal AR signaling. Aside from the effects of AR function in androgen insensitive prostate cancer, it is worth revisiting the subject of testosterone production. It has been previously stated that the testes are the main site of testosterone production and that therapies that reduce testosterone production target the testes. Adrenal androgen synthesis is not affected by these therapies and it is greatly increased in androgen insensitive prostate cancer, thus compensating for the reduced testicular androgen production. In this case, a broad spectrum cytochrome P450 inhibitor such ketokonazole, may be useful. However, the side effects are extensive so its utility is limited [48]. The androgen receptor participates in androgen insensitive prostate cancer through several mechanisms that are summarized in Figure 2.3. It can be amplified, creating a hypersensitivity to even low amounts of circulating androgens [49]. The AR can also become promiscuous. Mutations can be introduced into the ligand binding domain that can allow for aberrant AR activation by factors other than androgens, such as estrogen or other steroid hormones [50]. Mutations can also be found in coregulators that can allow for increased AR activity. The AR can become an outlaw receptor by being transactivated by other signaling pathways. Peptide growth factors like IGF-1, KGF and Il-6 can phosphorylate and activate the AR through other signal transduction pathways in the absence of androgen [51]. Activation of other pro-
proliferative and anti-apototic can bypass the AR pathway, thus eliminating any dependence on androgens for these physiological effects. These other pathways may not be activated under normal physiological conditions. In the absence of AR function, BCL2, an anti-apoptotic protein is produced. BCL2 is not expressed in normal prostate epithelium [37]. The AR develops these modified functions as a survival mechanism and the presence of these functions determines to a large extent how effective treatment will be.

2.4 Animal Models of Prostate Cancer

Cell culture experiments are economic and useful in providing preliminary and mechanistic data that warrants further investigation. However, the utility of these models is limited. Conditions required for successful cell culture are artificial. Concentrations of media nutrients and gases are maintained at a constant, representing the “ideal” environment which is often supraphysiologic. Cell culture experiments often utilize a single, clonally identical cell line of a particular origin in a dish or flask. A particular organ in the human body may contain many types of tissues. These tissues may contain many types of cells. For example, the prostate consists of an epithelial layer surrounded by a stromal layer which contains fibroblasts and blood vessels, among other cell types. Typical cell culture eliminates paracrine and endocrine communication from surrounding and distant tissues. This is problematic particularly in prostate research because the paracrine and endocrine communications between prostatic epithelium, prostatic stroma and distant endocrine organs like the pituitary are elegantly interrelated [52]. A compound’s toxicity to surrounding and distant normal tissue is also speculative. Use of
only one cell type also eliminates any potential metabolic effects a distant organ may have on the compound used. For example, the roles of the kidney and liver in vitamin D metabolism are well documented. Furthermore, cell culture is often conducted on transformed cells which represent one end of the carcinogenic spectrum which is represented in Figure 2.4. This neglects lifetime exposure. Humans are often exposed to agents throughout the lifespan and a given agent may have different effects on a preneoplastic versus neoplastic tissue.

Animal experiments are time-consuming and expensive, but they are critical for fostering the transition from bench to bedside. Although animal experiments are crucial, inherent differences exist between the anatomical properties of the human and mouse prostate gland and are shown in Figure 2.5. The earliest in vivo prostate cancer experiments were performed in rodent models exposed to carcinogens or hormones. Recent technologies such as tissue specific promoters and conditional gene deletions have enabled researchers to study many more genetic defects, producing a surge in the generation of disease models [53]. Certain models have shown utility in treatment but not prevention. Other models may produce prostate phenotypes or other key features that are not entirely identical and relevant to the human disease. Indeed, each model has both strengths and weaknesses. For example, possibly the most popular mouse model, the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model develops cancer as a result of Simian Virus 40 (SV40) T antigen expression [54]. The expression of this transgene results in many genetic defects, including p53 and Rb loss. This “multi-hit” effect is desirable because prostate cancer is generally not the result of a single genetic mutation. However, this model is virally transformed, which may not be relevant to
human disease. Additionally, this model is very aggressive and progresses to advanced
cancer very rapidly, which does not follow the human disease course. There are many
existing mouse models that are useful for investigating the stepwise progression of
prostate cancer. Each has its strengths and weeknesses. Table 2.1 presents several of the
common mouse models of prostate cancer and lists their characteristics. However, few of
these models have been fully characterized with respect to the molecular events unique to
cancer.

2.5 Prostate Cancer Incidence and Etiology

Since 2001, cancer has become the leading cause of death in individuals younger
than 85 years, surpassing heart disease. It is estimated that 1.5 million new cases of
cancer were diagnosed in 2009 in the United States with males representing slightly more
cases than females [1]. Cancer was also estimated to cause approximately 560,000
deaths in 2009, again males having a higher death rate than females.

Prostate cancer is the most common malignancy among men, comprising
approximately 25% of all cancer cases. Nearly 200,000 new cases were diagnosed in the
United States in 2009, resulting in 30,000 deaths [1]. It is a disease of aging, with the
largest proportion of deaths being from those men older than 60. Furthermore, in
individuals 80 years or older, it is the second leading cause of cancer death, following
lung cancer [1]. Additional risk factors predispose one for prostate cancer development.
This includes race. For example, African Americans display the highest prostate cancer
incidence rates (248.5/100,000) while American Indian/Alaskan natives display the
lowest incidence (73.3/100,000). Prostate cancer death rates follow similar trends
although the lowest death rate is seen in Asian Americans and Pacific islanders [1]. Environmental factors may also impact prostate cancer development. Infectious agents and viruses [55] are implicated in chronic prostate inflammation, which may lead to prostate cancer. Many dietary factors can impact prostate cancer risk which will be subsequently discussed.

### 2.6 Diet and Prostate Cancer

Doll and Peto first hypothesized in 1981 [56] that improper diet and nutrition were a plausible risk factor for cancer development. It has been estimated that up to 50% of all cancers could be prevented by modifying these lifestyle risk factors [57]. There are many dietary factors that are thought to influence prostate cancer development. However, little conclusive evidence implicates any one factor or food as a preventative or causative agent for prostate cancer. Several will be discussed in this section. Bioactive lipids, calcium and vitamin D will be presented in subsequent sections as they are a focus of this dissertation.

Selenium and vitamin E, were recently evaluated as a nutritional chemopreventive regimen in the SELECT trial (Selenium and Vitamin E Cancer Prevention Trial). Incidental epidemiological evidence suggested that both of these compounds, thought to act as antioxidants against the cancer process, were protective against prostate cancer. These data were conducted from vitamin E and lung and selenium and skin cancer prevention trials in which men displayed 32% and 52% fewer cases of prostate cancer [58, 59]. Unfortunately, the SELECT trial showed no benefit of either selenium or vitamin E or a combination of both [60]. The five year cancer diagnosis rate for men in
the placebo group was 4.43%, the selenium only group was 4.56%, the vitamin E only group, 4.95% and the selenium and vitamin E group, 4.56%.

Lycopene is the carotenoid that is responsible for the red color of tomatoes. It has received substantial attention in prostate cancer prevention [61]. The HPFS showed that increased tomato consumption was associated with a decreased risk of developing prostate cancer [62]. Lycopene inhibits the growth of cells in culture and the growth of tumors in animal models [63]. Interestingly, a study in the NMU model of rat carcinogenesis showed that lycopene containing whole tomato diets were more effective at reducing prostate cancer than diets supplemented with only lycopene, suggesting that other compounds in the tomato are impacting prostate cancer development.

2.7 Bioactive Lipids and Prostate Cancer

2.7.1 Epidemiological Evidence

Dietary lipids have long been implicated in the development of prostate cancer. Increased dietary fat is thought to increase prostate cancer risk. Additionally, the type of fat consumed is thought to play a prominent role in the development of prostate cancer. Diets rich in omega-3 polyunsaturated fatty acids (PUFAs), such as those found in many species of cold water fish, are proposed to possess anti-prostate cancer activities. This association was prompted by the observation that populations such as Japanese men, who consume large amounts of cold water fish, display low prostate cancer mortality rates [64]. Data from epidemiological studies suggest that men who consume no fish have an increased risk of developing prostate cancer when compared to men who consume moderate to high amounts of fish [65, 66]. Likewise, diets enriched in omega-6 PUFAs,
like those present in many ruminant animal products and vegetable oils, may be cancer promoting. Indeed, men who consume the typical “Western” diet which is high in meat, red meat and dairy products, are at a higher risk for advanced prostate cancer [2].

2.7.2 Molecular Mechanisms of Bioactive Lipids

Prostate cancer incidences are higher in cultures that adhere to the “Western” dietary pattern, which possesses many pro-carcinogenic characteristics. The western diet contains low levels of omega-3 PUFAs (polyunsaturated fatty acids) like eicospentaenoic acid (EPA) and docosahexaenoic acid (DHA), and high levels of omega-6 PUFAs like linoleic acid (LA). Both linoleic acid and a-linolenic acid (ALA) are deemed essential because the human body can not produce them on its own and are required for normal physiological functions such as neurological development and blood clotting. ALA can be further metabolized to EPA and DHA. Supplementation with both EPA and DHA has been shown to inhibit growth of prostate cancer cells in culture [67]. Likewise, linoleic acid (LA), the most abundant omega-6 PUFA in the Western type diet, promotes growth of cancer cells [4] (summarized in Figure 2.6).

Dietary PUFAs impact many cellular pathways associated with cancer [68-70] including inflammation and angiogenesis. However, considerable interest has been placed on their effects on the cyclooxygenase family (COX-1 and COX-2) and lipoxygenase family (5-LO, 12-LO, 15-LO-1 and 15-LO-2) of enzymes. These enzymes catalyze the formation of compounds called eicosanoids. Importantly, the tumor environment displays an inherent dysregulation of these enzymes to favor conditions that promote carcinogenesis [71]. Both enzyme activities and products are subject to a
carefully orchestrated regulation by the ratio of omega-3 and omega-6 PUFAs [72-74]. LA can be metabolized through the lipoxygenase (15-LO-1) pathway to the mitogenic 13-S-Hydroxyoctadecadienoic acid (13-S-HODE). It can also further be metabolized to arachidonic acid (AA). AA can then serve as a substrate for COX-2, thus producing prostaglandin E2 (PGE2). PGE2 promotes inflammation, enhances cell proliferation, stimulates cell migration and invasion and induces angiogenesis [74]. EPA and DHA can serve as substrates for the COX and LO enzymes as well. EPA is metabolized to prostaglandin E3 (PGE3) and 15-hydroxyeicospentanoic acid (15-HEPE) through COX-2 and 15-LO-1, respectively. PGE3 and 15-HEPE both possess anti-carcinogenic properties [74]. The ratio of omega-6/omega-3 PUFAs is also of importance due to the fact that omega-3 PUFAs can compete with omega-6 PUFAs for COX and LO binding and activity. Western diets typically possess a ratio exceed 16:1 while the optimal ratio is postulated to be about 4:1 [75]. If the ratio is optimal, the balance will be shifted to production of anticarcinogenic compounds. Additionally, omega-3 PUFAs can inhibit the metabolism of omega-6 PUFAs further favoring an anticarcinogenic milieu. Recent data also indicate that EPA and DHA can form compounds called resolvins that are anti-inflammatory [76].

2.7.3 In Vivo Evidence

Omega-3 PUFA enriched diets are found to be inhibitory, whereas omega-6 PUFA enriched diets are found to be promotional in several animal models of prostate cancer. Although in vivo studies provide evidence for the role of dietary lipids in prostate cancer, the majority of studies have been conducted in xenograft models [77]. These
models can be considered tumorigenesis models rather than carcinogenesis models in that the cells injected are already transformed, neglecting the early stages of the cancer process. Transgenic carcinogenesis models are more relevant to human disease in that they represent the whole cancer development spectrum. However, few transgenic animal studies exist and their results are conflicting. A recent study [3] showed that a diet high in omega-3 fatty acids was protective in the Phosphatase and Tensin Homologue on chromosome 10 (PTEN)/Nkx3.1 compound mutant mouse model of prostate cancer. In contrast, another recent study performed in the Erb overexpressing PTEN +/- model [78], no effect of omega-3 fatty acids was seen on prostate cancer development. Discrepancies in the results of these two studies can largely be attributed to dietary lipid content and composition and animal model selection. Evidence has been presented that shows that transgene expression in several animal models is correlated with dysregulated expression of lipid metabolizing enzymes such as COX-2 [79], further emphasizing the importance of model selection.

2.8 The Role of Calcium and Vitamin D in Prostate Cancer

2.8.1 Epidemiology

Several laboratories have published evidence showing that high dietary calcium intake has a negative impact on prostate cancer risk. These studies show that diets rich in calcium from dairy products as well as high dietary calcium intake from foods or supplements is associated with increased prostate cancer risk. The Health Professional’s Follow-up Study of over 50,000 men, showed that high calcium intake was associated with increased risk of advanced cancer (relative risk (RR) = 2.97, intake > 2000 mg/d vs.
< 500 mg/d) and metastatic prostate cancer (RR = 4.57) [5]. Similarly, in a study of Swedish men, calcium intake increased the risk of prostate cancer (RR = 1.91 for intake >1183 mg/d vs. < 825 mg/d) and metastatic tumors (RR = 2.64) [80]. This is of concern because the prostate cancer promoting effects of calcium seen in these studies are within the levels of intake recommended to protect bone health and prevent osteoporosis [81].

Evidence from a number of laboratory and human studies are converging to support the hypothesis that suboptimal vitamin D status promotes prostate carcinogenesis. In fact, this data has led to Institute of Medicine to reconsider the dietary requirements for vitamin D in the United States. It has been hypothesized that vitamin D deficiency is the critical underlying factor for increased prostate cancer risk. This is due to the fact that advancing age, African-American race or dark skin pigment, and northern latitudes (shown in Figure 2.8) are all risk factors for prostate cancer. Each of these risk factors is associated with decreased UV-induced vitamin D synthesis in the skin [82].

Several groups have attempted to establish a relationship between vitamin D metabolites/biomarkers and prostate cancer risk in humans. Ahonen et al. [82] found a 70% increase of prostate cancer risk in men with 25 hydroxyvitamin D (25-OH D) levels below the median. This was especially true for men < 52 y who entered the study with low serum 25-OH D (odds ratio = 3.5). Corder et al. [83] found that circulating 1,25(OH)₂D levels were significantly lower in men with prostate cancer. They also found that prostate cancer risk was decreased in men with higher 1,25(OH)₂D levels, especially if serum 25-OH D levels were low. Another study in Finnish and Norwegian men [84] showed that low vitamin D status measured within 10 years of a prostate cancer diagnosis with prostate cancer, increased risk of prostate cancer by nearly 4 fold.
Several studies have emerged that refute the protective effects of vitamin D against prostate cancer. Studies in men from sun-rich regions where vitamin D insufficiency is uncommon, did not detect a relationship between serum vitamin D metabolites and prostate cancer [85-87]. The Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) showed that the highest serum levels of vitamin D were surprisingly associated with risk for developing more aggressive disease (in order of lowest to highest quintile of vitamin D 406, 479, 780, 633 and 544 rate per 100,000 person years) [88]. The European Prospective Investigation into Cancer and Nutrition (EPIC) performed a nested case-control analysis of 652 prostate cancer cases versus 752 controls and found that there was no association of serum vitamin D with prostate cancer (odds ratio 1.28 for highest versus lowest quintile) [89]. Although not uniform, the majority of the data suggest that high serum 25-OH D or high serum 1,25(OH)₂D are associated with a lower risk of prostate cancer.

2.8.2 Vitamin D Endocrinology and Metabolism

The metabolism of vitamin D has been long studied and is well established. Vitamin D, whether from the diet or produced in skin, is hydroxylated in the liver to form 25-OH D. This is considered a reliable marker of vitamin D status and is commonly used to clinically assess vitamin D levels [90]. Further activation of 25-OH D to 1,25(OH)₂D must take place in the kidney before the hormone is biologically active.

The classical physiological role of vitamin D was thought to be regulation of bone formation and blood calcium homeostasis. This was refuted upon the discovery that nearly every type of cell in the body expresses the vitamin D receptor. Furthermore,
prostate cancer cell lines, along with primary prostate epithelial cells, express 1alpha-hydroxylase [91]. Thus, they possess the ability to metabolize 25-OH D3 to the biologically active 1, 25 \((\text{OH})_2\) D3. Vitamin D can induce genes in the prostate that are associated with growth arrest, inhibition of metastasis and other cellular signaling proteins [92]. High calcium suppresses prostatic conversion of 25-0H D3 to the active form of vitamin D, thereby decreasing the size of the pool of active vitamin D. This may provide mechanistic evidence as to why high levels of dietary calcium may increase prostate cancer risk through negating any of the protective effects of vitamin D. Figure 2.8 summarizes the vitamin D endocrine system, calcium regulation and biological effects of vitamin D.

### 2.8.3 Molecular Mechanisms

Localized prostatic metabolism of 25-OH D to 1,25\((\text{OH})_2\)D may be a critical process for vitamin D mediated prostate cancer prevention (Fig 2.8). Importantly, circulating levels of 25-OH D are strongly influenced by race, latitude/geography, seasonal sunlight, and dietary vitamin D intake [7, 93] and that high circulating levels of 25-OH D may result in local production of 1,25\((\text{OH})_2\)D by the prostate. It has also been shown that the 1alpha-hydroxylase gene is hypermethylated in malignant human prostate lesions as compared to benign lesions [94], leading to decreased expression and enzymatic activity. These data support the hypothesis that both high serum 25-OH D and 1,25\((\text{OH})_2\)D are protective against prostate cancer. In contrast, high dietary calcium and low vitamin D synthesis or intake counters this protective process.
Due to the potential hypercalcemic effects of high levels of vitamin D the role of vitamin D status on prostate carcinogenesis or prevention in transgenic animal models has received almost no investigation. A multitude of studies have examined the effects of non-calcemic vitamin D analogs. However, a small number of studies have been conducted and will be reviewed here. One murine study [95, 96] found that a low calcium (0.05%) and vitamin D (500 IU/kg diet) diet increased epithelial cell proliferation 5-fold in the dorsal lobe of the prostate and that this could be prevented by increased dietary vitamin D (to 2300 IU/kg diet). This suggests that low vitamin D status increases prostate cell turnover and proliferation, thereby rendering epithelial cells more susceptible to DNA damaging processes. However, this study used a “Western” diet which contained many other dietary variables, possibly confounding the effects of vitamin D. Another study conducted in compound mutant Nkx3.1;PTEN transgenic mice, showed that 46/ng/kg/day of vitamin D inhibited preneoplastic lesion formation but not invasive cancer formation [97]. However, this study used surgically implanted osmotic pumps to deliver vitamin D as opposed to dietary treatment. Furthermore, blood levels of vitamin D metabolites were not measured, failing to establish an association between serum levels and cancer development. Interestingly, a recent study by Mordan-McCombs et al. [98] used androgen-driven, T antigen transformed mutant mice to show that high dietary calcium, which reduced circulating vitamin D, has no effect on prostate tumor development. However, they showed that high dietary calcium reduced circulating testosterone levels in this model, possibly decreasing transgene expression, and therefore inherent phenotypic penetrance.
The hormonally active form of vitamin D, 1,25(OH)₂D, alters cell biology by activating nuclear vitamin D receptor (nVDR)-mediated gene transcription. The nVDR is expressed in both the central and peripheral zones of the human prostate [99]. Many studies have shown that in cultures of primary or transformed prostate cells, 1,25(OH)₂D or its analogs can inhibit proliferation, promote differentiation and apoptosis, and limit invasiveness of cells [100]. Interestingly, vitamin D has been shown to modulate the IGF1 axis [101, 102], a prominent growth factor signaling pathway in prostate cancer. Several studies have shown that 1,25(OH)₂D mediated growth inhibition is directly associated with nVDR levels [103, 104], demonstrating that disruption of the nVDR signaling pathway in proliferating epithelium may increase cancer risk. Increased epithelial cell proliferation has been observed in both the distal colon and mammary glands of nVDR knockout mice [105, 106]. A recent study by Mordan-McCombs et al. [107] examined the effects of nVDR deletion in a transgenic murine prostate cancer model. They found that tumors progressed more rapidly and lesions were more severe and exhibited higher proliferative rates in mice lacking the nVDR. However, when testosterone levels were increased, there was no effect of nVDR status, suggesting an interaction between androgens and vitamin D. Overall, these data illustrate the importance of nVDR mediated signaling in the control of epithelial cell growth and prostate cancer pathogenesis and prevention.

It has been hypothesized that high vitamin D levels might overcome the increased risk associated with high dietary calcium intake and the suppression of renal 1,25(OH)₂D production [108] by presumably increasing prostatic conversion of 25-OH D. To date, the only study that has directly tested this hypothesis was performed in the LNCaP tumor
xenograft model. Neither higher dietary vitamin D (2900 IU/kg diet) nor low dietary calcium (0.04%) altered tumor growth [109]. However, LNCaP cells are derived from a poorly differentiated prostate cancer lymph node metastasis and possess virtually no 1alpha-hydroxylase enzymatic activity. These results, together with those from Banach-Petrosky et al. [97] which showed no effect of vitamin D on advanced cancer, suggest that study of the calcium and vitamin D axis may be more relevant in a prevention setting.

2.9 Summary

Prostate cancer is a disease which, due to its latency, can be significantly modulated by exposure to lifestyle and environmental risk factors. However, little conclusive evidence implicates any one dietary factor as a bona fide prostate cancer chemopreventive agent. The epidemiologic data are suggestive of dietary patterns that may be protective against prostate cancer and a plethora of cell culture data provides mechanistic data. Unfortunately, in depth animal studies that address these dietary patterns and factors are lacking and serve to provide a large gap in our understanding of the role of nutrients in prostate carcinogenesis. Until these studies have been completed and sufficient preclinical data are achieved, clinical trials of nutrients or dietary patterns can not be completed and these questions will remain unanswered. The subsequent chapters of this dissertation provide thorough characterization of an animal model of prostate cancer and employ the model in studies addressing the roles of bioactive lipids, vitamin D and calcium in prostate carcinogenesis.
Figure 2.1: Molecular events and interactions in prostate development. Taken from [31].
Figure 2.2: The hallmarks of cancer. Taken from [32].
Figure 2.3: Androgen Receptor involvement in androgen insensitive prostate cancer. Taken from [110].
Figure 2.4: Molecular events in prostate cancer development and progression. Taken from [111].
Figure 2.5: Comparative anatomy of the human and mouse prostate. Taken from [112].
<table>
<thead>
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<th>Model</th>
<th>Characteristics</th>
<th>Ref.</th>
</tr>
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<td>TRAMP</td>
<td>Large, poorly-differentiated neuroendocrine tumors by 16 weeks of age; lymph node, liver, lung metastasis.</td>
<td>[113]</td>
</tr>
<tr>
<td>cPTEN</td>
<td>Invasive carcinoma by 30 weeks of age; lymph node, lung metastasis.</td>
<td>[114]</td>
</tr>
<tr>
<td>Nkx3.1;PTEN</td>
<td>High-grade PIN at 6 months with invasion by 12 months of age; lymph node metastasis.</td>
<td>[20]</td>
</tr>
<tr>
<td>Hi-Myc</td>
<td>PIN at 2 weeks of age; invasive carcinoma at 6-12 months of age; lymphovascular invasion.</td>
<td>[115]</td>
</tr>
<tr>
<td>LADY</td>
<td>Invasive neuroendocrine tumors by 9 months of age; lymph node, liver, lung metastasis.</td>
<td>[116]</td>
</tr>
<tr>
<td>Apt121/Rbf</td>
<td>PIN at 4 weeks of age; microinvasive carcinoma by 8 months of age.</td>
<td>[117]</td>
</tr>
</tbody>
</table>

**Table 2.1- Summary of commonly used transgenic murine prostate cancer models.**
Figure 2.6: Proposed mechanism of the role of omega-6 and omega-6 fatty acids in carcinogenesis.
Figure 2.7: Map of prostate cancer mortality rates. Taken from http://www3.cancer.gov/atlasplus/.
Figure 2.8: Vitamin D metabolism and actions. Adapted from [92].
CHAPTER 3

THE IMPACT OF RB FAMILY PROTEIN DELETION ON PROSTATE CARCINOGENESIS AND BIOMARKERS IN A TRANSGENIC MODEL (RBF/TGAPT121)

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3.1 Abstract

Well characterized rodent models of prostate carcinogenesis permit useful evaluation of chemopreventative and therapeutic measures. The recently developed transgenic \( Rbf/TgAPT_{121} \) model \cite{117} is a variation of the well-known TRAMP model, in that it uses the SV40 transformation system and is driven by the androgen-responsive probasin promoter. However, only the 121 N-terminal amino acids of the large T-antigen are expressed, allowing for more specific inactivation of the pRb family of proteins, a common genetic mutation in human prostate cancer.

Histopathological examination of prostate tumor stage and grade was evaluated in male \( Rbf/TgAPT_{121} \) mice at 1, 2, 3, 4, 5, 6, 8 and 12 months of age. Proliferation and apoptosis were evaluated as relevant biomarkers of prostate carcinogenesis.

\( Rbf/TgAPT_{121} \) mice developed hyperproliferative and dysplastic lesions with focal areas of carcinoma progressing to microinvasion but without evidence of metastatic disease. Prostatic intraepithelial neoplasia (PIN) was observed by one month of age. High-grade PIN with microinvasive adenocarcinoma was first observed at 3 months of age. By 6 months, 100% of mice displayed microinvasive adenocarcinoma. As anticipated, proliferative and apoptotic indices increased with advancing histopathologic grade. While TRAMP mice develop aggressive prostate tumors that progress to poorly differentiated adenocarcinoma, \( Rbf/TgAPT_{121} \) mice typically develop atypical hyperplasia, PIN and early localized adenocarcinoma.

In comparison to the TRAMP model, the \( Rbf/TgAPT_{121} \) model is relevant to the early stages of prostate carcinogenesis. This model will be useful for examination of
dietary and chemopreventive agents that target the earlier phases of prostate carcinogenesis.

3.2 Introduction

Prostate cancer is considered a disease of aging in that preneoplastic lesions, termed Prostatic Intraepithelial Neoplasia (PIN) have been noted in men as early as 20 years of age [118]. The molecular mechanisms leading to the development and progression of prostate cancer are poorly understood. However, numerous studies have shown that members of the Rb family of proteins are commonly dysregulated in human prostate cancer [36]. These proteins- pRb, p107 and p130, have overlapping functional characteristics. The Rb proteins bind to E2F transcription factors. When Rb is lost, E2F is released, resulting in the transcription of genes associated with cellular proliferation and evasion of apoptosis.

Rodent models have been generated to disrupt of the Rb family of proteins to both investigate their function and model human prostate cancer. The TRAMP mouse is a widely used transgenic model of prostate cancer as it develops preneoplastic lesions that progress to invasive adenocarcinoma and distant metastases by 30 weeks of age [113]. The recently developed transgenic Rb/TgAPT121 model [117, 119] is a variation of the well-known TRAMP model in that it uses an SV40 transformation system, and is driven by the androgen-responsive probasin promoter. However, in this model, only the 121 N terminal amino acids of the large T-antigen are expressed. This allows for specific inactivation of the Rb family proteins pRb, p107 and p130 in androgen responsive prostate epithelium, leaving p53 and other targets of the SV40 large T antigen intact. Hill
et al reported that these mice demonstrate enhanced prostatic epithelial proliferation and apoptosis resulting in PIN and adenocarcinoma [117].

This study was designed to characterize the histopathological changes during the course of prostate carcinogenesis in the \(Rbf/Tg\text{APT}_{121}\) murine model. We present here a subclassification of PIN during the carcinogenic process and delineate the proliferative and apoptotic indices of each. The \(Rbf/Tg\text{APT}_{121}\) model will will be useful for the evaluation of chemopreventative and chemotherapeutic interventions that interact with Rb mediated signaling or the proliferative and apoptotic signals that occur during the early stages of prostate carcinogenesis.

### 3.3 Materials and Methods

#### 3.3.1 Animals and diets

Derivation and identification of \(Rbf/Tg\text{APT}_{121}\) transgenic mice was previously described by Hill et al. 2005 [117]. For this analysis, 78 male \(Rbf/Tg\text{APT}_{121}\) transgenic mice and 20 non-transgenic B6D2F1 mice were randomly grouped and sacrificed at the age of 1, 2, 3, 4, 5, 6, 8, and 12 months. TRAMP mice (n=30) were generated as previously described [113] for the TRAMP comparison arm and were sacrificed at when palpable tumors reached 2 cm or when they reached 27 weeks of age. The mice were fed the AIN-76A (Research diets D10001), semi-purified, nutritionally adequate, and devoid of phytochemicals diet. Distilled water and diet was provided ad libitum. The study was completed in the ULAR facilities within the OSU College of Medicine and Comprehensive Cancer Center, and Purdue University according to the Institutional
Laboratory Animal Care and Use Committee-approved protocols at respective universities.

3.3.2 Tissue Collection

At sacrifice, the prostate, bladder and seminal vesicles were removed en bloc and fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol. The tissues were dissected into three parts as recommended by the Bar Harbor Classification of Mouse Prostate Pathology. At trimming, a transverse section through the urethra was made to include dorsal (DP), lateral (LP) and ventral lobes (VP) of the prostate. Subsequently both pieces were embedded in paraffin with cut surfaces down. Seminal vesicle and anterior prostate (AP, coagulating gland) were embedded without trimming and sectioned frontally to examine the entire length of the gland. The tissues were processed and embedded in paraffin using standard techniques. Tissues were sectioned at 4.5 micron thickness, and stained with H&E by an automated slide stainer (Leica Autostainer XL, Leica Microsystems Nussloch GmbH, Heidelberger, Germany) for histopathologic examination.

3.3.3 Grading and Staging

The histopathology of embedded tissues from all mice was assessed by scoring sections stained with hematoxylin and eosin (H&E). Prostatic lobes, including DP, LP, VP, and AP were assessed in accordance with established criteria from the Bar Harbor Prostate Pathology Consortium [120]. Histologic sections were examined and the worst lesion seen in each prostatic lobe was classified as normal, hyperplasia, prostatic
intraepithelial neoplasia (PIN), well-differentiated/microinvasive carcinoma (WD),
moderately-differentiated carcinoma (MD), poorly-differentiated carcinoma (PD) or
phylloides-like (Phyl). PIN lesions were sub-classified as later described. H&E sections
of the lymph nodes, lungs, and liver were evaluated for the presence or absence of
apparent metastatic lesions.

3.3.4 Immunohistochemistry

Immunohistochemical analysis was conducted on formalin-fixed paraffin
embedded tissue sections (4.5 micron). Antigen retrieval for all antibodies was performed
by boiling the slides in citrate buffer (Antigen Retrieval Citra Plus, BioGenex, San
Ramon, CA) for 15-30 minutes. The subsequent steps were completed in an OptiMax
Automated Cell Staining System (BioGenex, San Ramon, CA) at room temperature.
Endogenous peroxidase was inhibited by incubation in peroxide blocking solution (Dako
Cytomation, Carpinteria, CA) for 5 min followed by 60 min incubation with the
following primary antibody: SV40 T antigen (DP02, 1:100, mouse monoclonal antibody,
Calbiochem, San Diego, CA), Synaptophysin (sc-9116, 1:100, rabbit antibody, Santa
Cruz, Santa Cruz, CA), CD31 (sc-1506, 1:200, goat antibody, Santa Cruz), Ki67
(M7249, 1:50, rat monoclonal antibody, Dako Cytomation). After three washes with
PBS, for SV40 T antigen and synaptophysin, sections are incubated with a matched
labeled polymer-HRP (Dako Cytomation); for CD31 and Ki67, biotinylated secondary
antibodies and Vector ABC Elite kit (Vector Laboratories, Burlingame, CA) were used.
Color was developed by 10 min incubation with DAB chromogen solution (Vector
Laboratories). Slides were counterstained with Mayers hematoxylin for 2 min and mounted. Negative controls include normal serum and omission of primary antibody.

### 3.3.5 Apoptosis Measurement

Apoptosis was assessed using ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Bellerica, MA) which utilizes terminal deoxynucleotidyl transferase (TdT) enzyme to add modified nucleotides to fragmented DNA. Briefly, tissues were deparaffinized in xylene and ethanol and pretreated with proteinase K. Endogenous peroxidase was quenched and sections were equilibrated before treatment with the TdT enzyme. After incubation with TdT, anti-digoxigenin conjugate was added to detect levels of labeled nucleotide. Color was developed using peroxidase substrate and slides were counterstained with methyl green and mounted.

### 3.3.6 Image Capture and Analysis

Three representative images without necrosis or artifact were captured from each tissue section at 400 x magnification using bright field microscopy (Nikon ECLIPSE E 800, Tokyo, Japan) by a digital camera (Spot RT, Diagnostic Instrument, Inc., Sterling Heights, MI). The area of image captured from a 400 x field is 0.37 mm² in our imaging system. Optical settings were constant for all images. The captured images were then transmitted to a computer and saved as JPEG files to be further analyzed by image analysis software (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).
For quantification of proliferative indices, image analysis of the Ki67 immunostained sections of prostate involved the following steps. The epithelial compartment was separated from non-epithelial compartment (primarily stoma and lumens/secrections). Only the nuclei in the epithelial compartment were evaluated for Ki67 staining. The percentage of positive nuclei was then calculated based on the following formula: labeling index (%) = L / (L+C) x 100, where L= labeled cells (red) and C = counterstained, unlabeled cells (yellow). This procedure was employed for the quantitative evaluation of wild type normal structure and various lesions (hyperplasia, PIN I-IV, and carcinomas) in \textit{Rbf/TgAPT121} mice. For analysis of apoptotic indices, the same image analysis approach was conducted on the ApopTag stained tissue sections.

3.3.7 Statistical Analysis

Data were compared among groups by one way ANOVA, using GraphPad Prism 4 Statistical Software (GraphPad Software, Inc. San Diego, CA). Data are presented as means ± SEM and values were considered significantly different at p < 0.05. Differences in proliferation and apoptosis between mice of different groups were evaluated by the Tukey’s Multiple Comparisons Post-Hoc test (p < 0.05 is considered of statistical significance).

3.4 Results

3.4.1 Body weight and UGT weight increase as a function of age and cancer progression
Both body and UGT weight increased as a function of age in \textit{Rhf/TgAPT\textsubscript{121}} mice (Figure 3.1A). The pronounced increase in UGT weight that occurs at 12 months is predominantly due to the disproportional increase in the size and weight of the seminal vesicles. This increase is likely due to a blockage of the ducts and fluid buildup as there were no apparent hyperplastic changes to the seminal vesicles. The relative ratio of the UGT weight to body weight is represented in Figure 3.1B.

### 3.4.2 Characterization of the carcinogenic process in \textit{Rhf/TgAPT\textsubscript{121}} mice

The inactivation of the pRb Family of proteins in the \textit{Rhf/TgAPT\textsubscript{121}} genetically engineered mouse model induces prostate epithelial hyperplasia (Figure 3.2 C and D) and prostate intraepithelial neoplasia (PIN) (Figure 3.2 E and F) and demonstrates progressive prostate carcinogenesis over 6 months (Figure 3.2 G and H). We have observed the highest incidence of disease in the anterior prostate and thus all results are presented in terms of the anterior prostate unless otherwise noted. Normal prostate was characterized histologically by a single flat layer of epithelial cells. The nuclei are centrally located and the nucleus to cytoplasm ratio is approximately equal. The stromal layer is thin, continuous, and tightly packed (Figure 3.2 A and B). Hyperplasia was characterized by increased crowding, layering or piling of normal cells (Figure 3.2 C and D) that display an approximately equal nuclear:cytoplasmic ratio. The stromal layer remains thin and unreactive. PIN is a premalignant lesion where cells display an increased nuclear:cytoplasmic ratio and increased hyperchromaticity. Generally, greater than one cell layer is present. PIN presents itself in several forms in the \textit{Rhf/TgAPT\textsubscript{121}} mouse and therefore warrants a more thorough classification scheme (see below). Carcinoma is
represented by the presence of more dysplastic cells and stromal reaction. The defining characteristic is the presence of individual cells, nests of cells, or small glands in the stromal layer. These cells have broken through the basement layer, away from the gland of origin.

### 3.4.3 Sub-classification of PIN in \textit{Rbf/TgAPT121} Mice

PIN lesions are considered to be the earliest recognizable indication of cancer. PIN lesions may often progress to an invasive lesion. Typically, in experiments using mouse models of prostate carcinogenesis, lesions are classified as normal, PIN and cancer. However, in the \textit{Rbf/TgAPT121} model, several sub classifications of PIN are observed that represent different degrees of severity and hence the blanket term “PIN” is not useful. Sub classification of PIN in the \textit{Rbf/TgAPT121} model is described here.

**PIN I** (Figure 3.3A-C) is characterized by cells with hyperchromatic, oblong nuclei and prominent nucleoli. These cells have an increased nuclear:cytoplasmic ratio and are common to all types of PIN. The epithelial layer appears “crowded” as opposed to the even spaced epithelial layer of normal tissue. Stroma is still thin, continuous, and tightly packed. These simple flat lesions are often interspersed among areas of normal grade tissue.

**PIN II** (Figure 3.3D-F) is characterized by the presence of the same cells seen in PIN I. These cells demonstrate a piling and papillary organization. However, layering is not as extensive as in subsequent grades of PIN. Epithelial cells remain oriented toward basement membrane and no stromal reaction is seen.
PIN III (Figure 3.3G-I) contains the common PIN epithelial cell. However, nuclei begin to lose orientation toward basement membrane. Epithelial cells form a papillary pattern that projects into, but does not fill the lumen. Some stromal reaction may be seen but is not extensive.

PIN IV (Figure 3.3J-L) is characterized by the typical hyperproliferative PIN cells filling and possibly expanding the lumen. The outline and border of the gland are consistent. Significant stromal reaction is present. Cellularity is less organized as evident by the advanced loss of orientation. An independent mass of cells may be present and driving gland herniation. However, the epithelium is still encapsulated in the stromal layer. There is no evidence of invasion. The full spectrum of PIN lesions may be present in a given sample (Figure 3.3M).

3.4.4 Distribution of histological grade over time in the Rbf/TgAPT_{121} mouse.

The proportion of each histological grade (normal, hyperplasia, PIN I, PIN II, PIN III, PIN IV and carcinoma) is represented as a percentage over 1-12 months (Figure 3.4). Hyperplasia to PIN I lesions have been observed in Rbf/TgAPT_{121} mouse prostates at one month. At two months the prostates contain lesions of PIN II and PIN III. PIN IV lesions appear at three months. Well-differentiated adenocarcinoma were present at 4 months. At the age of 6 months or older, all mice had developed well-differentiated adenocarcinoma.

3.4.5 Differences in Carcinogenesis between Prostate Lobes

Although the effects of T antigen expression on prostate carcinogenesis are most robust in the anterior prostate of the Rbf/TgAPT_{121} model, cancer is apparent in the dorsal
(Figure 3.5C), lateral (Figure 3.5F) and ventral (Figure 3.5I) lobes, albeit to a lesser extent. We did not note the presence of the full spectrum of PIN classifications in these other lobes so they will not be presented here. Tufting, micropapillary, and cribriform PIN lesions have been observed in other lobes in this model (Figure 3.5 B, E, H).

### 3.4.6 Microinvasion in the \textit{Rbf/TgAP}T_{121} Mouse Prostate

Microinvasive carcinoma is the earliest recognizable form of invasive carcinoma in \textit{Rbf/TgAP}T_{121} mouse model, with penetration of malignant cells through the basement membrane of PIN-involved glands into the surrounding stroma. Invasion takes on several forms in this model. Microinvasion is recognized by the extension of individual tumor cells or small nests (Figure 3.6 B, C, G) or acini (Figure 3.6 A, D, E, F, and H) of cytologically atypical cells into the thin rim of stroma. It is distinguished from invasive carcinoma by the greater extent of the invasive focus of carcinoma in the latter.

### 3.4.7 Variants of adenocarcinoma.

During the advancing stages of adenocarcinoma that occur in the murine prostate by 12 months of age, a variety of histologic patterns can be observed. Some unusual patterns may be considered as a variant or subtypes of prostatic adenocarcinoma, such as signet-ring cell, ductal, phylloides and others. Occasionally one can see necrosis in the middle of adenocarcinoma similar to comedonecrosis (Gleason pattern 5) in human prostate carcinoma (Figure 3.7).

### 3.4.8 Proliferation and Apoptosis in \textit{Rbf/TgAP}T_{121} Lesions
Quantification was conducted on expression of Ki67 (Figure 3.8) and ApopTag (Figure 9) detection in control epithelium and hyperplastic, PIN I-IV, and carcinogenic lesions. The proliferative index is unique to the histopathologic grade (ANOVA, p<0.0001). In the normal prostate epithelium, the percentage of proliferating cells remains relatively low (2.93 ± 0.45%). As anticipated, the proliferative index increases in hyperplasia (26.27 ± 3.418%) and PIN I (64.08 ± 1.62%). Interestingly, from the peak in a PIN I lesion, the proliferative index is lower in PIN II (45.87 ± 4.09%) and PIN III (35.19 ± 2.78%) before increasing again in PIN IV (46.70 ± 2.79%) and Cancer (59.72 ± 1.42%). As demonstrated in Figure 3.8, the proliferative regions in the PIN lesions are located at the margin of the gland and the less proliferative areas in the center of the gland. The apoptotic index is also impacted by the histopathologic grade (ANOVA, p<0.0001) with the apparent increase in apoptosis with advancing grade. The increase in apoptosis is apparent even in regions of prostate epithelial hyperplasia (7.25 ± 1.41%) compared to normal epithelium (0.97 ± 0.11%). The apoptotic index is maximal in the areas well differentiated adenocarcinoma (14.57 ± 1.47%), the most advanced lesion identified in the Rbf/TgAPT121 model. Representative photomicrographs of the ApopTag staining in each lesion are presented in Figure 3.9.

3.4.9 Expression of biomarkers in Rbf/TgAPT121 and TRAMP mice

As the Rbf/TgAPT121 model was generated as a variant of the widely used TRAMP model, proliferation, apoptosis and transgene expression were evaluated in lesions from the anterior prostate. Immunohistochemical analysis was conducted for these biomarkers of carcinogenesis in wild-type (Figure 3.10A-D), Rbf/TgAPT121 (Figure
3.10E-L) and TRAMP (Figure 10M-X) mice. H&E staining was conducted to assess architecture (Figure 3.10A,E,I,M,Q,U), Ki67 for assessment of proliferation (Figure 3.10B,F,J,N,R,V), ApopTag to measure apoptosis (Figure 10C,G,K,O,S,W) and SV40 T-antigen IHC for transgene expression (Figure 3.10D,H,L,P,T,X) was conducted. Normal tissue from wild-type mice (B) displays a very low proliferative index with only a fraction of cells proliferating (arrows). In the Rbf/TgAPT121 model, as lesions progress to PIN (F) and carcinoma (J), proliferation markedly increases (arrows). The apoptotic index in wild-type normal tissue (C) is very low as well. In Rbf/TgAPT121 PIN (F) and carcinoma (J), apoptosis increases (arrows). As expected, SV40 T-antigen expression is nonexistent in wild-type mice (D). Expression is increased in Rbf/TgAPT121 PIN (H, arrows) and nearly all cells are positive in Rbf/TgAPT121 carcinoma (L, arrows). Similarly, TRAMP PIN displays elevated rates of proliferation (N, arrows) apoptosis (O, arrows) and SV40 T-antigen expression (P, arrows). Proliferative and apoptotic indices, along with SV40 expression are even more elevated in both TRAMP well-differentiated (R,S,T, arrows) and poorly-differentiated (V,W,X, arrows) tumors. Note the presence of SV40 positive epithelial cells in surrounding blood vessels (X, arrowheads).

3.4.10 Neuroendocrine phenotype

To examine whether Rbf/TgAPT121 prostate lesions display a neuroendocrine phenotype, we performed immunostaining on sections representing different stages of cancer in both the Rbf/TgAPT121 and TRAMP mice with an antibody against synaptophysin, a marker of neuroendocrine differentiation. As shown in Figure 3.11, synaptophysin was only detected in the neuroendocrine cells (A and C, arrows), neuron
(B) and nerve bundles (C, arrowhead) within the prostate glands of Rbf/TgAPT121 mice. Consistent with prior reports [121], in intact TRAMP mice, synaptophysin expression is detected in small foci in PIN (E) and well-differentiated carcinoma (G), but markedly increases in poorly differentiated regions (E, H).

3.5 Discussion

The Rbf/TgAPT121 mouse is a genetically modified murine model similar to the TRAMP mouse. Both transgenic models rely on androgen-induced expression of the SV40 T antigen for oncogenic transformation. However, the Rbf/TgAPT121 mouse differs from the TRAMP mouse in that Rbf/TgAPT121 mice express only the 121 N terminal amino acids of the large T antigen. This results in the functional deletion of the Rb family of proteins. TRAMP mice express both full length large and small t antigens. These genetic variations elicit distinct differences in resulting T-antigen directed carcinogenesis. Deletion of pRB family proteins induces prostate intraepithelial neoplasia (PIN) as early as 4 weeks of age and demonstrates progressive prostate carcinogenesis over 30 weeks in the Rbf/TgAPT121 model. Rbf/TgAPT121 mice show increased proliferative index and cell turnover compared to wild type mice as the initial histopathologic phenotype.

PIN is the earliest recognizable form of cancer. PIN is represented by the presence of hyperproliferative epithelial cells confined to the glandular space. These cells appear abnormal in that they stain stronger than normal cells, due to their increased DNA and protein synthetic activity. The increased DNA synthetic activity results in a greater nuclear to cytoplasmic ratio, presence of prominent nucleoli, and increased
heterochromatin, giving these cells an “atypical” appearance. We observed the appearance of PIN at the onset of puberty and high grade PIN at 4 weeks in the Rbf/TgAPT121 mouse. This is in contrast to the Nkx3.1+/- PTEN +/- mouse that develops PIN at 6 months of age [20], the TRAMP model at 12 weeks of age [113], the cPTEN model by 29 weeks of age [114], and the HiMyc model at 2 weeks of age [115]. PIN in the Rbf/TgAPT121 mouse presents in forms ranging from simple flat lesions to complex papillary lesions. Lesions in these models all progress to locally invasive carcinoma. However, the worst lesion seen in the Rbf/TgAPT121 model is a well differentiated or microinvasive adenocarcinoma whereas the worst lesion in TRAMP is an aggressive poorly differentiated adenocarcinoma. Importantly in the Rbf/TgAPT121 model, invasion through the basement membrane is typically only seen after the gland lumen is filled with epithelial cells. TRAMP lesions can become invasive at even at the lowest grade of PIN. This suggests that Rbf/TgAPT121 lesions are mainly proliferative in nature and do not acquire the genetic abnormalities necessary for invasion until later in the time course of carcinogenesis. Additionally, TRAMP lesions metastasize to the liver, lungs and lymph nodes, Nkx3.1+/- PTEN +/- lesions metastasize to lymph nodes and cPTEN lesions metastasize to lymph nodes and lungs. Metastasis is not noted in the Rbf/TgAPT121 model.

Tumors develop as a result of an imbalance between proliferation and apoptosis. Proliferative and apoptotic indices are much higher Rbf/TgAPT121 mouse model compared to normal prostate tissue. The earliest time point that mice were sacrificed in this study was 4 weeks of age, or weaning age. We observed low grade PIN and hyperplasia at this time point. None of the tissues examined were histologically normal.
We speculate that due to the high prevalence of PIN at this early age, we would see hyperplasia and PIN at ages as young as 2 weeks.

Prostate epithelium is comprised of basal cells, secretory luminal cells and a small subset of neuroendocrine cells. Neuroendocrine differentiation is seen in a subset of primary prostate cancers and metastases. Several studies suggest a correlation between increased neuroendocrine differentiation and worse prognosis [122]. However, the significance of neuroendocrine differentiation and its role in human disease progression is unclear and its prognostic significance is questionable [123]. It is well-established that the poorly differentiated tumors in the TRAMP model display features consistent with the neuroendocrine phenotype. In fact, this is a common criticism of the TRAMP model due to the limited evidence addressing the prognostic value of neuroendocrine differentiation. Therefore, TRAMP carcinogenesis is inherently different from human carcinogenesis in this respect. Therefore, the neuroendocrine phenotype is an undesirable phenotypic characteristic in a transgenic mouse model. Neuroendocrine differentiation is not seen in the Nkx3.1+/- PTEN +/- model, the cPTEN model or the HiMyc model. We and others [117] have not observed this phenomenon in the Rbf/TgAPT121 model.

3.6 Conclusions

The Rbf/TgAPT121 mouse is an appropriate model for studying the early stages of prostate carcinogenesis, namely the development of PIN and the transition from PIN to early microinvasive cancer. It is a suitable model for studies focusing upon very early events in prostate carcinogenesis associated with dysregulation of proliferation and
apoptosis. This model may prove useful in chemopreventive studies utilizing dietary agents where the effects on cancer progression are frequently early and subtle.

3.7 Acknowledgements

Our gratitude is extended to Kimberly Carter and Valerie DeGroff for technical support.

Contract Support: These studies were supported by The National Institutes of Health and National Cancer Institute grant R01 CA 101113 and the American Institute for Cancer Research grant (20020852), 05A131 grant to Drs. Clinton and Fleet.
Figure 3.1- Increases in body weight and UGT weight of Rbf mice. A, Body Weight (square label) and UGT weight (circle label) increase in mice between 1 and 12 months of age. B, The ratio of UGT weight to body weight (%) increases through six months of age.
Figure 3.2- Histological representation of the carcinogenic process in anterior prostates of Rbf/TgAPT121 mice. Inactivation of the pRb induces widespread hyperplasia and PIN that progresses to adenocarcinoma with time. A and B, wild type (WT) normal prostate displays a single layer of luminal epithelial cells and a thin stromal cell layer. C and D, the luminal cells of Rbf/TgAPT121 mice display hyperplasia. E, PIN showing focal involvement of the epithelium (black arrows) with nuclear stratification, enlargement, and atypia. F, more advanced PIN. G, microinvasive carcinoma (arrows) and H, well-differentiated adenocarcinoma. Nests of tumor cells extending into the stroma show primitive gland formation (arrows). H&E stain, Scale bars = 50 µm.
Figure 3.3- Histological representation and characterization of PIN in anterior prostates of Rbf/TgAPT_{121} mice. PIN I (A–C), represents a simple flat crowded lesion, containing hyperchromatic, oblong cells (arrows). Note the presence of hyperplastic foci (arrowheads) that contain crowded normal looking cells. PIN II (D–F), is represented by a papillary organization of hyperchromatic PIN cells. Typically, greater than one layer is present. PIN III (G–I), is a more extensive papillary organization of the hyperchromatic, oblong PIN cells. The luminal space is decreased by the extension of these papillary structures. The nuclei lose orientation and cellular disorganization is increased. PIN IV (J–L), typical PIN cells fill and expand the lumen. There is a stromal reaction and the outline of the gland is distorted. However, no invasion is present. H&E stain, Scale bars = 50 µm.
Figure 3.4- Distribution of histological grade over time in the *Rbf/TgAPT121* mouse. *Rbf/TgAPT121* mouse prostates progress to hyperplasia and PIN I at one month. At two months the prostates show lesions of PIN II and PIN III. PIN IV lesions appear at three months. Mice begin to develop well-differentiated adenocarcinoma at 4 months of age. At the age of 6 months or older, all mice developed well-differentiated adenocarcinoma.
Figure 3.5- Histological representation of carcinogenesis in dorsal (A-C), lateral (D-F) and ventral (G-I) lobes in Rbf/TgAP121 mouse prostates. Wild-type, normal gland (A,D,G), note the single layer of round epithelial cells and thin stromal layer; PIN lesions present as cribriform (B), tufting (E) and micropapillary forms (H); carcinoma (C,F,I). H&E stain. Scale bars = 50 µm.
Figure 3.6 - Histological representation of microinvasion in anterior (A and B), dorsal (C and D), lateral (E - G) and ventral (H) lobes in Rbf/TgAPT121 mouse prostates. A and B, microinvasion (arrows) occurring in association with PIN (arrowheads) in AP. C, microinvasion (arrows) occurring in association with PIN (arrowheads) in DP, small nest of atypical cells with hyperchromatic nuclei without evident glandular formation are invading into the stroma. D, microinvasive carcinoma with evident glandular formation (arrows) occurring in association with PIN (arrowheads) in DP. E and F, microinvasion (arrows) occurring in association with PIN (arrowheads) in LP. G and H, microinvasion (arrows) occurring in association with PIN (arrowheads) in VP. H&E stain, Scale bars = 50 µm.
Figure 3.7- Prostatic adenocarcinoma shows variety of histologic lesions. A. Necrosis in the middle of adenocarcinoma; B. Intraductal carcinoma; C. Phyllodes pattern; D. Signet-ring cell differentiation and E. Appearance of large cancer cells with giant nucleoli.
Figure 3.8- Proliferation index in anterior prostates in wild type and *Rbf/TgAPT*<sub>121</sub> mice. Representative Ki-67 immunostaining and quantification (mean ± SEM) in the *Rbf/TgAPT*<sub>121</sub> anterior prostate lesions ranging from normal epithelium, hyperplasia, PIN I, PIN II, PIN III, PIN IV, and adenocarcinoma.
Figure 3.9- Apoptosis in anterior prostates in wild type and Rbf/TgAPT_{121} mice. Representative ApopTag staining and quantification (mean ± SEM) in the Rbf/TgAPT_{121} anterior prostate lesions ranging from normal epithelium, hyperplasia, PIN I, PIN II, PIN III, PIN IV, and adenocarcinoma.
Figure 3.10 - Comparative immunohistochemical analysis of cancer progression in wild-type, Rbf/TgAPT121, and TRAMP mouse anterior prostates. Representative images of the architecture (H&E), proliferation (Ki-67), apoptosis (ApopTag), and transgene expression (SV40 T-antigen) in the normal prostate epithelium, PIN and WD adenocarcinoma lesions in Rbf/TgAPT121 model, and PIN, well differentiated and poorly differentiated lesions in the TRAMP model. Scale bars = 50 µm.
Figure 3.11- Synaptophysin is not present in Rbf/TgAPT121 prostate tumor tissue. Synaptophysin staining in Rbf/TgAPT121 and TRAMP prostate tumor tissues. Synaptophysin is only detected in the neuroendocrine cells (A), neuron (ganglia) (B) and nerve bundle (C) within the prostate glands of Rbf/TgAPT121 mice. In TRAMP mice, synaptophysin expression is detected in small foci in PIN (E, arrow) and well-differentiated carcinoma (G). Synaptophysin expression was robustly elevated in poorly differentiated carcinoma regions (E, arrowheads and C, arrows). Synaptophysin is also positive in some neurons in ganglia (F). Scale bars = 50 µm.
CHAPTER 4

PROSTATE CARCINOGENESIS IN THE TRAMP MODEL IS RESISTANT TO THE TUMOR SUPPRESSIVE EFFECTS OF OMEGA-3 FATTY ACIDS IN THE CONTEXT OF A HIGH-FAT DIET.

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4.1 Abstract

Epidemiological studies indicate that diets rich in omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), are associated with a reduced risk of prostate cancer. It is hypothesized that these fatty acids exert their effects through a suppression of prostaglandin E₂ (PGE₂) and enhancement of prostaglandin E₃ (PGE₃) synthesis, subsequently reducing proinflammatory signals and/or enhancing antiproliferative/proapoptotic signaling. We investigated the effects of high fat diets containing lipids derived from omega-3 rich fish oil compared to corn oil on prostate carcinogenesis in the murine TRAMP model. Male mice (4 week old) were placed on semipurified diets containing 46% of calories from fish oil (FO) or corn oil (CO) for 23 weeks. Prostate carcinogenesis was not altered by lipid source in the context of a high fat diet. However, prostatic fatty acid profiles mimicked dietary fatty acid exposure. Prostate PGE₂ was reduced by the FO diet in wild-type mice, but no changes in PGE₂ were seen in the prostates of TRAMP mice. In parallel, PGE₃ was increased in response to the FO diet as compared to the CO diet in both wild-type, and TRAMP mice. These data indicate that TRAMP mice may acquire additional genetic survival mechanisms that confer resistance to the protective effects of an omega-3 rich- fish oil diet.

4.2 Introduction

Dietary lipids have long been implicated in the development of prostate cancer, yet definitive data regarding a role for specific fatty acid profiles, cholesterol, lipid sources, or total fat have not emerged. Prostate cancer incidences are higher in cultures that adhere to the “Western” dietary pattern, which posseses many pro-carcinogenic
characteristics. Of particular interest are the essential fatty acids which are classified as omega-3 or omega-6 polyunsaturated fatty acids (PUFAs). The western diet contains low levels of omega-3 PUFAs like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and high levels of omega-6 PUFAs like linoleic acid (LA). Both EPA and DHA have been shown to inhibit growth of prostate cancer cells in culture [4] whereas LA promotes growth of cancer cells [67].

Dietary PUFAs impact many cellular pathways associated with cancer [68-70] but considerable interest has been placed on their effects on the cyclooxygenase family (COX-1 and COX-2) and lipoxygenase family (5-LO, 12-LO, 15-LO-1 and 15-LO-2) of enzymes. Importantly, the tumor environment displays an inherent deregulation of these enzymes to favor conditions that promote carcinogenesis [71, 124]. Both enzyme activities and products are subject to a carefully orchestrated regulation by the ratio of omega-3 and omega-6 PUFAs. LA can be metabolized through the lipoxygenase (15-LO-1) pathway to the mitogenic 13-S-Hydroxyoctadecadienoic acid (13-S-HODE) or can be converted to arachidonic acid (AA). AA can then serve as a substrate for COX-2, thus producing prostaglandin E2 (PGE2). PGE2 promotes inflammation, enhances cell proliferation, stimulates cell migration and invasion and induces angiogenesis. EPA and DHA can serve as substrates for COX and LO enzymes as well. EPA is metabolized to prostaglandin E3 (PGE3) and 15-hydroxyeicosapentanoic acid (15-HEPE) through COX-2 and 15-LO-1, respectively. PGE3 and 15-HEPE both possess anti-carcinogenic properties. The ratio of omega-3/omega-6 is important because omega-3 PUFAs can compete with omega-6 PUFAs for COX and LO binding and activity. If the ratio is ideal, the balance will be shifted to production of anticarcinogenic compounds. Additionally,
omega-3 PUFAs can inhibit the metabolism of omega-6 PUFAs [72, 73], further favoring an anticarcinogenic milieu.

Overall, results of in vivo studies are conflicting and epidemiological findings have been largely inconclusive with regards to the omega-3/omega-6 dietary pattern and prostate carcinogenesis [125]. Furthermore, although in vivo studies provide evidence for the role of dietary lipids in prostate cancer, the majority of studies have been conducted in xenograft models [77, 126, 127]. Due to the inconsistent nature of the existing data, we have chosen to investigate the effects of varying lipid source of a high fat diet in the transgenic TRAMP murine model of prostate cancer. Rather than attempt to provide conclusive evidence of the role of fatty acids in prostate carcinogenesis, we asked specifically how this process is impacted by varying fatty acid source in the context of a high fat diet. We show here that TRAMP carcinogenesis is resistant to the effects of omega-3 fatty acids in this context. Contrary to what was expected, dietary fatty acid source had no impact on COX-2 catalyzed prostaglandin production in TRAMP mice. Prostatic fatty acid levels mimic those seen in the diet, suggesting that TRAMP mice acquire genetic defects in PUFA metabolism that render them resistant to the protective effects of omega-3 PUFAs.

4.3 Materials and Methods

4.3.1 Animals

C57BL/6J TRAMP mice were originally obtained as a kind gift from Dr. Norman Greenberg [113] and were maintained as a hemizygote colony. TRAMP [C57xFVB] F1
mice were generated by crossing C57BL/6J TRAMP hemizygotes with wild-type FVB/n mice. All genotyping was performed by PCR on tail DNA. All mice were maintained according to Institutional Laboratory Animal Care and Use Committee (ILACUC) approved protocols at the Science Park-Research Division, University of Texas, MD Anderson Cancer Center.

4.3.2 Diet

Diets were formulated by Research Diets (New Brunswick, NJ) and were based on the AIN-76A semi-purified rodent diet (Table 4.1). 4 week old male TRAMP and wild-type FVB/n mice were randomly distributed into experimental and control groups (n=30 per group). Mice were fed with either corn oil (CO) or fish oil (FO) supplemented AIN-76A diets until the animals were 27 weeks old. The CO and FO diets provided approximately 46% of calories from fish oil and corn oil, respectively. Diet formulations were stored at -20°C and mice were provided with new diet every 3 days to limit potential oxidation of fatty acids. Throughout the experiment the animals had access to drinking water and diets ad libitum. Fresh diets were provided and food intakes were recorded every other day.

4.3.3 Tumor Studies

All transgenic mice were weighed and palpated for tumors weekly starting at 12 weeks of age. Mice were necropsied when palpable tumors reached 2 cm in diameter, or when they had reached 27 weeks of age. Mice that died prior to 30 weeks from causes unrelated to prostate tumor growth were censored from the analyses. At necropsy the
dorsal (DP), lateral (LP), ventral (VP), and anterior (AP) lobes of the prostate were microdissected into individual lobes whenever possible. The techniques for dissection of the prostate lobes were in concordance with the 2001 Bar Harbor Pathology Workshop [120]. Prostate lobes, tumor tissues, lymph nodes, and sections of lung and liver were fixed in 10% neutral buffered formalin and paraffin-embedded for histopathological examination or were flash-frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

4.3.4 Histopathology

The histopathology of embedded tissues from all mice was assessed by scoring hematoxylin and eosin (H&E) stained sections. All sections were coded, blinded and randomized prior to independent analysis. Prostate lobes, including DP, LP, VP, and AP were assessed in accordance with established criteria from the Bar Harbor Prostate Pathology Consortium [120]. Histologic sections were examined and the most advanced lesion identified in each prostatic lobe was classified as normal, prostatic intraepithelial neoplasia (PIN), well-differentiated/microinvasive carcinoma (WD), moderately-differentiated carcinoma (MD), poorly-differentiated carcinoma (PD) or phylloides-like (Phyl). H&E sections of lymph nodes, lungs, and liver were assessed for the incidence of apparent metastatic lesions.

4.3.5 Prostaglandin Extraction and Analysis

All chemicals used in prostaglandin and fatty acid extraction and analysis were purchased from Sigma unless otherwise noted. Frozen tissue (25–50 mg) was ground
using a liquid-nitrogen-cooled mortar (Fisher). Samples were then transferred to sealed microcentrifuge tubes, and three times the volume of ice-cold PBS buffer containing 0.1% BHT and 1 mM EDTA was added. The sample was then homogenized by an Ultrasonic Processor (Misonix, Farmingdale, NJ) at 0 °C for 3 min. A 100-μl aliquot of the homogenate was transferred to a glass tube (13×100 mm) and subjected to extraction of eicosanoids using a modification of the protocol by Kempen et al. [128]. Briefly, 20-μl aliquots of 1 N citric acid and 10 μl of deuterated PGE2 (100 ng/ml) were added to the samples. Eicosanoids were then extracted with 1 ml of hexane:ethyl acetate (1:1, v/v). Samples were centrifuged at 1800×g for 10 min at 4 °C. The upper organic layer was collected, and the organic phases from three extractions were pooled and evaporated to dryness under nitrogen at room temperature. All extraction procedures were performed at minimum light levels under cold conditions (4 °C). Samples were then reconstituted in 100 μl of methanol:ammonium acetate buffer (10 mM at pH 8.5; 70:30, v:v) before LC/MS/MS analysis. The protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA).

Extracted prostaglandins were quantified by LC/MS/MS using a Quattro Ultima tandem mass spectrometer (Waters Corp., Milford, MA) equipped with an Agilent HP 1100 binary pump HPLC inlet (Agilent Technologies, Palo Alto, CA). Prostaglandins were separated using a 2 x 150 mm Luna 3 μ phenyl-hexyl analytical column (Phenomenex, Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate, pH 8.5, and methanol. Column temperature was maintained at 50°C, and samples were kept at 4°C during analysis. Individual analytes were detected using electrospray negative ionization and multiple reaction monitoring of the transitions m/z 351 →271 for PGE2,
m/z 349 \rightarrow 269 \text{ for PGE}_3, \text{ and m/z } 355 \rightarrow 275 \text{ for PGE}_2\text{-d}_4. \text{ Fragmentation of all compounds was performed using argon as the collision gas at a collision cell pressure of } 2.10 \times 10^{-3} \text{ Torr.}

**4.3.6 Fatty Acid Analysis**

Lipids were extracted from dorsal prostate samples using the Folch method\cite{129}. Briefly, samples were homogenized, and extracted twice with a 2:1 (v:v) Chloroform: ice cold methanol/Beta-hydroxy toluene (50µg/mL) solution and potassium chloride(KCl)(0.88%). The samples were then centrifuged for 10 minutes at 1000xg, 4°C. Methanolation reagent (1:4 tetra methyl glycine:methanol, v/v) was added to each sample for preparation of fatty acid methyl esters. KCl (0.88%) and n-hexane were added to each sample and two more extractions were performed by removing the upper aqueous phase, and combined. Samples were evaporated under nitrogen at room temperature. The resulting sample was then reconstituted in n-hexane and fatty acid methyl esters were analyzed by gas chromatography (HP 5890 equipped with FID and 30-m Omegawax capillary column, Supelco Chromatography Products). Fatty acids were identified using authentic standards (Matreya) and quantified by determining areas under identified peaks (ChemStation Software; Packard Instrument Company, Meriden, CT)

**4.3.6 Statistical Analysis**

Data are presented as means \( \pm \) standard deviation (SD). Inter-group comparisons of prostate cancer incidence, histologic grade, fatty acid and prostaglandin levels were made using Student’s t-test. Survival curves were compared using Kaplan-Meier
analysis, and the difference between groups was estimated using the log-ranked test. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA). A p-value of < 0.05 was considered statistically significant. All analyses were performed using GraphPad software (Chicago, IL).

4.4 Results

4.4.1 Impact of dietary lipid composition on TRAMP tumorigenesis.

The effects of dietary lipid composition or prostate tumorigenesis were determined by feeding male TRAMP mice high fat diets containing 46% kcals from corn oil (CO) or fish oil (FO), until the animals were 30 weeks old or until tumors measured 2 cm. There were no significant differences in body weights or food intake between animals consuming FO or CO diets (Figure 4.1). The dietary lipid composition had no significant effect on palpable tumor development in TRAMP mice (Figure 4.2). Accordingly, there was also no significant effect on tumor size or weight (data not shown). Furthermore, dietary lipid source had no effect on prostate histopathology or metastasis (Figure 4.3).

4.4.2 Influence of dietary lipid composition on prostatic prostaglandin production.

To determine the effects of dietary lipid source on endogenous prostaglandin production, PGE$_2$ and PGE$_3$ levels were compared in wild-type versus TRAMP mice using LC/MS/MS analysis (n=6-7). PGE$_2$ production in the prostate of wild-type mice
was higher in those fed the CO diet compared to the FO diet (6.83 vs. 1.28 ng/mg protein, p < .05), but diet elicits no difference in TRAMP mice (1.23 vs. 1.24 ng/mg protein) (Figure 4.4). As shown in Figure 4.5, PGE3 levels were lower in those fed the CO diet as compared to the FO diet in both wild-type (0.27 vs. 0.82 ng/mg protein p < .05), and TRAMP mice (0.10 vs. 0.30 ng/mg protein p < .05).

4.4.3 Dietary influence on prostate fatty acid profile.

To validate fatty acid bioavailability to the prostate and to examine the effect of altering dietary fatty acid composition on prostatic fatty acid profile, we performed fatty acid analysis on TRAMP mouse dorsal prostate tissues (n=3) subjected to CO or FO diets. As shown in Figure 4.6, tissue lipid patterns mimicked lipid composition of the respective diet. Prostates from mice on the CO diet had higher levels of linoleic acid (C18:2n-6) than those on the FO diet. Prostates from the FO group had higher levels of eicosapentenoic acid (C20:5n-3) and docosahexenoic acid (C22:6n-3), whereas mice on the CO diet displayed undetectable amounts of EPA. Arachidonic acid (C20:4n-6) levels from mice on the FO diet were half of those on the CO diet.

4.5 Discussion.

We have shown that dietary lipid source in a high-fat diet does not impact prostate carcinogenesis in the TRAMP model. Neither the presence of palpable tumor or prostate lesion severity was reduced in response to a high fish oil diet. Contrary to what was expected, the high fish oil diet had no effect on the synthesis of mitogenic PGE2 and only elicited a modest increase in the synthesis of the non-mitogenic PGE3. However,
prostatic lipid composition mimicked dietary fatty acid patterns, suggesting that resistance to the effects of fish oil is conferred by a perturbation in lipid signaling downstream of fatty acid incorporation into prostate tissue.

To our knowledge only two other studies have specifically examined the effects of PUFAs in transgenic mouse models of prostate carcinogenesis. In accordance with our results, Vissapragada et al. [78] showed that dietary omega-3 fatty acids had no effect on prostate cancer development in the compound mutant ErbB-2 overexpressing/ Pten +/- mouse. In contrast to what we have observed in the TRAMP model, Berquin et al. [3] found a protective effect of omega-3 fatty acids in the prostate-specific PTEN-knockout model. However, the discrepancies between the results of this study and study that demonstrates the prostate protective effects of omega-3 fatty acids may largely be explained by differences in dietary composition.

One difference between the study we conducted and others is the level of total fat in the diet. The Berquin et al. study diet consisted of 13% fat, which provided 30% of calories from fat. Our diets contained 24.6% fat, which provided 46.1% of calories from fat. It is possible that the potential effects of omega-3 PUFAs were blunted by the high levels of fat used in our study. The study by Berquin et al. differs also in respect to the ratios and amounts of dietary omega-6 and omega-3 PUFAs. They utilized three diets (high omega-3, low omega-3 and high omega-6) whose ratios omega-6:omega-3 PUFAs were 1:1, 20:1, and 40:1 respectively. In contrast, the ratios of our corn oil diet and fish oil diet were 6:1 and 1:1, respectively. The wider range of omega-6:omega-3 ratios in their study may have allowed for more of an experimental effect. Likewise, differences in dietary fatty acid profile, and hence prostatic fatty acid profile, may have contributed
to discrepancies in results. Both of our diets contained higher levels of omega-6 PUFAs than even their high omega-6 diet. Therefore, it is probable that even our fish oil diet was cancer promoting in this respect. Other studies have shown that total fat levels [2, 130], omega-6:omega-3 ratios [77, 127], and total PUFA levels [131] are critical determinants of omega-3 chemopreventive efficacy.

This and previous studies from our laboratory have shown that TRAMP mice acquire additional survival mechanisms that render them resistant to the proposed chemopreventive effects of a diet high in fish oil. We have shown that COX-2, a pivotal PUFA metabolizing enzyme, is decreased in poorly differentiated TRAMP tumors [79]. This is in accordance with human data that show a weak relationship between COX-2 expression with prostate cancer [132-134]. The loss of a prominent omega-3 PUFA target in an aggressive model of murine prostate cancer may therefore nullify the effects.

Our results do not imply that fish oil is not an effective prostate cancer chemopreventive agent. Rather, they suggest that further carefully designed studies in transgenic models be conducted. We have shown that perhaps, the TRAMP mouse is not the ideal model for studying the effects of a fish oil enriched, high fat diet on prostate carcinogenesis. However, we do provide evidence that this type of diet is effective at modulating parameters associated with the carcinogenic process, suggesting some potential benefit to humans. Fish oil may be beneficial in the proper context but further studies must be conducted to define those parameters.
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| Total                          | 812.58| 3902 | 812.55| 3902 |

**Table 4.1-** High fat diet composition.
Figure 4.1- Weekly bodyweights of mice fed CO or FO diets. Mice were fed diets that provided 46% of kcals from either corn oil or fish oil. Diets were based on the standard AIN-76 formula and supplemented with the respective lipid source. Diet has no effect on average bodyweight determined by weekly measurements.
Figure 4.2 – The effect of variable dietary lipid source in a high fat diet on TRAMP prostate tumorigenesis. Figure 2 represents the percentage of TRAMP mice free of a palpable prostate tumor (2 cm diameter) over time. Dietary lipid source has no impact on palpable tumor development in TRAMP mice. Tumor presence was not significant at 21 weeks (p>.20) or 27 weeks (p=1.0) using Fisher’s test.
Figure 4.3- The effect of variable dietary lipid source in a high fat diet on TRAMP histopathologic grade. FO diet did not limit tumor progression in TRAMP mice as determined by the histological grading of the most advanced lesion and location of metastics. WD- well-differentiated adenocarcinoma; PD-poorly-differentiated adenocarcinoma; LN Mets- lymph node metastasis; Other mets- liver, lung metastasis. N-normal, PIN- prostatic intraepithelial neoplasia, and MD- moderately-differentiated adenocarcinoma were not included on the graph because these histological grades were not observed in this analysis.
Figure 4.4 - Prostaglandin E₂ content in the prostate tissue  The fish oil diet resulted in lower levels of proinflammatory, mitogenic PGE₂ in wild-type mice (p<.05) but not in TRAMP mice. Prostaglandin E₂ concentrations were determined using LC/MS/MS. N=6-7. Results are presented as means ± SD. * Indicates a significant effect (p<.05) of dietary treatment. # Indicates a significant effect (p<.05) of genotype.
Figure 4.5 - Prostaglandin E₃ content in the prostate tissue  The fish oil diet resulted in higher levels of the non-mitogenic PGE₃ in both wild type and TRAMP mice (p<.05). Prostaglandin E₃ concentrations were determined using LC/MS/MS. N=6-7. Results are presented as means ± SD. * Indicates a significant effect (p<.05) of dietary treatment. # Indicates a significant effect (p<.05) of genotype.
Figure 4.6 – TRAMP prostatic fatty acid pattern mimics dietary fatty acid pattern. TRAMP Dorsal prostate fatty acid profiles resemble dietary fatty acid profiles. Levels of fatty acid methyl esters of interest are shown as a percentage of quantifiable, identified gas chromatograph peaks. C18:2n6- linoleic acid; C20:4n6- arachidonic acid; C20:5n3- eicosapentaenoic acid; C22:6n3-docosahexaenoic acid. Relative fatty acid patterns were determined using the Folch method of lipid extraction and gas chromatography. N=3. Results are presented as means ± SD. * Indicates a significant difference (p<.05) between dietary treatment for each individual fatty acid.
CHAPTER 5

THE INTERACTIONS OF DIETARY VITAMIN D AND CALCIUM ON PROSTATE CARCINOGENESIS (RBF) AND BONE HEALTH IN MICE.

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5.1 Abstract

Accumulated evidence from laboratory and human investigations supports the hypothesis that “low” vitamin D status (from dietary intake or UV induced synthesis) enhances prostate cancer risk. Although less consistent, several studies suggest that high calcium status is also associated with increased prostate cancer risk. Given that prostate cancer is a disease of aging, it is critical to define vitamin D and calcium levels that will protect against prostate cancer yet will not compromise bone health. In this study, we examined the effects of varying dietary vitamin D and calcium on the carcinogenic process in the transgenic Rbf murine model with a variant of the SV40 large -T antigen driven by the androgen responsive probasin promoter. These mice develop hyperproliferative and dysplastic lesions with focal areas of adenocarcinoma. A 3 x 3 factorial design experiment was completed in mice fed one of 9 diets (n = 30 per diet) varying in dietary vitamin D (25, 150 or 1,000 IU/kg) or calcium (0.2%, 0.5% or 1.5% calcium) from 4 - 28 weeks of age. Food intake and growth were similar in all treatment groups. We have shown that levels of circulating 25(OH)D are more sensitive to dietary vitamin D levels while circulating 1,25(OH)2D is more responsive to dietary calcium levels. Our analysis suggests that increasing dietary vitamin D lowered the risk of prostate adenocarcinoma (incidence of adenocarcinoma: 95%, 86% and 69% for 25, 150 and 1000 IU /kg, respectively) while increasing the percentage of mice with precancerous PIN as the most advanced lesion. Although less striking, increased levels of dietary calcium are associated with a trend towards advanced prostate pathology, particularly in the setting of low dietary vitamin D. However, femur bone analysis showed that
increasing dietary calcium increased bone mineral density while vitamin D demonstrated a very modest impact on the bone.

5.2 Introduction

In 1998, a provocative report from the Health Professional’s Follow-up Study (HPFS) suggested that dietary calcium, from foods or from calcium supplementation, was associated with an increased risk of prostate cancer [5]. In parallel, the HPFS and many other epidemiologic studies also suggested that lower vitamin D status is associated with a greater risk of prostate cancer [80, 83]. Numerous studies performed using cell culture corroborate the epidemiological observation of vitamin D protection against prostate cancer [7]. However, to our knowledge, only two studies have examined the effects of vitamin D on a transgenic model of prostate cancer [95, 97]. The effects of calcium in cell culture and animal models are less well studied. Nevertheless, the controversy regarding calcium and prostate cancer risk, and how vitamin D may impact this relationship, has not been resolved.

This controversy also raises an important public health concern- What levels of vitamin D and calcium are optimal and safe for both prostate and bone health? Given that prostate cancer is a disease of aging and the role of vitamin D and calcium in bone health, it is critical to take both of these parameters into consideration.

We propose that diets rich in calcium, suppress renal production of 1,25(OH)2D, reduce serum and prostate 1,25(OH)2D concentrations, and thereby promote carcinogenesis. We further propose that diets high in vitamin D, which exceed traditional requirements for vitamin D, work independently of the renal production of 1,25(OH)2D and will overcome the cancer-promoting effects of high calcium diets.
To test our hypothesis, we used the Rbf mouse model with prostate epithelial cell specific expression of a SV40 large T antigen modified to specifically inactivate the Rb family of cell cycle control proteins [117]. In contrast to the TRAMP model that progresses to poorly differentiated adenocarcinoma [113], the Rbf mouse is more relevant to the earlier stages of carcinogenesis with the majority of pathology consistent with hyperplasia, intraepithelial neoplasia (PIN) and early localized adenocarcinoma occurring over 30 wks. In addition, given the relationship between vitamin D and calcium status and bone health, it is critical to determine levels of dietary vitamin D and calcium intake that will be chemopreventive against prostate cancer yet will not compromise bone health.

We performed a 3x3 factorial experiment, where diets contained one of 3 levels of vitamin D (25, 150 or 1000IU) and one of three levels of calcium (0.2%, 0.5% and 1.5%) for a total of 9 diets (Figure 1a). At weaning, we assigned 30 male Rbf mice to each of 9 dietary groups and fed the diets for 20 - 24 weeks. Distilled water and diet was provided *ad libitum*. The experiment was completed in 3 replicates, of 10 mice per diet (n = 30 per group). Food intake was monitored twice per week. Mice were sacrificed at 24 - 28 wks of age.

5.3 Materials and Methods

5.3.1 Identification of Rbf transgenic mice.

Transgenic Rbf mice were identified by PCR amplification of a 160-bp fragment using the following primers 5’-GAATCTTTGCAGCTAATGGACC-3’ and 5’-
GCATCCAGAAGCTCCAAAG-3’ and digit-derived genomic DNA as template. Cycling profile was 94 °C for 2 minutes; 35 cycles of 94 °C for 20 seconds, 62 °C for 45 seconds, and 72 °C for 45 seconds; and final incubation of 72 °C for 2 minutes. Rbf/TgAPT121 mouse lines were maintained by crossing to nontransgenic B6D2F1 mice.

5.3.2 Study design and diets.

This study was completed in the ULAR/IACUC approved animal facilities within the OSU College of Medicine. At weaning, male Rbf1 transgenic mice were randomly assigned to shoebox cages and housed in a controlled temperature environment with a 12 hr light/dark cycle in rooms lacking UV light in the wavelength necessary to produce vitamin D in the skin (290 - 320 nm). To assess the effects of vitamin D and calcium on both prostate and bone health, we performed a 3x3 factorial experiment, where diets contained one of 3 levels of vitamin D (25, 150 or 1000IU) and one of three levels of calcium (0.2%, 0.5% and 1.5%) for a total of 9 diets (Table 5.1). Dietary composition is represented in Table 5.2. We assigned 30 male Rbf mice to each of 9 dietary groups and fed the diets for 20 - 24 weeks. Distilled water and diet was provided ad libitum. The experiment was completed in 3 replicates, of 10 mice per diet (n = 30 per group). Food intake was monitored twice per week. Mice were sacrificed at 24 - 28 wks.

5.3.3 Tissue collection.

At sacrifice, the prostate, bladder and seminal vesicles were removed en bloc and fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol.
The tissues were dissected into three parts as recommended by the Bar Harbor Classification of Mouse Prostate Pathology [120]. At trimming, a transverse section through the urethra was made to include both dorsolateral and ventral lobes of the prostate. Subsequently both pieces were embedded in paraffin with cut surfaces down. Seminal vesicle and anterior prostate (coagulating gland) were embedded without trimming and sectioned frontally to examine the entire length of the gland. The tissues were processed and embedded in paraffin using standard techniques. Tissues were sectioned at 4 micron thickness, mounted and stained with H-E by an automated slide stainer (Leica Autostainer XL, Leica Microsystems Nussloch GmbH, Heidelberger, Germany) for histopathologic examination.

Femurs were excised and all tissue was scraped from the bone using a scalpel. Femur length and width at midshaft was measured using calipers. Fixed femora were scanned using a PIXImus densitometer (Lunar, GE-Healthcare, Madison, WI). Measured variables included bone mineral content (BMC, g) and bone mineral density (BMD, g/cm²). Contact radiographs were taken using a digital specimen radiography system (piXarry 100 DSR, Bioptics, Inc., Tucson, AZ). The image was digitalized by Biopix image acquisition software. Peripheral quantitative computed tomography (pQCT) was conducted on femora using a XCT Research SA+ machine with software version 5.40 (Norland Stratec Electronics, Pforzheim, Germany). pQCT was used to measure bone area and volumetric BMD at the mid-femur (cortical bone site) and at the distal femur (30% position of the total length measured from the distal femur as a trabecular bone-rich site). Analysis was performed using the following parameters: Threshold 1: 450, Threshold 2: 750, Peel Mode: 2, Contour Mode: 1.
5.3.4 Vitamin D and metabolite measurement.

Serum levels of vitamin D metabolites (1,25(OH)2D and 25-OH D) were assessed by RIA (IDS Inc., Fountain Hills, AZ). For 1,25D, samples are delipidated and 1,25D extracted from potential cross-reactants by incubation for 3 hours with a specific solid phase monoclonal anti-1,25D. The immunoextraction gel is then washed and purified 1,25D eluted directly into glass assay tubes. Reconstituted eluates and calibrators are incubated overnight with sheep anti-1,25D. 125I 1,25D is added and incubation continued for 1 hrs. Separation of bound from free is achieved by a short incubation with anti-sheep IgG cellulose followed by centrifugation, decantation and counting. 25D is extracted with a solution of sodium hydroxide and acetonitrile. Purification, separation and counting are performed in a similar manner to the method for 1,25D. Bound radioactivity is inversely proportional to the concentration of 1,25D and 25D.

5.3.5 Histopathology-grading and staging.

The histopathology of embedded tissues from all mice was assessed by grading sections stained with hematoxylin and eosin (H&E). Prostatic lobes, including DP, LP, VP, and AP were assessed in accordance with established criteria from the Bar Harbor Prostate Pathology Consortium. Histologic sections were examined and the worst lesion seen in each prostatic lobe was classified as normal, prostatic intraepithelial neoplasia (PIN), well-differentiated/microinvasive carcinoma (WD), moderately-differentiated carcinoma (MD), poorly-differentiated carcinoma (PD) or phylloides-like (Phyl). Lymph
nodes, lungs, and liver were assessed for metastases by scoring H&E sections for the presence or absence of apparent metastatic lesions.

5.3.6 Statistic Analysis.

Data were compared among groups by one way ANOVA, using graphPad Prism 4 Statistical Software (GraphPad Software, Inc. San Diego, CA). Data are presented as means ± SEM unless otherwise noted, and values were considered significantly different from controls at P < 0.05.

5.4 Results.

5.4.1 Impact of calcium and vitamin D on serum vitamin D metabolites.

We first assessed serum levels of vitamin D metabolites by RIA. Serum levels of the storage form of vitamin D, 25(OH)D are significantly increased with greater dietary vitamin D intake (Figure 5.1). At 0.2% dietary calcium levels, vitamin D increased serum 25(OH)D (47.82± 4.29 for 25 IU, 93.11± 8.66 for 150 IU, and 155.55± 11.73 for 1000 IU. All results are in nmol/L). Vitamin D also increased serum 25(OH)D t 0.5% dietary calcium levels (57.79± 5.60 for 25 IU, 83.59± 5.64 for 150 IU, and 168.13± 8.68 for 1000 IU. All results are in nmol/L). Similarly, at 1.5% dietary calcium levels, vitamin D increased serum 25(OH)D (55.49± 7.19 for 25 IU, 85.63± 7.48 for 150 IU, and 134.87± 12.51 for 1000 IU. All results are in nmol/L). Thus, dietary calcium has a minimal effect on circulating 25(OH)D. Conversion of vitamin D to its active form, 1,25(OH)2D, is responsive to the extremes of dietary calcium levels (Figure 5.2). At 25
IU of vitamin D, serum levels of 1,25(OH)2D were decreased by calcium (247.84±24.08, 159.57±48.27, 79.18±31.29 at 0.2%, 0.5% and 1.5% levels of dietary calcium, respectively. All results are in nmol/L) Calcium also decreased serum levels of 1,25(OH)2D at 150 IU of dietary vitamin D (287.31±73.35, 188.65±42.86, 73.73±32.78 at 0.2%, 0.5% and 1.5% levels of dietary calcium, respectively. All results are in nmol/L). Similarly, at 1000 IU of vitamin D, serum levels of 1,25(OH)2D were decreased by calcium (320.45±64.17, 248.21±48.85, 128.98±44.23 at 0.2%, 0.5% and 1.5% levels of dietary calcium, respectively. All results are in nmol/L). Thus calcium directly regulates the biologically active form of vitamin D.

5.4.3 Calcium and vitamin D effects on prostate histopathology.

To assess the affects of Vitamin D and calcium on the development and progression of prostate cancer, we performed H&E analysis on prostate tissue sections. Sections were graded using established criteria. Representative images of Rbf prostate histopathology are shown in Figure 5.3. As shown in Figure 5.4, vitamin D reduced invasive cancer in a dose dependent manner (main effects for 25 IU vitamin D, 150 IU vitamin D and 1000 IU vitamin D on invasive cancer incidence were 95.3%, 86.4% and 68.8% respectively). Calcium tended to worsen prostate pathology; however the effects were less robust (main effects for 0.2% calcium, 0.5% calcium and 1.5% calcium are 79.7%, 82.1% and 88.7%, respectively. Cancer risk was highest in the 25 IU Vitamin D/1.5% calcium group (100.0%). Decreasing calcium in the lowest vitamin D setting (25 IU) had only a modest effect on cancer incidence (for 0.5% calcium and 0.2% calcium, 93.1% and 92.9%, respectively). At the moderate vitamin D level (150 IU) cancer
incidence was and 80.1%, 85.2% and 93.1% at calcium levels of 0.2%, 0.5% and 1.5%, respectively. The lowest cancer risk (65.4%) was seen at the highest vitamin D (1000 IU) and lowest calcium levels (0.2%). However, at this level of vitamin D, increasing calcium to both 0.5% and 1.5% had a minimal effect on cancer incidence (67.9% and 73.1%, respectively).

Increased vitamin D also increased the proportion of mice that demonstrated normal or PIN histology (Figure 5.4), suggesting a delay in the carcinogenic cascade (main effects for 25 IU vitamin D, 150 IU vitamin D and 1000 IU vitamin D on PIN incidence were 4.7%, 13.7% and 31.2% respectively). Again, calcium tended to worsen prostate pathology, but the effects were minimal (main effects for 0.2% calcium, 0.5% calcium and 1.5% calcium on PIN are 20.3%, 17.9% and 11.3%, respectively). PIN incidence was highest in the 1000 IU Vitamin D/0.2% calcium group (34.6%). Increasing calcium in the highest vitamin D setting (1000 IU) had only a modest effect on PIN (for 0.5% calcium and 1.5% calcium, 32.1% and 26.9% incidence respectively). At the moderate vitamin D level (150 IU) PIN incidence was and 19.2%, 14.8% and 6.9% at calcium levels of 0.2%, 0.5% and 1.5%, respectively. The lowest PIN incidence (0%) was seen at the lowest vitamin D (25 IU) and highest calcium levels (1.5%). At 25 IU vitamin D, PIN incidence was 6.9% and 7.1% for 0.5% and 0.2% calcium, respectively.

5.4.4 Calcium and vitamin D effects on bone.

Femurs from Rbf mice were excised and bone mineral density was assessed using a bone mineral densitometer. At 25IU of dietary vitamin D, calcium increased bone mineral density (0.0635± 0.0010 for 0.2% calcium, 0.0674± 0.0009 for 0.5% calcium and 0.0693± 0.0009 for 1.5% calcium). Bone mineral density was also increased by dietary
calcium at 150IU dietary vitamin D (0.0640± 0.0009 for 0.2% calcium, 0.0669± 0.0011 for 0.5% calcium and 0.0692± 0.0011 for 1.5% calcium). Similarly, at 1000IU of dietary vitamin D, calcium increased bone mineral density (0.0655± 0.0009 for 0.2% calcium, 0.0640± 0.0009 for 0.5% calcium and 0.0684± 0.0010 for 1.5% calcium). In contrast to the effects of calcium and vitamin D on prostate health, these results suggest that bone health was much more dependent on calcium.

5.5 Discussion.

This is the first study in an animal model of prostate carcinogenesis to address the hypothesis derived from human epidemiologic studies that high dietary calcium and low vitamin D enhance risk of prostate cancer. We have shown that serum levels of the storage form of vitamin D, 25(OH)D are more responsive to levels of dietary vitamin D than levels of dietary calcium. Conversely, the conversion of 25(OH)D to 1,25(OH)2D of vitamin D is more tightly controlled by dietary calcium than dietary vitamin D. Prostate cancer incidence is reduced by vitamin D. However, calcium has a minimal effect on increasing prostate cancer. This suggests that prostatic conversion of 25(OH)D to 1,25(OH)2D is responsible for the protective effects of vitamin D. This is in contrast to bone mass, which is more responsive to calcium than vitamin D.

Our study supports the hypothesis that high dietary vitamin D has a beneficial effect on the early stages of prostate carcinogenesis in vivo and that high calcium diets may modestly attenuate the anti-carcinogenic effects of 1,25(OH)2D while having a beneficial impact upon bone density. The interactions of dietary vitamin D and calcium on prostate cancer risk and bone health appear to be unique for each “target”, an
observation that will have impact upon efforts to define optimal intake for human populations or individuals.

5.6 Acknowledgements

Our gratitude is extended to Kimberly Carter and Valerie DeGroff for technical support and Dr.s. Jun-Ge Yu and Sanjay Bhave for tissue biosample collection. This study was supported by NCI/NIH R01 CA 101113, PI: Fleet J; Co-PI: Clinton SK American Institute for Cancer Research (20020852), AICR 05A131, PI: Clinton SK
### Table 5.1 - Study Design

Each dietary group contains ~30 mice to examine the effect of diet on androgen-driven prostate carcinogenesis and 10 mice to examine the effect of diet on Ca/Bone homeostasis and normal prostate biology.

<table>
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<tr>
<th>DIETARY LEVEL</th>
<th>25 IU Vitamin D3</th>
<th>150 IU Vitamin D3</th>
<th>1,000 IU Vitamin D3</th>
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<tr>
<td>0.2% Calcium</td>
<td>Diet A 0.2% Ca, 25 IU VD</td>
<td>Diet B 0.2% Ca, 150 IU VD</td>
<td>Diet C 0.2% Ca, 1,000 IU VD</td>
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<td>0.5% Calcium</td>
<td>Diet D 0.5% Ca, 25 IU VD</td>
<td>Diet E 0.5% Ca, 150 IU VD</td>
<td>Diet F 0.5% Ca, 1,000 IU VD</td>
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<tr>
<td>1.5% Calcium</td>
<td>Diet G 1.5% Ca, 25 IU VD</td>
<td>Diet H 1.5% Ca, 150 IU VD</td>
<td>Diet I 1.5% Ca, 1,000 IU VD</td>
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### Table 5.2 Dietary Composition

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<th>1000 IU Vitamin D</th>
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<tr>
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</tr>
<tr>
<td>Total</td>
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</table>

**25 IU Vitamin D**: 0.2 % Ca, 0.2 % P, 0.75 % P
**100 IU Vitamin D**: 0.2 % Ca, 0.2 % P, 0.75 % P
**1000 IU Vitamin D**: 0.2 % Ca, 0.2 % P, 0.75 % P

### Nutrient Values

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<th>1000IU</th>
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</tr>
<tr>
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<tr>
<td>%RDA</td>
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<tr>
<td>P, g/mg</td>
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<tr>
<td>K, g/mg</td>
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Figure 5.1-Vitamin D and calcium interactions on serum concentrations of 25(OH)D (nmol/L) (n=10/group). Serum levels of 25(OH)D are significantly increased with greater dietary vitamin D intake. Dietary calcium has a minimal effect on circulating 25(OH)D (p<.05 for main effect of vitamin D)
Figure 5.2- Vitamin D and calcium interactions on serum concentrations of 1,25(OH)2D (pg/mL) (n=10/group). Conversion of vitamin D to its active form, 1,25(OH)2D, is responsive to the extremes of dietary calcium levels (p<.05 for main effect of calcium).
Figure 5.3- Histological and immunohistochemical representation of the carcinogenic process in the mouse anterior prostate (Rbf). The normal mouse anterior prostate is characterized by a single thin layer of uniform epithelial cells with a low proliferative and apoptotic index. Murine PIN is the earliest histopathological change and characterized by crowding of epithelial cells showing nuclear atypia and increased proliferative and apoptotic rates. The progression to microinvasive cancer in the APT121/Rbf model is characterized by numerous small glands that have invaded the basement membrane and often seen within the matrix. Proliferation and apoptosis are further increased as the carcinogenic process progresses.
Figure 5.4- Vitamin D and calcium interactions on prostate cancer histopathology in anterior prostates of 28 week old Rbf mice (n = 30/group). Vitamin D reduced invasive cancer in a dose dependent manner (main effects for 25 IU vitamin D, 150 IU vitamin D and 1000 IU vitamin D on invasive cancer incidence were 95.3%, 86.4% and 68.8% respectively). Calcium tended to worsen prostate pathology; however the effects were nonsignificant. (p = .001 for main effect of vitamin D on cancer incidence by chi-square analysis). Increased vitamin D also increased the proportion of mice that demonstrated normal or PIN histology (main effects for 25 IU vitamin D, 150 IU vitamin D and 1000 IU vitamin D on PIN incidence were 4.7%, 13.7% and 31.2% respectively). Calcium tended to worsen prostate pathology, but the effects were nonsignificant. (p = 0.0003 for main effect of vitamin D on PIN histology incidence by chi-square analysis)
Figure 5.5- Vitamin D and calcium interactions on bone mineral density of 28 week old Rbf mice (n = 29/group). Calcium increases bone mineral density in a dose-dependent manner.
CHAPTER 6

EPILOGUE

Many epidemiological studies suggest that dietary factors or patterns can modulate prostate carcinogenesis. Additionally, cell culture data supports the protective role of many of these factors. However, no public health recommendations have been made in regards to dietary preventive measures that are effective against prostate cancer. This is due, in part, to paucity in carefully controlled animal studies. Until these studies have been completed, no clinical trials of these dietary measures can be warranted.

The objective of this dissertation was to evaluate the mechanisms whereby several dietary factors will influence prostate carcinogenesis. Furthermore, it provides data to better understand the process, and to define molecular biomarkers that can be used in subsequent human studies examining the relationship between diet and prostate cancer. The first study characterized two popular murine models of prostate cancer with respect to time course of carcinogenesis and biomarker expression. The second study utilized this data to investigate the effects of varying lipid source in the context of a high fat diet on prostate carcinogenesis in the TRAMP model. The results of this study suggest that the TRAMP model is resistant to the prostate cancer preventive effects of fish oil. The final study investigated the effects of vitamin D and calcium on serum vitamin D
metabolites and prostate cancer and bone health in the Rbf transgenic murine model. This study showed that prostate health was more dependent on dietary vitamin D while bone health was more dependent on calcium. Furthermore, vitamin D seems to protect against prostate cancer in a high calcium setting, providing evidence supporting optimal levels of vitamin D and calcium for both bone and prostate health.

These studies provide critical mechanistic data that will foster the transition from preclinical trials to controlled clinical trials. These clinical trials, in turn will potentially provide rationale for public health recommendations for dietary preventive measures against prostate cancer. Currently, no such measures exist. Data from epidemiological studies are suggestive and cell culture studies are supportive. However, there is little animal study data to provide the crucial link. The completed studies from this dissertation attempt to form a link in the cases of bioactive lipids, calcium and vitamin D.

Several questions still remain to be answered. How do other animal models respond to the outlined dietary treatments? Are there better existing animal models of prostate cancer? As stated previously, there are a vast number of animal models that remain to be fully characterized. Which models are best for prevention or treatment? We have attempted to characterize the molecular events associated with carcinogenesis in two animal models. However, many other pathways, such as inflammation, remain to be investigated. How may our dietary treatments affect these parameters? What are the genetic changes associated with our dietary treatments?

These questions provide the rationale for conducting future studies to provide conclusive evidence for dietary recommendations that will protect against prostate cancer.
REFERENCES


