Targeting Estrogen- and Hedgehog-Signaling Pathways in Prostate Cancer

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by

ANNA ŚLUSARZ

Dr. Dennis B. Lubahn, Dissertation Supervisor

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The undersigned, appointed by the Dean of the Graduate School,

have examined the dissertation entitled

Targeting Estrogen- and Hedgehog-Signaling Pathways in Prostate Cancer

Presented by Anna Ślusarz

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

Dr. Dennis Lubahn _____

Dr. Cynthia Besch-Williford _____

Dr. William Folk _____

Dr. Venkataseshu Ganjam _____

Dr. Mark Martin _____

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17βHSD: 17β-hydroxysteroid dehydrogenase

22RV1: Human prostate cancer cell line

3α-HSD: 3α-hydroxysteroid oxidoreducase

3β-HSD: 3 β-hydroxysteroid oxidoreducase

AhR/ARNT: dimer that activates transcription of several genes involved in metabolism of foreign chemicals, including CYP1A1, CYP1B1, and NADP(H)

AKR1c3: 17β -hydroxysteroid dehydrogenase type 5, 3α-hydroxysteroid dehydrogenase type 2

Akt: 3-gene family that are serine/threonine-specific protein kinases

Ap1: Transcription factor

AR: Androgen Receptor

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BERKO: Mice lacking functional ER-β

BOC: Brother of CDO gene

BODIPY: boron-dipyrromethene, a fluorescent dye

FRET: Förster resonance energy transfer or fluorescence resonance energy transfer

BPH: Benign Prostatic Hyperplasia

BSA: Bovine Serum Albumin

cAMP: Cyclic adenosine monophosphate, a second messenger

CDO: Transmembrane protein involved in hedgehog signaling in *Drosophila*

c-Jun: Transcription factor

CK1: Casein kinase 1, serine-threonine selective protein kinases

CM: Conditioned Medium containing Shh-peptide

Cyc: Cyclopamine

CYP: Cytochrome P450 related enzymes

DBD: DNA Binding Domain

DES: Diethylstilbesterol

Dhh: Desert hedgehog

DHT: 5α-dihydrotestosterone

Disp: Dispatched, membrane receptor important for Hh Signaling

DPN: Diarylpropionitrile

DU145: Human prostate cancer cell line

E2: 17β-Estradiol

EBP: Estrogen Binding Proteins

EGCG: Epigallocatechin 3-gallate

eNOS: endothelial NOS or nitric oxide synthase 3

ER: Estrogen Receptor

ErbB-2: This gene also known as Her-2/neu, encodes for an epidermal growth factor receptor

ErbB-3: This gene encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases

ERE: Estrogen Response Element

ERK: Extracellular signal-related kinases, also known as MAP kinases

ERWT : Mice with both estrogen receptors present

ERα: Estrogen Receptor Alpha

ERαKO: Estrogen Receptor Alpha Knock Out Mice, lacking a functional ERα

ERβ: Estrogen Receptor Beta

ERβKO: Estrogen Receptor Beta Knock Out Mice, lacking a functional ERβ

FBS: Fetal Bovine Serum

G_0: Quiescent phase of the cell cycle

G_1: Part of the cell cycle where interphase, cytokinesis, and DNA synthesis take place

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene used as a control gene in many experiments

Gli1: Hedgehog gene that is most commonly associated with pathway activity

GliBS-reporter: Gli1 binding site driving a luciferase reporter

GLUT: Glucose Transporter

GnRH: Gonadotropin-releasing hormone (GnRH), also known as Luteinizing-hormone releasing hormone (LHRH), responsible for the release of FSH and LH from the anterior pituitary

GPR30: G-protein Coupled Receptor 30, an integral membrane protein with high affinity for estrogen. Also known as G Protein-coupled Estrogen Receptor 1, GPER.

GSK3beta: Glycogen synthase kinase-3β is a proline-directed serine-threonine kinase

GSTP1: Glutathione Sulfotranserase-pi

HHMI: Howard Hughes Medical Institute

Hip: Hh-interacting Protein

HIV: Human Immunodeficiency Virus

HMG-CoA Reductase: 3-hydroxy-3-methyl-glutyral CoA Reductase

HPC1: Hereditary Prostate Cancer 1 Gene

HPCX: Hereditary Prostate Cancer X-linked Gene

HPTE: 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane

HRP: Horseradish Peroxidase

HYP: Hyperplasia

IC_{50}: Half-maximal Inhibitory Concentration

ICI: ICI 182,780 or fulvestrant

Ihh: Indian Hedgehog

Ki67: Cellular marker for proliferation

KO: Genetic technique whereby a specific gene is made inactive, or "knocked-out"

LADY: Transgenic mouse prostate cancer model in which the large T antigen gene containing the deletion mutation d1 2005 (removing expression of small t antigen) is driven by the prostate specific probasin promoter. It was created after the TRAMP model, and was named after the classic Disney movie "Lady and the Tramp."

LBD: Ligand Binding Domain or Hormone Binding Domain

LH: Luteinizing hormone

LHRH: Luteinizing hormone releasing hormone gene

LNCaP: Human prostate cancer cell line that is androgen-sensitive

MDC: Moderately Differentiated Carcinoma

MMP: Matrix metalloproteinases are zinc-dependent endopeptidases

NADPH: Nicotinamide adenine dinucleotide phosphate

NAO: Neoxanthin

NE: Neuroendocrine

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells is a protein complex that controls the transcription of DNA

p38: a MAPK, MAPK11

P450: Cytochrome P450

p53: transcription factor known to be a tumor supressor

p73: a protein related to the p53, involved in cell cycle regulation, and induction of apoptosis

PBS: Phosphate Buffered Solution

PBTag: Probasin-driven T-antigen

PC3: Human prostate cancer cell line

PC3M: A reportedly more metastatic variant of the PC3 cell line

PCaP: Predisposing for Prostate Cancer Gene

PDC: Poorly Differentiated Carcinoma

PI3K: Phosphoinositide 3-kinases, family of related intracellular Signal transducer enzymes

PIN: Prostatic Intraepithelial Neoplasia

PKA: Protein Kinase A, activated by cAMP

PPT: Propyl Pyrazole Triol

PSA: Prostate Specific Antigen

Ptch: Patched

Rb: Retinoblastoma

Real-time RT-PCR:

RM-9: mouse prostate cancer cell line derived from ras + myc transformed mouse (C57BL/6) prostate carcinomas

RT-PCR: Reverse-Transcriptase Polymeraes Chain Reaction

SAG: Smoothened Agonist

SERMs: Selective Estrogen Receptor Modulators

Shh: Sonic Hedgehog

shRNA: short hairpin RNA

siRNA: small interfering RNA

Ski: Skinny Hedgehog

Smo: Smoothened

SUFU: Suppressor of Fused

SV40: Simian vacuolating virus 40 or Simian virus 40

TACE: chlorotrianisene

TGF-β: Transforming Growth Factor Beta

THC: R,R-tetrahydrochyrsene

TRAIL: TNF-related Apoptosis Inducing ligand

Hh: Hedgehog

TRAMP: TRansgenic Adenocarcinoa of the Mouse Prostate

TRAMP-C2: prostate tumor cell line derived in 1996 from a TRAMP mouse.

VEGF: Vascular Endothelial Growth Factor

WDC: Well Differentiated Carcinoma

WT: Wild type

Abstract

Prostate cancer is the second most frequent cause of cancer deaths in men, and the risk to develop cancer increases with age. Since the lifespan of the society is progressively increasing, the importance of preventing the development of this common disease is becoming more urgent. The prevention and therapy options for non-metastatic prostate cancer are available but limited. For the androgen-insensitive, metastatic form of prostate cancer there still is no effective therapy.

Our laboratory has an estrogen receptor based perspective on prostate cancer. We are using a transgenic mouse as an animal model to study the development and treatment of prostate cancer. We are also investigating a selection of botanical compounds that have been implicated as cancer protective either by traditional medicine or in modern cancer research.

The TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) model enables us to study the specific roles of estrogen receptors in prostate cancer progression. The expression of the two estrogen receptors alpha (ERα) and beta (ERβ) in TRAMP mouse prostates switches upon progression from non-metastatic to metastatic tumor phenotype. During earlier stages of cancer, ERβ is the main estrogen receptor present, as the phenotype of the cancer changes to the more neuro-endocrine like, the expression of ERβ decreases and ERα seems to remain the only active receptor.

Initial studies in the Lubahn lab and elsewhere led us to **hypothesize** a protective role for ERβ in prostate cancer tumorigenesis. The presence of ERβ only seems to prevent or slow down the incidence of (PDC) poorly differentiated carcinoma in contrast

to ERα, which promotes the metastatic, neuro-endocrine like phenotype. We have found though a study in double transgenic mice, that were WT and KO for estrogen receptors alpha or beta on a TRAMP background, that ERαKO mice did not develop poorly differentiated carcinoma (75% reduction compared to control animals), however ERβKO mice had significantly increased PDC incidence and had double the PDC compared to animals WT for both receptors.

We were also interested in cancer protective properties of a variety of botanical compounds, reported in the literature to be potentially beneficial for prostate health, and widely bought through health food stores around the country. We specifically investigated apigenin, baicalein, curcumin, EGCG, genistein, quercetin, and resveratrol, both *in vitro* and *in vivo*. All seven compounds were able to delay prostate cancer cell growth of both human (LNCaP, PC3, and PC3M) and mouse (TRAMP-C2) prostate cancer cell lines. All seven compounds combinations were also able to inhibit or delay prostate cancer incidence by up to 80%, specifically at the well differentiated carcinoma stage, when fed to TRAMP mice. The protective effects were only present in ERWT mice, indicating a need for both receptors for these compounds to act on the prostate cancer incidence.

I introduced a new pathway to the lab, the hedgehog signaling pathway, which has been recently found to play a role in prostate cancer, specifically in metastatic cancer. Several botanicals used in the lab were able to inhibit the hedgehog pathway as indicated by decreasing Gli1 levels. With IC_{50} values ranging from <1μM to 25μM these compounds demonstrated hedgehog pathway inhibition by deceasing *Gli1* mRNA concentration by up to 95% and down regulating Gli-reporter activity by 80%. Also, both

estrogen and ICI inhibit *Gli1* mRNA in TRAMP-C2 cells, and Gli-reporter activity in Shh Light II cells.

My research sheds light on an additional mechanism by which phytoestrogens are potentially protecting against cancer. My work suggests a potential new treatment target for addressing both slow and fast growing prostate cancers. Based on data presented here, we propose that a combination of ERα antagonists, ERβ agonists and selected botanicals should present a comprehensive prostate cancer remedy.

I - Background

Prostate Cancer

Prostate cancer, after skin cancer, is the second most common form of cancer in men in America. According to the Prostate Cancer Foundation over 186,000 men were diagnosed with prostate cancer in 2008, and almost 30,000 men will die from the disease (1). After lung cancer, prostate cancer is the leading cause of cancer-related deaths among men in the U.S. It is estimated that there are over 2 million American men currently living with prostate cancer. The chance of having prostate cancer increases rapidly after age 50. More than 70% of all prostate cancers are diagnosed in men over the age of 65. It is still unclear why this increased risk with age occurs for prostate cancer (2).

Risk factors for prostate cancer may be found in both genetic and environmental areas. While there are no genes so far that have been reported to cause prostate cancer (3), but there are some which are correlated with a higher chance of developing the disease. Epidemiological studies showed, for example, that a polymorphism in the CYP1A1 gene, causing increased activity of the cytochrome P450 enzyme involved in the metabolism of estrogens to form reactive catechol estrogens or quinone/semiquinone intermediates, like 2-hydroxy-(OH)-estrogens, makes the carriers of this allele have a higher incidence of prostate cancer (4). There are also genetic markers correlated with a higher prostate cancer risk like HPCX (Hereditary Prostate Cancer X-linked) (5), HPC1 (Hereditary Prostate Cancer 1) (6), PCaP (Predisposing for Prostate cancer) (6), and other predisposition loci (3, 7, 8), but together these genetic factors are found responsible for

only 9% of all cancer cases (9, 10). Additional risk factors include environmental factors (11), and maybe more importantly for this dissertation, dietary factors (12), to be discussed in more detail later in this chapter.

Estrogen Signaling Pathway

Estrogen receptors are ligand-activated transcription factors that belong to the large family of steroid or nuclear receptors together with the receptors for androgen, progesterone, glucocorticoid, and a whole range of orphan receptors whose ligands are still to be found (13).

Currently two types of estrogen receptors have been identified, alpha (ERα) cloned in 1986 (14-16), and beta (ERβ) cloned a decade later (17). There are three major isoforms described for ERα and at least 5 for ERβ **(Figure I-1)**.

The classic mechanism of action for estrogen receptors was proposed in 1962 by Elwood Jensen (18), who used tritiated estradiol to demonstrate the nuclear localization of the ER (19). The classic estrogen, 17β-estradiol, which is synthesized in the ovaries in response to hypothalamic-pituitary-gonadal signaling, is circulating in the blood in either free form or bound to steroid binding globulins. It can cross the cell membrane and bind to cytosolic or nuclear localized estrogen receptor molecules. Bound ER in the nucleus, forms homo- or heterodimers, and binds an estrogen response element (ERE) on promoters of target genes **(Figure I-2)**. They are able to recruit co-regulators and the basal transcription machinery, to initiate or block target gene transcription, to mediate its response.

Non-genomic responses to estradiol are mediated through a membrane bound estrogen receptor, as proposed by Ellis Levin (20), and results in ERK, PI3K, cAMP, and eNOS pathway activation, and can cross-talk, i.e. synergize with nuclear responses, by activating ER though phosphorylation. In addition, there are other reported proteins that can bind and/or respond to estrogens, like the controversial orphan G-coupled receptor GPR30 (21).

Besides the classic estrogen, 17β-estradiol, which is the reference ER "agonist" there are a range of estrogen metabolites, botanical compounds with estrogenic properties and synthetic estrogen receptor ligands **(for structures see Figure I-3)**. ICI 182,780 or fulvestrant is a pure estrogen receptor antagonist, binding both ERs with nanomolar affinities and targeting them for degradation (22). In addition to the pure agonist and antagonist which are defined both by response relative to 17β-estradiol and the structural positioning of the switch-helix 12, selective estrogen receptor modulators (SERMs) (23) elicit their action in a tissue- and promoter dependent manner **(Figure I-3)** (24).

Estrogen Receptor Mouse Models

The ERαKO mouse was generated by my adviser, Dr. Dennis Lubahn using homologous recombination in mouse embryonic stem cells to disrupt the ERα gene (25, 26). We have established large breeder colonies of these animals. Estrogen insensitivity is evident in these mice, however male and female sexual differentiation and development occurs (26). The recently cloned ERβ has been found in the ERαKO mice prostate and other tissues (27-29). Other non-ERα, non-ERβ estrogen-response proteins have not been fully characterized but may mediate specific effects of estrogens in cells (30, 31). We

also have established colonies of ERβKO mice generated by similar techniques (32). These mice are fertile, although females have smaller litters (33), the males however, show no overt phenotype as accepted by most, but not all. Jan Åke Gustafsson reports a range of abnormalities in ERβKO mice including hyperplastic prostate (34), as well as impaired hearing (35, 36), brain development (37), and social behavior (38). We further developed a double transgenic mouse model with ERαKO or ERβKO mice on the TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate, described later in this chapter) (39) background to elucidate the contributions of each receptor to prostate cancer incidence as well as study mechanisms of action of potential estrogenic compounds.

Estrogens and Breast Cancer

Breast cancer, which is the oldest form of cancer described, dating back 1600 BC (40), is another hormone cancer, and shares some parallels with prostate cancer.

Elwood Jensen was a pioneer in distinguishing between patients with ER-positive and ER-negative tumors, and thus predicting which patients are likely to respond to estrogen ablation therapy, and which should rather receive more aggressive radio- and chemotherapy (41).

For ER-positive breast cancer, the two hormones of choice were raloxifen and tamoxifen, which have also been proposed to be used in a preventative regimen for postmenopausal women with increased risk for developing breast cancer (42).

Estrogens and Prostate Cancer

Chemical castration that removed androgens through the use of estrogens was first described by Charles B. Huggins in 1941 (43), and his work was honored in 1966 with a Nobel Prize. Andrzej W. Schally and Roger Guillemin subsequently described the role of Gonadotropin-Releasing Hormone in reproduction, and were honored by the Nobel Prize in 1977. Their work lead to the development of GnRH receptor agonists such as leuroprolide and goserelin for prostate cancer treatment which block testosterone synthesis (44, 45).

Estrogen therapy, principally the use of a nonsteroidal estrogen diethylstilbestrol (DES), was a suggested practice in earlier days for prostate tumor treatment (46-48). Its primary mode of action is through feedback on the anterior pituitary with suppression of gonadotropin secretion and subsequent decrease in testosterone production by Leydig cells of the testis. This would in turn cause a decrease in androgens required for hormone dependent cancer (49). However, direct effects of DES through ER are also possible, since both estrogen receptors are expressed in the prostate. It has not been fully determined whether DES works through a classical ERα pathway, through ERβ or another estrogen-dependent mechanism.

Due to the increased risk of dying from heart disease or stroke after DES treatment, it is no longer used in therapy (50, 51). The main reason however, to ban the use of DES was its detrimental effect on children whose mothers were using the widely prescribed drug to prevent miscarriage during pregnancy (52).

Transdermal estradiol introduced by means of patches has been reported to reduce testosterone levels after 3 weeks of treatment with an average 95 percent reduction in

PSA, without the cardiovascular toxicity observed up to 14 months after treatment (53, 54).

Other synthetic estrogens, like conjugated estrogens (Premarin), ethinyl estradiol (Estinyl), medroxyprogesterone acetate (Provera), chlorotrianisene (TACE), have been tested for their potency in suppressing the production of luteinizing hormone and thus lower the testosterone production. All, except TACE, were able to lower LH production, but unfortunately they also resulted in significant cardiovascular toxicity, and were not an acceptable alternative to DES (55).

The actions mediated by the estrogen receptors α and β in the prostate seem to be of opposite character (56-58). In the rodent prostate, ERβ has been reported to be present in the epithelial cells throughout the normal organ. It has been also reported to be expressed in prostate cancer metastases (59). ERα on the other hand is expressed at low levels in the stromal cells with decreasing levels across the specific lobes from lateral to dorsal to ventral (60), but not in the epithelium. Ricke *et al.* recently proposed that *in situ* estrogen production is contributing to carcinogenesis of the prostate, since aromatase KO mice did not develop tumors in the described testosterone+estradiol induced prostate cancer model (61). ERα is also proposed to be necessary for prostate cancer development, since mice lacking the alpha receptor did not develop cancers (62).

In BPH, benign prostatic hyperplasia, the levels of ERα have been reported to increase compared to normal prostate (63).

The expression of both estrogen receptors seen in our mouse model undergoes a transition during prostate cancer progression. TRAMP mice with prostatic hyperplasia (score 2, non-cancer) do not express ERα but have detectable ERβ. Once the tumor

progresses through the poorly differentiated carcinoma stage (score 6), ERβ expression is absent in most cells and the major receptor present remains ERα (unpublished data, Dr. Besch-Williford).

Stromal - epithelial cell communication in the prostate

There is a significant amount of paracrine signaling between the stromal cells and the epithelial cells in the prostate, critical for both development and tumorigenesis. Even though prostate cancer is primarily arising in the epithelial cells of the prostate, Cunha *et al.* propose the prostate stroma to be the determining factor in benign vs. malignant growth of the prostate (64). Abnormal stromal cells derived from a human carcinoma stimulated epithelial proliferation and induced carcinoma when combined with benign epithelium (65). Studies with recombinant prostate tissues containing androgen receptor (AR) positive and negative mesenchyme and epithelium demonstrated that even though the androgen responsive cells are present in the epithelium, there is no requirement for an epithelial androgen receptor. An AR- mesenchyme combined with AR+ epithelium will not respond to androgens, and thus fail to develop properly (66). Hedgehog signaling, to be described later, provides a mechanism for paracrine stromal cell to epithelial cell (and vice versa) communication.

Androgens in the prostate

The development and growth of the prostate gland is dependent on the presence of androgens. An observation by the Scottish surgeon John Hunter in 1786 that castrated bulls had small prostates, led to the treatment of prostate cancer by castration in the next

century. Prostate cancer was first described in 1853 (67), and first reports of castrations came from Hugh H. Young at Johns Hopkins Hospital in 1904 (68-71).

The main androgen, testosterone is synthesized by the Leydig cells in the testis and by the adrenal gland. In the prostate, testosterone is metabolized by 5α-reductase to the more potent 5α-dihydrotestosterone (DHT), which binds to the androgen receptor (AR) to induce AR's transcriptional activity. Androgen ablation and blockage of the AR are used to halt androgen responsive prostate cancers. At first this blocks proliferation in the epithelial cells but eventually, the cancer escapes the androgen-dependency, and spreads. The prostatic stroma also expresses AR, but is not dependent on androgens. After androgen withdrawal, the AR expression in the stroma is lost, but there is no effect on stromal growth (72, 73).

The suggested neoadjuvant hormonal therapy for prostate cancer today combines a luteinizing hormone releasing hormone (LHRH) agonist combined with radiation therapy for 24 months, or a combination of LHRH agonist plus antiandrogen for six months (74).

Recently, intratumoral de-novo synthesis of testosterone within the prostate has been proposed as one mechanism (75), by which androgen-deprived tumors maintain androgen-related gene expression in the prostate (76) in the absence of exogenous androgens.

Anatomy of the prostate

The diverse phenotypes of prostate cancer cells can be seen in human prostate cancer patients. The most common, slow growing cancers arise in the epithelium. The

basic cell types making up the prostatic epithelium are the secretory luminal cells, basal cells, and endocrine cells. Currently the androgen sensitive luminal cells are seen as the origin of common adenocarcinoma (77). The majority of the tumor mass consists of exocrine cells, which are closely related to the secretory cell type within the transitional zone of the prostate epithelium. But about 10% of all human adenocarcinomas of the prostate have extensive multifocal neuroendocrine differentiation present (78). The neuroendocrine cells themselves are androgen insensitive, do not proliferate, and reside in a quiescent G_0 state, which makes the resistant to many therapeutic procedures. They produce detectable serum markers like Chromogranin A, B, and C, but also growth factors that can stimulate the proliferation of adjacent tumor cells (78).

The human prostate gland consists of three distinct anatomical zones, the transitional, central and peripheral zones, and each contains several distinct epithelial cell systems, the basal, secretory luminal and endocrine cells (77). Secretory luminal cells express the androgen receptor (AR), and require continuous support by circulating androgens. Basal cells are androgen independent, but a small basal cell population expressess the androgen receptor and remains androgen responsive (79). The maturation process of basal cell into luminal cells is induced by androgens, and counterbalances estrogen-induced basal cell hyperplasia. In the normal prostate 70% of proliferating epithelial cells express basal cell-specific cytokeratins, and the remaining 30% of cycling cells are found in the secretory epithelium (80). Neuroendocrine cells lack proliferative activity and represent a post-mitotic, terminally differentiated cell population (81).

The mouse prostate is divided into four distinct lobes: the ventral prostate, the dorsal prostate, the lateral prostate and the anterior prostate (coagulating gland) (82). These lobes function independetly to supply protein to the seminal fluid, with the anterior, dorsal and lateral prostate having a similar secretory profile, while the proteins secreted by the ventral prostate are quite distinct (83). The dorsolateral prostate of the mouse can be compared with the human peripheral zone as far as the origins of PIN (prostatic intraepithelial neoplasia) are concerned (84). Benign prostatic hyperplasia originates usually in the periurethral, transitional zone in human (85).

TRAMP Mouse Model

The animal model used in our lab is known as TRAMP for TRansgenic Adenocarcinoma of the Mouse Prostate, and was developed by placing the SV40 large and small T-antigen genes under the control of the rat probasin promoter. Probasin has been shown to be highly and specifically expressed in prostate (86). The transgene known as PBTag, is highly expressed throughout the prostate. This oncoprotein is known to abrogate tumor suppressor functions of p53 and Retinoblastoma (Rb) and thereby drive the cell through the normal cell cycle checkpoints. In our hands ~60-85% of ER wild type /TRAMP mice develop prostate cancer spontaneously within 5 months. The strength of the TRAMP model is that tumorigenesis can be studied from normal through metastatic cancer (http://thegreenberglab.fhcrc.org) so that prevention studies can be performed, unlike common human cancer xenograft models in mice, which are useful models for prostate cancer therapy but not prevention **(Figure I-4)**. We finished our studies at five

months so we can observe a range of different tumor stages and can detect nuanced changes resulting from the various dietary treatments **(Figure I-5) (87)**.

To study the TRAMP model *in vitro*, we are using the TRAMP-C2 prostate tumor cell line (CRL-2731). The cell line was derived in 1996 from a heterogeneous 32 week primary tumor in the prostate of a PB-Tag C57BL/6 (TRAMP) mouse (88). The cells were reported not to express the Tag antigen (SV40 T), but I was able to detect large and small Tag mRNA by RT-PCR. According to ATCC's specification the cells express the Androgen Receptor (AR). They also express both estrogen receptors ERα and ERβ as was determined in our lab by RT-PCR **(Figure IV-1).**

Poorly Differentiated ("Neuroendocrine like") Carcinoma (PDC) in TRAMP mice

In our TRAMP studies, we have found that PDC arises predominately in the ventral lobe of the prostate, as also reported by Paula and Greenberg, 2003 (89). The PDC we observe is characterized by being heterogeneous for positive staining with synaptophysin and starts as a focus of neuroendocrine cell proliferation, then progressively penetrates the tubules, and proliferates in the stroma, encroaching on adjacent tubules and eventually surrounding the urethra (See progression in **Figure I-4 A to D**). The incidence of PDC has been reported to increase with androgen ablation (90, 91). In our TRAMP studies, the incidence of well differentiated carcinoma (WDC) was predominantly found in the dorsal lateral prostate and in the ERαKO casein group was significantly ($P< 0.00001$) higher compared to ERα WT casein. On the other hand, the ERαKO casein group has a significantly ($p<0.0006$) lower frequency of PDC compared to ERα WT casein **(Table II-1)**. However, the incidence of PDC was higher in ERβKO

than in ERβWT casein. One speculation is that ERα and ERβ have opposing roles in the prostate in regard to prostate cancer and that ERα facilitates the development of PDC, while ERβ plays a protective role in prostate cancer. This role is supported in the literature (34, 92, 93). The balance between ERα and ERβ seems to have a significant role in the development of PDC.

Cancer Stem Cells

The existence of cancer stem cells has been proposed more that 40 years ago, but first conclusive evidence for such was not published until 1997 in leukemia (94).

Stem cells have the capacity to divide endlessly without acquiring mutations due to a protective mechanism allowing for parental strand maintenance (95). They also have a mechanism to protect the cells from random mutations in the parental DNA strand mediated by p53-dependent stem cell apoptosis and G1 arrest by transforming growth factor (TGF-β) (96).

Stem cells are pluripotent and characterized by their ability to produce different cell lineages. The cancer stem cell hypothesis proposes two possible mechanisms of cancer origin: oncogenic mutations may inactivate the constraints on normal stem cell expansion (97) or mutations lead to a continuous proliferating cell pool of more differentiated cells, that no longer enter a postmitotic differentiated state and expand accumulatig more and more mutations (98).

Stem/progenitor cells residing in niches in the prostate, growth restricted during androgen-responsive growth both in the normal and hyperplastic prostate, could be the source of the aggressive non-responsive phenotype that occurs after initial hormonal or

surgical treatments. Targeting stem cell niches, the specific microenvironment that enables cells to maintain self-renewing properties is a potential secondary treatment option (99). The hedgehog signaling pathway has been proposed to play a crucial role in human cancer stem cell renewal and tumorigenicity and may be responcible for tumor reoccurrences after radiation therapy (100). Suggested therapies targeting cancer stem cells would include induction of the stem cell into a more differentiated state or inhibition of the self-renewing stem cell state (101). Analogues of the hedgehog inhibitor cyclopamine are in phase II human trials for several cancers (16 trials to date on clinicaltrials.gov). Hedgehog therapy in pancreatic cancer is being monitored based on its property to reduce aldehyde dehydrogenase, a stem/progenitor marker (102).

Botanicals and Cancer

It has been recognized that a Western high-fat diet is increasing the risk to develop prostate cancer (103, 104). Other nutrients have a protective effect against prostate cancer, like lycopenes found in tomatoes, which act as antioxidants (105-107) and have been correlated with epidemiological significantly decreased prostate cancer incidence. On the other hand foods or food supplements rich in calcium are thought to cause prostate cancer (108). The soy isoflavones daidzein and genistein are also believed to reduce prostate cancer risk (109, 110). We have selected seven botanical compounds that have been implicated to have cancer protective properties. These compounds have been also suggested to have estrogenic and/or antiestrogenic properties, and where thus of highest interest to us. These compounds include apigenin, baicalein, curcumin, EGCG, genistein, quercetin, and resveratrol (for structures see **Figure III-10)**. A study from our

lab has shown all 7 have estrogen binding activity (111). More recently, we have also focused our attention on cyclopamine, a compound isolated from corn lily, which can inhibit the hedgehog signaling pathway.

Apigenin

Apigenin is a flavone, found in various flowers and vegetables, among others in chamomile. Apigenin itself, or extracts from its plants of origin have been used in prostate cancer research *in vivo* (112-115), and *in vitro* (111, 116-122). Mechanistically, it is proposed to act as a potent inhibitor of CYP2C9 (123), and thus affect drug metabolism, inhibitor of CYP1B1 (124), casein kinase 2 (117, 125, 126), topoisomerases I and II (127, 128), oncogenic proline-directed protein kinase FA (PDPK FA) (129), and 17beta-hydroxysteroid oxidoreductase (130). It is selectively proapoptotic (131-134), and inhibits synthesis of the inflammatory mediators nitric oxide and prostaglandin E2 (135), reducing inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression (136). Apigenin induces HER2/neu degradation (137-139). It inhibits HIF-1 alpha and VEGF expression through the Akt pathway (120, 140), inhibits fatty acid synthase activity (139, 141), and induce glutathione (GSH) depletion (142). It has been reported to have estrogenic activity (128, 130, 143, 144) acting specifically through ERβ (122) (siRNA against ERβ abrogated apigenin antiproliferative effect, but not that of genistein in DU145, and MDA-MB-231)

Mechanism of action of novel agents (145)

Baicalein

Baicalein and baicalin, are the most common flavonoid components of scutellaria extracts, and have shown antiproliferative and proapoptotic activities against various cancer cells (146-148), specifically prostate cancer (111, 116, 149-153). Baicalein has also shown a favorable effect in cisplatin-induced cell death of human glioma cells (154). Mechanistically it has been shown to inhibit prostaglandin E2 production (155), 12-lipoxygenase (156, 157), 5α-reductase (158), and aromatase (CYP19) (159). It has been shown to down-regulate MMPs (160), androgen receptor expression (151, 161), and decrease tumor volume *in vivo* (162).

Curcumin

Curcumin, found in the Indian curry spice tumeric, was first isolated from the perennial plant *Curcuma longa* in 1910 (163).

It has been reported to degrade androgen receptor (164, 165), and is able to inhibit constitutive NFκB activation (165), and lead to inhibition of cancer proliferation and the inflammatory biomarker PSA (prostate specific antigen) expression (166). It is proapoptotic (111, 167) through inhibition of PI3K/Akt pathways and upregulation of p53 (168). Inhibits MMPs 2 and 9 (169), inhibits tumor growth *in vivo* (169-171), in the TRAMP (172). It can block prostate cell motility and invasion by down-regulating the expression and activity of CC motif ligand 2 (CCL2) (173). Curcumin has anti-inflammatory (174, 175) and chemoprotective properties and has been found to attenuate

AhR/ARNT-mediated CYP induction by dioxin (176), inhibit glyoxalase 1 (177). It enhanced the activities of Phase 2 detoxification enzymes of xenobotic metabolism, including glutathione transferase (178) and NADPH:quinone reductase (179). It also inhibited pro-carcinogen activating Phase 1 enzymes such as cytochrome P450 1A1 (180). Curcumin is a potent antioxidant (181, 182), with a unique conjugated structure that includes two phenols and an enol form of a β-diketone, which might give it a typical radical trapping ability and a chain-breaking anti-oxidant activity (183). It has been tested as an anti-HIV agent (184, 185).

Synthetic curcumin analogues are being tested in breast and prostate cancer treatment (186, 187).

EGCG

Epigallocatechin 3-gallate is the most abundant catechin found in green tea. It inhibits prostate cancer *in vitro* (188-191), *in vivo* (192, 193) models, and has been used in clinical trials (194).

EGCG is proapoptotic (111, 188, 195), inhibits fatty acid synthase activity (141), VEGF(194), 5α-reductase (158), AR and PSA expression (196) in prostate cancer cells. It regulates cell and mitochondrial membranes, and thus motility and viability, through interactions with cellular zinc (189, 197). It is able to inhibit MAP kinases and activator protein 1 (AP-1) (198-200), inhibit NFκB activation and nuclear translocation (201). It has been reported to inhibit EGFR-mediated pathways (202, 203), among others in TRAMP mice (204). EGCG was able to inhibit uPA (205), MMPs 2 and 9 *in vitro* and *in vivo*. Some of its cancer protective effects have been suggested to be mediated through

the 67kDa laminin receptor, which is expressed in a variety of tumor cells, and binds EGCG with nanomolar affinities (206). EGCG acts as an anti-oxidant (190), promotes translocation of insulin-sensitive glucose transporter (GLUT) 4 into skeletal muscle (207). It has been shown to act synergistically with tamoxifen to inhibit growth of ER- breast cancer cell lines *in vitro* (208), and with curcumin *in vitro* and *in vivo* (209). Several studies on EGCG and HIV have been described, as discussed by Nance & Shearer (210), but there is not yet enough evidence for anti-viral properties of green tea.

Genistein

Epidemiologic studies suggested a lower incidence in prostate cancer among Asian men, compared to men in the Western world. A correlation with diet, especially the high consumption of soy in the Asian countries has been proposed as the main variable (211-213).

Genistein (4',5,7-trihydroxyisoflavone) is the main phytoestrogen found in soy (214). It has a structure similar to 17β-estradiol, and is a natural ligand for the estrogen receptors, with a binding affinity (K_i) of 2.6 nM for ERα and 0.3 nM for ERβ (215). Although genistein has a ~30 fold higher affinity for ERβ compared to ERα, it has been shown to have a potency only 4-5 times higher when acting through ERβ compared to ERα, and although it is a full agonist with ERα, (107-130% of E_2 activity on ERE-luciferase assays), it does display only partial agonist character with ERβ, indicating that the ligand might not induce an optimal agonist conformation with ERβ (216).

Besides having estrogenic character (111), genistein also acts through other mechanisms. Some of its non-estrogen receptor mediated cellular responses include

potent inhibition of tyrosine kinases (217-219), stabilization of topoisomerase II, and transcriptional regulation of transforming growth factor β. Genistein has been reported to inhibit tyrosine kinase activity by competing with ATP (220). It can act as a phytoestrogen (111, 221), and alter the activity of enzymes involved in steroid metabolism: HMG-CoA reductase (222, 223), 5α-reductase (224), 17β-hydroxysteroid dehydrogenase (225), aromatase (226), and other P450 pathways (227). There have been reports of genistein's acting as an antioxidant (228, 229), and its ability to alter DNA methylation (230, 231). Recently two papers were published, describing the anti-metastatic effect of genistein (232, 233). Interestingly, genistein will increase the bioavailability of EGCG *in vitro* and *in vivo* (234).

Quercetin

Quercetin is a flavonol found in a variety of plants, among others citrus fruits, buckwheat and onions. It can act as an anti-inflammatory agent (235) and anti-oxidant (236-238). Quercetin induces Ap1 in PC3 cells (239) and inhibits expression and activity of androgen receptor (240, 241) through activation of c-Jun (242). It has been reported to inhibit fatty acid synthase activity (141), 5α-reductase (158, 243), ErbB-2 and ErbB-3 (244), and AKR1C3 (also known as 17beta-hydroxysteroid dehydrogenase type 5 or 3alpha-hydroxysteroid dehydrogenase type 2) (245), which have been found to be over-expressed in prostate cancer. Quercetin, and genistein also represent potent non-competitive inhibitors of sulfotransferase 1A1 (or P-PST) (159). Quercetin has been shown to inhibit prostate cancer growth *in vitro* (246-249) and *in vivo* (250-252).

Interestingly both genistein and quercetin have been shown to activate the mutant androgen receptor (T877A) in LNCaP cells (253).

Resveratrol

Resveratrol has been reported to inhibit prostate cancer *in vitro* (188, 254-256), and *in vivo* (170, 257-260). It has the ability to down-regulate androgen receptor (242, 261), at the post-translational level (262) and to bind Glutathione sulfotransferase-pi (GSTP1) and estrogen receptor-beta (ER-beta) (263). It inhibits the formation of depurinating estrogen-DNA adducts, that are thought to play a role in initiating breast and prostate cancer (264).

It acts proapoptotic (188, 265) though inhibition of PI3K/Akt pathways (266, 267), and inhibition of NFκB (268), CK1 (188), and interactions with TRAIL (TNF-related apoptosis inducing ligand) (266, 269, 270). It also has the ability to inhibit Src tyrosine kinase activity (271) and stimulate prostaglandin release in prostate cancer cells (272). Resveratrol has been also reported to act as an antioxidant (238) and anti-inflammatory agent in prostate cancer (273).

Cyclopamine

In the 1950s several one-eyed lambs were born on an Idaho farm, and it took scientists from the Department of Agriculture 11 years to link the birth defects to wild corn lilies (*Veratrum californicum*), on which the pregnant sheep were grazing during droughts (274). In 1965 researchers isolated the responsible compound 11-deoxojervine, and named it cyclopamine (275). It took another 25 years to link the newly discovered

hedgehog signaling pathway to the old teratogen cyclopamine (276). Mutations in Sonic Hedgehog resulted in holoprosencephaly in mice (277), and humans (278). Knocking out one of the pathway components, the trans-membrane receptor Smoothened resulted in one-eyed mice. The phenotype could be copied through *in utero* cyclopamine exposure (279). Beachy *et al.* demonstrated subsequently that cyclopamine could directly bind and thus inhibit Smoothened (280).

Hedgehog Signaling Pathway

The hedgehog signaling pathway is important in proliferation and developmental patterning of many tissues, including the prostate. The pathway works by allowing the coordination of epithelial-mesenchymal interactions and subsequent differentiation (281, 282).

The pathway takes its name from the activating ligand, a secreted protein – hedgehog. In vertebrates there are 3 homologues with tissue specific and overlapping expression and function – Indian, Desert, and Sonic Hedgehog. In absence of the ligand, the receptor – Patched is constitutively repressing another transmembrane protein – Smoothened **(Figure I-5).** Upon ligand binding, Patched releases Smoothened from its inhibited state in the cytosol. Smoothened then can migrate to the tip of the primary cilium, where it interacts with the Gli family transcription factors which are activated and can now migrate to the nucleus to execute the hedgehog response (283-290) **(Figure I-6)**.

Sonic hedgehog is synthesized as a 45kDa precursor, which is then cleaved autoproteolytically to generate a 19kDa N-terminal signaling peptide (291). During cleavage cholesterol is covalently attached to the C-terminus of the peptide to anchor it in

the cell membrane (292). A second lipid modification that incorporates palmitic acid occurs at the N-terminal cysteine that is exposed after signal peptide cleavage (293). This step is catalyzed by the transmembrane acyltransferase Skinny hedgehog (Ski) (294). Dispatched (Disp), a 12-pass transmembrane protein is needed to release the hedgehog protein from the cell membrane (295).

The lipid modifications on hedgehog are required to maintain a signaling gradient, that results in the proper cell fate and patterning (296). The way the hedgehog ligand moves between origin and target cell is still being debated (297). It could form micelle-like structures with its lipid moieties embedded in the core. It has been proposed that lipoprotein particles could carry hedgehogs between cells for long-range transport (297) and potentially systemically. The transport of Hh among tissues also requires heparan-sulfate (298). Perlecan, an extracellular matrix proteoglycan is a recent discovered modulator of the sonic hedgehog signaling in prostate cancer (299), with its main function of limiting the spread of ligand, and increasing local concentrations (300). Vertebrates have an additional transmembrane protein, Hh-interacting protein (Hip), which binds to Hh proteins and reduces their range of movement (301), and is down-regulated in advanced prostate cancer (302, 303).

Once it arrives at its destination, hedgehog binds to its receptor molecule Patched, a 12 transmembrane receptor with structural homology to the sterol sensing domain of HMGCoA reductase, and sterol pumps (304). Binding of Hh is also facilitated by the transmembrane protein CDO and Brother of CDO (BOC) (305). Upon ligand binding, Patched releases Smoothened from its inhibition, which is of catalytic rather than stoichiometric character and is potentially regulated through small molecules (306).

Oxysterols (307) and vitamin-D3 derivatives (308) have been proposed as these mediators. Smoothened, a 7-pass transmembrane protein, which in its repressed state is residing in cytoplasmic vesicles, is now translocating to the primary cilium, which requires a functional intraflagellar transport machinery (309). It is in the primary cilium, where the activation and processing of Gli transcription factors occurs (310). There are 3 Gli zinc-finger transcription factors in vertebrates that mediate the hedgehog response. Gli1 is reported to exist only in a full length activator form; Gli2, can act as full length activator or truncated repressor form (311); and Gli3, which has been reported to only act as a repressor by some (312), but not others, which claim, that it directly activates the Gli1 promoter (313). Suppressor of Fused (SuFu) is another negative regulator of the pathway, sequestering Gli1 in the cytoplasm in the absence of ligand signal (314, 315), and mutations of this gene have been found in prostate cancer patients (303, 316). It has been found to recruit GSK3beta to process Gli3 (317). Other kinases, specifically PKA (318), CK1 (319) and p38 MAPK (320, 321) have been reported as negative regulators of the pathway.

Besides the "classical" hedgehog signaling pathway described briefly here, there is emerging evidence of hedgehog pathway components interacting with other pathways, like Patched binding directly to Cyclin B1 and caspases to prevent cell proliferation or non-Gli mediated pathway responses (322) or its interactions with TGFβ signaling (323).

The hedgehog signaling pathway is crucial in cell fate and patterning, and its role in prostate development has been studied by several laboratories (324, 325).

Hedgehog Signaling and Prostate Cancer

Hedgehog signaling has been found to be important in a number of tumors including basal cell carcinomas (326), medulloblastomas (327), gliomas (328), sarcomas (329), tumors of the digestive tract (330), small cell lung cancers (331) and pancreatic carcinomas (332), and prostate cancer (328, 333-338). In brief, the hedgehog signaling pathway proteins Sonic Hedgehog, Patched, Gli and sometimes Smoothened were elevated in metastatic prostatic tumors versus normal prostates or non-metastatic tumors. Interestingly, hedgehog pathway inhibitors, either cyclopamine or anti-Shh antibodies, were able to inhibit *in vitro* the growth of several well known human prostatic cell lines, including LNCaP, PC3, 22RV1 and DU145. More importantly, *in vivo* in multiple xenograft models these same inhibitors worked extraordinarily well to inhibit tumor growth and metastases (303, 335, 337, 339). In control experiments, where Gli1 was over expressed in various tumor lines, increased *in vivo* tumor growth was observed and because Gli1 is downstream of the inhibitors used, the inhibitors did not inhibit growth of these Gli1-over expressing tumors.

Estrogen and hedgehog signaling is reviewed in chapter IV.

General Overview

Chapter II describes the roles of both estrogen receptors, alpha and beta, on cancer incidence in the TRAMP mouse. Results from a series of animal studies with ERα- and ERβWT and KO mice demonstrated the ERαKO genotype to be protective against the aggressive, androgen independent form of prostate cancer, while ERβKO mice where twice as likely to develop this metastatic form of cancer. Also, genistein had a cancer protective effect, specifically at the more common, slow growing cancer that was dose dependent, and required the presence of both estrogen receptors.

In **chapter III** I present data for the presence of autocrine hedgehog signaling in epithelial human and mouse prostate cancer cells. I also demonstrate how selected botanical compounds inhibit hedgehog signaling at the Gli1 level, which is one of the mechanisms they might work through to protect against cancer.

Chapter IV explores the impact of estrogens on the hedgehog signaling pathway. Oxysterols, cholesterol derivatives that have been recently proposed to act as SERMs, and be responsible for a wide range of unexplained estrogen effects, have now been found to stimulate the hedgehog signaling pathway. Both, estrogen and the antiestrogen ICI are able to inhibit hedgehog signaling in our assays, and the botanical compounds from chapter III are potentially working though one or both estrogen receptors.

In **chapter V** I am proposing future studies that would test some of the hypothesis arising from my data and my proposed working model. Specifically, a combinational therapy could be developed and tested in the TRAMP model to target both, slow growing, non-metastatic, and fast growing, androgen-independent prostate cancer subtypes, using hedgehog-inhibiting botanicals and ERα antagonists, and ERβ agonists.

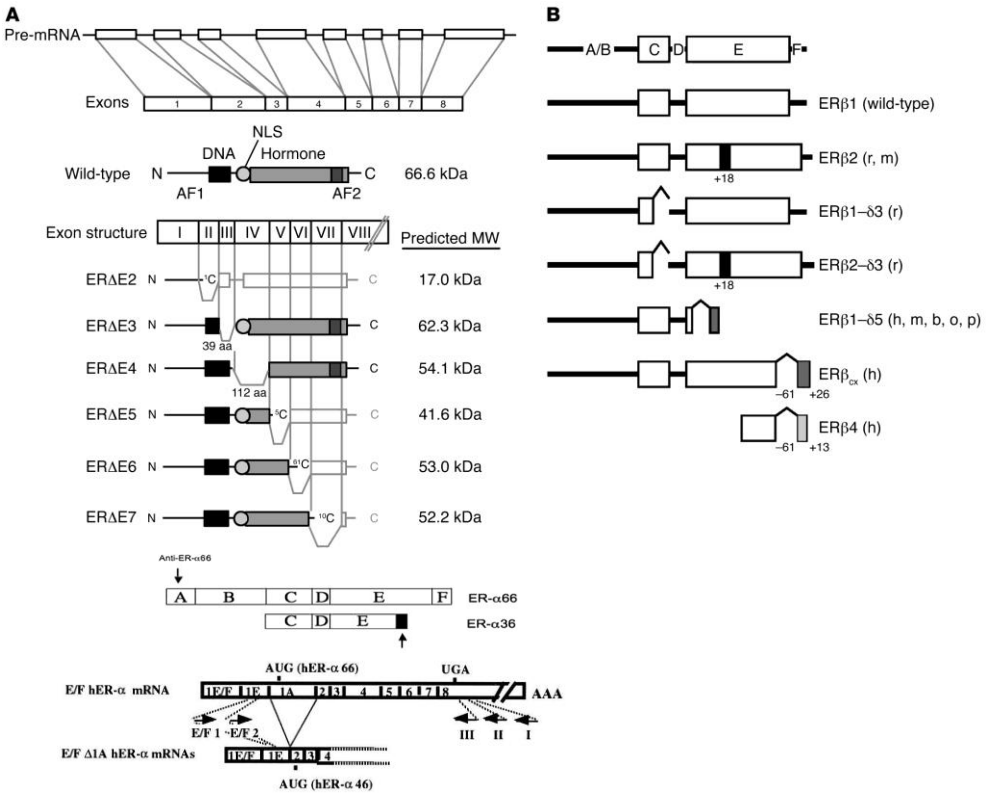


Figure I-1: Exon structure, primary transcript, and common mRNA splice variants of the ER.

(A) The most common splice variants of ERα are expressed in multiple tissues and arise from deletions of internal exons, resulting in truncated proteins lacking segments of the DNA-binding domain (DBD) or hormone-binding (ligand-binding) domain (LBD) of the receptor. Most variant isoforms possess little transcriptional activity, with the exception of ERαΔE5, which binds DNA but lacks most of the LBD, resulting in low levels of constitutive activity in some cell lines. ERαΔE3, ERαΔE5, and ERαΔE7 variants have demonstrated a dominant-negative effect on transcriptional activity mediated by wild-type ER. Adapted from Bollig 2000 (340) and Couse 2006 (341). ERα36 (342) and ERα46 (343) as described by Zhao-Yi Wang and Frank Gannon, respectively.

(B) Mammalian ERβ variants identified in humans (h), rats (r), mice (m), cows (b), sheep (o), and pigs (p). ERβ (ERβ1) possesses both a DBD (C domain) and an LBD (E domain). ERβ2 codes for a variant that contains an additional 18 amino acids in the LBD, while ERβ1-δ3 lacks exon 3 and therefore part of the DBD. ERβ2-δ3 contains both of these variations. ERβ1-δ5 lacks exon 5, and in $ER\beta_{CX}$, the C-terminal 61 amino acids are replaced by a unique sequence of 26 amino acids. ERβ4 is truncated at both the N and the C termini. In humans, variants lacking exon 2, exon 4, exon 6, and exon 7 also exist. Adapted from Leung 2006 (344).

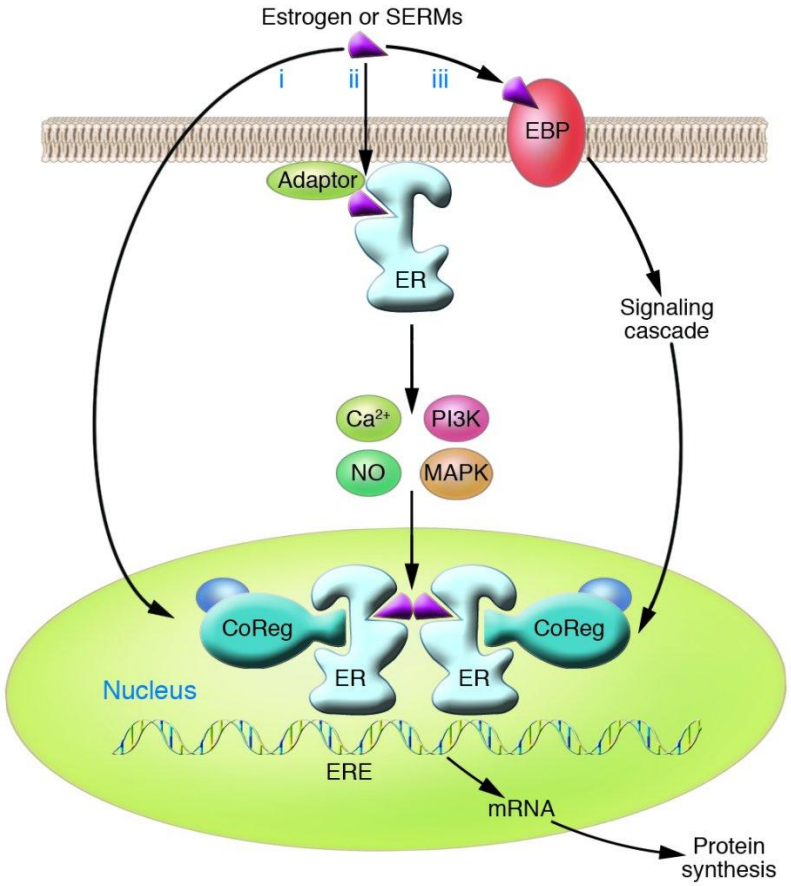


Figure I-2: Models of estrogen action adapted from Deroo & Korach 2006 (345).

In the "classical" pathway of estrogen action (i), estrogen or other selective estrogen receptor modulators (SERMs) bind to the estrogen receptor (ER), a transcription factor that regulates transcription of target genes in the nucleus by binding to estrogen response element (ERE) regulatory sequences in target genes and recruiting coregulatory proteins (CoRegs). Rapid or "nongenomic" effects of estrogen may also occur through the ER located in or adjacent to the plasma membrane (ii), which may require the presence of "adaptor" proteins, which target the ER to the membrane. Activation of the membrane ER leads to a rapid change in cellular signaling molecules and stimulation of kinase activity, which in turn may affect transcription. Lastly, other non-ER membrane-associated estrogen-binding proteins (EBPs) may also trigger an intracellular response (iii).

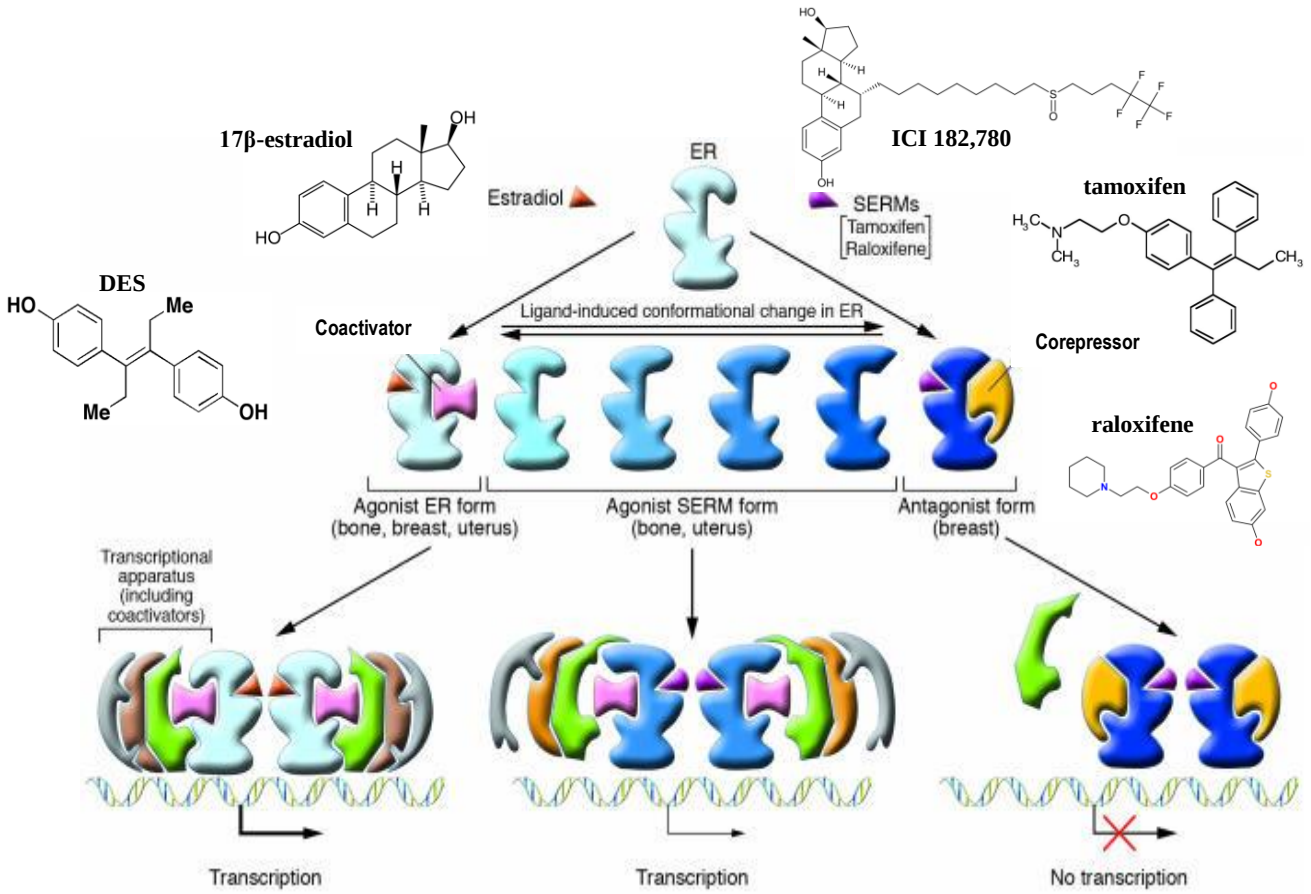


Figure I-3: Differential ER structure and coactivator recruitment by ER agonists, antagonists, and SERMs.

Upon binding ER ligands such as estradiol or SERMs, the receptor undergoes a conformational change, allowing the ER to exist in a spectrum of conformations from active to inactive depending on the nature of the bound ligand. This conformation, in turn, regulates the recruitment of specific transcriptional coregulatory proteins and the resulting transcriptional apparatus. Coactivators such as SRC1 bind to the active (agonist-bound) form of the receptor and activate transcription, while corepressors interact with the antagonist-bound receptor, inhibiting transcription. Depending on the cellular and promoter context, both unique and overlapping sets of genes may be regulated by various ligands. Adapted from McDonnell 2002 (346).

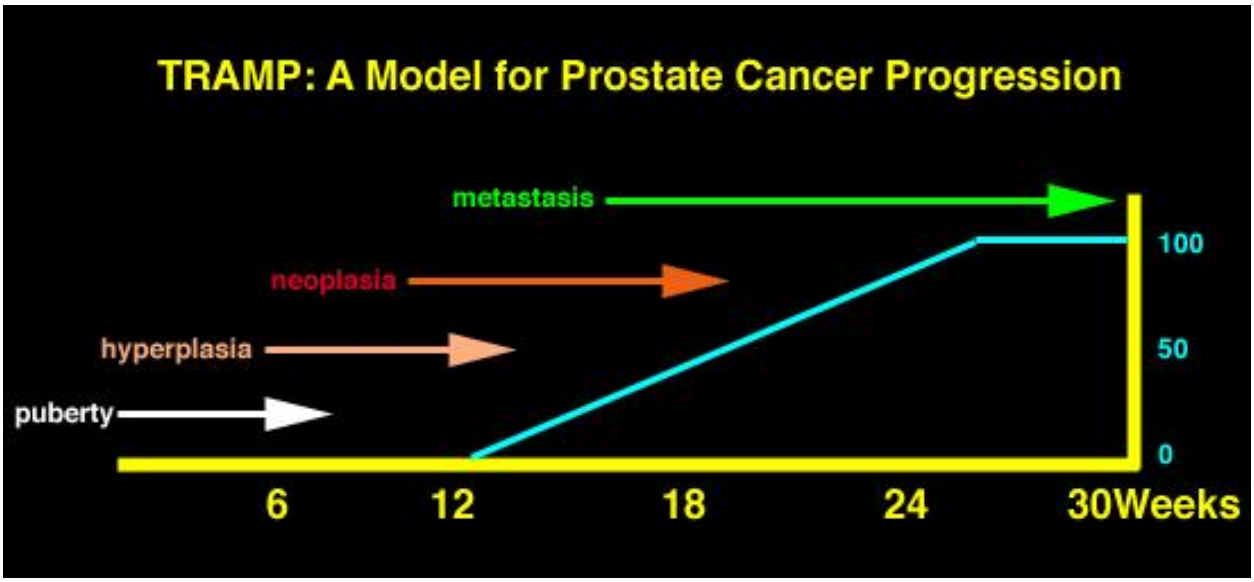


Figure I-4: Model for prostate cancer progression in TRAMP mice.

Adapted from the Norman Greenberg lab webpage: http://thegreenberglab.fhcrc.org

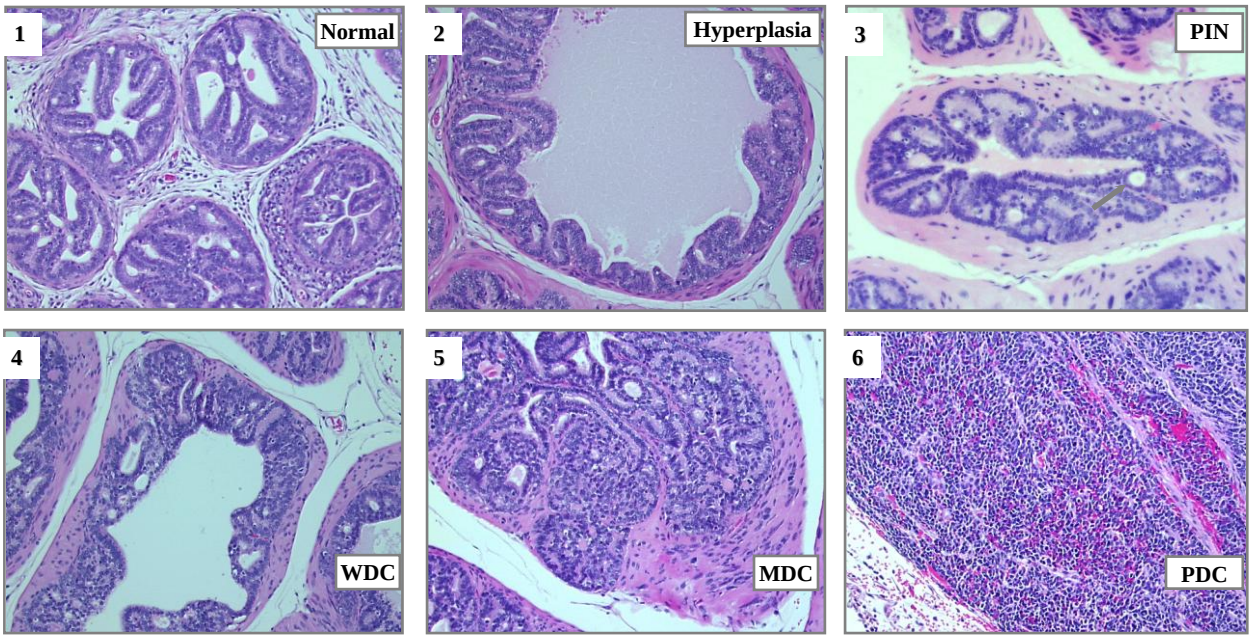


Figure I-5: Histological classification of prostate tumor progression in the ERαKO TRAMP mouse. Slides and scoring by Dr. Besch-Williford (87); descriptions from http://thegreenberglab.fhcrc.org/research/TRAMPhistopath

1. Normal prostate, well defined tubules, single layer of epithelial cells.
2. Hyperplasia, more numerous epithelial cells with retention of single layer and thin muscle wall
3. PIN – prostatic intraepithelial neoplasia: epithelial tufting, elongated nuclei, micropapillary projections, cribriform structures, nuclear stratification, increased mitosis, increased apoptosis
4. Well differentiated carcinoma: increased quantity of small glands, desmoplastic response, stromal thickening, round nuclei, fewer hyperchromatic nuclei than PIN, increase mitosis, increased apoptosis, inflammation frequent
5. Moderately well differentiated carcinoma: relatively solid growth, glandular architecture +/-
6. Poorly differentiated carcinoma: anaplastic, sheets of cells, pleiomorphic cells, irregular nuclei, glands often trapped, highly vascularized, often hemorrhagic, areas of necrosis, very little cytoplasm

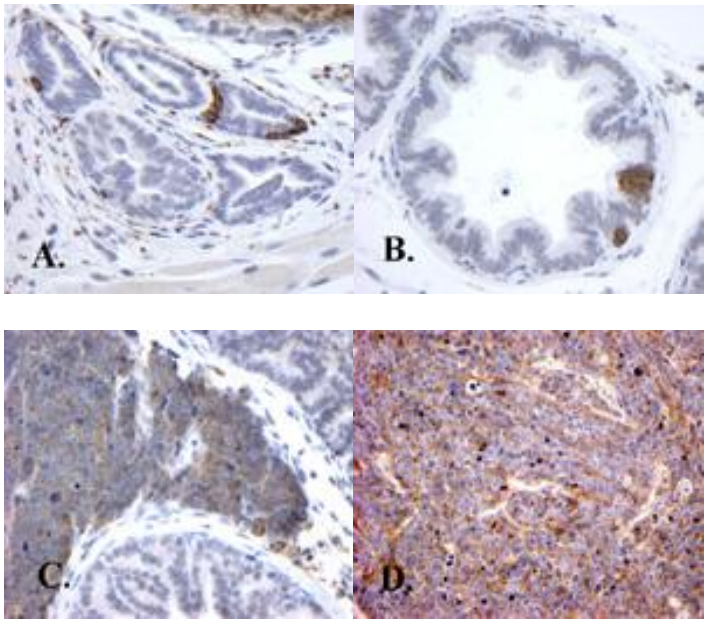


Figure I-6: Neuroendocrine-like tumor.

Slides and legend by Dr. Besch-Williford

Sections of mouse prostate probed with antibody to synaptophysin. **(A)**: Basal cells in the prostate ducts are positive for synaptophysin, a marker of neuroendocrine cells. The prostate secretory epithelium is synaptophysin negative. **(B)**: A focus of neuroendocrine cell proliferation, the earliest stage of neuroendocrine-like neoplasia. **(C)**: The neoplastic neuroendocrine cells penetrate through the wall of the prostate tubule and proliferate in the stroma, encroaching adjacent tubules and eventually surrounding the urethra **(D)**.

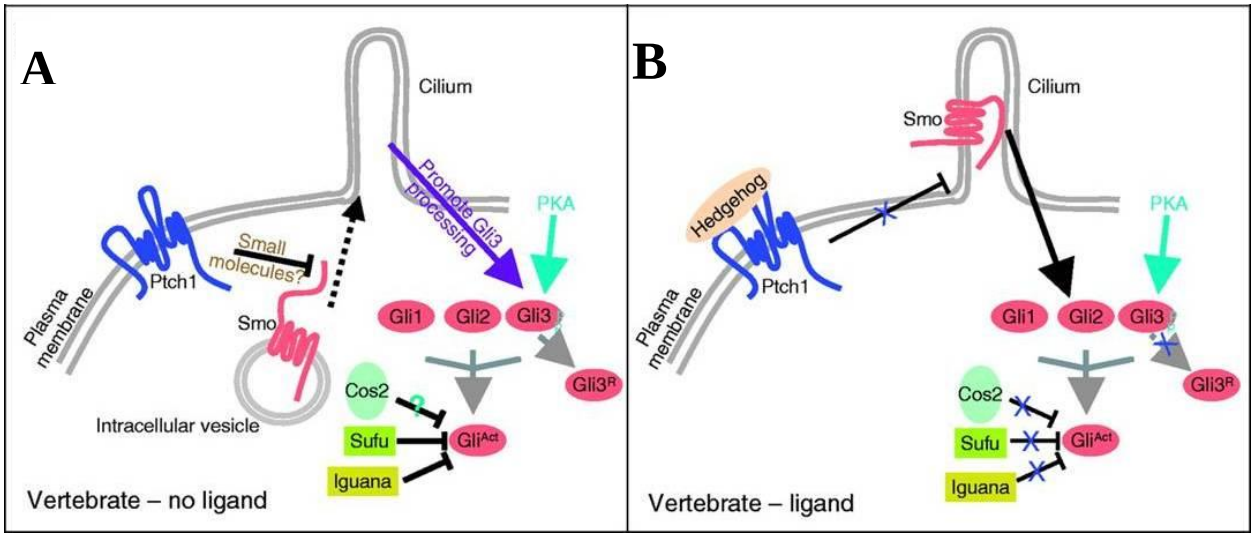
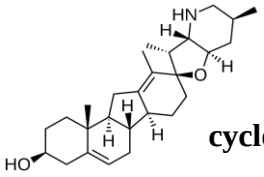


Figure I -5: The Hedgehog pathway in vertebrates.



cyclopamine

In the absence of ligand, Patched will inhibit Smoothened, through an unknown mechanism, possibly enzymatically or by use of small inhibitory molecules (A). Upon hedgehog binding, the inhibition of Smoothened is released, and it is now free to translocate to the primary cilium and activate Gli transcription factors (B). Cyclopamine can directly bind to Smoothened and prevent it from entering the cilium. Interestingly, even thought there is a significant conservation of pathway members, specifically Smoothened, cyclopamine will not inhibit the pathway in Drosophila (347).

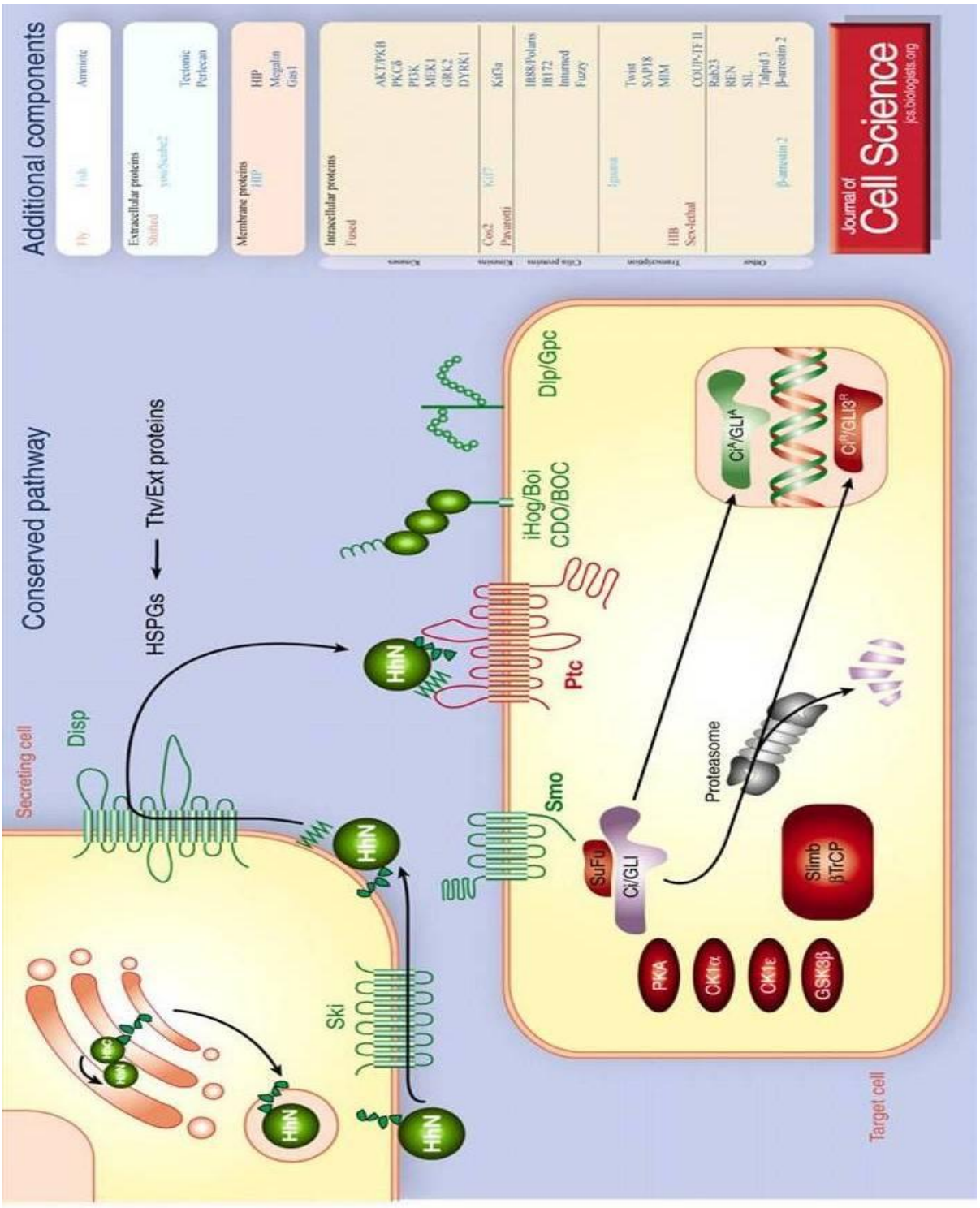


Figure I-6: Conserved hedgehog pathway - adapted from Varjosalo & Taipale 2007 (348).

II - Genistein Reduces Incidence of Prostate Cancer in TRAMP mice through Estrogen Receptor Dependent Mechanism

Overview

The purpose of this animal study was to test the cancer preventative role of genistein in the TRAMP prostate cancer model and to elicit its mechanism through the use of Estrogen Receptor-alpha and -beta KO mice. We expected genistein to prevent and delay cancer incidence in WT animals in a dose dependent manner, which is what we observed. Our **hypothesis** was that genistein worked primarily through ERβ. We also expected to see a absent or reduced protective effect of genistein in the ERβKO animals, but not, or to a lesser degree in the ERαKO mice.

We were surprised to see that genistein only affected the WDC incidence, but had no effect on PDC. Another unexpected result was the fact that genistein had no effect in either the ERα- or ERβKOs. We were really intrigued by the very strong genotypic effects on cancer incidence, a protective effect in ERαKO mice, which got only 5% PDC, and an increase in the incidence of aggressive PDC in ERβKO mice.

Introduction

Dietary consumption of soy products has long been associated with reduced incidence of various diseases. Considerable epidemiological evidence supports the observation that soy foods promote health and reduce chronic ailments including cardiovascular disease and osteoporosis (349, 350). Genistein has been implicated as cancer preventative through epidemiological studies (351, 352) as well as targeted *in vitro* (353) and *in vivo* (354-356) studies. The cancer incidence for breast (357) and prostate (358) cancer is significantly lower in cultures with high soy consumption (359) and high serum genistein levels.

Estrogen therapy, specifically DES treatment, has been used in prostate cancer treatment since 1941 (43, 360-363). The importance of estrogens and estrogen receptors in the prostate has been recognized both in the organ development (364) and disease (365).

ERα is expressed in the stromal compartment of the normal adult mouse prostate, while ERβ can be found in the prostatic epithelial cells (366). In the rat prostate ERβ is expressed in both cell types at birth, but steadily declines in the mesenchyme, and reaches maximal adult levels in the epithelium by day 90, upon completion of differentiation (367). Neonatal estrogen exposure has profound effects on prostate development both in the rodent (29, 368-371) and human (372).

Genistein has a multitude of documented mechanisms of action, here we wanted to investigate to what degree its cancer protective effects could be attributed to its

estrogenic activity. By using both ERα- and ERβKO mice in conjunction with mice with both receptors intact we were able to demonstrate that actually both receptors are required for genistein to have an effect in preventing prostate carcinogenesis.

Materials and Methods

TRAMP mouse studies

The TRAMP model was developed by placing the SV40 large and small T-antigen gene under the control of the androgen regulated rat probasin promoter, which has been shown to be highly and specifically expressed in the mouse prostate (39, 373). TRAMP mice spontaneously develop prostate tumors starting at the age of three months and they can be used to study the progression of the disease, as well as prevention and treatment options. Male TRAMP mice on a C57BL6/J background were raised in-house. All University of Missouri institutional guidelines for animal care and use were followed.

To generate the ERα/βKO-TRAMP mice, female C57Bl/6J mice, heterozygous for the ERα or ERβ gene and positive for the PBTag Transgene (TRAMP) were crossed with male C57Bl/6J mice that were heterozygous for the ERα/β gene and negative for the PBTag transgene. All breeder pairs were maintained on a casein-based diet (AIN 93G) with casein from MP Biomedical LLC (Aurora, Ohio). ERWT, ERαKO and ERβKO offspring of this breeding scheme, that were positive for the PBTag transgene, were used in this study.

The mice were housed in pairs in micro-isolator cages and given free access to food and water. A daily light:dark cycle of 12:12h was used, with ambient temperature and humidity set at 21°C, and 50%, respectively. Animals were monitored weekly for body

weight and tumor burden. The mice were fed the casein-based diet (AIN93G) until weaning, and then randomly assigned to groups fed either the control casein diet or the experimental genistein diet which consisted of the same base diet AIN93G (374) to which 300mg/kg or 750 mg/kg genistein (LC Laboratories, Woburn, MA) has been added. The mice were maintained in the study from 5-6 weeks until 5 months (18-22 mice per treatment group). The concentration of genistein was selected after analyzing serum concentrations of mice consuming a range of diets from 0 gm genistein / kg diet to 500 mg genistein / kg diet in 100 mg increments (data not shown). The goal was to provide a concentration of dietary genistein which resulted in serum concentrations below 1-5μM range. Tyrosine kinase inhibition will not occur (375), but these concentrations will saturate both estrogen receptors.

At 5 months mice were euthanized and tissues were collected. The reproductive tract (testes, vas deferens, empty urinary bladder, seminal vesicles and prostate lobes), testes, and prostates were weighed. At the time of collection, a portion of each prostate was fixed in neutral buffered formalin and paraffin embedded for histological analysis and the remainder snap-frozen in liquid nitrogen and stored at -80^{O}C. Tissues sections were stained with hematoxylin and eosin, and examined by light microscopy for assessment of cancer stages (376). Prostates were scanned by trained veterinary pathologists who were unaware of the treatment groups and staged as either (A) normal, (B) hyperplastic, (C) prostatic intraepithelial neoplasia (PIN), (D) well-differentiated carcinoma (WDC), (E) moderately well-differentiated carcinoma (MDC), (F) poorly differentiated carcinoma (PDC) " neuro-endocrine- like carcinoma" **(Figure I-2)**. This protocol follows a procedure described previously (87, 89).

Immunohistochemistry:

Slides were prepared using heat mediated antigen retrieval. Immunohistochemistry reactions were performed using the DakoCytomation Autostainer (Dako, Carpinteria (CA). Primary antibodies were used in the following dilutions: SV40 Tag Pab101 1:200 (Pharmingen), Chromogranin A&B 1:50 (RDI), Synaptophysin 1:100 (Dako), AR RG-21 1:50 (Upstate), ERα MC-20 1:300 (Santa Cruz, CA), ERβ PPG5/10 1:50 (Serotec). Scoring was done by Dr. Besch-Williford.

Cell Culture:

The TRAMP-C2 cell lines was obtained from ATCC (http://www.atcc.org). PC3M cells (377), which is a highly metastatic form of PC3 was obtained from our in house cell core (www.biotech.missouri.edu/cic).

Mouse prostate cancer TRAMP-C2 and human prostate cancer PC3M cell lines were maintained in RPMI 1640 media supplemented to contain 10% fetal bovine serum (U.S. Bio-Technologies, Parkerford, PA), 4.5g/mL Glucose, 4mM L-glutamine , 100μM Non-essential Amino Acids, 10mM HEPES, 1mM Sodium Pyruvate, and 1% Penicillin/ Streptomycin (all from Invitrogen, Carlsbad, CA).

Protein Assay:

All compound treatments for the growth assessment consisted of a 72-hour time course in phenol red-free RPMI 1640 medium, supplemented with 10% charcoal-stripped FBS. Cells were seeded in 12-well plates and adjusted to phenol-red free medium for 24 hours.

TRAMP-C2 cells were treated at 30-40% confluency, PC3M cells at 40%, which allowed the controls to reach 100% by day 3. Cells were lysed with 1N NaOH and left overnight. Protein assays to measure overall cell protein concentration were performed using Bio-Rad Dc kit (Bio-Rad, Hercules, CA), with absorbance measured at 699nm. Compounds were used at concentrations ranging from 1μM to 100μM in half log increments. Each experiment was performed at least three times in duplicate. Total cellular protein correlates well with thymidine uptake in prostate cancer cells and is a reliable assay to measure cell growth (111).

Statistical Analysis

The stages of tumor incidence were classified into non-cancer stages (Normal, Hyperplasia and PIN) and cancer stages (WDC, MDC, and PDC or neuro-endocrine like carcinoma). Tumor incidence data was analyzed as a 2 x 2 factorial with genotype (ERαWT or ERαKO) and diet (casein or genistein) as main effects using χ^2 test, after consultation with Dr. Lamberson. Values that achieved $p<0.05$ were considered to be significantly different. Comparison within the same tumor stage among different dietary groups and genotypes has also been done. Body weight, reproductive tract weight, testicular weight, and prostate weight were analyzed using a two-sample t-test, assuming unequal variance. GraphPad Prism4 software was used to perform the analysis (www.graphpad.com, La Jolla, CA).

Results

Genistein's effect on cancer incidence in TRAMP

Genistein significantly reduced overall cancer incidence in ERWT animals compared to control diet in a dose dependent manner **(Table II-1)**. Specifically WDC incidence was extremely decreased from 49% in the control to 18% with low dose (300mg/kg) and 8% with high dose (750mg/kg) genistein. No effect on PDC incidence were observed, contrary to what was reported before (356). Genistein had no effect in ERαKO or ERβKO animals **(Table II-2)**.

Estrogen Receptor status and cancer incidence

Approximately 70% of TRAMP mice wild type for both estrogen receptors developed cancer by 5 months of age, with WDC incidence of ~50% and PDC incidence of ~20%. Mice lacking ERα had an increased WDC incidence at ~85% however they have a greatly reduced PDC incidence with only 5%. ERβKO TRAMP mice however have over 40% PDC, indicating a possible protective role for ERβ in tumorigenesis, or a tumorigenic role of ERα, or a combination of both **(Table II-2)**.

Immunohistochemistry - Estrogen Receptor profile changes with cancer progression

In order to have a better understanding of the involvement of estrogen receptors in cancer progression we have performed immunohistochemical analyses of hormonal receptor expression in the prostates from TRAMP mice. We have also looked at the expression of the neurondocrine markers chromogranin A and synaptophysin, which have

been proposed to be expressed by the aggressive neuroendocrine-like tumors, and hat are being investigated as biomarkers for metastatic prostate cancer in patients (378-381).

Shown in **Figure II-4** are representative tissue samples from two ERWT/TRAMP mice (4448 and 4264) fed genistein with HYP (top) from the dorsal lobe or PDC (bottom) from the ventral lobe. Hyperplastic prostate epithelium does not express chromogranin and synaptophysin, both cytoplasmic neuroendocrine (NE) markers, nor ERβ. There is expression of ERβ and androgen receptor in epithelial and stromal cells, as has been reported for normal prostate epithelium in man and mouse. The SV40 immunoreactivity confirms the expression of the transgene in the nucleus of the prostate epithelium. The immunoreactivity to this panel of antibodies of the HYP prostate was not different from those fed casein. As shown in Figure II-4, bottom, these markers differed in PDC tissues. There was no difference between casein and genistein fed mice. In contrast to the hyperplastic prostate, neuro-endocrine carcinoma in this TRAMP model did express neuroendocrine markers, with 80% of sections expressing synaptophysin, and 30% expressing chromogranin. Androgen receptor reactivity diminished from occasional weak positives to uniformly negative. Transgene expression was maintained in NE cells. Interestingly, the pattern of estrogen receptor immunoreactivity switched, with nearly complete loss of ERβ expression and gain of ERα expression. The pattern of ERα immunoreactivity was predominantly in clusters of NE cells scattered throughout the tumor mass and not in all NE cells. In data not shown, ERα expression was also observed in PIN and well-differentiated adenocarcinoma cells in both diet groups. Hence, distinct cancer origins appear to occur in the TRAMP prostate and these are independent of diet.

We will further our exploration of these events in order to more fully relate this animal model to human prostate cancer.

Weights differences

High dose genistein in the wildtype and in ERαKO mice significantly increased total bodyweights **(Figure II-1)**, with 28.8±0.22g and 29.74±0.52g in the casein fed WT and αKO vs. 30.18±0.68g and 32.38±0.81g in the high dose genistein fed WT and αKO mice **(Table II-4)**. It has been reported that genistein contributes to increased lean body mass in neutered cats (382), and food intake and total body mass in quails (383).

ERαKO mice had significant lower testes weights which is corresponding to previous reports (384) and the observed lower fertility in both ERαKO mice (385) and one human male (386, 387). There is still some controversy regarding the main ER in the testis - Gustafsson claims ERβ is main receptor in testis (388), Kathenellenbogen votes for ERα (389). We did not observe any differences in testis between ERWT and ERβKO mice.

Total prostate weights did not vary between groups due to significant variance in tumor size, specifically within the PDC tumors **(Figure II-3A)**. When separated into two groups with a cut-off weight of 1.5g however, there were visible pattern with significant differences between groups. The separation by weight was used to determine whether there were differences between groups within the same cancer stage. It is a gross way to characterize the proliferation of the tumors, and will be followed up by Ki67 staining.

Prostates over 1.5g, which were exclusively PDCs, were most highly represented in the ERWT casein group - 7/175, and 6/81 in the ERWT low genistein group. The

weights of those large tumors were highest in the ERWT low genistein group, followed by ERWT casein, and ERWT high genistein being same as ERβKO genistein. This is interesting to note that even though ERβKO mice had a significantly higher PDC incidence relative to control their tumor weights were not higher. ERαKO mice had only 1 tumor above 1.5g corresponding to the overall absence of PDC in ERαKOs (n=105) **(Figure II-3B)**. Within the prostates under 1.5g, ERαKO mice had significant higher weight compared to the other genotypes **(Figure II-3C)**, corresponding to the increased WDC incidence in ERαKO mice **(Table II-2)**.

Genistein inhibits prostate cancer cell growth *in vitro*

We and others have previously reported on the inhibitory growth effect of genistein on prostate cancer cells – LNCaP and PC3 (111, 218). Here we showed how genistein is able to inhibit cell growth in mouse TRAMP-C2 cells starting at 100nM and human PC3M cells starting at 10nM **(Figure II-5)**.

Estradiol or ICI alone will not inhibit prostate cancer cell growth, together however they will.

Neither 17β-estadiol, nor the pure anti-estrogen ICI alone are able to inhibit prostate cancer cell growth in TRAMP-C2 or PC3M cells. When administered together however, they will decrease total cellular protein in both cell lines in a dose dependent manner, starting with 1nM E2 + 100nM ICI **(Figure II-5)**, for discussion see chapter IV.

Discussion

We were able to confirm our hypothesis of genistein being protective against prostate tumorigenesis **(Figure II-2)**, however our data showed that it is not solely acting through ERβ. The fact that genistein had no effect on total cancer incidence in either ERKO animal group indicates that genistein requires both estrogen receptors present in order to enact its protective action.

A surprising observation that came out of the first animal study with ERWT and ERαKO mice, was further confirmed with follow-up studies **(Table II-3)**, was that of the opposing roles the two estrogen receptors were playing in carcinogenesis. ERαKO animals had almost no PDC indicence (4%), and ERβKO mice had double the PDCs (42%) compared with WT mice (22%) **(Table II-1)**.

It is also possible that the genotypic effect that we observed is dominant over any dietary treatment. In our many parallel dietary studies with mixes of flavones and isoflavones in TRAMP mice, we were not able to see any dietary effect in ERαKO animals compared to animals WT for both receptors (data not shown).

Comparing the prostate weights by stage between the groups, we observed an interesting phenomenon. Besides of having an increased WDC incidence, which is responsible the overall increase in cancer, the ERαKO mice had significantly higher tumor weights compared to both ERWT and ERβKO mice. It is interesting still that even though it seems as they had a more aggressively growing cancer, it did not progress to the

poorly differentiated carcinoma stage. Studies with aged ERαKO mice showed that they did not develop more PDC up to 8 months (data not shown).
ERβKO mice were also bearing a surprise in it they had smaller tumors, and less tumors over 1.5g than control even though they had double the PDC incidence. It would be interesting to see whether those PDC tumors differed in other aspects, like expression of neuroendocrine markers or proliferation markers.

The immunohistochemical analysis is in agreement with our new hypothesis about the importance of ERα and ERβ in cancer progression. The non-cancerous, hyperplastic prostate did express ERβ, but not ERα. It also expressed AR, corresponding with the initial responsiveness to androgens in the prostate. In the PDC however, the expression pattern switches to ERα-positive cells that lack ERβ. The androgen is also lost at this stage as is the androgen responsiveness. The picture of the protective ERβ and tumorigenic ERα is very suggestive.

Another player in the prostate is the DHT metabolite, 5-androstene-3β, 17β-diol, which is able to bind to both estrogen receptors *in vitro* (215), with affinities of 6nM and 2nM, respectively, however it was reported to preferentially activate ERβ. It is possible, that the protective effect against PDC in the ERαKO mice is mediated through 5-androstene-3β, 17β-diol via ERβ. Removal of the "good" receptor in the ERβKO mouse leads thus to a doubling in PDC incidence **(Figure II-6)**.

We also observed an antiproliferative effect of genistein *in vitro*, both in human and mouse prostate cancer cells **(Figure II-5)**. We do not see the initial stimulatory effect of low dose genistein, as observed in PC3 and LNCaP by us ((111) and data not shown), and others.

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERWT	Casein	175	2 (1%)	10 (6%)	41 (23%)	88 (50%)	0	34 (19%)
ERWT	Genistein 300mg/kg	81	1 (1%)	13 (16%)	29 (36%)	15 (19%)	1 (1%)	22 (27%)
ERWT	Genistein 750mg/kg	25	0	0	17 (68%)	2 (8%)	0	6 (24%)
ERαKO	Casein	80	0	3 (4%)	4 (5%)	68 (85%)	1 (1%)	4 (5%)
ERαKO	Genistein	25	0	0	1 (4%)	23 (92%)	1 (4%)	0
ERβKO	Casein	51	0	0	13 (25%)	18 (35%)	0	20 (39%)
ERβKO	Genistein	23	0	0	5 (22%)	8 (35%)	0	10 (43%)

Table II-1: Effect of diet and genotype on the incidence of prostate cancer in 5 month old TRAMP mice.

Fisher's exact test two-tailed P-value

PDC incidence: ERWT vs ERαKO 0.0021

ERWT vs ERβKO 0.0027

Very statistically significant genotype effect.

No significant dietary effect.

WDC incidence: within ERWT low and high dose genistein vs casein 0.0001, extremely significant

Within casein ERWT vs ERαKO >0.0001, extremely significant

ERWT vs ERβKO 0.0572, not quite significant

Within ERWT casein vs low genistein >0.0001, extremely significant

Casein vs high gensitein >0.0001, extremely significant

Genotype	Diet	n	Tumor Stage	
			Non-Cancer	Cancer
ERWT	Casein	175	53 (30%)	122 (70%)
ERWT	Genistein 300mg/kg	81	43 (53%)***	38 (47%)***
ERWT	Genistein 750mg/kg	25	17 (68%)***	8 (32%)***
ERαKO	Casein	80	7 (9%)***	73 (91%)***
ERαKO	Genistein 300mg/kg	25	1 (4%)**	24 (96%)**
ERβKO	Casein	51	13 (25%)	38 (75%)
ERβKO	Genistein 300mg/kg	23	5 (22%)	18 (78%)

Table II-2: Effect of diet and genotype on the incidence of prostate cancer in 5 month old TRAMP mice.

Non-cancer was defined as normal, hyperplasia, and prostatic intraepithelial neoplasia.

Cancer was defined as well-, moderately-, and poorly differentiated carcinoma.

Each group was compared with ERWT casein.

** $p<0.005$ – very statistically significant; *** $p<0.001$ extremely statistically significant.

1. ERαKO genistein 2x2 study 1033-01

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERαWT	Casein	25	2 (8%)	4 (16%)	1 (4%)	13 (52%)	0	5 (20%)
ERαWT	Genistein 300mg/kg	28	1 (4%)	10 (36%)	7 (25%)	6 (21%)	0	4 (14%)
ERαKO	Casein	29	0	1 (3%)	0	25 (86%)	1 (3%)	2 (7%)
ERαKO	Genistein 300mg/kg	25	0	0	1 (4%)	23 (92%)	1 (4%)	0

mice collected 5/7/2001-12/6 2001

deaths: WT cas 4888 died at 5 months

αKO cas 4880 died at 5 months

2. **Pygeum study 1001-04**

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERαWT	Casein	16	0	0	6 (37.5%)	8 (50%)	0	2 (12.5%)

mice collected 12/9/2003 – 5/1/2004

deaths: WT cas 9805 died at 4½ months

3. Isoflavone 7 Mix study 1023-03

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERαWT	Casein	22	0	1 (4%)	2 (9%)	14 (64%)	0	5 (23%)
ERαKO	Casein	20	0	1 (5%)	4 (20%)	15 (75%)	0	0

mice collected 4/13/2003-12/2/2003

deaths: αKO casein 9178 died at 4½ months

4. Daidzein PCSpez study 1009-02

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERαWT	Casein	27	0	5 (18.5%)	8 (30%)	9 (33%)	0	5 (18.5%)
ERαKO	Casein	31	0	1 (3%)	0	28 (90%)	0	2 (6%)

mice collected 1/9/2002-3/3/2003

deaths: WT cas 880 died at 4¾ months

WT cas 9004 died at 5 months

5. ERβKO genistein 2x2 study 1042-04

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERWT	Casein	29	0	0	8 (28%)	14 (48%)	0	7 (24%)
ERWT	Genistein 300mg/kg	25	0	3 (12%)	10 (40%)	1 (4%)	1 (4%)	10 (40%)
ERβKO	Casein	25	0	0	4 (16%)	8 (32%)	0	13 (52%)
ERβKO	Genistein 300mg/kg	23	0	0	5 (22%)	8 (35%)	0	10 (43%)

mice collected 3/27/2004-7/8/2005

deaths: βKO cas 1539 large tumor, culled at 4½ months

6. Hi-lo genistein 1084-07

Genotype	Diet	n	Tumor Stage					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERWT	Casein	28	0	0	7 (25%)	16 (57%)	0	5 (18%)
ERWT	Genistein 300mg/kg	28	0	0	12 (43%)	8 (29%)	0	8 (29%)
ERWT	Genistein 750mg/kg	25	0	0	17 (68%)	2 (8%)	0	6 (24%)

mice collected 6/21/2007-9/8/2007

deaths: WT hi gen 5153 culled at 4½ months due to poor health

7. New berko 1049-09								
Genotype	**Diet**	**n**	**Tumor Stage**					
			Non-Cancer			**Cancer**		
			Normal	**HYP**	**PIN**	**WDC**	**MDC**	**PDC**
ERWT	Casein	28	0	0	9 (32%)	14 (50%)	0	5 (18%)
ERβKO	Casein	26	0	0	9 (35%)	10 (38%)	0	7 (27%)
mice collected 11/16/2007-2/27/2008 deaths: βKO casein 9928 died at 4¾ months								

Table II-3: Tumor incidence for TRAMP mice split by separate study.

		Weights (gm)			
Genotype	**Diet**	**Body**	**Repro. Tract**	**Prostate**	**Testes**
ERWT	Casein	28.80 ± 0.22^{a}	1.41 ± 0.10^{a}	0.40 ± 0.10^{a}	0.21 ± 0.004^{a}
ERWT	$Genistein^{low}$	28.92 ± 0.33^{a}	1.78 ± 0.26^{ad}	0.81 ± 0.27^{a}	0.20 ± 0.004^{a}
ERWT	$Genistein^{high}$	30.18 ± 0.68^{b}	1.35 ± 0.18^{a}	0.28 ± 0.17^{a}	0.21 ± 0.005^{a}
ERαKO	Casein	29.74 ± 0.52^{a}	3.02 ± 0.18^{b}	0.33 ± 0.03^{a}	0.17 ± 0.011^{b}
ERαKO	Genistein	32.38 ± 0.81^{c}	3.98 ± 0.24^{c}	0.43 ± 0.03^{a}	0.18 ± 0.018^{ab}
ERβKO	Casein	28.16 ± 0.52^{a}	2.20 ± 0.32^{d}	0.54 ± 0.19^{a}	0.20 ± 0.003^{a}
ERβKO	Genistein	28.49 ± 0.42^{a}	1.76 ± 0.36^{ad}	0.83 ± 0.39^{a}	0.21 ± 0.006^{a}

Table II-4: Body and organ weights of mice from different dietary groups.

Means ± SEM. Values within columns with different letter superscripts are significantly different, $p<0.05$.

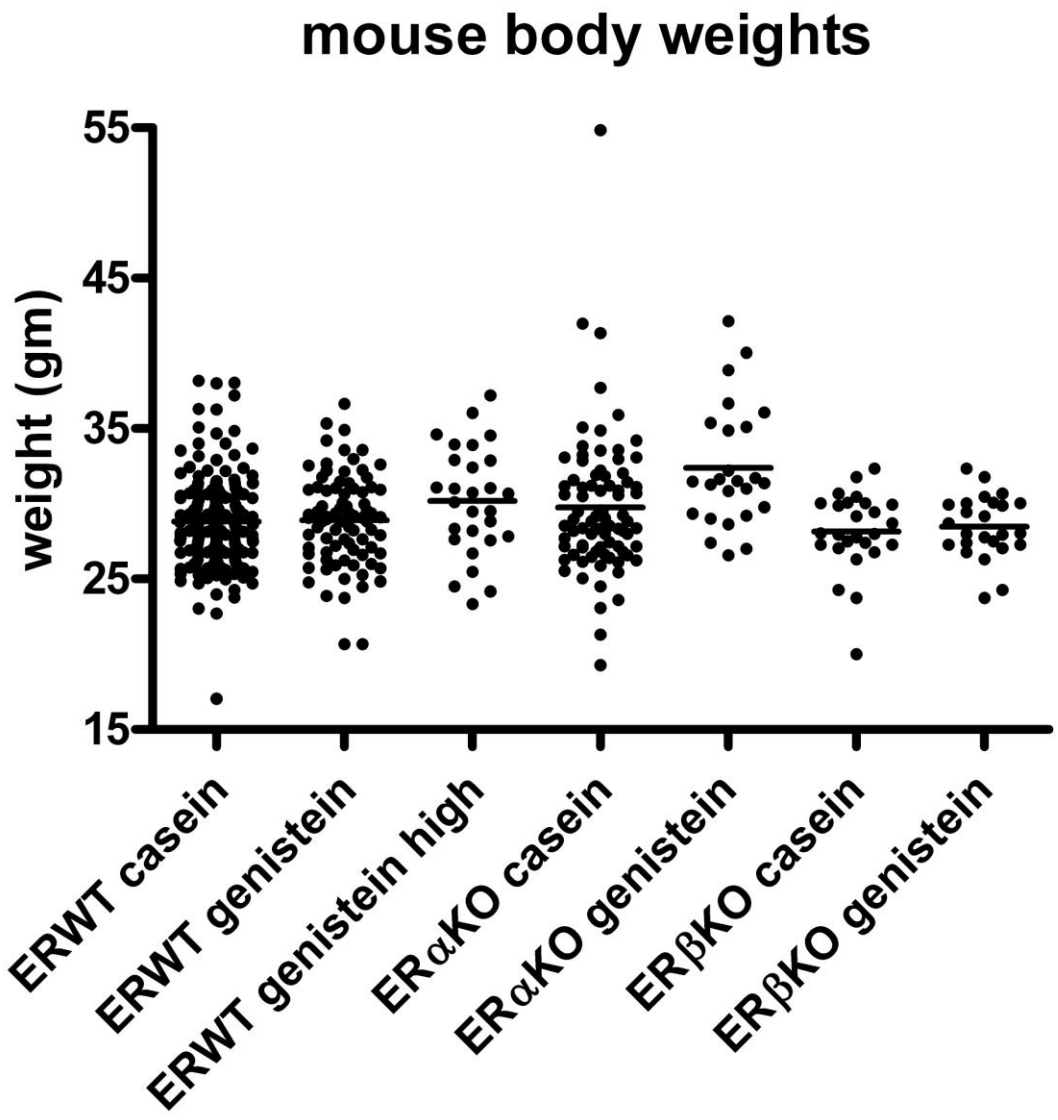


Figure II-1: Mouse body weight from all studies combined.

For statistics between groups see Table II-4.

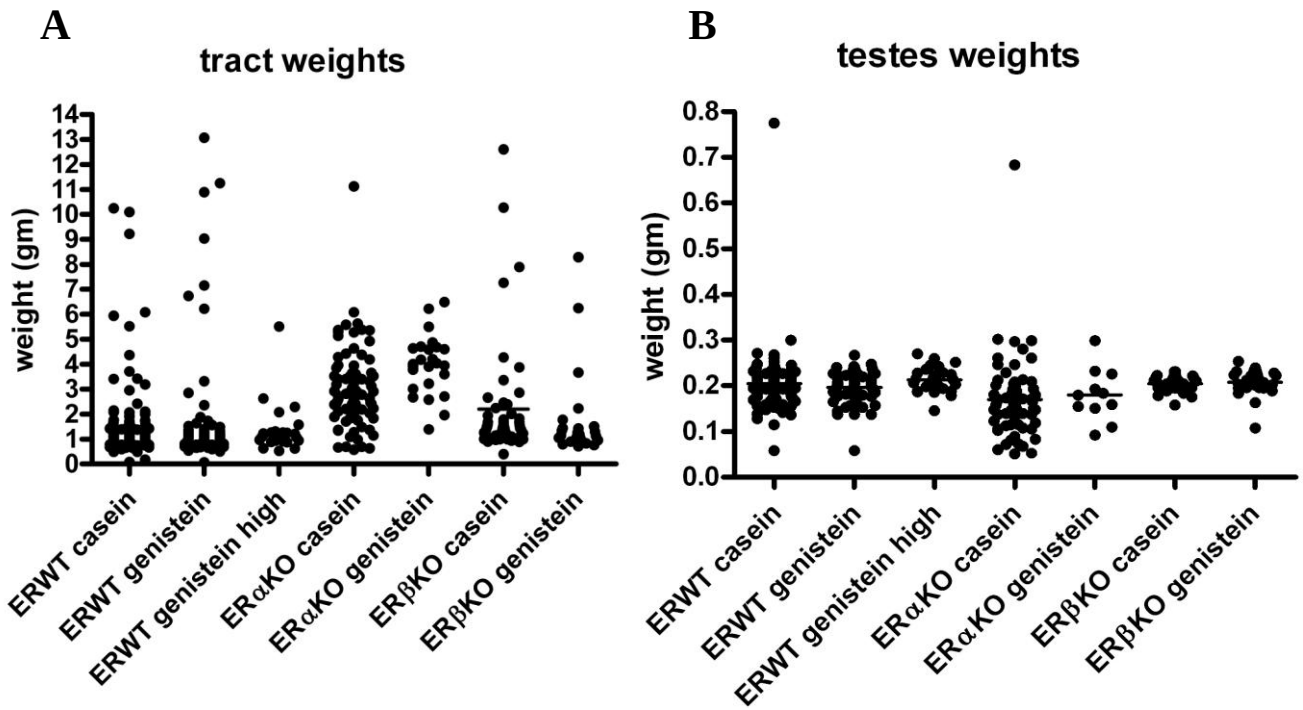


Figure II-2: TRAMP mouse urogenital tract (A) and testes (B) weights.

ERαKO mice have significant lower testes weights.

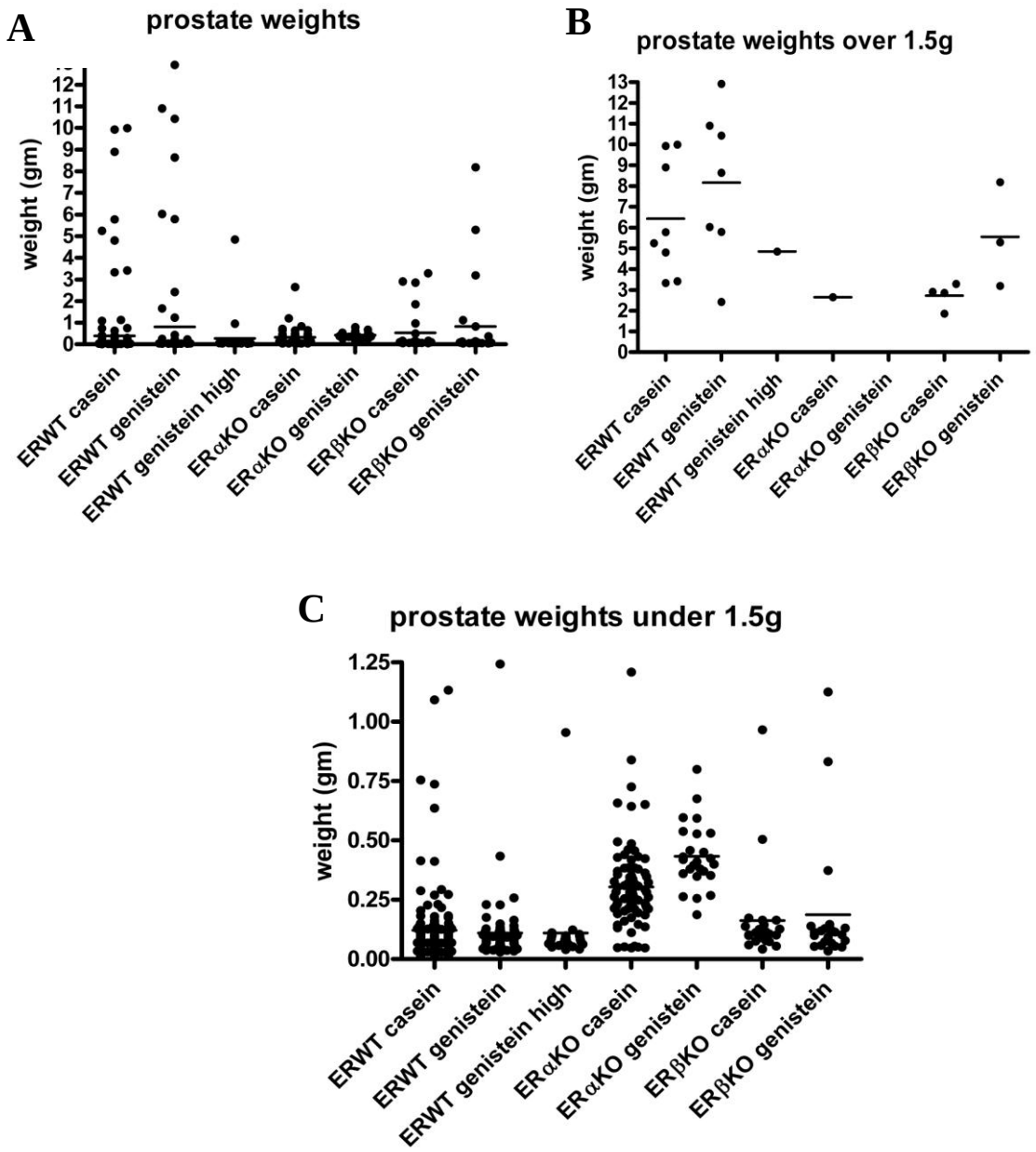
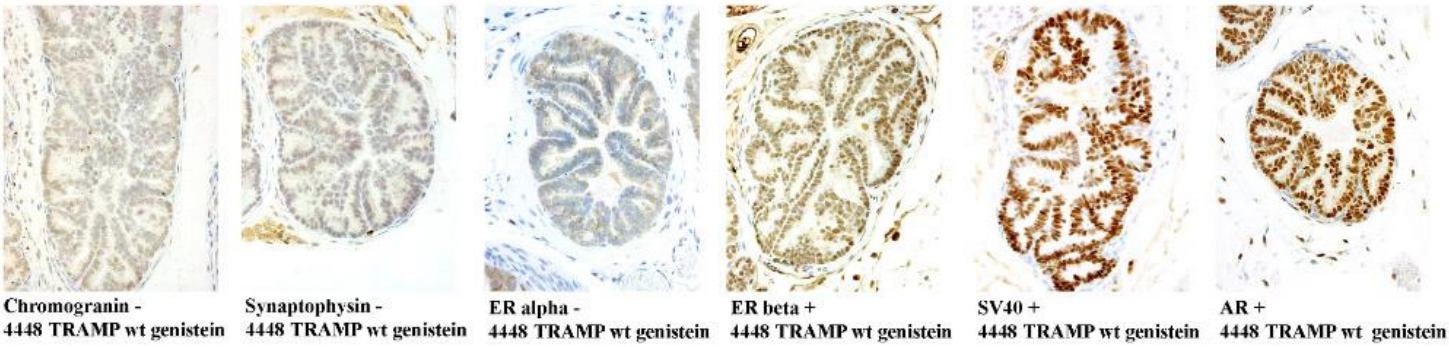


Figure II-3: Weights for 5 month old TRAMP mouse prostates

When combined as in **(A),** there are no significant differences in prostate weights, when we applied a cut-off of 1.5g for very large prostate, which were predominantly PDCs, we obtained a more differentiated pattern for prostate weight above 1.5g **(B)**, and under 1.5g **(C)**. ERWT mice on casein and low dose genistein.

Top: Prostatic Hyperplasia



Bottom: Poorly Differentiated Carcinoma (PDC)

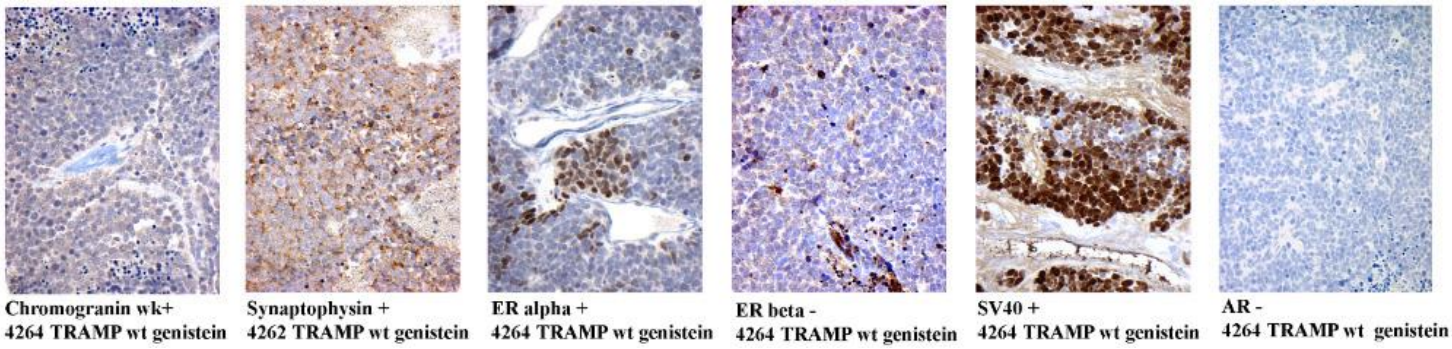


Figure II-4: Immunohistochemistry for T-antigen, neuron-endocrine markers, and sex-steroid receptors in TRAMP prostates.

In both panels, the brown regions represent immunoreactivity. The immunoreactivity to this panel of antibodies of the prostate was not different from those fed casein.

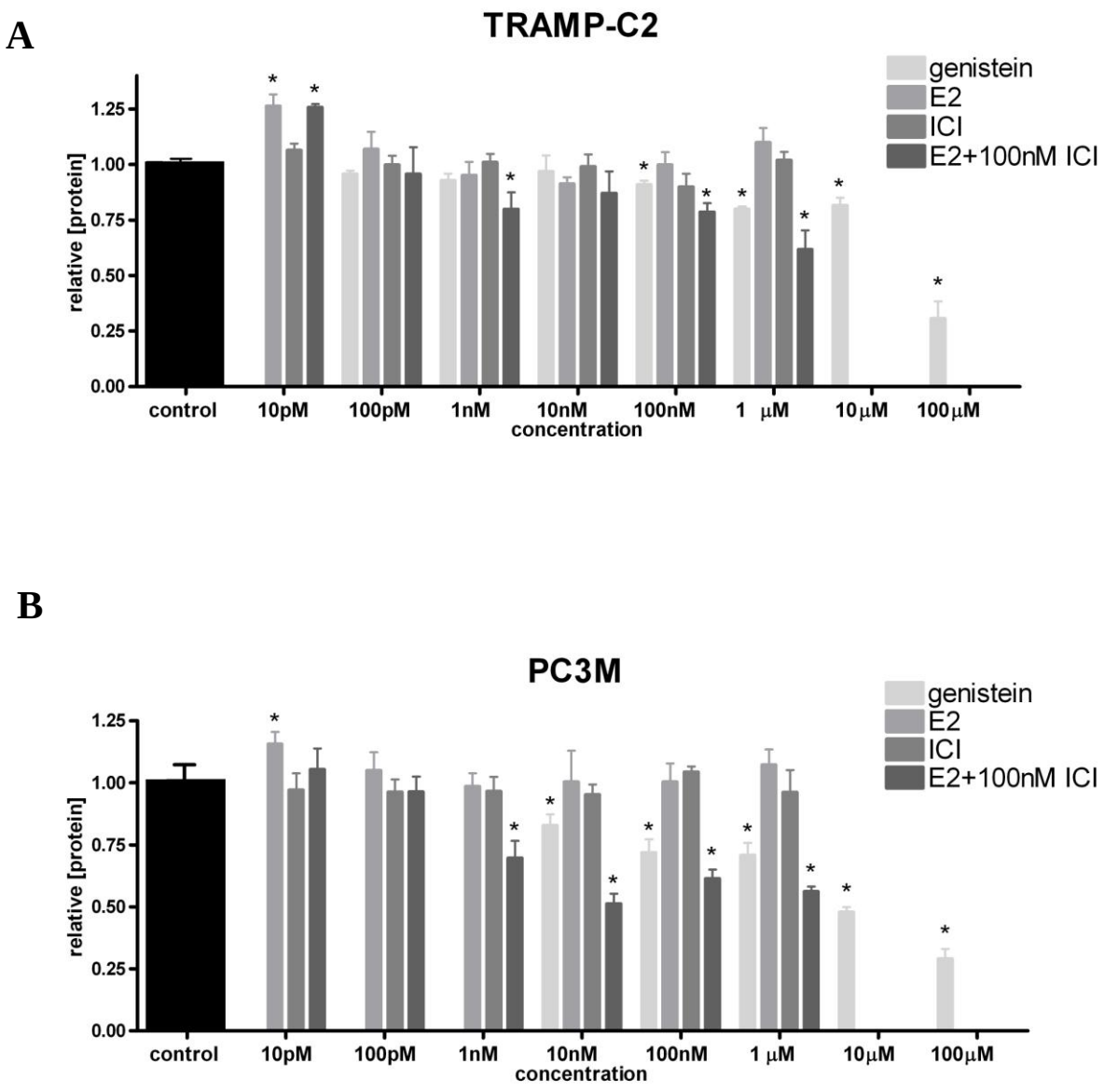


Figure II-5: Effects of Genistein, E2 and ICI on prostate cancer cell growth.

TRAMP-C2 **(A)** and PC3M **(B)** cells were treated for 72 hours with genistein, E2, ICI, or E2+100nM ICI. Cells were lysed with 1N NaOH for 24 hours, following an assay for total cellular protein.

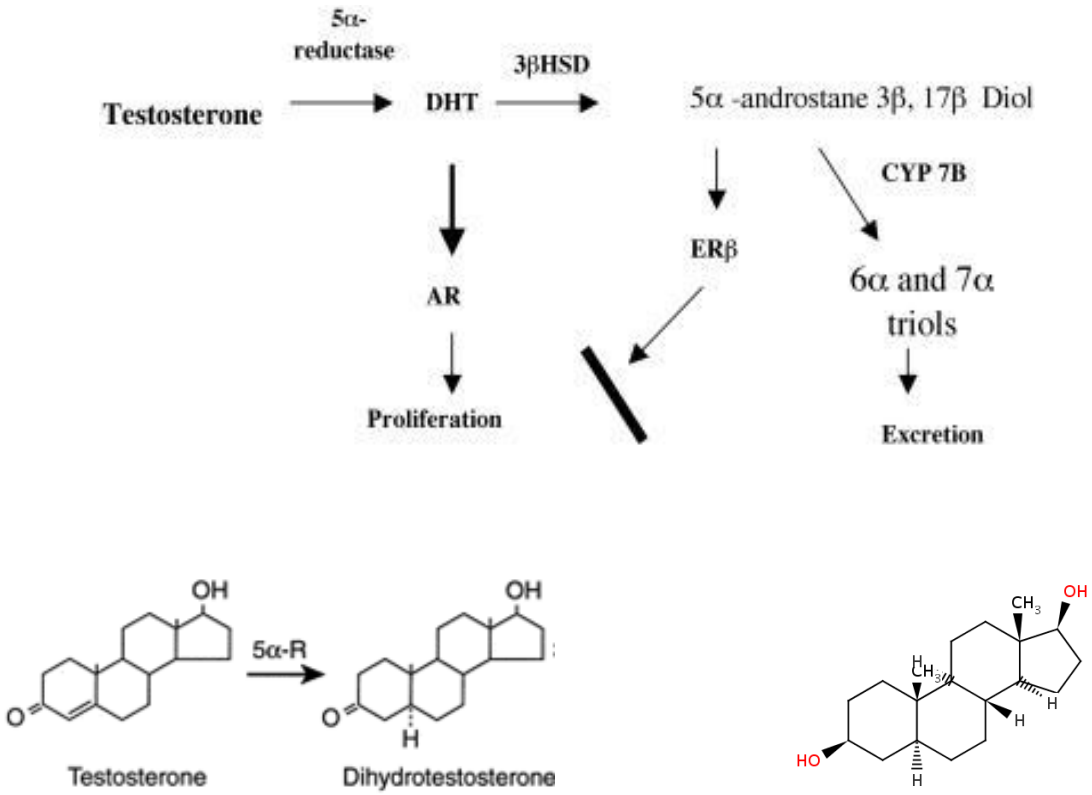


Figure II-6: 5α-androstane-3β,17β-diol in the prostate.

5α-androstane-3β,17β-diol can be converted from its androgen precursor, 5α-dihydro-testosterone (DHT), by a variety of steroid metabolizing enzymes, including: 17ß-hydroxysteroid dehydrogenase (17ßHSD), 3α-hydroxysteroid oxidoreductase (3αHSD), and 3ß hydroxysteroid oxidoreductase (3ßHSD) (390).

III - Common Botanical Compounds Inhibit the Hedgehog Signaling Pathway in Prostate Cancer.

Overview

Many botanical compounds have been proposed to prevent cancer. We investigated the cancer treatment and prevention abilities of apigenin, baicalein, curcumin, EGCG, genistein, quercetin, and resveratrol, both *in vivo* in TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) mice, as well as *in vitro* in prostate cancer cell lines.

In our experiments we **hypothesized** that these seven compounds act similarly to the hedgehog antagonist, cyclopamine, a teratogenic plant alkaloid, which had been previously shown to "cure" prostate cancer in a mouse xenograft model by inhibiting the hedgehog signaling pathway. With IC_{50} values ranging from <1μM to 25μM these compounds demonstrated hedgehog pathway inhibition by deceasing *Gli1* mRNA concentration by up to 95% and down regulating Gli-reporter activity by 80%.

We show that four compounds, genistein, curcumin, EGCG, and resveratrol, inhibit hedgehog signaling as monitored by real-time RT-PCR analysis of *Gli1* mRNA concentration or by Gli-reporter activity. Three compounds, apigenin, baicalein, and quercetin, decreased *Gli1* mRNA concentration but not Gli-reporter activity.

Our results show that these compounds are also able to reduce or delay prostate cancer *in vivo* in TRAMP mice. All 7 compounds, when fed in combination as pure compounds or as crude plant extracts, inhibit well differentiated carcinoma of the prostate

by 58% and 81%, respectively. *In vitro* we show that all 7 compounds also inhibit cell growth in human as well as mouse prostate cancer cell lines. *In vitro* experiments show also that low concentrations of each botanical, which are not quite inhibitory to *Gli1* when used alone, act synergistically, when used in combinations corresponding to mixes used in the mouse diets in our *in vivo* studies.

Mechanistically, we propose the hedgehog-signaling pathway to be a direct or indirect target of these compounds. These botanicals at pharmacological concentrations are potentially safer and less expensive alternatives to cyclopamine and its pharmaceutical analogs for cancer therapy.

Introduction:

Prostate cancer remains the second most commonly diagnosed cancer in the USA. According to the Prostate Cancer Foundation, for every three men diagnosed with cancer, one will be diagnosed with prostate cancer. It is also the second leading cause of cancer deaths of men in the United States (1). Because prostate cancer typically develops later in life, identifying botanical compounds that delay the progression of this disease will have a positive impact on quality of life, and reduce healthcare costs of the aging population.

It is well known that diet and other environmental factors can greatly reduce the risk of cancer incidence. In particular, dietary phytoestrogens and antioxidants have been implicated in protecting against cancer (111, 391). We have selected a group of 7 botanical compounds that have been reported by our lab and others to have prostate cancer protective activities (111, 128). They have been widely used in traditional medicine and in dietary supplements that are currently available in the United States (392). Those compounds include apigenin from *Matricaria recutita* (chamomile), baicalein from *Scutellaria baicalensis* Georgi (Chinese skullcap), curcumin from *Curcuma longa* (turmeric), EGCG (Epigallocatechin 3-gallate) from *Camellia sinensis* Kuntze (green tea), genistein from *Glycine max* (soy), quercetin from *Ginkgo biloba* and resveratrol from *Vitis vinifera* (grape).

We have previously reported on the cancer preventive effect of each of these 7 compounds in PC3 and LNCaP human prostate cancer cell lines (111). Here we show

that all 7 can individually also inhibit growth of the mouse prostate cancer cell line TRAMP-C2 (88).

Pursuant to these results, we wanted to determine whether these seven botanical compounds would impact the hedgehog signaling pathway, which, through its inhibitor cyclopamine, has been recently found to be important in prostate cancer and its treatment (303, 335, 337, 339).

The hedgehog (Hh) signal-transduction pathway is crucial to the growth, survival and organization of many cells, tissues and organs. Dysregulation of the Hh signal-transduction pathway has been implicated in several cancers, including human prostate cancer (335, 337, 339, 393). The pathway is activated by one of three types of Hedgehog protein, Sonic, Desert or Indian. These are secreted glycoproteins that bind to their membrane receptors, Patched1 or Patched2, which in turn relieve the inhibition of another trans-membrane protein, Smoothened. Smoothened mediates its actions via three transcription factors in the Gli family, specifically Gli1, Gli2 and Gli3 proteins **(Figures I-5 & I-6)**. Several types of cancer, including medulloblastomas, lung, esophageal, skin, pancreas, and prostate cancer, show greatly increased hedgehog pathway activation. Therefore, inhibition of Gli function might be a promising prevention and therapeutic target in certain tumors.

We found that four of the seven botanical compounds can inhibit the hedgehog-signaling pathway *in vitro*, both in the prostate cancer TRAMP-C2 cells, as well as in an established hedgehog pathway assay in Shh Light II cells.

We propose that the prostate cancer preventative effects of these dietary botanicals may result from inhibition of the hedgehog pathway, and that they potentially represent an inexpensive, safe and effective alternative to cyclopamine in cancer prevention and treatment.

Materials and Methods:

Diet formulations and sources:

Curcumin, baicalein and soy isoflavones were provided by Dr. Acharan Narula (Narula Research, Chapel Hill, NC). EGCG, resveratrol, and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO). Apigenin and genistein were purchased from LC Laboratories (Woburn, MA). Sencha leaves, curcuma longa roots, yucca roots, saw palmetto, chamomile flowers, and gingko were purchased from Frontier Natural Products Co-op. (Norway, IA). Experimental diets were based on the AIN93G formulation (374). All diets contained the same formulation, with addition of botanical ingredients substituted by weight for cornstarch. The controlled diet contained no added botanicals (Casein). The Pure 3 Diet contained 10g/kg curcumin, 100mg/kg EGCG and 10mg/kg resveratrol; the Pure 4 Diet contained 15mg/kg apigenin, 150mg/kg baicalein, 250mg/kg genistein, and 1g/kg quercetin; the Pure 7 Diet included all 7 pure compounds at the same level as in the 3 and 4 mix diets; and the Crude 7 Diet contained 1% w/w of the following dried herbs: sencha leaves, curcuma longa roots, yucca roots, saw palmetto (in lieu of *Scutalaria baicalensis*), chamomile flowers, gingko and soy isoflavone extract. The herbs were ground in a coffee mill prior to mixing into the diets and care was taken to ensure

complete uniform mixing of all ingredients. The concentrations of pure compounds represent a high-end dietary intake in humans, and were scaled proportionally for mice.

TRAMP mouse studies:

The TRAMP model was developed by inactivating two tumor suppressing genes, Rb and p53, by placing the SV40 large and small T-antigen gene under the control of the androgen regulated rat probasin promoter, which has been shown to be highly and specifically expressed in the mouse prostate (39, 373). TRAMP mice spontaneously develop prostate tumors starting at the age of three months and they can be used to study the progression of the disease, as well as prevention and treatment options.

Male TRAMP mice on a C57BL6/J background were raised in-house. All University of Missouri institutional guidelines for animal care and use were followed. The mice were housed in pairs in micro-isolator cages and given free access to food and water. A daily light:dark cycle of 12:12h was used, with ambient temperature and humidity set at 21°C, and 50%, respectively. Animals were monitored weekly for body weight and tumor burden. The mice were fed the specific diets from 5 weeks until 5 months (18-22 mice per treatment group). Prostates were collected, formalin fixed and paraffin embedded. Tissues sections were stained with hematoxylin and eosin, and examined by light microscopy for assessment of cancer stages (376).

All TRAMP mice were wild type for estrogen receptors except for the Crude 7 study, in which both casein control and experimental mice were heterozygous for ERα (394). The cancer incidence did not vary between WT and ER heterozygous mice.

Cell Culture:

All cell lines were obtained from ATCC (http://www.atcc.org).

Mouse prostate cancer TRAMP-C2 and human prostate cancer PC3 cell lines were maintained in RPMI 1640 media supplemented to contain 10% fetal bovine serum (U.S. Bio-Technologies, Parkerford, PA), 4.5g/mL Glucose, 4mM L-glutamine , 100μM Non-essential Amino Acids, 10mM HEPES, 1mM Sodium Pyruvate, and 1% Penicillin/ Streptomycin (all from Invitrogen, Carlsbad, CA).

Shh Light II cells (JHU-68) were maintained in Dulbecco's modified Eagle's medium with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.4 mg/ml G-418, 0.15 mg/ml Zeocin (Invitrogen), and 10% fetal bovine serum. This mouse embryonal NIH 3T3 cell line contains a stably transfected luciferase reporter with 8 copies of the consensus Gli binding site derived from the mouse Hepatocyte Nuclear Factor-3β (JHU-73 pGL3B/8XgliBS-lc-luc 5'-GAACACCCA-3') (395).

The purified compounds used in tissue culture experiments were obtained from the following suppliers: Apigenin (LC laboratories), Baicalein (Indofine Chemical Company, Hillsborough, NJ), Curcumin (Sigma), Cyclopamine (Toronto Research Chemicals and LC Laboratories), EGCG (Sigma), Genistein (Sigma), Quercetin (Sigma), Resveratrol (Sigma).

Mouse recombinant Shh was obtained from R&D Systems (Minneapolis, MN) cat. # 464-SH. All compounds were dissolved in DMSO, except: LC Lab cyclopamine was dissolved in ethanol; Shh was dissolved in PBS with 0.1% BSA. In each experiment the

controls and all the treatments contained all vehicles used. All treatments were conducted in phenol red-free medium with charcoal-stripped serum.

Protein Assay:

All compound treatments for the growth assessment consisted of a 72-hour time course in phenol red-free RPMI 1640 medium, supplemented with 10% charcoal-stripped FBS. Cells seeded in 12-well plates and adjusted to phenol-red free medium for 24 hours. TRAMP-C2 cells were treated at 30-40% confluency, PC3 cells at 50%, which allowed the controls to reach 100% by day 3. Cells were lysed with 1N NaOH and left overnight. Protein assays to measure overall cell protein concentration were performed using Bio-Rad Dc kit (Bio-Rad, Hercules, CA), with absorbance measured at 750 nm. Compounds were used at concentrations ranging from 1μM to 100μM in half log increments. Each experiment was performed at least three times in duplicate. Total cellular protein correlates well with thymidine uptake in prostate cancer cells and is a reliable assay to measure cell growth (111).

RNA isolation:

Total RNA was isolated from the TRAMP-C2 and PC3 cell line using the RNeasy kit (QIAGEN, Valencia, CA). From 1-5x 10^6 cells we obtained between 25-35μg total RNA. RNA concentration was determined using the ND 1000 Spectrophotometer v3.1, NanoDrop Technologies (Wilmington, DA). RNA integrity was monitored on a 1% agarose gel.

RT-PCR:

700ng of RNA was used in each RT-PCR reaction. RT-PCR was performed using the SSIII one-step RT-PCR system from Invitrogen with the following primers: Mouse GAPDH forward 5'-agcctcgtcccgtagacaaaat-3'; reverse 5'-ccgtgagtggagtcatactgga-3', mouse Shh forward 5'-gtgagctttggattcatagtagacc-3'; reverse 5'-aattacaaccccgacatcatattta-3', mouse Gli1 forward 5'-aggaggaaagagagatccttcagt-3'; reverse 5'-agtcaatagctatagtgagccatgc-3', mouse Gli2 forward 5'-gttcacacacatatggcttctctc-3'; reverse 5'-catcatatcaacaatgaacacatcc-3', mouse Gli3 forward 5'-tctcattggaatgtgttctgtttt-3'; reverse 5'-cattcatggagaaaagaaggagtt-3'. The following program was used for the reverse transcription and amplification: 55˚C for 30 min., 94˚C for 2 min., followed by 31 (GAPDH), 35 (Gli1 and Gli3), or 40 (Shh and Gli2) cycles of 94˚C for 15 sec., 58˚C (GAPDH, Gli1 and Gli3) or 55˚C (Shh and Gli2) for 30 sec., 68˚C for 1 min., followed by the final extension step of 68˚C for 5 min.

Real Time RT-PCR:

Relative expression of mRNA was measured by one-step real-time RT-PCR with TaqMan EZ-RT-PCR kit (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System. Real-time RT primers and probes were designed using the Primer Express software (Applied Biosystems). The candidate primers were verified using RT-PCR. Mouse GAPDH: forward 5'- cagagacggccgcatctt-3', probe 6FAM-ttgtgcagtgccagcctcgtcc-TAMRA, reverse 5'-ccgaccttcaccattttgtctaC-3'. Mouse Gli1 forward 5'-gcatgggaacagaaggactttc-3', probe 6FAM-tgccctttgccaccaagcca –TAMRA, reverse 5'-ccctgggaccctgacataaa-3'. Mouse Patched1: forward: 5'-

ccctaacaaaaattcaaccaaacc-3', probe 6FAM-tgatgtggcccttgttttgaatggtg-TAMRA, reverse 5'-gcatatacttcctggataaaccttgac-3'. Human GAPDH: forward 5'-atggaaatcccatcaccatctt-3', probe 6FAM-cgccccacttgattttgg-TAMRA, reverse 5'-caggagcgagatcc-3'. Human Gli1: forward 5'-gcaggtgcgtcttcaggttt-3', probe 6FAM-cgaggcgtgagtatgacttccggc-TAMRA, reverse 5'-gccacacaagtgcacgtttg -3'. Human Patched1: forward 5'-gggtggcacagtcaagaaca-3', probe 6FAM-cgtcagcgcccatgccctg-TAMRA, reverse 5'-gggagtcattaactggaacatggt -3'. The relative expression of mRNA was measured by one-step real-time RT-PCR with TaqMan EZ-RT-PCR kit (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System using the following program: 2 min at 50˚C, 30 min at 60˚C, 5 min at 95˚C, followed by 45 cycles of 20 sec. at 94˚C and 1 min. at 62˚C.

Each reaction was performed in triplicate for at least 3 individually isolated RNA samples per treatment. Data were analyzed using the ΔΔCT method (396).

Reporter Assay:

Gli activity in the Shh Light II cell line was assayed after 24 hours or 48 hours of treatment with selected compounds in phenol red-free DMEM supplemented with 0.5% charcoal-stripped serum using the Dual-Luciferase Reporter Assay System (Promega Madison, WI). Each experiment was performed at least in triplicate.

Western Blot Analysis:

Cells were lysed using passive lysis buffer (Epitomics), scraped on ice, sonicated or passed through a 26G needle and spun for 30 min at 13K rpm at 0˚C. Cell lysates were collected and stored at -80˚C. Mouse prostates were disrupted in liquid N_2 or using a

tissue homogenizer in TEG buffer. Tissues were spun for 10 min at 1.2K rpm at 4˚C, lysates were spun again for 30 min at 13K rpm at 0˚C. Tissue lysates were collected and stored at -80˚C.

Rabbit polyclonal antibody against mouse Gli1 (ab7523, 1:2000, Abcam, Cambridge, MA discontinued and, Q59214R, 1:2000, Biodesign, Saco, MA), and against β-actin (C4-HRP, 1:10^5 Santa Cruz Biotechnology, Santa Cruz, CA) were used according to manufacturer's recommendations. Western blot bands were scanned to Adobe Photoshop.

Statistical Analysis:

GraphPad Prism 4 was used to calculate p-values (http://www.graphpad.com).

In vivo studies were analyzed with the chi-square test, comparing each treatment vs. control diet for each cancer stage. *In vitro* results were analyzed using the t-test. A p-value <0.05 was considered statistically significant.

Results:

Botanical Compounds inhibit TRAMP-C2 cell growth.

All 7 botanical compounds tested were able to inhibit mouse prostate cancer cell growth in the TRAMP-C2 cell line with IC_{50} values between 20-30μM **(Table III-1)**. Compounds were used at concentrations ranging from 1μM to 100μM in half log increments. The maximal inhibition varied between 25% and 70%, with genistein showing the strongest effect followed by cyclopamine > curcumin = resveratrol > quercetin > EGCG > baicalein > apigenin. The inhibition of total cellular protein by genistein is significant starting at concentrations as low as 1μM.

Botanical Compounds decrease basal and Shh-stimulated *Gli1* mRNA in TRAMP-C2 cells.

The botanical compounds were able to inhibit hedgehog pathway signaling as measured by real-time RT-PCR analysis of basal *Gli1* mRNA concentrations in TRAMP-C2 cells after a 24 and 72 hour treatment (**Figure III-4 and Table III-1)**. Resveratrol had the strongest potency, with an IC_{50} value <1μM, followed by apigenin, baicalein and cyclopamine. Curcumin had the highest efficacy, reaching maximal inhibition of 95%, followed by cyclopamine with 85%. Baicalein and resveratrol produced the smallest change in *Gli1* concentrations, with 35% maximal inhibition at 30μM and 10μM, respectively.

The IC_{50} values for *Gli1* inhibition were in each case lower than the IC_{50} value for growth inhibition, potentially indicating that inhibition of multiple pathways in addition to the Hh signaling pathway is needed to alter cell growth. We and others have reported previously that the 7 botanical compounds are able to act on other non-hedgehog signaling pathways (111). All compounds are also able to decrease Gli1 protein concentrations in TRAMP-C2 cells after a 24 hour treatment **(Figures III-4C and III-5).**

It has been reported that there is a requirement for functional primary cilia for the hedgehog pathway to be active (289). In culture however, cells will not develop a primary cilium until they become confluent (397). We thus performed a time course experiment with cyclopamine and genistein, starting with cells that were already confluent. Since the data showed that all seven compounds inhibited cell growth, we

analyzed the Hedgehog pathway to determine if the inhibition was an apparent secondary effect and simply due to reduced cell confluency. Beginning near 100% confluency and investigating the cells after 2, 4, 8, 24 and 72 hour time points, we were able to observe a quick inhibition of hedgehog activity thus excluding the possibility of an indirect growth retardation effect of the compounds. 30μM cyclopamine was able to cause a significant decrease in *Gli1* mRNA concentration after only 2 hours of treatment. 50μM genistein decreased *Gli1* mRNA significantly after 4 hours **(Figure III-6)**. These results confirm the relatively fast-acting hedgehog inhibitory abłility of genistein is independent of growth and a subsequent change in cilia or cell confluency.

We next tested whether *Gli1* mRNA expression in the TRAMP-C2 cell line could be increased with the pathway agonist Sonic Hedgehog. Treatment of TRAMP-C2 cells with 0.5μg/mL N-terminal Shh peptide caused a 25fold elevation in *Gli1* mRNA that was inhibited by 3μM cyclopamine**.** 5μM genistein was able to reproducibly decrease this stimulated *Gli1* mRNA **(Figure III-5)**.

Botanical Compounds decrease Shh-stimulated Gli-reporter activity in Shh Light II cells.

To independently confirm the hedgehog inhibitory effects of the seven botanical compounds hown in **Table III-10**, we tested them in the widely used hedgehog responsive fibroblast cell line Shh Light II (395, 398). Four of the 7 compounds were able to decrease Shh peptide-stimulated Gli-reporter activity. The cyclopamine positive control had the strongest effect at 30μM, followed by curcumin, EGCG, genistein, and

resveratrol. Apigenin, baicalein and quercetin, were not able to inhibit the pathway in this system **(Figure III-8)**.

To test the general significance of our observations, we selected genistein, based on cost, availability and purity, to analyze further its functionality in a human prostate cancer cell line – PC3. Genistein can significantly inhibit PC3 growth starting at 10μM **(Figure III-9A)**, with an IC_{50} of 40μM. At 10μM genistein is also able to significantly inhibit *Gli1* and *Patched1* mRNA concentrations in the cells by over 50% **(Figure III-9B)**.

Botanical Compounds prevent tumorigenesis in TRAMP mice.

Due to economical reasons we were not able to test each compound individually. For these initial studies the compounds were grouped based on their structure. The non-flavones/non-isoflavones – curcumin and resveratrol plus EGCG were used together in Pure 3 Diet. The flavones/isoflavones - apigenin, baicalein, genistein and quercetin, were used together in Pure 4 Diet **(Figure III-10)**. To examine the combined effects, all 7 pure compounds were used in the Pure 7 Diet. To further explore a natural exposure to these compounds, crude plant materials or extracts from which the pure compounds were derived were combined in the Crude 7 Diet. Saw palmetto, another prostate herbal was chosen in lieu of *Scutalaria baicalensis* because of initial difficulties in obtaining the latter.

We tested the combinations of compounds used in the diets *in vitro* in the TRAMP-C2 cells, with concentrations of each individual compound low enough to where

no significant inhibition of *Gli1* mRNA is seen. When combined, all mixes used result in significant reduction of *Gli1* mRNA **(Figure III-11)**. Synergistic effects of some of these compounds have been reported previously in prostate cancer cell lines (399).

TRAMP mice fed the experimental diets grew similarly and consumed similar amounts of each diet throughout the study **(Figure III-12)**. No group weight mean varied more than 7% from overall weight mean. After 5 months, prostates were staged according to severity of lesions (376). At this age, no normal prostates were seen, as observed with earlier studies, and a wide range of cancer stages was present. The relative incidence hyperplasia (HYP), prostatic intraepithelial neoplasia (PIN), well differentiated carcinoma (WDC), moderately differentiated carcinoma (MDC) and poorly differentiated carcinoma (PDC) were quantified by histological examination by a trained Veterinary Pathologist who was blinded to the treatments. All experimental diets significantly decreased cancer incidence, defined as WDC, MDC and PDC when compared to control diet **(Table III-2)**. Within the specific cancer stages, all diets decreased WDC incidence in the TRAMP mice, with Pure 4 Diet (apigenin, baicalein, genistein and quercetin), and Crude 7 Diet having the strongest effect **(Table III-3)**. No protective effects were observed in ERαKo mice.

Discussion

In the fall of 2004 four papers were published that profoundly altered the outlook for prostate cancer treatment (303, 335, 337, 339). The authors independently reported that advanced human prostate cancer specimens, and metastases showed elevated Hedgehog pathway activity. These studies found that the Smoothened-binding and –

inhibiting compound, cyclopamine, an alkaloid isolated from *Californium veratrum*, was able to inhibit human prostate cancer cell proliferation *in vitro* and was able to cure mouse xenograft models of prostate cancer (303, 337, 339, 400). 3-4 weeks of high dose cyclopamine injections caused tumor regression without reoccurrence after cessation of treatment. Thus, cyclopamine is potentially a promising treatment for prostate cancer. However, the high cost of cyclopamine make it an unrealistic drug for wide scale use (401). Several companies are working to develop small molecule hedgehog pathway antagonists, some of which are now entering phase 2 clinical trials.

We were interested in testing cyclopamine in our TRAMP mice, to see whether it is able to cure SV40 T/t antigen induced prostate tumors *in vivo*. Unfortunately, the price and availability of cylopamine at the time made it impossible to conduct extensive animal studies. Our previous investigations revealed that several botanical compounds that inhibit prostate cancer cell proliferation *in vitro* (111) when used in the diet, also delayed prostate cancer incidence *in vivo* in TRAMP mice **(Table III-2)**. Since there are many known possible mechanisms of action for botanical compounds potentially useful as cancer preventative agents, we decided to test the hypothesis that these selected seven compounds, that were able to reduce cancer incidence in our TRAMP mice, would have the ability to inhibit Hedgehog signaling. We found that all 7 botanical compounds had cancer preventative action when fed in combination to TRAMP mice. There was a significant reduction in overall cancer incidence, with significant effects at the well differentiated carcinoma stage with the Pure 4 and Crude 7 Diet **(Table III-2 and III-3)**. After obtaining these results with our combination diets the Lubahn lab will further test

some of the compounds individually. Genistein (355, 356, 402), EGCG (191, 403-408), resveratrol (260), and apigenin (113), and recently curcumin (172) have all been previously tested individually, and only high dose genistein (250-500mg/kg) (356) has been reported to effect PDC. Thus we will further test cyclopamine, along with curcumin and genistein as treatments for already established tumors in TRAMP mice.

We were very interested in testing the effects of the 7 compounds on hedgehog signaling *in vitro* and *in vivo*. For our *in vitro* studies we used the TRAMP-C2 cell line derived from a primary prostate tumor of a 32-week old TRAMP mouse (88) to study the mechanism of action of those compounds, as well as the Shh Light II cell line. Our results show that four of seven compounds, namely genistein, curcumin, EGCG, and resveratrol, inhibited the Hedgehog-signaling pathway in our assays comparable to the control compound cyclopamine.

Why did not all seven compounds work in both these *in vitro* hedgehog assays?

Each of the 7 compounds inhibited prostate cancer cell growth in both human and in mouse cell lines. It was however interesting to see the discrepancy in hedgehog pathway inhibition between *Gli1* mRNA inhibition in TRAMP-C2 cells and Gli-reporter activity in Shh Light II cells. Three out of seven compounds – apigenin, baicalein, and quercetin, inhibit strongly, even at low concentrations *Gli1* mRNA. However, in Shh Light II we could not observe any inhibition with concentrations as high as 30μM with apigenin, baicalein or quercetin.

Potential reasons these three compounds are not inhibiting the hedgehog-signaling pathway in both assays could be due to their direct vs. indirect effects on the pathway, with the possibility of cell specific characteristics from additional unknown pathways' crosstalk with the hedgehog pathway. For example, in melanomas interactions between Gli1 and the Ras-MEK/AKT pathways have been observed (409). Finally, a recent report presents data that in some cell lines hedgehog pathway activation at the level of Gli1 and Gli2 can be inhibited by TGF-β inhibitors, but *not* by cyclopamine (410).There are many significant steps between the hedgehog signal at the cell membrane and the Gli-regulated transcription response in the nucleus, with a variety of potential signaling interactions not yet fully explored. Also, all of the targets of botanical compounds have not been completely determined. Genistein for example, which has been the most extensively studied, regulates a large range of molecular and enzymatic activities which may interact with hedgehog signaling (see chapter I).

The changes in enzyme activities could differentially alter an endogenous inhibitor that is absent in one of the cells, or the different media used to culture the two cell lines could impinge on a hedgehog crosstalking pathway. Potential candidates for such inhibitors or modulators are cholesterol derivatives and precursors whose concentrations vary widely between cell types and culture media, and which can be removed with different efficiencies by charcoal-stripping sera. We are using charcoal-stripped media for all our treatments, with the charcoal removing most cholesterol compounds, like hedgehog activating oxysterols (307, 411), or sex steroids, like androgens, which also have been shown to inhibit hedgehog signaling in prostate cancer (412, 413).

Crosstalk with the hedgehog-signaling pathway is still under active investigation, and there are still open questions regarding good models for studying the pathway. Tumors from TRAMP mice and TRAMP-C2 cells clearly demonstrate Hedgehog signaling which makes them appropriate for studying prostate cancer responses to hedgehog antagonists. It had been proposed that tumor cells of epithelial origin, would not have a functional autocrine hedgehog signaling (414, 415), our TRAMP-C2 cells, however, show hedgehog signaling that is both inducible and inhibitable **(Figure III-5)**.

All of our *in vitro* cell culture experiments have been conducted with cells of epithelial origin. By the current dogma, Hedgehog signaling consists of communication between different cell types. The ligand (Hh) is produced by epithelial cells at mesenchymal interfaces and signals to the adjacent mesenchyme through its receptor Patched. (281, 416, 417). Many cancer cells however undergo an epithelial-mesenchymal transition with cancer progression, which could be what we are seeing in our cells. While Zhang *et al.* (418) showed a lack of demonstrable autocrine hedgehog signaling in human prostate cancer cell lines, we have observed cyclopamine inhibiting PC3 and LNCaP cell growth (data not shown), as well as decreased *Gli1* and *Patched1* mRNA in PC3 cells by genistein **(Figure III-9B)**. These differences in observation might be due to higher starting confluencies (419), or different components in the medium. We are using charcoal-stripped media for all our treatments, with the charcoal removing most cholesterol compounds, like hedgehog activating oxysterols (307, 411), or sex steroids, like androgens, which have been shown to inhibit hedgehog signaling in prostate cancer (412, 413, 420).

We also looked for hedgehog pathway activation in TRAMP mice on different backgrounds. We saw a higher Gli1 mRNA and protein concentration in primary tumors from C57/FVB crosses compared to pure C57JBL6 (data not shown). This agrees with the observation, that FVB mice and C57/FVB crosses are more prone to metastasis than C57, and hedgehog pathway activity being greatly elevated in metastases relative to primary tumors. Interestingly, C57BL6 mice carry a Patched polymorphism when compared to FVB mice. This polymorphism makes them highly resistant to development of skin squamous carcinomas (421).

The LADY model is an alternative prostate cancer model to TRAMP. Gipp *et al.* reported a lack of increased hedgehog signaling markers during tumor development in the transgenic LADY prostate cancer mouse model (422). The LADY mice have been created using the androgen-regulated probasin driving the large T antigen on a CD-1 background. Although the tumors are very fast growing they rarely produce metastases (423). The lack of metastatic potential might be explained by the low hedgehog activity in this model, and stress the importance of strain background for studying the Hh signaling pathway.

Lu Yuan in the Lubahn lab recently sequenced the Patched gene from CD-1 mice to test for the cancer/metastasis resistance polymorphism, and surprisingly CD-1 carry the wild type allele, like FVB, so our hypothesis stating that the lack of hedgehog activity in the LADY model could be explained by the less aggressive mutant Patched allele was incorrect.

The SV40 large/small T antigen disrupts the actions of Rb and p53. Since both the TRAMP tumors and the TRAMP-C2 and Shh Light II cells express the T antigens, it is interesting to note, that mutations in p53 occur frequently alongside mutations in the Hedgehog signaling pathway. Their effects tend to be additive making animals more susceptible to cancer (424). There has been a recent report on a regulatory loop between p53 and Gli1 that has to be evaluated in regard to our model (425).
p53 mutations are seen frequently with Patched mutations in basal skin cancer (426, 427). On the other hand, Gli-induced skin (428) and gastric (429) tumors have wildtype p53, suggesting that Gli1 is sufficient in tumor initiation, and does not require additional p53 mutations. Constitutively activated mutants of Smoothened, which causes over-expression of Gli1 and Gli2, have been shown to inhibit the accumulation of the tumor suppressor protein p53 (430). It has been recently reported that p63 and p73, but not p53, over-expression can induce Shh expression (431).

We have analyzed tumors for TRAMP mice for *Gli1* mRNA and Gli1 protein by western blot and so far we have observed a high variance in Gli1 expression among and within treatments **(Figure III-13)**. This could be due to the nature of our sample material, since PDC tumors tend to be non-homogenous and may contain large necrotic areas. We are planning to perform immuno-histochemical analysis on the tissues as soon as a lot-to-lot consistent Gli1 antibody becomes available in larger quantities.

The doses of our botanical compounds shown to be effective *in vitro* are at apparent pharmacological levels. However, reports on free genistein levels (aglycone) in serum and prostate of rats fed genistein diets vary. Dalu *et al.* (432) reported bioavailable genistein to be comparable between serum and dorsolateral prostate in Lobund-Wistar rats fed 250mg/kg and 1g/kg genistein diets at ~18 and 150nM, respectively. Chang *et al.* however reported higher concentrations of aglycone genistein in several tissues including the prostate compared to serum (433). In their hands, Sprague-Dawley Rats fed 100mg/kg and 500mg/kg genistein diets reached prostate concentrations of bioavailable genistein as high as 400 and 500nM, compared to 6-30 and 60-300nM in serum, respectively. These values are already in the range of genistein's effect on prostate cancer cell growth inhibition and *Gli1* mRNA reduction in TRAMP-C2 cells, which were significant, starting at 100nM. Thus, dietary concentrations may be sufficient to reach at least partial hedgehog inhibition because of the ability of prostates to concentrate some compounds.

In our *in vivo* study with TRAMP mice three of the 7 compounds, curcumin, EGCG and resveratrol, were present in Pure 3 Diet which showed an over 50% reduction in PDC incidence **(Table III-3)**, which is not significant due to the small number of animals displaying PDC. We have seen that low doses (300mg/kg) of genistein significantly reduce WDC incidence (data not shown), while high doses (500mg/kg) of genistein have been reported to decrease PDC incidence in TRAMP mice (356). These four compounds potentially are strong alternatives to cyclopamine. They are significantly cheaper and safer, and have been used in traditional diets and in dietary supplements, that are available in the US.

Although the compounds have been extensively used, there only has been a limited number of reproductive safety studies conducted, that would shed light on the potential teratogenicity of those compounds indicating a cyclopamine-like effect. Genistein did not cause any fetal malformations when fed to pregnant rats up to 1000mg/kg/day (434). Resveratrol has been reported to act as a anti-teratogenic compound (435), as well as curcumin (175, 436). Feeding pregnant rats diets supplemented at 14,000 ppm EGCG during organogenesis was non-toxic to dams or fetuses (437).

Our findings of hedgehog inhibition with genistein, curcumin, EGCG and resveratrol potentially provide available, safer and more affordable anticancer treatments, in hedgehog-signaling-driven cancer types. Additionally, they help provide better understanding of the mechanisms by which traditional herbal medicines and dietary supplements may be working to prevent and treat cancers.

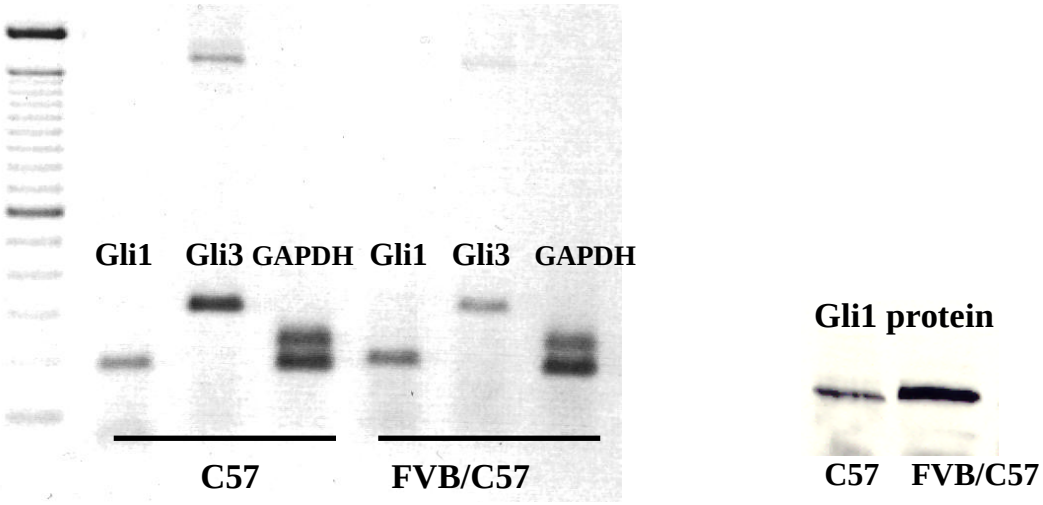


Figure III-1: TRAMP mice express Gli transcription factors.

Both strains of TRAMP used in the lab, the C57 and the F1 cross FVB/C57 mice express Gli1 and Gli3 mRNA as determined by RT-PCR (A), and Gli1 protein as determined by western blot by Dr. Mary Sakla (B). The FVB/C57 cross, which is more aggressive and more susceptible to metastasis has a higher expression of Gli1, the positive pathway executor, and a lover expression of Gli3 which is acting as a repressor.

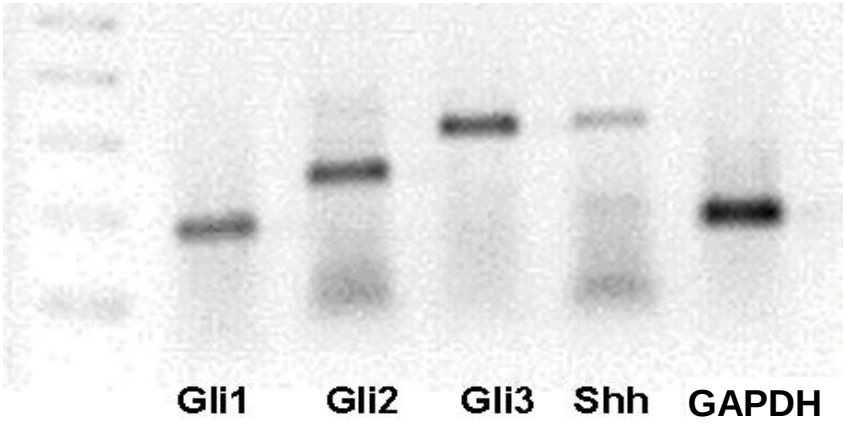


Figure III-2: TRAMP-C2 cells express Hedgehog pathway components.

TRAMP-C2 pathways express Gli1, Gli2, Gli3, and Shh mRNA as shown by RT-PCR.

Botanical	**IC_{50}**	**Maximal *Gli1* inhibition**
Apigenin	< 10μM	60% at 30μM
Baicalein	< 10μM	35% at 30μM
Curcumin*	10-20μM	95% at 100μM
Cyclopamine	3-10μM	85% at 30μM
EGCG	10-25μM	80% at 100μM
Genistein	20μM	75% at 50μM
Resveratrol	< 1μM	35% at 10μM
Quercetin*	10μM	55% at 30μM

* 24 hours

Table III-1: Botanical compounds inhibit Hedgehog pathway activity in TRAMP-C2 cells. *Gli1/GAPDH* mRNA concentrations indicating Hedgehog pathway activity after 72 or 24 hours of treatment with various botanicals were determined by real-time RT-PCR.

Diet	N	Phenotype	
		Non-Cancer	Cancer
Casein	**22**	3 **(13.6%)**	19 **(86.4%)**
Pure 3	**20**	10 **(50%)**	10 **(50%)***
Pure 4	**18**	12 **(66.7%)**	6 **(33.3%)*****
Pure 7	**19**	11 **(57.8%)**	8 **(42.2%)****
H-Casein	**20**	5 **(25%)**	15 **(75%)**
H-Crude 7	**21**	15 **(71.4%)**	6 **(28.6%)****

Table III-2: Incidence of prostate tumorigenesis in TRAMP mice fed various botanical compounds.

Male TRAMP mice were started on diets at weaning, and sacrificed at 5 months.

Pure 3 = Curcumin + Resveratrol + EGCG

Pure 4 = Apigenin + Baicalein + Genistein + Quercetin

Pure 7 = Pure 3 + Pure 4

Crude 7 = Soy + sencha leaves + tumeric + yucca roots + saw palmetto + chamomile flowers + gingko; H-mice were heterozygous for ER α.

Non-cancer defined as normal, hyperplasia, and prostatic intraepithelial neoplasia.

Cancer defined as well-, moderately-, and poorly differentiated carcinoma.

* p-value<0.05; **p-value<0.01; ***p-value<0.001; χ^2-test was performed to compare cancer vs. non-cancer incidence for each treatment vs. casein diet.

Genotype	Diet	N	Phenotype					
			Non-Cancer			Cancer		
			Normal	HYP	PIN	WDC	MDC	PDC
ERWT	**Casein**	**22**	0	1 **(4.5%)**	2 **(9%)**	14 **(63.6%)**	0	5 **(22.9%)**
ERαKO		**18**	0	0	4 **(22%)**	14 **(78%)**	0	0
ERWT	**Pure 3**	**20**	0	2 **(10%)**	8 **(40%)***	8 **(40%)**	0	2 **(10%)**
ERαKO		**19**	0	0	4 **(21%)**	15 **(79%)**	0	0
ERWT	**Pure 4**	**18**	0	8 **(44.5%)****	4 **(22.2%)**	2 **(11.1%)****	0	4 **(22.2%)**
ERαKO		**20**	0	1 **(5%)**	4 **(20%)**	14 **(70%)**	0	1 **(5%)**
ERWT	**Pure 7**	**19**	0	0	11 **(57.8%)****	5 **(26.3%)***	0	3 **(15.9%)**
ERαKO		**19**	0	0	1 **(5%)**	14 **(74%)**	1 **(5%)**	3 (**16%)**
ERαH	**Casein**	**20**	0	0	5 **(25%)**	10 **(50%)**	0	5 **(25%)**
	Crude 7	**21**	0	0	15 **(71.5%)***	2 **(9.5%)***	0	4 **(19%)**

Table III-3: Incidence of prostate tumorigenesis in TRAMP mice fed various botanical compounds for each tumor stage.

Male TRAMP mice were started on diets at weaning, and sacrificed at 5 months.

Pure 3 = Curcumin + Resveratrol + EGCG

Pure 4 = Apigenin + Baicalein + Genistein + Quercetin

Pure 7 = Pure 3 + Pure 4

Crude 7 = Soy + sencha leaves + tumeric + yucca roots + saw palmetto + chamomile flowers + gingko; H-mice were heterozygous for ER α.

χ^2 – test was performed for each treatment relative to control diet within each tumor stage. * p-value<0.05; **p-value<0.01.

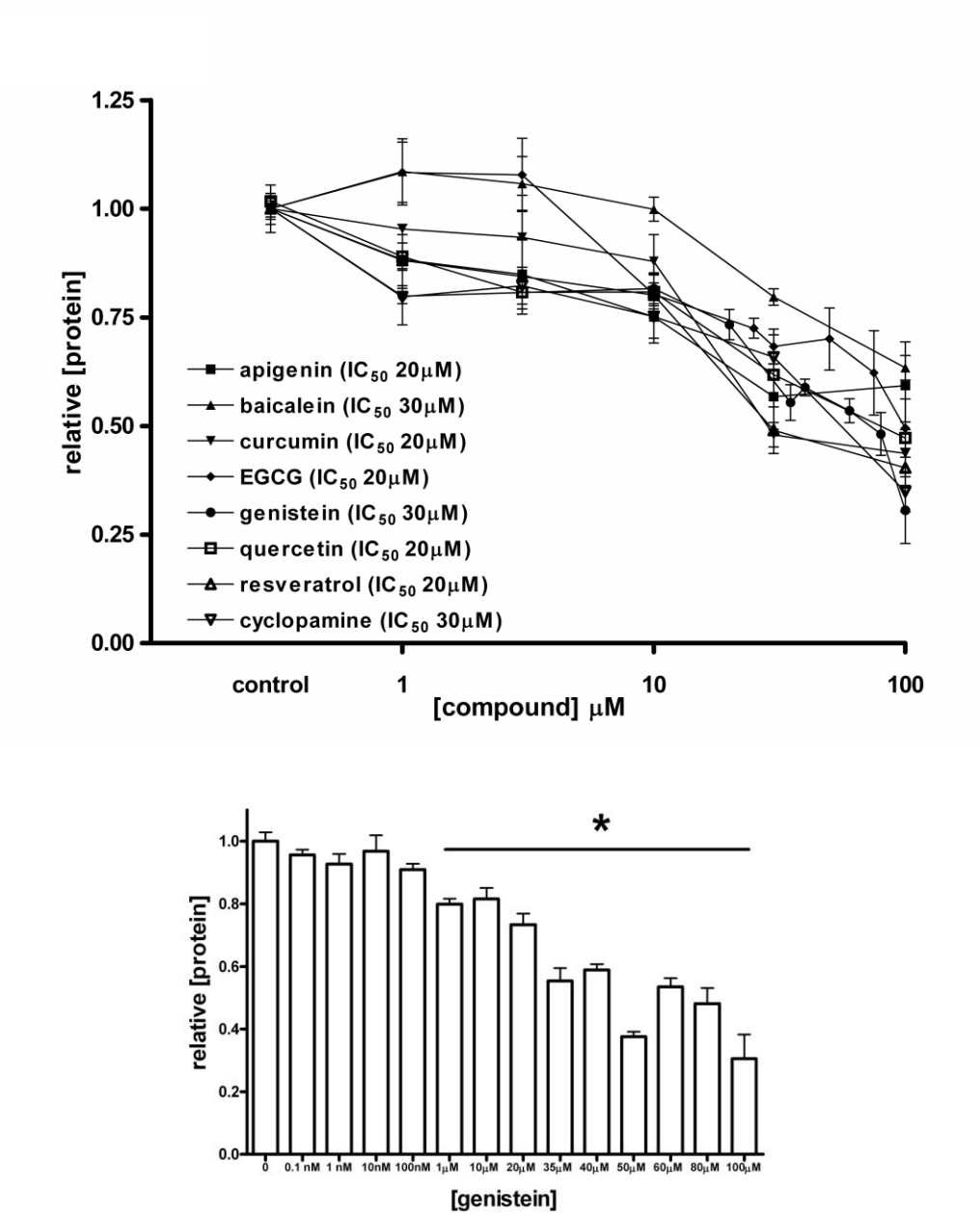


Figure III-3: Botanical compounds inhibit TRAMP-C2 cell growth.

Mouse prostate cancer cell growth was determined based on total protein concentration after a 72 hour treatment with each compound relative to control treatment. Compounds were used at concentrations ranging from 1μM to 100μM in half log increments, genistein was tested down to 100pM Each experiment was performed at least three times in duplicate

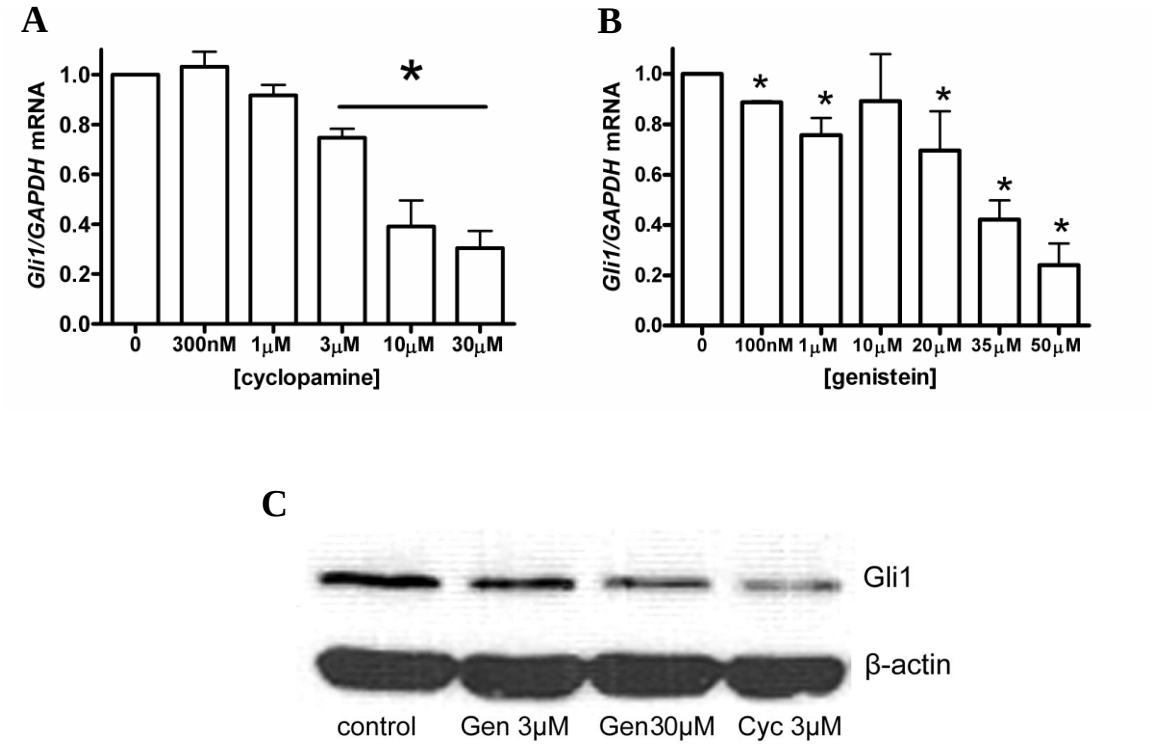


Figure III-4: Botanical compounds inhibit Hedgehog pathway activity in TRAMP-C2 cells. Relative *Gli1* mRNA concentrations after 72 hours of (A) cyclopamine and (B) genistein treatment as determined by real-time RT-PCR. *GAPDH* was used as control. (C) Gli1 protein decreases after 24 hours of treatment with cyclopamine and genistein (here: Biodesign antibody). T-test was performed to determine p-value. * $p<0.05$, error bars indicate SD.

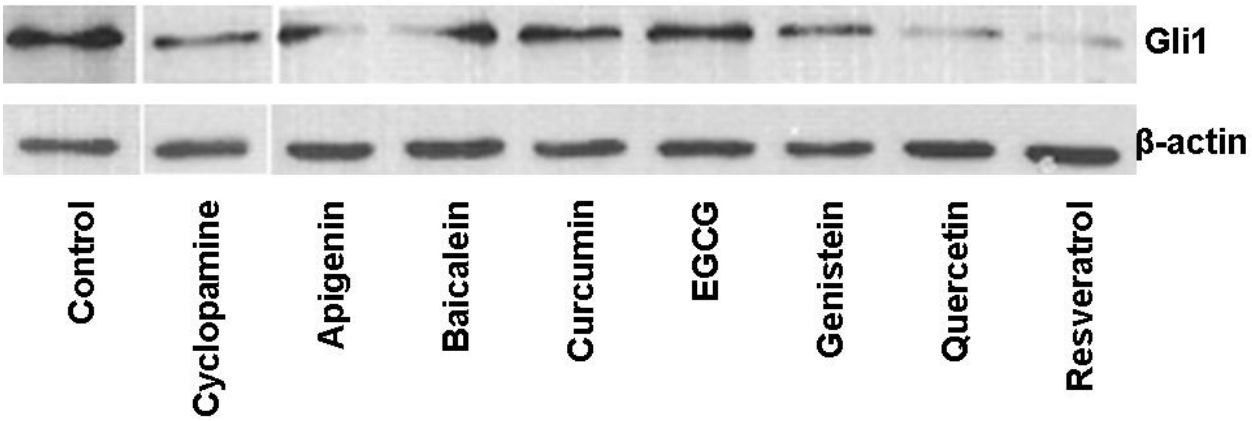


Figure III-5: Botanical compounds decrease Gli1 protein in TRAMP-C2 cells.

Representative western blot of TRAMP-C2 cell lysates analysed for Gli1 (here: Biodesign antibody) and β-actin after a 24 hour treatment with 3μ cyclopamine and 10μM of all other botanicals.

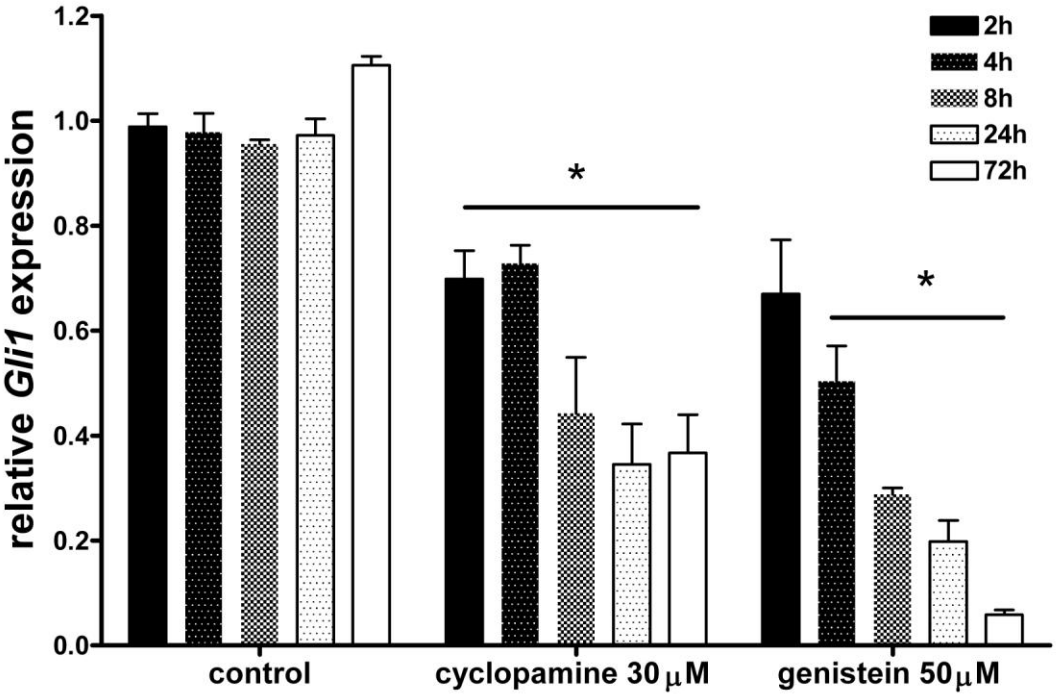


Figure III-6: *Gli1* mRNA in TRAMP-C2 cells by real-time RT-PCR after time course treatments with cyclopamine and genistein.

Sub-confluent TRAMP-C2 cells were treated with 50μM genistein or 30μM cyclopamine. RNA was isolated after 2, 4, 8, 24, and 72 hours. *Gli1* mRNA was measured relative to *GAPDH* mRNA. Each experiment was performed in triplicate. T-test was performed to determine p-value. * $p < 0.05$, error bars indicate SD.

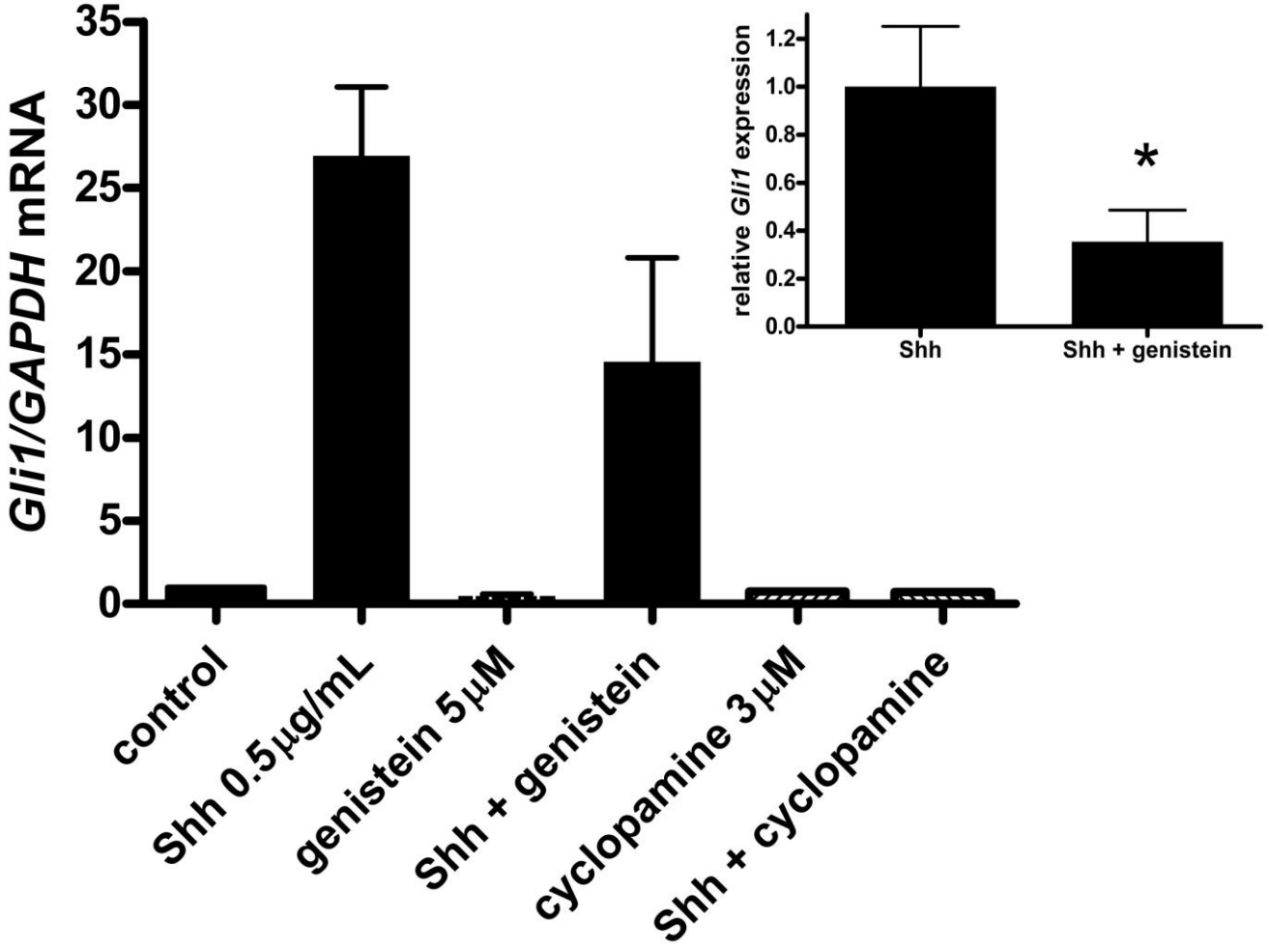


Figure III-7: Shh can stimulate *Gli1* mRNA concentrations in TRAMP-C2 cells, genistein can inhibit the stimulated *Gli1* expression.

TRAMP-C2 cells were treated with mouse recombinant 0.5μg/mL Shh for 24 hours. Co-treatment with 5μM genistein resulted in significant reduction of *Gli1* mRNA concentrations. Shh stimulation varied significantly between experiments. Insert shows genistein inhibition of Shh-stimulated *Gli1* expression normalized to 0.5μg/mL Shh-stimulated state within individual experiments, since the stimulation by Shh varied between 3-8 fold between experiments. T-test was performed to determine p-value. * $p<0.05$, error bars indicate SD.

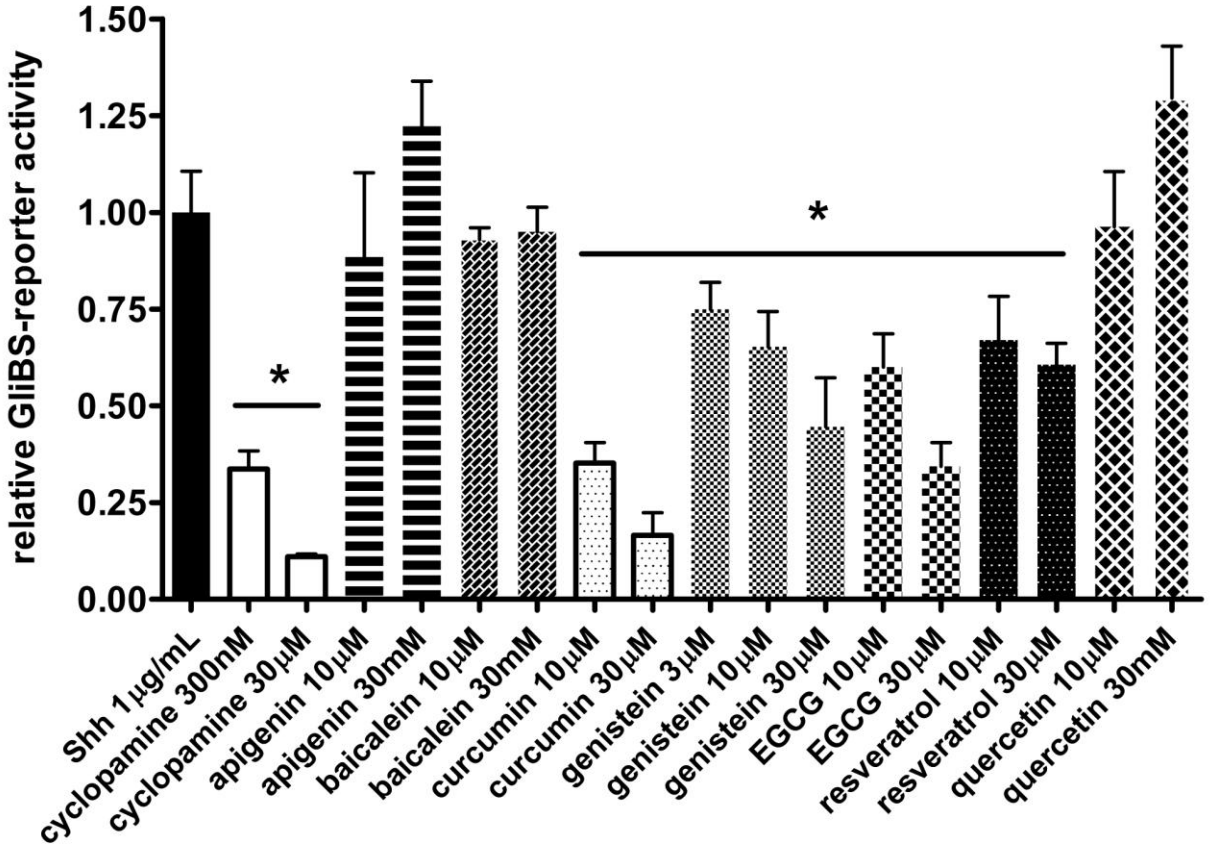


Figure III-8: Botanical compounds inhibit Shh-stimulated Gli-responsive promoter in Shh Light II cell line.

Shh Light II cells were treated with various compounds in full medium supplemented with 0.5% FBS in the presence of 1μg/mL Shh for 24 hours before cells were lysed and subjected to luciferase assay to assess the regulation of the Gli-responsive promoter. Each experiment was performed at least in triplicate. T-test was performed to determine p-value. * $p<0.05$, error bars indicate SD.

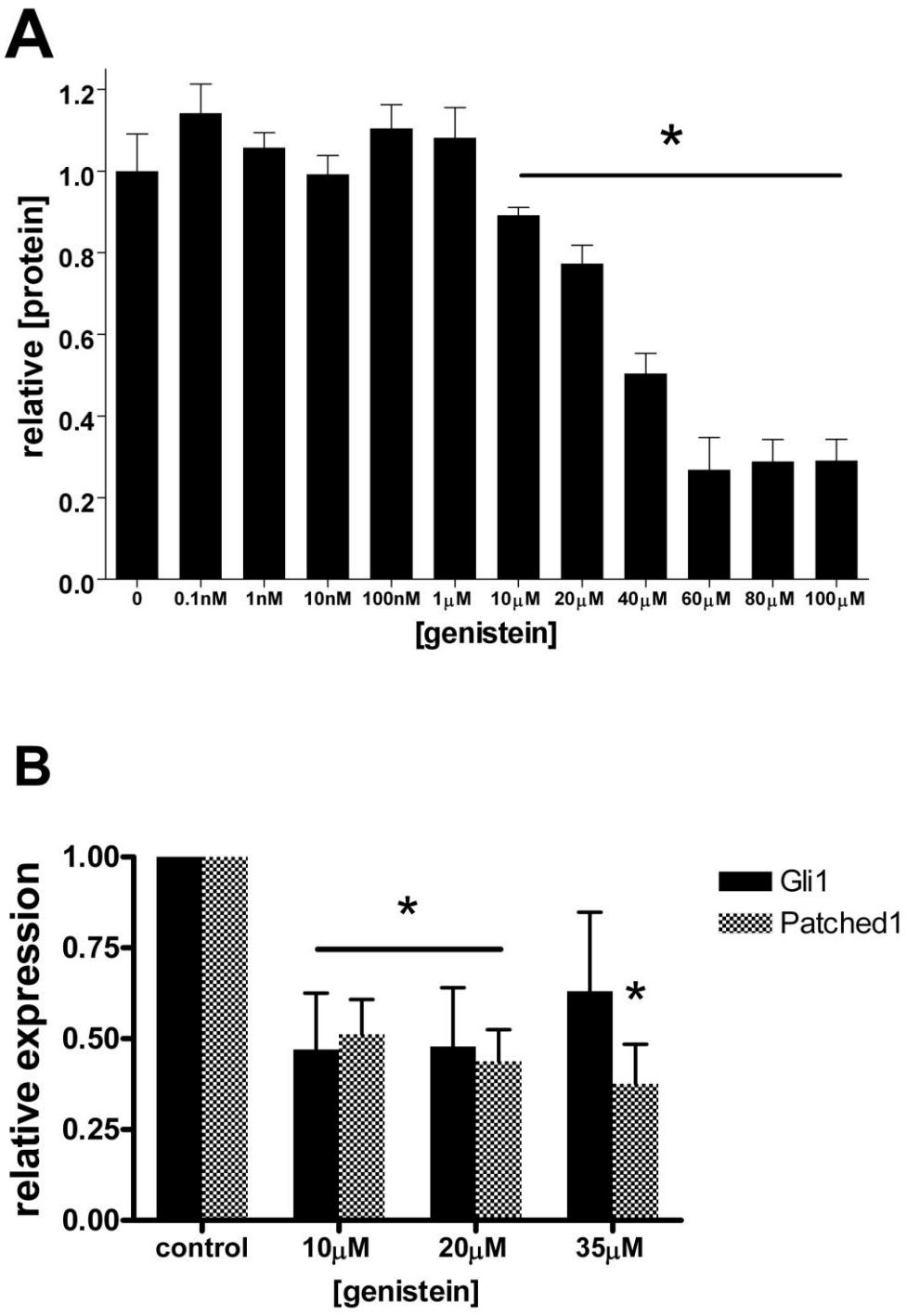


Figure III-9: Genistein inhibits cell growth and Hedgehog pathway activity in PC3 cells.

(A) Total protein after 72 hours genistein treatment. (B) *Gli1* and *Patched1* mRNA concentrations after 72 hours genistein treatment as determined by real-time RT-PCR. *GAPDH* mRNA was used as control. Each experiment was performed at least in triplicate. T-test was performed to determine p-value. * $p<0.05$, error bars indicate SD.

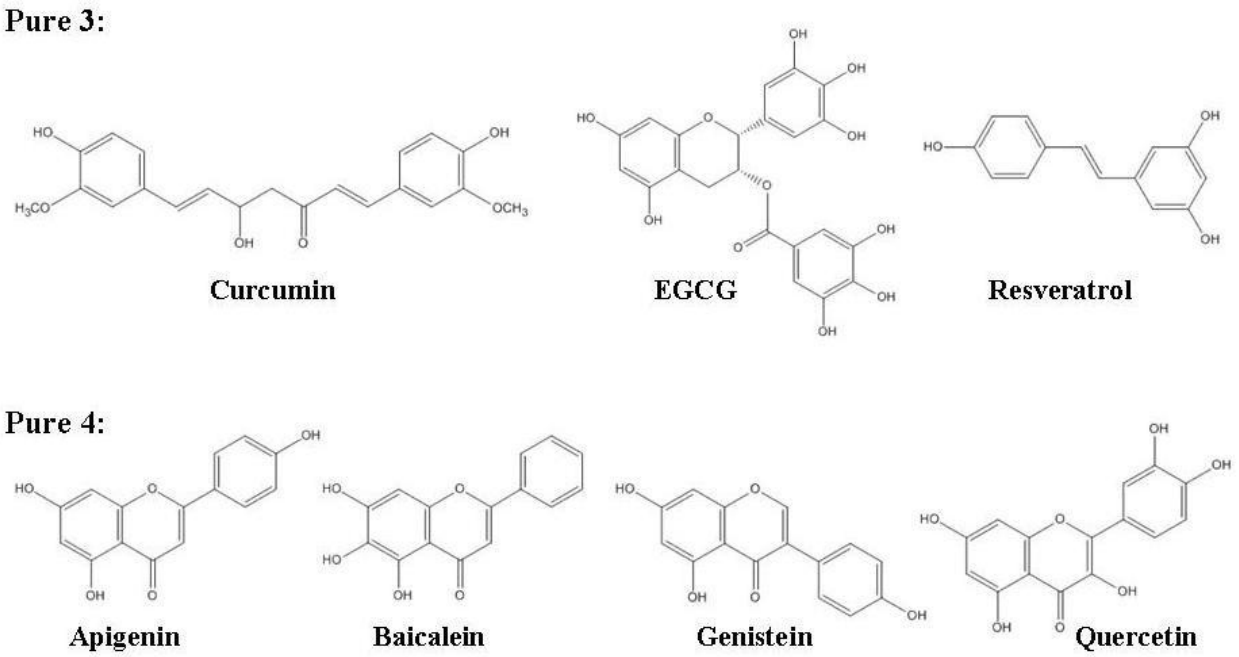


Figure III-10: Structures on botanical compounds used in mouse diets.

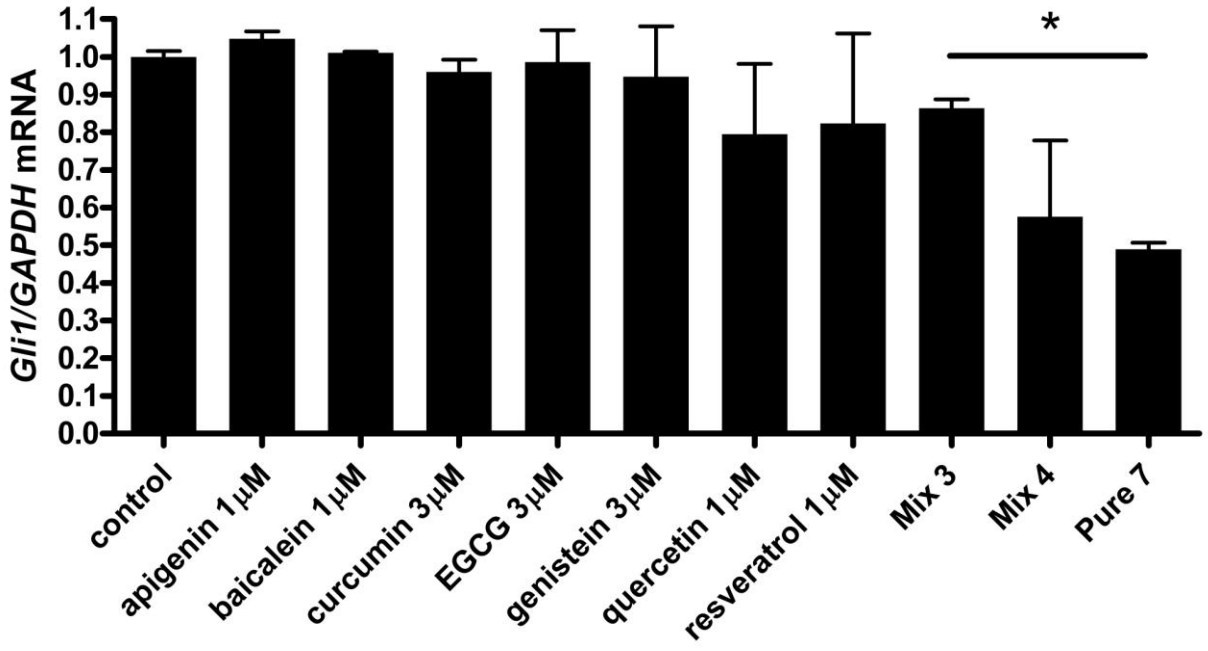


Figure III-11: Botanicals act synergistically to inhibit *Gli1* mRNA in TRAMP-C2 cells.

TRAMP-C2 cells were treated for 24 hours with selected botanical or mix of botanicals. Total RNA was assayed for relative *Gli1* mRNA concentration changes.

Mix 3 = Curcumin + Resveratrol + EGCG

Mix 4 = Apigenin + Baicalein + Genistein + Quercetin

Pure 7 = Pure 3 + Pure 4

Each experiment was performed twice in triplicate. * p-value<0.05 (T-test)

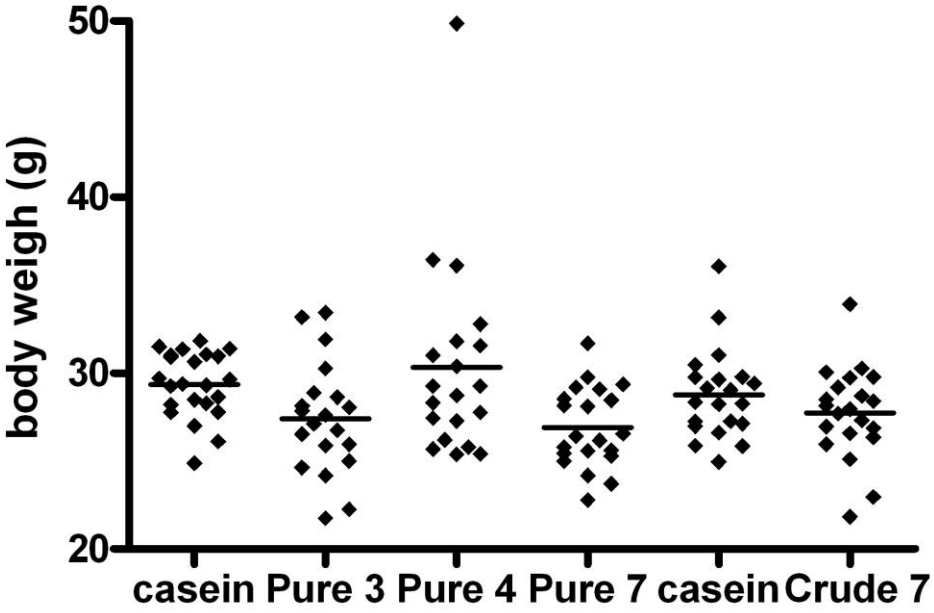


Figure III-12: Total body weights of mice at the end of study.

Pure 3 (p= 0.0164) and Pure 7 (p=0.0003) were statistically lower than casein control mice. No group mean varied more than 7% from total mean.

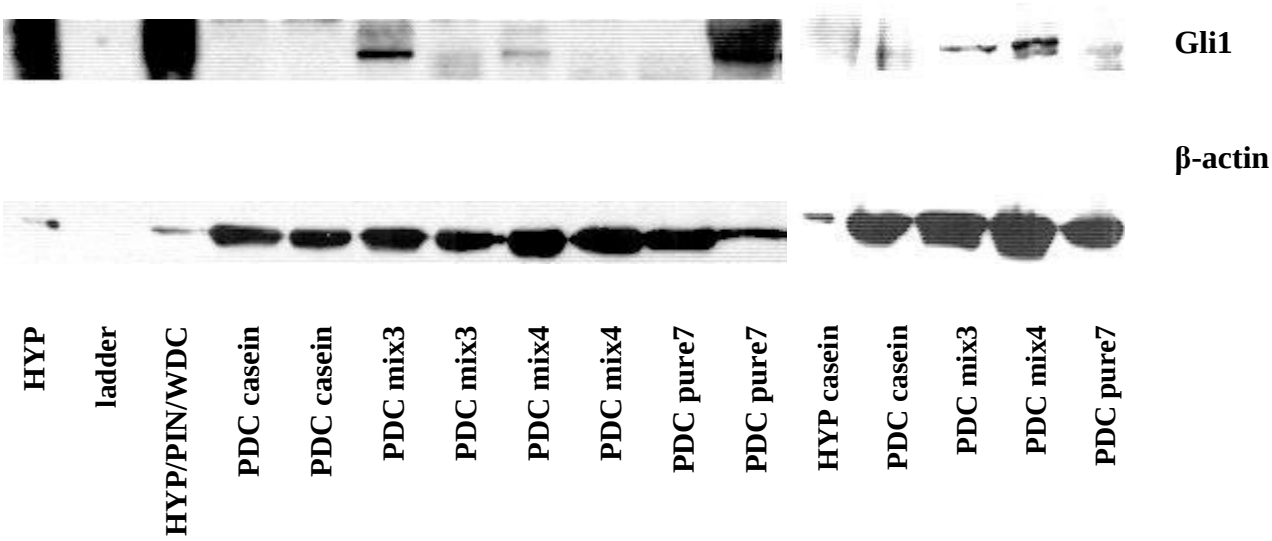


Figure III-13: Gli1 protein expression varies in prostates of TRAMP mice.

Total cellular protein from frozen mouse prostates were analyzed by western blot assays with primary antibodies to Gli1 (biodesign), and β-actin (Santa Cruz). Each well was loaded with 40μg protein.

IV - Crosstalk between Estrogen- and Hedgehog Signaling Pathways

Overview

The botanical compounds, that we were testing as described in chapter III and that were able to inhibit hedgehog signaling in prostate cancer cell lines and partially in Shh Light II cells, all display estrogenic actions. They were either reported to bind to the estrogen receptors and/or act as an agonist/antagonist in functional assays. I was thus interested to test our **hypothesis** that these compounds were acting through one or both of the estrogen receptors, and further test how classic estrogens and antiestrogens would affect the hedgehog pathway.

17β-estradiol was able to inhibit Gli1 mRNA and protein in TRAMP-C2 cells. It was also able to decrease Sonic Hedgehog- and Smoothened Agonist (SAG)-induced Gli-reporter activity in Shh Light II cells in a dose-dependent manner. Surprisingly, the pure ER antagonist ICI was also able to inhibit Gli1 mRNA in the TRAMP-C2 cells.

Further indications for estrogenic regulation of hedgehog signaling could be demonstrated through ERβ-binding oxysterols that were able to induce *Gli1* mRNA in TRAMP-C2 cells.

Differential inhibition of Shh- vs. SAG-induced Gli-reporter activity by 17β-estradiol and EGCG suggests a possible target for these at a step in the pathway between the Shh-binding Patched and SAG-binding Smoothened (see **Working Model Figure IV-10**).

Introduction

There have been a variety of publications suggesting some crosstalk between hedgehog-signaling and estrogen-signaling pathways. Estrogen influences hedgehog signaling in the thymus, where estrogen deficient Aromatase KO mice displayed impaired thymocyte development and displayed increased Smoothened expression (438). Estrogenization of neonatal rat prostates differentially altered expression of various hedgehog proteins in the pathway, specifically decreasing Shh, Patched, Gli1and 3 expression, which caused a lobe specific blockage of ductal branching in the dorsal and lateral prostate (281). One of the ligands, Indian Hedgehog, is target of PR, which itself is ER regulated (439-441).

Both, estrogen- and hedgehog-signaling pathways also share downstream targets, for example E-cadherin which is a target of the Hh pathway via Snail (442, 443), and is regulated by ER via MTA3 and Snail (443-445). However, regulation by estradiol was not observed in promoter studies with human sonic hedgehog (446).

A recent connection appeared when two laboratories reported the cholesterol metabolites oxysterols as novel activators of the Hedgehog signaling pathway (307, 411).
At the same time a different two laboratories published work on 27-Hydroxycholesterol, one of the oxysterols reported to activate hedgehog signaling, being an endogenous SERM (selective estrogen receptor modulator) (447, 448). These two oxysterol SERM papers report binding/response to ERs in the low micromolar range. In our lab, Nicholas Starkey and Sara Drenkhahn have found that 25- and 27-OHC in ^{3}H-estradiol competition binding assays, bind ERβ 100x better than ERα, with a K_d of ~ 20nM (data not shown).

Initially it was reported that oxysterols bind directly to Smoothened (307). Later, however, a different lab found that 20- and 22-OHC are probably not binding directly to Smoothened, or at least not to the same region as cyclopamine, since they were unable to displace BODIPY-cyclopamine from Smoothened as measure by a FRET assay (411).

We **hypothesize** that oxysterols are binding to the missing link between Patched and Smoothened. Since oxysterols do not bind directly to Smoothened, there must be another oxysterol-binding protein between Patched and Smoothened – we propose it to be ERβ.

Materials and Methods

Chemicals and constructs:

The purified compounds used in tissue culture experiments were obtained from the following suppliers: Apigenin (LC laboratories), Baicalein (Indofine Chemical Company, Hillsborough, NJ), Curcumin (Sigma), Cyclopamine (Toronto Research Chemicals and LC Laboratories), EGCG (Sigma), Genistein (Sigma), Quercetin (Sigma), Resveratrol (Sigma). Mouse recombinant Shh was obtained from R&D Systems (Minneapolis, MN) cat. # 464-SH. All compounds were dissolved in DMSO, except: LC Lab cyclopamine was dissolved in ethanol; Shh was dissolved in PBS with 0.1% BSA. In each experiment the controls and all the treatments contained all vehicles used. All treatments were conducted in phenol red-free medium with charcoal-stripped serum.

GliBS-reporter was obtained from the ATCC.

Cell culture

TRAMP-C2 and Shh Light II cells were obtained from the ATCC (www.atcc.org).

HEK293 stably transfected with N-Shh were a kind gift from Dr. Beachy, HHMI, Stanford University.

TRAMP-C2 cells were maintained in RPMI 1640 RPMI 1640 media supplemented to contain 10% fetal bovine serum (U.S. Bio-Technologies, Parkerford, PA), 4.5g/mL Glucose, 4mM L-glutamine , 100μM Non-essential Amino Acids, 10mM HEPES, 1mM Sodium Pyruvate, and 1% Penicillin/ Streptomycin (all from Invitrogen, Carlsbad, CA).

Shh Light II cells (JHU-68) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% NBCS, 0.4mg/mL G418, 0.15mg/mL zeocin, 4mM L-glutamine, HEPES, and adjusted to contain 1.5g/L sodium pyruvate. This mouse embryonal NIH 3T3 cell line contains a stably transfected luciferase reporter with 8 copies of the consensus Gli binding site derived from the mouse Hepatocyte Nuclear Factor-3β (JHU-73 pGL3B/8XgliBS-lc-luc 5'-GAACACCCA-3') (395). The responsiveness of these cells to hedgehog activators and inhibitors is very sensitive. In order to maintain the ability to respond the cells need to be subcultured between 70%-80% confluency in a ratio not higher than 1:8. 50K and 100K cells/well were then seeded in 24-well plates and incubated for 72 hours in a pre-treatment medium (DMEM supplemented with maintenance medium w/o G418 or zeocin). At the time of treatment all of the cells are super-confluent, often forming multi-layered clusters. Cells are treated for 48 hours with selected compound in treatment medium (phenol-red free DMEM supplemented with 10% charcoal-stripped NBCS, HEPES, L-glutamine, and NaPyruvate) GliBS-reporter activity was assayed using the Dual Luciferase System, Promega.

HEK 293 N-Shh cells were maintained in DMEM, supplemented with 10% FBS, Pen/Strep, and G418 (Geneticin) 400 mg/ml. For preparation of conditioned medium, cells were seeded in 150cm^2 flasks and grown to ~80-90% confluency. Cells were washed with unsupplemented DMEM and supplemented with 35mL DMEM with 2% FBS, P/S, but *NO* geneticin. Cells were maintained for another 24-30 hours, medium was collected and passed through a 0.22μM filter. Conditioned medium was stored at -80°C for up to 6 months.

RNA isolation and RT-PCR:

Total RNA was isolated from the TRAMP-C2 cells using the RNeasy kit from QIAGEN. From 1-5x 10^6 cells we obtained between 25-35μg total RNA. RNA concentration was determined using the ND 1000 Spectrophotometer v3.1, NanoDrop Technologies. 700ng of RNA was used in each RT-PCR reaction. RT-PCR was performed using the SSIII one-step RT-PCR system from Invitrogen. The following primers were used: mouse GAPDH: forward 5'-cagagacggccgcatctt-3', reverse 5'-ccgaccttcaccattttgtctac-3'. mouse ERα: (NM007956 coding redion 183-1982, 599aa) forward 576: 5'-gtgccctactacctggagaacgag-3', reverse 793: 5'-cagaccccataatggtagccagag-3' (exon2-3, product size 128bp) and forward 1208: 5'-tgaagcctcaatgatgggcttatt-3', reverse 1665: 5'-ccatcaggtggatcaaagtgtctg-3' (exon 5-8, product size 458bp); mouse ERβ: (NM 010157, coding region 348-1997, 549aa and NM 207707, coding region 348-2051, 567aa) forward 1464: 5'-ctcatctttgctccagacctcgtt-3', reverse 1736: 5'-actcttcgaaatcacccagaccag-3' (exon 5-8 ± 6, product size 273bp for NM 010157 and 327bp for NM 207707) and forward 1669: 5'-gtagccggaagctgacacacctat-3', reverse 1959: 5'-tactgtcctctgtcgagcagcact-3' (exon 8-9, product size 291) Mouse uterus was used as positive control for ERα expression, mouse ovary for ERβ. The following program was used for the

reverse transcription and amplification: 55˚C for 30 min., 94˚C for 2 min., followed by 25-40 cycles of 94˚C for 15 sec., 55˚C for 30 sec., 68˚C for 1 min., followed by the final extension step of 68˚C for 5 min. RT-PCR products were visualized on a 1% agarose gel with ethidium bromide staining and UV detection.

Transfection assay

GliBS-reporter (JHU-73) was obtained from the ATCC and independently from Dr. Reiter's laboratory, UCSF. 2 clones from each construct, grown up in our lab, were used to confirm the results. TRAMP-C2 cells were seeded in 24-well plates in phenol-red free full medium supplemended with 10% charcoal-stripped FBS. Cells were transfected at 60-80% confluency with 500ng of GliBS-reporter, 10ng renilla, and 100ng GliBS-luciferase reporter or control vector. Cells were treated 24 hours after transfection, and assayed for luciferase and renilla activity after another 24 hours using the Dual Luciferase Assay from Promega.

For real-time RT-PCR, western blot and luciferase reporter assays see chapter III.

Results

TRAMP-C2 cells express both estrogen receptors.

TRAMP-C2 cells express both, ERα and ERβ, as determined by RT-PCR and western blot **(Figure IV-1)**, and are therefore an appropriate model to study the effects of botanical and estrogenic compounds as mediated by estrogen receptors.

Oxysterols stimulate *Gli1* mRNA expression in TRAMP-C2 cells.

20-, 22-, 25-, and 27-OHC are able to significantly increase *Gli1* mRNA concentration in TRAMP-C2 cells after 24 hour treatments, comparable to the N-terminal Shh peptide and the Smoothened agonist SAG **(Figure IV-3)**. This 2-8 fold stimulation of *Gli1* obtained with 10μM of each compound in our mouse epithelial cell line is comparable to the ~10 fold stimulation by a mix of 5μM 20- and 22OHC reported in a pluripotent mesenchymal cell line – M2 (411). Genistein is able to inhibit the 25-, and 27OHC-induced stimulation of *Gli1* mRNA **(Figure IV-4)**.

Estrogen and antiestrogens inhibit *Gli1* mRNA expression in TRAMP-C2 cells.

To test whether the observed inhibition of *Gli1* mRNA was possible through one of the estrogen receptors, we tested 17β-etradiol, which is binding and acting as an agonist with both estrogen receptors at comparable concentrations. 10nm E2 was able to significantly inhibit both *Gli1* mRNA and Gli1 protein expression in TRAMP-C2 cells **(Figure IV-5 and IV-6).**

In order to establish whether genistein exhibits its inhibitory action on the Hedgehog pathway via the estrogen receptor. I used the pure antiestrogen ICI to block both ERs and hopefully prevent genistein from inhibiting *Gli1* mRNA. Surprisingly, at a high dose (1μM) ICI itself was able to significantly inhibit Gli1 mRNA, and has an additive effect in co-treatment with genistein. Also a lower dose of 100nM ICI which did not inhibit by itself was acting additive with genistein **(Figure IV-5)**.

To determine which ER receptor is responsible for this inhibitory effect, I used the ERα specific agonist PPT, and the ERβ specific agonist DPN. At 10nM both compounds showed high variance in inhibitory actions, with PPT inhibiting significantly, and DPN having a not quite significant effect **(Figure IV-5)**.

GliBS reporter experiments yield surprising but suggestive results.

To confirm the effects of the estrogens and phytoestrogens on the mRNA modulation of the hedgehog pathway TRAMP-C2 cells were transfected with a Gli Binding Site-luciferase reporter. Surprisingly, I was not able to stimulate this Gli reporter with either overexpression of mouse Gli1 or the Sonic Hedgehog ligand **(Figure IV-7)**. Also cyclopamine, or any of the phytoestrogens, were not able to inhibit the Gli reporter, even though they were able to inhibit *Gli1* mRNA **(Table III-1 and Figure III-2)** and Gli1 protein **(Figure III-3)** in the TRAMP-C2 cells. The only significant effects were obtained with 25- and 27-OHC, and those were again opposite to what I have seen in real-time RT-PCR assays, since both compounds were inhibiting the reporter by ~50% **(Figure IV-7)**. It is possible that the Gli1 protein, which is significantly higher expressed in TRAMP-C2 that it is, even in stimulated Shh Light II cells (western blot data not shown), is at its maximum. The less stable *Gli1* mRNA is more responsive to both inhibition and stimulation, but the protein is not.

In Shh Light II cells, which are expressing both estrogen receptors as determined by RT-PCR **(Figure IV-1)**, 17β-etradiol had no effects on the baseline reporter activity (data not shown), but had significant inhibitory effects on both, SAG- and Shh-conditioned medium (CM)-stimulated GliBS-reporter activity, starting at 100pM **(Figure IV-8)**.

When I tested the seven botanicals compounds from chapter III under the new stimulatory conditions (see materials and methods) with SAG and Shh-conditined medium used to stimulate the reporter, only baicalein, curcumin, and resveratrol were able to inhibit both Shh-conditioned medium- and SAG-induced GliBS-reporter activity. Apigenin had no effect with SAG, and a stimulatory effect with CM. EGCG had a stimulatory effect with SAG, but strongly inhibited CM. Genistein had a greatly variable response depending on cell number (to be discussed later), but had overall no effect on pathway activity in this assay. Quercetin was slightly inhibitory with SAG, but had no effect with CM **(Figure IV-9)**.

Discussion:

We were able to confirm our hypothesis that estrogens and phytoestrogens are able to inhibit the hedgehog signaling pathway. We demonstrated that selected oxysterols which can bind ER could stimulate *Gli1* mRNA expression in our mouse prostate cancer cell line **(Figure IV-3)**, and that the observed stimulation was inhibitable by genistein **(Figure IV-4)**. And since the cells also express both estrogen receptors **(Figure IV-1)**, they offer a great tool to study estrogen's impact on the hedgehog pathway.

All estrogens used, the pure agonist 17β-etradiol, the pure antagonist ICI 182,780, as well as the ERα specific agonist PPT, and the ERβ specific agonist DPN were able to inhibit *Gli1* mRNA in TRAMP-C2 cells **(Figure IV-5)**. Estradiol was also able to inhibit Gli1 protein in TRAMP-C2 **(Figure IV-6)** and Gli-reporter activity in Shh Light II cells **(Figure IV-8)**. This offers strong evidence for estrogenic regulation of hedgehog signaling and Gli1 expression. It is

thus possible that the phytoestrogens could be also working though the estrogen receptors to elicit its effect on the pathway.

Treatments with all seven botanicals were tried under a variety of conditions. In previous experiments with Shh Light II cells we were not able to obtain consistent stimulation with the N-terminal Shh peptide, with stimulation varying between 3 and 8 fold between experiments. There were reports however, specifically from Dr. Beachy's lab that reported 50-100 fold stimulation in the reporter activity. After adjusting our protocol (see materials and methods) we were able to obtain 20-40 fold stimulations with SAG and Shh-conditioned medium, however still not with the Shh peptide. Because of the variability and expense, we have stopped using the peptide.

With these new conditions, the seven phytoestrogens did partially confirm previous results with the purified N-terminal Shh-peptide **(Figure III-7 and IV-9)**. Apigenin had no or slightly stimulatory effects like previously observed, baicalein was now inhibitory with both SAG and CM, curcumin and resveratrol did inhibit, like they did with Shh, quercetin had no or slightly inhibitory effects like previously observed. Genistein had no significant effect, when I combined all results, which was due to significant differences between experiments, mainly we believe because slight differences in cell number caused significant changes in other confluency dependent pathways crosstalking with hedgehog signaling. Even though the Shh Light II cells were super-confluent at the time of treatment, and were incubated with the compounds for another 48 hours, a two-fold change in starting cell number made all the difference between 60% inhibition to 50% stimulation by genistein. The differences in variability might be suggesting different mechanisms these compounds might be working through.

EGCG was the most surprising compound with a very stable response regardless of cell density it had a stimulatory effect with SAG and a strongly inhibitory with Shh-conditioned medium. This differential response might shed light on its mechanism of action, specifically on the location of the estrogen receptor - if it is acting through ER. Shh and Shh-conditioned medium presumably too are acting directly through the Patched membrane receptor. SAG however is activating the pathway through direct binding to Smoothened, which is downstream of Patched. Thus the point of action of EGCG would be in between those two, potentially through a membrane associated estrogen receptor **(Figure IV-10)**.

We saw this discrepancy in inhibition pattern between CM- and SAG-stimulated activity again with high doses (1 and 10μM) of 17β-estradiol **(Figure IV-8)**. The affinities for E2 in the membrane associated ER should not differ from the nuclear receptor. For the reported isoforms of ERβ, ERβ1, 2, and 5 are all expressed in the prostate and prostate cancer cells lines. ERβ5 has the lowest reported K_d of ~25nM, and for ERβ2, which has additional 18 amino acids in the ligand binding domain binding has not been determined (344). A heterodimer between different isoforms might explain the discrepancy between binding affinities and responses observed. A previous graduate student in the Lubahn lab, Dr. Xiaohui Yuan, has found that ERβ binding affinities can differ as much as 50 fold between cell types. We now believe that these differences might be explained by different concentrations of oxysterols present in the different cell types, a hypothesis that is part of Sara Drenkhahn's dissertation.

It was also surprising to see the ER antagonist ICI inhibit the hedgehog pathway just like estradiol did **(Figure IV-5)**. However, in data from TRAMP-C2 and PC3M cells, in which neither E2 nor ICI alone, but a combination of both was able to inhibit prostate cancer cell growth **(Figure II-5)**, we have reason to speculate that the effects of these are mediated by a membrane ER. Ellis Levin has published data suggesting that ERα and ERβ work as a heterodimer in the membrane (449)**.** Thus perhaps ICI binds to one half of the heterodimer and estradiol bind to the other.

ICI has been reported to bind and target nuclear ER for proteosomal degradation (22), but not membrane ER (Seminar S17-1 by Dr. PE Micevych at ENDO 2009) (450). Unpublished observations from Dr. Hannink's lab suggested that ICI can also target ER to the plasma membrane. Thus it is possible that the synergistic effect of E2 with ICI could be explained through a two-step mechanism, in which ICI targets ER to the membrane, or enriches the membrane ER pool, and then E2 and the other botanicals can act upon it.

A membrane ER, positioned between Patched and Smoothened, as the EGCG and high dose E2 data suggests, could be the missing link in explaining how Patched is able to inhibit Smoothened. The lack of protective effects of genistein in TRAMP mice in the absence of either receptor, as described in chapter II, is consistent with the idea that the membrane ER would have to function as an ERα/ERβ heterodimer **(Figure IV-10)**. We also cannot exclude the involvement of other estrogen binding proteins like the G-protein coupled receptor 30 (GPR30), which has been reported to bind E2 and phytoestrogens in the micromolar range (451). Also, ICI will act as an agonist with GPR30 (452), or when the ER receptors are tethered to the AP-1 (453-455), Sp1 (456, 457), and STAT5 (458) transcription factors in the nucleus.(459).

Obviously, many additional questions have arisen that need following up in order to more clearly understand estrogen- and hedgehog-signaling crosstalk. Some of those questions will be discussed in the Future Studies, chapter V.

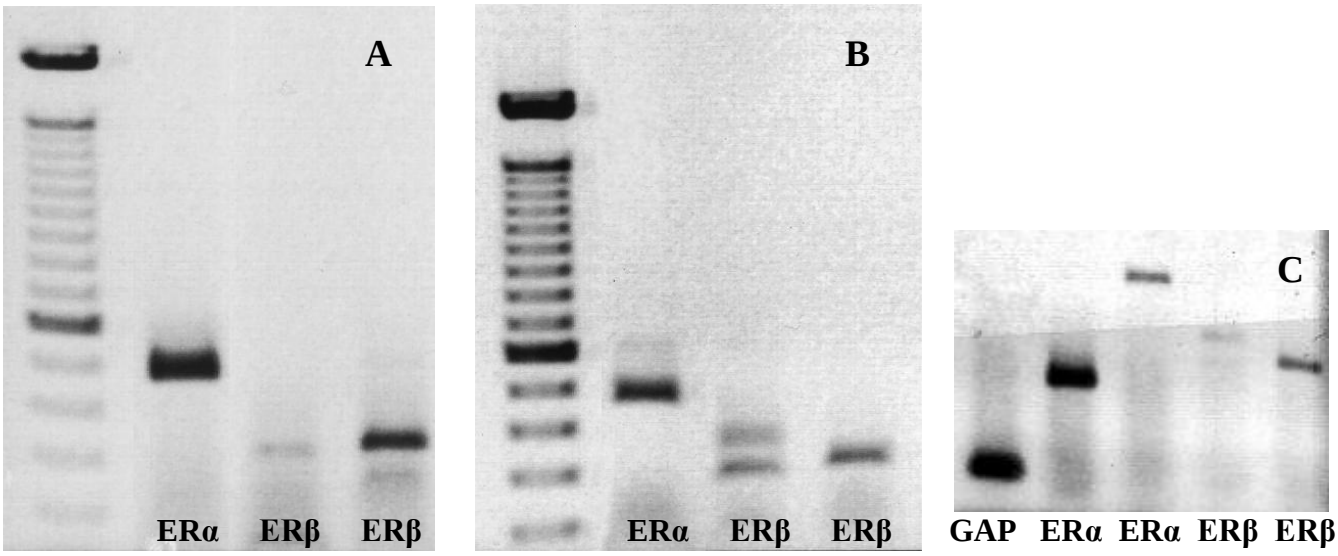


Figure IV-I: TRAMP-C2 and SHH Light II cells express both estrogen receptors.

A *ERα* and *ERβ* mRNA in TRAMP-C2; **B** positive control: *ERα* mRNA in mouse uterus; *ERβ* mRNA in mouse ovary. ERα primers span exons 5-8, ERβ primers span exons 5-8 and 8-9. **C** Shh Light II cells express *ERα* mRNA, and low concentration of *ERβ* mRNA.

ERα's presence could be determined using primers spanning exons 2-3 (shown for Shh Light II only) and 5-8, and that of ERβ using primers spanning exons 5-8 and 8-9. Using the primer set spanning exons 5-8 we found that only one isoform of ERβ is present in the TRAMP-C2 cell line (NM 010157) which is missing exon 6, whereas 2 isoforms are present in the mouse ovary (NM 010157 and NM 207707).

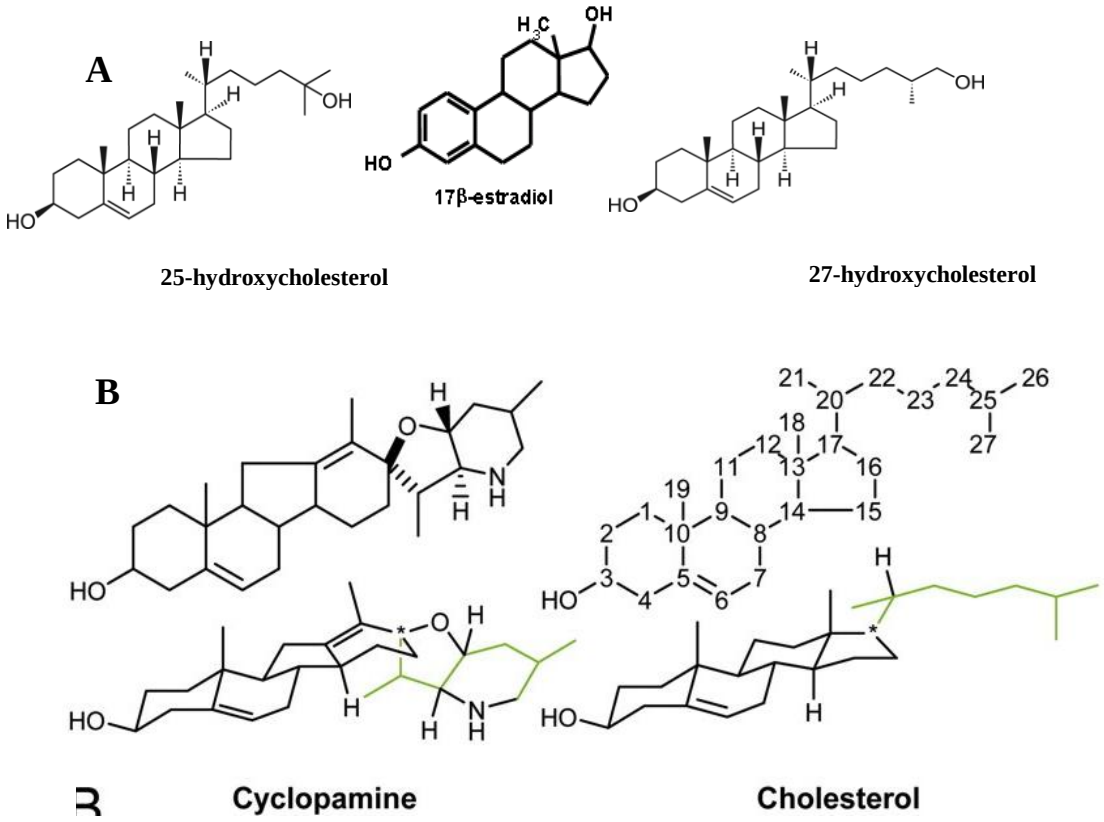


Figure IV-2: Oxysterols as novel hedgehog pathway stimulators (307)

Structures of 17β-estratiol, the newly described SERMs - 25- and 27-OHC (A), cyclopamine and cholesterol (B). This paper compared the structures of hedgehog-activating oxysterols to cyclopamine, suggesting that they would both bind Smoothened, and thus regulate the pathway. A different lab shortly thereafter demonstrated, that 20-, and 22-OHC were unable to displace cyclopamine from its binding site on Smoothened (411).

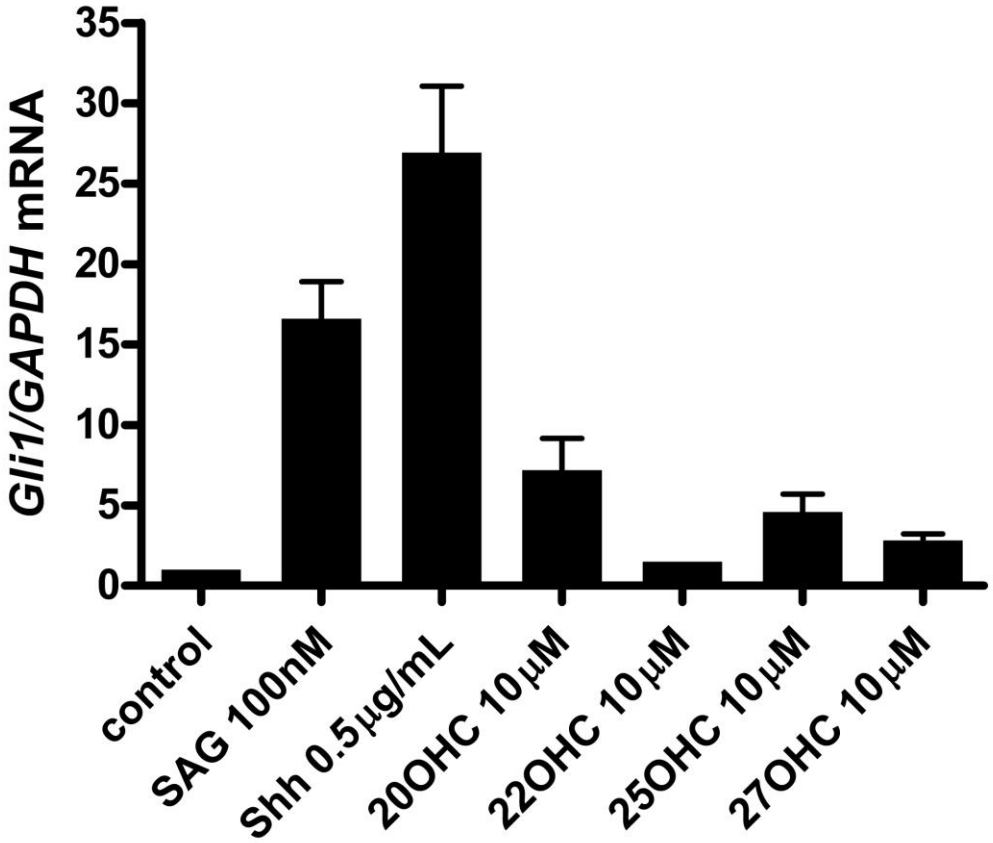


Figure IV-3: Selected oxysterols stimulate *Gli1* mRNA in TRAMP-C2 cells.

20-, 22-, 25-, and 27-OHC are able to stimulate Gli1 mRNA concentrations in TRAMP-C2 cells after a 24 hour treatment, as did the control compounds SAG and Shh. All treatments resulted in statistically significant stimulation.

Each experiment was performed at least in triplicate.

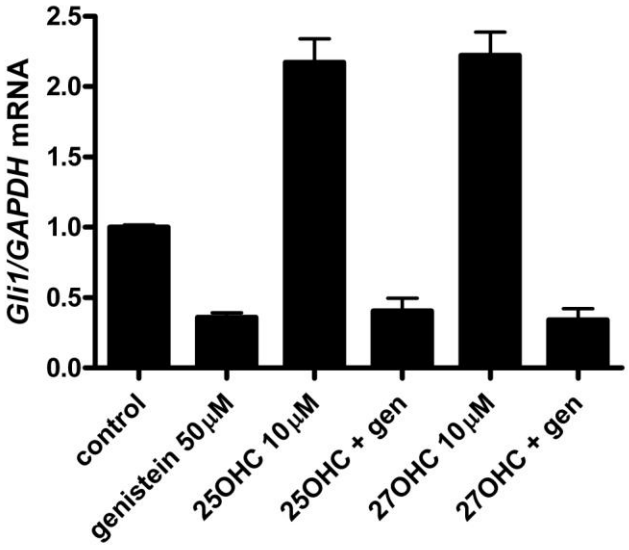


Figure IV-4: Genistein inhibits stimulation by 25-, and 27OHC in TRAMP-C2 cells.

TRAMP-C2 cells were treated for 24 hours with oxysterols +/- 50μM genistein. Both oxysterols are significantly stimulating *Gli1* mRNA, and genistein is able to significantly inhibit, both the basal and stimulated state.

Each experiment was performed at least in triplicate.

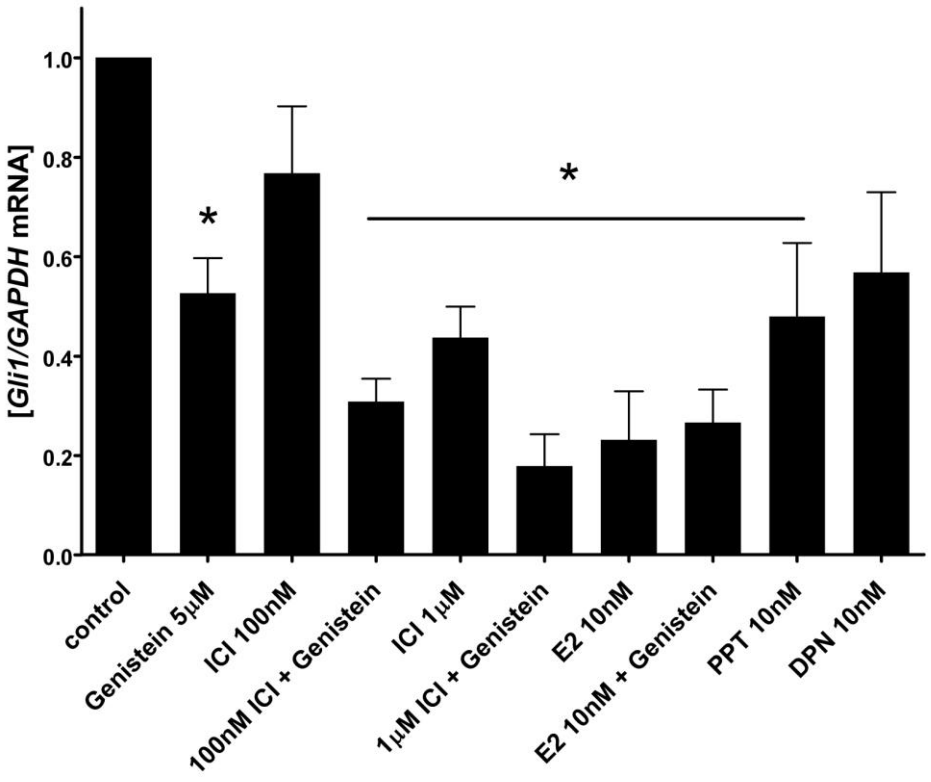


Figure IV-5: Estrogens and antiestrogens inhibit *Gli1* mRNA in TRAMP-C2 cells.

TRAMP-C2 cells were treated for 24 hours with selected compounds. * $p<0.05$, t-test.

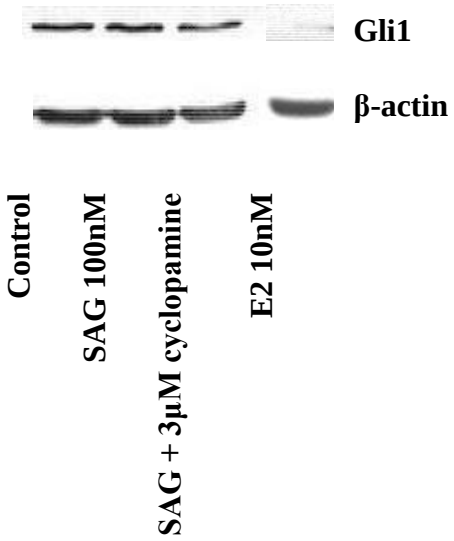


Figure IV-6: E2 inhibits Gli1 protein in TRAMP-C2 cells.

Representative western blot showing TRAMP-C2 cells treated for 24 hours with vehicle, SAG +/- cyclopamine and 10nM 17β-estradiol. E2 was able to significantly reduce Gli1 expression.

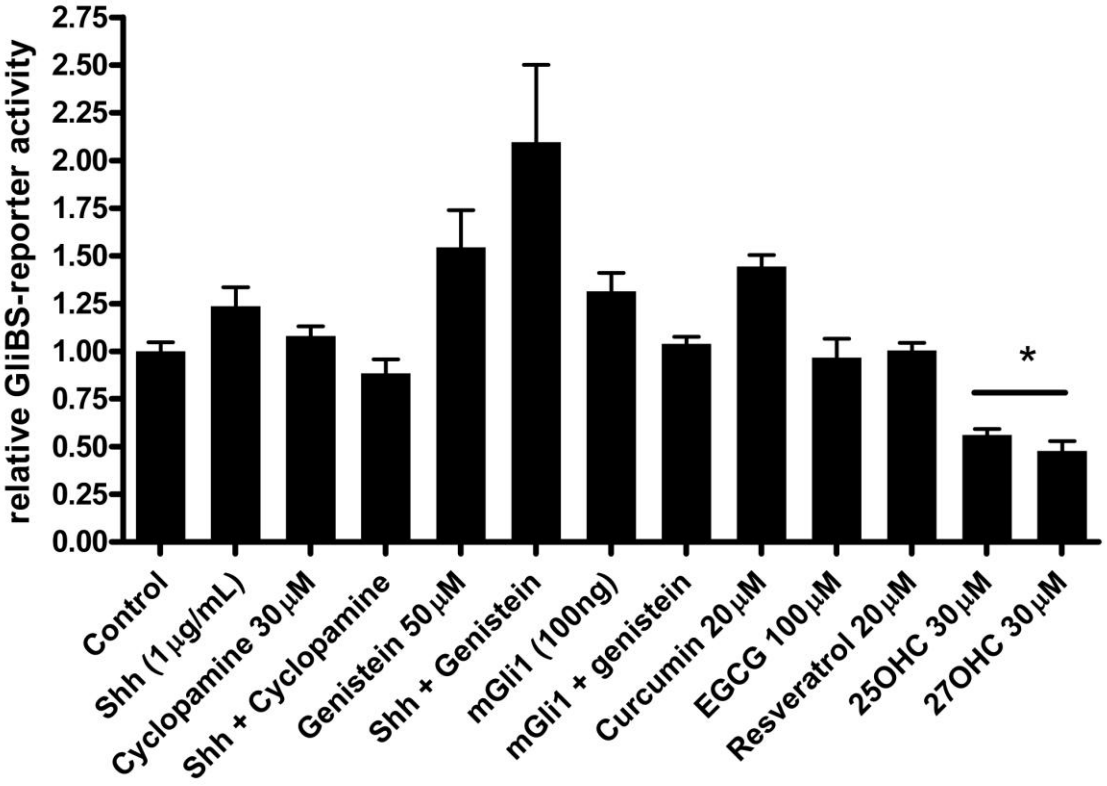


Figure IV-7: GliBS-reporter in TRAMP-C2 cells.

TRAMP-C2 cells were transfected with GliBS-luciferase reporter and treated with various compounds. Surprisingly, the only compounds having significant effects on Gli activity were 25- and 27OHC, and those were both inhibiting. * $p<0.05$, t-test.

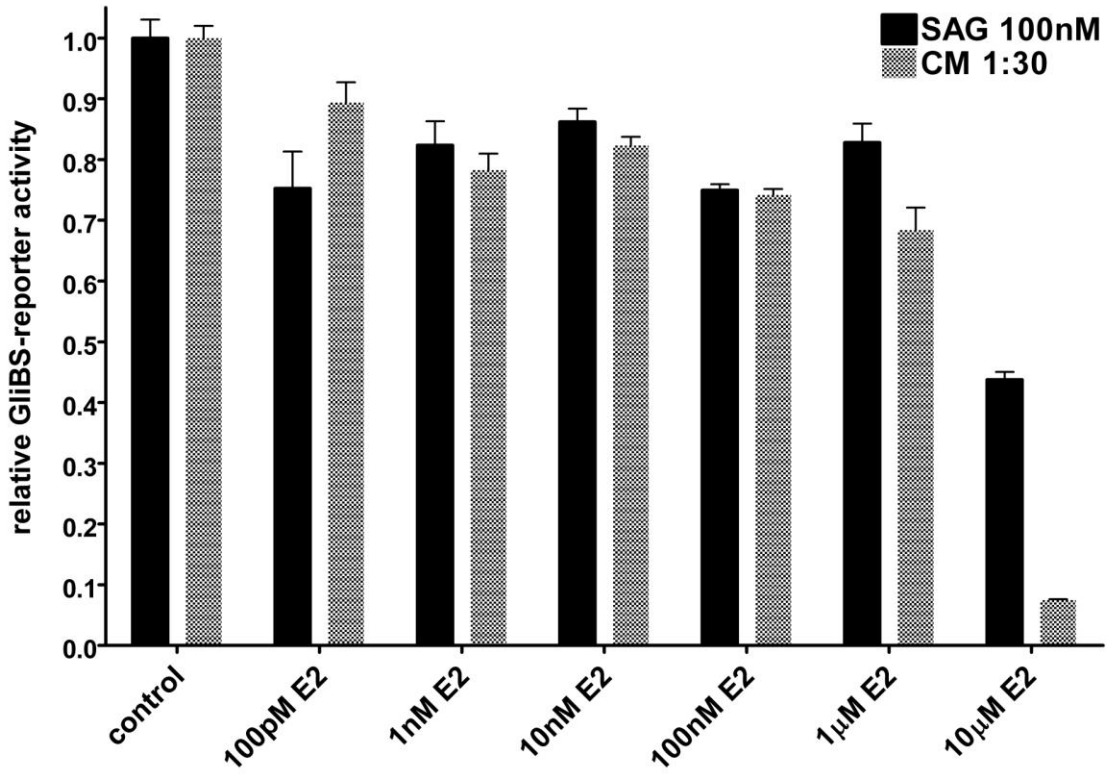


Figure IV-8: 17β-etradiol is significantly inhibiting SAG- and CM-stimulated Gli-reporter activity in Shh Light II cells.

Shh Light II cells were treated for 48 hours with 100pM to 10μM E2 +/- SAG or Shh-conditioned medium. E2 had no effects on baseline (data not shown) but did significantly inhibit stimulated GliBS-reporter activity. All treatments $p < 0.05$, t-test.

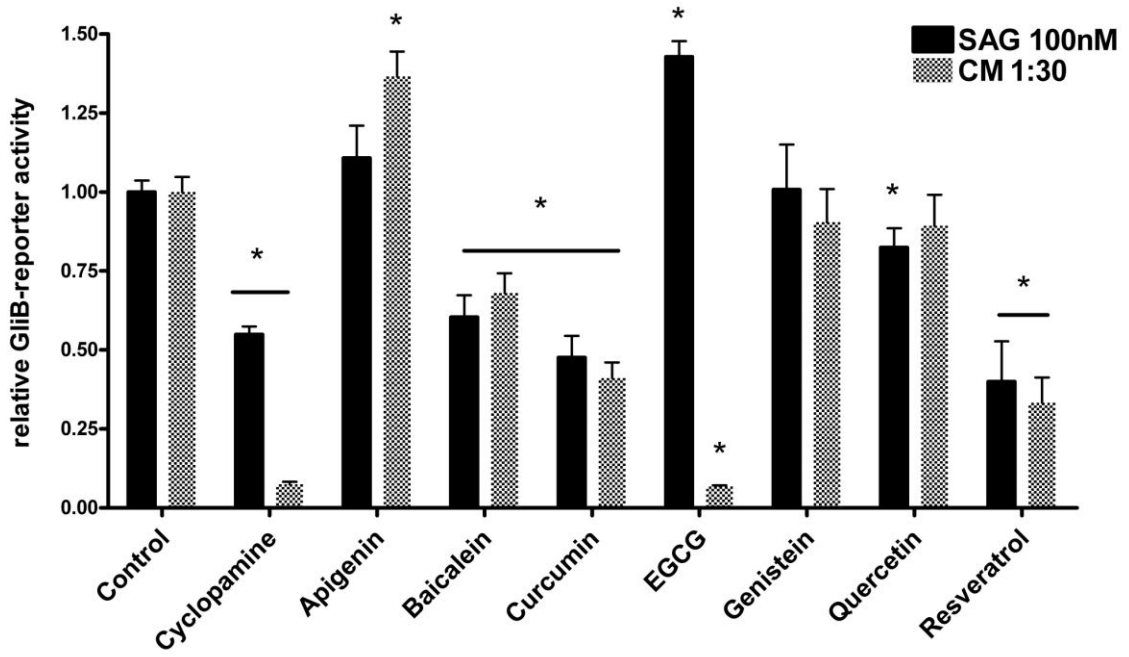


Figure IV-9: Botanical compounds with SAG and conditioned medium in Shh Light II cells.

Each compound was tested at 10μM, except cyclopamine which was used at 1μM.

* p-value < 0.05, t-test

Working Model

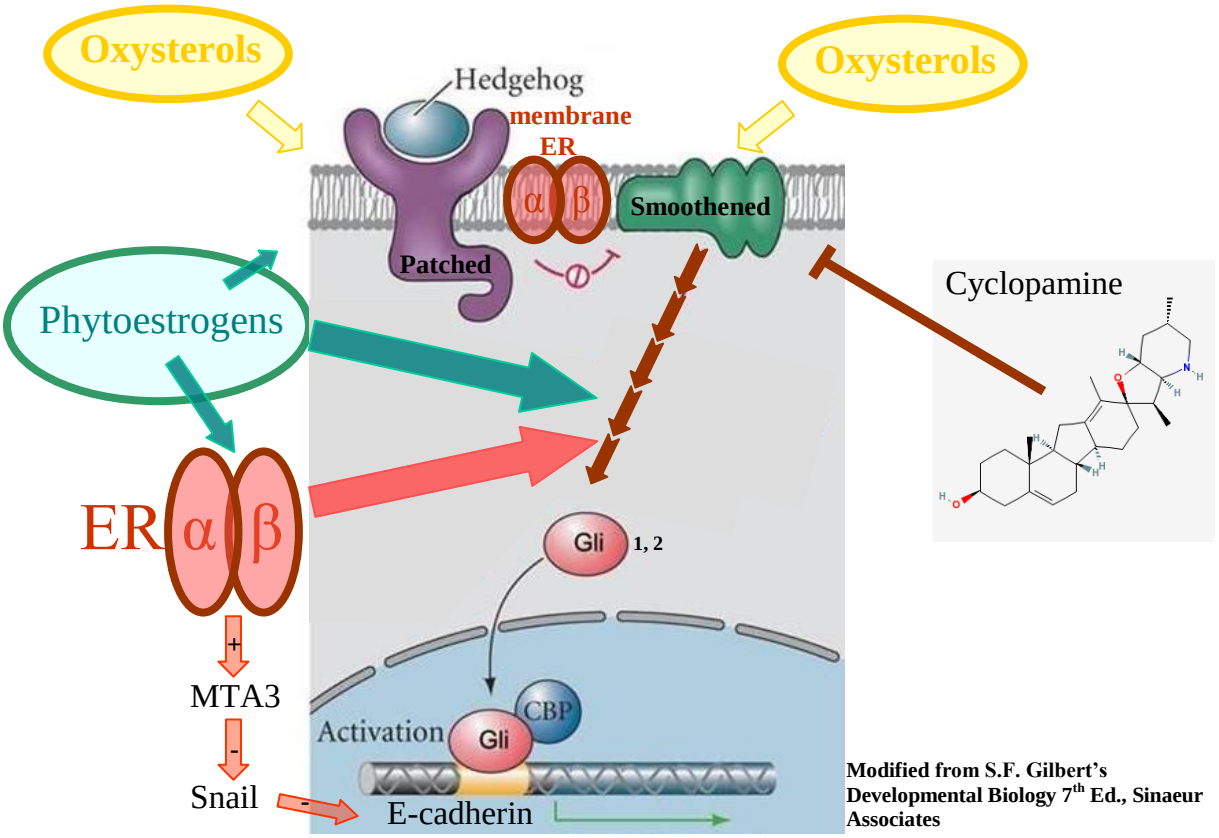


Figure IV-10: Working model of how botanicals might modulate hedgehog signaling pathway.

Chapter V - Future Studies

While much has been accomplished over the course of my work, there are still questions that remain open, and new questions which arose as new data became available. Each of these questions might develop into a project by itself.

From the genistein TRAMP studies with ERα- and ERβKO mice described in Chapter II, it is still left to rule out developmental consequences of missing estrogen receptors and their contribution to the protective vs. more PDC-prone genotype. Conditional KO mice, in which the gene for each estrogen receptor is turned off after completion of development, and preferably in the prostate only, would be ideal. The burden to breed triple transgenic mice to set up this study makes it undoable at this point. A possibly better solution would be the use of compounds selective for each receptor. At this point we have considered two compounds for our purposes that would, we hypothesize, recreate the results from our ERKO study, the ERα specific agonist PPT (propyl pyrazole triol) (460), and the ERβ specific agonist DPN (diarylpropionitrile). As mentioned before on of the problems with DPN is its low selectivity for ERβ over ERα, which is only about 70fold (461). Pilot-studies will be necessary to determine the optimal ER selective dosage. Another available compound to further distinguish between the dual roles of the receptors, specifically to answer the question whether ERα is the "driving force" for PDC, and is it required or necessary for PDC. This question could be answered by using the ERα antagonist MPP (methyl-piperidino-pyrazole) (462). And finally, as a proof of concept we could try to make the PDC incidence even higher by using the ERbeta-selective antagonist/ERalpha-agonist R,R-THC (R,R-tetrahydrochrysene (463), or the metoxychlor metabolite 2,2-bis(p-hydroxyphenyl)-

1,1,1-trichloroethane (HPTE) which was previously shown to have selective agonist activity through ERα and antagonist activity through ERβ and androgen receptor (AR) (464, 465).

To confirm that genistein is acting on prostate cancer *in vivo*, and specifically *in vitro* it is necessary to silence each receptor individually using siRNA or shRNA. If genistein is in fact requiring an ERα /ERβ heterodimer, knocking out either receptor would abolish its inhibitory effects, both on cell growth as well as on hedgehog signaling.

As far as genistein is concerned there are still other questions remaining. Genistein is showing slight stimulation of prostate cancer cell growth *in vitro*, at low concentrations, at 100pM-1μM with PC3 (Figure III-9) and LNCaP (111) cells, but not in TRAMP-C2 or PC3M (Figure II-5), also been reported by others. But even though there is undisputable evidence that genistein has protective effects on low grade tumors (111) and disputable evidence about its protection againts PDC (356), it has been observed that genistein even though protective against primary tumors, induces metastasis to lymph nodes, both in immuno-compromised mice as well as in C57/Bl6 mice with orthotopic syngeneic tumors from RM-9 cells (466). Genistein has been also shown to promote neuro-endocrine differentiation in human prostate LNCaP cells, as demonstrated by increased expression of neuro-endocrine markers in genistein treatment-surviving cells (467).

All this could mean that cells that escape genistein inhibition are of more aggressive character, and therefore combination treatment is needed, with for example cyclopamine, or one of the cheaper botanical compounds described in this dissertation, like EGCG, which should target hedgehog signaling in aggressive metastasis-prone tumor cells, and with an estrogen receptor modulator, like MPP and/or DPN, to target ER's protective effects.

To clearly demonstrate the need for one or both estrogen receptors in the inhibition of hedgehog signaling, we need to knock-down each receptor. siRNA studies targeted against ERα and ERβ are underway. From the differential responses with our botanical compounds in different assays it is apparent, that they are not necessarily working through the same mechanism. It is therefore necessary to test all of them without one or both estrogen receptors to confirm/exclude their inhibitory effects though the ER.

A great natural and cheap tool that can be used to study the pre- and post- Smoothened hedgehog signaling is EGCG, which displayed strong inhibitory effects on Shh-conditioned medium-stimulated, but not SAG-stimulated hedgehog pathway activity. These effects need to be further tested in the prostate cancer cells, specifically PC3, PC3M and TRAMP-C2, with and without estrogen receptors present.

Dose response with ICI, DPN and PPT should be completed, both in TRAMP-C2 and the Shh Light II cell line to have a full profile of their action to generate IC_{50} data and therefore correlations with their estrogenic activities and ER binding affinities. Also full dose responses are being performed by Sara Drenkhahn for the selected oxysterols, both with and without pre-stimulation with SAG and Shh-conditioned medium. It would be interesting to test different cell lines for their oxysterol content, and see whether it correlated with their differential responsiveness to E2 and hedgehog. There is a whole new layer of combinations appearing, if we look at the synthesis of oxysterols. It has been proposed that mutations in the cholesterol pathway lead to perturbation of hedgehog signaling, due to decreased oxysterol pools. But now, the question arises, could both estrogen (468, 469) and androgen signaling in the prostate modulate cholesterol metabolism (470) and oxysterol production, which could modulate both hedgehog- and estrogen-signaling.

To answer the question about the roles of membrane ER in hedgehog signaling, it will be necessary to use impermeable E2, BSA or HRP linked, and assay short term responses to E2, ICI, and the selected botanicals, as well as hedgehog responses. Since the relative amount of membrane ER is small compared to nuclear fractions, it might be difficult to assay the changes in ICI induced membrane migration of ER. It would be really interesting to assay for the specific isoforms of ERα and ERβ, and their involvement in hedgehog responses.

We have not discussed the thought of the involvement of the controversial membrane ER called GPR30 in our results. Does GPR30 interact with hedgehog signaling? Additionally, Ellis Levin has evidence for mitochondrial ER protein to be responsible for E2 response in breast cancer, he does not think it is GPR30.

The interesting combinational effect with E2 + ICI also requires to be followed up upon, specifically to answer whether it has a similar synergistic effect on the hedgehog signaling pathway, both in the TRAMP-C2 and Shh Light II cells. It would be also good to conclude whether it involves membrane ERs.

We cannot forget that the overall goal was to cure prostate cancer, all types of prostate cancer. With our current results, it seems that a combination treatment consisting of ER modulators and botanicals may potentially be useful in preventing and treating both types of cancer, WDC and PDC in the prostate, at least in the TRAMP.

Appendix - Spinach extracts in prostate cancer treatment

Overview

The main focus of our lab was to find novel botanical compounds, preferably with estrogenic properties that have been implicated in prostate cancer or cancer prevention and test them both, in our TRAMP mouse model as well as more recently in our hedgehog assays. Two of the compounds coming through the lab were spinacetin and patuletin, two flavones found in spinach, that had structural similarities with luteolin, a "legendary" compound acting through membrane ERs, but supposedly not nuclear ERs (Dr. Charlie Wang, personal communication).

Dr. Lubahn obtained on a rather adventurous expedition to Spinach-country Fayetteville, Alma, AR large amounts of custom grown spinach. Dr. Rottinghaus' lab to isolated the two candidate compounds from it – spinacetin and patuletin.

We have subsequently tested these compounds in our lab, and found them to bind selectively to ERβ, but not ERα 66, 46, or 36, as originally expected. Both compounds were able to inhibit prostate cancer cell growth and showed promising results in our hedgehog assays, inhibiting *Gli1* mRNA and the Gli-reporter.

Introduction

We set out to find botanical compounds that would inhibit PDC in the TRAMP mice. To date all of our botanicals were able to decrease WDC incidence but had no effect on PDC. There were published reports that genistein (356), EGCG, green tea catechins (404) or green tea (403) and spinach extracts (471) were able to decrease PDC incidence in TRAMP mice.

Our results do not confirm the effects of genistein in our TRAMP mouse system. EGCG was used in the drinking water in the described studies, we did not use EGCG alone, only in a dietary mix together with curcumin and resveratrol, and we did observe a 66% reduction in PDC incidence, which was not significant due to small animal numbers. Other labs saw reduction in PIN, but not PDC in TRAMP mice supplemented with EGCG (5).

Neoxanthin, a compound present in Spinach leaf (*Spinacia oleracea L.*), has been found to have potent apoptotic and anti-prostate cancer cell activity in the low micro-molar range (472-474). Spinach leaf (*Spinacia oleracea*) is a rich source of many flavonoids, carotenoids, neoxanthin, lutein, Vitamin C, and Vitamin E (471-473, 475). A water-soluble, natural antioxidant extract (NAO) of spinach has been shown to slow the tumorigenic progression especially in high grade tumors (PDC) in TRAMP mice (471), prostatic carcinoma (472, 476, 477) and liver (478) cell lines. NAO has been also shown to alleviate effects of oxidative/nitrosative damage in early neoplastic lesions in TRAMP mice (408).

However, neoxanthin is rather unstable. Two flavones, patuletin and spinacetin **(Figure V-1)**, found in high amounts in glucuronated forms in the spinach, are potentially the more likely contributors to spinach's anticancer activity (479). We were additionally interested in these two flavones, due to their structural similarity to luteolin, an estrogenic compound (480-482) with

interesting selectivity for estrogen receptors reported (483). Luteolin has been reported to inhibit angiogenesis (484, 485), growth of melanoma (486), and prostate cancer (487) cell lines. I decided to test the ability of luteolin, patuletin, and spinacetin to inhibit TRAMP-C2 and PC3 growth and also their effects on hedgehog signaling.

Materials and Methods:

Spinach, spinacetin, patuletin

Spinach was harvested, strains 380 and 415. Freeze dried spinach leaves were mixed with mQ autoclaved water in a 2:3 ratio, blended to obtain homogenous mixture, and double filtered through fast flow whatman filter paper. The yield was 65% for strain 380 and 45% for strain 415. Samples were given to Dr. Rottinghaus for further purification and isolation of patuletin and spinacetin. After first HPLC column purification, crude patuletin contained 49,000 ppm, spinacetin was 5,575 ppm, and was tested in protein, hedgehog, and binding assays at 0.1 and 1%. After initial positive results in all assays, we received highly purified compounds. Luteolin was obtained from Sigma, St. Louis, MO.

For Cell culture, real-time, and luciferase assays details see chapter IV.

Results and discussion

Both spinach compounds, patuletin and spinacetin, were able to inhibit mouse prostate cancer cell growth, as well as hedgehog signaling as measured by a decrease in *Gli1* mRNA concentrations **(Figure V-2)**. In an independent assay Sara Drenkhahn was testing both compounds for inhibition of Shh-stimulated Gli-reporter in Shh Light II cells **(Figure V-3)**. In all

assays, patuletin was the more potent compound, with 300nM patuletin and 1μM spinacetin significantly inhibiting *Gli1* mRNA in TRMAP-C2 cells.

From the results of our ERα- and ERβKO we would expect an ERβ agonist to prevent PDC incidence, luteolin was supposed to act primarily through ERβ (488). We therefore expected that the structurally similar patuletin and spinacetin would be ERβ-selective as well, and the cancer protective actions of spinach could be attributed to the estrogenic activity of those compounds.

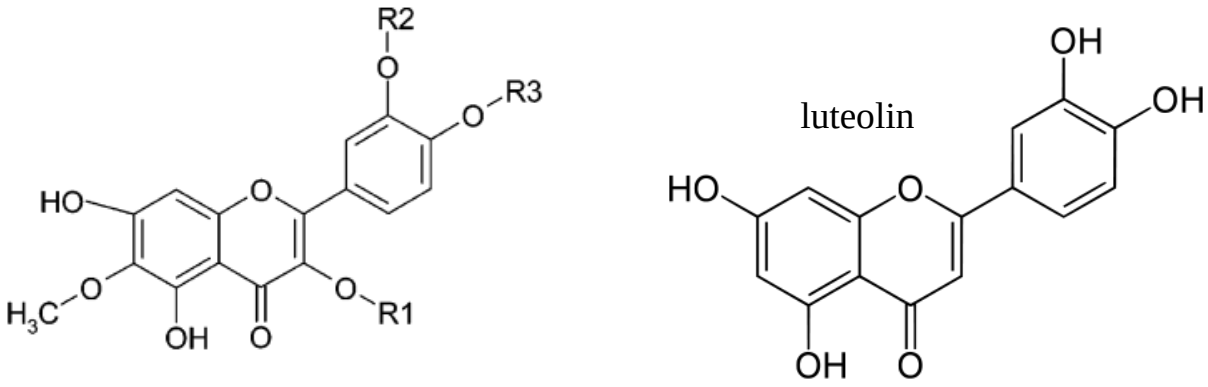
Nick Starkey in our lab, was able to confirm ERβ-selectivity of luteolin, and was able to demonstrate binding to ERβ at nanomolar concentrations, compared with micromolar affinites to ERα (data not shown).

Since both compounds were able to inhibit prostate cancer cell growth, as well as inhibit Hedgehog signaling as determined by decreased Gli1 mRNA in TRAMP-C2 cells, and decreased Gli-reporter activity in Shh Light II cells (work done by Sara Drenkhahn), we set out to test spinach extracts *in vivo* in our TRAMP mice.

The results of our mouse studies were very surprising. The PDC incidence in the FVB/C57 mice was significantly higher in the control group than expected - 41%, compared to 19% in pure C57B6, and 30% from previous FVB/C57 wheat diet studies. Although the PDC incidence in the ERβKO mice was comparable to previous results with pure C57 TRAMP mice, 48% and 41% respectively, we did not observe an increase in PDC due to the high baseline in the control group. Whole dried spinach extracts at 0.2 and 2% showed slight although not quite

statistically significant protection in WDC incidence in the WT animals, but an increased WDC incidence in the KOs (again, not statistically significant). There were no statistically significant effects in the PDC incidence, but trends suggested increased PDC incidence in the WTs, and protection in the KOs.

These results are slightly discouraging, but do not exclude potential protective effects from purified spinach extracts like patuletin and spinacetin. It would be still useful to test these two compounds, possibly in a TRAMP-C2/TRAMP isograft model, or a xenograft model for hedgehog inhibiting and prostate cancer treatment potential.



Compound 1 "Patuletin" R1 = β-D-glucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = H; R3 = H
Compound 2 "Spinacetin" R1 = β-D-glucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH_3; R3 = H
Compound 3 "Patuletin" R1 = β-D-2" feruloylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = H; R3 = H
Compound 4 "Spinacetin" R1 = β-D-2" *p*-coumaroylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH_3; R3 = H
Compound 5 "Spinacetin" R1 = β-D-2"feruloylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH_3; R3 = H
Compound 6 "Spinacetin" R1 = β-D-glucosyl (1→6) β-D-glucoside; R2 = CH_3; R3 = H
Compound 7 "Jaceidin" R1= CH_3; R2 = CH_3; R3 = glucuronic acid

Figure A-I: Flavonoids in spinach (479) and luteolin.

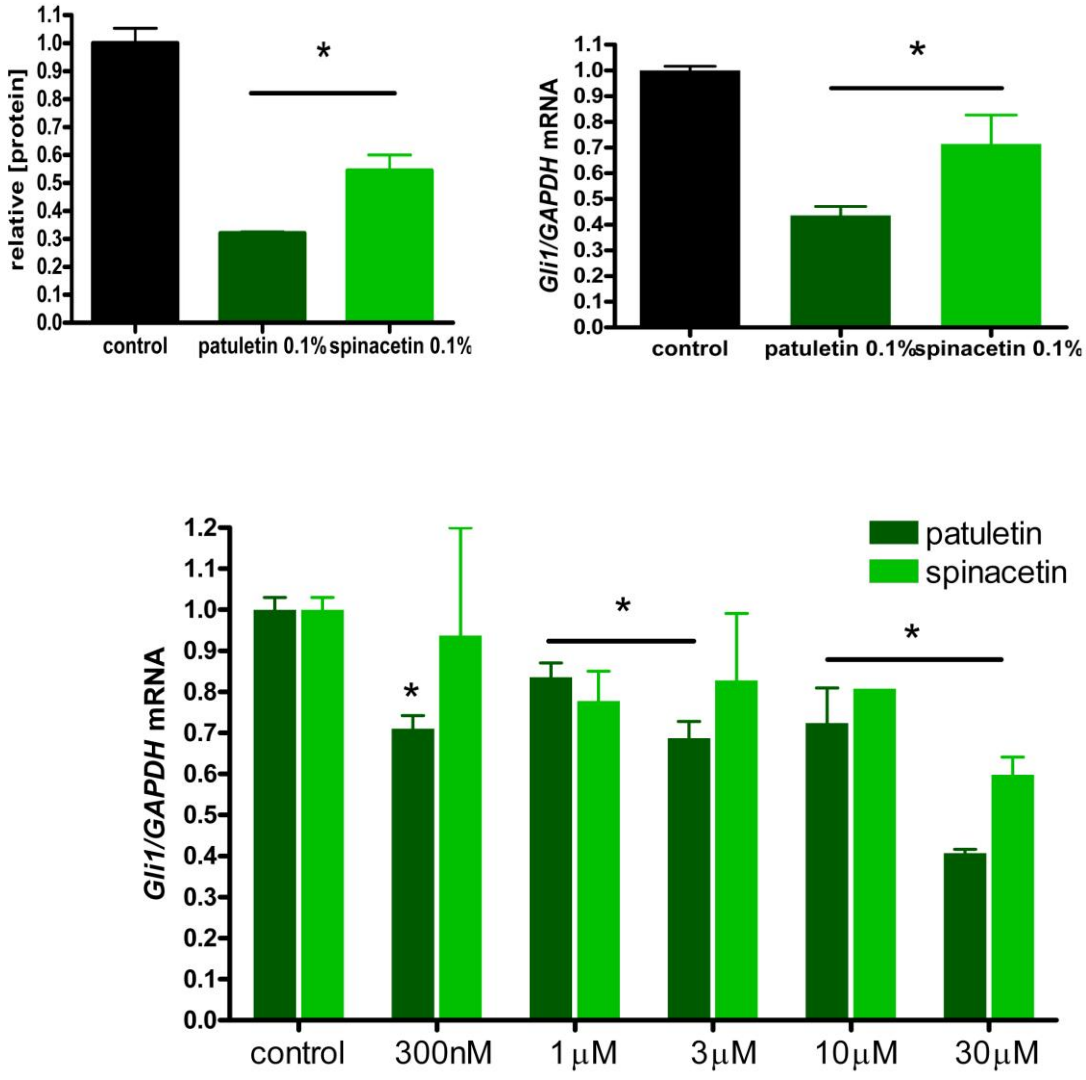


Figure A-2: Patuletin and Spinacetin inhibit cell growth and *Gli1* in TRAMP-C2 cells.

Crude 0.1% patuletin and spinacetin inhibit total cellular protein (A) and Gli1 mRNA (B) after a 72 hour treatment of TRAMP-C2 cells. (C) Highly purified patuletin and spinacetin inhibit *Gli1* mRNA less potent than the crude extract.

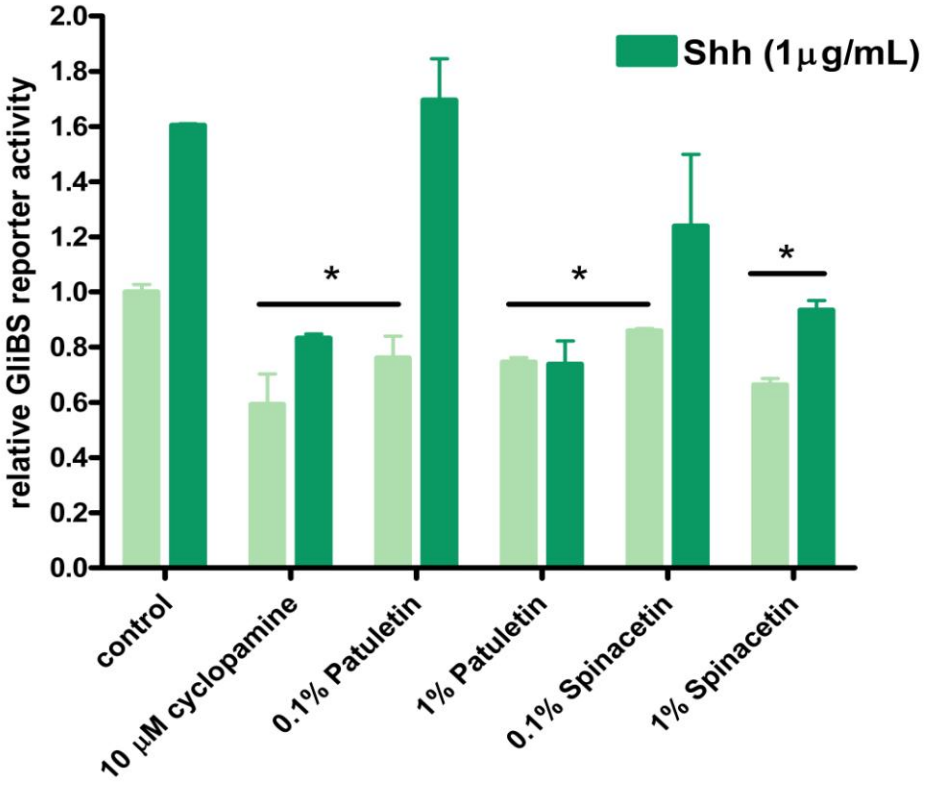


Figure A-3: Patuletin and Spinacetin inhibit GliBS reporter activity in SHH Light II cells (done by Sara Drenkhahn).

SHH Light II cells were treated for 24 hours with cyclopamine, patuletin, and spinacetin +/- N-Shh peptide, before being lysed, and assayed for luciferase activity. * $p<0.05$

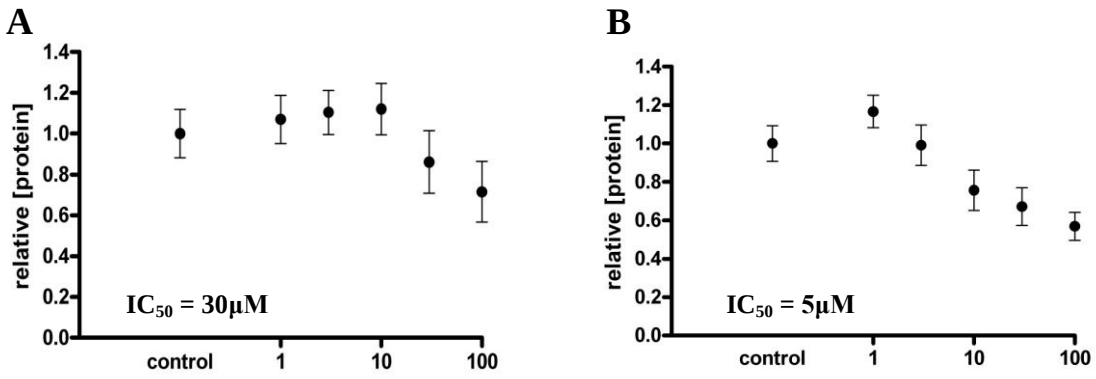


Figure A-4: Luteolin inhibits prostate cancer cell growth *in vitro*.

Luteolin inhibits TAMP-C2 **(A)** and PC3 **(B)** cell growth as measured by total protein after a 72 hours treatment.

Control Diet		HYP	PIN	WDC	PDC
	WT	0/27	1/27	15/27	11/27
	KO	0/25	5/25	8/25	12/25
0.2% Diet					
	WT	0/23	5/23	7/23	11/23
	KO	0/23	3/23	11/23	9/23
2% Diet					
	WT	0/27	4/27	8/27	15/27
	KO	0/23	2/23	13/23	8/23

Control Diet		HYP	PIN	WDC	PDC
	WT	0%	4%	55%	41%
	KO	0%	20%	32%	48%
0.2% Diet					
	WT	0%	22%	30%	48%
	KO	0%	13%	48%	39%
2% Diet					
	WT	0%	15%	30%	55%
	KO	0%	9%	57%	35%

Table A-1: Preliminary results from TRAMP spinach study (done by Dr. Glenn Jackson).

References:

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008;58(2):71-96.
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VITA

Anna Ślusarz was born in Warsaw, Poland on October 19th 1978. She completed her secondary education in Vienna, Austria. In July 2001, she received the Magistra Philosophiae Degree in Interpreting from the University of Vienna, Austria. In May 2003, she received the Masters of Arts Degree in German Literature form the University of Missouri, Columbia. Anna entered the Department of Biochemistry at the University of Missouri in August 2003. She began to work as a Ph.D. student under the guidance of Dr. Dennis Lubahn in May 2004. Her research has been focusing on estrogen- and hedgehog-signaling pathways in prostate cancer. She will continue her scientific training as a research fellow in Dr.Lubahn's lab for a short period of time while she is searching for a position at a cancer research facility elsewhere.

My dissertation research has resulted in the following papers:

1. Common Botanical Compounds Inhibit the Hedgehog Signaling Pathway in Prostate Cancer. Anna Ślusarz, Nader S. Shenouda, Mary S. Sakla, Sara K. Drenkhahn, Acharan S. Narula, Ruth S. MacDonald, Cynthia L. Besch-Williford, Dennis B. Lubahn ***Cancer Research Under Review***

2. Genistein Reduces Incidence of Prostate Cancer in TRAMP mice through Estrogen Receptor Dependent Mechanism. Anna Ślusarz, Glenn Jackson, Kevin Day, Nader Shenouda, Norman M Greenberg, Andrew Bauer, Leslie Newton, Kimberly Jordan, Jim Browning, Ruth S. MacDonald, Cynthia L. Besch-Williford, Dennis B. Lubahn ***About to be submitted***

3. Estrogenic regulation of the Hedgehog Signaling Pathway in Prostate Cancer. Anna Ślusarz, Sara K. Drenkhahn, Dennis B. Lubahn ***Manuscript in preparation***

4. Effects of nanosecond pulsed electric fields on human prostate cancer cell line – LNCaP. Vinitha Donthula, Bruno Camps Raga, Naz E. Islam, Anna Ślusarz, Dennis B. Lubahn, Venkataseshu Ganjam **IEEE Transactions on Dielectrics and Electrical Insulation Vol. 16, Issue 5, pp. 1311-1316, October 2009**

Patent pending 12/106,993 *and* **PCT/US2008/061055:** PHYTOESTROGENS AS REGULATORS OF HEDGEHOG SIGNALING AND METHODS OF THEIR USE IN CANCER TREATMENT; initial submission April 20, 2007