Metabolomic Identification of Hyaluronan as a Target of Dietary Flavonoid Fisetin in Prostate Cancer

By

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In memory of my father

To my mother, brother and sister
With love and eternal appreciation

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TABLE OF CONTENTS

Page

LIST OF FIGURES AND TABLES

Page

ABSTRACT

Metabolomic Identification of Novel Targets of Fisetin in Prostate Cancer

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Prostate cancer (PCa) is the most prevalent disease affecting males in Western countries. With an estimated 180,890 new cases and 26,120 deaths in 2016, PCa remains a formidable challenge. Due to limitations of the known targeted therapies, prevention approaches with small molecules targeting multiple biochemical pathways appears to be the most practical way to decrease the disease incidence and burden. We and others have previously shown that fisetin has therapeutic potential against many cancer types. Here, we explored further the mechanism(s) of action of fisetin in PCa using a global metabolomics approach. HPLC-ESI-MS analysis of tumor xenografts from fisetin treated animals identified several metabolic targets. We focused our studies on hyaluronan (HA) as the metabolite was significantly altered in two different fisetin-treated PCa xenograft models. Elevated HA levels are associated with disease progression in certain cancer types. Biological responses triggered by HA mainly depend on the HA polymer length where HMM-HA represses mitogenic signaling and has anti-inflammatory properties while LMM-HA promotes proliferation and inflammation. Our preliminary observations in various PCa models of tumor progression revealed that increased HA levels correlated with

increase in HAS2 & 3 and HYAL1, 2 & 4 levels. We evaluated the efficacy of dietary flavonoid fisetin against HA in *in-vitro* and *in-vivo* transgenic TRAMP mouse model of PCa progression. SEC-MALS was performed to analyze the molar mass (*M*w) distribution of HA. Fisetin downregulated intracellular and secreted HA levels both *in-vitro* and *in-vivo*. Silencing HAS2 and HAS3 in PCa cells resulted in significant decrease in cell proliferation and invasion suggesting proliferative function of these genes in PCa. Fisetin inhibited HAS and HYALs involved in the turnover of HA, respectively. This was associated with cessation of HA synthesis and repressed the degradation of the available HMM-HA. SEC-MALS analysis of intact HA revealed that PCa cells and transgenic TRAMP tumor tissues have abundance of HMM-HA and decreased levels of LMM-HA upon fisetin treatment. In contrast, *M*w analysis of secreted HA fragment sizes in serum of TRAMP animals and PCa cells media revealed abundance of LMM-HA concomitant with decreased HMM-HA levels. Taken together, our findings establish the efficacy of fisetin as a non-toxic HA synthesis inhibitor which has the potential to be explored further for the management of PCa, using well-designed clinical trials.

CHAPTER 1

Introduction

Dietary Polyphenols in Prevention and Treatment of Prostate Cancer

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Abstract

PCa is the most prevalent disease affecting males in many Western countries, with an estimated 29,480 deaths in 2014 in the US alone. Incidence rates for PCa deaths have been decreasing since the early 1990s in men of all races/ethnicities, though they remain about 60% higher in African Americans than in any other group. The relationship between dietary polyphenols and the prevention of PCa has been examined previously. Although results are sometimes inconsistent and variable, there is a general agreement that polyphenols hold great promise for the future management of PCa. Various dietary components, including polyphenols, have been shown to possess anti-cancer properties. Generally considered as non-toxic, dietary polyphenols act as key modulators of signaling pathways and are therefore considered ideal chemopreventive agents. Besides possessing various anti-tumor properties, dietary polyphenols also contribute to epigenetic changes associated with the fate of cancer cells and have emerged as potential drugs for therapeutic intervention. Polyphenols have also been shown to affect post-translational modifications and microRNA expressions. This article provides a systematic review of the health benefits of selected dietary polyphenols in PCa, especially focusing on the subclasses of polyphenols, which have a great effect on disease prevention and treatment.

Introduction

PCa is the most prevalent cancer in the male population in Western countries. Based on recent evidence, it is the second leading cause of cancer-related death among men in the US [1]. Age, family history, genetic factors, lifestyle, environmental influences, and diet are some of the most important risk factors associated with PCa. Rising incidence rates of PCa have been observed over the last few decades, largely due to screening and early detection procedures [2]. Recently, diet-derived polyphenols have received tremendous attention among nutritionists, food scientists, and consumers for their health-promoting effects, including their use in the chemoprevention of PCa [3]. The health effects of dietary polyphenols depend directly on the amount consumed and on their bioavailability, which is influenced by chemical structure (polymerization, esterification, acetylation, methylation, and esterification), food matrix, and excretion back into the intestinal lumen. Furthermore, neither the absorption efficacies of all polyphenols are the same nor are their effects on the various signaling pathways that they modulate. The absorption efficacy is dependent on the hepatic enzymes and the composition of the intestinal microflora within the human body [4, 5, 6]. Polyphenols have also been reported to modulate key proteins in the signaling cascades related to differentiation, proliferations, metastasis and apoptosis [7, 8].

Dietary polyphenols are naturally occurring food compounds found in fruits, vegetables, cereals and beverages. To date, more than 8000 compounds have been identified in the human diet based on their chemical structures [9]. These molecules are identified as the secondary metabolites of plants that contain one or more hydroxyl (–OH) groups attached to -ortho, -meta or -para positions on a benzene ring. These metabolites are generally involved in defense against ultraviolet radiation, the effects of various environmental pollutants, and hostility from pathogens [10]. The long-term consumption of a polyphenol-rich diet has shown promise against

cardiovascular diseases (CVDs), neurodegenerative diseases, diabetes, cancer, and many others in epidemiological studies as shown in **Figure 1.1**. This review focuses on the current understanding of the biological effects of selected dietary polyphenols, which are being reported as instrumental in their effect on treatment and prevention of PCa.

The following key words were used in the initial search strategy using PubMed: polyphenols, diet, natural products, PCa, tumor and chemoprevention; the search was augmented by a profound exploration of polyphenols involved in the treatment and prevention of PCa.

General Structure and Classes of Dietary Polyphenols

Polyphenols are polyhydroxylated phytochemicals and share common chemical structures such as conjugated closed rings and hydroxyl groups [11]. Most abundant polyphenols found in diets may be classified into various groups as a function of their chemical structure and orientation of the number of phenol rings bound to one another. They are subdivided into four main subclasses: phenolic acids, stilbenes, curcuminoids and flavonoids, of which phenolic acids and flavonoids account for 30% and 60% respectively [12, 13]. The different subclasses and general chemical structures of the polyphenols are illustrated in **Figure 1.2**.

Phenolic acids are further categorized into hydroxy-benzoic and hydroxy-cinnamic acids. Phenolic acids account for about a third of the polyphenolic compounds in our diet and are found in all plant material, but are particularly abundant in acidic-tasting fruits. Caffeic acid, gallic acid and ferulic acid are some common phenolic acids. Flavonoids are the most abundant polyphenols in human diet and share a common basic structure consisting of two aromatic rings, which are bound together by three carbon atoms that form an oxygenated heterocycle. Biogenetically, one ring usually arises from a molecule of resorcinol, and the other ring is derived from the shikimate

pathway [12, 13]. Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. Most stilbenes in plants act as anti-fungal phytoalexins, compounds that are synthesized only in response to infection or injury. The most extensively studied stilbene is resveratrol, which we have discussed below.

Curcumin and various analogs of curcumin contain the linear diarylheptanoid curcuminoid. These compounds are natural phenols and produce a pronounced yellow color. The different chemical groups increase the solubility of curcuminoids, making them more suitable for drug formulation. The known cellular, molecular, and biochemical actions of dietary polyphenols have been summarized in **Figure 1. 3**.

Dietary Polyphenols in PCa

This section provides an overview of selected dietary polyphenols (based on their subclasses) which have been used in studies directed towards PCa prevention and treatment.

1. Phenolic Acids

Phenolic acids are composed of hydroxy-cinnamic and hydroxy-benzoic acids and account for 30% of dietary polyphenols [7]. They are ubiquitous to plant material and sometimes present as esters and glycosides. They have anti-oxidant activity as chelators and free radical scavengers with special impact over hydroxyl (–OH) and peroxyl radicals, superoxide anions, and peroxynitrites. Gallic acid, one of the most studied and promising compounds in PCa research, belongs to the hydroxy-benzoic group. Gallic acid is the precursor of many plant-derived tannins, while cinnamic acid is the precursor of hydroxy-cinnamic acids [14,15].

1.1. *Anacardic Acid*

Anacardic acid (AA; 6-pentadecylsalicylic acid) is the active phenolic lipid found in the *Amphipterygium adstringens* plant. It possesses anti-inflammatory, anti-cancer, anti-oxidative and anti-microbial functions. The bark of this plant is widely used in traditional medicines for treatment of gastric ulcers, gastritis and stomach cancers [16]. In PCa, AA is reported as a natural inhibitor of non-specific histone acetyltransferase and has been shown to inhibit prostate tumor angiogenesis by targeting the proto-oncogene tyrosine-protein kinase (Src)/focal adhesion kinase (FAK)/rhodopsin (Rho) guanosine triphosphate (GTP)ase signaling pathway [17]. AA affects multiple steps of tumor angiogenesis including endothelial cell viability, migration, adhesion, and differentiation both *in-vitro* and *in-vivo*. The AA-mediated effect and mechanism on PCa cells is based on its ability to inhibit cell proliferation and induce G1/S cell cycle arrest and apoptosis. AA inhibits androgen receptors (AR), activates tumor suppressor protein p53 and cyclin-dependent kinase (CDK) inhibitor-1/p21, and regulates the transcription of other related target genes [18].

1.2. *Caffeic Acid*

Caffeic acid (CA; 3,4-dihydroxycinnamic) is one of the hydroxy-cinnamate metabolites universally present in plant tissues. CA is found in many food sources, including coffee drinks, blueberries, apples and cider. Besides acting as a cancer inhibitor [19,20], it also possesses anti-oxidant and anti-bacterial activities *in-vitro* and can contribute to the prevention of atherosclerosis and other CVDs [21]. CA has been reported to inhibit AR signaling and subsequent inhibition of cell proliferation of human androgen-dependent PCa cells.

Some derivatives of CA have also shown potent cytotoxic and anti-proliferative effects and dihydrotestosterone (DHT)-stimulated prostate specific antigen (PSA) secretion [22]. CA-

phenyl ester (CAPE) enhances anti-proliferative and cytotoxic effects of docetaxel (DOC) and paclitaxel (PTX) in PCa cells attributed to CAPE augmentation of DOC and PTX proapoptotic effects in addition to CAPE-induced alterations in estrogen receptors (ER)-α and ER-β abundance [23,24]. CAPE significantly reduced protein kinase-B/Akt, extracellular signal-regulated kinases (ERK), and ER-α phosphorylation. CAPE-mediated inhibition of Akt phosphorylation was more prominent in cells expressing ER-α such as PC3 compared to LNCaP. CAPE suppressed the proliferation of LNCaP, DU145, and PC3 human PCa cells in a dose-dependent manner.

Overexpression of Akt1 and c-Myc significantly blocked the antiproliferative effects of CAPE. CAPE administration may be useful as an adjuvant therapy for cancers that are driven by the p70S6K and Akt signaling networks [25]. CAPE, a known inhibitor of NFκB can inhibit interleukin (IL)-6 secretion induced by tumor necrosis factor (TNF)-alpha, thereby suppressing signal transducers and activators of transcription (STAT)-3 translocation [26]. CAPE treatment suppressed proliferation, colony formation, and cell cycle progression in PC3 cells. CAPE decreased protein expression of cyclin D1, cyclin E, SKP2, c-Myc, Akt1, Akt2, Akt3, total Akt, mammalian target of rapamycin (mTOR), B-cell lymphoma (Bcl)-2, retinoblastoma protein (Rb), as well as phosphorylation of Rb, ERK1/2, Akt, mTOR, glycogen synthase kinase (GSK)3α, GSK3β, and PDK1, but increased protein expression of KLF6 and p21Cip1 in PC3 cells [27]. Taken together, evidence shows that CA has multiple protective effects, which can be further explored and developed towards PCa chemoprevention.

1.3. *Ellagic Acid*

Ellagic acid (EA; 4, 4', 5, 5', 6, 6'-Hexahydroxydiphenic acid) is a polyphenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries, and blackberries. It has anti-carcinogenic, anti-oxidant and anti-fibrosis properties. It is responsible for more than 50% of the anti-oxidant activity of pomegranate juice and for the beneficial effects of EA in PCa [28, 29, 30, 31]. EA treatment of LNCaP cells induced a significant decrease in heme oxygenase (HO)-1 and -2, cytochrome P450 (CYP) 2J2 expression, and vascular endothelial growth factor (VEGF) and osteoprotegrin (OPG) levels. Similarly, CYP4F2 and CYPA22 were significantly downregulated by EA treatment, suggesting that EA interfered with multiple biological processes involved in angiogenesis and metastasis in PCa cells [32].

Recently, apoptotic pathways involved in EA-mediated chemoprevention were reported. Apoptosis was induced by downregulation of anti-apoptotic proteins, SIRT1, HuR, and HO-1. EA modulated apoptosis inducing factor (AIF), resulting in an increase in ROS levels and caspase (CASP)-3, while reducing transforming tumor growth factor (TGF)-β and IL-6 [33]. EA reduced proliferation by inhibiting mTOR and decreasing levels of β-catenin. EA slightly decreased matrix metalloproteinase (MMP)-2 but had no effect on MMP-9 in PC3 cells. Non-toxic concentration of EA was shown to inhibit invasion and motility of PCa cells through its action on protease activity [34]. Treatments with EA induced differentiation by causing significant reduction in chromogranin-A, p-Rb, DNMT-1, and p-Akt levels, along with increased p75 neurotrophin receptor expression. EA also induced DNA damage in PCa cells in a dose-dependent manner [35]. Pomegranate juice (PJ) containing EA, along with other components, has been shown to inhibit PCa metastasis.

Two initial exploratory clinical studies investigating proprietary pomegranate products reported a trend of effectiveness in increasing PSA doubling time in patients with PCa [36, 37]; however, another clinical study did not support these results [38]. Recently, a group evaluated the PJ blends to investigate the contrasting clinical evidence between these two studies. Their results showed that daily doses of PJ in the latter study contained very little concentrations of gallic acid and punicalagin compared to the concentrations found in the earlier two studies. The authors confirmed that not just pomegranate but the amount of co-active compounds in the PJ blend along with EA was responsible for its clinical effectiveness [39].

1.4. *Gallic Acid*

Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) is ubiquitously present either in free form or, more commonly, as a constituent of tannins, namely gallotannins [40]. Some of the natural products found in nature that are rich in GA are strawberries, pineapples, bananas, lemons, red and white wines, gallnuts, sumac, witch hazel, tea leaves, oak bark and apple peels [41]. Biologically, GA possesses anti-bacterial, anti-viral, anti-inflammatory, and anti-oxidant properties [41, 42, 43, 44]; anti-melanogenic activity is also present via the inhibition of tyrosinase activity [45]. Anti-cancer activity of GA has been reported in leukemia, oral tumor and esophageal cancer cells [46, 47]. GA inhibited cell viability in DU145 and 22Rv1 PCa cells in a dose-dependent manner via induction of apoptosis [48].

Regarding GA's ability against PCa, studies have shown both anti-cancer and cancer chemopreventive effects in human PCa DU145 cells *in- vitro* and the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, respectively [49,50]. GA inhibited the tumor growth in DU145 and 22Rv1 PCa xenografts in nude mice and decreased microvessel density, as compared

to controls in both models [51]. Penta-O-galloyl-beta-d-glucose (5GG), which consists of a glucose molecule on which five –OH groups are esterified with GA, has been shown to suppress tumor growth via inhibition of angiogenesis [52] and STAT-3 activity in PCa cells [53]. 5GG arrested cells at the G1 phase, induced apoptosis, inhibited lipopolysaccharide-induced NFκB activation, fatty acid synthase (FAS) expression and suppressed cell invasion by reducing MMP-9 expression [54].

Mechanistic studies of 5GG-mediated regulation of MMP-9 showed activation of EGF-induced c-jun N-terminal kinase and subsequent suppression of NFκB nuclear translocation. 5GG also reduced epidermal growth factor receptor (EGFR) expression through the proteasome pathway and suppressed invasion and tumorigenesis in nude mice implanted with PC3 cells [55]. 5GG's role as a novel inhibitor of DNA polymerases was studied and the results showed that 5GG induced PCa S-phase arrest through DNA replicative blockage and induced G1 arrest via cyclin D1 downregulation [56]. Another analog of GA, theaflavin-3-3'-digallate (TF3), and 5GG together showed inhibition of rat liver microsomal 5alpha-reductase activity, which catalyzes the conversion of testosterone to a more active androgen, DHT which then subsequently binds to AR and functions inside the nucleus to regulate specific gene expression. Furthermore, TF3 and 5GG reduced androgen-responsive LNCaP cell growth, inhibited expression of AR, and lowered androgen-induced PSA and FAS protein levels.

2. Stilbenes

Stilbenes or stilbenoids are a well-known class of naturally occurring polyphenols. Stilbenes are chemically characterized by their core structure of 1,2-diphenylethylene. Most stilbenes are stress metabolites produced in plants and act as anti-fungal phytoalexins, compounds that are

only synthesized in response to an infection or injury. These plant defense compounds have tremendous potential in biological and cellular processes applicable to human health [57]. Stilbenes are reported to be potentially important cancer chemoprotective agents, being able to inhibit cellular events associated with carcinogenesis, including tumor initiation, promotion, and progression [58].

2.1. *Piceatannol*

Piceatannol (PT; trans-3, 4, 3', 5'-tetrahydroxystilbene) is a naturally occurring polyphenol present in rhubarb, berries, peanuts, sugar cane, wine and grape skins. PT, a metabolite biotransformed from resveratrol (RSV), has been demonstrated to exert anti-inflammatory, anti-carcinogenic and cardioprotective effects [59]. In silico and biochemical analyses have identified quinone reducatase 2 (QR2) as a target of PT. PT-mediated inhibition of cell proliferation and induction of apoptosis was comparable to RSV. PT interacted with QR2 at the same site as RSV, forming an H-bond with asparagine-161. The anti-cancer effect of PT observed in PCa cells was shown to be QR2-dependent, as PT-mediated inhibition of proliferation and QR2 activity were much lower in QR2-knockdown cells relative to QR2 expressing cells. The study suggested PCa prevention by RSV to be partially attributed to its conversion to PT [60].

PT inhibits the migration/invasion of DU145 PCa cells possibly mediated by decrease in IL-6/STAT-3 signaling [61]. PT delayed G1 cell cycle progression of DU145 cells via the inhibition of CDK2 and CDK4 [62]. PT was found to induce apoptosis in DU145 human PCa cells via activation of extrinsic death receptors and intrinsic mitochondrial-dependent pathways [63]. Recently a study showed that inhibition of MMP-9 by PT decreased the invasive potential of DU145 cells. PT inhibits TNF-α-induced invasion by suppression of MMP-9 activation via

Akt-mediated NFκB pathways in DU145 PCa cells [64]. Another study showed in vivo evidence that PT, when administered orally, inhibits tumor formation, growth, and diminished cell colonization in LNCaP PCa xenografts [65]. PT has been shown to suppress the activation of some transcription factors including NFκB, which plays a central role as a transcriptional regulator in response to cellular stress caused by free radicals, ultraviolet radiation, cytokines, or microbial antigens.

PT inhibits Janus kinase-1 (Jnk-1), a key member of the STAT pathway crucial in controlling cellular activities in response to extracellular cytokines, and is involved in inflammation and carcinogenesis. The anti-tumor, anti-oxidant, anti-inflammatory, and pharmacological properties of PT suggest that PT might be a potential biomolecule for PCa prevention; however, more data are needed on its bioavailability and toxicity in humans [66].

2.2. *Pterostilbene*

Pterostilbene (PTER; trans-3,5-dimethoxy-4-hydroxystilbene), an anti-oxidant found mainly in berries and grapes, has gained much attention due to its chemopreventive and potential therapeutic effects reported in a variety of cancer types [67]. PTER-isothiocyanate, a conjugate of PTER inhibits the AR-regulated pathways in PCa cells. The conjugate significantly repressed cell proliferation, induced apoptosis by modulating phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/ERK pathways, arrested cell cycles, abrogated DHT induced activation, and down regulated AR expression in LNCaP cells [68]. PTER treatment inhibited cell proliferation in a dose-dependent manner in p53 wild type LNCaP and p53 null PC3 cells. PTER activated adenosine monophosphate-activated protein kinase (AMPK) in both p53 positive and negative human PCa cells, resulting in a decrease in activity and expression of

lipogenic enzymes FASN and acetyl-CoA carboxylase (ACC). PTER increased the expression level of p53 and subsequently enhanced the expression level of p21, resulting in cell-cycle arrest in LNCaP cells. It is proposed that induction of p21 promoted growth arrest and exerted a protective affect after AMPK activation [69].

PTER induced apoptosis, cell cycle, and PSA in the human androgen-responsive LNCaP cells [70]. The effects of PTER against PCa were also studied in highly metastatic androgen-independent LNCaP cells and showed that PTER is an effective inducer of apoptosis based on flow cytometry and microscopic analysis of cell surface morphology. The authors investigated PTER's effect upon three specific markers of mitochondrial apoptosis—Bcl-2, BAX and CASP-3—and found that pterostilbene decreased Bcl-2 expression by 2- to 2.5-fold and increased expression of BAX and CASP-3 by 2- and 3-fold, respectively [70]. This study reported PTER inhibits cell viability in LNCaP cells and causes cell cycle arrest at the G1/S-phase after 72 h of treatment. Furthermore, the anti-carcinogenic effects of PTER were seen upon two CDK inhibitors, CDNK1A and CDNK1B, which are essential to G1/S-phase regulation. PTER was found to up-regulate both CDNK1A and CDNK1B at a concentration of 25 μM in LNCaP cells. PTER treatment inhibited elevated PSA mRNA expression in LNCaP cells with a minimal concentration of 1 μM [70]. Further, PTER treatment inhibited elevated PSA levels that were hormonally induced groups, DHT and 17β-estradiol. PTER decreased Akt activation, MMP expression, and further contributed to anti-carcinogenesis. Akt and MMP are both associated with cancer cell proliferation and metastasis and down-regulated expression of the cancer marker α-methylacyl-CoA racemase.

A recent study demonstrated that dietary stilbenes are effective regulators of metastasis-associated protein (MTA1)/nucleosome, remodeling deacetylase-mediated p53 acetylation,

apoptosis, and angiogenesis in PCa xenografts [71]. MTA1 has the additional advantage of being sensitive to pharmacologically safe dietary compounds. On the basis of strong *in-vitro* and in vivo evidence, the authors proposed PTER to be explored as a lead compound for potent target-specific treatment of MTA1-overexpressing advanced PCa. PTER increased glutathione (GSH) peroxidase, GSH reductase and total GSH by 1.4-, 1.6-, and 2.1-fold, respectively. Furthermore, PTER increased levels of ROS by 5-fold and nitric oxide production by 6-fold. These findings indicated that PTER modified the anti-oxidant profile of PCa cells, leading to a cellular environment that is conducive to apoptosis [72]. Based on these cumulative findings, PTER possesses potent effects in both hormonal-responsive and hormonal-independent PCa *in-vitro* and *in-vivo*, suggesting its chemotherapeutic implications in PCa.

2.3. *Resveratrol*

Resveratrol (RSV; 3, 4', 5-trihydroxystilbene), one of the best studied stilbenes, is found largely in grapes, blueberries, peanuts, pistachios and hops. A product of grapes, red wine also contains significant amounts of RSV [10, 73]. RSV exists both in cis- and trans-stereoisomeric forms of which the trans-isomer is biologically active [74, 75]. RSV induces a broad range of effects on cell phenotypes. Ample evidence on RSV indicates inhibition of cancer cell growth, induction of cell cycle arrest, and apoptosis in various PCa cell lines [76, 77, 78]. RSV is known to induce differentiation in certain cell types [79, 80, 81].

COX-2 catalyzes the conversion of free arachidonic acid to prostaglandins, which can stimulate cell proliferation, promote angiogenesis, and suppress apoptosis, all of which promote malignancy [82, 83, 84]. RSV expresses anti-inflammatory activity by directly inhibiting COX-2 activity and suppressing NFκB by up-regulating MAPK-phosphatase-5 [85, 86]. RSV has also

been reported to reduce expression of MMPs, which are responsible for tumor invasion and metastasis and also decreases the levels of VEGF, resulting in angiogenesis inhibition [87, 88, 89, 90, 91]. RSV has the ability to increase sensitivity of PCa cells to ionizing radiation, which has potential, in combination with radiotherapy, for clinical applications [92, 93, 94, 95, 96, 97]. Another recent report suggests Zn, in combination with RSV, as a novel approach for PCa management. Zn is abundantly available in healthy prostates, but with PCa progression, it reduces significantly [98]. RSV, in combination with Zn, was reported to increase the total cellular Zn and intracellular free labile Zn in normal human prostate epithelial cells [99]. In addition, an increase of Zn levels in plasma was reported in healthy adult rats administered with RSV. These studies suggest that RSV may influence Zn homeostasis, possibly via enhancing intracellular Zn accumulation [100]. The anti-cancer potential of RSV has been summarized in many *in-vitro* and *in-vivo* studies previously published [101]. RSV is well tolerated, but an optimal dose has not yet been determined. Another study recently confirmed that even though RSV has shown anti-cancer potential in various experimental studies reported to date, there is so far no concrete evidence to support the use of the compound for PCa treatments outside of clinical trials. The main reason for this caveat is that there is not enough clinical evidence to justify a recommendation for the prophylactic administration of RSV [102].

3. Curcuminoids

Curcuminoids, curcumin, and their structurally related compounds are comprised of phenolic yellowish crystalline powder and are used to provide flavor and color to spice blends. Nutraceuticals (foods with medicinal potential) are prepared and consumed all across the world and are active in the prevention and treatment of various diseases including PCa [103]. Curcuminoids found in turmeric contain three principal components—curcumin,

demethoxycurcumin and bisdemethoxycurcumin—of which curcumin is the most abundant and potent [104,105,106,107].

3.1. *Curcumin*

Curcumin and its derivatives have been reported to possess anti-inflammatory, anti-oxidative and anti-carcinogenic properties [108]. Curcumin was shown to inhibit proliferation in both androgen-dependent and androgen-independent PCa cell lines [109]. Curcumin inhibited several cell signaling pathways including NFκB, TNFR pathways. Curcumin and its derivatives demonstrated anti-cancer properties by inhibiting enzymes like COX-2, MMPs, mTOR, protein kinase C, and EGFR [110,111,112,113,114]. Curcumin inhibits PCa cell viability and induces cell apoptosis. The authors report that curcumin downregulates the expression of the inhibitor of DNA binding (Id)-1 mRNA and protein in PC3 cells, a key signaling molecule in PCa carcinogenesis and metastatic progression [115]. Curcumin was shown to inhibit proliferation and migration of human PCa cells.

Curcumin significantly suppressed phosphorylation of ERK1/2 and VEGF expression modulating the osteopontin/integrin-αvβ3 signaling pathway. It also caused MMP-9 activation associated with angiogenesis via regulation of secretion of VEGF and angiostatin in PC3 cells [116]. Curcumin analogues have been reported to be more effective in inhibiting human PCa cells and to retard the growth of human PC3 xenografts in immuno-compromised mice, as compared to curcumin alone [117,118]. Curcumin as a modulator of ER activity is an effective agent and has demonstrated protection against PCa invasion and metastasis [119]. Several *in-vitro* and *in-vivo* studies have provided evidence regarding the efficacy of curcumin against PCa; however, further studies directed towards the development of curcumin analogues/nanoparticles

are needed, through which bioavailability of curcumin may be enhanced for prevention or reducing the development of PCa [120,121].

3.2. *Demethoxycurcumin and Bisdemethoxycurcumin*

Demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), analogs of curcumin, have been reported to modulate inflammatory signaling and cell proliferation to the same extent as curcumin. The relative potency for suppression of TNF-induced NFκB activation reported is curcumin > DMC > BDMC, suggesting the critical role of methoxy groups on the phenyl ring of curcumin. DMC and BDMC induced GSH to a similar extent as curcumin. Production of GSH correlates with suppression of NFκB activation and induction of cell proliferation through a ROS independent mechanism [122]. DMC has been reported to show the most efficient cytotoxic effects on PC3 cells. DMC activates AMPK and decreases activity of lipogenic enzymes FASN and ACC. DMC downregulates heat-shock protein (HSP)-70 and increases the activity of CASP-3. In addition, DMC treatment activates AMPK in PCa cells, which, in turn, regulated the HSP70/EGFR pathways. These findings demonstrate that AMPK pathways have a significant influence on DMC-mediated inhibition of tumor viability [123]. DMC inhibits migration of PC3 cells in both a dose- and time-dependent manner. DMC has also been reported to prevent against proliferation and apoptosis of PCa cells via CASP-3 routes. The activity of MMP-2 is suppressed, suggesting correlation between migration and invasion of PCa cells [124].

4. Flavonoids

Flavonoids comprise over 4000 varieties and account for about 60% of structurally-related dietary polyphenols, which are widely present in plants and ingested in varying degrees in the diet. Their chemical structure contains 2-benzene rings linked to three carbon atoms that form an

oxygenated heterocycle [125]. Flavonoids are classified into flavonols, flavones, isoflavones, anthocyanidins, chalcones, and dihydrochalcones. The flavonols themselves are subdivided into cathechins, proanthocyanadins, theaflavins, and thearubigins [126]. Several beneficial properties have been attributed to these dietary compounds, including anti-oxidant, anti-inflammatory, and anti-carcinogenic effects. Flavonoids have shown potential to protect against viral infections, as well as several diseases such as diabetes, CVDs, inflammatory and neurological diseases [127,128].

4.1. *Apigenin*

Apigenin (APG; 4',5,7,-trihydroxyflavone) is a naturally occurring plant flavone abundantly present in common fruits and vegetables such as grapefruits, plant-derived beverages, parsley, onions, chamomile, oranges, tea and wheat sprouts. The most common source of APG consumed as a single ingredient in herbal tea is chamomile, prepared from the dried flowers of *Matricaria chamomilla* [129,130]. Recently, APG has been increasingly recognized as a cancer chemopreventive agent. Numerous studies have explored the possible cancer chemopreventive effect of APG based on its potent anti-oxidant, anti-mutagenic, anti-inflammatory, anti-viral and purgative effects [131]. The promising role of APG was summarized in various cancers including PCa [132].

In-vitro and *in-vivo* studies indicate that APG mediated growth inhibitory responses are due to the inhibition of histone deacetylases (HDACs), specifically HDAC1 and HDAC3. This effect was observed both at the protein level as well as localized hyperacetylation of histone H3 on the p21 promoter, a condition that manifests HDAC-mediated therapeutic resistance [133]. APG induced up-regulation of p21, followed by subsequent inhibition of polo-like kinase (PLK)-

1 transcription, resulting in apoptosis of PCa cells [134]. APG acts as an inhibitor of adenine nucleotide translocator (ANT)-2, an ADP/ATP translocator which up-regulates death receptors (DR)-5 at the post-transcriptional level and sensitizes malignant PCa tumor cells to apoptosis-inducing ligands (Apo2L)/TNF-related apoptosis-inducing ligands (TRAIL), whereas ANT-2 silencing leads to the enhancement of Apo2L/TRAIL mediated apoptosis [135]. APG treatment of androgen-refractory human PCa PC3 and DU145 cells resulted in dose-dependent reduction of X-linked inhibitor of apoptosis protein (X-IAP), c-IAP1, c-IAP2, and survivin protein levels. APG resulted in decreased cell viability and induction of apoptosis accompanied by decreases in Bcl-xL, Bcl-2 and increases in the active form of BAX proteins. APG resulted in inhibition of class 1 HDACs and HDAC1 protein expression, thereby increasing the acetylation of Ku70 and the dissociation of BAX resulting in apoptosis of PCa cells [136].

APG effectively suppresses PCa progression in spontaneous TRAMP mice by attenuating insulin-like growth factor (IGF)-1/IGF binding protein-3 signaling associated with inhibition of p-Akt and p-ERK1/2, resulting in inhibition of invasion and progression of PCa. APG showed marked inhibition of VEGF, urokinase-type plasminogen activator, MMP-2, and MMP-9, which coincided with tumor growth inhibition and complete absence of metastasis in TRAMP mice [137]. A study was performed to investigate inhibitory effect of APG on TGF-β-induced VEGF production. The authors reported that APG inhibited VEGF along with TGF-β1-induced phosphorylation via mothers against decapentaplegic homolog (Smad)-2/3 and Src/FAK/Akt pathways, providing insight into a novel molecular mechanism underlying the anti-angiogenic potential of APG [138]. Recently, APG has been reported to inhibit PCa progression in the TRAMP mouse model via targeting PI3K/Akt/forkhead box FoxO pathways. APG-treated mice showed decreased phosphorylation of Akt and FoxO3a, which correlated with reduced binding

of FoxO3a. It also reduces proliferation by lowering Ki-67 and cyclin D1 along with an increase in FoxO responsive proteins like BIM and p27/Kip1 [139]. Oxidative stress is linked to a progression of PCa and human prostate is vulnerable to DNA damage due to oxidation. APG has been shown to preferentially accumulate in the nuclear matrix, binding particularly to the nucleic acid bases, and has the ability to reduce oxidative DNA damage in prostate epithelial cells [140].

4.2. *Epigallocathechin-3-gallate*

Green tea is an aqueous mixture of dried unfermented leaves of *Camellia sinensis* and has shown to possess anti-mutagenic, anti-bacterial, hypocholesterolemic, anti-oxidant, anti-tumor and cancer preventive properties. Green tea is comprised of polyphenolic compounds like epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). The possible cancer-preventive activity of green tea constituents has been studied extensively, by us and others [141]. Many *in-vitro* and *in-vivo* studies have reported that consumption of green tea polyphenols (GTP) is associated with decreased risk and/or slower progression of PCa [142,143]. GTP inhibits prostate carcinogenesis by modulating one or more cell signaling pathways (NF-κB/MAPK/IGFR/COX-2), inhibiting many protein kinases, and suppressing the activation of transcription factors [144].

Among catechins, EGCG has been shown to be the most powerful with an anti-oxidant activity about 25–100 times more potent than that of vitamins C and E [145]. A vast amount of scientific literature is present showing the potential health benefits of EGCG attributable to green tea consumption. Various mechanisms have been proposed for the biological activities of EGCG such as anti-oxidant action, apoptosis induction, cell-cycle arrest, modulation of carcinogen-metabolizing enzymes, inhibition of mitotic signal transduction through modulation of growth

factor receptor binding, and inhibition of DNA methylation [144]. In this context, recent studies showed that EGCG induced PCa cell death via downregulation of ID2 and up-regulation and stabilization of p53 [146]. EGCG promoted apoptosis associated with expression of CASP-9a splice variants in PCa cells, both alone and in combination with cisplatin [147,148]. EGCG provided protection against inflammation by suppressing proinflammatory cytokines, MMPs -2 and -9, independent of the AR expression and p53 status in PCa cells [149].

Green tea has higher concentrations of polyphenols, while black tea consumption has been shown to increase phenolic acids levels. Though clinical and pre-clinical studies provide evidence of green tea showing stronger chemopreventive effects as compared to black tea, concrete evidence from epidemiological studies is missing [150]. Additionally there still remain concerns about the bioavailability of EGCG and toxicity associated with its long-term use in clinical settings. Our group recently employed a nanochemoprevention approach involving chitosan-based nanoencapsulation of EGCG (Chit-nanoEGCG) for PCa cell growth inhibition, primarily addressing issues related to its bioavailability. Chit-nanoEGCG significantly inhibited tumor growth and suppressed PSA serum levels in athymic nude mice xenografted with 22Rv1 cells. In addition, there was significant induction of apoptosis and inhibition of tumor proliferation as evidenced by ADP-ribose polymerase cleavage, increase in BAX protein, decrease in Bcl-2, activation of CASPs, and reduction in proliferative markers Ki-67 and PCNA in the Chit-nanoEGCG treated groups as compared to control groups [151].

4.3. *Fisetin*

Fisetin (FST; 3,7,3',4'-tetrahydroxyflavone) belongs to the flavonol subgroup of flavonoids, along with quercetin, myricetin and kaempferol. FST is primarily present in fruits and

vegetables, such as strawberries, apples, persimmons, grapes, onions, and cucumbers [152]. Cell culture studies show that FST exerts anti-proliferative effect on human PCa cells. We have shown that FST selectively decreases the viability of LNCaP, 22Rv1, and PC3 cells with minimal effects on normal prostate epithelial cells [153,154]. FST induces apoptosis, cell cycle arrest, and inhibits androgen signaling and tumor growth in PCa models both *in-vitro* and i*n-vivo*. FST suppressed cell proliferation by hypophosphorylation of eukaryotic translation initiation factor 4E-binding protein-1 and induced autophagic cell death in PCa cells through suppression of mTORC1 and mTORC2 complexes. In addition, FST acts as a dual inhibitor of mTORC1/C2. FST also activated the mTOR repressor TSC2 (tuberous sclerosis 2), commonly associated with inhibition of Akt and activation of AMPK [155].

TRAIL plays an important role in the defense against tumor cells. FST sensitizes androgen dependent LNCaP and androgen independent DU145 and PC3 cells to TRAIL-induced death. In addition, FST augmented TRAIL-mediated cytotoxicity and apoptosis in LNCaP cells by activating the extrinsic receptor-mediated and intrinsic mitochondrial apoptotic pathways. FST increased the expression of TRAIL-R1 and decreased NFκB activity [156]. Recently, we showed that FST inhibits YB-1, an important transcription factor that promotes epithelial-to-mesenchymal transition (EMT) in PCa. YB1 is overexpressed in PCa and has a functionally inverse relationship with e-cadherin, which is a marker for EMT. During PCa, endogenous EMT occurs which leads to induction of YB-1, which induces a mesenchymal phenotype both *in-vitro* and *in-vivo*. FST binds to the cold shock domain of YB-1 protein as shown by in silico docking studies and surfaces as an inhibitor of YB1 phosphorylation and MTA-1 expression. FST also inhibits EGF and TGF-β induced YB-1 phosphorylation and EMT in PCa cells [157].

Collectively, all these studies provide ample evidence that FST could be developed as an effective agent against PCa.

4.4. *Proanthocyanidins*

Proanthocyanidins (PAC), commonly known as condensed tannins, are found abundantly in various plants and foods and contribute to organoleptic properties such as bitterness and astringency [158]. Food and nutritional supplements rich in PAC are known to have benefits in health promotion. PAC is primarily enriched in apple peel, red kidney beans, pinto beans, cacao beans, cocoa, grape seeds, blueberries, several nuts (peanuts, hazelnuts, etc.), sorghum , and cinnamon [159]. Cellular mechanisms involved in regulation of human PCa cells via blueberry fractions have been previously reported. MMPs are major mediators of extracellular matrix degradation and play an important role in PCa metastasis [160]. PAC, one of the primary flavonoids present in the blueberry fraction along with anthocyanin, caused down-regulation of MMP activity and up-regulation of endogenous tissue inhibitors of MMP's (TIMP) activity in DU145 cells. The authors also reported the possible involvement of protein kinase C (PKC) and MAPK-associated events with a PAC-mediated decrease of MMP-2, -9 and increase in TIMP-1, -2 [161].

Another study examined the inhibitory effects of PAC isolates from wild and cultivated blueberries on proliferation of androgen dependent LNCaP and androgen independent DU145 cells. Differences in cell growth inhibition profile of LNCaP and DU145 cell lines indicated that PAC primarily affects the growth of androgen-dependent growth of PCa cells [162]. PAC isolated from cranberries via column chromatography was tested on DU145 tumor implants in athymic nude mice. PAC showed significant reduction in growth of the tumors explant cells *in-*

vitro and induced a complete regression in DU145 tumor implants in vivo [163]. Studies on PAC isolates from grape seeds demonstrated similar inhibitory effects on human PCa cells. Anti-proliferative and pro-apoptotic effects on LNCaP cells were primarily associated with decreased expression of androgen receptors. PAC mediated inhibition of CDKs, cyclins, and activation of tumor suppressor's p21 and p27 was observed in both LNCaP and PC3 cells, along with changes in the Bcl-2/BAX ratio which favors apoptosis. PAC also induced cellular differentiation by increasing MAPK p44/42 [164]. In androgen-independent PCa, urokinase plasminogen activator (uPA) is implicated in cell migration and cancer metastasis. Treatments of PC3 cells with PAC-rich grape seed extract (PAC-GSE) have shown to regulate uPA expression and cell migration in a dose-dependent manner. Additional *in-vitro* studies showed that PAC-GSE repressed DNA-binding activity of NFκB which in turn decreased NFκB-dependent uPA transcription [165]. Additional *in-vitro* studies have found that PAC is an inhibitor of apoptosis suppressor proteins, NFκB, PI3K/Akt pathway, cytokines, angiogenesis factors, and many other molecular targets, which may contribute to anti-proliferative and pro-apoptotic effects in PCa and many other cancers [166]. Recently, a cancer prevention study II (CPS-II) was reported showing PAC intake and its inverse relationship of PCa risk in a cohort of US men, suggesting its potential efficacy against this deadly disease [167].

Limitations and Future Directions

Our understanding of the molecular aspects of PCa has progressed a lot in the recent years, but the overall PCa incidence and mortality remains a significant concern. Taking all the present findings to date into consideration, further research in PCa treatment and prevention remains critically important to target this deadly disease. A major limitation for the effectiveness of polyphenols in disease prevention is their bioavailability. It differs greatly among various

polyphenols and the most abundant ones in our diet are not necessarily those having the best bioavailability profile. The identification and quantification of polyphenol metabolites with a focus on their potential biological activity should be the emphasis in future. To improve the bioavailability, dedicated strategies need to be implemented and it is necessary to determine whether these strategies actually translate into increased biological activity. Suitable animal models and appropriate doses should be used to demonstrate the true health benefits of dietary polyphenols before clinical trials in humans are initiated.

With current advancements in available technologies, it is now possible to interpret specific molecular events responsible for the anti-tumor effects of each individual polyphenol. This could allow present and future investigators to design preclinical studies to establish the scientific basis upon which more human studies can be planned. This will potentially help eliminate any existing disagreements with regards to past epidemiological and clinical studies. Motivated research on polyphenols and its anti-tumor effects will identify new molecules that can be studied and used for PCa prevention and treatment, both alone and in combination with existing therapies. Despite the various health benefits, polyphenols need to undergo similar analyses used for development of new therapeutic drugs. The results from such approaches shall determine the pharmacokinetics profiles of the compounds, as well as confirm the presumed interactions with other molecules. Several polyphenols possess synergistic characteristics with cancer chemotherapeutic agents. Hence, an appropriate combination of polyphenols with existing chemotherapeutics will lead to a reduction in side effects without decreasing the chemotherapeutic effects. Furthermore, dietary polyphenols are promising molecules for chemoprevention of PCa as they are safe and inexpensive, especially in patients at increased risk of PCa due to their genetic background or long-term exposure to carcinogens.

Conclusions

Studies in literature provide ample evidence that polyphenols have the potential to prevent PCa risk. Patients diagnosed with PCa have depleted antioxidant levels in blood [168] and increased levels of lipid peroxidation [169,170]. Dietary polyphenols in plasma have been shown to influence PCa risk by regulating inflammatory genes and repairing oxidative DNA damage [171,172]. In addition, there are also interactions between different dietary polyphenols, which could modify PCa risk through both anti-oxidant and non-anti-oxidant mechanisms [173]. The effect of dietary polyphenols on PCa remains inconclusive until well designed clinical trials are initiated to prove their efficacy in humans. The development of PCa is driven mainly by signaling pathways; hence, multi-targeted therapy approach should be employed to evade and avoid drug resistance. Further precise studies are needed to find the specific target of each polyphenol so that a combination regimen could be developed. Thus, the association of dietary polyphenols and their influence on PCa risk in target populations and patients renders a very promising tool for prevention and treatment of PCa.

Figure 1.1

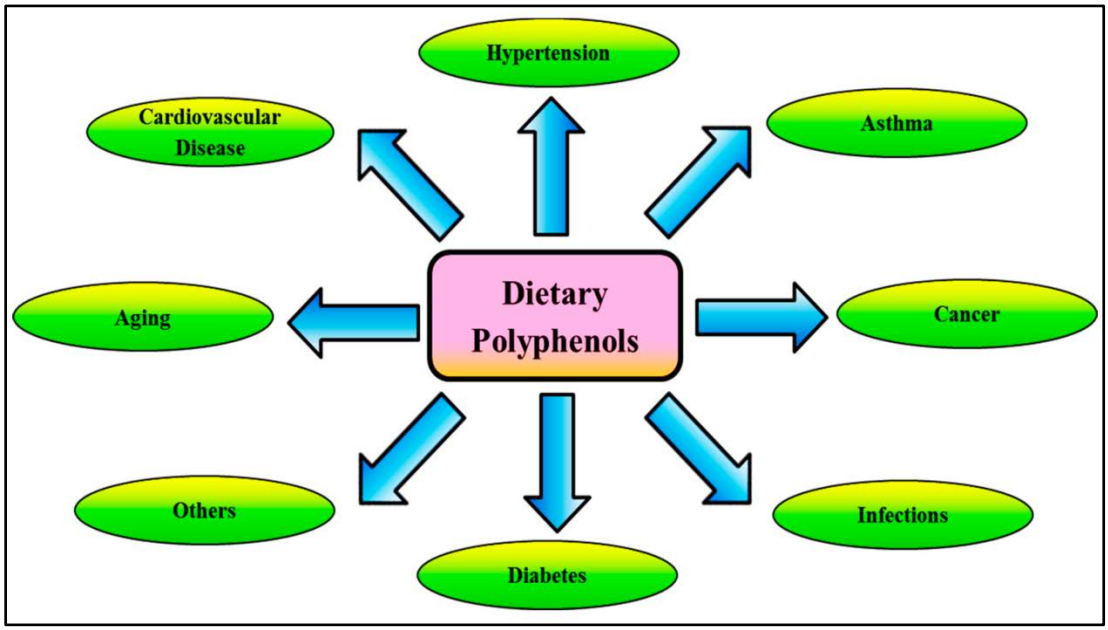

Figure 1.1. Beneficial health effects of dietary polyphenols. Polyphenols have been widely explored and are potent antioxidants. Polyphenols neutralize the destructive reactivity of undesired reactive oxygen species (ROS)/reactive nitrogen species (RNS) produced during the metabolic processes in the human body.

Figure1.2

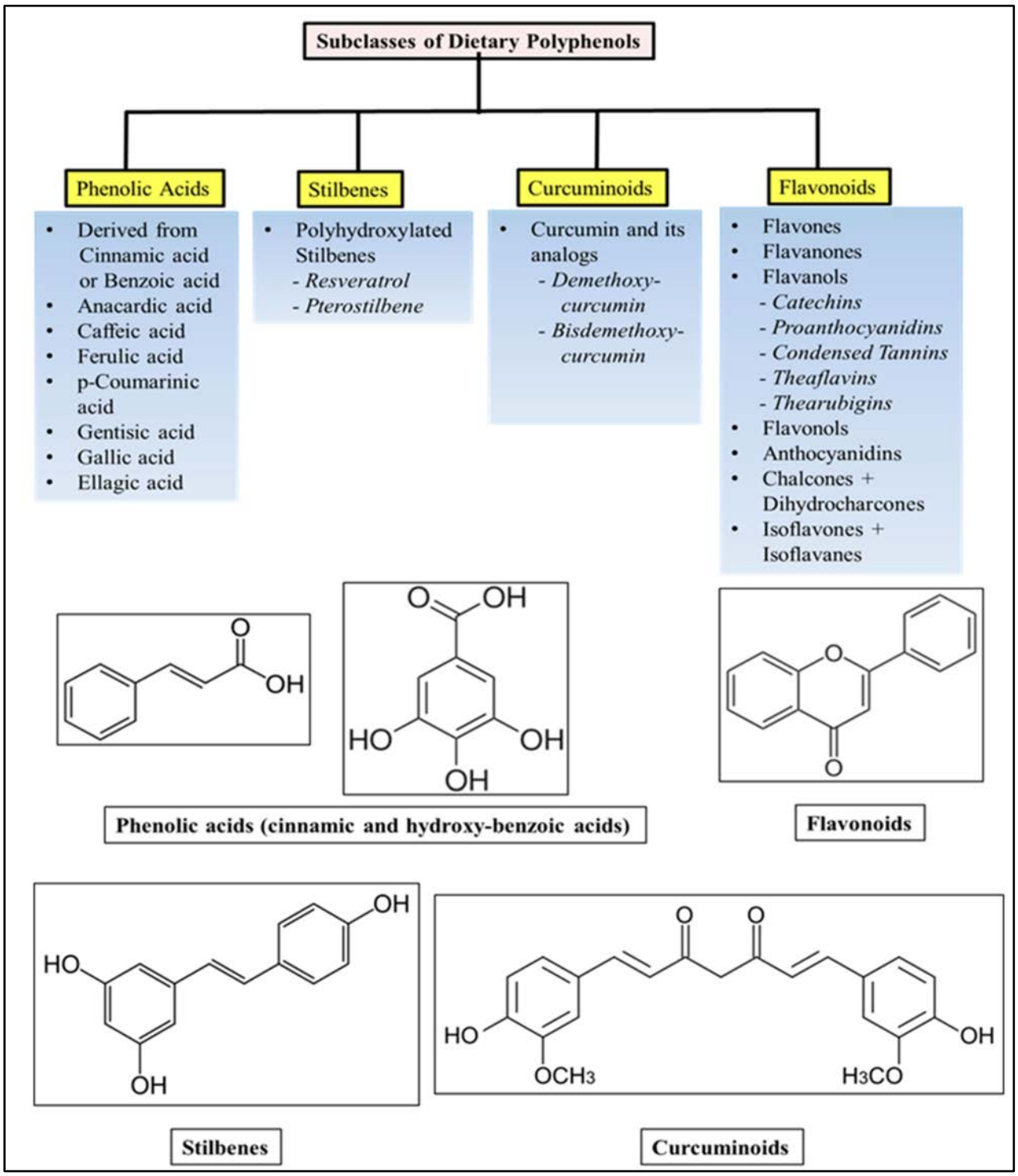

Figure 1.2. Subclasses and general structures of dietary polyphenols.

Figure 1.3

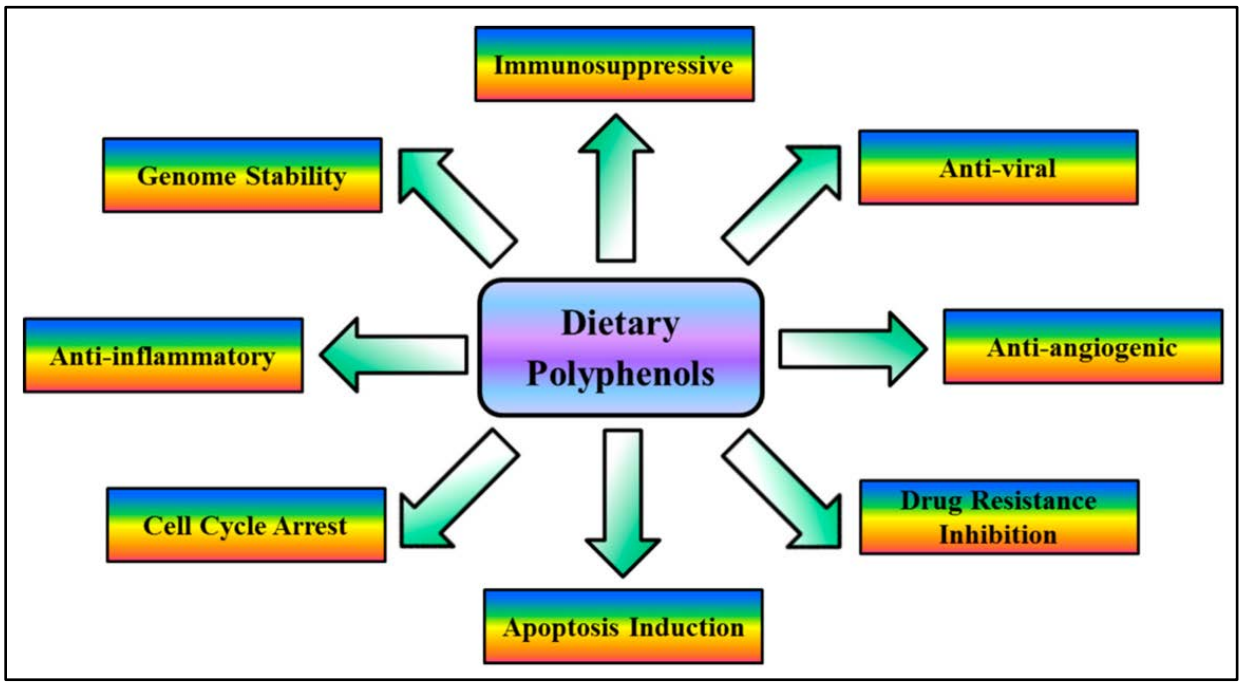

Figure 1.3. Cellular, molecular, and biochemical actions of dietary polyphenols. Dietary polyphenols target signaling molecules, including growth factors, transcription factors, cytokines, enzymes, and genes regulating apoptosis. Dietary polyphenols play an important role in inflammation, apoptosis, angiogenesis and auto-immune diseases.

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CHAPTER 2

Dietary Flavonoid Fisetin for Cancer Prevention and Treatment

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Abstract:

Cancer remains a major public health concern and a significant cause of death worldwide. Identification of bioactive molecules that have the potential to inhibit carcinogenesis continues to garner interest among the scientific community. In particular, flavonoids from dietary sources are the most sought after because of their safety, cost-effectiveness and feasibility of oral administration. Emerging data has provided newer insights into understanding the molecular mechanisms that are essential to identify novel mechanism-based strategies for cancer prevention and treatment. Dietary flavonoid fisetin (3,3′,4′,7-tetrahydroxyflavone) found in many fruits and vegetables has been shown in preclinical studies to inhibit cancer growth through alteration of cell cycle, inducing apoptosis, angiogenesis, invasion and metastasis without causing any toxicity to normal cells. Although data from *in-vitro* and *in-vivo* studies look convincing, well designed clinical trials in humans are needed to conclusively determine the efficacy across various cancers. This review highlights the chemopreventive and therapeutic effects, molecular targets and mechanisms that contribute to the observed anti-cancer activity of fisetin against various cancers.

Introduction

Cancer remains as one of the leading causes of death worldwide [1]. It is a major public health concern with about 1,658,370 new cases expected to be diagnosed in 2015 [2]. Oncogenesis is a multistep process in which a normal cell acquires alteration at the cellular, genetic and epigenetic level to progressively transform into a cancer cell. These transformations progresses to hyperproliferation, unlimited replication potential, evading apoptosis, sustained angiogenesis, invasive potential and metastasis [3]. Despite advances in diagnostic and therapeutic approaches over the years, cancer continues to be a formidable challenge [1]. This is largely because of the growing urbanization as well as cancer-associated lifestyle choices including unhealthy dietary habits. Among all cancers, lung cancer remains as the leading cause of deaths among men and women followed by PCa (PCa) in men and breast cancer in women [4]. Mono-targeted therapies are currently available but can induce toxicity and side effects due to a specific single target. Other limitations of targeted therapies include ineffective targeting, resistance and ever increasing cost of treatment [5, 6]. As cancer is a multifactor disease, it may require prevention/treatment approaches with compounds that are able to target multiple biochemical and molecular pathways. This appears to be the most practical method to decrease the disease incidence and burden [5, 7]. Cancer chemoprevention, a rapidly evolving field of preventive oncology, focusses on the use of synthetic, pharmaceutical and/or natural agents to entirely inhibit, retard or reverse the process of carcinogenesis [8]. Few of the many advantages of using natural agents for chemoprevention comprise of safety, efficacy, ease of availability, affordability, potential to overcome resistance to other traditional therapies and anticancer drugs [7]. Fisetin is one such agent with a potential to target multiple signaling pathways in cancer cells without presenting any toxicity to normal cells.

Fisetin and Cancer

Chemoprevention is an emerging, appealing and an innovative strategy for the management of cancer. While fruits and vegetables are an abundant source of many cofactors, vitamins, minerals, the phytochemicals constituents known as flavonoids have special ability to target several key cellular events involved in the development of cancer [9, 10]. Flavonoids are the most abundant polyphenols consumed in human diet and are classified into flavonols, flavones, isoflavones, anthocyanidins, theaflavins, and thearubigins. Several studies have demonstrated that flavonoids possess anti-oxidant, anti-inflammatory and chemopreventive properties. The compound 3, 7, 3', 4'-tetrahydroxyflavone is a natural flavone commonly referred to fisetin. The chemical structure and basic properties of fisetin are shown in **Figure 2.1**.

Major fisetin containing food sources include strawberries, apples, persimmons, grapes, onions, and cucumbers [11] as shown in **Table 2.1**. Fisetin affects multiple molecular and signaling pathways depending on the cancer type as shown in **Figure 2.2**. Among all the multiple biological effects fisetin possesses, its anti-cancer potential has been recently explored making it a promising agent for cancer prevention and therapy. This review summarizes the cellular effects of fisetin in an attempt to elucidate its preventive and therapeutic potential against various cancers.

Fisetin and Lung Cancer

Lung cancer is the leading cause of cancer-related deaths in the United States. It accounts for 13% of all cancers diagnosed and 27% of all cancer related deaths [1, 2]. An important obstacle to non-small cell (NSC) lung cancer chemotherapy is the development of resistance to a widely used chemotherapeutic drug cisplatin. Fisetin has shown antiproliferative, apoptotic and antiangiogenic properties in lung cancer cells [12]. Fisetin was reported to reverse the acquired cisplatin-resistance in A549-CR lung cancer cells. Fisetin (40μM) in combination with cisplatin (10μM) showed intense suppression of cell viability and induction of apoptosis as compared to cells treated with fisetin and cisplatin alone, possibly via inactivating MAPK pathways as well as suppressing Survivin expression [13]. Fisetin was shown to induce apoptosis in NSC lung cancer via mitochondrial-mediated pathways. Fisetin induced DNA fragmentation, ROS generation and apoptosis in NCI-H460 cells via a reduction in Bcl-2 and increase in Bax expression. Fisetin treatment increased cleavage of caspase-9 and caspase-3 thereby increasing caspase-3 activation [14]. Similarly, fisetin supplementation alleviated mitochondrial dysfunction and induced apoptosis by the upregulation of Bax/Bcl-2 ratio, thereby leading to cytochrome-c release and activation of caspase-9, caspase-3 leading to apoptotic cell death during benzo(a)pyrene (B(a)P)-induced lung cancer in-vivo [15]. Fisetin (25 mg/kg body weight) decreased histological lesions and levels of lipid peroxidation and modulated the enzymatic and non-enzymatic anti-oxidants in B(a)P-treated Swiss Albino mice [16].

We observed that fisetin treatment (5-20μM) inhibits cell growth and colony formation in A549 NSC lung cancer cells. Fisetin activated tumor suppressor PTEN and negatively regulated protein synthesis by phosphorylation of AMPKα. Fisetin also inhibited PI3K/Akt/mTOR signaling pathway in NSC lung cancer cells. Inhibitors of this pathway have entered preclinical

and clinical trials and suggest fisetin as a promising candidate drug for therapeutic intervention in lung cancer [17]. Fisetin in combination with CPA was shown to inhibit angiogenesis using a Matrigel plug assay. Combination of fisetin (223mg/kg) with CPA (30mg/kg) produced a marked inhibition of tumor growth (92%) in LLC-bearing mice as compared to animals treated with fisetin or CPA alone. This study provided the first evidence that fisetin exhibits antiangiogenic and anticancer activities in mice bearing LLC [18]. Another study showed that fisetin inhibits adhesion, migration, and invasion in A549 lung cancer cells by downregulating uPA, ERK1/2, and MMP-2. Treatment with fisetin also decreased the nuclear levels of NF-κB, c-Fos, c-Jun and AP-1 and inhibited NF-κB binding. Taken together, fisetin demonstrated promising results against lung cancer both *in-vitro* and *in-vivo*.

Fisetin and PCa

PCa (PCa) is the most frequently diagnosed cancer in men with an estimated 220,800 new cases. It is the second-leading cause of cancer deaths in men with 27,540 deaths in 2015 in the US alone [1, 2]. Treatment and prevention of PCa with fisetin is an active area of research. Cell culture studies show that fisetin exerts anti-proliferative effect on human PCa cells. Our laboratory has previously shown that treatment of LNCaP cells with fisetin caused inhibition of PCa by G1-phase cell cycle arrest, modulating CKI–cyclin–CDK network and induction of apoptosis [19]. Fisetin induced apoptosis and cell cycle arrest in LNCap cells, and inhibited androgen signaling and tumor growth in athymic nude mice implanted with androgen receptor-positive 22Rv1 cells [20]. Fisetin suppressed cell proliferation by hypophosphorylation of 4E-binding protein-1 and induced autophagic cell death in PCa cells through suppression of mTORC1 and mTORC2. Fisetin acts as a dual inhibitor of mTORC1/C2 and activated the mTOR repressor TSC2, commonly associated with inhibition of Akt and activation of AMPK

[21]. TRAIL plays an important role in the defense against tumor cells. Fisetin sensitized TRAIL-resistant androgen-dependent LNCaP and the androgen-independent DU145 and PC3 PCa cells to TRAIL-induced death [22]. Additionally, in androgen-independent PCa cell lines the cytotoxic and apoptotic effects of TRAIL in combination with fisetin are lowest compared to androgen dependent LNCaP cells. Inhibition of NF-κB activation by fisetin in LNCaP cells augmented the apoptotic effect of TRAIL. These findings confirm that the down-regulation of NF-κB sensitizes PCa cells to TRAIL *in-vitro* [22].

We recently reported that fisetin inhibits YB-1, an important transcription factor that promotes EMT in PCa. YB-1 is overexpressed in PCa whereas E-cadherin, a marker for EMT is downregulated. During PCa progression, forced YB1 expression induced a mesenchymal phenotype both *in-vitro* and *in-vivo.* Fisetin binds to the cold shock domain of YB-1 protein and was reported as an inhibitor of YB1 phosphorylation and MTA-1 expression. Fisetin also inhibits EGF and TGF-β induced YB-1 phosphorylation and EMT in PCa cells [23]. TMFol, a structural analogue of fisetin and quercetin exhibited chemopreventive efficacy superior to fisetin alone both *in-vitro* and *in-vivo*. TMFol inhibited cell growth in 22Rv1, TRAMP C2, PC-3 and LNCaP cells. It also slowed tumor development in nude mice bearing TRAMP C2 and 22Rv1 cells [24]. Recently, we showed that fisetin binds to β-tubulin and disrupts microtubule dynamics in PCa cells by enhancing tubulin polymerization. Fisetin was able to arrest cells in G2/M phase, inhibits cell proliferation, invasion, migration, cell viability, colony formation, and decrease the P-gp protein in multidrug resistant NCI/ADR-RES cells. These *in-vitro* results establish fisetin as a microtubule targeting agent and shows potential to be developed as an adjuvant with microtubule targeting based therapies [25]. Collectively, all these studies provide ample evidence that fisetin by targeting multiple pathways could be developed as an effective agent against PCa.

Fisetin and Melanoma/Skin Cancer

Melanoma is the deadliest form of skin cancer due to its high likelihood of producing metastasis. With an estimated 73,870 new cases, melanoma will account for an estimated 13,340 skin cancer deaths in 2015 alone making it an important area for research [1, 2]. We reported for the first time that fisetin has the potential to inhibit human melanoma by disrupting the Wnt/β-catenin/Mitf signaling. *In-vitro* studies showed that that decrease in β-catenin levels, induction of β-TrCP and a reduction of Mitf mRNA and protein levels are possible mechanisms for fisetin-mediated suppression of Wnt signaling in 451Lu human melanoma cells. Interestingly, the *in-vitro* effects were reflected in in-vivo studies which showed that fisetin significantly inhibited tumor growth in 451Lu melanoma xenografts that was associated with decreased Mitf levels [26]. Another study showed that a 4′MF, a mono-methyl analogue of fisetin is a potent inhibitor of SIK2 and strongly induced melanogenesis in B16F10 melanoma cells. By modulating SIK2 signaling with 4′MF, the authors reported CREB-mediated transcription via TORC1 activation in-vivo [27]. A non-melanoma skin cancer study revealed that fisetin inhibited growth and induced apoptosis via increase in Bax, Bak and Bad protein expressions in A431 cells. Fisetin treatment also resulted in G2/M arrest, modulation in Bcl-2 family proteins (Bcl-2, Bcl-xL and Mcl-1), disruption of mitochondrial potential, and activation of caspases and cleavage of PARP [28].

Another group investigated the photochemopreventive effect of fisetin for the management of UVB-induced skin malignancies in SKH-1 mouse model of skin cancer. Their findings revealed that topical application of fisetin (250 and 500 nmol) to SKH-1 hairless mice skin after UVB exposure results in significant decrease in leukocyte infiltration, inflammatory markers (MPO, COX2, and PGE2), cytokines (TNFα, IL-1β, and IL-6) and proliferation

markers (PCNA and cyclin D1). Fisetin also inhibited the PI3K/AKT/NFκB signalling which is associated with UVB-induced inflammation, cell survival and proliferation. Fisetin was found to cause no damage to the mouse skin and increased protein expression of p53 and p21 after UVB treatment [29]. The MAPK (BRAF-MEK-ERK) pathway is a key regulator of melanoma cell invasion and potential targets for melanoma treatment and prevention. Fisetin treatment (5-20μM) resulted in inhibition of cell invasion in A375, SK-MEL-28, RPMI-7951, SK-MEL-119 and Hs294T melanoma cells. BRAF mutated cells were found to be more sensitive due to a decrease in phosphorylation of MEK1/2 and ERK1/2. Fisetin inhibited the activation of IKK leading to a reduction in the activation of the NF-κB signaling pathway. Fisetin also stimulated melanoma cell phenotype transition from mesenchymal to epithelial as observed by decrease in mesenchymal markers (N-cadherin, vimentin, snail, fibronectin) and increase in epithelial markers (E-cadherin and desmoglein) [30]. We showed that fisetin inhibits A375 human melanoma cell growth in monolayer and 3D cultures. Fisetin inhibited melanoma progression in a 3D melanoma skin model with downregulation of mTOR, Akt and upregulation of TSC. Fisetin inhibited A375 and 451Lu melanoma cell proliferation by binding to p70s6K with higher affinity than mTOR [30].

A combinational treatment study of melatonin and fisetin demonstrated enhanced antitumor activity of fisetin. The combination significantly led to inhibition of melanoma cell viability, migration, colony formation and induced greater apoptosis when compared to fisetin alone. These effects were associated with activation of cytochrome-c/caspase-dependent apoptosis and inhibition of p300/NF-κB-mediated COX-2 and iNOS expression [31]. We showed involvement of ER stress and activation of extrinsic and intrinsic apoptotic pathways with fisetin in human melanoma. Fisetin inhibited ROS and augmented NO generation in A375

melanoma cells. Fisetin (20-80μM) mediated apoptosis was accompanied with transient autophagy and induction of ER stress evidenced by increased IRE1α, XBP1s, ATF4 and GRP78 levels in A375 and 451Lu cells. Silencing of AMPK failed to prevent cell death indicating that fisetin induced cytotoxicity is mediated through both AMPK-dependent and -independent mechanisms [32]. Recently, another combinatorial approach of using fisetin with sorafenib (RAF inhibitor) demonstrated inhibition of melanoma cell proliferation, induction of apoptosis and inhibition of tumor growth in athymic nude mice implanted with BRAF-mutated melanoma cells. The combination treatment resulted in enhanced apoptosis, cleavage of caspase-3 and PARP, expression of Bax and Bak, inhibition of Bcl-2 and Mcl-1, and inhibition of expression of PI3K, phosphorylation of MEK1/2, ERK1/2, AKT and mTOR. Fisetin treatment resulted in greater reduction of tumor growth and inhibition of the MAPK/PI3K pathways in A375 and SK-MEL-28 cell xenografts when compared to fisetin and sorafenib alone suggesting combination therapy to be more effective than monotherapy [33]. A follow up study showed that combination of fisetin with sorafenib effectively inhibited EMT and augmented the anti-metastatic potential of sorafenib by reducing MMP-2 and MMP-9 proteins in melanoma cell xenografts [34]. Collectively these studies reflect the potential of fisetin alone as well as in combination with other established agents for management of melanoma.

Fisetin and Colorectal Cancer

Colorectal cancer is the third most common cancer in both men and women. With an estimated 93,090 cases of colon cancer and 39,610 cases of rectal cancer expected to be diagnosed, 49,700 deaths from colorectal cancer are expected to occur in 2015 [1, 2]. A comparative study of various flavonoids was performed showing their efficacy on proliferation, cytotoxicity, and apoptosis in Caco-2 and HT-29 human colon cancer cells [35]. Fisetin (0-60μM) was shown to

inhibit activity of CDKs dose-dependently leading to cell cycle arrest in HT-29 human colon cancer cells. Fisetin inhibited both cell growth and DNA synthesis at 72h, cell cycle progression at 8h, and G2/M phase arrest after 24h. Fisetin treatment decreased activities of CDK2 and CDK4 via decreased levels of cyclin-E, cyclin-D1 and increase in p21 (CIP1/WAF1) levels. This study indicated that inhibition of cell cycle progression in HT-29 cells with fisetin treatment can be explained by modification of CDK activities [36]. Another follow up study attempted to characterize the actual mechanism of fisetin mediated apoptosis in HCT-116 colon cancer cells. Fisetin (5-20μM) induced DNA condensation, cleavage of PARP and activation of caspase-9, -7, and -3 in HCT-116 cells. Anti-apoptotic proteins Bcl-xL and Bcl-2 were downregulated whereas pro-apoptotic proteins Bak and Bim were upregulated that induced the mitochondrial translocation of Bax. Fisetin increased p53 protein levels, and the inhibition of p53 expression by small interference RNA resulted in a decrease in the fisetin-induced translocation of Bax to the mitochondria, release of mono- and oligonucleosome in the cytoplasm, and PARP cleavage. This study provided a molecular basis for using fisetin as an apoptosis stimulatory agent via activation of caspases and induction of p53 resulting in translocation of Bax to mitochondria [37].

Overexpression of COX-2 and Wnt signalling has been known to play roles in colorectal cancer. We reported that fisetin (30–120 μM) induces apoptosis in colon cancer cells by inhibiting COX-2 and Wnt/EGFR/ NF-κB -signaling pathways. Fisetin induced apoptosis and downregulation of COX-2 protein expression without affecting COX-1 and inhibited the secretion of prostaglandin E2 in HT-29 and HCT116 colon cancer cells. Fisetin treatment inhibited Wnt/EGFR/ NF-κB signaling via downregulation of β-catenin, TCF-4, cyclin D1 and MMP-7 suggesting its promising role in colon cancer prevention [38]. In another study fisetin treatment was found to radiosensitize human colorectal cancer cells which are resistant to

radiotherapy. Pre-treatment of p53-mutant HT-29 cells with fisetin enhanced the radiosensitivity by causing accumulation of cells in the radiosensitive G2/M phase, suppressing cellular DNA repair capacity, thereby increasing radiation-induced double-strand breaks. Fisetin pre-treatment augmented radiation-induced pro-apoptotic p38 MAPK activation and ultimate shut down of pro-survival signals [39]. A recent combinatorial study showed that 10-20μM NAC enhances fisetin mediated apoptosis in COLO25 colon cancer cells when compared to fisetin treatment alone. Combined treatment of fisetin with NAC increased cleaved caspase-3, PARP, reduced mitochondrial membrane potential with induction of caspase-9 in COLO25 cells. NAC sensitization to fisetin-induced apoptosis was also identified in various other cells like HCT-116, HT-29, and HCT-15 suggesting a novel strategy to treat colon cancer [40]. Fisetin is poorly soluble in water and it's difficult for intravenous administration. To address this issue, a novel approach was adapted by preparing nanoassemblies of polymeric micelles which can encapsulate fisetin leading to improved therapeutic effect in colon cancer. The polymeric micelle encapsulation demonstrated a sustained and prolonged *in-vitro* release, enhanced cytotoxicity, cellular uptake, and apoptosis. Fisetin micelles were also shown to exhibit increased tumor apoptosis, suppress proliferation and anti-angiogenesis activities [41]. Taken together, fisetin demonstrated promising results against colon cancer.

Fisetin and Bladder Cancer

With an estimated 74,000 new cases and 16,000 deaths alone in 2015, bladder cancer remains as a concern. Bladder cancer incidence is about 4 times higher in men than in women and almost 2 times higher in white men than in black men [1, 2]. A recent study reported that fisetin induced apoptosis in human bladder cancer via upregulation of p53 and down-regulation of NF-κB activity, causing a change in the ratio of pro- and anti-apoptotic proteins. Results from this study

revealed that fisetin inhibits the proliferation of T24 and EJ cells by inducing apoptosis and blocking cell cycle progression in the G0/G1 phase by significantly increasing p53, p21 proteins, and decreasing the protein levels of cyclinD1, cyclinA, CDK4, and CDK2. This in-vitro study showed that activation of p53 and inhibition of the NF-κB play important roles in the fisetin-induced apoptosis in bladder cancer cells [42].

A follow up in-vivo study using a rat bladder cancer model induced by MNU suggested that p53 activation and NF-κB inhibition play important roles in the fisetin-induced apoptosis in bladder cancer. Furthermore, fisetin inhibited tumor growth and bladder carcinogenesis in MNU-initiated rats without any toxicity [43]. These findings identify the in-vivo chemopreventive efficacy of fisetin and suggest that fisetin could be developed as an effective chemopreventive agent against bladder cancer.

Fisetin and Breast Cancer

With an estimated 231,840 new diagnoses and 40,730 deaths, breast cancer ranks as the second cause of cancer deaths in women after lung cancer [1, 2]. A study published in 2012 extensively investigated the cytotoxicity and apoptotic effects induced by fisetin in MCF-7 and MDA-MB-231 breast cancer cells. Fisetin was found to exhibit anti-cancer activity in caspase-3 deficient MCF-7 cells. Additionally, in MCF-7 cells, fisetin induced a novel form of atypical apoptosis and triggered plasma membrane rupture, mitochondrial depolarization, activation of caspase-7, -8, and -9, and PARP cleavage. These atypical features of apoptosis were due to caspase-3 deficiency in MCF-7 cells. Furthermore, fisetin inhibited autophagy which promoted cell death in MCF-7 cells [44]. These few preliminary studies suggest that bioactive actions of fisetin hold promise against breast cancer.

Fisetin and Leukemia

A limited number of studies have examined the potential of fisetin against leukemia. Leukemia is a cancer of the bone marrow and blood and is classified into four main groups according to cell type and rate of growth: acute lymphocytic (ALL), chronic lymphocytic (CLL), acute myeloid (AML), and chronic myeloid (CML). With an estimated 54,270 new cases and 24,450 deaths, leukemia still remains a health concern [1, 2]. A study involving seven structurally related flavonoids was conducted to evaluate their biological activities on human leukemia cell line HL-60. Among all flavonoids, fisetin showed the most cytotoxic effects and a combination of wogonin and fisetin being the most-potent apoptosis inducers. The combination caused rapid and transient induction of caspase-3/CPP32, cleavage of PARP and decrease in anti-apoptotic protein Mcl-1. However, the combination treatment did not have any effect on Bcl-2, Bcl-xL, and Bad. ROS production was decreased in apoptosis induced by the combination treatment along with enhanced Ca(2+)-dependent endonuclease activity. This study showed an interesting correlation between antioxidant activities of fisetin combined with wogonin towards flavonoids-induced apoptosis [45]. DNA topoisomerases (topo) are the target of several drugs commonly used in cancer chemotherapy. These drugs induce topo-DNA complexes with either topo I or topo II that eventually trigger cell death [46].

An increased risk for developing leukemia has been observed in patients treated with some topo II inhibitors. Effect of many flavonoids including fisetin was evaluated on topo I and topo II in K562 human leukemia cells at various concentrations and exposure times. Fisetin induced neither topo I- nor topo II-DNA complexes, but behaved as a catalytic inhibitor of both enzymes. These results suggest that fisetin acts as an inhibitor of DNA topoisomerases I and II in leukemia cells [46]. Recently, a combination effect of fisetin and hesperetin was reported

elucidating their role and mechanism(s) of action in human HL-60 acute promyelocytic leukemia cells. Fisetin and hesperetin induced apoptosis along with inhibited cell proliferation, induced G2/M arrest, disrupted the mitochondrial potential and increased caspase-3 activity. Microarray gene profiling of treated cells revealed some important biological pathways including MAPK, DNA binding signaling pathways and genes involved in cell proliferation, division, and apoptosis [47]. Another similar study demonstrated the effect of fisetin and hesperetin combination on human K562 CML cells. Combination treatment significantly modulated genes involved in cell proliferation, cell division, apoptosis, cell cycle, and various other cellular processes such as replication, transcription, and translation. Microarray gene profiling analysis revealed genes involved in JAK/STAT pathway, KIT receptor , and growth hormone receptor signaling to be potential candidates of fisetin-hesperetin combination for targeted CML therapy [47].

Fisetin and Cervical Cancer

With an estimated 12,900 new cases and 4,100 deaths, cervical cancer is currently one of the leading causes of mortality in women [1, 2, 48]. A preliminary study reported that fisetin induced apoptosis in human cervical cancer HeLa cell via activation of caspase-3, -8, and cleavage of PARP. Additionally, fisetin treatment induced a sustained activation of pERK1/2. Inhibitor of ERK1/2 or transfection with mutant ERK1/2 expression vector reversed the fisetin induced apoptosis. This study showed that apoptosis induction by fisetin in HeLa cells is via ERK1/3-mediated activation of caspase-3/caspase-8 dependent pathway. In-vivo tumor xenograft experiments in mice revealed that fisetin significantly reduced tumor growth [49]. Another study reported that fisetin inhibits migration and invasion of SiHa and CaSki human cervical cancer cells. This study provided strong evidence for the molecular mechanism of fisetin in inhibiting

aggressive phenotypes by downregulating uPA gene expression via interrupting the p38 MAPK-dependent NF-κB signaling pathway [50]. A novel combination approach of fisetin with sorafenib on human cervical cancer cell lines showed significant antitumor effect both *in-vitro* and *in-vivo*. The combination treatment induced apoptosis in HeLa and SiDR5-treated HeLa cells via caspase-3 and -8 activation and was accompanied by marked loss of mitochondrial potential. In addition, animal studies using a HeLa xenograft model demonstrated that combined fisetin and sorafenib treatment was clearly superior to sorafenib treatment alone. This combined fisetin and sorafenib treatment approach reveals a novel therapeutic strategy for future clinical development in advanced cervical cancer [51].

Future Prospects and Conclusion

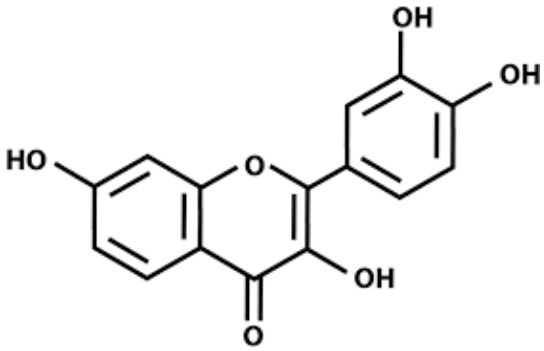
Cancer is a heterogeneous disease that uses multiple signaling pathways to survive and continues to be one of the leading causes of death. Single-agent-targeted therapies have rarely cured patients with cancer. To effectively halt tumor development and progression, a drug that can target multiple deregulated proteins and pathways would be ideal. Dietary flavonoid fisetin has shown tremendous potential in preclinical settings to target multiple deregulated proteins, signaling pathways and regulates wide variety of cell functions against various cancers. Fisetin disrupts Wnt signaling and results in cell cycle arrest. It inhibits the YB-1 binding protein to suppress epithelial to mesenchymal transition and thus prevents invasion and migration of cancer cells. By physically interacting with the mTOR molecule, fisetin inhibits signaling involved in cell survival thus explaining its inhibitory effect on cellular growth and proliferation. Fisetin binds to and disrupts microtubule dynamics and acts as a stabilizing agent with effects far superior to paclitaxel. To put the chemopreventive potential of fisetin to clinical use, well designed clinical trial interventions at right doses (as shown in **Table 2.2**) in right population are

required, which actually mirror the animal modeling data from which they were derived. Before actual clinical trials are conducted it is important that toxicological profile of fisetin is comprehensively investigated. Unfortunately, no toxicity data on fisetin currently exists to justify its potential use in humans. Also, the pharmacokinetic and bioavailability issues have to be studied in detail before phase I and II trials are initiated.

The debate on cancer chemoprevention attracts a lot of attention with dietary components showing promise in preclinical setting but lack of effect when used in clinical trials. Several critiques downgrade the importance phytochemicals based on their multiple biological effects, targets and signaling pathways. However, we strongly feel that there is a lot of potential for cancer chemoprevention provided that if modelled in the right way, it can offer an effective alternate strategy for the management of cancer especially for high-risk individuals [52]. Development of drug resistance is one of the most prominent limitations in the development of targeted therapies. A combination of targeted therapies with more traditional therapies could be a potential key to the problem of resistance. However, in overcoming drug resistance issues related to toxicity and high treatment cost remains an impediment in the development of FDA-approved anticancer drugs. There is enough strong preclinical evidence that fisetin shows tremendous promise as an anticancer agent and warrants clinical trials either to be administered alone or in combination with available anticancer drugs.

Figure 2.1

A



B

CAS Number	**528-48-3**
Molecular Structure	**$C_{15}H_{10}O_6$**
Molecular Mass	**286.24 g/mol**
Melting Point	**330°C (626°F; 603°K)**
Aqueous Solubility	**Insoluble**
Solubility	• **0.5mg/ml in DMSO:PBS (1:1; pH 7.2)** • **5mg/ml in ethanol** • **30mg/ml in DMSO & DMF**

Figure 2.1. Structure and basic properties of fisetin. **(A)** The chemical structure of fisetin (3, 7, 3', 4'-tetrahydroxyflavone). **(B)** Major physical and chemical properties of fisetin.

Figure 2.2

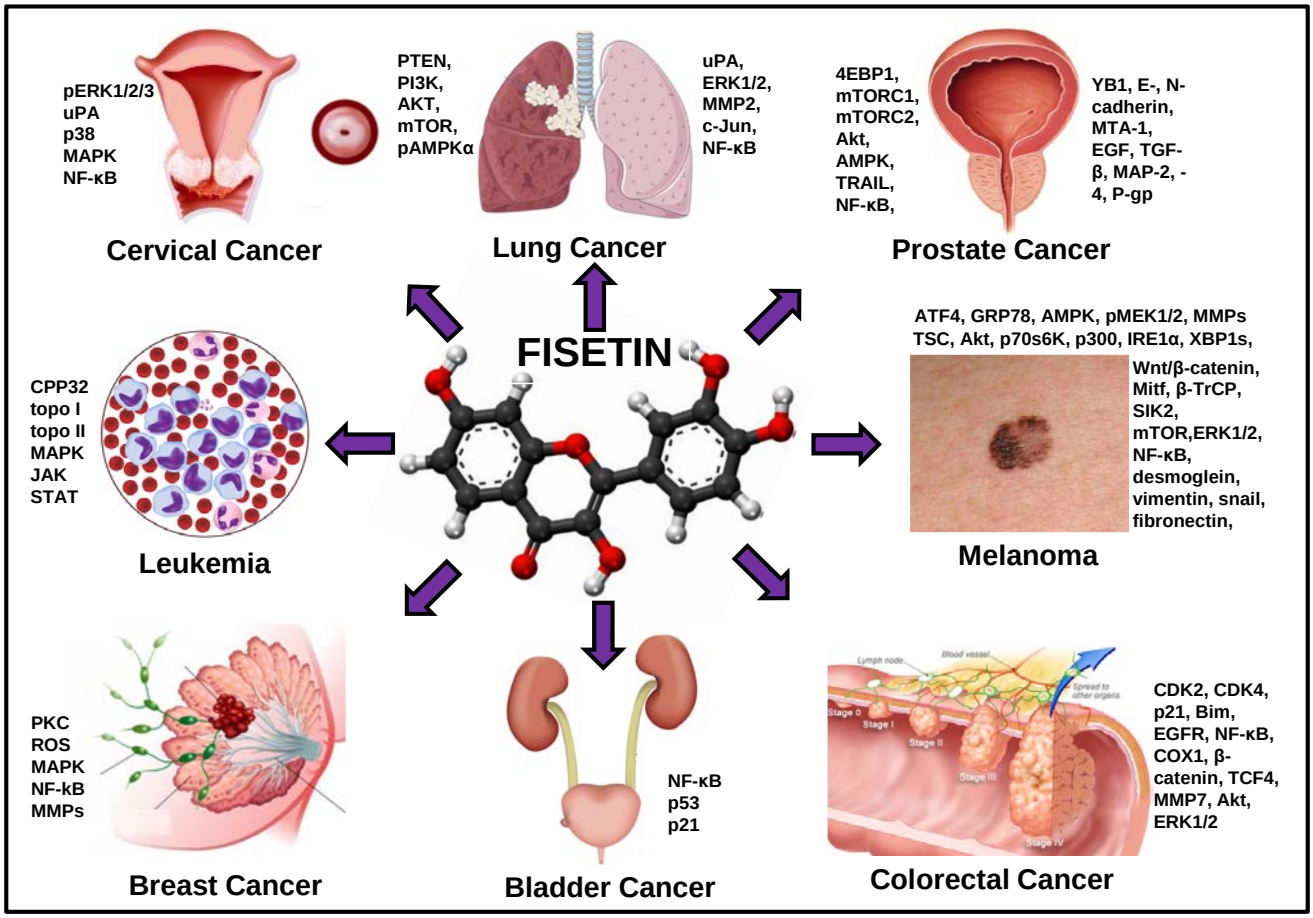


Figure 2.2. Molecular targets of fisetin in various cancers. Fisetin interacts with multiple cellular targets by binding to and interacting with several molecular targets fisetin regulates a wide variety of cell functions. Fisetin disrupts Wnt signaling and results in cell cycle arrest. It inhibits the YB-1 binding protein to suppress epithelial to mesenchymal transition and thus prevents invasion and migration of cancer cells. By physically interacting with the mTOR molecule, fisetin inhibits signaling involved in cell survival thus explaining its inhibitory effect on cellular growth and proliferation. Fisetin binds to and disrupts microtubule dynamics and acts as a stabilizing agent with effects far superior to paclitaxel.. However across all cancers, mTOR and NF-κB appear to be the most comonly affected pathways.

Table 2.1

Dietary Food Sources	Fisetin Concentration (μg/g dry weight)
Strawberry	160
Apple	26.9
Persimmon	10.6
Lotus Root	5.8
Onion	4.8
Grape	3.9
Kiwi	2.0
Peach	0.6
Cucumber	0.1
Tomato	0.1

Table 2.1. Dietary sources of fisetin. The concentration of fisetin (dry weight basis) was measured in freeze-dried vegetables and fruits after acid hydrolysis of the parent glycosides. Adapted from Kimira et al. [53]

Table 2.2

Cancer	Animal Models	Fisetin Dosage	Outcome of the Study	Ref
Lung Cancer	B(a)P-induced	25mg/kg b.wt., orally	Inhibited tumor growth by modulating activities of mitochondrial enzymes and induced apoptosis	**15**
	Lewis lung carcinoma cell xenograft	223 mg/kg b.wt., i.p	Inhibited angiogenesis, tumor growth	**18**
PCa	22Rv1 cell xenograft	40 mg/kg b.wt. (1 mg/animal), i.p.	Inhibited androgen signaling, tumor growth and reduced serum PSA levels	**20**
	NB26 cell xenograft	40 mg/kg b.wt. (1 mg/animal), i.p	Inhibited YB1 phosphorylation and TGF-β induced EMT	**23**
Melanoma	451Lu cell xenograft	45 and 90 mg/kg b.wt. (1 mg and 2 mg/animal), i.p	Inhibited tumor growth, decreased Mitf levels	**26**
Skin Cancer	SKH-1 hairless mice model	250 and 500 nmol/animal; topical application	Reduced inflammation and proliferation with decreased leukocytic infiltration and cytokines	**29**
Bladder Cancer	MNU-induced rat model	200mg/kg b.wt., i.p	Inhibited carcinogenesis, induced apoptosis via p53 activation and NF-κB inhibition	**43**
Cervical Cancer	HeLa cell xenograft	4 mg/kg b.wt., orally	Reduced tumor growth, induced apoptosis via mitochondrial and DR5-dependent caspase signaling	**51**

Table 2.2. Fisetin dosage and efficacy in preclinical studies against various cancers.

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CHAPTER 3

Hyaluronan as a Biomarker in Prostate Cancer

Abstract

Conventional tumor markers have limited value for diagnosis and treatment in PCa and developing novel circulating tumor markers therefore need to be further explored. HA, is a high molecular weight polysaccharide present in the extracellular matrix (ECM), and has been reported to increase with disease progression across certain cancer types including PCa. Like many ECM molecules, the function of the native HA is altered by fragmentation, which is promoted by oxygen/nitrogen free radicals and release of hyaluronidases within the tumor microenvironment. Smaller HA fragments are pro-inflammatory and activate signaling pathways that promote survival, migration, and invasion within both tumor and host cells through binding to HA receptors such as CD44 and RHAMM. In the present study, we identified HA levels to be a prognostic indicator with increased levels associated with tumor progression in PCa. We then assessed the intracellular and extracellular HA level in PCa cells/media and athymic nude mice xenografted with NB11 and NB26 cells. We further evaluated the HA levels associated with prostate carcinogenesis using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model and tumor samples from human patients. We observed significant increase in HA levels across all PCa models both *in-vitro* and in-vivo. Notably, our *in-vitro* studies showed that both HAS and HYAL enzyme levels were found to be upregulated in cancer cells when compared to normal RWPE cells. Similar increase in HAS and HYAL levels were observed in PCa xenografts, TRAMP animals and human patients. Stable colonies of HAS2/HAS3-silenced PC3 and NB26 cells exhibited a significant decrease in HA levels. Of great importance, HAS2/HAS3 silencing in PCa cells significantly inhibited PCa cell proliferation and invasion. Our observations suggest that inhibiting HAS2 and HAS3 in PCa could be used as a therapeutic target for delaying prostate oncogenesis.

Introduction

PCa is the most commonly diagnosed male malignancy due to its unique and heterogeneous nature. Currently, a major unmet clinical need exists to develop biomarkers that enable indolent disease to be distinguished from aggressive disease. The prostate is an abundant secretor of glycoproteins of all types, and alterations in glycans are, therefore, attractive as potential biomarkers and therapeutic targets. Despite progress over the past decade in profiling the genome and proteome, the PCa glycoproteome remains relatively understudied. [1]. Hyaluronan (HA) is a ubiquitous high molecular weight glycosaminoglycan polymer required for growth, development, cell motility, and cushioning of joints [2, 3]. Elevated levels of HA are associated with various pathologies, such as arthritis, inflammation, and several cancers [4-6], including prostate [7, 8]. Melanoma cells selected for high expression of HA were more metastatic when injected into nude mice than cells that expressed low amounts of HA [9]. Furthermore, overexpression of HA biosynthetic enzymes in tumor cell lines has been shown to increase tumorigenicity and metastatic potential [10, 11].

Steady-state levels of HA are maintained by the cumulative efforts of two families of enzymes: the HA synthases (HAS-1,-2,-3 in mammals) [12] and the hyaluronidases (HYAL-1,-2,-3,-4) [13]. HA synthases are integral membrane enzymes that synthesize HA polymers of 105 to 107 daltons, simultaneously secreting them to the extracellular space as they are polymerized. Retention of HA at the cell surface may result either from association of nascent polymers with the HAS enzyme during actively up-regulated HA synthesis and/or from specific cell-surface HA receptors cross-linking and weaving the polymers into a pericellular matrix [14-16]. Dissolution of HA from the extracellular space requires processing of the polymers into smaller oligomeric

units by the hyaluronidases [17]. The respective functions of enzymes and receptors in maintenance of such matrices are not well understood.

Overexpression of HA synthases in several cell lines has been shown to stimulate tumorigenicity and metastasis [10, 11, 18, 19]. Hyal1 overexpression has been implicated in metastasis [20] and invasive tumor growth [21], and in glioma cells, excess production of HA requires counterbalance by HYAL expression to promote growth [22]. Hyal1 is a secreted protein found in plasma, urine, and intracellularly in lysosomes [17]. Its activity profile is acidic, suggesting it may function with maximal efficacy in acidic microenvironments such as those created locally within developing tumors. HYAL1 converts polymeric HA to oligomeric species, some of which have potent biological function. Depending on molecular mass, the effect of HA exposure to cells can be antiproliferative (polymeric HA) [23], angiogenic (large HA oligomers) [23-26], or apoptotic (small HA fragments) [27, 28]. The outcome of concurrent increased HA production and processing on a tumor cell could therefore vary with differential expression of HAS and HYAL isozymes.

In this report, we studied the production of HA levels in PCa models both *in-vitro* and in-vivo. We observed the presence of increased HA levels associated with tumor progression that was due to upregulation of HAS2 and HAS3 enzymes. The intrinsic rate of proliferation and invasion in PCa cells was significantly reduced by HAS2 and HAS3 antisense inhibition. Here, we conclude that HA levels are increased in PCa and are associated with prostate carcinogenesis. We also report that increased expression of HAS2 and HAS3 with disease progression is responsible for the increased HA levels. Inhibition of HAS2 and Has3 exhibited decreased rate of proliferation and invasion in PCa cells which could be further explored to delay PCa progression.

Results

Intracellular and Extracellular HA levels are upregulated in PCa

To identify new molecular markers in PCa, we designed comprehensive metabolic profiling of NB11 and NB26 cell xenografts. NB11 and NB26 cells are unique tumorigenic PCa cells derived from exposure of non-tumorigenic RWPE1 to N-methyl-N-nitrosourea [29]. We selected these unique PCa cells NB11 and NB26 due to their ability to form rapid and reproducible tumors. Aqueous and organic extracts were isolated from the tumor tissues and analyzed using HPLC-ESI-MS in both positive and negative scan modes. The total ion chromatogram (TIC) resulted in excellent separation between the two tissue groups (n=6). The XCMS online analysis of the TIC revealed a list of newly identified metabolites. The quasimolecular ions were confirmed and the exact masses of the monoisotopic molecular weights were used to search the METLIN database. Total metabolites identified in NB11 cell xenografts were 2086. Similarly, a total of 2203 metabolites were identified in NB26 cell xenografts.

To obtain a comprehensive global metabolic signature for this discovery based approach, we considered a liberal p-value of 0.1 as significant for this dataset. Among identified metabolites, an m/z of 776.25 stood out as a single metabolite HA. The relative abundance when plotted revealed increased abundance in both xenograft groups while NB26 xenografts had a higher abundance when compared to NB11 xenograft group (**Figure 3.1A**). To validate this increased abundance of HA, we next performed immunofluorescence staining for HA in NB11 and NB26 tumor tissues. However, as previously noted, we observed that NB26 xenografts had higher HA levels when compared to the NB11 group. This data suggests that HA levels increase with progression as NB26 cells are more malignant than NB11 cells (**Figure 3.1B**). To further

confirm our finding, we did a comparative immunofluorescence staining for HA in PCa cells normal RWPE cells. We observed that PCa cells showed increased abundance of HA when compared to normal cells. NB11, NB26, PC3, DU145 and 22Rυ1 cells showed increased HA staining as compared to the normal RWPE cells (**Figure 3.1C**). Next, we evaluated intracellular and extracellular HA levels using a solid phase human & mouse sandwich HA ELISA in cells, media, mouse serum and human patient serum. Our *in-vitro* data showed that all PCa cells have significant higher intracellular HA levels when compared to normal RWPE cells. Similarly, secreted HA levels were significantly higher in cell media (**Figure 3.1D**). Secreted HA levels in mouse and human patient serum were significantly lower in non-diseased (hyperplasia) stage but significantly increased with progression during various stages of PCa (**Figure 3.1E**). Next, we performed immunohistochemistry for HA on wild-type and transgenic TRAMP animal tissues to investigate HA levels with progression of disease from non-diseased (8-week) to diseased (24-week) stage and compare them to their respective wild-type controls. The transgenic TRAMP animals showed similar HA expression at 8-weeks in both groups but the 24-week transgenic animals showed a much higher expression as compared to its respective wild-type control. A representative picture is shown (**Figure 3.1F**). Similarly, in human patient samples, we found the tumor tissue obtained from diseased patients have relative much higher abundance of HA when compared to their respective normal tissue. A representative picture of HA abundance in two human patients is shown (**Figure 3.1G**).

HAS2 and HAS3 are upregulated in PCa

To investigate the levels of HA synthases and their effect on HA levels, we evaluated the mRNA levels of HAS enzymes in all MNU and PCa cells and compared them to the normal RWPE cells. We observed that MNU cells (NA22, NB14, NB11 and NB26) and PCa cells (DU145, PC3

and LnCAP) had higher HAS2 and HAS3 mRNA expression when compared to the normal RWPE cells except 22Rυ1 cells where the expression was unchanged. Previous studies in breast cancer has reported HAS1 to maintain baseline HA levels and in PCa cells was found to be not significantly affected as compared to HAS2 and HAS3 (**Figure 3.2A**). The changes in transcriptional levels of HAS2 and HAS3 (data not shown) were reflected in protein expression which was markedly upregulated in both MNU and PCa cells (**Figure 3.2B**).

Immunohistochemical analysis of NB11 and NB26 xenograft tissues also revealed a similar increase in HAS2 and HAS3 mRNA levels (**Figure 3.2C**). We further examined the HAS2 and HAS3 mRNA expression in wild-type (non-diseased) and transgenic TRAMP (diseased) animals. We observed that the mRNA expression of HAS2 and HAS3 is significantly upregulated in transgenic TRAMP animals and increases even further with disease progression when compared to their wild-type animals (**Figure 3.2D**). The changes in transcriptional levels of HAS2 and HAS3 were reflected in protein expression which was also increased in transgenic animals at 20 and 24-weeks (**Figure 3.2E**). Next we observed a similar amplification of HAS2 and HAS3 at both transcriptional and translation levels in cancer patients when compared to their respective normal patients (**Figure 3.2F**).

HYAL1, HYAL 2 and HYAL4 are upregulated in PCa

The role of HYALs is to break the high molecular weight HA into smaller fragments. This is important to maintain a constant HA turnover in the body with constant synthesis and degradation. qPCR analysis of MNU and PCa cells for HYAL1-4 exhibited that HYAL1 and HYAL4 are significantly upregulated among the others (HYAL2 and HYAL3) when compared to the normal RWPE cells (**Figure 3.3A**). To further confirm our in-vitro data, we estimated the

HYAL protein expression in normal vs cancerous human patients. We observed that HYAL1, HYAL2 and HYAL4 were significantly upregulated in cancerous human patients (representative picture shown from one patient) when compared to the respective normal controls (**Figure 3.3B**). The changes in transcriptional levels in human patients were reflected in protein expression of HYAL2 and HYAL4 which were significantly augmented in cancer patients when compared to their respective normal human tissue samples (**Figure 3.3C**).

HAS2 and HAS3 silencing decreased cell proliferation and invasion of PCa cells

Next, we evaluated HAS2 and HAS3 mRNA expression in PC3 and NB26 HAS2 and Has3 knock-out cells (data not shown). As expected, silencing HAS2 in PC3 cells resulted in decreased mRNA expression of HAS2 gene when compared to the scrambled. Similarly, upon HAS3 silencing, we observed a marked downregulation of HAS3 mRNA expression in PC3 cells. However, an interesting observation was that upon silencing HAS2 gene, the expression of HAS3 gene significantly increased in PC3 cells. Likewise, upon HAS3 silencing, we observed a marked increase in HAS2 gene expression. Similar effect of HAS2 and HAS3 silencing in PC3 cells were observed in another NB26 PCa cells (**Figure 3.4A**). The changes in transcriptional levels were reflected in protein expression of HAS2 and HAS3 in PC3 cells (**Figure 3.4B**). We presume that a complementary mechanism comes into effect that alleviates the burden of HA synthesis on one gene in the absence of the other.

To investigate the functional importance of the HAS genes, we performed cell proliferation and invasion assays. Our data shows that in HAS2 knock-out PC3 cells, we observed a significant decrease in cell proliferation and invasion. Similar effect of decreased proliferation and invasion was observed in another HAS2 knock-out NB26 cells (**Figure 3.4C**).

Taken together, these findings suggest the HAS2 and HAS3 genes are associated with important cellular events like proliferation and invasion that are normally associated in cancer cells with increased HA levels.

Discussion

Employing *in-vitro* and *in-vivo* PCa models, we report that HA levels are increased and significantly correlate with PCa progression. This finding was further validated from serum and tissues from human patients with PCa where significantly higher levels of HA were observed in tumors when compared to the adjacent normal tissues. There are many conflicting opinions about the significance of large accumulation of HA in tissues as well as serum and its possible role in cancer (31). In addition, the functional importance of HA synthesis enzymes is unknown with context of PCa progression. Our study indicated that the enzymes HAS2 and HAS3 which are primarily responsible for the HA synthesis are upregulated in PCa and play a crucial role in cellular events such as proliferation and invasion associated with prostate carcinogenesis. In PCa, the burden of HA synthesis falls upon HAS2 and HAS3. Increased HA synthesis is normally associated with increased HYAL activity with disease progression. The HYALs are responsible for the degradation of high-molar-mass (HMM)-HA, that are synthesized at a steady state in the biological system into smaller fragments. While large molecules of HA promote tissue integrity and quiescence, the generation of breakdown products enhances signal transduction, contributing to the pro-oncogenic behavior of cancer cells (33). Interestingly, our data clearly shows increase in HYAL levels across all PCa progression model as well as in tumors of humans compared to the adjacent normal tissue. On basis of these findings, we speculated that the simultaneous increase in HA synthesis and degradation with PCa progression results in a constant turnover of small molecular weight fragments of HA. These low molecular weight HA are known to bind with HA receptors such as CD44, LYVE1, RHAMM etc and activate the downstream signaling leading to unfavorable cellular events like invasion, proliferation and EMT that are common in cancer cells as compared to normal cells.

To further explore the increased HA levels in PCa, we investigated the underlying role of HAS2 and HAS3 in PCa. HAS1 is reported to maintain basal level of HA synthesis in breast cancer but did not alter significantly as compared to HAS2 and HAS3 in PCa (33). HAS2 is catalytically active and reported to be the most important HA synthase enzyme that synthesizes HA, predominantly HMM-HA. HAS3 on the other hand, is reported to synthesize medium range HA and appears to play minor role as recently studied in other cancers (33). Our data suggests that silencing HAS2 and HAS3 in NB26 and PC3 cells results in altered morphogenesis, decreased cell proliferation and cell invasion. HAS2 silencing in both PC3 and NB26 cells resulted in significant suppression in cell invasion when compared to HAS3 silenced cells. Interestingly, HAS3 silencing resulted in a significant suppression in cell proliferation rate when compared to the HAS2 silenced PCa cells suggesting diverse functions of these genes in PCa.

In summary, the present study provides valuable data supporting the role of HA in progression of PCa. Furthermore, our observations in human patients tumor samples and serum, revealed higher expression of HA synthase enzymes (HAS2 and HAS3) as well HA degradation (HYAL1, HYAL2 & HYAL4). Similar data in other PCa models suggests a possible role of high HA turnover associated with disease progression. HAS2 and HAS3 inhibition significantly affected PCa cell proliferation and invasive potential. This data suggests diverse function of these genes and can be explored further in PCa prevention and treatment. However one limitation of this study was that we were unable to determine the exact size of HA molecules upon HAS2 and HAS3 silencing, which can hold significant relevance on understanding the functional role of these genes and correlate them with PCa progression. Future experiments on examining the size analysis of HA fragment are planned in our laboratory. Overall, this study

provides valuable data showing increased HA levels that can possible serve as a biomarker in PCa prognosis and HAS2 and HAS3 as genes involved in prostate oncogenesis.

Materials and Methods

Materials

Antibodies were obtained from Cell Signaling Technology (Danvers, MA), Abcam (Cambridge, MA) and Santa Cruz Biotechnology (Dallas, TX) and a list is provided in **Table 3.A**. Quantitative PCR (qPCR) primers were synthesized and obtained from DNA synthesis laboratory at the University of Wisconsin Biotechnology Center. The sequences of oligonucleotides used are listed in **Table 3.B**. Puromycin antibiotic for stable cell selection was obtained from Sigma (St. Louis, MO).

Plasmids

Control (sc-108060), HAS2 (sc-45328-SH) and HAS3 shRNA (sc-45295-SH) plasmids were obtained from Santa Cruz Biotechnology (Dallas, TX) and used as per manufacturer's protocol.

Animals

TRAMP animals were obtained as described previously [30]. Housing and care of the animals was approved by the University of Wisconsin's Research Animal Resource Committee in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Cell culture

Non-tumorogenic RWPE1 (CRL-11609), tumorogenic NB11 (CRL-2851) and NB26 (CRL-2852) cell lines were directly obtained from ATCC (Manassas, VA) in 2013 while PC3 (CRL-1435), DU145 (HTB-81), LnCAP (CRL-1740) and 22Rυ1 (CRL-2505) cell lines were obtained in 2012. ATCC ensures cell lines authenticity using morphology, karyotyping, and PCR based approaches which includes assays to detect cytochrome C oxidase I gene (COI analysis) to rule

out inter-species contamination and short tandem repeat (STR) DNA profiling to rule out intra-species contamination. Cell lines were immediately resuscitated upon receipt and frozen in aliquots in liquid nitrogen. Once thawed, early passage cells were cultured within 3 months from a frozen vial of the same batch of cells. Cells were routinely tested to ensure there was no mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza). RWPE1, NB11 and NB26 cells were cultured in KSFM from Thermo-Fisher Scientific (Grand Island, NE), supplemented with HKGS and 1% penicillin–streptomycin. PC3, LnCAP, 22Rυ1 and DU145 cells were cultured in RPMI 1640 from Gibco (Carlsbad, CA), with 10% FBS and 1% penicillin–streptomycin. The cells were then incubated at 37 °C with 5% CO2 in a humid environment. Upon 80-90% confluency, cells and media were harvested for further studies.

Western blotting

Whole cell lysates from cells. tumor tissues and prostate were prepared and western blot analysis was performed as described previously [31]. Densitometric measurements of the bands were done with image analysis software using the Biorad ChemiDoc MP imaging system.

HA ELISA

Human and Mouse HA ELISA kit were obtained from TSZ ELISA (Waltham, MA). HA levels in mouse tissues, prostate, serum, human PCa cells, cell media and human serum were measured according to the manufacturer's protocol.

RNA isolation and qPCR analysis

Total RNA was extracted from cells and animal tissues using RNeasy kit (Qiagen, Germantown, MD), and reverse transcribed with iScript Reverse transcription supermix kit (Biorad, Hercules,

CA). cDNA was amplified using gene specific primers as per the protocol described previously [32].

Immunoflourescence analysis

RWPE, NB11, NB26, PC3, 22Rυ1 and DU145 cells and xenograft animal slides were incubated with HA antibody as per protocol as described previously [32]. After rinsing in PBST, slides were incubated with fluorescent-conjugated secondary antibody at 1:500 dilution in blocking buffer. Slides were then rinsed in PBST, and sections were mounted with ProLong Gold Antifade reagent containing DAPI (Invitrogen) and left in dark overnight. Slides were imaged with the Andor Revolution XD spinning-disk confocal microscope using a 20X/40X/1.4 NA oil objective with identical exposures and gains for each antibody stained.

Immunohistochemistry analysis

Immunocytochemical analysis of NB11 and NB26 xenograft tissue sections were done as per protocol described previously (33).

Cell Proliferation and Invasion Assays

QCM ECMatrix cell invasion assay (colorimetric; ECM550) was obtained by EMD Millipore (Billerica, MA). The BrdU assay was obtained from Cell Signaling (Danvers, MA). All assays were used as per manufacturer's instructions. Absorbance was read at respective wavelengths using a Synergy 2 multi-detection microplate reader (BioTek, VT).

Transfection and generation of stable antisense HAS transfectants PCa cells

For plasmids transfections, NB26 and PC3 cells were transfected with 15μg each of control shRNA (scrambled), HAS2 shRNA and HAS3 shRNA using nucleofection technology, Lonza

(Anaheim, CA) and allowed to recover in complete standard culture medium for 24-48 hours before assaying. Prior to using puromycin antibiotic, we titrated the selection agent (0-0.5-1.0-2.5-5.0 μg/ml) to determine the optimal dose (0.5 μg/ml) where 100% of non-transfected cells were killed. For generating stable colonies of HAS2/HAS3 transfected cells of NB26 and PC3 cells were cultured in 0.5 μg/ml puromycin supplemented medium every 48 hours for the next 28 days to obtain stable transfected cells. After 28 days, the silencing of the gene of interest was verified in both cell lines by estimating the expression of HAS2 and HAS3 RNAs and protein via RT-PCR and western blotting. Once established, stable transfectants were maintained in the parental medium supplemented with 0.5 μg/ml puromycin for further studies.

HPLC-ESI-MS analysis

Aqueous and organic metabolites were isolated from NB11 and NB26 tumor xenograft tissues using a methanol/water and dichloromethane/water extraction as described previously [33]. Metabolite extracts (10 μL) were then diluted to 50 μL with 0.1% formic acid in water. Untargeted metabolomics using HPLC-ESI-MS was performed with a 10uL injection on an Agilent 6210 ESI-TOF mass spectrometer with Agilent 1200 series HPLC (Santa Clara, CA). Solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The HPLC column was an Agilent Zorbax SB C18 1.8um, 2.1mm i.d. x 50 mm length (Santa Clara, CA). Metabolites were gradient-eluted at a flow rate of 0.25 mL/min starting at 2%B with a 1 minute hold, ramping to 50% B at 35 min, then to 95% B at 40 min, returning to 2% B at 42 min, and re-equilibrating at 2%B for 18 min. Electrospray was performed in positive-ion (+) mode at 3.6kV and fragmentor at 130V. Metabolite detection was performed over the m/z range 50-1700 by summing 10013 transients/scan (0.89 scans/sec). Blank injections (10 μL) of 20% MeOH, 0.08% formic acid in water were made between sample injections.

Raw data acquired using HPLC-ESI-MS system was processed by Xcalibur software which provides an appropriate format for further data analysis. The structured data files were then uploaded on XCMS Online (https://xcmsonline.scripps.edu), a high-quality cloud-based platform linked with METLIN (https://metlin.scripps.edu), to facilitate metabolite identification using a repository of metabolite and tandem mass spectrometry information of known compounds.

In-vivo tumor xenograft model

7-8 week old athymic (nu/nu) male nude mice (Harlan, USA) were xenografted as previously described [32]. We selected unique PCa cells NB11 and NB26 due to their ability to form rapid and reproducible tumors. Each group had 12 animals as replicates. All animals were sacrificed when tumors reached a volume of 1200 mm^3. Samples were collected and stored at -200^oC until further analysis.

Spontaneous PCa progression (TRAMP) model

Eight-week-old wild-type and transgenic TRAMP mice (n=30) were randomly divided into two groups for 5 different time points from 8-12-16-20-24 weeks. Each time point/group contained 3 animals respectively. The first group of wild-type (n=15) animals served as the un-diseased control. The second group served as diseased animals with PCa progression from 8-24 weeks respectively. Throughout the experiment, the animals had access to chow diet ad libitum. Animals in all groups were observed weekly for body weight, tumor progression by abdominal palpation and survival. At the termination of the experiment at their respective time points, blood samples were collected by “mandibular bleed” and serum was separated and stored at -20^oC until further analysis. The prostate was excised under a dissecting microscope and snap frozen in liquid nitrogen for further analysis.

Statistical analysis

Microsoft Excel software was used to calculate the mean and standard error of the mean (SEM). For the HPLC-ESI-MS metabolomics study, a liberal p value of 0.1 was considered significant. Two-tailed, Student's t-test was used to assess statistical significance. Data points in all the other studies represent mean ± SEM, and p values < 0.05 were considered significant.

Figure 3.1A

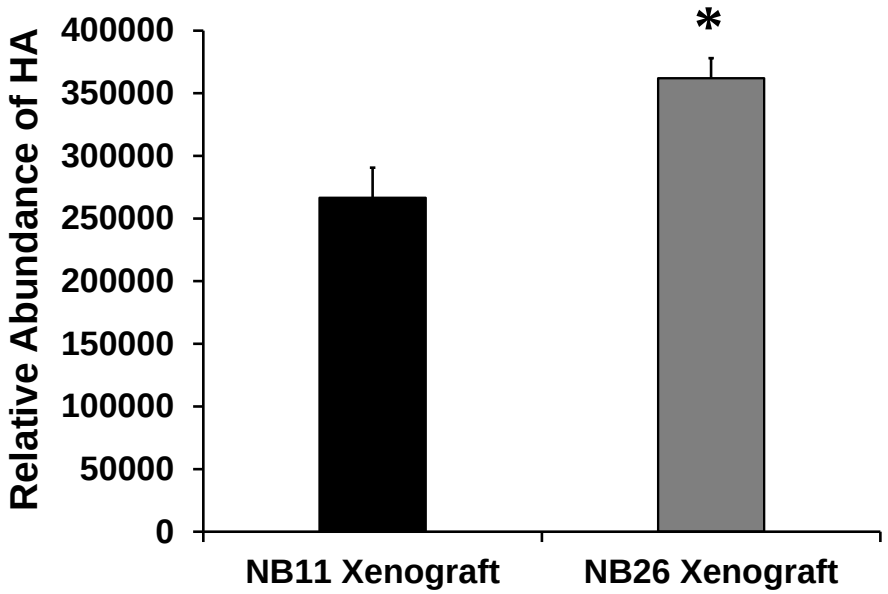

Figure 3.1A. Comparative plot of relative abundance of HA between NB11 and NB26 xenografts. Error bars represent mean±SEM among six biological replicates.

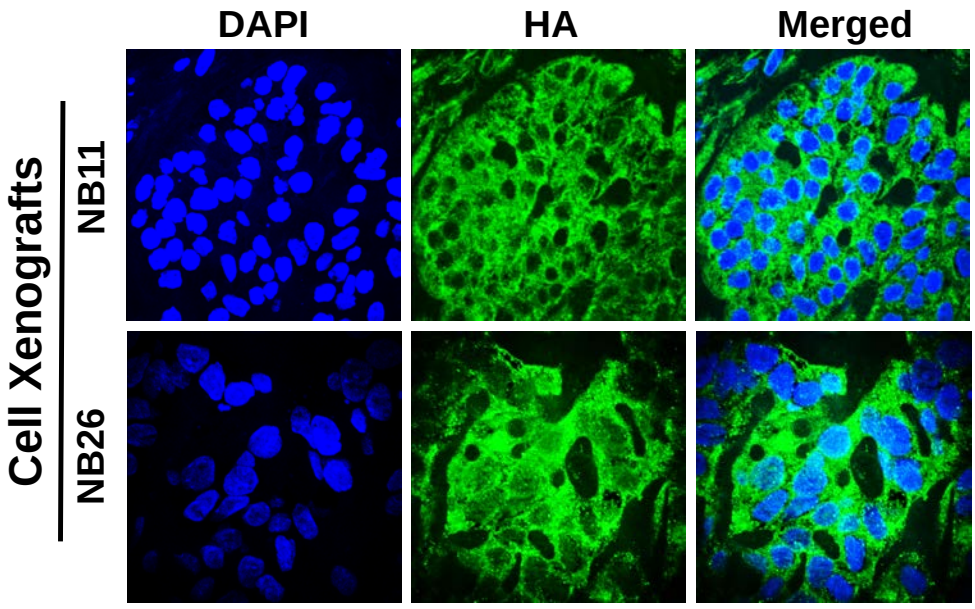

Figure 3.1B. Representative images showing immunofluorescence for HA in NB11 and NB26 xenografts. Tumor tissues were harvested and each of the six biological replicates was performed in triplicates for HA staining. Images were captured by a confocal microscope as described in materials and methods. Scale bar, 30 μm. Magnification for NB11 images are at 20X and NB26 images at 40X. DAPI was used as a nuclear staining control.

Figure 3.1C

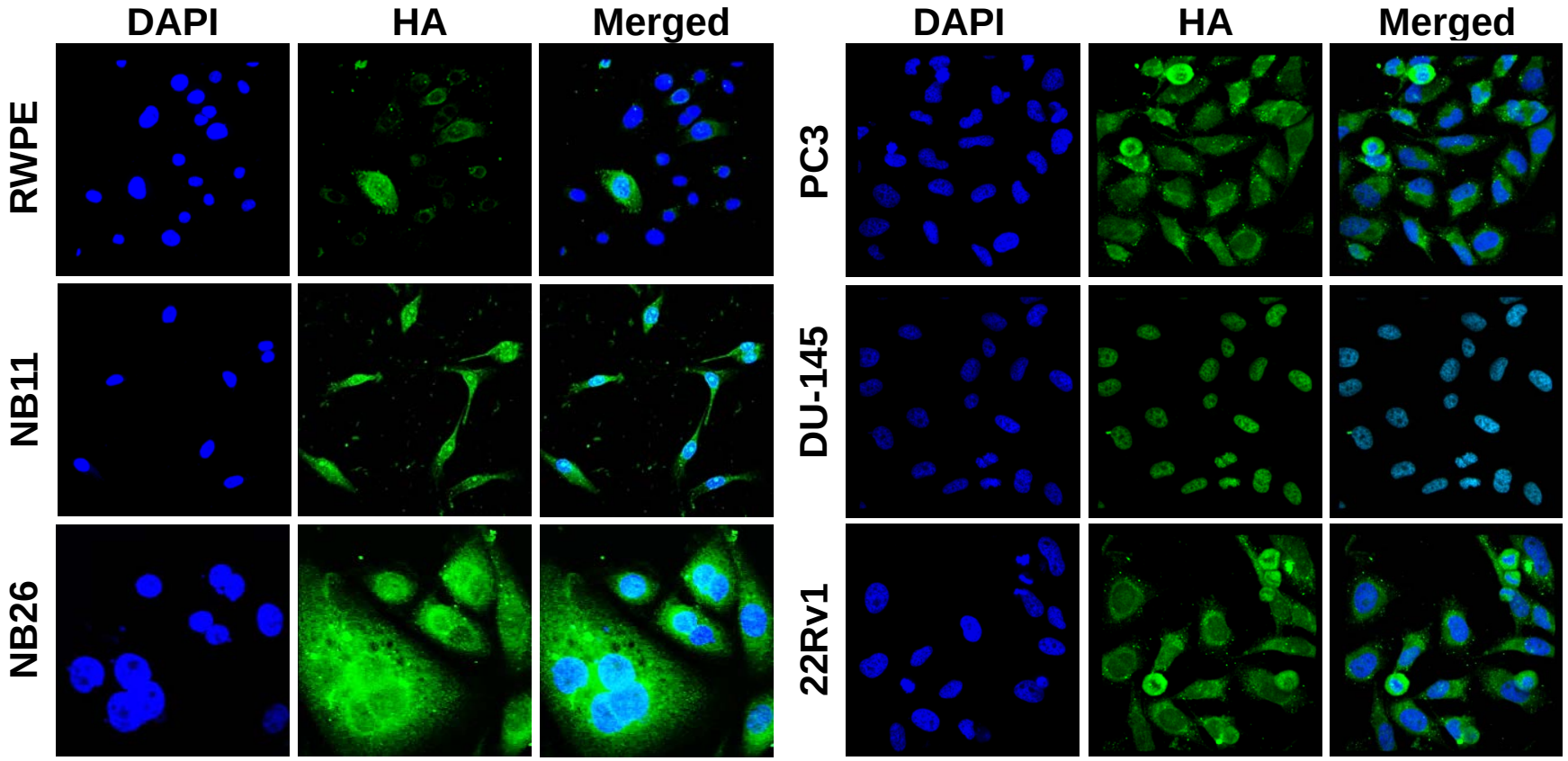

Figure 3.1C.Representative images showing immunofluorescence for HA in RWPE (normal), NB11, NB26, PC3, 22Rv1 and DU145 PCa cells. Three biological replicates for each were subjected to staining and studied in triplicate. Images were captured by a confocal microscope as described in methods. Scale bar, 30 μm. Magnification for RWPE and NB11 images are at 20X and for NB26, PC3, 22Rv1 and DU145 images at 40X. DAPI was used as a nuclear staining control.

Figure 3.1D

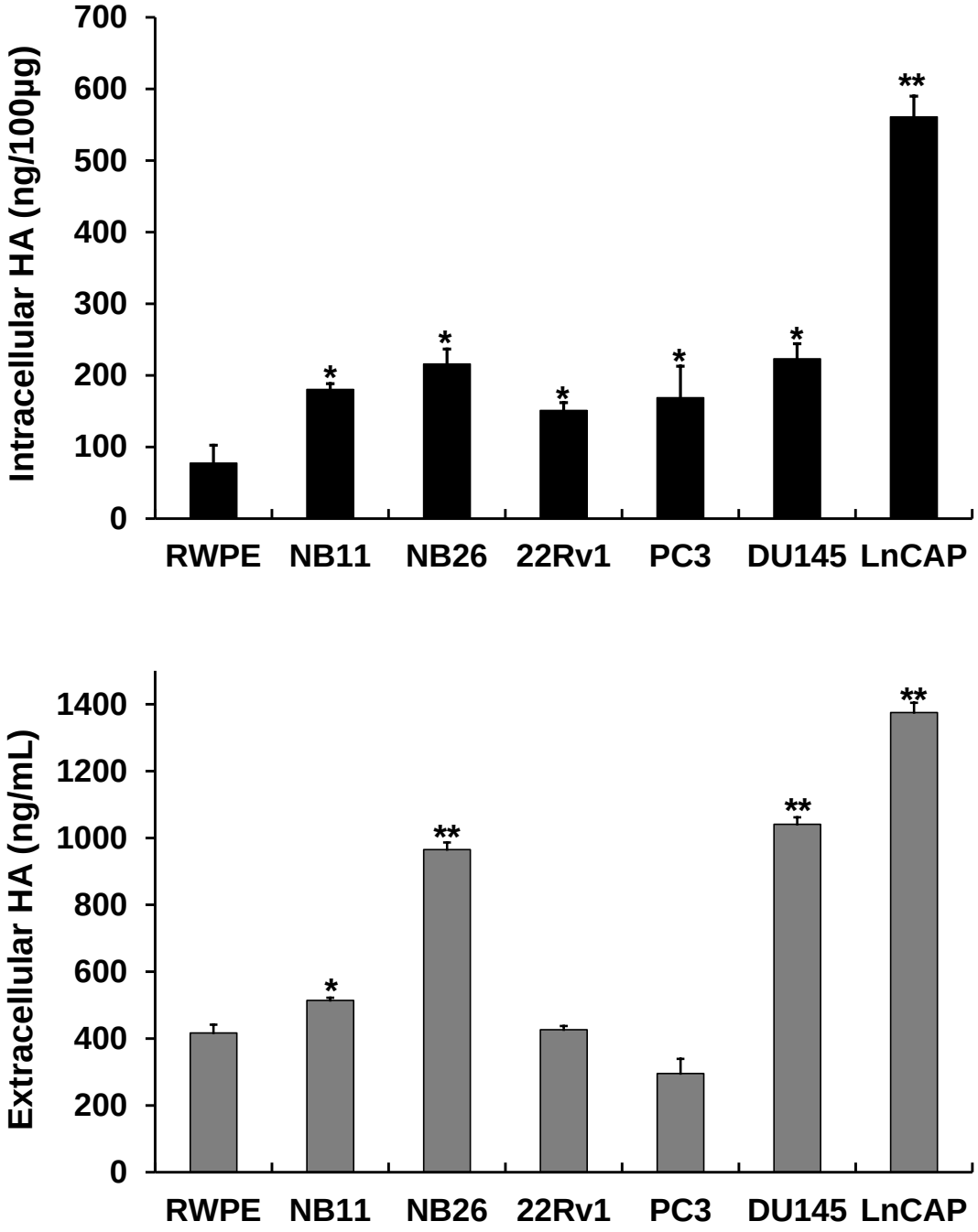

Figure 3.1D. Histogram showing intracellular (top) and extracellular (bottom) HA levels in NB11, NB26, 22Rv1, LnCAP, PC3 and DU145 cells. Error bars represent mean±SEM among three independent experiments/group and each of the three biological replicate was performed in triplicate to measure HA levels using ELISA (*P $\leq$ 0.05; **P $\leq$ 0.01). All intracellular samples were normalized using 100 μg protein of starting whole cell lysates. Likewise, all extracellular samples were normalized using 1 ml of collected cultured media.

Figure 3.1E

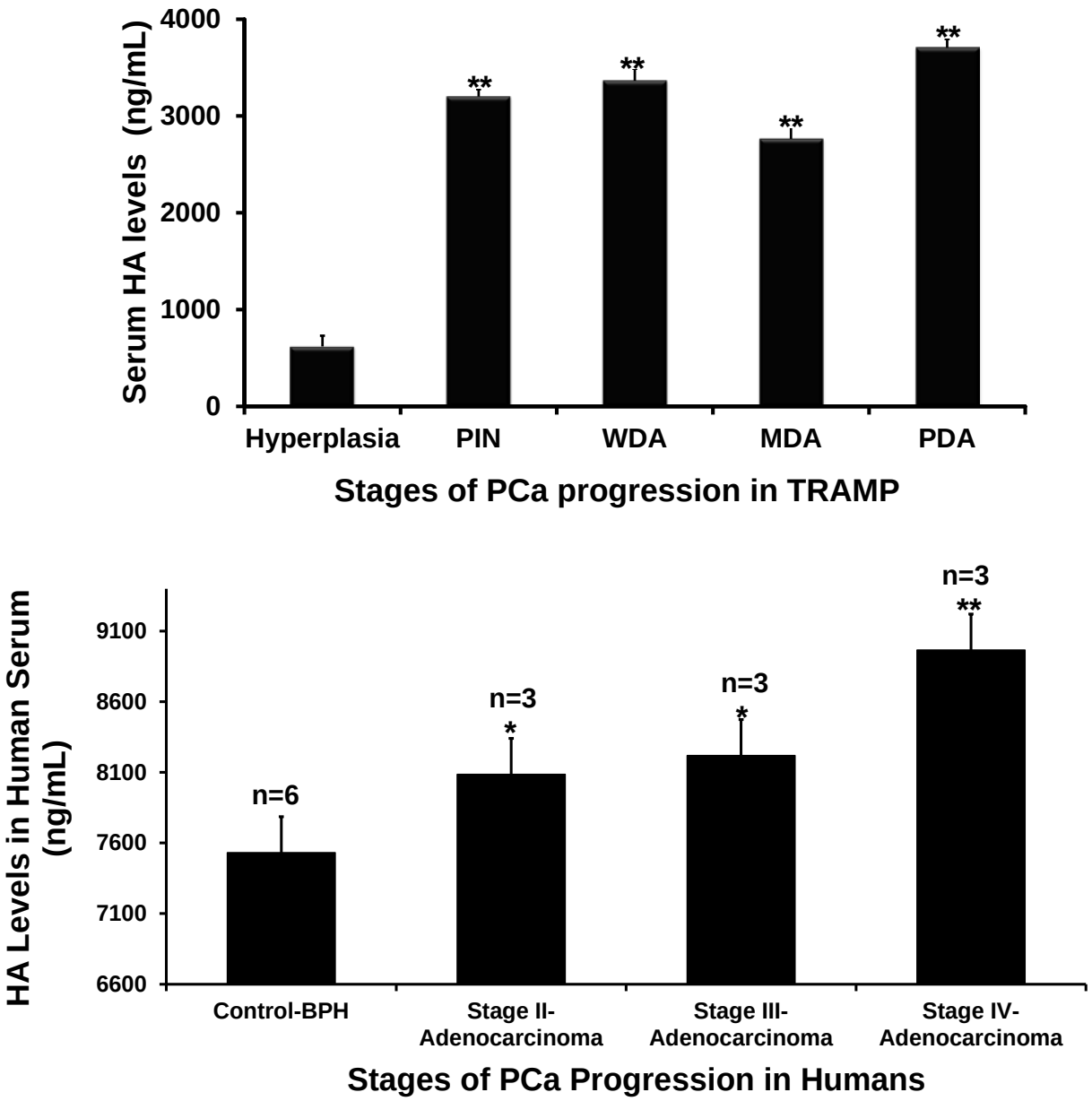

Figure 3.1E. Histogram showing serum HA levels secreted in TRAMP mice serum (top) and human patients (bottom) with increasing stages of PCa progression. PIN-prostatic intraepithelial neoplasia, WDA-well differentiated carcinoma, MDA-moderately differentiated carcinoma, PDA-poorly differentiated carcinoma. Error bars represent mean±SEM among three biological replicates/group and each biological replicate was performed in triplicate for HA levels using ELISA ((*$P \leq 0.05$;**$P \leq 0.01$). All secreted HA were normalized using 1 ml of mouse and human serum.

Figure 3.1F

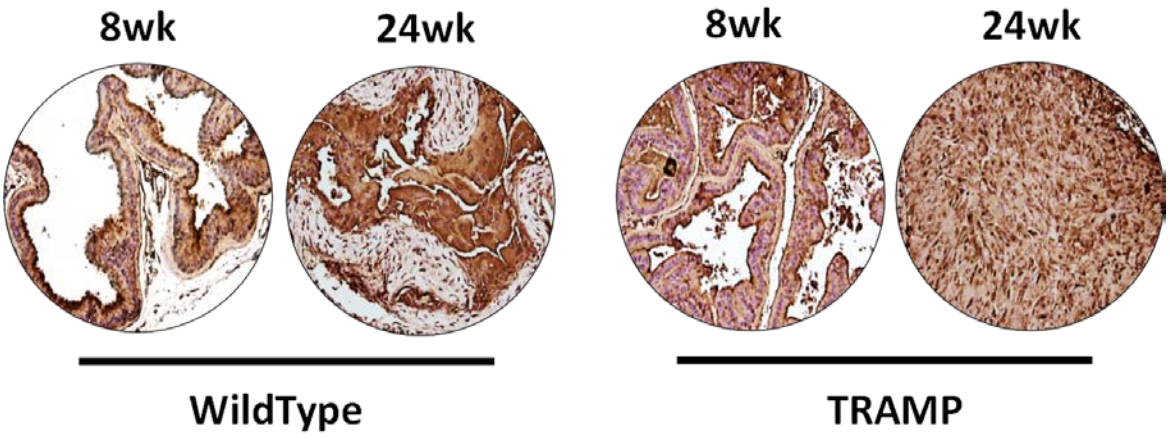

Figure 3.1F. Representative photomicrographs comparing immunohistochemical staining for HA in wild-type (non-diseased) and transgenic TRAMP (cancerous) animals at 8 & 24 weeks. Three biological replicates for each were subjected to staining and performed in triplicate. Magnification: 20X for all images.

Figure 3.1G

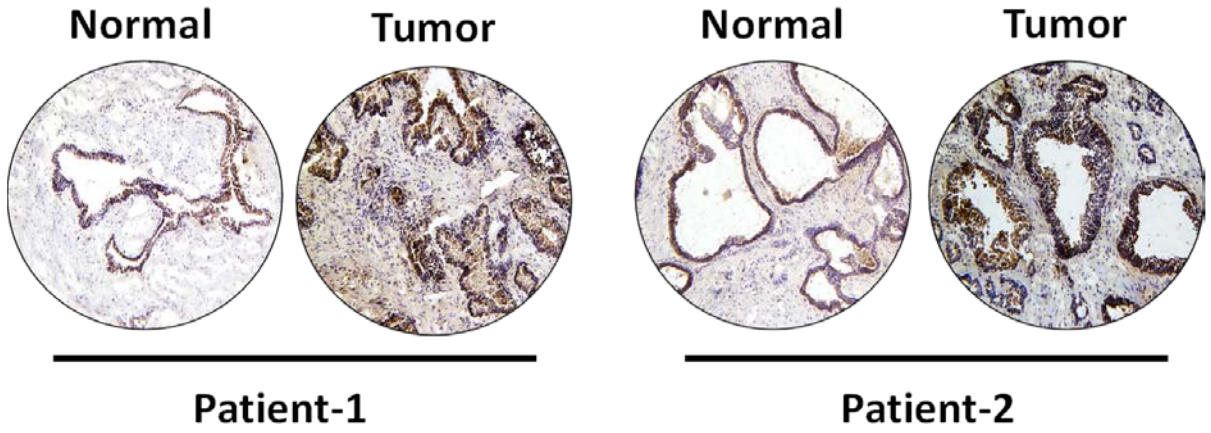

Figure 3.1G. Representative photomicrographs comparing immunohistochemical staining for HA in normal tissues (non-diseased) and tumor tissues (cancerous) in two human patients. Three biological replicates for each were subjected to staining and performed in triplicate. Magnification: 20X for all images.

Figure 3.2A

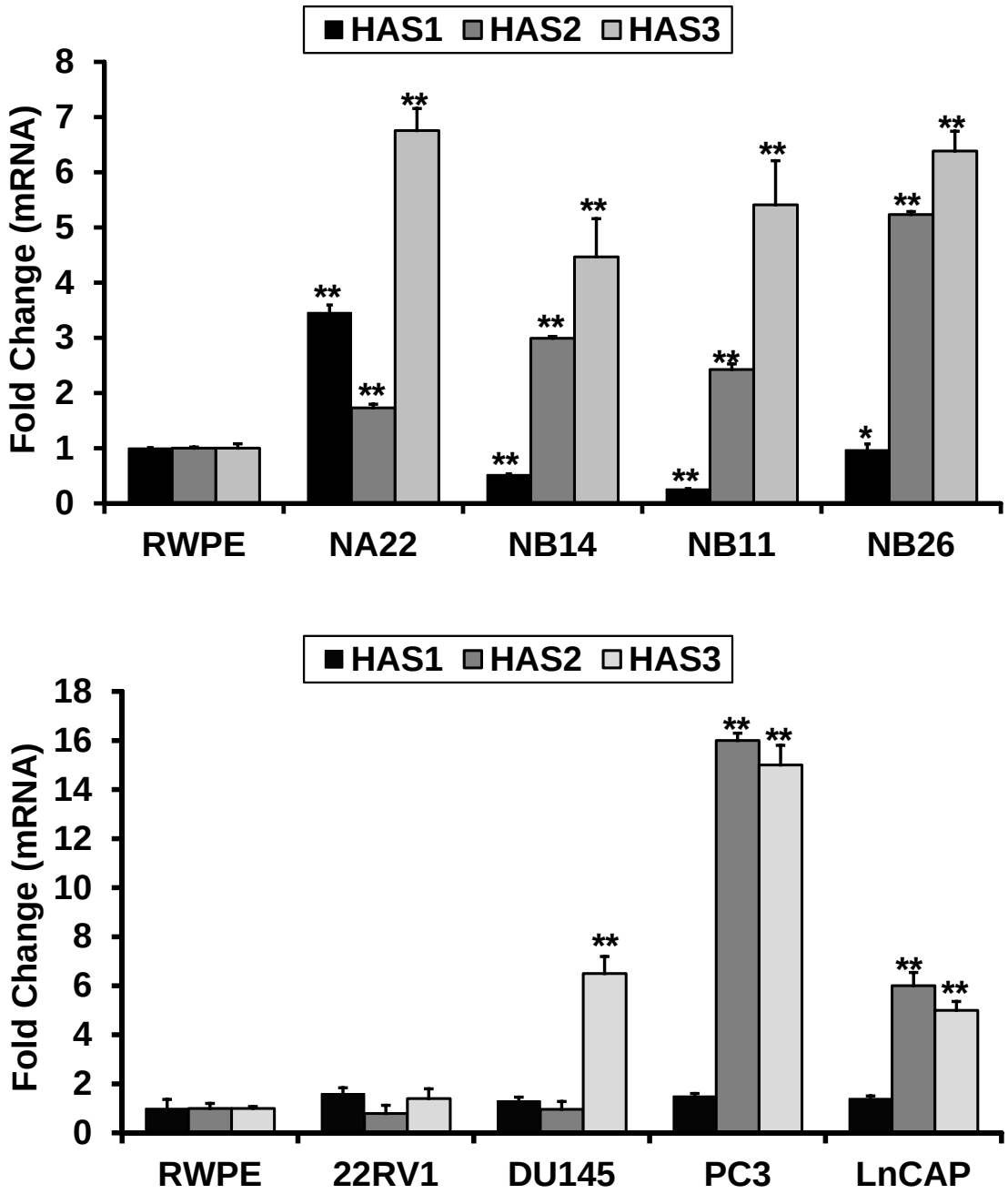

Figure 3.2A. Histograms represent relative HAS (1, 2, & 3) mRNA expression in RWPE (normal), MNU cells (NA22, NB14, NB11 and NB26) and PCa cells (22Rv1, DU145, PC3 and LnCAP). Gene expression was measured at 6 hour by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate. Statistical difference was seen in gene expression when compared to the respective control RWPE (*$P \leq 0.05$, **$P \leq 0.01$).

Figure 3.2B

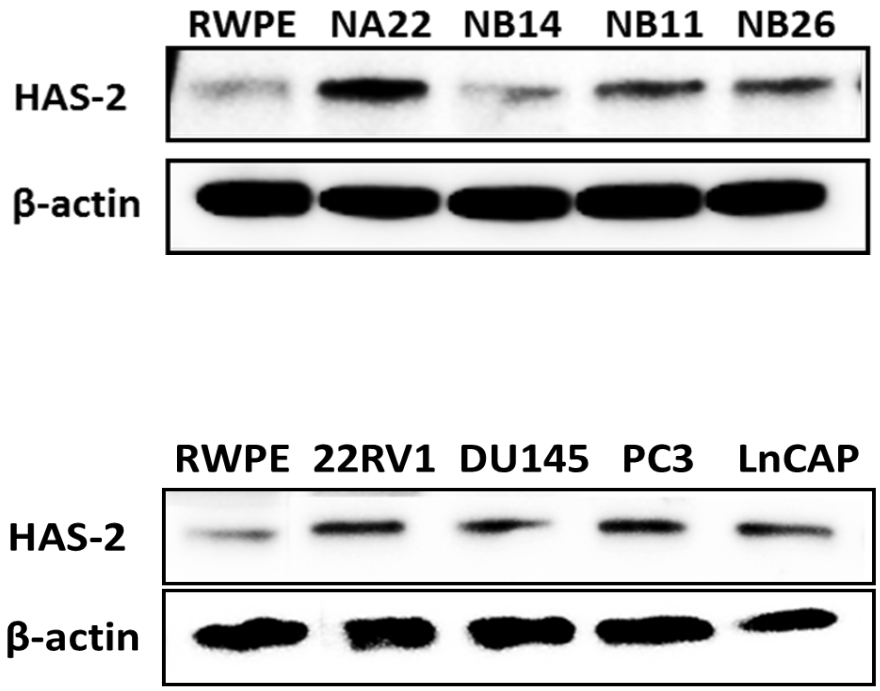

Figure 3.2B. Immunoblot images of HAS2 protein expression in RWPE (normal), MNU cells (NA22, NB14, NB11 and NB26) and PCa cells (22Rv1, DU145, PC3 and LnCAP). Three independent experiments were performed and each experiment was analyzed in triplicate. β-actin was used as a loading control.

Figure 3.2C

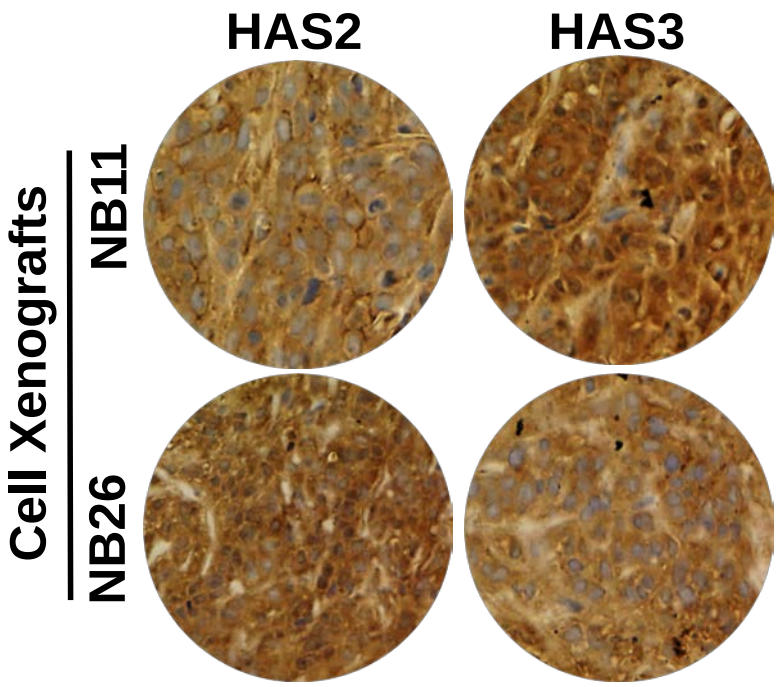

Figure 3.2C. Representative photomicrographs showing immunohistochemical staining for HA in NB11 and NB26 xenograft tissue sections. Three biological replicates for each were subjected to staining and performed in triplicate. Magnification: 40X for all images

Figure 3.2D

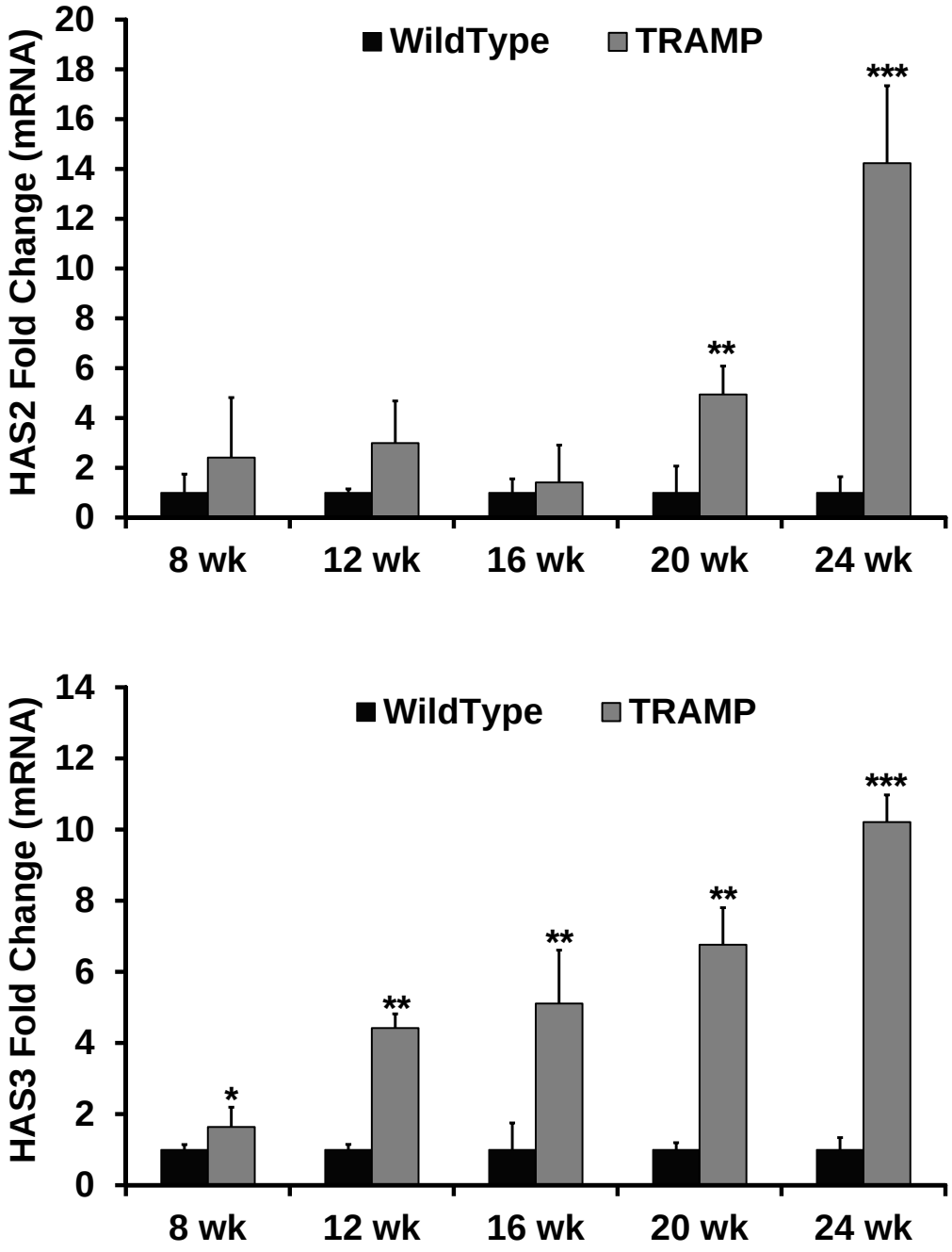

Figure 3.2D. Histograms represent relative HAS2 & HAS3 mRNA expression in wild-type (non-diseased) and transgenic TRAMP (cancerous) animals with disease progression (8-12-16-20-24 weeks). Gene expression was measured by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate. Statistical difference was seen in gene expression when compared to their respective wild-type controls (*P $\leq$ 0.05, **P $\leq$ 0.01).

Figure 3.2E

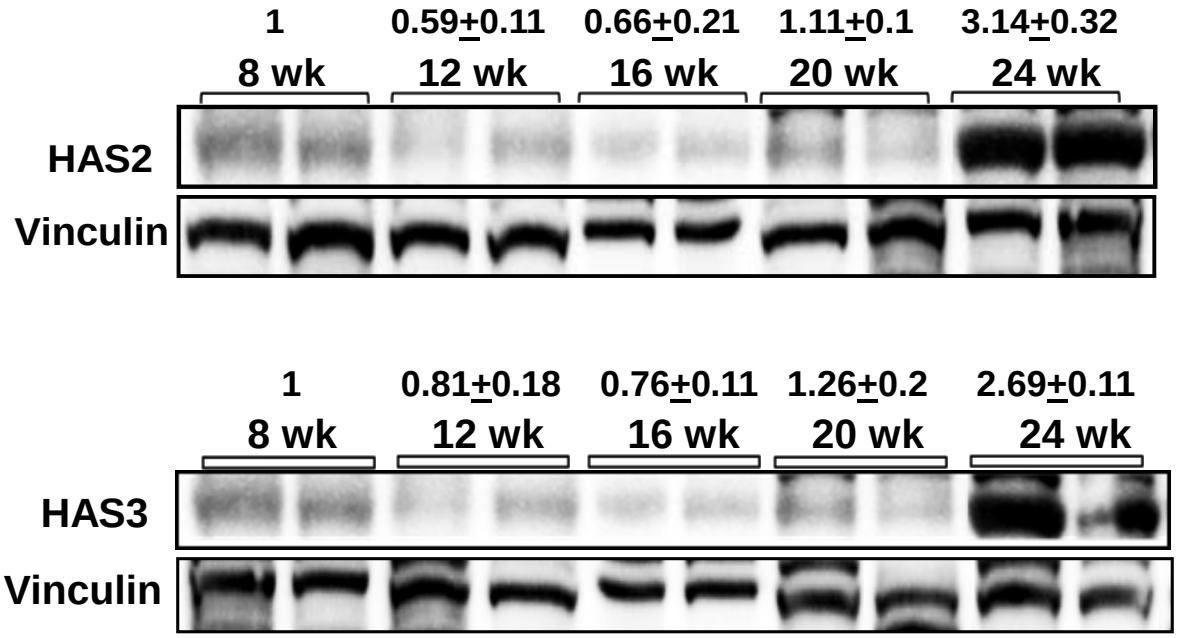

Figure 3.2E. Representative immunoblot images showing HAS2 and HAS3 protein expression in transgenic TRAMP animals with disease progression (8-12-16-20-24 weeks). Three independent experiments were performed and each experiment was analyzed in triplicate. For every time point, two mice blots are shown as representative images. Vinculin was used as a loading control. Quantitative densitometry was analyzed with vinculin expression and can be seen above the time points as measurements normalized to 8-week animals.

Figure 3.2F

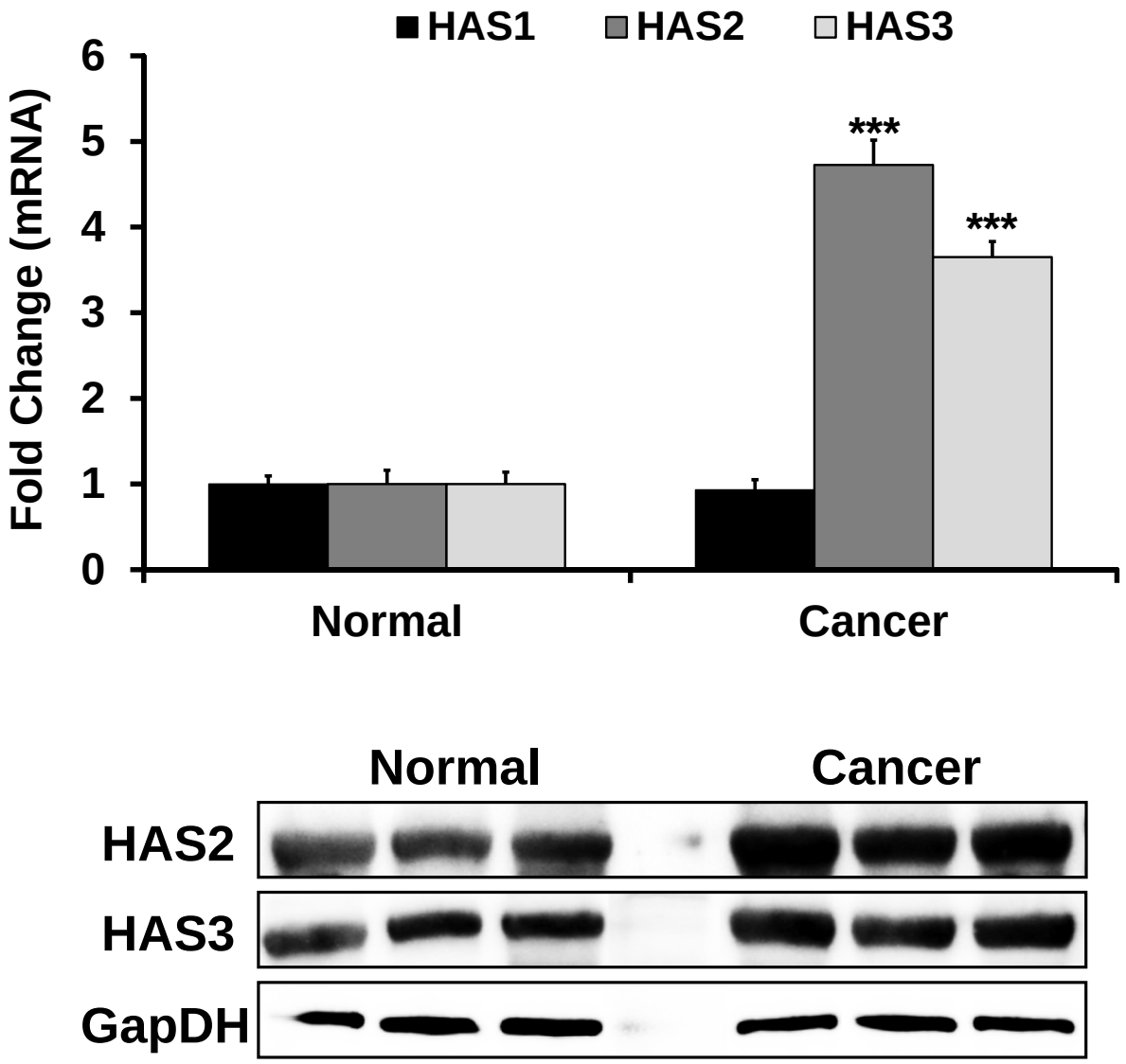

Figure 3.2F. Histograms represent relative HAS (1, 2, & 3) mRNA expression (top) and immunoblot images showing HAS2 and HAS3 protein expression in normal vs tumor tissues in human patients. Gene expression was measured by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three biological patients and each experiment was performed in triplicate. Statistical difference was seen in tumor tissues when compared to their respective control (***$P \leq 0.001$). Immunoblot images are from three different biological replicates, each performed in triplicates and a representative blot is shown.

Figure 3.3A

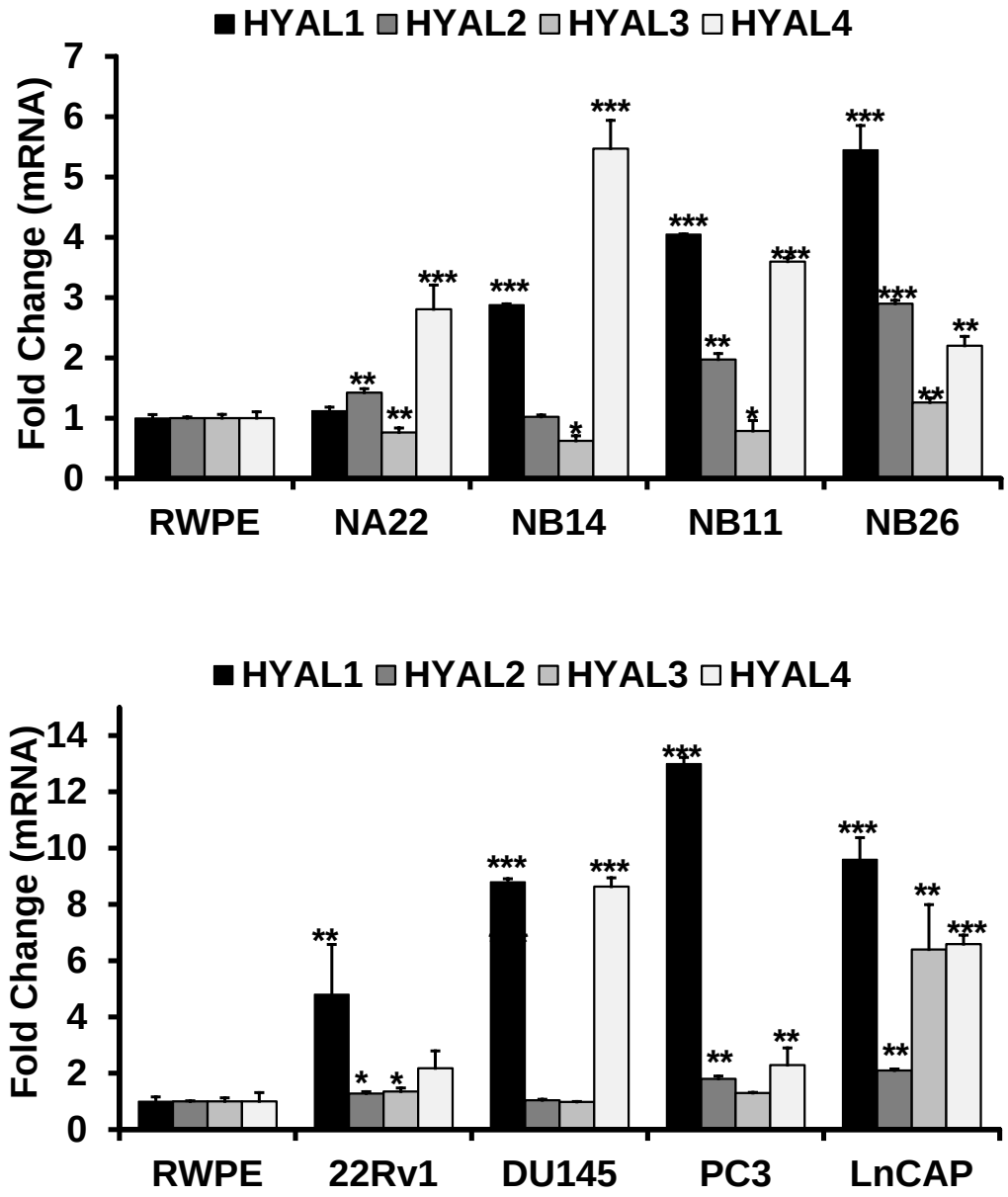

Figure 3.3A. Histograms represent relative HYAL (1, 2, 3 and 4) mRNA expression in RWPE (normal), MNU cells (NA22, NB14, NB11 and NB26) and PCa cells (22Rv1, DU145, PC3 and LnCAP). Gene expression was measured by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate. Statistical difference was seen in gene expression when compared to RWPE as control (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001).

Figure 3.3B

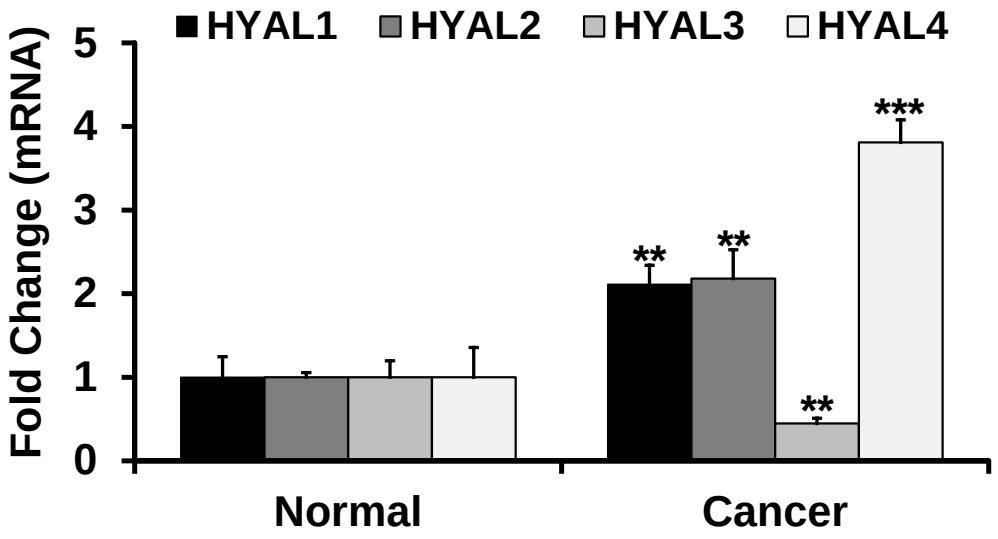

Figure 3.3B. Histograms represent relative HYAL (1, 2, 3 and 4) mRNA expression in normal vs tumor tissues in human patients. Gene expression was measured by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three biological patients and each experiment was performed in triplicate. Statistical difference was seen in tumor tissues when compared to their respective control (**P $\leq$ 0.01, ***P $\leq$ 0.001).

Figure 3.3C

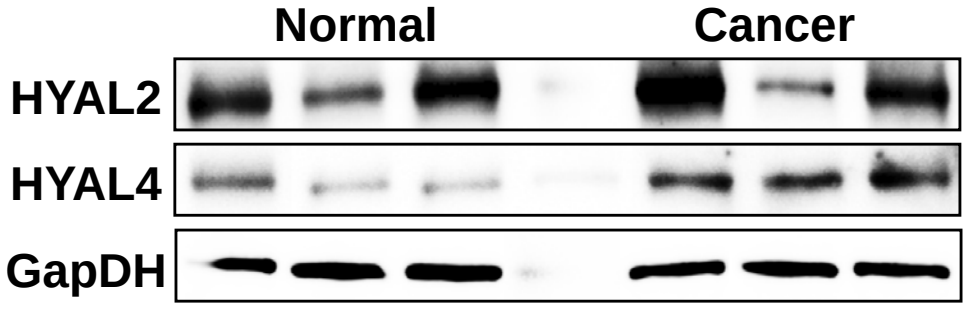

Figure 3.3C. Immunoblot images represent relative HYAL (2 & 4) protein expression in normal vs tumor tissues in human patients. Immunoblot images are from three different biological patients, each performed in triplicates and a representative blot is shown.

Figure 3.4. HAS2 and HAS3 silencing decreased cell proliferation and invasion of PCa cells.

Figure 3.4A

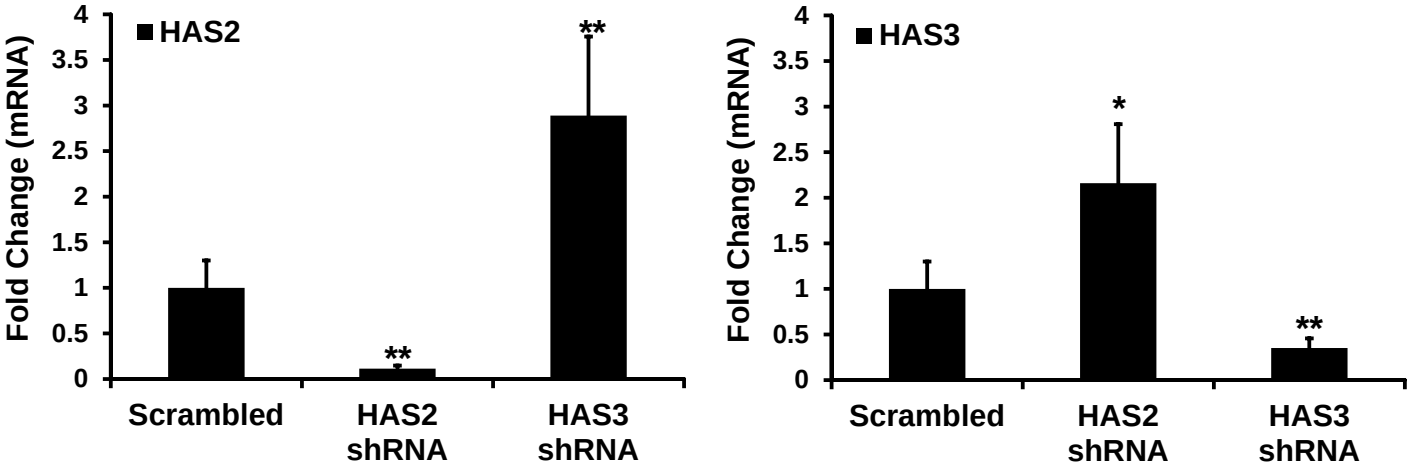

Figure 3.4A. Histograms represent relative HAS2 and HAS3 mRNA expression in PC3 cells stable transfectants with HAS2 and HAS3 shRNA. Gene expression was measured by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate. Statistical difference was seen in stable transfectants when compared to their respective scrambled (*$P \leq 0.05$, **$P \leq 0.01$)

Figure 3.4B

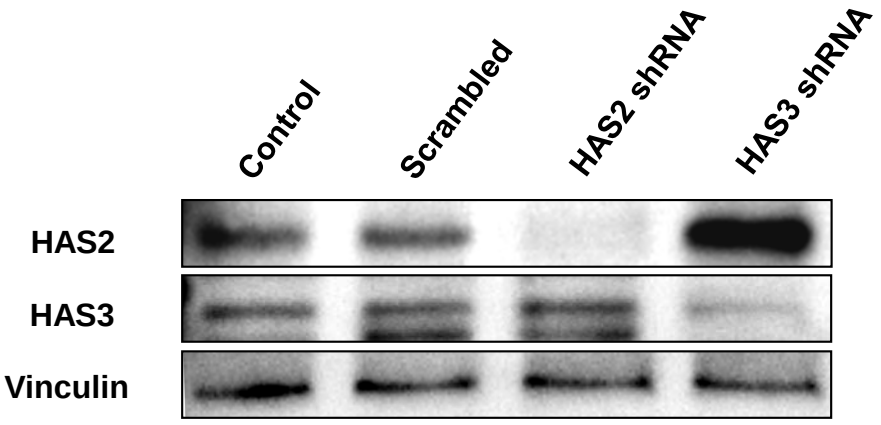

Figure 3.4B. Immunoblot images showing relative HAS2 and HAS3 protein expression in PC3 cells stable transfectants with HAS2 and HAS3 shRNA. Immunoblot images are from three different independent experiment, each performed in triplicates and a representative blot is shown.

Figure 3.4C

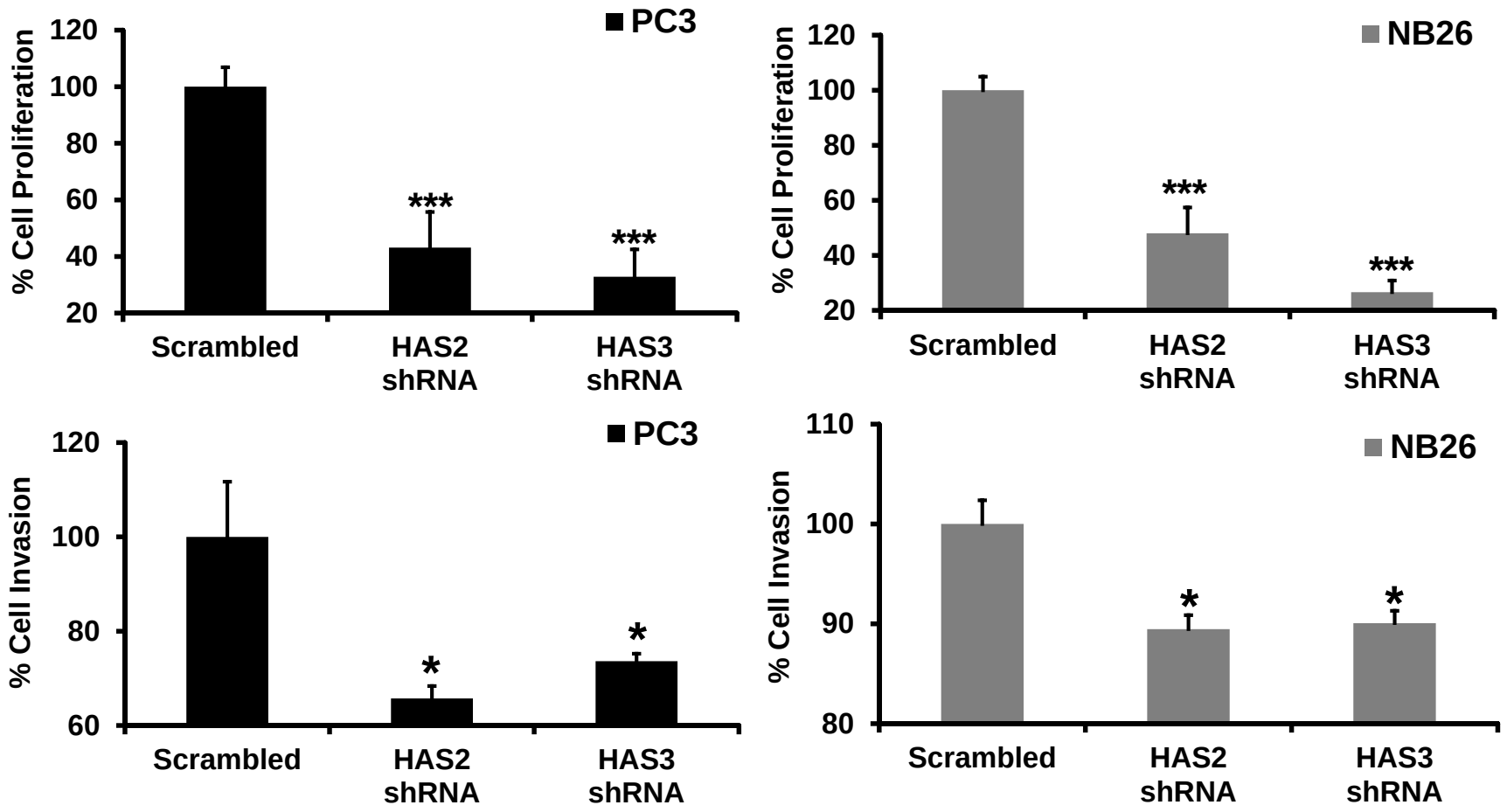

Figure 3.4C. Histograms showing cell proliferation (top) and cell invasion (bottom) in PC3 and NB26 cells. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate. Statistical difference was seen in stable transfectants when compared to their respective scrambled (*$P \leq 0.05$, ***$P \leq 0.001$)

Table 3.A.

Antibody Name	Manufacturer	Reference
HA	Abcam	ab53842
HAS1	Santa Cruz	sc23145
HAS2	Abcam	ab140671
HAS3	Abcam	ab128816
HYAL1	Pierce	pa527901
HYAL2	Pierce	pa524223
HYAL3	Abcam	ab116454
HYAL4	Abcam	ab116547
β-Actin	Cell Signaling	4967

*Antibodies were used as per manufacturer's dilutions

Table 3.A. List of antibodies used for immunoblot and immunofluorescence analysis.

Table 3.B.

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
HAS1	CTGCGATACTGGGTAGCCTTCA	CCAGGAACTTCTGGTTGTACCAG
HAS2	GTCATGTACACAGCCTTCAGAGC	ACAGATGAGGCTGGGTCAAGCA
HAS3	AGCACCTTCTCGTGCATCATGC	TCCTCCAGGACTCGAAGCATCT
HYAL1	GACACGACAAACCACTTTCTGCC	ATTTTCCCAGCTCACCCAGAGC
HYAL2	GGACCTCATCTCTACCATTGGC	CTTTGAGGTACTGGCAGGTCTC
HYAL3	GCAGTCCATTGGTGTGAGTGCA	CCAAGGTGTCCACCAGGTAGTC
HYAL4	GGGTGCGTGAATCACTGAGGAT	CAACGCAGCACTTTCTCCTATGG
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
18sRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 3.B. Sequences of primers used for qPCR analysis

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CHAPTER 4

Fisetin Increases Abundance of High-Molecular-Mass Hyaluronan Conferring Resistance to Prostate Oncogenesis

Manuscript now in revision in Carcinogenesis 2016

Abstract

We and others have previously shown that fisetin, a plant flavonoid, has therapeutic potential against many cancer types. Here, we examined the probable mechanism of its action in PCa using a global metabolomics approach. HPLC-ESI-MS analysis of tumor xenografts from fisetin treated animals identified several metabolic targets with HA as the most affected. Efficacy of fisetin on HA was then evaluated *in-vitro* and also *in-vivo* in the transgenic TRAMP mouse model of PCa. SEC-MALS was performed to analyze the molar mass (*M*w) distribution of HA. Fisetin treatment downregulated intracellular and secreted HA levels both *in-vitro* and *in-vivo*. Fisetin inhibited HA synthesis and degradation enzymes, which led to cessation of HA synthesis and also repressed the degradation of the available high-molecular-mass (HMM)-HA. SEC-MALS analysis of intact HA fragment size revealed that cells and animals have more abundance of HMM-HA and less of low-molecular-mass (LMM)-HA upon fisetin treatment. Elevated HA levels have been shown to be associated with disease progression in certain cancer types. Biological responses triggered by HA mainly depend on the HA polymer length where HMM-HA represses mitogenic signaling and has anti-inflammatory properties while LMM-HA promotes proliferation and inflammation. Similarly, *M*w analysis of secreted HA fragment size revealed less HMM-HA is secreted that allowed more HMM-HA to be retained within the cells and tissues. Our findings establish that fisetin is an effective, non-toxic, potent HA synthesis inhibitor, which increases abundance of anti-angiogenic HMM-HA and could be used for the management of PCa.

Introduction

Tumor microenvironment plays a major role during PCa development. Interactions between the microenvironment and cancer cells are important for migration, metastasis and survival (1). A major component of the extracellular matrix (ECM) in the tumor microenvironment is glycosaminoglycan, hyaluronan (HA). HA is a non-sulfated, linear polymer composed of repeating disaccharides of glucuronic acid (GlcUA) and N-acetyl glucosamine units (GlcNAc) (2). HA is synthesized at the cell surface by the membrane-bound enzyme HA synthase (HAS). Three HAS enzymes HAS1, HAS2 and HAS3 are involved in the production of HA, whereas HA degradation is controlled by hyaluronidases (HYALs), six of which have been identified (3). Increased HA levels in various cancers including PCa, correlates with malignant progression and poor survival. These high HA levels are associated with high expression of HAS and low expression of HYALs (4-6).

HA regulates several cellular functions (7,8). The native, high-molecular-mass (HMM)-HA (~10^7 Da) can be broken down into smaller fragments in response to glycosidase activity and environmental parameters like pH and reactive oxygen species (ROS) (9-12). High and low molecular weight forms of HA provoke distinct anti-inflammatory and pro-inflammatory effects upon binding to CD44 and can deliver either proliferative or anti-proliferative signals in various cell types (13,14). Studies have shown that HMM-HA exhibited anti-angiogenic effects both *in-vitro* and *in-vivo* (2,15). On contrary, the lower molecular-mass (LMM)-HA stimulated cell proliferation, exhibited pro-inflammatory and pro-angiogenic effects in various studies (14,16,17).

In recent years considerable progress has been made in identifying a specific inhibitor for HA synthesis and such inhibitor would not only help elucidate the function of HA, but also can

be used for treatment of diseases with elevated HA levels (18-22). 4-methylumbelliferone (4-MU) is one of the well-known and potent HA synthesis inhibitor and its role has been explored extensively in various cancers (19,21-26) but the IC50 of 4-MU to inhibit HA synthesis in prostate and other cancers is as high as 400 µM (~70 µg/mL) (19,22). Therefore, identifying non-toxic inhibitors that effectively suppress HAS levels and its production will be important. Generally considered as non-toxic, dietary flavonoids act as key modulators of signaling pathways and are therefore considered desirable chemopreventive agents (27,28). Fisetin, found in many fruits and vegetables belongs to the flavonol subgroup of flavonoids and has shown potential against PCa (29-34).

In this study, we identified HA as a unique target of fisetin in PCa cells, tumor xenografts, and in the TRAMP mouse model. We utilized SEC-MALS for a quick, accurate and quantitative determination of different molecular masses of HA both in-vitro and in-vivo.

Results

Identification of HA as a unique target of fisetin in PCa

To identify new biomarkers in PCa, we designed a comprehensive metabolic profiling of fisetin treated NB11/NB26 tumor xenografts. NB11 and NB26 cells are unique tumorogenic PCa cells derived from non-tumorgenic RWPE1 cells upon exposure to N-methyl-N-nitrosourea (37). We selected these unique PCa cells NB11 and NB26 for determining the in-vivo effects of fisetin due to their ability to form rapid and reproducible tumors. Aqueous and organic extracts were isolated from the tissue samples and analyzed using HPLC-ESI-MS in both positive and negative scan modes. The total ion chromatogram (TIC) resulted in excellent separation between the two tissue groups (n=6; **Figure 4.1A**). The XCMS online analysis of the TIC revealed a list of newly identified metabolites. The quasimolecular ions were confirmed and the exact masses of the monoisotopic molecular weights were used to search the METLIN database. Total metabolites identified were 2086, out of which 1203 were downregulated and 883 were upregulated between vehicle control and fisetin treated NB11 xenografts. Similarly, a total of 2203 metabolites were identified in control vs fisetin treated NB26 xenografts, out of which 1654 were downregulated and 549 were upregulated (**Figure 4.1B**).

To obtain a comprehensive global metabolic signature for this discovery based approach, we considered a liberal p-value of 0.1 as significant for this dataset. Among identified metabolites, an m/z of 776.25 stood out as a single metabolite HA (**Figure 4.1B**). The relative intensity of the identified HA peak (RT=2546.73 min) exhibited a significant decrease in fisetin treated NB26 xenografts (**Figure 4.1C**) and plotted as a function of relative abundance (fold change = 76.09) in both fisetin treated xenograft groups (**Figure 4.1D**). We next performed immunofluorescence staining for HA in NB11 and NB26 tumor tissues and observed that fisetin

treated animals exhibited significantly decreased HA expression as compared to the untreated controls (**Figure 4.1E**).

Fisetin decreases intracellular and extracellular HA abundance in PCa cells

We performed immunofluorescence staining for HA in NB11, NB26, PC3 and DU145 PCa cells treated with or without fisetin (40 µM, 48 hour). Fluorescence intensity in all four cell types suggested that fisetin treated cells showed significantly decreased HA expression as compared to the untreated controls (**Figure 4.2A** and **Figure 4.2B**). Next, we evaluated intracellular and extracellular HA upon fisetin treatment (40 µM, 48 hour) using a solid phase sandwich human HA ELISA assay. Our data showed that intracellular HA levels significantly decreased in fisetin treated PCa cells (**Figure 4.2C**). Similarly, secreted HA levels in the fisetin treated group were significantly lower than the untreated control (**Figure 4.2D**).

It was further noted that PCa cells secrete more HA in the media and retain less HA within the cell (**Figure 4.2C**, **Figure 4.2D**). PC3 and NB26 cells treated with or without fisetin (5-40 µM) showed a similar decrease in secreted HA levels in a dose dependent manner. We found that fisetin decreases HA levels secreted in the media in a dose dependent manner and enables retention of more HA inside the cell with increasing doses (**Figure 4.2E**). Also, fisetin treatment (40 µM, 48 hour) significantly decreased cell proliferation in NB11 and NB26 cells (**Figure 4.2F**). Fisetin was able to decrease both intracellular and extracellular HA levels signifying HA as a potential target of fisetin in PCa.

Fisetin decreases HA levels that are associated with inhibition of disease progression in TRAMP mouse model

The *in-vitro* results of NB11/NB26 cells prompted us to look for similar effects of fisetin in another mouse model of PCa. The TRAMP model is a spontaneous PCa progression model

which closely mirrors the pathogenesis of human PCa (38,39). Compared with vehicle treated, fisetin treated animals of 24 weeks exhibited decreased hyperplasia (**Figure 4.3A**) in the prostate. We found no significant change in body weight of the animals between the vehicle and fisetin treated groups as shown (**Figure 4.3B**), indicating that fisetin is not associated with any significant adverse side effects. We also observed less palpable tumors in fisetin treated animals when compared to the vehicle treated controls. Many of the fisetin treated animals had well differentiated adenocarcinoma with no evidence of poorly differentiated carcinoma with disease progression (**Figure 4.3C**).

Next, we performed immunofluorescence staining for HA in TRAMP prostate tissues and compared HA expression intensity among control and fisetin treated groups. Fluorescence intensity showed that the vehicle treated control animals demonstrated increased expression with disease progression that was significantly reduced in fisetin treated animals (**Figure 4.3D**, **Figure 4.3E**). Next, we evaluated the effect of fisetin on secreted HA levels in TRAMP mouse serum using a mouse HA ELISA. Our results revealed that serum HA levels increased with disease progression and fisetin treated animals showed significantly lower serum HA levels as compared to the respective controls (**Figure 4.3F**).

Fisetin reduces HA synthesis and degradation enzyme levels *in-vitro* and *in-vivo*

Previous studies indicate that elevated HAS and HYAL levels are responsible for the high turnover (both synthesis and degradation) of HA in advanced disease state and may cooperate together in cancerous growth. To determine whether fisetin has any effect on HA synthesis and degradation, we evaluated mRNA levels of HAS and HYAL enzymes in NB11 and NB26 cells with or without fisetin treatment (40 μM) for 6 hour. Fisetin treated cells exhibited a significant decrease in HAS and HYAL mRNA levels (**Figure 4.4A**). The change in transcriptional levels of

HAS and HYALs were reflected in protein expression which were markedly reduced upon fisetin treatment between the two cell types (**Figure 4.4B** and **Figure 4.4C**).

qPCR analysis of PCa tumors of TRAMP tissues revealed a similar significant decrease in HAS2 and HAS3 mRNA levels in fisetin treated mice as compared to the vehicle treated controls with increasing weeks of disease progression (**Figure 4.4D**). Previous studies have shown that native HA can be broken down into smaller fragments either via enzymatic (via HYALs) or non-enzymatic catabolism (via ROS). Since fisetin decreases enzymatic catabolism via reduction in HYAL levels (**Figure 4.4A**, **Figure 4.4B**) both in-vitro/in-vivo, we investigated the role of non-enzymatic catabolism in fisetin treated cells by measuring ROS levels in both PC3 and NB26 cells. Increase in ROS levels can breakdown the native HMM-HA into smaller molecular weight fragments. Our data shows that fisetin increased ROS levels significantly in both PC3 and NB26 cells (**Figure 4.4E**).

Fisetin increases abundance of HMM-HA in PCa cells

In non-diseased conditions, native HA is present as HMM-HA (Mw~10^7 Da) whereas in diseased condition, it gets broken down into LMM-HA. HMM-HA is known to have anti-angiogenic properties; however, LMM-HA has shown to be pro-angiogenic. Since the biological functions of HA is size dependent, we first evaluated the molar mass distribution profile of HA fragments in normal vs cancerous cells. Intracellular HA was collected from normal RWPE-1 and PCa PC3 cells by employing the PEGNAC HA size protocol. The isolated HA was then used for SEC-MALS analysis to identify and compare the molar mass of HA fragment in both cell types. The molar mass vs elution volume chromatograms of SEC-MALS analysis and the Mw of HA fragments showed that normal RWPE1 cells predominantly exhibited higher levels of HMM-HA fragment cluster and no detectable LMM-HA cluster. In PC3 cells, we observed

lower levels of HMM-HA fragment cluster and higher levels of LMM-HA (**Figure 4.5A**, **Table 4.5A)** that could contribute to the pro-oncogenic nature of cancer cells.

Next, we determined the effect of fisetin on molecular mass profile of HA fragments in PCa cells. Intracellular and extracellular HA with or without fisetin treatment were isolated from PC3 cells/culture media and analyzed using SEC-MALS. We observed an increased abundance of intracellular HMM-HA fragment cluster in fisetin treated cells when compared to the untreated control cells. Even the intracellular LMM-HA fragments in fisetin treated cells were found to have comparatively higher Mw than the untreated control cells (**Figure 4.5B**, **Table 4.5B**). Interestingly, we observed that PC3 cells release more HMM-HA in the media whereas; no detectable HMM-HA was secreted in media of fisetin treated cells suggesting retention of anti-angiogenic HMM-HA fragments within the cells.

Fisetin increases abundance of HMM-HA conferring resistance to prostate oncogenesis in TRAMP mouse model

TRAMP (n=15) and TRAMP+Fisetin (n=12) animals were selected randomly and vehicle or fisetin was administered beginning at 8 weeks of age and continued until 24 weeks old respectively. We measured the HA fragment sizes in vehicle vs fisetin treated animals. The light scattering MALS data in vehicle treated TRAMP animals showed a decreased abundance of HMM-HA fragment cluster with increased abundance of pro-angiogenic LMM-HA fragment clusters. Interestingly, in fisetin treated animals, we observed increased abundance of anti-angiogenic HMM-HA fragment clusters and lower abundance of LMM-HA fragment clusters (**Figure 4.6A**, **Table 4.6A**). Studies have shown that increased HMM-HA levels are directly associated with resistance to oncogenesis (15,40,41). These results suggest a novel role of fisetin

in increasing the abundance of intracellular HMM-HA which is associated with resistance to PCa progression.

Serum analysis of vehicle treated TRAMP animals revealed higher secretion of anti-angiogenic HMM-HA and lower secretion of LMM-HA fragment clusters. Notably, serum analysis of fisetin treated animals showed lower secretion of anti-angiogenic HMM-HA and higher secretion of pro-angiogenic LMM-HA fragment clusters (**Figure 4.6B**, **Table 4.6B**). Taken together, these findings clearly show that fisetin inhibits development of PCa in TRAMP mouse model.

Discussion

Our study demonstrates that fisetin, which is found in many fruits and vegetables, is a potent HA synthesis inhibitor and can be developed for management of PCa. We and others have previously reported that fisetin is an effective proapoptotic agent with anti-invasive and anti-angiogenic properties (29-34). The inhibition of HA synthesis by fisetin is significant considering the fact that targeting the HA pathway with other agents has met with limited success. Only 4-MU has been found to be the most promising till date (19). D-mannose has shown promise *in-vitro* although at very high concentrations of 20mM (42). Similarly, a curcumin analogue was shown to inhibit HA export from fibroblasts at 5 µM (43); however antitumor activity of both these agents has not been evaluated at these doses. Sulfated HA has been reported to display anti-tumor activity by blocking HA signaling but its oral bioavailability and evidence in experimental in-vivo models has not yet been evaluated (18). 4-MU, which is a known HA synthesis inhibitor, is effective at high concentrations. The IC50 of 4-MU is 400 µM whereas fisetin inhibits HA synthesis at 40 µM, a 10-fold lower dose. The *in-vitro* IC50 dose of fisetin translates to 40 mg/kg body weight (~1mg/day) in-vivo which is less than the dose required in humans (1.5-2g/day) as

dietary supplement. At these low doses, fisetin exhibits no detectable toxicity as revealed by xenograft and TRAMP studies. We observed that fisetin (1mg/animal/day) significantly reduced tumor growth and delayed progression of PCa in the TRAMP mouse model. Fisetin acts as a potent HA synthesis inhibitor and exhibits significant dose advantage when compared to 4-MU both in-vitro and in in-vivo PCa models.

Accumulation of HA is associated with progression of various cancers including PCa. Nevertheless, there are inconsistent views regarding the significance and relevance of HA secreted in serum. Studies in breast cancer have shown that the total serum HA levels reflect disease progression (44) while other studies have shown that the serum HA level does not have any prognostic significance with disease (45). Our results suggest that fisetin significantly decreases both cellular and secreted HA levels in *in-vitro/in-vivo* and shows promise in prevention of other cancers which are associated with increased HA levels.

HAS enzymes synthesize HA while the HYALs degrade it into smaller fragments. This constant HA turnover is important for the maintenance of tissue homeostasis, and ~30% of HA is replaced by newly formed HA every 24 hour (46). Importantly, the presence of reactive oxygen species (ROS) is known to enhance HA turnover (47-49). Our results show that fisetin treatment significantly inhibits both HAS and HYAL enzymes and halts the synthesis of new HA and also prevents the degradation of previously synthesized HA. Fisetin treatment increased ROS levels *in-vitro* but did not enhance HA turnover in PCa cells.

HA is a “dynamic” molecule with a constant turnover in many tissues via rapid metabolism leading to HA fragments of various sizes: HMM-HA, LMM-HA and o-HA. The innumerable oncogenic functions of HA mainly depend on its molecular size (2,5,9,11,13,14,50). In this study, we used SEC-MALS analysis to accurately measure the molecular distribution of

HA size fragments in all biological samples. While large molecules of HA promote tissue integrity and quiescence, the generation of breakdown products enhances signal transduction, contributing to the pro-oncogenic behavior of cancer cells. Previous studies (15,40,41) have revealed that cancer resistance in naked mole rat derives from the abundant production of HMM-HA. On the contrary, LMM-HA has been proven to play a crucial role in breast cancer and seems to be a more promising molecular biomarker than total HA for detecting breast cancer metastasis (50). Our in-vivo results showed that during PCa progression in TRAMP animals, there is increased pool of HMM-HA that further breaks down into LMM-HA. This suggests higher activity of HYAL enzymes leading to higher degradation with disease progression. Increased ROS levels in diseased state may also contribute to the breakdown of HMM-HA into smaller fragments. Upon fisetin treatment, we observed an increase in accumulation of HMM-HA both *in-vitro/in-vivo*. This increase of HMM-HA fragments in presence of fisetin mimics the HA fragment profile of the non-diseased state which unveils a novel effect of fisetin. This effect is because fisetin significantly decreases both HAS and HYAL levels. In this condition, the HA synthesis process is halted and no new HMM-HA is produced. At the same time with reduced activity of HYALs upon fisetin treatment, there seems to be very little or no degradation of the preexisting HMM-HA. Fisetin treatment induces ROS levels but surprisingly, increased ROS levels do not seem to contribute towards HA degradation as evidenced by larger accumulation of HMM-HA upon fisetin treatment *in-vitro*. Hence, the HMM-HA stays intact within the cells and tissues upon fisetin treatment. Another possible reason for this phenomenon could be fisetin's ability to directly or indirectly catalyze and reassemble the LMW-HA fragments into HMW-HA fragments which requires further exploration. Based on our findings, anti-angiogenic HMM-HA is increased in non-diseased states, which undergoes breakdown with progression of disease and

larger pool of pro-angiogenic LMM-HA accumulates. Fisetin reduces the larger pool of LMM-HA and increases the abundance of HMM-HA which mimics the HA fragment profile of the non-diseased state. The precise mechanism underlying the actual role of fisetin and its ability to directly or indirectly restructure the smaller HA fragments into larger fragments requires further exploration.

In summary, our study demonstrates fisetin, as a non-toxic, orally bioavailable, potent HA synthesis inhibitor in PCa. Fisetin treatment enables accumulation of a larger pool of anti-angiogenic HMM-HA and lowers level of pro-angiogenic LMM-HA which leads to reduction of HA signaling. The doses of fisetin used in our in-vivo studies were comparable with those in human use as a dietary supplement. Hence, by inhibiting HA synthesis, fisetin displays significant potential for prevention and treatment of PCa in preclinical models.

Materials and methods

Materials

Fisetin was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were obtained from Cell Signaling Technology (Danvers, MA), Abcam (Cambridge, MA) and Santa Cruz Biotechnology (Dallas, TX) and a list is provided in **Table 4.A**. Quantitative PCR (qPCR) primers were synthesized and obtained from DNA synthesis laboratory at the University Of Wisconsin Biotechnology Center. The sequences of oligos used are listed in **Table 4.B**.

Animals

TRAMP animals were obtained as described previously (35). Housing and care of the animals was approved by the University of Wisconsin's Research Animal Resource Committee in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Cell culture

Non-tumorogenic RWPE1 (CRL-11609), tumorogenic NB11 (CRL-2851) and NB26 (CRL-2852) cell lines were directly obtained from ATCC (Manassas, VA) in 2013 while PC3 (CRL-1435) and DU145 (HTB-81) cell lines were obtained in 2012. ATCC ensures cell lines authenticity using morphology, karyotyping, and PCR based approaches which includes assays to detect cytochrome C oxidase I gene (COI analysis) to rule out inter-species contamination and short tandem repeat (STR) DNA profiling to rule out intra-species contamination as tabulated in **Table 4.C**. Cell lines were immediately resuscitated upon receipt and frozen in aliquots in liquid nitrogen. Once thawed, early passage cells were cultured within 3 months from a frozen vial of the same batch of cells. Cells were routinely tested to ensure there was no mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza). RWPE1, NB11 and NB26 cells were cultured in KSFM from Thermo-Fisher Scientific (Grand Island, NE), supplemented with

HKGS and 1% penicillin–streptomycin. PC3 and DU145 cells were cultured in RPMI 1640 from Gibco (Carlsbad, CA), with 10% FBS and 1% penicillin–streptomycin. The cells were then incubated at 37 °C with 5% CO2 in a humid environment. For time-dependent studies, cells (70% confluent) were treated with fisetin dissolved in DMSO (0–40 μM) for specified time points at 37 °C in media and harvested for further studies.

BrdU cell proliferation assay

The BrdU assay from Cell Signaling (Danvers, MA) was used as per the manufacturer's instructions to measure cell proliferation. Absorbance was read at 450 nm using a Synergy 2 multi-detection microplate reader (BioTek, VT).

HPLC-ESI-MS analysis

Aqueous and organic metabolites were isolated from control and fisetin treated tumor xenograft tissues using a methanol/water and dichloromethane/water extraction as described previously (36). Metabolite extracts (10 μL) were then diluted to 50 μL with 0.1% formic acid in water. Untargeted metabolomics using HPLC-ESI-MS was performed with a 10uL injection on an Agilent 6210 ESI-TOF mass spectrometer with Agilent 1200 series HPLC (Santa Clara, CA). Solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The HPLC column was an Agilent Zorbax SB C18 1.8um, 2.1mm i.d. x 50 mm length (Santa Clara, CA). Metabolites were gradient-eluted at a flow rate of 0.25 mL/min starting at 2%B with a 1 minute hold, ramping to 50% B at 35 min, then to 95% B at 40 min, returning to 2% B at 42 min, and re-equilibrating at 2%B for 18 min. Electrospray was performed in positive-ion (+) mode at 3.6kV and fragmentor at 130V. Metabolite detection was performed over the m/z range 50-1700 by summing 10013 transients/scan (0.89 scans/sec). Blank injections (10 μL) of 20% MeOH, 0.08% formic acid in water were made between sample injections.

Raw data acquired using HPLC-ESI-MS system was processed by Xcalibur software which provides an appropriate format for further data analysis. The structured data files were then uploaded on XCMS Online (https://xcmsonline.scripps.edu), a high-quality cloud-based platform linked with METLIN (https://metlin.scripps.edu), to facilitate metabolite identification using a repository of metabolite and tandem mass spectrometry information of known compounds.

In-vivo tumor xenograft model

7-8 week old athymic (nu/nu) male nude mice (Harlan, USA) were xenografted as previously described (30). We selected unique PCa cells NB11 and NB26 for determining the in-vivo effects of fisetin due to their ability to form rapid and reproducible tumors. After injected, twelve animals were then randomly divided into two groups with 6 animals each. The first group of animals received 30 µL i.p. injection of PBS/DMSO (1:1) and served as control. The animals of second group received i.p. injection of fisetin (40mg/kg ~ 1mg/animal) in 30 µL of PBS/DMSO (1:1) twice weekly. All animals were sacrificed when tumors reached a volume of 1200 mm3 in the control group. Samples were collected and stored at -20oC until further analysis.

Spontaneous PCa progression (TRAMP) model

Eight-week-old transgenic TRAMP mice (n=27) were randomly divided into two groups for 5 different time points from 8-12-16-20-24 weeks. Each time point/group contained 3 animals respectively. The first group of TRAMP (n=15) animals received 50 µL PBS/DMSO (1:1) and served as the control. The second group (n=12) received fisetin (1mg/animal) dissolved in 50 µL PBS/DMSO (1:1) thrice weekly, beginning at 8 weeks of age and continued until the animals were 12,16,20 and 24 weeks old respectively. Throughout the experiment, the animals had access to chow diet ad libitum.

Animals in all groups were observed weekly for body weight, tumor progression by abdominal palpation and survival. At the termination of the experiment at their respective time points, blood samples were collected by "mandibular bleed" and serum was separated and stored at -20oC until further analysis. The prostate was excised under a dissecting microscope and snap frozen in liquid nitrogen for further analysis.

Western blotting

After treatment with fisetin (40 μM: 48 hour), whole cell lysates were prepared and western blot analysis was performed as described previously (29). Densitometric measurements of the bands were done with image analysis software using the Biorad ChemiDoc MP imaging system.

HA ELISA

Human and Mouse HA ELISA kit were obtained from TSZ ELISA (Waltham, MA). HA levels in mouse serum and human PCa cells treated with or without fisetin (40 μM:48 hour) were measured according to the manufacturer's protocol.

RNA isolation and qPCR analysis

Total RNA was extracted from cells and animal tissues using RNeasy kit (Qiagen, Germantown, MD), and reverse transcribed with iScript Reverse transcription supermix kit (Biorad, Hercules, CA). cDNA was amplified using gene specific primers as per the protocol described previously (30).

Intracellular ROS assay

The OxiSelect™ Intracellular ROS Assay Kit obtained from Cell Biolabs Inc. (San Diego, CA) provides a cell-based assay for measuring primarily hydrogen peroxide, along with hydroxyl, peroxyl and other ROS levels within a cell. Cells treated with or without fisetin (40 μM) for specified times were processed as per the manufacturer's instructions. Hydrogen Peroxide was

used as a positive control in the assay. Fluorescence was evaluated on a Synergy H1 (BioTek) multi-mode microplate reader at 480/530 nm (excitation/emission) using Gen5 2.0 software (BioTek).

Immunoflourescence analysis

NB11, NB26, PC3 and DU145 cells treated with fisetin (40 μM: 24 hour), xenograft and TRAMP animal slides were incubated with HA antibody as per protocol as described previously (30). After rinsing in PBST, slides were incubated with fluorescent-conjugated secondary antibody at 1:500 dilution in blocking buffer. Slides were then rinsed in PBST, and sections were mounted with ProLong Gold Antifade reagent containing DAPI (Invitrogen) and left in dark overnight. Slides were imaged with the Andor Revolution XD spinning-disk confocal microscope using a 20X/40X/1.4 NA oil objective with identical exposures and gains for each antibody stained.

SEC-MALS analysis

Cells with or without fisetin treatment were collected over time and pooled in order to isolate enough HA to be detected in SEC-MALS analysis. HA was isolated using the PEGNAC HA size protocol (available online). This method sequentially removes proteins, nucleic acids and isolate HA from cells, media, tissues or any biological samples. Separation of HA molecular fragments was carried out by SEC-MALS on a Superose 6, 10/300 GL column in series with a Superose 12, 10/300 GL column (GE Healthcare, Pittsburgh, PA) attached to a Waters HPLC system (Waters Corporation, Milford, MA 01757). A solution containing 20 mM imidazole and 50 mM NaCl at pH 7.0 was used as an eluting buffer. All the samples were filtered through a 0.22 μm filter. Sample injection volume was 100 μL, and nominal flow rate was 0.5 mL/min. The chromatography system consisted of columns, a UV detector (Waters, model 2998, Milford,

MA) operating at 280 nm, a DAWN-DSP MALS photometer Wyatt Technology, Santa Barbara, CA) fitted with a helium-neon laser (λ=632.8 nm) and a K-5 flow cell, and a DRI detector (Waters, model 2414, Milford, MA). The electronic outputs of the UV, DRI, and MALS were sent to a Dell computer. The data were processed with ASTRA (version 4.0) software. The DRI response factor was measured by injecting a series of known NaCl concentrations into the detector with the syringe pump. This response factor was obtained from the slope of the linear plot between NaCl concentration and DRI response. The factor to correct the Rayleigh ratio to 90° for instrument geometry was obtained by measuring the LS intensity of filtered (0.025 μm) HPLC quality toluene at 90°. The responses to LS intensity of the photodiodes arrayed around the scattering cell were normalized to the diode at 90° with a BSA sample (monomeric BSA with a nominal molecular weight of 66 kDa).

Statistical analysis

Microsoft Excel software was used to calculate the mean and standard error of the mean (SEM). For the HPLC-ESI-MS metabolomics study, a liberal p value of 0.1 was considered significant. Two-tailed, Student's t-test was used to assess statistical significance. Data points in all the rest of studies represent mean ± SEM, and p values < 0.05 were considered significant.

Figure 4.1. Identification of HA as a unique target of fisetin.

Figure 4.1A

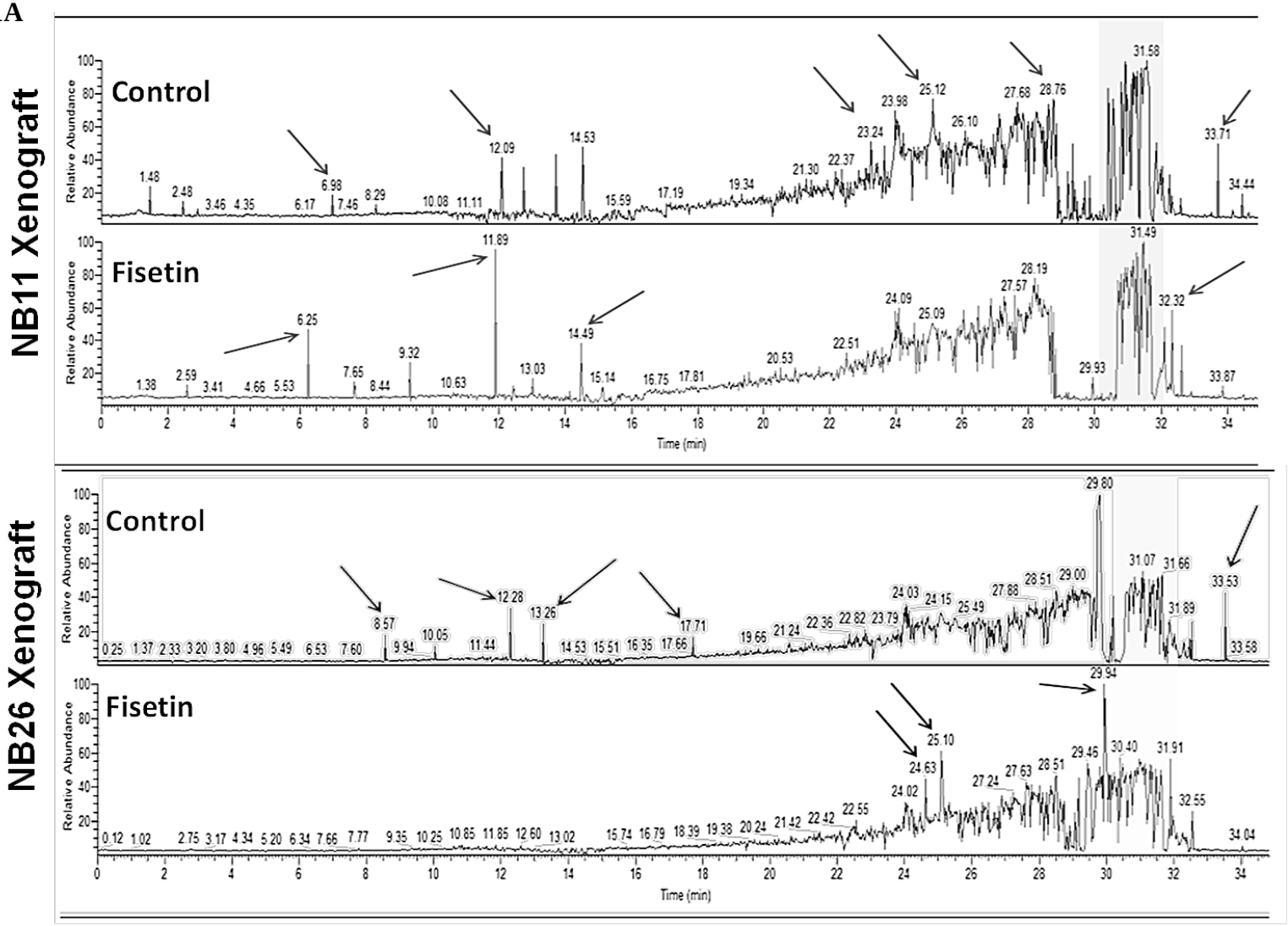

Figure 4.1A. Total ion chromatogram (TIC) of control vs fisetin treated NB11 and NB26 xenograft tissues (n=6 animals/group) obtained by positive-ion HPLC-ESI-MS. Arrows represents unique peaks found between the two groups. All six biological replicates showed similar analytical separation between the control and treated groups and a representative picture is shown.

Figure 4.1B

Sr. No	Name	Fold Change	pvalue	MZ-med	RT-med	NB11-Control	NB26-Control	NB11-Fisetin	NB26-Fisetin
60	M426T2516	1.33	0.0823	426.38	2516.06	506362.23	458206.64	375059.25	351749.18
61	M499T2551	7.11	0.0825	499.43	2551.44	13401.58	8097.09	66184.94	86771.49
62	M416T665	1.22	0.0836	416.23	664.57	156841.39	145152.94	181843.51	185960.66
63	M924T2548	2.03	0.0837	924.27	2548.41	26735.61	36857.23	63362.93	65747.29
64	M307T637	2.50	0.0860	307.12	637.34	81088.94	38937.45	168641.77	131975.48
65	M791T1703	1.57	0.0864	791.46	1702.67	77525.51	78790.74	116467.78	129026.58
66	M191T1648	2.12	0.0874	191.14	1648.49	671148.27	668572.70	1314799.90	1521572.11
67	M270T663	3.67	0.0907	270.04	662.94	2833.02	12583.08	31278.47	25312.56
68	M530T2346	1.58	0.0914	530.37	2345.82	90882.46	75247.46	119239.41	142476.21
69	M618T2493	1.75	0.0931	618.47	2493.21	673178.32	656188.09	334637.98	426653.39
70	M157T39	1.23	0.0933	157.17	39.24	5016996.69	4532728.10	5738061.15	5976580.70
71	M777T2547	76.09	0.0954	777.23	2546.73	266618.7157	361956.33	7144.21*	11258.36*
72	M191T638	1.64	0.0961	190.53	637.88	12902.4332	13469.92	20237.77	23106.38
73	M340T2368	12.63	0.0963	339.56	2367.84	7735.377385	20533.89	149996.85	207111.49
74	M226T2358	1.40	0.0967	226.16	2357.66	178282.0097	203334.77	130099.65	142473.68
75	M99T2801	1.04	0.0999	99.07	2800.63	210874.2821	208497.13	202347.97	202646.83
76	M527T44	1.92	0.1002	527.12	44.11	26365.48505	16832.15	43896.55	38938.64
77	M639T2338	1.60	0.1003	639.39	2337.74	298425.1529	219678.35	438370.94	388721.48
78	M683T2335	1.59	0.1010	683.41	2335.36	202781.7558	167849.04	292922.51	294576.18
79	M279T2017	4.78	0.1011	278.61	2017.18	25678.82495	16737.81	116968.56	85835.81
80	M324T2414	1.57	0.1023	324.27	2413.66	139868.203	169328.92	110230.14	86575.13

Figure 4.1B. Metabolites of NB11 and NB26 xenograft tissues with or without fisetin treatment (n=6 animals/group) analyzed using untargeted metabolomics (HPLC-ESI-MS) in positive-ion mode. A representative list of 20 metabolites identified from the METLIN database is shown; m/z=776.25 highlighted in yellow was identified as HA (Student's *t*-test; *P $\leq$ 0.1). For the complete list of metabolites, please see Supplementary Table 4.8 (MS excel file).

Figure 4.1C

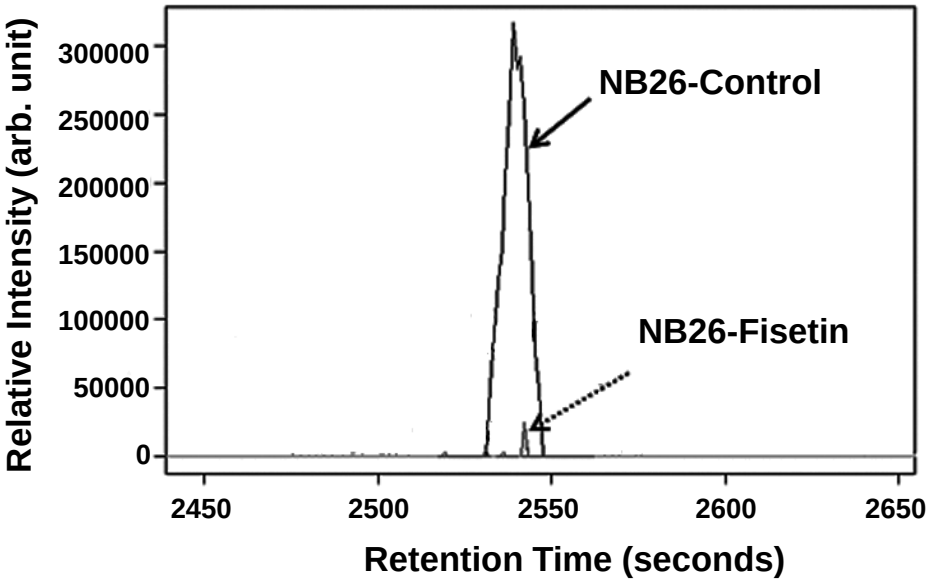

Figure 4.1C. HPLC separation (RT=2546.73) and relative intensity of HA metabolite peak identified in NB26 xenografts between control vs fisetin treated animals. All six biological replicates showed similar analytical separation between the control and treated groups and a representative picture is shown.

Figure 4.1D

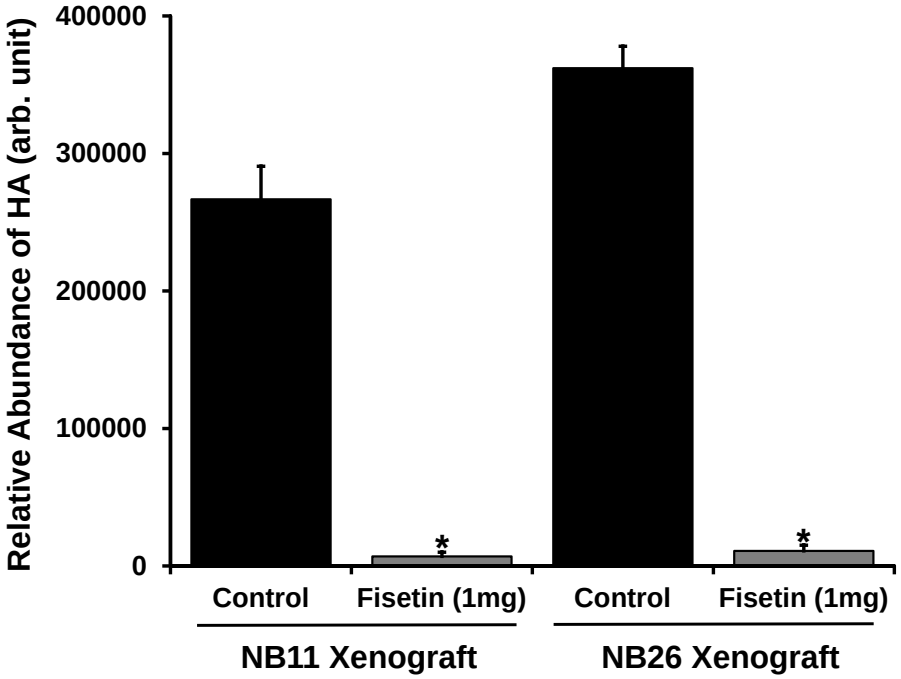

Figure 4.1D. Comparative plot of relative abundance of HA between control and fisetin treated animal groups in NB11 and NB26 xenografts. Error bars represent mean±SEM among six biological replicates (fisetin dose was 1mg/animal thrice weekly; *$P \leq 0.1$

Figure 4.1E

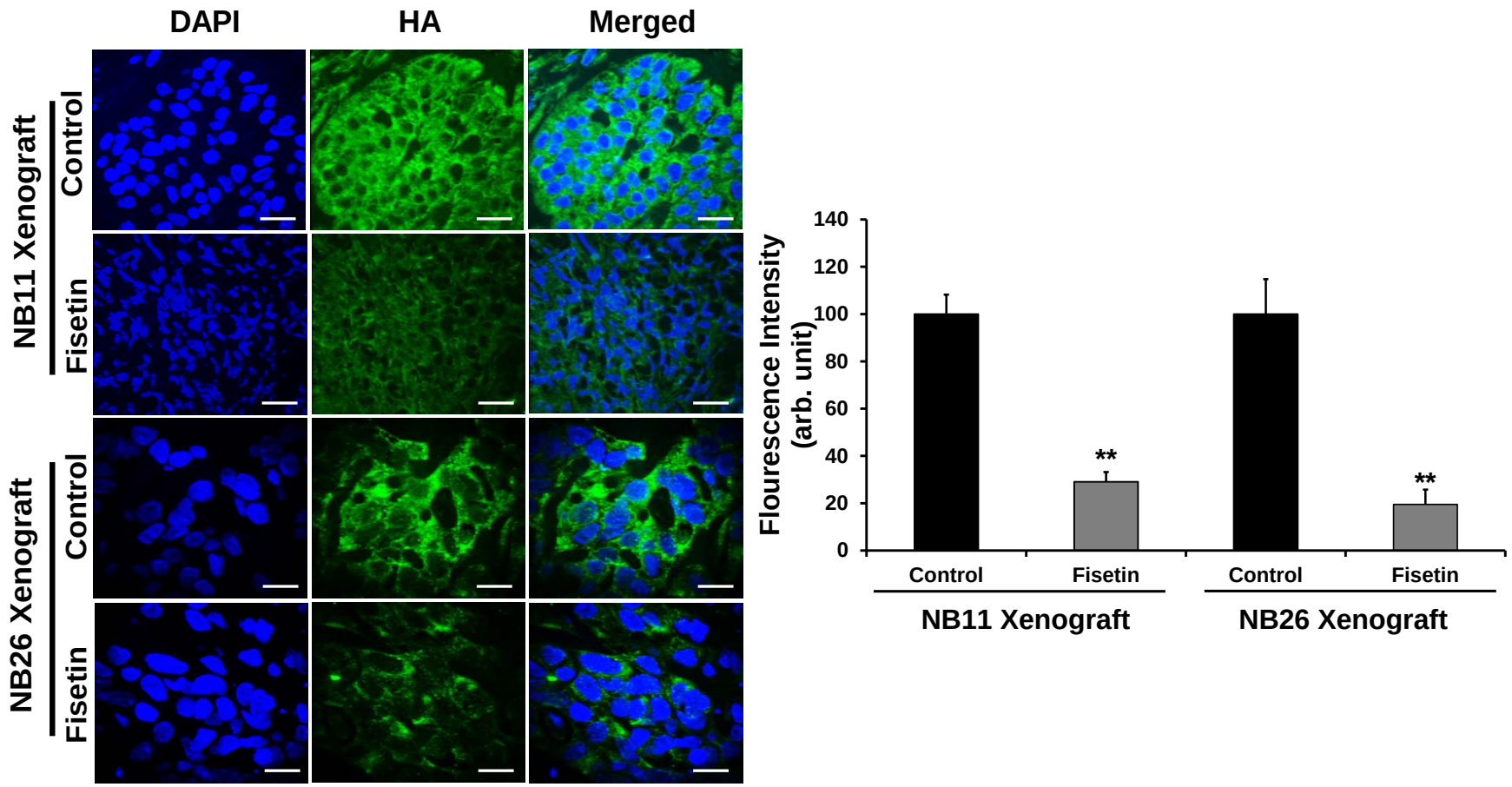

Figure 4.1E. Representative images showing immunofluorescence (left) for HA between control and fisetin treated NB11 and NB26 xenografts. Tumor tissues were harvested and each of the six biological replicates was performed in triplicates for HA staining. Images were captured by a confocal microscope as described in materials and methods. Scale bar, 30 μm. Magnification for NB11 images are at 20X and NB26 images at 40X. DAPI was used as a nuclear staining control. Fluorescence intensity was measured and plotted (right). Statistical difference was seen in fisetin treatment group when compared to the respective control group (**P $\leq$ 0.01).

Figure 4.2. Fisetin decreases abundance of HA in PCa cells.

Figure 4.2A

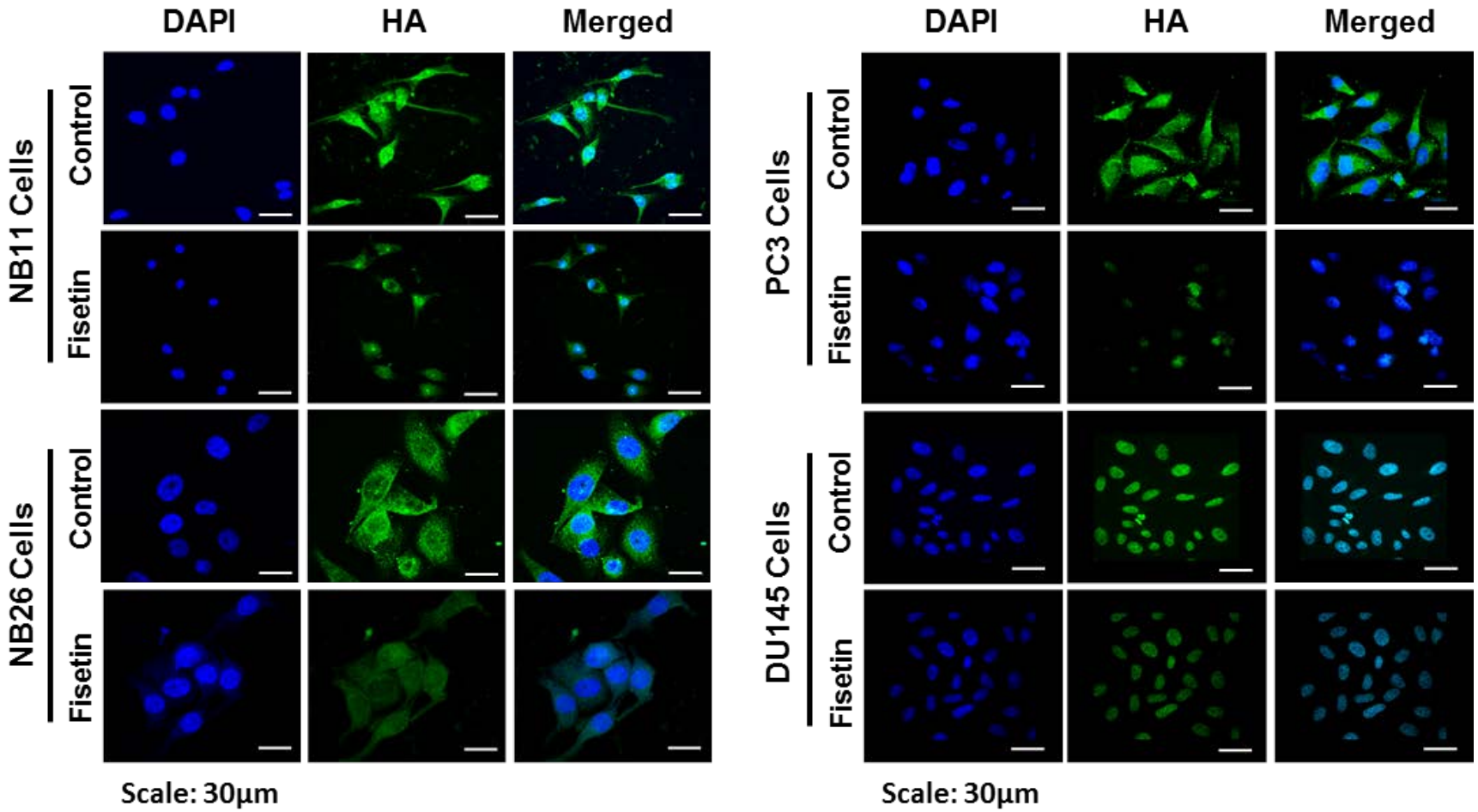

Figure 4.2A.Representative images showing immunofluorescence for HA in NB11, NB26, PC3 and DU145 cells with or without fisetin (40 μM) treatment for 48 hour. Three biological replicates for each were subjected to staining and performed in triplicate. Images were captured by a confocal microscope as described in methods. Scale bar, 30 μm. Magnification for NB11 images are at 20X and for NB26 images at 40X. DAPI was used as a nuclear staining control. Fluorescence intensity was measured and plotted (See **Figure 4.2B**).

Figure 4.2B

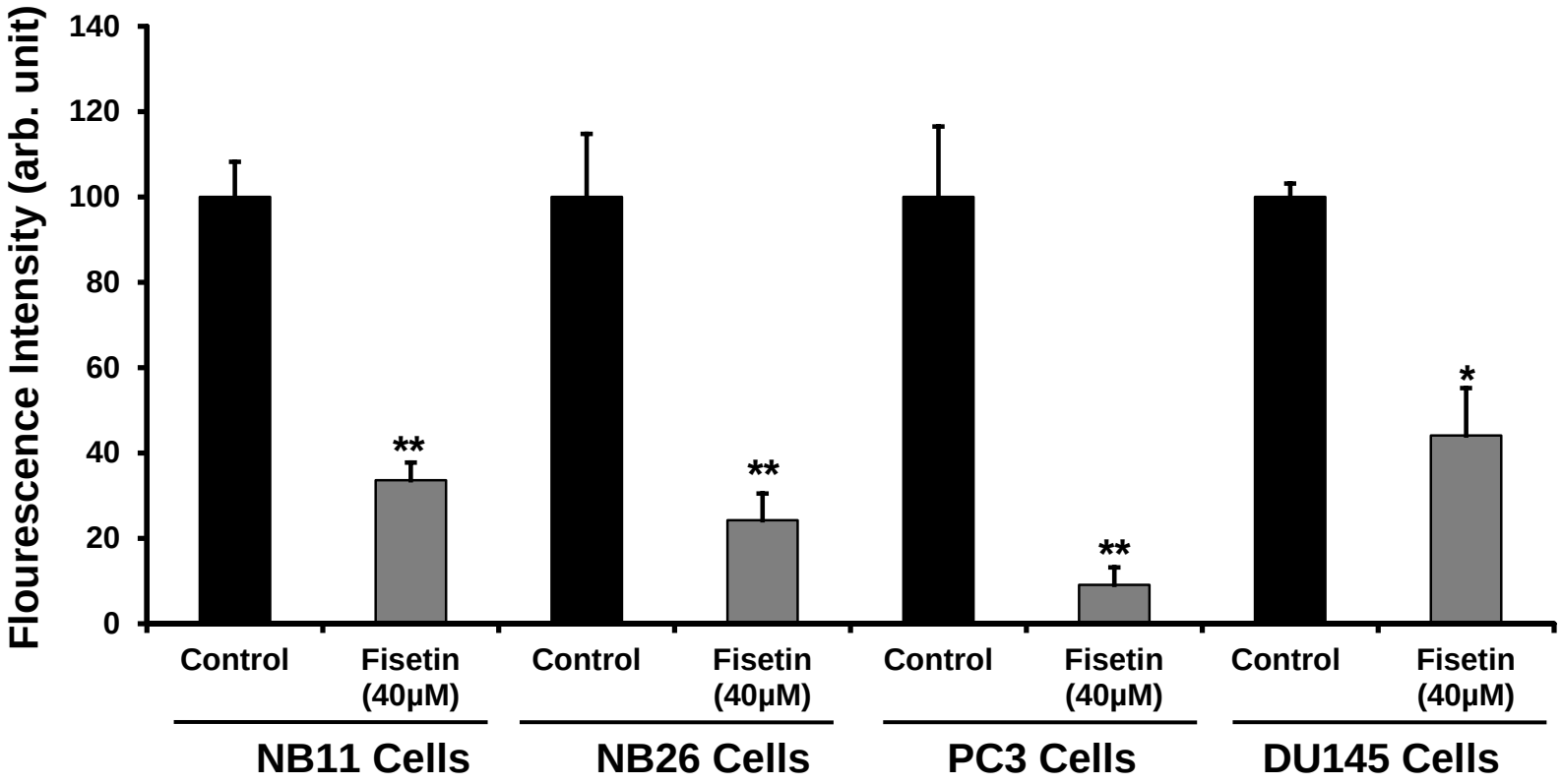

Figure 4.2B. HA expression plotted as relative fluorescence intensity (arb.unit) of NB11, NB26, PC3 and DU145 cells with or without fisetin treatment (40 μM) for 48 hour as shown in **Figure 4.2A**. Error bars represent mean±SEM between 3 biological replicates/group. Each biological replicate was performed in triplicate. Fisetin treatment showed significant difference when compared to the respective control groups (*P < 0.05, **P < 0.01).

Figure 4.2C

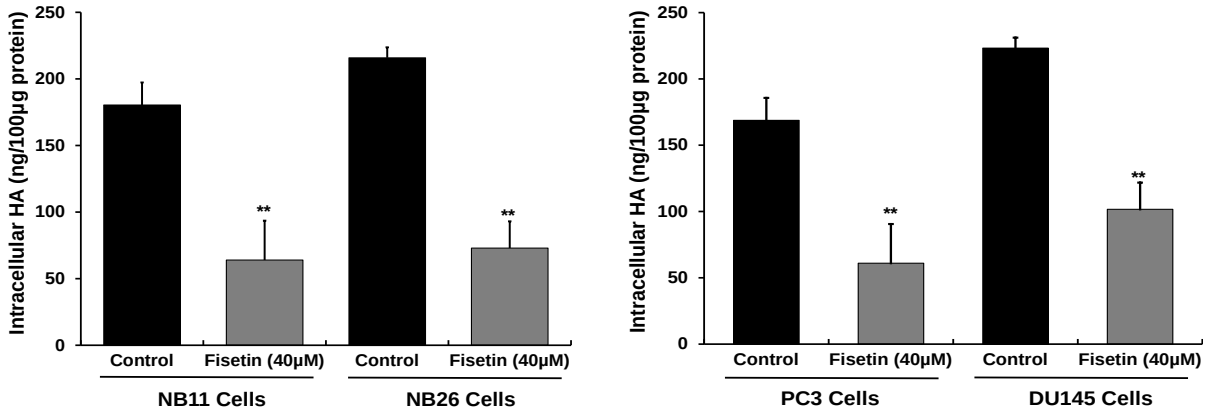

Figure 4.2C. Histogram showing intracellular HA levels in NB11, NB26, PC3 and DU145 cells measured with or without fisetin (40 μM) treatment for 48 hour. Error bars represent mean±SEM among three independent experiments/group and each of the three biological replicate was performed in triplicate to measure HA levels using ELISA (**$P \leq 0.01$). All samples were normalized using 100 μg protein of starting whole cell lysates.

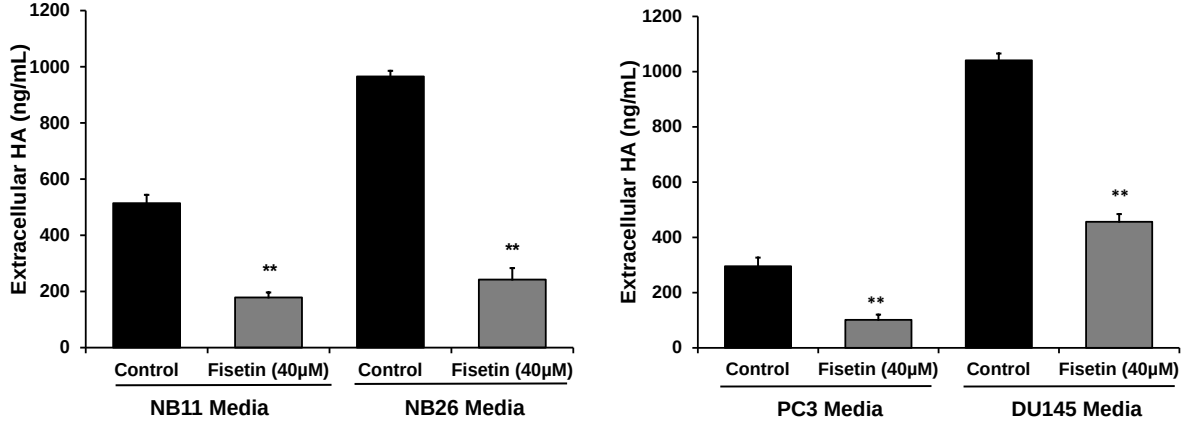

Figure 4.2D. Histogram showing extracellular HA levels secreted in NB11, NB26, PC3 and DU145 cell culture media with or without fisetin treatment (40 μM) for 48 hour. Error bars represent mean±SEM among three independent experiments/group and each of the three biological replicate was performed in triplicate to measure HA levels using ELISA (**P $\leq$ 0.01). All samples were normalized using 1 ml of collected cultured media.

Figure 4.2E

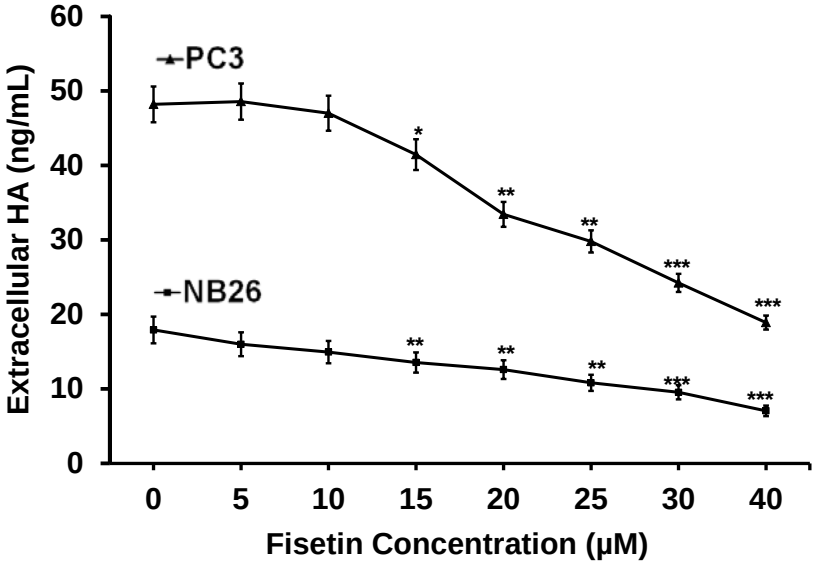

Figure 4.2E. Dose dependent effect of fisetin on secreted HA levels in PC3 and NB26 cell cultured media. Errors bars represent mean±SEM between 3 biological replicate/group. Each biological replicate was performed in triplicate. Increasing doses of fisetin (5-40 μM) significantly decreased HA levels when compared to the control group (*P $\leq$ 0.05, ** P $\leq$ 0.01, ***P $\leq$ 0.001).

Figure 4.2F

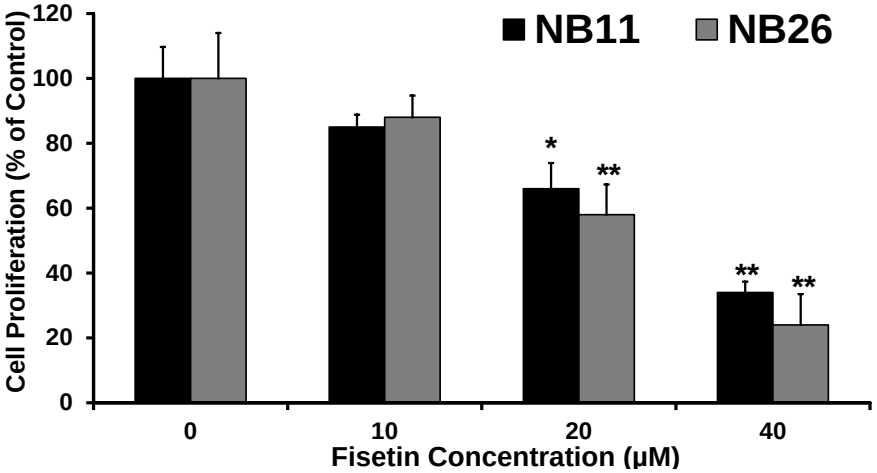

Figure 4.2F. Cell proliferation assay via BrdU incorporation in NB11 and NB26 cells treated with or without fisetin at indicated concentrations for 48 hour. Error bars represents mean±SEM showing statistical analysis between three independent assays performed in triplicate (*P $\leq$ 0.05, **P $\leq$ 0.01).

Figure 4.3. Fisetin decreases HA levels in-vivo

Figure 4.3A

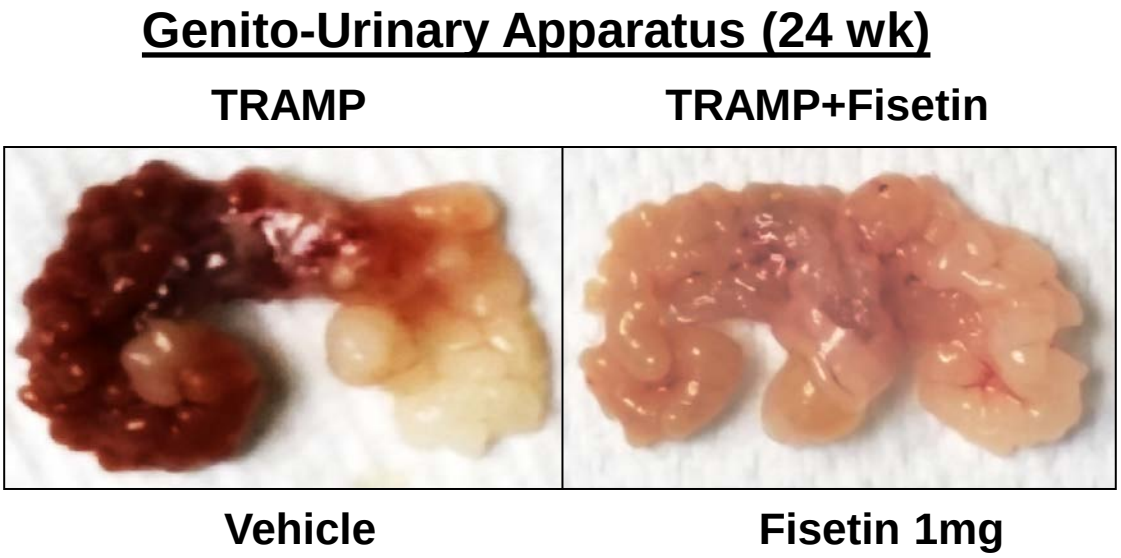

Figure 4.3A. Representative photomicrographs of the GU apparatus taken from control and fisetin-supplemented TRAMP mice after 24 weeks of age.

Figure 4.3B

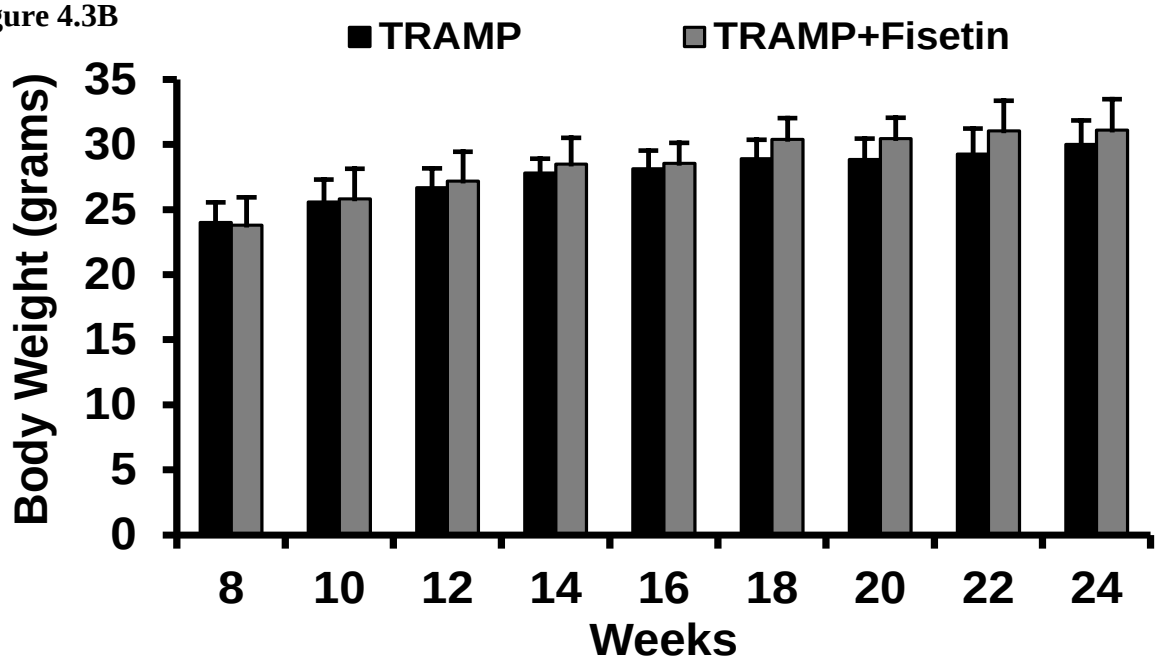

Figure 4.3B. Average body weight (grams) of TRAMP mice between the vehicle control and fisetin treatment group. Fisetin administration started at 8 weeks and continued until 24 weeks and body weight of animals was measured thrice weekly. Error bars represent mean±SEM of biological replicates between the control (n=15) and fisetin (n=12) groups.

Figure 4.3C

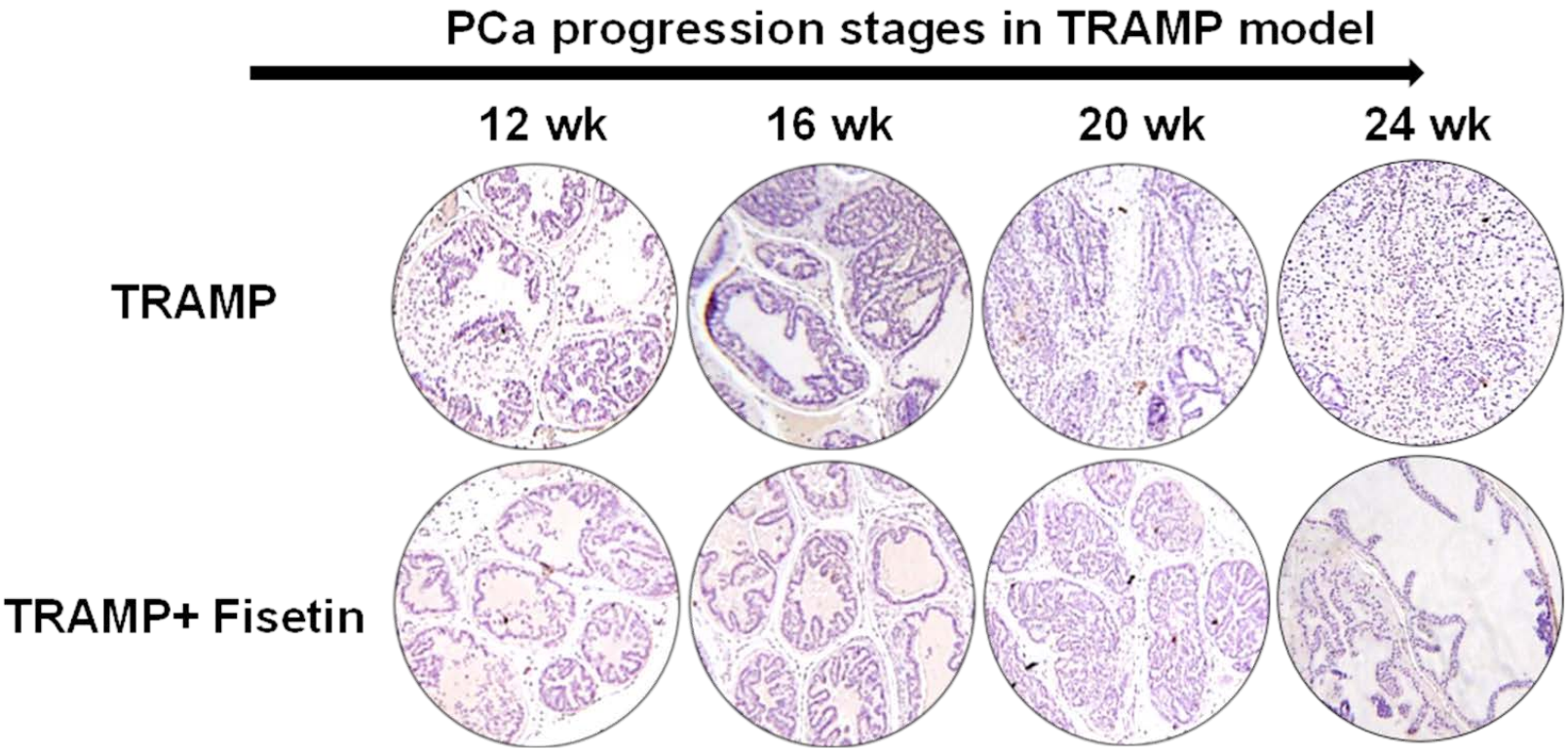

Figure 4.3C. Representative photomicrograph showing prostate histology in control and fisetin treated TRAMP mice at 12, 16, 20 and 24 week (wk). Magnification 10x; Scale=100 μM).

Figure 4.3D

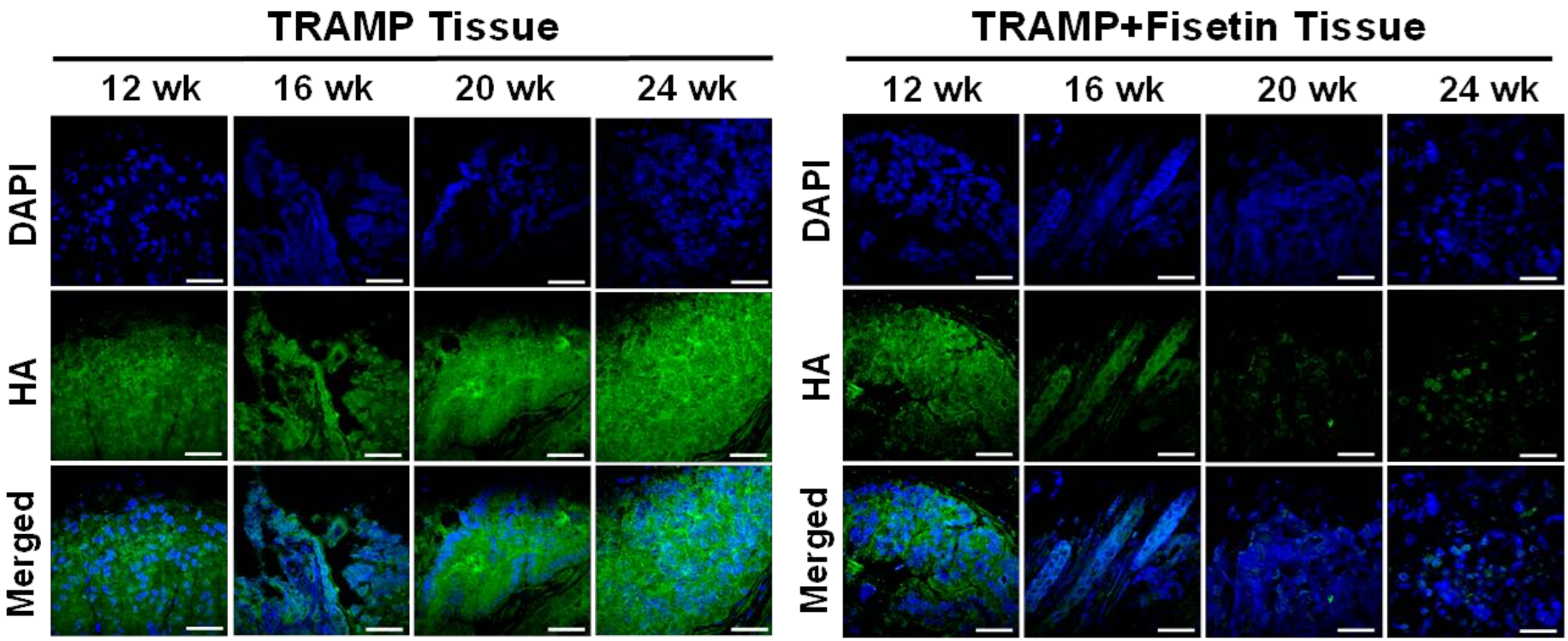

Figure 4.3D. Representative images showing immunofluorescence for HA between TRAMP and TRAMP+Fisetin groups with increasing age and PCa progression. Prostatic tumor tissues were harvested from three biological replicates and performed in triplicate for HA staining. Images were captured by a confocal microscope as described in materials and methods. Scale bar, 30 μm; Magnification: 20X. DAPI was used as a nuclear staining control.

Figure 4.3E

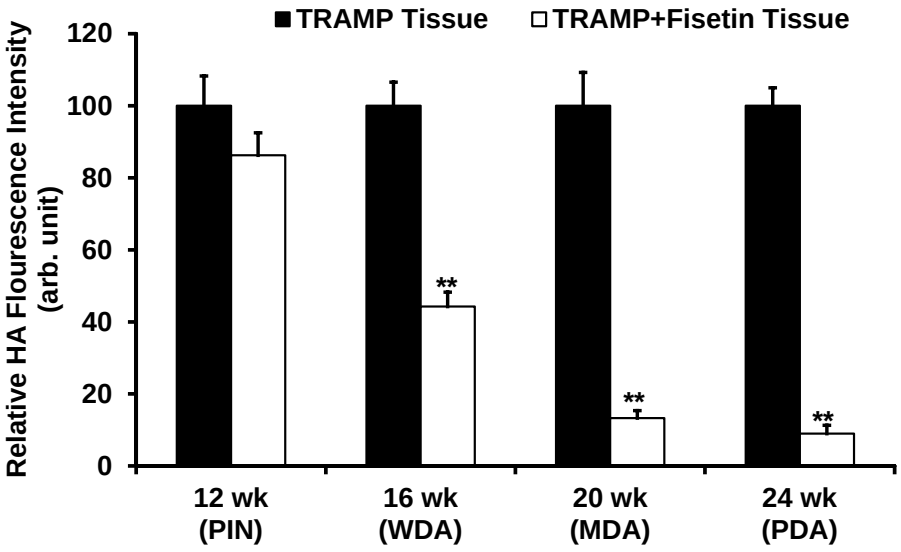

Figure 4.3E. HA expression in **Figure 4.3D** graphed as relative fluorescence intensity (arb.unit) between control and fisetin treated TRAMP animals with increasing age and PCa progression. Error bars represent mean±SEM of three biological replicates/group, and each replicate was performed in triplicate for HA staining. Statistical differences were seen in fisetin treatment groups when compared to the respective control group (**P $\leq$ 0.01).

Figure 4.3F

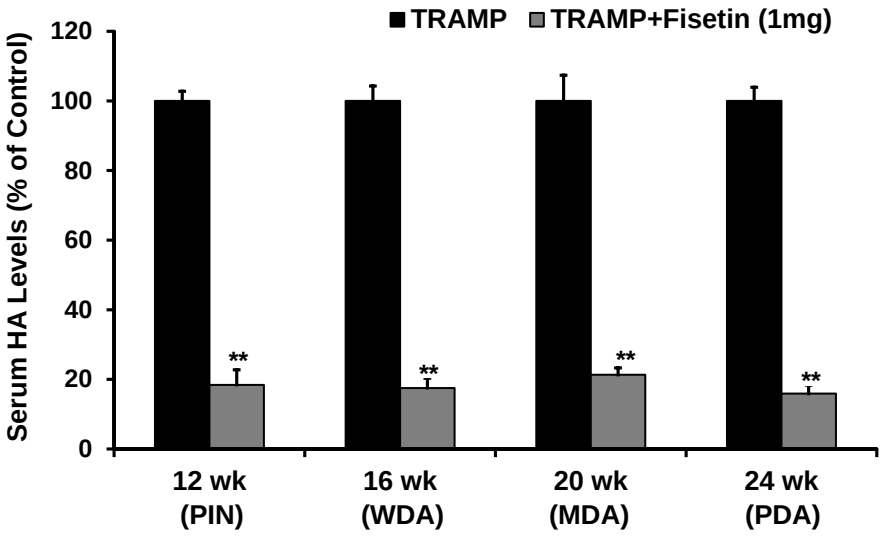

Figure 4.3F. Histogram showing HA levels (% control) secreted in TRAMP mice serum between vehicle and fisetin treated groups (1 mg/animal; 3 times/week) with increasing stages of PCa progression (PIN-prostatic intraepithelial neoplasia, WDA-well differentiated carcinoma, MDA-moderately differentiated carcinoma, PDA-poorly differentiated carcinoma). Error bars represent mean±SEM among three biological replicates/group and each biological replicate was performed in triplicate for HA levels using ELISA (**P $\leq$ 0.01).

Figure 4.4 Fisetin reduces HA synthesis and degradation enzymes both *in-vitro/in-vivo*

Figure 4.4A

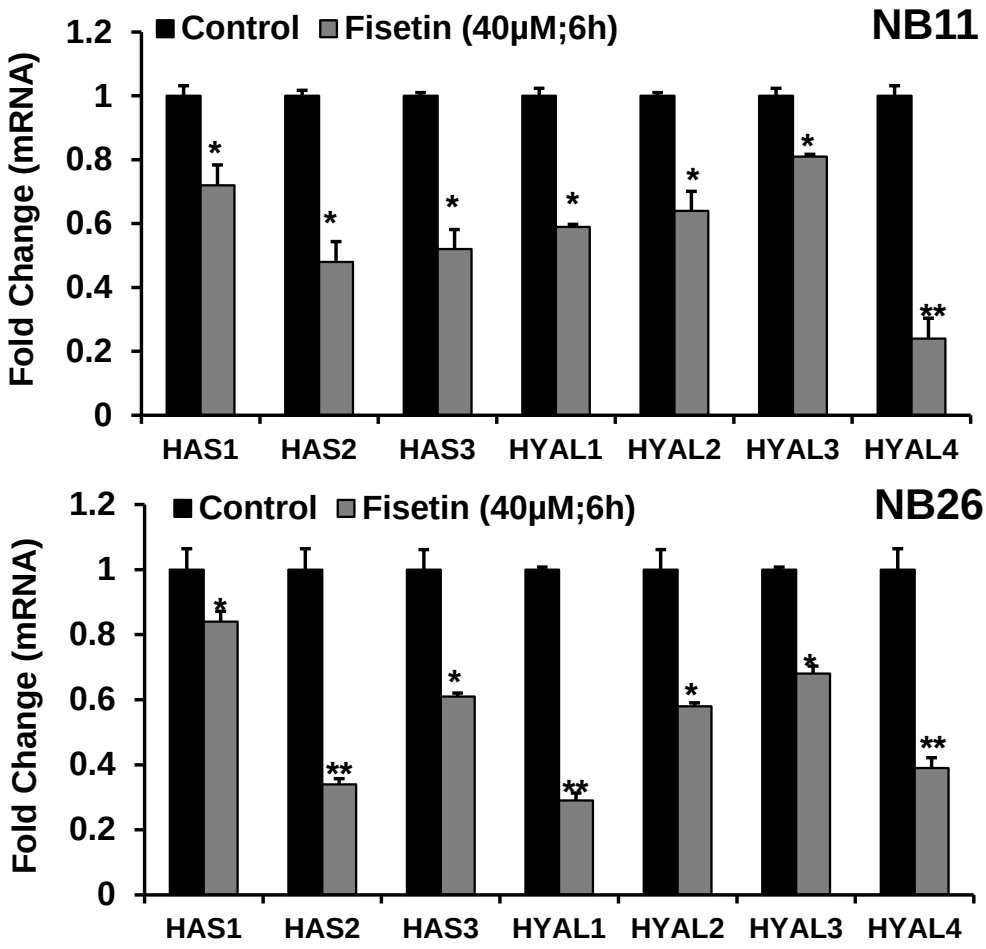

Figure 4.4A. Histograms represent relative HAS (1, 2, & 3) and HYAL (1, 2, 3, & 4) mRNA expression in NB11 and NB26 cells treated with or without fisetin (40 μM). Gene expression was measured at 6 hour by qPCR and normalized to housekeeping control, GapDH. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate. Statistical difference was seen in gene expression on fisetin treatment when compared to the respective control (*P $\leq$ 0.05, **P $\leq$ 0.01).

Figure 4.4B

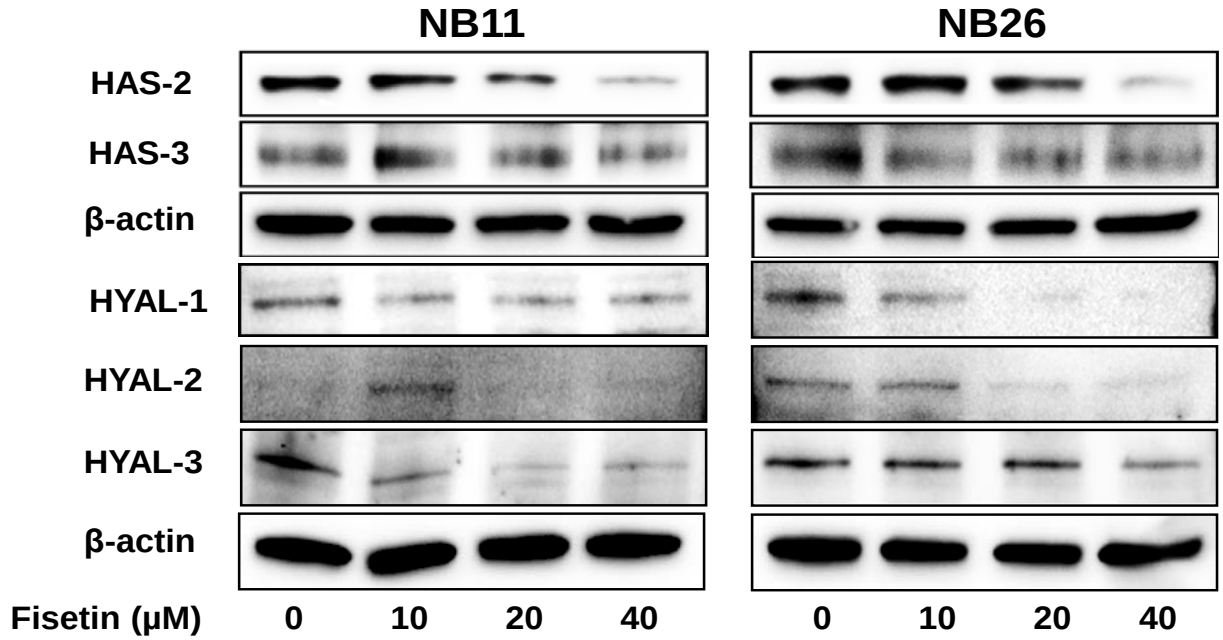

Figure 4.4B. Immunoblot images of NB11 and NB26 cells treated with or without increasing doses of fisetin (0-10-20-40 μM) for 48 hour and HAS (2 & 3) and HYAL (1, 2, & 3) protein expression were measured. Three independent experiments were performed and each experiment was analyzed in triplicate. β-actin was used as a loading control. Quantitative densitometry was analyzed and plotted (See **Figure 4.4C**).

Figure 4.4C

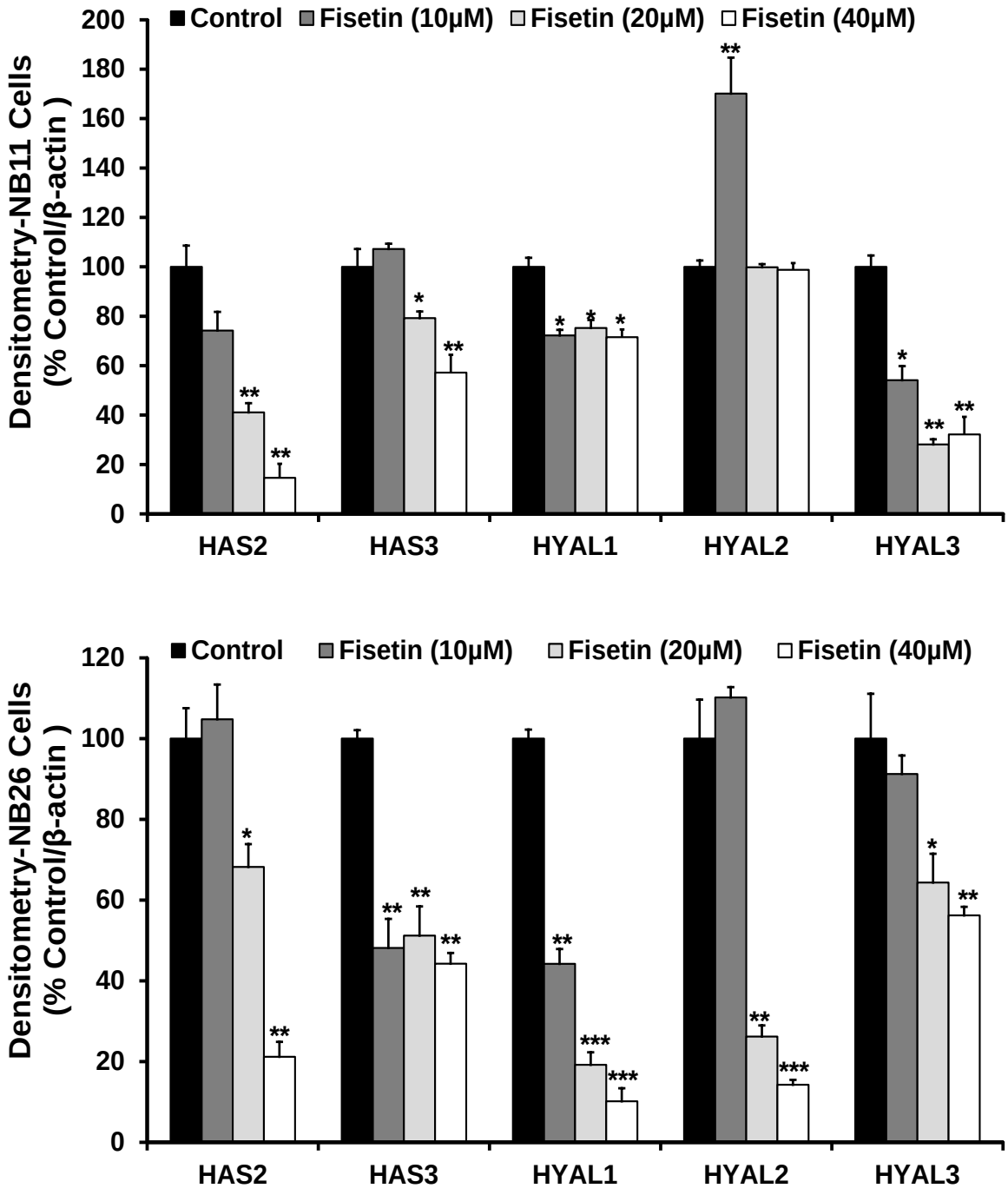

Figure 4.4C. Histogram showing quantitative densitometry of protein expressions of HAS (2 & 3) and HYAL (1, 2 &3) as shown in Figure 4B. Error bars represent mean±SEM between 3 biological replicates/group. Each biological replicate was performed in triplicate and normalized to loading control β-actin. Statistical difference was seen upon fisetin treatment (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001).

Figure 4.4D

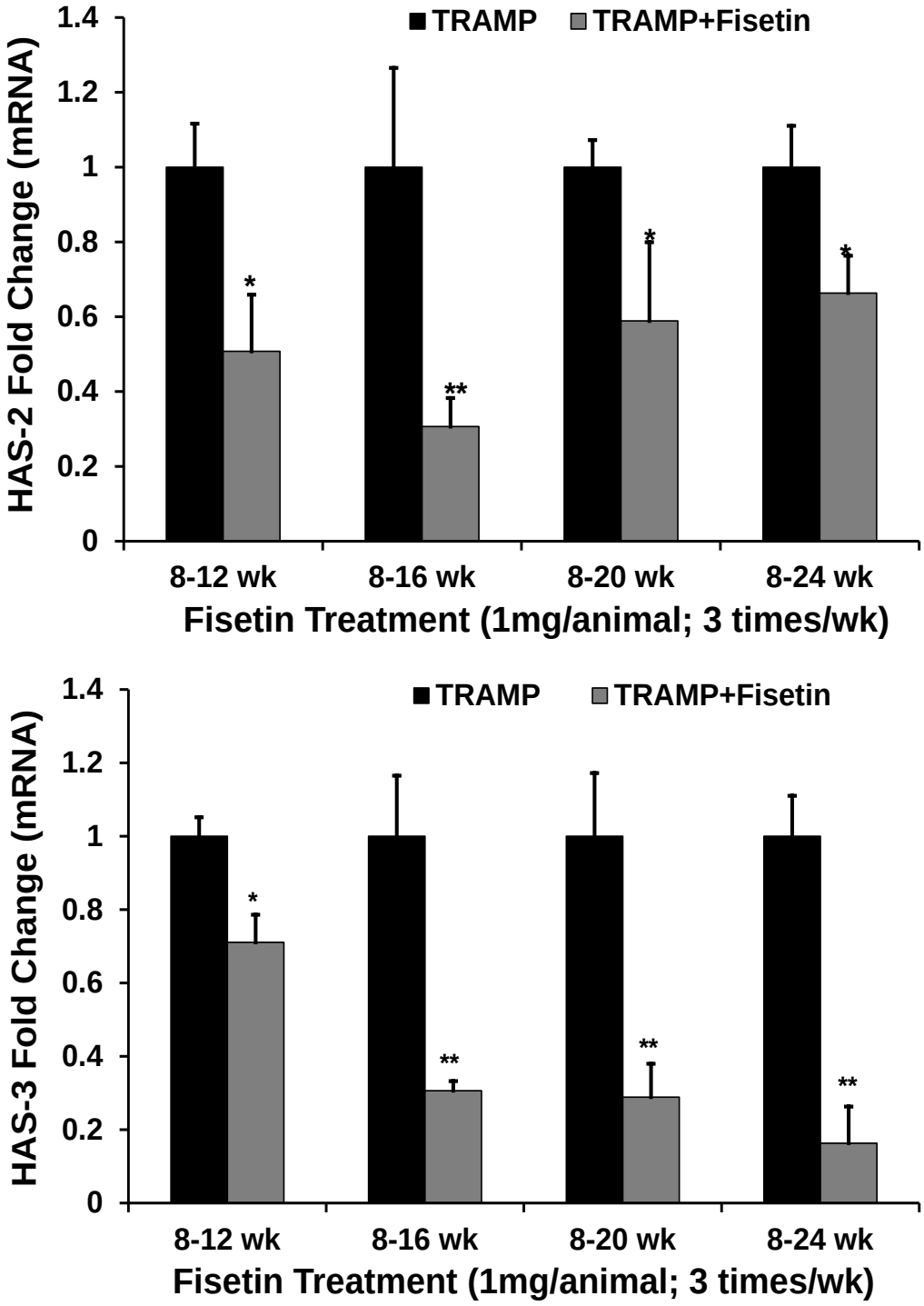

Figure 4.4D. Histograms represents relative HAS2 and HAS3 mRNA expression measured by qPCR in prostatic tissues of control and fisetin (1 mg/animal thrice weekly) treated TRAMP animals with increasing age and PCa progression. 18sRNA was used as a housekeeping gene for normalization. Error bars represent mean±SEM among three independent experiments and each experiment were performed in triplicates (*$P \leq 0.05$, **$P \leq 0.01$).

Figure 4.4E

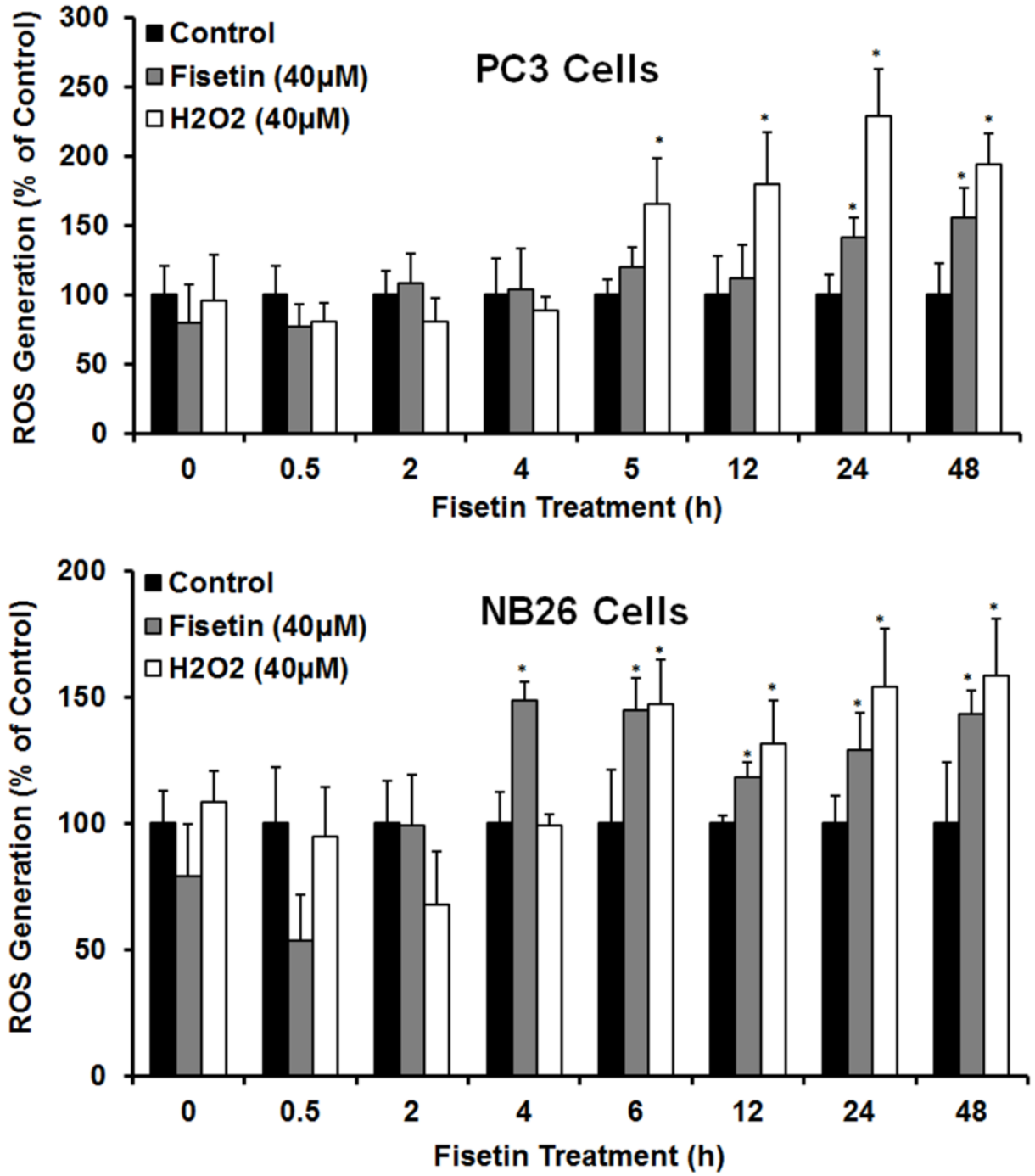

Figure 4.4E. Histogram showing intracellular ROS generation levels (% control) with or without fisetin treatment (40 μM) in a time dependent manner (0-0.5-2-4-6-12-24-48 hour). H2O2 (40μM) was used as a positive control. Error bars represent mean±SEM among three independent experiments and each experiment was performed in triplicate (*P $\leq$ 0.05).

Figure 4.5. Fisetin increases the abundance of HMM-HA in PCa cells.

Figure 4.5A

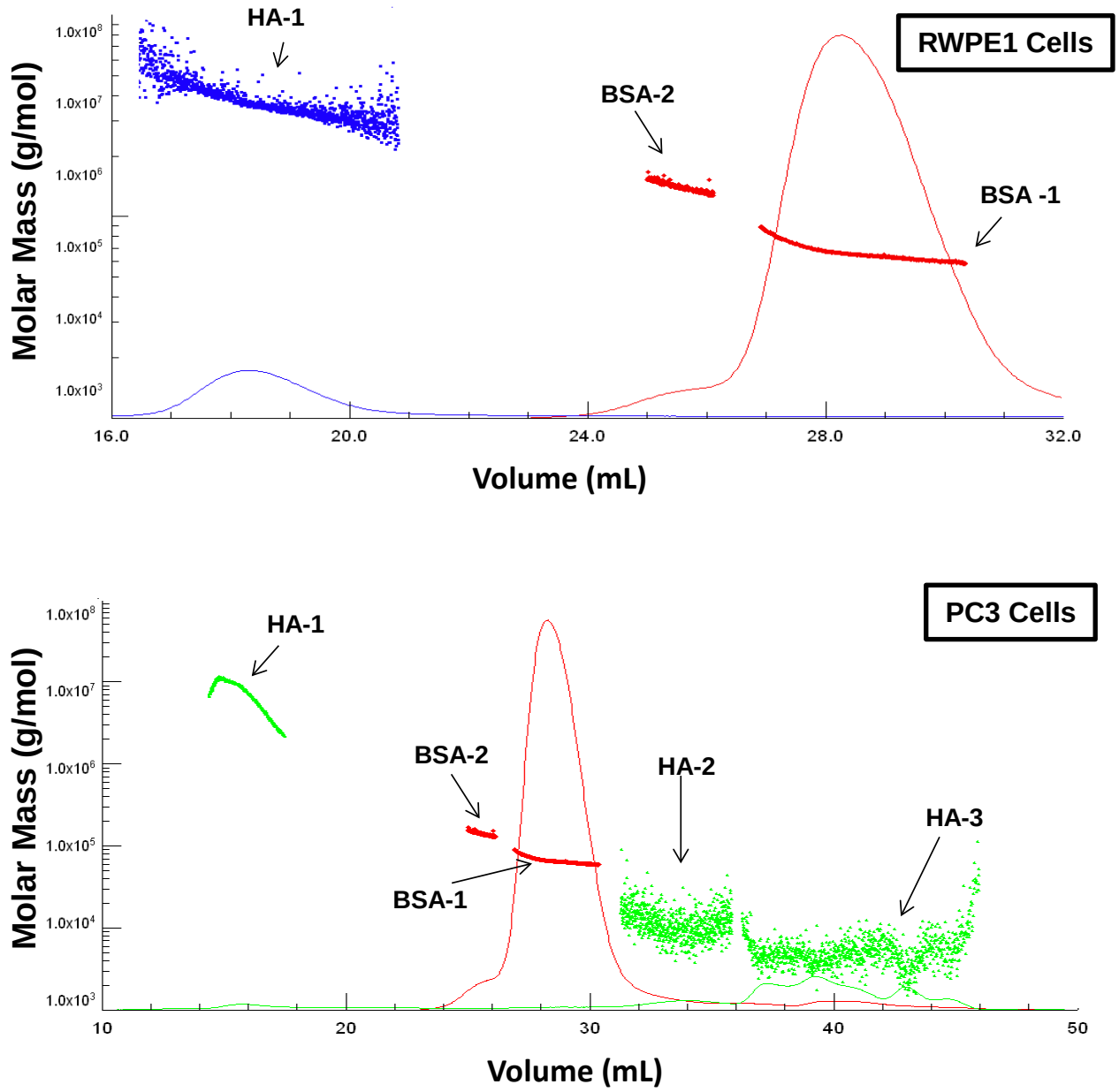

Figure 4.5A. SEC-MALS chromatogram showing molar mass distribution profile of HA fragments between normal RWPE1 and cancerous PC3 cells. Refractive index traces (solid line) and the molar mass values calculated from the light scattering data (dotted clusters) are shown, indicating the relative amount and size of HA present in RWPE1 (blue dot clusters) and PC3 cells (green dot clusters). BSA was used as an internal standard (red dotted clusters). HA was isolated from three independent experiments and analyzed using SEC-MALS.

Table 4.5A

PCa Cells	HA-1 (Cluster-1) M_w[a] (g/mol) mDa	HA-2 (Cluster-2) M_w[a] (g/mol) kDa	HA-3 (Cluster-3) M_w[a] (g/mol) kDa
RWPE1	**50.60±3.3**	ND[b]	ND[b]
PC3	5.70±0.41	**121.28±13.2**	**5.14±1.1**

[a]M_w from MALS (mean of three determinations ± SE); [b]ND, not detected. **Bold values are HA clusters with higher abundance**. Pure HA was isolated from normal and PCa cells, vacuum dried and resuspended in elutant buffer for 30 mins. The samples were then filtered and used for SEC-MALS analysis. Internal Standard BSA-1 (monomer) = 66 kDa and BSA-2 (dimer) = 132 kDa.

Table 4.5A. Average molar mass (M_w) of HA fragments in PCa cells analyzed using SEC-MALS.

Figure 4.5B

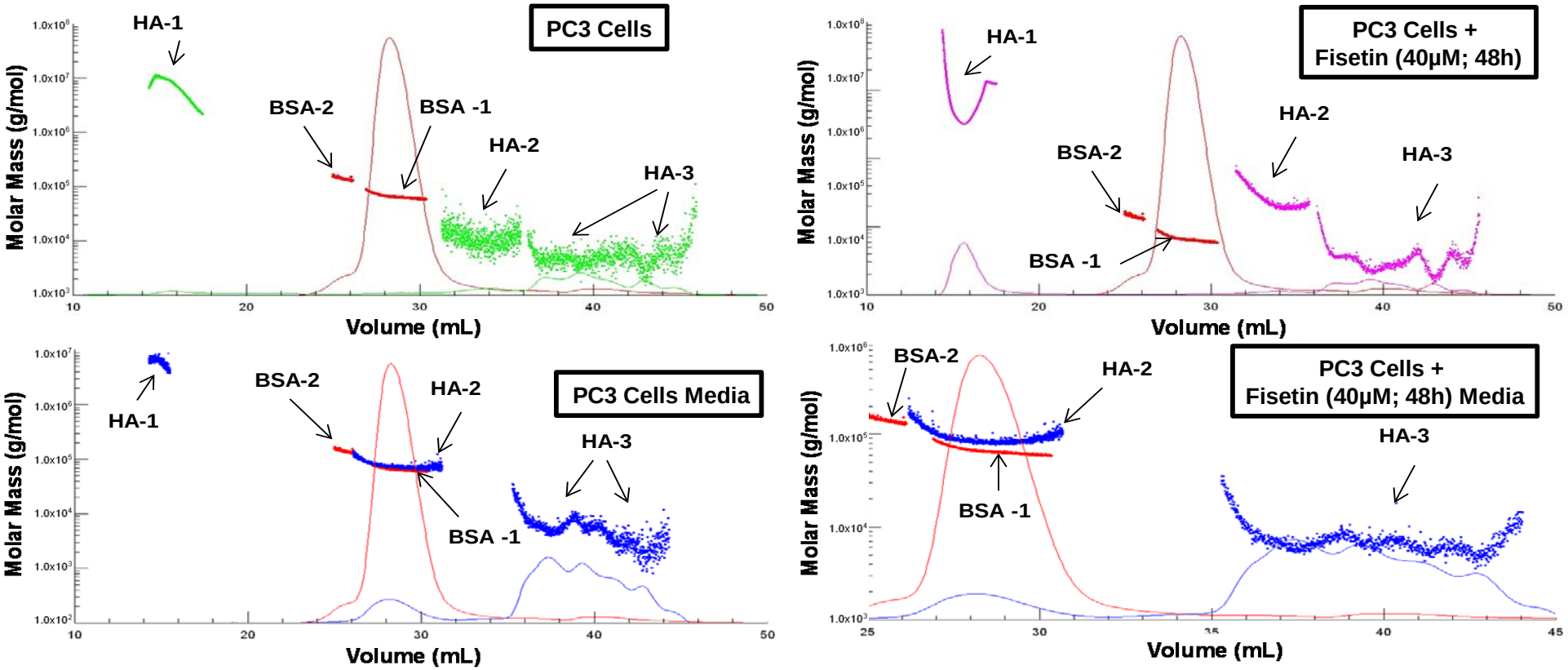

Figure 4.5B. Intra-and extracellular HA was isolated from cells and culture media with or without fisetin treatment (40 μM; 48 hour) and analyzed using SEC-MALS to generate a molar mass distribution profile of HA fragments. Refractive index traces (solid line) and the molar mass values calculated from the light scattering data (dotted clusters) are shown, indicating the relative amount and size of HA present in PC3 cells (green dot clusters, top left) and PC3 cells+Fisetin (40 μM, 48 hour; pink dot clusters, top right). Similarly, secreted HA sizes are shown in the cell culture media of PC3 cells (blue dot clusters, bottom left) and PC3 cells+Fisetin (40 μM, 48 hour; blue dot clusters, bottom right). BSA was used as an internal standard (red dotted clusters). HA was isolated from three independent experiments and analyzed using SEC-MALS.

Table 4.5B

PCa Cells/Media	HA-1 (Cluster-1) M_w[a] (g/mol) mDa	HA-2 (Cluster-2) M_w[a] (g/mol) kDa	HA-3 (Cluster-3) M_w[a] (g/mol) kDa
PC3 Control	5.70±0.5	**121.28 ±12.2**	**5.14±0.6**
PC3+Fisetin	**7.91±1.1**	260.1±19.1	32.2±4.4
PC3 Control Media	**5.97±0.9**	76.1±6.5	56.5±2.6
PC3+Fisetin Media	ND[b]	**64.6±3.3**	**30.3±1.1**

[a]M_w from MALS (mean of three determinations ± SE); [b]ND, not detected. **Bold values are HA clusters with higher abundance**. Pure HA was isolated from cells and media with/without fisetin treatment (40µM; 48 hour), vacuum dried and resuspended in elutant buffer for 30 mins. The samples were then filtered and used for SEC-MALS analysis. Internal Standard BSA-1 (monomer) = 66 kDa and BSA-2 (dimer) = 132 kDa.

Table 4.5B. Average molar mass (M_w) of HA fragments in PCa cells (intracellular) and media (secreted/extracellular) analyzed using SEC-MALS

Figure 4.6. Fisetin increases abundance of HMM-HA in TRAMP mouse model.

Figure 4.6A

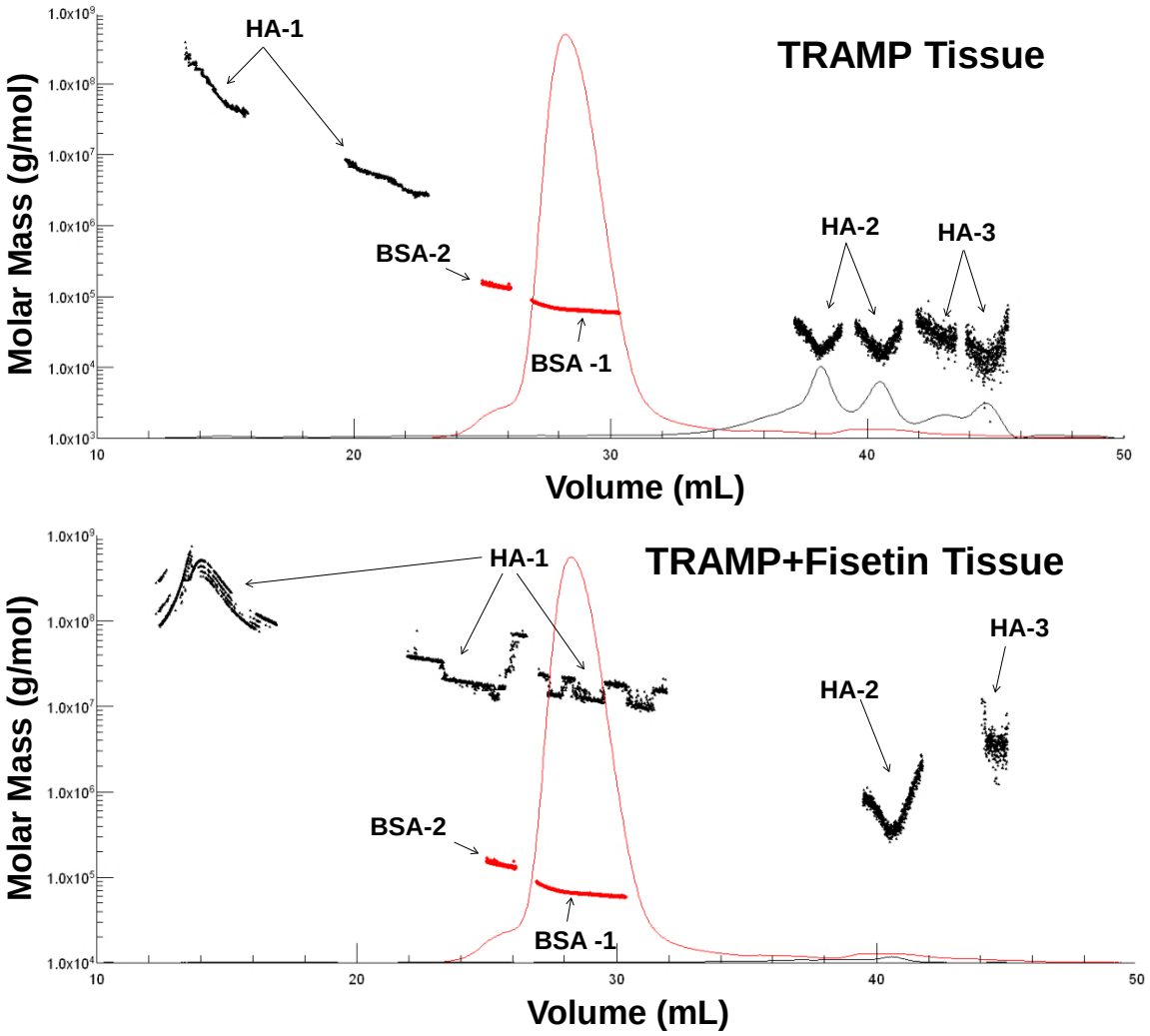

Figure 4.6A. Intracellular HA was isolated from tumors (top) of 24 week TRAMP and TRAMP+Fisetin animals (n=3 biological replicates/group) and analyzed using SEC-MALS.Refractive index traces (solid line) and the molar mass values calculated from the light scattering data (dotted clusters) are shown, indicating the relative amount and size of intact HA present in both animal groups. BSA was used as an internal standard (red dotted clusters).

Table 4.6A

Prostate Tissue	HA-1 (Cluster-1) M_w[a] (g/mol) mDa	HA-2 (Cluster-2) M_w[a] (g/mol) kDa	HA-3 (Cluster-3) M_w[a] (g/mol) kDa
24 Week TRAMP	11.80±1.5 2.3±0.8 1.2±0.11	**21±3** **23±4**	**15±3.1**
24 Week TRAMP + Fisetin	**125±12.1** **7.2± 2.2** **3.7±1.2**	892.2±44.1	18±2.1 16±3.3 24±1.9

[a]M_w from MALS (mean of three determinations ± SE). **Bold values are HA clusters with higher abundance**. HA was isolated from 24 week TRAMP prostate from control vs fisetin treated groups with disease progression, vacuum dried and resuspended in elutant buffer for 30 mins. The samples were then filtered and used for SEC-MALS analysis. Internal Standard BSA-1 (monomer) = 66 kDa and BSA-2 (dimer) = 132 kDa

Table 4.6A. Average molar mass (M_w) of HA fragments of 24 week TRAMP prostate tissue analyzed using SEC-MALS

Figure 4.6B

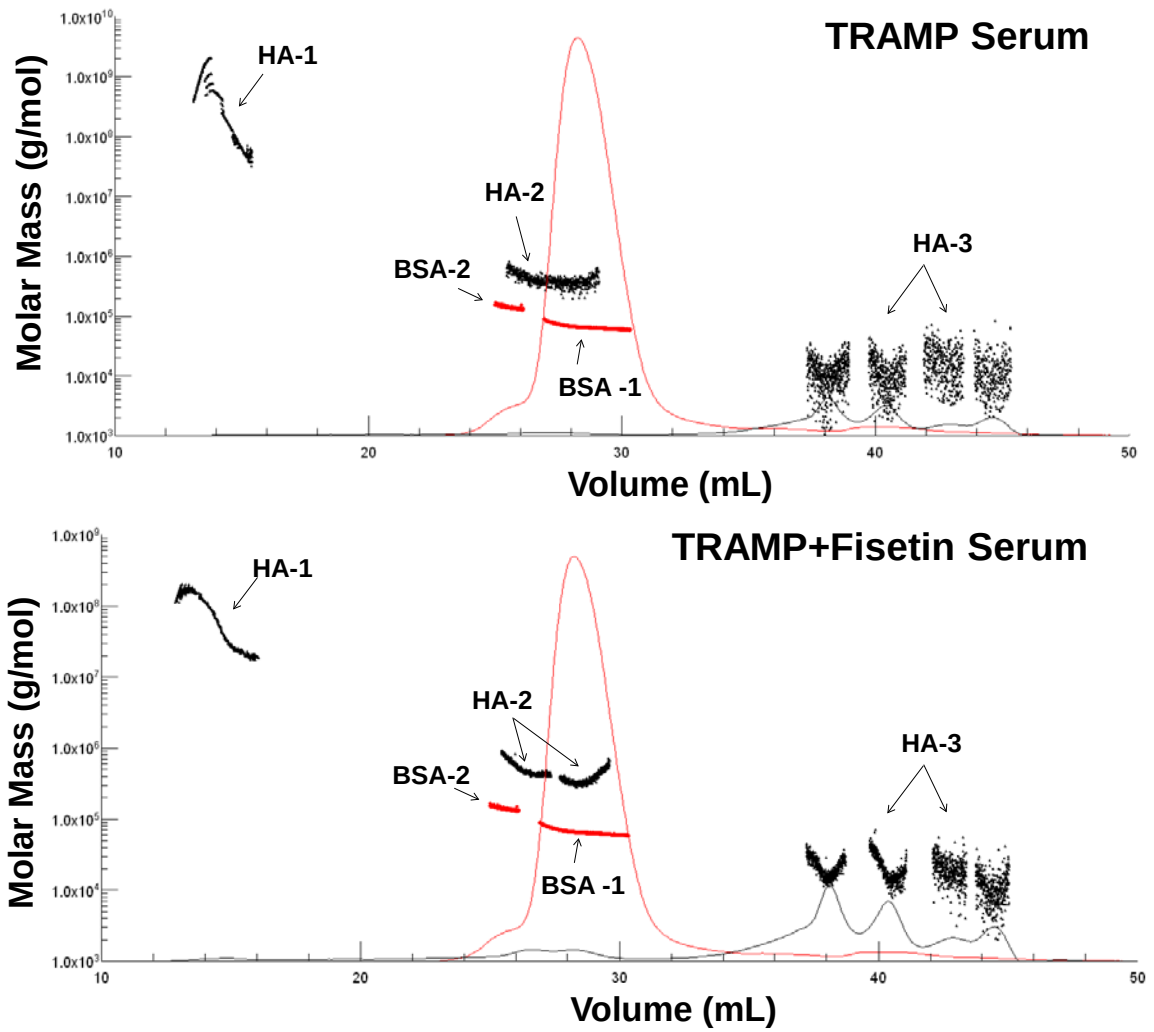

Figure 4.6B. Extracellular HA (bottom) was isolated from serum of 24 week TRAMP and TRAMP+Fisetin animals (n=3 biological replicates/group) and analyzed using SEC-MALS. Refractive index traces (solid line) and the molar mass values calculated from the light scattering data (dotted clusters) are shown, indicating the relative amount and size of secreted HA in serum of both animal groups. BSA was used as an internal standard (red dotted clusters).

Table 4.6B

Serum	HA-1 (Cluster-1) M_w[a] (g/mol) mDa	HA-2 (Cluster-2) M_w[a] (g/mol) kDa	HA-3 (Cluster-3) M_w[a] (g/mol) kDa
24 Week TRAMP	**26.1±1.2**	185.1±21.12 61.5±11.1	28.7±4.2 26.8±4.2
24 Week TRAMP + Fisetin	16±1.1 1.2± 0.2	**153.4±32.1** **54.6±12.1**	**5.2±2.1** **5.3±3.3** **7.3±1.9** **10.1±2.3**

[a]M_w from MALS (mean of three determinations ± SE). **Bold values are HA clusters with higher abundance**. HA was isolated from 24 week TRAMP serum from control vs fisetin treated groups with disease progression, vacuum dried and resuspended in elutant buffer for 30 mins. The samples were then filtered and used for SEC-MALS analysis. Internal Standard BSA-1 (monomer) = 66 kDa and BSA-2 (dimer) = 132 kDa.

Table 4.6B. Average molar Mass (Mw) of HA fragments of 24 week TRAMP serum analyzed using SEC-MALS

Table 4.A

Antibody Name	Manufacturer	Reference
HA	Abcam	ab53842
HAS1	Santa Cruz	sc23145
HAS2	Abcam	ab140671
HAS3	Abcam	ab128816
HYAL1	Pierce	pa527901
HYAL2	Pierce	pa524223
HYAL3	Abcam	ab116454
HYAL4	Abcam	ab116547
β-Actin	Cell Signaling	4967

*Antibodies were used as per manufacturer's dilutions

Table 4.A. List of antibodies used for immunoblot and immunofluorescence analysis.

Table 4.B

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
HAS1	CTGCGATACTGGGTAGCCTTCA	CCAGGAACTTCTGGTTGTACCAG
HAS2	GTCATGTACACAGCCTTCAGAGC	ACAGATGAGGCTGGGTCAAGCA
HAS3	AGCACCTTCTCGTGCATCATGC	TCCTCCAGGACTCGAAGCATCT
HYAL1	GACACGACAAACCACTTTCTGCC	ATTTTCCCAGCTCACCCAGAGC
HYAL2	GGACCTCATCTCTACCATTGGC	CTTTGAGGTACTGGCAGGTCTC
HYAL3	GCAGTCCATTGGTGTGAGTGCA	CCAAGGTGTCCACCAGGTAGTC
HYAL4	GGGTGCGTGAATCACTGAGGAT	CAACGCAGCACTTTCTCCTATGG
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
18sRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 4.B. Sequences of primers used for qPCR analysis

Table 4.C

Cell line	ATCC #	Disease	Sex	Tissue	Authentication	Receipt and Authentication Month & Year	Usage after authentication
RWPE1	CRL-11609	Normal	M	prostate	STR, Karyotype	May 2013	<3 months
NB11	CRL-2851	Normal	M	prostate	STR, Karyotype	May 2013	<3 months
NB26	CRL-2852	Normal	M	prostate	STR, Karyotype	May 2013	<3 months
PC3	CRL-1435	Grade IV, adenocarcinoma	M	prostate; derived from metastatic site: bone	STR, Karyotype	July 2012	<3 months
DU145	HTB-81	Carcinoma	M	prostate; derived from metastatic site: brain	STR, Karyotype	July 2012	<3 months

Table 4.C. ATCC cell lines characterization and authentication.

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CHAPTER 5

Conclusions and Future Directions

From the experiments described in this dissertation, several important findings were discussed with respect to the role of HA abundance in the tumor microenvironment which negatively influenced mouse and human outcome in PCa. The main objective of this dissertation was to identify novel metabolic targets of fisetin and to establish if the identified target can be validated across various models of PCa and can possibly serve as a molecular marker for PCa prognosis. To this end, we identified HA and established its role in PCa progression. Next, we established the functional role of HAS2 and HAS3 in cell proliferation and invasion of PCa cells. We further identified fisetin, as a potent HA synthesis inhibitor. We developed a robust SEC-MALS method to accurately measure the molecular size of HA fragments across biological samples, *in-vitro* and *in-vivo*. The innumerable oncogenic functions of HA typically depend on its molecular size which is influenced by the constant turnover of HA with either increased HA synthesis or HA degradation or both. Using SEC-MALS, we further reported the comparative molecular size within the normal and diseased condition. Our findings suggest that anti-angiogenic HMM-HA is increased in non-diseased conditions due to increased HA synthase and decreased hyaluronidase levels. However, with onset of disease progression , the degradation enzymes are upregulated and large pool of pro-angiogenic LMM-HA accumulates. Fisetin treatment of PCa cells reduced the larger pool of LMM-HA and increased the abundance of HMM-HA which mimics the HA fragment profile of the non-diseased state which is the highlight of this work.

PCa is a heterogeneous disease that uses multiple signaling pathways to survive and continues to be one of the leading causes of death in men. Single-agent-targeted therapies have rarely cured patients with cancer. To effectively halt tumor development and progression, a drug that can target multiple deregulated proteins and pathways would be ideal. Dietary flavonoid fisetin has shown tremendous potential in preclinical settings to target multiple deregulated proteins, signaling pathways and regulates wide variety of cell functions against various cancers. Fisetin disrupts Wnt signaling and results in cell cycle arrest. It inhibits the YB-1 binding protein to suppress epithelial to mesenchymal transition and thus prevents invasion and migration of cancer cells. By physically interacting with the mTOR molecule, fisetin inhibits signaling involved in cell survival thus explaining its inhibitory effect on cellular growth and proliferation. Fisetin binds to and disrupts microtubule dynamics and acts as a stabilizing agent with effects far superior to paclitaxel. Findings from this dissertation work showcases fisetin as a potent HA synthesis inhibitor and can regulate HA signaling. To put the chemopreventive potential of fisetin to clinical use, well designed clinical trial interventions at appropriate doses in high risk population are essential. Efforts should be targeted to select populations which can mirror the data that has previously been obtained from animal studies to gauge the efficacy of the compound. Before actual clinical trials are conducted it is important that toxicological profile of fisetin is comprehensively investigated. Also, the pharmacokinetic and bioavailability issues have to be studied in detail before phase I and II trials are initiated.

The debate on cancer chemoprevention attracts a lot of attention with dietary components showing promise in preclinical setting but lack of effect when used in clinical trials. Several critiques downgrade the importance of phytochemicals based on their multiple biological effects, targets and signaling pathways. However, we strongly feel that there is a lot of potential for

cancer chemoprevention by dietary agents provided that they are studied in appropriate populations. Importantly, these agents offer an effective alternate noninvasive strategy for the management of cancer especially for high-risk individuals. Development of drug resistance is one of the most prominent limitations in the development of targeted therapies. A combination of targeted therapies with more traditional therapies could be a potential key to the problem of resistance. However, in overcoming drug resistance issues, toxicity and high treatment cost remains an impediment in the development of FDA-approved anticancer drugs. There is enough strong preclinical evidence that fisetin shows tremendous promise as a therapeutic anticancer agent and warrants clinical trials in humans either to be administered alone or in combination with available anticancer drugs.

The PCa tumor microenvironment is dynamic, complex and poorly understood. While more studies are required to have a better understanding of the complexity of the mechanisms, it is clear that several inflammatory components like HA, play a role not only in tumor cell development but also, in extracellular matrix modulation, which in turn, promote a more aggressive tumor microenvironment. The work described in this dissertation provides additional support for the role of HA in prostate carcinogenesis and suggests that men suffering with invasive carcinoma with high hyaluronan levels may effectively benefit from fisetin consumption.